CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Terpenoids are a structurally diverse group of natural products. More than 25,000 representatives with a variety of biological functions have been reported in the plant kingdom (Sacchettini and Poulter, 1997). Most of terpenoids play an important role as therapeutic agents such as paclitaxel (Taxol®) from *Taxus brevifolia*, ginkgolides from *Ginkgo biloba*, eleutherobin from a soft coral, etc.

Terpenoids are derived from the unique C 5 atoms, the isoprene unit. It has been currently reported that the origins are provided from two different pathways: the classical mevalonate (MVA) pathway and the recently discovered deoxyxylulose phosphate (DXP) pathway. For the latter DXP pathway, the isoprene unit is formed from the condensation of pyruvate and glyceraldehydes 3-phosphate, catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), yielding the first intermediate 1-deoxy-D-xylulose-5-phosphate (DXP). Further, DXP is intramolecular rearranged to a branch polyol sugar, 2C-methyl-D-erythritol 4-phosphate (MEP), catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr). The MEP is then transformed to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) consecutively, catalyzed by IspD, IspE, IspF, IspG and IspH. (for review see Eisenreich *et al.*, 2004). Since the DXP pathway has distributed naturally in eubacteria, malaria parasite and higher plants, therefore, the enzymes involved in the DXP pathway are targets for novel antibacterial, antimalarials and herbicides drug discovery (Lichtenthaler, 1999 and Kaiser *et al.*, 2007).

In higher plants, many genes involved in the DXP pathway have been reported. The benefit of usage the biosynthetic gene is to increase the secondary metabolite production by manipulating the gene either in the intact plant or plant cell cultures. Several studies reported the function and regulatory role of the genes involved the DXP pathway in the terpenoid biosynthesis in higher plant. For example, the investigation of *dxr* gene transcripts from *Lycopersicon esculentum* concluded that *dxr* gene responsible for regulation the carotenoid production, however, non rate-limiting step (Rodriguez-Concepcion *et al.* 2001). In *Coleus forskohlii* cell culture, the *dxr* gene showed to regulate the forskolin biosynthesis and be able to inhibit by fosmidomycin (Engprasert *et al.*, 2005). The transgenic *Lavendula latifolia* by incorporating the *dxs* gene from *Arabidopsis thaliana* enhanced the essential oil production (Munoz-Bertomeu *et al.*, 2006). Those studies are intensifying the regulatory role of on the target genes in terpeniods biosynthesis.

Among Thai medicinal plant, *Croton stellatopilosus* Ohba (formerly name *C. sublyratus* Kurz.) or plau-noi is the only source of plaunotol, which is a potent cytoprotective

antipeptic ulcer (Ogiso et al., 1978). It acts by inhibiting the growth of Helicobacter pylori (Koga et al., 1996). Plaunotol is an acyclic diterpene alcohol and composes of four isoprene units attached in head to tail fashion (Ogiso et al., 1978). The isoprene units of plaunotol skeleton have been originated from the DXP pathway (Wungsintaweekul and De-Eknamkul, 2005). The biosynthesis of plaunotol was first studied from the group of De-Eknamkul and his coworkers since 1997. The geranylgeraniol 18-hydroxylase catalyzes the final committed step from the molecule of geranylgeraniol to plaunotol (Tansakul and De-Eknamkul, 1998). Recently, the phosphatase was reported to catalyze the step of dephosphorylation yielding geranylgeraniol from geranylgeranyl diphosphate (Nualkaew et al., 2006). For the early step in the plaunotol biosynthesis, it is unfortunately lack of knowledge that has been reported.

Therefore, the objective of this work aims to study the dxr gene in the DXP pathway presence in C. stellatopilosus leaves. The dxr gene will be cloned by homology base and RACE (rapid amplification of cDNA ends) method and the dxr gene will be characterized for the function. The regulatory role of dxr gene will be investigated in terms of the transcription level in relation to the amount of plaunotol in various parts of C. stellatopilosus. The results obtained from this study will be useful for understanding the plaunotol biosynthetic pathway, which leads to enhance the plaunotol production either in intact plant or plant cell culture by metabolic engineering.

