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Natural and engineered production of taxadiene with taxadiene synthase[†]

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Abstract

Taxadiene synthase (TXS) is the rate-limiting enzyme in the biosynthesis of paclitaxel, an important anticancer compound. TXS catalyzes the conversion of the diterpene precursor geranylgeranyl pyrophosphate (GGPP) into the diterpene taxadiene. Due to the importance of taxadiene in the overall biosynthetic pathway of paclitaxel biosynthesis, the enzyme TXS has been the subject of intense scientific and engineering investigations. The crystal structure of TXS was recently elucidated, thereby providing an atomic blueprint for future protein engineering efforts. Metabolic engineering of TXS for taxadiene product in different microbial and plant organisms have also been extensively performed, culminating in the high titer production in *Escherichia coli*. Additional aspects of taxadiene production by TXS will be discussed in the review, including metabolic regulation in native host and possible production by endophytic fungal hosts.

Keywords: Taxadiene synthase, Metabolic regulation, *Taxus*, Fungus, Metabolic engineering, Protein engineering

Introduction

Paclitaxel (Taxol) is a highly effective diterpenoid for the treatment of different types of cancers (Kingston 2007). Sources of paclitaxel include extraction from *Taxus* plant, total synthesis, semi-synthesis, isolation from plant cell culture and possible production from fungal endophytes. Paclitaxel production from the native producing plant source is very low (~ 1g/10kg plant tissue) (Suffness and Wall 1995). As a result, the amount of paclitaxel harvested from 2-4 mature trees is not sufficient to treat a single patient (Suffness and Wall 1995). Total synthesis of paclitaxel requires at least 40 steps (Nicolaou et al. 1994) and despite recent improvements (Mendoza et al. 2012), is still of overall low yield and prohibitively costly. Semi-synthesis using the paclitaxel precursor, 10-deacetylbaccatin III which is isolated from the yew needles, still requires substantial purification effort from the plant source (Baloglu and Kingston 1999). The use of *Taxus* plant cell culture is promising but the yields have been unstable and impurities can interfere with extraction of the desired compound (Jennewein and Croteau 2001). Currently, both semi-synthesis and cell culture are used for the commercial supply of paclitaxel, but these routes still depend on substantial plant resources that increase the cost of paclitaxel and can be environmentally unsustainable (Frense 2007). On the other hand, discovery of paclitaxel-producing endophytic fungi (Zhou et al. 2010) has led to hope that production in a microbial host through fermentation methods may be possible. Unfortunately to date, the production level has remained unstable and very low, while a microbial biosynthetic pathway has remained elusive. Collectively, the importance of paclitaxel as anticancer drug, the costly synthesis of paclitaxel, as well as the prospects of using a microbial host for the production, have led to intense efforts in the metabolic engineering of paclitaxel production in various hosts.

A widely used strategy for engineering paclitaxel biosynthesis has been to focus on enhancing metabolic flux to increase the precursor pool size. These efforts have led to cloning of the necessary genes and understanding the early pathway enzymes that are involved in terpenoid accumulation, including the rate-limiting enzyme in paclitaxel biosynthesis, taxadiene synthase (TXS) (Koepp et al. 1995). In general, terpene synthases (cyclases) such as TXS

operate at metabolic branch points involved in the regulation of terpenoid pathway flux, and catalyze the first committed step leading to specific terpene metabolites. TXS catalyzes the cyclization of geranylgeranyl pyrophosphate (GGPP) to taxadiene, and the low catalytic activity of TXS in plants suggests that the cyclization of GGPP is slow relative to subsequent oxygenations steps (Hezari et al. 1995). This finding has led to the hypothesis that increasing TXS activity in paclitaxel-producing hosts can lead to overall increase in paclitaxel production levels. Considerable efforts have been made to reconstruct and metabolically engineer this rate-limiting step in model organisms including microbial hosts (Ajikumar et al. 2010; DeJong et al. 2006; Huang et al. 2001), and higher plant hosts (Besumbes et al. 2004; Cha et al. 2012). Biochemical efforts to understand TXS function have also been successful in recent years, including the first X-ray structure of TXS that revealed a novel, modular architecture of the enzyme (Koksal et al. 2011). The availability of the structure opens the door to future protein engineering efforts aimed at increasing the catalytic activities of the enzyme.

