

Mechanistic investigations of class II diterpene cyclases

by

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Chapter I. Introduction

Terpenoids form the largest class of natural products with over 55,000 known. These compounds are particularly widespread in plants where they play vital roles in defense signaling or growth and development. These terpenoid natural products have also been found to be pharmacologically active. Terpenoids begin with the 5-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These 5-carbon building blocks are synthesized through either the mevalonic acid (MEV) pathway which proceeds from acetyl-CoA and is present in the cytosol, or the 2-methylerythritol-4-phosphate (MEP) which begins with the condensation of pyruvate and glyceraldehyde-3-phosphate and is found in plant plastids (Rohmer 2008, Christianson 2006). These 5-carbon isoprenoids then undergo 1'-4 condensation reactions with allylic prenyl diphosphates via isopentenyl diphosphate synthases (IDSs) to form longer chain terpenoids (Crick et al. 2000) in increments of five carbons including, monoterpenes (10-carbon), sesquiterpenes (15-carbon), diterpenes (20-carbons), and triterpenes (30-carbons). These longer chain terpenoids will undergo cyclization reactions to form a very diverse library of hydrocarbon backbones with ring structures.

Labdane-related diterpenoids

The Labdane-related diterpenoids (LRDs) which proceed from the dual cyclization of the 20-carbon (*E,E,E*)-geranylgeranyl diphosphate (GGPP) are the focus for this thesis project. The LRDs comprise a vast group of the terpenoid natural products, over 12,000 are known and are involved in the metabolism of plants in pathways such as the biosynthesis of gibberellin phytohormones (Yamaguchi 2008). The LRDs all contain a core decalin ring structure obtained upon the initial cyclization of GGPP by a class II diterpene cyclase to a

labdadienyl/copalyl diphosphate (CPP). Depending on the enzyme and the pro-chiral conformation it enforces onto the GGPP, there can be a variety of stereoisomers of CPP. The three most widely known stereoisomers are *normal-*, *ent-* and *syn-*CPP (Figure 1). *Syn-ent-*CPP has not been observed, but the stereochemistry has been shown in natural products from the *Calceolaria* genus of plants (Garbarino et al. 2004). The resulting stereoisomers of CPP can be further cyclized through an ionization-initiated mechanism catalyzed by a class I diterpene cyclase where the pyrophosphate group is ionized with a Mg^{2+} ion and subsequent cyclization occurs, resulting in class I products such as the abietadienes, kaurenes, pimaradienes, and others (Peters 2010).

The diterpene cyclases

The class II diterpene cyclases contain a highly conserved catalytic DXDD motif (Christianson 2006). This catalytic motif sits on an alpha helix deep in active site cleft pointing directly in to the cleft space (Figure 3). This motif has been studied in the class II diterpene cyclase in *Arabidopsis thaliana* (AtCPS) which catalyzes the production of *ent*-CPP. It was found that mutating either the first or middle aspartate in the motif had significant effects on the catalytic activity, with a much larger decrease with the mutation of the middle Asp than the first Asp. It was also hypothesized that the last Asp is required to activate the middle Asp for catalysis (Prisic et al. 2007). The same effect is seen when mutating the middle Asp in the DXDD motif of AgAS (Peters et al. 2001). This AgAS mutant D404A, which abolishes class II activity, is used throughout the rest of this thesis projects for several control experiments.

The class I diterpene cyclases have a similar conserved DDXXD motif and another less conserved (N,D)DXX(S,T)XXE motif (Zhou and Peters 2009). These motifs are

assumed to be involved in binding Mg^{2+} which is then used to ionize the pyrophosphate group on CPP. After the pyrophosphate group is ionized subsequent cyclizations can occur which give rise to further varied products. This reaction can also proceed without a subsequent cyclization and simply use a direct deprotonation after removal of the pyrophosphate. There can also be further variations such as methyl shifts to give rise to a large variety of compounds produced by class I diterpene cyclases (Peters 2010). It has been shown in AgAS that conversion of the first Asp621 of the DDXXD motif to Ala the class I activity is effectively abolished (Peters et al. 2001). This D621A mutant of AgAS, like the D404A mutant was used throughout this thesis project for control experiments.

Class II diterpene cyclases

There is still much not understood about the class II diterpene cyclase mechanism even though the catalytic motif has been identified. For instance, these enzymes seem to contain some type of inhibitory regulation that is dependent on the concentration of Mg^{2+} present. *In vitro* assays show AtCPS exhibits biphasic kinetic properties in which the absence of Mg^{2+} and the presence of exceeding Mg^{2+} activity is not present or begins to decrease. However this property is not observed in AgAS. Alignments show a conserved His in *ent*-CPP cyclases like AtCPS, but in diterpene cyclases that produce *normal*-CPP this residue is conserved as an Arg. When these residues are switched between AtCPS and AgAS, the biphasic kinetic property is observed in AgAS and AtCPS retains activity in excess Mg^{2+} (Mann et al. 2010). It was originally thought that this Mg^{2+} inhibition was using a direct effect on the catalytic DXDD motif. However that hypothesis has changed after the crystal structures of AtCPS and AgAS were determined showing those residues as being pointing out of the active site cleft. The mechanism of this inhibition is now currently not

well understood, but it is thought that upon the action of Mg^{2+} binding to His331 causes a rumple in the active site cleft disrupting the spacing of GGPP with the DXDD motif.

These class II diterpene cyclases contain putative aromatic boxes in the active site cleft that form around the middle Asp in the catalytic DXDD motif (Figure 3). It is currently unclear what the role is of these aromatic residues, but they are assumed to assist the middle catalytic Asp in the protonation of GGPP. Because this initial step requires a large amount of energy the enzyme needs to have some system to force the GGPP into close proximity to allow the protonation to happen. It is not quite clear exactly how this happens; either the aromatic box interacts with the π -electrons of GGPP or just sterically forces it into position. But, it is possible that when Mg^{2+} binds to His331 in AtCPS, the rumple in the active site mentioned above could potentially be the disruption of this aromatic box rendering the enzyme energetically unable to protonate GGPP.

There is also a conserved counter ion in the class II diterpene cyclases that is positioned very near to the catalytic Asp (Figure 3). This residue is thought to help stabilize the middle Asp via hydrogen-bonding. This residue is conserved as an Asn in plant class II cyclases, but in bacterial class II cyclases it is conserved as a His. Mutational analysis on this Asn in AgAS caused a 100-fold decrease in catalytic activity suggesting the counter ion as having a catalytic role (Zhou et al. 2012). The difference in conservation between the plants and bacteria is not well understood either, but further understanding on this difference in counter ion could give insights into the evolution of the class II cyclases. This counter ion could also be affected by the Mg^{2+} inhibition mentioned above.

Class II diterpene cyclases also use a catalytic base in order to deprotonate C-8 during catalysis (Scheme 1). Wendt proposed earlier for triterpene cyclases that this

deprotonation would need to occur via a His or water molecule (Wendt 2005). Other work done on a Copal-8-ol diphosphate synthase in *Cistus creticus* suggested either a water molecule or hydroxyl anion must be used for deprotonation (Falara, Pichersky and Kanellis 2010). It is not completely certain what residue, in general, would be used as a general catalytic base for these enzymes. However, data in this thesis project seems to suggest that these enzymes have a His that serves as this base which fits the model that Wendt suggested.

There have been two crystal structures (Figure 2) determined to date for these diterpene cyclases, AgAS and AtCPS (Köksal et al. 2011). The crystal structures show a high amount of structural homology between the two enzymes, both having $\gamma\beta\alpha$ domains, although the α domain of AtCPS is inactive. Almost all of the diterpene cyclases contain these three domains. However, only in the bifunctional cyclases such as AgAS are all three domains active. In class II diterpene cyclases, the α domain is inactive, whereas in the class I cyclases the γ and β domains are inactive. These crystal structures have given insights into the structure-function relationships of these enzymes and have served as the foundation to the research for this thesis project.

Thesis Outline

For this thesis project I examined the structure-function relationships of class II diterpene cyclases using a site-directed mutagenesis approach. I experimented with the His as a possible catalytic base in AtCPS, AgAS, and a class II diterpene cyclase in *Oryza sativa* (OsCPS4) which catalyzes the production of *syn*-CPP. To date, I was able to show that mutating the relevant His in both AtCPS and AgAS changes the product outcome of both of these enzymes with the mutant versions catalyzing the production of a novel hydroxylated form of CPP in their respective stereo isomeric forms. Also, I have made some intriguing

initial observations on a Tyr-His dyad in AgAS (Figure 3). All *normal*-CPP synthases have a conserved Tyr, or in a few cases Phe, in the active site cleft. Mutating the relevant Tyr in AgAS to a Phe changed the product outcome from *normal*-CPP to *syn*-CPP which was quite intriguing, but more work in this area needs to be done before it is clear why such a minute change in the active site cleft causes the production of a different stereoisomer of CPP.

This thesis is set up to show my progress throughout my research project and to serve as a backbone of manuscripts for the eventual publication of my results. Chapter II is going to be a manuscript of a communication paper to be submitted to *Angewandte Chemie*. Chapter III is a manuscript of results following up on the chapter II manuscript and addition of new data to form an article to eventually be submitted to a scientific journal. Chapter IV will be a short manuscript on some new data that will serve as a basis for a new project in the lab after I leave. And chapter V will be a general conclusion summarizing what I accomplished in my research project, future directions, and acknowledgements.

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Scheme 1. General cyclization of GGPP with class II and class I diterpene cyclases.

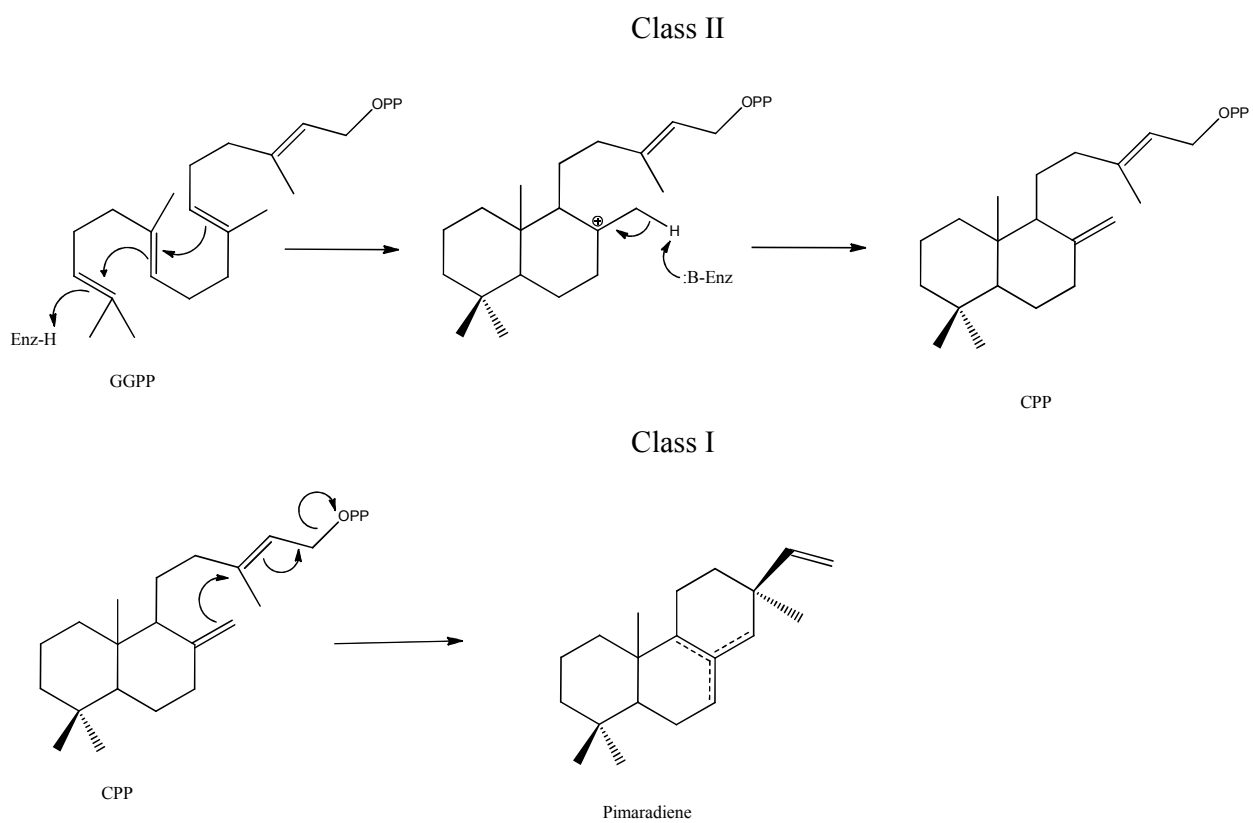


Figure 1. Structures of the different stereoisomers of CPP and the carbon number assignments.