1.2 Review of literature
1.2.1 The biosynthetic pathway of terpenoids
1.2.1.1 The classical mevalonate (MVA) pathway

The classical route for the formation of the C_5 building blocks of terpenoid biosynthesis in plants via the reactions of the mevalonate pathway, first demonstrated in yeast and mammals (for review see Porter and Spurgeon, 1981). This well-characterized sequence (Fig. 1.1) starts from acetyl-CoA as precursor. Sequentially, two molecules of acetyl-CoA are condensed to acetoacetyl CoA by thiolase. The latter compound is then condensed with another molecule of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. Later, HMG-CoA is reduced to mevalonic acid in the presence of NADPH by HMG-CoA reductase. This enzyme attracted and great interest since it catalyzes the rate-limiting step in MVA pathway thus became a key target for interruption of cholesterol biosynthesis. The series of HMG-CoA reductase inhibitors such as mevastatin, lovastatin, simvastatin and pravastatin are highly effective hypochloesterolemic agents (Spurgeon and Porter, 1981). Mevalonic acid is phosphorylated twice to form mevalonic 5-diphosphate via mevalonic monophosphate. Mevalonic 5-diphosphate is then decarboxylated and dehydrated to form isopentenyl diphosphate. Isopentenyl diphosphate is isomerized to dimethylallyl diphosphate by isomerase (Spurgeon and Porter, 1981).
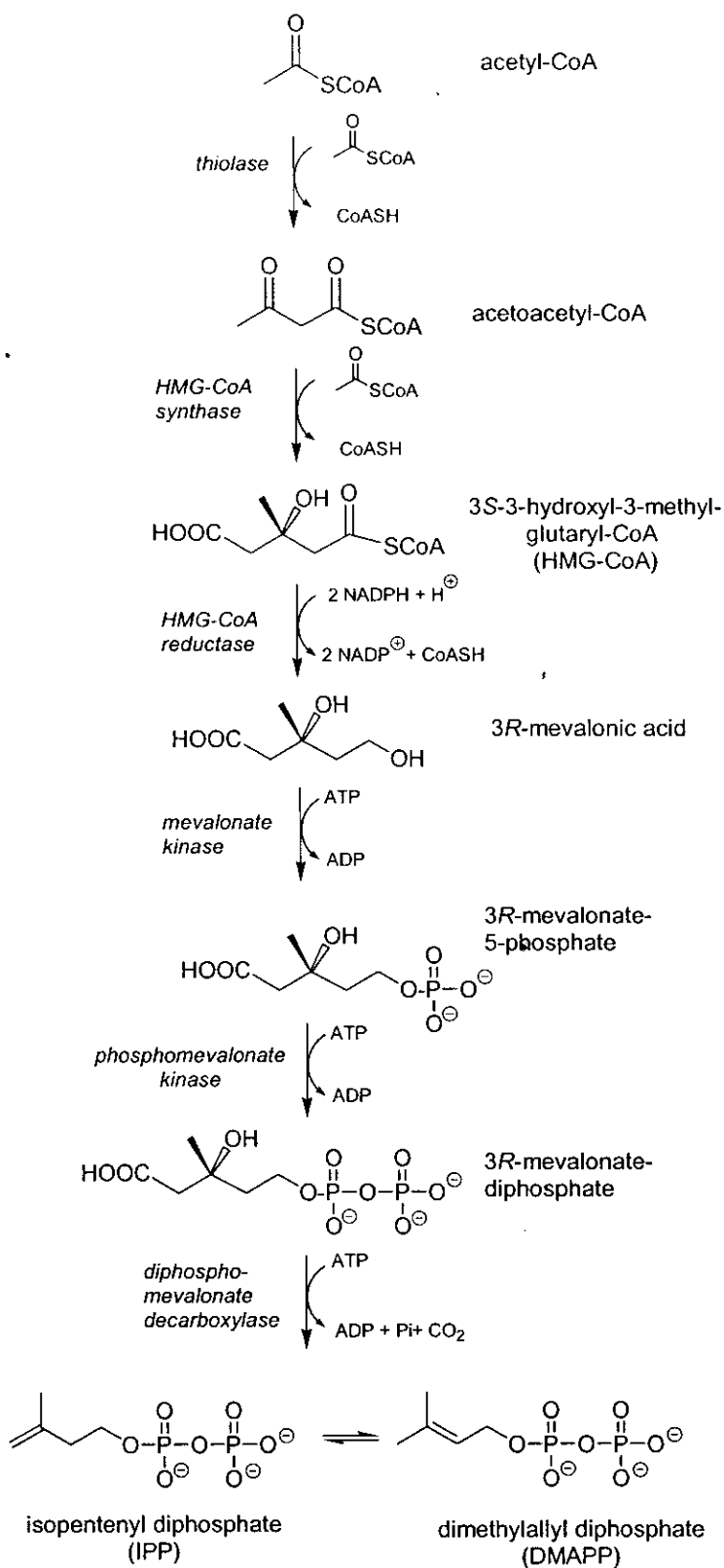
acetyl-CoA

