

## Increasing Complexity of a Diterpene Synthase Reaction with a Single Residue Switch

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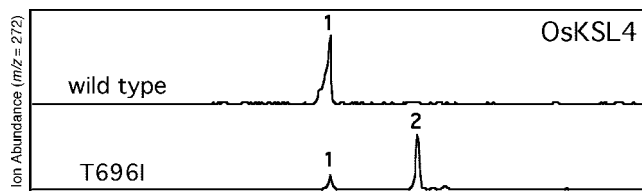
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Terpene synthases often catalyze complex reactions involving an intricate series of carbocation intermediates. The resulting, generally cyclical structures provide initial hydrocarbon frameworks that underlie the astonishing structural diversity of the enormous class of terpenoid natural products (>50,000 known),<sup>1</sup> and these enzymes often mediate the committed step in their particular biosynthetic pathway. Accordingly, how terpene synthases specify product outcome has drawn a great deal of attention.<sup>2</sup> Recent reports demonstrate that changes in a small number of amino acid residues can substantially alter specificity, although without generally increasing complexity of the catalyzed reaction and resulting product.<sup>3</sup> In previous work, we have shown that mutational introduction of a hydroxyl group at specific positions within diterpene synthase active sites can “short circuit” complex cyclization and rearrangement reactions, resulting in the production of “simpler” diterpenes.<sup>3e,g</sup> Here we demonstrate that the converse change, substitution of an Ile for Thr at the relevant position in a native pimaradiene synthase, leads to a dramatic increase in reaction complexity. Product outcome is shifted from the tricyclic *syn*-pimara-7,15-diene (**1**) to a rearranged tetracycle, aphidicol-15-ene (**2**). This result has implications for our understanding of the structure–function relationships underlying the reaction mechanism, engineering, and evolution of terpene synthases.

Previously we found that substitution of a particular conserved Ile by Thr was sufficient to convert diverse *ent*-kaurene specific (>95%) synthases to the specific production of *ent*-pimara-8(14),15-diene.<sup>3e</sup> In addition, the abietadiene synthases involved in conifer resin acid production contain a nearby conserved Ala (Supporting Figure), substitution of which by Ser similarly leads to specific formation of pimaradienes rather than the original rearranged abietane tricycles.<sup>3g</sup> Accordingly, replacement of these particular aliphatic amino acids by residues containing a hydroxyl group can “short circuit” complex cyclization/rearrangement reactions. In particular, the mutant diterpene synthases presumably deprotonate the pimar-15-en-8-yl<sup>+</sup> intermediate formed by initial (tri)cyclization of their specific bicyclic copalyl/labdadienyl diphosphate (CPP) substrate. In both cases, the targeted residue can be modeled near the 8-yl carbocation position, with the shift in exact position presumably due to the differing stereochemistry of the relevant CPP substrate. Hence, a carbocation proximal aliphatic residue seems to be necessary, but possibly is not sufficient, for more complex reactions.

There is a *syn*-pimara-7,15-diene synthase (OsKSL4)<sup>4</sup> involved in rice antifungal phytoalexin (momilactone) biosynthesis that contains a Thr in place of the aforementioned Ile found in *ent*-kaurene synthases, consistent with the identical C9 configuration of *ent*- and *syn*-CPP (**3**). The mechanistically important Ala identified in abietadiene synthases also is conserved in OsKSL4, as well as *ent*-kaurene synthases. OsKSL4 is only distantly related to the rice *ent*-kaurene synthases (<60% amino acid identity) and

