สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกจากใบอินทนิลน้ำ
Lagerstroemia speciosa Linn.

นางสาว วรรษยา วิเศษศักดากร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547
ISBN 974-53-1888-4

BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Lagerstroemia speciosa* Linn.

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
Faculty of Science
Chulalongkorn University
Acadamic Year 2004

ISBN 974-53-1888-4

Thesis Title	BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Lagerstroemia speciosa* Linn.
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นางสาววรรษยา วิเศษศักดากร: สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากใบอินทนิลน้ำ *Lagerstroemia speciosa* Linn. (BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Lagerstroemia spesiosa* Linn.) อาจารย์ที่ปรึกษา : ผศ.ดร.พลกฤษณ์ แสงวณิช, อาจารย์ที่ปรึกษาร่วม : รศ.ดร.ประกิตติ์สิน สีหนนทน์ 107 หน้า. ISBN.

งานวิจัยนี้ทำการแยกสารออกฤทธิ์ทางชีวภาพที่สร้างจากราเอนโดไฟต์ที่แยกได้จากใบอินทนิลน้ำ *Lagerstroemia speciosa* Linn. พบว่าราเอนโดไฟท์ไอโซเลต K_BK5 สามารถสร้างสารที่มีฤทธิ์ทางชีวภาพในการยับยั้ง *B. subtilis* การศึกษาลักษณะทางสัณฐานวิทยาพบว่าราเอนโดไฟท์ไอโซเลต K_BK5 ไม่สร้างสปอร์ จึงจัดอยู่ในกลุ่ม Mycelia sterilia จึงทำการจำแนกประเภทโดยการวิเคราะห์ลำดับนิวคลีโอไทด์ ในบริเวณ ITS (internal transcribed spacer) ของ rDNA พบว่ามีความใกล้เคียงทางวิวัฒนาการกับ Fungal endophyte MS6 IS133 ในการศึกษาเพื่อหาสารทุติยภูมิจากราเอนโดไฟท์ไอโซเลต K_BK5 พบว่าเมื่อทำการแยกสารบริสุทธิ์จากน้ำหมักเชื้อและเซลล์ของราเอนโดไฟท์ไอโซเลต K_BK5 ด้วยวิธีทางโครมาโทกราฟีและการตกผลึก พบว่าได้สารบริสุทธิ์ 2 ชนิดและสารผสม 2 ชนิด การพิสูจน์โครงสร้างทางเคมีของสารเหล่านี้โดยอาศัยสมบัติทางกายภาพ ทางเคมี ข้อมูลทางทางสเปกโทสโกปี และการวิเคราะห์ทางเอ็กซ์เรย์ คริสตอลโลกราฟี ร่วมกับการเปรียบเทียบข้อมูลที่มีรายงานมาแล้ว สามารถพิสูจน์โครงสร้างได้ คือ triglyceride (สารผสม 1), deoxyaustrocortirubin (สารผสม 2), austrocortinin (สารบริสุทธิ์ 1) และสารชนิดใหม่ (สารบริสุทธิ์ 2) จากการทดสอบฤทธิ์ต้านจุลินทรีย์พบว่า สารผสม 1 แสดงฤทธิ์ต้านแบคทีเรีย *B. subtilis* ด้วยค่า MIC 250 μg/ml และพบว่า สารผสม 1 และ สารบริสุทธิ์ 1 มีฤทธิ์ต้านอนุมูลอิสระอย่างสูง

ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา....2547..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4572472023 : BIOTECHNOLOGY

KEYWORD : Endophytic fungi / *Croton sublyratus* / *Bipolaris spicifera* / Curvulin / Emodin.

WATSAYA WISETSAKDAKORN : BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Lagerstroemia speciosa* Linn. THESIS ADVISOR : ASSIST PROF. Polkit Sangvanich, Ph.D. ,THESIS COADVISOR : ASSOC.PROF.PRAKITSIN SRIHANONTH,Ph.D 107 pp. ISBN 974-17-6096-5

The purpose of this research was to isolate bioactive compounds from endophytic fungi isolated from *Lagerstroemia speciosa* Linn. leaves. Fungal isolate K_BK5 was selected for bioactive compounds due to this isolate produced active compounds against *Bacillus subtilis* ATCC 6633. Based on morphology, the fungus isolate K_BK5 was identified as Mycelia sterilia. Based on nucleotide sequencing of ITS region, it was closely related to Fungal endophyte MS6 IS133. In this study we investigated for secondary metabotites of fungus isolate K_BK5. Chromatographic techniques and crystallization method were used to purify bioactive compounds from sabouraud's dextrose culture broth and mycelia. Two compounds and two mixtures were isolated and identified. The structures of these compounds were characterized using their physical and chemical properties, spectral data and x-ray crystallographic analysis and comparison with literatures. Three compounds were Triglyceride (mixture 1), deoaustrocortirubin (mixture 2), austrocortinin (compound 1) and unknown (compound 2). The pure compound and mixture were tested for antioxidant activity. Deoaustrocortirubin and austrocortinin showed high potentially of antioxidant activity with EC_{50} 30.17 and 23.91 respectively. Deoaustrocortirubin inhibits *B. subtilis* with the MIC value of 250 μg/ml.

Student's signature……..……

Field of study…...Biotechnology…… Advisor's signature……..…

Academic year….2004…… Co-advisor's signature…….……

ACKNOWLEDGEMENTS

I wishes to express my gratest appreciation to my thesis advisor, Assistant Professor Dr. Polkit Sangwanich, and my thesis coadvisor, Associate Professor Dr. Prakitsin Sihanonth for their excellent instruction, valuable advice, guidance, encouragement and support throughout this dissertation.

I am particulary grateful to chairman of thesis committee, Professor Dr. Sophon Roengsumran, Associate Professor Dr. Amorn Petsom, and Assistant Professor Dr. Nattaya Ngamrojnavanich as committee and for their guidance, editorial assistance and comments.

The special thanks to Assistant Professor Dr. Surachai Ponpakakul for guidance, helpful discussion and valuable suggestions throughout the research study.

I wishes to express my sincere gratitude and thanks to Professor A.J.S. Whalley at Liverpool John Moores University, United Kingdom, , Dr.Surang Thienhiran at Royal Forest Department, and Mr. Ek Sangvichien at Ramkhamhaeng University, Associate Professor Dr. Wichai Rerdchewasart at Department of Biology, Chulalongkorn University, for their constant help, and advice for my research.

I would like to thank Dr. Jittra Karnchanaprayudh at Department of Botany, Chulalongkorn University, for her help in DNA sequencing and suggestion for fungi characterization.

I would like to thank Miss Somjintana Taveepanich, Mr. Jatupol Liangsakul, Mr. Sophon Kaeothip and members of Research Center for Bioorganic Chemistry (RCBC) for their advice, encouragement, helpfulness and kind friendship.

I also wishs to express my sincere appreciation to The Programe of Biotechnology, and Department of Chemistry, Faculty of Science, Chulalongkorn university for providing facilities during my study. The financial support to conduct this research from the Graduate School of Chulalongkorn University is also gratefully acknowledged.

Finally, I wishes to express my gratest gratitude is expressed to my family and especially my parent and my sisters for their love, understand, moral support, encouragement and continuing support throughout this graduate study.

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LIST OF SCHEME

LIST OF ABBREVIATIONS

$[\alpha]_D^{20}$	= Specific rotation at 20° and Sodium D line (589 nm)
ATCC	= American Type Culture Collection, Maryland, U.S.A
br s	= broad singlet (for NMR spectral data)
°C	= degree Celsius
^{13}C-NMR	= carbon-13 nuclear magnetic resonance
$CDCl_3$	= deuterated chloroform
$CHCl_3$	= chloroform
CD_3OD	= deuterated methanol
cm	= centimeter
COSY	= ^{1}H-^{1}H correlation spectroscopy
CFU	= Colony forming unit
δ	= chemical shift
d	= doublet (for NMR spectral data)
dd	= doublet of doublet (for NMR spectral data)
dt	= doublet of triplets (for NMR spectral data)
ε	= molar absorptivity
EIMS	= electron impact mass spectroscopy
Eq	= equatorial
EtOAc	= ethyl acetate
g	= gram
HMBC	= ^{1}H-^{13}C hetronuclear corration
HMQC	= ^{1}H- detected heteronuclear multiple quantum coherence
^{1}H-NMR	= proton nuclear magnetic resonance
Hz	= hertz
IR	= infrared spectroscopy
l	= liter
μl	= misrelate

LIST OF ABBREVIATIONS (CONTINUED)

λ_{max}	= wavelength of maximum absorption
$[M+H]^+$	= protonated molecular ion
m	= multiplet (for NMR spectral data)
MEA	= Malt extract agar
MHB	= Mueller- Hinton broth
MeOH	= methanol
MIC	= Minimum inhibitory concentration
mg	= milligram
μg	= microgram
MHz	= megahertz
ml	= milliliter
mm	= millimeter
ν_{max}	= wave number at maximum absorption
NMR	= nuclear magnetic resonance
No.	= Number
ppm	= part per million
s	= singlet (for NMR spectral data)
SEM	= scanning electron microscope
t	= triplet (for NMR spectral data)
TLC	= thin layer chromatography
UV	= Ultraviolet

CHAPTER I

INTRODUCTION

Fungi play important role in biodiversity because they have world wide distribution and successfully exploit many different habitats (Hawksworth, 1991). Endophytic microorganisms are to be found in virtually every plant on earth. Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas (Strobel et. al., 2004).

Endophytic fungi are organism which live almost entirely within the leaves and stems of apparently healthy host plants, doing so asymptomatically, causing no visible signs of infection (Isaac, 1992). They commonly live in intercellular spaces of living plant hosts. They may provide their hosts with metabolites and other potentially useful bioactive compounds. Endophytic fungi, are recognized as one of the most chemically promising groups of fungi in terms of diversity, pharmaceutical, and agricultural potential (Dreyfuss and Chapela, 1994, Strobel and Long, 1998).

Since endosymbiotic microbes must benefit biochemically with host tissues to obtain nutrients, overcome host defenses, and defend host tissues, it is likely that many endophytes produced secondary metabolites to perform key roles related to the survival of the microbe and symbiotic unit. Thus, endophytic habitat is a niche that has been continued exploration (Bacon et. al., 2000). Endophytic fungi had been known as the important resource for novel metabolites to antibacterial, antifungal, and cytotoxicity activities (Tan and Zou, 2000). Application of endophytes included potential biological control agents, sources of novel metabolites for medicine, plant protection, and

industrial uses, and as research model systems for investigation of host parasite interactions and evolution in natural systems (Bacon et. al., 2000).

At present, It is increasing concern about health problem with antibiotic drug resistance on the rise and unusual microbial threats emerging, the need for more effective antimicrobial agents is increasing. In addition, increasing food production with the world population expanding rapidly, but severe plants loss due to plant diseases. Which application of the synthetic fungicides may take long timelines to be degraded completely causing heavy toxicity to human, animals and environment. The diversity, biological activity and secondary metabolite production of fungal endophyte with host plants should be investigated to discovery new biologically active secondary metabolites for a goal of both pharmaceutical and agrochemical industries. It's needed to develop and search for new drugs with high potentially compounds from natural bioresources. In the last decade, the interest for endophytic fungi as potential source of novel bioactive compound has increased.

The forests of the world are sources of these microbial endophytes. Thailand locate in a tropical rainforest region with many varieties of plants that may support endophytes. In this research, *Lagerstroemia speciosa* Linn. leaf was employed as a plant source of fungi because *Lagerstroemia speciosa* Linn. is used in local Thai herbal medicine used for diabetes. *Lagerstroemia speciosa* Linn. Leaf have been exported to reduce diabetic symptoms in genetically diabetic mice (Type II, KK-Ay) (Shizuoka, 1999). Consequently, the endophytic fungi isolated from *Lagerstroemia speciosa* Linn. leaf may also produce potentially bioactive compounds.

Objectives

1.To isolate the endophytic fungi from healthy mature leaves of *Lagerstroemia speciosa* Linn.

2.To determine biological activities of the extracts from endophytic fungal cultures, by microbiological activity test and thin layer chromatographic method.

3.To identify a selected endophytic fungal isolate by using classification based on morphology and nucleotide sequence of ITS regions of rDNA.

4.To isolate and characterize the bioactive compounds from a selected endophytic fungal isolate.

5.To elucidate the structural formula of the isolated bioactive compounds.

6.To evaluate the biological activity of the bioactive compounds obtained.

CHAPTER II

LITERATURES REVIEW

2.1 Endophytic fungi

Endophytic microbes are an intriguing group of organisms associated with various tissues and organs of terrestrial and some aquatic plants, and are the subject of increasing interest to mycologists, ecologists, and plant pathologists. In general , endophytic infections are inconspicuous, the infect host tissues are at least transiently symptom less (Stone, Bacon, and White, 2000). However, the possibility that they may become pathogenic when the host is stressed (Stone, 1990; Carroll, 1988).

Fungal endophytes are fungi that invade and reside within host tissues or cells and cause unapparent and asymptomatic infection for all or part of their life cycle (Wilson, 1995). Fungal endophytes are form internal localized infections in either intercellular or intracellular space of usually above-ground plant tissues such as leaves, stem and bark (Suske and Acker, 1989), but also occasionally in roots, and are distinguished from mycorhizae by lacking external hyphae or mantels. Most of these endophytes are horizontally transmitted via spores, a much smaller fraction, mostly found in grasses, form systemic infections in above-ground tissue. Some of these are vertically transmitted via hyphae growing into seeds (Saikkonen et al., 1998)

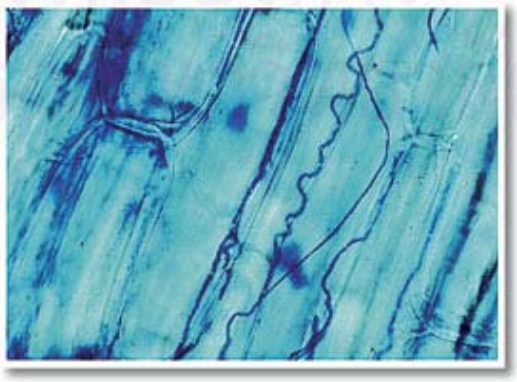


Figure2.1 Microscopic picture of tall fescue leaf sheath cells with endophyte mycelium (twisted, corkscrew structure) growing between cells.

In the past few decades, endophytic fungi have attracted great attention for five main reasons.

First, Infections of land plants by endophytes are ubiquitous, having been found throughout a broad range of host orders, families, and genera worldwide, are extremely abundant and representing a very diverse array of terrestrial and a aquatic habitats. Although, endophytic fungi have been found in almost plant groups, however, fungal endophytes of grasses (Poaceae) and sedges (Cyperaceae and Juncaceae) are probably the most extensive studied group (Clay, 1988). Report on the presence of endopytes in vascular plants, other than grasses, have focused mainly on ericaceous, dicotyledonous plants and conifers (Rodrigues, 1996).

Second, endophytes may produce mycotoxins, or otherwise also alter other physiological, developmental or morphological properties of host plants such that competitive abilities are enhanced, especially in stressful environments (Clay et.al., 1988, 1990; Bacon, 1993; Malinowski and Belesky, 1999).

Third, It seems obvious that endophytes are a rich and reliable source of genetic diversity and may represent previously undescribed species. Finally, novel microbes (as defined at the morphological and or molecular levels) often have associated with them novel natural products. This fact alone helps eliminate the problems of dereplication in compound discovery (Strobel et al., 2004). Systemic grass endophytes are restricted to clavicipitaceous members of the Balansiae (Ascomycota). Taxonomically, endophtyes from woody plants are usually member of the Ascomycota but may also include member of the Basidiomycota, Deuteromycota, and Zygomycota (Leuchtmann, 1992; Petrini, 1986; Sinclair and Cerkauskas, 1996).

Fouth, The Promise of Bioactive Compounds from Endophytes. Needs for New Natural Products. There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and will have a minor environmental impact, respectively. This search is driven by the development of resistance in infectious microorganisms (e.g., *Staphylococcus*, *Mycobacterium*, *Streptococcus*) to existing drugs and by the menacing presence of naturally resistant

organisms. The ingress to the human population of new disease-causing agents such as AIDS, Ebola, and SARS requires the discovery and development of new drugs to combat them. Not only do diseases such as AIDS require drugs that target them specifically, but new therapies are needed for treating ancillary infections which are a consequence of a weakened immune system. Furthermore, others who are immunocompromised (e.g., cancer and organ transplant patients) are at risk of infection by opportunistic pathogens, such as *Aspergillus*, *Cryptococcus*, and *Candida*, that normally are not major problems in the human population. Finally, because of safety and environmental problems, many synthetic agricultural agents have been and currently are being targeted for removal from the market, which creates a need to find alternative ways to control farm pests and pathogens. Novel natural products and the organisms that make them offer opportunities for innovation in drug and agrochemical discovery. Exciting possibilities exist for those who are willing to venture into the wild and unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes, their biology, and potential usefulness (Strobel et al., 2004).

Fifth, Tan and Zou believe the reason some endophytes produce certain photochemical, originally characteristic of the host, might be related to a genetic recombination of the endophyte with the host that occurred in evolutionary time. This is a concept that was originally proposed as a mechanism to explain why *T. andreanae* may be producing taxol. Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price (Tan and Zou, 2000).

2.2 Association of endophytic fungi and plant

Endophytic microorganisms are to be found in virtually every plant on earth. Symbiotic associations between microorganisms and plants are ancient and fundamental, and many examples of complex and highly specific symbioses between plants and microbes have been described. Results of their interaction were increase capacity of a plant to resist disease and increase survival of plant from natural environment by producing bioactive compounds of plant growth promoting, antibacterial, antifungal and insecticidal to enhance the plant growth (Strobel and Long, 1998).

Endophytic fungi colonize living plant tissues by penetration of fungal hyphae between plants cells or may also grow intracellular and must obtain nutrient materials through this intimate contact with the host. (Isaac, 1992). Endophytic fungi are asymptomatic and may be described as mutualistic (Clay, 1999). The major features of mutualistic symbiosis include the lack of destruction of most cells or tissues, nutrient or chemical cycling between the fungus and host, enhanced longevity and photosynthetic capacity of cells and tissues under the influence of Infection, enhanced survival of the fungus, and a tendency toward greater host specificity than seen in nectrophic infection (Lewis,1973). A comparison of the fitness of the host and fungus when living independently in contrast to their fitness when living in association is the major mean determining whether a specific symbiotic association is mutualistic or parasitic (Lewis,1974).

By definition, endophytic colonization or infection cannot be considered as causing disease, since a plant disease is an interaction between the host, parasite, vector and the environment over time, which results in the production of disease signs and/or symptoms. The distinction between endophyte and a pathogen is not always clear. A mutation at a single genetic locus can change a pathogen to nonpathogenic endophytic organism with no effect on host specificity (Freeman and Rogriguez,1993). Many pathogens undergo an extensive phase of asymptomatic growth corresponding to

colonization and then latent infection before symptoms appear. Many pathogens of economically important crops many be endophytic or latent in weeds (Raid and Pennypacker,1987). Alternately, nonpathogenic endophytic organisms may play a role as biocontrol agents(Freeman and Rogriguez,1993). Both endophytic and latent infection fungi can infect plant tissues and become established after penetration. However, infection dose not imply the production of visible disease symptoms (Redin and Carris,1996).

Figure 2.2 The five interactive components that can be involved in a symptomatic plant disease. (Redin and Carris,1996).

2.3 Interaction between endophytic fungi and plants

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

In physiological terms relatively little is known about the endophytic interactions between host and fungus and it is not easy to see how a host plant may benefit from such a relationship. Endophytic associations do not lead to the development of disease symptoms but do result in some morphological and physiological changes in host

tissues which increase the survival and vigor of the plants concerned. Such physiological enhancement would be likely to increase the capacity of a plant to resist disease. It has also been suggested that endophyte-infected plants are more tolerant of water stress and recover more quickly than uninfected individuals (Belesky *et al*., 1987), although it is not clear quite how photosynthetic rates are affected by the presence of endophytes (Clay, 1990). There are suggestions that endophytes may produce plant growth regulators, which may alter the normal developmental pattern of the host plant (Porter *et al*., 1985). Reports of secondary metabolite production by endophytes, e.g. alkaloids and antibiotics, which affect a range of herbivores, have attracted a great deal of attention. It has also been suggested that endophytes provide the plant with a chemical defense mechanism (Isaac, 1992).

Mutualism has been the prevailing conceptual framework under which the evolution and ecology of endophytes have been viewed and interpreted. Discovery of severe biological effects of endophytes in grasses on livestock, such as toxicosis and hoof gangrene (Clay, 1988, 1990; Ball, Pedersen, and Lacefield, 1993), and on of invertebrate pest species (Clement et al., 1996, 1997; Bultman and Murphy, 1998) led to the concept of grass endophytes as plant mutualisms, primarily by deterring herbivores as “acquired defenses” (Cheplick and Clay, 1988). Defense against vertebrate and invertebrate herbivores, and also against plant pathogens, purportedly results from production of multiple alkaloid compounds by endophytes, at least in agronomic grasses (Siegel et al., 1990; Siegel and Bush, 1996, 1997). In turn, host plants provide endophytic fungi with a protective refuge, nutrients, and --in the case of vertically transmitted endophyte--dissemination to the next generation of hosts. Other studies of agronomic grasses show that endophytes provide other fitness-enhancing properties for their hosts, such as increasing plant competitive abilities, many by increasing efficiency of water use (Bacon, 1993; Elmi and West, 1995). Furthermore, because alkaloids from endophytes are often concentrated in seed, vertically transmitted grass endophytes also may deter seed predators and increase seed dispersal (Wolock-Madej and Clay, 1991; Knoch, Faeth, and Arnott, 1993).

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

Furthermore, little information exists relative to the biochemistry and physiology of the interactions of the endophyte with its host plant. It would seem that many factors changing in the host as related to the season and other factors including age, environment, and location may influence the biology of the endophyte. All of these interactions are probably chemically mediated for some purpose in nature. An ecological awareness of the role these organisms play in nature will provide the best clues for targeting particular types of endophytic bioactivity with the greatest potential for bioprospecting (Strobel et al., 2004).

2.4 Endophytes and Biodiversity

Of the myriad of ecosystems on earth, those having the greatest general biodiversity of life seem to be the ones also having the greatest number and most diverse endophytes. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface; yet, they harbor over 60% of the world's terrestrial biodiversity. In addition, each of the 20-25 areas identified as supporting the world's greatest biodiversity also supports unusually high levels of plant endemism. As such, one would expect with high plant endemism there also should exist specific endophytes that may have evolved with the endemic plant species. Biological diversity implies chemical diversity because of the constant chemical innovation that is required to survive in ecosystems where the evolutionary race to survive is most active. Tropical rainforests

are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds. Bills et al. describe a metabolic distinction between tropical and temperate endophytes through statistical data that compare the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other substrata, as illustrated by the discovery of cyclosporin A. This observation suggests the importance of the host plant as well as the ecosystem in influencing the general metabolism of endophytic microbes. (Strobel et al., 2004)

2.5 Host specificity

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

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

2.6 Botanical Aspects of L*agerstroemia speciosa* Linn.

2.6.1 Description

Common name: Queen's flower, pride of India, queen's crape myrtle, banana.

Family: Lythraceae.

Height : 40 to 60 feet

Spread : 30 to 40 feet

Crown uniformity : symmetrical canopy with a regular (or smooth) outline, and individuals have more or less identical crown forms.

Crown shape : round ; upright ; vase shape

Crown density : moderate

Growth rate : fast

Texture : medium

Foliage

Leaf arrangement : opposite / sub opposite

Leaf type : simple

Leaf margin : entire

Leaf shape : elliptic (oval) ; oblong ; obviate

Leaf venation : branchidodrome ; pinnate

Leaf type and persistence : semi evergreen

Leaf color : green

Leaf fall color : red

Flower

Flower color : lavender ; pink ; purple ; purplish - pink

Flower characteristics : summer flowering ; very showy

Fruit

Fruit shape : oval ; round , about one inch long and splits in six pieces when mature.

Fruit length : <5 inches

Fruit covering : dry or hard

Fruit color : brown

Fruit characteristics : does not attract wildlife ; inconspicuous and showy ; no significant litter problem ; persistent on the tree

Seed : small and have winged flaps.

Trunk and Branches

Trunk / bark / branches : bark is thin and easily damaged from mechanical impact ; droop as the tree grows, and will require pruning for vehicular or pedestrian clearance beneath the canopy ; routinely grown with, or trainable to be grown with several trunks but can be trained to grown with a single trunk no thorns.

Current year twig color : brown ; green

Current year twig thickness : medium ; thin

Occur area : south east Asian areas including the Philippines, India, Malaysia, southern China and Australia.

