ชีวสังเคราะห์ของไอโซปรีนอยด์ในคัลลัสเพาะเลี้ยงของเปล้าน้อย

นาง บุปผาชาติ พตด้วง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ISOPRENOID BIOSYNTHESIS IN CALLUS CULTURES OF *CROTON SUBLYRATUS*

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmaceutical Chemistry and Natural Products
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By	Mrs. Buppachart Potduang
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บุปผาชาติ พตด้วง: ชีวสังเคราะห์ของไอโซปรีนอยด์ในคัลลัสเพาะเลี้ยงของเปล้าน้อย. (ISOPRENOID BIOSYNTHESIS IN CALLUS CULTURES OF *CROTON SUBLYRATUS*) อ. ที่ปรึกษา : รศ. ดร. วันชัย ดีเอกนามกูล, 88 หน้า. ISBN 974-346-732-7.

การศึกษาที่มาของไอโซปรีนอยด์ (isoprenoid)ในต้นเปล้าน้อยได้ดำเนินการโดยการป้อนน้ำตาลกลูโคสที่ติดฉลากให้แก่ใบและเนื้อเยื่อเพาะเลี้ยงสีเขียวที่อยู่ในสภาพคัลลัส(callus) ผลที่ได้พบว่าวิถีชีวสังเคราะห์ของหน่วยไอโซปรีนมาจากทั้ง mevalonate pathway และ non-mevalonate pathway จากผลการวิเคราะห์หาสารเปลาโนทอล(plaunotol)และจากภาพถ่ายด้วยกล้องจุลทรรศน์อิเลคตรอนของเนื้อเยื่อจากใบพบว่าใบเปล้าน้อยมีการสะสมสารเปลาโนทอลเห็นเป็นลักษณะคล้ายหยดน้ำมันอยู่ในคลอโรพลาสต์ (chloroplasts) ของกลุ่มเซลล์พาลิเสด (palisade cells)ในเนื้อเยื่อชั้นกลาง(mesophyll layer)ของใบ การป้อนน้ำตาลกลูโคสที่ติดฉลากด้วยสารรังสีที่คาร์บอนตำแหน่งที่ 1 คือ [1-^{14}C]glucose ให้ใบเปล้าน้อย พบว่ามันสามารถรับเอาสารติดฉลากเข้าสู่โมเลกุลของสารเปลาโนทอลได้ในระดับต่ำ สำหรับคัลลัสเพาะเลี้ยงพบว่าไม่สร้างสารเปลาโนทอลแต่สามารถสร้างสารไฟโตสเตอรอล(phytosterols) ซึ่งมีเบต้า-ซิโตสเตอรอล(β-sitosterol)และสติกมาสเตอรอล(stigmasterol)ผสมกัน เมื่อป้อนน้ำตาลกลูโคสที่ติดฉลากด้วยคาร์บอนไอโซโทปตำแหน่งที่ 1 คือ [1-^{13}C]glucose พบว่าคัลลัสเพาะเลี้ยงสามารถรับเอาคาร์บอนไอโซโทปเข้าสู่โมเลกุลของสารไฟโตสเตอรอลทั้งสองชนิดได้ในระดับสูง จากนั้นทำการสกัดแยกสารติดฉลากที่ได้แล้วนำไปศึกษาด้วยวิธีคาร์บอน-13นิวเคลียร์แมกเนติก รีโซแนนซ์สเปคโตรสโคปี(^{13}C nuclear magnetic resonance spectroscopy, ^{13}C NMR) พบว่าสารผสมไฟโตสเตอรอลมีรูปแบบการรับเอาคาร์บอนไอโซโทปเข้าสู่โมเลกุล(^{13}C labeling pattern) เป็นไปตามวิถีชีวสังเคราะห์ของหน่วยไอโซปรีนจากทั้ง mevalonate pathway และ non-mevalonate pathway เนื่องจากที่ผ่านมามีรายงานการศึกษาเกี่ยวกับวิถีชีวสังเคราะห์ของสารไฟโตสเตอรอลว่ามาจาก mevalonate pathway เป็นเส้นทางหลัก งานวิจัยนี้จึงได้องค์ความรู้ใหม่ที่บ่งชี้ว่าสารไฟโตสเตอรอลสามารถมีวิถีชีวสังเคราะห์มาจาก non-mevalonate pathway ได้ด้วยเช่นกัน

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ ลายมือชื่อนิสิต .....
ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่ปรึกษา .....

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BUPPACHART POTDUANG : ISOPRENOID BIOSYNTHESIS IN CALLUS CULTURES OF *CROTON SUBLYRATUS.* THESIS ADVISOR : ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D. 88 pp. ISBN 974-346-732-7.

The leaves and callus cultures of *Croton sublyratus* Kurz were used to determine glucose uptake in experiments to study the original source of isopentenyl diphosphate (IPP) biosynthesis, the universal isoprene unit was possibly formed from either the classical mevalonate pathway or the novel non-mevalonate pathway. The leaves appeared to accumulate plaunotol as oil globules within the chloroplasts of palisade mesophyll cells as observed under electron microscope operation of subcellular organelles of plaunotol-containing tissues, which was confirmed by plaunotol detection in specific leaf tissues. [1-^{14}C]glucose uptake into a leaf showed low incorporation into plaunotol. The callus cultures, on the other hand, did not seem to produce plaunotol but it was found highly active in producing a mixture of two phytosterols, namely stigmasterol and β-sitosterol. [1-^{13}C]glucose uptake into the callus cultures showed a very high incorporation of the labeled glucose into both forms of phytosterol. Isolation of the labeled products followed by ^{13}C NMR analysis showed that the phytosterols had their ^{13}C labeling patterns corresponding to the biosynthetic pathways of both the mevalonate pathway and non-mevalonate pathway. Since the biosynthesis of phytosterols has so far been reported to be mainly from the mevalonate pathway, therefore, this study provides original information on the biosynthesis of phytosterols via the novel non-mevalonate pathway.

Field of study Pharmaceutical Chemistry and Natural Products. **Student's signature**.....
Academic year 2000 **Advisor's signature**.....

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Finally, deepest thanks from the heart are expressed to the author's parents, her husband and daughters for their loves, support and understanding throughout the study.

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ABBREVIATIONS

[1-^{13}C]	=	carbon position 1 of the molecule is labeled with carbon 13
[1-^{14}C]	=	carbon position 1 of the molecule is labeled with radioactive carbon 14
2,4-D	=	2,4--dichlorophenoxyacetic acid
β-	=	beta-
B5	=	Gamborg *et al* (1968) medium
BA	=	6-benzylaminopurine; N^6-benzyladenine
cm	=	centimeter (s)
$CDCl_3$	=	deuterated chloroform
$CHCl_3$	=	chloroform
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
1-D	=	one dimensional
2,4-D	=	2,4-dichlorophenoxyacetic acid
d	=	doublet (for NMR spectra)
dd	=	doublet of doublets (for NMR spectra)
e.g.	=	for example
etc.	=	et cetera
Fig	=	figure
g	=	gram (s)
^{1}H NMR	=	proton nuclear magnetic resonance
Hz	=	Hertz
hr	=	hour (s)
in vitro	=	culture of living material literally "in glass"
IPP	=	isopentenyl diphosphate
DMAPP	=	dimethylallyl diphosphate
J	=	coupling constant
kinetin	=	kinetin-6-furfurylaminopurine
l	=	liter (s)
lb	=	pound (s)
m	=	meter (s)
mg	=	milligram
ml	=	milliliter
mm	=	millimeter
MS	=	Murashige and Skoog (1962) medium

n-	=	normal
N	=	normality
NAA	=	α-naphthaleneacetic acid
NaOH	=	sodium hydroxide
nm	=	nanometer
No.	=	number
pH	=	The negative logarithm of the concentration of hydrogen ions.
ppm	=	part per million
Rf	=	distance spot moved/distance solvent moved (TLC)
rpm	=	revolutions per minute
s	=	singlet (for NMR spectra)
SD	=	standard deviation
S or S	=	^{13}C-labeled phytosterol mixture of β-sitosterol and stigmasterol
t	=	triplet (for NMR spectra)
TLC	=	thin layer chromatography
U or U	=	unlabeled phytosterol mixture of β-sitosterol and stigmasterol
UV	=	ultraviolet light
w/v	=	weight/volume (concentration)
w/w	=	weight/weight (concentration)
^{o}C	=	degree Celsius
δ	=	chemical shift
μCi	=	microcurie (S) (10^{-6} Curie)
μg	=	microgram (S) (10^{-6} g)
μl	=	microliter (S) (10^{-6} l)
λ_{max}	=	maximum absorption wavelength
/	=	per, or divided by
%	=	percent (parts per 100) ; percentage

CHAPTER I

INTRODUCTION

Isopentenyl diphosphate (IPP, Figure 1) is widely known to be the fundamental five-carbon precursor (isoprene unit) of all terpenoid compounds. The acetate-mevalonate pathway (Figure 2A) has long been assumed to be the exclusive biosynthetic route used in all organisms for the biosynthesis of IPP. The enzymology and regulation of this pathway have been well studied, particularly in mammalian systems where it is responsible for the biosynthesis of cholesterol. There are also numerous studies demonstrating that the acetate-mevalonate pathway is also operated in plants, specifically in the case of sterol and phytoalexin synthesis (Weissenborn *et al*., 1995 ; Stermer *et al*., 1994).

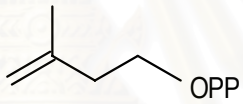


Figure 1 The structure of isopentenyl diphosphate (IPP)

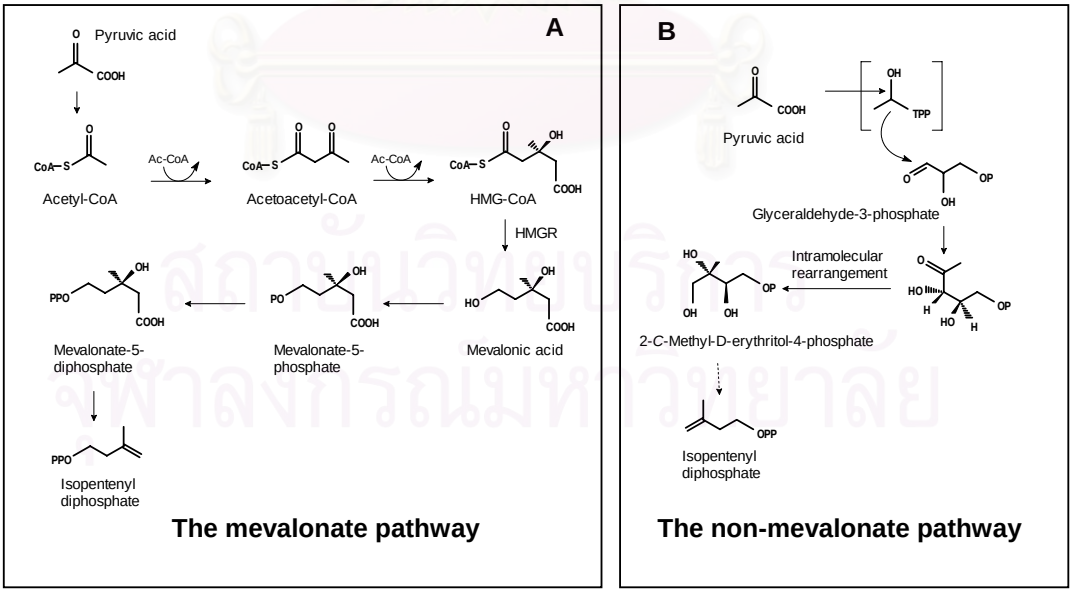


Figure 2 The biosynthetic pathways of IPP based on the well-known mevalonate pathway (A) and the newly found non-mevalonate pathway (B).

Recently, the existence in nature of a non-mevalonate pathway (Figure 2B) for the biosynthesis of certain terpenoids has been demonstrated (for review, see Lichtenthaler *et al*., 1997), based on ^{13}C-labelling patterns that are incompatible with the operation of the acetate-mavalonate pathway. This non-mevalonate pathway was originally demonstrated in a variety of eubacteria, including *Escherichia coli* (Rohmer *et al*., 1993). Subsequent work has established that this non-mevalonate pathway is responsible for the biosynthesis of isoprene (Zeidler *et al*., 1997 ; Schwender *et al*., 1997), monoterpenes (Eisenreich *et al*., 1997) and diterpenes (Eisenreich *et al*., 1996 ; Knoss *et al* ; 1997) in plants, as well as of carotenoids and the phytyl side chain of chlorophyll (Schwender *et al*., 1997; Lichtenthaler *et al*., 1997), all of these plant terpenoids are of origin from plastids.

This newly discovered non-mevalonate pathway starts with the condensation of pyruvic acid with glyceraldehyde-3-phosphate to yield deoxyxylulose-5-phosphate. (Sprenger *et al*., 1997 ; Losi *et al*., 1998). Intramolecular rearrangement of deoxyxylulose-5-phosphate (Arigoni *et al*., 1997) yields a branched five-carbon intermediate that subsequently undergoes a series of reactions to yield either IPP or dimethylallyl diphosphate (DMAPP) (Figure 2B). Based on the ^{13}C-labelling pattern from glucose, 2-*C*-methyl-D-erythritol-4-phosphate has been proposed to be an early intermediate of this pathway (Duvold *et al*., 1997). Confirmation of this non-mevalonate pathway will require characterization of the intermediates and enzymatic steps of this novel pathway. Although the non-mevalonate labelling patterns of terpenoids have been demonstrated in plants as mentioned above, these studies have been carried out only in a limited taxonomically families of plants. Particularly, not many tropical plants have been investigated. Furthermore, more and more findings have suggested that plants contain multiple, parallel pathways for terpenoid biosynthesis, and the subcellular compartmentation of these pathways (i.e. in plastids)

plays a central role in regulating the biosynthesis of the wide variety of terpenoids (Mc Caskill and Croteau, 1998). Therefore, more information from other sources of plants is still needed for a better understanding of terpenoid metabolism in plants.

Among Thai tropical plants, *Croton sublyratus* Kurz (Thai name: Plaunoi), which belongs to the family Euphorbiaceae, is particularly interesting. Presently, the leaves of *C. sublyratus* are well-known as being raw material for manufacturing an antipeptic-ulcer drug, namely Kelnac® which contains plaunotol as an active ingredient (Ogiso *et al*., 1985). Plaunotol (Figure 3) was isolated from this plant since 1978 (Ogiso *et al*.). It is an acyclic diterpene alcohol which has been found to be accumulated in the leaves with the content up to 0.5% (w/w) of dry weight (Vongchareonsathit and De-Eknamkul, 1998). A number of esters of plaunotol have also been found in *C. sublyratus* leaves (Kitazawa *et al*., 1982). In addition, other phytochemical studies of this plant have shown that the leaves contain at least 5 diterpenelactones (Plaunol A-E) (Kitazawa *et al*., 1980) and a few cyclic diterpene alcohols (Kitazawa *et al*., 1981).

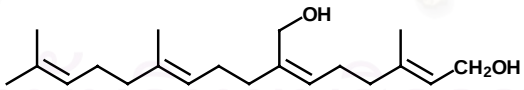


Figure 3 The structure of plaunotol

Recently, biosynthetic studies (Tansakul and De-Eknamkul, 1998) have shown that plaunotol is biosynthesized from geranylgeranyl diphosphate (GGPP) by using two enzymatic reactions (Figure 4). First, GGPP is hydrolyzed by a phosphatase to form geranylgeraniol

(GGOH). Second, GGOH is hydroxylated at C-18 by a specific 18-hydroxylase to form plaunotol. The discovery of the latter has led to a patent in the United States that suggests the possibility of using the enzyme for plaunotol production (De-Eknamkul and Tansakul, 1999).

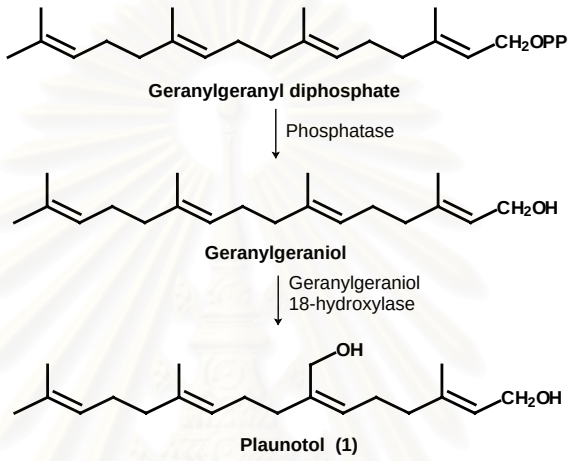


Figure 4 The last two steps of plunotol biosynthesis

Although the biosynthesis of plaunotol has been clarified at the enzyme level, the subcellular localization of these enzymes and the final product plaunotol has not yet been investigated. The information is extremely important since it can lead to the whole picture of plaunotol biosynthesis in Plaunoi plant. Recently, a rapid TLC-densitometric method for plunotol detection and analysis was developed (Vongchareonsathit and De-Eknamkul, 1998). This technique appears to be as sensitive as gas chromatographic method and allows plaunotol of less than 0.1 mg to be detected. The technique will allow the detection of plaunotol in different tissue types of *C. sublyratus* leaf, which is also part of the research work of this study.

The structure of plaunotol (Figure 3) is ideal to be used for studying the biosynthesis of IPP in Plaunoi plant. Its acyclic diterpenoid molecule allows easy interpretation of ^{13}C-

labelling patterns to conclude whether the precursor IPP is formed *via* either mevalonate or non-mevalonate pathway. However, the success will depend very much on the efficiency of ^{13}C-incorporation experiments. The incorporation rate of ^{13}C-precursors into plaunotol must be high enough so that the enrichment of ^{13}C in particular atoms in plaunotol molecule is detectable by ^{13}C-NMR. This is usually a problem for ^{13}C-incorporation experiments into intact plants or differentiated parts (i.e. leaves, roots etc.) which usually end up with low incorporation. With tissue (*in vitro*) cultures, on the other hand, high incorporation of a precursor into the product is normally observed, and it is obviously the material of choice.

Plant tissue and cell cultures of *C. sublyratus* have been established previously to study their potential to produce plaunotol. It has been shown that callus cultures of this plant could accumulate plaunotol under the presence of gelling agents in the medium (Morimoto and Murai, 1989). For cell suspension culture, it has been reported to produce geranylgeraniol (Kitaoka *et al*., 1989). Although the results appear to support our present proposal, we have never repeated these experiments and both plaunotol and geranylgeraniol have not been found in our *C. sublyratus in vitro* cultures.

In the present study, callus cultures of *C. sublyratus* reestablished in our laboratory were used for ^{13}C-incorporation experiments in order to answer the possible biosynthetic pathway of IPP operating in this plant.

CHAPTER II

HISTORICAL

1. *Croton sublyratus* Kurz.

1.1 Botanical Aspect of *Croton sublyratus* Kurz.

Croton sublyratus Kurz. or Plaunoi (Thai-name) (Figure 5) is in the family 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 sepals, five petals 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 metres 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).

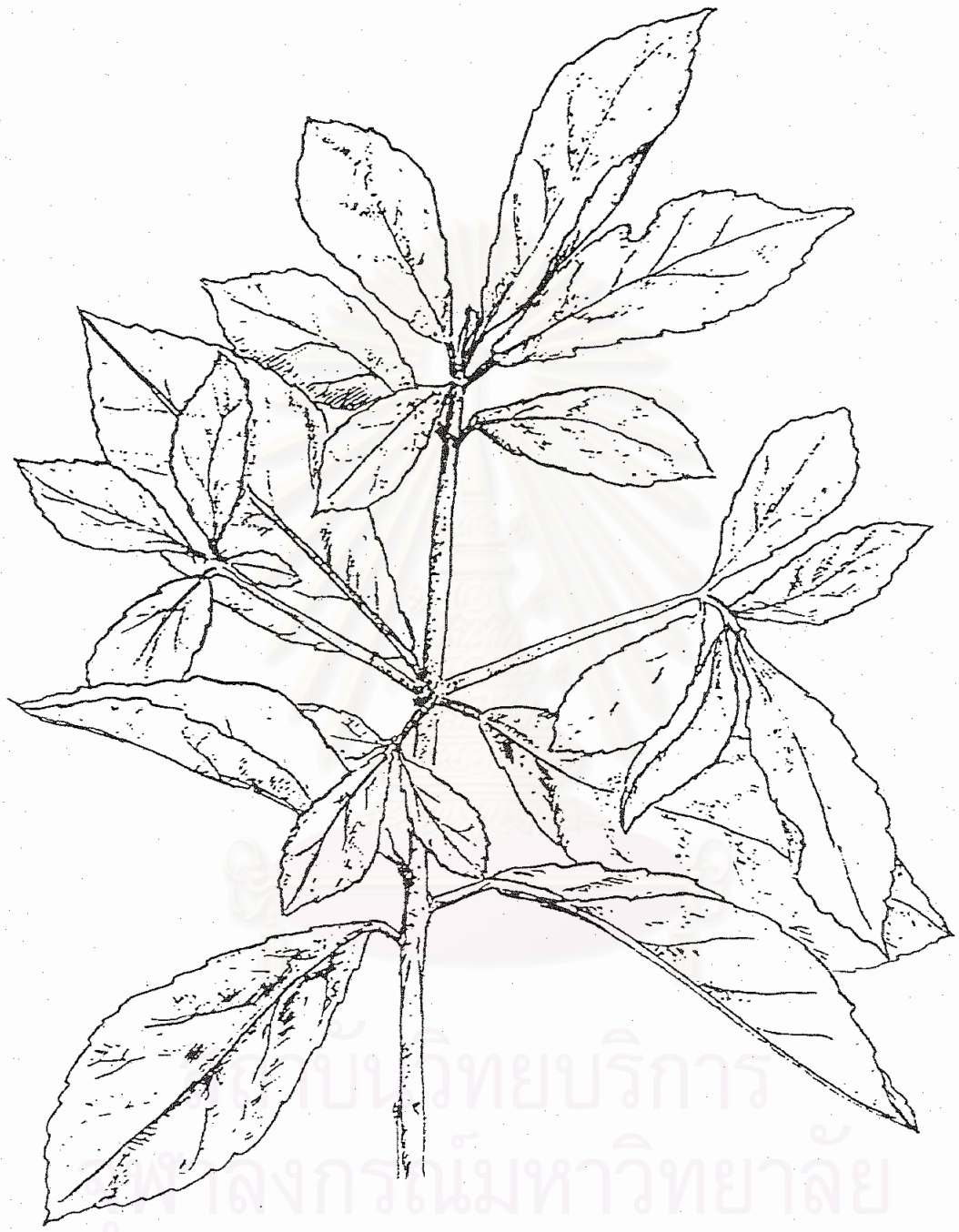


Figure 5 *Croton sublyratus* Kurz (Euphorbiaceae).

1.2 Medicianl Uses of *C. sublyratus*

1.2.1 Traditional Uses

C. sublyratus (Plaunoi) is aThai medicinal plant used as an anthelmintic and a dermatologic agent for skin diseases (จุฬาลงกรณ์มหาวิทยาลัย, คณะเภสัชศาสตร์, ภาควิชาเภสัชพฤกษศาสตร์, 2530; Dhavadee Ponglux., *et al*, 1987). The parts of stem, bark and leaf have been used as an antidiarrheal and to normalize menstruation, whereas its flower has been used as an 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 together for various purposes such as stomachic, anthelmintic, emmenagogue, digestant, transquillizer and carminative. They also have been used for the treatment of lymphatic, pruritic, leprosy, tumor and yaws (ประเสริฐ พรหมมณี และคณะ , 2531; นันทวัน บุณยะประภัศร, 2532).

1.2.2 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 factors by increasing gastric mucosal blood flow, promoting mucous and prostaglandin 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).

1.3 Chemical Constituents of *C. sublyratus*

In 1978, Ogiso and coworkers have isolated plaunotol from the stem of *C. sublyratus* and identified it as an antipeptic ulcer substance (Ogiso *et al*., 1978). This made it interesting to continue research on the isolation of 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 1

Table 1 Chemical constituents of *C. sublyratus*

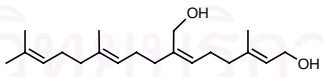
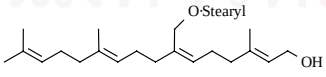
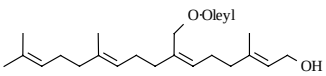
Chemical Group	Chemical substance	Chemical structure	Reference
Acyclic Diterpene	Plaunotol (18-hydroxy geranylgeraniol)		Ogiso *et al*.,1978
	Geranylgeraniol Ester A		Kitazawa *et al*., 1982
	Geranylgeraniol Ester B		Kitazawa *et al*., 1982

Table 1 (continued)

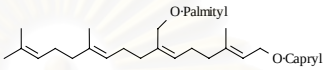
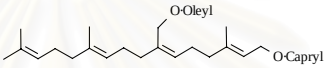
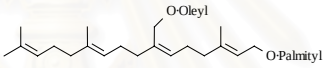
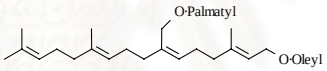
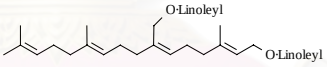
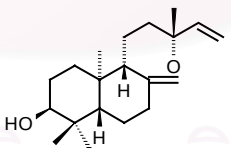
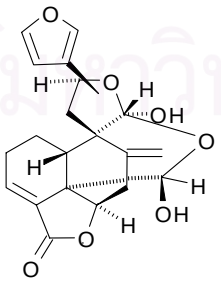
Chemical Group	Chemical substance	Chemical structure	Reference
Acyclic Diterpene	Geranylgeraniol Ester C		Kitazawa *et al*., 1982
	Geranylgeraniol Ester D		Kitazawa *et al*., 1982
	Geranylgeraniol Ester E		Kitazawa *et al*., 1982
	Geranylgeraniol Ester F		Kitazawa *et al*., 1982
	Geranylgeraniol Ester 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 1 (continued)

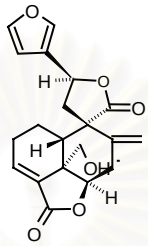
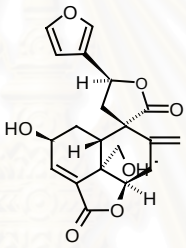
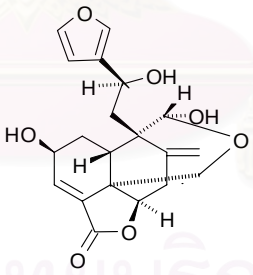
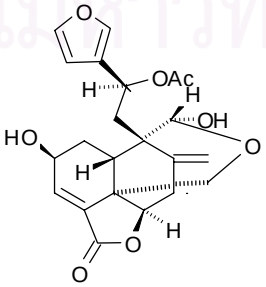
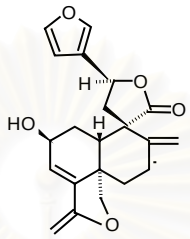
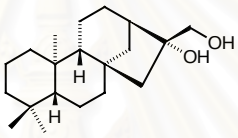
Chemical Group	Chemical substance	Chemical structure	Reference
Clerodane Diterpene	Plaunol B		Kitazawa *et al*., 1979; Kitazawa *et al*., 1980
	Plaunol C		Kitazawa *et al*., 1980
	Plaunol D		Kitazawa *et al*., 1980
	Plaunol E		Kitazawa *et al*., 1980

Table 1 (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

2. The Biosynthesis of Plaunotol

Although the structure of plaunotol has been known for almost twenty years, its biosynthetic pathway has been investigated just recently (Tansakul and De-Eknamkul, 1998). Based on its structure, the biosynthesis of plaunotol in this plant could be simple since the compound is a 18-hydroxy derivative of geranylgeraniol (GGOH), a common precursor of all natural diterpenoids. It is well documented that GGOH is biosynthesized *via* the terpenoid pathway and its immediate precursor is geranylgeranyl diphosphate (GGPP) (Loomis and Croteau, 1981). Therefore, it is reasonable to propose that plaunotol is biosynthesized from GGPP by two steps of enzymatic reactions (Figure 4). First, GGPP is hydrolysed by a phosphatase enzyme to form GGOH . Second, GGOH is hydroxylated at C-18 position by a specific 18-hydroxylase to form plaunotol.

