CHAPTER ONE

LITERATURE REVIEW

Introduction

Polyketides, a large family of natural products, show broad range of biological and pharmacological properties. They are used as antibiotics, antifungals, antitumor, immunosuppressants, anticholesterolemics, antiparasitics, coccidiostatics, animal growth promotants and natural insecticides (Hranueli *et al.* 2001). Polyketides are widely distributed in bacteria, fungi, and plants (Shen and Kwan 2002). The complex polyketide, especially, macrolide erythromycin, inhibits protein ribosome assembly during synthesis. It is considered an important clinically anti-microbial drug against respiratory tract infections (Yu *et al.* 1996). Side effect of drug and development of antibiotic-resistant strains requires the continual discovery of new agents. *Streptomyces* species produce the largest number of secondary metabolites with a variety of biological activities. Over forty percentages of polyketides are isolated from *Streptomyces* species. The strategies concerning drugs development are based upon genetically manipulations. Much of the current research on polyketide biosynthesis is divided to two methods. First, the unparalleled biological activities are recovered to be enormous commercial value of natural products, which remain the most successful candidates for new drug discovery. Second, the extraordinary structure, mechanism and catalytic reactivity of PKSs allow the generation of novel compounds by combinatorial biosynthesis methods as called 'unnatural' natural products (Khosla 1997). More recently, picromycin-related semisynthetic ketolide antibiotics have demonstrated promising potential in combating multi-drug-resistant pathogens (Agouridas *et al.* 1998). The 14-membered ring macrolide

picromycin is catalyzed by the hydroxylation of narbomycin in *Streptomyces venezuelae* (Graziani *et al.* 1998). This research, we attempt to generate *Streptomyces* species, particularly *S. narbonensis*, carrying recombinant plasmid by the genetic manipulation. *S. narbonensis* produces the precursor (narbomycin) which is converted to picromycin, the high potential bioactive compound. The recombinant *Streptomyces* might be a producer for high purity and high quantity of picromycin production.

Streptomycetes

Streptomyces spp. is higher bacteria in actinomycetes genera. The genus *Streptomyces* are gram-positive, aerobic and spore-forming actinomycetes, possess DNA with a high guanine-plus-cytosine content and form extensive branching substrates and aerial mycelia (Hopwood 1999). Members of the genus *Streptomyces* are most important bacterial producers of antibiotics, accounting for about 75 percent of the commercially and medically useful antibiotics. Clinically important antibiotics produced by a *Streptomyces* species include streptomycin, the tetracycline, vancomycin, kanamycin, rifamycin, chloramphenicol, imipenem and erythromycin (Ketchum 1988). The Actinomycetes mainly *Streptomyces* species produce many of important antibiotics and other classes of biologically active secondary metabolites but it is useful to put this statement in context. As seen from **Table 1.1**, actinomycetes do indeed make some two – thirds of know antibiotics that are produced by microorganisms.

The secondary metabolites are nonessential for growth of the producing organism, at least under the condition and are indeed typically made after the phase of most active vegetative growth the producer is entering a dormant or reproductive stage. Their range of biological actives is wide, including the inhibition or killing of other microorganism (Challis and Hopwood 2003). Advantages of secondary metabolites of *Streptomyces* are

antibacterial drugs such as tetracycline, macrolide (erythromycin), aminogycosidase (Streptomycin), kanamycin, antifungal agent like candidin and nystatin, and anticancer drugs such as adrimycin. Amongst them, polyketides is the largest class. Polyketides are a structurally diverse family of natural products with a broad range spectrum of biological activities . The polyketides are produced by *Streptomyces* as shown in **table 1.2.**

Table 1.1 Approximate numbers of secondary metabolites produced by different groups of organisms, as of 1994 (Kieser *et al.* 2000)

Source	Bioactive metabolites			"inactive"
	antibiotics	other	total	metabolites
Non–actinomyces bacteria	1400 (12%)	240 (9%)	1640 (11%)	2000-5000
Actinomyces	7900[1] (66%)	1220 (61%)	9120[1] (61%)	8000-10,000
Fungi	2600 (22%)	1540 (51%)	4140 (28%)	15,000-25,000
Total microorganisms	11,900 (100%)	3000 (100%)	14,900 (100%)	25,000-40,000

[1]In each category, nearly 80% were found in *Streptomyces* and 20% in other actinomycetes.