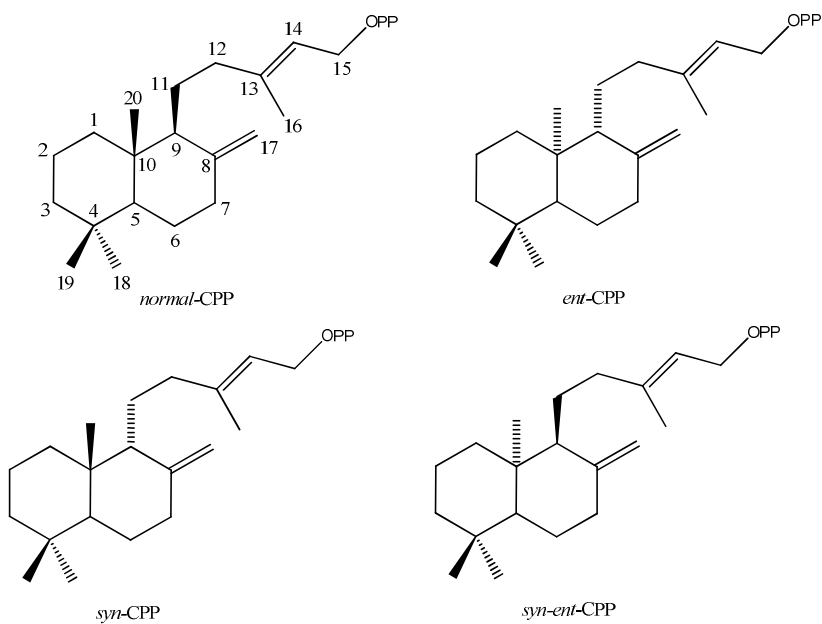


Figure 2. *Crystal structures of AgAS and AtCPS*

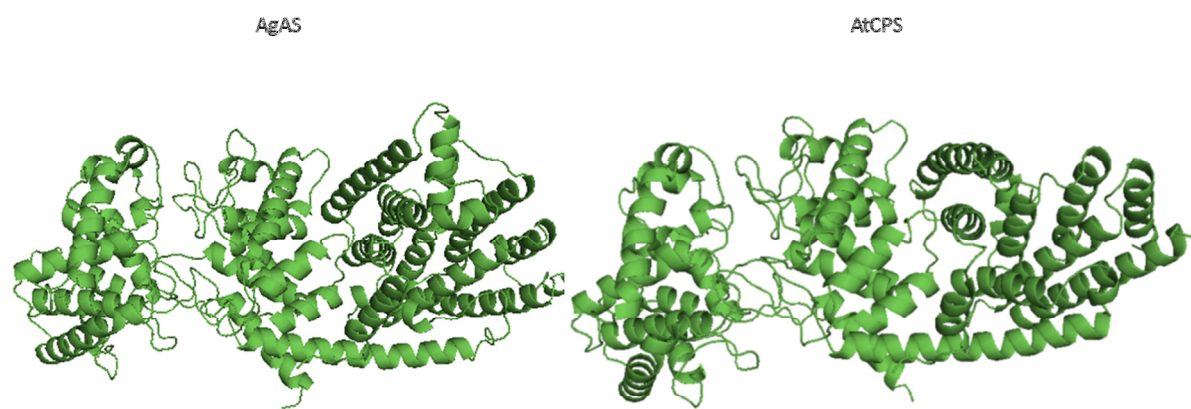
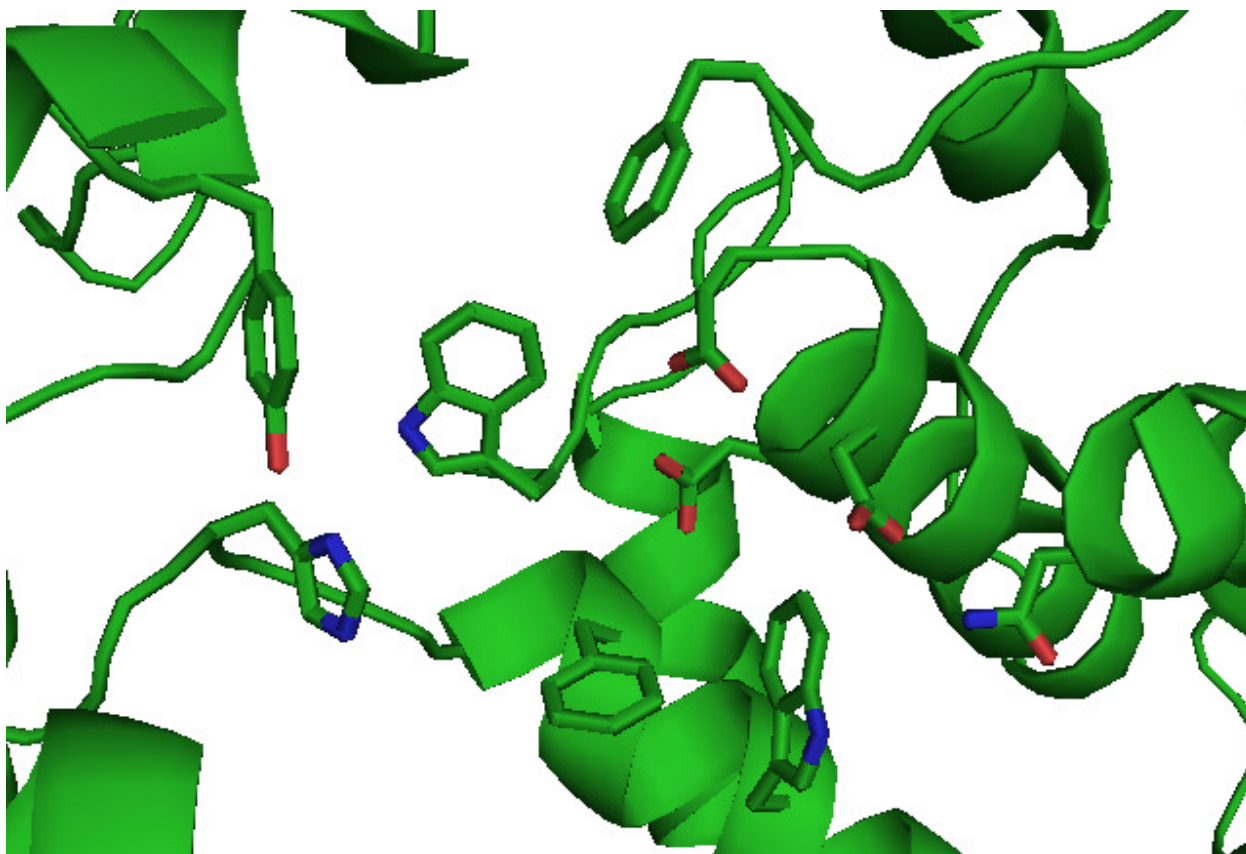


Figure 3. Class II active site of AgAS showing the DXDD motif, aromatic box, and Y-H dyad.



Chapter II. Changing the product outcome of a class II diterpene synthase in *Arabidopsis thaliana* via a single residue switch

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Diterpene synthases catalyze complex cyclization/rearrangement reactions resulting in hydrocarbon scaffolds that are lead compounds in an enormous class of natural products, of which over 50,000 are known. The largest class of these diterpenoid natural products is the labdane related diterpenoids (LRDs). LRDs can proceed via the class II protonation-initiated dual cyclization and subsequent deprotonation of (E,E,E)-geranylgeranyl diphosphate (GGPP) resulting in a specific stereoisomer of labdadienyl/copalyl diphosphate (CPP). This primary bicyclic structure can undergo further rearrangement and/or cyclization via the class I diphosphate ionization initiated reaction.⁴ The protonation-initiated cyclization in class II diterpene cyclases depends on a catalytic DXDD motif with the middle Asp acting as the catalytic acid that protonates GGPP (scheme 1).^{1,5}

Crystal structures for the class II *Arabidopsis thaliana* diterpene cyclase (AtCPS)³ involved in gibberellin phytohormone biosynthesis and the bifunctional class II/I *Abies grandis* abietadiene synthase (AgAS) involved in more complex resin-acid biosynthesis have recently been determined and have given prolific insights into the complexities of the catalytic mechanisms of these enzymes. For instance, these enzymes have homologous structural motifs and conserved aromatic residues contained in the cleft of the active site.

Here we demonstrate that a substitution of an Ala for a conserved active site His (Figure 1A) in AtCPS has a striking effect on the product output of the enzyme. The mutant enzyme catalyzes the production of a novel hydroxylated version of *ent*-CPP. This result provides some marked insights into the structure-function relationships of class II diterpene cyclases, specifically the role of the relevant His in their active sights and their ability to incorporate water into the reaction.

Previous work has shown a diterpene cyclase in the angiosperm *Cistus creticus* to produce a novel oxygen containing LRD. It was found that the purified enzyme in *Cistus creticus* (CcCLS) when combined with GGPP *in vitro* produced a novel labda-13-ene-8 α -ol diphosphate (copal-8-ol diphosphate) or labda-13-ene-8 α ,15-diol upon dephosphorylation.² It has been shown previously that incubation of this copal-8-ol diphosphate with recombinant AgAS will result in manoyl oxide epimers.^{6,2}

Upon examination of the crystal structure of AtCPS and the alignment showing a His as well conserved among diterpene cyclases which produce *ent*-CPP, we hypothesized that the His served as the catalytic base for the proposed mechanism (Scheme 1a). Wendt proposed earlier that a His or water molecule was responsible for deprotonation in an oxidosqualene cyclase.^{10,11} Depending on the pro-chiral conformations the active sight forces on GGPP proposed earlier⁴, we had hypothesized this catalytic base would need to be in a position that would be in close proximity to C-8 of GGPP allowing for deprotonation and that position would be specific to the conformation GGPP adopts in the active site cleft. In the crystal structure, (Figure 1B) His263 is shown to be positioned in the active site far enough away from the catalytic aspartate motif proposed to be responsible for protonation of

GGPP⁵, allowing it to potentially be positioned near C-8 making it a probable candidate for the catalytic base in the enzyme. Furthermore, in the surrounding area of His263, there are no other residues that could very well serve as a catalytic base. Therefore, we hypothesize that this residue, His263, is acting as the catalytic base for deprotonation of the labda-13-en-8-yl⁺ intermediate (Scheme 1).

This His263 is well conserved among class II diterpene cyclases involved in gibberellin phytohormone biosynthesis (Figure 1A). Therefore, assuming this His263 was the catalytic base and is well conserved, we converted this residue to an Ala via site-directed mutagenesis, as described earlier⁷, on AtCPS to acquire an AtCPS H263A mutant. This mutant was then transferred to a pGG expression vector via directional recombination giving a dual expression vector for GGPP and AtCPS H263A. This was then transformed into C41 expression cells and subjected to our *E.coli* metabolic engineering system described earlier⁸. This same experiment was done with wild-type AtCPS. Because we hypothesized His263 as being the catalytic base we expected all catalytic activity to be terminated. However, AtCPS strikingly catalyzed the production of an entirely different product with a later retention time on GC-MS analysis (Figure 2). The same experiment was then performed with the class II diterpene cyclase from *Nicotiana glauca* (NgCPS) which catalyzes a copal-8-ol diphosphate having the normal (9S, 10S) stereochemistry. Astonishingly, we found the AtCPS H263A mutant to be catalyzing the same product as NgCPS. Presumably, this is due to addition of a water molecule or a hydroxyl anion (scheme 1B) as suggested earlier.² The GC-MS analysis showed the major product peak for AtCPS H263A having a much later retention time than wild-type AtCPS, but had the same retention time as the major product from NgCPS (Figure

2). The GC-MS data also showed similar fragmentation patterns for both NgCPS and AtCPS H263A products, indicating that AtCPS H263A was catalyzing the formation of the same version of copal-8-ol diphosphate as NgCPS (supplemental data). However, we hypothesized that the AtCPS H263A still retained the (9R, 10R) stereochemistry, as mentioned before,⁴ catalyzing the production of *ent*-copal-8-ol diphosphate (1) since AtCPS is an *ent*-CPP synthase, whereas NgCPS catalyzes a *normal*-copal-8-ol diphosphate. Metabolic engineering experiments were then done with NgCPS and AtCPS H263A being co-transformed with AgAS D404A, a mutant that abolishes class II activity in AgAS.⁵ These experiments showed conversion to epimers of manoyl oxide with NgCPS, but not with AtCPS H263A (supplemental data). This further confirmed the stereochemistry of the mutant AtCPS H263A product as being *ent*-copal-8-ol diphosphate (Scheme 1B). We also performed metabolic engineering experiments with AtCPS H263A and an *ent*-kaurene synthase from *Cucurbita maxima* (CmKS) and this showed the production of *ent*-manoyl oxide (supplemental).

The catalytic activity is affected by this mutation, as would be expected if His263 is the catalytic base. We performed *in vitro* assay experiments on AtCPS H263A and NgCPS as described earlier⁹ with a few changes to the extraction process as discussed in the supplemental material. We used 300nM enzyme and 20uM substrate in an overnight assay, AtCPS H263A only converted 3.79% of GGPP into product. This low amount of turnover is presumably due to a much more delicate deprotonation mechanism and possibly some steric hindrance in positioning a water molecule within a feasible distance from C-8 for hydroxylation in AtCPS H263A.