thiolase

CoASH

acetoacetyl-CoA

HMG-CoA synthase

CoASH

3S-3-hydroxyl-3-methyl-glutaryl-CoA (HMG-CoA)

HMG-CoA reductase

$2\ NADPH + H^{\oplus}$

$2\ NADP^{\oplus} + CoASH$

3R-mevalonic acid

mevalonate kinase

ATP

ADP

3R-mevalonate-5-phosphate

phosphomevalonate kinase

ATP

ADP

3R-mevalonate-diphosphate

diphospho-mevalonate decarboxylase

ATP

$ADP + Pi + CO_2$

isopentenyl diphosphate (IPP)

dimethylallyl diphosphate (DMAPP)

Figure 1.1 The classical mevalonate (MVA) pathway

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1.2.1.2 The deoxyxylulose phosphate (DXP) pathway

Recently, the DXP pathway was completely elucidated in *Escherichia coli*. The initial step in the DXP pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by the condensation of pyruvate and D-glyceraldehyde 3-phosphate, catalyzed by Dxs (Broers, 1994; Schwarz, 1994). The *dxs* gene encoding this enzyme was first cloned from *E. coli* (Lois *et al.*, 1998; Sprenger *et al.*, 1997). This enzyme has a typical thiamine-binding motif and needs both thiamine and a divalent cation such as Mg^{2+} or Mn^{2+} for enzyme activity.

In the second step DXP is transformed into 2C-methyl-D-erythritol-4-phosphate (MEP). The formation of MEP from DXP is synthesized in a single step by rearrangement DXP to an intermediate with a branched carbon skeleton, 2C-methyl-D-erythrose-4-phosphate (MEOP), followed by reduction using NADPH (Takahashi *et al.*, 1998). The enzyme required Mg^{2+} or Mn^{2+} as cofactor.

The next step of the DXP pathway is the conversion of MEP to 4-(diphosphocytidyl)-2C-methyl-D-erythritol (CDP-ME) in a cytidine triphosphate (CTP) dependent reaction catalyzed by enzyme encoded *ygbP* gene (now designated *ispD*), which is present in many bacteria as well as in *Arabidopsis thaliana* (Rohdich *et al.*, 1999).

The further step in the pathway is the ATP-dependent phosphorylation of the 2-hydroxy group of CDP-ME converting into 4-(diphosphocytidyl)-2C-methyl-D-erythritol-2-phosphate (CDP-ME2P) by an enzyme namely CDP-ME kinase encoded *ychB* gene (now designated *ispE*) which was identified in the genome of *E. coli* and many other organisms (Lüttgen *et al.*, 2000).

The next step in the pathway was also identified using bioinformatics. Genome analyses had shown that many putative orthologues of the *E. coli ygbB* gene (now designated *ispF*) were linked or fused to putative orthologues of *ispD* (Rohdich *et al.*, 1999 and Herz *et al.*, 2000). Based on these findings, the *E. coli ispF* gene was expressed and the recombinant protein shown to catalyze the conversion of CDP-ME2P into 2C-methyl-D-erythritol 2,4-cyclodiphosphate (Herz *et al.*, 2000).

