ราเอนโดไฟต์ที่แยกจากใบและกิ่งยอ *Morinda citrifolia* L.

นางสาว ศรัณยา คุ้มวงษา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ENDOPHYTIC FUNGI ISOLATED FROM *Morinda citrifolia* L. LEAVES AND TWIGS

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นางสาวศรัณยา คุ้มวงษา : ราเอนโดไฟต์ที่แยกจากใบและกิ่งยอ *Morinda citrifolia* L. (ENDOPHYTIC FUNGI ISOLATED FROM *Morinda citrifolia* L. LEAVES AND TWIGS) อ. ที่ปรึกษา : ศ. ดร. โสภณ เริงสำราญ, อ. ที่ปรึกษาร่วม : รศ. ดร. ประกิตติ์สิน สีหนนท์, 154 หน้า. ISBN 974-17-6243-7

จากตัวอย่างใบและกิ่งยอ *Morinda citrifolia* L. ที่สุ่มเก็บจาก 4 จังหวัดในประเทศไทย สามารถแยกราเอนโดไฟต์ได้ 178 ไอโซเลต เมื่อทดสอบฤทธิ์ต้านจุลินทรีย์ของราเอนโดไฟต์ที่แยกได้ พบว่าราเอนโดไฟต์ไอโซเลต LSS06 ซึ่งเพาะเลี้ยงบนอาหารแข็งมอลท์สกัดมีฤทธิ์ยับยั้งจุลินทรีย์ทดสอบดีที่สุด เนื่องจากเป็นราเอนโดไฟต์ไอโซเลตเดียวที่สามารถยับยั้งการเจริญของจุลินทรีย์ทดสอบได้ทั้งแบคทีเรียแกรมบวกคือ *Bacillus subtilis* และ *Staphylococcus aureus* แบคทีเรียแกรมลบ *Escherichia coli* และยีสต์ *Candida albicans* จากการจัดจำแนกสายพันธุ์โดยศึกษาลักษณะทางสัณฐานวิทยาและการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ ITS ของ rDNA พบว่าราเอนโดไฟต์ไอโซเลต LSS06 คือ *Nodulisporium* sp. เมื่อทำการแยกสารสกัดด้วยเอธิลแอซิเตตจากน้ำเลี้ยงเชื้อของราเอนโดไฟต์ไอโซเลต LSS06 ที่เพาะเลี้ยงในอาหารเลี้ยงเชื้อเหลวมอลท์สกัด ได้ของผสม B1 และ ของผสม B2 ส่วนสารสกัดด้วยเอธิลแอซิเตตจากเส้นใยแยกได้ ของผสม M1 ของผสม M2 และ สารบริสุทธิ์ M1 เมื่อวิเคราะห์โครงสร้างของสารที่แยกได้โดยอาศัยคุณสมบัติทางกายภาพและเทคนิคทางสเปกโตรสโกปี พบว่า ของผสม B1 และของผสม B2 ยังไม่สามารถบอกสูตรโครงสร้างได้ ของผสม M1 คือ ไดกลีเซอไรด์ที่ประกอบด้วยกรดไขมันอิ่มตัว 3 ชนิดคือ กรดไมริสติก กรดปาล์มิติก และกรดสเตียริก และกรดไขมันไม่อิ่มตัว 3 ชนิดคือ กรดปาล์มิโตเลอิก กรดโอเลอิก และกรดลิโนเลอิก ของผสม M2 คือ กรดคาร์บอกซิลิกอิ่มตัวโซ่ตรง C_{16}–C_{18} สารบริสุทธิ์ M1 คือ D-mannitol นำสารที่แยกได้มาทดสอบฤทธิ์ทางชีวภาพพบว่า ของผสม B1 มีฤทธิ์ยับยั้ง *B. subtilis* ATCC 6633 โดยมีค่า MIC เท่ากับ 1000 µg/ml แต่ไม่มีฤทธิ์ยับยั้งเซลล์มะเร็งทดสอบ ส่วนสารชนิดอื่นไม่มีฤทธิ์ยับยั้งจุลินทรีย์ทดสอบ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....
ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4472415123: MAJOR BIOTECHNOLOGY

KEY WORD: Endophytic fungi / *Morinda citrifolia* L. / *Nodulisporium* sp. / Fatty acid / D-Mannitol

SARANYA KHUMWONGSA : ENDOPHYTIC FUNGI ISOLATED FROM *Morinda citrifolia* L. LEAVES AND TWIGS. THESIS ADVISOR : PROF. SOPHON ROENGSUMRAN, Ph.D., THESIS COADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D., 154 pp. ISBN 974-17-6243-7

Endophytic fungi 178 isolates were isolated from leaves and twigs of *Morinda citrifolia* L. collected from 4 provinces of Thailand. Endophytic fungal isolate LSS06 cultured on malt extract agar had the best antimicrobial activity because it was the only isolate that inhibited the growth of tested gram positive bacteria *(Bacillus subtilis* and *Staphylococcus aureus*), gram negative bacteria (*Escherichia coli*), and yeast (*Candida albicans*). Based on morphology and nucleotide sequencing analysis of ITS regions of rDNA, endophytic fungal isolate LSS06 was identified as *Nodulisporium* sp. Isolation of EtOAc extract crude of the culture broth of endophytic fungal isolate LSS06 cultivated in malt extract broth gave mixture B1 and mixture B2. Isolation of EtOAc extract crude of the mycelia gave mixture M1, mixture M2 and compound M1. The structure elucidation of these compounds was achieved by analysis of their spectroscopic data and physical properties. The structures of mixture B1 and mixture B2 were still unidentified. Mixture M1 was diglyceride which composed of 3 saturated fatty acids including myristic acid, palmitic acid, and stearic acid and 3 unsaturated fatty acids which were palmitoleic acid, oleic acid and linoleic acid. Mixture M2 was a mixture of saturated C_{16}-C_{18} long chain carboxylic acid. Compound M1 was identified as D-mannitol. From the biological activity testing of isolated compounds, mixture B1 inhibited growth of *B. subtilis* ATCC 6633 with the MIC value of 1000 µg/ml but no cytotoxicity, while other compounds did not inhibited any tested microorganisms.

Field of study Biotechnology Student'ssignature……...

Acadamic Year 2004 Advisor's signature……..

Co-advisor's signature……

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Saranya Khumwongsa

October 2004

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

ATCC	= American Type Culture Collection, Maryland, U.S. A
^{o}C	= Degree Celsius
^{13}C-NMR	= Carbon-13 nuclear magnetic resonance
$CDCl_3$	= Dueterated chloroform
$CHCl_3$	= Chloroform
cm	= centimeter
COSY	= Correlation Spectroscopy
δ	= NMR chemical shift
DEPT	= Distortionless Enhancement by Polarization Transfer
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
D_2O	= Deuterium Oxide
ε	= Molar absorptivity
EI-MS	= Electron Impact Mass Spectrometry
ESI-MS	= Electrospray Ionization 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)

LIST OF ABBREVIATIONS (continued)

M^+	= Molecular ion
MCzA	= Malt Czapek Agar
MEA	= Malt Extract Agar
MeOH	= Methanol
mg	= milligram
MHz	= Megahertz
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
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

LIST OF ABBREVIATIONS (continued)

w = weak (for IR spectral data)

YES = Yeast Extract Sucrose agar

λ_{max} = the wavelength at maximum absorption (UV)

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

CHAPTER I

INTRODUCTION

The fungi are a very large and diverse group of organisms. It has been estimated that there may be as many as 1.5 million different fungal species on our planet (Hawksworth and Rossman, 1987). The fungi can be found throughout an environment such as in the fresh water, sea water, soil, decay, dung, include in the living plant and animal tissue. For the fungi which live inside plant tissue are called "endophytic fungi".

Endophytic fungi, are recognized as one of the most chemically promising groups of fungi in terms of diversity, pharmaceutical, and agricultural potential (Dreyfuss and Chapela, 1994, Strobel and Long, 1998). 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).

Of all of the world's plants, it seems that only a few grass species have had their complete complement of endophytes studied. As a result, the opportunity to find new or interesting endphytes and their bioactive compounds among the myriad of plants is great.

Thus, in this research the Thai medicinal plant, *Morinda citrifolia* L. had been used as a plant source for isolating endophytic fungi because the bark, stem, root, leaf, and fruit of this plant have been used traditionally as a folk remedy for many diseases. Moreover, scientific research revealed that the fruit juice of *Morinda citrifolia* L. contains a polysaccharide-rich substance that has been reported to have antitumor activity in the mice implanted with Lewis lung peritoneal carcinoma (Hirazumi et al., 1994, 1996, 1999). Damnacanthal, an anthraquinone from root of this plant has been found to be a new inhibitor of *ras* function and to help to suppress the activated *ras*-expressing tumors (Hiramatsu, 1993). The extract of plant roots have also been found to possess a

significant, dose-dependent, central analgesic activity in the treated mice (Younos et al., 1990). Consequently, the endophytic fungi isolated from *Morinda citrifolia* L. may also produce potentially bioactive compounds.

Objectives

1. To isolate the endophytic fungi from *Morinda citrifolia* L. leaves and twigs.
2. To determine antimicrobial activity of the isolated endophytic fungi.
3. To identify a selected endophytic fungal isolate.
4. To isolate and characterize the bioactive compounds from a selected endophytic fungal isolate.
5. To evaluate the biological activity of the bioactive compounds.

CHAPTER II

LITERATURES REVIEW

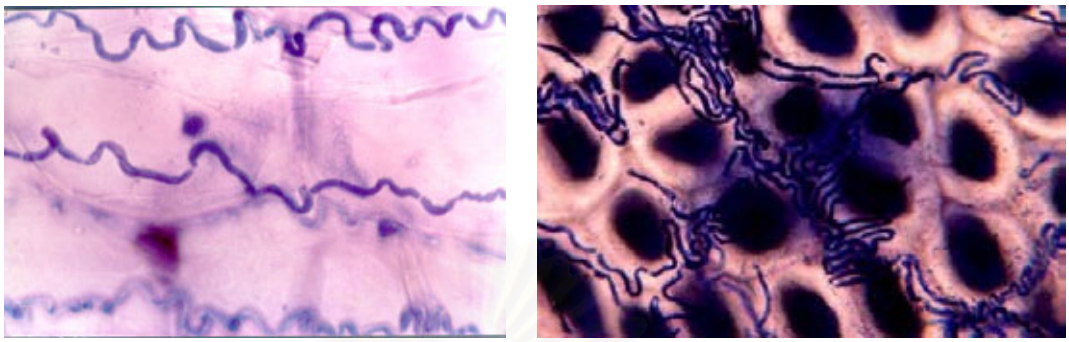
2.1 General consideration of Endophytic fungi

According to the most prevalent definition, fungal endophytes are 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 is stressed (Stone, 1990; Carroll, 1988). 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). In the past few decades, endophytic fungi have attracted great attention for two main reasons.

First, 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 endopytes in vascular plants, other than grasses, have focused mainly on ericaceous, dicotyledoneous plants and conifers (Rodrigues, 1996).

Fungal endophytes are 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 are distinguished from mycorrhizae by lacking external hyphae or mantels. Most of these endophytes are 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) (Figure 2.2).



(a) (b)

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

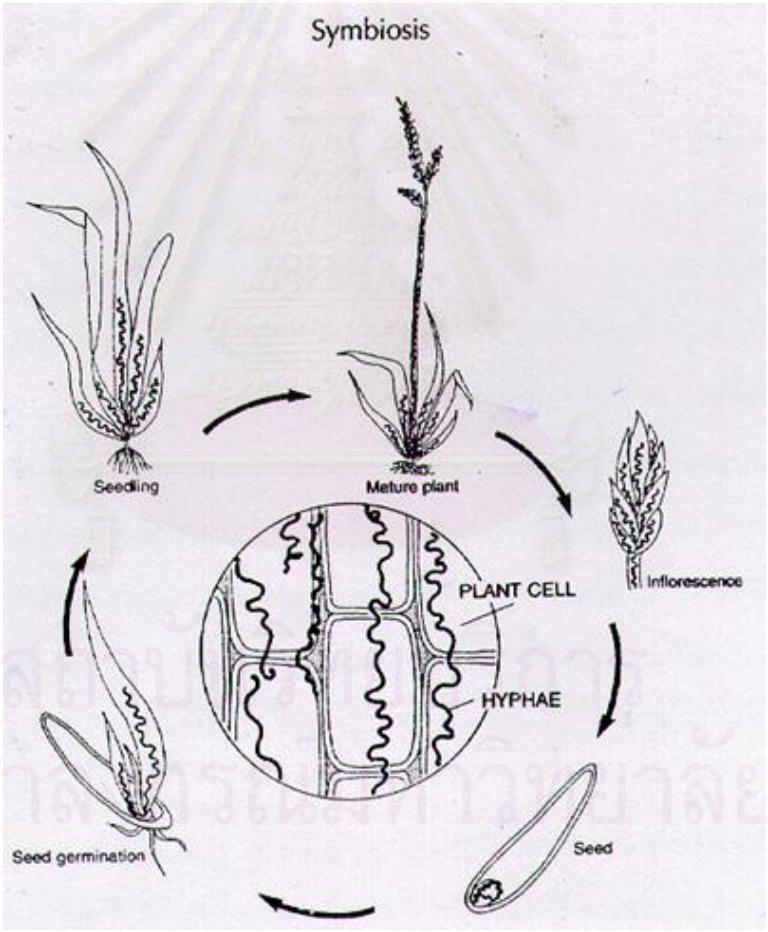


Figure 2.2 Life cycle of *Acremoium coneophiaum*, an endophytic fungus of grass. The fungus only produces hyphae that grow between plant cells (Paracer and Ahmadjian, 2000).

Second, endophytes may produce mycotoxins, or otherwise alter host physiology and morphology. Endophytic mycotoxins are thought to benefit their woody plant hosts as “inducible defenses” against insect herbivores (Carroll, 1988, 1991), and their grass hosts as “acquired plant defenses” (Cheplick and Clay, 1988) against both vertebrate and invertebrate herbivores. Endophytes may also alter other physiological, developmental or morphological properties of host plants such that competitive abilities are enhanced, especially in stressful environments (Clay, 1988, 1990; Bacon, 1993; Malinowski and Belesky, 1999).

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).

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 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). 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 nad 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).

2.3 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.

2.3.1 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.

2.3.1.1 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.

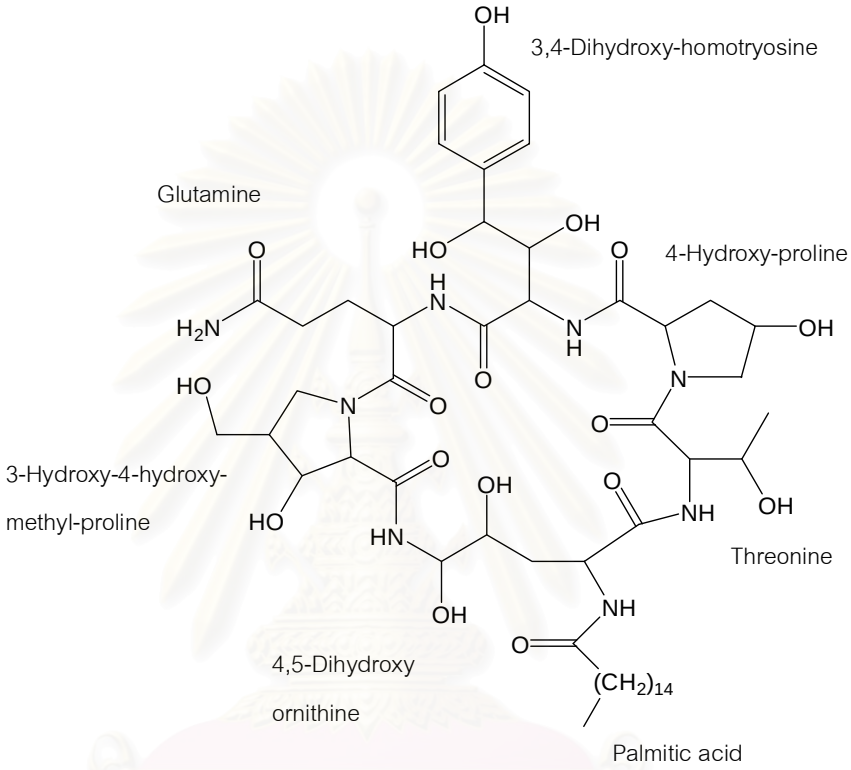


Figure 2.3 Cryptocandin, an antifungal lipopeptide obtained from the endophytic fungus *Cryptosporiopsis* cf. *quercina.*

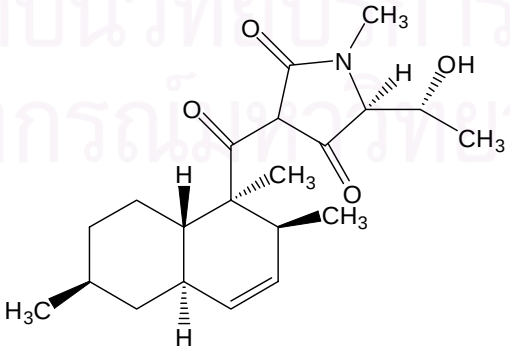


Figure 2.4 Cryptocin, a tetramic acid antifungal compound found in *Cryptosporiopsis* cf. *quercin*a.

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.

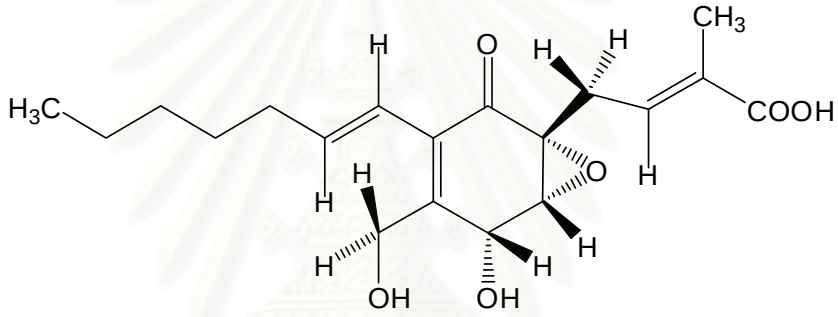


Figure 2.5 Ambuic acid, a highly functionalized cyclohexenone produced by a number of isolates of *Pestalotiopsis microspora* found in rainforests around the world. This compound possesses antifungal activity and has been used as a model compound for the development of solid-state NMR methods for the 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.

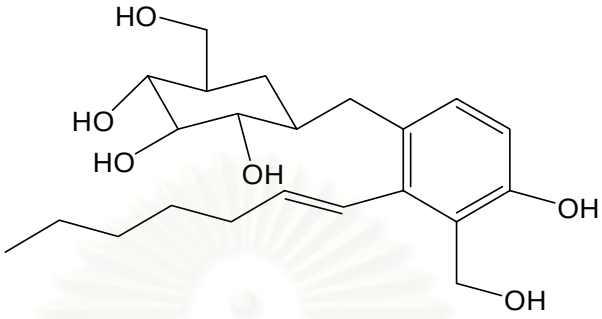


Figure 2.6 Pestaloside, a glucosylated aromatic compound with antifungal properties from *Pestalotiopsis microspora*.

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.

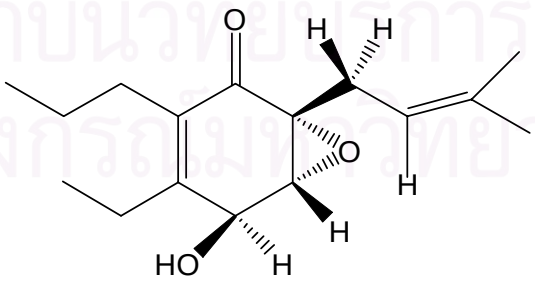


Figure 2.7 Jesterone, a cyclohexenone epoxide from *Pestaliotiopsis jesteri* with antioomycete activity.

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., 2001).

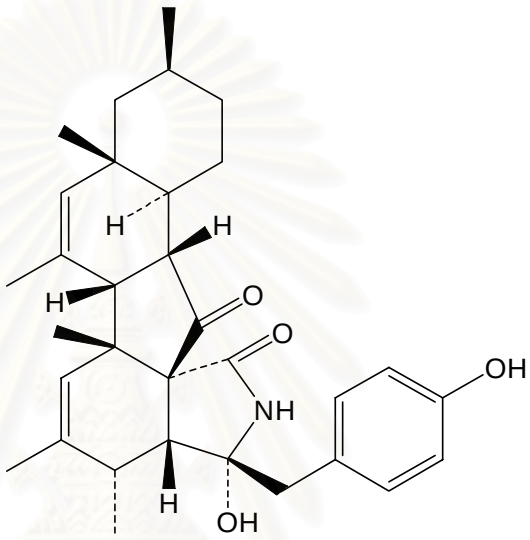


Figure 2.8 Phomopsichalasin, the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring from an endophytic *Phomopsis* sp. with antibacterial activity.

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).

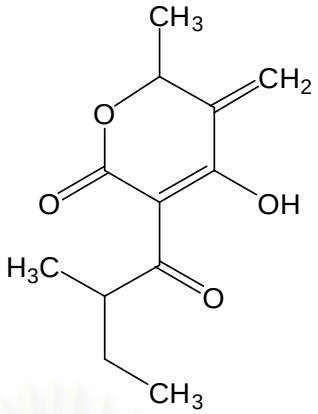


Figure 2.9 CR377, a new pentaketide antifungal agent from endophytic *Fusarium* sp.

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).

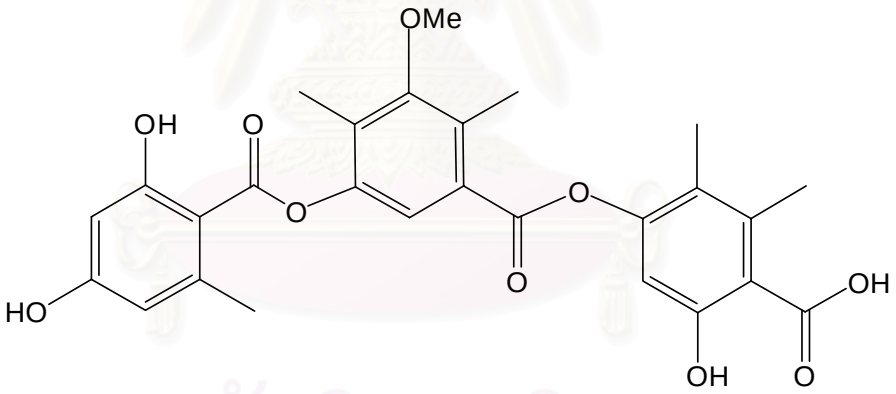


Figure 2.10 Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays antibacterial activity.

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).

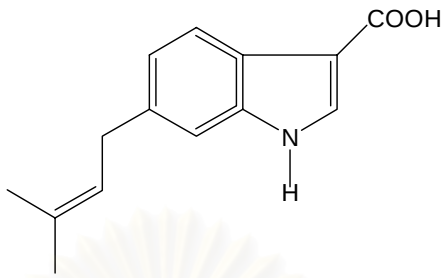
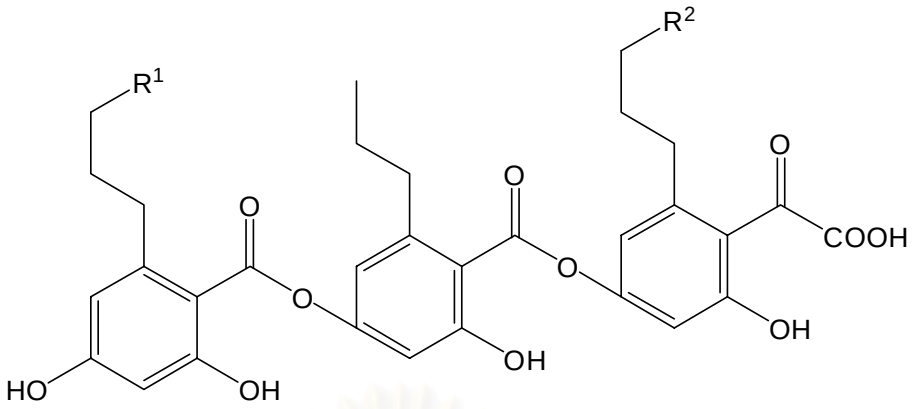


Figure 2.11 6-isoprenylindole-3-acetic acid, a indole derivative from *Colletotrichum* sp., an endophytic fungus isolated from *Artemisia annua* with activity against human pathogenic fungi, bacteria and was fungistatic to plant pathogenic fungi

2.3.1.2 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.



cytonic acid A R^1 = Et, R^2 = H
cytonic acid B R^1 = H, R^2 = Et

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

2.3.1.3 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 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).

2.3.2 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., 1993; 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.

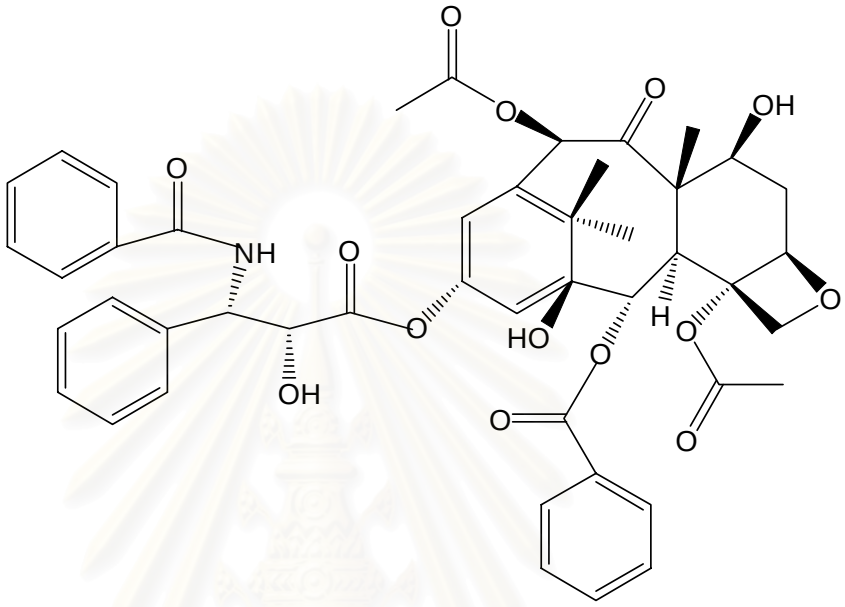


Figure 2.13 Taxol, the world's first billion-dollar anticancer drug produced by many endophytic fungi. It, too, possesses outstanding antioomycete activity.

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).

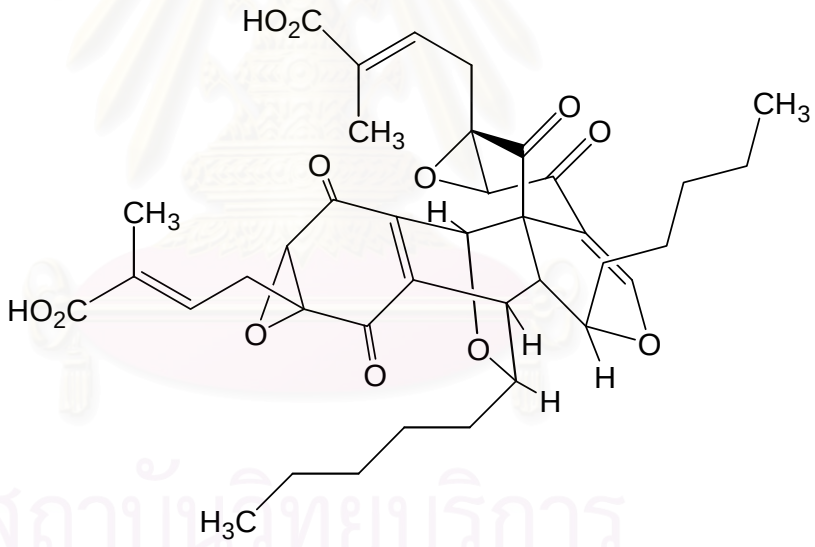
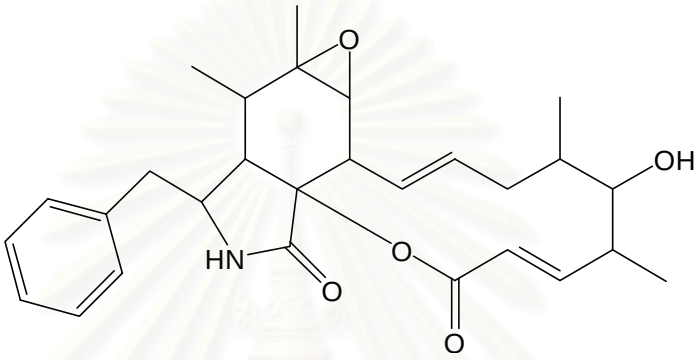


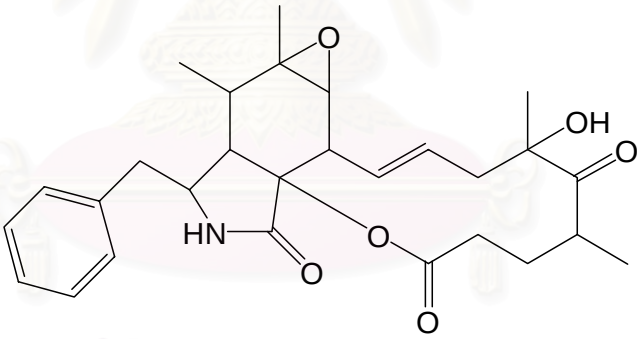
Figure 2.14 Torreyanic acid, an anticancer compound, from *Pestalotiopsis microspora.*

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.

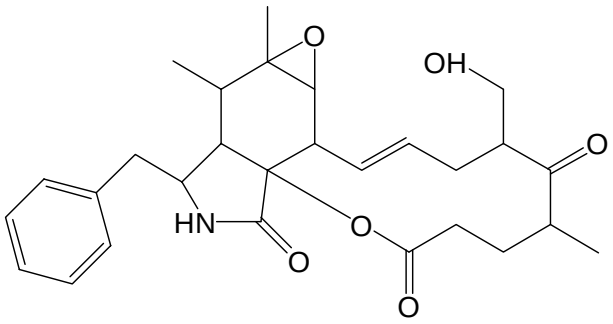


Cytochalasin 1

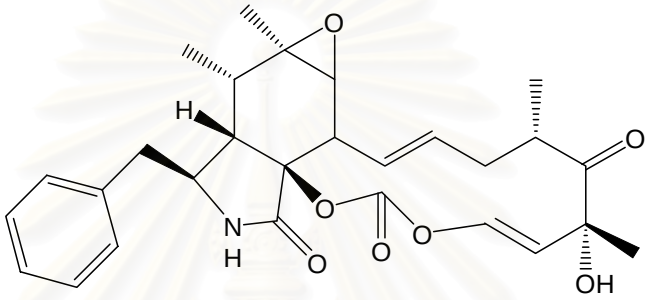


Cytochalasin 2

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)

2.3.3 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., 2003a). 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).

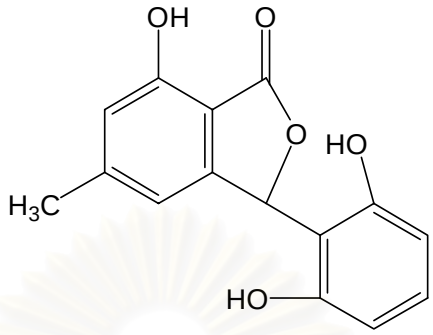


Figure 2.16 Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain, isolated from *Terminalia morobensis* growing on the north coast of Papua New Guinea.

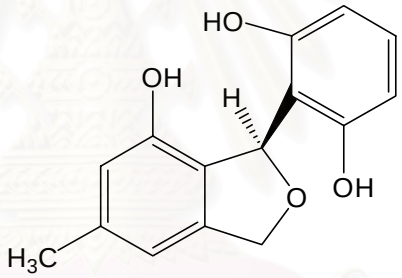
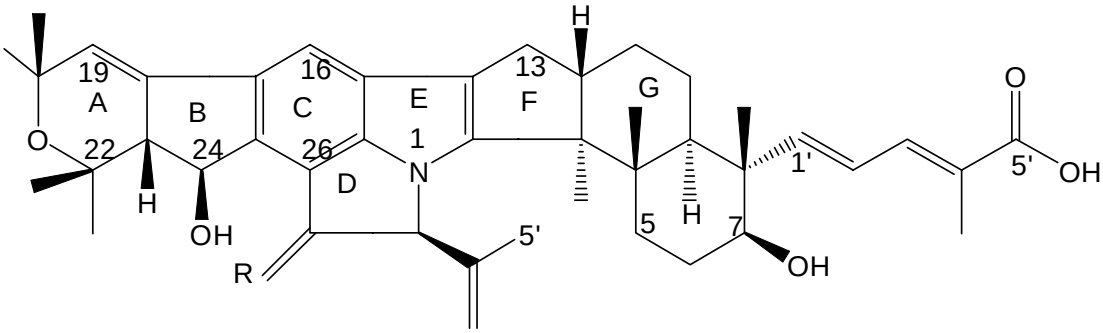


Figure 2.17 Pestacin, produced by *Pestalotiopsis microspora.* It, too, is an antioxidant.

2.3.4 Products of Endophytic fungi with Insecticidal Activities

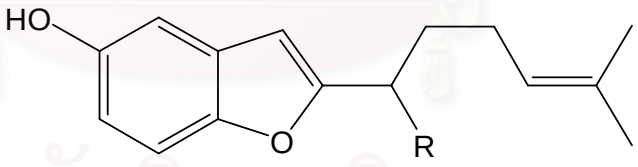
Bioinsecticides are only a small part of the insecticide field, but their market is increasing (Demain, 2000). Several endophytes are known to have anti-insect properties. Nodulisporic acids (Figure 2.18), novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, 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.

2.3.5 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, 1999).

2.3.6 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 *T. 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).

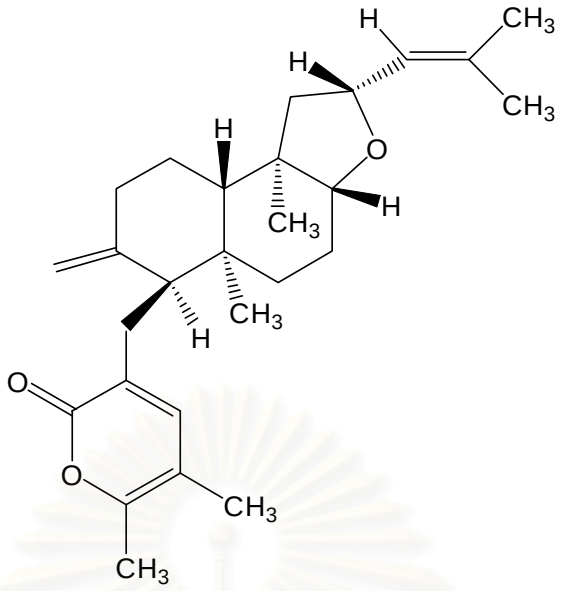


Figure 2.20 Subglutinol A, an immunosuppressant produced by an endophytic *Fusarium subglutinans* strain.

2.4 Rational for plant selection

Generally, it seems that novel taxa of these microbes are the most likely source of novel bioactive molecules such as antifungal agents, antibiotics, immunosuppressants, and other molecules of interest and importance. It is important to understand the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms at the genus, species, or biotype level as well as ones making novel bioactive products.

The most difficult problem, however, is to properly choose those among thousands of plant species on the planet to be the most fruitful foe study. Thus, since the number of plant species in the worlds is so great, creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying bioactivity (Mittermeier et al., 1999). A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is used. Several reasonable hypotheses govern this plant selection strategy and these follows:

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

ii. Plants that have an ethnobotanical history (use by indigenous peoples) that is related to the specific uses or application of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, may have nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).

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

iv. Plants growing in areas of great biodiversity, its follow, also have the prospect of housing endophytes with great biodiversity.

Just as plants from a distinct environmental setting are considered to be a promising source of novel endophytes and their compounds, so too are plants with an unconventional biology. For example an aquatic plant, *Rhyncholacis penicillata*, was collected from a river system in southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles (Strobel et al., 1999b) These environmental insults created many portals through which common phytopathogenic oomycetes could enter the plant. Still, the plant population appeared to be healthy, possibly due to protection from an endophytic product. This was the environmental biological clue used to pick this plant for a comprehensive study of its endophytes.

In addition, it is worthy of note that some plants generating bioactive natural products have associated endophytes that produce the same natural products. Such is the case with taxol, a highly functionalized diterpenoid and famed anticancer agent that is found in each of the world's yew tree species (*Taxus* spp.) (Suffness, 1995). In 1993, a novel taxol-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* was isolated and characterized (Strobel et al., 1993).

2.5 Plant sample

Morinda citrifolia L. is a medicinal plant which is native from Southeastern Asia to Australia (Morton, 1992), and now has a pantropical distribution from tropical Asia to

Polynesia such as in Thailand, India, Malaysia, Indonesia, Australia, Philippines, and Polynesia, This plant is called “Indian mulberry”, “cheese fruit” in England, “canary wood” in Australia, mengkudu, bengkudu, cangkudu in India, “ba ji tian” in China, “nhau” in Vietnam, “nhoo banz” in Lao, “mengkuda besar”, “mengkuda jantan” in Malaysia, “tumbongaso”, “bangkoro” in Philippines, “noni” in Tahiti and Hawaii. In Thailand called “yo ban” in central and northeast, “matasue” in north, and “yaeyai” in Maehongsorn province (มงคล แก้วเทพ, 2544).

