CHAPTER I

LITERATURE REVIEW

Introduction

Streptomyces, a genus of order Actinomycetale represented by gram positive, is a large group of filamentous bacteria, like fungi. The DNA base compositions of bacteria in actinomycetes group are high G+C content, about 60-70 %. The most interesting property of *Streptomyces* is its ability to produce secondary metabolites (Hopwood 1979). Polyketides are such a group of secondary metabolites found in bacteria, fungi and plants. The polyketides such as plant flavonoids and bacterial macrolides are medically important because of their pharmacologically important activities including antimicrobial, antifungal, antiparasitic, antitumor and antibiotic. Erythromycin, a macrolide antibiotic produced by *Saccharopolyspora erythrea*, play an important role in treatment of several infectious diseases including pneumonia, sore throat and whooping cough (Cupp-Vickery and Poulos 1997). Because of, its side effects, development of a new generation of erythromycin needs knowledge and understanding of biosynthetic process. The biosynthesis of polyketides is composed of condensation step of acyl CoA precursors, followed by a cycle of three reactions : ketoreduction, dehydration and enoyl reduction, which are catalyzed by polyketide synthases (PKS) and tailoring step that modified a polyketide structure. The tailoring step of polyketide antibiotics is one or more oxidation reactions catalyzed by a cytochrome P-450 hydroxylase. This process converts inactive polyketides to an active form. The aims of this research are to screen and identify cytochrome P-450 hydroxylase gene of *Streptomyces* species isolated in Thailand. We are interested in *picK* cytochrome P-450 hydroxylase catalyzing the hydroxylation

of narbomycin to picromycin in *S. venezuelae*, and use it as a probe for finding cytochrome P-450 hydroxylases in order to generate novel polyketides.

Streptomyces as Bioactive Compound Producers

Streptomyces are soil bacteria ubiquitous in nature. They colonize in soil as a vegetative hyphal mass which differentiate into spore . Spores are a semi-dorminant stage in the life cycle that can survive in soil for long periods. Spores can resist to low nutrient and water availability whereas the mycelial stage is sentitive to drought (Kieser *et al.* 2000). The life cycle of *Streptomyces* is described as follows : First, the germination of rod-shaped or oval immobile spores results in the formation of 1-4 hyphae per spore. Hyphae only grow on the tip but do not branch. Since there is no cross-wall formed in vegetative hyphae, a polynucleated filament is being formed. It results in vegetative mycelium with actually 0.5 -1.0 μm in diameter. Second, Once colonies become aging, aerial mycelia are formed and spores are produced above the surfaces of the colonies. Finally, *Streptomyces* spores is brought about by the formation of cross walls in polynucleated aerial mycelium resulting in cell wall formation. Individual cells then differentiates directly into spores which are still surrounded by a sheath leading to the formation of a spore chain. The classification of *Streptomyces* is often done according to the shape, arrangement of aerial mycelium and colonial pigment (Brock 1974).

The most interesting property of *Streptomyces* is its ability to produce secondary bioactive metabolites, valuable in medicine, agriculture, and unique biochemical tools (Hopwood 1979). Two-thirds of known antibiotics are produced by bacteria groupep in Actinomycetes, and nearly 80% of antibiotics are made by genus *Streptomyces* (Kieser *et al.* 2000). *Streptomyces spp.* produce not only antibiotics but also antitumor, immunosuppressive and cholesterol-lowering agents (Demain 1999). For examples, erythromycin, amphotericin B,

and avermectin are antibacterial, antifungal and antihelmintic agents produced by *Saccharopolyspora erythraea*, *S. nodosus* and *S. avermitilis*, respectively. Doxorubicin and mitomycin C are antitumors synthesized by *S. peucetius* and *S. lavendulae*. Rapamycin and FK506 are immunosuppressants produced by *S. hygroscopicus*. Pravastatin is a drug for lowering blood cholesterol which produced by *S. carbophilus*.

Polyketide Biosynthesis of *Streptomyces*

Polyketides are a group of secondary metabolites found in bacteria, fungi and plants. They possess a pharmacologically important activities. Polyketides are usually classified upon the basis of their chemical structures in to two groups : the aromatic polyketides with one to six aromatic rings, and the complex polyketides, subdivided into macrolides, ansamycins, polyenes and polyethers (Hranueli *et al.* 2001). The chemical structures of the two groups of polyketides are shown in Figure 1.

