Biosynthesis of Taxol

A Research Plan for Enzyme Optimization and Project Submission for the iGEM Competition

The production of Taxol has a long history of biosynthesis literature but is currently incomplete. Taxol, however is of important use as a cancer therapeautic and decreased production cost would benefit the treatment of those throughout the year. This report provides an overview of the current state of known enzymes in the Taxol biosynthesis pathway, outline of existing unknowns and an experimental overview for the production, characterization and consolidation known enzymes into one plasmid. Recommendations are also made for highest impact enzyme optimization projects.

Background

Taxol, generically called as paclitaxel, is small molecule metabolite that has been shown to work on many forms of cancer (Kingston, 2007). Taxol promotes the formation of microtubule assembly in cells which disrupts tubulin and creates cytotoxicity (Schiff, Fant, & Horwitz, 1979). Further, even synthetic variations on the taxol structure have been tested and shown to have chemical activity, such as doxetaxel (Kingston, 2007).

The efficacy of the drug in treating various cancers has created a boom in demand for taxol and a supply shortage to keep up. In order to understand the current state of taxol production and the need for it today, this section will outline the history of Taxol discovery, usage and synthesis to understand the market as it is now.

History

Taxol was originally derived from the bark of the Pacific yew tree, Taxus brevifolia, a slow growing tree found in the Pacific Northwest stretching from Alaska to Northern California. Taxol was first purified and isolated from bark extract in 1971 (Bocca, 1998; M. C. Wani, 1971). However, isolation from natural sources is hindered not only by the slow growth of the Pacific yew tree but by the low concentration of the drug in its bark, only 100 mg of Taxol per kg of bark (Bocca, 1998).

Interest in Taxol as a drug was low until the discovery of its mechanism in 1979, with clinical trials beginning in 1983 (Donehower, 1996). Taxol was first approved for second line ovarian cancer treatment starting in 1992 and later for first line treatment starting in 1998 (Pai-Scherf). It has also been approved for breast, adenocarcinoma of the pancreas and non-small cell lung cancers (Food and Drug Adminisitration, 2013).

Current Production Strategies

As mentioned previously, natural production of Taxol suffers from extremely low production weigh and slow growth rate. This includes both the Pacific yew but also the Chinese yew tree, which is still being harvested. This has evolved from harvesting wild specimens to cultivation of specific specimens. For example, in 2004 a Chinese company had planted 30 km^2 of Chinese yew (Meštrović, 2014).

Further methods have made use of chemical and semi-chemical methods. The first pure chemical synthesis of taxol was achieved by 1994. However, the intricate stereoisomerism and multistep production kept the production and yield rates very low and left cost of production high. A similar production method makes use 10-deacetylbaccatin from the European yew as a start of a semisynthetic method of production (Bocca, 1998).

A final interesting mode of production makes use of plant cell fermentation for the production of Taxol. This makes use of plant cell cultures with the inherent enzymes expressed for the production of Taxol without the addition of 10-deacetylbaccatin. This creates a means of creating Taxol effectively and efficiently with no natural product harvesting (Phyton Biotech, 2016). It provides a novel technology that allows for future production, but the efficiency of this method compared to simpler and more optimized fermentation organisms like bacteria have not been compared because of the proprietary nature of the fermentation set-up.

The current state of the production methods moves closer to an efficient and economical method. With the movement to fermentation based methods of production show that the scope of this project seems relevant within the movement of the market despite the decades since the proposal of such a system. The movement to bacterial total biosynthesis would make use of the

development of plant cell fermentation while making use of a more established system to make the discovery more accessible and possibly even cheaper.

Cost and Accessibility

Current production of Taxol makes the cost per dosage cycle as $458, which relates to about $5,000 per patient with breast cancer (Havrilesky, 2012). This number however does not make comment to the system of care that allows for its implementation. Not included in the cost of the pill is the cost of personnel to administer the drug, manage side effects and verify drug effectiveness. It also does not a comment on the cost of the system that allows for the storage and delivery of Taxol injections and preparations for the patient to make use of the drug.