The next enzyme was studied, *IspG* gene of *E. coli* was expressed and this protein was shown to catalyze the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate. The last step, *IspH* protein catalyzes the subsequent transformation of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into a mixture of IPP and DMAPP (Rohdich *et al.*, 2003). The late steps in the DXP pathway was studied with an IPP isomerase-disruptant of *E. coli* confirmed that IPP isomerase is not essential for growth in this organism. This finding indicates that IPP and DMAPP are synthesized through independent routes in the late steps of the DXP pathway.
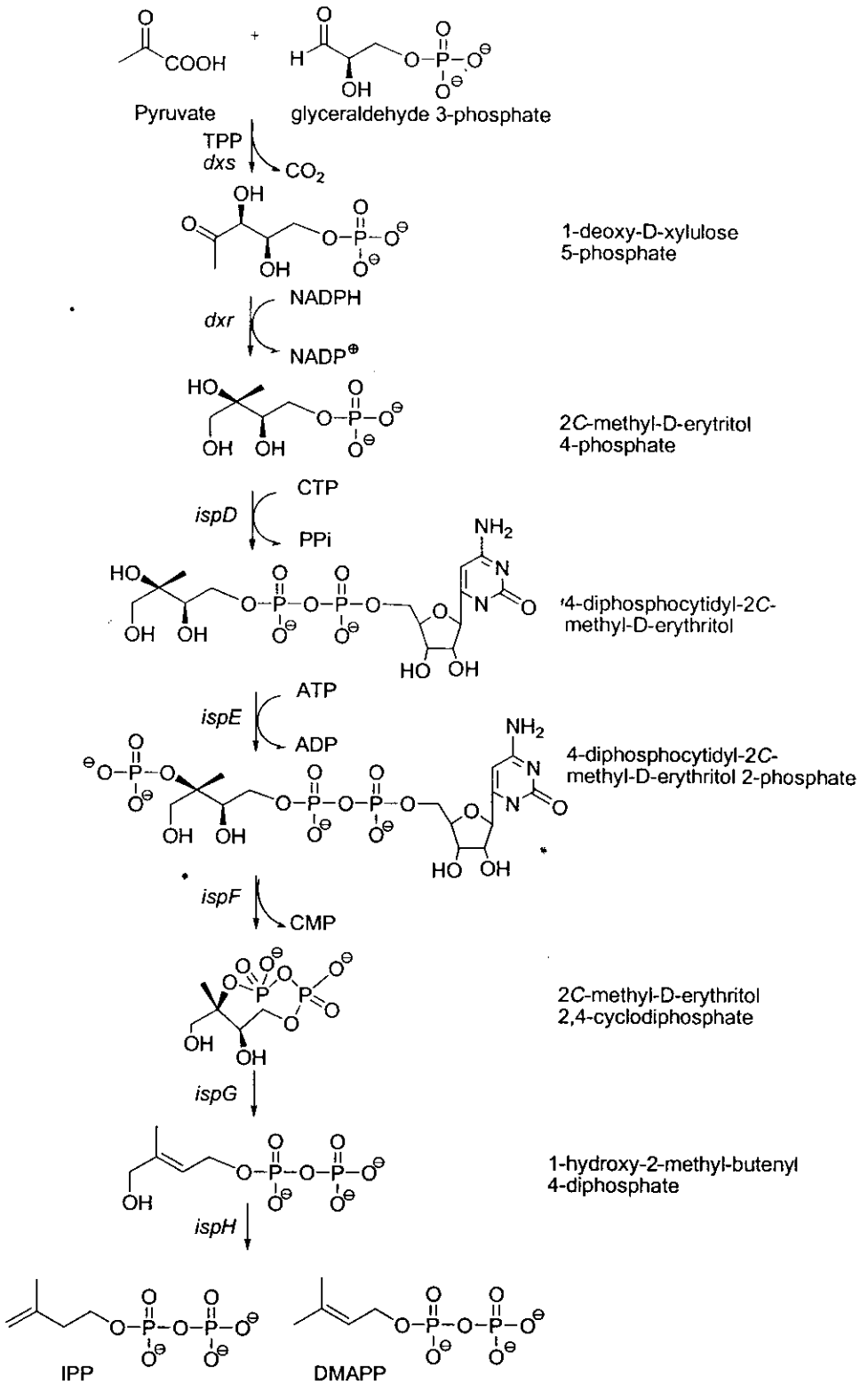
Figure 1.2 The deoxyxylulose phosphate (DXP) pathway