In Thailand, it is commonly known as In-ta-nin-nam. In the Philippines in particular, dry leaves and flowers of Lagerstroemia Speciosa, Linn. or Pers. are decocted and taken as a drink. This drink is also well known as a folk medicine against diabetes

(Suzuki et al., 1999).

2.6.2 Traditional uses

- roots are used for stomach problems.
- *Lagerstroemia speciosa* leaves as an aid in lowering blood glucose. Findings in this scientific area were corroborated by in vitro studies on enhancing glucose transporter by corosolic acid (Murakami et al., 1993), and recently lagerstroemin, flosin B and reginin A (Hayashi et al., 2002), and on inhibiting the digestive enzyme by polyphenolic substances (Suzuki et al., 1999). The hypoglycemic effect of *Lagerstroemia speciosa* has been evaluated with significant results genetically in diabetes KK-Ay mice (Kakuda et al., 1996) and human subjects (Judy et al., 2003). Xanthine oxidase XOD inhibitor for the prevention and treatment of hyperuricemia.

- Bark and leaves were used for treatment of dysentery, diarrhea, blood in urine, diabetes and malaria.
- The reddish brownwood was used for home building, furniture, boots, etc.

(A)

(B) (C)

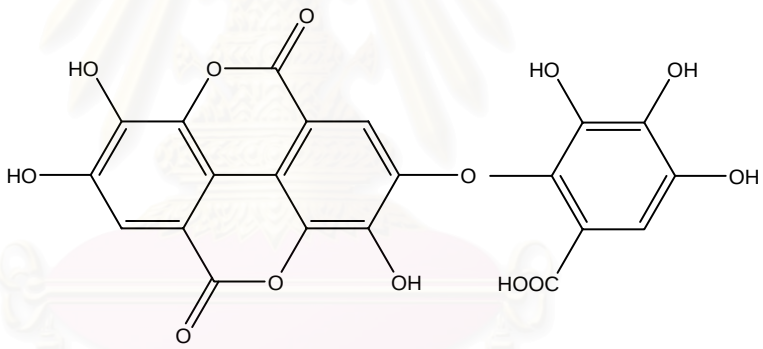
Figure 2.3 A) Leaf of *Lagerstroemia speciosa* Linn.

B) Fruits of *Lagerstroemia speciosa* Linn.

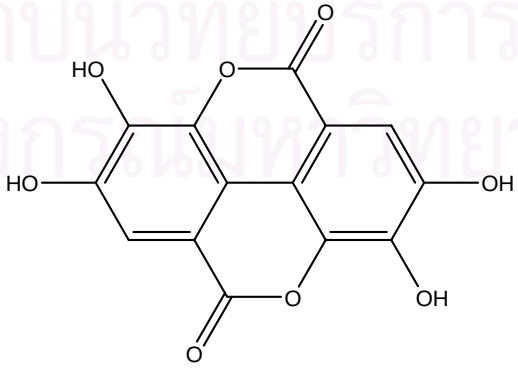
C) Flowers of *Lagerstroemia speciosa* Linn.

2.6.3 Chemical constituents

Photochemical examination of *Lagerstroemia speciosa* Linn. leaves gives triterpenoid : corosolic acid, maslinic acid (Judy et al., 2003) and three active ellagitannins : lagerstroemin, flosin B and reginin A (Hayashi et al., 2002) and tannin : tannic acid and polyphenolic substances : valoneic acid dilactone (VAD) and ellagic acid (EA) (Unno et al., 2004). Terpenoids and sterols : a new natural product, 31-norlargerenol acetate , along with known compounds 24-methylenecycloartanol acetate, cycloeucalenol acetate, the diterpenes tinotufolins C and D, lutein, phytol, sitosterol, and sitosterol acetate (Ragasa et al., 2005). Keto fatty acid (9-ketooctadec-cis-11-enoic acid) were found from oil of seeds(Chirag et al., 1990). Structures of some compounds from *Lagerstroemia speciosa* Linn. are shown in Figure 2.5.

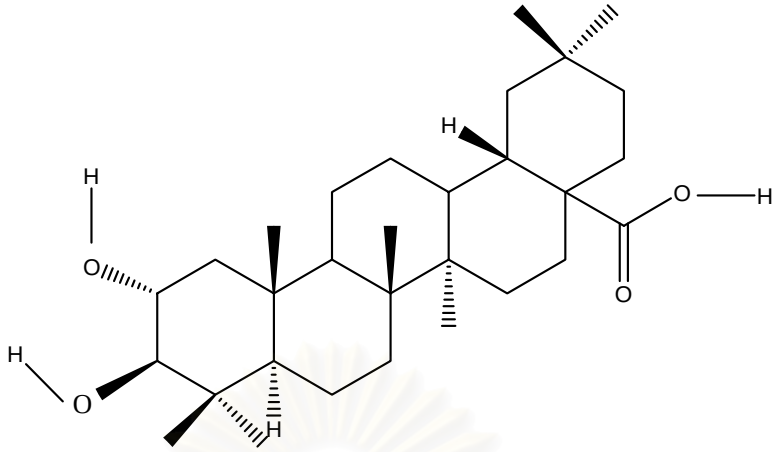


Valoneic acid dilactone (VAD)

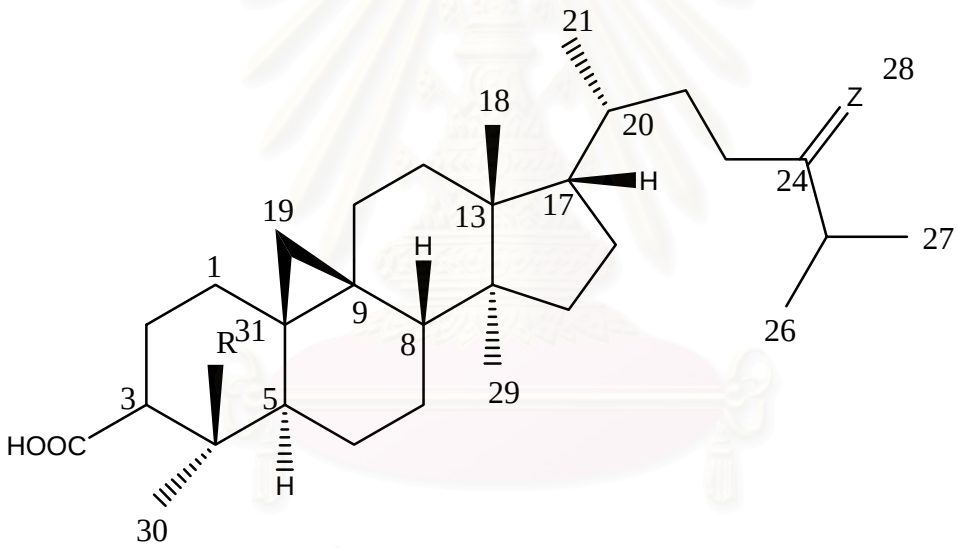


Ellagic acid (EA)

Figure 2.4 Structure of some compounds from *Lagerstroemia speciosa* Linn.



Maslinic acid



1 R = CH_3 , Z = CH_2

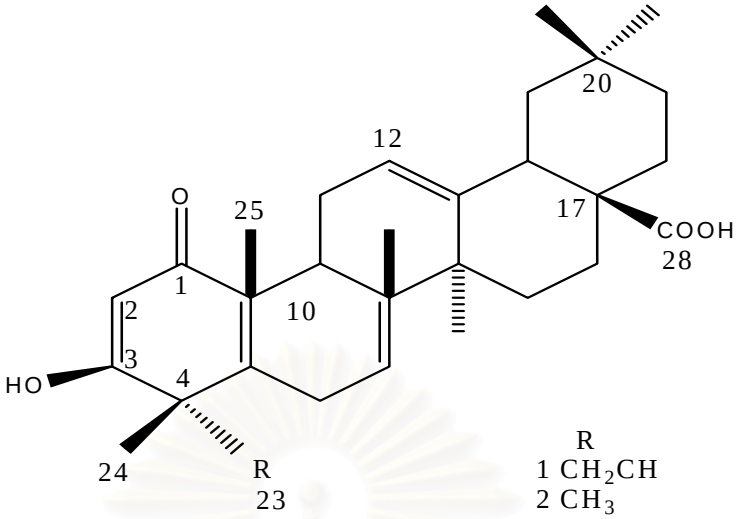
2 R = H , Z = CH_2

3 R = CH_3 , Z = O

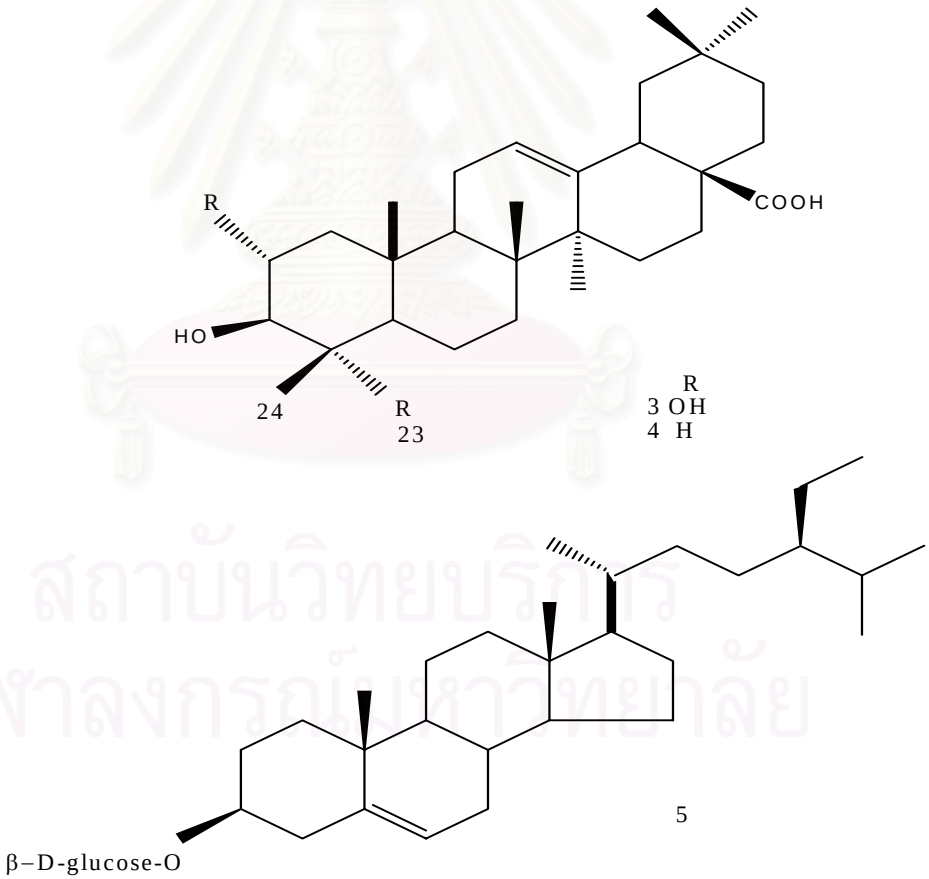
4 R = H , Z = O

24-Methylenecycloartanol acetate (1), Cycloeucalenol acetate (2), Largerenol acetate (3), 31-Norlargerenol acetate (4)

Figure 2.4 Structure of some compounds from *Lagerstroemia speciosa* Linn.(continued)

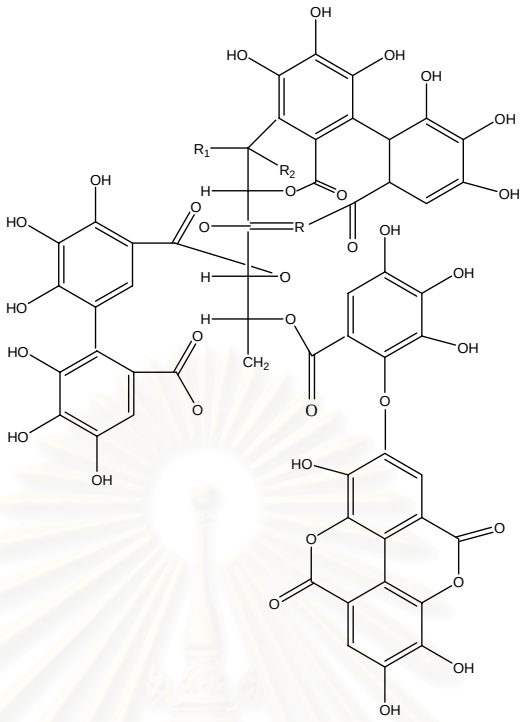


Ursan (1), Virgatic acid (2)



Colosolic acid (3), Ursolic acid (4), and b-Sitosterol glucoside (5)

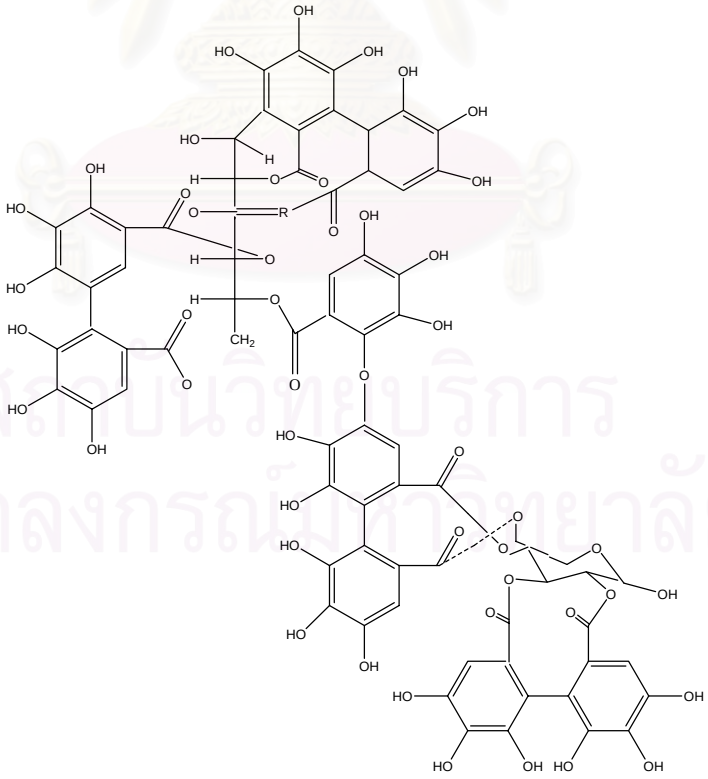
Figure 2.4 Structure of some compounds from *Lagerstroemia speciosa* Linn.(continued)



1 R_1 = OH, R_2 = H

2 R_1 = H, R_2 = OH

Lagerstroemin (1), Flosin B(2)



Reginin A

Figure 2.4 Structure of some compounds from *Lagerstroemia speciosa* Linn.(continued)

2.7 Study of secondary metabolites from endophytic fungi

Microorganisms, in particular fungi, are important sources of secondary metabolites. Fungal secondary metabolites are a diverse group of compounds produced by a wide range of different fungi. Endophytic fungi are a potent source of novel chemistry and biology to assistive helping solve not only human health, but plant and animal health problem as well. Endophytic fungi reside in the tissue between living plant cells. The relationship that established with the plant varies from symbiosis borderline on pathogenic. As a result, the opportunity to find new and interesting endophytic fungi among the myriad of plants is great. Sometimes extremely unusual and valuable organic substances are produced by these endophytic fungi (Tan and Zou, 2000).

The fungi provide us with an enormous variety of strange and wonderful 'secondary metabolites', some of which have profound biological activities that we can exploit. Secondary metabolites are those that are not essential for vegetative growth in pure culture. Secondary metabolism occurs as growth rate declines and during the stationary phase, and often is associated with differentiation and sporulation (Carlile *et al*., 2001).

Recently, fungal endophyte research has focused on screening of secondary metabolites that exhibit interesting biologically activities compounds such as antibacterial, antifungal, antiviral, algicidal, herbicidal, insecticidal, antifeedant, antioxidant and anticancer drugs. For example, three new cytochalasins were discovered in *Hypoxylon fragiforme* because one of them, L-696,474 (18-dehydroxy cytochalasin H), was a potent competitive inhibitor of HIV-1 viral protease (Dombrowski *et al*., 1992). Echinocandin analogues, potent lipopeptide inhibitors of fungal β-1,3 glucan synthase, have been isolated form *Cryptosposiopsis* sp. (Noble *et al*., 1991; Tscherter and Dreyfuss, 1982). Furthermore, a novel lactone with potential antiherpes and antimicrobial properties was discovered in an endophytic *Microsphaeropsis* strain

(Tscherter *et al*., 1988). Additionally, a new cyclodepsipeptide antihelminthic was produced by sterile fungus isolated from *Camellia japonica* L.

(Sasaki *et al*., 1992). The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi are summarized in Appendix C

CHAPTER 3

EXPERIMENTS

3.1 Instruments and equipments

3.1.1 Chromatographic techniques

3.1.1.1 Analytical thin-layer chromatography (TLC)

Technique	: one dimension ascending
Adsorbent	: silica gel 60 F_{254} coated on aluminum sheet (E. Merck)
Layer thickness	: 0.2 mm
Size	: 20×20 cm^2
Distance	: 5 cm
Temperature	: laboratory temperature (25-30 ^{o}C)
Detection	: 1. Visual detection under ultraviolet light at wavelengths 254 and 365 nm.
	2. Visual detection in iodine vapor.
	3. Visual detection under daylight after spraying with vanillin reagent (Dissolve 1 g of vanillin in a mixture of 95 ml ethanol and 4 ml of concentrated sulfuric acid) and heating until the colors developed.

3.1.1.2 Column chromatography

Adsorbent : Silica gel 60 No. 1.09385.1000 (230-400 Mesh ASTM) (E. Merck).

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

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

Elution step : Eluted by increasing polarity of solvents from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH, MeOH.

Detection : Fractions were examined by TLC technique in the same manner as described in section 3.1.1

3.1.1.3 Gas Chromatography

Fatty acid were analyzed on 3 mm glass column of 15% DEGS on 60/80 mesh chromasorb S AW at 180 ^{o}C using Shimadzu 15A Gas Chromatography (Shimadzu Ltd.,Kyoto)

3.1.2 Spectroscopy

3.1.2.1 Ultraviolet-visible Spectrophotometer (UV-VIS)

The UV-VIS spectra were recorded on a Perkin Elmer Lambda 25 UV-VIS Spectrophotometer.

3.1.2.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760x Fourier Transform Infrared Spectrophotometer Impact 410. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

3.1.2.3 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; Merck's chloroform-*d* ($CDCl_3$), Merck's deuterium oxide (D_2O), Wilmad's methanol-*d* (MeOD) and Merck's 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 (s) ppm (^{13}C) for DMSO-d_6.

3.1.2.4 Mass spectrometer

The mass spectra were recorded on a MALDI-TOF BIFLEX-BRUKER GERMANY

3.1.2.5 X-Ray Diffractometer

The X-ray diffractometer were obtained on a BRUKER SMART CCD diffractometer at Department of Physics, Faculty of Science and Technology, Thammasart University.

3.1.2.6 Optical rotation

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

3.1.2.7 Melting point

The melting points were examined using a Stuart scientific melting point SMP1 apparatus

3.2 Chemical reagents

All commercial grade solvents are obtained from A.C.S. Xenon Limited Partnership, used in this research such as hexane, chloroform, ethyl acetate and methanol, were purified by distillation prior to use. The reagent grade solvents such as Merck's hexane, Merck's chloroform, Merck's ethyl acetate and Merck's methanol ,were used for re-crystallization.

3.3 Culture media

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

The medium for growing tested bacteria was nutrient medium (agar and broth). Yeast-malt extract medium (agar and broth) was used for growing tested yeast.
The media's formula are shown in Appendix A.

3.4 Sites of plant sampling

Healthy leaves of *Lagerstroemia speciosa* Linn. were collected from 5 area which were Rama IX Garden, Bangkok; Kasetsart university campus, Bangkok; Amphoe

Maung, Surin province; Amphoe Maung, Kumpangpetch province; Amphoe Nayone; Trang province. The leaves were preserved in a plastic bag at 4°C in a refrigerator until processing.

3.5 Fungal isolation and culture methods

The plant samples were cleaned and the endophytic fungi were isolated by using surface sterilization method which was modified from the method described by Blodgett et al., (2000).

The leaves sections (ϕ 6 mm.) were cut from the petiole, middle, end rib, side rib, and lamina. The leaf sections of *Lagerstroemia speciosa* Linn. collected from each source were used to isolate endophytic fungi. The disks were surface sterilized by sequential immersion in 95 % ethanol for 1 minute, in sodium hypochlorite (5% available chlorine) for 3 minutes and then transferred to 95 % ethanol for 30 seconds. Finally washed 2 times in sterilized water and the sterilized leaf pieces were surfaced dried with sterile papers and immediately placed on the surface of Potatoes Dextrose Agar (PDA) Petri dishes.

The Petri dishes were incubated at room temperature (25-30°C) and examined periodically for fungal mycelium from leaves under a stereomicroscope. Outgrowing were purified and transferred into new Petri dishes containing PDA by hyphal tip transfer. Fungal colonies development, were observed daily, and they were subcultured to new media to obtain pure cultured. The purity of isolated fungal mycelium were checked under light microscope. Fungal isolates were used for further study.

3.6 Culture collection of isolated endophytic fungi

Agar pieces(ϕ 7 mm.) were cut from the growing edge of fungal colonies grown on PDA. Five to ten pieces of agar blocks were put in a glass vials containing 5-ml sterile deionized water and kept at room temperature (25°C). The deionized water was steriled by autoclaving twice at 121 °C for 15 minutes.

3.7 Determination of antimicrobial activities of the isolated fungi

3.7.1 Antimicrobial activities

The isolated endophytic fungi were examined for antimicrobial activities against tested microorganisms as listed in Table 3.1.

Table 3.1 Tested microorganisms for antimicrobial assays

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

Viable counts of the standardized inoculums of test bacteria and yeast which was adjusted turbidity to match to 0.5 McFarland standard (OD_{625} = 0.08-0.1 nm) were performed.

3.7.2 Preparation of endophytic fungi for antimicrobial activities

Each endophytic fungal isolates were cultivated on five media which were Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), and Sabouraud's Dextrose Agar (SDA) at room temperature (25-30°C) for 21 days. Then, the agar cultures of each fungal endophyte isolate that grew on each medium were then cut into 7 mm diameter of small cylinder by a flamed cork hole borer for antimicrobial activity test.

3.7.3 Preparation of tested bacterial inoculums

Tested bacteria were grown on Nutrient Agar (NA) for 24 h at 37°C. Four to five isolated colonies which showed similar morphologic type were picked up by sterile loop and were then transferred to a tube containing 4 to 5 ml of Nutrient Broth (NB) and incubated at 37°C for 2-6 h, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of 0.5 McFarland standards (OD 0.08-0.1 at 625 nm.)

3.7.4 Preparation of tested yeast inoculums

Yeast was grown on Yeast-Malt Extract agar (YMA) for 24-48 h at room temperature (25-30°C). Four to five isolated colonies which showed similar morphologic type were were picked up by sterile loop and were then transferred to a tube containing 4 to 5 ml of Yeast-Malt Extract broth (YMB) and incubated at room temperature for 2-6 h. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of 0.5 McFarland standards (OD 0.08-0.1 at 625 nm.)

3.7.5 Antimicrobial assays for isolated endophytic fungi

The isolated endophytic fungi were grown on various culture media agar the same as described in section 3.6.2 and then were investigated for their antimicrobial activity by fungal disk dual culture method (Howell and Stipanovic, 1980).

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

allowed to dry for 3-5 minutes. The culture agar disks of each isolated entophytic fungal in section 3.6.2 were placed on the inoculated plates and pressed firmly into the agar with needle to ensure complete surface contact with tested microorganism plates and then incubated at 37°C, respectively for 24 h. Inhibition zone around the fungal disk was measured in mm.

3.8 Identification of endophytic fungi

3.8.1 Morphological identification

A. Macroscopical features

Colony characteristic of specimens such as shape, size, color, margin, pigment, and others were studied using stereomicroscope on a Leica MZ6.

B. Microscopical features

The microscopic analyses were based on morphological structure by light microscopy on an Olympus CH2 research microscope using a 40x dry objective.

3.8.2 Molecular Identification for species level

Selected endophytic fungi strain K_BK5 was identified by molecular techniques. Because it had the highest activities against *B. subtilis*, and produce some metabolites that change color of some culture media into red, so was chosen for further study. Sequences of internal transcribed spacer (ITS) regions of rDNA from isolated endophytic fungi were sent for identification by molecular methods at Macrogen, Inc. in Seoul, South Korea.

