ENGINEERING OF GERANYL DIPHOSPATE *C*-METHYLTRANSFERASE FOR THE DEVELOPMENT OF NEW DITERPENOID PRECURSORS

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ABSTRACT

ENGINEERING OF GERANYL DIPHOSPATE C-METHYLTRANSFERASE FOR THE DEVELOPMENT OF NEW DITERPENOID PRECURSORS

Terpenoids constitute the most diverse family of natural products. They are involved in several biological functions and are used in medical and industrial applications. The key to their diverse biological activities is their structural diversity. Terpenoids are synthesized in three stages, all of which contribute to generation of structural diversity. In the terpenoid biosynthetic pathways, terpene synthases generate larger linear terpenoid precursors from smaller units via condensation reactions, terpene cyclases transform precursors via cyclization reactions, and then tailoring enzymes modify terpenoid products via addition of functional groups. Recently discovered geranyl diphosphate *C*-methyltransferase (GPPMT) from *Streptomyces coelicolor* A3(2) is able to modify a linear monoterpenoid precursor, geranyl diphoshate (GPP), to produce a non-canonical terpenoid precursor, 2-methylgeranyl diphosphate. Modification of GPP by GPPMT is the first example of modification of a canonical linear isoprenoid precursor in nature. This study aims to achieve enzymatic synthesis of novel methylated non-canonical diterpenoid precursors, such as 2-methylgeranylgeranyl diphosphate (2MGGPP) by engineering GPPMT. The novel non-canonical precursors may later be utilized by cyclases to enhance the diversity of the terpenome. For example, taxadiene synthase could utilize 2MGGPP to generate variants of taxadiene, the precursor of the leading anti-cancer drug paclitaxel (Taxol®). Candidate mutants predicted to use GGPP as substrate were selected via *in silico* analysis of GPPMT structure. These mutations were introduced using the Quick-change site-directed mutagenesis. Mutant genes were expressed in *E.coli* strains. Mutant proteins were purified by Fast Protein Liquid Chromatography. Catalytic activities of mutants against canonical terpenoid precursors were determined by SAM methyltransferase assay.

ÖZET

YENİ DİTERPEN ÖNCÜLLERİ GELİŞTİRMEK AMACIYLA GERANİL DİFOSFAT *C*-METİLTRANSFERAZ ENZİMİNİN DEĞİŞTİRİLMESİ

İzoprenoidler yada terpenler olarak da bilinen terpenoidler, doğal ürünlerin en çeşitli ailesini oluştururlar. Birçok biyolojik işlevlere sahiptirler ve çeşitli endüstride ve tıpta kullanılırlar. Terpenoidlerin çeşitli biyolojik aktiviteleri yapısal çeşitliliklerinden kaynaklanır. Terpenoidler az sayıda öncülden üç safhada sentezlenirler, bu safhaların hepsi, yapısal çeşitliliğin oluşmasına katkıda bulunur. Terpenoid biyosentez yolaklarında, terpen sentaz enzimleri küçük birimlerden daha büyük doğrusal terpenoid öncüllerini kondensasyon tepkimeleri ile oluşturur, terpen siklaz enzimleri öncülleri halkalılaştırma tepkimeleri ile dönüştürür ve sonrasında ise rütuş enzimleri terpenoid ürünlere fonksiyonel gruplar ekleyerek modifiye eder. Son yıllarda keşfedilen *Streptomyces coelicolor* A3(2) kaynaklı geranil difosfat *C*-metiltransferaz (GPPMT) enziminin standart dışı olan 2-metilgeranil difosfatı (2MGPP) üretmek için standart bir doğrusal monoterpenoid olan geranil difosfatı (GPP) modifiye ettiği gösterilmiştir. GPP'nin GPPMT tarafından modifikasyonu, standart doğrusal izoterpenoid öncülünün modifikasyonun doğadaki ilk örneğidir. Bu çalışma GPPMT'nin değiştirilmesi ile 2-metilgeranilgeranil difosfat (2MGGPP) gibi diğer standart olmayan diterpenoidlerin enzimatik sentezini başarmayı amaçlamaktadır. Sonrasında, standart olmayan bu öncüller diterpen siklazlar tarafından terpenom (toplam terpen içeriği) çeşitliliğini artırmak için kullanılabilirler. Örneğin, taksadiyen sentaz yaygın bir kanser ilacı olan paklitaksel'in (Taksol®) öncülü olan taksadiyen'in değişik şekillerini üretmek için 2MGGPP'yi kullanabilirler. Substrat olarak GGPP'yi kullanacağı düşünülen aday GPPMT mutantları, GPPMT'nin yapısının bilgisayar ortamındaki analizleri ile seçildi. Bu mutasyonlar QuickChange bölgeye yönlendirilmiş mutasyon protokolü ile yapıldı. Mutant genler *E.coli* suşları kullanılarak ifade edildi. Proteinler hızlı protein sıvı kromatografisi ile saflaştırıldı. GPPMT mutantlarının terpenoid öncüllere karşı katalitik aktiviteleri SAM metiltransferaz yöntemi ile belirlendi.

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ABBREVIATIONS

GPPMT	Geranyl Diphosphate *C*-Methyltransferase
IPP	Isopentenyl Diphosphate
DMAPP	Dimethylallyl Diphosphate
GPP	Geranyl Diphosphate
FPP	Farnesyl Diphosphate
GGPP	Geranylgeranyl Diphosphate
GFPP	Geranylfarnesyl Diphospate
HPP	Heptaprenyl Diphospate
SAM	*S*-Adenosyl-L-Methionine
SAH	*S*-Adenosyl-L-Homocysteine
PDB	Protein Data Bank

CHAPTER 1

INTRODUCTION

1.1. Introduction

Terpenoids, also known as isoprenoids or terpenes, constitute the largest, the most diverse and the most significant family of natural products found in all life forms that possess nearly 70000 identified members ("Dictionary of Natural Products: http://dnp.chemnetbase.com,"). They serve numerous functions in biological systems; in plants as hormones, in signaling as pheromones and fragrance molecules; in natural pigmentation as beta-carotene and lycopene; in electron transport as plastoquinone; in biological membrane dynamics as beta-sitosterol and sterol; in photosynthesis as a part of chlorophyll molecule; in chemical defense as toxins, antimicrobials, antifungals and phytoalexins; in post-translational modification of proteins as prenyl groups (Davis & Croteau, 2000; Gershenzon & Dudareva, 2007). Terpenes are also significant in terms of economic value; they are used as medicinal ingredients including anti-cancer or antibiotic agents or as raw materials for various chemical industries (Davis & Croteau, 2000).

All terpenoids are derived from a few precursors such as five-carbon isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP) (Figure 1.1). Two cascades for production of the precursors are known. One is the mevalonate pathway, which operates in eucaryotes, archaebacteria, and cytosols of higher plants. The other is a recently discovered pathway, the nonmevalonate pathway, which is used by many eubacteria, green algae, and chloroplasts of higher plants. In the first stage, larger linear terpenoid precursors are generated by addition of a number of IPP molecules to a DMAPP molecule (Cane, 1999). In the second stage, cyclic or acyclic terpenoids are generated by transformation of these linear terpenoid precursors. In the third stage, terpenoid structures are diversified by addition of various functional groups to the cyclic or acyclic terpenoid cores (Figure 1.1) (Cane & Ikeda, 2012).

1.2. Generation of Linear Precursors

In first stage of the terpenoid biosynthesis, two kinds of condensation mechanism are used. One is that head-to-tail condensation of smaller precursors yield larger isoprenoid diphosphates. For instance, enzyme-catalyzed addition of IPP to DMAPP in a "head to tail" fashion gives rise to major isoprenoid diphosphates. The other is that head-to-head condensation of linear precursors yield larger non-phosphorylated isoprenoids (Davis & Croteau, 2000). On the other hand, terpenoids generally contain number of carbon atoms in the multiple of five and are named accordingly based on C5 rule as hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), sesquar- (C35), and tetra- (C40) terpenoids (Figure 1.1) (Cane, 1999).