As to date, we have not yet been able to fully characterize this mutant with k_m and k_{cat} parameters. But, because this mutant enzyme was re-engineered to catalyze a hydroxylated version of its wild-type counterpart, it was no real surprise that the rate of catalysis was affected so dramatically. The mechanism for positioning a water molecule or a hydroxyl anion requires a larger number of events than having just the basic His in place to deprotonate C-8 and form *ent*-CPP. It is possible if the His263 is acting as a catalytic base and is taken out then a chain of water molecules may need to be employed to provide a route for the proton to be transferred until it reaches a true proton acceptor. This would be a rate-limiting step for the reaction.

We will want to further study this residue in AtCPS by substituting in other relevant residues such as, Gly, to see if that data correlates with our current data further enforcing our hypothesis that the His is the catalytic base and the Ala mutant is deficient in deprotonation, but decreases the steric hindrance in the active site in order to incorporate a water molecule or hydroxyl anion to position itself in close proximity to C-8. Also, we will search out relevant His residues in other class II diterpene cyclases which are involved in the biosynthesis of other natural products to understand if this proposed catalytic base residue is true for *syn*-CPP and *normal*-CPP synthases. If we find that our hypotheses are correct this will provide a much clearer understanding of how the class II diterpene synthases catalyze the formation of CPP, specifically in reference to which residue acts as the catalytic base, which, from our experimental results, we propose to be a His.

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Supporting information available: Methods and data from structural analysis.

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Figure Legends

Figure 1: A) An alignment showing *ent*-CPP synthases and the relevant conserved His. B) a picture from the crystal structure of AtCPS³ showing the position of His263 in relation to the catalytic DXDD motif found among all class II diterpene cyclases.

Figure 2: GC-MS chromatograms of wild-type AtCPS, AtCPS H263A, and NgCPS. 1=*ent*-CPP, 2=*ent*-copal-8,15-diol, 3=*normal*-copal-8,15-diol.

Scheme 1. Cyclization of GGPP by wild-type AtCPS and AtCPS H263A

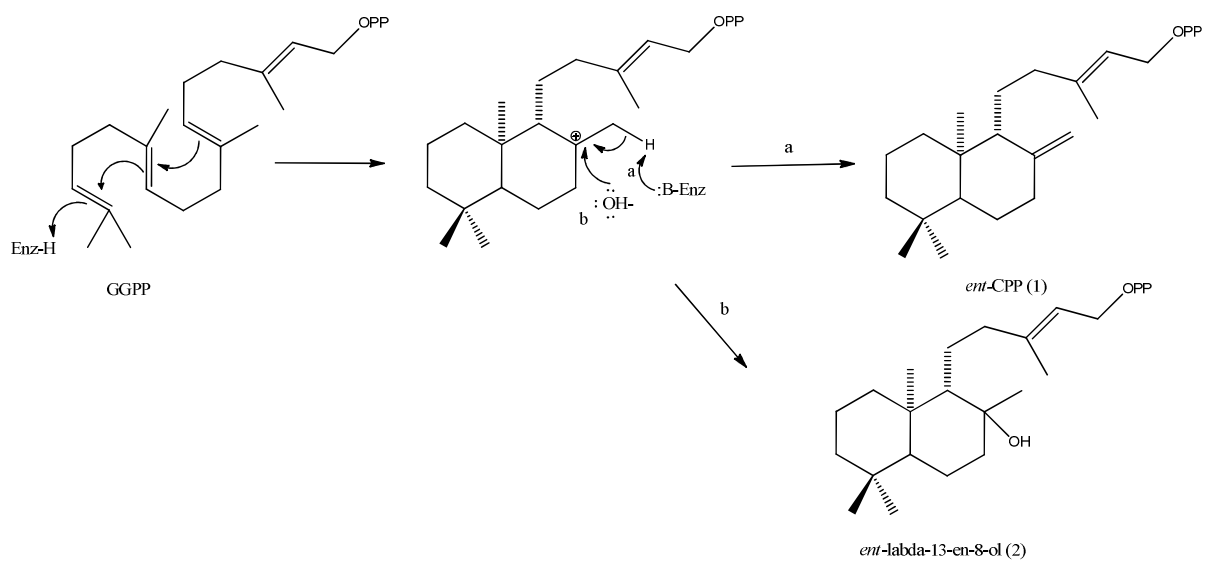


Figure 1.

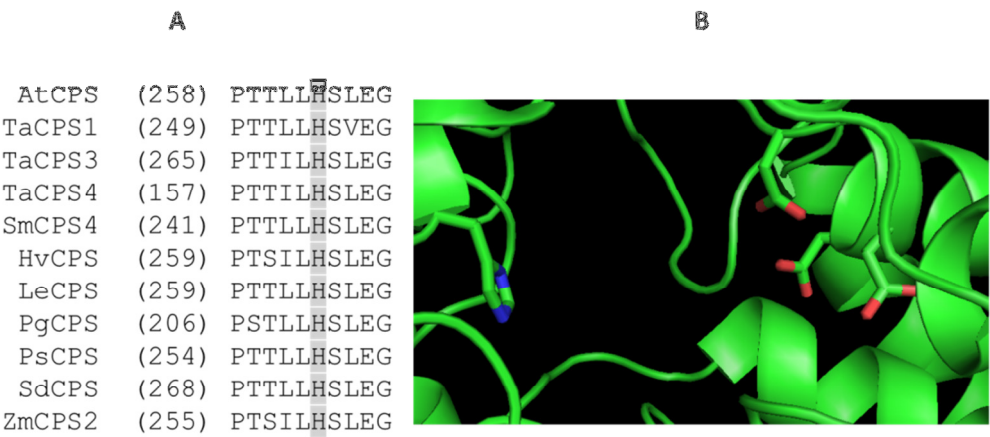
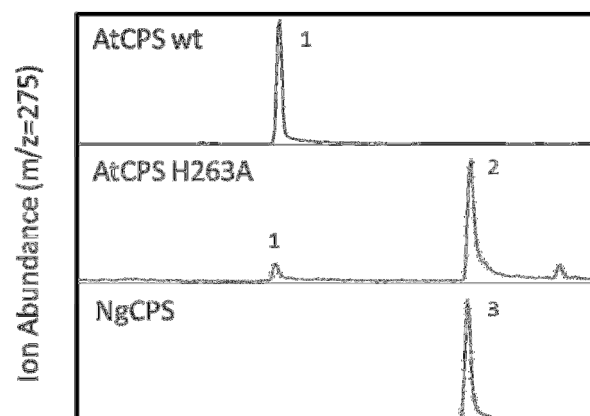
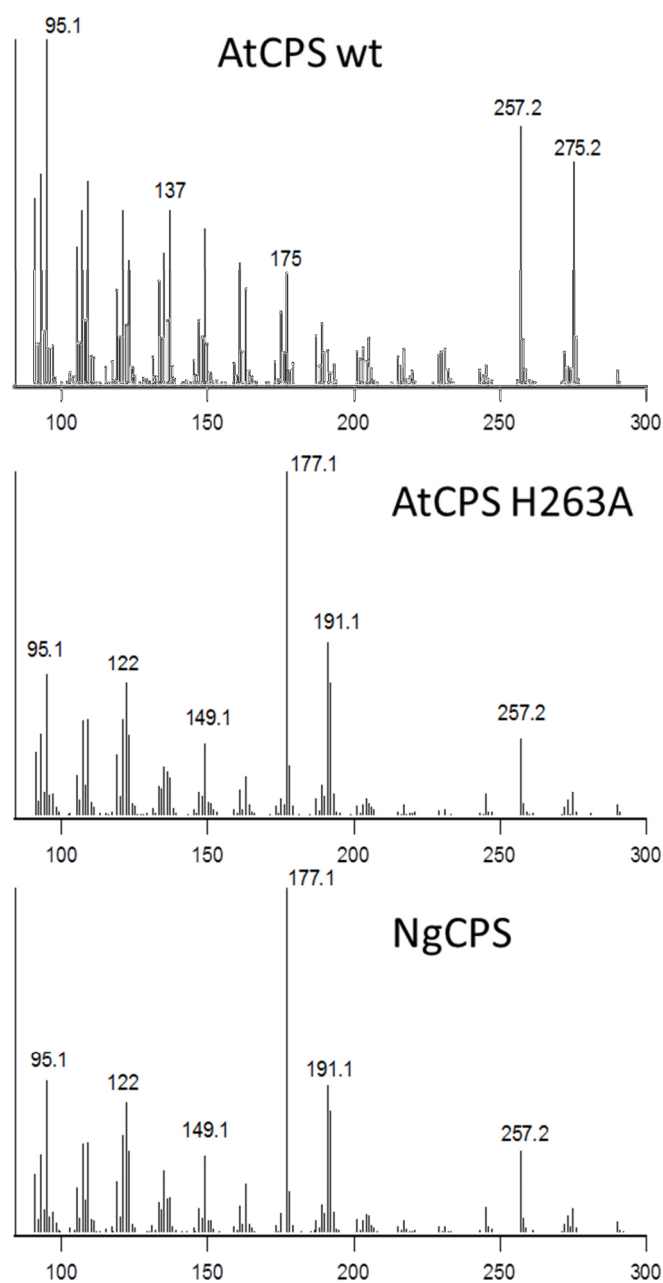


Figure 2.

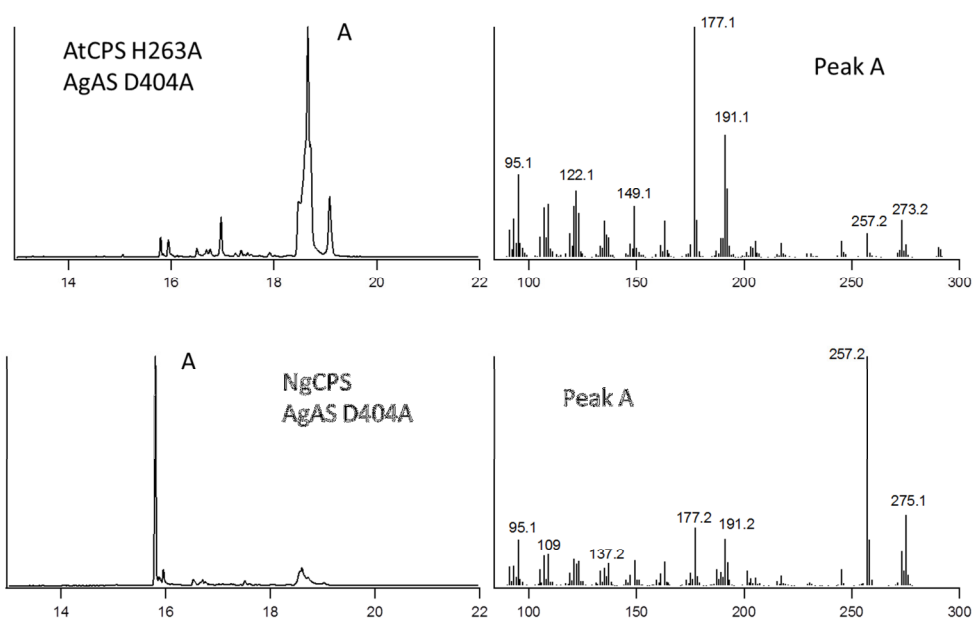
Supplemental Information

Supporting Data

Mass Spectra of AtCPS, AtCPS H263A, and NgCPS peaks corresponding to the major product from Figure 2.

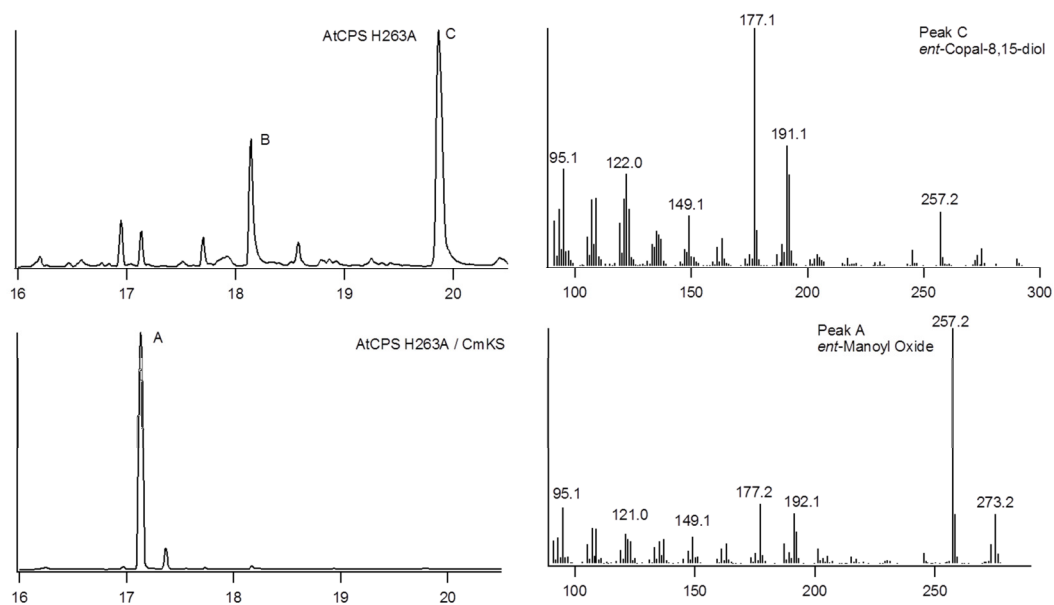


AtCPS H263A and NgCPS were also coupled with AgAS D404A in our *E. Coli* metabolic engineering system to further suggest that AtCPS H263A product retained *ent* stereochemistry. Here are the chromatograms and mass spectra of the two experiments.



