# CHAPTER II

# LITERATURE REVIEW

## 2.1. Biotransformation

Biotransformation is a biological process whereby an organic compound is modified into a recoverable product by simple, chemically defined reactions catalyzed by enzymes contained in the cells. Microbial, plant and animal cells can supply the enzymes for transformation, but microbes surpass plant and animal cells in several respects. Their high surface-volume ratio confers rapid growth and high rates of metabolism leading to efficient transformation of substrate added. Moreover the microbial world, rich in species, provides a varied assortment of enzymes for a tremendous variety of reactions on many classes of compounds, and with the facility to adapt to the artificial environment imposed by technical and economic requirements [21]. Innumerable microbiologically catalyzed reactions of the most diverse types have been described in **Table 2.1.**

Enzymatically catalyzed reactions may be superior to those catalyzed chemically in relation to the following properties [23]:

a) **Reaction specificity:** the catalytic activity of an enzyme is, as a rule, limited to one type of reaction, so that no side reactions take place.

b) **Regiospecificity:** enzyme reactions generally take place specifically in relation to the position of the reaction in the substrate molecule.

c) **Stereospecificity:** enzymes distinguish between the enantiomers of a racemic mixture and therefore, in many cases, transform only one enantiomeric form exclusively or at least preferentially. If in an enzymatic reaction a new center of asymmetry arises, as a rule the product is optically active, i.e., one of the possible enantiomers is formed exclusively or at least preferentially.

d) **Mild reaction condition:** enzyme reactions take place in aqueous solutions at low temperatures (< 40 °C) and at pH values in the neighborhood of 7.

e) **Lowering of the activation energy:** in the enzyme-substrate complex, the activation energy can be lowered to such an extent that even certain non-activated positions in the substrate molecule are accessible to selective

transformation under mild reaction conditions and positions in the molecule that ordinarily do not react because of insufficient activation.

**Table 2.1** lists types of reactions that have been carried out with microbial cells [22].

| | |
|---|---|
| Hydroxylation | Oxidation |
| Hydrolysis | Esterification |
| Methylation | Demethylation |
| Condensation | Hydration |
| Decarboxylation | Amination |
| Amidation | Phosphorylation |
| Racemization | Isomerization |
| Epoxidation | Reduction |
| Acylation | Halogenation |
| Transglycosidation | Epimerization |
| Dehydration | Deamination |
| Cleavage of C-C bonds | |

Because of these favorable properties, biotransformations are opening up the following operation, given as examples, which are possible by chemical methods only *via* complicated round about pathways or not at all: selective functionalization of nonactivated C atoms and transformation of one functional group among several groups of similar reactivity. Moreover, an advantage over chemical processes are generally run in aqueous media which may pollute less than the organic solvents used in chemical conversions and economic means of obtaining compounds which would be expensive to produce chemically. As the result, biotransformation provided an impetus to the development of many transformations that have operated or are still operating in industry such as pharmaceutical industries.

For example, one of the earliest known bioconversions involves the manufacture of vinegar from ethanol by the acetic bacteria. The rapid and near-quantitative conversion

is characteristic of certain aerobic bacteria of the genus *Acetobacter* or *Gluconobacter* [22]. Cortisone can be synthesized chemically from deoxycholic acid, but process requires 32 steps, many of which must be carried out under extreme conditions of temperature and pressure, with the resulting product costing over $200 per gram. The major difficulty in chemically synthesizing cortisone is the need to introduce an oxygen atom at the number 11 position of the steroid ring; this can be accomplished by microorganisms. The fungus *Rhizopus arrhizus*, for example, hydroxylates progesterone, forming another steroid with the introduction of oxygen at the number 11 position [24] **(Fig 2.1).**
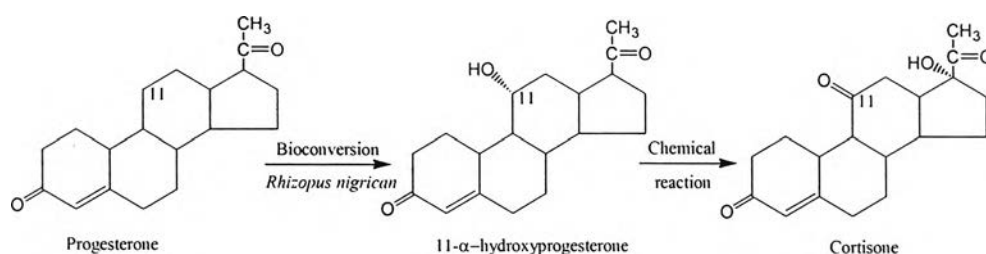