3.9 Cultivation and metabolite extraction

3.9.1 Growth curve of selected endophytic fungus strain K_BK5

Selected endophytic fungus strain K_BK5 was cultivated on SDA at room temperature (25-30 °C) for 1 week. Five pieces of agar culture were transferred aseptically into each 250 ml. Erlenmeyer flasks containing 100 ml of Sabouraud's Dextrose Broth (SDB). The flasks were incubated at room temperature (25 – 30°C) under static conditions. Mycelium dry weights were obtained by harvesting the mycelium on pre-activated (at 65 °C for 48 hr), and pre-weighted Whatman no. 4 filter paper. The mycelium was dried at 65 °C for 48 hr in an oven and weighted again. The difference between initial and final weight was take as dry weight. The culture filtrate was tested for antimicrobial activity by the agar well diffusion method (Weaver,Angel and Botlomley, 1994). Wells were made in the agar by removing disk cut (7 mm of diameter) with a flamed cork hole borer. One hundred μl of culture broth extracts and mycelium extracts were pipette into the agar wells. This was absorbed by the media surrounding the wells. The test microorganisms were incubated at 37°C for 24 hours in the same manner as described in section 3.6.5. Inhibition zones around the wells were measured in mm every 2 days.

3.9.2 Cultivation

Selected endophytic fungal K_BK5 was chosen for the study of metabolites and cultivated on SDA at room temperature (25-30 °C) for 1 week. The agar culture was cut with flamed cork borer (diameter 7 mm). Five disks were inoculated into 250 ml. Erlenmeyer flasks containing 100 ml of Sabouraud's Dextrose Broth (SDB) medium. The cultures were incubated at room temperature (25-30°C) for 14 days.

3.9.3 Crude extraction

The culture broth of selected endophytic fungal K_BK5 (4 L) was filtered through 4 layer of cotton gauze and exhaustively pressed. The filtrate was extracted with an equal volume of ethyl acetate (EtOAc) for 5 times. The EtOAc extracted layers were combined and dehydrated with anhydrous sodium sulfate. The solvent was evaporated using a rotary evaporator at 35°C. The crude EtOAc extract of broth was collected.

The fungal mycelia were blended and extracted 10 times with EtOAc in ultrasonic bath and filtered through filter paper (Whatman no.4). The EtOAc layers were combined and dehydrated with anhydrous sodium sulfate. The solvent was evaporated and using a rotary evaporator at 35°C. The crude EtOAc extract of mycelium was collected.

3.9.4 Antimicrobial activity of crude culture broth and mycelium extract

The crude extracts of the culture broth and mycelium of the endophytic fungus isolate K_BK5 were investigated for their antimicrobial activity by the agar well diffusion method as described in section 3.8.1.

3.10 Isolation of metabolites of crude culture broth and mycelium extracts

The broth and mycelium crude extracts of isolate K_BK5 were purified by column chromatography and eluted by increasing polarity of solvents from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH, MeOH. Fractions (50 ml each) were collected and examined. Fraction was examined by TLC on silica gel plates with hexane, hexane and ethyl acetate mixture, ethyl acetate, ethyl acetate and methanol mixtures, and methanol as the developing solvent. Fractions with the same TLC pattern were pooled and dried. The results from the isolation of crude culture broth and mycelium extracts and the biological activity of each pool fraction are described in Chapter 4.

3.11 Biological activities test

3.11.1 Antimicrobial activities test

3.11.1.1 Antimicrobial activities for the crude extracts and pool fractions

Evaluation of the antimicrobial activities of the crude extracts and fractions were determined for their antimicrobial activity by showing the clear zone with testes organisms (*B. subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231).

A. Preparation of samples

Each of the culture broth extract and separated fractions (4 mg) was dissolved in 1 ml of 10% DMSO in sterile distilled water. All samples were kept in a refrigerator at 4 ^{o}C for bioassay.

B. Preparation of bacterial inoculums

Bacterial inoculums was prepared in the same manner as described in section 3.6.3.

C. Preparation of yeast inoculums

Yeast inoculums was prepared in the same manner as described in section 3.6.4.

D. Inoculation of the test plate

Sterile cotton applicators were immersed in the inoculums suspension and pressed lightly against the tube wall to remove excess moisture. The agar was

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

E. Antimicrobial activity of the crude extracts and pool fractions

The crude extracts and fractions were investigated for their antimicrobial activity by the agar well diffusion method as described in section 3.8.1. Chemical constituents were investigated by TLC. The TLC results were monitored by UV (254 and 365 nm), iodine vapor and vanillin/H_2SO_4 reagent.

3.11.1.2 Antimicrobial activity of pure compounds

Evaluation of the antimicrobial activities of pure compounds was determined by the antimicrobial susceptibility test micro dilution method (Wood and Washington,1995). Antimicrobial activities were performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231.

A. Preparation of pure compounds

One mg of pure compounds and antibiotic drug standards were dissolved in 1 ml of 10% DMSO in sterile distilled water. Antibiotic drug standards (Streptomycin, Pennicilin, Kanamycin) were used as positive controls.

B. Preparation of bacterial tested inoculums

Bacterial inoculums was prepared in the same manner as described in section 3.6.3. The final inoculums was diluted with MHB to obtain the turbidity of the bacterial suspension with OD 0.1 at 625 nm, containing approximately 10^6 CFU/ml.

C. Preparation of yeast tested inoculums

Yeast inoculums was performed in the same manner as described in section 3.6.4. The final inoculums was diluted with YEB to obtain the turbidity of the yeast suspension with OD 0.1 at 625 nm, containing approximately 10^5 CFU/ml.

D. Assay procedure

Solutions of pure compounds were diluted with Mueller-Hinton Broth (MHB) and YEB for assays of a bacterial and antifungal (yeast form) activity respectively. 50 μl of pure compound was dispensed into each well in sterile microtitre plates (96-well bottom well). 50 μl of the final adjusted microbial suspension was inoculated into each well (Final inoculums size of bacterial and yeast was approximately 2.5 x10^5 and 2.5 x 10^4 CFU/ml, respectively). 100 μl of medium only was as the sterility control. A100 μl volume of medium and microbial inoculums mixture acted as the growth control. Microbial microlitre plates were incubated at 37°C and room temperature for bacterial and yeast, respectively.

E. Reading of microtitre plates assays

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

3.11.2 Cytotoxic test

Cytotoxic test were carried out at the Institute of Biotechnology and Genetic Engineering. Chulalongkorn University. Bioassay of cytotoxic activity againts human

tumor cell culture in *vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltetrazolium bromide) calorimetric method (Carmichael et al., 1987).

3.11.3 Antioxidant assay

1. Prepared DPPH solution at 200 μM in absolute ethanol (freshly prepare) and kept in the dark by covered with aluminum foil until use.
2. Prepared Crude sample extract and vitamin E (Positive control) in absolute ethanol as stock solutions. Pipette crude extract and vitamin E into each tube (covered tube with aluminum foil). The appropriate concentration of sample was added in various concentration such as 200, 100, 80, 60, 40, 20, 0 μg/ml (n = 3-5). Ratio of dilution is shown in Table3.2

Table 3.2 Ratio of dilution of sample from stock solution (1 mg/ml) with absolute ethanol (n = 3)

Concentration (μg/ml)	200	100	80	60	40	20	50	0
Vol. of crude extract (1 mg/ml)	200 μl	100μl	50 μl	25 μl	20 μl	10μl	5 μl	0 μl
Vol. of absolute ethanol	0 μl	100 μl	150 μl	175 μl	180 μl	190 μl	195 μl	200 μl

3. Added 800 μl of DPPH solution into each tube. Thus it has about 1 ml final volume.
4. The mixture was shaken vigorously and incubated at 37°C for 30 min.
5. Added 200 μl of mixture solution to 96 well plate. The absorbance was measured at 517 nm.
6. The absorbance of 200 μl DPPH solution was also measured at 517 nm.
7. Measurement was performed in triplicate in at least three independent experiments.
8. Result was analyzed by SPSS program.
9. Calculation for % inhibition (Paolo et al., 1999)

$$\% \text{ inhibition} = [(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100$$

A_{DPPH} = absorbance of DPPH after pipette 200 μl

A_{sample} = absorbance of DPPH after adding in each concentration of testing sample after minus concentration value of sample before adding DPPH 200 μl

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

The total of forty two isolates of endophytic fungi, which were obtained from healthy leaves of *Lagerstroemia speciosa* Linn. are shown in Table 4.1. Outgrowing of endophytic fungal mycelium from leaves samples were examined periodically under a stereomicroscope as shown in Figure 4.1. Colony morphology of some endophytic fungi are shown in Figure 4.2-4.13. Endophytic fungi were identified by microscopic method, it was found that there are 40 isolates of Mycelia sterile, 1 isolates of *Fusarium* sp. and 1 isolates of *Phomopsis* sp.

Table 4.1 Number and isolate of endophytic fungi from various provinces

Name of province	Number of endophytic fungi Isolates	Code of Endophytic fungi isolates
Kasetsart university, Bangkok	5	K_BK1 to 5
Rama IX Garden, Bangkok	6	S_BK1 to 6
Surin	11	SR1 to 11
Kumpangpetch	8	KP1 to 8
Trang	12	TG1 to 12
total	42	

The results present on examples of associations between endophytic fungi and plants. It presents that plants are habitat of endophytic fungi. From the research everyplace of the healthy leaves have isolated endophytic fungi. Number of endophytic fungi isolates effect on many factors such as abundant rainfall, soil, climate, environment and ecologically habitat of plants. These factors have influence to different number of endophytic fungi isolates(Strobel et al., 2003). Isolated endophytic fungal from 5 areas have different characteristic and diversity in each area. From the results Trang is highest number of endophytic fungi isolates. It maybe location has abundant rainfall.

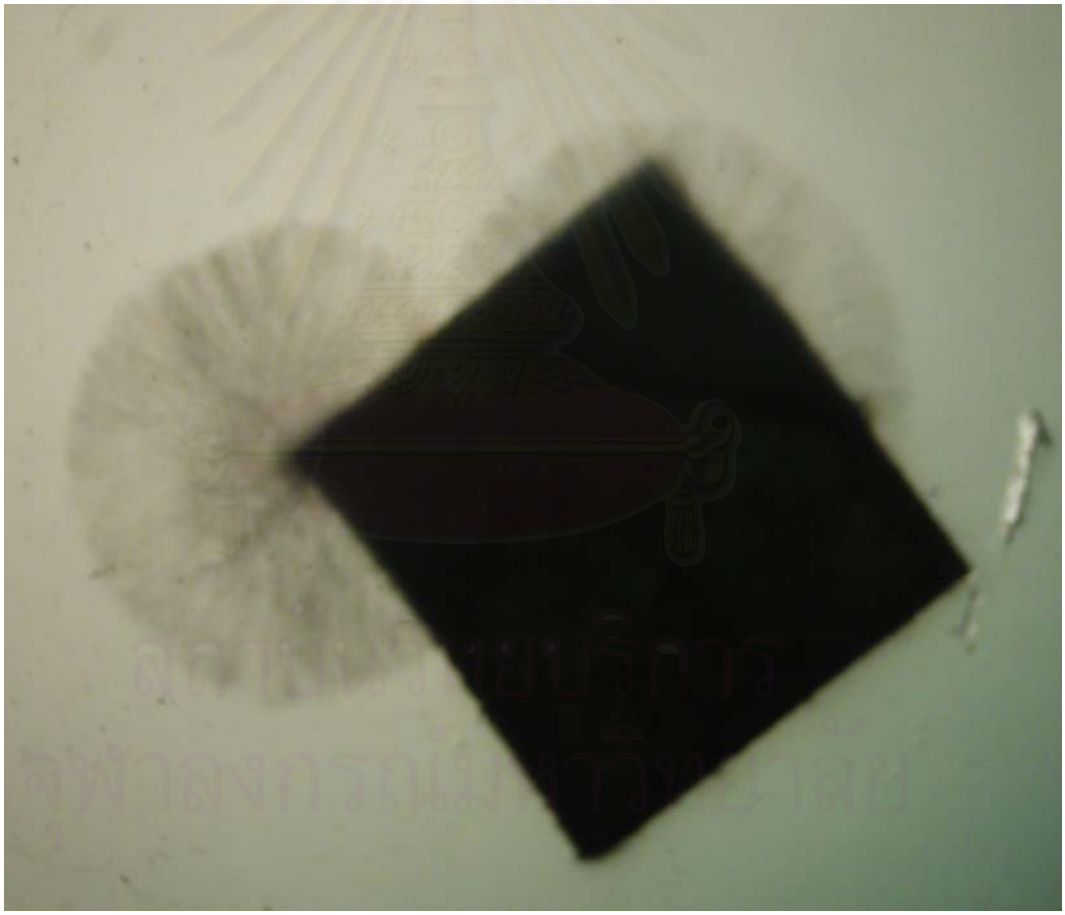


Figure 4.1 Outgrowing of endophytic fungal mycelium from leaves samples and were examined periodically under a stereomicroscope every 2 days.

Figure 4.2 Colonial morphology characteristic of endophytic fungus strain,K_BK4, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.3 Colonial morphology characteristic of endophytic fungus strain,S_BK3, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

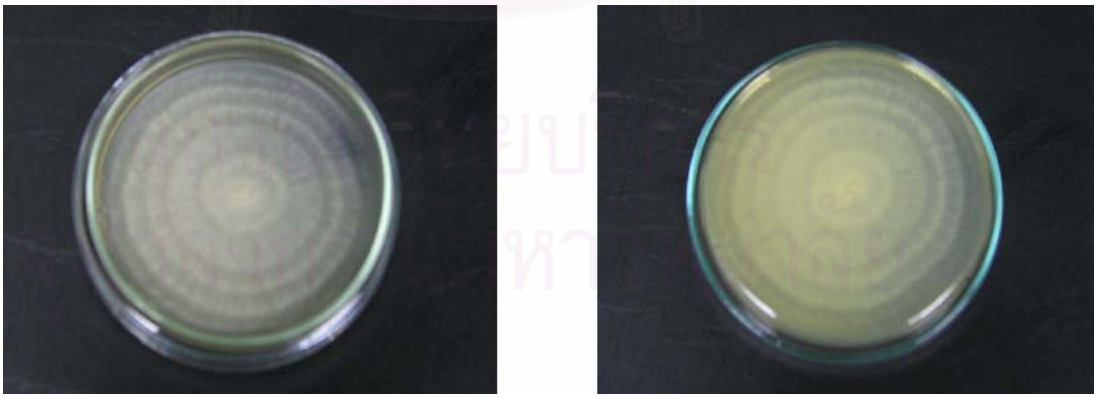


Figure 4.4 Colonial morphology characteristic of endophytic fungus strain,S_BK6, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.5 Colonial morphology characteristic of endophytic fungus strain,SR3, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

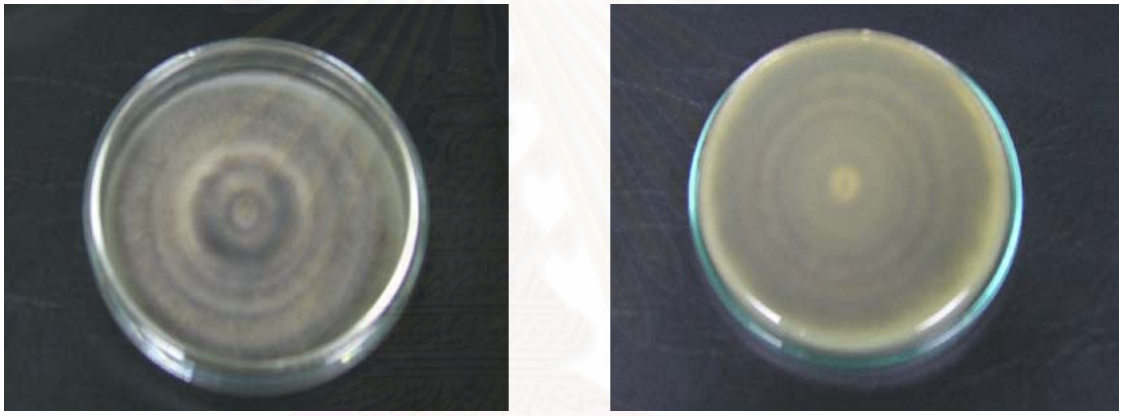


Figure 4.6 Colonial morphology characteristic of endophytic fungus strain,SR6, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.7 Colonial morphology characteristic of endophytic fungus strain,SR8, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.8 Colonial morphology characteristic of endophytic fungus strain,KP2, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.9 Colonial morphology characteristic of endophytic fungus strain,KP3, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.10 Colonial morphology characteristic of endophytic fungus strain,KP5, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Figure 4.11 Colonial morphology characteristic of endophytic fungus strain,KP7, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

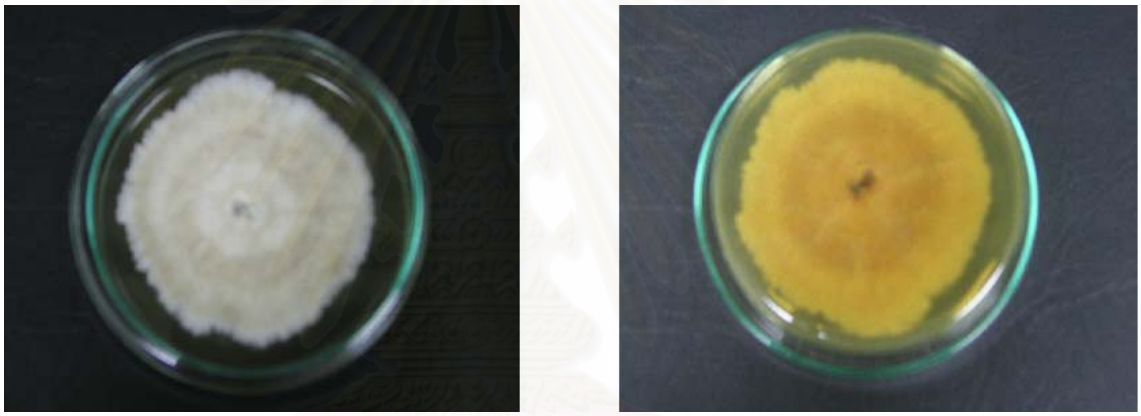


Figure 4.12 Colonial morphology characteristic of endophytic fungus strain,TG6, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

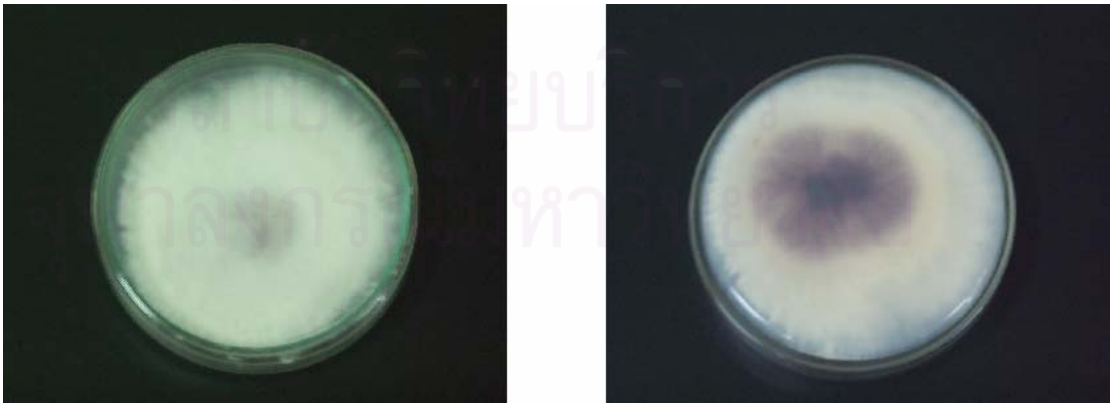


Figure 4.13 Colonial morphology characteristic of endophytic fungus strain,TG10, on PDA media after cultivation for 3 weeks at room temperature. Appearance on top side (A), and on reverse side (B).

Table 4.2 Characteristics of colony and identification of endophytic fungi from *Lagerstroemia speciosa* Linn. Leaves.

Isolated endophytic fungi strain	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Pigment color in culture media	
K_BK1	Absence of elevation	white	colorless	Mycelia sterilia
K_BK2	Absence of elevation	orange	colorless	Mycelia sterilia
K_BK3	Absence of elevation	grey	colorless	Mycelia sterilia
K_BK4	Cottony	White and green	colorless	Mycelia sterilia
K_BK5	Absence of elevation	white	red	Mycelia sterilia
S_BK1	Absence of elevation	white	colorless	Mycelia sterilia
S_BK2	Absence of elevation	green	colorless	Mycelia sterilia
S_BK3	Powdery	white	colorless	Mycelia sterilia
S_BK4	Absence of elevation	dark black	colorless	Mycelia sterilia
S_BK5	Powdery	grey	colorless	Mycelia sterilia
S_BK6	Absence of elevation	white	colorless	Mycelia sterilia
SR1	Powdery	green	colorless	Mycelia sterilia
SR2	Powdery	white	colorless	Mycelia sterilia
SR3	Powdery	white	colorless	*Phomopsis* sp.
SR4	Absence of elevation	white	colorless	Mycelia sterilia
SR5	Absence of elevation	brown	colorless	Mycelia sterilia
SR6	Absence of elevation	dark brown	colorless	Mycelia sterilia
SR7	Absence of elevation	white	colorless	Mycelia sterilia
SR8	Cottony	white and black	colorless	Mycelia sterilia
SR9	Powdery	white	colorless	Mycelia sterilia
SR10	Powdery	yellow	colorless	Mycelia sterilia
SR11	Powdery	orange and dark	colorless	Mycelia sterilia
KP1	Powdery	grey	colorless	Mycelia sterilia
KP2	Cottony	white and green	colorless	Mycelia sterilia
KP3	Absence of elevation	white	colorless	Mycelia sterilia
KP4	Powdery	white	colorless	Mycelia sterilia

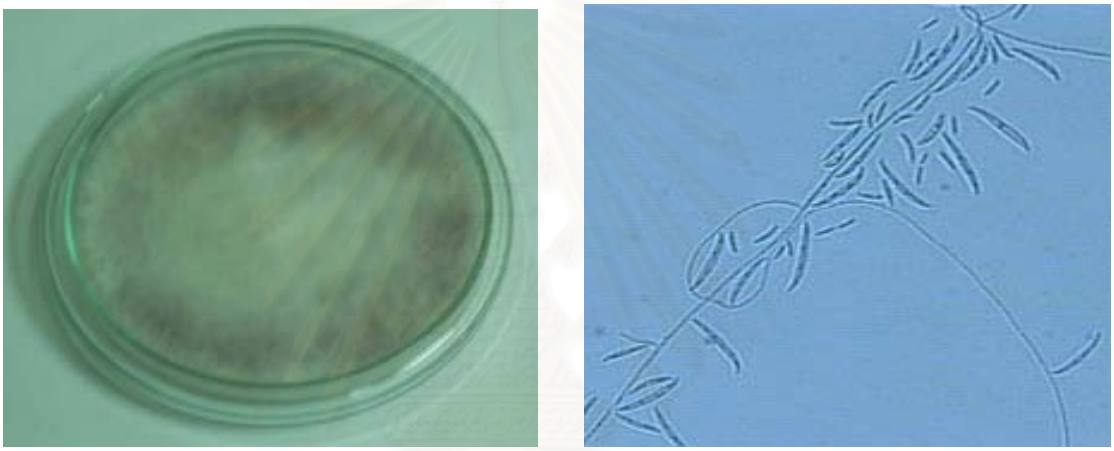
Table 4.2 Characteristics of colony and identification of endophytic fungi from *Lagerstroemia speciosa* Linn. Leaves. (continued)

Isolated endophytic fungi strain	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Pigment color in culture media	
KP5	Cottony	white	colorless	Mycelia sterilia
KP6	Absence of elevation	grey	colorless	Mycelia sterilia
KP7	Cottony	White and green	colorless	Mycelia sterilia
KP8	Absence of elevation	grey	colorless	Mycelia sterilia
TG1	Absence of elevation	dark blown	colorless	Mycelia sterilia
TG2	Cottony	white	colorless	Mycelia sterilia
TG3	Absence of elevation	blown	colorless	Mycelia sterilia
TG4	Absence of elevation	grey	colorless	Mycelia sterilia
TG5	Absence of elevation	grey	colorless	Mycelia sterilia
TG6	Absence of elevation	white	yellow	Mycelia sterilia
TG7	Cottony	black	colorless	Mycelia sterilia
TG8	Absence of elevation	grey	colorless	Mycelia sterilia
TG9	Absence of elevation	grey	colorless	Mycelia sterilia
TG10	Absence of elevation	white and purple	colorless	*Fusalium* sp.
TG11	Cottony	grey	colorless	Mycelia sterilia
TG12	Absence of elevation	white	colorless	Mycelia sterilia

4.2 Detailed characters of isolated endophytic fungal species

FUSARIUM strain

Colony color was white and purple. Mycelium composed of hyaline, septate, branched hyphae. Conidial masses typically formed in sporodochia or in pionnotes or sometimes scattered in the mycelium. Conidiophores simple or branched once or repeatedly. Conidia of two types: microconidia and macroconidia.

