CHAPTER II

HISTORICAL

1. Botanical Aspects of the *Croton* Genus.

The genus *Croton* belongs to the family of Euphorbiaceae. This genus comprises of 800 species (Mabbeley, 1987) growing in the warmer part of the world. They are herbs , shrubs or trees , usually monoecious , sometimes dioecious with pubescence stellate or scally. Leaves usually alternate . Inflorescences mostly in terminate spike or racemes. Sepals usually 5-10, small, petals present at least in the staminate flower; stamen 5 to many , incurved in the bud; ovary densely stellate hairy, 3-celled, 1 ovule in each cell (Bailey, 1963), styles very large, usually bifid, and again devided spreading; fruit globose more or less 3- lobed always stellate-hairy cocci hard, 2-valved, dehiscent; seeds 3, ovoid, testa crustaceous, endosperm fleshly (Trimen, 1974).

According to Tem Smithinand (1980) and ลีนา ผู้พัฒนพงศ์ และ ธวัชชัย วงศ์ประเสริฐ, (2530), the occurrence of the species of the genus *Croton* found in Thailand can be summarized as listed in Table 1

Table 1 *Croton* spp. found in Thailand.

Scientific name	Plant Habit	Thai-name (Source)
1. *Croton argyratus* Bl. (*C. budopensis* Gagnep).	Shrub/ Shrubby tree	เปล้า plao (Prachuap Khiri Khan)
2. *C. birmanicus* Muell.Arg.	Shrub	บะกัง bakang (Phrae)

Table 1 (continued)

Scientific name	Plant Habit	Thai-name (Source)
3. *C. cascarilloides* Raeusch. (*C. cumingii* Muell. Arg.; *C. pierrei* Gagnep)	Shrub	เปล้าเงิน plao ngoen (Songkhla); เปล้าน้ำเงิน plao nam ngoen (Prachuap Khiri Khan)
4. *C. caudatus* Geisel	Climber	กระดอหดใบขน krado hot bai khon (Chantaburi)
5. *C. columnalis* Airy Shaw	Shrub	เปล้าคำ plao kham (Sukhothai)
6. *C. crassifolius* Geisel (*C. tomentosus* Muell.Arg)	Under Shrub /Shrub	ปังคี pang khee ; พังคี phang kee (Chaing Mai)
7. *C. hutchinsonianus* Hoss.	Shrub and Shrubby tree	เปล้าแพะ plao phae (Northen)
8. *C. joufra* Roxb.	Shrub	เปล้าน้อย plao noi (Lampang)
9. *C. longisimus* Airy Shaw.	Shrub	เปล้าน้อย plao noi (Lampang)
10. *C. oblongifolius* Roxb	Tree	คะวู khwa wuu. (Karen-Kanchanaburi); เซ่งเค่คัง seng- khe - khang, สะกะวา, sa-kaa-waa; ส่ากูวะ saa-kuu- wa (Karen -Mae hong son); เปาะ poh (Kamphaeng Phet); เปล้าหลวง plao uang (Northen); เปล้าใหญ่ plao yai (Central); ห้าเยิ่ง haa-yoeng(shan -Mae Hong Son.);
11. *C. robustus* Kurz. (*C. siamensis*)	Shrubby tree	เปล้าเลือด plao lueat (Lampang)

Table 1 (continued)

Scientific name	Plant Habit	Thai-name (Source)
12. *C. rottleri* Geisel (*Chrozophora rottleri* Juss. ex Spreng)	Herb	ถั่วนา thua naa(Ratchaburi) ; พญามุตติต้น (Petchaburi); มะพร้าวห้าว ma praao haao (Central); หญ้ารักนา (Chaing Mai)
13. *C. sublyratus* Kurz.	Shrubby tree	เปล้าน้อย plaunoi (Central)
14. *C. tiglium* Linn.	Exotic Shrubby tree.	มะข่าง makhaang; มะคัง makhang ; มะตอด matot ;หมากทาง maakthang; หัสคืน haskhuen (Northen); ลูกผลาญศัตรู luuk plaan sattruu; สลอด salot; สลอดต้น salot ton ; หมากหลอด marklot(Central); หมากยอง (Shan -Mae Hong Son.); Croton oil plant
15. *C. trachycaulis* Airy Shaw.	Shrub	ขี้อ้น khee on (Prachuap Khiri Khan)

These plants grow naturally in every part of Thailand. However only *C. sublyratus* has been reported to contain the anti-ulcer substance of plaunotol (Ogiso *et al.*, 1981; Ogiso *et al.*, 1985; Apacha Vongcharoensathit, 1994).

2. *Croton sublyratus* Kurz.

2.1 Botanical Aspect of *Croton sublyratus* Kurz.

Croton sublyratus Kurz. or Plaunoi (Thai-name) (Figure 1) is in the family of Euphorbiaceae. This plant is a deciduous shrub or tree, 2-3.5 m. high, shoots rusty-scurfy. The leaves are simple, alternate, 4-6 cm wide, 10-15 cm long; cordate at the narrow base, very shortly petioled obovate to almost lyrate oblong obtuse or accuminate repand-serrulate beneath glabrous or with scabous nerve and raceme stellate-tomentose. Young leaves are dark brown and inflorescence. Petiole is stout, 6-12 mm long. The flowers are small, perfect and raceme. Flowering is up the scar of leaf with near shoot. Staminate flower has five lanceolate with acuminate sepal, five petal with stellate rim, long stellate base and stamens 15-20 glabrous. Pistillate flower is similar to staminate flower, no petal and ovary is densely stellate tomentose, brown-yellow with short styles. The fruit are capsules small 3 lobed crustaceous sparsely pubescent and 3-5 mm long. The seeds are 2-3 mm long, white-brown and smooth (ลีนา ผู้พัฒนพงศ์, 2530; ลีนา ผู้พัฒนพงศ์ และ ธวัชชัย วงศ์ประเสริฐ, 2530).

The propagation of *C. sublyratus* includes budding (to form plantlet from root), and cutting (เปรมจิต นาคประสิทธิ์,บรรณาธิการ, 2526; สำนักงานคณะกรรมการวิจัยแห่งชาติ, 2533). For cultivation, *C. sublyratus* is planted approximately 250-256 plants in one rai area with the distance of 2.5X2.5 metre for each. Its young leaves are annually harvested 2-3 times after three years or up to ten years of cultivation. By average, the productivity of *C. sublyratus* leaves is about 625-750 kg of dry weight per one rai area (ณรงค์ เพ็งปรีชา, 2530).

2.2 The Uses of *C. sublyratus.*

2.2.1 Traditional Uses

C. sublyratus (Plaunoi) is a Thai medicinal plant used as anthelmintic and dermatologic agent for skin disease (จุฬาลงกรณ์มหาวิทยาลัย, คณะเภสัชศาสตร์, ภาควิชาเภสัชพฤกษศาสตร์, 2530; Dhavadee Ponglux., *et al*, 1987). The parts of stem, bark and leaf have been used as antidiarrheal and normalize menstruation, whereas its flower has been used as anthelmintic (มหิดล, มหาวิทยาลัย, คณะเภสัชศาสตร์, 1990). Firewood of plaunoi has been used for postpartum (เปรมจิต นาคประสิทธิ์, บรรณาธิการ, 2526). In addition, plaunoi and plau-yai (*C. oblongifolius* Roxb.) have been used joinly in many Thai drugs as stomachic, anthelmintic, emmenagogue, digestant, transquillizer, carminative. They also have been used for treatment of lymph, pruritic, leprosy, tumor and yaws (ประเสริฐ พรหมมณีและ คณะ , 2531; นันทวัน บุณยะประภัศร, 2532).

