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Altering the Regiospecificity of C6 Indole Prenyltransferase Enzymes Towards Drug Development

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Dedicate this thesis to my parents

ABSTRACT

Altering the Regiospecificity of C6 Indole Prenyltransferases Towards Drug Development

by Ahmed R Aoun

Prenyltransferase (PT) biocatalysts are late-stage tailoring enzymes that modify natural products. PTs catalyze the attachment of prenyl moieties to natural product acceptors using pyrophosphate donors. This prenyl modification in small molecules leads to changes in structural and biological activities. Understanding the structural insights as well as the mechanisms by which PTs function allows us to utilize PTs as a unique approach towards drug development. PriB PT is an example of aromatic PTs that has been characterized previously as a L-tryptophan (L-Trp) C6 *C*-prenyltransferase (C6-*C*-PT). Analysis of PriB binding pocket, highlighted key residues that might play an important role in determining the regiospecificity of the enzyme and their mechanisms. Hence, we hypothesized that site-directed mutagenesis of one of these residues will alter the enzyme regiospecificity and/or its permissiveness leading to variation in the pharmacokinetics and biological activities of small molecules. Site-directed mutagenesis approach was used to engineer PriB PT as a model for C6-PTs and the purified mutant proteins were produced and purified. *In vitro* reactions of the purified engineered proteins have shown altered regiospecificity toward substrate acceptors when compared to wild type. The long-term goal for this study is to utilize the permissiveness of PriB enzyme for drug diversification and determine the activity of the mutant PriB enzymes. This study will also shed some light on the mechanistic insights of aromatic C6-PTs.

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INTRODUCTION

1.1. The Progress of Natural Products Discovery

Natural products (NPs), have been used as different treatment options for many severe diseases. The majority of the active ingredients of medicines are obtained from natural sources, such as plants, animals, fungi, and microorganisms (Newman & Cragg, 2016). The diversity in the chemical structures and the efficacy of NPs have served as essential approaches to understanding the cell physiology (Wilkinson & Micklefield, 2007). Many pharmaceutical companies, during the last few decades, have shifted to synthetic compounds rather than NPs in drug discovery due to several limitations including traditional NPs screening techniques which frequently led to the rediscovery of already known compounds and inconsistency of NP production from their natural sources. Furthermore, the minute quantities of NPs obtained from their natural sources, which requires large quantities of the original source, high labor costs for extraction and purifications, as well as time-consuming processes are additional hurdles. Moreover, the complexity of natural products makes the characterization of their structures a challenging task not preferable for medicinal chemists (Lam, 2007).

Nevertheless, the innovation of NP discovery methods, such as new methodology in production, purification, and characterization, has influenced numerous pharmaceutical industries to regain interest in this field. Furthermore, with the development of new screening assays and targets combined with the new chemical entities that NPs provide, have increased the interest in NP discovery (Katz & Baltz, 2016). For example, Novartis Institutes for BioMedical Innovation techniques led to the discovery of novel bioactive compounds, such as anticancer Englerin A,

Argyrin B, and antibacterial antiparasitic. Cyclomarin A (Schmitt et al., 2016). Mutational biosynthesis also is another approach for the development of novel NPs through the insertion of analogs of NP intermediates into biosynthetic pathways; for instance, the production of nikkomycin antibiotic from novobiocin analogs (Weist & Süssmuth, 2005).

During the last three decades, there have been two fundamental scientific progressions that had an essential impact on NP discovery. These advancements, include the detailed understanding of NP biosynthesis, and the development of genetic tools for *Streptomycetes*. The diverse NP structures are synthesized through a complex system of enzymatically catalyzed metabolic pathways. Enzymes from natural product biosynthetic pathways have become fascinating approaches for modifying the bioactivities of these complex molecules especially after the evolution of NPs biosynthesis technology and the identification of biosynthetic gene clusters and encoding enzymes (Ncube et al., 2017) (Medema et al., 2015). In addition, *Streptomycetes* have exposed a significant and adjustable group of bacteria for the production of diverse valuable secondary metabolites. These secondary metabolites have brought attention in numeral medicinal companies and academia after the development of recombinant DNA (rDNA) technology. These advancements include plasmid vectors, protoplast transformation, and engineering strategies as well as the development of analytical approaches such as proteomics and metabolomics (Bekker et al., 2014) (Bro & Nielsen, 2004) (Katz & Baltz, 2016). Improvement of DNA cloning and sequencing have assisted in understanding the biochemical pathways of NPs via resolving and predicting the enzymes that are involved in the biosynthesis from NPs genes sequences or gene cluster. Clustering enables the cloning of the whole NP biosynthesis pathway into vectors and heterologous hosts and the production of NPs combinatorial biosynthesis.

Combinatorial biosynthesis is a method to manufacture a sequence of elected potential drugs for high-throughput screening. This approach has been used for the generation of "unnatural" NPs especially those biosynthesized by nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) genes, which are involved in the biosynthesis of polyketides (PKs) and nonribosomal peptides (NRPs), respectively, possess essential antibiotic, anticancer, and immunosuppressant activities (Menzella & Reeves, 2007) (Yin et al., 2007) (Weissman, 2015).

1.2. Tailoring Enzymes and Chemoenzymatic Reactions

The decoration of NPs by chemical moieties such as hydroxyls, methyls, sugars, and halogens is crucial for the modification of these compounds and leads to the enhancement of their pharmacological activities and properties. These groups are added by a group of enzymes that are encoded within the biosynthetic gene cluster of the NP and are collectively called tailoring enzymes. Enzymes, such as methyltransferases, cytochrome P450s, halogenases, glycosyltransferases, prenyltransferases, are examples of tailoring enzymes (Lin et al,. 2012). Tailoring enzymes can be utilized as valuable tools for the targeted modification of molecules and drugs and can complement chemical methods to generate novel compounds (Alwaseem et al,. 2018) (S. Mori et al,. 2019) (Newmister et al., 2018) (Hughes et al., 2017).

Enzyme catalysis is one of the powerful tools to modify small molecules and has become frequently used in the chemical industry and scientific research due to multiple reasons. Biocatalysts are efficient, usually produce regiospecific products, under greener, milder, and safer conditions for the modification of bioactive compounds (González-Sabín et al., 2011) (Wells et al., 2012). Enzymes are highly efficient and catalyze specific reactions often with high yield in

aqueous conditions with minimum side products (Romney et al, 2018). Chemoenzymatic reactions, enzymatic modification, along with synthetic methods, has succeeded to produce biologically active compounds such as analogs of the antibiotics daptomycin, vancomycin, and pristinamycin (Grünewald & Marahiel, 2006).

1.3. Prenyltransferase Enzymes

Prenyltransferase (PT) biocatalysts are tailoring enzymes used to catalyze the attachment of prenyl moieties from pyrophosphate substrates (donors) to various aliphatic or aromatic acceptors via nucleophilic substitution (Figure 1). Examples of natural pyrophosphate donor substrates include the pyrophosphates of dimethylallyl (DMAPP, C_5), geranyl (GPP, C_{10}), farnesyl (FPP, C_{15}), or geranylgeranyl (GGPP, C_{20}). Acceptor substrates include low and high molecular weight small molecules, nucleic acids, peptides, and proteins (Winkelblech, Fan, & Li, 2015). Furthermore, protein prenyltransferases are significant for the post-translational modification of proteins in living organisms and demonstrate essential drug targets (Heide, 2009).

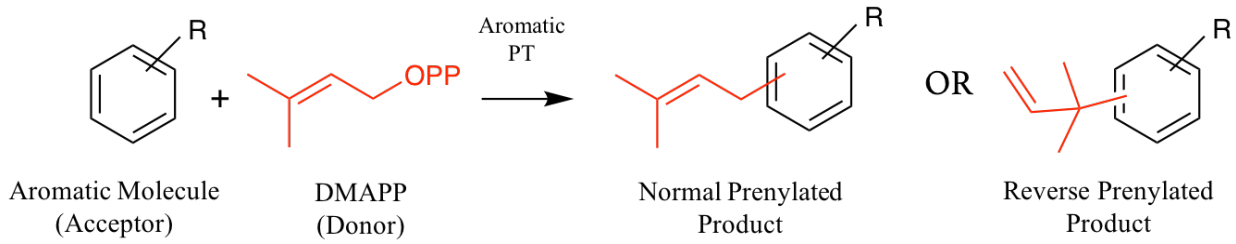

Figure 1. The catalyzed reaction of aromatic PTs

The S_N1 reaction of dimethylallyl pyrophosphate (DMAPP) is initiated by the release of the pyrophosphate moiety as a good leaving group and the cleavage of carbon-oxygen (C_1-OPP) bond leading to cationic intermediates. This cationic intermediate is stabilized by charge resonance over

C_1 and C_3 of the prenyl group (Figure 2). The prenyl cation attaches to the nucleophiles in the indole ring (acceptor) via C_1 or C_3 to form normal and reverse prenylation, respectively (Walsh, 2014).

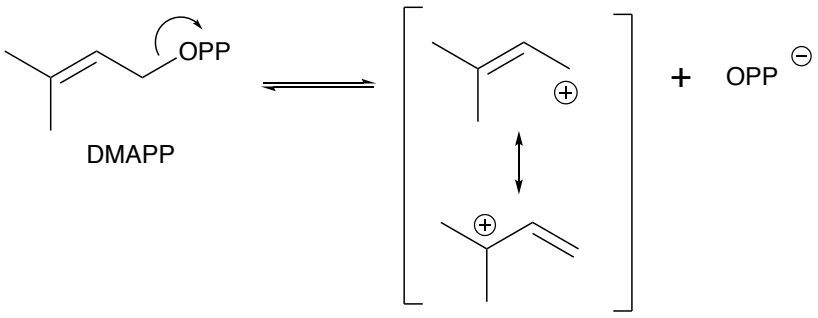

Figure 2. Release of OPP group lead to carbocation intermediate on C_1 or C_3

Dimethylallyltryptophan synthase (DMATS) superfamily are aromatic PTs (ArPTs) and is one of the most investigated enzyme subgroups. They share sequence homology with the DMATSs involved in the biosynthesis of ergot alkaloids in *Claviceps purpurea* (Tsai et al., 1995). Until 2015, more than 40 such enzymes have been determined and characterized biochemically (Winkelblech et al., 2015). The majority of the DMATS superfamily members are involved in the biosynthesis of prenylated indole alkaloids and modify indole derivatives as acceptors including, tryptophan and tryptophan-containing cyclic dipeptides as substrates (Winkelblech et al., 2015). One interesting feature of DMATSs and other ArPTs is their relatively broad acceptor permissiveness allowing them to accommodate various substrates. Thus, using these enzymes in drug modification could, in some instances, provide a more efficient and environmentally friendly approach alternative to synthetic modification (Fan & Li, 2013).

1.4. Importance of Prenylation and Regiospecificity

Many published studies demonstrated the bioactivity of prenylated natural products (Alhassan et al., 2014). The advantages of the presence of a prenyl moiety in various natural compounds include enhancing the target protein binding affinity as well as improving the interaction with cell membrane/cell wall compared to the nonprenylated counterparts (Botta et al., 2005). Prenylation in small molecules leads to changes in structural and biological activities. Prenylation opens the door for a unique approach towards drug development and provides new chemical libraries for pharmacological screening (Botta et al., 2005). Biosynthesis of many biologically active compounds in animals and plants depends on aromatic secondary metabolite prenylation (Alhassan et al., 2014). Examples of prenylated bioactive compounds include novobiocin, clorobiocin, fumigaclavine C, fumitremorgin B, sophoraflavanone G, cyclomarin C, and roquefortine C (Figure 3).

