CHAPTER II

LITTERATURE REVIEWS

2.1 *Croton oblongifolius* Roxb. and *Ent*-kaurenoic acid (*Ent*-kaur-16-ene-19-oic acid)

Croton oblongifolius Roxb., locally known as Plao-yai or Plao luang, is a medium size deciduous tree belong to the Euphorbiaceae Family (Figure 1.) (Roengsumran et al., 1998). It is widely distributed throughout Thailand and known as a Thai traditional herb. All parts of this plant have been prescribed for many symptoms such as the fruit is used to treat dysmenorrheal, the seed is used as a purgative, the bark is used to treat dyspepsis and the root is used to treat dysentery. According to its medicinal properties, *C. oblongifolius* have been widely studied and the chemical constituents have been isolated and characterized. Many diterpenoids have been investigated such as Pimarane (Aiyar and Seshadri, 1970), Isopimarane (Aiyar and Seshadri, 1971), Clerodane (Aiyar and Seshadri, 1972), Cembrane (Roengsumran et al., 1999a), Labdan (Roengsumran et al., 1999b), Halimane (Singtothong, 1999), Cleistanthane (Siriwat, 2000) and Kaurane diterpenoid (Sirimongkhon, 2000).

As part of our studies on the phytochemistry and biological activities of *Croton oblongifolius* Roxb. from Kui buri, Prachuap Khiri Khan province, Thailand, we have examined the hexane extract of stem bark of *C. oblongifolius*. From this research we were able to isolate a large amount of *ent*-kaurenoic acid. *Ent*-kaurenoic acid, *ent*-kaur-16-en-19-oic acid (Figure 2), a tetracyclic diterpenoid can be obtained in a good yield from many plants such as, *Xylopia frutescens* (Annonaceae) (Takahashi et al., 1995), *Wedelia Paludosa* (Asteraceae) (Vieira, Takahashi and Boaventura, 2001) and *Capaifera langsdrffii* Desf. (Leguminosae) (Cavalcanti et al., 2006), and *C. oblongifolius* Roxb. (Sirimongkhon, 2000).

2.2 Biological activities of *ent*-kaurenoic acid

The literature surveys have extensively shown that *ent*-kaurenoic acid exerts several biological activities. The important role of *ent*-kaurenoic acid is as the intermediate in the biosynthetic pathway of gibberellin, the plant growth stimulating

hormone found in plants (Stumpf and Conn, 1980) and microorganisms (Turner, 1971). The biosynthetic pathway for Gibberellin (GA12) is shown in Figure 3. Kaurenoic acid not only acts as the intermediate in gibberellin biosynthesis but also exhibits a gibberellin like activity but at a low level.

Figure 1. *Croton oblongifolius* Roxb.

Figure 2. Chemical structure of *ent*-kaur-16-en-19-oic acid

Figure 3. The biosynthesis pathway of Gibberillin (GA12) (Stumpf and Conn, 1980)

This kaurene has also been shown to display anti-parasitic activity again the protozoan flagellate *Trypanosoma cruzi*, the causative agent of Chagas' disease, which affects more than 90 million people in the world (Alves et al., 1995). *T. cruzi* infect the blood by insect vector. To limit disease the insect vector has been control but the transmission of disease by blood transfusion is a concern. This study was undertaken with the aim of developing new chemoprophylactic agents to treat banked blood. Alves et al. reported that *ent*-kaurenoic acid derived from *Mikania obtusata* D. C. (Asteraceae) showed activity against trypomastigate blood form of *T. cruzi* at IC_{50} of 0.5 mg/ml. Furthermore, the diterpenoids isolated from the root of *Viguiera aspillioides*, (-)-trachyloban-19-oic acid and (-)-kauran-16α-ol and (-)-kaur-16-en-19-ol, have also reported against this parasite (Costa, Albuquerque and Vidhnewski, 1966). In 2002, Vieira et al. described the preparation of some derivative of kaurenoic acid aiming at the improvement of the trypanocidal activity (Vieira, Takahashi and Boaventura, 2002). The results showed one of twelve new derivatives of kaurenoic acid exerted the activity higher than kaurenoic acid itself but still showed the lytic activity on erythrocytes. The rest of the compounds showed the level of activity similar to that of kaurenoic acid, but with out lysis.

One of the interesting biological activities of kaurenoic acid is anti-microbial activity. A study revealed kaurenoic acid showed the activity against several micro-organism especially pathogenic strains. Pedmaja et al. (1995) reported anti-bacterial activities of *ent*-kaurenoic acid against *Staphylococcus pyogenes* and *Pseudomonas pyocyaneae* and moderate activity against *Bacillus brevis, Salmonella typhi, Escherichia coli,* and *Klebsiella aerogenes*. In the same study, the anti-fungal and sporicidal activities were also reported. It showed that to inhibit growth of *Aspergillus, Penicillium* and *Trichophyton* even at 50 μg /ml, inhibitory to *Candida* at 100 μg/ml and to *Microsporum* and *Epidermophyton* at 250 μg /ml. The inhibition of spore germination produced by *ent*-kaurenoic acid was 66% in the case of *Fusarium lateritium* and 62% in the case of *Cercospora henningsii* at a concentration of 250 μg/ml. In 2000, the anti-microbial activities of *ent*-kaurenoic acid was further evaluated, it was shown to be active against five Gram positive bacteria; *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus* and *Clavibacter michiganensis* with MIC values that ranged from 16 to 125 μg /ml (Rezende et al., 2000). By the studies of Zgoda-Pol and co-worker (Zgoda-Pols et al., 2002), the anti-

bacteria activities of *ent*-kaurenoic acid and *ent*-trachyloban-19-oic acid have been described. Both were identified as the compounds against the bacteria *Staphylococcus aureus* and *Mycobacterium smegmatis*.

It addition to its antimicrobial and anti-parasitic activities, *ent*-kaurenoic acid also exerts anti-inflammatory action (Paiva et al., 2003). It has been reported as an anti-inflammatory agent by evaluation on rat colitis induced by acetic acid. The results indicated kaurenoic acid prevents tissue damage in the rat model of acetic acid colitis. Moreover, in the caregenan-induced edema test, kaurenoic acid showed the slightly less active than phenylbutazone the inflammatory agent (Ambrosio et al., 2006).

More recently, Cavalcanti, et al. described that kaurenoic acid has DNA damaging activity (Cavalcanti et al., 2006). This study evaluated its potential genotoxicity against Chinese hamster lung fibroblast (V79) cell in vitro. The data suggested exposure of V79 cell to higher concentration of kaurenoic acid (30 and 60 μg/ml) caused significant increases in cell damage.