1.2.1.3 Cross-talk between two independent IPP generating pathways in plants

As mentioned earlier, building blocks of terpenoids in higher plants, the isoprene unit can be supplied from MVA pathway and DXP pathway (Arigoni and Schwarz, 1999; Lichtenthaler, 1999; Eisenreich et al., 1998). The MVA route has been reported to operate in the cytoplasm for the formation of sterols, triterpenes and sesquiterpenes whereas the alternate DXP pathway occurs in the chloroplasts for the formation of terpenoids required for photosynthetic machinery (carotenoids, phytol, prenyl side chain of plastoquinone) and various groups of monoterpenoids, diterpnoids, abscisic acid, tocopherol and phylloquinone (Fig. 1.3) (Rohmer, 1999; Bick and Lange, 2003). This compartmentation is, however, not always clear cut based on observations in various labeling experiments (Adam and Zapp, 1998; Itoh et al., 2000; Yang and Orihara, 2002). It can be suggested that exchange of common intermediates such as IPP, DMAPP, GPP and FPP might occur between the two compartments as described by the "Cross-talk theory" (Bick and Lange, 2003). The extent of this crosstalk depends on the species as well as on the presence and concentration of exogenous precursors.

A powerful strategy for quantitative assessment of the differential contribution of the two isoprenoid pathways has been established using the labeled glucose as well as labeled 1-deoxy-D-xylulose and mevalonate as precursors in whole plants, plant tissue cultures or cultured plant cells (Schuhr et al., 2003). For instance, feedings of [1-^{13}C]glucose and [1-^{13}C]1-deoxy-D-xylulose into chamomile flower have been reported. Analysis of the labeling patterns using quantitative ^{13}C-NMR spectroscopy of isolated bisabololoxide-A and chamazulene showed that the two of the isoprene building blocks were predominantly formed via the DXP pathway, whereas the third unit was of mixed origin (Adam and Zapp, 1998 and Adam et al., 1999). Various labeled precursors were also studied in Scenedesmus obliquus, Lemna gibba, Hordeum vulgare, Daucus carota, cell cultures of Taxus chinensis and Mentha x piperita (Schwender et al. 1996; Lichtenthaler et al. 1997; Eisenreich et al., 1996). Those studies revealed the actual contribution of the MVA and DXP pathway, which in agreement with the explanation of "Cross-talk theory).

1.2.2 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr)

Gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) in the DXP pathway was firstly reported by Seto and his group in 1998. By the mutagenesis approach, genes responsible for the DXP pathway were cloned by using a strategy to prepare E. coli mutants with a metabolic block(s) between DXP and MEP (Kuzuyama et al., 1998; Takahashi et al., 1998). The mutant that required 2C-methyl-D-erythritol for growth was

screened. By complementation of these mutants, only the *yaeM* gene from *E. coli* was cloned and the gene product was purified and characterized (Takahashi *et al.*, 1998). The *yaeM* gene was later renamed to *dxr* or *ispC* (Takahashi *et al.*, 1998).

1-Deoxy-D-xylulose 5-phosphate reductoisomerase or 2*C*-methyl-D-erythritol 4-phosphate synthase (EC 1.1.1.267) is belonging to the reductoisomerase family. It catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate to 2*C*-methyl-D-erythritol 4-phosphate in the presence of NADPH, a co-substrate. It requires a divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}) for its activity (Fig. 1.3). Beyond the discovery *dxr* gene and gene product in *E. coli*, homologous proteins were expressed from various bacteria, plants and protozoon (Eisenreich *et al.*, 2004).
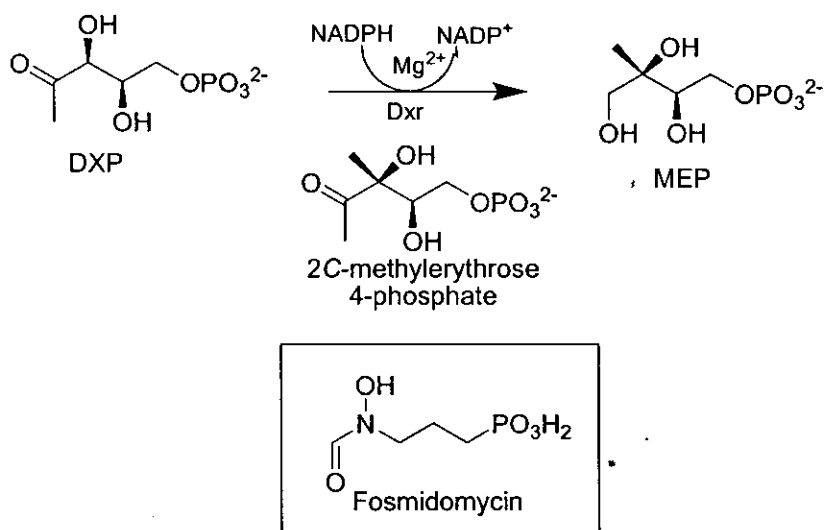