2.2.2 Therapeutic Uses of Plaunoi

The leaves of *C. sublyratus* have been used as raw material for extracting plaunotol, the antipeptic ulcer substance. Plaunotol has been registered with the World Health Organization (WHO) under the code CS-684. Its tradename is Kelnac® which has been manufactured by Sankyo Co., Ltd. (Ogiso *et al.*, 1985; Department of Medical Information, Sankyo Co.,Ltd, 1993.). Kelnac® has been reported to enhance the mucosal protective factor by increasing gastric mucosal blood flow, promoting mucous and protaglandin production in the gastric mucosa and increasing gastric mucosal resistance. Furthermore, it has been found to exert a profound therapeutic effect in gastric ulcer (Department of Medical Information, Sankyo Co., Ltd, 1993).

2.3 Chemical Constituents of *C. sublyratus*

In 1978, Ogiso and coworkers have isolated plaunotol from the stem of *C. sublyratus* Kurz. and identified as an antipeptic ulcer substance (Ogiso *et al.*, 1978). This made it interesting to continue the research on isolation of the chemical constituents from *C. sublyratus*. Until now several diterpene compounds have been isolated and identified. They can be classified into 4 types as follows:

1. Acyclic Diterpenes
2. Labdane Diterpenes
3. Clerodane Diterpenes
4. Kaurane Diterpenes

The compounds in these four groups and their chemical substances are shown in Table 2

Table 2 Chemical constituents found in *C. sublyratus*

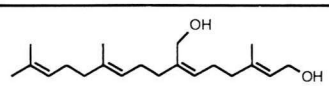
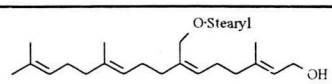
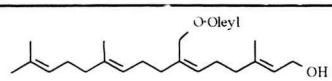
Chemical Group	Chemical substance	Chemical structure	Reference
Acyclic Diterpene	Plaunotol (18-hydroxy geranylgeraniol)		Ogiso *et al.*,1978
	Geranylgeraniol Ester A		Kitazawa *et al.*, 1982
	Geranylgeraniol Ester B		Kitazawa *et al.*, 1982

Table 2 (continued)

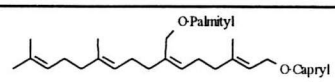
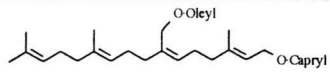
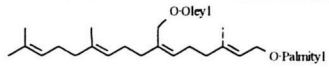
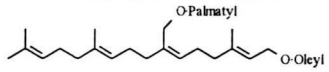
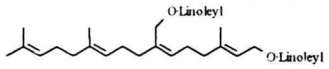
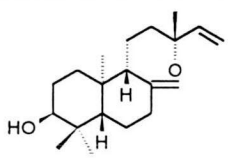
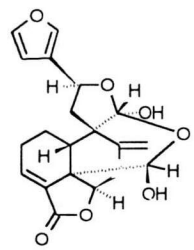
Chemical Group	Chemical substance	Chemical structure	Reference
Acyclic Diterpene	Geranylgeraniol Ester C		Kitazawa *et al.*, 1982
	Geranylgeraniol Ester D		Kitazawa *et al.*, 1982
	Geranylgeraniol Ester E		Kitazawa *et al.*, 1982
	Geranylgeraniol Ester F		Kitazawa *et al.*, 1982
	Geranylgeraniol Ester G		Kitazawa *et al.*, 1982
Labdane Diterpene	*ent*-3α-hydroxy-13-epimanool		Kitazawa and Ogiso ,1981
Clerodane Diterpene	Plaunol A		Kitazawa *et al.*, 1979; Kitazawa *et al.*, 1980

Table 2 (continued)

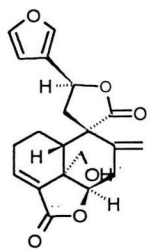
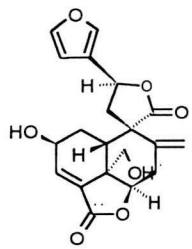
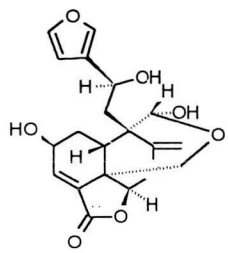
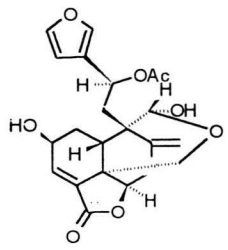
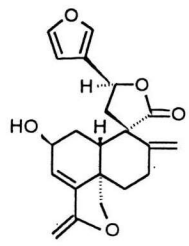
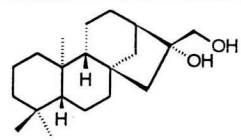
Chemical Group	Chemical substance	Chemical structure	Reference
Clerodane Diterpene	Plaunol B		Kitazawa *et al.*, 1979; Kitazawa *et al.*, 1980
	Plaunol C		Kitazawa *et al.*, 1980
	Plaunol D		Kitazawa *et al.*, 1980
	Plaunol E		Kitazawa *et al.*, 1980

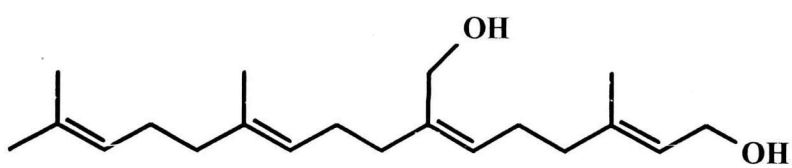
Table 2 (continued)

Chemical Group	Chemical substance	Chemical structure	Reference
Clerodane Diterpene	Plaunolide		Takahashi *et al.*,1983
Kaurane Diterpene	*ent*-16β,17-dihydroxy kaurane		Kitazawa and Ogiso ,1981

3. Plaunotol

3.1 Structure and Chemical Properties

Plaunotol is an acyclic diterpene alcohol occurring in the leaves of *C. sublyratus*. Its chemical name is (Z,E,E)-2-(4,8-dimethyl-3,7-nonadienyl)-6-methyl-2,6-octadiene-1,8-diol (Budavari,1989) or (E,Z,E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol or 18-hydroxygeranylgeraniol (Ogiso *et al.*, 1978). It has a formular of $C_{20}H_{34}O_2$ and molecular of 306.256 (Ogiso *et al.*,1978). The structure of plaunotol is shown as follow:



Plaunotol occurs as light yellow oil, having aromatic odor and a bitter taste. It is soluble in methanol, acetone, ethylacetate, dioxane, ether, chloroform, benzene, toluene or vegetable oil, but it is practically insoluble in water (Budavari, 1989; Department of medicinal Information ,Sankyo Co., Ltd., 1993).