The cytotoxic and embryotoxic effects of kaurenoic acid are including in the interesting biological properties. The *ent*-kauranoic acid isolated from *Copaifera langsdorffii* oleo-resin, were studied for this activity (Costa-Lotufo et al., 2002). The study showed the effects of kaurenoic acid in developing sea urchin (Lytechinus variegatus) embryos, on tumor cell growth in microculture tetrazolium (MTT) test and on mouse and human erythrocytes in hemolytic assay. Continuous exposure of embryos to kaurenoic acid starting immediately after fertilization inhibited the first cleavage (IC_{50}: 84.2 mM) and progressively induced embryo destruction at the IC_{50} of 44.7 mM and 10 mM for blastulae and larvae stages, respectively. In MTT assay, kaurenoic acid at a concentration of 78 mM produced growth inhibition of CEM leukemic cells by 95%, MCF-7 breast and HCT-8 colon cancer cells by 45% each. Further, it induced a dose-dependent hemolysis of mouse and human erythrocytes with an EC_{50} of 74.0 and 56.4 mM, respectively.

Recently, four *ent*-kaurene diterpenes (*ent*-kaurenoic acid, *ent*-3β-hydroxy kaur-16-ene, *ent*-kaur-16-en-3α,19-diol and ent-17-hydroxykaur-15-en-19-oic acid) isolated from the leaves of *Laetia thamnia* L. (Henry et al., 2006) together with the methyl ester of *ent*-kaurenoic acid and acetate diester of *ent*-kaur-16-en-3α,19-diol have been reported. All compounds were evaluated for cytotoxicity against human

prostate (22Rv1, LNCaP), colon (HT29, HCT116, SW480, SW620), and breast (MCF-7) tumor cells at concentrations ranging from 6 to 50 mg/ml. The kaurenes showed activities in all cell lines tested but most sensitivity were affected on prostate cells, 22 Rv1 cells and LNCaP cells. The results reveal that *ent*-kaurenoic acid and its methyl ester showed activities on 22 Rv1 cells at IC_{50} 5.03 mg/mL. and 6.81 μg/ml , respectively. The *ent*-3β-hydroxykaur-16-ene and *ent*-17-hydroxykaur-15-en-19-oic acid showed activities on LNCaP cell at IC_{50} =12.83 mg/mL and 17.63 μg/ml, respectively. Furthermore, by the study of Zhang et al. (2004), *ent*-kaurenoic acid and *ent*-kaur-19-ol-17-oic acid were found to inhibit the proliferation of HLC cell line SMMC-7721 (Zhang et al., 2004).

The biological assay which was also exerted by kaurene type diterpenoid is antiplasmodic and relaxant actions on smooth muscle. Kauradienoic acid the active compound isolated from zoapatle leave reproduced the stimulation and relaxant effects which were observed in the *in vitro* model. Additionally, Bejar et al. (1984) stated that kauradienoic acid exerted inhibitory action on the contractility induced by acetylcholine, prostaglandin $F_{2\alpha}$ and oxytocin and it also acts as a Ca^{+} antagonist in myometrium. Further studied have described the effect of kaurene type diterpenoids; kaurenoic acid, kauradienoic acid and 16α-hydroxy-*ent*-kaurenoic acid, inhibited the maximal contraction induced by serotonin, oxytocin and acetylcholine on rat uterus. Moreover, the effect of kaurenoic acid on vascular smooth muscle contraction was investigated. (Tirapelli et al., 2004).

As part of our research, *ent*-kaur-16-en-19-oic acid was identified as an Na^{+},K^{+}-ATPase inhibitor which exhibits an IC_{50} of 2.2 x 10^{-5} M against crude enzyme Na^{+},K^{+}-ATPase from rat brain (Ngamrojnavanich et al., 2003) and shown to possess cytotoxic activities against P388 cells line and five tumor cell lines including Hep-G2 (hepatoma), Chago (lung), SW620 (colon), Kato-3 (gastric) and BT474 (breast).

In 2004, eleven *ent*-kauranes were subjected to assay on the generation of superoxide anion (O_2^-) by human neutrophiles (Yang et al., 2004). The superoxide anion (O_2^-) is considered to play an important role in injury to some organs to prevent human diseases. The results reveal that *ent*-kaurenoic acid could significantly increase O_2^- production. Furthermore, the effect of *ent*-kaurenoic acid on nitric oxide generation by NR8383 macrophages in response to lipopolysaccharide (LPS) was also examined.

Kaurenoic acid and the methyl ester of 15-hydroxy-dihydro-kaurenoic acid and 15-keto-dihydrodro kaurenoic acid were tested in vitro on human sperm motility and viability (Valencia et al., 1986). The result showed that kaurenoic acid and both of its derivatives played weak to negligible capacity for killing human sperms.

Anthelmintic activity of *ent*-kaurenoic acid was identified by testing against the cattle parasite *Haemonchus contortus*. Kaurenoic acid can kill all the worms with a death time of, 7 min at 0.25% concentration, whereas the time taken by mebendazole, as the standard drug, at the same concentration was 4 min. The insecticidal activity against the sweet potato weevil *Cylas formicarius* of this compound was also shown in the same report. The mortality shown by the 1% solution of *ent*-kaurenoic was 50% on the 7th day (Pedmaja et al., 1995).

One of the interesting biological activities of kaurene type diterpenoid which was investigated is anti-HIV activity. In 1996, kaurane derivative from the fruit of *Annona squamosa*, 16β,17-dihydroxy *ent*-kaur-19-oic acid, was found to show significant inhibition of HIV replication in K9 lymphocyte cell (Wu et al., 1996). The result revealed that the diterpene inhibited HIV replication at IC_{50} = 0.8 μg/ml. Additionally, the phytochemical analysis of the fruits of *Annona glabra* yielded in 13 kaurane derivatives, which were evaluated of their anti-HIV activities. Among these, methyl-16α-hydro-19-al-*ent*-kauran-17-oate exhibited mild activity against HIV replication in H9 lymphocyte cells, and 16α-17-dihydroxy-*ent*-kauran-19-oic acid showed significant inhibition of HIV-reverse transcriptase (Chang et al., 1998).

In addition to *ent*-kaurenoic acid, the other kaurane type diterpenoids have been investigated and evaluated for their biological activities such as antimicrobial activity (Rezende et al., 2000), cytotoxic activity (Hou et al., 2001), anti-HIV activity (Bruno et al., 2002), antifeedant activity (Bruno et al., 2001), trypanocidal activity (Vieira, Takahashi and Oliveira, 2002), anti-platelet aggregation activity (Yang et al., 2002), inhibitory activity on vascular smooth muscle contraction (Muller et al., 2003), apoptosis induction in human leukemia cell (Kondoh et al., 2004) and anti-cancer activity (Zhamg et al., 2004).

Because of a number of interesting biological activities of *ent*-kaur-16-en-19-oic acid and its derivatives, this highly active phytochemical compounds are worthy of further studies. Several reports indicated that hydroxyl substiuents along the skeletal of kaurane diterpenoid improved their biological activitiest (Vieira, Takahashi

and Boaventura, 2000). For this reason, much attention has been paid on the synthesis of hydroxylated kauranes to obtain more biological active compounds. Now a day, the numbers of investigation of kaurane diterpenoids from both natural source and synthesis has attracted increasing attention in order to screen for the pharmacologically useful agents. The chemical reaction may be used as an alternative way to hydroxylate the compounds but this may require functionalizations at non-activated carbon atom. This type of transformation is often very difficult to achieve with reasonable yield and regioselectivity using classical chemical reaction. Biotransformation is one of the few methodologies available for fuctionalization of inactive carbon atoms making it possible to carry out reaction that have no equivalent in conventional chemistry.