2.5.1 Botanical description

Morinda citrifolia L. belongs to the family Rubiaceae, an evergreen shrub or small crooked tree that grows 3 to 8 meter in height (Perry and Metzger 1980; Groenendijk, 1993). It is cultivated wildly especially along occurring from the plains up to an altitude of 500 m. The leaves are simple, arranged in opposite pair, entire and are broadly elliptic to oblong in shape. They are dark shiny green in color. An average leaf measures about 30-35 cm in length and 13-15 cm in width. At each leaf axile there is a green tongue-like shaped stipule measuring about 1.5 cm in length. The bark of this tree is grayish in color, shallowly fissured and has quadrangular branchlets. The flowers are born on inflorescence that look like fleshy rounded heads (resembling the fruits), 1 to 1.5 cm in diameter, the calyx is truncate. The flowers are bisexual, fragrant with funnel-shaped corollas, the corolla is white. The stamens are inserted at the mouth of the corolla. The fruits are fleshy, very uneven drupes, more or less oblong in shape, white or greenish-white, and 3 to 10 cm in length and results from coalescence of the inferior ovaries of many closely packed flowers. It has a foul taste and a soapy smell when mature (สมพร ภูติยานันต์, 2546; อำไพวรรณ ประทุมธารารัตน์, 2540; Quisumbling, 1951). The pictures of leaves, twigs, flower, and fruit of *Morinda citrifolia* L. are shown in Figure 2.21.

Figure 2.21 Some parts of *Morinda cirtifolia* L.;(a) leaves, (b)twigs, (c) flowers, and (d) fruit.

2.5.2 Chemical constituents

In previous chemical studies, some anthraquinones, including damnacanthal, 7-hydroxy-8-methoxy-2-methyl-anthraquinone, morenone 1, and morenone 2, have been found in the roots of *Morinda citriflia* L. (Rusia and Srivastava 1989; Jain, Ravindra, and Srivastava, 1992).

In the seeds, ricinoleic acid has been found (Daulatabad, Mulla, and Mirajkar, 1989). From the heartwood, two known anthraquinones (morindone and physcion) and one new anthraquinone glycoside have been isolated (Srivastava and Singh, 1993).

Studies on the chemical components of the flowers have resulted in the identification of one anthraquinone glycoside and two flavone glycosides (Tiwari and Singh, 1977; Singh and Tiwari, 1976).

The volatile compounds in the ripe fruit are characterized by a large amount of carboxylic acids, especially octanoic acid and hexanoic acid (Farine et al., 1996). Several nonvolatile compounds have also been identified in fruit, including acetyl derivatives of asperuloside, glucose (Levand and Larson, 1979), glycosides of octanoic and hexanoic acid (Wang et al., 1999), 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanosyl-β-D-glucopyranose, 6-*O*-(β-D-glucopyranosyl)-1-*O*-hexnoyl-β-D-glucopyranose and 3-methylbut-3-enyl 6-*O*-β-D-glucopyranosyl-β-D-glucopyranoside (Wang et al., 2000).

The chemical constituents in the leaves of this plant are comprise of β-sitosterol, ursolic acid (Ahmad and Bano,1980), iridoid glycosides as citrifoliniside A and citrifolinin B, five flavonol glycosides including quercetin-3-*O*-β-D-glucopyranoside, kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside,quercetin-3-*O*-α-L-hamnopyranosyl-(1 →6)-β-D-glucopyranoside,quercetin-3-*O*-β-D-rhamnopyranosyl-(1 →2)-[α-L-rhamnopyranosyl-(1 →6)]-β-D-galacopyra-noside, and kaempferol-3-*O*-β-D-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galacopyranoside, asperulosidic acid and asperuloside (Sang et al., 2001a, 2001b).

2.5.3 Traditional uses

The bark has reddish purple and brown dye for in making batik. A yellowish dye is also extracted from the roots used to dye cloth (Leister, 1975). Very young leaves are cooked as vegetables and eaten with rice in Java and Thailand; mature leaves are wrapped around fish before cooking and then eaten with the cooked fish.The fruit of this plant were also used as foods in time of famine. On native premises this plant is very frequently cultivated not only valuable as a vegetable but also as a medicine (วันดี กฤษณพันธ์, 2538).

The roots are used as laxative (วุฒิ วุฒิธรรมเวช, 2540). In India, the roots are used as a cathartic (มงคล แก้วเทพ, 2544). A decoction of the root is used as febrifuge, tonic, and roborant in China and Japan. In Taiwan, the root decoction is given to treat dysentery, the pounded leaf is applied to ulcers and knife wounds (Perry and Metzger,

1980). In Vietnam roots serve to treat stiffness and tetanus and have been proven to combat arterial tension (Groenedijk, 1993).

The leaves are used as anti-diarrhea, febrifuge. In Philippines the leaves, when fresh, are applied on ulcers to affect a rapid cure. The sap of the leaves is anti-arthritic. In India the leaves are used as a healing application to wounds and ulcers and administered internally as a tonic and febrifuge. In Malaysia, the leaves are heated and applied to the chest or to the abdomen, for cough, enlarge spleen, in nausea, colic fever. (มงคล แก้วเทพ, 2544; Quisumbing, 1951).

The over-ripe fruit is used as an emmenagouge in Philippines and India (Perry and Metzger, 1980). In Thailand, traditional healers have used the fruit for symptomatic relief of nausea and vomiting (วันดี กฤษณพันธ์, 2538).

The bark decoction is used as an astringent in India and as a febrifuge in Malaysia (มงคล แก้วเทพ, 2544).

2.5.4 Pharmacological studies

The fruit juice of *Morinda citrifolia* L. contains a polysaccharide-rich substance that has been reported to have antitumor activity in the Lewis lung peritoneal carcinoma model (Hirazumi et al., 1994, 1996, 1999). Damnacanthal, an anthraquinone from root of this plant has been found to be a new inhibitor of *ras* function and to help to suppress the activated *ras*-expressing tumors (Hiramatsu, 1993). The extracts of plant roots have also been found to possess a significant, dose-dependent, central analgesic activity in the treated mice (Younos et al., 1990).

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments and equipments

3.1.1 Ultraviolet-visible Spectrophotometer (UV-VIS)

The UV-VIS spectra were recorded on a Hewlett Packard 8453 spectrophotometer.

3.1.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 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.

3.1.3 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H-NMR, ^{13}C-NMR, DEPT, 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; chloroform-*d* ($CDCl_3$), deuterium oxide (D_2O), and dimethylsulfoxide (DMSO-d_6) were used in NMR experiments. Reference signals were the signals of residual protonated solvents at δ 7. 26 (s) ppm (^{1}H) and 77.1 (t) ppm (^{13}C) for $CDCl_3$, 4.79 (s) ppm (^{1}H) for D_2O, and 2.50 (t) ppm (^{1}H) and 39.5 (sept) ppm (^{13}C) for DMSO-d_6.

3.1.4 Mass spectrometer

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

3.1.5 Gas Chromatography

The gas chromatograph were analyzed on 3 mm glass column of 15 % DEGS on

60/80 mesh Chromasorb S A W at 180°C using Shimadzu 15 A Gas Chromatograph.

3.1.6 Melting point

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

3.2 Chemical reagents

3.2.1 Solvents

All commercial grade solvents, used in this research such as hexane, chloroform, ethyl acetate and methanol, were purified by distillation prior to use. The reagent grade solvents were used for re-crystallization.

3.2.2 Other chemicals

1. Merck's silica gel 60 GF 254 for thin-layer chromatography Art. 7730 was used as adsorbents for Quick column chromatography.

2. Scharlau's silica gel 60, 0.04-0.06 mm for flash chromatography (230 - 400 mesh ASTM) was used as adsorbents for column chromatography.

3. Merck's TLC aluminum sheets, silica gel $^{60}F_{254}$ precoated 25 sheets, 20x20 cm^2, layer thickness 0.2 mm were used as adsorbent for TLC analysis.

4. TLC spots were visualized under ultraviolet light at wavelengths 254 and 365 nm, in iodine vapour, and under daylight after spraying with vanillin reagent (Dissolve 0.5 g vanillin in 95 ml ethanol and add 4.5 ml concentrated sulfuric acid) and heating until the colors developed.

3.3 Culture media

Potatoes Dextrose Agar (PDA) was used for the endophytic fungi isolation. PDA, Malt Extract Agar (MEA), Malt Czapek Agar (MczA), Yeast Extract Sucrose Agar (YES), and Sabouraud's Dextrose Agar (SDA) were used for observation morphology and determination antimicrobial activities of isolated endophytic fungi.

The medium for growing tested bacteria was nutrient medium (agar and broth). Yeast-malt extract medium (agar and broth) was used for growing tested yeast.

The media's formula was shown in appendix A.

3.4 Plant samples collection

Healthy leaves and twigs of *Morinda citrifolia* L. were collected from 4 provinces of Thailand, including Bangkok, Samutsongkhram, Kanchanaburi, and Khonkaen. The leaf and twig samples were preserved in a plastic bag at 4°C in a refrigerator until processing.

3.5 Isolation and cultivation of endophytic fungi

The plant samples were cleaned then the endophytic fungi were isolated using the surface sterilization method which was modified from the method described by Petrini (1986).

The leaf sections (ϕ 6 mm.) were cut from the petiole, middle, end rib, side rib, and lamina. The twig sections (1x1 cm) were cut from the plant samples. The leaf sections 100 pieces and twig sections 20 pieces of *Morinda citrifolia* L. collected from each source were isolated endophytic fungi. Both the leaf and the twig sections were surface sterilized by immersing in 95 % ethanol for 1 min. Then, the leaf sections and the twig sections were immersed in 5% sodium hypochlorite and 10% sodium hypochlorite respectively for 5 min and then were transferred to 95 % ethanol for 30 sec. They were finally washed twice in sterile distilled water and the sterilized leaf and twig pieces were then surfaced dried with sterile papers and immediately placed on the surface of Potatoes Dextrose Agar (PDA) Petri dishes.

All Petri dishes were incubated at room temperature (25-30°C) and examined every day for fungal mycelium from leaves and twigs under a stereomicroscope. Out growing were purified and transferred into new Petri dishes containing PDA by hyphal tip transfer. The purity of isolated endophytic fungi was determined by colony morphology.

3.6 Determination of antimicrobial activities of the isolated endophytic fungi

3.6.1 Tested microorganisms for antimicrobial activities

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

Table 3.1 Tested microorganisms for antimicrobial assays

Type of tested microorganisms		Reference strains
Bacteria	Gram positive rod bacterium	*Bacillus subtilis* ATCC 6633
	Gram positive cocci bacterium	*Staphylococcus aureus* ATCC 25923
	Gram negaitive rod bacterium	*Escherichia coli* ATCC 25922
	Gram negaitive rod bacterium	*Pseudomonas aeruginosa* ATCC 27853
Fungi	Yeast form	*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 nm) 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 tested 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

3.6.2 Preparation of endophytic fungi for antimicrobial activities

Each endophytic fungal isolate was cultivated on five media which were Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), and Sabouraud's Dextrose Agar (SDA) at room temperature (25-30°C) for 14 days. Then, the agar cultures of each fungal endophyte

isolate that grew on each medium were cut to the disk with a flamed 7 mm cork borer and were removed from cork borer hole by sterile needle.

3.6.3 Preparation of tested bacterial inoculum

Tested bacteria were grown on Nutrient Agar (NA) for 24 h at 37°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.

3.6.4 Preparation of tested 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 Yeast-Malt Extract broth (YMB) and incubated at room temperature for 6-8 h. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of 0.5 McFarland.

3.6.5 Antimicrobial assays for isolated endophytic fungi

The isolated endophytic fungi cultured on PDA, MEA, MCzA, YES, and SDA were investigated for their antimicrobial activity by fungal disk dual culture method (Howell and Stipanovic, 1980).

Within 15 minutes of adjusting the density of the inoculum, a sterile cotton swab on a wooden applicator stick was dipped into the standardized inoculum suspension. The excess fluid was removed by rotating the swab with firm pressure against the inside of the tube above the fluid level. The dried surface of Nutrient agar plate for test bacterial and Yeast-malt extract agar plate for test yeast were inoculated by streaking the swab in three difference planes, by rotating the plate approximately 60° each time, to ensure an even distribution of the inoculum.

The surface of the medium was allowed to dry for 3-5 minutes. Then, the agar culture disks of each endopytic fungal isolate (from 3.6.2) were placed on the inoculated plates and pressed firmly into the agar with needle to ensure complete contact with the

agar. Bacteria and yeast plates were incubated at 37°C and room temperature, respectively for 24 h. Inhibition zone around the fungal disk was measured in mm.

3.7 Identification and classification of endophytic fungal isolate LSS06

3.7.1 Morphological identification

3.7.1.1 Macroscopical features

Colony Characteristics of endophyitc fungal isolate LSS06, for example, shape, size, color, margin, pigment, and others were studied.

3.7.1.2 Microscopical features

3.7.1.2.1 Preparation of specimens for light microscope

The specimens for light microscopy were mounted in lactophenol-cotton blue or lactophenol aniline blue for observation the characteristic spore arrangements and other characteristics necessary for a definitive identification on an Olympus CH2 research microscope. Examine the preparation microscopically, first under the low-power (10x) objective and the under high-power (40x), or under oil immersion (100x) if suspicious fungal structures were seen. The methods of preparing cultures for microscopic examination are described as in the following sections.

3.7.1.2.1.1 Preparation of the fungal mycelia on glass slides by teasing mycelia

Fungal mycelia were dug out from a small portion of the fungal colony and some of the subsurface agar by dissecting needles. Placed the mycelial fragment on a microscope slide in a drop of lactophenol aniline blue, teased the mycelia apart by the needles, and covered with a coverslip. Gentle pressured on the surface of the coverslip with the eraser end of pencil for disperse the mount.

3.7.1.2.1.2 Preparation of the fungal mycelia on glass slides by Scoth tape preparation

With the unfrosted, clear cellophane tape, pressed the sticky side gently but firmly to the surface of the colony, picking up a portion of the aerial mycelium. This operation always was performed under a biologic safety hood and gloves were worn. Care must also be taken that the exposed fingers did not come in contact with the mold surface. The preparation was made by placing a drop of lactophenol aniline stain on a microscope slide, stuck one end of the tape to the surface of the slide adjacent to the drop of stain. The stretched the tape over the stain, gently lowering it so that the mycelium becomes permeated with stain. Pull the tape taut and then stuck the opposite end to the glass, avoiding as much as possible the trapping of air bubbles.

3.7.1.2.1.3 Preparation of slide culture

When permanent slide mounts are desired for further study, the microslide culture technique is recommended. The technique was as follows:

1. Placed a round piece of filter paper or gauze flat into the bottom of a sterile petri dish. Placed a pair of thin glass rods or applicator sticks cut to length to fit on top of the filter paper to serve as supports for a glass microscope slide.

2. Placed a 1x1 cm block or plug of PDA or MEA on the surface of the microscope slide.

3. Inoculated the margins of the agar plug in four places with a small portion of the colony tobe studied, using a straight inoculating wire or the tip of a needle

4. Gently heated a coverslip by passing it quickly through the flame and immediately placed it directly on the surface of the inoculated agar block. Heating the coverslip produces a tight seal between the bottom of the coverslip and the surface of the agar, which is briefly melted by the warm glass.

5. Pipeted a small amount of water into the bottom of the Petri dish to saturate the filter paper or the guaze. Placed the lid on the Petri dish and incubated the assembly at room temperature (or 30^{o}C) for 3 to 5 days.

6. When growth visually appears to be mature, the coverslip can be gently lifted from the surface of the agar with a pair of forceps, taking care not to disrupt the mycelium adhering to the bottom of the coverslip any more than necessary.

7. Placed the coverslip on a small drop of lactophenol aniline blue applied to the surface of a second glass slide. The mount can be preserved for further study by rimming the outside margins of the coverslp with clear fingernail polish.

8. After the coverslip had been removed from the agar block, the agar block itself can be removed by prying it away from the glass slide with an applicator stick. This operation was performed over a beaker containing 95% ethanol, into which the agar blocks were allowed to fall. The mycelium adhering to the surface of the original glass slide after the block was removed can also be stained with lactophenol aniline blue and a coverslip overlaid, serving as a second stained mount. Again, the mount can be preserved for further study by rimming the outside margins of the coverslp with clear fingernail polish, as previously described.

3.7.1.2.2 Preparation of specimens for scanning electron microscope

Endophytic fungal isolate LSS06 cultured on MEA was sent for observation and photography with scanning electron microscope at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

The cultures of endophytic fungi isolate LSS06 were cut into 1x1 cm and fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodim 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.

3.7.2 Molecular Identification

Sequences of internal transcribe spacer (ITS) regions of rDNA (Figure 3.1) from endophytic fungal isolate LSS06 were sent for identification by molecular methods at the Asian Natural Environmental Science Center, the University of Tokyo, Japan.

3.7.2.1 DNA extraction

Mycelia of endophytic fungal isolate LSS06 were dried with silica gel and kept at 4°C for further study. Genomic DNA was prepared from the dried samples by homogenization in 1.5 ml tubes with a FastPrep FP120 homogenizer (Savant, faxmingdale, NY, USA) and extraction with cetyltrimethylammonium bromide (CTAB) as described by Zhou et al. (1999). Fungal DNA extract was applied in CTAB buffer (2% CTAB, 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4M NaCl and 0.5% 2-mercaptoethanol) at 65°C for 1 h, extracted with phenol chloroform-isoamyl alcohol (25:24:1,v/v), then extracted with phenol chloroform-isoamyl alcohol mixture (24:1, v/v) twice. Fungal DNA was pricipitated with isopropanol and centrifuged at 8000 rpm for 5 min. Fungal DNA was dissolved in 100 µl TE buffer (10mM Tris-HCl (pH8.0) and 1mM EDTA) and kept at -30°C for further study.

3.7.2.2 ITS amplification

The ITS region of endophytic fungal isolate LSS06 was amplified with the primers ITS1F and ITS4. One primer was labeled with Texas red fluorescent dye (Genset KK, Kyoto, Japan) at the 5'-end for analysis with a sequencer. The primer pairs comprised labeled ITS1F and ITS4, ITS4, ITS1F and labeled ITS4. Fragments amplified by primers ITS1F and ITS4 were designated as ITS_{1F-4}. Twenty microliters of reaction mixture contained 5 ng template DNA, 0.2 mM each dNTP, 1xPCR buffer, 1.5 mM Mg^{2+}, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 µM of the primer pair. The amplification reactions were performed in a thermal cycler (TP 3000; Takara Shuzo, Tokyo, Japan). Amplification was started at 94°C for 9 min, followed by 38 cycles of a denaturing step at 94°C for 1 min, an annealing step at 51°C for 1 min, and an extension step at 72°C for 1 min, and ended with an additional 5-min extension step at 72°C. Two kinds of labeled ITS_{1F-4} were used in the following terminal-RFLP analysis. Three microliters ITS_{1F-4} was digested with 5U restriction endonuclease (*Alu*I or *Hin*fI) at 37°C for 8 h. After tenfold dilution, polymerase chain reaction (PCR) products of ITS and their restricted fragments were denatured at 94°C for 5 min and eletrophoresed on 6% Long Panger acrylamide gels (FMC Bioproducts, Pockland, ME, USA), with 6.1 M urea, and 1,2xTBE (0.1 M Tris (hydroxymethyl) aminomethane, 3.0 mM

ethylene diaminetetraacetic acid (EDTA), and 0.1 M boric acid), in a sequencer (SQ-5500E; Hitachi, Tokyo, Japan) (Kanchanaprayudh et. al., 2003).

3.7.2.3 DNA Sequencing

ITS_{1F-4} regions were amplified from the representative sample of isolated endophytic fungal. Amplified ITS_{1F-4} fragments were cloned using pT7 Blue vectors (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* strain XL1-Blue MRF. Legation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Texas Red (Hitachi) in and SQ-5500E sequencer (Kanchanaprayudh et. al., 2003).

ITS_{1F-4} sequences were automatically aligned with fungi ITS sequences obtained from GenBank DNA database, available from: http://www.ddbj.nig.ac.jp.

Primers for amplification and sequencing of ITS region and ITS2 sequence of rRNA gene.

ITS1F CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

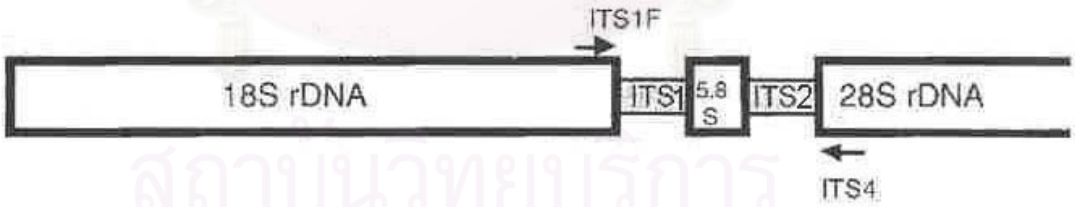


Figure 3.1 Schematic representation of ITS regions of rDNA

3.8 Determination of growth profile and antimicrobial activities of culture filtrate from endophytic fungal isolate LSS06

Endophytic fungal isolate LSS06 was grown on MEA at room temperature (25-30°C) for 2 weeks. The agar culture was cut into 7 mm diameter disks by a flamed cork borer. Five disks of the agar culture of fungal isolate LSS06 were inoculated into 250 ml

Erlenmeyer flasks containing 100 ml of MEB medium. The cultures were incubated at room temperature (25-30°C) under static condition for 38 days.

Mycelial dry weights were obtained by harvesting the fungal mycelia on dried (at 80°C for 24 h) and pre-weighted Whatmann no. 93 filter paper. The fungal mycelia were dried at 80°C for 24 h in an oven and weighted again. The mycelial dry weights were calculated from the difference between dry weights of filter paper before and after used to harvest the fungal mycelia.

Antimicrobial activities of culture filtrate of fungal isolate LSS06 during the time of cultivation were performed 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, by agar well diffusion method which was modified from the method described by Pereda-Miranda et al. (1993).

3.8.1 Preparation of tested bacterial inoculum

The bacterial inoculum were prepared in the same manner as described in section 3.6.3.

3.8.2 Preparation of tested yeast inoculum

The yeast inoculum was prepared in the same manner as described in section 3.6.4.

3.8.3 Inoculation of the tested plate

Within 15 minutes of adjusting the density of the bacteria or yeast inoculum, a sterile cotton swab on a wooden applicator stick was dipped into the standardized inoculum suspension. The excess fluid was removed by rotating the swab with firm pressure against the inside of the tube above the fluid level. The dried surface of Nutrient agar (NA) plate for test bacterial and Yeast-malt extract agar (YMA) plate for test yeast were inoculated by streaking the swab in three difference planes, by rotating the plate approximately 60° each time, to ensure an even distribution of the inoculum. The surface of the medium was allowed to dry for 3-5 min.

3.8.4 Application of culture filtrate

The NA and YMA plates that were already inoculated with tested bacteria and yeast, respectively were cut with a flamed 7 mm diameter cork borer to make the wells in the agar. One hundred µl of culture filtrate was applied into the agar wells. The culture filtrate was absorbed by the media surrounding the wells. Bacteria and yeast plates were incubated at 37°C and room temperature, respectively for 24 h. Inhibition zones around the wells were measured in mm.

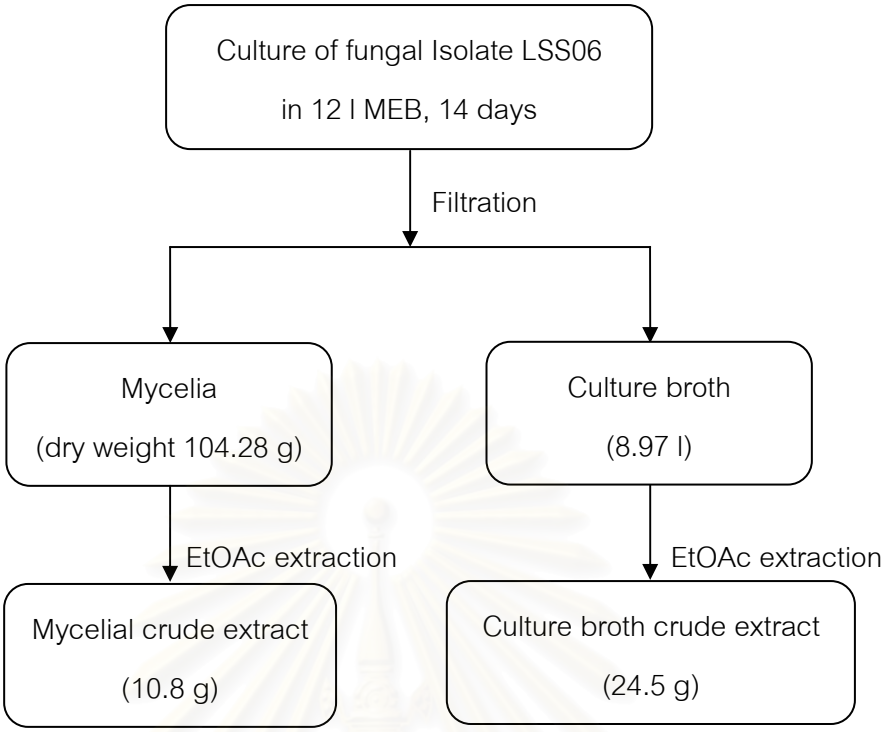
3.9 Cultivation and metabolites extraction of endophytic fungal isolate LSS06

3.9.1 Cultivation of endophytic fungal isolate LSS06 for producing secondary metabolites

Endophytic fungal isolate LSS06 cultured on MEA had the best antimicrobial activities because of this it was selected for study of the metabolites. Fungal isolate LSS06 was cultivated on MEA at room temperature (25-30°C) for 2 weeks. The agar cultures of this fungal isolate were cut into 7 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 12 l of MEB. The flasks were incubated statically at room temperature (25-30°C) for 14 days.

3.9.2 Extraction of secondary metabolites from culture broth and mycelia of endophytic fungal isolate LSS06

Cultivation broth of fungal isolate LSS06 in 12 l MEB was filtered through filter paper (Whatman No. 93). The culture filtrate (8.97 l) and mycelia (104.28 g dry weight) were extracted exhaustively with ethyl acetate (EtOAc). The EtOAc layer was collected and evaporated the solvent by using a rotary evaporator under reduced pressure at 35°C gave a brown viscous residue from culture broth extract 24.5 g and from mycelial extract 10.8 g. The extraction of the cultivation broth and mycelia of the endophytic fungal isolate LSS06 is shown in the scheme 3.1.



Scheme 3.1 Diagram of method for extraction of culture broth and mycelia of endophytic fungal isolate LSS06 by EtOAc

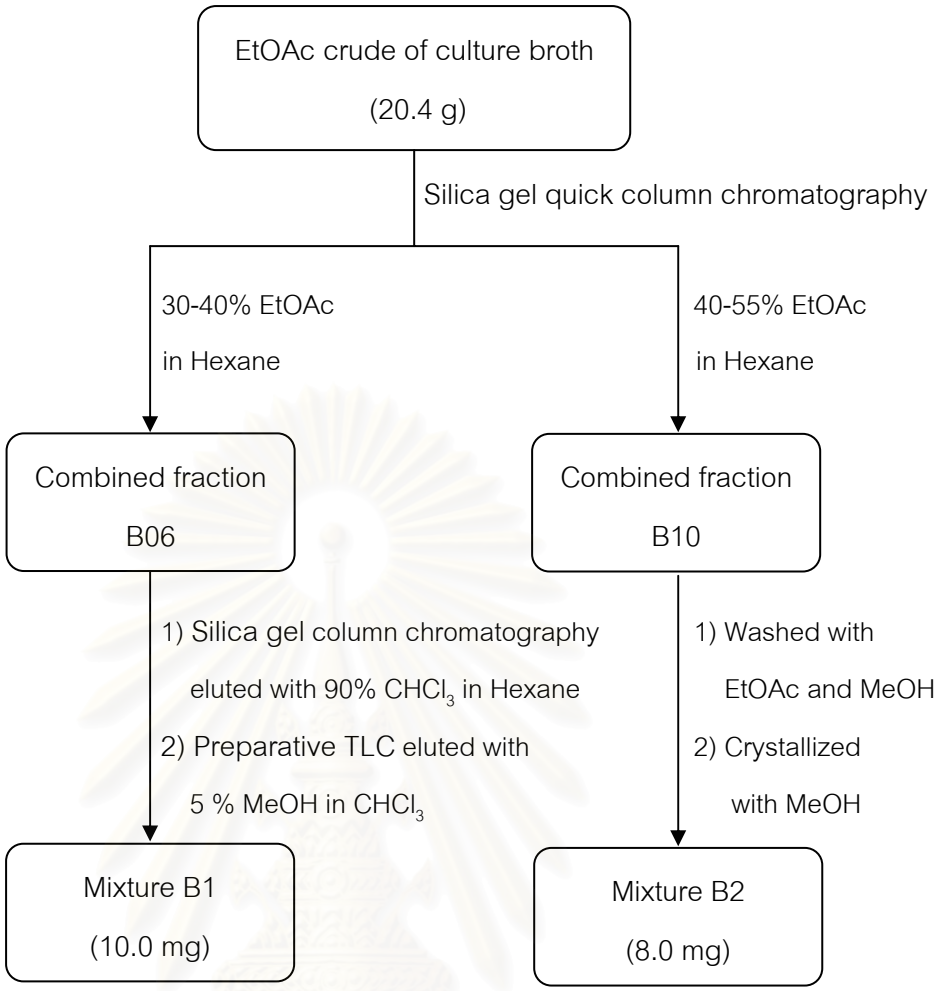
3.10 Isolation and characterization of metabolites of endophytic fungal isolate LSS06

3.10.1 Isolation and characterization of metabolites from culture broth crude extract

EtOAc crude of culture broth extract of endophytic fungal isolate LSS06 (20.4 g) was fractionated by chromatography on a silica gel quick column (silica gel 283 g). Elution systems were Hexane, Hexane-EtOAc gradients, EtOAc, EtOAc-MeOH gradients, and MeOH. Each fraction (250 ml) was collected and examined. The fractions were combined by silica gel TLC. Fractions with the same TLC pattern were combined and dried. The results from the separation and purification of culture broth EtOAc extract crude are shown in Table 3.3 and Scheme 3.2.

Table 3.3 Characteristics of separation fraction of EtOAc crude of culture broth extract of endophytic fungal isolate LSS06

Combined fraction	Fraction No.	Eluents	Appearance	Weight (g)
B01	1	100% Hexane	Yellow viscous liquid	0.0029
B02	2-7	10-20% EtOAc in Hexane	Yellow brown viscous liquid	0.0169
B03	8-11	20% EtOAc in Hexane	Yellow brown viscous liquid	0.6981
B04	12-16	20-25% EtOAc in Hexane	Yellow brown viscous liquid	0.6963
B05	17-21	25% EtOAc in Hexane	Yellow brown viscous liquid	0.5991
B06	22-32	30-40% EtOAc in Hexane	Yellow brown viscous liquid	4.0387
B07	33-37	40% EtOAc in Hexane	Yellow brown viscous liquid	2.4581
B08	38-42	40% EtOAc in Hexane	Yellow brown viscous liquid	1.5157
B09	43-49	40% EtOAc in Hexane	Red brown viscous liquid	1.6626
B10	50-71	40-55% EtOAc in Hexane	Red brown amorphous solid with red brown viscous liquid	3.0332
B11	72-94	55-80% EtOAc in Hexane	Red brown viscous liquid	1.7339
B12	95-122	80% EtOAc in Hexane-100% EtOAc	Red brown viscous liquid	2.5967
B13	123-143	10-30% MeOH in EtOAc	Black brown viscous liquid	3.0142
B14	144-163	30% MeOH in EtOAc-100% MeOH	Black brown viscous liquid	2.1753



Scheme 3.2 Diagram of isolation procedure for EtOAc crude of culture broth extract of endophytic fungal isolate LSS06

The yellow brown viscous liquid combined fraction B06 (1.0 g) was purified further by silica gel column chromatography eluted with 90% $CHCl_3$ in Hexane; then, preparative TLC eluted with 5 % MeOH in $CHCl_3$ was performed to yield mixture B1 as yellow viscous liquid (10 mg);

λ_{max} ($CHCl_3$) (ε): 242 (4629) nm;

ν_{max} (Kbr): 3420 (broad, m), 2918 and 2848 (w), 1715 (s), 1653 and1443 (w), 1306, 1279, and 1104 (w) cm^{-1};

ESI-MS: $[M+Na]^+$ *m/z* 381;

δ_H ($CDCl_3$, 400 MHz): 6.62 (1H, dd, *J* = 7.2, and 15.2 Hz), 6.18 (1H, d, *J* = 15.6 Hz), 4.06 (2H, d, *J* = 11.2 Hz), 3.93 (2H, d, *J* = 11.2 Hz), 3. 91 (1H, s), 3.76 (1H, s), 3.65 (1H, d, *J* = 6.4 Hz), 1.57 (1H, s) ppm;

δ_C ($CDCl_3$, 100 MHz): 165.7 (C), 141.2 (CH), 125.4 (CH), 101.9 (C), 73.2 (CH), 68.0 (C), 65.4 (CH_2), 55.6 (CH), 52.0 (CH_3), 24.1 (CH_3) ppm.

The red brown amorphous solid with red brown viscous liquid combined fraction B10 (3.03 g) was washed with EtOAc and MeOH and followed by crystallization from MeOH to afford mixture B2 as white amorphous solid (8.0 mg);

m.p.: 203-205 °C; λ_{max} (MeOH) (ε): 202 (1383) nm;

ν_{max} (Kbr): 3424 and 3485 (broad, s), 2935 (w), 1782 (s), 1445 and1365 (w), 1146 and 1112 (w) cm^{-1};

ESI-MS: $[M+Na]^+$ *m/z* 287;

δ_H (DMSO-d_6, 400 MHz): 5.32 (1H, d, *J* = 5.2 Hz), 5.09 (1H, d, *J* = 4.0 Hz), 4.80 (1H, dd, *J* = 5.6, and 6.4 Hz), 4.71 (1H, d, *J* = 6.8 Hz), 4.70 (1H, d, *J* = 2.8 Hz), 4.67 (1H, d, *J* = 5.6 Hz), 4.56 (1H, dd, *J* = 3.2, and 4.8 Hz), 3.49 (1H, d, *J* = 5.2 Hz), 2.95 (2H,dd, *J* = 5.2, and 17.6 Hz), 2.30 (2H, d, *J* = 17.6 Hz), 2.17 (3H, s), 1.22 (3H, s) ppm;

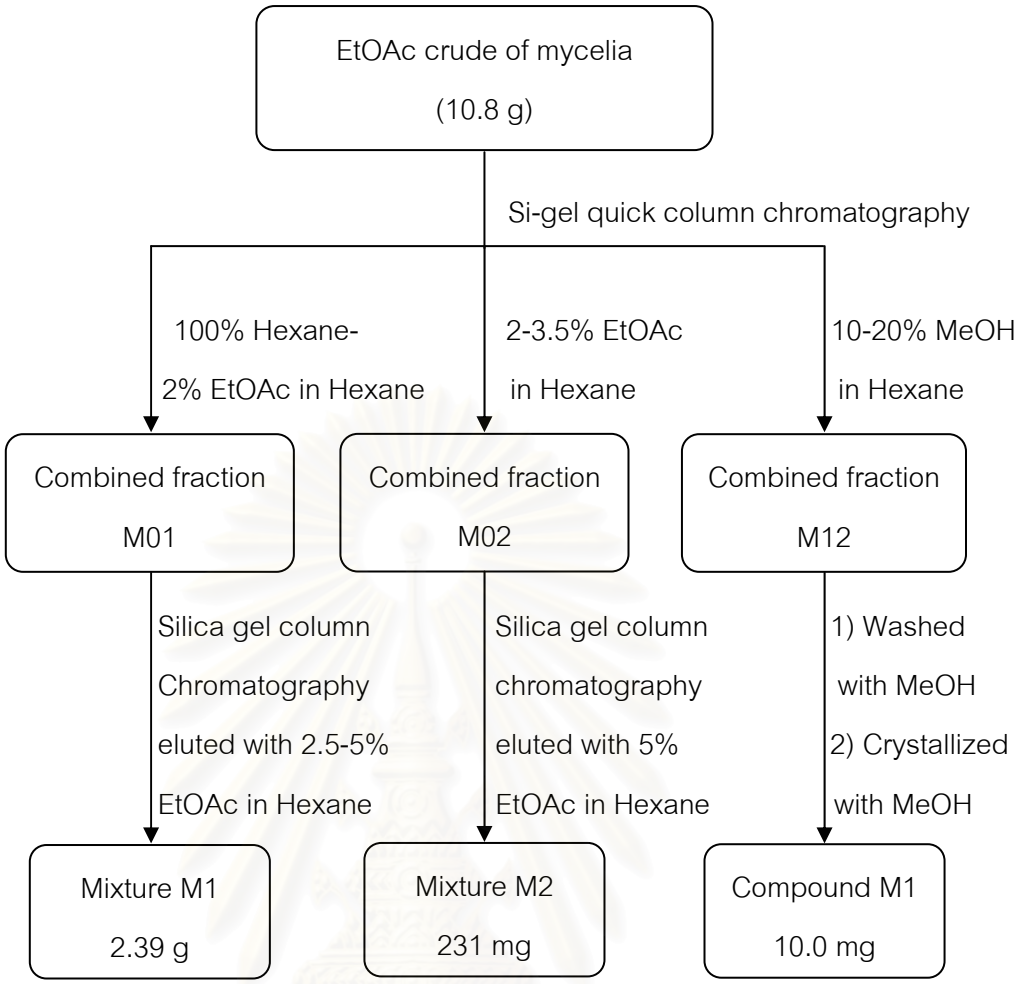
δ_C (DMSO-d_6, 100 MHz): 176.4 (C), 176.3 (C), 106.6 (C), 101.4 (CH), 100.5 (CH), 86.6 (CH), 84.4 (C), 83.7 (CH), 77.9 (C), 76.3 (CH), 76.1 (CH), 74.2 (CH), 69.8 (CH), 37.0 (CH_2), 28.3 (CH_3), 20.0 (CH_3) ppm.