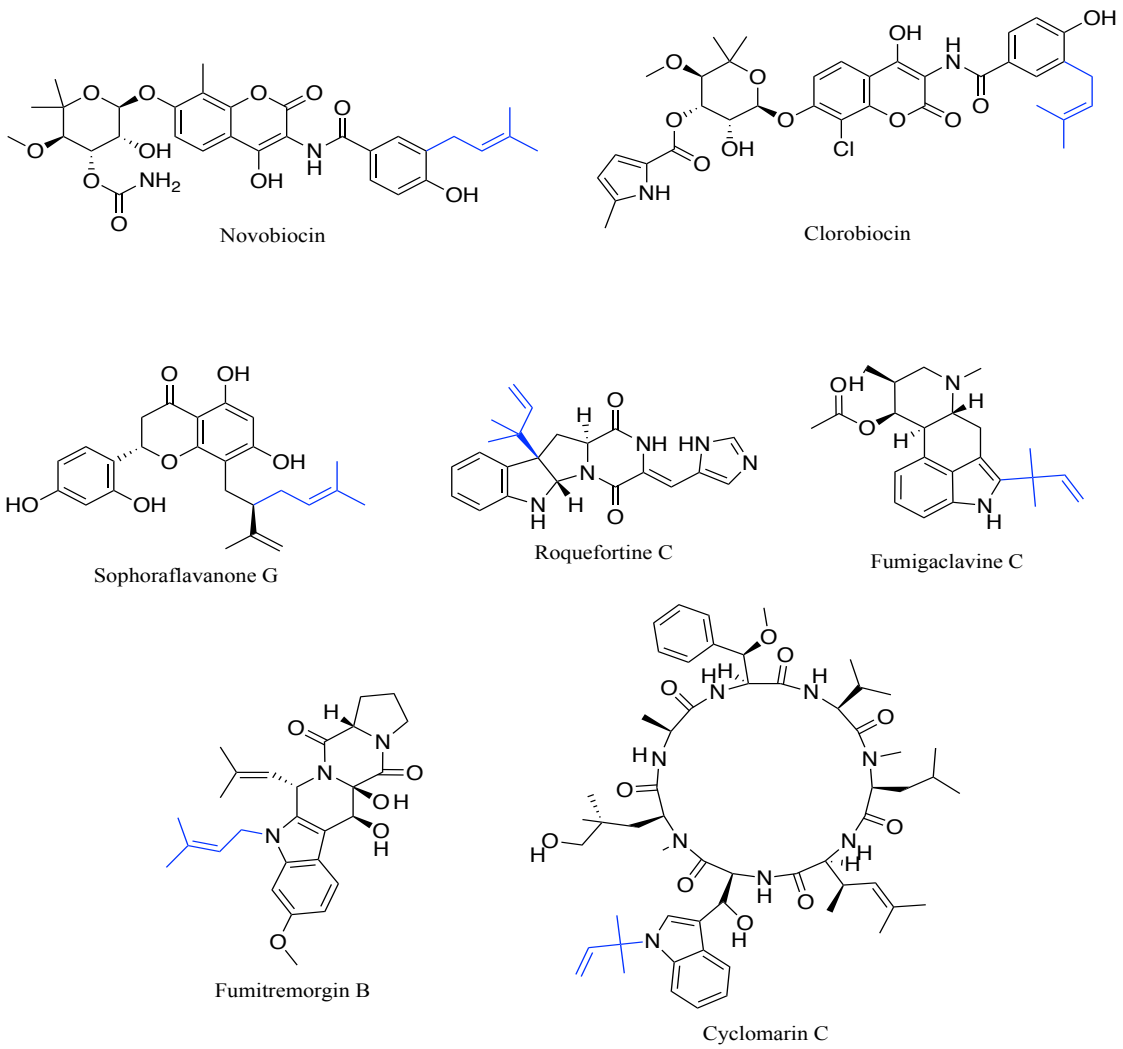

Figure 3. Examples of prenylated active compounds

One study examined the antiproliferative activity of nonprenylated naringenin and prenylated flavonoids [(Xanthohumol and four prenylflavonoids: 8-prenylnaringenin, 6-prenylnaringenin isoxanthohumol, and α,β-dihydroxanthohumol) (Figure 4)] against different human cancer cell line (Bartmańska et al., 2018). The outcome of this study exhibited that prenylated naringenins had higher anticancer activity compared to nonprenylated naringenin. Moreover, this study pointed out the significance of the position of prenylation, where there were changes in activity and specificity between 6-prenyl- and 8-prenylnaringenin. The C-8 isomer showed higher

antiproliferative activity, except breast cancer cell line, where 6-prenyl analog exhibited higher activity and selectivity (Bartmańska et al., 2018). Furthermore, other previous study showed that adding prenyl group to the cyclic lipopeptide antibiotic daptomycin increased the antimicrobial activity against gram-positive bacteria compared to nonprenylated daptomycin (Elshahawi et al., 2017). Furthermore, the mode of prenylation, normal versus reverse, also affects the activity of the drugs (Elshahawi et al., 2017).

Another advantage of PTs is their ability to catalyze carbon-carbon bond formation. Generally, different PTs have been characterized as capable of catalyzing the transfer of the prenyl moiety to all carbon positions of the indole ring. Noticeably, PTs catalyze the formation of a carbon-carbon bond between the indole ring and the prenyl group in milliseconds (Tanner, 2015). Carbon-carbon bond formation in organic synthesis is a crucial step to build up complex structure compounds from smaller molecules. Generating complex products have tremendous applications. Nevertheless, it is very challenging to approach this via synthetic methods because it requires intricate approaches for chiral synthesis (for non-aromatic moieties), multiple protection and deprotection steps as well as high temperature and pressure requirement (Schmidt et al., 2016).

Thus, this indicates the significance of prenyl moieties for improving the pharmacological activity for many natural products and utilizing PTs to modify drugs.

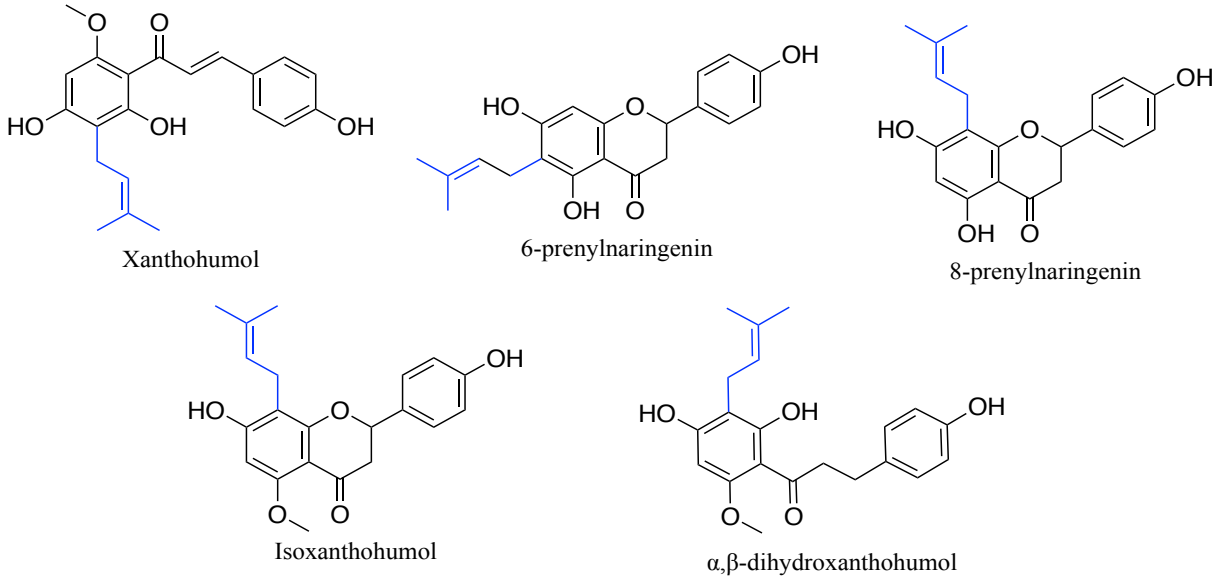

Figure 4. Examples of prenylated flavonoids that showed anticancer activity

1.5. Enzyme Engineering

Biochemical reactions in aqueous media are fundamental for life; however, in synthetic chemistry, the existence of water in chemical reactions could lead to negative impacts, including minimizing yields and productivity. Certainly, removing even minute quantities of water from chemical reactions is sometimes a challenging task for most chemists (Arnold, 2018). Most reactions in chemical syntheses require organic solvents, which produce toxic wastes that affect safety and the environment. Therefore, replacement of reactions running in organic solvents with substitutes that work in aqueous media is a hot topic in research. Moreover, water-based reactions provide an essential boundary between chemistry and biology, which allow making chemical modifications of biomolecules. This leads to the improvement of biotherapeutic medicines such as bioactive peptides, drug conjugated antibody, biopharmaceuticals, and small molecule derivatives from

natural products. Mainly, none of these accomplished would be achievable without aqueous reactions because most biological molecules are unstable and insoluble in organic media (Romney et al., 2018).

Recently, several pharmaceutical and biotechnological industries started incorporating natural enzymes for many fundamental reactions. Yet, many natural biocatalysts do not exhibit the desired stability and efficiency to proceed with the industrial requirements (Kokkonen et al., 2019). Nowadays, the advancement of biotechnology has brought new techniques to improve and produce enzymes with desirable properties. Protein engineering such as site-directed mutagenesis is one of the approaches that can alter amino acids residues at specific enzyme sites. In the last two decades, enzyme engineering has succeeded in modifying enzymes with higher activities than wild-type enzymes (Bilal et al., 2018). Furthermore, enzyme engineering demonstrated the ability to improve the stability and catalytic activity towards non-native substrates (Bornscheuer et al., 2012) (Almhjell et al., 2018). Enzyme engineering has also led to catalyzing reactions that were restricted only by synthetic catalysts (Hammer et al., 2017) (Zhang et al., 2019). Various engineered enzymes have exhibited significant improvement in selectivity and activity during biological reactions (Hammer et al., 2017) (Cho et al., 2019).

Previous studies on the PTs engineering showed that site-directed mutagenesis on some PT amino acid residues changed the catalytic activity and improved the acceptance of other substrates. One study published in 2017 showed that site-directed mutagenesis of a DMATS superfamily member, FtmPT1 (Gly115 and Tyr205 to other residues), led to altering the prenylation position and increase of permissiveness towards nonnative acceptors (Zhou et al., 2016). Another study

published in 2015, demonstrated that a mutant FgaPT2 (Lys174 to Phe) had altered the substrate preference (L-Trp) to another substrate, L-Tyr (Fan et al., 2015).

Other researches have also studied the role of other active site residues to improve the substrate permissiveness, Fan and colleagues have shown enhancing the activity of aromatic prenyltransferase FgaPT2 acceptance toward cyclic dipeptide substrate, R244 has been replaced with other amino acids. Notably, mutation of R244 with amino acid leucine significantly improved the product yield with cyclic dipeptide substrate comparing with other mutants; however, the result of R244 mutants which tested with the native substrate L-Trp decreased or abolished the activity. Clearly, this outcome showed the importance of R244 as a key residue to maintain the L-Trp substrate by hydrogen bonding to the carboxylate group of L-Trp (Fan & Li, 2016). Moreover, in a previous study, site-directed mutagenesis on TleC and MpnD aromatic PTs have changed the enzyme activity and the preferences of prenyl group length of the substrate. The structural analysis of the binding pocket of both enzymes demonstrated that Trp97, Ala173 in TleC have replaced with Tyr80, Met159 in MpnD. In TleC, mutation of Trp97 to Tyr and Ala173 to Met have affected the donor preferences comparing with TleC wild-type. The Trp97 to Tyr replacement enhanced the activity of C_5 DMAPP substrate preferences than the preferred donor C_{10} GPP. Moreover, the Ala173 Met mutant increased the acceptance toward C_5 DMAPP, whereas C_{10} GPP is no longer accepted. In MpnD PT mutation of Tyr80 to Trp and Met159 to Ala increased the C_{10} prenylation and whereas the prenylation of C_5 decreased compared with wild-type (T. Mori et al., 2016). Structure alignment of PriB PTs with the other previous studied PTs have shown similarity among them. This suggests that to perform alteration of substrate (donors/ acceptors) selectivity for PriB, mutagenesis based on the previously mentions studies could be performed.

1.6. PriB Prenyltransferases

PriB PT is an aromatic PTs that was characterized from the genome of a bacterial strain *Streptomyces sp.* (actinomycetes), which was separated from the soil near a thermal vent of Ruth Mullins coal fire in the Appalachian Mountains (Elshahawi et al., 2017; Wang et al., 2015). PriB protein belong to the DMATS superfamily characterized with a barrel shape (β-barrel) composed of $\alpha\beta\beta\alpha$ units. The PriB is composed of a cylindrical β-sheet arranged around a ring of solvent-exposed α-helices (Figure 5) (Elshahawi et al., 2017). PriB was characterized as an L-Trp C6 *C*-prenyltransferase (C-PT) (Elshahawi et al., 2017, Fan, Winkelblech, & Li, 2015). PriB's permissiveness is not limited to acceptors but also extends to donor substrates. PriB is one of few DMATS enzymes that is capable of accommodating not only DMAPP and L-Trp (native donor and acceptor, respectively), but also various non-native donors (geranyl, farnesyl, geranylgeranyl and other synthetic diphosphates) and other aromatic acceptor substrates including, anthranilic acid, naphthalenes, anthraquinones, phenazines, and D-Trp with high conversion percentages. PriB PT was also found to prenylate the drug pindolol (Visken®) and the antibiotic daptomycin (Cubicin®) producing a derivative with higher activity (Elshahawi et al., 2017).

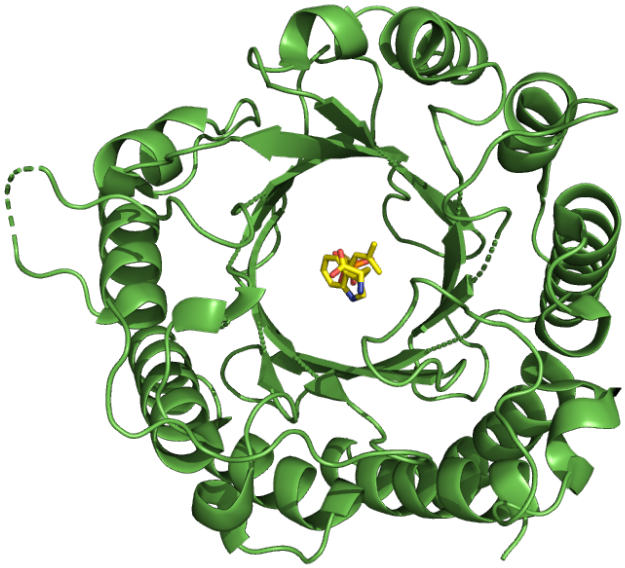

Figure 5. The X-ray ternary crystal structure of PriB enzyme with substrates, L-Trp (acceptor) and DMSPP (donor analog) in yellow sticks

In this study, the examination of the binding pocket of PriB X-ray crystal structure (Figure 6) showed that both the native donor and acceptor are in proximity with multiple residues including His312, Tyr380, Leu110, Trp165, Tyr181, Tyr364, Arg316, Phe220, and Leu293.

