

Importance of Inherent Substrate Reactivity in Enzyme-Promoted Carbocation Cyclization/Rearrangements

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carbocations · enzymes · inherent reactivity ·
intrinsic reactivity · terpenes

*Dedicated to Roald Hoffmann on the
occasion of his 80th birthday*

The importance of inherent substrate reactivity for terpene synthase enzymes is discussed, with a focus on recent experimental tests of predictions derived from computations on gas-phase reactivity of carbocations.

1. Amazing Enzymes, Spring-Loaded Substrates, Simple Mechanistic Models

The power of enzymes to promote otherwise unfavorable chemical transformations is astounding. However, the origins of this ability are still far from fully understood. Many roles have been ascribed to enzymes, ranging from selective stabilization of transition state structures by electrostatic effects^[1] to geometric and vibrational promotion of quantum mechanical tunneling,^[2] to binding of otherwise unfavorable conformations of reactants that resemble key transition state structures.^[3] The importance of the inherent/intrinsic reactivity of enzymatic substrates to biologically relevant reactions is often overshadowed by studies focused on enzymatic catalysts. It is perhaps wise to take a more balanced tack and not assume that an enzyme must modulate substrate reactivity *throughout* a multistep reaction that occurs within its interior. Evidence for the importance of inherent substrate reactivity for terpene-synthase-promoted reactions is discussed herein. The inherent (gas phase!) reactivity of carbocations putatively generated in terpene synthase interiors has been examined in detail using quantum chemistry (generally, density functional theory, DFT),^[5] and recent experimental studies making use of labeled enzymatic substrates validate the idea that this inherent reactivity is indeed expressed in biological contexts. While no mechanistic scheme can be proven to be true, truth is not the goal of these studies. Instead “validation” here refers to confirmation that a given mechanistic model, which involves the expression of inherent substrate reactivity, is predictive.^[4]

2. The Most Circumstantial Sort of Evidence

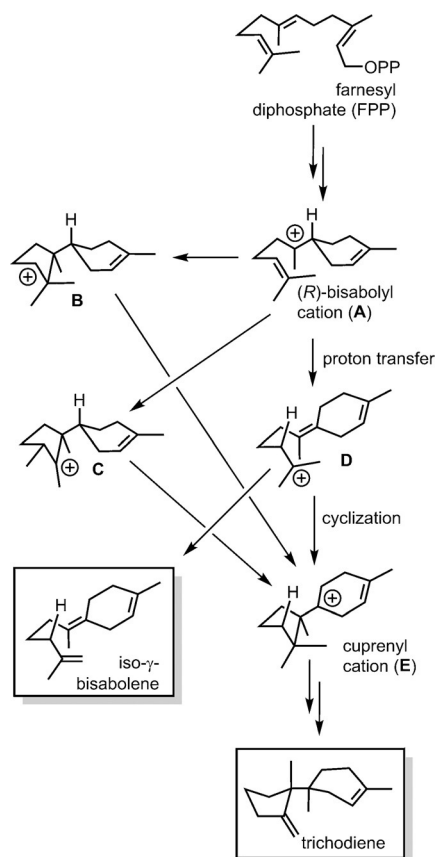
2.1. Short-Circuiting Multi-Step Pathways

The by-products formed in reactions carried out by terpene synthases, wild-type or mutant versions, have traditionally been taken as evidence for the formation of particular carbocation intermediates. While such evidence is circumstantial at best, in that such by-products could be formed by different mechanisms than those leading to major products, it does at least indicate that certain carbocations are likely generated in a given active site. Such carbocations may be true minima on potential energy surfaces (PES) or may instead occur elsewhere along reaction coordinates.^[5] Two reactions for which unexpected intermediates along pathways to major products were predicted by theory are described below.^[6]

