เมแทบอไลต์ของราเอนโดไฟต์ในใบเปล้าน้อย *Croton sublyratus* Kurz.

นายสุชน ดีจังวิภาต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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METABOLITES OF ENDOPHYTIC FUNGI IN *Croton sublyratus* Kurz. LEAVES

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สุชน ดีจังวิภาต: เมแทบอไลต์ของราเอนโดไฟต์ในใบเปล้าน้อย *Croton sublyratus* Kurz. (METABOLITES OF ENDOPHYTIC FUNGI IN *Croton sublyratus* Kurz. LEAVES) อ. ที่ปรึกษา : รศ.ดร. นาตยา งามโรจนวณิชย์, อ. ที่ปรึกษาร่วม : ผศ.ดร. สุรชัย พรภคกุล, 84 หน้า.

วัตถุประสงค์ของงานวิจัยนี้ก็เพื่อแยกเมแทบอไลต์จากราเอนโดไฟต์ไอโซเลต Bkk3 ที่ได้จากใบเปล้าน้อย โดยอาศัยการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ ITS1-5.8S-ITS2 ของ rDNA บ่งชี้ว่าราเอนโดไฟต์ไอโซเลต Bkk3 คือ *Diaporthe* sp. นำส่วนสกัดหยาบไดคลอโรมีเทนของอาหารเลี้ยงเชื้อเหลวสารสกัดมอลท์ของราเอนโดไฟต์ไอโซเลต Bkk3 มาทำการแยกสารบริสุทธิ์โดยเทคนิคโครมาโทรกราฟีได้สารใหม่ 1 ชนิด ที่เรียกชื่อว่า diaporthichalasin และสารที่มีการรายงานไว้แล้ว 2 ชนิด คือ pycnidione และ 5-methylmellein พิสูจน์ทราบโครงสร้างโดยการวิเคราะห์ข้อมูล UV, IR, MS และ NMR ยืนยันโครงสร้างของ diaporthichalasin โดยการวิเคราะห์ด้วย single crystal X-ray crystallography Diaporthichalasin นั้นแสดงฤทธิ์การยับยั้งการทำงานของเอนไซม์ CYP3A4 ที่ดีด้วยค่า IC_{50} เท่ากับ 0.626 μM และแสดงฤทธิ์ยับยั้งเซลล์มะเร็งทดสอบ BT474 (เต้านม), CHAGO (ปอด), HEP-G2 (ตับ), KATO-3 (กระเพาะอาหาร) และ SW620 (ลำไส้ใหญ่) ด้วยค่า IC_{50} เท่ากับ 18.01, 13.94, 11.29, 9.86 และ 0.93 μM ตามลำดับ ขณะที่ pycnidione แสดงฤทธิ์ยับยั้งการทำงานของเอนไซม์ CYP3A4 ด้วยค่า IC_{50} เท่ากับ 465 μM และแสดงฤทธิ์ยับยั้งเซลล์มะเร็งทดสอบ BT474 (เต้านม), CHAGO (ปอด), HEP-G2 (ตับ), KATO-3 (กระเพาะอาหาร) และ SW620 (ลำไส้ใหญ่) ด้วยค่า IC_{50} เท่ากับ 18.25, 1.28, 11.72, 1.37 และ 12.92 μM ตามลำดับ อย่างไรก็ตามสารทั้ง 3 ชนิดนี้ไม่มีฤทธิ์การยับยั้งเชื้อจุลินทรีย์ทดสอบ *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATTC 27853 และ *C. albicans* ATCC 10231

ภาควิชา..... เคมี..... ลายมือชื่อนิสิต.....สุชน ดีจังวิภาต.....

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The purpose of this research was to isolate metabolites of endophytic fungus isolate Bkk3 obtained from *Croton sublyratus* Kurz. leaves. Based on nucleotide sequences of ITS1-5.8S-ITS2 regions of rDNA, the fungus isolate Bkk3 was identified as *Diaporthe* sp. The dichloromethane extract of malt extract broth of the fungus Bkk3 was purified by chromatographic techniques to afford a novel compound named diaporthichalasin together with two known compounds, pycnidione and 5-methymellein. Their structures were elucidated by analysis of UV, IR, MS, and NMR. The structure of diaporthichalasin was confirmed by single crystal X-ray crystallographic analysis. Diaporthichalasin exhibits significantly potent inhibition of CYP3A4 with an IC_{50} value of 0.626 μM and shows cytotoxic activity against BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) with the IC_{50} values of 18.01, 13.94, 11.29, 9.86 and 0.93 μM, respectively while pycnidione inhibits CYP3A4 with the IC_{50} value of 465 μM and displays cytotoxic activity against BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) with the IC_{50} values of 18.25, 1.28, 11.72, 1.37 and 12.92 μM, respectively. However all of three compounds do not show antimicrobial activity against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATTC 27853 and *C. albicans* ATCC 10231.

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LIST OF ABBREVIATIONS

$[\alpha]_D^{20}$	Specific rotation at 20 °C and Sodium D line (589 nm)
ATCC	American Type Culture Collection, Maryland, U.S.A
cm	centimeter
mm	millimeter
μm	micrometer
MHz	megahertz
TLC	thin layer chromatography
kg	kilogram
g	gram
mg	milligram
MeOH	methanol
EtOAc	ethyl acetate
CH_2Cl_2	dichloromethane
mp	melting point
KBr	potassium bromide
ν_{max}	the reciprocating wavelength (IR spectrum)
λ_{max}	the wavelength at maximum absorption (UV-VIS)
cm^{-1}	unit of wave number
s	strong (IR)
m	medium (IR)
w	weak (IR)
°C	degree Celsius
L	Liter
ml	milliter
R*f*	rate of flow in chromatography
ppm	part per million
m/z	mass to charge ratio
MS	Mass Spectrometer
HRMS	High Resolution Mass Spectrometry
δ	chemical shift
NMR	Nuclear Magnetic Resonance

^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
COSY	Correlated Spectroscopy
NOESY	Nuclear Overhauser Enhancement Spectroscopy
J	coupling constant
d	doublet (for NMR spectrum)
dd	double of doublet (for NMR spectrum)
s	singlet (for NMR spectrum)
br s	broad singlet (for NMR spectral data)
SDA	Sabouraud's Dextrose Agar
YES	Yeast Extract Agar
PDA	Potato Dextrose Agar
MEA	Malt extract agar
CMA	Corn meal agar
NB	Nutrient broth
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

CHAPTER I

INTRODUCTION

Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from natural sources. Despite chemodiversity in plants offers a valuable source for lead discovery, many diseases remain which are presently untreatable. Therefore, the search for novel secondary metabolites requires continual search and should concentrate on organisms that inhabit novel biotopes. Endophytic fungi inhabit such a biotope. These fungi reside in the living tissues of the host plant and typically causing no apparent symptoms of disease. The colonization and propagation of endophytic fungi may in some ways offer significant benefits to their host plants by producing a plethora of substances that provide protection and survival value to the plants, such as enhancement of stress-, insect- and disease-resistance, productivity improvement, and herbicide activities. These facts indicate that endophytic fungi are recognized as one of the most chemically promising groups of fungi in terms of diversity and pharmaceutical potential.

Most mycologists agree that fungal diversity likely peaks in tropical forests. Additionally, Thailand has been considered as one of the world's regions of origin and diversity of species. To my knowledge, little is known about the bioactive compounds from endophytic fungi in many of the Thai medicinal plants. This background information led mine to speculate that Thai medicinal plants might constitute another source for screening novel metabolites derived from endophytic fungi.

In summary, this thesis describes the isolation, structure elucidation, and bioactivities of metabolites from the endophytic fungus, *Diaporthe* sp. which was isolated from leaves of *Croton sublyratus* Kurz. in Bangkok, Thailand. The identification of the fungus is also discussed.

Objectives:

1. To isolate, and select the endophytic fungi in *Croton sublyratus* Kurz. leaves from Bangkok
2. To extract, separate, and purify the metabolites from a selected endophytic fungal isolate
3. To elucidate the structure of the metabolites
4. To evaluate the biological activity of the metabolites

CHAPTER II

LITERATURE REVIEWS

2.1 Endophytic Fungi and Technical terms

It is estimated that up to ninety five percent of the world's fungi are unknown to science [*1*]. One of the most likely, relatively unexplored, habitats of the novel fungi are the world's rainforests. The kingdom Fungi is a large and diverse group which has a range of sizes from unicellular microscopic forms to large mushrooms and tree bracket fungi. The unicellular fungi are called **yeasts**. However, most fungi are not unicellular yeasts but instead grow as an extensive system of branched filament. Individual filaments are called **hyphae** and the filamentous mass is the **mycelium.** Not only can fungi be found inhabiting surfaces, but also within the organs and tissue of plants. Fungi that exist within the tissues are known as **endophytes**.

Endophytes are microorganisms that colonize and cause unapparent, asymptomatic infections in healthy plant tissues. Because of the symptomless, endophytic fungi are difficult to detect and can be determined by microscopic examination of the tissue as well as by isolation in pure culture. To accomplish the isolation, the plant part is surface-sterilized and then a segment is placed on a standard cultural medium and incubated. With time, the endophytic fungus will grow onto the culture medium [*2*].

Endophytic fungi have been found in all plant species examined to date, including algae [*3*], mosses [*4*], ferns [*5*], and conifers [*6*], as well as angiosperms, including grasses [*7*], palms [*8*], and a variety of dicotyledonous shrubs [*9*] and trees [*10*]. As a matter of fact, endophytes are important components of microbial biodiversity. Commonly, several to hundreds of endophytes species can be isolated from a single plants, among them, at least one species showing host specificity. The environmental conditions under which the host is growing also affect the endophyte population [*11*], and the endophyte profile may be more diversified in tropical areas. For example, Arnold and co-workers [*12*] isolated 418 endophyte morphospecies

(estimated 347 genetically distinct taxa) from 83 healthy leaves of *Heisteria concinna* and *Ouratea lucens* in a lowland tropical forest of central Panama, and proposed that tropical endophytes themselves could be hyperdiverse with host preference and spatial heterogeneity.

In general, all endophytes can reproduce asexually, and most undergo sexual reproduction as well. Both asexual and sexual reproductions involve the production of unicellular reproductive units called **spores**. Often only one method of reproduction is observable at a specific point in time or under specific environmental conditions. In these cases, mycologists have devised two names for the particular fungus. The sexual stage often called **perfect or teleomorph stage**, and asexual stage often designated as the **imperfect or anamorph stage**. **Holomorph** describes the whole fungus, encompassing both stages. Commonly, asexual reproduction is more importance for the propagation of the species because it results in the production of numerous individuals, and particularly since the asexual cycle is usually repeated several times during the season, whereas the sexual stage of many fungi is reproduced only once a year. On the other hand, sexual reproduction is the most evolutionarily conserved means of reproduction and thus used to classification. This system is complicated and often confusing but can be a useful method for mycologists trying to identify an endophyte. Within the division of true fungi (Eumycota), there are five classes of the division classified by mode of sexual reproduction (Table 2.1). Fungi that do not produce a teleomorph stage (or in cases in which it has yet to be observed) are placed into the Deuteromycota. If sexual reproduction ever observed in a *Deuteromycetes* fungus, then it will be reclassified [*13*].

Table 2.1 The Division Eumycota of the Kingdom Fungi [*13*]

Classes	Sexual Reproduction
Oomycetes	Oospore
Zygomycetes	Zygospores
Ascomycetes	Ascospores
Basidiomycetes	Basidiospores
Deuteromycetes	Absent

However, many endophytes are not to be classified because most of them can not produce spores in **broth**, a liquid culture medium that contain all the nutrients required by a fungus. To some degree this taxonomic problem is now being approached using a novel technology termed **Phylogenetic Character Mapping**. The phylogenetic character mapping method involves combining molecular and morphological data with statistical support to assist in fungal identification. The rDNA genes are frequently targeted for fungal taxonomy because they are highly conserved regions of molecular information. In addition, the rDNA genes are found in all organisms with a common function evolutionary origin. Finally, the rDNA genes are a non-protein-coding region, and they represent a single nuclear copy that is easy to amplify by Polymerase Chain Reaction (PCR) techniques [*14*].

2.2 Endophytic Fungi and Host Plant Interactions

Endophytes colonizing inside plants tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain functional metabolites. Endophyte-infected plants often grow faster than non-infected ones. This effect is at least in due to the endophytes' production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances [*15*]. Furthermore, endophytes could have enhanced the hosts' uptake of nutrition elements such as nitrogen [*16*] and phosphorous [*17*]. Tan and Zou [*15*] studied in the growth promotion of the host plant. They found that a culture broth of *Colletotrichum gloeosporioides*, an endophytic fungus of *Artemisia annua* L., can promote the growth of the host callus (Figure 2.1).

Figure 2.1 An *Artemisia annua* endophyte culture liquid can significantly promote the growth of host callus incubated on MS medium without (Left) and with 10% endophyte cultured liquid (Right) [*15*].

Certain endophytes improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens and/or herbivores including some insects feeding on the host plant. Endophyte-infected grasses usually posses an increased tolerance to drought and aluminum toxicity [*18*]. Furthermore, some endophytes are able to provide the host plant with protection against some nematodes [*19*], mammal [*20*], and insect herbivores [*21*] as well as bacterial and fungal pathogens [*22*]. Some endophytes are capable of enhancing the host's allelopathic effects on other species co-growing nearby, usually being competitors for the nutrition and the space [*23*]. This could be the reason why some plants with special endophytes are usually competitive enough to become dominant species in successional fields [*24*].

Recently, a very interesting report by Redman and co-workers [*25*] showed that endophytic fungi called *Curvularia* sp. also enhanced thermotolerance to plants, *Dichanthelium lanuginosum.* They found in the absence of thermal stress, endophyte-colonized (symbiotic) and endophyte-free (nonsymbiotic) plants showed no measurable growth or developmental differences. When root zones were heated with thermal tape at 50 ^{o}C, nonsymbiotic plants became shriveled and chlorotic; the tissue becomes yellow because the chlorophyll has been destroyed. In contrast, symbiotic plant tolerated constant 50 ^{o}C for 3 days and intermittent soil temperatures as high as 65 ^{o}C for 10 days. All nonsymbiotic plants died during the 65 ^{o}C heat regimes, whereas symbiotic plants survived (Figure 2.2A). The endophyte was reisolated from surface sterilized roots and leaves of all surviving plants, indicating both the fungus and the host were protected from thermal stress. Moreover, they also field-test symbiotic and non-symbiotic seedlings in pasteurized geothermal soil collected and returned in May 2001 (Figure 2.2B). By May 2002, symbiotic plants were greener with greater root and leaf mass than those of nonsymbiotic plants in soils below 40 ^{o}C. In soils above 40 ^{o}C, nonsymbiotic plants did not survive while symbiotic plants thrived. The beneficial effect of fungal symbiosis increased with soil temperatures, demonstrating that *Curvularia* sp. provided thermal protection for *D. lanuginosum*. In addition to thermotolerance, several possible symbiotic mechanisms could confer thermotolerance. In planta, the fungal endophyte produces cell wall melanin that may dissipate heat along the hyphae and/or complex with oxygen radicals generated during heat stress. Alternatively, the endophyte may act as a "biological trigger" allowing

symbiotic plants to activate stress-response systems more rapidly and strongly than nonsymbiotic plants.

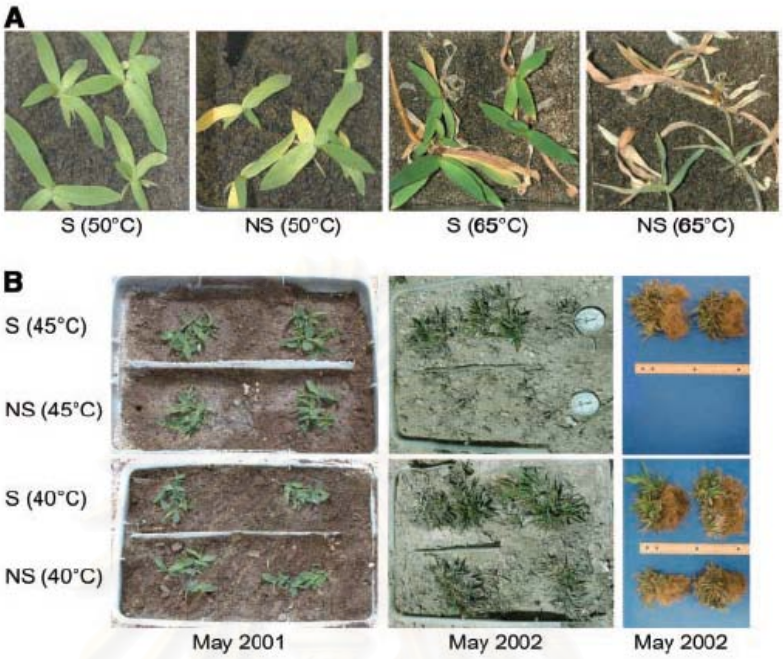


Figure 2.2 Representative symbiotic (with *Curvularia* sp.) and nonsymbiotic *D. lanuginosum* plants with rhizosphere temperatures of [*25*]

(a) 50 ^{o}C for 3 days or 65 ^{o}C for 10 days under laboratory conditions.

(b) 40 ^{o}C or 45 ^{o}C soil under field conditions.

NS = Non-Symbiotic plants, S = Symbiotic plants

2.3 Endophytic Fungi and Metabolites

Nowadays, endophytic fungi have proven to be a rich source of novel organic compounds, some of which have beneficial biological activities [*26, 27*]. A recent comprehensive study has indicated that 51% of bioactive substances isolated from endophytic fungi were previously unknown [*27*]. Additionally, in comparison to surface fungi, fungal plants pathogens, and fungal soil isolates relatively few secondary metabolites have been isolated from endophytic fungi [*15*].

Hence, the endophytic fungi are expected to be a potential source for intelligent screening and fulfill criteria.

Tan and Zou [*15*] reviewed the chemodiversity of endophytes before year 2000. In their review, metabolites of endophytic fungi were described by functional groups, i.e., alkaloids, steroids, terpenoids, isocoumarin derivatives, quinones, flavonoids, phenylpropanoids and lignans, peptides, phenol and phenolic acids, aliphatic compounds, chlorinated compounds, and others.

Strobel and Daisy [*26, 27*] reviewed the methods and rationale for plant selection used to provide the best opportunities to discover novel bioactive products, and these are as follows.

1. Plants from unique environmental settings, especially those with an unusual biology and possessing novel strategies for survival, are seriously considered for study.

For example, an aquatic plant, *Rhyncholacis penicillata*, was collected from a river system in southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles. These environmental insults created many pathogens could enter the plant. But, the plant population appeared to be healthy, possibly due to production from an endophyte product. This was the clue used to pick this plant for a study of its endophytes. Eventually, an unusual and potent antifungal strain of *Serratia marcescens* was isolated from *R. penicillata*. This endophyte was shown to produce oocydinA, a novel antioomycetous compound, having the properties of a chlorinated macrocyclic lactone (Figure 2.3). Currently, oocydinA is being considered for agricultural use to control the ever-threatening presence of oomycetous fungi such as *Pythium* sp. and *Phytophthora* sp. [*28*].

2. Plants that have an ethnobotanical history (use by indigenous people) that is related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local people or via local literature.

3. Plants that are endemic, having an unusual longevity, or that have occupied a certain ancient land mass, such as Gondwanaland, are also more likely to lodge endophytes with active natural products than other plants.

4. Plants growing in areas of great biodiversity, it follows; also have the prospect of housing endophytes with great biodiversity.

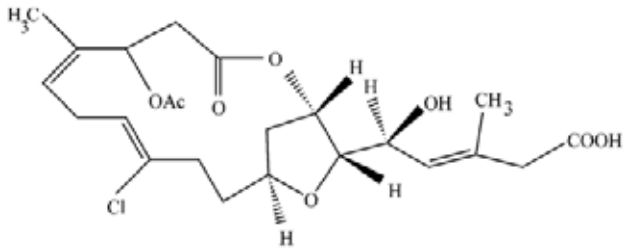


Figure 2.3 Oocydin A, a chlorinated macrocyclic lactones isolated and characterized from strain of *Serratia marcescens*, obtained from *Rhyncholacis penicillata* [*28*].

In addition, it is worthy of note that some plants generating bioactive natural products have associated endophytes that produce the same natural products. Such is the classical case with taxol. It may be learned that the healing powers of the botanical source, in fact, may have nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).

2.3.1 Taxol-producing Fungi, Classic and Significance

Hardly any natural compound has captured as much attention among the scientific community as “taxol”, a natural bioactive diterpene derivative and a widely used anticancer drug, was first identified from the Pacific yew *Taxus brevifolia* in 1963. Its chemical structure was determined three years later (Figure 2.4). After more than ten years’ use in clinics, its anti-tumor range has been expanding [*29*].

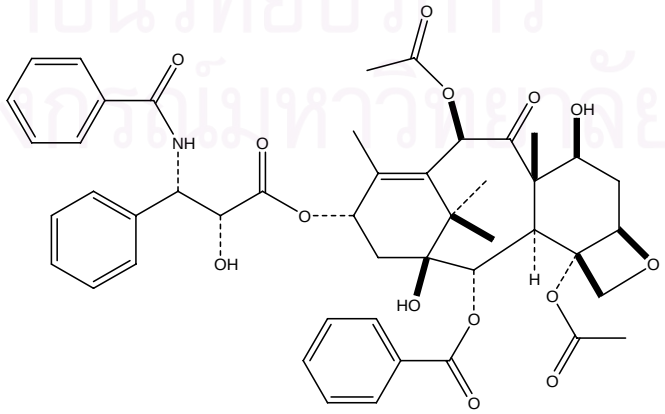


Figure 2.4 Structure of Taxol [*29*]

However, the production of taxol can not meet the demand of the growing market by problem with the low content in yew tress, approximately 7,000 kg; equal to 2,000-2,500 yew trees can produce only 1 kg taxol [*30*]. This makes taxol a financial burden for many patients. To overcome this problem, including ecological reasons, scientists have been seeking new ways to improve the production of the drug, such as, total synthesis [*31*], yew tissue-culture technique [*32*], and cell suspension culture [*33*]. Problems still exist in these process, for instance, low yield of taxol, long production period, and cell easily become brown and die when the culture is scaled-up, respectively. Thus, no practical applications have been reported so far. This current technique to produce taxol is semi-synthesis [*34*]. Although the productivity has been improved with semi-synthesis, virtually, it is quite similar regarding the consumption of yew trees. After all, it is clear that the environment problem may be difficult to solve if it rests on the extraction of taxol from yew trees alone.

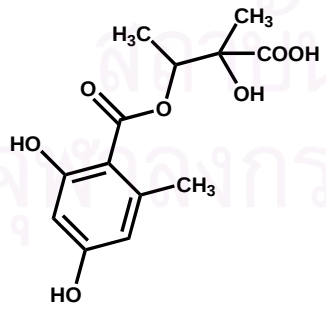
The most significant searching for new routes to improve taxol production might be the discovery that an endophytic fungus (*Taxomyces andreanae*) colonizing in yew trees also produced taxol [*35*]. Strobel, Stierle A. and Stierle D. [*36*] found that taxol accumulated in culture at low level of 24-25 ng/l. Afterwards, a number of taxol-producing fungi were identified all over the world. For example, *P. microspora,* isolated from *T. wallicbiana*, produces 60-70 µg/l taxol in culture, approximately 1,000 times higher than *T. andreanae* [*37*]. Li and co-workers [*38, 39*] discovered that 9 of 16 strains of *P. microspora* from larches produce taxol. One of them can secrete as much as 1,487 ng/l in the medium. Fungi producing taxol were isolated in both the inner and outer bark of *Taxodium distichum* [*39*]. One isolate of endophytic taxol-producing fungi in Austeria, *P. guepinii*, was obtained from *Wollemia nobilis* [*40*]. Moreover in China, a variety of taxol-producing fungal endophytes have been documented from *Taxus* sp., following the first record from *T. yunnanensis* by Qiu and Co-workers [*41*]. One strain representing a new genus in china, *Nodulisporium sylviforme*, was isolated by Zhou and co-workers [*42*]. These discoveries indicate that endophytic fungi are abundant resources of taxol and are valuable source for both basic research and scale-up interest.

In terms of environmental friendly, endophytic fungi have some advantages over yew trees, for instance, short-life-cycle and easy cultivation. In practice, it opens a new way to make this important drug by fermentation of fungi. On the other hand, study on these fungi has its own significance. For example, the fact that *Taxus* sp. and their endophytes produce the same secondary metabolite raises questions: Is the biosynthetic pathway of taxol in fungi identical to that in *Taxus* sp.? Do the enzymes involved in taxol biosynthesis and the genes encoding for them in fungi and plants have any evolutional co-relationship? Who is the true producer of taxol? Further study on these fungi will solve all of questions and the cost of the treatment with taxol will fall greatly as expected once the fermentation of taxol succeeds [*43*].

2.3.2 Metabolites of the Genus *Phomopsis*/*Diaporthe*

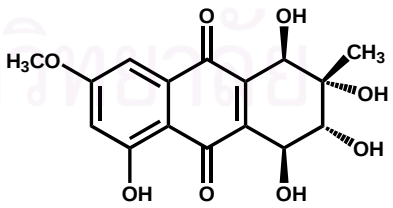
All perfect stages of *Phomopsis* species have been proved to belong to the genus *Diaporthe*. The genus *Phomopsis* has been known to be a rich source of bioactive secondary metabolites of diverse structures.