Table 1.2 The polyketides are produced from Streptomycetes. (Kieser *et al.* 2000)

Antibiotic	**Producer**	**Chemical class**[1]	**Target**[2]	**Application**
Avermectin	*S. avermitilis*	Macrolide (PK)	Chloride ion channels	Antiparasitic
Candicidin	*S. hriseus*	Polyene macrolide (PK)	Membrane (pore former)	Antifungal
Chlortetracycline	*S. aureofaciens*	Tetracycline (PK)	R	Antibacterial
Daunorubicin (daunomycin)	*S. peucetius*	Anthracycline (PK)	DNA intercalation	Antitumour
Doxorubicin (adriamycin)	*S. peucitius* var. *caesus*	Anthracycline (PK)	DNA intercalation	Antitumour
Erythromycin	*Sac. erythraea*	Macrolide (PK)	R	Antibacterial
FK506 (tacrolimus)	*S. hygroscopicus*	Macrolide (PK)	Binds to FK protein	Immunosup-pressant
Lasalocid	*S. lasaliensis*	Polyether (PK)	Membrane (ionophore)	Anticoccidial; growth promotant
Lincomycin	*S. hygroscopicus*	Macrolide (PK)	Chloride ion channels	Antiparasitic
Mithramycin	*S. argillaceus*	Aureolic acid (PK)	DNA alkylation	Antitumour
Monensin	*S.cinnamonensis*	Polyether (PK)	Membrane (ionophore)	Anticoccidial; growth promotant
Natamycin	*S. nataensis*	Tetraene polyene (PK)	Membrane (ionophore)	Antifungal
Nystatin	*S. noursei*	Polyene macrolide (PK)	Membrane (ionophore)	Antifungal
Oleandomycin	*S. antibioticus*	Macrolide (PK)	R	Antibacterial
Oxytetracycline	*S. rimosus*	Tetracycline (PK)	R	Antibacterial
Pristinamycin	*S. pristinaespiralis*	Peptidic macrolactine + polyunsaturated macrolactone (PK)	R	Antibacterial
Rapamycin	*S. hygroscopicus*	Macrolide (PK)	Binds to FK protein	Immunosup-pressant
Salinomycin	*S. albus*	Polyether (PK)	Membrane (ionophore)	Anticoccidial; growth promotant
Spinosyns	*Sac. sponosa*	Tetracyclic macrolide(PK)	Unknown	Insecticidal
Sporamycin	*S. graminofaciens*	Macrolide (PK)	R	Antibacterial
Tetracycline	*S. aureofaciens*	Tetracycline (PK)	R	Antibacterial
Tyrodin	*S. fradiae*	Macrolide (PK)	R	Growth promotant

[1]PK = polyketide

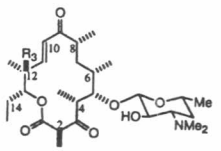
[2]R = binds to ribosomes and thus inhibits protein synthesis

Polyketide

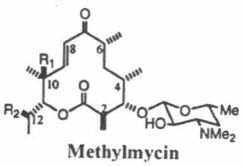
Polyketides are a large family of natural products found in plants, fungi and bacteria. In plants, e.g. colors of the flowers and flavors of the higher plants are polyketides. They have structural diversity. The smallest polyketide is 6-methylsalicylic acid and the largest is brevitoxin B (50 carbon atoms in chain) (Hopwood and Sherman 1990). The polyketide can be structurally classified into four major groups: aromatics (e.g., doxorubicin, tetracycline, etc.), macrolides (e.g., erythromycin and rapamycin, etc.), polyethers (e.g., monensin, salinomycin, etc.), and polyenes (e.g. amphotericin, candicidin, etc.) (Shen 2000). The chemical structures of polyketides are shown in **Figure 1.1**.

The biosynthesis of polyketide is sequential reactions catalyzed by polyketide synthase (PKSs) which produced a long chain of carbon atom by condensation of small organic acids, such as acetic acid and malonic acid. The carbon chain is extended by a building unit in the form of activated derivatives, called coenzyme A (CoA) ester. This includes acetyl CoA and malonyl CoA. The number and type of the building units make the variation in polyketide core structure is catalyzed by ketoacyl synthase (KS). Finally, the full saturation with hydrogen atoms in the structure is catalyzed by enoly reductase (Hopwood 2004).

