BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* IN CHULALONGKORN UNIVERSITY CAMPUS

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สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* ในจุฬาลงกรณ์มหาวิทยาลัย

นางสาว ณัฐจิรา อ้นนวล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of study	Industrial Microbiology
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นางสาวณัฐจิรา อ้นนวล : สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* ในจุฬาลงกรณ์มหาวิทยาลัย (BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* IN CHULALONGKORN UNIVERSITY CAMPUS) อ. ที่ปรึกษา : รศ. ดร. ประกิตติ์สิน สีหนนทน์, 173 หน้า. ISBN 974-53-1999-6

งานวิจัยนี้ทำการศึกษาสารออกฤทธิ์ทางชีวภาพที่สร้างจากราเอนโดไฟต์ที่แยกจากส่วนใบอ่อน, ใบแก่, กิ่ง และเปลือกของเปล้าใหญ่ที่ปลูกภายในบริเวณจุฬาลงกรณ์มหาวิทยาลัย คัดแยกราเอนโดไฟต์โดยผ่านวิธีการฆ่าเชื้อที่ผิวนอกและวางบนอาหารแข็งมอลท์สกัด สามารถแยกราเอนโดไฟต์ได้ 47 ไอโซเลต เมื่อทดสอบฤทธิ์ต้านจุลินทรีย์ทดสอบของราเอนโดไฟต์ที่แยกได้โดยวิธี Agar well diffusion method พบว่าราเอนโดไฟต์ไอโซเลต CuLm17 สามารถสร้างสารซึ่งมีฤทธิ์ยับยั้งจุลินทรีย์ทดสอบได้ดี จากการจัดจำแนกสายพันธุ์โดยศึกษาลักษณะทางสัณฐานวิทยา พบว่าราเอนโดไฟต์ไอโซเลต CuLm17 คือ *Penicillium* sp. เมื่อทำการศึกษาสารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ไอโซเลต CuLm17 โดยเลี้ยงในอาหารเหลวมอลท์สกัด และสกัดด้วยเอธิลแอซิเตต แล้วแยกสารให้บริสุทธิ์โดยวิธีทางโครมาโทกราฟีและการตกผลึก ได้สารบริสุทธิ์ 2 ชนิด คือ สาร ME1 และ สาร ME2 เมื่อวิเคราะห์โครงสร้างของสารที่แยกได้โดยอาศัยคุณสมบัติทางกายภาพและเทคนิคทางสเปกโตรสโกปี (IR, MS, UV, ^{1}H, ^{13}C NMR และ 2D NMR) รวมทั้งยืนยันโครงสร้างของสาร ME1 โดยเทคนิค X-ray พบว่าสารบริสุทธิ์ ME1 คือ folipastatin และสารบริสุทธิ์ ME2 คือ unguinol เมื่อนำมาทดสอบฤทธิ์ทางชีวภาพ พบว่าสาร folipastatin มีฤทธิ์ยับยั้ง *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 และ *P. aeruginosa* ATCC 27853 โดยมีค่า MIC เท่ากับ 164.5, 657.9, 328.9 และ 328.9 µM ตามลำดับ รวมทั้งมีฤทธิ์ยับยั้งเซลล์มะเร็งทดสอบ HEP-G2 (ตับ), SW 620 (ลำไส้ใหญ่), CHAGO (ปอด), KATO-3 (กระเพาะอาหาร) และ BT474 (เต้านม) โดยมีค่า IC_{50} เท่ากับ 15.7, 15.3, 14.5, 15.8 และ 22.6 µM ตามลำดับ และสาร unguinol มีฤทธิ์ยับยั้ง *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 และ *P. aeruginosa* ATCC 27853 โดยมีค่า MIC เท่ากับ 191.7, 1533.7, 1533.7 และ 1533.7 µM ตามลำดับ รวมทั้งมีฤทธิ์ยับยั้งเซลล์มะเร็งทดสอบ KATO-3 (gastric) โดยมีค่า IC_{50} เท่ากับ 21.2 µM แต่สารทั้ง 2 ชนิดไม่มีฤทธิ์ต้านอนุมูลอิสระ งานวิจัยนี้เป็นครั้งแรกที่แยกและศึกษาฤทธิ์ทางชีวภาพของ folipastatin และ unguinol จากราเอนโดไฟต์ *Penicillium* sp..

ภาควิชา .....จุลชีววิทยา.....ลายมือชื่อนิสิต.....

สาขาวิชา.....จุลชีววิทยาทางอุตสาหกรรม...ลายมือชื่ออาจารย์ที่ปรึกษา.....

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This research investigated bioactive compounds produced by endophytic fungi isolated from healthy young and mature leaves, branches and barks of *Croton oblongifolius* from sites on Chulalongkorn University. Endophytic fungi 47 isolates were isolated from plant samples by surface sterilization method and placed on malt extract agar. Endophytic fungal isolate CuLm17 was chosen for study of bioactive compounds because this isolate produced the compounds that were active against a large number of test microorganisms with highest activities. Based on morphology endophytic fungal isolate CuLm17 was identified as *Penicillium* sp. Ethyl acetate (EtOAc) crude mycelial extract of *Penicillium* sp. was separated by chromatographic techniques and was purified by crystallization. Isolation and purification of EtOAc crude mycelial extract gave two pure compounds. The structure elucidation of these compounds was achieved by a combination of spectroscopic methods (IR, MS, UV, ^{1}H, ^{13}C NMR and 2D NMR), physical properties and the structure of compound ME1 was confirmed by X-ray analysis. It found that compound ME1 was folipastatin and compound ME2 was unguinol. Moreover, folipastatin was inhibitory against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 with MIC value of 164.5, 657.9, 328.9 and 328.9 µM, respectively and displayed high cytotoxic activity against HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) with IC_{50} value of 15.7, 15.3, 14.5, 15.8 and 22.6 µM, respectively . Unguinol was inhibitory against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 with MIC value of 191.7, 1533.7, 1533.7 and 1533.7 µM, respectively and displayed cytotoxic activity against KATO-3 (gastric) with IC_{50} value of 21.2 µM. On the other hand, folipastatin and unguinol were not shown antioxidant activity. This research was the first report for isolation and studying bioactive activities of folipastatin and unguinol from *Penicillium* sp. endophyte.

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CONTENTS

Page

Page

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF SCHEMES

LIST OF ABBREVIATIONS

ATCC	= American Type Culture Collection, Maryland, U.S. A
°C	= Degree Celsius
^{13}C-NMR	= Carbon-13 nuclear magnetic resonance
$CHCl_3$	= Chloroform
cm	= centimeter
COSY	= ^{1}H -^{1}H Correlation Spectroscopy
δ	= NMR chemical shift
d	= doublet (for NMR spectral data)
dd	= doublet of doublets (for NMR spectral data)
ddd	= doublet of doublet of doublets (for NMR spectral data)
DMSO-d_6	= Dueterated dimethylsulfoxide
ε	= Molar absorptivity
EI-MS	= Electron Impact Mass Spectrometry
EtOAc	= Ethyl acetate
g	= gram
HMBC	= ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation
^{1}H-NMR	= Proton Nuclear Magnetic Resonance
HSQC	= ^{1}H-^{13}C Heteronuclear Single Quantum Correlation
Hz	= Hertz
IR	= Infared
ITS	= Internal Transcribe Spacers
J	= Coupling constant
l	= liter
m	= multiplet (for NMR spectral data)
m	= medium (for IR spectral data)
M^+	= Molecular ion
MEA	= Malt Extract Agar
MeOH	= Methanol
mg	= milligram

LIST OF ABBREVIATIONS (continued)

MHz = Megahertz
MIC = Minimum inhibitory concentration
min = minute
ml = milliliter
mm = millimeter
MS = Mass Spectroscopy
m/z = mass to charge ratio
nm = nanometer
NMR = Nuclear Magnetic Resonance
NOESY = Nuclear Overhauser Enhancement Spectroscopy
OMA = Oat Meal Agar
PDA = Potato Dextrose Agar
ppm = Part per million
PCR = Polymerase Chain Reaction
q = quartet (for NMR spectral data)
s = singlet (for NMR spectral data)
s = strong (for IR spectral data)
SDA = Sabouraud's Dextrose Agar
SEM = Scaning Electron Microscpoe
sept = septet (for NMR spectral data)
sp. = species
t = triplet (for NMR spectral data)
TLC = Thin Layer Chromatography
µl = microliter
µg = microgram
UV = Ultraviolet
w = weak (for IR spectral data)
YES = Yeast Extract Sucrose agar
λ_{max} = the wavelength at maximum absorption (UV)

LIST OF ABBREVIATIONS (continued)

ν_{max} = wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

Fungi are fundamental to the health and prosperity of every terrestrial ecosystem and are essential to the sustainability of biodiversity. It has been estimated that there may be as many as 1 million different fungal species on our planet (Hawksworth and Rossman, 1987). In the past century, many of the 0.1 million fungi that have been described were those associated with various higher organisms as either parasites or saprophytes on dead and dying biological materials (Strobel, 2003). Thus, the question, where are the remaining 0.9 million fungi. In the past few decades, plant scientists have begun to realize that plants may serve as a reservoir of untold numbers of organisms known as endophytes (Bacon and White, 2000).

By definition, these microorganisms live in the intercellular spaces of plant tissues. Some of these endophytes may be producing bioactive substances that may be involved in a host-endophyte relationship. As a direct result of the role may ultimately be shown to have applicability in medicine.

There are approximately 300,000 different plant species on our planet. And of the several hundred of these that the scientists have examined, each individual plant has one or more endophytic microbes. Those plants growing in unique environmental settings, having ethnobotanical uses, having extreme age or interesting endemic locations generally produce novel endophytic fungi. Consequently, the opportunity to find new and interesting endophytic fungi amoung myriads of plants in different settings and ecosystems is great.

Endophytic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industial arenas. As an example, the fungal endophytes, *Taxomyces andreanae*, *Pestalotiopsis microspora* and several other fungi isolated from each the bark of the world's yew tree species (*Taxus* spp.) are potential new source of the anticancer drug taxol (Strobel et al., 1993). In addition, an endophytic fungus, *Colletotrichum* sp., which

isolated from herbaceous plant *Artemisia annua*, produced the antibacterial and antiphytopathogenic fungal compounds (Lu et al.,2000). A worldwide scientific effort to isolate endophytes and study their natural products is now under way.

Thus, in this research the Thai medicinal plant, *Croton oblongifolius*. has been used as a plant source for isolating endophytic fungi because it is believed that all parts of the this plant can be used in the treatment of many ailments. For example, the leaves can be used as a tonic, the flowers are used as a teniacide, the fruits are used to treat dysmenorrhea, the seeds are used as a purgative, the bark is used to treat dyspepsia, and the roots are used to treat dysentery (เสงี่ยม พงษ์บุญรอด, 2502).

Objectives

1. To isolate and identify the endophytic fungi found within healthy young and mature leaves, branches, and barks of *Croton oblongifolius* in Chulalongkorn University.
2. To determine antimicrobial activity of the isolated endophytic fungi.
3. To identify a selected endophytic fungal isolate by using classification based on morphology.
4. To extract, isolate and purify the bioactive compounds from a selected endophytic fungal isolate.
5. To elucidate the structural formula of the isolated bioactive compounds.
6. To evaluate the biological activity of the bioactive compounds.

CHAPTER II

LITERATURES REVIEW

2.1 General consideration of Endophytic fungi

According to the most prevalent definition, fungal endophytes (endo=inside + phyte=plant) were fungi that live within their host plant tissues for at least part of their life cycle without causing any symptoms of diseases (Petrini, 1991; Carroll, 1988). This does not, however, preclude the possibility that they may become pathogenic when the host was stressed (Stone, 1990; Carroll, 1988). Endophytes, in contrast to epiphytes, were contained entirely within the substrate plant. Endophytic colonization or infection cannot be considered as causing disease, since a plant disease is an interaction between the host, parasite, vector and the environment over time which results in the production of disease signs and/or symptoms. The distinction between an endophyte and a pathogen was not always clear. A mutation at a single genetic locus can change a pathogen to nonpathogenic endophytic organism with no effect on host specificity (Freeman and Rogriguez, 1993). Many pathogens undergo an extensive phase of asymtomatic growth corresponding to colonization and then latent infection before symptoms appear. Additionally many pathogens of economically important crops may be endophytic or latent in weeds (Cerkauskas, 1988; Cerkauskas *et al*., 1983; Hartman *et al*., 1986; Hepperly *et al*., 1980; Kulik, 1984; McLean and Roy, 1988; Raid and Pennypacker, 1987). Alternately, nonpathogenic endophytic organisms may play a role as biocontrol agents (Freeman and Rogriguez, 1993). Both endophytic and latent infection fungi can infect plant tissues and become established after penetration. However, infection does not imply the production of visible disease symptoms (Redin and Carris, 1985). The biological and ecological diversity of endophyes is reflected in the varying emphasis and heterogeneity of concepts among researchers concerned with studying them. Often the term “endophyte” and “endophytic“ are used with particular meaning by different workers and for particular groups of hosts and microbes (Stone, Bacon, and White, 2000).

Members of the Ascomycota, Basidiomycota, Deuteromycota, and some Oomycota have been isolated as endophytes. Endophytic fungi have been isolated from phanerogams in alpine, temperate and tropical regions, although the plants of the Coniferae, Ericaceae and Germineae have been most intensively sampled (Clay, 1991; Petrini, 1986; Siegal *et al*., 1997). Accumulating evidence suggests that endophytes represent a large reservoir of genetic diversity and a rich source of heretofore undescribed species. Systemic grass endophytes are restricted to clavicipitaceous members of the Balansiae (Ascomycota). Taxonomically, endophtyes from woody plants are usually member of the Ascomycota but may also include member of the Basidiomycota, Deuteromycota, and Zygomycota (Leuchtmann, 1992; Petrini, 1986; Sinclair and Cerkauskas, 1996).

Growing evidence indicates that endophytes are found in a wide range of plant groups including mosses, ferns, lichens, orchids, grasses and trees (Marchisio et al., 1985; Clay, Hardy, and Hammond, 1988; Petrini, Hake, and Dreyfus, 1990; Petrini, Fisher, and Petrini, 1992; Weber, 1995; Kowalski and Rolf, 1996), are extremely abundant and are often very diverse (Stone and Petrini, 1997; Schulthess and Faeth, 1998). Although, endophytic fungi have been found in almost plant groups, however, fungal endophytes of grasses (Poaceae) and sedges (Cyperaceae and Juncaceae) are probably the most extensive studied group (Clay, 1988, 1989). Report on the presence of endophytes in vascular plants, other than grasses, have focused mainly on ericaceous, dicotyledoneous plants and conifers (Rodrigues, 1996).

Fungal endophyte were form internal localized infections in either intercellular or intracellular space of usually above-ground plant tissues such as leaves, stem and bark (Figure 2.1) (Suske and Acker, 1989), but also occasionally in roots, and were distinguished from mycorrhiza by lacking external hyphae or mantels. Most of these endophytes were horizontally transmitted via spores, a much smaller fraction, mostly found in pooid grasses, form systemic infections in above-ground tissue. Some of these are vertically transmitted via hyphae growing into seeds (Saikkonen et al., 1998)

Some fungal endophyte-grass associations, such as that between the perennial rye grass *Lolium perenne* and the fungus *Epichloe* (anamorph *Acremonium*), are

common and widespread in natural populations. The association has become very close and the endophyte invades the flowers, is incorporated in the seeds, and passed to the next generation of host grass. The life cycles of endophytic fungi in grasses are shown in the Figure 2.2 (Carlile *et al*., 2001).

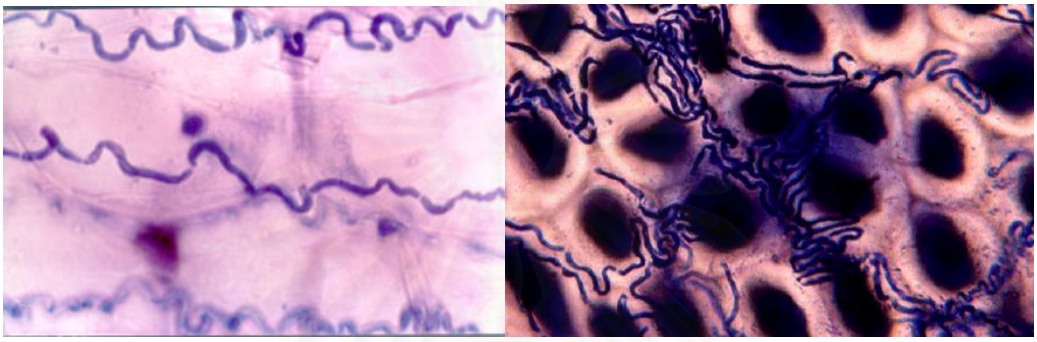


Figure 2.1 Endophytic fungi in plant tissues; in leave tissue (a), and in seed (b).

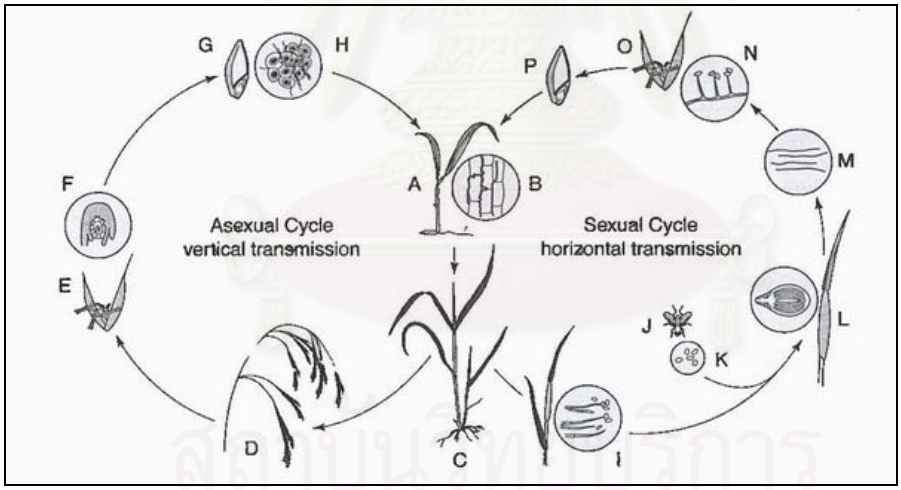


Figure 2.2 The life cycles of endophytic fungi in grasses. Left: in some fungus-endophyte associations the fungus is transmitted from parent to progeny by vertical transmission down the generations. The fungus does not sporulate at all on the surface of the plant (D), but its mycelium is passed to the next generation exclusively throught infection of the ovule (E,F) and seeds (G,H) by vegetative mycelium (B) present in the tissues of the host grass. This is the sole mode of transmission in some grass-endophyte associations, for

example *Neotyphodium lolii* in the perennial rye grass *Lolium perenne.* It results in the inheritance of clones of the endophyte, unchanged by sexual genetic recombination. Molecular phylogenetic studies comparing ribosomal or ribosomal or nuclear gene sequences in grasses and their endophytes show that the fungus and grass species in such associations have often evolved in parallel. Right: The fungus is transmitted from one grass plant to another by means of spores produced on the fungal reproductive structure (stroma). A stroma forms (I) on which the fungus's sexual structures, receptive hyphae and spermatia are produced. Insects (j) carry spermatia (K) from one stroma to another, effecting cross-fertilization which is followed by the production of ascospores in perithecia (L) developing in the stroma on the grass. The thread-like ascospores (M) are wind dispersed to grass flowers, where they germinate and produce secondary conidia (N) which may infect ovules (O) and developing seeds (P) of a new host after entering through stigmata. As a result the next generation of plants is infected by new strains of the endophyte. A typical example of such an association is *Epichloe typhina* infecting the cocksfoot grass *Dactylis glomerata.* Several grass endophyte associations, for example *Epichloe festucae* on red fescue, *Festuca rubra*, are capable of both seed and ascospore transmission, the balance depending on the environmental conditions or genotypes involved (Carlile *et al*., 2001).

Endophytes are considered plant mutualists: They receive nutrition and protection from the host plant while the host plant may benefit from enhanced competitive abilities and increased resistance to herbivores, pathogens, and various abiotic stresses. Practical applications of endophytes include potential biological control agents, sources of novel metabolites for medicine, plant protection, and industrial uses, and as research model system for investigation of host-parasite interactions and evolution in natural systems (Stone, Bacon, and White, 2000).

2.2 Host specificity

The degree of host specificity which operates in endophytic fungi was not yet clear. Some species were commonly occurring and may be isolated from various host plant species and from different locations with differing environmental conditions. In general terms, the geographical occurrence of endophytes was related to the distribution of host species. In some cases almost all individuals in a plant population may be infected by endophytes. *Cladosporium* spp., *Nodulisporium* spp. and *Pleospora* spp. were common. Some endophytes, however, do not show such a wide species range and were often isolated from plants of the same family or closely related families. Other species were only rarely detected (Isaac, 1992).

The degree to which endophytes were tissue or organ specific is also not yet clear. Some species were most commonly isolated from similar tissues, particularly the endophytes of conifer needles (Carroll and Petrini, 1983). In other cases the occurrence was less distinct. However, only limited surveys had been carried out to date (Isaac, 1992).

2.3 Endophytic mutualism

2.3.1 Effects of endophyte infections

Many endophytes lived almost entirely within the host plant tissues, often without causing any visible signs of infection. Fungal hyphae penetrate between plant cells or may also grow intracellularly and must obtain nutrient materials through this intimate contact with the host. The occurrence of specialized feeding structures had not been reported in these fungi. Hyphae were sometimes quite wide in diameter (10-15μm) when in association with plant cells and may be distorted, irregular or bulbous in form. In some instances considerable amounts of fungal biomass are supported in host plant tissues. In physiological terms relatively little was known about the endophytic interactions between host and fungus and it is not easy to see how a host plant may benefit from such a relationship. Endophytic associations do not lead to the development of disease symptoms but do result in some morphological and

physiological changes in host tissues which increase the survival and vigour of the plants concerned. Such physiological enhancement would be likely to increase the capacity of a plant to resist disease. It has also been suggested that endophyte-infected plants are more tolerant of water stress and recover more quickly than uninfected individuals (Belesky *et al*., 1987), although it is not clear quite how photosynthetic rates are affected by the presence of endophytes (Clay, 1989). There are suggestions that endophytes may produce plant growth regulators, which may alter the normal developmental pattern of the host plant (Porter *et al*., 1985). Reports of secondary metabolite production by endophytes, e.g. alkaloids and antibiotics, which affect a range of herbivores, have attracted a great deal of attention. It has also been suggested that endophytes provide the plant with a chemical defense mechanism (Isaac, 1992).

2.3.2 Effects on insects

One of the main reasons for the recent increase in interest in endophytic fungi had been the realization that endophyte infections have a marked effect on the grazing of insect pests. The presence of endophytes makes plant tissues unacceptable and unpalatable to insects such that infected tissues are avoided. Additionally, infected tissues may gave rise to toxic effects on the insects causing poor larval growth and development, reduction in reproduction capacity or death of individuals. Therefore having more far-reaching effects on insect populations (Isaac, 1992).

A range of species of insects had been reported to be negatively affected by endophyte-infected grasses, including crickets, aphids, armyworms and flour beetles. Much of this information has led to the suggestion that endophytic fungi may be suitable biocontrol agents for the protection of grass species (Clay, 1989). A range of field and laboratory feeding experiments with endophyte-infected grasses saw that these were toxic to insects and led to a reduction in the rate of growth and development (Isaac, 1992).

An interesting example of the effects of endophytes on insects is provided by the interaction between elm bark beetles and elm trees. The beetles attack elm trees by burrowing into the inner bark tissues and in doing so infection the trees with spores of

Ceratocystis ulmi, which causes Dutch Elm disease which is responsible for the death of many elm trees. A correlation has been demonstrated between the presence of the endophyte *Phomopsis oblonga* and the demise of the bark beetles. Feeding on endophyte-infected wood led to a reduction in the reproductive capacity and a decline in the beetle populations (Webber, 1981).

A number of endophytic fungi isolated from needles of Canadian fir trees were shown to produce secondary metabolites in culture. These metabolites were toxic to spruce budworms, both decreasing growth rate and increasing mortality of larvae (Miller, 1986). It is not clear to what extent such toxins were produced *in vivo* or the degree of protection which is afforded to the conifers from this source. The presence of the endophytic fungus *Rhabdocline parkeri* in Douglas-fir needles (Carroll, 1986, 1988) incresed the mortality of gall-forming midge larvae (*Contarinia*). Toxic metabolites from the fungus were implicated as the responsible agents (Isaac, 1992).

2.3.3 Effects on other herbivores

Although toxicoses induced in domestic herbivores had been know and related to grazing fodder for many years, it is only recently that the observed effects have been correlated with the presence of fungal endophytes in pasture grass populations. Endophytes in grasses, responsible for poisoning mammalian stock had now been positively identified. Major examples of such problems had been encountered in grazing areas. In the southern USA the effects of fescue toxicosis had been known for many years. Cattle grazing on infected tall fescue (*Festuca arunginacea*) can develop symptoms including poor weight gain, increased body temperature, lameness, gangrene and limb loss. The effects develop particularly in the summer during hot, dry conditions when the livestock are under most physiological stress. A further example is the condition known as ryegrass staggers, typified by muscular spasms and, in severe cases, an inability to even stand. These symptoms have developed in sheep in New Zealand, and have been correlated with grazing on endophyte-infected *Lolium perenne*. These effects incur substantial economic losses annually (Isaac, 1992).

Although such conditions had been attributed to the presence of fungal endophytes the exact causes are not entirely clear. The endophytes of grasses were Clavicipitaceous fungi and were therefore likely to produce alkaloids, indeed the symptoms of the induced toxicoses are related to those of ergot poisoning. It has now been shown that alkaloids are present in the tissues of these host plants. However, alkaloids had been reported to occur in healthy plant tissues only extremely rarely. Fungi belonging to *Balansia* species do produce alkaloids in culture. It was likely that these were the cause of the toxic effect in mammals (Isaac, 1992).

2.3.4 Plant resistance to microbial pathogens

An increase in host plant vigour will probably enhance the plants inherent ability to resist disease. It has also been suggested that the presence of endophytic fungal infections may protect a host plant from some potentially virulent pathogens (Carroll, 1988). Infected perennial ryegrass plants were apparently protected, to some degree, from *Puccinia coronata* since the development of rust pustules was less, per unit leaf area, than for uninfected plants (Clay, 1989). It had also been suggested that some endophytic species may be antagonistic towards other fungal species. Such antagonism has been demonstrated in culture, and culture filtrates from the grass endophyte *Acremonium coenophialum*, had been shown to have inhibitory effects on the growth of potential grass pathogens (White and Cole, 1985).

2.3.5 Endophytic strategies

Although investigations concerning endophytic associations were limited, the available evidence suggests that endophytes had evolved from plant pathogenic fungi. Many of the endophytes which have been described are very closely related to virulent pathogens and occur on the same, or related, host plants also. For example, on grasses *Acremonium coenophialum* is related to the pathogen *Epichloe typhina* and occurs on a similar range of host plants. *Rhabdocline parkeri* is a frequently isolated endophyte of conifers and had been shown to infect Douglas fir (Sherwood-Pike et al., 1986; Stone, 1987, 1988). In autumn, germinating conidia became attached to the needle surface by

mucilage and produce penetration pegs by which the plant tissues were invaded. Hyphae form intracellularly within individual epidermal cells and remain there for a long time (2-5 years), not proliferating further until needle senescence. As the host tissues age, the fungus then invades surrounding tissues, forming structures somewhat akin to haustoria. Sporulation occurs after needle abscission. *Rhabdocline parkeri* is related to the plant pathogens *R. wierii* and *R. pseudotsugae*, and, in fact all these species may infect the same tree simultaneously. However the distinctions between endophytic and pathogenic relationships were often not clear. Some pathogenic species may show a long period of apparently endophytic growth in a host plant before symptom development occurs. Additionally, the growth requirements of endophytic species indicate the utilisation of a limited range of materials as substrates, a characteristic which was often associated with pathogenic species (Isaac, 1992).

Two strategies of endophytic mutualism had been described (Carroll, 1986, 1988). In grasses, many fungal species have been identified which do not leave the host plants at reproduction and do not produce any external fruiting bodies. Spread of the fungus is achieved by vegetative growth of hyphae into the ovules of the host so that dispersed seed is already infected by these fungi. This has been termed constitutive mutualism (Carroll, 1986). Such endophytes infect aerial parts of the plant systemically, commonly developing a large biomass of fungal mycelium in host tissues. These species do not appear to harm the host plant but often produce toxins which may have important deterrent effects on grazing herbivores and may therefore provide protection to the plant. In some instances the presence of these endophytes within ovule tissues results in host plant sterilisation and therefore the cost of this association to the plant in high. However, endophyte-induced sterility may result in more vigorous vegetative growth. Grasses may produce more vegetative tillers enhancing competitive ability in some ecological situations. It is very likely that the metabolic and physiological drains on host plant reserves and energy supplies are also high, since a large fungal biomass is supported by the plant (Isaac, 1992).

An alternative strategy, adopted by some endophytic species, is inducible mutualism, in which a looser relationship is formed with the host plant (Carroll, 1986,

1988). In these associations the distribution of the endophyte in plant tissues is patchy and probably affected by ageing in the host. These endophytes normally inhabit senescent host tissues and only penetrate metabolically active regions when the plant is stressed. Herbivore wounding of tissues leads to endophyte invasion of active regions. The toxins produced by endophyte activity then give rise to destruction in the herbivore population. There may be of little benefit to the host plant directly, although it has been suggested that the host population may benefit, albeit often in the long term, as a result of the reduction in herbivory (Carroll, 1988). Here, the metabolic cost to the plant is relatively small (Isaac, 1992).

2.4 Interaction between endophytic fungi and plants

The interactions between fungi and plant host are often variable among and within population and communities. Biologically and ecologically, endophytes represent a diversity of nutritional modes from biotrophic parasites to interim or facultative saprotrophs, and associations with their hosts span the continuum from biotrophic mutualists and benign commensals to nectotrophic, and antagonistic pathogens.

Mutualism has been the prevailing conceptual framework under which the evolution and ecology of endophyes have been viewed and interpreted. Discovery of severe biological effects of endophytes in grasses on livestock, such as toxicosis and hoof gangrene (Clay, 1988, 1990; Ball, Pedersen, and Lacefield, 1993), and on of invertebrate pest species (Clement et al., 1996, 1997; Bultman and Murphy, 1998) led to the concept of grass endophytes as plant mutualists, primarily by deterring herbivores as “acquired defenses” (Cheplick and Clay, 1988). Endophytes defense against vertebrate and invertebrate herbivores, and also against plant pathogens, purportedly results from production of multiple alkaloid compounds by endophytes, at least in agronomic grasses (Siegel et al., 1990; Siegel and Bush, 1996, 1997). In turn, host plants provide endophytic fungi with a protective refuge, nutrients, and in the case of vertically transmitted endophyte dissemination to the next generation of hosts. Other studies of agronomic grasses show that endophytes provide other fitness-enhancing

properties for their hosts, such as increasing plant competitive abilities, many by increasing efficiency of water use (Bacon, 1993; Elmi and West, 1995). Furthermore, because alkaloids from endophytes are often concentrated in seed, vertically transmitted grass endophytes also may deter seed predators and increase seed dispersal (Wolock-Madej and Clay, 1991; Knoch, Faeth, and Arnott, 1993).

Fungal endophytes in grasses were thus considered prototypical mutualists. “Endophyte” quickly became synonymous with “mutualist” (Petrini et al, 1992; Bacon and Hill, 1996) at least in grasses (Stone and Petrini, 1997), and the primary driving selective force behind the mutualist was (Cheplick and Clay 1988; Clay, 1988), still is (Clay, 1997; Leuchtman, 1992), considered defense against herbivores.

Similarly, Carroll (1988) proposed that endophytes of woody plants provide a defensive role for the host plant because they produce a wide array of mycotoxins and enzymes that can inhibit growth of microbes and invertebrate herbivores (Petrini et al, 1992; Stone and Petrini, 1997). Because endophytes of woody plants are diverse and have shorter life cycles than their perennial host plants, defense via endophytes is considered a mechanism by which long-lived woody plants could keep pace evolutionarily with shorter generational and hence, presumably more rapidly evolving invertebrate herbivores (Carroll, 1988).

2.5 Natural products from endophytic fungi

The following section shows some examples of natural products obtained from endophytic fungi and their potential in the pharmaceutical and agrochemical areanas.

2.5.1 Products of endophytic fungi as antibiotics

Antibiotics are defined as low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms (Demain, 1981). Often, endophytic fungi are a source of these antibiotics. Natural products from endophytic fungi have been observed to inhibit or kill a wide variety of harmful disease-causing microorganisms including, but not limited to, phytopathogen,

as well as bacteria, fungi, viruses, and protozoans that affect humans and animals. Described below are some examples of bioactive products from endophytic fungi.

2.5.1.1 Antibacterial and antifungal compounds

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia. On petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. A unique peptide antimycotic, termed crytocandin, was isolated and characterized from *C. quercina* (Strobel et al., 1999). This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid: 3-hydroxy-4-hydroxymethylpropine (Figure 2.3). The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins (Walsh, 1992). As is generally true, not one but several bioactive and related compounds are produced by an endophytic microbe. Thus, other antifungal agents related to cryptocandin are also produced by *C.* cf. *quercina.* Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea.* Cryptocandin and its related compounds are currently being considered for use against a number of fungal-causing diseases of the skin and nails.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (Figure 2.4). This unusual compound possesses potent activity against *Pyricularia oryzae,* he causal organism of one of the worst plant diseases in the world, as well as a number of other plant pathogenic fungi (Li et al., 2000). The compound was generally ineffective against a general array of human pathogenic fungi. Nevertheless, with a minimum inhibitory concentration against *P. oryzae* of 0.39 µg/ml, this compound is being examined as a natural chemical control agent for rice blast and is being used as a model to synthesize other antifungal compounds.

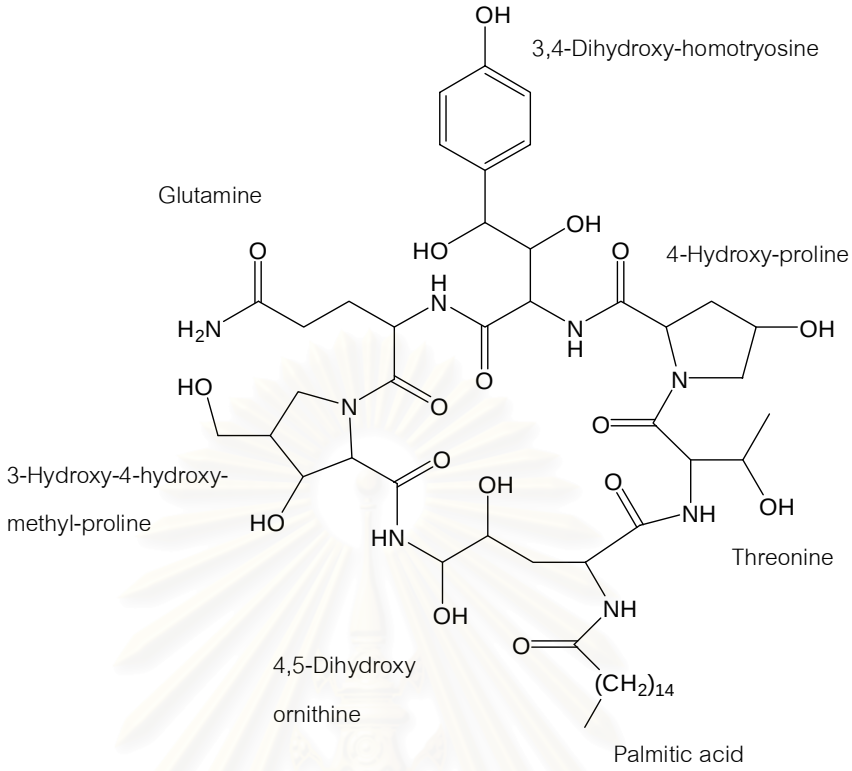


Figure 2.3 Cryptocandin, an antifungal lipopeptide obtained from the endophytic fungus *Cryptosporiopsis* cf. *quercina.*

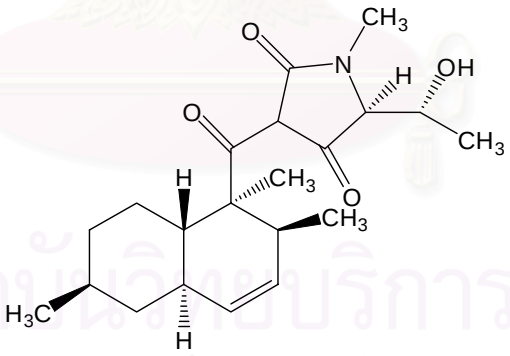


Figure 2.4 Cryptocin, a tetramic acid antifungal compound found in *Cryptosporiopsis* cf. *quercin*a.

Pestalotiopsis microspora is a common rainforest endophyte. It turns out that enormous biochemical diversity does exist in this endophytic fungus, and many secondary metabolites are produced by various strains of this widely dispersed organism (Li et al., 1996, Strobel et al., 1996, Strobel, 2002a, Strobel, 2002b). One such secondary metabolite is ambuic acid, an antifungal agent, which has been recently

described from several isolates of *P. microspora* found as representative isolates in many of the world's rainforests (Figure 2.5) (Li et al., 2001). This compound as well as another endophyte product, therein, have been used as models to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules.

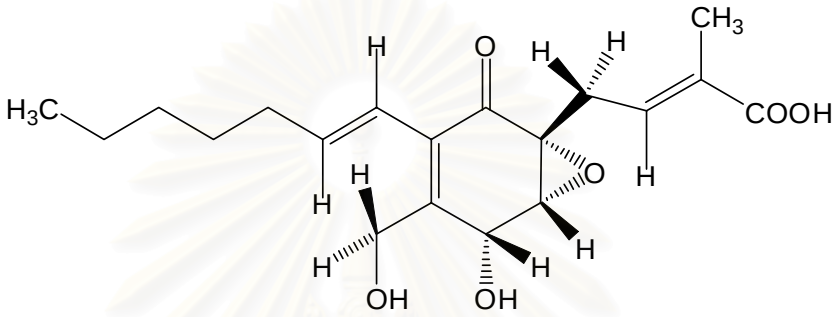


Figure 2.5 Ambuic acid, a highly functionalized cyclohexenone produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity and has been used as a model compound for the development of solid-state NMR methods for the structural determination of natural products (Harper et al., 2001, 2003).

A strain of *P. microspora* was also isolated from the endangered tree *Torreya taxifolia* and produces several compounds having antifungal activity including pestaloside, an aromatic β-glucoside (Figure 2.6), and two pyrones, pestalopyrone and hydroxypestalopyrone (Lee et al., 1995). These products also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. microspora* (endophytic on *Taxus brevifolia*) include two new caryophyllene sesquiterpenes, pestalotiopsins A and B (Pulici et al., 1996a). Additional new sesquiterpenes produced by this fungus are 2-α-hydroxydimeninol and a highly functionalized humulane (Pulici et al., 1996b, 1996c). Variation in the amount and kinds of products found in this fungus depends on both the cultural conditions and the original plant source from which it was isolated.

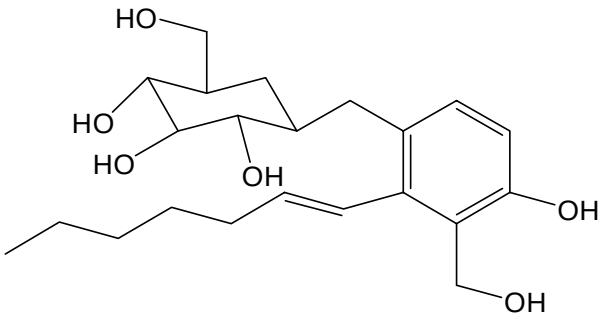


Figure 2.6 Pestaloside, a glucosylated aromatic compound with antifungal properties from *Pestalotiopsis microspora*.

Pestalotiopsis jesteri is a newly described endophytic fungal species from the Sepik river area of Papua New Guinea, and it produces jesterone (Figure 2.7) and hydroxyjesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi (Li and Strobel, 2001). These compounds are highly functionalized cyclohexenone epoxides. Jesterone, subsequently, has been prepared by organic synthesis with complete retention of biological activity (Hu et al., 2001). Jesterone is one of only a few products from endophytic microbes in which total synthesis of a bioactive product has been successfully accomplished.

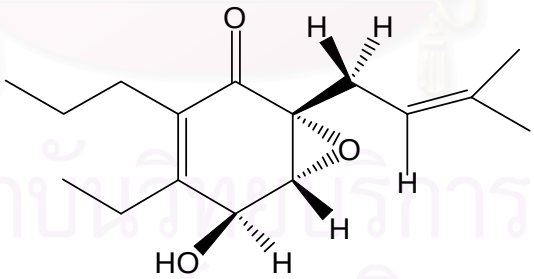


Figure 2.7 Jesterone, a cyclohexenone epoxide from *Pestaliotiopsis jesteri* with antioomycete activity.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp. (Figure 2.8), represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 µg/disk) against *Bacillus subtilis,*

Salmonella gallinarum, and *Staphylococcus aureus.* It also displays a moderate activity against the yeast *Candida tropicalis* (Horn et al., 1995).