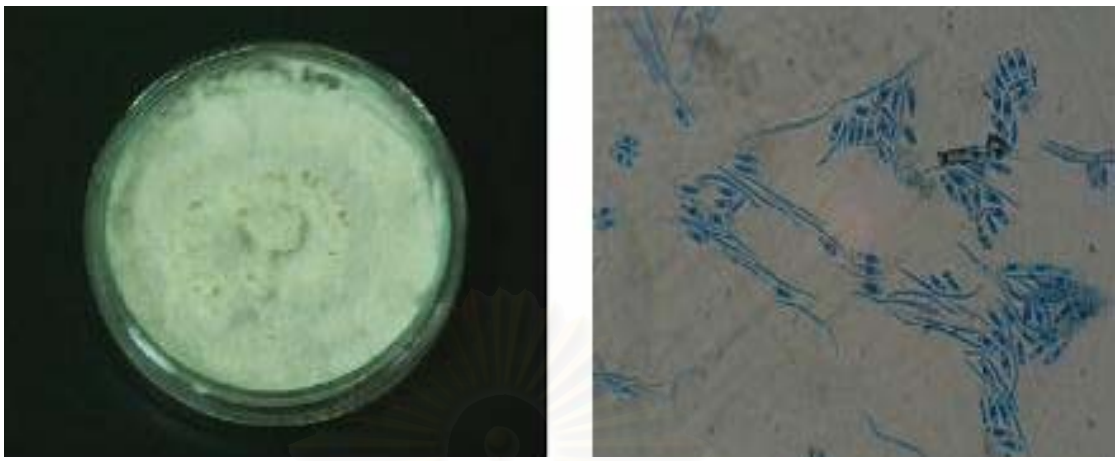


(a) (b)

Figure 4.14 *Fusarium* sp. (a) Culture on MEA (10 days). (b) Macroconidia.

PHOMOPSIS strain

Mycelium immersed, branched, septate, hyaline to pale brown. Conidiophores branched and septate at the base and above, occasionally short and only 1-2 septate, more frequently multiseptate and filiform, hyaline, formed from the inner cells of the locular walls. Conidia of two basic type, but in some species with intermediates between the two: α-conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end) but sometimes with more guttules, aseptate; β-conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate.



(a) (b)

Figure 4.15 *Phomopsis* sp. (a) culture on MEA (7-10 days) (b) α and β conidia (100x)

4.3 Antimicrobial activities of isolated endophytic fungi

By dual-culture agar diffusion assay as described in section 3.6.5, 23 isolates (54.76%) have antimicrobial activities. The number of active endophytic fungi isolates exhibiting activities against bacteria *B. subtilis*, *S. aureus, E. coli, P. aeruginosa* were 14(33.33%), 11(26.19%), 5(11.90%), 2(4.76%) isolates respectively. Five isolates (11.90%) were found to inhibit *C. albicans*. The high percentage of active endophytic fungi isolates have confirmed that fungal endophytes are source of bioactive compounds (Strobel et al., 1998). From the results, gram positive bacteria (*B. subtilis* and *S. aureus*) have antimicrobial activities in higher percentage than gram negative bacteria (*E. coli* and *P. aeruginosa*) and pathogen yeast (*C. albicans*). It's because of gram positive bacteria have more sensitive.

The endophytic fungi isolates that exhibited antimicrobial activities when they were cultured on different media, are shown in Table 4.2. The endophytic fungi isolates were grown on various media in order to observe cultural morphology and activity. Every media normally support the growth of fungal isolates. The different media has influenced to the type and intensity of antimicrobial activities. The main factors of media effect are type and intensity of C-source and N-source that is different in each media that effect to

expression of antimicrobial activity. The highest numbers of active isolates against all test organisms were found when they were grown on YES. The specific of fungal isolates with culture media supports the maximum antimicrobial activity. Using numerous culture media probably will be increasing the opportunity to obtain more antimicrobial activities. Table 4.2 showed effect of culture media on antimicrobial activities. Activities were classified according to the diameter of clear zone after inoculated with a small pieces of the grow fungus of isolate endophytic fungi.

Table 4.3 Summarized of culture media on antimicrobial activities from fungal isolated

Isolated endophytic fungal strain	Media	Clear zone of testes organisms				
		B.subtilis	*E. coli*	*P.aeruginosa*	*S.aureus*	*C.albicans*
K_BK1	MCA	-	+	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
K_BK4	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	+	-
K_BK5	MCA	-	-	-	-	-
	MEA	++++	-	-	-	-
	SDA	++++	-	-	-	-
	PDA	+++	-	-	-	-
	YES	-	-	-	-	-
S_BK3	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	++	-	-	-	++
	PDA	-	-	-	-	-
	YES	+	-	-	-	+
S_BK5	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	+	-	-	-	-

Table 4.3(continued)

Isolated endophytic fungal strain	Media	Clear zone of testes organisms				
		B.subtilis	*E. coli*	*P.aeruginosa*	*S.aureus*	*C.albicans*
S_BK6	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	++	-
SR3	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	+	-	-	+	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
SR6	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	++	-	-	-	-
SR7	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	+	-
SR8	MCA	+	++	-	-	-
	MEA	-	-	-	++	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
SR10	MCA	-	-	-	++	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	++	-	-	+	-
KP1	MCA	+	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-

Table 4.3(continued)

Isolated endophytic fungal strain	Media	Clear zone of testes organisms				
		B.subtilis	*E. coli*	*P.aeruginosa*	*S.aureus*	*C.albicans*
KP3	MCA	-	-	-	++	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
KP4	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	+	+	+	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
KP6	MCA	++	-	-	-	-
	MEA	++	-	-	+	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
KP8	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	++	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
TG2	MCA	-	-	-	-	-
	MEA	+	-	-	-	-
	SDA	++	-	-	++	++
	PDA	-	-	-	-	-
	YES	-	-	-	+	+
TG4	MCA	-	-	-	-	-
	MEA	-	+	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-
TG6	MCA	-	-	-	-	-
	MEA	++	+	-	-	-
	SDA	-	+	-	+	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	-

Table 4.3(continued)

Isolated endophytic fungal strain	Media	Clear zone of testes organisms				
		B.subtilis	*E. coli*	*P.aeruginosa*	*S.aureus*	*C.albicans*
TG7	MCA	-	-	-	-	-
	MEA	-	-	-	-	+
	SDA	-	-	-	-	+
	PDA	-	-	-	-	+
	YES	++	-	-	-	+
TG10	MCA	+	-	+	++	-
	MEA	-	-	-	+	-
	SDA	+	+	-	+	-
	PDA	-	-	-	++	-
	YES	+	-	-	+	-
TG11	MCA	-	-	-	-	-
	MEA	-	-	-	-	-
	SDA	-	-	-	-	-
	PDA	-	-	-	-	-
	YES	-	-	-	-	+
TG12	MCA	-	-	-	-	-
	MEA	-	-	-	-	+
	SDA	-	-	-	-	-
	PDA	-	-	-	-	+
	YES	-	-	-	-	+

Activities were classified according to the diameter of the point of application of the sample

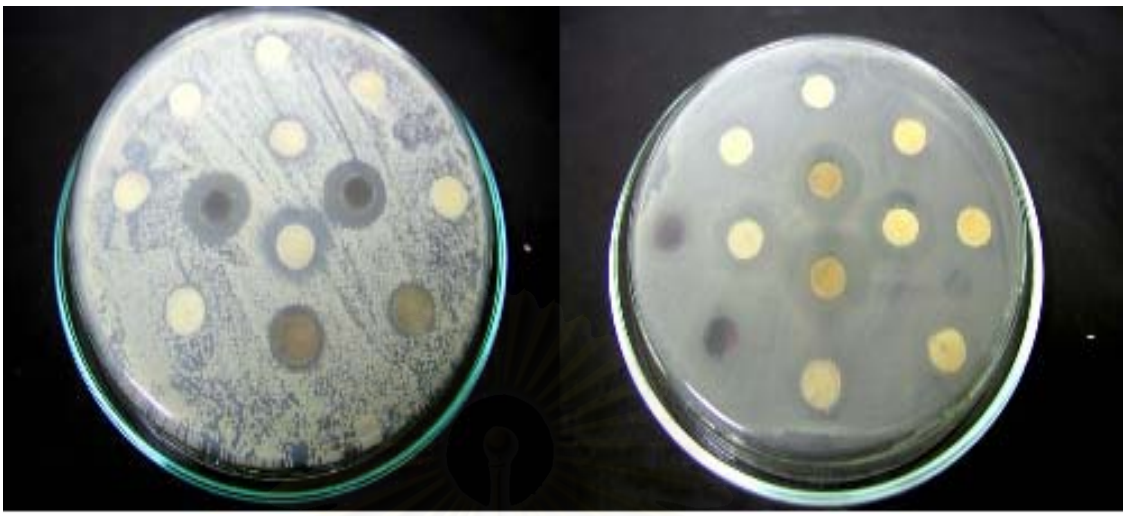
++++ = more than 30 mm

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

\+ = Inhibition more than 8 mm

\- = No inhibition



(a) (b)

Figure 4.16 Dual-culture diffusion exhibit against (a) C.albicans and (b) E.coli of activity of endophytic fungus isolated

Summarize antimicrobial activity of active endophytic fungus isolates and specific culture media are shown in Figure4.17.

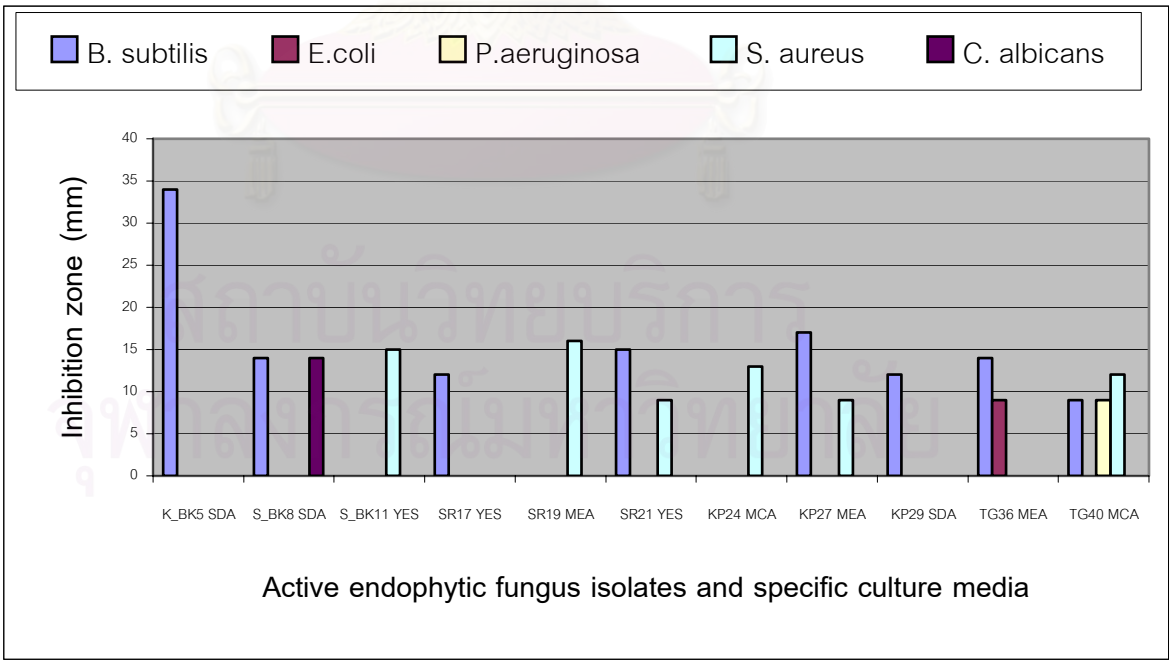


Figure 4.17 Inhibition zone (mm) measured from the agar block of the 11 active endophytic and specific culture media

From the result, most of the isolates have not broad activity to test microorganisms. The Half of active endophytic fungi have activity to against one test microorganisms. The others have specific activity to few microorganisms mostly with *B. subtilis* and *S. aureus*. Fungus isolate K_BK5 was chosen for study bioactive compounds. Because it was the highest active activities against *B. subtilis* ,and produce some interesting metabolites that change color of some culture media into red which supports the maximum production of secondary metabolites. So it is interesting to select fungus isolate K_BK5 to further study.

4.4 Identification of fungal endophyte K_BK5

4.4.1 Morphology identification

Selected endophytic fungal strain K_BK5 did not produce conidium or spore formation on common mycological media including Potato Dextrose Agar, Malt Extract Agar, Sabouraud Dextrose Agar, Yeast Extract Sucrose Agar, Mait Czapek Agar. Therefore, fungal strain K_BK5 was classified as Mycelia sterilia. Colony characteristic of the isolate K_BK5 was shown in Figure 4.18-4.19 colony color was white and absence of elevation, but colony color was gray on Mait Czapek Agar. Mycelium composed of hyaline, septate, branched hyphae. Mycelia characteristics was shown in Figure 4.20

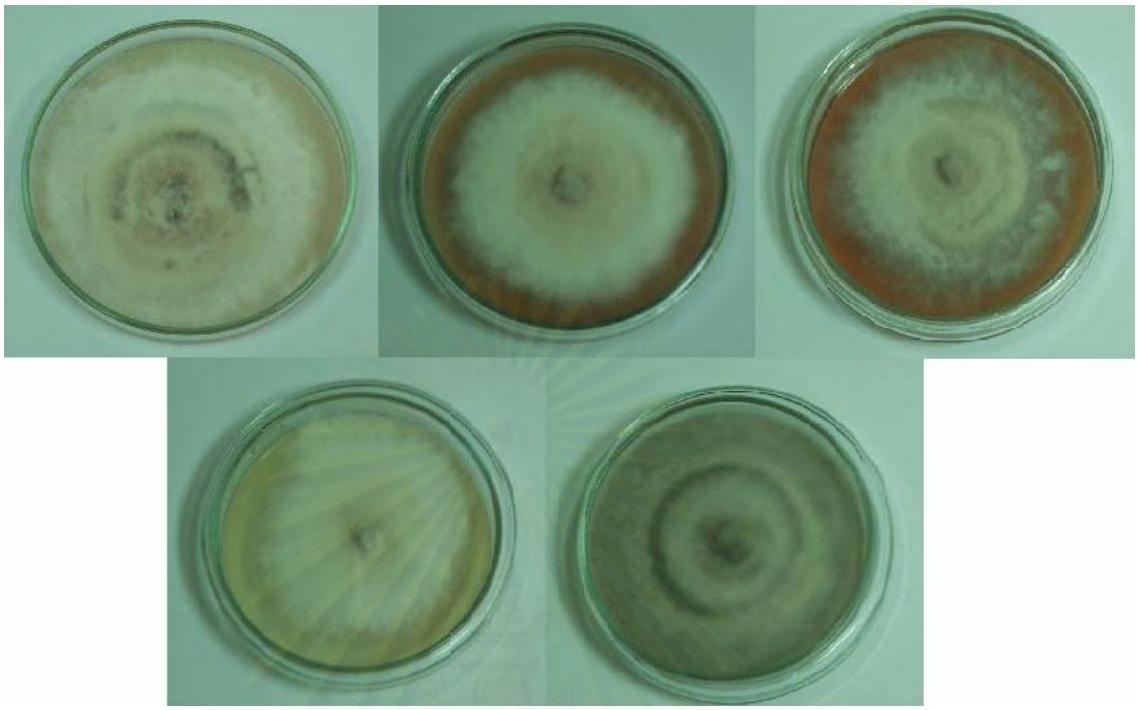


Figure 4.18 Colony characteristic of endophytic fungus isolate K_BK5 was grown on 5 different media at room temperature for 21 days on top side.

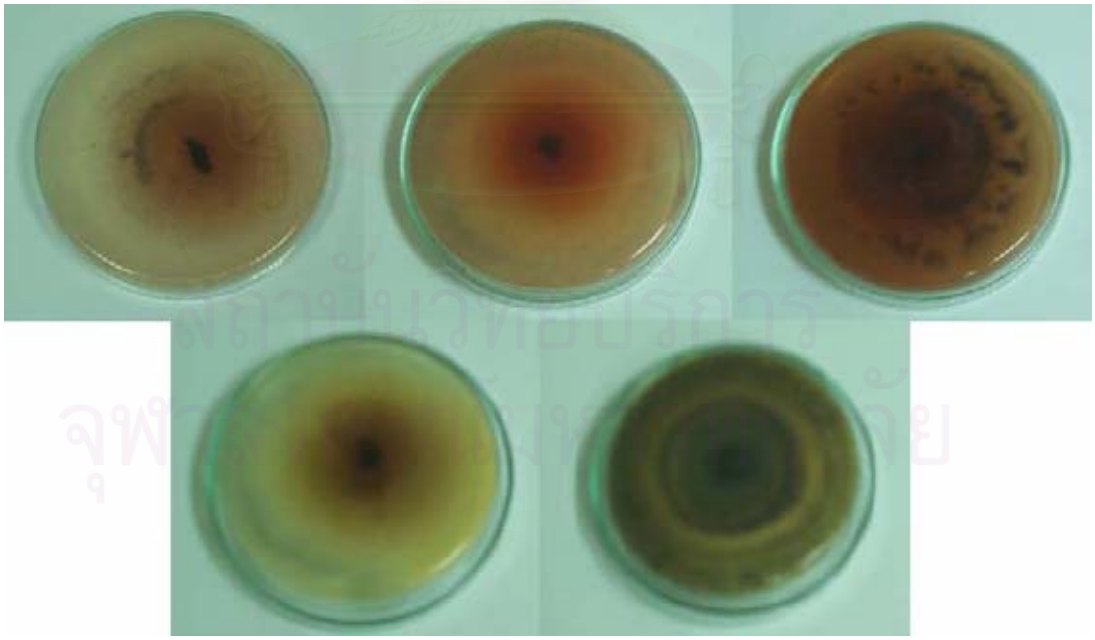


Figure 4.19 Colony characteristic of endophytic fungus isolate K_BK5 was grown on 5 different media at room temperature for 21 days on reverse side.

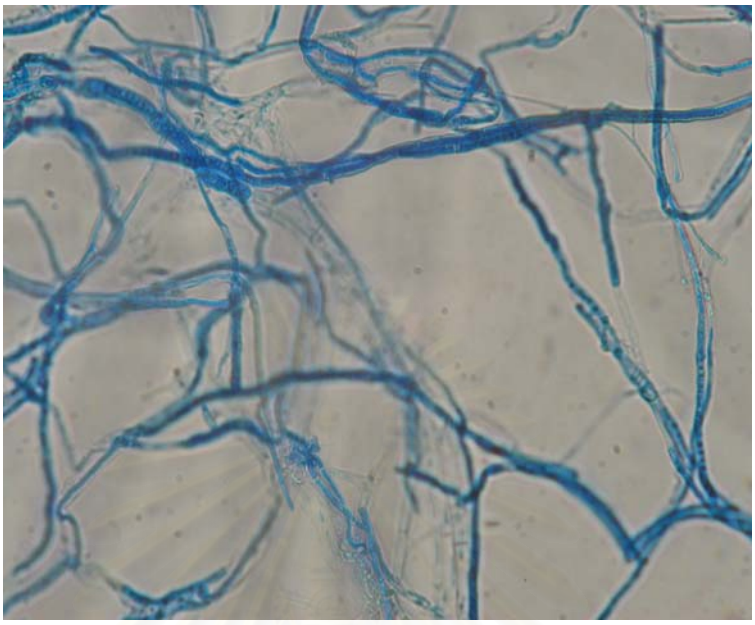


Figure 4.20 Mycelia characteristics of endophytic fungus isolate K_BK5

4.4.2 Molecular identification

Molecular methods of identification was also performed. The nucleotide sequence of the ITS region of rDNA is conserved. It can be used to delineate species relationships and separated taxonomy from class to species (Mitchell et al., 1995).

The rDNA ITS region of isolate K_BK5 was amplified with the conserved fungal primer ITS1t and ITS4. Isolate K_BK5 produced a single ITS band. The length of corresponding fragment was 583 bp, containing a part of the 18S; ITS1, 5.8S and 28S rDNA

1

5'AGTNCGGTGG TGACAGCGGA GGGATCATTC AGAGTTATCC AACTCCCAAA
CCCATGTGAA CATATCTCTT TGTTGCCTCG GCGCAAGCTA CCCGGGACCT
CGCGCCCCGG GCGGCCCGCC GGCGGACAAA CCAAACTCTG TTATCTTCGT
TGATTATCTG AGTGTCTTAT TTAATAAGTC AAAACTTTCA ACAACGGATC
TCTTGGTTCT GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT
GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC
ATTAGTATTC TAGTGGGCAT GCCTGTTCGA GCGTCATTTC AACCCCTAAG
CACAGCTTAT TGTTGGGCGT CTACGTCTGT AGTGCCTCAA AGACATTGGC
GGAGCGGCAG CAGTCCTCTG AGCGTAGTAA TTCTTTATCT CGCTTTTGTT
AGGCGCTGCC CCCCCGGCCG TAAAACCCCC CATTTTTTCT GGTTGACCTC
GGATCAGGTA GGAATACCCG CTGAACTTAA GCATATCAAT AGGCCGGAGG
AANNNNNNN NNN 3'

583

Figure 4.21 Nucleotide sequences of partial 18s region, complete ITS region of endophytic fungal strain K_BK5

A blast search was performed to find a similar sequence to ITS region of selected endophytic fungal strain K_BK5 using Genbank DNA database. The result suggested that 98.324% of sequence of ITS region is similar to DNA sequence of Fungal endophyte MS6 IS133, endophytic fungi that isolated from Hong Kong. The comparison a alignment as shown in Figure 4.18

>>AF413049|AF413049.1 Fungal endophyte MS6 IS133 18S rib (558 nt)
initn: 2037 init1: 1492 opt: 2559 Z-score: 2044.9 bits: 388.2 E(): 1.7e-105
banded Smith-Waterman score: 2559; 98.324% identity (99.435% ungapped) in 537 nt overlap (3-535:23-558)

```
10          20        30
AGTNCGGTGGTGA--CAGCGGAGGGATCATTACAGAGTTA
: :: ::::::  ::::::::::::::::::::::::::
AF4130 AAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACAGAGTTA
10        20        30        40        50        60

40        50        60        70        80        90
TCCAACTCCCAAACCCATGTGAACATATCTCTTTGTTGCCTCGGCGCAAGCTACCCGGGA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4130 TCCAACTCCCAAACCCATGTGAACATATCTCTTTGTTGCCTCGGCGCAAGCTACCCGGGA
70        80        90       100       110       120

100       110       120       130       140       150
CCTCGCGCCCCGGGCGGCCCGCCGGCGGACAAACCAAACTCTGTTATCTTCGTTGATTAT
:::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::
AF4130 CCTCGCGCCCC-GGCGGCCCGCCGGCGGACAAACCAAACTCTGTTATCTTCGTTGATTAT
130        140       150       160       170

160       170       180       190       200       210
CTGAGTGTCTTATTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4130 CTGAGTGTCTTATTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
180       190       200       210       220       230

220        230        240       250       260       270
ATG-AAGAACGCAGCG-AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
::: :::::::::::: ::::::::::::::::::::::::::::::::::::::::::::
AF4130 ATGAAAGAACGCAGCGAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
240       250       260       270       280       290

280       290       300       310       320       330
GAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4130 GAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTC
300       310       320       330       340       350

340       350       360       370       380       390
ATTTCAACCCCTAAGCACAGCTTATTGTTGGGCGTCTACGTCTGTAGTGCCTCAAAGACA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4130 ATTTCAACCCCTAAGCACAGCTTATTGTTGGGCGTCTACGTCTGTAGTGCCTCAAAGACA
360       370       380       390       400       410

400       410       420       430       440       450
TTGGCGGAGCGGCAGCAGTCCTCTGAGCGTAGTAATTCTTTATCTCGCTTTTGTTAGGCG
:::::::::::::::::::::::::::::::::::::::::::::::::: :::::::::
AF4130 TTGGCGGAGCGGCAGCAGTCCTCTGAGCGTAGTAATTCTTTATCTCGCTTCTGTTAGGCG
420       430       440       450       460       470

460       470       480       490       500       510
CTGCCCCCCCGGCCGTAAAACCCCCCATTTTTTCTGGTTGACCTCGGATCAGGTAGGAAT
:::::::::::::::::::::::: ::::::::::::::::::::::::::::::::::
AF4130 CTGCCCCCCCGGCCGTAAAACCCCCAATTTTTTCTGGTTGACCTCGGATCAGGTAGGAAT
480       490       500       510       520       530

520       530       540       550       560
ACCCGCTGAACTTAAGCATATCAATAGGCCGGAGGAANNNNNNNNN
::::::::::::::::::
AF4130 ACCCGCTGAACTTAAGCAT
540       550
```

Figure 4.22 Alignment data of ITS region of isolate K_BK5 and 1 reference taxa

4.5 Determination of growth profile and antimicrobial activity of endophytic fungal strain K_BK5

Growth profile of fungal endophyte isolate K_BK5 was observed from mycelium dry weight. The raw data of mycelium dry weight are shown in appendix C. Cultivation of fungal isolate K_BK5 was done in SDB medium for 40 days. Figure 4.23 show the growth profile and antimicrobial activity against *B.subtilis* of fungal isolate K_BK5 at day 0 to day 40.