Tsantrizos [*44*] reported a list of all known metabolites in this genus. He classified metabolites by fungal species. The chemical structures and biosynthetic origin of these compounds are just as diverse as their biological activity. Thus, some representative examples of general interest in natural product chemistry will be shown in Figure 2.5.



Phomopzin

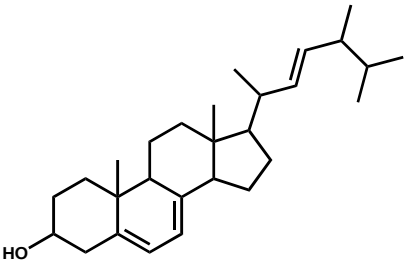
Metabolite of *P. helianthi*



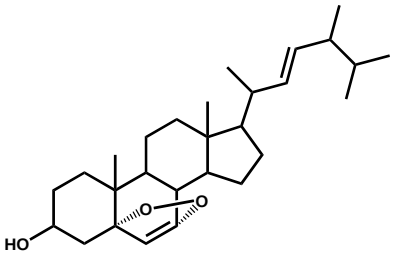
Altersolanol A

Metabolite of *P. juniperovora*

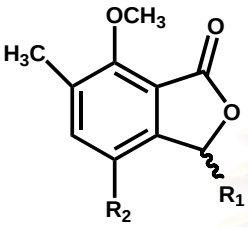
Figure 2.5 continued [*44*]



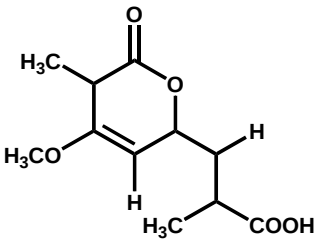
Ergosterol



Ergosterol peroxide

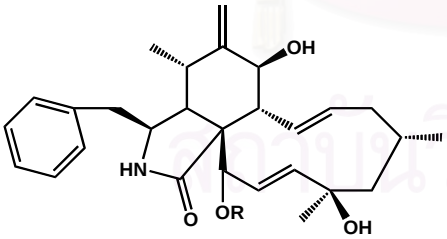


Convolvulanic acid A: R_1 = OH, R_2 = CO_2H
Convolvulanic acid B: R_1 = H, R_2 = CO_2H
Convolvulol: R_1 = H, R_2 = CH_2OH



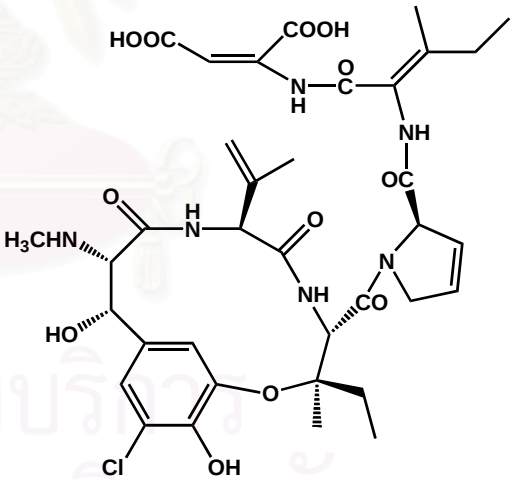
α-pyrone convolvupyrone

Metabolites of *P. convolvulus*



Cytochalasin H: R = Ac
Cytochalasin J: R = H

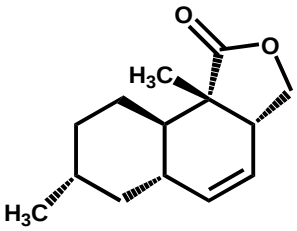
Metabolites of *P. pasalli*



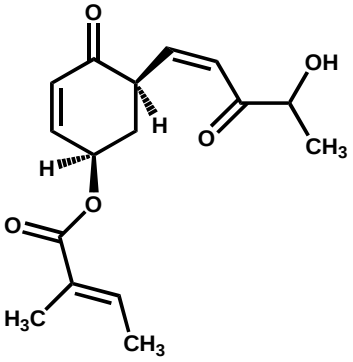
Phomopsin A

Metabolite of *P. leptostromiformis*

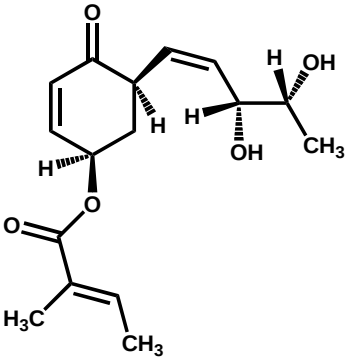
Figure 2.5 continued [*44*]



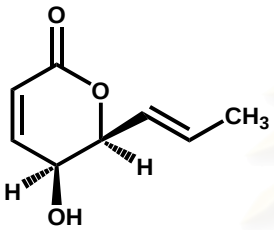
Oblongolide



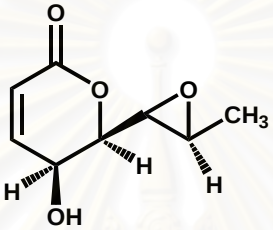
Phomopsolide A



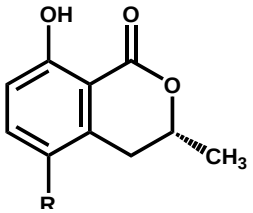
Phomopsolide B



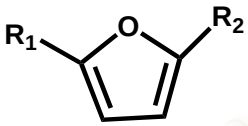
Phomoplactone



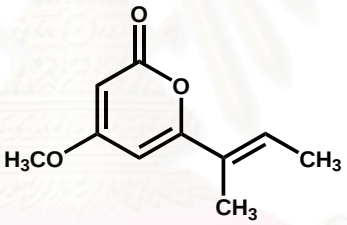
Asperline



5-Methylmellein: R = CH_3
Mellein-5-carboxylic acid derivative: R = CO_2H

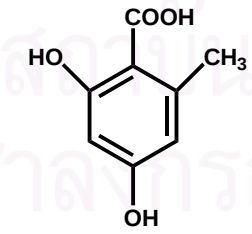


2-Furoic acid: R_1 = H, R_2 = CO_2H
2,5-Dicarboxylic acid derivative:
R_1 = R_2 = CO_2H

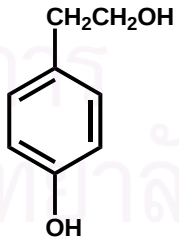


Nectriapyrone

3-Nitropropanoic



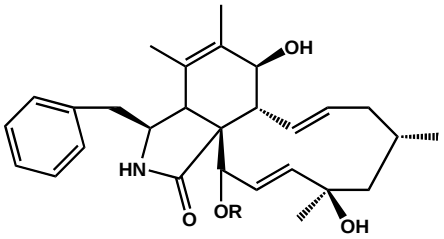
Orsellinic acid



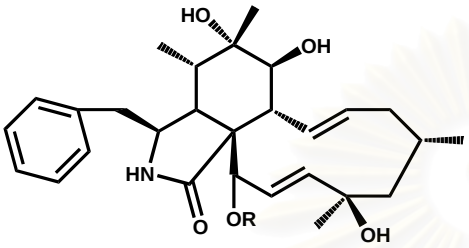
4-Hydroxyphenylethanol

Metabolites of *P. oblonga*

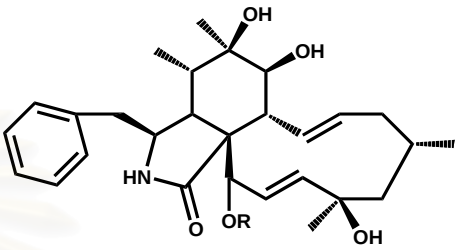
Figure 2.5 continued [*44*]



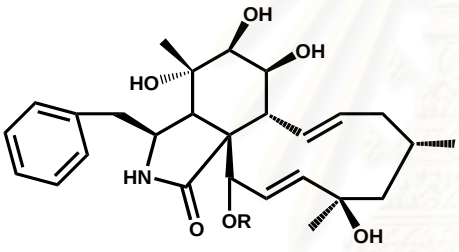
Cytochalasin N: R = Ac
Cytochalasin O: R = H



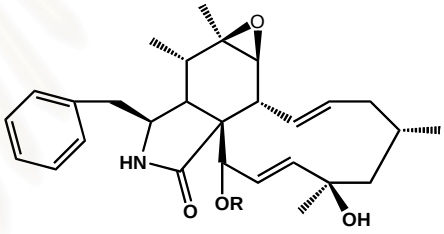
Cytochalasin P: R = Ac
Cytochalasin Q: R = H



Cytochalasin R



Cytochalasin S



Epoxycytochalasin H: R = Ac
Epoxycytochalasin J: R = H

Metabolites of Unidentified species

Figure 2.5 Classification metabolites by fungal species [*44*]

In 1995, Horn and co-workers [*45*] discovered a novel antimicrobial agent phomopsichalasin (Figure 2.6). This compound was isolated from an endophytic *Phomopsis* sp. (MF6031, Merck Microbial Resources Culture Collection) fermented on shredded wheat. Phomopsichalasin showed mainly antibacterial activity in disk diffusion assays. At 4 μg/disk phomopsichalasin exhibited zones of 12 mm against *Bacillus subtilis*, 11 mm against *Salmonella gallinarum*, and 8 mm against *Staphylococcus aureus*. It also exhibited an 8 mm zone against the yeast *Candida*

tropicalis. Phomopsichalasin represents the first cytochalasin-type compound with a three ring system replacing the cytochalasin macrolide ring.

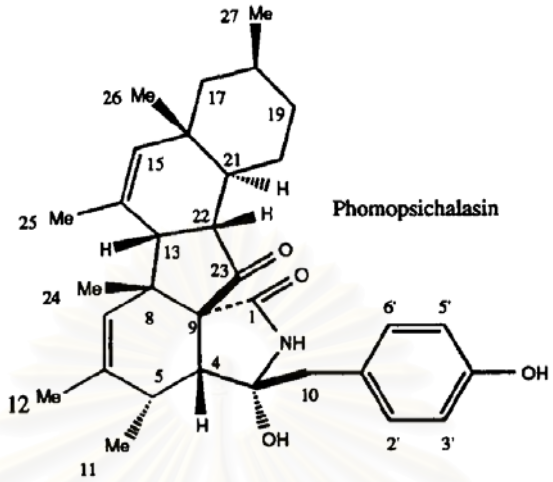


Figure 2.6 Structure of Phomopsichalasin [*45*]

In 2000, Brady and co-workers [*46*] studied endophytic fungi collected in the Guanacastle Conservation Area of Costa Rica. Organic extracts from cultures of endophytic fungi were screened for antibiotic activity. Two endophytes CR200 (*Cytospora* sp.) and CR146 (*Diaporthe* sp.) were found to have potent antibiotic activity. Bioassay-guided fractionation of the extracts from these fungi led to the identification of cytosporones D and E, antibacterial active trihydroxybenzene lactones, and three related but inactive metabolites (Figure 2.7). Moreover, they proposed biosynthetically linked by a hypothetical cytosporone A-like intermediate that cytosporone required reduction at C-15 and C-9 followed by closure of the lactone at C-9.

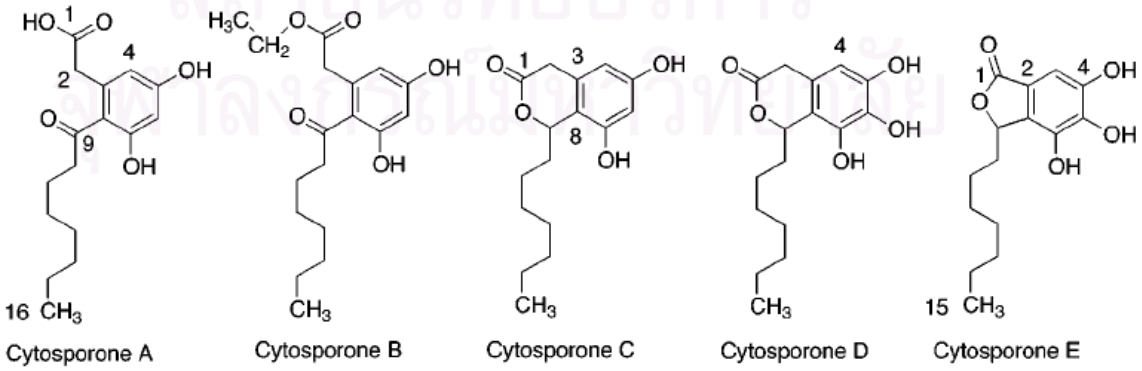
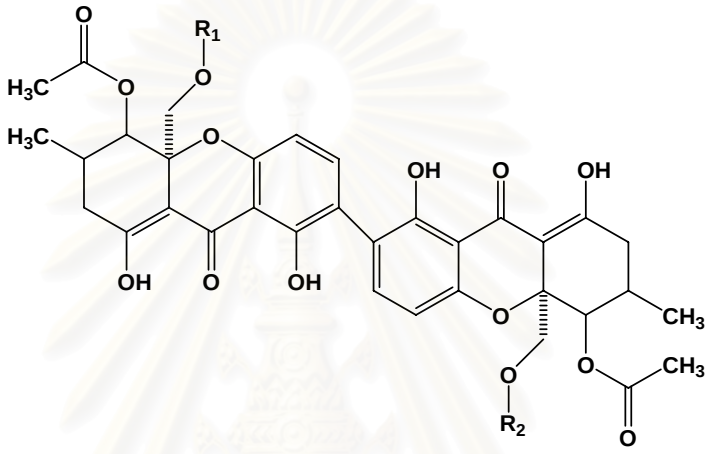


Figure 2.7 Structures of Cytosporones [*46*]

In 2001, Wagenaar and Clardy [*47*] found three new cytotoxic dimers, named dicerandrols A, B, and C (Figure 2.8) which were produced by the endophyte *Phomopsis longicolla* isolated from an endangered mint. Three compounds also exhibited antibacterial activity against both *Staphylococcus aureus* and *Bacillus subtilis* but are inactive against the fungus *Geotrichum candidum* and the yeast *Saccharomyces cerevisiae*. Dicerandrol A was more active than B and C respectively. So, the antibacterial activity of these compounds decreased upon successive acetylation.



Dicerandrol A, $R_1 = R_2 = H$

Dicerandrol B, $R_1 = Ac$, $R_2 = H$

Dicerandrol C, $R_1 = R_2 = AC$

Figure 2.8 Structures of Dicerandols [*47*]

In 2001, Isaka and co-workers [*48*] reported two novel xanthone dimers, Phomoxanthones A and B. Two compounds were isolated from the endophytic fungus *Phomopsis* sp. BCC1323 (BIOTECH Culture Collection). The endophyte was isolated from a teak leaf at Mee Rim district, Chaingmai Province, Northern Thailand. A deacetyl derivative was prepared by treatment of Phomoxanthones A with concentrated H_2SO_4/MeOH. These structures are related to secalonic acids which are C2-C2' dimers of tetrahydroxanthone. Phomoxanthones A and B are the first cases of naturally occurring C4-C4' and C2-C4' tetrahydroxanthone dimers. In addition, phomoxanthones A and B exhibited significant activity against *Plasmodium falciparum* and *Mycobacterium tuberculosis*, although weaker than standard drugs. These compounds were also cytotoxic to two cancer cell lines (KB, BC-1). The

deacetyl analogue of Phomoxanthones A was inactive all assays. This may due to the low lipophilicity of analogue compared to those Phomoxanthones A and B (Figure2.9).

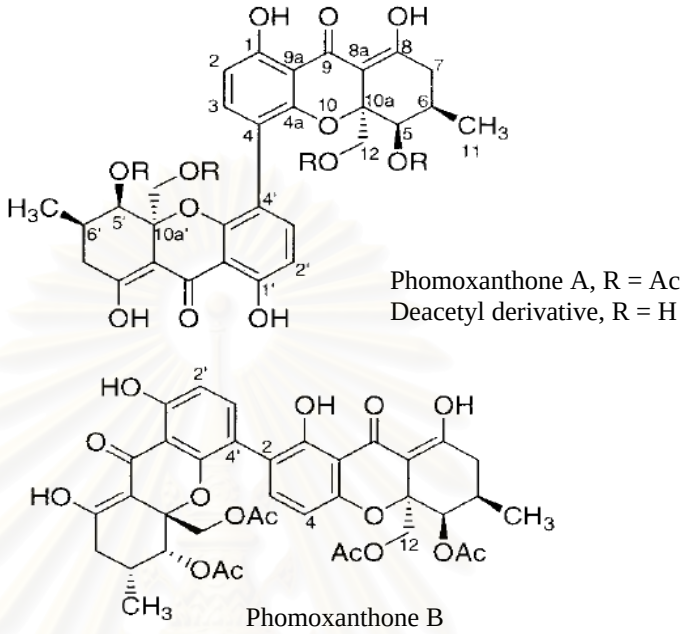


Figure 2.9 Structures of Phomoxanthones [*48*]

In 2004, Daniela and co-workers [*49*] reported a new anti-inflammatory metabolite from an endophyte *Phomopsis* sp. of the medicinal plant *Erythrina cristagalli* called Phomol, a polyketide lactone (Figure 2.10). The anti-inflammatory activity was tested in different receptor gene assays (TNF-α, STAT1/STAT2, and NF-κB) and in an ear edema model in mice. In the receptor gene assays phomol exhibited no activity, whereas showed interesting anti-inflammatory activity in the mouse ear assay.

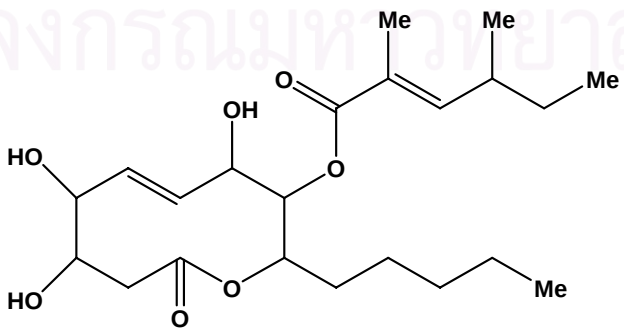
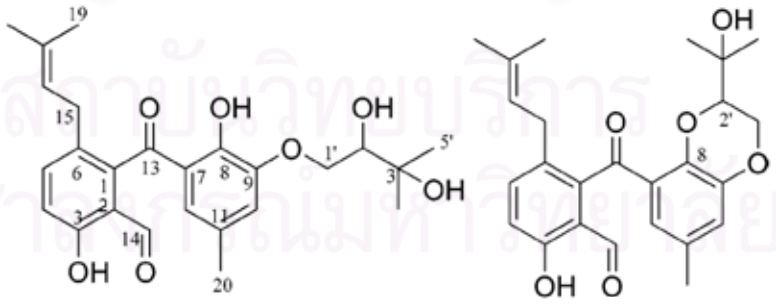


Figure 2.10 Structure of Phomol [*49*]

In 2004, Schwarz and co-workers [*50*] isolated 3-hydroxypropionic acid (3HPA, OH-CH_2CH_2-COOH) which obtained from submerged cultures of several endophytic fungi isolated from above-ground plant organs. This compound showed selective nematicidal activity against the plant-parasitic nematode *Melodiogyne incognita* with LD_{50} values of 12.5-15 μg/ml. Activity against the saprophytic *Caenorhabditis elegans* was fivefold lower. No antimicrobial, cytotoxic or phytotoxic effects were observed. Propionic acid and d- and l- lactic acids were not active against either nematode species. According to identification of the producers the five endophytes producing 3HPA thus belonged to two different families (Diaporthaceae and Melanconidaceae).

In 2005, Zhang and co-workers [*51*] screened their library of natural product extracts for inhibitors of parasite cGMP-dependent protein kinase (PKG) because parasite PKG has been recently validated as a biochemical target for the treatment of coccidiosis. They performed high-throughput bioassay guided fractionation of those extracts with whole cell activity using the PKG enzyme assay. They found that the natural product extract from endophytic fungus *Diaporthe* sp., which isolated from a plant sample collected in Tenerife Canary Islands (Spain), hit this assay. This effort led to the discovery of new anticoccidial lead benzophenones, tenellones A and B (Figure 2.11).



Tenellone A Tenellone B

Figure 2.11 Structures of Tenellones [*51*]

In 2005, Dai and co-workers [*52*] discovered thirteen new metabolites, namely oblongolides B-M (2-13) and 4-[5-(1-hydroxyethyl)furan-2-yl]-4-oxobutanoic acid (14), together with the six known compounds phomopsolide B (15), alternariol dimethyl ether (17), the mycotoxin alternariol (18), ergosterol (19), and 5α,8α-epidioxyergosterol (20) were isolated from the endophytic fungus *Phomopsis* sp., which was obtained from the plant *Melilotus dentata* from the shores of the Baltic Sea (Germany). The new biologically active norsesquiterpene γ-lactones (2-13) differ in their degree of substitution, saturation, and substituent pattern from the known oblongolide (1). Thus, it represents a potential lead structure in particular for herbicidal and antifungal properties. The interesting Ulm bark beetle repellent property of a selected series of the oblongolides is currently under investigation (Figure 2.12).

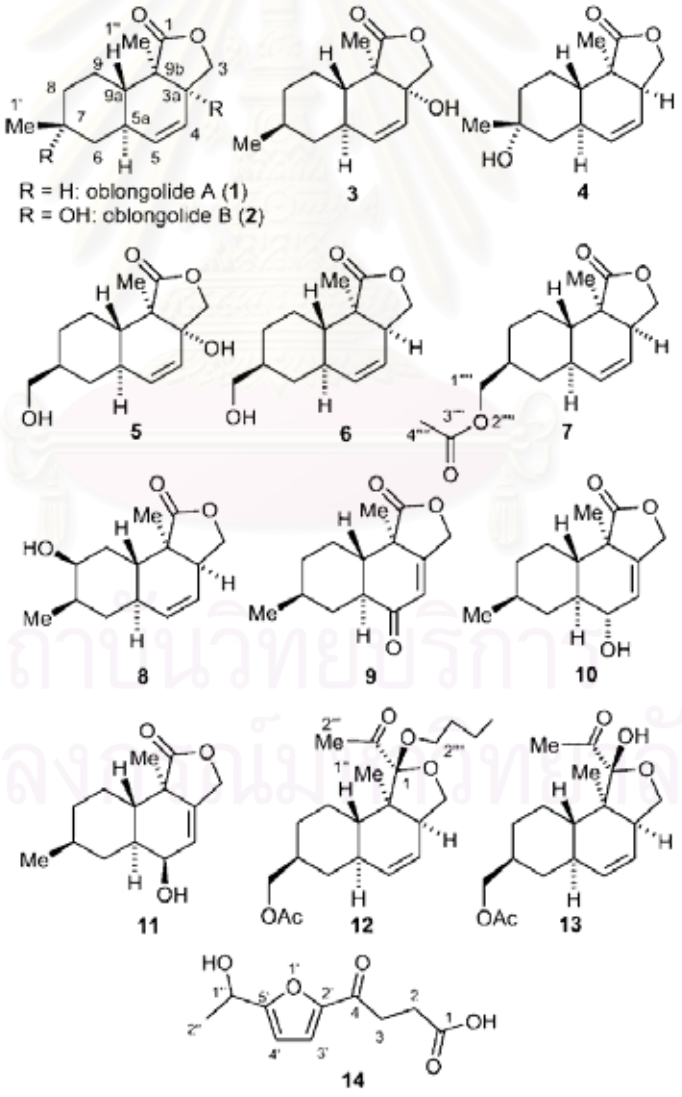


Figure 2.12 Metabolites isolated from the broth of *Phomopsis* sp. [*52*]

In 2005, Dai and co-workers [*53*] reported six new metabolites, phomosines D-G (18-21), 6-hydroxy-6-isopropylcyclohex-1-enecarboxylic acid (22) and (1a*S*, 3*R*,4*R*,4a*R*,6*S*,7*R*,8a*S*)-7-chloro-3,6-dihydroxy-3,4a,8,8-tetramethyl-octahydro-1a*H*-naptho[1-b]oxirene-4-carboxylic acid (23) were isolated together with seven known compound (15-17, 24-27) (Figure 2.13) from endophytic fungus *Phomopsis* sp. strain No.711, was isolated from *Adenocarpus foliolosus* from Gomera. The antibacterial, fungicidal, and herbicidal properties of the new compounds were evaluated. In summary, the investigated *Phomopsis* sp. No.711 shows remarkable biodiversity in the production of bioactive secondary metabolites in two ways. The first is exemplified in the functional group transformation of one basic skeleton ranging from changements in oxidation states, alkylation or acylation degree to chain and ring-closed forms. The second way is the diversity expressed in the production of products of different ring systems generated by different biosynthetic pathways.