**Figure 2.1** Cortisone production using a microorganism.

Since then numerous investigators have identified other fungi, bacteria and actinomycetes which will accomplish these reactions. These studies led to significant reduction in the cost of corticosteroids (<$1/g) and stimulated an intense effort to find new transforming culture [24]. The successful biotransformations of steroids provided an impetus to development of many transformations that have operated a huge variety of organic compounds; such as alkaloid, antibiotics, amino-acids including terpenoids.

## 2.2 Diterpenoids and their biological activities in *C. oblongifolius.*
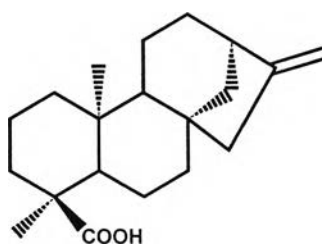
The diterpenoids are a large class of natural products possessing a variety of skeletal types. They display a wide range of biological activities. Amongst the diterpenoids are tumor inhibitory substances, antibiotics, hypertensive agents, sweeteners, perfumery intermediates, antioxidant, antifeedants and plant growth hormones [25].

*Croton oblongifolus* Roxb. (Euphorbiaceae) is a perennial herb widely distributed throughout Thailand. It has been used as a traditional medicine for many

applications such as to alleviate dysmenorrheal, as a purgative, and to treat dyspepsia and dysentery. Additionally, this plant has been used in conjunction with *C. sublyratus* to treat gastric ulcers and gastric cancers. Recently, our investigation on the chemical constituents of this plant, which available in various parts of Thailand, have been widely studied and found many diterpenoid compounds such as cembrane diterpenoid, clerodane diterpenoid, labdane diterpenoid and kaurane diterpenoid [4-6].

In 2003, Ngamrojanavanich and co-workers isolated (-)-*ent*-kaur-16-en-19-oic acid, from the stem bark of *C. oblongifolius* collected from Ampur Kuiburi, Prachuab Kirikhan Province [8] and along with its semi-synthetic derivatives, (-)-methyl kaur-16-en-19-oate, (-)-kaur-16-en-19-ol, (-)-16β,17-epoxykauran-19-oic acid and (-)-17-hydroxykaur-15-en-19-acid, the $Na^+,K^+$-ATPase inhibitory activity were investigated.

**Kaurane diterpenoids**



(-)-*ent*-Kaur-16-en-19-oic acid

Kaurane diterpenoids are a very important class of natural products widespread in the plant kingdom. These compounds are the biosynthetic precursors of gibberellins. the well-known plant growth hormones. A number of important biological activities have been associated with kaurane diterpenoids, such as antimicrobial, antiparasitic, insect antifeedant, cytotoxicity, anti-HIV, anti-inflammatory, and antifertility activities [7]. Kaurenoic acid can be isolated in good yield from many plants such as *Croton oblongifolius* Roxb., *Xylopia frutescens* (Annonaceae) [26] and *Wedelia paludosa* (Asteraceae) [27]. (-)-*ent*-Kaur-16-en-19-oic acid is a typical kaurane representative possessing several biological activities. It is known as precursors of the gibberellin biosynthesis which was a plant growth hormone. In 1966 Mori *et al.* reported that total synthesis of *ent*-kaur-16-en-19-oic acid with appreciable gibberellin-like

activity [28]. *ent*-Kaur-16-en-19-oic acid and its derivatives have been reported for many biological activities such as antibacterial, antihepatotoxic, uterotonic, antitumor and trypanomicidal activities [29-31]. Moreover in 1996, Wu *et al.* found that 16β,17-dihydroxy-ent-kauran-19-oic acid showed significant activity against HIV replication in H9 lymphocyte cells with an $EC_{50}$ value of 0.8 μg/ml [32] and in 1998, Chang *et al.* reported that 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 [33]. In 2003, *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 [8] 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 2005, Chaichantipyuth and coworkers found six new labdane-type diterpenoids, *ent*-3-oxomanoyl oxide, *ent*-1,2-dehydro-3-oxomanoyl oxide, *ent*-3α-hydroxymanoyl oxide, *ent*-1β-hydroxy-3-oxomanoyl oxide, *ent*-1,2-dehydro-12α-hydroxy-3-oxomanoyl oxide and *ent*-1β,3α-dihydroxymanoyl oxide, from *C. oblongifolius* specimen from Loei Province. These compounds gave effective cytotoxicity against cancer cell lines [9].

