สารยับยั้งจุลชีพที่สร้างโดยราเอนโดไฟต์จากใบและกิ่งเสม็ดขาว *Melaleuca cajuputi*

นายทินกร รสรื่น

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ANTIMICROBIAL SUBSTANCES PRODUCED BY ENDOPHYTIC FUNGI FROM *Melaleuca cajuputi* LEAVES AND TWIGS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ทินกร รสรื่น: สารยับยั้งจุลชีพที่สร้างโดยราเอนโดไฟต์จากใบและกิ่งเสม็ดขาว *Melaleuca cajuputi* (ANTIMICROBIAL SUBSTANCES PRODUCED BY ENDOPHYTIC FUNGI FROM *Melaleuca cajuputi* LEAVES AND TWIGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุรชัย พรภคกุล, 134 หน้า.

วัตถุประสงค์ของงานวิจัยนี้เพื่อแยกหาสารยับยั้งจุลินทรีย์ที่สร้างโดยราเอนโดไฟต์ที่แยกจากใบและกิ่งของต้นเสม็ดขาว *Melaleuca cajuputi* Powell. ที่เก็บจาก 4 จังหวัด คือ กระบี่ นครศรีธรรมราช พัทลุง และตรังแยกราเอนโดไฟต์ได้ 56 ไอโซเลตโดยวิธีการฆ่าเชื้อที่ผิวนอก ทำการทดสอบฤทธิ์การยับยั้งจุลินทรีย์ด้วยวิธี Dual culture agar diffusion technique พบว่าราเอนโดไฟต์ 26 ไอโซเลตที่มีฤทธิ์ยับยั้งจุลินทรีย์ และราเอนโดไฟต์ที่เลี้ยงในอาหาร YES เป็นอาหารเลี้ยงเชื้อที่เหมาะสมในการเมทาบอไลต์ที่มีฤทธิ์ยับยั้งจุลินทรีย์ ราเอนโดไฟต์ไอโซเลต PTYL6 เป็นราเอนโดไฟต์ที่ถูกเลือกมาศึกษาเพราะมีฤทธิ์ยับยั้งจุลินทรีย์ในการยับยั้ง *Escherichia coli* ATCC 25923 และ *Candida albicans* ATCC 10231 โดยมีบริเวณยับยั้ง 20 และ 21 มม.ตามลำดับโดยอาศัยสัณฐานวิทยาของราและการวิเคราะห์ลำดับ DNA บริเวณ ITS1 ITS2 และ 5.8S ของยีน rRNA ราเอนโดไฟต์ PTYL6 จัดเป็น *Fusarium* sp. เพาะเลี้ยงรานี้ในอาหารเลี้ยงเชื้อเหลว Yeast Extract Sucrose Broth (YEB) และทดสอบสารเมแทบอไลต์ที่ยับยั้งจุลินทรีย์ แยกส่วนสกัดหยาบเอธิลอะซิเตดจากเส้นใยได้สารใหม่ 1 ชนิด คือ 6-benzyl-3-isopropyl-1-methylpiperazine-2,5-dione และสารที่ทราบโครงสร้างแล้ว 1 ชนิด คือ ergosterol รวมทั้งได้สาร uracil จากส่วนสกัดหยาบเอธิลอะซิเตดจากน้ำเลี้ยง ทดสอบฤทธิ์การยับยั้งจุลินทรีย์ของสารทั้ง 2 ชนิด ด้วยวิธี the minimum inhibitory concentration method (MIC) โดยใช้ streptomycin และ ketoconazole เป็น positive control พบว่า 6-benzyl-3-isopropyl-1-methylpiperazine-2,5-dione มีฤทธิ์ยับยั้งเชื้อ *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923 และ *Pseudomonus aeruginosa* ATCC 27853 ได้ที่ค่า MIC 3.91, 7.82, 15.63, 31.25 และ 31.25 µg/ml และ ergosterol peroxide มีฤทธิ์ยับยั้งเชื้อ *Bacillus subtilis* ATCC 6633 และ *Escherichia coli* ATCC 25922 ได้ที่ค่า MIC 250 และ 250 µg/ ml ตามลำดับ

สาขาวิชา .....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....ทินกร รสรื่น.....

ปีการศึกษา.....2552.....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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TINNAKORN ROSRUEN: ANTIMICROBIAL SUBSTANCES PRODUCED BY ENDOPHYTIC FUNGI FROM *Melaleuca cajuputi* LEAVES AND TWIGS
THESIS ADVISOR :ASSOC. SURACHAI PORNPAKAKUL, Ph.D., 134 pp.

The purpose of this research was to investigate antimicrobial agents produced by endophytic fungi of *Melaleuca cajuputi*. Leaves and twigs of this plant collected from 4 provinces including Krabi, Nakhonsithammarat, Phatthalung and Trang were isolated using surface sterilization technique to afford 56 isolates of endophytic fungi. All fungal isolates were examined their antimicrobial metabolites using dual culture agar diffusion technique. It was found that there are twenty-six isolates produced antimicrobial metabolites and YES medium was an appropriate medium for production of the antimicrobial metabolites of those endophytic fungi. Isolate PTYL 6 was selected for the study because it produced metabolites against *Escherichia coli* ATCC 25923 and *Candida albicans* ATCC 10231 with inhibition zone of 20 and 21 mm, respectively. On basis of fungal morphology including microscopic and macroscopic features and molecular identification the fungal isolate PTYL6 was identified as *Fusarium* sp. This fungus was cultured in YEB and its antimicrobial metabolites were investigated. The EtOAc extract of mycelia was isolated to give a new compounds, 6-benzyl-3-isopropyl-1-methylpiperazine-2,5,dione and a known compound ergosterol while uracil was obtained from broth EtOAc and MeOH extract. Antimicrobial activities of two compounds were determined by the minimum inhibitory concentration (MIC) method using streptomycin and ketoconazole as positive control. The results showed that 6-benzyl-3-isopropyl-1-methylpiperazine-2,5-dione exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25923 and *Pseudomonus aeruginosa* ATCC 27853 with MIC values of 1.96, 62.5 and 31.25 µg/ml, respectively, and ergosterol exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 with MIC values of 250 and 250 µg/ml, respectively.

Field of study.....Biotechnology.....Student's signature...Tinnakorn Rosruen.....
Academic year.....2009.....Advisor's signature...S. Pornpakakul.....

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LIST OF ABBREVIATIONS

$[\alpha]_D$	optical rotation
ATCC	American Type Culture Collection
bp	base pair
^oC	degree Celsius
cm^{-1}	reciprocated centimeter (unit of wave number)
^{13}C NMR	carbon-13 nuclear magnetic resonance
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	doublet doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
EI	Electron impact
g	gravity (NMR)
h	hour
HMBC	Heteronuclear Multiple Bond Cerrelation
^1H NMR	proton nuclear magnetic resonance
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
IR	infared
J	coupling constant
m	multiplet (NMR)
m	medium (IR)
M^+	molecular ion
MHz	megahertz
mg	milligram
min	minute
mL	milliliter (s)
MS	mass spectroscopy
m/z	mass to change ratio
nm	namometer
No.	number
NOESY	Nuclear Overhauser Enhancement Spectroscopy

ppm	part per million
q	quartet
s	singlet (NMR)
sp.	species
t	tripet (NMR)
TLC	thin layer chromatography
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet
μg	microgram
μL	microliter
δ	Chemical shift
λ_{max}	the wavelength at maximum absorption (UV)
ν_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

Currently, new antimicrobial agents need for human due to problems from resist antibiotic drug action. Drug resistance in bacteria and the tremendous increase in the incidence of fungal infection in the world's population each only underscore our inadequacy to cope with these medical problems. Thus scientists are interested to discover new compounds form the new origin of natural sources product.

Endophytes are microorganisms which live in the intercellular spaces of stems, petioles, roots and leaves of plants which for all or part of their cycle invade the tissue of living plants and cause unapparent and asymptomatic infections entirely with plant tissues (Wilson, 1995). Their relationship with the host plant are symbiotic and probably mutualistic (Lu *et al.*, 2000, Strobel *et al*., 2001).

Nowadays studies found that endophytes are source of new compounds from natural product for useful in pharmaceutical potentials, industry and agricultural and the bioactive compounds produced by endophytes have their have relationships to the production of bioactive compounds of the host plant. Only these few plants have ever been completely studied relative to their endophytic biology (Strobel *et al*., 2001).

The opportunity to find new and interesting endophytic fungi is great and the products that they make are beginning to show some potential for human use.

Endophytes are the greatest number and the most biodiversity microorganisms. Ultimately, biodiversity implies chemical diversity because of the constant new compounds that exists in ecosystems where the evolutionary race to survive. Tropical rainforests are a remarkable example of this type of environment. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds (Strobel *et al.*, 2003).

Melaleuca cajuputi is a Thai medicinal plant in mangrove forest. It has volatiles from leaves and twigs, and various pharmacological activities. Therefore, it is interesting to investigate the bioactive compounds from local plants.

In this research, leaves and twigs of *Melaleuca cajuputi* were used as a source for isolation of endophytic fungi and some fungi were investigated on their secondary metabolites.

Objectives

1. To isolate endophytic fungi from leaves and twigs of *Melaleuca cajuputi*.
2. To investigate antimicrobials produced by the isolated endophytic fungi.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction to endophyte

2.1.1 Definition

Endophytes are microorganisms which live in the intercellular spaces of stems, petioles, roots and leaves of plants which for all or part of their cycle invade the tissue of living plants and cause unapparent and asymptomatic infections entirely with plant tissues (Wilson, 1995). An endophyte is an organism, often a bacterium or fungus that lives within a plant for at least part of its life without causing apparent disease. Endophytes are ubiquitous and have been found in all the species of plants. Studied to date; however, relationships between endophyte and the host plant are not well understood.

Endophytes may be transmitted either vertically (directly from parent to offspring directly) or horizontally (from individual to unrelated individual). Vertically transmitted fungal endophytes are asexual and transmit via fungal hyphens penetrating the host's seeds (e.g. *Neotyphodium*). Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutuality. Conversely, horizontally transmitted fungal endophytes are sexual and transmit via spores that can be spread by wind and insect vectors. Since they spread similar to pathogens, often horizontally transmitted endophytes are closely related to pathogenic fungi (Saikkonen *et al.*, 1999).

Endophytes may benefit host plants by preventing pathogenic organisms from colonizing them. Extensive colonization of the plant tissue by endophytes creates a "barrier effect", where the local endophytes outcompete and prevent pathogenic organisms from taking hold. Endophytes may also utilize chemicals which inhibit the growth of competitors, including pathogenic organisms. Some endophytes could be reliable sources of materials of the agricultural and pharmaceutical such as Taxol,

production of *Taxomyces andreanae*, endophytic fungus of Pacific yew (Stierle, 1993).

2.1.2 Endophytes of plants

Plants are extraordinarily common and widespread source of organic energy, thus likely that a huge array of fungus interact with plants. A diverse suite of fungus can be isolated from apparently healthy tissue, and many have never been associated with disease. Others may cause disease when environmental conditions change. Yet others appear to be associated with initial degradation of the leaf following senescence. Endophytic fungi and host plant relationship are believed to be complex and probably vary from host to microbe. Relationship between endophytic fungus and host plant are hereditary one, and that the fungus passes from one generation to another through the seed. The fungus enters the seedling from the seed and spreads throughout the plant, entering new tissues as they arise (Owen and Hundlry, 2004).

2.2 Endophytic fungi

2.2.1 Definition

The fungi are found inside the host plant for most of their life cycle and capable of symptomless in healthy plant tissues, called “endophytic fungi” (Pereira *et al*., 1993; Stone *et al*., 2004) (Figure 2.1). These fungi are associated with roots, stems, leaves, flowers and seeds (Rodriguez *et al.,* 2005) and also a mutuality relationship with host plant (Saikkonen *et al*., 1998). However, endophytic fungi can be a pathogens and saprophytes (Fisher and Petrini, 1992). All kind of plants include trees, grass, algae and herbs are sources of the endophytic fungi (Huang *et al*., 2001) and isolated a lot of endophyte species from a single plant (Tan and Zou, 2001).

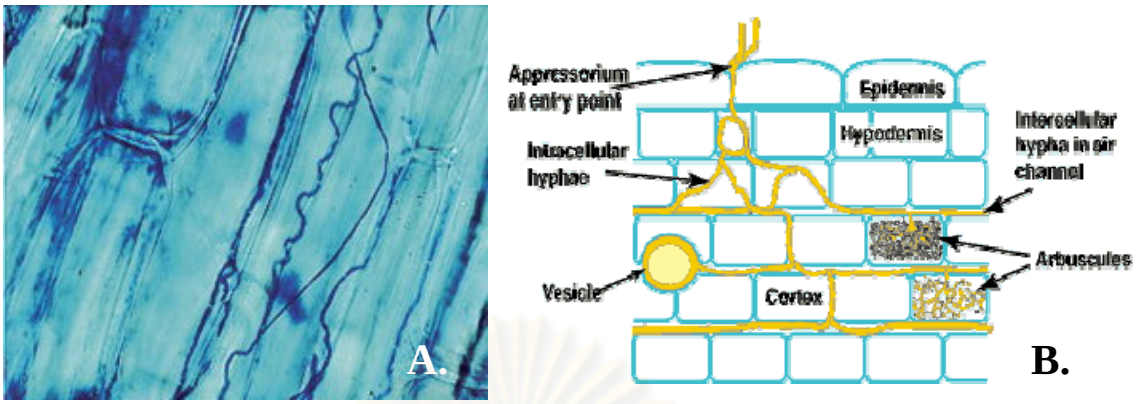


Figure 2.1 **A.** Microscopic picture of tall fescue leaf sheath cells with endophyte mycelium growing between cells. (http://www.noble.org/Index.html) **B.** Endophyte in intercellular space (http://www.uark.edu/depts/agronomy/west/3113/lec.endophyte.html)

2.2.2 Bioactive source

Endophytic fungi, in many ways, have been inquired to novel secondary metabolites after they were found in 1904 by Darnel (Tan and Zou, 2001). Some of endophytic fungi were prolific the bioactive compounds which a feasibility source of novel metabolites for medicinal and agricultural industrial (Strobel *et al*., 2004). This is an attractive reason of endophytic fungi studying in the past few decades (Faeth, 2002).

Tan and Zou (2001) inspected groups of the secondary metabolites from endophytic fungi as alkaloids (amines-amide, indole derivatives and pyrrolizidines), steroids, terpenoids (sesquiterpens and diterpenes), isocoumarin derivatives, quinines, flavonoids, phenylpropanoids, peptides, phenol-phenolic acids and aliphatic compound. More recently, they reported the phytohormones from endophytic fungus, *Collectotrichum gloeosporioides*, which induced the growth of host callus.

2.3 Community and ecology

In most cases their relationship with the host plant is symbiotic and probably mutalistic (Owen and Hundley, 2004). Ecological observations, coupled with phylogenetic and molecular analyses of foliar endophytic fungi, have provided glimpses of the degree of specialization between endophytic fungi and host plant interaction (Saikkonen *et al.*, 2004).

However, endophytic fungi can be a pathogens and saprophytes (Fisher and Petrini, 1992). All kind of plants include trees, grass, algae and herbs can be found the endophytic fungi (Huang *et al*., 2001) and isolated a lot of endophyte species from a single plant (Tan and Zou, 2001).

Group of Endophyte in general

2.3.1. Clavicipitales (Endobiotics & Epibiotics) Non-Clavicipitaceous Endophytes

Included in the Clavicipitales (Ascomycota) are several fungi that form associations with grasses. The associations include a pathogenic epibiotic (outside living tissue) fungus (e.g. *Claviceps*, *Myriogenospora*), a partly endobiotic fungus with surface fruiting structures (some *Balansia* spp), and an entirely endobiotic fungus with unknown sexual stages (the agronomically important *Neotyphodium*). The fungi have the following characteristics:

- annual infection
- overian parasite
- fruiting body is entirely fungal
- found on many grasses and a few sedges
- seed is replaced by a fungal sclerotium.

A certain degree of specificity was in the establishment of endophytes in plant population. Thus, species lists obtained for a given host specific taxa. In the last few years, community ordination analyses have shown that endophyte community ordination analyses have shown that endophyte communities are usually specific at the host species level. (Bacon and White, 2000)

2.3.2. Neotyphodium (Grass Endophytes) Clavicipitaceous Grass Endophytes

-systemic infection of leaves, stems and seeds

-perennial

-no sexual stage in Neotyphodium

-plants colonised by either fungus flower successfully

Most investigations on host specific establishment of grass-endophyte symbioses rely on artificial inoculation experiment carried out by introducing

mycelium in wounds inflicted to the host tissue. Those endophyte communities are usually specific at the host species level.

In all cases, the fungus contains toxic alkaloids. Thus consumption of the plant poisons the herbivore. Toxicity is most common where grass is short and growing rapidly (for example after rain in spring). The signs of consumption include staggering and unsteadiness. Once the toxin has passed through the system, the individual recovers, unless further toxin is ingested. (Bacon and White, 2000; Bischoff and White, 2005).

Endophyte is a naturally occurring fungus whose complete life cycle occurs within grasses such as perennial ryegrass and tall fescue. The endophyte fungus grows between the cells of the host plant, drawing nutrients from it but in return conferring resistance to insect pests, drought tolerance, and protection from overgrazing – a relationship known as symbiosis. The endophyte life cycle showed in Figure 2.2.

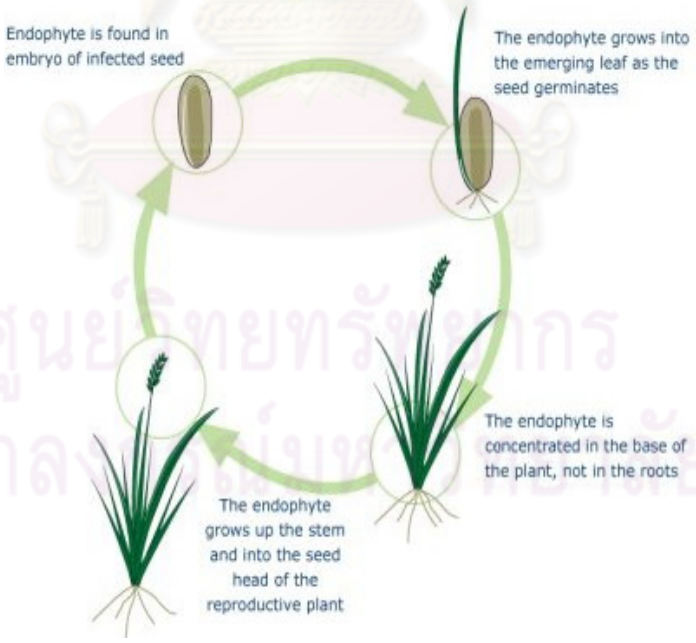


Figure 2.2 The endophyte life cycle
(http://www.grasslanz.com/content/endophytes.html)

Some of fungi may live as saprotrophs which obtain the energy from nonliving organic materials, dead plants or animals, their parts, or their wastes (Moore-Landecker, 1982; Garraway and Evans, 1984). When the fungi are associated with other organisms, in which called “symbiosis” and the partners are “symbionts” (Moore-Landecker, 1982). Sometimes symbiosis has been comprised to mutualistic, both partners benefiting (Carlile and Watkinson, 1994) or parasitic that one partner is absorbed the nutrients from host organisms (Garraway and Evans, 1984).

The fungi diversity is spreading in several habitats such as terrestrial, aquatic, animals and plants (Moore and Frazer, 2002). The plant habitats of fungi include living vascular plants, dead vascular plants and nonvascular plants (Hawksworth and Mueller, 2005).

2.4 Biodiversity of endophyte

Endophytes represent a huge diversity of microbial adaptations that have developed in special and sequestered environments, and their diversity make them an exciting fled of study in the search for bioactive compounds and novel sources of effective new drugs (Owen and Hundley, 2004). Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land’s surface, yet, they harbor over 60% of the world’s terrestrial biodiversity (Mittermeier *et al.*, 1999). As such, one would expect that areas having high plant endemism also possess specific endophytes that may have evolved with the endemic plant species.

Host specialization in highly diverse tropical forests may be limited by the low local abundance of suitable hosts. Because mangrove plant species are a valuable source of useful metabolites, their endophytes have gained more importance. For example research about endophyte in mangrove forest. Suryanarayanan and Kumaresan in 2000 isolated endophytic from ten plants of each host in an estuarine mangrove forest (south of Chennai, in Tamil Nadu, India). Four halophytes belonging to three dicotyledon families, *Acanthaceae*, *Aizoaceae* and *Chenopodiaceae* were screened for the presence of fungal endophytes 36 species of fungi, in groups *Camarosporium, Colletotrichum*, *Phomopsis*, *Phyllosticta* and *Sporormiella.* In 2001, Kumaresan and Suryanarayanan also studied endophytic fungi in mangrove

community from seven dominant mangrove species of an estuarine mangrove forest in south India Mitosporic fungi, ascomycetes and sterile mycelia were obtained. The endophyte assemblage of each mangrove species was dominated by different endophyte species. Then, mangrove plant species are a valuable source of secondary metabolites from their endophytes that interest.

2.5 Study of bioactive compounds of endophytic fungi

Many researches of fungal metabolites have been investigated to cytotoxic, mutagenic, teratogenic, immunosuppressive, enzyme inhibitory, etc. Between 1993 and 2001, 1,500 compounds were found and more than half of these had antibacterial, antifungal or antitumor activity (Keller *et al*., 2005).

Endophytic fungi, in many ways, have been inquired to novel secondary metabolites after they were found in 1904 by Darnel (Tan and Zou, 2001). Some of endophytic fungi was prolific the bioactive compounds and which a feasibility source of novel metabolites for medicinal and agricultural industrial (Strobel *et al*., 2004).

2.5.1 Antibiotics agents

Compounds from endophytic metabolite have been observed to inhibit a wide variety of harmful disease-causing agents including but not limited to, phytopathogens, bacteria, fungi, viruses and protozoans that affect human and animal (Strobel and Daisy, 2003).

2.5.1.1 Antimicrobial agent

Cytosporone E was isolated from the broth of the endophytic fungi *Cytospora* sp. and *Diaporthe* sp. (was synthesized as a racemic mixture). The key step in the synthesis is the Meyers *ortho*-alkylation of a chiral aromatic oxazoline. Preliminary antibiotic activity shows antibiosis against Gram-positive bacteria (Hall *et al*., 2004).

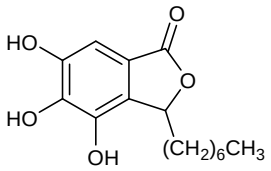


Figure 2.3 Structure of Cytosporone E

Two new fusicoccane diterpencs, named Pcriconicins A and B, with antibacterial activities were isolated by bioassay-guided fractionation from an endophytic fungus *Periconia* sp., collected from small branches of *Taxus cuspidata*. The structures of the new compounds were deter-mined by combined spectroscopic methods (Geo *et al*., 2008).

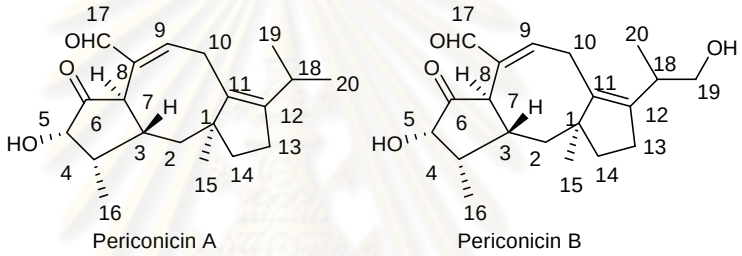


Figure 2.4 Structures of Periconicin A and Periconicin B

2.5.1.2 Antifungal

Antifungal metabolites extracted from *Colletotrichum gloeosporioides*, an endophytic fungus in *Cryptocarya mandioccana* Nees**.** The strong antifungal activity against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum* of the compounds from endophytic fungi *C. gloeosporioides* in *C. mandioccana* could protect the host by producing metabolites, which may be toxic or even lethal to phytopathogens, and highlights the potential of endophytic fungi in producing bioactive metabolites (Maysa *et al.*, 2006).

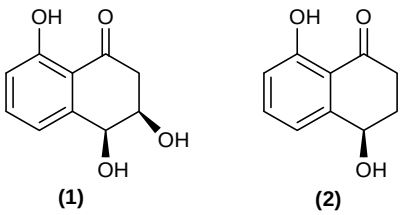


Figure 2.5 Structure of affording cis-4-hydroxy-6-deoxyscytalone (1) and (4R)-4, 8-dihydroxy-α-tetralone(2)

Glucoside derivatives, xylarosides A (1) and B (2), were isolated from the broth extract of the endophytic fungus *Xylaria* sp. PSU-D14 along with two known compounds, sordaricin (3) and 2,3-dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one (4). Compound 3 (Sordaricin) exhibited moderate antifungal activity against *Candida albicans* ATCC90028 with a MIC value of 32 µg/ml (Pongcharoen *et al*., 2008).

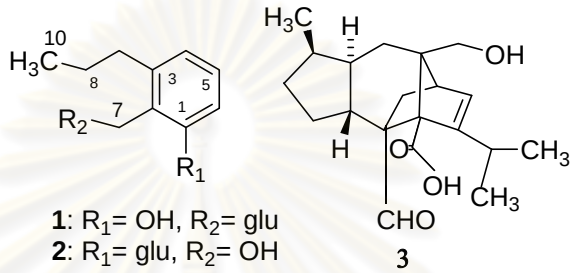


Figure 2.6 Structure of xylarosides A (1) and B (2) and sordaricin(3)

2.5.1.3 Antiviral

Two novel compounds in group tridepside isomer, called "Cytonic acids A and B" have been isolated from endophytic fungus *Cytonaema* sp.

The novel tridepside isomer of Human cytomegalovirus or hCMV is a ubiquitous opportunistic pathogen protease inhibitors using high throughput screening (Guo *et al.*, 2000).

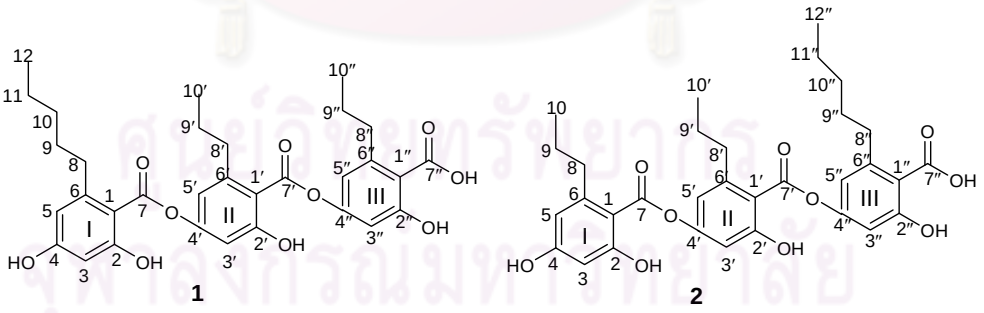


Figure 2.7 Structure of acids A (1) and B (2)

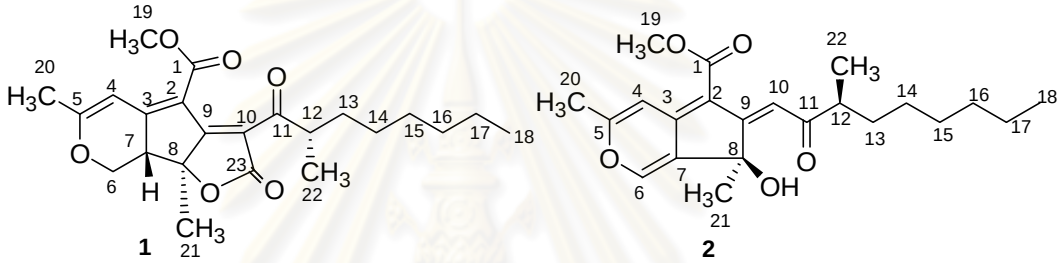
2.5.1.4 Volatile antibiotic

The phytopathogen, *Crinipellis perniciosa*, causal agent of Witche's Broom disease was inhibited by endophytic fungal community of cacao (Rubini *et al*., 2005). Volatile antimicrobials from endophytic fungus, *Muscodor albus* which isolated from

small limbs of *Cinnamomum zeylanicum* or common name called “cinnamon tree” (Strobel *et al*., 2001).

2.5.2 Anticancer and antitumor agents

The common endophytic fungi found in the world’s yews are *Psetalotiopsis* spp. such as *P. microspora* that produced paclitaxel, an anticancer agent (Strobel *et al*., 1996). Sequoiatones A and B, antitumor metabolites, were isolated from the fungus *Aspergillus parasiticus*, a redwood endophyte (Stierle and Bugni, 1999).



1 **2**

Figure 2.8 Structures of Sequoiatones A (**1**) and B (**2**)

Podophyllotoxin, a precursor to anticancer drugs, was produced by endophytic fungus isolated from *Podophyllum peltatum* (Eyberger *et al.*, 2006).

Figure 2.9 Structure of Podophyllotoxin from endophytic fungus *Podophyllum peltatum*

2.5.3 Antidiabetic agents

An metabolite, called 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. 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 (Zhang *et al*., 1999).

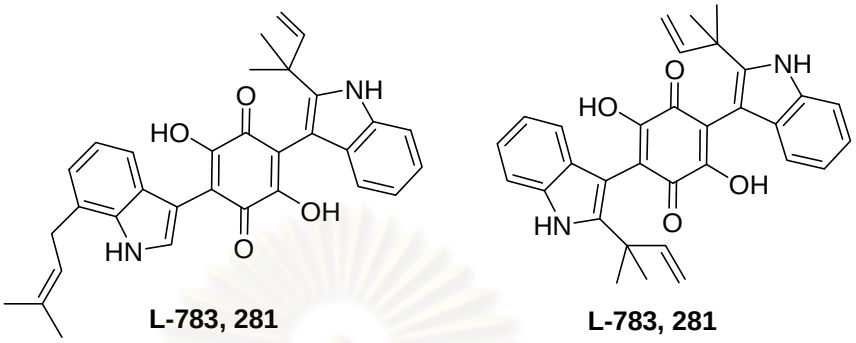


Figure 2.10 Structures of L-783,281 (1) and L-767,827 (2)

2.5.4 Antimalarial agents

Pullularin A-D, four novel cyclohexadepsipeptides, were isolated from the endophytic fungus *Pullularia* sp., isolated from a leaf of *Culophyllum* sp. (Guttiferae) collected in Narathiwat Province, Thailand.

Pullularin A exhibited activities against the malarial parasite Plasmodium falciparum K1 (Isaka *et al*., 2007).

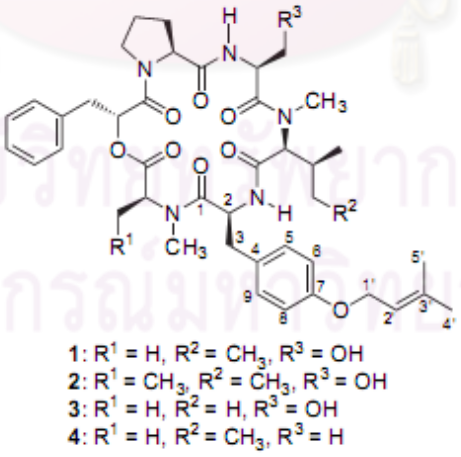


Figure 2.11 Structures of Pullularin A-D (1-4)

2.5.5 Antioxidants agents

The unnamed endophytic fungus from *Podophyllum hexandrum* was possessed the metabolite podophyllotoxin that fined application as antioxidant (Puri, *et al*., 2006). Secondary metabolite, Graphislactone A, from cultures of endophyte, *Cephalosporium* sp. IFB-E001 which isolated from *Trachelospermum jasminoides* was assayed for *in vitro* antioxidant activity and free radical-scavenging agents. The activity of Graphislactone A against DPPH radicals at IC_{50} for DPPH radicals was 2.9 μg/ ml. (Song *et al.*, 2005).

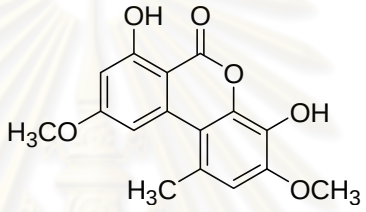


Figure 2.12 Structure of Graphislactone A

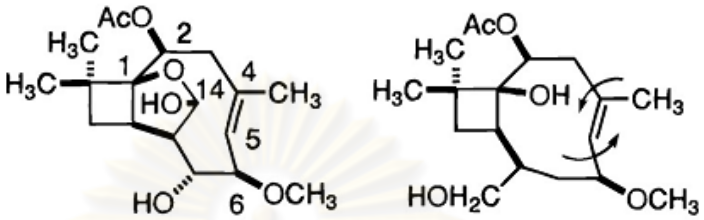
2.5.6 Biological control

Fungal endophytes isolated from healthy *Theobroma cacao* tissues were screened in vitro for antagonism against major pathogens of cacao. They showed in vitro antagonism against *Moniliophthora roreri* (frosty pod rot), *Phytophthora palmivora* (black pod rot) *and Moniliophthora perniciosa* (witches broom).