Polyketide biosynthesis in *Streptomyces* is carried out by complex biochemical processes catalyzed by enzymes. The enzymes which make a diversity of polyketide backbone are polyketide synthase complexes (PKS) (Hopwood 2004). PKS are catagorized in to three types(Hranueli *et al.* 2001) : Type I PKS is very large multifunctional proteins or modules, Each module plays role only once in biosynthetic process. The type I PKS is responsible for synthesis of macrolides, such as erythromycin, rapamycin, oleandomycin, nystatin and amphotericin B. Type II PKS, consists of monofunctional proteins. They are the aggregate of small polypeptides carrying an individual with a single catalytic activity. They are used iteratively during the biosynthetic process (Hranueli *et al.* 2001). Type II PKS catalyzes the formation of polyketides that requires aromatization and cyclization. This type of PKS is involved in the biosynthesis of aromatic polyketides, for examples : actinorhodin, tetracenomycin and doxorubicin. Type III PKS

is responsible for synthesis of chalcones and stilbenes in plants and polyhydroxy phenols in bacteria. Unlikewise other type of PKS, chalcone synthase is composed of small protein with a single polypeptide chain. These PKS is involved in the biosynthesis of precursors for flavonoids (Hopwood 2004; Hranueli *et al.* 2001).

In general, the biosynthesis of polyketide is the sequential condensation of small organic acids such as acetic and malonic acid reactions catalyzed by PKS to produce a long chain of carbon atoms. First step of the biosynthesis is the conversion of acetic and malonic acid to activated derivartives (acely CoA and malonyl CoA) by acyl transferases (Shen 2003). Then acetyl CoA and malonyl CoA are in condensation repeat reaction catalyzed by ketosynthase. The condensation steps produce a four carbon chain attached to ACP. The condensation cycle with malonyl CoA or other extender units are required to extend the length of the carbon chain. The number and the types of building units make the variety of polyketide backbones. The other biosynthetic steps are the three-steps of reduction. The reduction reactions catalyzed by ketoreductase convert keto groups to hydroxyl that appear at the alternate carbon atom in the growing carbon chain. Dehydration of some hydroxyl group attached to the carbon chain results in double bond formation in the structure. Finally, full saturation with the enoyl reduction of double bond by enoly reductase (Hopwood and Sherman 1990; Hopwood 2004). The complete carbon chain detaches from the PKS and forms macrocyclic, aromatic or complex structure by cyclization. The polyketide backbone is further by post polyketide synthase tailoring steps (e.g. methylation, modified by glycosylation and hydroxylation). These post-polyketide modifications are significant to enhance the maturity and bioactivity of polyketide (Betlach *et al.*1998).

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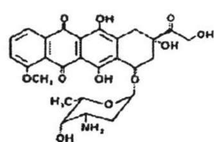
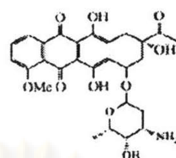
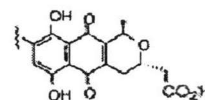
I. Aromatic polyketides

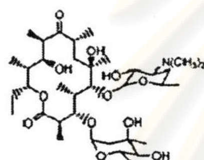

Doxorubicin

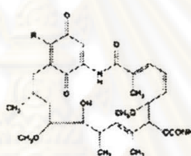

Nogalamycin

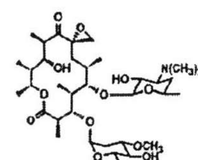

Actinorhodin

II. Complex polyketides

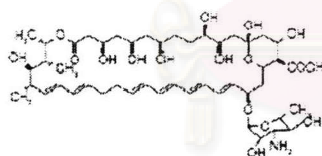
A) Macrolides and ansamycins

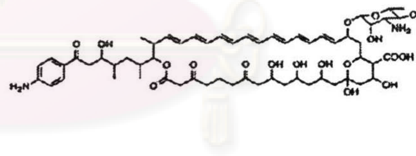

Erythromycin


Ansamycin


Oleandomycin

B) Polyenes


Nystatin


Candicidin D

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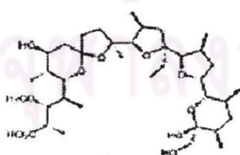
C) Polyethers


Monencin A

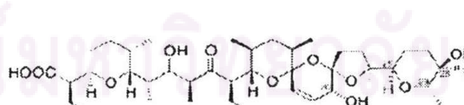

Salinomycin

Figure 1. Aromatics and complexes polyketides (Hopwood 1997; Khosla 1997; Brautaset *et al.* 2000; Leadlay *et al.* 2001)

Post-Polyketide Synthase Tailoring Enzymes

Many of bioactive polyketides contain methyl groups in their structures, which are introduced methyl groups during biosynthesis. However, all methylations are not due to the action of methyltransferases. In the case of the polyketides, some of methyl groups derives from the carboxylic acids used as starter or extender units during the condensation reactions. In other cases, the action of specific methyltransferases is responsible for the methylation reactions. Although O-, N- and C-methylations occur in the polyketide most of them is O-methylation. Such a methylation appears in the biosynthesis of tylosin, erythromycin, tetracenomycin and daunomycin. Most methyltransferases participating in antibiotic biosynthesis use *S*-adenosylmethionine as a cofactor (Lozano *et al.* 2000). In erythromycin biosynthesis, the methylase uses *S*-adenosylmethionine for the methylation of the 3-OH of mycarose on erythromycin C to yield erythromycin A. In addition, the methylase also catalyzes directly the conversion of erythromycin D to erythromycin B (Carreras *et al.* 2002).