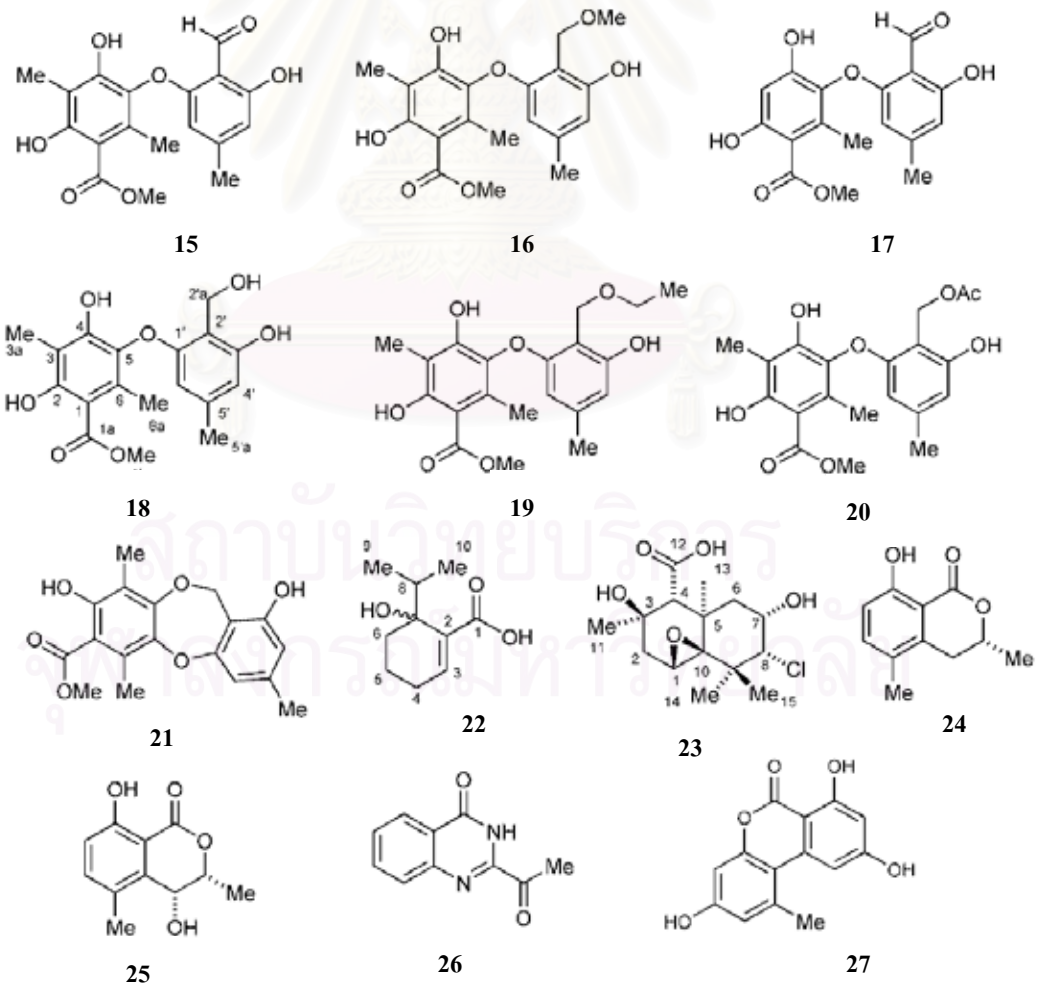


Figure 2.13 Structures of compounds isolated from *Phomopsis* sp. [*53*]

In 2006, Agusta, Ohashi and Shibuya [*54*] cultivated six species of endophytic filamentous fungus *Diaporthe* sp. which were isolated from the young stems of the tea plant *Camella sinensis*. And it was found that one of two filamentous fungus *Diaporthe* sp., which is closely related to *D. phaseolorum*, strain SW-93-13 produced two bisanthraquinones named epicytoskyrin and 1,1'-bislunatin (Figure 2.14). In contrast, another endophytic fungus closely related to *D. phaseolorum* from the same plant does not produce two bisanthraquinones, but has a capacity to transform catechins possessing 2*R*-phenyl substitution into the corresponding 3,4-*cis*-dihydroxyflavan derivatives [*55*, *56*].

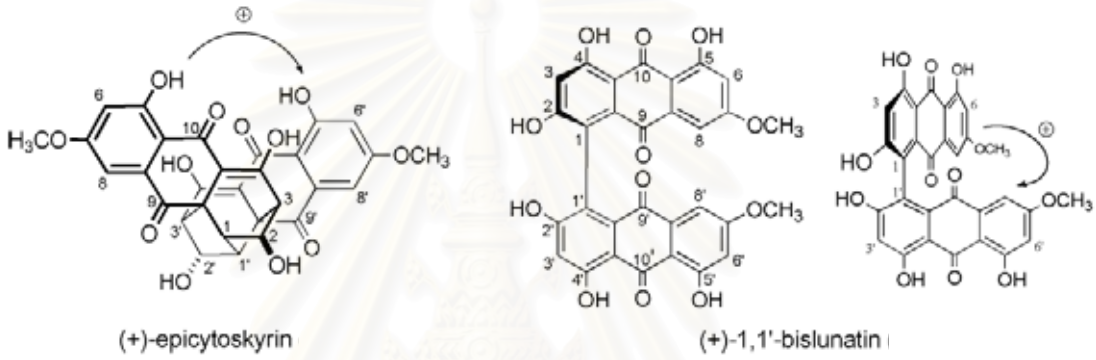


Figure 2.14 Structures of metabolites from Diaporthe sp. [*54*]

2.4 *Croton sublyratus* Kurz. [*57*]

Croton sublyratus Kurz. 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 acuminate repand-serrulate beneath glabrous or with scabrous nerve and raceme stellate-tomentose. Yong 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.

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.

Until now several diterpenes have been isolated and identified. The compounds are shown in Table 2.2

Table 2.2 Chemical constituents of *Croton sublyratus* Kurz.

Chemical Group	Chemical substance (part of plant's isolated)	References
Diterpene lactones	Plaunol A (stem) Plaunol B (stem) Plaunol C (stem) Plaunol D (stem) Plaunol E (stem)	*58*
Furanoid diterpene	Plaunolide (stem)	*59*
Diterpene alcohols	Plaunotol (leaf) *ent*-13α-hydroxy-13-epimanool (stem) *ent*-16β,17-dihydroxykaurane (stem)	*60* *61*
Esters of 18-hydroxy-geranylgeraniol	Steric acid (whole plant) Oleic acid (whole plant) Caprylic acid-oleic acid (whole plant) Caprylic acid-palmitic acid (whole plant) 2-Palmitic-olenic acid (whole plant) Linoleic acid-linolenic acid (whole plant)	*62*

CHAPTER III

EXPERIMENTS

3.1 Chemicals

3.1.1 Solvents

All commercial grade solvents used in this research, such as hexane, chloroform, dichloromethane, ethyl acetate and methanol, were distillated prior to use. The reagent grade solvents were used for thin layer chromatography and crystallization.

3.1.2 Other chemicals

3.1.2.1 Merck's silica gel 60 Art. 1.09385.9025 (230-400 mesh ASTM) were used as adsorbent for normal column chromatography.

3.1.2.2 Merck's TLC aluminium sheet, silica gel $60F_{254}$ Art. 1.05554.0001 procoated 25 sheets, 20x20 cm^2, layer 0.2 mm was used to monitor the fractions and preparative TLC.

3.2 General experiments

3.2.1 Melting point

The melting points were recorded on a Fisher-Johns melting point apparatus.

3.2.2 Specific optical rotation

All specific optical rotation, $[\alpha]_D$, were measured at 589 nm (the sodium D line), 20 ^oC by a Perkin-Elmer 341 polarimeter.

3.2.3 Ultraviolet - visible Spectrophotometer (UV-VIS)

UV-VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in chloroform and methanol.

3.2.4 Fourier Transform-Infrared Spectrophotometer (FT-IR)

FT-IR spectra were recorded on a Nicolet Impact 410 spectrophotometer. Spectra of solid samples were recorded as NaCl or KBr pellets. Liquids can be placed as a thin film between two salts plates made of NaCl or KBr.

3.2.5 Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 400 and 100 MHz, respectively, on a Varian Model Mercury 400 MHz in deuterated solvents. Chemical shifts protons were reported relative to residual solvents peaks the values 7.26(1) ppm for chloroform ($CDCl_3$), 2.49(5) ppm for dimethylsulfoxide-d_6 (DMSO-d_6), 4.78(1) and 3.30(5) ppm for methanol-d_4 (CD_3OD) and 4.67 ppm for deuterium oxide (D_2O).

3.2.6 Mass Spectrometer (MS)

High resolution mass spectra (ESI-TOF-MS) were performed by Mass Spectrometer LCT, Micromass UK Limited.

3.2.7 Rotary Evaporator

Buchi rotary evaporator was used for the rapid removal of large amounts of volatile solvents.

3.3 Culture Media

Culture medium used for isolation and cultivation for study metabolites of bioactive compounds was Malt Extract Medium (agar and broth). Potato Dextrose Medium, Yeast Extract Medium, Corn Meal Medium and Sabouraud Dextrose Medium were used for observation characteristics of selected endophytic fungal isolate. The composition and preparation of culture media are described in Appendix A.

3.4 Isolation of Endophytic fungal isolates

Endophytic fungal isolates were isolated from healthy leaves of *Croton sublyratus* (Euphorbiaceae), collected from plants growing in the open field of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Leaves of *Croton sublyratus* were washed under tap water, and then soaked in 95% ethanol for 1 minute followed by surface sterilization in 10% Clorox® (5% available chlorine) for 5 minutes and washing 2 times with sterile distilled water. With sterilization techniques, the sterile leaves were excised into small pieces (1 x 1 cm^2) and placed on Malt Extract Agar (MEA) medium for fungal germination, then maintained in a culture room at

ambient temperature (25-30 ^{O}C). Under these conditions, endophytes mycelia germinate from the leaf pieces.

For endophytes purification, outgrowing mycelia were transferred into new petri dishes containing MEA by hyphal tip transfer. Endophytes were incubated for 10 days at room temperature and were subcultured 2-3 times, which resulted in pure cultures. Purity was determined by colony morphology. Fungal isolates were used for further study.

3.5 Selection of Endophytic fungal isolates

A total of thirty of unidentified endophytic fungal isolates were studied (Bkk1-30). They were divided into two groups, the first group (Bkk1-16) was isolated by Monthika Pothavorn in 2004 [*63*] and the second group (Bkk17-30) was isolated in this research. All isolated were screened the production of metabolites using conventional procedure. Based on their ^{1}H-NMR spectra and number of spots on a TLC plate of dichloromethane (CH_2Cl_2) crude extract, the endophytic fungal isolate Bkk3 was selected for the investigation of secondary metabolites.

3.5.1 Preparation of CH_2Cl_2 crude extract

Pure endophytic fungal isolates were cultivated on Malt Extract Agar (MEA) for 1-2 weeks depend on growth rates. Two plates of each isolate were cut into small pieces. Every pieces of agar culture were transferred into 500 ml Erlenmeyer flask, and then extracted with CH_2Cl_2 (250 ml x 3) in ultrasonic bath. The solvent was evaporated using a rotary evaporator at 25 ^{o}C to yield crude extract.

3.5.2 Monitoring of TLC plate

3.5.2.1 Visual detection under ultraviolet light at wavelengths of 254 and 365 nm.

3.5.2.2 Visual detection in iodine vapor.

3.5.2.3 Visual detection under daylight after spraying with vanillin reagent (Dissolve 1 g vanillin in 95 ml ethanol and add 4 ml concentrated sulfuric acid) and heating until the colors developed.

3.6 Identification of selected endophytic fungal isolate Bkk3

3.6.1 Macroscopic structure examination

Characters such as shape, size, color and others were observed.

3.6.2 Microscopic structure examination

3.6.2.1 Preparation of specimen for light microscope

Materials preparation for slide culture technique was done onto V-shape glass rod onto filter paper in Petri dishes. The materials were sterilized in autoclave. Malt Extract Agar was poured in sterile Petri dishes and the agar was cut into 1 x 1 x 0.3 cm size, then aseptically put on prepared slide glass and inoculated the culture of Bkk3 on middle of four edges of agar, after that covered a piece of agar with cover slide and poured sterile distilled water for moisture inside the Petri dish, incubated at room temperature (25-30 ^{o}C) until fungi grew onto glass slide and cover slip. Picture of slide culture preparation is shown in Figure 3.1.

Semipermanent slides (from slide culture) for light microscopy were mounted in lactophenol-cotton blue for observations of spore production. The microscopic mycelial structure was examined under light microscope model Olympus CH_2.

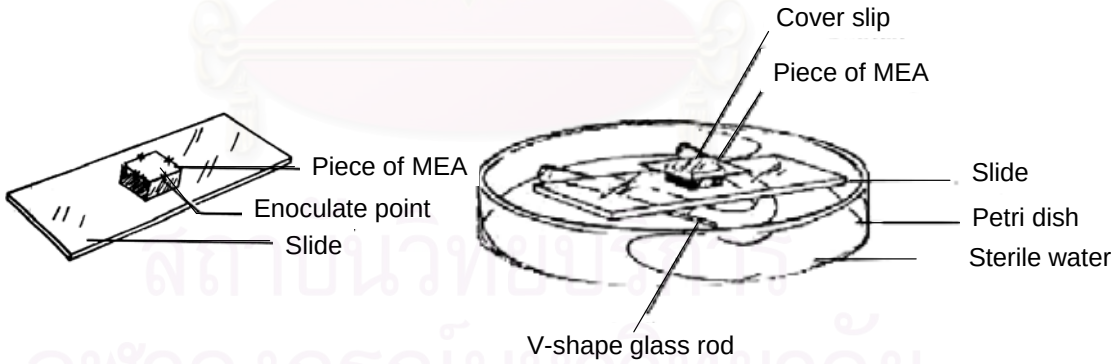


Figure 3.1 Slide culture preparation

3.6.2.2 Preparation of DNA analysis

Total DNA was extracted from fungal mycelium grown in Potato Dextrose Broth (PDB) using DNeasy® Plant Mini Kit (Qiagen). Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC)

were used to amplify the ITS1-5.8S-ITS2 region from total DNA extracted [64]. The thermal cycle program was as follows: 3 min at 95 ^{o}C followed by 30 cycles of 50 s at 95 ^{o}C, 40s at 45 ^{o}C and 40s at 72 ^{o}C, with a final extension period of 10 min at 72 ^{o}C. The amplified DNA was purified and directly subjected to sequencing reactions using primers ITS5 and ITS4. BLASTN 2.2.10 was used to search for similar sequences in the GenBank [*65*]. DNA sequence similarity was determined by ClustalW (1.82) multiple sequence alignment program [*66*]. Phylogenetic relationship was estimated using PAUP* (v 4.0 b10) [*67*].

3.7 Cultivation and extraction of endophytic fungal isolate Bkk3

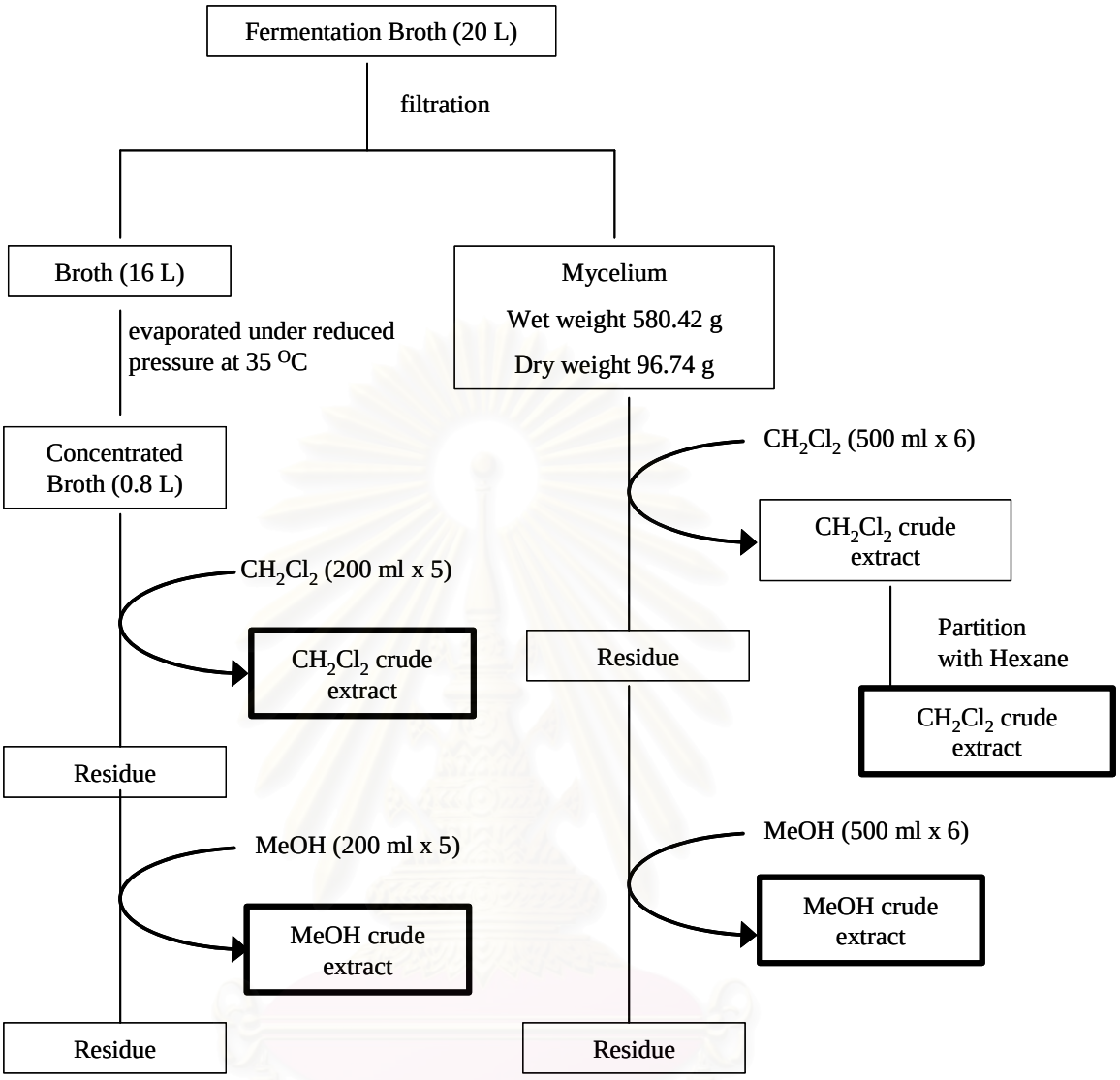
3.7.1 Cultivation

The isolate Bkk3 was grown on MEA at room temperature (25-30 ^{o}C) for 7 days. The agar was then cut into 8 mm diameter with a flamed cork hole borer. Five pieces of agar cultures were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of MEB (x 200), and then statically incubated at room temperature for 60 days.

3.7.2 Metabolite extraction of isolate Bkk3

The culture broth (20 L) was filtered through filter paper (Whatman No. 1) to separate the broth supernatant and mycelia. The filtrate (broth) was evaporated under reduced pressure at 35 ^{o}C. The concentrated broth was extracted with 200 ml of dichloromethane (CH_2Cl_2) 5 times. The CH_2Cl_2 layers were collected, dried over anhydrous sodium sulfate and then evaporated under reduced pressure at 25. The residue broth was extracted again with 200 ml of methanol (MeOH) 5 times. The MeOH layers were collected, dried over anhydrous sodium sulfate and then evaporated under reduced pressure at 35 ^{o}C.

The mycelia were blended and extracted with 500 ml of CH_2Cl_2 (x 6) in ultrasonic bath. The CH_2Cl_2 layers were collected and concentrated to dryness under reduced pressure at 25 ^{o}C. The dry CH_2Cl_2 crude extract was partitioned with hexane followed by evaporation. The residue mycelia were extracted again with an equal volume of MeOH. The MeOH layers were concentrated to dryness under reduced pressure at 35 ^{o}C. Priority of the crude extracts for isolation was selected based on their dry weight and ^{1}H-NMR spectra to column chromatography. The extraction of the mycelia and culture broth of isolate BKK3 is shown in Scheme 3.1.



Scheme 3.1 Diagram of extraction of the mycelia and fermentation broth of Bkk3

3.8 Separation of metabolites from endophytic fungal isolate Bkk3

The dichloromethane crude extracts of the isolate Bkk3 designated as DCM and DCB respectively for mycelia and broth supernatant. The DCM was subjected to silica gel column chromatography and eluted with hexane-dichloromethane and dichloromethane-methanol in a stepwise fashion. The DCB was eluted with hexane-ethyl acetate and ethyl acetate-methanol, respectively. Similar fractions were combined on the basis of TLC with detection by UV light and vanillin/H_2SO_4 reagent.

3.9 Determination of biological activities

3.9.1 Assay of CYP3A4 Inhibition

CYP activity was based on nifedipine oxidation. Various amounts (0-10 mM, final concentration) of samples in 1 µl of DMSO were added to 192 µl of solution containing 100 mM phosphate buffer (pH 7.4) containing 50 µM nifedipine (Wako Pure Chemical Industries, Ltd. (Osaka, Japan)), 5 mM glucose-6-phosphate (Oriental Yeast Co., Ltd. (Tokyo, Japan)), 0.5 mM β-NADP+ (Oriental Yeast Co., Ltd.), 0.5 mM $MgCl_2$, and 4.3 µg/ml glucose-6-phosphate dehydrogenase (Oriental Yeast Co., Ltd.) and incubated at 37 °C for 5 min. CYP3A4 (Gentest Co. (Woburn, MA)) was also pre-incubated in 7 µl of the buffer at 37 °C for 5 min and added to the sample solution. After the incubation at 37 °C for 1 h, the reaction was quenched by the addition of 100 µl of MeOH. After adding 3.7 µg of 6-methoxycarbonyl-5-methyl-7-(2-nitrophenyl)-4,7-dihydrofuro[3,4-*b*]pyridin-1-(3*H*)-one in 1 µl of DMSO as an internal standard, the reaction mixture was extracted with 1 ml of ether, and the ether layer was evaporated. The residue was dissolved in 100 µl of MeOH, and an aliquot (20 µl) was analyzed by reverse-phase HPLC (column, TSK-gel ODS-120T, 4.6 mm i.d. x 150 mm; mobile phase, 64% MeOH-H_2O; flow rate, 1.0 ml/min; detection, UV 254 nm); retention times: 2.9 min for the internal standard, 4.0 min for the nifedipine metabolite (nifedipine pyridine), and 5.5 min for nifedipine. The value of IC_{50}, the concentration required for 50% inhibition of CYP3A4 activity, was calculated from the data of triplicate measurements.

3.9.2 Cytotoxicity test

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed into replicate 96-well culture plates in 100-µl volumes using a repeating pipette. Following a 24-h incubation at 37^oC, 5% CO_2, 100% relative humidity, 100 µl of culture medium, culture medium containing the sample was dispensed into the appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) “background” determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ ml PBS was sterilized and

filtered through 0.45-µl filter units. MTT working solutions were prepared just prior to culture application by dilution of MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 µl) was added to each culture well, resulting in 50 µl MTT/ 250 µl total medium volumes; and cultures were incubated at 37 ^{o}C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 µl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 µl of DMSO using a pipette. Following formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

Cell line growth and growth inhibition were expressed in terms of mean (+/- 1 SD) absorbance units and / or percentage of control absorbance (+/- 1 SD %) following subtraction of mean “background” absorbance.

Samples were also tested for cytotoxic activity towards 5 cell lines, which contain HEP-G2 (hepatoma), SW620 (colon), Chago (lung), KATO-3 (gastric) and BT 474 (breast) following the experimental method of bioassay of cytotoxic activity.

3.9.3 Antimicrobial activity test

Microorganisms used for antimicrobial activity assay were *Bacillus subtilis* ATTC 6633, *Staphyllococcus aureus* ATTC 25923, *Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853. Pure colonies bacteria were inoculated into 5 ml of Nutrient Broth (NB) and inoculated at 37^{o}C for 24 h. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of 0.5 McFerland. *Candida albicans* ATTC 10231 were grown on Yeast-malt Extract Broth (YEM) and incubated at room temperature for 24 h. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of a 0.5 McFerland.

Sterile cotton applicators were immersed in the inoculum suspension and pressed lightly against the tube wall to remove excess moisture. The agar was inoculated by streaking the swab across the entire surface. This was repeated twice, turning the plate 60 degree between each streaking. 2 mg of pure compound was dissolved in 1 ml of 10% DMSO in sterile distilled water. Streptomycin and Captan (1µg/ml) were used as positive control for bacteria and yeast, respectively. Then

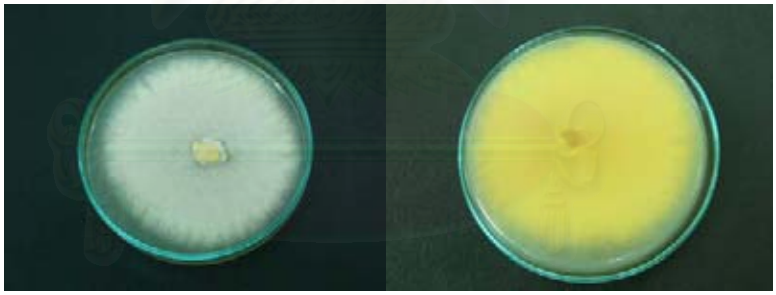
sterile paper disc (size 6.0 mm,Whatman) were placed on microorganism test plate. 100 µl of pure compound (2 mg/ml) was pipetted onto paper dics. Microorganism test plates were incubated at 37^{o}C for 24 h, followed by observation the clear zone.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Identification of endophytic fungal isolate Bkk3

The culture of endophytic fungal isolate Bkk3 was whitish mycelium on Malt Extract Agar and did not produce spores (Figure 4.1). In common mycological media, the isolate Bkk3 did not produce spores on different media, including Potato Dextrose Medium, Yeast Extract Medium, Corn Meal Medium and Sabouraud Dextrose Medium. Moreover, the isolate Bkk3 did not develop any fruiting body or conidia after cultivation for 2 months at 25 ^{o}C on banana leaf agar. This condition is suggested for promoting sporulation [*68*]. Furthermore, the slide culture of the isolate Bkk3 was observed only hyphae and septal structures. Therefore, the isolate Bkk3 was classified as mycelia sterile, and nucleotide sequences of rDNA genes provided an attractive approach in its taxonomy.