Plaunotol shows its infrared spectrum with absorption bands at 3300 (O-H strech), 1665 (C=C strech), 1440 (C-H bend), 1380 (C-H bend) and 1000 (C-O strech) cm^{-1} (Ogiso *et al.*,1978; Sato *et al.*, 1988; Inou, *et al.*, 1990). Its nuclear magnetic resonance (^{1}H-NMR) spectrum shows signals due to four vinyl methyl groups at δ1.58 (6H, s), and δ1.66 (6H, s), six allyl methylene groups at δ1.9-2.3 (12H, m), two hydroxy groups at δ3.94 (2H, s) and δ3.97 (2H, d), and four olefinic protons at δ5.0-5.3 (4H, m) (Ogiso *et al.*, 1978; Ogiso, *et al.*, 1985). Plaunotol has been reported to have its molecular ion at *m/e* 306.255 (M^+, Calcd. 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 ; Ogiso *et al.*, 1985).

3.2 Extraction, Isolation and Purification of Plaunotol from *C. sublyratus* Leaves.

Extraction and isolation of plaunotol from *C. sublyratus* stems (Ogiso *et al.*, 1978; Ogiso *et al.*, 1985) and leaves (Sunuta Cajesanun, 1991; Nilubol., 1992) for the preparation of antipeptic ulcer drug have been reported. The isolation of plaunotol from the stem is described below (Figure 4).

The crude drug is firstly extracted with acetone under reflux. After evaporation of the acetone, the residue is extracted with 80% aqueous methanol and washed with n-hexane. The concentrated methanol layer is then dissolved in benzene. After washing with aqueous sodium hydrogen carbonate solution, the benzene fraction

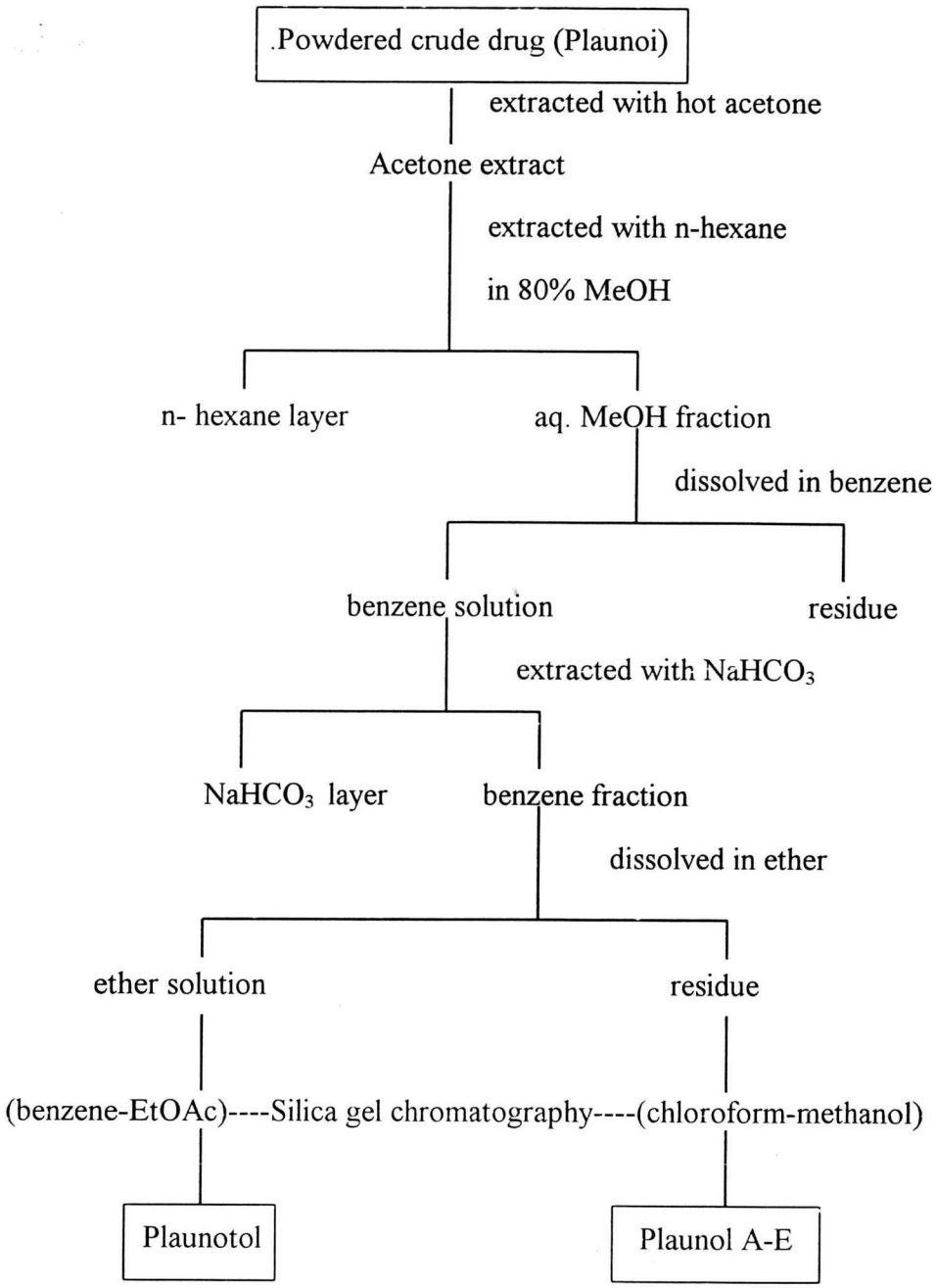


Figure 4 Extraction and isolation of plaunotol from *C. sublyratus* stems (Ogiso *et al.*, 1985).

is evaporated and extracted with ether. From this step ether soluble fraction and its residue are obtained. Plaunotol is isolated from the ether soluble fraction by column chromatography on silica gel using the eluents of benzene and ethylacetate. Plaunol A-E are isolated from the residue by column chromatography on silica gel using the eluent of chloroform and methanol (Ogiso *et al.*,1978; Ogiso *et al.*,1985).

For the leaves, the extraction of plaunotol from the dried ground leaves is performed with 95% ethanol (Sununta Cajesanun, 1991; Nilubol, 1992). The concentrated ethanol is mixed with deionized water and extracted with chloroform. The chloroform fraction is mixed with carbon powder in order to adsorb chlorophyll and other impurities. After filtration and evaporation, the dried substance is dissolved in 80% ethanol and washed with n-hexane. The concentrated hexane layer is refluxed with sodium hydroxide and extracted with hexane. Plaunotol in hexane solution is mixed with deionized water, isolated by filtration through Fuller Earth and evaporated to dryness (Sununta Cajesanun,1991; Nilubol, 1992). This process yields 15-17g of plaunotol from 6 kg of dried ground leaves of *C. sublyratus* . The obtained plaunotol is approximately 80-90% pure (Sununta Cajesanun, 1991). For drug manufacturing, further purification of the plaunotol fraction has been performed by using column chromatography on silica gel G60 and G40 with n-hexane, n-hexane:ethylacetate and ethylacetate as eluent. Plaunotol obtained after this process is about 90% pure (Sununta Cajesanun, 1991).