The most common antagonistic mechanism was simple competition for substrate. They showed clear antibiosis against *M. roreri, P. palmivora*, and *M. perniciosa*. One isolate of Trichoderma was observed to be parasitic on *M. roreri*. Endophyte species that were common in the host plants under natural conditions often are good colonizers and grow fast in vitro whereas antibiosis producers usually appear to be relatively rare in nature, tend to grow slowly in vitro, and often are not good colonizers (Mejia *et al.*, 2008).

2.5.7 Immunosuppressive agents

For example Pestalotiopsins A and B in culture broth of *Pestalotiopsis* sp., isolated as an endophytic fungus from *Taxus brevifolia* (or common name Yew tree) produced immunosuppressive (Pulici *et al.*, 1996).



1 **2**

Figure 2.13 Structures of Pestalotiopsins A (1) and B (2)

Pestalotiopsis leucothes, an endophytic fungus isolated from *Tripterygium wilfordii*, a new source of immunomodulatory compounds for the treatment of human immune mediated diseases. The immunomodulatory effects of three compounds designated as BS, GS and YS were obtained from endophytivc fungi (Kumar *et al*., 2005).

2.5.8 Insecticide agents

The bioinsecticidal activities have been found in endophytic fungi such as an endophyte*, Muscodor vitigenus* from *Paullinia paullinioides*. This fungus was presented naphthalene, an insect repellent (Daisy *et al*., 2002). Another endophytic fungus, *Phomopsis phaseoli* isolated from leaf of the tropical tree produced 3-hydroxypropionic acid that was nematicide metabolites (Schwarz, 2004).

2.5.9 Endophytes products as other bioactive agents

The fungal neurotoxin, lolitrem B, is an indole diterpene produced by the endophyte *Neotyphodium lolii*, which was inhibited *hSlo*–large conductance calcium activated potassium channels. This is a new tool for studying the functional properties of human BK channel expressed in human embryonic kidney cells (Dalziel *et al.*, 2005). Cytochalasin F, an inhibitor of photosynthesis, was extracted from *Geniculosporium* sp. that isolated *Teucrium scorodonia* (König *et al*., 1999).

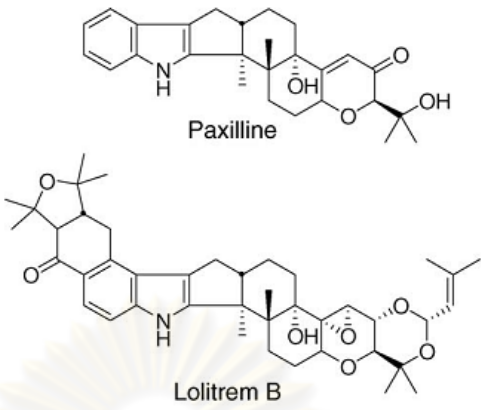


Figure 2.14 Structures of Paxilline and Lolitrem B

2.6 Plant sample (*Melaleuca cajuputi)*

2.6.1 Plants Classification (Species identify)

Kingdom: *Plantae* – Plants

Subkingdom: *Tracheobionta* – Vascular plants

Super division**:** *Spermatophyta* – Seed plants

Division: *Magnoliophyta* – Flowering plants

Class**:** *Magnoliopsida* – Dicotyledons

Subclass: *Rosidae*

Order**:** *Myrtales*

Family**:** *Myrtaceae* – Myrtle family

Genus**:** *Melaleuca* L. – melaleuca

Species**:** *Melaleuca cajuputi* Powell – cajeput

Synonyms': *M. lecadendron* Linn., *M. leucadendra* Linn. Var.*minor* Duthie.

Common name: Cajuput Oil Tree, Paper Bark Tree, Swamp Tea, Milk Wood

English: Cajuput, White Tea Tree, White Wood

Indonesia: Kayu putih tree

Malaya: Kelar

Thailand: Samed, Samedkaw, Med

Vietnam: Kayu putih

Distribution: Tropical Asia, Australia, Central America and Florida.

2.6.2 Description

An evergreen tree or shrub is very common in coastal areas. The woody tree, with a narrow crown, can reach 25 m in height and 40 cm in diameter. The shrub straggles along the ground.

Bark: Thick, soft, shaggy, white, like paper.

Leaves: 4-8 cm long, 1-2 cm wide, oil-containing.

Flowers: White, fragrant with a fluff of long stamens, copious nectar flow.

Flowering: Two periods a year from January to April and from July to August.

Honey: Amber colored with a tendency for further darkening, with a strong and distinctive smell.

Wood: Hard and fairly heavy.

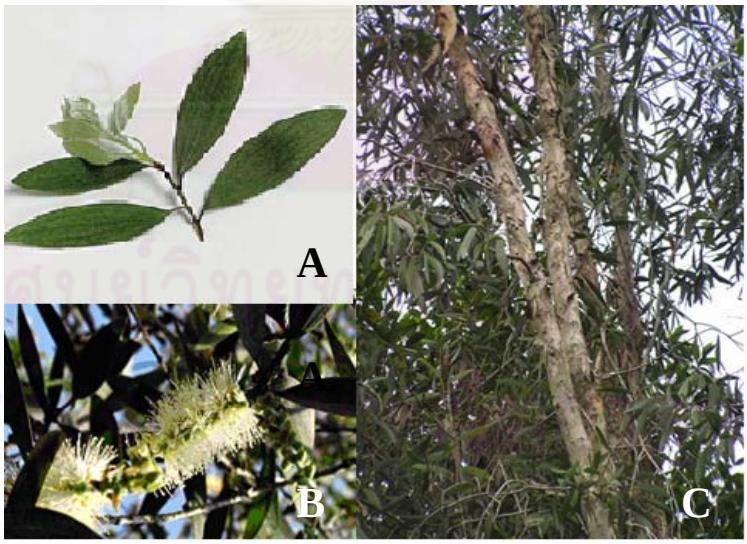


Figure 2.15 Plant sample *Melaleuca cajuputi*

A. Leaves and twigs of cajuput tree
B. Flower of cajuput tree (http:// schoolnet.nectec.or.th)
C. Trunk and branch of cajuput tree

2.6.3 Uses

Shrub: oil extract from the leaves is used in medicine, as an insect repellent, and in soap manufacturing.

Tree trunks: used in construction and for fuel wood.

Bark: used as smoker fuel. Also used as packing material.

Honey: tens of tones of honey are harvested from Apis dorsata colonies by the rafter beekeeping technique. Up to 100 tones are harvested by professional Apis mellifera beekeepers each year.

2.6.4 Medicinal used

Cajuput Essential oil is extracted from the twigs and leaves of Cajuput tree. The scientific name of Cajuput tree is *Melaleuca Cajuputi*. Chief components of Cajuput Oil are Caryophyllene, Alpha Pinene, Beta Pinene, Limonene, Alpha Terpinene, Alpha Terpineol, Gamma Terpinene, Terpinolene, Terpineol, Cineole, Cymene, Linalool, Myrcene etc. The process of extraction of Cajuput Oil involves steam distillation of its twigs and leaves, which is quite similar to that of extraction of Eucalyptus Oil.

1). Antiseptic and Bactericide These are perhaps the most priced properties of Cajuput Oil. It is very efficient in fighting infections from bacteria, virus and fungi, such as tetanus (bacteria), influenza (virus) and infectious diseases like cholera, typhoid. It can be externally applied on cuts and wounds from rusty iron to protect against tetanus, until vaccine is taken.

2). Insecticide and Vermifuge: Very efficient in killing and driving away insects. Its insecticidal properties are so strong that its diluted solution can be sprayed or it can be vaporized with the help of a vaporizer to drive away mosquitoes, ants etc. (of course, not the cockroaches) from the rooms

3). Decongestant and Expectorant: Just like the Eucalyptus Oil, it is also an expert decongestant and expectorant. Being a decongestant, it gives immediate relief

in congestion of nose, throat and other respiratory organs as well as in coughs, infection and resultant inflammation of throat and respiratory tracts such as laryngitis (for larynx), pharyngitis (for pharynx) and bronchitis (for bronchi).

4). Cosmetic and Tonic: It smoothens and brightens skin and keeps it free from infections, thus used extensively in cosmetics, anti septic creams. It also tones up the skin and hence used as a tonic.

5). Stimulant and Sudorific: It stimulates the organs, gives a warming effect, promotes circulation and activates secretions. Being a stimulant, it also stimulates the Eccrine glands promoting perspiration and thus serving as a Sudorific

6). Analgesic: It is analgesic in nature, for example it reduces feeling of pain. When applied locally, such as on an infected tooth in case of a toothache or rubbed on forehead in case of headache or on sprains, it gives quick relief. If ingested, it can reduce pain in joints, headache and muscles, etc., in case of fever and cold.

7). Febrifuge: It helps reducing fever by fighting infection as well as by bringing sweat, which cools down the body.

8). Carminative: Cajuput Oil can give you a good relief from the gas trouble as it has carminative properties. It restricts formation of gas as well as helps remove the gas already formed in the intestines.

9). Anti Neuralgic: Neuralgia is a very painful situation in which the almost the entire oral zone, including the throat, ears, tonsils, base of the nose, larynx and pharynx and the surrounding areas suffer from severe pain due to compression of the Gloss pharyngeal or the ninth cranial nerve by the surrounding blood vessels which tend to swell from chewing, eating, laughing, shouting or any other excitement or movement in that region. Here Cajuput Oil can give immense relief. It, being an analgesic, reduces the feeling of pain on the one hand and on the other, cools down or relaxes the blood vessels reducing their swelling, thereby relieving the pressure on the ninth cranial nerve, giving immediate relief from the pain.

10). Other Benefits: Cajuput Oil is Anti Spasmodic and relieves cramps. It, being a stimulant, acts as an Emenagogue too and opens menstruations. Being a detoxifier, it helps cure pimples or acne and as an anti inflammatory, gives relief in inflammation (Patil *et al.*, 2008).

2.6.5 Compounds

Many compounds from *M. cajuputi* were investigated and have been reported in many research. Nerolidol, an antifeeding sesquiterpene alcohol, farnesol and geraniol from *M. leucadendron* (Doskotch *et al*., 1980). Doricin, a compound from the seed extract, were effective antifeedants utilizing larvae of fall armyworm, *Spodoptera frugiperda*, and European corn borer, *Ostrina nubilalis* (Powell *et al*., 1991).

Crude chloroform obtained ursolic acid, a triterpene, and methanol extracts obtained two stilbenes, piceatannol and oxyreseratrol of the fruits of *M. leucadendron* strongly inhibited histamine release from rat mast cells induced by compound 48/80 or Concanavalin A (Tsuruga *et al*., 1991).

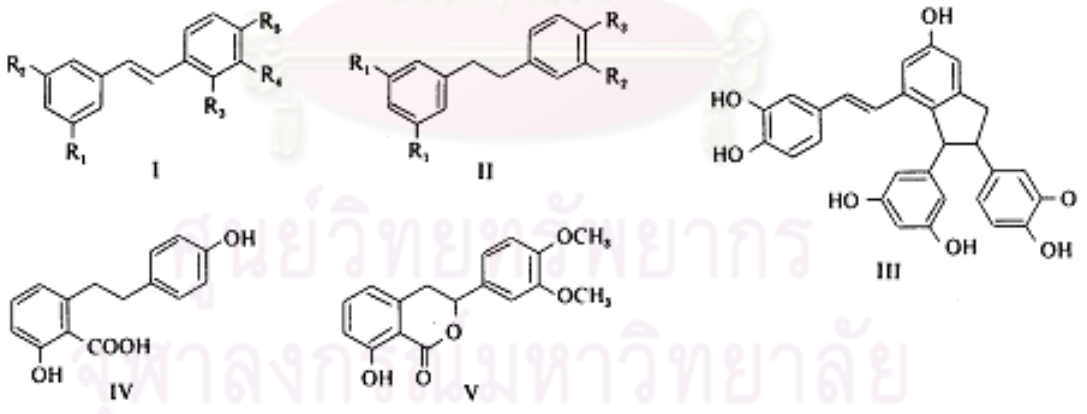


Figure 2.16 Structure of biological active constituents from heartwood of *M. leucadendron*

Yoshida *et al*. (1996) reported new hydrolysable tannin which was isolated from dried fruits of *M. leucadendron* and characterized as 1,2-di-O-galloyl-3-O-digalloyl-4,6-O-(S)-hexahydroxydiphenoyl-β-D-glucose, based on the chemical and

pectral evidence. Nine known hydrolysable tannins, as well as known stilbene glycosides and triterpenes, were also isolated.

Three novel triterpenoid esters (3*β*-*cis*-*p*-coumaroyloxy-2*α*-hydroxyursa-12,20(30)-dien-28-oic acid,*cis*-and *trans*-3β-caffeoyloxy-2α-hydroxyurs-12-en-28-oic acids) were isolated from ethyl acetate fraction of the acetone extract of the leaves of *M. leucadendron* and obtained the five known triterpenes, ursolaldehyde, ursolicacid, 2*α*-hydroxyursolic acid, 3*β*-*cis*-*p*-coumaroyloxy-2*α*-hydroxyurs-12-en-28-oic acid and 3*β*-*trans*-*p*-coumaroyloxy-2*α*-hydroxyurs-12-en-28-oic acid. The structures of the new compounds were determined by spectral methods to be 3*β*-*cis*-*p*-coumaroyloxy-2*α*-hydroxyursa-12, 20(30)-dien-28-oic acid, *cis*-and*trans*-3β-caffeoyloxy-2α-hydroxyurs-12-en-28-oic acids (Lee C.K., 1998a).

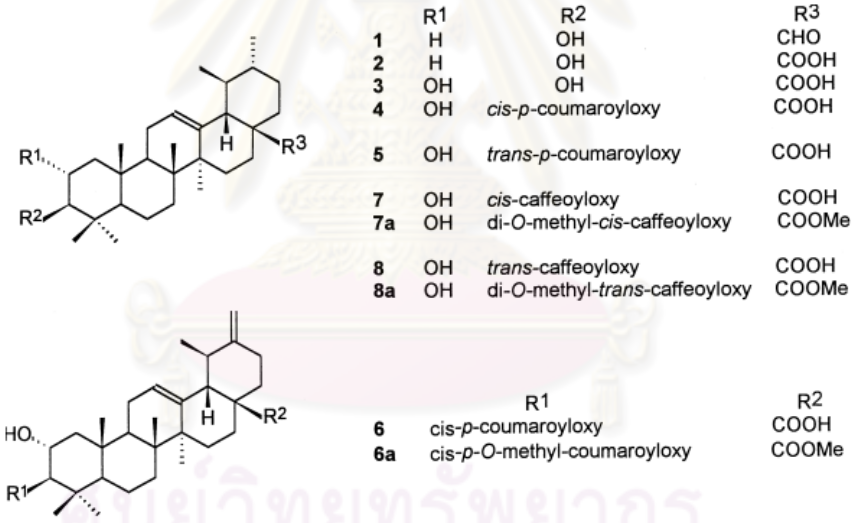
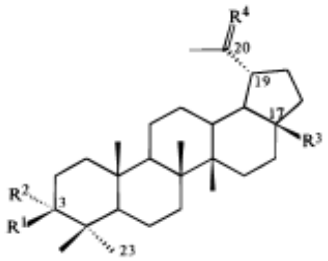


Figure 2.17 Structure of triterpenolds from leaves of *M. leucadendron*

The extracted from the leaves of *M. leucadendron* L., with Me_2CO obtained a new lupane-type nortriterpene and 13 known compounds. Based on chemical and spectral methods, the structure of the new compound was 28-norlup-20(29)-ene-3*β*,17 *β* -diol while the known compounds were identified as (2E,6E)-farnesol, phytol, squalene, all oaromadendrene, ledene, palustrol, viridiflorol, ledol, betulinaldehyde, betulinicacid,3-acetyl-lup-20(29)-en-28-oicacid,3-oxolup-20(29)-en 28 oicacid, and platonic acid (Lee C.K., 1998b).



	R^1	R^2	R^3	R^4
(1)	OH	H	OH	CH2
(2)	OAc	H	OH	CH2
(3)	OH	H	CHO	CH2
(4)	OH	H	COOH	CH2

Figure 2.18 Structure of lupine derivatives from leaves of *M. leucadendron*

The Me_2CO extract of heartwood of *M. leucadendron* gave four new triterpenes, eupha-7,24-diene-3,22-diol(1), 20-taraxastene-3R,28-diol(2),3R,27-dihydroxy-28,20-taraxastanolide(3)and3R-hydroxy-13(18)-oleanene-27,28-dioicacid(4) (Lee *et al.*,1999).

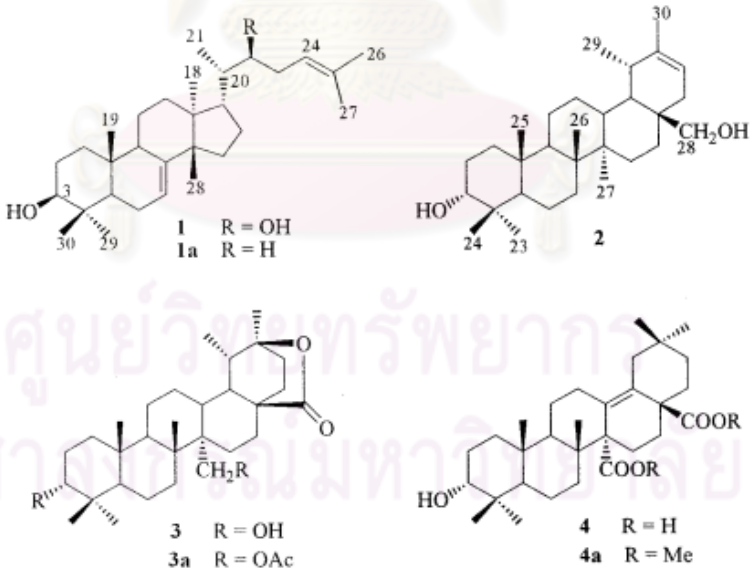
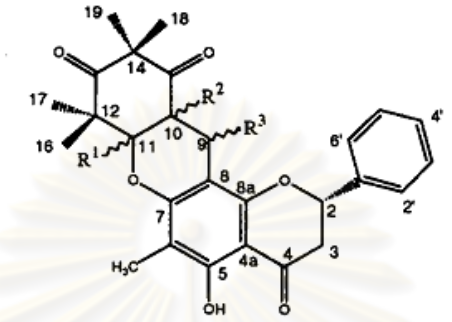


Figure 2.19 Structure of four new triterpenes from heartwood of *M. leucadendron*

The novel β-triketone flavanones, leucadenone A-D, have been isolated from crude acetone extracted of the leaves of *M. leucadendron* L. The structures of 1-4 were determined by NMR spectral and X-ray analysis (Lee C. K., 1999).



	R^1	R^2	R^3
1	β-OH	α-H	α-Ph
2	α-OH	β-H	β-Ph
3	α-OH	α-H	α-Ph
4	α-OH	β-H	α-Ph

Figure 2.20 Structure of four new β-triketone flavanones from leaves of *M. leucadendron*

M. cajuputi was studied on antioxidant and antibacterial activities. Dried fruits were extracted by solvents to obtain 3 crude extracts, including hexane, dichloromethane and methanol crude extracts. Evaluation of antioxidant activity, the methanol and crude extract exhibited the highest scavenging on DPPH radical. The methanol crude also the highest reducing power with EC_{50} extract contained the highest total phenolic compounds. For tested antibacterial activity using disc diffusion method against *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* and *Sarcina* sp., the dichloromethane and methanol crude extracts inhibited the growth of all bacteria tested whereas the hexane crude extract displayed the inhibitory activity against 3 strains of bacteria, including *E. coli*, *P. aeruginosa* and *Sarcinar* sp. (Chairgulprasert *et al*., 2007).

Table 2.1 Total phenols and EC_{50} values of extracts from *M. cajuputi* fruits.

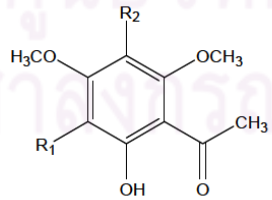
Extract	MIC (mg/mL) for bacteria				
	S. aureus	*B. subtilis*	*Sarcina* sp.	*E. coli*	*P. aeruginosa*
Hexane	-	-	40	100	80
Dichloromethane	40	40	100	40	40
Methanol	100	40	20	40	40

(-) not detected

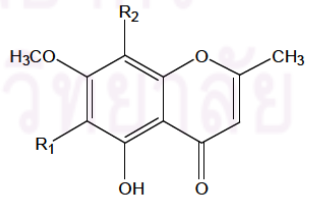
Table 2.2 The minimum inhibitory concentration (mg/mL) for antibacterial activity of *M. cajuputi* fruits.

Crude Extracts	Total phenols (mg GA/g of extract)	EC_{50} (mg/mL)	
		DPPH	Reducing Power
Hexane	32.8 ± 1.8	7.37 ± 0.24	11.10 ± 0.10
Dichlorometha	50.0 ± 2.0	0.26 ± 0.02	9.73 ± 0.06
Methanol	168.0 ± 1.7	0.03 ± 0.00	0.26 ± 0.01

Pullaput and Other (2007) investigated the crude hexane extracted from the leaves of *Melaleuca cajuputi* and afforded six compounds through chromatographic techniques. Their structures were determined by spectroscopic data analysis and comparisons of spectroscopic data with those previously reported, some of which showed antimicrobial and antimicrobial activity (Pullaput *et al.*, 2007).



Compound **1** : R_1 = H, R_2 = CH_3
Compound **5** : R_1 = CH_3, R_2 = CH_3
Compound **6** : R_1 = H, R_2 = H



Compound **2** : R_1 = H, R_2 = CH_3
Compound **3** : R_1 = CH_3, R_2 = CH_3
Compound **4** : R_1 = H, R_2 = H

Figure 2.21 Structure of compounds **1-6** of the crude hexane extract from the leaves of *Melaleuca cajuputi*

There are many researches about volatile leaf oil of *M. cajuputi* (Cajuput oil) for example in research. Sixteen samples of dried cajuput leaves from 9 provinces of Thailand, were collected and distillated by Water and Steam Distillation. Antimicrobial activities of cajuput oils in Thailand were tested using a paper disc diffusion method against 2 fungi; *Trichophyton mentagrophytes* and *Aspergillus niger* and 2 bacteria; *Staphylococcus aureus* and *Streptococcus* sp. It was found that all oils could inhibit growth of the microbes and there was different in degree of inhibition. The highest inhibition activity of the oils was to *T. mentagrophytes* and to *S. aureus*, respectively (Dilokkunanant *et al*., 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Instruments

3.1.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

FT-IR spectra were recorded on a Nicolet Impact 410 FT-IR. Potassium bromide (KBr) was used to form a pellet with the solid samples. The liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

3.1.2 Mass spectrometer (MS)

HRESIMS were performed on a Micromass LCT (LC/MS) at National Center for Genetic Engineering and Biotechnology (BIOTECH), National Science and Technology Development Agency Building (NSTDA).

3.1.3 Melting point

Melting points were examined using a Electrothermal Mel-Temp® melting point apparatus.

3.1.4 Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H and ^{13}C NMR data were performed on Varian Model Mercury +400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Deuterated solvents chloroform-*d* ($CDCl_3$), methanol-d_4 (CD_3OD), DMSO-d_6 and deuterium oxide (D_2O) were used for NMR experiments and chemical shifts (δ) were referenced the signals of residual solvents at δ 7.26 (^{1}H) and 77.0 (^{13}C) for $CDCl_3$, at 4.87 (^{1}H) for CD_3OD, at 2.50 (^{1}H) for DMSO-d_6 and at 4.79 (^{1}H) for D_2O.

3.1.5 Optical rotation

The optical rotations were measured on a Perkin-Elmer Model 341 Polarimeter, using a sodium lamp at wavelength 589 nm.

3.1.6 UV-VIS spectrometer

UV-VIS spectra were measured in the appropriate solvent and recorded on a Varian cary 50 probe UV-VIS spectrophotometer.

3.2 Chemicals

3.2.1 Solvent

The solvents used for column chromatography were commercial grade and were distilled prior to use.

3.2.2 Deuterated solvents

The deuterated solvents for NMR experiments including chloroform-*d* ($CDCl_3$), Methanol-*d* (CD_3OD), DMSO-d_6 ($(CD_3)_2SO$) and deuterium oxide (D_2O) were purchased from Merck.

3.2.3 Other Chemicals

- Silica gel 60 (0.040-0.063 mm) No. 1.09385.2500, Merck, Damstadt, Germany
- TLC aluminium sheets, silica gel 60 F_{254} (Merck, Damstadt, Germany
- Sephadex LH-20, GE Healthcare Bio-Science, Sweden
- Clorox® (5.25% NaOCl)

3.3 Plant samples Collection

Healthy leaves of *Melaluca cajuputi* were carefully collected from 4 provinces; Krabi, Nakhonsithammarat, Phatthalung and Trang. Fresh specimens were kept in a plastic bag and then immediately brought to the laboratory and processed within 24 h after collection.

3.3.1 Isolation endophytic fungi

Endophytic fungi were isolated using the surface sterilization technique which was modified from the method described by Petrini (1982). Plant leaves and twigs were washed in running tap water and dried in laminar air flow. The leaves were cut into a diameter 8 mm and twigs were cut into 10 mm rods. The sample were surface sterilized respectively in 70% EtOH for 1 minute, a solution of 5% Clorox® for 3 minute and 70% EtOH for 1 minute followed by rinsing twice with sterile distilled water. The surface sterilized samples were dried on sterile filter papers and put on Potato dextrose agar (PDA) and incubated at room temperature. The mycelium growth of endophytic fungi was examined every day under a stereomicroscope. The fungal hyphal tips were transferred to the new PDA plate and cultured in same conditions.

3.3.2 Culture media for endophytic fungi cultivation

Potato Dextrose Agar (PDA) was used for the endophytic fungi isolation. Endophytic fungi morphology observation and determination of antimicrobial activities of isolated endophytic fungi were carried out by culture in PDA, Malt extract agar (MEA) Corn meal agar (CMA), Sabouraud's dextrose agar (SDA) and Yeast Extract Sucrose Agar (YES).

The culture media for bacteria were Nutrient agar (NA). Yeast-malt extract agar (YMA) was used for growing yeasts. The formula for culture media was shown in Appendix A.

3.4 Screening of endophytic fungi for antimicrobial activity

Screening of endophytic fungi for antimicrobial activity was tested by dual culture agar diffusion technique which was modified from the method described by Weaver *et al.*, 1994.

3.4.1 Cultivation of endophytic fungal isolates

Each endophytic fungus were cultured in five media (PDA, MEA ,CMA, SDA and YES) at room temperature (at 25-30 ^{o}C) for 14 days.

3.4.2 Preparation of test microorganisms

The test microorganisms for antimicrobial activity are listed in Table 3.1

Table 3.1 Tested microorganisms for antimicrobial activity assay

Type of tested microorganisms	**Reference stains**
Gram positive bacterium	*Bacillus subtilis* ATCC 6633
	Staphylococcus aureus ATCC 25923
Gram negative bacterium	*Escherichia coli* ATCC 25922
	Pseudomonus aeruginosa ATCC 27853
Yeast	*Candida albicans* ATCC 10231

3.4.2.1 Preparation of bacterial inoculums

The test bacteria were *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Bacteria were grown on NA at 37^{o}C for 24 h. Selected single colonies were inoculated into NB (5 mL) and incubated at 37^{o}C for 4-8 h, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of a 0.5 McFarland (OD 0.08-0.1 at 625 nm).

3.4.2.2 Preparation of yeast inoculums

Candida albicans ATCC 10231 was grown on YMA at room temperature for 24 h. Selected single colonies were inoculated into YMB (5 mL) and incubated at room temperature for 4-8 h, depending on the growth rate. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of a 0.5 McFarland (OD 0.08-0.1 at 625 nm).

The colony forming unit/ml (CFU/ml) values of the test microorganisms used for antimicrobial assay in study shown in Table 3.2

Table 3.2 Quantity of the standardized inoculums of the test microorganisms

Type of tested microorganisms	**Quantity(CFU/ml)**
Bacillus subtilis ATCC 6633	5.2 x 10^6
Staphylococcus aureus ATCC 25923	4.5 x 10^6
Escherichia coli ATCC 25922	4.9 x 10^6
Pseudomonus aeruginosa ATCC 27853	4.7 x 10^6
Candida albicans ATCC 10231	3.9 x 10^6

3.4.3 Inoculation of the test plate

The test plates were inoculated by streaking the swab across the entire surface. This was repeated twice, turning the plate 60 degree between each streaking. The surface of the medium was allowed to dry for 3-5 minutes.

3.4.4 Application of antimicrobial by dual culture agar diffusion technique

The cultures on agars were cut with flamed cork borer (8 mm diameter) and then placed on test plate. All plates were incubated at 37 ^{o}C overnight. Inhibition zones were measured in mm.

3.5 Determination of growth profile and antimicrobial activity of culture filtrate from endophytic fungi isolate PTYL6

Endophytic fungi isolate PTYL6 was grown in YEA at room temperature (at 25-30 ^{o}C) for 14 days. The agar culture was cut into 75 mm diameter disks with flamed cork borer. Three disks of the agar culture of fungi isolated PTYL6 were inoculated in to 250 ml Erlenmeyer flask (x3) containing 100 ml of YEB medium. The cultures were incubated at room temperature (at 25-30 ^{o}C) under static condition for 18 days.

Mycelia were harvested by filtering through pre-weighted Whatman no.1 filter paper. The fungal mycelia were dried in oven at 55 ^{o}C to dryness and weighted again. The dried weight of mycelia was calculated.

The cultures of fungal isolate PTYL6 at various cultivation times were filtered through Whatman no.1 filter paper and antimicrobial activity of the filtrates were examined against 5 test microorganisms as presented in Table 3.1 using agar well diffusion methods described by Pereda-Miranda *et al.* (1993).

3.5.1 Preparation of tested bacterial inoculums

The bacterial inoculums were prepared in the same manner as described in section 3.4.2.1

3.5.2 Preparation of tested yeast inoculums

The yeast inoculums were prepared in the same manner as described in section 3.4.2.2

3.5.3 Inoculation of the test plates

Within 15 minute of adjusting the density of the bacteria or yeast inoculum a sterile cotton swab on a wooden applicator was dipped into the cell suspension which the turbidity equal to a 0.5 McFarland (OD 0.08-0.1 at 625 nm). 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 different planes, by rotating the plate approximately 60^{o} each time, to ensure and even distribution of the inoculums. The surface of medium was dried for 3-5 minutes.

3.5.4 Application of culture filtrate

The NA and YMA plates that were already inoculated with tested bacterial and yeast respectively were cut with a flamed 7 mm diameter cork borer to make wells in the agar. The culture filtrates (each 20 µl) were transferred into the well. Bacterial plates were incubated at 37^{o}C for 24 hours while yeast plates were incubated at room temperature for 24 hours. Inhibition zone around the well were measured in mm.

3.6 Identification of the selected endophytic fungi

3.6.1 Morphological identification of selected endophytic fungi

Endophytic fungi were characterized on the basis of morphological identification, macroscopic features (e.g., shape, size, color, margin, pigment) and microscopic features observed by compound microscopy a stereomicroscopy (e.g., spores, mycelia). The fungi were identified as described by Barron (1977), Von Arx (1981) and Barnett and Hunter (1998).

3.6.2 Molecular identification of the selected endophytic fungi

selected endophytic fungi were characterized based on morphological identification in section 3.2.2 and molecular identification. Cultivation of selected endophytic fungi in PDB (100 mL) at room temperature for a few weeks under static condition was filtered through filter paper (Whatman No. 1). Mycelia of endophytic fungi were kept at 4^{o}C for genomic DNA extraction as described by Zhou *et al.* (1999).

3.6.2.1 DNA extraction

The mycelia were homogenized in 1,000 µL of a solution of washing buffer (0.1 M Tris-HCl (pH 8.0), 2% 2-mercaptoethanol, 1% polyvinylpyrrolidone and 0.05 M ascorbic acid) with a pestle in a mortar. Then the sample was transferred to 1.5 mL micro centrifuged tube and a mixture of the sample and the washing buffer was centrifuged at 15,000 g for 3 min. After removal of the supernatant, the pellet was washed 4-5 times using the washing buffer and centrifugation at 15,000 g for 3 min. DNA was then extracted from the washed pellet by adding 700 µL of cetyltrimethylammonium bromide (CTAB) lysis buffer and incubation in water bath at 65^{o}C for 1 h followed by extracted twice with a mixture of chloroform and isoamyl alcohol (24:1, v/v). Fungal DNA was precipitated in ice bath with isopropanol and centrifuged at 4^{o}C, 8000 rpm for 10 min. After removal of the supernatant, 80% cool ethanol was added to wash the fungal DNA. TE 100 µL(Buffer solution in molecular biology, especially in procedures involving DNA or RNA. "TE" is derived from its components: Tris, a common pH buffer, and EDTA) was added to dissolve the fungal DNA and kept at -20^{o}C. The genomic DNA was checked by 1.2% agarose gel (ISC Bioexpress) electrophoresis.