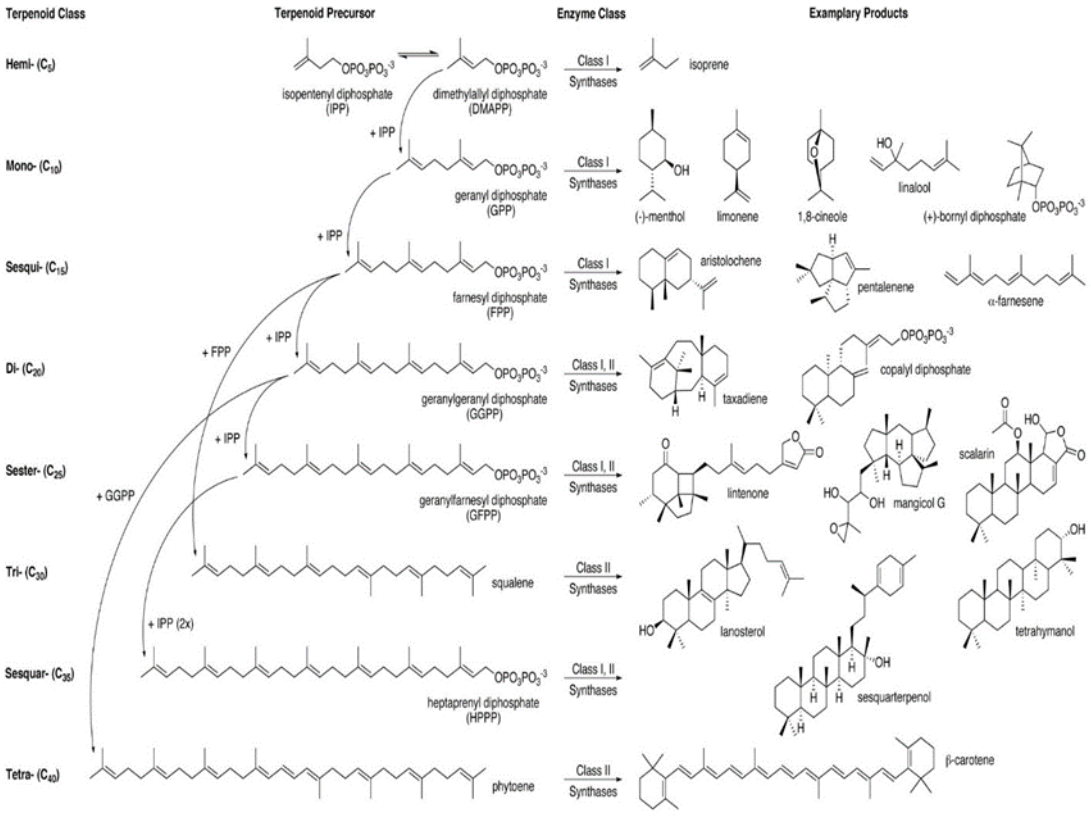

Figure 1.1. Overview of terpenoid biosynthesis. Formation of terpenoid precursors are shown with examples of cyclic or acyclic terpenes derived from them

1.3. Generation of (A)Cyclic Terpenoid Cores

Terpene synthases or cyclases generate numerous and various cyclic or acyclic terpenoid products through utilization of limited number of linear precursors (Christianson, 2008; Allemann, 2008). Terpene cyclases are classified into two groups in general. These are class I and class II cyclases; their strategies are different in terms of initiating catalysis. Take class I cyclases, the cyclases split the diphosphate group from the hydrocarbon part of the substrate in order to form a carbocation in the initial step of the reaction, as called ionization dependent. Take class II cyclases, the cyclases create the initial carbocation through protonating the hydrocarbon part in order to trigger catalysis, leaving the diphosphate group intact, as called protonation dependent (Davis & Croteau, 2000).

1.4. Derivation of A(Cyclic) Terpenoid Cores

After generation of cyclic or acyclic terpenoid intermediates by terpene cyclases, they undergo several enzymatic modifications by using several tailoring enzymes, such as benzoyl, acetyl- or methyltransferases and cytochrome P450 oxygenases. The enzymes take a role in the diversity mechanism of terpenoids through oxidation and functional group transfers to the core structure in order to retouch the terpenoid core structure. Thus, the enzymes give the natural products their final structures. On the other hand, the modifications deal with alteration of the number of carbon atoms, functionalization of the core hydrocarbon skeleton, and crossover between terpenoids and other natural product classes (alkaloids, polyketides, etc.) that are also significant for further especial structures (Walsh & Fischbach, 2010).

1.4.1. Generating Diversity in Terpenoid Biosynthesis

Three general mechanisms determine diversity of terpenome (total terpenoid content of a cell or an organism). Generation of linear terpenoid precursors from IPP and DMAPP constitutes the first layer of structural diversity observed in the terpenome. For example, seven new linear terpenoid precursors (GPP, FPP, GGPP, GFPP, squalene, HPP, phytoene) are generated from two starting molecules (IPP and DMAPP) (Figure

1.1). Second layer of diversity is created by terpene cyclases through generation of various cyclic or acyclic terpenoid products from limited number of linear precursors. For example, limonene, menthol, 1, 8-cineole, bonyl diphosphate are all generated from GPP by various terpenoid cyclases (Figure 1.1). After terpenoid cyclases, modification of terpenoid core structures by tailoring enzymes (hydroxylases and transferases) constitutes the third layer of diversity. Tailoring enzymes give the natural products their final structures via oxidation of and functional group transfers to the core structures.

Recent discovery of *S*-adenosyl methionine (SAM) dependent methylation of canonical terpenoid precursors enables an additional (fourth) layer of diversity in terpenoid biosynthesis by increasing the number and variety of terpenoid precursors available to terpene synthases. Theoretically, methylation of other canonical terpenoid precursors may yield more diversity in the terpenome (Ariyawutthiphan et al., 2011).

1.5. Methylation of Linear Terpenoid Precursors

Terpenoids are present in various livings, such as both terrestrial and marine plants even fungi. Recently, it has been explored that terpenes are also generated through some types of bacteria. For example, soil-dwelling gram-positive organisms, such as some *Actinomycetes* and *Streptomyces* species. Although some microbial terpenes, such as geosmin, is significant in terms of the smell of the moist soil, have been known very well for a long time; however, structure and mechanistic of a few terpenoids have been characterized and known recently (Cane & Ikeda, 2012). For instance, geosmin is a cleaved sesquiterpene that is crucial in terms of dealing with the characteristic odor of moist soil and associated with unpleasant off-flavors in water, furthermore it dealing with of taste of wine and fish (Buttery & Garibaldi, 1976). Some microorganisms such as some *Streptomyces* and *Cyanobacteria* species and fungi can produce the terpenoid. In the biosynthesis of geosmin, the C15-intermediate germacradienol subjected to an elimination reaction in order to form C12-geosmin and a C3-acetone molecule (Jiang et al., 2006, Jiang et al., 2007 and Jiang & Cane, 2008). Additionally, complex sterol methyl transferases (SMT) convert olefins into separate C-methylated products through a one-carbon modification of fungal and plant steroids by the *S*- adenosyl-L-methionine (SAM)-dependent methylation reaction at C-24 (Nes, 2003; Nes, 2007).

In addition to the modified examples, a significant modified terpenoid is the volatile C11-terpenoid 2-methylisoborneol produced by certain bacteria and cyanobacteria. Although the volatile C11-terpenoid alcohol that contaminates drinking water and infects aqua flora and fauna, it is responsible for both odor of freshly turned soil and pleasant earthy aromas of Brie and Camembert cheeses (Buttery & Garibaldi, 1976; Martin et al, 1988; Jüttner et al, 2007; Karahadian et al, 1985; McCallum et al. 1998). It was shown that additional methyl group comes from methionine through *S*-adenosyl methionine (SAM) dependent methylation (Bentley & Meganathan, 1981). However, it has been explored recently that the methyl groups are originated before the cyclization of the terpenoid precursor (Dickschat et al, 2007; Komatsu et al, 2008; Wang et al, 2008). Biosynthesis of 2-methylisoborneol is a rare example of utilizing acyclic precursor such as geranyl diphospate (GPP) substrate to generate a non-canonical monoterpenoid precursor such as 2-methylgeranyl pyrophosphate (2MGPP) (Dickschat et al, 2007; Wang et al, 2008) (Figure 1.2).

Several studies have shown that some *Streptomyces* and *Cyanobacteria* species encode a SAM-dependent geranyl diphosphate methyltransferase (GPPMT) and 2-methylisoborneol synthase (MIBS) (Komatsu et al, 2008; Wang et al, 2008; Wang et al, 2011). In 2-methylisoborneol biosynthesis, firstly, geranyl pyrophosphate *C*-methyltransferase (GPPMT, encoded by the *SCO7701* gene) catalyzes methylation of GPP in order to form non-canonical diterpenoid precursor, 2MGPP, in an S-adenosyl methionine (SAM) dependent manner. Afterwards, 2MGGP is cyclized by methylisoborneol synthase to produce 2-methylisoborneol (Ariyawutthiphan et al., 2011; Köksal et al., 2012) (Figure 1.2). In spite of the fact that numerous methyltransferases exist to modify proteins, nucleic acids and several intermediates, GPPMT is the first example of a methyltransferase that modifies an acyclic terpenoid precursor in a natural pathway (Martin et al., 2002; Köksal et al., 2012).

Employing GPPMT in 2-methylisoborneol biosynthesis illustrates a novel strategy to change the carbon stoichiometry and diversify the terpenome, by modification of linear terpenoid precursors rather than cyclic terpenoid products (Ariyawutthiphan et al., 2012).

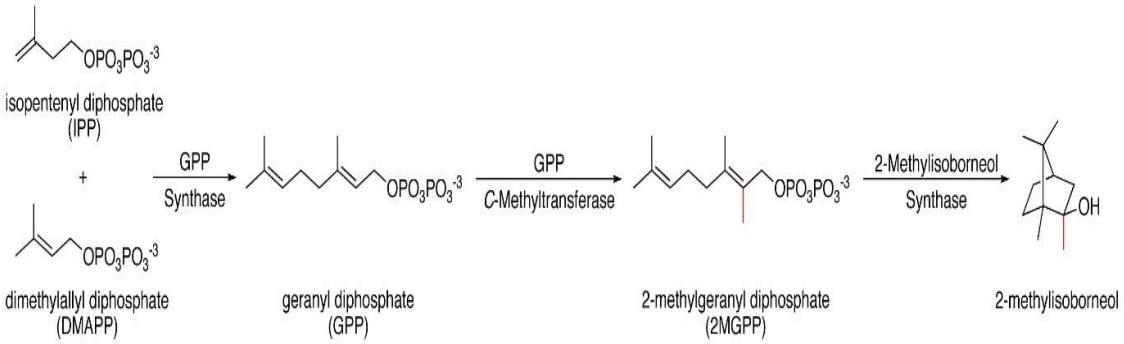

Figure 1.2. Biosynthesis of 2-methylisoborneol. The additional methyl group in 2MGPP is red.