This shows that with the AtCPS H263A/AgAS D404A experiment there was no turnover by AgAS D404A, whereas with NgCPS/AgAS D404A there was turnover by AgAS D404A to form manoyl oxide as a product.

AtCPS H263A was also coupled with CmKS in our metabolic engineering system. The *ent*-CPP accepting CmKS took the *ent*-copal-8-ol diphosphate from AtCPS H263A and formed *ent*-manoyl oxide. This allowed us to conclude that the mutant was still producing product with *ent* stereochemistry.



Materials and Methods

Metabolic Engineering

All chemicals were purchased from Fischer Scientific (Pittsburgh, PA) unless otherwise noted. For the metabolic engineering plasmids from AtCPS, AtCPS H263A, and NgCPS in the pENTR vector were cloned into the pGG expression vector which codes for GGPP synthase. These pGG plasmids were co-transformed into C41 *E.Coli* cells with the pIRS plasmid as well which contains the enzymes involved in the MEP pathway^[1]. AgAS D404A was cloned into the pDEST14 expression vector and co-transformed with the other mutants for the coupled experiments described above. These were grown in 40mL of TB media to OD 0.6-0.8. Then cooled to 16°C and induced to final concentrations of Isopropyl β -D-1-thiogalactopyranoside (IPTG) (0.5mM), Phosphate buffer (0.1M), MgCl₂ (1mM), and pyruvate (50mM). These were grown at 16°C for three days then 50mL of hexanes were added and the mixture was shaken and allowed to sit overnight at 4°C. The hexane layer was extracted and dried under N₂. The dried sample was re-suspended in 100uL of fresh hexanes and was ran on a varian 3900 gas chromatograph with a Saturn 2100T mass spectrometer and injected onto an HPMS5 column with a CP-8400 auto sampler. Data was processed into figures using Igor pro 6.20 technical graphing software.

Enzymatic Assays

For enzymatic analysis all GGPP was purchased from Isoprenoids, Lc. (Tampa, FL). The GGPP was purchased as the trisammonium salt as 1mg/mL in a solution of Methanol/10mMNH₄OH. Calf intestinal alkaline phosphatase (CIP) was purchased from

New England Biolabs, Inc. (Ipswich, MA). All cloning, purifications, assays, and data analysis were carried out as described before^[2] with some modifications to the extraction procedure. In the case of AtCPS H263A, after dephosphorylation the product contains two hydroxyl groups. We used ethyl acetate for extracting the product for its increased polarity as opposed to hexanes. Each sample was extracted three times with approximately 1mL of ethyl acetate. These were then dried under N₂ and re-dissolved in 50uL of methanol. These samples were then run on an Agilent 6890N Network GC System with an HPMS1 column and a 7683 Series Injector.

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Chapter III. Investigating the role of a His as possible catalytic base in diterpene cyclases

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Abstract

Class II diterpene cyclases catalyze the protonation-initiated dual cyclization of (*E,E,E*)-geranylgeranyl diphosphate (GGPP). This reaction proceeds through a labda-13-en-8-yl⁺ intermediate which is subsequently deprotonated into a labdadienyl/copalyl diphosphate (CPP) by a general base or water (scheme 1a). Here, we investigated His348 in Abietadiene synthase from *Abies grandis* (AgAS) as the possible general base. We converted this His to an Ala and found that the product outcome changed to a *normal*-copal-8-ol diphosphate. This result is similar to His mutant found earlier in *Arabidopsis thaliana* ent-CPP synthase (AtCPS) and further suggests that these class II diterpene cyclases use a His as the general base for deprotonating the labda-13-en-8-yl⁺ intermediate.

Introduction

Terpenoids are synthesized via the five-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). An isoprenyl diphosphate synthase, or prenyltransferase, utilizes these five-carbon precursors to catalyze the production of longer-chain terpenoids such as geranyl diphosphate (C-10), farnesyl diphosphate (FPP) (C-15), and GGPP^[1]. These longer chain terpenoids act as substrates for terpene cyclases, and though the

number of these linear terpenoid substrates is limited, they can be cyclized and rearranged to form a backbone of precursors to the production of a vast range of natural products^[2].

The thirty-carbon terpenoid, squalene, is formed by the condensation of two FPP molecules via a squalene synthase^[3]. This can be further cyclized by protonation-initiation into hopene by a squalene-hopene cyclase (SHC)^[4]. SHC's contain a conserved catalytic DXDD motif which was shown through site-directed mutagenesis to be essential for catalysis^[5]. This DXDD motif is thought to be responsible for protonating the terminal double bond of squalene to initiate polycyclization into hopene. Wendt proposed later using crystallographic data of SHC from *Alicyclobacillus acidocaldarius*^[6] that the middle Asp in the motif was responsible for protonation and there was a His close by that increased acidity of the middle Asp^[7]. Wendt also earlier reported a His232-Tyr503 base dyad in oxidosqualene-lanosterol cyclase (OSC) and suggested that this was responsible for deprotonating the lanosterol carbocation^[8].

Diterpene cyclases have been suggested to be descended from SHC's. There is structural homology among both SHC's and diterpene cyclases^[9]. For instance, the recently obtained crystal structures from the *Abies grandis* bi-functional diterpene cyclase (AgAS)^[10] which produces *normal*-CPP then subsequently abietadiene, and AtCPS^[11] both show conserved γ , β , and α domains. SHC's contain the γ and β domains similar to all class II diterpene cyclases. Diterpene cyclases catalyze complex cyclization and rearrangement reactions producing lead compounds important for the biosynthesis of natural products. For instance, the biosynthesis of gibberellin phytohormones which are essential for growth and development in higher plants^[12] proceeds from the initial production of *ent*-CPP by a class II diterpene cyclase^[13].

The class II diterpene cyclases also contain a conserved DXDD motif^[2] in which the middle Asp is thought to protonate the terminal double bond of GGPP and the first and last Asp's increase the acidity of the first Asp^[14] like the SHC's. The class I diterpene cyclases contain a similar DDXXD motif where the Asp's act as Mg²⁺ binding sites to promote the ionization of the diphosphate moiety of CPP and initiate the class I reaction^[2]. Earlier mutagenesis studies on these motifs in AgAS showed two important mutants to use in order to split the class II and class I activity of the bifunctional enzyme. AgAS D404A abolishes class II activity and AgAS D621A abolishes class I activity allowing for specific use singly as a class I or class II enzyme respectively^[15]. After the protonation-initiated dual cyclization to the labda-13-en-8-yl⁺ intermediate, there is a subsequent deprotonation by a catalytic base or water molecule.

Earlier work on a class II diterpene cyclase in *Cistus creticus* (CcCLS) showed the production of a novel hydroxylated version of CPP^[16]. Even more recent work on a *cis*-abienol synthase from *Abies balsamea* also showed the production of a hydroxylated version of CPP in its class II activity. The subsequent class I activity then causes the ionization of the diphosphate moiety and subsequent deprotonation without any further cyclization leading to production of *cis*-abienol^[17].

Previously we have shown through mutational analysis that in AtCPS, mutating His263 in the active site cleft to an Ala produced the same hydroxylated version of CPP as CcCLS, but with opposite stereochemistry. This finding gave us some marked insights into the mechanism of these class II diterpene cyclases and the role of a His as a possible general base. AgAS also has a His348 that is across the active site cleft from the DXDD motif like AtCPS (Figure 1). Here we performed mutational analysis on His348 and found a similar

result to that of AtCPS H263A where AgAS D621A:H348A mutant catalyzed the production of *normal*-copal-8-ol diphosphate. This result reinforces our previous hypothesis that these class II diterpene cyclases use a His as the general base to deprotonate the C-8 carbocation of the labda-13-en-8-yl⁺ intermediate.

Experimental Procedures

General- all GGPP was purchased from Isoprenoids, LC (Tampa, FL). All molecular biology reagents were purchased from Invitrogen (Grand Island, NY) and all other chemicals, unless specified otherwise, were purchased from Fischer Scientific (Loughborough, Leicestershire, UK).

Enzymatic analysis- Site-directed mutagenesis was carried out via PCR amplification of pENTR (Gateway, Invitrogen) clones using overlapping primers. The acquired mutant genes were verified by complete sequencing then transferred to pDEST17, pDEST14, and/or pGG expression vectors. pDEST14 is an untagged expression vector and was only used with AgAS D404A. For product profiling, AgAS H348A, AgAS D621A:H348A, AtCPS H263G, NgCPS, and AtCPS H263I, AtCPS H263L, AtCPS H263V were all cloned in the pGG vector and were implemented into our previously described metabolic engineering system^[18]. His-tag purification was done with the pDEST17 vectors which encode a 6xN-terminal His-tag. This allowed purification using a nickel-nitrilotriacetic acid superflow resin (Thermo Scientific, Rockford, IL) and it was used according to the manufacturer's instructions resulting in the enzymes being >95% pure estimated by SDS-PAGE gel. AgAS D621A:H348A assays and percent conversion calculation were done as previously described^[19] with the assay samples being extracted three times with approximately 1mL of

ethyl acetate, dried with N₂, and re-dissolved in 50uL of methanol before GC-flame ionization detection.

Results

We mentioned previously that we wanted to explore additional mutants to AtCPS His263. We previously reported that we were particularly interested in making a Gly mutant at this position to see if that would correlate with our hypothesis that the reaction is being quenched by addition of a water molecule or possibly a hydroxyl anion after taking out the proposed base. We successfully made AtCPS H263G and found that it catalyzed the production of the same product as AtCPS H263A from the metabolic engineering experiments as according to GC-MS (Figure 2). Expanding on this study we also made Val, Leu, and Ile mutants of the His263 residue. In the metabolic engineering these mutants showed no measurable production of a copal-8-diphosphate or *ent*-CPP (Figure 3).

We superimposed the crystal structures of AtCPS and AgAS and noticed that His263 of AtCPS and His348 of AgAS are in quite similar positions (Figure 1). We hypothesized at this point that this His348 could be working as a base in AgAS similar to that of AtCPS. We performed site-directed mutagenesis on this residue to obtain AgAS D621A:H348A. This was implemented into our metabolic engineering system along with NgCPS and AgAS D621A and we found the AgAS D621A:H348A to be catalyzing the production of a copal-8-ol diphosphate (scheme 1b) (Figure 4). From the GC-MS analysis you can see that AgAS D621A:H348A also still manages to catalyze the production of trace amounts of CPP along with its major production of copal-8-ol diphosphate.

In order to determine if this mutant retained *normal* stereochemistry we co-transformed AgAS D621A:H348A with AgAS D404A in *E.Coli* and implemented those into

our metabolic engineering system. Previously it has been shown that mixing copal-8-ol diphosphate in an assay with AgAS D404A the resulting product is manoyl oxide^[20] (scheme 1c), which is what we saw in our experiment with the two enzymes together in the GC-MS analysis (Figure 5). The metabolic engineering experiment done with AgAS D621A:H348V and AgAS D621A showed the mutant as well as the pseudo wild-type enzyme catalyzing the production of the same product, *normal*-CPP (Figure 6).

In vitro assays were carried out for AgAS D621A:H348A using 300nM enzyme and 20uM substrate. AgAS D621A:H348A converted 37.49% of GGPP into *normal*-copal-8-ol, whereas AtCPS H263A, as previously stated only converted, 3.79% of GGPP into *ent*-copal-8-ol. As to date, we still have not been successful in obtaining full kinetic parameters for these enzymes. However, these numbers are consistent with the hypothesis that a His is acting as the catalytic basic residue, and they give insight into the enzyme's ability to incorporate a water molecule into the catalyzed reaction which presumably overtakes the initial protonation as the rate-limiting step.

Discussion

Class II diterpene cyclases catalyze the protonation-initiated bicyclization of GGPP which is the committed step in the biosynthesis of labdane-related diterpenoids. Class I diterpene cyclases catalyze the ionization-initiated reaction to produce further specialized compounds expanding on the variety of these products. There is still much to be studied on these diterpene cyclases. Here we have begun to show that the catalytic basic residue for these enzymes is likely to be a His that would be located near C-8 of bound GGPP. When looking at the crystal structures of AgAS and AtCPS it is interesting that His348 of AgAS and His263 of AtCPS are located in different positions in their respective active site clefts.

