สารยับยั้งจุลชีพจากราเอนโดไฟต์ *Aspergillus terreus* ที่แยกจากเหง้าไพล *Zingiber cassumunar* Roxb.

นางสาว สุรีย์ หมาดอาหิน

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ANTIMICROBIAL AGENTS FROM ENDOPHYTIC FUNGI *Aspergillus terreus* ISOLATED FROM *Zingiber cassumunar* Roxb. RHIZOMES.

Miss Suree Mat-arhin

สถาบันวิทยบริการ
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สุรีย์ หมาดอาหิน: สารยับยั้งจุลชีพจากราเอนโดไฟต์ *Aspergillus terreus* ที่แยกจากเหง้าไพล *Zingiber cassumunar* Roxb. rhizomes (ANTIMICROBIAL AGENTS FROM ENDOPHYTIC FUNGI *Aspergillus terreus* ISOLATED FROM *Zingiber cassumunar* Roxb. RHIZOMES) อ.ที่ปรึกษา: ผศ.ดร. สุรชัย พรภคกุล, 154 หน้า.

The purpose of this research was to isolate antimicrobial compounds from endophytic fungi isolated from *Zingiber cassumunar* rhizomes obtained from Surin, Supanburee, Songkhla and Pathumthanee province. The endophytic fungi were isolated using surface-sterilization technique to give 48 isolates including 59 % Mycelia sterilia, 35% *Fusarium* sp., 4% *Aspergillus* sp. and 2% *Schlerotium* sp. All isolates were examined antimicrobial activity using agar plate bioassay. The results showed that 10 isolates of the fungi inhibited tested microorganisms. Isolate ZcSR5-4 was selected for the study because it exhibited activity against *Bacillus subtilis* and *Escherichia coli* with inhibition clear zone 1.50 and 2.50 cm, respectively while others showed less activity. Based on morphology and nucleotide sequencing of Internal Transcribed Spacer (ITS) regions of rDNA, isolate ZcSR5-4 was identified as *Aspergillus terreus*. Mycelium and broth of isolate ZcSR5-4 were isolated by extraction and chromatographic technique to give 3 known compounds. On the basis of physical properties and spectroscopic data, they were elucidated as butyrolactone I, butyrolactone III and butyrolactone IV. These compounds were examined Inhibitory Concentrations (MICs). Butyolactone I inhibited *B. subtilis* ATCC6633, *S. aureus* ATCC25923, *E. coli* ATCC25922 with the MICs values of 62.5 μg/ml (147.41μM) and *C. albicans* ATCC10231 with the MICs values of 15.63 μg/ml (36.86 μM); butyrolactone III inhibited all tested microorganisms and butyrolactone IV inhibited *B. subtilis* ATCC6633, *S. aureus* ATCC25923, *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853 with the MICs values of 7.82 (17.77 μM), 31.25 (71.02 μM), 62.50 (142.04 μM) and 62.50 μg/ml (142.04 μM), respectively. In addition, all compounds were inactivity on cytotoxicity against 5 tumor cell lines.

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LIST OF ABBREVIATIONS

°C	= Degree Celsius
^{13}C-NMR	= Carbon-13 nuclear magnetic resonance
$CDCl_3$	= Dueterated chloroform
$CHCl_3$	= Chloroform
cm	= centimeter
COSY	= Correlation Spectroscopy
δ	= NMR chemical shift
d	= doublet (for NMR spectral data)
dd	= doublet of doublets (for NMR spectral data)
DMSO-d_6	= Dueterated dimethylsulfoxide
D_2O	= Deuterium Oxide
ε	= Molar absorptivity
EI-MS	= Electron Impact Mass Spectrometry
ESI-MS	= Electrospray Ionization Mass Spectrometry
EtOAc	= Ethyl acetate
g	= gram
HMBC	= ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation
^{1}H-NMR	= Proton Nuclear Magnetic Resonance
HSQC	= ^{1}H-^{13}C Heteronuclear Single Quantum Correlation
Hz	= Hertz
IR	= Infared
ITS	= Internal Transcribe Spacers
J	= Coupling constant
l	= liter
m	= multiplet (for NMR spectral data)
m	= medium (for IR spectral data)
M^+	= Molecular ion

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MEA	= Malt Extract Agar
MeOH	= Methanol
mg	= milligram
MHz	= Megahertz
min	= minute
ml	= milliliter
mm	= millimeter
MS	= Mass Spectroscopy
m/z	= mass to charge ratio
nm	= nanometer
NMR	= Nuclear Magnetic Resonance
NOESY	= Nuclear Overhauser Enhancement Spectroscopy
Na	= Sodium
PDA	= Potato Dextrose Agar
ppm	= Part per million
PCR	= Polymerase Chain Reaction
Plai	= *Zingiber cassumunar* Roxb.
q	= quartet (for NMR spectral data)
s	= singlet (for NMR spectral data)
s	= strong (for IR spectral data)
SEM	= Scanning Electron Microscope
sept	= septet (for NMR spectral data)
sp.	= species
t	= triplet (for NMR spectral data)
TLC	= Thin Layer Chromatography
μl	= microliter
μg	= microgram
UV	= Ultraviolet
w	= weak (for IR spectral data)
YES	= Yeast Extract Sucrose agar
λ_{max}	= the wavelength at maximum absorption (UV)
ν_{max}	= wave number at maximum absorption (IR)

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CHAPTER I

INTRODUCTION

Infectious disease is the principal cause of death worldwide, microorganisms being responsible for more than 13 million humans dying annually from respiratory, diarrhoeal and malarial infections (Beardsley, 1995). With the advent of antibiotics 50 years ago, scientists made sweeping predictions heralding the end of death and suffering from infectious diseases. During the past 25 years, however, the increasing incidence of drug resistance in pathogenic microbes which many people have looked for the new antimicrobial compounds of biological origin from wide variety organisms and many natural habitats.

In 1993, Stierle et al. (1993) reported the first paclitaxol producing endophytic fungus *Taxomyces andreanae*. Although the yield of pacliaxol is only as low as 24-50 ng/l, this finding causes scientists' great interest. Ever since, there have been many reports on the isolation of endophytic fungi that increasing interest has focused on the biology and chemistry of endophytic microorganisms.

The nature and biological role of endophytic fungi with their plant host is variable. Endophytic fungi are known to have mutualistic relations to their hosts, often protecting plants against herbivory, insect attack or tissue invading pathogens (Siegel et al., 1985; Clay, 1986; Yang et al., 1994). In some instances the endophyte may survive as a latent pathogen, causing or quiescent infections for a long period and symptoms only when physiological or ecological conditions favors virulence (Carroll, 1986)

Endophytic fungi and plant are closely related. Endophytic fungi can produce compounds similar or even identical to those produced by the host plant. Regina and Edson (2002) reported the endophytic fungus *Penicillium sp.* of *Melia azadarach*, which is a well-known limonoid producer, produced meroterpenes that it is similar to the partial structures of some lomonoids.

Currently, Barbara et al. (2002) reported ratio of novel biological metabolites in many reports and novel biological metabolites from endophytes. It shows that endophytes isolation can give new species of fungi and sources of novel biological

metabolites because of high biodiversity. Endophytic fungi can produce interested compound including antimicrobials, anticancer, antipesticides, antioxidance, biofumigant, antinematode etc. (Tan and Zou, 2001).

Endophytic fungi produce secondary metabolites that foster great medicinal or agricultural potentials. Kongsaeree et al. (2003) reported isolation and structure elucidation of antimalarial dihydroisocoumarin from endophytic fungus of *Crassocephalum crepidioides*. John et al. (1997) isolated two new 5-hydroxybenzofuran derivatives obtained by bioassay-directed fractionation of an extract of cultures of endophyte from wintergreen, *Gaulthria procumbens* L., which are toxic to spruce budworm, *Christoneura fumiferana*

Zingiber cassumunar Roxb., commonly known as Plai, is a Thai herbal plant which is a very interesting plant. It has been exploited for medicinal purposes in Thailand and Southeast Asia for centuries. Plai has long been regarded by Thai massage therapists as one of those oils necessary to have in their kit to combat joint and muscle problems. There are many reports which Plai produce antimicrobial compounds (อรัญญาและจีรเดช, 2005). This research may lead to selection of endophytic fungus isolates that are a potential source of antimicrobial compound.

Therefore, the main objectives of this research are as follow:

1. To isolate endophytic fungi from rhizomes of *Zingiber cassumunar* Roxb. that produce antimicrobial agents
2. To isolate and purify antimicrobial compounds from endophytic fungi

Additions, the scope of research are as follow:

1. To isolate endophytic fungi from healthy rhizomes of *Zingiber cassumunar* Roxb. from Songkhla, Supanburee, Patumthanee and Surin provinces.
2. To screen endophytic fungi for antimicrobial activities.
3. To identify a selected endophytic fungal isolate using classification based on morphology and molecular biology.
4. To extract, isolate and purify antimicrobial compounds of a selected endophytic fungal isolate.
5. To elucidate the structures of the isolated antimicrobial compounds.
6. To evaluate the biological activity of the isolated antimicrobial compound.

CHAPTER II

LITERATURE REVIEWS

2.1 Fungal biotechnology

Some 1.5 million species of fungi are believed to exist all over the world, of which only about 72,000 (less than 5%) have been describes so far (Hawksworth, 1991; Hawksworth et al., 1995). These display a wide variety of morphological forms ranging from the microscopic unicellular yeasts to multicellular macroscopic mushrooms. The vegetative structure of a vast majority of the fungi consists of thin-walled, transparent, branched or unbranched hyphae. In a number of simple fungi (especially yeasts and chytrids), the vegetative structure consists of a single, microscopic cell, spherical, ellipsoidal, tubular or irregular in shape. However, the uniqueness of the fungi lies in (Charaya and Mehrotra, 1997)

1. Their ability to produce a surprisingly large variety (conferring upon them the ability to colonise and degrade a huge variety of substrates)
2. Their potential to synthesise an amazing variety of metabolites with the biological activity

Technologies based on the degradative or synthetic activities of the fungi have become an integral part of the human society and, hence, of our commercial set up as well. Current commercial products of the fungi include amino acid, antibiotic, alcoholic beverages, fuel, biopesticide e. g. Charaya and Mehrotra (1997) said "The highly advanced form of fungal biotechnology we have at our command today has involved over a long period of time from the "art of the biotechnologist". All the modern high technologies for utilizing the fungi products and their genomes began with their use by different human societies solely on the basis of their empirical experiences. Their scientific exploitation at the organism level, subcellular level and finally at genome level constitute different steps in the evolution of fungal biotechnology."

From the report on the National Seminar on Recent Advances in Mycology (Meeting Report Recent Advances in mycology, 2005), current studies revealed that

endophytic fungi are neither incidental residents nor latent pathogens of plant hosts (Suryanarayanan, R.M.V. College, Chennai); they possess high metabolic versatility and produce novel secondary metabolites of industrial importance. Dr. Padmanaban (IMTECH, Chandigarh) emphasized the need to invent and conserve new and novel fungal resources to meet the requirement of rapidly developing biotechnology.

This clearly shows that there is unrivalled chemical diversity which could give us novel leads to molecules for the generation of new medicine. The sales of medicines in the top ten therapeutic classes report globally demand of medicines that show on table

Table 2.1 Sales of medicines in the top ten therapeutic classes (World health organization, 2004)

Class	Total Sales (US $ billion)
Anti-ulcers	19.5
Cholesterol & triglyceride reducers	18.9
Antidepressants	15.9
Non- steroidal anti-inflammatory drugs (NSAIDs)	10.9
Antihypertensive drugs (Ca^{2+} antagonists)	9.9
Antipsychotics	7.7
Oral antidiabetics	7.6
ACE inhibitors (plain)	7.5
Antibiotics (cephalosporins and combinations)	6.7
Systematic antihistamines	6.7
Total	111.3

The medicines in the top ten therapeutic classes account for one-third of all sales and the ten-selling medicines for one-eight of the world pharmaceutical market. As discussed earlier, it presents a picture of what have been the major points in the filed of fungal biotechnology and endophytic fungal are the sources of new medicine product that many demands from one.

2.2 Fungal endophytes

2.2.1 Endophyte discovery

2.2.1.1 Origin of tall fescue.

Tall fescue (*Festuca arundinacea* Schreb.), a native of Europe but is of minor importance there as perennial ryegrass (*Lolium perenne* L.) is favored because of its higher nutritive quality, thriving in most of this area because of mild temperatures and good rainfall. Tall fescue was introduced to the USA in the 1800's but was not planted widely until the 1940's and 1950's. It is the most important cultivated pasture grass in the USA, occupying over 35 million acres (Buckner and Bush, 1979).

2.2.1.2 Fescue toxicity problems.

Tall fescue soon gained a reputation for livestock health problems, resulting in poor animal performance (Pratt and Haynes, 1950). Since then, it has been found that three syndromes are associated with fescue toxicity (Stuedemann and Hoveland, 1988).

1. In the upper south, cattle symptoms include elevated respiration rate, and gangrene that resulted in loss of hooves, tails, and ears. This syndrome, known as fescue foot, was first described by Cunningham (1949) in New Zealand.
2. Where high rates of nitrogen fertilizer are applied to tall fescue pastures, hard fat accumulates along the bovine intestinal tract, resulting in upset digestion and difficult births (Bush et al., 1979; Stuedemann et al., 1975).
3. The syndrome has general symptoms of failure to shed the winter hair coat, intolerance to heat, poor animal gains reduced milk production, and low conception rates (Hoveland et al., 1983; Stuedemann and Hoveland, 1988).

These symptoms are most severe in warm weather. The problems are serious with cattle losses widespread throughout the tall fescue area. Beef cattle losses in the USA have been conservatively estimated at well over $600 million annually from reduced calf births and lower weaning weights (Hoveland, 1993).

2.2.1.3 Determining the cause of toxicity problems and findings endophyte.

Research was concentrated on external plant fungi, plant alkaloids, toxins produced in the rumen, and anions during the 1950's-1970's (Bush et al., 1979). Of the many alkaloids found in tall fescue, perloline was thought to be a major factor and a breeding program in Kentucky developed low-perloline lines. Unfortunately, low-perloline tall fescue was no more successful than other approaches in solving the toxicity problem.

For several years, Dr. J.D. Robbins, a toxicologist at the USDA Russell Research Center in Athens, GA, had become convinced that fescue toxicity involved a fungus because the symptoms were similar to that from ergot toxicity (Robbins, 1983). In 1973, Robbins and his associates C.W. Bacon and J.K. Porter isolated three species of Balansia fungi from a toxic tall fescue pasture in north central Georgia (Bacon et al., 1975).

These fungi were endophytic (live within the host plant) and not virulent to their grass host. This group of fungi was first associated with grass toxicosis of cattle and goats in India (Nobindro, 1934). Toxicological studies demonstrated these endophytic fungi species in Georgia were toxic and had the potential for ergot alkaloid synthesis (Porter et al., 1979). These findings resulted in the hypothesis that an endophyte was the causal agent in fescue toxicosis and stimulated further research (Bacon, 1995).

Later, these scientists became aware of research by Neil (1940) in New Zealand that confirmed a fungal endophyte in tall fescue and perennial ryegrass, suspecting that it might produce a toxic compound. The first discovery of a grass endophyte was reported in 1898 by Vogl in Germany (White et al., 1993). The dependence of the endophyte on seed transmission for dispersal was demonstrated by Sampson (1933) in Wales.

2.2.2 Endophytic fungi description

The termed endophyte is appled to fungi (or bacteria) which live within plant tissue, for all or part of their life cycle and cause no apparent infections. This definition excludes the mycorrhizal fungi but dose not imply that endophytic fungi are not cultivable on artificial media. Some species of endophytic fungi have been identified as source of anticancer, antidiabetic, insecticidal and immunosuppressive

compounds (Maheshwari, 2006). For Endophytic fungi description, it characterizes endophytic fungi in diversity aspects from many reports as below

2.2.2.1 Types of endophytic fungi

First discovery of endophytic fungi was found in grass, but it found many reports studied fungal endophytes in tree, shrubs, and ferns. Separating of endophytic fungi of mycologists have many aspect example differences of host plant, differences of landscapes, difference of climates, differences of compound from its. For this report, it separates types of endophytic fungi from differences of host plant.

2.2.2.1.1 Grass endophytes

Endophyte is a fungus found in many grass species. The fungal stands grow between the plant cells, and transmit themselves to the next grass generation by growing into the developing seed head and then growing into the subsequent grass seedling.

-Taxonomy and specificity (Saikkonen et al., 1998; Clay, 1990)

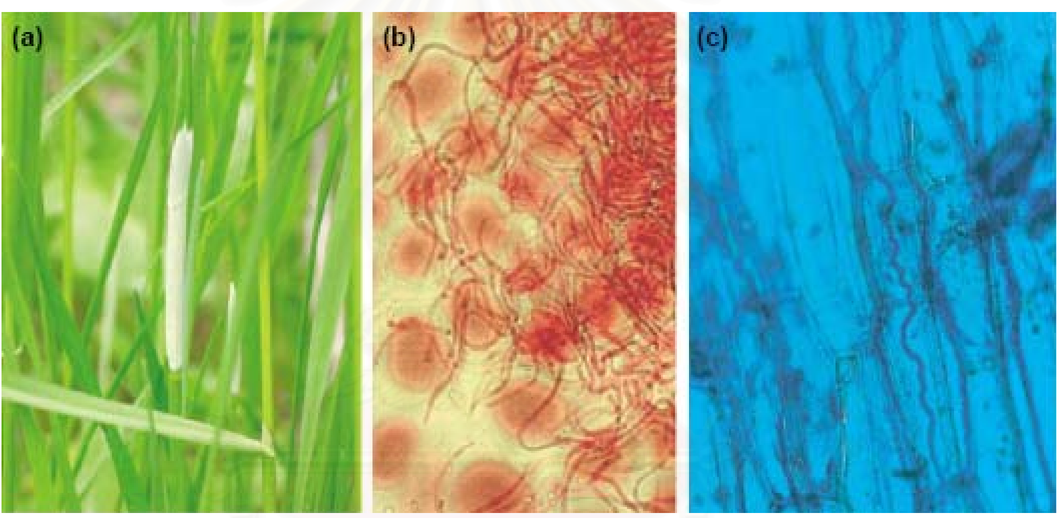
Systematic grass endophytes are restricted to clavicipitaceous member of the tribe Balansiae (Ascomycotina) and infected at least 80 genera and 300 species. A few other genera of systematic endophytes occur in pooid or non-pooid grasses. The taxonomy of the group is based on the morphology of fruiting structures on host plant and the type of conidia and ascospores produced. Endophyte genera include *Atkinsonella* (two species), *Balansia* (approximately 15 species), *epichloe* (less than 10 species), and *Myriogenospora* (two species) but most studies of endophyte-host grass interactions have involved Neophydium (asexual form of Epichloe) and Epichloe

-Systematic versus localized infections

Few systematic endophyte-grass associations have been studies intensively for within-plant distribution. In *Neophydium*-infected Arizona fescue plant, for example, infection of individual tillers is variable, ranging from 10% to 100%. Even in the intensively studied, *Neophydium*-infected cultivars of tall fescue and perennial ryegrass, uninfected seeds are often produced by infections within plants.

-Reproduction (Muller and Krauss, 2006)

Grass endophytes, such as Epichloe, have a sexual and an asexual life cycle. The sexual form, Epichloë, has a parasitic nature. This form causes choke disease (figure 2.1a), which suppresses flower and seed production in grasses, and is transmitted horizontally to new host plant. The asexual form, *Neophydium* (previously Acromonium), is seen as a mutualist of grasses, causing no visible symtomps and being transmitted vertically through the seeds of the host plant. *Neophydium* can be observed as hyphal accumulations in infected grass seeds (figure 2.1b) or as single, long and often convoluted hyphae that grow between the cells of the host's leaf tissue (figure 2.1c)



Figures 2.1 Clavicipitalean endophytes (Clavicipitaceae; Ascomycota) that are associated with grasses have two life forms that have resulted in dual nomenclature for this group of fungi.

(a) The sexual-type Epichloe reproduces by forming a stroma structure on the stem of the host plant, thereby suppressing the formation of host inflorescences and seeds (choke disease). Epichloe is transmitted horizontally via conidiae whose transportation to another stroma requires an insect vector. Mating between two different mating types of the fungus occurs in this second stroma.

(b) The asexual symptomless form of Clavicipitalean endophytes are called Neotyphodium, and can be detected in the seed as an agglomeration of short hyphae within the aleuron layer. The endophyte propagates vertically with the seed of its host.

(c) Epichloe and Neotyphodium both live intercellularly within the host's leaf tissue, forming sparsely branched and typically convoluted hyphae that grow parallel to the long axis of the plant cells.

2.2.2.1.2 Woody endophytes

Endophytic fungi have been found in all woody plants that have been examined for endophytes. Studies of fungal endophytes in tree, shrubs, and fern show that individual species and even individual plants typically harbor scores of fungal species.

-Taxonomy and specificity

Taxonomically, endophytes from woody plants are usually members of the Ascomyticona but may also include members of the Basidiomycotina, Deuteromycotina, and Oomycetes. Endophytes in woody plants are more diverse than grass endophytes in terms of genera and species, as expected given the much broader taxonomic range of host plants, At least at the generic level, woody plant endophytes have wider host ranges than the grass endophytes and, thus, appear to be more generalized than grass endophyte.

-Systematic versus localized infections

Infections by endophytes of woody plants are usually highly localized within leaves, petiole, bark, or stem. However, under certain conditions, such as when leaves age or senesce, localized infections can become more widespread, although the term endophyte may no longer apply because the infections becomes external. Ryegrass, uninfected seeds are often produced by infections within plants.

-Reproduction (Muller and Krauss, 2006)

Many nonsystemic endophytes in woody plants are transmitted horizontally via asexual spores. However, the frequency of asexual reproduction of endophytic fungi has not been extensively studied in woody plants. Because endophytes of woody plants have been found in seed and acorns, endophytes of woody plants may also be transmitted vertically and maternally via seed. This possibility is supported by at least anecdotal evidence in non grass host.

2.2.3 Comparison of endophytic fungi and parasite switching.

Endophytes of both grasses and woody plants are thought to have evolved from parasitic and pathogenic fungi. Woody plant endophytes are closely related to pathogenic fungi, and presumably evolved from them via an extension of latency periods and a reduction of violence (Saikkonen, 1998). The current report, Kogel et al. (2006) describes symbiotic development of biotrophic endophytes and pathogen. Because endophytes posses structural similarities with pathogens, they are the object of a host's non self recognition (see figure 2.2). Addition, cell wall penetration by a fungal instruder is normally accompanied by the release of plant-derived elicitor-active molecules. Hence, endophytes must avoid or overcome non-specific resistance responses to achieve successful penetration by reprogramming the invaded cell to accommodate infection structures.

The similarity of recognition of endophytes and parasite by plant indicated that a potential common basis might have been specified during the evolution of symbioses. Interestly, a small plant derived molecule that induced branching in a mycorrhizal fungus was identified sterigmatone. As related molecules are also detected by the parasite weeds striga and orobanche, similar molecule seem to play important roles during both friendly and unfriendly interactions.

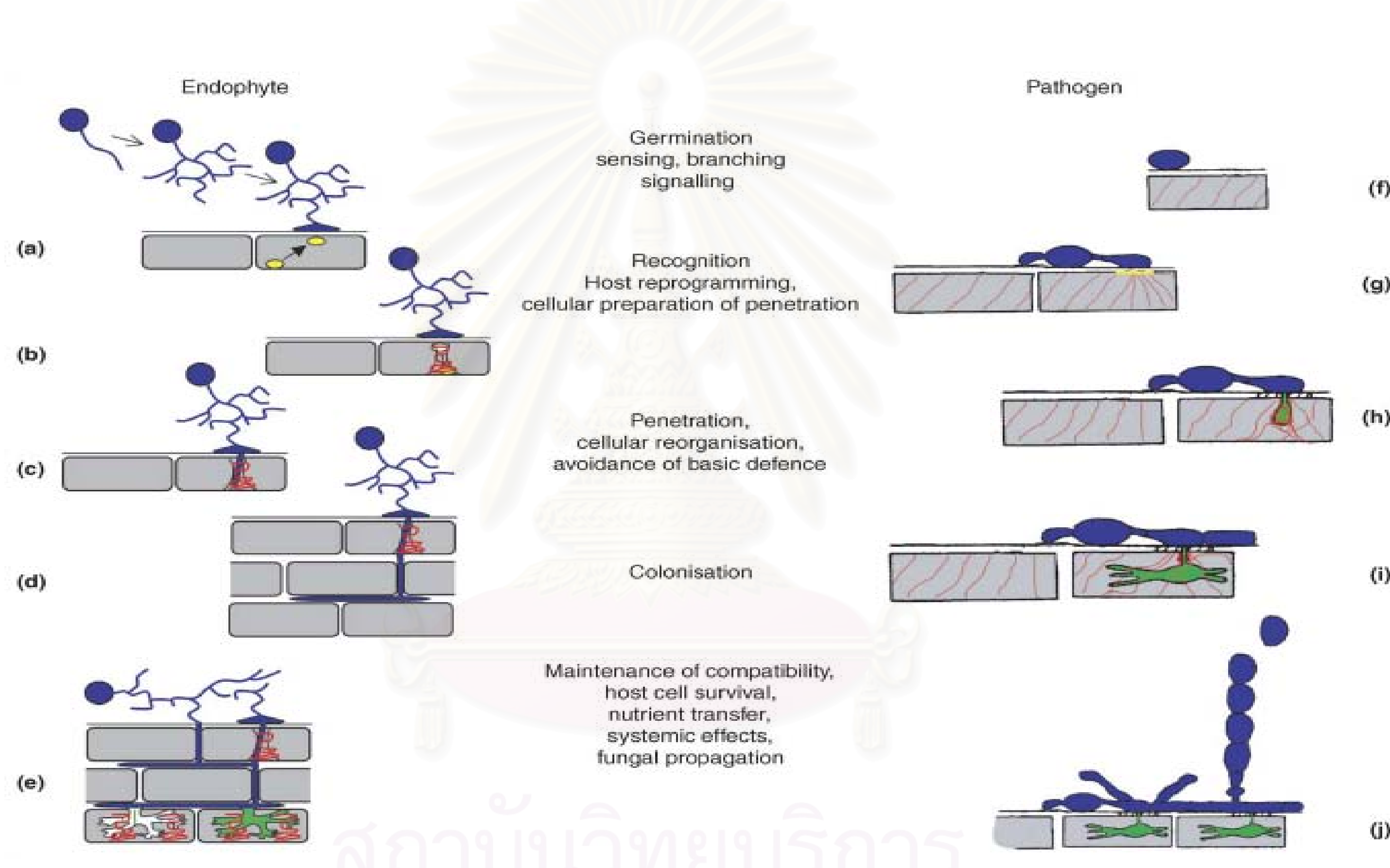


Figure 2.2 Shown symbiotic developments of biotrophic endophytes and pathogens (Kogel et al., 2006).

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(a) Once AM spores germinate and the germ tube approaches a root, apical dominance is abandoned and the branching of hyphae is triggered by 5-deoxy-strigol (Akiyama et al., 2005). Upon physical contact, the fungus forms an appressorium, which appears to induce the movement of the plant nucleus towards the contact site.

(b) Cytoskeletal elements and the endoplasmic reticulum form the pre-penetration apparatus along the axis of nuclear movement (Genre et al., 2005).

(c) The structure is entered by an infection hypha, from which

(d) Colonization of root cortex begins. Initial infestation is accompanied by a balanced induction of plant defence genes.

(e) When the fungus finally reaches the inner cortex, it penetrates the cell wall and builds up a tree-like hyphal structure, the arbuscule. Arbuscule-containing cells have specific cytoskeletal structures and accumulate ROS. While arbuscules develop and decease, the fungus spreads further in the root and also colonises the surrounding soil. There it takes up mineral nutrients, which are transported into the root and exchanged for carbohydrates.

(f) Once a powdery mildew fungus germinates, it forms an appressorium for host cell wall penetration.

(g) Appressoria seem to release signals for the formation of membrane domains (yellow) into which host susceptibility factors and defence factors are recruited (Bhat et al., 2005). In a compatible interaction, the host nucleus transiently migrates to the site of attempted penetration (not shown) and some actin filaments (red) polarise toward this site.

(h) During penetration, host cell membrane is formed around the fungal feeding structure (green), which is closely enveloped by actin filaments and led by a ring of actin around the growing tip (Opalski et al., 2005).

(i) When the haustorium matures, a meshwork of cortical actin is maintained around the haustorial neck, whereas actin polarisation resolves.

(j) Eventually, the parasite establishes secondary haustoria and fulfils its lifecycle by producing a new generation of conidia.

2.2.4 Isolation of Endophytes

Techniques for endophyte isolation and culture have been developed gradually over time. Bacon and white (1994) have written an excellent review on staining, media and procedure for analyzing endophytes. Endophytes can be isolated from various plant parts such as seeds, leaf and stem.

It is important to understand the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms as well as ones making novel bioactive products. Thus, since the number of plant species in the world is so great, creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying bioactivity (Mittermeier et al., 1999).

A specific rationale for the collection of each plant for endophyte isolation and natural-product discovery is used. Several reasonable hypotheses govern this plant selection strategy and these are as follows.

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

2. Plants that have an ethnobotanical history (use by indigenous peoples) that are related to the specific uses or applications of interest are selected for study.

3. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, may have nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).

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

5. Plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity.

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However, most procedures for isolating endophytes are relatively simple and routine for anyone skilled in basic microbiological technique. One of the critical needs for isolating endophytic fungi is obtaining fresh plant material. The need for preventing desiccation must be balance against the need for adequate aeration

1. Methods slow down tissue death.

Prolonged transport in sealed plastic bags should be avoided if possible. Study paper bags, wax paper bags, or zip-locked, perforated bags designed for vegetable storage work well for transport and temporary storage of most types of plant tissues.

2. Methods minimize the growth of endophytes, contaminating fungi and bacteria.

The plant and plant parts collected for studying endophytic communities should look apparently healthy, in order to minimize the compounding effect because of plant pathogenic and sporic species. Young tissue is appropriate for isolation as older tissues often contain many additional fungi that make isolation of slow growing fungi difficult (Bacon and white, 1994)

In addition, selected isolation procedures are listed in Table 2.2 and 2.3 a standard method utilizes dips in both EtOH and EtOCl (bleach).

Table 2.2 Isolation of endophytes: sterilization times and concentration of the NaOCl solutions used surface sterilization. The following sequential steps are involved;

A) first dip in 96% EtOH for 1 min (lichen and mosses for 30 sec)

B) sterilization in NaOCl

C) second dip in 96% EtOH for 30 sec.

Plant species	Minutes in NaOCl	NaOCl dilution	Refference
Lichens	1	1:5	Petrini (1986)
Moss	1	1:5	Petrini (1986)
Fern	3	1:5	Dreyfuss & Petrini(1984)
Conifer needles	5	1:2	Carroll & Carroll (1987)
Confer twigs	7	1:2	Carroll & Carroll (1987)
Monocots-leaves & *culms* (Triticum)	3	1:5	Petrini (1986)
Dicots (general)	3	1:5	Petrini (1986)
Erica leaves	3	1:5	Petrini (1986)
Erica stem	5	1:5	Petrini (1986)
Rhododendron	3	1:5	Petrini (1986)
Vaccinium	3	1:5	Petrini (1986)

Table 2.3 Selected examples of isolation procedures for endophytes from woody plants (Bills, 1996).

Tissues/Hosts	Dissection of Tissue	Surface Sterilization
Ulex-spines, stems	Pines and 2 cm stem	1 min 96% EtOH, 3min 3.25% NaOCl, 0.5 min 96% EtOH
Fagus-bark	2 mm borer	propane torch or none
Alnus-xylem and bark	1 cm stem sections, bark and xylem cultered separately	35% peracetic acid
Castanea & *Quercus*-bark	5 mm arch punch	0.525% NaOCl 10 min
Picea-mature stems	sawing followed by chisel extraction	Only extraction tools sterilized
Picea-roots	1 cm segments	sink washing, ultrasound, serial washing
Fraxinus, Quercus, Fagus-twigs	2 cm segments, bark and xylem cultured separately	ethanol flaming
Carpinus-bark	1 cm leather punch	0.525% NaOCl for 3 min., flaming in alcohol lamp
Rhizophora-seedlings	1.1 cm cork borer of hypocotyls and radicle	Sterile sea-water rinse, 0.1% HgCl2 in 5% EtOH
Licuala-leaves	3 mm discs from veins and interveins	1 min 96% EtOH, 10 min 3.25% NaOCl, 0.5 min 96% EtOH
Euterpe-leaves	3 mm discs from vains and intervains	1 min 75% EtOH, 10 min 3.25% NaOCl, 0.5 min 75% EtOH

Surface-sterilized plant tissues are plated in an appropriate medium amended with antibiotics and Rose Bengal and incubated at room temperature with periodic light and darkness. Incubated plates are checked after 1 week of incubation at regular intervals for fungal development. If the colony is very small and there is a risk of engulfment by other colonies, it needs to be subcultured. Subcultured isolates are generally maintained at room temperature for many weeks to study morphological and other characteristics (Ajit et al., 1998).

Most endophytes will sporulate after a few weeks at 15 to 21 C^0, either in darkness or in daylight on 1% malt extract agar (MEA). For nonsporulating isolates, various methods to induced sporulation are use exposure at 8 C^0 under fluorescent or near-ultraviolet light with a 12 hours dark-light cycle or incubation at 4 C^0 in darkness may induce spolulation in some fungi.

Transferring nonspolulating isolates to natural media such as corn meal agar, V8 agar potato carrot agar, or on chemically defined media such as Czapek's agar, peptone dextrose agar, etc. may induce spoluration. Sometimes it is necessary to grow the fungus on host substrate to induce sporulation. This accomplished several different ways.

The easiest method is to sterilize pieces of host tissue for 20 minutes at 120 C^0 and place these in the medium. For some host tissue it is desirable to sterilize using irradiation or propylene oxide. A decoction of host leaves, brewed up in a manner similar to marking tea, may by add to the medium.

Other methods can be designed, but should try mimicking the situation in nature as closely as possible.

2.2.5 Antimicrobial agents from endophytic fungi

Endophytes have also been recognized as a repository of novel metabolites of pharmaceutical importance (Tan and Zou, 2001). The discovery that an endophytic fungus (*Taxomyces andreanae*) colonizing *Taxus brevifolia* also produced the anticancer drug Taxol was a huge surprise (Stierle et al., 1993). Thus, traditional Chinese herbs may provide us with a profuse potential source of endophytic fungi for finding new therapeutic drugs. The following section shows some examples of antimicrobial agents and other bioactive agent from endophytic fungi and their potential in the pharmaceutical and agrochemical arenas.

The past, the major source of antimicrobial agents came from soil-borne microorganisms. It now appears that an enormous, relatively untapped source of microbial diversity is the microbial endophytes. Some of endophytes may produce antimicrobial (antibacterial, antifungal, antiviral, etc.) substances that may be involved in the host-endophyte relationship. These items describe the studying endophytes and their antimicrobial agent.

From an endophytic of the eastern larch, a novel ester metabolite was isolated as antibacterial agent against *Vibrio salmonicida*, *Psudomonas aeruginosa* and *Strephylococcus areus* (Findlay et al., 1995)

Phomopsichalasin (Zau and Tan, 1999), a novel cytochalasan with an isoindolelone moiety fused to a 13-membered tricyclic system, was characterized from the culture of an endophytic *Phomopsis sp.* originating from twigs of *Salix gracilostyla* var. *melanostachys* (Horn et al., 1995). In disk diffusion assay, this metabolite was show to be antibacterial against Bacillus subtilis, *Strephylococcus aureus* and *Salmonella gallinarum*, and antifungal against the human pathogenic yeast *Candida albican*.

A new indole derivative 6-isoprenylindole-3-carboxylic acid was characterized from the A. annua endophyte *Collectotrichum sp*. It shows moderate antibacterial activity against the Gram-positive bacteria Bacillus subtilis, *Strephylococcus aureus*, *Sarcina lutea* and Gram-negative bacterium *Psudomonas sp*. Furthermore this product is also inhibitory to the growth of some crop phytopatogenic fungi *Phytopthera capisici*, *Rhizoctonia cerealis* and *Gaeumannomyces graminis* var. *tritici* (Lu et al., 2002).

Bioactive metabolites from *Aspergillus fumingatus* CY018 in *Cynodon dacylon* leaves, asperfumoid, fumigaclavine C, fumitremugin C, physcion and helvolic acid were shown to inhibit *C. albicans* with MICs of 75.0, 31.5, 62.5, 125. 0 and 31.5 g/ml , respectively (Liu et al., 2004) The other endophytic fungi from this plant, Rhizoctonia sp. Cy064 were cultured and isolated a new benzophenone, name rhizoctonic acid, together with three known compound monomethylsulochrin, ergosterol and 3b,5a,6b-trihydroxyergosta-7,22-diene. All four metabolites were subjected to a more detailed in vitro assessment of their antibacterial action against five isolated and reference (ATCC 43504) *Helicobacter pylori* strains (Ma et. al., 2004).

In 2006, two new metabolites named 6-oxo-de-O-methyllasiodiplodin and (E)-9-etheno-lasiodiplodin, with tree known compounds lasiodiplodin, de-O-methyllasiodiplodin, and 5-hydroxy-de-O-methylasiodiplodin, were isolated from the mycelium extract of brown algae endophytic fungus (No. ZZF36) obtained from the South China Sea. Lasiodiplodins and derivatives of lasiodiplodins had antimicrobial activities. (Yang et al., 2006)

Maysa et al., 2006 reported two compound named cis-4-hydroxy-6-deoxyscytalone and (4R)-4,8-dihydroxy-α-tretalone from *Colletrotricum gloeosporioides* in *Cryptocarya mandioccana* Nee. (Lauracrae) that inhibited the phytopathogenic fungi *C. cladosporioides* and *C. sphaerospermum*.