1.6. Structure and Mechanism of GPPMT

Recently, high-resolution structure of GPPMT from *Streptomyces coelicolor A3 (2)* have been determined in conjugated with *S*-adenosyl-L-homocysteine (SAH) and substrate GPP and with SAH and GSPP (its substrate analogue) (Ariyawutthiphan et al., 2011; Köksal et al., 2012; Ariyawutthiphan et al., 2012). In the meantime, GPPMT possess low-level structural similarities to other SAM-dependent methyltransferases (Martin & McMillan, 2002. The most similar methyltransferases to GPPMT in terms of sequence, are rebeccamycin sugar 4′-O- methyltransferase RebM from *Lechevaleria aerocolonigenes* (20% identical) and mycolic acid cyclopropane synthase MmaA2 from *Mycobacterium tuberculosis* (22% identical) (Köksal et al., 2012; Singh et al., 2008). GPPMT is crystallized as a trimer of dimers, namely as a hexameric quaternary structure (Köksal et al., 2012).). Structure motif of GPPMT resembles Rosmann fold structural protein motif (Rao & Rosmann, 1973) (Figure 1.3) 5α helices flanking a central 7-stranded β sheet constitute the core α/β structure of GPPMT (Figure 1.3). On the other hand, the SAM binding site and GPP substrate-binding site are in different locations. Take the SAM binding site, which is defined by residues from αZ2, following polypeptide loop and the N-terminal region of α/β core (Figure 1.3). Take GPP binding site, which extends towards the C-terminal region of the protein and is surrounded by residues from αZ3, αD1, αE1, αE2 and the loops following αZ2, αZ3, β4, and β5 (Figure 1.3).

It is possible to manipulate the GPP binding pocket without compromising cofactor binding because the hydrocarbon tail of GPP (and GSPP) is extended away from the SAM binding site. In this deep binding pocket, the hydrocarbon tail makes van der Waals

interactions with hydrophobic side chains of different sizes and shapes. The size of GPP binding pocket is limited mainly due to the presence of larger non-polar residues at the end of the cavity. Some mutations in the restricted site could provide some opportunities in order to enable accommodation of larger terpenoid substrates such as FPP, and GGPP (Köksal et al., 2012).

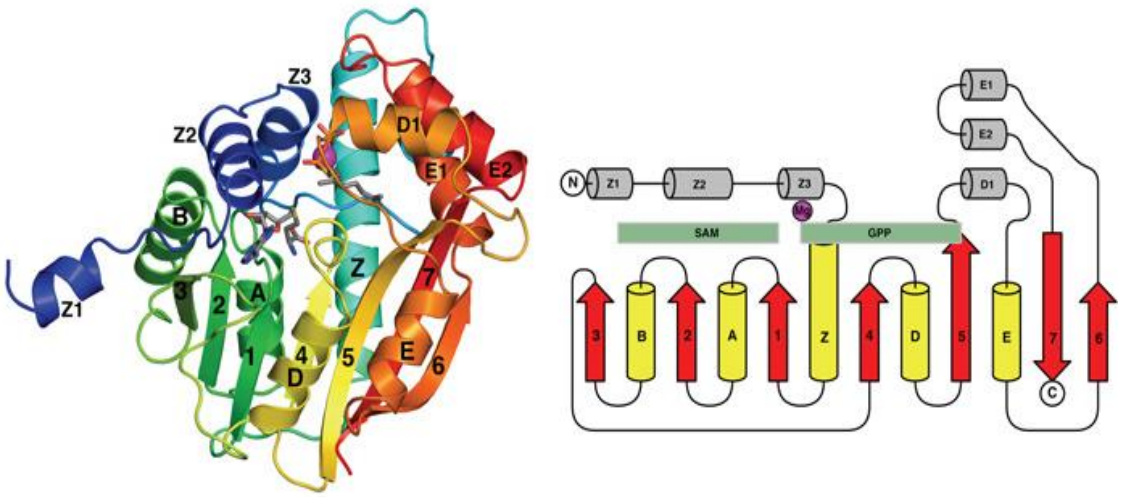

Figure 1.3. Structure of geranyl pyrophosphate C-methyltransferase (GPPMT). (a) Ribbon diagram of GPPMT with secondary structure elements labeled according to Martin & McMillan (2002). Substrate analogue (GSPP) and the product form of the cofactor (SAH) are displayed as stick figures. The Mg^{2+} ion is displayed as magenta sphere. (b) Topology diagram of GPPMT. Strands of the central β sheet are colored red, flanking α helices yellow, and additional α helices beyond the core Rossmann fold gray. The general regions of the SAM and GPP/GSPP binding sites are shown as green boxes, and the Mg2+ binding site is represented as a magenta sphere.

1.7. Importance of Engineering of GPPMT

Chemical diversity of terpenoids corresponds to various and several products, such as active/inactive ingredients of medicines, commodity chemicals, food products and biofuels (Bohlman & Keeling, 2008). The chemical and structural diversity of terpenoids should be maintained and improved, since the applications are significant for drug discovery efforts and bioactive natural products (Chou et al., 2011).

SAM dependent methylation of canonical terpenoid precursors provides an additional and a novel layer of in terpenoid biosynthesis thereby increasing the number and variety of terpenoid precursors. Despite the fact that some non-canonical terpenoid precursors such as 2MGPP are rare in nature, some terpenoid cyclases utilize such precursors in substrate binding pocket in order to catalyze the modified terpenoids (Cane & Tsantrizos, 1996; Vedula et al, 2007) that are fluorinated and methylated sesquiterpenoids (Miller et al, 2007; Miller et al, 2009). Catalytic production of methylated non-canonical terpenoid precursors, such as 2MGGPP could undergo newer versions of terpenoids using terpenoid cyclases. Significant intermediate steps such as alkyl transfers, methyl migrations, hydride shifts and alkyl transfers observed in a typical terpenoid cyclization reaction can be rerouted through stabilizing a specific carbocation over the other by presence of substituents. Thereby, enzymatic modification of a terpenoid precursor possesses the potential to modify its subsequent cyclization steps because of presence of an additional methyl group and create even more diversity in the terpenome (Köksal et al, 2012). Lastly, structure-based engineering of GPPMT could break new ground to improve novel terpenoid biosynthesis strategies and production of possible crucial products associated with medicine, active/inactive ingredients, commodity chemicals, biomaterials, biofuels, and alternative to petrochemicals (Bohlman & Keeling, 2008).

CHAPTER 2

MATERIALS AND METHODS

2.1. *In Silico* Design and Analysis of Candidate GPPMT Mutants

High-resolution structures of GPPMT in complex with SAH and GPP (PDB ID: 3VC2) and with SAH and GSPP (PDB ID: 3VC1) have been determined recently (Ariyawutthiphan et al., 2012; Köksal, 2012). The size of GPP binding pocket is restricted because of the presence of larger non-polar residues at the end of the cavity. Smaller amino acids were substituted for larger residues to expand the substrate-binding pocket. For example, alanine (A), valine (V) substituted for tyrosine (Y), phenylalanine (F). *In silico* analysis of these mutations were carried out manually by using *COOT* program. The mutations were single, double and triple amino acid mutations, such as Y284A, Y284G, Y277A, Y277V, Y277L, Y277I, T253A Y277A, T253A Y277G, Y284G I53A I200A and Y253A Y277G. The types of mutations could enable binding of smaller or larger canonical terpenoid precursors, such as GGPP at GPPMT active site.

2.2. Mutagenesis GPPMT Genes

A clone of wild type geranyl diphosphate methyltransferase from *S. coelicolor* with a 20-residue N- terminal hexahistidine tag and linker (GPPMT) in the pET28a+ plasmid (Novagen Inc.). The candidate GPPMT mutants were prepared using the quick-change site-directed mutagenesis protocol (Qiagen Inc., USA) with designed mutagenic primers (i.e., GPPMT_Y284A_F (31bp78°C): GACGGCTCCTTCCAGGCCGTCCTG ATCGCGG, GPPMT_Y284A_R (31bp78°C): CCGCGATCAGGACGGCCTGGAAG GAGCCGTC). The protocol consists of four steps. In the first step, mutagenic primers were designed containing desired mutation. Second step is temperature cycling; denaturing plasmid and annealing the mutagenic primers containing desired mutation through nonstrand-displacing function property of high fidelity *Pfu* DNA polymerase (2.5 U/µl) (Invitrogen). The polymerase provides extend primers without displacing primers, also hot start DNA polymerase (0.02U/µl) (Solis Biodyne) was used for the purpose.