This is presumably due to the earlier suggested pro-chiral conformations which GGPP adopts upon binding in the active site of class II diterpene cyclases^[13]. GGPP takes on a chair-chair-“normal” pro-chiral conformation in AgAS, whereas in AtCPS, it adopts a chair-chair-“antipodal” pro-chiral conformation. These two different conformations would presumably put C-8 of GGPP in different positions making it a logical assumption that the catalytic basic residue would be in a different position in each type of enzyme. The results found here correlate with that assumption.

In order to further understand this hypothesis we would like to pursue the basic residue in one of the class II diterpene cyclases in *Oryza sativa* (OsCPS4) which catalyzes the production of *syn*-CPP, and further investigating these residues in the other class II diterpene cyclases as well. We logically assume that the catalytic basic residue would be in an even different position in OsCPS4 than the two described here since GGPP undergoes chair-boat”normal” pro-chiral conformation upon binding to the active site in order to catalyze the production of *syn*-CPP.

It is also interesting that the *in vitro* analyses for AgAS D621A:H348A and the previous analysis for AtCPS H263A show AgAS D621A:H348A as clearly being much more active indicating it has greater proficiency for allowing a water molecule in the active site. When looking at the crystal structures, there is a close by Tyr287 in AgAS that could be acting to facilitate the positioning of a water molecule, whereas in AtCPS, the closest residue is Val323. We would like to further study this discrepancy between the two enzymes by performing mutagenesis experiments on AgAS Tyr287, i.e. to Phe or His. And perform mutagenesis on AtCPS His263 and Val323, by studying whether a Thr could help recover

some catalytic activity with a close by hydroxylated side-chain possibly increasing the capacity of AtCPS to incorporate water into the catalyzed reaction.

It is very peculiar that AgAS D621A:H348V would still catalyze the production of *normal*-CPP. The results for AgAS D621A:H348A would suggest that the catalytic base is this His, but the result for AgAS D621A:H348V would suggest that His348 is not the specific base residue used in the catalyzed reaction. Even though the Ala mutant and other coupled experiments with the class I enzymes correlate nicely with the data obtained from AtCPS H263A it is more unclear, at least in the case of AgAS, which residue would be the base in the class II reaction. There seems to be something more complex happening in the case of AgAS than the seemingly simpler mechanism of AtCPS. We hypothesize here that the answer to that question must lie in the close-by Tyr287 and the two residues are working together to form a base dyad.

Acknowledgement

We would like to thank the NIH for funding this project with a grant to Dr. Reuben J. Peters.

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Scheme 1. A) production of *normal*-copalyl diphosphate through labda-13-8-yl⁺ intermediate. B.) production of *normal*-labda-13-en-8-ol diphosphate or copal-8-ol diphosphate through the labda-13-en-8-yl⁺ intermediate. C.) subsequent class I reaction producing manoyl oxide.

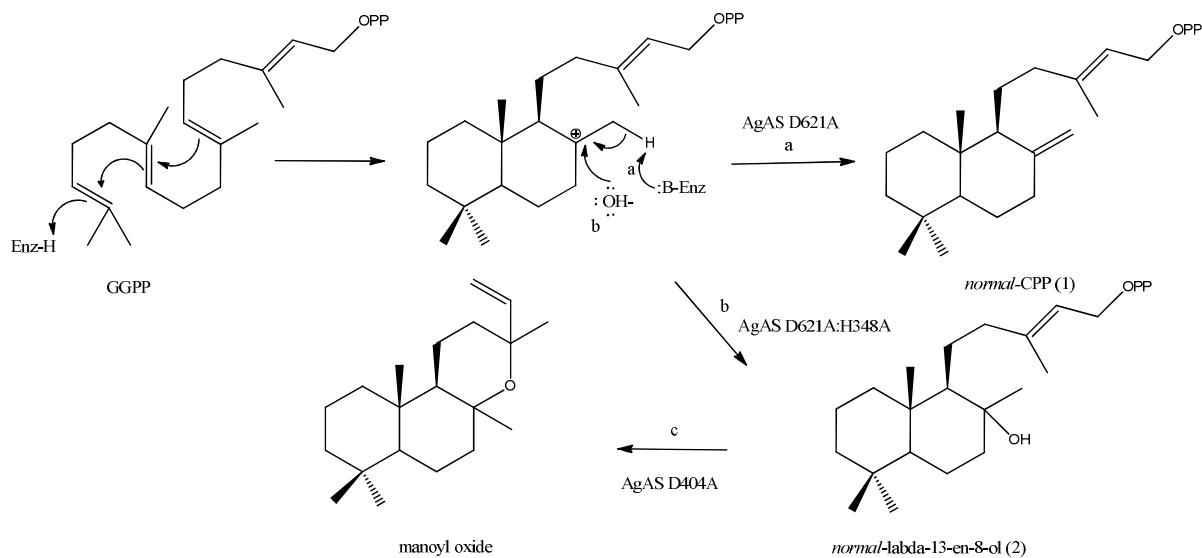


Figure 1. Overlapping crystal structure of AtCPS^[11] and AgAS^[10] showing His348 of AgAS and His263 of AtCPS.

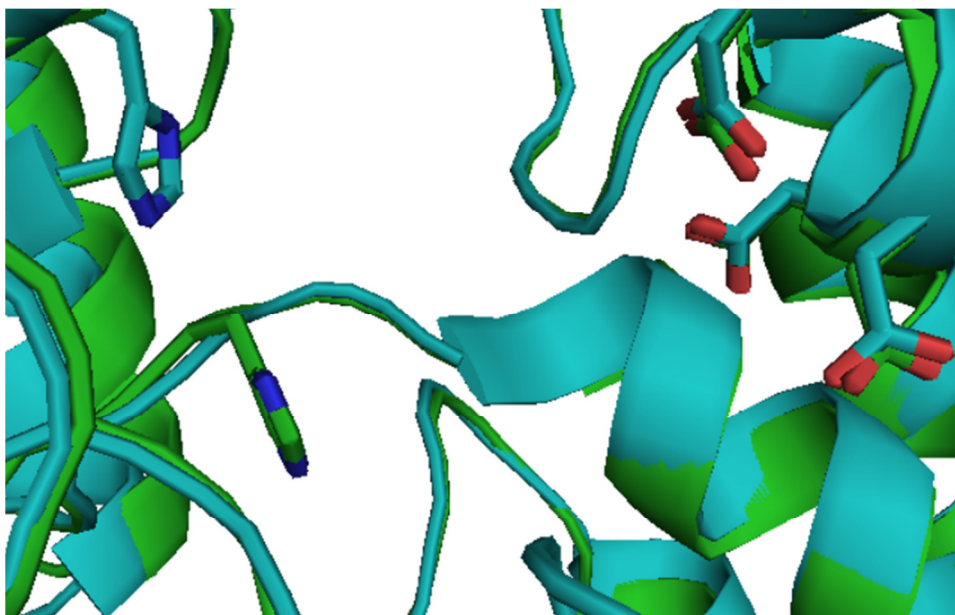


Figure 2. GC-MS analysis of AtCPS H263A and H263G with chromatograms on top and mass spectra on bottom. Peak A of both is geranylgeraniol (GGOH) and peak B of both is *ent*-copal-8,15-diol. The products were dephosphorylated prior to analysis.

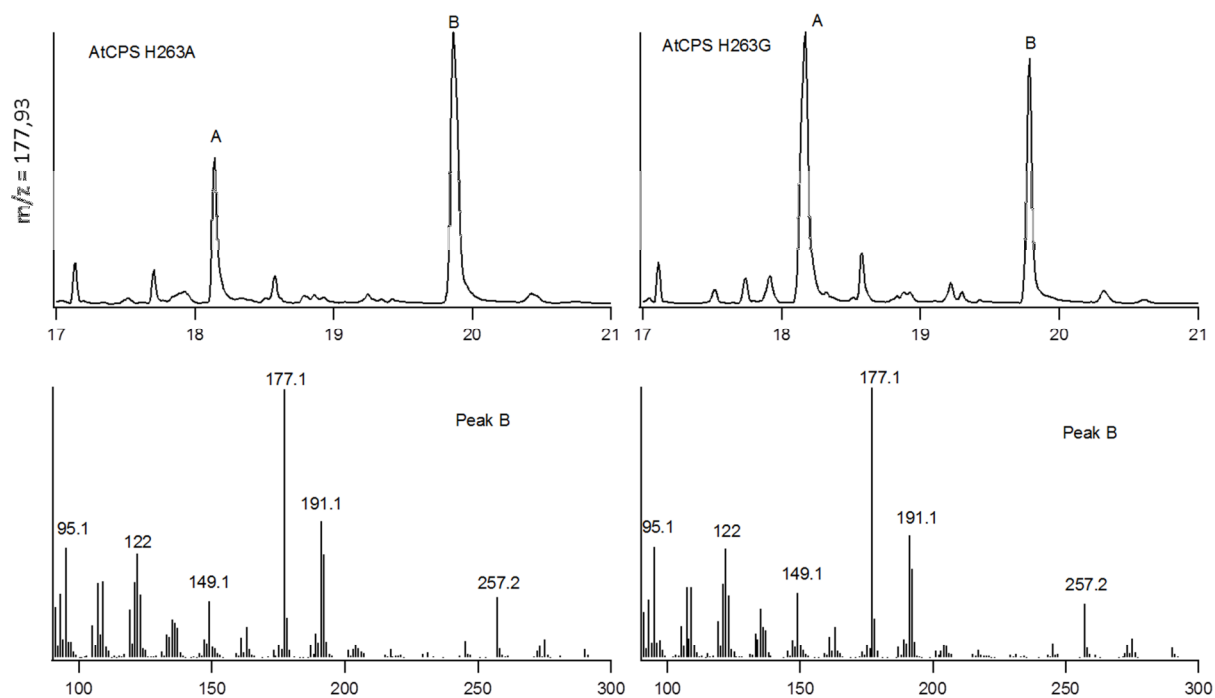


Figure 3. GC-MS analysis of AtCPS H263I, H263L, and H263V all with GGOH standards showing each mutant unable to catalyze the dual cyclization of any GGPP. Chromatograms on the left and mass-spectra on the right.

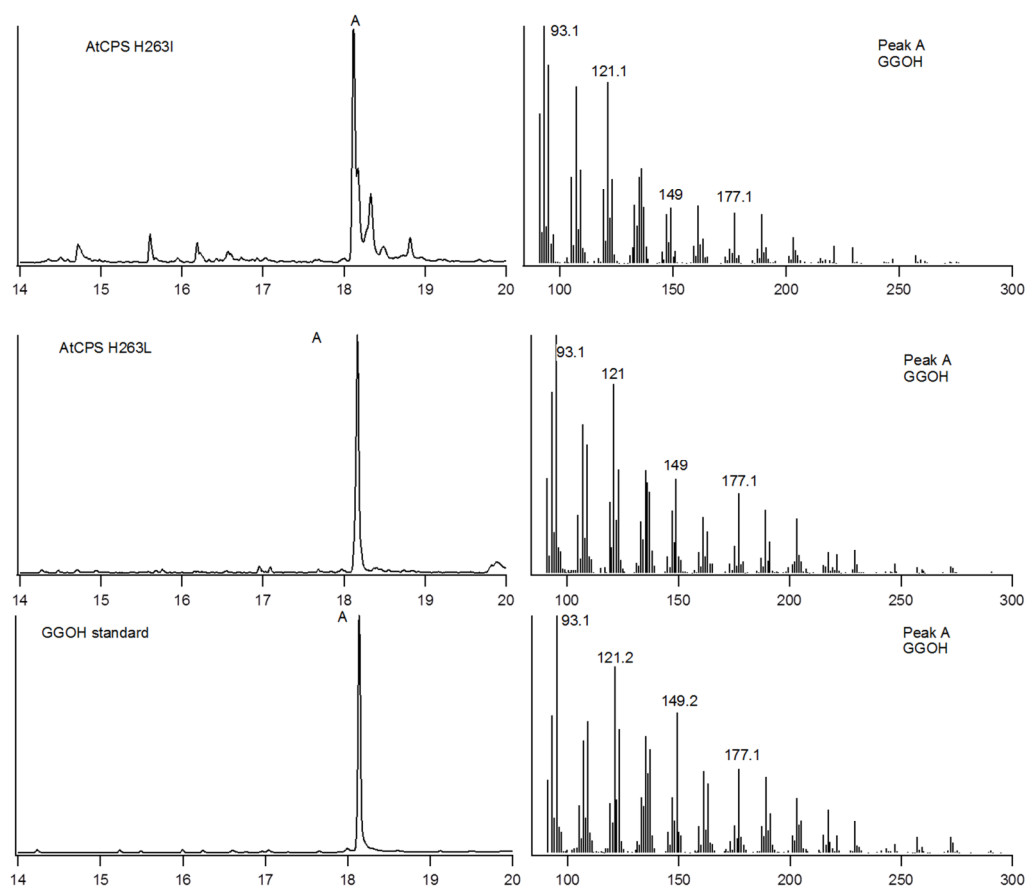


Figure 3. GC-MS analysis of AtCPS H263I, H263L, and H263V all with GGOH standards showing each mutant unable to catalyze the dual cyclization of any GGPP. Chromatograms on the left and mass-spectra on the right.