2.2.6 Application of endophytic fungi

2.2.6.1 MaxP™ endophyte

MaxP™ is a registered trademark of Grasslanz, It is a new tall fescue endophyte available to farmers to improve the robustness of tall fescue plants, enabling them to better handle the pressures imposed by pests and moisture stress. Tall fescue with MaxP™ endophyte offers improved persistence compared with tall fescue without endophyte. Unlike ryegrass endophyte, MaxP™ endophyte produces a group of compounds called lolines (table 2.4). Generally, major endophyte compounds are

- Peramine – a natural insecticide that acts as a deterrent to some important pests (e.g. Argentine Stem Weevil); non-toxic to stock.
- Lolitrem B – provides protection against some insects; causes summer ryegrass staggers.
- Ergovaline – a compound that can reduce animal performance in moderate to high amounts but provides greater Black Beetle resistance.

Lolines are completely safe for animals but toxic to insects, providing protection against Argentine Stem Weevil, Black Beetle, Pasture Mealy Bug and Cutworm as well as many other insect pests. MaxP™ can improve the drought tolerance of tall fescue. In areas where there are few insect pests and limited moisture stress, MaxP™ may not be essential, but may still improve production.

Animal Production of MaxP™ inoculated grass has undergone rigorous animaltesting prior to its release and has been found to produce no negative effects on animal health and production. In addition, the persistence of MaxP™ tall fescue has been very good, out-yielding perennial ryegrass three years after sowing, and having considerably higher plant density than the same fescue without endophyte (Figure 2.3).

Tall fescues with MaxP™ endophyte have been tested throughout New Zealand since 1997. Trials consistently show significant yield and persistence advantages to tall fescues with MaxP™ endophyte. In Kerikeri, Northland, MaxP™ tall fescue produced 21–81% more dry matter per year than the same cultivar without endophyte. This benefit is more pronounced through late summer and autumn, when yield increases of up to 180% have been measured. Much of the increased performance is due to the resistance MaxP™ provides against the destructive insects Black Beetle and Argentine Stem Weevil. These insects reduce the growth and persistence of tall fescue without endophyte (Available from http://www.reidfarmers.co.nz/assets/seed/2006_ Pasture _Options_1.pdf).

Table 2.4 Comparison of main compound producing from MaxP™ and Nil endophyte (Available from http://www.reidfarmers.co.nz/assets/seed/2006_ Pasture _Options_1.pdf).

Endophyte type	Grass Species	Endophyte compound			
		Peramine	Loliterm B	Ergovaline	Lolines
MaxP™	Tall fescue	Produce	Not produce	Not produce	Produce
Nil endophyte	Tall fescue	Not produce	Not produce	Not produce	Not produce

Figure 2.3 Shown grass with MaxP™ endophyte and grass without Endophyte (Available from http://www.reidfarmers.co.nz/assets/seed/ 2006_ Pasture _Options_1.pdf).

2.2.6.2 Muscodor™

In the late 1990s, Strobel found *Muscodor albus* endophytes. The endophytic fungi were isolated from *Zinnamon zeylanicum* barks that produces a mixure of volatile organic compounds including ester, alcohol, acid and lipid. The volatile organic compounds of *M. albus* kill many of the Postharvest pathogen such as below

-*Botrytis cinerea* (gray mold)

-*Colletotrichum acutatum* (anthracnose)

-*C. coccodes (anthracnose)*

-*Geotrichum candidum* (sour rot)

-*G. citri-aurantii* (sour rot of citrus)

-*Monilinia fructicola* (brown rot of stone fruits)

-*Penicillium digitatum* (green mold of citrus)

-*P. expansum* (blue mold of pome fruits)

-*Rhizopus sp.* (Rhizopus rot or leak)

Later component of volatile organic compounds were analized by Gas chromatography that found many compound comparison area following below table

Table 2.5 Shown Analysis of volatiles of *Muscodor albus* in boxes by GC/FID (Available from http://www.mbo.org/2005/MBAO%202005%20pdfs/Preplant/11/Walgenbach.pdf).

Compound	Retention Time (min)	% area
Ethyl propionate	5.65	9.6
Ethyl isobutyrate	6.69	0.7
Methyl 2-methylbutyrate	7.83	0.3
Ethyl butyrate	8.78	0.1
Isobutyl alcohol	10.14	1.4
2-Methylbutyl acetate	11.04	2.4
2-Methyl-1-butanol	13.68	48.5
Isobutyric acid	25.26	14.9
Phenethyl alcohol	32.90	5.7

The term “mycofumigation” has been applied to the practical aspects of this fungus. The first practical demonstration of its effects against a pathogen was the mycofumigation of covered, smut-infected barley seed for a few days resulting in 100% disease control. This technology is currently being developed for the treatment of fruits in storage and in transit.

AgarQuest, of Davis, CA, USA, is in full scale development of *M. albus* for numerous agricultural applications with the anticipated release of an agricultural product in 2006 and the products are paper pad with dehydrated Muscodor culture (figure 2.4). In addition, the test of dehydrated Muscodor culture in strawberry and other vegetables are good activity of pathogen inhibition (figure 2.5).

Figure 2.4 Paper pad with dehydrated Muscodor culture. (Available from http://www.mbo.org/2005/MBAO%202005%20pdfs/Preplant/11/Walgenbach.pdf).

Figure 2.5 Shown Muscodor controls fruit rot (Gray mold on strawberries) (Available from http://www.mbo.org/2005/MBAO%202005%20pdfs/ /Preplant/11/Walgenbach.pdf).

2.3 Description of *Zingiber cassumunar* Roxb. (อรัญญาและจีรเดช, 2005; Wanauppathmkul, 2003)

Zingiber cassumunar, Roxb. (Zingiberaceae) commonly called Plai in Thai, an annual herb, is widely distributed in Thailand. The rhizome part of the herb has yellow to green color with fleshy thick texture containing multiple sessile tubers. The plant has leaves stem 1 to 1.5 m tall, leaves distichous, oblong-lanceolate 20 to 30 cm long and 2 to 8 wide, pubescent below: ligule very short bilobed, pubescent; sheath glabrous or hairy, inflorescences scapose; peduncle 8 to 30 cm long, with pubescent sheaths, spike ovoidellipsoid, bracts greenish red, narrowly obovate or rhomboid, 2.5 to 3.5 cm long; bracteole shorter than bract, ovate, 3-dentate, calyx truncate, glabrous,

corolla tube ca. 2.5 cm long, pale yellow, dorsal lobe cymbiform, lateral lobe linear-lanceolate, labellum pale yellow, suborbicular, apex emarginated, lateral lobe ovate-oblong, appendage slightly longer than anther; stamen pale yellow. (Fig. 2.1)

Figure 2.6 *Zingiber cassumunar* Roxb.; a) rhizomes, b) leaves and c) flower (Available from http://aoki2.sigunma-u.ac.jp/BotanicalGarden/HTMLs /Zingiber-cassumunar.html and http://www.terebess.hu/tiszaorreny /fuszer/thai.html)

3.1 Chemical constituents

Essential oil of Plai is steam distilled from the rhizome and has a pale amber color. The scent is a cool, green peppery one with a touch of a bite. Active chemicals: sabinene (25-45%), γ-terpinene (5-10%), α-terpinene(2-5%), terpinen-4-ol (25-45%), and (E)-1-(3',4'-dimethoxyphenyl)butadiene- in text as DMPBD (1-10%) (อรัญญาและจีรเดช, 2005). Some constituents from Zingiber cassumunar, Roxb. are

- Monoterpenes
- Arylbitanoids
- Cyclobutyl derivative
- Curcuminoid derivatives
- Naphthoquinones
- Cyclohaxane derivatives

It were shown on table 2.6, 2.7, 2.8, 2.9, 2.10 and 2.11

Table 2.6 Monoterpenes from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003)

Item	Structure	Biological activity	References
1.	Terplnen-4-ol	Anti-inflammatory, Antimicrobial	Kuroyanagi et al.(1980), Taroeno et al. (1991), Pongprayoon et al. (1996/97)
2	Sabinene	Antimicrobial	Casey TE et al. (1971), Taroeno et al. (1991), Pongprayoon et al. (1996/97)

Table 2.7 Arylbitanoids from Zingiber cassumunar Roxb. (Wanauppathmkul, 2003)

Item	Structure	Biological activity	References
1.	(E)-1-(3',4'-Dimethoxyphenyl)butadiene = DMPBD	Anti-inflammatory	Kuroyanagi et al. (1980), Tuntiwachwuttikul P et al. (1981), Ozaki et al. (1991), Taroeno et al. (1991), Pongprayoon et al. (1996/97), Jeenapomgsa R et al. (2003)

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Table 2.7 Arylbitanoids from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003) (Continued)

Item	Structure	Biological activity	References
2	(E)-1-(3',4'-Dimethoxyphenyl)but-1-ene	Anti-inflammatory Analgesic	Kuroyanagi et al. (1980) Tuntiwachwuttikul et al. (1981) Ozaki et al. (1991) Brophy and Zwaving (1991)
3.	(E)-4-(3',4'-Dimethoxyphenyl)but- 3-en-1-ol	Uterine relaxant, Anti-inflammatory Smooth muscle relaxant, Anti-histamine	Amatayakul et al. (1979) Tuntiwachwuttikul et al. (1980) Kuroyanagi et al. (1980) Kanjanapothi et al. (1987) Taroeno et al. (1991) Masuda and Jitoe (1995), Pongprayoon et al. (1996/97)
4.	(E)-4-(3’,4’-dimethoxyphenyl)but-3-enyl acetate	Anti-inflammatory	Amatayakul et al. (1979) Tuntiwachwuttikul et al. (1980) Taroeno et al. (1991) Masuda and Jitoe (1995) Pongprayoon et al. (1996/97)

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Table 2.7 Arylbitanoids from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003) (Continued)

Item	Structure	Biological activity	References
5.	(E)-4-(3',4'-dimethoxyphenyl)but-3-enyl palmitate	-	Kuroyanagi et al. (1980), Tuntiwachwuttikul et al. (1981)
6.	(E)-3-hydroxy-1-(3',4'-dimethoxyphenyl)but-1-ene	-	Masuda and Jitoe (1995)
7.	(E)-2-hydroxy-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol	-	Masuda and Jitoe (1995)
8.	(E)-2-methoxy-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol	-	Masuda and Jitoe (1995)
9.	1-(3',4'-dimethoxyphenyl)butane	-	Taroeno et al. (1991)

Table 2.7 Arylbitanoids from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003) (Continued)

Item	Structure	Biological activity	References
10.	(E)-4-(4’-hydroxy-3’-methoxyphenyl)but-2-en-1-ol	-	Masuda and Jitoe (1995)
11.	(E)-4-(4’-hydroxy-3’-methoxyphenyl)but-3-enyl acetate	-	Masuda and Jitoe (1995)
12.	4-(2’,4’,5’-trimethoxyphenyl)but-1,3-diene	-	Tuntiwachwuttikul et al. (1981)
13.	(E)-1-(2’,4’,5’-trimethoxyphenyl)but-1-ene	-	Kuroyanagi et al. (1980), Tuntiwachwuttikul et al. (1981)

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Table 2.8 Cyclobutyl derivative from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003)

Item	Structure	Biological activity	References
1.	cis-1,2-bis(E)-3”,4”-dimethoxystyryl cyclobutane	-	Jitoe et al. (1993)

Table 2.9 Curcuminoid derivatives from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003)

Item	Structure	Biological activity	References
1.	Curcumin	Anti-oxidative, Anti-inflammatory Anti-tyrosinase Anti-microbial	Dechatiwongse and Yoshihira (1973)
2.	Cassumunin A	Anti-oxidative, Anti-inflammatory	Masuda et al. (1993)
3.	Cassumunin B	Anti-oxidative, Anti-inflammatory	Masuda et al. (1993)
4.	Cassumunin C	Anti-oxidative, Anti-inflammatory	Masuda et al. (1993)

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Table 2.9 Curcuminoid derivatives from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003) (Continued)

Item	Structure	Biological activity	References
5.	Cassumunarin A	Anti-oxidative	Jitoe et al. (1994)
6.	Cassumunarin B	Anti-oxidative	Jitoe et al. (1994)
7.	Cassumunarin C	Anti-oxidative	Jitoe et al. (1994)

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Table 2.10 Naphthoquinones from *Zingiber cassumunar* Roxb.
(Wanauppathmkul, 2003)

Item	Structure	Biological activity	References
1.	2-methoxy-8-(3',4'-dimethoxyphenyl)-1,4-naphthaquinone	-	Amatayakul et al. (1979) Dinter and Hansel (1980) Kuroyanagi et al. (1980)
2.	2-methoxy-8-(2',4',5'-trimethoxyphenyl)-1,4-naphthaquinone	-	Dinter and Hansel (1980) Kuroyanagi et al. (1980)

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Table 2.11 Cyclohaxane derivatives from *Zingiber cassumunar* Roxb.
(Wanauppathmkul, 2003)

Item	Structure	Biological activity	References
1.	tran-3-(2',4',5' trimethoxyphenyl)-4-[(E)-3''',4'''-dimethoxystyryl]cyclohax-1-ene	-	Jitoe et al. (1993)
2.	cis-3-(2',4',5'-trimethoxyphenyl)-4-[(E)-3''',4'''-dimethoxystyryl] cyclohax-1-ene	-	Kuroyanagi et al. (1980)
3.	tran-3-(3',4'-trimethoxyphenyl)-4-[(E)-3''',4'''-dimethoxystyryl] cyclohax-1-ene	-	Kuroyanagi et al. (1980), Jitoe et al. (1993)
4.	tran-3-(3',4'-dimethoxyphenyl)-4-[(E)-2''',4''',5'''-trimethoxystyryl] cyclohax-1-ene	-	Kuroyanagi et al. (1980)

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Table 2.11 Cyclohaxane derivatives from *Zingiber cassumunar* Roxb. (Wanauppathmkul, 2003) (Continued)

Item	Structure	Biological activity	References
5.	cis-3-(2’,4’,5’-trimethoxyphenyl)-4-[(E)-2’’’,4’’’,5’’’-trimethoxystyryl] cyclohax-1-ene	Anti-inflammatory	Amatayakul et al. (1979), Tuntiwachwuttikul et al. (1980), Kuroyanagi et al. (1980), Pongprayoon et al. (1996/97)
6.	cis-3-(3’,4’-dimethoxyphenyl)-4-[(E)-3’’’,4’’’ -dimethoxystyryl] cyclohax-1-ene	Anti-inflammatory	Amatayakul et al. (1979), Kuroyanagi et al. (1980), Tuntiwachwuttikul et al. (1981), Pongprayoon et al. (1996/97)
7	cis-3-(3’,4’-dimethoxyphenyl)-4-[(E)-2’’’,4’’’,5’’’ -trimethoxystyryl] cyclohax-1-ene	Anti-inflammatory	Amatayakul et al. (1979), Kuroyanagi et al. (1980), Tuntiwachwuttikul et al. (1981), Jitoe et al. (1993) Pongprayoon et al. (1996/97)

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3.2 Biological activity (อรัญญาและจีรเดช, 2005)

3.2.1 Anti-inflammatory

The most active component, (E)-1 (3, 4-dimethoxyphenyl) butadiene (DMPBD) in Plai oil was twice more potent than the reference anti-inflammatory drug (diclofenac)

Using the model of carrageenan-induced hind paw oedema in rats, individual assessment of the topical anti-inflammatory activity of the five components of Plai oil demonstrated that DMPBD, terpinene-4-ol and terbinene significantly inhibited oedema formation, whereas sabinene and γ-terbinene were in active up to 6 mg.

The anti-inflammatory action and analgesic action is the result of DMPBD, contained in Plai oil.

DMPBD from Plai dose-depently inhibited the rat ear edema induced by ethyl phyenylpropionate (EPP), arachidonic acid (AA) and 12-o-tetra decanoylphorbol 13-acetate (TPA). It was more potent than any other standard anti-inflammatory drugs in EPP-induced edema, IC50 of DMPBD and oxyphenbutazone were 21 and 136 nmol per ear, respectively in AA-induced edema, whereas DMPBD was 11 times more potent than diclofenac in TPA induced edema (IC50 = 660 and 7200 pmol per ear, respectively). DMPBD and diclofenac inhibited the rat paw edema induced by carrageenan, but not by platelet activating factor (PAF).

The anti-inflammatory effect of compound D, (E)-4(3'-4',-dimethoxyphenyl) but-3-en-2-ol, mediated prominently on the acute phase of inflammatory. It exerted marked inhibition on carrageenan-induced rat paw edema and on the exudates formation, leukocyte accumulation and prostaglandin biosynthesis in carrageenan-induced rat pleurisy. Compound D elicited analgesic activity when tested on acetic acid-induced writhing response in mice.

3.2.2 Immunomodulating activity

In vitro phagocytosis assay by NBT test on mouse macrophage, Plai oil at 0.625 mg-ml gave a stimulation index of 1.3 of the control. This indicated the possible immunimodulating activity of this oil.

3.2.3 Antioxidant

The most active antioxidant fraction from Plai oil was found to contain three potent antioxidants, cassumunar A, B and C which where show by spectral methods to represent a new type of complex curcumin.

For free redicle acavenging (DPPH method) and tryosinase inhibition activity, although Plai oil did not give any free radicle acavanging activity, it showed some tryosinase inhibition (mushroom tryosinase). This suggested that Plai oil can be used together with other tryosinase inhibitiors for whitening cosmetic products.

3.2.4 Antimicrobial activity

For antimicrobial activity, Plai oil demonstrated potent antimicrobial activity using the agar diffusion assay. The mean diameters of inhibition zone (mm) against oral *Candida albicans* from ATCC 10231 and the 17 out 19 clinical oral isolates were 57.0 and 55.5 cm respectively, whereas the mean diameters of the inhibition zone against *Candida albicans* from ATCC 10321 and the clinical isilates of the positive controls (Nystatin and chlorhexidine) were 49.0, 44.5, and 55.0, 40.5 cm respectively. This indicated the possible application of Plai oil in gargle formulations.

3.2.5 Insecticide

Extracts from Plai, when incorporated into articial diets, displayed significant insecticidal activity in chronic feeding bioassays at concentrations of 2500 ppm. Bioassay-guided isolation afforded two phenylbutanoids from rhizomes of Plai which had LD50 values against neonate larvae of 121 and 127 ppm respectively, in the chronic feeding bioassay.

3.2.6 Anticancer

The cytotoxic potential of subfractions of *Zingiber cassumunar* agains a panel of human cancer cell lines [human lung (A549), colon (Co12), stomach (SNU-638)] are presented**.**

A bioassay-guided fractionation of the active chloroform extracts of the rhizomes of *Zingiber cassumunar*, Roxb. Led to the isolation of a potential catatonic principle.

3.3 Pharmacological activities (อรัญญาและจีรเดช, 2005)

An amount of 10% of Plai oil in proper diluents in massage oil in aromatherapy is used as analgesic and local anesthetic, anti-neuralgic, anti-inflammatory, sprains and strains, torn muscle and ligaments.

An amount of 10% of Plai oil in cream, gel or blend with other oil in proper oil base is used in aromatherapy for post operative surgical knee to reduce inflammatory, pain and swelling.

Plai has a cooling action on the inflamed areas. It is used with Tarragon or Rosemary and Cypress, which appeared to reduce the intensity of the attacks of asthma. It has an antihistaminic effect.

With digestive upsets, Plai oil along with oil of Black Pepper, Orange and Terragon have been used to counter irritable bowel syndrome. This blend was used across the abdomen after each bowel movement or anytime when there any cramping or pain in the abdominal area. Within three applications, all problems calmed down.
An amount of 10 % of Plai oil blended with oil from Linden Blossim, Marjoram and Orange when applied across the lower back and front abdominal area every 15 minutes for menstrual craming after three applications, all aramps and blood clot diminished.

The innovation Development Fund (IDF), the Postgraduate Education and Research Program in Chemistry (PERCH) and many leadind herbal companies, have the goal to export Plai products to both local and global markets. IDF and PERCH have succeeded in the development of a new “Plai” (*Zingiber cassumunar* Roxb.) through “collaborative innovation” between herbal companies. The project benefited from PERCH’s Plai research which has developed the substances having the trademark Plaitanoid^{TM} will be exported in the forms of Plaitanoids^{TM} essential oil, Plaitanoid^{TM} liquid extract and Plaitanoids^{TM} powder extract for make-up, shampoo, toothpaste and SPA industry products.

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments and equipments

3.1.1 Ultraviolet-visible Spectrophotometer (UV-VIS)

The UV-VIS spectra were recorded on a Varian Cary 50 Probe spectrophotometer. The samples were diluted by solvent to appreciated concentration.

3.1.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thin film by drop liquid sample about 1-2 drop on a sodium chloride (NaCl) cell then covered another cell to fit.

3.1.3 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, NOESY, HSQC, and HMBC spectra were recorded on a Varian Spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. Deuterated solvents; chloroform-*d* ($CDCl_3$), deuterium oxide (D_2O), and dimethylsulfoxide (DMSO-d_6) were used in NMR experiments. Reference signals were the signals of residual protonated solvents at δ 7. 26 (s) ppm (^{1}H) and 77.1 (t) ppm (^{13}C) for $CDCl_3$, 4.79 (s) ppm (^{1}H) for D_2O, and 2.50 (t) ppm (^{1}H) and 39.5 (sept) ppm (^{13}C) for DMSO-d_6.

3.1.4 Mass spectrometer

The mass spectra were recorded on a LCT of Micromass UK Limited, and used searching for molecular weight of compound on high resolution electrospray/time of flight (HSES/TOF)

3.1.5 Optical rotation

Optical rotations were measured on a Pergin Elmer 341 polarimeter, using a sodium lamp at wavelength 589 nm.

3.2 Chemical reagents

3.2.1 Solvents

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

3.2.2 Other chemicals

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

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

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

4. TLC spots were visualized under ultraviolet light at wavelengths 254 and 365 nm, in iodine vapour, and under daylight after spraying with vanillin/H_2SO_4 reagent (a solution of 0.5 g of vanillin dissolved in a solution of 95 ml ethanol and 4.5 ml concentrated sulfuric acid) and heating until the colors developed.

5. Merck's Sodium hypochlorite 12-14 % chlorine were used as detergent of surface sterilization.

3.3 Culture media

Potatoes Dextrose Agar (PDA) was used for the endophytic fungi isolation and observation morphology and determination antimicrobial activities of isolated endophytic fungi. In addition, Malt Extract Agar (MEA) and Yeast Extract Sucrose Agar (YES) were used for only observation morphology and determination antimicrobial activities of isolated endophytic fungi.

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

In addition, the medium for identification *Aspergillus* sp. was Czapek Yeast Agar (CYA), Czapek yeast agar with 20% sucrose (CY20s)

The all media's formula was shown in appendix A.

3.4 Plant samples collection

Healthy *Zingiber cassumunar* Roxb rhizomes were collected from four province of Thailand including Songkhla, Suphanburee, Surin, and Phatumtanee. The rhizome samples were preserved in a plastic bag at 4 ^{o}C in a refrigerator until processing.

3.5 Isolation and cultivation of endophytic fungi

Endophytic fungi isolation from plant samples were processed using the modified surface sterilization of Wilson and Carroll (1994):

3.5.1 The rhizome samples were washed in running tap water and dried in laminar flow. Each rhizome was cut to 2.5 x 2.0 x 1.5 (width x long x height) mm^3 sections and then surface sterilized by immersing in 95 % ethanol for 1 min. After that the rhizome sections were immersed in sodium hypochlorite 12-14% chlorine for 5, 10, 15 min and then transferred to 95 % ethanol for 30 sec. They were finally washed twice in sterile distilled water then surfaced dried with sterile papers.

3.5.2 Sterilized rhizome sections were cut again on sterilized papers in laminar flow to 2.0 X 1.5 X 1.0 (width x long x height) mm^3. The rhizomes placed on the surface of Potatoes Dextrose Agar (PDA) on Petri dish controls for 20 min and then transfer the section to the other PDA dish. All Petri dishes were incubated at room temperature (25-30°C) and examined every day for fungal mycelium from rhizomes under a stereomicroscope. The isolated fungi were purified after that transferred pure fungi into new Petri dishes containing PDA by hyphal tip transfer. They were incubated at room temperature and purity was determined by colony morphology.

3.6 Identification and classification of endophytic fungal isolate ZCSR5-4 and another isolates

3.6.1 Morphological identification

3.6.1.1 Macroscopic characteristics

Endophytic fungal were cultured in various media including Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA) and Yeast Extract Sucrose Agar (YES). Each isolates were observed Colony Characteristics, for example, shape, size, color, margin, pigment, and others were studied.

3.6.1.2 Microscopic characteristics

Preparation of the specimens for light microscope

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

1. A piece of filter paper placed into a sterile Petri dish then a pair of thin glass rods were placed to serve as supports for a glass slide.

2. Placed a 1x1 mm block or plug of PDA or MEA on the surface of the microscope slide. The margins of the agar plug in four places were inoculated with small mycelia of the studied colony, using the tip of a needle. Gently heated a coverslip by passing it quickly through the flame and immediately or autoclaved coverslip, placed it directly on the surface of the inoculated agar block.

3. Added sterilized water in the Petri dish to moist filter paper. Then placed the lid on the Petri dish and incubated at room temperature (or 30 ^{o}C) for 3 to 5 days.

4. When growth visually appears to be mature. Prepared slide by drop phenol cotton blue on its then put the coverslip from Petri dish to the slide with a pair of forceps.

After the coverslip had been removed from the agar block, lower agar slide were prepared again by removed agar block and stained with phenol cotton blue. The mount can be preserved for further study by rimming the outside margins of the coverslip with clear fingernail polish.

Identification of an endophytic fungus ZcSR5-4

ZcSR5-4 isolate was preliminary observed for the characteristic spore arrangements and it was identified as *Aspergillus* sp. This fungus was further characterized using the *Aspergillus* sp. identification of Maren (2002) as following:

1. Spores of the fungus were streak by needle on PDA agar slant and incubated at room temperature for 7 day. Stock cultures were suspended in a medium consisting of 0.2 % agar and 0.05 % tween 80 after that pipetted spore suspensions into sterilized grass vials. Then 2 µl aliquots are placed on each of three equidistant points on the agar plate using a micropipetor

2. The condition of cultured endophytic fungus ZcSR5-4 was describes as:

2.1 Cultured on Czapek yeast extract Agar (CYA25) and incubated for seven days at 25 ^{o}C

2.2 Cultured on Czapek yeast extract Agar (CYA37) and incubated for seven days at 37 ^{o}C

2.3 Cultured on Czapek yeast extract Agar with 20 % sucrose (CY20s) and incubated for seven days at 25 ^{o}C

2.4 Cultured on Malt extract Agar (MEA) incubated for seven days at 25 ^{o}C

3. Macroscopic characteristics (diameter, color, conidia, mycelium, exudate, pigment, soluble pigment) and microscopic characteristics (stripe, vesicle, seriation, conidia) were observed and noticed the results (see 3.6.1.2)

4. Identification key was used with data of macroscopic and microscopic characteristics.

3.6.2 Molecular identification of selected endophytic fungus

3.6.2.1 DNA extraction

For DNA extraction, the procedure described by Zhou et al. (1999) was employed using Cetyltrimethylammonium bromide (CTAB) method. Briefly, about 10 mg of mycelium were thawed and homogenized in 1,000 µl 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. Moved the sample to 1.5 ml microcentrifuged tube and centrifuged the sample in washing buffer at 15,000 g for 3 min.

The pellet was washed many times by homogenization in the washing buffer and centrifuged at 15,000 g for 3 min. DNA was then extracted from the wash pellet by adding 700 µl of 2X CTAB solution into its and incubated at 65 ^oC for 1 h. Extracted the pellet again using 700 µl phenol chloroform-isoamyl alcohol mixtures (25: 24: 1, v/v), then centrifuged the suspension at 15,000 g for 8 min. The supernatant was extracted using same phenol chloroform-isoamyl alcohol mixtures (25: 24: 1, v/v) and centrifuged the mixtures at 15,000 g for 8 min then moved the supernatant into new micro centrifuged tube.

After that, nucleic acid were precipitated by adding 700 µl isopropanol into the supernatant and put its in ice water for 30 min then centrifuged the microcentrifuged tube at 15,000 g for 8 min. Poured the supernatant into wasted beaker then suspended the pellet by adding 500 µl of 70 % ethanol then centrifuged at 8,000 g for 5 min. Dried the DNAs at room temperature and resolubilized its in 100 µl of sterilized distilled water, then treated its with 1 µl of 10 mg/ml ribonuclease solution after that incubated suspension at room temperature for 30 min.

Precipitated the DNAs again by adding 60 µl of PEG solution (20 % polyethylene glycol (6,000) and 2.5 M NaCl) and put it's in ice water for 30 min then centrifuged the suspension at 15,000 g for 5 min. Poured the supernatant into wasted beaker and dried pellet at room temperature. the DNAs were resuspened in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and stored its at -30^oC until use.

3.6.2.2 PCR amplification and electrophoresis

The ITS region of DNAs were amplified with the primers ITS1 and ITS4. PCR amplification was performed in 10 µl reaction mixtures which shown in table 3.1

Table 3.1 Components of mixtures were used in Polymerase Chain Reaction (PCR) reaction

Compound	**Final concentration**	**Volume** (µl)
10X PCR buffer without $MgCl_2$	1X	1.0
2 mM dNTP mixed	0.2 mM	1.0
5u/µl *Taq* DNA polymerase	0.5 units/10µl	0.1
25 mM $MgCl_2$	2.5 mM	1.0
20 µM Primer I (ITS1F)	1 µM	0.5
20 µM Primer II (ITS4)	1 µM	0.5
DNA template	-	1.0
Sterilized distilled water	-	4.9
Total volume		10

The amplified reactions were performed in a Thermal cycler. The conditions of reaction were described as below.

Initial denaturation	94	^oC	5 min	
Amplification				
Denaturation	94	^oC	1 min	38 Cycles
Annealing	51	^oC	1 min	
Extension	72	^oC	1 min	
Final Extension	72	^oC	5 min	
Hold	4	^oC		

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PCR products were load into 1.5 % agarose gels in TBE buffer setting power supply at about 100 volt for 6o min. After staining with ethidium bromide, band patterns were visualized on a UV transilluminator using 312 nm wavelengths.

3.6.2.3 DNA sequencing

Amplified ITS_{1f-4} fragments were cloned using PCR-Script™ Amp Cloning Kit and extracted plasmid using FastPlasmid™ Mini Kit. The sequences were analyzed at Macrogen, Inc. Seoul, South Korea using ABI 3730 xl automatic sequencer. ITS region of DNA was automatically aligned with fungi. ITS sequences obtain from Genbank DNA database (http://www.ddbj.nig.ac.jp) Using BLAST program version 2.1.

3.7 Selection of antimicrobial properties endophytic fungi isolates.

The isolated endophytic fungi were investigated for their antimicrobial activities against the tested microorganisms as showed in Table 3.2 which was modified from the Agar plate bioassay method described by Hopton and Hill (1987):

Table 3.2 Tested microorganisms used for antimicrobial activity.

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

Viable counts of the standardized inoculum of test bacteria and yeast which adjusted turbidity matched to 0.5 McFarland standard (OD_{625} = 0.08-0.1 nm) were performed. The Colony forming unit/ml (CFU/ml) values of test microorganisms used for antimicrobial assay in this study are shown in Table 3.3.

Table 3.3 Quantity of standardized inoculum of tested microorganisms

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

3.7.1 Preparation for tested bacterial inoculums

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

3.7.2 Preparation for tested yeast inoculums

Yeast was grown on Yeast-Malt Extract agar (YMA) for 24-48 h at room temperature (25-30 ^{o}C). With a sterile wire loop, the tops of four or five isolated colonies of a similar morphologic type were transferred to a tube containing 4 to 5 ml of Yeast-Malt Extract broth (YMB) and incubated at room temperature for 6-8 h. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of 0.5 McFarland.

3.7.3 Preparation of endophytic fungi for antimicrobial activities

Each endophytic fungal isolate was cultivated on three media which were Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA) and Yeast Extract Sucrose agar (YES) at room temperature (25-30 ^{o}C) for 14 days. Then the agar cultures of each fungal endophyte isolate that grew on each medium were cut to the disk with a flamed 7 mm cork borer and were removed from cork borer hole by sterile needle.

3.7.4 Screening for antimicrobial activity of endophytic fungal isolates

The isolated endophytic fungi cultured on PDA, MEA, and YES were investigated for their antimicrobial activity by agar plate bioassay methods (Hopton and Hill, 1987).

A sterile cotton swab on a wooden applicator stick was dipped into the standardized inoculum suspension. The excess fluid was removed by rotating the swab. The dried surface of Nutrient agar plate for test bacterial and Yeast-malt extract agar plate for test yeast were inoculated by streaking the swab in three difference directions by rotating the plate approximately 60 ^{o}C each time, to ensure an even distribution of the inoculum.

The surface of the medium was allowed to dry for 3-5 minutes. Then the agar culture disks of each endopytic fungal isolate (from 3.7.3) were placed on the inoculated plates and pressed firmly into the agar with needle to ensure complete contact with the agar. Bacteria and yeast plates were incubated at 37 ^{o}C and room temperature, respectively for 24 h. Inhibition zone around the fungal agar was measured in mm.

3.8 Determination of growth profile and production of antimicrobial of the fungus ZcSR5-4 in MEB

3.8.1 Measurement of growth of the fungus ZcSR5-4

3.8.1.1 Preparation endophytic fungal isolate ZcSR5-4

Pulled and drew spores of endophytic fungal isolate ZcSR5-4 to slant PDA with needle then incubated at room temperature (25-30 ^{o}C) for 7-14 days.

3.8.1.2 Preparation spore suspension

Stock cultures from 3.8.1.1 were suspended in 1 % of tween 80 in distilled water and filtrated by a sterile filter cloth. Fungal spores were counted by

Haemacytometer and adjusted spore suspension of $2.5x10^6$-$2.5 x10^7$ spore/ml (spore calculating was shown in appendix B.

3.8.1.3 Growing of mycelium

Pipetted 1 ml spores suspension from 3.8.1.2 into 250 ml Erlenmeyer flasks containing 100 ml of MEB medium. The cultures were incubated at room temperature (25-30 °C) under static condition for 45 days. The fungal mycelia were harvested every 3 days. Mycelium and broth were separated by filtering through Whatmann no. 93 filter paper. The mycelia were dried in oven at 60 °C for 48 h and weighted its. The mycelial dry weights were calculated from the difference between dry weights of filter paper before and after harvested mycelium of cultures. Determination of the growing has been done 4 replicates.

3.8.2 Antimicrobial activity of each harvested culture

Each filtrate from 3.8.1.3 were tested against tested microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albican*s ATCC 10231, by agar well diffusion method which was modified from Pereda-Miranda et al. (1993) procedures as below.

3.8.2.1 Preparation of bacterial inoculum

The bacterial inoculums were prepared in the same manner as described in section 3.7.1.

3.8.2.2 Preparation of yeast inoculum

The yeast inoculums were prepared in the same manner as described in section 3.7.2.

3.8.2.3 Examination of antimicrobial activity of cultured broth

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

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

3.9 Extraction secondary metabolite of endophytic fungal isolate ZcSR5-4

3.9.1 Cultivation of endophytic fungal isolate ZcSR5-4 for producing secondary metabolites

Stock cultures of endophytic fungal isolate ZcSR5-4 was suspended in 1% tween 80 in distilled water then filtrated by a sterile filter cloth after that counted and adjusted spores to $2.5x10^6$-$2.5\ x10^7$ spore/ml (see 3.8.1-3.8.1.2). One ml of spore suspension was transferred to 250 ml Erlenmeyer flasks containing 100 ml of total 30 L MEB medium and incubated at room temperature (25-30 ^oC) under static condition for 15 days

3.9.2 Extraction of secondary metabolites from culture broth and mycelia of endophytic fungal isolate ZcSR5-4

ZcSR5-4 in 30 L MEB was filtered by filter paper (Whatman No. 93). The cultured broth (26l) was evaporated to concentred broth (1.5l) using a rotary evaporator under reduced pressure at 35 ^oC. The broth was extracted with hexane, ethyl acetate (EtOAc) and methanol (MeOH), respectively. Each extracted solvent was evaporated using a rotary evaporator under reduced pressure at 35 ^oC to give hexane crude as a brown viscous residue (6.27 g), brown viscous ethyl acetate crude (33.52 g) and black brownish methanol crude (71.03 g), respectively

The mycelia of fungi were divided using blender in laminar flow and then lyophilized to dryness (256.72 g dry weight). The dried mycelia were extracted with hexane, ethyl acetate (EtOAc) and methanol (MeOH), respectively. Each extracted solvent was collected and evaporated using a rotary evaporator under reduced pressure at 35 ^oC to give hexane crude as a brown viscous residue (6.27 g), brown viscous ethyl acetate crude (33.78 g) and black brownish methanol crude (42.95 g),

respectively. The extraction of cultured endophytic fungus ZcSR5-4 is shown in Figure 3.1.