The cheaper creation of Taxol will lower the cost of the drug and its production but this cost is not the only thing holding back treatment. To understand how lowering the cost of drug production will translate in to more available treatment is beyond the scope of this report. However, it is recommended that a secondary project focusing on the cost of Taxol treatment, from healthcare to purely treatment times and durations be considered and modelled to know of the creation of a cheaper Taxol would actually affect those who cannot afford Taxol under current systems and production.

Pathway

The creation of Taxol requires the implementation of as many as 19 enzymes to produce the shape and features of a functional molecule. This pathway starts with a mevalonate pathway in order to create isopentyl diphosphate, which is then extended to geranylgeranyldiphosphate,

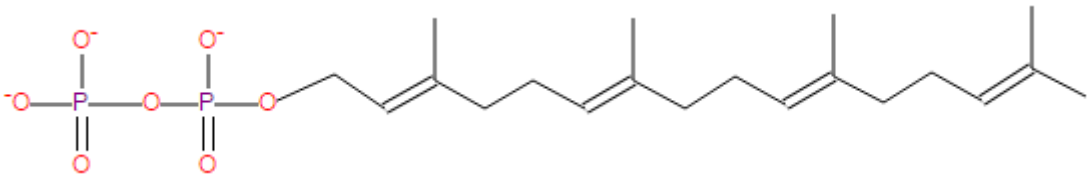

Figure 1: Geranylgeranyl diphosphate, an unlooped precursor to taxol

However, at this point, the formation of the basic ring structure begins. This happens using a synthase to close the ring onto itself. With this ring structure largely in place, the next step consists of largely of oxidations along the ring, including an oxatane ring structure, as well as the addition of the "Taxol sidechain," a side chain that features two benzene rings, an amide link, an ester link and a hydroxyl group.

The framework of this project will be primarily focused on these later steps which include the oxidation events and especially the addition of the sidechain. As such, this project starts by assuming a cell line that can already produce taxa-4(20),11-dien-5α-ol, which is the step after taxa-4,11-diene is oxidized by the T5α-H taxadiene 5α-hydroxylase. The following section will go through the known enzymes and gaps in knowledge in the creation of taxol's sidechain and oxidations. These will include associated kinetics and the reactions associated with each enzyme.

The order of enzymes examined will begin with the addition of the acetyl group and benzoyl group to the Taxol rings. Then there will be an overview on the largely known oxidation

steps along the Taxol rings, then the addition of an acetyl group to the 10' hydroxyl group. Finally, the production and addition of the Taxol sidechain will be outlined.

A chart of relative kinetics of the enzymes recommended for the project is provided on the following page (Table 1). The range of turnover rates leads to the emphasis on the development of certain enzymes, especially Taxane 2α-O-benzoyltransferase, with a k_{cat} of only 0.003 s^{-1}, or 0.19 min^{-1}. The possible projects will be explored in greater detail after the exploration of each enzyme. An overview is below (Figure 2).