A basic amino acid residue suggests that it could be playing a role in the catalysis of the C6 nucleophilic substitution reaction (Murakami et al., 2006; Guillén Schlippe & Hedstrom, 2005; Metzger et al., 2009). Furthermore, we determined that the closest position of the nitrogen atom of the imidazole ring of His312 is with C6 L-Trp (3.7 Å) (Figure 7). Also, the structure alignments of PriB with other non C-6 PTs, AnaPT (C3-reverse, PDB ID 4LD7) TleC (C7-reverse, PDB ID 4YZL), MpnD (C7-reverse, PDB ID 4YLA), FgaPT2 (C3-normal, PDB ID 3I4X), FtmPT1(C2-normal, PDB ID 3O2K), and CdpNPT (C3-reverse, PDB ID 4E0U) showed the H312 in PriB are aligned with Tyr residues in these enzymes (Figure 9). Thus, we propose that site-directed mutagenesis of PriB His312 residue into other amino acid residues will alter the enzyme

regiospecificity. The goal of this work is to investigate the role of His312 in the regiospecificity of PriB and other C-6 indole PTs. This work will also shed some light on the mechanistic insights of aromatic PTs and other C-6-C-PTs, and the outcome of this study would be to utilize the altered regiospecificity.

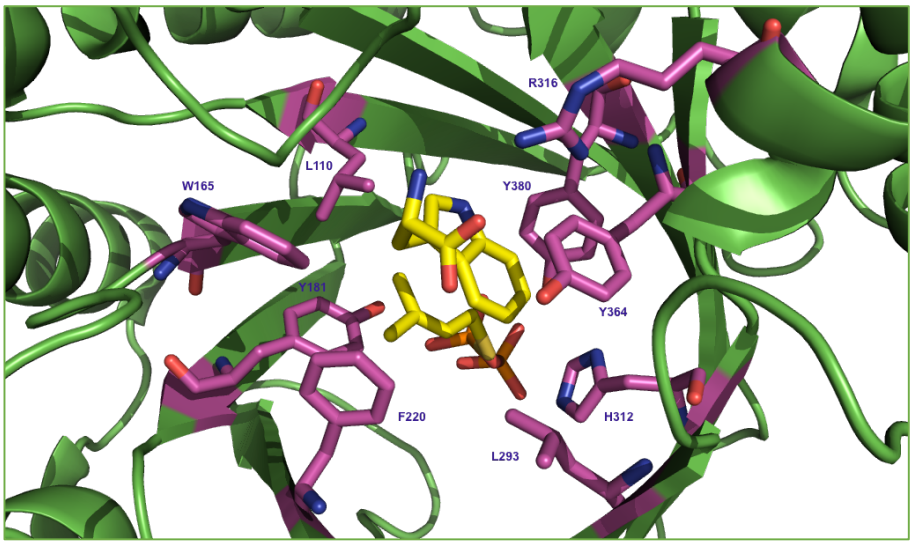

Figure 6. Exhibit close up view of the binding pocket, the binding pocket shows that both L-Trp and DMSPP (yellow sticks) in close proximity to each other and to PriB multiple residues (purple sticks).

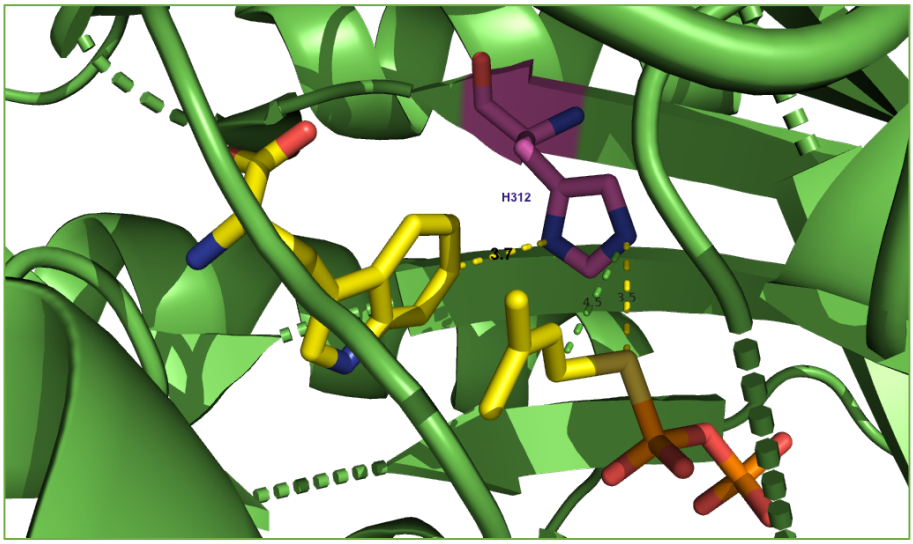

Figure 7. Show the nearest position of nitrogen atom of imidazole ring in His312 residue is with C6 L-Trp (3.7 Å).

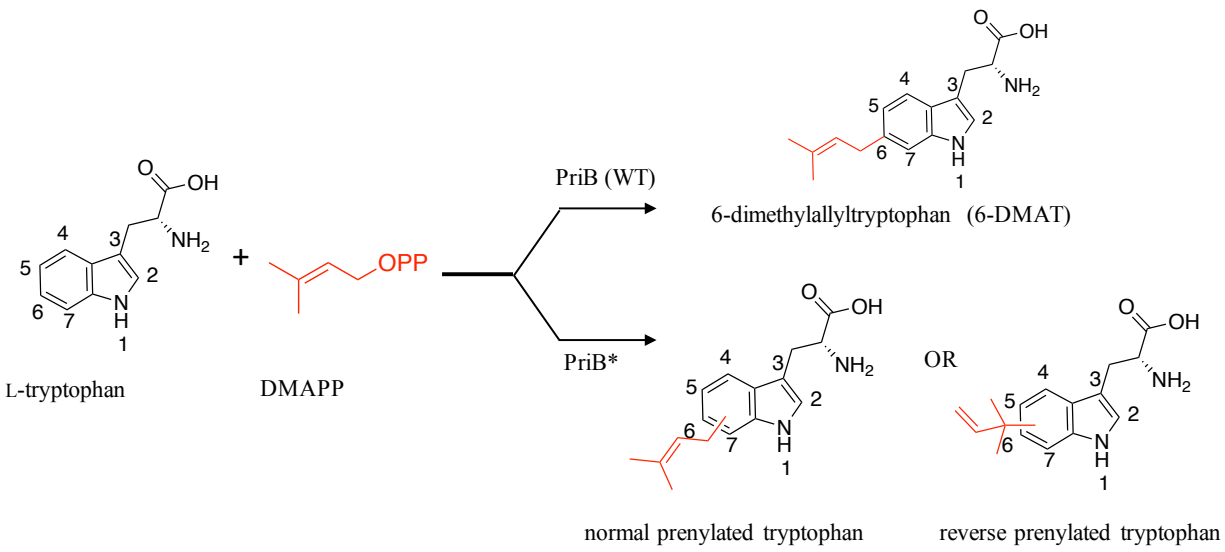

Figure 8. The catalyzed reaction of PriB PTs (WT), and the hypothetical reaction for mutant PriB

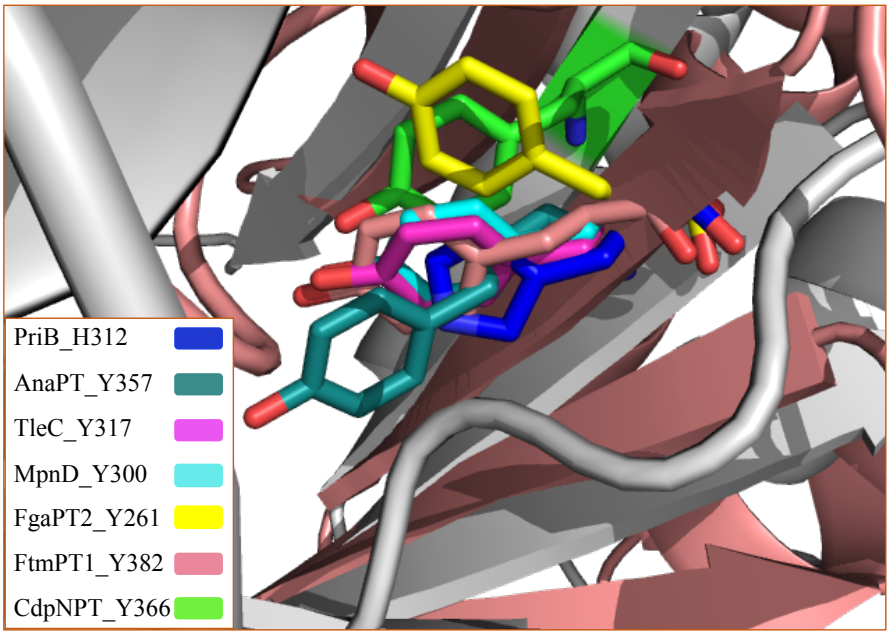

Figure 9. Structure alignment of PriB (C6-normal, PDB ID 5INJ), AnaPT (C3-reverse, PDB ID 4LD7) TleC (C7-reverse, PDB ID 4YZL), MpnD (C7-reverse, PDB ID 4YLA), FgaPT2 (C3-normal, PDB ID 3I4X), FtmPT1(C2-normal, PDB ID 3O2K), and CdpNPT (C3-reverse, PDB ID 4E0U) indole PTs showing conserved His312 in C-6-C-PTs representing another evidence of the involvement of His312 residue in the C6 prenylation regiospecificity. The crystal structures of PTs have been downloaded from Protein Data Bank, www.rcsb.org

Chapter 2
METHODS

2.1. Strains and Materials

PriB plasmid was obtained as a gift from the University of Kentucky, Center for Pharmaceutical Research and Innovation, Natural Products Repository. *E. coli* 5α and BL21 (DE3) competent cells were purchased from New England Biolabs. All reagents and chemicals were purchased from Sigma-Aldrich or Fisher Scientific. LB Broth (Miller) and LB Broth with agar (Lennox) were obtained from Sigma Aldrich (St. Louis, Missouri). DNA primers were obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa). All mutations were sequence verified at Retrogen, Inc (San Diego, California) or ACGT, Inc (Wheeling, Illinois). Gene analysis and alignments were performed using Geneious 11.1.5.

2.2. Plasmid Purification

The PriB plasmid was transformed into *E. coli* 5α cells and streaked on a plate containing LB agar and kanamycin with final concentration 80 μg mL^{-1}. The transformed cells were incubated at 37°C for 16 hr followed by inoculation into liquid medium (LB broth with kanamycin) at 37°C with 250 rpm for 16 hr. The cell pellet was collected by centrifugation at 4,500 ×g for 10 min. Genomic DNA was purified using Monarch® Nucleic Acid Purification Kits (New Englands BioLabs®) following the manufacturer's protocol. DNA quality and quantity were assessed using gel electrophoresis and Nanodrop (Thermo Scientific NanoDrop 2000 1-position Spectrophotometer).

2.3. Site Directed Mutagenesis

In order to perform site-directed mutagenesis, the primers of PriB_H312 were designed using NEBase Changer tool, as shown in Table 1 and were used for mutagenesis. Q5® Site-Directed

Mutagenesis Kit (New Englands BioLabs®) was performed following the manufacturer's protocol and transformed into 5α cells. Purified plasmids were isolated, purified and quantified. Quality of purified plasmids were assessed by measuring the $A_{260/280}$ and $A_{260/230}$. Plasmids of suitable quantity and high quality were sent for DNA sequencing. The correct plasmids, as confirmed by sequencing, were transformed into BL21(DE3) incubated onto LB agar with kanamycin (80 μg mL^{-1}) plates and incubated overnight at 37°C. After checking the cells growth, one colony from each strain was inoculated into 15 mL culture tube containing 4 mL liquid medium (LB with kanamycin, 80 μg mL^{-1}) and incubated for 16 hr, at 37°C, 250 rpm. Glycerol stocks of each construct containing 15% glycerol were stored in at −80°C for future use.