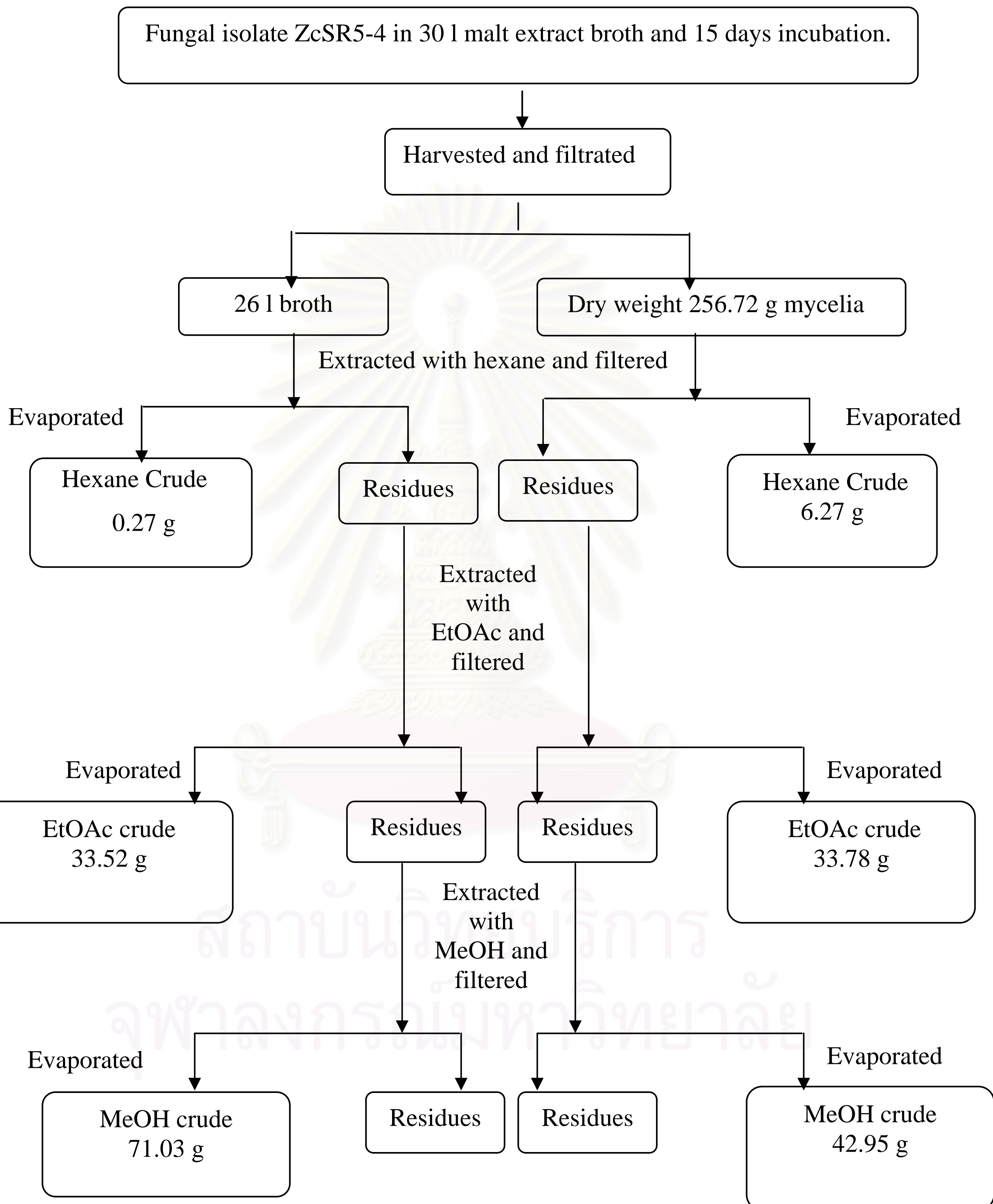

Figure 3.1 Diagram of methods for extraction of the culture broth and mycelia of endophytic fungal isolate ZcSR5-4

3.10 Selection of ZcSR5-4 isolated mycelia and broth crudes in preliminary

3.10.1 Examination of Antimicrobial Properties

Each crudes from 3.9.2 were tested inhibited tested microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231, by paper disc diffusion method which was modified from the method described by Mario et al. (1999) and Kunin and Ellis (2000).

3.10.1.1 Preparation of bacterial inoculum

The bacterial inoculums were prepared in the same manner as described in section 3.7.1.

3.10.1.2 Preparation of yeast inoculum

The yeast inoculums were prepared in the same manner as described in section 3.7.2.

3.10.1.3 Preparation of samples

The crude (20 mg) was dissolved with 1 ml of DMSO 10% in distilled water (20 mg/ml concentration).

3.10.1.4 Preparation of controlled groups

- Positive controlled group

Streptomycin and ketoconazole 10 μg/disc were used as the positive control for bacterial and yeast examination, respectively.

- Negative controlled group

Used DMSO 10 % in distilled water.

3.10.1.5 Antimicrobial activity test

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

The NA and YMA plates that were already inoculated with tested bacteria and yeast respectively were placed 7 mm diameter disc on the agar. 50,100 and 200 µg/disc concentration of the crudes were applied that means pipette 2.5, 5 and 10 µl of 20 mg/ml concentration into the disc, respectively. The samples were absorbed by the disc. Bacteria and yeast plates were incubated at 37 ^{o}C and at room temperature respectively for 24 h. Inhibition zones around the wells were measured in mm.

3.10.2 Chemical component analysis

3.10.2.1 Consideration from TLC

The crude was dissolved in an appropriate solvent and then analyzed by TLC. All TLC profiles were visualized under ultraviolet light at wavelengths 254 and 365 nm, in iodine vapor, and under daylight after spraying with vanillin/H_2SO_4 reagent and heating until the colors developed.

3.10.2.1 Consideration from NMR

The crude (10-20 mg) was dissolved in NMR solvent and analyzed on a Varian Spectrometer operated at 400 MHz for ^{1}H-NMR analysis.

3.11 Isolation of chemical compounds of ZcSR5-4 isolated endophytic fungi

3.11.1 Isolation of ethyl acetate crude from mycelium extract

20 g of mycelia EtOAc extracted crude of the endophytic fungus ZcSR5-4 (total 33.8 g) was fractionated by chromatography on a silica gel column (silica gel 350 g, height column 80 cm. ϕ column 5.0 cm), using Gradient elution system was successful with Hexane, Hexane and dichloromethane mixtures, dichloromethane, dichloromethane and MeOH mixtures, and MeOH. Each fraction (50 ml) was collected and examined. The fractions were combined by silica gel TLC with the appreciated developing solvent. Fractions with the same TLC pattern were combined and dried. The results from the separation of culture broth EtOAc crude extract were shown in Table 3.4

Table 3.4 Characteristics of separation fraction of EtOAc crude extract of mycelium of ZcSR5-4 isolated endophytic fungi

Combined fraction	Fraction number	Eluent	Appearance	Weights (g)
EM1	1-20	$CH_2Cl_3$50% in Haxane	Yellow oil	4.01
EM2	21-29	$CH_2Cl_3$50% in Haxane	Yellow oil	0.18
EM3	30-34	$CH_2Cl_3$60% in Haxane	Yellow oil	0.24
EM4	35	$CH_2Cl_3$60% in Haxane	Yellow brown viscous liquid	0.06
EM5	36-48	$CH_2Cl_3$70% in Haxane	Red orange viscous liquid	0.66
EM6	49-55	$CH_2Cl_3$80% in Haxane	brown viscous liquid	0.17
EM7	56-64	$CH_2Cl_3$90% in Haxane	brown viscous liquid	6.42
EM8	65-73	$CH_2Cl_3$90% in Haxane	brown viscous liquid	0.40
EM9	74-84	$CH_2Cl_3$90% in Haxane	brown viscous liquid	0.19
EM10	85-120	$CH_2Cl_3$100% in Haxane	brown viscous liquid	2.58
EM11	121-165	MeOH 10% in CH_2Cl_3	brown viscous liquid	0.14
EM12	166-195	MeOH 10% in CH_2Cl_3	brown viscous liquid	0.45
EM13	196-270	MeOH 20% in CH_2Cl_3	brown viscous liquid	0.09
EM14	271-320	MeOH 20% in CH_2Cl_3	Black brown viscous liquid	0.23
EM15	321-345	MeOH 20% in CH_2Cl_3	Black green solid	0.21
EM16	346-374	MeOH 30% in CH_2Cl_3	Black solid	0.49
EM17	375-399	MeOH 40% in CH_2Cl_3	Black solid	0.74
EM18	400-429	MeOH 50% in CH_2Cl_3	Black solid	0.38
EM19	430-490	MeOH 75% in CH_2Cl_3	Black solid	0.93
EM20	491-510	MeOH 75% in CH_2Cl_3	Black solid	0.19
EM21	511-530	MeOH 75% in CH_2Cl_3	Black solid	0.17
EM22	531-551	MeOH 75% in CH_2Cl_3	Black solid	0.28
EM23	552-600	MeOH 100%	Black solid	0.12
Total				18.04

3.11.2 Examination of Antimicrobial Properties of combined fractions.

Each combined fractions from 3.11.1 were tested against tested microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231, by paper disc diffusion method which was modified from the method described by Mario et al. (1999) and Kunin and Ellis (2000).

3.11.2.1 Preparation of bacterial inoculum

The bacterial inoculums were prepared in the same manner as described in section 3.7.1.

3.101.2.2 Preparation of yeast inoculum

The yeast inoculums were prepared in the same manner as described in section 3.7.2.

3.11.2.3 Preparation of samples

The crude (10 mg) was dissolved with 1 ml DMSO 10% in distilled water (10 mg/ml concentration).

3.11.2.4 Preparation of controlled groups

- Positive controlled group

Streptomycin and ketoconazole 10 μg/disc were used as the positive control for bacterial and yeast examination, respectively

- Negative controlled group

Used DMSO 10 % in distilled water

3.11.2.5 Antimicrobial activity test

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

The NA and YMA plates that were already inoculated with tested bacteria and yeast respectively were placed 7 mm diameter disc on the agar.

100 μg/disc concentration of the crudes were applied that pipetted 10 μl of 10 mg/ml (10 μg /ml) concentration into the disc. The samples were absorbed by the disc. Bacteria and yeast plates were incubated at 37 ^{o}C and at room temperature respectively for 24 h. Inhibition zones around the wells were measured in mm.

3.12 Purification of compound 1

The yellow brown viscous liquid compound (16.4 mg of compound 1) was isolated from combined fraction EM7 (6.43g) that eluted with Haxane 10% in CH_2Cl_3. Then, the combined fraction EM7 was eluted with MeOH 10% in $CHCl_3$ that was combined fraction EM7-8 (2.90 g). After that the combined fraction was applied using first TLC (Haxane 10% in $CHCl_3$) next, the second band of TLC (1.44 g, Rf = 0.05) was isolated again because of interested spectrum of ^1H NMR

The second band of TLC was divided 100 mg to isolate using second TLC (Haxane 10% in $CHCl_3$) and compound 1 was isolated from the divided part. While the remain crude of second TLC was purified in column chromatpgraphy again using $CHCl_3$ 100% then the compound 1 was purified (644.70 mg). Diagram of isolation steps compound 1 ws showed on Figure 3.2.

3.13 Purification of compound 2

The yellow brown viscous liquid compound (23.40 mg of compound 2) was isolated from combined fraction EM10 (2.58 g) that eluted with Haxane 10% in CH_2Cl_3. Then, the combined fraction EM10 was eluted with EtOAc 100% that was combined fraction EM10-2 (753 mg). After that the combined fraction was applied using column chromatography again (Haxane 40% in EtOAc- Haxane 20% in EtOAc) next, the combined fraction EM 10-2/3 (255 mg) was collected.

Compound 2 was purified from the combined fraction EM 10-2/3 using eluents that were Acetone 12.5% in $CHCl_3$ - Acetone 15.0% in $CHCl_3$ and Diagram of isolation steps compound 2 was showed on Figure 3.3.

3.14 Purification of compound 3

The brown viscous liquid compound (30.3 mg of compound 3) was isolated from combined fraction EM10 (2.58 g) that eluted with Haxane 10% in CH_2Cl_3. Then, the combined fraction EM10 was eluted with EtOAc 100% that was combined fraction EM10-2 (753 mg). After that the combined fraction was applied

using column chromatography again (Haxane 40% in EtOAc- Haxane 20% in EtOAc) next, the combined fraction EM 10-2/3 (255 mg) was collected.

Compound 3 was purified from the combined fraction EM 10-2/3 using eluents that were Acetone 20% in $CHCl_3$ and diagram of isolation steps of compound 3 were shown on Figure 3.3.

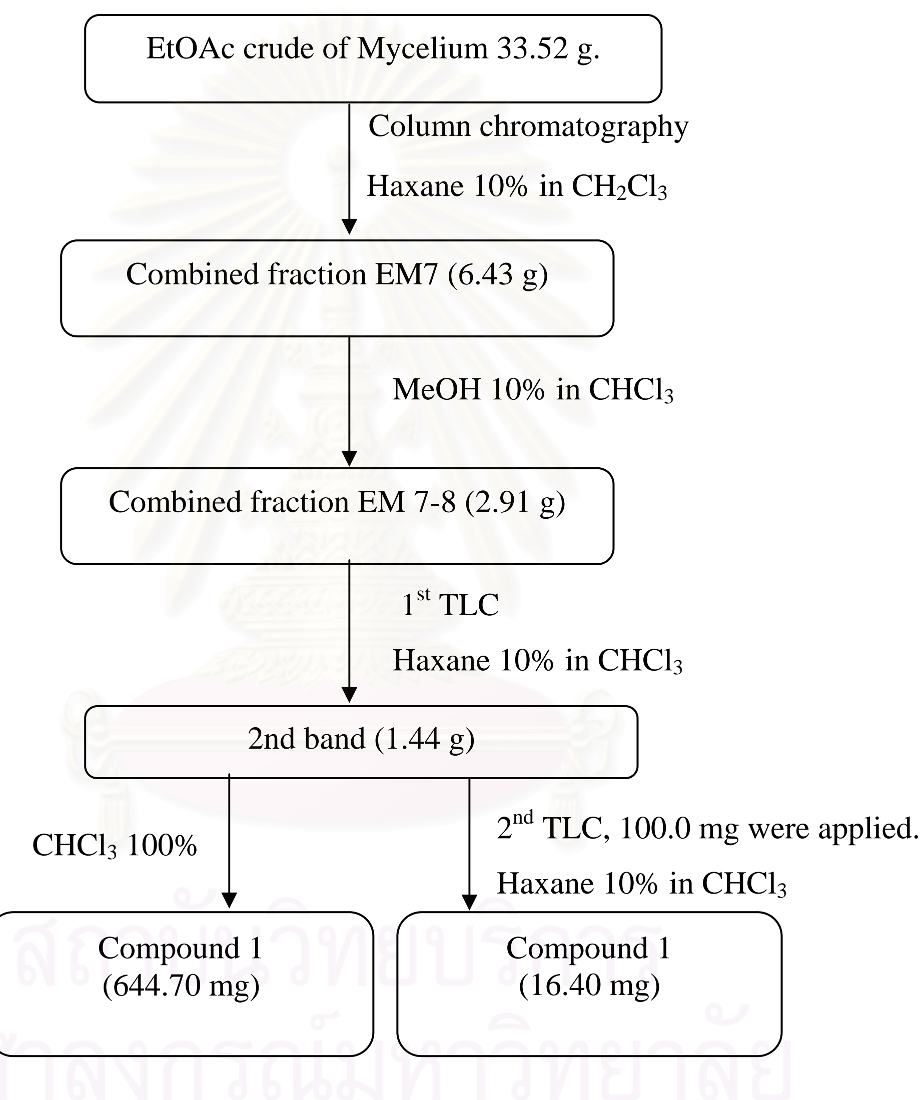

Figure 3.2 Diagram of isolation procedures for compound 1

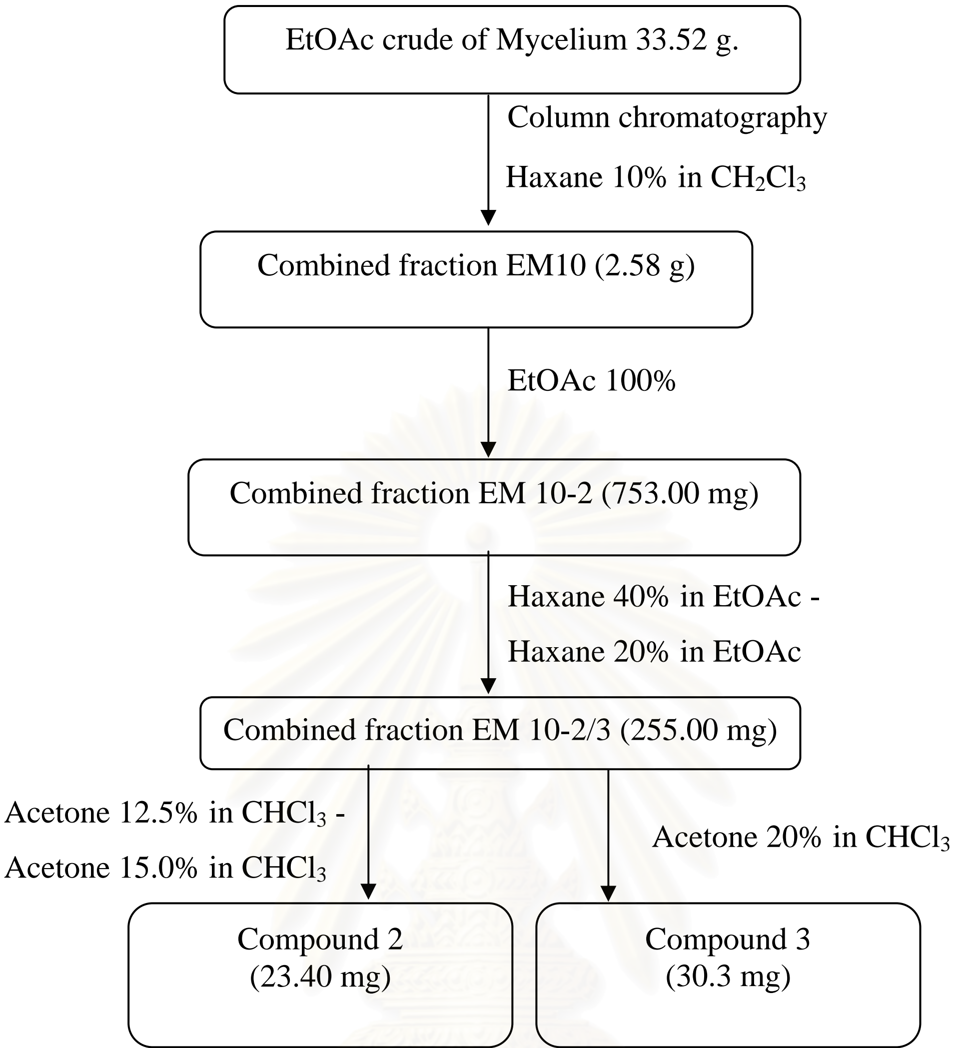

Figure 3.3 Diagram of isolation procedures for compound 2 and 3

3.15 Minimum inhibitory concentrations (MICs)

Pure and mixtures compounds were tested against tested microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231, by micro dilution broth susceptibility testing which was modified from Jennifer (2006) procedures as below.

3.15.1 Preparation of bacterial inoculums

The bacterial inoculums were prepared in the same manner as described in section 3.7.1 but the broths were instead by Muller Hinton Broth (HMB).

3.15.2 Preparation of yeast inoculums

The yeast inoculums were prepared in the same manner as described in section 3.7.2.

3.15.3 Preparation of tested compounds.

One mg of tested compounds was dissolved in 10 % DMSO (0.1ml) in 3.9 ml broth so first concentrations are 250 µg/ml. Concentrations of tested compounds ranges will be prepared one step higher than the final dilution ranges required. The final dilution ranges of 125, 62.5, 31.25, 15.63, 7.82, 3.91 and 1.96 µg/ml was required then ranges of 250, 125, 62.5, 31.25, 15.63, 7.82 and 3.91 were prepared by transferring 1.5 ml of first concentrations (250 µg/ml) to 1.5 ml broths in next tube (125 µg/ml), and next tubes with same volume until final concentrations (3.91 µg/ml) in sixth 1.5 ml broth tube.

3.15.4 Preparation of positive control.

The positive controls were Streptomycin and Chloramphenical for bacteria and Ketoconazole for yeast. The antibiotics were dissolved and diluted following as tested compounds.

3.15.5 Microdilution tests.

Planed position of tested compound and antibiotics dilutions in microtiter trays then labeled the 96 well sterile microtiter trays. The groups of microdilution tests are described as

Blank – 10 % DMSO in broths + microorganism suspensions.

Positives control – Antibiotic dilutions + microorganism suspensions.

Sample tests – Tested compound dilutions + microorganism suspensions.

50 µl of 10 % DMSO in broths (blank), antibiotic dilutions and tested compound dilutions were added in each well of sterile microtiter trays then added 50 µl of tested microorganism suspensions in same well so total volumes are 100 µl per well (three replicates per one dilution). Next, covered microtiter trays with lid and incubated at 37°C for 18-20 h.

3.15.6 Reading and interpretation

1. Reading of antimicrobials activities of tested compound were determined by measurement the turbidity of each well in the microtiter trays at 625 nm. The element is the Sunrise Microplate Reader (TECAN, AUSTRIA).

2. Noticed the absorbance of three replicates and averaged its. Interpretations of the results were described as.

- If the absorbance of tested compound and antibiotic dilutions are higher than blank so the concentration can’t inhibited tested microorganisms.

- If the absorbance of tested compound and antibiotic dilutions are lower than blank so the concentration can’t inhibited tested microorganisms.

The lowest concentration of tested compounds that inhibits growth of tested microorganisms was recorded as Minimum Inhibitory Concentrations or MICs (µg/ml).

3.16 Cytotoxicity assay

Pure compounds were sent to determine cytotoxicity at the Institute of Biotechnology and Genetic engineering, Chulalongkorn University. The bioassay of cytotoxicity activities were tested tumor cell lines inhibitions in vitro including HEP-G2 (hapatoma), CHAGO(lung), SW 620 (colon), KATO-3(gastric) and BT474 (breast).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Endophytic fungi from *Zingiber cassumunar* Roxb. rhizomes

Endophytic fungi isolation of from *Zingiber cassumunar* Roxb. were studied from five various Provinces in Thailand including Songkhla, Supanburee, Surin and Phatumtanee. Methods of endophytic fungi isolation were described as modified procedures from Wilson and Carroll (1994), surface sterilization were used the condition as 5 min 95% ethanol, varies 5, 10 and 15 min sodium hypochlorite 12-14 % chlorine, 30 sec 95 % ethanol and 2 times sterilized water, respectively. It's shown 48 isolates of endophytic fungi and group of endophytic fungi when varies 5, 10 and 15 min sodium hypochlorite 12-14 % chlorine that describe as below (see Table 4.1).

1. The number of endophytic fungi's isolation aren't different as Songkhla's endophytes found 3 isolates in various times of surface sterilization

2. The trend of quantity's endophytic fungi is decreasing when used long time of surface sterilization. It was found as endophyte of Patumthani and Surin are decreasing that 5 to 3 isolates and endophytes from Pathumthani found 6 to 3 isolates.

3. The quantity's endophytic fungi are variable in various times of surface sterilization. It's found as number of endophyte's Supanburee had 3, 5 and 4 isolates when used varies 5, 10 and 15 min sodium hypochlorite 12-14 % chlorine, respectively. It shown the times of surface sterilization aren't response in number of endophytic fungi from this isolation. Addition, differential plentifulness of position sterilized specimens and carefulness of surface sterilization were used that took it variably number of endophytes in spite of using difference surface sterilization

Table 4.1 The number of endophytic fungi from *Zingiber cassumunar* Roxb. rhizomes when sterilized by 12-14% chlorine in Sodiumhypochlorite in various times.

Provinces	Times of surface sterilization (min)	The number of endophytic fungi (isolates)	Code of endophytic fungi
Songkhla	5	3	ZcSK5-1 - ZcSK5-3
	10	3	ZcSK10-1 - ZcSK10-3
	15	3	ZcSK15-1 - ZcSK15-3
Supanburee	5	3	ZcSP5-1 - ZcSP5-3
	10	8	ZcSP10-1 - ZcSP10-8
	15	4	ZcSP15-1 - ZcSP15-4
Pathumthanee	5	5	ZcPT5-1 - ZcPT5-5
	10	3	ZcPT10-1 - ZcPT10-3
	15	3	ZcPT15-1 - ZcPT15-3
Surin	5	6	ZcSR5-1 - ZcSR5-6
	10	3	ZcSR10-1 - ZcSR10-3
	15	4	ZcSR15-1 - ZcSR15-4
Total(isolates)	-	48	-

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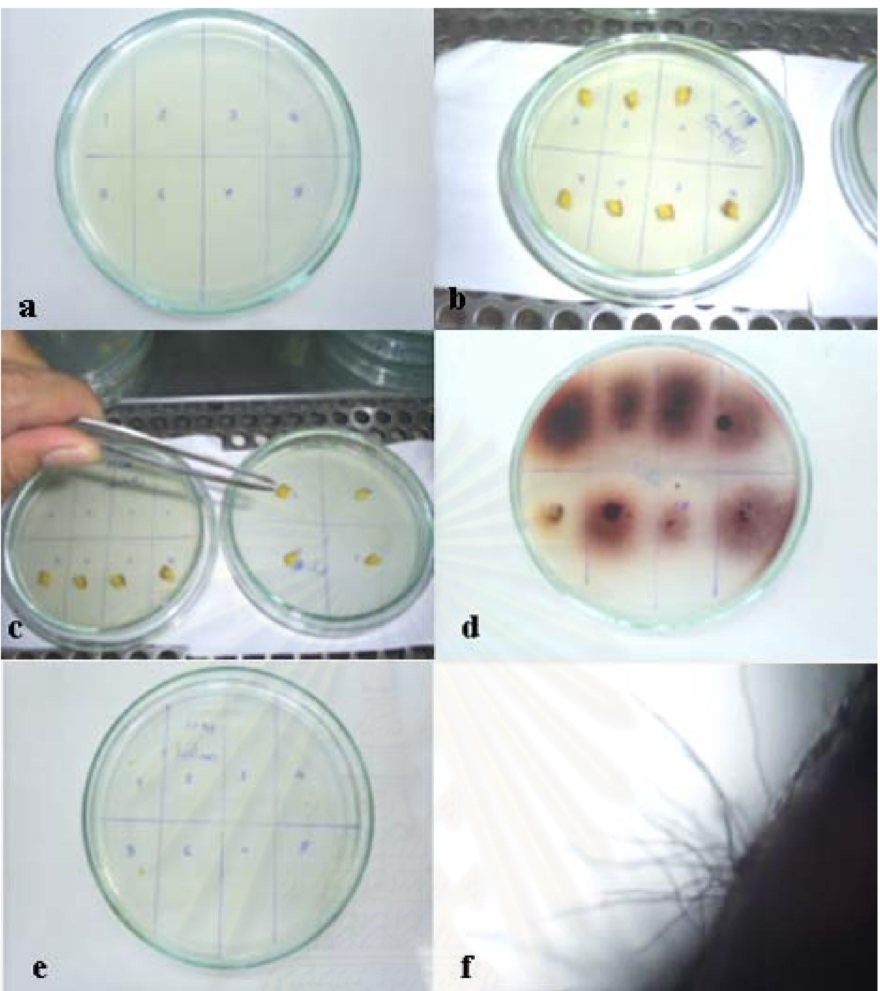


Figure 4.1 Shown isolation of endophytic fungi when observed contamination on controlled petri dish

a) Controlled petri dish

b) Sterilized specimens on controlled petri dish

c) When removed sterilized specimens to other dish

d) Epiphytic fungi on controlled petri dish

e) Epiphytic fungi and bacteria weren't found on controlled Petri dish.

f) Endophytic fungi grow early from sterilized specimen below microscope (40X)

Pure endophytic fungi were placed on various 3 media including MEA (Malt Extract Agar), PDA (Potato Dextrose Agar) and YES (Yeast Extract Sucrose agar). The fungi were incubated at room temperature until they were fully grew on petri dish

then observed characteristics of fungi consist of colony, colors, pigment and spore etc.(Table 4.3) Genus of endophytic fungi were identified preliminary below microscope that recited as following

1. Mycelia sterile

The most of endophytic fungi group from *Zingiber cassumunar* Roxb. rhizomes were mycelia sterilia. These fungi were found 59% (Figure 4.2) and appearances were colorful colony including white, black and brown and downy or non downey mycelia. When observed by slide culture below microscope, it were noticed only mycelium and spore weren't found. Septate or non septate of mycelium were characteristics of these fungi (Figure 4.3a and 4.3b).

2. *Sclerotium* sp.

These fungi were found only 1 isolate or 2% (Figure 4.2). It grown very fast, only 3 days full of white mycelium spread on Petri dish plate. When 7 day of *Sclerotium* sp. cultures observed sclerotia that is brownish rounded structure characteristics. Then slide culture of it was seen that was non-septate mycelium. This fungus normally a plant pathogenic that causes important diseases known as white mold, sclerotium Stem and root rot. Although it is plant pathogen but it sometimes actions as endophytic fungi (Figure 4.3c and 4.3d).

3. *Aspergillus* sp.

Aspergillus is a genus of anamorphic fungi reproducing by production of phialospores (conidia borne on phialindes). These fungi can identify below the light microscope. The endophytes are *Aspergillus* sp. by its distinctive conidiophore (Figure 4.3h and 4.3g) and this fungi were found in *Zingiber cassumunar* Roxb. rhizomes about 4% (Figure 4.2).

4. *Fusarium* sp.

Fusarium is a filamentous fungus widely distributed on plants and in the soil. It is found in normal mycoflora of commodities, such as rice, bean, and other crops. This fungi were found in *Zingiber cassumunar* Roxb. rhizomes about 35% (Figure 4.2).

Fusarium sp. from sterilized specimes found that some colonies were fast growing, pale or brightly colored and had a cottony aerial mycelium. The color of the mycelium varies from whitish to yellow, brownish, reddish shades. The Fusarium produces macroconidia from slender phialides. Macroconidia are fusiform- to sickle-shaped(Figure 4.3f and 4.3g).

Table 4.2 Characteristics of endophytic fungi were isolated from Plai

Endophytic fungi number	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color (aerial mycelium)	Color pigment production on the media	
ZcSK5-1	Round powdery	White	not produce	Mycelia Sterilia
ZcSK5-2	Smooth	Gray and black	not produce	*Fusarium sp.*
ZcSK5-3	Round powdery	Violet	not produce	*Fusarium sp.*
ZcSK10-1	Round powdery	White	not produce	Mycelia sterilia
ZcSK10-2	Cottony	Gray and black	black	Mycelia sterilia
ZcSK10-3	Powdery	White	not produce	Mycelia sterilia
ZcSK15-1	Like as flower	black	red	*Fusarium sp.*
ZcSK15-2	Powdery like as flower	White and yellow	yellow	*Fusarium sp.*
ZcSK15-3	Powdery like as cotton	Brown	not produce	*Fusarium sp.*
ZcSP5-1	Very powdery	White	not produce	Mycelia sterilia
ZcSP5-2	Smooth	White	not produce	Mycelia sterilia
ZcSP5-3	Powdery like as cotton	Gray	not produce	Mycelia sterilia
ZcSP10-1	Brown spore	Brown	yellow	*Aspergillus sp.*
ZcSP10-2	Round powdery	Violet	not produce	*Fusarium sp.*
ZcSP10-3	Powdery like as cotton	White and yellow	yellow	*Fusarium sp.*

Table 4.2 Characteristics of endophytic fungi were isolated from Plai (continued)

Endophytic fungi number	Endophytic fungi characteristics on the PDA medium			
	Colony characteristic	Colony color (aerial mycelium)	Color pigment production on the media	Fungal species
ZcSP10-4	Powdery like as cotton	brown	not produce	*Fusarium sp.*
ZcSP10-5	smooth	white	not produce	Mycelia sterilia
ZcSP10-6	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcSP10-7	Like as cotton	violet	not produce	*Fusarium sp.*
ZcSP10-8	Very powdery	orange	not produce	*Fusarium sp.*
ZcSP15-1	Powdery like as cotton	violet	not produce	*Fusarium sp.*
ZcSP15-2	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcSP15-3	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcSP15-4	Very powdery	black	not produce	Mycelia sterilia
ZcPT5-1	Powdery like as cotton	orange	not produce	*Fusarium sp.*
ZcPT5-2	Powdery like as cotton	White and yellow	not produce	Mycelia sterilia
ZcPT5-3	Powdery like as cotton	orange	orange	*Fusarium sp*
ZcPT5-4	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcPT5-5	smooth	black	not produce	Mycelia sterilia
ZcPT10-1	smooth	black	black	Mycelia sterilia
ZcPT10-2	Powdery like as cotton	white	not produce	Mycelia sterilia

Table 4.2 Characteristics of endophytic fungi were isolated from Plai (continued)

Endophytic fungi number	Endophytic fungi characteristics on the PDA medium			
	Colony characteristic	Colony color (aerial mycelium)	Color pigment production on the media	Fungal species
ZcPT10-3	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcPT15-1	Powdery like as cotton	black	black	Mycelia sterilia
ZcPT15-2	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcPT15-3	Powdery like as flower	gray	not produce	Mycelia sterilia
ZcSR5-1	smooth	brown	yellow	Mycelia sterilia.
ZcSR5-2	Powdery like as cotton	gray	not produce	Mycelia sterilia
ZcSR5-3	Powdery like as cotton	white	not produce	Mycelia sterilia
ZcSR5-4	Brown spore	brown	yellow	*Aspergillus terreus*
ZcSR5-5	Very powdery	white	not produce	*Sclerotium* sp.
ZcSR5- 6	Powdery like as cotton	White and yellow	not produce	*Fusarium sp.*
ZcSR10-1	Powdery like as cotton	white	not produce	Mycelia Sterilia
ZcSR10-2	Powdery like as cotton	violet	red	*Fusarium sp.*
ZcSR10-3	smooth	white	not produce	Mycelia Sterilia
ZcSR15-1	Powdery like as cotton	violet	not produce	*Fusarium sp.*
ZcSR15-2	Powdery like as cotton	black	not produce	Mycelia Sterilia
ZcSR15-3	powdery	white	not produce	Mycelia Sterilia
ZcSR15-4	Powdery like as flower	gray	not produce	*Fusarium sp.*

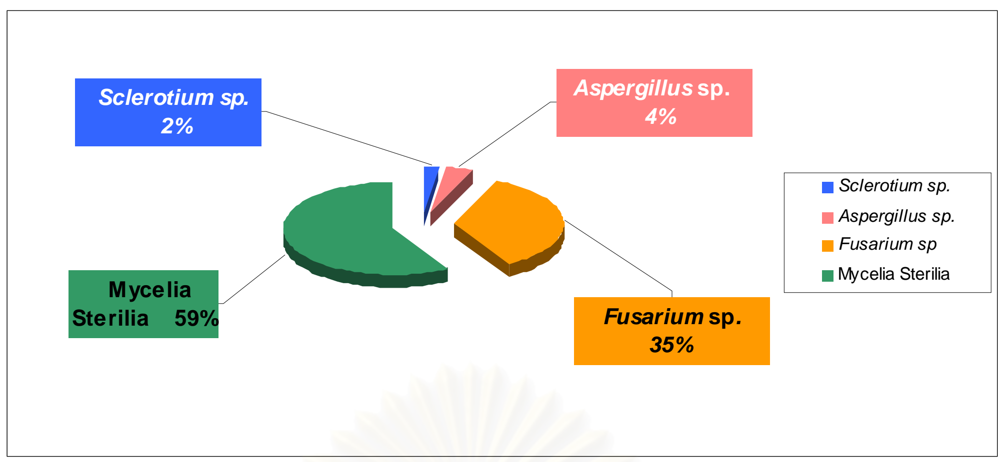


Figure 4.2 Percentages of genus of endophytic fungi were isolated from Plai

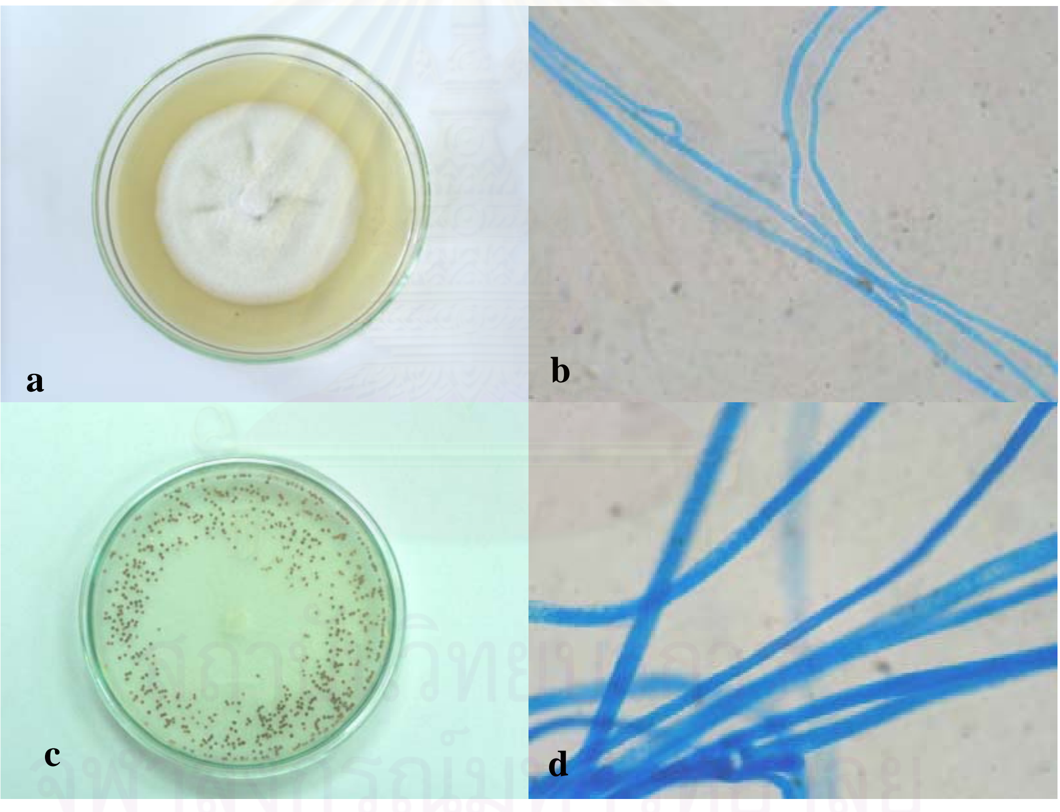


Figure 4.3 Colony characteristics and microcharacteristics of some endophytic fungi

a) Colony of mycelia sterilia showed white aerial mycelium

b) Mycelia of mycelia sterilia showed non septate mycelium (x 100)

c) Colony of *Sclerotium* sp. with schlerotia formation

d) Mycelia of *Sclerotium* sp. showed non septate mycelium (x 100)