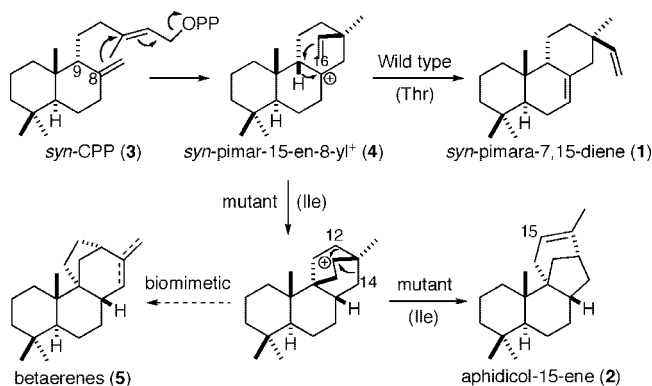


**Figure 1.** Effect of T696I mutation on OsKSL4 product outcome. Selected ion ( $m/z = 272$ ) chromatograms from GC–MS analysis (**1**, *syn*-pimara-7,15-diene; **2**, aphidicol-15-ene).

exhibits distinct substrate stereospecificity for *syn*- rather than *ent*-CPP. Furthermore, the gene for OsKSL4 is found in the rice genome near that for the *syn*-CPP synthase (OsCPS4),<sup>4</sup> along with those for a dehydrogenase and cytochromes P450 also involved in momilactone biosynthesis.<sup>5</sup> Such secondary metabolism gene clusters are unusual in plants and generally are taken as an indication of strong selective pressure for production of the resulting compound operating over evolutionarily long timescales.<sup>6</sup> Together these findings suggest OsKSL4 has served as a *syn*-pimaradiene specific synthase for an extended period of time, with no selective pressure to retain catalytic features in its active site for more complex reaction mechanisms. Accordingly, mutational analysis of OsKSL4 provides an opportunity to investigate the possibility that the previously identified carbocation proximal single residue change acts as a true switch controlling reaction complexity.

For this purpose, Ile was substituted for Thr at the previously identified functionally important position in OsKSL4. The product mix from reaction of the resulting OsKSL4:T696I mutant with *syn*-CPP was comprised of ~80% of a novel diterpene, along with ~20% of the original pimaradiene (Figure 1). Comparison of the yield from wild type or mutant diterpene synthases upon coexpression with geranylgeranyl diphosphate and CPP synthases has been correlated with relative catalytic efficiency *in vitro*,<sup>3e,g</sup> and such comparative analysis with OsKSL4:T696I demonstrate this mutation has only minimal effect on yield (less than 2-fold decrease) and, presumably, enzymatic activity.

To obtain sufficient amounts of the novel diterpene product for structural analysis, we incorporated use of the “bottom half” of the heterologous mevalonate dependent isoprenoid pathway from yeast, along with mevalonate supplementation, using a previously described pMBI plasmid,<sup>7</sup> in conjunction with a modular metabolic engineering system we recently developed.<sup>8</sup> The resulting recombinant bacterial strain produced ~1 mg diterpenes/L culture, enabling isolation of sufficient amounts of the novel diterpene product of OsKSL4:T696I for structural determination by NMR, demonstrating that this predominant enzymatic product corresponds to aphidicol-15-ene (see Supporting Information). Notably, this appears to be the first report of an aphidicolene specific diterpene synthase.

**Scheme 1.** Cyclization of *syn*-CPP by OsKSL4

The production of aphidicolene by OsKSL4:T696I represents significant extension of the original direct deprotonation of the pimar-15-en-8-yl<sup>+</sup> (4) intermediate formed by initial (tri)cyclization mediated by wild type OsKSL4. In particular, to form aphidicolene the initial pimaranyl<sup>+</sup> intermediate must undergo a 1,2-hydride shift from C9 to C8, secondary (tetra)cyclization, and Wagner-Meerwein ring rearrangement of the initial secondary carbocation tetracycle to the more stable tertiary aphidicolanyl carbocation that undergoes terminating deprotonation (Scheme 1).