Table 1. *Designed primers in order to mutate PriB_H312. Residues targeting His312 residue are underlined.*

DNA Primer	Sequences
PriB_H312A-F	5'-CTA CAC CCT CGC TGT GCC GGT CCG CGA CTA CGT CCG G-3'
PriB_H312R-F	5'-CTA CAC CCT CAG AGT GCC GGT CCG CGA CTA CGT CCG-3'
PriB_H312N-F	5'-CTA CAC CCT CAA TGT GCC GGT CCG CGA CTA C-3'
PriB_H312D-F	5'-CTA CAC CCT CGA TGT GCC GGT CCG CGA CTA C-3'
PriB_H312C-F	5'-CTA CAC CCT CTG TGT GCC GGT CCG CGA CTA CGT CCG G-3'
PriB_H312Q-F	5'-CTA CAC CCT CCA AGT GCC GGT CCG CG-3'
PriB_H312E-F	5'-CTA CAC CCT CGA AGT GCC GGT CCG CGA CTA CG-3'
PriB_H312G-F	5'-CTA CAC CCT CGG TGT GCC GGT CCG CGA CTA CGT CCG G-3
PriB_H312I-F	5'-CTA CAC CCT CAT TGT GCC GGT CCG CGA CTA CGT CCG G-3'
PriB_H312L-F	5'-CTA CAC CCT CTT AGT GCC GGT CCG CGA CTA CGT CCG G-3'
PriB_H312K-F	5'-CTA CAC CCT CAA AGT GCC GGT CCG CGA CTA C-3'

PriB_H312M-F	5’-CTA CAC CCT CAT GGT GCC GGT CCG CGA CTA CGT CCG GCA CGA CGG-3’
PriB_H312F-F	5’-CTA CAC CCT CTT TGT GCC GGT CCG CGA CTA CGT CCG G -3’
PriB_H312P-F	5’-CTA CAC CCT CCC TGT GCC GGT CCG CGA CTA CGT CC-3’
PriB_H312S-F	5’-CTA CAC CCT CTC AGT GCC GGT CCG CGA CTA CGT CCG GCA C-3’
PriB_H312T-F	5’-CTA CAC CCT CAC TGT GCC GGT CCG CGA CTA CGT CCG GCA C-3’
PriB_H312W-F	5’-CTA CAC CCT CTG GGT GCC GGT CCG CGA CTA CGT CCG G-3’
PriB_H312Y-F	5’-CTA CAC CCT CTA TGT GCC GGT CCG CGA C-3’
PriB_H312V-F	5’-CTA CAC CCT CGT AGT GCC GGT CCG CGA CTA CGT CCG GCA CG -3’
PriB_H312X-R	5’-CCG CTG GGC CGC CCG GTC-3’
6DMATSsa_H284Y-F	5’-CTT TAC CTT GTA TAT TCC GGT TCG TGA TTA TG-3’
6DMATSsa_H284Y-R	5’-CCG CTC GGA CGA CCA CTG-3’
6DMATSsv_H287Y-F	5’-TTA TAC GCT GTA TGT TCC CGT AAG-3’
6DMATSsv_H287Y-R	5’-CCA GAA GGA AGG CCA GTT-3’
6DMATSmo_H329Y-F	5’-TCT GAT CGC GTA TGT TTC TCT GCG-3’
6DMATSmo_H329Y-R	5’-CCA ACA CCG TCA CGC AGC-3’
6DMATSmo_Y277H-F	5’-TTA CTC TAT CCA TGT TCC GAT CCG TTC TTA CGT TAC C-3’
6DMATSmo_Y277H-R	5’-CCA ACC GGA CGG TCC GCA-3’

2.4. Protein Production

LB broth (1L) supplemented with kanamycin (80 μg mL^{-1}) was inoculated with 0.3% (v/v) of an overnight PriB_H312_X (Phe, Lys, Arg, Trp, Tyr) *E. coli* BL21 (DE3) at 37°C with shaking (250 rpm). Cultures were induced at OD_{600} of ~ 0.6–0.8 with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM final concentration) and allowed to grow for an additional 16 hr at 23°C. The cells were centrifuged, harvested and stored at −80°C until used. The proteins of other mutant

PriB_H312 plasmids were expressed following the same procedure, but on a small scale using 250 mL baffled flasks containing 100 mL LB/kanamycin.

2.5. Protein Purification

All following steps were carried out on the ice. Cells were allowed to thaw then subsequently lysed by sonication (Virtis VirSonic 475 with a microtip, 100W, 10 × 10 sec pulses, 20 sec between pulses). Insoluble debris was removed by centrifugation at 15,000 ×g for 1 hr. The supernatants were collected and filtered using 0.22 μm filters, and the desired N-His6-PriB mutant fusion proteins were purified via HiTrap nickel-nitrilotriacetic acid (Ni-NTA, Sigma) affinity chromatography using standard protocols with NGC™ 10 Medium-Pressure Chromatography Systems (BIO-RAD). Buffer exchange of each sample were performed using a PD-10 column (GE Healthcare) eluted with 50 mM Tris, 100 mM NaCl, pH 8.0 to yield the desired mutant PriB proteins. Fractions were collected and concentrated using Amicon Ultra Centrifuge columns 30,000 MWCO (EMD Millipore) and stored in 50 mM Tris, 100 mM NaCl, glycerol 10%, pH 8.0 at −80°C. Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Purity and presence of proteins were confirmed by SDS-PAGE gel (Blot™ 4-12% Bis-Tris Plus, Invitrogen) electrophoresis. The images of the gels were taken by ChemiDoc^{TM} Imaging system (Bio-Rad).

2.6. HPLC Methods

HPLC Method A—The method was developed on Shimadzu UHPLC (RX-SHMADZU-9020-Instrument). SUPELCO® Titan C-18 (1.9 μm, 15 cm × 2.1 mm) column (Sigma-Aldrich) [7% B for 2 min, gradient of 7% to 100% B for 6 min, 100% B for 3 min, 100% B to 7% B over 20

second, 7% B for over 4 min (A = ddH2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid) flow rate = 0.4 mL/min; A280]. Injection volume 20 µL.

HPLC Method B —The method was developed on Bruker Q-TOF LC/MS (HPLC). SUPELCO® Titan C-18 (1.9 µm, 5 cm × 2.1 mm) column (Sigma-Aldrich) [7% B for 2 min, gradient of 7% B to 100% B for 5 min, 100% B for 3 min, 100% B to 7% B for 20 second, 7% B for over 4 min (A = ddH2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid) flow rate = 0.4 mL/min; A280]. Injection volume 10 µL.

HPLC Method C— The method was developed on Shimadzu analytical HPLC (Prominence i, LC-2030 3D). Supelco Ascentis® C-18 (5 µm, 25 cm × 10 mm) column (Sigma-Aldrich) [17% B for 140 min, isocratic, (A = ddH2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid) flow rate = 4 mL/min; A280].

2.7. *In Vitro* Characterization of Mutant Proteins

Mutant PriB in vitro assays were conducted in PCR tubes (200 µL) in a volume of 25 µL Tris 50 mM (pH 8.0) containing a final concentration of 0.5 mM L-Trp, 0.5 mM DMAPP, and 1 µM PriB mutant. After pre-incubation of the reaction mixture at 37°C for 10 min, the reactions were initiated by the addition of enzyme and allowed to proceed for 16 hrs at 37°C. Reactions with positive and negative reactions with and without the wild type of PriB respectively were performed under the same conditions. The reactions were quenched by the addition of 25 µL MeOH and mixing followed by centrifugation (22,000 ×g, 15 min, 4°C) to remove precipitated protein. The supernatants were analyzed by HPLC-PDA using Method A and mass using Method B were used to calculate conversion rate based on the area of the substrate and the prenylated product peaks.

2.8. Large Scale *in Vitro* Reaction for Mutant Protein

For isolation and full characterization of prenylated L-Trp analogs, 1 mL reactions (×50) in 1.5 mL Eppendorf tubes containing 2.5 mM acceptor (L-Trp), 2.5 mM DMAPP, 12.5 μM mutant enzyme in 50 mM Tris (pH 8.0) were incubated for 16 hr at 37°C. Reactions were combined, terminated by mixing with an equal volume of MeOH, centrifuged to remove precipitated protein and dried under reduced pressure.

2.9. Generation of 6-DMATS$_{xx}$ Mutants

Codon optimized constructs of 6-DMATS$_{Mo}$, 6-DMATS$_{Sa}$, and 6-DMATS$_{Sv}$ were synthesized by Twist Bioscience (San Francisco, CA) and ligated into pET28a plasmids. Plasmids were purified as described with PriB. Site-directed mutagenesis was performed in the active site of other C-6 ArPTs including 6-DMATS$_{Sa}$_H284Y, 6-DMATS$_{Sv}$_H287Y, and 6-DMATS$_{Mo}$ (Y277H, H329Y, and double mutant Y277H_H329Y). The corresponding 6-DMATS$_{xx}$ proteins were produced and purified with the same methods described for PriB-H312X mutants. Proteins production were qualitatively and quantitatively assessed as described previously.

Chapter 3

RESULTS and DISCUSSION

3.1. Site Directed Mutagenesis

Saturation mutagenesis has been performed on PriB_H312 to mutate it to all other 19 amino acid residues. Thus we obtained PriB_H312X where X = Ala (A), Cys (C), Asp (D), Glu (E), Phe (F), Gly (G), Ile (I), Lys (K), Leu (L), Met (M), Asn (N), Pro (P), Gln (Q), Arg (R), Ser (S), Thr (T), Val (V), Trp (W) & Tyr (Y). The DNA mutants of the mutated PTs were confirmed by DNA sequencing (Figure 10).

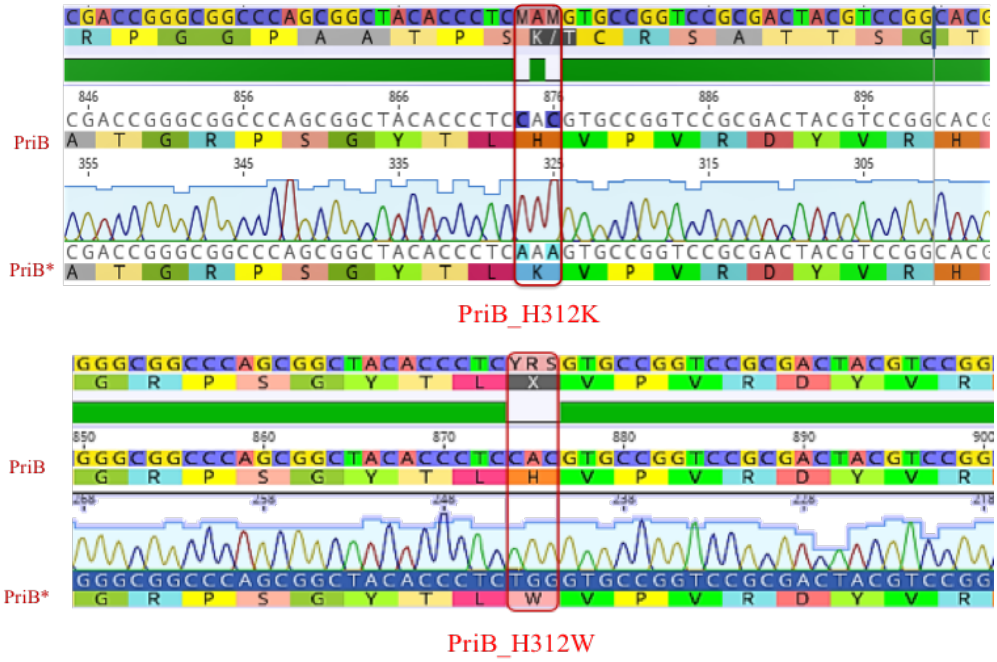

Figure 10. Representative sequencing results of some amino acids (Lys, Trp) showing successful mutations.

3.2. Protein Production and Purification

Attempts to produce all 19 PriB_H312X mutants were performed followed by purification using Ni-NTA resin column (Figure 11). The mutant proteins culture yield (other than Tyr mutant) were between 2 – 5 mg L^{-1}. The final concentrations of the mutated proteins were between 8 – 12 mg

mL^{-1}. The PriB mutant proteins were analyzed by SDS-PAGE (Figure 12). Only two mutants (PriB_H312D and PriB_H312P) did not show the protein bands. This lack of protein production indicates possible toxicity or stability issues. The molecular weight of the PriB was consistent with the calculated weight (43.34 kDa).

