CHAPTER II

HISTORICAL

1. Botanical Aspects of *Croton sublyratus* Kurz

Croton sublyratus Kurz (Fig. 1) or Plau-noi (Thai-name) is in the family of Euphorbiaceae (ภาควิชาเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, 2530; ลีนา ผู้พัฒนพงศ์ และธวัชชัย วงศ์ประเสริฐ, 2530; Ponglux *et al.*, 1987). 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 narrowed base, very shortly petioled obovate to almost lyrate oblong obtuse or acuminate repand-serrulate beneath glabrous or with scabrous nerves and recemes stellate-tomentose. Young leaves are dark brown and inflorescense. Petiole is stout, 6-12 mm long. The flowers are small, perfect and receme. 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 fruits 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 และธวัชชัย วงศ์ประเสริฐ, 2530; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; Hooker, 1973).

C. sublyratus grows extensively in tropical areas, especially those near by the Andaman sea such as Indonesia, Malaysia, Thailand, Burma

Figure 1 *Croton sublyratus* Kurz (Euphorbiaceae).

and the south of China. The survey on various plants related to *C. sublyratus* was relatively extensive in Thailand (ณรงค์ เพ็งปรีชา, 2530). There are several species belonging to the genus *Croton* in Thailand, including Plau-noi (*C. sublyratus* or *C. joufra* or *C. kerrii*), Plau-luat (*C. hatchinsonianus*), Plau-yai (*C. oblongifolius* or *C. poilanei*), Plau-namngern or Plau-kradat (*C. cascarilloides*). These plants grow naturally in every part of Thailand. However, only *C. sublyratus* has been found to contain anti-ulcer substances (ณรงค์ เพ็งปรีชา, 2530; Ogiso *et al.*, 1981; Ogiso *et al.*, 1985). Thai *C. sublyratus* or Plau-noi is found to be native to the Thai provinces of Prachin Buri, Prachuap Khiri Khan and the border near Burma of Kanchanaburi (สำนักงานคณะกรรมการวิจัยแห่งชาติ, 2533; ณรงค์ เพ็งปรีชา, 2530; ลีนา ผู้พัฒนพงศ์ และธวัชชัย วงศ์ประเสริฐ, 2530; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; วีณา วิรัจฉริยากูล และคณะ, 2533).

The propagation of *C. sublyratus* includes budding (to form plantlet from root), seedling, and cutting (สำนักงานคณะกรรมการวิจัยแห่งชาติ, 2533; เปรมจิต นาคประสิทธิ์, บรรณาธิการ, 2528). It has been reported that *C. sublyratus* plants from Prachuap Khiri Khan have more anti-ulcer substances than the plants from Prachin Buri and that the leaves contain higher content than the stems, branches and roots (ณรงค์ เพ็งปรีชา, 2530; พนิดา แสงทอง, 2528; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; วีณา วิรัจฉริยากูล และคณะ, 2533). As a result, this plant has been cultivated in 7000 rai in Prachuap Khiri Khan area by Sankyo Co., Ltd, a Japanese pharmaceutical firm (ณรงค์ เพ็งปรีชา, 2530; นันทวัน บุณยะประภัศร, บรรณาธิการ, 2532; วีณา วิรัจฉริยากูล และคณะ, 2533).

In the plantation, *C. sublyratus* is grown approximately 250-256 plants on a one rai area with the distance of 2.5 x 2.5 meter for each plant. 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. The Uses of *C. sublyratus*

2.1 Traditional Uses

C. sublyratus (Plau-noi) is a Thai flok medicine for anthelmintic and dermatologic agent for skin disease (ภาควิชาเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, 2530; ลัดดาวัลย์ บุญรัตนกรกิจ, 2535; Ponglux *et al.*, 1987). The plant parts of stem, bark and leaf have been used as antidiarrheal and normalize menstruation whereas its flower is used as anthelmintic (คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล, 1990). Firewood of Plau-noi is used for postpartum (เปรมจิต นาคประสิทธิ์, บรรณาธิการ, 2528). In addition, it has been reported that Plau-noi and Plau-yai (*C. oblongifolius* Roxb.) are used joinly in many Thai drugs, such as stomachic, anthelmintic, emmenagogue, digestant, transquilizer, carminative, treatment of lymph, pruritic, leprosy, tumor and yaws (ประเสริฐ พรหมมณี และคณะ, 2531; นันทวัน บุณยะประภัศร, บรรณาธิการ, 2532).

2.2 Therapeutic Uses

The leaves of *C. sublyratus* are used as material for extracting an antipeptic ulcer substance, namely Plaunotol. Plaunotol has been registered with the World Health Organization (WHO) under the code CS-684. Its trade name is Kelnac which has been manufactured in two forms of soft gelatin capsule and micro granule by Sankyo Co., Ltd. (ณรงค์ เพ็งปรีชา, 2530; นันทวัน บุณยะประภัศร, บรรณาธิการ, 2532; พนิดา แสงทอง, 2528; วีณา วิรัจฉริยากูล และคณะ, 2533).

Kelnac enhances the mucosal protective factors by the increase in gastic mucosal blood flow, promotion of mucous and prostaglandin production in the gastric mucosa, and increase in 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).

3. Chemical Constituents of *C. sublyratus*

Since 1978, when Ogiso *et al.* isolated and identified plaunotol as an antipeptic ulcer substance from the stems of *C. sublyratus* Kurz (Ogiso *et al.*, 1978), the research on isolation of the constituents from *C. sublyratus* has continued. The groups of compounds found in this plant include diterpene lactones, furanoid diterpene, diterpene alcohols and esters of diterpene alcohol. The list of these compounds and their chemical structures is shown in Table 1.