Figure 1.3 The catalytic action of Dxr

Naturally, Dxr protein does not utilize 1-deoxy-D-xylulose. It should be noted that the successful incorporation of 1-deoxy-D-xylulose into terpenoids of various organisms required a phosphorylation step catalyzed by the kinase (Wungsintaweekul *et al.*, 2001). The enzyme prefers to use NADPH rather than NADH. It transfers the pro-s proton from NADPH and is therefore belonging to the class B dehydrogenases (Arigoni *et al.*, 1999; Radykewicz *et al.*; 2000; Proteau *et al.*, 1999). The K_M of DXP and V_{max} values are 171 µM and 18 µmol min^{-1}mg^{-1} (turnover number 13 s^{-1} per subunit). It utilizes NADPH as cofactor with a K_M of 25 µM (Wungsintaweekul, 2001).

Fosmidomycin [3-(N-formyl-N-hydroxyamino) propyl phosphate] inhibits 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Kuzuyama *et al.*, 1998). The inhibitory effect of fosmidomycin on 1-deoxy-D-xylulose 5-phosphate reductoisomerase can be explained by a structural similarity of fosmidomycin to 2*C*-methyl-D-erythrose 4-phosphate, a

putative intermediate of the enzyme reaction. Fosmidomycin had been reported as a mixed-type inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* with a *K*i value of 38 nM (Kuzuyama *et al.*, 1998). For 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Zymomonas mobilis*, fosmidomycin served as a competitive inhibitor with a *K*i value of 600 nM (Grolle *et al.*, 2000). Currently, 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Arabidopsis thaliana* was inhibited by fosmidomycin with *K*i value of 85 nM (Rohdich *et al.*, 2006).

The structure of the *E. coli* Dxr protein has been published (Reuter *et al.*, 2002; Yajima *et al.*, 2002; Steinbacher *et al.*, 2003; Mac Sweeney *et al.*, 2005). Each subunit of the homodimer consists of an N-terminal dinucleotide binding domain, a connecting domain with the catalytic site and C-terminal helical domain (Fig. 1.4). The structure of a complex with NADPH (Yajima *et al.*, 2002) confirmed the essential role of Gly-14, Glu-231, His-153, His 209 and His-257 in the catalytic process. Glu-231 of the *E. coli* enzyme is important in the conversion of DXP into MEP, and His-153, His-209 and His-257 are part of the binding site of DXP to the enzyme (Kuzuyama *et al.*, 2000).

In plants, two different groups have cloned the genes encoding Dxr from *Arabidopsis thaliana* (Schwender *et al.*, 1999) and *Mentha piperita* (Lange and Croteau, 1999), and expressed those in *E. coli*. Carretero-Paulet and coworkers have cloned a single copy gene (cDNA) from *A. thaliana* encoding the Dxr (Carretero-Paulet *et al.*, 2002). The protein sequence analysis predicted the presence of an N-terminal transit peptide located to plastids, with a conserved cleavage site, and a conserved proline-rich region at the N-terminus of the mature protein (Rodriguez-Concepcion *et al.*, 2001, Carretero-Paulet *et al.*, 2002, Totte *et al.*, 2003, Engprasert *et al.*, 2005; Yao *et al.*, 2006). From *M. piperita* (Lange and Croteau, 1999) have shown that the enzyme can be inhibited by fosmidomycin, as reported in some other plant species (Zeidler *et al.*, 1998; Fellermeier *et al.*, 1999; Lichtenthaler, 2000; Engprasert *et al.*, 2005).