In addition to methylation, aglycone of polyketide is furnished with different carbohydrates. The sugar moieties impart polar functionality to aglycone structures that are often hydrophobic and poorly water soluble. Glycosylation occurs in both the macrolide and the tetracyclic subclasses of polyketide antibiotics (Walsh *et al.* 2003). In the case of erythromycin, glycosyltransferase next catalyzes attachment of mycarose to the C-3 group hydroxyl of erythronolide B to yield -mycarosylerythronolide B, which is further glycosylated with desosamine at the C-5 hydroxyl by desosaminyltransferase to form erythromycin D (Carreras *et al.* 2002). Other post-polyketide modification usually involved the hydroxylation reaction. Polyketide cytochrome P-450 hydroxylase catalyzes the site-specific oxidation of the polyketide core structure to form an active antibiotics. EryF and EryK are cytochrome P-450 hydroxylases that catalyze hydroxylation of 14-carbon macrolactone. EryF hydroxylates at C-6 of 6-

deoxyerythronolide B to yield erythronolide B, and EryK catalyzes the hydroxylation of glycosylated compound erythromycin D at C -12 to erythromycin C. The biological importance of the hydroxylation is represented by increase in antibiotic potency (Betlach *et al.*1998).

Picromycin Biosynthesis of *Streptomyces venezuelae*

Streptomyces venezuelae produces 14-membered ring (picromycin and narbomycin) and 12-membered macrolactone ring macrolide antibiotics. These metabolites are derived from 1 acetate and 5 propionate building blocks for 12-membered ring (methymycin and neomethymycin). Picromycin and narbomycin are derived from narbonolide by addition of one or more propionate unit to the polyketide chain (Graziani *et al.* 1998; Xue and Sherman 2001). Macrolactones are synthesized by modular polyketide synthases that consists of five genes : *pikAI*, *pikAII*, *pikAIII*, *pikAIV* and *pikAV* for biosynthesis of 10-deoxymethynolide and narbonolide (Xue *et al.* 1998; Xue and Sherman 2001). The biosynthesis of the aglycones are shown in Figure 2.

The downstream of *pikA* gene cluster is responsible for post-polyketide biosynthesis including desosamine glycosylation and hydroxylation.The *des* genes which are responsible for desosamine glycosylation encode desosamine glycosyltransferase for addition of a desosamine sugar to 10-deoxymethynolide and narbonolide to yield YC-17 and narbomycin respectively. Nearby the *des* genes, *picK* encoding a cytochrome P-450 hydroxylase is responsible for the C-12 hydroxylation of narbomycin to picromycin (Graziani 1998). Interestingly, PicK is exclusively cytochrome P-450 hydroxylase in the *pik* cluster that utility 12- and 14-membered ring macrolide substrates (Xue *et al.*1998; Zhang and Sherman 2001). The hydroxylation reactions catalyzed by the PicK are depicted in Figure 3.

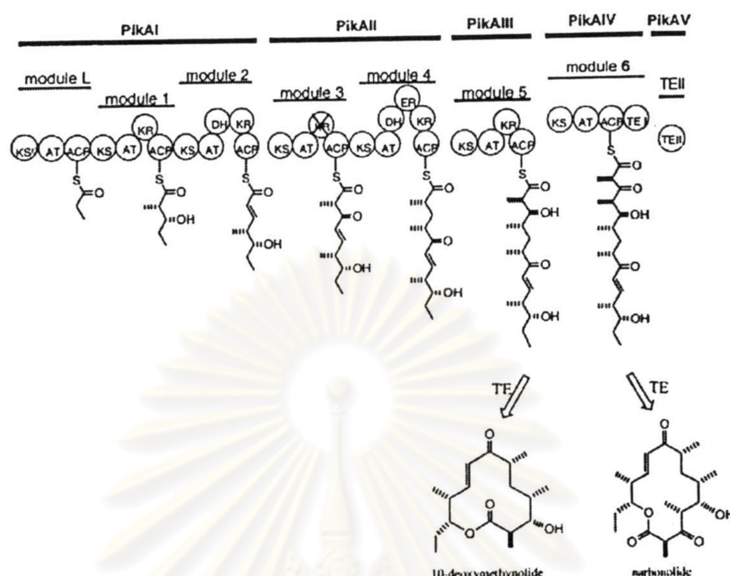


Figure 2. Polyketide biosynthesis in the Pik PKS system. Each circle represents an enzymatic domain in PKS protein. ACP, acyl carrier protein; KS, β-ketoacyl-ACP synthase; KS^Q, a KS-like domain; AT, acyltransferase; KR, β-ketoacyl ACP reductase; DH, β-hydroxyl-thioester dehydratase; ER, enoyl reductase; TEI, thioesterase domain; TEII, type II thioesterase (Xue and Sherman 2001).