3.3 Detection and Determination of Plaunotol

Plaunotol from *C. sublyratus* has been identified by thin layer chromatographic method (Ogiso *et al.*,1981; Apacha Vongcharoensathit, 1994) and

determined for its content by gas chromatography (Ogiso *et al.*, 1981; Morimoto and Murai,1989; Sununta Cajesanun,1991) and by TLC Densitometry (Apacha Vongcharoensathit, 1994).

Thin-layer chromatography (TLC) has been reported to performed on silica gei 60 F254 plate using a number of systems as developing solvent. These include benzene and ethyl acetate (1:1) (Ogiso *et al.*,1981), 20% ether in chloroform (Sununta Cajesanun, 1991) and chloroform: n-Propanol (24:1), (24:0.5), (24:0.5) (Apacha Vongcharoensathit, 1994). Vanillin-sulphuric acid solution in ethanol (Ogiso *et al.*, 1981) and iodine vapour (Apacha Vongcharoensathit, 1994) were used for detection of the spots.

Gas-liquid chromatography has been performed to determine the plaunotol content. The chromatographic column has been reported to be a glass column packed with 2% OV-225 on Chromosorb G (Ogiso *et al.*, 1981) or 2% silicone OV-17 uniport HP (Sununta Cajesanun, 1991). Carrier gas is N_2 and detecter is FID (Ogiso *et al.*, 1981; Sununta Cajesanun, 1991).

TLC densitometric method has also been used for quantitative analysis of plaunotol (Apacha Vongcharoensathit, 1994). This technique has been developed carefully to maximize separation of plaunotol from other constituents in plaunoi crude extract by using silica gel 60F 254 plate with triple development of developing system, chloroform:n-propanol (24:1), (24:0.5), (24:0.5). The TLC chromatogram is produced by TLC densitometer using the wavelength 220 nm.

3.4 Plaunotol Content in *C. sublyratus* Leaves

The quantitative analysis of plaunotol in *C.sublyratus* leaves in Thailand has been previously reported by Apacha Vongcharoensathit in 1994. These plants' leaves from various sources in Thailand contained highly variable plaunotol contents, ranging from 0.139 to 0.786%(w/w). The majority of the samples have plaunotol in the range 0.3-0.4%(w/w). In the same study, it has been reported that there is no clear correlation between the plaunotol content and geographic conditions. In addition, the leaves from the young plants and mature plants have been found to have no significant differences in their plaunotol content. It has been therefore concluded that the age of these plants have not had much effect on the plaunotol in the leaves (Apacha Vongcharoensathit, 1994).

3.5 Synthesis of plaunotol

The total synthesis of plaunotol bearing an E,Z,E configuration has been successfully achieved by of the method developed by Corey and Yamamoto (1970) (Figure 5). This synthetic route involved a stereospecific sequence for trisubstituted olefin having allylic alcohol via β-oxido phosphonium ylide. Reaction of phosphonium iodide (**1**) (prepared by Coates' procedure) with aldehyde (**2**) (obtained by ozonolysis of geranyl-2-tetrahydropyranyl ether) in the presence of n-butyllithium in tetrahydrofuran gives a Wittig betaine (**3**). Subsequent reactions of the betain (**3**) with sec-butyllithium and dried paraformaldehyde followed by treatment of the resulting tetrahydropyranyl ether (**4**) with acid give the desired compound bearing (E,Z,E)-configuration of plaunotol (Ogiso *et al.*,1978; Ogiso *et al.*,1985).

In 1988, Sato and coworkers have reported the total synthesis of plaunotol from geraniol derivative in short steps with high stereoselectivity (Figure 6) (Sato *et al.*, 1988). The synthesis of plaunotol involves direct Wittig reaction employing α-

alkoxy ketones (**5a, 5b**) and a phosphorus ylide (**6**). The requisite α-alkoxyketone (**5**) having a geranylacetone skeleton can be prepared either by three-carbon elongation from geranyl sulfide or by regioseletive oxidation of geranylacetone as shown in Figure 6A and B. α-Benzyloxyketone (**5a**) is prepared by the reaction of glycidyl benzyl ether (**7**) with α-lithiogeranyl phenyl sulfide followed by oxidation (Figure 6A). An alternative ketone, α-tetrahydropyranyloxy ketone (**5b**) can be prepared from geranylacetone (**9**) in 3 steps (Figure 6B). Reaction of geranylacetone (**9**) with lithiumdiisopropylamide are quenched with trimethylsilyl chloride to form a terminal enolates (**10a**) and inner enolate (**10b**). The mixture of two enolates (**10a,10b**) is then subjected to oxidation with m-chloroperbenzoic acid and acid hydrolysis to form α-hydroxy ketone (**11**). α-tetrahydropyranyloxyketone (**5b**) is obtained by protection of ketol (Sato *et al.*,1988).

For phosphorus ylide (**6**), phosphonium iodide (**17**) is a precursor which is prepared from gerany benzyl ether (**12**) as shown in Figure 6C. Regioselective epoxidation of the terminal double bound of (**12**) give aldehyde (**14**) and treated with sodium borohydride to furnish benzyloxy alcohol (**15**). Phosphonium iodide (**17**) is obtained by the conventional method from **15** via the corresponding tosylate and iodine (**16**) and converted into phosphorus ylide (**6**) by treatment with butyllithium in THF-HMPA (Sato *et al.*,1988).

The direct Wittig olefination of α-alkoxy ketones (**5a,5b**) with ylide (**6**) affords the product **18a,18b** which is subjected Na/NH_3 reduction to give the plaunotol (Sato *et al.*, 1988).

