CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Equipment.

Authentic plaunotol was obtained from Kelnac® soft gelatin capsules which are manufactured by Sankyo Co., Ltd ,Tokyo, Japan. Standard geranylgeraniol, farnesol, geraniol were purchased from Signa Co. St Loius, MO , USA.

[1-^{3}H] Geranylgeranyl pyrophosphate, Triammonium salt, (specific activity = 19.3 Ci/mmol, 0.5 mCi/ml) was purchased from Du Pont Wilmington, USA.

Tricine, glycine, ethylenediamine tetraacetic acid (EDTA), trisma base, bovine serum albumin (BSA), β-nicotinamide adenine dinucleotide phosphate (reduced form), tetrasodium salt (NADPH), β-nicotinamide adenine dinucleotide, (reduced form), disodium (NADH), *dl*-dithiothreitol (DTT) were purchased from Sigma Chemical Company, St Loius , Mo., USA. Disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) and sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) were purchased from Famitalia, Milan, Italy. Protease inhibitor cocktail tablets were purchased from Boehringer Manheim (Germany). Coomassie brilliant G-250 (for protein checking) was purchased from Bio-Rad Laboratories, Richmo, Ca., USA. Liquid nitrogen was perchase from Thailand Industrial Gases, Samutprakarn, Thailand.

Organic solvents were all reagent grade or better. Water was triple deionized.

Thin layer chromatography plates, were both Silica gel 60 F254 on aluminium sheets and Silica gel 60 F254 S on flatten-glass (For preparative TLC) were purchased from Merck (Damstadt, Germany).

Detection of Radioactive was performed by using TLC Linear analyzer LB 284/285 (Berthold, Germany).

The Chemical Ionization Mass spectra (CI-MS) were performed on a Varian Saturn GC/MS, (Varian, USA) and infrared spectra (IR) were obtained from Perkin-Elmer FT-IR spectrometormeter 1760X. Both belong to the Scientific Technological Research Equipment Centre, Chulalongkorn University (STREC CU).

The following equipment was used for the enzyme work: High speed Refrigerated Centrifuge Model CR20B3 (Hitashi), microcentrifuge Model 235C (Fisher Scientific), Shaker model TYP VX2 (Janke&Kunkel), Microplate Reader Model 450 (Bio-Rad), Multi-block heater (Lab-line), Centricon-10 Concentrators (Amicon).

Densitometric technique was performed by using Shimadzu Dual Wavelength TLC scanner Model CS-930.

Electron Micrograph are obtained by Trasmission Electron Microscope model TEM-200CX (Jeol, Japan), with Accelerating voltage = 80 kvolt, at The Scientific Technological Research Equipment Centre, Chulalongkorn University (STREC CU).

2. Plant Materials

The leaves of *C. sublyratus* used in this study were obtained from the plant growing in the open field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3. Preparation of Crude Enzyme Extracts and Microsomal Fractions from the Leaves of *C. sublyratus*

Fresh leaves (30 g) of *C. sublyratus* were quick frozen using liquid nitrogen and ground in a pre-cooled mortar. The resulting fine powder was added with 60 ml extraction buffer (83 mM tricine-NaOH pH 7.8, containing 5 mM β-mercapto ethanol, 0.4 M sucrose, 10 mM EDTA, 1 mM DTT 10 mM $MgCl_2$, 10 mg/ml BSA and protease inhibitor 1 tab/ 50 ml) and allowed to thaw and stirred for 20 min at 4° C. The suspension was pressed through four layers of cheese-cloth and the filtrate was centrifuged at 3000 *g* for 10 min in high speed refrigerated centrifuge. The supernatant part was used as "crude enzyme extracted"

For microsomal enzyme preparation, the 3,000 *g* supernatant was further centrifuged at 20,000 *g* for 20 min. The 20,000 *g* pellet obtained was resuspended in 10 ml of 0.1 M tricine-NaOH pH 7.8, containing 5 mM β-mercaptoethanol, 1 mM DTT, 0.2 M sucrose, 1 mM EDTA and 15 % glycerol. The resulting supernatant was called "microsomal fraction".

4. Detection of Enzyme Activity by Using Radioactively Labelled Substrate.

The enzyme activity in the crude enzyme extract which is responsible for the later-step biosynthesis of plaunotol was examined in the reaction mixture containing 0.045 nM [1-^{3}H] geranylgeranyl pyrophosphate (280,000 dpm), 83 mM tricine, pH 7.8, 0.8 mM NADPH and 100 µl enzyme solution in a total volume of 150 µl. The

heated microsomal fraction which was boiled for 30 min was used as control. After 60 min incubation at 30°C, the reaction mixture was subjected directly to TLC (Silica gel 60 F254) using the solvent system of chloroform : n-propanol (24:1). The TLC plate was finally scanned to produced radiochromatograms by TLC-radioscanner (Linear analyzer(B 284/285, Berthold, Germany).

In the study of the enzyme activity in different centrifugal pellets, the solutions of 3,000 *g* pellet, 20,000 *g* pellet or 20,000 *g* supernatant was used as the enzyme solution. The incubation time of this study was 60 min. For the time-course study using the 20,000 *g* pellet solution, the incubation time was varied (5, 30, 60, 120 and 180 min.). After 60 min incubation, or during a time course of study of reaction product formation, the incubation mixtures were extracted by ether before subjecting to TLC plate. The same solvent system was used for separating products. The TLC plate was then scanned with TLC Linear analyzer.