Table 1 Chemical constituents and structure found in *C.sublyratus.*

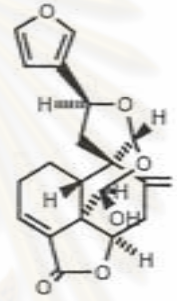
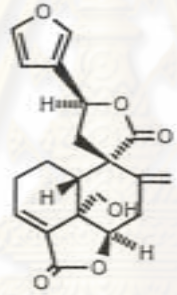
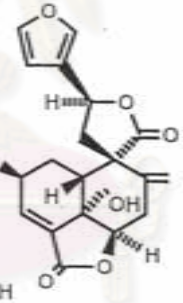
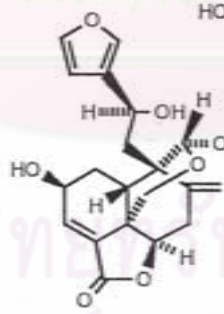
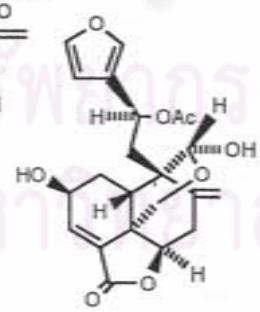
Chemical group	Chemical substance	Chemical structure	Reference
Diterpene lactones	Plaunol A		Kitazawa *et al.*,1979; Kitazawa *et al.*,1980
	Plaunol B		Kitazawa *et al.*,1979; Kitazawa *et al.*,1980
	Plaunol C		Kitazawa *et al.*,1980
	Plaunol D		Kitazawa *et al.*,1980
	Plaunol E		Kitazawa *et al.*,1980

Table 1 (continued).

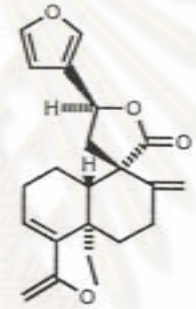
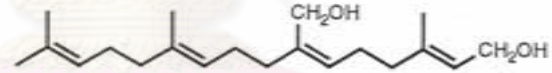
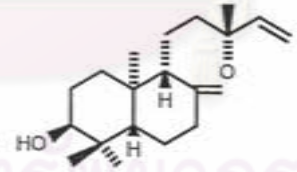
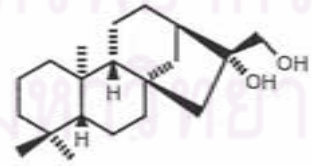
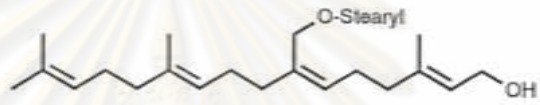
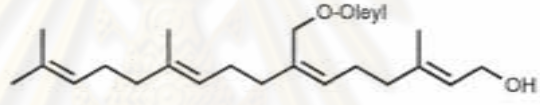
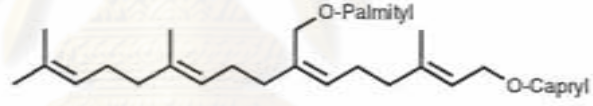
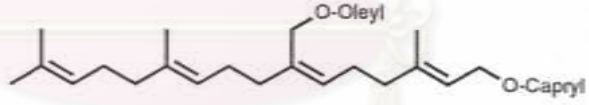
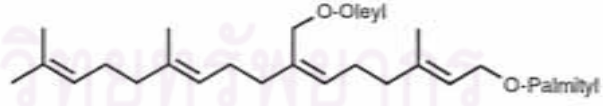
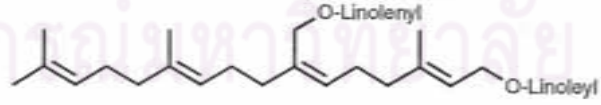
Chemical group	Chemical substance	Chemical structure	Reference
Furanoid diterpene	Plaunolide		Takahashi *et al.*, 1983
Diterpene alcohols	Plaunotol (18-hydroxygeranylgeraniol)		Ogiso *et al.*,1978
	ent-13α-hydroxy-13-epimanool		Kitazawa and Ogiso,1981
	ent-16β,17-dihydroxykaurane		Kitazawa and Ogiso,1981

Table 1 (continued).

Chemical group	Chemical substance	Chemical structure	Referenece
Esters of 18-hydroxygeranylgerniol	Steric acid		Kitazawa *et al.*, 1982
	Oleic acid		Kitazawa *et al.*,1982
	Caprylic acid-pamitic acid		Kitazawa *et al.*,1982
	Caprylic acid-oleic acid		Kitazawa *et al.*,1982
	2Palmitic-oleic acid		Kitazawa *et al.*,1982
	Linoleic acid-linolenic acid		Kitazawa *et al.*,1982

4. Plaunotol

4.1 Structure and Chemical Properties

Plaunotol is an acyclic diterpene alcohol present in the leaves of *C. sublyratus*. Its chemical name is (E, Z, E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol. 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 in Fig. 2.

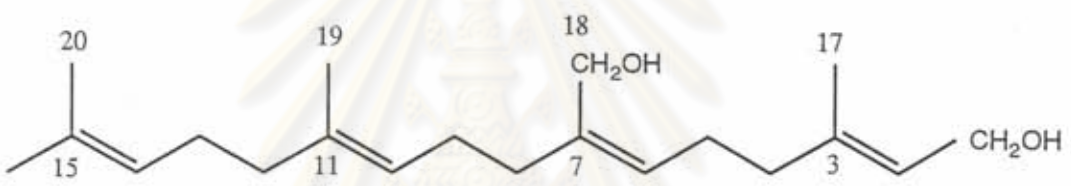


Figure 2 The chemical structure of plaunotol.