**Labdane diterpenoids**
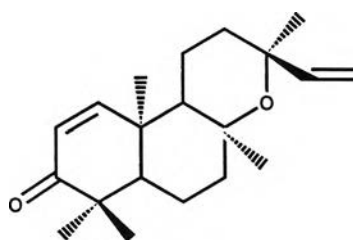


*ent*-1,2-dehydro-3-oxomanoyl oxide

Because of a number of interesting biological activities of *ent*-kaur-16-en-19-oic acid and *ent*-1,2-dehydro-3-oxomanoyl oxide have been reported, therefore, novel derivatives of these two compounds indicated that they were worthy of further studies.

## 2.3 Literature of biotransformation of diterpenoids.

The use of microbiological transformations in modifying substrate molecules has attracted increasing attention. In particular advantage has been taken of the specific hydroxylating ability of certain microorganisms to prepare synthetically or pharmacologically useful compounds. This approach presents a number of advantages. The introduction of a hydroxyl at unactivated sites allows ready entry to sections of the molecule otherwise inaccessible, unutilized material from the incubation can often recycle and in general the technique is relatively simple. The main limitation however lies in the inability to predict for any one molecule the site(s) of hydroxylation. Experiments directed towards an understanding of the factors controlling the direction of hydroxylation, *i.e.* the relationship between structure and hydroxylation pattern, have been undertaken for a group of steroids.

### 2.3.1 Literature of biotransformation of *ent*-kaur-16-en-19-oic acid.

Towards similar ends in 1973, Beilby *et al.* have initiated a study of microbial transformation of *ent*-kaur-16-en-19-oic acid by *Rhizopus nigricans, Calonectria decora, Aspergillus ochraceous,* microorganism extensively studied in steroid hydroxylation [11] and in 1977, Ghisalberti *et al.* had summarized of these results parted of Beilby *et al.* and concerned with a full description of microbial transformation. They found that *C. decora* can convert the acid into the 7,15-dihydroxylated acid in 30% yield and minor quantities of the 7α- and 15α-monohydroxylated compounds. *R. nigricans* afforded a monohydroxyl compound in 25% yield which was found to be identical with the known 7β-hydroxylated compound and variable amounts of the 16,17-dihydroxy acid. The 16,17-dihydroxy acid was the only metabolite which could be isolated from the incubation of 1 with *A. ochraceous* [12] (**Fig 2.2**).
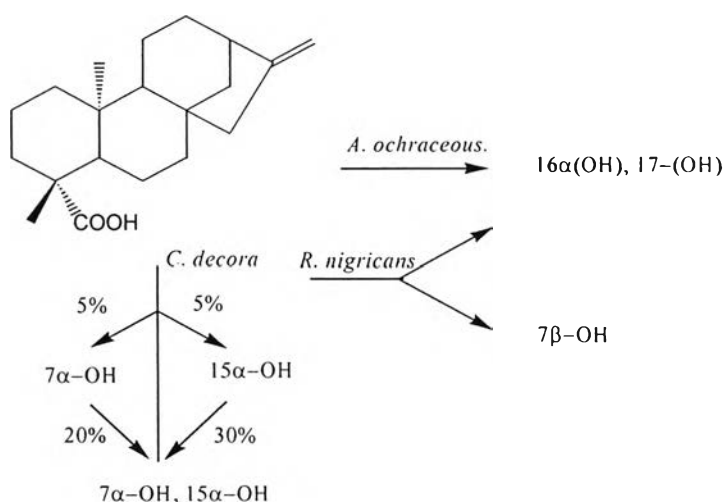
**Figure 2.2** Microbiological transformations of *ent*-kaur-16-en-19-oic acid by