Due to the availability of *C. sublyratus* plants in Thailand, the whole plant has been used as a source for searching the enzyme activity of GGOH-18-hydroxylase. The leaf part

was chosen as the material for the study since it accumulates plaunotol and is potentially the site of plaunotol biosynthesis. With this plant material, the activity of geranylgeraniol-18-hydroxylase, a novel enzyme catalysing the C-18 hydroxylation of geranylgeraniol (GGOH) to plaunotol, was discovered in the cell-free extract (Tansakul and De-Eknamkul, 1998). This enzymatic formation of plaunotol was correlated with both incubation time and the amount of microsomal protein used. The enzyme activity could be increased by adding NADPH and by heating the 20,000 *g* microsomal fraction prior to the incubation. The pH optimum for the enzyme activity was 5.0. Observation of the heated 20,000 *g* microsomal fraction under electron microscope revealed the presence of particles with the diameter ranging from 20 to 40 nm.

3. Plaunotol Production in Plant Tissue Cultures

Plant tissue and cell culture techniques 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 found to accumulate in the suspension cultures of *C. sublyratus* (Kitaoka *et al*., 1989).

4. The Biosynthetic Pathways of Isoprene Unit

The fundamental five-carbon precursor of all terpenoids is isopentenyl diphosphate (IPP, Figure 1). The acetate-mevalonate pathway (Figure 2A) has long been assumed to be the exclusive biosynthetic route used in all organisms for the biosynthesis of IPP, and the enzymology and regulation of this pathway have been well studied, particularly in mammalian systems, where it is responsible for the biosynthesis of cholesterol. Extensive studies have emphasized the regulatory role of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGR) in the acetate-mevalonate pathway. HMGR is considered to be the most heavily regulated enzyme in mammalian metabolism (Goldstein and Brown, 1990), and there are numerous studies demonstrating that it is also closely regulated in plants, specifically in the case of sterol and phytoalexin synthesis (Weissenborn *et al*., 1995 ; Stermer *et al*., 1994).

Until recently, the acetate-mevalonate pathway has been assumed to be the exclusive source of IPP utilized for terpenoid biosynthesis throughout nature. However, the existence of a non-mevalonate pathway (Figure 2B) for the biosynthesis of certain terpenoids has been demonstrated (and recently reviewed in detail (Lichtenthaler *et al*., 1997)), based on ^{13}C-labelling patterns that are incompatible with the operation of the acetate-mevalonate pathway. This non-mevalonate pathway was originally demonstrated in a variety of eubacteria, including *Escherichia coli* (Rohmer *et al*., 1993), as well as for diterpene biosynthesis in the primitive plant *Ginkgo biloba* (M. K. Schwarz, Ph.D. thesis, ETH, Zurich, Switzerland, 1994). Work with a series of *E. coli* mutants blocked at defined points in glycolysis established that the condensation of an activated two-carbon intermediate derived from pyruvic acid with glyceraldehyde-3-phosphate (GAP) is the probable first step of this non-mevalonate pathway (Rohmer *et al*, 1996), Subsequent work has established that this non-mevalonate pathway is responsible for the biosynthesis of isoprene (Zeidler *et al*. 1997 ; Schwender *et al*., 1997), monoterpenes (Eisenreich *et al*., 1997) and diterpenes (Eisenreich *et al*., 1996 ; Knoss *et al*., 1997) in plants, as well as of carotnoids and the phytyl side chain of chlorophyll (Schwender *et al*., 1997 ; Lichtenthaler *et al*, 1997), all of these plant terpenoids are of plastidial origin. This pathway is evolutionarily ancient and widespread, being present in a variety of eubacteria (Rohmer *et al*., 1993), Streptomyces (Seto *et al*, 1996), green algae (Schwender *et al*., 1996) and liverworts (Adam *et al*., 1998), as well as in higher plants (Zeidler *et al*., 1997 ; Eisenreich *et al*., 1997 ; Eisenreich *et al*., 1996 ; Lichtenthaler *et al*., 1997). The intital product of this pathway, deoxyxylulose-5-phosphate, is also utilized for both thiamine (vatamin B_1) (David *et al*, 1982) and pyridoxyl (vitamin B_6) (Hill et al., 1989) biosynthesis. The transketolase responsible for the synthesis of deoxyxylulose-5-phosphate from glyceraldehyde-3-phosphate and pyruvate has recently been cloned from *E. coli* by Sprenger *et al*. (Sprenger *et al*., 1997) and Boronat and co-workers (Lois *et al*., 1998). The corresponding gene has also recently been cloned from higher plants and shown to encode a protein with a typical plastidtargeting sequence, as expected for a nuclear-encoded plastidial enzyme (Lange *et al*., 1998). Intramolecular rearrangement of deoxyxylulose-5-phosphate (Arigoni *et al*., 1997) yields a branched five-carbon intermediate that subsequently undergoes

a series of reactions (probably involving both redox reactions and dehydrations, and an additional phosphorylation) to yield either IPP or dimethylallyl diphosphate (DMAPP). Based on the ^{13}C-labelling pattern from glucose, 2-*C*-methyl-D-erythritol-4-phosphate has been proposed to be an early intermediate of this pathway (Duvold *et al*., 1997). It is important to note that there is no reason to assume that IPP is the ultimate product of the non-mevalonate pathway. Depending on the sequence of reactions, this pathway could as easily produce DMAPP as the final product, which could yield IPP through the action of IPP isomerase. Confirmation of the final product(s) of the non-mevalonate pathway will require characterization of the intermediates and enzymatic steps of this novel pathway.

5. Subcellular Organization of Terpenoid Metablism

Plant contain multiple, parallel pathways for terpenoid biosynthesis, and the subcellular compartmentation of these pathways plays a central role in regulating the biosynthesis of the wide variety of terpenoids. In light of recent developments demonstrating the existence of the non-mevalonate pathway for terpenoid biosynthesis in plastids, the following generalizations might be made about the subcellular compartmentation of terpenoid formation in plants : the cytoplasmic acetate-mevalonate pathway is primarily responsible for the synthesis of sterols and sesquiterpenoids, whereas the plastids (employing the non-mevalonate pathway) are responsible for the synthesis of isoprene, carotenoids, monoterpenes and diterpenes (Figure 6). However, there is substantial evidence in the literature that this is probably an oversimplification of the subcellular compartmentation of terpenoid biosynthesis in plants because of the possible exchange of cytoplasmic and plastidial metabolites.

Although the non-mevalonate labelling patterns of plastid-derived terpenoids have been demonstrated in a taxonomically diverse selection of plants, these studies have been carried out only in selected tissues at certain developmental stages (i.e. either tissue cultures (Eisenreich *et al*., 1996 ; Lichtenthaler *et al*., 1997 ; Arigoni *et al*., 1997) or mature photosynthetic tissue (Zeidler *et al*., 1997 ; Eisenreich *et al*., 1997). In contrast, floral tissues

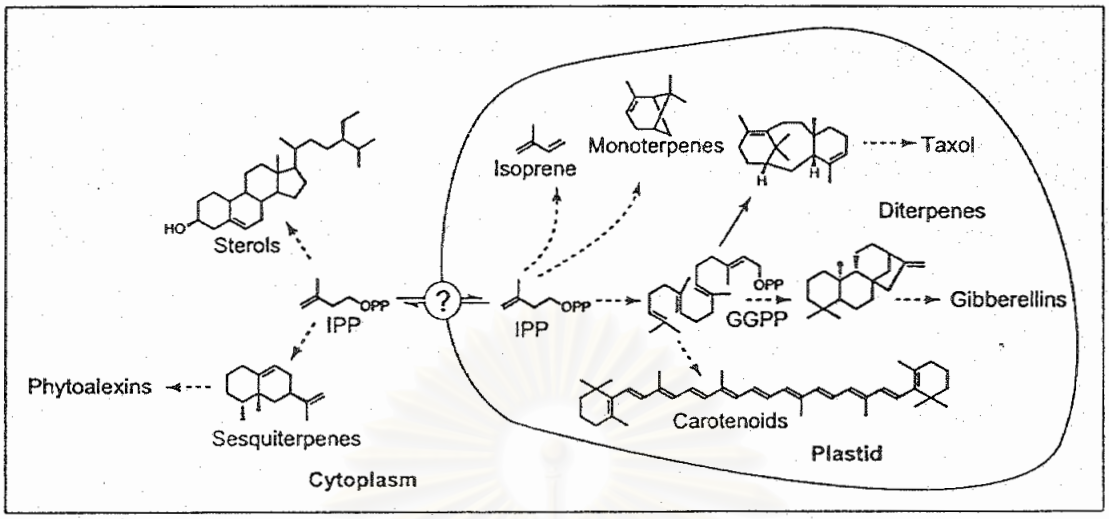


Figure 6 Examples of the different families of terpenoids produced by plants. Sesquiterpenoid phytoalexins and sterols (triterpenes) are produced in the cytoplasm ; isoprene, monoterpenes, diterpenes and carotenoids (tetraterpenes) are produced in the plastid ; independent pathways exist for the cytoplasmic and plastidic synthesis of isopentenyl diphosphate (IPP). Dashed arrows indicate multiple enzymatic steps. There is a variable amount of exchange of isopentenyl diphosphate between subcellular compartments, depending on tissue type and developmental stage (from TIBTECH August 1998, Vol. 16).

may synthesize monoterpenes using the cytoplasmic acetate-mevalonate pathway. Early studies with rose petals demonstrated a very rapid and efficient incorporation (>20% in less than 1 h) of [^{14}C]mevalonic acid into monoterpene glycosides (Francis *et al.*, 1969). Related to this observation, linalool synthase, a monoterpene synthase responsible for the production of the major component of the floral scent of *Clarkia breweri*, is encoded by a gene that apparently does not specify a plastid-targeting sequence characteristic of other (plastidic) monoterpene synthases (Dudareva *et al.*,1996). Monoterpene and sesquiterpene biosynthesis has been examined in intact secretory cells isolated from the essential-oil-producing plant peppermint (*Mentha piperita*). Using a variety of radiolabelled substrates, it has been demonstrated that both monoterpene and sesquiterpene biosynthesis by the secretory cells rely exclusively on a plastid-derived pool of IPP (McCaskill *et al.*, 1995). Thus, although it has recently been shown that monoterpene biosynthesis in peppermint relies on the plastidic non-mevalonate pathway (Eisenreich et al., 1997), it appears that the sesquiterpenes of the essential oils may also be derived from this pathway and not from the

cytoplasmic acetate-mevalonate pathway (Eisenreich et al., 1997), as might be expected based on the cytoplasmic location of sesquiterpene biosynthesis. This suggestion has recently been supported by studies on sesquiterpene biosynthesis in chamomile (*Chamomilla recutita*) (Adam and Zapp, 1998) in which a heterogeneous labeling pattern of the sesquiterpenes was observed : the initial two isoprene units (IPP) incorporated into the farnesyl diphosphate (FPP) precursor were derived exclusively from the non-mevalonate pathway, whereas the last IPP incorporated was derived from both the acetate-mevalonate and the non-mevalonate pathways. Similarly, small but significant labeling of sterols by [^{13}C]deoxyxylulose has been observed, again inconsistent with strict formation of a cytosolic terpene by the acetate-mevalonate pathway (Arigoni *et al*., 1997). Both of these studies indicate that, *in vivo*, there can be exchange of prenyl diphosphates between subcellular compartments (Figure 6), although there are clearly significant differences in the degree of exchange, depending upon the tissue type and, probably, developmental stage. Therefore, it may not be possible to make broad generalizations about the biosynthetic origin of specific families of terpenoids because, in many cases, there may not be strict segregation of terpenoid biosynthetic intermediates within subcellular compartments. Finally, several studies have demonstrated an apparent developmental shift in the preferred substrates used by chloroplasts for the synthesis of plastidic terpenoids (Heintze *et al*., 1990 ; Heintze *et al*., 1994 ; Hoppe *et al*., 1993). In particular, isolated, immature spinach chloroplasts show approximately five times more incorporation of [^{14}C]acetate into chloroplast terpenoids than [^{14}C]pyruvate (Heintze *et al*., 1994). This argues in favour of a plastidic form of the acetate-mevalonate pathway, albeit one that may only be active during a short period of development in immature plastids.

Although largely circumstantial, these studies collectively provide evidence that there may, in certain tissues, be a significant amount of exchange of IPP (or other prenyl diphosphates) between subcellular compartments, that the relative activities of each pathway and the extent of exchange of IPP between compartments is likely to be developmentally regulated, and that there may be a plastidic form of the acetate-mevalonate pathway that is

active only at certain developmental stages. This apparent flexibility of terpenoid biosynthesis in plants is probably a consequence of the requirement to coordinate and regulate the synthesis of a complex array of terpenoids from a single common substrate (IPP).

CHAPTER III

MATERIALS AND METHODS

1. Plant Materials

The leaves of *C. sublyratus* (Euphorbiaceae) used in this study were obtained from plants growing in the open field of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. A voucher specimen is deposited in the Herbarium, Royal Forest Department in Bangkok, Thailand under no. 21867.

2. Chemicals

[1-^{14}C]glucose with specific activity of 56.0 mCi/mmol , radioactive concentration of 308 μCi/ml , was purchased from Amersham, Buckinghamshire, England.

[1-^{13}C]glucose was a gift from Professor Dr. Meinhart H. Zenk of the University of Halle, Halle, Federal Republic of Germany.

3. Transmission Electron Microscopy

Young fresh leaves of *C. sublyratus* were cut into small pieces (1.0 x 1.0 mm) and fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4^{o}C for 1 hr. The specimens were post-fixed in 1% osmium tetroxide at 4^{o}C for 2 hr. They were then dehydrated in a graded series of acetone solutions (30%, 50%, 70%, 90% 15 min each and 100% 10 min 3 times) and infiltrated using a mixture of acetone and Spurr's medium at the ratios of 2:1 for 3 hr, 1:2 for 3 hr and in pure Spurr's medium for another 3 hr. The resulting samples were embedded in liquid paraffin wax for at least 8 hr at 70^{o}C. Blocks of specimens were sectioned at 60-90 nm thickness by ultramicrotome. The sections were viewed and photographed under a JEOL JEM-200 CX TEM at 80 kv.

4. Separation of Leaf Tissues Containing Palisade and Spongy Mesophyll Cells

From a young fresh leaf of *C. sublyratus*, the upper layer of the leaf was removed using a pair of sharp forceps to separate the leaf into 2 parts, these being dorsal and ventral sides. The dorsal (upper) side of the leaf was obtained as small pieces of approximately 1 x 1 cm in size whereas the ventral side was obtained as the whole under-portion of the leaf. Observation under light microscope indicated the presence of mainly palisade mesophyll cells in the dorsal part and mainly spongy mesophyll cells in the ventral part. The two leaf tissue parts were separated from five fresh leaves to give sufficient tissues for plaunotol analysis.

5. Detection of Plaunotol in Leaf Tissues and in Tissue Cultures

The presence of plaunotol in the leaf tissues and various *in vitro* cultures was analyzed by the TLC-densitometric method as described previously (Vongchareonsathit and De-Eknamkul, 1998). Leaf tissues obtained from dorsal sides, ventral sides, whole fresh leaves, and tissues from callus or cell suspension cultures were each extracted with 10 ml of boiling 95% ethanol. After filtering through Whatman no. 1 filter, 0.5 μl of each aliquot was spotted onto a TLC plate (silica gel 60 F_{254}, 0.2 mm thickness). The plate was developed three times using the same solvent system of chloroform : n-propanol, 96:4 with 10 cm height of the solvent front. The TLC plate was then scanned using a TLC densitometer (Shimadzu Model CS-930) at a wavelength of 220 nm.

6. Callus Cultures

6.1 Plant Materials

Young leaves (5-6 cm long) of *Croton sublyratus* Kurz. were collected from the plant cultivated in the open field of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

6.2 Nutrient Media

A standard basal medium used in this study was MS (Murashige and Skoog, 1962) of which the composition and preparation are described in Table 2. The

compounds used in the preparation of MS stock solutions were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Water used for media preparation was deionized and redistilled.

Sucrose from Mitrpol Co. Ltd.was added for carbon supply.

Agar from Difco Laboratories (Detroit Michigan, USA) was added as gelling Agent.

Supplemented plant growth regulators were purchased from Gibco Laboratories (New York, USA) as follows :

Auxin :	2,4-D	or	2,4-Dichlorophenoxyacetic acid
	NAA	or	α-Naphthaleneacetic acid
Cytokinin :	Kinetin	or	Kinetin-6-furfurylaminopurine
	BA	or	6-Benzylaminopurine

The medium for callus induction was supplemented with 2.0 mg/l 2,4-D and 0.1 mg/l kinetin. The first subculture medium for callus proliferation was supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l BA. The subculturing medium for green callus induction was supplemented with 1.0 mg/l 2,4-D ; 0.1 mg/l BA and 0.1 mg/l NAA.

The media were adjusted to volume by deionized and redistilled water, then adjusted to pH 5.8 with 1N sodium hydroxide solution or 1N hydrochloric acid solution. 0.8% w/v agar was added as gelling agent and heated while stirring until the agar was dissolved and clear media solution was obtained. The media were transferred to sterile subculture bottles, 20 ml each, tightly closed the lids and sterilized by autoclaving at 121° C (15 lb/in^2) for 15-20 minutes. The media were cooled down to room temperature before used.

6.3 Callus Induction and Proliferation

Young fully expanded leaves of *Croton sublyratus* were washed under tap water, then soaked in 70% ethanol for 3 minutes followed by surface sterilization in

0.5% sodium hypochlorite for 5 minutes and washing 5 times with sterile distilled water. With sterilization techniques, the sterile leaves were excised into pieces, 5 x 5 mm^2 each with short cuttings on the surface, and placed on MS agar medium(0.8% w/v agar) for callus induction (MS supplemented with 3% w/v sucrose, 2.0 mg/l 2,4-D and 1.0 mg/l kinetin), then maintained in a culture room at 25 ± 2 °C under controlled 16 hours of photo period (2000 lux). Under these conditions, the callus was formed in 2 weeks.

For callus proliferation, the calli formed were subcultured 2-3 times, every 3 weeks, on MS agar medium (0.8% w/v agar) containing 3% w/v sucrose, 1.0 mg/l 2,4-D and 1.0 mg/l BA, which resulted in high growth rate friable, soft and yellowish tissues.

Table 2 Composition and preparation of MS agar (Murashige and Skoog ,1962)

MS in stock solution	concentration stock solution used	amount of
water q.s.		1000 ml
stock 1 (Macronutrients)	**g/1000 ml**	50 ml
NH_4NO_3	33.0	
KNO_3	38.0	
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
stock 2 (Micronutrients)	**mg/100 ml**	1 ml
H_3BO_3	620	
$MnSO_4.H_2O$	1,690	
$ZnSO_4.7H_2O$	860	
$Na_2MoO_4.2H_2O$	25	
$CuSO_4.5H_2O$	2.5	
$CoCl_2.6H_2O$	2.5	
stock 3 (Ca stock)	**g/100 ml**	5 ml
$CaCl_2.2H_2O$	8.7	
stock 4 (KI stock)	**mg/100 ml**	1 ml
KI	75	
stock 5 (Vitamins)	**mg/100 ml**	10 ml
Thiamine HCl	8	
i-Inositol	10,000	
stock 6 (Fe-EDTA stock)	**g/500 ml**	5 ml
Na_2EDTA	3.73	
$Fe_2SO_4.7H_2O$	2.78	
Plant growth regulators		as required
Sucrose		30 g
pH		5.8
Agar		8 g

6.4 Green Callus Induction

To induce green callus for feeding experiments, the 3 weeks old proliferated calli were subcultured 2-3 times, every 3 weeks, on MS agar medium (0.8% w/v agar) for green callus induction supplemented with 3% w/v sucrose ; 1.0 mg/l 2,4-D ; 0.1 mg/l BA and 0.1 mg/l NAA. The relatively yellowish green callus was maintained at the same conditions as above.

7. Cell Suspension Cultures

Cell suspension cultures of *C. sublyratus* were initiated from the established green callus cultures. The callus tissues were placed in a 250-ml Erlenmeyer flask containing 50 ml of MS liquid medium with 3% w/v sucrose ; 1.0 mg/l 2,4-D ; 0.1 mg/l BA and 0.1 mg/l NAA., on a rotary shaker at 120 rpm at 25 $\pm$ 2 °C. These cell suspensions were subcultured on the same medium every 3 weeks by adding 20 ml of culture to 50 ml of fresh medium.

8. Product Detection

8.1 Sample Preparation

Callus tissues of 1 g fresh weight, was extracted with 2 ml solution of 95% ethanol + 10% sodium hydroxide (9:1) under reflux at 80°C for 1 hour. The hydrolysate was then partitioned with 1 ml n-hexane for three times and the combined n-hexane extract was evaporated to dryness under vacuum. The dried n-hexane extract was used directly for TLC densitometric analysis.

8.2 Thin-Layer Chromatography Densitometric Analysis

The dried n-hexane extract obtained above was dissolved in 40 µl absolute ethanol and applied on a precoated TLC plate of silica gel 60 F254 on aluminium sheets obtained from Merck(Damstadt, Germany). The plate was developed in a solvent system of chloroform : n-propanol 24:1. The corresponding TLC chromatogram was produced by TLC densitometer (Shimadzu Model CS-930) at a single wavelength of 220 nm, deuterium lamp, with setting parameters as follows :

PHOTO MODE SELECT : 1. **1** = ABSORPTION REFLECTION

SCAN WIDTH : 1. **10** = "X" WIDTH (mm)

2. **3** = DELTA "Y" 0.1

SLIT WIDTH : 1. LEFT = **7**

2. RIGHT = **2**

SIGNAL PROCESS : 1. **3** = LINEARIZER

2. **2** = ACCUMMULATION NO. 8

3. **0** = BACKGROUND CORRECT OFF

4. **1** = SIGNAL AVERAGE 4

OUTPUT MODE SELECT : 1. **1** = ORDINATE X 1

2. **2** = ABSCISSA X 1

3. **1** = CURVE AND PRINT

4. **1** = DRIFT LINE ON

PEAK DETECT : 1. **1** = 1

2. **1** = DRIFT LINE ON

3. **1** = AREA

4. **1** = HIGH SENSITIVITY

5. **500** = MINIMUM AREA

6. **2** = MINIMUM WIDTH 10

PROGRAM SCAN PARAMETERS : 1. **220** = WAVELENGTH 1 (nm)

2. **220** = WAVELENGTH 2 (nm)

3. **20** = "X" START POSITION (mm)

4. as required = "Y" START POSITION (mm)

5. **150** = "Y" END POSITION (mm)

6. **20** = LANE DISTANCE (mm)

7. **9** = TOTAL LANES

The visualisation of the spots can be obtained by exposing the developed TLC plate with iodine (I_2) vapor in an iodine tank.

9. Time-Course Study of Culture Growth and Product Formation of *C. sublyratus* Callus Cultures

Approximately 0.05 g fresh weight of 3-week-old tissues were transferred to each jar containing MS agar medium (0.8% w/v agar) for green callus induction supplemented with 3% w/v sucrose ; 1.0 mg/l 2,4-D ; 0.1 mg/l BA and 0.1 mg/l NAA. The jars were maintained in a culture room at 25 ± 2°C under controlled 16 hours of photo period (2000 lux). Two jars were taken, for duplicate, every 2 days for callus cultures harvesting and fresh weight recording. The fresh tissues were then extracted and analyzed for phytosterol formation as described in the sections of 8.1 and 8.2 respectively.

10. Incorporation Experiments

10.1 Incorporation Experiments of *C. sublyratus* Leaves

5 μl (1 μCi) of D-[1-^{14}C]glucose was put into an eppendorf to be diluted to 200 μl with distilled water. A young leaf of *C. sublyratus* was immersed in the solution for 3 days. During this period of time, 200 μl of distilled water was added when the solution was about to dry. After 3 days, the leaf was extracted under reflux at 80^{o}C in 95% ethanol for 1 hour and the extract was separated by TLC using the conditions described in the section of Product Detection (8.1 and 8.2). The resulting TLC plate was then analyzed for radioactivity by using Linear Analyzer LB 284/285 (Berthod, Germany). In this study, leaves with different sizes were used to observe the relationship between ages and sizes of the leaves versus ^{14}C incorporation.

10.2 Incorporation Experiments of *C. sublyratus* Callus Cultures

Incorporation experiments were performed in 50-ml jars using the solution of 0.4% (w/v) [1-^{13}C]glucose (99% ^{13}C enrichment), with a parallel experiments of the solution of 0.4% (w/v) unlabeled glucose as a control. The green callus tissues (approximately 5 g wet weight) were incubated with 2 ml of each glucose solution and incubated at 25$\pm$2^{o}C under controlled photoperiod of 16 hours for 3 days. Approximately 50 jars of ^{13}C incorporated

callus were carried out in this study. One of the jars was also added with 1μCi of [1-^{14}C]glucose in order to monitor the uptake and incorporation of glucose into the phytosterol products. Based on the radioactive monitoring, ^{13}C incorporation of the callus was found to maximize after 3 days of the experiment. Therefore, the 60 jars each of both the [1-^{13}C]glucose and unlabeled glucose incorporation experiments were all harvested for the callus followed by extraction and isolation of the phytosterols.