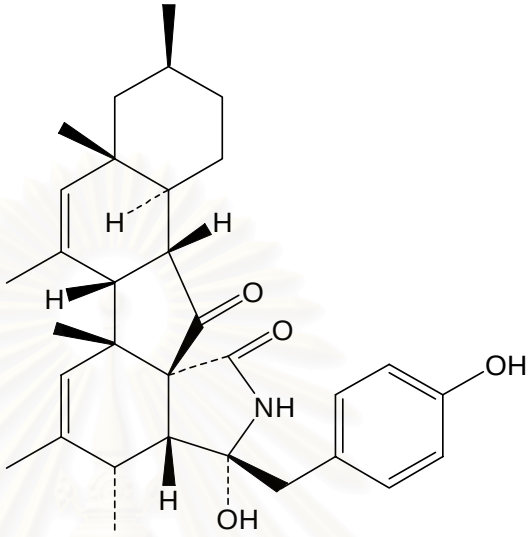


Figure 2.8 Phomopsichalasin, the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring from an endophytic *Phomopsis* sp. with antibacterial activity.

An endophytic *Fusarium* sp. from the plant, *Selaginella pallescens*, collected in the Guanacaste Conservation Area of Costa Rica, was screened for antifungal activity. A new pentaketide antifungal agent, CR377 (Figure 2.9), was isolated from the culture broth of the fungus and showed potent activity against *Candida albicans* in agar diffusion assays (Brady and Clardy, 2000).

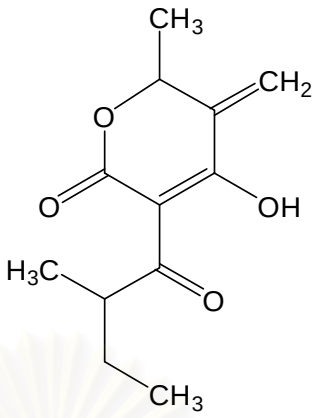


Figure 2.9 CR377, a new pentaketide antifungal agent from endophytic *Fusarium* sp.

Colletotric acid (Figure 2.10), a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays antibacterial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou et al., 2000).

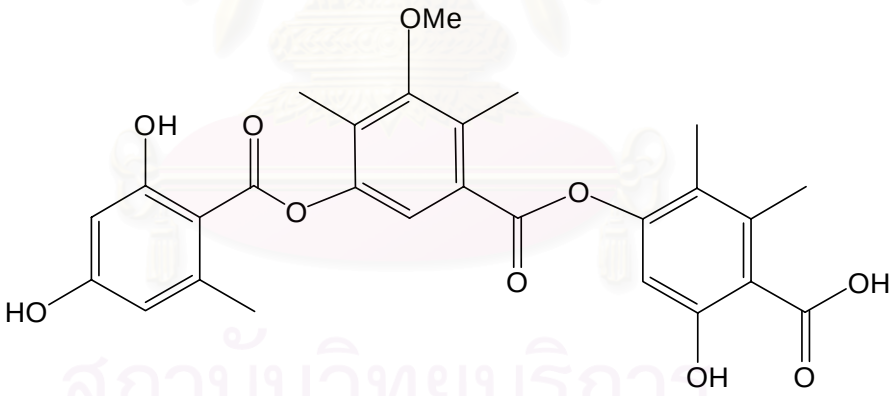


Figure 2.10 Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays antibacterial activity.

Colletotrichum sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed antimicrobial activity as well. *A. annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. The *Colletotrichum* sp. found in *A.*

annua produced a new indole derivative 6-isoprenylindole-3-acetic acid (Figure2.11), not only metabolite with activity against human pathogenic fungi and bacteria but also metabolite that was fungistatic to plant pathogenic fungi (Lu et al., 2000).

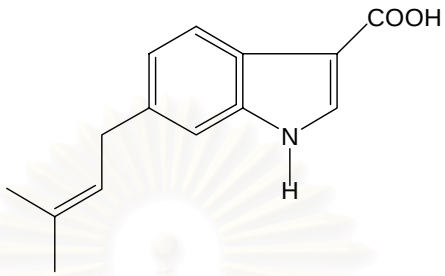


Figure 2.11 6-isoprenylindole-3-acetic acid, a indole derivative from *Colletotrichum* sp., an endophytic fungus isolated from *Artemisia annua* with activity against human pathogenic fungi, bacteria and was fungistatic to plant pathogenic fungi

2.5.1.2 Antiviral compounds

Another fascinating use of products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B (Figure 2.12), have been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures were elucidated as *p*-tridepsides isomers by MS and NMR methods (Guo et al, 2000). It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The fact, however, that some compounds have been found already is promising. The main limitation to compound discovery to date is probably related to the absence of common antiviral screening systems in most compound discovery programs.

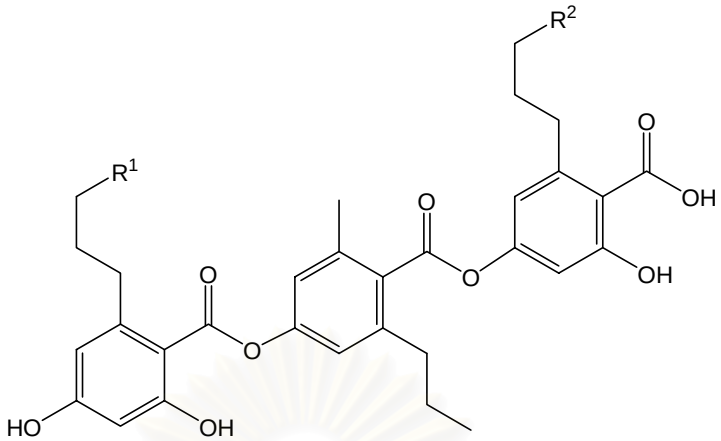


Figure 2.12 Cytonic acids A and B, two novel human cytomegalovirus (hCMV) protease inhibitors from the endophytic fungus *Cytonaema* sp.

2.5.1.3 Volatile antibiotics from endophytic fungi

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) (Woropong et al., 2001). This xylariaceaous (non-spore producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds. The majority of these compounds have been identified by GC/MS, synthesized or acquired, and then ultimately formulated into an artificial mixture. This mixture was not only mimicked the antibiotic effects of the volatile compounds produced by the fungus but also was used to confirm the identity of the majority of the volatiles emitted by this organism (Strobel et al., 2001). Each of the five classes of volatile compounds produced by the fungus had some microbial effects against the test fungi and bacteria, but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active. The composition of the medium on which *M. albus* grows dramatically influences the kind of volatile compounds that are produced (Ezra and Strobel, 2003). The ecological implications and potential practical benefits of the “mycofumigation” effects of *M. albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon

be illegal in the United States. The potential use of mycofumigation to treat soil, seeds, and plants may soon be a reality. The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits, and other plant parts in storage and while being transported.

Using *M. albus* as a screening tool, it has now been possible to isolate other endophytic fungi producing volatile antibiotics. The newly described *M. roseus* was twice obtained from tree species growing in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus* (Woropong et al., 2002). In addition, for the first time, a non-muscodor species (*Gliocladium* sp.) was discovered as a volatile antibiotic producer. The volatile components of this organism are totally different than those of either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8]-annulene, formerly used as a rocket fuel and discovered for the first time as a natural product. However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or comprehensive as that of the *Muscodor* spp. (Stinson, Ezra, and Strobel, 2003).

2.5.2 Products of endophytic fungi with anticancer activities

Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes (Figure 2.13). Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia* (Wani et al., 1971; Suffness, 1995).

The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well. The presence of taxol in yew species prompted the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel taxol-producing endophytic fungus, *Taxomyces andreanae*, was discovered in *Taxus brevifolia* (Strobel et al., 1993). The most critical line of evidence for the presence of taxol in the culture fluids of this fungus was the electrospray mass spectrum of the putative taxol isolated from *T. andreanae.* In electrospray mass spectroscopy, taxol usually gives two peaks,

one at *m*/*z* 854 which is $[M + H]^+$ and the other at *m*/*z* 876 which is $[M + Na]^+$. Fungal taxol had a mass spectrum identical to that of authentic taxol. Then, ^{14}C labeling studies showed the presence of fungal-derived taxol in the culture medium (Stierle, Strobel, and Stierle, 1993). This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and many other plants to yield endophytes producing taxol.

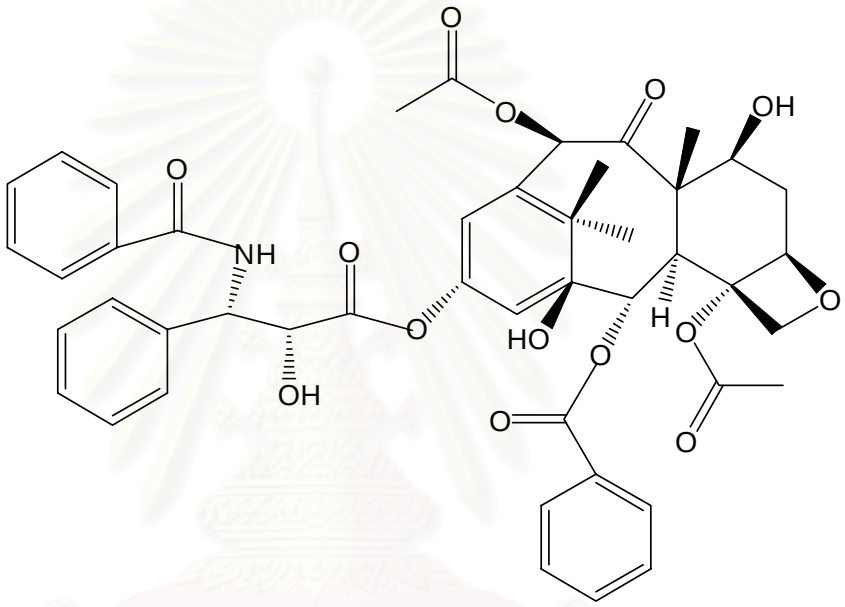


Figure 2.13 Taxol, the world's first billion-dollar anticancer drug produced by many endophytic fungi. It, too, possesses outstanding antioomycete activity.

Some of the most commonly found endophytes of the world's yews and many other plants are *Pestalotiopsis* spp. (Li et al., 1996, Strobel et al., 1996, Strobel, 2002a, Strobel et al., 2002b). One of the most frequently isolated endophytic species is *Pestalotiopsis microspora* (Strobel, 2002a). An examination of the endophytes of *Taxus wallichiana* yielded *P. microspora*, and a preliminary monoclonal antibody test indicated that it might produce taxol. .After preparative TLC, a compound was isolated and shown by spectroscopic techniques to be taxol. Labeled (^{14}C) taxol was produced by this organism from several ^{14}C precursors that had been administered to it (Strobel et al., 1996). Furthermore, other *P. microspora* isolates were obtained from a bald cypress tree

in South Carolina and also were shown to produce taxol (Li et al., 1996). This was the first indication that endophytes residing in plants other than *Taxus* spp. were producing taxol. Therefore, a specific search was conducted for taxol-producing endophytes on continents not being known for any indigenous *Taxus* spp. This included investigating the prospects that taxolproducing endophytes exist in South America and Australia. From the extremely rare and previously thought to be extinct Wollemi Pine (*Wollemia nobilis*), *Pestalotiopsis guepini* was isolated, which was shown to produce taxol (Strobel et al., 1997) . Also, quite surprisingly, a rubiaceous plant, *Maguireothamnus speciosus*, yielded a novel fungus, *Seimatoantlerium tepuiense*, that produces taxol. This endemic plant grows on the top of the tepuis in the Venzuelan-Guyana border in southwest Venezuela (Strobel et al., 1999a). Furthermore, fungal taxol production has also been noted in *Periconia* sp. (Li et al., 1998) and *Seimatoantlerium nepalense*, another novel endophytic fungal species (Bashyal et al., 1999). Simply, it appears that the distribution of those fungi making taxol is worldwide and is not confined to endophytes of yews. The ecological and physiological explanation for the wide distribution of fungi making taxol seems to be related to the fact that taxol is a fungicide, and the most sensitive organisms to it are plant pathogens such as *Pythium* spp. and *Phytophthora* spp. (Young et al., 1992). These pythiaceous organisms are some of the world's most important plant pathogens and are strong competitors with endophytic fungi for niches within plants. In fact, their sensitivity to taxol is based on their interaction with tubulin in a manner identical to that in rapidly dividing human cancer cells. (Young et al., 1992). Thus, bona fide endophytes may be producing taxol and related taxanes to protect their respective host plant from degradation and disease caused by these pathogens.

Other investigators have also made observations on taxol production by endophytes, including the discovery of taxol production by *Tubercularia* sp. isolated from the Chinese yew (*Taxus mairei*) in the Fujian province of southeastern mainland China (Wang et al., 2000). At least three endophytes of *Taxus wallichiana* produce taxol including *Sporormia minima* and *Trichothecium* sp. (Shrestha et al., 2001). Using HPLC and ESIMS, taxol has been discovered in *Corylus avellana* cv. Gasaway (Hoffman et al., 1998). Several fungal endophytes of this plant (filbert) produce taxol in culture (Hoffman

et al., 1998). It is important to note, however, that taxol production by all endophytes in culture is in the range of sub-micrograms to micrograms per liter. Also, commonly, the fungi will attenuate taxol production in culture, with some possibility for recovery, if certain activator compounds are added to the medium (Li et al., 1998). Efforts are being made to determine the feasibility of making microbial taxol a commercial possibility. The greatest prospect of making microbial taxol a commercial reality may be the discovery of endophytes that make large quantities of one or more taxanes that could then be used as platforms for the organic synthesis of taxol or one of its anticancer relatives.

Torreyanic acid, a selectively cytotoxic quinone dimmer and potential anticancer agent, was isolated from a *P. microspora* strain (Figure 2.14). This strain was originally obtained as an endophyte associated with the endangered tree *Torreya taxifolia* (Florida torreya) as mentioned above (Lee et al., 1996). Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5-10 times more potent cytotoxicity in those lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis. Recently, torreyanic acid has been successfully synthesized by application of a biomimetic oxidation/dimerization cascade (Li, Johnson, and Porco, 2003).

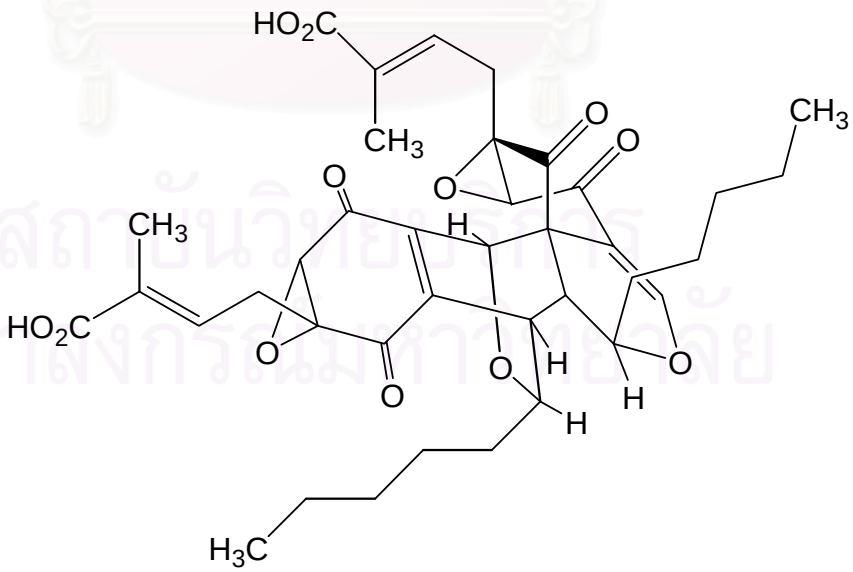
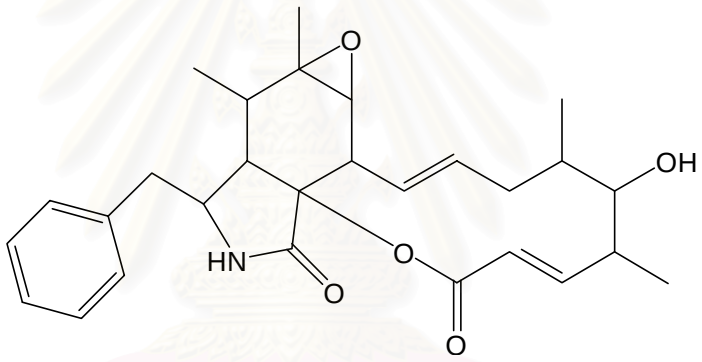
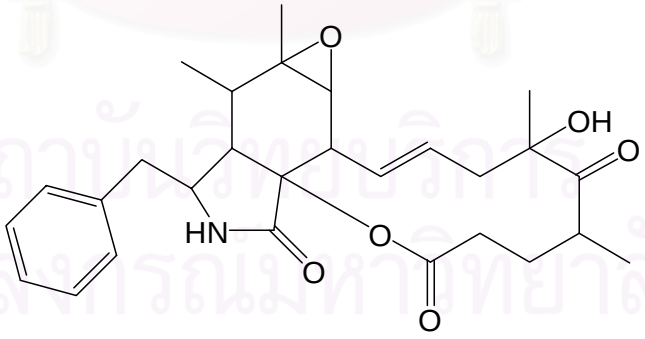


Figure 2.14 Torreyanic acid, an anticancer compound, from *Pestalotiopsis microspora.*

Alkaloids are also commonly found in endophytic fungi. Such fungal genera as xylaria, phoma, hypoxylon, and chalara are representative producers of a relatively large group of substances known as the cytochalasins (Figure 2.15), of which over 20 are now known (Wagenaar et al., 2000). Many of these compounds possess antitumor and antibiotic activities, but because of their cellular toxicity, they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from *Rhinocladiella* sp. as an endophyte on *Tripterygium wilfordii.* These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins (Wagenaar et al., 2000). Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi, and this provides an example of the fact that redundancy in discovery does occur, making dereplication an issue even for these under-investigated sources.

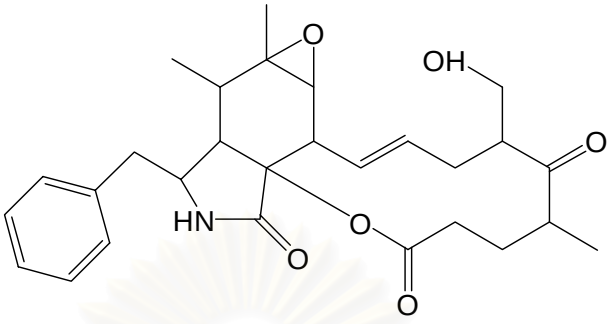


Cytochalasin 1

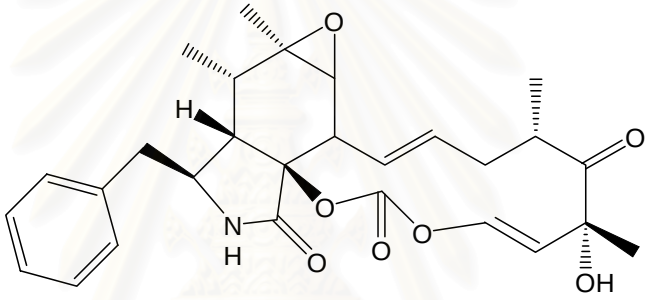


Cytochalasin 2

Figure 2.15 Cytochalasin 1,2, 3, and E, these compounds were isolated from a culture of endophytic fungus *Rhinocladiella sp.*, possess antitumor and antibiotic activities, but because of their cellular toxicity, they have not been developed into pharmaceuticals.



Cytochalasin 3



Cytochalasin E

Figure 2.15 (continued)

2.5.3 Products of endophytic fungi with antioxidants activities

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage system of Papua New Guinea (Strobel et al., 2002; Harper et al., 2003). Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity based on its structural similarity to the flavonoids (Figure 2.16). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution (Strobel et al., 2002). Pestacin (Figure 2.17) was later described from the same culture fluid,

occurring naturally as a racemic mixture and also possessing potent antioxidant activity (Harper et al., 2003). The proposed antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C-H bond and, to a lesser extent, through O-H abstraction. The antioxidant activity of pestacin is at least 1 order of magnitude more potent than that of trolox, a vitamin E derivative (Harper et al., 2003).

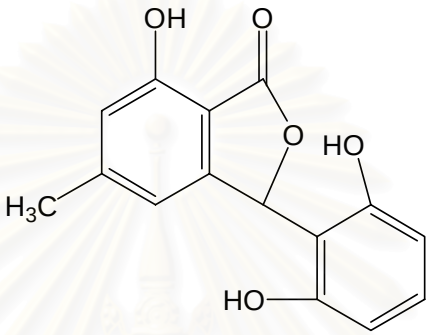


Figure 2.16 Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain, isolated from *Terminalia morobensis* growing on the north coast of Papua New Guinea.

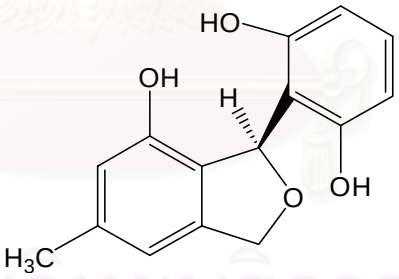
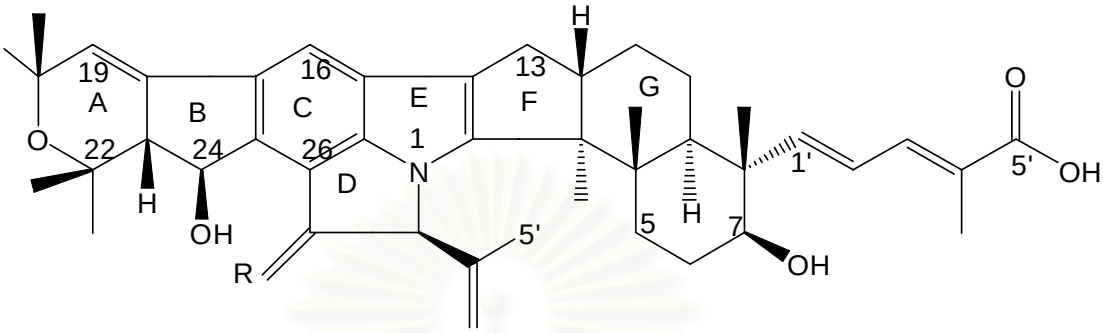


Figure 2.17 Pestacin, an antioxidant produced by *Pestalotiopsis microspora.*

2.5.4 Products of endophytic fungi with insecticidal activities

Bioinsecticides are only a small part of the insecticide field, but their market is increasing (Demain, 2000). Several endophytes are known to have anti-insect properties. Nodulisporic acids (Figure 2.18), novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, work by activating insect glutamate-gated chloride channels. The first nodulisporic acids were isolated from an endophyte, a

Nodulisporium sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for additional *Nodulisporium* spp. or other producers of more potent nodulisporic acid analogues (Bills et al., 2002).

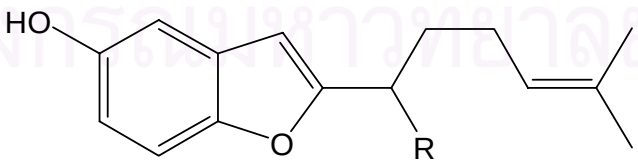


R=O, Nodulisporic acid A

R=H_2, Nodulisporic acid B

Figure 2.18 Nodulisporic acid A and B, novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly. The first nodulisporic acids were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides*.

Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). The two new compounds, 5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran (Figure 2.19), both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm (Findlay et al., 1997).



1 R=O
2 R=OH

Figure 2.19 Two new compounds, (1) 5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and (2) 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran,

isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*), both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm

Another endophytic fungus, *Muscodor vitigenus*, isolated from a liana (*Paullina paullinioides*) yields naphthalene as its major product. Naphthalene, the active ingredient in common mothballs, is a widely exploited insect repellant. *M. vitigenus* shows promising preliminary results as an insect deterrent and has exhibited potent insect repellency against the wheat stem sawfly (*Cephus cinctus*) (Daisy et al., 2002a; Daisy et al., 2002b). As the world becomes wary of ecological damage done by synthetic insecticides, endophytic research continues for the discovery of powerful, selective, and safe alternatives.

2.5.5 Products of endophytic fungi with antidiabetic activities

A nonpeptidal fungal metabolite (L-783,281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo (Zhang et al., 1999). This compound acts as an insulin mimetic but, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783,281 in two mouse models of diabetes resulted in significant lowering in blood glucose levels. These results may lead to new therapies for diabetes (Bensky and Gamble, 1993).

2.5.6 Products of endophytic fungi with immunosuppressive activities

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *Taxus wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinols A and B (Figure 2.20) (Lee et al., 1995a). Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) and thymocyte proliferation (TP) assays with an IC_{50} of 0.1 μM. In the same assay systems, the famed immunosuppressant drug cyclosporine A, also a fungal metabolite,

was roughly as potent in the MLR assay and 10^4 more potent in the TP assay. Still, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants (Lee et al., 1995a).

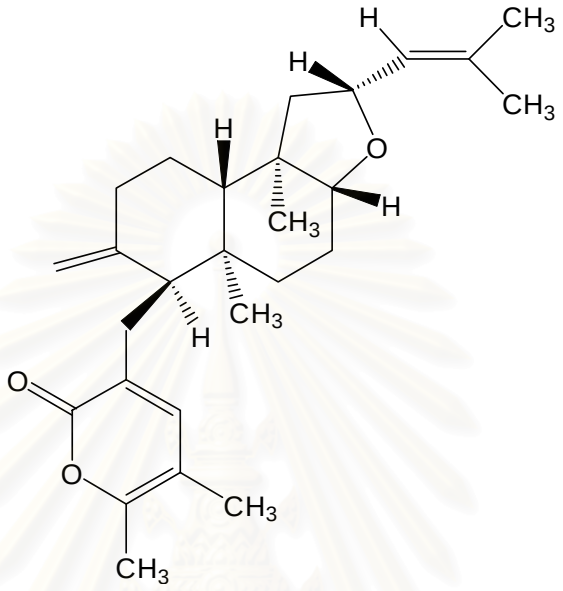


Figure 2.20 Subglutinol A, an immunosuppressant produced by an endophytic *Fusarium subglutinans* strain.

2.6 Plant sample

Croton (or "Plao") (ลัดดาวัลย์ บุญรัตนกรกิจ, 2535) belongs to the family Euphorbiaceae, which have many species widely distributed in Thailand. Many species are commonly used in folk medicine. For example, Plao Nam Ngoen (*C. cascarilloides* Raeusch.) can be used as an antifebrile, Plao Lueat (*C. robutus* Kurz.) can be used as an antianemic agent, and Plao Noi (*C. sublyratus* Kurz.) can be used as antiulceric agent (โรงเรียนแพทย์แผนโบราณ วัดพระเชตุพน, สมาคม. 2521).

2.6.1 Botanical aspects of *Croton oblongifolius*

Croton oblongifolius is a medium sized deciduous tree in the Euphorbiaceae family. There are about 700 species in this family. In Thailand, it is commonly called Plao Yai (central) or Plao Luang (Northern). It is distributed throughout forests or shrubland below 700 meters above sea level. Its calyx and ovary are clothed with minute orbicular silvery scales. Leaves are 5.6-12.0 by 13.0-24.0 cm in size. The shape of the leaf blade is oblong-lanceolate. Its flowers are pale yellowish green and solitary in the axials of minute bracts on long erect racemes. The male flowers are located in the upper part of the raceme and the females in the lower part. The male flowers are slender and have pedicels of 4.0 mm in length. The calyx is more than 6.0 mm long and segments are woolly. The twelve stamens are inflexed in bud and the length of the filaments is 3.0 mm. In female flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely ciliated margins. The diameter of the fruit is less than 1.3 cm, slightly 3-lobed and clothed with small orbicular scales and quite smooth on the back (เต็ม สมิติ นันท์, 2523; ลีนา ผู้พัฒนพงศ์, 2530). The leaf, flower, and stem bark of *Croton oblongifolius* are shown in Figure 2.21.

Figure 2.21 Leaf (A), flower (B) and stem bark (C) of *Croton oblongifolius*

The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi were summarized in Table 2.1 and Figure 2.22.

Table 2.1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
1	Taxol	*Taxomyces andreanae*	*Taxus brevifolia*	Anticancer	Strobel *et al*., 2003, Stierle and Strobel,1995, Stierle *et al*., 1993, Strobel and Stierle, 1993
		Stegolerium kukenani	*Stegolepis guianensis*	Anticancer	Strobel *et al*., 2001
		Aspergillus niger	*Taxus chinensis*	Anticancer	Wang *et al*., 2001
		Tubercularia sp.	*Taxus mairei*	Anticancer	Strobel *et al*., 2003, Wang *et al*., 2000
		Pestalotiopsis microspora	*Taxus wallachina*	Anticancer	Strobel *et al*., 2003, Metz *et al*., 2000, Li *et al*., 1998, Strobel *et al*., 1996
			Taxodium distichum	Anticancer	Li *et al*.,*1996*
		Periconia sp.	*Torreya grandifolia*	Anticancer	Li *et al*., 1998
		Pestalotiopsis guepinii	*Wollemia nobilis*	Anticancer	Strobel *et al*., 1997

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
2	1,3,5,7 cyclooctatetraene or [8]annulene	*Gliocladium* sp.	*Eucryphia cordifolia*	Antimicrobial	Stinson *et al*., 2003
3 4	Lactones 1893 A Lactones 1893 B	Endophytic fungus No. 1893	*Kandelia candel*	Cytotoxic	Chen *et al*., 2003
5	Pestacin	*Pestalotiopsis microspora*	Rainforest	Antioxidant and antimycotic	Harper *et al*., 2003
6 7 8	7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one 7-Butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one 7-Butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one	*Geotrichum* sp.	*Crassocephalum crepidioides*	Antimalarial, antituberculous and antifungal	Kongsaeree *et al*., 2003

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
9	Brefeldin A	*Paecilomyces* sp. and *Aspergillus clavatus*	*Taxus mairei and Torreya grandis*	Cytotoxic	Wang *et al*., 2002
10	Isopestacin	*Pestalotiopsis microspora*	*Terminalia morobensis*	Antifungal and antioxidant	Strobel *et al*., 2002
11 12 13	Preaustinoid A Preaustinoid B Alkaloid verruculogen	*Penicillium* sp.	*Melia azedarach*	Bacteriostatic	Santos and Rodrigues-Fo, 2002
14	Ambuic acid	*Pestalotiopsis* spp., *Monochaetia* sp.	Rainforests	Antifungal	Li *et al*., 2001
15 16	Jesterone hydrosy-jesterone	*Pestalotiopsis jesteri*	*Fragraea bodenii*	Antioomycete	Li *et al*., 2001
17 18 19	Preussomerin G Preussomerin H Preussomerin I	Mycelia sterila	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn *et al*., 2001

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
20 21 22	Preussomerin J Preussomerin K Preussomerin L	Mycelia sterila	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn *et al*., 2001
23 24 25	Dicerandrols A Dicerandrols B Dicerandrols C	*Phomopsis longicolla*	*Dicerandra frutescens*	Antibiotic and cytotoxic	Wagenaar and Clardy, 2001
26	Microcarpalide	Unidentified endophytic fungus	*Ficus microcarpa*	Microfilament disrupting agent	Ratnayake *et al*., 2001
27	Nomofungin	Unidentified endophytic fungus	*Ficus microcarpa* L.	Microfilament disruptin agent and cytotoxic	Ratnayake *et al*., 2001
28	Isoprenylindole-3-carboxylic acid	*Collectotrichum* sp.	*Artemisia annua*	Antibactirial and antifungal	Lu *et al*., 2000

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
29	3beta,5alpha-Dihydroxy-6beta-acetoxy-ergosta-7,22-diene	*Collectotrichum* sp.	*Artemisia annua*	Antibactirial and antifungal	Lu *et al*., 2000
30	3beta,5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7,22-diene				
31	Indole-3-acetic acid (IAA)	*Epichloe/Neotyphodium* spp.	Grasses	Antifungal	Yue *et al*., 2000
32	Indole-3-ethanol (IEtOH)				
33	Methylindole-3-carboxylate				
34	Indole-3-carboxaldehyde				
35	Diacetamide				
36	Cyclonerodiol				
37	Colletotric acid	*Colletotrichum gloeosporioides*	*Artemisia mongolica*	Antimicrobial	Zou *et al*., 2000

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
38	CR377, pentaketide	*Fusarium* sp.	*Selaginella pallescens*	Antifungal	Brady and Clardy, 2000
39 40 41 42	Cytochalasin 1 Cytochalasin 2 Cytochalasin 3 Cytochalasin E	*Rhinocladiella* sp.	*Tripterygium wilfordii*	Cytotoxic	Wagenaar *et al*., 2000
43	Cryptocandin	*Cryptosporiopsis* cf. *quercina*	*Tripterigeum wilfordii*	Antimycotic	Strobel *et al*., 1999
44 45	Geniculol Cytochalasin F	*Geniculosporium* sp.	*Teucrium scorodania*	Antialgal	Konig *et al*., 1999
46 47	Sequoiatones A Sequoiatones B	*Aspergillus parasiticus*	*Sequoia sempervirens*	Antitumor	Stierle *et al*., 1999
48	Terpendole M	*Neotyphodium lolii*	*Lolium perenne*	neurotoxins	Gatenby *et al*., 1999
49 50 51	Tricin (1) 7-O-(B-D-glucopyranosyl) tricin Isoorientin (3)	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998

Table 2.1 (continued)

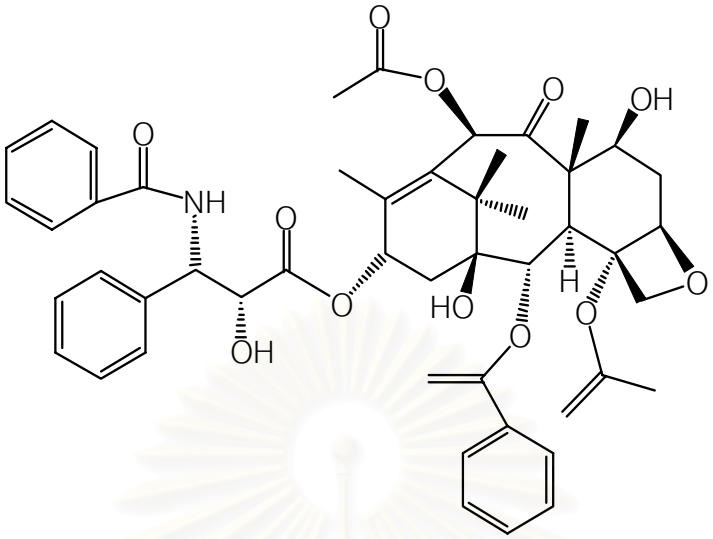
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
52	7-O-[α-L-Rhamnopyranosyl(1-6)-β-D-glucopy-ranosyl]tricin	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998
53	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxic	Berny *et al*., 1997
54	Leucinostatin A	*Acremoium* sp.	*Taxus baccata*	Antifungal and anticacer	Strobel *et al*., 1997
55 56 57 58	Oreganic acid (1) Trimethyester (2) Desulfated analog (3) Desulfated analog (4)	Endophytic fungus (MF 6046)	*Berberis oregana*	Anticancer	Jayasuriya *et al*., 1996
59 60	Pestalotiopsins A Pestalotiopsins B	*Pestalotiopsis* sp.	*Taxus brevifolia*	-	Pulici *et al*., 1996
61 62	(R)-mellein (-)-mycorrhizin A	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
63	2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995
64	(+)-cryptosporiopsin				
65	4-epi-ethiosolide				
66	Altersolanol A	*Phoma* sp.	*Taxus wallachiana*	Antibacterial	Yang *et al*., 1994
67	2-hydroxy-6-methylbenzoic acid				
68	Preussomerin D	*Hormonema dematioides*	Conifer wood	Antifungal	Polishook *et al*., 1993
69	Lolitrem C	*Acremonium lolii*	*Lolium perenne*	Neurotoxic and insect antifeedant	Rowan *et al*., 1993
70	Peramine R=H				
71	Diacetylperamine R=Ac				
72	Paxilline				
73	Loline alkaloid				
74	Ergovaline				

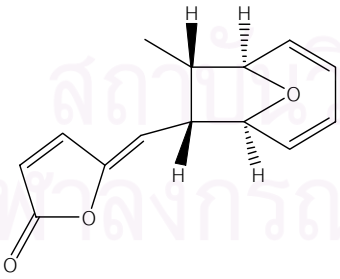
Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
75	Lysergic acid	*Acremonium coenophialun*	*Festuca arundinacea*	Toxin	Garner *et al*., 1993
76	Isolysergic acid				
77	Pospalic acid				
78	Lysergol				
79	Lysergic acid amide				
80	Lysergic acid diethyl-amide				
81	Lycergic acid-2-propanolamide or (Ergonovine)				

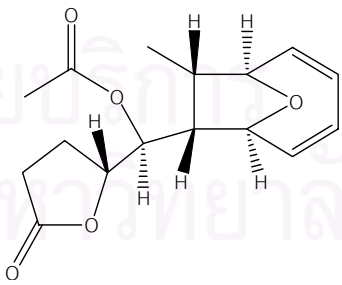


[1] Taxol

[2] 1,3,5,7 cyclooctatetraene or (8)-annulene

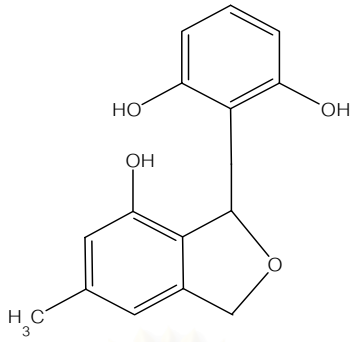


[3] Lactones 1893 A

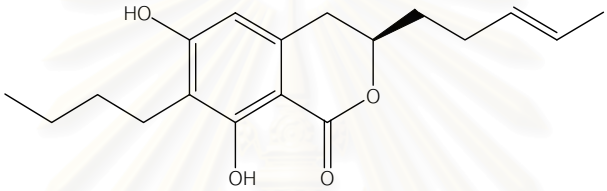


[4] Lactones 1893 B

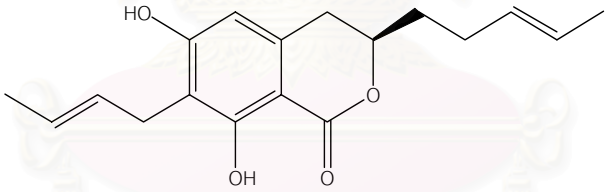
Figure 2.22 Structure of secondary metabolites of endophytic fungi



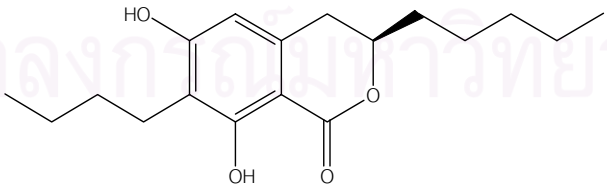
[5] Pestacin



[6] 7-Butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



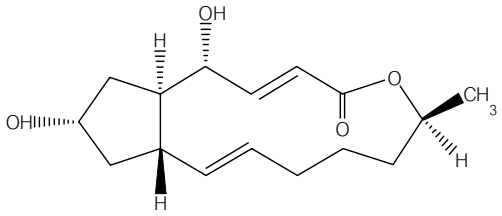
[7] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



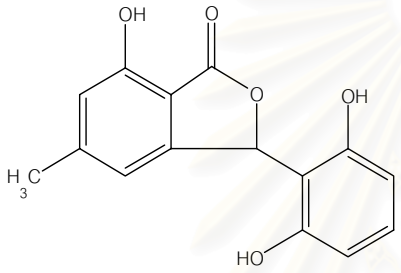
[8] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

Dihydroisocumarins [6-8]

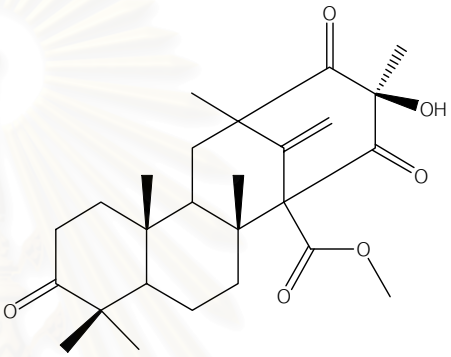
Figure 2.22 (continued)