Third step is *Dpn* I digestion; mutated primers contain nicked circular strands, Dpn I digested enzyme (EUR_X) was used for non-mutated parental DNA incubate @ 37°C for 16 hrs. and 1 Unit of the enzyme was used for 50 µl PCR reaction volume. Fourth step is bacterial transformation; transform the circular and nicked DNA to XL1-Blue competent cells in order to repair the nicks in the mutagenic plasmid. (Greene & Jerpseth, 1993; Papworth et al., 1996). In the meantime, 1-2 µl sterile β-mercaptoethanol was added to 100-µl competent cells mixture to increase efficiency of transformation (Hanahan, 1983).

2.3. Expression of Mutant GPPMT Genes

2.3.1. Wild Type GPPMT Gene Expression

High-level expression of candidate GPPMT mutants were performed using *Escherichia coli* BL21 (DE3) cells according to wild type GPPMT expression (Köksal et al., 2012). Initially, protein was expressed using *Escherichia coli* BL21 (DE3) cells (Stratagene Inc.), *Escherichia coli* BL21-GoldpLysS (DE3) (Agilent Inc.) and E. coli Overexpress C41 (DE3) (Lucigen Inc.). Transformed cell cultures were grown in 1 L flasks containing 0.5 L of Terrific Broth (Fisher) (4 mL glycerol per L of media was added before autoclaving media) supplemented with 50 µl/ml of kanamycin at 37 °C. At an OD600 of 0.8−0.9 (Thermo, Multiscan Spectrum), cultures were equilibrated at 18 °C and expression was induced by 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (CarboSynth Inc.) for 16 hrs. Cells were harvested through centrifugation (Sigma 6K15) at 7000 g for 15 min in 500 mL bottles. Approximately, 4 g cell pellet was obtained per 0.5 L of culture. Afterwards, the pellet was suspended in 2X mL Lysis Buffer [96 % E-Buffer (50 mM K_2HPO_4 pH 8.0, 10 % Glycerol, 5 mM β-mercaptoethanol (β-ME), 300 mM NaCl, 100 uM phenylmethanesulfonyl fluoride (PMSF), 4 % I-Buffer (50 mM K_2HPO_4 pH 8.0, 10 % Glycerol, 5 mM β-ME, 300 mM NaCl, 100 uM phenylmethanesulfonyl fluoride (PMSF), 250 mM Imidazole)] at 4 °C, vortexed intermittently; kept on ice all times, and then it was added lysozyme (0.25 mg/mL final concentration) (Bioshop Inc.) and DNase I (0.01 mg/mL final concentration) (Applichem). Suspension was shaken/rotated for 30 min at 4 °C. Cells were disrupted by sonication on ice with a large probe at medium power (5 cycles of 60 sec ON 60 sec OFF,

50% bursts, 60% power) (Branson Inc.). Cell debris was cleared by centrifugation twice at 20000 g for 1hr.

2.3.2 Gene Expression Test

Gene expression test was used to check the expression level of mutant genes at different conditions such as bacterial strain (i.e., BL21(DE3), BL21-Gold pLysS(DE3), C41(DE3)), culture medium (i.e., LB, TB), expression temperature (i.e., 37 °C, 25 °C, 18 °C, 15 °C, 10 °C), expression duration (i.e., 3, 6, 9, 15, 20 hrs), and IPTG concentration (i.e., 1, 0.5, 0.25, and 0.125 mM) according to Invitrogen manual.

Transformed BL21-Gold pLysS (DE3) competent cells were streaked out on plates with 50 µl/ml of kanamycin and chloramphenicol at 37 °C for selection. Following, transformed BL21-Gold pLysS(DE3) cells were grown in 5ml LB with 50 µl/ml of kanamycin and chloramphenicol and grown overnight at 37 °C. Next day, 300 mL LB media (containing 50 µg/ml kanamycin and chloramphenicol) was inoculated with 1 ml of preculture at 37 °C. At an OD600 of 0.7−0.8 (Thermo, Vario Skan Flash), culture was transferred to ice bath for 30 s to equilibrate culture to the temperatures indicated. Expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside as indicated above. Just before induction, and 2, 5, 10, 15, and 20 hrs after induction, 50 mL sample was taken from culture into a tube and pelleted at 7000 g for 10 min. Cell pellets were stored at -80 °C. Later, frozen cell pellets were thawed on ice and weighed, resuspended in 2X ml ND-buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 5 % Glycerol) with 1 mg/mL lysozyme, and incubated and shaken for two hrs. at 4 °C. Subsequently, the cells are sonicated twice (30 s ON 30 s OFF) using micro tip of the sonifier (Bandelin). Cell debris was pelleted at 18000 g for 1 hr. supernatant was taken into a clean microcentifuge tube as Non-Denaturing Lysate (NDL). On the other hand, the pellet was resuspended in 500 µl D-Buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 5 % Glycerol and 6 M Guanidine-HCl) and spun down at 18000 g for 1 hr. Supernatant was taken into a clean microcentifuge tube as Denaturing Lysate (DL) and stored at 4 °C. In order to analyze expression with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 20 µL non-denaturing lysate sample and 20 µL 1x Laemmle's sample buffer were mixed and heated for 5 min at 99 °C in order to load 5 µL of this mixture to SDS-PA Gel. For denaturing lysates, trichloroacetic acid (TCA)

precipitation was performed (Sanchez et al., 2008). 100-µL denaturing lysate sample and 100 µL of 10% TCA were mixed and incubated on ice for 20 min, centrifuged at 18000 g for 15 min. Subsequently, the pellet washed with 100 µL of ice-cold ethanol, dried, dissolved in Laemmle's sample buffer, heated for 5 min at 95 °C, and 5 µL of this mixture was loaded to gel immediately. SDS-PA gel was stained with Coomassie Stain (0.1% Coomassie R250, 10% acetic acid, 40% methanol) and destained by destaining solution (20% methanol, 10% acetic acid). This procedure was repeated for each of the GPPMT mutants.

2.3.3 Plasmid Stability Test

Plasmid stability test shows if expression plasmids are stable or not. Plasmids of four GPPMT mutants such as I200A Y284G, I53A Y284G, Y284A and Y284G were analyzed through the method according to pET system manual (8th ed., 1999, Novagen Inc.). Before induction with IPTG at OD600 is approx. 0.6, 180 µl aliquot of the cell culture was taken in order to make a serial dilution of the cell suspension at total volume 200 µl, including a $10^{5}$and 10^{-6} dilution. 50 µl of the diluted culture at a 10^{-5} dilution was plated on

LB plate + IPTG

LB plate + IPTG + antibiotic

while 50 µl of the diluted culture at a dilution 10^{-6} dilution on

LB plate

LB plate + antibiotic

and incubated the plates overnight a 37°C.

If plasmid of interest is stable, almost all cells will constitute colonies both on the LB plate and on the LB plate + antibiotic; less than 2% of the cells can constitute a colony on the LB plate + IPTG; and less than 0.01% can form a colony on the LB plate + antibiotic + IPTG. If a lot of colony formation was observed on IPTG containing plates compared to only antibiotic containing plates, plasmid of interest is said to be unstable. Cells containing an expression plasmid do not grow on plate very well since they are induced with IPTG and use all their resources to produce recombinant protein.

2.3.4 "Plating" Method for Culture Inoculation

If the heterologous protein is toxic for the cells, higher expression levels can be obtained by using the plating method. A single colony was taken from plate containing transformed BL21(DE3) cells with WT GPPMT or I200A/Y284A mutant plasmid. Subsequently, the colony was suspended in 200 ml sterile water and vigorously shaken and the suspension was plated on an LB plate containing Kanamycin and incubated at 37°C for 16 hrs. Following, all colonies were scraped-off and suspended in 300 ml LB medium. The culture was induced at an OD600 of 0.4−0.5 by 0.25 mM IPTG for 16 hrs (Suter-Crazzolara & Unsicker, 1995).

2.4. Purification of the Expressed Proteins

Fast protein liquid chromatography (FPLC) purification of candidate GPPMT mutants were performed according as wild type GPPMT using immobilized metal affinity chromatography (IMAC) (Köksal et al., 2012). After breaking of expressed cells as indicated above, the clear supernatant was filtered by 0.2-micron cellulose acetate syringe filter (VWR). Lysate was applied to 5 mL HiScale 16 column (GE Healthcare Bio-Sciences AB) packed with Ni-NTA resin (Pierce) and pre-equilibrated with buffer E (50 mM K_2HPO_4 pH 8.0, 10 % Glycerol, 5 mM β-ME, 300 mM NaCl, 100 μM PMSF) at a flow rate of 1 mL/min using an ÄKTAprime plus FPLC system (GE Healthcare Bio-Sciences AB). The loaded column was washed first with 50 ml buffer E and then with buffer E containing 50 mM imidazole in total volume 30 ml. The protein was eluted with a 50 mL gradient from 50 to 250 mM imidazole (Alfa Aesar) in buffer E at a flow rate of 2 mL/min. Afterwards, selected fractions were collected, analyzed, and confirmed with SDS-PAGE. Some samples (i.e., GPPMT Y284A) have precipitated so the precipitate was filtered and concentrated by concentration tubes (Sarthorius Stedium Biotech.). Buffer E of GPPMT proteins exchanged with GPPMT (50 mM PIPES pH 6.7, 20 % Glycerol, 5 mM β-ME, 100 mM NaCl and 15 mM $MgCl_2$) buffer by using buffer exchange (desalting) column (GE Healthcare Bio-Sciences AB).