*Rhizopus nigricans, Calonectria decora, Aspergillus ochraceous.*

In 1968, Hanson *et al.* investigated the transformation of *ent*-13-hydoxy-kaur-16-en-19-oic acid (steviol) by *Gibberella fujikuroi* to 6α,7β,13-trihydoxy-kaur-16-en-19-oic acid [13] **(Fig 2.3)**.
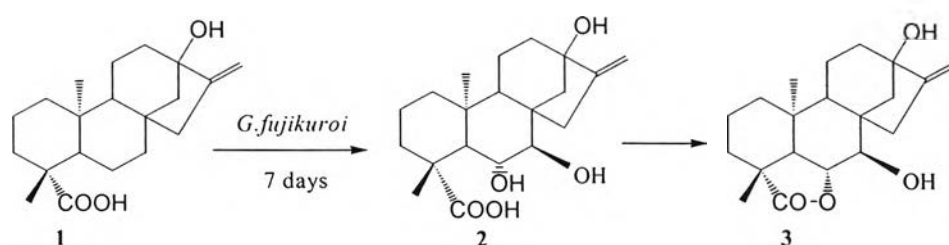


**Figure 2.3** Microbiological transformations of *ent*-13-hydoxy-kaur-16-en-19-oic acid by *Gibberella fujikuroi*.

In 1999, Silva *et al.* carried out the transformation of *ent*-kaur-16-en-19-oic acid by *Rhizopus stolonifer*. Incubation for 7 days yielded two metabolites, *ent*-7α-hydroxy-kaur-16-en-19-oic acid and *ent*-12β-hydroxy-kaur-9(11),16-dien-19-oic acid, which were isolated as a result of hydroxylation and hydroxylation/dehydrogenation. Incubation for 15 days also afforded *ent*-16β,17-dihydroxy-kauran-19-oic acid. It is interesting to note

the role of *ent*-7α-hydroxy-kaur-16-en-19-oic acid as an intermediate in gibberellin biosynthesis and as a plant growth hormone as well as the anti-HIV activity of *ent*-16β,17-dihydroxy-kauran-19-oic acid [14] **(Fig 2.4)**.
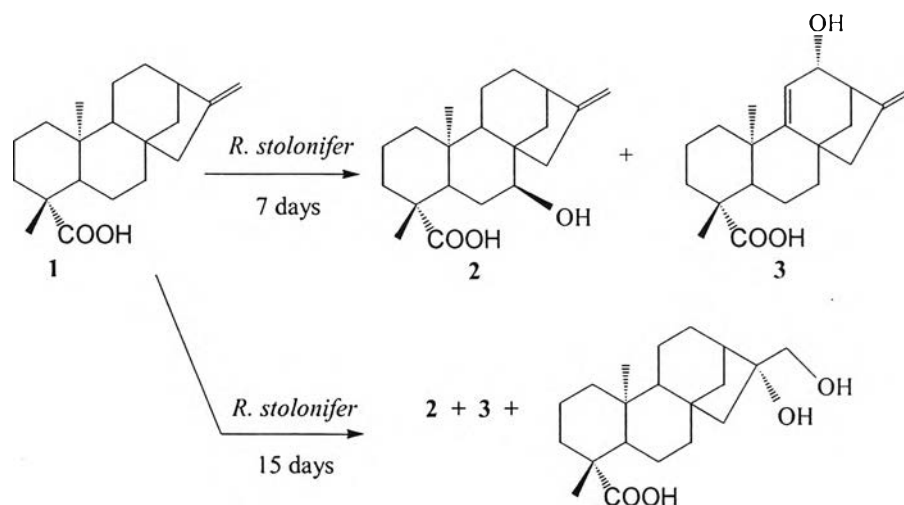