Plaunotol occurs as pale yellow to light brown viscous liquid, having a slightly characteristic oder 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 Medical 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}. Its proton magnetic resonance (PMR) spectrum of plaunotol shows signals due to for 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 olelinic protons at δ 5.0–5.3 (4H,m). For mass spectrum, plaunotol shows the 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).

4.2 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 (Nilubol, 1992; Sununta Cajesanun, 1991) for the preparation of antipeptic ulcer drug have been reported.

Form the stems, the crude drug is first extracted with acetone under reflux (Fig. 3). After evaporation of the solvent, 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 an aqueous sodium hydrogen carbonate solution, the benzene solution is evaporated and the residue is extracted with ether. Plaunotol is isolated from the ether solution fraction by a column chromatography on silica gel using benzene and ethyl acetate as eluent. The remaining ether residue is chromatographed on silica gel using chloroform and methanol giving several furanoditerpenes (Ogiso *et al.*, 1978; Ogiso *et al.*, 1985).

Form the leaves, the extraction of plaunotol from the dried ground leaves has been performed with 95% ethanol (Nilubol, 1992; Sununta Cajesanum, 1991). The concentrated ethanol is mixed with deionized water and extracted with chloroform. The chloroform fraction is then mixed with carbon powder in order to adsorb chlorophyll and other impurities. After filtration and evaporation, the dried substance is dissolved in 80% ethanol

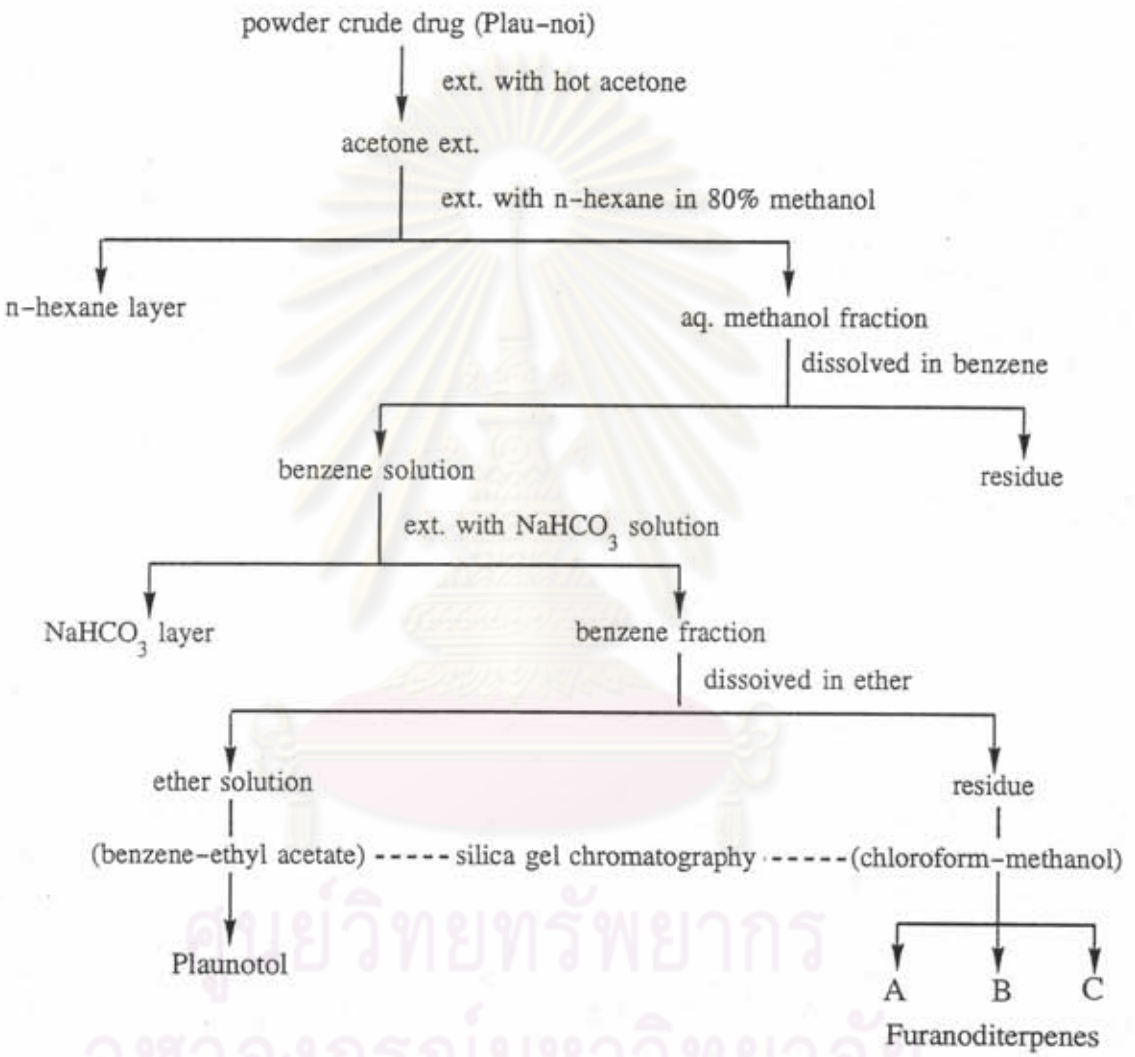


Figure 3 Extraction and isolation of plaunotol from *C. sublyratus* stems (Ogiso *et al.*, 1978).

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 (Nilubol, 1992; Sununta Cajesanun, 1991). This process has been found to be able to obtain 15-17 g of plaunotol from 6 kg of dired 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 was performed by using column chromatography on silica gel G60 and G40 with n-hexane, n-hexane : ethyl acetate and ethyl acetate as eluent. Plaunotol obtained after this process is about 90% pure (Sununta Cajesanun, 1991).