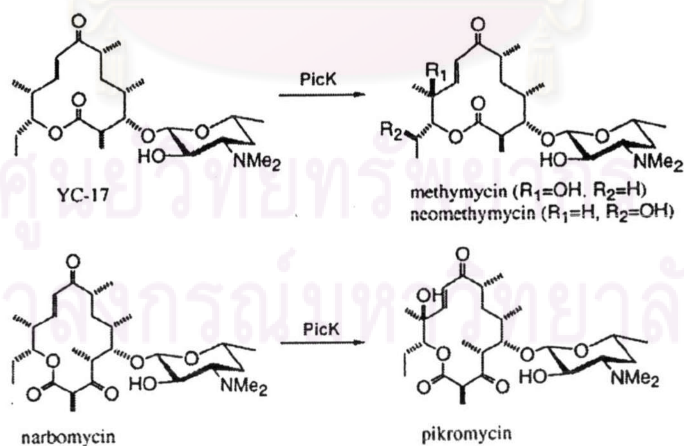


Figure 3. Hydroxylations catalyzed by PicK cytochrome P-450 hydroxylase of *S. venezuelae* (Xue and Sherman 2001).

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Importance of Cytochrome P-450 Monooxygenases

The cytochrome P-450 (called P-450) is a superfamily of *b*-type heme containing protein (Miles *et al.* 2000) involved in the metabolism of a wide variety of both exogenous and endogenous compounds. They act as a terminal oxidase in multicomponent electron transfer chain (Degtyarenko 1995). P-450s are found in a wide variety of organisms and play roles in drug metabolism and steroid hormone synthesis in mamalian cells. In plants, they involve in herbicide resistance and defense mechanism against insects. Moreover, P450 enzymes are involved in antibiotic biosynthesis in fungi and bacteria (e.g. streptomycetes) (Miles *et al.* 2000). All cytochrome P-450s of eukaryotic organisms are bound to the membranes of endoplasmic reticulums and mitochondria whereas most bacterial P-450s are water-soluble (Omura 1999).

The cytochrome P-450s were first recognized by Martin Klingenberg who studied the spectrophotometric properties of pigments in a microsomal fraction prepared from rat livers. When addition of a reducing agent (sodium dithionite) to a diluted microsome suspension previously gassed with carbon monoxide, a unique absorption spectrum with a maximum wavelength at 450 nm was detected. This spectrum with a λmax at 450 nm is unique amongst hemeproteins and serves as the signature of P-450 (Hasler *et al.* 1999; Guengerich 1991). Nomenclature of P-450s established by CYP nomenclature committee. The name cytochrome P-450 is derived from the fact that the proteins have a heme group and an unusual spectrum. The P-450 enzymes are characterized by a maximum absorption wavelength of 450 nm in the reduced state in the presence of carbon monoxide. Naming a P-450 gene include the italicized symbol "CYP" , denoting cytochrome P-450, and Arabic number designating the P-450 family, a letter indicating the subfamily when two or more subfamilies are known to exist within that family, and an Arabic numeral representing the individual gene. After the gene number is used

to denote a pseudogene. If no second subfamily or second gene exists in a family, the subfamily and gene number need not be included, e.g., CYP5 or CYP19. The same nomenclature for the corresponding gene product (enzyme) is recommended, e.g., italicized " CYP1A1" or "CYP17" (Nelson *et al.* 1993). The CYP families are involved in the metabolism of endogenous substances, such as fatty acids, prostaglandins, steroid and thyroid hormones. Isoenzymes in the same family must have >40% homology in their amino acid sequence and members of the same subfamily must have >55% homology (Badyal and Dadhich 2001).

Based on the protein components which mediated tranfer of electrons to P-450s, are devided into three classes as follows. Class I P-450 (most system of the bacterial and eucaryotic mitochondrial) consists of there components; an FAD or FMN-containing flavoprotein (NADPH or NADH-dependent reductase), an iron sulfur protein, and the P-450. Class II P-450 system (most eykaryotic microsome) the flavoprotein containing both FAD and FMN (NADPH-dependent P-450 reductase) and the P-450. Bacterial P-450 monooxygenase (P-450_{sca}) from *Streptomyces carbophilus* is exceptionally classified in this class. Class III P-450 system, for example P-450_{BM-3} from *Bacillus megaterium*. The flavoprotien and the P-450 are fused in a single polypeptide as one protein system (Miles *et al.* 2000; Degtyarenko and Archakov 1993). These three classes of P-450 are illustrated in Figure 4. Both microsomal and mitochondrial eukaryotic P-450s utilize NADPH as an electron donor of the monooxygenation reaction whereas most bacterial P-450s recieve electron from NADH (Omura 1999).