2.3 Biotransformation

The use of microbial and enzyme as biocatalysts for key step in the synthesis of organic molecule is gaining popularity at a great rate (Parales et al., 2002). This technique is termed biotransformation or bioconversion. Biotransformation is a biological process whereby an organic compound is modified into a recoverable product by simple, chemically defined reaction catalyzed by enzymes contained in the cell (Lock and Kristiansen, 1987). It is now widely employed as an important tool in the industrial synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates and food ingredients (de Carvalho and da Fonseca, 2006). The biotransformations allow the production of regio- and stereoselective compound under mild condition. Furthermore, it can accomplish some reactions which are difficult to achieve by using conventional chemical methods (Urlacher, Lutz-Wahl and Schmid, 2004). This makes biotransformation an interesting method which is useful in synthetic organic chemistry (enzyme in organic synthesis) to achieve single-step of multi-step reactions with shortening of chemical step synthesis. Microbial, plant and animal cells can supply the enzymes for biotransformation. Plant cells possess many kinds of biotransformation reaction but it grows slowly in artificial culture and the transformation may require several weeks to obtain the desirable product. In the case of animal cell, the cell culture or organ preparation is complex to operate even on the laboratory scale, however, it may be useful in some of the biotransformation reactions such as the biotransformation of 11β-hydroxycorticosterone to corticosterone by beef adrenals (Figure 4) (Vezina, 1987) but after discovered of the transformation by *R. nigricans*, it became a conventional method in industry. The microbial transformation surpasses plant and animal cell in several respects. Their high surface-volume ratio confers rapid growth and rates of metabolism loading to give efficient transformation of the substrate added. Moreover the microbial world, rich in species, provides a varied assortment of enzymes for a vast variety of reaction on many classes of compounds, and with the facility to adapt the artificial environment imposed by technical and economic requirement. The proportional used of microbial in biotransformations were revealed by de Carvalho et al. in the review of biotransformation of terpenes (de Carvalho and da Fonseca, 2006). It indicated that nearly two-thirds of manuscripts published on production and

/or biotransformation of terpenes in last decade, the biocatalyst used were either bacteria or fungi (Figure 5).

Figure 4. Hydroxylation of deoxycorticosterone to corticosterone

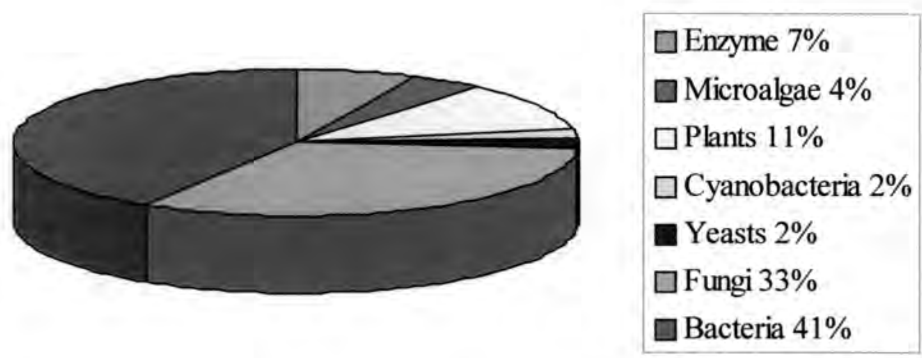

Figure 5. Number of paper published on various biocatalyst types in the last ten years

Nowadays, microbes are used as chemical regents for the preparation of key intermediates needed in organic synthesis. Many types of chemical reaction can be carried out by microorganisms. The scope of chemical reaction types mediated by microorganisms is shown in Table 1 (Leuenberger, 1990)

The salient features by which biocatalysts are favorably distinguished from common chemical catalysts are founded on their ability to provide stereo and regio-selective compounds, their ability to produce pure isomers compared with racemic mixtures. Furthermore, high reaction rates are obtained under mild reaction conditions which leading to energy safe and a large impact in reducing environmental pollution compared to chemical procedures. The main advantages of biotransformation are summarized in the following (Faber, 1997; Leuenberger, 1990; Prave et al., 1987):

Reaction specificity

The catalytic activity of an enzyme is usually limited to one type of reaction yielding a homogeneous product. As a result, reactions generally tend to be cleaner and laborious purification of product(s) from impurities due to side-reactions (or by-product respectively) can largely be omitted.

Stereospecificity

The reaction site of an enzyme represents a complex, three-dimensional and asymmetric environment which enables the enzyme to display high selectivity with respect to its substrate and even to distinguish between the enantiomers of a racemic mixture. Thus, in many cases, enzymes transform only one enantiomeric form exclusively or at least preferentially. On the other hand, if an enzyme reaction gives rise to a new center of asymmetry, as a rule the product is optically active, i.e., that only one of the possible enantiomers is formed. The product is therefore optically active. Thus, the chiral building block became the general rule in pharmaceutical, agrochemical and food industry. The majority of flavor and pharmaceutical compounds are racemic, usually only one of the enantiomers has the desired activity. The other enantiomers may be inactive or it may have the unwanted activity or side effect. The chirality is very important in fragrance and favors because different enantiomer may cause quite different odor. The examples of odor properties with different enantiomer were shown in Table 2.

Regiospecificity

Enzyme reactions are generally take place in the highly specific to the position of its substrate molecule. This holds true even if several groups of equivalent or similar reactivity are present in the substrate molecule. The enzyme converted regioselectively only one of the available functional group, affording a homogeneous product. The classical example is the bioconversion step in the technical synthesis of vitamin C. (Leuenberger, 1990), in the process *Acetobacter suboxydans* was used to transform D-sobital to L-sorbose (Figure 6) which is the intermediate for vitamin C production.

Mild reaction conditions

The activation energy of chemical reactions is significantly lowered by enzyme/substrate interactions. Thus, enzymes display high catalytic activities even under mild reaction conditions. Enzymes act in a pH range of about 5-8, typically around 7, in temperature range of 20-40 °C, preferably at around 30 °C and at normal pressure. Thus, the harsh and energy consuming reaction conditions used for chemical catalysis can be avoided and even labile molecules can be converted without undesired decomposition or other side reactions.

Environmentally acceptable

In contrast to chemical method, in which toxic metal catalysts were needed for reactions, biocatalysts are completely degraded in the environmental. Furthermore, enzyme reactions generally run in aqueous media which may pollute less than the organic solvents used in chemical conversion.