3.6.2.2 Internal transcribed spacer region

The ITS region was amplified in a total volume of 50 µL comprising of approx. 100 ng genomic DNA, 1x PCR Master Mix (Fermentas), and the primer ITS1f (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990). The PCR amplification was performed in a thermocycler (T Gradient 96, Biometra) with 94^{o}C for 5 min, followed by 38 cycles of 94^{o}C for 1 min, 51^{o}C for 1 min and 72^{o}C for 1 min, with a final extension of 72^{o}C for 5 min. PCR product was purified and

subcloned with PCR-Script^{TM} Amp Cloning Kit (Stratagene) following the manufacturer's protocol.

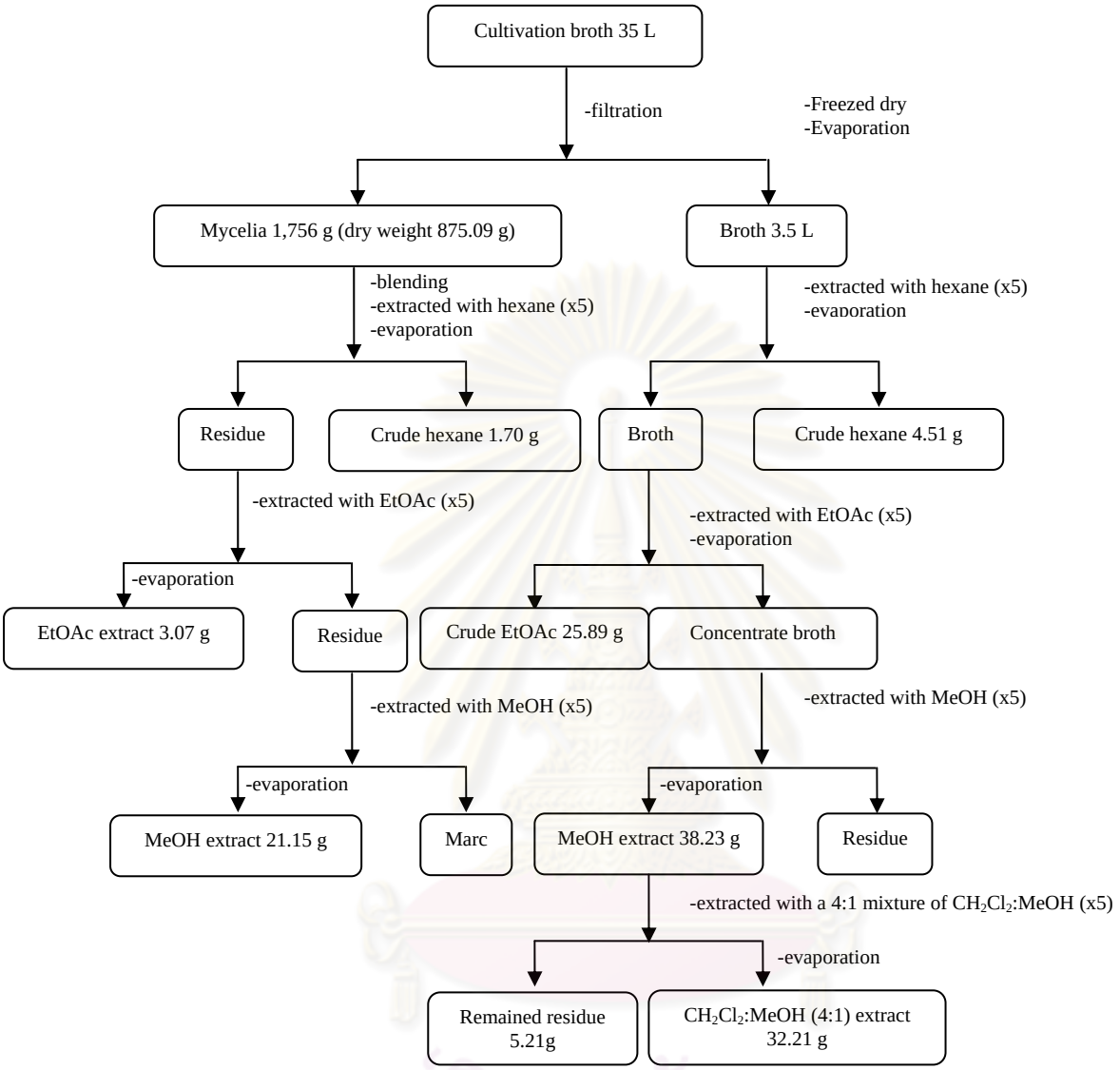
3.6.4.3 DNA Sequencing

The DNA sequences were analyzed at Macrogen (Seoul, South Korea) using the same primers as for amplification.

3.7 Cultivation and chemical investigation of the selected endophytic fungi

The culture of the endophytic fungus PTYL6 grown on YEA at room temperature for 7-14 days was cut into 7.5 cm diameter disks by a flamed cork borer. Three disks were inoculated into 250 mL Erlenmeyer flask containing 100 mL of YEB. After cultivation under static condition at room temperature for 18 days total of cultured media volume (35 L) were filtered through filter paper (Whatman No. 1). The filtrate was concentrated by a rotary evaporation under reduced pressure at 37^{o}C and Freeze dryer to give a viscous liquid (2500 mL) and then the filtrate was partitioned with an equal volume of hexane (x5) and EtOAc (x5) respectively. The viscous liquid was extracted with MeOH (x5). The combined MeOH extracts were evaporated under reduced pressure to give MeOH crude as brown viscous oil (4.52 g). After that crude MeOH extract with CH_2Cl_2 : MeOH = 4 : 1 obtain crude as brown viscous oil (215 mg) and residue crude as dark brown viscous (4.52 g). The hexane extract and EtOAc extract were evaporated to give hexane crude as brown viscous oil (215 mg) and EtOAc crude as dark brown viscous (4.52 g).

Fungal mycelia (1585.85 g of wet weight) were ground using blender and dried in hot air oven at 55 ^{o}C then extracted with hexane (x5), EtOAc (x5) and MeOH (x5), respectively. After evaporation of the solvents under reduced pressure the hexane crude, the EtOAc crude and the MeOH crude were obtained as a yellow viscous oil (1.70 g), as a yellow viscous residue (3.07 g) and as a brown viscous oil (21.15 g) respectively. Extraction of mycelia and broth of endophytic fungus isolate PTYL6 were showed in Scheme 3.1. All of the crude was kept in a refrigerator at -20^{o}C in the dark before further studied.



Scheme 3.1 Extraction of mycelia and broth of endophytic fungus isolate PTYL6.

3.8 Chemical investigation metabolite from endophytic fungus PTYL6

3.8.1. Chemical investigation of EtOAc extract of mycelia of the fungus PTYL6

During evaporation of the solvent compound **1** was obtained as white-yellow solids in the crude extract. The white-yellow solids were washed by 70% CH_2Cl_2 in MeOH to give compound 1 as white solid (93.7mg). The EtOAc extract (497 mg) was isolated by silica gel column chromatography eluted with increasing polarity from hexane, EtOAc and MeOH. The similar fractions were combined on the basis of TLC profile to give 12 combined fractions as shown in Table 3.3. The combined fractions 6MyEt-6, 7, 8, 9, 10 and 11 was isolated by silica gel column chromatography eluted by hexane EtOAc and MeOH to afford compound 1 (12.5 mg). Total amount of compound 1 from the precipitate during evaporation of the solvent and from chromatography was (116.2 mg) which was then characterized by NMR, MS and optical rotation.

The white solids in yellow liquid oil obtained in the combined fractions 6MyEt 3-5 were washed by 90% Hexane in EtOAc followed by crystallization from Hexane and EtOAc to give compound **2** (96.2 mg) as white solids. Compound was subjected to spectroscopic analysis including NMR, MS and optical rotation.

Table 3.3 The combined fractions obtained from isolation of the EtOAc extract of mycelia using silica gel column chromatography

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
6MyEt-1	1-7	hexane:EtOAc (95:5)-hexane:EtOAc (90:10)	Yellow viscous liquid	225.00
6MyEt-2	8-19	hexane:EtOAc (90:10)-hexane:EtOAc (80:20)	Yellow viscous liquid	36.10
6MyEt-3	20-28	hexane:EtOAc (80:20)-hexane:EtOAc (75:25)	Yellow viscous liquid	27.10
6MyEt-4	29-35	hexane:EtOAc (75:25)	Yellow viscous liquid	25.30

Table 3.3 (Continued)

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
6MyEt-5	36-39	hexane:EtOAc (70:30)	Yellow viscous liquid	71.20
6MyEt-6	40-48	hexane:EtOAc (70:30)	Yellow viscous liquid	112.30
6MyEt-7	49-59	hexane:EtOAc (70:30)-hexane:EtOAc (65:35)	Yellow viscous liquid	57.00
6MyEt-8	60-69	hexane:EtOAc (65:35)-hexane:EtOAc (60:40)	White solid and brown viscous liquid	93.00
6MyEt-9	70-78	hexane:EtOAc (60:40)	Brown viscous liquid	22.00
6MyEt-10	79-86	hexane:EtOAc (55:45)-hexane:EtOAc (50:50)	Yellow viscous liquid	135.20
6MyEt-11	87-119	hexane:EtOAc (60:40)-EtOAc:MeOH (50:50)	Yellow viscous liquid	73.00
6MyEt-12	120-145	EtOAc:MeOH (50:50)-MeOH (100)	Yellow viscous liquid	37.50

3.8.2 Chemical investigation of EtOAc extract of the broth of the fungus PTYL6

The EtOAc extract was divided into five portions including 6BrEt (510 mg), 7BrEt (512 mg), 8BrEt (512 mg), 9BrEt (508 mg) and 10BrEt (507 mg) and then each portion was subjected to Sephardex LH20 column chromatography eluted with increasing polarity from MeOH and MeOH/H_2O. The similar fractions were combined on the basis of TLC profile to give 17, 15, 17, 15 and 15 combined fractions, respectively, as shown in Table 3.4-3.8.

Table 3.4 The combined fractions obtained from isolation of the EtOAc extract of broth (portion of 6BrEt) using Sephardex LH-20 column chromatography.

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
6BrEt-1	1-3	MeOH (100)	Brown liquid	47
6BrEt-2	4-5	MeOH (100)	Brown liquid	116
6BrEt-3	6	MeOH (100)	Brown liquid	590
6BrEt-4	7-9	MeOH (100)	Brown liquid	230
6BrEt-5	10-11	MeOH (100)	Brown liquid	308
6BrEt-6	12-14	MeOH (100)	Orange yellow liquid	103
6BrEt-7	15-17	MeOH (100)	Orange yellow liquid	1080
6BrEt-8	18-20	MeOH (100)	Red brown liquid	140
6BrEt-9	21-24	MeOH (100)	Red brown liquid	571
6BrEt-10	25-29	MeOH (100)	Brown liquid	171
6BrEt-11	30-31	MeOH (100)	Brown liquid	848
6BrEt-12	32-34	MeOH (100)	Brown liquid	305
6BrEt-13	35-36	MeOH (100)	brown viscous liquid	251
6BrEt-14	37-38	MeOH : H_2O (90:10)	Brown viscous liquid	149
6BrEt-15	39-44	MeOH : H_2O (80:20)	brown viscous liquid	114
6BrEt-16	45-70	MeOH : H_2O (80:20)	brown viscous liquid	93
6BrEt-17	70-80	MeOH : H_2O (70:30)	Black viscous liquid	812

Table 3.5 The combined fractions obtained from isolation of the EtOAc extract of broth (portion of 7BrEt) using Sephardex LH20 column chromatography.

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
7BrEt-1	1-3	MeOH (100)	Brown liquid	65
7BrEt-2	4	MeOH (100)	Brown liquid	15
7BrEt-3	5-6	MeOH (100)	Brown liquid	48
7BrEt-4	7-8	MeOH (100)	Orange yellow liquid	169
7BrEt-5	9-12	MeOH (100)	Orange yellow liquid	41
7BrEt-6	13-15	MeOH (100)	Orange yellow liquid	208
7BrEt-7	16-18	MeOH (100)	Orange yellow liquid	451
7BrEt-8	19-21	MeOH (100)	Orange yellow liquid	347
7BrEt-9	22-23	MeOH (100)	Red brown liquid	1320
7BrEt-10	24-28	MeOH (100)	Red brown liquid	957
7BrEt-11	29-30	MeOH (100)	Brown liquid	305
7BrEt-12	31-39	MeOH (100)	Brown liquid	971
7BrEt-13	40-47	MeOH : H_2O (90:10)	brown viscous liquid	156
7BrEt-14	48-60	MeOH : H_2O (80:20)	Brown viscous liquid	343
7BrEt-15	61-70	MeOH : H_2O (70:30)	Black viscous liquid	517

Table 3.6 The combined fractions obtained from isolation of the EtOAc extract of broth (portion of 8BrEt) using Sephardex LH20 column chromatography.

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
8BrEt-1	1-5	MeOH (100)	Brown liquid	135
8BrEt-2	6-7	MeOH (100)	Brown liquid	1243
8BrEt-3	8-10	MeOH (100)	Orange yellow liquid	274
8BrEt-4	11-15	MeOH (100)	Orange yellow liquid	501
8BrEt-5	16-18	MeOH (100)	Orange yellow liquid	568
8BrEt-6	19-20	MeOH (100)	Orange yellow liquid	501
8BrEt-7	21	MeOH (100)	Red brown liquid	93
8BrEt-8	22-24	MeOH (100)	Red brown liquid	1304
8BrEt-9	25-27	MeOH (100)	Red brown liquid	668
8BrEt-10	28-30	MeOH (100)	Red brown liquid	1468
8BrEt-11	31-32	MeOH (100)	Brown liquid	494
8BrEt-12	33-35	MeOH (100)	Brown liquid	51
8BrEt-13	36-38	MeOH (100)	Brown viscous liquid	95
8BrEt-14	39-40	MeOH : H_2O (90:10)	Brown viscous liquid	186
8BrEt-15	41-42	MeOH : H_2O (80:20)	Brown viscous liquid	78
8BrEt-16	43-44	MeOH : H_2O (80:20)	Brown viscous liquid	387
8BrEt-17	45-70	MeOH : H_2O (70:30)	Black viscous liquid	2241

Table 3.7 The combined fractions obtained from isolation of the EtOAc extract of broth (portion of 9BrEt) using Sephardex LH20 column chromatography

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
9BrEt-1	1-9	MeOH (100)	Brown liquid	730
9BrEt-2	10-11	MeOH (100)	Brown liquid	1100
9BrEt-3	12-18	MeOH (100)	Brown liquid	590
9BrEt-4	19-24	MeOH (100)	Yellow liquid	941
9BrEt-5	25-28	MeOH (100)	Yellow liquid	209
9BrEt-6	29-37	MeOH (100)	Orange yellow liquid	45
9BrEt-7	38-39	MeOH (100)	Orange yellow liquid	39
9BrEt-8	40-43	MeOH (100)	Yellow liquid	120
9BrEt-9	44-49	MeOH (100)	Yellow liquid	401
9BrEt-10	50-55	MeOH (100)	Yellow liquid	214
9BrEt-11	56-62	MeOH (100)	Yellow liquid	140
9BrEt-12	63-67	MeOH (100)	Yellow liquid	208
9BrEt-13	68-70	MeOH : H_2O (90:10)	Yellow liquid	210
9BrEt-14	71-75	MeOH : H_2O (80:20)	Yellow liquid	101
9BrEt-15	76-80	MeOH : H_2O (70:30)	Brown viscous liquid	953

Table 3.8 The combined fractions obtained from isolation of the EtOAc extract of broth (portion of 10BrEt) using Sephardex LH20 column chromatography.

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
10BrEt-1	1-7	MeOH (100)	Yellow liquid	260
10BrEt-2	8-11	MeOH (100)	Brown liquid	450
10BrEt-3	12-18	MeOH (100)	Brown liquid	350
10BrEt-4	19-20	MeOH (100)	Yellow liquid	1160
10BrEt-5	21-36	MeOH (100)	Yellow liquid	340
10BrEt-6	37-42	MeOH (100)	Orange yellow liquid	195
10BrEt-7	43-48	MeOH (100)	Brown liquid	290
10BrEt-8	49-53	MeOH (100)	Brown liquid	260
10BrEt-9	54-58	MeOH (100)	Brown liquid	350
10BrEt-10	59-61	MeOH (100)	Brown liquid	250
10BrEt-11	62-64	MeOH (100)	Brown liquid	150
10BrEt-12	65-67	MeOH (100)	Brown liquid	320
10BrEt-13	68-71	MeOH : H_2O (90:10)	Yellow liquid	235
10BrEt-14	72-78	MeOH : H_2O (80:20)	Yellow liquid	278
10BrEt-15	79-80	MeOH : H_2O (70:30)	Brown viscous liquid	352

3.8.3 Chemical investigation of MeOH crude extracted from culture broth of the fungus PTYL6

The MeOH extract of broth (38.23 g) was re-extracted with a 4:1 mixture of CH_2Cl_2 and MeOH followed by evaporation of the solvents to obtained CH_2Cl_2:MeOH (4:1) extract (521 mg). The MeOH crude extracted was then separated by Sephardex LH20 column chromatography eluted with increasing polarity from MeOH to MeOH/H_2O. The similar fractions were combined on the basis of TLC profile to give 14 combined fractions as shown in Table 3.9.

Table 3.9 The combined fractions obtained from isolation of the CH_2Cl_2:MeOH (4:1) extract of the MeOH extract of broth using Sephardex LH20 column chromatography.

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
6BrMe-1	1-10	MeOH (100)	Brown liquid	13
6BrMe-2	11-13	MeOH (100)	Brown liquid	1025
6BrMe-3	14-16	MeOH (100)	Brown liquid	51
6BrMe-4	17-19	MeOH (100)	Brown liquid	490
6BrMe-5	20-21	MeOH (100)	Brown liquid	52
6BrMe-6	22-25	MeOH (100)	Brown liquid	21
6BrMe-7	26-27	MeOH (100)	Brown liquid	45
6BrMe-8	28-29	MeOH (100)	Yellow liquid	59
6BrMe-9	30-33	MeOH (100)	Red brown liquid	39
6BrMe-10	34-35	MeOH (100)	Red brown liquid	15
6BrMe-11	36-37	MeOH (100)	Brown liquid	15
6BrMe-12	38-49	MeOH : H_2O (90:10)	Brown liquid	29
6BrMe-13	50-55	MeOH : H_2O (80:20)	Black liquid	21
6BrMe-14	56-70	MeOH : H_2O (70:30)	Black liquid	53

3.8.4 Chemical investigation of portion 6BrMr of the residue from re-extraction of MeOH extract of the broth of the fungus PTYL6 with a 4:1 mixture of CH_2Cl_2 and MeOH

The residue (37.5 g) from re-extraction of MeOH extract of the broth of the fungus PTYL6 with a 4:1 mixture of CH_2Cl_2 and MeOH was divided into three portions including 6BrMr (989 mg), 7BrMr (1005 mg), and 8BrMr (1008 mg). Each portion was separated by Sephardex LH20 column chromatography eluted with MeOH and MeOH/H_2O (90:10). The similar fractions were combined on the basis of TLC profile to give 17, 15 and 15 combined fractions, respectively, as shown in Table 3.9. The combined fractions 6BrEt6-7, 7BrEt4-5 and 8BrEt4-5 eluted by MeOH (100)

were precipitated from 10% H_2O in MeOH to give compound **3** (116.2 mg) as white solids. Compound 3 was characterized by NMR, MS and optical rotation.

Table 3.10 The combined fractions obtained from isolation of portion 6BrMr of the residue from re-extraction of MeOH extract of the broth of the fungus PTYL6 with a 4:1 mixture of CH_2Cl_2 and MeOH using Sephardex LH20 column chromatography.

Fraction code	Fractions	Eluents	Appearance	Weight (mg)
6BrMr-1	1-2	MeOH (100)	Brown liquid	25
6BrMr-2	3-5	MeOH (100)	Brown liquid	680
6BrMr-3	6-7	MeOH (100)	Brown liquid	51
6BrMr-4	8-10	MeOH (100)	Brown liquid	490
6BrMr-5	11-12	MeOH (100)	Brown liquid	52
6BrMr-6	13	MeOH (100)	Brown liquid	21
6BrMr-7	14-17	MeOH (100)	Brown liquid	45
6BrMr-8	18-22	MeOH (100)	Yellow liquid	59
6BrMr-9	23-24	MeOH (100)	Red brown liquid	39
6BrMr-10	25-26	MeOH (100)	Red brown liquid	15
6BrMr-11	27	MeOH (100)	Brown liquid	15
6BrMr-12	28-30	MeOH (100)	Brown liquid	29
6BrMr-13	31-39	MeOH (100)	Black liquid	21
6BrMr-14	40-43	MeOH (100)	Black liquid	53
6BrMr-15	44-50	MeOH : H_2O (90:10)	Black liquid	17
6BrMr-16	51-57	MeOH : H_2O (80:20)	Black liquid	23
6BrMr-17	58-60	MeOH : H_2O (70:30)	Black liquid	12

Table 3.11 The combined fractions obtained from isolation of portion 7BrMr of the residue from re-extraction of MeOH extract of the broth of the fungus PTYL6 with a 4:1 mixture of CH_2Cl_2 and MeOH using Sephardex LH20 column chromatography.

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
7BrMr-1	1-3	MeOH (100)	Brown liquid	25
7BrMr-2	4-5	MeOH (100)	Brown liquid	680
7BrMr-3	6-7	MeOH (100)	Brown liquid	51
7BrMr-4	8-10	MeOH (100)	Brown liquid	490
7BrMr-5	11-12	MeOH (100)	Brown liquid	52
7BrMr-6	13-14	MeOH (100)	Brown liquid	21
7BrMr-7	15-16	MeOH (100)	Brown liquid	45
7BrMr-8	17-18	MeOH (100)	Yellow liquid	59
7BrMr-9	19	MeOH (100)	Red brown liquid	39
7BrMr-10	20-21	MeOH (100)	Red brown liquid	15
7BrMr-11	22-26	MeOH (100)	Brown liquid	15
7BrMr-12	27-35	MeOH (100)	Brown liquid	29
7BrMr-13	36-38	MeOH : H_2O (90:10)	Black liquid	21
7BrMr-14	39-45	MeOH : H_2O (80:20)	Black liquid	53
7BrMr-14	39-45	MeOH : H_2O (80:20)	Black liquid	75

Table 3.12 The combined fractions obtained from isolation of portion 8BrMr of the residue from re-extraction of MeOH extract of the broth of the fungus PTYL6 with a 4:1 mixture of CH_2Cl_2 and MeOH using Sephardex LH20 column chromatography.

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
8BrMr-1	1-2	MeOH (100)	Brown liquid	25
8BrMr-2	3-7	MeOH (100)	Brown liquid	680
8BrMr-3	9-12	MeOH (100)	Brown liquid	51
8BrMr-4	13	MeOH (100)	Brown liquid	490
8BrMr-5	14-15	MeOH (100)	Brown liquid	52
8BrMr-6	16-17	MeOH (100)	Brown liquid	21
8BrMr-7	18-19	MeOH (100)	Brown liquid	45
8BrMr-8	20-24	MeOH (100)	Yellow liquid	59
8BrMr-9	25-26	MeOH (100)	Red brown liquid	39
8BrMr-10	20-21	MeOH (100)	Red brown liquid	15
8BrMr-11	22-26	MeOH (100)	Brown liquid	15
8BrMr-12	27-35	MeOH (100)	Brown liquid	29
8BrMr-13	36-38	MeOH : H_2O (90:10)	Black liquid	21
8BrMr-14	39-45	MeOH : H_2O (80:20)	Black liquid	53
8BrMr-15	46-70	MeOH : H_2O (70:30)	Black liquid	17

3.9 Antimicrobial activity

3.9.1 Paper disk diffusion method

The disk diffusion method (Jorgensen, 1999) was used to determine the antimicrobial activity.

1). Preparation of samples

The isolated metabolites were weighed and dissolved in 1 mL of a solution of 10% DMSO and 0.5% Tween 80 in sterile distilled water.

2). Preparation of bacterial inoculum

Bacterial inoculum was prepared as the same manner described in 3.2.3.3.2.

3). Preparation of yeast inoculum

Yeast inoculum was prepared as the same manner described in 3.2.3.3.3.

4). Inoculation of the test plate

Bacteria and yeast were inoculated in the same manner as described in 3.2.3.3.4.

5). Application of secondary metabolites

A solution of the isolated metabolites (10 µL) was pipetted into the 7 mm diameter sterile paper disks (Whatman) which were allowed to dry in the sterile Petri dish. Then the paper disks were placed on the test plate. Bacteria plates were incubated at 37^{o}C while yeast plates were incubated at room temperature for 24 h.

3.9.2 Bioautography method (Rahalison *et al.*, 1991)

3.9.2.1 Preparation of test microorganisms

1). Preparation of bacterial inoculums

The bacterial inoculums were prepared in the same manner as described in section 3.4.2.1.

2). Preparation of yeast inoculum

The yeast inoculum was prepared in the same manner as described in section 3.4.2.2.

3.9.2.2 Preparation of test sample

20 x 20 cm Silica gel 60 sheets (Merck, Darmstadt, Germany) were used. Samples were prepared in concentration 10 mg/ml, sport 10 µL in each sample with capillaries (0.1 mg/spot). TLC plates were developed with a 4.7:0.3 mixture of $CHCl_3$ and MeOH and dried under air flow.

3.9.2.3 Spayed test microorganism

The developed TLC plates were sprayed with test microorganism prepared in section 3.8.1. The TLC plates were then incubated in a water-vapor chamber (high humid condition) at room temperature for 24 h. Inhibition zone on the TLC plates was visualized by spraying MTT reagent (methylthiazolyltetrazolium bromide) on TLC plates followed by drying under air flow.

3.9.3 Evaluation of antimicrobial activity of pure compound by Minimum Inhibitory Concentration Method (MIC) (Jorgensen, 1999)

Minimum inhibitory concentration (MIC), in microbiology, is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents (Andrews, 2001). The procedures of MIC were described below.

1). Preparation of samples

1 mg of pure compounds and antibiotic drug standards were dissolved in 2 ml of 10 % DMSO in sterile distilled water. Antimicrobial (Streptomycin) and antifungal (Ketoconazole) agents were used as positive controls.

2). Preparation of bacterial inoculum

Bacterial inoculum was prepared as the same manner described in 3.2.3.3.2.

3). Preparation of yeast inoculum

Yeast inoculum was prepared as the same manner described in 3.2.3.3.3.

4). Inoculation of the test plate

Bacteria and yeast were inoculated in the same manner as described in 3.2.3.3.4.

5). Assay procedure

Solutions of pure compounds and antibiotic drug standards were diluted with Nutrient broth (NB) and Yeast-malt extract broth (YMB) for assays of antimicrobials activity. A solution of pure compounds (50 µL) was dispensed into each well in sterile 96-well plates. Fifty µL of the final adjusted microbial suspension was inoculated into each well. One hundred µL of medium only was used as the growth control. A 100 µL volume of medium and microbial inoculums mixture acted as the growth control. Microbial 96-well plates were incubated bacteria at 37^{o}C while yeast plates were incubated at room temperature.

6). Reading of antimicrobials assay

Antimicrobials activities were determined by measuring the turbidity of each well in the microtitre plates by using Sunrise micro plate reader (TECAN, AUSTRIA) before and after incubation. The lowest concentration of pure compounds showed complete inhibition of growth was recorded as minimum inhibitory concentration (MIC).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

The healthy and mature leaves of *Melaleuca cajuputi* were collected from 4 provinces. Isolation of endophytic fungi using surface sterilization (Petrini 1982) was performed by soaking in 90% EtOH for 1 minute, in a solution of 5% NaOCl (Clorox®) for 3 minutes, in 90% EtOH for 1 minute and rinsing twice with sterile distilled water to give fifty six endophytic fungal isolates as shown in Table 4.1.

Table 4.1 Number of endophytic fungi isolated from leaves of *Melaleuca cajuputi* Powell.

Province (Code) / Part of tree (Code)	Krabi (KB)	Nakhon sithammarat (NS)	Phatthalung (PT)	Trang (TR)	Total(isolate)
Old Leaves (OL)	3	5	7	5	20
Young Leaves (YL)	4	4	6	5	19
Twigs (TW)	3	7	4	3	17
Total(isolate)	10	16	17	13	56

4.2 Identification and classification of endophytic fungi

Each fungal isolate was grown on 5 agar media, Potato Dextrose Agar (PDA), Malt extract agar (MEA) Corn meal agar (CMA), Sabouraud's dextrose agar (SDA) and Yeast Extract Sucrose Agar (YEA) for 14 days at room temperature. A total of 56 isolates of endophytic fungi were classified using colony morphology such as sexual

state, spores (sexual and asexual). Fifty six fungal isolates were identified as *Alternaria* sp. (1 isolates-KBOL3), *Aspergillus* sp. (1 isolates-TRTW3), *Colletotrichum* sp. (1 isolates-NSYL4), *Fusarium* sp. (5 isolates-PTOL6, PTOL6, KBYL3, NSTW1 and NSYL5), *Pestalotia* sp. (5 isolates-PTOL1, PTOL3, PTYL3 and KBYL4), *Phomopsis* sp. (4 isolates-KBOL2, NSTW6 , TROL4 and TRYL4) and the fungi in Xylariaceae family (6 isolates), while other isolates of endophytic fungi were classified as mycelia sterilia due to no conidia or sporulate produced by those fungi. The results of the identification of the endophytic fungi from 4 provinces were shown in Table 4.2.

Table 4.2 Identification of endophytic fungi

Species	**Number of endophytic fungi (isolates)**
Mycelia Sterilia	33
Alternaria sp.	1
Aspergillus sp.	1
Colletotrichum sp.	1
Fusarium sp.	5
Pestalotia sp.	5
Phomopsis sp.	4
Xylariaceae	6
Total	56

4.3 Screening the secondary metabolite production of the endophytic fungi

Antimicrobial activity of 56 endophytic fungal isolates grown on Potato Dextrose Agar (PDA), Malt extract agar (MEA) Corn meal agar (CMA), Sabouraud's dextrose agar (SDA) and Yeast Extract Sucrose Agar (YES) were examined against 5 tested microorganisms (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231) using dual agar diffusion method with diameter 75 mm (Jorgensen, 1999). The results were shown in Table 4.3. The number of

endophytic fungi isolates from four provinces which produced antimicrobial metabolites was presented in Table 4.4 and Figure 4.1.

Table 4.3 Dual agar diffusion assay on antimicrobial activity of metabolites produced by endophytic fungi.

Code of Isolated Endophytic fungi[#]	Culture medium	Inhibition zone diameter (mm) against tested microorganisms*				
		B. subtilis ATCC 6633	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*C. albicans* ATCC 10231
KBOL1	CMA	-	-	-	-	2.10
KBOL2	SDA	-	-	-	1.45	2.00
	MEA	1.40	0.90	-	-	-
KBYL1	YEA	-	-	-	-	1.0
KBYL3	MEA	1.25	2.05	1.80	1.55	1.70
NSOL1	SDA	1.50	1.50	1.30	1.40	1.25
NSYL3	SDA	-	-	1.65	-	1.50
	YEA	-	-	-	-	1.20
NSYL4	YEA	1.30	1.80	1.00	-	1.60
PTOL4	SDA	1.60	-	-	-	2.10
PTOL5	MEA	-	1.00	-	-	2.30
	YEA	0.90	-	-	-	-
PTOL6	YEA	1.75	-	-	-	2.05
PTYL1	YEA	-	1.30	1.00	-	-
PTYL2	PDA	1.40	-	-	-	1.80
	CMA	-	-	-	-	1.80
PTYL3	CMA	-	-	-	-	1.60
	MEA	-	-	-	-	1.60
PTYL5	MEA	-	-	-	-	1.10
PTYL6	MEA	-	1.90	-	-	2.00
TROL1	CMA	-	2.10	2.00	-	
TROL2	MEA	-	-	1.10	-	1.80
TROL3	MEA	1.90	-	-	0.90	-
	SDA	-	-	0.80	-	-
TROL5	PDA	-	-	-	-	1.05
	SDA	-	-	-	-	1.20
	YEA	-	-	-	-	1.55
TRTW1	YEA	-	-	1.30	-	-
TRTW2	YEA	0.90	1.05	1.00	1.00	0.80
	SDA	1.60	-	1.60	-	1.70
TRYL1	MEA	-	-	-	-	1.85
TRYL2	MEA	-	-	-	-	1.20
TRYL3	YEA	-	0.90	-	-	-
TRYL4	SDA	-	-	1.40	-	-
TRYL5	PDA	-	-	-	-	0.90

* The inhibition diameter was an average of horizontal and vertical diameter and diameter of the fungal mycelia on gar was 7.5 mm; symbol "-" means no inhibition zone;

[#] The codes for the fungal isolates: the first two letters denote the abbreviation of provinces (see Table 4.1); two letters after the abbreviation of provinces denote part of plant (see Table 4.1) and number denote isolate number. For example, PTYL6 denote isolate number 6 isolated from young leaves of plant collected from Phatthalung province.