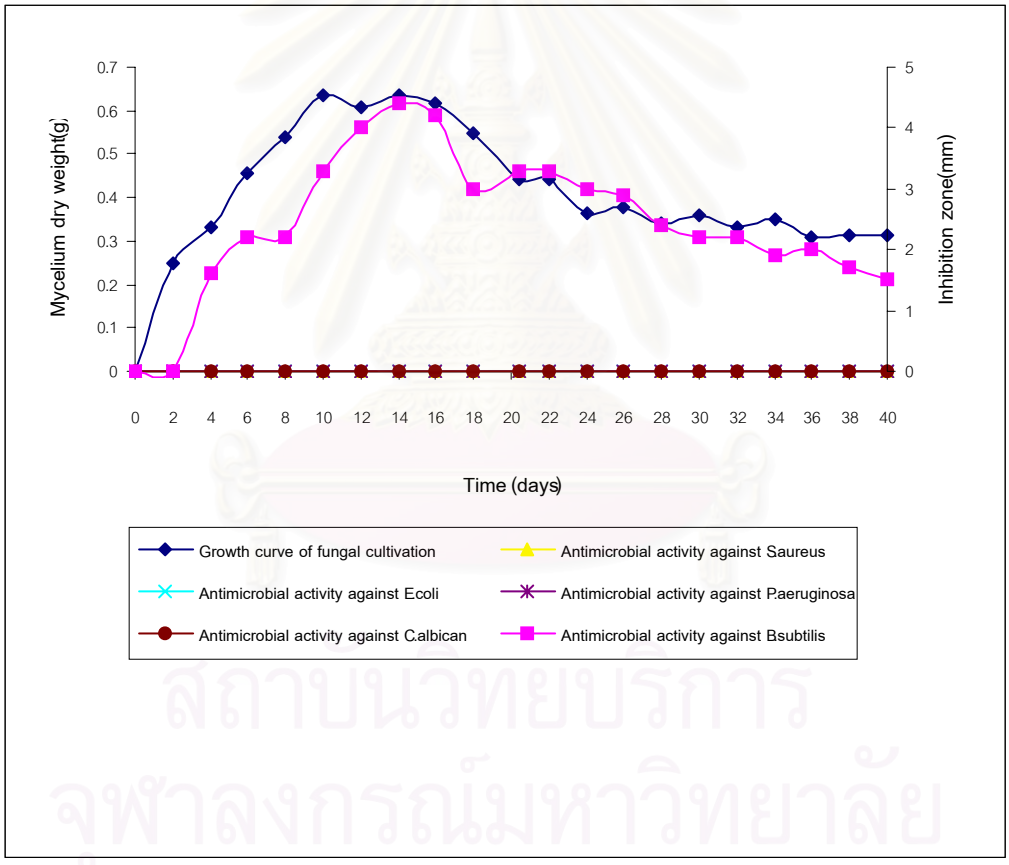


Figure 4.23 Growth profile and antimicrobial activity of endophytic fungal strain K_BK5

Fungal isolate K_BK5 grow in log phase. Then the mycelium increased up to highest amount within 10 days. The growth of mycelium was in the form of stationary phase at date 10 to date 16 and continued to death phase in 22 days. From the results, While fungi was growing it produced primary metabolite be red pigment and have antimicrobial activity against *B.subtilis* at the same time. The fungi produced maximum antimicrobial activity during date 12 to date 16 that base on stationary phase. It has the best high activity on date14 static and gently down when continued to death phase. The NMR spectrum (as described in appendix E) and TLC pattern bands of date 6, date14 and date 28, which is sample of log phase, stationary phase and death phase respectively, are similarly. Most of every date have activity against *B.subtilis.* It indicates that fungal isolate K_BK5 have potentially produced some bioactive compounds, but amount is different in each phase time of growing.

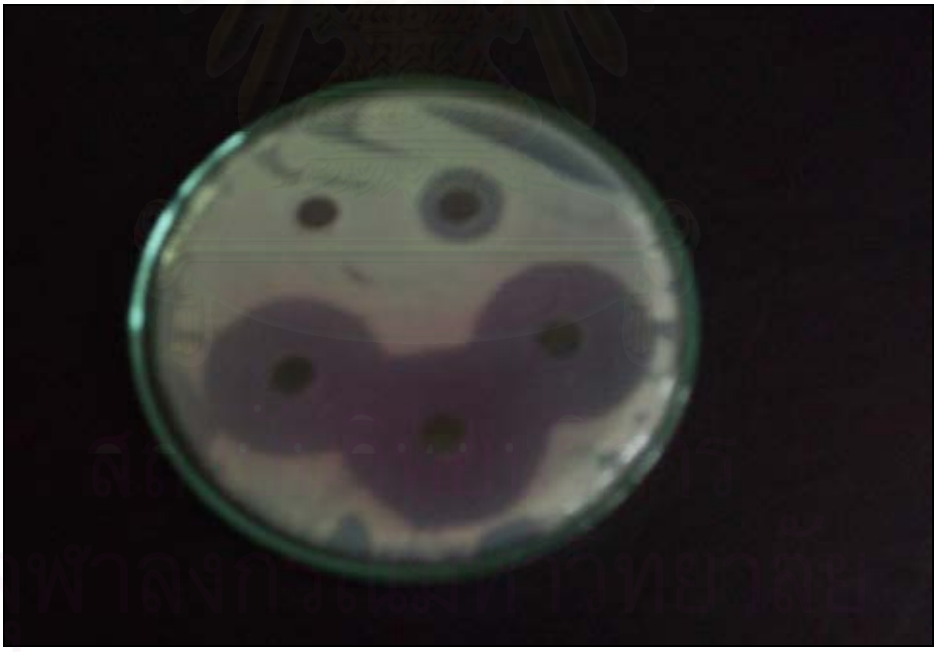


Figure 4.24 Inhibition growth against Bacillus subtilis of date2, 4, 6, 8 and 10 by endophytic fungal strain K_BK5

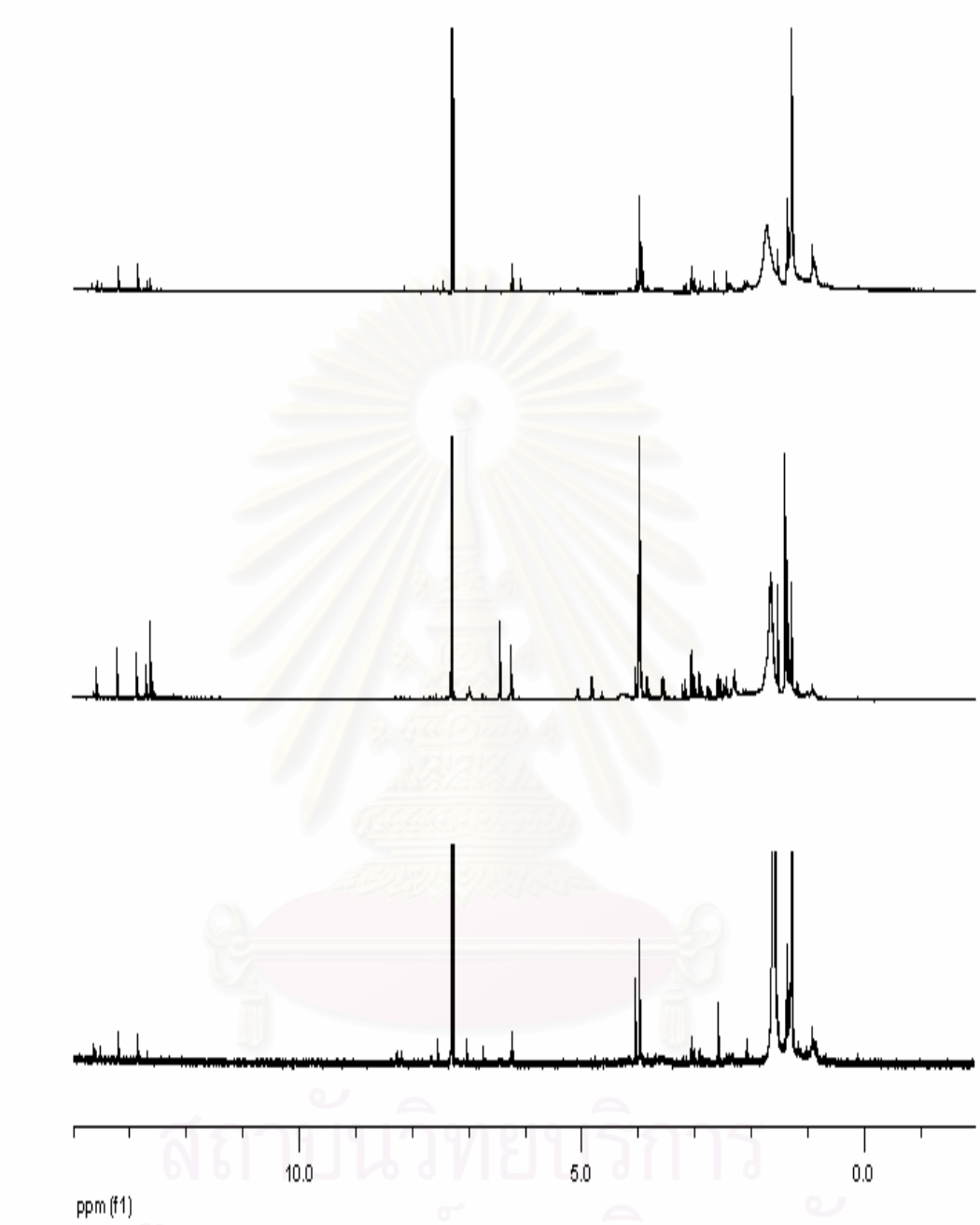
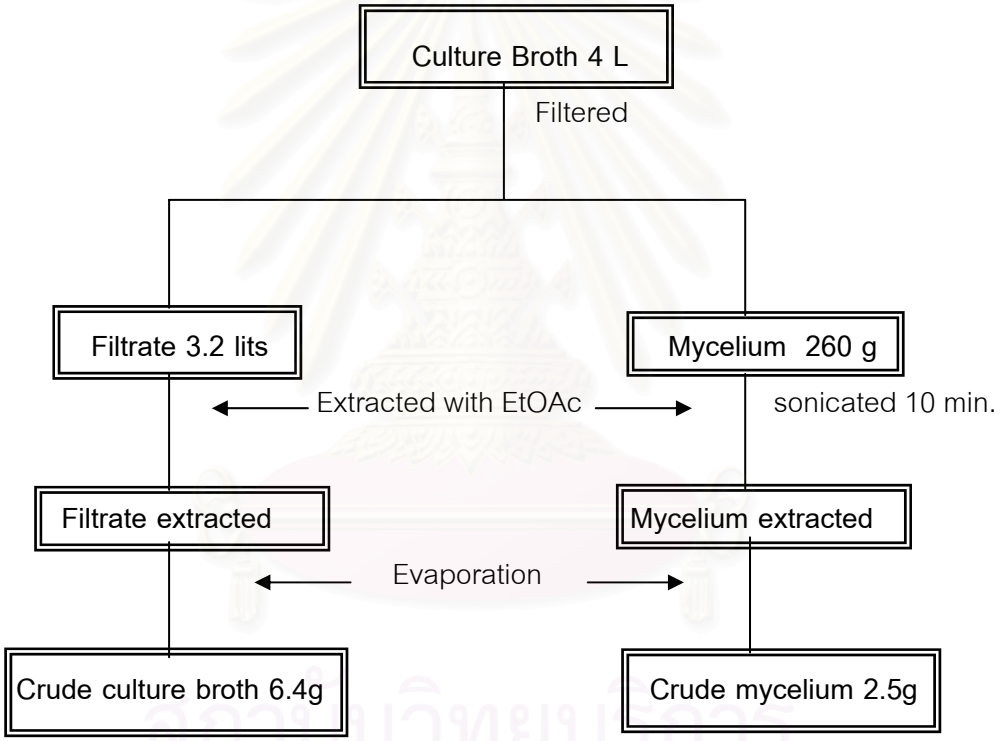


Figure 4.25 The NMR spectrum of date 6 (top), date14 (middle) and date 28 (bottom)

4.6 Cultivation and extraction

Four liters of endophytic fungal strain K_BK5 was cultivated in SDB. A 6.4 and 2.5 g of EtOAc crude ,were obtained as a red powder solid, were collected from cultivation broth and mycelium, respectively. The extraction of the fermentation broth and mycelia of the endophytic fungus isolate K_BK5 is shown in Scheme 4.1



Scheme 4.1 Amount (g) of crude extract of culture broth and mycelium of endophytic fungal strain K_BK5

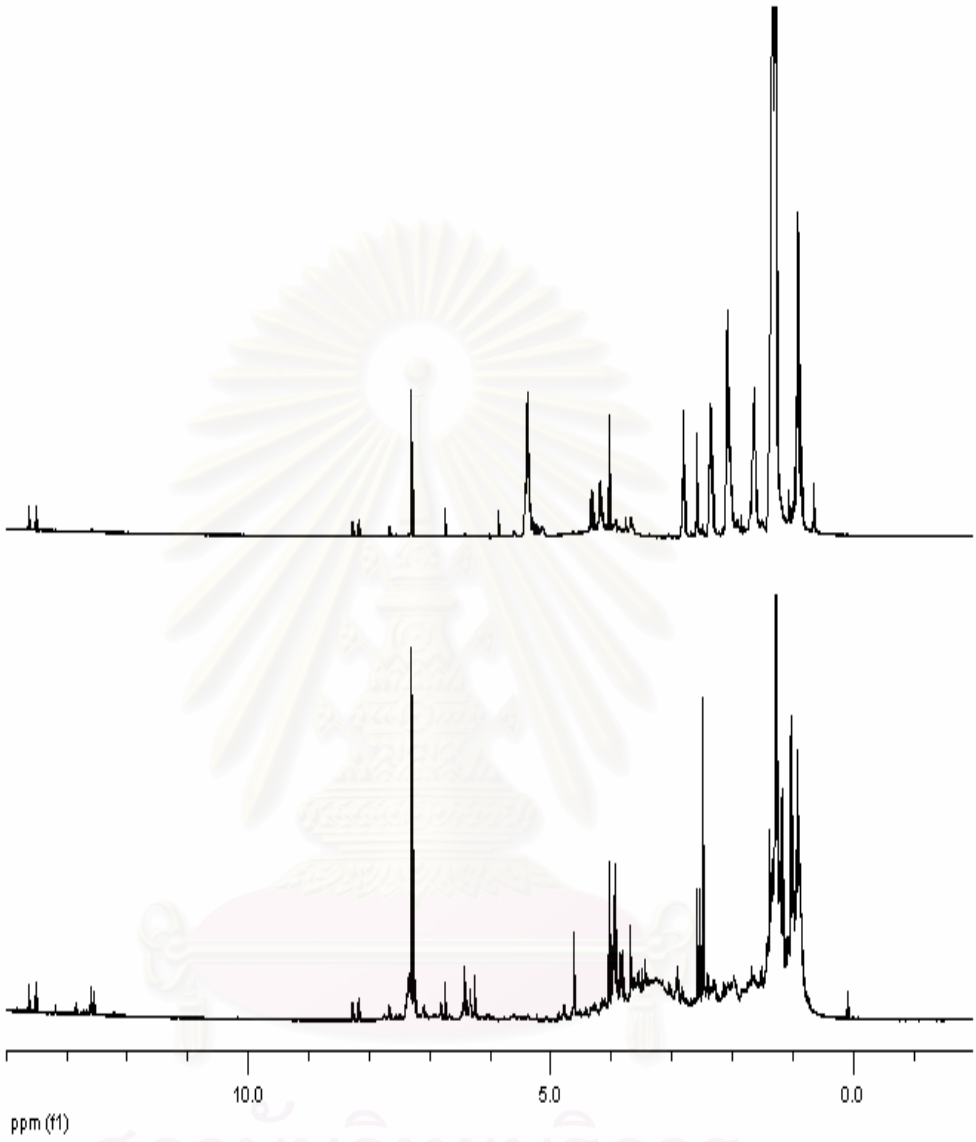


Figure 4.26 The NMR spectrum of crude broth and mycelium of endophytic fungal strain K_BK5

The NMR spectrum of crude broth and mycelium from endophytic fungal strain K_BK5 was similarly. According, cultivation broth and mycelium have TLC pattern bands as same as.

4.7 Isolation of fermentation broth and mycelium of endophytic fungal strain K_BK5

The ethyl acetate crude of fermentation broth (6.4 g) and mycelium (2.5 g) of fungus isolate K_BK5 was subjected to column chromatography using silica gel 200 g and 100 g, respectively and eluted by increasing polarity of solvents from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH. The pooling fraction of the broth and mycelium crude were presented in Table 4.4 and Table 4.5 respectively.

Table 4.4 Characteristic of fraction of broth crude extract by ethyl acetate of endophytic fungus strain K_BK5

Fraction code	Eluents	Fraction No.	Appearance	Weight (mg)
B01	Hex : EtOAc 100:0→ 90:10	1-36	Yellow viscous liquid	145
B02	90:10→80:20	37-54	Orange brown viscous liquid	72
B03	70:30	55-120	Orange solid	251
B04	70:30→60:40	121-187	Red brown viscous liquid	146
B05	55:45→30:70	188-258	Red viscous liquid	183
B06	30:70→20:80	259-293	Red viscous liquid	1272
B07	20:80→15:85	293-307	Red viscous liquid	347
B08	15:85→10:90	308-321	Red powder	211
B09	100	322-346	Red powder	323
B10	EtOAc : MeOH 95:5	347-373	Dark red viscous liquid	285
B11	90:10→85:15	374-445	Dark red viscous liquid	128
B12	85:15→80:20	446-468	Dark red viscous liquid	346
B13	70:30→50:50	469-526	Dark red viscous liquid	200
B14	50:50→30:70	527-581	Violet viscous liquid	429
B15	30:70→10:90	582-595	Violet viscous liquid	236
B16	0:100	596-620	Violet viscous liquid	144

Table 4.5 Characteristic of fraction of mycelium crude extract by ethyl acetate of endophytic fungus strain K_BK5

Fraction code	Eluents	Fraction No.	Appearance	Weight (mg)
M01	Hex : EtOAc 100:0→ 85:15	1-24	Yellow viscous liquid	63
M02	80:20	37-54	Orange solid	22
M03	70:30	55-120	Orange solid	186
M04	70:30→60:40	121-187	Red brown viscous liquid	84
M05	60:40→50:50	188-258	Red viscous liquid	63
M06	40:60→30:70	259-293	Red viscous liquid	48
M07	20:80→10:90	293-307	Red viscous liquid	69
M08	10:90→100	308-321	Red viscous liquid	47
M09	10:90→100	322-346	Red powder	71
M10	EtOAc : MeOH 100:0→95:5	347-373	Dark red powder	72
M11	90:10→85:15	374-445	Dark red viscous liquid	128
M12	85:15→80:20	446-468	Dark red viscous liquid	477
M13	80:20→40:60	469-526	Dark red viscous liquid	184
M14	40:60→30:70	527-581	Violet viscous liquid	78
M15	30:70→10:90	582-595	Violet viscous liquid	225
M16	0:100	596-620	Violet viscous liquid	89

4.8 Purification and physical characterization of isolated compounds

4.8.1 Purification and properties of mixture 1

Mixture 1 was obtained from the elution of silica gel column chromatography of broth and mycelium crude with 90% and 85% ethyl acetate in hexane respective.

FT-IR spectrum : ν_{max} 3449 (br, m), 2927 and 2850 (s), 1750 (s), 1576 and 1462 (m), 1161 (s) cm^{-1}. (Figure E.20, Appendix E)

^{1}H-NMR spectrum ($CDCl_3$ 400MHz.): δ 0.80, 1.27, 1.55, 1.98, 2.32, 4.14, 4.25, 5.26 ppm. (Figure E.21, Appendix E)

^{13}C-NMR spectrum ($CDCl_3$ 400MHz.): δ 13.9, 22.5, 24.6, 26.9, 27.0, 28.8, 28.9, 29.1(4) , 29.1 (7), 29.2 (0), 29.2 (4), 29.3, 29.4 (0), 29.4 (9), 29.5 (4), 29.5 (8), 31.8, 33.8, 33.9, 61.9, 68.7, 129.4, 129.7, 173.0 ppm. (Figure E.22, Appendix E)

4.8.2 Purification of mixture 2

Mixture 2 was obtained from the elution of silica gel column chromatography with 70% ethyl acetate in hexane and 80% ethyl acetate in hexane and was purified by re-crystallization with ethyl acetate and methanol to obtain a red powder from combined fraction B06 was 14 mg. Re-crystallization with DMSO to obtain a red crystal was 10 mg. Mixture 2 showed a single spot at the R_f value 0.70 on TLC plate using 100% ethyl acetate as the mobile phase. TLC spot was visualized with UV lamp (254 and 365 nm) and with I_2. Mixture 2 is soluble in methanol.