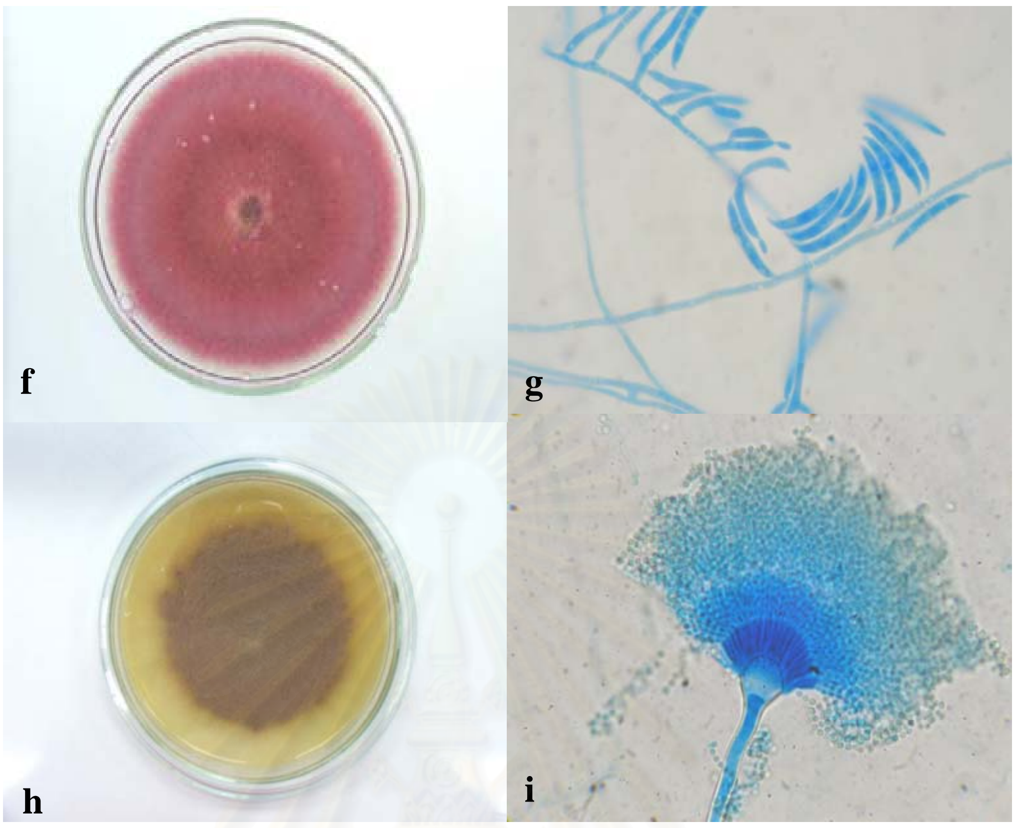


Figure 4.3 Colony characteristics and microcharacteristics of some endophytic fungi (continued)

f) Colony of *Fusarium* sp. showed red aerial mycelium.

g) Macrospore of *Fusarium* sp. showed sickle-shaped (x 100)

h) Colony of *Aspergillus* sp. with brown conidia formation

i) Conidia formation of *Aspergillus* sp. (x 100)

4.2 Selection endophytic fungi with antimicrobials test.

When cultured endophytes on various 3 media including MEA, PDA and YES for 2 weeks, The endophytes were tested antimicrobial activity followed to Howell and Stipanovic (1980) methods and compared antimicrobials activity of all endophytic fungi, it found that antimicrobials actions for 10 isolates including isolate ZcSP10-3, ZcSK15-3, ZcSP10-4, ZcSP10-6, ZcPT15-1, ZcSR5-1, ZcSR5-2, ZcSR5-4, ZcSR5-5 and ZcSR15-2. Especially, endophytes ZcSR5-4 on MEA inhibited *B. subtilis* with 1.5 cm. of clear zones and inhibited *E. coli* with 2.5 cm of clear zones 5 (Table 4.3 and Figure 4.4)

Table 4.3 Antimicrobial activities of endophytic fungi were tested with tested microorganisms

Endophytic fungi number	Culture medium	Inhibition zone diameters (cm) /Tested microorganisms				
		Positive gram bacteria		Negative gram bacteria		yeast
		B.subtilis ATCC 6633	*S.aureus* ATCC 25923	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853	*C.albicans* ATCC 10231
ZcSK15-3	PDA	1.5±0	-	1.7±1	-	-
ZcSP10-3	PDA MEA YES	1.5±1 1.4±0 0.9±0	- - -	1.0±1 - -	- - -	- - -
ZcSP10-4	PDA YES	1.5±0 1.5±1	- -	- -	- -	- -
ZcSP10-6	PDA	1.5±0	-	-	-	-
ZcPT15-1	PDA	1.5±0	-	1.0±0	-	-
ZcSR5-1	PDA MEA YES	1.7±1 1.4±0 0.9±0	- - -	- - -	- - -	- - -
ZcSR5-2	PDA YES	2.0±1 0.9±0	- -	1.5±0 -	1.2±1 -	- -
ZcSR5-4	PDA MEA YES	1.2±0 1.5±0 1.2±1	- - -	1.2±0 2.5±1 1.6±0	- - -	- - -
ZcSR5-5	MEA	-	-	-	1.0±0	-
ZcSR15-2	PDA	0.9±1	-	-	-	-

- = No inhibition

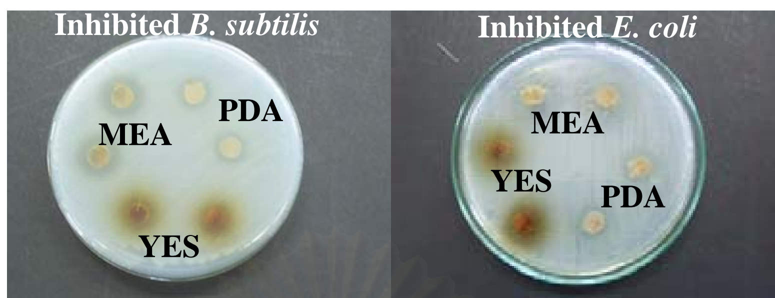


Figure 4.4 Fungal discs for determination of antimicrobial activities of endophytic fungal isolate ZcSR5-4 culture on PDA, MEA and YES. Culture disc on MEA inhibited *B. sutilis* (a) and *E. coli* (b) with high clear zones.

Because of high antimicrobial activity of endophytes ZcSR5-4, then its were selected to cultured 45 old day in erlenmeyer flasks containing 100 ml MEB about 6 l (kept sample every 3 day). When kept endophytes ZcSR5-4 in MEB flasks (4 replicates), measured growth curve, pH and tested antimicrobial activity exchanges. It analyzed characteristics of ZcSR5-4 that describe following (Figure 4.5)

1. Growth curve (see blue line in Figure 4.5a)

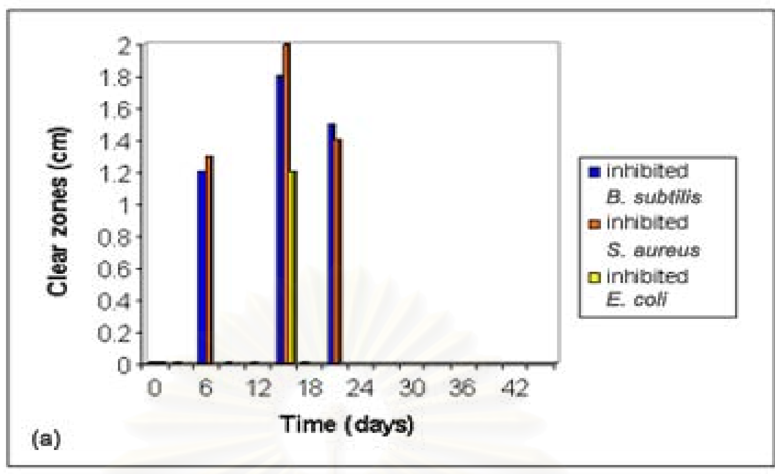
The growth curve was measured using dry weight per 1 ml of MEB medium (g/ml). It found that 3-9 old days of fungi were exponential phases after that 9-27 old days of fungi were stationary phase next 30-45 old days of fungi were death phase. Generally, Antimicrobial agents or secondary metabolites may be produced in stationary phase.

2. pH of broth (see pink line in Figure 4.5a)

The pH of broth started at 5.03 afterthat it decreased to minimum pH at 3.54. Since ninth day, it increased continually near first pH. However, the graph changed narrow ranges.

3. Antimicrobial activity (see bar graph in Figure 4.5b)

When tested activity of broth every 2 week (at 6th, 15th, 21st, 27th, 36th, 42th day) found maximum antimicrobial activity at 15 old days of fungus ZcSR5-4 that was stationary phase of these fungus. Broth of ZcSR5-4 inhibited *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 *and E.coli* ATCC 25922 with 1.8, 2.0 and 1.2 cm diameters, respectively at 15 old days of fungus ZcSR5-4 (Figure 4.6)



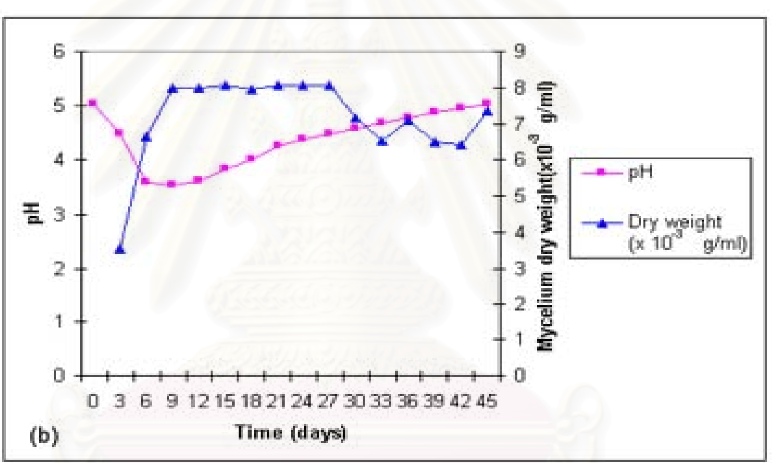


Figure 4.5 Growth profiles and antimicrobial activities of endophytic fungal isolate ZcSR5-4. Growth profile and pH (a) and antimicrobial activities of culture filtrate of endophytic fungal isolate ZcSR5-4 (b).

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Figure 4.6 Antimicrobial activity of endophyte ZcSR5-4 broth showed inhibition against *B. subtilis* (a) *E. coli* (b) *S. aureus* (c), respectively

4.3 Identifications of selected endophytic fungi

Primarily, details of *Aspergillus sp.*, it is a genus of anamorphic fungi reproducing by production of phialospores (conidia borne on phialindes). It is characterized by its distinctive conidiophores (Figure 4.7). The base of the conidiophores usually forms a ‘T’ or ‘L’ shape where it connects with the vegeTable hyphae. This is commonly called the ‘foot cell’ even though it is not a separate cell. The stipe extends from the foot cell and may be quite short (50 µm or less) to several millimeters in length. The apex of stripe expends into vesicle. Vesicles may have various characteristics shape (Figure 4.7)

In some species, the conidia–bearing phialides arise directly from the vesicle. These cells are called uniseriate. In others species, there is a second layer of cells between the vesicle and the phialides. These cells are called metulae and aspergilli with metulae are referred to as biseriate species. An important character that distinguishes *Aspergillus* from several closely related genera is that the phialies/metulae arises simultaneously on the vesicle.

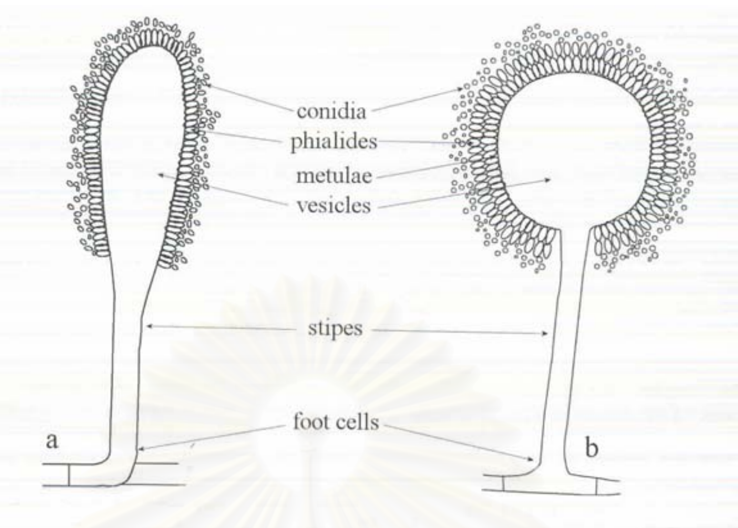

Figure 4.7 Conidiophores of a) *Aspergillus clavatus* (uniseriate) and b. *Aspergillus flavas (*biseriate) (Klinch, 2000)

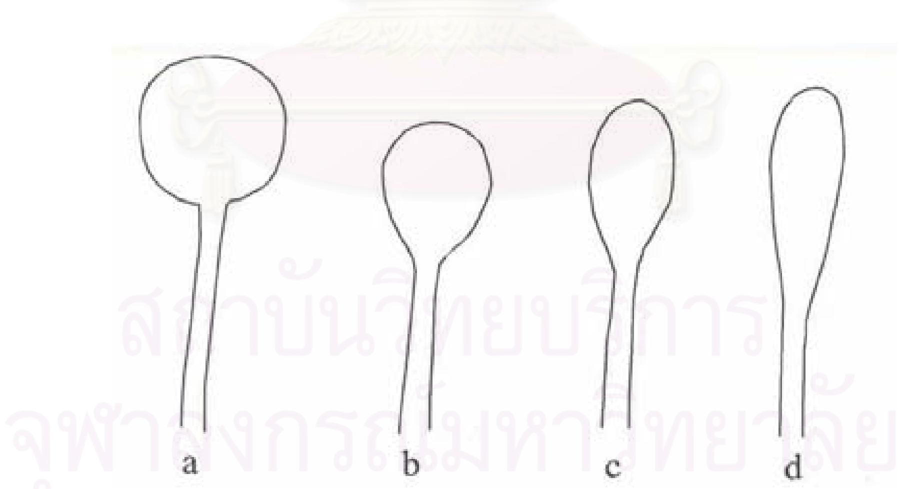

Figure 4.8 Some common vesicle shapes (Klinch, 2000) :
a) globose or spherical, b) pyriform, c) spathulate and d) clavate

Endophytes isolated ZcSR5-4 was prepared to study below light microscope (100X). The endophyte is *Aspergillus sp*. by its distinctive conidiophore (Figure 4.7) then identified its carefully using “Identification of common *Aspergillus* sp.” book of Klinch (2002). The endophytes necessary characterized to species for the next part (isolation of metabolites from the fungi).

4.3.1 Observation of Macroscopic characteristics of selected endophytes

Macroscopic characters are important in subgeneric classification in the genus *Aspergillus*. Conidial colors as observed on Petri plates serve as a major subgeneric classification characters. Colors vary from black to white to yellow to green to blue. A color guide is useful for determining this character. Colonies may also produce droplets of liquid (exudates) on the surface of the colony. Colored pigment may be produced in the agar on the reverse of the colony (reverse color).

Some of these may be soluble enough to extend beyond the edge of the colony (soluble pigment). Colony diameter is sometimes a useful character, as species in the genus vary in ability to grow at different water potentials and at different temperatures. Colony diameter is measured by holding the plate up to the light and measuring from one edge of mycelial growth to the opposite edge.

Cultured *Aspergillus sp.* on various medias then kept its in the dark for 7 days, in each condition following codes as

- CyA25 means cultured its on Czapek Yeast Agar and incubated at 25 ^{o}C.
- MEA means cultured its on Malt Extract agar and incubated at 25 ^{o}C.
- CyA37 means cultured its on Czapek Yeast Agar and incubated at 37 ^{o}C
- Cy20s means cultured its on Czapek Yeast Agar with 20% Sucrose at 25 ^{o}C.

Morphology's ZcSR5-4 were found that had 5.5 - 7.0 cm diameters, white mycelium, yellow colony on top and reverse on MEA media but brown colony on top and reverse on others medias. In addition, Colonies of ZcSR5-4 produced exudates on the surface of the colony when cultured on CyA25 and CyA37 with golden colors and soluble pigment of endophyte were observed only colors on CyA25 and MEA with yellow color. Then, the details of macroscopic characteristics were noticed and shown on Tables 4.4 and Figure 4.6. Finally, the macroscopic characteristics were used partly in *Aspergillus* sp. identification of Klinch (2002).

Table 4.4 Endophytes ZcSR5-4 on four various media

Fungal characteristics	Media			
	CyA25	MEA	CyA37	Cy20s
Colony diameter (cm.)	6.5	5.5	7.0	7.0
color	brown	yellow	brown	brown
conidia	brown	yellow	brown	brown
mycelium	white	white	white	white
exudates	golden	-	golden	-
reverse	brown	yellow	brown	brown
Soluble pigment	yellow	yellow	-	-

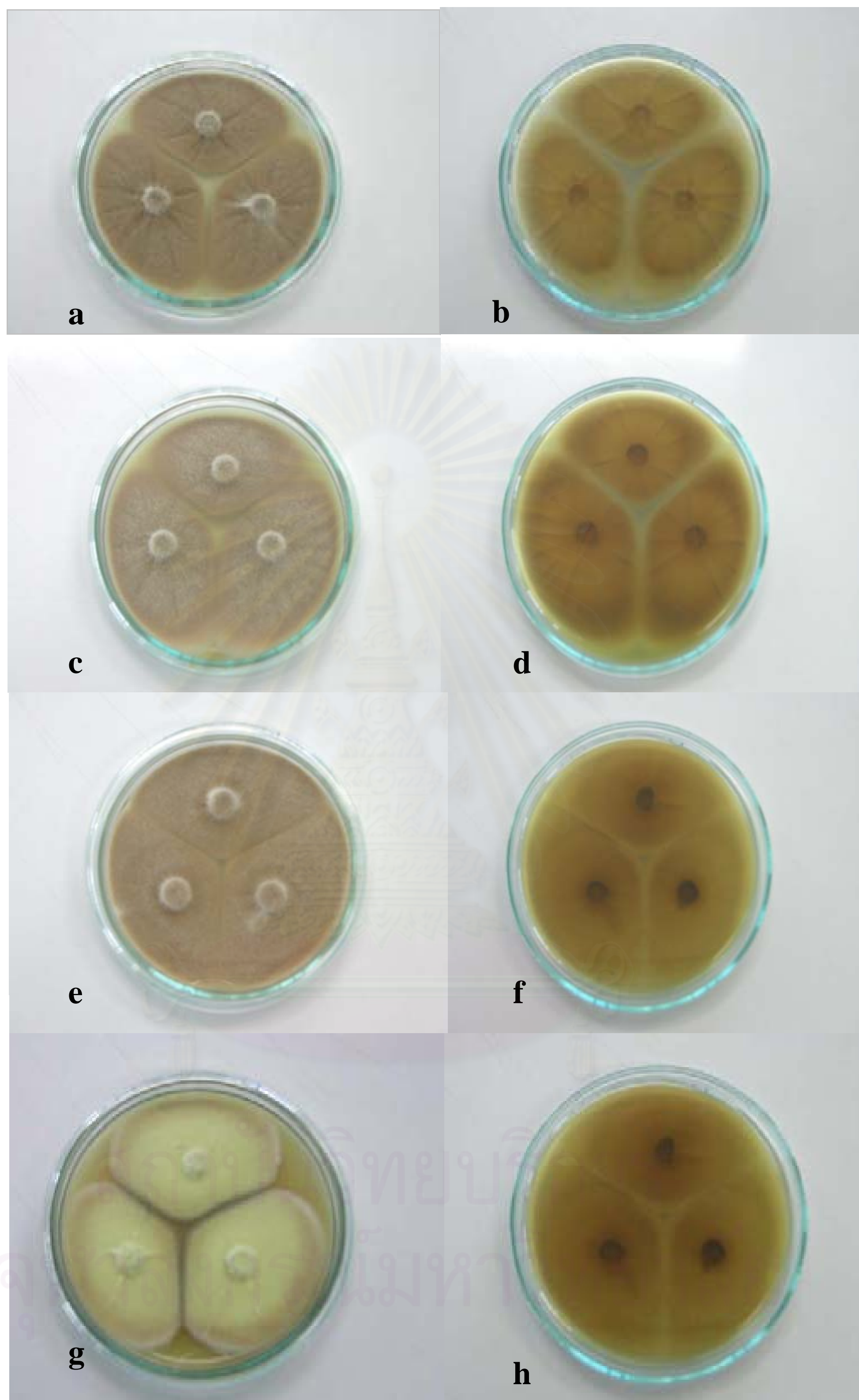

Figure 4.9 Colony characteristics of seven days old cultures of endophytes ZcSR5-4 grow on CyA37, CyA25, Cy20s and MEA (a, c, e and g on top view; b, d, f and h on bottom view)

4.3.2 Observation Microscopic charecteristics of selected fungus.

Endophyte ZcSR5-4’s morphology were observed below light microscope and Scanning Electron Microscope (SEM). The results were recorded including stripe, vesicle, conidia, conidiophores and conidial head. Firstly, the characters of ZcSR5-4 were 75-95 µm (100x) tall and 3.0 µm(100x) wide of stripe, biseriate with groups of conidiogenous cells arising from metulae, 12 µm in diameter and pyriform shape of vesicles, 3.0 µm wide , globose, smooth walled with conidia, hyphae septate, smooth, highly branched, Hulle cells, sclerotia, and cleistothecia absent.

Cultured the fungi on PDA for 5 day then prepared fungi to study morphology of ZcSR5-4 below Scanning Electron Microscope (SEM). The results showed that can see smooth walled and 7.0 µm wide of stripes, clearly. In addition, Klinch reported specific conidia of each *Aspergillus sp*. can used to conidia were used to identify species of Aspergillus. Conidia characteristic of endophytes ZcSR5-4 were identical of Aspergillus *terreus* (Figure 4.11a, b). The Conidiophores were compact compact column (Figure 4.11c, d).

When compared morphology of endophytes ZcSR5-4 and *Aspergillus terreus* Thom (Klich, 2002) found similarly that shown on Table 4.5

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Table 4.5 Comparison of microscopic characteristics of endophytes ZcSR5-4 and *Aspergillus terreus* Thom (Klinch, 2002).

Morphology characteristics	Endophytes ZcSR5-4	*Aspergillus terreus* Thom (Klinch, 2002)
Stipe - length - width - surface texure - color	75-95 µm (100x) 7.0 µm (SEM) Smooth walled uncolour	25-100µm (100x) 4-7 µm (100x) Smooth walled uncolour
Vesicle - wide - shape	12 µm (100x) Pyriform	12 -22 µm (100x) Spherical or pyriform
Conidia - diameter - shape - surface texture	3.0 µm (100x) globose Smooth walled	2-2.5µm (100x) globose Smooth walled
Groups of conidiogenous cells arising from metulae	biseriate	biseriate
Conidial head	Compact column	Compact column

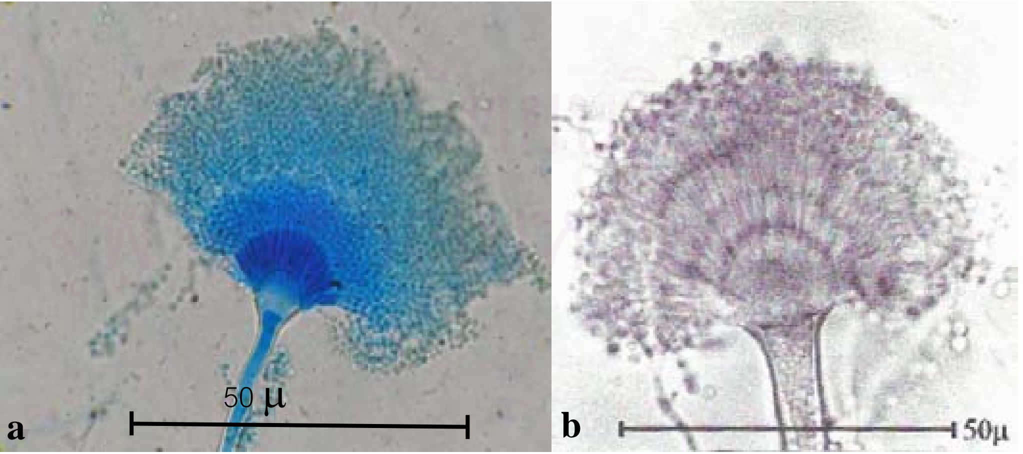

Figure 4.10 Light micrograph of structure conidia formation of (a) endophyte ZcSR5-4 (100x) and (b) *Aspergillus terreus* (Klinch, 2002)

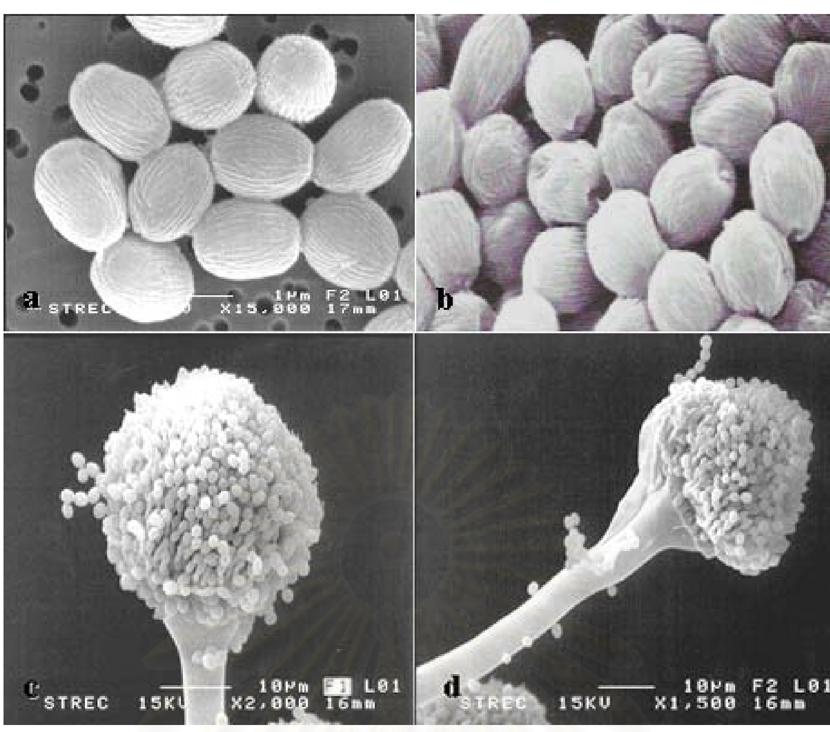

Figure 4.11 Scanning electron micrograph of ornamentation surface

(a) Conidia of endophytes ZcSR5-4 (x15,000)

(b) Conidia of *Aspergillus terreus* (Klinch, 2002)

(c,d) Structure of conidia forms of endophytes ZcSR5-4 (x1,500)

c d

4.3.3 Taxonomy of endophyt ZcSR5-4

The result of morphology of endophytes *Aspergillu sp*. with macroscopic characteristics and microscopic characteristics were noticed, next identified the fungi using key of Klinch (2002) and were described as following.

1.Predominantly biseriate 2

1.Predominantly uniseriate 43

2. Colony diameter greater than 45 mm on CyA25 and/or MEA 3

2. Colony diameter less than 45 mm on CyA25 and/or MEA 22

3. Conidia in shades of green to blue green on CyA25 4

3. Conidia not green on CyA25 11

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11. Conidial heads predominantly uniseriate, conidia not ornamented with dark-coloredtubercles 12
11. Conidial heads variable, uniseriate and biseriate both usually present,conidia ornamemted with dark-colored tubercles 16

16. Conidia 4-6 µm long .... *A. ostianus*
16. Conidia predominantly 3.5 µm or less in length 17

17. Conidial heads compecly columnar, conidia in carmel to cinnamon colors, colony diameter on CyA37 predominantly 55-70 mm .... ***A. terreus***
17. Conidial heads radiate when young, conidia in yellow to ochraceus colors, colony diameter on CyA37 usually less than 55-70 mm 18

Taxonomy of ZcSR5-4 identified as *Aspergillus terreus.* However, study of molecular techniques for identify fungi are necessary, then the research using ITS region of DNAs that were amplified with the primers ITS1 and ITS4, clone gene and analyzed the sequences by Blast Program to confirm species of Aspergillus.

4.3.4 Molecular identification of selected endophytic fungi

Endophytic fungi isolated ZcSR5-4 was sent at at Macrogen, Inc. Seoul, South Korea using ABI 3730 xl automatic sequencer.

The rDNA ITS region of isolate ZcSR5-4 was amplified with the conserved fungal primer ITS_{1F} and ITS_4. The length of corresponding fragment was 604 bp that shown in Figure 4.12

1

5’ TTAAGTTCAG TACCTGATCC CGGGTATCCC GAGGTCAACC
TGGAAAAAAA CAAGTTGCAA ATAAATGCGT CGGCCGGGCC
TACGGAGCGG AACGGCGGGC GCGACGAAGC CCCATACGCT
CGAGGACCGG ACGCGGTGCC GCCGCTGCCT TTCGGGCCCG
TCCCCCGGGA GCCGGGGGAC GAGGGCCCAA CACACAAGCC
GGGCTTGAGG GCAGCAATGA CGCTCGGACA GGCATGCCCC
CCGGAATACC AGGGGGCGCA GTGTGCGTTC AAAGACTCGA
TGATTCACTG AATTCTGCAA TTCACATTAG TTATCGCATT
TCGCTGCGTT CTTCATCGAT GCCGGAACCA AGAGATCCAT
TGTTGAAAGT TTTAACTGAT TGCAAAGAAT CACACTCAGA
CTGCAAGCTT TCAGAACAGG GTTCATGTTG GGGTCTCCGG
CGGGCACGGG CCCGGGGGCG AGTCGCCCCC CGGCGGCCAG
CAACGCTGGC GGGCCCGCCG AAGCAACAAG GTACAATAGT
CACGGGTGGG AGGTTGGGCC ATAAAGACCC GCACTCGGTA
ATGATCCTTC CGCAGGTTCA CCTACGGAAA CCTTGTTACG
ACTT 3’

604

Figure 4.12 Nucleotide sequences of endophytic fungal isolate ZcSR5-4.

A blast search was performed to find a similar sequence to ITS region of fungal isolate ZcSR5-4 in the Genbank DNA database, available from: http://www.ddbj.nig.ac.jp. The result revealed that ITS region of endophytic fungal isolated ZcSR5-4 had 99.00 % identity to *Aspergillus terreus* isolate wb464 Accession number AF455426 as showed in Figure 4.13.

>gi|21666855|gb|AF455426.1| *Aspergillus terreus* isolate wb464 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
Length=624

Score = 1148 bits (579), Expect = 0.0
Identities = 603/604 (99%), Gaps = 0/604 (0%)
Strand=Plus/Minus

ZcSR5-4 1
TTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGGAAAAAAACAAGTTGCAA 60
::
wb464 546
TTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGGAAAAAAACAAGTTGCAA 547

ZcSR5-4 61
ATAAATGCGTCGGCGGGCGCCGGCCGGGCCTACGGAGCGGAAGACGAAGCCCCATACGCT 120
::
wb464 546
ATAAATGCGTCGGCGGGCGCCGGCCGGGCCTACGGAGCGGAAGACGAAGCCCCATACGCT 487

ZcSR5-4-12
CGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGCCGGGGGAC 180
::
wb464 486
CGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGCCGGGGGAC 427

Figure 4.13 Aliment data of apart of the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2

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ZcSR5-4 181

GAGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCC 240

::

wb464 426

GAGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCC 367

ZcSR5-4 241

CCGGAATACCAGGGGGCGCAGTGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAA 300

::

wb464 366

CCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAA 307

ZcSR5-4 301

TTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCAT 360

::

wb464 306

TTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCAT 247

ZcSR5-4 361

TGTTGAAAGTTTTAACTGATTGCAAAGAATCACACTCAGACTGCAAGCTTTCAGAACAGG 420

::

wb464 246

TGTTGAAAGTTTTAACTGATTGCAAAGAATCACACTCAGACTGCAAGCTTTCAGAACAGG 187

ZcSR5-4 421

GTTCATGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCGAGTCGCCCCCCGGCGGCCA 480

::

wb464 186

GTTCATGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCGAGTCGCCCCCCGGCGGCCAG 127

Figure 4.13 Aliment data of apart of the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (continued)

ZcSR5-4 481

CAACGCTGGCGGGCCCGCCGAAGCAACAAGGTACAATAGTCACGGGTGGAGGTTGGGCC540

::

wb464 126

CAACGCTGGCGGGCCCGCCGAAGCAACAAGGTACAATAGTCACGGGTGGAGGTTGGGCC 67

ZcSR5-4 541

ATAAAGACCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACG 600

::

wb464 66

ATAAAGACCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACG 7

ZcSR5 601

ACTT 604

:::::

wb464 6

ACTT 3

Figure 4.13 Aliment data of apart of the internal transcribed spacer1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (continued)

Base on morphology identification using macroscopic characteristics and microscopic characteristics and nucleotide sequencing analysis of ITS regions of rDNA, endophytic fungal isolate ZcSR5-4 was identified as *Aspergillus terreus*, clearly

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Aspergillus terreus is distributed worldwide in soil but is mare abundant in tropical and subtropical regions than temperate regions, and more common in cultivated soils and forests than grassland. This is one of the few Aspergillus species that occurs in greater than expected numbers of reports from cultivated soils. It is common stored crops and has been isolated from other foodstruffs, and from indoor environments (Klinch, 2002).

Isolations of biological and new compound from *Aspergillus terreus* have been well studied. The Aspergillus were often isolated from soils after that the researchers found this Aspergillus from new sources for example unhulled rices, algae etc. The studies of compound from *Aspergillus terreus* were found for a long time.

Nobutoshi et al. (1973) reported new butanolides and seven yellow acidic substances that were isolated by chromatographic separation of the Et_2O extract of the acidified culture filtrate after culturing *Aspergillus terreus* IAM2054 in Czapek dox medium for 3 weeks at 27°C. After that Nobushi et al. (1975) were isolated six pulvinone derivatives that was new compound from same *Aspergillus terreus.*

Quadrone was obtained from *A. terreus* NRRL 11,156 from the n-butanol extract from broth and quadrone was active against KB cell in vitro (Calton et al., 1978) and reported in vivo against P388 lymphocytic leukemia in mice (Geran et al., 1972).

In 1983, Akira et al. isolated terrecyclic acid that was new sesquiterpene antibiotic from *Aspergillus terreus* Thom No. 14. Ling et al. (1979) were found territrem A and B in *Aspergillus terreus* chloroform crude. A thin layer chromatographic plate was used in this isolation, which was then developed in benzene-ethyl acetate (7:3 v/v). The ascending development distance was 10 cm and the blue fluorescent bands of R_f 0.43 and 0.33 corresponding to territrem A and B, respectively.

Terami et al. (1997) isolated terpeptin from the cultured broth of *Aspergillus terreus* 95F-1 and the compound was a novel peptide having cell cycle inhibitory activity

However, the biological and new compounds were found in *Aspergillus terreus* since 2000, continually.

When Rao et al. (2000) required to develop lovastatin producing from *A. terreus* DRCC152. They isolated a new butyrolactone-IV along with two known butyrolactones-I and butyrolactones II from the ethyl acetate extract of solid substrate of *A. terreus* and the new compound had cytotoxic activities.

In 2002, Kim et al. found terrulactone A that was isolated from the solid state fermentation of *A. terreus* Fb000501. It is new sesquiterpene lactone type meroterpenoid and the compound has acetyl cholinesterase inhibitors that attracted particular attention for treatment of the Alzhimer-type demania.

In 2003, Lee et al. cultured the marine algilocous fungus *A. terreus*. They isolated novel chiral dipyrrolobenzoquinone derivatives, terreusinone. The broth extract (EtOAc) revealed a moderate UV-A absorbing activity and was separated by assay-guided fractionation using repeated salica gel flash chromatography and HPLC.

In same year, Kithsiri et al. (2003) isolated novel cyclopententenedione, asterredione along with two new terrecyclic acid A derivatives, (+)-5(6)-dihydro-6-methoxyterrecyclic acid A and (+)-5(6)-dihydro-6-hydroxyterrecyclic acid A, and five known compounds as terrecyclic acid, quadrone betulinan A, asterriquinone D and asteriquinone C-1.

Later year, Fernando et al. (2004) isolated novel metabolite from *A. terreus*, named terreinol and used [1-^{13}C]-D-glucose as a labeled precursor resulted in formation of this metabolite.

The current reports of metabolites from *Aspergillus terreus* in 2005 have three reports. Four new sesterpenoids, terretonins A-D, and a new alkaloid, asterrelenin together with five known compounds, were isolated from the ethyl acetate extract of solid state fermented culture of *A. terreus* As3.3955 (Li et al., 2005). Next, *Aspergillus terreus* Fb000501 was carried out in solid state of moisture wheat-bran and isoterreulactone A was isolated from ethyl acetate crude. In addition, it inhibited acetylcholinesterase with an IC_{50} value of 2.5 μM.