4.3 Detection and Determination of Plaunotol

Plaunotol from *C. sublyratus* has been identified by thin-layer chromatographic method (Ogiso *et al.*, 1981) and determined for its content by gas chromatography (Morimoto and Murai, 1989; Ogiso *et al.*, 1981; Sununta Cajesanun, 1991).

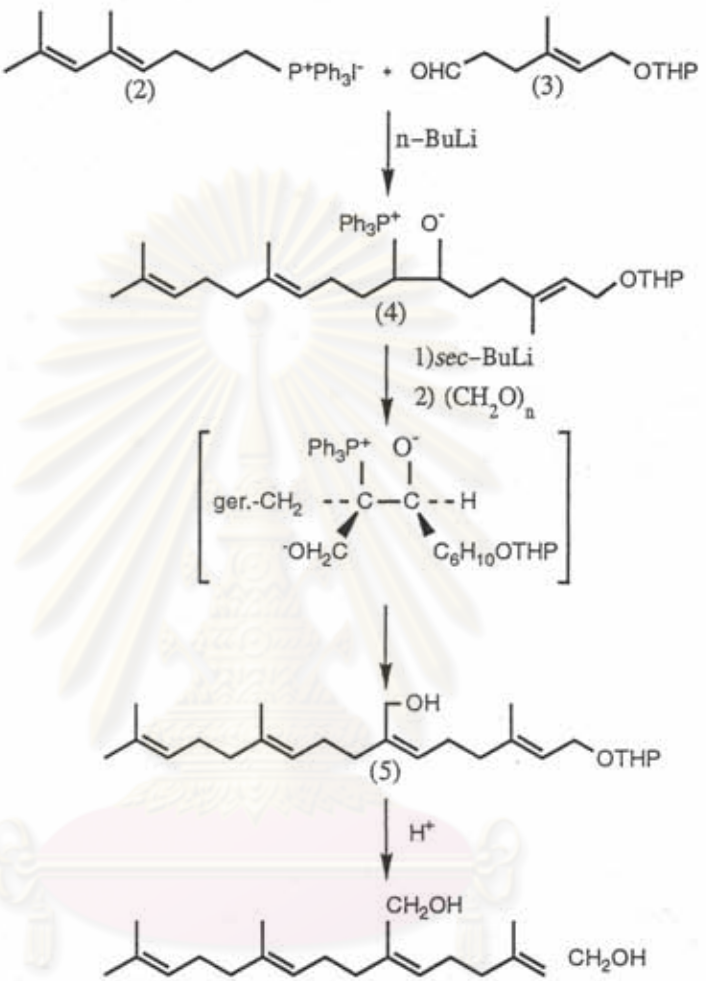
Thin-layer chromatography (TLC) was preformed on silica gel 60 F254 plate using benzene and ethyl acetate (1:1) (Ogiso *et al.*, 1981) or 20% ether in chloroform (Sununta Cajesanun, 1991) as developer.Vanilin-sulfuric acid solution in ethanol was used for detection of the spots (Ogiso *et al.*, 1981).

Gas-liquid chromatography used for determination of plaunotol was preformed by using 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 N_2 and detector FID (Ogiso *et al.*, 1981 ; Sununta Cajesanun, 1991).

4.4 Systhesis of Plaunotol

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

In 1988, Sato *et al.* have reported that total synthesis of plaunotol from geraniol derivative in short steps with high stereoselectivity (Fig. 5). The synthesis of plaunotol (1) involves direct Wittig reaction employing α-alkoxy ketones (2a, 2b) and a phosphorus ylide (3). The requisite α-alkoxy ketones (2) having a gerahylacetone skeleton can be prepared either by three-carbon elongation form geranyl sulfide or by regioselective oxidation of geranylacetone as shown in Fig. 5A and B. α-Benzyloxy ketone (2a) is prepared by the reaction of glycidyl benzyl ether (4) with α-lithiogeranyl phenyl sulfide followed by oxidation (Fig. 5A). An alternative ketone, α-tetrahydropyranyloxy ketone (2b) can be prepared from geranylacetone (6) in 3 steps (Fig. 5B). Reaction of geranylacetone (6) with lithiumdiisopropylamide are quenched with trimethylsilyl chloride to from a terminal enotate (7a) and inner enolate. The mixture of two enolates



Plaunotol (1)

Figure 4 Synthesis of plaunotol by application of the method developed by Corey and Yamamoto (1970).