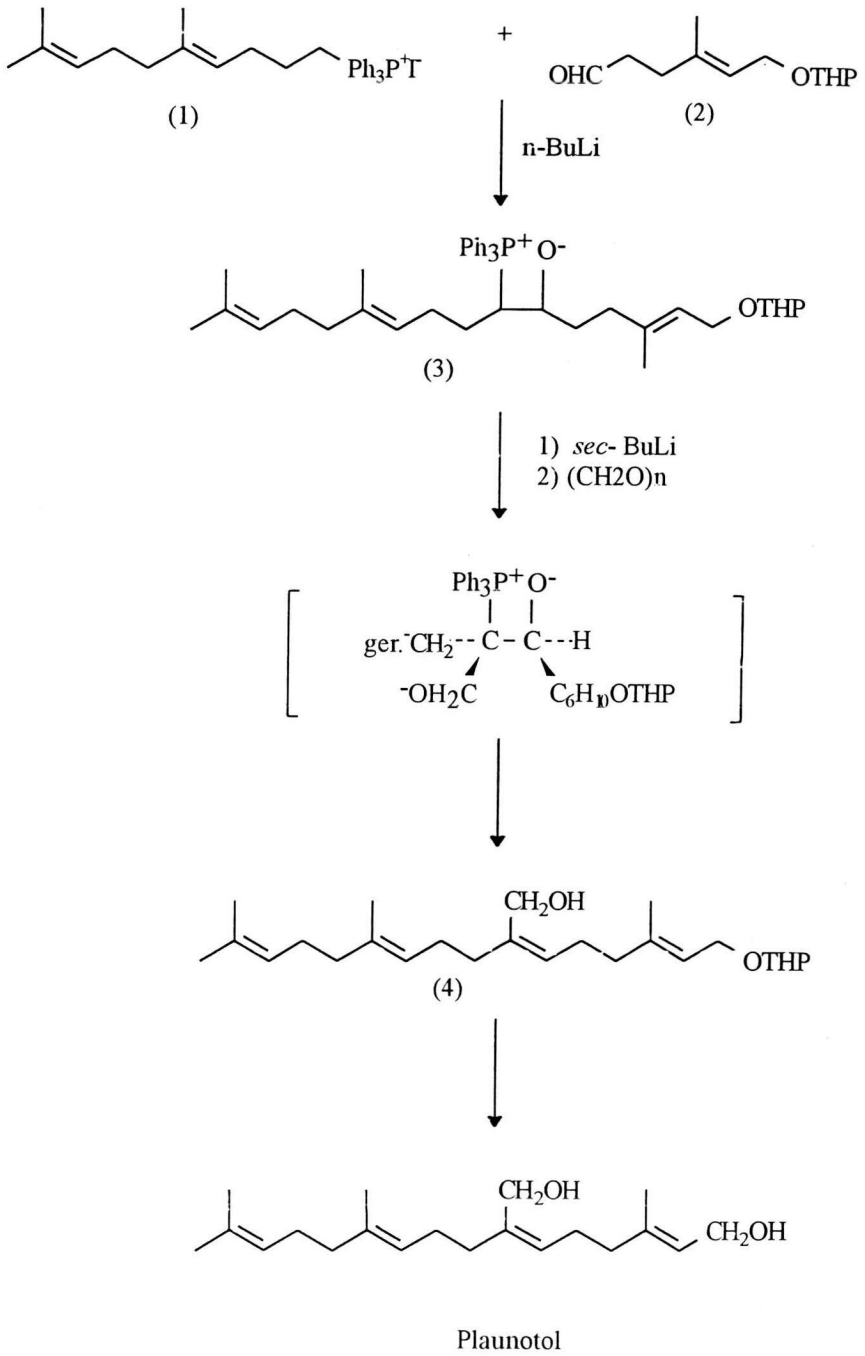


Figure 5 Synthesis of palunotol by application of the method developed by Corey and Yamamoto (1970)

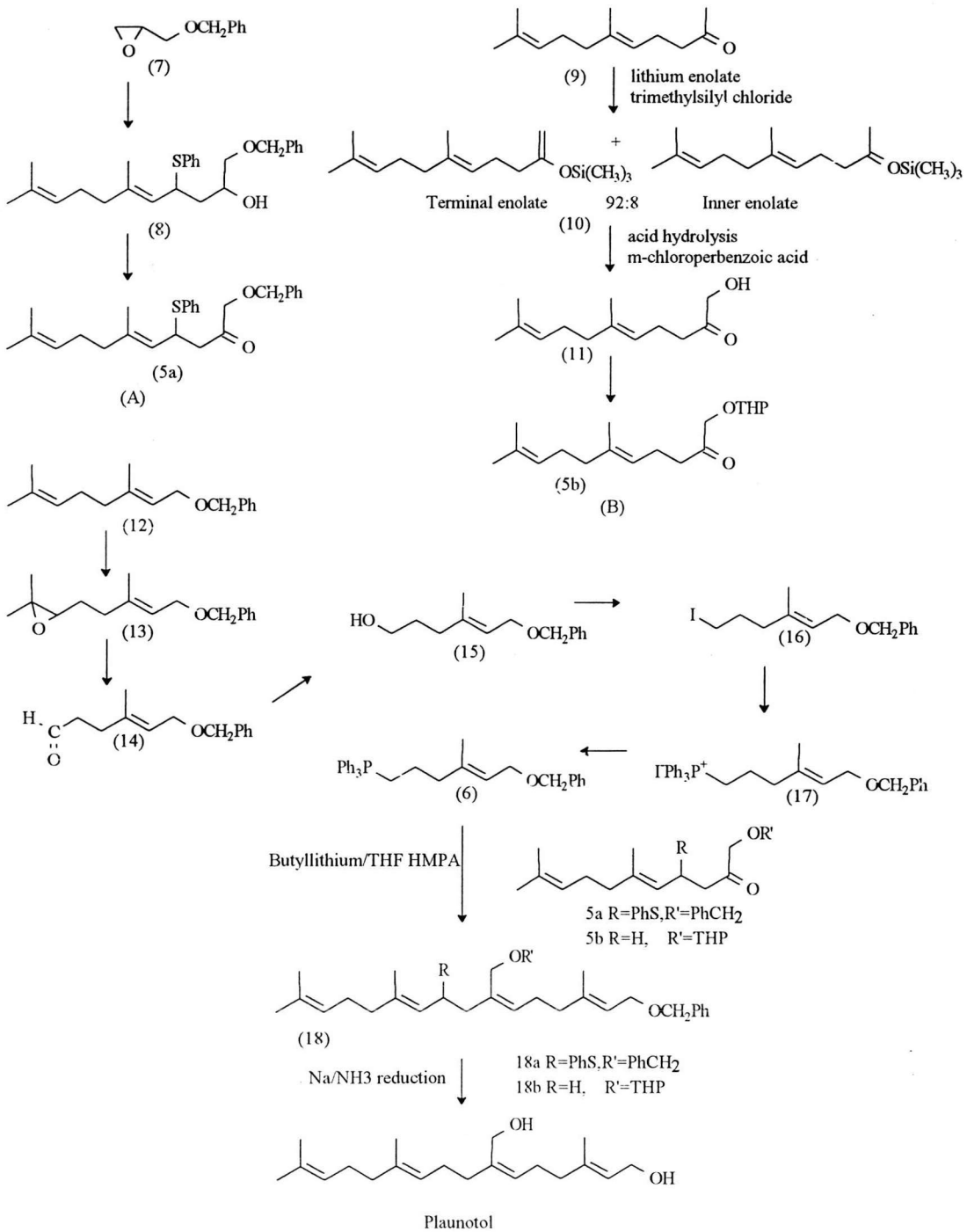


Figure 6 Synthesis of plaunotol by the stereoselective direct Wittig olefination to α-alkoxy ketones having geranylacetone skeleton (Sato et al., 1988)

In 1994, Takayanaki has clarified the conditions which allows the Horner-Emmons reaction of phosphonitrile to proceed Z-selectively. The reaction has been applied to a stereoselective synthesis of plaunotol as shown in Figure 7.