Last, Cazar et al. (2005) isolated three main metabolites identified as terreic acid, butyrolactone I and lovastatin from *A. terreus* Thorn var. *terreus* that isolated from an Ecuador soil sample. Then, they synthesized three compounds from butyrolactone I as butyrolactone 4', 4''-diacetate and 3'-(3-Methylbutyl) - butyrolactone II and final compound as butyrolactone III. The total compounds were tested biological activities that described as below

1. Terreic acid, butyrolactone I, butyrolactone 4', 4''-diacetate and 3'-(3-Methylbutyl) - butyrolactone II inhibited phytopathogenic bacteria *Erwinia carotovora* with IC_{50} of 5 and 4-18 μg/ml

2. 3'-(3-Methylbutyl) - Butyrolactone II inhibited *Psudomonas syringae* with IC_{50} of 21 μg/ml and *Botrytis cinera* with MIC of 15.6 μg/ml.

3. Butyrolactone I inhibited germination of the dicot *Lactuca sativa* with an IC_{50} of 5×10^{-5} M when compared the IC_{50} of reference herbicide acetochlor that showed 1×10^{-5} M.

4. Inhibition of 3'-(3-Methylbutyl) - butyrolactone II and butyrolactone III on *Panicum millaceum* germination and growth was stronger than butyrolactone I and butyrolactone 4', 4''-diacetate.

However compound isolation from *A. terreus* was studied worldwide. The researcher found biological compounds or novel compounds from these fungi, continually. Differences of source of fungi, conditions of culturing example media, temperatures and static or shakes conditions, biological tests and procedures of compounds isolations are factor to found surprised compounds from this fungi.

Preliminary, the selected fungi is *Aspergillus terreus*. From below reasons, this research expects to find antimicrobial compounds or new compounds from *Aspergillus terreus* isolated ZcSR5-4, the endophytic fungi from *Zingiber cassumunar* Roxb. rhizomes.

4.4 Cultivation and extraction of endophytic fungi isolate ZcSR5-4

4.4.1 Isolation and purification of antimicrobial compound in ethyl acetate crude of mycelium

Main one compound was isolated from the ethyl acetate crude of mycelium chemical structure of this compound were determined by analyses of spectroscopic data, including IR, UV, NMR and Mass spectra, as well as by comparison their spectral data with those of published values.

4.5 Structure elucidation of the pure compound from endophytic fungus isolate ZcSR5-4.

4.5.1 Structure elucidation of compound 1.

Compound 1 was yellow viscous liquid. The structure of compound 1 was elucidated by using spectroscopic techniques.

λ_{max} (nm), MeOH (log ε) : 303.02(3.55) (Figure C 1.1 in Appendix C)

The IR spectrum of compound 1 is shown in Appendix C Figure 1.2 and the absorption peaks were assigned as Table 4.6. Its spectrum indicated important absorption band at 3360.87 cm^{-1}(OH stretching), 1730.43 cm^{-1} (C=0 stretching), 1617.39, 1508.70, 1447.83 and 1386.96cm^{-1} (C=C stretching vibration in aromatic compound)

Table 4.6 The IR absorption band assignment of compound 1

Wave number(cm^{-1})	Intensity	Assignment
3360.87	Broad	OH stretching
1730.43	Strong	C=0 stretching
1617.39, 1508.70, 1447.83 and 1386.96	Medium to strong	C=C stretching vibration in aromatic compound

The ^{1}H-NMR spectrum (Figure C 1.3 in Appendix C) of compound 1 possessed two methyl proton at 1.58 and 1.64 ppm, three methylene proton at 3.11, 3.46 and 3.52 ppm, a methoxy proton at 3.74 ppm, a olefinic proton at 5.07 and aromatic proton at 6.50, 6.57, 6.90 and 7.60 ppm that showed parasubstitution structure.

δ_H ($CDCl_3$, 400 MHz): δ (ppm) 3.46 (d, 1H, J=14.8), 3.52 (d, 1H, J=14.8), 6.50(d, 1H, J=10.8), 7.60(d, 1H, J=8.4), 6.57(d, 1H, J=8.8), 6.90(d, 2H, J=8.8), 6.90(d, 2H, J=8.8), 6.57(d, 1H, J=8.8), 3.11(d, 2H, J=7.2), 1.64(s, 3H), 1.58(s, 3H), 3.74(s, 3H) (Figure 1.3 in Appendix C)

The ^{13}C-NMR spectrum (Figure C1.4 in Appendix C) of compound 1 showed 22 signals.

δ_C ($CDCl_3$, 100 MHz): δ (ppm) 169.69(s), 137.46(s), 129.53(s), 86.18(s), 169.97(s), 38.60(t), 124.49(s), 131.76(d), 126.93(s), 153.03(s), 114.99(d), 129.53(s), 121.93(s), 129.06(d), 116.06(d), 157.03(s), 116.06(d), 129.06(d), 28.61(t) , 121.93(d), 133.88(s), 25.66(q), 17.68(q), 53.65(q). (Figure 1.4 in Appendix C)

The HR/ES-TOF mass spectrum (Figure C 1.10 in Appendix C) showed the $[M+Na]^+$ at 447.14. Then DBE (Double bond equivalent) calculation showed

DBE = C-0.5(H+X-N)+1 = 24-0.5(24)+1 = 13

It is assumed that this compound contain carbon, protons and oxygen and then the molecular formular is $C_{24}H_{24}O_7$

The information from 2D-NMR techniques, including HSQC (Figure C1.5 in Appendix C), HMBC (Figure C1.6 in Appendix C), COSY (Figure C1.7 in Appendix C), NOESY (Figure C1.8 in Appendix C) and TOCSY (Figure C1.9 in Appendix C)

Optical rotation of compound 1 showed $[\alpha]_D^{24.5}$ = +83 (c=0.01, $CHCl_3$)

Optical rotation of Butyrolactone I showed $[\alpha]_D^{25}$ = +82 (c=0.2, $CHCl_3$) (Cazar et al., 2005)

Compound 1 showed spectral data identical to that of Butyrolactone 1 which was reported in the literature (Cazar et al., 2005). The ^{1}H-NMR and ^{13}C-NMR signal of compound 1 and butyrolactone I are presented in Table 4.14

Table 4.7 Comparison of ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound 1 and Butyrolactone I (Cazar et al., 2005)

position	Compound 1		Butyrolactone I	
	δ_C	δ_H	δ_C(400 MHz)	δ_H(100 MHz)
1	169.69(s)	-	169.55(s)	-
2	137.46(s)	-	137.37(s)	-
3	128.69(s)	-	128.51(s)	-
4	86.18(s)	-	86.21(s)	-
5	169.97(s)	-	169.91(s)	-
6	38.60(t)	3.46 (d, 1H, J=14.8)	38.67(t)	3.58(d, *J*=14.7)
	-	3.52 (d, 1H, J=14.8)	-	3.52(d, *J*=14.2)
1’	124.49(s)	-	124.60(s)	-
2’	131.76(d)	6.50(d, 1H, J=10.8)	131.85(d)	6.51(d, J=2.0)
3’	126.93(s)	-	126.82(s)	-
4’	153.03(s)	-	153.16(s)	-
5’	114.99(d)	6.51(d,1H,J=10.8)	115.12(d)	6.52(d, J=7.8)
6’	129.53(s)	7.60(d, 1H, J=8.4)	129.61(d)	6.58(dd, J=7.8 and 2.0)
1’’	121.93(s)	-	122.06(s)	-
2’’	129.06(d)	6.57(d, 1H, J=8.8)	129.53(d)	7.65(d, J= 8.8)
3’’	116.06(d)	6.90(d, 2H, J=8.8)	116.09(d)	6.90(d, J=8.8)
4’’	157.03(s)	-	156.98(s)	-
5’’	116.06(d)	6.90(d, 2H, J=8.8)	116.09(d)	6.90(d, J=8.8)
6’’	129.06(d)	6.57(d, 1H, J=8.8)	129.53(d)	7.65(d, J= 8.8)
1’’’	28.61(t)	3.11(d, 2H, J=7.2)	28.86(t)	3.12 (d, J=7.3)
2’’’	121.62(d)	5.07(t, 1H, J=7.2)	121.63(d)	5.08(brt, J= 7.3)
3’’’	133.88(s)	-	134.10(s)	-
4’’’	25.66(q)	1.64(s, 3H)	25.68(q)	1.65(s)
5’’’	17.68(q)	1.58(s, 3H)	17.17(q)	1.70(s)
5-OMe	53.65(q)	3.74(s, 3H)	53.60(q)	3.80(s)

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

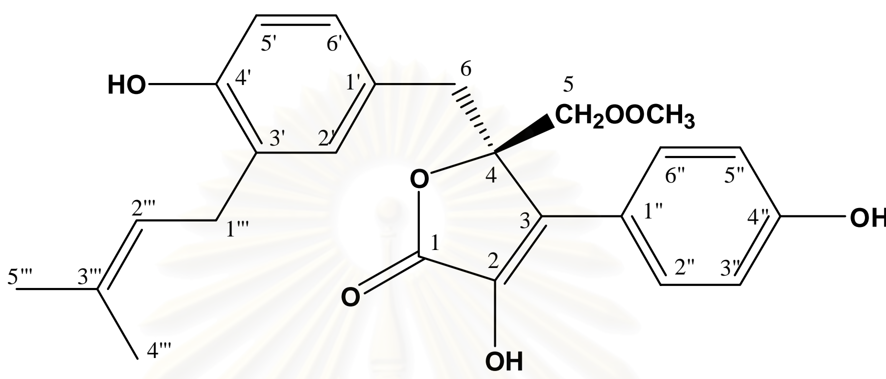

Figure 4.14 The structure of compound 1

Butyrolactone I (BL), a low molecular weight (MW 424) CDK specific inhibitor derived from *Aspergillus terreus* var. *africanus* IFO 8835, was first described in 1977 (Kiriyama et al., 1977). BL has been found to exhibit antiproliferative activity on a human lung cancer cell line (Nishio, 1996)

Butyrolactone I inhibited phytopathogenic bacteria *Erwinia carotovora* with IC50 of 5 µg/ml. Butyrolactone I inhibited germination of the dicot *Lactuca sativa* with an IC50 of 5 x 10-5 M when compared the of IC50 reference herbicide acetochlor that showed 1 x 10-5 M. (Cazar et al., 2005)

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4.5.2 Structure elucidation of compound 2.

Compound 2 was yellow viscous liquid. The structure of compound 1 was elucidated by using spectroscopic techniques.

λ_{max} (nm), MeOH (log ε): 300.05(3.75) (Figure C 2.1 in Appendix C)

The IR spectrum of compound 2 is shown in Appendix C Figure 5 and the absorption peaks were assigned as Table 4.8. Its spectrum indicated important absorption band at 3306.70 cm^{-1}(OH stretching), 1739.13 cm^{-1} (C=0 stretching), 1613.04, 1500.00, 1439.13 and 1378.26 cm^{-1} (C=C stretching vibration in aromatic compound)

Table 4.8 The IR absorption band assignment of compound 2

Wave number(cm^{-1})	Intensity	Assignment
3306.70	Broad	OH stretching
1739.13	Strong	C=0 stretching
1613.04, 1500.00, 1439.13 and 1378.26	Medium	C=C stretching vibration in aromatic compound

The ^{1}H-NMR spectrum (Figure C 2.3 in Appendix C) of compound 2 possessed two methyl proton at 1.17 and 1.24 ppm, four methylene proton at 3.37, 3.46, 2.55 and 2.80 ppm , one methoxy proton at 3.70, three aromatic proton at 6.52, 6.84 and 7.52 ppm that showed parasubstitution structure.

. δ_H ($CDCl_3$, 400 MHz): δ (ppm) 3.37 (d, 1H, J=14.4), 3.46 (d, 1H, J=14.8), 6.52(s, 1H), 6.84(d, 1H, J=8.8), 7.52(d, 1H, J=8.8), 7.52(d, 1H, J=8.8), 2.55(dd, 1H, J=4.4 and 17.2), 2.80(dd, 1H, J=4.8 and 17.2), 3.69(t, 1H, J=5.2), 1.17(s, 3H), 1.24(s, 3H), 3.70(s, 3H) (Figure 2.3 in Appendix C).

The ^{13}C-NMR spectrum (Figure C 2.4in Appendix C) of compound 1 showed 20 signals.

δ_H ($CDCl_3$, 100 MHz): δ (ppm) 169.26(s), 137.41(s), 129.06(s), 85.92(s), 169.74(s), 38.64(t), 122.26(s), 132.09(d), 124.72(s), 156.58(s), 116.02(d), 132.09(s), 118.14(s), 129.50(d), 31.00(t), 69.56(d), 69.91(s), 24.59(q), 22.41(q), 53.95(q).

The HR/ES-TOF mass spectrum (Figure C 2.10 in Appendix C) showed the $[M+Na]^+$ at 463.63. Then DBE (Double bond equivalent) calculation showed

DBE = C-0.5(H+X-N)+1 = 24-0.5(24)+1 = 13

It is assumed that this compound contain carbon, protons and oxygen and then the molecular formular is $C_{24}H_{24}O_8$.

The information from 2D-NMR techniques, including HSQC (Figure C 2.5 in Appendix C), HMBC (Figure C 2.6 in Appendix C), COSY (Figure C 2.7 in Appendix C), NOESY (Figure C 2.8 in Appendix C) and TOCSY (Figure C 2.9 in Appendix C)

Optical rotation of compound 2 showed $[\alpha]_D^{24.5}$ = +89 (c=0.01, $CHCl_3$).

Optical rotation of Butyrolactone I showed $[\alpha]_D^{25}$ = +78 (Keiichi et al., 1983).

Compound 2 showed spectral data identical to that of Butyrolactone III which was reported in the literature (Cazar et al., 2005). The ^{1}H-NMR and ^{13}C-NMR signal of compound 2 and butyrolactone III are presented in Table 4.9

Table 4.9 Comparison of [13] C-NMR and ^{1}H-NMR chemical shifts of compound 2 and Butyrolactone III (Cazar et al., 2005)

position	Compound 2		Butyrolactone III	
	δ_C	δ_H	δ_C(400 MHz)	δ_H(100 MHz)
1	169.26(s)	-	168.13(s)	-
2	137.41(s)	-	138.34(s)	-
3	129.06(s)	-	127.75(s)	-
4	85.92(s)	-	84.94(s)	-
5	169.74(s)	-	169.96(s)	-
6	38.64(t)	3.37 (d, 1H, J=14.4)	38.24(t)	3.53(d, J=14.2)
	-	3.46 (d, 1H, J=14.8)		3.48(d, J=14.2)
1’	122.26(s)	-	123.43(s)	-
2’	132.09(d)	6.52(s, 1H)	131.35(d)	6.5(Id, J=2.2)
3’	124.72(s)	-	124.82(s)	-
4’	156.58(s)	-	156.45(s)	-
5’	116.02(d)	6.84(d, 1H, J=8.8)	114.83(d)	6.50(d, J=8.1)
6’	132.09(s)	6.52(s, 1H)	131.35(d)	6.57(dd, J=8.0 and 2.0)
1’’	118.14(s)	-	121.23(s)	-
2’’	129.50(d)	7.52(d, 1H, J=8.8)	129.02(d)	7.62(d, J= 8.8)
3’’	116.02(d)	6.84(d, 2H, J=8.8)	116.07(d)	6.91(d, J=8.8)
4’’	156.58(s)	-	158.12(s)	-
5’’	116.02(d)	6.84(d, 1H, J=8.8)	116.07(d)	6.91(d, J=8.8)
6’’	129.50(d)	7.52(d, 1H, J=8.8)	129.02(d)	7.65(d, J= 8.8)
1’’’	31.00(t)	2.55(dd, 1H, J=4.4 and 17.2)	26.63(t)	2.37m
	-	2.80(dd, 1H, J=4.8 and 17.2)	-	-
2’’’	69.56(d)	3.69(t, 1H, J=5.2)	65.44(d)	2.41m
3’’’	69.91(s)		58.32(s)	-
4’’’	24.59(q)	1.17(s, 3H)	23.31(q)	1.24(s)
5’’’	22.41(q)	1.24(s, 3H)	23.31(q)	2.25(s)
5-OMe	53.95(q)	3.70(s, 3H)	53.52(q)	3.78(s)

After elucidation of compound 2 by 2D NMR techniques, the chemical shift on ^{1}H-NMR spectrum of compound 2 and butyrolactone III was compared signal by signal. This result indicated that the structure of compound 2 identical to butyrolactone 2. Thus, it could be concluded that compound 2 was butyrolactone III. The structure is presented in Figure 4.15

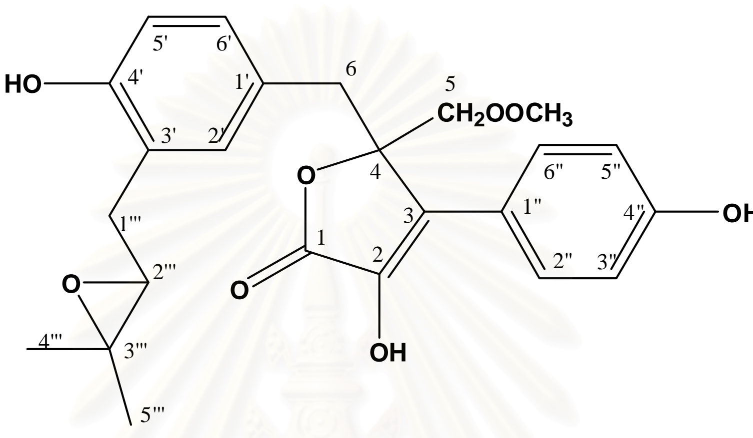

Figure 4.15 The structure of compound 2.

Butyrolactone III was synthesized from Butyrolactone I by epoxidation of the prenyl group with m- chloperbenzoic acid (Keiichi, 1983). Inhibition of butyrolactone III on *Panicum millaceum* germination and growth was stronger than butyrolactone I and butyrolactone 4’, 4’’-diacetate.(Cazar et al., 2005)

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4.5.2 Structure elucidation of compound 3.

Compound 3 was yellow viscous liquid. The structure of compound 3 was elucidated by using spectroscopic techniques.

λ_{max} (nm), MeOH (log ε) : 300.98(3.62) (Figure C 3.1 in Appendix C)

The IR spectrum of compound 3 is shown in Appendix C Figure 5 and the absorption peaks were assigned as Table 4.10. Its spectrum indicated important absorption band at 3295.65 cm^{-1}(OH stretching), 1730.43 cm^{-1} (C=0 stretching), 1613.04, 1526.09, 1491.30 and 1439.13 cm^{-1} (C=C stretching vibration in aromatic compound)

Table 4.10 The IR absorption band assignment of compound 3

Wave number(cm^{-1})	Intensity	Assignment
3295.65	Broad	OH stretching
1730.43	Strong	C=0 stretching
1613.04, 1526.09, 1491.30 and 1439.13	Weak	C=C stretching vibration in aromatic compound

The ^{1}H-NMR spectrum (Figure C 3.3 in Appendix C) of compound 3 possessed 1.14, 1.22, 1.28, 2.18, 2.64, 2.98, 3.50, 3.75, 4.49, 6.48, 6.57, 6.65, 6.89 and 7.58 ppm.

The ^{13}C-NMR spectrum (Figure C 3.4 in Appendix C) of compound 3 showed 48 signals.

The HR/ES-TOF mass spectrum (Figure C 3.10 in Appendix C) showed the $[M+Na]^{+}$ at 463.13. Then DBE (Double bond equivalent) calculation showed

DBE = C-0.5(H+X-N)+1 = 24-0.5(24)+1 = 13

It is assumed that this compound contain carbon, protons and oxygen and then the molecular formular is $C_{24}H_{24}O_{8}$.

The information from 2D-NMR techniques, including HSQC (Figure C 3.5 in Appendix C), HMBC (Figure C 3.6 in Appendix C), COSY (Figure C 3.7 in Appendix C), NOESY (Figure C 3.8 in Appendix C) and TOCSY (Figure C 3.9 in Appendix C)

Optical rotation of compound 3 showed $[\alpha]^{24.5}$ = +85 (c=0.01, $CHCl_3$).

Optical rotation of Butyrolactone IV showed $[\alpha]^{27}_{D}$ = +92 (c=0.7, EtOAc) (Rao et al., 2000).

Compound 3 showed HMBC spectral data identical to that of compound 2 which had one point difference. At 85.92 ppm of ^{13}C-NMR showed on HMBC spectral data of compound 3 but not showed on HMBC spectral data of compound 2. This results described as the point had C-C correlation of position 21 carbon and position 18 carbon.

After elucidation of compound 3 by 2D NMR techniques, this result indicated that the structure of compound 3 identical to butyrolactone IV. Thus, it could be concluded that compound 3 was butyrolactone IV. The structure is presented in Figure 4.16

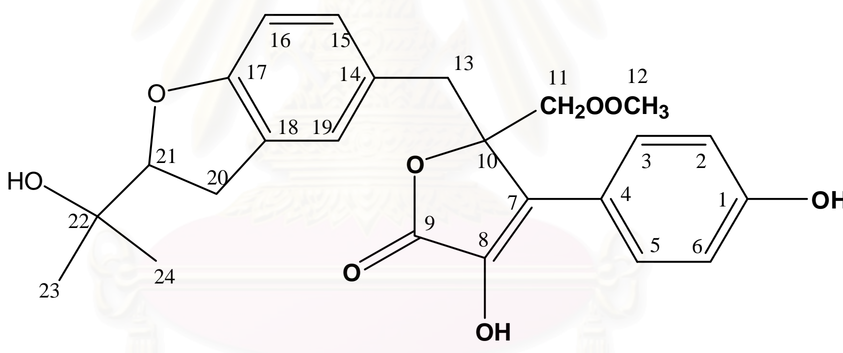

Figure 4.16 The structure of compound 3

Rao et al. (2000) required to develop lovastatin producing from *A. terreus* DRCC152. They isolated a new butyrolactone-IV along with two known butyrolactones-I and butyrolactones II from the ethyl acetate extract of solid substrate of *A. terreus* and the new compound had cytotoxic activities.

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4.6 Biological activity

4.6.1 Antimicrobial activity of the crude extracts from endophytic fungi isolate ZcSR5-4.

The antimicrobial activity of crude extracts from endophytic fungus isolate ZcSR5-4 was evaluated by the disc diffusion method. All crude extract were examined at a concentration 10 mg/ml and dissolved in 10 % DMSO in sterile distilled water and drop solution of crude extract on paper disc (50, 100 and 200 µg/disc, diameter 7.0 cm. The antimicrobial activity was calculated from the inhibition zone(cm) of test microorganism, including the bacteria strains *B. subtilis*, *S. aureus*, *E. coli*, *P aeroginosa* and fungi, yeast form strain *C. albican.* Antimicrobial extracts is shown in Figure 4.17 and Table 4.15.

The results were described as only ethyl acetate crude of mycelium and broths have antimicrobial activity. The 50 µg/disc ethyl acetate crude of mycelium inhibited *B. subtilis* and *S. aureus* with both 0.8±0 cm clear zones, the 100 µg/disc ethyl acetate crude of mycelium inhibited both *B. subtilis* and *S. aureus* with 0.9±0 cm clear zones and the 200 µg/disc ethyl acetate crude of mycelium inhibited both *B. subtilis* and *S. aureus* with 1.5±1 and 1.4±1 cm, respectively.

The 50 µg/disc ethyl acetate crude of broth inhibited *B. subtilis* with 0.9±0 cm clear zones, the 100 µg/disc ethyl acetate crude of broth inhibited both *B. subtilis* and *S. aureus* with 1.0±1 and 0.9±1 cm clear zones, respectively and the 200 µg/disc ethyl acetate crude of broth inhibited *B. subtilis* and *S. aureus* with both 1.3±1 and 1.1±0 cm, respectively.

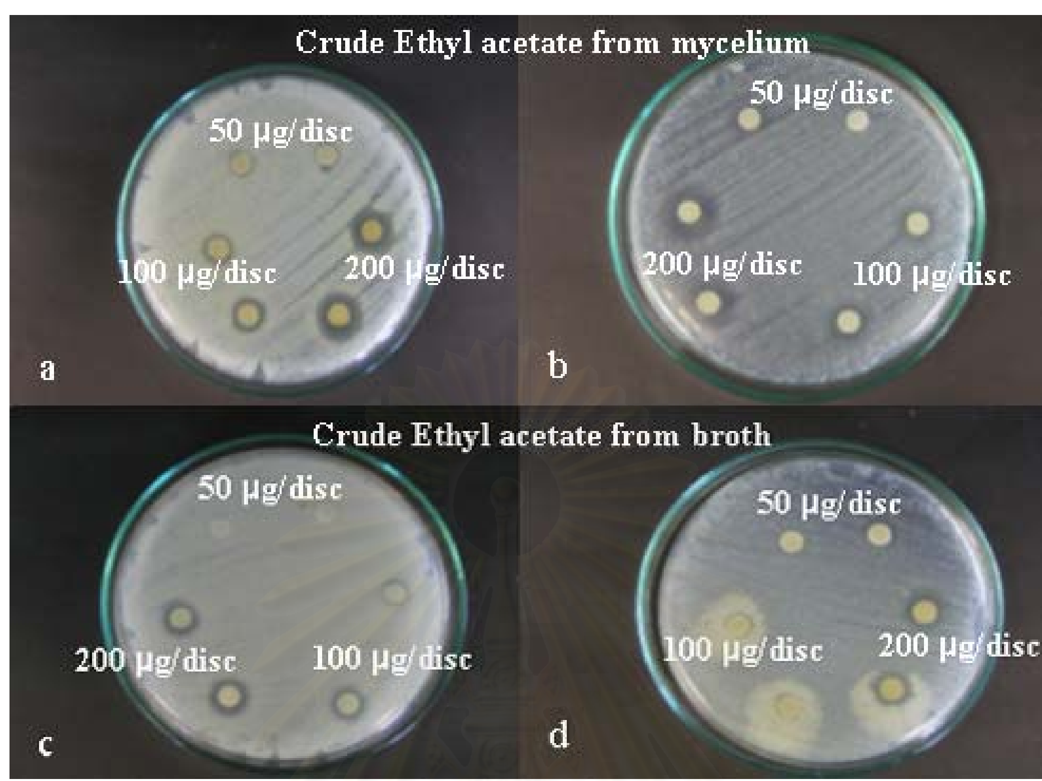

Figure 4.17 200 µg/disc of EtOAc crude extract of mycelium inhibited *B. subtilis* (a) and *S. aureus* (b), respectively. 200 µg/disc of EtOAc crude extract of culture broth inhibited *B. subtilis* (c) and *S. aureus* (d), respectively.

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Table 4.12 Antimicrobial activities of crude extract of endophytic fungal isolate ZcSR5-4

Crude extract	Inhibition zones(cm)				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeroginosar*	*C. albican*
Crude Haxane (Mycelium)					
50 µg/disc	-	-	-	-	-
100µg/disc	-	-	-	-	-
200µg/disc	-	-	-	-	-
Crude Ethyl acetate(Mycelium)					
50 µg/disc	0.8±0	0.8±0	-	-	-
100µg/disc	0.9±0	0.9±0	-	-	-
200µg/disc	1.5±1	1.4±1	-	-	-
Crude methanol (Mycelium)					
50 µg/disc	-	-	-	-	-
100µg/disc	-	-	-	-	-
200µg/disc	-	-	-	-	-
Crude Haxane (Broth)					
50 µg/disc	-	-	-	-	-
100µg/disc	-	-	-	-	-
200µg/disc	-	-	-	-	-
Crude Ethyl acetate(Broth)					
50 µg/disc	0.9±0	-	-	-	-
100µg/disc	1.0±1	0.9±0	-	-	-
200µg/disc	1.3±1	1.1±0	-	-	-
Crude methanol (Broth)					
50 µg/disc	-	-	-	-	-
100µg/disc	-	-	-	-	-
200µg/disc	-	-	-	-	-

- = no inhibition

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4.6.2 Antimicrobial activity of the fraction from crude extract.

Antimicrobial activities of the combined fractions of endophytic fungi isolate ZcSR5-4. The antimicrobial activity of crude extracts from endophytic fungus isolate ZcSR5-4 was evaluated by the disc diffusion method. All crude extract were examined at a concentration 10 mg/ml and dissolved in 10 % DMSO in sterile distilled water and drop solution of crude extract on paper disc (100 µg/disc). The antimicrobial activity was calculated from the inhibition zone(cm) of test microorganism, including the bacteria strains *B. subtilis*, *S. aureus*, *E. coli*, *P aeroginosa* and fungi, yeast form strain *C. albican.* Antimicrobial extracts is shown in Figure 4.18 and Table 4.16.

At 100 µg/disc concentration, combined fraction EM5, EM6, EM7, EM8, EM9, EM10, EM11 and EM12 inhibited *B. subtilis* with 0.9±0, 0.9±0, 2.0±1, 1.0±0, 0.9±0, 1.1±1, 0.8±0 and 0.8±0 cm, respectively and only EM7, EM8 and EM10 inhibited *S. aureus* with 2.0±0, 0.9±0 and 0.9±1 cm, respectively.

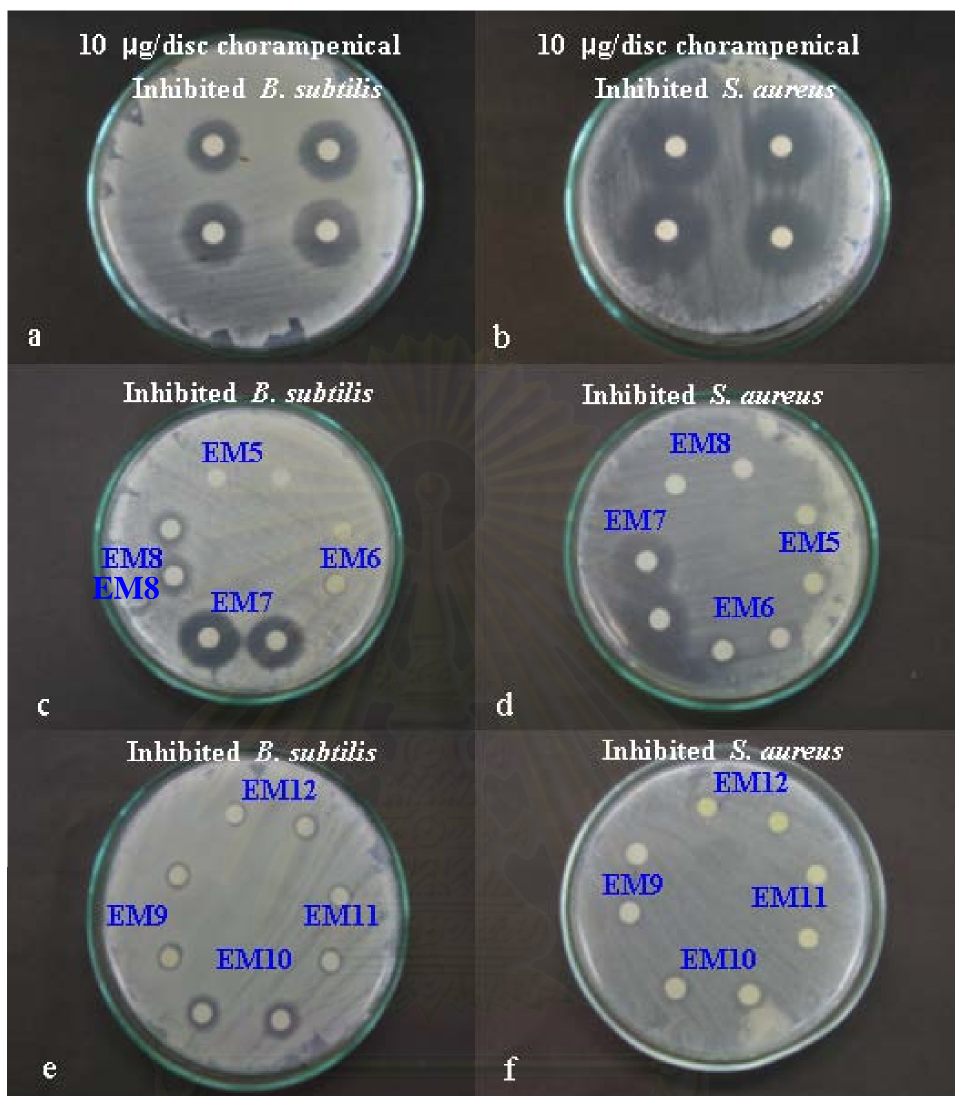

Figure 4.18 Positive control of 10 µg/disc chorampenical inhibited *B. subtilis* (a) and *S. aureus* (b). 100 µg/disc of combined fractions number EM7 and EM8 inhibited *B. subtilis* (c), combined fractions number EM7 inhibited *S. aureus* (d). Combined fractions number EM10 inhibited *B. subtilis* (e) and *S. aureus* (f).

Table 4.13 Antimicrobial activities inhibited tested microorganisms of the combined fractions from siliga gel chromatography of EtOAc crude of mycelium extract of endophytic fungal isolate ZcSR5-4.

Combined fraction (100 microgram/disc)	Inhibition zone (cm)				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeroginosa*	*C. albican*
EM1	-	-	-	-	-
EM2	-	-	-	-	-
EM3	-	-	-	-	-
EM4	-	-	-	-	-
EM5	0.9±0	-	-	-	-
EM6	0.9±0	-	-	-	-
EM7	2.0±1	2.0±0	-	-	-
EM8	1.0±0	0.9±0	-	-	-
EM9	0.9±0	-	-	-	-
EM10	1.1±1	0.9±1	-	-	-
EM11	0.8±0	-	-	-	-
EM12	0.8±0	-	-	-	-
EM13	-	-	-	-	-
EM14	-	-	-	-	-
EM15	-	-	-	-	-
EM16	-	-	-	-	-
EM17	-	-	-	-	-
EM18	-	-	-	-	-
EM19	-	-	-	-	-
EM20	-	-	-	-	-
EM21	-	-	-	-	-
EM22	-	-	-	-	-
EM23	-	-	-	-	-

- = no inhibition

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4.6.3 Antimicrobial activity of pure compound

The antimicrobial activity of pure compounds was evaluated by the antimicrobial suspension test, broth microdilution method. The pure compound was examined at a concentration 3.91 – 125 µg/ml. The results were shown that Butyolactone I inhibited *B. subtilis* ATCC6633, *S. aureus* ATCC25923*, E. coli* ATCC25922 with the MICs values of 62.5 µg/ml (147.41µM) and *C. albicans* ATCC10231 with the MICs values of 15.63 µg/ml (36.86 µM).

Butyrolactone III inhibited *B. subtilis* ATCC6633, *S. aureus* ATCC25923*, E. coli* ATCC25922, *P. aeruginosa* ATCC27853 and *C. albican* ATCC10231 with the MICs values of 62.50 (142.04 µM), 31.25 (71.02 µM), 125.00 (284.08 µM), 62.50 (142.04 µM) and 125 (284.08 µM) µg/ml, respectively.

Butyrolactone IV inhibited *B. subtilis* ATCC6633, *S. aureus* ATCC25923, *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853 with the MICs values of 7.82 (17.77 µM), 31.25 (71.02 µM), 62.50 (142.04 µM) and 62.50 µg/ml (142.04 µM), respectively. Antimicrobial activity of pure compounds is presented in Table 4.14

Table 4.14 Broth microdilution method for antimicrobial activities of pure compounds.

Compound	Tested microorganisms and MIC (µg/ml), (µM)				
	Gram positive bacteria		Gram negative bacteria		Yeasts
	B. subtilis ATCC6633	*S.aureus* ATCC25923	*E. coli* ATCC25922	*P. aeruginosa* ATCC27853	*C. albican* ATCC10231
Compound 1	62.50 (142.04)	62.50 (142.04)	62.5 (142.04)	-	15.63 (36.86)
Compound 2	62.50 (142.04)	31.25 (71.02)	125 (284.08)	62.50 (142.04)	125 (284.08)
Compound 3	7.82 (17.77)	31.25 (71.02)	62.50 (142.04)	62.50 (142.04)	-
Streptomycin	3.97 (6.83)	15.63 (26.88)	31.25 (53.73)	31.25 (53.73)	ND
chorampenical	1.92 (5.94)	3.79 (11.73)	15.63 (48.39)	15.63 (48.39)	ND
Ketoconazole	ND	ND	ND	ND	7.81 (14.71)

ND= not determined

- = no against tested microorganism at 125 µg/ml

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4.6.4 Cytotoxic activity

The in vitro activity of pure compounds from fungal isolate ZcSR5-4 was tested against 5 cell lines including, HEP-G2 (hapatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast). The results had not cytotoxic activity.

CHAPTER V

CONCLUSION

Forty eight endophytic fungi were isolated from rhizomes of *Zingiber cassumunar* Roxb. Plant samples were collected from Surin phatumthanee, Songkhla and Supanburee province of Thailand. Endophytic fungi were isolated using the surface sterilization method. A total of 48 isolates (100%) of endophytic fungi were identified. All fungal endophytes were identified as belonging to typical genera of endophytes such as 59 % *sterilia mycelia*, 35% *Fusarium* sp., 4% *Aspergillus terreus* and 2% *schlerotium* sp.

Preliminary, antimicrobial activities of these endophytes were tested by Agar plate bioassay method. The fungi were prepared from 2 weeks old culture. The cork of fungi were placed on agar plat with tested against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomanas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231. 10 fungal endophytes have antimicrobial activity especially fungal endophytic isolate ZcSR5-4 had high inhibition zones.

Fungal isolate ZcSR5-4 was identified as *Aspergillus terreus* based on morphological and nucleotide sequencing of ITS region, respectively. Fungal isolate ZcSR5-4 was chosen for further study for growth curve profiles, pH and antimicrobial activity. The stationary phase was reached at 9-27 old days and high antimicrobial activity was measured at 15 days.

The antimicrobial compounds from broth and mycelium of fungi were extracted. The mycelia ethyl acetate extracts were activated against most number of test microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923. In addition, ZcSR5-4 culture broth ethyl acetate extracts also actived against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923.