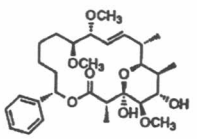
1) Macrolide



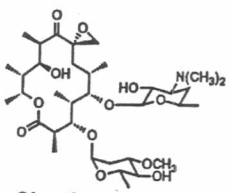
picromycin



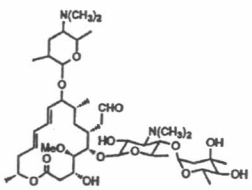
Methylmycin



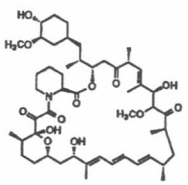
Soraphen A



Oleandomycin

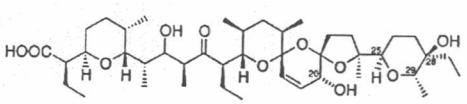


Spiramycin

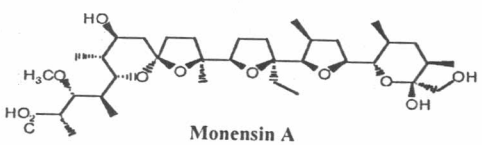


Rapamycin

2) Polyether

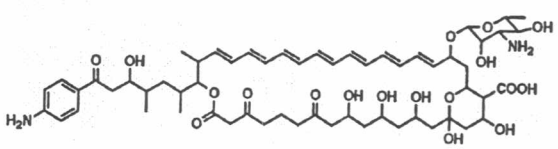


Salinomycin

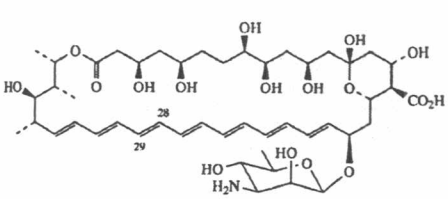


Monensin A

3) Polyene



Candicidin D



Amphotericin B

Figure 1.1 The chemical structure of polyketide such as macrolide, polyether, polyene and aromatic. (Hopwood 1997; Khosla 1997; Shen 2000; Cortes *et al.* 2002; Aparicio *et al.* 2003; Izumikawa *et al.* 2003)

4) Aromatic

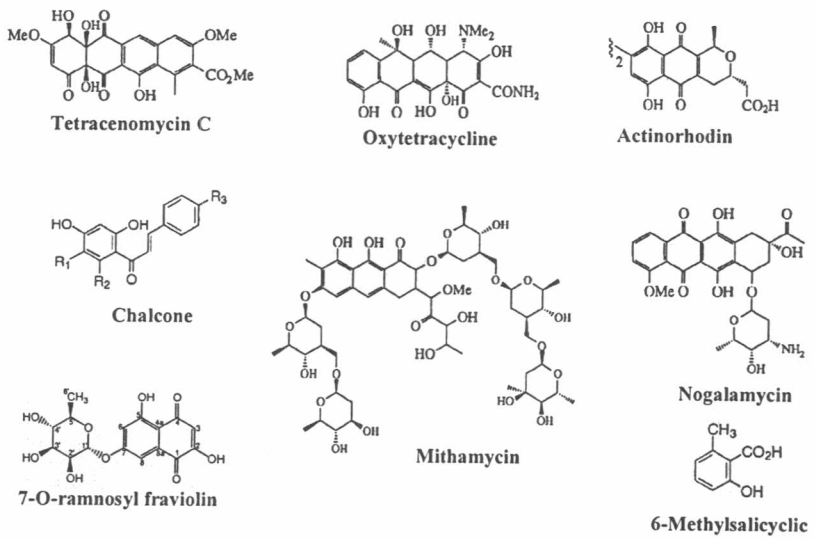


Figure 1.1 (Continued) The chemical structure of polyketide such as macrolide, polyether, polyene and aromatic. (Hopwood 1997; Khosla 1997; Shen 2000; Cortes et al. 2002; Aparicio et al. 2003; Izumikawa et al. 2003)

Polyketide is able to be classified according to type of polyketide synthases. They can be categorized in three groups of PKSs: Type I PKSs are multidomain mega-polypeptides enzyme that is organized in modules. Each module is responsible for a single cycle of polyketide carbon chain extension. Therefore, there is a one-to-one correspondence between the product structure and the active domains in modular PKSs (**Figure 1.2A**). They concern with producing a large lactone ring of polyketides in their structure, called macrolide. It is a significant member of antibiotics. There are a great many of antibiotics in macrolide group, for example erythromycin, narbomycin and picromycin (Hranueli *et al.* 2001). Moreover, the structure of polyene and polyether are synthesized by the modular PKSs, such as amphotericin (Aparico *et al.* 2003) and salinoycin (Izamikawa *et al.* 2003). Type II PKS, the products in this type is a polyaromatic ring. These compounds include oxytetracycline, actinorhodin, tracenomycin and daunorubicin. The aggregates of small polypeptides, each having a single catalytic activity that is used iteratively during the biosynthetic process (**Figure 1.2B**). The biosynthesis of these compounds is considerably more complex than that for the macrolides, since a single set of PKS enzymes is involved in iterative chain lengthening. A programming of biosynthesis is controlled by a set of three or four enzymes biosynthesis of Type II (Sexena *et al.* 2003). And the last, Type III PKSs has been recently discovered in bacteria and belongs to the plant chalcone synthases (CHSs) and stilbenes, polyhydroxy phenol in bacteria (Funa *et al.* 1999). These are homodimeric proteins and structurally and mechanistically quite distinct from type I and type II PKSs. CHSs are small proteins with a single polypeptide chain, responsible for biosynthesis of starting materials for many flavonoids (**Figure 1.2C**). These structures are aromatic polyketides that are often monocyclic or bicyclic (Hopwood 2004).