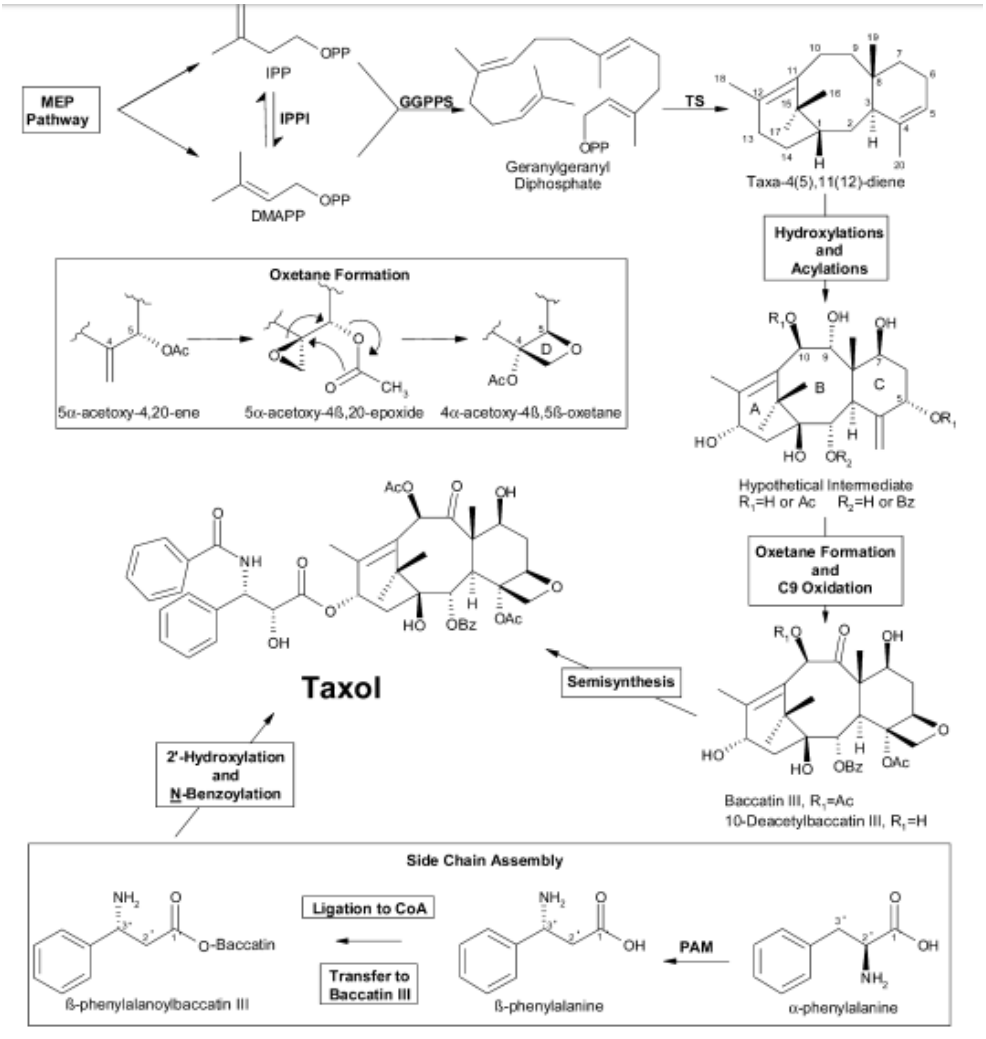

Figure 2: (Figure from previous paper) An overview of the production pathway of Taxol starting from geranylgeranyl diphosphate through Taxol (Croteau, Ketchum, Long, Kaspera, & Wildung, 2006).

Name	**Shortened**	**K_m**	**k_{cat}**
Taxadienol acetyl transferase	TAT,TAX19	5.0 uM (taxadienol); 5.5 uM (acetyl-CoA)	29.59 s^{-1}
benzoate-CoA ligase	badA	4.4 uM	28 s^{-1}
Taxane 2α-O-benzoyltransferase	TBT,mTBT	110 uM	0.003 s^{-1}
10-deacetylbaccatin III-10-O-acetyl transferase	DBAT, mDBAT, TAX10	57.6 uM (deacetylbaccatin III)	0.59 s^{-1}
Phenylalanine aminomutase	PAM	45 uM (phenylalanine)	0.015 s^{-1}
Tyrocidine synthase I (S563A)	TycA	62.3 uM (phenylisoserine); 804 uM (CoA)	0.05 s^{-1}
Phenylpropanoyltransferase	BAPT, TAX7	5.6 uM (phenylpropanoyl-CoA); 4.9 uM (beta-phenylalanoyl-CoA); 68 uM for baccatin III	0.0583 s^{-1}
3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase	DBTNBT, TAX10	420 uM (debenzoyl-taxol); 400 uM (benzoyl-CoA)	1.5 s^{-1}

Table I: An overview of the kinetic constants of enzymes investigated for the Taxol biosynthesis process. These include kinetic constants found from biochemistry literature

Taxadienol acetyl transferase (TAT)

taxa-4(20),11-dien-5α-ol + acetyl-CoA → taxa-4(20),11-dien-5-α-yl acetate + coenzyme A

K_m: 4.2 uM for taxa-…-dien-…-ol, 5.5 uM for acetyl-CoA (Walker, et al., 1999).

k_{cat}: 29.59 s^{-1} (Chau, Walker, Long, & Croteau, 2004)

TAT makes use of the endogenous metabolite acetyl-CoA to add an acetyl group to the backbone of the Taxol ring using the hydroxyl group just added to the ring. This enzyme is one of the more straight-forward cases, with well-established metabolites and chemistry pairing with among the best metabolic kinetics of the enzymes explored. The production of TAT as produced from the engineered TAX19 strain provides a basis for which to incorporate into further processes. Further work is largely unnecessary.