**Figure 2.4**   Microbiological transformations of *ent*-kaur-16-en-19-oic acid by
*Rhizopus stolonifer*.

In 2000, Vieira *et al*. described the biotransformation of methyl *ent*-17-hydroxy-16β-kauran-19-oate by *Rhizopus stolonifer*. Incubation for 11 days yielded two novel metabolites, methyl *ent*-9α,17-dihydroxy-16β-kauran-19-oate and methyl *ent*-7α,17-dihydroxy-16β-kauran-19-oate were isolated as a result of hydroxylation [15] **(Fig 2.5)**.
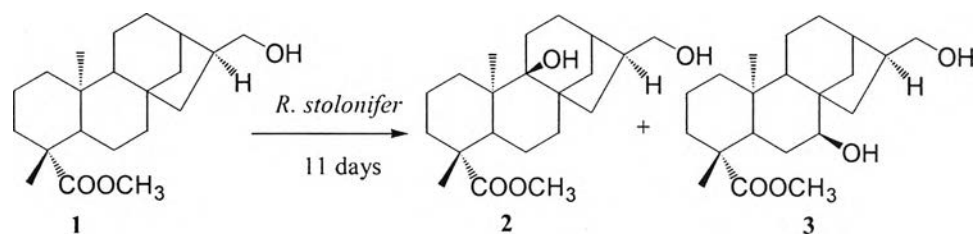
**Figure 2.5** Microbiological transformations of methyl *ent*-17-hydroxy-16β-kauran-19-oate by *Rhizopus stolonifer*.

In 2002, our preliminary investigation revealed the biotransformation of *ent*-kaur-16-en-19-oic acid with *Aspergillus niger*. Incubation for 7 days gave a novel metabolite, *ent*-(7β,11α)-dihydroxy-1-oxo-kaur-16-en-19-oic acid [16] **(Fig 2.6)**.
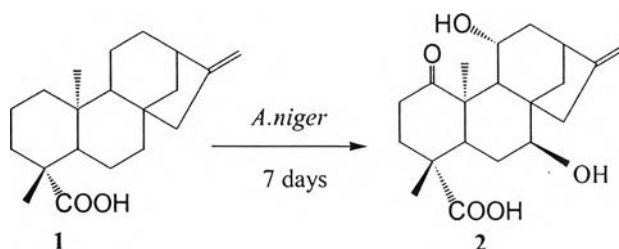


**Figure 2.6** Microbiological transformations of *ent*-kaur-16-en-19-oic acid with *Aspergillus niger*.

## 2.3.2 Literature of biotransformation of *ent*-13-*epi*-manoyl oxide.

In 1994, Garcia-Granados *et al.* described the biotransformation of *ent*-manoyl oxides with *Curvularia lunta*. *Ent*-manoyl oxides, epimeric at C-13 which deriving from abundant natural diterpenes isolated from several spanish *Sideritis* species (Labiatae), have been extensively investigated, using incubations with *Curvularia lunta* in order to obtain highly functionalized derivatives comparable to forskolin, but belonging to the enantiomeric series, for biological testing. Incubation for 10 days gave four novel metabolite, *ent*-6β-hydroxy, *ent*-1α-hydroxy, *ent*-11β-hydroxy and $\Delta^1$-derivatives [17] **(Fig 2.7)**.
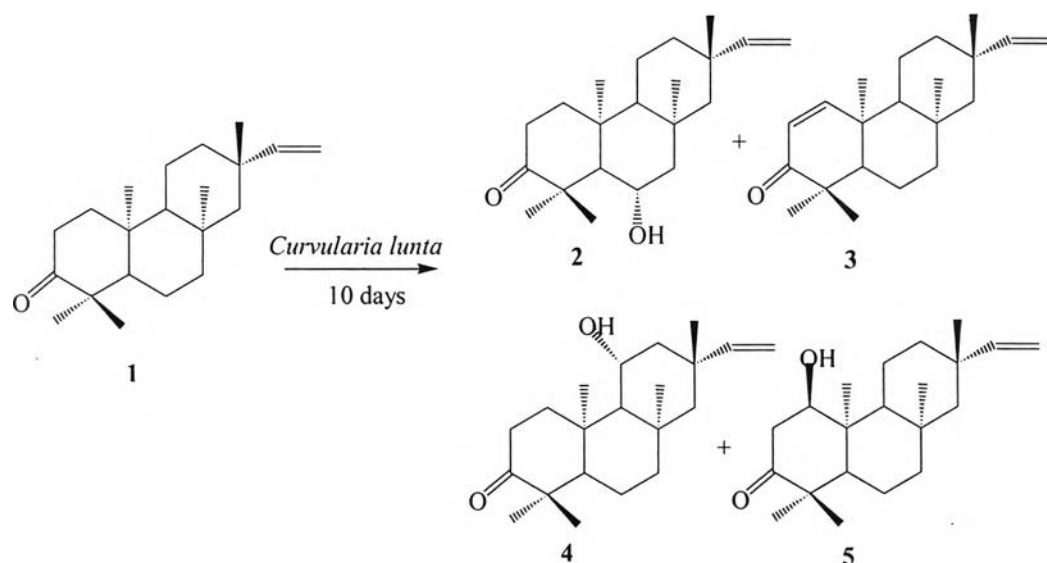
**Figure 2.7** Microbiological transformations of *ent*-13-*epi*-3-keto manoyl oxide with *Curvularia lunta*.