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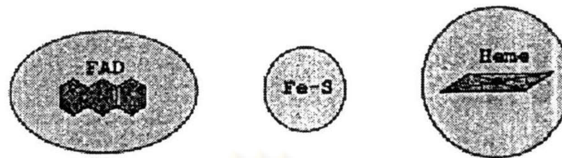
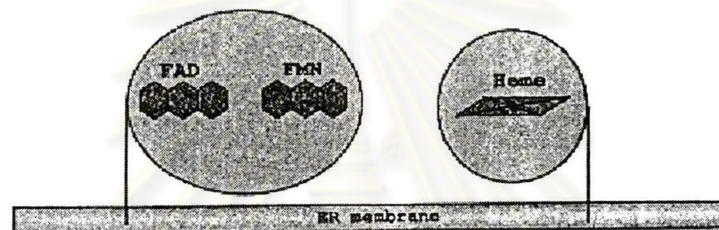
(A) Class I



(B) Class II



(C) Class III

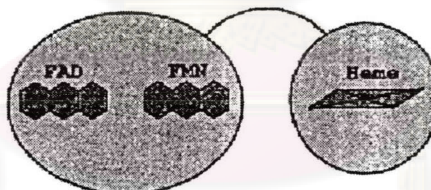


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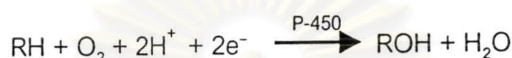
Figure 4. Classification of P-450 system. (A) Class I, bacterial and eucaryotic mitochondrial P-450 systems have three components : a flavodoxin reductase (FAD), an iron-sulfur (flavodoxin), and the P-450. (B) Class II, eucaryotic microsomal P-450 systems include the diflavin reductase and the P-450. (C) Class III, one component P-450 system that is the fusion of diflavinreductase and P-450 fused in a single polypeptide (Miles *et al.* 2000).

I. Mechanism of Cytochrome P-450 Enzymes

The most common reaction catalyzed by P-450 is hydroxylation. Moreover, they catalyze epoxidation, especially in detoxification system, with yield unstable products that react to nucleophilic groups of macromolecules to initiate biological effects (Degtyarenko and Archakov 1993). The hydroxylation reaction is shown as following :



$$RH + O_2 + 2H^+ + 2e^- \xrightarrow{P\text{-}450} ROH + H_2O$$

The P-450 hydroxylation reaction requires two electrons provided by redox partner proteins, dioxygen and two protons. The reaction is carried out through an activated oxygen species that effects controlled insertion of an oxygen atom into substrate. The catalytic cycle of P-450 is represented in Figure 5. The cycle is initiated by the substrate (RH) binding over the surface of the heme, which usually induces a change in the spin state of the iron from low to high spin (step 1). The heme iron is reduced from ferric (Fe^{3+}) to ferrous (Fe^{2+}) by the transfer of one electron from a redox partner (step 2) and O_2 binds rapidly (step 3). Once the second electron transfer to the heme, it in results in dioxygen bond cleavage and the formation of a transient oxy-ferryl intermediate as well as the release of water (step 4 and 5). Finally, the reactive oxyferryl species attacks the substrate, resulting in the monooxygenation of the compound (step 6, 7 and 8) (Miles *et al.* 2000; Guengerich 1991; Poulos 1995).

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II. Functions of Cytochrome P-450s

Cytochrome P-450 is essential for sterol biosynthesis in eukaryotic cells, which is an essential important of the plasma membrane. Some prokaryotes, which utilize organic hydrocarbons as the sole carbon and energy sources contain extremely high concentrations of special forms of cytochrome P-450s as found in *P.putida* which carries camphor-oxidizing P-450 (P-450cam) (Omura 1999). The P-450s are grouped as an important superfamily of enzymes involved in