In transgenic peppermint (*M. piperita*), overexpressing Dxr led to an increase of essential oil monoterpenes in its leaf tissues compared to the wild type. Gene silencing of partial *dxr* gene of the engineered peppermint plants led to a reduction of essential oil accumulation (Mahmoud and Croteau, 2001). A positive correlation was found between the accumulation of apocarotenoids in mycorrhizal roots from monocots with increase in Dxr transcript (Walter *et al.*, 2000), and similar observation was also reported with indole alkaloids accumulation in *Catharanthus roseus* cell suspension culture (Veau *et al.*, 2000). However, in tomato (*L. esculentum*) fruit during ripening, neither Dxr transcripts nor protein level increased, despite the massive carotenoids accumulation, suggesting a non-limiting role for Dxr in this system (Rodriguez-Concepcion *et al.*, 2001). Later, the expression level of *dxr* gene in relation to terpenoid production were reported from *Gingko biloba* (Gong *et al.*, 2005), *Elaeis guineensis* (Khemvong and Suvachittanont, 2005) and *Camptotheca accuminata* (Yao

et al., 2007). It has been demonstrated that *dxr* gene associated with terpenoid biosynthesis and the gene product does not exhibited as rate-limiting step in the DXP pathway.

1.2.3 *Croton stellatopilosus* Ohba

Figure 1.4 *Croton stellatopilosus* Ohba (Euphorbiaceae)

In 1972, Airy Shaw from Kew garden introduced the name of *C. sublyratus* Kurz. for Plau-noi (Thai name), characterized by distinctly obovate-panduriform leaf shape (Airy Shaw, 1972). This plant belongs to the Euphorbiaceae family. More than 20 years ago, this plant has been reported that it is the only source of antipeptic ulcer (Ogiso *et al.*, 1987). However, in 2001, Esser and Chayamarit has changed plant name from *C. sublyratus* Kurz. to *C. stellatopilosus* Ohba (Esser and Chayamarit, 2001). Because of the characteristic of obovate leaves and scattered hairs containing stellate-dendritic trichomes on the leave blade was strong evidence to other species, not *C. sublyratus* Kurz. (Esser and Chayamarit, 2001). Thus, name of plaunoi has been changed to *C. stellatopilosus* Ohba.

1.2.4 Plaunotol: Structure and chemical properties

The chemical name of plaunotol is (*E, Z, E*)-7-hydroxymethyl-3, 11, 15-trimethyl-2, 6, 10, 14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol. It has a molecular formula of $C_{20}H_{34}O_2$ and molecular mass of 306.2546 (Ogiso *et al.*, 1978). The structure of plaunotol is shown in Figure 1.6.
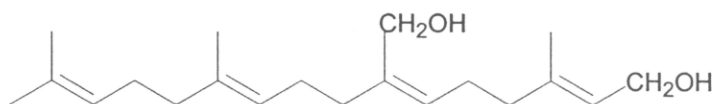

Figure 1.5 The chemical structure of plaunotol

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Plaunotol occurs as pale yellow to light brown viscous liquid, having a slightly characteristic odor and a bitter taste. It is soluble in methanol, ethanol, acetone, ethyl acetate, dioxane, ether, chloroform, toluene or vegetable oil, but is practically insoluble in water (Department of Medicinal Information, Sankyo Co., Ltd., 1993).

For physicochemical properties, plaunotol shows its infrared spectrum with absorption band at 3300, 1665, 1440, 1380 and 1000 cm^{-1}. The proton magnetic resonance (PMR) spectrum of plaunotol shows signals due to the vinyl methyl groups at δ 1.9-2.3 (12H, m), two hydroxymethyl groups at δ 1.58 (6H, s) and δ 1.66 (6h, s), six allyl methylene groups at δ 1.9-2.3 (12H, m), two hydroxymethyl groups at δ 3.94 (2H, s) and δ 3.97 (2H, d), and four olefinic protons at δ 5.0-5.3 (4H, m). For mass spectrum, plaunotol shows the molecular ion at m/e 306.2585 (M^+, calculated for $C_{20}H_{34}O_2$ 306.256) and also other main peaks at m/e 288, 270, 121, 81 and 69 (base) (Ogiso et al., 1978).

1.2.5 Plaunotol: Quantitative analysis

The determination of plaunotol content has been reported that Thin-layer chromatography (TLC) densitometric method (Vongchareonsathit and De-Eknamkul, 1998) and gas chromatography method (Moromoto and Murai, 1989; Ogiso et al., 1981) can be used.