Benzoate-CoA ligase (BadA)

ATP + benzoate + CoA <-> AMP + diphosphate + benzoyl-CoA

K_m: 1 uM (Egland, Gibson, & Harwood, 1995); 4.4 uM (Thornburg, Wortas-Strom, Nosrati, Geiger, & Walker, 2015)

k_{cat}: 28 s^{-1}.

BadA does not interface directly with the Taxol production pathway directly but is involved in the production of a metabolite used for the addition of the benzoyl group to the Taxol backbone, benzoyl-CoA. Benzoyl-CoA does not exist in the metabolome of E. coli, nor does benzoate. However, benzoate is a low cost metabolite and can be bought and added to fermentation if necessary for the project after the Thornburg optimization.

With benzoate added, BadA as a strain from Rhodopseudomonas palustris. The strain of BadA is efficient and features favorable kinetic constants. The enzyme provides another straight-forward case and optimization on BadA is very low priority.

Taxane 2α-O-benzoyltransferase mutant (mTBT)

10-deacetyl-2-debenzoylbaccatin III + benzoyl-CoA → 10-deacetylbaccatin III + coenzyme A

K_m: 110 uM (Nawarathne & Walker, 2010)

K_{cat}: 0.19 min^{-1} (Nawarathne & Walker, 2010)

This step occurs after the additional oxidation events on the Taxol backbone. It is the final step in the production of 10-deacetyylbaccatin III, which is used in many downstream Taxol production methods. It depends on the oxidation of the 2' carbon by a later discussed. The benzoyl-CoA produced by BadA is used to add a benzoyl ring to the 2' hydroxyl group using standard CoA chemistry.

The enzyme and gene included in this report is an engineered mutant that has improved kinetics over the wild type from *Taxus cuspidata* Japanese yew. That said however, it is still the slowest of the enzymes described in this report. Therefore, it is possible that there is something inherently slow in the addition of the benzoyl ring to the Taxol backbone, but optimization of this speed may make one of the most effective enzyme optimization projects for the overall product. However, in-lab production of substrate will require the characterization of oxidation enzymes to obtain their product. This would need to be considered and overcome for large-scale characterization and optimization projects.

10-deacetylbaccatin III-10-O-acetyl transferase (DBAT)

10-deacetylbaccatin III + acetyl-CoA → baccatin III + coenzyme A

Km: 57.6 uM for 10-deacetylbaccatin III (Ondari & Walker, 2008)

kcat: 0.58 s^{-1} (Ondari & Walker, 2008)

DBAT provides the final acetylation to the Taxol backbone. It makes use of well characterized substrates, both 10-deacetylbaccatin III and acetyl-CoA to make for easier characterization. The product is also well characterized. It makes use of the 10' carbon hydroxyl group to add the acetyl group via the CoA thioester mechanism.

The turnover rate of DBAT is still lower than other enzymes such as TAT, although by no means the lowest. By terms of enzyme function, the enzyme is not of highest priority for optimization, but because of the availability and high degree of characterization of the substrates and product the enzyme may be an easy project for learning methods and verifying kinetics to better characterize DBAT for iGEM submission and for general results verification projects.

Ring Oxidations

The largest unknown in the production of the Taxol backbone structure is the many different oxidation events. This includes the multiple hydroxyl groups in the backbone but it also includes the oxetane ring that is necessary for the function of Taxol. The hydroxyl groups have been hypothesized to be performed by endogenous P450 cytochromes. These however, need to be found and characterized for the project to move forward. The scale of finding these enzymes is very large. Each of these enzymes, if properly characterized and understood could result in publications and advances in the development of Taxol. The creation of a full suite of oxidation P450 cytochromes would be a massive movement forward towards the total biosynthesis of Taxol in bacteria.

Exploration and testing for the production of these hydroxyl groups is among the most important subprojects in the Taxol biosynthesis project and thus should likely include multiple subprojects looking at exploring P450 domains which can be interchanged for the different oxidation events (Croteau, Ketchum, Long, Kaspera, & Wildung, 2006). The beginnings of this project are already underway in the work of a fellow undergrad working under Professor Mike Lynch.