UVλ^{EtOH}_{max}nm (log ε) : 300 (4.01)

FT-IR spectrum : ν_{max} 3449 (br, m), 2927 and 2850 (s), 1750 (s), 1576 and 1462 (m), 1161 (s) cm^{-1}. (Figure E.24, Appendix E)

^{1}H-NMR spectrum ($CDCl_3$ 400MHz.): δ 1.26, 1.82, 2.78, 2.92, 2.94, 3.84, 3.87, 3.94, 6.64, 6.12, 12.74, 13.09, 13.41, 13.52 ppm. (Figure E.25, Appendix E)

^{13}C-NMR spectrum ($CDCl_3$ 400MHz.): δ 25.17, 29.74, 29.94, 34.22, 56.75, 56.77, 70.56, 71.40, 107.0, 109.56, 110.08, 127.35, 135.58, 137.61, 160.66, 161.97, 177.4, 183.8 ppm. (Figure E.26, Appendix E)

MS spectrum (m/z) : 326. (Figure E.31, Appendix E)

4.8.3 Purification of compound 1

Compound 1 was obtained from the elution of silica gel column chromatography with 30% ethyl acetate in hexane and was purified by re-crystallization with ethyl acetate to obtain a red crystal from combined fraction B03 and M03 was 27 mg and 7 mg respectively. Compound 1 showed a single spot at the R_f value 0.87 on TLC plate using 70% ethyl acetate in hexane as the mobile phase. TLC spot was visualized with UV lamp (254 and 365 nm) and with I_2. Compound 1 is soluble in organic solvent such as dichloromethane, ethyl acetate and chloroform.

m.p. 240°C

UVλ^{EtOH}_{max}nm (log ε) : 261 (4.50)

FT-IR spectrum : ν_{max} 3449 (broad, m), 2927 and 2850 (s), 1750 (s), 1576 and 1462 (m), 1161 (s) cm^{-1}. (Figure E.4, Appendix E)

^{1}H-NMR spectrum ($CDCl_3$ 400MHz.): δ 2.58, 4.04, 6.27, 7.65, 8.15, 8.24, 13.48, 13.59 ppm. (Figure E.5, Appendix E)

^{13}C-NMR spectrum ($CDCl_3$ 400MHz.): δ 22.0, 56.7, 106.2, 106.9, 112.66, 127.0, 127.3, 131.8, 133.2, 135.6, 145.2, 150.1, 157.4, 160.6, 184.7, 187.6 ppm. (Figure E.6, Appendix E)

MS spectrum (m/z) : 285 $[M+H]^+$. (Figure E.11, Appendix E)

4.8.4 Purification of compound C2

Compound 2 was obtained from the elution of silica gel column chromatography with 100% ethyl acetate and was purified by re-crystallization with ethyl acetate and methanol to obtain a red crystal from combined fraction B06 was 3 mg. Compound 2 showed a single spot at the R_f value 0.45 on TLC plate using 100% ethyl acetate as the mobile phase. TLC spot was visualized with UV lamp (254 and 365 nm) and with I_2. Compound 2 is soluble in methanol .

m.p. 220°C

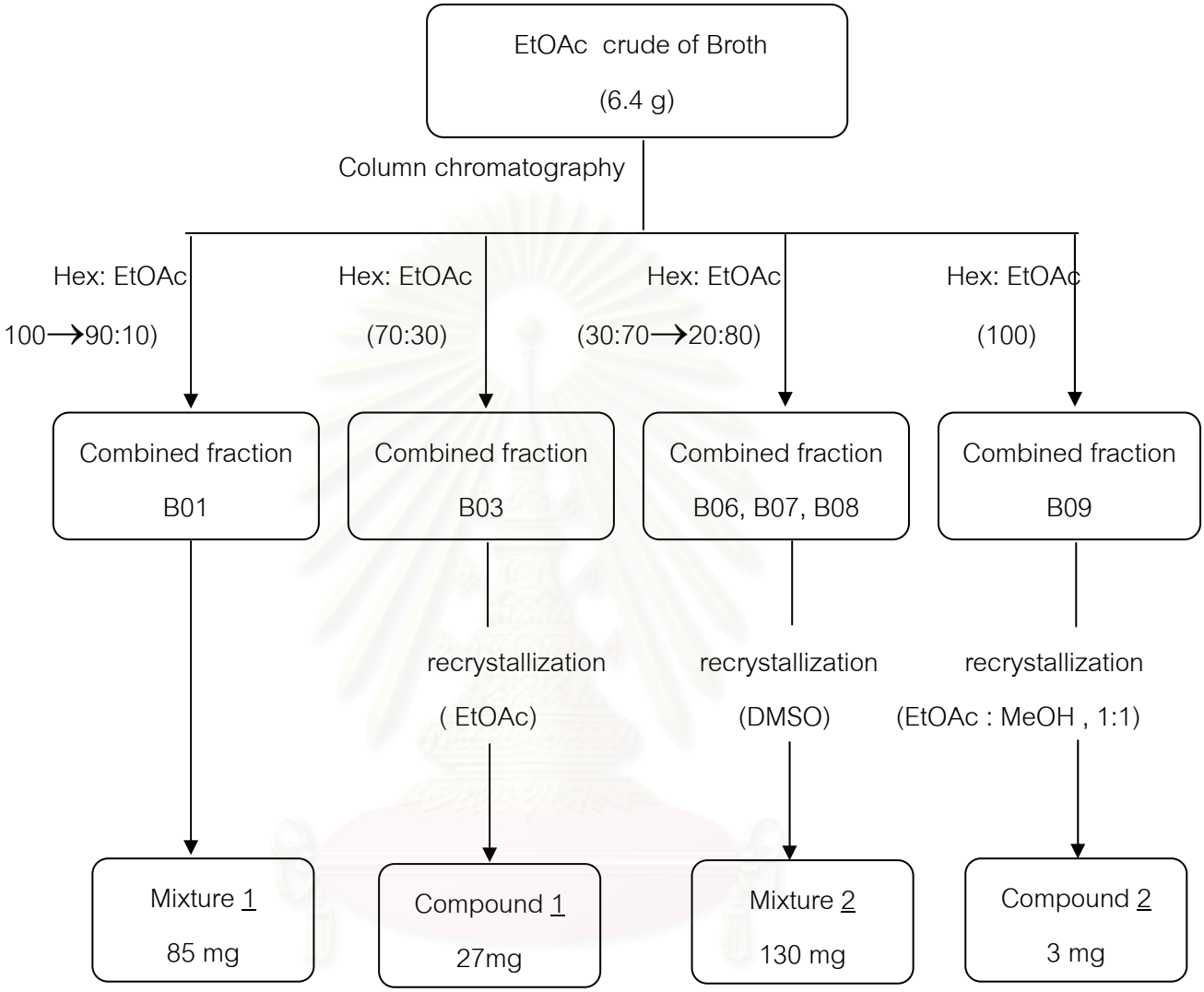
UVλ^{MeOH}_{max}nm (log ε) : 280 (4.31)

FT-IR spectrum : ν_{max} 3449 (br, m), 2927 and 2850 (s), 1750 (s), 1576 and 1462 (m), 1161 (s) cm^{-1}. (Figure E.12, Appendix E)

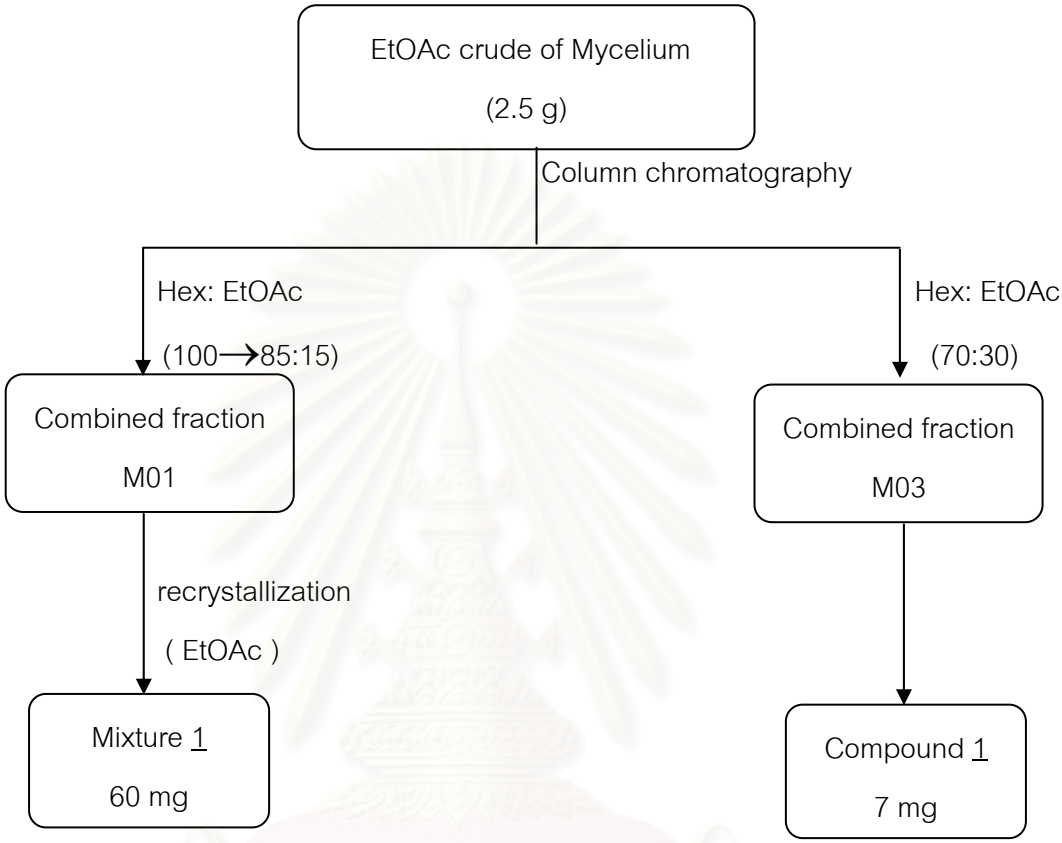
^{1}H-NMR spectrum ($CDCl_3$ 400MHz.): δ 1.34, 1.38, 1.69, 2.19, 2.32, 2.38, 2.44, 3.48, 3.81, 3.92, 4.78, 6.48 ppm. (Figure E.13, Appendix E)

^{13}C-NMR spectrum ($CDCl_3$ 400MHz.): δ 25.6, 40.7, 40.8, 46.2, 48.5, 55.2, 70.1, 72.3, 73.9, 98.6, 107.9, 127.2, 137.2, 155.5, 155.5, 158.3, 158.4, 202.3 ppm. (Figure E.14, Appendix E)

MS spectrum (m/z) : 324 (Figure E.19, Appendix E)



Scheme 4.2 Diagram of isolation procedure for EtOAc crude extract of broth of endophytic fungal strain K_BK5



Scheme 4.3 Diagram of isolation procedure for EtOAc extract crude of mycelium of endophytic fungal strain K-BK5

EtOAc crude extract of endophytic fungal strain K_BK5 produced metabolites as Mixture 1 36.25 mg/l, Compound 1 8.5 mg/l, Mixture 2 32.5 mg/l and Compound 2 0.75 mg/l.

4.9 X-ray Diffraction

Red crystal of compound 1 was recrystallized from ethyl acetate and identified by X-ray diffraction analyses. All data were collected at room temperature using graphite monochromated Mo Kα Radiation (lambda = 0.71069 °A) on BRUKER SAMART CCD diffractrometer. The data were corrected for Lorentz and polarization effects. The crystal data of compound 1 were given in Appendix F.

The structure were solved by direct methods using SHELXLS-97 and refined by full matrix least-squares on F^2 using SHELKLS-97 with anisotropic thermal parameters for all non-hydrogen atoms. All hydrogen atoms were found from difference Fourier maps and were included in refinement. The fraction coordinates of non-hydrogen atom and selected bond distances and angles of compound 1 were listed in Appendix F.

4.10 Structure elucidation of the purified compounds

4.10.1 Structure elucidation of Compound 1

The IR spectrum of compound 1 (Figure-E.4 in Appendix E) revealed the presence of alcohol group according to the broad absorption band between 3101 to 3689 cm^{-1} and the medium absorption band at 2916 and 2845 cm^{-1} suggested methyl and methylene group stretching vibration. In addition, there is the presence of C=O stretching according to the strong absorption band at 1587 and C=C stretching according to the strong absorption band at 1472 cm^{-1} and C-O stretching vibration of alcohol at 1230 cm^{-1}. The IR spectrum of compound 1 is summarized in Table 4.6

Table 4.6 The IR absorption band assignment of compound 1.

Wave number (cm^{-1})	Intensity	Vibration
3417	Broad	O-H stretching vibration of alcohol
2845,2916	Medium	C-H stretching vibration of $-CH_3$, $-CH_2$
2366	Weak	C=O stretching vibration of carbonyl group
1587	Strong	C=O stretching vibration of carbonyl group
1472	Strong	C=C stretching vibration of aromatic ring
1230	Strong	C-O stretching vibration of alcohol
955	Medium	C-H out of plane bending vibration
813	Strong	C-H out of plane bending vibration

The ^{1}H-NMR spectrum (Table 4.7, Figure E.5, in Appendix E) of compound 1 revealed in addition to methyl and methoxy proton singlet at δ2.58(3H,s) and 4.64(3H,s) ppm, and signals from aromatic protons in a 1,2,4-arrangement at 7.65(1H,d, *J*=8 Hz), 8.15(1H,s) and 8.24(1H,d, *J*=8 Hz) ppm, an isolated aromatic proton at δ6.27(1H,s), and two *peri*-hydroxyl groups at δ13.48 and 13.59 ppm.

The ^{13}C-NMR spectrum (Table 4.7, Figure E.6, in Appendix E) of compound 1 showed 16 signals, which two carbonyl carbon at 187.63,and 184.65 ppm. Four methine carbon signals at 135.6, 127.3, 127.0, and 106.9 ppm., a methyl carbon at 22.0 ppm and a methoxy carbon at 56.6 ppm. The signal at 106.2, 112.7, 131.8, 133.2, 145.2, 150.1, 157.4, and 160.6 ppm. were quaternary carbon.

The MS spectrum (Figure E.11 in Appendix E) showed the $[M+H]^+$ ion peak at m/z 285. The mass spectrum indicated that it possesses the molecular weight 284.

The information from 2D-NMR technique; HSQC correlation (Table 4.8, Figure E.7 in Appendix E), HMBC(Table 4.8, Figure E.8 in Appendix E),COSY(Table 4.8, Figure E.9 in Appendix E) and NOESY correlation (Table 4.8, Figure E.10 in Appendix E) were used to assist the interpretation of compound 1 structure.

Table 4.7 The HSQC spectral data of Compound 1

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
22.0(q)	2.58s
56.7(q)	4.64s
106.2(s)	-
106.9(d)	6.27s
112.7(s)	-
127.0 (d)	8.24d (*J*=8)
127.3(d)	8.15s
131.8(s)	-
133.2(s)	-
135.6(d)	7.65d (*J*=8)
145.2(s)	-
150.1(s)	-
157.4(s)	-
160.6(s)	-
184.7(s)	-
187.6(s)	-
	13.48s
	13.59s

Table4.8 The correlation of gHSQC, g HMBC, COSY and NOESY of compound 1

Position	δ_C	δ_H	COSY	NOESY	HMBC
1	150.1(s)	13.48(s)	-	-	C-1, C-2, C-12
1a	112.7(s)	-	-	-	-
2	157.4(s)	-	-	-	-
3	106.9(s)	6.72(1H, s)	-	OCH_3-2(4.64)	C-1, C-2, C-4, C-4a
4	160.6(s)	13.59(s)	-	-	C-4, C-4a
4a	106.2(s)	-	-	-	-
5	127.0(d)	8.24(1H, d, *J*=8)	H-2(7.65) H-8(8.15)	H-6(7.65)	C-6, C-9a, C-10
5a	131.8(s)	-	-	-	-
6	135.6(d)	7.65(1H, d, *J*=8)	H-8(8.15) H-5(8.24)	CH_3-7(2.58) H-5(8.24)	C-8, C-10a
7	145.2(s)	-	-		-
8	127.3(d)	8.15(1H, s)	CH_3-7 (2.58),H-6(7.65), H-5(8.24)	CH_3-7(2.58)	C-6, C-9, C-10a
8a	133.2(s)	-	-	-	-
9	187.6(s)	-	-	-	-
10	184.7(s)	-	-	-	-
2-OCH_3	56.7(q)	4.64(3H,s)	-	H-3(6.72)	C-2, C-3
7-CH_3	22.0(q)	2.58(3H,s)	H-8(8.15)	H-6(7.65) H-8(8.15)	C-6, C-8, C-10a

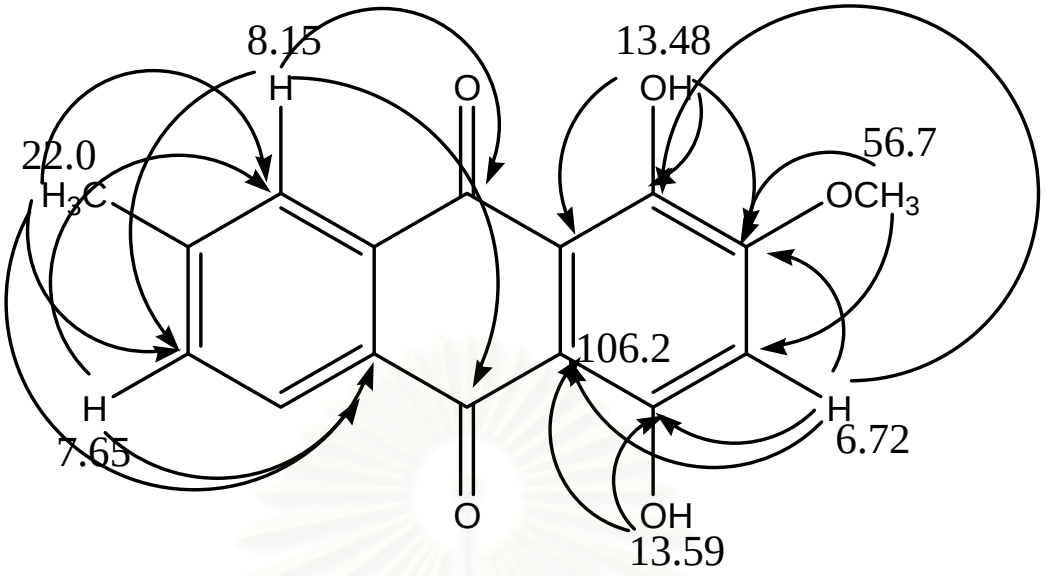


Figure 4.27 The HMBC correlation of compound 1

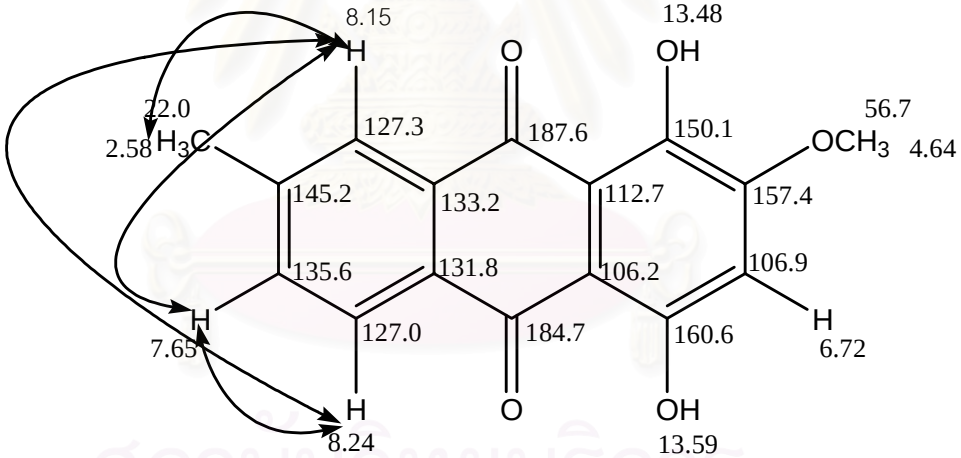


Figure 4.28 The COSY correlation of compound 1

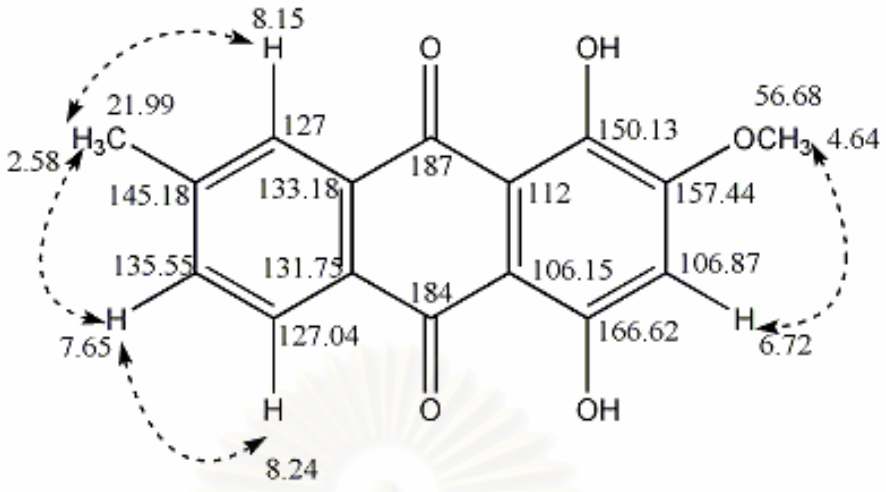


Figure 4.29 The NOESY correlation of compound 1

Compound 1 showed spectral data similar to that of Austrocortinin, (1,4-Dihydroxy-2-methoxy-7-methylanthracene-9,10-dione) from fruit bodies of a red Australian toadstool belonging to the genus *Cortinarius* (Michelle et al., 1985) and pigments from the fungus Dermocybe splendida (Melvyn et al., 2000). From the literature, Austrocortinin was a red crystals ($C_{16}H_{12}O_5$), m.p. 237-240 °C; UVλ^{EtOH}_{max}nm (log ε) : 210 (4.51), 262.5 (4.50), 302 (3.09), 458sh (3.85), 482 (3.93), 514 (3.76); IR ν_{max}^{KBr}cm^{-1} : 1593, 1620. The ^{1}H-NMR signal of compound 1 and Austrocortinin are presented in Table 4.9.

Table 4.9 The ^{1}H-NMR spectral data of compound 1 (in CD_3OD) and Austrocortinin (in CD_3OD)

Position	δ_H(ppm)	
	Compound 1	Austrocortinin
1	13.48(1H, *s*, *peri*-OH)	13.46(1H, *s*, *peri*-OH)
1a	-	-
2	-	-
3	6.72(1H, *s*)	6.70(1H, *s*)
4	13.59(1H, *s*, *peri*-OH)	13.58(1H, *s*, *peri*-OH)
4a	-	-
5	8.24(1H, *d*, *J*=8)	8.24(1H, *d*, *J*=8.1)
5a	-	-
6	7.65(1H, *d*, *J*=8)	7.63(1H,*dd*,*J*=8.1,1.9)
7	-	-
8	8.15(1H, *s*)	8.13(1H, *s*)
8a	-	-
9	-	-
10	-	-
2-OCH_3	4.64(3H,*s*)	4.01(3H,*s*)
7-CH_3	2.58(3H,*s*)	2.55(3H,*s*)

Moreover, the structure of compound 1 was also confirmed by X-ray diffraction analysis, which indicated that compound 1 was identical to Austrocortinin. The ORTEP drawing of compound 1 is shown in Figure4.28.

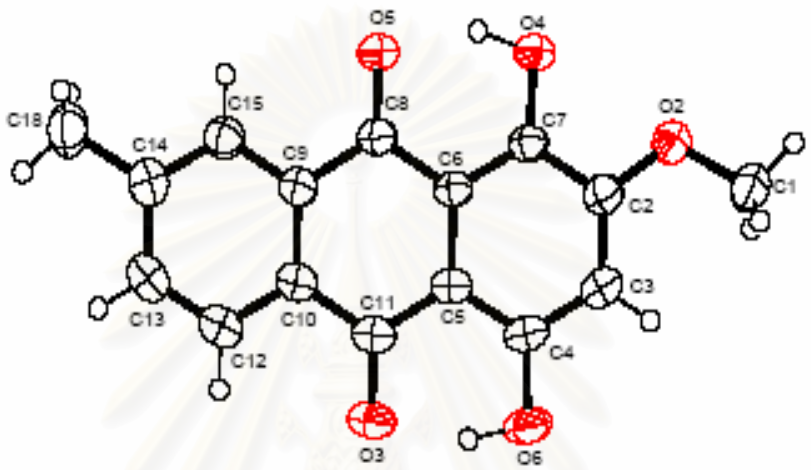


Figure 4.30 The ORTEP drawing of compound 1

From all spectral data (IR, H and C NMR spectra including 2D-NMR, mass spectrum and X-ray diffraction analysis), it was concluded that compound 1 is Austrocortinin. The structure IS presented in Figure 4.28.

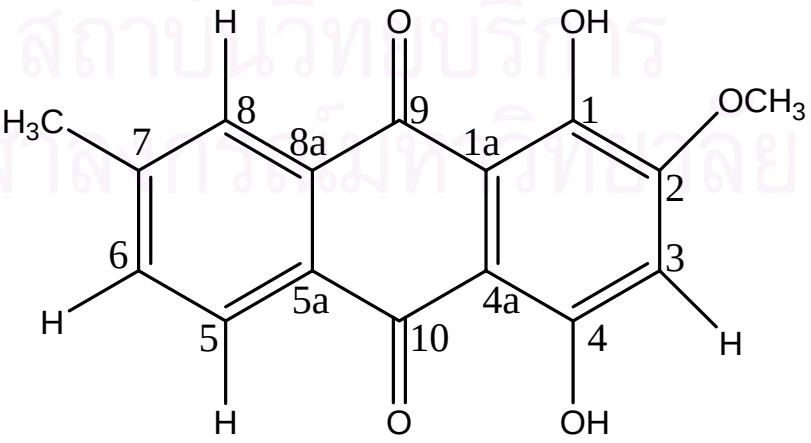


Figure 4.31 The chemical structure of compound 1

4.10.2 Structure elucidation of compound 2

The IR spectrum of compound 2 (Figure E.12 in Appendix E) revealed the presence of alcohol group according to the broad absorption band between 3019 to 3689 cm^{-1} and the medium absorption band at 2845 and 2927 cm^{-1} suggested methyl and methylene group stretching vibration. In addition, there is the presence of the carbonyl stretching according to the medium absorption band at 1636 cm^{-1}. The IR spectrum of compound 2 is summarized in Table 4.10.

Table 4.10 The IR absorption band assignment of compound 2.

Wave number (cm^{-1})	Intensity	Vibration
3411	Broad	O-H stretching vibration of alcohol
2845,2927	Medium	C-H stretching vibration of -CH_3, -CH_2
1728	Weak	C=O stretching vibration of carbonyl group
1636	Strong	C=O stretching vibration of carbonyl group
1423	Medium	C=C stretching vibration
1265, 1042	Medium	C-O stretching vibration
813	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum (Table 4.11, Figure E.13, in Appendix E) of compound 2 revealed in addition to methyl and methoxy proton singlet at δ1.34(3H,s), δ3.92 ppm. and an isolated aromatic proton at 6.48 (1H,s) ppm. Moreover, it possessed five methylene groups at δ1.38 (2H, dd, *J*=12.4 and 12.6 Hz), 1.69 (2H, q, *J*=12.4 Hz),

2.32(2H, ddd, *J*=4, 4.4 and 12.8 Hz), 2.38 (2H,ddd) and 3.81(2H, s) ppm. and four methine groups at δ2.19(1H, ddd, *J*=3.6 Hz), 2.44(1H, dd, *J*=3.2 and 12 Hz), 3.48(1H, dd, *J*=4.8 and 12 Hz), and 4.78(1H, d, *J*=10 Hz).