3.10.2 Isolation and characterization of metabolites from mycelial crude extract

EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 (10.8 g) was fractionated by chromatography on a silica gel quick column (silica gel 283 g). Elution systems were Hexane, Hexane-EtOAc gradients, EtOAc, EtOAc-MeOH gradients, and MeOH. Each fraction (250 ml) was collected and examined. Fraction combination was performed by silica gel TLC. Fractions with the same TLC pattern were combined and dried. The results from the isolation of EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 are shown in Table 3.4 and Scheme 3.3.

Table 3.4 Characteristics of separation fraction of EtOAc crude of mycelial extract of endophytic fungal isolate LSS06

Combined fraction	Fraction No.	Eluents	Appearance	Weight (g)
M01	1-20	100% Hexane-2% EtOAc in Hexane	Yellow oily liquid with white amorphous solid	2.8520
M02	21-34	2-3.5% EtOAc in Hexane	Pale yellow wax solid	0.4600
M03	35-52	5-8% EtOAc in Hexane	Red brown viscous liquid	0.4920
M04	53-69	8-12% EtOAc in Hexane	Red brown viscous liquid	0.1019
M05	70-81	12-17% EtOAc in Hexane	Red brown viscous liquid	0.0712
M06	82-91	17-25% EtOAc in Hexane	Red brown viscous liquid	0.0978
M07	92-103	25-35% EtOAc in Hexane	Red brown viscous liquid	0.1403
M08	104-114	35-40% EtOAc in Hexane	Red brown viscous liquid	0.2386
M09	115-130	45-55% EtOAc in Hexane	Red brown viscous liquid	0.4329
M10	131-148	55-70%EtOAc in Hexane	Red brown viscous liquid	0.4391
M11	149-178	70% EtOAc in Hexane-5% MeOH in EtOAc	Red brown viscous liquid	1.9778
M12	179-188	10-20% MeOH in EtOAc	Red brown amorphous solid with red brown viscous liquid	1.0593
M13	189-213	20% MeOH in EtOAc-100% MeOH	Red brown viscous liquid	1.4048



Scheme 3.3 Diagram of isolation procedure for EtOAc crude of mycelial extract of endophytic fungal isolate LSS06

The yellow oily liquid with white amorphous solid combined fraction M01 (2.85 g) was purified further by silica gel column chromatography eluted with 2.5-5% EtOAc in Hexane to yield mixture M1 as yellow oily liquid with rancid odor (2.39 g);

ν_{max} (NaCl): 3464 (broad, m), 2926 and 2857 (s), 1743 (s), 1458 and 1373 (s), 1161 (s) cm^{-1};

δ_H ($CDCl_3$, 400 MHz): 5.34 (broad, s), 5.26 (broad, s), 4.29 (dd, *J* = 4.0, and 8.0 Hz), 4.14 (m), 2.32 (m), 2.01 (d, *J* = 5.6 Hz), 1.60 (broad, s), 1.37-1.17 (m), 0.88 (t, *J* = 8.4 Hz) ppm;

δ_C ($CDCl_3$, 100 MHz): 173.0 (C), 129.7 (CH), 129.4 (CH), 68.7 (CH), 61.9 (CH_2), 33.9 (CH_2), 33.8 (CH_2), 31.8 (CH_2), 29.6 (CH_2), 29.5(4)* (CH_2), 29.5(0) (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2(4) (CH_2), 29.2(0) (CH_2), 29.1(7) (CH_2), 29.1(4) (CH_2), 29.0 (CH_2), 28.9 (CH_2), 28.8 (CH_2), 27.0 (CH_2), 26.9 (CH_2), 24.6 (CH_2), 22.5 (CH_2),13.9 (CH_3) ppm.

* The number in parenthesis after δ_C value was the second decimal notation value.

The yellow waxy solid combined fraction M02 (460 mg) was purified further by silica gel column chromatography eluted with 5% EtOAc in Hexane, yielded mixture M2 as white waxy solid with rancid odor (231 mg);

ν_{max} (Kbr): 3466 (broad), 2920 and 2852 (s), 1705 (s), 1467 and 1409 (w), 1294 (w) cm^{-1};

MS (EI 70 eV) *m/z*: 284 $[C_{18}H_{36}O_2]^+$, 256 $[C_{16}H_{32}O_2]^+$, 241, 227, 213, 199, 185, 171, 157, 143, 129, 115, 97, 87, 73, 69, 57;

δ_H ($CDCl_3$, 400 MHz): 2.34 (4H, t, *J* = 7.6 Hz), 2.07-1.97 (m), 1.77-1.57 (m), 1.52-1.17 (m), 0.87 (6H, *J* = 6.4 Hz) ppm;

δ_C ($CDCl_3$, 100 MHz): 180.1 (C), 34.2 (CH_2), 32.0 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29. 4 (CH_2), 29.2 (CH_2), 24.8 (CH_2), 22.8 (CH_2), 14.2 (CH_3) ppm.

$CH_3(CH_2)_{14\text{-}16}COOH$

Mixture M2

The red brown amorphous solid with red brown viscous liquid combined fraction M12 (1.05 g) was washed with MeOH and followed by crystallization from MeOH to afford compound M1 as white amorphous solid (10.0 mg);

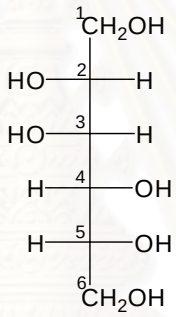
m.p.: 169-170 °C; λ_{max} (H_2O): no absorption;

ν_{max} (Kbr): 3398 (br, s), 2943 and 2900 (s), 1433 (s), 1277, 1083, and 1013 (m) cm^{-1};

ESI-MS: $[M+Na]^+$ *m/z* 205.4;

δ_H (D_2O, 400 MHz): 3.59 (2H, H-1 and H-6, dd, *J* = 6.0, and 11.6 Hz), 3.68 (2H, H-2 and H-5, ddd, *J* = 2.4, 6, and 14.8 Hz)), 3.72 (2H, H-3 and H-4, d, *J* = 8.8 Hz), 3.79 (2H, H-1 and H-6, dd, *J* = 2.4, 11.6 Hz) ppm;

δ_C (D_2O, 100 MHz): 70.6 (CH), 69.1 (CH), 63.1 (CH_2) ppm.



Compound M1

3.11 Study of mycelial metabolites of endophytic fungal isolate LSS06 cultivated in different media

Endophytic fungal isolate LSS06 cultured in various media were determined the mycelial metabolites. Endophytic fungal isolate LSS06 was cultivated on five media, including Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), and Sabouraud's Dextrose Agar (SDA) at room temperature (25-30°C) for 2 weeks. The agar culture of endophytic fungal isolate LSS06 was cut into 7 mm diameter disks using a flamed cork borer. Five pieces of agar culture of fungal isolate LSS06 on each medium were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of the broth of each medium which were Potatoes Dextrose Broth (PDB), Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Yeast Extract Sucrose Broth (YESB), and Sabouraud's Dextrose Broth (SDB). The flasks were incubated statically at room temperature (25-30°C) for 14 days. Several flasks of culture were prepared to obtain 2 l of each medium. The cultivation broth of isolate LSS06 in 2 l of each media was filtered through a filter paper (Whatman No. 93). The mycelia of endophytic fungal isolate LSS06 were extracted exhaustively with EtOAc. EtOAc crude of mycelial extract of fungal isolates LSS06 cultured in PDB, MCzB, MEB, YESB, and SDB were fractionated by chromatography on a silica gel quick column (silica gel 283 g). Elution systems were Hexane, Hexane-EtOAc gradients, EtOAc, EtOAc-MeOH gradients, and MeOH. Each fraction (50 ml) was collected and examined. Fractions with the same TLC pattern were combined and dried. Isolated metabolites were purified by the same procedure as described in section 3.10.2.

3.12 Determination of antimicrobial activities of crude extracts and combined fractions

The antimicrobial activities of the crude extracts and combined fractions were performed against *B. subtilis* ATCC 6633*, S. aureus* ATCC 25923*, E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albican*s ATCC 10231. Ten mg of crude extracts and each combined fraction were dissolved in 1 ml of 10% DMSO in sterile distilled water and determined antimicrobial activities by the agar well diffusion method in the same manner as described in section 3.8.1 to 3.8.4.

3.13 Evaluation of biological activities of isolated metabolites

3.13.1 Antimicrobial assay

Antimicrobial activities of isolated metabolites against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 were determined by the microdilution broth susceptibility testing which was modified from Gerlach, Jones, and Barry(1983).

3.13.1.1 Preparation of tested compound

Four mg of the isolated metabolites were dissolved in 2 ml of 10% DMSO in sterile distilled water for used in the assay.

3.13.1.2 Preparation of tested bacterial inoculum

The test bacteria were grown on Nutrient Agar (NA) for 24 h at 37°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 Muller Hinton Broth (MHB) (Hi media) and incubated at 37°C for 2-8 h, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with MHB to match the turbidity of 0.5 McFarland (OD_{625} = 0.08-0.1 nm) to obtain a cell suspension containing approximately 10^6-10^7 CFU/ml.

3.13.1.3 Preparation of tested 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 Yeast-Malt Extract broth (YMB) and incubated at room temperature for 6-8 h. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of 0.5 McFarland (OD_{625} = 0.08-0.1 nm) to obtain a cell suspension containing approximately 10^6 CFU/ml.

3.13.1.4 Procedure for antimicrobial assay

A solution of the isolated metabolites (2 mg/ml) was two fold serial diluted with MHB and YMB for antibacterial and antifungal (yeast form) assays,

respectively. Fifty µl of isolated metabolite solution was dispensed into each well in sterile microtiter plates (96-well bottom wells). Fifty µl of the final adjusted inoculum suspension was inoculated into each well. The concentration range of tested compound was 1000-31.25 µg/ml. The final concentration of the inoculum of tested bacteria and yeast was approximately $2.5x10^5$-10^6 and $2.5x10^5$CFU/ml, respecticely. One hundred µl of medium only was as the sterility control. A 100 µl volume of the medium and inoculum mixture acted as the growth control. Inoculated microtiter plates were incubated at 37°C for 18-24 h.

Antibacterial and antifungal (yeast form) activities were determined by measuring the turbidity each well in the microtiter plates by using the Sunrise microplate reader (TECAN, AUSTRIA) after incubation. The lowest concentration of isolated metabolites that inhibits growth of test microorganisms was recorded as minimal inhibitory concentration (MIC).

3.13.2 Cytotoxicity assay

Isolated metabolites were sent to determine cytotoxicity at the Institue of Biotechnology and Genetic Engineering. 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 °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 $^{\circ}$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.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

Endophytic fungi were isolated from *Morinda citrifolia* L. leaves and twigs collected from 4 provinces in Thailand, including Bangkok, Samutsongkhram, Kanchanaburi, and Khonkaen. Leaf sections 100 pieces and twig sections 20 pieces of *Morinda citrifolia* L. collected from each source were used for isolation endophytic fungi.

One hundred and seventy eight endophytic fungal isolates were isolated from leaves and twigs of *Morinda citrifolia* L. Endophytic fungal 116 isolates were isolated from leaf sections (29 % of total leaf sections) and 62 isolates were isolated from twig sections (77.5 % of total twig sections). The percentage of endophytic fungi isolated from twig sections was higher than that from leaf sections. This result is in accordance with most of the investigations undertaken with other host plants from other localities in which twigs tended to support a greater frequency of internal fungal colonization (Norio et al., 1999). This may be owing to the twigs 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. The examples of the isolated endophytic fungi from leaf and twig sections of *Morinda citrifolia* L. are shown in Figure 4.1 and 4.2, respectively.

Table 4.1 The number and code of the isolated endophytic fungi from leaves and twigs of *Morinda citrifolia* L.

Source of endophytic fungi	Endophytic fungi isolated from leaf sections		Endophyic fungi isolated from twig sections	
	Number of isolates (% of total sections)	Code of isolated endophytic fungi	Number of isolates (% of total sections)	Code of isolated endophytic fungi
Bangkok	22 (22)	LBK01-22	13 (65)	TBK01-13
Samutsongkhram	28 (28)	LSS01-28	17 (85)	TSS01-17
Kanchanaburi	31 (31)	LKC01-31	16 (80)	TKC01-16
Khonkaen	35 (35)	LKK01-35	16 (80)	TKK01-16
Total	116 (29% of total sections)		62 (77.5% of total sections)	
	178			

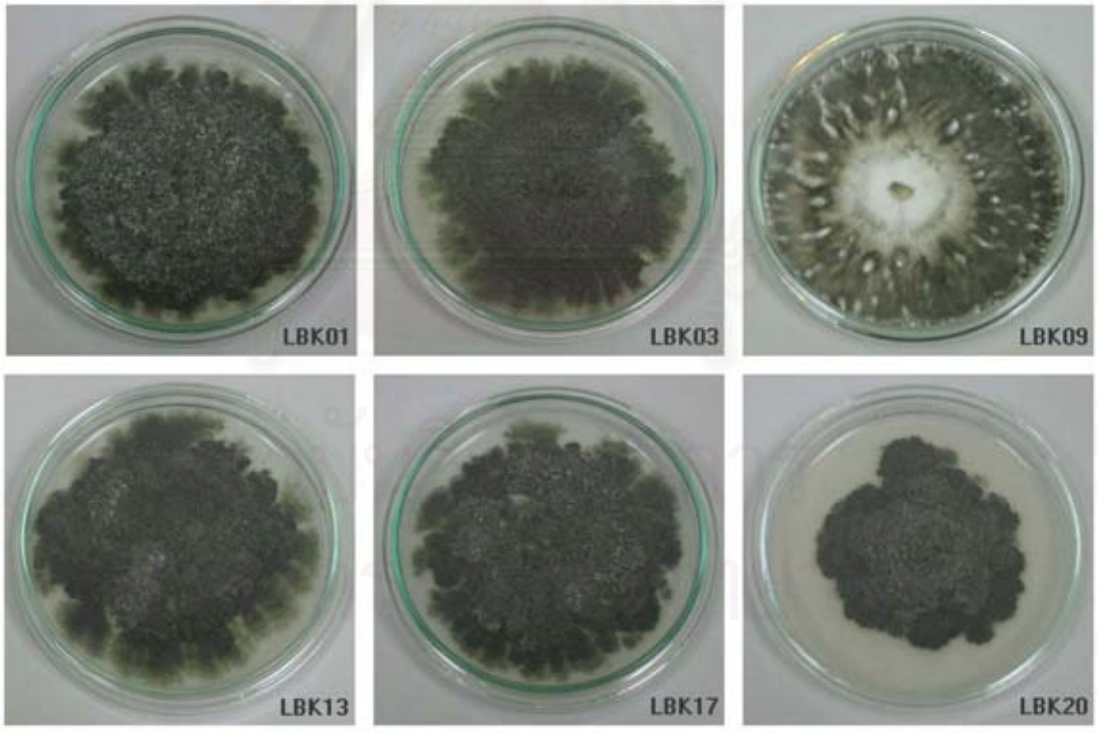


Figure 4.1 Colony characteristic of some endophytic fungi isolated from leaf sections of *Morinda citrifolia* L., after cultivation on PDA for 2-4 weeks at room temperature. The code of each isolate is shown in each picture.

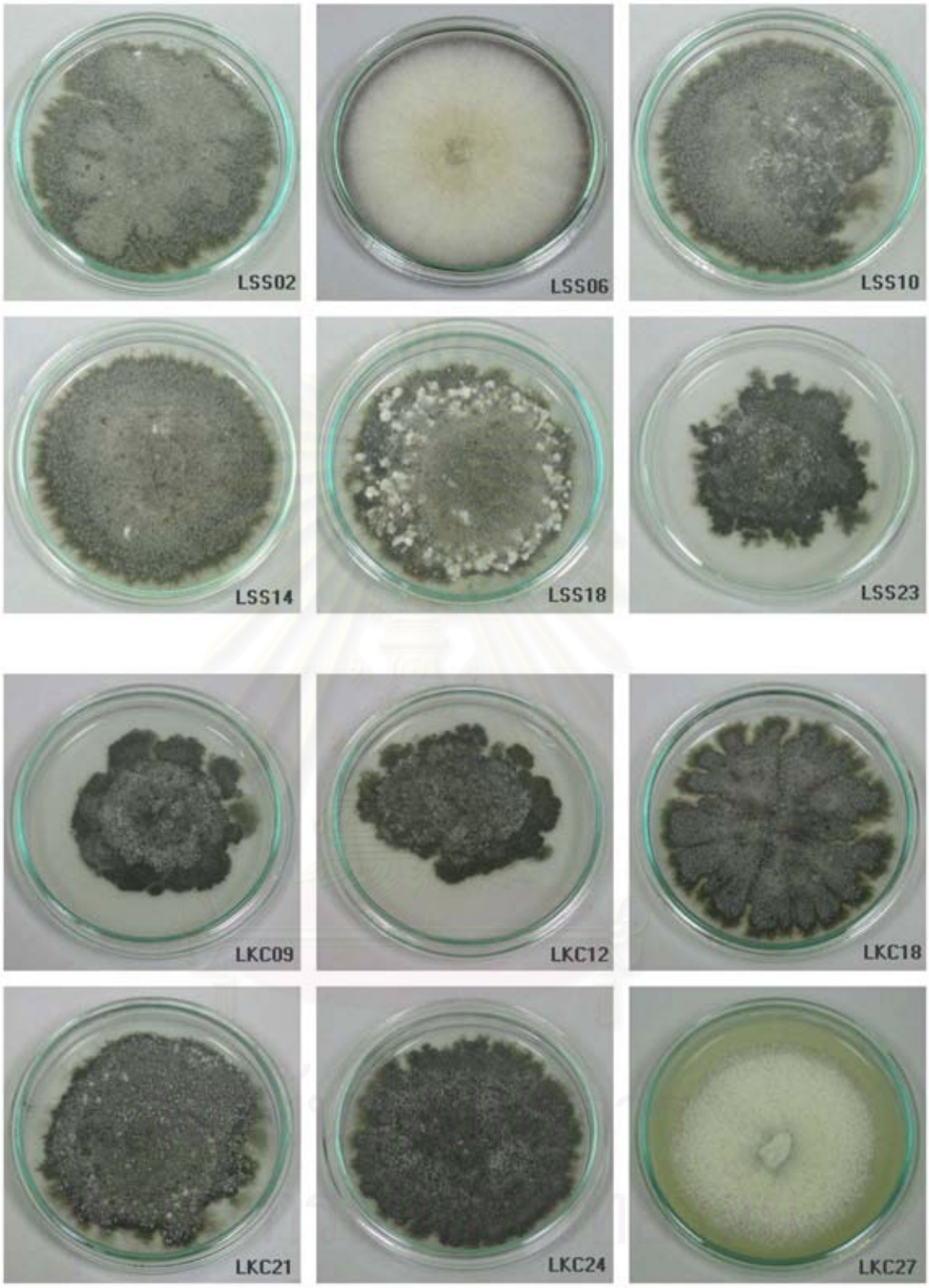


Figure 4.1 (continued)

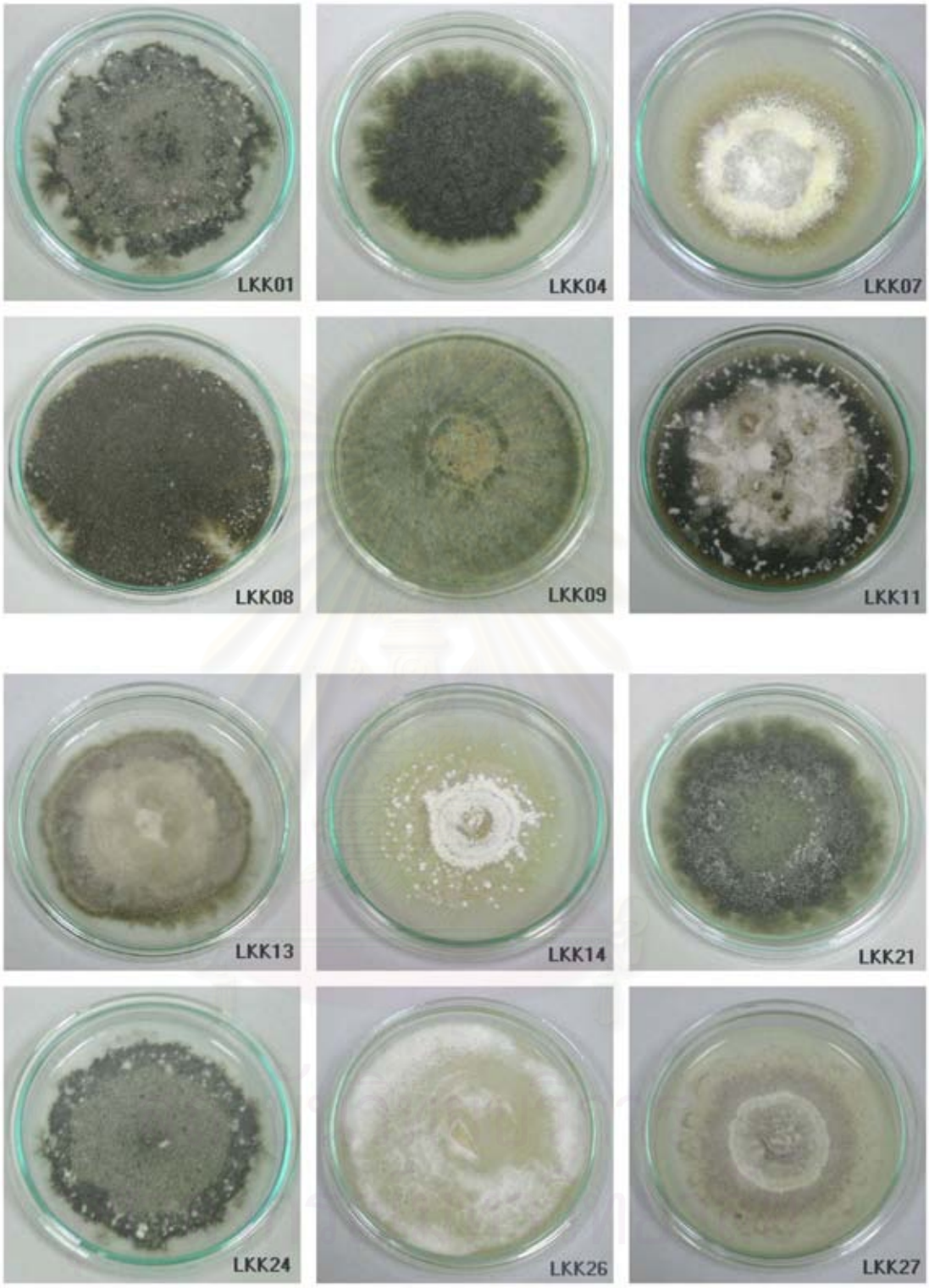


Figure 4.1 (continued)

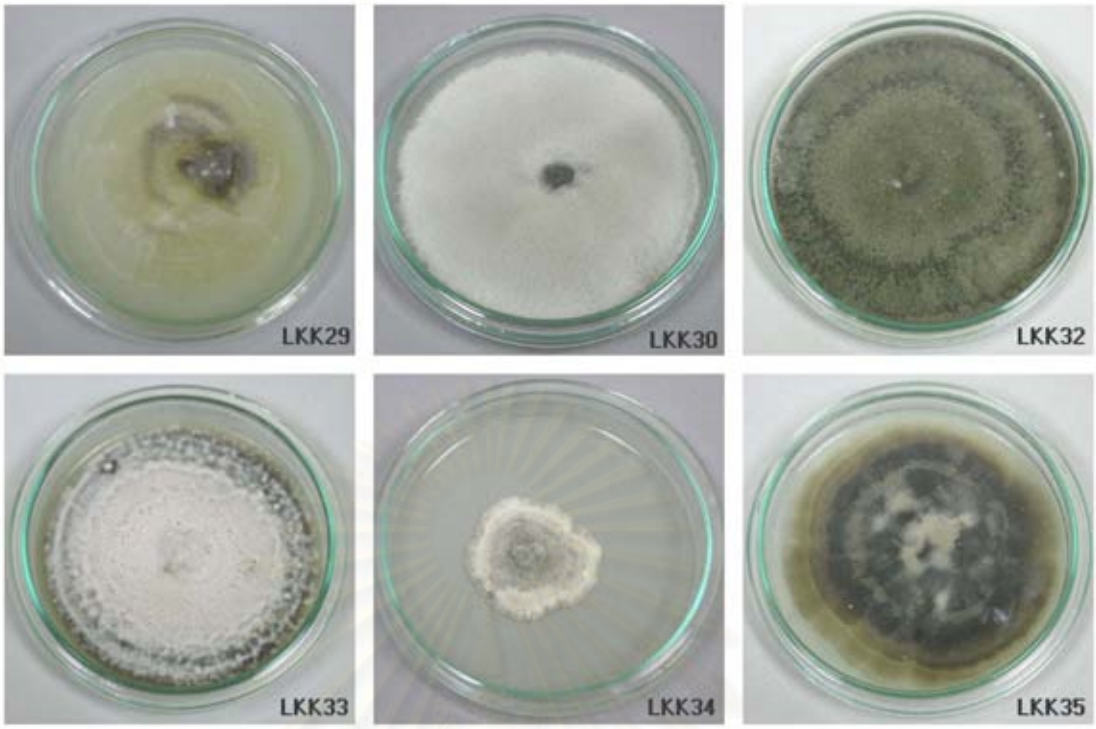


Figure 4.1 (continued)

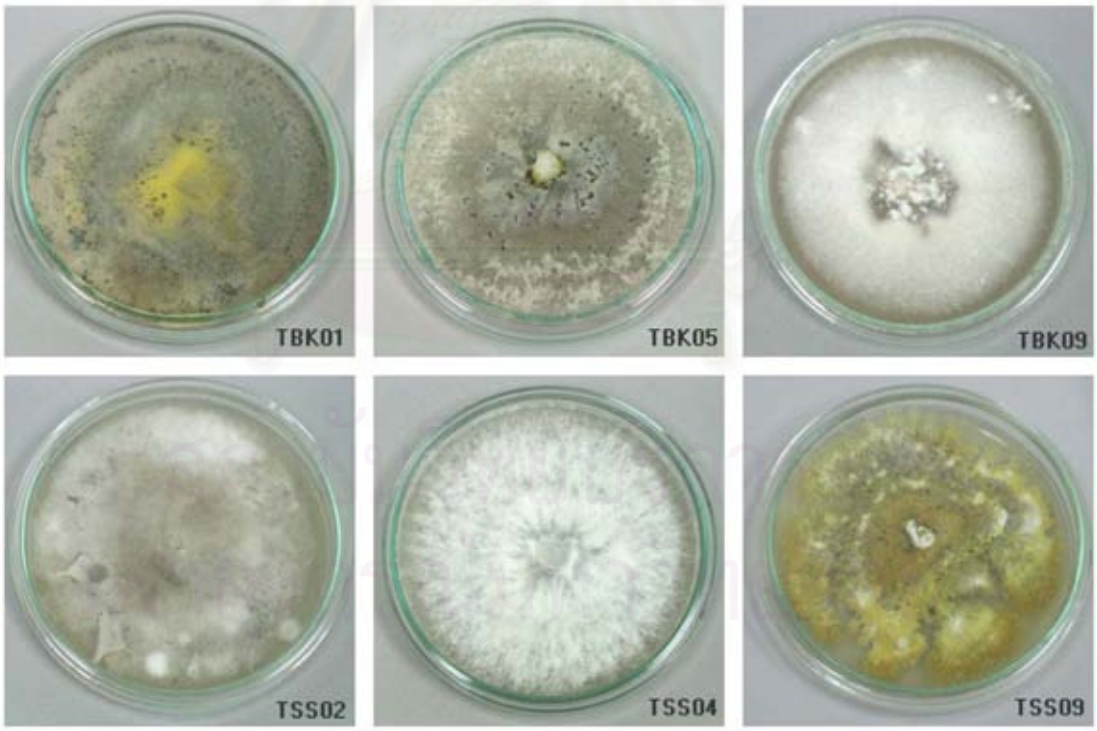


Figure 4.2 Colony characteristic of some endophytic fungi isolated from leaves sections of *Morinda citrifolia* L., after cultivation on PDA for 2-4 weeks at room temperature. The code of each isolate is shown in each picture.

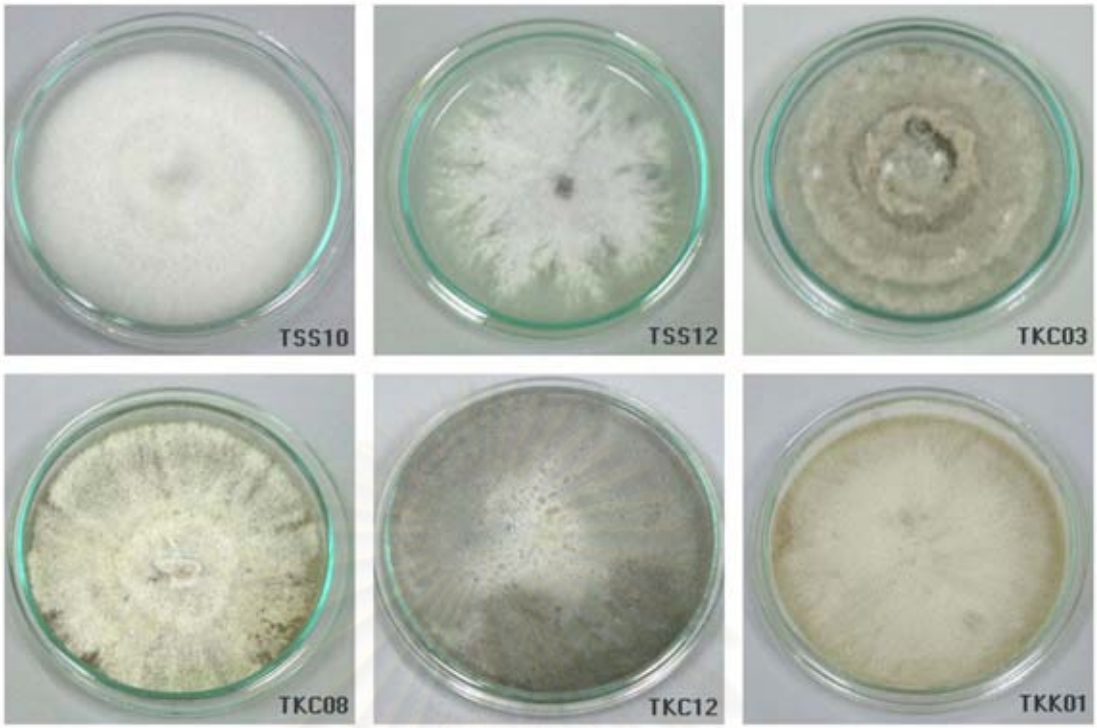


Figure 4.2 (continued)

4.2 Determination of antimicrobial activities of the isolated endophytic fungi

The isolated endophytic fungi cultured on five media, including Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), and Sabouraud's Dextrose Agar (SDA) were determined antimicrobial activities by fungal disk dual culture method against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231.

The antimicrobial activities of isolated endophytic fungi which were active against at least one tested microorganisms are shown in Table 4.2. The results showed that endophytic fungal 44 isolates (24.7 % of total 178 isolates) 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 14.0%, 11.8%, 1.7%, 6.2%, and 7.9%, respectively (Table 4.3 and Figure 4.3). This indicated that *B. subtilis* ATCC 6633, the gram positive rod bacterium, was more sensitive to isolated endophytic fungi than other tested microorganisms.

Moreover, the results showed that MEA gave the highest number of active isolates against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922. SDA gave the highest number of active isolate against *P. aeruginosa* ATCC 27853. MEA and MCzA gave equally highest number of active isolate against *C. albicans* ATCC 10231. This revealed that culture medium had effect on antimicrobial activities of isolated endophytic fungi as showed Figure 4.3. These results are in harmony with most of the investigations undertaken with other fungi and other culture media in which the composition of growth medium affected bioactive metabolites production. As reported by Tkacz et al. (1993), the production medium which appeared to be magnesium limited, supported good pneumocandin production by *Zalerion arboricola*. The carbon to nitrogen ratio of media has been an important variable influencing the production of secondary metabolites by soil fungi (Monaghan et al., 1995).

Table 4.2 The antimicrobial activities of active endophytic fungal isolates.

Code of Isolated Endophytic fungi	Culture mediium	Inhibition zone diameters (mm) /Tested microorganisms				
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
LBK09	PDA	+11	+11	-	-	-
LBK12	PDA	-	-	-	-	+20
	MEA	+13	-	-	-	-
	MCzA	+11	-	-	-	-
	SDA	-	-	-	-	+11
TBK01	MEA	+9	+11	-	-	-
	MCzA	-	-	-	-	+13
	SDA	+10	-	-	-	-
	YES	-	+10	-	-	-
TBK03	SDA	+9	-	-	-	-
TBK04	MEA	-	-	-	+10	-
	SDA	-	-	-	+10	-

Table 4.2 (continued)

Code of Isolated Endophytic fungi	Culture medium	Inhibition zone diameters (mm) / Tested microorganisms				
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
TBK06	SDA	-	-	-	+11	+24
TBK07	PDA	+16	-	-	-	-
	MCzA	-	-	-	+10	-
	SDA	-	-	-	+14	-
	YES	-	-	-	+12	-
TBK08	MCzA	-	-	-	-	+14
TBK11	PDA	+10	-	-	-	+14
	SDA	+10	-	-	-	-
TBK13	MCzA	+18	-	-	-	-
	YES	-	-	-	+14	-
LSS04	MEA	-	+11	-	-	-
LSS06	MEA	+15	+15	+14	-	+12
TSS01	MEA	-	-	-	-	+9
TSS03	YES	+15	-	-	-	-
TSS04	MEA	-	+10	-	-	-
TSS07	MEA	-	+9	-	-	-
TSS08	MCzA	+12	-	-	-	-
	YES	+9				
TSS09	PDA	+.9	+11	-	-	+9
	MEA	-	-	-	-	+12
TSS10	MEA	+10	+11	+10	-	-
TSS14	PDA	-	+18	-	-	-
	SDA	+14	-	-	-	-
TSS16	MEA	+15	+9	-	-	-
LKC05	MEA	+10	-	+10	-	-
	SDA	-	-	-	+12	-
LKC07	YES	-	-	-	+13	-

Table 4.2 (continued)

Code of Isolated Endophytic fungi	Culture medium	Inhibition zone diameters (mm) / Tested microorganisms				
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
LKC11	MCzA	-	-	-	-	+18
LKC13	SDA	-	+18	-	-	-
LKC28	SDA	+13	-	-	-	-
TKC03	MEA	+10	+09	-	+12	-
TKC05	SDA	-	+11	-	-	-
TKC07	YES	+09	-	-	-	-
LKK03	MCzA	-	-	-	+11	-
LKK05	SDA	-	-	-	+10	-
LKK06	MCzA	-	+13	-	-	-
LKK07	SDA	-	+16	-	-	+10
LKK13	SDA	-	+11	-	-	+11
LKK15	MEA	+12	+11	-	-	-
LKK19	MEA	+9	+10	-	-	+10
LKK27	MEA	+10	-	-	-	-
	MCzA	-	+11	-	-	+13
LKK34	MEA	-	-	-	-	+13
	MCzA	-	-	-	-	+17
	SDA	-	-	-	-	+11
	YES	-	-	-	-	+18
TKK03	MEA	+10	-	-	+14	-
	MCzA	-	-	-	+12	-
TKK04	MCzA	-	+11	-	-	-
TKK09	PDA	+10	-	-	-	-
	MCzA	+8	-	-	-	-
TKK10	MEA	+9	+9	-	-	-
	MCzA	+11	+12	-	-	-
	SDA	+11	+13	-	-	-

Table 4.2 (continued)

Code of Isolated Endophytic fungi	Culture medium	Inhibition zone diameters (mm) / Tested microorganisms				
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
TKK12	YES	-	-	-	+12	-
TKK16	SDA	+10	-	-	-	-

- = No inhibition zone

Table 4.3 The amount of active endophytic fungal isolates

Tested microorganisms		Number of active isolates (isolates)	Percent of active isolate (%)
Gram positive bacteria	*B. subtilis* ATCC 6633	25	14.0
	S. aureus ATCC 25923	21	11.8
Gram negative bacteria	*E. coli* ATCC 25922	3	1.7
	P. aeruginosa ATCC 27853	11	6.2
Yeast	*C. albicans* ATCC 10231	14	7.9

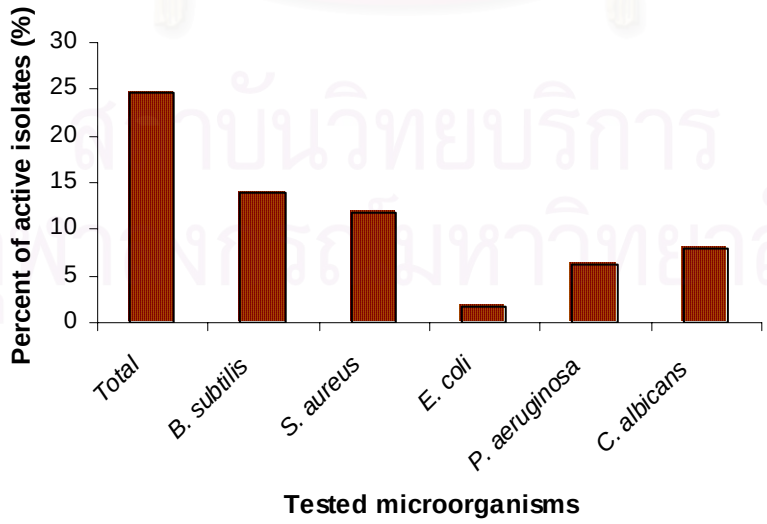


Figure 4.3 The amount of active endophytic fungal isolates

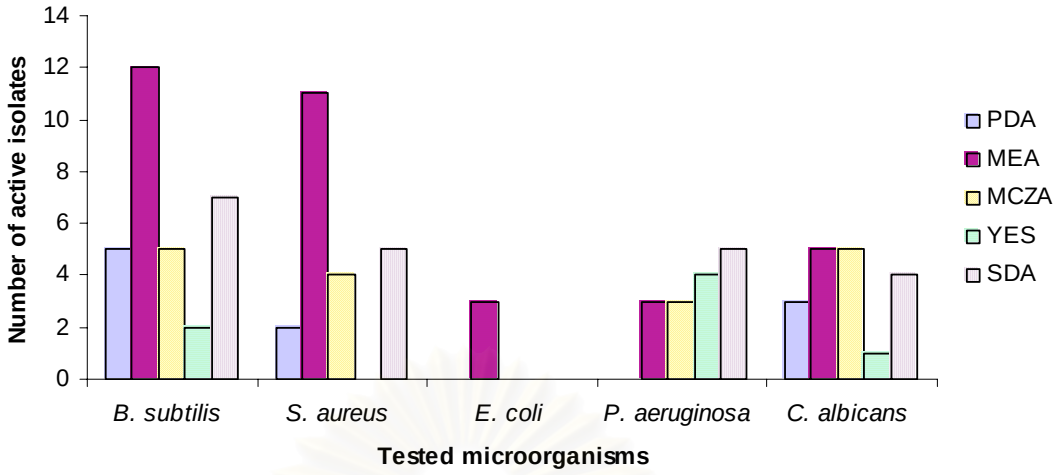


Figure 4.4 The effect of the culture medium on antimicrobial activities of the isolated endophytic fungi.