The oxetane ring however does not have a well characterized and agreed upon mechanism. It is possible that this is enabled by another P450 (Croteau, Ketchum, Long, Kaspera, & Wildung, 2006). The unknown of even the mechanism makes exploration of this enzyme exceptionally interesting but the method was not found within the scope of research for this report.

Phenylalanine aminomutase (PAM)

(3S)-α –phenylalanine (aka L-phenylalanine) → (3R)-β-phenylalanine

$\mathbf{K_m}$ = 45 uM for 2S-α-phenylalanine (Walker, Klettke, Akiyama, & Croteau, 2004)

$\mathbf{k_{cat}}$ = 0.015 s^{-1} (Walker, Klettke, Akiyama, & Croteau, 2004)

PAM is the first step in the creation of the Taxol side chain. It makes use of a natural alpha amino acid, L-phenylalanine, for modification into an unnatural stereoisomer. This takes the form of moving the amine group to the adjacent carbon. The reaction, therefore, does not need other metabolites for the reaction to move forward. The enzyme however, is slow acting with a turnover rate of only 0.015 s^{-1}. Because this is a slow reaction and has a well characterized substrate, PAM is a good candidate for exploring enzyme engineering.

Tyrocidine synthase I (S563A) (TycA-S563A)

(3R)-β-phenylalanine + CoA + ATP ⟶ (3R)-3-amino-3-phenylpropanoyl-CoA + AMP + Diphosphate

K_m: 62.3 uM for (R)-beta-phenylalanine; 804 uM for CoA (Muchiri & Walker, 2012)

k_{cat}: 3.00 min^{-1} for (R)-beta-phenylalanine; 0.90 min^{-1} for CoA (Muchiri & Walker, 2012)

TycA-S563A is an enzyme that loads the beta-phenylalanine onto the CoA thioester in order to add to the Taxol backbone. This enables the addition of this to the 13' carbon of the Taxol backbone to become the ring structure. The turnover rate for the enzyme is among the lowest, but TycA-S563A also features a high K_m with regard to CoA. This mutant was shown to half the CoA Michaelis constant but reducing this number could be reduced further as the concentration of coenzyme A is only 1.4 mL (Bennett, et al., 2009) and CoA is a common cofactor in the Taxol pathway. This would, however, only be a consideration for a more pressing problem of increasing turnover rate.

TycA-S563A also catalyzes the production of phenylisoserinyl-CoA, although with a lower turnover rate (Muchiri & Walker, 2012). This rate is still faster than mTBT but would greatly benefit from further enzyme optimization for this substrate. However, this substrate promiscuity allows for the given enzymes to complete the pathway regardless of the later addition of a 2'-hydroxylase or phenylisoserine production.

Phenylpropanoyltransferase (BAPT, TAX7)

baccatin III + (3R)-3-amino-3-phenylpropanoyl-CoA → N-debenzoyl-(3'-RS)-2'-deoxytaxol + coenzyme A

K_m: 5.6 uM for (3R)-3-amino-3-phenylpropanoyl-CoA , 68 uM for baccatin III (Thornburg, Development of a Four-Step Semi-Biosynthesis of Taxol Biosynthesis: Tyrocidine Synthetase A Catalyzes the Production of Phenylisoserinyl CoA and Other Amino Phenylpropanoyl Thioesters, 2015);

2.4 uM for baccatin III and 4.9 uM beta-phenylalanoyl-CoA (Walker, Fujisaki, Long, & Croteau, 2002)

k_{cat}: 0.0583 s^{-1} (Thornburg, Development of a Four-Step Semi-Biosynthesis of Taxol Biosynthesis: Tyrocidine Synthetase A Catalyzes the Production of Phenylisoserinyl CoA and Other Amino Phenylpropanoyl Thioesters, 2015)

BAPT is the enzyme that adds the C13 Taxol side chain partially formed to the Taxol backbone. It makes use of CoA chemistry to add the side chain to the 13' hydroxyl group of baccatin III. The reaction takes place very slowly and features the second lowest turnover rate of any known enzyme in the pathway. This, again paired with the well-known structure and availability of the substrate make the enzyme another good candidate for enzyme engineering.