2.2. Trichodiene Synthase

Trichodiene synthase produces the sesquiterpene trichodiene from farnesyl diphosphate (FPP; Scheme 1).^[7] The pathway proposed to lead to trichodiene, before computations on the relevant carbocations were carried out, involves the conversion of the bisabolyl cation (**A**) to cation **B** via cyclization, and then conversion of cation **B** to cation **E** by way of a 1,5-hydride transfer.^[7] This mechanism is consistent with previous experiments in which the migrating hydrogen atom was labeled with deuterium. Quantum chemical computations indicate, however, that such a pathway is not likely on energetic grounds.^[8] First, carbocation **B** appears not to be a minimum on the PES. Cation **C** is a minimum and can be formed directly from **A** and then converted directly to **E**, but this pathway has a predicted barrier of approximately 20 kcal mol⁻¹. An alternative pathway that involves an intramolecular proton transfer (**A**→**D**→**E**), which is also consistent with labeling experiments, was predicted to have a much lower barrier, approximately 8 kcal mol⁻¹. Iso- γ -bisabolene

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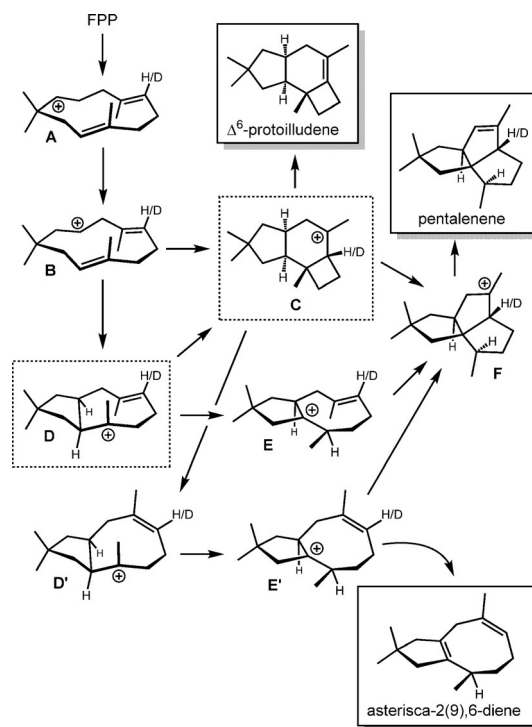


Scheme 1. Pathways to trichodiene branching from the bisabolyl cation. Letters correspond to those used in Ref. [8a] to facilitate comparisons.

(Scheme 1) is a known natural product,^[9] suggesting that cation **D** is likely biosynthetically relevant. Although iso- γ -bisabolene has not yet been reported as a product of trichodiene synthase, it has been observed to be produced by an organism that produces trichodiene (*F. verticillioides*).^[10]

2.3. Pentalenene Synthase

Pentalenene synthase produces the sesquiterpene pentalenene from FPP (Scheme 2).^[11] The mechanism for pentalenene formation, proposed before computations on the



Scheme 2. Pathways to pentalenene. Letters correspond to those used in Ref. [12b] to facilitate comparisons.

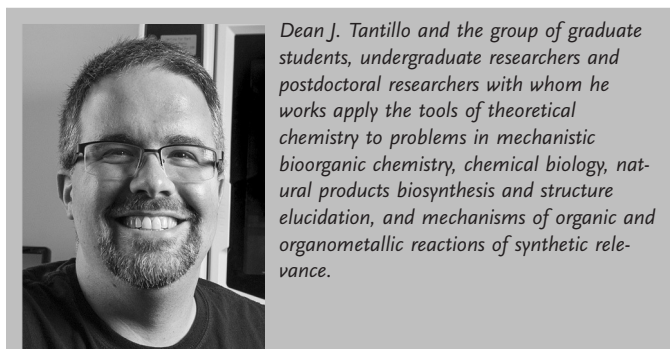
relevant carbocations were carried out, involves the sequence **B**→**D**→**E**→**F**.^[11] However, cation **D** was found not to be a PES minimum through computations.^[12] Instead, a mechanism involving cation **C** (first proposed as **B**→**C**→**F** but later revised as **B**→**C**→**D'**→**E'**→**F**) was predicted to be preferred. While Δ^6 -protoilludene, a product of direct deprotonation of cation **C** (Scheme 2), was known to be produced in substantial quantities by mutant pentalenene synthases,^[11] it was not, before quantum chemical calculations were carried out, thought to be found along the pathway to pentalenene. The revised mechanistic model provides a straightforward explanation for formation of **C**: mutations stop the pathway to pentalenene prematurely rather than inducing a different pathway to form cation **C**.