Table1. Classification of chemical reaction types catalyzed by enzymes (Leuenberger, 1990)

Oxidations	Hydroxylation, epoxidation, dehydrogenation of C-C bonds. Oxidation of alcohols and aldehydes, oxidative degradation of alkyl, carboxyalkyl or oxoalkyl chains, oxidative removal of substituents, oxidative deamination, oxidation of hetero-functions, oxidative ring fission
Reductions	Reduction of organic acids, aldehydes, ketones and hydrogenation of C=C bonds, reduction of hetero - functions, dehydroxylation, reductive elimination of substituents.
Hydrolysis	Hydrolysis of esters, amines, amides, actones, ethers, lactams etc. Hydration of C=C bonds and epoxides.
Condensation	Dehydration, O- and N-acylation, glycosidation, esterifi - cation, lactonization, amination
Isomerization	Migration of double bonds or oxygen functions, racemization, rearrangements.
Formation of	C-C bonds or hetero-atom bonds.

Table 2. Properties of enantiomers of some terpene compounds

Monoterpene	Enantiomer	Fragrance
Carvone	(*R*)-(-)	Spearmint
	(*S*)-(+)	Caraway
Limonene	(*R*)-(-)	Orange
	(*S*)-(+)	Turpentine
α-Pinene	(1*R*,5*R*)-(+)	Slightly minty
	(1*S*,5*S*)-(-)	Pine tree
Menthol	(1*R*,3*R*,4*S*)-(-)	Minty
	(1*S*,3*S*,4*R*)-(+)	Phenolic

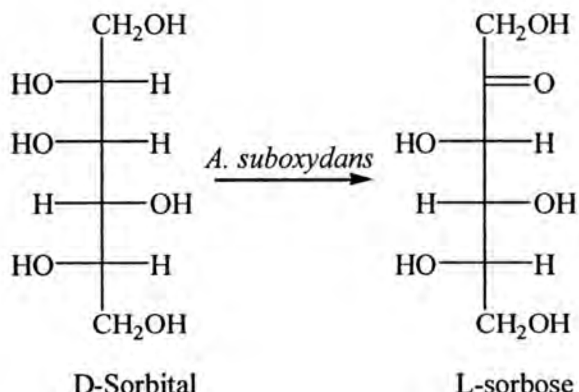

Figure 6. Regioselective oxidation of D-sorbital

Not only has biotransformation been used for chemical synthesis, but it has also served as a model for the evaluation of drug metabolism. Microbial model are useful in predicting, and sometime necessary for preparing the metabolites of drugs administered (Parshikov et al., 2001). Moreover, another fact of microbial transformations is that it embraces the degradation of fungicides, insecticides, and herbicide. Here the object is not to prepare useful, recoverable products, but to seed contaminated areas with microbes capable of converting pollutants in to innocuous compounds, in an attempt to improve the environment (Winkelmana, 1992).

According to these favorable properties of biotransformation processes, this method is now widely employed in the chemical and pharmaceutical industries and, essentially (Owen, 1960), it uses microorganisms to carry out single-step or multi-step transformation of organic compounds that are not easily accomplished by conventional chemical methods. The production of steroid drugs and hormones is one of the best examples of the successful application of microbial technology in large scale industrial processes (Fernandes et al., 2003). The manufactured steroid compounds have a wide range of therapeutic purposes, namely as anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic and contraceptive agents etc. The complex structure of the steroid molecule requires complicated, multi-step schemes for the chemical synthesis of steroid compounds. Microbial steroid conversions are performed in mild temperature and pressure conditions and can provide an efficient alternative to chemical synthesis for the development of manufacturing processes.

A most important microbiological transformation in steroids from an industrial viewpoint was the introduction of a hydroxyl group at carbon 11 of progesterone. This transformation was first reported by Peterson and Murray in 1952 (Peterson and Murray, 1952), and it led to economical production of cortisone and its derivatives from 11α-hydroxyprogesterone. The first reaction is a typical microbial bioconversion by the fungus *Rhizopus nigricans* formation of 11α-hydroxyprogesterone from progesterone. This highly specific oxidation bypasses a difficult chemical synthesis. All the other steps from progesterone to cortisone are performed chemically (Figure 7). Because of this specificity, the number of steps in manufacturing process can be drastically reduced. Discovery of 11α-hydroxylation not only reduced the reaction

required for cortisone manufacture from 36 to 11 steps but also reduced the cost of corticosteroids from $200 to <$ 1 per gram (Fogarty and Kelly, 1990).

To present day, a large number of publication in steroid biotransformation have been reported (Fernandes et al., 2003; Iizuka and Naito, 1981; Mahato, Banerjee and Podder, 1989; Mahato and Marjumda, 1993). The steroid industry thus couples the chemical and biological approaches taking advantage of the best aspects of each. A sample of this evidenced is give in Figure 8.

The success of steroids biotransformation has provided an impetus to the development of many transformations of various organic compounds including diterpenoids. Systematic studies of steroid microbiological hydroxylation have led to their rationalization in term of geometrical relationships between a binding site and site of hydroxylation. Whilst some diterpenoids possess a formal similarity to the steroid, their steriochemical differences have shed further light on the geometrical requirements for hydroxylation. Steroid and terpenoid bioconversion, particularly hydroxylation, is an area in which biocatalysis is very useful. Some of their products contained novel compounds or displayed potent biological activity. However, the main limitation lied in the inability to predict for any one molecule to the site(s) of hydroxylation. Thus, the screening method was normally performed in every organisms and substrates reaction.

progesterone → *R. nigricans* → 11α-hydroxyprogesterone → chemical reaction → hydrocortisone → chemical reaction → cortisone

Figure 7. Cortisone production using a microorganism (Fogarty and Kelly, 1990)