TXS is the first committed enzyme and could be rate-limiting in paclitaxel biosynthesis

Paclitaxel is biosynthesized from two major structural building blocks, a diterpenoid (baccatin III) and the phenylpropanoid side chain (phenylisoserine) (Fig. 1) (Croteau et al. 2006). Baccatin III is biosynthesized through the cyclization of the diterpene precursor GGPP into taxadiene, followed by extensive modification of the cyclic olefin product. The assortment of modifications on taxadiene includes numerous oxidation steps that start with hydroxylation at C5 by taxadien-5á-ol cytochrome P450; two acetylation and one benzoylation steps by acyl and aryl CoA-dependent transferases, respectively. Downstream modifications en route to paclitaxel include C9 oxidation, oxetane formation and side chain ligation with phenylisoserine at C13 (Croteau et al. 2006).

On the basis of taxoids co-occurrence (>400 identified), the taxadiene structure is the common nucleus in all taxane metabolites including paclitaxel. In earlier studies, enzyme preparation isolated from stem sections of the producing plant was able to convert labeled

GGPP in the presence of $MgCl_2$ to taxadiene; and the labeled taxadiene was confirmed to be incorporated into radiolabeled taxoids such as baccatin III and paclitaxel (Fig. 1) (Koepp et al. 1995). The responsible enzyme for taxadiene formation was subsequently purified from stem extracts and identified as TXS (Fig. 1) (Hezari et al. 1995). The rate of GGPP cyclization by TXS was very slow compared to other cyclases of gymnosperm and was thus proposed to be a rate-limiting step in paclitaxel pathway (Hezari et al. 1995). It has been shown that TXS activity (k_{cat}/K_M = 3.5 x 10^3 $S^{-1}M^{-1}$) (Hezari et al. 1995) is ~ two orders of magnitude less active compared to other plant terpene cyclases, with a much slower turnover rate (k_{cat}) (Table I).

TXS properties, mechanism and molecular structure

TXS was first isolated from *Taxus brevifolia* plant (Hezari et al. 1995). TXS was shown to be similar in properties to other plant terpene cyclases, dependence on Mg^{2+} for catalysis, optimum activity at slightly alkaline pH (Hezari et al. 1995) and a size of ~100 kDa (Kai et al. 2005). Subsequently, TXS was expressed as a soluble enzyme in *E. coli* (Williams et al. 2000b) and purified by hydroxyapatite chromatography and anion-exchange chromatography.

The TXS protein sequence shows three alpha-helical domains, which is a common feature shared by most plant diterpene synthases (Bohlmann et al. 1998). The C-terminus domain is sequentially similar to Class I terpenoid synthase (Fig. 2a) such as farnesyl diphosphate synthase (Tarshis et al. 1994) and geranylgeranyl pyrophosphate synthase (GGPPS) (Chang et al. 2006). The two N-terminal domains are sequentially similar to Class II terpenoid cyclases, such as the triterpene squalene cyclases (Fig. 2a) (Thoma et al. 2004). Class I and II terpenoid cyclases have two different substrate activation mechanisms; Class I activates the diterpene substrate by ionization of the allylic diphosphate ester; and class II activates its substrate by protonation and general acid catalysis through an aspartic acid rich motif (DXDD) (Christianson 2008). Comparison of TXS with plant diterpene synthases reveals that TXS lacks the DXDD motif present in other class II terpenoid synthase in the N-terminal domains, suggesting that TXS catalytic

activity should reside in the C-terminus Class I terpenoid synthase (Hezari et al. 1995; Thoma et al. 2004). The mechanism of TXS catalysis was extensively studied by Croteau and coworkers (Williams et al. 2000a). Cyclization of GGPP to taxa-4(5),11(12)-diene involves an electrophilic mechanism with the formation of verticillen-12-yl carbocation intermediate. This is followed by two deprotonation steps (Lin et al. 1996); first, deprotonation at C11 followed by intramolecular hydrogen transfer and C7 protonation in the B/C ring junction closure generates the taxen-4-yl carbocation; second, deprotonation of taxen-4-yl carbocation at C5 forms taxadiene (Lin et al. 1996; Williams et al. 2000a).