Table 4.4 The number of endophytic fungi isolates from four provinces producing antimicrobial metabolites.

Province	Number of endophytic fungi	Number of endophytic fungi which exhibited antimicrobial activities	Percentage of active isolates (%)
Krabi	10	4	40
Nakhon sithammarat	16	3	18.75
Phatthalung	17	8	47.06
Trang	13	11	84.62
Total	**56**	**26**	**-**

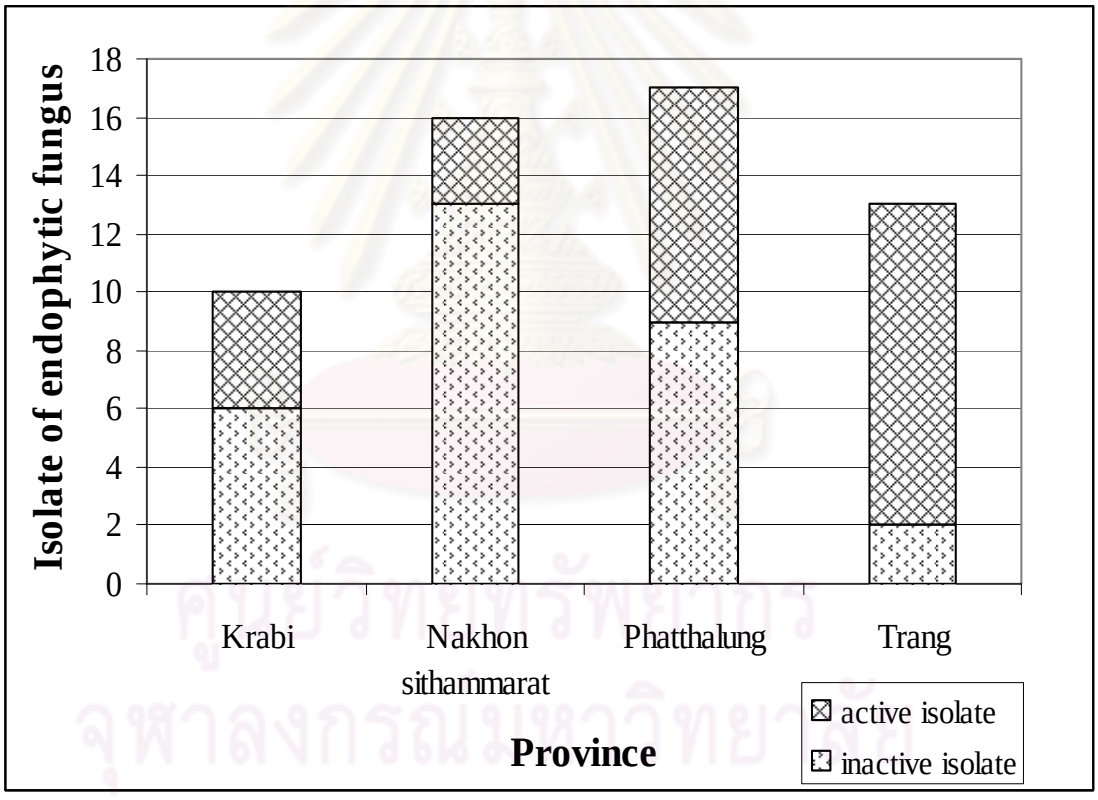


Figure 4.1 Isolates of active and inactive of endophytic fungi isolate inhibiting antimicrobial activity from four provinces.

Table 4.5 Number of endophytic fungi produced antimicrobial metabolites against 5 microorganisms.

Test microorganisms		Number of active isolates	Percentage of active isolates (%)
Gram + bacteria	*Bacillus subtillis*	**9**	**16.08**
	Staphylococcus aureus	**6**	**10.71**
Gram – bacteria	*Escherichia coli*	**11**	**19.64**
	Pseudomonas aeruginosa	**10**	**17.86**
Yeast	*Candida albicans*	**20**	**35.71**

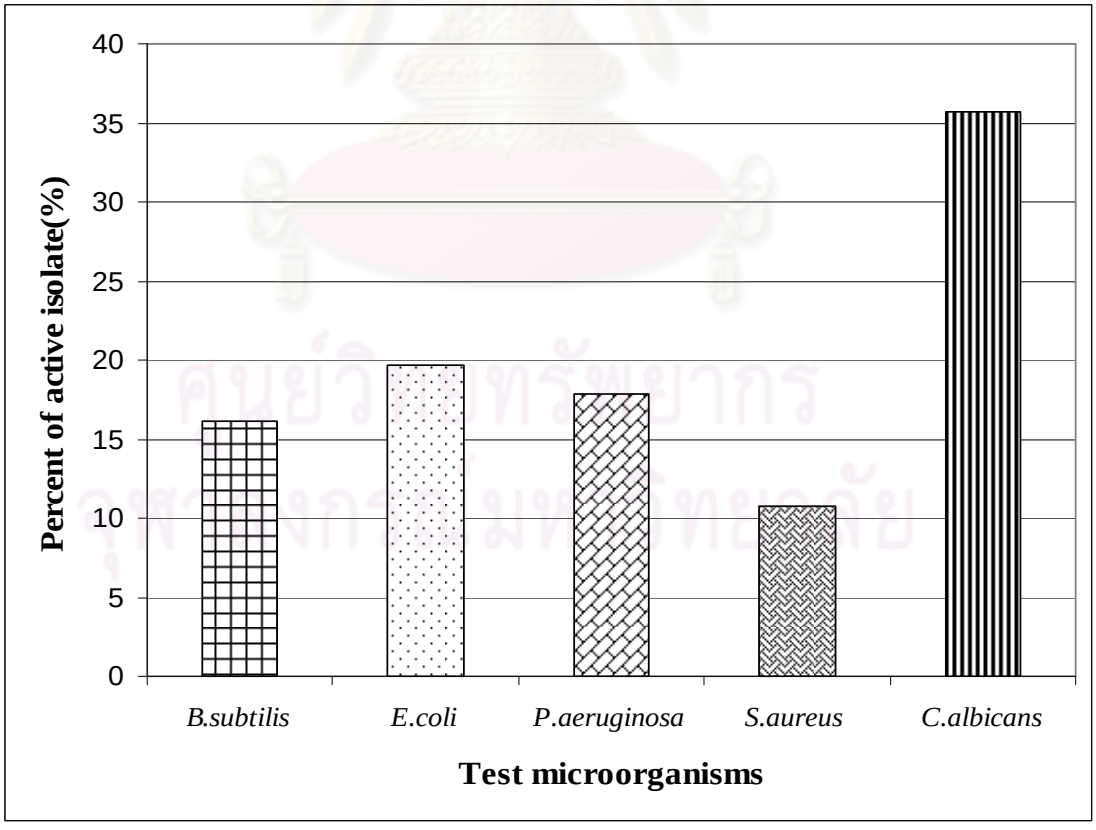


Figure 4.2 Percent of active of endophytic fungal isolates inhibiting antimicrobial activity against five microorganisms.

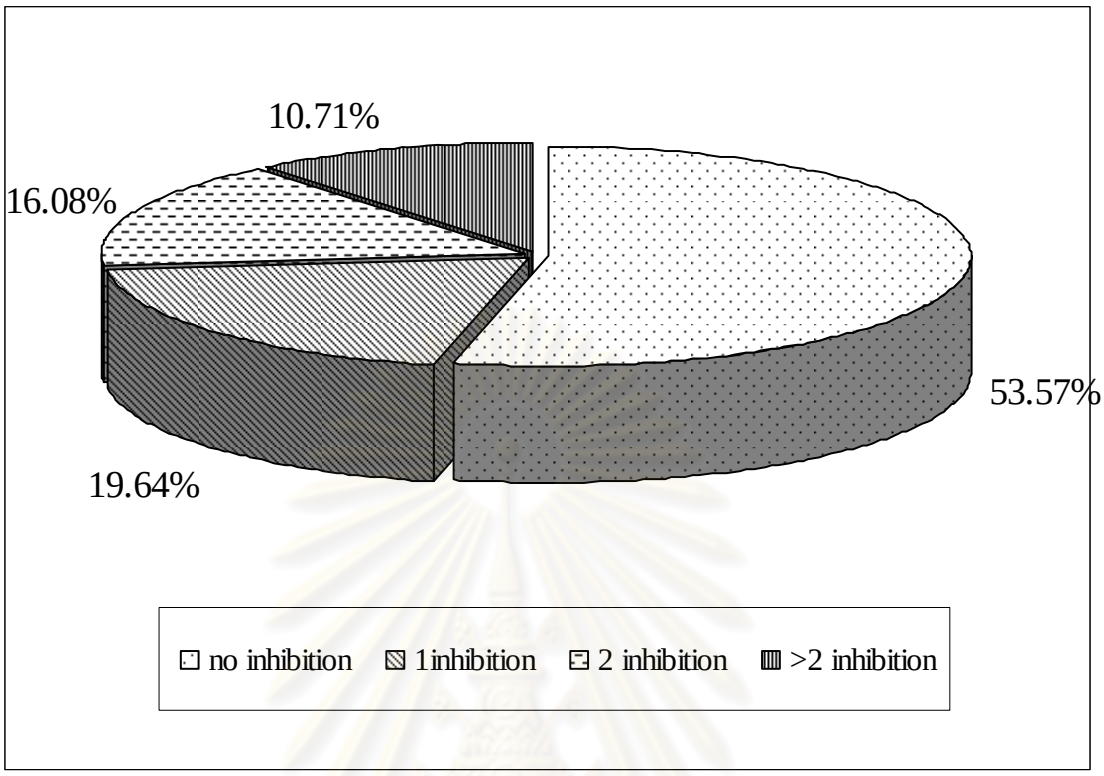


Figure 4.3 Percentage of active of endophytic fungal isolates exhibiting antimicrobial activity against one, two and ,ore than two test microorganisms.

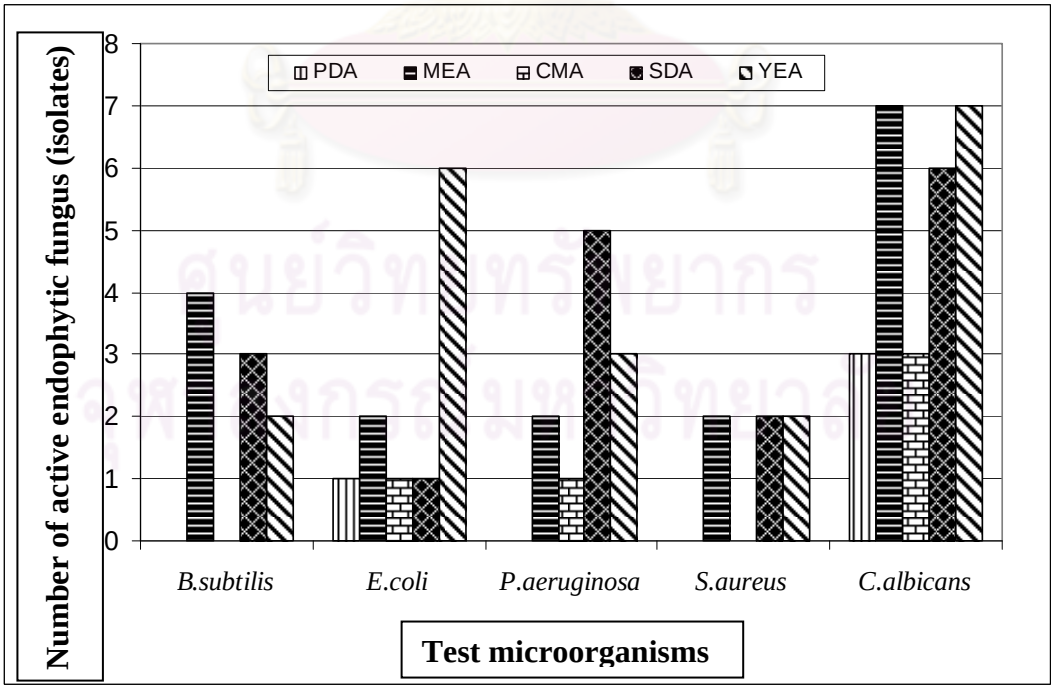
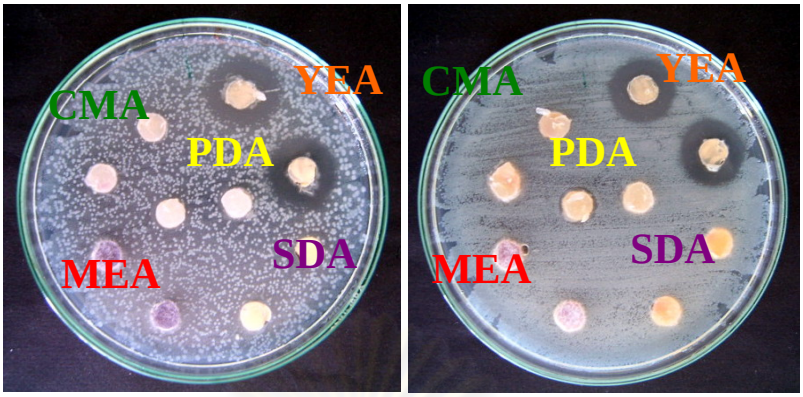
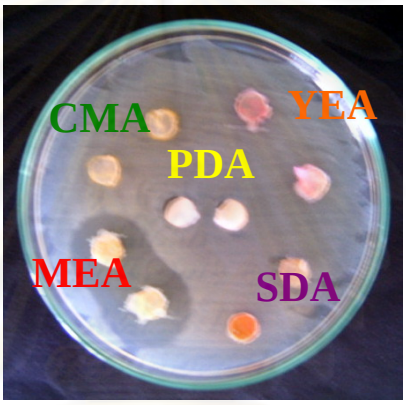


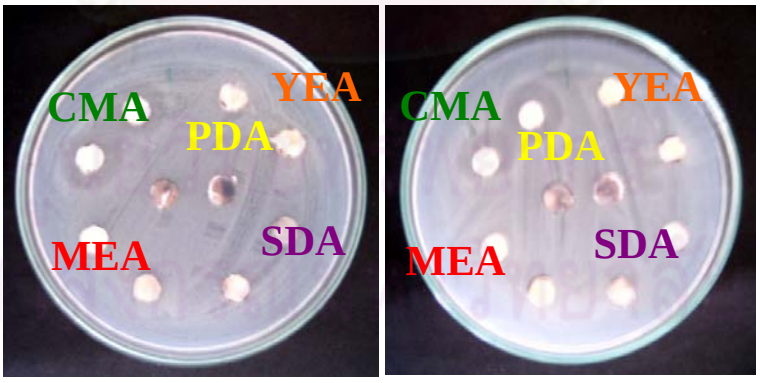
Figure 4.4 Metabolites of endophytic fungal isolates cultured on five media exhibiting antimicrobial activity against each test microorganism.



(A) PTYL6 against *Escherichia coli* and *Candida albicans*

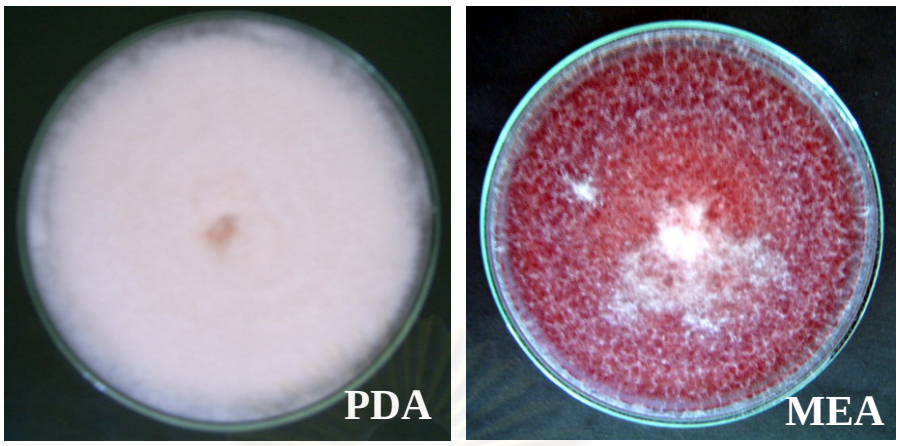


(B) PTOL5 against *Candida albicans*

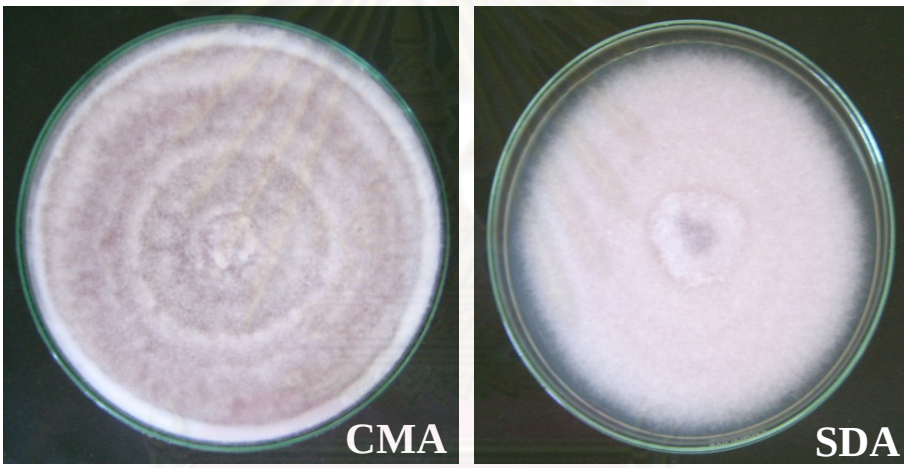


(C) TROL1 against *Escherichia coli* and *Pseudomonas aeruginosa*

Figure 4.5 Dual culture activity assay of endophytic fungal isolates PTYL6, PTOL5 and TROL1 cultured on five media exhibiting antimicrobial activity against test microorganisms.



Potato Dextrose Agar (PDA) Malt extract agar (MEA)



Corn meal agar (CMA) Sabouraud's dextrose agar (SDA)

Yeast Extract Sucrose Agar (YEA)

Figure 4.6 Colony of endophytic fungus isolate PTYL6 grown on 5 agar media for 14 days at room temperature.

4.4 Identification of the endophytic fungi PTYL6

PTYL6 was identified based on fungal morphology and analysis of the DNA sequence of the ITS region. Fungal isolate PTYL6 produced microconidia and macroconidia (Figure 4.6) which was identified as *Fusarium* sp. (Fournier and Magni, 2002)

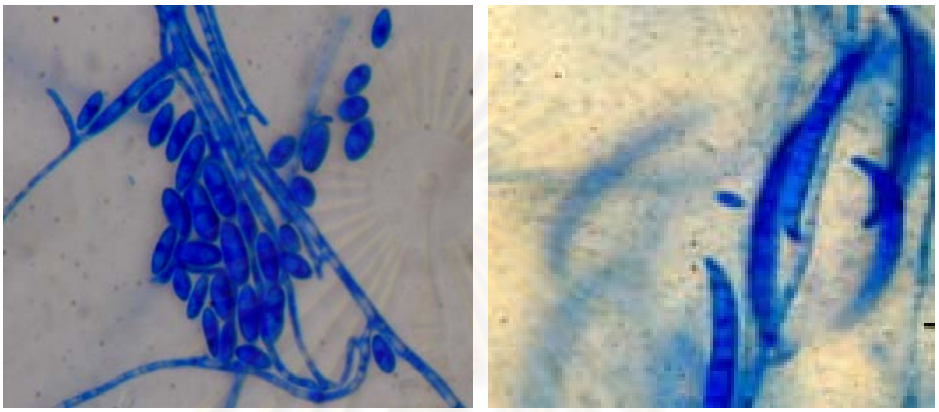


Figure 4.7 Spore of endophytic fungus isolate PTYL6
Microconidia (A) Macroconidia (B)

Total DNA was extracted from fungal mycelium grown in PDB followed in 3.2.4.1. Primers ITS1f and ITS4 were used to amplify the ITS1-5.8S-ITS4 region from total DNA extracted. The thermal cycle program was as follows: 94^{o}C for 5 min, followed by 38 cycles of 94^{o}C for 1 min, 51^{o}C for 1 min and 72^{o}C for 1 min, with a final extension of 72^{o}C for 5 min. The amplified DNA was purified and directly subjected to sequencing by Macrogen (Seoul, South Korea) using the same primers as for amplification. BLASTN 2.2.15 was used to search for similar sequences in the GenBank.

The ITS fragment length of fungal isolate PTYL6 was 531 bp fragment as shown in Figure 4.3. The nucleotide sequence data of PTYL6 was submitted in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers EF 453121.1. A blast search was performed to find a similar sequence to ITS region of fungal isolate PTYL6 in the GenBank DNA database. The ITS region of isolate PTYL6 was similar to 93% identity of *Fusarium* sp. Alignment data of ITS region of isolates PTYL6 was showed in Appendix D.

```
5′TTAAGTTCAG CGGGTATTCC TACCTGATCC GAGGTCAACC TTGATAAATT
  AGGGGTTTTA CGGCAGGGGA CCGGTCCAAC TAATAGGCGA GATAATATTT
  ACTACGTCTA GAGTGTGAAC CGACTCCGCC ACTAATTTTA AGGGGCTACC
  GCCATACGGT AGGCCCCCAA CGCTAAGCAA CAGAAGGCTT AAGGGTTGAA
  ATGACGCTCG AACAGGCATG CCCACTAGAA TACTAATGGG CGCAATGTGC
  GTTCAAAGAT TCGATGATTC ACTGAATTCT GCAATTCACA TTACTTATCG
  CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT CCGTTGTTGA
  AAGTTTTAAC TTATTTAGTT GTAATTCAGA TATCCAGTAA TTAAACAGAG
  TTTAATGGGG CGCCGGCGGG CTTACCCGTG CCTACCGGGT AGGCACTTAC
  AGGTAAGTGC ACTACAGGGT AGGTACGACC CGCCGAGGCA ACGTTAGGTA
  TGTTCACATG GGGTTTGGGA GTTATAAACT CTTTAATGAT CCCTCCGCTG
  GTTCACCAAC GGAGACCTTG TTACGACTT 3′
```

Figure 4.8 Nucleotide sequences of partial 18S region, complete ITS region of the isolate PTYL6.

4.5 Growth profile and biological activity test of culture broth

4.5.1 Growth profile and antimicrobial activity test of PTYL6 culture broth

The endophytic fungus PTYL6 was cultured into 250 mL flask containing 100 mL of YEB and culture under static condition at room temperature for 18 days. The culture broth was filtered through filter paper (Whatman No. 1). The mycelia were measured the cell mass and antimicrobial activity of fermentation broth was examined using agar well diffusion method (Jorgensen, 1999) in 7.5 diameter of each well against 5 microorganisms. The results are presented in Table 4.6, and Figure 4.9 and 4.10.

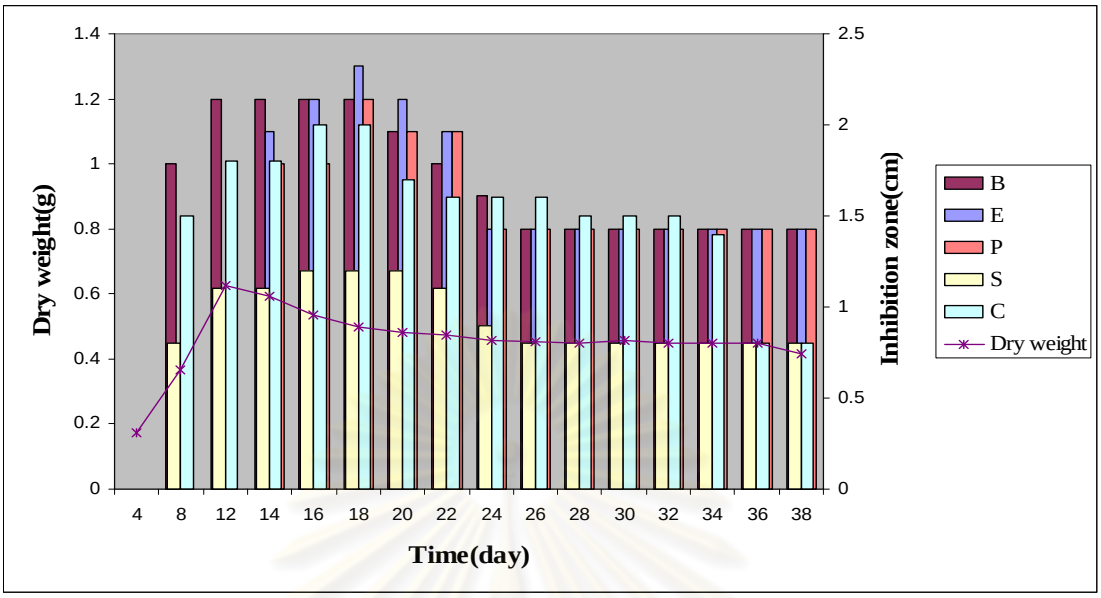
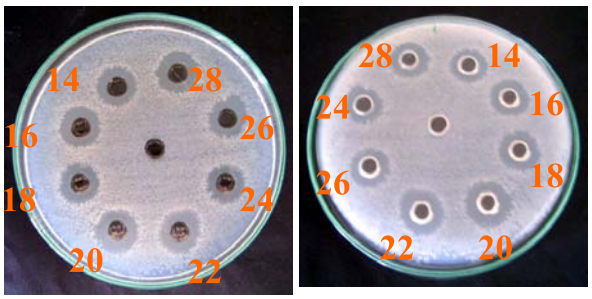


Figure 4.9 Growth profile of PTYL6 and antimicrobial activity of its fermentation broth

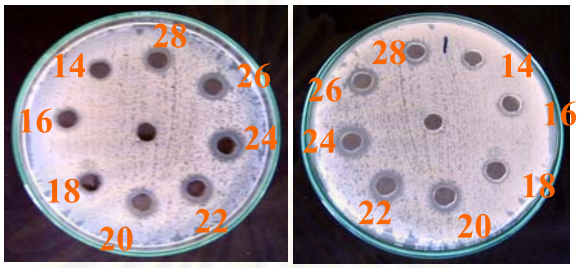
Table 4.6 Biological activity test of PTYL6 culture broth against tested microorganisms

PTYL6 culture broth (days)	Inhibition zone diameter (mm) against tested microorganisms*				
	B. subtilis ATCC 6633	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*C. albicans* ATCC10231
8	-	1.00	-	0.80	1.50
10	-	1.10	-	1.00	1.50
12	-	1.20	-	1.10	1.90
14	1.10	1.20	1.00	1.10	1.80
16	1.20	1.20	1.00	1.20	2.00
18	1.30	1.20	1.20	1.20	2.00
20	1.20	1.10	1.10	1.20	1.95
22	1.10	0.80	1.10	1.10	2.00
24	0.80	0.80	0.80	0.80	1.70
26	0.90	0.80	0.80	0.90	1.80
28	0.80	0.80	0.80	0.80	1.50
30	0.80	0.80	0.80	0.80	1.80
32	0.80	0.80	0.90	0.80	1.60
34	0.80	1.00	0.80	0.80	-
36	0.80	0.90	0.80	0.80	-
38	0.80	1.00	0.80	0.80	-

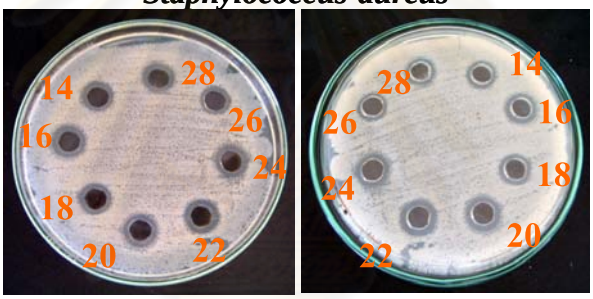
- : no inhibition zone: *Activity was determined by agar well diffusion method



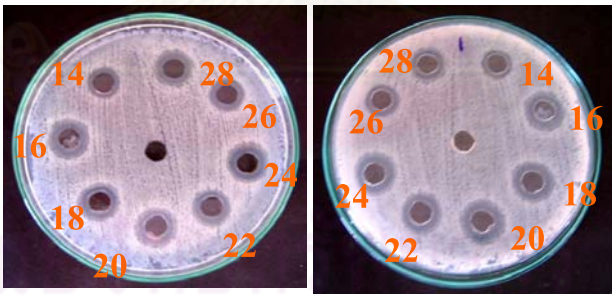
Bacillus subtilis



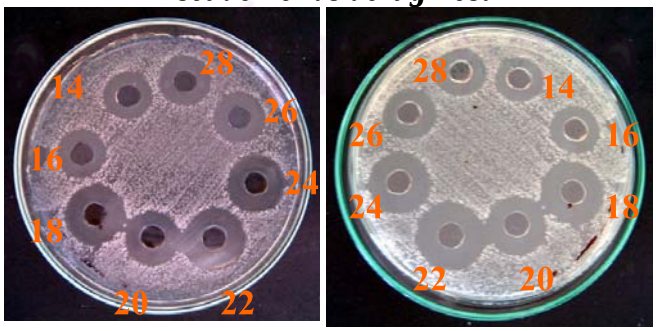
Staphylococcus aureus



Escherichia coli



Pseudomonas aeruginosa



Candida albicans

Figure 4.10 Metabolites in PTYL6 culture broth against tested microorganisms using agar well diffusion method

4.6 Chemical investigation of the metabolites of the fungi PTYL6

After the endophytic fungus PTYL6 was cultivated in yeast extract sucrose broth (YEB) under static condition at room temperature for 18 days the whole culture (35 L) was filtered through filter paper (Whatman no. 1). The filtrate (~35 L) was concentrated by rotary evaporator under reduced pressure and by freeze dryer to obtain 2500 mL of the concentrated filtrate and then partitioned with an equal volume of hexane, EtOAc and MeOH, respectively. After removal of the solvents hexane, EtOAc and MeOH extracts were obtained in 4.51 g, 25.89 g, 5.21 g and 38.23 g, respectively. The MeOH crude extract was further extracted with a 4:1 mixture of CH_2Cl_2 and MeOH followed by removal of the solvent to yield 5.21 g of a CH_2Cl_2-MeOH (4:1) extract and 32.21 g of residual MeOH extract, respectively.

Fungal mycelia were dried in hot air oven at 55 ^{o}C for 24 hours and extracted successively with hexane, EtOAc and MeOH to give the hexane extract (1.70 g), as yellow viscous oil, the EtOAc extract (3.07 g) as orange viscous residue and MeOH extract (21.15g) as brown viscous oil respectively. Extraction diagram of mycelia and broth of endophytic fungus PTYL6 were shown in Figure 3.2

4.6.1. Characterization of compound 1

Compound **1** was obtained from precipitation during evaporation of the solvent of the EtOAc extract of mycelia and from the elution of column chromatography with 40% hexane in EtOAc (combined fractions 6MyEt 6, 7, 8, 9 and 10) and further purified by washing with 70% to 50% hexane in EtOAc, give compound **1** as a white solid which was soluble in EtOAc, $CHCl_3$ and MeOH

$[\alpha]_{D}^{20} + 9^{\circ}$ (*c* 0.2, $CHCl_3$)

^{1}H-NMR spectrum: δ ($CHCl_3$, 400 MHz) 0.643 (2H, *d*, *J* = 6.4 Hz), 0.721 (2H, *d*, *J* = 1.9 Hz), 2.111(1H, *hept*, *J* = 6.8 Hz), 2.885 (1H, s), 3.14 (3H, *dd*, *J* = 2.8 Hz), 4.522 (1H, *brs*), 7.059 (1H, *d*, *J* = 5.6 Hz), 7.237 (1H, *d*, *J* = 5.6 Hz) and 7.244 (1H, s) ppm.

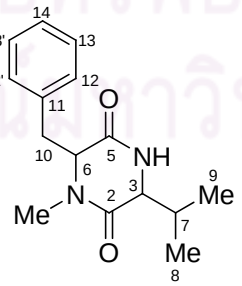
^{13}C-NMR spectrum: δ ($CHCl_3$, 400 MHz) 13.94**,** 17.49**,** 29.34, 31.31, 36.32**,** 62.30**,** 80.68**,** 127.68**,** 128.21, 128.62, 128.69**,** 129.61**,** 134.85, 166.03, 167.62 ppm.