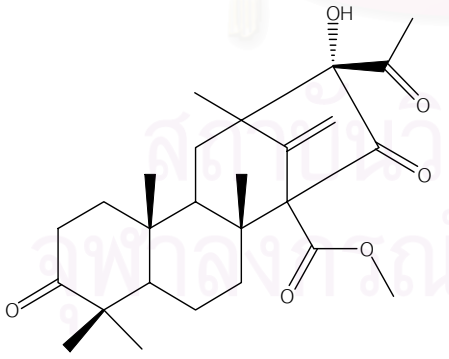
[9] Brefeldin A



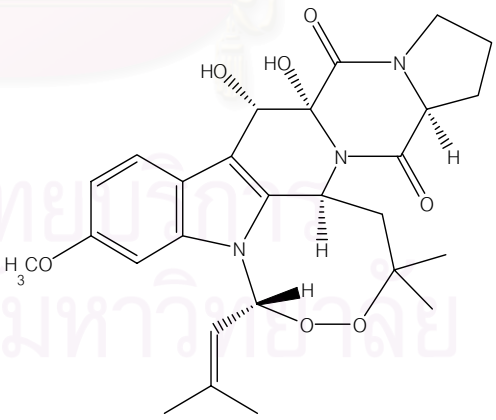
[10] Isopestacin



[11] Preaustinoid A

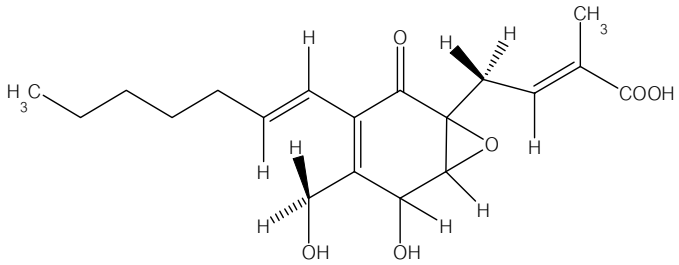


[12] Preaustinoid B

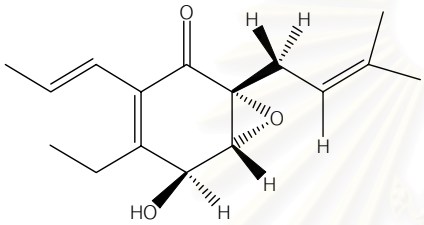


[13] Alkaloid verruculogen

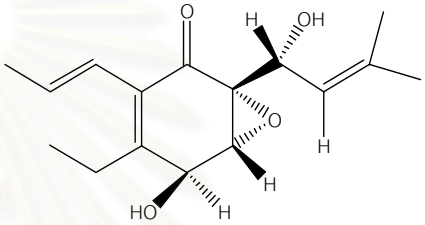
Figure 2.22 (continued)



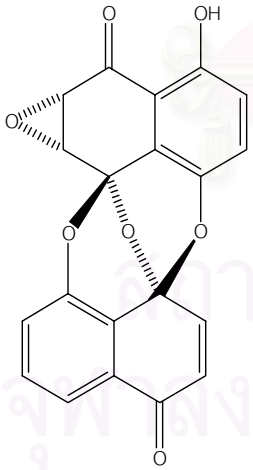
[14] Ambuic acid



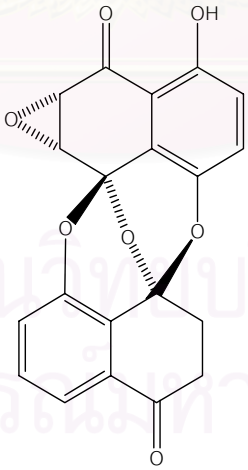
[15] Jesterone



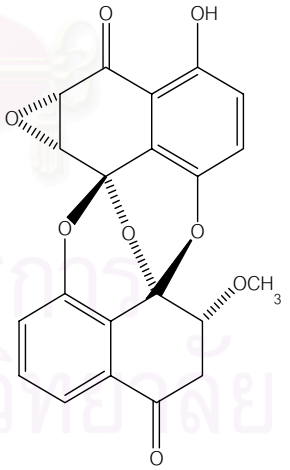
[16] Hydroxy-jesterone



[17] Preussomerin G

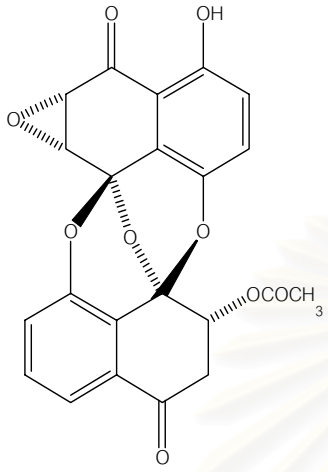


[18] Preussomerin H

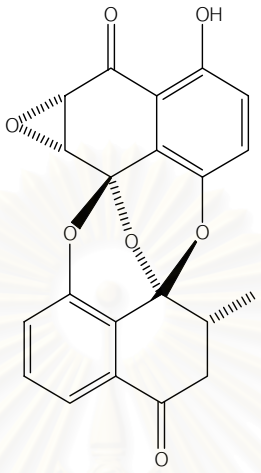


[19] Preussomerin I

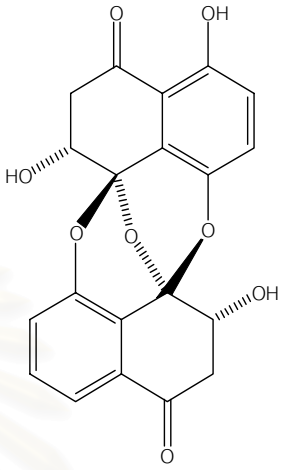
Figure 2.22 (continued)



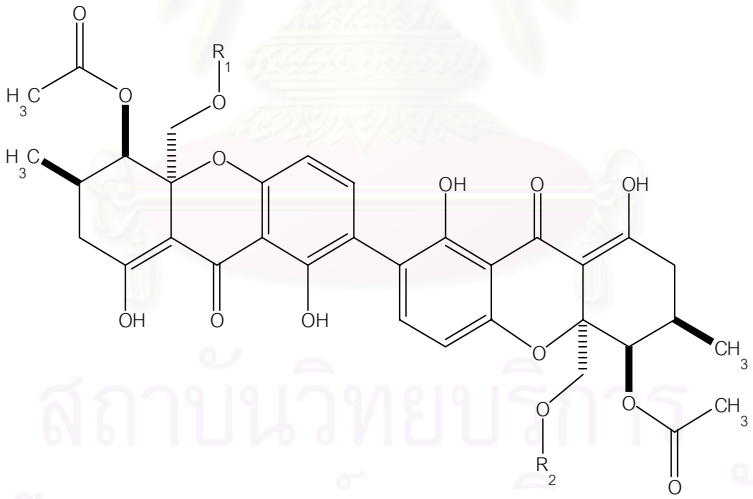
[20] Preussomerin J



[21] Preussomerin K



[22] Preussomerin L

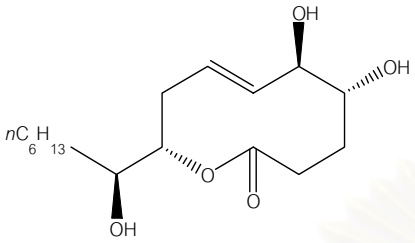


[23] Dicerandrols A, R1=R2=H

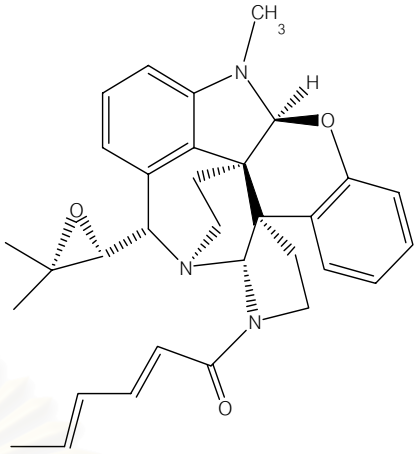
[24] Dicerandrols B, R1=Ac, R2=H

[25] Dicerandrols C, R1=R2=Ac

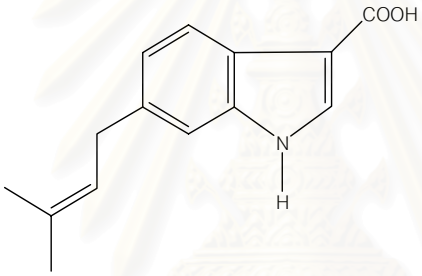
Figure 2.22 (continued)



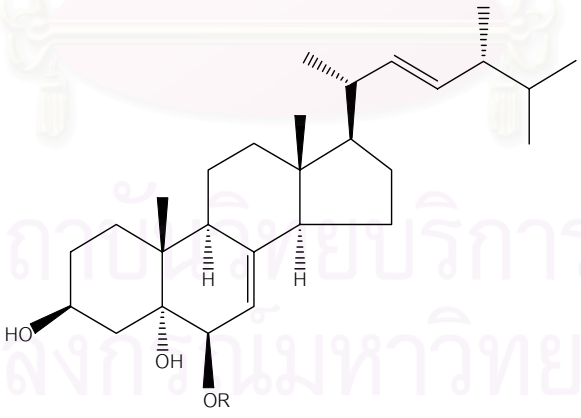
[26] Microcarpalide



[27] Nomofungin



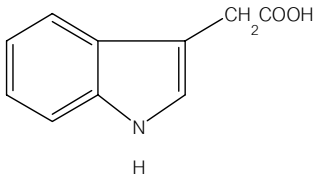
[28] Isoprenylindole-3-carboxylic acid



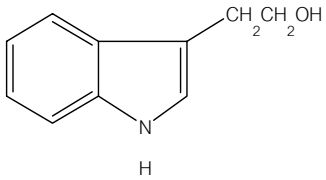
[29] 3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7, 22-diene, R=$COCH_3$

[30] 3beta, 5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7, 22-diene, R=$COCH_2C_6H_5$

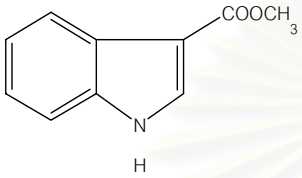
Figure 2.22 (continued)



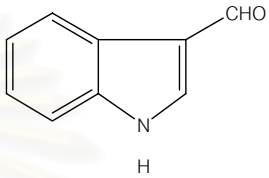
[31] Indole-3-acetic acid (IAA)



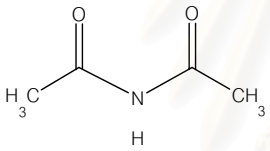
[32] Indole-3-ethanol (IEtOH)



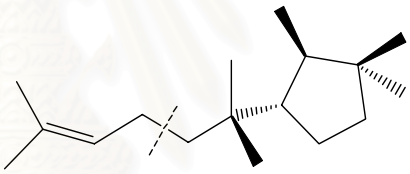
[33] Methylindole-3-carboxylate



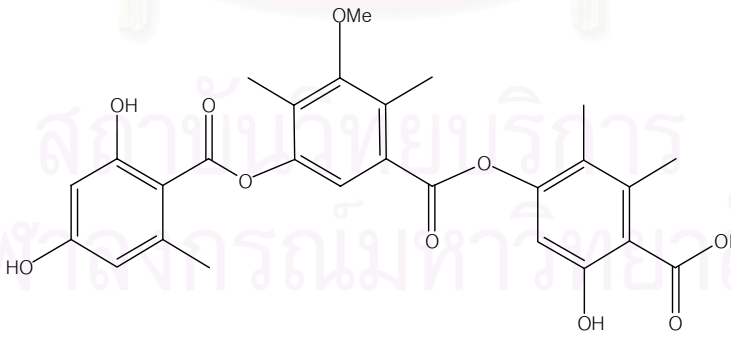
[34] Indole-3-carboxaldehyde



[35] Diacetamide

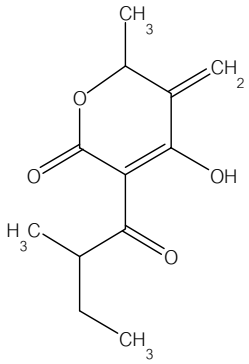


[36] Cyclonerodiol

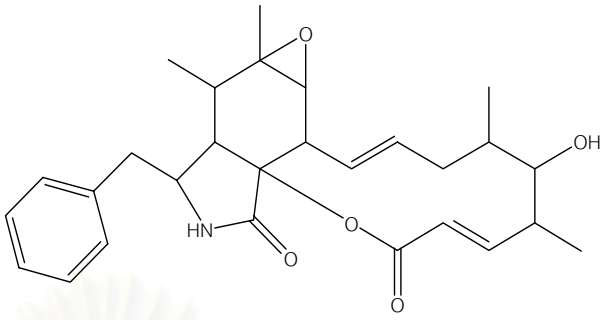


[37] Colletotric acid

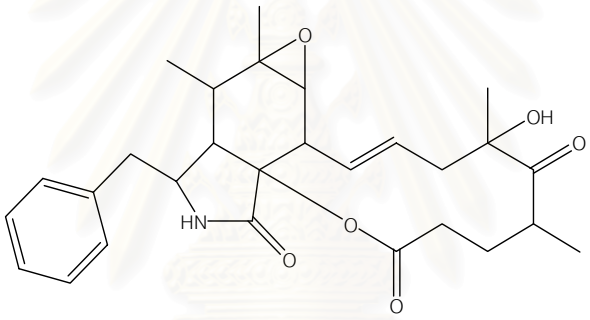
Figure 2.22 (continued)



[38] CR377, pentaketide



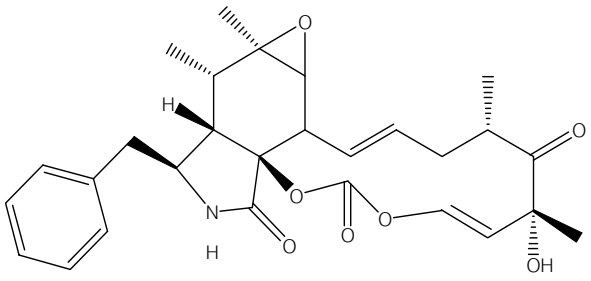
[39] Cytochalasin 1



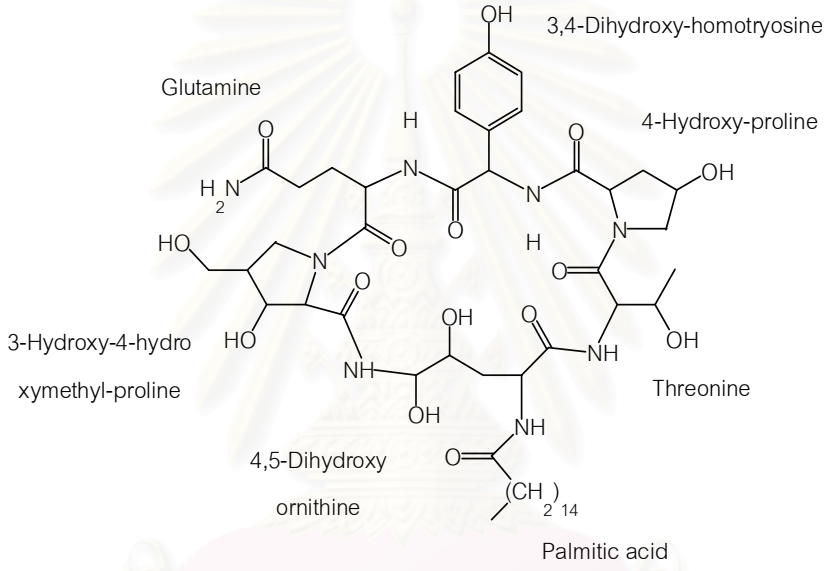
[40] Cytochalasin 2

[41] Cytochalasin 3

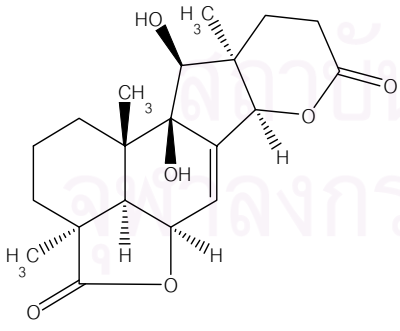
Figure 2.22 (continued)



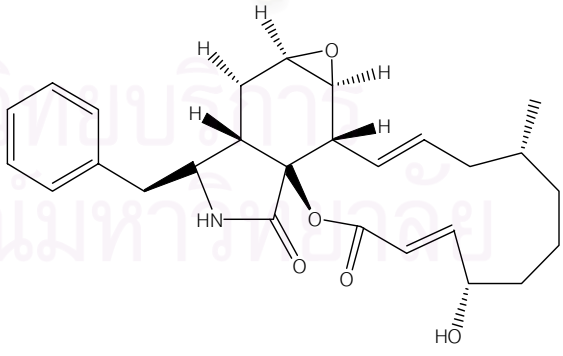
[42] Cytochanlasin E



[43] Cryptocandin

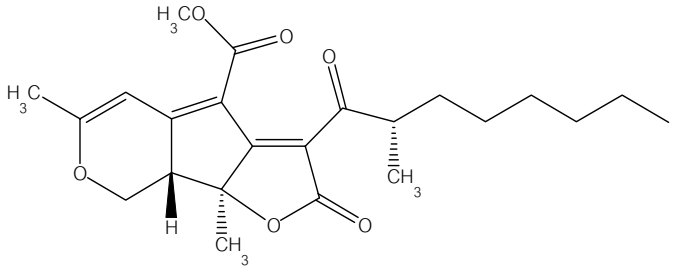


[44] Geniculol

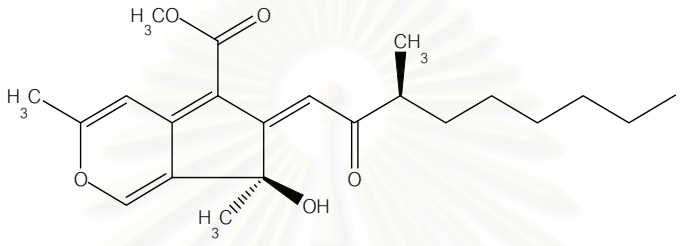


[45] Cytochalasin F

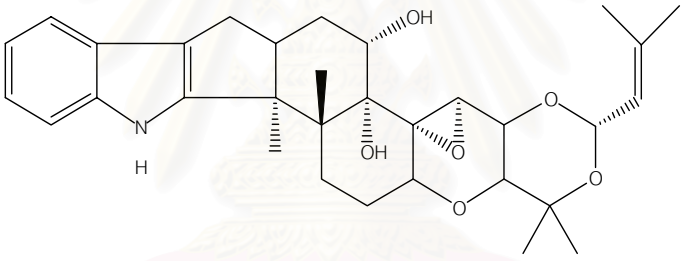
Figure 2.22 (continued)



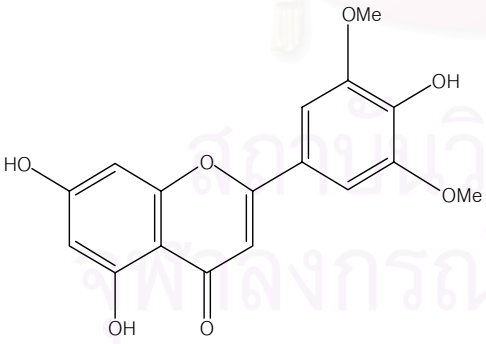
[46] Sequoiatones A



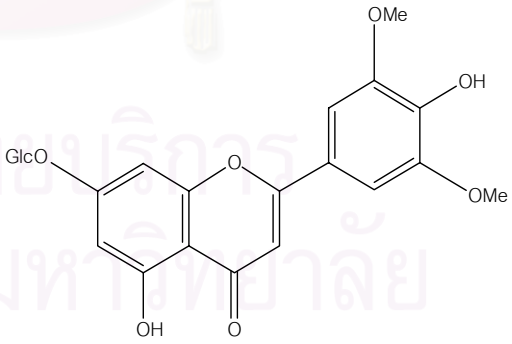
[47] Sequoiatones B



[48] Terpendole M

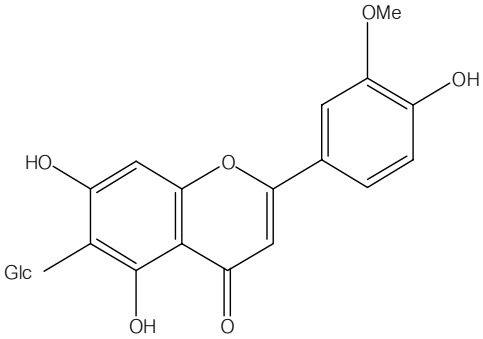


[49] Tricin

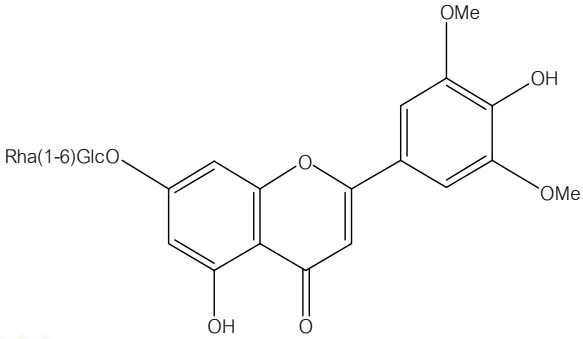


[50] 7-O-(B-D-glucopyranosyl)tricin

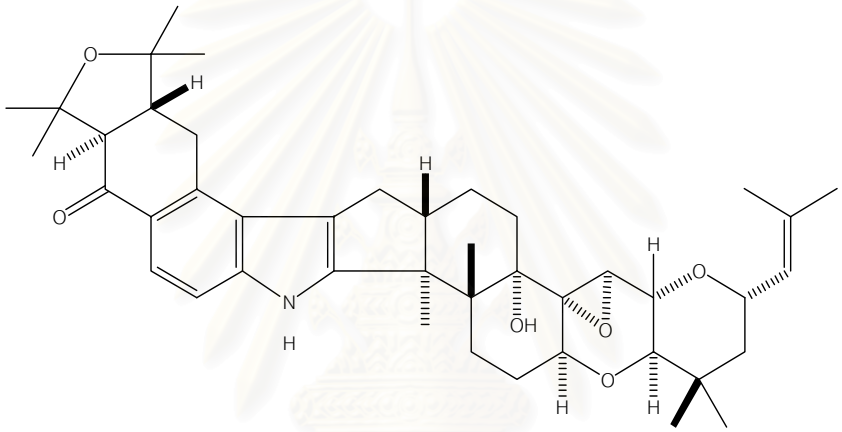
Figure 2.22 (continued)



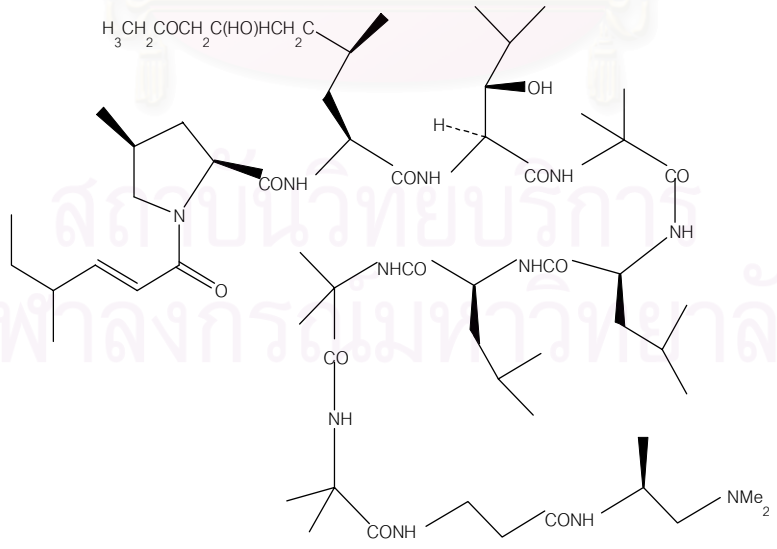
[51] Isoorientin



[52] 7-**o**-[**α**-L-Rhamnopyranosyl(1-6)-**β**-D-glucopy-ranosyl]tricin

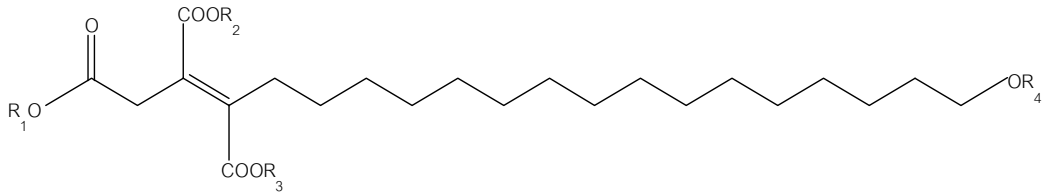


[53] Lolitrem B

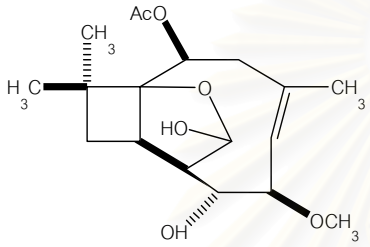


[54] Leucinostatin A

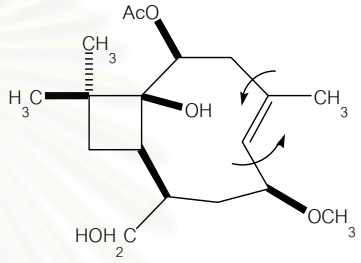
Figure 2.22 (continued)



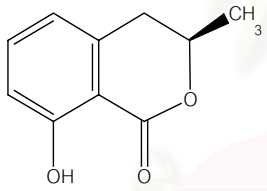
[55] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H
[56] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H
[57] 3: Desulfated analog, R1=R2=R3=CH3, R4=H
[58] 4: Desulfated analog, R1=R2=R3=R4=H



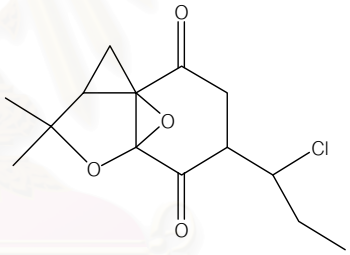
[59] Pestalotiopsins A



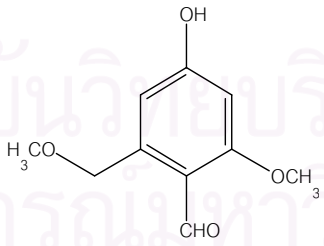
[60] Pestalotiopsins B



[61] (R)-mellein

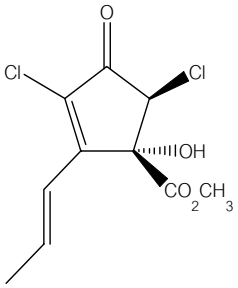


[62] (-)-mycorrhizin A

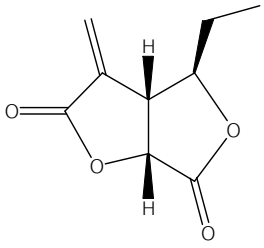


[63] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

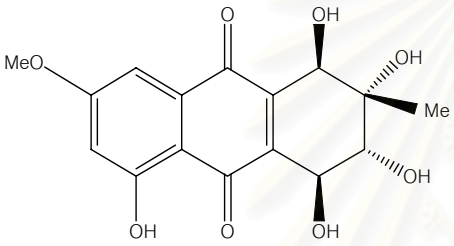
Figure 2.22 (continued)



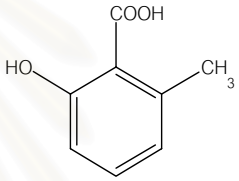
[64] (+)-cryptosporiopsin



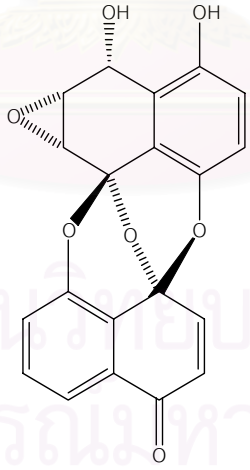
[65] 4-epi-ethiosolide



[66] Altersolanol A

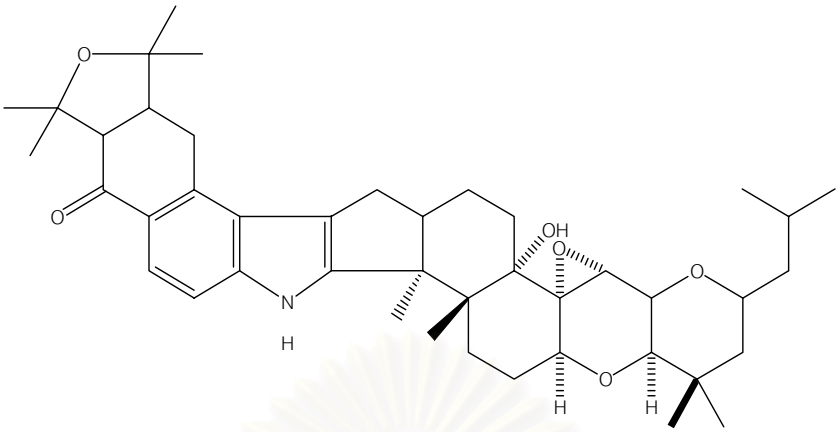


[67] 2-hydroxy-6-methyl benzoic acid

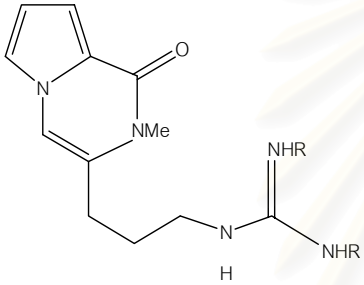


[68] Preussomerin D

Figure 2.22 (continued)

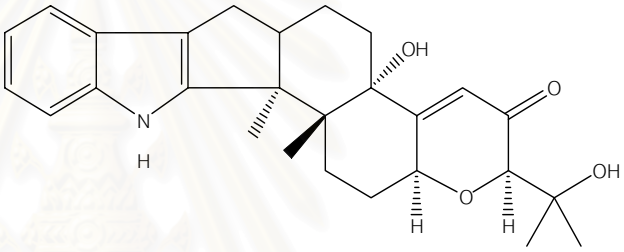


[69] Lolitrem C

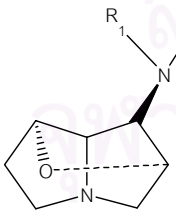


[70] Peramine R=H

[71] Diacetylperamine R=Ac

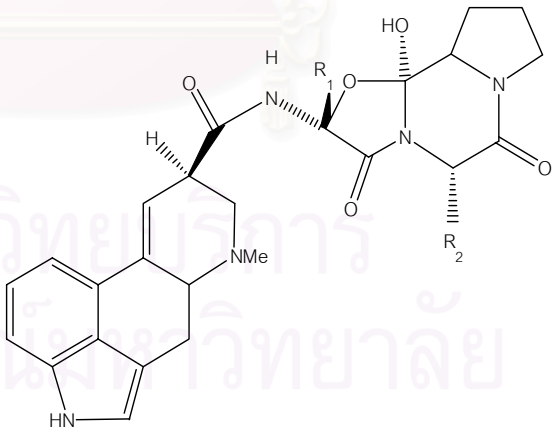


[72] Paxilline



[73] Loline alkaloid, R_1=H, Me,

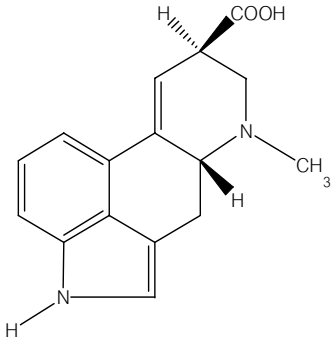
R_2= H, HCO, Ac



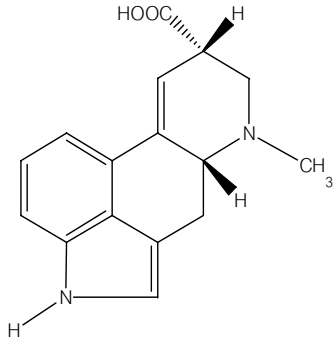
[74] Ergopeptine alkaloids

Ergovaline R_1=Me, R_2=i-Pr

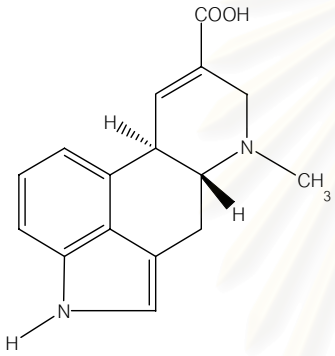
Figure 2.22 (continued)



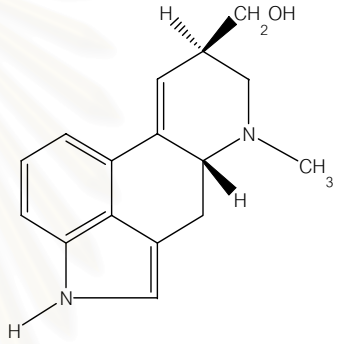
[75] Lysergic acid



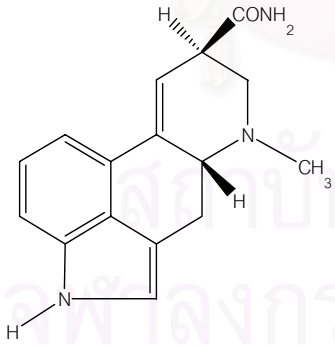
[76] Isolysergic acid



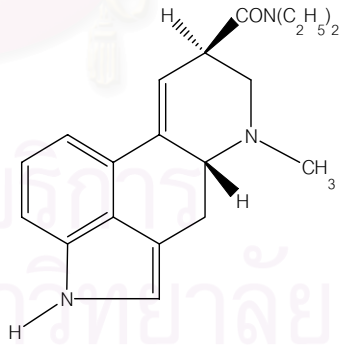
[77] Paspalic acid



[78] Lysergol

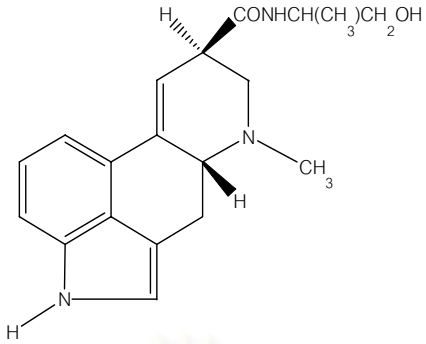


[79] Lysergic acid amide



[80] Lysergic acid diethyl amide

Figure 2.22 (continued)



[81] Lysergic acid-2-propanolamide (Ergonovine)

Figure 2.22 (continued)

CHAPTER III

MATERIALS AND METHODS

3.1 Plant samples collection

Healthy young and mature leaves, branches, and bark samples of Plao-Yai (*Croton oblongifolius*) were collected from sites on the campus of Chulalongkorn University, Bangkok, on October 10, 2003. Plant samples were kept in a plastic bag. Fresh specimens were processed for isolation endophytic fungi with in 24 hours after collection.

3.2 Culture media

Culture medium used for isolation, cultivation for study metabolites of bioactive compounds and morphology observation of endophytic fungi was malt extract medium (agar and broth). Potatoes Dextrose agar (PDA), Malt Extract Agar (MEA), Oat meal Agar (OMA), Yeast Extract Sucrose Agar (YES), and Sabouraud's Dextrose Agar (SDA) were used for observation morphology characteristics of selected endophytic fungal isolate.

Nutrient medium was used for antimicrobial activity test of bacteria and yeast-malt extract medium (agar and broth) was used for yeasts. Sabouraud's dextrose agar (SDA) and V8 agar were used for culturing dermatophytic fungi and plant phatogenic fungi, respectively. The formula of media are shown in Appendix A.

3.3 Isolation and cultivation of endophytic fungi

Endophytic fungi were isolated by using the surface sterilization method. Plant samples were washed in running tap water and dried in laminar air flow.

The leaves were cut into 6 mm of diameter disks in the middle, rib, and lamina. Young leaf sections 100 pieces and mature leaf sections 100 pieces of Plao-Yai *(Croton oblongifolius*) were surface sterilized by using modified technique which described by

Blodgett *et. al*, (2000). The samples were sequential immersed in 95% ethanol for 1 min, in Clorox® (5% available chlorine) for 5 min, in 95% ethanol for 30 sec and then rinsed twice with sterile distilled water.

The branches and barks were prepared for surface sterilization technique which modified from White, Drake and Martin technique (1996). The samples were cut into size of 1x1x0.5 cm. The branch sections 20 pieces and bark sections 20 pieces of Plao-Yai *(Croton oblongifolius*) were immersed in saturated calcium hypochlorite (20% available chlorine) for 5 minutes, and were rinsed twice with sterile distilled water.

The sterile pieces of plant samples were allowed to surface-dry on sterile filter papers in sterile petri dishes and were placed on malt extract agar (MEA). Negative controls were these plant samples which placed on MEA and immediately remove from MEA. All petri dishes were incubated at room temperature (25-30 ^{o}C) and examined periodically for fungal mycelium from plant tissues under a stereomicroscope. The regions of negative controls were examined to sure in the surface sterilization method. Outgrowing mycelia were purified and transferred into new petri dishes containing MEA by hyphal tip transfer. They were incubated for 30 days at room temperature. Fungal isolates were used for further study.

3.4 Identification and classification of endophytic fungi isolates

3.4.1 Morphological identification

A. Macroscopical studies

Characters for colony shape, size, color, margin, pigment, and others were studied under stereomicroscope on a Leica MZ6.

B. Microscopical studies

The microscopic analyses were based on observations by light microscopy on an Olympus CH2 research microscope using a 40x dry objective. Specimens were mounted in lactophenol aniline blue for observations of morphology and characteristics, and then identified.

C. Scanning electron microscope studies

Endophytic fungal isolate CuLm17 cultured on MEA was sent for observation and photography with scanning electron microscope at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

The specimen was prepared by cut into 1x1 cm and fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodelate buffer (pH7.2) for 2 h. Then, the samples were dehydrated under the serine concentration (70-95%) within 15 minutes. The samples were dried under critical point dried and coated with gold under sputter coater model. Changes of each fine immersed in absolute ethanol for 30 minutes for each twice and observed and photographed with a JSM-5410 LV scanning electron microscope.

3.5 Preservation of endophytic fungi

A. Storage under 15% glycerol

Fungal endophyte isolates were grown on MEA agar slants at room temperature (25-30 ^{o}C) for 1-2 weeks depending on the individual fungal growth rate. The mature cultures were then covered up to 10 mm height with sterile 15% glycerol and kept at 4 ^{o}C. The 15% glycerol was steriled by autoclaving at 121 ^{o}C for 15 minutes.

B. Storage in 15% glycerol

Six millimeter diameter of agar culture plugs were cut from the growing edge of fungal colonies grown on MEA. Five to ten pieces of agar plugs were put in a glass vials containing 5 ml sterile 15% glycerol and kept at -20 ^{o}C. The 15% glycerol was steriled by autoclaving twice at 121 ^{o}C for 15 minutes.

3.6 Fungal cultivation and metabolite extraction

3.6.1 Fungal cultivation

Each fungal endophyte isolate was grown on MEA at room temperature (25-30 ^{o}C) for 1-2 weeks depending on the individual fungal growth rate. The agar culture was then cut into 6 mm diameter disks by a flamed cork hole borer. Five pieces of agar

culture were inoculated into 250 ml Erlenmeyer flasks containing 100 ml malt extract broth(MEB). All cultures were incubated for 8 weeks at room temperature (25-30 ^{o}C) under static condition.

3.6.2 Metabolite extraction

The culture broth was filtered twice through a filter paper (Whatman No. 4). The culture broth was evaporated by using a rotary evaporator (Eyela, Type N-N series, Japan) under reduced pressure at 35 ^{o}C. The culture broth was extracted with ethyl acetate (culture broth : EtOAc in ratio 1:1) and kept at 4 ^{o}C for bioassays.

The fungal mycelium was extracted with ethyl acetate (wet mycelium 20 g : EtOAc 100 ml). The flasks of fungal mycelium were extracted on rotary shaker (200 rpm) at room temperature for overnight. The extracted solvent was filtered through filter paper (Whatman No. 4). The filtrate was then evaporated by using a rotary evaporator (Eyela, N-N series, Japan) under reduced pressure at 35 ^{o}C, and then transferred to a glass vial. The mycelium extracts were kept at 4 ^{o}C for bioassays.

3.7 Screening of endophytic fungi for their antimicrobial activities

Screening of endophytic fungi for their antimicrobial activities of culture broth extracts and mycelium extracts against test microorganisms were determined by the modified “agar well diffusion method” as describes in Weaver, Angel and Botlomley (1994) and Joseph, Dave and Shah (1998).

3.7.1 Test microorganisms for antimicrobial activities

The isolated endophytic fungi were investigated for their antimicrobial activities against test microorganisms as shown in Table 3.1.

Table 3.1 Test microorganisms for antimicrobial assays

Characteristic of test microorganisms		Reference strains
Bacteria	Gram positive rod	*Bacillus subtilis* ATCC 6633
	Gram positive cocci	*Staphylococcus aureus* ATCC 25923
	Gram negaitive rod	*Escherichia coli* ATCC 25922
	Gram negaitive rod	*Pseudomonas aeruginosa* ATCC 27853
Fungi	Yeast	*Candida albicans* ATTC 10231

Viable counts of the standardized inoculum of test bacteria and yeast which adjusted turbidity matched to 0.5 McFarland standard (OD_{625} = 0.08-0.1) were performed. The colony forming unit/ml (CFU/ml) values of test microorganisms used for antimicrobial assays in this study are shown in Table 3.2.