As summarized in Table 4.4 and Figure 4.5, antimicrobial activities of the isolated endophytic fungi were classified into 4 types as bacterial inhibition only (16.9%), yeast inhibition only (2.2 %), bacterial and yeast inhibition (5.6 %) and no antimicrobial activity against tested microorganisms (75.3 %). Isolated endophytic fungi 24.7 % only that had antimicrobial activities, this may be explainable. From Figure 4.1, the majority of endophytic fungi isolated from leaf sections often had the similar colony characteristic such as isolate LBK01, LBK03, LBK13, LBK 17, LBK20, LSS02, LSS10, LSS14, LSS18, LSS23, LKC09, LKC12, LKC18, LKC21, LKC24, LKK01, LKK04, and LKK08, and they did not exhibit any antimicrobial activities as a consequence the amount of active isolate was depreciated.

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Table 4.4 Classification of antimicrobial activities of the isolated endophytic fungi.

Antimicrobial activities against tested microorganisms	Isolated endophytic fungi	Total	%
Bacteria only	LBK09,TBK03, LSS04, TSS03, TSS04, TSS07,TSS08,TSS14,TSS16,LKC13, LKC 28,TKC05,TKC07,LKK06,LKK15, TKK04,TKK10,TKK16,TBK04, LKC07, LKK03,LKK05,TKK12,TBK07,TBK13, TSS10,LKC05,TKC03,TKK03,TKK09	30	16.9
Bacteria and yeast	LBK12,TBK01, TBK06,TBK11, LSS06, TSS09,LKK07,LKK13,LKK19, LKK27	10	5.6
Yeast only	TBK08, TSS01, LKC11,LKK34	4	2.2
No antimicrobial activity	LBK01,LBK02,LBK03,LBK04,LBK05, LBK06,LBK07,LBK08,LBK10,LBK11, LBK13,LBK14,LBK15,LBK16,LBK17, LBK18,LBK19,LBK20,LBK21,LBK22, TBK02,TBK05,TBK09,TBK10,TBK12, LSS01,LSS02,LSS03,LSS05,LSS07, LSS08,LSS09,LSS10,LSS11,LSS12, LSS13,LSS14,LSS15,LSS16,LSS17, LSS18,LSS19,LSS20,LSS21,LSS22, LSS23,LSS24,LSS25,LSS26,LSS27, LSS28,TSS02,TSS05,TSS06,TSS11, TSS12,TSS13,TSS15,TSS17,LKC01, LKC02,LKC03,LKCO4,LKC06,LKC07, LKC08,LKC09,LKC10,LKC12,LKC14, LKC15,LKC16,LKC17,LKC18,LKC19, LKC20,LKC21,LKC22,LKC23,LKC24, LKC25,LKC26,LKC27,LKC29,LKC30, LKC31,TKCO1,TKC02,TKC04,TKC06,	134	75.3

Table 4.4 (continued)

Antimicrobial activities against tested microorganisms	Isolated endophytic fungi	Total	%
No antimicrobial activity	TKC08,TKC09,TKC10,TKC11,TKC12, TKC13,TKC14,TKC15,TKC16,LKK01, LKK02,LKK04,LKK08,LKK09,LKK10, LKK1,LKK12,LKK14,LKK16,LKK17, LKK18,LKK20,LKK21,LKK22,LKK23, LKK24,LKK25,LKK26,LKK28,LKK29, LKK30,LKK31LKK32,LKK33,LKK35, TKK01,TKK02,TKK05,TKK06,TKKO7, TKK08,TKK11,TKK13,TKK14,TKK15		

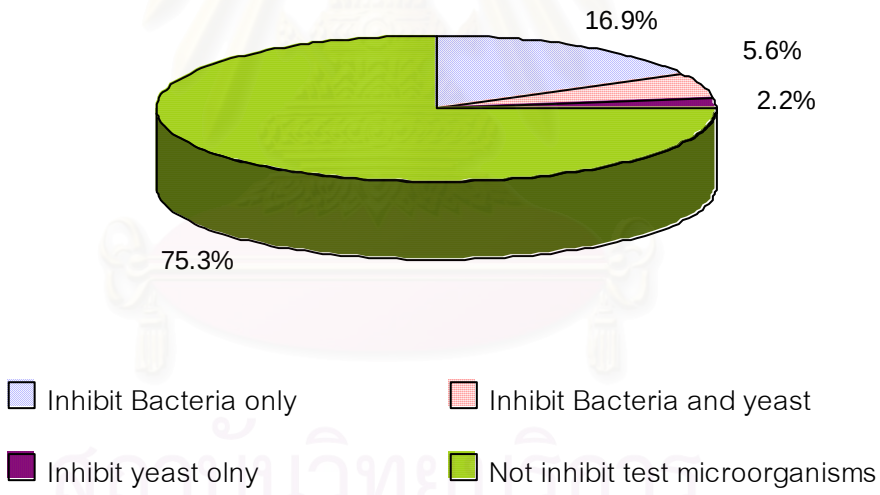


Figure 4.5 A summary of antimicrobial activities of isolated endophytic fungi

From Table 4.4, endophytic fungal 10 isolates which were LBK12, TBK01, TBK06, TBK11, LSS06, TSS09, LKK07, LKK13, LKK19, and LKK27 showed antimicrobial activities against both tested bacteria and yeast. Among of these fungi, endophytic fungal isolate LSS06 cultured on MEA (Figure 4.6) had the best antimicrobial activities because it was the only isolate that inhibited the growth of all types of tested microorganisms, including gram positive bacteria *(B. subtilis* ATCC 6633 and *S. aureus*

ATCC 25923), gram negative bacteria (*E. coli* ATCC 25922) and yeast (*C. albicans* ATCC 10231) (Figure 4.7) as showed in Figure 4.8. For this reason, endophytic fungal isolate LSS06 was selected for identification and isolation bioactive metabolites.

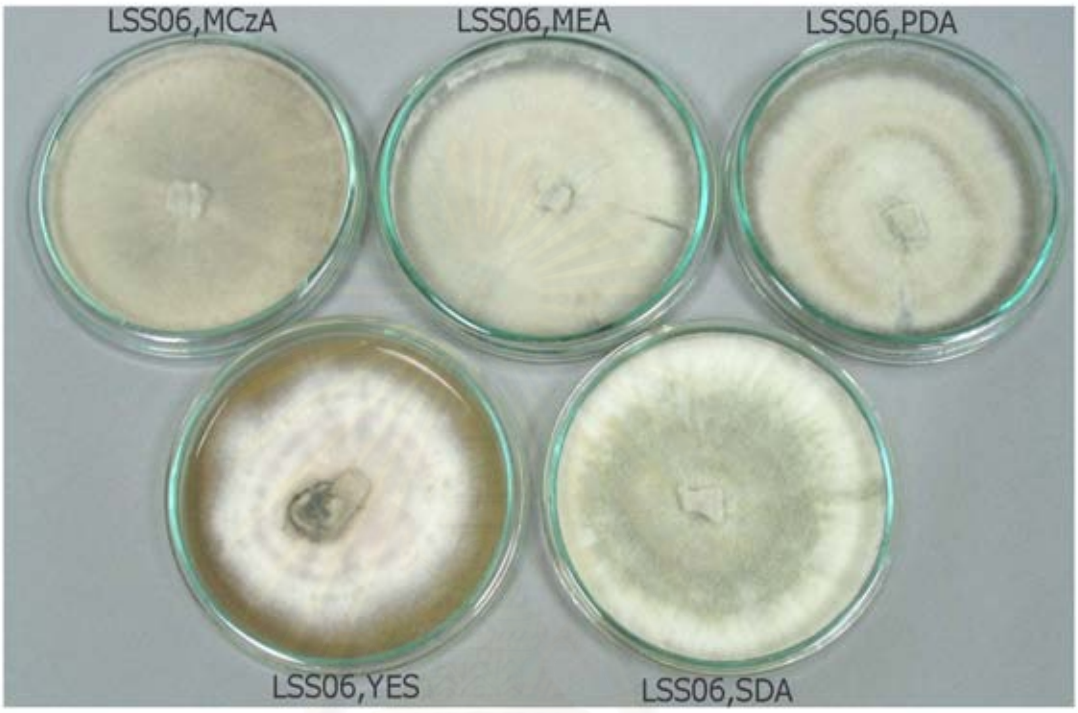


Figure 4.6 Colony characteristic of endophytic fungal isolate LSS06 cultured on five different media (MCzA, MEA, PDA, YES, and SDA) after cultivation for 14 days at room temperature.

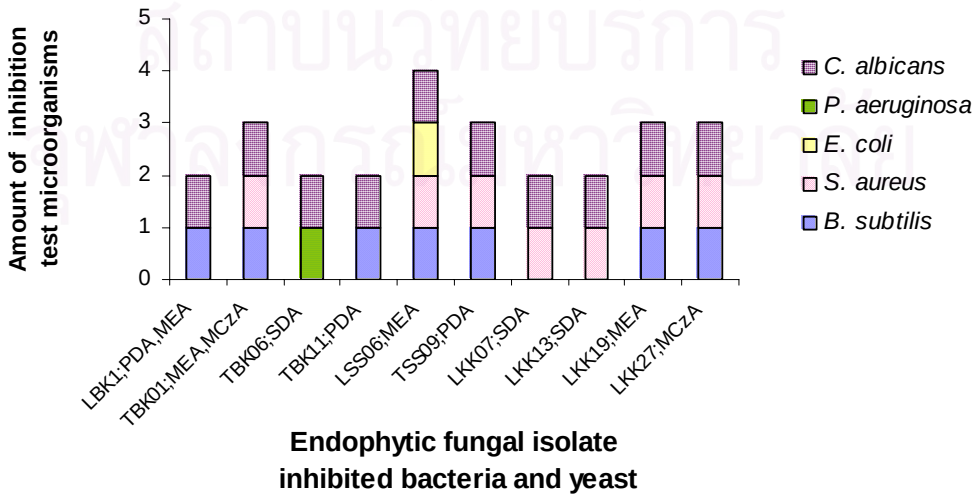


Figure 4.7 The antimicrobial activities of some active endophytic fungal isolates.

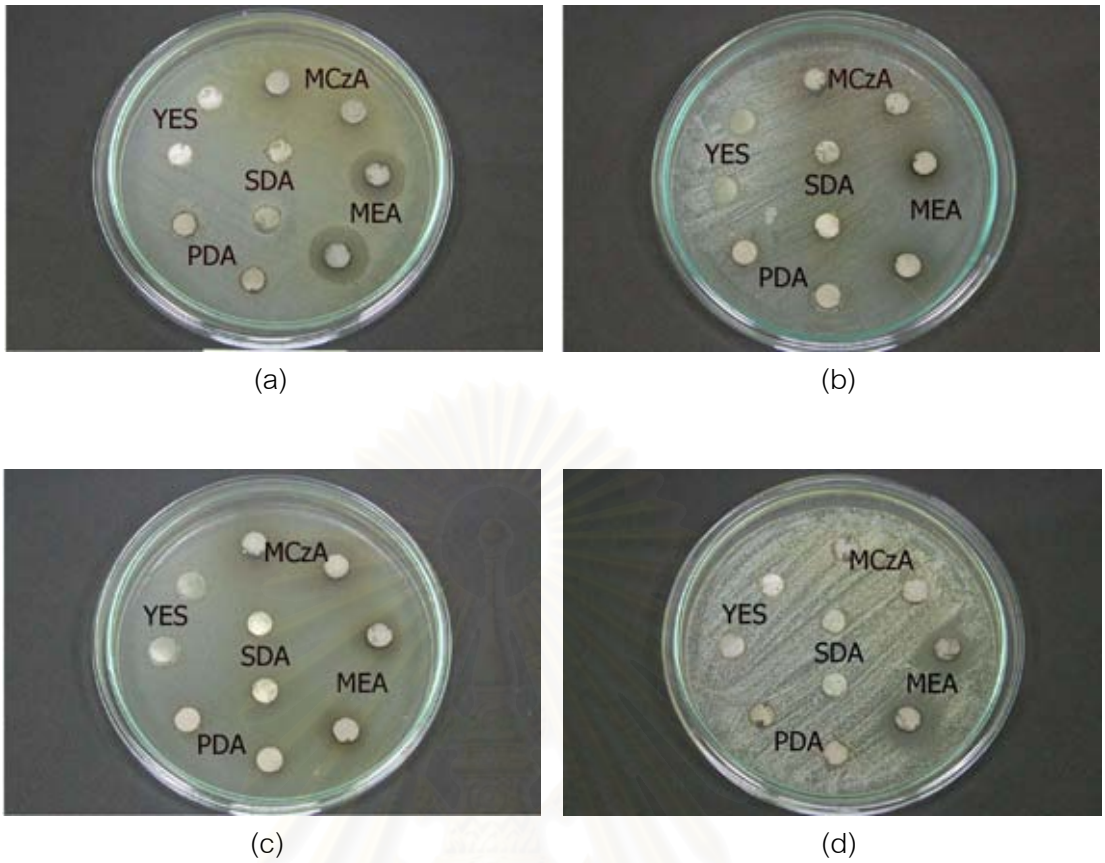


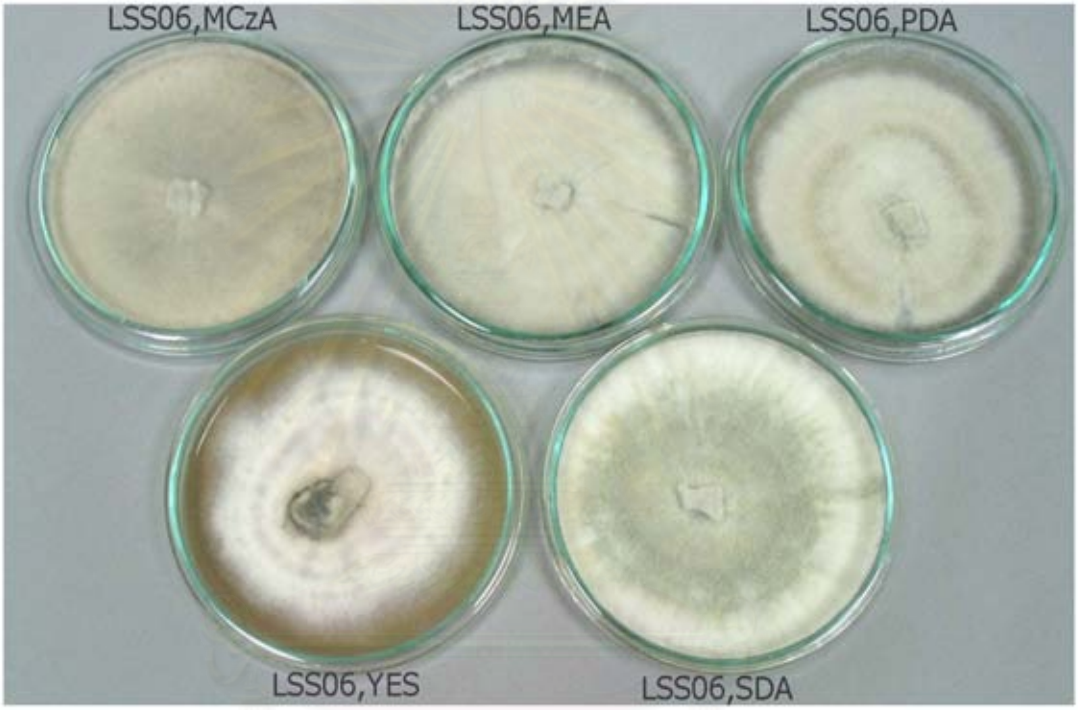
Figure 4.8 Fungal disk dual culture test for determination of antimicrobial activities of endophytic fungal isolate LSS06 cultured on five different media. Culture disks on MEA of endophytic fungi isolate LSS06 inhibited *B. subtilis* ATCC 6633 (a), *S. aureus* ATCC 25923 (b), *E. coli* ATCC 25922 (c), and *C. albicans* ATCC 10231 (d).

4.3 Identification and classification of endophytic fungal isolate LSS06

4.3.1 Morphological identification

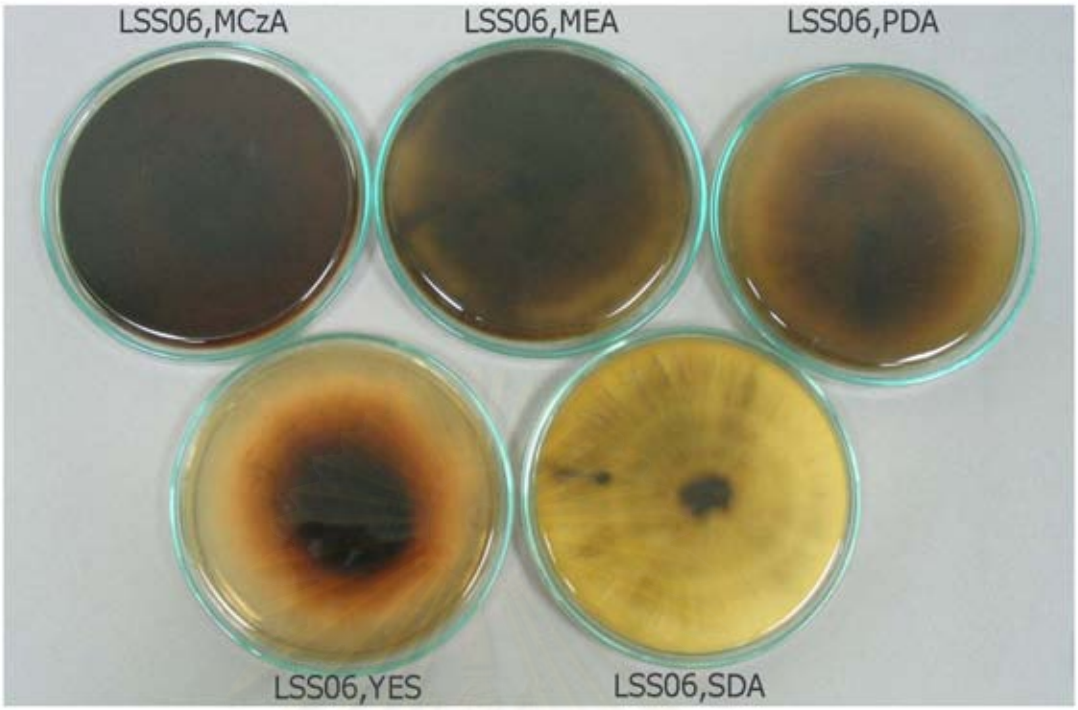
Endophytic fungal isolate LSS06 grew rapidly on Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapex Agar (MCzA), and Sabouraud's Dextrose Agar (SDA) rather than on Yeast Extract Sucrose agar (YES). On MEA, at room temperature, the fungal covered a 9-cm-diameter Petri dish within 1 week and the colony was pale brownish. Colony characteristic of endophytic fungal isolate LSS06 on PDA, MEA, MCzA, YES and SDA is shown in Figure 4.9.

Under the microscope, the mycelium of endophytic fungal isolate LSS06 was composed of septate hyphae. The conidiophores were septate, erect, flexous (wavy, not rigid), irregularly branched, and deposits of a brown material. Conidia were single celled and ellipsoidal, they arose singly and successively on denticles at the tips of conidiogenous cells. Colony characteristic on MEA and light microscopic characteristic of endophytic fungal isolate LSS06 are shown in Figure 4.10. Scanning Electron Microscopic characteristic of endophytic fungal isolate LSS06 is shown in Figure 4.11.



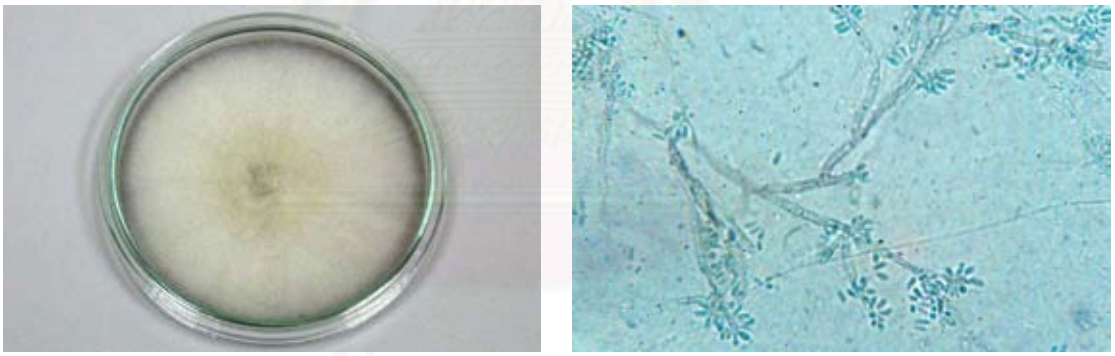
(a)

Figure 4.9 Colony characteristic of endophytic fungal isolate LSS06 cultured on five different media (MCzA, MEA, PDA, YES, and SDA) after cultivation for 14 days at room temperature. Appearance on the obverse side (a), and on the reverse side (b).



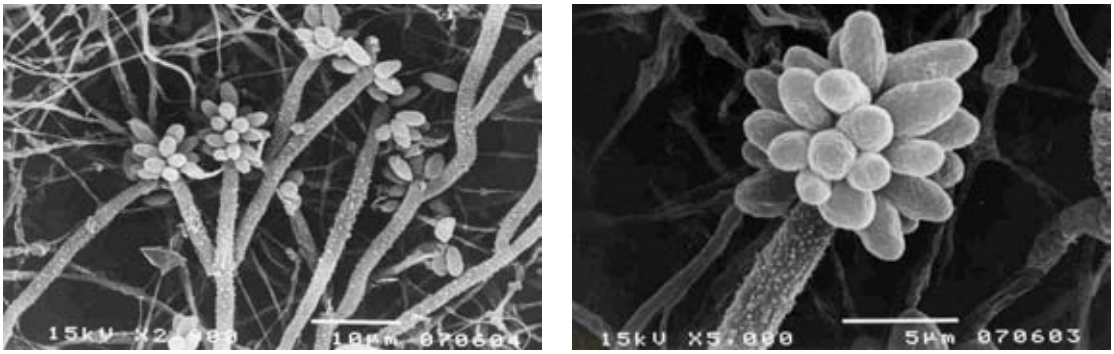
(b)

Figure 4.9 (continued)

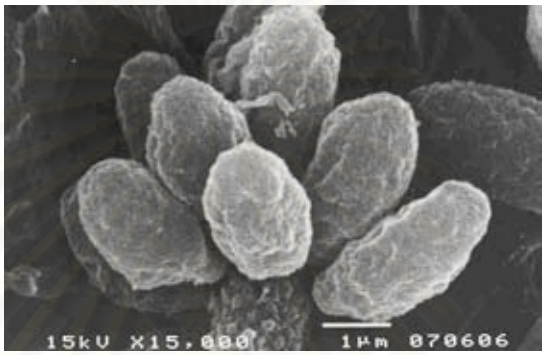


(a) (b)

Figure 4.10 Colonial and microscopic characteristic of endophytic fungal isolate LSS06. Cultured on MEA showing the pale brownish colony(a), and lactophenol cotton blue mount at magnification x100 of the fungal mycelia and conidial showing irregularly branched septate conidiophores, with denticles closely crowded of the conidia, and deposits of a brown material on the conidiophore (b).



(a) (b)



(c)

Figure 4.11 Scanning Electron Microscopic characteristic of endophytic fungal isolate LSS06. Slightly roughened, erect, flexuous (wavy not rigid) and irregularly branched conidiophores (bar = 10 µm) (a), closely crowded conidia at the tip of the conidiogenous cell (bar = 5 µm) (b), and single ellipsoidal conidia (bar = 1µm) with scaled on conidiophores (c).

4.3.2 Molecular Identification

Endophytic fungal isolate LSS06 was sent for identification by molecular methods at the Asian Natural Environmental Science center, the University of Tokyo, Japan.

The rDNA ITS region of isolate LSS06 was amplified with the conserved fungal primer ITS_{1F} and ITS_4. Isolate LSS06 produced a single ITS band. The length of corresponding fragment was 783 bp, containing a part of the 18S, ITS1, 5.8S and 28S rDNA is shown in Figure 4.12.

1

```
5′ CTTGGTCATT    TAGAGGAAGT    AAAAGTCGTA    ACAAGGTCTC
   CGTTGGTGAA    CCAGCGGAGG    GATCATTAAT    GAGTTACTAA
   ACTCCAAAAC    CCTTTGTGAA    CCTTACCGTC    GTTTCCTCGG
   CGCGTGCTGC    TGCTACCTGG    AGCTACCCTG    GAAGGCACCT
   ACCCTGTAGT    GGTTGTCTGC    CAAGAGCTAT    CATGTCAGGC
   ACCTACCTCT    GTAGTGGTTG    TCTGCCATGA    GCTGTCTTGG
   CAGGCACCTA    CCCTGTAGTG    GTTGTCTGCT    AAGAGTTATC
   CTAGCAGGCA    CCTACCCTGT    AGTAGTTGTC    TACCCTGGAG
   CTACCCTGTA    GCCGCGTGAA    GGCCCGCCGA    AGACCGTTAA
   ACTCTTGCTT    CTACAACTGT    ATCTCTGAAC    ACGTAACTGA
   AATAAGTTAA    AACTTTCAAC    AACGGATCTC    TTGGTTCTGG
   CATCGATGAA    GAACGCAGCG    AAATGCGATC    AGTAATGTGA
   ATTGCAGAAT    TCAGTGAATC    ATCGAATCTT    TGAACGCACA
   TTGCGCCCAT    TAGTATTCTA    GTGGGCATGC    CTATTCGAGC
   GTCATTTCAA    CCCTTAAGCC    CTGTTGCTTA    GTGTTGGGAG
   TCTACGGCTT    CGGCGTAGTT    CCTGAAAATC    AGTGGCGGAG
   TTAGGGTACA    CTCTCAGCGT    AGTAATCTCT    CTCGCTCGTG
   TGGTGGCCCT    GGCTGCTGGC    CGTAAAACCC    CCTATTTTCT
   AGTGGTTGAC    CTCGGATTAG    GTAGGAATAC    CCGCTGAACT
   TAAGCATATC    AATAAGCGGA    GGA 3′
                                783
```

Figure 4.12 Nucleotide sequences of endophytic fungal isolate LSS06, containing a partial of the 18S, ITS1, 5.8S and 28S rDNA.

A blast search was performed to find a similar sequence to ITS region of fungal isolate LSS6 in the Genbank DNA database, available from: http://www.ddbj.nig.ac.jp The results revealed that ITS region of endophytic fungal isolate LSS06 had 99.571% identity to *Nodulisporium* sp., as showed in Figure 4.13.

```
>> AF201751 | AF201751.1  Nodulisporium  sp. MF6321 internal (700 nt)
 initn: 2026 init1: 2026 opt: 3461 Z-score: 3511.0 expect()  1.7e-187
 99.571% identity in 700 nt overlap (60-758:1-700)

      30        40        50        60        70        80

       AACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTAATGAGTTACTAAACTCCAAAA

                                        ::::::::::::::::::::::::::::

AF2017                                  GGATCATTAATGAGTTACTAAACTCCAAAA

                                               10        20        30

      90       100       110       120       130       140

       CCCTTTGTGAACCTTACCGTCGTTTCCTCGGCGCGTGCTGCTGCTACCTGGAGCTACCCT

       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::

AF2017 CCCTTTGTGAACCTTACCGTCGTTTCCTCGGCGCGTGCTGCTGCTACCTGGAGCTACCCT

               40        50        60        70        80        90

     150       160       170       180       190       200

       GGAAGGCACCTACCCTGTAGTGGTTGTCTGCCAAGAGCTATCATGTCAGGCACCTACCTC

       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::

AF2017 GGAAGGCACCTACCCTGTAGTGGTTGTCTGCCAAGAGCTATCATGTCAGGCACCTACCTC

              100       110       120       130       140       150

     210       220       230       240       250       260

       TGTAGTGGTTGTCTGCCATGAGCTGTCTTGGCAGGCACCTACCCTGTAGTGGTTGTCTGC

       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::

AF2017 TGTAGTGGTTGTCTGCCATGAGCTGTCTTGGCAGGCACCTACCCTGTAGTGGTTGTCTGC

              160       170       180       190       200       210

      270       280       290       300       310       320

       TAAGAGTTATCCTAGCAGGCACCTACCCTGTAGTAGTTGTCTACCCTGGAGCTACCCTGT

       ::::::::::::::::::::::::::::::::::::::::: :::::::::::::
AF2017 TAAGAGTTATCCTAGCAGGCACCTACCCTGTAGTAGTTGTCTACCATGGAGCTACCCTGT

              220       230       240       250       260       270
```

Figure 4.13 Alignment data of a part of the 18S, ITS1, 5.8S and 28S rDNA of endophytic fungal isolate LSS06 and 1 reference taxa.

```
      330       340       350        360       370       380
       AGCCGCGTGAAGGCCCGCCGAA-GACCGTTAAACTCTTGCTTCTACAACTGTATCTCTGA
       :::::::::::::::::::: ::::::::::::::::::::::::::::::::::::
AF2017 AGCCGCGTGAAGGCCCGCCGAAGGACCGTTAAACTCTTGCTTCTACAACTGTATCTCTGA
              280       290       300       310       320       330
      390       400       410       420       430       440
       ACACGTAACTGAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2017 ACACGTAACTGAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
              340       350       360       370       380       390
      450       460       470       480       490       500
       AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2017 AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
              400       410       420       430       440       450
      510       520       530       540       550       560
       TTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATTCGAGCGTCATTTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2017 TTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATTCGAGCGTCATTTC
              460       470       480       490       500       510
      570       580       590       600       610       620
       AACCCTTAAGCCCTGTTGCTTAGTGTTGGGAGTCTACGGCTTCGGCGTAGTTCCTGAAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2017 AACCCTTAAGCCCTGTTGCTTAGTGTTGGGAGTCTACGGCTTCGGCGTAGTTCCTGAAAA
              520       530       540       550       560       570
      630       640       650       660       670       680
       TCAGTGGCGGAGTTAGGGTACACTCTCAGCGTAGTAATCTCTCTCGCTCGTGTGGTGGCC
       :::::::::::::::::::::::::::::::::::: ::::::::::::::::::::
AF2017 TCAGTGGCGGAGTTAGGGTACACTCTCAGCGTAGTAATATCTCTCGCTCGTGTGGTGGCC
              580       590       600       610       620       630
```

Figure 4.13 (continued).

```
      690       700       710       720       730       740

       CTGGCTGCTGGCCGTAAAACCCCCTATTTTCTAGTGGTTGACCTCGGATTAGGTAGGAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2017 CTGGCTGCTGGCCGTAAAACCCCCTATTTTCTAGTGGTTGACCTCGGATTAGGTAGGAAT

              640       650       660       670       680       690

      750       760       770       780

       ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
       ::::::::::
AF2017 ACCCGCTGAA
                  700
```

Figure 4.13 (continued).

Based on colony characteristic, microscopic characteristic, and nucleotide sequencing analysis of ITS regions of rDNA, endophytic fungal isolate LSS06 was identified as *Nodulisporium* sp.

Nodulisporium species comprise a small proportion of the fungal biota. This genus is most closely related to *Geniculosporium*, *Hansfordia*, and *Calcarisporium*. No information is available regarding health effects, or toxicity. Allergenicity has not been studied. They may be identified on surfaces by tape lifts and tease mounts from bulk samples. Spores do not have distinctive morphology and would be categorized as "other colorless" on spore trap samples. They are commonly isolated and grow relatively readily in culture (Bills and Polishook, 1992; Jesenska, Pieckova, and Bernat, 1992 and 1993). The unique cultural characteristics of *Nodulisporium* spp., including the ability to grow faster at 37°C than at 25°C in the plate culture, the presence of a chemically undefined sweet, aromatic odor, deposits of a melanin-like material on the conidiophore, and the abundant production of a dark brown soluble pigment in plate culture and fermentation broth. In culture, this fungal can only be propagated in the vegetative state because the germinating conidia fail to develop more than a single cell beyond the germtube stage (Polishook, Ondeyka, and Dombrowski, 2001).

Nodulisporium is reported as the anamorphs of at least 10 genera (e.g., *Biscogniauuxia* Kuntze, *Camillea* Fr. and *Hypoxylon* Bull.) of the Xylariaceae, a large

and diverse family of upwards of 40 genera (Whalley 1996). The anamorph genus *Nodulisporium* is characterized by long, mononematous conidiophores with holoblastically produced conodia in a sympodial sequence which gives rise to the characteristic nodulose appearance of the conidiogenous cell (Jong and Rogers, 1972).

Nodulisporium spp. are often associated with dying plants and woody debris. Also they are frequently isolated from living plants (Rollinger and Langenheim, 1993). In spite of their common occurrence, only a few metabolites have been reported to be produced by cultures of these fungi (Aldrige et al.,1975; O' Leary and Hanson,1982; Hanson, O' Leary, and Wadsworth, 1983; Dombrowski et al., 1992; Igarashi et al., 1993).

4.4 Determination of growth profile, and antimicrobial activities of culture filtrate from fungal isolate LSS06

Endophytic fungal isolate LSS06 cultivated in MEB medium for 38 days at room temperature was observed the growth from mycelial dry weight. The culture filtrate of fungal isolate LSS06 was determined antimicrobial activities by the agar well diffusion method. Figure 4.14 showed the growth profile and antimicrobial activities of culture filtrate of fungal isolate LSS06. The raw data of mycelial dry weight and inhibition zone of culture filtrate of endophytic fungal isolate LSS06 cultivated in MEB are shown in Table B1, appendix B.

Endophytic fungal isolate LSS06 grew into mid log phase within 8 days and gradually grew into stationary phase within 14 days. After 18 days, the growth of fungal isolate LSS06 was decreasing into death phase.

The culture filtrate of fungal isolate LSS06 showed antimicrobial activities against *B. subtilis* ATCC 6633 and *E. coli* ATCC 25922 at day 6. In day 8 the culture filtrate of this fungus inhibited *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 , and *C. albicans* ATCC 10231 and showed highest activities at day 14 continued to day 20 and decreased after day 20 until day 38. The culture filtrate exhibited the highest antimicrobial activities when fungal isolate LSS06 grew into the stationary phase of growth. This is because many antibiotic compounds are secondary growth products that are excreted into the medium only after the culture enters stationary growth (Fhurmann, 1994).