The ^{13}C-NMR spectrum (Table 4.11, Figure E.14, in Appendix E) of compound 2 showed 19 signals, which carbonyl carbon at 202.29 ppm. Five methine carbon signals at 40.8, 46.2, 72.3, 73.9, 98.6 ppm., methelene carbons at 28.7, 40.7, 48.5. A methyl carbon at 25.6 ppm and a methoxy carbon at 55.2 ppm. The signal at 70.1, 107. 8, 127.2, 137.2, 155.5, 155.5, 158.3, 158.4, 202.3 ppm. were quaternary carbon.

The MS spectrum (Figure E.19 in Appendix E) showed the $[M+Na]^+$ ion peak at m/z 339. The mass spectrum indicated that it possesses the molecular weight 324.

The information from 2D-NMR technique; HSQC correlation (Table 4.12, Figure E.15 in Appendix E), HMBC(Table 4.12, Figure E.16 in Appendix E),COSY(Table 4.12, Figure E.17 in Appendix E) and NOESY correlation (Table 4.12, Figure E.18 in Appendix E) were used to assist the interpretation of the structure of compound 2.

Table 4.11 The HSQC spectral data of Compound 2

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
25.6(q)	1.34s
28.7(t)	1.69q(*J*=12.4)
	2.32ddd(*J*=4,4.4and12.8)
40.7(t)	1.38dd(*J*=12.4,12.6)
	2.37ddd
40.8(d)	2.19ddd(*J*=3.6)
46.2(d)	2.44dd(*J*=3.2,12)
55.2(s)	3.92s
70.1(s)	-
73.0(d)	4.78d(*J*=10)
73.9(d)	3.48dd (*J*=4.4,10)
98.6(d)	6.48s
107.8(s)	-
127.2(s)	-
137.2(s)	-
155.5(s)	-
158.3(s)	-
202.3(s)	-

Table4.12 The correlation of gHSQC, g HMBC, COSY and NOESY of compound C2

Position	δ_C	δ_H	COSY	NOESY	HMBC
1	137.2(s)	-	-	-	-
1a	127.2(s)	-	-	-	-
2	155.5(s)	-	-	-	-
3	98.6(d)	6.48(1H, s)	-	H-2(3.92)	C-1, C-2, C-4, C-4a
4	158.3(s)	-	-	-	-
4a	107.8(s)	-	-	-	-
5	28.7(q)	1.69(2H, q, *J*=12.4) 2.32(2H, ddd, *J*=4,4.4 and12.8)	H-5(2.32), H-10a(2.44), H-6(3.48) H-5, H-6(3.48)	H-8(2.37)	C-6, C-7, C-10a C-6, C-7, C-8
5a	46.2(d)	2.44(1H, dd, *J*=3.2 and12)	H-5(1.69), H-9a(2.19)	H-8(1.38), H-9(4.78)	C-1,C-1a,C-2, C-3,C-4
6	73.9(d)	3.48(1H, dd, *J*=4.4and10.4)	H-5(2.37), H-5(1.69)	H-8(1.38), H-5(2.32)	C-5,C-7
7	70.1(s)	-	-	-	-
8	40.7(t)	1.38(2H, dd, *J*=12.4 and12.6) 2.37(2H, ddd)	H-9a(2.19), H-8(2.37) H-8(1.38),H-9a(2.19),	H-1(2.44), H-9(4.78) H-5(1.69)	C-8, C-9 C-5, C-6, C-7, C-8, C-10a
8a	40.8(d)	2.19(1H, ddd, *J*=3.6)	H-8(1.38), H-8(2.37), H-9(4.78)	-	C-8
9	43.0(d)	4.78(1H,d, *J*=10)	H-9a(2.14)	H-8(1.38), H-10a(2.44)	C-1, C-1a, C-4a, C-8
10	202.3(s)	-	-	-	C-6, C-7, C-10
2-OCH_3	55.2(s)	3.92(s)	-	H-3(6.48)	C-6,C-8
7-CH_3	25.6(q)	1.34(3H,s)	-	H-1(2.44), H-1(3.48), H-1(4.78)	C-6, C-7, C-8

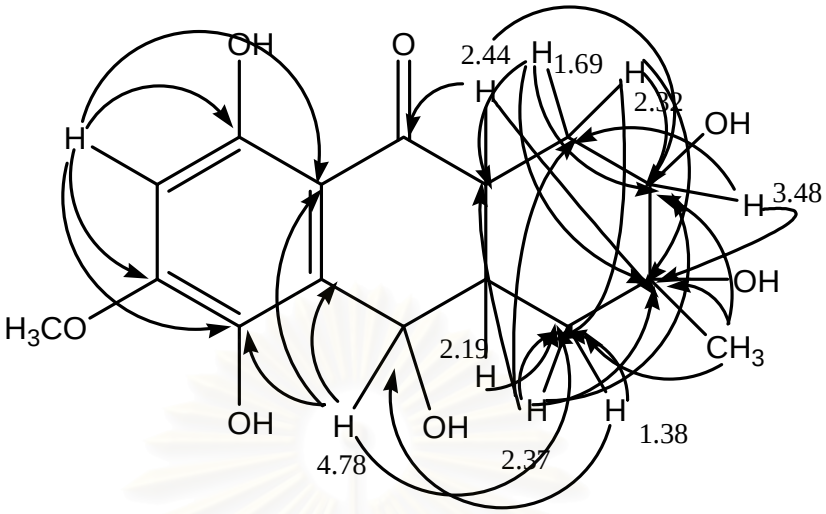


Figure 4.32 The HMBC correlation of compound 2

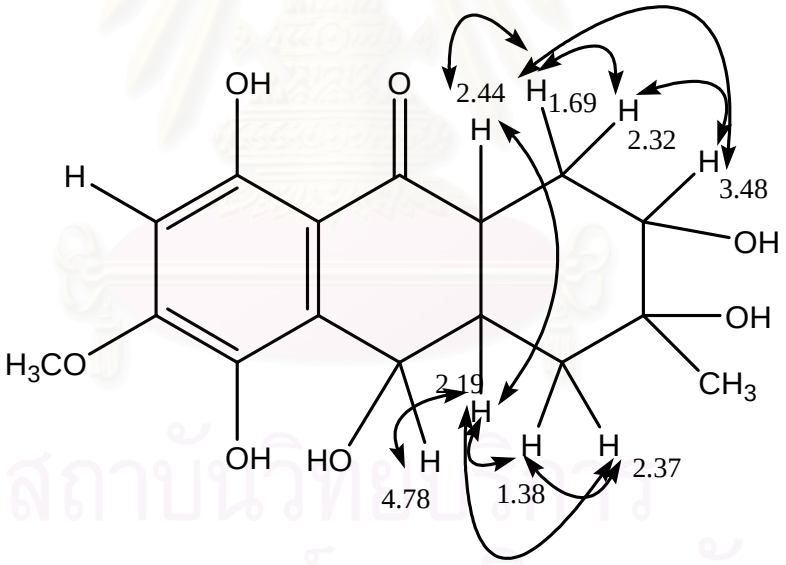


Figure 4.33 The COSY correlation of compound 2

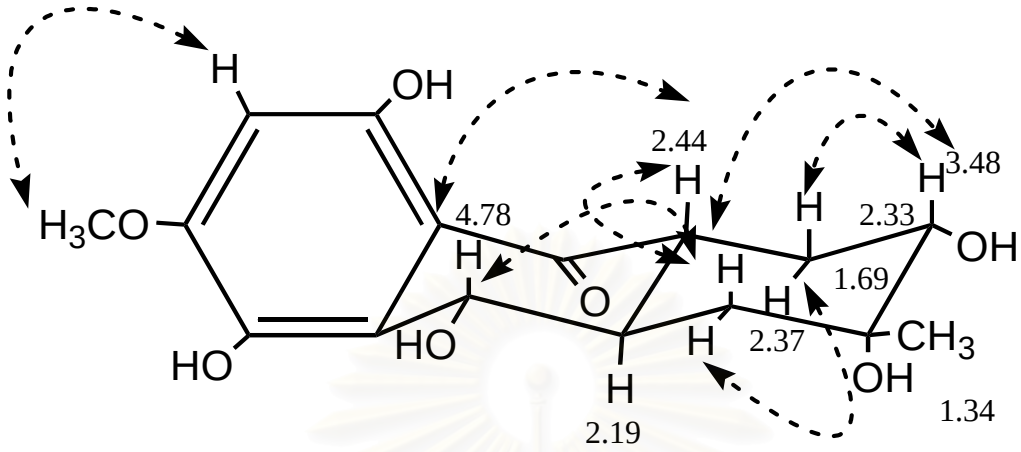


Figure 4.34 The NOESY correlation of compound 2

After elucidation of compound 2 by 2D NMR technique. This result indicated that the structure of compound 2 is identical to 1,4,6,7,9-Pentahydroxy-2-methoxy-7-methylanthracene-10-dione. Thus, it could be concluded that compound 2 was a 1,4,6,7,9-Pentahydroxy-2-methoxy-7-methy-5,6,7,8,8a,9-hexahydroanthracene10(10aH)-one. The structure is presented in Figure 4.32.

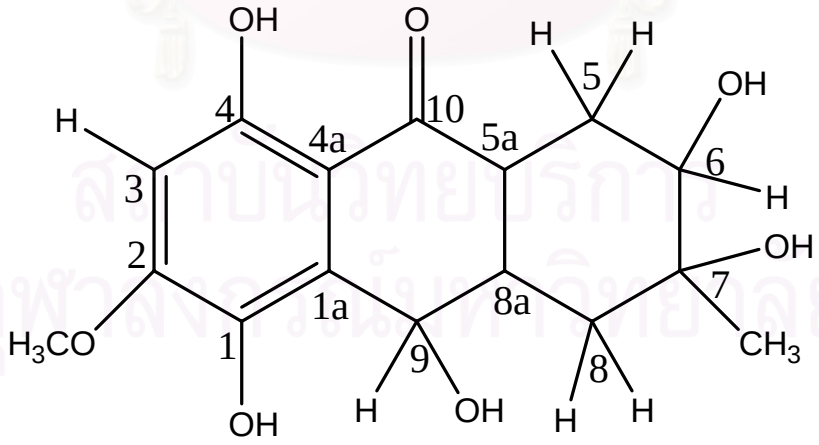


Figure 4.35 The chemical structure of compound 2

4.10.3 Structure elucidation of mixture 1

Mixture 1 (145 and 63 g) was obtained as yellow oily liquid with rancid odor from silica gel column chromatography of combined fraction B01 and M01, eluted with 0-10% and 0-15% EtOAc in Hexane, respectially.

The IR spectrum of mixture 1 (Figure E.20, appendix E) showed important absorption bands at 3449 cm^{-1} of O-H stretching vibration of hydroxyl group , 2927 and 2850 cm^{-1} of C-H stretching vibration, 1750 cm^{-1} of C=O vibration of ester, 1576 and 1462 cm^{-1} of C-H bending and 1167 cm^{-1} of C-O stretching vibration of ester. The IR absorption bands of mixture B1 were assigned as shown in Table 4.13

Table 4.13 The IR absorption bands assignment of mixture 1

Wave number (cm^{-1})	Intensity	Tentative assignment
3449	Broad, Medium	O-H stretching vibration of hydroxyl group
2927,2850	Sharp, Strong	C-H stretching vibration
1750	Sharp, Strong	C=O stretching vibration of ester
1576,1462	Sharp, Medium	C-H bending vibration
1167	Sharp, Medium	C-O stretching vibration of ester
721	Sharp, Weak	C-H rocking mode of $-(CH_2)_n-$

The ^{1}H-NMR spectrum (Figure E.21 in appendix E) of mixture 1 indicated that it possesses a methyl proton at δ 0.80 ppm, three methylene protons at 1.27, 1.55, and 1.98 ppm, a methylene proton attached to oxygen (-OCH_2-) at 2.32 ppm, two methine protons attached to oxygen (-O-C*H* -) at 4.14, 4.25, and two olefinic protons at 5.26 and 5.30 ppm.

The ^{13}C-NMR spectrum (Figure E.22 in appendix E) of mixture 1 showed one methyl carbon signal at δ 13.9 ppm, nineteen methylene carbon signals at 22.5, 24.6, 26.9, 27.0, 28.8, 28.9, 29.0, 29.1 (4), 29.1 (7), 29.2 (0), 29.2 (4), 29.3, 29.4 (0), 29.4 (9), 29.5 (4), 29.5 (8), 31.8, 33.8, and 33.9 ppm, methylene carbon attached to oxygen at

61.9, methine carbons attached to oxygen at 68.7, two olefinic carbon signals at δ 129.4 and 129.7 ppm, and the carbon signal at δ 173.0 ppm should be the carbonyl group of ester.

The structure of mixture 1 was elucidated base on the spectroscopic data and the comparison with the retention time of standard methyl ester of those fatty acid by gas chromatography(Table 4.14). The relative percentages of fatty acids were determined by the area of the peaks in the chromatograms. Chromatogram of mixture 1 is shown in Figure D4 in appendix D and comparison their spectral data with those of published values results is shown in Table 4.15.

Table 4.14 GC Retention time of mixture 1 and standard methyl esters (Std.-ME) of those fatty acids.

Fatty acid	Retention time(min)	
	Std.-ME	Mixture M1
C14:0	5.697	5.900
C16:0	10.295	10.756
C16:1	11.292	12.600
C18:0	18.999	19.967
C18:1	21.558	22.733
C18:2	27.005	28.457
C18:3	34.258	37.583

Table 4.15 The chemical structure and the name of fatty acids composition of mixture 1

Chemical name	Common name	Symbol	Relative fatty acids composition (%) of total fatty acids
SATURATED			
Methyl myristate	Myristic acid	C14:0	1.039
Methyl palmitate	Palmitic acid	C16:0	36.406
Methyl palmitoleate	Stearic acid	C18:0	11.691
UNSATURATED			
Methyl palmitoleate	Palmitoleic acid	C16:1	0.541
Cis-methyl oleate	Oleic acid	C18:1	34.927
Methyl linoleate	Linoleic acid	C18:2	12.581
Methyl linolenate	Linolenic acid	C18:3	1.345

From all data, mixture 1 should be triglyceride which composed of three saturated fatty acids including, myristic acid, palmitic acid, and stearic acid and four unsaturated fatty acids including palmitoleic acid, oleic acid, linoleic acid, and linolenic acid. The major components of saturated and unsaturated fatty acid in mixture 1 were palmitic acid and oleic acid, respectively and found high percentage of linoleic acid and linolenic acid. These are useful unsaturated fatty acid which the human body cannot synthesized itself.

4.10.4 Structure elucidation of Mixture 2

The IR spectrum of mixture 2 (Figure E.24 in Appendix E) revealed the presence of alcohol group according to the broad absorption band between 3038 to 3665 cm^{-1} and the medium absorption band at 2916 and 2850 cm^{-1} suggested methyl and methylene group streching vibration. In addition, there is the presence of the carbonyl stretching according to the strong absorption band at 1592 cm^{-1}. The C-O streching vibration of hydroxyl groups at 1271 cm^{-1} . The IR spectrum of mixture 2 is summarized in Table 4.16

Table 4.16 The IR absorption band assignment of mixture 2

Wave number (cm^{-1})	Intensity	Vibration
3368	Broad	O-H streching vibration of alcohol
2916,2850	Medium	C-H streching vibration of -CH_3, -CH_2
1703	Weak	C=O streching vibration of carbonyl group
1592	Strong	C=O streching vibration of aromatic ring
1434	Medium	C=C streching vibration of aromatic ring
1271	Medium	C-O streching vibration
1021	Weak	C-O streching vibration
813	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum (Table 4.10, Figure E.25 in Appendix E) of mixture 2 possessed three methyl groups at δ1.26(3H),2.94(3H,s) and 3.87(3H,s) ppm, four methylene groups at δ1.28(2H,s), 2.78(2H,d,*J*=18.4Hz),2.92(2H,d,*J*=18.4Hz) and 2.94(2H,s) ppm. and four methine groups at δ3.84(1H), 3.94(1H,s), 6.12(1H,s), and 6.64(1H) ppm. Four hydroxyl protons were signal at δ12.74, 13.09, 13.41 and 13.52 ppm.

The ^{13}C-NMR spectrum (Table 4.10, Figure E.26 in Appendix E) of mixture 2 showed 16 signals, which two carbonyl carbon at 187.63, 184.65 ppm. Four methine carbons signal at 135.55, 127.30, 127.04, 106.87 ppm., a methyl carbon at 21.99 ppm and a methoxy carbon at 56.63 ppm.

The MS spectrum (Figure E.31 in Appendix E) showed the $[M+Na]^{+}$ ion peak at m/z 326. The mass spectrum indicated that it possesses the molecular weight 304.

The NMR spectrum of mixture 2 was found to have peaks of major compound in mixture 2 and another peaks were compared signal by signal with compound 1. Therefore, mixture 2 was a mixture of compound 1 with other substances.

The information from 2D-NMR technique; HSQC correlation (Figure E.28 in Appendix E), HMBC(Table 4.7, Figure E.28 in Appendix E),COSY(Table 4.7, Figure E.29 in Appendix E) and NOESY correlation (Table 4.7, Figure E.30 in Appendix E). After deleting signal of compound 1, 2D-NMR information were used to assist the interpretation of the structure of mixture 2.

Table 4.17 The HSQC spectral data of Mixture 2

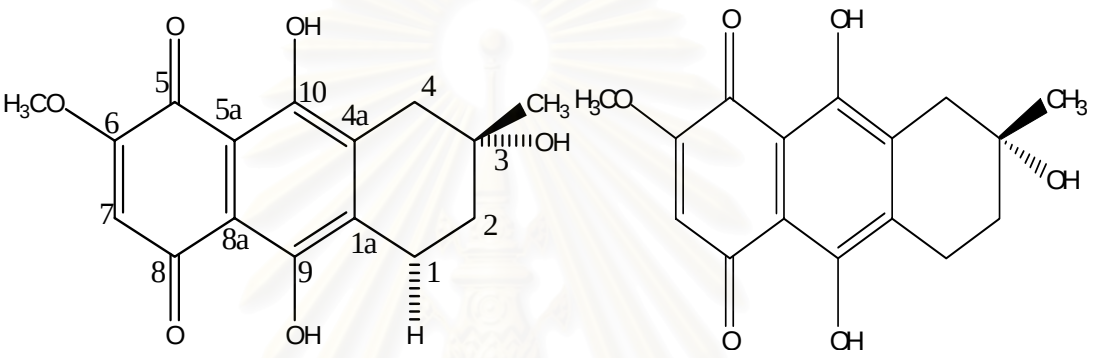
^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
25.2(q)	1.26s
29.8(t)	1.82m
29.9(t)	2.94m
34.2(t)	2.78d(*J*=18.4)
	2.92d(*J*=18.4)
56.8 (q)	-
70.6(s)	3.87s
71.4(d)	3.84s
109.6(d)	6.12s
110.1(s)	-
127.4(s)	-
135.6(s)	-
137.6 (s)	-
160.7(s)	-
162.0(s)	-
177.2(s)	12.74s
184.0(s)	13.09s

Because mixture 2 was a mixture of a few anthraquinone. The major compound of mixture 2 can interprete from 2D-NMR. Mixture 2 showed spectral data similar to that of Deoxyaustrocortirubin from pigments from the fungus Dermocybe splendida (Melvyn et al., 2000).. From the literature, 1-Deoxyaustrocortirubin was a red needles ($C_{16}H_{16}O_6$), m.p. 208-214°C; $[\alpha]_D^{20}$: -59+_10°C ($CHCL_3$; *c*0.049) UVλ_{max}^{EtOH}nm (log ε) : 226 (4.53), 304 (4.01), 472 (3.91), 501 (4.00), 538 (3.84) ; IR $\nu_{max}^{KBr}cm^{-1}$: 3449, 1598. The ^{1}H-NMR signal of mixture 2 and deoxyaustrocortirubin are presented in Table 4.18.

Table 4.18 The ^{1}H-NMR spectral data of mixture 2 (in CD_3OD) and deoxyaustrocortirubin (in CD_3OD)

Position	δ_H(ppm)	
	Mixture 2	Deoxyaustrocortirubin
1	-	-
1a	-	-
2	1.82(2H, m)	1.73(2H,ddd,*J*=13.8,7.7,7.7) 1.95(2H,dddd,*J*=13.8,5.5,5.5,1.8)
	-	-
4	2.78(2H, *d, J*=18.4) 2.92(2H, *d, J*=18.4)	2.7(2H, brd, *J*=18.9) 2.86-2.91(2H, m)
4a	-	-
5	-	-
5a	-	
6	-	-
7	6.12(1H, s)	6.21(1H, s)
8	12.74(1H, *s*, *peri*-OH)	12.88(1H, *s*, *peri*-OH)
8a	13.09(1H, *s*, *peri*-OH)	13.23(1H, *s*, *peri*-OH)
9	-	
10	-	-
6-OCH_3	3.87(3H,*s*)	3.93(3H, s)
3-CH_3	1.26(3H,*s*)	1.43(3H, *s*)

After elucidation of mixture 2 by 2D NMR technique, the chemical shift on ^{1}H-NMR spectrum of mixture 2 and deoxyaustrocortirubin were compared signal by signal. This result indicated that the structure of major compound of mixture 2 is identical to deoxyaustrocortirubin. The possible structure of mixture 2 and deoxyaustrocortirubin are presented in Figure 4.22



Mixture 2 Deoxyaustrocortirubin

Figure 4.36 The chemical structure of mixture 2 and Deoxyaustrocortirubin

4.11 Biological activities

4.11.1 Antimicrobial activity of crude extract and pool fractions

The antimicrobial activity of crude extract and pool fractions were evaluated by the agar well diffusion method as described in section 3.8.4. The crude extract and fraction were examined at a concentration of 4 mg/ml. The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganism. Antimicrobial activity of crude extract and fractions separated from crude broth and mycelium are shown in Table 4.18, Table 4.19 and Table 4.20, respectively.

Table 4.19 Antimicrobial activities of the crude extracts from fungus isolate K_BK5

Test microorganisms	Inhibition of crude extract against tested microorganisms	
	Culture broth	Mycelium
	4 mg/ml	4 mg/ml
B. subtilis	++++	++++
S. aureus	-	-
E. coli	-	-
P. aureuginosa	-	-
C. albicans	-	-
S. cerevisiae	-	-

Activities were classified according to the diameter of the point of application of the sample

++++ = more than 30 mm

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

\+ = Inhibition zone more than 8 mm

\- = No inhibition

Table 4.20 Antimicrobial activities of each fraction of culture broth extracts

Fraction	Inhibition activities against test microorganisms					
code	*B. subtilis*	*S.aureus*	*E.coli*	*P.aeruginosa*	*C. albicans*	*S. cerevisiae*
B01	-	-	-	-	-	-
B02	-	-	-	-	-	-
B03	+	-	-	-	-	-
B04	++++	-	-	-	-	-
B05	++++	-	-	-	-	-
B06	++++	-	-	-	-	-
B07	++++	-	-	-	-	-
B08	++++	-	-	-	-	-
B09	++++	-	-	-	-	-
B10	+++	-	-	-	-	-
B11	+++	-	-	-	-	-
B12	++	-	-	-	-	-
B13	++	-	-	-	-	-
B14	+	-	-	-	-	-
B15	+	-	-	-	-	-
B16	+	-	-	-	-	-

Activities were classified according to the diameter of the point of application of the sample

++++ = more than 30 mm

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

\+ = Inhibition zone more than 8 mm

\- = No inhibition

Table 4.21 Antimicrobial activities of each fraction of mycelium extracts

Fraction	Inhibition activities against test microorganisms					
code	*B. subtilis*	*S.aureus*	*E.coli*	*P.aeruginosa*	*C. albicans*	*S. cerevisiae*
M01	-	-	-	-	-	-
M02	-	-	-	-	-	-
M03	+	-	-	-	-	-
M04	++++	-	-	-	-	-
M05	++++	-	-	-	-	-
M06	++++	-	-	-	-	-
M07	++++	-	-	-	-	-
M08	++++	-	-	-	-	-
M09	++++	-	-	-	-	-
M10	++++	-	-	-	-	-
M11	+++	-	-	-	-	-
M12	+++	-	-	-	-	-
M13	++	-	-	-	-	-
M14	+	-	-	-	-	-
M15	+	-	-	-	-	-
M16	+	-	-	-	-	-

Activities were classified according to the diameter of the point of application of the sample

++++ = more than 30 mm

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

+ = Inhibition zone more than 8 mm

- = No inhibition

Results indicated that endophytic fungal strain K_BK5 produce biologically active compounds in both cultivation broth and mycelium. The crude cultivation broth, mycelium and pool fractions have red pigment and have high potentially active compounds against *B. subtilis* ATCC 6633.