Chromatographic was used to isolate antimicrobial compounds from mycelium extracts of endophytic fungi isolate ZcSR5-4. The structures of isolated compounds were elucidated using their physical properties and spectroscopic techniques. Compound 1 was identified as Butyrolactones I, compound 2 was identified as Butyrolactones III and compound 3 was identified as Butyrolactones IV.

Antimicrobial activities and cytotoxicity of isolated compounds were tested. It was found that compound 1 inhibited *B. subtilis* ATCC6633, *S. aureus* ATCC25923*, E. coli* ATCC25922 at MIC 62.5 µg/ml and *C. albican* ATCC10231 at MIC 15.63 µg/ml.Compound 2 inhibited all tested microorganisms and compound 3 inhibited only bacteria as MIC 7.82,31.25, 62.5 and 62.5 µg/m in *B. subtilis* ATCC6633, *S. aureus* ATCC25923, *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853, respectively. In addition, all compounds were inactived on cytotoxicity against 5 tumor cell lines.

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Appendices

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APPENDIX A

MEDIA

The media were prepared by sterilization in autoclave at 121 °C for 15 minutes. pH was adjusted with NaOH or HCl before addition of agar and sterilization.

1. Malt extract agar (MEA)

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

2. Yeast-malt extract agar (YMA)

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

3. Nutrient agar (NA)

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

4. Potato dextrose agar (PDA)

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

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

6. Yeast extract sucrose agar (YES)

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

7. Czapek Yeast Ager with 20% sucrose (CY20S)

Preparing Czapek concentration (with trace metal)

$NaNO_3$	30.0	g
KCl	5.0	g
$MgSO_4.7H_2O$	5.0	g
$FeSO_4.7H_2O$	0.1	g
$ZnSO_4.7H_2O$	0.1	g
$CuSO_4.5H_2O$	0.05	g
Distilled water	1000	ml

K_2HPO_4	1.0	g
Czapek concentration	10.0	ml
Powdered Yeast Extract	5.0	g
Sucrose	200.0	g
Agar	15.0	g
Distilled water	1000	ml

8. Czapek Yeast Ager

K_2HPO_4	1.0	g
Czapek concentration	10.0	ml
Powdered Yeast Extract	5.0	g
Agar	15.0	g
Distilled water	1000	ml

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Appendix B

Spore counting using a Haemacytometer

Haemacytometer, A graduated glass microscope slide and cover slip used for counting red blood cells, ideal for counting microorganism and microalgae (unicellular phytoplankton). For fungi cultivation, it is essential to known the spore concentration of all formulation to be used in the laboratory or in the field. The methods of spore counting using a haemacytometer are described as below **(http://web.agri.cmu.ac.th/ppath/course/360301/lesson5_inoculation_prepare.htm).**

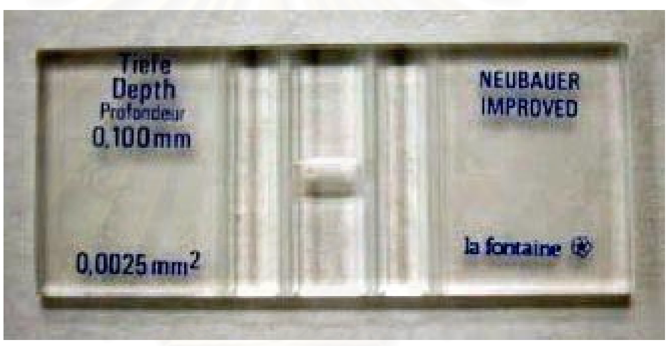

Figure B1 A haemacytometer

1. Preparing the haemacytometer, the haemacytometer must be dry and free of grease such as finger print etc. Grease should be removed using a tissue and alcohol.
2. Dropped a sample of the suspension (added 0.1 % of tween 80) into haemacytometer with a pipette. Carefully deposit an aliquot (about 10 µl) of spore suspension in the notch and watch the sample is drawn by capillary action into the chamber.
3. Place hemacytometer on microscope stage and use field at 400 x magnification. (Figure B2)
4. Count the spores in the small squares and smallest squares including A, B, C, and E area for spore's counting (figure B3)

4.1 If there are small spores, count at E area including 25 small squares and 16 smallest squares of each small square.

$$E \text{ area} = 25 \times 16 \times 1/400 \text{ mm}^2$$

Calculate volume of E area in mm^3 (0.1 depth of haemacytometer).

$$= 25 \times 16 \times 1/400 \times 1/10 \text{ mm}^3 \qquad = 0.1 \text{ mm}^3$$

If the total counting's spores of E area was N in 0.1 mm^3
and 1000 mm^3 (1ml) have spores as below :

$$= \text{N} \times 1000 \times 1/0.1 \text{ spores /ml} \qquad = \text{N} \times 10^4 \text{ spores/ml}$$

If there are big spore, should be count at all A, B, C, D and E area. The number of spores were counted and averaged by 5 (N/5) before spore calculating.

$$= \text{N /5} \times 1000 \times 1/0.1 \text{ spores/ml} \qquad = \text{N/5} \times 10^4 \text{ spores/ml}$$

4.2 If there are more than 100 cells per small square, dilute suspension and count the diluted sample, again. Dilution factor of a suspension will be 0.1 (0.1 ml of stock + 0.9 ml of water). If count of spores at A, B, C, D and E area was 90 therefore:

$$\text{Spore/ml} = \text{N /5} \times 1000 \times 1/0.1 \text{ spores} \qquad = \text{N/5} \times 10^4$$
$$= 90/5 \times 1000 \times 1/0.1 \times 0.1 \qquad = 4.5 \times 10^5.$$

4.3 The concentrations were adjusted to concentrations ranging from 2.5×10^6 to 2×10^7 spores/ml.

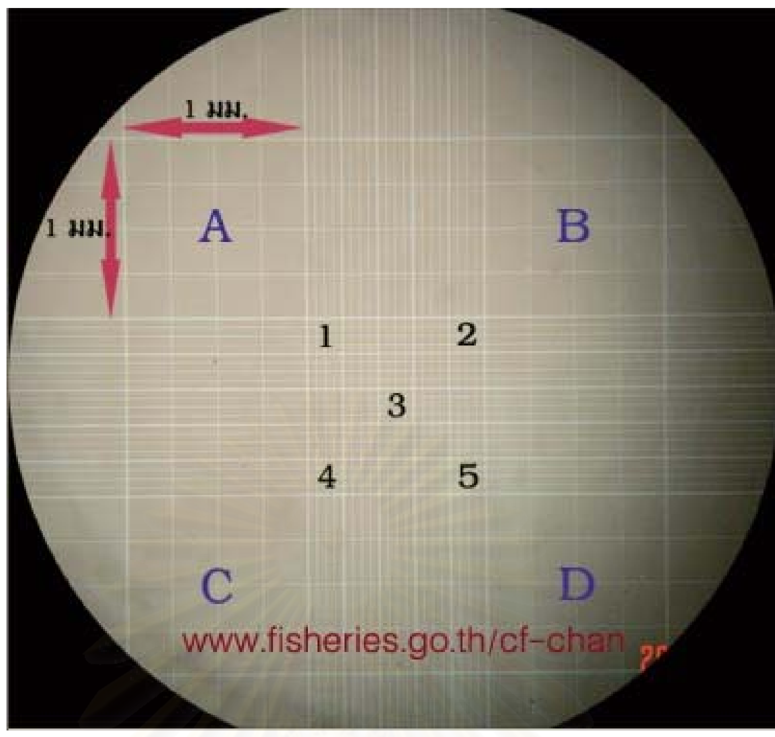

Figure B2 A haemocytometer field at 400 x magnification.

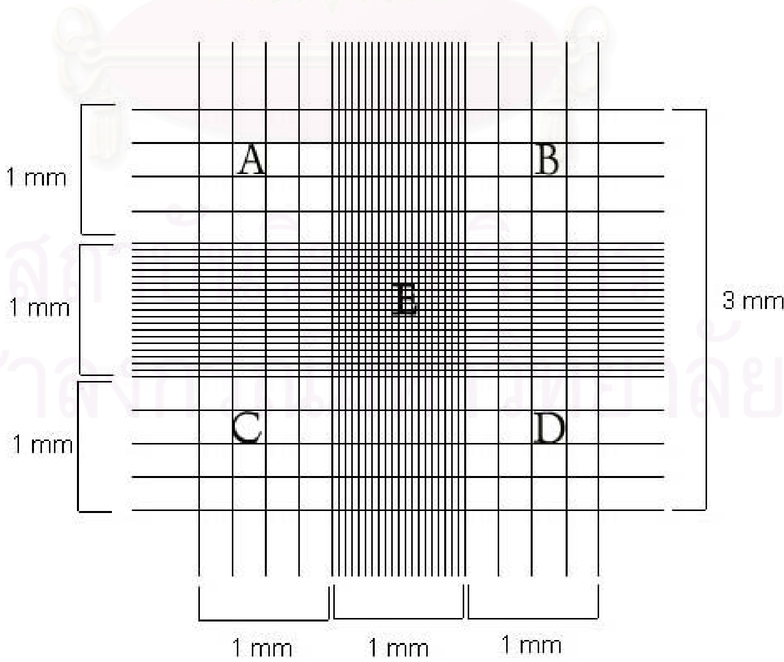

Figure B3 A, B, C, D and E areas in Haemacytometer for spore counting

Appendix C

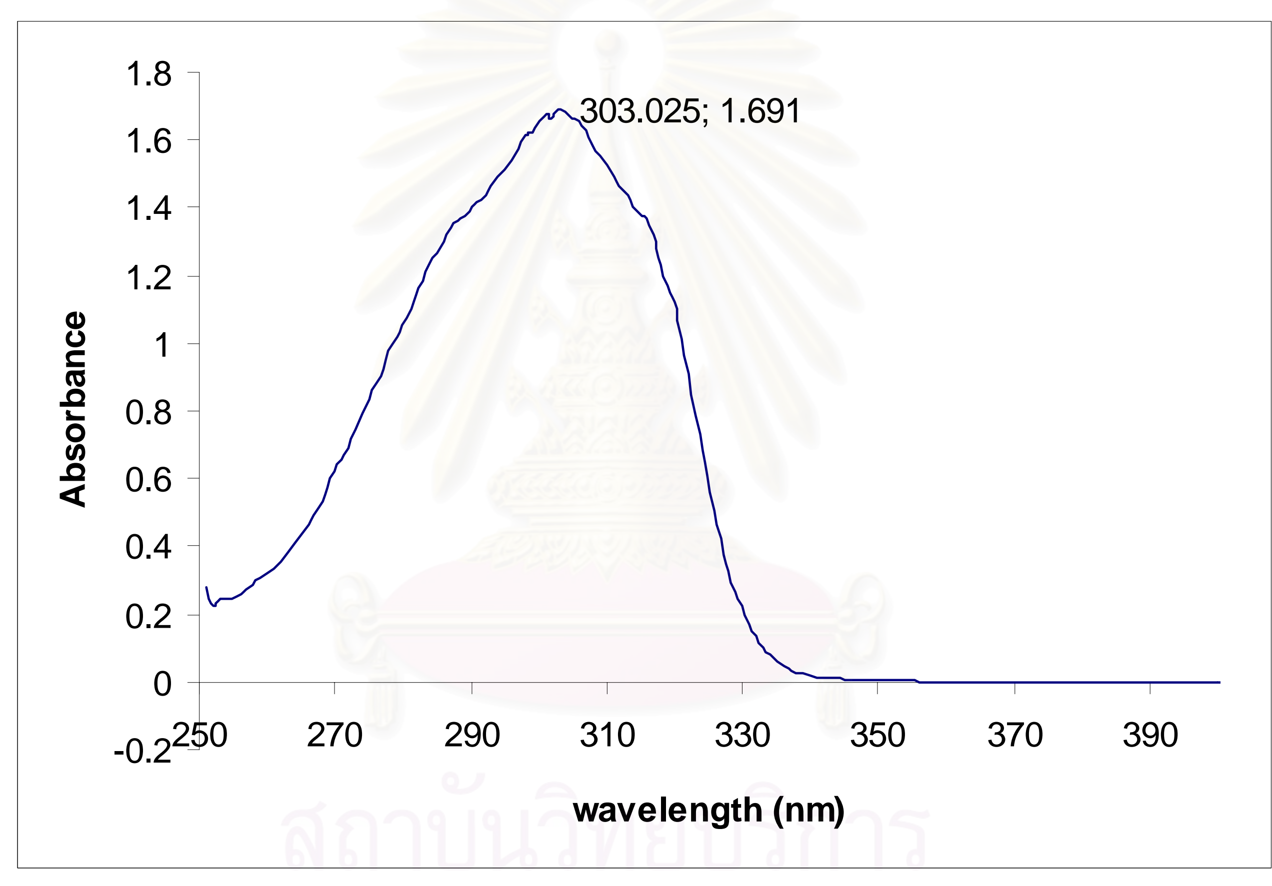

Figure C1.1 UV spectrum of Compound 1

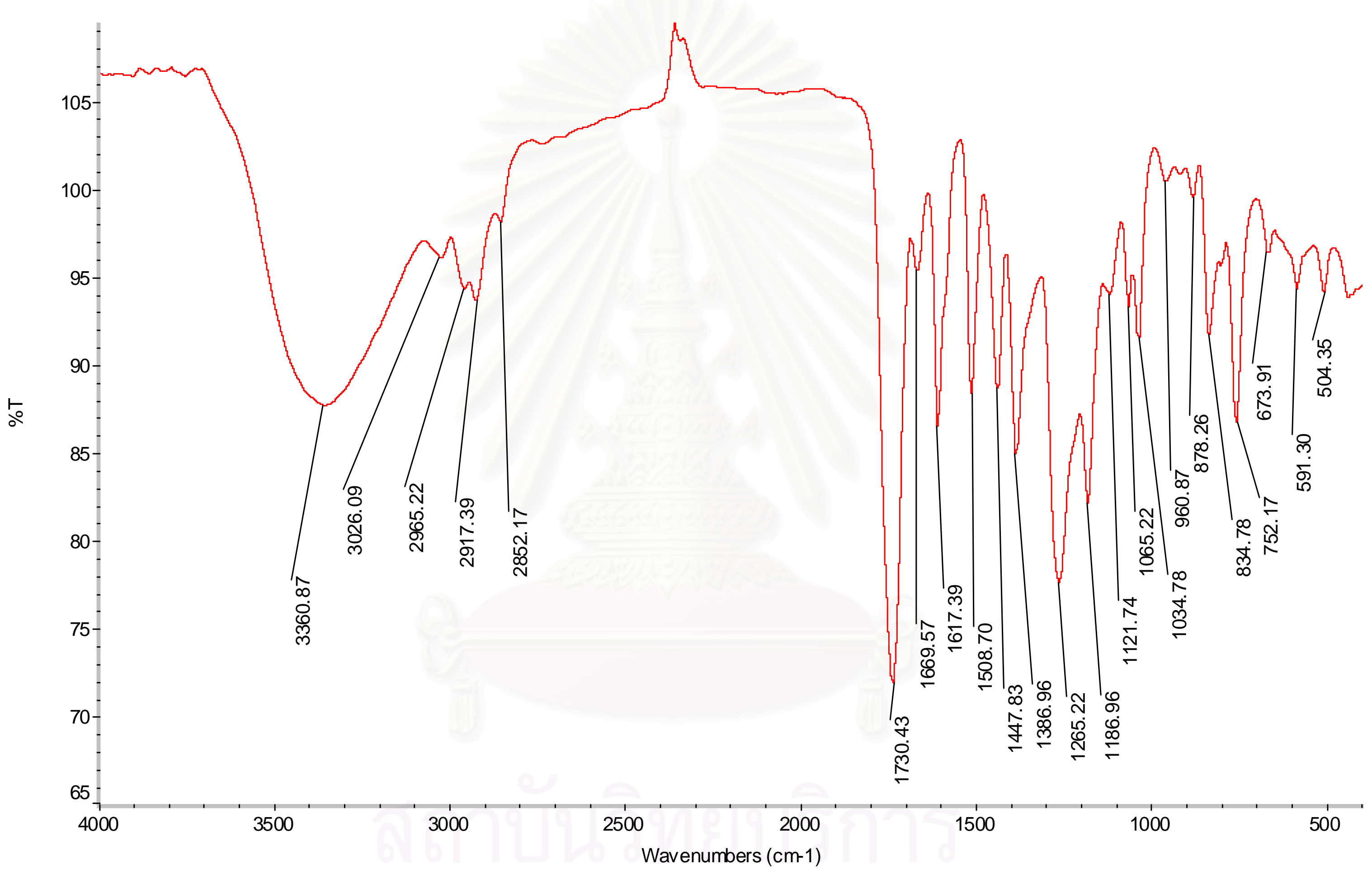

Figure C1.2 IR spectrum of Compound 1

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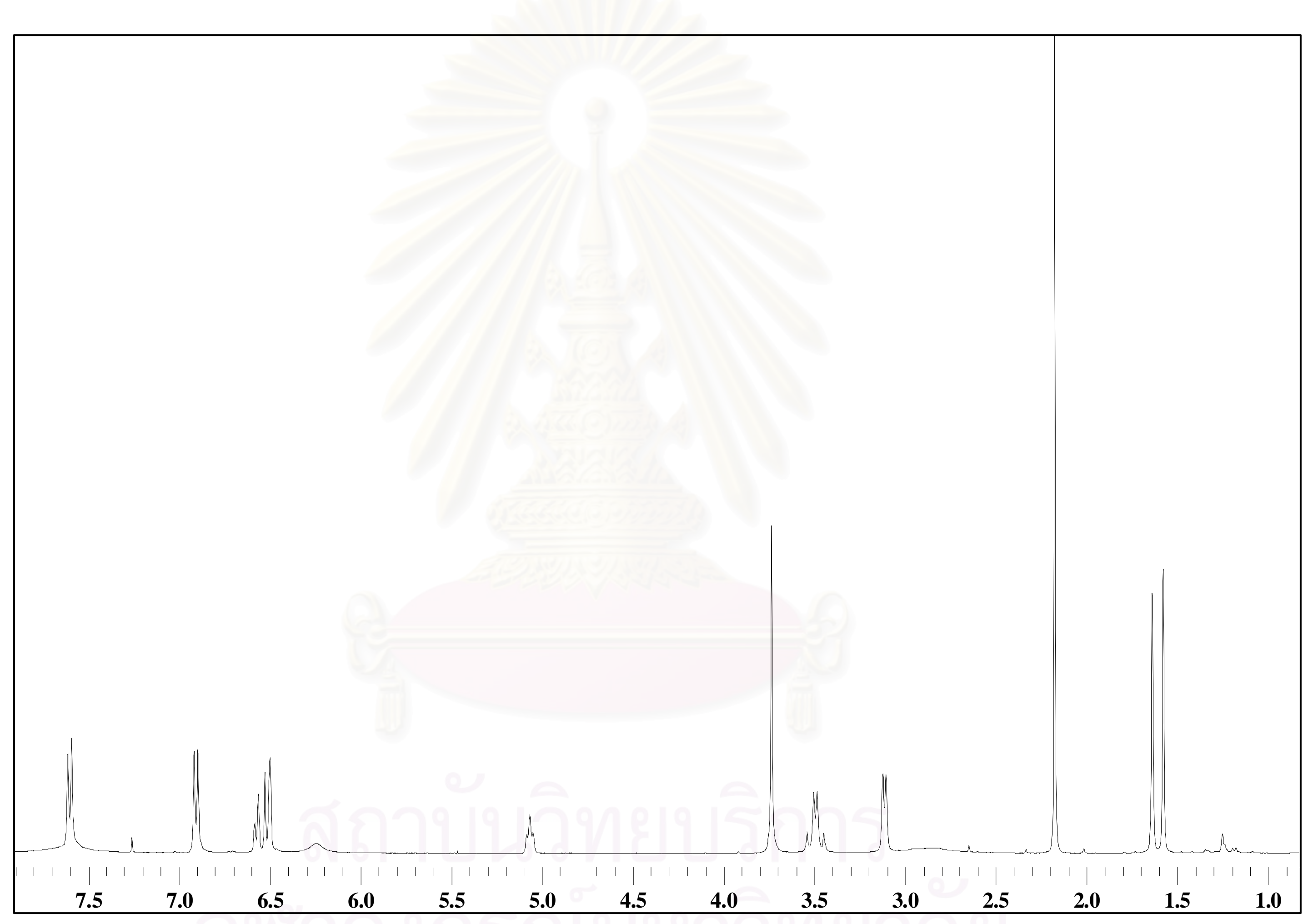

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Figure C1.3 ^{1}H-NMR spectrum of Compound 1

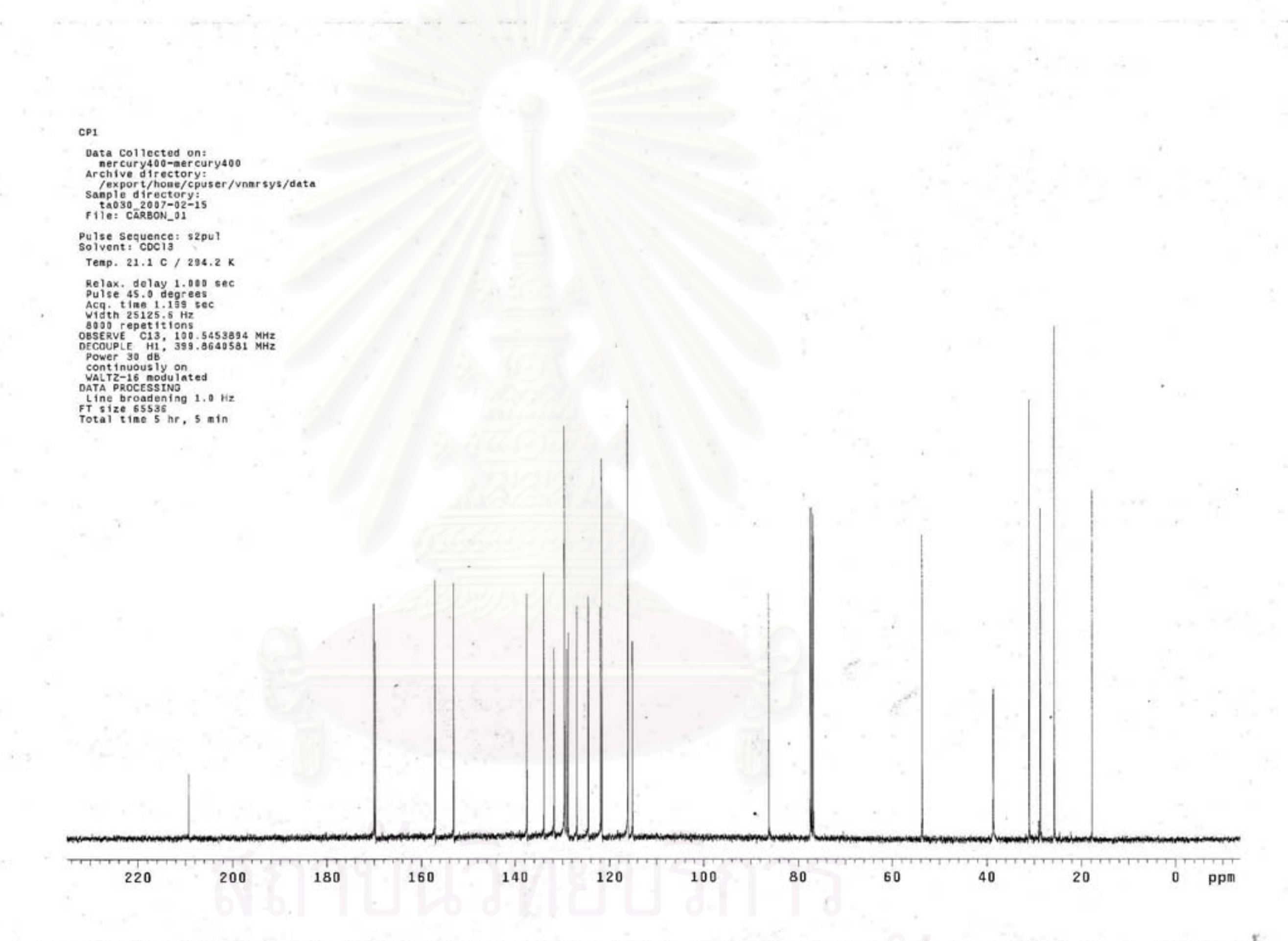

Figure C1.4 ^{13}C-NMR spectrum of Compound 1

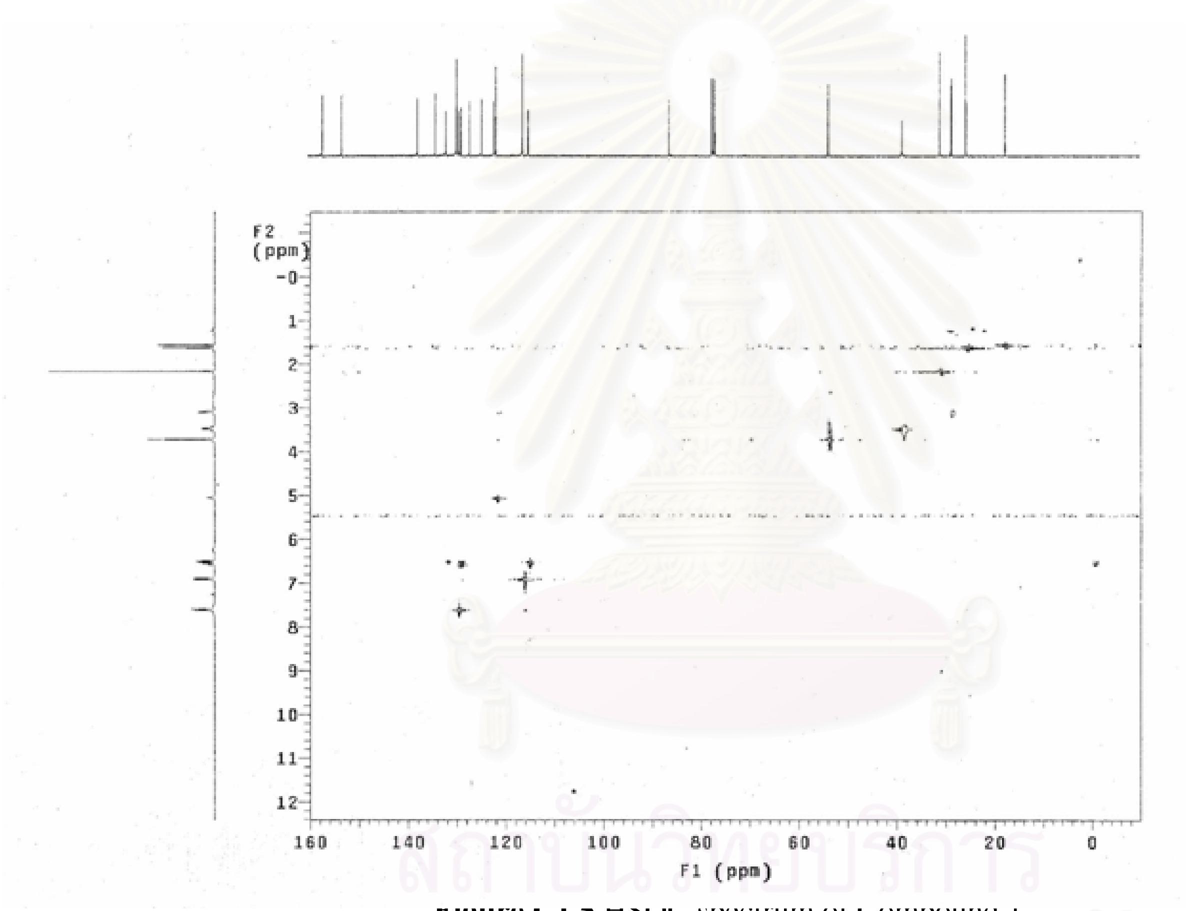

Figure C1.5 HSQC spectrum of Compound 1

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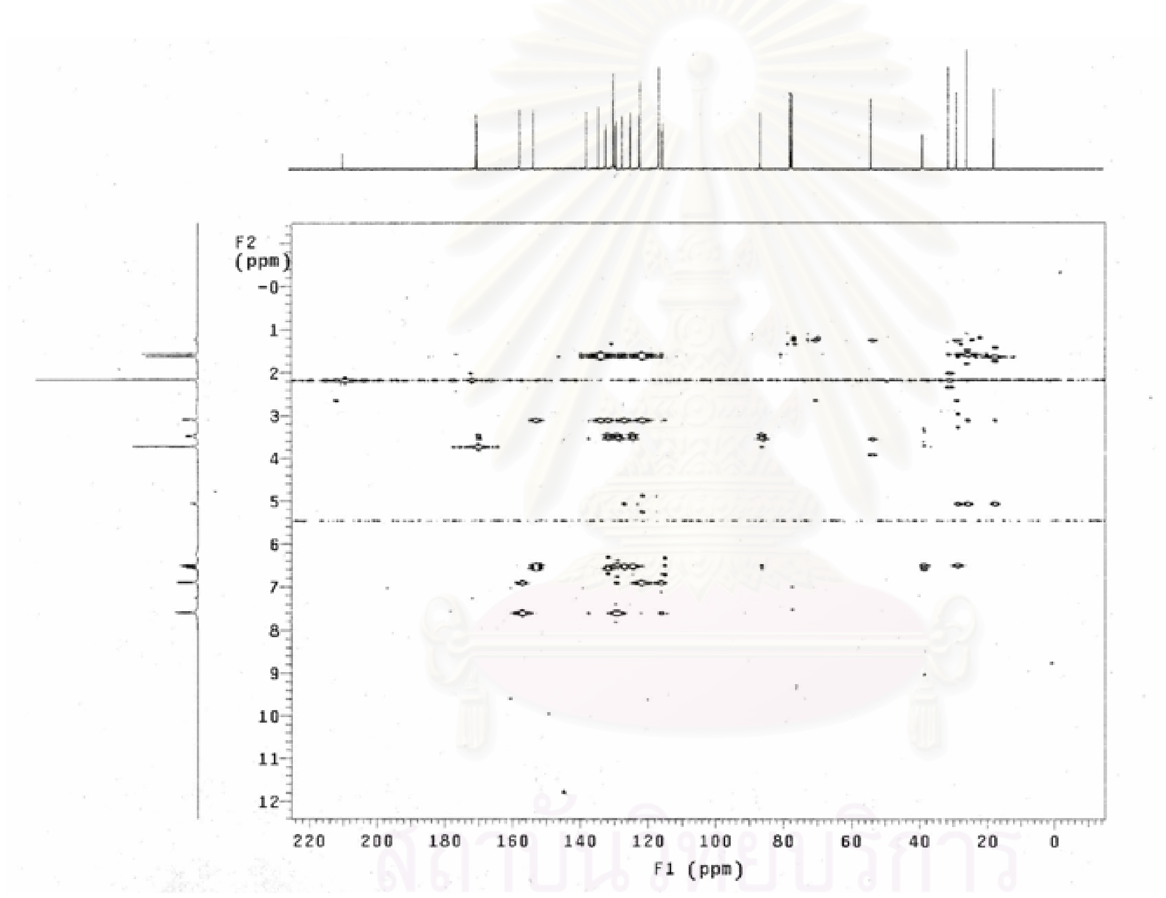

Figure C1.6 HMBC spectrum of Compound 1

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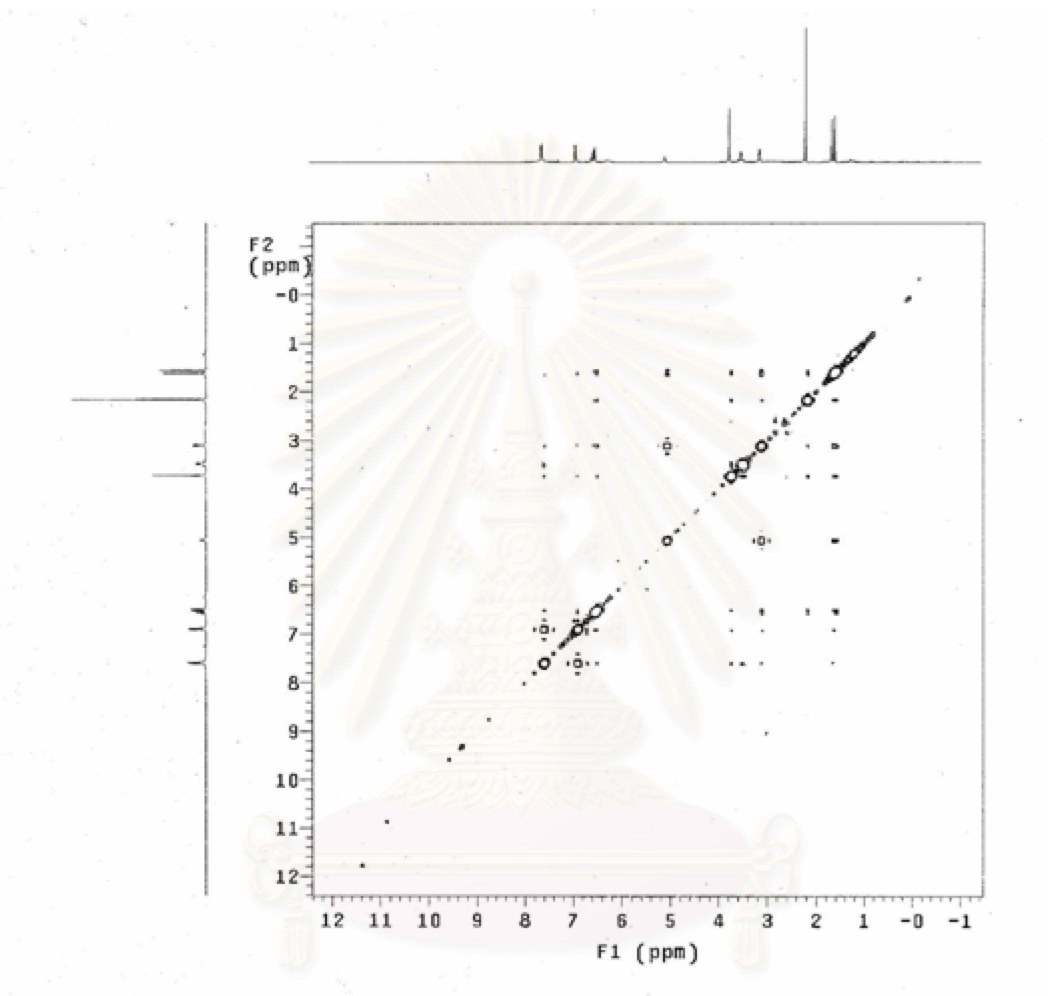

Figure C1.7 COSY spectrum of Compound 1

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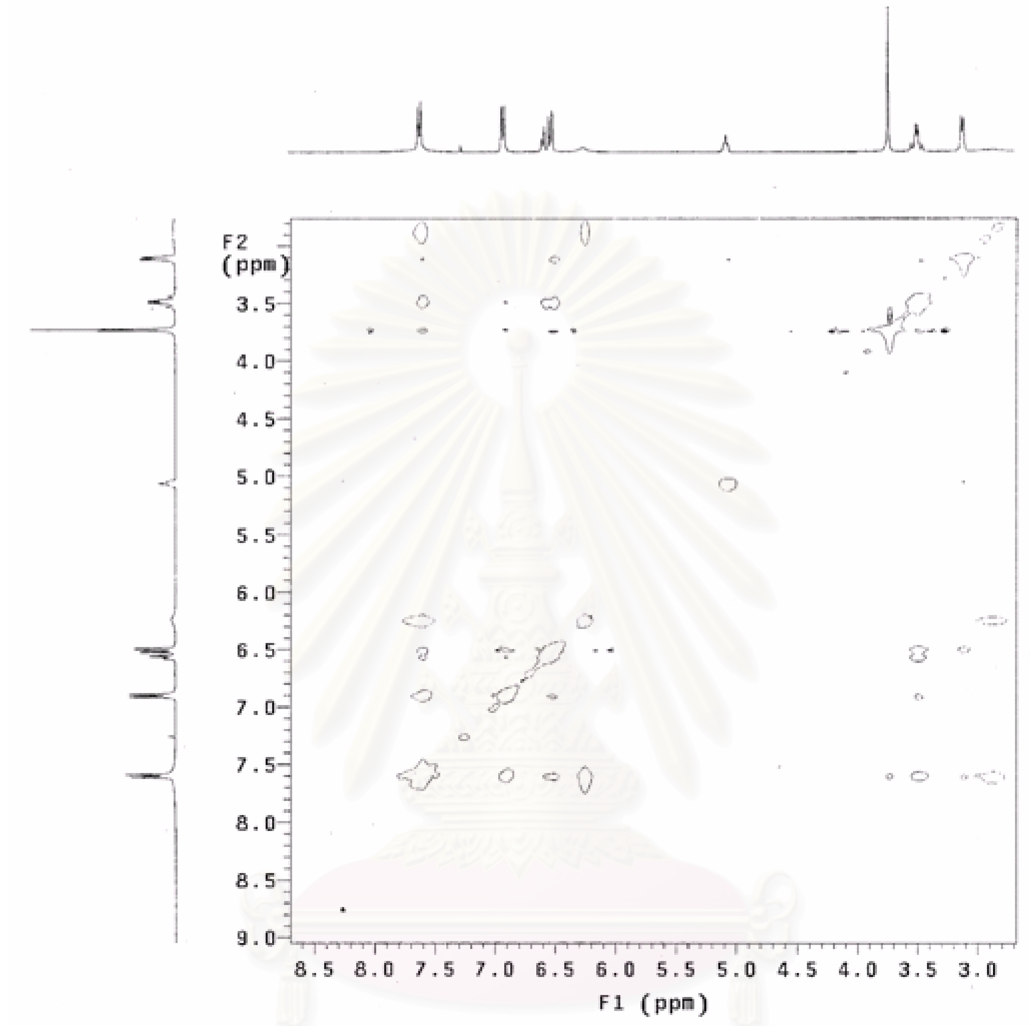