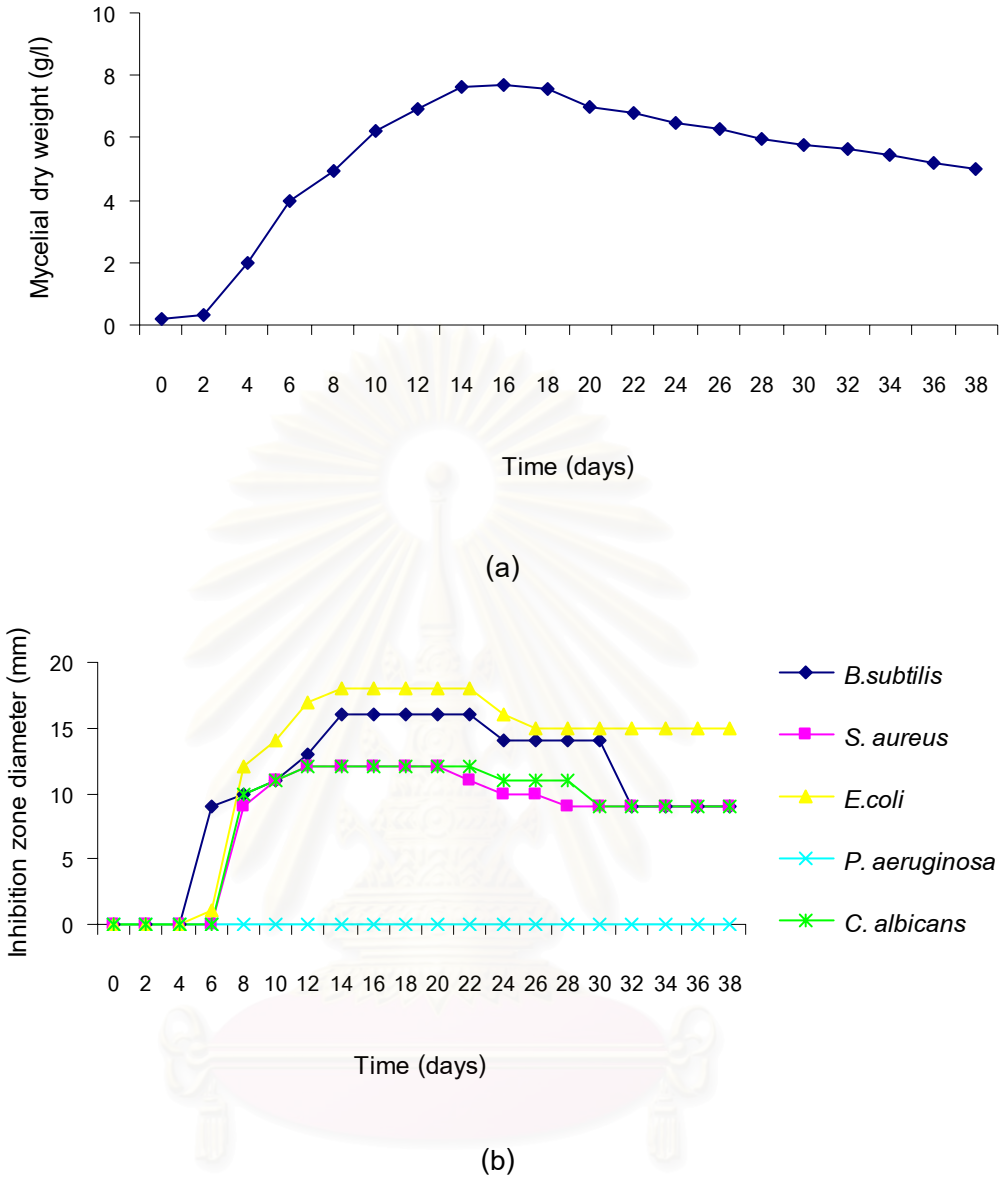


Figure 4.14 Growth profile and antimicrobial activities of endophytic fungal isolate LSS06. Growth profile (a), and antimicrobial activities of culture filtrate of endophytic fungal isolate LSS06 (b)

4.5 Cultivation and metabolites extraction of endophytic fungal isolate LSS06

According to the result of antimicrobial activities of the isolated endophytic fungi showed that endophytic fungal isolate LSS06 cultured on MEA was active against type of tested microorganisms more than the other fungal isolates so endophytic fungal isolate LSS06 was selected for study bioactive compounds.

Endophytic fungal isolate LSS06 was cultivated statically in 12 l of Malt Extract Broth (MEB) at room temperature for 14 days because it grew into stationary phase of growth and had the highest antimicrobial activities at 14^{th} of cultivation. Culture broth was filtered and gave 8.97 l of culture broth free mycelium and 104.28 g of mycelial dry weight. Extraction with EtOAc yielded culture broth and mycelial crude extract of 24.5 g and 10.8 g, respectively.

4.6 Isolation and characterization of metabolites of endophytic fungal isolate LSS06

EtOAc crude of culture broth and mycelial extract of endophytic fungal isolate LSS06 cultivated in 12 l MEB was separated and purified. Chemical structures of isolated metabolites were elucidated by their spectroscopic data, including IR, UV, NMR and MS spectra with physical properties, and also by comparison their spectral data and physical properties with the references.

4.6.1 Isolation and characterization of metabolites from culture broth crude extract

EtOAc crude of culture broth of endophytic fungal isolate LSS06 was separated by silica gel quick column chromatography. Combined fraction B06 (1g), which inhibited *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans* ATCC 10231 and had more amount than other combined fractions, was purified further by silica gel column chromatography, eluted with 90 % $CHCl_3$ in Hexane and followed by preparative TLC, eluted with 5% MeOH in $CHCl_3$ to yield mixture B1 (10 mg, 0.04% by weight of EtOAc crude of culture broth extract). Combined fraction B10 (3.03 g) was washed with EtOAC and MeOH and re-crystallized with MeOH to give mixture B2 (8 mg, 0.03% by weight of EtOAc crude of culture broth extract). The structure elucidation of mixture B1 and mixture B2 is presented in section 4.6.1.1 and 4.6.1.2, respectively.

4.6.1.1 Structure elucidation of mixture B1

Mixture B1 (10 mg) was obtained as yellow viscous liquid from combined B06, purified by silica gel column chromatography eluted with 90 % $CHCl_3$ in Hexane and followed by preparative TLC, eluted with 5% MeOH in $CHCl_3$.

The IR spectrum of mixture B1 is shown in Figure C2 in appendix C and the absorption bands were assigned as shown in Table 4.5. The IR spectrum indicated important absorption band at 3420 cm^{-1} of O-H stretching vibration of alcohol, at 2918 and 2848 cm^{-1} of C-H stretching vibration, 1715 cm^{-1} of C=O vibration of carbonyl group, at 1653 cm^{-1} of C=C vibration of double bond, at 1443 cm^{-1} of C-H bending vibration, at 1306, 1279, and 1104 cm^{-1} of C-O stretching vibration. IR absorption bands of mixture B1 are assigned in Table 4.5.

Table 4.5 The IR absorption bands assignment of mixture B1

Wave number (cm^{-1})	Intensity	Tentative assignment
3420	Broad, Medium	O-H stretching vibration of alcohol
2918 and 2848	Sharp, Weak	C-H stretching vibration
1715	Sharp, Strong	C=O vibration of carbonyl group
1653	Sharp, Weak	C=C vibration of double bond
1443	Sharp, Weak	C-H bending vibration
1306, 1279, and 1104	Sharp, Weak	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure C3 in appendix C) of mixture B1 showed eight proton signals which belong to methyl protons at δ 1.57 ppm, methyl protons attached to oxygen (-O-CH_3 -) at 3.76 ppm, methylene protons attached to oxygen (-O- CH_2 -) at 3.93 and 4.06 ppm, two methine protons attached to oxygen (-O-C*H* -) at 3.65 and 3.91 ppm and two olefinic protons at 6.18 and 6.62 ppm.

The ^{13}C-NMR spectrum (Figure C4 in appendix C) of mixture B1 showed ten signals, which six signals belong to sp^3 carbon at 24.1 to 73.2 ppm, two signals of sp^2 carbon at 125.4 and 141.2 ppm, and the signal of carbonyl group of ester at 165.7

ppm. The assigned carbon signals based on the ^{13}C-NMR spectrum were confirmed by the information obtained from the DEPT spectrum (Figure C5 in appendix C).

DEPT analysis (Figure C5 in appendix C) of mixture B1 showed two methyl carbons at 24.1 and 52.0 ppm, a methylene carbon at 65.4 ppm and four methine carbons at 55.6, 73.2, 125.4, and 141.2 ppm, which indicated that the carbon signals at 68.0 and 101.9 ppm were quaternary.

The LC-MS spectrum (Figure C10 in appendix C) showed the $[M+Na]^+$ ion at *m/z* 381, so mixture B1 had the molecular weight of 358.

The information from 2D-NMR techniques, HSQC correlation are shown in Table 4.5 and Figure C6 in appendix D. The HMBC, COSY, and NOESY spectra of mixture B1 are shown in Figure C7-C9 in appendix C.

Table 4.6 HSQC spectral data of mixture B1

^{13}C-NMR (ppm)	^{1}H-NMR (ppm)(coupling constant (Hz))
165.7 (s)	-
141.2 (d)	6.65dd (*J*=7.2,15.2)
125.3 (d)	6.21d (*J*=15.6)
101.8 (s)	-
73.1 (d)	3.94s
68.0 (s)	-
65.4 (t)	4.09d (*J*=11.2), 3.96d (*J*=11.2)
55.5 (d)	3.68d (*J*=6.4)
52.0 (q)	3.79s
24.0 (q)	1.60s

The structure of mixture B1 is undefined and still under investigation. However, the major compound in mixture B1 should be unsaturated non-aromatic, containing ten or more than ten carbons, with the hydroxyl group and carbonyl group substitution in the molecule.

4.6.1.2 Structure elucidation of mixture B2

Mixture B2 (8 mg) was obtained as white amorphous solid from crystallization combined fraction B10 with MeOH and had m.p. of 202-205 °C.

The IR spectrum of mixture B2 is shown in appendix C, Figure C12, the IR absorption spectrum indicated important absorption band at 3429 and 3368 cm^{-1} of O-H stretching vibration of alcohol, at 2935 cm^{-1} of C-H stretching vibration, 1782 cm^{-1} of C=O vibration of carbonyl group, at 1445 and 1365 cm^{-1} of C-H bending vibration, and at 1146 and 1112 cm^{-1} of C-O stretching vibration. The absorption bands of mixture B2 are assigned as shown in Table 4.7

Table 4.7 The IR absorption bands assignment of mixture B2

Wave number (cm^{-1})	Intensity	Tentative assignment
3429 and 3368	Broad, Strong	O-H stretching vibration of alcohol
2935	Sharp, Weak	C-H stretching vibration of CH_2, CH_3
1782	Sharp, Strong	C=O stretching vibration of carbonyl
1445 and 1365	Sharp, Weak	C-H bending vibration
1146 and 1112	Sharp, Weak	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure C13 in appendix C) of mixture B2 showed the proton signal of methyl protons at 1.22 and 2.17 ppm, methylene protons at 2.30, and 2.95 ppm, methine protons attached to oxygen (-O-C*H* -) at 3.49, 4.56, 4.67, 4.70, 4.71, 4.80, 5.09, 5.32 ppm.

The ^{13}C-NMR spectrum (Figure C14 in appendix C) of mixture B2 showed the signals of sp^3 carbon at 20.0 to 101.4 ppm, which belong to two methyl carbons at 20.0 and 28.3 ppm, methylene carbons at 37.0 ppm, and two carbon signals of the carbonyl group at 176.4 and 176.3 ppm. The assigned carbon signals based on the ^{13}C-NMR spectrum were confirmed by the information obtained from the DEPT spectra (Figure C15 in appendix C).

DEPT experiments (Figure C15 in appendix C) of mixture B2 showed the signals of methine carbons at 69.8, 74.2, 76.1, 76.3, 83.7, 86.6, 100.5, and 101.4 ppm,

which indicated that the carbon signals at 84.4, 77.9 and 106.6 ppm were quaternary.

The LC-MS spectrum (Figure C20 in appendix C) showed the $[M+Na]^+$ ion at *m/z* 287, so, mixture B2 had the molecular weight of 264.

The information from 2D-NMR techniques, HSQC correlation are shown in Table 4.8 and Figure C16 in appendix C. The HMBC, COSY, and NOESY spectra of mixture B2 are shown in Figure C17-C19 in appendix C.

Table 4.8 HSQC spectral data of mixture B2

^{13}C-NMR (ppm)	^{1}H-NMR (ppm)(coupling constant (Hz))
176.4(s)	-
176.3(s)	-
106.6(s)	-
101.4(d)	5.09d (*J*=4.0)
100.5(d)	5.32d (*J*=5.2)
86.6(d)	4.67d (*J*=5.6)
84.4(s)	-
83.7(d)	4.70d (*J*=2.8)
77.9(s)	-
76.3(d)	4.80dd (J=5.6, 6.4)
76.1(d)	4.71d (*J*=6.8)
74.2(d)	3.49d (*J*=5.2)
69.8(d)	4.56dd (*J*=3.2, 4.8)
37.0(t)	2.30d (*J*=17.6), 2.95dd(*J*=5.2,17.6)
28.3(q)	2.17s
20.0(q)	1.22s

The structure of compounds in mixture B2 were under investigation. However, mixture B2 should be non-aromatic and a mixture of two closely related structure which had the carbon attached to oxygen, hydroxyl group and carbonyl group substitution in the molecule.

4.6.2 Isolation and characterization of metabolites from mycelial crude extract

EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 was separated by silica gel quick column chromatography. Combined fraction M01 (2.85 g) was purified further by silica gel column chromatography eluted with 2.5-5% EtOAc in Hexane to yield mixture M1 (2.39 g, 22.1% by weight of EtOAc crude of mycelial extract). Combined fraction M02 (460 mg) was purified further by silica gel column chromatography eluted with 5% EtOAc in Hexane to give mixture M2 (231 mg, 2.1 % by weight of EtOAc crude of mycelial extract). Combined fraction M12 was washed with MeOH and re-crystallized with MeOH to give Compound M1 (10 mg, 0.09 % by weight of EtOAc crude of mycelial extract). The structure elucidation of mixture B1 and mixture B2 is presented in section 4.6.2.1 and 4.6.2.2, respectively.

4.6.2.1 Structure elucidation of mixture M1

Mixture M1 (2.39 g) was obtained as yellow oily liquid with rancid odor from silica gel column chromatography of combined fraction M01 eluted with 2.5-5% EtOAc in Hexane.

The IR spectrum of mixture M1 (Figure C21, appendix C) showed important absorption bands at 3464 cm^{-1} of O-H stretching vibration of hydroxyl group , 2926 and 2855 cm^{-1} of C-H stretching vibration, 1743 cm^{-1} of C=O vibration of ester, 1458 and 1373 cm^{-1} of C-H bending and 1161 cm^{-1} of C-O stretching vibration of ester. The IR absorption bands of mixture B1 are assigned as shown in Table 4.9

Table 4.9 The IR absorption bands assignment of mixture M1

Wave number (cm^{-1})	Intensity	Tentative assignment
3464	Broad, Medium	O-H stretching vibration of hydroxyl group
2926,2857	Sharp, Strong	C-H stretching vibration
1743	Sharp, Strong	C=O stretching vibration of ester
1458,1373	Sharp, Medium	C-H bending vibration
1161	Sharp, Weak	C-O stretching vibration of ester
724	Sharp, Weak	C-H rocking mode of $-(CH_2)_n-$

The ^{1}H-NMR spectrum (Figure C22 in appendix C) of mixture M1 indicated that it possesses a methyl protons at δ 0.88 ppm, three methylene protons at 1.17-1.37, 1.60, and 2.01 ppm, a methylene proton attached to oxygen (-OCH_2-) at 2.32 ppm, two methine protons attached to oxygen (-O-C*H* -) at 4.14, 4.29, and two olefinic protons at 5.26 and 5.30 ppm.

The ^{13}C-NMR spectrum (Figure C23 in appendix C) of mixture M1 showed one methyl carbon signal at δ 13.9 ppm, nineteen methylene carbon signals at 22.5, 24.6, 26.9, 27.0, 28.8, 28.9, 29.0, 29.1(4)*, 29.1(7), 29.2 (0), 29.2 (4), 29.3, 29.4, 29.5, 29.5 (4), 29.6, 31.8, 33.8, and 33.9 ppm, methylene carbon attached to oxygen at 61.9, methine carbons attached to oxygen at 68.7, two olefinic carbon signals at δ 129.4 and 129.7 ppm, and the carbon signal at δ 173.0 ppm should be the carbonyl group of ester (*The number in parenthesis after δ_C value was the second decimal notation value).

The structure of mixture M1 was elucidated base on the spectroscopic data and the comparison with the retention time of standard methyl ester of those fatty acid by gas chromatography (Table 4.10, Figure C24 in appendix C).

Table 4.10 GC Retention time of mixture M1 and standard methyl esters (Std.-ME) of those fatty acids.

Fatty acid	Retention time(min)	
	Std.-ME	Mixture M1
C14:0	8-9	8.967
C16:0	16-17	16.923
C16:1	19-20	19.524
C18:0	32-33	32.408
C18:1	35-36	36.160
C18:2	43-44	44.272

The GC data revealed that mixture M1 consist of three saturated fatty acids including myristic acid, palmitic acid, and stearic acid and three unsaturated fatty acids including palmitoleic acid, oleic acid, and linoleic acid.

Table 4.11 showed the chemical structure, the name, the amount of fatty acids composition in mixture M1.

Table 4.11 The chemical structure and the name of fatty acids composition of mixture M1

Systematic name	Common name	Symbol	Structure	Relative fatty acids composition (%) of total fatty acids	Weight per mycelial dry weight (mg/g)
SATURATED					
Tetradecanoic acid	Myristic acid	C14:0	$CH_3(CH_2)_{12}COOH$	0.44	0.10
Hexadecanoic acid	Palmitic acid Stearic acid	C16:0	$CH_3(CH_2)_{14}COOH$	34.73	7.90
Octadecanoic acid		C18:0	$CH_3(CH_2)_{18}COOH$	15.71	0.18
UNSATURATED					
9-exadecenoic acid	Palmitoleic acid	C16:1	$CH_3(CH_2)_5CH=CH-(CH_2)_7COOH$	0.79	3.60
9-Octadecenoic acid	Oleic acid	C18:1	$CH_3(CH_2)_7CH=CH-(CH_2)_7$-COOH	37.47	8.58
9,12-Octadece-noic acid	Linoleic acid	C18:2	$CH_3(CH_2)_4(CH=CHCH_2)_2-(CH_2)_6COOH$	2.63	0.60

From all data, mixture M1 should be diglyceride which composed of three saturated fatty acids, including myristic acid, palmitic acid, and stearic acid and three unsaturated fatty acids which were palmitoleic acid, oleic acid, and linoleic acid. The major components of saturated and unsaturated fatty acid in mixture M1 were palmitic acid and oleic acid, respectively.

4.6.2.2 Structure elucidation of mixture M2

Mixture M2 (231 mg) was obtained as white waxy solid from silica gel column chromatography of combined fraction M02 eluted with 5% EtOAc in Hexane.

The IR spectrum of mixture M2 is shown in Figure C25, appendix C and IR absorption presented band of O-H stretching vibration of carboxylic acid at 3466 cm^{-1}, the absorption band at 2920 and 2852 cm^{-1} of C-H stretching vibration of $-CH_2$, CH_3, the absorption band of C=O stretching vibration of carboxylic acid at 1705 cm^{-1}, the absorption band of C-H bending vibration of CH_2, CH_3 at 1467 and 1409 cm^{-1}, and the absorption band at 1294 cm^{-1} of C-O stretching vibration of carboxylic acid. The IR absorption bands of mixture M2 are summarized in Table 4.12.

Table 4.12 The IR absorption band assignment of mixture M2

Wave number (cm^{-1})	Intensity	Tentative assignment
3466	Very broad	O-H stretching vibration of carboxylic acid
2920,2852	Sharp, Strong	C-H stretching vibration of $-CH_2$, CH_3
1705	Sharp, Strong	C=O stretching vibration of carboxylic acid
1467,1409	Sharp, Weak	C-H bending vibration of CH_2, CH_3
1294	Sharp, Weak	C-O stretching vibration of carboxylic acid

The ^{1}H-NMR spectrum (Figure C26 in appendix C) of mixture M2 showed the proton signal of a methyl protons at δ 0.87 ppm, a methylene protons at 1.52-1.17, 1.77-1.57, and 2.07-1.97 ppm, and a methylene protons attached to a carboxyl group at 2.34 ppm.

The ^{13}C-NMR spectrum (Figure C27 in appendix C) of mixture M1 showed a signal of methyl carbons appeared at 14.2 ppm, ten methylene carbon signals at δ 22.8, 24.8, 29.2, 29.4, 29.5, 29.6, 29.7, 29.8, 32.0, a d 34.2 ppm, and the carbon signal at δ 180.1 ppm should be the carbonyl group of carboxylic acid.

The EI-MS spectrum (Figure C28 in appendix C) of mixture M2 showed the M^+ ion peak at *m/z* 284 and 256. They were separated by 28 mass units ($-CH_2-CH_2-$) and they presented the fragmentation pattern of long chain hydrocarbon.

From all data, the structure of mixture M2 should be long chain carboxylic acid C_{16-18} or a mixture of palmitic acid (C16:0) and stearic acid (C18:0). The chemical structure of mixture M2 is shown in Figure 4.15.

$CH_3(CH_2)_{14-16}COOH$

Long chain carboxylic acid

Figure 4.15 The chemical structure and name of mixture M2

4.6.2.3 Structure elucidation of compound M1

Compound M1 (10 mg) was obtained as white amorphous solid, m.p. 169-170°C, from washing and crystallization of combined fraction M12 with MeOH.

The IR spectrum of compound M1 as showed in Figure C30, appendix D revealed a strong broad absorption band at 3398 cm^{-1} which was corresponded to the O-H stretching vibration of hydroxyl functional group, and at 1088,1023 cm^{-1} which was C-O stretching vibration of hydroxyl group. In addition, the C-H stretching and C-H bending vibration of $–CH_2$ was observed at 2942 cm^{-1}. IR absorption band of compound M1is summarized in Table 4.13.

Table 4.13 The IR absorption band assignment of compound M1

Wave number (cm^{-1})	Intensity	Tentative assignment
3398	Broad, Strong	O-H stretching vibration of C-OH
2943, 2900	Sharp, Strong	C-H stretching vibration of $-CH_2$
1433	Sharp, Strong	C-H bending vibration of $–CH_2$
1277, 1083, and 1013	Sharp, Medium	C-O stretching vibration of C-OH

The ^{1}H-NMR spectrum (Figure C31 in appendix C) of compound M1 possessed signals of methylene protons attached to oxygen (-O- C*H*$_2$ -) at 3.59 and 3.79 ppm, and methine protons attached to oxygen (-O-C*H* -) at 3.68 and 3.72 ppm.

The ^{13}C-NMR spectrum (Figure C32 in appendix C) of compound M1 exhibited a total three carbon signals at δ 63.1, 69.1, and 70.6 ppm. The assigned carbon signals based on the ^{13}C-NMR spectrum were confirmed by the information obtained from the DEPT spectra (Figure C33 in appendix C).

DEPT experiments (Figure C33 in appendix C) of compound M1 showed one methylene signal at 63.1 ppm and two methine carbon signals at 69.1, and 70.6 ppm.

Compound M1 possessed ^{13}C-NMR spectral data identical to that of D-mannitol (Kurakake, Osada, and Komaki, 1997) as showed in Table 4.14.

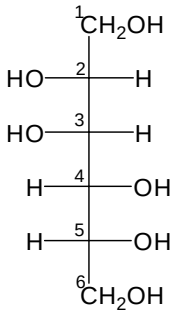
Table 4.14 ^{13}C-NMR chemical shift of compound M1 and D-mannitol (Kurakake, Osada, and Komaki, 1997)

Position	δC (ppm)	
	Compound M1	D-mannitol
1,6	63.12	62.78
2,5	70.67	70.40
3,4	69.11	68.84

* Compound M1 and D-mannitol were measured in D_2O.

The LC-MS mass spectrum (Figure C34 in appendix C) of compound M1 showed the $[M+Na]^+$ ion peak at *m/z* 205.4. The mass spectrum indicated that compound M1 possesses the molecular weight 182.4. If it is assumed that this compound contains carbons, protons, and oxygens, then the molecular formula of $C_6H_{14}O_6$ can be established. The molecular formula; $C_6H_{14}O_6$ of this compound indicated no degree of unsaturation.

From the literature, D-mannitol is the alcohol form of D-mannose which had molecular weight 182 and mp.168-169°C (Cao, Ma, and Mizuno, 1996). Considerably, the information obtained from UV, IR, NMR and MS spectra along with physical properties such as melting point led to the deduction that compound M1 is D-mannitol. The chemical structure is shown in Figure 4.16.



D-mannitol

Figure 4.16 The structure and chemical name of compound M1

D-Mannitol, white crystalline powder, is a straight-chain alcohol having 6 hydroxyl groups with slightly sweet taste. It has a property of limited water solubility and remains powdery and granular on long storage. It is widely used in medicine and as a dietary supplement. It is used as a dietetic sweetener and in medical tests of renal function.

Natural D-mannitol was often isolated from plant parts. For example, Cambie et al. (1991) had isolated D-mannitol from the heartwood of the Fijian species *Euphorbia fidjiana*. Pomilio, Gonzalez, and Eceizabarrena (1995) isolated D-mannitol from hole plants of *Nierembergia hippomanica*, a plant native to Argentina. D-mannitol was also isolated from microorganisms, especially from fungi, such as from the mycelium free PD broth in which *Aspergillus niger* was grown for nine days extracted with EtOAc (Nair and Burke, 1988) and from the heat-dried fruiting body of Chinese mushroom, *Hohenbuehelia serotina* extracted with EtOAc (Cao, Ma, and Mizuno, 1996). This is the first report of the isolation of D-mannitol from mycelia of endophytic fungal *Nodulisporium* sp. isolate LSS06 extracted with EtOAc.

4.7 Study of mycelial metabolites of endophytic fungal isolate LSS06 cultivated in different media

Metabolites from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 cultivated in five different media, including Potatoes Dextrose Broth (PDB), Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Yeast Extract Sucrose Broth (YESB), and Sabouraud's Dextrose Broth (SDB) were compared using g/g mycelial dry weight.

The amount of isolated metabolites from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 cultivated in five media was different as showed in Table 4.15. The highest yield of mixture M1 (diglyceride) and mixture M2 (long chain carboxylic acid) was obtained from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 cultivated in MEB and YES, respectively. Compound M1 (D-mannitol) was obtained from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 cultivated in MEB only.

Diglyceride and long chain carboxylic acid were classified as lipid. It is well known that many fungi are able to accumulate large amounts of cellular lipids. Mycelial lipids of many fungi have been studied in detail (Hartman, Morice, and Shorland, 1962; Yusef, Therlfall, Goodwin, 1965; Stone and Hemming, 1968; Lopez and Burgos, 1976; Renaud, Safe, and Subden, 1976). The lipid composition in fungi varies with the cultural conditions and supply of nutrients (Fisher, Brown, and Holloway, 1978; Chopra and Khuller, 1983). In the same way, the results in this study suggested that growth medium influenced to lipid composition in the mycelia of endophytic fungal isolate LSS06.

Table 4.15 The effect of growth medium on the amount of isolated mycelial metabolites of endophytic fungal isolate LSS06

Growth medium	Amount of mixture M1 (Diglyceride)		Amount of mixture M2 (Long chain carboxylic acid)		Amount of compound M1 (D-mannitol)	
	g/g mycelial dry weight	% by weight of mycelial dry weight	g/g mycelial dry weight	% by weight of mycelial dry weight	g/g mycelial dry weight	% by weight of mycelial dry weight
PDB	0.0127	1.27	0.0022	0.22	-	-
MEB	0.0502	5.02	0.0048	0.48	0.0001	0.01
MCzB	0.0058	0.58	-	-	-	-
YESB	0.0092	0.92	0.0110	1.10	-	-
SDB	0.0282	2.82	0.0031	0.31	-	-

4.8 Determination of antimicrobial activities of crude extracts and combined fractions of endophytic fungal isolate LSS06

EtOAc crude of culture broth and mycelial extract of endophytic fungal isolate LSS06 and the combined fractions from silica gel column chromatography of them 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 10 mg/ml of (1 mg/ ϕ 7 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 EtOAc crude of culture broth and mycelial extract of endophytic fungal isolate LSS06 and the combined fractions of them are presented in Figure 4.17-4.18 and Table 4.16-4.18.

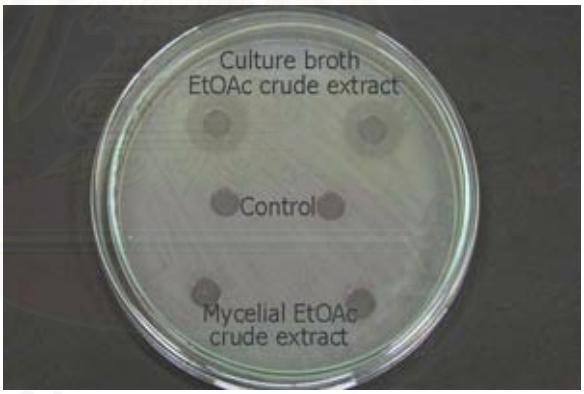
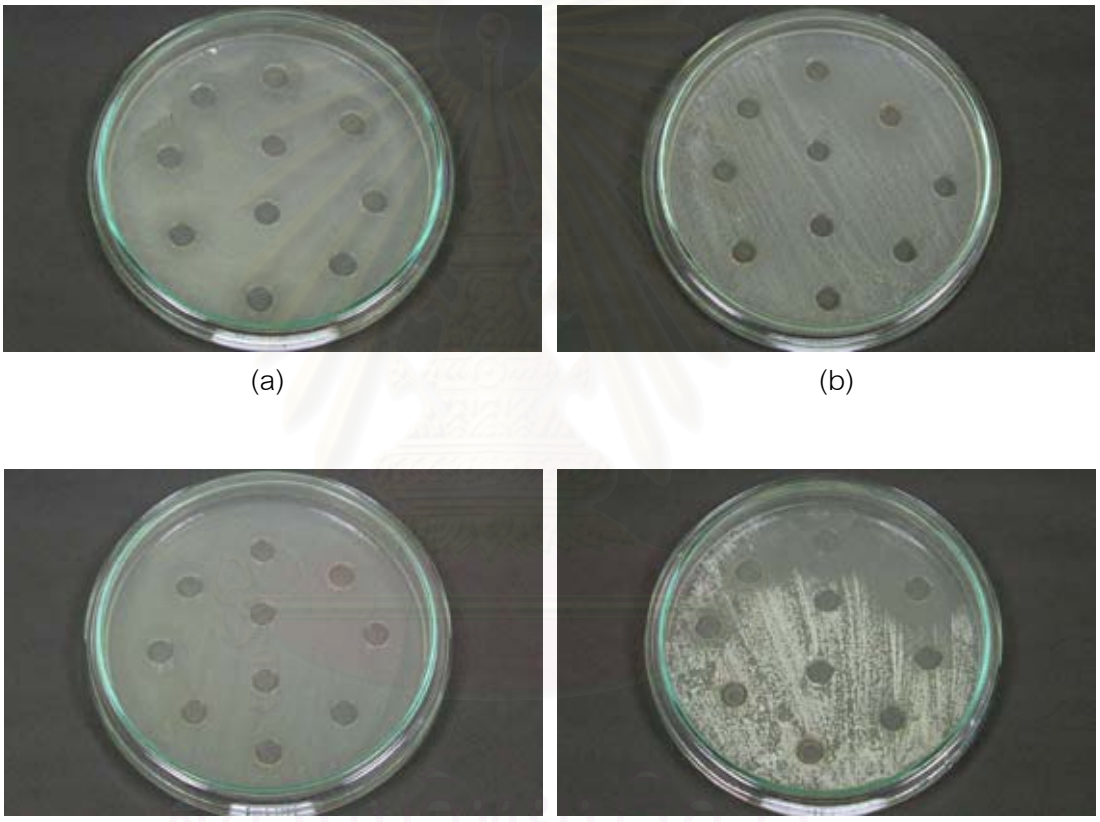


Figure 4.17 Antimicrobial activities of EtOAc crude of culture broth and mycelial extract of endophytic fungal isolate LSS06 against *B. subtilis* ATCC 6633.

Table 4.16 Antimicrobial activities of EtOAc crude of culture broth and mycelial extract of endophytic fungal isolate LSS06

Crude extract	Inhibition zone diameter (mm)				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
Culture broth	15	8	11	-	8
Mycelia	-	-	-	-	-

- = No inhibition zone



(a) (b)

(c) (d)

Figure 4.18 Antimicrobial activities of some combined fractions from silica gel column chromatography of EtOAc crude of culture broth extract of endophytic fungal isolate LSS06 against *B. subtilis* ATCC 6633 (a), *S. aureus* ATCC 25923 (b), *E. coli* ATCC 25922 (c), and *C. albicans* ATCC 10231(d).

Table 4.17 Antimicrobial activities against tested microorganisms of the combined fractions from silica gel column chromatography of EtOAc crude of culture broth extract of endophytic fungal isolate LSS06.

Combined fraction	Inhibition zone diameter (mm)				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
B01	ND	ND	ND	ND	ND
B02	9	14	-	-	20
B03	9	-	-	-	-
B04	10	-	-	-	-
B05	10	-	14	-	-
B06	14	11	19	-	12
B07	12	11	19	-	12
B08	14	11	19	-	12
B09	13	10	17	-	11
B10	13	-	14	-	-
B11	12	-	11	-	-
B12	10	-	10	-	-
B13	-	-	-	-	-
B14	-	-	-	-	-

ND = Not determined, - = No inhibition zone

Table 4.18 Antimicrobial activities against tested microorganisms of the combined fractions from silica gel column chromatography of EtOAc crude of mycelia extract of endophytic fungal isolate LSS06

Combined fraction	Inhibition zone diameter (mm)				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
M01	-	-	-	-	-
M02	-	-	-	-	-
M03	-	-	-	-	-
M04	-	-	-	-	-
M05	-	-	-	-	-
M06	-	-	-	-	-
M07	-	-	-	-	-
M08	-	-	-	-	-
M09	-	-	-	-	-
M10	-	-	-	-	-
M11	-	-	-	-	-
M12	-	-	-	-	-
M13	-	-	-	-	-

- = No inhibition zone

As reported in Table 4.16, EtOAc crude of culture broth extract had antimicrobial activities against 4 of 5 tested microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans* ATCC 10231. On the contrary, the results in Table 4.16 and 4.18 showed that EtOAc crude of mycelial extract and combined fraction from silica gel column chromatography of EtOAc crude of mycelial extract exhibited no activity against any tested microorganisms. These results suggested that, antimicrobial metabolites of endophytic fungal isolate LSS06 (*Nodulisporium* sp.) were secreted into the culture broth rather than accumulated within mycelium. The bioactive metabolites of *Nodulisporium* sp. from other plants were also isolated from culture broth extract (not from mycelial extract) (Cole and Kirksey, 1975; Monaghan et al., 1995).

From Table 4.17, the combined fraction B01 was not determined for its antimicrobial activities because it was obtained in small amount. The combined fraction B06-B09 had antimicrobial against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans* ATCC 10231 similar to EtOAc crude of culture broth extract. The combined fraction B06 was isolated and purified for the bioactive metabolites.

4.9 Evaluation of biological activities of isolated metabolites from endophytic fungal isolate LSS06

4.9.1 Antimicrobial activity

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 4.19.

Table 4.19 MIC of antimicrobial activities of isolated metabolites from endophytic fungal isolate LSS06.

Compound	MIC(µg/ml)				
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC10231
Mixture B1	1000	>1000	>1000	>1000	>1000
Mixture B2	>1000	>1000	>1000	>1000	>1000
Mixture M1	ND	ND	ND	ND	ND
Mixture M2	ND	ND	ND	ND	ND
M1	>1000	>1000	>1000	>1000	>1000

ND = Not determined

The results showed that mixture B1 inhibited growth of *B. subtilis* ATCC 6633 with the MIC of 1000 µg/ml. Mixture B1 had only slightly antimicrobial activity against *B. subtilis* ATCC 6633. On the other hand, mixture B2 and compound M1 did not show any antimicrobial activities.

4.9.2 Cytotoxic activity

The *in vitro* cytotoxic activity of isolated metabolites from endophytic fungal LSS06 against 5 cell line including HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) is shown in Table 4.20 as the minimum concentration of 50 % inhibitory activity (IC_{50}).

Table 4.20 Cytotoxic activity of isolated metabolites from endophytic fungal isolate LSS06.

Compound	IC_{50} (µg/ml)				
	HEP-G2 (hepatoma)	SW 620 (colon)	CHAGO (lung)	KATO-3 (gastric)	BT474 (breast)
Mixture B1	>10	>10	>10	>10	>10

As reported in Table 4.20, mixture B1 had no any cytotoxic activity. Mixture B2 was not determined for its cytotoxic activity because it was obtained in small amount.

From structure elucidation, mixture M1 was identified as diglyceride and mixture M2 was identified as C_{16}-C_{18} long chain carboxylic acid. Both compounds were classified as lipids which no potential to have antimicrobial and cytotoxic activity. For this reason, mixture M1and mixture M2 were not determined for both antimicrobial and cytotoxic activities. In the same way, compound M1 was not determined for its cytotoxic activity because it was D-mannitol which classified as sugar alcohol and known that it no cytotoxicity.

As far as we knew, only a few bioactive metabolites have been reported to be produced by cultures of *Nodulisporium* spp. from other sources. As reported by Cole and Kirksey (1975), desmethoxyviridol, a toxin from *Nodulisporium hinnuleum* isolated

from peanut seed produced in Virginia during 1972 growing season. This toxin had an oral median lethal dose of 4.2 mg/kg in 1-day-old cockerels and produced plant-growth regulating and phytotoxic effects in plant systems. Dombrowski et al., (1992) reported about slightly HIV-1 protease inhibition of *Nodulisporium* sp. L-6, endophytic fungi from live foliage of *Chamaecyparis thyoides*. Nodulisporic acid A (NAA) is an indole-diterpene metabolite produced by *Nodulisporium* sp. (MF5954, ATCC 74245), an endophytic fungus isolated from a woody plant. NAA exhibits potent, insecticidal activities against the mosquito larvae of *Aedes aegypti*, larvae of the blowfly *Lucilia seracata*, and it is a potent, long-lasting, nontoxic systemic orally-active agent that kill fleas on dog (Ondeyka, et al., 2002). Novel chlorosis-inducing substances, AB5046A and B (2-butyryl-3,5-dihydroxy-cyclohex-2-ene-1-one and 2-acetyl-3,5-dihydroxy-cyclohex-2-ene-1-one, repectively) were isolated from the culture broth of a fungal *Nodulisorium*. AB5046A and B induced chlorosis against Japanese barnyard millet in vitro. The cholorosis activity of these compounds was stronger against monocotyledon than dicotyledons (Igarashi et al., 1993).