Obverse **Reverse**

Figure 4.1 Colony morphology of endophytic fungal isolate Bkk3

Further efforts to taxonomically classify the endophytic fungal isolate Bkk3 was carried out with molecular method by determining the nucleotide sequence of ITS1-5.8S-ITS2 region of rDNA gene. Nucleotide sequence of 5.8S region is highly conserved, so it is used for the phylogenetic analysis at the higher taxonomic levels (Phylum and Class). The highly variable internal transcribed spacers (ITS1 and ITS2) were used for phylogenetic analysis at lower taxonomic levels (orders to species) [*14*].

The optimization of PCR condition was previously described in the experiments section (3.5.2.2). Sequencing of the PCR product amplified from chromosomal DNA of the isolate Bkk3 resulted in a 524 base pair fragment as shown in Figure 4.2.

```
....|....| ....|....| ....|....| ....|....| ....|....|
        10         20         30         40         50

GCGGAGGGAT CATTGCTGGA ACGCGCCCCA GGCGCACCCA GAAACCCTTT
GTGAACTTAT ACCTTACTGT TGCCTCGGCG TACGCTGGCC CCCAGGGGTC
CCTCTGTCTA CAGAGGAGCA GGCACGCCGG CGGCCAAGTT AACTCTTGTT
TTTACACTGA AACTCTGAGA AAAAACAAAA ATGAATCAAA ACTTTCAACA
ACGGATCTCT TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA
GTAATGTGAA TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT
TGCGCCCTCC GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCAAC
CCTCAAGCCT GGCTTGGTGT TGGGGCACTG CCTGTAAAAG GGCAGGCCCT
CAAATCTAGT GGCGAGCTCG CCAGGACCCC GAGCGTAGTA GTTAAACCCT
CGCTTTGGAA GGCCCTGGCG GTGCCCTGCC GTTAAAACCC CAACTCTTTG
AAAATTTGAC CTCGGATCAG GTAG
```

Figure 4.2 Nucleotide sequence of the partial 18S ribosomal DNA gene (1-10), complete internal transcribed spacer 1 (11-188) - 5.8S ribosomal DNA gene (189-346) – internal transcribed spacer 2 (347-505), and partial 28S ribosomal DNA gene (506-524) of the isolate Bkk3

The complete ITS1-5.8S-ITS2 sequences of the isolate Bkk3 was used as the query sequence to search for similar sequences from GenBank. It revealed *Diaporthe phaseolorum* as the closest match with 96% sequence identity. Phylogenetic analysis with 19 known close species hit (*Diaporthe* and *Phomopsis*, its anamorph, 90-96% sequence identities) using maximum parsimony placed it in the same clade with *Diaporthe phaseolorum* with 80% bootstrap support. These results suggested that the isolate Bkk3 should be a sterile *Diaporthe* sp. The DNA sequence of ITS1-5.8S-ITS2 of the isolate Bkk3 has been submitted to GenBank with accession numbers of DQ435767.

This study shows that the isolate Bkk3 could be species of *Diaporthe* and its anamorph, *Phomopsis*, whose several members were known to be plant pathogens often associated with diseases of agricultural crop plants and produce. Among the best known examples are fungi which cause post-harvest fruit-rot, such as *P. viticola*, a pathogen of grapes and vines, *P. citri*, a pathogen of citrus fruits and trees, and the seedborn pathogens of soybeans (*P. longicolla* and *P. phaseoli*) and lupines (*P. leptostomiformi*) [*44*].

Despite of these evidences of plant pathogenic nature of *Phomopsis* sp. and *Diaporthe* sp., there are two reasons to believe that the isolate Bkk3 is considered as an endophytic fungus. First, it is capable of living as a symptompless endophyte for prolonged periods within its host plant, *Croton subrylatus* Kurz. Second, it does not produce sexual reproductive units such as spores either normal media (PDA, MEA, YEA, CMA and SDA) or promoting medium (banana leaf agar). The type of symbiosis exhibited between plant and endophyte is often related to the reproductive mode of the endophyte. For example, vertically transmitted endophytes are asexual and transmit via fungal hyphae penetrating the host's seeds. Evolutionary theory predicts these endophytes are closely related to evolve toward strong mutualism, since their reproductive fitness is intimately tied to that of their host plant. Conversely, horizontally transmitted endophytes are sexual and transmit via spores that can be spread by wind and/or insect vectors. Therefore, these endophytes can evolve a more pathogenic lifestyle since they can escape their host plant.

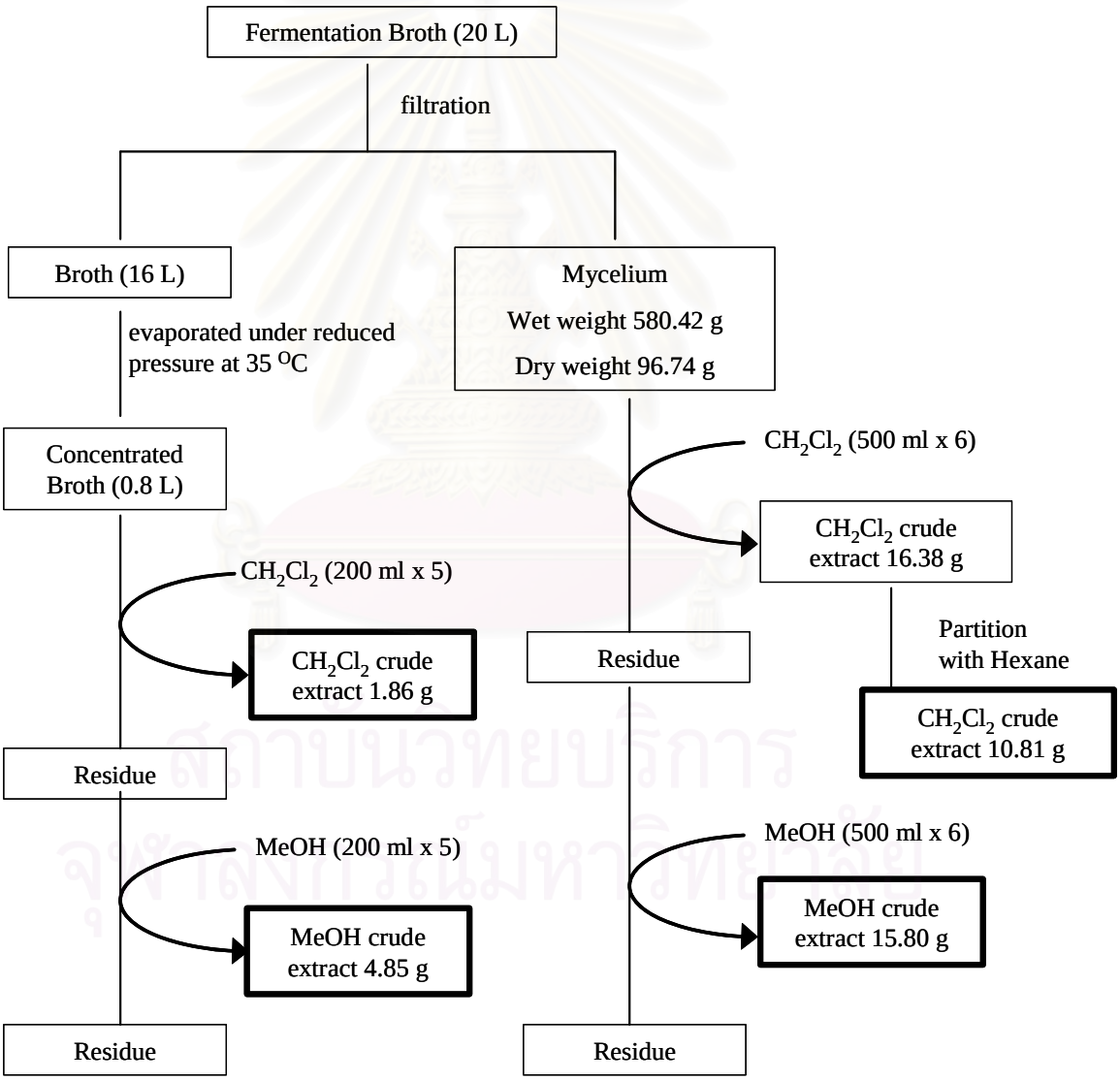
4.2 Cultivation and extraction of endophytic fungal isolate Bkk3

Micelia of the filamentous endophytic fungus *Diaporthe* sp. Bkk3 growing on MEA was inoculated into a 250 mL Erlenmeyer flask (x 200) containing 100 mL of malt extract broth culture medium and cultured statically at room temperature. The isolate Bkk3 grow rapidly during 50 days. A period from 55 to 65 days, the growth rate remains constant. But after 70 days, it starts to decline.

Typically the period of time when the size of the microbial population increases exponentially is called Log Phase, whereas, the period during which the average population size remains constant is known as Stationary Phase. During the log phase, fungi synthesize the primary metabolites for growing process. In contrast, in stationary phase, the population of fungi may deplete the environment of an essential

nutrient, such as carbon and energy and/or a required vitamin. As a result, the secondary metabolites are synthesized by fungi in order to survive. To fully obtain secondary metabolites, 60 days which are an average period of stationary phase in this study were selected for cultivation.

After cultivation for 60 days, twenty liters of whole broth was filtered through filter paper (Whatman no. 1) to separate the broth supernatant and mycelia. The mycelia and broth were extracted with hexane, dichloromethane and methanol, respectively. The results of extraction of the mycelia and broth of the isolate Bkk3 is shown in Scheme 4.1.



Scheme 4.1 The results from extraction of the mycelia and fermentation broth of Bkk3

4.3 Separation of metabolites from endophytic fungal isolate Bkk3

The dichloromethane crude extracts of mycelia and broth supernatant designated as DCM and DCB respectively, using wet packing and dry loading method and eluted by increasing polarity. The results are shown in Table 4.1 and 4.2

Table 4.1 Fraction obtained from silica gel column of DCM (10.81 g)

Eluents	Fraction No.	Appearance	Weight (mg)
70% hexane in CH_2Cl_2	1-9	Yellow oil	12.4
70% hexane in CH_2Cl_2	10-15	Yellow oil	77.8
70% hexane in CH_2Cl_2	16-20	Yellow oil	50.2
70% hexane in CH_2Cl_2	21-29	Yellow oil	88.0
50% hexane in CH_2Cl_2	30-34	Orange oil	10.7
50% hexane in CH_2Cl_2	35-49	Orange oil	570
50% hexane in CH_2Cl_2	50-60	Orange viscous liquid	42.8
100% CH_2Cl_2	61-90	Orange viscous liquid	547
1% MeOH in CH_2Cl_2	91-100	White solid with oil	234
1% MeOH in CH_2Cl_2	101-130	Yellow viscous liquid	301
2% MeOH in CH_2Cl_2	131-142	Yellow viscous liquid	24.9
2% MeOH in CH_2Cl_2	143-161	White solid with oil	124
2% MeOH in CH_2Cl_2	162-166	White solid with oil	98.0
2% MeOH in CH_2Cl_2	167-183	Red viscous liquid	78.2
2% MeOH in CH_2Cl_2	184-193	Orange solid with oil	610
2% MeOH in CH_2Cl_2	194-202	Red viscous liquid	448
3% MeOH in CH_2Cl_2	203-220	White solid with oil	1600
3% MeOH in CH_2Cl_2	221-233	Brown viscous liquid	300
5 to 10%MeOH in CH_2Cl_2	234-290	Brown viscous liquid	741
10% MeOH in CH_2Cl_2	291-300	Brown viscous liquid	630
50 to 100% MeOH in CH_2Cl_2	301-350	Brown viscous liquid	770

Table 4.2 Fraction obtained from silica gel column of DCB (1.86 g)

Eluents	Fraction No.	Appearance	Weight (mg)
80% hexane in EtOAc	1-25	Yellow viscous liquid	11.9
80% hexane in EtOAc	26-35	Yellow viscous liquid	3.40
50% hexane in EtOAc	36-55	Yellow viscous liquid	3.60
50% hexane in EtOAc	56-60	Yellow viscous liquid	3.10
40% hexane in EtOAc	61-70	Yellow viscous liquid	4.72
40% hexane in EtOAc	71-81	Brown viscous liquid	5.70
40% hexane in EtOAc	82-91	Brown viscous liquid	8.60
40% hexane in EtOAc	92-101	Brown viscous liquid	8.20
40 to 10% hexane in EtOAc	102-112	Brown viscous liquid	25.6
10% hexane in EtOAc	113-120	Brown viscous liquid	10.7
10% hexane in EtOAc	121-129	Brown viscous liquid	14.4
10% hexane in EtOAc	130-134	Brown viscous liquid	11.0
100% EtOAc	135-141	Brown viscous liquid	49.1
100% EtOAc	142-158	Red brown viscous liquid	127
100% EtOAc	159-195	Red brown viscous liquid	268
2%MeOH in EtOAc	196-224	Red brown viscous liquid	71.9
2%MeOH in EtOAc	225-235	Black viscous liquid	28.8
2 to 5% MeOH in EtOAc	236-272	Black viscous liquid	17.4
5 to 10% MeOH in EtOAc	273-295	Black viscous liquid	54.4
10 to 100% MeOH in EtOAc	296-300	Black viscous liquid	40.2
100% MeOH	300-320	Black viscous liquid	64.2

4.4 Purification of metabolites from endophytic fungal isolate Bkk3

Combined fraction number 203-220 of DCM possesses the highest yield (1600 mg) and exhibites interesting ^{1}H-NMR pattern. Then it was re-crystallized with dichloromethane-methanol to give a white solid of compound **I** (1300 mg).

Combined fraction number 184-193 of DCM possesses a high yield (610 mg) and exhibites interesting ^{1}H-NMR pattern. Then it was re-crystallized with diethyl ether to give a pale orange solid of compound **II** (300 mg).

Combined fraction number 194-202 of DCM (448 mg) and the filtrate of combined fraction number 184-193 of DCM (290 mg) shows similar patterns of ^{1}H-NMR spectral data. Both of them were combined, and further purified by silica gel column using 1.5% methanol in dichloromethane as mobile phase to give a white solid of compound **I** (250 mg) and a pale orange solid of compound **II** (325 mg).

Combined fraction number 91-100 of DCM possesses a moderate yield (234 mg) but exhibits interesting ^{1}H-NMR pattern. Then it was purified by preparative TLC (Merck's TLC aluminium sheet, silica gel 60F_{254} Art. 1.05554.0001) followed by re-crystallization to give compound **III** as colorless crystalline compound (10.2 mg).

Combined fraction number 143-161 (124 mg) and 162-166 (98.0 mg) of DCM shows similar patterns of ^{1}H-NMR spectral data. Both of them were combined, and further purified by re-crystallized with dichloromethane to give a mixture of white crystalline compound (33.4 mg). These compounds are known as ergosterol and ergosterol peroxide which are a component of fungal cell membranes.

Furthermore, compound **I** could be crystallized from acetronitrile in the presence of a small amount of water. In DCB (and some combined fraction of DCM), pure compounds could not be separated because of the complexity of mixtures and/or low yields. Several chromatographic separations were applied such as preparative reversed phase TLC, flash column chromatography, size exclusion chromatography (Sephadex LH-20), and HPLC. However, problems remain unsettled for these methods, for instance, peaks composed of more than one substance in HPLC.

In summary, there are three unknown compounds assigned as compound **I** (1550mg), **II** (625 mg) and **III** (10.2 mg). Chemical structures of these compounds were determined by the analysis of spectroscopic data including IR, NMR, MS and X-ray crystallographic data.

4.5 Structure elucidation of isolated compounds from endophytic fungal isolate Bkk3

4.5.1 Structure elucidation of compound I (raw data *see* Appendix B)

The compound **I** was obtained as white solid: mp 169-170 ^{o}C; $[\alpha]_D^{20}$ -135 (*c* 0.14, MeOH). The ESI-TOF MS of the compound **I** displayed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 526.2927 (calculated for $C_{32}H_{41}NO_4Na^+$ 526.2933). The UV spectrum in MeOH of the compound **I** showed λ_{max} (log ε) 233 (3.51) and 277 (3.04) nm. The IR adsorption spectrum of compound **I** exhibited ν_{max} (KBr) at 3409, 3339, 2948, 2361, 1722, 1687, 1509, 1448, 1378, 1226, 1104, 1026, 857, and 770 cm^{-1}. Thirteen degrees of unsaturation were indicated by the molecular formula.

^{1}H-NMR data presented in Table 4.3 indicated the presence of a *para*-substituted benzene ring and six methyl groups. The ^{13}C NMR (Table 4.3) data also displayed two double bonds, two carbonyl groups and aromatic ring which accounted for eight degrees of unsaturation, thus the remaining degrees of unsaturation must be due to five additional rings.

The HMBC experiment (Table 4.3) with assistance of COSY, TOCSY and NOESY (Figure 4.3) led to the structure of **I**. The large coupling constant of H-22 with H-21 ($J_{H\text{-}22/H\text{-}21} \approx 12$ Hz) and the observed NOEs between H-22 and H-13 and between H-13 and the methyl protons of C-24 in the NOESY experiment suggested that H-22 was axially orientated and occupied the same face as H-13 and the methyl protons of C-24. The observed NOEs between H-4 and the methyl protons of C-11 and between H-4 and H-10 in the NOESY experiment revealed that they occupied the same faces. Due to non-observation of NOEs between H-21 and the methyl protons of C-26 and between the methyl protons of C-26 and the methyl protons of C-27, it was suggested that the methyl protons of C-26 were on the opposite face to H-21 and the methyl protons of C-27. The configurations of **I** were established thereby except the configurations at C-9.

Fortunately, compound **I** could be crystallized from acetonitrile in the presented of a small amount of water and the complete relative configuration of **I** were finally established by X-ray crystallographic analysis (Figure 4.4).

Table 4.3 NMR spectral data for compound **I** in DMSO-d_6

Position	δ_C	δ_H (mult; *J*, Hz)	HMBC (H→C)	NOESY
1	174.83	-		
2	-	8.58 (s)	1, 3, 4, 9	
3	87.99	-		
4	49.27	2.47 (s)	3, 5, 6, 8, 9, 11, 23	11
5	28.82	2.03 (m)	3, 4, 6, 7, 9, 11	
6	134.77	-		
7	126.13	5.08 (s)	5, 8, 9, 12, 13	12, 24, 25
8	43.64	-		
9	63.62	-		
10	44.07	2.86 (s)	3, 4, 1', 2', 4'	2'
11	20.04	0.71 (d, 7.2)	4, 5, 6	4
12	22.44	1.58 (s)	5, 6, 7	
13	50.14	2.72 (d, 8.0)	7, 8, 14, 15, 21, 22, 24	22, 24
14	128.13	-		
15	137.95	5.36 (s)	13, 16, 17, 21, 25	25, 26
16	35.56	-		
17	47.80	0.58 (dd, 12.4 and 12.0)	16, 18, 19, 26, 27	
		1.41 (br d, 12.8)	16	
18	26.72	1.54 (m)		26
19	35.37	0.46 (br q, 12.4)		
		1.62 (br d, 9.2)		
20	22.99	0.98 (be q, 12.4)		
		1.37 (m)		
21	40.34	1.32 (dd, 12.8 and 13.2)	26	
22	48.75	2.03 (dd, 12 and 8.4)	8, 9, 13, 14, 16, 21	13
23	218.95	-		
24	25.49	1.47 (s)	7, 8, 9, 13	7, 13, 25
25	24.98	1.82 (s)	13, 14, 15	7, 15, 24
26	19.52	0.74 (s)	15, 16, 17, 21	15, 18
27	22.66	0.75 (d, 7.6)	17, 18, 19	
1'	126.69	-		
2' and 6'	131.60	7.09 (d, 8.4)	10, 3', 4'	10
3' and 5'	114.83	6.67 (d, 8.4)	1', 4'	
4'	155.93	-		
5'				
6'				
OH	-	5.63 (s)		
OH	-	9.26 (br s)		

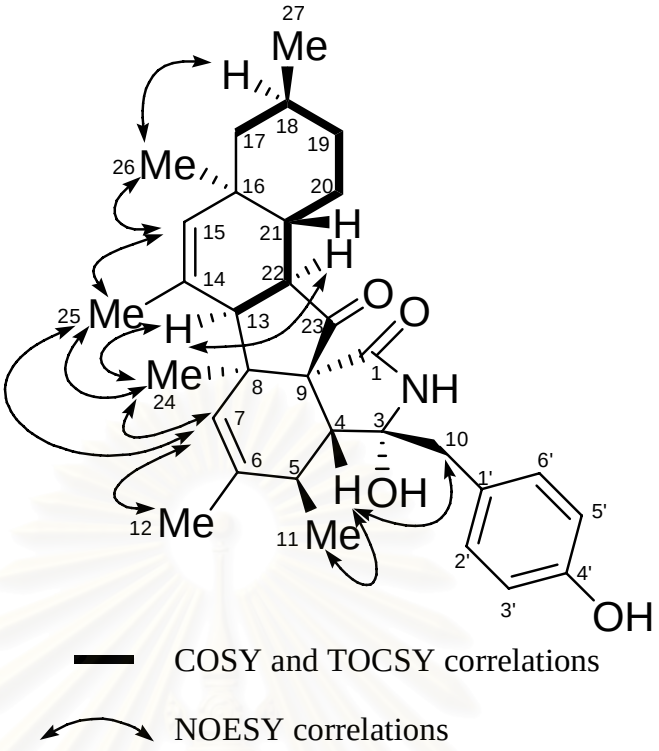


Figure 4.3 COSY, TOCSY, and NOESY correlations of compound **I**

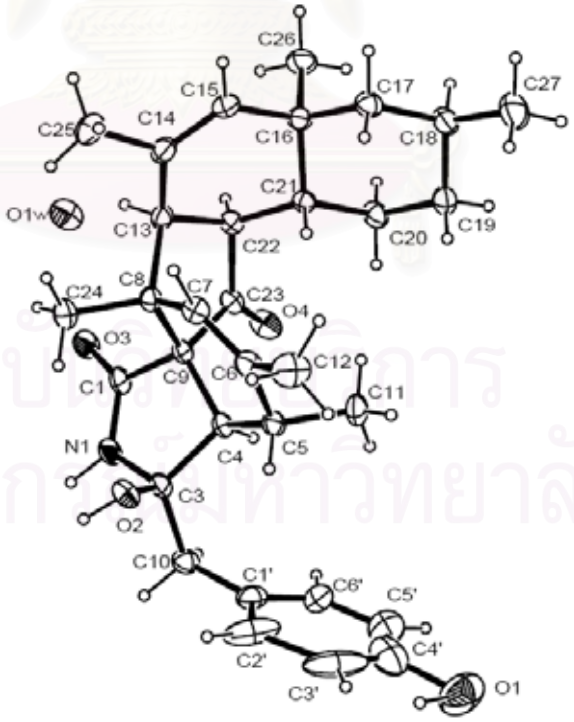


Figure 4.4 X-ray crystal structure of compound **I**

It was revealed that compound **I** was a novel compound, diaporthichalasin, which was an isomer of the previous known phomopsichalasin [*45*], an antimicrobial agent from an endophytic *Phomopsis* sp. Further support for the difference of two isomers came from the fact that diaporthichalasin showed a different specific optical rotation with a strong negative optical rotation, $[\alpha]_D^{20}$ -135 (*c* 0.14, MeOH), compared with phomopsichalasin, $[\alpha]_D^{20}$ -7.16 [*45*] and diaporthichalasin exhibited no antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Psuedomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 at concentration lower 125 μg/ml using microtiter plate broth dilution assay.

4.5.2 Structure elucidation of compound II (raw data *see* Appendix C)

The compound **II** was obtained as a pale orange solid: mp 286 ^oC (decompo); $[\alpha]_D^{20}$ +317 (*c* 0.045, $CHCl_3$). The ESI-TOF MS of the compound **II** displayed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 549.2844 (calculated for $C_{33}H_{41}O_7$ at *m/z* 549.2852). The UV spectrum in MeOH of the compound **II** showed λ_{max} (log ε) 260 (4.58) and 363 (4.23) nm. The IR adsorption spectrum of compound **II** exhibited ν_{max} (KBr) at 3860, 3734, 3669, 3608, 3161, 2948, 1700, 1630, 1591, 1523, 1443, 1388, 1280, 1154, 1084, 993, 897, 774 and 667 cm^{-1}. Sixteen degrees of unsaturation were indicated by the molecular formula.

Complete ^1H and ^{13}C NMR assignment for compound **II** were shown in Table 4.4. Both of the spectra exhibited considerable symmetry because of two identically substituted tropolone/pyran rings fused with one 11-membered ring consisting of three isolated proton spin systems. The ^{13}C NMR data also displayed fourteen tropolone-ring carbons and two olefinic carbons which accounted for sixteen degrees of unsaturation.

The HMBC experiment (Table 4.4, Figure 4.4) with assistance of COSY, TOCSY and NOESY (Figure 4.5) led to the structure of **II**. The last coupling constant of H-25 with H-26 ($J \approx 11$ Hz) suggested that they were axially oriented. In the NOESY experiment, the cross peak observed between H-8 and H-26 and between H-26 and the methyl proton of C-33 revealed that they were on the same faces. Since non-observation of NOEs between H-8 and the methyl protons of C-35 and between

H-24 and the methyl protons of C-33, this indicated that both pyran rings were trans-fused to the 11-membered ring.