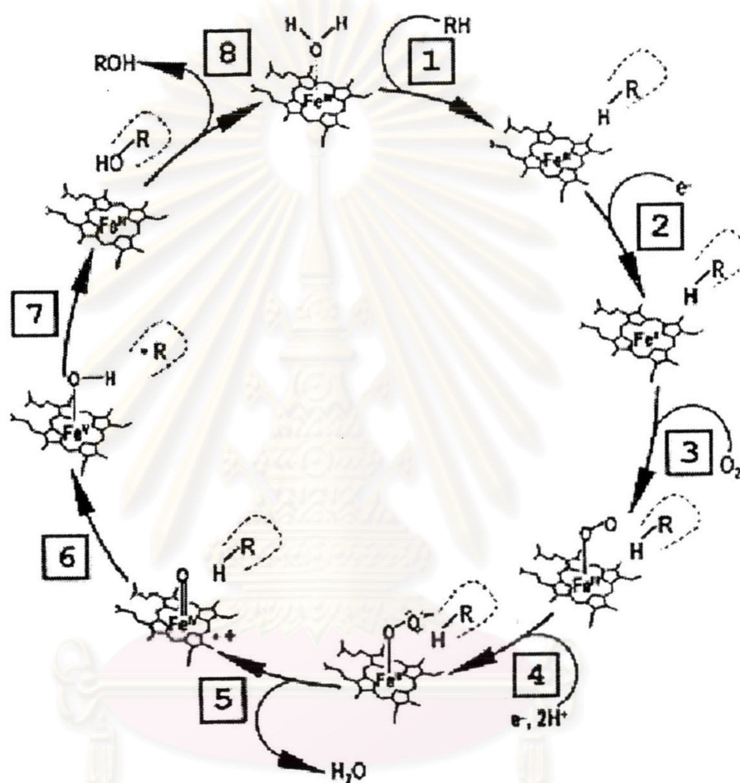

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Figure 5. The catalytic cycle of cytochrome P-450 (Miles *et al.* 2000).

metabolism of drugs, foreign chemicals, cholesterol, steroids and other important lipids. The role of P-450 in biosynthesis and catabolism of steroids and other sterols (cholesterol of animals, ergosterol of fungi, and phytosterols of plants) is the biocatalyst in the oxidative removal of 14a-methyl group from the intermediate compounds (lanosterol in the synthesis of cholesterol and ergosterol, and obtusiferiol in the synthesis of phytosterols). This oxidative reaction is the common essential step for the formation of all functional sterols. In addition, the oxygenase reactions (mainly hydroxylation in the biosynthesis of various steroid hormones and various lipid compounds) including prostaglandins, leuko-trienes and eicosanoids (Omura 1999). Moreover, P-450s are play an important roles in metabolism of xenobiotic compounds and drugs. The major role of P-450 in drug metabolism is bioavailability. The second role of P-450 is the catalytic oxidation of drugs to their active forms. The reaction involves the conversion of pro-drug to an active forms (Guengerich 2000).

Polyketide cytochrome P-450 genes, found in streptomycetes are located in macrolide or polyketide biosynthesis gene clusters. They catalyze stereo- and regio- specific oxidation of the polyketide precursors leading to make structural diversity of the molecule (Lamb *et al.* 2003). For example, the biosynthesis of erythromycin, EryF, is responsible for hydroxylation of 6-deoxyerythronolide B to erythronolide B. EryK encoded by *eryK* gene catalyzes the hydroxylation of erythromycin D to erythromycin C. PicK encoded by picK gene in *Streptomyces venezuelae*, catalyzes the hydroxylation of narbomycin to picromycin and catalyzes the hydroxylation of methylmycin to neomethymycin. The biosynthesis of antihelmintic avermectin in *Streptomyces avermitilis*, AveE catalyzes the hydroxylation of furan ring formation at C6, C7 and C8 to avermectin (Ikeda *et al.* 1999). OleP, P-450 of *Streptomyces antibioticus*, catalyzes the oxidation of C-8 of oleandomycin lactone ring to oleandomycin (Olano *et al.* 1998). AmphN and AmphL from *Streptomyces nodosus* catalyze the hydroxylation reactions in

the post-modification step of polyene amphotericin B biosynthesis (Caffrey *et al.* 2001). P-450 in *Streptomyces peucetius*, DoxA, catalyzes three oxidation steps in the biosynthesis of doxorubicin (Walczak *et al.* 1999). The biological importance of resulting oxidation particulary hydroxylation of compounds by P-450 enzymes is siginificant increasing in antibiotic potency (Lamb *et al.* 2003; Parajuli *et al.* 2004).

III. Structures of Cytochrome P-450 Enzymes

P-450s, approximately 500 amino acids, carry a cysteine residue located near the carboxy-terminus and hydrophobic residues near the amino-terminus of the enzyme. The cysteine provides an essential thiol-ligand for the heme iron. (Hasler *et al.* 1999).

For several years of studies of the P-450 structures, the structures of P-450, P-450_{cam} (camphor hydroxylase) from *Pseudomonas putida* (Poulos *et al.* 1987), P-450_{terp} (α-terpenoel hydroxylase) from *Pseudomonas* sp., P-450_{BM-3} (fatty acid hydroxylase) from *Bacillus megaterium*, and P-450_{eryF} (6-deoxyerythronolide B hydroxylase) from *Saccharopolyspora erythraea* have been resolved (Miles *et al.* 2000; Hasemann *et al.* 1995). All P-450 enzymes show low sequence identity ($<20\%$) but they reveal the close structural similarity in the term of topology. The P-450 structures have common and similar secondary structures elements. The structures are arranged in to a similar series of α-helices and β-sheets, with the well-conserved α-helices denoted A, B, B', C, D, E, F, G, H, I, J, K and L as represented in Figure 6. In addition, they show highly conserved motifs of cytochrome P-450. The motifs are the FXXGXXXCXG signature of heme-binding domain and the A (A,G,) X (E,D) T signature of oxygen-binding motif (Guengerich 2000).