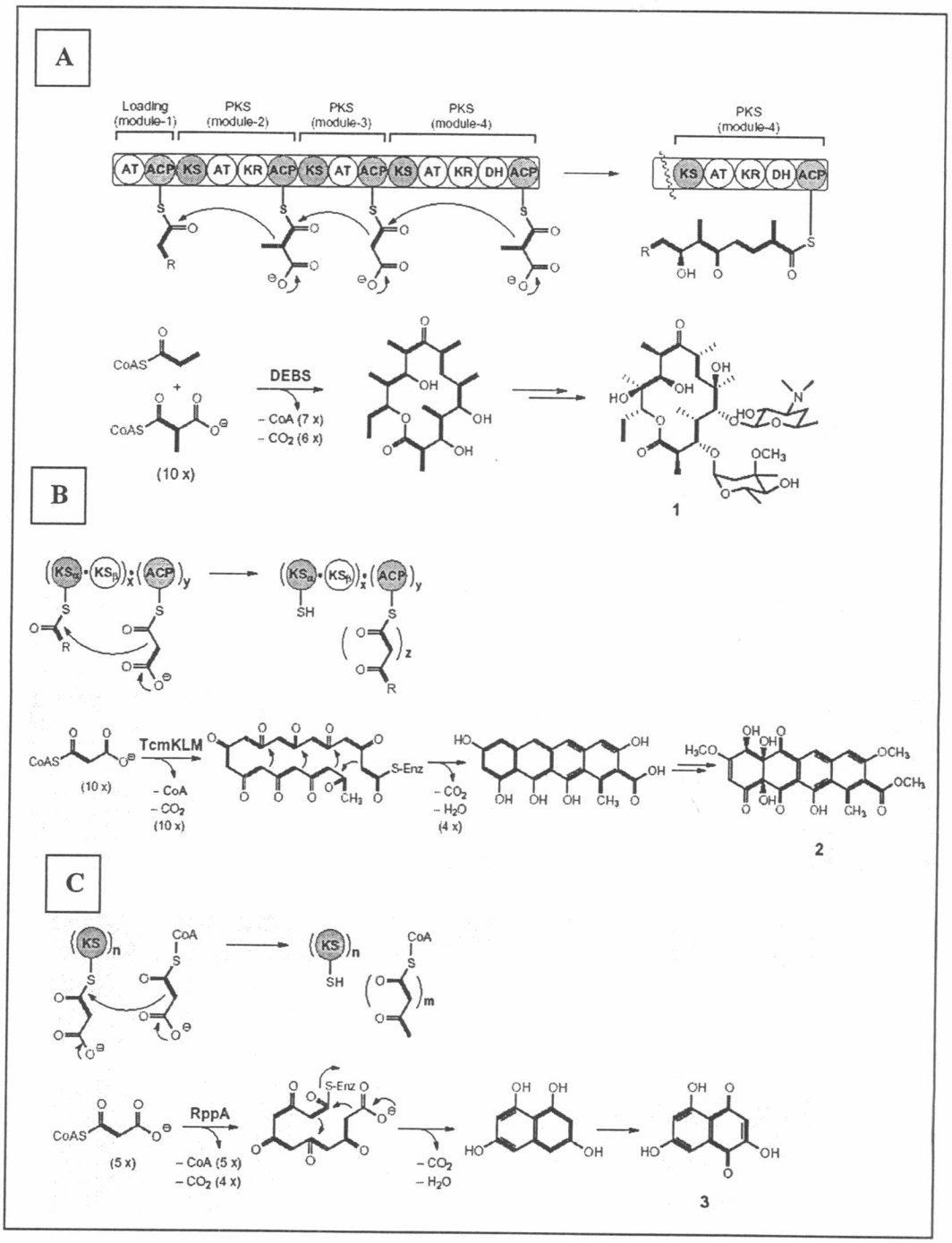


Figure 1.2 Polyketide synthesis. Schematics representation of (A) genes encoding the type I polyketide synthase responsible for the production of erythromycin A (B) type II polyketide synthase responsible for the production of tetracenomycin (C) type III polyketide synthase responsible for the production of flavolin (Shen 2003). (AT, KS, ACP, KR and DH are acyl transferase, ketoacyl synthase, acyl carrier protein, ketoreductase and dehydratase, respectively)

Then the final length of carbon chain is produced, it leaves the PKSs and form the macrocyclic ring by cyclization. The last step of biosynthesis is the tailoring step or post-PKSs. This process is conversion of polyketides to an active form by hydroxylation and/or glycosylation so it is a significant step to produce antibiotics. This step is catalyzed by tailoring enzymes such as glycosyltransferase and cytochrome P450 hydroxylase. The structural variability among polyketide is due to post-PKS synthetic steps that the post-PKS are modifying their structure. The modification are often necessary to impart or enhance the specific biological activity of the molecule (Kendrew *et al.* 1997; Tang and McDaniel 2001).