Christianson and coworkers solved the X-ray crystal structure of TXS in 2011 (Koksal et al. 2011), which provided a detailed atomic view of the catalytic mechanism of TXS. As expected, the active site of TXS is located in the C-terminus which binds GGPP substrate and three Mg^{2+} ions forming TXS-Mg^{2+}-Substrate complex. The final deprotonation step at C5 during taxadiene biosynthesis is catalyzed by either a product-assisted or substrate-assisted mechanism (Koksal et al. 2011). Taxadiene can be oriented in two different orientations in the TXS active site, either rendering a phenolic hydroxyl group of the active site Y688 to function as a general base (Fig. 2b) or exposing the inorganic pyrophosphate group (PPi) to function as base (Fig. 2c) (Koksal et al. 2011). In both orientation, the H5â atom of taxen-4-yl carbocation would be oriented towards the base prior to taxadiene release from TXS active site (Koksal et al. 2011). The active site of TXS is larger than the volume of the product taxadiene, and is more similar in size to the larger diterpenoid substrate. This partially explains the promiscuity of TXS in ligand binding and the generation of 20% of the alternative isomer taxa-4(20),11(12)-diene (Jin et al. 2005).

Taxus plant TXS and its metabolic regulation

In *Taxus* plant, the production of paclitaxel and activities of TXS are metabolically regulated in producing cells (Ketchum and Croteau 2006). Activities of the pacemaker enzyme, such as TXS, and metabolites flux of a pathway must be coordinated with the needs of the cells and organism for the final products.

The major control of the level of enzymes is determined by the differential expression of their genes. Tissue expression pattern of TXS showed the highest signals in leaves, lowest signals in stem and no signal in fruits (Kai et al. 2005). A frequently used strategy to increase the flux towards paclitaxel biosynthesis is alteration of the rate of transcription (Emes and Dennis 1997). The examples of elicitors used to enhance the expression of TXS and the production of paclitaxel in cell culture at the transcription level include methyljasmonate (MJ), silver nitrate (SN) and ammonium ceric sulphate (ACS) (Kai et al. 2005). However, overproduction of by-products in *Taxus* plant cell culture upon unspecific and global transcription up-regulation can repress paclitaxel production or accelerate paclitaxel degradation (Frense 2007).

Regulation of TXS activity in plant can also be achieved through controlling the precursor flux. The primary precursor in terpenoid biosynthesis is isopentenyl pyrophosphate (IPP) (Aharoni et al. 2006). Two possible biosynthetic pathways for IPP in plants paclitaxel are known; the cytosolic mevalonic acid pathway and the plastidic methylerythritol phosphate (MEP) pathway (Fig. 3). Several metabolic inhibitors studies were used to identify the source of IPP in paclitaxel biosynthetic pathway including the use of carrier protein inhibitors which inhibit metabolite translocation and translation (cycloheximide and Streptomycin) (Srinivasan et al. 1996), mevastatin (an inhibitor of cytoplasmic HMGR enzyme) and chlorocholine (an inhibitor of sterol biosynthesis) (Srinivasan et al. 1996), fosmidomycin (non-mevalonate pathway inhibitor) and mevinolin (mevalonate pathway inhibitor) (Palazón et al. 2003), and the metabolite translocation inhibitors; sodium pyrophosphate (NaPP) and D, L- glyceraldehyde (DLG) (Wang et al. 2003). Results from these studies suggested that incorporation of IPP in paclitaxel

biosynthesis occurs mainly in the plant plastids, while cytosolic IPP is incorporated only at the late growth stage of the plant.

Increasing IPP biosynthesis in plant cells have been achieved through the overexpression of deoxyxylulose-5-phosphate (DXP) synthase and reductoisomerase (the rate-limiting enzymes in the MEP pathway) (West 1997). When plants expressing TXS under CaMV35S promoter were crossed with plants overexpressing DXP synthase or DXP reductoisomerase, taxadiene titers were increased by 6.5 times and 13 times, respectively in comparison with plants overexpressing TXS alone (Aharoni et al. 2005).