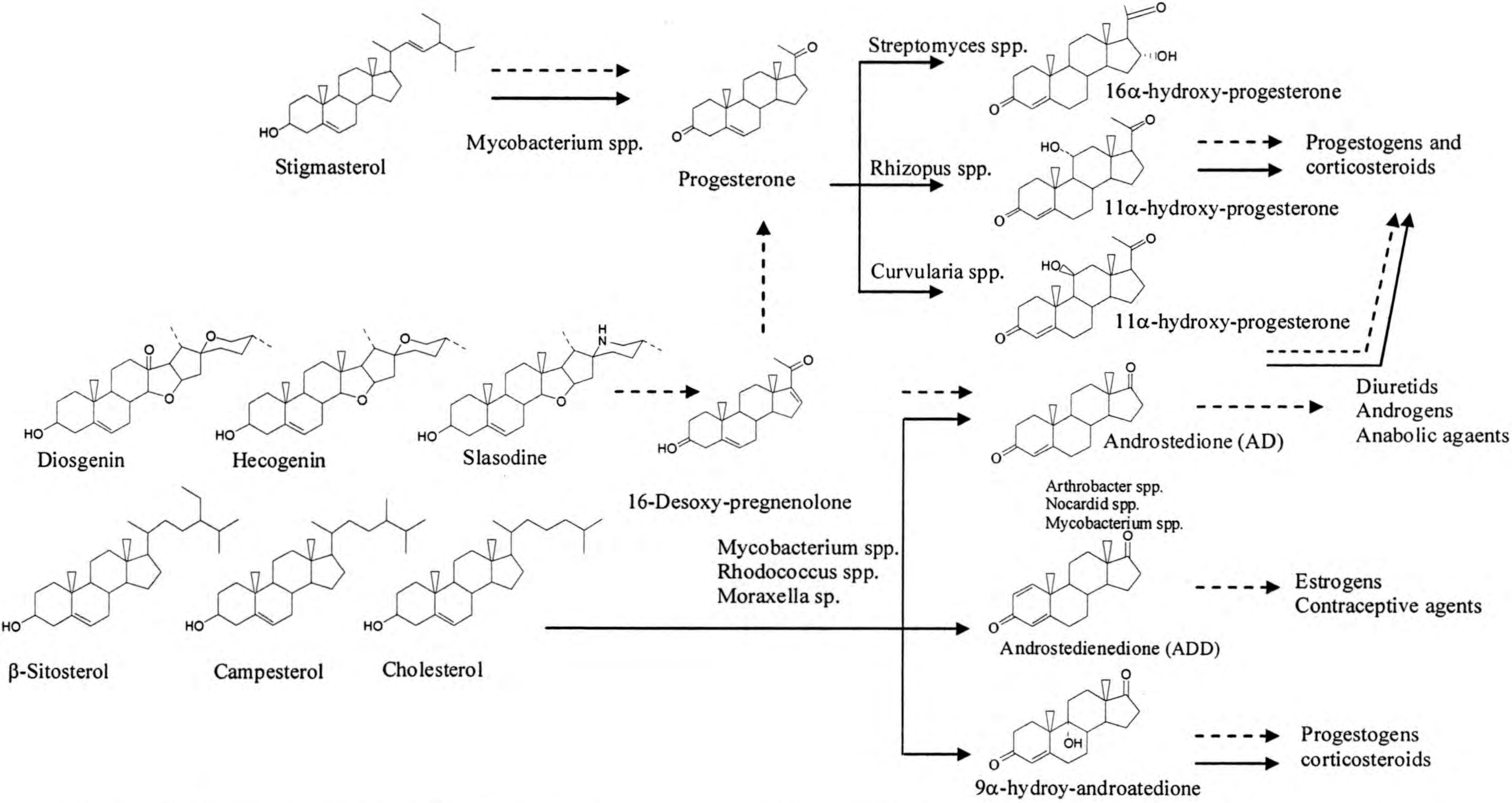

Figure 8. An overview of steroid production, from raw materials to finished products. Full lines indicate bioconversion whereas dashed lines indicate chemical transformation.(Fernandes et al., 2003)

2.4 Biotransformation of *ent*-kaurenoic acid by fungi

In recent years, a considerable number of studies exploring the microbial hydroxylation of karene diterpenoids have been reported. The use of fungi to achieve the biotransformation of these compounds furnishing the metabolites in good yields and some products displayed enhance biological activity and /or investigated as novel compounds.

From the literatures survey, in 1973, Beilby and coworker reported the microbial transformation of *ent*-kaur-16-en-19-oic acid (kaurenoic acid) by the three fungi; *Rhizopus nigricans*, *Calonectria decola*, and *Aspergillus ochraceous,* the microorganism extensively studied in steroid hydroxylation. This report investigated the biotransformation of four substrates containing the *ent*-kaurane skeleton and two substrates with *ent*-beyerene skeleton. The biotransformation gave the hydroxylation products with hydroxyl group which was shown in Table 3. In the case of *ent*-kaurenoic acid, the hydroxyl groups have been incorporated at C-7 by *R. nigricans*, C-15, 7 by *C. decola* and C-16, 17 by *A. ochraceous*, respectively (Beilby et al., 1973).

A further report by Ghisalberti et al. (1977), summarized the results from the previous study of Beilby in 1973 and gave a full description of the microbiological transformation of kaurane and the evidence for structural assignment. The report showed the transformation of kaurenoic acid by three fungi (Figure 9). *Calonectria decola* can convert the acid into 7,15-dihydroxylated acid in 30 % yield and minor qualities of 7α- and 15α-monohydroxylated compounds. *Rhizopus nigricans* afforded a monohydroxy compound, 7β-hydroxylated compound, in 25 % yield and variable amount of 16, 17-dihydroxy acid. The 16, 17-dihydroxy acid was the only metabolite which could be isolated from incubation of kaurenoic acid with *Aspergillus ochraceous* (Ghisalberti et al., 1977). Never the less, this report also showed the results of microbial transformation of three other *ent*-kaurene diterpenes which present the oxygenation at C-19, ent-16-oxo-17-nor-kauran-19-oic acid and ent-19-hydroxy-16-oxo-17-kaurane. The C-19 oxygenated was interesting because of the importance of 19-oxygenated kauranes as precursors of gibberellin. The results reveal three fungi which were successful in incorporating hydroxyl group in to the skeletal of both 19-oxygenated kaurenes.

Table 3. Biotransformation of *ent*-kaurane and *ent*-beyerene by *A. ochraceous*, *C. decora* and *R. nigricans* (Beilby et al., 1973)

Substrates	Products (%)		
	A. ochraceous	*C. decora*	*R. nigricans*
1)	16α-OH (10)	1β-OH (10) 7β-OH (10)	1β-OH (20) 7β-OH (20)
2)	13-OH (5) 13,16α-OH (5)	1β-OH (5) 7β-OH (15) 7α-OH (40)	1β-OH (30) 7β-OH (30) 7α-OH (5)
3)	16β,17-OH (20)	15β-,7β-OH (30) 15β-OH (5) 7β-OH (5)	7α-OH (25)
(4)	6α-OH (30) 7β-OH (25)	7β-OH (40)	1β-OH (25) 7β-OH (35)
5) R=H ; 6) R=CH_3	3α-OH	6α-OH (50)	-