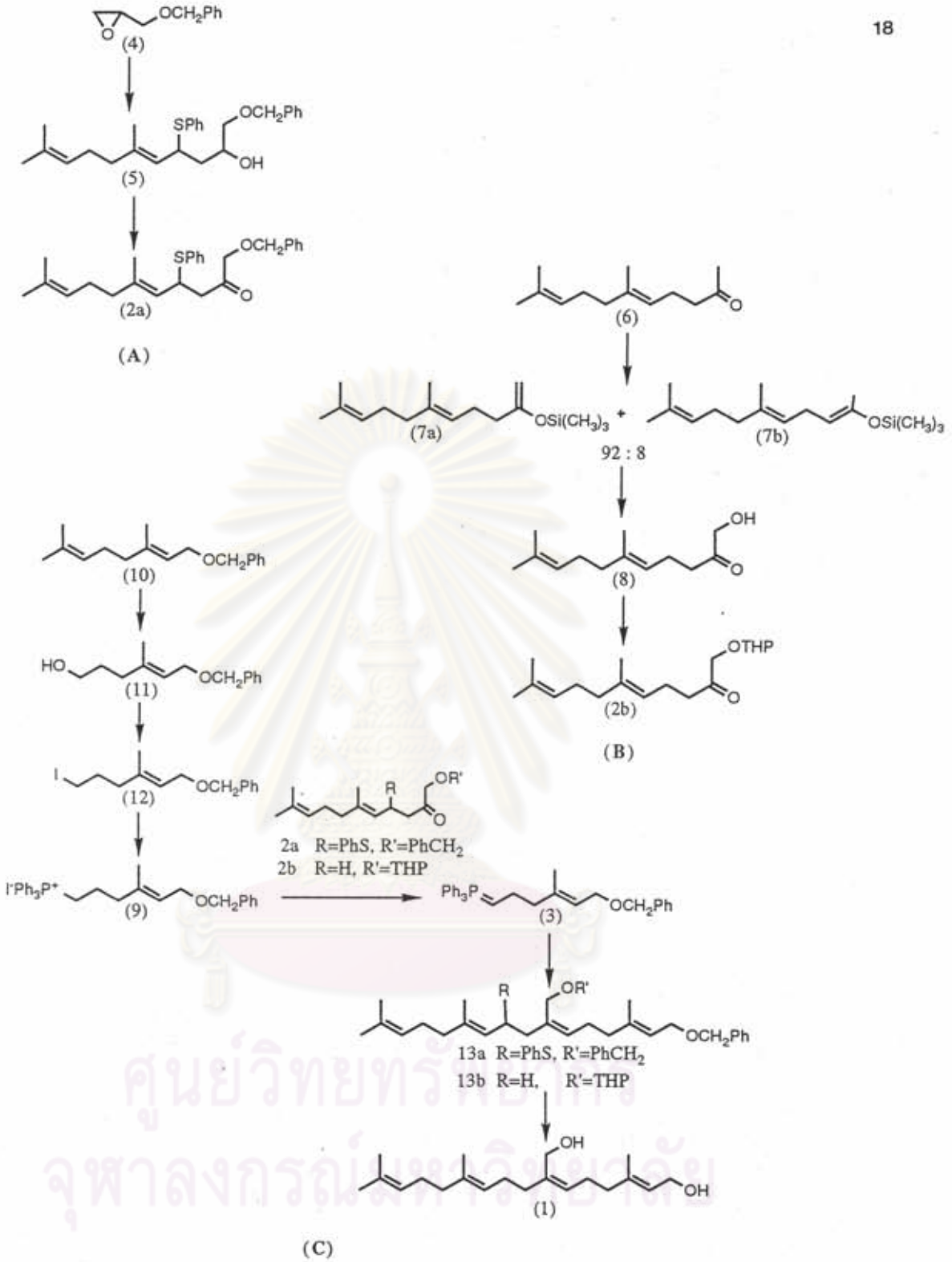


Figure 5 Synthesis of plaunotol by the application of the stereoselective direct Wittig olefination to α-alkoxy ketones having geranylacetone skeleton.

(7a, 7b) is then subjected to oxidation with m-chloroperbenzoic acid and acid hydrolysis to form α-hydroxy ketone (8). α-Tetrahydropyranyloxyketone (2b) is obtained by protection of ketol (8) (Sato *et al.*, 1988).

For phosphorus ylide (3), phosphonium iodine (9) is a precursor which is prepared from geranyl benzyl ether (10) as shown in Fig 5C. Regioselective epoxidation of the terminal double of 10 give aldehyde and treated with sodium borohydride to furnish benzyloxy alcohol (11). Phosphonium iodine (9) is obtained by the conventional method from 11 via the corresponding tosylate and iodine (12) and converted into phosphorus ylide (3) by treatment with butyllithium in THE-HMPA (Sato *et al.*, 1988).

The direct Wittig olefination of α-alkoxy ketones (2a, 2b) with ylide (3) affords the product 13a, 13b, which is subjected Na/NH_3 reduction to give the plaunotol (1) (Sato *et al.*, 1988).

4.5 Biological Activities of Plaunotol

Plaunotol, the active constituent of a commerical drug named Kelnac, is a mucosal protective factor-enhancing antiulcer agent (Department of Medical Information, Sankyo Co, Ltd., 1993). There have been reports on the effects of plaunotol on acute gastric or duodenal ulcers and on chronic gastric ulcers in animals (Ogiso *et al.*, 1985).

For the acute ulcers, plaunotol has been found to possess inhibitory effect on the ulceration induced by reserpine and stress in mice and also on the ulceration induced by stress, aspirin, indomethacin, pylòric ligature and cysteamine in rats, as well as aspirin in dogs (Department of Medical Information, Sankyo Co., Ltd, 1993; Ogiso *et al.*, 1985). Therefore, plaunotol appears to have 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 factors (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 doses 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 inhibites gastric secretion, increases the blood flow of gastric mucosa, facilitates biosynthesis of mucosal substances and prostaglandins in the mucosa and protects the breakdown of the mucous barrier (Department of Medical Information, Sankyo Co., Ltd., 1993; Ogiso *et al.*, 1985).

5. The Biosynthetic Pathway of Diterpenes

The biosynthesis of diterpenes starts from the formation of isopentenyl diphosphate which is formed from acetyl CoA (Goodwin and Mercer, 1983; Luckner, 1990). An important intermediate of this biosynthetic pathway is mevalonic acid (Fig. 6) which is converted to isopentenyl diphophate, the active molecule for the formation of all terpenes. As shown in Fig. 7, isopentenyl diphosphate is first isomerized by an isomerase enzyme to form 3,3-dimethylallyl diphosphate. 3,3-Dimethylallyl diphosphate is then condensed with three units of isopentenyl diphosphate to

form geranylgeranyl diphosphate. This geranylgeranyl diphosphate acts as starter molecule for the biosynthesis of various diterpenes including acylic 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 on the involved biosynthetic pathway. However, geranylgeraniol has been found to accumulate in the cell suspension cultures of *C. sublyratus* (Kitaoka, Nagashima and Kamimura, 1989). As a result, it is likely that plaunotol (18-hydroxygeranylgeraniol) is simply formed by a one-step 18-hydroxylation of geranylgeraniol (Fig. 8).