Table 4.4 NMR spectral data for compound **II** in $CDCl_3$

Position	δ_C	δ_H (mult; *J*, Hz)	HMBC (H→C)	NOESY
1	112.30	6.92 (s)	2, 3, 6, 29	
2	162.71	-		
3	173.20	-		
4	125.48	7.16 (s)	2, 3, 5, 6, 30	
5	151.31	-		
6	118.48	-		
7	34.18	2.85 (dd, 5.2 and 17.2)	5, 6, 8, 27, 29	
		2.37 (d, 16.0)	8	
8	31.81	1.82 (br, s)	6, 7, 9, 10, 26, 27	26
9	46.41	0.78 (dd, 4.0 and 14.4)	7, 8, 10, 11, 27	
		1.79 (d, 15.2)		
10	34.82	-		
11	143.89	5.80 (d, 16.8)	9, 10, 12, 13	
12	125.71	5.68 (ddd, 4.4, 10.4 and 15.6)	10, 11, 13, 14	
13	46.13	2.73 (br dd, 2.4 and 13.2)	11, 12, 13, 24	
		2.52 (dd, 10.8 and 13.2)	11, 12, 13	
14	80.35	-		
16	160.13	-		
17	113.32	6.95 (s)	16, 18, 19, 22	
18	163.00	-		
19	172.47	-		
20	124.58	7.13 (s)	18, 19, 22	
21	150.37	-		
22	122.27	-		
23	32.86	3.38 (dd, 13.6 and 17.2)	14, 16, 21, 24	
		2.42 (dd, 4.0 and 17.2)		
24	41.28	2.20 (m)		
25	30.04	1.54 (dd, 11.6 and 12.0)	14, 23	
		2.23 (m)		
26	70.64	4.22 (d, 11.2)	24, 27	8, 33
27	81.84	-		
29	159.33	-		
30	27.34	2.38 (s)	5	
31	29.06	1.07 (s)	9	
32	27.00	1.10 (s)	11	
33	19.19	1.41 (s)	14, 24	26
34	27.28	2.41 (s)	21	
35	15.94	1.14 (s)	27	

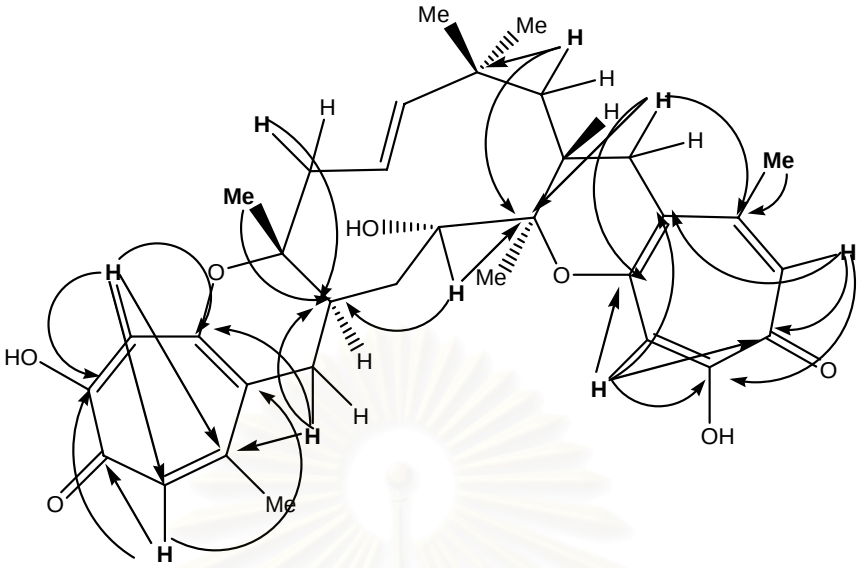


Figure 4.4 Selected HSQC correlations of compound **II**

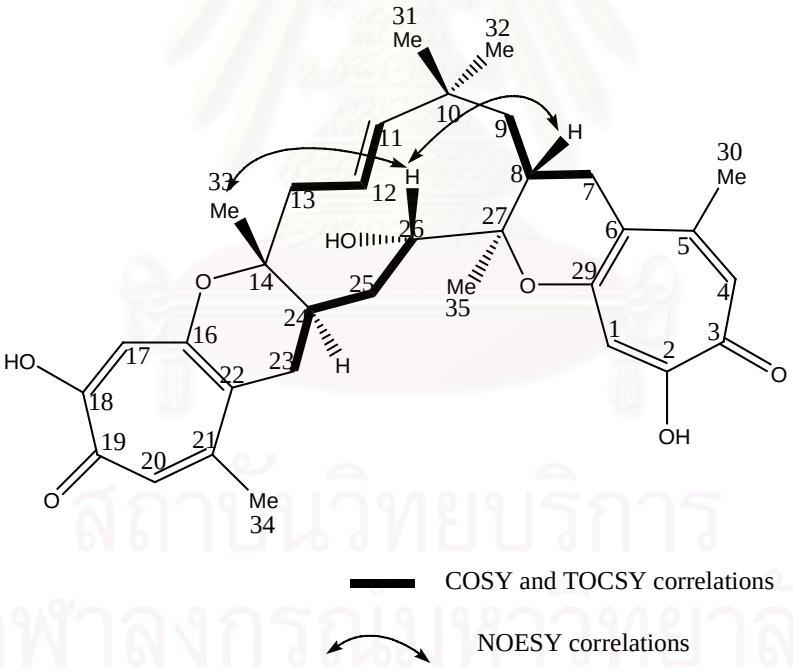


Figure 4.5 COSY, TOCSY, and NOESY correlations of compound **II**

Comparison of the spectroscopic data of compound **II** with that published of the known pycidione [*69*, 70], indicated that compound **II** was identical with the reported compound.

4.5.3 Structure elucidation of compound III (raw data *see* Appendix D)

The compound **III** was obtained as colorless crystals: mp 127 ^{o}C; $[\alpha]_D^{20}$ -110 (*c* 0.23, $CHCl_3$). The ESI-TOF MS of the compound **III** displayed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 193.0870 (calculated for $C_{11}H_{12}O_3H^+$ 193.0864). The UV spectrum in MeOH of the compound **III** showed λ_{max} (log ε) 250 (3.80) and 325 (3.61) nm. The IR adsorption spectrum of compound **III** exhibited ν_{max} (KBr) at 3266, 2984, 2841, 1645, 1572, 1513, 1443, 1300, 1227, 1160, 1117, 1021, 961 and 812 cm^{-1}. Six degrees of unsaturation were indicated by the molecular formula.

NMR data presented in Table 4.5 indicated the presence of a *para*-substituted benzene ring and two methyl groups. Detailed analysis of 2D ^{1}H and ^{13}C spectrum, including COSY, NOESY (Figure 4.6) and HMBC (Figure 4.7) confirmed the structure of compound **III**.

Table 4.5 NMR spectral data for compound **III** in $CDCl_3$

Position	δ_C	δ_H (mult; *J*, Hz)	HMBC (H→C)	NOESY
1	170.34	-		
3	75.42	4.68 (ddq, 3.6, 11.2 and 6.4)		4
4	31.92	2.94 (dd, 3.6 and 16.4)	5, 9, 10	3
		2.72 (dd, 11.2 and 16.4)	3, 5, 9, 10, 11	11
5	124.90	-		
6	137.93	7.28 (d, 8.4)	8, 10, 12	
7	115.69	6.82 (d, 8.4)	5, 8, 9	
8	160.49	-		
9	108.08	-		
10	137.04	-		
11	20.92	1.55 (d, 6.4)	3, 4	4
12	18.09	2.19 (s)	5, 6, 10	
OH	-	11.00 (s)	7, 8, 9	

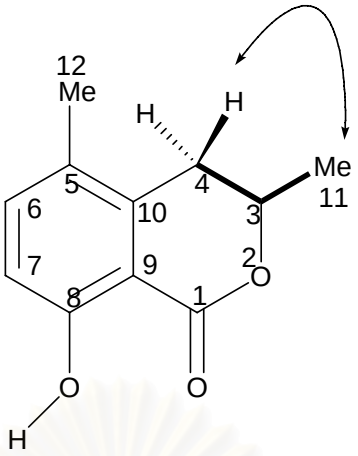


Figure 4.6 COSY, TOCSY, and NOESY correlations of compound **III**

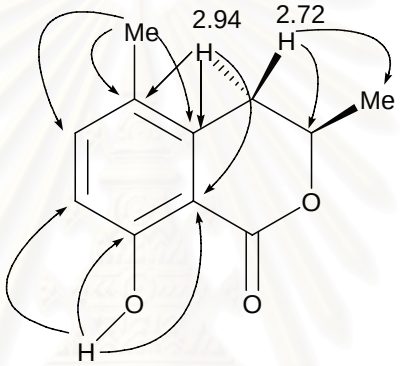


Figure 4.7 Selected HSQC correlations of compound **III**

The physicochemical properties of compound **III** were identical with those of 5-methylmellein [*71*].

4.6 Determination of biological activities

4.6.1 Assay of CYP3A4 Inhibition

Cytochrome P450 (CYP) enzymes have been mainly expressed in liver microsomes and are recognized to be responsible for drug metabolism, carcinogenesis, and degradation of xenobiotics. These enzymes constitute three families including CYP1, CYP2, and CYP3, which play an important role in the biosynthesis of lipids, steroids, and other secondary metabolites. CYP3A4 is the most abundant enzymes in human liver microsomes, metabolizing over 50% of drugs biotransformed by this enzyme. The majority of drug-drug interactions are metabolism-based and, of these,

most involve cytochrome P450. If a new chemical entity is a potent cytochrome P450 inhibitor, it may inhibit the metabolism of a co-administered medication, potentially leading to adverse clinical events. [*72*]

CYP3A4 activity was monitored by nifedipine oxidation with expressed human CYP3A4. Ketokonazole was used as positive control and exhibited inhibition of CYP3A4 with an IC_{50} value of 0.11 μM. Compound **I** exhibited significantly potent inhibition of CYP3A4 with IC_{50} value of 0.626 μM, while the IC_{50} value of **II** was 465 μM.

4.6.2 Cytotoxicity test

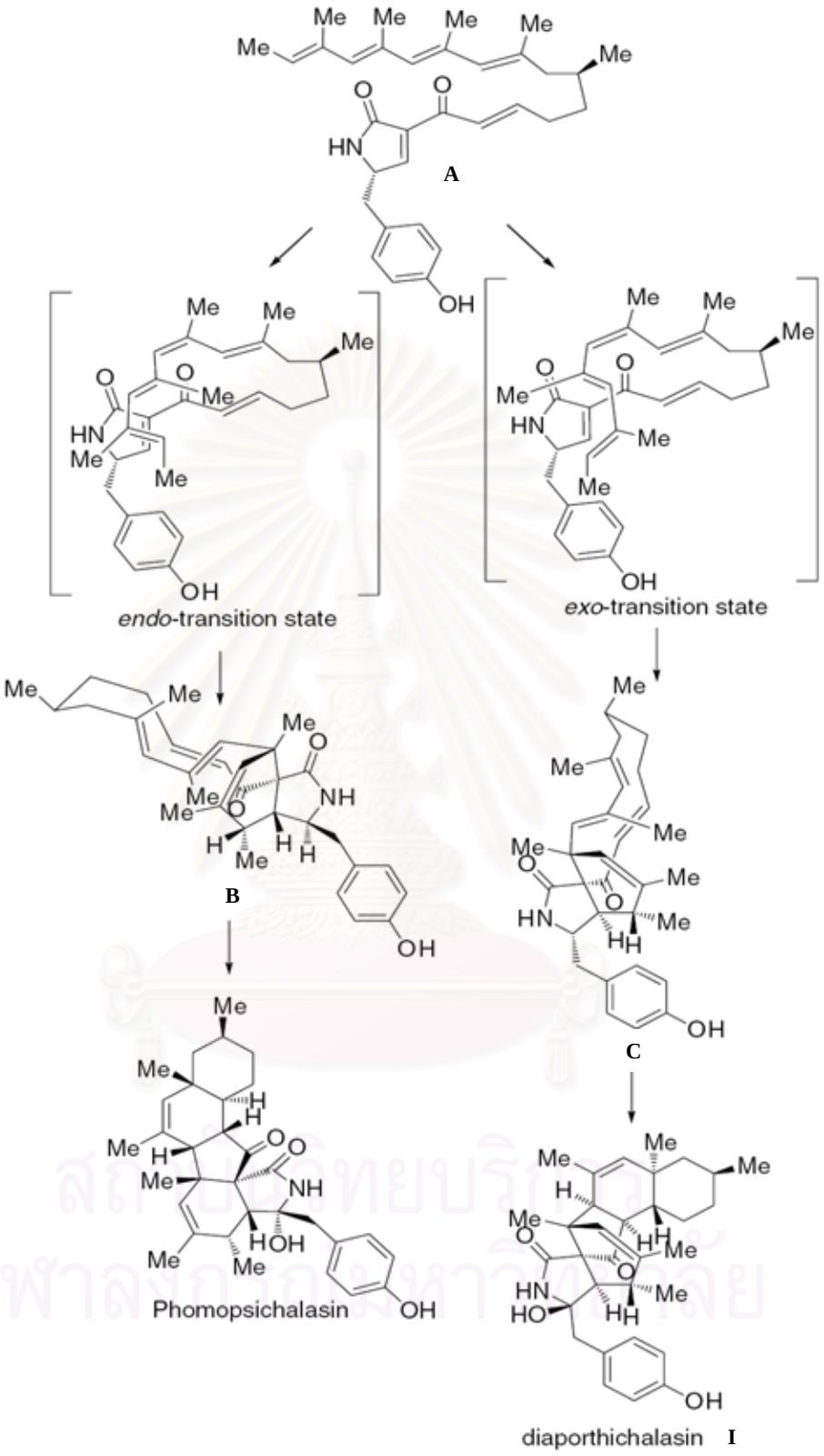
The results indicated that compound **I** showed cytotoxic activity against BT474 (breast), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) with the IC_{50} values of 18.01, 13.94, 11.29, 9.86 and 0.93 μM, respectively while compound **II** displayed cytotoxic activity against BT474 (breast), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) with the IC_{50} values of 18.25, 1.28, 11.72, 1.37 and 12.92 μM, respectively. Compound **III** was inactive against those five cell lines**.**

4.6.3 Antimicrobial activity test

All of three compounds did not show antimicrobial activity against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATTC 27853 and *C. albicans* ATCC 10231. at < 125 μg/mL using microtiter plate broth dilution assay.

4.7 Proposed biosynthetic pathway for compound I

In a general biosynthetic pathway of cytochalasins, Vederas and Tamm suggested that cytochalasin-type metabolites are constructed from an amino acid and a polyketide unit [*73*], On the basis of cytochalasin biosynthesis, the phenol moiety in **A** is derived from tyrosine and the fused rings of **A** are created by double Diels–Alder closure [*74*]. The perhydroisoindole moiety of diaporthichalasin (compound **I**) is thought to arise through an exo-selective intramolecular Diels–Alder reaction similar to chaetochalasin A [*75*] while the perhydroisoindole moiety of phomopsichalasin is thought to arise through an endo-selective intramolecular Diels–Alder reaction. The Diels–Alder reactions of putative **B** and **C** provide the cycloadducts, which then undergo hydroxylation to give phomopsichalasin and diaporthichalasin (compound **I**), respectively (Scheme 4.2).



Scheme 4.2 Proposed biosynthetic pathway for compound **I**

CHAPTER V

CONCLUSION

The endophytic fungus isolate Bkk3 was isolated from *Croton sublyratus* Kurz. leaves. Based on nucleotide sequences of ITS1-5.8S-ITS2 regions of rDNA, the fungus isolate Bkk3 was identified as *Diaporthe* sp. In the present investigation, afford a novel compound (compound **I**) named diaporthichalasin together with two known compounds, pycnidione (compound **II**) and 5-methymellein (compound **III**). Diaporthichalasin exhibits significantly potent inhibition of CYP3A4 with an IC_{50} value of 0.626 μM and shows cytotoxic activity against BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) with the IC_{50} values of 18.01, 13.94, 11.29, 9.86 and 0.93 μM, respectively while pycnidione inhibits CYP3A4 with the IC_{50} value of 465 μM and displays cytotoxic activity against BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) with the IC_{50} values of 18.25, 1.28, 11.72, 1.37 and 12.92 μM, respectively. However all of three compounds do not show antimicrobial activity against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATTC 27853 and *C. albicans* ATCC 10231.

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APPENDICES

APPENDIX A

MEDIA

The media were prepared by sterilization in the autoclave at 121 ^{o}C for 15 minutes.

1. Malt extract agar (MEA)

Malt extracts	20.0	g
Peptone	1.0	g
Glucose	20.0	g
Distilled water	1000	ml
Agar	15.0	g

2. Yeast extract sucrose agar (YEA)

Yeast extracts	20.0	g
Sucrose	150.0	g
Agar	15.0	g
Distilled water	1000	ml

3. Nutrient agar (NA)

Peptone	5.0	g
Beef extract	3.0	g
Distilled water	1000	ml
Agar	15.0	g

4. Sabouraud's dextrose agar (SDA)

Peptone	10.0	g
Dextrose	40.0	g
Distilled water	1000	ml
Agar	15.0	g

5. Corn Meal Agar (CMA)

Corn meal	20.0	g
Peptone	20.0	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water	1000	ml

6. Potato dextrose agar (PDA)

Potato, peeled and diced	200	g
Glucose	20.0	g
Agar	15.0	g
Distilled water	1000	ml

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by streaming and sterilize by autoclaving at 121 °C for 15 minutes.

7. Yeast-malt extract agar (YMA)

Glucose	10.0	g
Peptone	5.0	g
Yeast extracts	3.0	g
Malt extracts	3.0	g
Agar	15.0	g
Distilled water	1000	ml