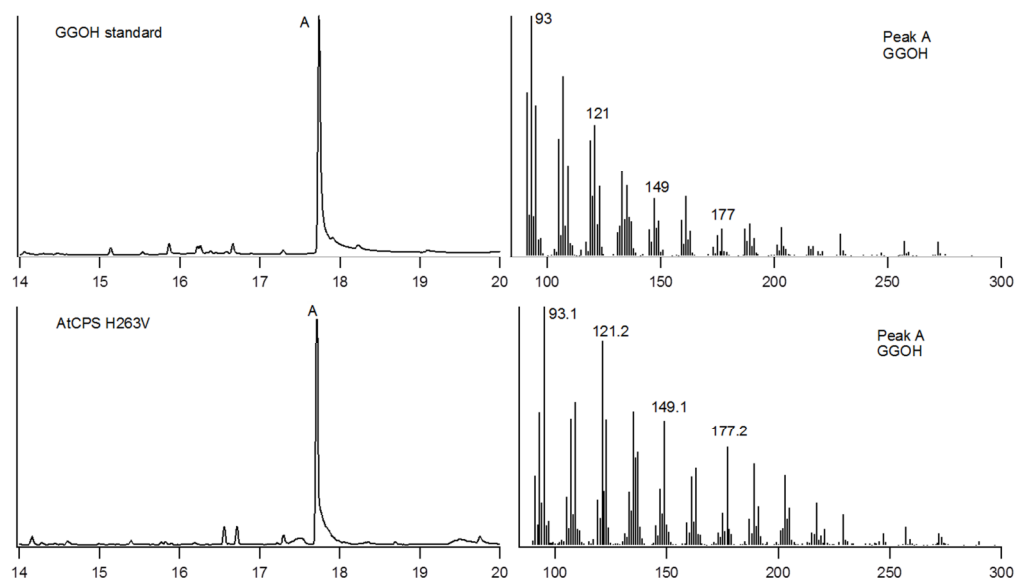


Figure 4. GC-MS analysis of AgAS D621A:H348A, NgCPS, and AgAS D621A. AgAS D621A:H348A peak A = GGOH, peak B = copalol (COH), and peak C = normal-copal-8,15-diol. NgCPS peak A = *normal*-copal-8,15-diol. AgAS D621A peak A = *normal*-COH. Chromatograms on the left and mass spectra on the right.

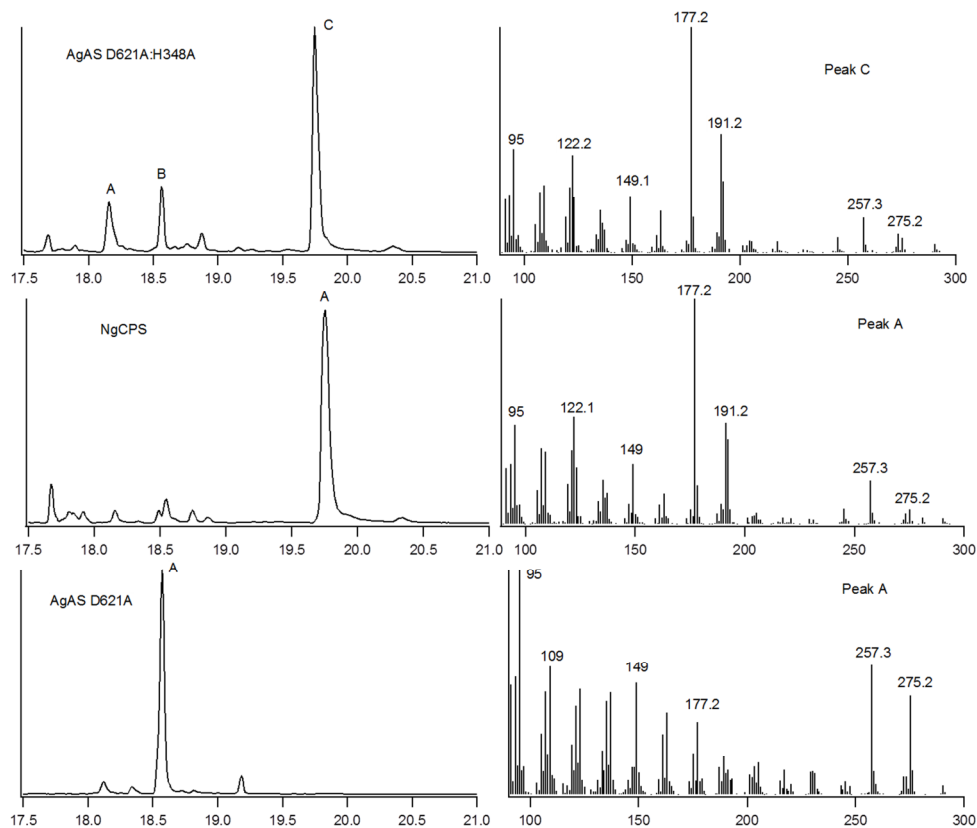


Figure 5. GC-MS analysis of AgAS D621A:H348A and NgCPS both coupled with AgAS D404A. In both instances peak A = manoyl oxide and peak B = *normal*-copal-8,15-diol leftover from unreacted *normal*-copal-8-ol diphosphate. Chromatograms on the left and mass spectra on the right.

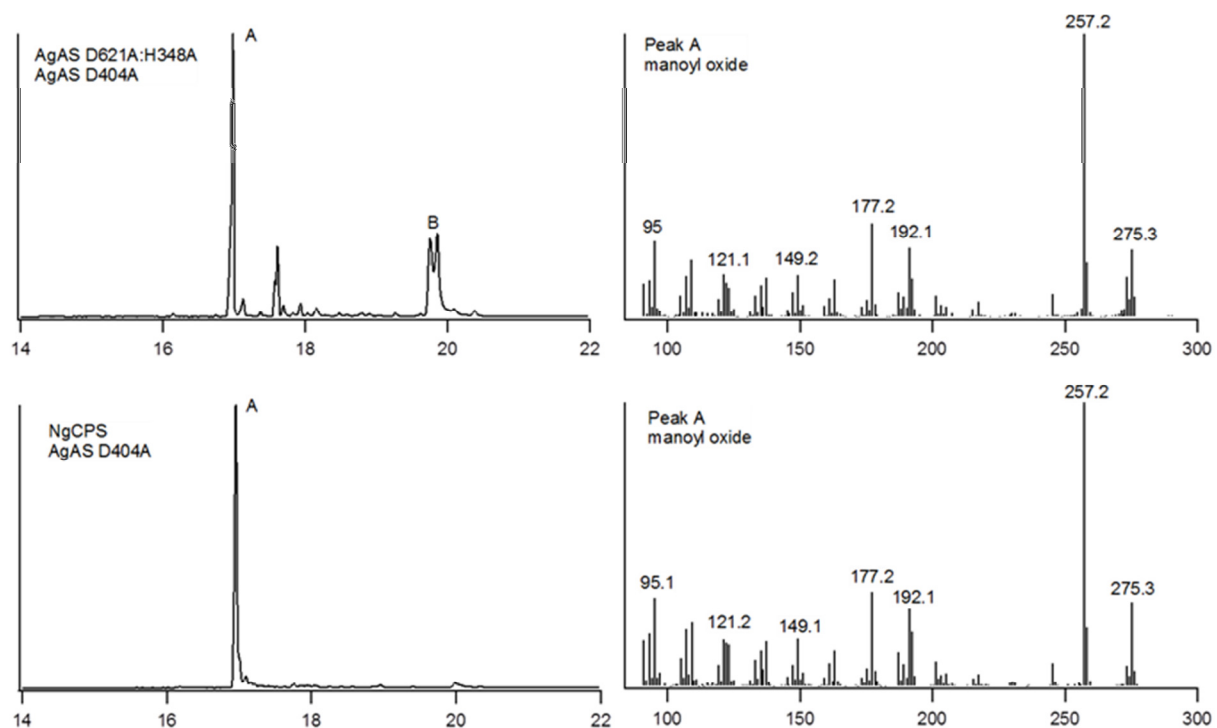
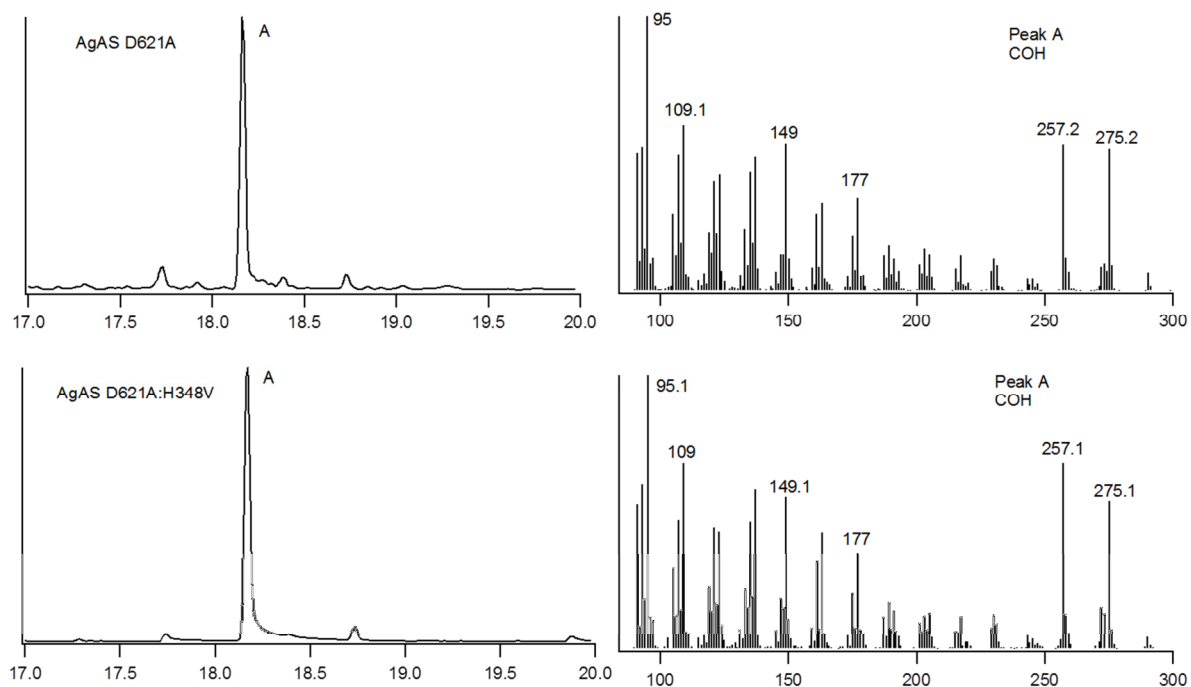


Figure 6. GC-MS analysis for AgAS D621A:H348V compared with AgAS D621A. In both cases peak A = *normal*-copalol. Chromatograms on the left and mass spectra on the right.



Chapter IV. Investigating the Tyr287-His348 base dyad in *Abies grandis* abietadiene synthase

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Abstract

Diterpene cyclases catalyze complex cyclization reactions producing lead compounds for natural product biosynthesis. The class II diterpene cyclases catalyze the committed protonation-initiated bicyclization of (*E,E,E*)-geranylgeranyl diphosphate (GGPP) to specific stereoisomers of labdadienyl/copalyl diphosphate (CPP) (scheme 1a)^[1]. Here, we study a Tyr287-His348 base dyad in abietadiene synthase from *Abies grandis* (AgAS) by mutagenesis. The previous mutations shown on His348 produced a novel hydroxylated version of (CPP) by allowing the addition of a water molecule for reaction quenching. Here we studied the effects of Tyr287 mutations and found an intriguing result with the substitution of a Phe residue altering the stereochemistry of the native CPP formed in the class II active site of AgAS.

Introduction

The class II diterpene cyclases catalyze the production of three observed stereoisomers of CPP, *normal* (9S,10S), *ent* (9R,10R), *syn* (9S,10R)^[1]. *Syn-ent* (9R,10S) is also a possible stereoisomer, but *syn-ent*-CPP has not been observed, only certain natural

products from the *Calceolaria* genus of plants have been observed with this stereochemistry^[2]. These initially bicyclized CPPs can be cyclized further in an ionization-initiated mechanism in a class I diterpene cyclase where the diphosphate moiety is ionized by Mg^{2+} bound to the Asp rich motif in the active site cleft^[1, 3]. These compounds have been shown to be important for metabolism in plants. For instance, *ent*-CPP is involved in the biosynthesis of gibberellin phytohormones which are important for plant growth and development^[1, 4].