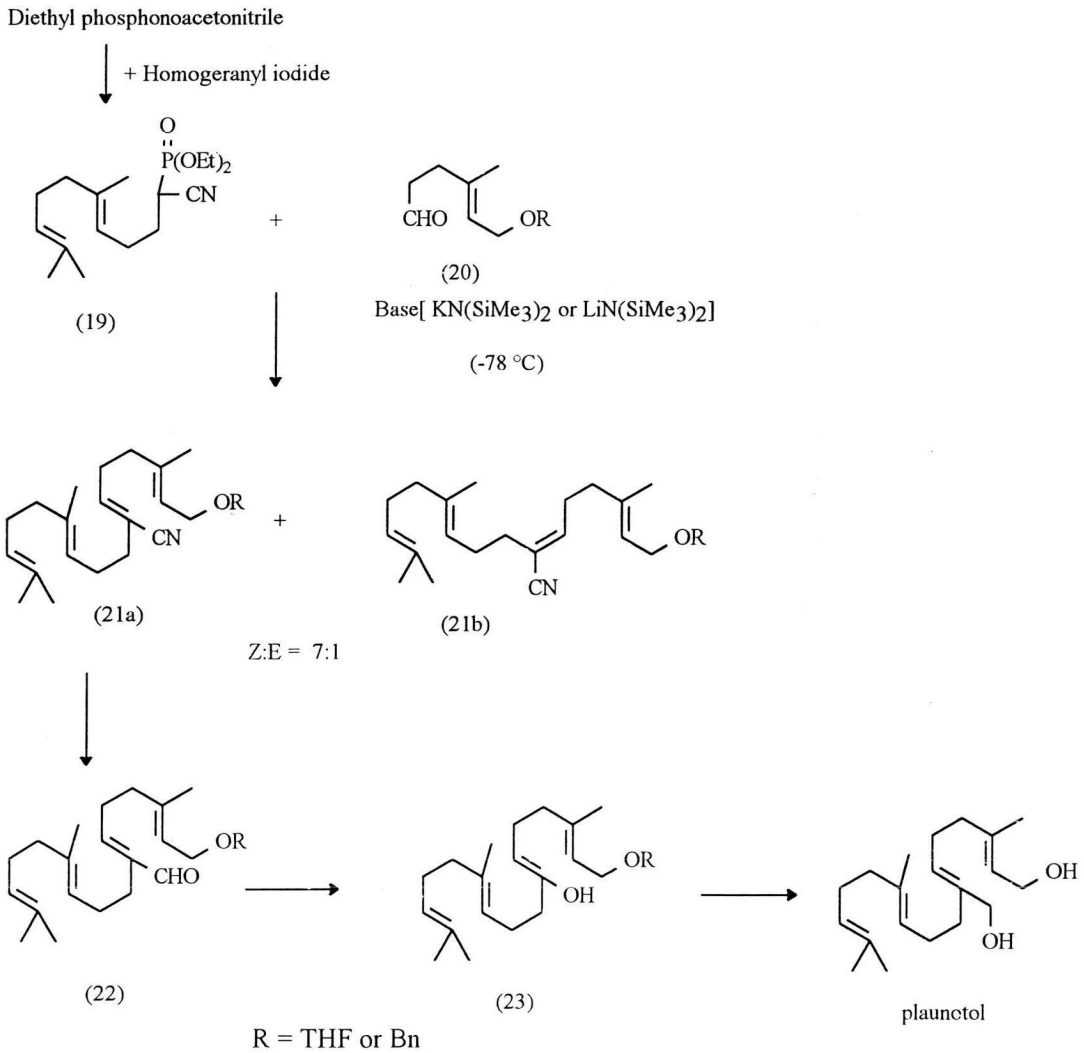


Figure 7 Synthesis of plaunotol by application of Horner-Emmons Reaction

The Horner-Emmons reaction of phosphonitrile (**19**), which has prepared by alkylation of diethyl phosphonoacetonitrile with homogeranyl iodide (Corel and Jautelat, 1968), with aldehyde (**20**) has proceeded smoothly at low temperature (-78°C) and give desired conjugated nitrile **21a** with high selectively. The isomer is easy to separate by SiO_2 column chromatography.

The synthesis of plaunotol from conjugate nitrile (**21a**) has involves the reduction of nitrile (**21a**) to alcohol (**23**) and deprotection. Thus, after DIBAL reduction of nitrile group in **21a**. to **22** and its isomer, which were separated by flash SiO_2 column chromatography. Finally, $NaBH_4$ reduction of **22** followed by removal of the protecting group afford plaunotol (Takayanaki, 1994).

3.6 Biological Activities of Plaunotol

Plaunotol, the active constituent of a commercial drug named Kelnac®, is a mucosal protective factor-enhancing antiulcer agent (Department of medicinal Information, Sankyo Co., Ltd., 1993). The effects of plaunotol on acute gastic or duodenal ulcers and on chronic gastric ulcers in animals have also been reported (For review, see Ogiso *et al.*, 1985).

For the acute ulcers, plaunotol has been found to possess inhibitory effects on the ulceration induced by reserpine and stress in mice at an oral dose 30 and 100 mg/kg/day, respectively (Ogiso *et al.*, 1985; Anon, 1987), and also on the ulceration induced by stress, aspirin , indomethacin, pyrolic ligature and cysteamine in rats at an oral dose 30, 100, 300, 300 and 600 mg/kg/day, respectively (Ogiso *et al.*, 1985), as well as aspirin in dogs at an oral dose 300 mg/kg/day (Department of medicinal Information, Sankyo Co., Ltd., 1993; Ogiso *et al.*, 1985). Therefore, plaunotol has broader anti-ulcer spectrum than other anti-ulcer drugs such as cetraxate, gefarnate

and sucralfate which have been regarded as potentiators of the mucosal protective factor (Ogiso *et al.*, 1985).

For chronic ulcers, plaunotol has been shown in rats to reduce ulcer size of the mucosa and increase mucosal regeneration of acetic acid-induced gastric ulcer at oral dose of 30 to 300 mg/kg/day (Ogiso *et al.*, 1985). In the rats with clamping-induced ulcer, plaunotol has been shown to reduce the defect of mucosa and increase the healing index, the mucosal regeneration index and the degree of collagen fiber proliferation at the base of ulcer at oral dose of 30 and 100 mg/kg/day (Ogiso *et al.*, 1985). In dogs, plaunotol has been found to reduce the acetic acid-induced gastric ulcer size at the oral doses of 3 to 30 mg/kg/day (Ogiso *et al.*, 1985).

For the mode of action of plaunotol , it has been proposed that plaunotol inhibits gastric secretion, increases the blood flow of gastric mucosa, facillitates biosynthesis of mucosal substances and prostaglandins in the mucosa. Furthermore, plaunotol could protect the breakdown of mucous barrier (Ogiso *et al.*, 1985; Department of medicinal Information ,Sankyo Co.,Ltd., 1993).

Very recently plaunotol has been reported to inhibit hypergastrinemia induced by long-term omeprazole administration in humans (Kaneko, *et al* , 1995).