Table 3.2 Quantity of standardized inoculum of test microorganisms

Test microorganisms	Quantity (CFU/ml)
Bacillus subtilis ATCC 6633	6.1×10^6
Staphylococcus aureus ATCC 25923	6.9×10^6
Escherichia coli ATCC 25922	2.1×10^7
Pseudomonas aeruginosa ATCC 27853	3.4×10^7
Candida albicans ATTC 10231	1.0×10^6

3.7.2 Test for antimicrobial activities

A. Preparation of samples

Ten milligrams each of culture broth extract and mycelium extracts was dissolved in 1 ml of 10% DMSO in sterile distilled water. All samples were kept at 4 ^oC for bioassay.

B. Preparation of test bacterial inoculum

Bacteria were grown on Nutrient agar (NA) for 24 h at 37 ^{o}C. With a sterile wire loop, the tops of four or five isolated colonies of a similar morphologic type were transferred to a tube containing 4 to 5 ml of Nutrient Broth (NB) and incubated at 37°C for 2-8 h, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of 0.5 McFarland.

C. Preparation of yeast inoculum

Yeast was grown on Yeast Malt Extract agar (YMA) for 24-48 h at room temperature (25-30°C). With a sterile wire loop, the tops of four or five isolated colonies of a similar morphologic type were transferred to a tube containing 4 to 5 ml of Nutrient Broth (NB) and incubated at 37°C for 2-8 h, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of 0.5 McFarland.

D. Inoculation of the test plate

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. The surface of the medium was allowed to dry for 3-5 minutes.

E. Application of culture broth extracts and mycelial extracts

Wells were made in the agar by removing disks cut (6 mm diameter) cut with a flamed cork hole borer. One hundred µl of culture broth extracts , mycelial extracts and negative contol (10% DMSO in sterile distilled water) was pipetted into the agar wells. This was absorbed by the media surrounding the wells. Bacteria and yeast plates were incubated at 37 ^{o}C and room temperature, respectively for 24 h. Inhibition zones around the wells were measured in mm with a ruler.

3.8 Determination of metabolites profile of the extracts from endophytic fungi

0.5 milligrams of the culture broth extracts and mycelium extracts in the section 3.6.2 were dissolved with methanol (1 ml) for determination of metabolites profile of the extracts by using TLC technique that is described in later. Elution systems were 50% Hexane in EtOAc, 100% EtOac, 10% MeOH in EtOAc.

Analytical thin-layer chromatography (TLC)

Technique : one dimension ascending
Adsorbent : silica gel F_{254} coated on aluminium sheet (E. Merck)
Layer thickness : 250 µm
Distance : 5 cm
Temperature : laboratory temperature (25-30 ^{o}C)
Detection : 1. Visual detection under ultraviolet light at wavelengths 254 and 365 nm
2. Visual detection in iodine vapor
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.

3.9 Cultivation and metabolites extraction of endophytic fungal isolate CuLm17

3.9.1 Cultivation of endophytic fungal isolate CuLm17 for producing secondary metabolites

Endophytic fungal isolate CuLm17 was chosen for the study of metabolites and cultivated on MEA at room temperature (25-30 ^{o}C) for 2 weeks. The agar cultures of this fungal isolate were cut into 6 mm diameter disks using a flamed cork borer. Five pieces of agar culture were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract broth (MEB). Several flasks of culture were prepared to obtain 18 l of MEB. The culture flasks were incubated statically at room temperature (25-30°C) for 8 weeks.

3.9.2 Solvent

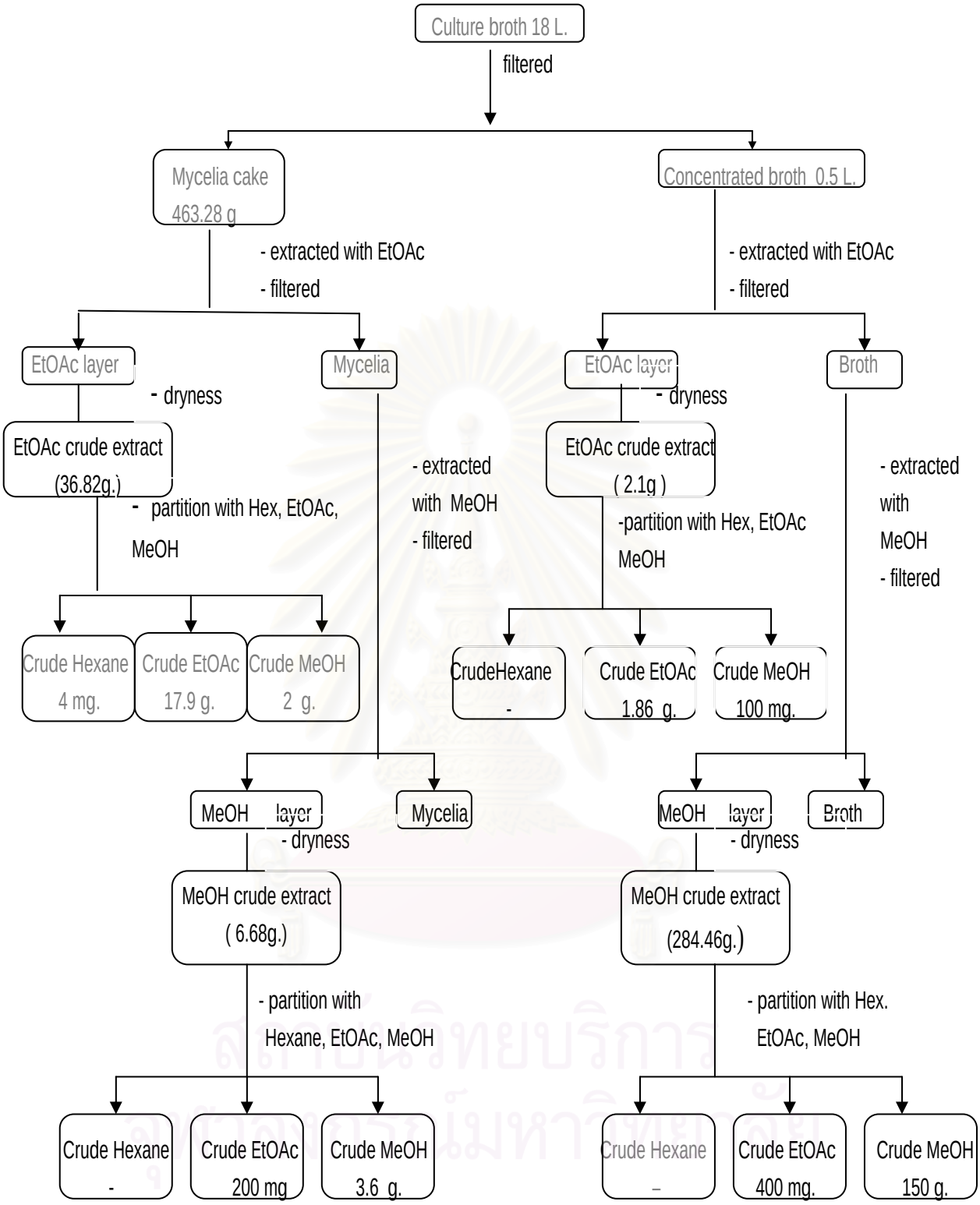
All solvents used in this research such as hexane, ethyl acetate (EtOAc) and methanol (MeOH) were commercial grade and were purified prior to use by distillation. The reagent grade solvents were used for recrystallization.

3.9.3 Extraction of secondary metabolites from culture broth and mycelia of endophytic fungal isolate CuLm17

The cultivation broth of Isolate CuLm17 (18 l) was filtered through filter paper (Whatman No.4). The filtrate broth was evaporated to concentrated broth (0.5 l) under reduced pressure at 35 ^{o}C and extracted with an equal volume of EtOAc 20 times. The EtOAc layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C and partitioned with hexane, EtOAc and MeOH gave crude hexane, crude EtOAc and crude MeOH extract. The crude hexane extract was obtained as none. The crude EtOAc extract was obtained as a dark brown viscous liquid (1.86 g). The crude MeOH extract was obtained as a brown viscous liquid (100 mg). The residue broth was extracted again with an equal volume of MeOH. The MeOH layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C and partitioned with hexane, EtOAc and MeOH gave crude hexane, crude EtOAc and crude MeOH extract. The crude hexane extract was obtained as none. The crude EtOAc extract was obtained as a mixture of brown solid and dark brown viscous liquid (400 mg). The crude MeOH extract was obtained as a mixture of brown solid and dark brown viscous liquid (150 g).

Mycelial cake 463.28 g was extracted with 2 liters EtOAc and filtered through filter paper (Whatman No. 4). The EtOAc layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C. The dry crude EtOAc extract (36.82 g.) was partitioned with hexane, EtOAc and MeOH gave crude Hexane, crude EtOAc and crude MeOH extract. The crude hexane extract was obtained as white oily liquid (4 mg) . The crude EtOAc extract was obtained as a mixture of yellow solid and brown viscous liquid (17.9 g). The crude MeOH extract was obtained as a dark brown viscous liquid (2 g). The residue mycelia was extracted again with an equal volume of MeOH. The MeOH layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C and partitioned with hexane, EtOAc and MeOH gave crude hexane, crude EtOAc and crude

MeOH extract. The crude hexane extract was obtained as none. The crude EtOAc extract was obtained as a dark brown viscous liquid (400 mg) and the crude MeOH extract was obtained as a mixture of brown solid and dark brown viscous liquid (150 g). All crude extracts were collected for antimicrobial activity test (according to section 3.7) and selected to column chromatography. The extraction of the cultivation broth and mycelia of the endophytic fungus isolate CuLm17 is shown in Scheme 3.1.



Scheme 3.1 Extraction of culture broth and mycelia of endophytic fungus isolate CuLm17

3.10 Chromatographic techniques

3.10.1 Column chromatography

Flash column chromatography

Adsorbent : Silica gel 60 (No. 7734) particle size 63-200 µm (70-230 Mesh ASTM) (E. Merck).

Packing method : Wet packing, the adsorbent was suspended in an eluent and then poured into a column, set it tight by using air pump before used.

Loading method : Dry loading: The sample was mixed in a small amount of a silica gel and then applied gently on the top of the column.

Detection : Fractions were examined by TLC technique in the same manner as described in section 3.10.1.

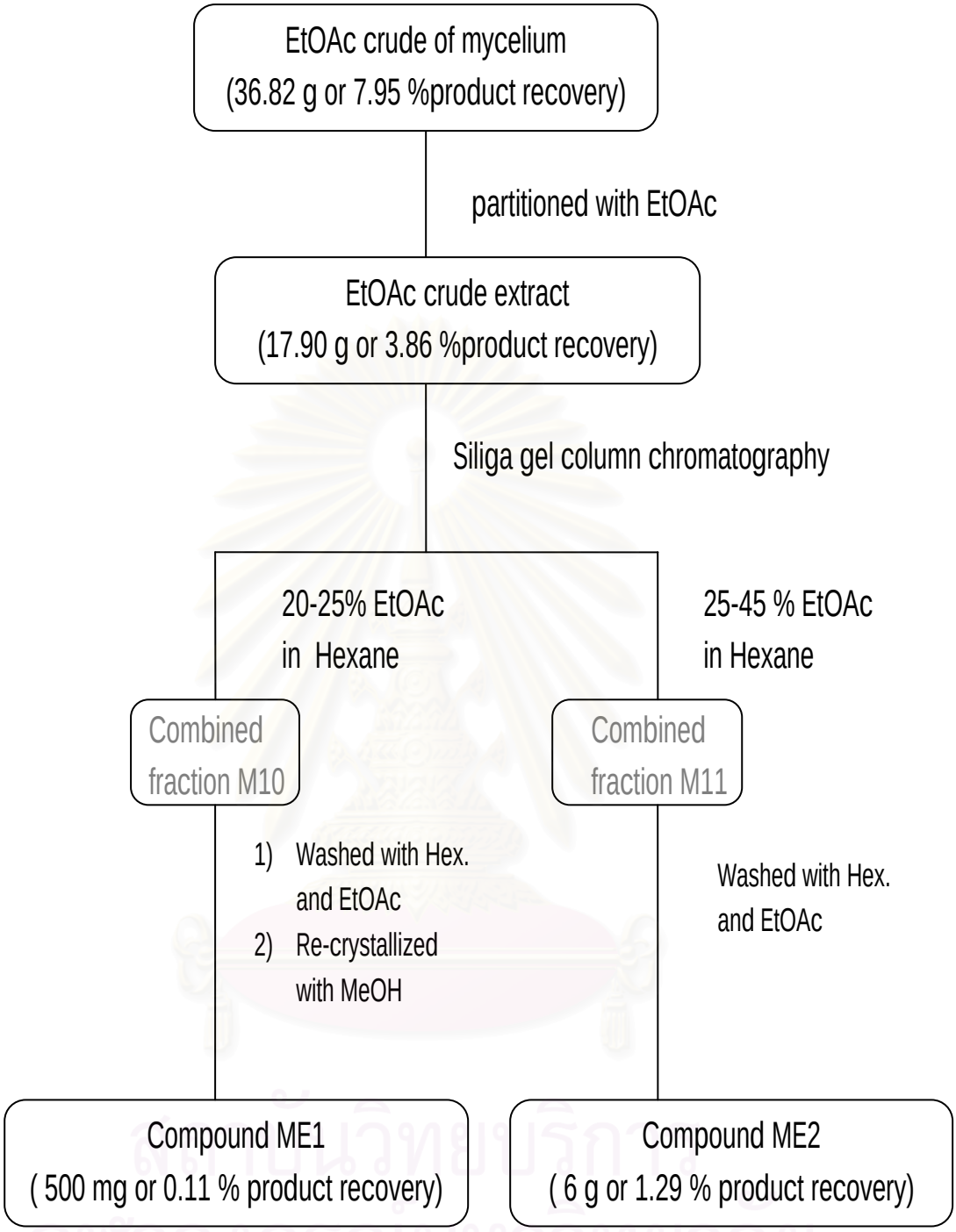
3.11 Isolation and purification of bioactive compounds of endophytic fungal isolate CuLm17

3.11.1 Isolation and purification of bioactive compounds in crude EtOAc from mycelial extract

The mycelium EtOAc crude from endophytic fungal isolate CuLm 17 (36.82 g) was partitioned with EtOAc (400 ml, 7 times) and then evaporated to give a mixture of yellow solid and brown viscous liquid (17.9 g). The EtOAc crude obtained from partition of the mycelium EtOAc crude was subjected to column chromatography (silica gel, 375 g), using wet packing and dry loading method and eluented by increasing polarity. 25 ml of each fraction was collected and examined by TLC. Fractions with the same TLC pattern were combined and evaporated the solvents. The results were presented in Table 3.3 and Scheme 3.2. The biological activity of each pool fraction was examined and described in Chapter 4.

Table 3.3 The results from separation of EtOAc crude from mycelial extract.

Combined fraction	Fraction No.	Eluents	Appearance	Weight (mg)
M01	1-46	100% Hexane-10%EtOAc in Hexane	White viscous liquid	4.3
M02	47-57	10-15% EtOAc in Hexane	Yellow viscous liquid	7.6
M03	58-65	15% EtOAc in Hexane	Yellow brown viscous liquid	4.8
M04	66-70	15% EtOAc in Hexane	Yellow brown viscous liquid	2.5
M05	71-88	15% EtOAc in Hexane	Yellow brown viscous liquid	3.4
M06	89-92	15% EtOAc in Hexane	Yellow brown viscous liquid with white viscous liquid	2.6
M07	93-104	15% EtOAc in Hexane	Amorphous white solid in yellow viscous liquid	1.7
M08	105-116	20% EtOAc in Hexane	Amorphous white solid in yellow viscous liquid	4.3
M09	117-124	20% EtOAc in Hexane	Amorphous white solid in yellow viscous liquid	5.4
M10	125-148	20-25% EtOAc in Hexane	Colorless crystal in yellow viscous liquid and white viscous liquid	540
M11	149-203	25-45% EtOAc in Hexane	Amorphous white solid in yellow viscous liquid	6100
M12	204-209	45% EtOAc in Hexane	Brown viscous liquid	9.3
M13	210-236	55% EtOAc in Hexane	Black brown viscous liquid	28.1
M14	237-250	65% EtOAc in Hexane	Amorphous white solid	2.6
M15	251-254	75% EtOAc in Hexane	Amorphous white solid in yellow viscous liquid	4.3
M16	255-280	75-90% EtOAc in Hexane	Dark brown viscous liquid	31.6
M17	281-291	90% EtOAc in Hexane-100% EtOAc	Dark brown viscous liquid	22.2
M18	292-303	100% EtOAc -10% MeOH in EtOAc	Black brown viscous liquid	16.2
M19	304-378	10-70% MeOH in EtOAc	Black brown viscous liquid	36.9
M20	379-428	70-100% MeOH in EtOAc	Black brown viscous liquid	49.7



Scheme 3.2 Diagram of isolation procedure for EtOAc crude which partition with EtOAc of mycelium of endophytic fungi isolate CuLm17

3.11.2 Isolation of bioactive compounds in crude EtOAc from culture broth extract

The EtOAc crude obtained from partition of the endophytic fungal isolate CuLm17 culture broth EtOAc extract (1.86 g) was subjected to column chromatography (silica gel, 77.30 g), using wet packing and dry loading method. Eluents of increasing polarity from hexane to MeOH were used. Elution systems were Hexane, Hexane-EtOAc gradients, EtOAc, EtOAc-MeOH gradients, and MeOH. Each Fractions (25 ml) were collected and examined by TLC. Fractions with the same TLC pattern were combined and evaporated the solvents. The results were presented in Table 3.4. The biological activity of each pool fraction was examined and described in Chapter 4.

Table 3.4 The results from separation of EtOAc crude from culture broth extract.

Combined fraction	Fraction No.	Eluents	Appearance	Weight (mg)
B01	1-17	100% Hexane-10% EtOAc in Hexane	White viscous liquid	1.1
B02	18-22	15% EtOAc in Hexane	Yellow viscous liquid	2.4
B03	23-25	15-20% EtOAc in Hexane	Yellow viscous liquid	1.3
B04	26-34	20-25% EtOAc in Hexane	Yellow viscous liquid	3.2
B05	35-37	25-30% EtOAc in Hexane	Amorphous yellow solid in yellow viscous liquid	2.1
B06	38	30% EtOAc in Hexane	Orange brown viscous liquid	1
B07	39-41	30% EtOAc in Hexane	Yellow viscous liquid	2.1
B08	42-45	35% EtOAc in Hexane	Amorphous yellow solid in yellow viscous liquid	48.7
B09	46-51	35-40% EtOAc in Hexane	Amorphous yellow solid in yellow viscous liquid	34.1
B10	52-53	50% EtOAc in Hexane	Amorphous orange brown solid in yellow viscous liquid	3.6
B11	54-59	50-55% EtOAc in Hexane	Orange brown viscous liquid	7.4
B12	60-62	60% EtOAc in Hexane	Amorphous orange solid in orange brown viscous liquid	2.8
B13	63-69	60-70% EtOAc in Hexane	Brown viscous liquid	5.7
B14	70-75	80-90% EtOAc in Hexane	Brown viscous liquid	8.4
B15	76-81	90% EtOAc in Hexane-100% EtOAc	Brown viscous liquid	9.5
B16	82-87	5-10% MeOH in EtOAc	Black brown viscous liquid	6.9
B17	88	10% MeOH in EtOAc	Black brown viscous liquid	1.6
B18	89-93	20-30% MeOH in EtOAc	Black brown viscous liquid	11.8
B19	94-99	30-40% MeOH in EtOAc	Black brown viscous liquid	16.3
B20	100-101	40-50% MeOH in EtOAc	Black brown viscous liquid	2.1
B21	103-105	50-60% MeOH in EtOAc	Black brown viscous liquid	8.6
B22	107-117	70% MeOH in EtOAc-100% MeOH	Black brown viscous liquid	24.7
B23	118-124	2-7% Acetic acid in MeOH	Black viscous liquid	17.9

3.12 Crystallization

The compound ME1 was crystallized from a mixture of hexane and EtOAc. It was dissolved in MeOH until saturation, and then EtOAc was added. The solution was left standing at room temperature.

3.13 Spectroscopy

3.13.1 UV-VIS spectrometer

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

3.13.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

3.13.3 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H-NMR, ^{13}C-NMR, COSY, NOESY, HSQC, and HMBC spectra were recorded on a Varian spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. Deuterated solvents; methanol-*d*4 (CD_3OD) were used in NMR experiments. Reference signals were the signals of residual protonated solvents at δ 3.35 ppm (^{1}H) and 49.0 ppm *sept* (^{13}C) for CD_3OD.

3.13.4 Mass spectrometer

The mass spectra were recorded on a Bruker-Franzen Analytik Gmbh Mass Spectrometer Model Esquire-LC 1.4 g.

3.14 Melting point

Melting points were examined using a Fisher-John melting point apparatus.

3.15 X-ray crystallographic analysis

White crystal of compound ME1 was recrystallized from methanol and was identified by x-ray diffraction analysis. All data were collected at room temperature using graphite monochromated Mok ∞ Radiation (lamda = 0.71069 ^{0}A) on BRUKER SAMART CCD diffractrometer. The data were corrected for Lorentz and polarization effects. The crystal data of compound ME1 were corrected for Lorentz and polarization effects. The structure were solved by direct methods using SHELXLS-97 and refined by full metrix least-squares on F^{2} using SHELXLS-97 with anisotropic thermal parameter for all non-hydrogen atoms. All hydrogen atoms were found from difference Fourier maps and were included in refinement.

3.16 Characterization of pure compound from endophytic fungal isolate CuLm17

3.16.1 Characterization of compound ME1

The colorless crystal in yellow viscous liquid and white viscous liquid combined fraction M10 (540 mg) was washed with Hexane and EtOAc and re-crystallized with MeOH to yield compound ME1 as colorless crystal (500 mg). Compound ME1 has melting point (m.p.) 249.2-249.8 ^{0}C and showed a single spot at the R_f value 0.43 on TLC plate using 25%$CHCl_3$ in Hexane as the mobile phase. TLC spots were visualized with UV lamp (254 nm), iodine vapour and spraying with vanillin reagent. Compound ME1 is soluble in MeOH, DMSO and slightly soluble in EtOAc, CH_2Cl_2 and $CHCl_3$.

UV : λ_{max} (MeOH) (ε) 211 (52000) nm, 278 (12000) nm. (Figure B1 in Appendix B)

FT-IR spectrum (KBr) : ν_{max} 3439 (broad, s), 2344 and 2050 (w), 1636 (m), 1418, 1265, 1129 (w) cm^{-1}. (Figure B2 in Appendix B)

EI-MS spectrum (70 eV) : *m/z* (M^{+}) 380 (31), 365 (35), 337 (16), 311 (29), 309 (38), 281 (17), 193 (19), 192 (100), 191 (78), 177 (66), 176 (42), 175(33), 161 (21), 149 (17), 115 (15), 91 (25), 83(17), 77(14) and 55 (8). (Figure B10 in Appendix B)

δ_H (CD_3OD, 400 MHz) : 6.52 (1H, s), 5.41 (1H, dq, *J* = 1.2 and 6.8),

1.73 (3H, dq, *J* = 7.2), 1.90 (3H, s), 2.16 (3H, s), 6.43 (1H, s), 5.55 (1H, dq, *J* = 1.2 and 6.8), 1.81 (3H, d, *J* = 6.8), 2.08 (3H, s), 2.18 (3H, s) ppm. (Figure B3 in Appendix B)

δ_C (CD_3OD, 100 MHz) : 148.7 (s), 159.8 (s), 113.7 (s), 162.3 (s), 111.1 (s), 111.6 (d), 136.0 (s), 123.8 (d), 12.9 (q), 16.5 (q), 7.6 (q), 165.7(s), 152.5 (s), 114.9 (s), 143.6 (s), 142.4 (s), 136.3 (s), 111.3 (d), 134.3 (s) , 124.9 (d), 12.7 (q), 16.9 (q) and 7.9 (q) ppm. (Figure B4 in Appendix B)

3.16.2 Characterization of compound ME2

The white amorphous solid in yellow viscous liquid combined fraction M11 (6.1 g) was washed with Hexane and EtOAc to yield compound ME2 as white amorphous solid (6 g). Compound ME2 has melting point (m.p.) 126-126.5 ^oC and showed a single spot at the R_f value 0.35 on TLC plate using 25%$CHCl_3$ in Hexane as the mobile phase. TLC spots were visualized with UV lamp (254 nm), iodine vapour and spraying with vanillin reagent. Compound ME2 is soluble in EtOAc, $CHCl_3$, CH_2Cl_2 and slightly soluble in Hexane.

UV : λ_{max} (MeOH) (ε) 210 (39310) nm. (Figure B11 in Appendix B)

FT-IR spectrum (KBr) : ν_{max} 3497 and 3385 (broad, m), 2918 (w), 1707 (s), 1571 and 1419 (s), 1337 (m) , 1252 and 1155 (s), 1057 (m) cm^{-1}. (Figure B12 in Appendix B)

EI-MS spectrum (70 eV) : *m/z* (M^+) 326 (6), 312 (20), 311 (100), 383 (22), 255 (29), 241 (17), 227 (10), 161 (10),152 (19), 151 (64), 77 (16) and 51 (7). (Figure B19 in Appendix B)

δ_H (CD_3OD, 400 MHz) : 6.45 (1H,s), 5.56 (1H,s), 1.85 (3H,d, *J* =6.8), 2.08 (3H,s), 2.17 (3H,s), 6.36 (1H,d, *J* =2.4), 6.55 (1H,d, *J* =1.2) and 2.41 (3H,s) ppm. (Figure B13 in Appendix B)

δ_C (CD_3OD, 100 MHz) : 152.44 (s), 110.82 (d), 135.82 (s), 141.02 (s), 143.47 (s), 114.91 (s),132.87 (s),125.11 (d), 12.63 (q), 16.48 (q), 7.95 (q), 111.85 (s), 163.98 (s), 104.47 (d), 162.04 (s), 115.33 (d), 144.89 (s), 164.27 (s), 19.85 (q) ppm. (Figure B14 in Appendix B)

3.17 Evaluation of biological activities of isolated metabolites

3.17.1 Antimicrobial activity of pool fractions

Evaluation of the antimicrobial activity of the fractions was determined by the agar well diffusion method (Weaver, Angel and Botlomley, 1994) in the same manner as described in section 3.7. Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169 and *C. albicans* ATCC 10231

3.17.2 Antimicrobial activity of pure compounds

Evaluation of the antimicrobial activity of pure compounds was determined by the antimicrobial susceptibility test broth microdilution method. Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169, and *C. albicans* ATCC 10231.

A. Preparation of pure compounds and antibiotic drug standards

Four mg of pure compounds and antibiotic drug standards were dissolved in 1 ml of 10% DMSO in sterile distilled water and kept in a refrigerator at 4 ^{o}C for bioassay. Antibacterial (Streptomycin and Penicillin G) and antifungal (Ketoconazole) compound were used as positive controls.

B. Preparation of bacterial inoculum

A bacterial inoculum was prepared in the same manner as described in section 3.7.3. The final inoculum was diluted with NB to obtain a cell suspension containing approximately 10^6 CFU/ml.

C. Preparation of yeast inoculum

A yeast inoculum was performed in the same manner as described in section 3.7.4. The final inoculum was approximately 10^5 CFU/ml.

D. Assay procedure

Solutions of pure compounds and antibiotic drug standards were diluted

with Mueller-Hinton Broth (MHB) and YMB for assays of antibacterial and antifungal (yeast form) activity respectively. Fifty µl of pure compound was dispensed into each well in sterile microtiter plates (96-well bottom wells). Fifty µl of the final adjusted microbial suspension was inoculated into each well (Final inoculum size of bacterial and yeast was approximately $2.5X10^5$ and $2.5X10^4$ CFU/ml, respectively). One hundred µl of medium only was as the sterility control. A 100 µl volume of medium and microbial inoculum mixture acted as the growth control. Microbial microtiter plates were incubated at 37 ^{0}C and room temperature for bacterial and yeast, respectively.

E. Reading of microtiter plates assays

Antibacterial and antifungal (yeast form) activites were determined by measuring the turbidity each well in the microtiter plates by using the Sunrise microplate reader (TECAN, AUSTRIA) before and after incubation. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibitory concentration (MIC).

3.17.3 Cytotoxicity test

Isolated metabolites were sent to determine cytotoxicity at the Institue of Biotechnology and Genetic Engineering, Chulalongkorn University. The bioassay of cytotoxic activities against 5 tumor cell lines *in vitro*, including HEP-G2 (hepatoma), CHAGO (lung), SW 620 (colon), KATO-3 (gastric) and BT474 (breast) was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry assay and survival of the cell lines was detected by spectrophotometer at 540 nm.

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2), 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 ^{0}C, 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.

3.18 Antioxidant activity of pure compounds

Test for antioxidant activity

A. Preparation of DPPH

DPPH solution were prepared at 200 µM in absolute ethanol (freshly prepare) and kept in dark by covered with aluminum foil until use.

B. Preparation of pure compounds and positive control

Compound ME1, compound ME2 and vitamin E (Positive control) were prepared in absolute ethanol as stock solutions. Pipetted crude extract and vitamin E in to each tube (covered tube with aluminum foil). The appropiate concentration of sample were added in vary concentration such as 200, 100, 50, 25, 20, 10, 5, 0 µg/ml with volumn as 200 µl.

C. Assay procedure

Added 800 µl of DPPH solution into each tube. Thus it has about 1 ml final volume. The mixture was shaken vigorously and incubated at 37^0 c for 30 min. And then added 200 µl of mixture solution to 96 wellplate. The absorbance of mixture solution and 200 µl DPPH solution were measured at 517 nm. Measurement were performed in triplicate in at least three independent experiments. Calculation for % inhibition according to this formula % inhibition = $[(A_{DPPH} - A_{sample})/ A_{DPPH}] \times 100$

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

Endophytic fungi were isolated from Plao-Yai *(Croton oblongifolius*) young and mature leaves, branches and bark samples collected from sites on the campus of Chulalongkorn University, Bangkok. Young leaf sections 100 pieces, mature leaf sections 100 pieces, branch sections 20 pieces and bark sections 20 pieces of Plao-Yai *(Croton oblongifolius*) were used for isolation endophytic fungi.

Forty seven endophytic fungal isolates were isolated from young and mature leaves, branches and bark samples of Plao-Yai *(Croton oblongifolius*). Seven isolates of endophytic fungi were isolated from young leaf sections (7% of total young leaves), 25 isolates were isolated from mature leaf sections (25 % of total mature leaves), 6 isolates were isolated from branch sections (30 % of total branches), 9 isolates were isolated from bark sections (45 % of total barks). The percentage of endophytic fungi isolated from branch and bark sections was higher than those from leaf sections. This result is in accordance with most of the investigations undertaken with other host plants from other localities in which branches and barks tended to support a greater frequency of internal fungal colonization (Norio et al., 1999). This may be owing to the branches and barks had been exposed longer than leaves and therefore received higher amounts of inoculum. The number and the code of isolated endophytic fungi are shown in Table 4.1.

Table 4.1 The number and code of the isolated endophytic fungi from young and mature leaves, branches and barks of Plao-Yai *(Croton oblongifolius*).

Plant morphology	Number of isolates (% of total plant morphology)	Code of isolated endophytic fungi
Young leaf	7 (7)	CuLy01-07
Mature leaf	25 (25)	CuLm01-25
Branch	6 (30)	CuBr01-06
Bark	9 (45)	CuBa01-09
Total	47	

4.2 Characterization and identification of isolated endophytic fungi

Each fungal isolate was grown on MEA, for 2 weeks at room temperature. Colony morphology of the 47 fungal isolates is shown in Figures 4.1-4.4. A total of 47 isolates of endophytic fungi were identified. Fungal isolates were identified as belonging to typical genera of endophytes such as *Penicillium, Curvularia, Trichoderma and Phomopsis* for 4, 2, 3 and 1 isolates, respectively. The remaining of 37 isolates of endophytic fungi did not sporulate on media and therefore were recorded as mycelia sterilia. The results and summary of identification of endophytic fungi is shown in Tables 4.2 and 4.3, respectively.

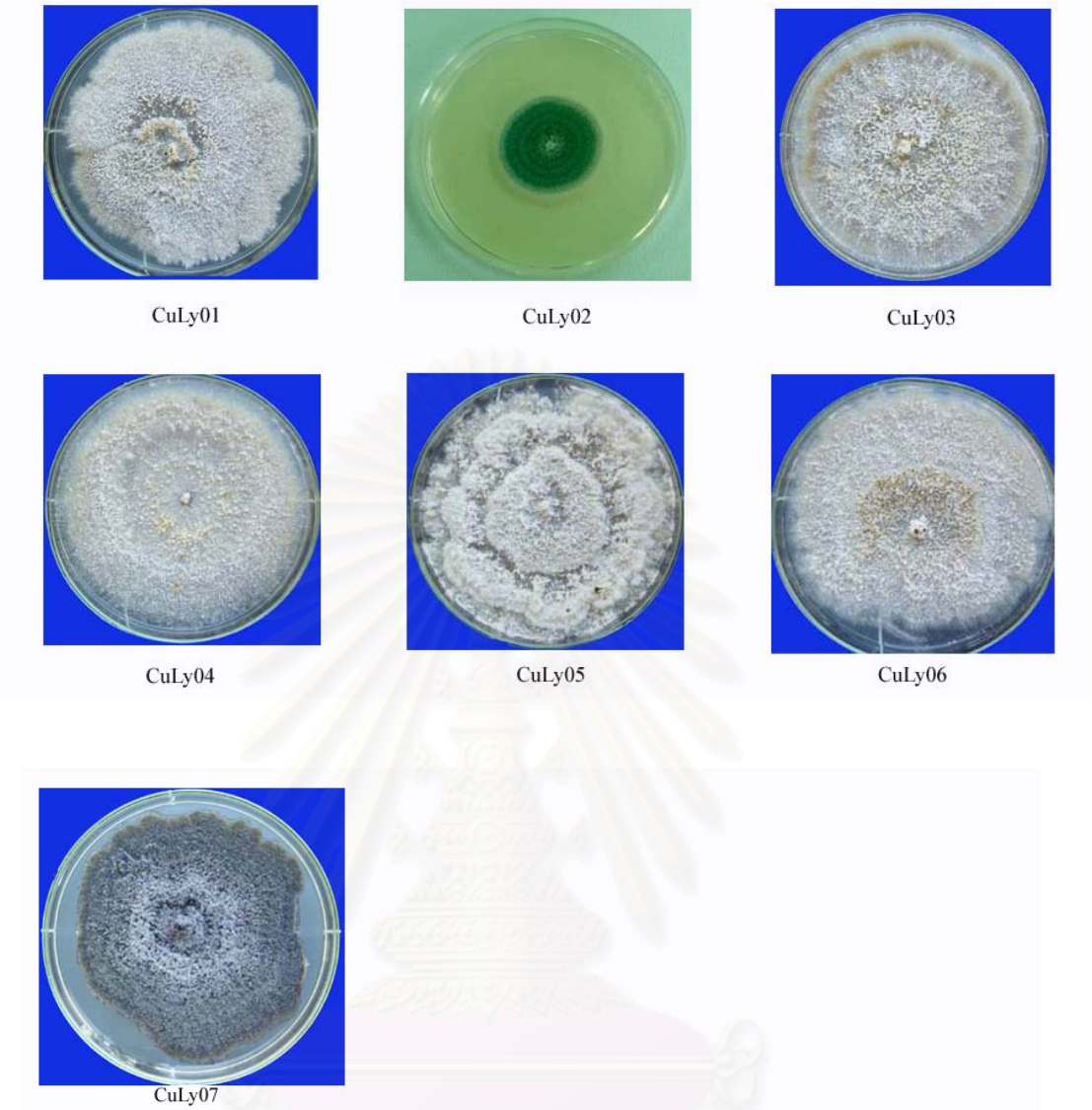


Figure 4.1 Colony characteristics of endophytic fungal isolates, CuLy01-06, on MEA after cultivation for 10 days at room temperature. Isolate number is shown in each picture.

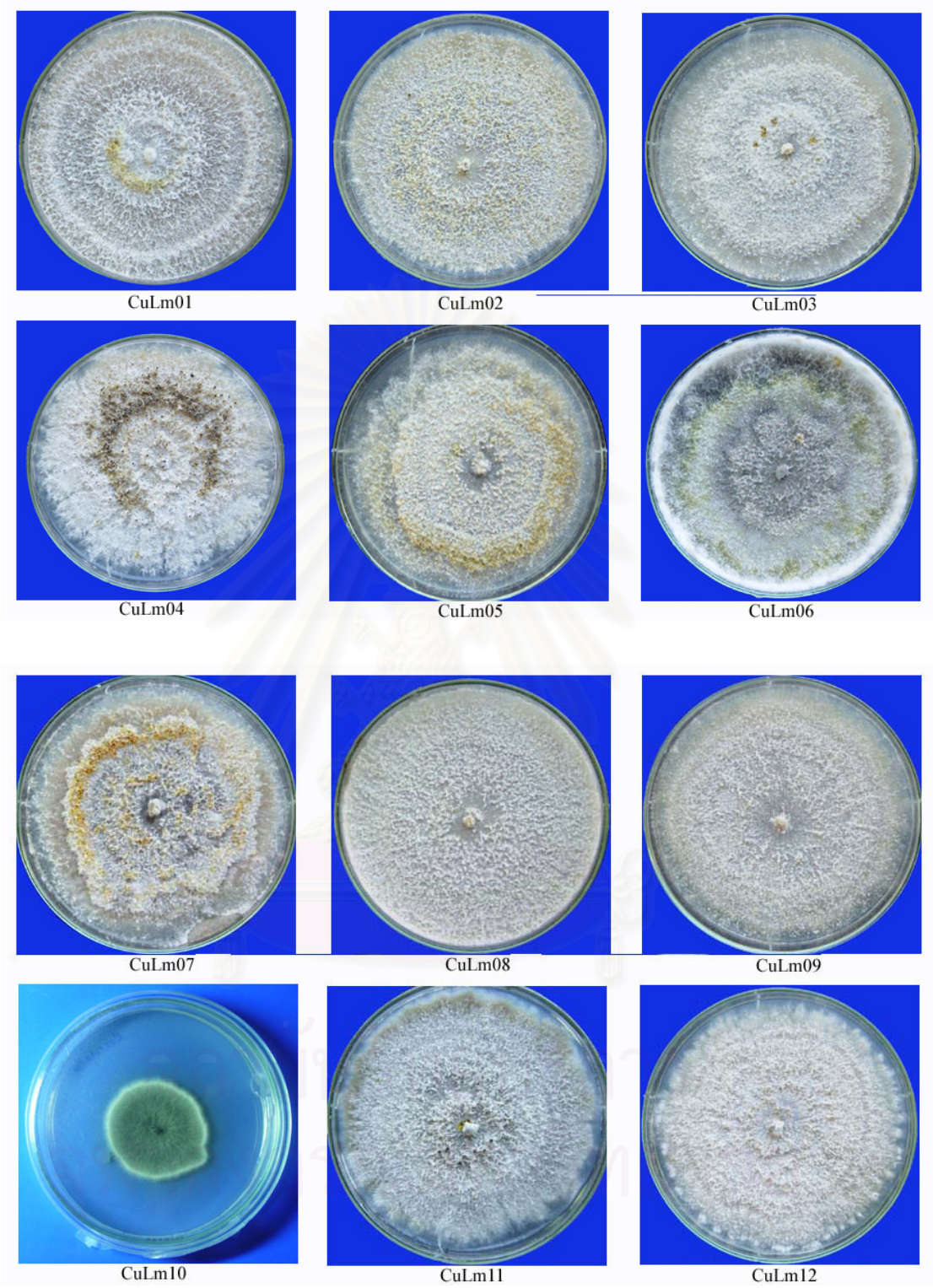


Figure 4.2 Colony characteristics of endophytic fungal isolates, CuLm01-25, on MEA after cultivation for 10 days at room temperature. Isolate number is shown in each picture.