R_f 0.43 developed on silica gel TLC using 60% hexane in EtOAc as the mobile phase and the spot was visualized with UV lamp (254, 365 nm).

Table 4.7 ^{1}H, ^{13}C and 2D (HMBC) NMR data for compound **1**

Position	δ_C		δ_H **(int., mult., *J* in Hz)[a]**	**HMBC (H→C)**
1	-	N	-	-
2	166.03	CO	-	-
3	80.68	CH	2.91 (1H, s)	C-7, C-8, C-9
4	-	N	-	-
5	167.62	CO	4.54 (1H, *brs*)	-
6	62.30	CH	2.13(1H, *hept*, 6.8)	-
7	29.34	CH	2.78 (1H, s)	C-8, C-9
8	17.49	CH_3	0.74 (2H, *d*, 7.2)	C-3
9	13.94	CH_3	0.66 (2H, *d*, 6.8)	C-3
10	36.32	CH_2	3.16 (1H, *dd*, 2.8, 14)	C-12, C-12′, C-5
-	-	-	3.22 (obsecued by the residual solvent of CD_3OD)	-
11	134.85	C	-	-
12	128.69	CH	7.08 (1H, *d*, 5.2)	-
12′	129.61	CH	7.08 (1H, *d*, 5.2)	C-12, C-14
13	127.68	CH	7.26 (2H, *d*, 5.6)	C11, C-13
13′	128.21	CH	7.26 (2H, *d*, 5.6)	-
14	128.62	CH	7.16 (1H, *d*, 1.6)	C-12′
Me-N	31.31	CH_3	2.95 (2H, s)	-

The information from ^{1}H-NMR, ^{13}C-NMR and 2D-NMR (HMBC techniques) were used to assist the interpretation of compound **1**.



6-benzyl-3-isopropyl-1-methylpiperazine-2,5-dione

Figure 4.11 Structure of compound **1.**

4.6.2 Characterization of compound 2

Compound **2** was obtained from the elution of column chromatography with 85% hexane in EtOAc and further purified by washing with 90% hexane in EtOAc to give compound **2** as a white solid and dissolved in EtOAc, $CHCl_3$ and MeOH

$[\alpha]_D^{20}$ -129° (*c* 0.2, $CHCl_3$)

^{1}H-NMR spectrum ($CHCl_3$, 400 MHz) (Figure D 1-3)important peaks at δ_H 5.56, 5.38, 5.19 and 5.18 ppm, six methyl groups at δ_H 1.03, 0.93, 0.90, 0.82, 0.82 and 0.62 ppm, a proton of CH-OH at 3.63 and proton of methylene and methane at 2.47-1.24 in the ring and side chain.

^{13}C-NMR spectrum ($CHCl_3$, 100 MHz) (Figure D 1-3) important peaks showed six olefinic carbons at δ_C 141.37, 139.77, 135.56, 131.94, 119.37 and 116.26 ppm, six methyl carbons at δ_C 21.09, 19.96, 19.65, 17.61, 16.27 and 12.04 ppm and a carbon of heteroatom CO-OH at 70.44 ppm.

EI MS spectrum at *m/z* = 396 and the molecular formula is $C_{28}H_{44}O$

R_f 0.87 developed on silica gel TLC using 60% hexane in EtOAc as the mobile phase and the spot was visualized with UV lamp (254, 365 nm).

Compound 2 showed spectral data identical to that of ergosterol which is reported in the literature. The 1H-NMR signals of compound 2 and ergosterol are presented in Table 4.8.

Table 4.8 Comparison of 1H-NMR spectrum of compound **2** and ergosterol.

Position	Compound 2	Ergosterol
1	38.35	38.4
2	31.97	32.0
3	70.44	69.5
4	40.77	40.5
5	139.76	140.5
6	119.56	119.2
7	116.25	116.5
8	141.37	140.4
9	46.21	46.3
10	37.01	37.0
11	21.09	21.0
12	39.05	39.2
13	42.80	42.8
14	55.69	54.4
15	22.98	22.9
16	28.29	28.1
17	55.69	55.8
18	12.04	11.6
19	16.27	15.8
20	40.43	40.3
21	19.64	19.2
22	131.94	132.0
23	135.55	135.8
24	42.80	42.8
25	33.07	33.0
26	19.64	19.5
27	21.09	20.8
28	17.60	17.2

After elucidation of compound **2** by the chemical shift on ^{1}H-NMR spectrum of compound **2** and ergosterol were compared signal by signal. This result indicated that the structure of compound **2** is identical to ergosterol. Thus, it could be concluded that compound **2** was ergosterol. The structure is presented in Figure 4.12.

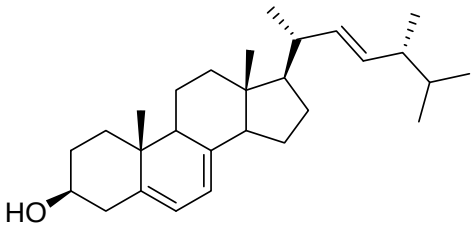


Figure 4.12 Structure of compound **2.**

Ergosterol is a component of fungal cell membranes, serving the same function that cholesterol serves in animal cells. The presence of ergosterol in fungal cell membranes coupled with its absence in animal cell membranes makes it a useful target for antifungal drugs. Ergosterol is also present in the cell membranes of some protists, such as trypanosomes (Roberts *et. al.*, 2003)**.**

Smith and Korn (1968) Report, the sterol fraction of *Acanthamoeba* (Neff) contains 60% 7-dehydrostigmasterol and 40% ergosterol. The sterols were characterized by infrared and ultraviolet spectroscopy, gas chromatography, specific reactions, and massspectral analysis. Sterols constitute 5% of the lipids and 15% of the neutral lipids in this species.

In 2006, He *et al.* reported ergosterol is produced by fungi, *Saccharomyces cerevisiae* YEH56 (pHXA42) with increased capacity of ergosterol formation was constructed by combined over expression of sterol C-24(28) reeducates and sterol acyltransferase in the yeast strain YEH56.

4.6.3 Structure elucidation of compound 3

Compound **3** was obtained from the elution of column chromatography with 100% MeOH and further purified by washing with 10% H_2O in MeOH to give compound **3** as a white solid and dissolved in MeOH and DMSO.

^{1}H-NMR spectrum (DMSO, 400 MHz) 5.46 (1H, dd, *J*= 0.60 and 2.6 Hz, 5-H) and 7.40 (1H, d, J = 5.6, 6-H) ppm;

^{13}C-NMR spectrum (DMSO, 100 MHz) (δ, ppm): 100.7 (C-5), 142.7 (C-6), 152.0 (C-2) and 164.3 (C-4) ppm;

R_f 0.67 developed on silica gel TLC using 80% $CHCl_3$ in MeOH as the mobile phase and the spot was visualized with UV lamp (254 nm).

The EI-MS mass spectrum (HR/ES-TOF MS spectrum) of compound 3 showed $[M+H]^+$ *m/z*: 113.0370; cal 113.0351

The information from 1H-NMR, 13C-NMR and 2D-NMR including gHMBC, gHMBC and gNOESY techniques were used to assist the interpretation of compound **3**. The 1D and 2D NMR data of compound 3 are presented in Table 4.9.

Table 4.9 Data 1D and 2D of compound **3**

Position	^{13}C-NMR (δ_C)	^{1}H-NMR (δ_H)	gHMBC	gCOSY	gNOESY
1	NH	10.88	-	H-6	H-6
2	152.0	-	-	-	-
3	NH	11.04	-	-	H-6
4	164.3	-	-	-	-
5	100.7	5.46 (1H, dd, *J*= 0.60 and 2.6 Hz, 5-H)	C-4,C-6	H-6	H-6
6	142.7	7.40 (1H, d, J = 5.6, 6-H)	C-2, C-4,C-5	H-1, H-5	H-1,H-3,H-5

After elucidation of compound **3** by the data 1D and 2D of compound **3** result indicated that the structure of compound **3** is identical to uracil. Thus, it could be concluded that compound **3** was uracil. The structure is presented in Figure 4.12.

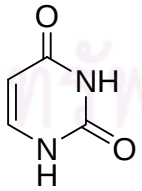


Figure 4.13 Structure of compound **3.**

4.7 Antimicrobial activity test

4.7.1 Paper disk diffusion method

Crude extract were evaluated the antimicrobials activity against 5 microorganisms by the disk diffusion method (Jorgensen, 1999). The result was showed the diameter of inhibition zone (mm) in Table 4.10.

Table 4.10 Antimicrobial activities of the extracts of mycelia and culture broth of the fungi PTYL6.*

Crudes (conc; 100 µg/mL /disk)	**Inhibition zone (mm)#**				
	B. subtilis	***E. coli***	***P. aeruginos***	***S. aureus***	***C. albicans***
Mycelium Hexane	-	-	-	-	-
Mycelium EtOAc	1.40	1.10	1.40	1.50	1.80
Mycelium MeOH	1.00	1.00	0.80	1.00	-
Broth Hexane	-	-	-	-	0.80
Broth EtOAc	1.50	1.10	1.40	1.50	2.00
Broth MeOH	1.50	1.00	1.50	1.50	1.40
Broth residue	1.20	1.10	1.00	1.10	1.20
10%DMSO in H_2O	-	-	-	-	-

* Antimicrobial activities were performed by paper disc diffusion assay
# The inhibition diameter was an average of horizontal and vertical diameter and diameter of paper disc was 8 mm; symbol "-" means no inhibition zone.

4.7.2 Minimum inhibition concentration methods

Compound 1, 2 and 3 were further determined minimum inhibition concentration (MIC) using microdilution method (Jorgensen, 1999)

The concentration of all compound were investigated as 250 µg/mL and then dilution two-fold to 0.0488 µg/mL (last concentration). MIC result were exhibited the lowest concentration of pure compound showing complete inhibition of growth (Table 4.19).

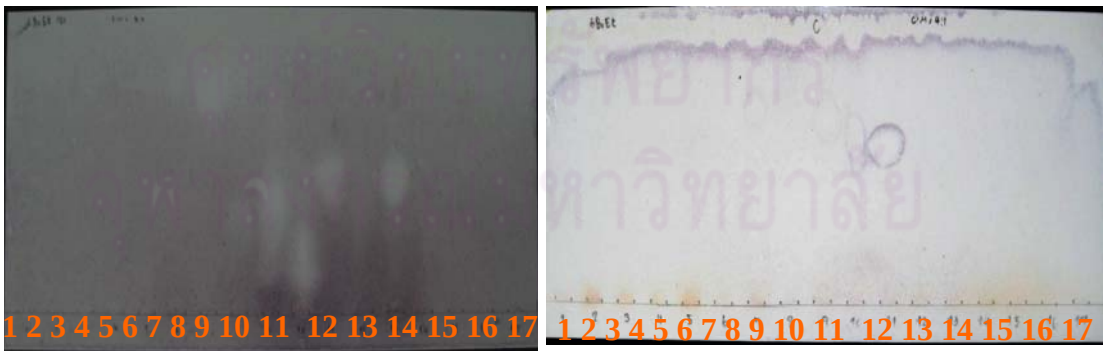
Table 4.11 Minimum inhibition concentration (MIC) of compounds **1-3**[*]

Test microorganism	MIC (μg/mL)[#]		
	Compound 1	**Compound 2**	**Compound 3**
B. subtilis	3.91	250	-
E. coli	7.89	250	-
P. aeruginos	31.25	-	-
S. aureus	31.25	-	-
C. albicans	15.63	-	-

[*] Minimum inhibition concentration (MIC) was determined by microdilution method (Jorgensen, 1999)
[#] - means no inhibition zone

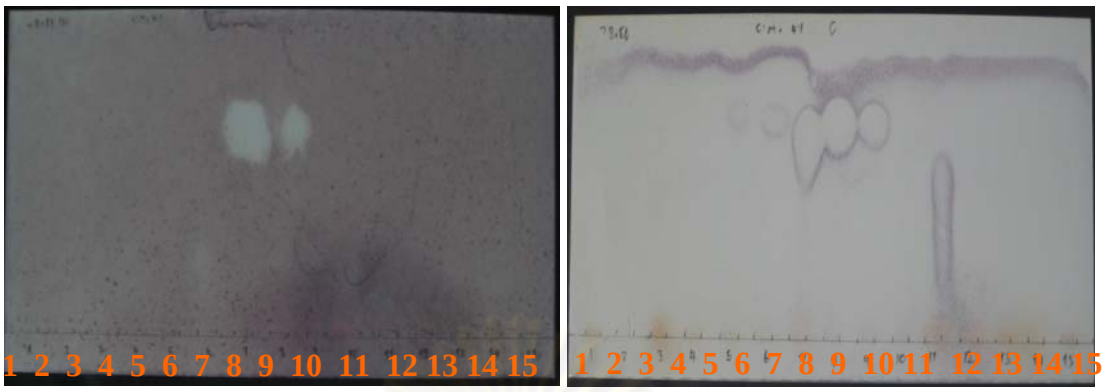
4.8 Antimicrobial activity test of combined fractions from isolation of EtOAc extract, CH_2Cl_2-MeOH (4:1) extract and MeOH extract residue of broth

The combined fractions from isolation of EtOAc extract, CH_2Cl_2-MeOH (4:1) extract and MeOH extract residue of broth by Sephadex LH-20 column chromatography were analyzed by TLC developed by $CHCl_3$-MeOH (4:1) and antimicrobial metabolites were examined using bioautography method (Rahalison *et al.*, 1991). Also minimum inhibition concentration (MIC) of those combined fractions was determined using microdilution method (Jorgensen, 1999). The results were presented in Figures 4.14-4.18 and Table 4.12.



Staphylococcus aureus *Candida albicans*

Figure 4.14 Bioautography of the sixth portion of the combined fractions from the isolation of EtOAc extract of broth.

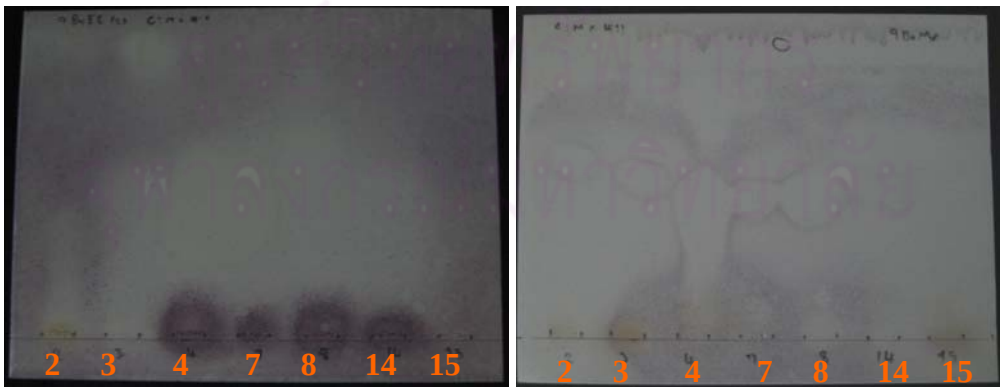


Staphylococcus aureus *Candida albicans*

Figure 4.15 Bioautography of the seventh portion of the combined fractions from the isolation of EtOAc extract of broth.

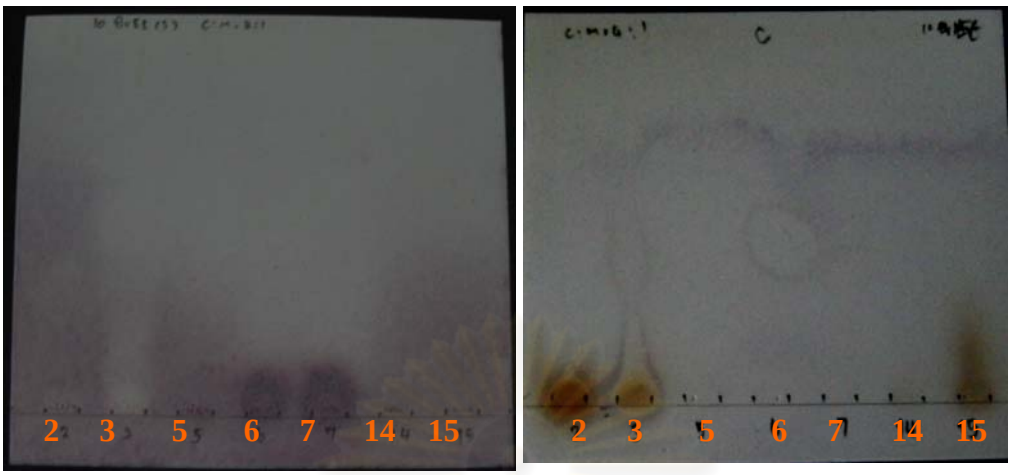
Staphylococcus aureus *Candida albicans*

Figure 4.16 Bioautography of the eighth portion of the combined fractions from the isolation of EtOAc extract of broth.



Staphylococcus aureus *Candida albicans*

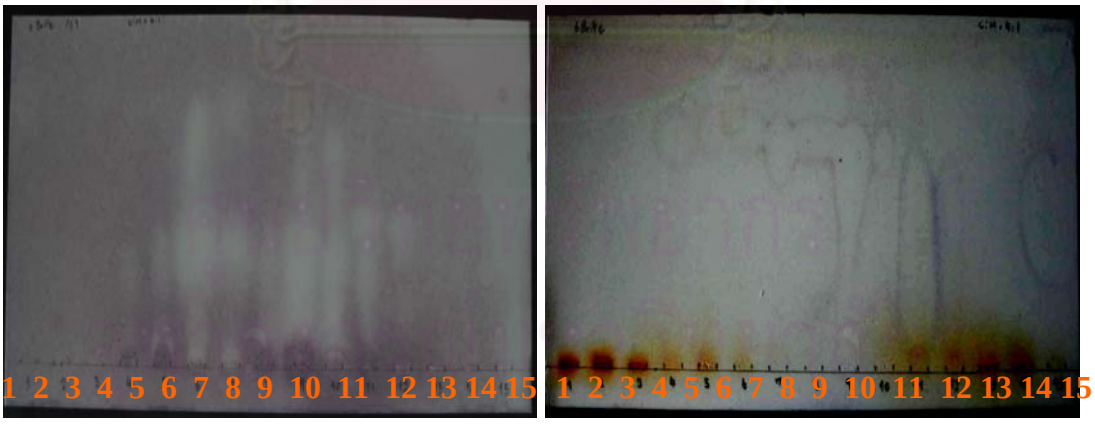
Figure 4.17 Bioautography of the ninth portion of the combined fractions from the isolation of EtOAc extract of broth.



Staphylococcus aureus *Candida albicans*

Figure 4.18 Bioautography of the tenth portion of the combined fractions from the isolation of EtOAc extract of broth

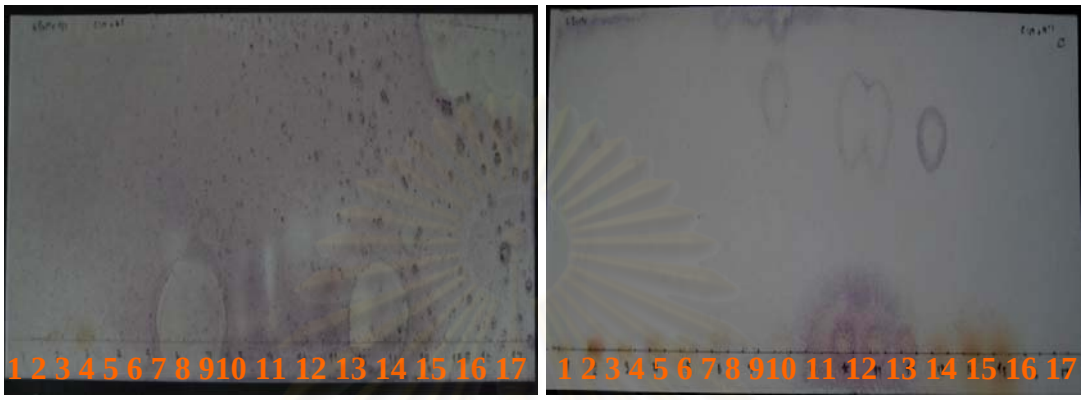
From bioautography of the combined fractions from the isolation of EtOAc extract of broth as shown in Figures 4.14-4.18, it showed that the antimicrobial metabolites were at R_f 0.75-0.53 and 0.50-0.38.



Staphylococcus aureus *Candida albicans*

Figure 4.19 Bioautography of the combined fractions from the isolation of CH_2Cl_2-MeOH (4:1) extract of broth

From bioautography of the combined fractions from the isolation of CH_2Cl_2-MeOH (4:1) extract of broth as shown in Figure 4.19, it showed that the antimicrobial metabolites were at R_f 0.78-0.57 and 0.63-0.28.

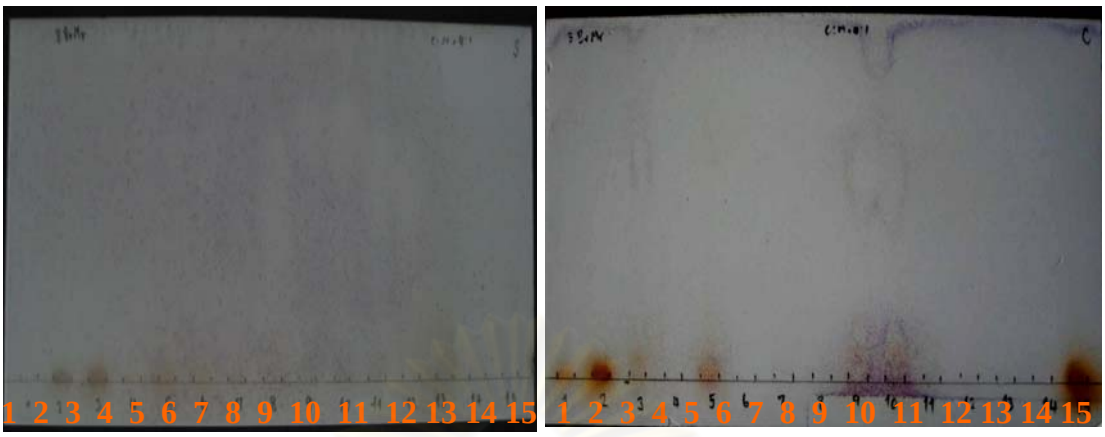


Staphylococcus aureus *Candida albicans*

Figure 4.20 Bioautography of the sixth portion of the combined fractions from the isolation of MeOH extract residue of broth

Staphylococcus aureus *Candida albicans*

Figure 4.21 Bioautography of the seventh portion of the combined fractions from the isolation of MeOH extract residue of broth.



Staphylococcus aureus *Candida albicans*

Figure 4.22 Bioautography of the eighth portion of the combined fractions from the isolation of MeOH extract residue of broth.

From bioautography of the combined fractions from the isolation of CH_2Cl_2-MeOH (4:1) extract of broth as shown in Figures 4.20-4.22 it showed that the antimicrobial metabolites were at R_f 0.78-0.57 and 0.63-0.28.

Minimum inhibition concentration (MIC) of those combined fractions was determined using microdilution method (Jorgensen, 1999). The results (Tables 4.12) showed that those combined fractions were active against *Staphylococcus aureus* and *Candida albicans*. The fractions were active against *Staphylococcus aureus* and *Candida albicans* with MIC 25-50 ug/mL. However, some active fractions were isolated by silica gel TLC but they were so complicated to be isolated to give any pure compounds.

Table 4.12 The minimum inhibition concentration (MIC) using microdilution method of combine fraction from broth extract.

Fractions	Antimicrobial activity	
	S.aureus	*C. albicans*
6BrEt1	-	-
6BrEt2	-	-
6BrEt3	-	-
6BrEt4	-	-
6BrEt5	-	-
6BrEt6	-	-
6BrEt7	50	50
6BrEt8	-	-
6BrEt9	25	25
6BrEt10	25	25
6BrEt11	25	50
6BrEt12	-	-
6BrEt13	-	-
6BrEt14	-	-
6BrEt15	-	-
7BrEt1	-	-
7BrEt2	-	-
7BrEt3	-	-
7BrEt4	-	-
7BrEt5	200	200
7BrEt6	200	200
7BrEt7	25	25
7BrEt8	25	25
7BrEt9	25	25
7BrEt10	-	-
7BrEt11	25	50

Fractions	Antimicrobial activity	
	S.aureus	*C. albicans*
7BrEt12	-	-
7BrEt13	2	-
7BrEt14	-	-
7BrEt15	-	-
8BrEt1	-	-
8BrEt2	-	-
8BrEt3	-	-
8BrEt4	-	-
8BrEt5	-	-
8BrEt6	-	-
8BrEt7	500	100
8BrEt8	100	50
8BrEt9	-	-
8BrEt10	25	25
8BrEt11	-	-
8BrEt12	50	50
8BrEt13	500	100
8BrEt14	500	100
8BrEt15	-	-
8BrEt16	-	-
8BrEt17	-	-
9BrEt2	25	200
9BrEt3	50	100
9BrEt4	25	200
9BrEt5	-	-
9BrEt6	-	-

Table 4.12 (Continued)

Fractions	Antimicrobial activity	
	S.aureus	*C. albicans*
9BrEt7	500	200
9BrEt8	100	200
9BrEt9	-	-
9BrEt10	-	-
9BrEt11	-	-
9BrEt12	-	-
9BrEt13	-	-
9BrEt14	-	-
9BrEt15	-	-
10BrEt1	-	-
10BrEt2	500	200
10BrEt3	50	50
10BrEt4	-	-
10BrEt5	-	-
10BrEt6	500	200
10BrEt7	-	-
10BrEt8	-	-
10BrEt9	-	-
10BrEt10	-	-
10BrEt11	-	-
10BrEt12	-	-
10BrEt13	-	-
10BrEt14	-	-
10BrEt15	-	-
6BrMe2	-	-
6BrMe3	-	-
6BrMe 4	100	100
6BrMe 5	100	100
6BrMe6	50	100

Fractions	Antimicrobial activity	
	S.aureus	*C. albicans*
6BrMe7		
6BrMe8	50	50
6BrMe9	50	25
6BrMe10	25	25
6BrMe11	25	50
6BrMe12	50	25
6BrMe13	50	50
6BrMe14	-	-
6BrMr1	-	-
6BrMr2	-	-
6BrMr3	-	-
6BrMr4	-	-
6BrMr5	-	-
6BrMr6	-	-
6BrMr7	-	-
6BrMr8	-	-
6BrMr9	200	50
6BrMr10	-	-
6BrMr11	200	25
6BrMr12	200	25
6BrMr13	200	50
6BrMr14	500	50
6BrMr15	-	-
6BrMr16	-	-
6BrMr17	-	-
7BrMr1	-	-
7BrMr2	-	-
7BrMr3	-	-
7BrMr4	-	-

Table 4.12 (Continued)

Fractions	Antimicrobial activity	
	S.aureus	*C. albicans*
7BrMr5	-	-
7BrMr6	-	-
7BrMr7	500	50
7BrMr8	-	-
7BrMr9	500	50
7BrMr10	100	25
7BrMr11	100	25
7BrMr12	100	25
7BrMr13	100	50
7BrMr 14	-	-
7BrMr 15	-	-
8BrMr1	-	-
8BrMr2	-	-

Fractions	Antimicrobial activity	
	S.aureus	*C. albicans*
8BrMr3	-	-
8BrMr 4	500	50
8BrMr 5	-	-
8BrMr6	-	-
8BrMr7	-	-
8BrMr8	-	-
8BrMr9	-	-
8BrMr10	100	50
8BrMr11	100	25
8BrMr12	-	-
8BrMr13	-	-
8BrMr14	-	-
8BrMr15	-	-

CHAPTER V

CONCLUSION AND DISCUSSION

The endophytic fungi from *Melaleuca cajuputi* leaves and twigs. Plant samples were collected from 4 provinces; Krabi, Nakhon sithammarat, Phatthalung, and Trang. The total of 56 endophytic fungi isolates were characterized on the basis of morphological identification, microscopic and macroscopic features. Fifty six fungal isolates were identified as *Alternaria* sp. (1 isolates), *Aspergillus* sp. (1 isolates), *Colletotrichum* sp. (1 isolates), *Fusarium* sp. (5 isolates), *Pestalotia* sp. (5 isolates), *Phomopsis* sp. (4 isolates) and the fungi in Xylariaceae family (6 isolates). Other isolates of endophytic fungi were classified as mycelia sterilia.

The metabolites in culture broth produced by 56 isolates of the endophytic fungi were examined the antimicrobial activity against 5 microorganisms using an dual agar diffusion method. The fungal isolate were examined antimicrobial activity using dual culture agar diffusion technique in 5 strains for test antimicrobial activity. The results shows that antimicrobial activity of fungal were 16.08%, 10.71%, 19.64%, 17.86% and 35.71% for *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 *and Candida albicans* ATCC 10231, respectively. These indicated that *C. albicans* was more sensitive to the isolated endophytic fungi than other tested microorganisms. The fungus PTYL6 was interested for study to isolated antimicrobial compound because it exhibited activity against *E. coli* and *C. albicans* with clear zone 19.0 and 20.0 mm respectively.

Endophytic fungi, PTYL6 were selected for the further study because it exhibited activity against *E. coli* ATCC 25922, *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923. Based on morphology, the fungi isolate PTYL6 were found as the

family Fusarium. Sequence analysis of the rDNA, partial 18S ITS region of the isolates PTYL6 were 531. bp, respectively. The sequence results suggested that PTYL6 should tentatively be *Fusrium* sp.

Isolation secondary metabolites from mycelia MeOH extracted, broth EtOAc extracted and broth hexane extracted of PTYL6 which was found a new compounds; 6-benzyl-3-isopropyl-1-methylpiperazine-2,5,dione (1) and 2 known compounds ergosterol (2) obtained from mycelium EtOAc crude and uracil (3) obtained from broth EtOAc and MeOH exteract and residue crude.

REFERENCES

Bacon, C.W. and White, J. F. 2000. **Microbial endophytes**. New York: Marcel Dekker.

Barnett, H. L. and Hunter, B. B. 1998. **Ilustrated genera of imperfect fungi**. Minnesota: APS Press.

Barron, G. L. 1977. **General of hyphomycetes from soil**. New York: Noble.

Bensky, D. and Gamble, A. 1993. **Chinese herbal medicine materia medica**. Seattle: Eastland Press.

Bischoff, J. F. and White, J. F. 2005. **In The Fungal Community**. 3rd ed. (eds.) Dighton J, White JF and Oudemans P. Taylor & Francis.

Carlile, M. J. and Watkinson, S. C. 1994. **The fungi**. San Diego: Academic Press.

Carroll, G. 1988. Fungal endophytes in stems and leaves from latent pathogen to mutualistic symbiont. **Ecology.** 69: 2-9.

Chairgulprasert, V., Kasiwut, C., Hayee, K. and Prasertsongskun, S. 2007. Antioxidant and Antibacterial activities from fruits of *Melaleca cajuputi* Powell. 33rd **Congress on Science and Technology of Thailand.**

Clay, K., and Holah, J.1999. Fungal endophyte symbiosis and plant diversity in successional fields. **Science**. 285: 1742-1744.

Dalziel, J. E., Finch, S. C. and Dunlop, J. 2005. The fungal neurotoxin lolitrem B inhibits the function of human large conductance calcium-activated potassium channels. **Toxicol. Lett.** 155: 421-426.

Dilokkunanant, U., Sukatta, U., Rugthavorn, P., Mongkolsook, Y., Rattana, W., Tanasombat, M. and Haruthanasant. V, Antimicrobial activites of cajuput oils in Thailand. 34th Congress on Science and Technology of Thailand.

Doskotch, R. W., Cheng, H. Y., Odell, T. M. and Giraed, L. 1980. Nerolidol:An antifeeding sesquiterpene alcohol for Gypsy moth larvae from *Melaleca leucadendron*. **Chem. Eco.** 6(4): 845-858.

Feath, S. H. 2002. Are endophytic fungi defensive plant mutualists?. **Oikos.** 98: 25-36.

Fisher, P. J. and Petrini, O. 1992. Fungal saprobes and pathoens as endophytes of rice (*Oryza sativa* L.). **New Phytol.** 20: 137-143.

Fournier, J. and Magni, J. F. 2002. **Pyrenomycetes from southwestern France**[Online]. Available from: http://www.pyrenomycetes.free.fr/index.htm [2009, March 14]

Garraway, M. O. and Evans, R. C. 1984. **Fungal nutrition & physiology.** New York: John Wiley.

Guo, B. G., Dai, J. R., Huang, S. Y., Waichung, C. L. and Brad, K., 2000. Carte-cytonic acids A and B: Novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonaema* species. **J. Nat. Prod.** 63: 602-604.

Guo, B.,Wang, Y., Sun, X., and Tang, K. 2008. Bioactive Natural Products from Endophytes. **Appl. Biochem. Micro.** 44: 136–142.