Recently, characterization of a sesquiterpene synthase that produces asterisca-2(9),6-diene was described.^[13] This natural product would result from cation **E'** via deprotonation (Scheme 2), suggesting that the unusual *E*-to-*Z* isomerization proposed to occur during pentalenene formation on the basis of quantum chemical computations (**B**→**C**→**D'**) is indeed likely biologically relevant.

3. Site-Specific Labeling Experiments

3.1. Follow the Proton

In some cases, the sites where substrate protons end up in terpene products differ depending on which of two (or more) mechanisms is followed. In such cases, site-specific deuterium labeling of a substrate can rule out one (or more) of these

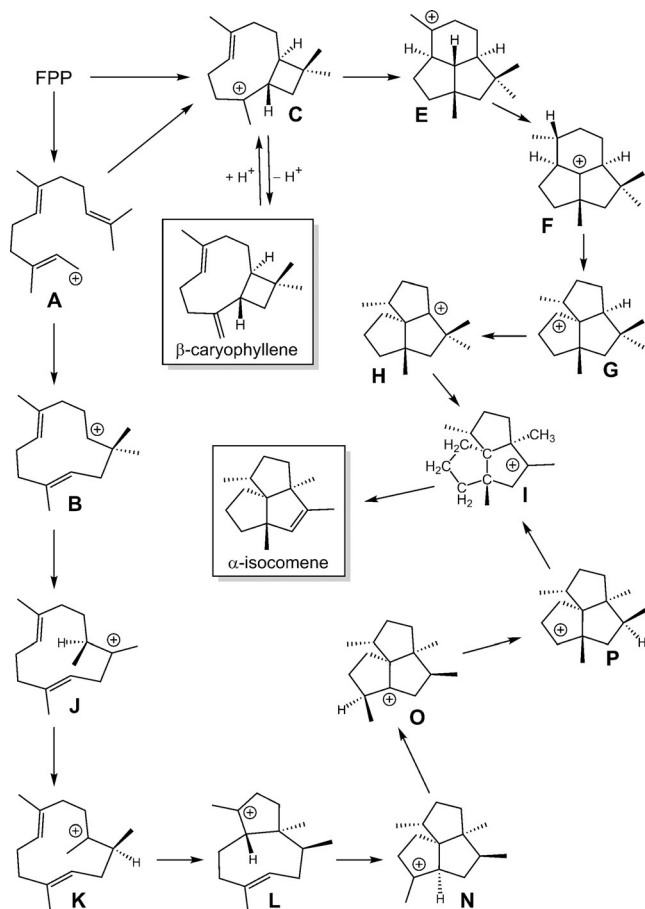


Dean J. Tantillo and the group of graduate students, undergraduate researchers and postdoctoral researchers with whom he works apply the tools of theoretical chemistry to problems in mechanistic bioorganic chemistry, chemical biology, natural products biosynthesis and structure elucidation, and mechanisms of organic and organometallic reactions of synthetic relevance.

mechanisms. This is a classic approach to probing terpene synthase mechanisms, but it has recently seen a renaissance due to the availability of new labeled substrates.^[14] Described below are four recent examples that showcase the power of this method.