APPENDIX B

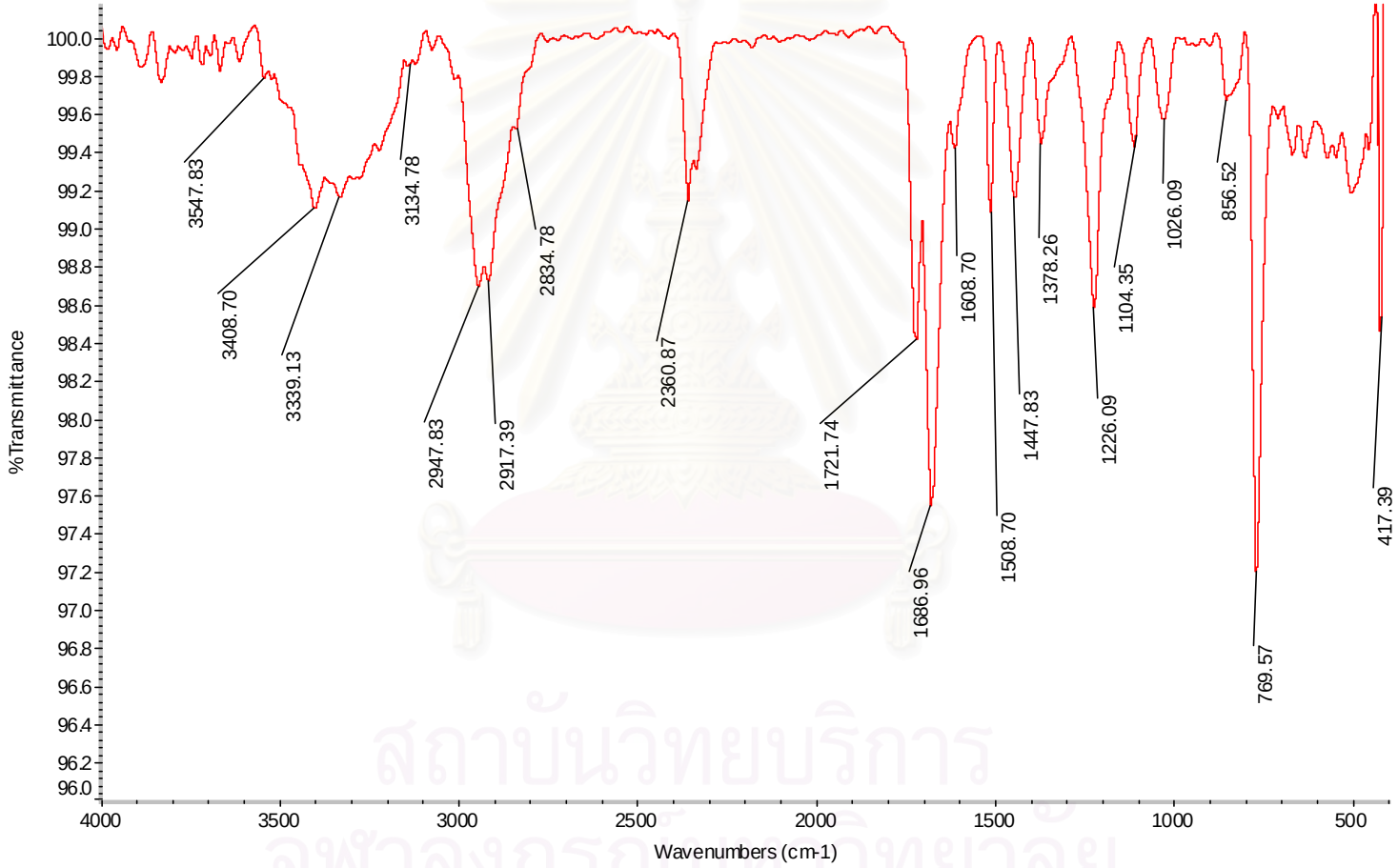

Figure B1 IR spectrum of compound **I**

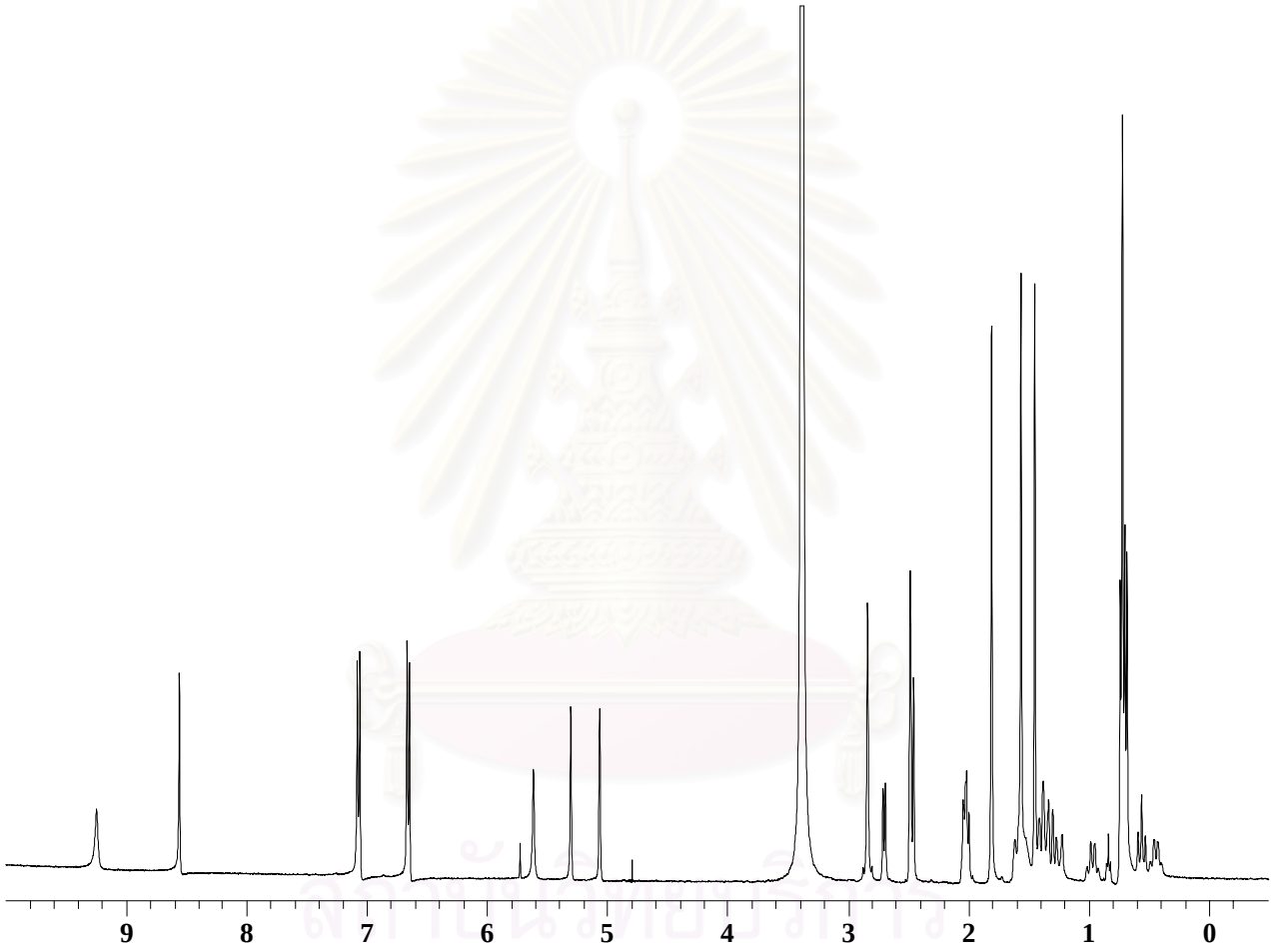

Figure B2 ^{1}H-NMR spectrum of compound **I**

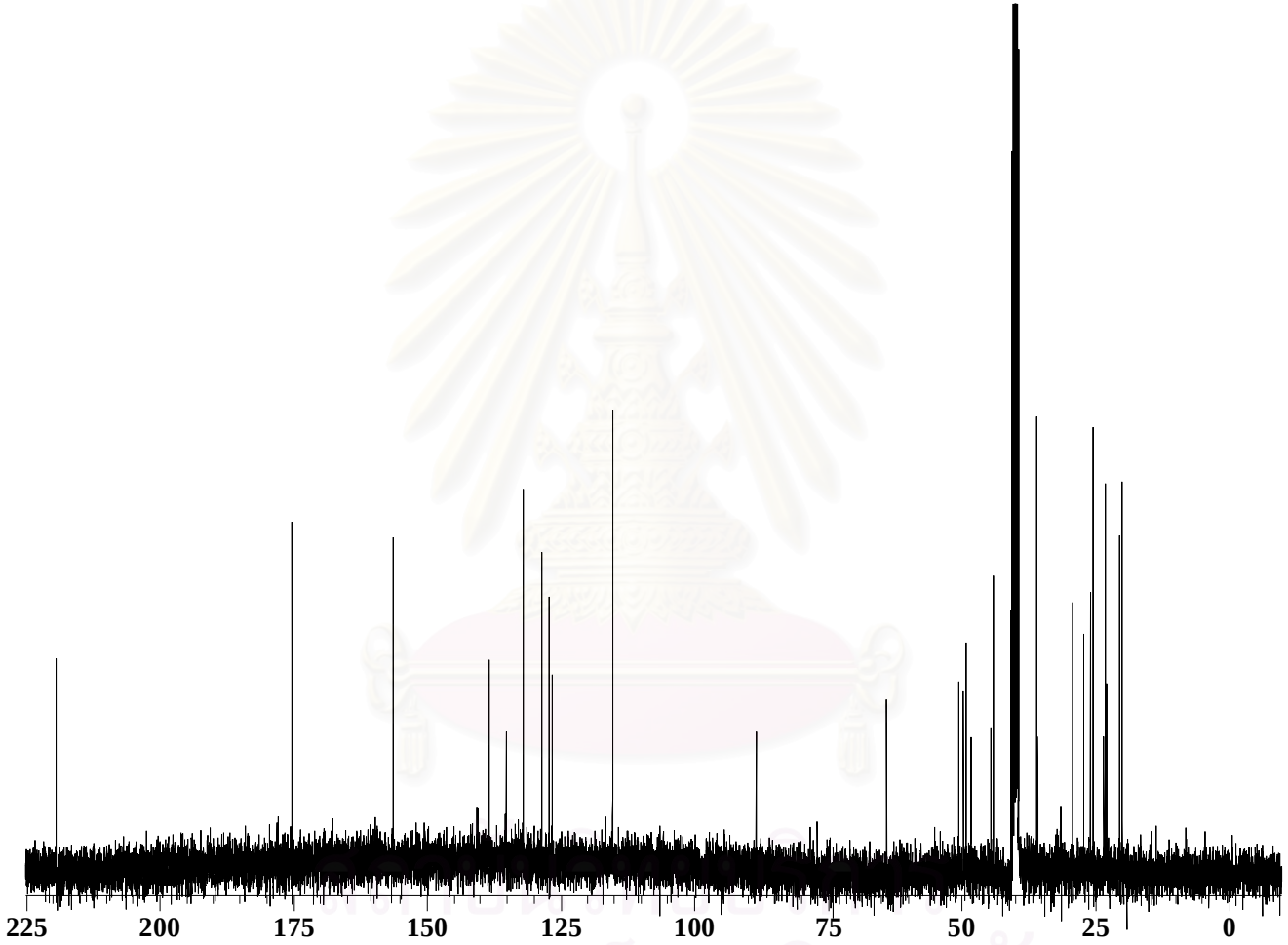

Figure B3 ^{13}C-NMR spectrum of compound **I**

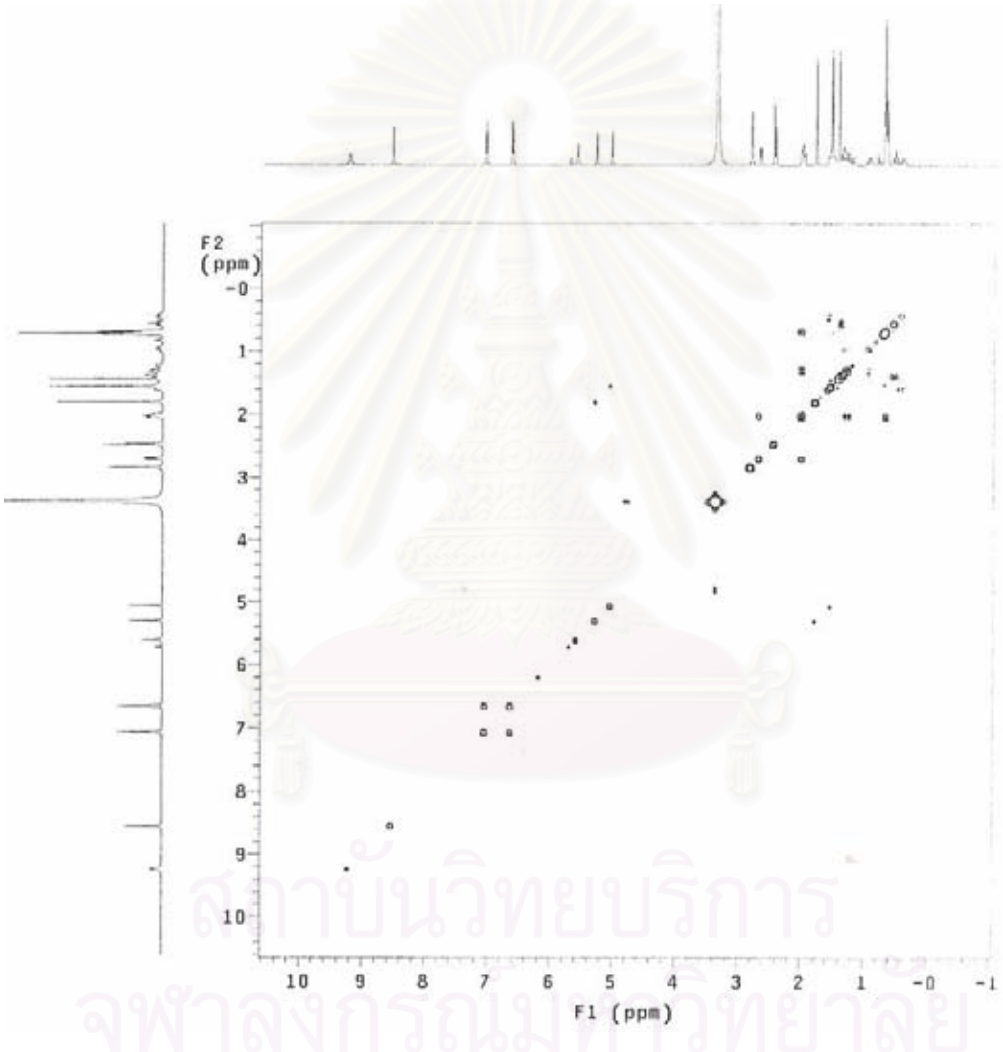

Figure B4 COSY spectrum of compound **1**

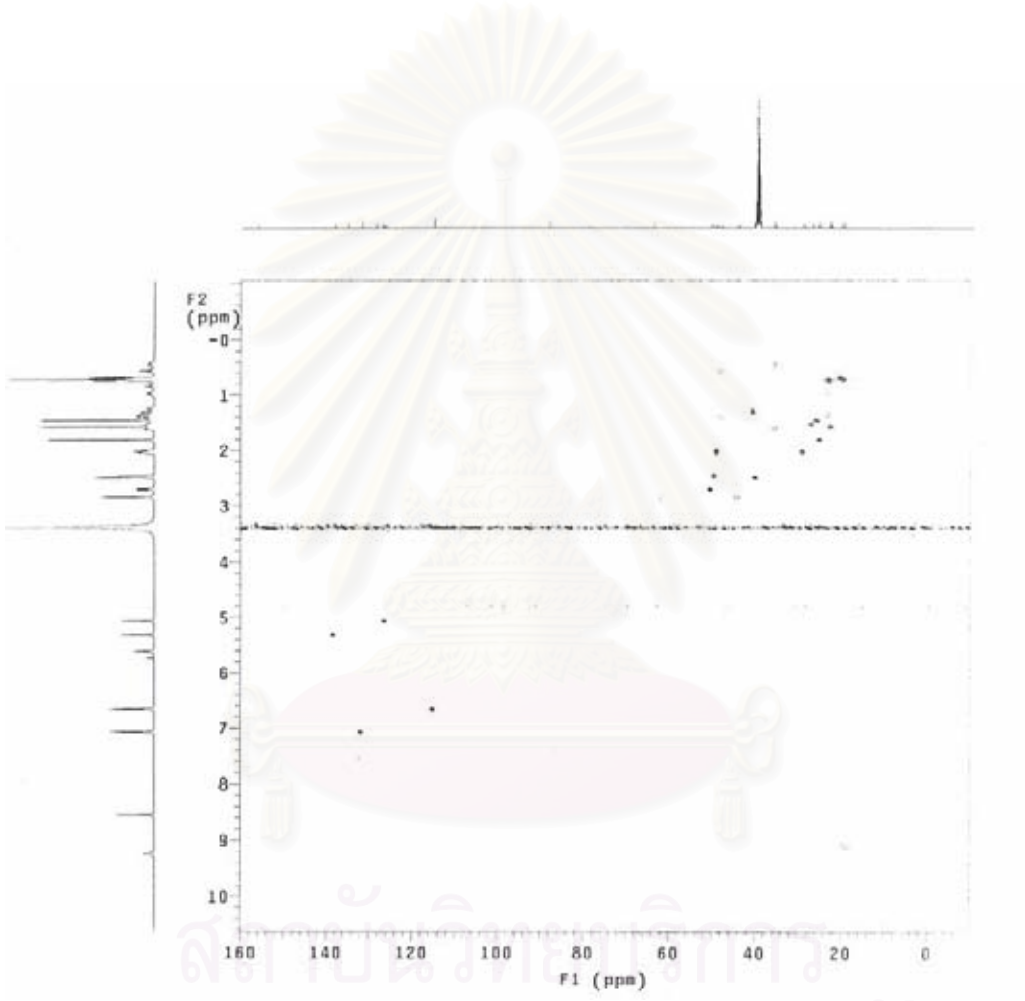

Figure B5 HSQC spectrum of compound **I**

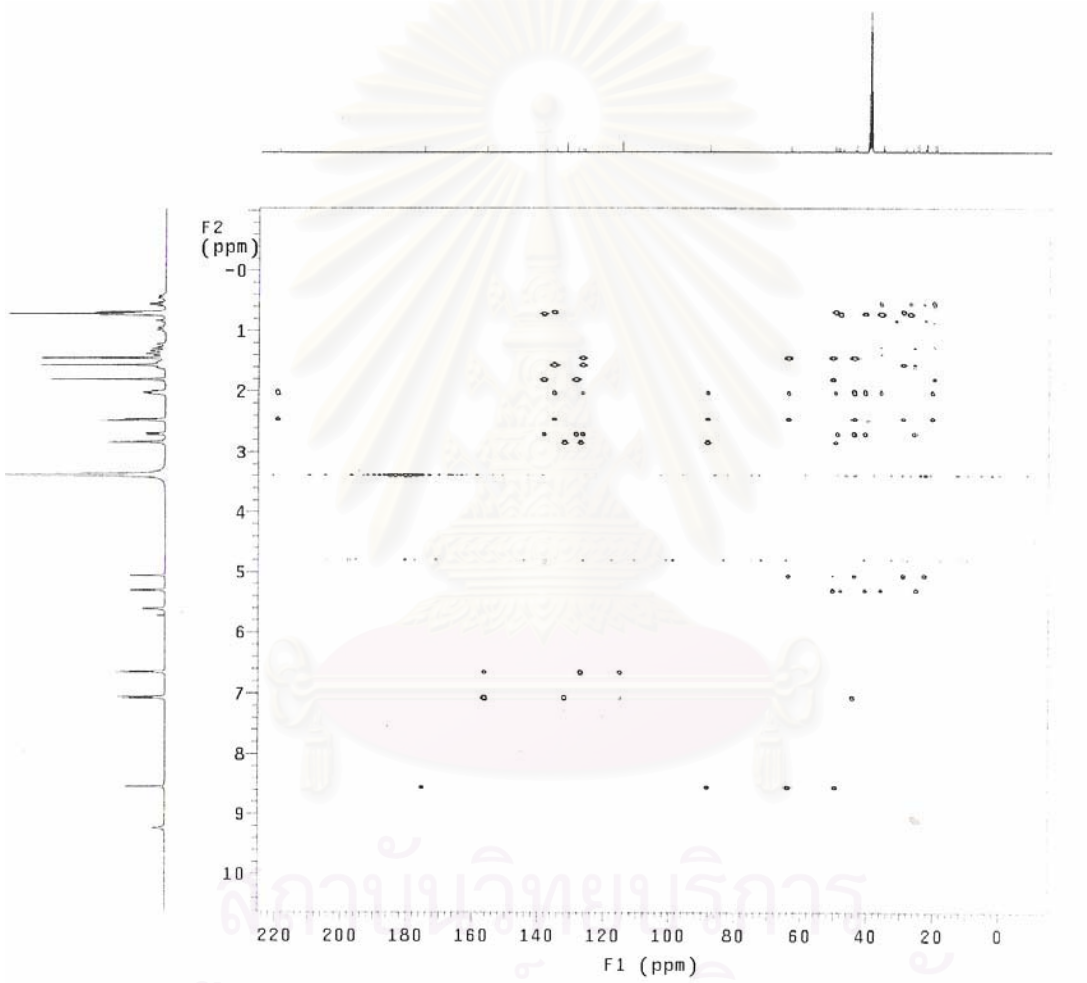

Figure B6 HMBC spectrum of compound **I**

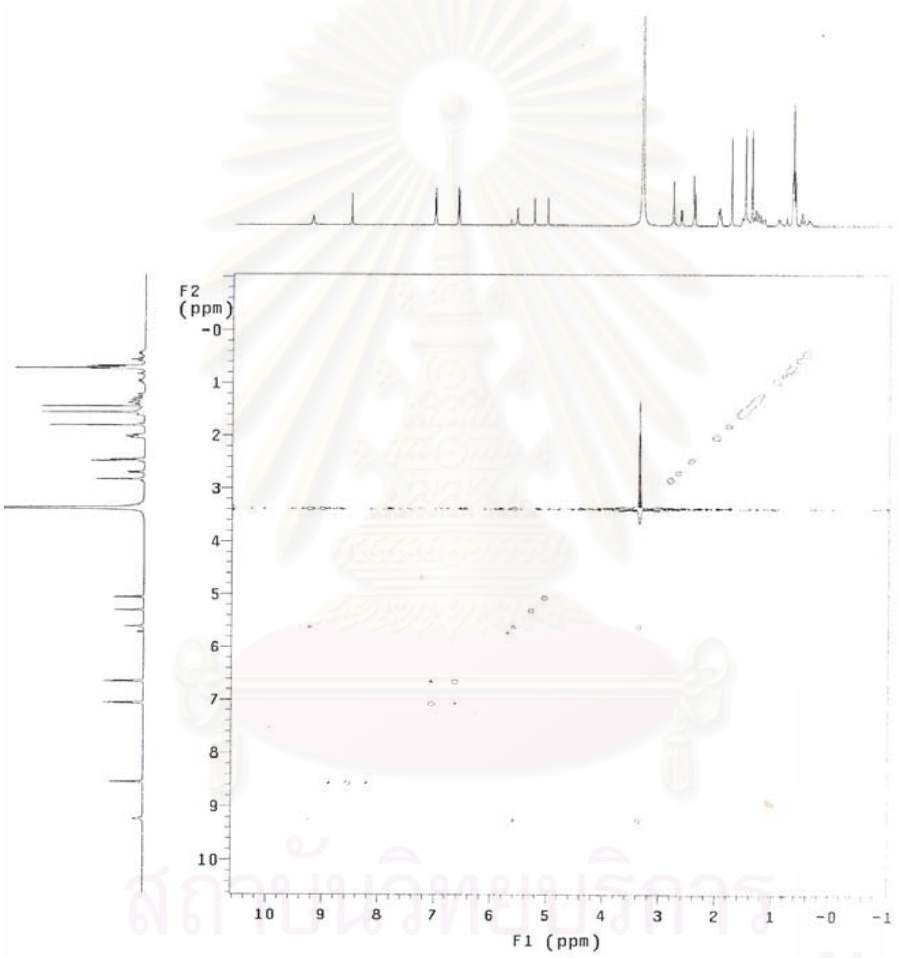

Figure B7 NOESY spectrum of compound **I**

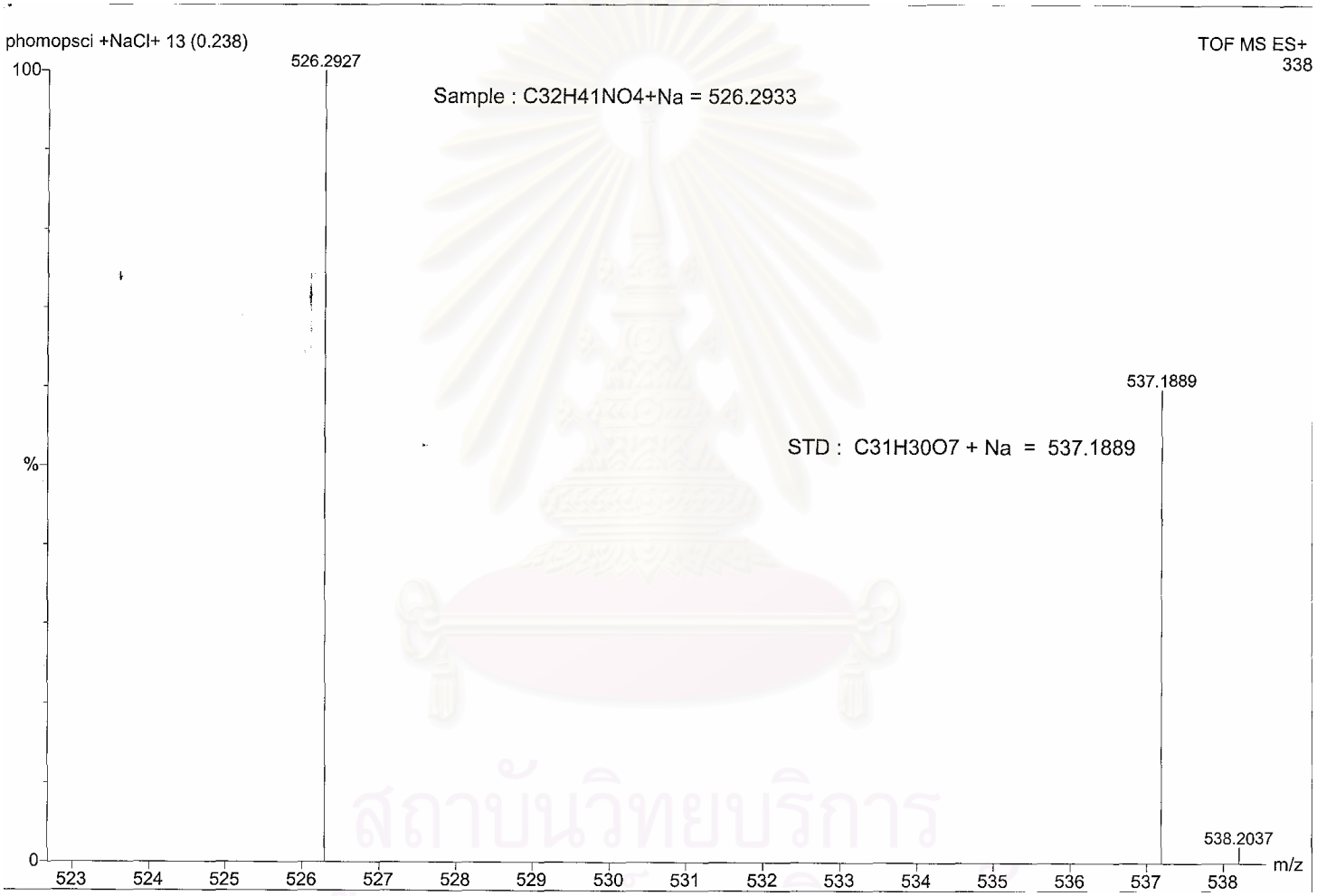

Figure B8 Mass spectrum of compound **I**

APPENDIX C

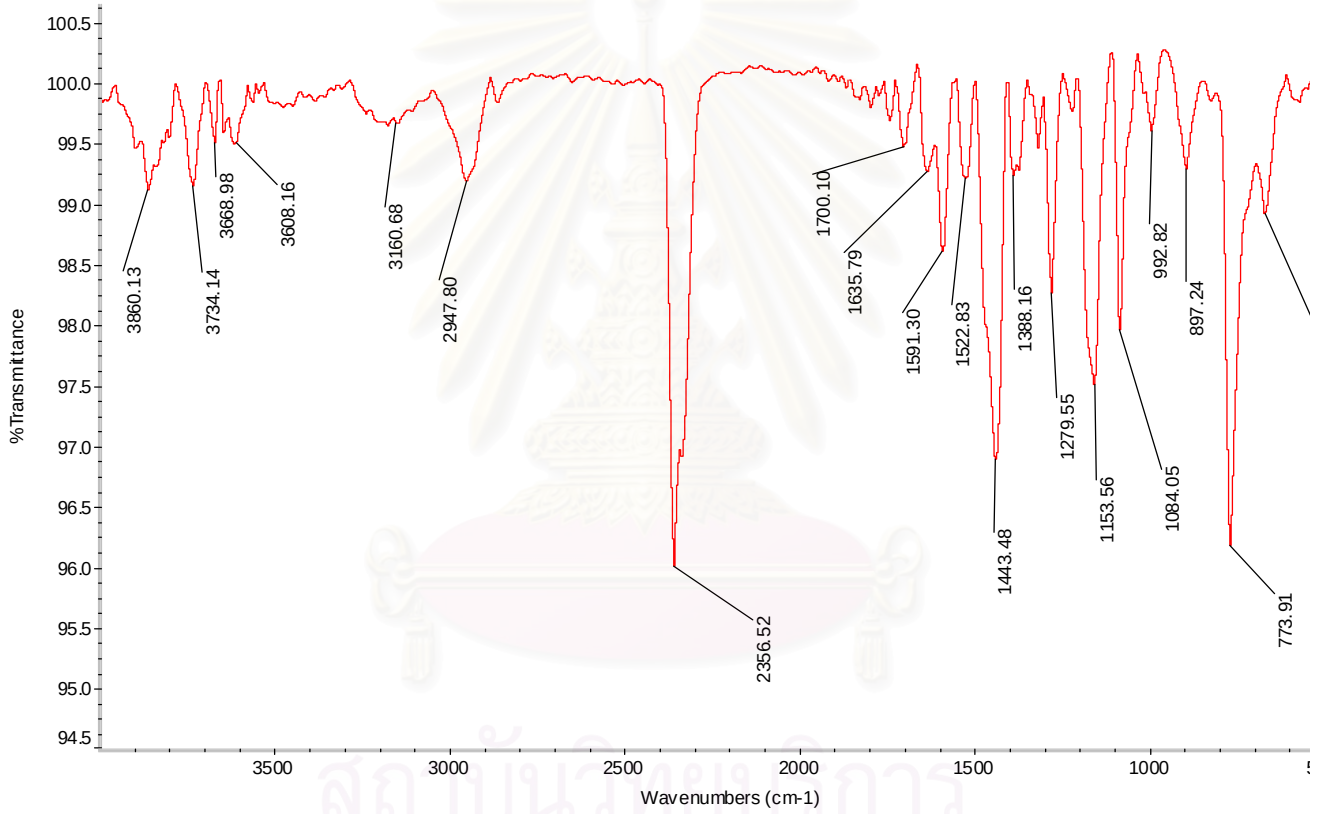

Figure C1 IR spectrum of compound **II**