Polyketide glycosylation attach the sugars to specific positions on the core structure. This step is involved by sugar biosynthesis enzymes and glecosyl recognition of its cellular target (Weynouth and Wilson, 1997). These sugars can be liked to the core structure as monosaccharide, disaccharide or oligosaccharide of variable sugar length through *C*-, *N*-, or (most often) *O*-glycosylation. The active sugar ate catalyzed by sugar biosynthesis enzymes which are attached to core structure by glycosyltransferase. Example, desosamine biosynthesis and glosyltransferase are observed in biosynthetic pathway of erythromycin (Summers *et al.*, 1997), Oleandomycin (Quiros *et al.*, 1998) and picromycin (Xue *et al.*, 1998)

Polyketide cytochrome P450 monooxygenase catalyzes the site-specific oxidation of the precursors to many polyketide antibiotics, including erythromycin, tylosin, mycinamicin, oleandomycin and methymycin (Betlach *et al.* 1998). The most conserved part of P450 sequence is the heme and oxygen binding sites (Guengerich 2001). The P450 monooxygenase system is found in polyketide synthetics, such as *eryF* and *eryK* (erythromycin PKS), *oleP* (oleandomycin PKS), *tylI* (tylosin), *mycG* (mycinamicin) and *picK* (picromycin, neomethymycin and methymycin). Moreover, the structure of

P450eryF has been crystallized and predicted to second and tertiary structures (Cupp-Vickery and Poulos 1997).

Cytochrome P450 monooxygenase

The P450 enzymes are hemoprotein monooxygenase in prokaryotes and eukaryotes. The wide spread of P450-containing monooxygenase systems are divided to two main types: bacterial/mitochondrial (type I) and microsomal (type II) P450 by the number of their protein components (Degtyarenko and Archakov, 1993). Mitochondrial and most of the bacterial P450 systems have three components: an FAD-containing flavoprotein (NADPH or NADH-dependent reductase), an iron-sulphur protein and P450. The eukaryotic microsomal P450 system contains two components; a flavoprotein containing both FAD and FMN (NADPH-dependent P450 reductase), and P450 (Degtyarenko 1995). The cytochrome P450s are named for absorption band at 450 nm of their carbon monoxide (CO) form (Omura and Sato 1964a). In the P450, the heme iron is proximally linked by cysteine-thiolate, and in the resting stage, the ferric iron is distally ligated by water (Cupp-Vickery and Poulos 1995).

They catalyze the monooxygenation of a variety of organic molecule. The most common reaction catalyze is hydroxylation (Poulos, 1995). Monooxygenases catalyze reaction in which only one of the two oxygen atom of O_2 is incorporated onto the organic substrate, the other being reduced to H_2O. Monooxygenases require two substrates to serve as reductants of the two oxygen atom of O_2. The main substrate accepts one of cosubstrate furnishes hydrogen atoms, to reduce the other oxygen atom to H_2O.

The general reaction equation for monooxygenase is

$$AH + BH_2 + O\text{-}O \longrightarrow A\text{-}OH + B + H_2O$$

Where AH is the main substrate and BH_2 the cosubstrate. Because most monooxygenases catalyze reaction in which the main substrate becomes hydroxylated, they are also called hydroxylases (Nelson and Cox 2000).

Cytochrome P450 catalyzes hydroxylation reactions in which an organic substrate RH is hydroxylated by one oxygen atom of O_2 to R-OH.

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O$$

The P450 reaction requires two electron (provided by redox partner protein), dioxygen and two protons. It proceeds via an activated oxygen species that effects controlled insertion of an oxygen atom into the substrate (Miles *et al.* 2000). One oxygen atom of O_2 incorporates to substrates and the other oxygen atom is reduced to H_2O by reducing equivalents which furnished by NADH or NADPH, but usually passed to P450 by an iron-sulphur protein. **Figure 1.3** shows a simplified outline of the action of cytochrome P450.