In all of these literatures, there was no reported on antibacterial and antifungal metabolites of *Nodulisporium* sp. In this study, it was found that endophytic fungal *Nodulisporium* sp. isolate LSS06 could produce some antibacterial and antifungal metabolies. Accordingly, it is very interesting to purify and characterize antibacterial and antifungal metabolites from the culture broth of endophytic fungal *Nodulisporium* sp. isolate LSS06 in the future work.

CHAPTER V

CONCLUSION

The objectives of this research were aimed at isolation of endophytic fungi from *Morinda citrifolia* L. leaves and twigs, determination of antimicrobial activities of the isolated endophytic fungi, identification of a selected endophytic fungal isolate based on morphology and nucleotide sequencing of ITS regions of rDNA, elucidation of the structure and evaluation of bioactivities of the isolated metabolites from a selected endophytic fungal isolate.

One hundred and seventy eight endophytic fungal isolates were isolated from surface sterilized leaves and twigs of *Morinda citrifolia* L. collected from 4 provinces in Thailand, including Bangkok, Samutsongkhram, Kanchanaburi, and Khonkaen. The percentage of isolates from twig sections is higher than that from leaf sections.

The isolated endophytic fungi cultured on five different media, including Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), and Sabouraud's Dextrose Agar (SDA) were determined for their antimicrobial activities by fungal disk dual culture method. Forty four isolates from one hundred and seventy eight isolates (24.7 %) had antimicrobial activity against at least one tested microorganism, they were active against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 at 14.0%, 11.8%, 1.7%, 6.2%, and 7.9%, respectively. The growth medium had shown the effect on antimicrobial activities of isolated endohytic fungi. MEA gave the highest activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922. SDA gave the highest activity against *P. aeruginosa* ATCC 27853. MEA and MCzA gave equally highest activity against *C. albicans* ATCC 10231. Antimicrobial activities of isolated endophytic fungi were classified into 4 types as bacterial inhibition only (16.9%), yeast inhibition only (2.2 %), bacterial and yeast inhibition (5.6 %) and no antimicrobial activity against tested microorganisms (75.3 %). Of forty four active endophytic fungal isolates, endophytic fungal isolate LSS06 cultivated on MEA had the

best antimicrobial activity because it was the only isolate that inhibited the growth of tested gram positive bacteria *(B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923), gram negative bacteria (*E. coli* ATCC 25922) and yeast (*C. albicans* ATCC 10231). Accordingly, endophytic fungal isolate LSS06 was selected for identification and isolation of bioactive metabolites.

Based on colony characteristic, microscopic characteristic, and nucleotide sequencing analysis of ITS regions of rDNA, endophytic fungal isolate LSS06 was identified as *Nodulisporium* sp.

Growth profile of endophytic fungal isolate LSS06 cultivated in Malt Extract Broth (MEB) was observed from mycelial dry weight. Endophytic fungal isolate LSS06 grew into mid log phase within 8 days and gradually grew into stationary phase within 14 days. After 18 days, the growth of fungal isolate LSS06 was decreasing.

EtOAc crude of culture broth and mycelial extract of endophytic fungal isolate LSS06 cultivated in MEB were separated and purified by silica gel column chromatography and crystallization techniques. Mixture B1 (10 mg) and Mixture B2 (8 mg) were obtained from EtOAc crude of culture broth extract. Isolation of EtOAc crude of mycelial extract gave mixture M1 (2.39 g), mixture M2 (231 mg) and pure compound M1 (10 mg). The structure elucidation of these compounds was achieved by analysis of their spectroscopic data and physical properties. The structures of mixture B1 and mixture B2 were still unknown. Mixture M1 was identified as diglyceride which composed of 3 saturated fatty acids, including myristic acid, palmitic acid, and stearic acid and 3 unsaturated fatty acids which were palmitoleic acid, oleic acid and linoleic acid. The major components of saturated and unsaturated fatty acid in mixture M1 were palmitic acid and oleic acid, respectively. Mixture M2 was a mixture of saturated C_{16}-C_{18} long chain carboxylic acid. Pure compound M1 was identified as D-mannitol.

Metabolites from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 cultivated in five different media, including Potatoes Dextrose Broth (PDB), Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Yeast Extract Sucrose Broth (YESB), and Sabouraud's Dextrose Broth (SDB) were compared using g/g mycelial dry weight. The highest yield of mixture M1 and mixture M2 was obtained from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 cultivated in MEB and YES,

respectively. For compound M1 (D-mannitol), it obtained from EtOAc crude of mycelial extract of endophytic fungal isolate LSS06 grown in MEB.

From biological testing of isolated metabolites, mixture B1 inhibited growth of *B. subtilis* ATCC 6633 with MIC 1000 µg/ml but no cytotoxicity against five tumor test cell lines *in vitro*. Other compounds showed no inhibition of any tested microorganisms.

Suggestions

1. The antimicrobial activities of isolated endophytic fungi should be tested on filamentous phytopathogenic fungi such as *Pyricularia oryzae*, *Fusarium oxysporum*, and *Pythium aphanidermatum*.

2. Cultivation conditions; carbon source, nitrogen source, temperature, and pH have affected the quantity and quality of bioactive metabolites from the selected endophytic fungi. Therefore, these cultivation conditions should be optimized.

3. Combined fraction B06 which separated from EtOAc crude of culture broth extract of endophytic fungal *Nodulisporium* sp. isolate LSS06, had antimicrobial activities. Consequently, it should be purified by other chromatographic technique, especially HPLC which may lead to the isolation of more antimicrobial metabolites. In addition, other combined fractions that showed antimicrobial activities should be purified and characterized.

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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 czapek agar (MCzA)

Czapek stock solution A	50.0	ml
Czapek stock solution B	50.0	ml
Malt extracts	40	g
Sucrose	30	g
Agar	15.0	g
Distilled water	1000	ml

Czapek stock solution A

$NaNO_3$	4.0	g
KCl	1.0	g
$MgSO_4.7H_2O$	10.0	g
$FeSO_4.7H_2O$	0.02	g
Distilled water	1000	ml

Keep in a refrigerator.

Czapek stock solution B

K_2HPO_4	20.0	g
Distilled water	1000	ml

Keep in a refrigerator.

2. 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

3. Nutrient agar (NA)

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

4. Sabouraud's dextrose agar (SDA)

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

5. 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.

6. Yeast extract sucrose agar (YES)

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

7. Yeast-malt extract medium (YMA)

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

APPENDIX B

TableB1 Raw data of mycelial dry weight and inhibition zone of endophytic fungus isolate LSS06

Time (days)	Mycelial dry weight (g/l)	Inhibition zone (mm)				
		Gram positive bacteria		Gram negative bacteria		Yeast
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
0	0.189	0	0	0	0	0
2	0.303	0	0	0	0	0
4	1.975	0	0	0	0	0
6	3.973	9	0	10	0	0
8	4.959	10	9	12	0	10
10	6.22	11	11	14	0	11
12	6.918	13	12	17	0	12
14	7.615	16	12	18	0	12
16	7.685	16	12	18	0	12
18	7.559	16	12	18	0	12
20	7.019	16	12	18	0	12
22	6.771	16	11	18	0	12
24	6.506	14	10	16	0	11
26	6.253	14	10	15	0	11
28	5.979	14	9	15	0	11
30	5.788	14	9	15	0	9
32	5.638	9	9	15	0	9
34	5.44	9	9	15	0	9
36	5.164	9	9	15	0	9
38	5.024	9	9	15	0	9

APPENDIX C

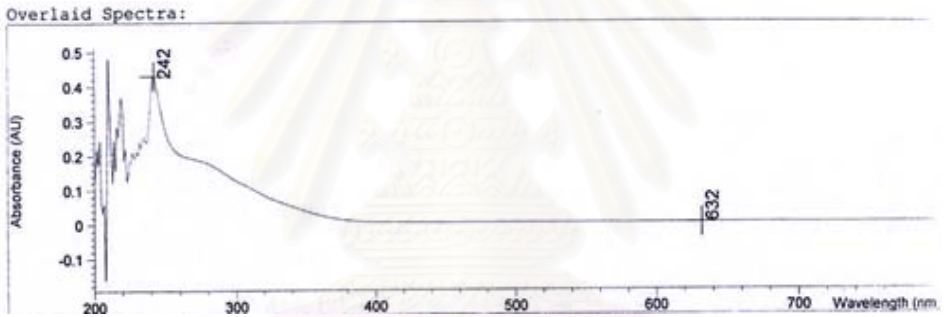
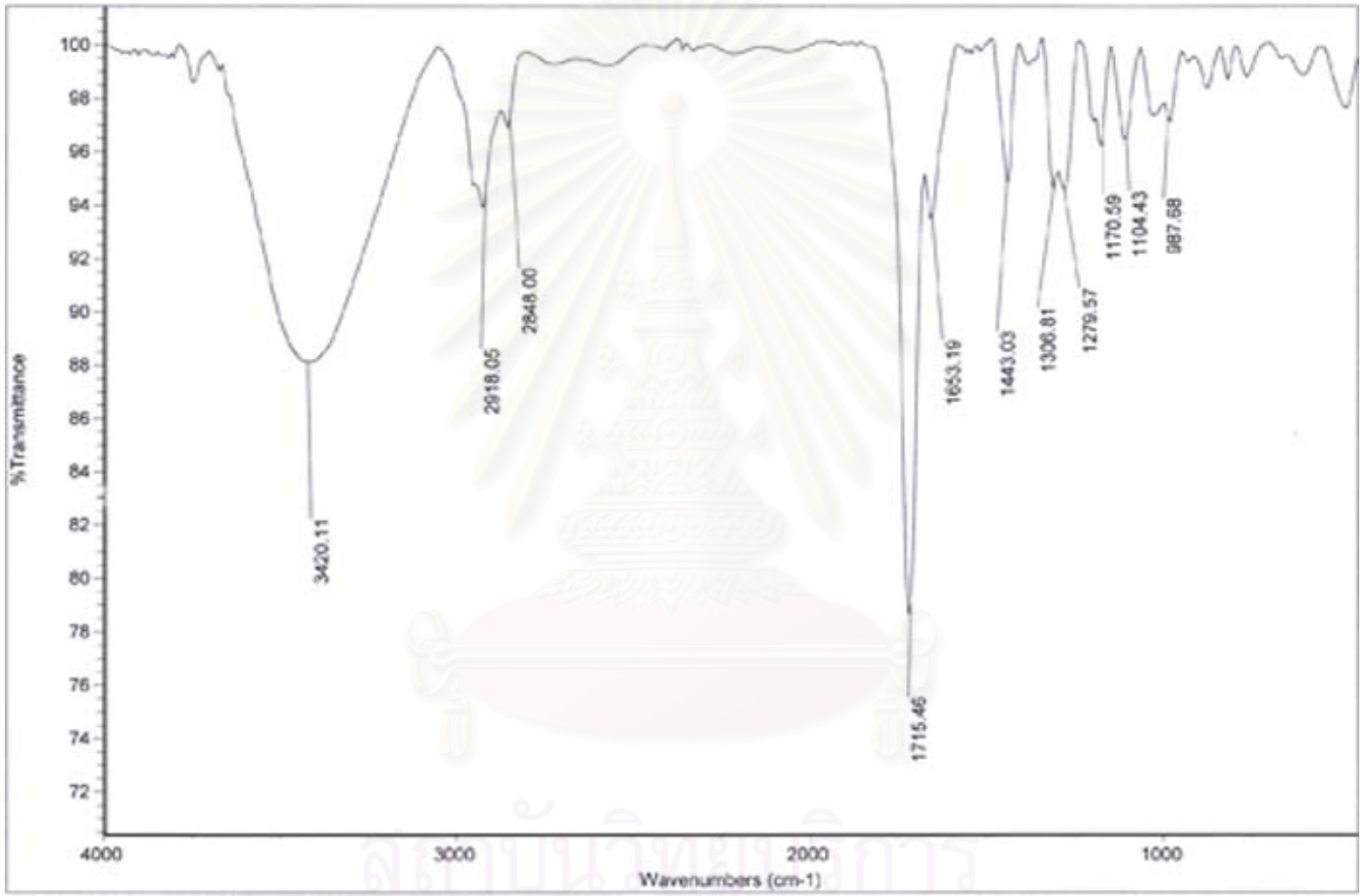