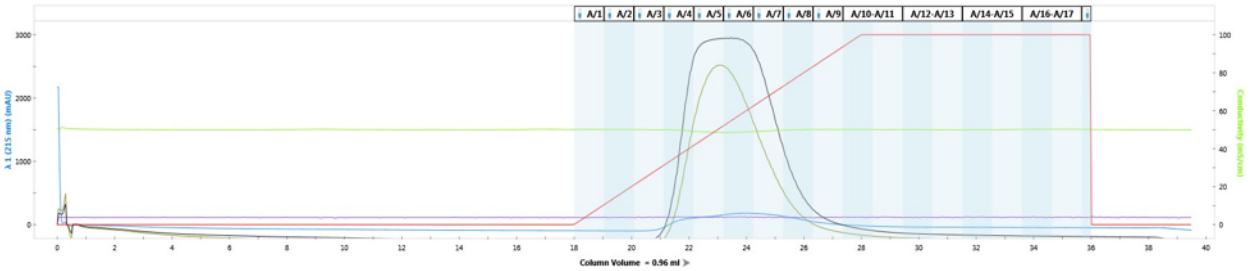

Figure 11. FPLC elution profile of PriB_H312Y crude protein. Column (Ni-NTA, Sigma) affinity chromatography. Buffer A: 10 mM Imidazole, 50 mM KH_2PO_4, 300 mM NaCl (pH 8.0). Buffer B: 250 mM Imidazole, 50 mM KH_2PO_4, 300 mM NaCl (pH 8.0). Flow rate: 1 mL/min

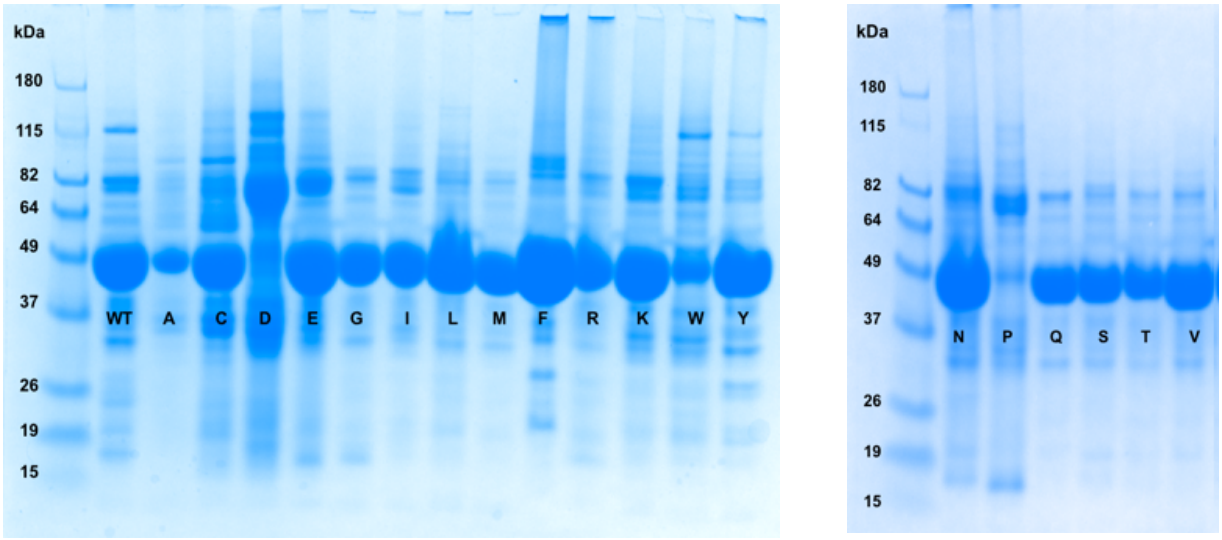

Figure 12. Analysis of the overproduced and purified PriB, and their mutants on SDS-PAGE

3.3. In Vitro Characterization of Mutant Proteins

HPLC analysis of the incubation mixture of L-Trp, DMAPP with PriB_H312Y protein showed that the enzymatic reaction produces more than one product in contrast to wild-type PriB which produces only one product 6-dimethylallyltryptophan (6-DMAT) (Figures 14 and 15) as previously reported (Elshahawi et al., 2017). Other PriB_H312X either lacked activity or produce minimal conversion than wild type except for PriB_H312X (G, E, and Q) where their conversion yield similar to wild type (Figure 13). Figure 16 exhibited LC-MS data of PriB (WT) and PriB_H312Y prenylated compound. The chromatogram PriB WT shows only one compound eluted at 5.7 min with observed mass $[M+H]^+$ 273.1602, whereas the PriB_H312Y shows 3 compounds in different retention time 5.3 min, 5.4 min, and 5.7 min, with observed mass $[M+H]^+$ 273.1588, 273.1596, and 273.1596 respectively. The molecular weight for the prenylated product is 272.1525.

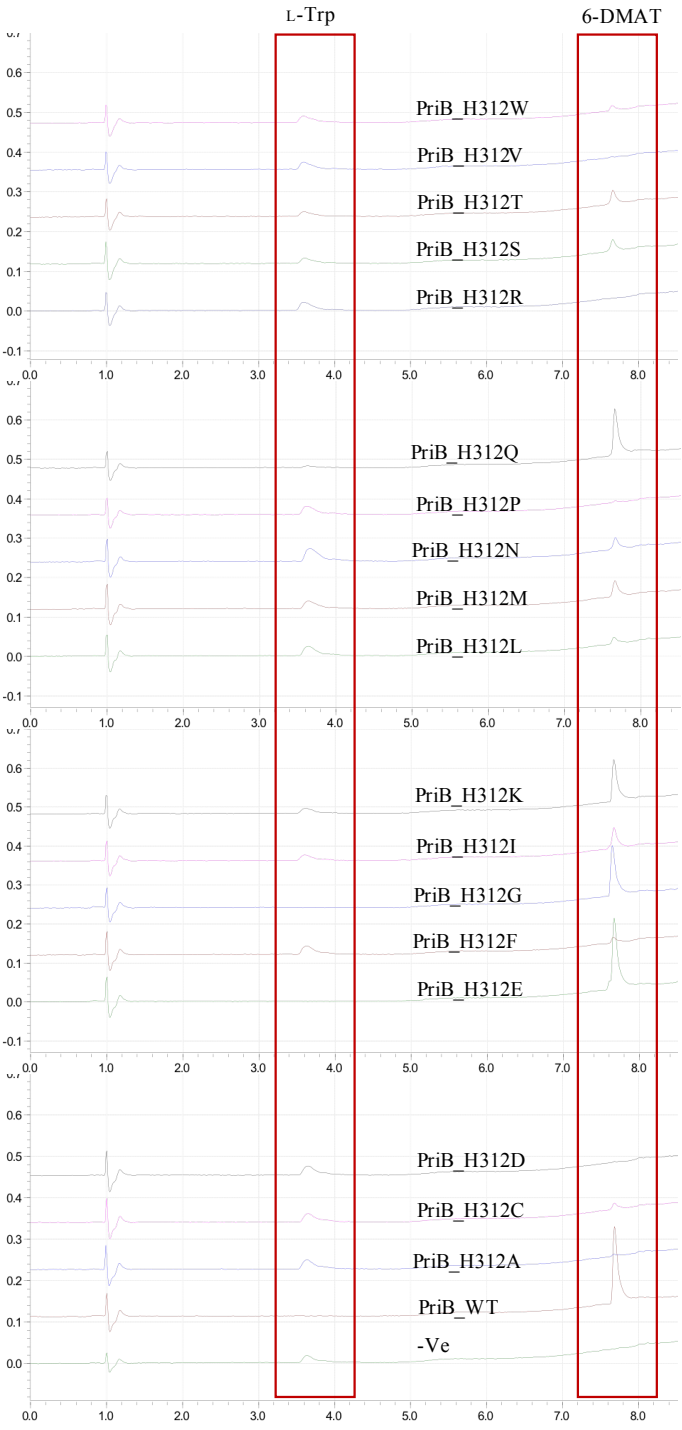

Figure 13. HPLC analysis of the PriB_H312X-mutants (other than Tyr) products, showed no new products other than C-6 prenylated products using Method A

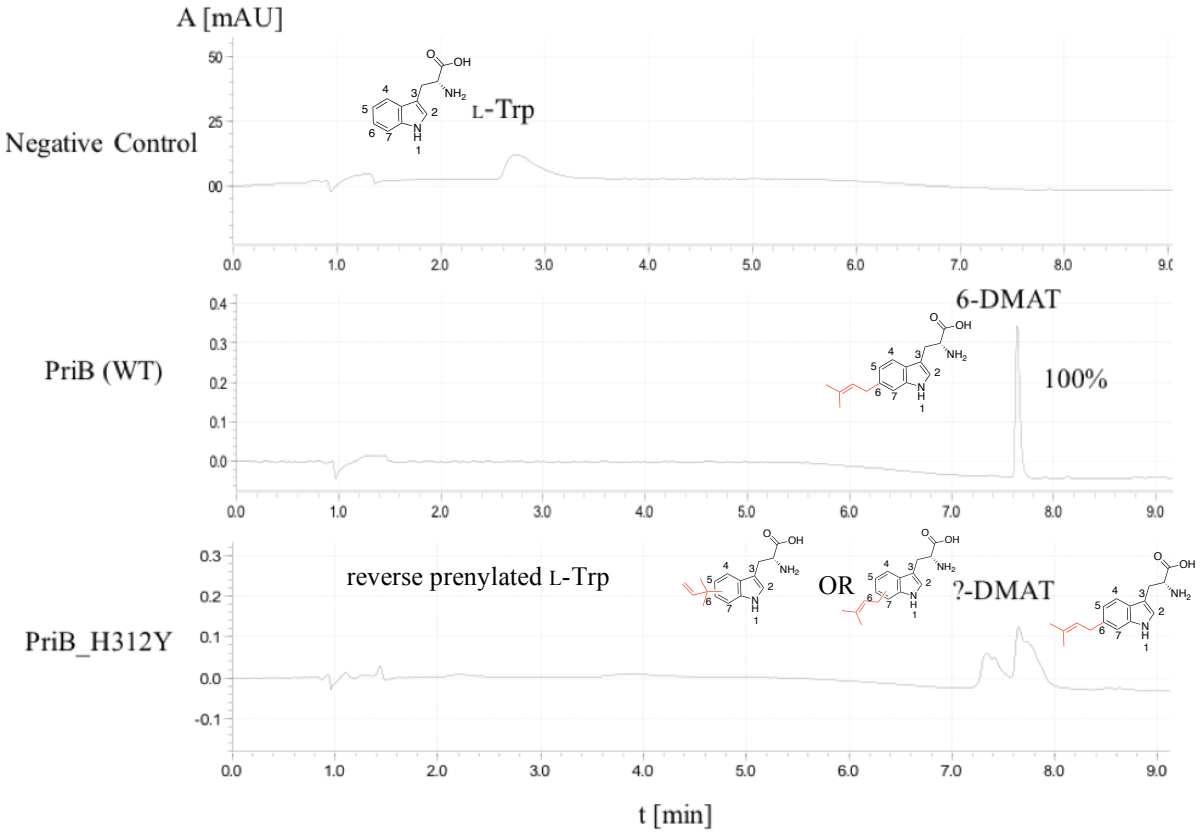

Figure 14. HPLC identification of the enzyme products of PriB and H\Y mutant. The enzymatic reaction consists of 50 mM Tris/ HCl (pH 8.0) including 0.5 mM L-Trp, 1 mM DMAPP, with 1 µM enzyme using Method A

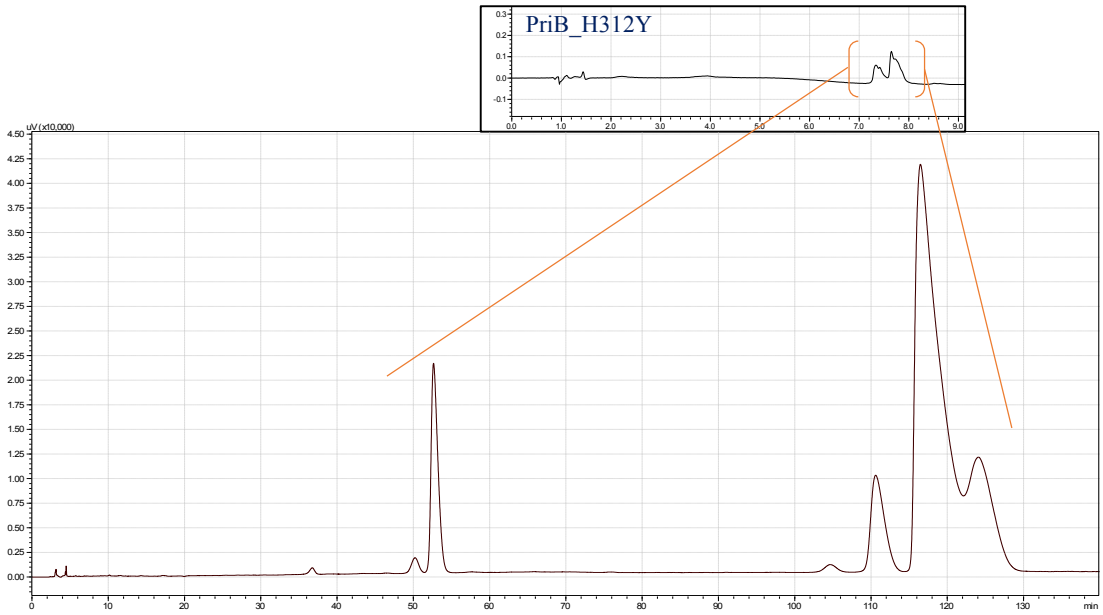

Figure 15. The enzyme products from the large-scale reaction was isolated and injected to HPLC (17% ACN, 0.1% FA for 140 min) to separate and collect the compounds using Method C