Fungal paclitaxel and possible fungal TXS

Since the discovery of paclitaxel-producing fungal endophyte, *Taxomyces andreanae* in 1993 (Stierle et al. 1993), and the discovery of other paclitaxel-producing fungi (Zhou et al. 2010), several groups have attempted to identify a possible fungal paclitaxel biosynthetic pathway. The goal has always been to find a set of genes that encode biosynthetic enzymes with similar functions as the plant paclitaxel pathway, including TXS. In preliminary experiments, PCR primers for the plant TXS gene were used to screen paclitaxel-producing fungal DNA and showed positive PCR products (Zhou et al. 2007). Recently, positive PCR products were identified from a different paclitaxel-producing fungus using plant TXS primers and the sequence of the amplified product showed 97% identity with the plant gene (Staniek et al. 2009). Similarly, another group recently detected positive PCR products for fungal TXS from different paclitaxel-producing fungi with ~40% similarity to plant TXS (Xiong et al. 2013). Working with a paclitaxel-producing fungus *Paraconiothyrium* SSM001, a protein with similar size as plant TXS was detected by Western blot using TXS-specific antibody (Soliman et al. 2013). The protein level could be varied by adding elicitors and was not detected in control fungus such as *Fusarium*.

On the contrary, a recent genome sequencing effort of three paclitaxel-producing fungi showed no encoded protein sequence with similarity to plant TXS, which led the authors to

conclude that the taxane derivatives from fungi could be a carryover from the plant tissue used to isolate the fungi (Heinig et al. 2013). While the fungal production of paclitaxel remains an unresolved and controversial issue, there are several pieces of evidence that suggest some fungi may indeed be able to produce paclitaxel. First, taxane derivatives other than paclitaxel were detected in paclitaxel-producing fungal extracts, indicating a possible autonomous biosynthetic pathway (Heinig et al. 2013). Second considering the small size of mycelia used to isolate the fungus followed by the large number of growth and scale-up steps, the carryover of plant metabolite into the fungal culture appears unlikely. Lastly, working with elicitors (Soliman and Raizada 2013) and inhibitors (Soliman et al. 2011), fungal paclitaxel production was increased and decreased respectively, also suggestive of a fungal autonomous pathway. All these indirect evidences suggest a possible separate pathway formed through convergent evolution with distinct enzymes to biosynthesize paclitaxel in the fungal species. Comparing the genome sequence of the paclitaxel-producing *Penicillium aurantiogriseum* NRRL62431 with 13 known paclitaxel biosynthetic genes in *Taxus* plant revealed potential fungal paclitaxel biosynthetic enzymes homologs with identity >30% to GGPPS and phenylalanine aminomutase (PAM) (Yang et al. 2014). Phylogenetic analysis suggested that these genes are independently evolved with no possibility of an horizontal gene transfer (Yang et al. 2014).

Whereas *Taxus* is the only plant genome that has been confirmed to encode a paclitaxel biosynthetic pathway, it is interesting to note that several endophytic fungi belonging to nearly 24 genera have been reported to produce paclitaxel *in vitro* in the absence of plants, and that list is growing (Zhou et al. 2010). However, with the limited information of biosynthetic genes from these paclitaxel-producing fungi, the true paclitaxel biosynthetic capabilities of these fungi remain an unexplored area of research (Stierle et al. 1993; Zhou et al. 2010). If the fungal paclitaxel biosynthetic pathway can be elucidated or even partially mapped, then metabolic engineering for microbial production of paclitaxel can become possible.

Phomactins, another class of fungal diterpenoids, was isolated from the marine fungus *Phoma sp*. (Sugano et al. 1991). Phomactins biosynthesis is similar to that of paclitaxel, and is proposed to involve the cyclization of GGPP to form phomactatriene, that is structurally similar to taxadiene. Isolation of phomacta-1(14),3(4),7(8)-triene and phomacta-1(15),3(4),7(8)-triene from *Phoma sp.* supported this proposed mechanism (Tokiwano et al. 2005). Additionally incubation of TXS with des-7-methylGGPP produced phomactatriene as major product (Chow et al. 2007). Identification of phomactatriene biosynthetic enzymes can perhaps aid the discovery of enzymes in possible fungal paclitaxel biosynthetic pathways.