11. Isolation and Purification of Phytosterols

Green callus cultures of *C. sublyratus* (approximately 300 g fresh weight) after 3 days of incorporation experiments were harvested and extracted under reflux at 80^{o}C, for 1 hour with 500 ml mixture solution of 95% ethanol and 10% sodium hydroxide solution (9:1). The extract was filtered and concentrated to a volume of 300 ml. The extract was then partitioned 3 times with n-Hexane 200 ml. The n-Hexane layers were combined and evaparated to dryness under reduced pressure. The residue was dissolved in absolute ethanol and further purified by preparative TLC on silica gel 60 F_{254} (precoated, Merck) using Benzene : ethylacetate (5:1, v/v) as mobile phase. The TLC plate was analyzed by TLC densitometric method at the wavelength of 220 nm to locate the highest peak. The selected band of preparative TLC was then cut off into pieces and eluted by vigorous shaking with absolute ethanol and taken to dryness under reduced pressure. The obtained product was further purified by preparative TLC one more time using the same procedure, and then analyzed by NMR spectroscopy.

12. NMR Spectrometry of Labeled Metabolites

^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, at 17^{o}C, using a Jeol JNM-A500 spectrometer of the Scientific and Technological Research Equipment Center, Chulalongkorn University. ^{13}C enrichment was determined by quantitative NMR spectroscopy.

CHAPTER IV

RESULTS

1. Cell Arrangement in Leaf Tissues of *C. sublyratus*

Figure 7A illustrates a transverse section of a *C. sublyratus* leaf. It clearly shows that cell arrangement of the leaf is of dorsiventral type (the upper and lower parts of the leaf have different cell structures). It can be seen that beneath the upper epidermis is a layer of vertically elongated palisade mesophyll cells. The lower half of the leaf contains the spongy mesophyll which is a loosely arranged network of cells of irregular shape and large air spaces. Below the spongy mesophyll cells is the lower epidermis. Inside the palisade mesophyll cells, the 2,300 x EM picture (Figure 7B) shows that the cells are packed with chloroplasts containing a number of oil globule-like structures. Similar subcellular structures were also found in the spongy cells connected with the palisade cells although to a much lesser extent.

2. Detection of Plaunotol in Leaf Tissues

The nature of the loose connection between leaf tissues of the palisade mesophyll cells (dorsal side) and the rest of spongy mesophyll cells (ventral side) (Figure 7A) allowed the two parts of the leaf to be separated from one another. This could be done by simply using forceps to remove the surface layer of the dorsal side from the leaves. Monitoring under light microscope showed that the two types of cells could be well separated by this physical separation technique.

Upon extraction and analysis for plaunotol from the separate tissue parts of the leaves, it was found that most of the compound resided in the palisade mesophyll cells. Only a small amount was detected in the spongy cells (Figure 8). The content of plaunotol, calculated by peak areas, found in the palisade cells (approx. 82%) appeared to be comparable with that found in whole leaves (Table 3). This strongly suggested that most plaunotol is accumulated in the palisade cells of the leaves of *C. sublyratus*.

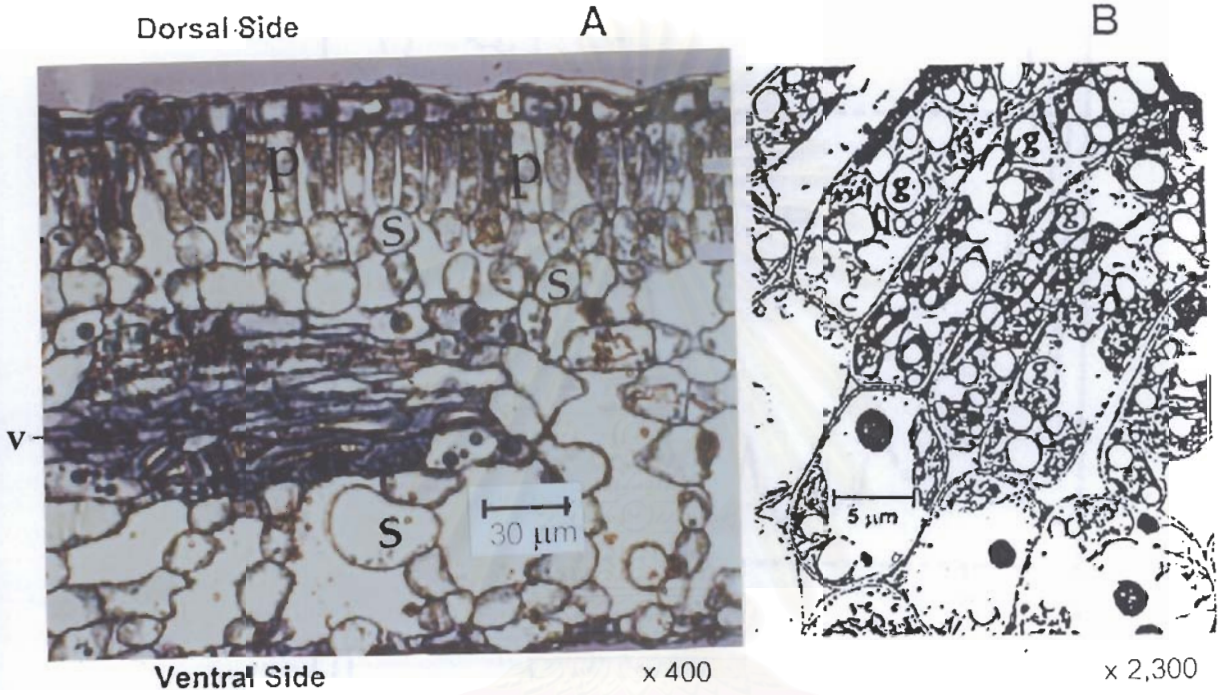


Figure 7 (A) A transverse section of a *C. sublyratus* leaf (x 400) showing palisade mesophyll cells (p), spongy mesophyll cells (s), a vein (v). (B) An electron micrograph (x2,300) of palisade mesophyll cells showing numbers of oil-like globules (g).

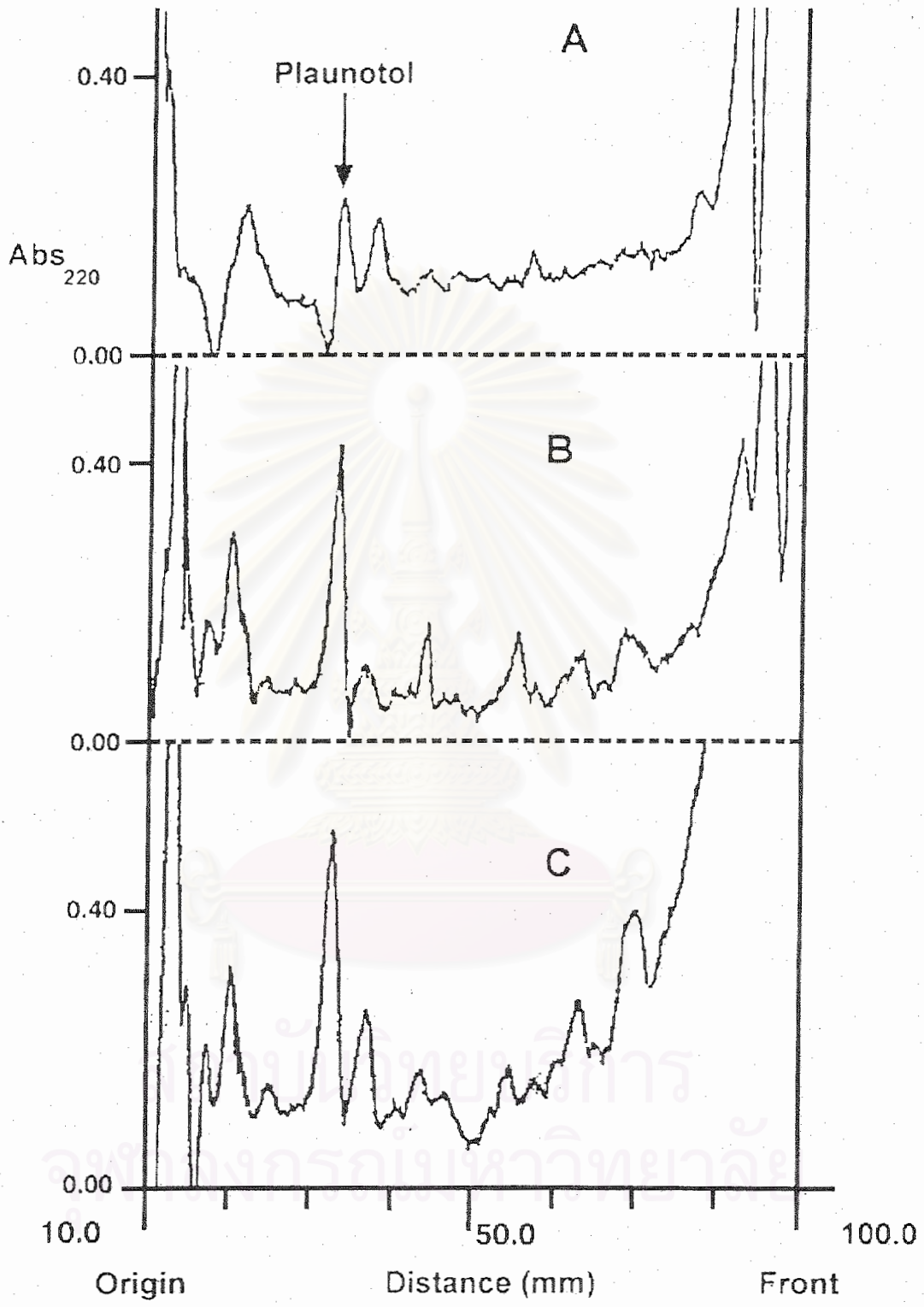


Figure 8 TLC-densitometric chromatograms of the ethanolic extracts from (A) spongy mesophyll tissues, (B) palisade mesophyll tissues, and (C) the whole leaf.

Table 3 Plaunotol content in the dorsal and ventral sides of *C. sublyratus* leaves comparing with the content in the whole leaves.

Leaf part	Plaunotol content* (mg per 5 leaves)	% Distribution
Whole leaf	1.60 ± 0.16	100.0
Dorsal side containing palisade mesophyll tissue	1.30 ± 0.20	82.1
Ventral side containing spongy mesophyll tissue	0.28 ± 0.03	17.9

3. Electron Micrographs of the Organelles Accumulating Plaunotol

Plaunotol is structurally an acyclic diterpenoid with twenty carbons and two hydroxyl groups in the molecule. It is relatively hydrophobic and dissolves well in non-polar solvents. Therefore, in the aqueous solution of normal cells, one would expect that the accumulation of plaunotol in the leaves should be in the form of oil globules. Examination of the EM picture in Figure 7B and detection of plaunotol in the leaf cells (Figure 8) strongly suggested that plaunotol is accumulated in the palisade cells of *C. sublyratus* leaves as oil globules.

To obtain more information on the organelles containing the oil globules, the EM pictures of the palisade mesophyll cells were observed at 3,800 times (Figure 9A) and 15,200 times (Figure 9B) magnification. Both pictures clearly showed that the sites containing plaunotol were located in the chloroplasts which are present in high numbers inside the palisade cells. Each chloroplast appeared to be able to accommodate 2-4 vesicles of various sizes.

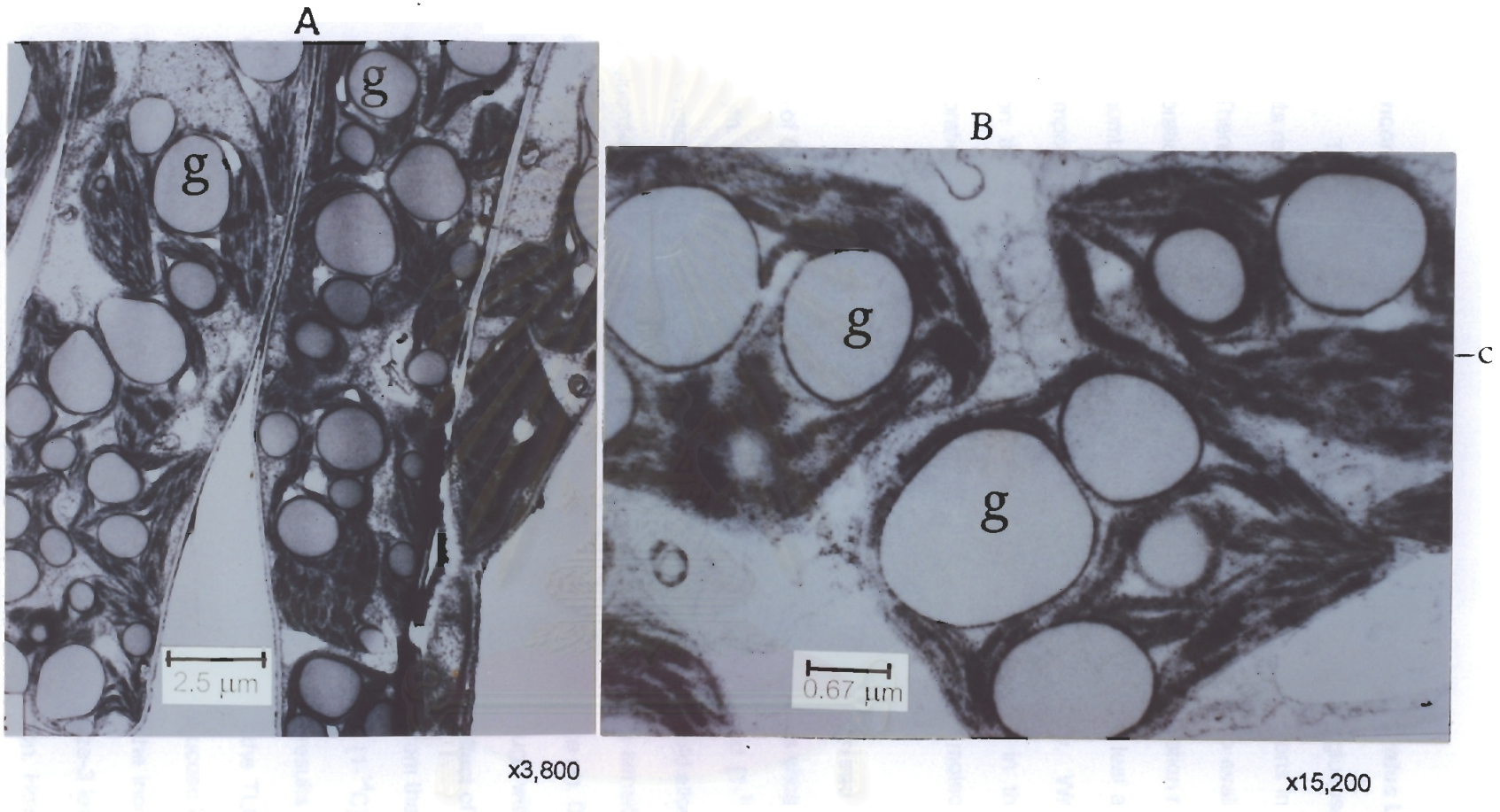


Figure 9 Electron micrographs of palisade mesophyll cells magnified 3,800 times (A), and 15,200 times (B) showing oil globules (g) in the chloroplasts (c).

4. Incorporation of [1-^{14}C]Glucose into Plaunotol in *C. sublyratus* Leaves

The ability of plant material to uptake and incorporate glucose into its terpenoid products reflects the possibility of studying original sources of carbons in the biosynthesis of IPP. Therefore, in the case of *C. sublyratus* leaf ; it is essential to evaluate its efficiency in incorporation of glucose metabolites into plaunotol. A high incorporation rate would result in a large number of labeled molecules of plaunotol accumulated in the leaf and thus would allow the compound to be determined easily by ^{13}C-NMR spectroscopy. With low incorporation rate, on the other hand, the unlabeled plaunotol accumulated in the leaf prior to the incorporation experiment would mask the ^{13}C-signals of the labeled molecules.

In order to evaluate the efficiency of glucose incorporation into plaunotol, the young leaves of *C. sublyratus* were first used in the experiment. The sizes chosen were 3.5 - 5 cm. in length. In this preliminary experiment, the radioactively labeled [1-^{14}C]glucose was first used instead of the stable isotope labeled [1-^{13}C]glucose. This would allow the incorporation of glucose to be monitored by TLC-radioscan which is much more sensitive and rapid. The results of the incorporation experiments showed that the leaf of size no. 0 and 1 (3.5 – 5 cm long) could incorporate the molecules of glucose into plaunotol though with a low rate (Figure 10). Another experiment was subsequently performed to see the effect of the leaf size on the efficiency of glucose incorporation. In this experiment, the leaves from the stages of relatively young (size 1-3) were collected. The leaves were incubated with [1-^{14}C]glucose for 3 days, followed by plaunotol extraction and TLC-radioscan analysis. The results showed, again, that the leaf of size 3 had the radiosignal at the spot of plaunotol on the TLC plate (Figure 11). The smaller size of leaves showed lower incorporation of [1-^{14}C]glucose into plaunotol. With more mature leaves (size 4-7), similar experiments showed that the incorporation of [1-^{14}C]glucose was even lower than the one of size 3 (Figure 12). The size-3 leaf therefore seemed to be the material for further use, with [1-^{13}C]glucose incorporation. However, to obtain the labeled plaunotol with sufficient amount for ^{13}C-NMR analysis, a much larger number of the size-3 leaves would be needed and the experiment would not be practical. The next experiment was therefore carried out by using the callus cultures of *C. sublyratus*.

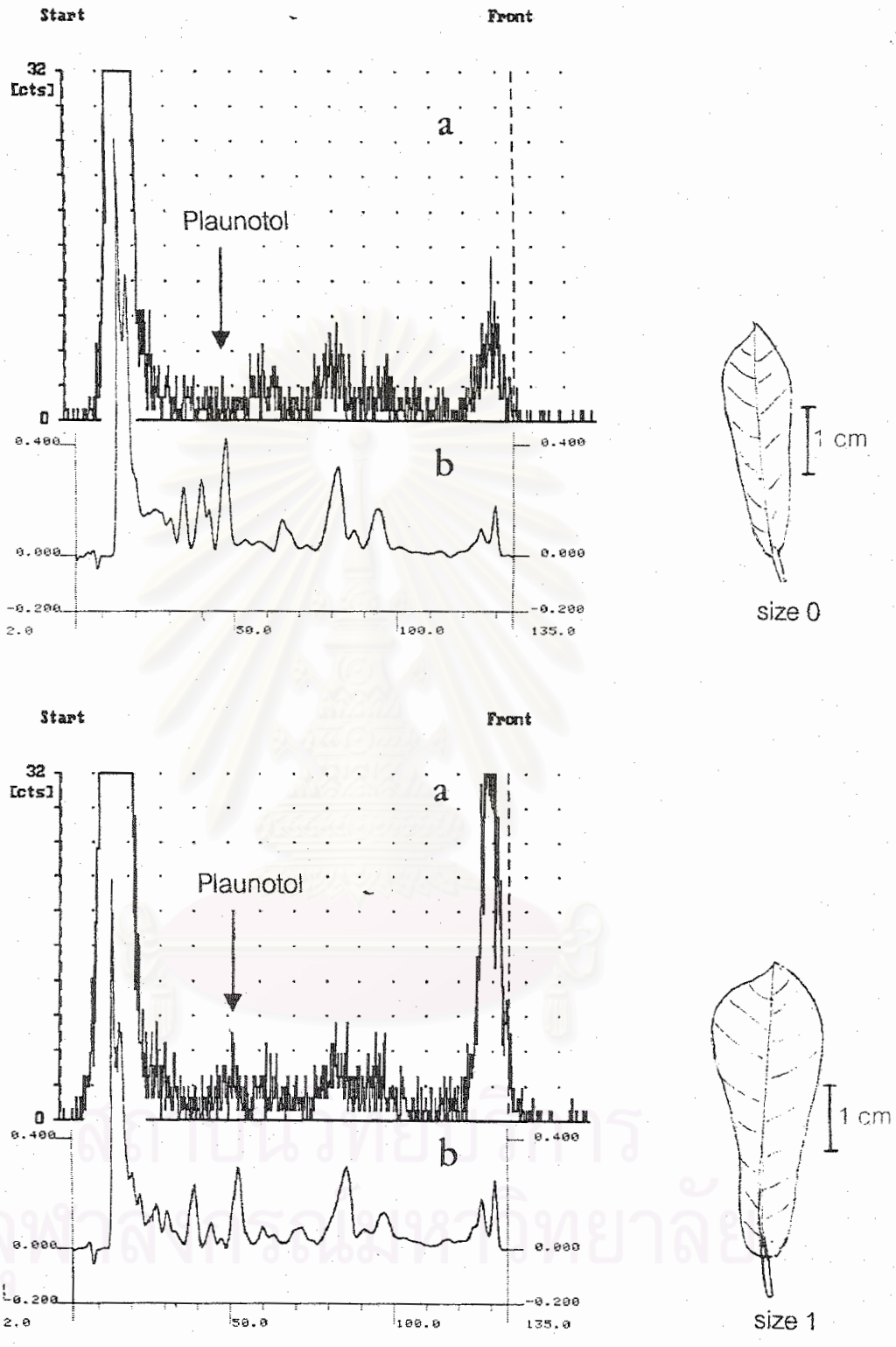


Figure 10 TLC-radiochromatogram (a) and TLC-densitometric chromatogram (b) of the leaf extracts obtained from the [1-^{14}C]glucose incorporation of size o-leaf (top) and size 1-leaf (bottom) of *C. sublyratus*. The position of plaunotol is indicated by an arrow.

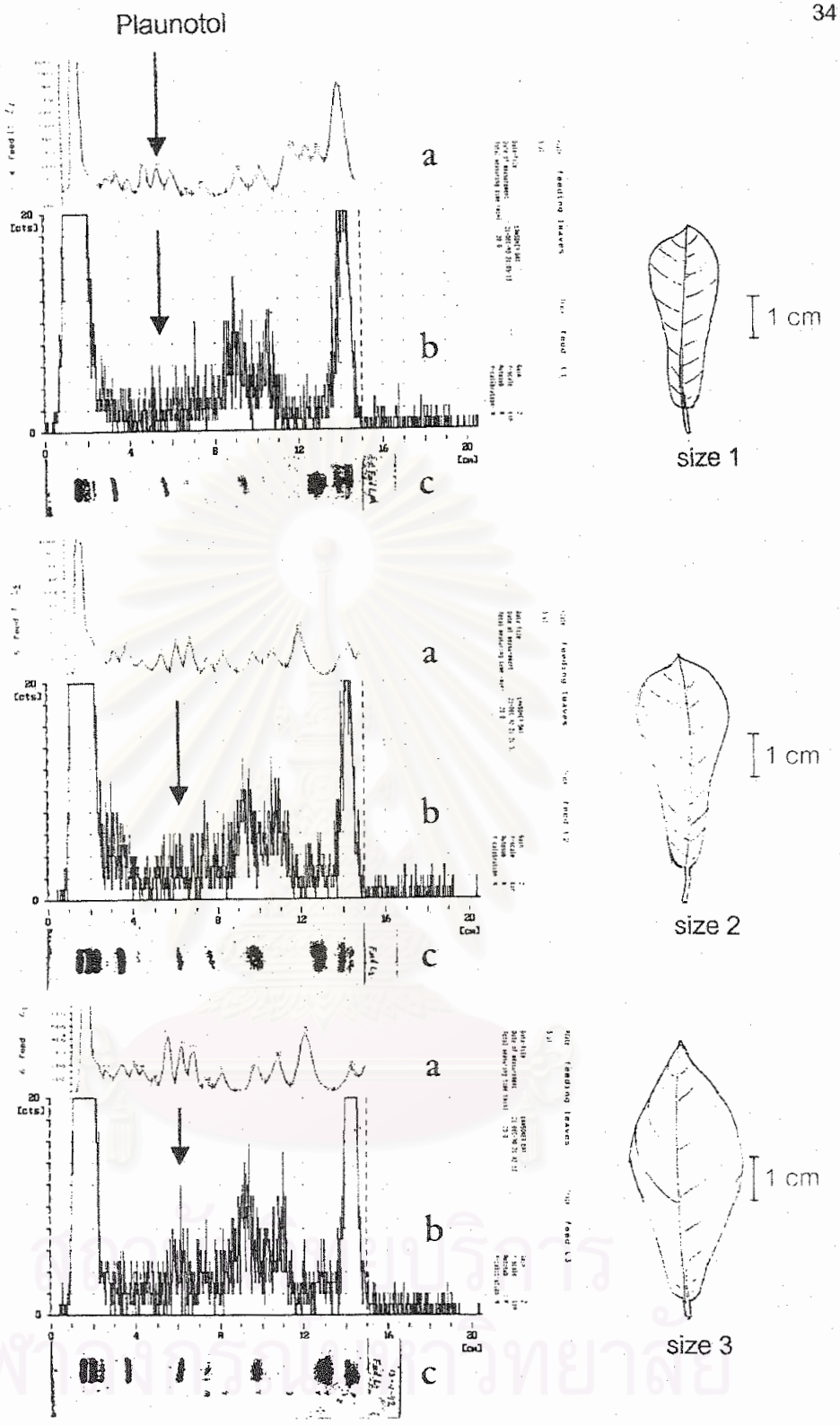


Figure 11 TLC-densitometric chromatogram (a), TLC-radiochromatogram (b), and TLC-plate being exposed with iodine vapor (c) of the leaf extracts obtained from [1-^{14}C]glucose incorporation of *C. sublyratus* leaves of size 1 (top), size 2 (middle) and size 3 (bottom). The position of plaunotol is indicated by an arrow.