TLC densitometric method was developed for the rapid and precise measurement of plaunotol. This method allows the active constituent to be determined in unpurified plant extracts and therefore allows a large-scale screening program in a plant population to be performed. TLC was performed on silica gel 60 F254 plate using benzene and ethyl acetate (1:1) (Ogiso et al., 1981) or 20% ether in chloroform or chloroform: n-propanol (96:4) (Vongchareonsathit and De-Eknamkul, 1998) as developer with 10 cm height of the solvent front. The TLC plate was then scanned by TLC densitometer under the wavelength of 220 nm.

Gas chromatography method can determine the plaunotol content in the microgram range. This method was performed by using a glass column packed with 2% OV17 on 60/80 Supelcoport, flow rate: 30 ml/min N_2, temperature program: from 235 °C (hold 2 min) to 250 °C (temperature rate 15 °C/min) and hold 10 min, injector temperature: 300°C, sample size 2 μl (Vongchareonsathit and De-Eknamkul, 1998).

1.2.6 Plaunotol biosynthesis

Plaunotol biosynthesis has been firstly reported from the group of De-Eknamkul and his coworkers in 1998. Plaunotol is derived from four isoprene units. One molecule of DMAPP and three molecules of IPP were attached by head to tail condensation, catalyzed by prenyltransferases (Fig. 1.6) (Spurgeon and Porter, 1981). Geranylgeranyl diphosphate (GGPP) is formed. GGPP is then dephosphorylated to GGOH by phosphatase enzyme (Nualkaew et al., 2005 and Nualkaew et al., 2006). Finally GGOH is hydroxylated at C-18 position, catalyzed by GGOH 18-hydroxylase (Fig. 1.7) (Tansakul and De-Eknamkul, 1998).

As mentioned earlier, the isoprene unit can be supplied from the MVA and/or DXP pathways. Therefore, the origin of isoprene unit in plaunotol skeleton was investigated. By feeding of isotopic glucoses revealed the isoprene unit was supplied from the DXP pathway dominantly without significant contribution from the MVA pathway. (Wungsintaweekul and De-Eknamkul, 2005).

Recently, localization of plaunotol has been investigated. The results showed that the storage site was in the palisade layer and plaunotol was kept as oil globule (Sitthithaworn et al. 2006). This data is also in agreement with the localization of the gene encoding GGPP synthase (Sitthithaworn et al. 2001). Evidence of the correlation of plaunotol production with the chlorophyll accumulation was supported that the plaunotol is biosynthesized and storage in the chloroplast (Morimoto and Murai, 1989).
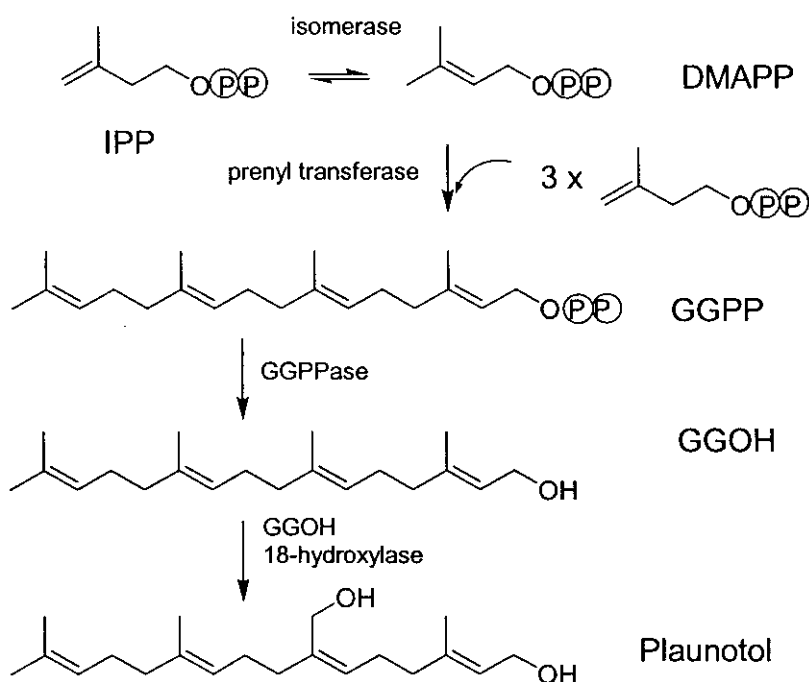

Figure 1.6 Biosynthesis pathway of plaunotol