Metabolic engineering of TXS

The isolation of TXS and the identification of its function in the cyclization of GGPP to taxadiene opened the way for its functional expression in heterologous organisms. Using *E. coli* as a platform, expression of TXS afforded 1.3 mg taxadiene /L culture (Huang et al. 1998). The production level of taxadiene was limited due to the insufficient supply of the terpene precursors. Optimization of terpenoid pathway in *E. coli* has led to significant increases in the level of taxadiene. Recently, using a multivariate-modular approach, Ajikumar et al successfully increased the production of taxadiene up to 1 g/L culture (Ajikumar et al. 2010). In this approach, the taxadiene biosynthetic pathway was divided into upstream and downstream modules; the upstream module comprised of the MEP pathway leading to IPP synthesis; and the downstream module is comprised of the *Taxus* plant GGPPS and TXS. Pairing both modules with different promoters and gene copy numbers led to the identification of a combination with a high level of taxadiene production (Ajikumar et al. 2010). Furthermore, comparison between *E. coli* variants used in this study revealed more metabolic engineering targets. Analysis of heterologous expression of the upstream and downstream modules in two different *E. coli* variants (K and B strains) was performed (Boghigian et al. 2012). *E coli* K strain showed 2.5-fold

greater production level of taxadiene than the B strain, with significant differences in pyruvate metabolism between the variants as a possible reason (Boghigian et al. 2012).

Heterologous expression of TXS in yeast has also been performed (Engels et al. 2008). IPP in yeast is biosynthesized through mevalonate pathway, which is used in the production of farensyl pyrophosphate (FPP) and GGPP. Expression of TXS alone in yeast does not produce significant amount of taxadiene because of the insufficient GGPP level due to competition of FPP metabolism in steroid biosynthesis (DeJong et al. 2006). The titer of taxadiene production increased from 0.2 mg/L to 8.7 mg/L by combined expression of different upstream genes with TXS (Engels et al. 2008). First GGPPS from *Sulfolobus acidocaldavus* was used because it allows the synthesis of GGPP through IPP without the need for FPP biosynthesis and thus does not compete directly with steroid biosynthesis. Second by using a truncated version of HMGR, the negative feedback inhibition through steroid production was alleviated. Third, inclusion of a mutant version of the transcription factor UPC2-1 allowed the exogenous steroid uptake under aerobic conditions thus increasing the terpenoid flux towards taxoid biosynthesis (Engels et al. 2008).

Heterologous expression of TXS in higher plants such as *A. thaliana* (60 µg taxadiene/ kg dry weight) has also been performed (Besumbes et al. 2004). However, TXS expression in *A. thaliana* was accompanied with a lethal phenotype due to siphoning of the GGPP precursor flux that decreased the levels of endogenous plastid isoprenoid products such as carotenoids and chlorophylls (Besumbes et al. 2004). Expression of TXS in tomato (Kovacs et al. 2007) by redirecting the GGPP from carotenoid to taxadiene biosynthesis afforded 160 mg taxadiene/kg fruit. However, expression of TXS in tomato led to a slow growth phenotype presumably due to disruption of gibberellins biosynthesis, which is also a diterpenoid product (Kovacs et al. 2007). TXS gene was successfully integrated into ginseng genome with a production level of 9.1 µg taxadiene /g dry weight without any phenotype changes (Cha et al. 2012). Ectopic expression of the TXS gene in *Nicotiana benthamiana* under control of the CaMV 35S promoter and

suppression of the phytoene synthase gene expression resulted in the increase of taxadiene production level up to ~40 µg/g dry weight (Hasan et al. 2014). Successful expression of TXS in *Physcomitrella* was also demonstrated with appreciable amount of taxadiene (0.05% fresh weight of tissue) and without alteration in the endogenous diterpenoid level (Anterola et al. 2009).

Protein engineering of TXS

Expression of foreign terpene synthases in engineered microbe can be problematic due to low activity, incorrect localization or limited solubility (Bruckner and Tissier 2013; Chang and Keasling 2006). Because terpene synthases including TXS are naturally evolvable and TXS protein structure has been solved to atomic resolution (Koksal et al. 2011), rational and directed evolution based protein engineering approaches can be applied to improving TXS function. Directed evolution strategies can increase the expression levels, solubility, catalytic activity and thermostability of this important enzyme (Dougherty and Arnold 2009). The difficulty in evolving TXS can be due to the lack of metabolite-dependent, high throughput screening. Lauchli and coworkers successfully designed a high throughput screening method for terpene cyclase by using an isoprenoid substrate modified with vinyl-methyl ether group (Lauchli et al. 2013). When an active enzyme variant reacts with the modified isoprenoid substrate, methanol is released and can be oxidized in the presence of alcohol oxidase (added to the reaction mixture) to produce formaldehyde. Formaldehyde reacts with Purpald to generate a blue-purple colorimetric output. This method was used to identify both more thermostable and more catalytically active terpene cyclase variant of BcBOT2 (a terpene cyclase from powdery mildew *Botrytis cinerea* fungus) out of a library of ~2800 mutants (Lauchli et al. 2013). Using a more rational approach, Yasuo et al. was able to design an algorithm based on the independent plasticity residues that contribute to molecular evolution of terpene synthases (Yasuo et al. 2006). Without the need of high throughput screening, they were able to obtain sesquiterpene

synthase with higher activity and selectivity. Combining both rational and evolutionary approaches can help in the near future to improve TXS properties in heterologous pathways.