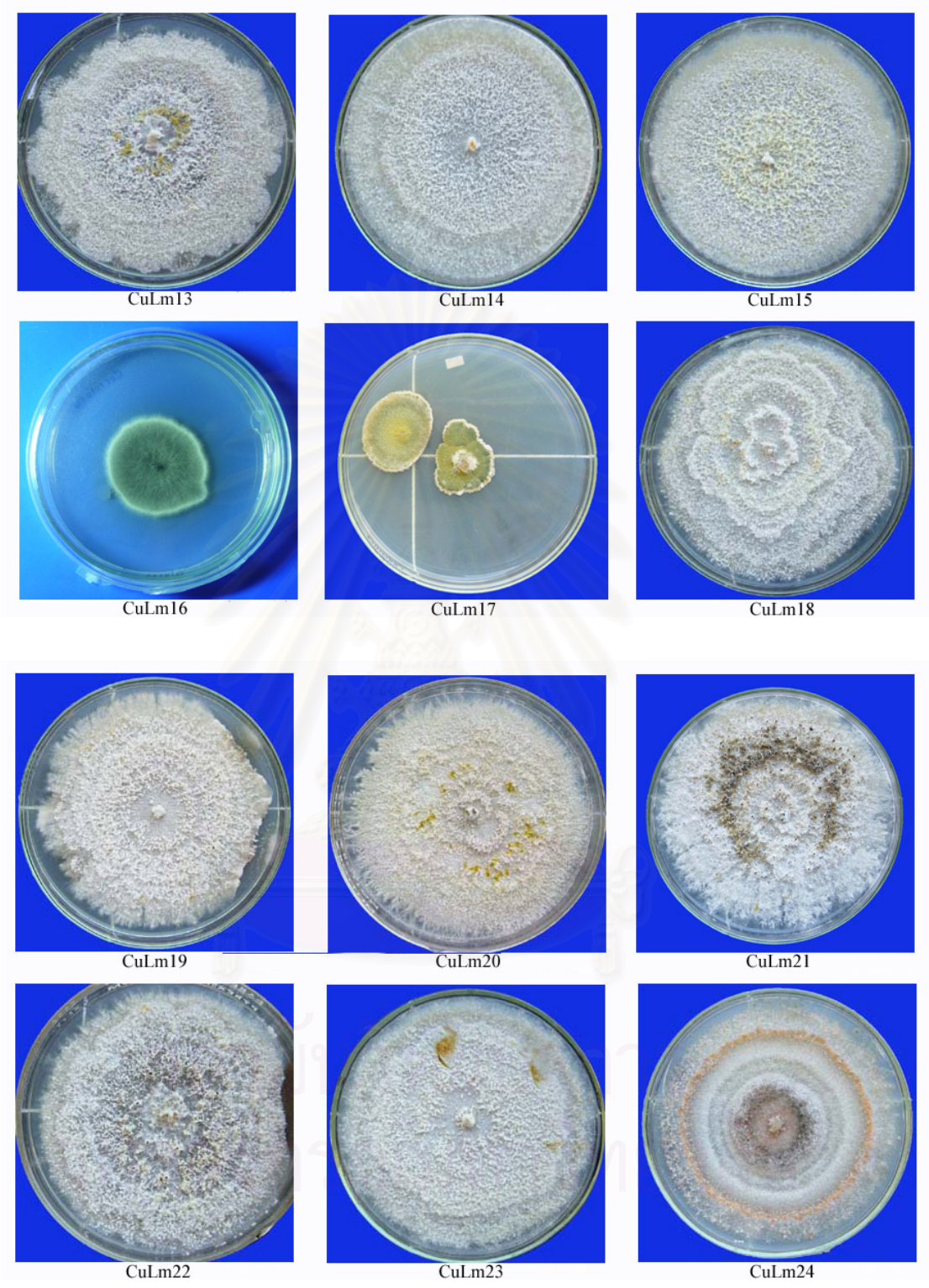
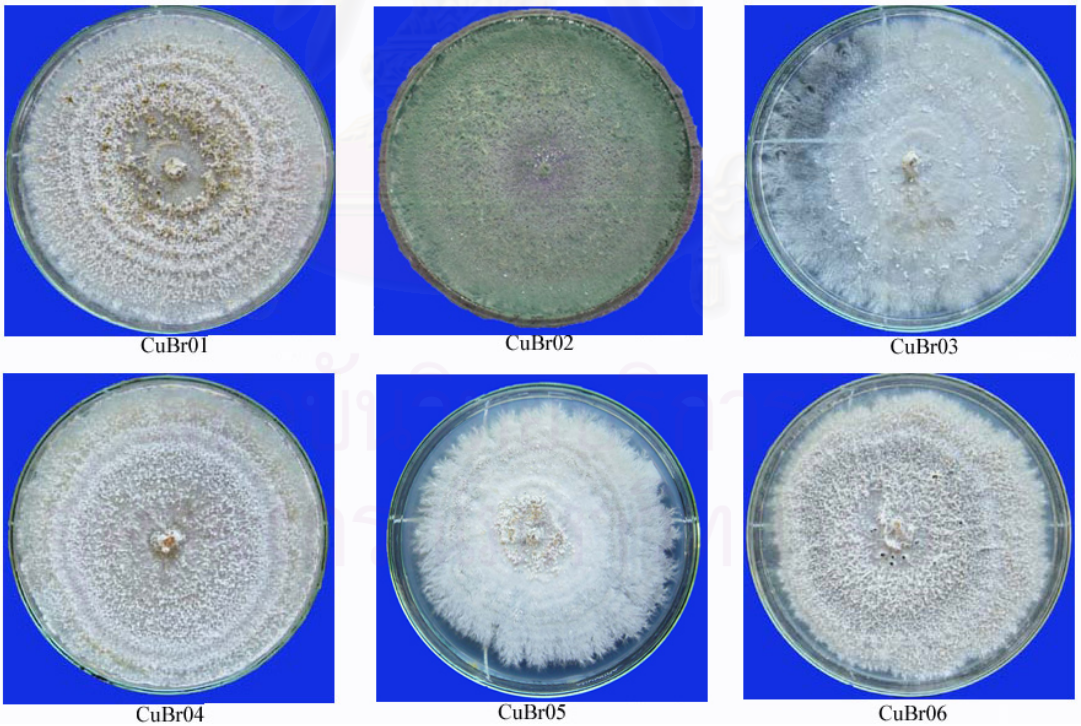


Figure 4.2 (continued)

CuLm25

Figure 4.2 (continued)



CuBr01 CuBr02 CuBr03

CuBr04 CuBr05 CuBr06

Figure 4.3 Colony characteristics of endophytic fungal isolates, CuBr01-06, on MEA after cultivation for 10 days at room temperature. Isolate number is shown each picture.

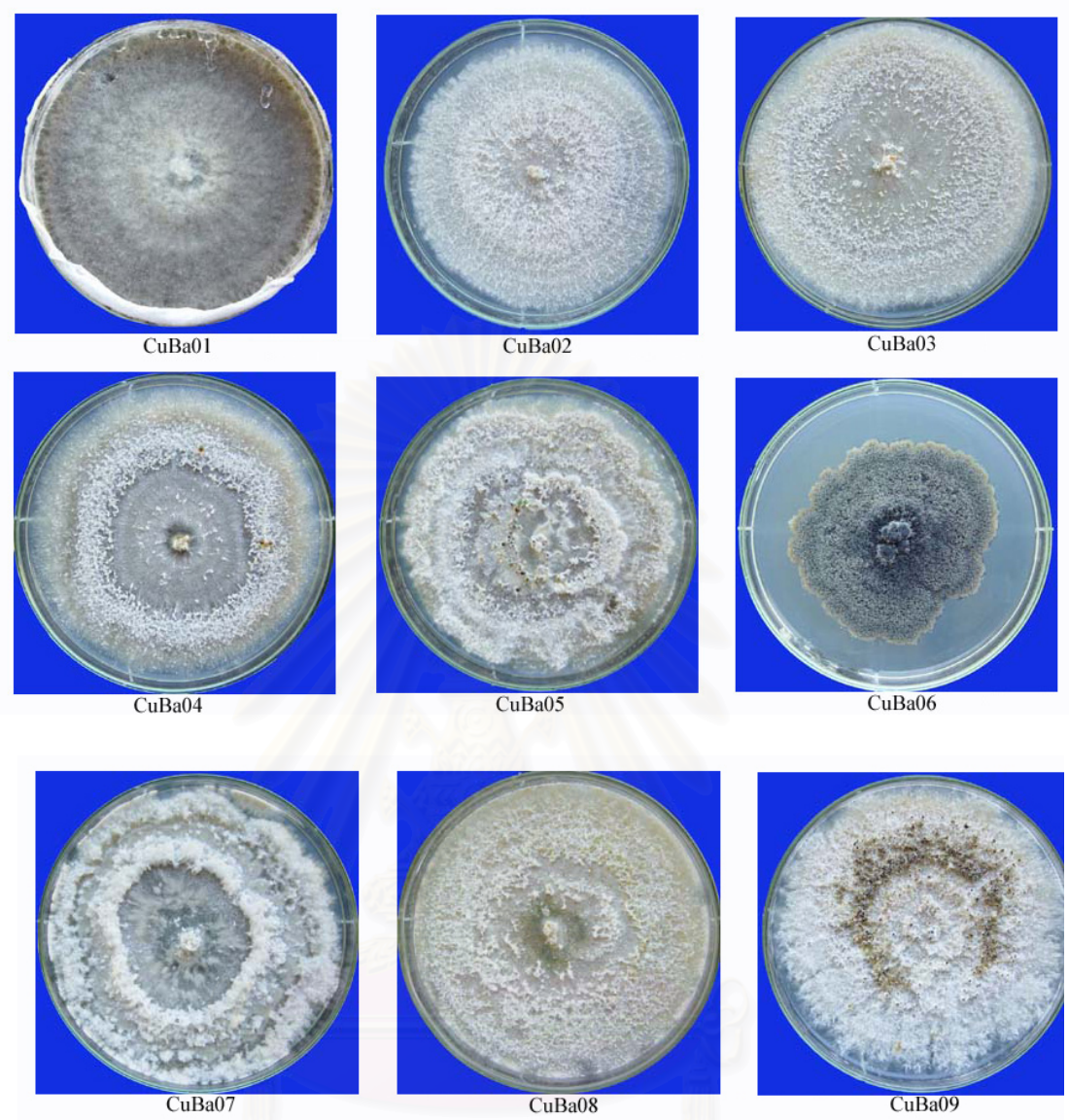


Figure 4.4 Colony characteristics of endophytic fungal isolates, CuBa01-09, on MEA after cultivation for 10 days at room temperature. Isolate number is shown in each picture.

Table 4.2 Identification of endophytic fungi

Plant morphology	Endophyte isolate	Species
Young leaf	CuLy01	*Penicillium* sp.
	CuLy02	*Penicillium* sp.
	CuLy03	Mycelia sterilia
	CuLy04	Mycelia sterilia
	CuLy05	Mycelia sterilia
	CuLy06	Mycelia sterilia
	CuLy07	Mycelia sterilia
Mature leaf	CuLm01	Mycelia sterilia
	CuLm02	Mycelia sterilia
	CuLm03	Mycelia sterilia
	CuLm04	*Phomopsis* sp.
	CuLm05	Mycelia sterilia
	CuLm06	Mycelia sterilia
	CuLm07	Mycelia sterilia
	CuLm08	Mycelia sterilia
	CuLm09	Mycelia sterilia
	CuLm10	*Curvularia* sp.
	CuLm11	Mycelia sterilia
	CuLm12	Mycelia sterilia
	CuLm13	Mycelia sterilia
	CuLm14	Mycelia sterilia
	CuLm15	*Penicillium* sp.
	CuLm16	*Curvularia* sp.
	CuLm17	*Penicillium* sp.
	CuLm18	Mycelia sterilia
	CuLm19	Mycelia sterilia
	CuLm20	Mycelia sterilia
	CuLm21	*Phomopsis* sp.

Plant morphology	Endophyte isolate	Species
Mature leaf	CuLm22	Mycelia sterilia
	CuLm23	Mycelia sterilia
	CuLm24	Mycelia sterilia
	CuLm25	Mycelia sterilia
Branch	CuBr01	Mycelia sterilia
	CuBr02	*Trichoderma* sp.
	CuBr03	Mycelia sterilia
	CuBr04	Mycelia sterilia
	CuBr05	Mycelia sterilia
	CuBr06	Mycelia sterilia
Bark	CuBa01	Mycelia sterilia
	CuBa02	Mycelia sterilia
	CuBa03	Mycelia sterilia
	CuBa04	Mycelia sterilia
	CuBa05	Mycelia sterilia
	CuBa06	Mycelia sterilia
	CuBa07	Mycelia sterilia
	CuBa08	Mycelia sterilia
	CuBa09	*Phomopsis* sp.

Table 4.3 Summary of identification of endophytic fungi

Isolated Endophytic fungi	Isolation frequency of plant morphology				Total of isolates
	Young leaf	Mature leaf	Branch	Bark	
Penicillium sp.	2	2	-	-	4
Curvularia sp.	-	2	-	-	2
Phomopsis sp.	-	2	-	1	3
Trichoderma sp.	-	-	1	-	1
Mycelia sterilia	5	19	5	8	37
Total (isolates)	7	25	6	9	47

The fungal genera found in young and mature leaves, branches, and barks of *Croton oblongifolius* were identified as *Penicillium* (CuLy01, CuLy02, CuLm1 and CuLm17), *Curvularia* (CuLm10 and CuLm16), *Trichoderma* (CuBr02), *Phomopsis* (CuLm04, CuLm21 and CuBa09) and mycelia sterilia. All genera of fungal isolate are well known common endophytes on many different plants. For example, *Penicillium* was found on *Pupulus Tremula* (O. Santamaria and J. J. Diez, 2005). *Curvularia* was found on agricultural crops (Ferdinand B. Apayo and Naomi G. Tangonan, 2003). *Phomopsis* was found on *Eucalyptus globulus* (Lu et al., 2000). *Trichoderma* was found on *Palicourea longiflora* (aubl.) rich and *Strychnos cogens* bentham (Antonia Queiroz Lima de Souza *et al,* 2004) . Mycelia sterilia was found on manuka (*Leptospermum scoparium*) and estuarine mangrove forest (*Sesuvium portulacastrum*) (Hu et al., 2001)

Characteristics of endophytic fungi isolates

PENICILLIUM (CuLy01, CuLy02, CuLm01 and CuLm17)

Colonies are usually fast growing, in shades of green, sometimes white, mostly consisting of a dense felt of conidiophores. Microscopically, chains of single-celled conidia (ameroconidia) are produced in basipetal succession from a specialized conidiogenous cell called a phialide. The term basocatenate is often used to describe such chains of conidia where the youngest conidium is at the basal or proximal end of the chain. In *Penicillium*, phialides may be produced singly, in groups or from branched metulae, giving a brush-like appearance known as a penicillus. The penicillus may contain both branches and metulae (penultimate branches which bear a whorl of phialides). All cells between the metulae and the stipes of the conidiophores are referred to as branches. The branching pattern may be either simple (non-branched or monoverticillate), one-stage branched (biverticillate-symmetrical), two-stage branched (biverticillate-asymmetrical) or three- to more-staged branched. Conidiophores are hyaline and may be smooth- or rough-walled. Phialides are usually flask-shaped, consisting of a cylindrical basal part and a distinct neck, or lanceolate (with a narrow basal part tapering to a somewhat pointed apex). Conidia are globose, ellipsoidal, cylindrical or fusiform, hyaline or greenish, smooth- or rough- walled. Sclerotia may be

produced by some species.

Endophytic fungal isolate CuLm17 grew rapidly on Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Oat Meal Agar (OMA), and Sabouraud's Dextrose Agar (SDA) rather than on Yeast Extract Sucrose agar (YES). Colonies are plane, mycelium white at the margins, elsewhere brightly coloured, yellowish green. Colonial and microscopic characteristic of endophytic fungal isolate CuLm17 showed in figure 4.5. Colony characteristic of endophytic fungal isolate CuLm17 on PDA, MEA, OMA, YES and SDA is shown in Figure 4.6. Scanning Electron Microscopic characteristic of endophytic fungal isolate CuLm17 is shown in Figure 4.7.

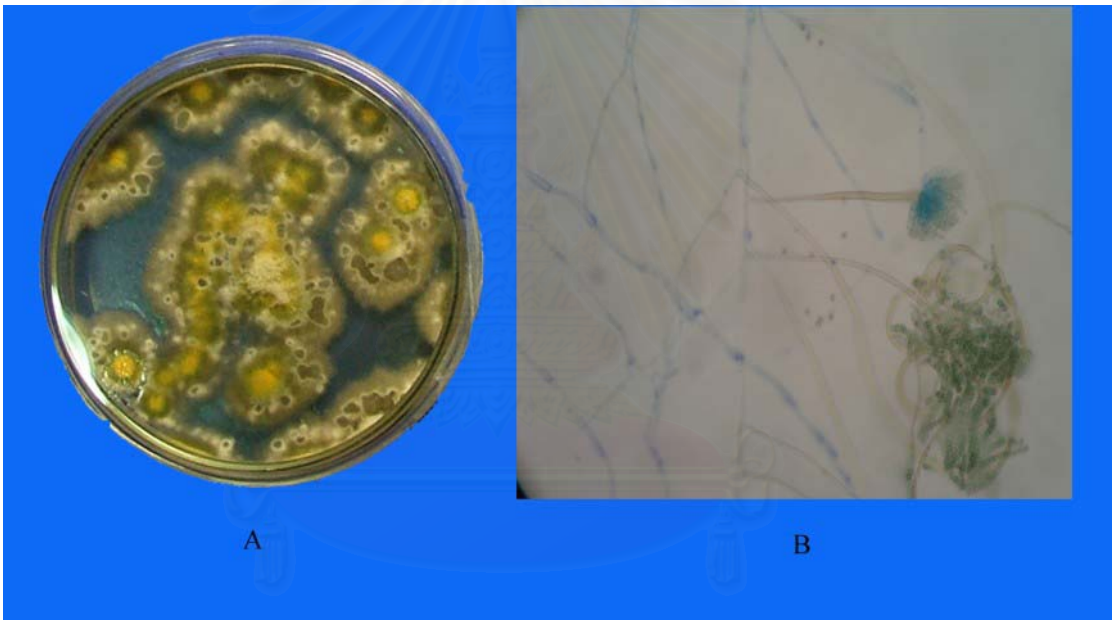


Figure 4.5 Colony (7 days) and microscopic characteristics of *Penicillium* sp.CuLm17

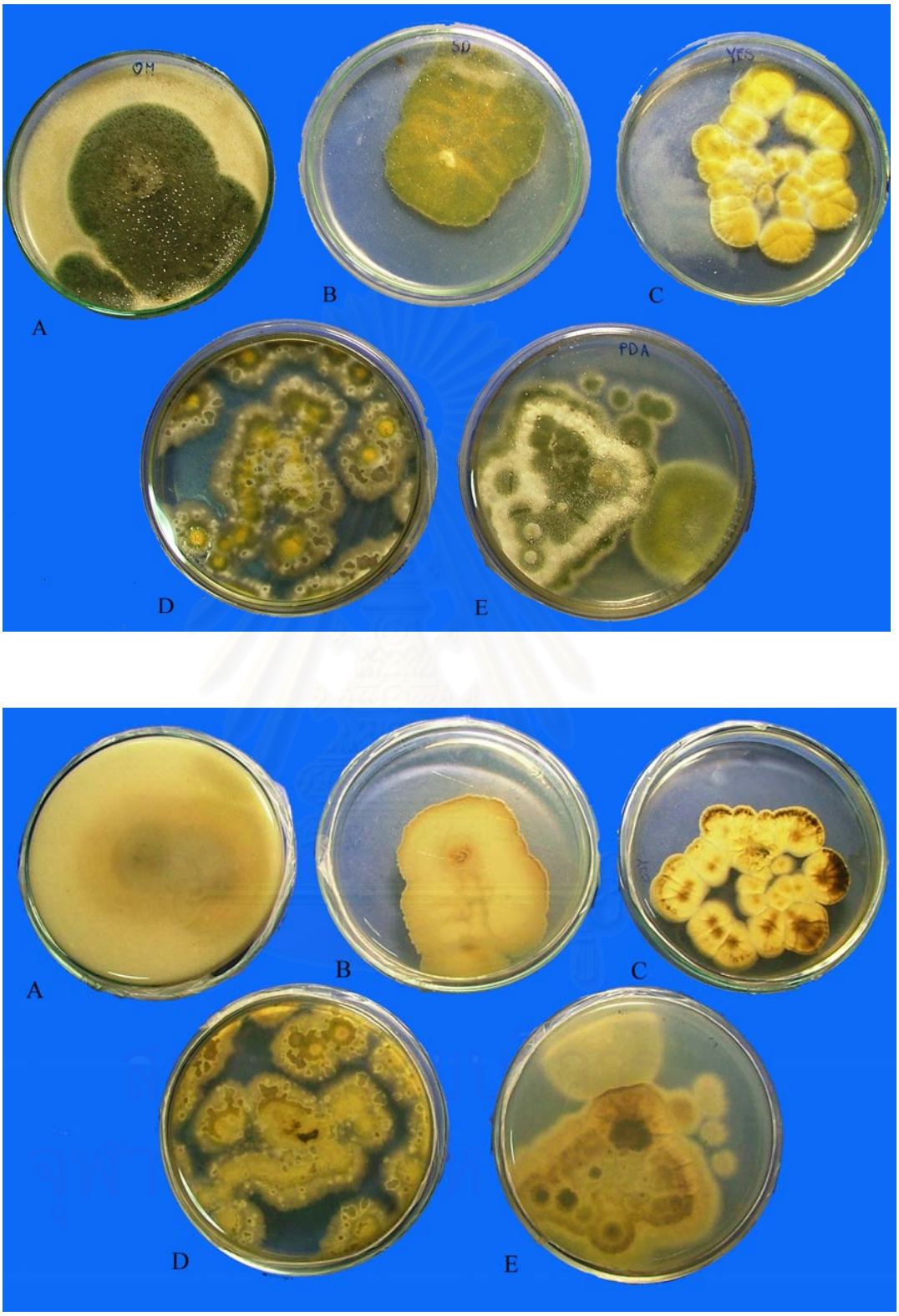


Figure 4.6 Colony characteristics of endophytic fungal isolate CuLm17. It was cultured on five different media (A=OMA, B=SDA, C=YES, D=MEA and E=PDA) after cultivation for 7 days at room temperature. Appearance on the obverse side (top), and on the reverse side (bottom).

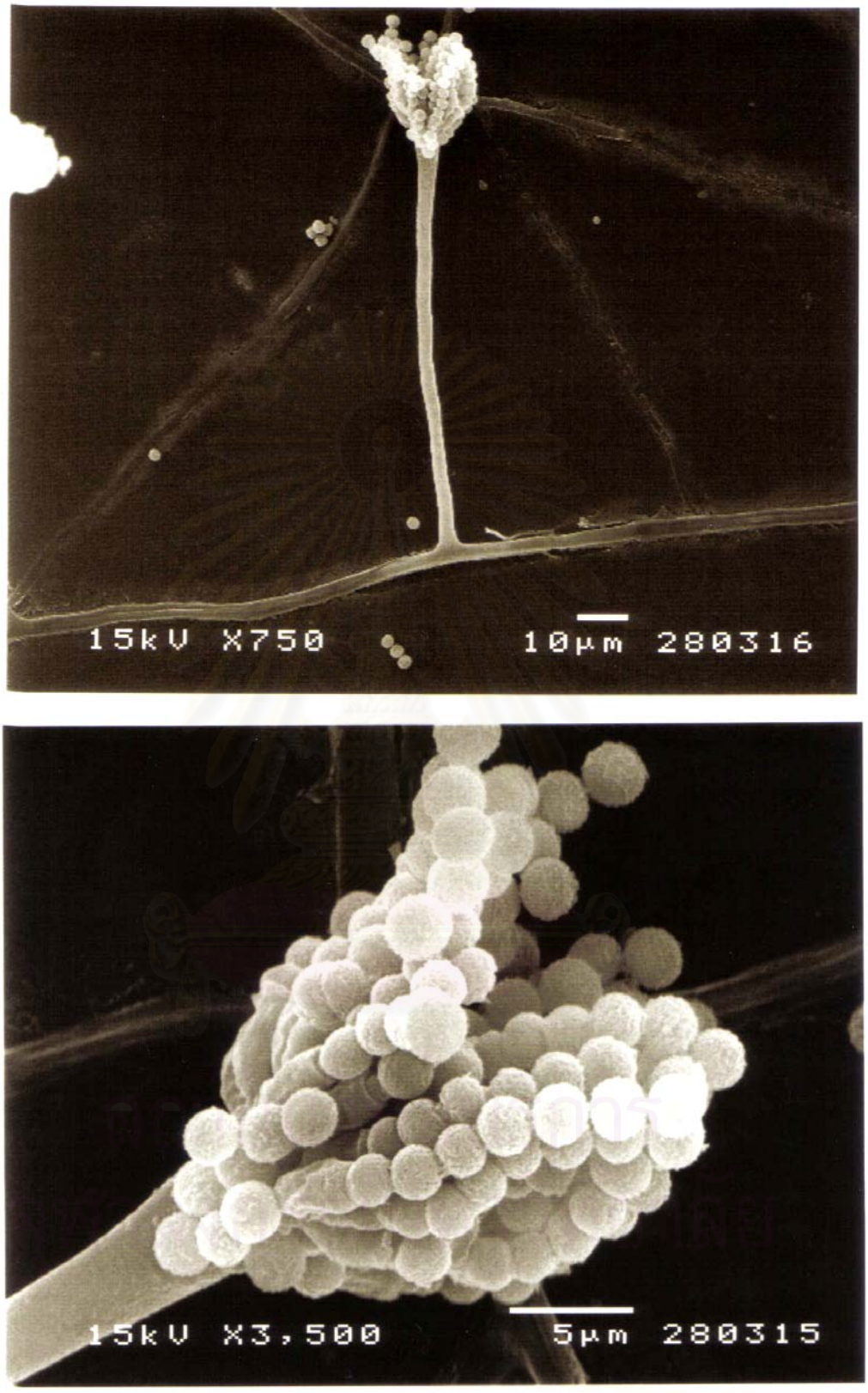
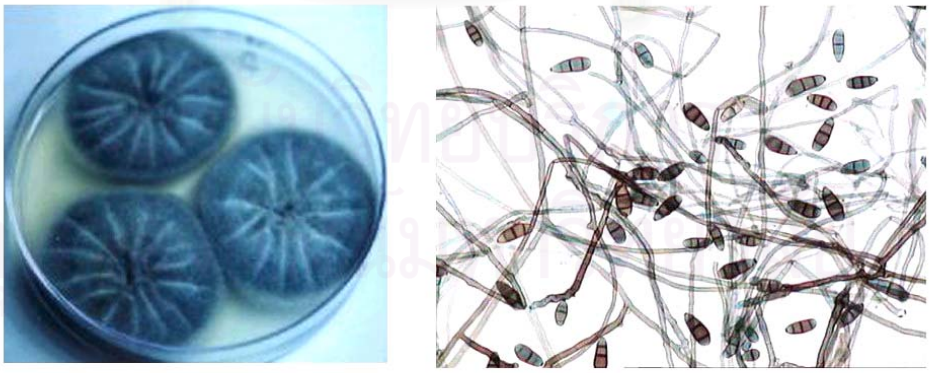


Figure 4.7 Scanning Electron Microscopic characteristic of endophytic fungal isolate CuLm17. (a), conidia on conidiophore (bar = 10 µm) (b), penicilli monovericillate (bar = 5 µm)

CURVULARIA (CuLm10 and CuLm16)

Curvularia produces rapidly growing, woolly colonies on potato dextrose agar at 25°C. From the front, the color of the colony is white to pinkish gray initially and turns to olive brown or black as the colony matures. From the reverse, it is dark brown to black. Septate, brown hyphae, brown conidiophores, and conidia are visualized. Conidiophores are simple or branched and are bent at the points where the conidia originate. This bending pattern is called sympodial geniculate growth. The conidia (8-14 x 21-35 µm), which are also called the poroconidia, are straight or pyriform, brown, multiseptate, and have dark basal protuberant hila. The septa are transverse and divide each conidium into multiple cells. The central cell is typically darker and enlarged compared to the end cells in the conidium. The central septum may also appear darker than the others. The swelling of the central cell usually gives the conidium a curved appearance. The number of the septa in the conidia, the shape of the conidia (straight or curved), the color of the conidia (dark vs pale brown), existence of dark median septum, and the prominence of geniculate growth pattern are the major microscopic features that help in differentiation of *Curvularia* spp. among each other. For instance, the conidia of *Curvularia lunata* have 3 septa and 4 cells, while those of *Curvularia geniculata* mostly have 4 septa and 5 cells. Colony and microscopic characteristic of *Curvularia* sp. showed in Figure 4.8.



(A) (B)

Figure 4.8 Colony and microscopic characteristics of *Curvularia* sp. Cultured on MEA (7 days) showing the pale brownish colony(A) , and lactophenol cotton blue mount at magnification 40x of the brown hyphae and poroconidia (B).

PHOMOPSIS (CuLm04, CuLm21 and CuBa09)

Mycelium immersed, branched, septate, hyaline to pale brown. Conidiomata eustromatic, immersed, brown to dark brown, septate or aggregated and confluent, globose, ampuliform or applanate, unilocular, multilocular or convoluted, thick-walled; walls of brown, thin- or thick-walled textura angularis, often somewhat darker in the upper region, lined by a layer of smaller-celled tissue. Ostiole single, or serveral in complex conidiomata, circular, often papillate. Conidiophores branched and septate at the base and above, occasionally short and only 1-2 septate, more frequently multiseptate and filiform, hyaline, formed from the inner cells of the locular walls. Conidiogenous cells enteroblastic, phialidic, determinate, integrated, rarely discrete, hyaline, cylindrical, apertures apical on long or short lateral and main branches of the conidiophores, collarette, channel and periclinal thickening minute. Conidia of two basic type, but in some species with intermediates between the two: α-conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end) but sometimes with more guttules, aseptate; β-conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate. Colony and microscopic characteristic of *Phomopsis* sp. showed in Figure 4.9.

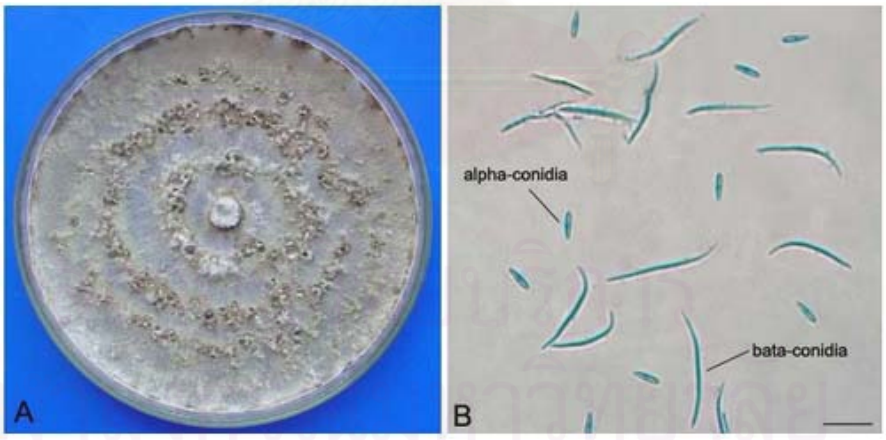


Figure 4.9 *Phomopsis* sp. A: Culture on MEA (14 days), and lactophenol cotton blue mount at magnification 40x of alpha and beta conidia (B).

TRICHODERMA (CuBr02)

Colonies of *Trichoderma* grow rapidly and mature in 5 days. At 25°C and on potato dextrose agar, the colonies are wooly and become compact in time. From the front, the color is white. As the conidia are formed, scattered blue-green or yellow-green patches become visible. These patches may sometimes form concentric rings. They are more readily visible on potato dextrose agar compared to Sabouraud dextrose agar. Reverse is pale, tan, or yellowish. Septate hyaline hyphae, conidiophores, phialides, and conidia are observed. *Trichoderma longibrachiatum* and *Trichoderma viride* may also produce chlamydospores. Conidiophores are hyaline, branched, and may occasionally display a pyramidal arrangement. Phialides are hyaline, flask-shaped, and inflated at the base. They are attached to the conidiophores at right angles. The phialides may be solitary or arranged in clusters. Conidia (3 µm in diameter, average) are one-celled and round or ellipsoidal in shape. They are smooth- or rough-walled and grouped in sticky heads at the tips of the phialides. These clusters frequently get disrupted during routine slide preparation procedure for microscopic examination. The color of the conidia is mostly green. Colony and microscopic characteristic of *Trichoderma* sp. showed in Figure 4.10.

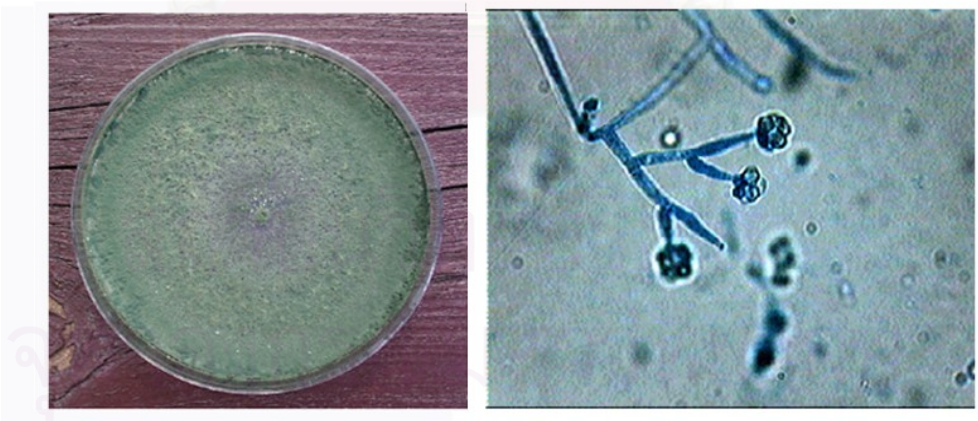


Figure 4.10 *Trichoderma* sp. A: Culture on MEA (10 days), and lactophenol cotton blue mount at magnification 100x conidia (B).

4.3 Crude extracts of endophytic fungi

Forty seven endophytic fungal isolates were grown in malt extract broth (MEB). Mycelium was separated from culture broth by filtration and extracted with Ethyl acetate. Culture broth was concentrated by evaporation, freeze dried and extracted with Ethyl acetate. From a total of 47 endophytic fungal cultures, a total of crude extracts 94 samples were obtained. The culture broth extracts and mycelium extracts were further tested for antimicrobial activity and metabolite profiles.

4.4 Enumeration of test microorganisms

Viable counts of bacteria and yeast were performed for standardized inocula whose turbidity matched a 0.5 McFarland standard. The CFU/ml values are shown in Table 4.4.

Table 4.4 Quantity of standardized inoculum of test microorganisms

Tested microorganisms	Quantity (CFU/ml)
Bacillus subtilis ATCC 6633	6.1×10^6
Staphylococcus aureus ATCC 25923	6.9×10^6
Escherichia coli ATCC 25922	2.1×10^7
Pseudomonas aeruginosa ATCC 27853	3.4×10^7
Candida albicans ATTC 10231	1.0×10^6

CFU: Colony forming unit

4.5 Determination of antimicrobial activities

Antimicrobial activities of culture broth extracts and mycelial extracts against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 were determined using the agar well diffusion method. The antimicrobial activities of the various isolates are shown in Figure 4.11 and Tables 4.5 - 4.12.

The antimicrobial activities of all isolated endophytic fungi are shown in Table 4.13 and Figure 4.12. The results showed that 92 crude extracts (97.87 % of total 94 crude extracts) had the antimicrobial activities against at least one tested microorganisms. They were active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 at 93.62%, 76.60%, 30.85%, 30.85%, and 89.36%, respectively. This indicated that *B. subtilis* ATCC 6633, the gram positive rod bacterium, was more sensitive to isolated endophytic fungi than other tested microorganisms.

As summarized in Table 4.14 and Figure 4.13, antimicrobial activities of the isolated endophytic fungi were classified into 6 types as no antimicrobial activity against test microorganisms (1.07%), inhibit 1 test microorganism only (3.19 %), inhibit 2 test microorganisms (18.09 %),inhibit 3 test microorganism (50 %), inhibit 4 test microorganisms (12.77%) and inhibit 5 test microorganisms (14.89 %).

The results in Table 4.15 and Figure 4.14 showed that 43 isolates of culture broth or/and mycelium extracts were active against *B. subtilis* ATCC 6633. Forty isolates showed activity with culture broth and mycelium extracts, 2 isolates showed activity with broth only and 1 isolates showed activity with mycelium only. Thirty-nine isolates of culture broth or/and mycelium extracts were active against *S. aureus* ATCC 25923. Thirty three isolates showed activity with culture broth and mycelium extracts, 2 isolates showed activity with broth only and 4 isolates showed activity with mycelium only. Twenty-one isolates of culture broth or/and mycelium extracts were active against *E. coli* ATCC 25922. Six isolates showed activity with culture broth and mycelium extracts, 8 isolates showed activity with broth only and 7 isolates showed activity with mycelium only. Nineteen isolates of culture broth or/and mycelium extracts were active against *P. aeruginosa* ATCC 27853. Nine isolates showed activity with culture broth and mycelium extracts, 6 isolates showed activity with broth only and 14 isolates showed activity with mycelium only. Fourty-three isolates of culture broth or/and mycelium extracts were active against *C. albicans* ATCC 10231. Fourty-two isolates showed activity with culture broth and mycelium extracts and 1 isolate showed activity with mycelium. Most biologically active compounds of these endophytic fungi isolates were produced and accumulated within mycelium and secreted into the culture broth.

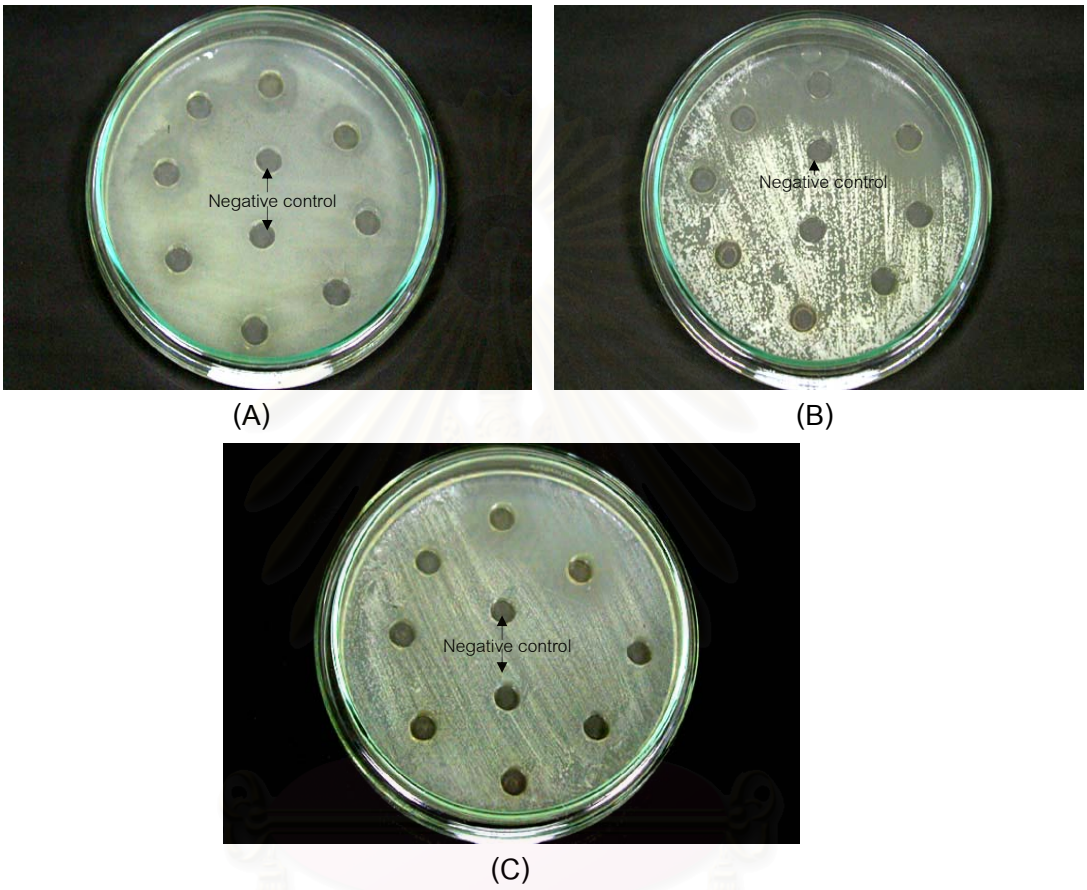


Figure 4.11 Agar well diffusion method for antimicrobial activity. A: Against *B. subtilis* ATCC 6633. B: Against *C. albicans* ATCC 10231. C: Against *S. aureus* ATCC 25923. Inhibition zone indicated growth inhibition. Negative control ; 10% DMSO in distilled water

Table 4.5 Antimicrobial activities of culture broth extracts of endophytic fungi isolated from young leaves

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuLy01	++	+	-	-	+
CuLy02	+	-	-	-	+
Culy03	++	+	-	-	+
CuLy04	+	-	-	-	+
CuLy05	+	-	-	-	-
CuLy06	+	+	-	+	+
CuLy07	++	+	-	-	+

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of culture broth extracts was 10mg/ml.