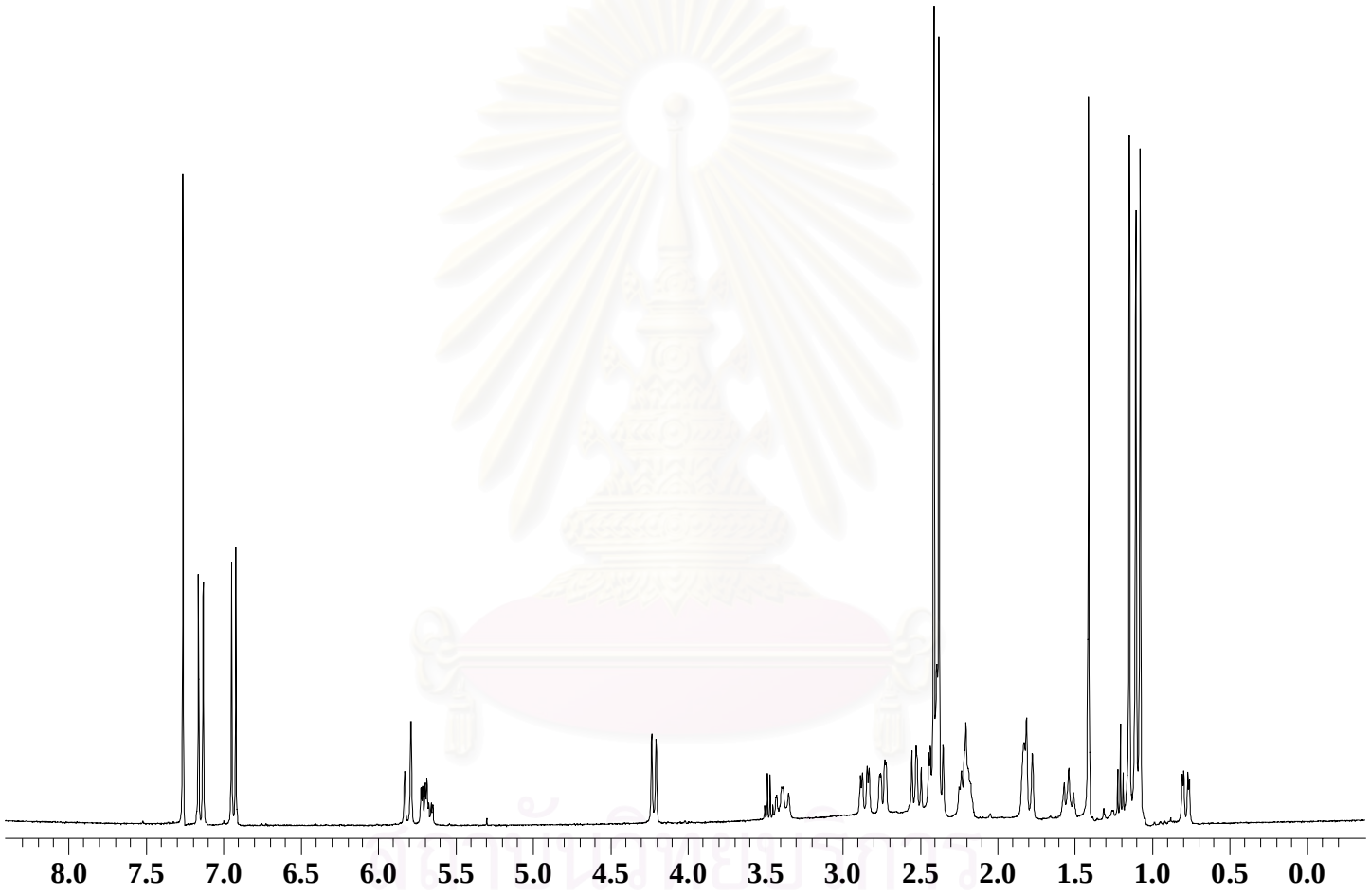

Figure C2 ^{1}H-NMR spectrum of compound **II**

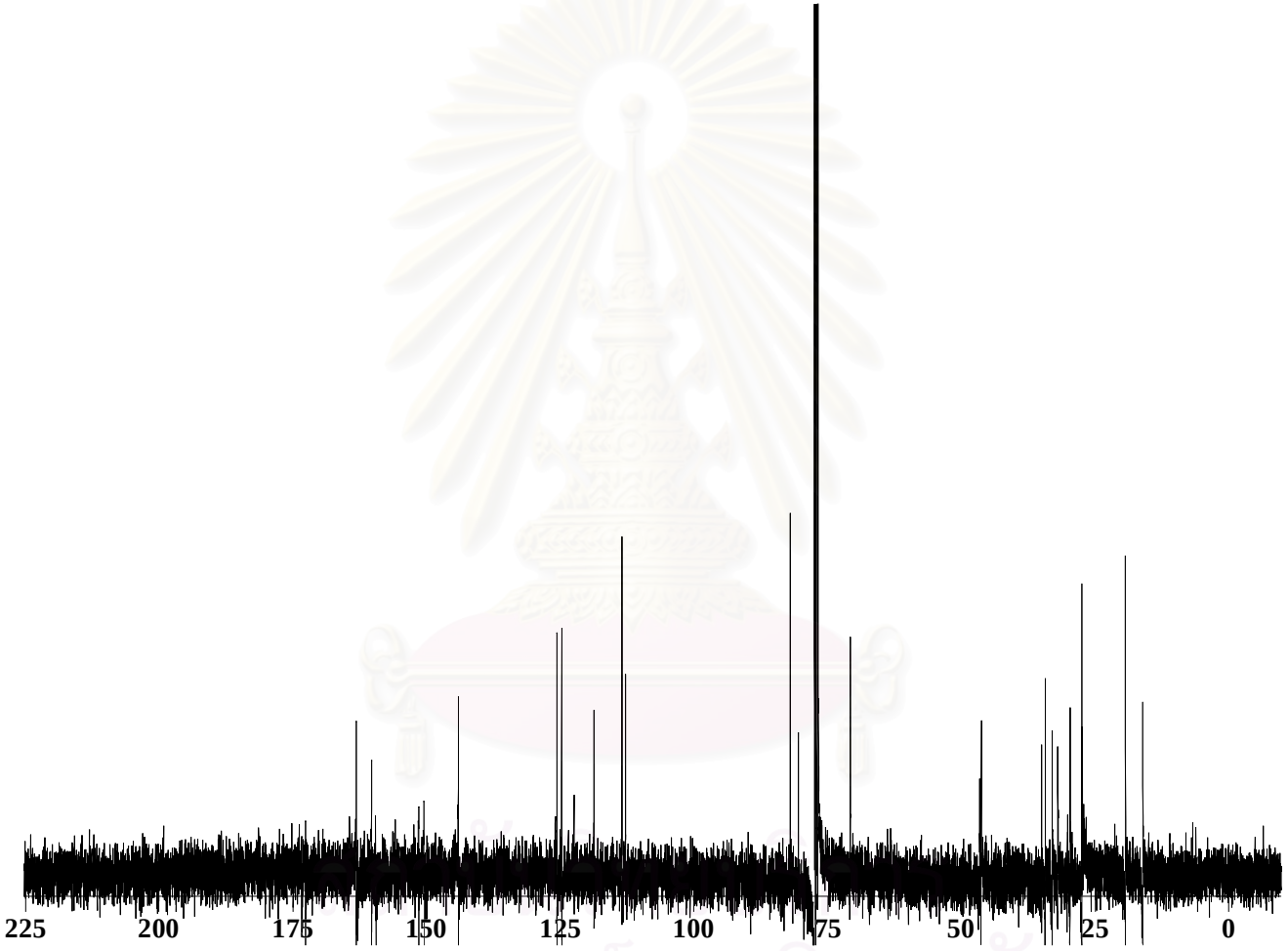

Figure C3 ^{13}C-NMR spectrum of compound **II**

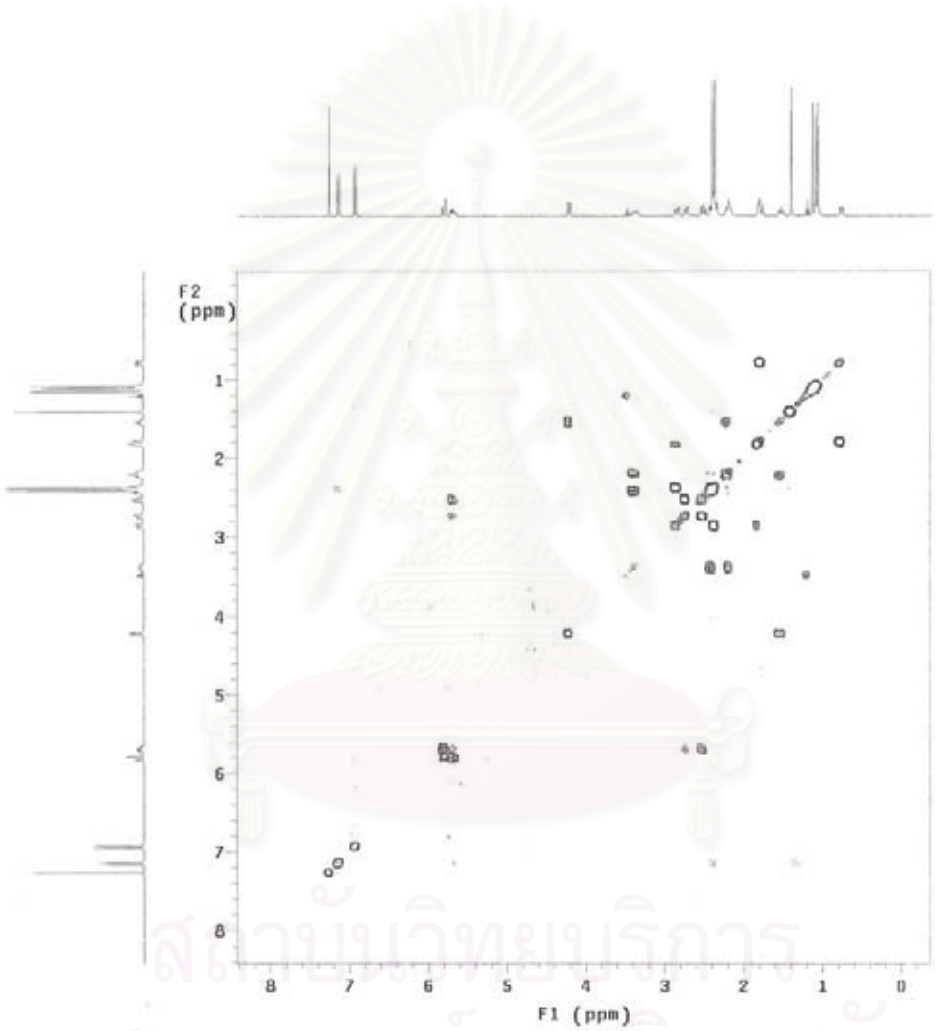

Figure C4 COSY spectrum of compound **II**

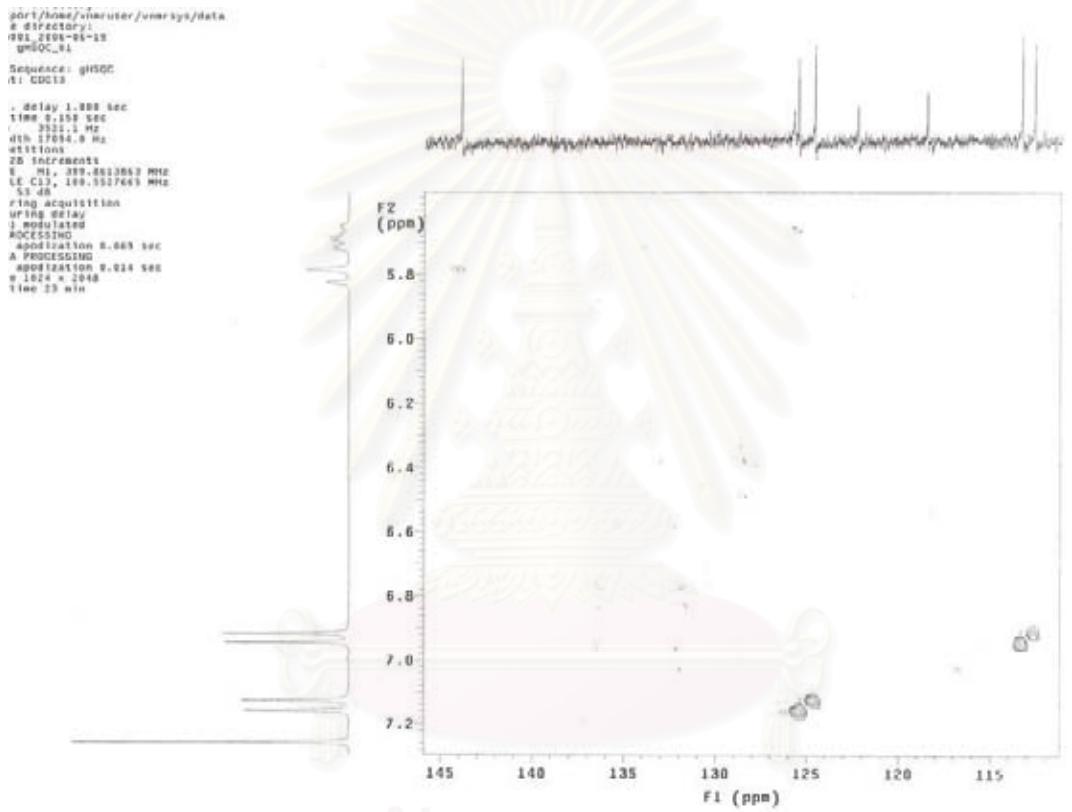

Figure C5 HSQC spectrum of compound **II**

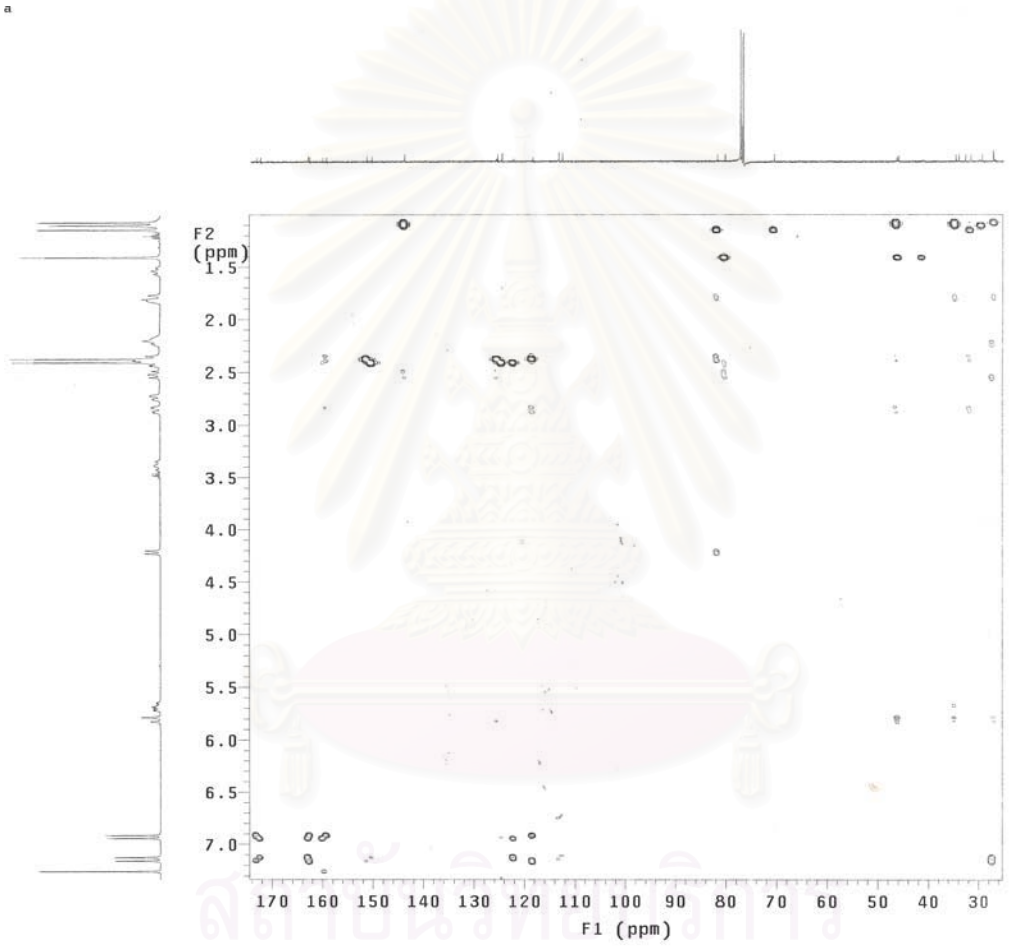

Figure C6 HMBC spectrum of compound **II**

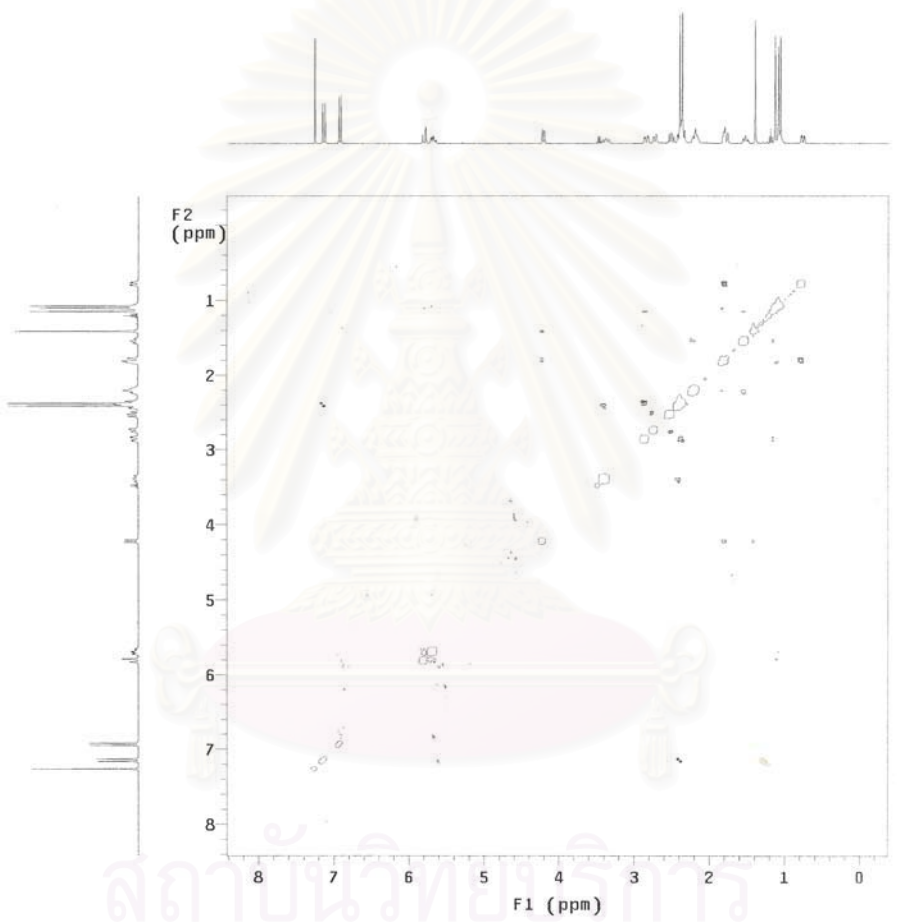

Figure C7 NOESY spectrum of compound **III**

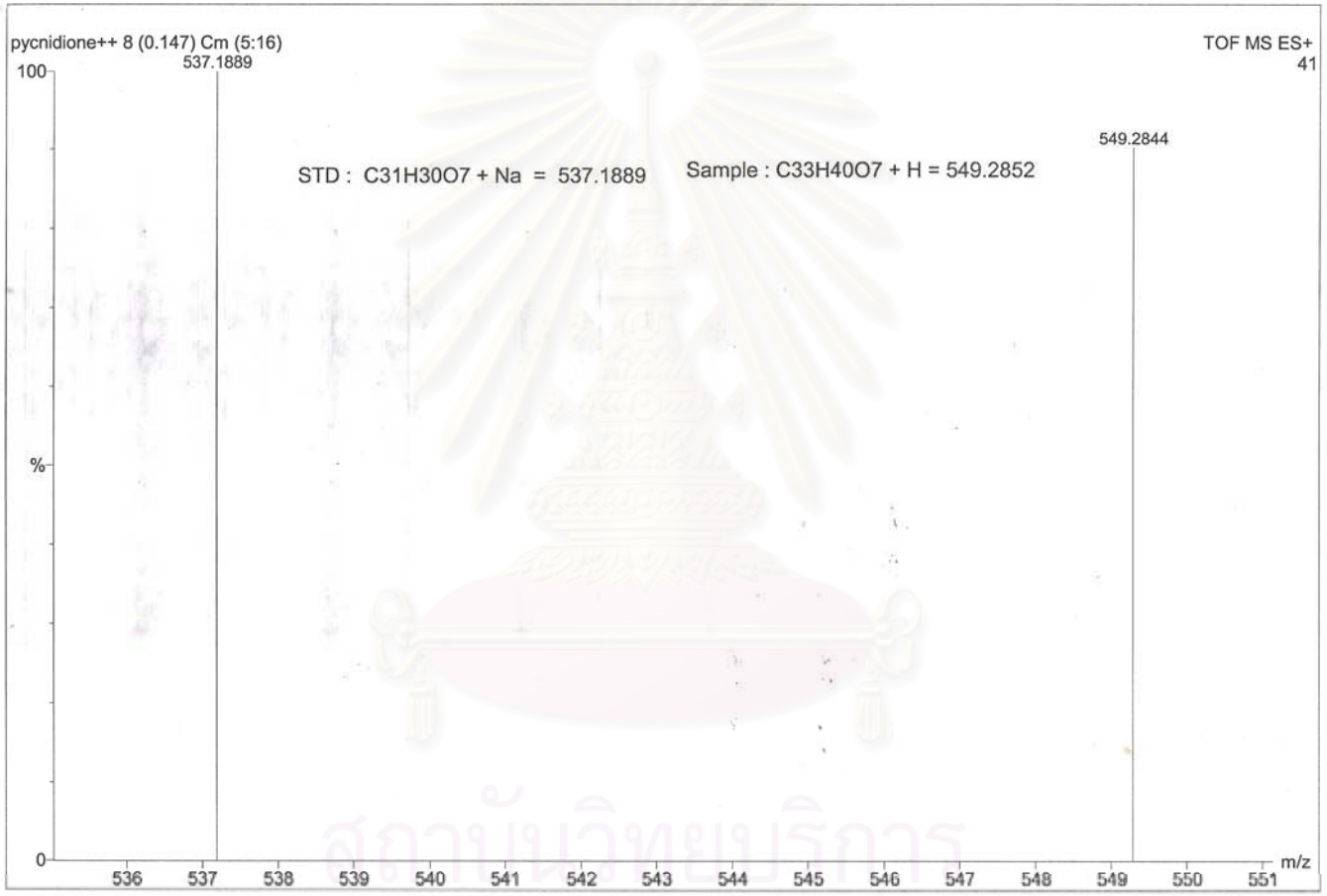

Figure C8 Mass spectrum of compound **II**

APPENDIX D

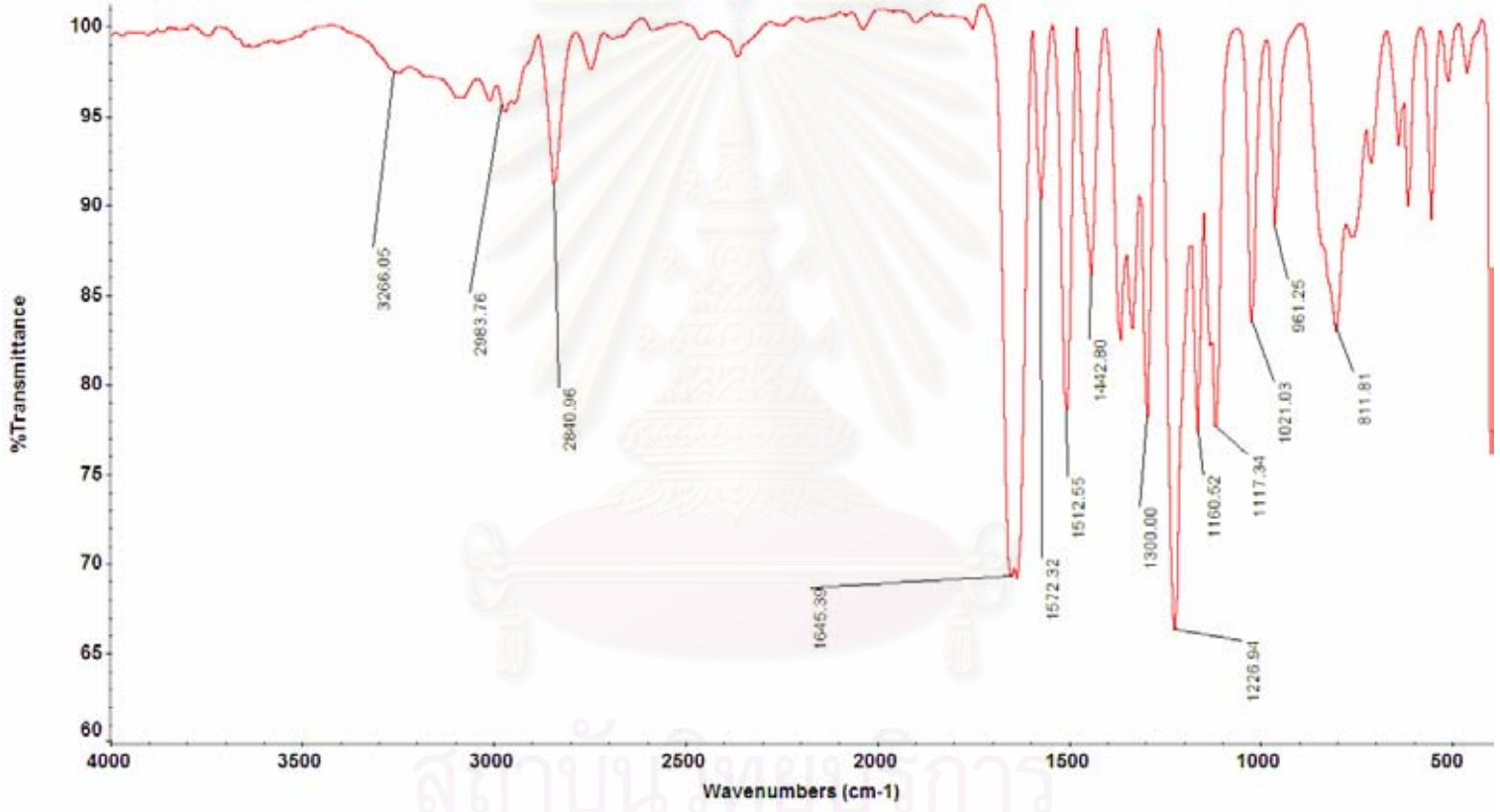

Figure D1 IR spectrum of compound **III**

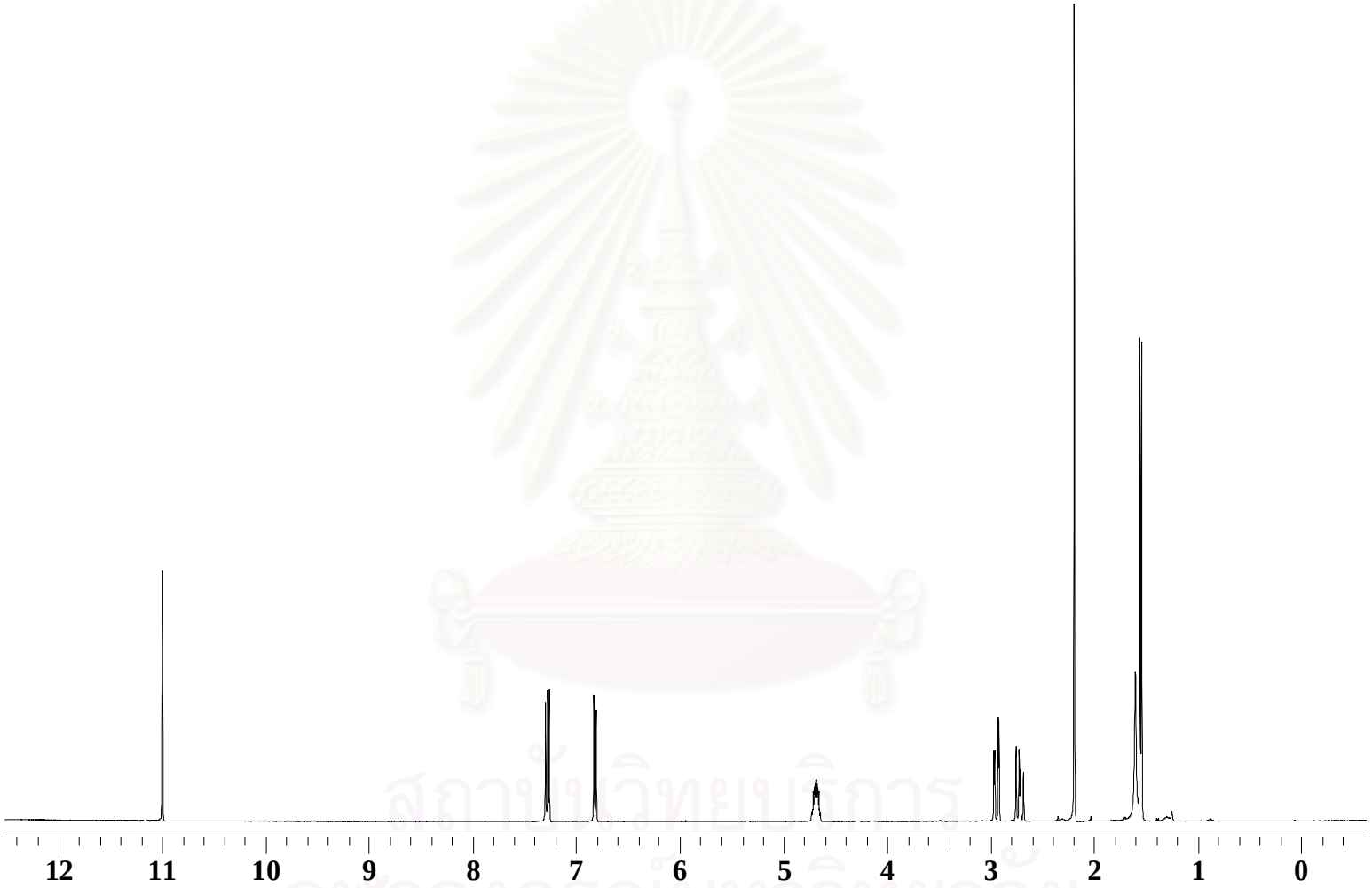

Figure D2 ^{1}H-NMR spectrum of compound **III**

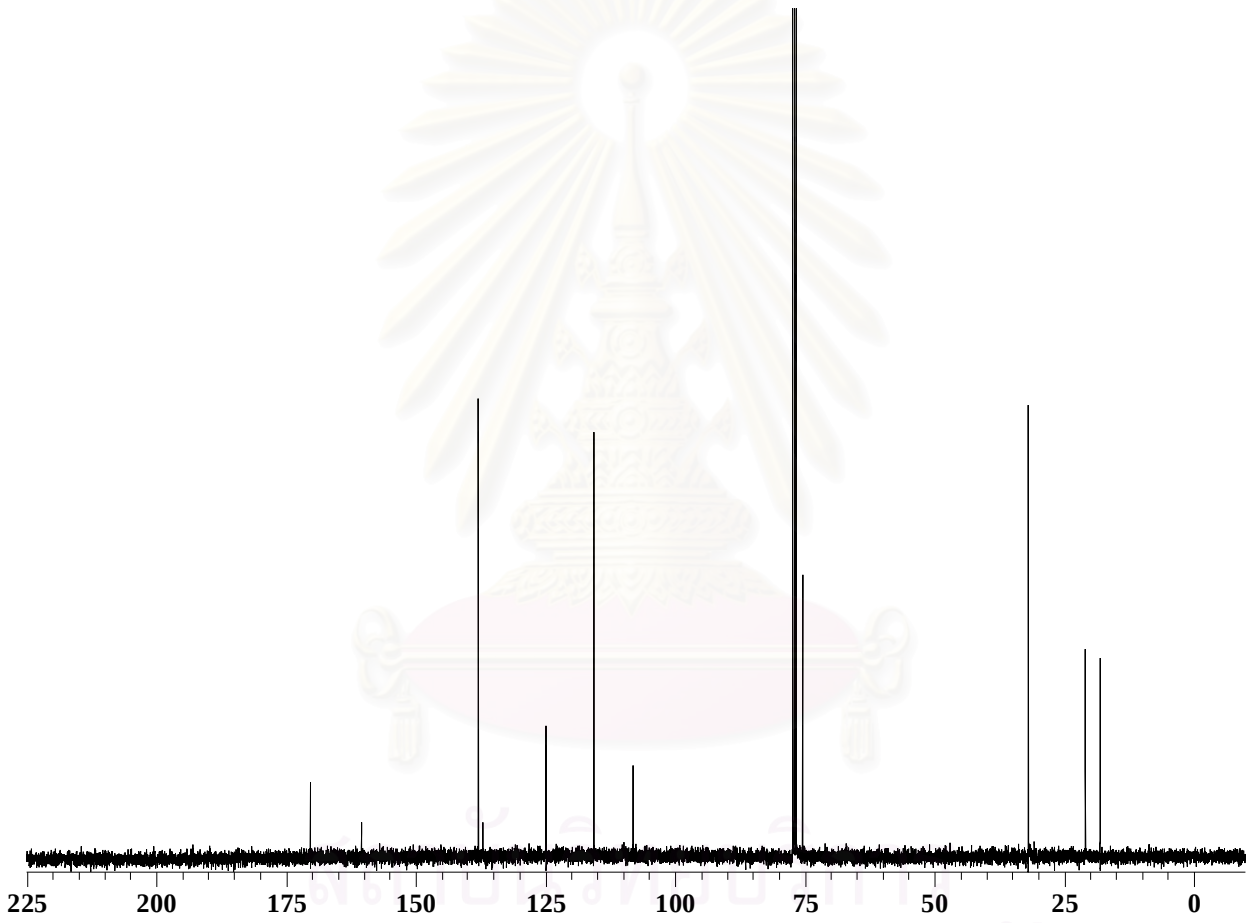