3.2. Isocomene Synthase

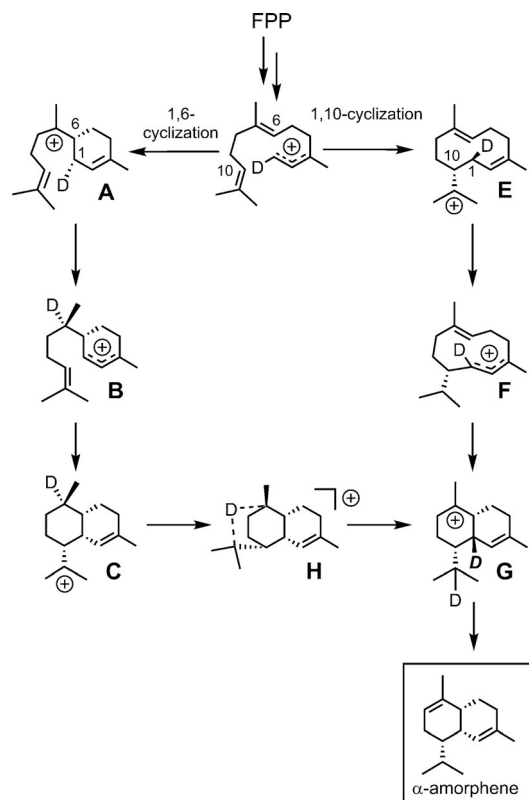
The sesquiterpene α -isocomene has been proposed to arise via either cation **C** or cation **L** in Scheme 3.^[15] Both options have been shown to be energetically viable on the basis of results from quantum chemical computations (the lowest energy pathways from **C** and **L** are shown in Scheme 3), but the pathway via cation **C** was predicted to be preferred.^[16] These pathways could be distinguished by labeling at the methine and methylene groups shown explicitly in cation **I**, since atoms of these groups would have different origins in the two possible pathways. A variety of deuterium-labeled substrates were fed to an isocomene synthase from chamomile by Köllner and co-workers,^[16] and the positions of deuterium atoms in the resulting products ruled out the pathway via cation **L**, consistent with the computational results on inherent carbocation reactivity. In addition, β -caryophyllene (Scheme 3) was observed as a by-product of the isocomene synthase examined.



Scheme 3. Pathways to α -isocomene. Letters correspond to those used in Ref. [16] to facilitate comparisons.

3.3. Amorphene Synthase

The amorphene sesquiterpenes (e.g., α -amorphene, Scheme 4) have generally been thought to arise via a pathway involving initial formation of a 10-membered ring (Scheme 4, right).^[17] The results of quantum chemical calculations

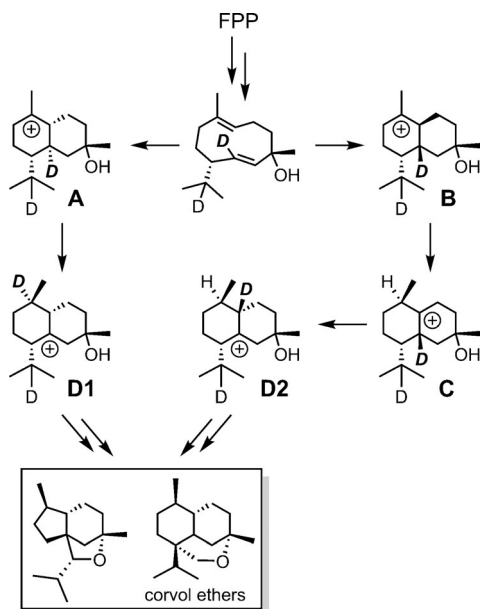


Scheme 4. Pathways to α -amorphene. Letters correspond to those used in Ref. [18] to facilitate comparisons.

indicated that such a pathway is energetically viable, but that an alternative pathway involving initial formation of a 6-membered ring (Scheme 4, left) is energetically preferred.^[18] Such a pathway was previously proposed as a route to other sesquiterpenes derived from cation **C**,^[17] but the facile conversion of **C** to **G** via **H** was predicted on the basis of theoretical results. A recent labeling study by Dickschat and co-workers supports the prevalence of the **A** $\rightarrow$ **B** $\rightarrow$ **C** $\rightarrow$ **H** $\rightarrow$ **G** pathway.^[19] The 1,6- and 1,10-cyclization routes to α -amorphene were distinguished by deuterium labeling at the position shown in Scheme 4. If the 1,10-cyclization pathway was followed, the deuterium label would end up attached to one of the carbons at the ring-fusion in cation **G** (bold *italics*) and, subsequently α -amorphene. However, the deuterium label was found to be located on the isopropyl group of isolated α -amorphene, consistent with the 1,6-cyclization pathway predicted using quantum chemistry.