Future perspectives

Paclitaxel is one of the most important plant natural product discovered in the 20th century. The potent and proven antitumor activities will make this an essential therapeutic compound moving forward. Because of this demand, there are considerable interests to increase the production of paclitaxel from plant sources, as well as installing the biosynthetic capabilities in microbial hosts. Significant challenges remain in both of these endeavors, especially in the complete identification of the pathways. The identification and characterization of TXS described in this review have spun multiple decades, culminating in the structural elucidation of TXS and gram-scale *E. coli* production of taxadiene in the last four years. It is anticipated that with new synthetic biology and plant transcriptomics tools, the next steps to fully map and reconstitute the pathway will be significantly more rapid. At the TXS front, the intrigue of possible fungal diterpene synthase that catalyze the same reaction will lead to genome mining of enzymes from sequenced endophytes; while structural-guided protein engineering and directed evolution efforts will enable the activity optimization of this important enzyme for paclitaxel synthesis.

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Figure legends

Figure 1: Overview of the paclitaxel biosynthetic pathway. IPP; Isopentenyl pyrophosphate, DMAPP; Dimethylallyl pyrophosphate. Solid arrow represents single step, and block arrows represent multiple steps.

Figure 2: Molecular structure of TXS and orientation of taxadiene in the active site of TXS. (a) Structure of *T. brevifolia* TXS is represented by three domains, the C-terminus Class I terpenoid in blue and the N-terminus Class II terpenoids in green and yellow; (b) Orientation of taxadiene (green) in the active site cavity exposing H5â atom of taxen-4-yl cation towards Y688; (c) Orientation of taxadiene (blue) in the active site cavity exposing H5â atom of taxen-4-yl cation towards pyrophosphate PPi anion. Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Koksal et al. 2011), copyright (2011)

Figure 3: The isoprenoid pathway in plants. MVA: mevalonate; MEP: 2-C-methyl-D-erythritol-4-phosphate; DXP: deoxyxylulose-5-phosphate; DXS: DXP synthase; DXR: DXP reductoisomerase; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GGPPS: geranylgeranylpyrophosphate synthase; Solid arrows represent one enzymatic step; and dashed arrows represents multiple enzymatic steps.

Table I: Comparison of TXS activity with other terpene cyclases

Catalytic property	**Taxadiene synthase** (Hezari et al. 1995)	**Plant Abietadiene synthase** (Peters et al. 2000)	**Fungal ent-kaurene synthase** (Kawaide et al. 2000)	**Plant Kaurene synthase** (Hillwig et al. 2011)	**Plant sesquiterpene synthase** (Hillwig et al. 2011)	***Arabidopsis thaliana* kaurene synthase** (Xu et al. 2007)
K_M of GGPP (μM)	3	3	8.7	6	1.7	0.3
k_{cat} (s^{-1})	0.0106	0.75	0.030	4.3	0.45	0.06
k_{cat}/K_M (s^{-1} M^{-1})	3.5×10^3	2.5×10^5	3.5×10^3	7.2×10^5	2.6×10^5	2×10^5