The enzyme in addition to working with phenylpropanoyl-CoA works with the similar phenylisoserinoyl-CoA, although with an even lower turnover rate. BAPT has a relative velocity of 0.4 for phenylisoserinoyl-CoA compared to phenylpropanoyl-CoA (Walker, Fujisaki, Long, & Croteau, 2002). Phenylisoserinoyl-CoA is phenylpropanoyl-CoA with an additional hydroxylation. The enzyme thus allows for the propagation of an earlier hydroxylation of the Taxol side chain. The phenylisoserinoyl-CoA or phenylisoserine could be spiked for early testing if the creation of the molecule cannot be achieved in the production strain.

However, this enzyme is in under patent in the form present here (Rodney Croteau, 1999). The patent is for 70% identical DNA to the gene. This is important to know going forward and will likely eliminate ability to work extensively on further uses of TAX7 and would

likely bar the submission of the gene to iGEM's standard registry. The patent does expire in 2019, however.

β-phenylalanoyl baccatin III-2'-hydroxylase (Tx)

$$\text{N-debenzoyl-(3'-RS)-2'-deoxytaxol} + \text{NADPH} + \text{oxygen} + H^+ \rightarrow \text{N-debenzoyl-(3'-RS)-taxol} + NADP^+ + H_2O$$

The final 2' oxidation step uses an enzyme that is not yet known. It has been hypothesized to be another P450 cytochrome hydroxylase. In natural production in Yew trees, it is likely that this reaction occurs after the addition of the side chain (Long & Croteau, 2005). However, isoserine addition does present an alternative to the natural mechanism. Further research could be performed on the isoserine hydroxylation enzyme to complete the pathway via an earlier hydroxylation but otherwise, the 2'-hydroxylase should be an extension of the P450 oxidation project.

β-phenylalanoyl baccatin III-2'-hydroxylase (DBTNBT)

$$\text{N-debenzoyl-(3'-RS)-taxol} + \text{benzoyl-CoA} \rightarrow \text{Taxol} + \text{CoA} + H^+$$

Km: 420 uM for N-debenzoyl-(3'-RS)-taxol, 400 uM for benzoyl-CoA (Walker, Long, & Croteau, The final acylation step in Taxol biosynthesis: Cloning of the taxoid C13-side-chain N-benzoyltransferase from Taxus, 2002)

kcat: 1.5 ± 0.3 s^{-1} (Walker, Long, & Croteau, The final acylation step in Taxol biosynthesis: Cloning of the taxoid C13-side-chain N-benzoyltransferase from Taxus, 2002)

The final step of the Taxol biosynthesis pathway is the addition of another benzoyl ring to the Taxol C13 side chain. It makes use of a second benzoyl-CoA from the BadA enzyme production and makes use of the same chemistry but with different substrate specificity for the later Taxol intermediate. The reaction progresses quickly with one of the higher turnover rates of the enzymes examined. It does however have a large Michaelis constant with respect to both N-

debenzoyl-(3'-RS)-taxol and benzoyl-CoA. The benzoyl-CoA sepeficity was observed to be a hundred fold higher than for mTBT. As such, a lower priority optimization project may look to lower the value of this constant to facilitate the rate of reaction in cases of lower substrate concentration.

Further, this enzyme is contained in the same patent as BAPT, expiring in 2019 (Croteau, Walker, Schoendorf, & Wildung, 2003). As such, work on this enzyme may be of lower priority due to the ambiguity of freedom to operate using the enzyme. Further analysis and investigation into fair use of the enzyme and history of patent enforcement can be further explored.

Key Enzyme Projects

In addition to overall cloning projects, the existing enzymes as found by published literature may be insufficient for the effective production of Taxol. In its current state, the enzymes have a wide range of turnover rates that would lead to the bottlenecks in the pathway even with high expression. As such, the enzyme optimization projects with the largest potential on the project are those with the lowest turnover rates. These are mTBT, PAM and TycA in order of ascending turnover rates.

mTBT optimizations can look into the difference in effective rates between mTBT and DBTNBT but care should be taken to avoid trespassing on the DBTNBT patent so as to later use and submit the new optimized enzyme. The substrate, 10-deacetyl-2-debenzoylbaccatin III, however, is not commercially available which may make large-scale assays difficult with creation of a production strain for the substrate.