3.4. Q-TOF Mass (LC-MS)

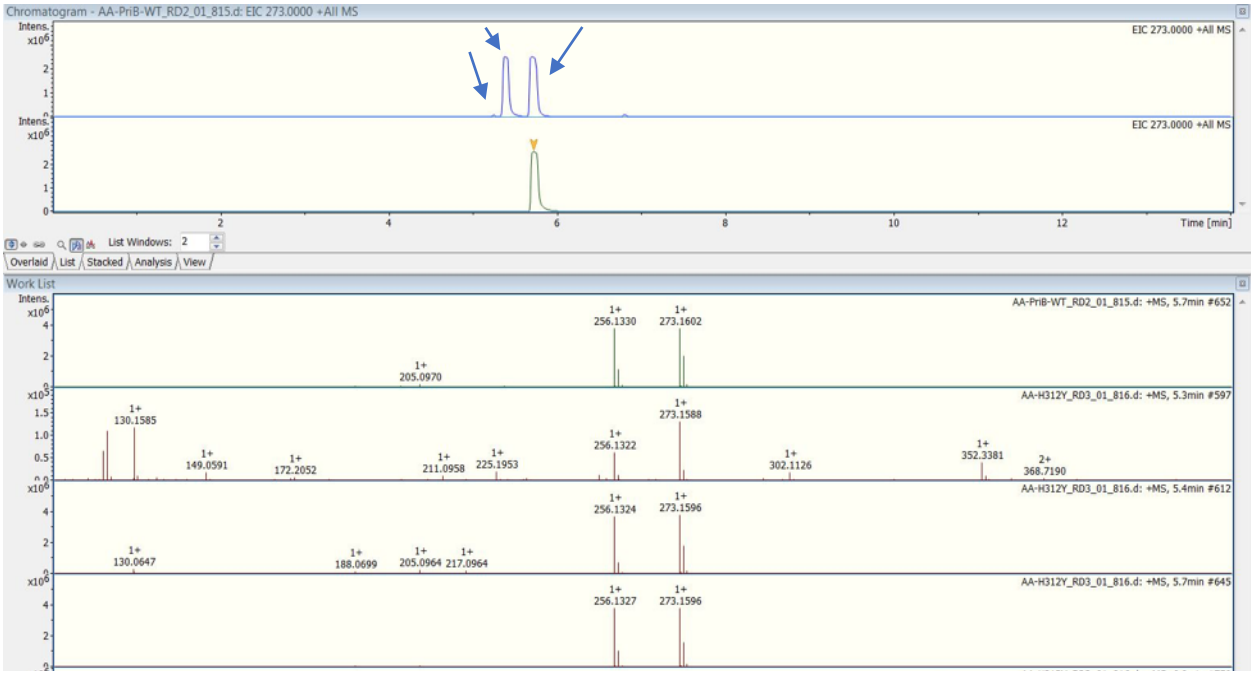

Figure 16. LC-MS spectra of PriB-WT and PriB-H312Y showed that the PriB-WT has only 1 prenylated compound with mass $[M+H]^+$ 273.1602. PriB-H312Y showed 3 compounds in different retention times (5.3 min, 5.4 min, and 5.7 min) with mass $[M+H]^+$ (273.1588), (273.1596), and (273.1596) respectively, using Method B**.**

3.5. Analysis of Binding Pockets of other C-6 PT

To see if the switch of the His residue to Tyr will provide the same results in other C-6-indole PTs could lead to the same PriB_H312Y results. Three C-6-indole PTs including 6-dimethylallyltryptophan synthase from bacteria *S. ambofaciens* (6-DMATS$_{Sa}$), 6-dimethylallyltryptophan synthase from bacteria *S. violaceusniger* (6-DMATS$_{Sv}$), and dimethylallyltryptophan synthase from bacteria *M. olivasterospora* (6-DMATS$_{Mo}$) were reported previously (Winkelblech & Li, 2014) (Winkelblech, Xie, & Li, 2016). However, no crystal structures are deposited in PDB. Thus, we generated homology models for the three proteins using SWISS-MODEL software. The structural alignment of these C-6-PTs have shown that His312 residue in PriB corresponds to H284 and H287 in 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$, respectively, but it corresponds to Y277 in 6-DMATSmo. However, 6-DMATS$_{Mo}$ has a His residue in the binding pocket (H329) which aligns with Tyr in the other analyzed 6-PTs (Figure 17).

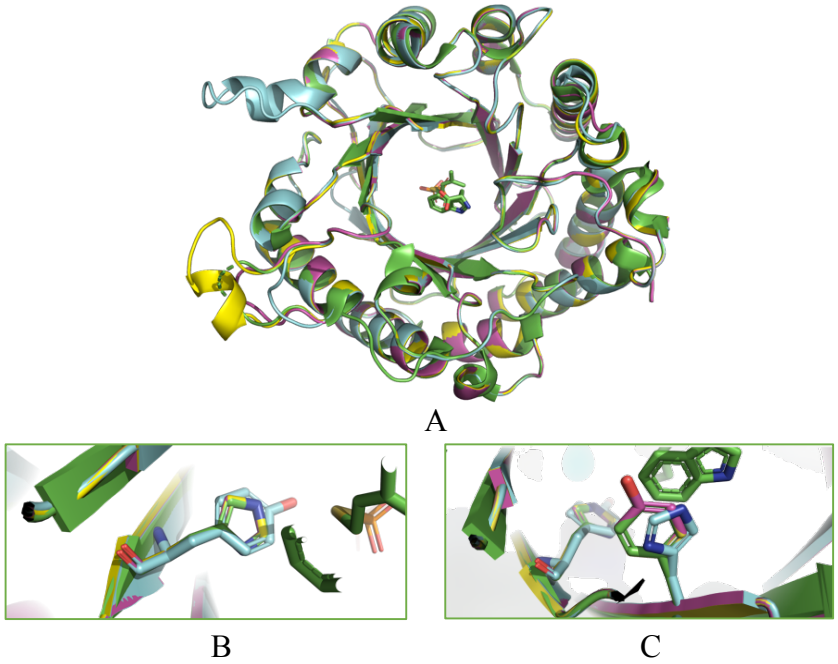

Figure 17. Structural alignment of PriB and homology models of 6-DMATS$_{Sa}$, 6- DMATS$_{Sv}$, and 6-DMATS$_{Mo}$. (B) A close up of His312 residue in PriB, and corresponding residues in other 6-DMATS enzymes showing the presence of His in 6- DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ but Tyr in 6-DMATS$_{Mo}$. (C) Histidine residue in 6-DMATS$_{Mo}$ aligns with a Tyr residue in the different region

3.6. Mutation of other C6-*C*-PTs

Site-directed mutagenesis was performed in the active site of other C-6 ArPTs including 6-DMATS$_{Sa}$_H284Y, 6-DMATS$_{Sv}$_H287Y, and 6-DMATS$_{Mo}$ (Y277H, H329Y, and double mutant Y277H and H329Y). The corresponding proteins were produced and purified. The presence of 6-DMATSx PTs (WT, and mutants) proteins were established via SDS-Page (Figure 18). The molecular weight of the 6-DMATS $_{Sa}$, 6-DMATS $_{Sv}$ and 6-DMATS$_{Mo}$ enzymes were 39.91 kDa, 40.87 kDa, and 40.59 kDa, respectively. HPLC analysis of the His mutant of the 6-indole-PTs utilizing the PriB_H312Y reaction condition, demonstrated His mutation to Tyr in these enzymes seems to produce compounds other than C-6 normal prenylated compound (Figure 19). Table 2 summarized the histidine\ tyrosine mutagenesis on the active site of PriB, 6-DMATS $_{Sa}$, 6-DMATS $_{Sv}$ and 6-DMATS$_{Mo}$, and their products.

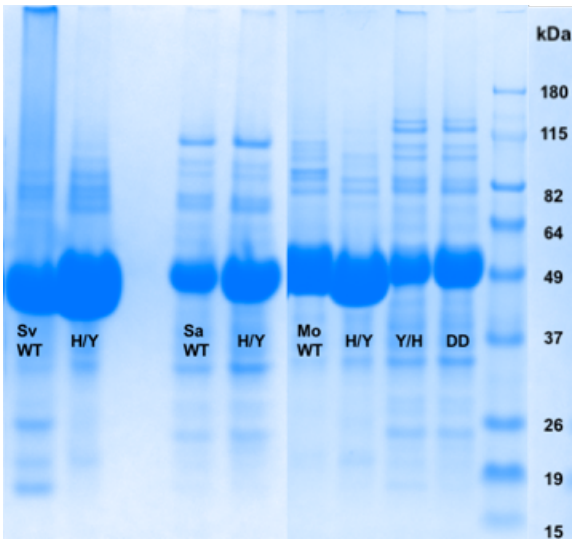

Figure 18. SDS-PAGE analysis for purified 6-DMATSx PTs and their mutant's proteins.

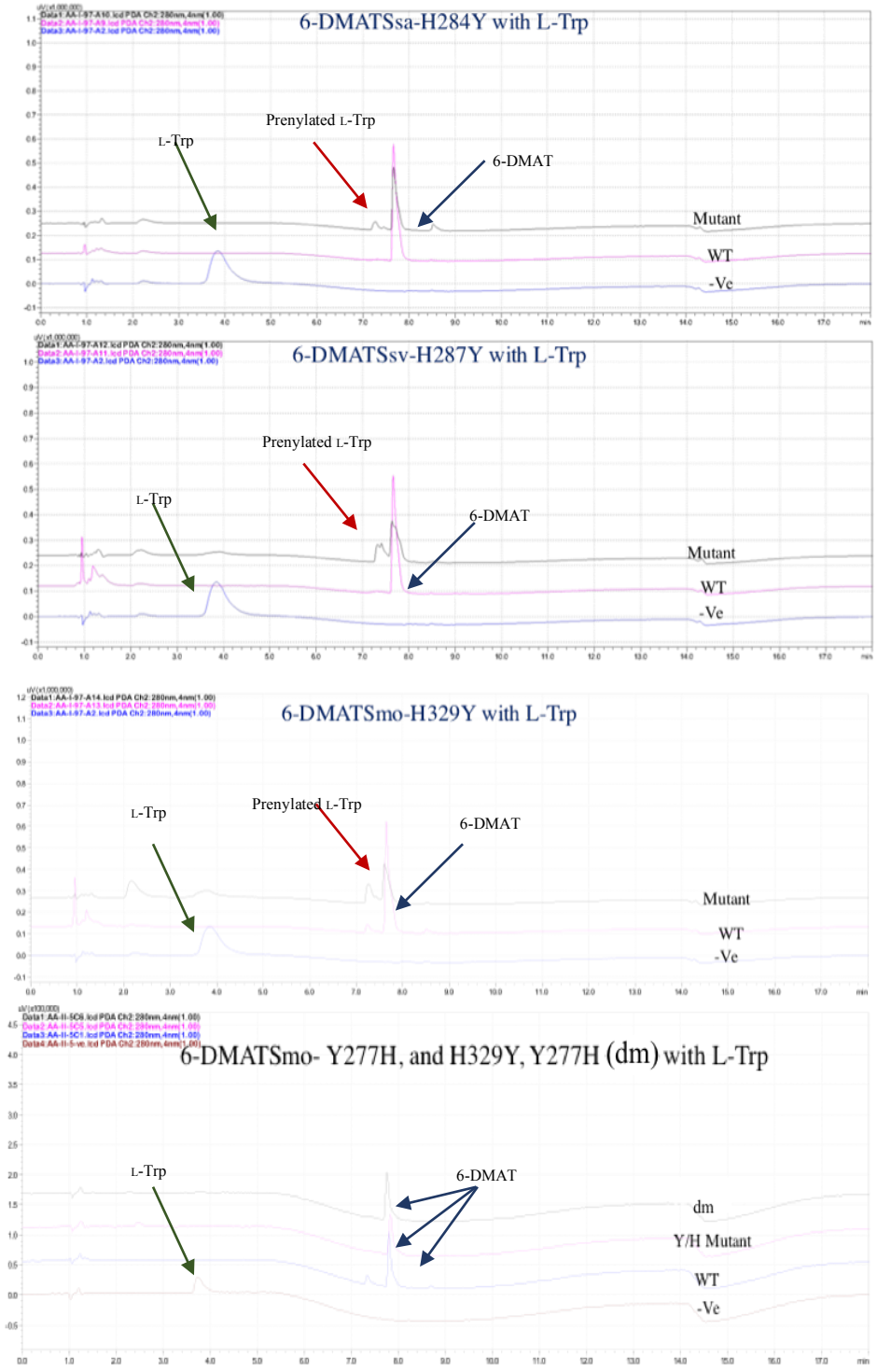

Figure 19. HPLC identification of the enzyme products of DMATS (sa, sv, mo) and their mutants. The enzymes (1 uM) were incubated (37°C in 16 hr) with 50 mM Tris/ HCl (pH 8.0), 1 mM DMAPP and 0.5mM of L-Trp using Method A

Table 2. *Summary of histidine and tyrosine mutations on ArPTs*

C-6 ArPT enzymes	PriB_H312 position	PriB_Y364 position	Products
PriB (WT)	His312	Tyr364	1 product
6-DMATS$_{Sa}$ (WT)	His284	Tyr336	1 product
6-DMATS$_{Sv}$ (WT)	His287	Tyr339	1 product
6-DMATS$_{Mo}$ (WT)	Tyr277	His329	More than 1 product
PriB_H312Y	Tyr312	Tyr364	~ 4 products
6-DMATS$_{Sa}$_H284Y	Tyr284	Tyr336	~ 4 products
6-DMATS$_{Sv}$_H287Y	Tyr287	Tyr339	~ 4 products
6-DMATS$_{Mo}$_Y277H	His277	His329	1 product
6-DMATS$_{Mo}$_H329Y	Tyr277	Tyr329	~ 4 products
6-DMATS$_{Mo}$_dm	His277	Tyr329	1 product

Structural analysis and site-directed mutagenesis have assisted in understanding the role of some residues within the PT active site as well as changing the regiospecificity or substrate selectivity. A previous study in FgaPT2 showed the role of acid-base residues during prenylation (Glu89 and Lys174) (Figure 21). FgaPT2_Glu89 probably have a significant role in enhancing the electrophilic substitution reaction of the L-Trp indole nucleus by forming a hydrogen bond between the indole N-H and the carboxylate group of Glu89. Mutation of Glu89 to alanine has completely abolished the activity, which shows the importance of this residue in the reaction mechanism (Metzger et al., 2009). FgaPT2_Glu89 corresponds to PriB_Glu94 (Figure 21). The Glu89 in FgaPT2 also corresponds to other PTs such as Glu116 in CdpNPT, Glu102 in FtmPT1, Glu89 in TleC and Glu106 in MpnD (Metzger et al., 2009). This shows the importance of Glu residue at

this position in most PTs. FgaPT2_Lys174 is placed conveniently in the catalytic binding pocket to catalyze prenylation during the formation of the final prenylated product. Furthermore, the indole ring of L-Trp substrate and the aromatic ring of Tyr345 in FgaPT2 sandwich the prenyl moiety of DMAPP. These interactions maintain the carbocation intermediate via cation–π interactions which expedite the first ionization stride (Tanner, 2015). Substitution of Lys174 to other residue diminished the activity of the biocatalysts. Interestingly, mutation of Lys174 to alanine has produced a new compound by changing the prenylation from normal C-4 to reverse C-3 (Luk et al., 2011).