Other work has shown that these class II diterpene cyclases can form other versions of CPP. Falara et al. showed a class II diterpene cyclase from *Cistus creticus* (CcCLS) catalyzing the production of copal-8-ol diphosphate^[5], a version of CPP where C-8 is hydroxylated presumably from addition of a water molecule or hydroxyl anion. More recent work on *Abies balsamea* revealed a *cis*-abienol synthase (AbCAS) that had bifunctional class II/I activity. In this study, Zerbe et al. revealed AbCAS to be catalyzing the production of copal-8-ol diphosphate and then *cis*-abienol after the subsequent ionization of the diphosphate moiety without further cyclization. They performed experiments using a version of AbCAS with class I activity abolished by converting a catalytic Asp to an Ala. Then they noted peaks in GC-MS analysis with longer retention times than what is normally seen with CPP after dephosphorylation^[6] corresponding to copal-8,15-diol which is dephosphorylated copal-8-ol diphosphate. Previously, we have shown that *Arabidopsis thaliana* copalyl diphosphate synthase (AtCPS) and AgAS can be re-engineered to produce C-8 hydroxylated versions of their respective stereo-isomeric CPP products. This is hypothesized to be the result of having mutated the corresponding catalytic basic residue to a residue with a smaller

side-chain to allow for a water molecule or hydroxyl anion to be positioned in close proximity to C-8 and perform a nucleophilic attack to quench the reaction (scheme 1b). This result provides us with a better understanding of class II diterpene cyclases and we are still in the process of finding a similar result for that of a *syn*-CPP synthase.

Diterpene cyclases are presumed to be descended from the more complex triterpene cyclases like squalene-hopene cyclase (SHC)^[7]. This is due to their general structural homology and use of similar catalytic mechanisms and amino acid motifs. For instance, both the SHC's and class II diterpene cyclases have DXDD motifs involved in the protonation of terminal double bonds to initiate cyclization^[8]. The homology of SHC's and other triterpene cyclases with class II diterpene cyclases is of great importance here as Wendt reported earlier a Tyr-His base dyad in a triterpene oxidosqualene-lanosterol cyclase (OSC) as being responsible for deprotonation^[9], which we observe a similar base dyad in AgAS (Tyr287-His348) (Figure 1a). Earlier results suggested that His348 is the basic residue responsible for deprotonating C-8 of the labda-13-en-8-yl⁺ intermediate. And preliminary *in vitro* results show that this mutation in AgAS retains more catalytic activity than the corresponding mutation in AtCPS. This discrepancy in catalytic efficiency between AgAS and AtCPS, we hypothesize, is due to the nearby Tyr287 in AgAS and its hydrophilic properties causing water molecules to be more efficiently positioned near C-8 of bound GGPP, which AtCPS does not have a hydrophilic residue in the immediate vicinity of its catalytic basic His. Interestingly, this Tyr is conserved among most class II diterpene cyclases producing *normal*-CPP, but with a few enzymes containing a Phe at this position (Figure 1b). Upon

mutational analysis of this Tyr287 in AgAS to a Phe we came across an intriguing result where the stereochemistry of the product had been altered to *syn*-CPP.

Results

AgAS Tyr287 was converted to Phe via site-directed mutagenesis. The resulting mutant, AgAS Y287F was checked by complete sequencing then cloned into the pGG expression vector. AgAS Y287F was then ran in our metabolic engineering system^[10] with OsCPS4, a *syn*-CPP synthase in *Oryza sativa*^[11]. According to GC-MS analysis, AgAS produced a product with the same retention time as OsCPS4 and the same MS pattern (Figure 2). In order to confirm the stereochemical change in product, AgAS Y287F was coupled with OsKSL4 in our metabolic engineering system. OsKSL4 is a pimaradiene-synthase from *Oryza sativa* that uses *syn*-CPP as substrate^[11]. The GC-MS analysis showed the production of *syn*-pimara-7,15-diene with both the AgAS Y287F/OsKSL4 and the OsCPS4/OsKSL4 experiments (Scheme 2) (Figure 3). Further studies were done on AgAS Tyr287 converting the residue to an Ala and His and these mutations terminated the catalytic activity of AgAS (Figure 4).

Discussion

These results provide some insight on the nature of the proposed base dyad in AgAS. With the previous results of the AgAS D621A:H348A mutant and the current results with AgAS D621A:Y287A it seems probable that these two residues are working together as the catalytic base. If Tyr287 is acting as the actual residue which deprotonates C-8, then that

helps explain the inconsistency in results we have observed between AgAS D621A:H348V and the Ile, Val, and Leu substitutions we made for AtCPS His263.

Wendt proposed earlier in OSC that it is both the residues essentially working together via hydrogen bonding in order to deprotonate carbocation intermediates. He also mentioned earlier experiments done on Tyr-His dyad in the OSC from *Saccharomyces cerevisiae*, where mutating the Tyr in the dyad caused the enzyme to not catalyze the cyclization of oxidosqualene. This result correlates with our result where mutating Tyr to Ala or His prevents the cyclization of GGPP. However, that does not explain the perplexing result we see with AgAS Y287F.

It is indeed strange that removal of a single hydroxyl group in the active site of AgAS could alter the activity of the enzyme to produce a different stereo-isomer of CPP. With other mutants of Tyr287 causing the activity of the enzyme to be abolished and other *normal*-CPP producing enzymes even containing a Phe at this position this result currently remains a mystery. However, if we look back to the earlier proposed pro-chiral conformations of bound GGPP in the active site cleft of class II cyclases^[1], it is possible to speculate that the removal of the dipole interactions allows the C-8 to shift downward from its position in the chair-chair “normal” conformation to induce the chair-boat “normal” conformation allowing production then of *syn*-CPP. This result does provide ground for some interesting future work. It would be interesting to perform mutagenesis experiments to obtain an AgAS Y287F H348A mutant to attempt to produce a *syn*-copal-8-ol diphosphate, since attempts in finding a mutation in OsCPS4 to cause this product alteration have been unsuccessful as to date.

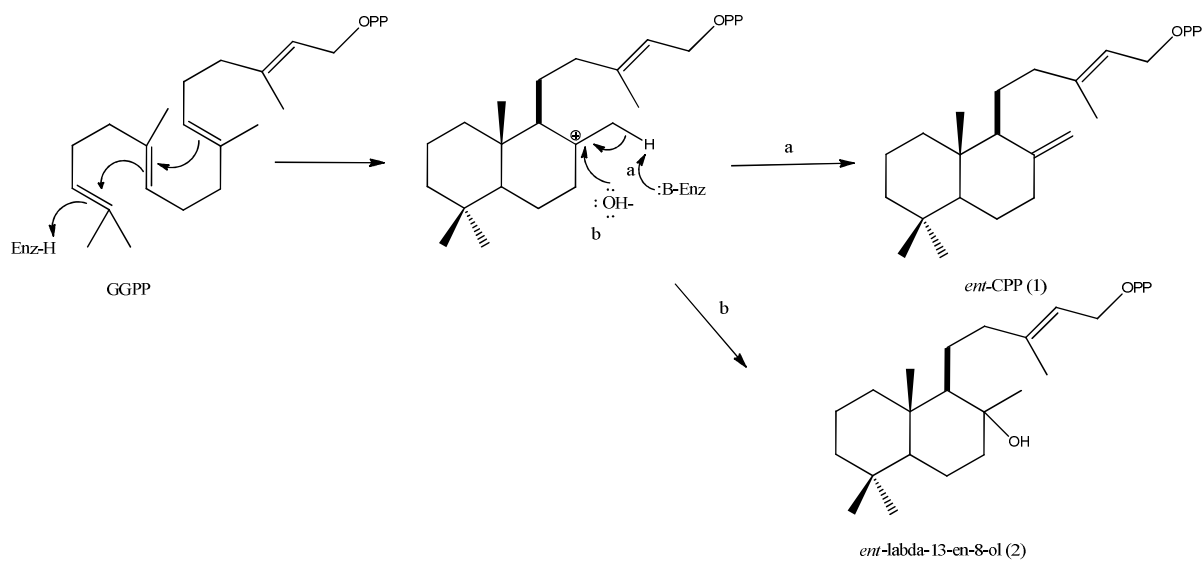
Acknowledgement

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Scheme 1. A.) production of CPP via the labda-13-en-8-yl⁺ intermediate with base deprotonation. B.) production of copal-8-ol diphosphate via the labda-13-en-8-yl⁺ intermediate with quenching by water molecule.



Scheme 2. Reaction scheme showing the production of *syn*-pimara-7,15-diene from GGPP

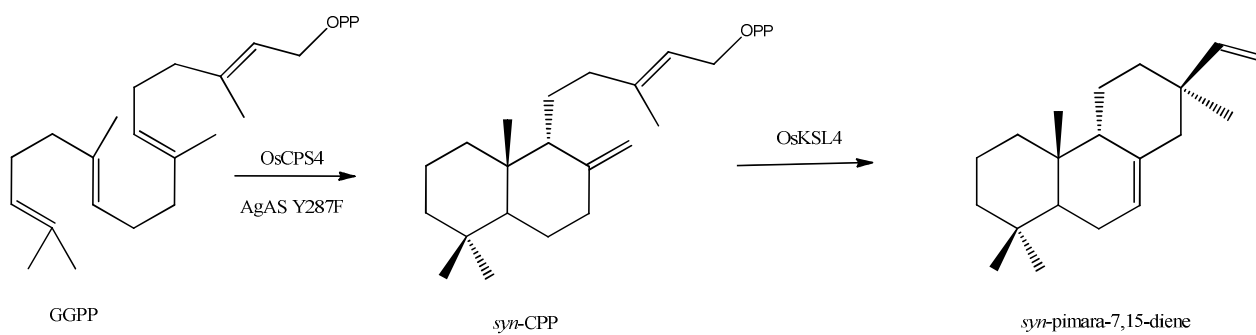


Figure 1. A.) crystal structure of AgAS^[12] showing the Tyr287-His348 base dyad. B.) alignment of class II diterpene cyclases involved in the production of *normal*-CPP showing the conserved Tyr and a less conserved Phe. Also showing NgCPS as containing a Leu at this conserved position.

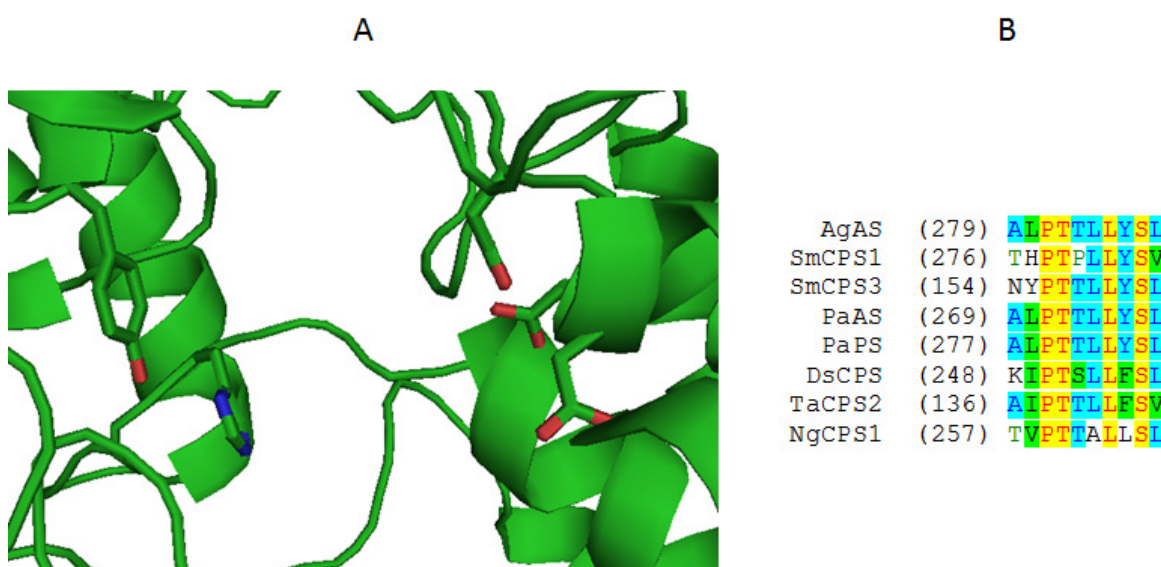


Figure 2. GC-MS analysis of AgAS Y287F and OsCPS4 showing the production of *syn*-CPP. Peak A = *syn*-copalol (COH) in both cases. Chromatograms on the left and mass spectra on the right.