Figure D3 ^{13}C-NMR spectrum of compound **III**

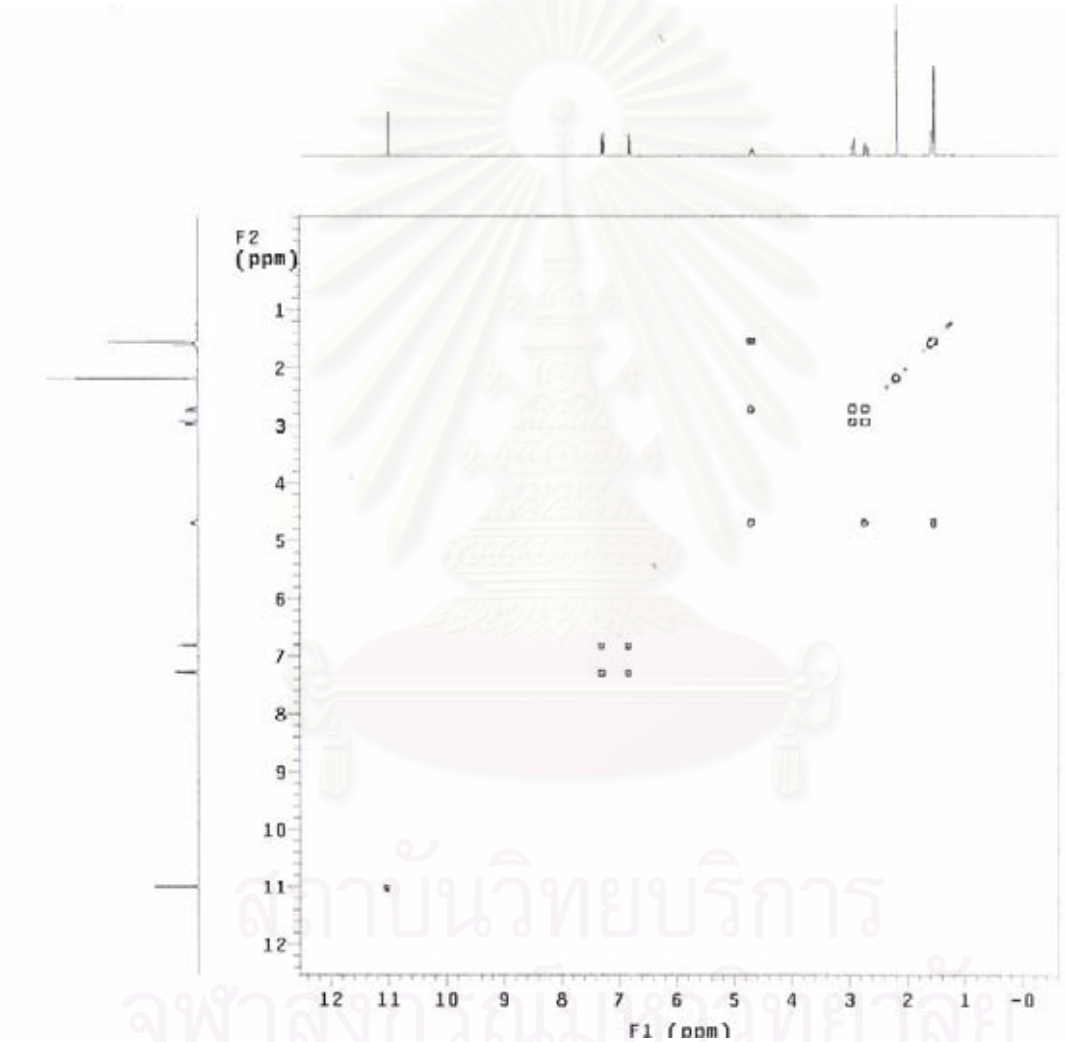

Figure D4 COSY spectrum of compound **III**

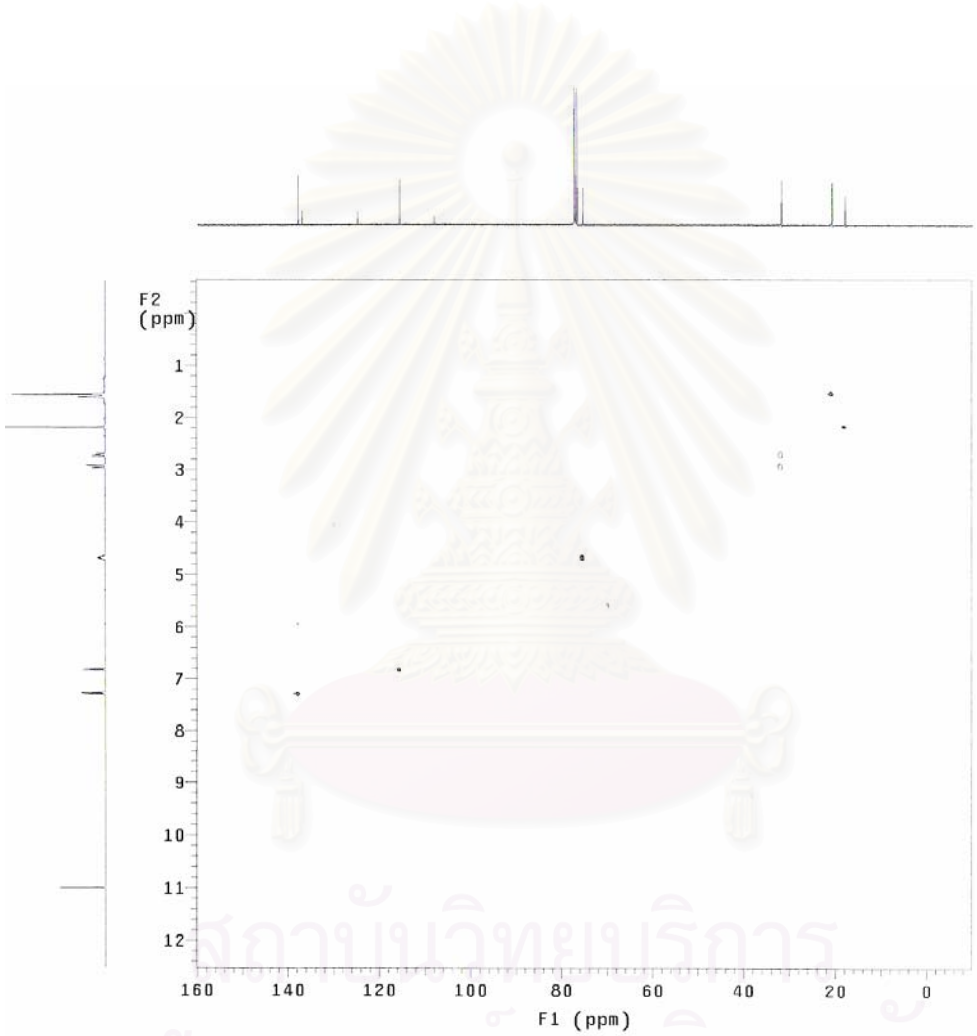

Figure D5 HSQC spectrum of compound **III**

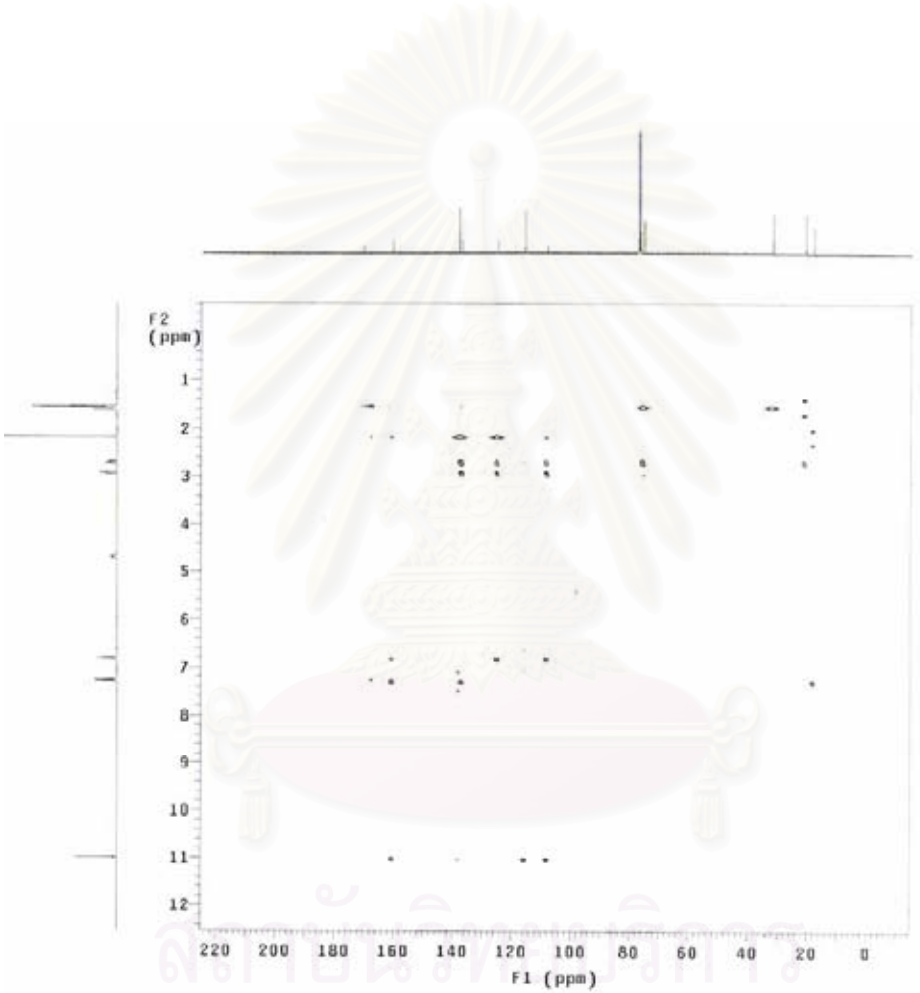

Figure D6 HMBC spectrum of compound **III**

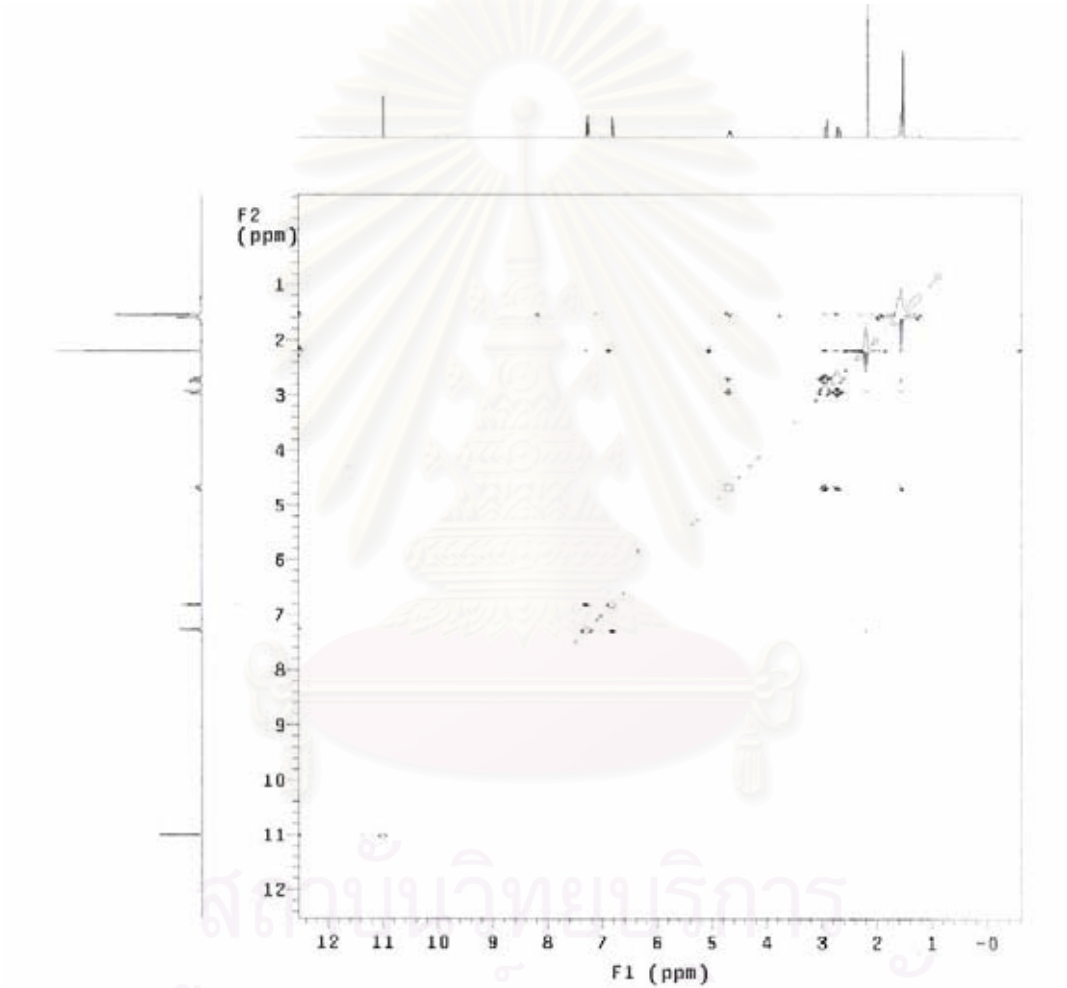

Figure D7 NOESY spectrum of compound **III**

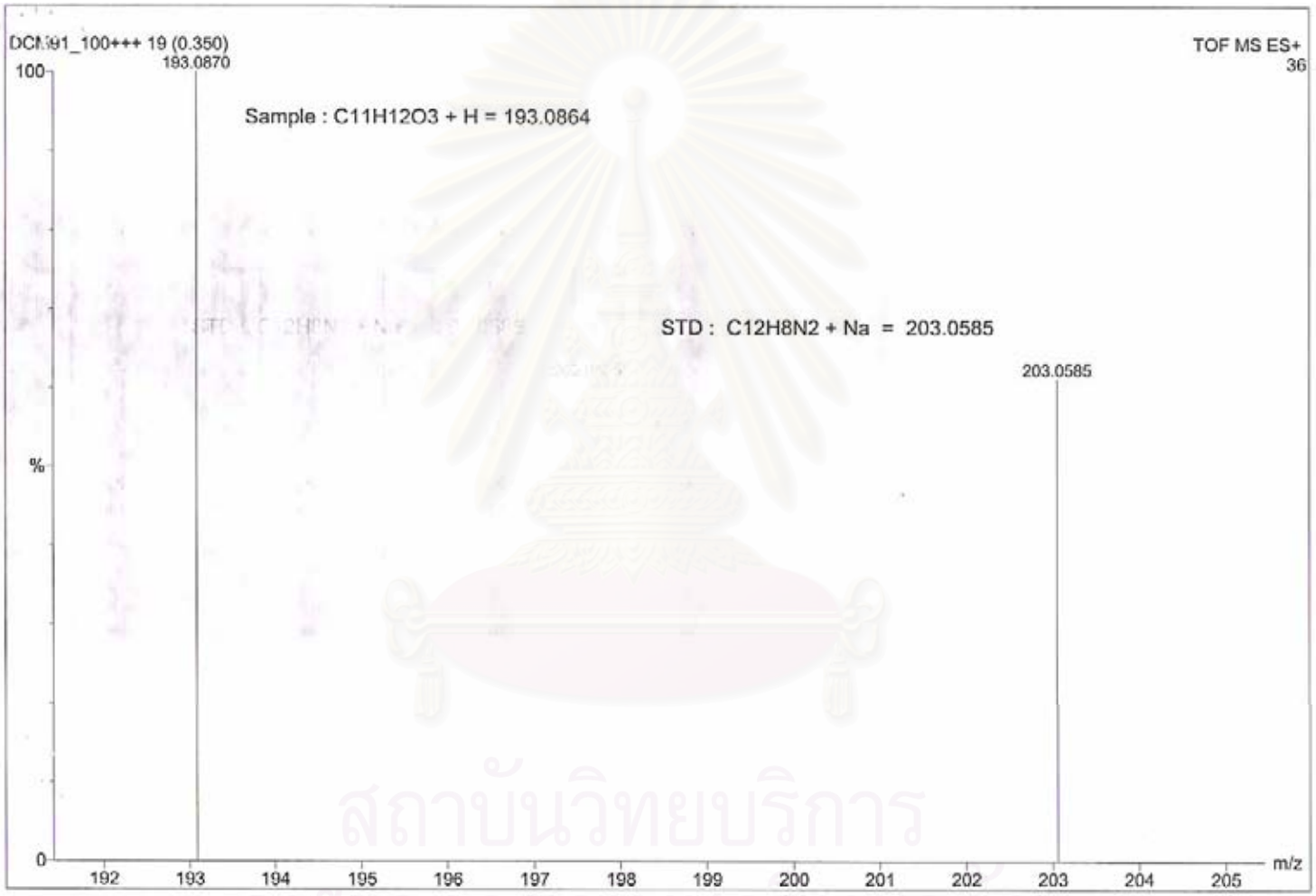

Figure D8 Mass spectrum of compound **III**

VITA

Mr. Suchon Deechangvipart was born on July 6, 1981 in Bangkok. He graduated with a Bachelor Degree of Science in Chemistry from Chulalongkorn University in 2002. Then he was admitted into a Master Degree program in Organic Chemistry at Department of Chemistry, Faculty of Science Chulalongkorn University in 2003.