5. Enzyme Assay for Geranylgeraniol-18-Hydroxylase

The enzyme activity of geranylgeraniol-18-hydroxylase was determined by using the technique of TLC-densitometry. The 20,000 g microsomal fraction was incubated in the standard reaction mixture containing 1 mM geranylgeraniol, 0.8 mM NADPH, 83 mM tricine buffer pH 7.8 and 100 µl of the microsomal fraction in a total volume 300 µl. After 30 min of incubation, the reaction mixture was extracted with ether and subjected to TLC plate (Silica gel G) in chloroform : n-propanol (24:1), (24:0.5), (24:0.5) (triple developement) in the same plate. The TLC plate was then scanned for chromatograms by using a TLC densitometer (Shimadzu CS-930) to which its conditions were set as followes :

Instrumental model	: Shimadzu Dual Wavelength TLC scanner Model CS-930
Lamp	: Deuterium (D2)
Determination Mode	: Absorption
Scan width	: X=10.0 mm
	: Y=0.50 mm
Sensitivity	: Medium
Slit width	: 1.2 x1.2 mm^2
Wavelength detector	: 210 nm

The amount of enzyme product formation was estimated from the area under the peak of plaunotol (Rf = 0.46) and the calibration curve of authentic plaunotol.

6. Protein Determination

The protein from microsomal fraction and BSA , which was used as protein standard (The linear range of microtiter plate assay was from 5 µg/ml to 30 µg/ml.), were determined by the method of Bradford (1976). The sample solution (160 µl) was mixed with dye reagent concentrate (Bio-Rad protein assay) (40µl) in separated 96-well microtiter plate and measure. The mixed solution was incubated at room temperature for 5 min. The absorbance was measured rapidly at wavelength. 595 nm using using Bio- Rad Model 450 Microplate Reader.

7. Preparation and Identification of Enzymatic Product

To identify the enzymatic product, total volume (250-fold) large scale incubation mixture was prepared. The reaction mixture (75 ml) contained 25 ml microsomal fraction 1 mM geranylgeraniol, 0.8 mM NADPH and 83 mM tricine-NaOH buffer, pH 7.8. After 3 hr, the incubation mixture was extracted five times with

ethylacetate. The pooled ethylacetate fraction was concentrated and fractionated on a preparative TLC of silica gel G in chloroform:n-propanol (24:1) (24:0.5) (24:0.5) (triple developement). The band of the product (Rf 0.46) was elute with chloroform and determined for its MS spectra by GC/MS mass spectrometer and its IR spectra by FT-IR compared to standard plaunotol.

The GC MS conditions was described as follows:

Instrumental model : Varian Star 3400 CX Gas Chromatography

Column : DB-5 MS 30x0.25 mm

Detector temperature : 250°C

Injector temperature : 250°C

Column temperature: initial temp =150°C hold 3 min

: rate 10 °C/min

: final temp =300°C hold 20 min

Methane was used as reagent gas in the chemical ionization mass spectro meter.

For IR spectroscopic method, the enzymatic product and authentic plaunotol were dissolved in chloroform. Each solution was handled in KBr liquid cell and the solvent was evaporated before operation.

8. The Study on Boiling of Microsomal Fraction

The micosomal fraction prepared as described previously (Section 3) was boiled at 100°C for 0, 5, 10, 20, 30, 45, 60 min. Each fraction was then incubated for 30 min in the presence of 1mM geranylgeraniol, 0.8 mM NADPH and 0.83 mM

tricine-NaOH buffer pH 7.8. After incubation, the mixture was determined for plauntol formation as described in section 5.

9. The Study on pH Optimum of Enzyme Activity

The 30-min boiled microsomal fraction was incubated with 1 mM geranylgeraniol and 0.8 mM NADPH in incubation buffer at various pHs, using 0.1M sodium acetate buffer for pH 4, 4.5 and 5.0, 0.1M sodium phosphate buffer for pH 6.5, 0.1M tricine buffer for pH 7.0, 7.8 and 8.0 and 0.1 M glycine buffer for pH 9.0 and 10.0 for 30 min at 30°C on the shaker. The incubation and enzyme assay were performed as described in section 5.

10. The Study on the Enzyme Specificity towards Shorter-Length Substrates

The 30-min boiled microsomal fraction was tested for the ability to hydroxylase other shorter-length terpenoids, including geraniol and farnesol compared to geranylgeraniol. The boiled microsomal fraction was assayed using the above terpenoids as substrates at a final concentration of 1 mM under the condition of 0.1 M sodium acetate buffer pH 5.0. After 30 min incubation, the amount of plaunotol formed in the reaction was determined as described in section 5.

11. Effect of Cofactors on the Enzyme Activity

The 30-min boiled microsomal fraction was incubated with 1 mM geranylgeraniol and 1 mM cofactor of either NADPH or NADH and the enzyme activity was determined as described in section 5.

12. Electron Micrograph of Boiled Microsomal Fraction

The 30 min-boiled microsomal fraction was centrifuged at 20,000 *g* for 20 min. The supernatant was then concentrated by Centricon-10 concentrator.

The concentrated boiled enzyme was dropped into grids of electron microscope, which were laid of formva (polyvinyl formva in $CHCl_3$) as supporting film and coated with carbon. After being stayed for 2-5 min, the excess specimen was removed. Phosphotungstic acid (1-2 %) was then dropped for staining. After staining for 1-2 min and the excess reagent was removed by using filter paper. The specimen was dried in dessicator before being observed for the microsomal appearance with transmission electron microscope.