Figure C1.8 NOESY spectrum of Compound 1

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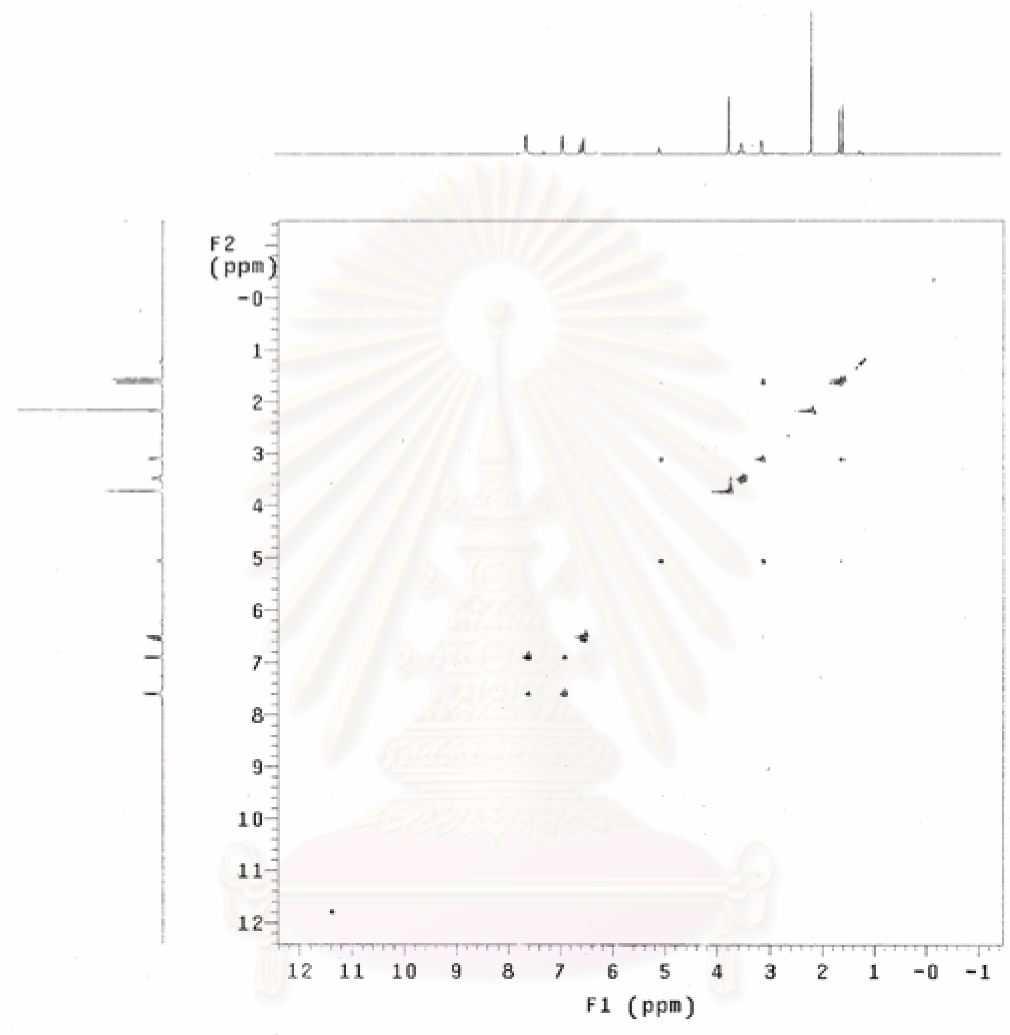

Figure C1.9 TOCSY spectrum of Compound 1

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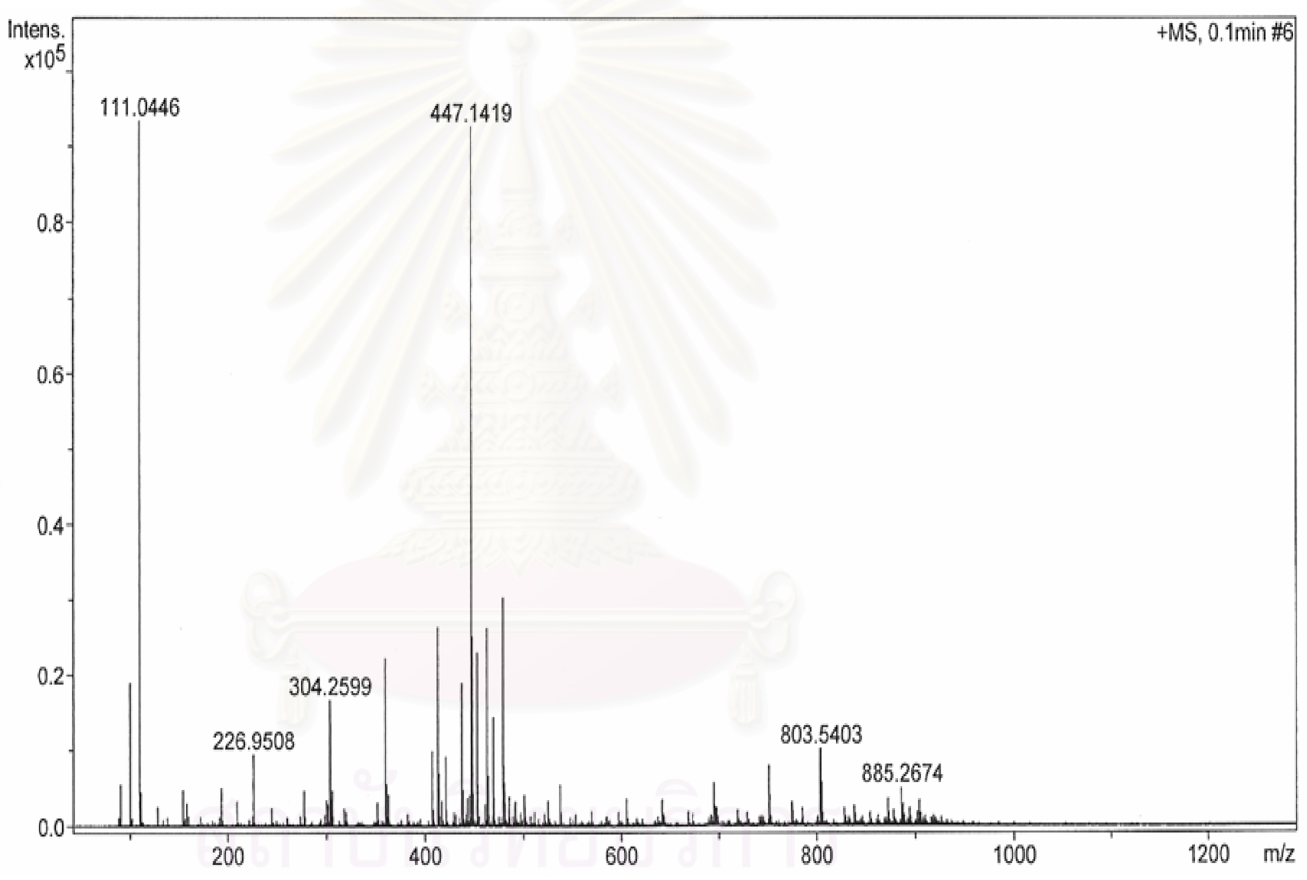

Figure 1.10 HR/ES-TOF MS spectrum of Compound 2

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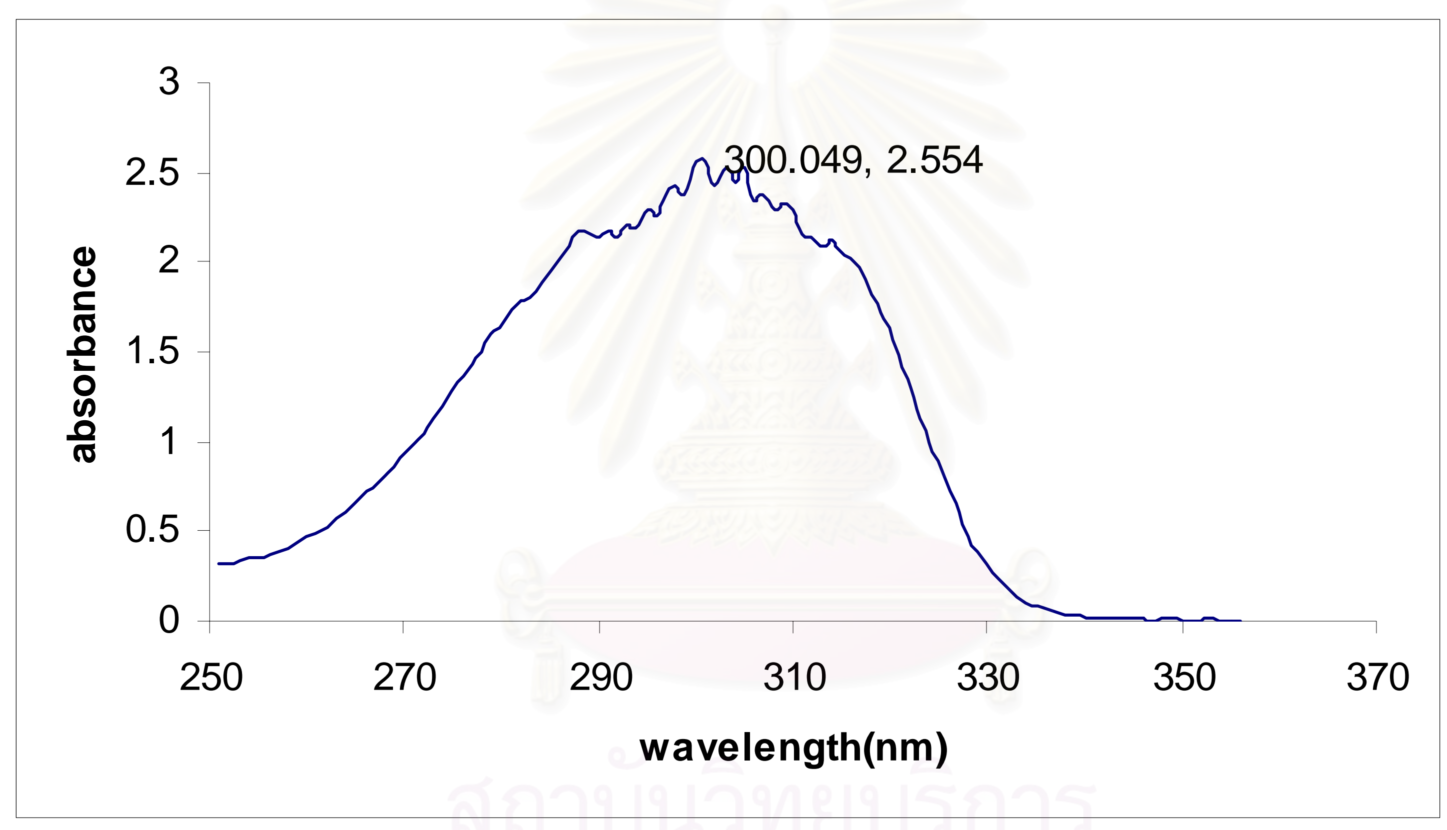

Figure C2.1 UV spectrum of Compound 2

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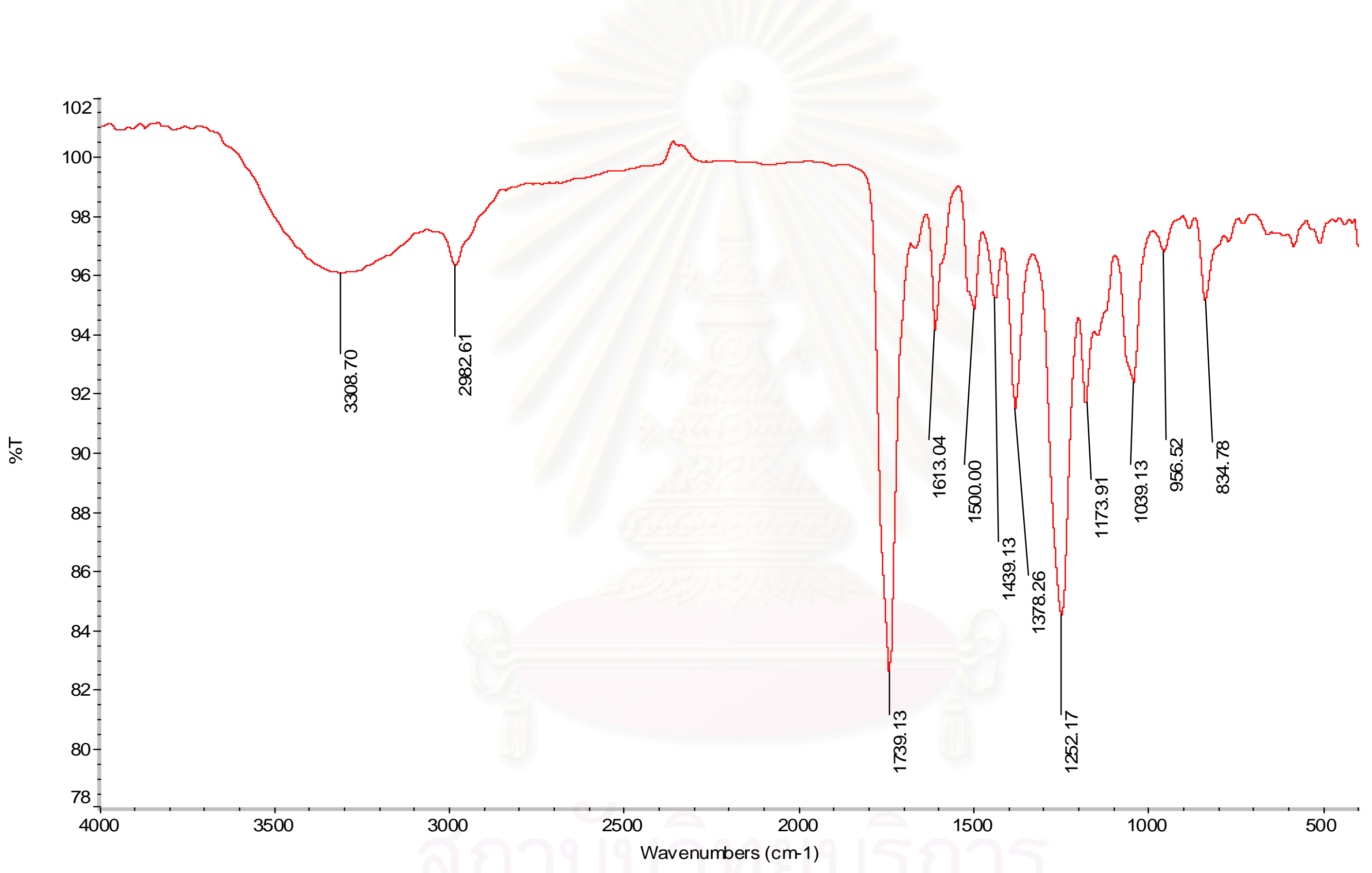

Figure C2.2 IR spectrum of Compound 2

Figure C1.2 IR spectrum of Compound 1

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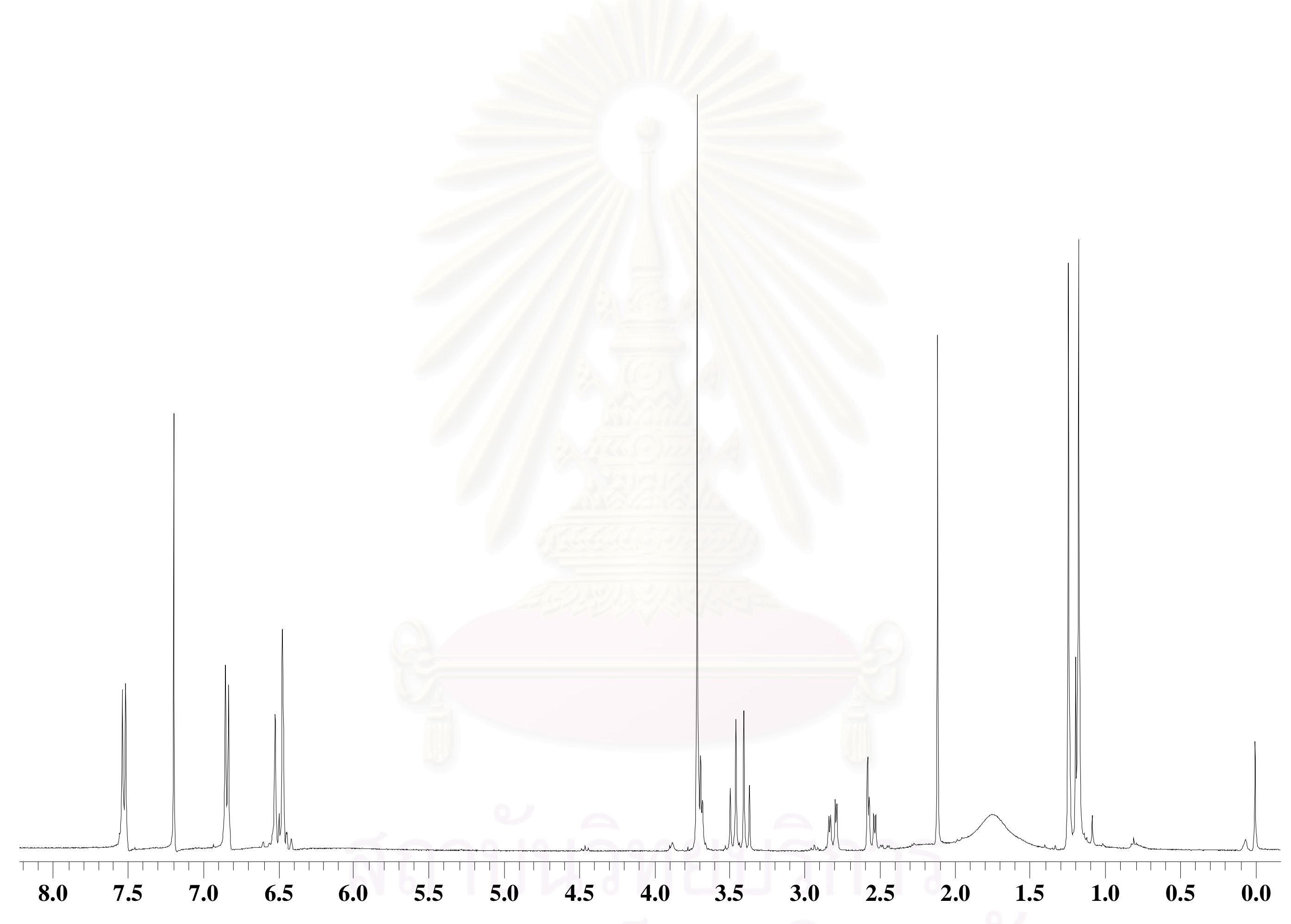

Figure C2.3 ^{1}H-NMR spectrum of Compound 2

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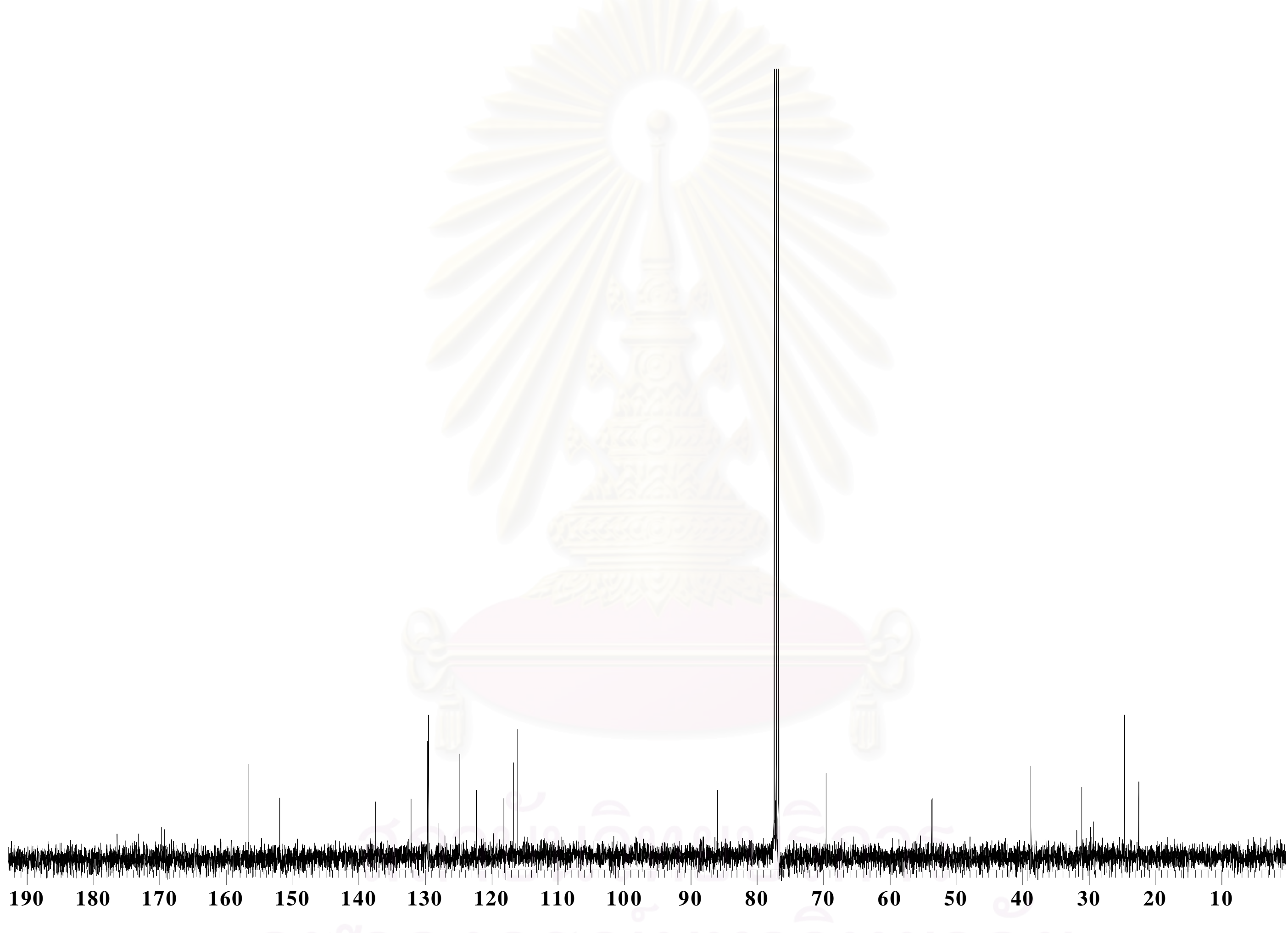
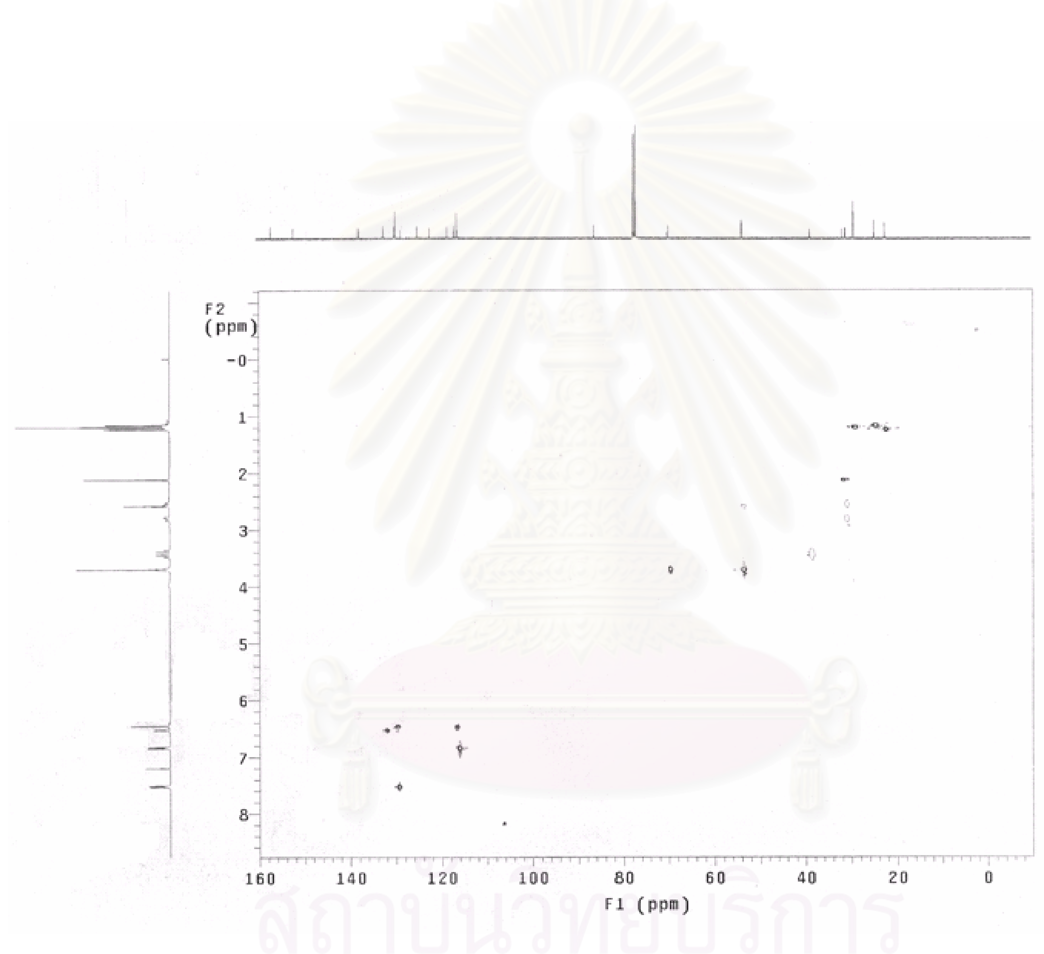


Figure C2.4 ^{13}C-NMR spectrum of Compound 2

จุฬาลงกรณ์มหาวิทยาลัย



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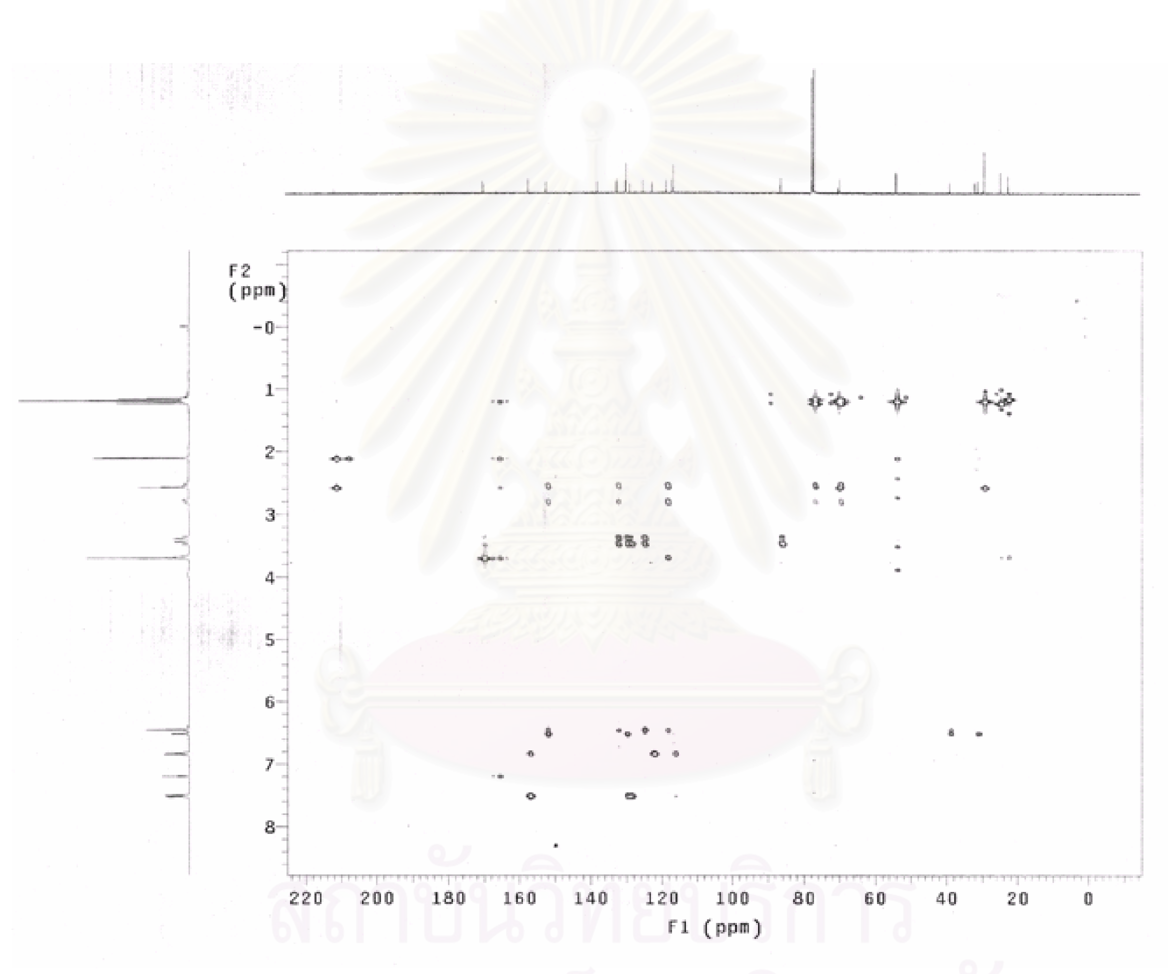
und 2

Figure C2.5 HSQC spectrum of Compound 2



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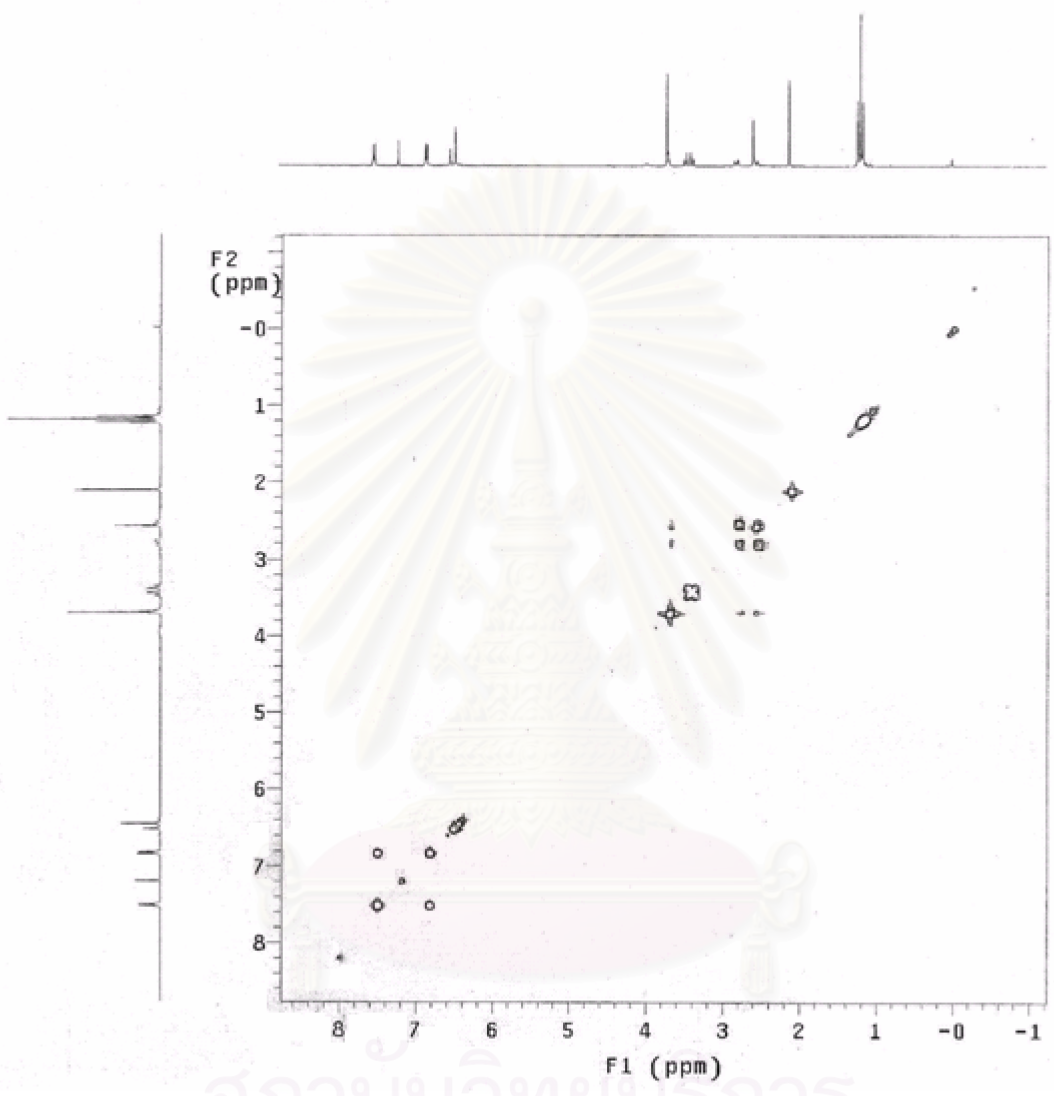

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Figure C2.7 COSY spectrum of Compound 2