Hall, L. D., Gould, N. D., Siddiqi, N. J., Kelly, N. G., Hoeferlin, L. A., Morrisonb, S. J. and Wyatta, J. K. 2005. Cytosporone E racemic synthesis and preliminary antibacterial testing. **Bioorgan. Med. Chem.** 13:1409–1413.

He, X. Guo, X., Liu, N. and Zhang, B. 2007. Ergosterol production from molasses by genetically modified *Saccharomyces cerevisiae*. **Appl. Microbiol. Biotechnol.** 75: 55–60.

Huang, Y., Wang, J., Li, G., Zheng, Z. and Su, W. 2001. Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxas mairei*, *Cephalataxus fortunei* and *Torreya grandis*. **FEMS Immuno. Med. Mic.** 31: 163-167.

Isaac, S. 1992. **Fungal-plant interactions.** Cambridge: The University Printing House. 266-327.

Jorgensen, J. H., Turnidge, J. D. and Washington, J. A. 1999. Antibacterial susceptibility test: dilution and diffusion method. **Manual of Clinical Microbiology,** pp. 1526-1542.

Keller, N. P., Turner, G. and Bennett, J. 2005. Fungal secondary metabolism-from biochemistry to genomics. **Nat. Rev. Mic.** 3: 937-947

Konig, G. M., Wright, A. D., Aust, H. J., Draeger, S. and Schulz, B. 1999. Geniculol a new biologically active diterpene from the endophytic fungus *Geniculosporium* sp. **J. Nat. Prod.** 62: 155-157.

Kumaresan, V. and Suryanarayanan, T. S. 2001. Occurrence and distribution of endophytic fungi in mangrove community. **Mycol. Res.** 105: 1388-1391.

Lee, C. K. 1998a. Uraane triterpenoids from leaves of *Melaleuca leucadendron*. **Phytochemistry.** 49 (4): 1119-1122.

Lee, C. K. 1998b. A new norlupene from the leaves of *Melaleuca leucadendron*. **J. Nat. Prod.** 61: 375-376.

Lee, C. K. and Chang, M. H. 1999. Four new triterpenes from the heartwood of *Melaleuca leucadendron.* **J. Nat. Prod.** 62: 1003-1005.

Lee, C. K. 1999. Leucadenone A-D the novel class flavanone from the leaves of *Melaleuca leucadendron* L. **Tetrahedron Lett.** 40: 7255-7259.

Lu, H., Zou, W. X., Meng, J. C., Hu, J., Tan, R. X. 2000. New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua.* **Plant Sci.** 151: 67–73.

Lund, B. M. L. and Lyon, G. L. 1975. Detection of inhibitors of *Erwinia carotovora* and *E. herbicola* on thin-layer chromatograms. **J. Chromatogr.** 110: 193-196.

Maysa, L. I., Geraldo, H. S., Helder, L. T., Henrique, C. T., Alberto, J. C., Vanderlan, S. B., Maria, C. M., Ludwig, H. P. and Araujo, A. R. 2006. Antifungal metabolites from *Colletotrichum gloeosporioides* an endophytic fungus in *Cryptocarya mandioccana* Nees (Lauraceae). **Biochem. Syst. Ecol.** 34: 822-824.

Mejia, L. C., Rojas, E. I., Maynaed, Z., Bael, S. V., Arnold, A. E., Hebbar, P., Samuels, G. J., Robbins, N. and Herre, E. J. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. **Biol. Control.** 46: 4–14.

Mittermeier, R. A., Meyers, N., Gil, P. R. and Mitterier, C. G. 1999. Hotspots Earth's biologically richest and most endangered ecoregions. Top-pan Printing Co: Tokyo, Japan.

Moore, L. E. 1982. **Fundaments of the fungi.** Englewood Cliffs: Prentice-Hall.

Owen, N. L. and Hundley, N. 2004. Endophytes the chemical synthesizers inside plants. **Science Progress.** 87 (2): 79-99.

Pereda, M. R., Hermandez, L., Villavicencio, M. J., Novelo, M. and Ibarra, P. 1993. Structure and stereochemistry of peotinolides A-C, novel antimicrobial and cytotoxic 5,6-dihydro-α-pyrones from Hyptis pectinata. **J. Nat. Prod.** 56: 583-593.

Petrini, O. 1996. Ecological and physiological aspects of host-specificity in endophytic fungi. In S.C. Redlin and L.M. Carris (eds.) **Endophytic fungi in grasses and woody plants. 2nd ed.** APS Press St. Paul, pp. 87-95.

Petrini, O. 1982. Endophytic fungi in evergreen shrubs in western Oregon: A preliminary study. **Can. J. Bot.** 60: 789-796.

Pulici, M., Sugawara, F., Koshino, H., Uzawa, J. and Yoshida, S., 1996. Pestalotiopsins A and B new caryophyllenes from an endophytic fungus of *Taxus brevifolia*. **J. Org. Chem.** 61: 2122-2124.

Pongcharoen, W., Rukachaisirikul, V., Phongpaichit, S., Kuhn, T., Pelzing, M., Sakayaroj, J. and Taylor, W. C. 2008. Metabolites from the endophytic fungus *Xylaria* sp. PSU-D14. **Phytochemistry.** 69: 1900-1902.

Puri, S. C., Nazir, A., Chawla, R., Arora, R., Riyaz, H. S., Amna, T., Ahmed, B., Verma, V., Singh, S., Sagar R., Sharma A., Kumar R., Sharma R. K., Qazi, G. N. 2006. The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. **J. Biotechnol** 122: 494-510.

Pullaput, Y., Fun, H. K., Chantrapromma, S., Boonnak, N. and Chantrapromma, K. 2007. Chemical constituent from the leaves of *Melaleuca cajup*uti Powell. **Congress on Science and Technology of Thailand 7th**.

Roberts, C. W., McLeod, R., Rice D. W., Ginger, M., Chance, M. L., Goad, L. J. 2003. Fatty acid and sterol metabolism potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. **Mol. Biochem. Parasit.** 126 (2): 129–42.

Rahalison, L., Hamuburger, M., Monod, M., Frenk, E., and Hostettann, K., 1994. Antifungal test in phytochrmical investigations: comparison of Bioautographic methods using phytopathogenic and human pathogenic fungi. Planta Med. 60: 41-44.

Rodrigues, K. F. and Petrini, O. 1997. Biodiversity of endophytic fungi in tropical regions. In K. D. Hyde (ed.). **Biodiversity of tropical microfungi.** Hong Kong: United League and Graphic Printing, pp. 57-69.

Saikkonen, K., Faeth, S. H., Helander, M. and Sullivan, T. J. 1998. Fungal endophytes: A continuum of interactions with host plants. **Annu. Rev. Ecol. Syst.** 29: 319-343.

Saikkonen, K., Helander, M., FaethS.H., Schulthess, F. and Wilson, D., 1999.Endophyte-grass-herbivore interactions:the case of Neotyphodium endophytes in Arizona fescue populations. **Oecologia.** 121: 411–420.

Saikkonen, K., Wali, P., Helander, M. and Faeth, S. H. 2004. Evolution of endophyte-plant symbioses. **TRENDS in Plant Science.** 9 (6): 275-280.

Schwarz, M., Kopcke, B., Weber, R. W. S., Sterner, O. and Anke, H. 2004. 3-hydroxypropionic acid as a nematicidal principle in endophytic fungi. **Phytochemistry.** 65: 2239-2245.

Schardl, C. L. and Leuchtmann, A. 2005. **In The Fungal Community.** 3rd ed. (eds) Dighton J, White JF and Oudemans P. Taylor & Francis.

Smith, F. R. and Korn, E. D. 1968. 7-Dehydrostigmasterol and ergosterol: the major sterols of an amoeba. **J. Lipid. Res.** 9: 405-408.

Stone, J. K., Polishook, J. D. and White, Jr. J. F. 2004. Endophytic fungi. In G. M. Mueller, G. F. Bills and M. S. Foster (eds.), **Biodiversity of fungi inventory and monitoring methods,** pp. 241-270

Strobel, G. and Daisy, B. 2003. Bioprospecting for microbial endophytes and their natural products. **Microbiol. Mol. Biol. R.** 67: 491-502.

Strobel, G., Dirske, E., Sears, J. and Markworth, C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. **Microbiology.** 147: 2943-2950.

Strobel, G., Yang, X., Sears, J. and Kramer, R. 1996. Taxol from *Pestalotiopsis microspora* an endophytic fungus of *Taxus wallachiana*. **Microbiology.** 142: 435-440.

Suryanarayanan, T. S. and Kumaresan, V. 2000. Endophytic fungi of some halophytes from an estuarine mangrove forest. **Mycol. Res.** 104: 1465-1467.

Stierle, A. A., Stierle, D. B. and Bugni, T. 1999. Sequoiatones A and B: Novel antitumor metabolites isolated from a redwood endophyte. **J. Org. Chem.** 64: 5479-5484.

Tan, R. X. and Zou, W. X. 2001. Endophytes: a rich source of functional metabolites.

The Royal Society of Chemistry. 18, 448–459.

Tsuruga, T., Chun, Y. T., Ebizuka, Y. and Sankawa, U. 1991. Biological activity constituents of *Melaleuca leucadendron.*: Inhibition of Induced Histamine Release from rat mast cells. **Chem. Pharm. Bull**. 39 (12): 3276-3278.

Von Arx, J. A. 1981. **The genera of fungi sporulation in pure culture.** Hirschberg2: Strauss & Cramer.

Weaver, R. W., Angle, J. S. and Botlomeley, P. S. 1994. Methods of Soil Analysis, part 2. microbiology and biochemical properties. **Madison: Soil Science Society of America.**

White, T. J., Bruns, T. D., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (eds.), **PCR protocols: a guide to methods and applications,** pp. 315-322. New York: Academic Press.

Wilson, D. 1995. Endophyte-the evolution of the term, a clarification of its use and definition. **Oikos** 73: 274-276.

Yoshida, T., Maruyama, T., Nitia, A., and Okuda, T., An Hydrolysable tannin and accompanying polyphennols from *Melaleuca leucadendron.* **Phytochemistry.** 42 (4): 1171-1173.

Zhang, B., Salituro, G., Szalkowski, D., Li, Z., Zhang, Y., Royo, I., Vilella, D., Dez, M., Pelaez, F., Ruby, C., Kendall, R. L., Mao, X., Griffin, P., Calaycay, J., Zierath, J. R., Heck, J. V., Smith, R. G. and Moller, D. E. 1999. Discovery of small moleculeinsulin mimetic with antidiabetic activity in mice. **Science.** 284: 974-981

Zhou, Z., Miwa, M. and Hogetsu, T. 1999. Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). **New. Phytol.** 144: 55- 63.

APPENDICES

Appendix A
Media

All of media were add distilled H_2O to a final volume of 1 liter and steriled in the autoclave at 121^{o}C for 15 minutes. The broth media did not add the agar in the formula.

1. Corn meal agar (CMA)

Suspened 17.0 g in 1 liter distilled H_2O

2. Malt extract agar (MEA)

Malt extracts	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	15.0 g

3. Nutrient agar (NA)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g

4. Potato dextrose agar (PDA)

Potato (pelled and diced)	200.0 g
Glucose	20.0 g
Agar	15.0 g

Boil 200 g of peels, dried potatoes for an hour in a liter of water. Filter, add the glucose and agar make up the filtrate to one liter.

5. Sabouraud's dextrose agar (SDA)

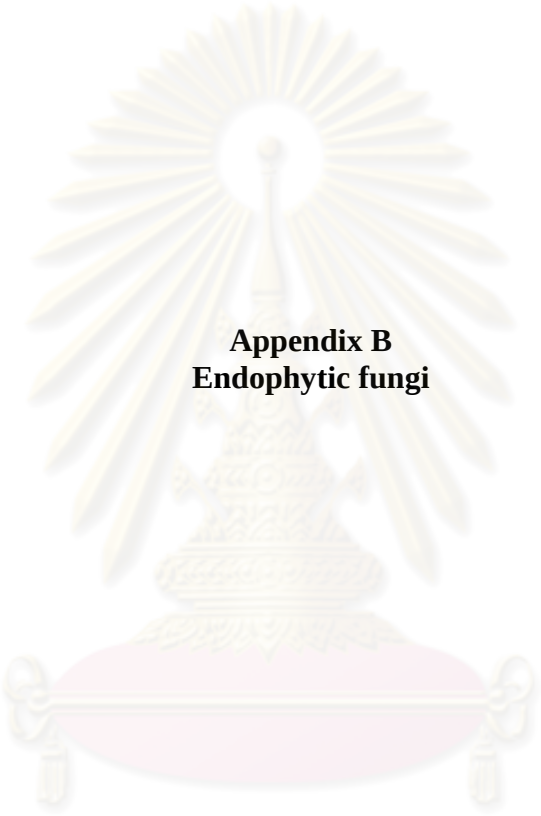
Peptone	10.0 g
Glucose	40.0 g
Agar	15.0 g

6. Yeast extract sucrose agar (YES)

Yeast extract	20.0 g
Sucrose	15.0 g
Agar	15.0 g

7. Yeast-malt extract agar (YMA)

Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g



Appendix B
Endophytic fungi

Table A.1 Charateristics of colony and identification endophytic fungi from leaves and twigs in Pattalung province.

Isolate	Fugal specie	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
PTTW1	Mycelia sterilia	PDA	0.7	Absence of elevation	White	Black
		MEA	0.7	Absence of elevation	Gray	Black
		SDA	0.7	Absence of elevation	Gray	Black
		CMA	0.7	Absence of elevation	White	-
		YES	0.7	Absence of elevation	White	Yellow
PTTW2	Mycelia sterilia	PDA	0.4	Cottony	White orange	Light orange
		MEA	0.4	Cottony	White orange	Light orange
		SDA	0.4	Cottony	White orange	Light orange
		CMA	0.4	Cottony	White	Light orange
		YES	0.3	Absence of elevation	White	Light orange
PTTW3	Mycelia sterilia	PDA	0.3	Powdery	White	-
		MEA	0.3	Powdery	White	-
		SDA	0.4	Powdery	White with purple	Brown red
		CMA	0.3	Powdery	White	-
		YES	0.4	Powdery	White	-
PTTW4	Mycelia sterilia	PDA	0.8	Absence of elevation	Gray with black	-
		MEA	0.8	Absence of elevation	Gray with black	-
		SDA	0.8	Absence of elevation	Gray with black	-
		CMA	0.8	Absence of elevation	Gray with black	-
		YES	0.8	Absence of elevation	Gray with black	-

Table A.1 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
PTOL1	*Pestalo-tia* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.4 0.3	Cottony Absence of elevation Cottony Cottony Cottony	White White White White White	Light yellow Light yellow Orange yellow Light yellow Orange yellow
PTOL2	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.2 0.4	Cottony Cottony Cottony Cottony Absence of elevation	White White Gray with white White White	Light yellow Light yellow Orange - Light yellow
PTOL3	*Pestalo-tia* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.4 0.5	Cottony Cottony Cottony Absence of elevation Cottony	White White White White White	- - Brown red - Brown yellow
PTOL4	Mycelia sterilia	PDA MEA SDA CMA YES	0.6 0.6 0.6 0.5 0.5	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White Gray Gray brown White Light pink	Yellow - Light orange - Light yellow
PTOL5	Mycelia sterilia	PDA MEA SDA CMA YES	0.5 0.4 0.4 0.4 0.2	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White White White White White	- - Brown - Light yellow

Table A.1 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
PTOL6	*Fusarium* sp.	PDA	0.5	Absence of elevation	Red with white	Red
		MEA	0.5	Absence of elevation	Red	Red
		SDA	0.5	Absence of elevation	Orange	Orange
		CMA	0.4	Absence of elevation	White	-
		YES	0.5	Absence of elevation	White orange	Light orange
PTOL7	Mycelia sterilia	PDA	0.5	Absence of elevation	White	-
		MEA	0.5	Absence of elevation	White	-
		SDA	0.5	Absence of elevation	Light brown	Brown
		CMA	0.5	Cottony	White	-
		YES	0.4	Absence of elevation	White	Light yellow
PTYL1	Mycelia sterilia	PDA	0.4	Cottony	White	Orange yellow
		MEA	0.4	Absence of elevation	White	-
		SDA	0.3	Absence of elevation	White	Orange yellow
		CMA	0.3	Cottony	White	-
		YES	0.2	Absence of elevation	White	Yellow
PTYL2	Mycelia sterilia	PDA	0.7	Absence of elevation	White	Yellow
		MEA	0.7	Absence of elevation	Gray	-
		SDA	0.5	Absence of elevation	Gray brown	Light orange
		CMA	0.7	Absence of elevation	White	-
		YES	0.5	Absence of elevation	Light pink	Light yellow
PTYL3	*Pestalotia* sp.	PDA	0.5	Cottony	White	-
		MEA	0.5	Cottony	White	-
		SDA	0.5	Absence of elevation	White	Brown
		CMA	0.4	Absence of elevation	White	-
		YES	0.5	Absence of elevation	White	Light yellow

Table A.1 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
PTYL4	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.3	Cottony Cottony Cottony Cottony Cottony	Red with white Red Orange White White orange	Red Red Orange - Light orange
PTYL5	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.3	Cottony Cottony Cottony Cottony Absence of elevation	White White Light orange White White	Light yellow - Brown red - Light yellow
PTYL6	*Fusa-rium* sp.	PDA MEA SDA CMA YES	0.4 0.4 0.3 0.3 0.2	Cottony Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White Red Light orange White White	Light yellow Red Orange yellow Light orange Brown red

Table A.2 Characteristics of colony and identification endophytic fungi from leaves and twigs in Krabi province.

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
KBTW1	Mycelia Sterilia	PDA	0.6	Cottony	Black	-
		MEA	0.6	Absence of elevation	Black	-
		SDA	0.6	Absence of elevation	Black	-
		CMA	0.5	Absence of elevation	Black	-
		YES	0.5	Absence of elevation	Gray with white	-
KBTW2	Mycelia Sterilia	PDA	0.5	Absence of elevation	White	-
		MEA	0.5	Absence of elevation	White	-
		SDA	0.5	Absence of elevation	Brown	Brown
		CMA	0.5	Absence of elevation	White	-
		YES	0.4	Absence of elevation	White	-
KBTW3	Mycelia sterilia	PDA	0.4	Cottony	White	Light orange
		MEA	0.4	Cottony	White	-
		SDA	0.4	Cottony	Light orange	Orange yellow
		CMA	0.4	Cottony	White	-
		YES	0.3	Cottony	Light pink	Light yellow
KBOL1	Mycelia sterilia	PDA	0.6	Absence of elevation	White	Yellow
		MEA	0.6	Absence of elevation	Gray	-
		SDA	0.6	Absence of elevation	Gray brown	Light orange
		CMA	0.5	Absence of elevation	White	-
		YES	0.6	Absence of elevation	White with pink	Light yellow
KBOL2	*Phomo-psis* sp.	PDA	0.6	Absence of elevation	White	-
		MEA	0.5	Absence of elevation	White	-
		SDA	0.5	Absence of elevation	White	Light pink
		CMA	0.5	Absence of elevation	White	-
		YES	0.6	Absence of elevation	White	-

Table A.2 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
KBOL3	*Alternaria* sp.	PDA MEA SDA CMA YES	0.4 0.4 0.5 0.5 0.5	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White White Orange White Brown with white	- - Orange - Brown red
KBYL1	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.3 0.4 0.4 0.3	Cottony Cottony Cottony Absence of elevation Absence of elevation	White White Light brown White White	- - Brown - Orange yellow
KBYL2	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.3	Cottony Cottony Cottony Cottony Absence of elevation	White White Light brown White White	Orange yellow - Brown red White Light orange
KBYL3	*Fusarium* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.5 0.5	Cottony Cottony Cottony Cottony Absence of elevation	White White White White White	- - Brown red - Light yellow
KBYL4	*Pestalotia* sp.	PDA MEA SDA CMA YES	0.6 0.5 0.5 0.5 0.5	Cottony Cottony Cottony Cottony Absence of elevation	White White White White White	- - Brown red - Light yellow

Table A.3 Characteristics of colony and identification endophytic fungi from leaves and twigs in Nakhon sithammarat province.

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
NSOL1	Mycelia sterilia	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.5 0.5	Cottony Cottony Cottony Cottony Cottony	Light brown Brown White White White and black	Black Brown Black White Black
NSOL2	Mycelia sterilia	PDA MEA SDA CMA YES	0.6 0.6 0.6 0.6 0.6	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White Red Yellow White and black White and black	- Red Black - Black
NSOL3	*Xylaria* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.5 0.4	Stroma Stroma Stroma Stroma Stroma	White black White black White White White	- - Brown - -
NSOL4	*Xylaria* sp.	PDA MEA SDA CMA YES	0.7 0.7 0.7 0.7 0.7	Stroma Stroma Stroma Stroma Stroma	White White black White White White	Black Black - - -
NSOL5	*Xylaria* sp.	PDA MEA SDA CMA YES	0.6 0.6 0.6 0.6 0.5	Stroma Stroma Stroma Stroma Stroma	White White White White White	- - Orange yellow - -

Table A.4 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
NSTW1	*Fusa-rium* sp.	PDA	0.4	Absence of elevation	Black with purple	-
		MEA	0.4	Absence of elevation	Black with purple	-
		SDA	0.4	Absence of elevation	White	Black brown
		CMA	0.2	Absence of elevation	White	Black
		YES	0.2	Absence of elevation	White with black	Light orange
NSTW2	Mycelia sterilia	PDA	0.6	Absence of elevation	Black	Black
		MEA	0.6	Absence of elevation	Black	Black
		SDA	0.6	Absence of elevation	Black	Black
		CMA	0.6	Absence of elevation	Black	Black
		YES	0.6	Absence of elevation	Black	Black
NSTW3	Mycelia sterilia	PDA	0.5	Absence of elevation	White	-
		MEA	0.5	Absence of elevation	White	-
		SDA	0.5	Absence of elevation	White	Black
		CMA	0.5	Absence of elevation	White	-
		YES	0.5	Absence of elevation	Light brown	Black
NSTW4	Mycelia sterilia	PDA	0.7	Cottony	Gray	Black
		MEA	0.7	Cottony	Gray	-
		SDA	0.7	Cottony	Gray	Light pink
		CMA	0.6	Cottony	Gray	-
		YES	0.4	Cottony	Gray	-
NSTW5	Mycelia sterilia	PDA	0.6	Absence of elevation	White	-
		MEA	0.6	Absence of elevation	White	-
		SDA	0.6	Cottony	Light pink	Orange
		CMA	0.6	Absence of elevation	White	-
		YES	0.5	Absence of elevation	White	-

Table A.4 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
NSTW6	Mycelia sterilia	PDA MEA SDA CMA YES	0.6 0.6 0.5 0.5 0.3	Cottony Cottony Absence of elevation Absence of elevation Absence of elevation	Black purple Black purple White White Black white	- - Black brown Black Light orange
NSYL1	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.4	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White White White White White	Light brown - Brown red - Light yellow
NSYL2	*Pestalo-tia* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.5 0.4	Cottony Cottony Absence of elevation Absence of elevation Absence of elevation	White White White White White	- - Brown - Light yellow
NSYL3	Mycelia sterilia	PDA MEA SDA CMA YES	0.7 0.7 0.7 0.4 0.4	Cottony Cottony Cottony Cottony Absence of elevation	White orange White orange White White White	Orange Light orange Orange - -
NSYL4	*Colletot richum* sp.	PDA MEA SDA CMA YES	0.6 0.6 0.6 0.5 0.5	Cottony Cottony Cottony Absence of elevation Absence of elevation	Brown white Yellow white White brown White Brown red	Brown red Yellow Brown red - Brown red

Table A.4 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
NSYL5	*Fusarium* sp.	PDA MEA SDA CMA YES	0. 6 0. 6 0. 5 0. 5 0. 3	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	Purple black Purple black White White Black white	- - Brown Black Light orange

Table A.5 Charateristics of colony and identification endophytic fungi from leaves and twigs in Trang province

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
TROL1	*Xylaria* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.3 0.3	Stroma Stroma Stroma Stroma Stroma	White White White White White	Black with white - Orange yellow Light yellow Light yellow
TROL2	*Xylaria* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.3 0.5 0.2	Stroma Stroma Stroma Stroma Stroma	White White White White White	- - - - -

Table A.5 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
TROL3	*Xylaria* sp.	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.3 0.3	Stroma Stroma Stroma Stroma Stroma	White Brown White White White White	Brown red - - - -
TROL4	Mycelia sterilia	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.5 0.4	Cottony Cottony Cottony Cottony Absence of elevation	White White White White White orange	- - - - -
TROL5	Mycelia sterilia	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.5 0.5	Cottony Cottony Cottony Cottony Absence of elevation	White White White White White	- - Brown red - Brown red
TRTW1	*Xylaria* sp.	PDA MEA SDA CMA YES	0.5 0.5 0.5 0.3 0.3	Absence of elevation Absence of elevation Absence of elevation Absence of elevation Absence of elevation	White White Light brown White White	Orange yellow Orange yellow Orange - Brown orange
TRTW2	Mycelia sterilia	PDA MEA SDA CMA YES	0.5 0.5 0.3 0.5 0.2	Absence of elevation Cottony Cottony Cottony Absence of elevation	White White White brown White White and black	- Light yellow Orange - Orange

Table A.5 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
TRTW3	*Aspergi-llus* sp.	PDA MEA SDA CMA YES	0.6 0.6 0.6 0.6 0.6	Cottony Cottony Cottony Cottony Absence of elevation	White White green White green Whitegreen White green	Yellow - - - -
TRYL1	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.4	Cottony Cottony Cottony Cottony Absence of elevation	White White White White White	Light brown - Brown - Light Yellow
TRYL2	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.3	Cottony Cottony Cottony Cottony Absence of elevation	White White Light Brown White White and black	Yellow - Light Brown White Light Yellow
TRYL3	Mycelia sterilia	PDA MEA SDA CMA YES	0.4 0.4 0.4 0.4 0.3	Cottony Cottony Absence of elevation Absence of elevation Absence of elevation	White White Light Brown White White	Yellow orange - Orange Light orange Light orange
TRYL4	*Phomo-psis* sp.	PDA MEA SDA CMA YES	0.6 0.6 0.6 0.5 0.5	Cottony Cottony Cottony Absence of elevation Absence of elevation	White White White White White	- - Brown red - Light brown

Table A.5 (continued)

Isolates	Fugal species	Media	Growth rate (mm /day)	Endophytic fungi characteristics		
				Colony characteristic	Colony colour	Colour pigment production on the media
TRYL5	Mycelia sterilia	PDA	0.5	Cottony	White	-
		MEA	0.5	Cottony	White	-
		SDA	0.5	Cottony	White	-
		CMA	0.5	Cottony	White	-
		YES	0.5	Cottony	White	-

CMA (Corn Meal Agar), MEA (Malt Extract Agar), PDA (Potato Dextrose Agar) SDA (Sabouraud's Dextrose Agar) and YEA (Yeast Extract Sucrose Agar)