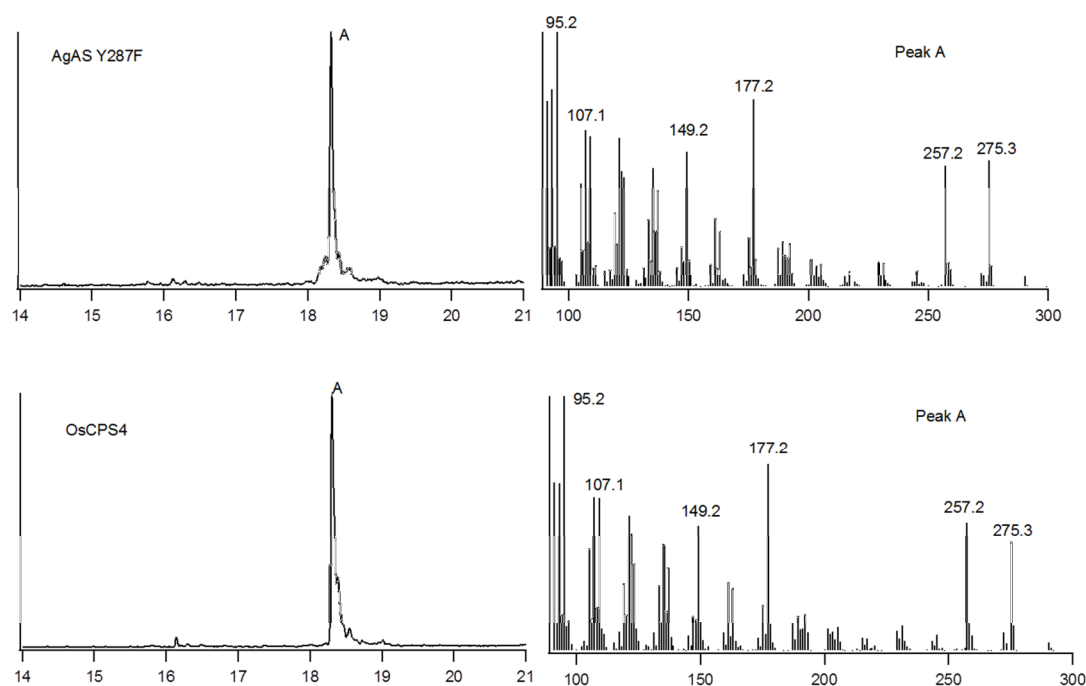


Figure 3. GC-MS analysis of AgAS Y287F and OsCPS4 both coupled with OsKSL4. Peak A for both shows the presence of *syn*-pimara-7,15-diene further suggesting that AgAS Y287F mutant produces *syn*-CPP. Chromatograms on the left and mass spectra on the right.

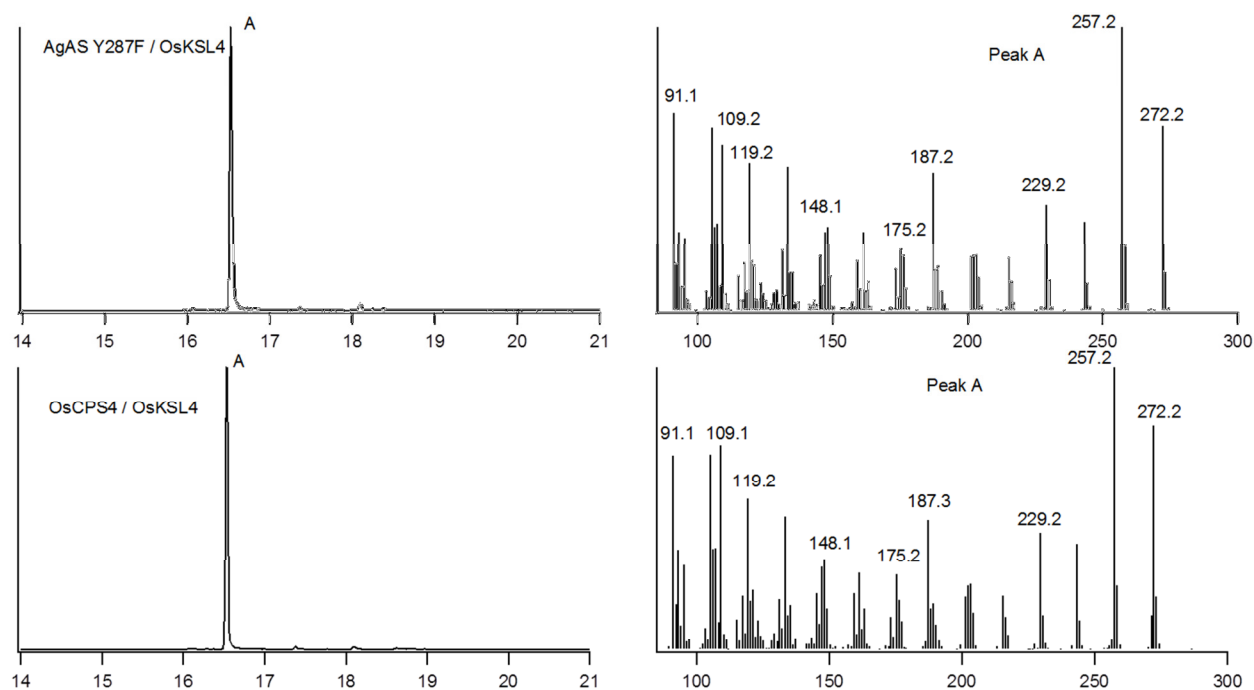


Figure 4. GC-MS analysis of AgAS D621A:Y287H and D621A:Y287A both with AgAS D621A showing that enzymatic activity of the Ala and His mutants is abolished with only the presence of GGOH in each of the mutants compared to the COH peak found for both AgAS D621A chromatograms.

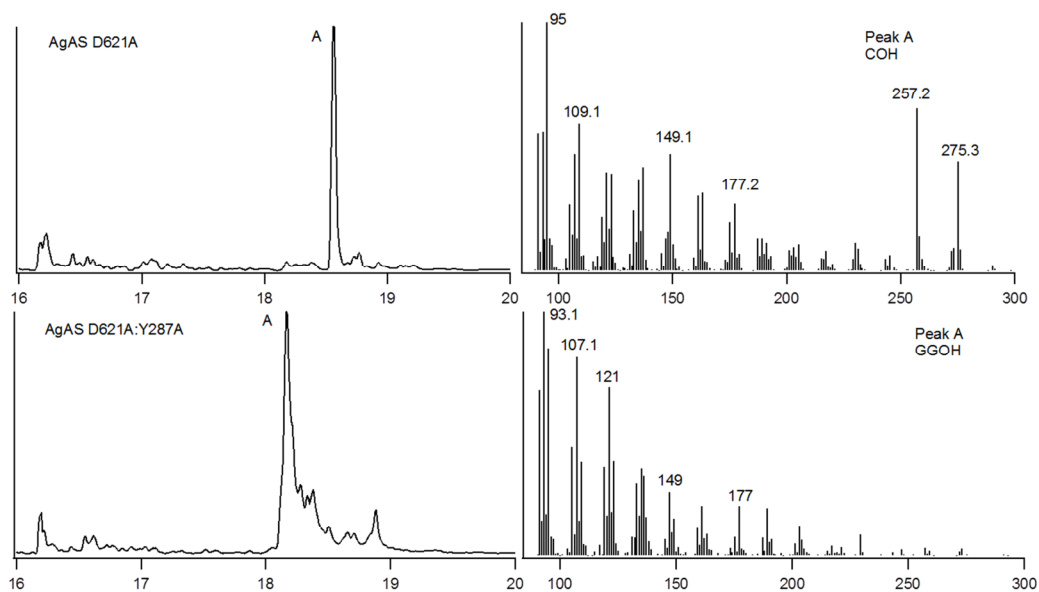
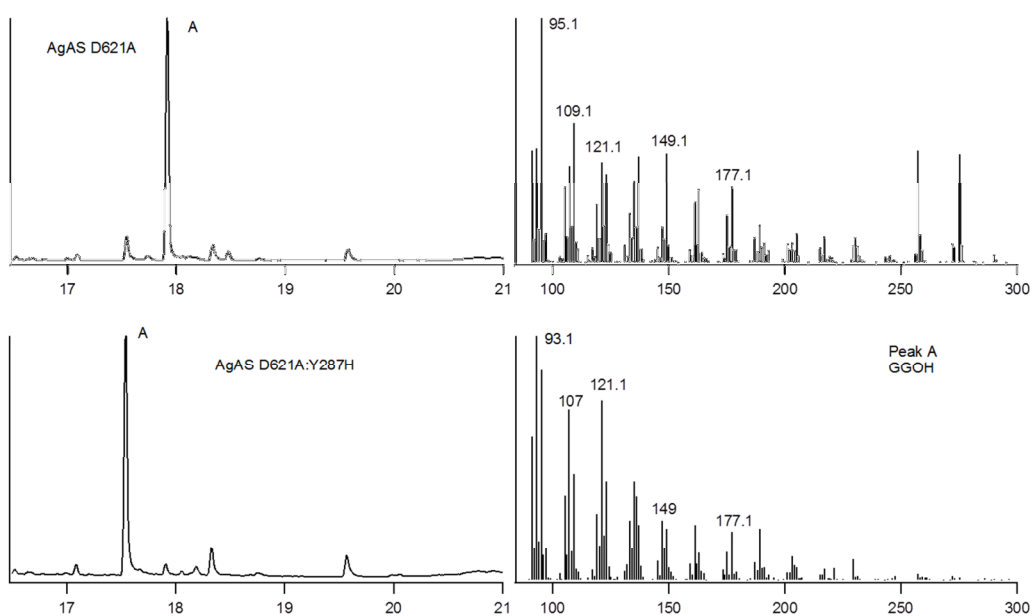


Figure 4. GC-MS analysis of AgAS D621A:Y287H and D621A:Y287A both with AgAS D621A showing that enzymatic activity of the Ala and His mutants is abolished with only the presence of GGOH in each of the mutants compared to the COH peak found for both AgAS D621A chromatograms.



Chapter V. Conclusion

The results presented here have given new insight into this still widely unknown class II diterpene cyclase mechanism. We set out to investigate the catalytic basic residue of these class II diterpene cyclases. We now believe with the evidence procured in this project that these class II diterpene cyclases use a His for their catalytic basic residue. With the consistency in data between AgAS and AtCPS on mutagenesis of just the His with AgAS able to be more versatile and efficient most likely due to the close by Tyr, it is likely that our hypothesis is correct.

With AtCPS we saw a single residue switch on His263 that alternated the product outcome from *ent*-copalyl diphosphate (CPP) to *ent*-copal-8-ol diphosphate. We hypothesized this to be because of the smaller side-chain of Ala allowing for a water molecule or hydroxyl anion to be positioned into close proximity of C-8. The water or hydroxyl anion performs a nucleophilic attack on the carbocation intermediate. We performed further metabolic engineering experiments to confirm stereochemistry and production of manoyl oxide. We also showed similar results with an Ala mutant in AgAS where there is a His348 that is in a position similar to the position of His263 in AtCPS. Our results showed that AgAS D621A:H348A was catalyzing the production of *normal*-copal-8-ol like that of CcCLS^[1] and AbCAS^[2] by the same mechanism we proposed for AtCPS H263A.

Interestingly, we noticed that AgAS D621A:H348A was catalyzing the production of copal-8-ol diphosphate more efficiently than that of AtCPS H263A *in vitro*. We hypothesized this to be due to Tyr287 being in close proximity to the His348 forming a

Tyr287-His348 base dyad similar to that proposed by Wendt in OSC^[3]. We hypothesized the hydroxyl group on the Tyr as being able to attract a water molecule or hydroxyl anion and position it more efficiently to quench the C-8 carbocation. We performed mutational analysis on this Tyr and found that it is important for catalysis. Converting the residue to His or Ala seemed to cut off all catalytic activity. However, when this residue was mutated to a Phe another alteration in product was observed. The stereochemistry of CPP was altered from *normal* (9S, 10S) to *syn* (9S, 10R). This is a very interesting result, however it is difficult at this point to speculate what might be happening and more analysis needs to be done on this dyad in relation to the rest of the active site in order to start answering some of the questions that have surfaced now.

Furthermore, in the case of AgAS, a larger side-chain residue in place of the supposed catalytic basic His does not seem to effect the catalyzed reaction too much according to the metabolic engineering results. This result itself seems to indicate that the His348 is not the catalytic base, at least by itself. At this point it may be that the Tyr287 is in a better position to deprotonate C-8 and its function is augmented by the presence of the His in the dyad which is what Wendt suggested for OSC^[3]. However, more work will need to be done to give some clearer understanding to this question.

So far, we have not yet uncovered a possible base residue for a *syn*-CPP synthase. We have uncovered this for both *ent* (AtCPS) and *normal* (AgAS) and it would be a very compelling case if we uncovered a His in a *syn*-CPP synthase that produces the same alteration in product. We are currently performing mutational analysis on OsCPS4, a *syn*-CPP synthase from *Oryza sativa*. Also, some screening needs to be done on other *ent*- and

normal-CPP synthases to see if His to Ala conversions will produce the same results among all other class II diterpene cyclases as we have found here in this project. Also, if a *syn-ent* (9R,10S) CPP synthase is ever discovered it would be interesting to perform similar experiments on that as well. Currently, no such enzyme has been discovered and the stereochemistry is only observed in certain natural products^[4]. It is very well possible, and we believe it to be likely, that all will have a His as the catalytic basic residue. And if these future experiments correlate with our current results it will be a significant addition to the knowledge of this field.

There is still much more to understand about class II diterpene cyclases. As mentioned before, they contain an aromatic “box”. Site-directed mutagenesis experiments will be done on conserved aromatic residues that form a “box” around the middle catalytic Asp of the DXDD motif found in class II diterpene cyclases^[5]. This aromatic “box” could be effected by the Mg²⁺ inhibition mentioned earlier^[6, 7]. Doing mutational and kinetic analysis on the Mg²⁺ inhibitory residue and the aromatic “box” to see if the aromatic residues are being disrupted by Mg²⁺ binding to the inhibitory residue.

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