In this study, we have analyzed the PriB PTs active site. Among all the key residues that surround the substrate, we have chosen the potential residue that could control the regiospecificity, which we proposed to be His312. Noticeably, histidine residue is conserved in other C-6 prenylation PTs, for instance, 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$. Also, the proximity of the basic residue His312 to C-6 of the indole ring in the L-Trp substrate, and the role of the basic residue histidine that seems to play a major role in the mechanism of reaction in ArPTs enzymes make it the first candidate for mutagenesis. Figure 20 demonstrates a proposed mechanism of the PriB enzymatic reaction based on the previous study on FgaPT2 enzyme. We propose that Glu94 will form a hydrogen bond with the nearby nitrogen atom of the Trp indole ring. Moreover, we propose that His312 plays a role as a general base by abstracting the proton from the indole ring during catalysis (Figure 21) as the case with FgaPT2_Lys174. It is worth to mention that histidine and tyrosine residues have shown significant roles in the catalysis mechanism in other enzymes (non-PT). For example, two histidine residues (His47, His12) have shown essential role in the acid-base reaction mechanism of sphingomyelinases D (spider venom) to hydrolyze sphingomyelin (Murakami et al., 2006). In addition, the tyrosine (Tyr166) residue also showed an important role in *E. coli* 2,4-dienoyl-CoA

reductase enzyme. The Tyr166 donate a proton to the C4 atom during substrate reduction. Remarkably, replacement of Tyr166 to phenylalanine generated a new compound 3-decenoyl-CoA instead of 2,4-dienoyl-CoA (Tu et al., 2008). These studies show the significance of His and Tyr residues in abstracting and donating hydrogens in catalysis.

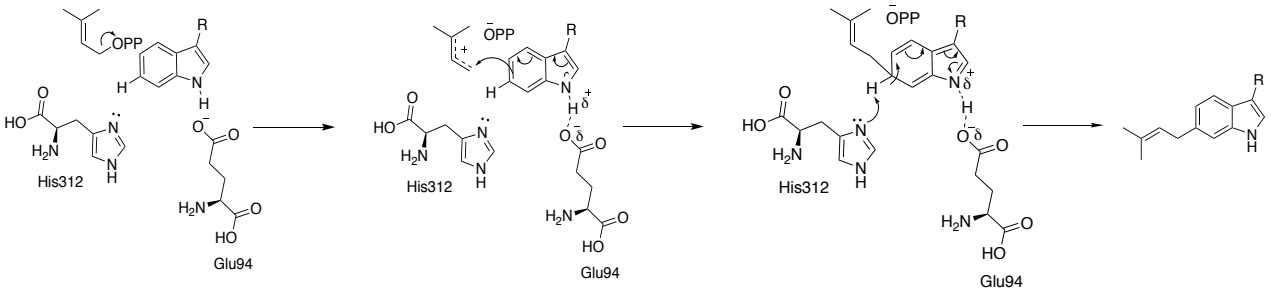

Figure 20. Theoretical enzymatic catalysis of DMATS in PriB

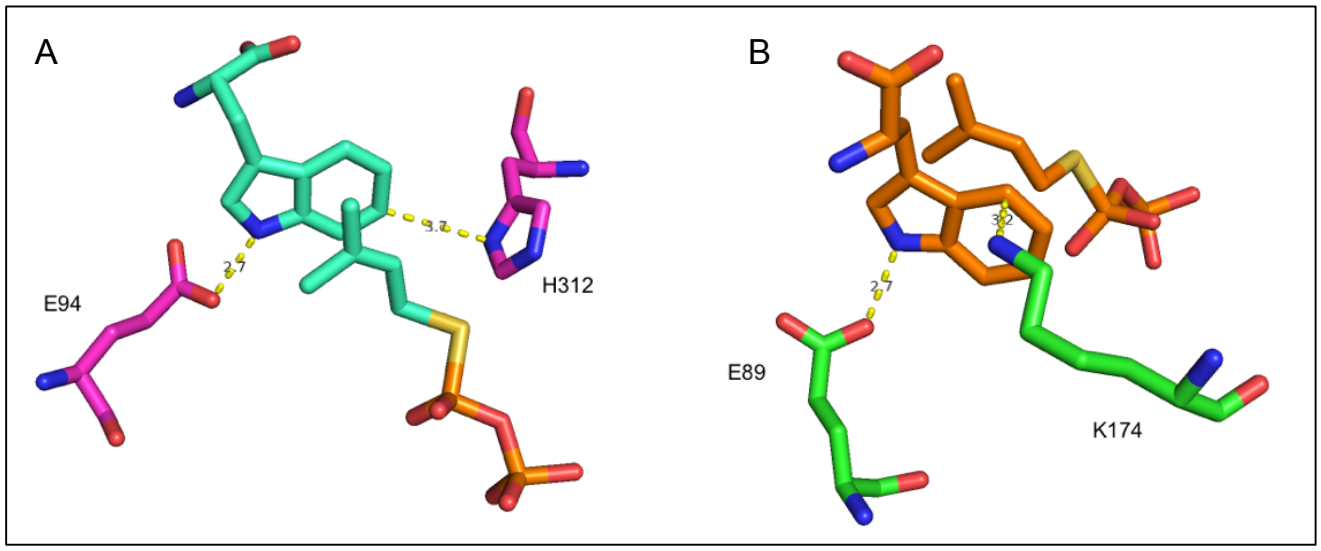

Figure 21. A (PriB), and B (FgaPT2) shows that both E94 (PriB) and E89 (FgaPT2) carboxylate group bind to indole N-H via hydrogen bond (2.7 Å for both enzymes), also the nearest amino acid residues H312 and K174 to the prenylation position C-6 (3.7 Å) for PriB and C-4 (3.2 Å) for FgaPT2

Figure 22 shows the structure alignment of PriB wt and PriB_H312Y. There are different possibilities that could explain the loss of regiospecificity in PriB_H312Y such as [1] PriB_His312

play a role in the catalytic reaction as proposed in Figure 20, is strategically distant from C-6 only on the indole ring and not other sites. Mutation of His to the larger aromatic ring in Tyr would extend to other C position such as C-5 and C-7. [2] the phenyl ring of Tyr (6- membered ring) differ than His imidazole ring (5- membered ring), therefore the π–π interactions of the Tyr residue could shift the substrate positions allow to produce different isomers. [3] His has been shown to abstract and donate protons during catalysis while Tyr role donates a proton. This suggests that a different mechanism might have occurred to produce the other derivatives. [4] His312 could be participating in attacking the DMAPP (donor) and stabilizing normal prenylation, whereas Tyr could stabilize normal/reverse or only reverse prenylation.

The rationale behind the fact that the activity of His312 mutants such as Gly, Glu, and Gln did not affect the catalytic activity is unclear, and it needs further study to understand the role of these mutations in catalysis.

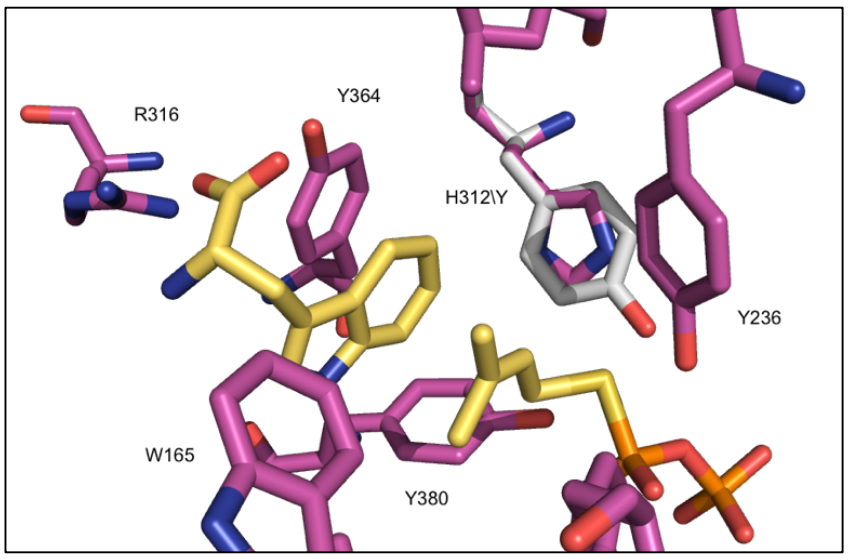

Figure 22. Structure analog of mutation of His312 to Tyr (gray stick) in the PriB active site

Chapter 4
CONCLUSION

This study exhibited the importance of His312 in the catalytic mechanism of PriB PTs. Structure-based enzyme engineering on PriB His312 to Tyr successfully altered the prenylation preference from C-6 normal to form other isomers. Also, His mutation on other C-6 indoles PTs 6-DMATS$_{Sa}$, 6-DMATS$_{Sv}$, and 6-DMATS$_{Mo}$ demonstrated other than single prenylated compound. Indeed, further work needed to finish this study, including purification and characterization of the newly formed compounds. Future goals would be to examine the permissiveness of mutant PriB enzymes for drug diversification purposes.

References

Alhassan, A. M., Abdullahi, M. I., Uba, A., & Umar, A. (2014). Prenylation of Aromatic Secondary Metabolites: A New Frontier for Development of Novel Drugs. *Tropical Journal of Pharmaceutical Research*, *13*(2), 307–314.

Almhjell, P. J., Boville, C. E., & Arnold, F. H. (2018). Engineering enzymes for noncanonical amino acid synthesis. *Chemical Society Reviews*, *47*(24), 8980–8997. https://doi.org/10.1039/c8cs00665b

Alwaseem, H., Frisch, B. J., & Fasan, R. (2018). Anticancer activity profiling of parthenolide analogs generated via P450-mediated chemoenzymatic synthesis. *Bioorganic & Medicinal Chemistry*, *26*(7), 1365–1373. https://doi.org/10.1016/j.bmc.2017.08.009

Arnold, F. H. (2018). Directed Evolution: Bringing New Chemistry to Life. *Angewandte Chemie International Edition*, *57*(16), 4143–4148. https://doi.org/10.1002/anie.201708408

Bartmańska, A., Tronina, T., Popłoński, J., Milczarek, M., Filip-Psurska, B., & Wietrzyk, J. (2018). Highly Cancer Selective Antiproliferative Activity of Natural Prenylated Flavonoids. *Molecules*, *23*(11), 2922. https://doi.org/10.3390/molecules23112922

Bekker, V., Dodd, A., Brady, D., & Rumbold, K. (2014). Tools for metabolic engineering in Streptomyces. *Bioengineered*, *5*(5), 293–299. https://doi.org/10.4161/bioe.29935

Bilal, M., Iqbal, H. M. N., Guo, S., Hu, H., Wang, W., & Zhang, X. (2018). State-of-the-art protein engineering approaches using biological macromolecules: A review from immobilization to implementation view point. *International Journal of Biological Macromolecules*, *108*, 893–901. https://doi.org/10.1016/j.ijbiomac.2017.10.182

Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., & Robins, K. (2012). Engineering the third wave of biocatalysis. *Nature*, *485*(7397), 185–194. https://doi.org/10.1038/nature11117

Botta, B., Monache, G. D., Menendez, P., & Boffi, A. (2005). Novel prenyltransferase enzymes as a tool for flavonoid prenylation. *Trends in Pharmacological Sciences*, *26*(12), 606–608. https://doi.org/10.1016/j.tips.2005.09.012

Botta, B., Vitali, A., Menendez, P., Misiti, D., & Delle Monache, G. (2005). Prenylated flavonoids: Pharmacology and biotechnology. *Current Medicinal Chemistry*, *12*(6), 717–739.