The tertiary structure has an approximate trigonal prism arrangement containing both α-helical and β-sheet regions. Helices dominate the structure, and many of these lie parallel to

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the plane of the heme. The heme is embedded between the I and L helices, the I helix lies distal to the heme and spans the diameter of the protein structure, the L helix lies proximal to the heme iron. The heme buries within the structure, and the substrate binding site lies on the distal face. The substrate binding sites of P-450 are different in size and shape, resulting from the nature of their substrates (Figure 7). For example, P-450_{BM-3} hydroxylates the long chain fatty acid substrate, and the substrate lies in the substrate binding site that extends from the surface of the enzyme to the distal face of the heme. On the other hand, the substrate binding site of P-450_{cam} is much deep seated and the substrate access channel is much less appearant. The P-450_{eryF} has a substrate binding region larger than the P-450_{cam} as a result from a network of hydrogen-bonded watermolecules in the binding site (Miles *et al.* 2000).

P-450_{eryF} is the enzyme that catalyzes the hydroxylation of the 6-deoxyerythronolide B (6-DEB) to erythronolide B as present in Figure 8. P-450_{eryF} is the only one P-450 responsible for macrolide antibiotic biosynthesis which has been crystallized and resolved the structure. The crystal structure of the substrate-bound P-450_{eryF} represent a useful model for other P-450 enzymes especially in macrolide biosynthesis which bind a large hydrophobic substrates. P-450_{eryF} has an α/β structure with 51% of the residues are in an α-helical configulation and 17% are involved in β-sheets (Cupp-Vickery and Poulos 1995). The overall structure of P-450_{eryF} looks similar to P-450_{cam} . However, the siginificant differences occur in the B' and F helices.

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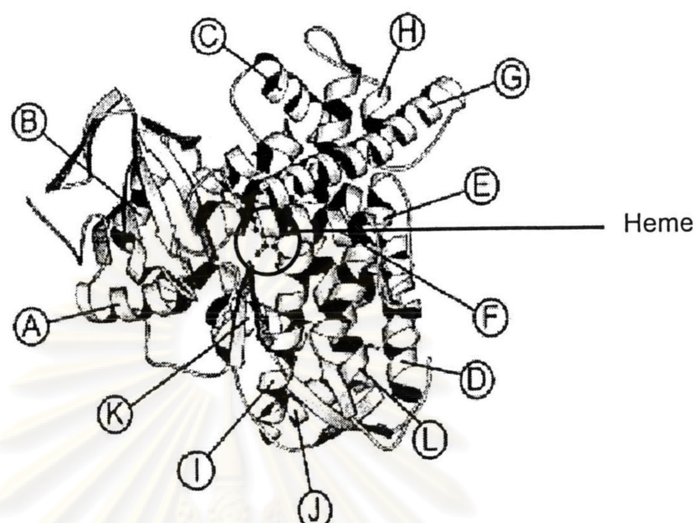

Figure 6. The representation of tertiary structure of cytochrome P-450_{BM-3}. The heme cofactor is visible as a ball-and-stick structure and helices A-L are labeled (Miles *et al.* 2000)

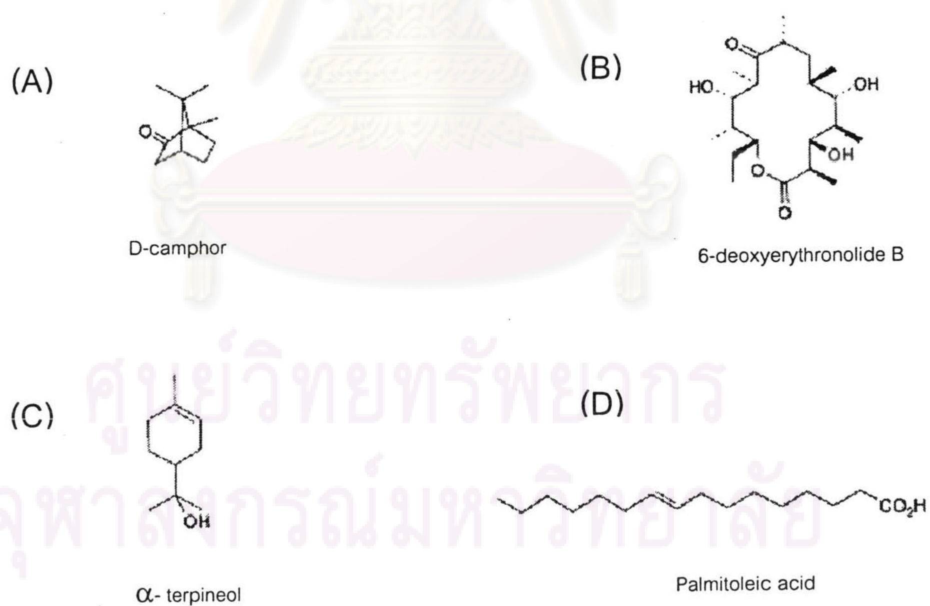

Figure 7. Substrates for the cytochrome P-450 (A) P-450_{cam} (B) P-450_{eryF} (C) P-450_{terp} (D) P-450_{BM-3} (Miles *et al.* 2000)