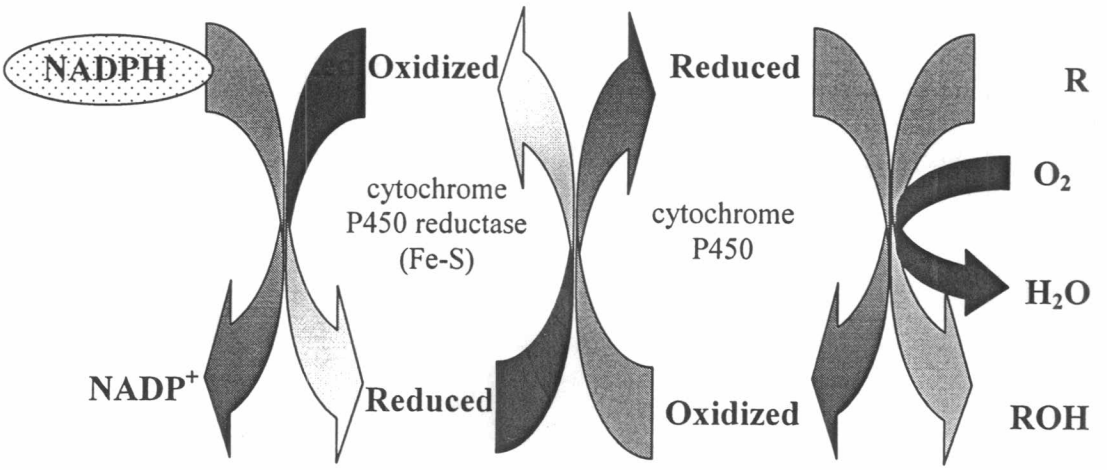


Figure 1.3 The action of cytochrome P450. Cytochrome P450 catalyzes hydroxylation of an organic substrate, RH which is hydroxylated to R-OH. The other oxygen atom is reduced to H_2O by reducing equivalents which furnished by NADH or NADPH, but usually passed to P450 by an iron-sulphur protein

The reaction cycle is represented in **Figure 1.4**. The cycle is initiated by substrate (RH) binding to the ferric P450 (1), permitting the first electron transfer (2) with consequent iron reduction in formation of the iron-peroxo species (4) and subsequently the oxyferryl hydroxylated product that diffuses from the active site to complete the cycle (7) (Segall, 1997 : Miles *et al.*, 2000).

The cytochrome P450s catalyzes the monooxygenation of aromatic and aliphatic compound. They are involved in metabolic processes, biosynthesis pathways, of both central and secondary metabolites. Both anabolic and catabolic, and collectively interact with an enormous variety of substrates (Demot and Parret, 2002). Many bacterial P450s are involved in oxidative biotransformation of natural compound. Several different biosynthetic P450s have been characterized during studies on the production of secondary metabolites; these are exemplified by the macrolide antibiotic erythromycin (Shafiee and Hutchinson 1988) anthelmintic avermectin (Ikeda *et al*, 1999), the anti-cancer agent epothilone (Tang *et al.*, 2000) and the phytotoxin thaxtomin (Healy *et al.*, 2002).

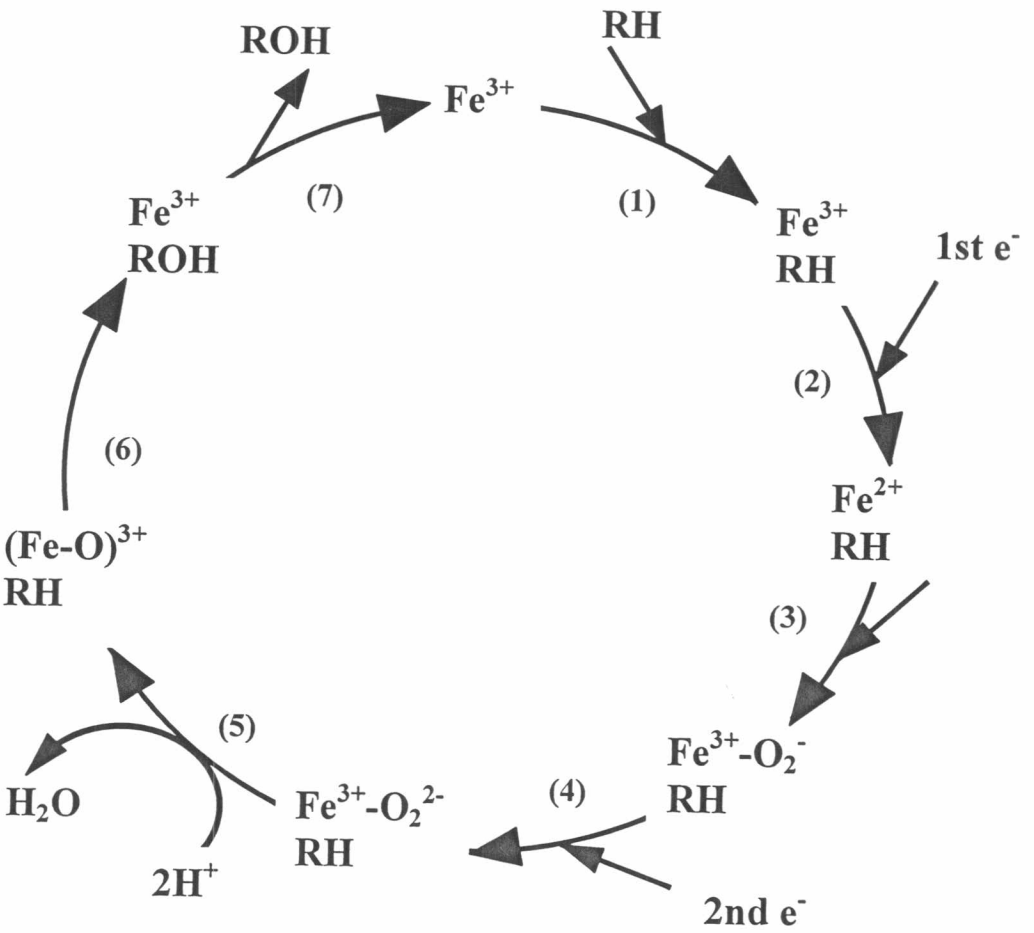


Figure 1.4 The catalytic cycle of cytochrome P450. (1) substrate binding, (2) the first reduction, (3) oxygen binding, (4) second reduction, (5) O_2 cleavage, (6) product formation and (7) product release.