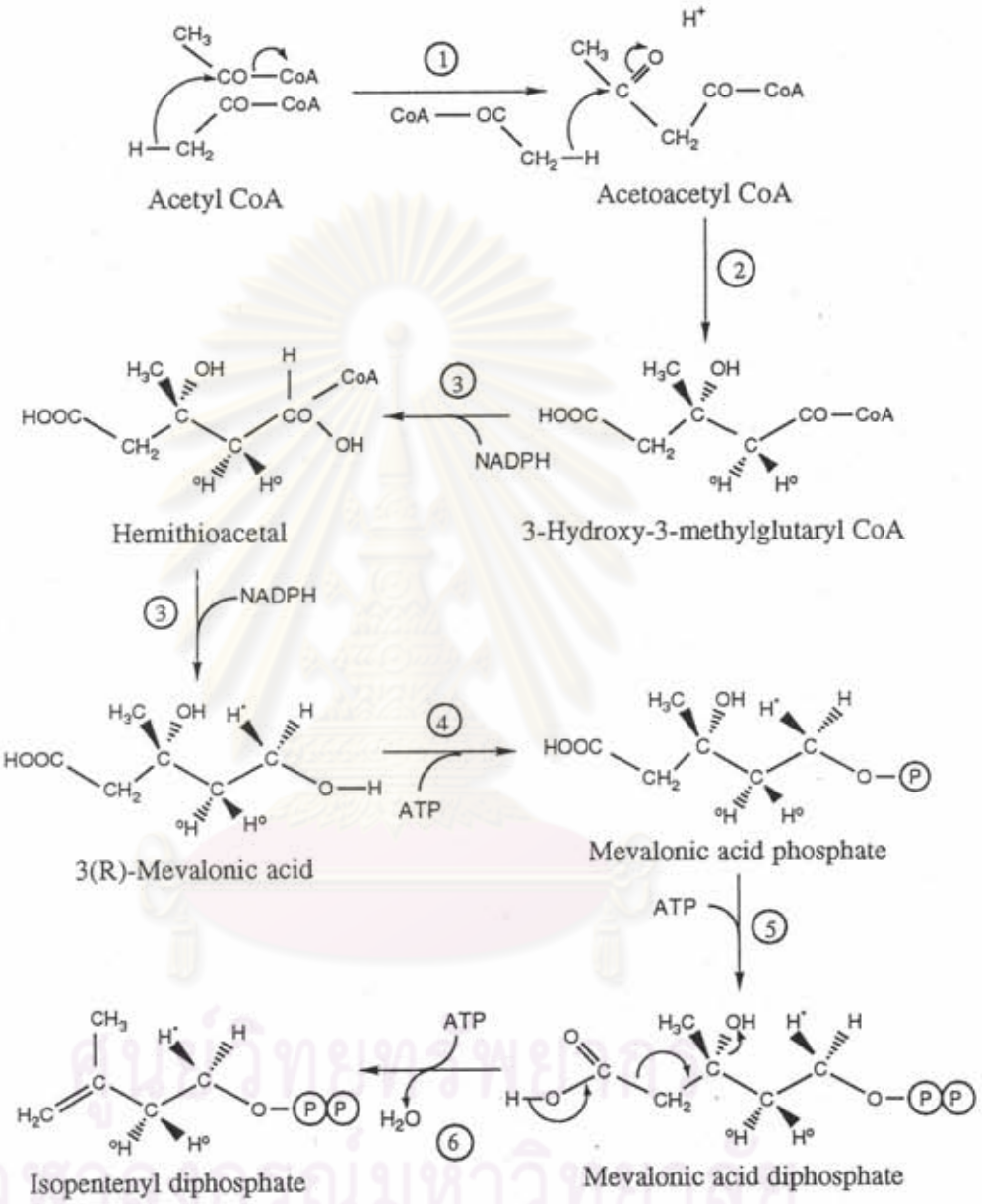


Figure 6 Biosynthesis of isopentenyl diphosphate form acetyl CoA. 1 Acetyl CoA acetyltransferase; 2 hydroxymethylglutaryl CoA synthase ; 3 hydroxymethylglutaryl CoA reductase ; 4 mevalonate kinase ; 5 phosphomevalonate kinase ; 6 diphosphomevalonate decarboxylase.