2.5. *In vitro* Analysis of Mutant Enzymatic Activities

Catalytic activities of candidate GPPMT mutants against GGPP were investigated by using SAM methyltransferase assay protocol (Calbiochem). Catalytic activities of T253A Y277A, T253A/Y277G, and Y284A against canonical terpenoid precursor GGPP were determined. Furthermore, G202A and G202F were tested for enzymatic activity against a smaller canonical terpenoid precursor, DMAPP. WT GPPMT was also tested against GPP by using the method.

In the assay, the removal of the methyl group from SAM forms *S*-adenosyl homocysteine, which is rapidly converted to *S*-ribosyl homocysteine and adenine by adenosyl homocysteine nucleosidase. Then, adenine is converted to hypoxanthine by adenine deaminase, which in turn is converted to urate and hydrogen peroxide. The rate of production of hydrogen peroxide is measured with a colorimetric assay by an increase in absorbance at 510 nm. In the assay, adenosyl homocysteine is used as a positive control (Figure 2.5).

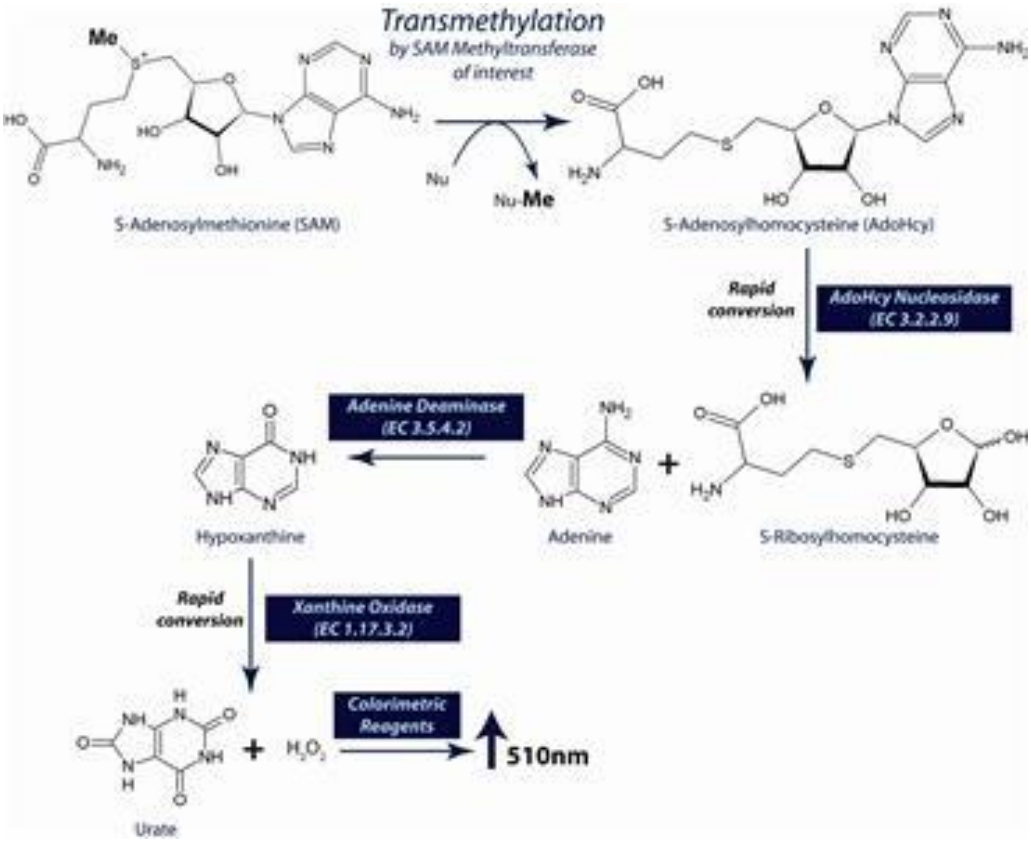

Figure 2.5. Transmethylation by SAM Methyltransferase of interest.

CHAPTER 3

RESULTS

3.1. *In Silico* Design, Analysis and Production of Candidate GPPMT Mutants

In silico analysis of candidate GPPMT mutants were carried out manually by using COOT program. The mutations were single, double and triple amino acid mutations, such as Y284A, Y284G, Y277A, Y277V, Y277L, Y277I, T253A/Y277A, T253A/Y277G, Y284G, I53A/I200A and Y253A/Y277G in order to bind GGPP or G202A, G202L, G202I, G202F, G202W, and G202V, in order to bind DMAPP substrates at GPPMT active site. Substrate binding pocket of WT GPPMT is restricted by these residues (Figure 3.1). Dr. Mustafa Köksal prepared the candidate GPPMT mutants using quick-change site-directed mutagenesis protocol (Qiagen Inc.) with designed mutagenic primers.

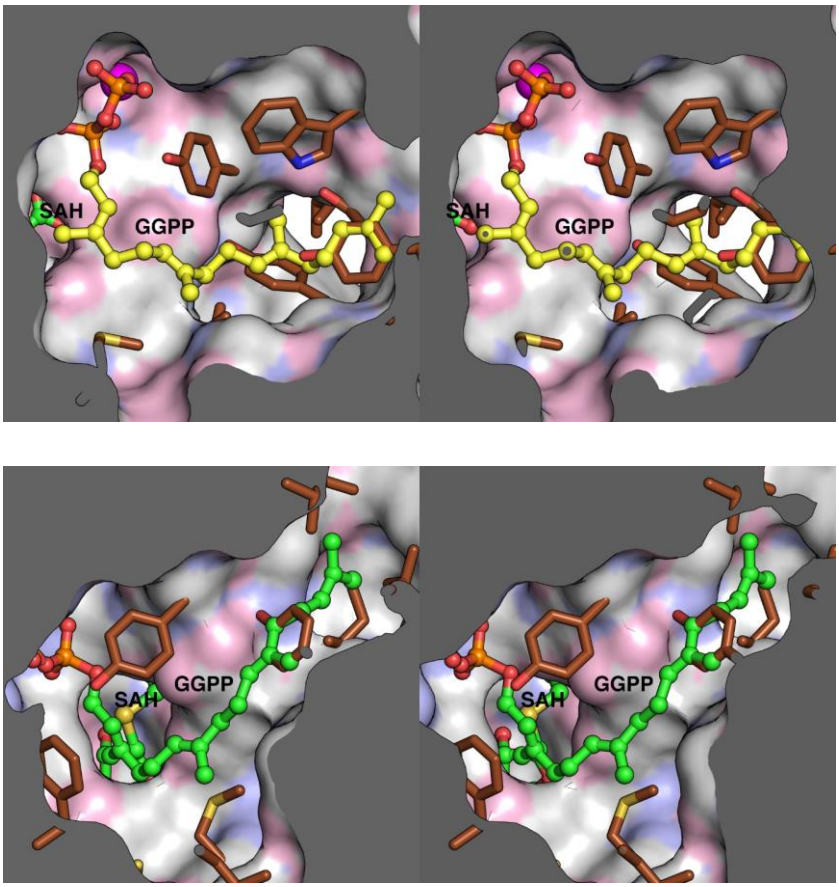

Figure 3.1. Stereographic view showing that cut-away surface diagram of substrate-binding pocket of GPPMT. SAH and GSPP are shown as ball-and-stick model (cut-away at a different level is shown for the SAH binding site). Some residues such as side chains of phenylalanine, tyrosine and tryptophan hinder GGPP in one conformation (upper panel) or in another conformation (lower panel). The cavity that surrounds the hydrocarbon tail of GSPP is mostly non-polar.

3.2. Expression of Wild Type GPPMT & Candidate GPPMT Mutants

High-level expression of candidate GPPMT mutants were performed using Escherichia coli BL21(DE3), Escherichia coli BL21-Gold pLysS(DE3) cells and Escherichia coli C41(DE3) cells according to wild type GPPMT expression. Cultures were equilibrated at 18 °C and expression was induced by 0.25 mM IPTG for 16 hrs. at an OD600 of 0.8−0.9 in Terrific Broth media (Köksal et al., 2012).

Furthermore, expression test was used to test the expression level of a gene at different conditions. The tests were carried out for seven GPPMT mutants that were not expressed very well (Y277A, Y277G, Y284G, T253S Y277G, T253S Y277A, Y284G

I53A and Y284G I200A). Results of tests were showed and analysed by SDS-PAGE (Figure 3.2.1).