Table 4.6 Antimicrobial activities of mycelial extracts of endophytic fungi isolated from young leaves

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuLy01	+	+	-	-	+
CuLy02	+	+	-	-	+
Culy03	+	-	+	-	+
CuLy04	++	-	-	-	+
CuLy05	+	+	-	-	-
CuLy06	+	+	+	+	+
CuLy07	+	-	-	-	+

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of mycelial extracts was 10mg/ml.

Table 4.7 Antimicrobial activities of culture broth extracts of endophytic fungi isolated from mature leaves

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuLm01	+	-	-	-	+
CuLm02	-	+	-	-	+
CuLm03	++	+	+	+	+
CuLm04	+	+	-	-	+
CuLm05	+	-	+	-	+
CuLm06	+	-	-	-	+
CuLm07	+	-	+	+	+
CuLm08	+	++	+	-	-
CuLm09	-	+	+	-	+
CuLm10	++	+	+	+	+
CuLm11	+	+	-	+	+
CuLm12	++	+	-	+	+
CuLm13	+	-	-	+	-
CuLm14	+	+	-	-	+
CuLm15	++	+	+	-	+
CuLm16	+	+	-	-	+
CuLm17	++	++	+	+	+
CuLm18	+	+	-	-	+
CuLm19	+	-	-	+	-
CuLm20	+	+	-	+	+
CuLm21	+	+	+	+	+
CuLm22	++	+	+	-	+
CuLm23	+	-	+	-	+
CuLm24	+	+	-	-	+
CuLm25	+	+	-	-	+

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of culture broth extracts was 10mg/ml.

Table 4.8 Antimicrobial activities of mycelial extracts of endophytic fungi isolated from mature leaves

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuLm01	+	+	-	-	+
CuLm02	-	++	-	-	+
CuLm03	+	+	-	-	+
CuLm04	+	+	+	+	+
CuLm05	+	-	-	-	+
CuLm06	++	+	+	-	+
CuLm07	+	-	-	+	+
CuLm08	+	+	-	-	-
CuLm09	+	+	-	-	+
CuLm10	+	+	-	-	+
CuLm11	+	++	+	+	+
CuLm12	+	+	-	-	+
CuLm13	+	-	-	-	-
CuLm14	+	+	-	-	+
CuLm15	++	+	+	+	+
CuLm16	+	+	-	-	+
CuLm17	++	++	++	+	+
CuLm18	+	++	-	-	+
CuLm19	+	-	-	-	-
CuLm20	-	+	-	-	+
CuLm21	++	+	+	+	+
CuLm22	+	+	-	-	+
CuLm23	+	-	+	-	+
CuLm24	+	+	-	-	+
CuLm25	++	+	-	-	+

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration mycelial extracts was 10mg/ml.

Table 4.9 Antimicrobial activities of culture broth extracts of endophytic fungi isolated from branches

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuBr01	+	+	-	-	+
CuBr02	+	+	-	-	+
CuBr03	+	-	+	-	+
CuBr04	++	++	+	+	+
CuBr05	+	++	-	+	+
CuBr06	-	+	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of culture broth extracts was 10mg/ml.

Table 4.10 Antimicrobial activities of mycelial extracts of endophytic fungi isolated from branches

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuBr01	+	++	++	+	+
CuBr02	+	+	-	-	+
CuBr03	+	-	+	-	+
CuBr04	++	+	-	-	+
CuBr05	+	+	-	+	+
CuBr06	-	+	-	-	+

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of mycelial extracts was 10mg/ml.

Table 4.11 Antimicrobial activities of culture broth extracts of endophytic fungi isolated from barks

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuBa01	-	+	-	+	+
CuBa02	+	+	-	-	+
CuBa03	+	+	+	+	+
CuBa04	++	-	-	-	+
CuBa05	+	+	-	-	+
CuBa06	+	+	-	-	+
CuBa07	+	+	+	+	+
CuBa08	+	+	-	-	+
CuBa09	++	+	-	-	+

Table 4.12 Antimicrobial activities of mycelial extracts of endophytic fungi isolated from barks

Isolated endophytic fungi strain	Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
CuBa01	-	+	-	+	+
CuBa02	+	++	-	-	+
CuBa03	+	+	+	+	+
CuBa04	+	-	-	-	+
CuBa05	++	+	+	-	+
CuBa06	+	+	-	-	+
CuBa07	+	++	+	+	+
CuBa08	+	+	-	-	+
CuBa09	+	+	-	+	+

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of culture broth and mycelial extracts was 10mg/ml.

Table 4.13 The amount of active endophytic fungal isolates

Test microorganisms	Number of active isolates (isolates)	Percent of active isolate (%)
B. subtilis ATCC 6633	88	93.62
S. aureus ATCC 25923	72	76.60
E. coli ATCC 25922	29	30.85
P. aeruginosa ATCC 27853	29	30.85
C. albicans ATCC 10231	84	89.36

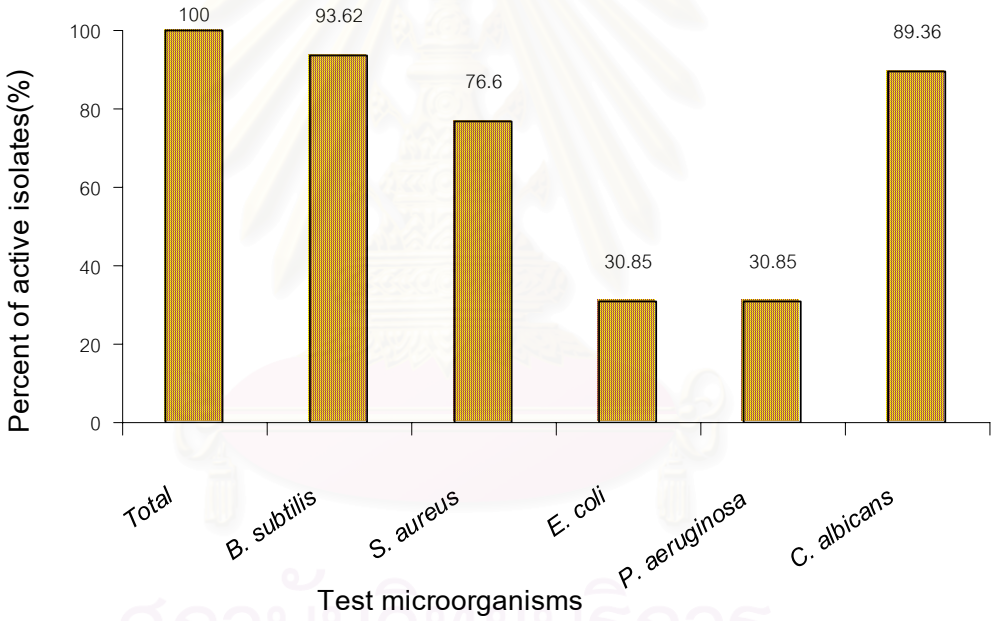


Figure 4.12 The amount of active endophytic fungal isolates

Table 4.14 The amount of endophytic fungal isolates showed antimicrobial activities against test microorganisms

Amount inhibited test microorganisms(species)	Number of active isolates (isolates)	Percent of active isolate (%)
0	2	2.13
1	3	3.19
2	17	18.09
3	47	50
4	12	12.77
5	14	14.89

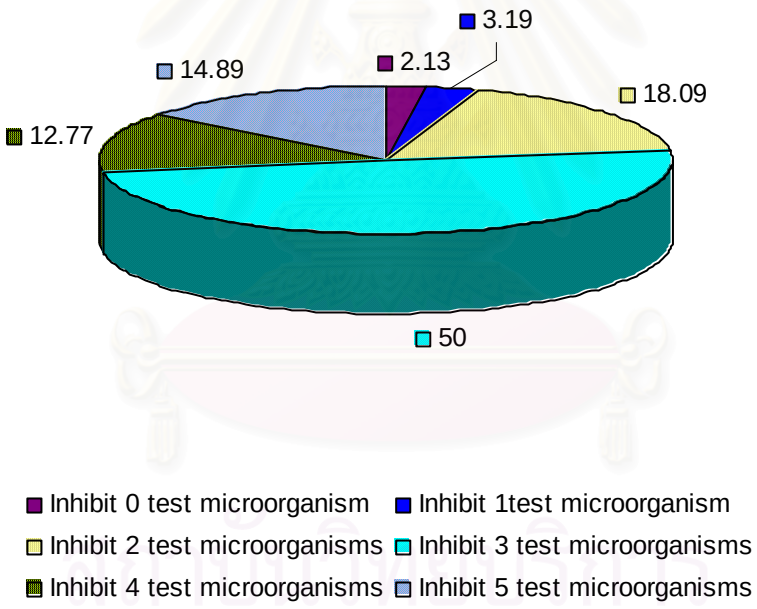


Figure 4.13 A summary of antimicrobial activities of isolated endophytic fungi.

Table 4.15 A summary of the agar well diffusion method assay results for the antimicrobial activities of endophytic fungi

Crude extract	Test microorganisms and number of active isolate frequency				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
Culture broth and mycelium	40	33	6	9	42
Culture broth only	2	2	8	6	0
Mycelium only	2	4	7	4	1
Total	44	39	21	19	43

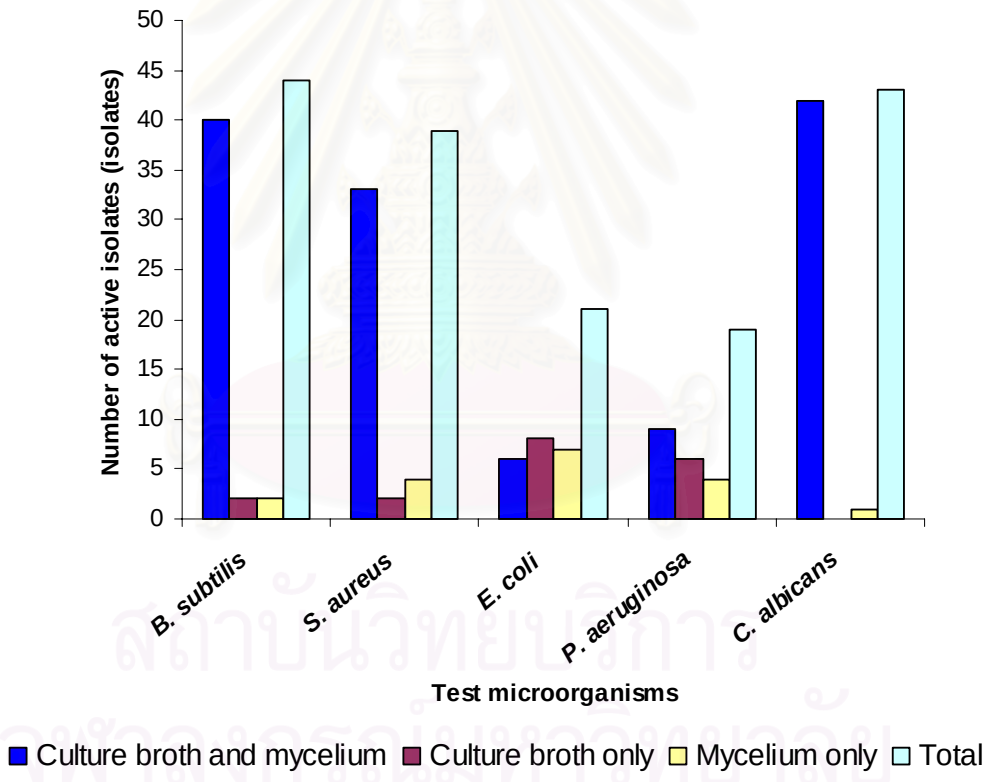


Figure 4.14 A summary of the agar well diffusion method assay results for the antimicrobial activity of crude extract from endophytic fungi.

Among of these fungi, endophytic fungal isolate CuLm17 had the best antimicrobial activities because it inhibited the growth of all types of tested microorganisms with highest activities, including gram positive bacteria *(B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923), gram negative bacteria (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and yeast (*C. albicans* ATCC 10231) as showed in Table 4.8 and Table 4.9. For this reason, endophytic fungal isolate CuLm17 was selected for identification and isolation bioactive metabolites.

In the recent, several researchers were performed for antimicrobial activities of endophytic fungi from many different plants. For example, Schulz *et al*. (2002) reported 80% of the endophytic fungi from plants inhibited at least one of the test organisms for antibacterial, fungicidal, algicidal or herbicidal activities and 51% biologically active substances isolated from endophytic fungi were previous unknown compound. These observations suggested that endophytic fungi may have pharmaceutical potential and could be a promising source for antimicrobial compounds.

4.6 Determination of metabolite profiles

Metabolite profiles of culture broth extracts and mycelium extracts were examined by TLC technique and detected under UV light at 254 and 365 nm, with iodine vapor and spraying with vanillin reagent then heating until the colors developed.

For metabolite profiles of culture broth extracts, the result showed variety of metabolite profiles. Most spots in the metabolite profiles from culture broth extracts were detected under UV light at 254 nm and spraying with vanillin reagent while some compounds showed spots when detected under UV light at 365 nm and with iodine vapor.

For metabolite profiles of mycelium extracts, the result showed variety of metabolite profiles. Most spots in the metabolite profiles from mycelium extracts were detected under UV light at 254 nm and spraying with vanillin reagent while some compounds showed spots when detected under UV light at 365 nm and with iodine vapor.

The crude extracts from endophytic fungal isolate CuLm17 showed various spots which

good separation in 50% hexane in EtOAC. Moreover, the metabolite profiles of CuLm17 crude mycelium extract showed a large spot when detected under UV light at 254 nm and spraying with vanillin reagent. TLC pattern of the crude extract samples were shown in Figure 4.15 and TLC pattern of the EtOAc crude extracts from endophytic fungal isolate CuLm17 were shown in Figure 4.16.

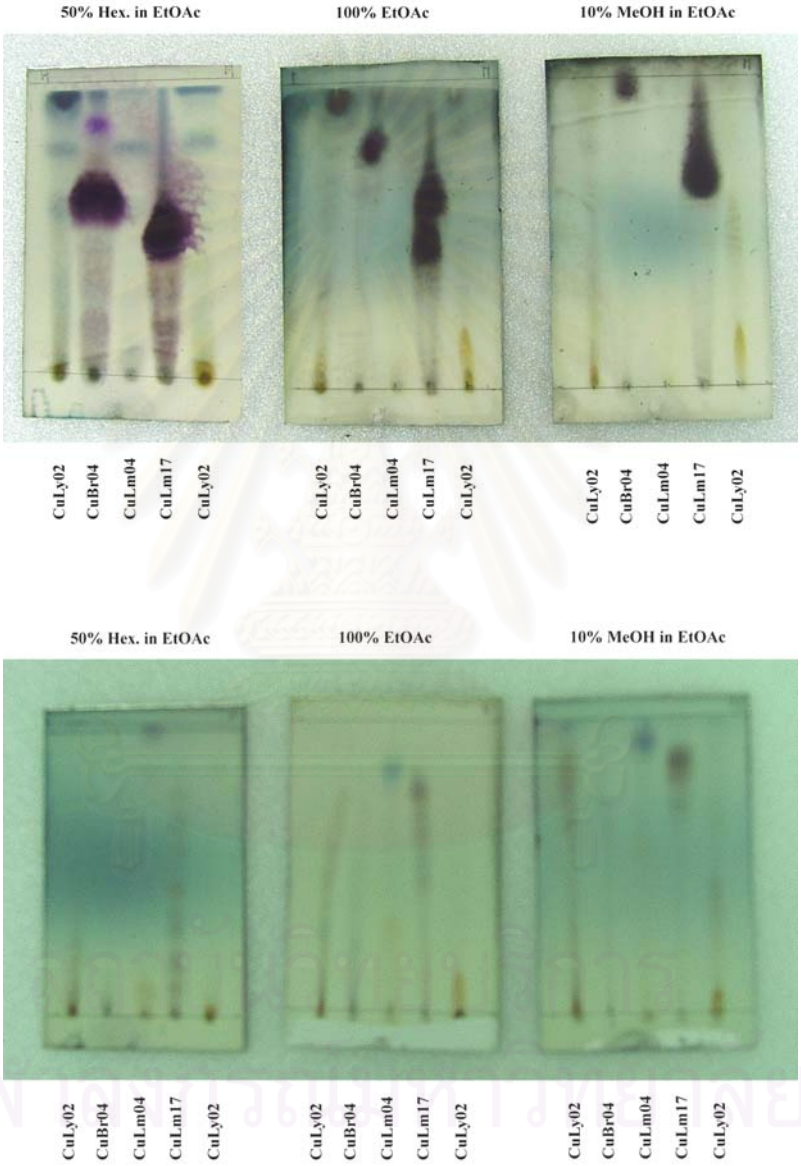


Figure 4.15 TLC pattern of the crude extract samples.

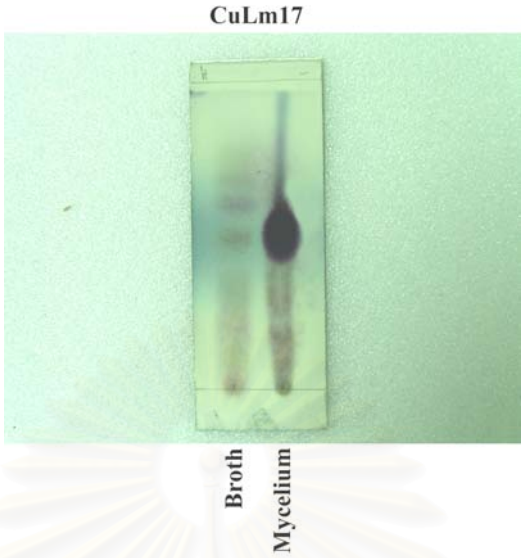


Figure 4.16 TLC pattern of the EtOAc crude extracts from endophytic fungal isolate CuLm17 when eluted with 50%hexane in EtOAc.

4.7 Cultivation and metabolites extraction of endophytic fungal isolate CuLm17

Endophytic fungal isolate CuLm17 was cultivated statically in 18 l of Malt Extract Broth (MEB) at room temperature for 8 weeks because it had the highest concentration of metabolites compound when examined by siliga gel TLC. Culture broth was filtered and gave 13.97 l of culture broth free mycelium and 463.28 g of mycelial dry weight to yield 4 mg of crude hexane, 17.9 g of crude EtOAc and 2 g of crude MeOH from EtOAc crude mycelium extracts, 200 mg of crude EtOAc and 3.6 g of crude MeOH from MeOH crude mycelium extracts, 1.86 g of crude EtOAc and 100 mg of crude MeOH from EtOAc crude culture broth extracts , 400 mg of crude EtOAc and 150 g of crude MeOH from MeOH crude cultivation broth extracts.

4.8 Determination of antimicrobial activities of the partitioned fractions from crude extracts

All crude extracts of culture broth and mycelium of endophytic fungal isolate CuLm17 were determined for their antimicrobial activities by agar well diffusion method. Both crude extract were dissolved in 10 % DMSO in sterile distilled water at

concentration of 10 mg/ml (1 μl/ 6 mm diameter agar well). The negative control in this assay was 10 % DMSO in sterile distilled water. The antimicrobial activities were evaluated from the inhibition zone diameter (mm) against tested microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231. Antimicrobial activities of all crude extracts of culture broth and mycelial of endophytic fungal isolate CuLm17 are presented in Table 4.16.

Table4.16 Antimicrobial activities of the partitioned fractions from crude extracts

Test microorganisms	Crude extracts/ Inhibition zone diameter(mm)											
	Broth						Mycelium					
	EtOAc extraction			MeOH extraction			EtOAc extraction			MeOH extraction		
	Hex	EtOAc	MeOH	Hex	EtOAc	MeOH	Hex	EtOAc	MeOH	Hex	EtOAc	MeOH
B. subtilis	ND	35	-	ND	8	18	ND	25	25	ND	11	10
S. aureus	ND	35	-	ND	7	10	ND	38	28	ND	19	25
E. coli	ND	8	8	ND	9	9	ND	9	8	ND	8	8
P. aeruginosa	ND	9	-	ND	7	9	ND	9	8	ND	11	9
C. albicans	ND	9	-	ND	7	8	ND	10	12	ND	-	8

- : No inhibition zone. ND : not determined = no antimicrobial test

Wells were made in the agar by removing disks cut (6 mm diameter). Concentration of crude extracts was 10 mg/ml.

As reported in Table 4.16, EtOAc crude extract which partitioned with EtOAc of culture broth and mycelium, MeOH crude extract which partitioned with MeOH of culture broth and mycelium and MeOH crude extract which partitioned with EtOAc of culture broth had antimicrobial activities against 5 test microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans* ATCC 10231. EtOAc crude extract which partitioned with EtOAc of culture broth and mycelium was chosen for further study for bioactive compounds. Because of these crude extracts were the highest active against a large number of test microorganisms.

4.9 Isolation and purification of bioactive compounds from endophytic fungal isolate CuLm17

EtOAc crude which partitioned with EtOAc of culture broth and mycelial extract of endophytic fungal isolate CuLm17 cultivated in 18 l MEB was separated and purified.

4.9.1 Isolation and purification of EtOAc crude from mycelial extract

The EtOAc crude which partitioned with EtOAc of mycelium (17.90 g) was separated by silica gel column chromatography (silica gel, 375.5 g) using eluents of increasing polarity from hexane to EtOAc to MeOH. The results from the separation of EtOAc crude of mycelial extract obtain twenty combined fractions. Combined fraction M10 (540 mg) was eluted with 20-25% EtOAc in Hexane. And then it was purified by washing with Hexane and EtOAc and re-crystallization with MeOH to yield compound ME1 (500 mg, 2.79% by weight of EtOAc crude of mycelium extract). Combined fraction M11 (6.1 g) was eluted with 25-45% EtOAc in Hexane. And then it was washed with Hexane and EtOAc to yield compound ME2 (6 g, 33.52% by weight of EtOAc crude of mycelium extract).

4.9.2 Isolation and purification of EtOAc crude from culture broth extract

The EtOAc crude which partitioned with EtOAc of culture broth (1.86 g) was separated by silica gel column chromatography (silica gel, 77.31 g) using eluents of increasing polarity from hexane to EtOAc to MeOH. The results from the separation of EtOAc crude of culture broth extract obtain 23 combined fractions.

4.10 Antimicrobial activities of the pool fractions from crude extracts

The combined fractions from silica gel column chromatography of endophytic fungal isolate CuLm17 were determined for their antimicrobial activities by agar well diffusion method. Both crude extract were dissolved in 10 % DMSO in sterile distilled water at concentration 1 mg/ml of (100 µl/ 6 mm diameter agar well). The negative control in this assay was 10 % DMSO in sterile distilled water. The antimicrobial activities were evaluated from the inhibition zone diameter (mm) against test microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 10231. Antimicrobial activities of the combined fractions are presented in Table 4.17 -4.18.

Table 4.17 Antimicrobial activities of the fractions of EtOAc crude from mycelia extracts

Fraction code	Inhibition zone diameters (mm) /Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
M01	-	-	-	-	-
M02	-	-	-	-	-
M03	-	-	-	-	-
M04	+	-	-	-	-
M05	-	+	-	-	-
M06	-	-	-	-	-
M07	-	-	-	-	-
M08	-	-	-	-	-
M09	-	-	-	-	+
M10	+	+	-	+	-
M11	+	+	+	-	-
M12	+	+	-	-	-
M13	-	-	-	-	-
M14	-	-	-	-	-
M15	-	-	-	-	-
M16	-	-	-	-	-
M17	-	-	-	-	-
M18	-	-	-	-	-
M19	-	-	-	-	-
M20	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of pool fractions was 1 mg/ml.

Table 4.18 Antimicrobial activities of the fractions of EtOAc crude from culture broth extracts

Fraction code	Inhibition zone diameters (mm) /Test microorganisms				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
B01	-	-	-	-	-
B02	-	-	-	-	-
B03	-	-	-	-	-
B04	+	-	-	-	-
B05	+	-	-	-	-
B06	-	-	-	-	-
B07	-	-	-	-	-
B08	+	-	-	-	-
B09	+	-	-	-	+
B10	+	-	+	-	-
B11	-	-	-	-	-
B12	+	-	-	-	-
B13	-	-	-	-	-
B14	-	-	-	-	-
B15	-	-	-	-	-
B16	-	-	-	-	-
B17	-	-	-	-	-
B18	-	-	-	-	-
B19	-	-	-	-	-
B20	-	-	-	-	-
B21	-	-	-	-	-
B22	-	-	-	-	-
B23	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition. Concentration of pool fractions was 1 mg/ml.

4.11 Structure elucidation of the pure compound from endophytic fungal isolate CuLm17

4.11.1 Structure elucidation of Compound ME1

Compound ME1 was purified by re-crystallization with MeOH and washing with hexane and EtOAc to obtain as white crystal (500 mg or 0.11 % product recovery). Chemical structures of compound ME1 was elucidated by its spectroscopic data, including IR, UV, NMR and MS spectra with physical properties.

IR spectrum of compound ME1 is shown in Figure B2 in appendix B and the absorption bands were assigned as shown in Table 4.19. The IR spectrum indicated important absorption band at 3439 cm^{-1} (O-H stretching vibration of alcohol), at 1636 cm^{-1} (C=O vibration of carbonyl group), at 1265 and 1129 cm^{-1} (C-O stretching vibration) .

Table 4.19 The IR absorption bands assignment of compound ME1

Wave number (cm^{-1})	Intensity	Tentative assignment
3439	Broad, Strong	O-H stretching vibration of alcohol
1740	Sharp, Weak	C=O vibration of carbonyl group
1265 and 1129	Sharp, Weak	C-O stretching vibration in esters

EI-MS spectrum (Figure B10 in appendix B) of compound ME1 showed the M^+ ion peak at *m/z* 380. The results from MS and ^{13}C-NMR indicated that molecular formula of compound ME1 was $C_{23}H_{24}O_5$.

^{1}H-NMR spectrum (Figure B3 in appendix B) of compound ME1 showed proton signals which belong to two aromatic protons at δ 6.43 and 6.52 ppm, two olefinic protons at 5.41 and 5.55 ppm, six methyl signals (-CH_3 -) at 1.73, 1.81, 1.90 2.08, 2.16 and 2.18 ppm .

The ^{13}C-NMR spectrum (Figure B4 in appendix B) of compound ME1 showed 23 signals, which two signals belong to carbonyl group of ester at 165.71 ppm, twelve signals of aromatic carbon at 162.32, 159.76, 152.51, 148.69, 143.56, 142.37, 136.29,

136.04, 134.26, 114.87, 113.74 and 111.28 ppm, four olefinic carbons at 124.93, 123.78, 111.57 and 111.12 ppm, and six signals of methyl group carbon at 7.6, 7.9, 12.71, 12.87, 16.45 and 16.85 ppm.

The information from 2D-NMR techniques including HSQC, HMBC, COSY, and NOESY (Fig B5-B8) were presented in Table 4.20 and elucidated the structure of compound ME1 as shown in Figure 4.17 and 4.18. Structure of folipastatin as shown in Figure 4.19.

Moreover, the structure of compound ME1 was also confirmed by X-ray diffraction analysis, which is shown in Figure 4.20. X-ray diffraction data are presented in Table 4.21- 4.24

Table 4.20 HSQC, HMBC and NOESY spectral data of compound ME1

Position	δC	δH	HMBC (H to C)	NOESY
2	142.4 (s)	-	-	-
3	143.6 (s)	-	-	-
5	165.7 (s)	-	-	-
6	111.3 (d)	-	-	-
7	162.3 (s)	-	-	-
8	113.7 (s)	-	-	-
9	159.8 (s)	-	-	-
10	111.6 (d)	6.52 (1H, s)	C-6, C-8, C-9, C-16	H-17(5.41)
11	148.7 (s)	-	-	-
12	114.9 (s)	-	-	-
13	152.5 (s)	-	-	-
14	111.1 (s)	6.43 (1H, s)	C-2, C-12, C-13, C-21	H-23 (1.81)
15	136.3 (s)	-	-	-
16	136.0 (s)	-	-	-
17	123.8 (d)	5.41 (1H, dq, *J* = 1.2 and 6.8)	C-11, C-18, C-19	H-10 (6.52), H-18 (1.73)
18	12.9(q)	1.73 (3H, d, *J* = 7.2)	C-16, C-17	H-17(5.41), H-19 (1.90)
19	16.4 (q)	1.90 (3H, s)	C-11, C-16, C-17	H-18 (1.73)
20	7.6(q	2.16 (3H, s)	C-7, C-8	H-23 (1.81), H-24 (2.08)
21	134.3 (s)	-	-	-
22	124.9 (s)	5.55 (1H, dq, *J* = 1.2 and 6.8)	C-15, C-23, C-24	H-14 (6.43), H-23 (1.81)
23	12.7 (q)	1.81 (3H, d, *J* = 6.8)	C-21, C-22	-
24	16.8 (q)	2.08 (3H, s)	C-15, C-21, C-22	H-20 (2.16), H-23 (1.81)
25	7.9(q)	2.18 (3H, s)	C-3, C-12, C-13	-

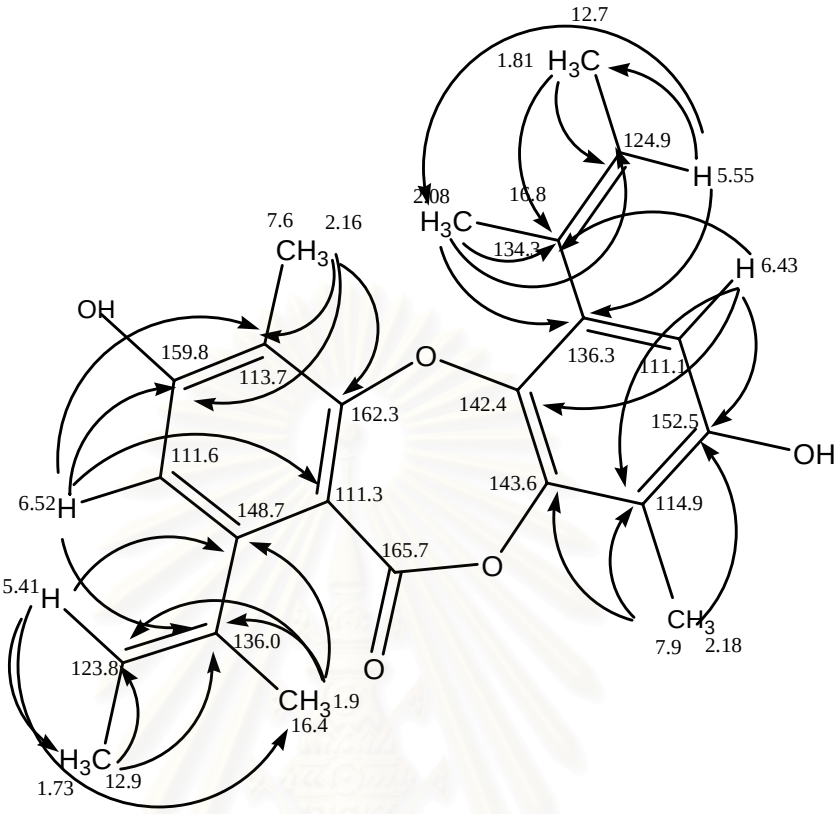


Figure 4.17 HMBC correlation of compound ME1

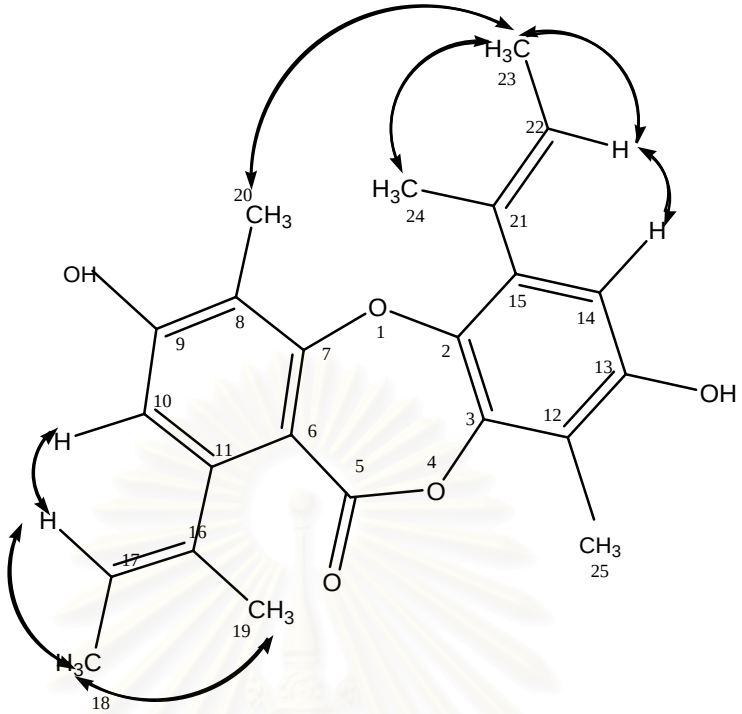


Figure 4.18 NOESY correlation of compound ME1

Considerably, this structure appears to be a possible for compound ME1. In view of its similarity to folipastatin.

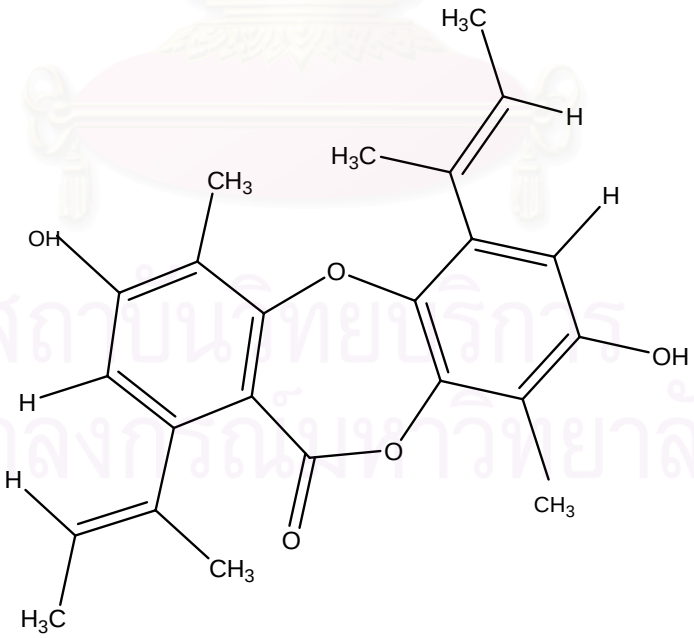


Figure 4.19 The structure of folipastatin

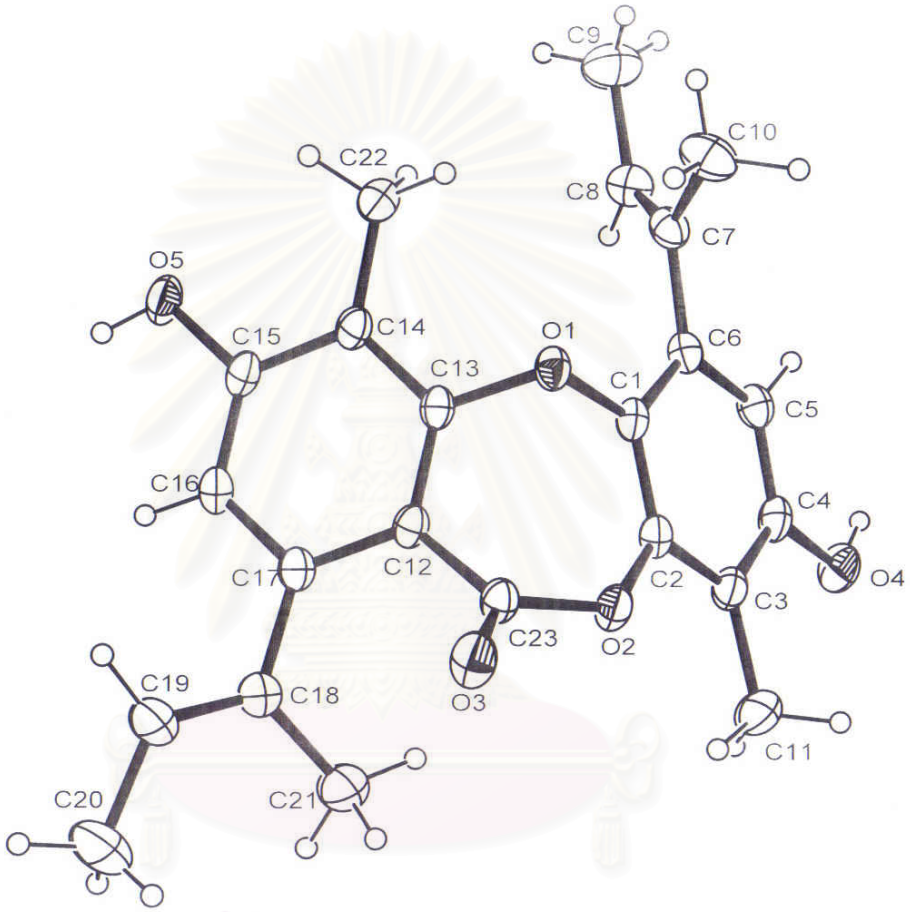


Figure 4.20 The ORTEP drawing of compound ME1

Table 4.21 Crystal data and structure refinement for compound ME1

Empirical formula	$C_{23}H_{24}O_5$
Formula weight	380.42
Temperature	293(2) K
Wavelength	0.71073 A
Crystal system, space group	monoclinic, P21/c
Unit cell dimensions	a = 11.2051(6) A alpha = 90 deg.
	b = 18.8714(10) A beta = 118.6920(10) deg.
	c = 10.8783(6) A gamma = 90 deg.
Volume	2017.84(19) A^3
Z, Calculated density	4, 1.252 Mg/m^3
Absorption coefficient	0.088 mm^-1
F(000)	808
Crystal size	0.57 x 0.49 x 0.25 mm
Theta range for data collection	2.16 to 26.37 deg.
Limiting indices	-13<=h<=13, -23<=k<=23, -13<=l<=13
Reflections collected / unique	15927 / 4118 [R(int) = 0.0192]
Completeness to theta = 26.37	99.8 %
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4118 / 0 / 325
Goodness-of-fit on F^2	1.033
Final R indices [I>2sigma(I)]	R1 = 0.0471, wR2 = 0.1357
R indices (all data)	R1 = 0.0533, wR2 = 0.1415
Largest diff. peak and hole	0.301 and -0.198 e.A^-3

Table 4.22 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for compound ME1.

	x	y	z	U(eq)*
C(1)	4688(1)	4970(1)	2108(1)	33(1)
C(2)	5842(2)	4780(1)	2033(1)	36(1)
C(3)	6514(2)	4140(1)	2550(2)	40(1)
C(4)	5976(2)	3698(1)	3192(2)	40(1)
C(5)	4805(2)	3874(1)	3240(2)	40(1)
C(6)	4130(1)	4511(1)	2696(1)	35(1)
C(7)	2832(2)	4670(1)	2703(2)	40(1)
C(8)	2794(2)	4685(1)	3898(2)	50(1)
C(9)	1583(3)	4821(2)	4089(3)	76(1)
C(10)	1613(2)	4772(1)	1284(2)	61(1)
C(11)	7724(2)	3931(1)	2396(2)	57(1)
C(12)	6130(2)	6293(1)	2414(1)	35(1)
C(13)	4896(1)	6181(1)	2405(1)	33(1)
C(14)	4415(2)	6620(1)	3094(2)	37(1)
C(15)	5308(2)	7154(1)	3914(2)	39(1)
C(16)	6568(2)	7256(1)	3991(2)	42(1)
C(17)	6989(2)	6848(1)	3209(2)	38(1)
C(18)	8308(2)	7011(1)	3243(2)	48(1)
C(19)	8608(2)	7676(1)	3127(3)	74(1)
C(20)	9892(3)	7932(2)	3146(5)	125(1)
C(21)	9266(2)	6415(1)	3425(3)	67(1)
C(22)	3021(2)	6548(1)	2940(2)	49(1)
C(23)	6381(2)	5910(1)	1368(2)	38(1)
O(1)	4074(1)	5630(1)	1583(1)	36(1)
O(2)	6316(1)	5196(1)	1290(1)	42(1)
O(3)	6652(1)	6213(1)	562(1)	53(1)
O(4)	6654(1)	3075(1)	3757(1)	53(1)
O(5)	4892(1)	7573(1)	4663(1)	50(1)

U(eq) is defined as one third of the trace of the orthogonalized *Uij* tensor.

Table 4.23 Bond lengths (A°) and angles [deg] for compound ME1.