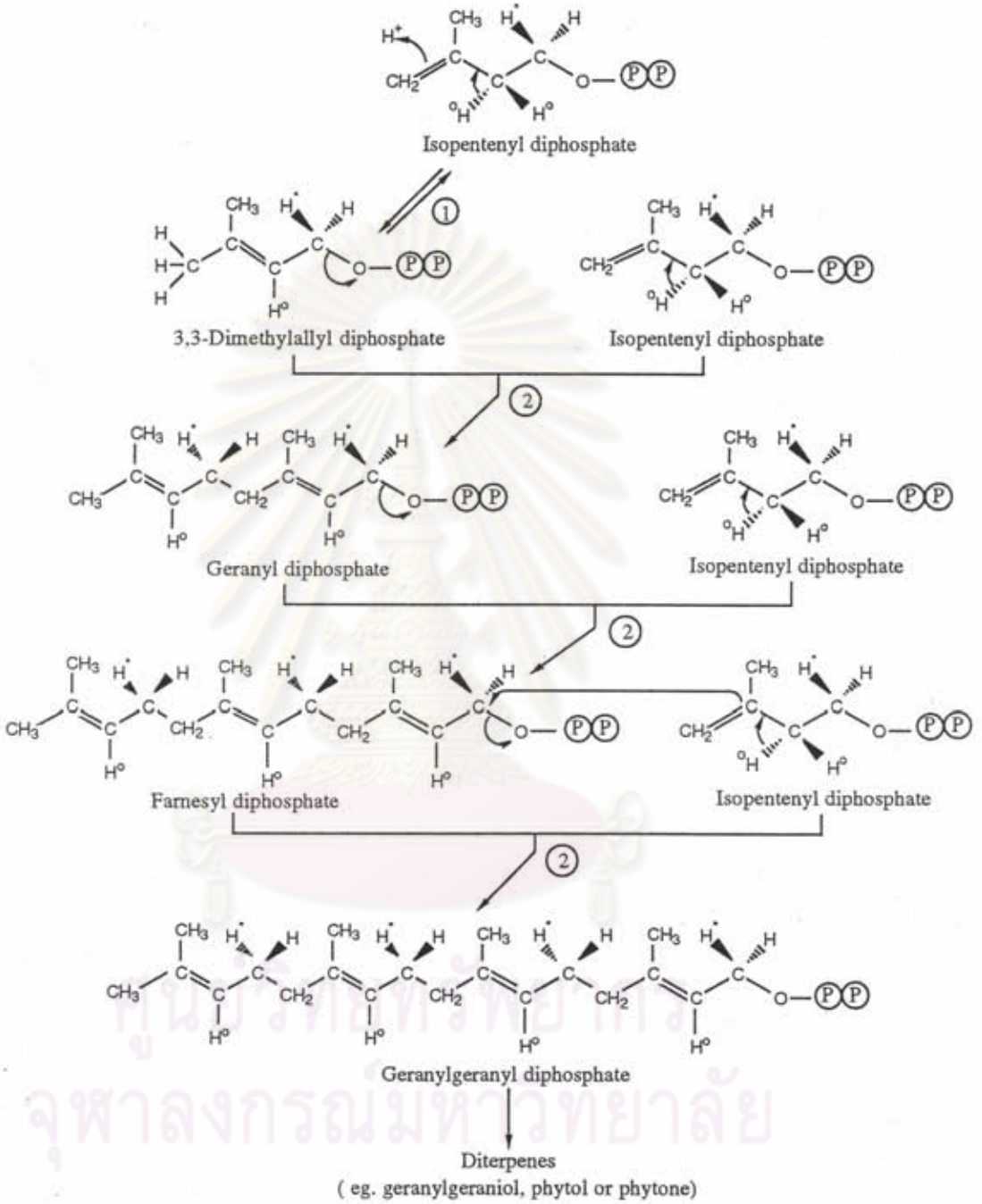


Figure 7 The biosynthetic pathway of diterpenes.

1 Isopentenyl diphosphste Δ-isomerase ; 2 prenyltransferase.

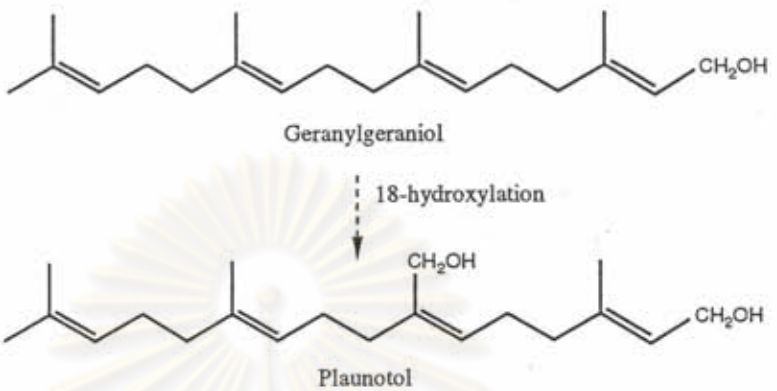


Figure 8 The hypothesis of plaunotol formation from geranylgeraniol.

6. Plant Tissue Culture for Plant Regeneration (Micropropagation)

Since White was successful in culturing tomato roots *in vitro* by supplying them with yeast extract in 1934 (White, 1934), plant tissue and cell culture techniques have been developed and used widely in many areas. Particularly, the application of tissue culture techniques for plant regeneration and commercial micropropagation (Hartmann and Kester, 1983). This method has become an important tool for research works in agriculture, horticulture and forestry. It involves the production of plants form very small plant parts, tissues, or cells grown aseptically in a test tube or other container where the environment and nutrition are regidly controlled. In addition, this tissue culture method has become an important alternative for more conventional propagation procedures for a wide range of plant species (Ammirato *et al.*, 1990; Bajaj, ed., 1988; Hartmann and Kester, 1983; Pierik, 1987; Yeoman, ed., 1986).

Plant regeneration through tissue culture or micropropagation (*In vitro*, vegetative propagation) has many advantages over conventional method (Bajaj, ed., 1988).

These include :

a) increase in the propagation rate of plants.

b) rapid multiplication of plants which in particular climate do not give seeds or whose seeds have a low germination capacity.

c) availability of plants throughout the year.

d) resistance of plants to insects, diseases, and herbicides.

e) uniform plants of a selected genotype.

f) production of uniform clones from highly heterozygous plants.

g) production of plants with changed genotype (tetraploids, haploids, hybrids).

h) conservation of genetic resources of species and threatened plants.

i) plant improvement by regeneration technique in conjunction with *in vitro* cell manipulation.

j) For medicinal plants, the rapid multiplication through tissue culture has a potential for production of various secondary metabolites (Hartmann and Kester, 1983) and helps to solve some theoretical problems connected with the biosynthetic pathways of chemical compounds in plants, and the relationship between organogenesis and secondary metabolite production (Bajaj, ed., 1988).