3.4. Corvol Ether Synthase

Two possible routes to the corvol ethers are shown in Scheme 5, one involving a 1,3-hydride shift and the other involving two sequential 1,2-H shifts.^[20] Quantum chemical computations were used to compare the barriers for both

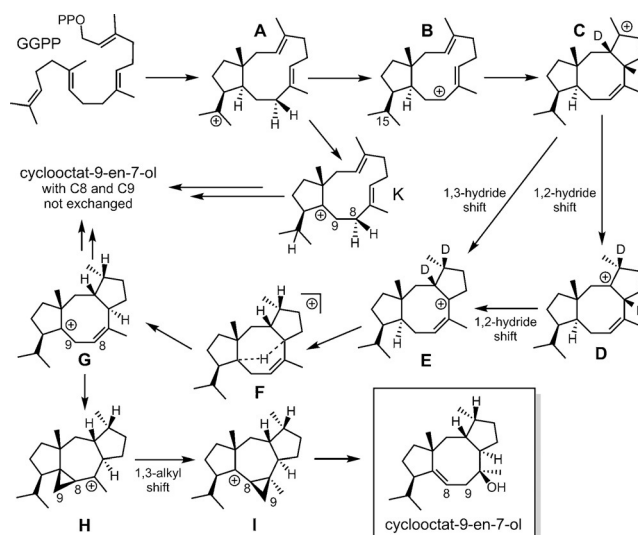


Scheme 5. Pathways to corvol ethers. Letters correspond to those used in Ref. [21] to facilitate comparisons.

pathways, and the results of these calculations indicated that the double 1,2-hydride shift pathway is preferred by approximately 5 kcal mol⁻¹.^[21] Which pathway is preferred in the presence of corvol ether synthase was also probed experimentally using a deuterium labeled substrate.^[21] FPP labeled with two deuterium atoms (Scheme 4; and one ¹³C, not shown) was incubated with corvol ether synthase. Depending on which of the two pathways was followed, one or the other of differently labeled carbocations **D1** and **D2** would presumably form and then be converted to the corvol ethers. The ether products observed had labeling patterns matching that of **D2**, indicating that the 1,3-hydride shift path, the path with an inherently higher barrier, was not followed.

3.5. Cyclooctatenol Synthase

Kuzuyama and co-workers reported that, surprisingly, the diterpenene cyclooctat-9-en-7-ol arises from a pathway in which carbons 8 and 9 have exchanged their positions as substrate is converted to product (Scheme 6).^[22] This conclusion was reached on the basis of results from experiments using ¹³C and deuterium-labeled substrates, carried out in advance of quantum chemical computations, and rules out previously proposed mechanisms involving cation **K** or mechanisms in which cation **G** is converted to cyclooctat-9-en-7-ol without rearrangement. Two subsequent theoretical studies confirmed that a pathway involving the intercon-



Scheme 6. Pathways to cyclooctatenol. Letters correspond to those used in Ref. [23a] to facilitate comparisons.

sion of cations **G**, **H** and **I** is energetically viable in the absence of an enzyme.^[23] Both studies also described predictions that sequential 1,2-hydride shifts are energetically preferred over a direct 1,3-hydride shift that converts cation **C** to cation **E** (as was predicted for corvol ether formation as well; Section 3.4). These predictions were tested by experiments in which a deuterium-labeled substrate would lead to differently labeled cations **E** (both possible positions of the deuterium label are shown in Scheme 6) and differently labeled final products. Isolated products had the deuterium label in the position consistent with the double 1,2-hydride shift pathway predicted on the basis of the inherent reactivity arguments described above.^[24]