Figure C1 UV spectrum of mixture B1



FigureC2 IR spectrum of mixture B1

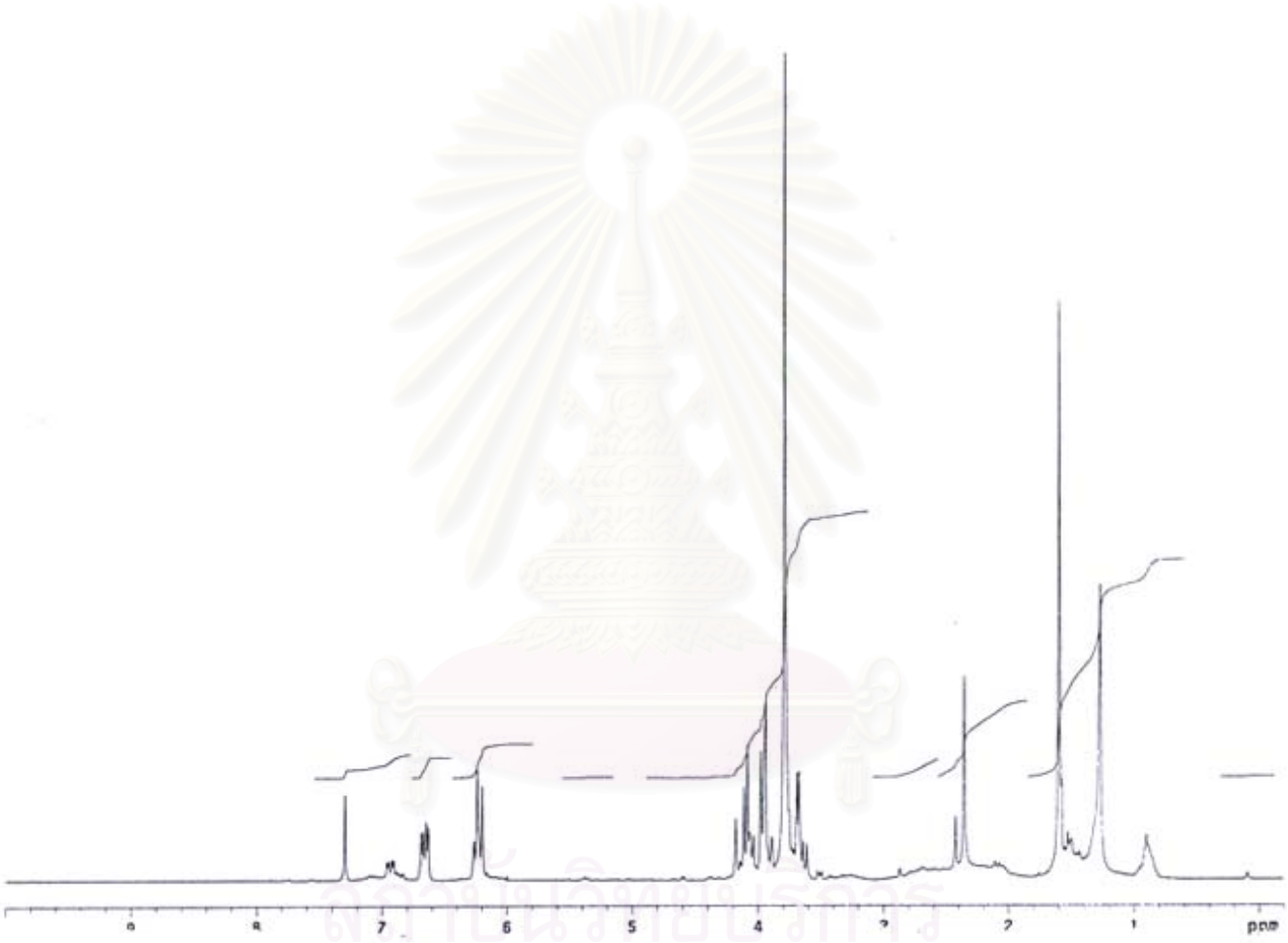


Figure C3 ^{1}H-NMR spectrum of mixture B1

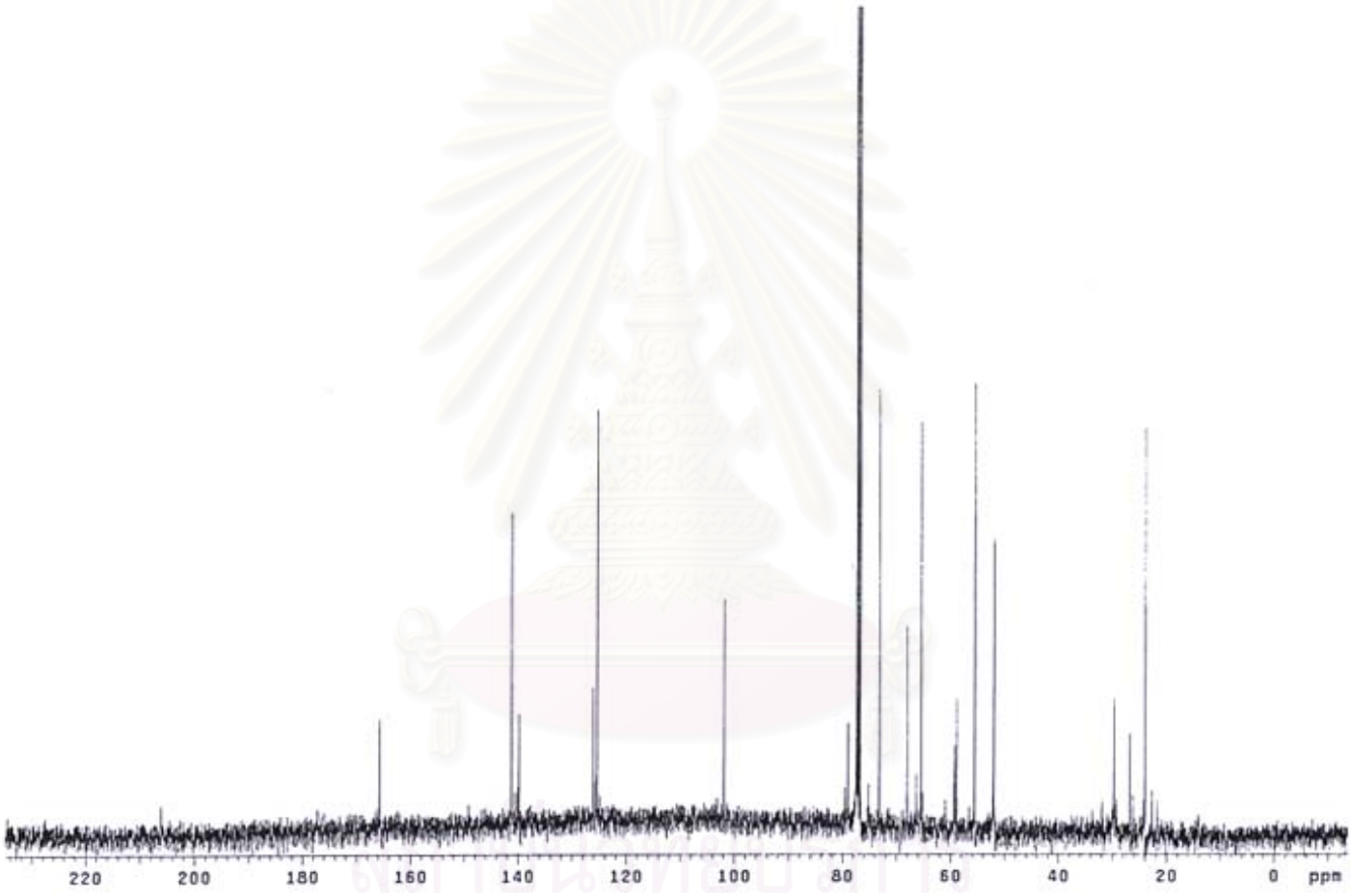


Figure C4 ^{13}C-NMR spectrum of mixture B1

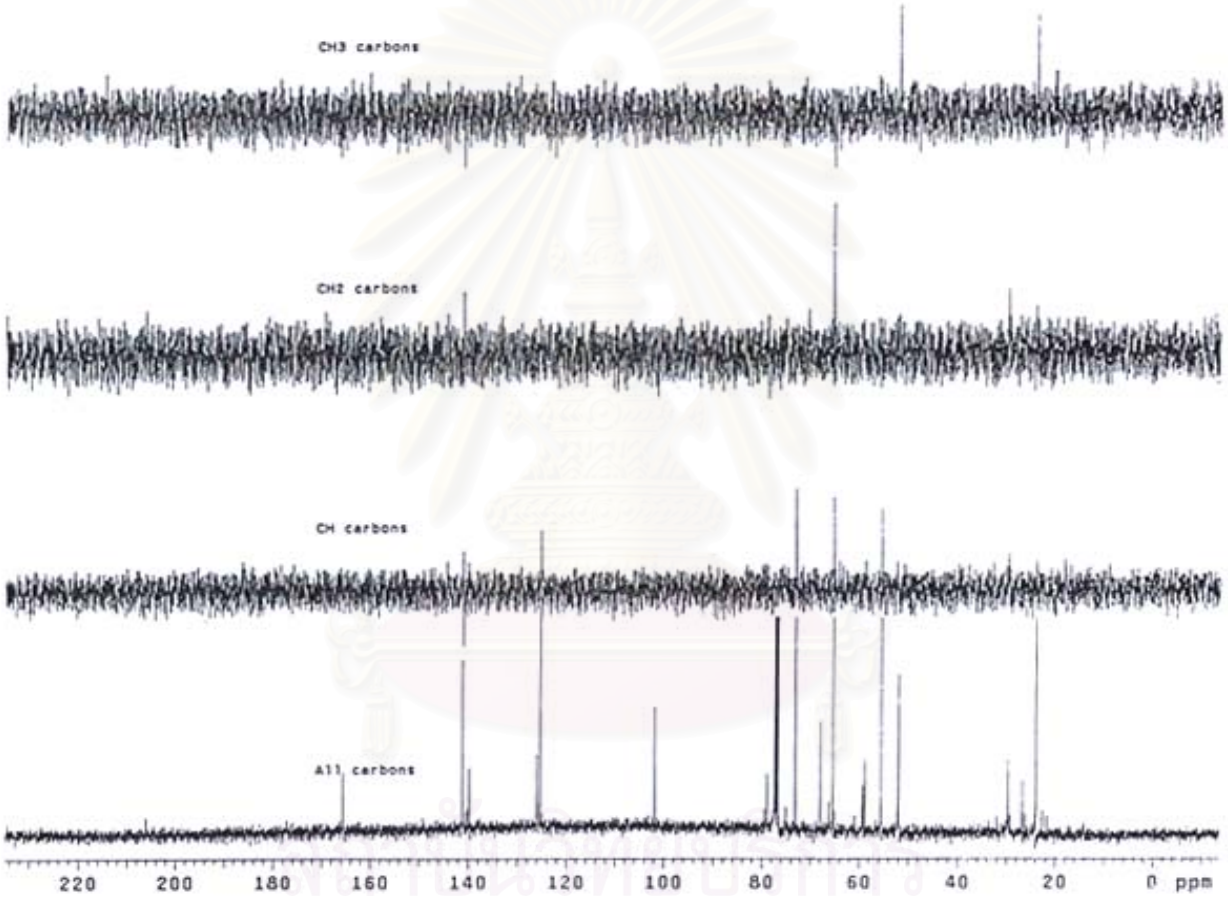


Figure C5 DEPT spectrum of mixture B1

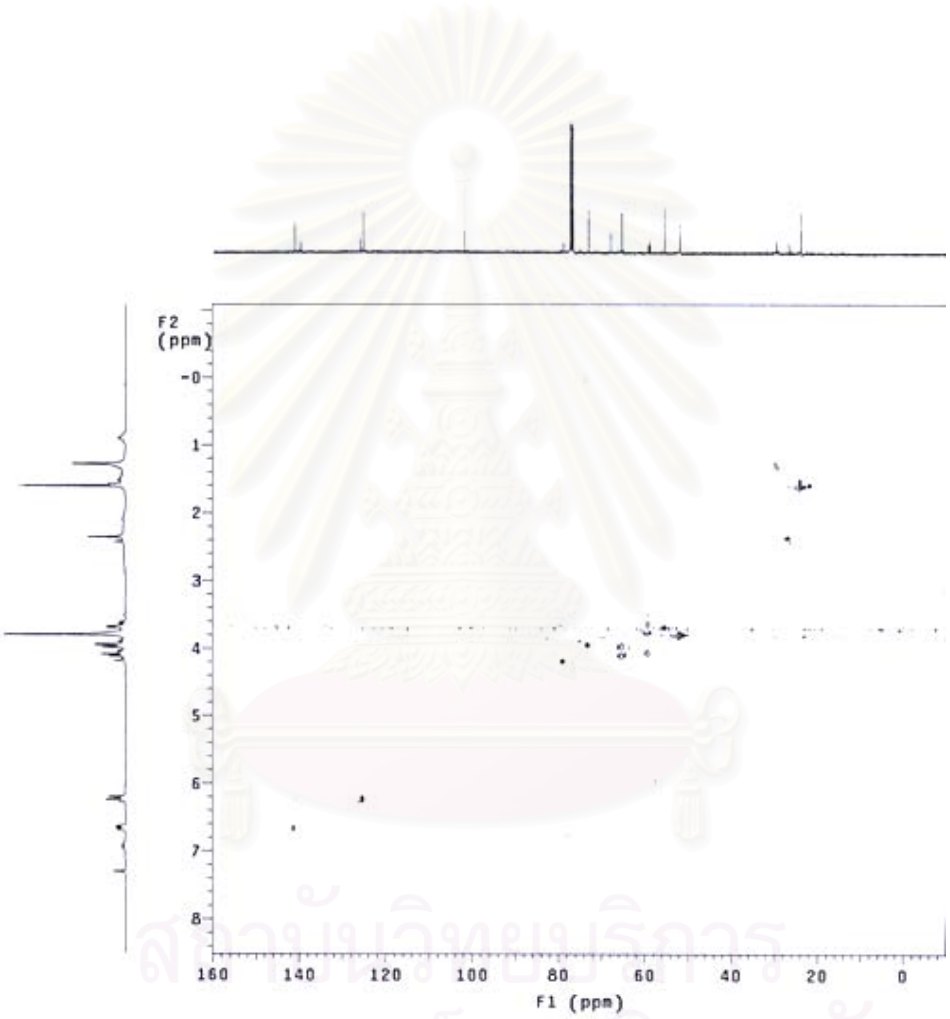


Figure C6 HSQC spectrum of mixture B1

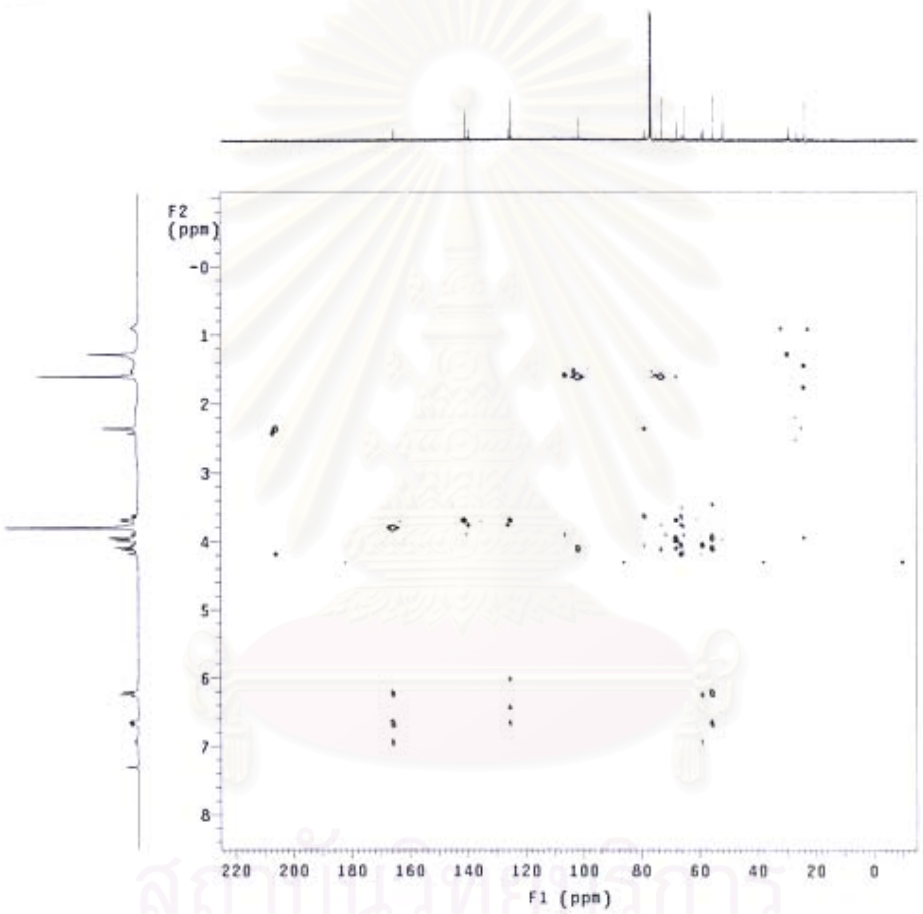


Figure C7 HMBC spectrum of mixture B1

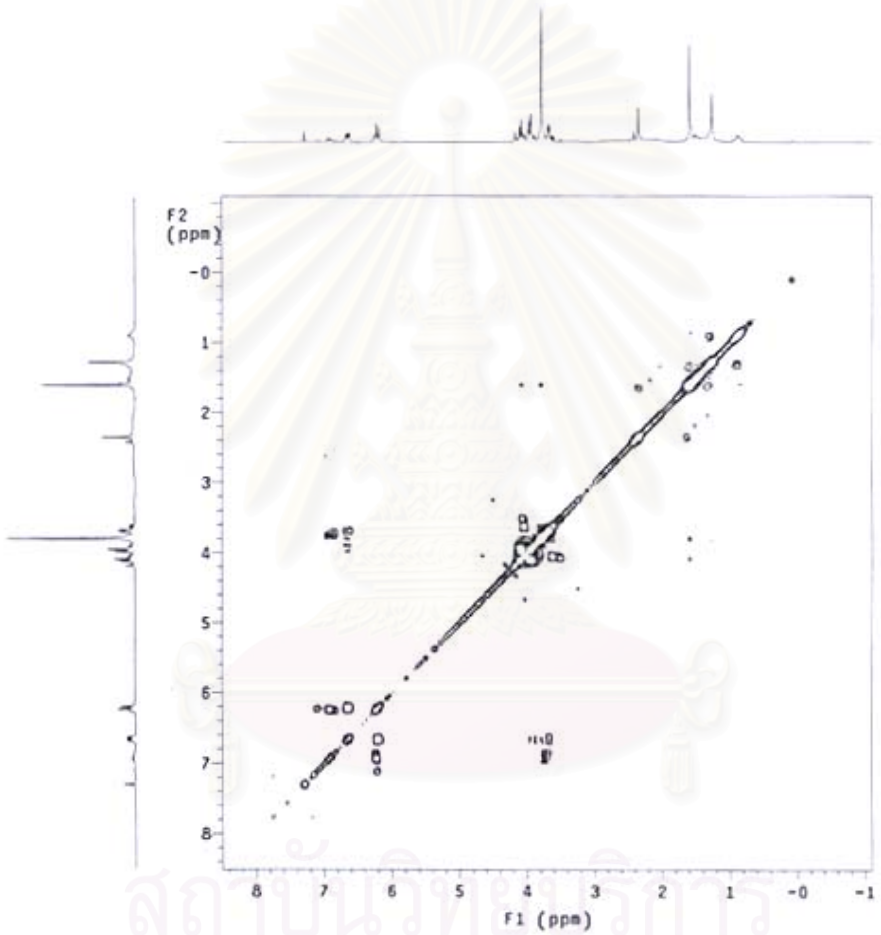


Figure C8 COSY spectrum of mixture B1

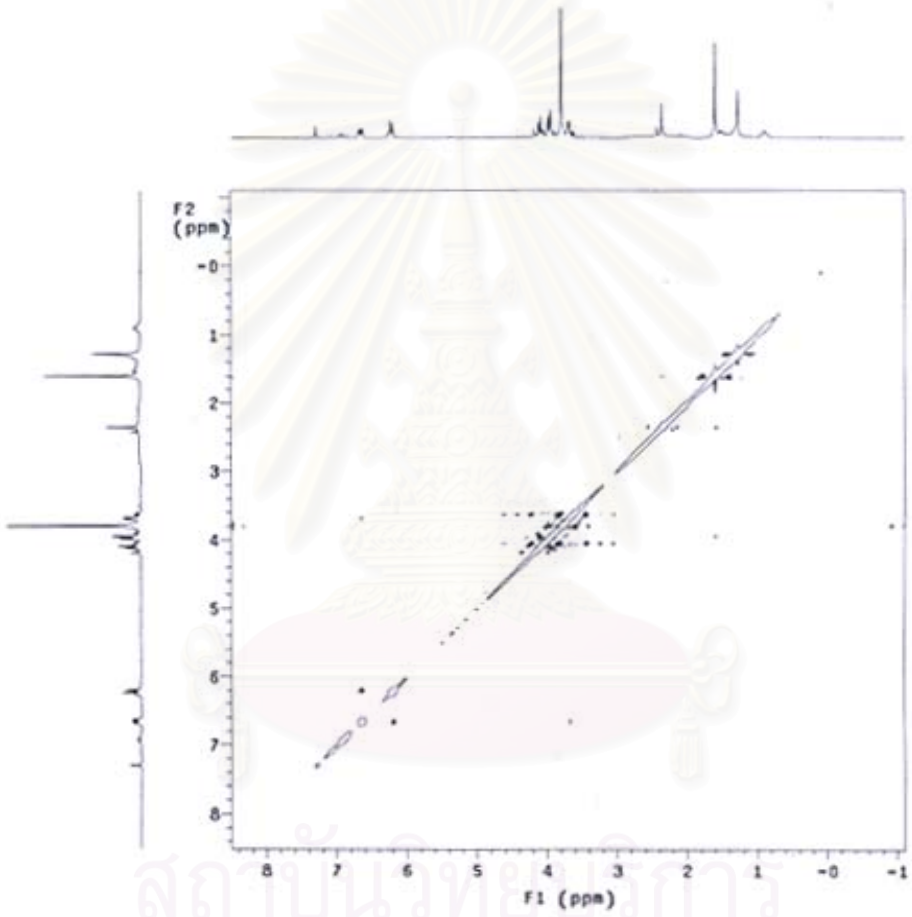


Figure C9 NOESY spectrum of mixture B1

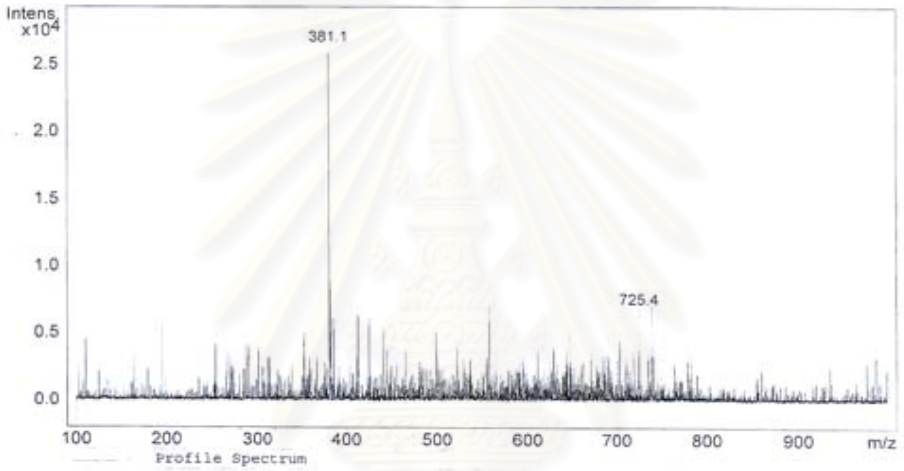


Figure C10 LC-MS spectrum of mixture B1

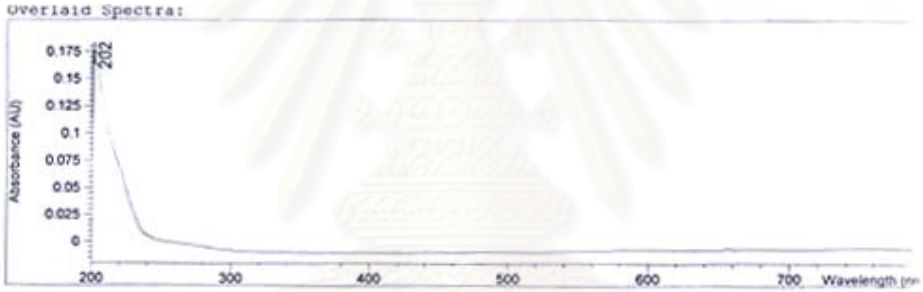


Figure C11 UV spectrum of mixture B2

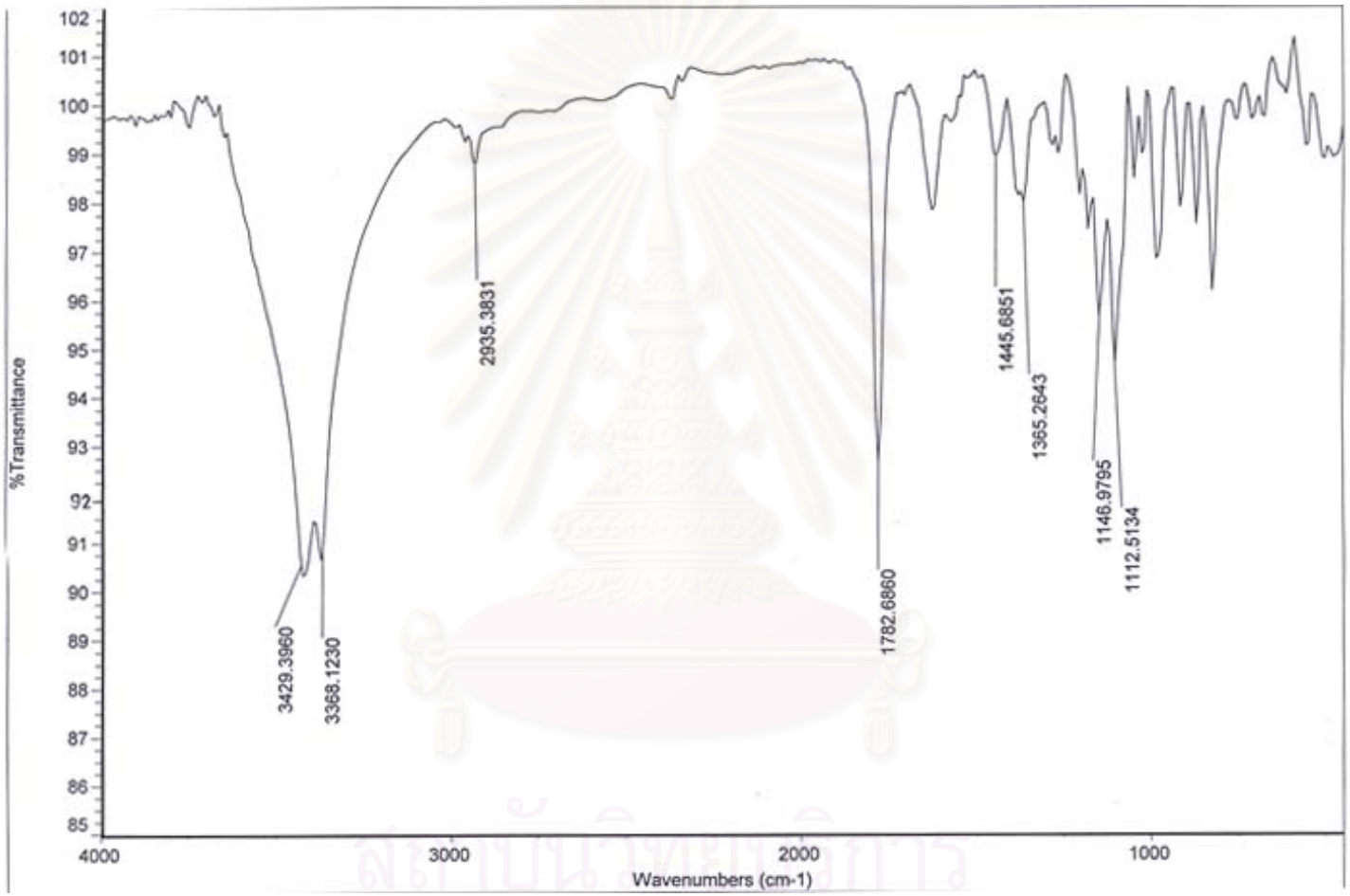


Figure C12 IR spectrum of mixture B2

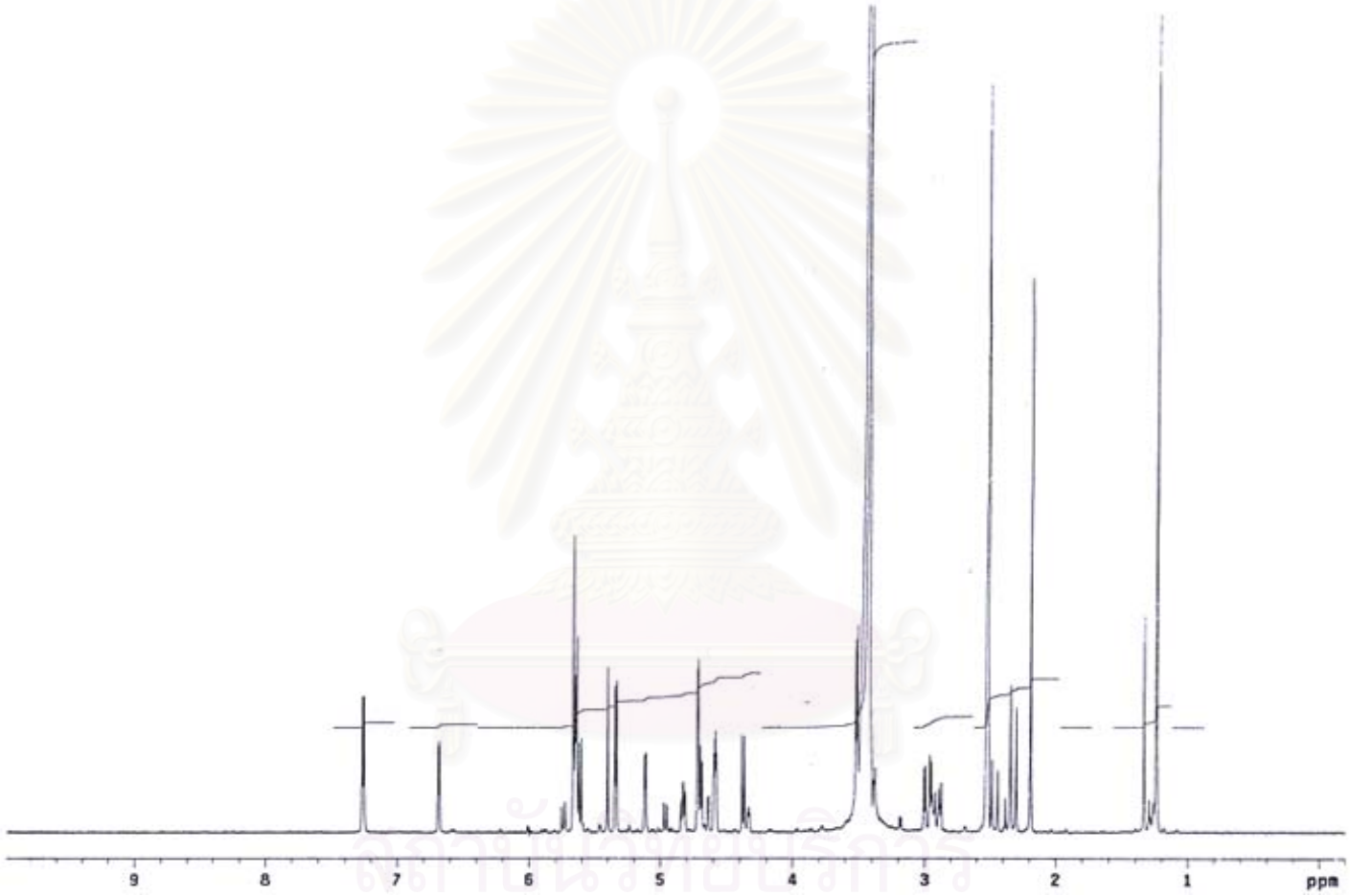


Figure C13 ^{1}H-NMR spectrum of mixture B2

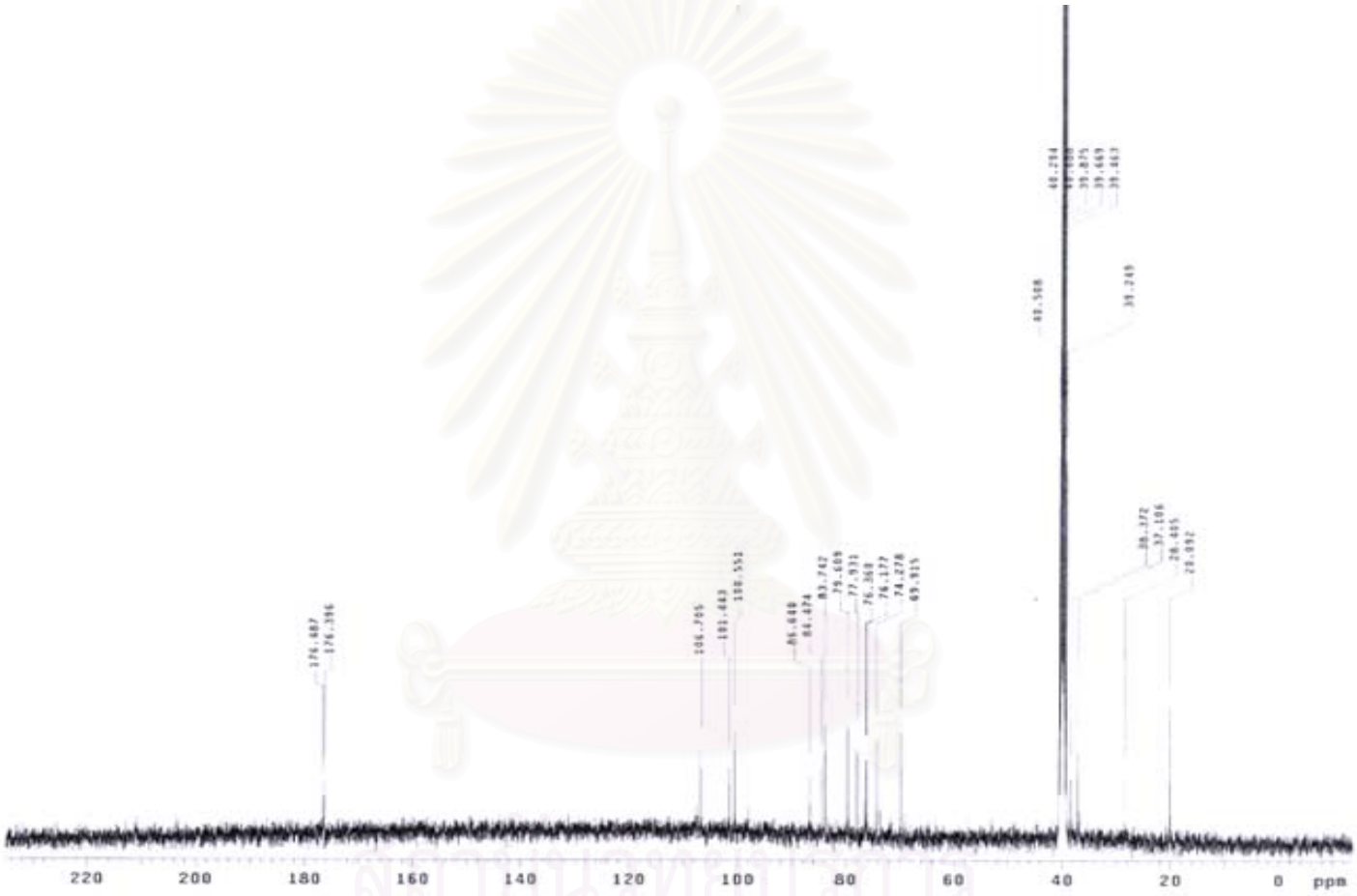


Figure C14 ^{13}C-NMR spectrum of mixture B2

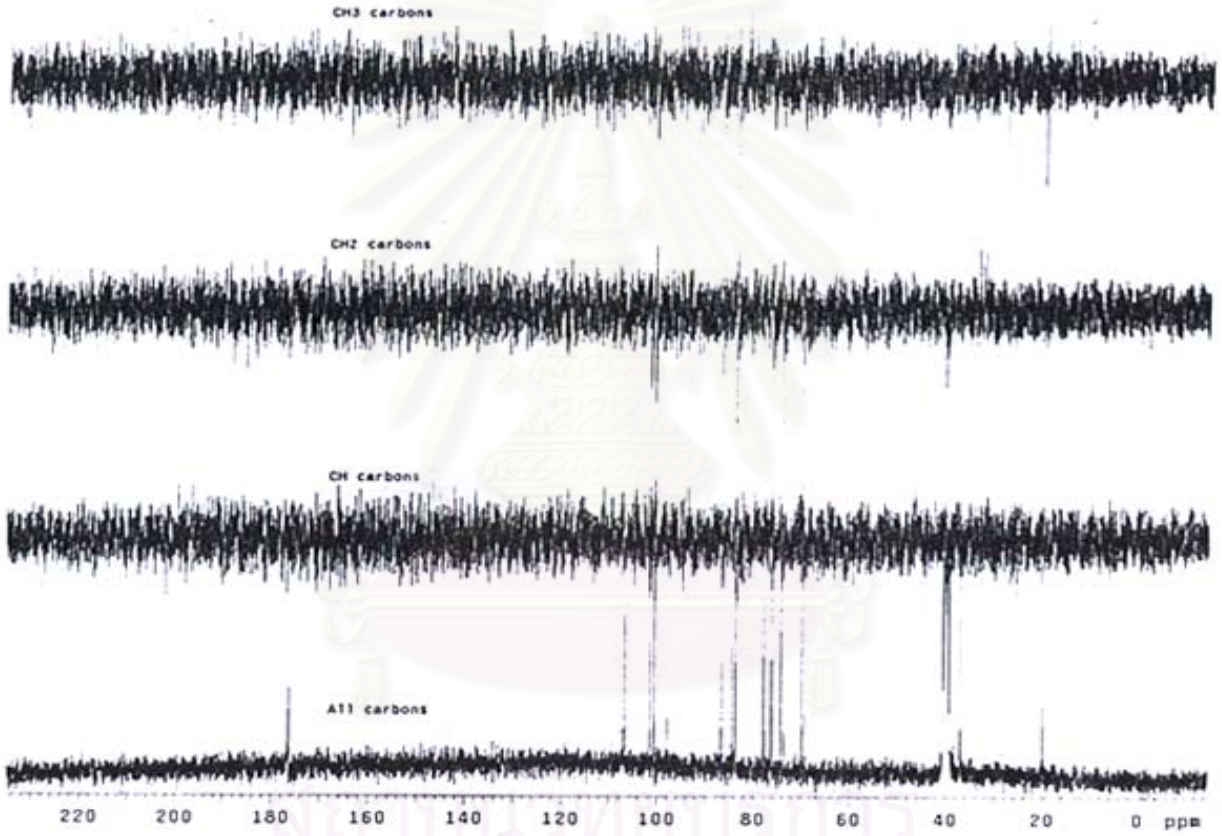


Figure C15 DEPT spectrum of mixture B2

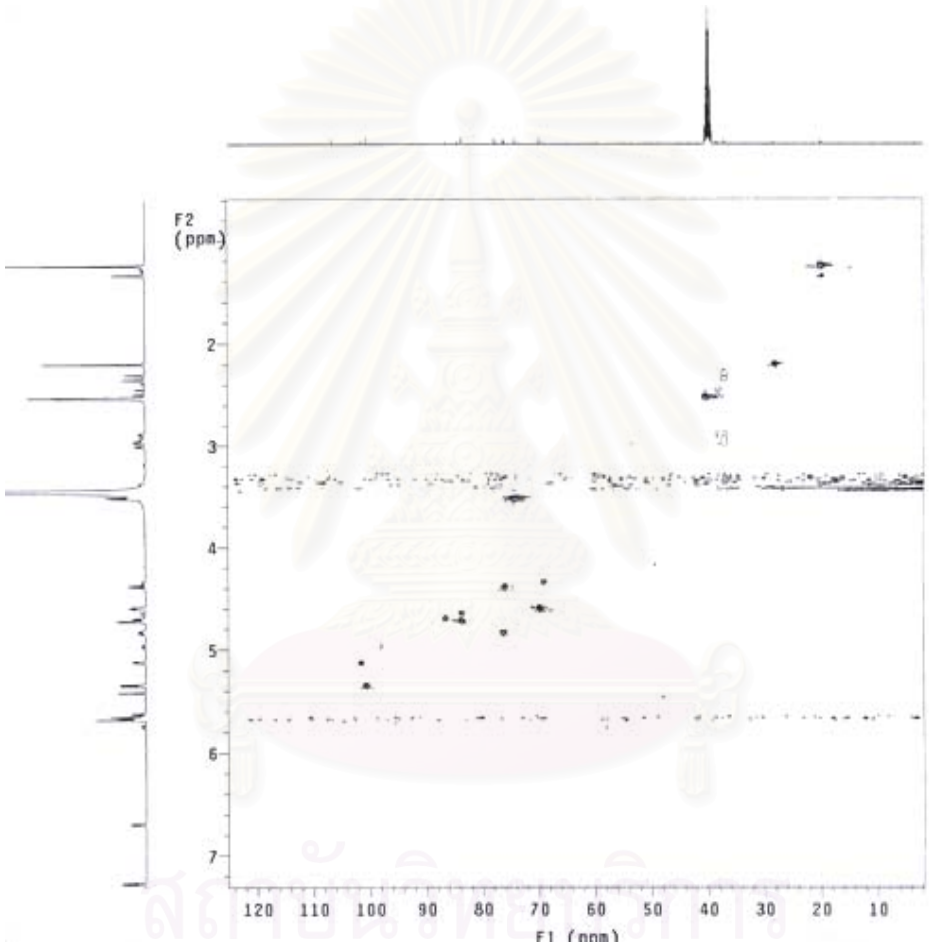


Figure C16 HSQC spectrum of mixture B2

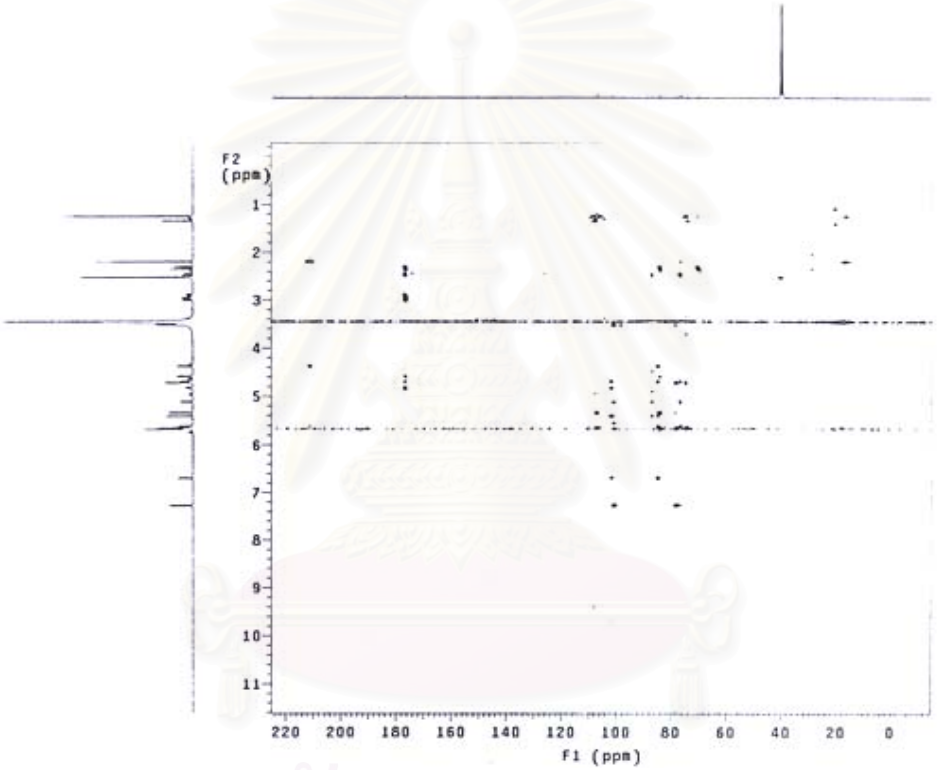


Figure C17 HMBC spectrum of mixture B2

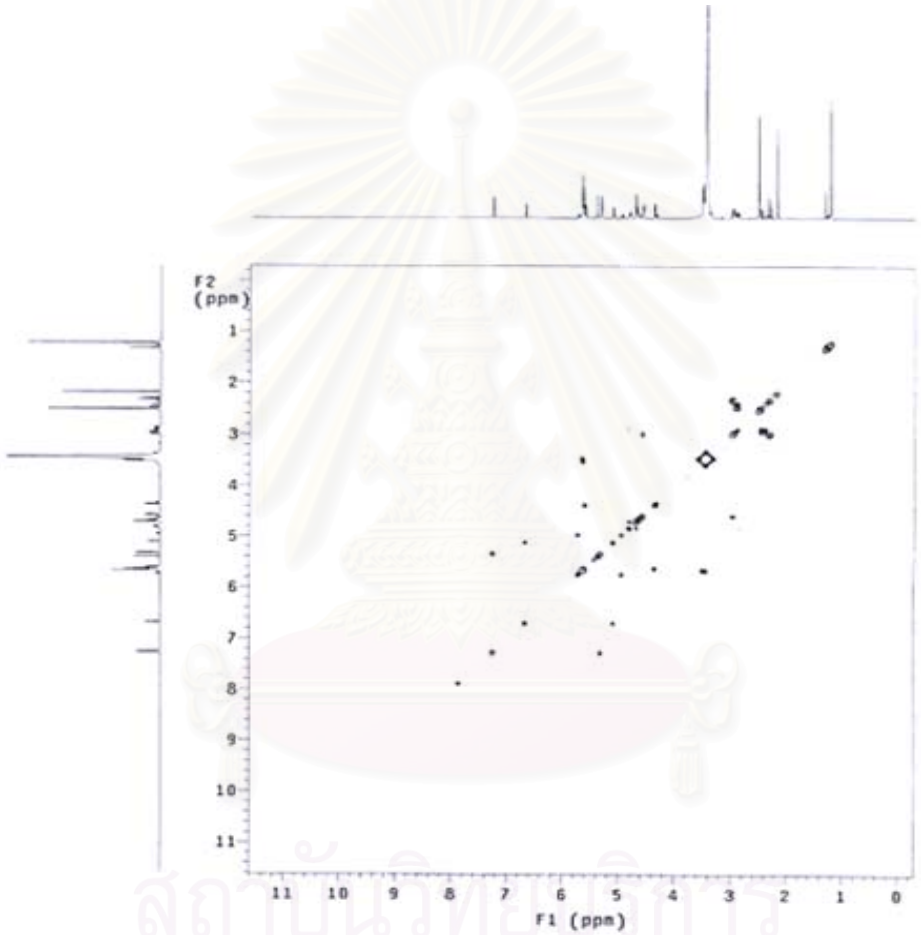


Figure C18 COSY spectrum of mixture B2

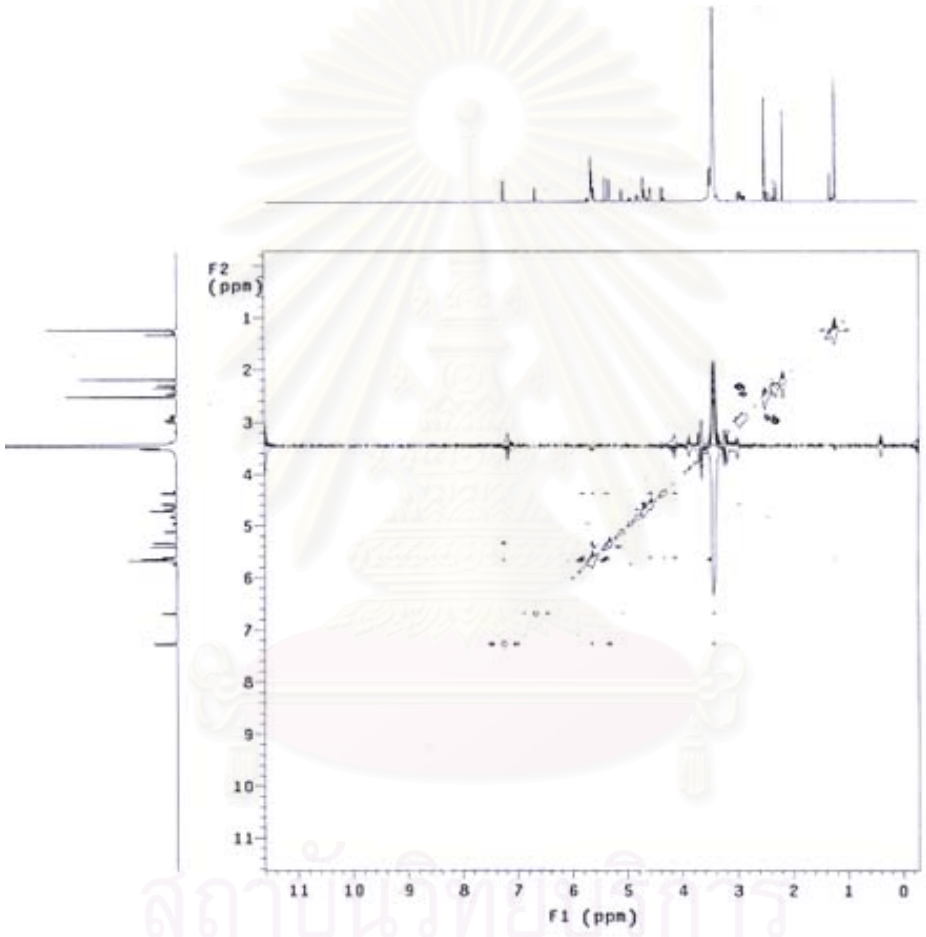


Figure C19 NOESY spectrum of mixture B2

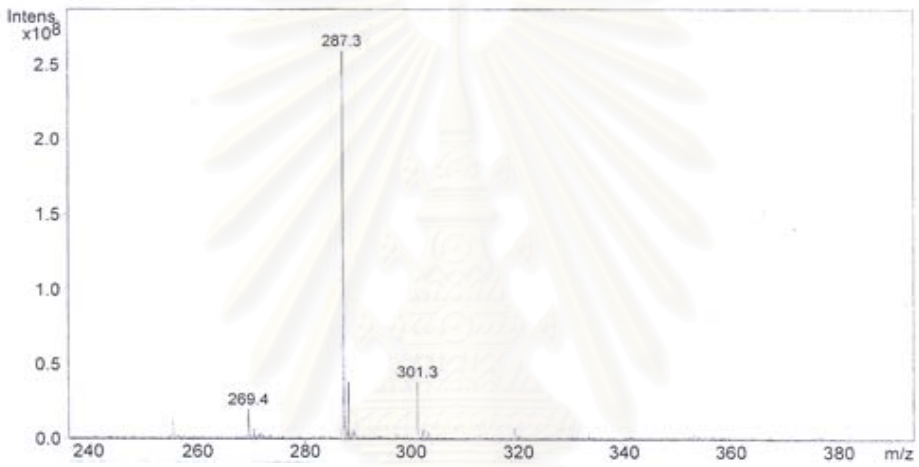


Figure C20 LC-MS spectrum of mixture B2