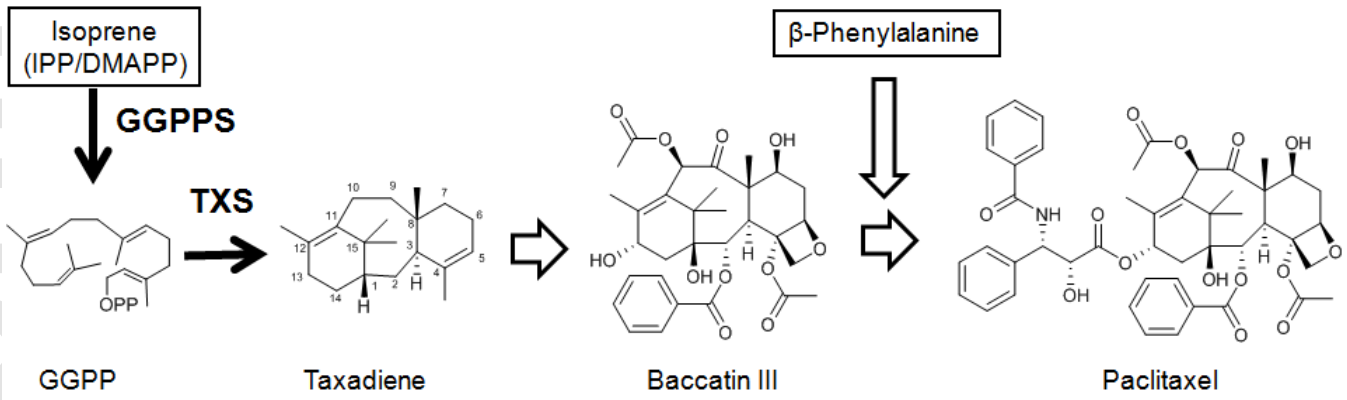

Figure 1

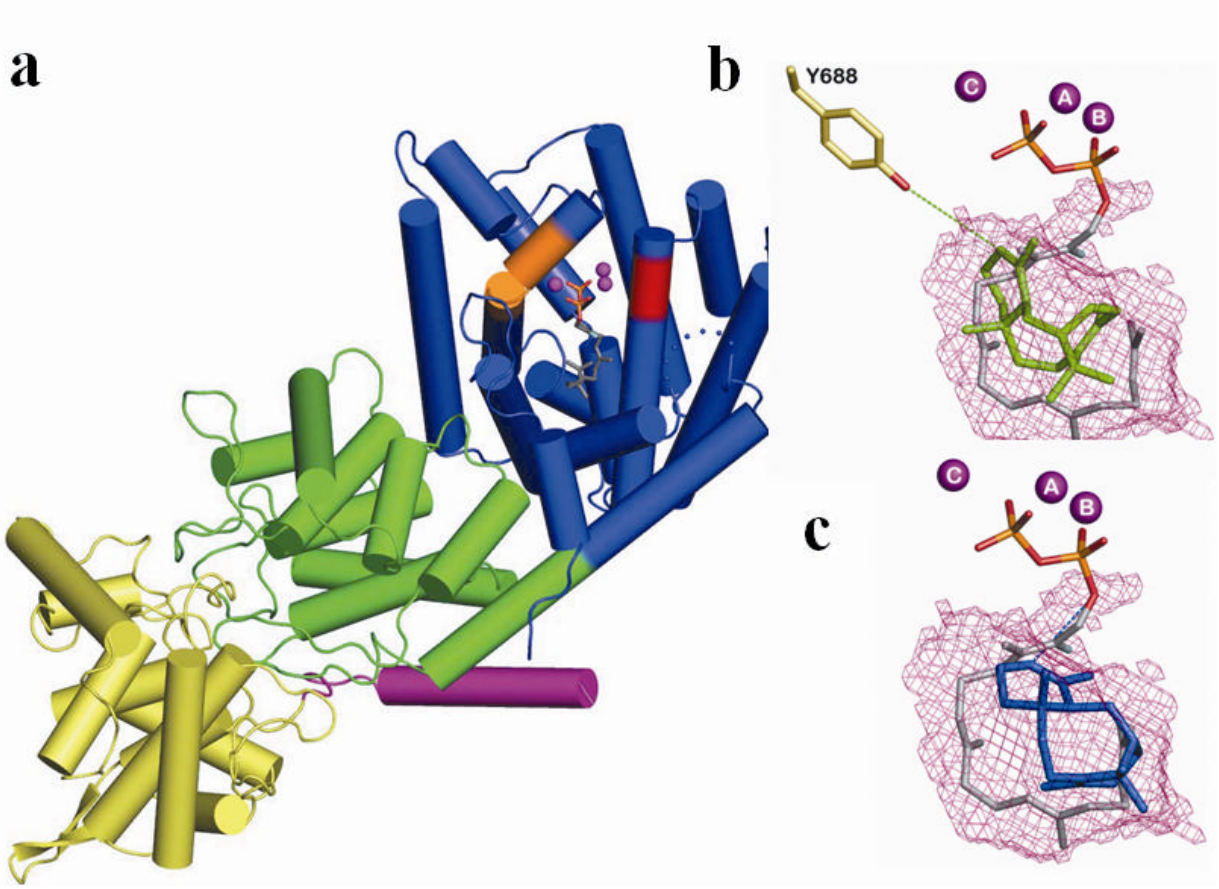