Bond Distances	(A°)
C(1)-C(2)	1.382(2)
C(1)-C(6)	1.3909(19)
C(1)-O(1)	1.4049(16)
C(2)-C(3)	1.392(2)
C(2)-O(2)	1.4013(17)
C(3)-C(4)	1.396(2)
C(3)-C(11)	1.496(2)
C(4)-O(4)	1.3739(18)
C(4)-C(5)	1.379(2)
C(5)-C(6)	1.391(2)
C(6)-C(7)	1.489(2)
C(7)-C(8)	1.321(2)
C(7)-C(10)	1.504(2)
C(8)-C(9)	1.490(3)
C(12)-C(13)	1.393(2)
C(12)-C(17)	1.405(2)
C(12)-C(23)	1.4844(19)
C(13)-C(14)	1.387(2)
C(13)-O(1)	1.3938(16)
C(14)-C(15)	1.400(2)
C(14)-C(22)	1.495(2)
C(15)-O(5)	1.3674(18)
C(15)-C(16)	1.387(2)
C(16)-C(17)	1.387(2)
C(17)-C(18)	1.492(2)
C(18)-C(19)	1.321(3)
C(18)-C(21)	1.501(3)
C(19)-C(20)	1.509(3)
C(23)-O(3)	1.2020(18)
C(23)-O(2)	1.3489(18)

Table 4.23 (continued)

Bond Distances	(A°)
C(2)-C(1)-C(6)	120.42(12)
C(2)-C(1)-O(1)	119.71(12)
C(6)-C(1)-O(1)	119.87(12)
C(1)-C(2)-C(3)	122.78(13)
C(1)-C(2)-O(2)	120.88(12)
C(3)-C(2)-O(2)	116.00(12)
C(2)-C(3)-C(4)	116.11(13)
C(2)-C(3)-C(11)	121.81(14)
C(4)-C(3)-C(11)	122.07(14)
O(4)-C(4)-C(5)	121.38(14)
O(4)-C(4)-C(3)	117.09(14)
C(5)-C(4)-C(3)	121.52(13)
C(4)-C(5)-C(6)	121.67(14)
C(5)-C(6)-C(1)	117.41(13)
C(5)-C(6)-C(7)	120.36(13)
C(1)-C(6)-C(7)	122.18(12)
C(8)-C(7)-C(6)	120.14(14)
C(8)-C(7)-C(10)	124.40(16)
C(6)-C(7)-C(10)	115.39(15)
C(7)-C(8)-C(9)	127.02(18)
C(13)-C(12)-C(17)	120.07(13)
C(13)-C(12)-C(23)	118.28(13)
C(17)-C(12)-C(23)	120.59(13)
C(14)-C(13)-O(1)	118.87(12)
C(14)-C(13)-C(12)	123.25(13)
O(1)-C(13)-C(12)	117.74(12)
C(13)-C(14)-C(15)	115.45(13)
C(13)-C(14)-C(22)	122.85(14)
C(15)-C(14)-C(22)	121.67(13)
O(5)-C(15)-C(16)	120.82(14)
O(5)-C(15)-C(14)	116.91(14)
C(16)-C(15)-C(14)	122.26(13)

Table 4.23 (continued)

Bond Distances	(A°)
C(15)-C(16)-C(17)	121.49(14)
C(16)-C(17)-C(12)	117.19(14)
C(16)-C(17)-C(18)	119.70(14)
C(12)-C(17)-C(18)	123.11(13)
C(19)-C(18)-C(17)	119.14(16)
C(19)-C(18)-C(21)	121.77(18)
C(17)-C(18)-C(21)	119.08(15)
C(18)-C(19)-C(20)	125.9(2)
O(3)-C(23)-O(2)	117.00(13)
O(3)-C(23)-C(12)	122.33(13)
O(2)-C(23)-C(12)	120.67(12)
C(13)-O(1)-C(1)	110.97(10)
C(23)-O(2)-C(2)	123.15(11)

Table 4.24 Hydrogen bonds for compound ME1 [A and deg.].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O(4)-H(4O)...O(5)#1	0.84(3)	2.39(3)	3.2110(19)	169(2)
O(5)-H(5O)...O(3)#2	0.81(3)	2.06(3)	2.8711(18)	171(3)

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,-y+1,-z+1 #2 x,-y+3/2,z+1/2

4.11.2 Structure elucidation of Compound ME2

Compound ME2 was purified by washing with hexane and EtOAc to obtain as white amorphous solid (6 g or 1.29% product recovery). Chemical structures of compound ME2 was elucidated by its spectroscopic data, including IR, UV, NMR and MS spectra with physical properties.

IR spectrum of compound ME2 is shown in Figure B12, appendix B and IR absorption presented band of O-H stretching vibration of alcohol at 3497 and 3385 cm^{-1}, the absorption band at 2918 cm^{-1} of C-H stretching vibration of $-CH_3$, the absorption band of C=O stretching vibration of carbonyl group at 1707 cm^{-1}, the absorption band of C=C stretch in aromatic compounds at 1571 cm^{-1}, the absorption band of C-H bending vibration in CH_3 group at 1337 cm^{-1} and the absorption band of C-O stretching vibration in esters at 1252, 1155 and 1057 cm^{-1}. The IR absorption bands of compound ME2 are summarized in Table 4.25.

Table 4.25 The IR absorption bands assignment of compound ME2

Wave number (cm^{-1})	Intensity	Tentative assignment
3497 and 3385	Broad, Medium	O-H stretching vibration of alcohol
2918	Sharp, Weak	C-H stretching vibration in $-CH_3$
1707	Sharp, Strong	C=O vibration of carbonyl group
1571	Sharp, Strong	C=C stretch in aromatic compounds
1337	Sharp, Medium	C-H bending vibration in CH_3 group
1252 and 1155	Sharp, Strong	C-O stretching vibration in esters
1057	Sharp, Medium	C-O stretching vibration in esters

EI-MS spectrum (Figure B19 in appendix B) of compound ME2 showed the M^+ ion peak at *m/z* 326. The results from MS and ^{13}C-NMR indicated that molecular formula of compound ME2 was $C_{19}H_{18}O_5$.

^{1}H-NMR spectrum (Figure B13 in appendix B) of compound ME2 showed three signals of aromatic protons at δ 6.36, 6.45 and 6.55 ppm, one olefinic proton at 5.58 ppm, four methyl protons (- C*H*$_3$ -) at 1.72, 1.79, 1.85 and 1.89 ppm.

^{13}C-NMR spectrum (Figure B14 in appendix B) of compound ME2 showed 19 signals, including one signal of carbonyl carbon of ester at 164.3 ppm, twelve signals of aromatic carbon at 104.47, 110.82, 111.85, 114.91, 115.22, 125.11, 132.87, 135.82, 141.02, 143,47, 144.89 and 152.44 ppm, two olefinic carbon at 124.9 and 134.3 ppm, and the signals of methyl group carbon at 7.95, 12.63, 16.48 and 19.85 ppm.

The information from 2D-NMR techniques including HSQC, HMBC, COSY, and NOESY (Fig B15-B18) were presented in Table 4.26 and elucidated the structure of compound ME2 as shown in Figure 4.21 and 4.22. Structure of unguinol as shown in Figure 4.23.

Table 4.26 HSQC, HMBC and NOESY spectral data of compound ME2

Position	δ_C	δ_H	HMBC (H to C)	NOESY
2	141.0 (s)	-	-	-
3	143.5 (s)	-	-	-
5	164.3 (s)	-	-	-
6	111.8 (s)	-	-	-
7	163.9 (s)	-	-	-
8	104.5 (d)	6.36(1H,d, *J* =2.4)	C-6, C-7, C-9	H-20 (2.08)
9	162.0 (s)	-	-	-
10	115.2 (d)	6.55(1H,d, *J* =1.2)	C-6, C-9	H-16 (2.41)
11	144.8 (s)	-	-	-
12	114.9 (s)	-	-	-
13	152.4 (s)	-	-	-
14	110.8 (d)	6.45(1H,s)	C-2, C-12, C-17	-
15	135.8 (s)	-		-
16	19.8 (q)	2.41(3H,s)	C6, C-10, C-11	H-10 (6.55)
17	134.3 (s)	-	-	-
18	124.9 (s)	5.56(1H,s)	C-19, C-20	H-19 (1.85)
19	12.6 (q)	1.85(3H,d, *J* =6.8)	C-17, C-18	H-18 (5.56), H-20 (2.08)
20	16.7 (q)	2.08(3H,s)	C-15, C-17, C-18	H-8 (6.36), H-19 (1.85)
21	7.95 (q)	2.17(3H,s)	C-3, C-12, C-13	-

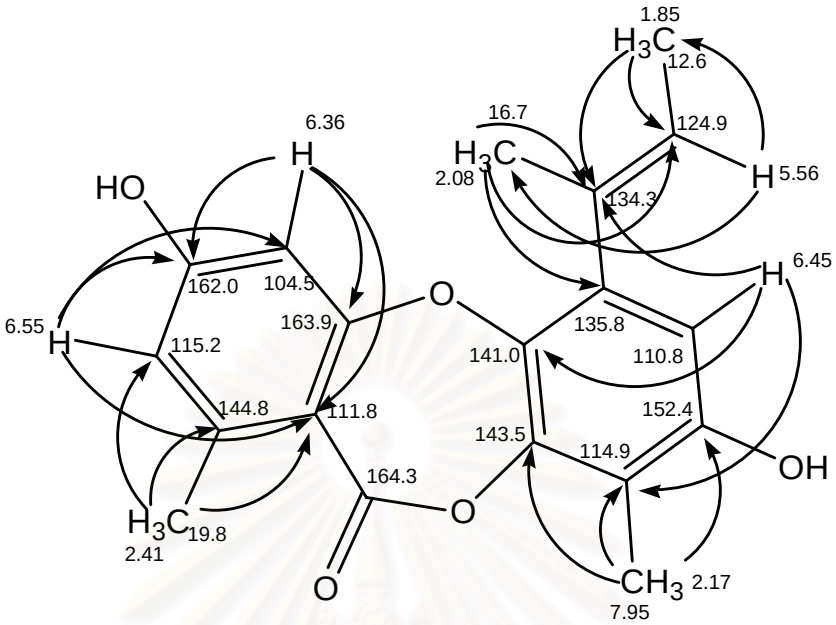


Figure 4.21 HMBC correlation of compound ME2

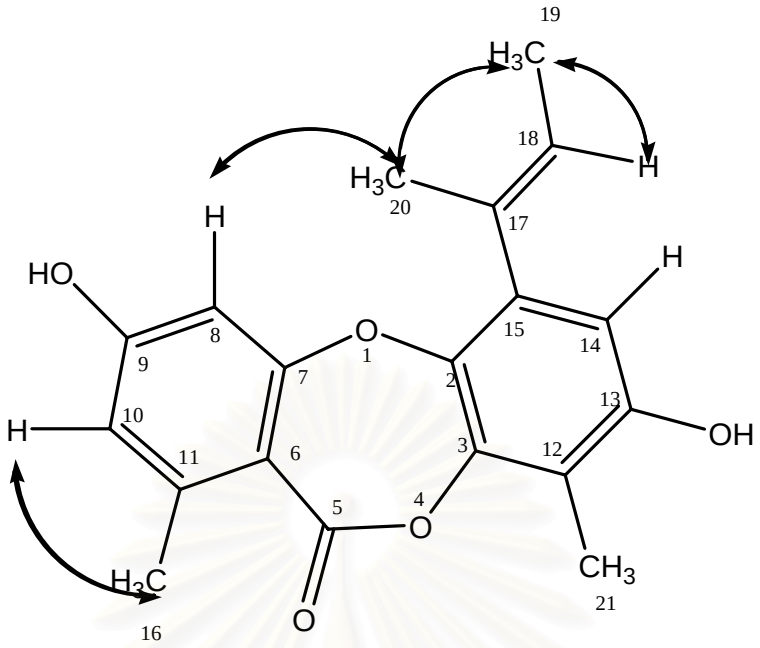


Figure 4.22 NOESY correlation of compound ME2

Considerably, this structure appears to be a possible for compound ME2. In view of its similarity to unguinol.

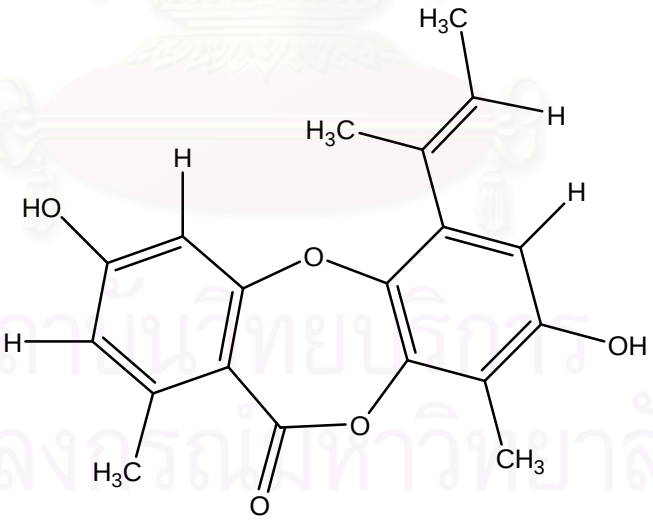


Figure 4.23 The structure of unguinol

4.12 Evaluation of biological activities of isolated metabolites from endophytic fungal isolate CuLm17

4.12.1 Antimicrobial activities of pure compounds

The isolated metabolites were evaluated for their antimicrobial activities by microdilution broth susceptibility test against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231. These compounds were examined in the concentration range of 1000-31.25 µg/ml. The lowest concentration of the compound that inhibited growth of test microorganisms is recorded as the minimal inhibitory concentration (MIC). Antimicrobial activity of isolated compounds is presented in Table 4.27.

Table 4.27 MIC of antimicrobial activities of isolated metabolites from endophytic fungal isolate CuLm17.

Compound	MIC(µM)				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC10231
Compound ME1	164.5	657.9	328.9	328.9	>2631.5
Compound ME2	191.7	1533.7	1533.7	1533.7	>3067.5
Streptomycin	ND	ND	1.34	5.36	ND
Penicillin G	43.83	43.83	ND	ND	ND
Ketoconazole	ND	ND	ND	ND	1728.9

- ND : not determined = no antimicrobial test

The results showed that compound ME1 was active against *B. subtilis* ATCC 6633 , *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 at the concentration 164.5, 657.9, 328.9 and 328.9 µM, respectively. Compound ME2 was active

against *B. subtilis* ATCC 6633 , *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 at the concentration 191.7, 1533.7, 1533.7 and 1533.7 μM, respectively.

4.12.2 Cytotoxic activity

The *in vitro* cytotoxic activity of isolated metabolites from endophytic fungal CuLm17 against 5 cell line including HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) is shown in Table 4.29 as the minimum concentration of 50 % inhibitory activity (IC_{50}).

Table 4.28 Cytotoxic activity of isolated metabolites from endophytic fungal isolate CuLm17.

Compound	IC_{50} (μM)				
	HEP-G2 (hepatoma)	SW 620 (colon)	CHAGO (lung)	KATO-3 (gastric)	BT474 (breast)
Compound ME1	14.7	15.3	14.5	15.8	22.6
Compound ME2	>10	>10	>10	21.2	>10
Doxorubicin	1.21	0.15	1.21	>10	>10

This result showed that compound ME1 displayed high cytotoxic activity against a total of five tested cancer cell lines, but compound ME2 only displayed cytotoxicity against KATO-3 cells.

4.13 Antioxidant activity

Compound ME1 and ME2 were not shown antioxidant activity because their activity less than 50% inhibition.

CHAPTER V

CONCLUSION

The objectives of this research were aimed at isolation of endophytic fungi from leaves, branches and barks of *Croton oblongifolius* in Chulalongkorn University campus, determination of antimicrobial activities of the isolated endophytic fungi, identification of a selected endophytic fungal isolate based on morphology, elucidation of the structure and evaluation of bioactivities of the isolated metabolites from a selected endophytic fungal isolate.

Forty seven endophytic fungi isolates were isolated by using the surface sterilization method. The percentage of isolates from branches and bark sections is higher than that from leaf sections.

Endophytic fungal isolates were identified as belonging to typical genera of endophytes such as *Penicillium, Curvularia, Trichoderma and Phomopsis* amount 4, 2, 3 and 1 isolate, respectively. The remaining of 37 isolates of endophytic fungi did not produce conidia or sporulate on media and were therefore recorded as mycelia sterilia.

Antimicrobial activities of these endophytes were tested by agar well diffusion method. Crude extracts were prepared from 8 weeks old culture. Ninety two culture broth crude extracts and mycelium crude extracts (97.87 % of total 94 crude extracts) had the antimicrobial activities against at least one tested microorganisms. They were active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 at 93.62 %, 76.60%, 30.85%, 30.85%, and 89.36%, respectively. This indicated that *B. subtilis* ATCC 6633, the gram positive rod bacterium, was more sensitive to isolated endophytic fungi than other tested microorganisms.

Endophytic fungal isolate CuLm17 was chosen for further study for bioactive compounds because the culture broth crude extract and mycelium crude extract were active against a large number of tested microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. cerevisiae* TISTR 5169, *C. albicans* ATCC 10231.

Based on colony characteristic and microscopic characteristic endophytic fungal isolate CuLm17 was identified as *Penicillium* sp.

Culture broth and mycelial extract of endophytic fungal isolate CuLm17 cultivated in MEB were extracted with EtOAc and MeOH and partitioned with Hexane, EtOAc and MeOH. EtOAc crude extract which partitioned with EtOAc of culture broth and mycelium was chosen for separation and purification by silica gel column chromatography and crystallization techniques. Because of these crude extracts were the highest active against a large number of test microorganisms. Folipastatin (500 mg) and unguinol (6 g) obtained from EtOAc crude of mycelium extract. The structure elucidation of these compounds was achieved by analysis of their spectroscopic data and physical properties. And the stucture of folipastatin was confirmed by X-ray analysis.

Antimicrobial activities and cytotoxicity of isolated compounds were tested. It was found that folipastatin was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerivisiae* TISTR 5169 and *C. albicans* ATCC 10231 with MIC value of 164.5, 657.9, 328.9 and 328.9 µM, respectively and unguinol was active against *B. subtilis* ATCC 6633 , *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 at the concentration 191.7, 1533.7, 1533.7 and 1533.7 µM, respectively. Moreover, it was found that folipastatin had high cytotoxic activity against HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) cell line with IC_{50} 14.7, 15.3, 14.5, 15.8 and 22.6 µM, respectively and unguinol was active against KATO-3 (gastric) with IC_{50} 21.2 µM.

Folipastatin and unguinol showed no antioxidant activity.

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APPENDICES

APPENDIX A
MEDIA

The media were sterilized by autoclaving at 121°C , 15 lb/in^2 for 15 minutes

1. Malt extract agar (MEA)

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

2. Nutrient agar (NA)

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

3. Sabouraud's dextrose agar (SDA)

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

4. Potato dextrose agar (PDA)

Potatoes, peeled and diced	200.0	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water	1000	ml

Boil 200 g of peels, diced potatoes for 1 hr in 1000 ml of distilled water. Filter, and adjust the filtrate to 1000 ml. Add the dextrose and agar and dissolve by steaming and sterilize by autoclaving at 121°C for 15 min.

5. Yeast extract sucrose agar (YES)

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

6. Oat meal agar (OMA)

Agar	15.0	g

7. V8 agar

V8 Vegetable juice	200	ml
Calcium carbonate	4.0	g
Distilled water	1000	ml
Agar	20.0	g

APPENDIX B

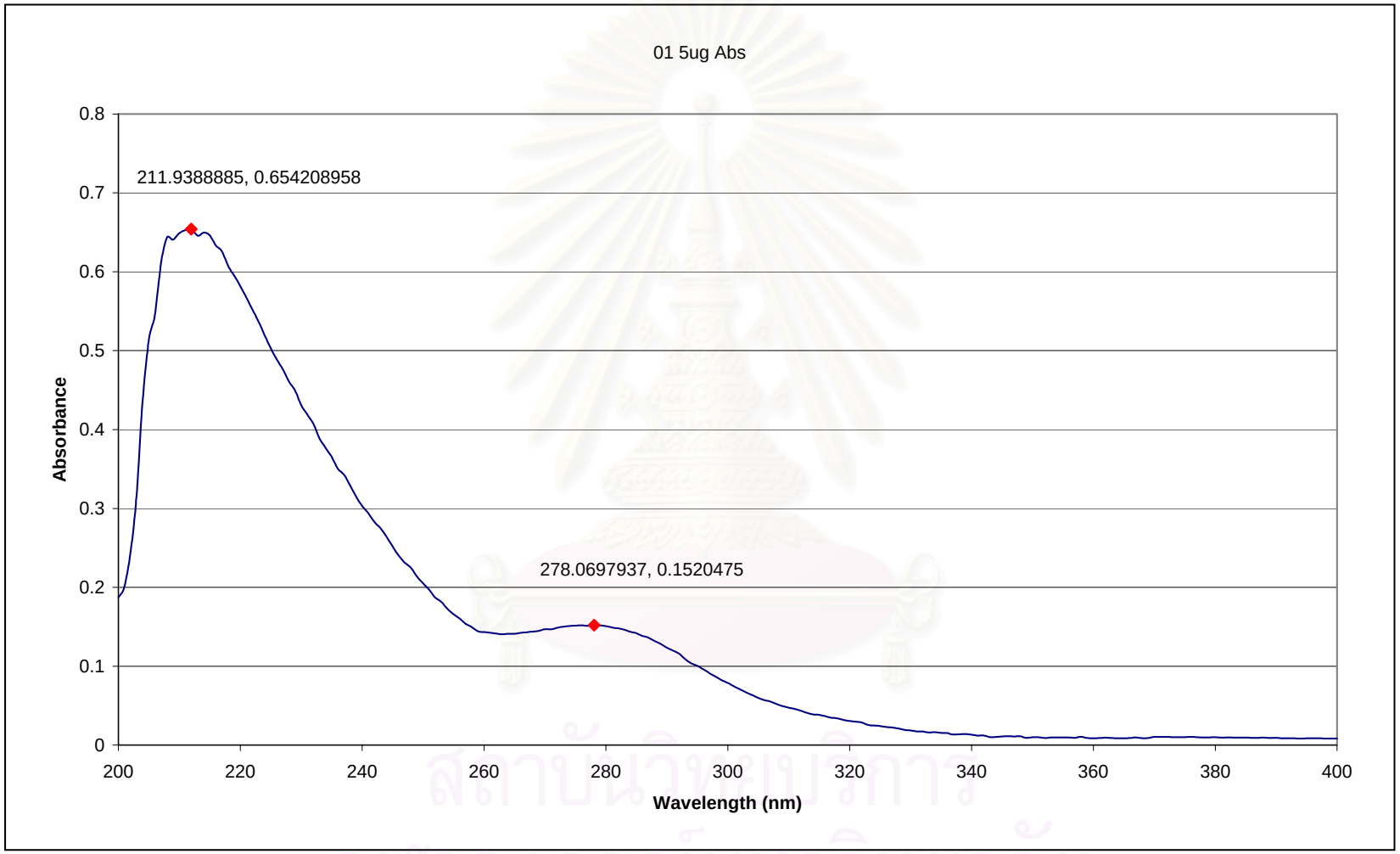
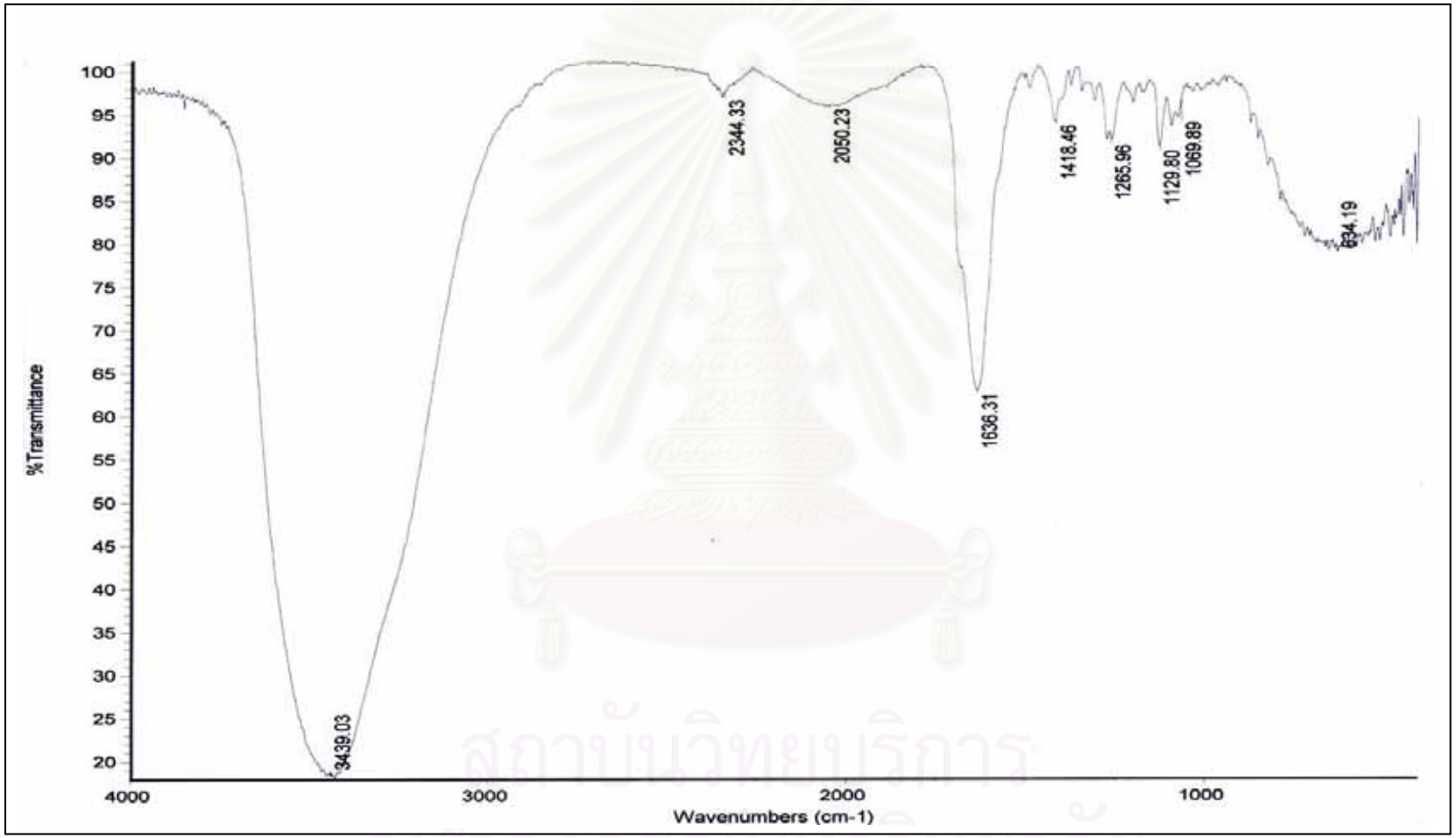