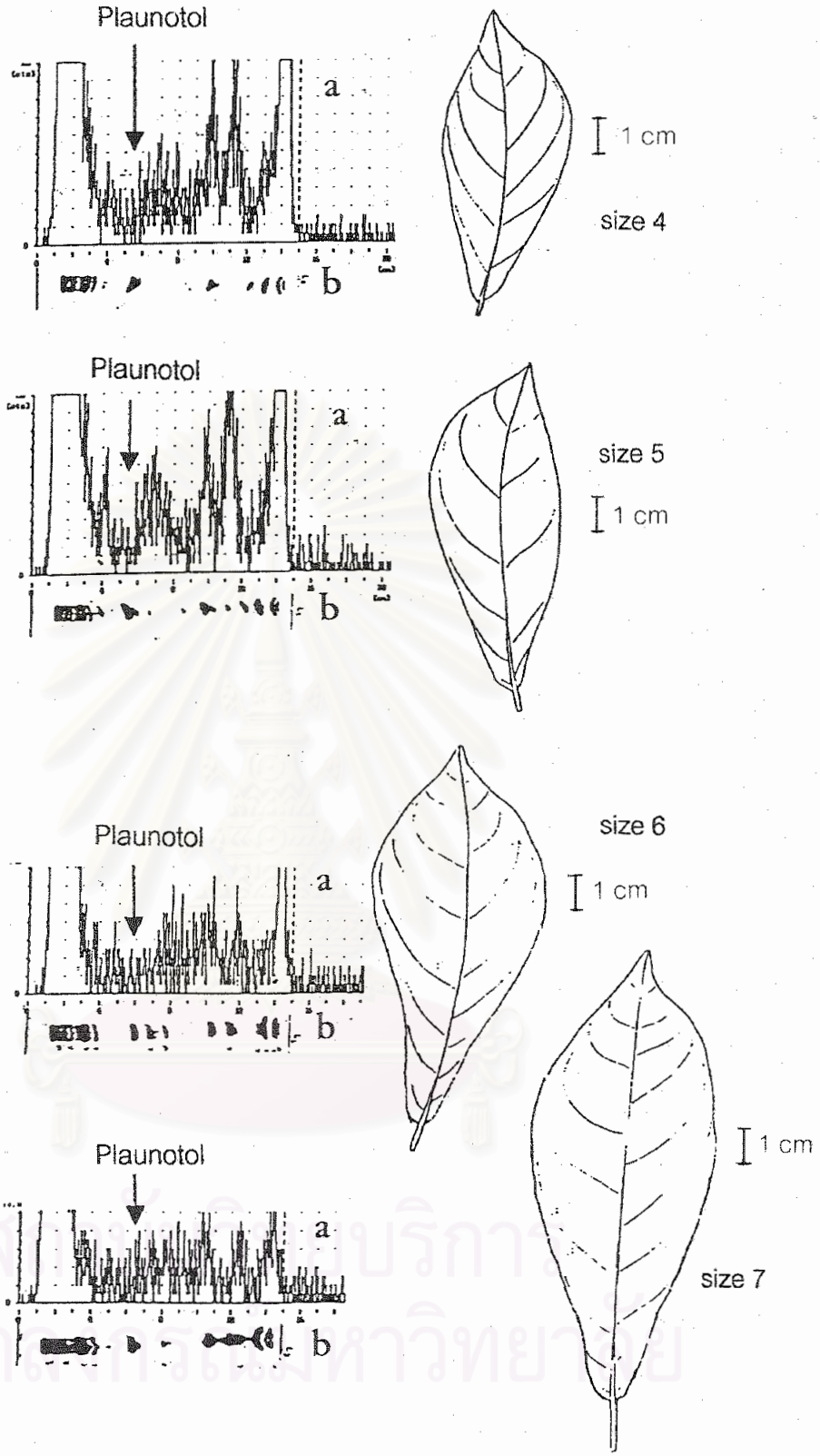


Figure 12 TLC-radiochromatogram (a), and TLC-plate being exposed with iodine vapor (b) of the leaf extracts obtained from [1-^{14}C]glucose incorporation of *C. sublyratus* leaves of size 4, 5, 6 and 7. The position of plaunotol is indicated by an arrow.

5. Establisment of Callus and Cell Cultures of *C. sublyratus*

5.1 Estabishment of *C. sublyratus* Callus Cultures

In a preliminary experiment, the effect of basal media on callus induction from *C. sublyratus* explants was studied. The young leaf explants were cultured on MS and B5 agar media containing **3% (w/v) sucrose**, 1.0 mg/l 2,4-D and 0.1 mg/l kinetin at 25$\pm$2^{o}C under controlled 16 hours of light period. Both MS and B5 media could induce callus formation from the leaf explants, but the developed calli appeared to grow more rapidly on MS medium than B5 medium. From these results, the leaf explants and the basal MS medium were chosen for subsequent studies. Firstly, the effect of the type and concentration of growth regulators on callus formation was investigated. It was found that callus formation was induced successfully on MS agar medium supplemented with the combination of 2,4-D (from 1.0 to 2.0 mg/l) and kinetin (from 0.5 to 1.0 mg/l). Especially, the combination of **2.0 mg/l 2,4-D and 1.0 mg/l kinetin** appeared to be the best **for callus formation**. As shown in Figure 13, the calli were initiated at the edge of leaf segments and formed as compact yellowish nodules after 2 weeks of incubation. The induced calli were **subcultured** successfully every 3 weeks on MS agar medium containing **3% (w/v) sucrose, 1.0 mg/l 2,4-D and 1.0 mg/l BA**. In this medium the callus had a high growth rate resulting in friable, soft and yellowish tissues (Figure 14A).

To obtain **green callus cultures**, the normal callus tissues were subcultured for a few times (3 weeks each) on MS agar medium containing **3% (w/v) sucrose, 1.0 mg/l 2,4-D, 0.1 mg/l NAA, and 0.1 mg/l BA.** Under these conditions, the yellowish callus gradually produced green pigment in the tissues (Figure 14B). These green calli were subsequently used for the analysis of plaunotol formation and for [1-^{13}C]glucose incorporation experiments.

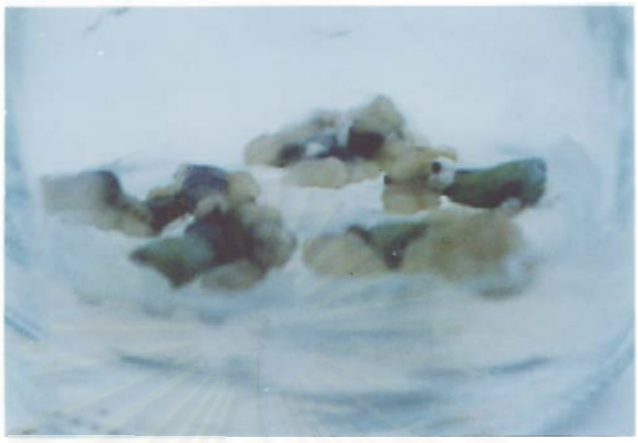


Figure 13 Induction of callus from the leaf explants of *C. sublyratus* on MS agar medium containing 2.0 mg/l 2,4-D and 1.0 mg/l kinetin.

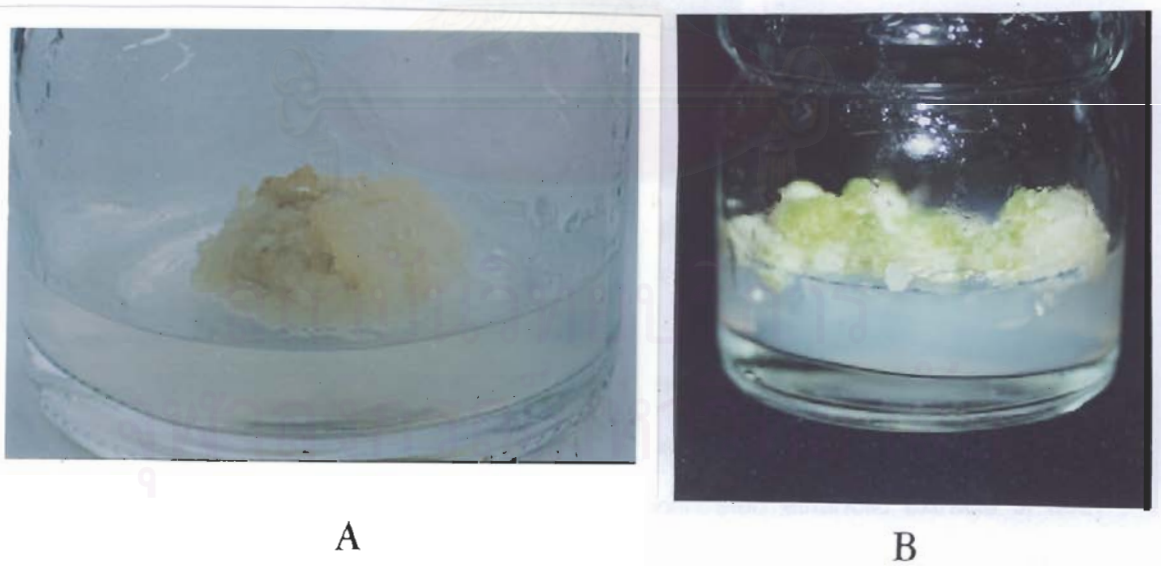


Figure 14 (A) Callus cultures of *C. sublyratus* on MS agar medium containing 1.0 mg/l 2,4-D and 1.0 mg/l BA. (B) Green callus cultures on MS agar medium containing 1.0 mg/l 2,4-D ; 0.1 mg/l BA, and 0.1 mg/l NAA.

5.2 Establishment of *C. sublyratus* Cell Suspension Cultures

Cell suspension cultures of *C. sublyratus* were obtained from the friable yellowish callus which were maintained by a regular subculture as described earlier. The callus tissues were separated into small aggregates before transferring into MS liquid medium containing 3% (w/v) sucrose , 1.0 mg/l 2,4-D; 0.1 mg/l BA and 0.1 mg/l NAA, and rotated at 120 rpm on a rotary shaker. The undifferentiated cultured cells under these conditions formed small yellowish aggregates (Figure 15). After stable cell suspension cultures were obtained, they were maintained in the same medium by subculture every 4 weeks.

Figure 15 Cell suspension cultures of *C. sublyratus* maintained in MS liquid medium containing 3% (w/v) sucrose, 1.0 mg/l 2,4-D ; 0.1 mg/l BA and 0.1 mg/l NAA.

6. Detection of Plaunotol in Callus and Cell Suspension Cultures of *C. sublyratus*

After a few passages of the callus and cell suspension cultures, they were examined for their ability to produce plaunotol. Concentrated ethanolic extracts of both culture types were prepared and their chemical constituents were separated by thin layer chromatography. Also, their TLC-densitometric chromatograms were produced by using TLC densitometer. In these experiments, the ethanolic extracts of the whole leaves were run parallel for comparison. The results of the TLC-densitometric chromatograms are shown in Figure 16. It clearly shows that plaunotol was not detected in the ethanolic extracts of the

callus (either yellow or green) and cell suspension cultures of *C. sublyratus*. The results are similar to previous reports that little or no plaunotol production was found in either callus cultures (Morimoto and Murai, 1989) or cell suspension cultures (Kitaoka, Nagashima and Kamimrua, 1989) of *C. sublyratus* .

7. Incorporation of [1-^{14}C] Glucose in the Callus Cultures of *C. sublyratus*

Although the callus cultures of *C. sublyratus* were not found to produce plaunotol, feeding experiments with [1-^{14}C]glucose were performed to observe the possibility of [1-^{14}C]glucose incorporation into any particular peak of the TLC chromatogram. In doing this, the callus cultures (3-weeks old) was exposed with 1 μCi D-[1-^{14}C]glucose (in water solution) for 3 days. The cells were then extracted under reflux with a solution of 95% ethanol and 10% NaOH (9:1). The crude ethanolic extract was then concentrated and partitioned with n-hexane. The hexane fraction was then concentrated and analyzed by both TLC-densitometer and TLC-radioscanner. It was found that *C. sublyratus* callus cultures had a very rapid incorporation of [1-^{14}C]glucose into a major unknown compound corresponding to the large peak with the Rf value of 0.54 (Figure 17). The observed high incorporation rate of [1-^{14}C]glucose into such a compound led us to pay attention to the identity of the compound. If the compound appeared to be either terpenoid or steroid type, study on the biosynthetic origin of isoprene unit was still possible. Therefore, the unknown labeled compound was subsequently isolated and identified.

In addition to the major peak, it was also found that the minor compound with its peak (Rf = 0.6) next to the major one was also labeled by [1-^{14}C] glucose. Its content, however, was too small to work out. Therefore, no attempt was made to isolate this compound.

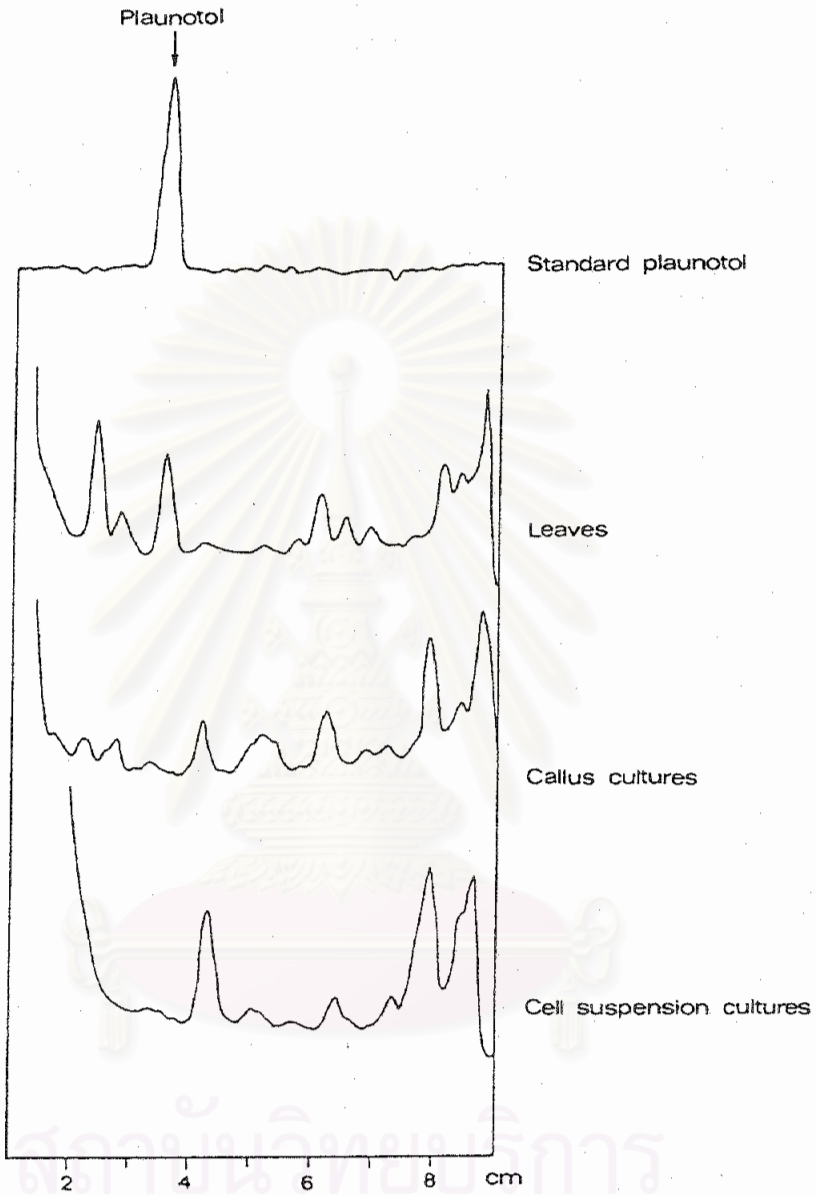


Figure 16 TLC-densitometric chromatograms of the extracts of leaves, callus and cell suspension cultures of *C. sublyratus*.

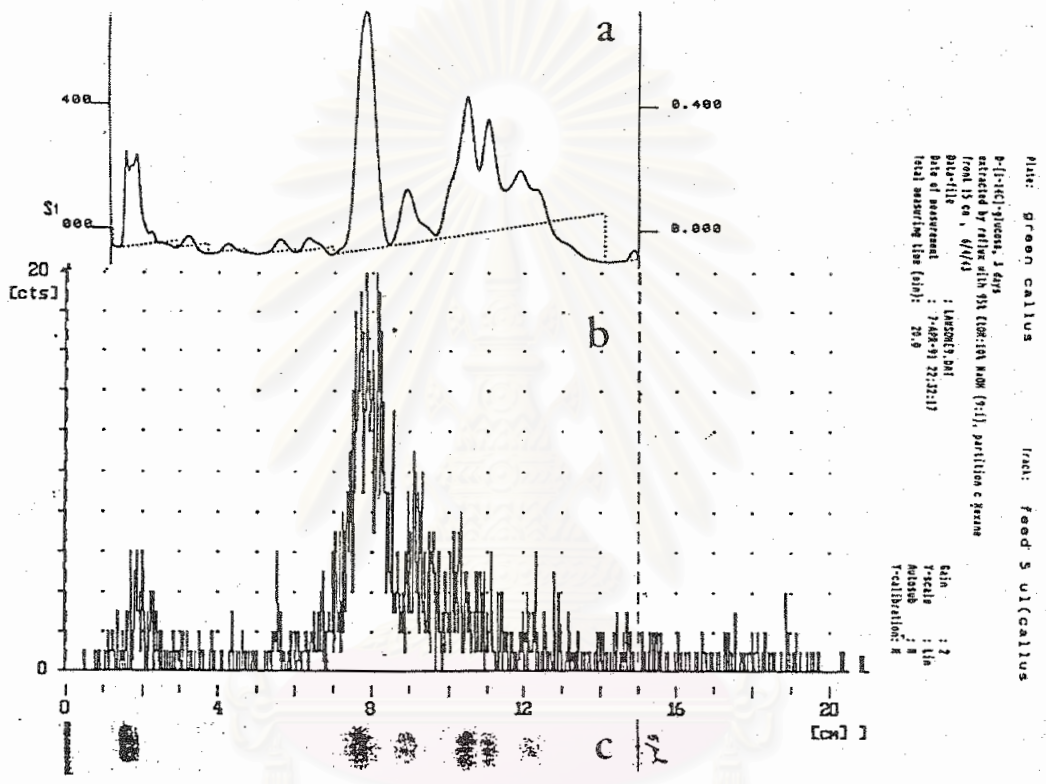


Figure 17 TLC-densitometric chromatogram (a), TLC-radiochromatogram (b), and TLC plate being exposed with iodine vapor (c) of the extract obtained from the 3 weeks old callus cultures of *C. sublyratus* after being incubated with [1-^{14}C]glucose for 3 days.

8. Product Identification of the Labeled Compound

To identify a major labeled compound found in the 3 weeks old callus cultures of *C. sublyratus* , approximately 300 gm fresh weight of the biomass was used for compound isolation. By preparative TLC as described in chapter III (Materials and Methods), a sufficient amount of pure compound was obtained for ^{1}H-NMR and ^{13}C-NMR spectroscopic analysis and identified as a mixture of β-sitosterol and stigmasterol (Figure 18). The resulting ^{1}H-NMR (Figure 19) and ^{13}C-NMR (Figure 20-21) spectra unambiguously corresponded with the data of the mixture reported previously (Wright *et al.*, 1978 ; Boonyaratavej, S. and Petsom, A., 1991). The ^{13}C NMR spectrum displayed 46 signals. Comparison of these data with reported values of β-sitosterol and stigmasterol is shown on Table 4.

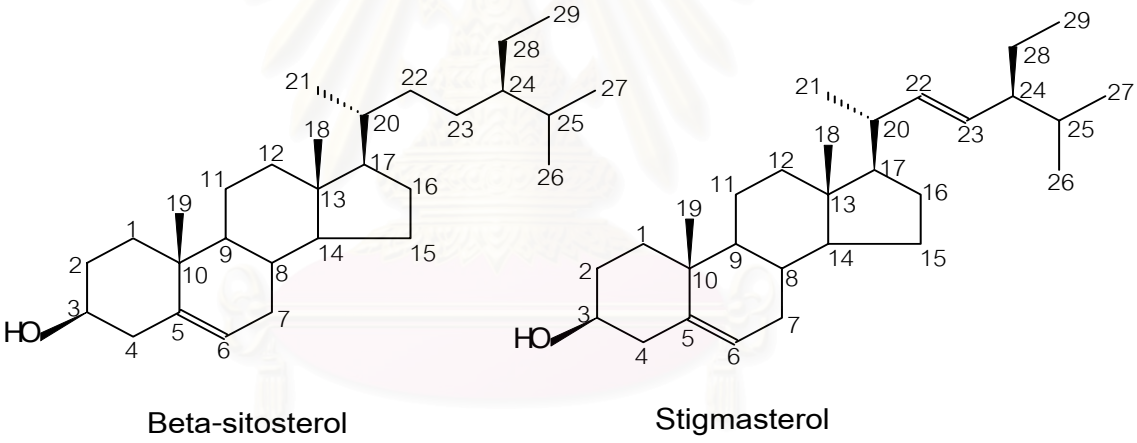


Figure 18 Chemical structures of β-sitosterol and stigmasterol

The isolated major labeled terpenoid could be deduced to be a mixture of β-sitosterol and stigmasterol in the ratio of 5: 5 or 1:1 by analysis of the integration steps of H-6, H-22, and H-23 in the ^{1}H NMR spectrum (Figure19). The signals at δ 5.00 (0.5H, dd, *J* = 15.0, 8.5 Hz), 5.13 (0.5H, dd, *J* = 15.3, 8.5 Hz) and 5.33 (1H, d, *J* = 5.2 Hz) were due to H-22 and H-23 of stigmasterol, and H-6 of β-sitosterol and stigmasterol respectively, with the integration steps ratio of 0.5: 0.5: 1.

Table 4 125.65 MHz ^{13}C NMR spectral data of the isolated major labeled compound (in $CDCl_3$) in comparison with reported values (Wright *et al.*, 1978) of β-sitosterol and stigmasterol.

Carbon	Chemical shift (ppm)			Assignments
position	β-sitosterol	Stigmasterol	Isolated compound	
1	37.31	37.31	37.22	$-CH_2-$
2	31.57	31.69	31.63	$-CH_2-$
3	71.69	71.81	71.80	-CH-OH
4	42.25	42.35	42.19 ; 42.26	$-CH_2-$
5	140.76	140.80	140.72	$=C<$
6	121.59	121.69	121.71	=CH-
7	31.92	31.94	31.87	$-CH_2-$
8	31.92	31.94	31.87	$>$CH-
9	50.17	50.20	50.10	$>$CH-
10	36.51	36.56	36.48	$>C<$
11	21.11	21.11	21.07	$-CH_2-$
12	39.81	39.74	39.74 ; 39.66	$-CH_2-$
13	42.33	42.35	42.26	$>C<$
14	56.79	56.91	56.73 ; 56.85	$>$CH-
15	24.32	24.39	24.29 ; 24.34	$-CH_2-$
16	28.26	28.96	28.24 ; 28.91	$-CH_2-$
17	56.11	56.06	56.02 ; 55.93	$>$CH-
18	11.87	12.07	11.84 ; 12.03	$-CH_3$
19	19.40	19.42	19.39	$-CH_3$
20	36.17	40.54	36.12 ; 40.48	$>$CH-
21	18.82	21.11	18.76 ; 21.07	$-CH_3$
22	33.95	138.37	33.91 ; 138.31	$-CH_2-$; =CH-
23	26.13	129.32	26.02 ; 129.25	$-CH_2-$; =CH-
24	45.85	51.29	45.81 ; 51.22	$>$CH-
25	29.18	31.94	29.11 ; 31.87	$>$CH-
26	19.84	21.26	19.80 ; 21.20	$-CH_3$
27	19.07	19.02	19.01 ; 18.96	$-CH_3$
28	23.09	25.44	23.04 ; 25.39	$-CH_2-$
29	12.32	12.27	11.97 ; 12.23	$-CH_3$