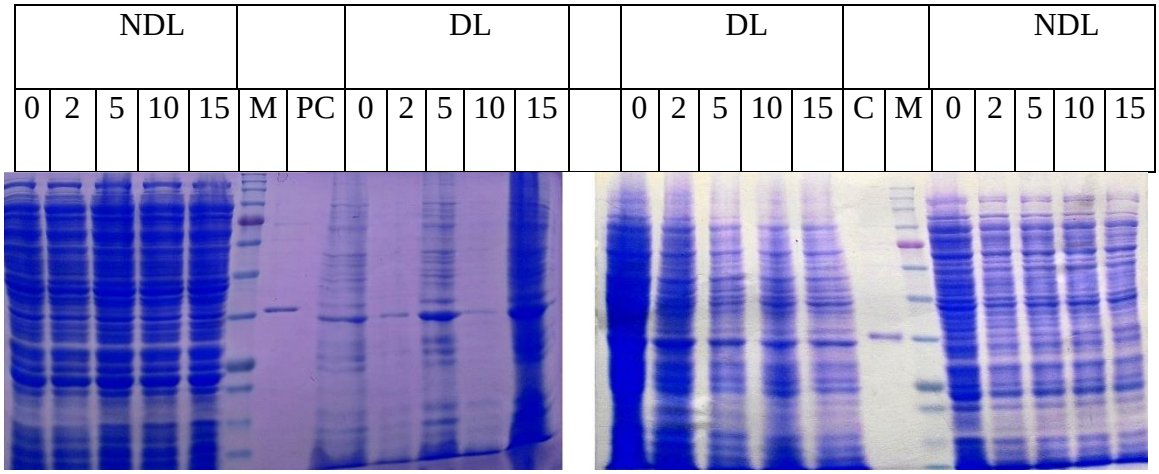

Figure 3.2.1. Gene expression test of GPPMT Y277V and T253A Y277G mutants subjected to gene expression test. It is obtained two lysate samples that are Non-Denaturing Lysate (NDL) and Denaturing Lysate (DL). Left panel shows NDL and DL of GPPMT Y277V at 0, 2, 5, 10 and 15 hour expression, at 1 mM IPTG, and 18 °C. Right panel shows NDL and DL of GPPMT T253A/Y277G at 0, 2, 5, 10 and 15 hour expression at 1mM IPTG, and 10 °C. PC indicates positive control.

After the expression tests, plasmid stability test was carried out according to pET system manual. Plasmids of four GPPMT mutants (I200A Y284G, I53A Y284G, Y284A and Y284G) were analyzed with this method. In the test, if plasmid of interest is stable, almost all cells will constitute colonies both on the LB plate and on the LB plate + antibiotic; less than 2% of the cells can constitute colonies on the LB plate + IPTG. If plasmid of interest is unstable, many colonies will be seen on IPTG containing plates. In the presence of IPTG, cells carrying expression plasmid do not grow because they dedicate their resources to the production of the recombinant protein instead of cell maintenance. Except for GPPMT I200A/Y284G all could constitute colonies on the LB plate + IPTG, which means all but GPPMT I200A/Y284G harbor unstable plasmids. (Figure 3.2.2).

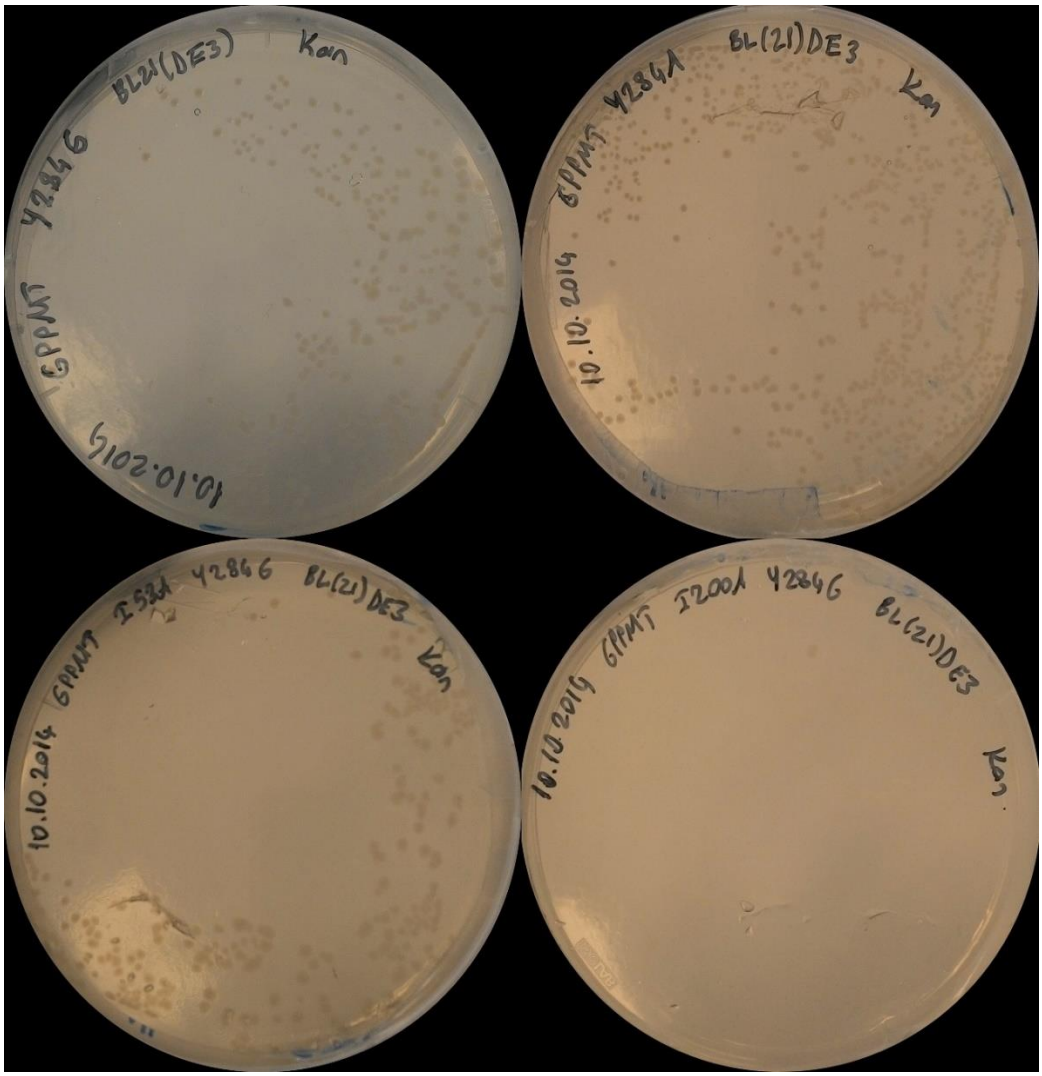

Figure 3.2.2. Plasmid stability test of GPPMT I200A/Y284G, I53A/Y284G, Y284A and Y284G. They were analyzed to verify stability of plasmid. The cells can constitute colonies on the LB plate + IPTG except for GPPMT I200A/Y284G. For GPPMT I53A/Y284G, Y284A, and Y284G, the situation reflects unstable plasmid of interest. It was seen that only GPPMT I200A/Y284G mutant is stable.

Following the plasmid stability test, when the heterologous is toxic for the cells, "plating" method is used in order to obtain higher expression yields. This method was used for GPPMT I200A/Y284G, I53A/Y284G and Y277A. The genes were expressed at an OD600 of 0.8−0.9, cultures were equilibrated at 18 °C and expression was induced by 0.25 mM IPTG for 16 hrs.

3.3. Purification of Wild Type GPPMT & Candidate GPPMT Mutants

Fast protein liquid chromatography (FPLC) purification of candidate GPPMT mutants were performed according to wild type GPPMT using immobilized metal affinity chromatography (IMAC) (Köksal et al., 2012). After breaking of expressed cells by using sonifier, the clear supernatant was filtered by 0.2-micron cellulose acetate syringe filter and applied to a pre-equilibrated 5 mL HiScale 16 column packed with Ni-NTA resin at a flow rate of 1 mL/min using an ÄKTAprime plus FPLC system.