Bro, C., & Nielsen, J. (2004). Impact of "ome" analyses on inverse metabolic engineering. *Metabolic Engineering*, *6*(3), 204–211. https://doi.org/10.1016/j.ymben.2003.11.005

Cho, I., Jia, Z.-J., & Arnold, F. H. (2019). Site-selective enzymatic C–H amidation for synthesis of diverse lactams. *Science (New York, N.Y.)*, *364*(6440), 575–578. https://doi.org/10.1126/science.aaw9068

Elshahawi, S. I., Cao, H., Shaaban, K. A., Ponomareva, L. V., Subramanian, T., Farman, M. L., … Singh, S. (2017). Structure and specificity of a permissive bacterial C-prenyltransferase. *Nature Chemical Biology*, *13*(4), 366–368. https://doi.org/10.1038/nchembio.2285

Fan, A., & Li, S.-M. (2013). One Substrate – Seven Products with Different Prenylation Positions in One-Step Reactions: Prenyltransferases Make it Possible. *Advanced Synthesis & Catalysis*, *355*(13), 2659–2666. https://doi.org/10.1002/adsc.201300386

Fan, A., & Li, S.-M. (2016). Saturation mutagenesis on Arg244 of the tryptophan C4-prenyltransferase FgaPT2 leads to enhanced catalytic ability and different preferences for

tryptophan-containing cyclic dipeptides. *Applied Microbiology and Biotechnology*, *100*(12), 5389–5399. https://doi.org/10.1007/s00253-016-7365-3

Fan, A., Winkelblech, J., & Li, S.-M. (2015). Impacts and perspectives of prenyltransferases of the DMATS superfamily for use in biotechnology. *Applied Microbiology and Biotechnology*, *99*(18), 7399–7415. https://doi.org/10.1007/s00253-015-6813-9

Fan, A., Zocher, G., Stec, E., Stehle, T., & Li, S.-M. (2015). Site-directed mutagenesis switching a dimethylallyl tryptophan synthase to a specific tyrosine C3-prenylating enzyme. *The Journal of Biological Chemistry*, *290*(3), 1364–1373. https://doi.org/10.1074/jbc.M114.623413

González-Sabín, J., Morán-Ramallal, R., & Rebolledo, F. (2011). Regioselective enzymatic acylation of complex natural products: Expanding molecular diversity. *Chemical Society Reviews*, *40*(11), 5321–5335. https://doi.org/10.1039/C1CS15081B

Grünewald, J., & Marahiel, M. A. (2006). Chemoenzymatic and Template-Directed Synthesis of Bioactive Macrocyclic Peptides. *Microbiology and Molecular Biology Reviews*, *70*(1), 121–146. https://doi.org/10.1128/MMBR.70.1.121-146.2006

Guillén Schlippe, Y. V., & Hedstrom, L. (2005). A twisted base? The role of arginine in enzyme-catalyzed proton abstractions. *Archives of Biochemistry and Biophysics*, *433*(1), 266–278. https://doi.org/10.1016/j.abb.2004.09.018

Hammer, S. C., Knight, A. M., & Arnold, F. H. (2017). Design and evolution of enzymes for non-natural chemistry. *Current Opinion in Green and Sustainable Chemistry*, *7*, 23–30. https://doi.org/10.1016/j.cogsc.2017.06.002

Heide, L. (2009). Prenyl transfer to aromatic substrates: Genetics and enzymology. *Current Opinion in Chemical Biology*, *13*(2), 171–179. https://doi.org/10.1016/j.cbpa.2009.02.020

Hughes, R. R., Shaaban, K. A., Zhang, J., Cao, H., Phillips, G. N., & Thorson, J. S. (2017). OleD Loki as a Catalyst for Tertiary Amine and Hydroxamate Glycosylation. *Chembiochem: A European Journal of Chemical Biology*, *18*(4), 363–367. https://doi.org/10.1002/cbic.201600676

Katz, L., & Baltz, R. H. (2016). Natural product discovery: Past, present, and future. *Journal of Industrial Microbiology & Biotechnology*, *43*(2), 155–176. https://doi.org/10.1007/s10295-015-1723-5

Kokkonen, P., Bednar, D., Pinto, G., Prokop, Z., & Damborsky, J. (2019). Engineering enzyme access tunnels. *Biotechnology Advances*. https://doi.org/10.1016/j.biotechadv.2019.04.008

Lam, K. S. (2007). New aspects of natural products in drug discovery. *Trends in Microbiology*, *15*(6), 279–289. https://doi.org/10.1016/j.tim.2007.04.001

Lin S, Huang T, & Shen B. (2012). Tailoring Enzymes Acting on Carrier Protein-Tethered Substrates in Natural Product Biosynthesis. *Methods in Enzymology*, *516*, 321–343. https://doi.org/10.1016/B978-0-12-394291-3.00008-3

Luk, L. Y. P., Qian, Q., & Tanner, M. E. (2011). A Cope Rearrangement in the Reaction Catalyzed by Dimethylallyltryptophan Synthase? *Journal of the American Chemical Society*, *133*(32), 12342–12345. https://doi.org/10.1021/ja2034969

Medema, M. H., Kottmann, R., Yilmaz, P., Cummings, M., Biggins, J. B., Blin, K., … Glöckner, F. O. (2015). Minimum Information about a Biosynthetic Gene cluster. *Nature Chemical Biology*, *11*, 625–631. https://doi.org/10.1038/nchembio.1890

Menzella, H. G., & Reeves, C. D. (2007). Combinatorial biosynthesis for drug development. *Current Opinion in Microbiology*, *10*(3), 238–245. https://doi.org/10.1016/j.mib.2007.05.005

Metzger, U., Schall, C., Zocher, G., Unsöld, I., Stec, E., Li, S.-M., … Stehle, T. (2009). The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(34), 14309–14314. https://doi.org/10.1073/pnas.0904897106

Mori, S., Pang, A. H., Thamban Chandrika, N., Garneau-Tsodikova, S., & Tsodikov, O. V. (2019). Unusual substrate and halide versatility of phenolic halogenase PltM. *Nature Communications*, *10*(1), 1255. https://doi.org/10.1038/s41467-019-09215-9

Mori, T., Zhang, L., Awakawa, T., Hoshino, S., Okada, M., Morita, H., & Abe, I. (2016). Manipulation of prenylation reactions by structure-based engineering of bacterial indolactam prenyltransferases. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms10849

Murakami, M. T., Freitas Fernandes-Pedrosa, M., de Andrade, S. A., Gabdoulkhakov, A., Betzel, C., Tambourgi, D. V., & Arni, R. K. (2006). Structural insights into the catalytic mechanism of sphingomyelinases D and evolutionary relationship to glycerophosphodiester phosphodiesterases. *Biochemical and Biophysical Research Communications*, *342*(1), 323–329. https://doi.org/10.1016/j.bbrc.2006.01.123

Ncube, B., Ndhlala, A. R., & Van Staden, J. (2017). Secondary Metabolism and the Rationale for Systems Manipulation. In S. Jha (Ed.), *Transgenesis and Secondary Metabolism* (pp. 45–65). https://doi.org/10.1007/978-3-319-28669-3_23

Newman, D. J., & Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products*, *79*(3), 629–661. https://doi.org/10.1021/acs.jnatprod.5b01055

Newmister, S. A., Romminger, S., Schmidt, J. J., Williams, R. M., Smith, J. L., Berlinck, R. G. S., & Sherman, D. H. (2018). Unveiling sequential late-stage methyltransferase reactions in the meleagrin/oxaline biosynthetic pathway. *Organic & Biomolecular Chemistry*, *16*(35), 6450–6459. https://doi.org/10.1039/c8ob01565a

Romney, D. K., Arnold, F. H., Lipshutz, B. H., & Li, C.-J. (2018). Chemistry Takes a Bath: Reactions in Aqueous Media. *The Journal of Organic Chemistry*, *83*(14), 7319–7322. https://doi.org/10.1021/acs.joc.8b01412

Schmidt, N. G., Eger, E., & Kroutil, W. (2016). Building Bridges: Biocatalytic C–C-Bond Formation toward Multifunctional Products. *ACS Catalysis*, *6*(7), 4286–4311. https://doi.org/10.1021/acscatal.6b00758

Schmitt, E. K., Hoepfner, D., & Krastel, P. (2016). Natural products as probes in pharmaceutical research. *Journal of Industrial Microbiology & Biotechnology*, *43*(2), 249–260. https://doi.org/10.1007/s10295-015-1691-9

Tanner, M. E. (2015). Mechanistic studies on the indole prenyltransferases. *Natural Product Reports*, *32*(1), 88–101. https://doi.org/10.1039/c4np00099d

Tsai, H. F., Wang, H., Gebler, J. C., Poulter, C. D., & Schardl, C. L. (1995). The Claviceps purpurea gene encoding dimethylallyltryptophan synthase, the committed step for ergot

alkaloid biosynthesis. *Biochemical and Biophysical Research Communications*, *216*(1), 119–125. https://doi.org/10.1006/bbrc.1995.2599

Tu, X., Hubbard, P. A., Kim, J.-J. P., & Schulz, H. (2008). Two Distinct Proton Donors at the Active Site of Escherichia coli 2,4-Dienoyl-CoA Reductase Are Responsible for the Formation of Different Products. *Biochemistry*, *47*(4), 1167–1175. https://doi.org/10.1021/bi701235t

Walsh, C. T. (2014). Biological Matching of Chemical Reactivity: Pairing Indole Nucleophilicity with Electrophilic Isoprenoids. *ACS Chemical Biology*, *9*(12), 2718–2728. https://doi.org/10.1021/cb500695k

Wang, X., Reynolds, A. R., Elshahawi, S. I., Shaaban, K. A., Ponomareva, L. V., Saunders, M. A., … Thorson, J. S. (2015). Terfestatins B and C, New p-Terphenyl Glycosides Produced by Streptomyces sp. RM-5–8. *Organic Letters*, *17*(11), 2796–2799. https://doi.org/10.1021/acs.orglett.5b01203

Weissman, K. J. (2015). The structural biology of biosynthetic megaenzymes. *Nature Chemical Biology*, *11*(9), 660–670. https://doi.org/10.1038/nchembio.1883

Weist, S., & Süssmuth, R. D. (2005). Mutational biosynthesis—a tool for the generation of structural diversity in the biosynthesis of antibiotics. *Applied Microbiology and Biotechnology*, *68*(2), 141–150. https://doi.org/10.1007/s00253-005-1891-8

Wells, A. S., Finch, G. L., Michels, P. C., & Wong, J. W. (2012). Use of Enzymes in the Manufacture of Active Pharmaceutical Ingredients—A Science and Safety-Based Approach To Ensure Patient Safety and Drug Quality. *Organic Process Research & Development*, *16*(12), 1986–1993. https://doi.org/10.1021/op300153b

Wilkinson, B., & Micklefield, J. (2007). Mining and engineering natural-product biosynthetic pathways. *Nature Chemical Biology*, *3*(7), 379–386. https://doi.org/10.1038/nchembio.2007.7

Winkelblech, J., Fan, A., & Li, S.-M. (2015). Prenyltransferases as key enzymes in primary and secondary metabolism. *Applied Microbiology and Biotechnology*, *99*(18), 7379–7397. https://doi.org/10.1007/s00253-015-6811-y

Winkelblech, J., & Li, S.-M. (2014). Biochemical investigations of two 6-DMATS enzymes from Streptomyces reveal new features of L-tryptophan prenyltransferases. *Chembiochem: A European Journal of Chemical Biology*, *15*(7), 1030–1039. https://doi.org/10.1002/cbic.201400046

Winkelblech, J., Xie, X., & Li, S.-M. (2016). Characterisation of 6-DMATS$_{Mo}$ from Micromonospora olivasterospora leading to identification of the divergence in enantioselectivity, regioselectivity and multiple prenylation of tryptophan prenyltransferases. *Organic & Biomolecular Chemistry*, *14*(41), 9883–9895. https://doi.org/10.1039/c6ob01803c

Yin, J., Straight, P. D., Hrvatin, S., Dorrestein, P. C., Bumpus, S. B., Jao, C., … Walsh, C. T. (2007). Genome-Wide High-Throughput Mining of Natural-Product Biosynthetic Gene Clusters by Phage Display. *Chemistry & Biology*, *14*(3), 303–312. https://doi.org/10.1016/j.chembiol.2007.01.006

Zhang, R. K., Chen, K., Huang, X., Wohlschlager, L., Renata, H., & Arnold, F. H. (2019). Enzymatic assembly of carbon-carbon bonds via iron-catalysed sp3 C-H functionalization. *Nature*, *565*(7737), 67–72. https://doi.org/10.1038/s41586-018-0808-5

Zhou, K., Zhao, W., Liu, X.-Q., & Li, S.-M. (2016). Saturation mutagenesis on Tyr205 of the cyclic dipeptide C2-prenyltransferase FtmPT1 results in mutants with strongly increased C3-prenylating activity. *Applied Microbiology and Biotechnology*, *100*(23), 9943–9953. https://doi.org/10.1007/s00253-016-7663-9