PAM acts on a common substrate which will make testing difficult. It however does not use any other cofactors in the reaction which makes kinetic assays dependent in some way on the

product directly. As the following reaction in the pathway is also slow, it makes it difficult to test indirectly using this enzyme. The relative low cost of the product, (3R)-β-phenylalanine, however means that an analytic method for this substrate could be developed for use in a kinetics assay.

TycA directly follows PAM in the pathway and thus a method for (3R)-β-phenylalanine could be useful for this project as well. The production of (3R)-3-amino-3-phenylpropanoyl-CoA also has many cofactors such as AMP and diphosphate that could be screened for as well as the product itself. The development of this assay and analytic method however is beyond the scope of this report. Additionally, and possibly separately, TycA could be developed to create phenylisoserinyl-CoA. This could represent a separate optimization project that could progress in parallel in order to better address and understand the function on the enzyme on phenylisoserine.

In addition to enzyme optimization, investigations can focus on designing and developing the P450 cytochrome enzymes necessary for the oxidations of the Taxol backbone. These are still widely unknown and investigation of these would likely contain more basic research than applied engineering. Looking at these could work from the directed mutation of CYP proteins of similar substrates but could also take the form of screening candidate genes from yew tree species and plant cell fermentations in order to track down and isolate the genes and proteins associated with each step. These experiments are of a larger scale and may not fit within the timeframe and scope of the project here developed. The identification of such a protein, however, would be critical to further progress in the production of Taxol in bacteria.

Additionally, the production pathway also offers alternative if the enzyme associated with the final hydroxylation is not found. The promiscuity of TycA and DBTNBT mean that if the production of phenylisoserine can be accomplished in cells, then the pathway can progress

without this unknown enzyme. Phenylisoserine is similar to (3R)-β-phenylalanine with the addition of a hydroxyl group. If an enzyme that catabolizes this reaction is also found, then the pathway can be completed. This does not eliminate an unknown but provides another possible route for completing the unknown pathway.

Deliverables

The goal of the project is the creation of taxol from a bacterial strain with inputs of benzoate. However, this is dependent on the location and description of the many oxidation steps of the taxol ring and of the side chain. These discoveries are the largest challenge presently to the complete biosynthesis of taxol. That said, the discovery of any of these individual enzymes, with characterization and associated amino acid sequence or cDNA could become a publication given the highly published history of taxol biosynthesis.

Beyond identifying unknown proteins, the project will also create part submissions of a library of Taxol associated genes for the Standard Registry of Biological Parts and an associated project submission to the iGEM competition. These parts will be processed for submission as well as for promotion and testing to better characterize the enzymes. Methods for isolation of enzymes and products as developed over the summer will be also relevant for submission. Further, the Taxol project can act as a springboard for non-laboratory projects associated with the background of Taxol and its use as a cancer medication.

Conclusions

The production of Taxol in bacteria is a case that has been a textbook example for the biomanufacturing. As such, there is extensive literature and continuing work to finish the pathway. Currently, the process is approaching complete knowledge but there are still unknowns. The identification of the proteins associated with the oxidation of the Taxol backbone, including identifying the mechanism of the oxetane ring formation, will finish the identification of the gene in the pathway.

After these are found, the problem will be one of finding and optimizing the pathway itself to make production as efficiently as possible. This project outlines an experimental method for the characterization, optimization and consolidation of known enzymes to move forward with this goal while the final enzymes are being found. This project works to make these genes more readily available for further investigation and use while looking at finding these unknown proteins.

The production of Taxol in bacteria will be a triumph of a body of literature that spans decades and would be noteworthy for this beyond practical applications. For practical applications however, Taxol production as described here would be added to a system of other production means and a complex medical system. The impact of production of Taxol is cannot be determined exactly without better understanding of the factors that go into its production, purification and use throughout the world, but the introduction of this new method will certainly make way for the cheaper production and increased access of Taxol throughout the world. Even if the product does not finish and only partial progress is made, the efforts of this project will continue to move towards this large and overarching goal.

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