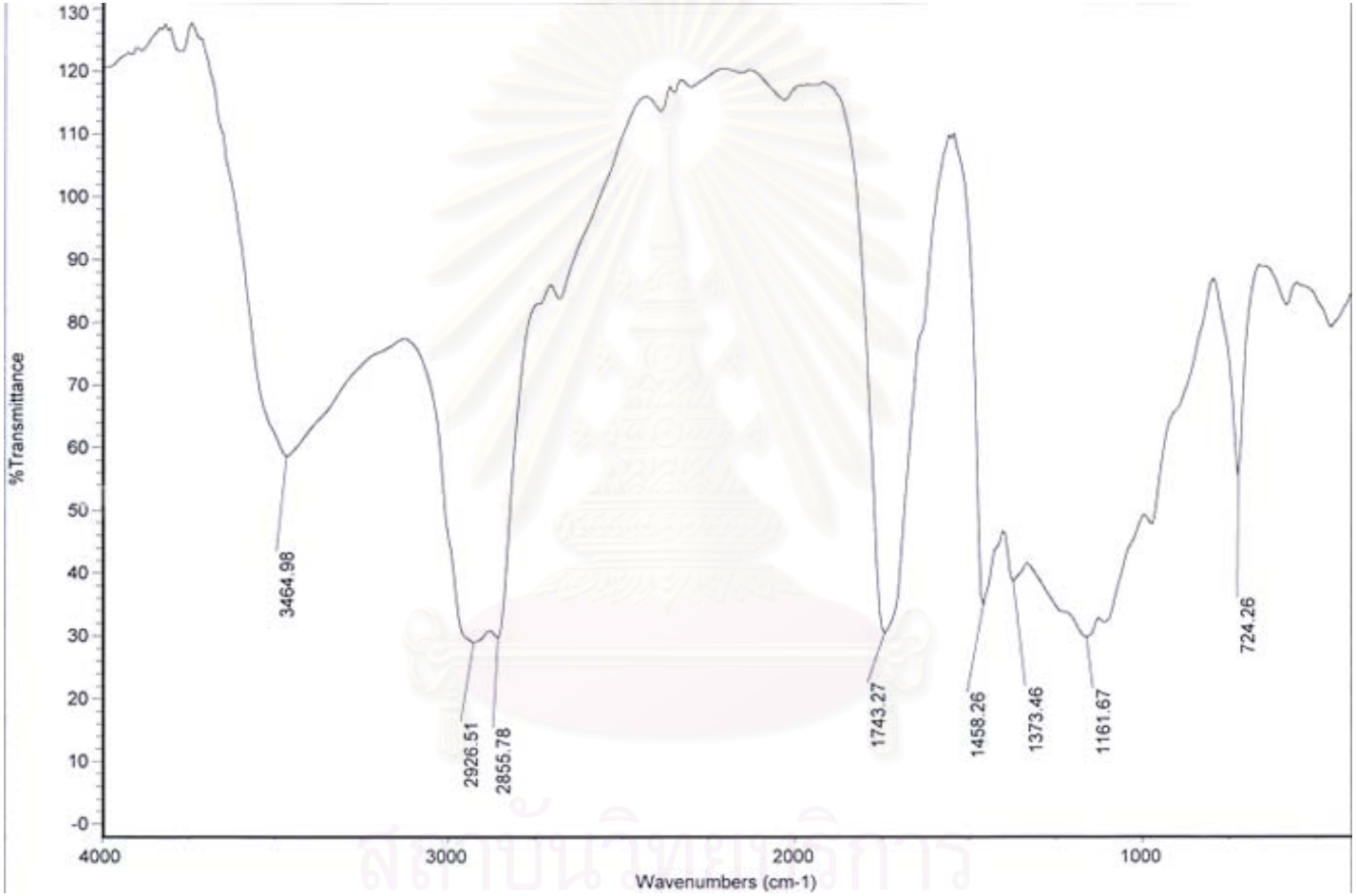


Figure C21 IR spectrum of mixture M1

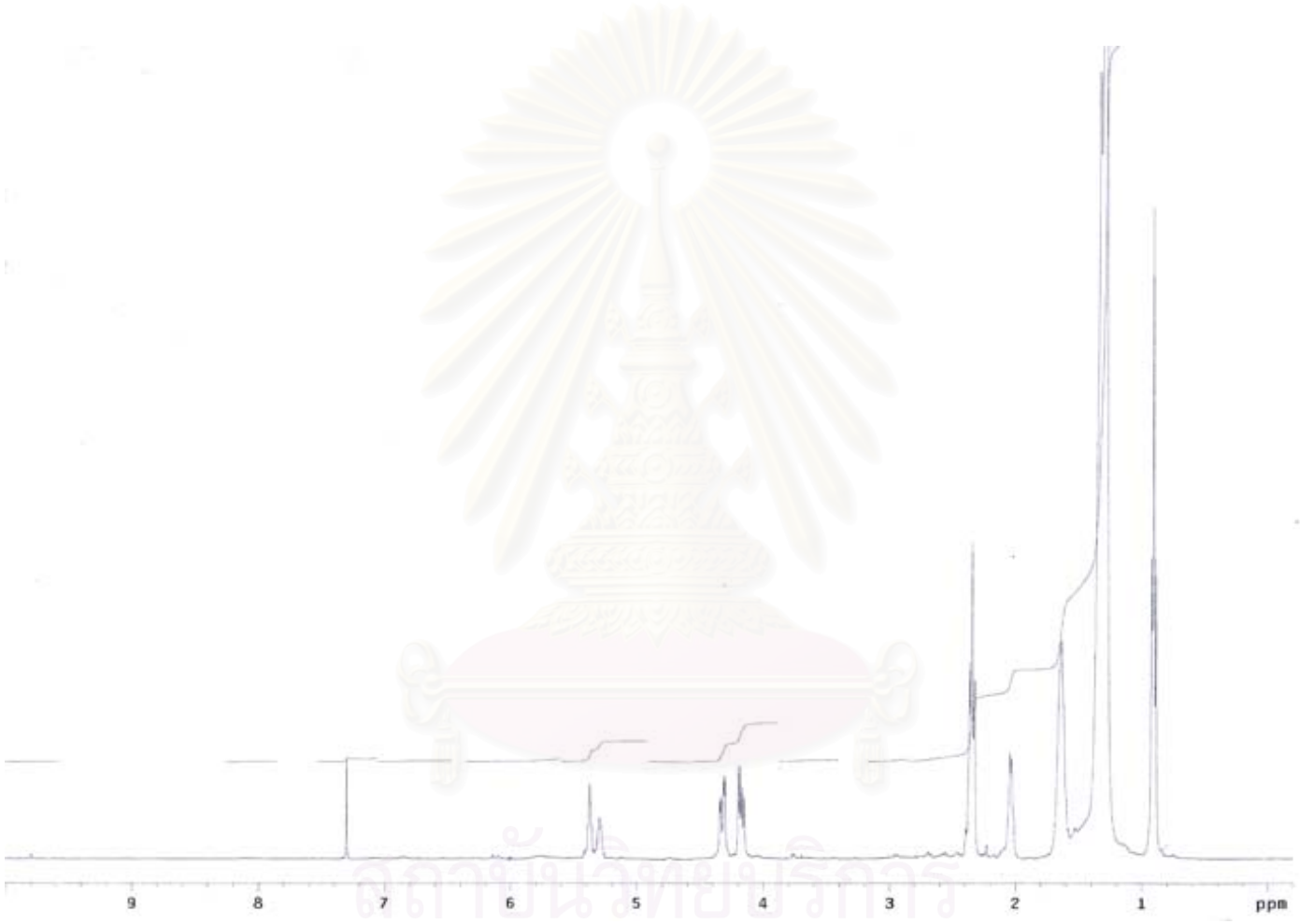


Figure C22 ^{1}H-NMR spectrum of mixture M1

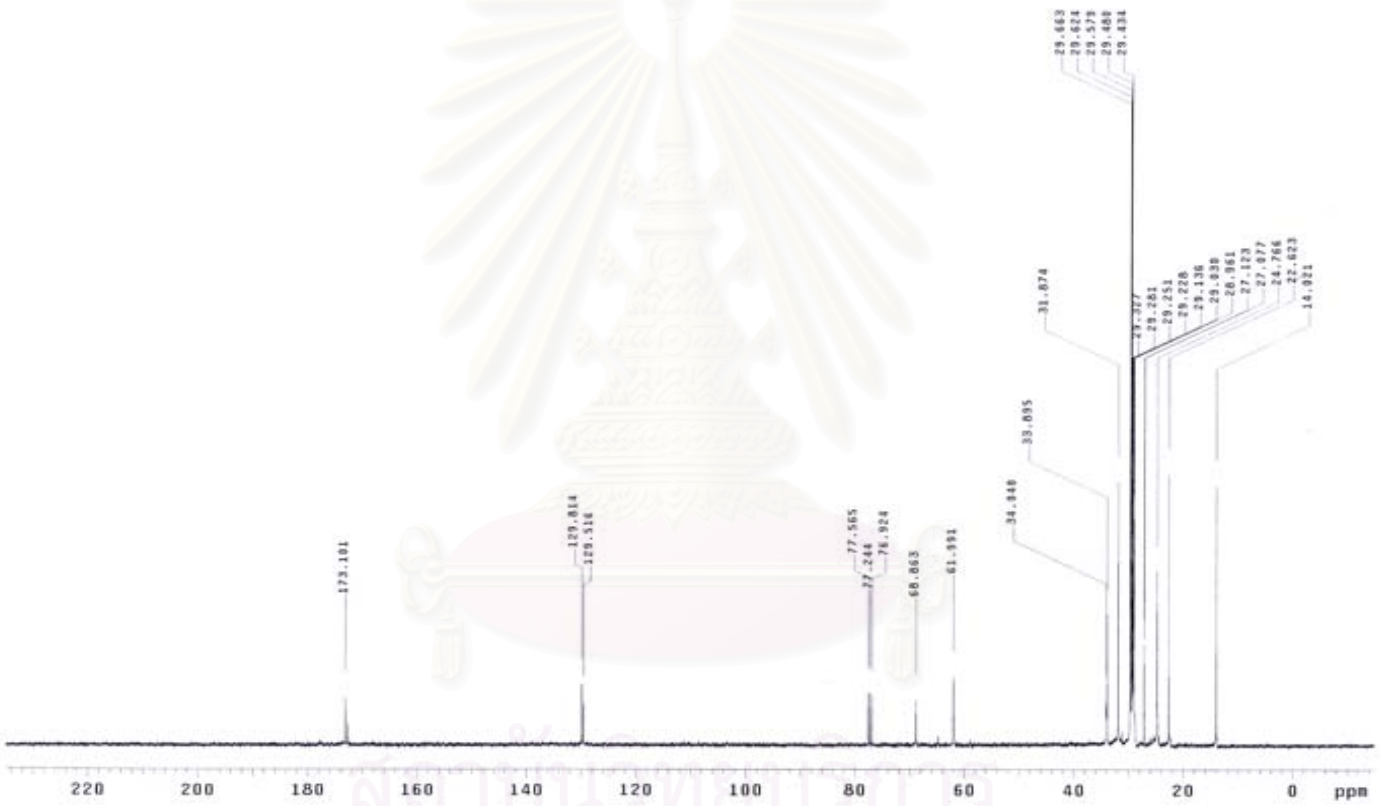


Figure C23 ^{13}C-NMR spectrum of mixture M1

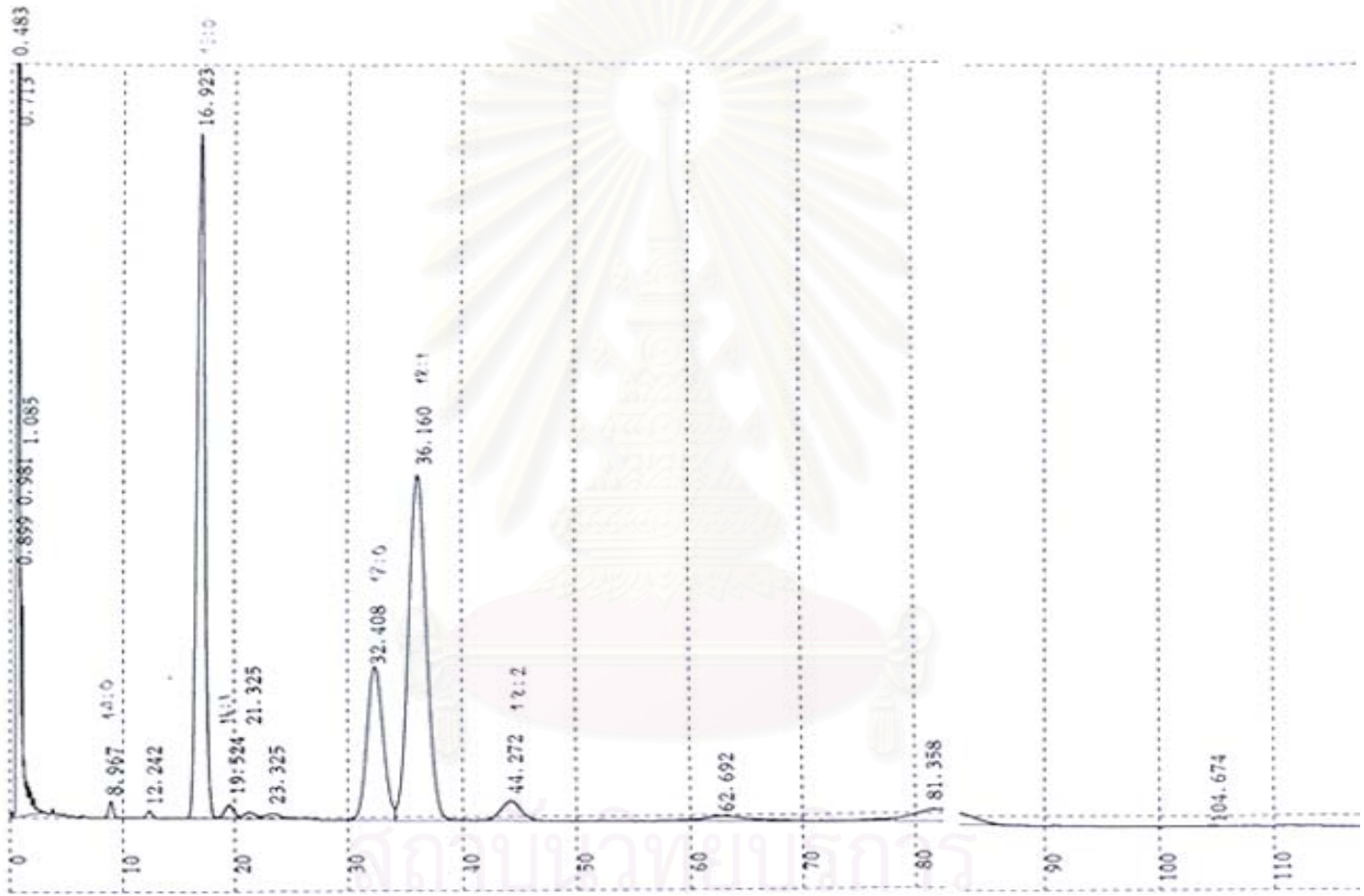


Figure C24 GC spectrum of mixture M1

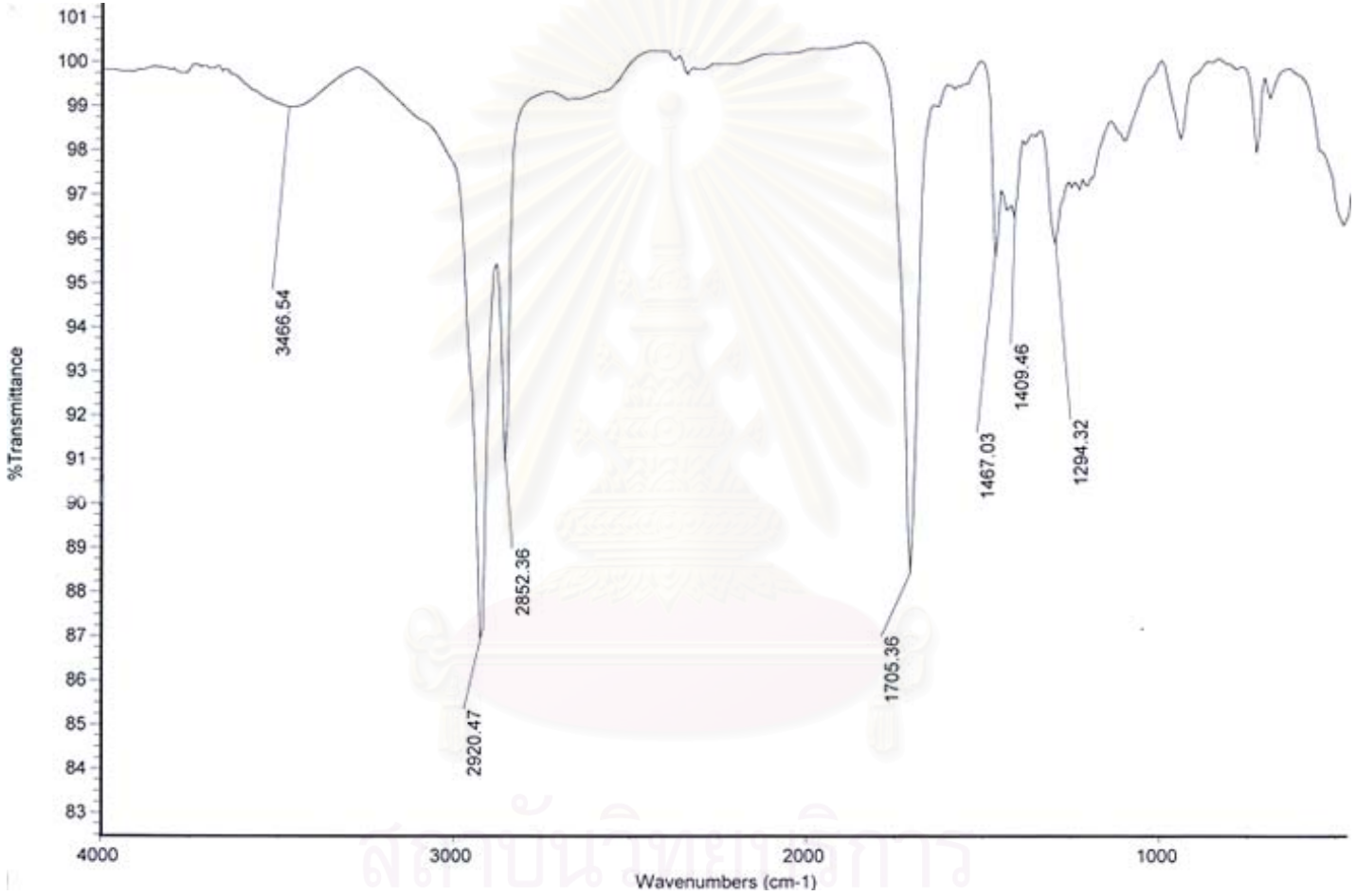


Figure C25 IR spectrum of mixture M2

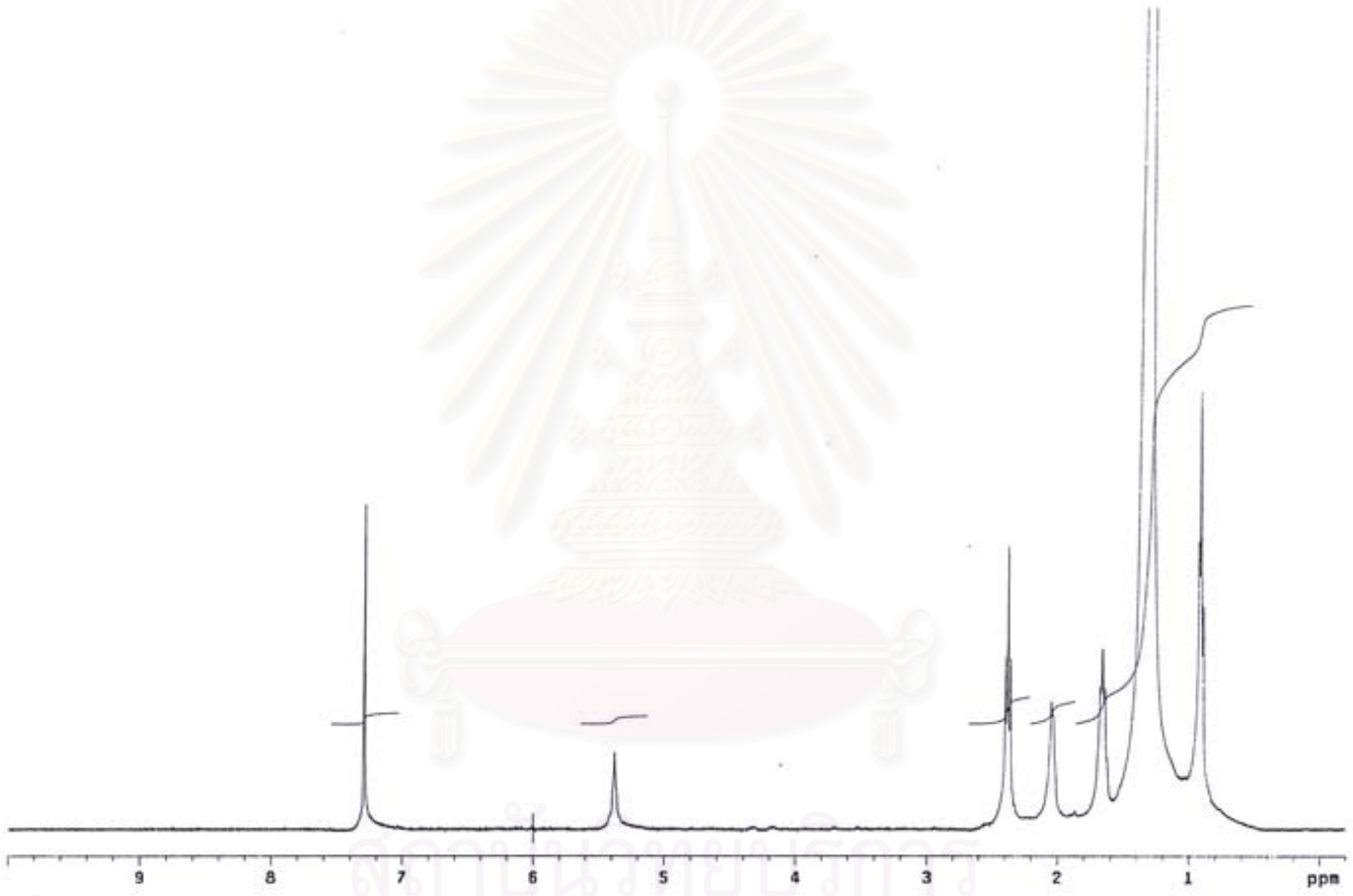


Figure C26 ^{1}H-NMR spectrum of mixture M2

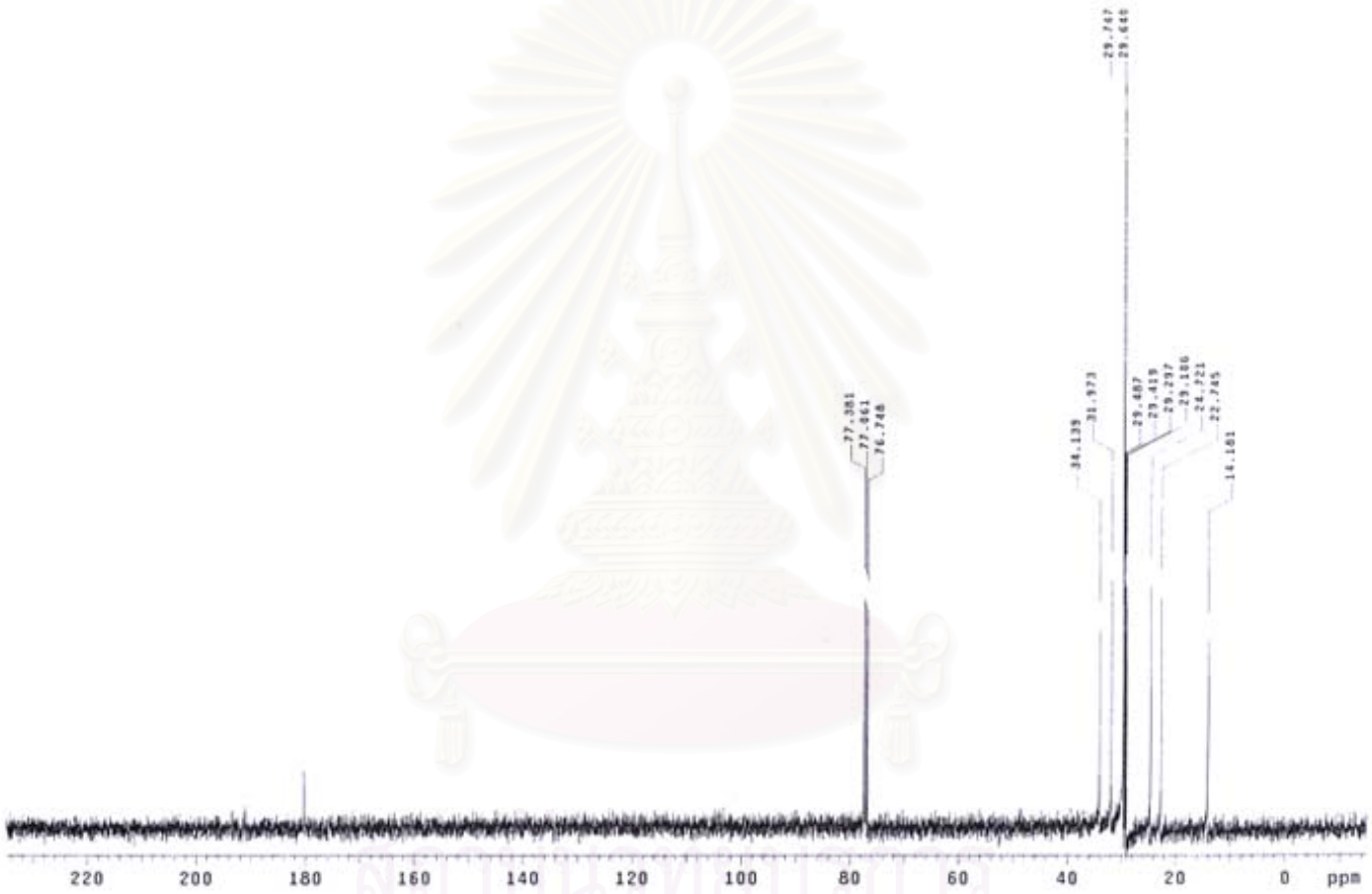


Figure C27 ^{13}C-NMR spectrum of mixture M2

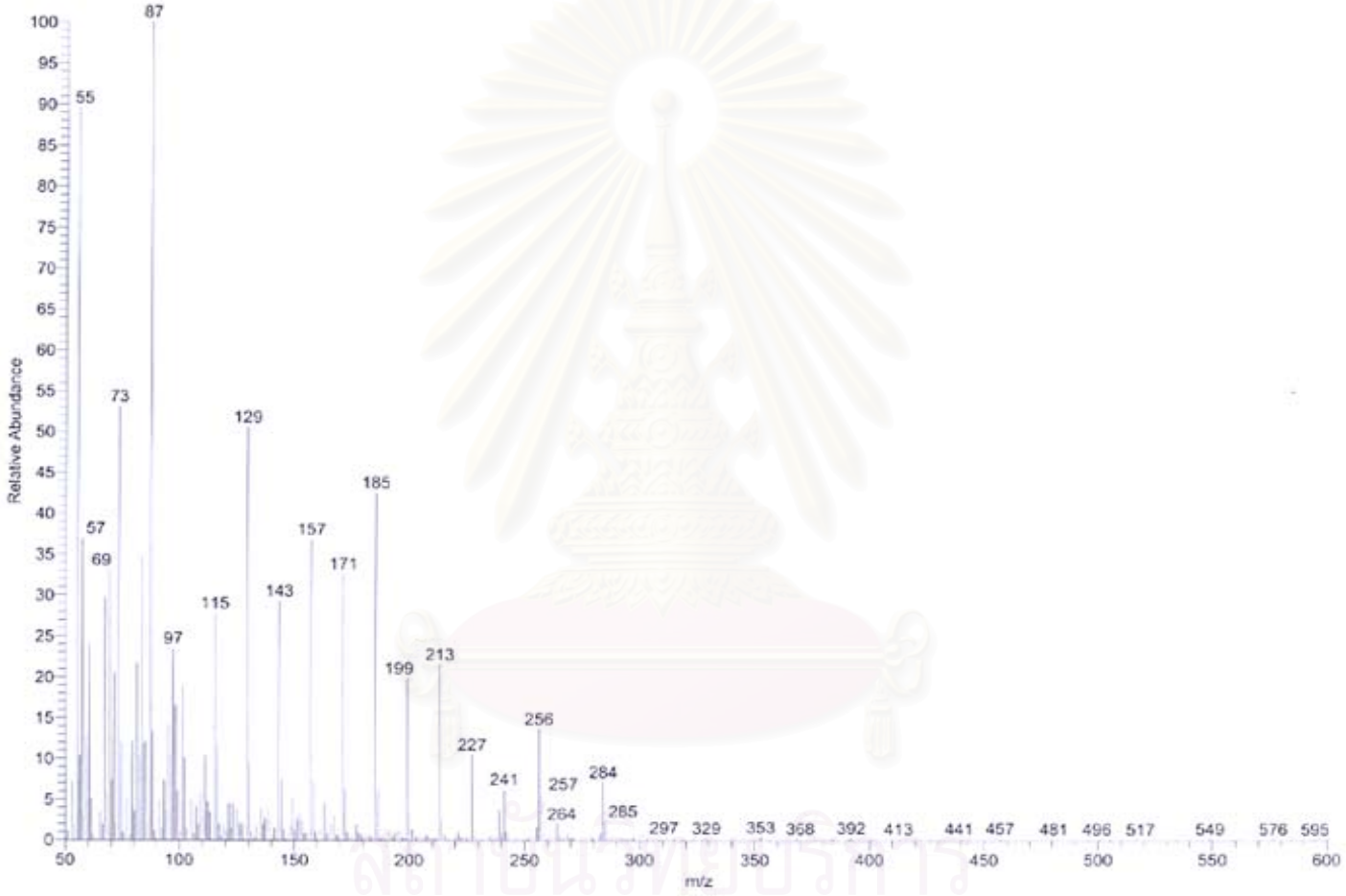


Figure C28 EI-MS spectrum of mixture M2

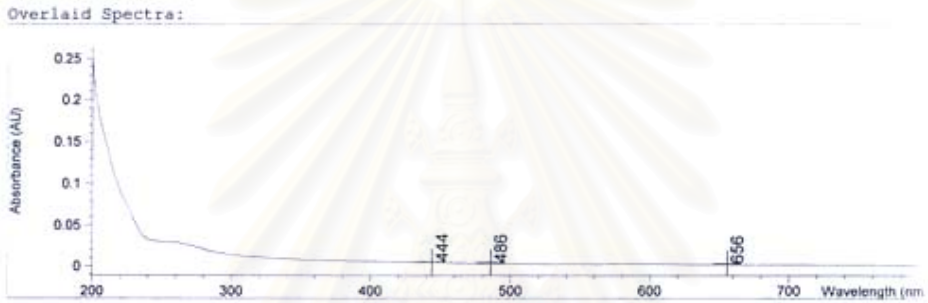


Figure C29 UV spectrum of compound M1

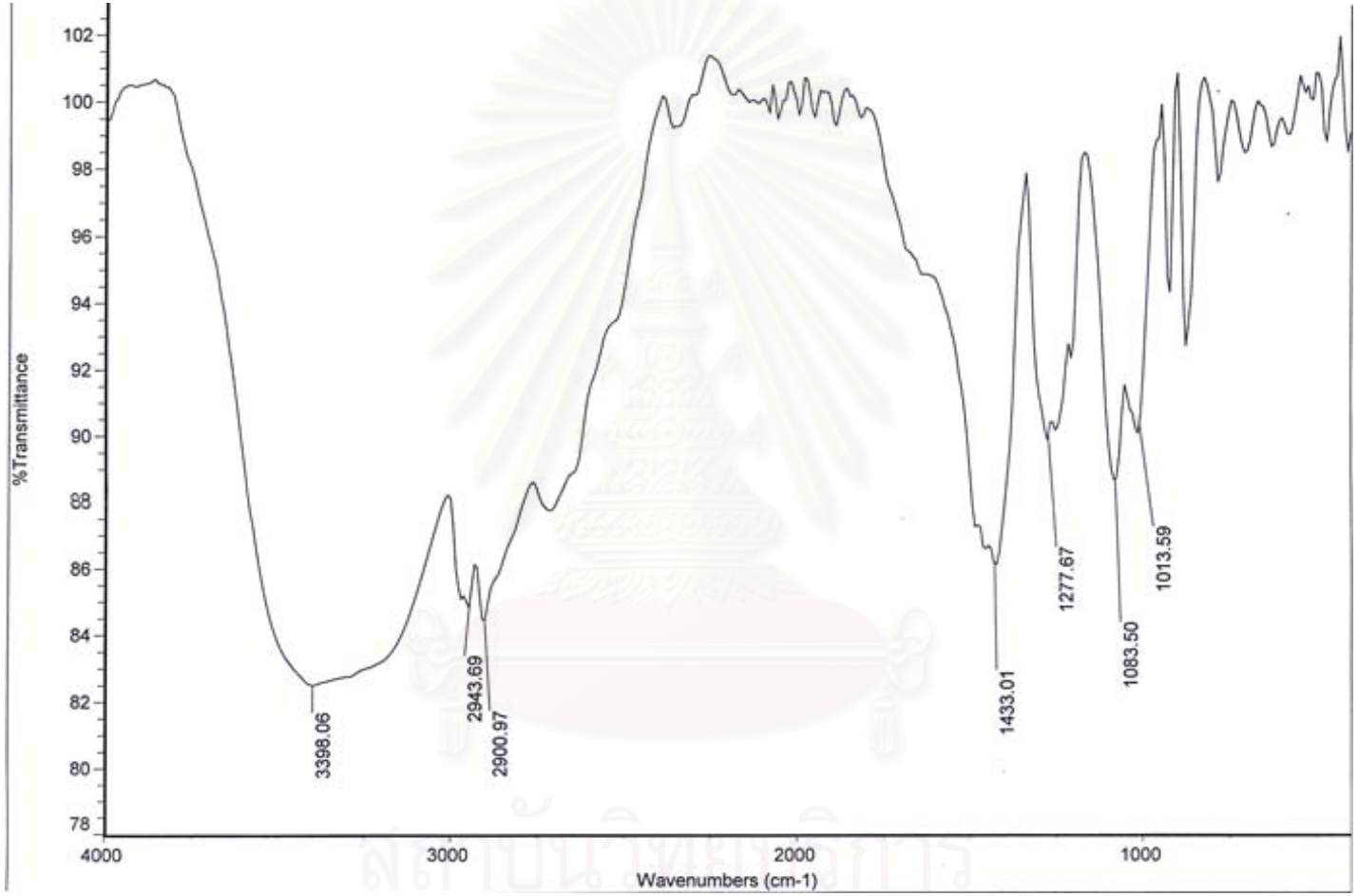


Figure C30 IR spectrum of compound M1

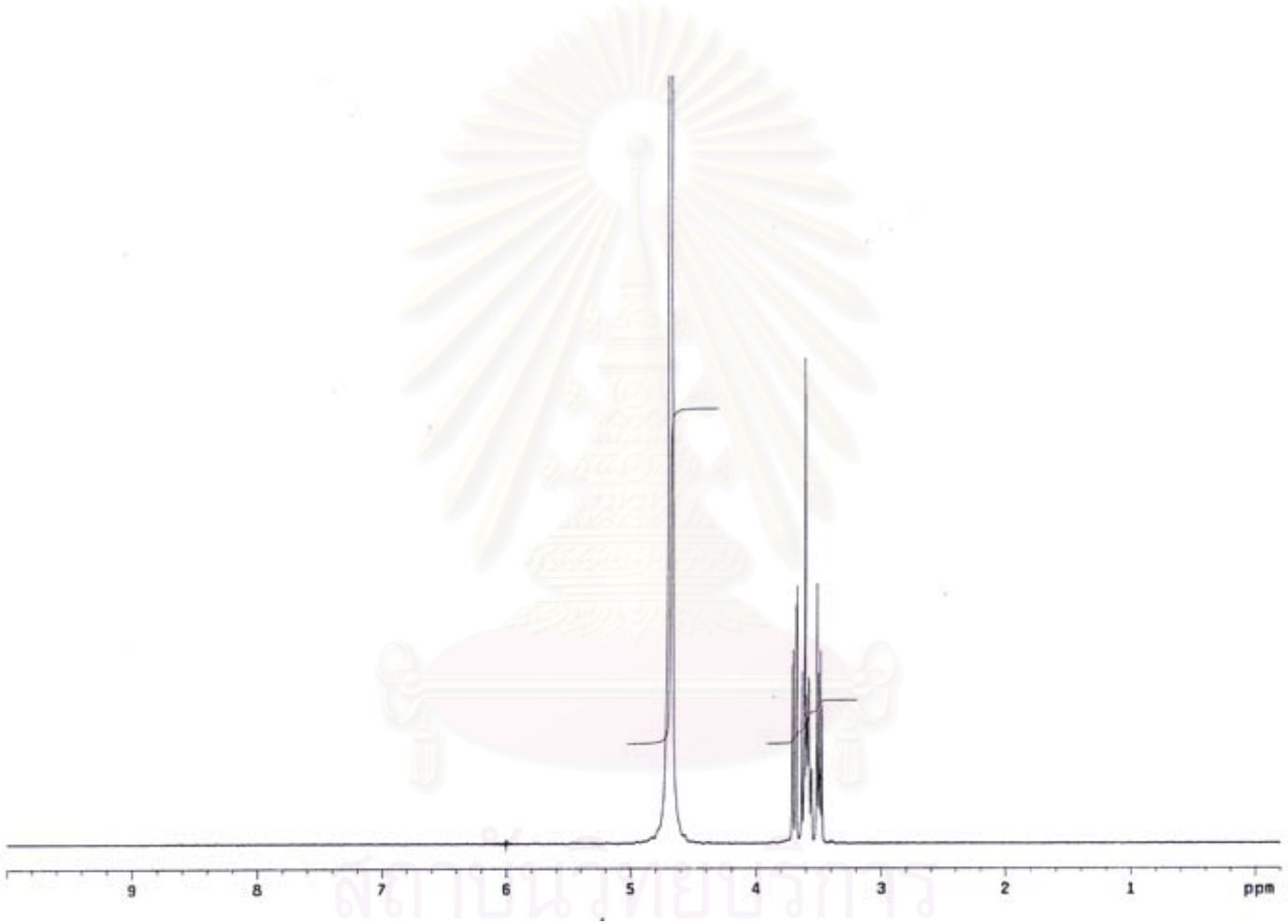


Figure C31 ^{1}H-NMR spectrum of compound M1

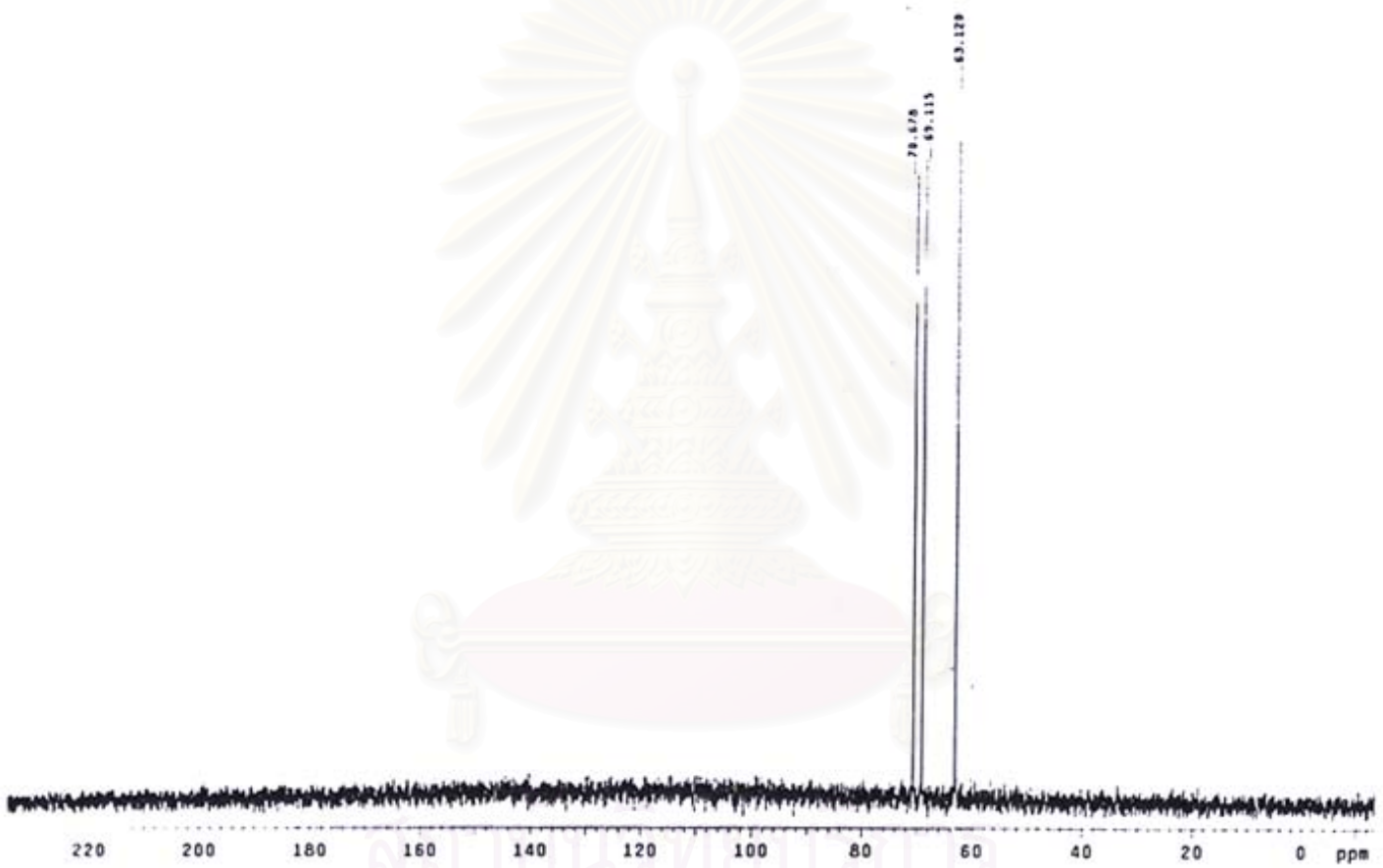


Figure C32 ^{13}C-NMR spectrum of compound M1

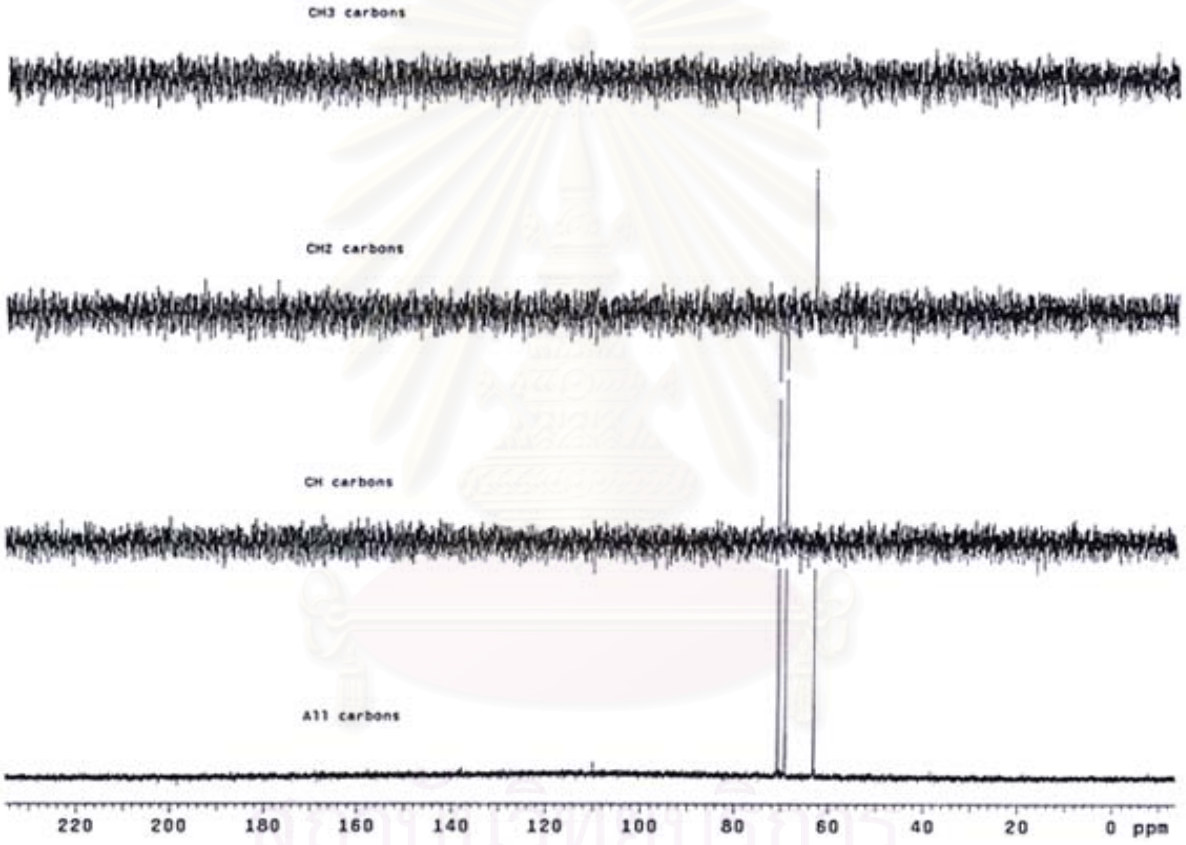


Figure C33 DEPT spectrum of compound M1

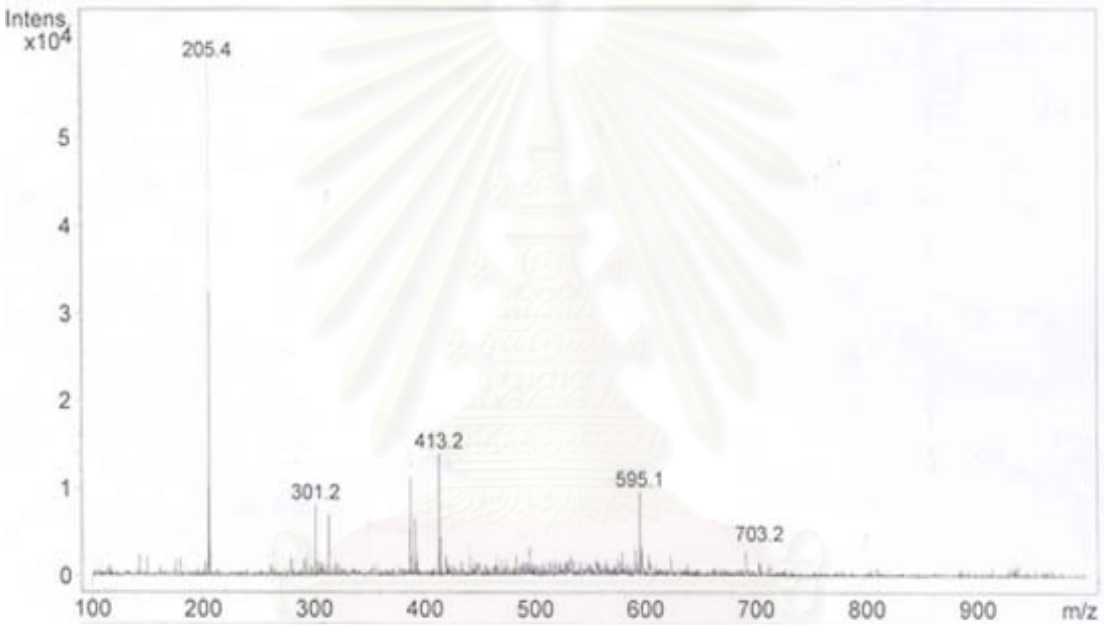


Figure C34 LC-MS spectrum of compound M1

BIOGRAPHY

Miss Saranya Khumwongsa was born on October 29, 1979 in Samutsongkhram province, Thailand. She graduated with Bachelor Degree of Science in Microbiology Department from the Faculty of Science, Chulalongkorn University, Thailand in 1999. She had been studying for a Master Degree of Science in Bioitechnology, Faculty of Science, Chulalongkorn University, Thailand since 2000.