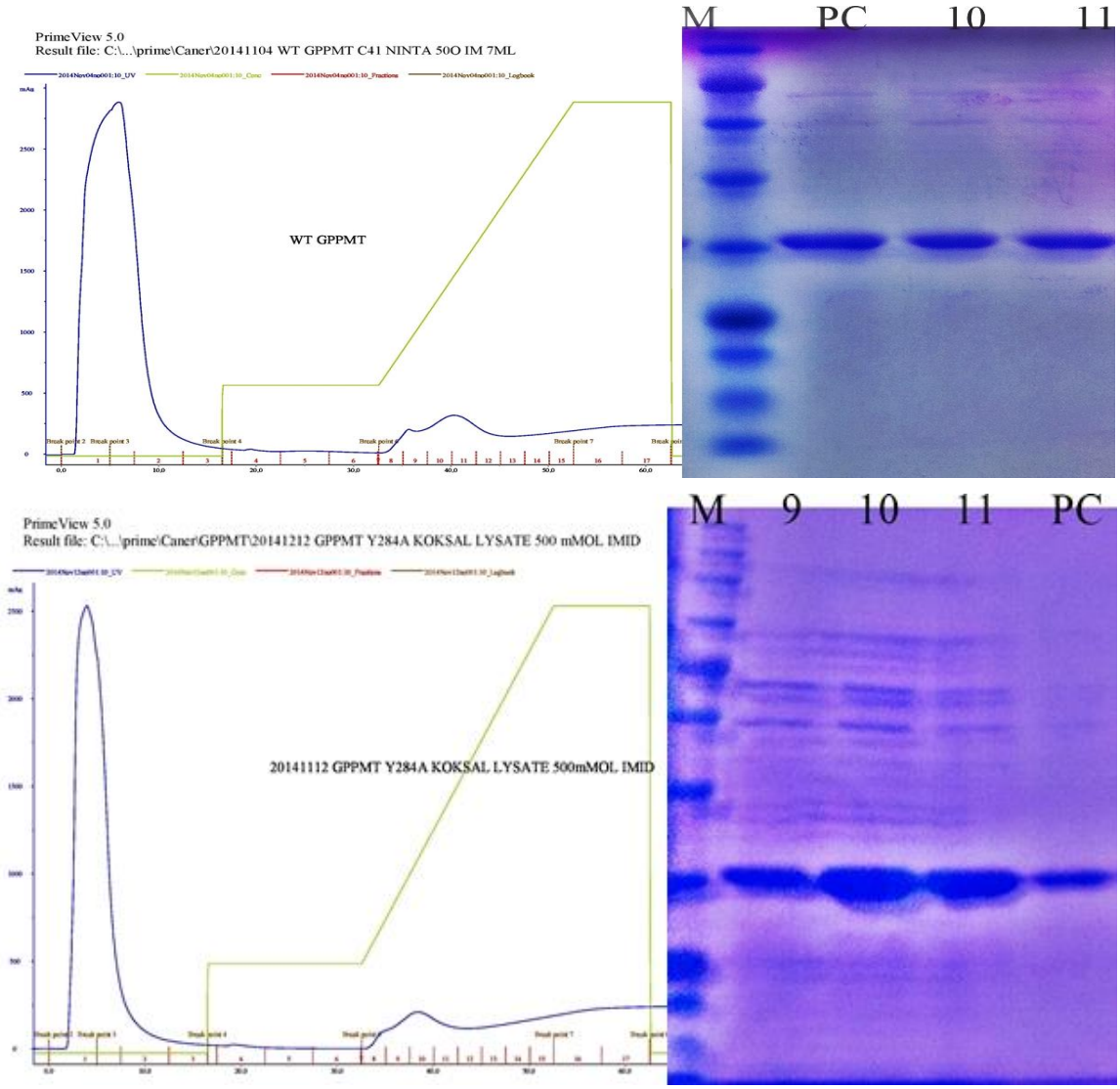

Figure 3.3. Sample purification chromatograms of WT GPPMT & Candidate Mutants. WT GPPMT and Y284A lysates were purified by FPLC and verified by SDS-PAGE. Numbers 9, 10, and 11 indicate fractions of FPLC, M indicates Protein Marker VI (10 -245 kDa, Applichem).

Table 3.1. GPPMT mutants for GGPP or DMAPP substrates and their expression results

List of mutants	Protein obtained	Protein not obtained
G202I	✓	
G202L	✓	
G202V	✓	
G202W	✓	
G202A	✓	
G202F	✓	
Y277A		X
Y277G		X
Y284G		X
Y284A	✓	
T253A Y277G	✓	
T253A Y277A	✓	
T253A Y277V		X
T253A Y277L	✓	
T253A Y277I	✓	
T253S Y277V		X
T253S Y277G		X
T253S Y277I		X
T253S Y277A		X
Y284G I53A		X
Y284G I200A		X

3.4. Desalting of Purified Proteins

After purification of the proteins, the proteins present in I-Buffer [50 mM K_2HPO_4 pH 8.0, 10 % Glycerol, 5 mM β-ME, 300 mM NaCl, 100 uM PMSF, 250 mM imidazole)]. The buffer is replaced with GPPMT assay buffer [50 mM PIPES pH 6.7, 20 % Glycerol, 100 mM NaCl at 1 M $MgCl_2$] by using HiTrap Desalting Column (GE Healthcare) (Figure 3.4).

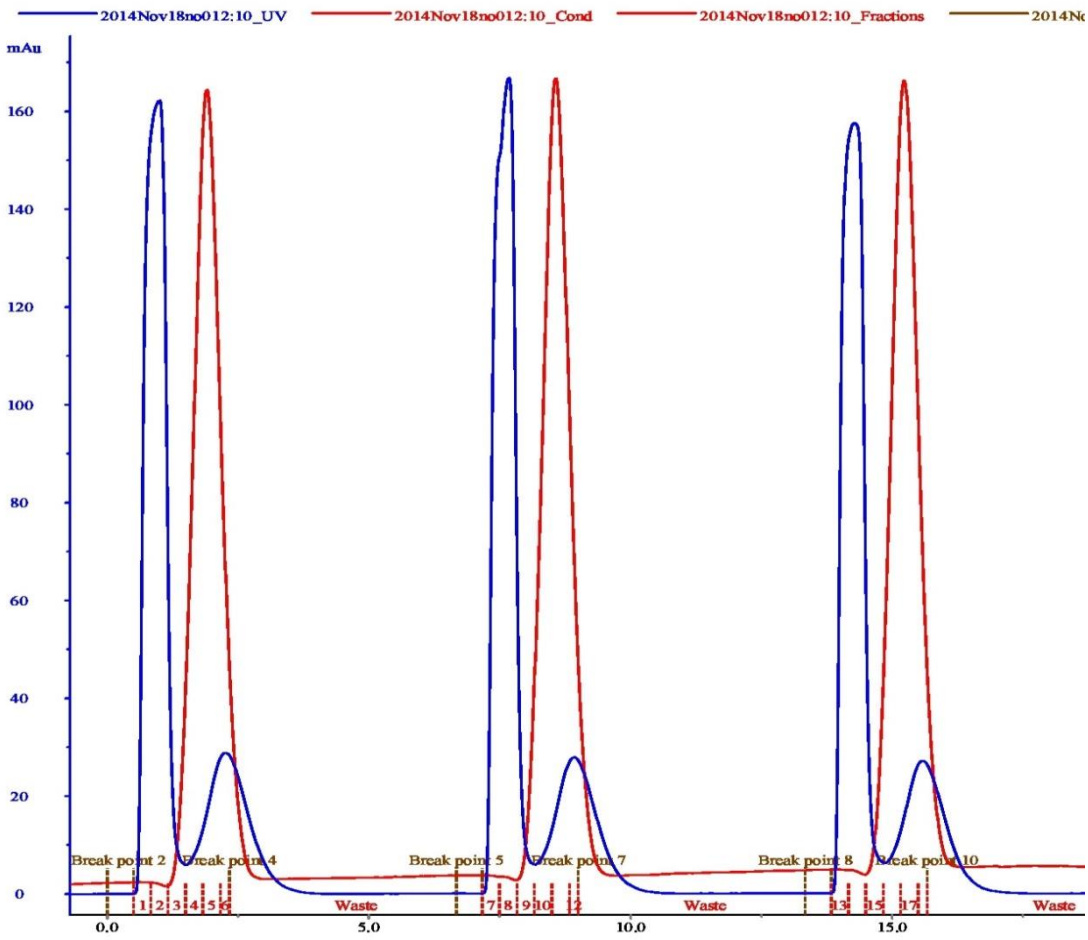

Figure 3.4. Desalting of GPPMT Y284A. Number 1 and 2 are fractions of FPLC containing protein of interest

3.5. Concentration of Purified Proteins

Some proteins need to be concentrated if it is necessary by using Vivaspin® centrifugal concentrators. Then, it was showed by SDS-PAGE (Figure 3.5).