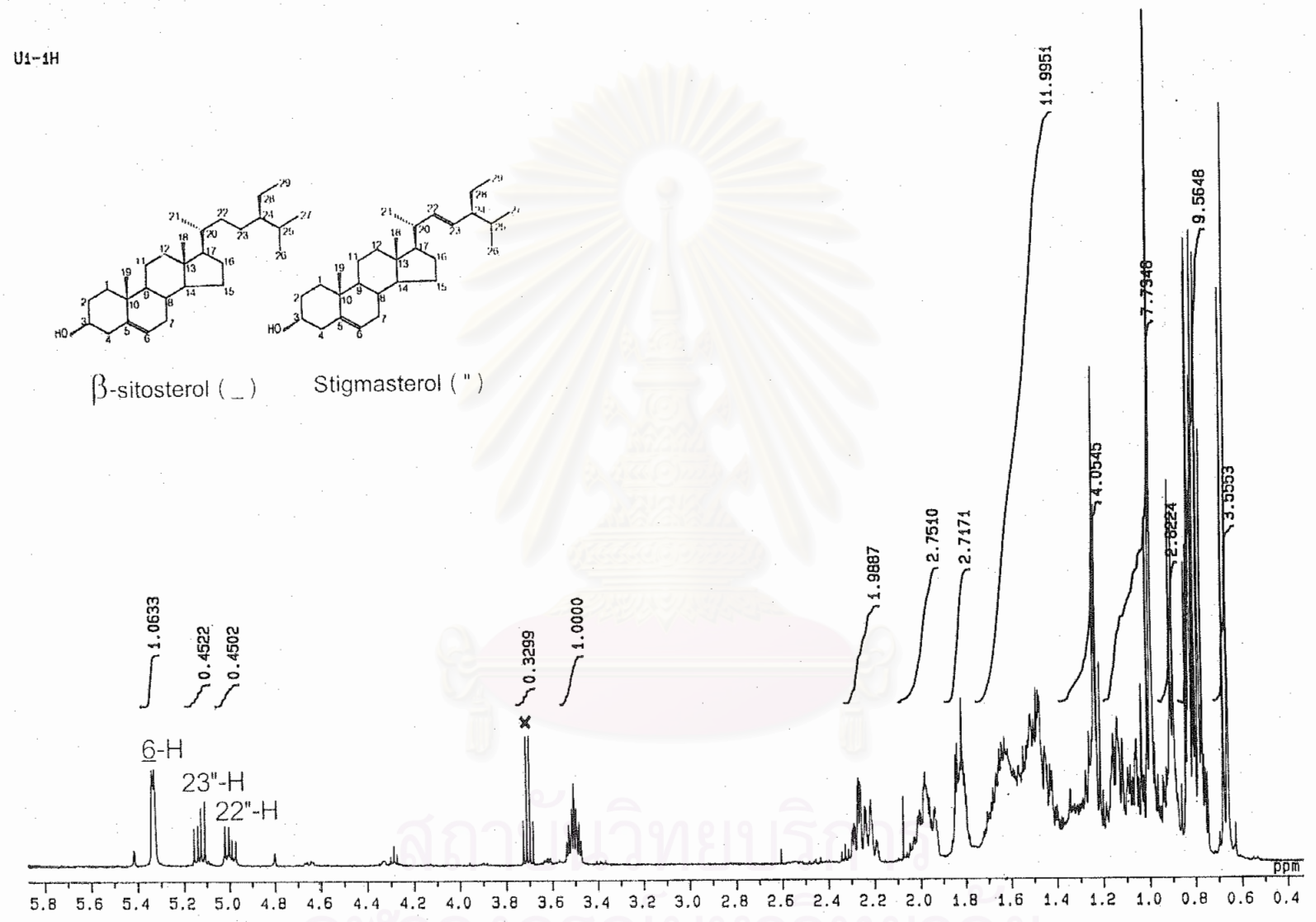
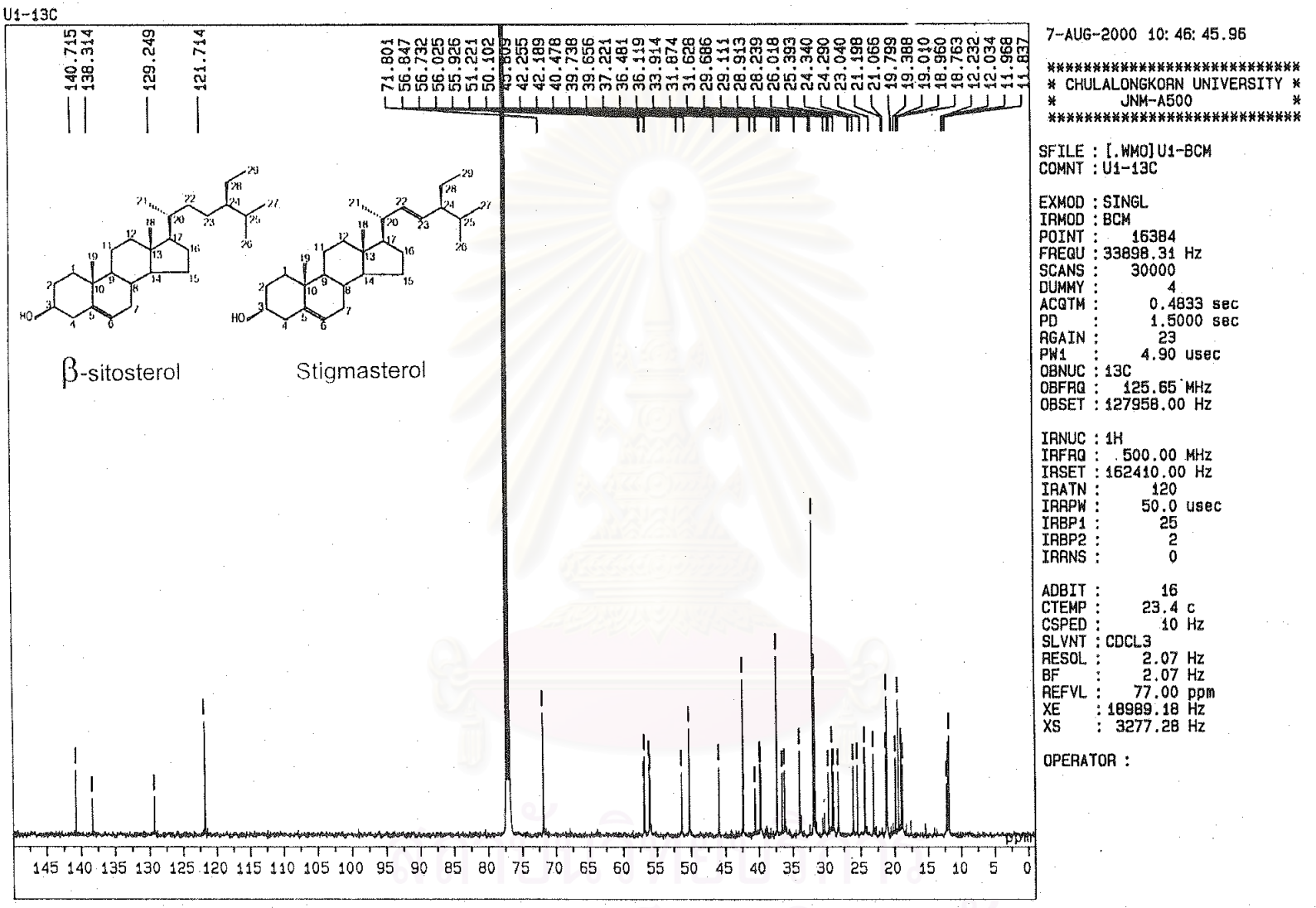


Figure 19 500 MHz ^{1}H NMR spectrum (in $CDCl_3$) of the major labeled compound found in the 3 weeks old green callus cultures of *C. sublyratus*.

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7-AUG-2000 10: 46: 45.96

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* CHULALONGKORN UNIVERSITY *
*        JNM-A500          *
**************************

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IRMOD : BCM
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SCANS :    30000
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PD    :   1.5000 sec
RGAIN :       23
PW1   :   4.90 usec
OBNUC : 13C
OBFRQ :  125.65 MHz
OBSET : 127958.00 Hz

IRNUC : 1H
IRFRQ :  500.00 MHz
IRSET : 162410.00 Hz
IRATN :      120
IRRPW :    50.0 usec
IRBP1 :       25
IRBP2 :        2
IRRNS :        0

ADBIT :       16
CTEMP :     23.4 c
CSPED :       10 Hz
SLVNT : CDCL3
RESOL :     2.07 Hz
BF    :     2.07 Hz
REFVL :    77.00 ppm
XE    : 18989.18 Hz
XS    :  3277.28 Hz

OPERATOR :
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Figure 20 125.65 MHz ^{13}C NMR spectrum (in $CDCl_3$) of the major labeled compound found in the 3 weeks old green callus cultures of *C. sublyratus*.

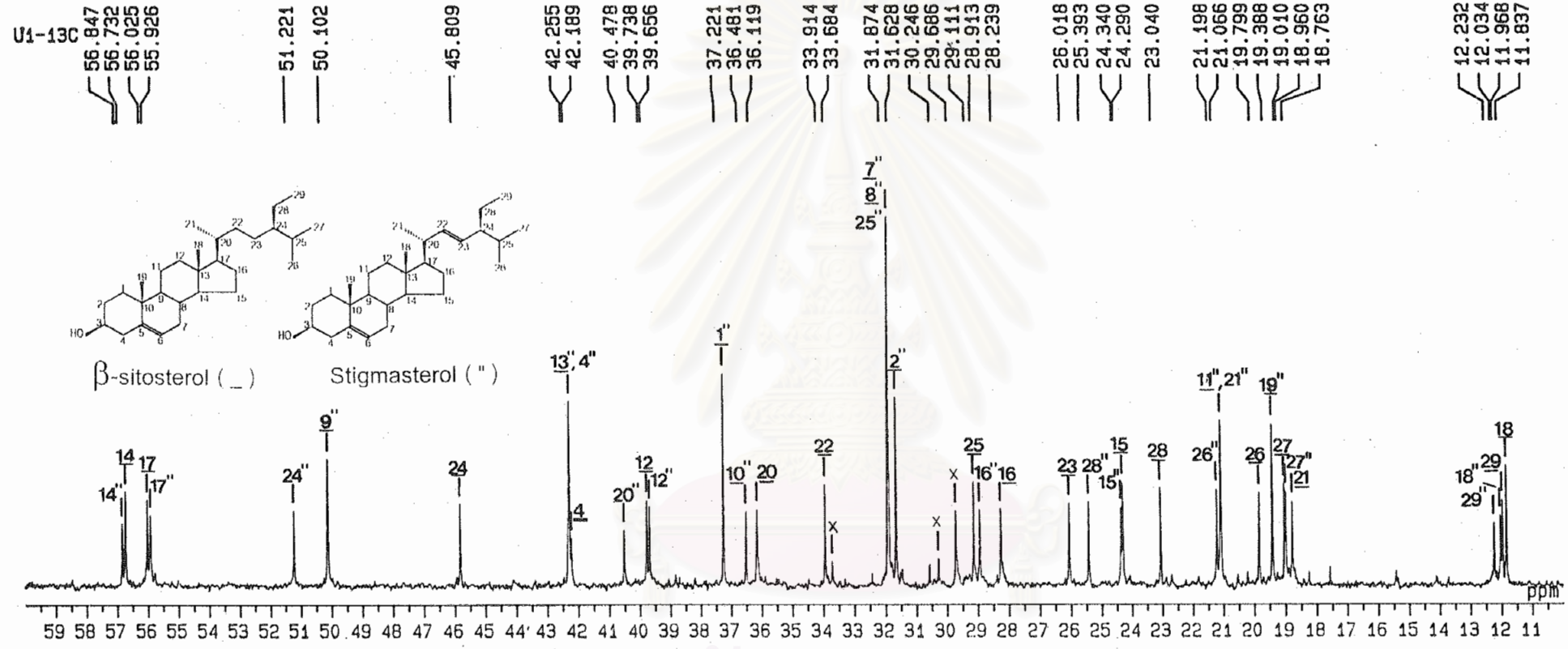
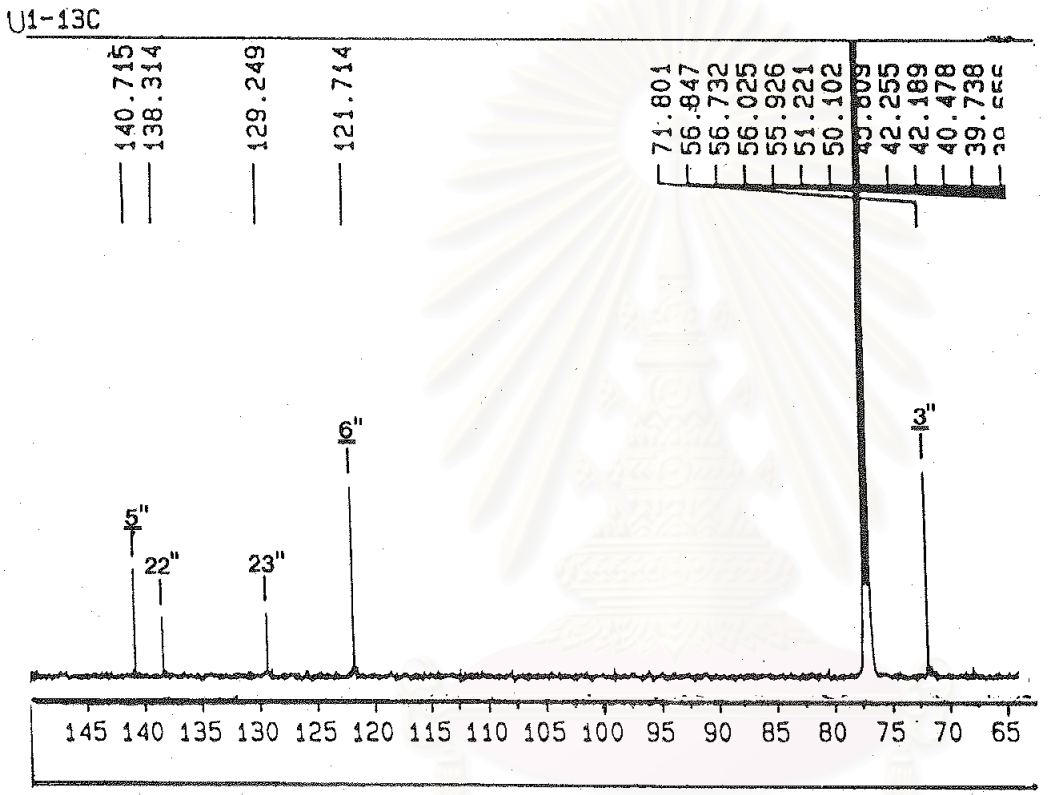
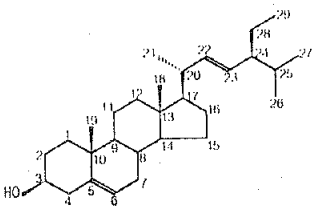
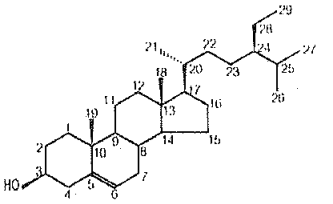


Figure 21 A 125.65 MHz ^{13}C NMR spectrum (in $CDCl_3$) of the major labeled compound found in the 3 weeks old green callus cultures of *C. sublyratus* (expanded from 11 to 59 ppm)





Stigmasterol (")



β-sitosterol (_)

Figure 21 B 125.65 MHz ^{13}C NMR spectrum (in $CDCl_3$) of the major labeled compound found in the 3 weeks old green callus cultures of *C. sublyratus* (expanded from 70-145 ppm)

9. Time-Course of Phytosterols Production in the Callus Cultures of *C. sublyratus*

Growth curve of *C. sublyratus* green callus cultures and production curve of phytosterols are shown in Figure 22A, corresponding to the results of TLC-densitometric chromatograms of callus extracts in Figure 22B. It can be seen that the growth reached maximum on day 21 as well as the production of phytosterols (β-sitosterol and stigmasterol). During the lag phase (day 1-9), the callus grew slowly with a little production of phytosterols. Although the ratio of phytosterols(mg)/ fresh weight of callus (gm) were relatively high during day 4-8, but the quantity of phytosterols was not high enough due to small biomass of the callus. The period from day 9-17 was the exponential phase of growth when callus proliferation was very active. The ratio of phytosterols/ fresh weight of callus was high from day 19-23 and reaching their highest peak on day 21. After day 21, the growth curve was slowly declined until the end of the 41 days of the culture cycle. The phytosterol production at this period, on the other hand rapidly declined and reached the lowest on day 27, then slowly enhanced during the end of the growth cycle.

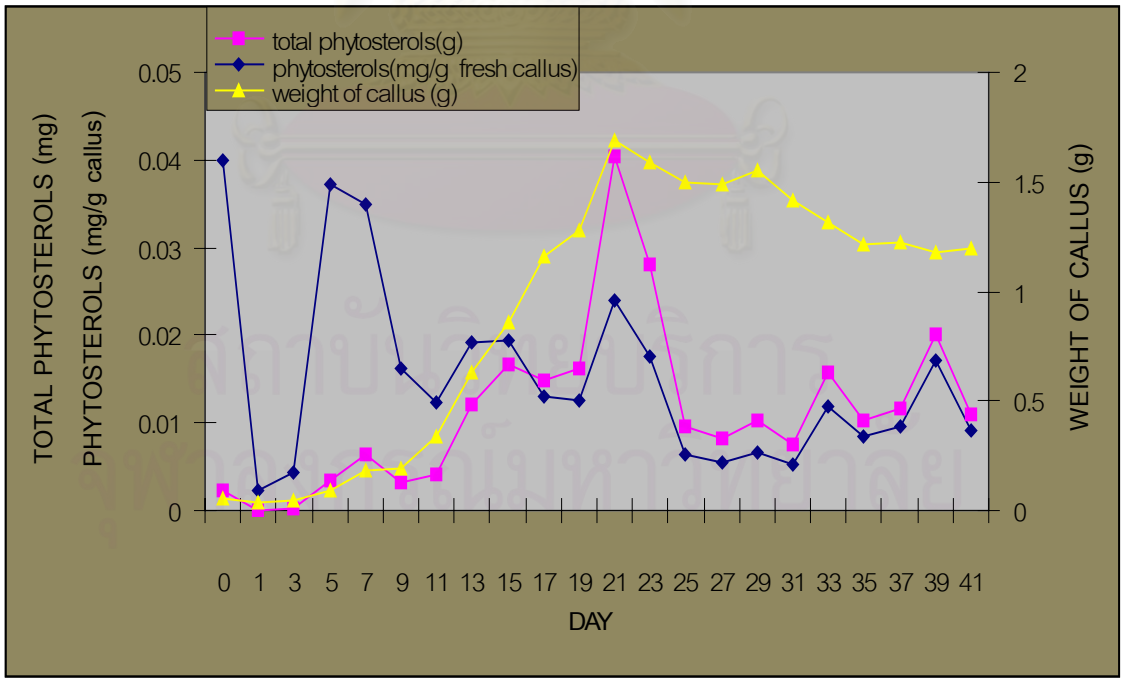


Figure 22A Growth curve of *C. sublyratus* green callus cultures and production curve of phytosterols.

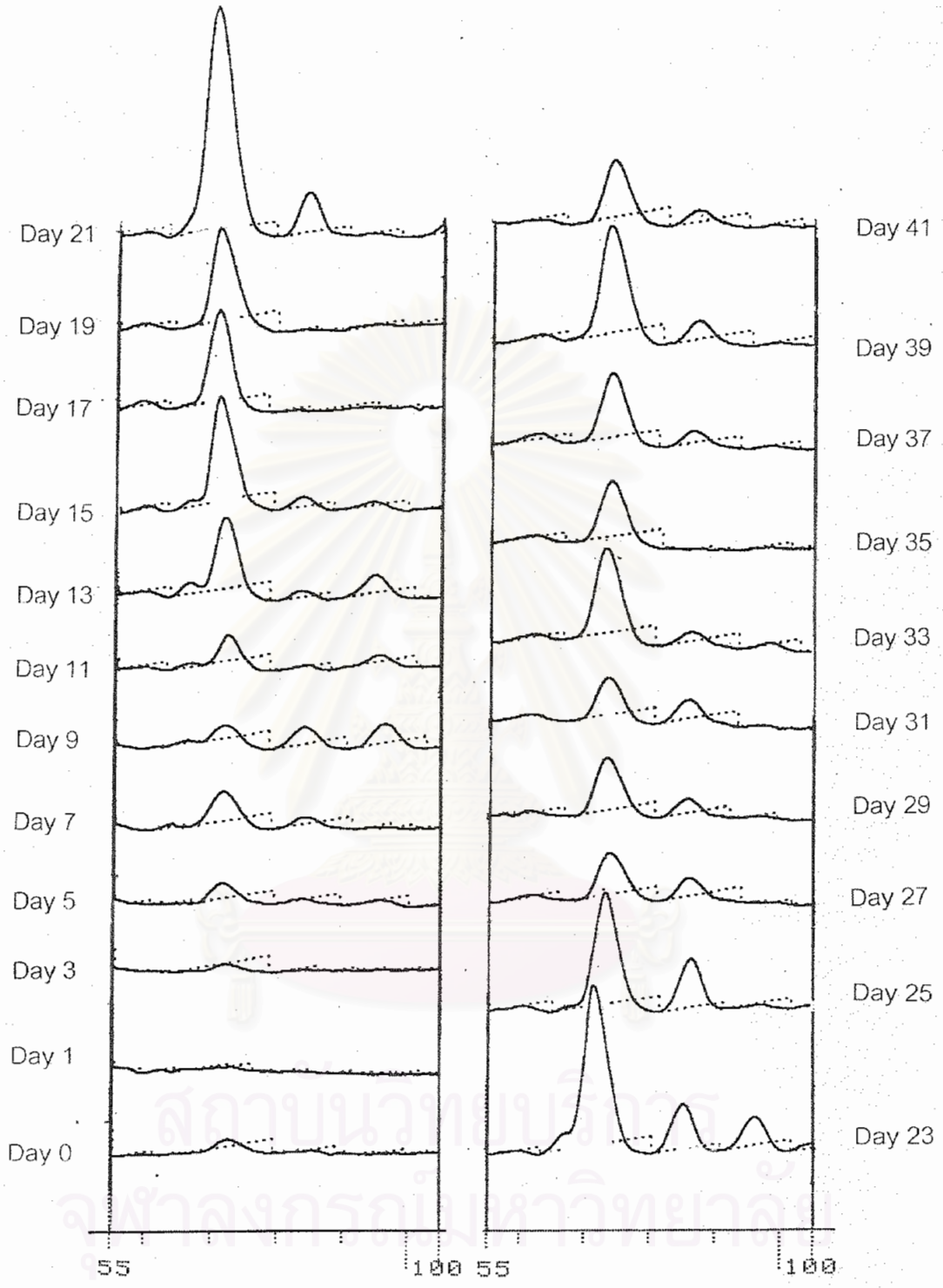


Figure 22 B TLC-densitometric chromatograms of a time-course of phytosterols production in the callus cultures of *C. sublyratus* from day 0 to day 41.

10. Optimization of [1-^{13}C]Glucose Incorporation into Phytosterols in *C. sublyratus* Callus Cultures

Although callus cultures of *C. sublyratus* were found to produce β-sitosterol and stigmasterol rather than plaunotol, the use of these callus cultures still allowed us to study the biosynthetic pathway of isoprene unit in this plant. It is widely known that steroid compounds are also biosynthesized from the isoprene unit. Therefore, the results of ^{13}C-labeling patterns of phytosterol molecules would also allow us to answer the question of isoprene origin.

In order to make sure that the amount of ^{13}C-enrichment in the phytosterol products was sufficient for NMR detection, optimization of [1-^{13}C]glucose incorporation was first carried out. In this experiment, two milliliters of 0.4% (w/w) [1-^{13}C]glucose with 99% ^{13}C enrichment was put into a sterile closed bottle containing 5 g of fresh callus by sterilization technique. The glucose solution was also added with 5 μl (1 μCi) of D-[1-^{14}C]glucose for monitoring the uptake and incorporation of glucose. This was necessary because this high glucose (0.4%) incorporation had not been investigated previously.

The results of a time-course of [1-^{13}C]glucose incorporation obtained from radio-monitoring of the radiolabeled phytosterols on a TLC plate showed that the highest incorporation of glucose was found on day 3 after the incubation. As shown in Figure 23, it can be seen that the radioactive peak of the phytosterols increased continuously from day 1 and reached a maximum point on day 3. From these results, the large-scale [1-^{13}C]glucose incorporation was, therefore, carried out for 3 days before harvesting the callus for compound extraction and isolation.

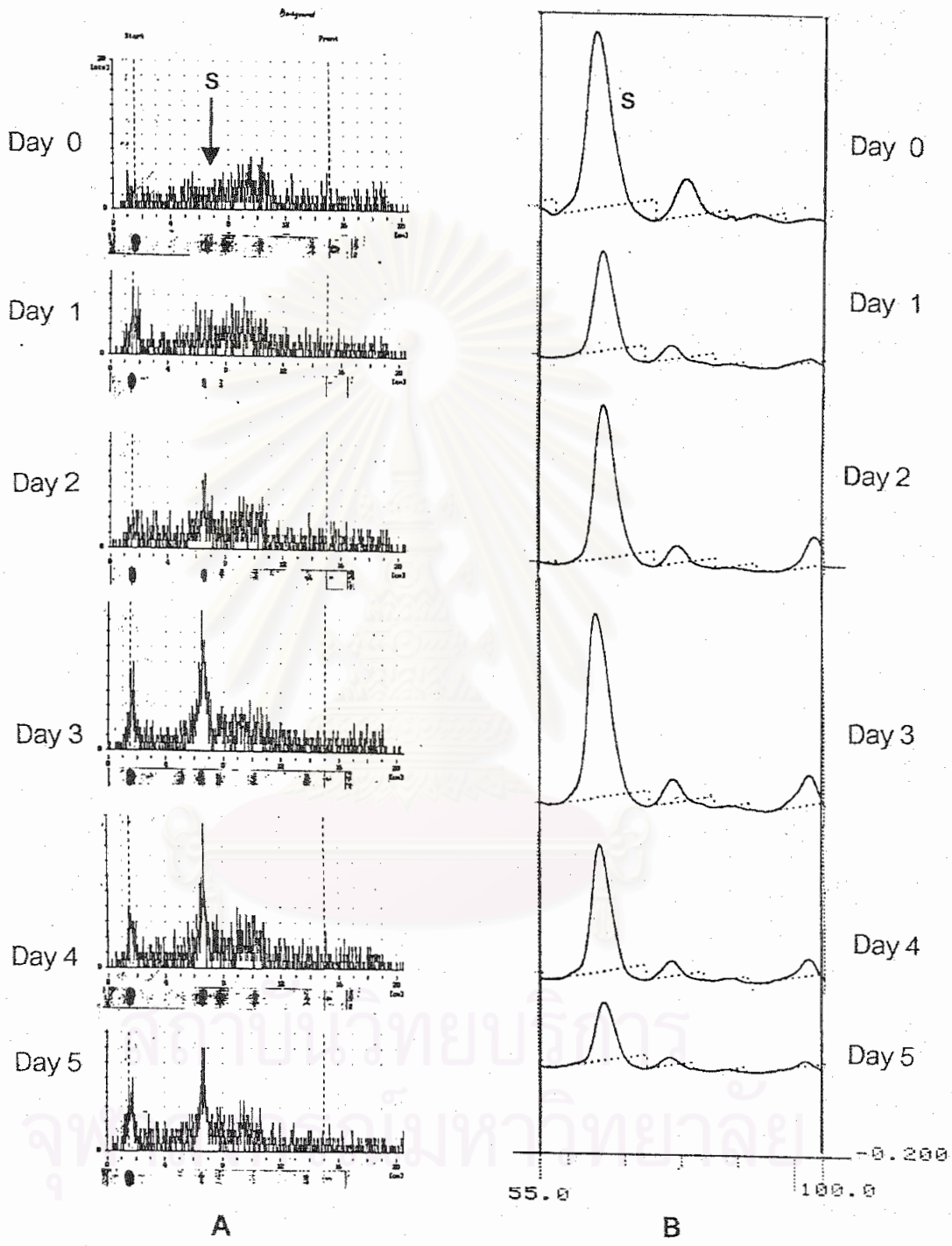


Figure 23 A time-course of [1-^{13}C]glucose incorporation experiment obtained from the radio-monitoring of the incorporation of [1-^{14}C] glucose into the phytosterols of *C. sublyratus* callus cultures. (A) TLC-radiochromatograms showing radioactive incorporation into the phytosterols during 1-5 days of the experiment. (B) TLC-densitometric chromatograms of the same time-course showing the highest production of the major labeled terpenoid compound **S** on day 3.

11. ^{13}C-Labeling Patterns of Phytosterols

After the 3-week-old callus cultures of *C. sublyratus* was incubated with **0.4% (w/w) [1-^{13}C]glucose** for 3 days, the callus tissues were harvested, extracted and isolated to obtain a mixture of ^{13}C-labeled β-sitosterol and stigmasterol (**S**). In parallel, another set of incorporation experiment with **0.4%(w/w) unlabeled glucose** was also performed to obtain a mixture of the same but non-labeled phytosterols (**U**). This control experiment was carried out in order to see the result of ^{13}C-enrichment more clearly when the ^{13}C-NMR patterns of the phytosterols obtained from the two conditions were compared to each other. Although it is generally difficult to separate β-sitosterol and stigmasterol from one another, it is fortunate that the ^{13}C-NMR data of each compound have been well studied (Wright *et al.*, 1978 ; Boonyaratavej, S. and Petsom, A., 1991) with the chemical shift value for each carbon completely assigned .