จุฬาลงกรณ์มหาวิทยาลัย

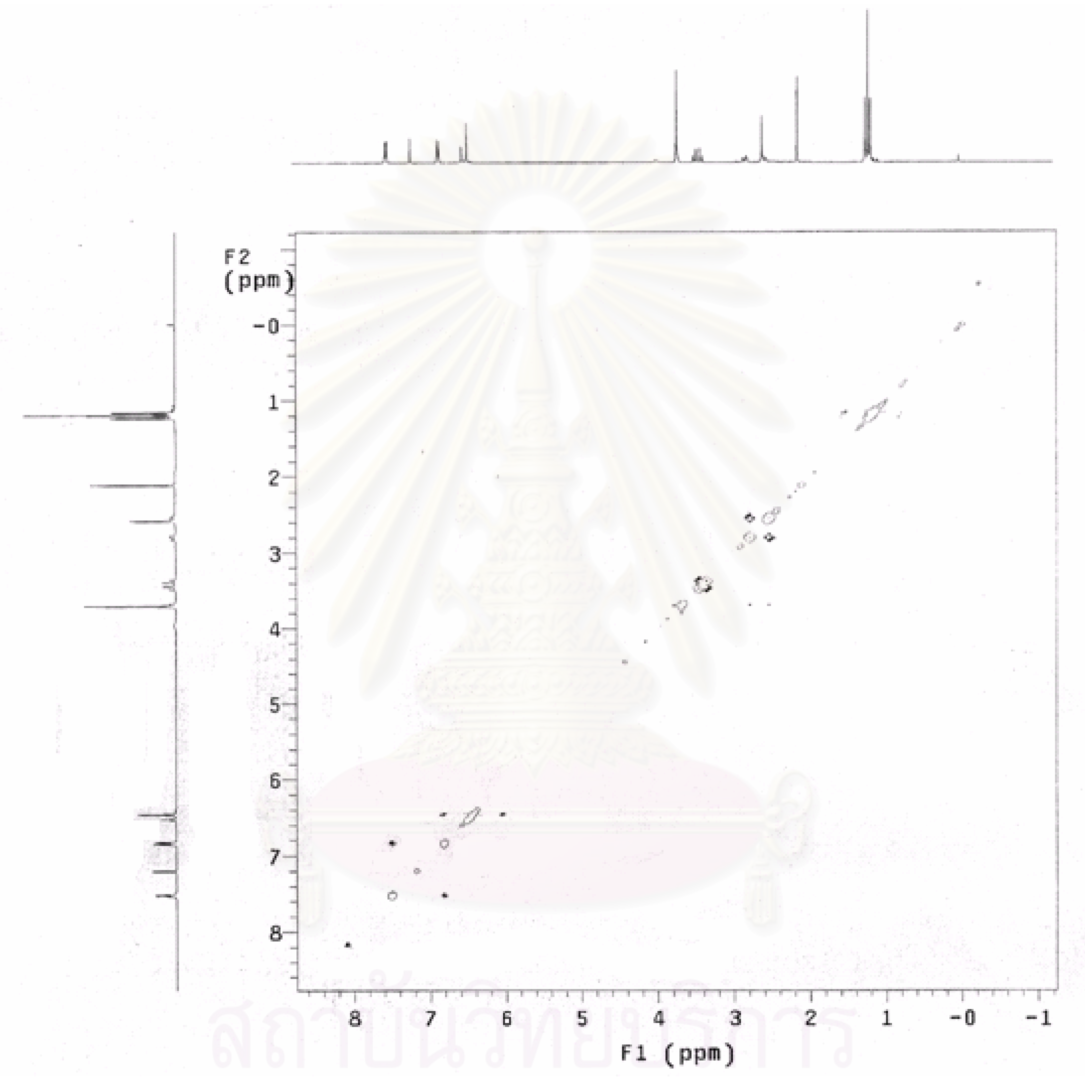

Figure C2.8 NOESY spectrum of Compound 2

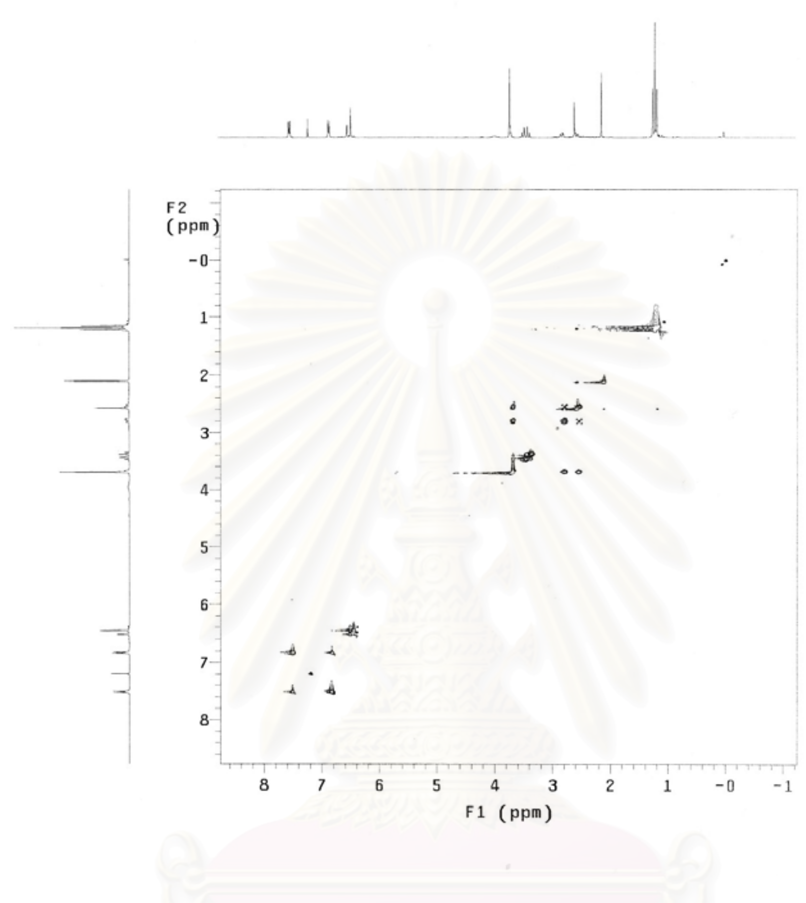

Figure C2.9 TOCSY spectrum of Compound 2

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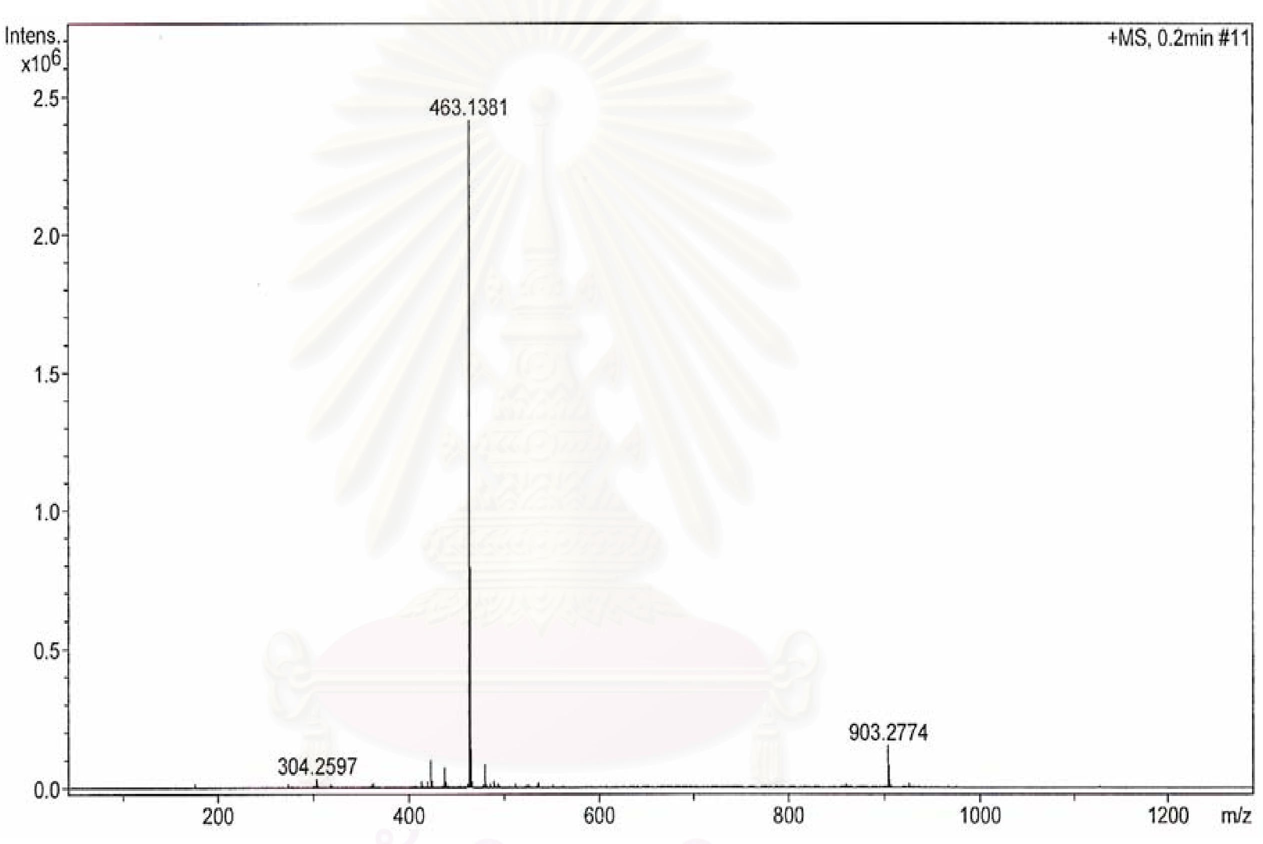

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Figure C 2.10 HR/ES-TOF spectrum of Compound 2

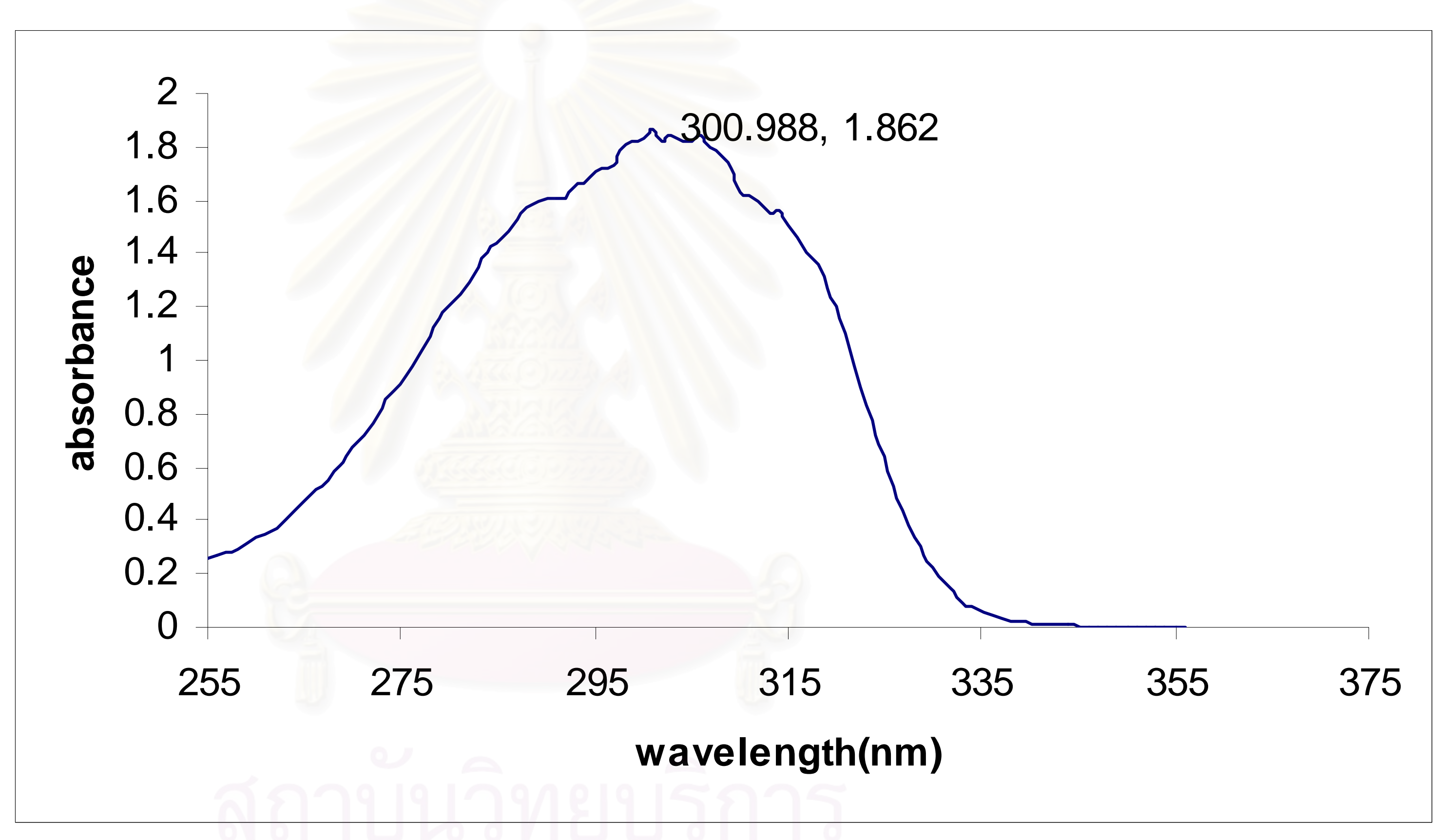

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Figure C3.1 UV spectrum of Compound 3

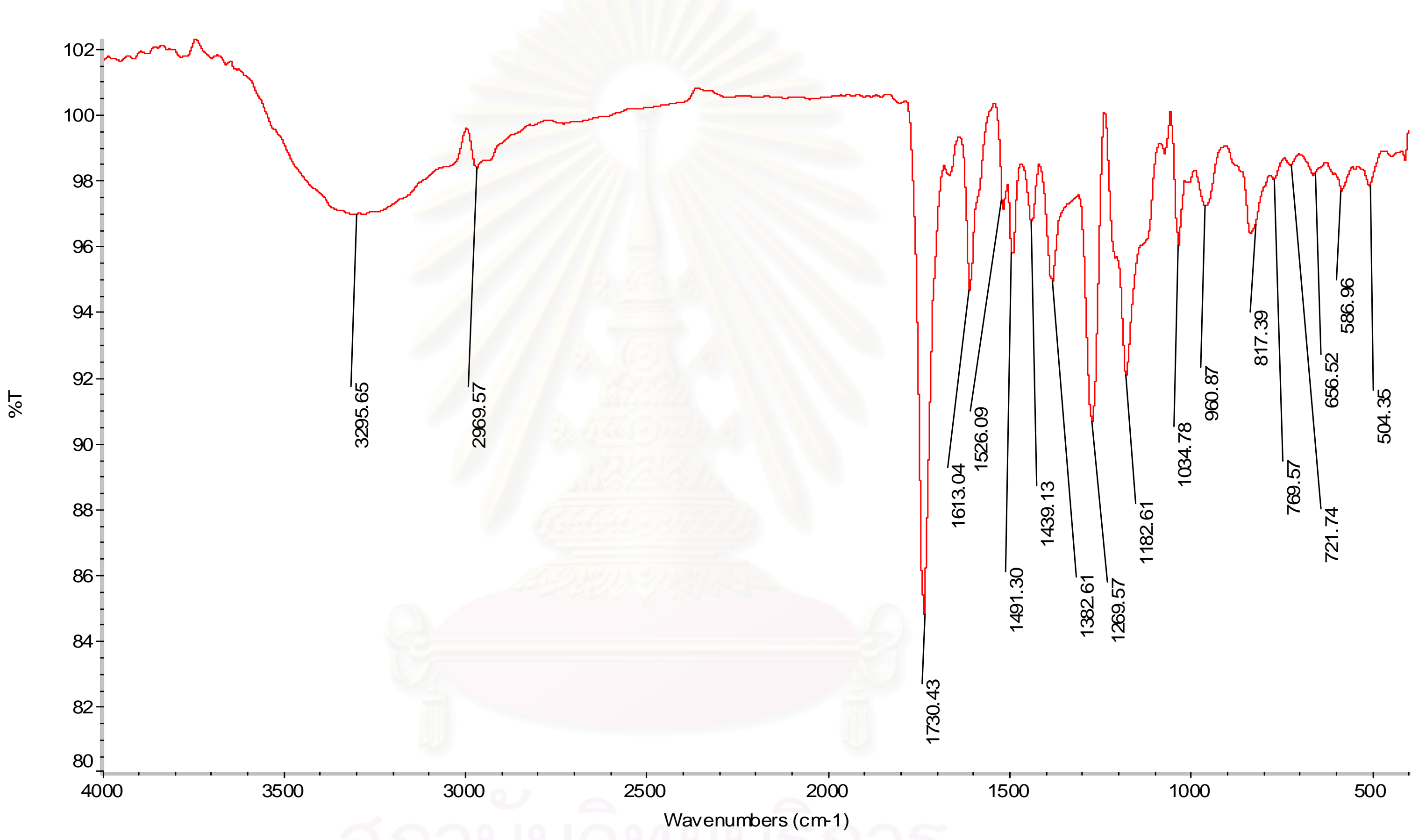

Figure C3.2 IR spectrum of Compound 3

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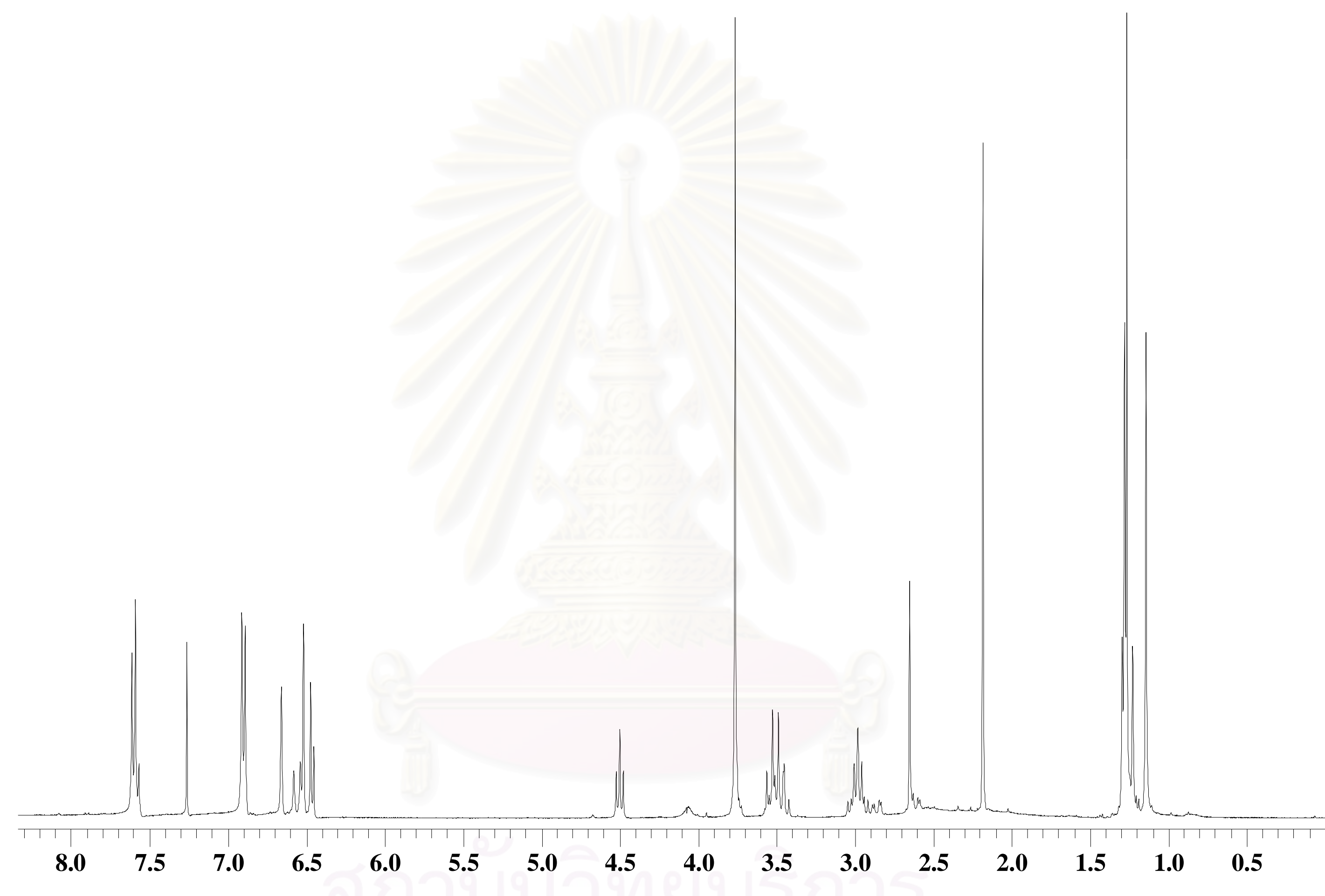

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Figure C3.3 ^{1}H-NMR spectrum of Compound 3

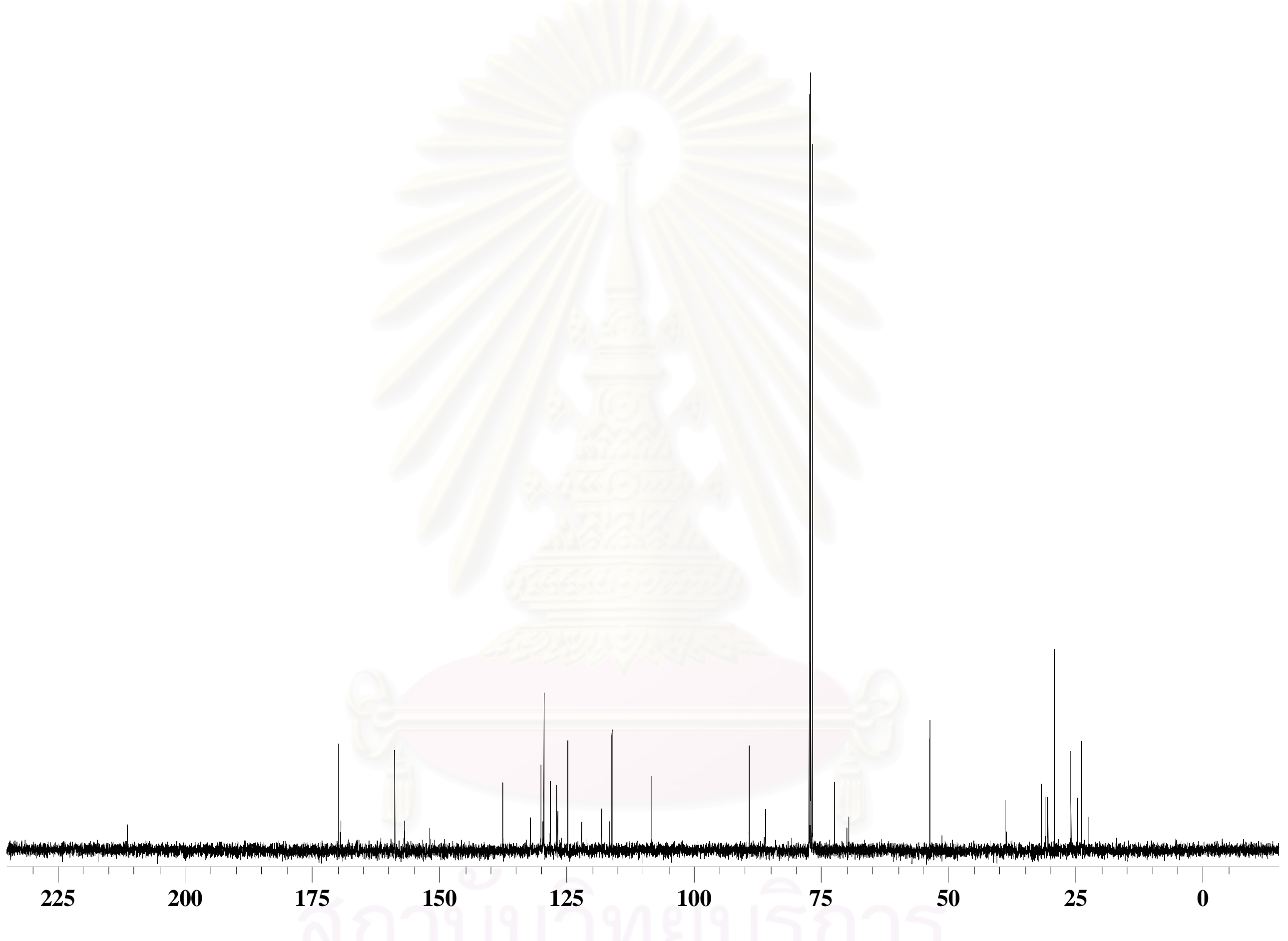

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Figure C3.4 ^{13}C-NMR spectrum of Compound 3

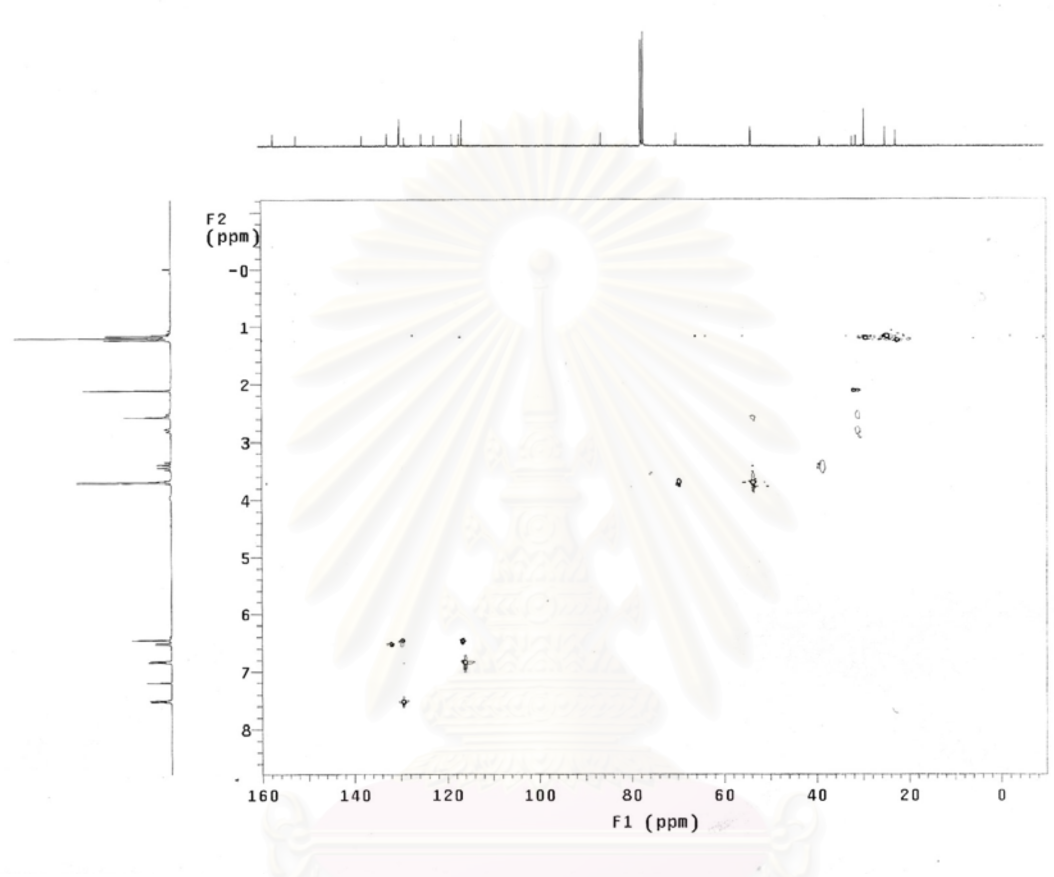

Figure C 3.5 HSQC spectrum of compound 3

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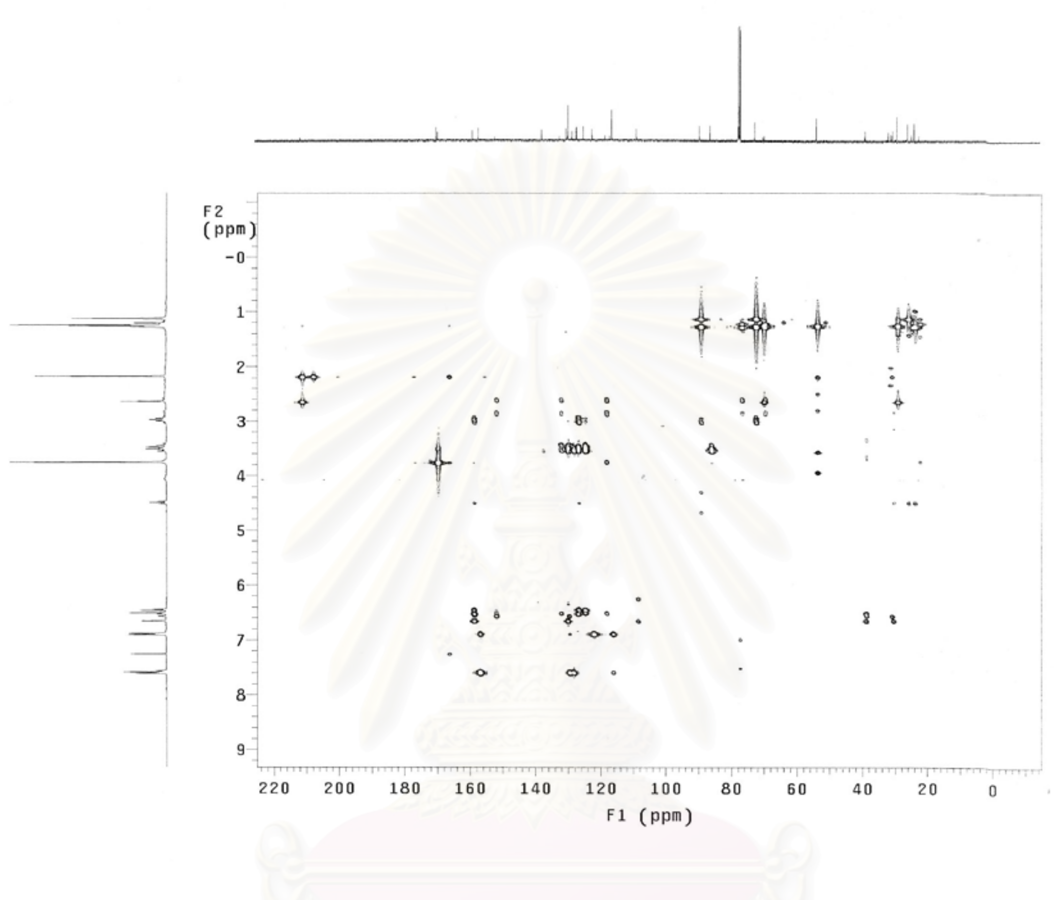

Figure C 3.6 HMBC spectrum of Compound 3

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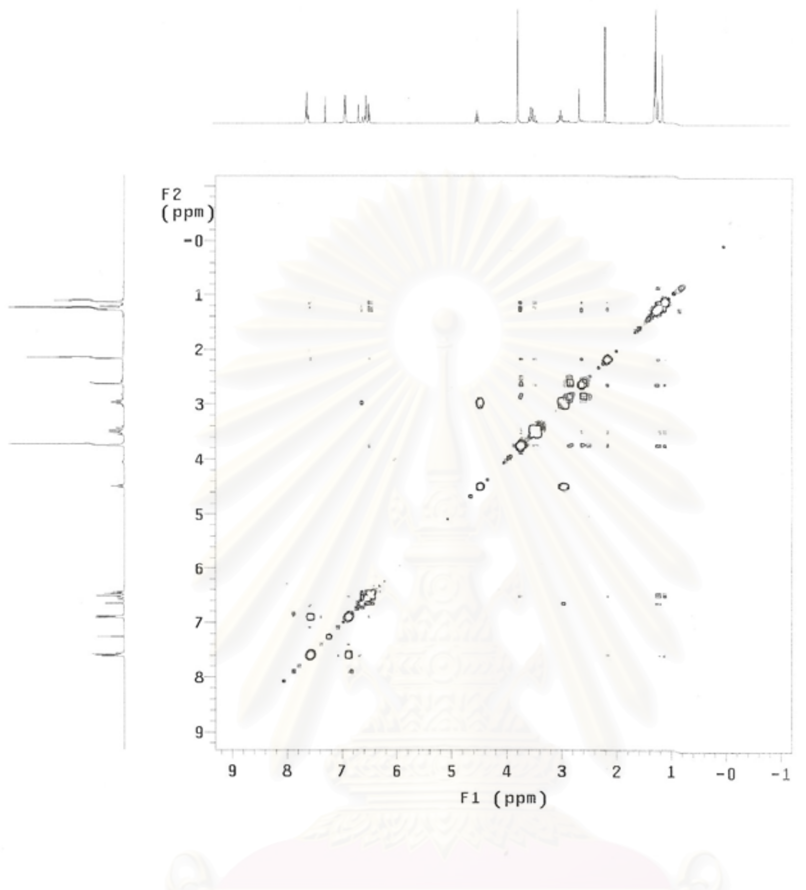

Figure C 3.7 COSY spectrum of Compound 3

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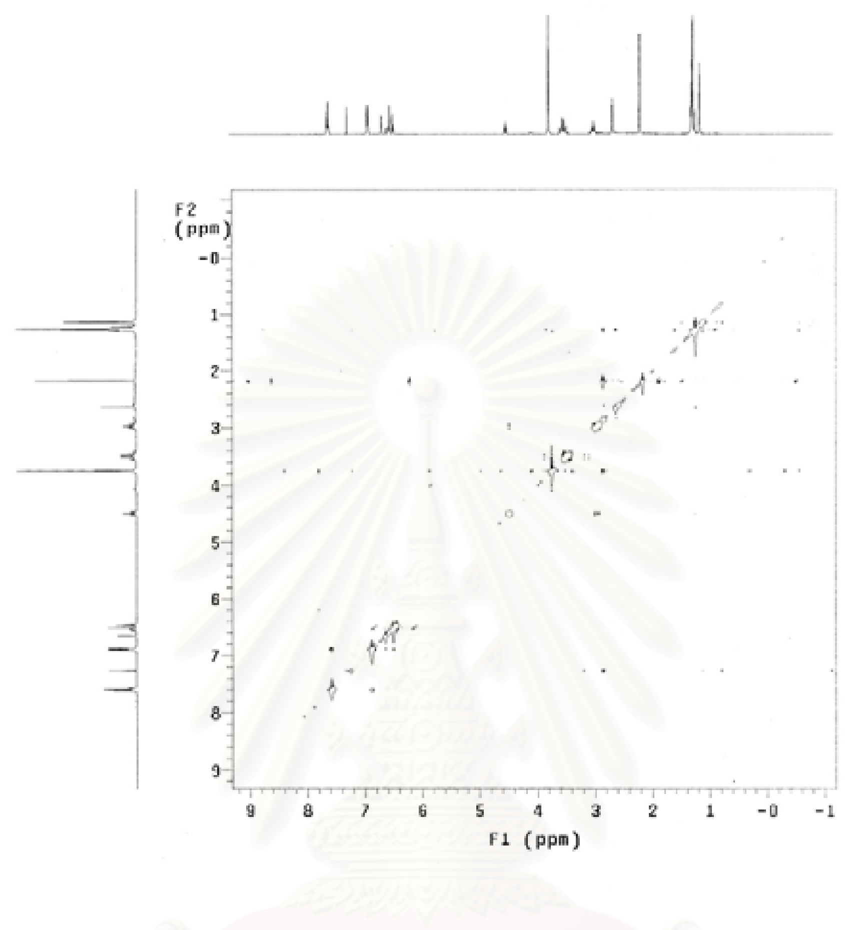

Figure C 3.8 NOESY spectrum of Compound 3

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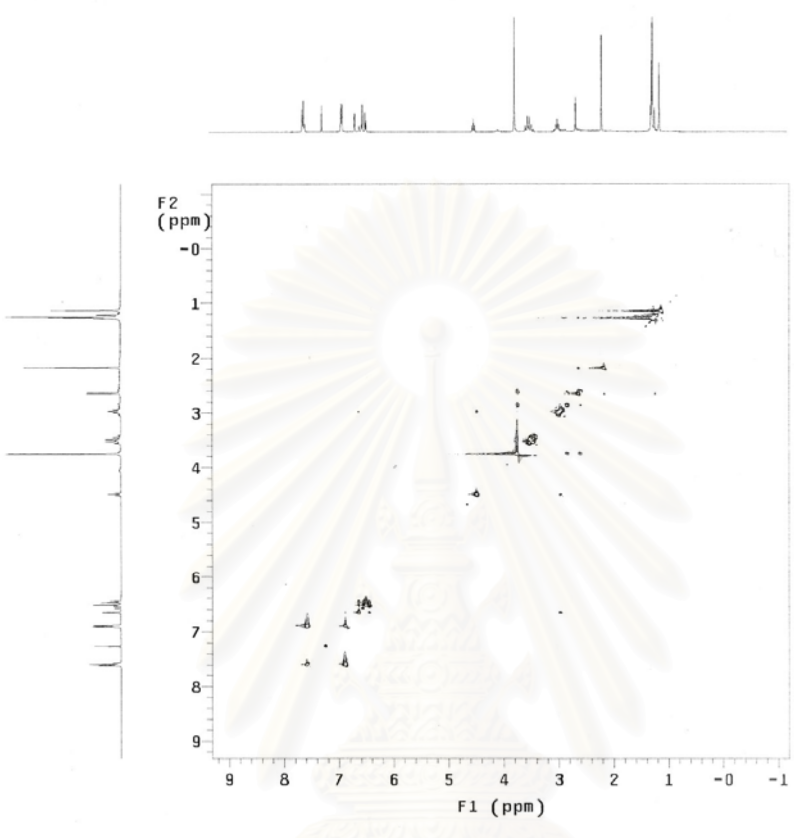

Figure C 3.9 TOCSY spectrum of Compound 3

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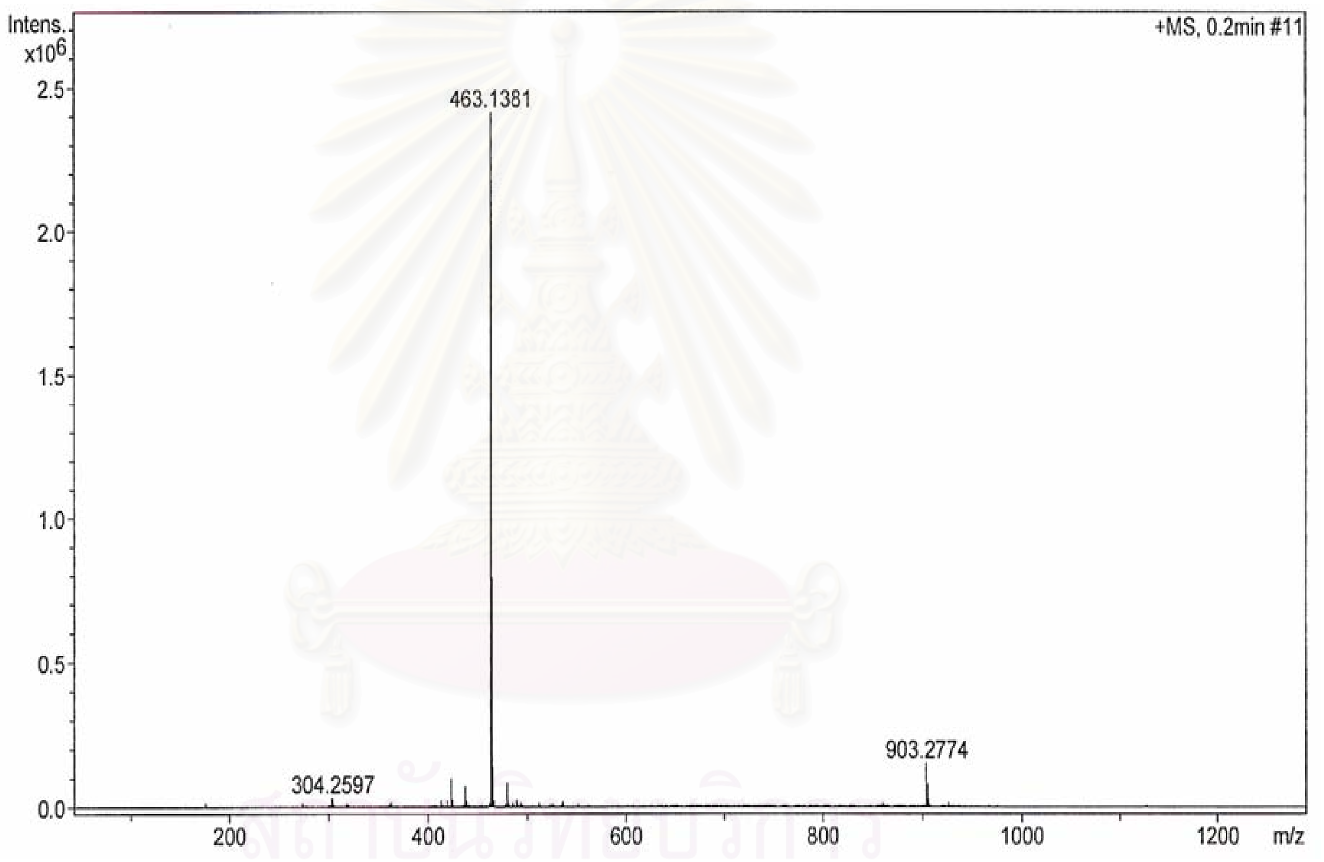

Figure 3.10 HR/ES-TOF MS spectrum of Compound 3

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VITA

Miss Suree Mat-arhin was born in December 15, 1979 in Songkhla province, Thailand. She graduated with Bachelor Degree of Science in Biotechnology Department from the Faculty of Science, Maejo University, Thailand in 2002. She had been studying for a Master degree of science in Biotechnology, Faculty of science, Chulalongkorn University, Thailand since in 2003.

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