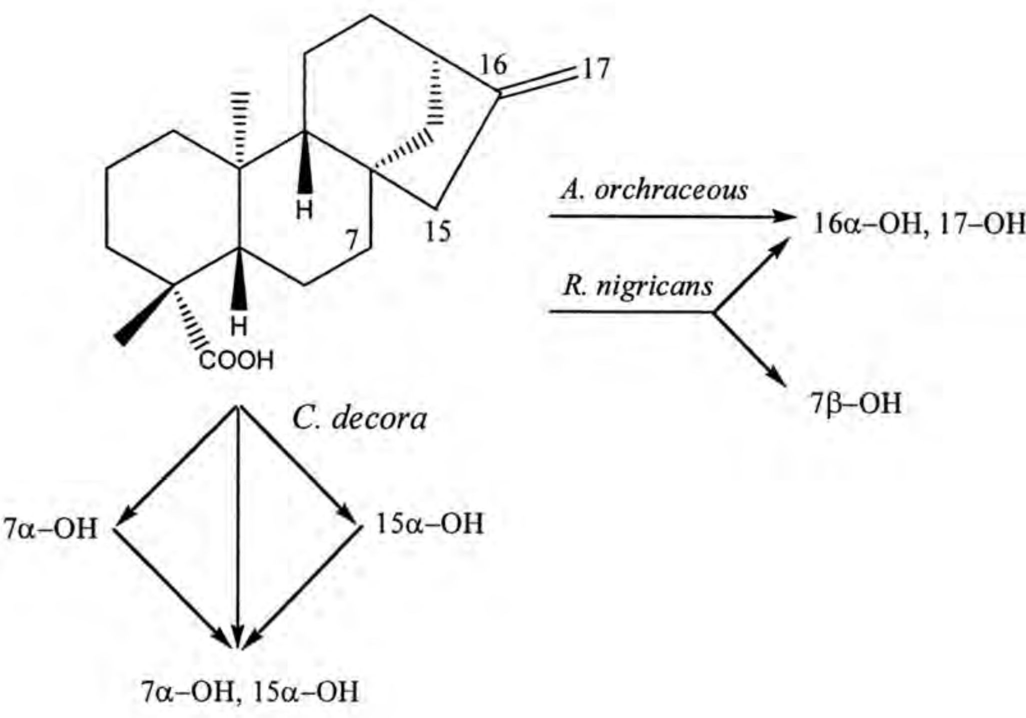

Figure 9. Microbiological transformations of *ent*-kaur-16-en-19-oic acid by *R. nigricans, C. decora and A. ochraceous* (Ghisalberti et al., 1977)

In 1999, Silva et al. carried out the transformation of *ent*-kaur-16-en-19-oic acid by *Rhizopus* stolonifer (Figure 10). After incubation for 7 days, two metabolites, *ent*-7*α*-hydroxy-kaur-16-en-19-oic acid (4.7%) and *ent*-12*β*-hydroxy-kaur-9(11), 16-dien-19-oic acid, were observed. In the longer period of incubation time (15 days) *ent*-16*β*, 17-dihydroxy-kauran-19-oic acid was also obtained. The products from this experiment are interesting to note that *ent*-7α-hydroxy-kaur-16-en-19-oic acid is an intermediate which plays a role in gibberellin biosynthesis and as a plant growth hormone. Furthermore, *ent*-16*β*, 17-dihydroxy-kauran-19-oic acid was found to associate with the compound correlated to anti-HIV activity (Silva et al., 1999).

In 2002, Punnapayak and coworker described the biotransformation of *ent*-kaur-16-en-19-oic acid by *Aspergillus niger*. The novel compound, *ent*-(7*β*,11*α*)-dihydroxy-1-oxo-kaur-16-en-19-oic acid, was produced after incubation for 7 days (Punnapayak et al., 2002). The transformation was shown in Figure 11.

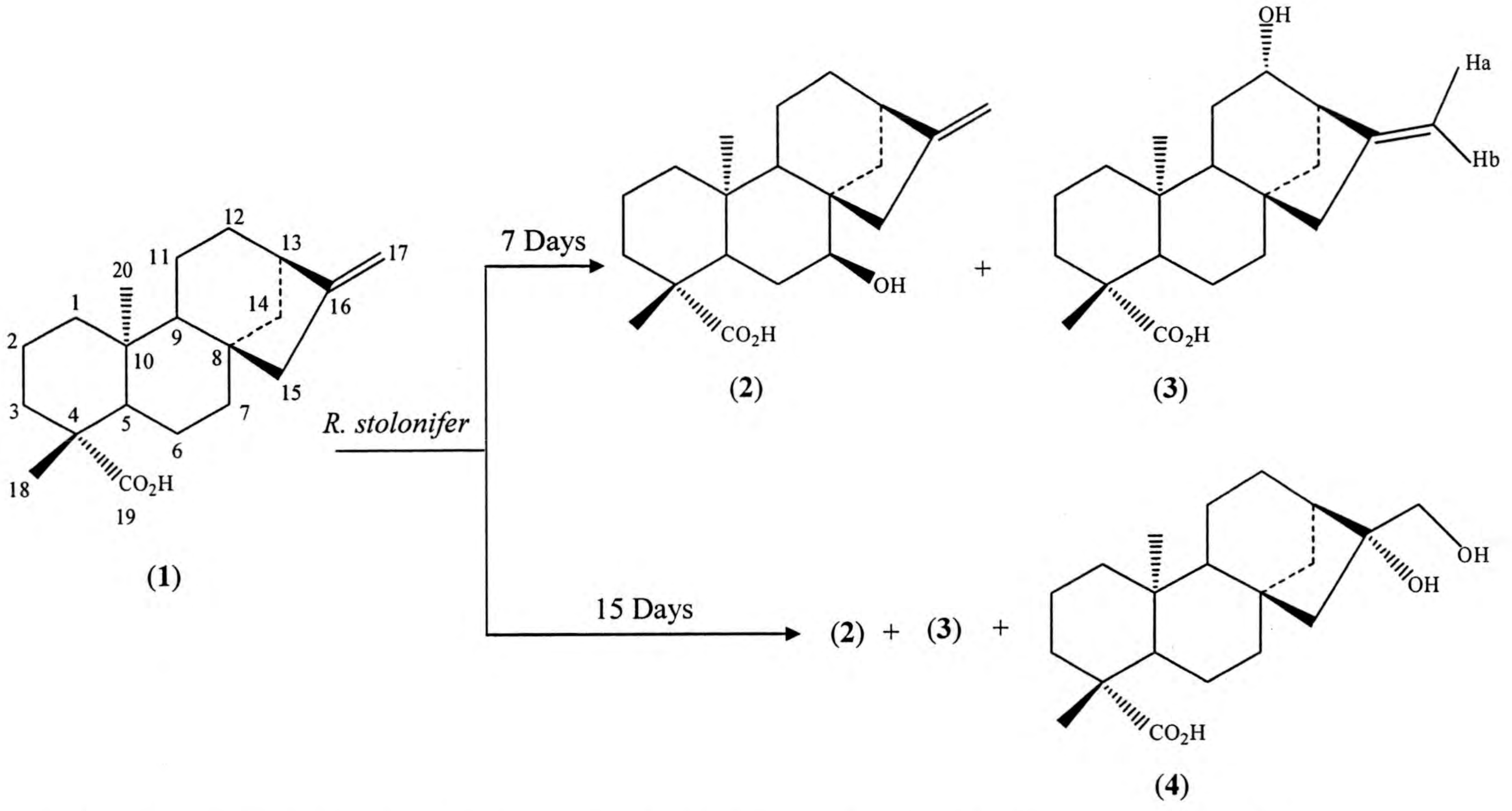

Figure 10. Biotransformation of *ent*-kaurenoic acid by *Rhizopus stolonifer* ; *ent*-kaurenoic acid **(1)**, *ent*-7α-hydroxy-kaur-16- en-19-oic acid **(2)**, *ent*-12β-hydroxy-kaur-9 (11),16-dien-19-oic acid **(3)**, and *ent*-16β,17-dihydroxy-kaur-19-oic acid **(4)** (Silva et al., 1999)

Figure 11. Biotransformation of *ent*-kaurenoic acid by *A. niger* (Punnapayak et al., 2002)

Not only kaurenoic acid was used as the substrate for synthesizing kauren derivatives but other karane diterpenoids were also used as well. Many fungi were selected as the biocatalyst in order to transform the kaurane derivative, for example, *Rhizopus nigricans* (Boaventura et al., 1995; Granados et al., 1990), *Curvularia lunata* (Granados, Martinaz and Ortiz, 1990), *Cephalosporium aphidicola* (Hanson, Hitchcock and Takahashi, 1995; Oliveira, Hanson and Takahashi, 1995), *Mucor plumbeus* (Boaventura et al., 1995), *Verticillium lecanii* (Vieira, Takahashi and Boaventura, 2002), *Aspergillus niger* (Yang et al., 2004) and *Gibberella fugikuroi* (Fraga et al., 1994; Fraga, Hernandez and Gonzalez, 1992; Fraga et al., 2005; Fraga, Hernández and Guillermo, 1996; Fraga, Hernandaz et al., 1993; Fraga, Tellado et al., 1993; Fraga et al., 1996).