Picromycin Biosynthesis

Picromycin biosynthesis pathway was catalyzed by polyketide synthase (picromycin synthase), and modified by desosamine glycosyltransferase and a cytochrome P450 monoxygenase. The gene cluster has been sequenced and 18 genes were identified including PKS locus, desosamine biosynthetic locus (*des*) and a cytochrome P450 hydrolase (*picK*)(Xue and Sherman 2001). The basically knowledge was described at above. The picromycin synthase, PICS catalyzed the condensation of proportion of precursor such as malonyl-CoA. Consequently, the products were core structures including the 14-membered ring macrolide narbonolide and 12- membered ring macrolide 10-deoxymethynolide (Yin *et al.* 2003). The post-PKSs were the tailoring enzymes including desosamine glycosyltransferase and a cytochrome P450 monoxygenase. The desosamine glycosyltransferase, Des synthesize and transfer a desosamine sugar to core structures yielded YC-17 and narbomycin (Zhao *et al.* 1998). The P450 monooxygenase converted narbomycin to picromycin and YC-17 to methymycin and neomethymycin via site-specific hydrolysation (Betlach *et al.* 1998). The steps of picromycin biosynthesis pathway were shown in **Figure 1.5**.

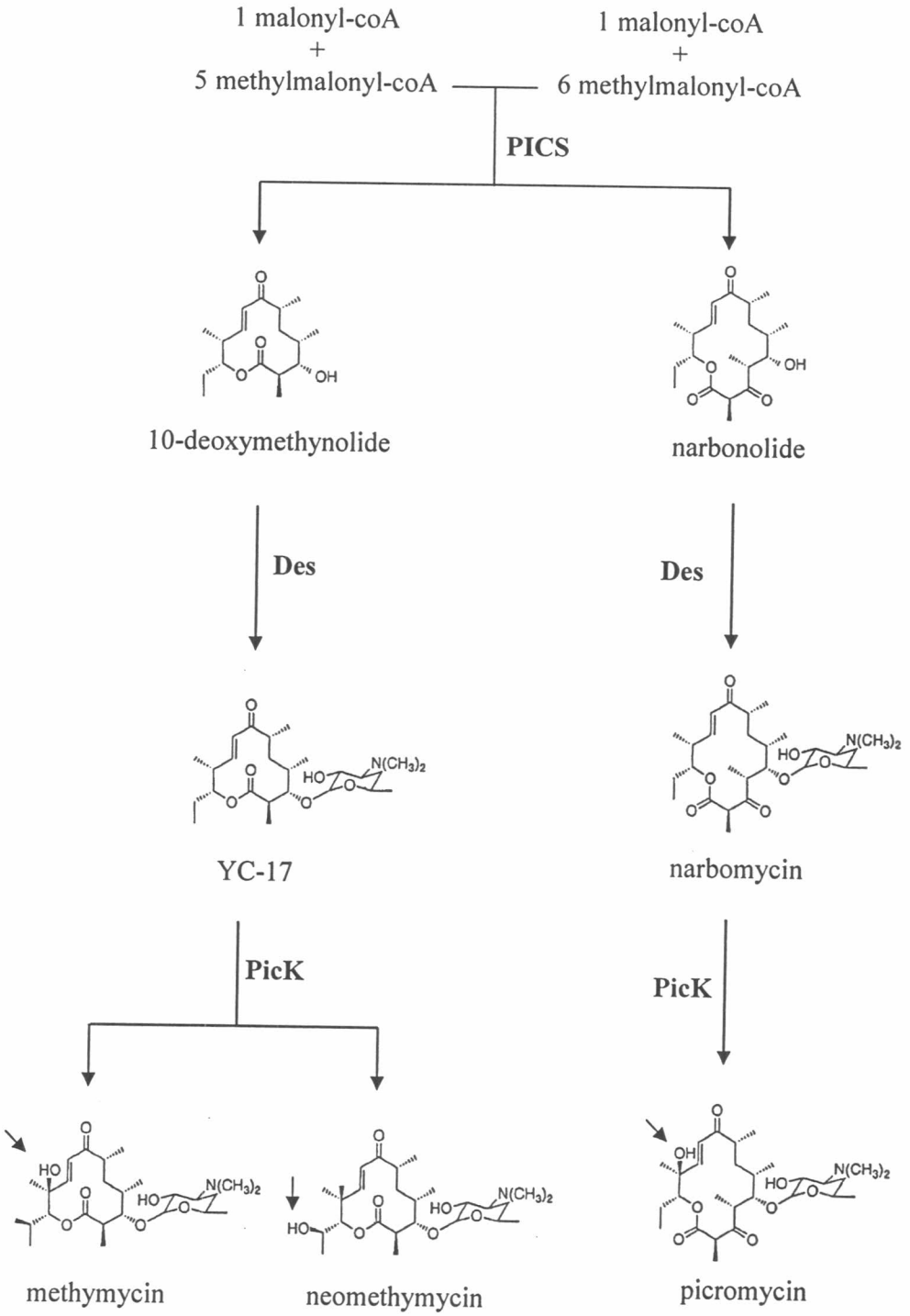


Figure 1.5 Biosynthesis of picromycin. (Modified from Xue *et al.* 1998 and Wilson *et al.* 2001) The picromycin biosynthase (PICS) catalyzed the precursor to core structures (narbonolide and10-deoxymethynolide), then the tailoring step modified of desosamine glycosyltransferase (Des) and a cytochrome P450 monoxygenase (PicK). The short arrows indicated the sited specific of P450.

Gene Expression of *Streptomyces*

The secondary metabolites of this group are important nature products, especially polyketides. The biologically synthesis are not enough to qualitative and quantitative application. Therefore, gene engineering is the necessary routes in biosynthetic pathway. The basically strategies are constructed the recombinant clones including cloning, expression, gene disruptions and site-directed mutagenesis that the ways are engineered for natural and "unnatural" natural products.

Cloning vectors and systems

The uses of cloning vector in *Streptomyces* are using phages and plasmids cloning vector. The different of *Streptomyces* replicons that have been developed into cloning vector differ in their sises, mode of replication, copy number and report host-ranges (Kieser *et al.* 2000).. The sample of cloning vectors was represented integrating, bi-functional plasmid vector. All the pIJ101-derived cloning vectors are currently used such as pIJ702, pIJ4090 and pIJ487. Moreover, the bi-functional plasmids are wild-spread applied to cloning genes from *Streptomyces* into *E. coli* hosts including pWHM series (Vara *et al.* 1989), pANT series (Dickens *et al.* 1997) and pUWL series (Wehmeier 1995).