Appendix C

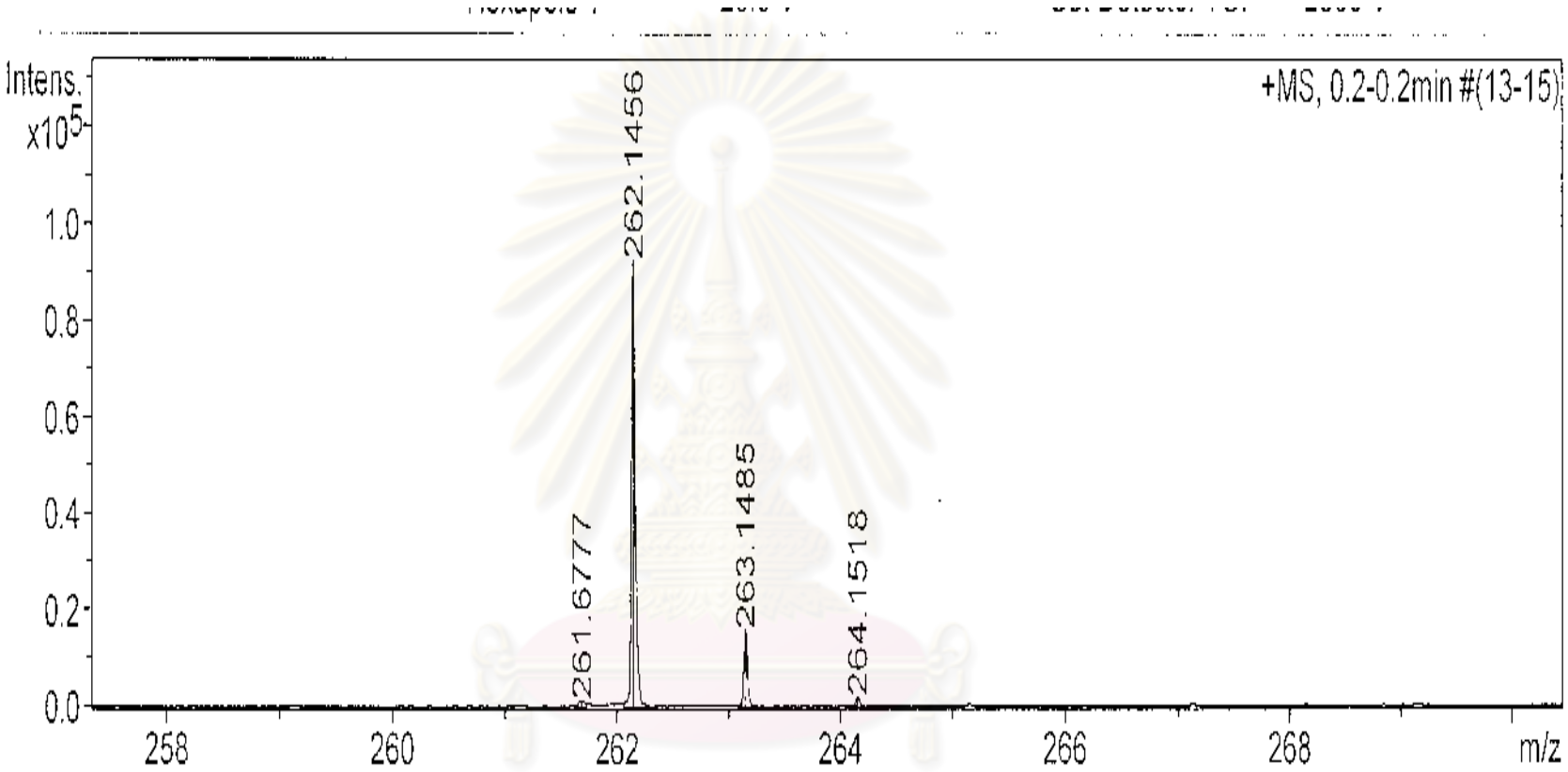


Figure C 1-1 Mass spectrum of Compound 1

103

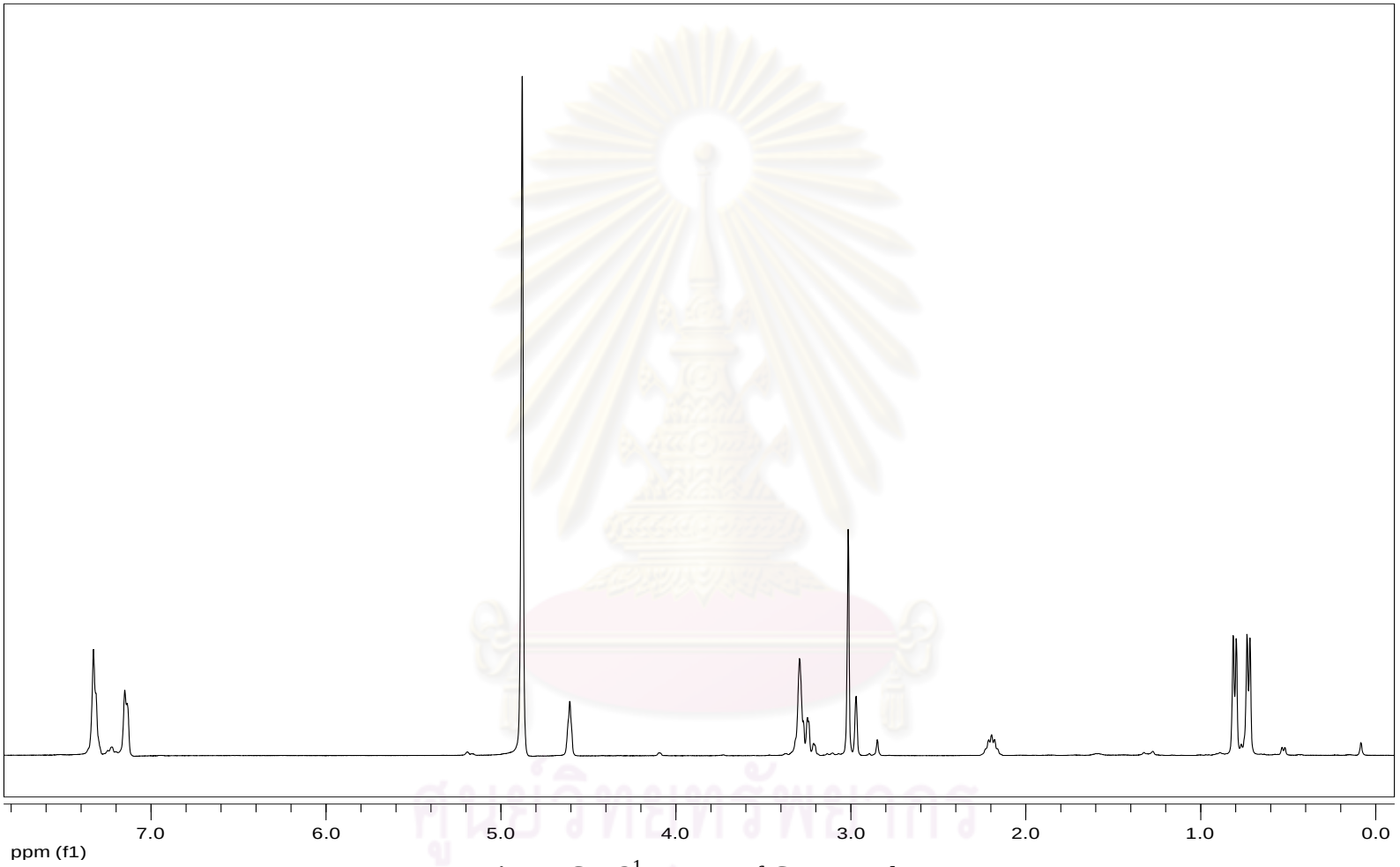


Figure C 1-2 ^{1}H NMR of Compound 1

104

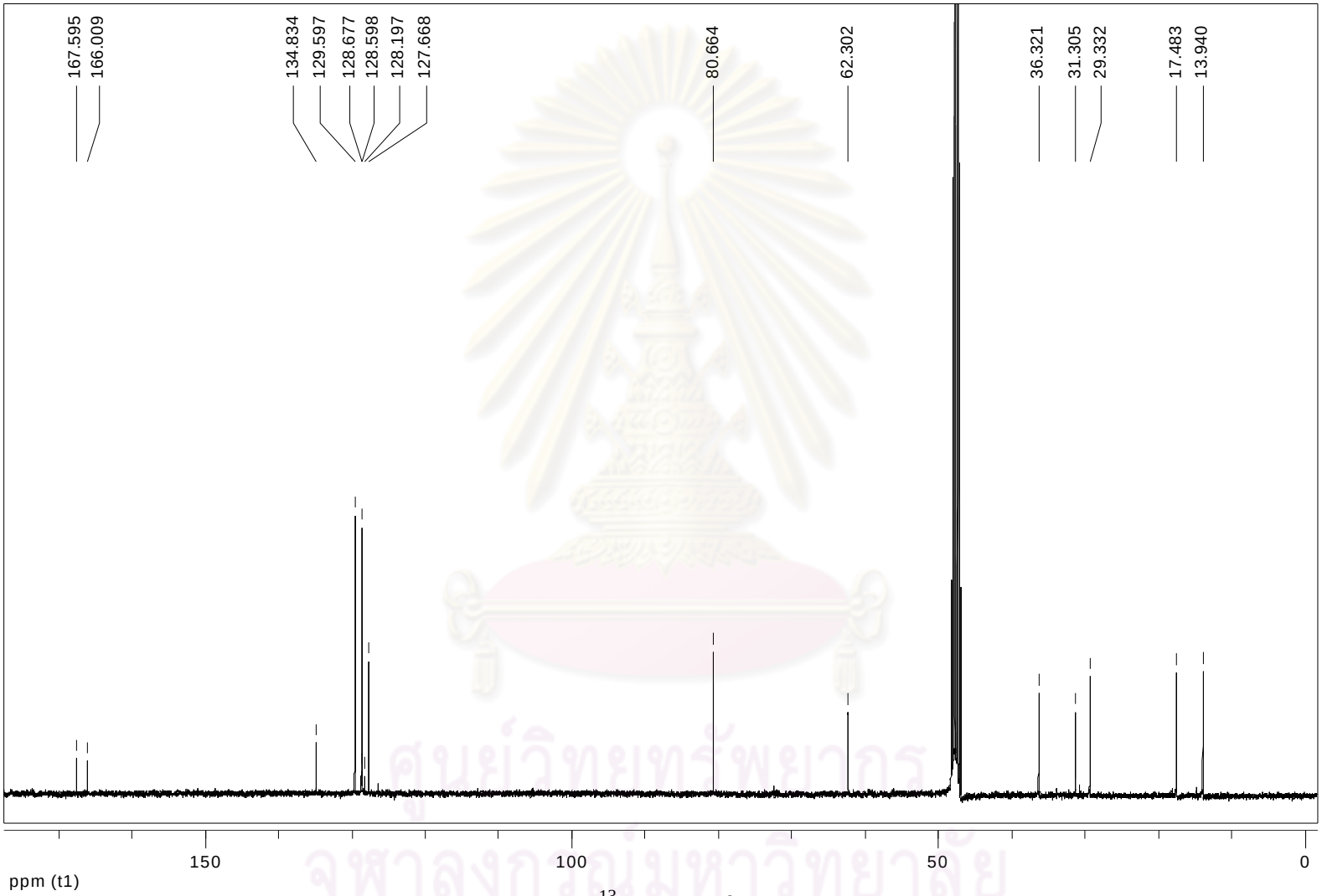


Figure C 1-3 ^{13}C NMR of compound 1

105

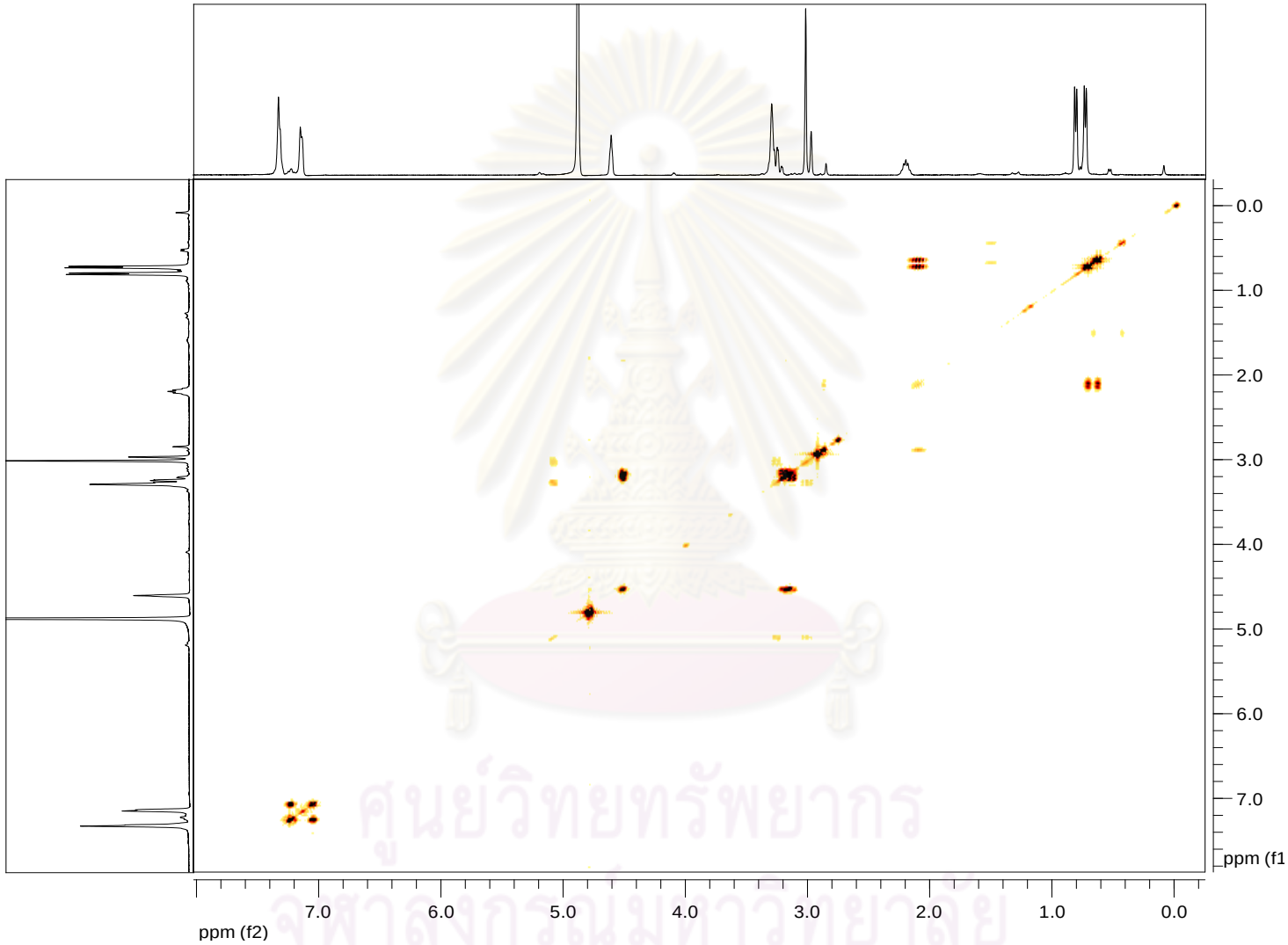


Figure C 1-4 gCOSY of compound 1

106

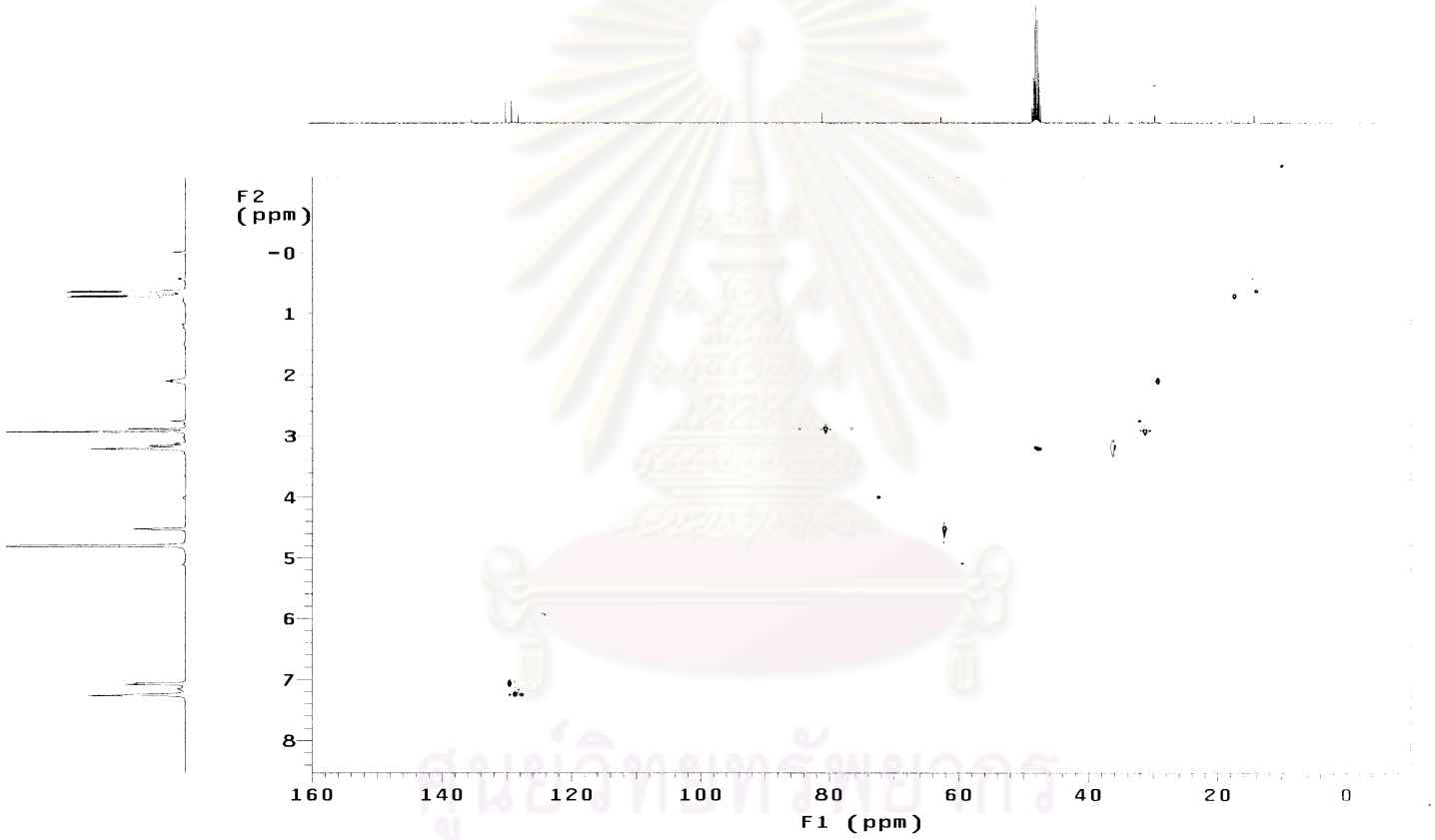


Figure C 1-5 gHSQC of compound 1

107

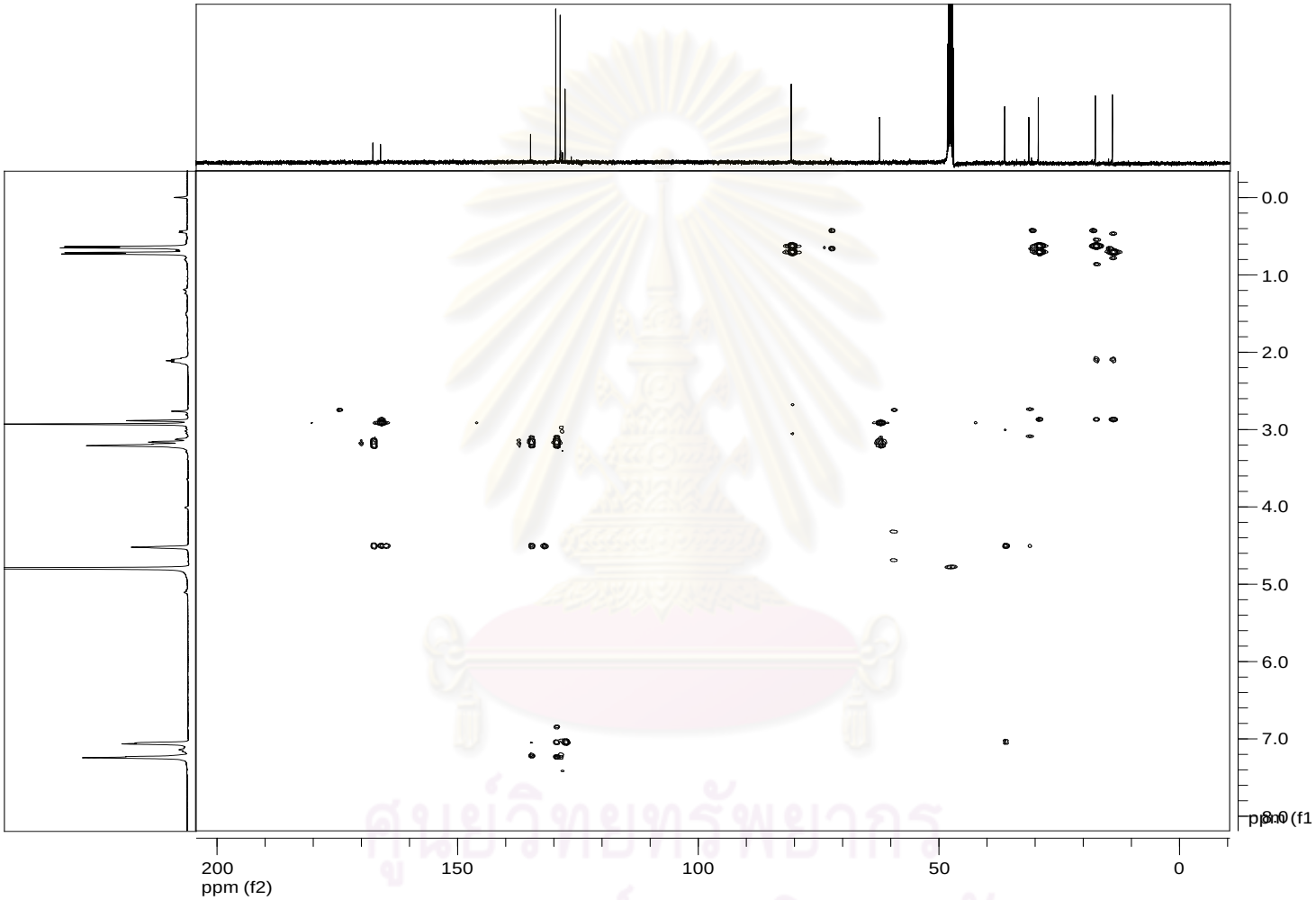


Figure C 1-6 gHMBC of compound 1

108

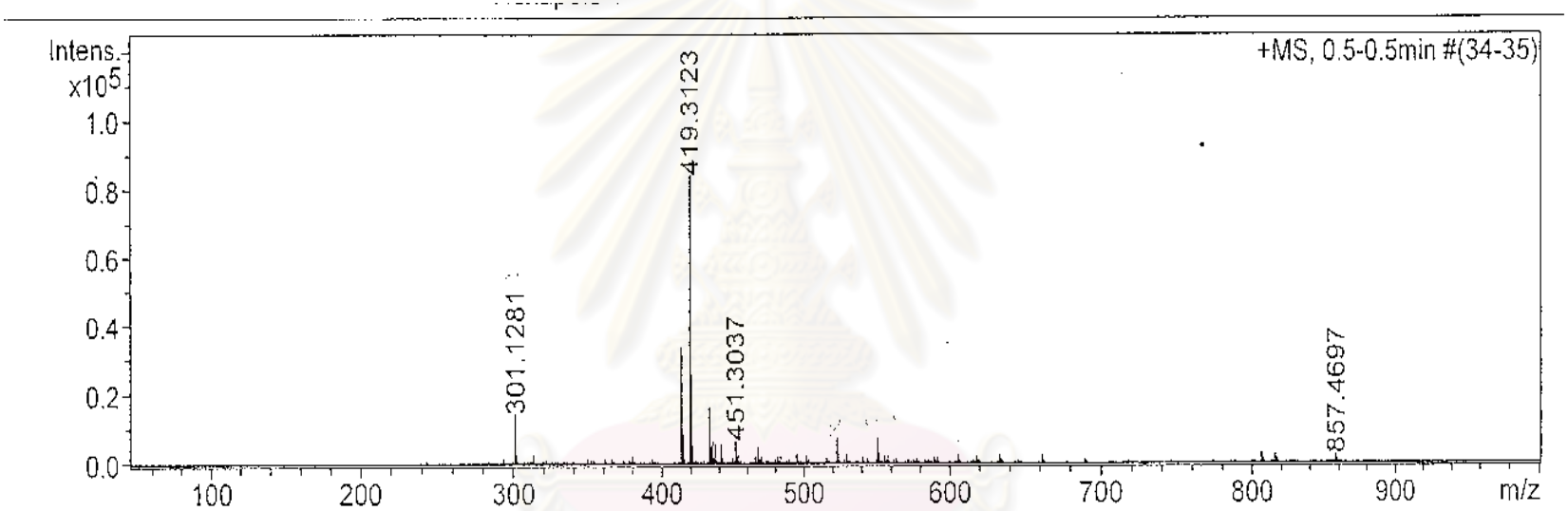


Figure C 2-1 Mass spectrum of Compound 1

109

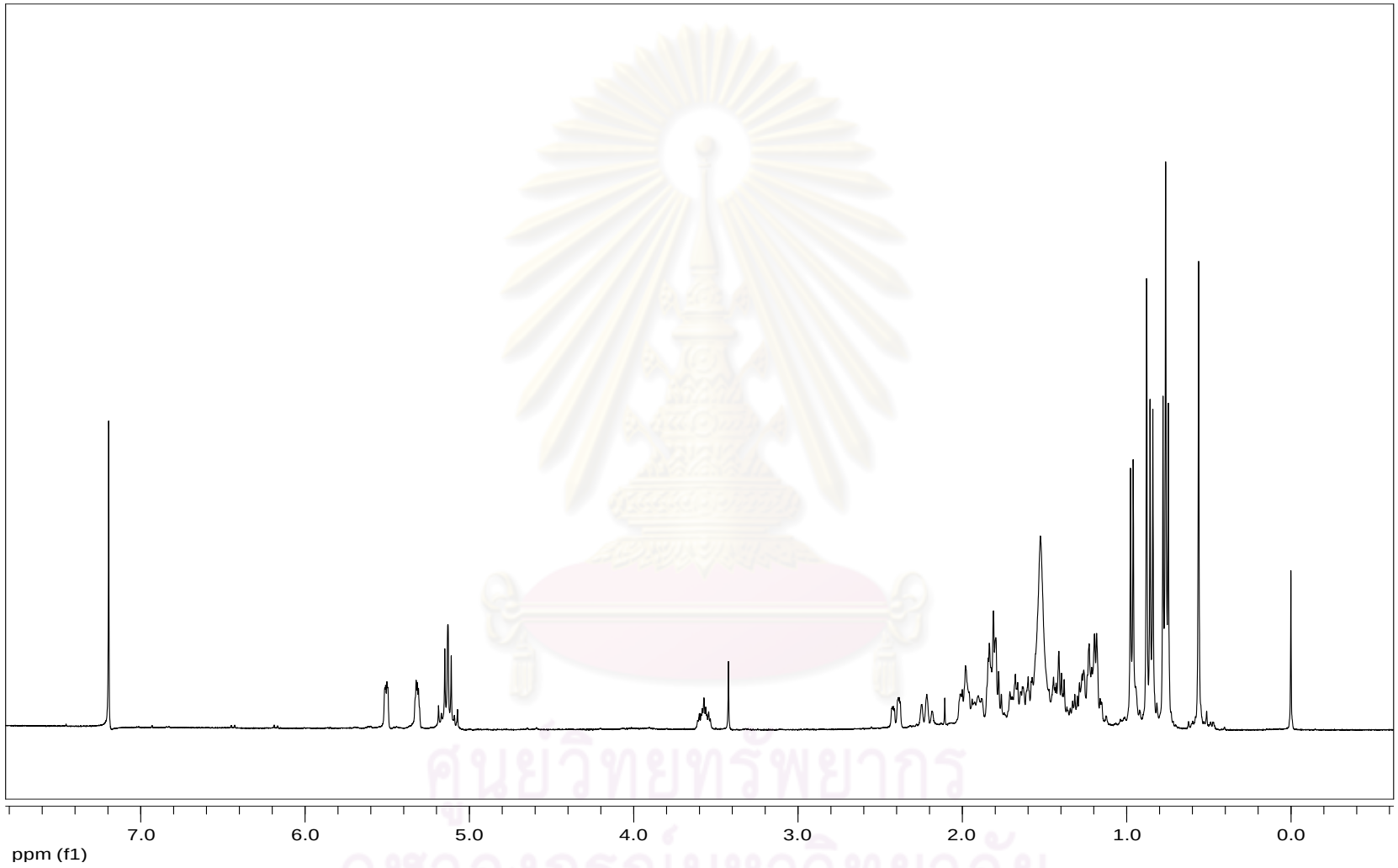


Figure C 2-2 ^{1}H NMR of Compound 2

110

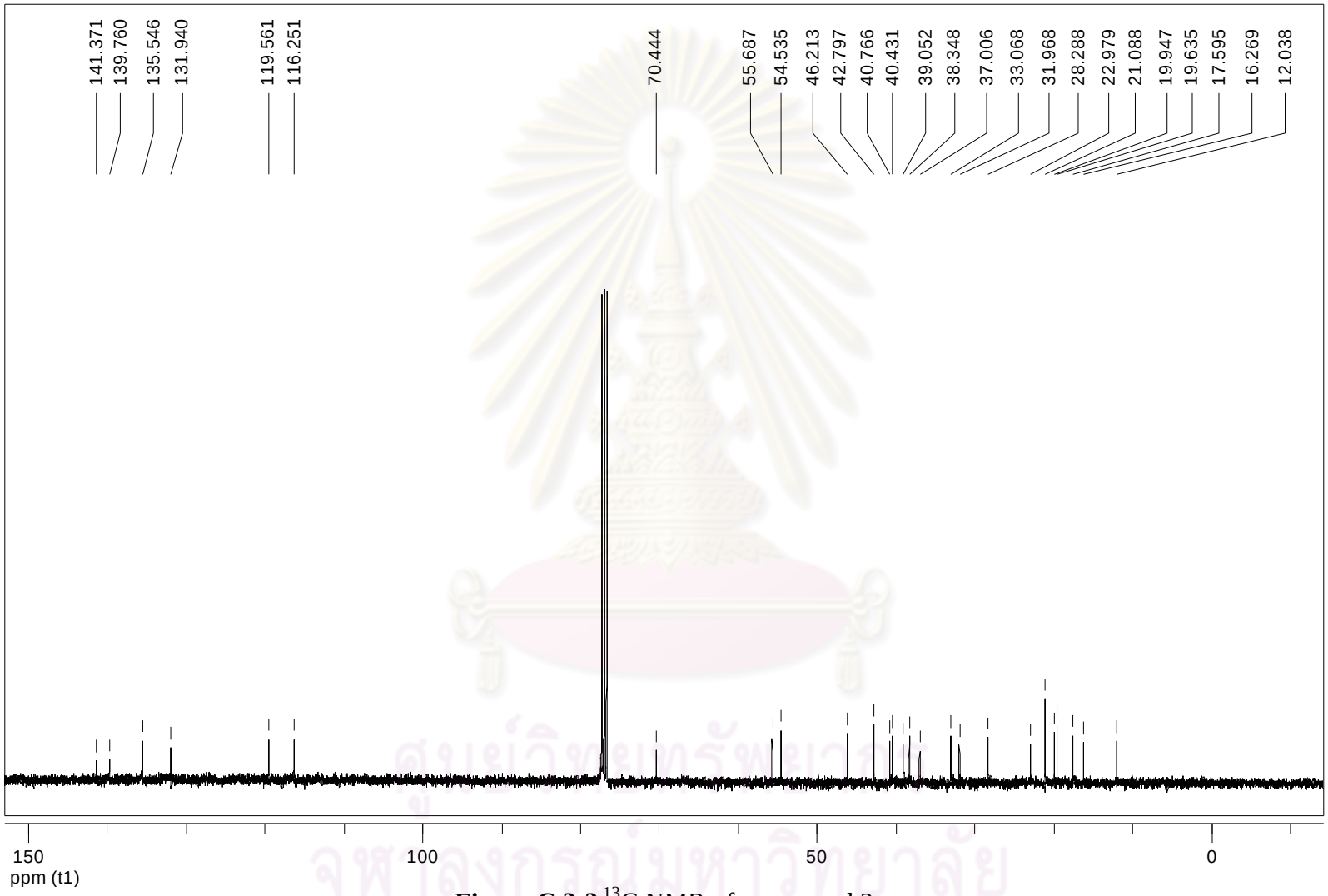


Figure C 2-3 ^{13}C NMR of compound 2

111

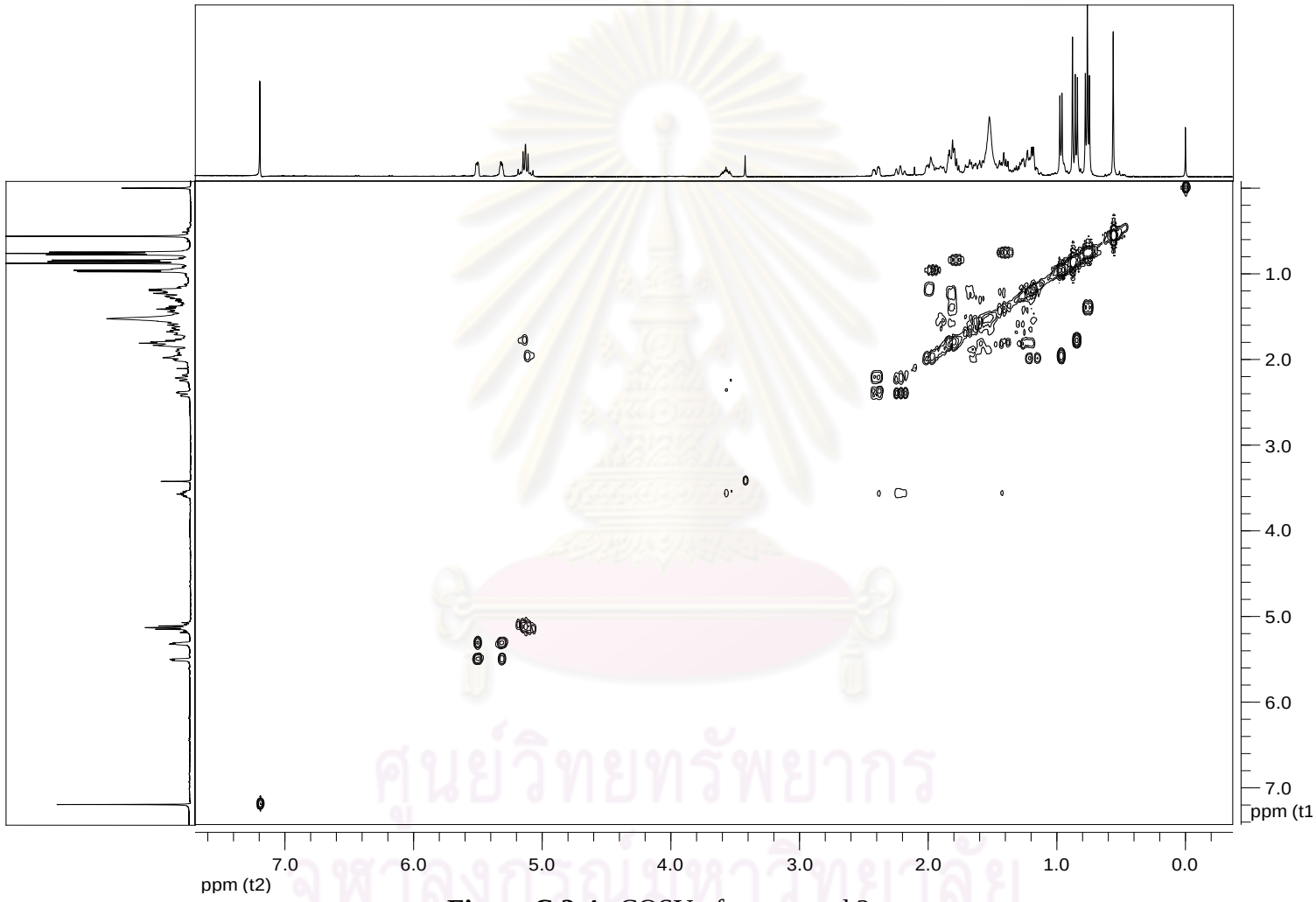


Figure C 2-4 gCOSY of compound 2

112

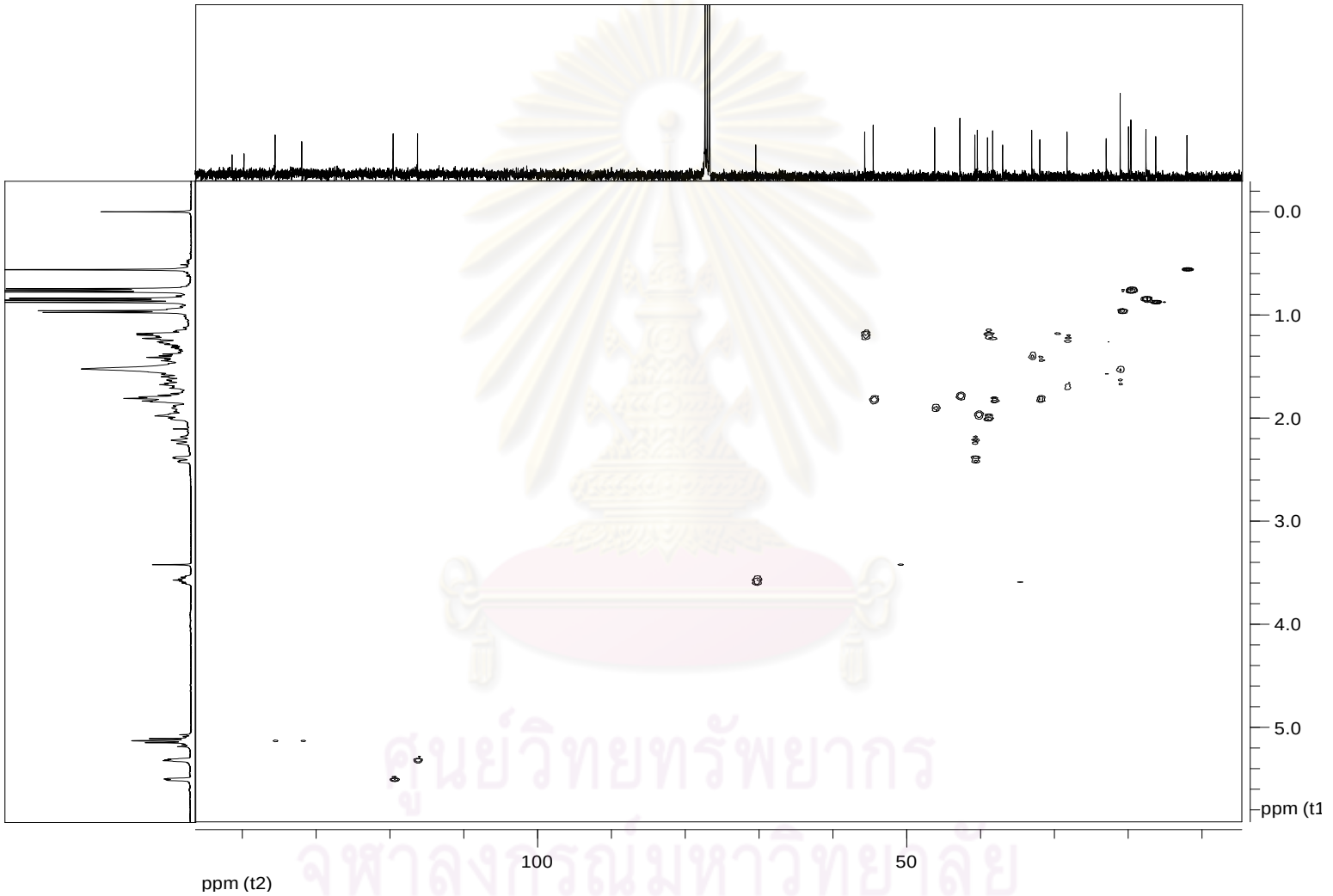


Figure C 2-5 gHSQC of compound 2

113

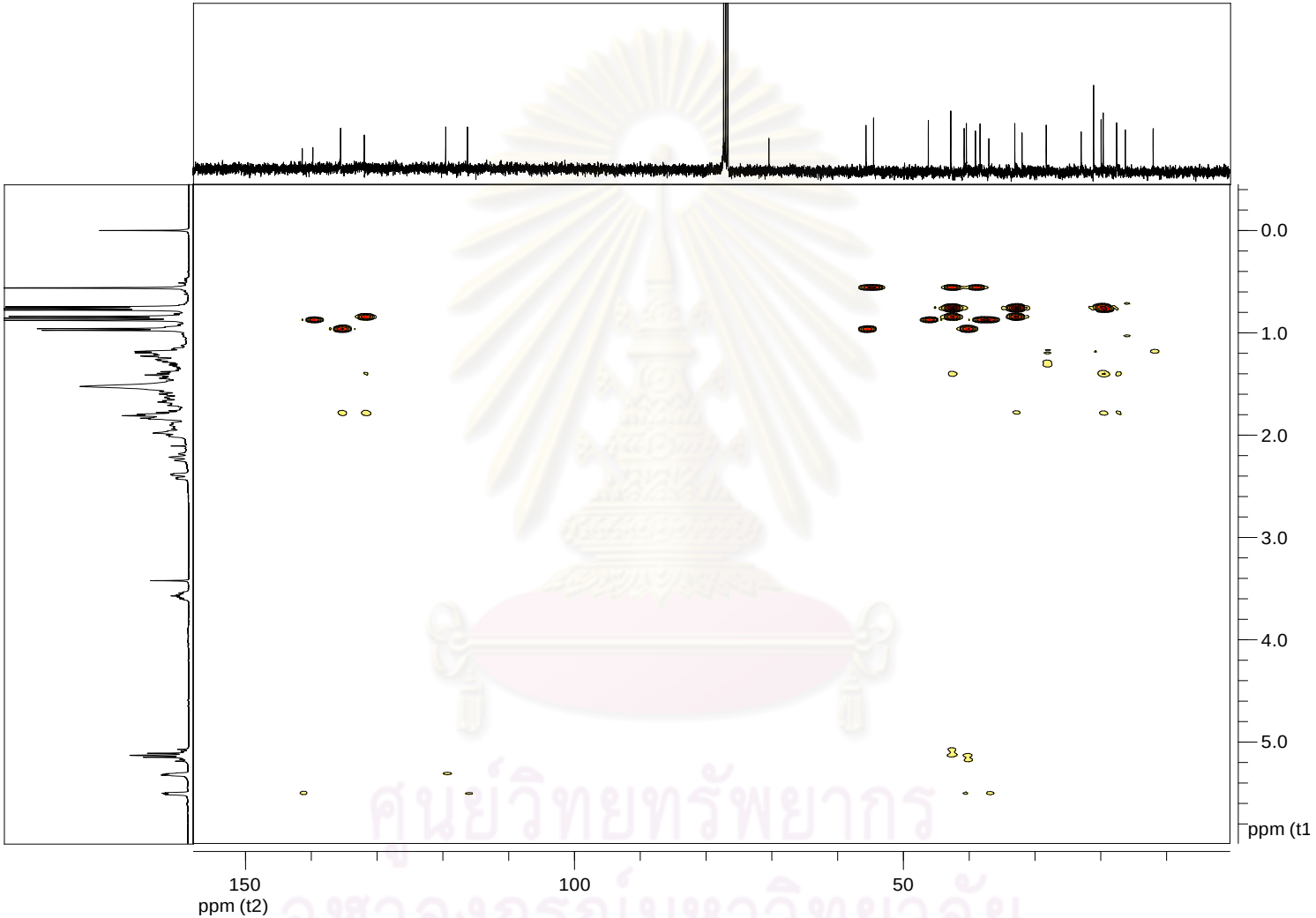


Figure C 2-6 gHMBC of compound 2

114

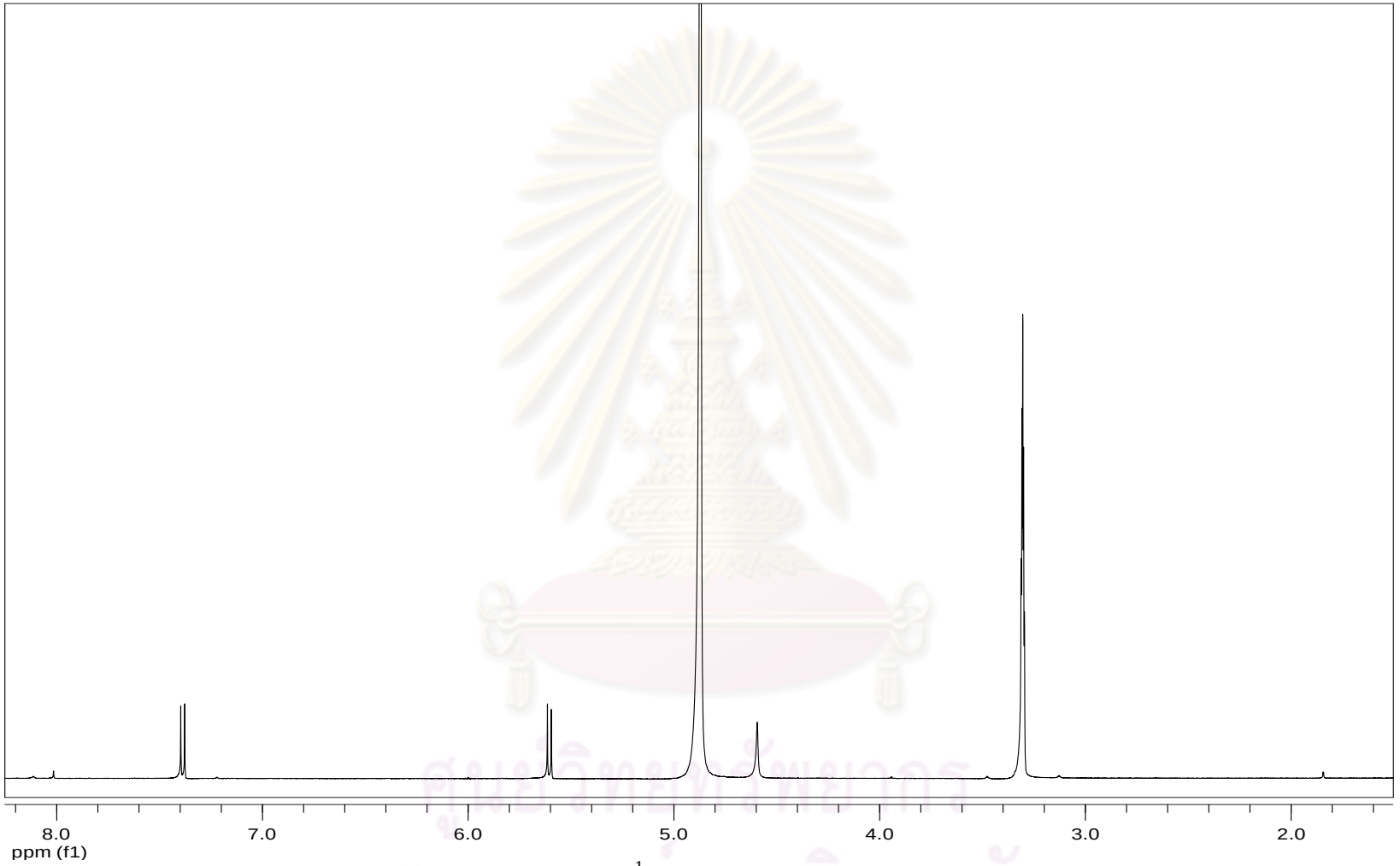


Figure C 3-1 ^{1}H NMR of Compound 3

115

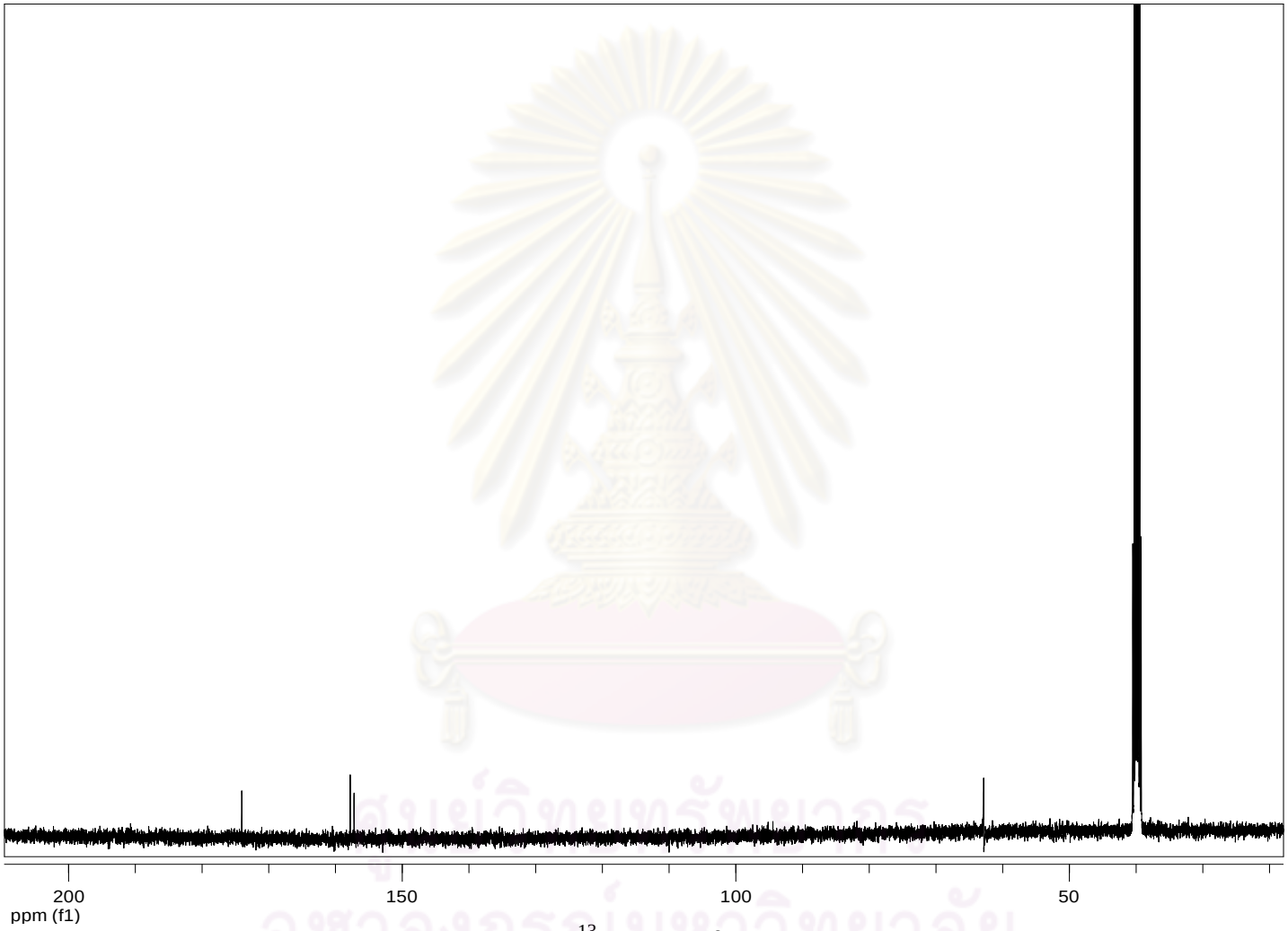


Figure C 3-2 ^{13}C NMR of compound 3

116

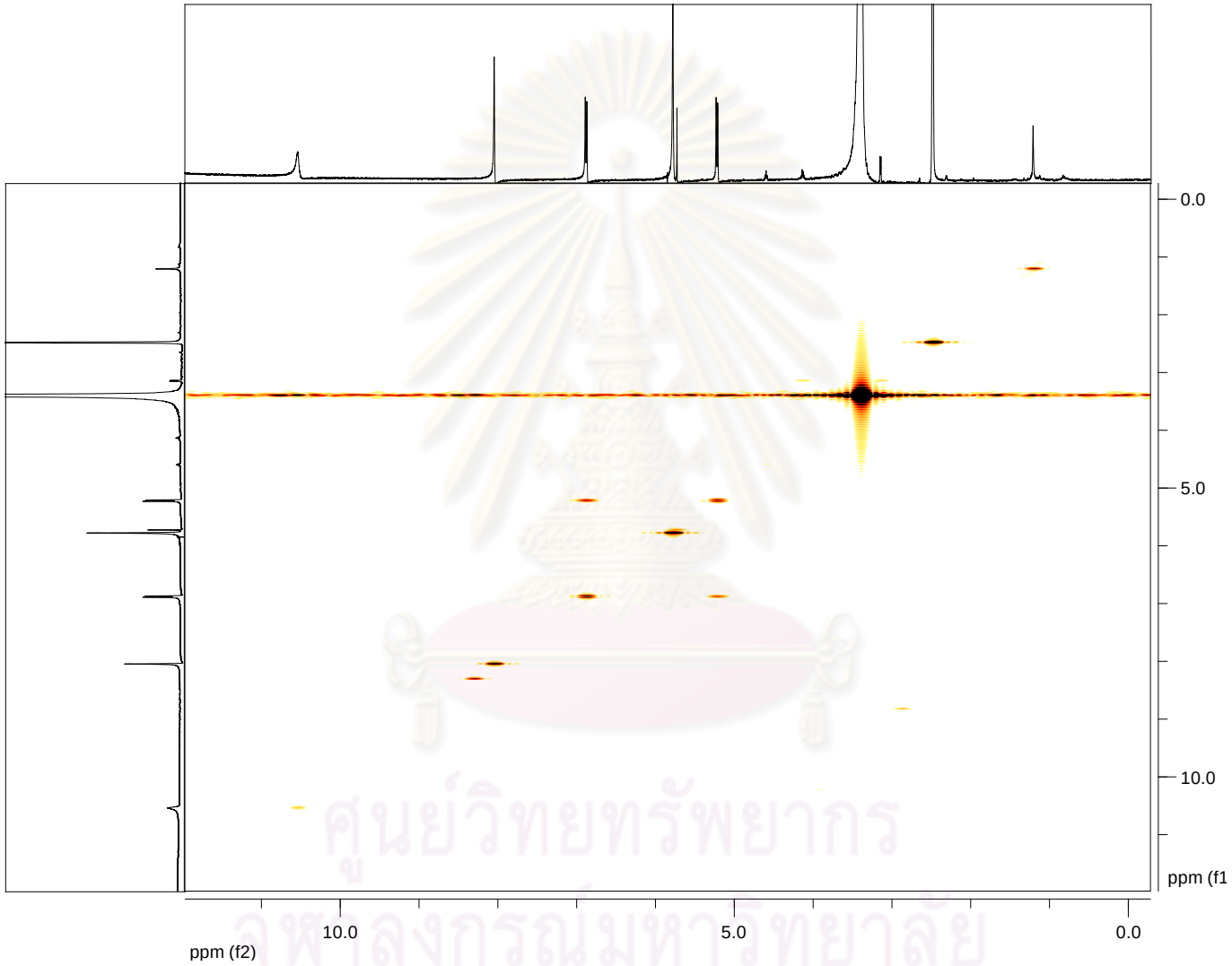


Figure C 3-3 gCOSY of compound 3

117

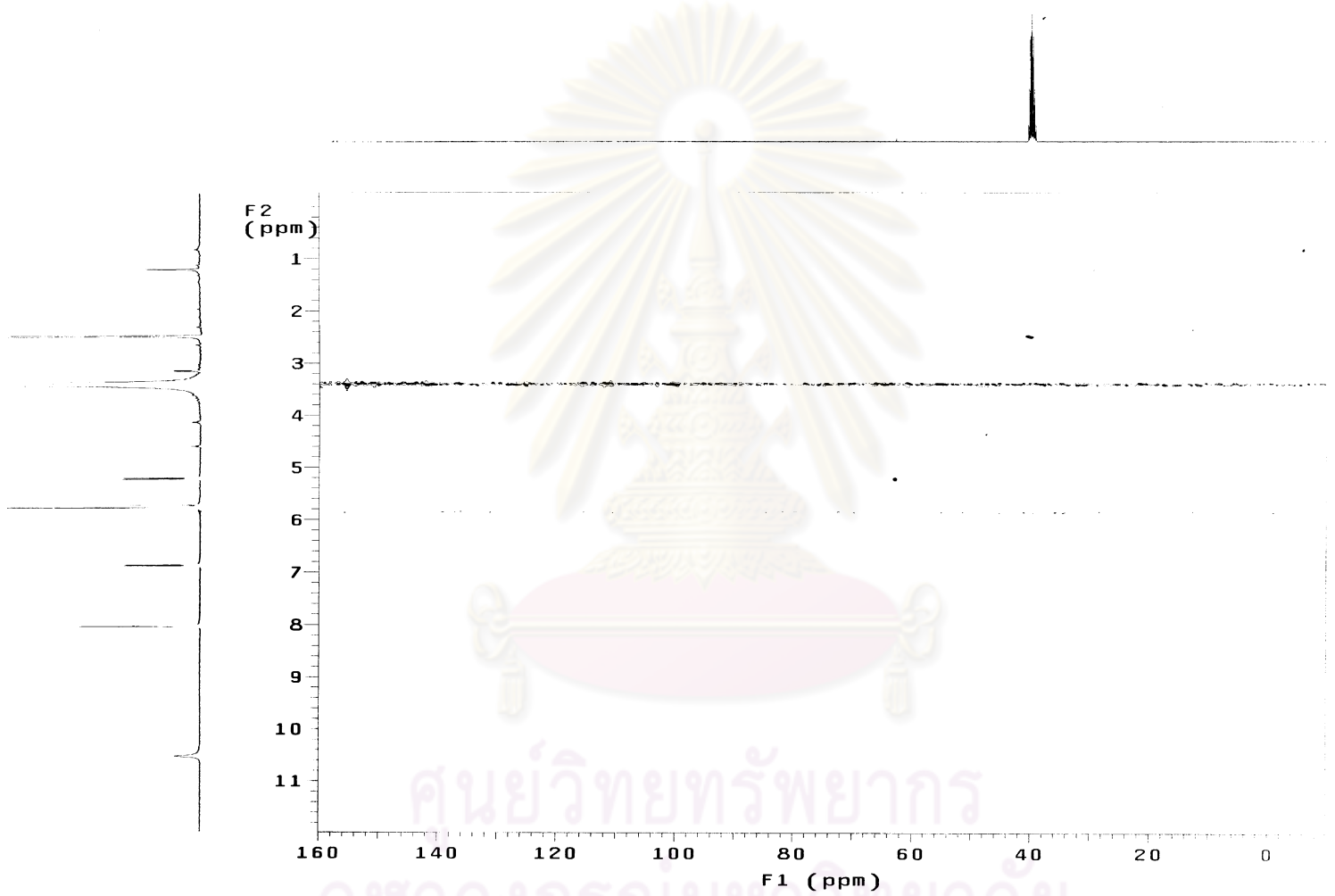


Figure C 3-4 gHSQC of compound 3

118

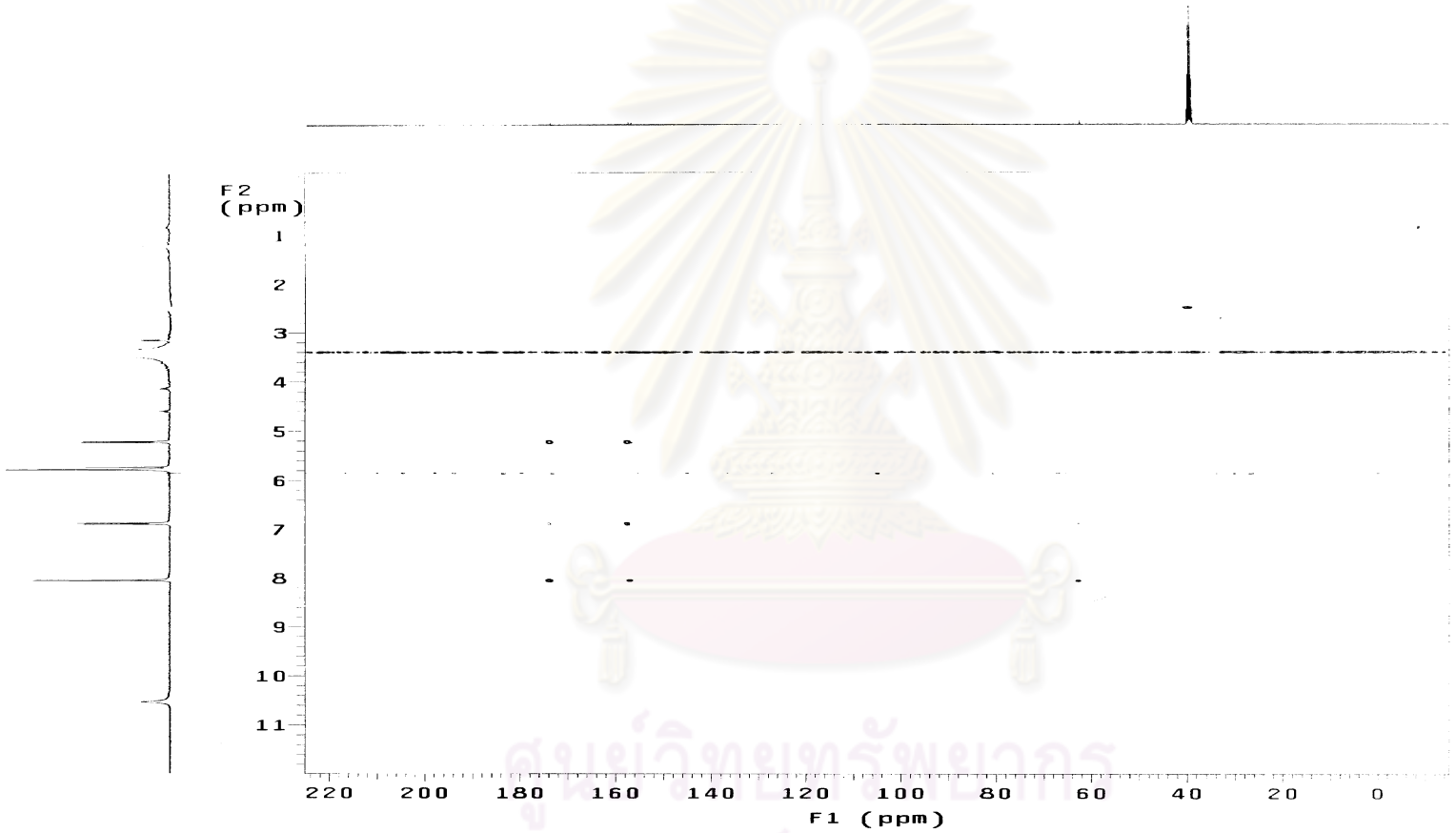


Figure C 3-5 gHMBC of compound 3

119

Appendix D
Alignment data

Sequences producing significant alignments:

EF453121.1
Fusarium sacchari strain NRRL 43543 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
869 86993%0.098%

AM162684.1
Uncultured Fusarium sp. 5.8S rRNA gene, ITS1, 26S rRNA gene (partial) and ITS2, clone (23)27
869 86993%0.098%

EU687129.1
Fungal endophyte isolate 516B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EU091040.1
Fusarium subglutinans isolate F2 internal transcribed space 1 gene, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EF453174.1
Gibberella moniliformis strain NRRL 43697 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EF453161.1
Fusarium sp. NRRL 43682 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EF453141.1
Fusarium sp. NRRL 43658 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EF453140.1
Fusarium sp. NRRL 43657 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EF453139.1
Fusarium sp. NRRL 43656 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EF453124.1
Gibberella moniliformis strain NRRL 43547 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

AY188916.1
Gibberella fujikuroi 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
863 86393%0.098%

EU151483.1
Gibberella moniliformis strain VP2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151482.1
Gibberella moniliformis strain SA3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151481.1
Gibberella moniliformis strain PI1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151480.1
Gibberella moniliformis strain GE1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151479.1
Gibberella moniliformis strain CHI1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151478.1
Gibberella moniliformis strain CA2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151477.1
Gibberella moniliformis strain SGV1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151475.1
Gibberella moniliformis strain LO1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151474.1
Gibberella moniliformis strain FR3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151473.1
Gibberella moniliformis strain CH2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151472.1
Gibberella moniliformis strain SAL4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151471.1
Gibberella moniliformis strain IV1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151470.1
Gibberella moniliformis strain BM1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151469.1
Gibberella moniliformis strain CM3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151468.1
Gibberella moniliformis strain A1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

EU151467.1
Gibberella moniliformis strain A1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AF455450.1
Gibberella sacchari isolate wb395 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898260.1
Gibberella moniliformis strain CNFM 960508 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898259.1
Gibberella moniliformis strain CNFM 960507 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898258.1
Gibberella moniliformis strain CNFM 960506 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898257.1
Gibberella moniliformis strain CNFM 960505 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898256.1
Gibberella moniliformis strain CNFM 960504 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898255.1
Gibberella moniliformis strain CNFM 960503 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898254.1
Gibberella moniliformis strain CNFM 960502 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898253.1
Gibberella moniliformis strain CNFM 960501 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898251.1
Fusarium subglutinans strain ATCC 38016 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898250.1
Gibberella fujikuroi strain ATCC 26263 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

AY898249.1
Gibberella fujikuroi strain ATCC 52539 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
861 86193%0.097%

X94175.1
Fusarium napiforme 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2)
861 86193%0.097%

X94168.1
Fusarium sacchari 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2)
861 86193%0.097%

X94166.1
Fusarium verticillioides 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2)
861 86193%0.097%

FJ612907.1
Fungal sp. ARIZ B086 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
859 85993%0.097%

U61682.1
Fusarium neoceras internal transcribed spacer ribosomal RNA
859 85988%0.099%

FJ744110.1
Gibberella circinata isolate 361-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85793%0.097%

EU314981.1
Gibberella moniliformis isolate FKCB-001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

EU314975.1
Gibberella moniliformis isolate FKCB-009 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

EF556217.1
Gibberella moniliformis strain bxq41213 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