According to the new publications in this field which have been continuously released, the reactions of the many fungi were also released and as much as various kind of activities, positions of hydroxyl group including yield of products by fungal were reported, researcher could be received more data for selecting the fungi which perform their desirable reactions. Accordingly, finding of the fungi with the desired hydroxylation activity becomes an important way to extend the study of this field. Many fungi have been reported as biocatalyst for steroid and terpenoid compounds but *Psilocybe cubensis* have never previously been used.

2.5 Magic mushroom and *Psilocybe cubensis*

Magic mushroom is the name defined for the mushrooms which provide psychoactive/halucinogenic compounds. The mushroom can elicit a wide range of bodily and mental effects including physical, sensory, emotion and intellectual. Hallucinogenic mushrooms have probably been in existence exactly as long as humanity. They have played a role in religions, psychotherapeutic ritual and spirit since ancient to present day. The principle hallucinogenic species belong to the class Basidiomycetes and can be divided into two subclasses. The first class contains biobenic acid and muscinol, and the second contains psilocybin and its related alkaloid. Psilocybe mushrooms is the mushroom contain psilocybin and/or psilocin, psychedelic tryptamines that are structurally similar to serotonin, a strong regulator of mood, state of mind, and consciousness. Several species of *Psilocybe* also contain the alkaloid baeocystin, which is a demethylated derivative of psilocybin. Other genera that contain psilocybin include *Conocybe*, *Copelandia*, *Gymnopilus*, *Inocybe* and Panaeolus. Hallucinogenic species of *Psilocybe* have a long history of use among the native peoples of Mesoamerica for religious communion, divination, and healing, from pre-Columbian times up to the present day.

Magic mushrooms were found to grow wide spread throughout of the world such as *Panaeolus cyanescens* was found in Hawaii, Mexico, Philippines, Eastern Australia, Thailand (Allen, 1992; Stamets, 1996), *Psilocybe cubensis* was found in Mexico, Cuba, America, Australia, India, Thailand, Vietnam, Japan (Tsujikawa et al., 2003; Allen, 1992; Stamets, 1996). Psilocybe mushroom are saprophytes which can be found in wide range of habitats: dungs, mosses, soils, grassland, or decaying wood debris.

One of the well known Psilocybe mushroom is *Psilocybe cubensis*. It has been classified as the following;

Psilocybe cubensis

Division: Basidiomycota
Class: Homobasidiomycetes
Order: Agaricales
Family: Strophariaceae
Genus: Psilocybe

Psilocybe cubensis (Figure 12) is a coprophilic fungus (one that prefers to grow on dung or manure soils) that often colonizes the dung of large herbivores, most notably cows and other grazing mammals such as goats. In Thailand, it often appears and grows directly on dung and the decomposed manure of domesticated water buffalo. The mushrooms are reddish-cinnamon brown to golden brown in color and they will turn bluish/purplish when bruised. Their caps are planar when fully mature, and their gills are adnate (horizontally attached to the stem) to adnexed (slightly indented at the attachment point) depending on the variety. The mycelia are like microscopic straws that look similar in appearance to foam or hoarfrost. The gills are closely spaced and contain microscopic dust-like dark purple spores that are self-propagating. This mushroom contains the hallucinogenic indole derivatives, psilocin and psilocybin (Stamets, 1996; Tsujikawa et al., 2003). The chemical structures of psilocin and psilocybin are showed in Figure 13.

Figure 12. *Psilocybe cubensis*

The biosynthetic pathway of psilocin and psilocybin required a hydroxylation step to transform the intermediate to psilocybin and psilocin. A possible pathway of psilocin and psilocybin biosynthesis is show in Figure14 (Agurell and Nilsson, 1968a; Agurell and Nilsson, 1968b).

Psilocin Psilocybin

Figure 13. Chemical structures of psilocin and psilocybin

tryptophan → tryptamine → *N*-methyltryptamine → N,N-dimethyltryptamine → psilocin → psilocybin

Figure 14. A possible pathway of psilocin and psilocybin biosynthesis as indicated by Agurell and Nilsson (Agurell and Nilsson, 1968a; 1968b)

In 1989, Gartch described the biotransformation of tryptamine in fruiting mycelium of *P. cubensis* (Gartz, 1989). The result showed that the enzyme systems in *P. cubensis* have a high hydroxylation and methylation capacity to convert added tryptamine to psilocin. Furthermore, the author suggested that many non-specific enzyme systems exist in this fungus which has the ability to oxidize exogenously added compounds as well as a normal, obligatory intermediate. It is interesting that this fungus probably achieves the biotransformation process in order to transform others organic compounds into hydroxylated products.

2.6 Enzymes in oxygenation reaction

The hydroxylated now become an important rule in chemical catalysis. The reaction has traditionally been achieved using whole-cell systems (Flitsch et al., 1999), but as more isolated enzyme systems and their three-dimensional structures are now becoming available, the study of biotransformation using isolated enzyme have been extensively interested. The direct oxygenation with regio- or enantiospecificity of the enzyme challenge many researchers to pay attention to the study of its mechanism in order to understand the mechanism in depth and lead to developing the process in which to obtain the most economical advantage. In the case of oxygenation of nonfunctional carbon atoms, the enzymes were found to have an activity to catalyze by direct incorporation of molecular oxygen into a substrate molecule are recognized as oxygenase (Faber, 1997). Oxygen-transfer into organic acceptor molecules may proceed through three different mechanisms, mono-oxygenase, di-oxygenase, and oxidase (Scheme 1).

Mono-oxygenase

Mono-oxygenases are characterized by their ability to introduce one of the atoms of molecular oxygen into an organic substrate, while the other is reduced at the expense of donor (usually NADH or NADPH) to form water. The most common of cofactors associated with the hydroxylase component of this enzyme complex are the heme-iron containing cytochrome P-450 (cyt P-450) system and flavin adenine dinucleotide (FAD). These enzymes are found in both prokaryotic and eukaryotic organisms (Omura, 1999), the catalytic cycle of cyt P-450 is shown in Figure 15 (Urlacher, Lutz-Wahl and Schmid, 2004). Cytochrome P-450 played an important role in hydroxylation reaction of several natural biosynthetic pathways and xenobiotic biotransformations (Parales et al., 2002). The reactions which can be catalyzed by mono-oxygenase are shown in Scheme 2.