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The B' helix of the P-450_{cam} is responsible in key interactions with camphor and is partly responsible for substrate-binding and site specificity for hydroxylation. In contrast, the repositioning of the B' helix in P-450_{eryF} enlarges the substrate- binding pocket and positions the side chains of the B' helix so that they do not make contact with the substrate. The F helix in P-450_{eryF} is shortened by two residues as compared to the helix of P-450_{cam} and is shifted away from the substrate binding site. The movement of the F helix is necessary to accommodate the rotation of the B' helix. Positional changes of B' and F helices enlarges the substrate binding pocket, which allows the binding of the bulky substrate such as 6-DEB. Another feature of P-450 is the I helix, which extends across entire molecule of the enzyme and has the conserved theronine residue. The I helix is proximal to the heme group where the catalytic function of the P-450s occurs. The difference between two P-450s is the presence of an alanine residue in P-450_{eryF} at the position homologous to that of Thr252 of P-450_{cam} in the conserved threonine. This residue is proposed to act as the proton donor during the reaction cycle. Nevertheless, the distal I helix of P-450_{eryF} undergoes a helical distortion similar to that of P-450_{cam} (Cupp-Vickery and Poulos 1995; Cupp-Vickery and Poulos 1997). The central region of I helix forms a pocket for binding molecular oxygen and has been proposed to play a role in proton delivery for catalysis. A model for proton delivery has evolved to include the delivery of a proton to iron-bound molecular oxygen via the hydroxyl oxygen of the conserved threonine in the I helix.

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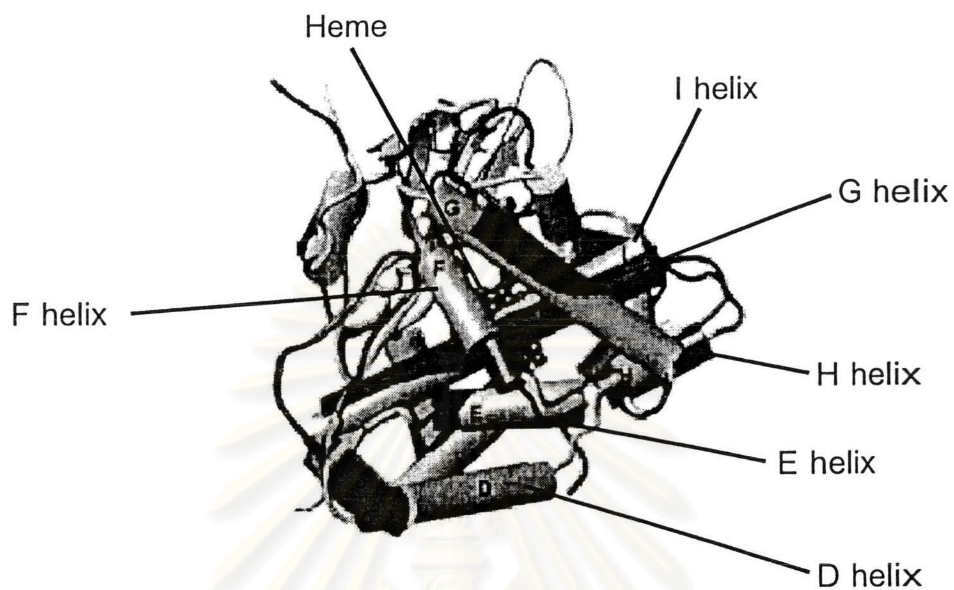

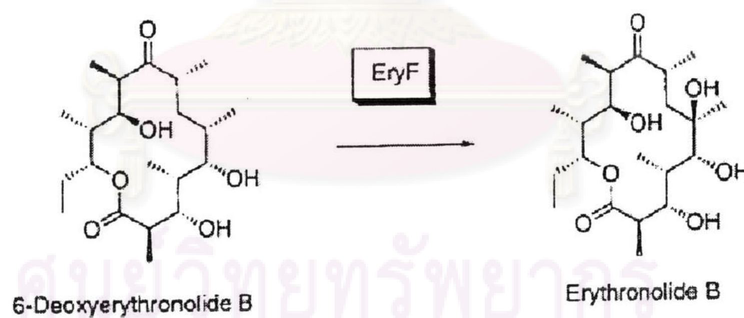

Figure 8. Crystal structure of EryF cytochrome P-450 hydroxylase and its hydroxylation reaction (Betlach *et al* 1998; Leys *et al* 2003).

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