CHAPTER I
INTRODUCTION

Geranylgeranyl diphosphate phosphatase is an enzyme that cleaves the diphosphate group from the universal diterpene intermediate of geranylgeranyl diphosphate (GGDP) to form geranylgeraniol (GGOH) (Nah et al., 2001; Tansakul and De-Eknamkul, 1999; Perez et al., 1980; Bansal and Vaidya, 1994). This enzyme has been shown to be involved in the biosynthetic pathway of an acyclic diterpene plaunotol in the well-known Thai medicinal plant Plau-Noi (*Croton stellatopilosus*) (Tansakul and De-Eknamkul, 1998). The pathway involves 2 steps downstream from GGDP: the dephosphorylation of GGDP to form GGOH, and the hydroxylation of GGDP to form plaunotol.

The proposed biosynthetic pathway of plaunotol looks very simple when it is compared with other bioactive terpenoid compounds such as taxol (Walker and Croteau, 2001), artemisinin (Bouwmeester et al., 1999), etc, which involve several steps. Therefore, it seems possible to study enzymes and genes of the plaunotol pathway followed by genetic manipulation of the genes to produce plaunotol by microorganisms instead of the plant.

For GGOH, besides being an intermediate in the plaunotol pathway, it is a well-known compound found to have apoptotic inducer activity to human tumor cell lines (Ohizumi et al, 1995), such as human lung adenocarcinoma A549 cells (Miquel et al., 1998); and human leukemia U937 cells (Masuda et al., 1997). It has been patented for the suspected activity of GGOH against PC3 human prostate carcinoma by Myers et al, 1997 as well. The mechanism of apoptosis induced by GGOH in human hepatoma cells has been proposed that GGOH plays a role in the activation of caspase cascade initiating from caspase-8 (caspase-8/-9/-3) causing DNA fragmentation and loss of mitochondrial transmembrane potential, which leaded into apoptotic cell death (Takeda et al., 2001).

In addition, GGOH has been of interest in cosmetic industry. It has been claimed that GGOH inhibits the esterification reaction of retinol in forming inactive retinyl esters and thus, it may be used to improve skin desquamation and epidermal differentiation (Burger et al., 1999). For other potential clinical uses, combination of

GGOH and HMG-CoA reductase inhibitors has been patented for the treatment of elevated blood cholesterol (Scolnick 1999).

So far, there has been a few reports on the production of GGOH for commercial proposes. The richest source of GGOH in plant is *Bixa orellana* (57% in oleoresin or 1.5 % of dry seed) (Pattenden and Jondiko, 1989). Its by-product after extraction of annatto (reddish-orange colorant which is a mixture of bixin and orelline) has been patented for GGOH production (Tan and Foley, 2002). The study using cell suspension culture of *Croton sublyratus* Kurz has showed the accumulation of GGOH in the lag or stationary phase of cell growth at 0.05% dry weight (Kitaoka et al., 1989), which is quite low yield. The alternative methods of the chemical synthesis of GGOH have also been reported (Bouzbouz, 1994; Mu and Gibbs, 1995; Mori et al., 1996).

Furthermore, various strategies of metabolic engineering for enhancing GGOH production have been tried. Those include i) Inhibition of *ent*-kaurene synthase (in *Gibberella fujikuroi*, Muramatsu and Obata, 2002). Since the enzyme is involved in the downstream of GGDP biosynthetic pathway, the suppression of this enzyme would result to a higher level of GGDP that leads to the accumulation of GGOH. ii) overexpressing of geranylgeranyl pyrophosphate synthase (GGPPS) gene in microorganisms by inactivation of squalene synthase gene. This has been shown to cause an increased yield of GGOH (Millis et al., 2000). iii) using genetically modified microorganisms with increased GGDP phosphatase action. For example, both *S. cerevisiae* and *E. coli* contain numerous phosphatase activities. By testing several phosphatases for efficient dephosphorylation of FDP or GGDP, one could select an appropriate phosphatase and express the gene encoding this enzyme in a production organism to enhance farnesol or GGOH production (Millis et al 2003). iv) overexpressing of prenyl phosphatase in recombinant yeast (*Saccharomyces cerevisae*), in which strain producing high level of the HMG CoA reductase, and contain a fusion gene of GGPPS and farnesyl pyrophosphate synthase. GGOH could be produced in this strain and could be secreted into an extracellular environment to reduce intracellular product concentration (Muramatsu et al., 2003).

In order to use the strategies iii and iv effectively in enhancing GGOH production, information about phosphatase enzyme involved in the biosynthesis of

GGOH is a prerequisite base. Therefore, the biosynthetic study on both the enzyme and genes involved in the biosynthetic of plaunotol in *C. stellatopilosus* should be first carried out. In this study, feeding experiments were performed to detect for enzymatic product according to the proposed biosynthetic pathway. Then the activity of GGDP phosphatase was purified from the fresh leaves of *C. stellatopilosus* in the same fraction that the activity of GGOH-18-hydroxylase was found (20,000 x g pellet) (Tansakul and De-Eknamkul, 1998). The GGDP phosphatase was then characterized by measuring the amount of GGOH in various conditions. Although there have been some reports of GGDP phosphatase activity in other organisms such as *Citrus sinensis* (Perez et al., 1980), *Oryza sativa* (Nah et al., 2001), and rat liver (Bansal and Vaidya, 1994), neither of them have been done in chloroplast containing fraction. Most of the study performed in the microsomal fraction, and detected only for GGOH by using non-polar TLC solvent system. In addition, the mechanism of the dephosphorylation of GGDP by the phosphatase whether it occurs in one or two steps was also investigated from *C. stellatopilosus*. Finally, the gene of prenyl diphosphate phosphatase from *C. stellatopilosus* was cloned and expressed in *E. coli*.
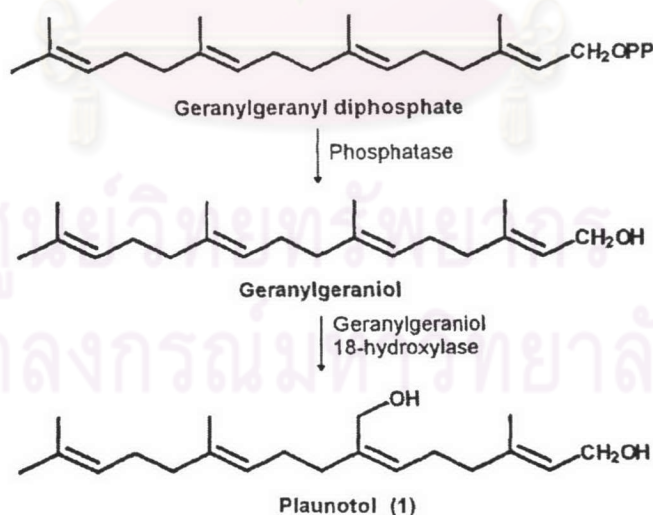

Figure 1 Proposed biosynthetic pathway for the formation of plaunotol.

(Tansakul and De-Eknamkul, 1998)