Figure 24 compares the ^{13}C-NMR spectra of the compounds obtained from the unlabeled glucose incorporation and [1-^{13}C]glucose incorporation (Figure 24A and 24B). It can be seen in general that there was an increase in the peak intensity of many carbons in the phytosterol molecules obtaining from the [1-^{13}C]glucose-incorporated callus. Particularly, there were **^{13}C-enrichment** at the positions of carbons **1, 2, 3, 5, 6, 7, 9, 11, 12, 13, 15, 16, 17, 18, 19, 21, 22, 23, 24, 26, 27, 28 and 29.** On the other hand, other carbon atoms of number 4, 10 and 20 were virtually not labeled.

For quantitative analysis, it was possible to calculate the degree of ^{13}C-enrichment based on the ^{13}C peak intensity values of the unlabeled glucose feeding. As shown in Table 5, it was found that the intensity **ratio of S:U for the enriched carbons** were generally **higher than** the value of **1.2**, whereas the S:U ratio of the non-enriched carbons were approximately 1.0 or lower.

Based on the results in Table 5, we could summarize the pattern of ^{13}C-enrichment in the molecules of stigmasterol and β-sitosterol as shown in Figure 25. Among these

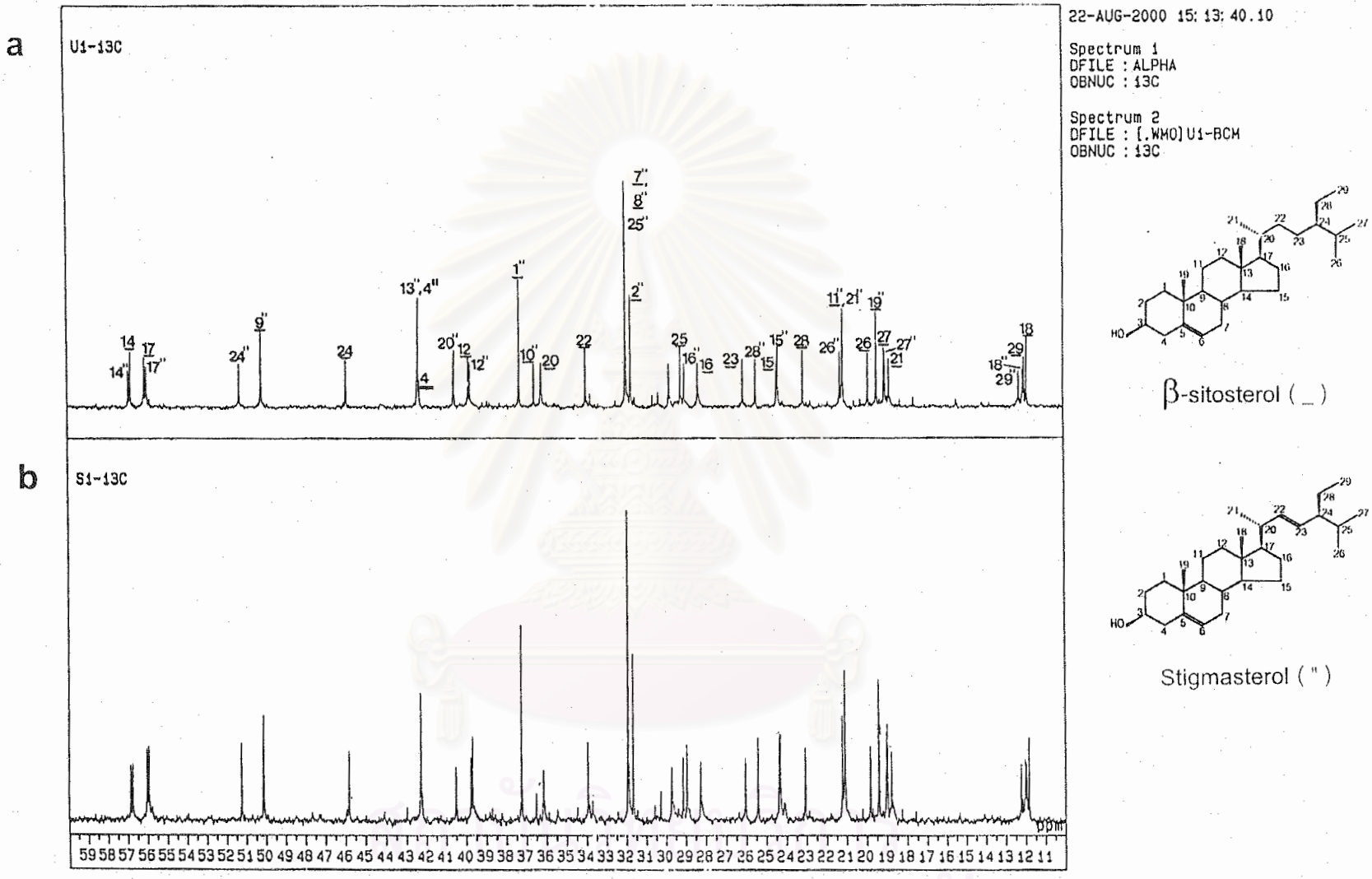
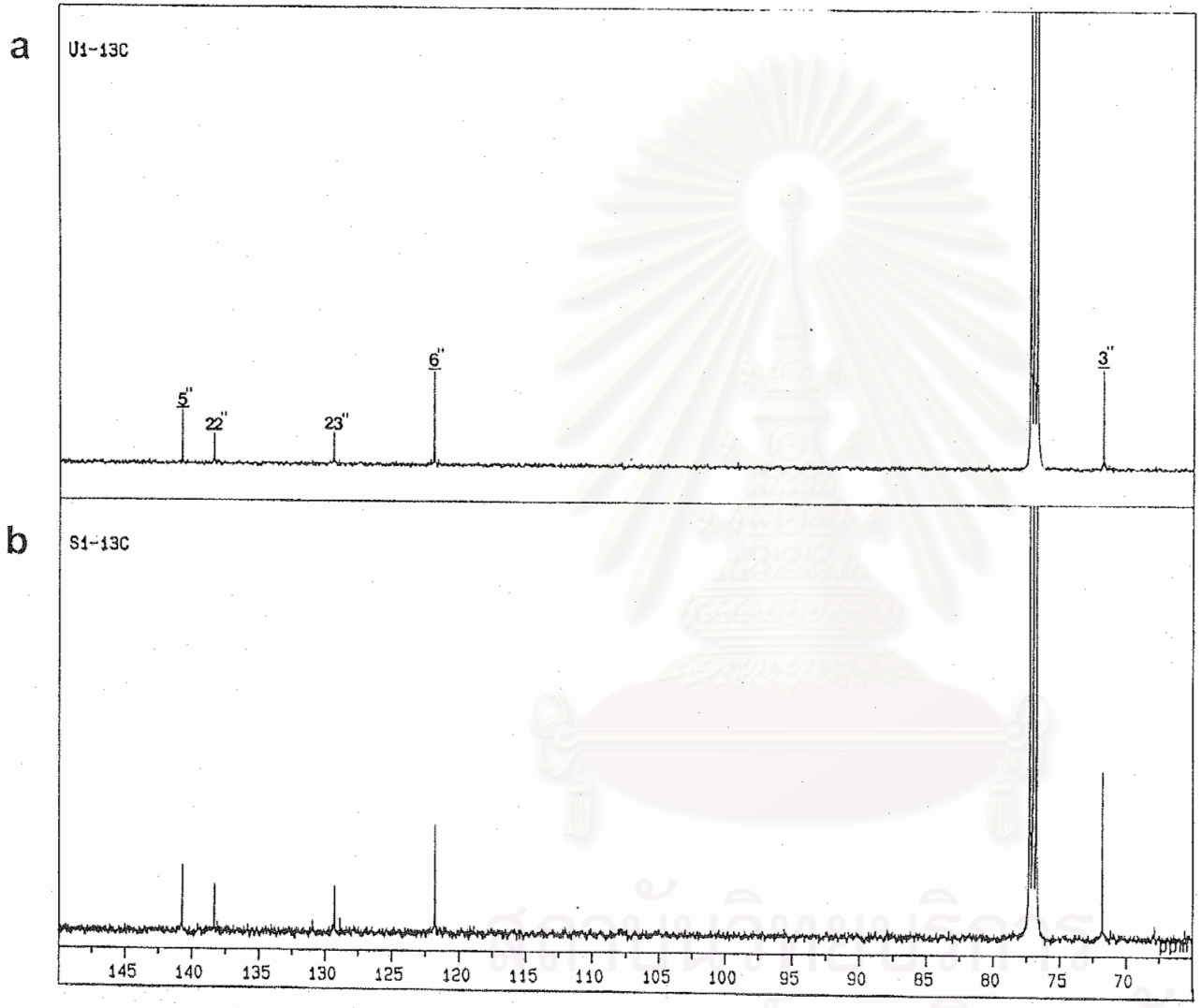
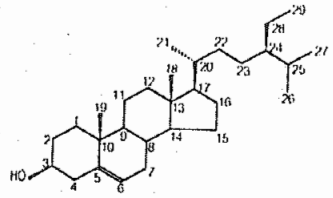
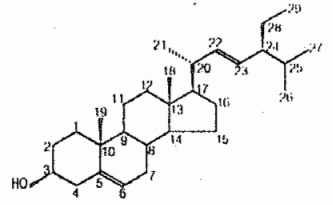


Figure 24A Comparison of 125.65 MHz ^{13}C NMR spectra (expanded from 11 to 58 ppm) of a mixture of phytosterols (β-sitosterol and stigmasterol) obtained from callus cultures of *C. sublyratus* : (a) unlabeled glucose incorporation, and (b) [1-^{13}C]glucose incorporation.



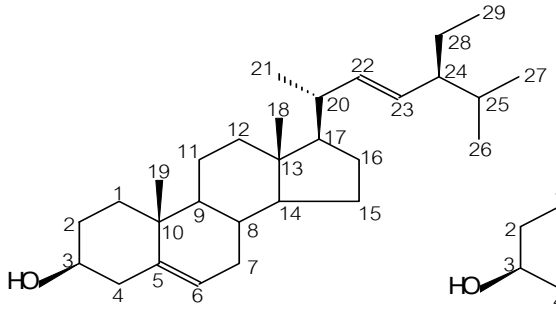


β-sitosterol (_)

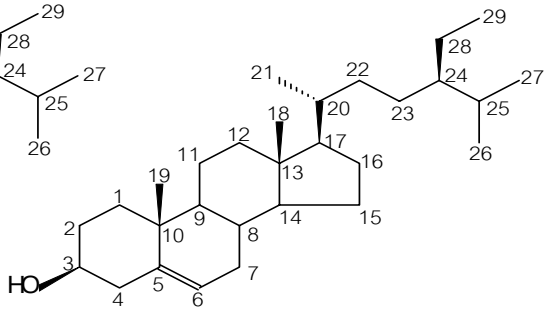


Stigmasterol (")

Figure 24B Comparison of 125.65 MHz ^{13}C NMR spectra (expanded from 70 to 145 ppm) of a mixture of phytosterols (β-sitosterol and stigmasterol) obtained from callus cultures of *C. sublyratus* : (a) unlabeled glucose incorporation, and (b) [1-^{13}C]glucose incorporation.



stigmasterol (symbol ")



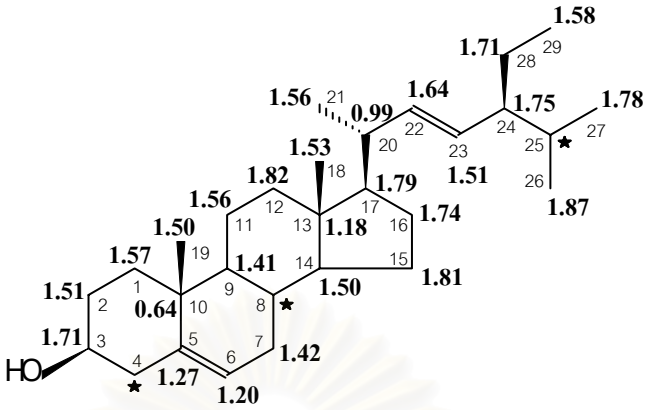
β-sitosterol (symbol _)

Table 5 The ^{13}C-NMR signal intensity of each peak related to the highest peak (100%, chemical shift 31.87 ppm) obtaining from both the [1-^{13}C]glucose incorporation (**S**) and unlabeled-glucose incorporation (**U**). Each intensity value was then calculated on the basis of the intensity of C4 of β-sitosterol (100%) which is not involved in the ^{13}C-enrichment from either mevalonate or non-mevalonate pathway . The ratio of S/U values are also shown to indicate the degree of enrichment at each peak. The carbon number with " means belonging to stigmaterol whereas _ belonging to β-sitosterol.

Carbon No.	Chemical shift (ppm)	S		U		ratio S/U
		Intensity (% of max)	Readjusted Intensity (4= 100%)	Intensity (% of max)	Readjusted Intensity (4= 100%)	
5"	140.72	18.616	208.583	20.918	164.501	1.27
22"	138.31	13.546	151.776	11.739	92.317	1.64
23"	129.25	13.157	147.417	12.453	97.932	1.51
6"	121.71	30.641	343.317	36.347	285.837	1.20
3"	71.8	46.839	524.807	38.997	306.677	1.71
14"	56.85	17.536	196.482	16.631	130.788	1.50
14	56.73	17.834	199.821	25.239	198.482	1.01
17	56.02	22.905	256.639	23.031	181.118	1.42
17"	55.93	23.884	267.608	19.004	149.450	1.79
24"	51.22	24.843	278.353	20.184	158.729	1.75
9"	50.1	33.743	378.073	33.996	267.348	1.41
24	45.81	22.726	254.633	21.897	172.200	1.48
13",4"	42.26	41.023	459.641	49.485	389.155	1.18
4	42.19	8.925	100.000	12.716	100.000	1.00
20"	40.48	17.337	194.252	24.933	196.076	0.99
12	39.74	20.502	229.714	22.757	178.964	1.28
12"	39.66	27.124	303.910	21.231	166.963	1.82

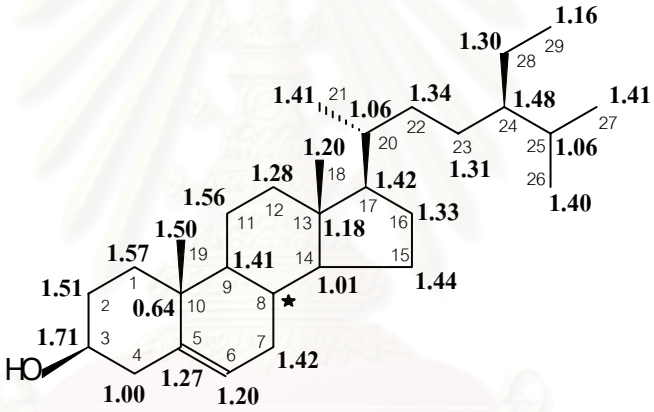
Table 5 (continued)

Carbon No.	Chemical shift (ppm)	S		U		ratio S/U
		Intensity (% of max)	Readjusted Intensity (4 = 100%)	Intensity (% of max)	Readjusted Intensity (4 = 100%)	
1"	37.22	62.591	701.300	56.924	447.656	1.57
10"	36.48	8.929	100.045	19.868	156.244	0.64
20	36.12	16.343	183.115	20.505	171.254	1.06
22	33.91	25.479	285.479	27.022	212.504	1.34
7",8",25"	31.87	100.000	1120.448	100.000	786.411	1.42
2"	31.63	53.571	600.235	50.560	397.609	1.51
25	29.11	20.601	230.824	27.617	217.183	1.06
16"	28.91	24.785	277.703	20.326	159.846	1.74
16	28.22	19.047	213.412	20.386	160.318	1.33
23	26.02	20.212	226.465	22.026	173.215	1.31
28"	25.39	26.886	301.244	22.360	175.841	1.71
15"	24.34	26.530	297.255	20.872	164.140	1.81
15	24.29	27.880	312.381	27.522	216.436	1.44
28	23.04	23.651	264.997	25.986	204.357	1.30
26"	21.2	33.709	377.692	25.621	201.486	1.87
11",21"	21.07	48.280	540.952	44.037	346.312	1.56
26	19.8	24.205	271.204	24.705	194.283	1.40
19"	19.39	45.459	509.345	43.130	339.179	1.50
27	19.01	26.627	298.342	26.981	212.182	1.41
27"	18.96	31.393	351.742	25.088	197.295	1.78
21	18.76	22.177	248.482	22.479	176.777	1.41
29"	12.23	18.351	205.613	16.579	130.379	1.58
18"	12.03	19.920	223.193	18.526	145.690	1.53
29	11.97	18.560	207.955	22.789	179.215	1.16
18	11.84	26.682	298.958	31.704	249.324	1.20



Stigmasterol

* not determined due to signal overlapping



β-sitosterol

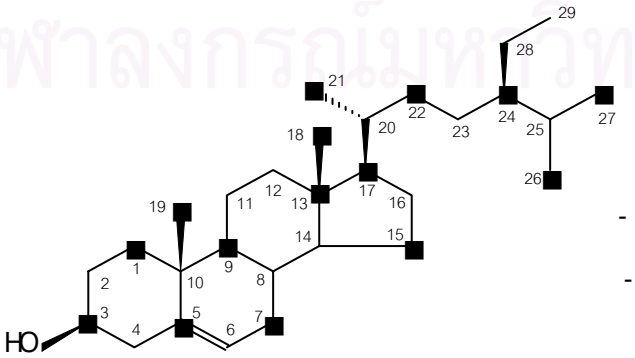
* not determined due to signal overlapping

Figure 25 Summary of the degree of ^{13}C-enrichment at various carbon atoms of stigmasterol and β-sitosterol. Both phytosterols were obtained as a mixture from *C. sublyratus* callus cultures which had been incorporated with [1-^{13}C]glucose. A control incorporation experiment was also carried out in parallel with unlabeled glucose. The value indicated at a particular carbon is the ratio of ^{13}C-NMR signal intensity of the ^{13}C-labeled : the non-labeled (**S:U**). The values at the carbons with * could not determined due to signal overlapping. The values obtained from the overlapping signals of the same carbons of both phytosterols are indicated to both carbons of the compounds (i.e. carbon numbers 1, 2, 3, 25, etc.). Clarification of the contribution to each value is given in the text.

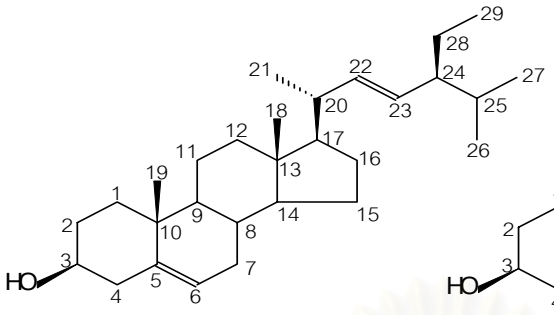
^{13}C-enrichment values, however, some of them were the results of more than one carbon signals and thus made it difficult to interprete the isoprene unit origin in *C. sublyratus* callus cultures. Analysis of the NMR data was therefore needed to clarify the observed ^{13}C-enrichment patterns of the phytosterols.

In order to digest the ^{13}C-enrichment data systematically, the ^{13}C-signal intensity ratios of **carbons number 12, 16, 20, 24, 26 and 27** of stigmasterol and β-sitosterol were first analyzed both in the conditions of unlabeled glucose incorporation (**U**) and [1-^{13}C]glucose incorporation (**S**). These carbon atoms had their own ^{13}C-signals with no overlapping from the signals of other carbons. It was found that in the **U** condition, all carbon atoms showed the ratio of **stigmasterol ^{13}C-signal : β-sitosterol ^{13}C-signal** close to the value of **1.0** (average 1.006±0.08) (Table 6). This suggested that the mixture of stigmasterol and β-sitosterol extracted from the callus cultures contained both sterols in almost equal amount. With the [1-^{13}C]glucose incorporation (**S**), on the other hand, the ratios were different and appeared to increase to an average value of **1.225 ± 0.114** (Table 6). Since both phytosterols should also be present in equal amount of 1:1 (like the U condition), it was concluded that **stigmasterol was enriched with ^{13}C approximately 1.23-fold more effective than β-sitosterol.**

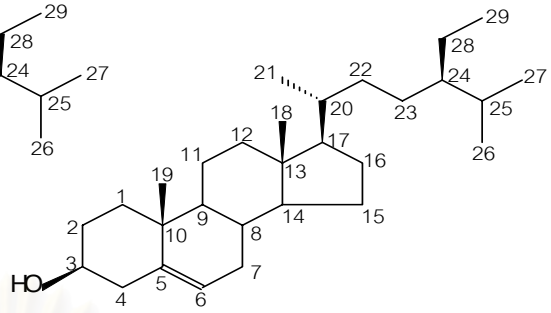
If considering the ^{13}C-labeling pattern of the phytosterols based on **mevalonate pathway**, one would expect the following pattern :



- β-Sitosterol
- $C_{22} = C_{23}$: Stigmasterol



Stigmasterol



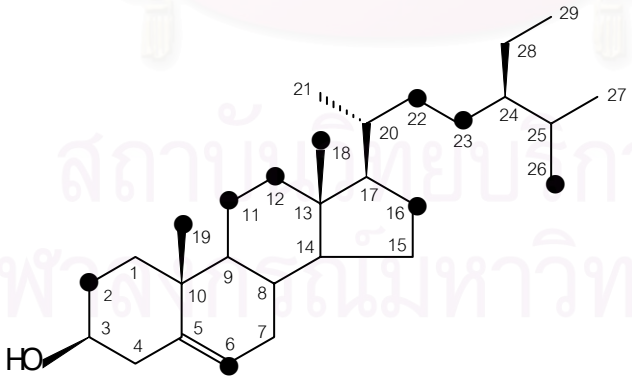
β-sitosterol

Table 6 The ratio of ^{13}C-NMR signal intensities of some carbons at the same numbering of stigmasterol and β-sitosterol. Both phytosterols were isolated as a mixture from *C. sublyratus* callus cultures after incorporation with unlabeled glucose or [1-^{13}C]glucose. The carbons selected for this calculation were those whose signals were **not overlapped** with other carbon signals in the ^{13}C-NMR spectra of both incorporation conditions.

	Incorporation of unlabeled glucose			Incorporation of [1-^{13}C]glucose		
Carbon no.	% Intensity of highest peak		**stigmasterol** / **β-sitosterol**	% Intensity of highest peak		**stigmasterol** / **β-sitosterol**
	stigmasterol	β-sitosterol		stigmasterol	β-sitosterol	
12	21.231	22.757	0.933	27.124	20.502	1.323
16	20.326	20.386	0.997	24.785	19.047	1.301
20	24.933	20.505	1.215	17.337	16.343	1.061
24	20.184	21.897	0.922	24.843	22.726	1.093
26	25.621	24.705	1.037	33.709	24.205	1.392
27	25.088	26.981	0.930	31.393	26.627	1.179
Average			1.006±0.080			1.225±0.114

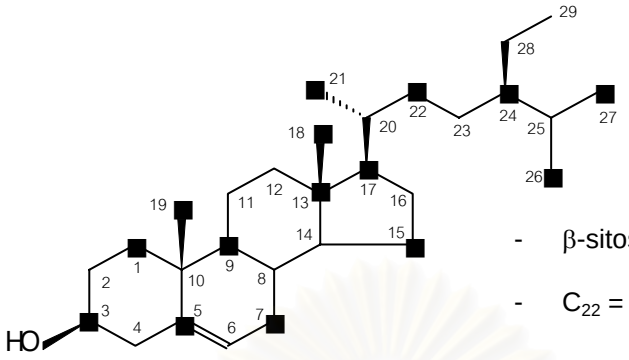
According to the enrichment values shown in Figure 25, it was obvious that all carbons in the above labeling pattern were enriched from the [1-^{13}C]glucose incorporation. These suggested that the isoprene units used for the phytosterols formation were biosynthesized *via* the mevalonate pathway. However, in order to obtain information on the average values of ^{13}C enrichment based on S/U ratio, the carbons number **15,17,24 and 26** of both stigmasterol and β-sitosterol were chosen for averaging their S/U ratio values. These carbons gave ^{13}C-NMR signals that were not overlapped by other carbon signals and therefore gave accurate values of ^{13}C enrichment of both sterols based on mavalonate pathway of isoprene biosynthesis. As shown in Table 7, it was found that the ^{13}C-enrichment of the labeled carbons in **stigmastorol** were relatively high with the average **S/U** value of **1.80±0.04** whereas the values of the same labeled carbons of **β-sitosterol** appeared to be lower significantly (average **1.43±0.04**). For the carbons number 1,3,5,7 and 9 in which their signals representing the combined effect of the two sterols, the average value of 1.48±0.13 was obtained.

If considering the ^{13}C-labeling pattern of the phytosterols based on **non-mevalonate pathway**, one would expect the following pattern :



- β-Sitosterol
- $C_{22} = C_{23}$: Stigmasterol

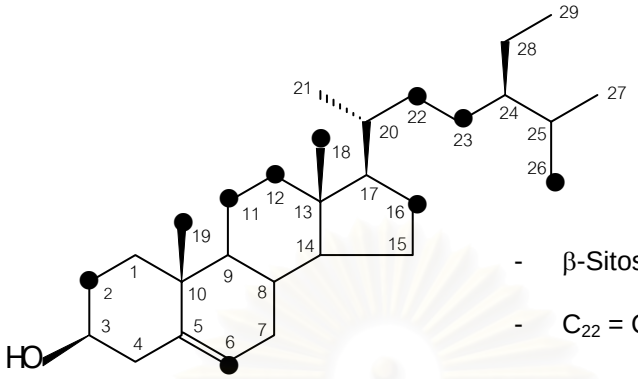
Again the ^{13}C enrichment values shown in Figure 25 also appeared to cover such a non-mevalonate pathway labeling pattern. This suggested that the non-mevalonate pathway was also functioning in the callus cultures of *C. sublyratus* to produce isoprene units for the biosynthesis of both phytosterols. Based on the results in Table 8 which were obtained in a similar way to those in Table 7, it was found that the ^{13}C-enrichment (**S/U**) of the labeled



- β-sitosterol
- $C_{22} = C_{23}$: Stigmasterol

Table 7 ^{13}C-Enrichment *via* the **mevalonate pathway** of some carbon atoms in the molecules of stigmasterol and β-sitosterol. The carbon atoms were selected based on the ones to be labeled by the mevalonate pathway of isoprene biosynthesis and also based on the ^{13}C-NMR signals of carbons that had no signal overlapping.