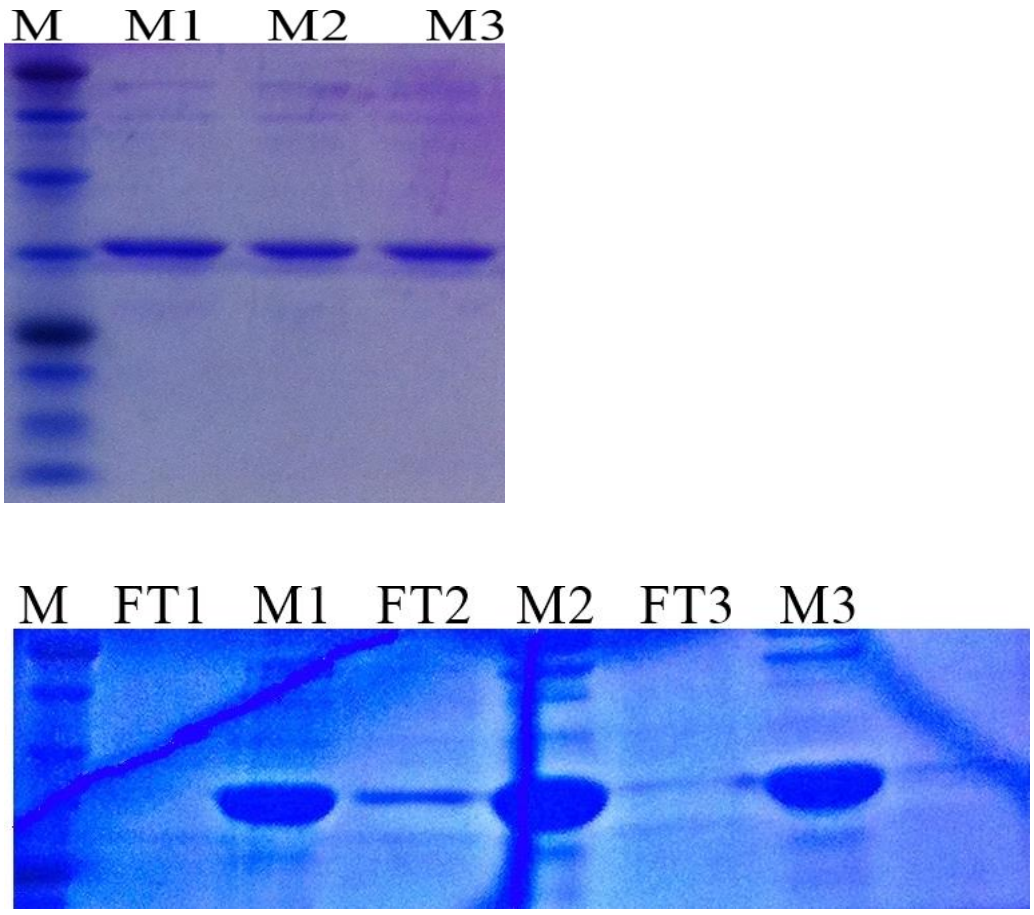

Figure 3.5. Concentration of GPPMT mutant proteins showed by SDS-PAGE. Upper panel indicates before concentration of samples, lower panel indicates after filter concentration process. M1, M2 and M3 indicates GPPMT Y284A, T253A/Y277A, and T253A/Y277G, respectively. FT indicates flow through (filtrate).

3.6. Enzyme-Linked Colorimetric SAM Methyltransferase Assay

Catalytic activities of candidate GPPMT mutants against GGPP, FPP and DMAPP were performed according to SAM methyltransferase assay protocol. The assays were carried out for WT GPPMT against GPP substrate, for GPPMT T253A/Y277A, T253A/Y277G, and Y284A mutants against GGPP substrate, and for GPPMT G202A

and G202F mutants against DMAPP substrate. WT GPPMT and G202A displayed enzymatic activity against respective substrates (Figure 3.6).

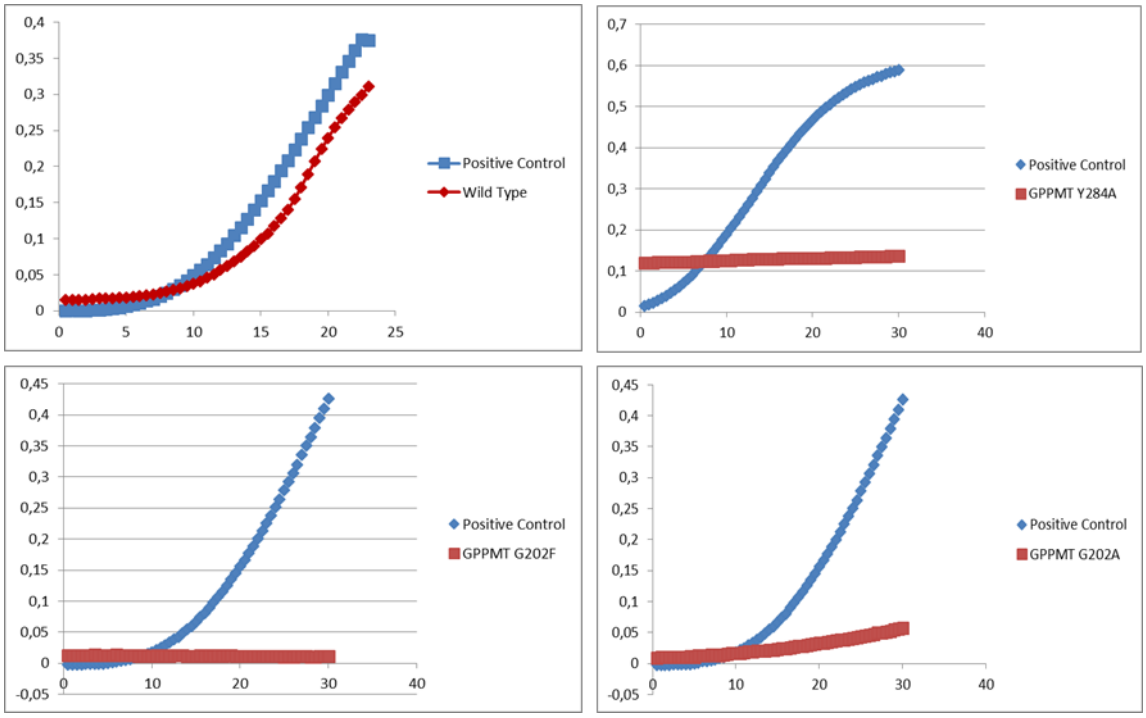

Figure 3.6. Catalytic activities of WT GPPMT and GPPMT mutants. Substrates are GPP for WT, GGPP for Y284A, and DMAPP for G202A and G202F.

3.6.1. GPPMT Activity Assay with GC-MS

GPPMT was also checked through product analysis using GC/MS. The assay consists of three steps (Figure 3.6.1). In the first step, GPPMT transfers the methyl- group to its substrate (i.e., GPP, GGPP, or DMAPP). Subsequently, pyrophosphate of the methylated product (and unreacted substrate) was cleaved by using alkaline phosphatase or acid treatment. Further, organic extraction with diethyl-ether:pentane (1:1) of the reactions were carried out and the products in the organic layer were concentrated. Then, possible products were analyzed was GS/MS.

It was seen that no pentane extractable products were detected in GC/MS, even positive controls were not detected in the GC/MS. Possible reasons are being investigated.

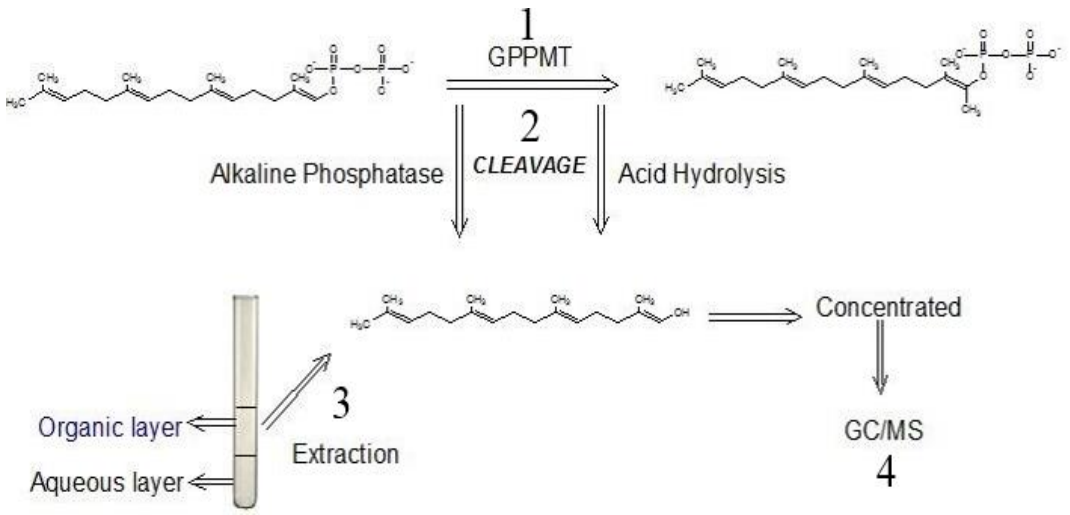

Figure 3.6.1. GPPMT Activity Assay with GC-MS

CHAPTER 4

DISCUSSION

Several GPPMT mutants were designed for binding to GGPP substrates. Three mutants could be purified as soluble proteins: GPPMT Y284A, T253A/Y277A, and T253A/Y277G. However, these mutants did not show enzymatic activity against GGPP. On the other hand, all of the mutants designed for binding to DMAPP substrates were purified as soluble proteins (G202A, G202V, G202L, G202I, G202F, and G202W). G202A and G202F mutants were tested for enzymatic activity against DMAPP, G202A mutant exhibits catalytic activity.

All of the mutants designed for GGPP precipitated in SAM methyltransferase assay buffer. WT GPPMT and GPPMT G202A mutant designed for DMAPP did not show precipitation. The buffer was altered with GPPMT buffer to overcome the issue; nevertheless, enzymatic activity was not detected. Possibly, most of the mutations destabilize the enzyme structure. To overcome these challenges, appropriate buffers may need to be optimized for each mutant. Structure of WT GPPMT should be analyzed again to create alternative GPPMT mutants for GGPP. On the other hand, G202A has enzymatic activity against DMAPP substrates. The product of this reaction needs to be confirmed by GC/MS and possible non-canonical methylated DMAPPs may be used for novel terpenoid products.

CHAPTER 5

CONCLUSION

Most of the GPPMT mutants expected to show activity against diterpenoid precursor (GGPP) were not stable at the conditions investigated in this study. Therefore, no enzyme activity was detected. Most of the mutations appear to destabilize the enzyme structure. A re-analysis of the GPPMT structure is underway to identify and create alternative mutants for GGPP. All of the GPPMT mutants expected to show activity against hemiterpenoid precursor (DMAPP) were stable, however, only G202A mutant displayed enzymatic activity among the mutants investigated. Investigation of other single mutants for DMAPP is underway. The product of DMAPP reaction with G202A is also being investigated.

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