These results indicate that the nature of the carbocation proximal residue acts as a true switch controlling product outcome. The presence of a hydroxyl containing side chain leads to predominant deprotonation of the pimaranyl<sup>+</sup> intermediate formed upon initial (tri)cyclization, whereas that of an aliphatic side chain enables predominant extension to more complex further cyclized and/or rearranged diterpenes. Given the expectation that terpene synthases act to stabilize many of the carbocation intermediates in their reaction cascades,<sup>2</sup> this result is somewhat counter-intuitive. Decreased polarity in the active site would have been expected to lead to loss of carbocation stabilization and, hence, less rather than more complex reaction mechanisms.

Although it is not readily evident why such a simple single residue switch leads to the intricate, yet specific, cascade of carbocationic intermediates observed here, fortuitously, extensive experimental and theoretical investigation of the cyclization of *syn*-copalol, as a biomimetic of *syn*-CPP cyclization, has been previously reported.<sup>9</sup> This study demonstrated that acid treatment (1.5 equiv. BF<sub>3</sub>·Et<sub>2</sub>O in CH<sub>2</sub>Cl<sub>2</sub>) of *syn*-copalol produced a variety of tricyclic pimaradienes as well as rearranged tetracycles. Beyond production of pimaradienes resulting from direct deprotonation of the pimar-15-en-8-yl<sup>+</sup> formed by initial (tri)cyclization (18% yield), a pimar-9(11),15-diene that must arise from deprotonation of the pimar-15-en-9-yl<sup>+</sup> intermediate from C9–C8 hydride shift (4% yield) was also observed. In addition, rearranged tetracycles resulting from subsequent secondary (tetra)cyclization and ring rearrangement (15% yield) were observed. Thus, even in inert organic solvent, *syn*-copalol readily undergoes an acid catalyzed extended cyclization and rearrangement reaction similar to that inferred for production of aphidicolene. Nevertheless, the rearranged tetracycles formed in this biomimetic cyclization reaction are of the betaerane type (e.g., 5), which differ from the enzymatically produced aphidicolene in regiochemistry of the concluding ring rearrangement (i.e., from C12 instead of C14). Furthermore, in contrast to the biomimetic cyclization reaction, OsKSL4:T696I predominantly produces the more complex rearranged tetracycle. Therefore, the mutant enzyme does exert significant control over the catalyzed reaction.

The inert nature of the aliphatic residue associated with formation of more complex products, both here and elsewhere,<sup>3e,g</sup> indicates the rationale for specificity of the inferred reaction mechanism lies elsewhere. In particular, secondary (tetra)cyclization requires that the  $\pi$  bond of C16 attacks the carbocation of the appropriate pimaranyl<sup>+</sup> intermediate (here C9 of the pimar-15-en-9-yl<sup>+</sup> arising from 1,2-hydride shift after initial (tri)cyclization — see Scheme 1). Clearly, these diterpene synthases dictate substrate conformations that promote such proximity of reacting carbons. In addition, as indicated by our previous results,<sup>3e,g</sup> the pyrophosphate coproduct from initiating ionization also may influence enzymatic reaction mechanisms in providing a stabilizing counterion to assist the energetically unfavorable tertiary to secondary carbocation transition occurring during (tetra)cyclization. Thus, our cumulative results provide strong evidence for the hypothesis that the major role of terpene synthases is to fold their substrate and then trigger ionization, simply providing a solvent-shielded template for the ensuing series of carbocation intermediates,<sup>10</sup> while additionally highlighting a role for the released pyrophosphate in driving the reaction toward intermediates wherein the carbocation is located proximal to this anionic coproduct. This provides an integrated view of terpene synthase catalysis that may assist rational engineering of these fascinating and important enzymes.

Beyond the mechanistic implications, the profound plasticity exhibited by diterpene synthases is consistent with the screening/diversity-oriented hypothesis of natural products metabolism.<sup>11</sup> Specifically, our results, both here and elsewhere,<sup>3e,g</sup> provide concrete examples of the ability of key single residue changes, requiring substitution of only a single nucleotide in each case, to quantitatively switch product outcome in these critical biosynthetic enzymes.

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**Supporting Information Available:** Methods and data from structural analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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