4. Isotopically Sensitive Branching

4.1. A Less Straightforward Experiment

Isotopically sensitive branching experiments have been applied to a variety of terpene-synthase-promoted reactions.^[25] In these experiments, a substrate analogue for which a particular atom is traded for an isotope (e.g., D for H) is fed to the enzyme and the ratio of products is compared to that formed when using the natural substrate. If kinetic isotope effects (KIEs)^[26] of different magnitudes are expected for the pathways that lead to different products, then the product ratio will differ when the isotopologue is used. If different mechanistic schemes lead to predictions of different changes to product ratios, then such an experiment can be used to rule out certain pathways. One such example is discussed below.

4.2. Pentalenene Synthase, Again

As described above (Section 2.3), the mechanism for formation of both pentalenene and Δ^6 -protoilludene pro-

posed on the basis of quantum chemical results involves cation **C** (Scheme 2). Importantly, the previously proposed mechanism for formation of pentalenene does not involve **C**. Thus, the new and previously proposed mechanisms differ in the branch point leading to pentalenene and Δ^6 -protoilludene. For the previously proposed mechanism, this is cation **D**, but for the new mechanism, this is cation **C**. Assuming that passing the branch point commits the substrate to formation of either pentalenene or Δ^6 -protoilludene, isotopically sensitive branching is expected for H/D substitution at the position shown in Scheme 2. In the traditional mechanism, secondary (i.e., small, if detectable at all) KIEs are expected for both branches. However, in the new mechanism, a secondary KIE is expected for the branch leading to pentalenene, while a primary (i.e., large) KIE is expected for formation of Δ^6 -protoilludene. Computations led to a prediction that, for the new mechanism, the ratio of pentalenene to Δ^6 -protoilludene should change by a factor of 1.5–2 (with Δ^6 -protoilludene formation being suppressed) upon substituting H for D; no significant change in product ratio was expected for the previously proposed mechanism. This prediction was tested experimentally for the H309A mutant of pentalenene synthase,^[12b] and a change in product ratio of approximately 1.9 was found, a result consistent with inherent carbocation reactivity being expressed in the enzymatic system, i.e., the previously proposed mechanism was not viable, as predicted on the basis of quantum chemical results.

5. Conclusions and Outlook

Without question, terpene synthases are essential for promoting formation of terpenes. They bind their substrates in relevant conformations, they facilitate carbocation generation, they shield said carbocations from premature quenching by, for example, water, and they direct the site of quenching. Nonetheless, the studies described here indicate that, at least in many cases, they need not intervene throughout the cyclization/rearrangement chemistry that a given carbocation would undergo in their absence (and the absence of solvent).

This is not a new concept, as evidenced by the following discussion between two giants of bioorganic chemistry, from a mid-20th century symposium on terpene biosynthesis:^[27]

Robinson: *Do you think an enzyme is needed at each of those first hypothetical stages?*

Eschenmoser: *I would not know.*

Robinson: *I should have thought it was not necessary. What you want is to give the enzyme a suitable place in the initiating mechanism and the intermediate stages, or at least some of them, can then take place automatically.*

Modern theory and experimentation are finally in place to address this fundamental mechanistic issue.

What the studies discussed in this Minireview have done is redefine the mechanistic questions that should be asked. One

need not look for an explanation, or mechanistic model, involving direct intervention by an enzyme when a substrate, if let be, will react as desired on its own. Theory can predict the portions of a reaction coordinate for which an enzyme must be engaged in active manipulation, allowing researchers to focus attention on those.

While terpene synthases are ubiquitous in nature, they are unusual in the chemistry they promote. Nonetheless, there is no reason to assume that other enzymes will not take full advantage of inherent substrate reactivity. Why evolve to alter something that may be useful?

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Conflict of interest

The author declares no conflict of interest.

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