Mono-Oxygenases

$$\text{Sub} + \text{DonorH}_2 + O_2 \longrightarrow \text{SubO} + \text{Donor} + H_2O$$

Cofactor-recycling

Di-oxygenases

$$\text{Sub} + O_2 \longrightarrow \text{SubO}_2$$

Oxidases

$$O_2 + 2e^- \longrightarrow O_2^{2-} \xrightarrow{+2H^+} H_2O_2$$

$$O_2 + 4e^- \longrightarrow 2O_2^{2-} \xrightarrow{+4H^+} 2H_2O_2$$

Scheme 1. Enzymatic oxygenation reaction (Faber, 1997)

$$\text{Sub} + O_2 + H^+ + \text{NAD(P)H} \longrightarrow \text{SubO} + \text{NAD(P)}^+ + H_2O$$

$$\text{—C—H} \longrightarrow \text{—C—OH}$$

H → OH

R

R

O

$$R_n\text{—X} \longrightarrow R_n\text{—X=O}$$

X= N, S, Se, P

O

R^1 R^2

O

O

R^1 R^2

Scheme 2. Mono-oxygenase catalyzed reactions

Many kinds of cyt P-450 have been characterized and the fungal cyt P-450 is one of the most interesting cyt P-450. The microbial oxidation involving a P450 mono-oxygenase is exploited industrially in the 11β- hydroxylation of Reichstein S, a progesterone derivative of corticosteroids production, using the fungi of the genus *Curvularia* (Urlacher, Lutz-Wahl and Schmid, 2004). Another example is the fungal cyt P-450 which responds to catalyze the 11α-hydroxylation of steroid molecule. This study was performed on hydroxylation of progesterone by *Rhizopus nigricans*. The isolation and partial characterization of this enzyme were performed (Kunic, Makovec and Breskvar, 2000; Makovec and Breskvar, 1998; Makovec and Breskvar, 2000). Furthermore, Cyt P 450 was investigated as the enzyme that catalyze the hydroxylation reaction to a number of compounds including kaurenoic acid. The reaction was examined in 1993 by Jame et al. (Jenning et al., 1993) and the characterization of kaurenoic acid hydroxylase from *Gibberella fugikuroi* was reported. The report indicated that the microsomal fraction exhibited to be the active fraction which converted [17-^{14}C] *ent*-kaurenoic acid to the oxidation products which were identified as *ent*-7α-hydroxykaurenoic acid and gibberellin A_{14} in presence of O_2 and NADPH. The properties of the enzyme from this study suggest that this kaurenoic acid hydroxylase of *G. fujikuroi* is cytochrome P-450 monooxygenase.

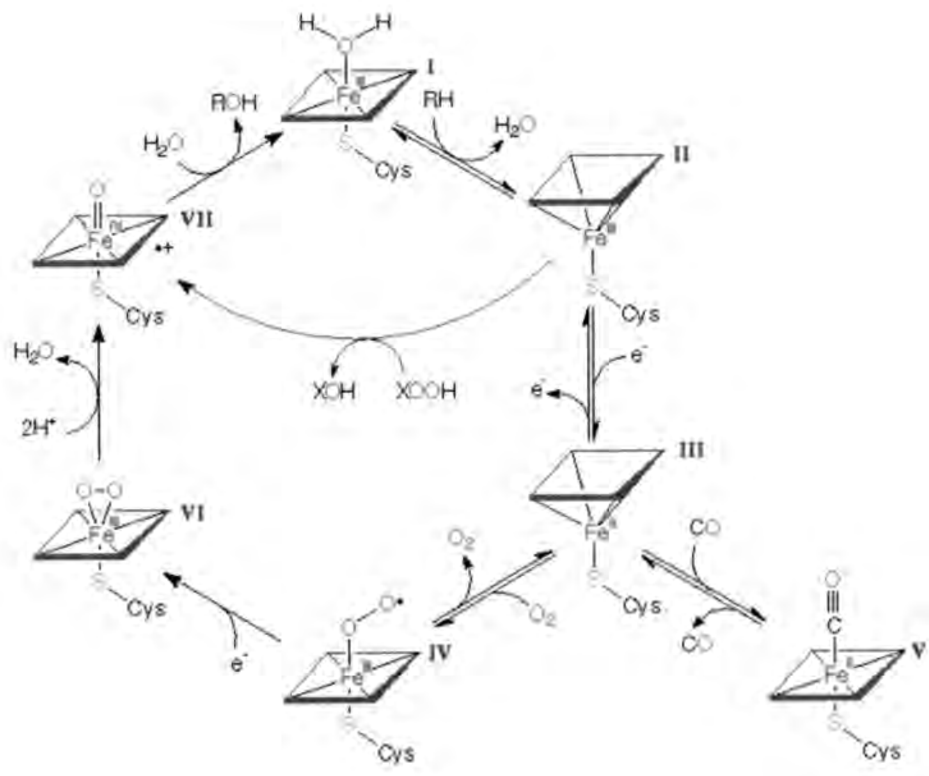

Figure 15. The catalytic cycle of cytochrome P 450 dependent mono-oxygenase (Urlacher, Lutz-Wahl and Schmid, 2004)

Di-oxygenase

Di-oxygenases simultaneously incorporate both oxygen atom of O_2 into the substrate. These enzymes are capable of transforming a range of substrate types (Su and Oliw, 1996) and these activities are of considerable practical interest due to their potential to destroy toxic, persistent pollutants, is a strategy known as bioremediation. The activities of bacterial dioxygenase enzymes were widely studied in *Pseudomonas sp.* in order to degrade the organic pollutants. Typical dioxygenase-reactions, during which two oxygen atoms are simultaneously transferred onto the substrate, are shown in Scheme 3.

Sub-H + O_2 —Di-oxygenase→ Sub-O-O-H (hydro-peroxide) —reduction, e.g. $NaBH_4$→ Sub-OH

Sub-H + O_2 —Di-oxygenase→ Sub(O–O) (endo-peroxide) —reduction, e.g. $NaBH_4$→ Sub(OH)(OH)

Benzene + O_2 —Di-oxygenase→ dioxetane —reductase, $[H_2]$→ Cis-glycol

Scheme 3. Di-oxygenase catalyzed reactions

Oxidase

Oxidases mainly catalyze the electron-transfer onto molecular oxygen. These reactions may proceed through a two- or four-electron transfer, involving either hydrogen peroxide or water as oxygen donor, respectively. These enzymes involve in bioconversions of several organic compounds. For example, the fungal lignin peroxidase can catalyze the oxidative degradation of biopolymer lignin as well as the oxidation of simple oxygenated aromatic compounds. Moreover, it has also been reported to involve in the benzylic hydroxylations, the oxidation of C=C bonds, the decarboxylation, and the oxidative cleavage of aromatic rings. The white-rot fungi, bacidiomycetes, were known as the major source of lignocellulotic enzyme. The mechanism pathway of lignin is shown in Scheme 4.

Scheme 4. Biodegradation of lignin