Expression vector and system

In Gram-positive bacteria, Streptomycetes obtain relatively small, uninterrupted gene which densely packed on the DNA of chromosomes, phages and plasmids. Streptomycetes are good at expressing gene (promoter, RBSs, CDSs and terminators) from low G+C organisms, but *Streptomyces* genes are usually more difficult to express *E. coli* because most promoters do not function, and translation may be

inefficient unless the initial amino acid codons are change to lower G+C alternatives (Binnie *et al.* 1997). In cases of *Streptomyces* genes are expressed in other hosts that called heterologous expression. The plasmids are usually constructed for particularly works. The assembly expression plasmids are composed by promoter, ribosome binding site, selective marker and essential region of *Streptomyces* such as pIJ series. Moreover, the expression systems are meditated to the compatible and secretory of used host. Most of the plasmids developed for high-level expression in *Streptomyces* are *Streptomyces – E. coli* shuttle vectors, based on derivatives of the multicopy pIJ101 replicon. The hosts *Streptomyces* are considered interesting candidates for the production of heterologous proteins for several reasons, including their efficient secretion mechanism by which the secreted proteins are localized into the culture supernatant (Anne and Van Mellaert 1993).

Biotechnological Application

The genus *Streptomyces* has renewed attention for genetic research in resent years. These bacteria produced a wide rage of hydrolytic enzymes capable of breaking down organic substrates, and yield diverse secondary metabolites. The study of bioactive natural-product biosynthesis has benefited significantly from the use of heteologous hosts (Pfeifer and Khosla 2001). The polyketides products were used gene engineering including rational designed and multiplasmids system led to combinatorial biosynthesis for novel products. The multiplasmid , three-plasmid system for heterologous expression of 6-deoxy-erythronolide B synthase (DEBS) was used by Xue abd coworker (Xue *et al.* 1999) and two-plasmid system was used for bioconversion of ε-rhodomucinone to rhodomycin D (Olano *et al.* 1999). The benefits of this system were demonstrated through production of a library of modified polyketide macrolactones. In the same way,

McDaniel and co-worker have engineered the modular PKSs of erythromycin (McDaniel *et al.* 1999) and picromycin PKSs were modified by post-PKSs that produce different macrolactones, the resulting strains produced desosaminylated derivatives (Tang and McDaniel 2001).

The aims of this study are construction of expression plasmid and study of heterologous expression of *picK* gene.