AY904065.1
Fusarium oxysporum 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

AM162679.1
Uncultured Fusarium sp. 18S rRNA gene, 5.8S rRNA gene, ITS1, 26S rRNA gene (partial) and ITS2, clone (59)3
857 85793%0.097%

DQ369912.1
Zea mays cultivar line T66 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
857 85793%0.097%

DQ297573.1
Fusarium sp. ZMS-555-17 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

DQ297572.1
Fusarium sp. ZMS-553-16 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

DQ297571.1
Gibberella moniliformis isolate ZMS-537-10A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

DQ297570.1
Gibberella moniliformis isolate ZMS-301-5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

DQ297559.1
Fusarium pseudonygamai isolate ZMS-539-10B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

DQ297554.1
Fusarium napiforme isolate ZMS-299-4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85793%0.097%

DQ297553.1
Fusarium napiforme isolate ZMS-295-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

AF158314.1
Fusarium sp. NRRL29124 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

AF158313.1
Fusarium sp. NRRL29123 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
857 85789%0.099%

EU151476.1
Gibberella moniliformis strain NOVb1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
856 85693%0.097%

EF577235.1
Fusarium proliferatum small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
856 85693%0.097%

AY533376.1
Gibberella moniliformis small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
856 85693%0.097%

DQ297555.1
Fusarium napiforme isolate ZMS-303-6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
856 85693%0.097%

X94167.1
Fusarium subglutinans 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2)
856 85693%0.097%

FJ614644.1
Fusarium sp. ATCC MYA-3973 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

FJ614643.1
Fusarium sp. ATCC MYA-3972 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU977254.1
Fungal endophyte sp. P1717C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
852 85289%0.099%

FJ466709.1
Fusarium oxysporum voucher UFMGCB_529 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85293%0.097%

FJ440700.1
Gibberella sp. FE 180 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU835475.1
Gibberella moniliformis strain L1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU272509.1
Fusarium proliferatum isolate 2705 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU272508.1
Fusarium proliferatum isolate 2704 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU272500.1
Fusarium proliferatum isolate 2696 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU714385.1
Gibberella sp. F14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU714384.1
Gibberella sp. F7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF694636.1
Fusarium sp. VegaE1-24 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence
852 85289%0.099%

EU314979.1
Gibberella moniliformis isolate FKCB-006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU314972.1
Gibberella moniliformis isolate FKCB-002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU314969.1
Gibberella moniliformis isolate FKCB-005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EU314968.1
Gibberella moniliformis isolate FKCB-008 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF505493.1
Uncultured endophytic fungus clone 66-17-11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF505471.1
Uncultured endophytic fungus clone 37-18-45 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF505469.1
Uncultured endophytic fungus clone 37-18-23 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF505462.1
Uncultured endophytic fungus clone 35-13-27 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF556212.1
Gibberella moniliformis strain bxq41208 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

EF534189.1
Gibberella moniliformis strain bxq33108 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence
852 85289%0.099%

EF483929.1
Gibberella moniliformis strain bxq512 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
852 85289%0.099%

DQ297569.1
Fusarium subglutinans isolate ZMS-547-14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

DQ297568.1
Fusarium subglutinans isolate ZMS-541-11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

DQ297567.1
Fusarium subglutinans isolate ZMS-309-9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

DQ297563.1

Fusarium pseudonygamai isolate ZMS-559-19 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

DQ297562.1
Fusarium pseudonygamai isolate ZMS-557-18 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

DQ297561.1
Fusarium pseudonygamai isolate ZMS-545-13B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

DQ297560.1
Fusarium pseudonygamai isolate ZMS-543-13A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

AF158306.1
Fusarium sp. NRRL25622 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
852 85289%0.099%

AY428795.1
Gibberella moniliformis strain FV4773 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence
852 85289%0.099%

AY428794.1
Gibberella moniliformis strain FV5146 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence
852 85289%0.099%

FJ605250.1
Gibberella moniliformis isolate UFMGCB 1229 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
850 85093%0.097%

EU314989.1
Gibberella moniliformis isolate FKCB-004 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
850 85092%0.097%

Fusarium sacchari strain NRRL 43543 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length=1047

Score = 869 bits (470), Expect = 0.0
Identities = 491/500 (98%), Gaps = 6/500 (1%)
Strand=Plus/Plus

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Sbjct  21
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Query  90
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Sbjct  498  CATATCAATAAGCGGAGGAA  517
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BIOGRAPHY

Mr. Tinnakorn Rosruen was born on October 2, 1983 in Bangkok, Thailand. He obtained a Bachelor Degree (Biotechnology) from the Faculty of Apply Science, King Mongkut's University of Technology North Bangkok in 2005. He has been studying for a Master of Program in Biotechnology, Faculty of Science, Chulalongkorn University since 2005.