Figure 2

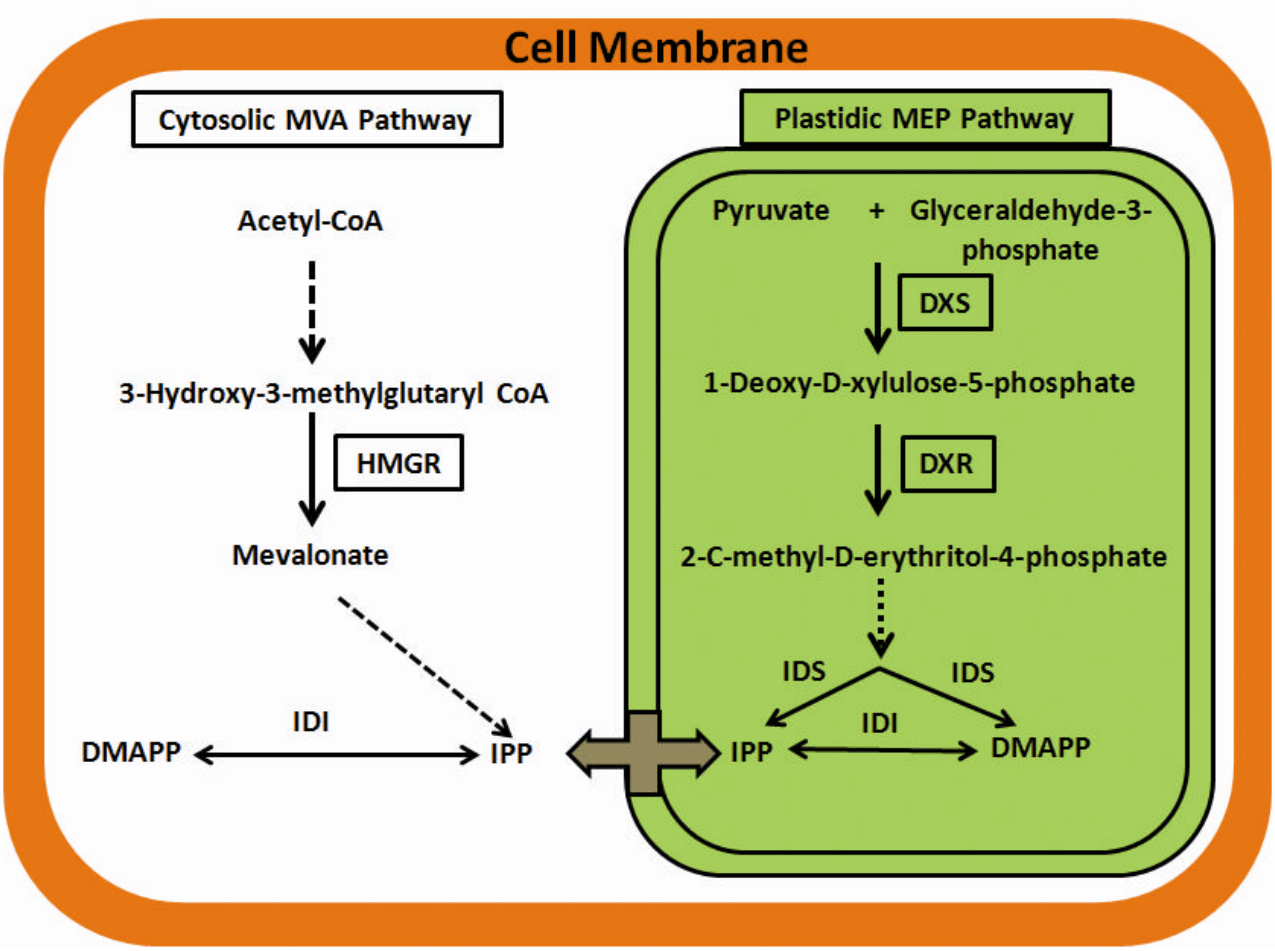

Figure 3