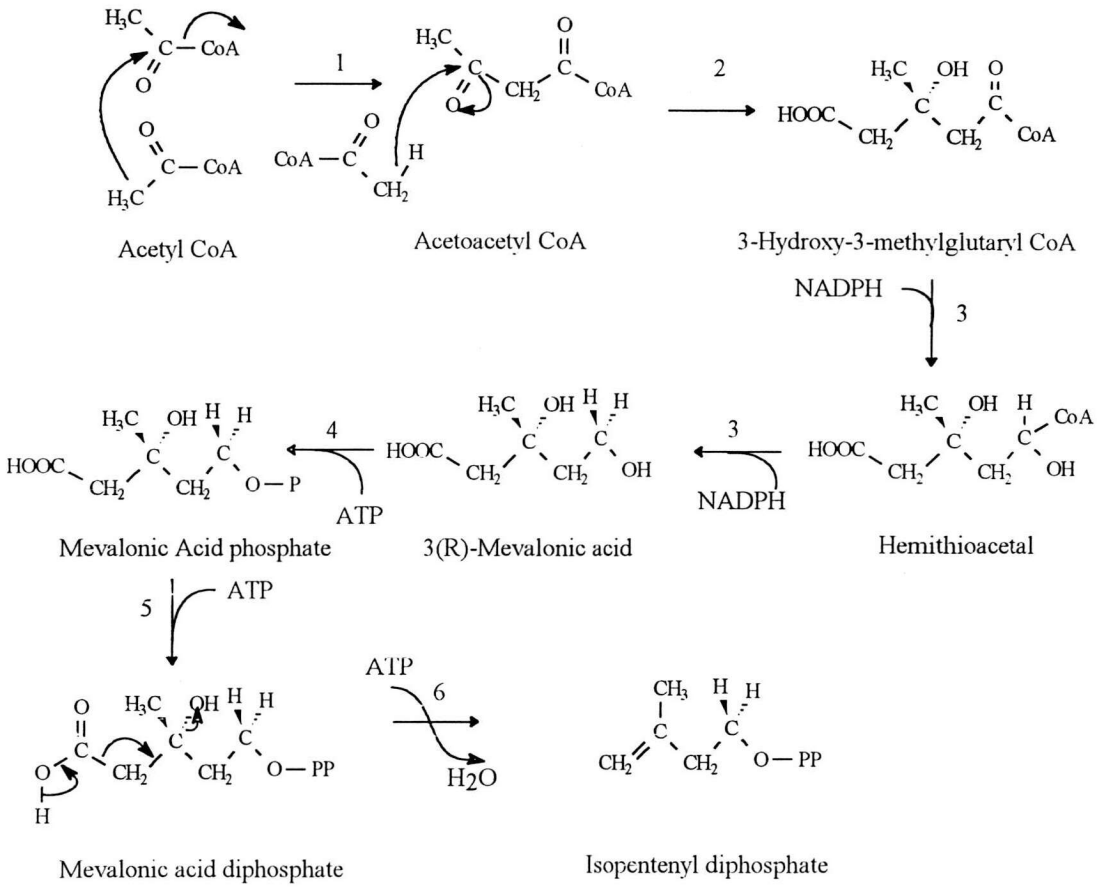
3.7 Plaunotol Production in Cell Cultures

Plant tissue and cell culture tecniques have been used to study the production of plaunotol in *in vitro* cultures of *C. sublyratus*. There has been only one report on cell cultures that showed plaunotol accumulation (Morimoto and Murai, 1989). Geranylgeraniol has also been founded to accumulate in the suspension cultures of *C. sublyratus* (Kitaoka, Nagashima and Kamimura, 1989).

4 The Biosynthetic Pathway of Diterpenes

The biosynthesis of diterpenes starts from the formation of isopentenyl diphosphate which is derived from acetyl CoA (Goodwin and Mercer, 1983; Luckner, 1990). An important intermediate of this biosynthetic pathway is mevalonic acid (Figure 8) which is converted to isopentenyldiphosphate, the active molecule for the formation of all terpenes as shown in Figure 9. Isopentenyl diphosphate is first isomerized by an isomerase enzyme to form 3,3-dimethylallyl diphosphate which is then condensed with three units of isopentenyl diphosphate to form geranylgeranyl diphosphate. This geranylgeranyl diphosphate acts as a starter molecule for the biosynthesis of various diterpenes including acyclic diterpenes such as geranylgeraniol ,phytol or phytone (Goodwin and Mercer, 1983; Luckner , 1990).

For the biosynthesis of plaunotol in *C. sublyratus* , there has been no report that involved biosynthetic pathway. However, geranylgeraniol has been found to accumulate in the suspension cultures of *C. sublyratus* (Kitaoka, Nagashima and Kaminura, 1989). As a result, it is likely that plaunotol (18-hydroxygeranylgeraniol) is simply formed by a one step 18-hydroxylation of geranylgeraniol .



1.= Acetyl CoA acetyltransferase
2.= Hydroxy methylglutaryl CoA synthase
3.= Hydroxy methyl glutaryl CoA reductase
4.= Mevalonate kinanse
5.= Phosphomevalonate kinase
6.= Diphosphomevalonate decarboxylase