Table 2 lists some plant species in which tissue culture techniques have been used successfully for regeneration (Ammirato *et al.*, 1990; Bajaj, ed., 1988; Hartmann and Kester, 1983; Pierik, 1987).

Table 2 *In vitro* regeneration studies on some plant species (Bajaj, ed., 1988).

Plant species	Explants	Results	Reference
Allium cepa	Meristem	Plants	Hussey, 1978
Atropa belladonna	Root	Embryoids, callus	Thomas and Street, 1970
Capsicum annuum	Embryo	Shoots, plantlets	Agrawal and Chandra, 1983
Catharanthus roseus	Leaf and stem	Shoots, roots	Ramawat, Bhansall and Arya, 1978
Coffea arabica	Seeding shoot tip	Shoots, plants	Kartha *et al.*, 1981
Digitalis lanata	Flower and filament	Callus, embryoids, plantlets	Kuberski *et al.*, 1984
Eucalyptus citriodora	Shoot tip	Shoots buds, shoots	Gerwal, Ahuja and Atal, 1980
Gardenia jasminoids	Node	Shoots, rooted plants	Dumanois, Godin and Bigot, 1984
Gossypium arboreum	Embryo	Plants	Gill and Bajaj, 1984
Mentha arvensis	Nodal segments	Callus, shoots, plantlets	Rech and Pires, 1986
Panax ginseng	Root	Callus, embryoids, plantlets	Chang and Hsing, 1980
Papaver somniferum	Hypocotyl	Embryoids, plantlets	Nessler, 1982
Pinus ponderosa	Embryo	Callus, buds	Ellis and Bilderbach, 1984
Rehmannia glutinosa	Shoot tip	Shoots, plantlets	Shoyama, Nagano and Nishioka, 1983
Zingiber officinale	Rhizome	Embryoids, plantlets	Hosoki and Sagawa, 1977

Although numerous advantages and benefits are associated with tissue culture micropropagation, the technique still has some problems. One of the problems is genetic stability of the regenerate that some *in vitro* vegetative propagations (e.g. adventitious shoot, callus culture, cell suspension culture, protoplast culture) may occur somaclonal variation and mutation (Ammirato *et al.*, 1990; Hartmann and Kester, 1983; Pierik, 1987). For example, variation in flower morphology in *Ornithogalum* plant regenerated from callus (Yeoman, ed., 1986). For other problems, one should consider the problems of loss of regenerative capacity by repeated subculturing of callus and cell suspension, difficulty of transfer for test tube to soil (Pierik, 1987) and development of specific methods for getting optimun results with each species (Ammirato *et al.*, 1990).

7. Review of the Studies on *C. sublyratus* Tissue Culture

Since Ogiso *et al.* (1978) first reported that *C. sublyratus* contained the antipeptic ulcer diterpene (plaunotol) which is used as anti-ulcer drug (Kelnac, Sankyo Co., Ltd., Tokyo). Plant tissue and cell culture techniques have been used to study the production of plaunotol in *in vitro* cultures of *C. sublyratus*. The study has been carried out in two ways. The first is the study of plant cells of *C. sublyratus* for formation of plaunotol. The second way is the rapid mutiplication of *C. sublyratus* plants by using micropropagation techniques. Various studies on the production of secondary metabolites in tissue culture and *in vitro* studies on regeneration of *C. sublyratus* are listed in Table 3 and 4.

Table 3 Studies on *in vitro* culture studies of *C. sublyratus* : for secondary metabolite production.

Explant source	Basal medium	Growth regulators (mg/l) and other supplements	Culture type	Production of secondary metabolites	Reference
Leaf	MS	NAA(2)+BA(0.2) +0.8% gellan gum	Callus	Plaunotol	Morimoto, 1989
Leaf (from seedling)	MS	NAA(2)+BA(0.2) +gellan gum or agarose	Callus	Plaunotol	Morimoto and Murai, 1989
Leaf	B5 (liquid)	NAA(0.027) +kinetin(0.023)	Cells	Geranylgeraniol	Kitaoka, Nagashima and Kamimura, 1989
Leaf	1/2MS	2,4-D(2)+BA(3)	Callus	Plaunotol (unstable)	ธราธร บุญแก้ว, 2534
Callus (from leaf)	1/2MS (liquid)	2,4-D(2)+BA(3)	Cell suspension	No plaunotol	ธราธร บุญแก้ว, 2534
Leaf	MS	NAA(2)+BA(0.2) +2.0% agarose	Callus	Plaunotol	ธราธร บุญแก้ว, 2534
Callus (from leaf)	MS (liquid)	NAA(2)+BA(0.2)	Cell suspension	No plaunotol	ธราธร บุญแก้ว, 2534

Table 4 Studies on plant regeneration of *C. sublyratus*.

Explant source	Basal medium	Growth regulators (mg/l) and other supplements	Response	Reference
Leaf or cotyledon (from seed)	MS	NAA(0.002–0.2) +BA(2–10)+0.2% gellan gum	Shoots	Murai, 1990; Murai, Akiyama and Morimoto, 1990
Cotyledon (from seed)	MS medium 1	NAA(2)+BA(2) +0.2% gellan gum	Callus	Murai, 1990; Murai, Akiyama and Morimoto, 1990
	MS medium 2	BA(1)+GA_3(0.05) +0.2% or 0.8% gellan gum	Multiple shoots	
Shoot (from callus)	MS (liquid)	IAA(0.02)+5g Biofiber BD_2	Roots and Plantlets	Murai, 1990; Murai, Akiyama and Morimoto, 1990