Carbon no.	Intensity (based on C4=100%)		S/U ratio	Average ^{13}C-Enrichment
	S	U		
Stigmasterol				
15	297.25	164.14	1.81	
17	267.61	149.45	1.79	**1.78±0.02**
24	278.35	158.73	1.75	
27	351.74	197.29	1.78	
β-Sitosterol				
15	312.38	216.44	1.44	
17	256.64	181.12	1.41	**1.44±0.03**
24	254.63	172.20	1.48	
27	298.34	212.18	1.41	
Signal overlapping carbons of both phytosterols				
1	701.30	447.66	1.57	
3	524.80	306.67	1.71	
5	208.58	164.50	1.27	**1.48±0.13**
7	1120.45	786.41	1.42	
9	378.07	267.35	1.41	



- β-Sitosterol
- $C_{22} = C_{23}$: Stigmasterol

Table 8 ^{13}C-Enrichment *via* the **non-mevalonate pathway** of some carbon atoms in the molecules of stigmasterol and β-sitosterol. The carbon atoms were selected based on the ones to be labeled by the non-mevalonate pathway of isoprene biosynthesis and also based on the ^{13}C-NMR signals of carbons that had no signal overlapping.

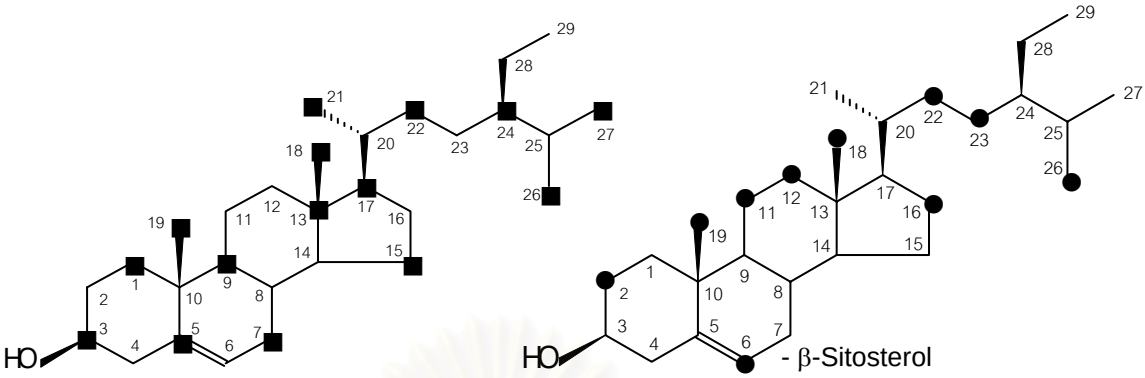
Carbon no.	Intensity (based on C4=100%)		S/U ratio	Average ^{13}C-Enrichment
	S	U		
Stigmasterol				
12	303.91	166.96	1.85	
16	277.70	159.84	1.74	**1.69±0.12**
23	147.42	97.93	1.51	
β-Sitosterol				
12	229.71	178.96	1.28	
16	213.41	160.32	1.33	**1.31±0.02**
23	226.46	173.21	1.31	
Signal overlapping carbons of both phytosterols				
2	600.23	397.61	1.51	**1.35±0.15**
6	343.32	285.84	1.20	

carbons (number **12,16 and 23**) in **stigmaterol** was **1.69+0.12**, and in **β-sitosteral** was **1.31±0.02**, both by average. For the signal overlapping carbons (number 2 and 6), the average value of 1.35±0.15 was obtained. Since these S/U values in Table 8 were slightly lower than those in Table 7, it was suggested that the formation of the isoprene unit from the mevalonate pathway was slightly faster than the formation from the non-mevalonate pathway.

In addition to the expected labeling patterns of the phytosterol carbons based on either the mevalonate or non-mevalonate pathway, there are four carbons in the molecules that are supposed to be labeled **from both pathways** of isoprene biosynthesis. Those are carbons number **18, 19, 22 and 26**. By analyzing the S/U ratios of these carbons (Table 9), it was found that ^{13}C-enrichment in the molecule of stigmasterol was also relatively higher (**1.68±0.13** in average) than of β-sitosterol (**1.31±0.07** in average).

Finally, there is the last group of carbons that are **not supposed to be labeled** by either pathway. These carbons included the numbers **4, 8, 14, 20 and 25.** The S/U ratios of these carbons are very important since their ^{13}C-enrichment should not be observed after the incorporation experiments. Unfortunately, the ^{13}C-NMR spectrum of the phytosterols mixture showed the signal overlapping of the carbons 4, 7, 8 and 25 of stigmasterol and carbons 8 of β-sitosterol. This situation made the determination of the S/U ratios for these signal overlapping carbons impossible. Consequently, only the carbons **10, 14 and 20 of stigmasterol** and carbons **4, 10**, **14, 20 and 25 of β-sitosterol** were used for the analysis. As shown in Table 10, all the carbons mentioned above showed absolutely **no ^{13}C-enrichment** based on their S/U values (~1.00). Only one exception observed was C-14 carbon which showed the value of 1.50. The C-10 carbon, on the other hand, appeared to have S/U only 0.64, strongly supporting the absence of ^{13}C-enrichment at this carbon.

From the S/U values of various carbons that were categorized according to the ^{13}C-labeling patterns, they could be used for estimating the **relative flow rate** of mevalonate



- β-Sitosterol

- $C_{22} = C_{23}$: Stigmasterol

Table 9 ^{13}C-Enrichment *via* both the **mevalonate** and the **non-mevalonate pathways** of the carbon atoms in the molecules of stigmasterol and β-sitosterol. The carbon atoms were selected based on the ones to be labeled by both pathways.

Carbon no.	Intensity (based on C4=100%)		S/U ratio	Average ^{13}C-Enrichment
	S	U		
Stigmasterol				
18	223.193	145.69	1.53	
22	151.78	92.32	1.64	**1.68±0.13**
26	377.69	201.48	1.87	
β-Sitosterol				
18	298.96	249.32	1.20	
22	285.48	212.50	1.34	**1.31±0.07**
26	271.20	194.28	1.39	
Signal overlapping carbon				
19	509.34	339.20		**1.50**

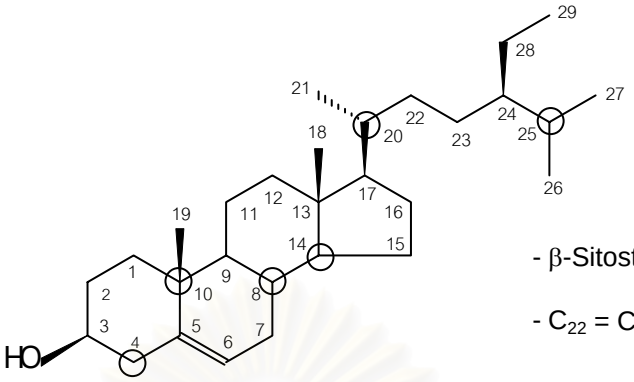


Table 10 The carbon atoms of stigmasterol and β-sitosterol showing **no significant enrichment** (except C-14 of stigmasterol) after the callus cultures of *C. sublyratus* was incorporated with [1-^{13}C]glucose. These carbon atoms were not supposed to be labeled by either the mevalonate or non-mevalonate pathway.

Carbon no.	Intensity (based on C4=100%)		S/U	Average
	S	U	ratio	^{13}C-Enrichment
Stigmasterol				
4	ND	ND	ND	
8	ND	ND	ND	
14*	196.48	130.788	1.50*	**1.00**
20	194.25	196.076	0.99	
25	ND	ND	ND	
β-Sitosterol				
4	100	100	1.00	
8	ND	ND	ND	
14	199.820	198.482	1.01	
20	183.115	171.253	1.06	**1.04**
25	230.823	217.183	1.06	
Signal overlapping carbon from both phytosterols				
10	100.045	156.244	0.64	

* Not included in the calculation for the average value owing to its unreasonably high S/U ratio.

and non-mevalonate pathways and the **relative labeling rate** of stigmasterol and β-sitosterol. These values are important for calculating the actual ^{13}C-enrichment value of a particular carbon in which its ^{13}C-enrichment is contributed by signal overlapping either from the same carbon number of different sterols or from the ^{13}C-enrichment by both mevalonate and non-mevalonate pathways.

In order to do this, the S/U values obtained from Tables 7 and 8 were used and the results of data processing are shown in Table 11. The results implied that the **flow rate ratio** of **mevalonate pathway : non-mevalonate pathway** for the biosynthesis of stigmasterol is 1.065 and of β-sitosterol is 1.091, or **1.078** by average. This means that, in *C. sublyratus* callus cultures, **the formation of isoprene unit by the mevalonate pathway is slightly faster than the non-mevalonate pathway** by a factor of 1.078.

For the biosynthesis of stigmasterol and β-sitosterol, the **relative enrichment rates of the two sterols** were also closed to each other. The value of 1.26 (S/U ratio of stigmasterol : S/U ratio of β-sitosterol) was obtained based on the mevalonate pathway calculation and the value of 1.29 was obtained based on the non-mevalonate pathway calculation. By averaging the two values which is 1.28, it can be interpreted that the **^{13}C-enrichment of stigmasterol is slightly faster than that of β-sitosterol** by a factor of **1.28** depending on the S/U ratio of stigmasterol and β-sitosterol.

Using the values of both the **relative flow rate of mevalonate : non-mevalonate (1.078)** and the **relative rate of ^{13}C-enrichment of stigmasterol : β-sitosterol (1.28)** into account, it was possible to recalculate the degree of enrichment of some carbons with signal overlapping more accurately. The results of this recalculation are shown in Table 12. Based on these results, the ^{13}C-enrichment of the phytosterol carbons contributed by the mevalonate and non-mevalonate pathways and by the different ^{13}C-enrichment rate for each phytosterol could be summarized as shown in Figures 26 and 27.

Table 11 The **relative metabolic flow of the metabolic pathways** of mevalonate : non-mevalonate and the **relative ^{13}C-enrichment of the two sterols**. The values were calculated based on ^{13}C-NMR signal intensities of some relevant carbon atoms. (see Tables 7 and 8)

Phytosterol (^{13}C-NMR signal of)	^{13}C-Enrichment (S/U ratio)		Relative flow of the two pathways (MVA/Non MVA
	MVA pathway	Non-MVA pathway	
Stigmasterol	1.80 ± 0.04	1.69 ± 0.12	1.065
β-Sitosterol	1.43 ± 0.04	1.31 ± 0.02	1.091
Relative enrichment of the two sterols (stigmasterol/β-sitosterol)	1.26	1.29	

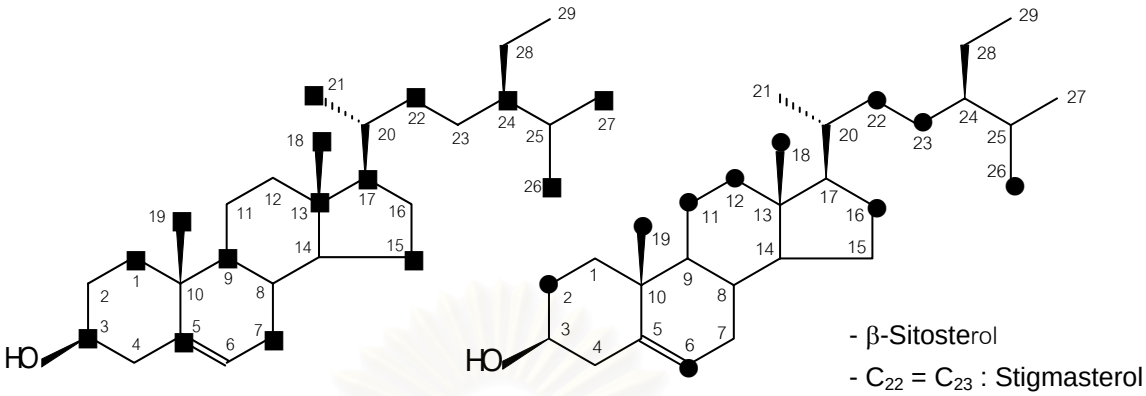


Table 12 Summary on various factors contributed to the apparent S/U values of the ^{13}C-NMR signal intensities of the spectra, and the adjusted S/U values of each factor. For non-signal overlapping carbons, the original S/U values did not need adjustment. The adjustment was based on the flow rate of MVAP : Non MVAP = 1.078 : 1 and the ^{13}C-enrichment of stigmasterol : β-sitostosterol = 1.28 : 1. MVAP = Mevalonate pathway and Non-MVAP = Non-mevalonate pathway.

Carbon no.	Chemical shift (ppm)	S/U ratio	Pathway involved	Signal overlapping	Adjusted S/U for the signal or pathway overlapping carbon			
					MVA pathway		Non-MVA pathway	
					stigm.	β-sitost.	stigm.	β-sitost.
5"	140.72	1.27	MVAP	Yes	5"=1.15	5=1.12		
22"	138.31	1.64	Both	No	22"=1.33		22"=1.31	
23"	129.25	1.51	Non-MVAP	No				
6"	121.71	1.20	Non-MVAP	Yes			6"=1.11	6=1.09
3"	71.8	1.71	MVAP	Yes	3"=1.40	3=1.31		
14"	56.85	1.50	not labeled	No				
14	56.73	1.01	not labeled	No				
17	56.02	1.42	MAVP	No				
17"	55.93	1.79	MAVP	No				
24"	51.22	1.75	MAVP	No				
9"	50.1	1.41	MAVP	Yes	9"=1.23	9=1.18		
24	45.81	1.48	MAVP	No				
13",4"	42.26	1.18	MAVP (13")	Yes	13"=1.10	13=1.08		
4	42.19	1.00	not labeled	No				
20"	40.48	0.99	not labeled	No				
12	39.74	1.28	Non-MVAP	No				
12"	39.66	1.82	Non-MVAP	No				
1"	37.22	1.57	MAVP	Yes	1"=1.32	1=1.25		
10"	36.48	0.64	not labeled	Yes				
20	36.12	1.06	not labeled	No				

Table 12 (Continued)

Carbon no.	Chemical shift (ppm)	S/U ratio	Pathway involved	signal overlapping	Adjusted S/U for the signal or pathway overlapping carbon			
					MVA pathway		Non-MVA pathway	
					stigm.	β-sitost.	stigm.	β-sitost.
22	33.91	1.34	Both	Yes		22=1.18		6=1.16
7",8",25"	31.87	1.42	MVAP (7")	Yes	7"=1.24	7=1.18		
2"	31.63	1.51	Non-MVAP	Yes			2"=1.29	2=1.22
25	29.11	1.06	-	No				
16"	28.91	1.74	Non-MVAP	No				
16	28.22	1.33	Non-MVAP	No				
23	26.02	1.31	Non-MVAP	No				
28"	25.39	1.71	not involved	No				
15"	24.34	1.81	MVAP	No				
15	24.29	1.44	MVAP	No				
28	23.0	1.30	not involved	No				
26"	21.2	1.87	Both	Yes	26"=1.45		26"=1.42	
11",21"	21.07	1.56	MVAP(21")and Non MVAP(11")	Yes	21"=1.29		11"=1.15	11=1.12
26	19.8	1.40	Both	Yes		26=1.21		26=1.19
19"	19.39	1.50	Both	Yes	19"=1.15	19=1.10	19"=1.14	19=1.11
27	19.01	1.41	MVAP	No				
27"	18.96	1.78	MVAP	No				
21	18.76	1.41	MVAP	No				
29"	12.23	1.58	Not involved	No				
18"	12.03	1.53	Both	No	18"=1.28		18"=1.25	
29	11.97	1.16	Not involved	No				
18	11.84	1.20	Both	Yes*		18=1.10		18=1.10

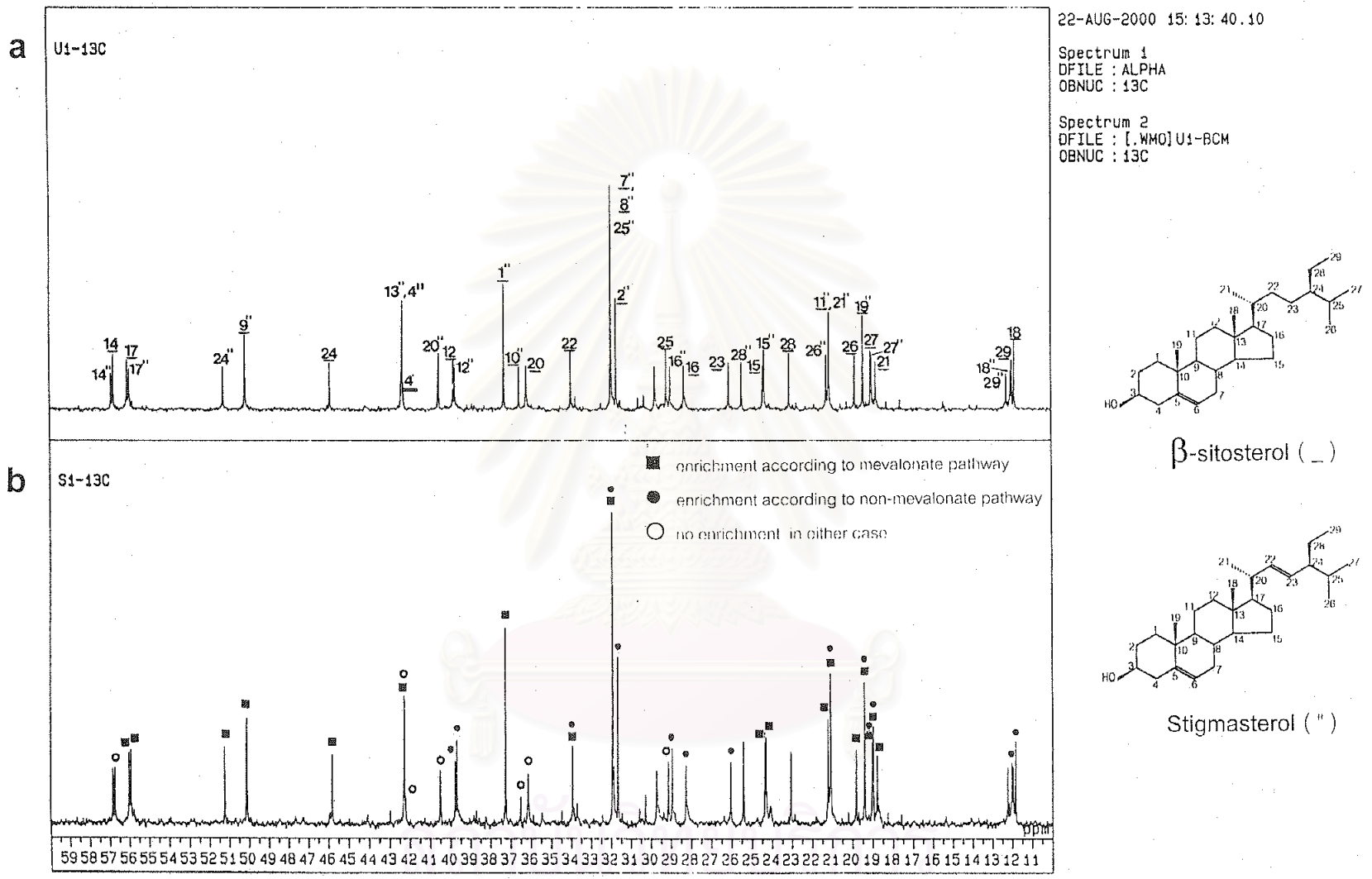


Figure 26 Comparative 125.65 MHz ^{13}C NMR spectra (expanded from **11 to 58 ppm**) showing ^{13}C enrichment of carbon atoms in the mixture of β-sitosterol and stigmasterol obtained from callus cultures of *C. sublyratus* : (a) incorporation with unlabeled glucose, and (b) incorporation with [1-^{13}C]glucose.

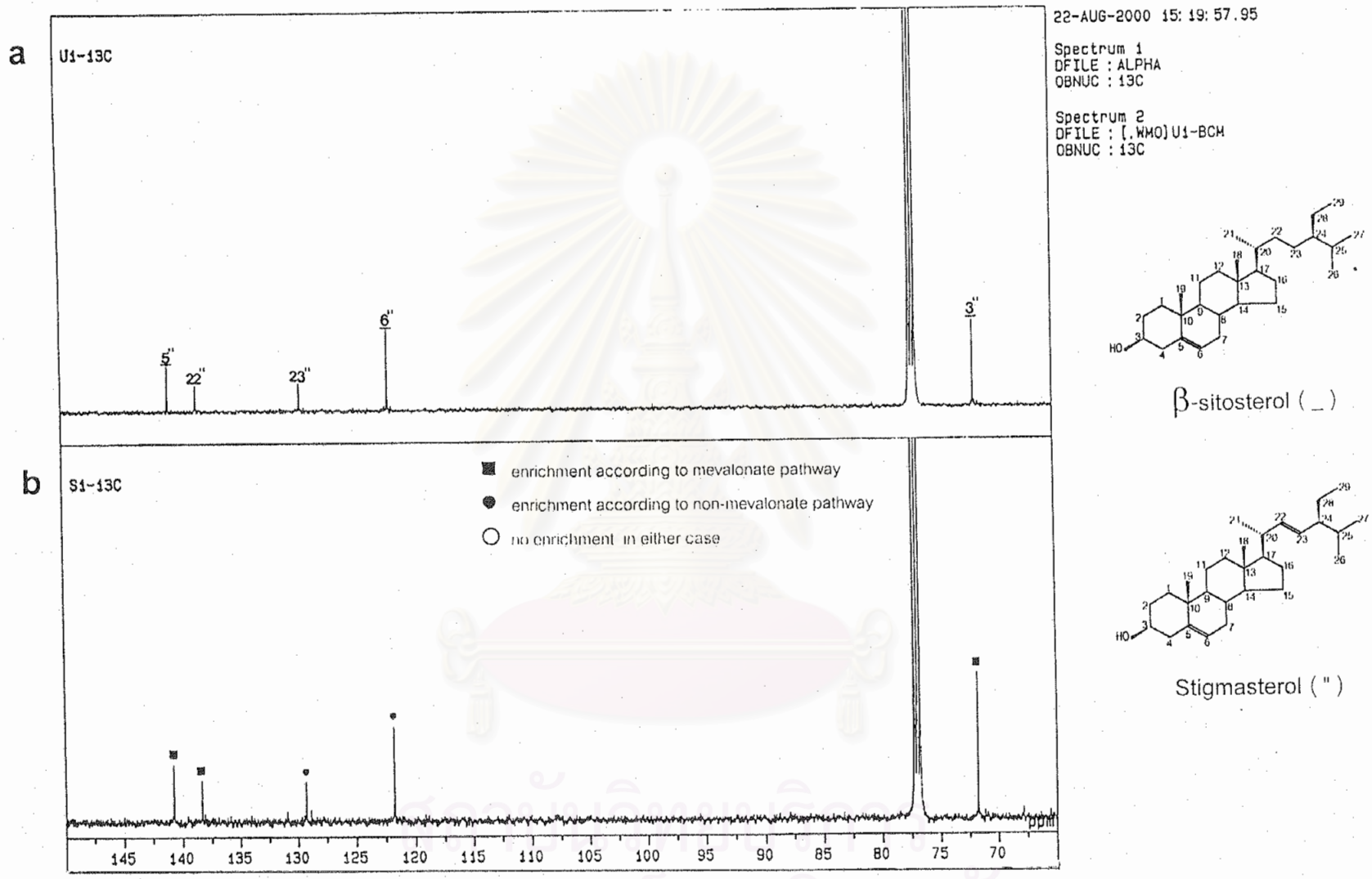
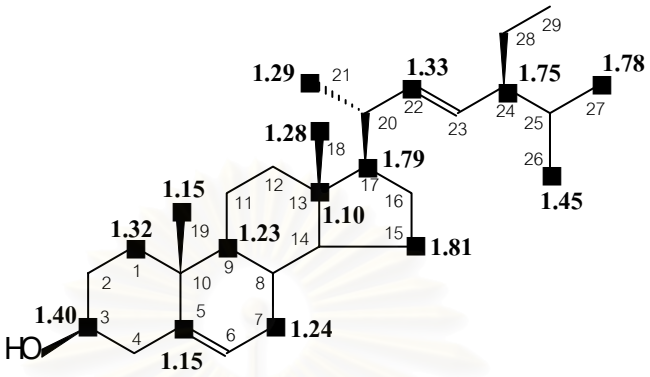


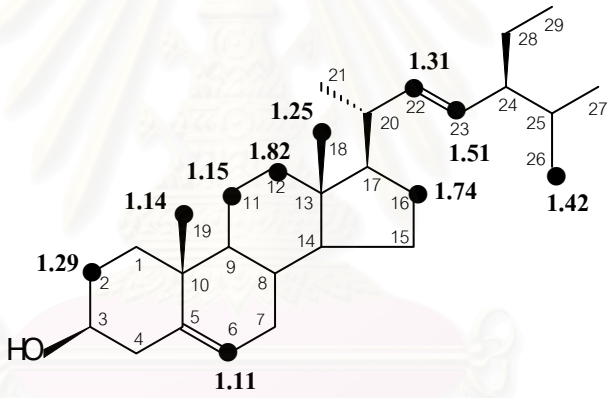
Figure 27 Comparative 125.65 MHz ^{13}C NMR spectra (expanded from **70 to 145 ppm**) showing ^{13}C enrichment of carbon atoms in the mixture of β-sitosterol and stigmasterol obtained from callus cultures of *C. sublyratus* : (a) incorporation with unlabeled glucose, and (b) incorporation with [1-^{13}C]glucose.