Figure 8 Biosynthesis of isopentenyl diphosphate

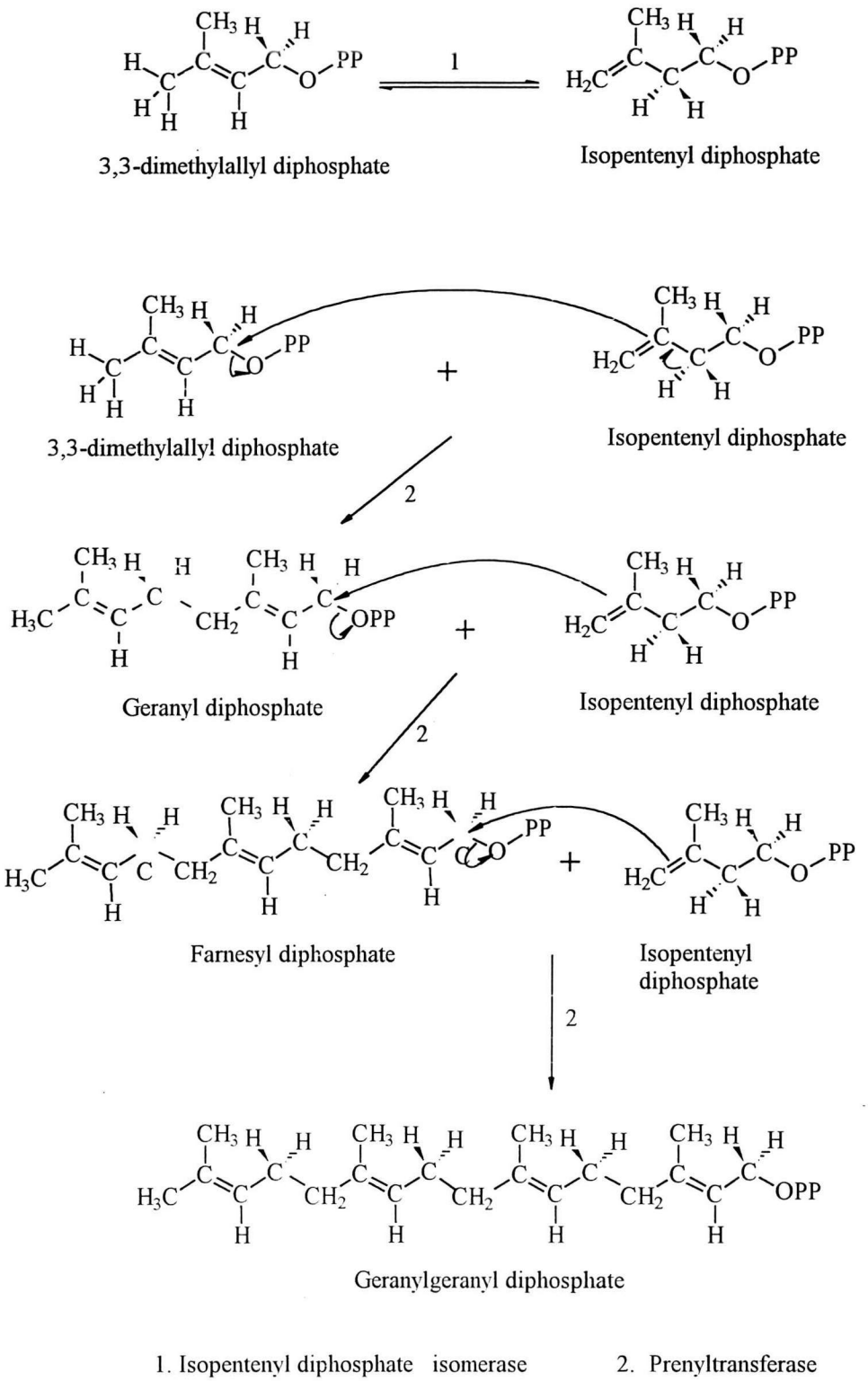


Figure 9 Biosynthesis of geranylgeranyl pyrophosphate

5 Hydroxylation in plants

The hydroxyl group is a common functionality found in all classes of natural product. Basically, there are two strategies for the introduction of a hydroxyl group into a substrate during metabolism (West,1980). One involves the addition of a preformed hydroxyl group originating in water to an electron-deficient center. There is no direct dependence of this type of reaction on molecular oxygen. The second type of reaction involves the synthesis of a hydroxyl group as a consequence of the oxygenation of a substrate with molecular oxygen or some species derived from molecular oxygen such as a peroxide.

The enzymes that catalyse the incorporation of oxygen from molecular oxygen in to organic substrates are called "oxygenase". The monooxygenases and dioxygenases are distinguished on the basis of whether only one or both of oxygen atoms of O_2 are incorporated in to the organic products. These monooxygenases are also refer to as mixed-function oxygenases (or sometimes are mixed-function oxidases) because of their simultaneous catalysis of both an oxygenation reaction and an oxidase reaction(the reduction of oxygen to water). The latter class of reactions usually occur in higher plant tissues.

The monooxygenases are found relating to cytochrome P-450 (The hemo protein). The catalytic cycle of these monooxygenase is shown in Figure 10. Step 1 indicates the binding of a substrate to oxidized cytochrome P-450. The second step involves the addition of one electron from external donor (such as NADPH or NADH)

to reduce the cytochrome to the ferrous state. Oxygen is bounded to the reduced cytochrome-substrate complex in step 3 to form a ternary complex. Step 4 is most likely the addition to the ternary complex of the second electron required in the overall process. Step 5 involves the di-protonation of one oxygen of the reduced complex to form water. The final step requires the cleavage of R-H bond in the substrate coupled with the insertion of and oxygen atom to form the hydroxylated product and regenerate oxidized cytochrome P-450 (West, 1980).

In the studies of hydroxylase both cell cultures and higher plants have been used as enzyme sources. For example, *Catharanthus roseus* G.Don seedlings have been used to study the hydroxylation of geraniol and nerol and the result revealed that geraniol-10-hydroxylase, in the form of monooxygenase, could catalyse the conversion of these monoterpenes into their corresponding 10-hydroxy derivatives (Meehan and Coscia, 1973; Madyastha, Meeehan and Coscia 1976). Etiolated mung bean seedlings (*Vigna radiata*) have been used to study the hydroxylation of cinnamic acid to *p*-coumaric acid by using cinnamic acid-4-hydroxylase presented in its seedlings (Mizutani, Ohta and Sato, 1993). In the biosynthesis of monoterpene compouds, the substrate, (-)-limonene has been incubated with different microsomal preparations from the epidermal oil gland of *Mentha piperita* ,*Mentha spicata* and *Perilla frutescens* . The result of hydroxylation activity of enzyme revealed that three different products were produced (Karp *et al.*, 1990). A microsomal preparation isolated from suspension cells of *Nicotiana tabacum* was able to catalyse the hydroxylation of terpineols and their acetates at the allylic positions (Tang and Suga, 1994).

All of those microsomal preparations described above have some similar characteristics. The hydroxylase activities of those enzymes depend strictly on the presence of NADPH as the reducing cofactor, molecular oxygen as the oxygen donor and relate to Cytochrome P-450.

In higher plants, cytochrome P-450 plays a crucial role in biosynthesis of phytohormone, sterols, flavonoids, phytoalexin and precursor for structural component (West, 1980; Donalson and Luster, 1991). Despite great effort to investigate these important functions, only a limited number of plant P-450s have so far been isolated because of difficulties in purification arising from low abundance of this class of cytochrome in plant cell.

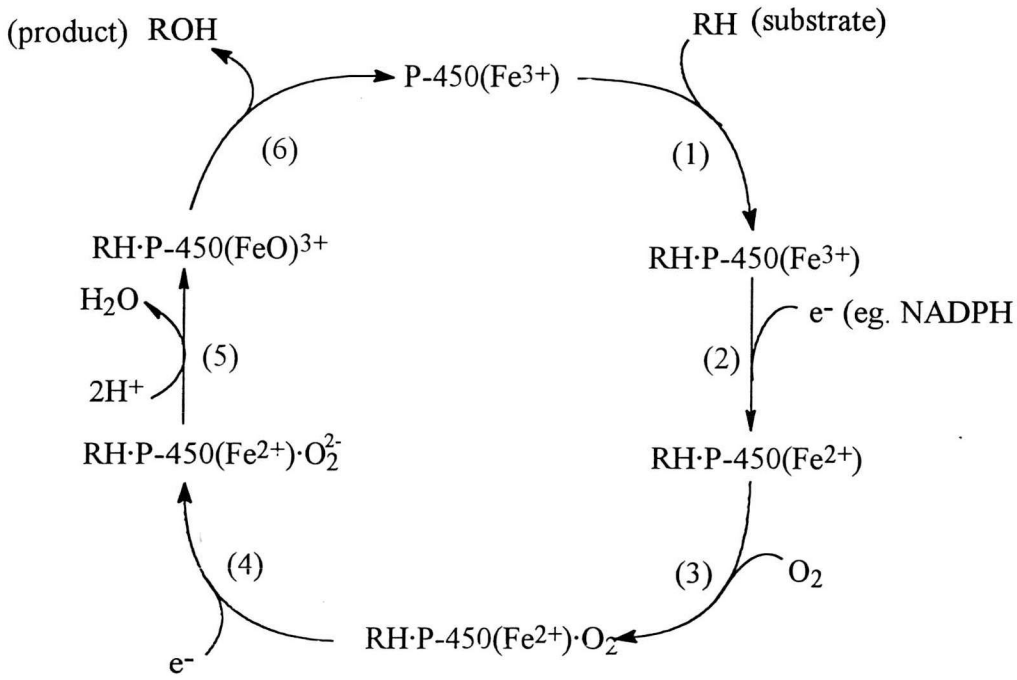


Figure 10 Representation of the catalytic cycle for Cytochrome P-450

(West, 1980)

P-450(Fe^{3+}) = Cytochrome P- 450 in Oxidized States

P-450(Fe^{2+}) = Cytochrome P- 450 in Reduced States