Figure B1 UV spectrum of compound ME1



FigureB2 IR spectrum of compound ME1

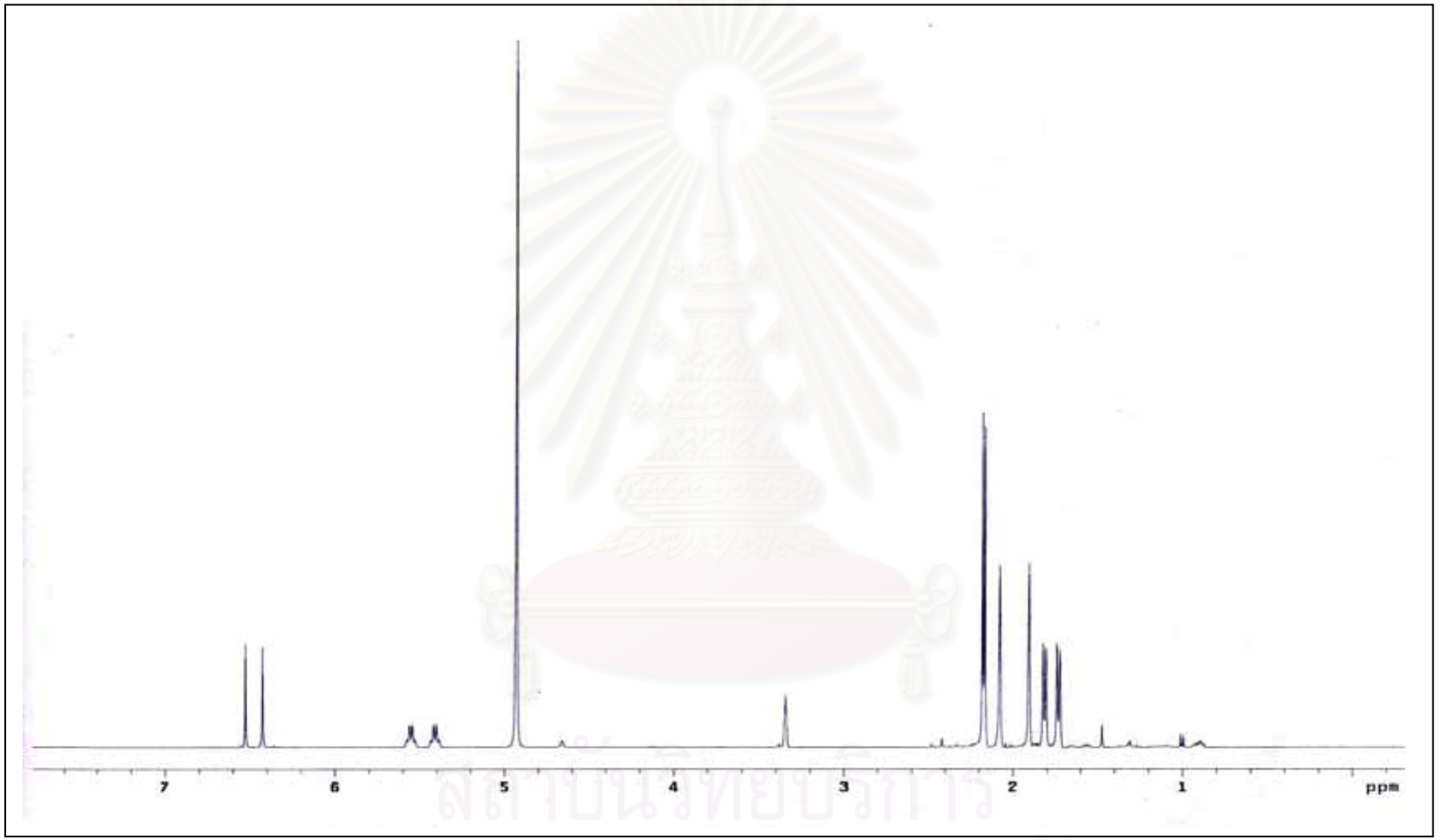


Figure B3 ^{1}H-NMR spectrum of compound ME1

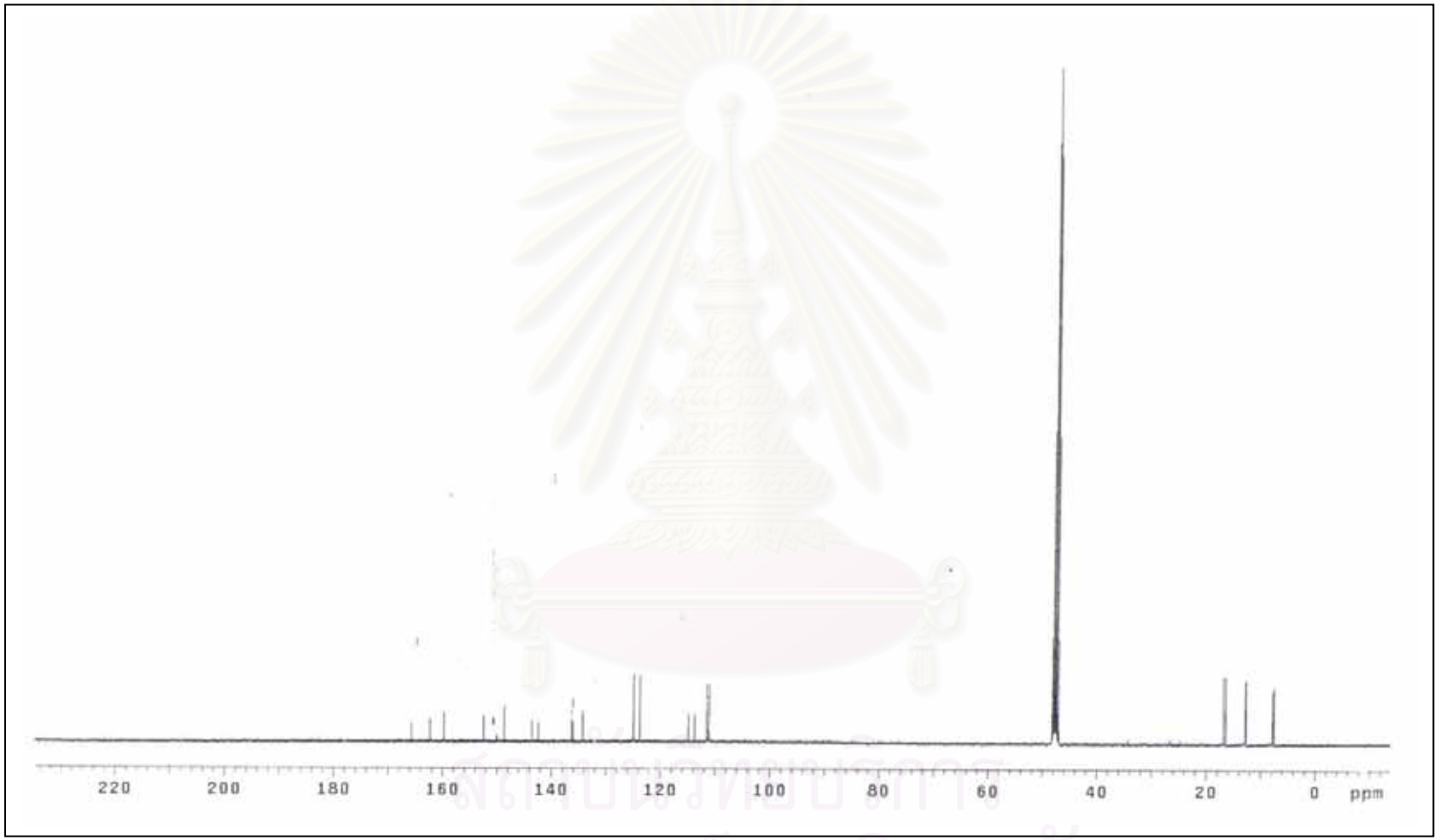


Figure B4 ^{13}C-NMR spectrum of compound ME1

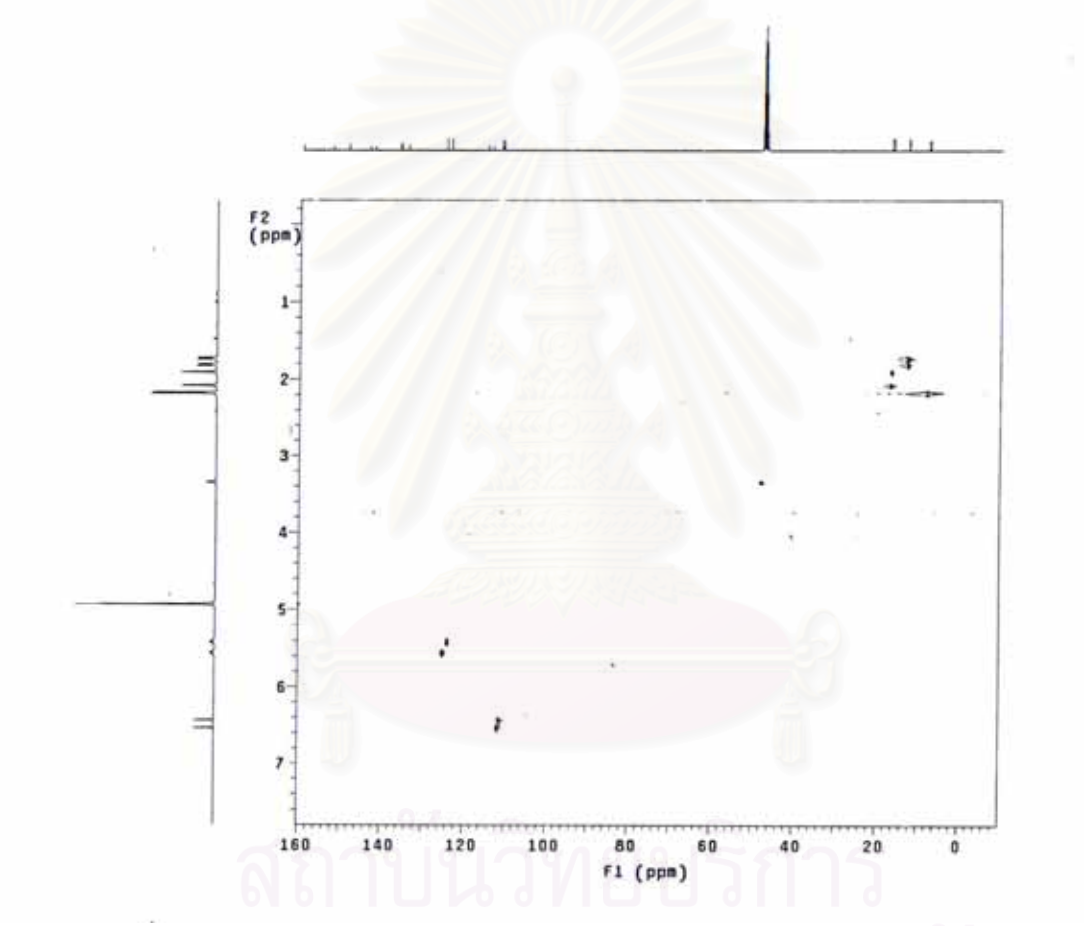


Figure B5 HSQC spectrum of compound ME1

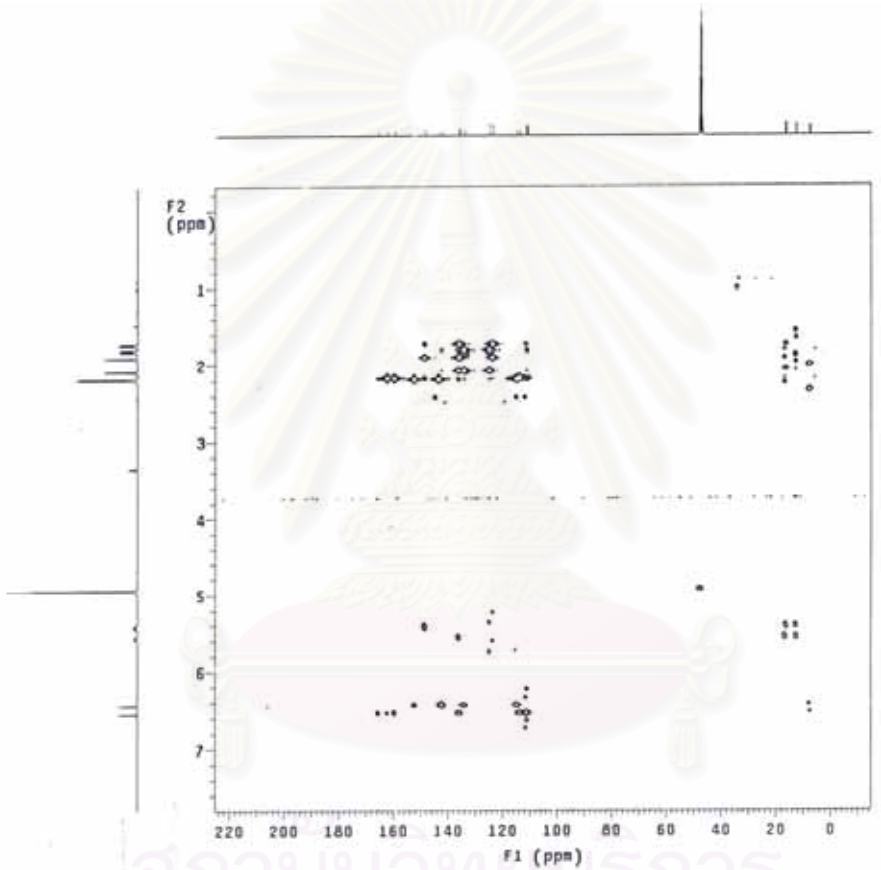


Figure B6 HMBC spectrum of compound ME1

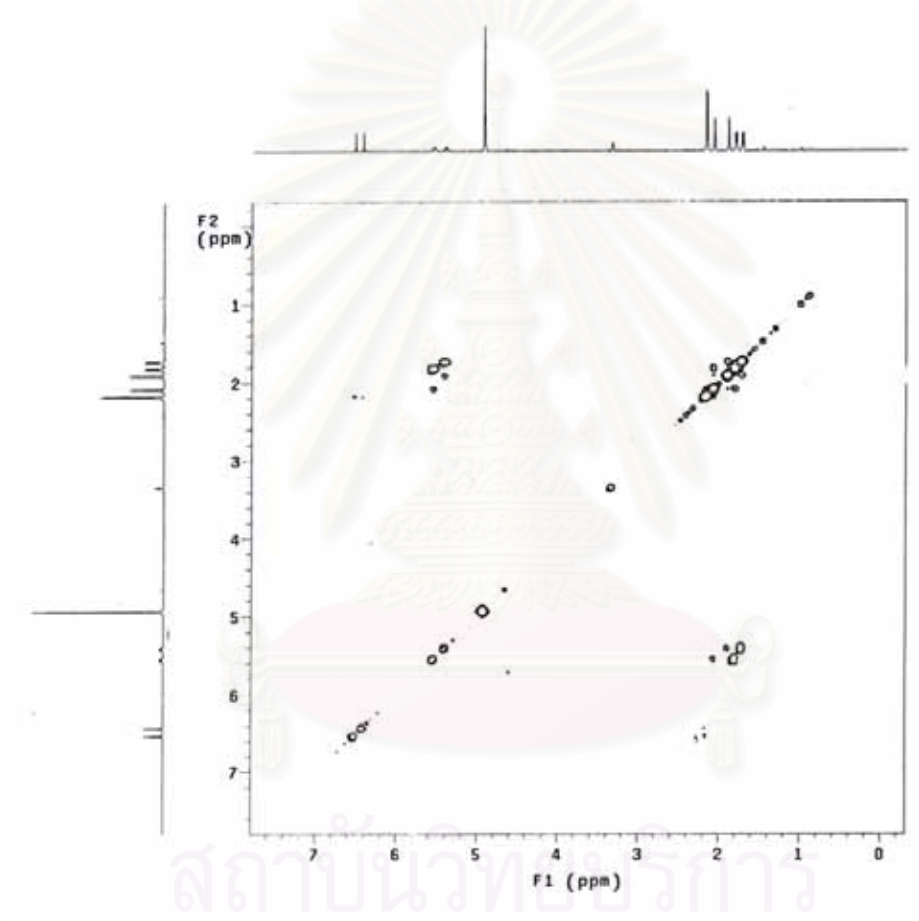


Figure B7 COSY spectrum of compound ME1

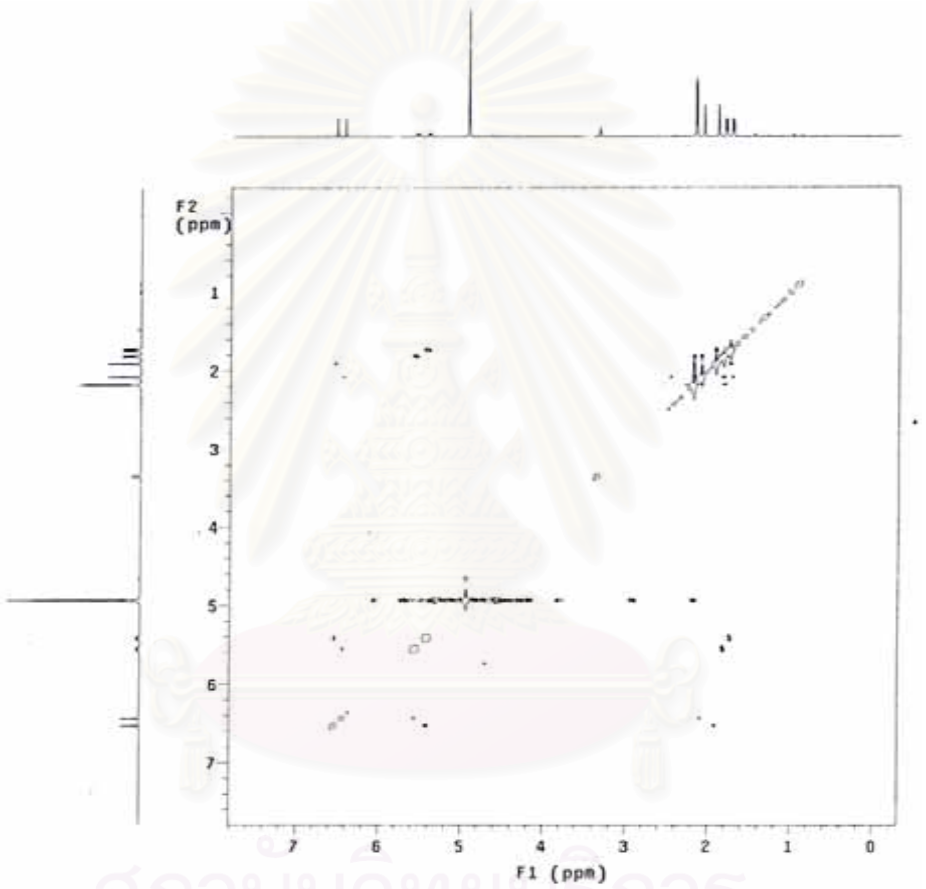


Figure B8 NOESY spectrum compound ME1

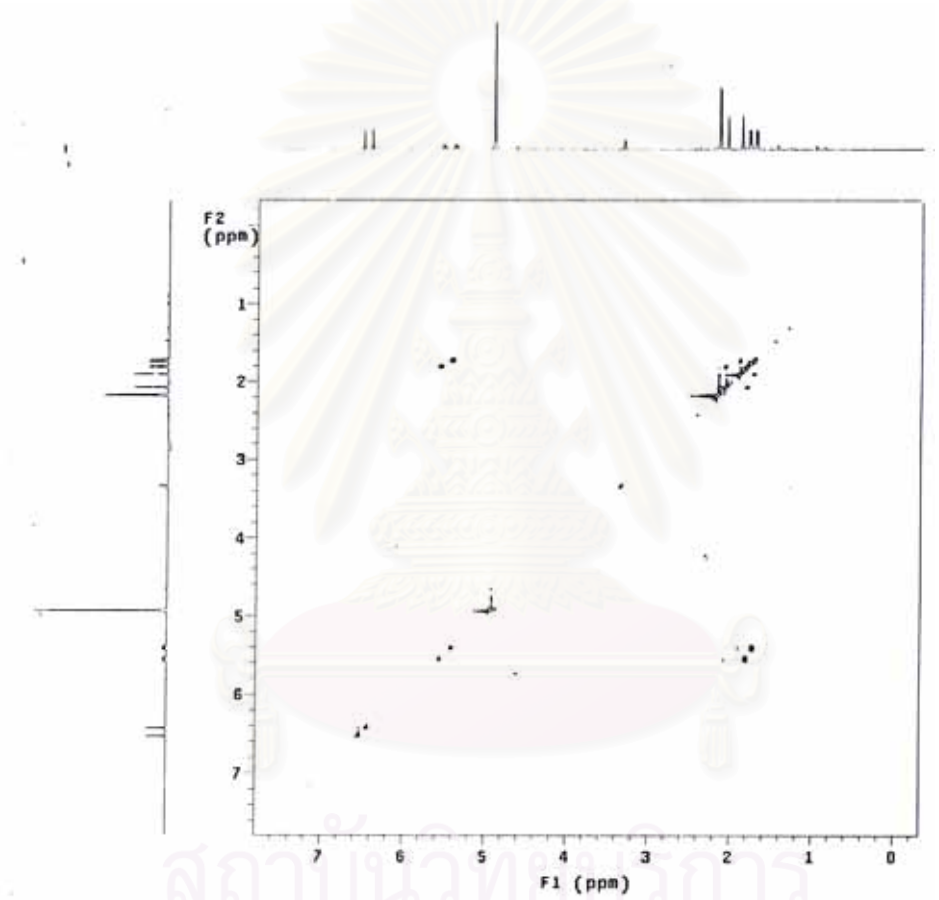


Figure B9 TOCSY spectrum of compound ME1

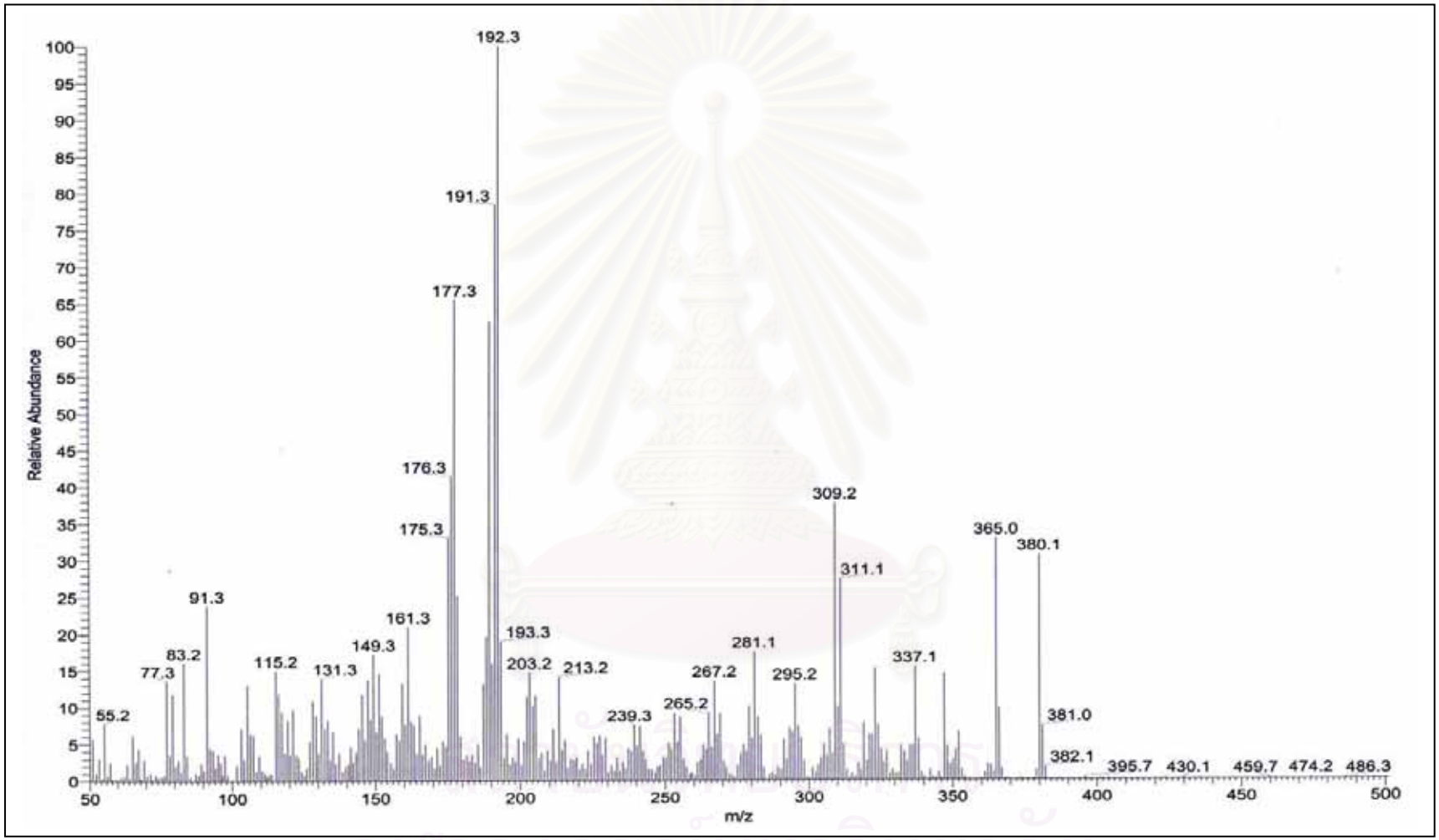


Figure B10 EI-MS spectrum of compound ME1

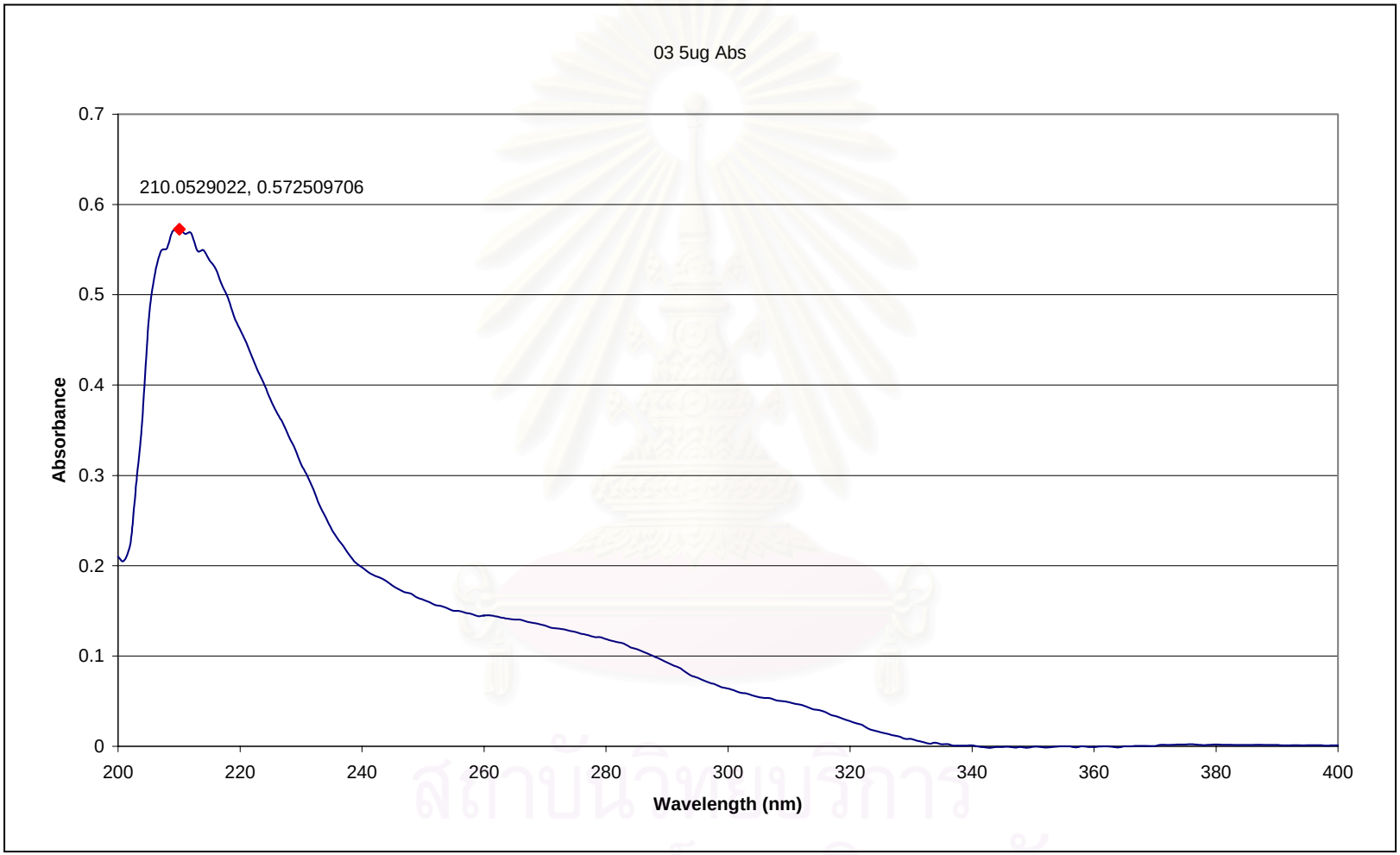


Figure B11 UV spectrum of compound ME2

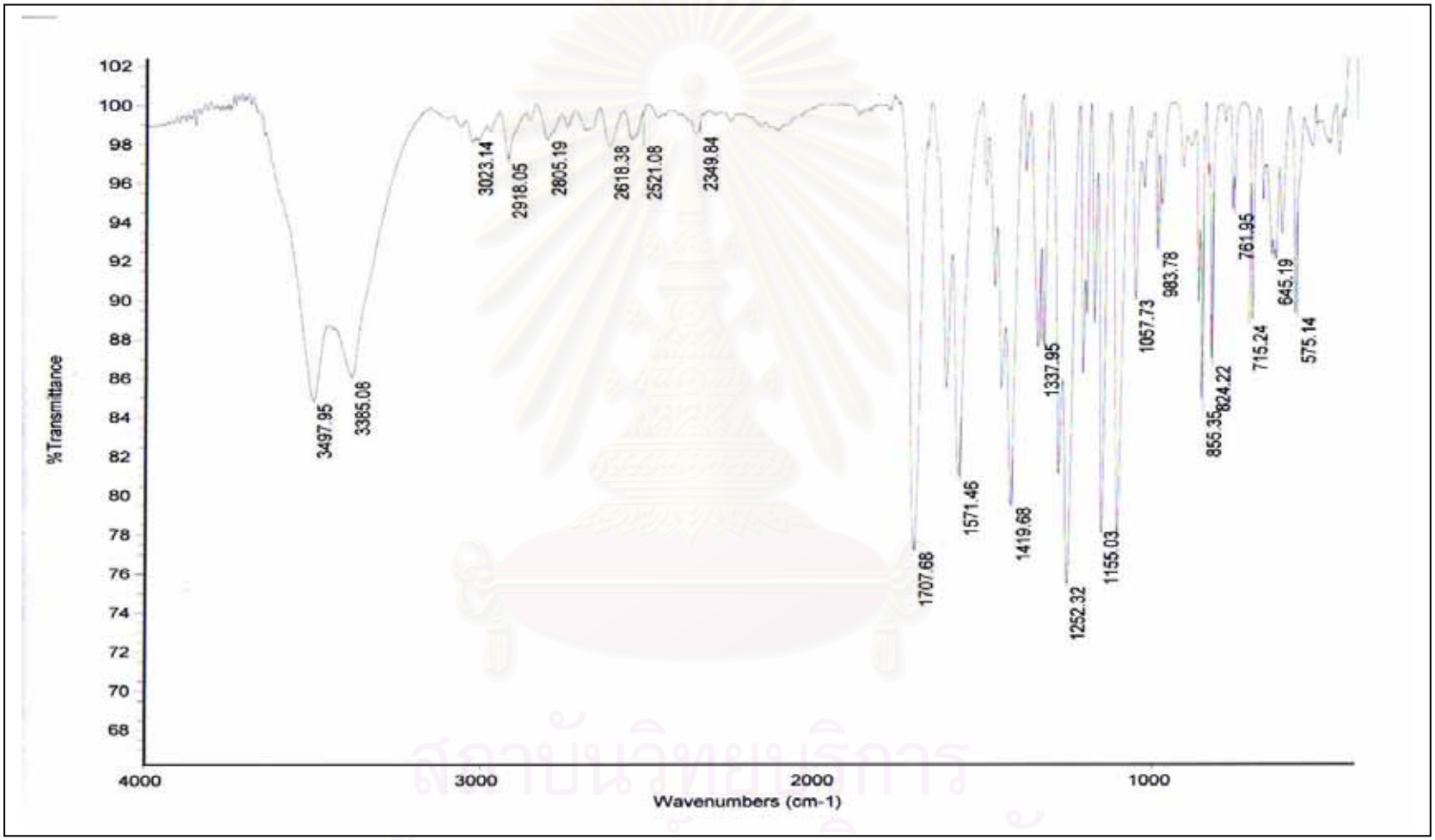


Figure B12 IR spectrum of compound ME2

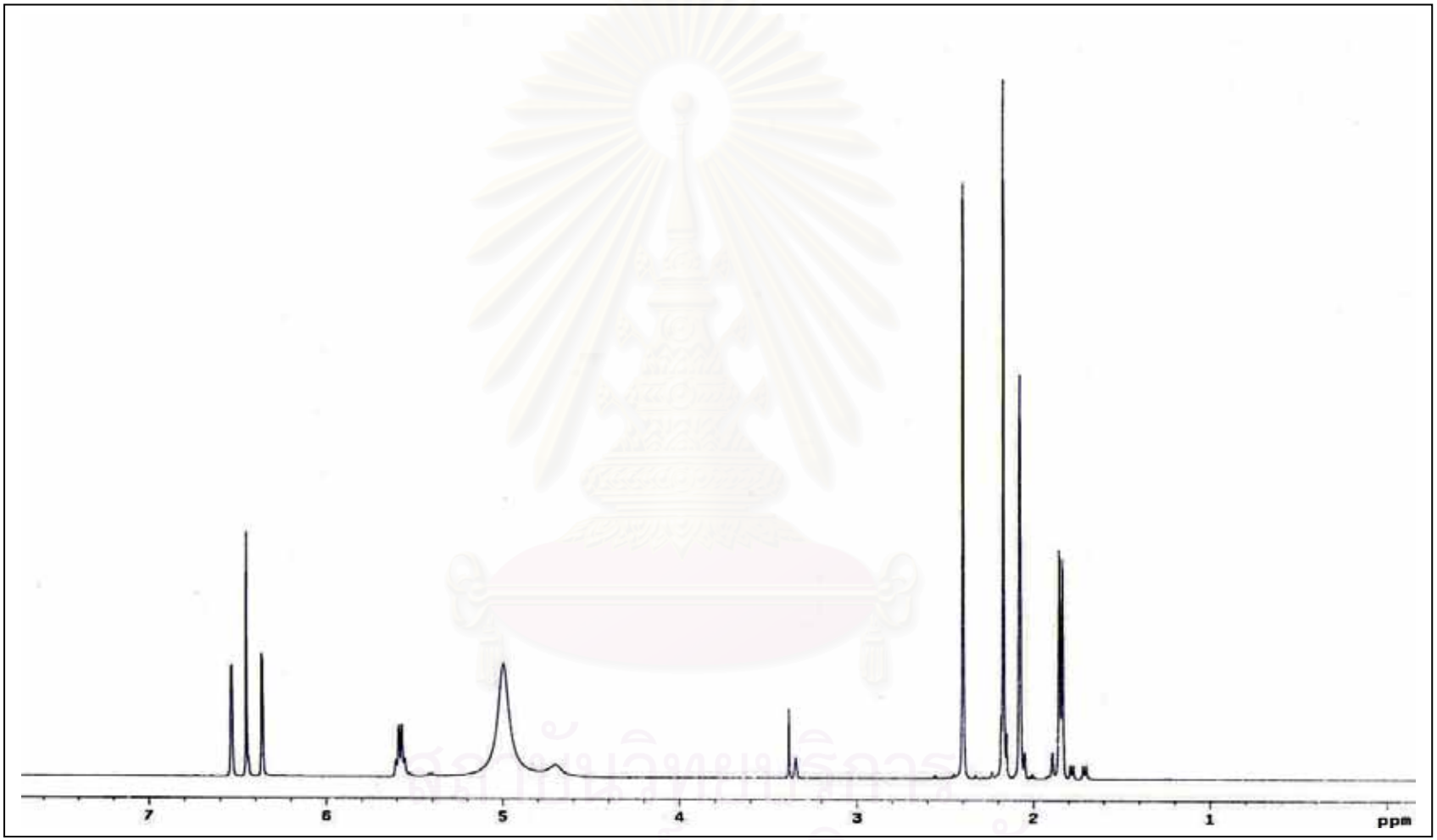


Figure B13 ^{1}H-NMR spectrum of compound ME2

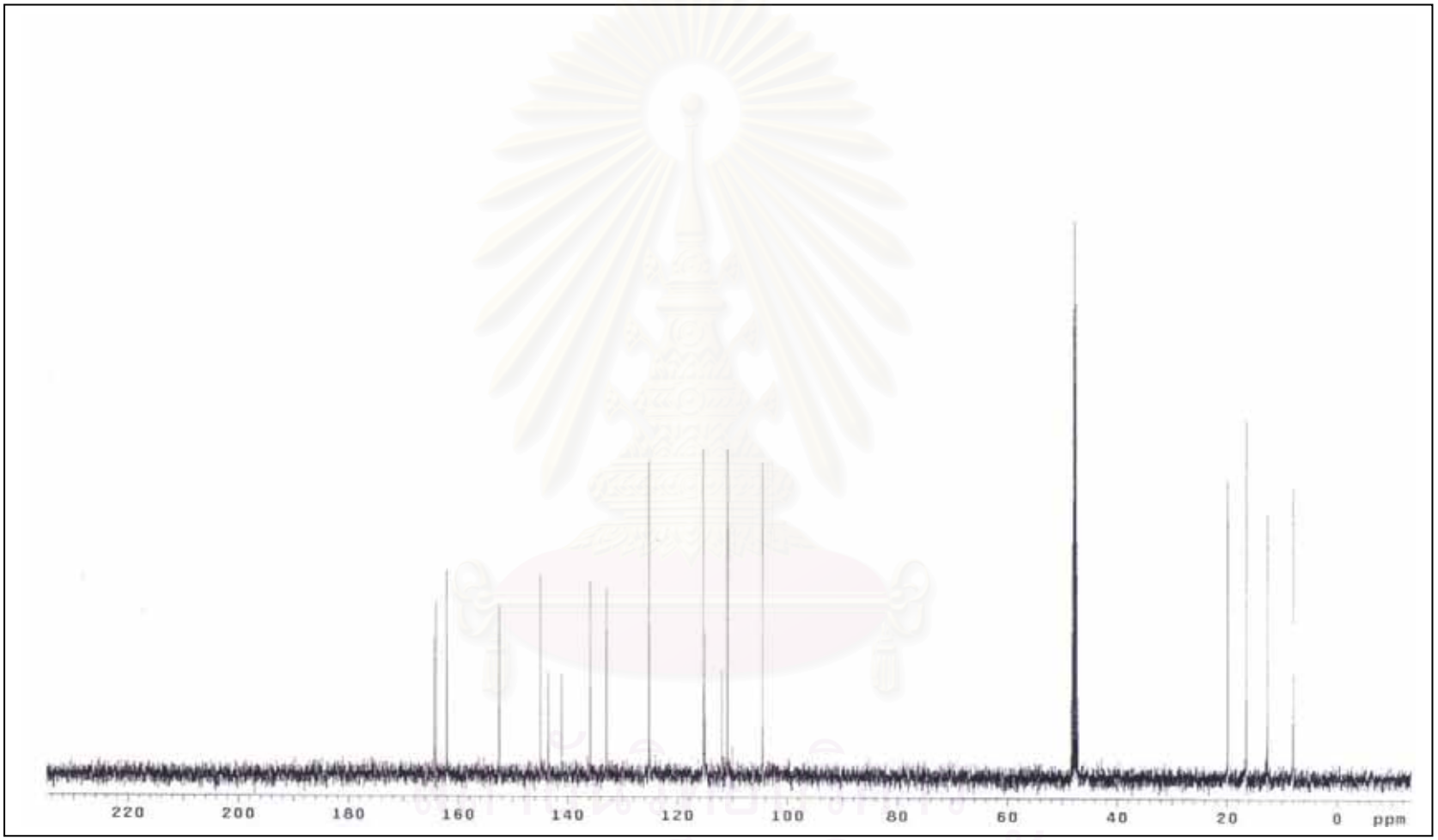


Figure B14 ^{13}C-NMR spectrum of compound ME2

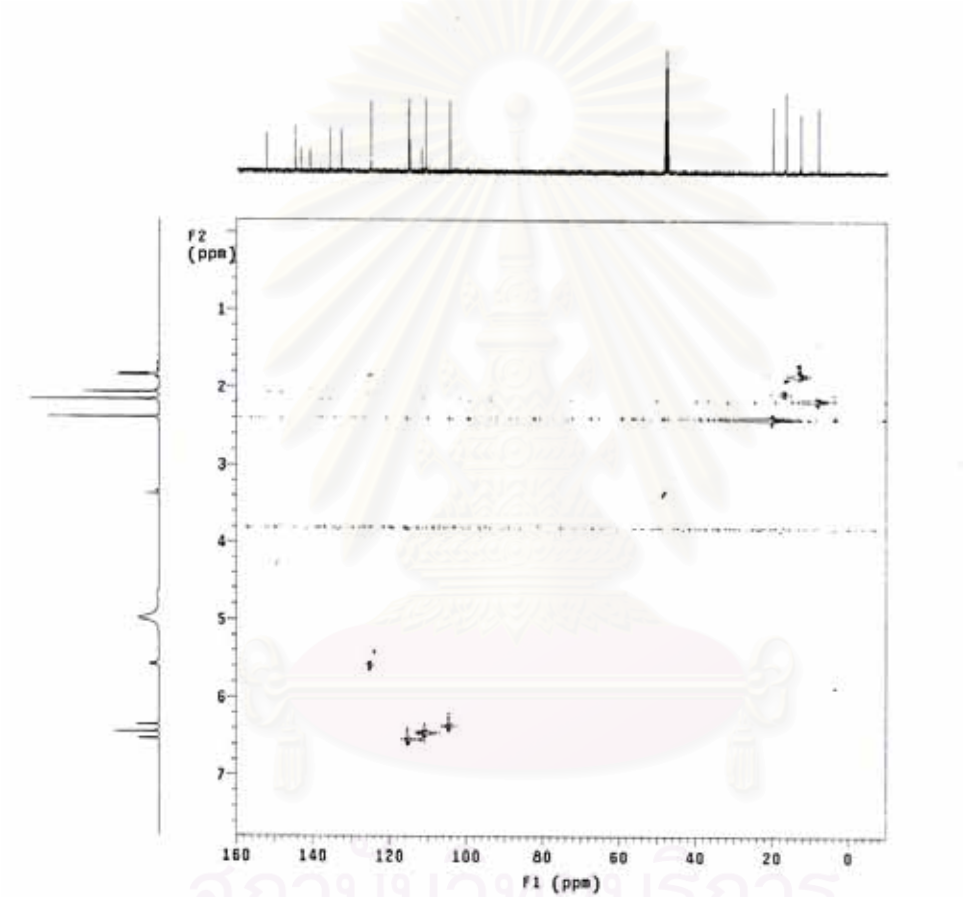


Figure B15 HSQC spectrum of compound ME2

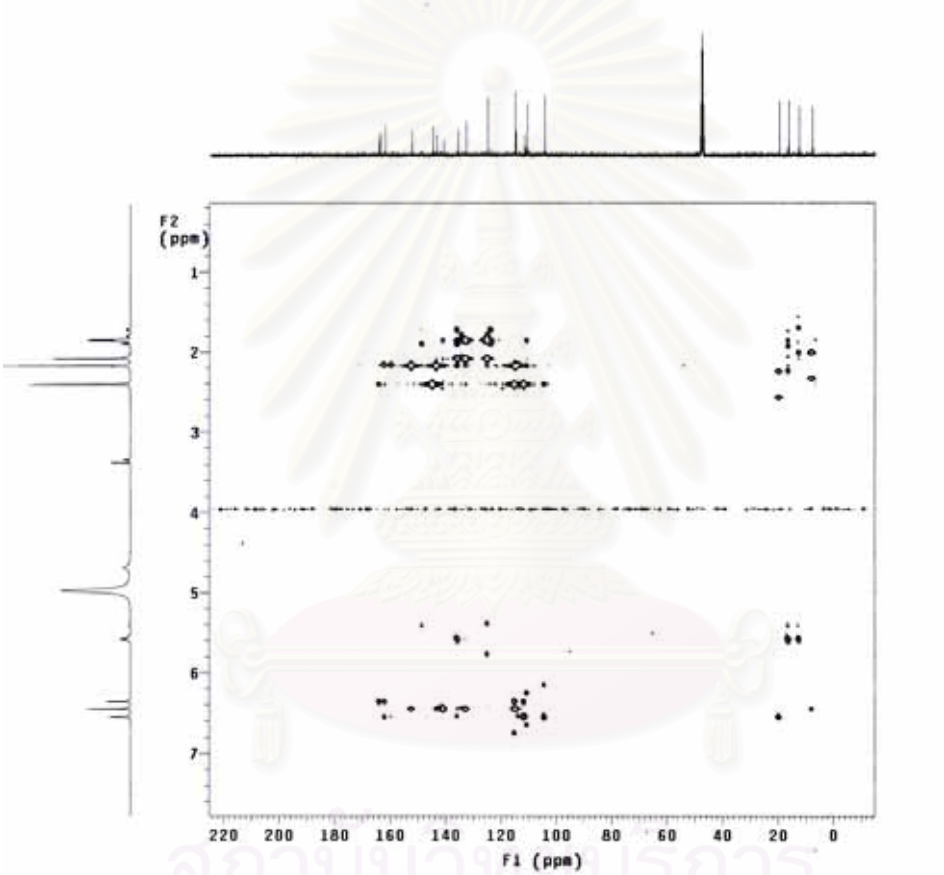


Figure B16 HMBC spectrum of compound ME2

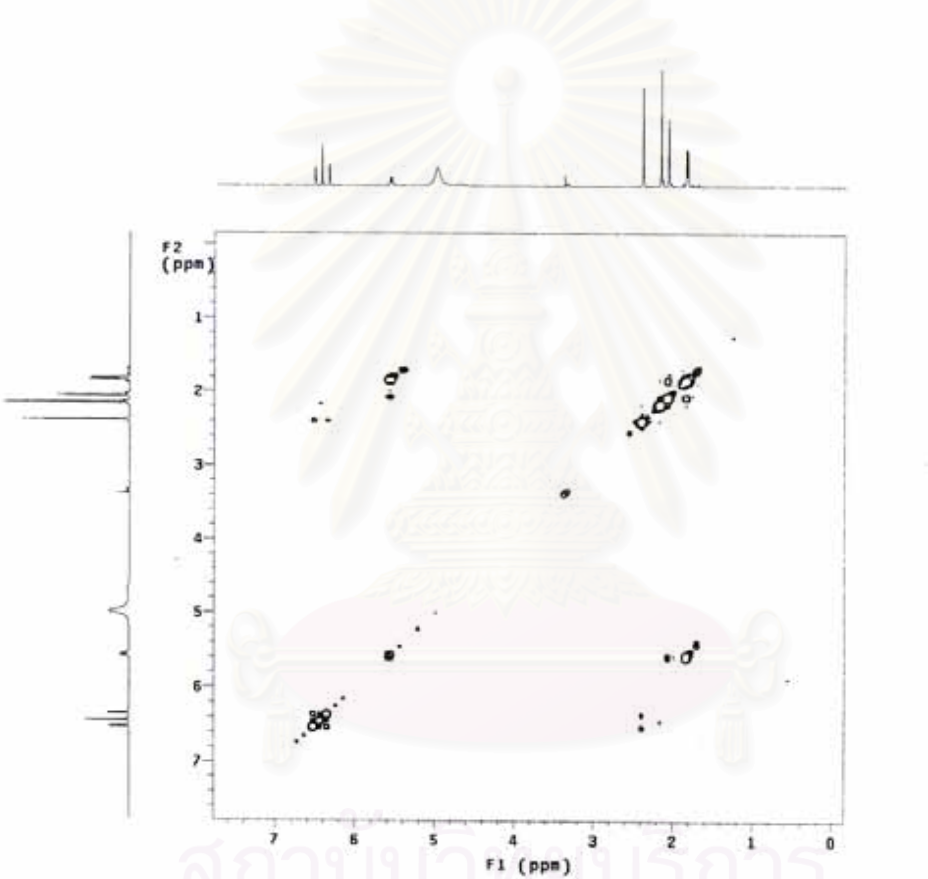


Figure B17 COSY spectrum of compound ME2

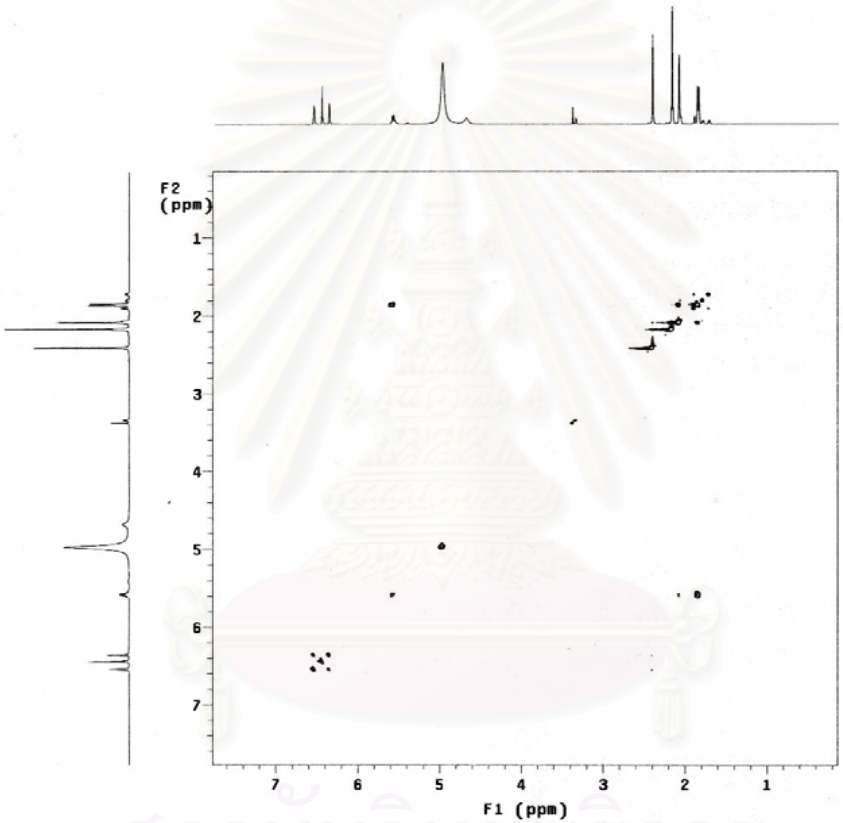


Figure B18 TOCSY spectrum of compound ME2

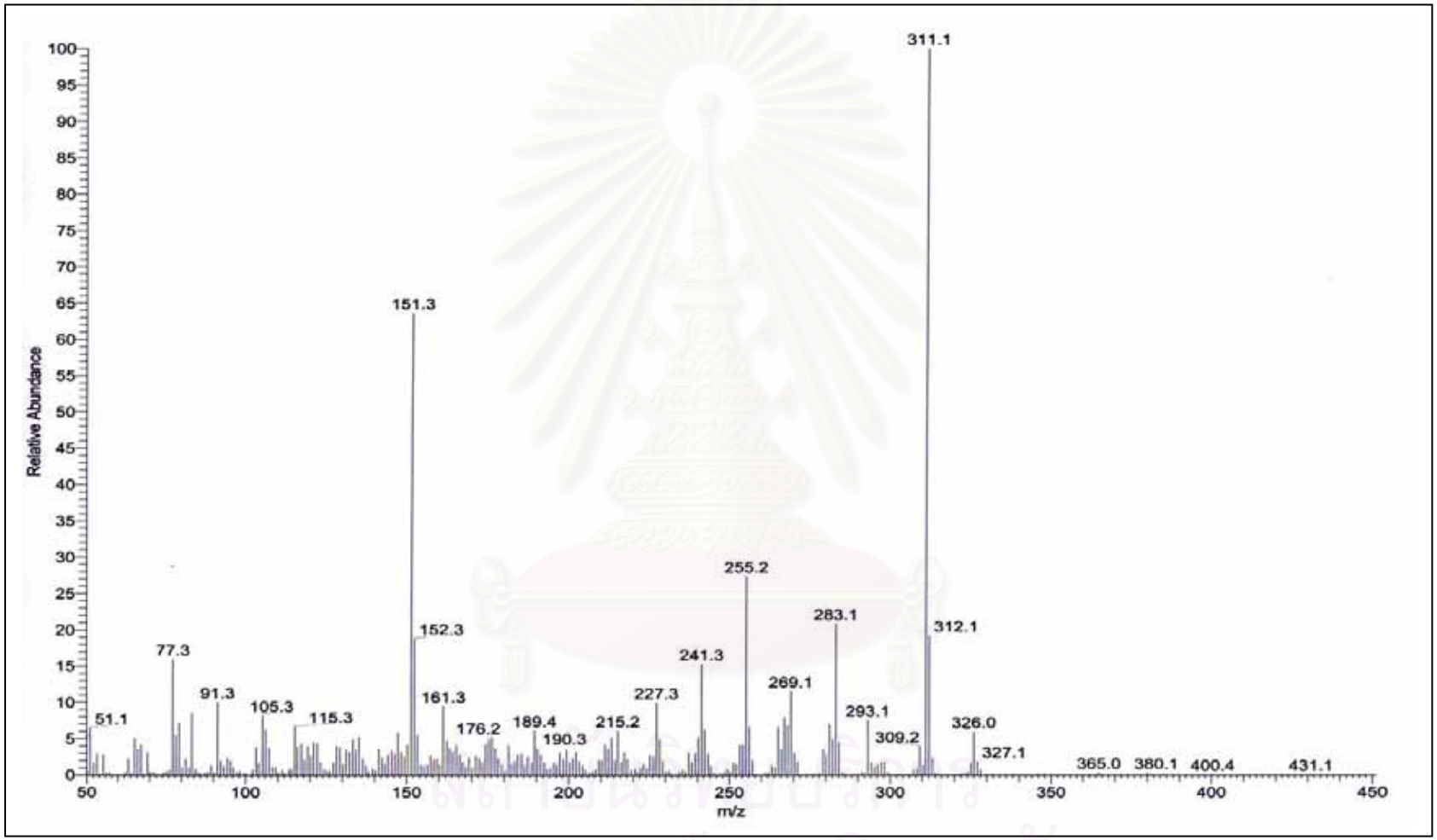


Figure B19 EI-MS spectrum of compound ME2

BIOGRAPHY

Miss Nutjira Onnuan was born on October 16, 1980 in Nonthaburi province, Thailand. She graduated with a Bachelor Degree of Science in Microbiology Department from the Faculty of Science, Kasetsart University, Thailand in 2001. She has been studying for a Master Degree of Science in Industrial Microbiology, Faculty of Science, Chulalongkorn University, Thailand since 2002.