Stigmasterol

A. ^{13}C-Enrichment Pattern by the Mevalonate Pathway



B. ^{13}C-Enrichment Pattern by the Non-Mevalonate Pathway



C. Carbons with no ^{13}C-Enrichment by either Pathway

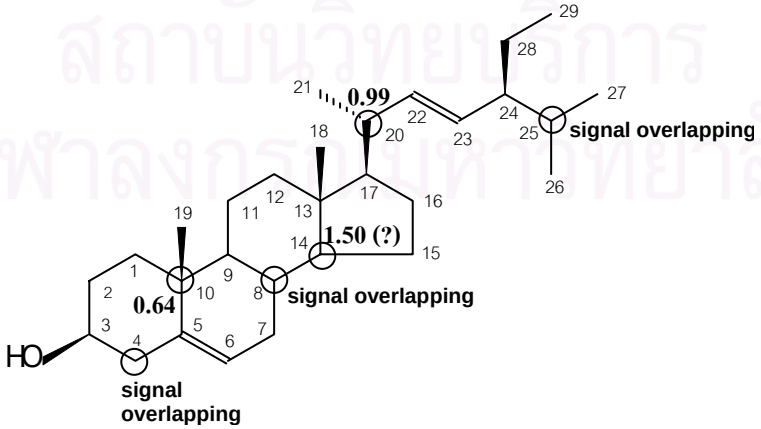
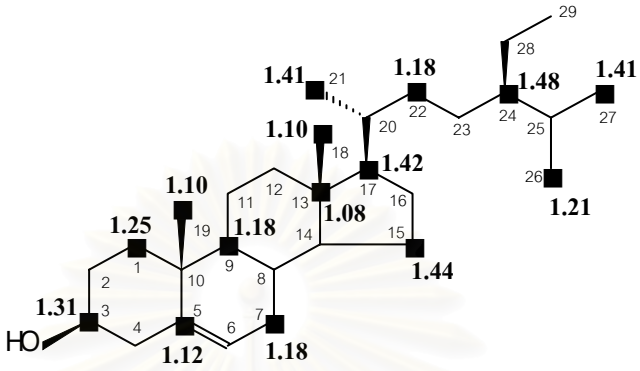


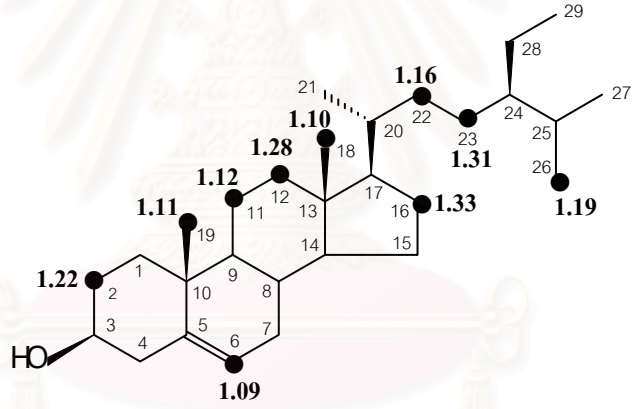
Figure 27A Summary of the contribution of mevalonate pathway and non-mevalonate pathway on the overall ^{13}C-enrichment of the carbon atoms in the molecule of stigmasterol.

β-sitosterol

A. ^{13}C-Enrichment Pattern by the Mevalonate Pathway



B. ^{13}C-Enrichment Pattern by the Non-Mevalonate Pathway



C. Carbons with no ^{13}C-Enrichment by either Pathway

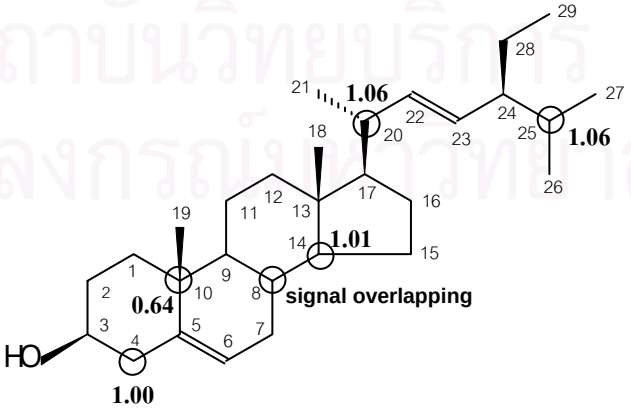


Figure 27 B Summary of the contribution of mevalonate pathway and non-mevalonate pathway on the overall ^{13}C-enrichment of the carbon atoms in the molecule of β-sitosterol.

CHAPTER V

DISCUSSION

1. Localization of Plaunotol in the Leaf of *Croton sublyratus*

We have shown that the leaf of *C. sublyratus* has a characteristic cell arrangement belonging to the dorsiventral type. This type of cell arrangement gives the leaf having distinct dorsal (upper) and ventral (lower) sides. Sequentially from the dorsal to ventral sides, the *C. sublyratus* leaf is composed of one layer of upper epidermis, one layer of closely connected palisade mesophyll cells, a loosely arranged network of spongy mesophyll cells and one layer of lower epidermis. Among these layers, the connection between the palisade mesophyll layer and the spongy mesophyll tissue seems to be relatively loose. This is apparently due to the limited contact between the two tissue types. As a result, the leaf can be separated physically into two parts along the weak connection border of the two layers. The resulting dorsal-side leaf containing palisade mesophyll cells and ventral-side leaf containing spongy mesophyll cells can then be used for preliminary study of plaunotol localization in the leaf tissues.

Analysis of plaunotol in both types of tissues strongly indicates that plaunotol is accumulated almost exclusively in the dorsal side which contains mainly palisade mesophyll cells. Under electron microscopic observation, the vertically elongated palisade mesophyll cells appear to be packed with chloroplasts. The chloroplasts are present in various sizes and each contains a few round-shaped structures similar to oil globules. These oil globules are very likely to be sites of plaunotol accumulation. This is due to the oil property of plaunotol which has twenty carbons and two hydroxyl groups in an acyclic linear form of structure. The presence of a high number of oil globules in the palisade mesophyll cells is also consistent with the high content of plaunotol (0.8%) found in the leaf (Vongchareonsathit and De-Eknamkul, 1998). Furthermore, it has been found in the green callus cultures of *C. sublyratus* that plaunotol accumulation and chloroplast development are closely associated

(Morimoto and Fukashi, 1989). Therefore, there is no doubt that plaunotol in *C. sublyratus* leaves is accumulated as oil globules in the chloroplasts of the palisade mesophyll cells.

It should be noted that the last two enzymes involved in the biosynthesis of plaunotol, namely phosphatase and geranylgeraniol-18-hydroxylase, have been found in the 20,000 g pellet fraction of *C. sublyratus* leaves (Tansakul and De-Eknamkul, 1998). The two enzymes have been shown to convert geranylgeranyl diphosphate effectively to plaunotol. Based on these results and our present study, it might be possible that the 20,000 g pellet fraction containing the two enzymes might be a part of the enzyme system of chloroplasts. Therefore, it is likely that the site of plaunotol biosynthesis is in the chloroplasts of the leaves of *C. sublyratus*.

2. Incorporation of [1-^{14}C] Glucose into Plaunotol in *C. sublyratus* Leaves

The accumulation of plaunotol in the chloroplasts which are also highly possible to be the site of plaunotol biosynthesis makes incorporation experiments rather difficult. In principle, a high degree of cellular compartmentation (i.e. plaunotol in the chloroplasts) makes a labeled compound taken up into a cell necessarily to pass barriers to reach its metabolic pathway, in order to be incorporated into the target product. This situation seemed to occur in the incorporation experiments of *C. sublyratus* leaf with [1-^{14}C]glucose since very low incorporation of the labeled compound into plaunotol was observed (Figures 10-12). Although the leaf of size 3 appeared to be the most effective for the feeding, the incorporation rate was still too low to label the product in the amount higher than the content of plaunotol already present in the leaf. Therefore, no attempt was made regarding the use of *C. sublyratus* leaf as material for study of the origin of isoprene unit in the plaunotol molecule.

3. Callus Cultures of *C. sublyratus* as Plant Material for [1-^{13}C]Glucose Incorporation Experiments

In this study, a callus culture of *C. sublyratus* has been established successfully from the leaf segments. The medium that induced callus formation was MS agar medium

containing 2.0 mg/l, 2,4-D and 1.0 mg/l kinetin. For subculture, this callus culture appears to grow well on MS agar medium containing 1.0 mg/l 2,4-D and 1.0 mg/l BA to form soft, friable and yellow tissues (Figure 14A). The friable calli are good for establishing cell suspension cultures using the same medium. The cell cultures showed small aggregates with yellow color (Figure 15). In terms of plaunotol formation, however, both callus and cell suspension cultures failed to produce plaunotol under various conditions tested in this study (Fiugure 16). It is possible that the composition of medium, type and concentration of growth regulators and other supplements are not suitable for plaunotol formation in both cultures.

It has been reported that plaunotol accumulation is observed in callus cultures of *C. sublyratus* grown on media containing gelling agents, especially gellan gum and agarose (Morimoto and Murai, 1989). Furthermore, the increase of chlorophyll content, slow growth and light have been found to stimulate plaunotol accumulation in *C. sublyratus* callus cultures (Morimoto and Murai, 1989). These results, however, have not been repeated in our study and, thus, plaunotol accumulation in either the callus or cell suspension has not yet been observed.

Charlwood and Rhodes (1990) have explained that the dedifferentiation of plant tissue *in vitro* to produce callus or suspension is usually accompanied by an apparent loss of ability to accumulate secondary compounds. This phenomenon is usually true as shown in many cases. This study, therefore, tried to increase the degree of differentiation by inducing the normal callus to be **green callus**. This was done by subculture the normal callus on MS agar medium containing 3% (w/v) sucrose, 1.0 mg/l 2,4-D, 0.1 mg/l NAA and 0.1 mg/l BA (Figure 14B).

The resulting green callus appears to produce phytosterols as its major metabolites. This was shown by a time-course study which exhibited a major peak of the phytosterol mixture during the 40-day-culture cycle of the green callus (Figure 22). The phytosterols were produced rapidly during the 3 weeks old of the culture age. Isolation and

structure identification found that the phytosterols were actually a mixture of stigmasterol and β-sitosterol.

Stigmasterol and β-sitosterol are commonly found in higher plants. Both are components of a complicated mixture of phytosterols and phospholipids of plant cell membranes. The presence of both sterols in high amount in the green *C. sublyratus* callus allowed us to study the isoprene origin although plaunotol was not produced in the cultures. Both phytosterols are well known to be biosynthesized by six isoprene units. Therefore, high incorporation of glucose into the phytosterols would be a good sign of success of the study. This was indeed the case when it was found that [1-^{14}C]glucose was rapidly and almost exclusively incorporated into the phytosterols (Figure 17). The green callus was therefore the material of choice for further study.

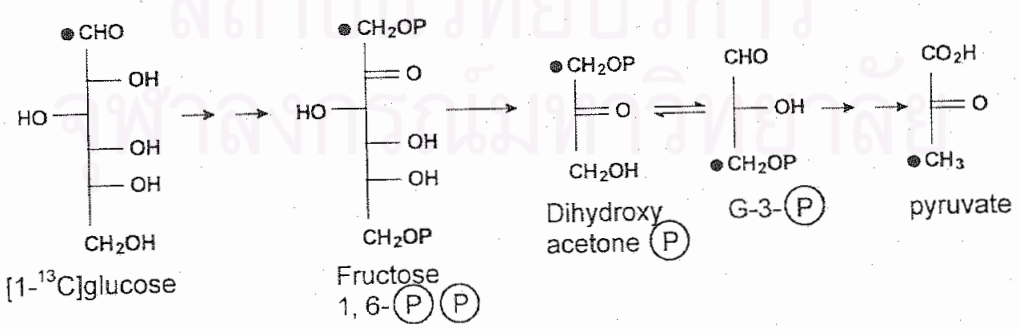
4. ^{13}C-Labeling Patterns of Stigmastrol and β-Sitosterol

We have shown in this study that incorporation of [1-^{13}C] glucose into *C. sublyratus* callus cultures during their highly active biosynthesis of phytosterol results in a ^{13}C-enrichment of some carbons in the molecules . This ^{13}C-enrichment is best expressed as a value of S:U ratio of each carbon, where **S** is the ^{13}C-NMR signal intensity of a phytosterol carbon obtained from [1-^{13}C] glucose incorporation and **U** is that obtained from unlabeled glucose incorporation. Incorporation of the unlabeled glucose under the same conditions as S is, therefore, a control in which its ^{13}C-NMR spectrum comes from the signals of the natural abundance of ^{13}C presence in each carbon atom of the phytosterol molecules. Moreover, under this control of U, the relative amount of stigmasterol : β-sitosterol should be the same as in the S condition. Therefore, the expression of S:U would reflect mainly the degree of ^{13}C-enrichment of a particular carbon. If the value is around 1.0, it means that there is no ^{13}C-enrichment. If the value is more than 1.0, ^{13}C-enrichment at that particular carbon would certainly occur. However, prior to obtaining the S:U ratio of each ^{13}C-peak, it is necessary to work out the **relative ^{13}C-signal intensity** of each peak to be based on a particular carbon that is not involved in any labeling process either by the mevalonate pathway or non-

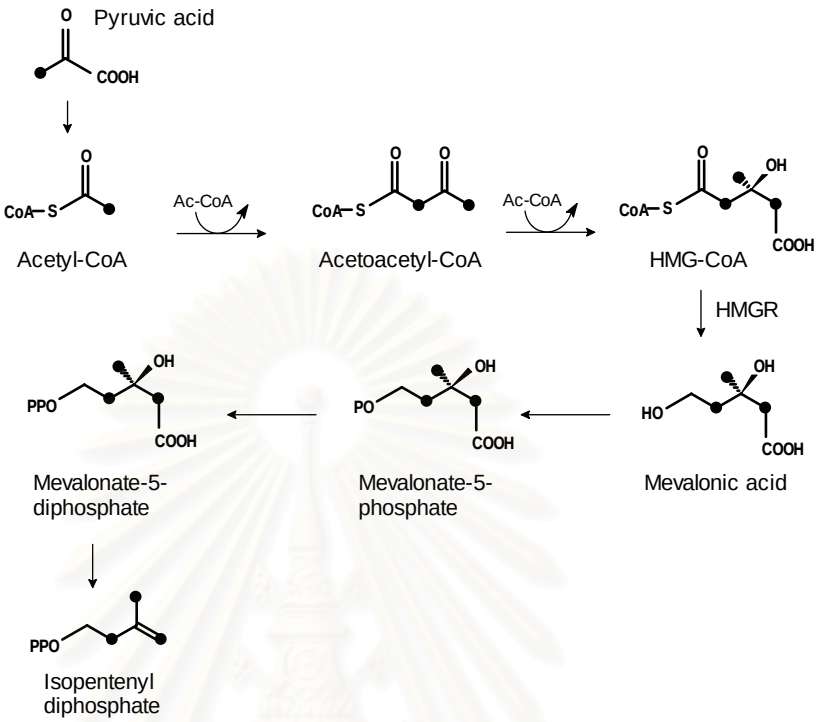
mevalonate pathway. In this study, the peak intensity of **C-4** carbon of β-sitosterol was chosen to have **100% intensity**. The intensities or other peaks related to C-4 were then recalculated. By this way, the values of S:U of all ^{13}C-signal peaks were obtained (Table 4). The results showed that 23 out of the 29 of both phytosterols were labeled by [1-^{13}C] glucose whereas less than 6 carbons were not labeled by the [1-^{13}C]glucose incorporation (Figure 25). Analysis on various aspects which possibly contributed to the distribution of ^{13}C-labeling in stigmasterol and β-sitosterol was then carried out as described in the "Results" section. The analysis has led to the following conclusion : First, the isoprene unit used for the formation of stigmasterol and β-sitosterol is **biosynthesized from both** mevalonate pathway and non-mevalonate pathway. Second, the mevalonate pathway and non-mevalonate pathway **operate with similar rate (1.1:1.0)** for the same isoprene unit formation in the callus cultures. Third, the ^{13}C-NMR signal **enrichment in the molecule of stigmasterol is faster** than the enrichment of β-sitosterol by the ratio 1.28:1.00, respectively.

The explanation on the conclusion can be summarized by the sequence of metabolic flows of [1-^{13}C] glucose after being taken up by *C.sublyratus* callus cultures as follows:

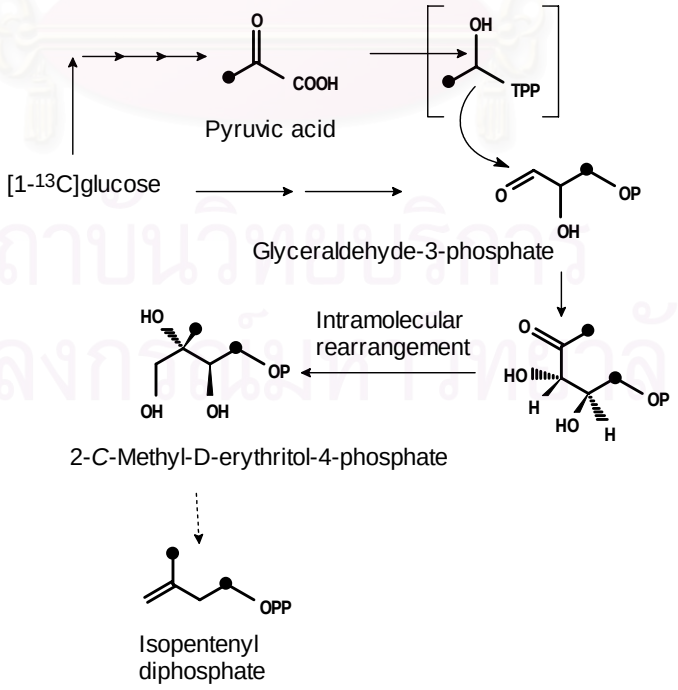
1. Conversion of [1-^{13}C]Glucose to Pyruvate by Glycolytic Pathway



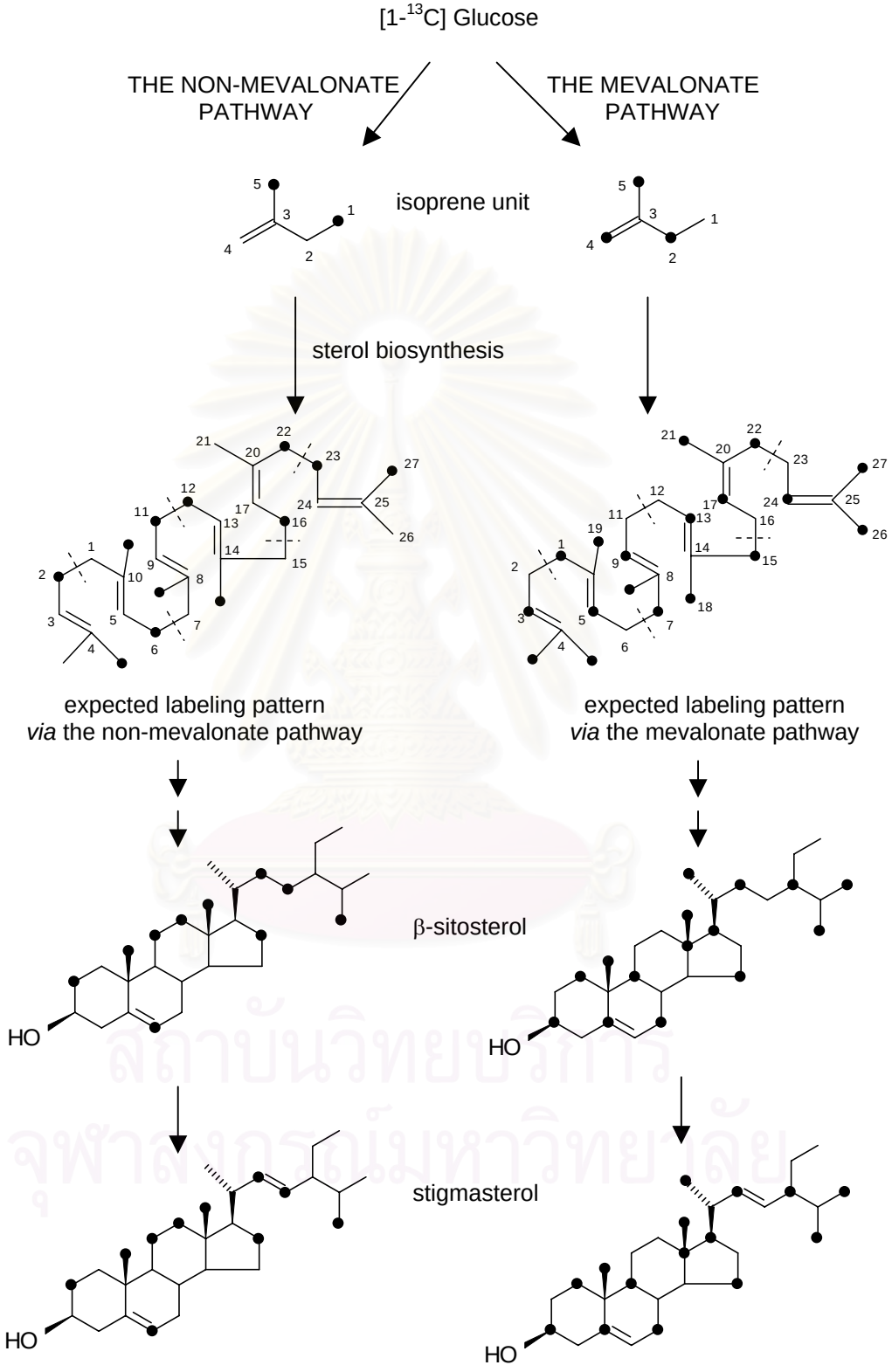
2. Biosynthesis of IPP from Pyruvate *via* the Mevalonate Pathway



3. Biosynthesis of IPP from Pyruvate and Glyceraldehyde-3-phosphase *via* the Non-Mevalonate Pathway



4. Biosynthesis of Phytosterols from IPP



Labeling patterns of ^{13}C-carbons expected from the operation of the mevalonate (right) and the non-mevalonate (left) pathways.

Therefore, it is clear from the metabolic flow of [1-^{13}C] glucose that various carbons in the phytosterols are due to the contribution of both mevalonate and non-mevalonate pathways in the isoprene biosynthesis.

In literature, the biosynthesis of sterols in plants has been reported to involve the mevalonate pathway, whilst other terpenoids can be formed concurrently *via* the non-mevalonate pathway (Rohmer, 1999). The efficient utilization of mevalonate for sterol biosynthesis has been reported in duckweed(*Lemna gibba*), yet poor incorporation of 1-deoxy-D-xylulose has been noted (Schwender, *et al*., 1997). The use of [1-^{13}C]glucose as a precursor has shown that three higher plants, duckweed, barley (*Hordeum vulgare*), and carrot (*Daucus carota*) possess two distinct routes for isoprene biosynthesis, producing their cytoplasmic sterols *via* the mevalonate pathway, but their chloroplast-bound terpenoids *via* the non-mevalonate pathway (Lichtenhaler etal., 1997). Thus, phytol, β-carotene, lutein and plastoquinone-9-all displayed the isoprene labeling patterns of non-mevalonate pathway, but β-sitosterol and stigmasterol had the isoprene labeling patterns of mevalonate pathway. This dichotomy in higher plants allows reasonable explanation for the many unexpected and inconclusive results concerning the biosynthesis of chloroplast isoprenoids which had mainly been interpreted *via* models involving compartmentation of the mevalonate pathway. The different pathways displayed may reflect the subcellular location of the terpenoid in question. Studies using plantlets and shoot cultures of *Marrubium vulgare* indicated that these plant's sterols were also produced by the mevalonate pathway, whilst labdane diterpenes arose *via* the non-mevalonate pahtway (Knoss *et al*., 1997).

Our results obtained from this study, therefore, do not support previous reports with respect to the origin of IPP since sterol biosynthesis is only through the mevalonate pathway. On the other hand, it shows clearly that both pathways contributed to the phytosterol biosynthesis. It is possible that subcellular compartmentation, in *C. sublyratus* callus cultures, is not well developed as is the case in the mature leaf. When the chloroplast's

non-mevalonate pathway is operating in the callus cultures, the IPP so formed can leak to the cytoplasm and is utilized by the sterol pathway.

5. Conclusion and Recommendation

Croton sublyratus (or Plaunoi) is an interesting indigeneous medicinal plant of Thailand. It is well-known as the only commercial source of plaunotol which is an acyclic diterpene alcohol possessing potent anti-peptic ulcer activity. The present investigation on isoprenoid biosynthesis in callus cultures of the plant, based on ^{13}C-labeling patterns analysis, leads to the conclusion that both mevalonate and non-mevalonate pathways operate for phytosterols biosynthesis. The non-mevalonate pathway indicates the existence of the gene encoding transketolase enzyme responsible for the synthesis of deoxyxylulose-5-phosphate, the initial product of this pathway, from glyceraldehyde-3-phosphate and pyruvate. Further study on the enzymology and regulation of this non-mevalonate pathway in *C. sublyratus* should be performed.

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VITA

Mrs. Buppachart Potduang was born on June 24th, 1956 in Saraburi Hospital, Saraburi, Thailand. She is the 4th child of six children in a family of government officers. Her mother is the late Mrs. Somboon Nira, a school teacher who was a cousin of the late Khun Luang Wijitwathakarn. Her father is Mr. Thawil Nira B.Sc. (in Forestry), a specialist who had been Head of the Phukae Botanical Garden, Saraburi, Thailand for 19 years (1953-1955 and 1963-1979). She spent her childhood happily in Uthai-Thani Province with her closely related aunt and uncle's family, Mrs. Arunee - Mr. Pissawong Nira, until she finished grade 4 from Anusitvidtaya School, then she went to live with her parents in Saraburi until finishing grade 6 from Pibulsongkloa 1 School, and came back to Uthai-Thani to finish grade 7 from Muang Uthai-Thani School. She was always the best pupil of her teachers and had been the first in classes all that time. She then got the 2nd in the examination for attending Satrividhaya School, Bangkok, and finished grade 12 from there. She obtained a B. Pharm. in 1979 from Chiangmai University Faculty of Pharmacy, Chiengmai, Thailand ; and a M. Sc. (Pharmacognosy) in 1981 from Chulalongkorn University Graduate School Department of Pharmacognosy, Bangkok, Thailand, where she was an advisee of Assistant Professor Bamrung Tantisewie. She has been married since 1982 to Mr.Yongpan Potduang, a pharmacist who is a son of the late M.R. Pongsri Kasemsri and Mr. Yan Potduang; and has two daughters, Miss Atchariya Potduang (age 17) and Miss Methilak Potduang (age 11). She used to work as a government officer, first as a specialist in food and drugs at the Office of the Consumer Protection Board (OCPB), the Ministry of the Prime Minister Secretariate, between 1982-1987; then as an expert in research project analysis at the National Research Council of Thailand (NRCT), the Ministry of Sciences, Technology and Environment, between 1987-1994, where she was an assistant secretary of two Boards, the Board of Chemical and Pharmaceutical Sciences, and the Board of Medical Sciences. She has changed her career and become a research officer at the Pharmaceuticals and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR) since 1994. She wishes to be a keen researcher of TISTR someday.