Moreover, Garcia-Granados *et al.* used the fungi *Cunninghamella elegans, Fusarium moniliforme* and *Rhizopus nigricans* to complete an earlier biotransformation study of this *ent*-manoyl oxide with the microorganism *Curvularia lunta*. Depending on the strain and the substrate function, a variety of dihydroxylated products bearing a newly introduced hydroxyl group at positions -1α, -1β, -3α, -6α, -7β, -11α, -12β, or -20 were obtained, together with trihydroxylated products, sometimes in substantial yields. Some of these derivatives exhibit significant antileishmanial activity [18].

In 1999, Fraga et al. studied the microbiological transformation of ribinone (*ent*-3-oxo-13-epi-manoyl oxide) by the fungus *Gibberella fujikuroi* led to compounds hydroxylated at C-1(α), C-6(β), C-11(β) or C-12(β), in addition to the 2,3-seco diacids [19] **(Fig 2.8).**
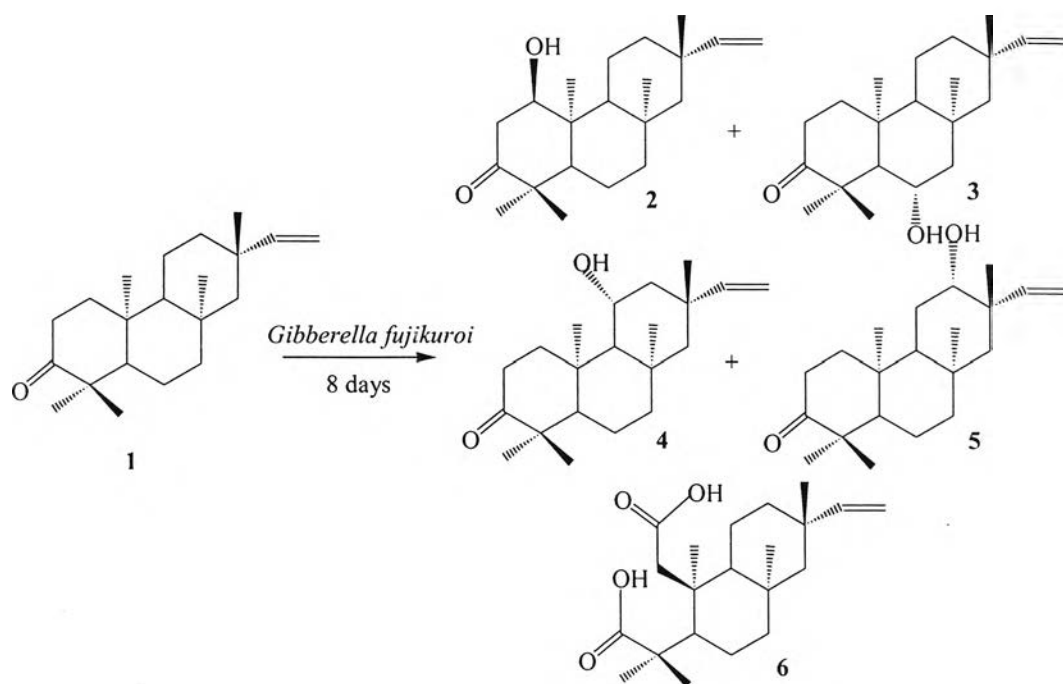
**Figure 2.8** Microbiological transformations of *ent*-3-oxo-13-*epi*-manoyl oxide with
*Gibberella fujikuroi.*

Furthermore, Fraga *et al.* describe the results of the biotransformation of ribenone with *Mucor plumbeus.* Epoxidation of the vinyl group constitutes the main reaction and there exists a preference for hydroxylation at C-1($\alpha$), C-6($\alpha$ or $\beta$) and C-12($\beta$) and to a lesser degree, at C-7($\alpha$) and C-11($\beta$) [20].