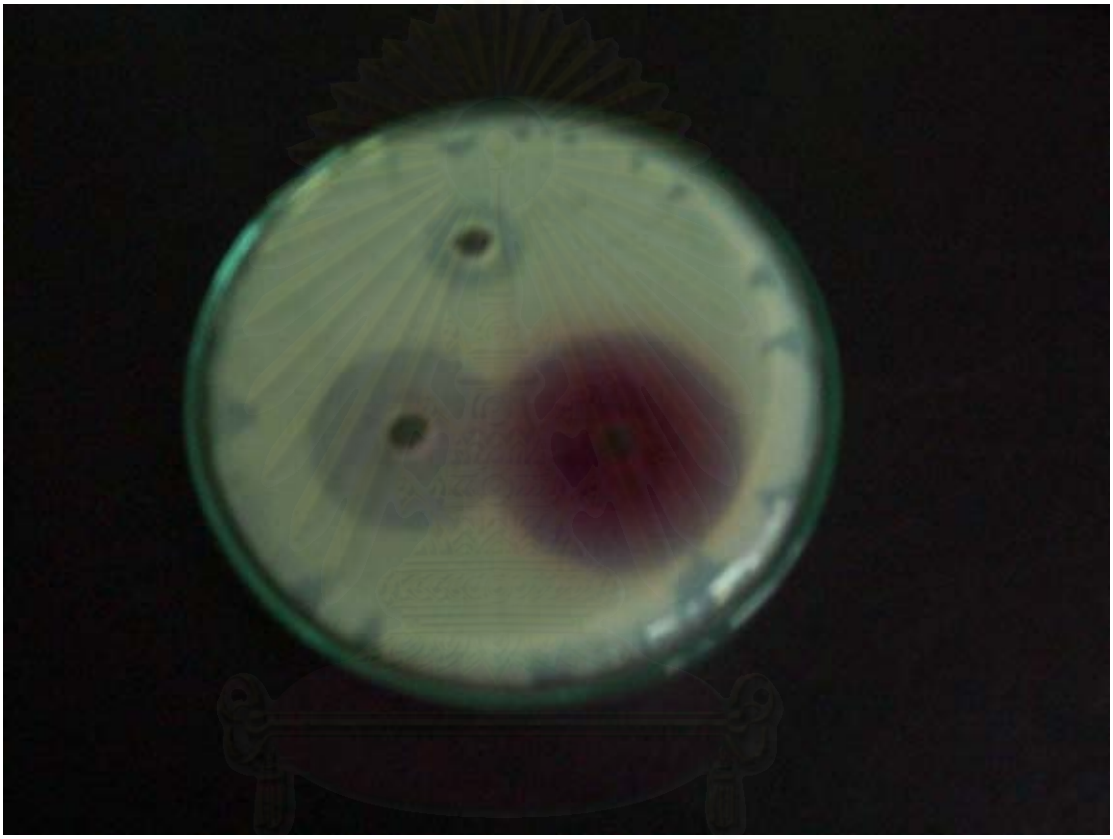


Figure 4.37 The agar well diffusion exhibition against *Bacillus subtilis* of fraction B15, B10 and B05.

4.11.2 Antimicrobial activities of pure compounds

The three metabolites isolated from endophytic fungal strain K_BK5 were subjected to antimicrobial assay. Compound 2 was not tested for antimicrobial activities because it was obtained in small amount. It was found that mixture 2 was active against *B. subtilis* ATCC 6633 with MIC 250 μg/ml. Mixture 1 and compound 1 showed no antimicrobial activity against 5 test microorganism as showed in Table 4.21

Table. 4.22 Broth micro dilution method for antimicrobial activities of pure compounds

Compound	Inhibition level against testes microorganisms and MIC (μg/ ml)				
	Gram positive bacteria		Gram negative bacteria		Yeasts
	B. subtils ATCC6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
Compound 1	-	-	-	-	-
Mixture 1	-	-	-	-	-
Mixture 2	250	-	-	-	-
Pennicillin G	15.62	500	ND	ND	ND
Streptomycin	ND	ND	1.95	7.81	ND
Ketoconasole	ND	ND	ND	ND	125

- = inactive

ND = Not determined

4.11.3 Cytotoxic activity

The *in vitro* cytotoxic activity of isolated metabolites from endophytic fungal strain K_BK5 against 5 cell line including, HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) was carried out. From the resulted, all metabolites showed no activity on cytotoxic activity against 5 tumor cell lines.

4.11.4 Antioxidant activity

All the four metabolites isolated of endophytic fungi strain K_BK5 were subjected to antioxidant assay and the data are reported in Table 4.22

Table 4.23 Antioxidant activities of metabolites from endophytic fungi strain K_BK5

Compound	EC_{50} (μg/ml), (μM/l)
Compound 1	23.91 (84.19)
Compound 2	-
Mixture 1	30.17
Mixture 2	-
Vitamin E	7.25 (13.86)

Figure 4.38 Micro dilution methods for antioxidant assay

From the results, compound 1 was identified as are anthraquinone called austrocortinin which is found in great variety in toadstools of the genus *Cortinarius* and *Dermocybe splendida*. We report here the isolation from endophytic fungi to which structure is assigned on chemical and spectroscopic grounds, and confirmed by single crystal X-ray analyses. This is the first report on antioxidant activity with EC_{50} 23.91 µg/ml. Compound 2 was a new 1,4,6,7,9-pentahydroxy-2-methoxy-7-methylanthracene-10-dione on chemical and spectroscopic grounds. From the comprehensive study of endophytic fungi it was reported that 51% of biologically active substance isolated from endophytic fungi were previously unknown (Shutz,2001). It is consistent with the previous idea that endophytic fungi are realized as a potential producer of novel secondary metabolites. From chemical and spectroscopic data it is found that mixture 2 was a mixture of anthraquinone. The major compound in mixture 2 was 1-deoxyaustrocortirubin. It is produced by the genus *Cortinarius* and *Dermocybe splendida*. 1-Deoxyaustrocortirubin has been reported to be effective in the growth inhibition of a variety of bacteria and fungi such as *Bacillus brevis, B. subtilis, Mucor miehei, Penicillium notatun*, and *Nematospora coryli*. The antioxidant activity of compound 2 was investigated to have EC_{50} 30.17 µg/ml.

CHAPTER V

CONCLUSION

Forty-two isolates of endophytic fungi were isolated from leaves of *Lagerstroemia speciosa* Linn. Endophytic fungal strain K_BK5 was chosen for further study because the isolate has the highest active against *B. subtilis* ATCC 6633. Fungal strain K_BK5 did not produce conidium or spore formation, therefore, it was classified as Mycelia sterilia. Fungal strain K_BK5 was identified as Fungal endophyte MS6 IS133 based on molecular identification on nucleotide sequencing of ITS region.

Chromatographic and crystallization techniques were used to isolate bioactive compounds from culture broth and mycelium extracts. The structures of pure compounds were elucidated by using their physical properties and spectroscopic techniques. Two bioactive compounds were isolated. Compound 1 was identified as austrocortinin by physical, spectroscopic and X-ray crystallographic methods. Compound 2 was identified as 1,4,6,7,9-Pentahydroxy-2-methoxy-7-methy-5,6,7,8,8a,9-hexahydroanthracene-10(10aH)-one. Moreover mixture 1 which was triglyceride and mixture 2 which was a mixture of deoxyaustrocortirubin and other compounds were also isolated, respectively.

Biological activities were performed for these compounds. Mixture 2 has antimicrobial activity against *B. subtilis* ATCC 6633 with MIC value of 250 (μg/ ml), and exhibited antioxidant activity with EC_{50} 30.17 μg/ ml. Mixture 1 and compound 1 showed no activity on antimicrobial activity and cytotoxity test. But compound 1 has exhibited high antioxidant activity with EC_{50} 23.91 μg/ ml. Compound 2 was not determined Biological activities because it was obtained in small amount.

REFERENCES

Thai

ธงชัย เปาอินทร์ และ นิวัตร เปา อินทร์. 2544. ต้นไม้ยาน่ารู้. พิมพ์ครั้งที่ 1 กรุงเทพมหานคร: บริษัทออฟเซ็ทเพรส.

English

Bacon, C. W. 1993. Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue. Agric. Ecosyst. Environ. 44: 123-141.

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

Ball, D. M., Pedersen, J. F., and Lacefield, G. D. 1993. The tall-fescue endophyte. Am. Sci. 81: 370-379.

Belesky, D.P., Devine, O.J., Pallas, Jr J.E. and Stringer, W.C. 1987. Photosynthetic activity of tall fescue as influenced by a fungal endophyte. Photosynthetica 21: 82-87.

Blodett, J.T., Swart, W.J., Louw, S. M. and Weeks, W.J. 2000. Species composition of endophytic fungi in *Amaranthus hybridus* leaves, petioles, stems, and roots. Mycologia 92(5): 853-859.

Brady, S.F. and Clardy, J. 2000. CR377, a new pentaketide antifungal agent isolated from an endophytic fungus. J. Nat. Prod. 63: 1447-1448.

Bultman, T. L., and Murphy, J. C. 1998. Do fungal endophytes mediate wound-induced resistance? In: White, J. E. Jr. (ed.), The evolution of endophytism. (in press). New York: Marcel Dekker.

Carmichael , J., Park J.G., Kramer, B.S., Steinberg, S.M., Collins, J.M., Minna, J.D.,and Gazdar A.F. 1987. Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. **Cancer Res. 47:5875 - 5879.**

Carroll, G.C. and Petrini, O. 1983. Patterns of substrate utilisation by some fungal endophytes from coniferous foliage. Mycologia 75: 53-63.

Carroll, G. 1988. Fungal Endophytes in Stems and Leaves: From Latent Pathogen to Mutualistic Symbiont. Ecology 69: 2-9.

Cheplick, G. P., and Clay, K. 1988. Acquired chemical defenses of grasses: The role of fungal endophytes. Oikos 52: 309-318.

Clay, K., Hardy, T. N., and Hammond, A. M. Jr. 1988. Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology 69: 10-16.

Clay, K. 1990. Fungal endophytes of grasses. Ann. Rev. Ecol. Syst. 21:275-297.

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

Chen, G., Lin, Y., Wen, L., Vrijmoed, L.L.P. and Gareth Jones, E.B. 2003. Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the south china sea coast. Tetrahedron 59: 4907-4909.

Chirag Mahmood Jehan, D. Daulatabad and Abdurrazzaque M. Mirajkar. 1990. A keto fatty acid from *Lagerstroemia speciosa* seed oil. Phytochemistry 29: 2323-2324.

Clement, S. L, Lester, D. G, Wilson, A.D., Johnson, R. C., and Bouton, J. H. 1996. Expression of Russian wheat aphid (Homoptera: Aphididae) resistance in genotypes of tall fescue harboring different isolates of Acremonium endophyte. J. Econ. Entomol. 89: 766-770.

Clement, S. L, Wilson, A.D., Lester, D. G, and Davitt, C.M. 1997. Fungal endophytes of wild barley and their effects on *Diuraphis noxia* population development. Entomol. Exp. Appl. 82: 275-281.

Dombrowski, A. W., Bills, G. F., Sabnis, G., Koupal, L., Meyer, R., Ondeyka, J. G., Glacobbe, R. A., Monagham, R. L., and Lingham, R. B. 1992. L-696474, a novel cytochalasin as an inhibitor of HIV-1 protease I. The producing organism and its fermentation. J. Antibiot. 45: 671-678.

Dreyfuss, M. M., Chapela, I. H. 1994. In Gullo, V. P. (ed.), The discovery of natural products with therapeutic potential, pp.49-80. Boston, MA: Butterworth-Heine-mann.

Elmi, A. A., West, C. P., 1995. Endophytic infection effects on stromatal conductance, osmotic adjustment and drought recovery of tall fescue. New Phytol. 131: 61-67.

Freeman, S., and Rodriguez, R.J. 1993. Genetic conversion of a fungal plant pathogen to an nonpathogenic, endophytic mutualist. Science 260: 75-78.

Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113-118.

Garner, G.B., Rottinghaus, G.E., Cornell, C.N. and Testereci, H. 1993. Chemistry of compounds associated with endophyte/grass interaction: ergovaline- and ergopeptine-related alkaloids. Agriculture, Ecosystems and Enviroment 44: 65-80.

Gatenby, W.A., Munday-Finch, S.C., Wilkins, A.L. and Miles, C. O. 1999. Terpendole M, a novel indole-diterpenoid isolated from *Lolium perenne* infected with the endophytic fungus *Neotyphodium lolii*. J. Agric. Food Chem. 47: 1092-1097.

Harper, J.K., Arif, A.M., Ford, E.J., Strobe, G.A., Porco, J.A., Tomor, D.P., Oneill,K.L., Heider, E. M. and Grant, D. M. 2003. Pestacin: a 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possesing antioxidant and antimycotic activities. Tetrahedron 59: 2471-2476.

Hawksworth, D.L. 1991. The fungal dimension of biodiversity: magitude, significance, and conversation. Mycological research 95:641-655.

Hayashi, T,. Maruyama, H,. Kasai, R,. Hattori, K,. Takasuga, S,. Hazeki, O,. Yamasaki, K, and Tanaka, T. 2002. Ellagitannins from Lagerstroemia speciosa as activators of glucose transport in fat cells. Planta Med, 68(2): 173-5.

Isaac, S. 1992. Fungal-Plant Interaction. 1st ed. London: Chapman & Hall.

Jayasuriya, H., Bills, G.F., Cascales, C., Zink, D.L., Goetz, M.A., Jenkins, R.G., Silverman, K.C., Lingham, R.B. and Singh, S.B. 1996. Oreganic acid: a potent novel inhibitor of ras farnesyl-protein transferase from an endophytic fungus. Bioorganic and Medicinal Chemistry Letters 6(17): 2081-2084.

Ju, Y., Sacalis, J.N. and Still, C.C. 1998. Bioactive flavonoids from endophyte-infected blue Grass (*Poa ampla*). J. Agric. Food Chem. 46: 3785-3788.

Knoch, T. R., Faeth, S. H., and Arnott, D. L. 1993. Endophytic fungi alter foraging and dispersal by desert seed-harvesting ants. Oecologia 95; 470-475.

Judy, W.V., Hari, S.P., Stogsdill, W.W., Judy, J.S., Naguib, Y.M. and Passwater ,R. 2003. Antidiabetic activity of a standardized extract (Glucosol) from Lagerstroemia

speciosa leaves in Type II diabetics. A dose-dependence study. J Ethnopharmacol. 87(1): 115-7.

Kanchanaprayudh, J., Zhou, Z., Yomyart, S., Sihanonth, P. and Hogetsu, T. 2003. Molecular phylogeny of ectomycorrhizal *Pisolithus* fungi associated with pine, dipterocarp, and eucalyptus trees in thailand. Mycoscience 44: 287-294.

Knoch, T. R., Faeth, S. H., and Arnott, D. L. 1993. Endophytic fungi alter foraging and dispersal by desert seed-harvesting ants. Oecologia 95; 470-475.

Kongsaeree, P., Prabpai, S., Sriubolmass, N., Vongvein, C. and Wiyakrutta, S. 2003. Antimalarial dihydroisocoumarins produced by *Geotrichum* sp., an endophytic fungus of *Crassocephalum crepidiodes*. J. Nat. Prod. 66: 709-711.

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

Krohn, K., Florde, U., John, M., Root, N., Steingrover, K., Aust, H., Draeger, S., Schulz, B., Antus, S., Simonyi, M. and Zsila, F. 2001. Biologically active metabolites from fungi. Part 16: New preussomerins J, K and L from and endophytic fungus: structure elucidation, crystal structure analysis and determination of absolute configuration by CD calculations. Tetrahedron 57: 4343-4348.

Kakuda, T,. Sakane, I,. Takihara, T,. Ozaki, Y,. Takeuchi, H, and Kuroyanagi, M. Hypoglycemic effect of extracts from Lagerstroemia speciosa L. leaves in genetically diabetic KK-AY mice. Biosci Biotechnol Biochem, 60(2): 204-8.

Murakami, C,. Myoga, K,. Kasai, R,. Ohtani, K,. Kurokawa, T,. Ishibashi, S,. Dayrit, F,. Padolina, WG, and Yamasaki, K. 1993. Screening of plant constituents for effect on glucose transport activity in Ehrlich ascites tumour cells. Chem Pharm Bull (Tokyo), 41(12): 2129-31.

Leuchtmann, A. 1992. Systematis, distribution, and host specificity of grass endophytes. Nat. Toxins 1:150-162.

Lewis, D.H.1973. Concept in fungal nutrition and the origin of biotrophy. Biol. Rev. 48:261-278

Lewis, D.H.1974. Micro-organisms and plants: The evolution of parasitism and muatualism. Pages 367-392 in : 24th Symposium of the Society for General Microbiology. M.J. Carlise, and J.J. Skehel, eds. Cambridge: Cambridge University Press.

Li, J., Strobel, G., Sidhu, R. and Hess, W.M. 1996. Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum*. Microbiology 142: 2223-2226.

Li, J., Sidhu, R.S., Bollon, A. and Strobel, G.A. 1998. Stimulation of taxol production in liquid cultures of *Pestalotiopsis microspora*. Mycol. Res. 102(4): 461-464.

Li, J., Sidhu, R.S., Ford, E.J., Long, D.M., Hess, W.M. and Strobel, G.A. 1998. The induction of taxol production in the endophytic fungus *Periconia* sp. from *Torreya grandifolia*. Journal of Industrial Microbiology and Biotechnology 20: 259-264.

Li, J.Y., Harper, J.K., Grant, D.M., Tombe, B.O., Bashyal, B., Hess, W.M. and Strobel, G.A. 2001. Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. and *Monochaetia* sp. Phytochemistry 56: 463-468.

Li, J.Y. and Strobel, G.A. 2001. Jesterone and hydroxy-jesterone antioomycetes cyclohexenone epoxides from the endophytic fungus *Pestalotiopsis jesteri*. Phytochemistry 57: 261-265.

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

Malinowski, D. and Belesky, D. P. 1999. *Neotyphodium coenophialum* – infection affects the ability of tall fescue to use sparingly available phosphorous. J. Plant Nutri. 22: 835-853.

Metz, A.M., Haddad, A. and Worapong, J. 2000. Induction of the sexual stage of *Pestalotiopsis microspora*, a taxol-producing fungus. Microbiology 146: 2079-2089.

Michelle, A.A., Melvyn G. and Richard J.S. 1985. Anthraquinones from the Genus Cortinarius. Phytochemistry 24: 2755-2758.

Noble, H.M., Langley, D., Sidebottom, P.J., Lane, S.J. and Fisher, P.J. 1991. An echinocandin from an endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in Pinus sylvestris and Fagus sylvatica. Mycol. Res. 95: 1439-1440.

Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plants tissues. In N.J.Fokkema and J. van den Heuvel (Eds), Microbiology of the Phyllosphere. 175-187. Cambridge, UK: Cambridge University Press.

Petrini, O., 1991. Fungal endophytes of tree leaves. In: Andrews, J. H. and Hirano, S. S. (eds), Microbial ecology of leaves, pp. 179-197. New York: Springer-Verlag.

Petrini, O., Fisher, P. J., and Petrini, L.E. 1992. Fungal endophytes of bracken (*Pteridium aquilinium*) with some reflection on biological control. Sydowia 44: 282-293.

Polishook, J.D., Dombrowski, A.W., Tsou, N.N., Salituro, G.M. and Curotto, J.E. 1993. Preussomerin D from the endophyte *Hormonema dematioides*. Mycologia 85(1): 62-64.

Poter, J.K., Bacon, C.W., Cutler, H.G., Arrendale, R.F. and Robbins, J.D. 1985. *In vitro* auxin production by *Balansia epichloe*. Phytochemistry 24: 1429-1431.

Pulici, M., Sugawara, F., Koshino, H., Uzawa, J. and Yoshida, S. 1996. PestalotiopsisA and B: New caryphyllenes from an endophytic fungus of Taxus brevifolia. J. Org. Chem. 61: 2122-2124.

Ragasa, Cy,. Ngo, HT, and Rideout JA,. 2005. Terpenoids and sterols from Lagerstroemia speciosa. J Asian Nat Prod Res, 7(1): 7-12.

Raid, R.N., and Pennypacker, S.P.1987. Weed as hosts for *Collectrichum coccodes*. Plant Dis. 71:643-646.

Ratnayake, A.S., Yoshida, W.Y., Mooberry, S.L. and Hemscheidt, T.K. 2001. Nomofungin: a new microfilament disrupting agent. J. Org. Chem. 66: 8717- 8721.

Ratnayake, A.S., Yoshida, W.Y., Mooberry, S.L. and Hemscheidt, T. 2001. The structure of microcarpalid, a microfilament disrupting agent from an endophytic fungus. Organic Letters 3(22): 3479-3481.

Redlin, S.C. and Carris, L.M. 1996. Endophytic fungi in grass and woody plants: systematic, ecology, and evolution. Minnesota:APS Press.

Rodrigues, K. F. 1996. Fungal endophytes of palms. In Redlin, S. C., and Carris, L. M (eds.), Endophytic fungi in grasses and woody plants, Systematics, ecology and evolution. pp. 121-132. St. Pual, Minnesota: APS Press.

Rowan, D.D. 1993. Loitrems, peramine and paxilline: mycotoxins of the ryegrass/ endophyte interaction. Agriculture, Ecosystems and Environment 44: 103-122.

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

Santos, R.M.G. and Rodrigues-Fo, E. 2002. Meroterpenes from *Penicillium* sp. Found in association with *Melia azedarach*. Phytochemistry 61: 907-912.

Schulz, B., Sucker, J., Aust, H.J., Krohn, K., Ludewig, K., Jones, P.G. and Doring, D. 1995. Biologically active secondary metabolites of endophytic *Pezicula* species. Mycol. Res. 99(8): 1007-1015.

Siegel, M. R., Latch, G. C.M., Bush, L.P., Fannin, F. F., Rowan, D.D., et al. 1990. Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. J. Chem. Ecol. 16: 3301-3305.

Siegal, M. R., and Bush, L. P. 1996. Defensive chemicals in grass-fungal endophyte associations. Recent Adv. Phytochem. 30: 81-118.

Siegal, M. R., and Bush, L. P. 1997. Toxin production in grass/endophyte association. In: Carroll, G.C., and Tudzynski, P. (eds.), The Mycota. V. Plant Relationships, Part B. pp.185-208. Berlin: Springer-Verlag.

Sinclair. J. B., and Cerkauskas, R. F. 1996. Latent infection vs. endophytic colonization by fungi. In: Redlin, S. C., and Carris, L. M. (eds.), Endophytic fungi in grasses and woody plants, Systematics, ecology and evolution. pp. 3-29. St. Pual, Minnesota: APS Press.

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

Stinson, M., Ezra, D., Hess, W.M., Sears, J. and Strobel, G. 2003. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Science 165: 913-922.

Stone, J. 1990. Endophytes as facultative pathogens. In Reisinger, A., and Bresinsky, A. (eds), Abstracts, Fourth International Mycological Congress, p. 158. Regensberg: The University.

Stone, J. K. and Petrni, O. 1997. Endophytes of forest trees: a model for fungus-plant interactions. In: Carroll, G. C. and Tudzynski, P. (eds), The Mycota. V. Plant Relationships. Part B., pp. 129-142. Berlin: Springer-Verlag.

Stone, K. J., Bacon, W. C., and White, F. J. Jr. 2000. An overview of endoophytivc microbes: Endophytism defined. In Bacon, W. C., and White, F. J. Jr. (eds.), Microbial endophytes, p. 4. New York: Marcel Dekker.

Stierle, A. and Strobel, G. 1995. The search for a taxol-producing microorganism among the endophytic fungi of the Pacific Yew, *Taxus brevifolia*. Journal of Natural Products 58(9): 1315-1324.

Stierle, A., Strobel, G. and Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific Yew. Science 260: 214-216.

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

Strobel, G.A., Hess, W.M., Li, J., Ford, E. and Sears, J. 1997. *Pestalotiopsis guepinii*, a taxol-producing endophytic of the Wollemi Pine, *Wollemia nobilis*. Aust. J. Bot. 45: 1073-1082.

Strobel, G. A., and Long, D. M. 1998. Endophytic microbes embody pharmaceutical potential. ASM News 64: 263-268.

Strobel, G.A., Miller, R.V., Martinez-Miller, C., Condron, M.M., Teplow, D.B. and Hess, W.M. 1999. Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. Microbiology 145: 1919-1926.

Strobel, G., Hess, W.M., Baird, G., Ford, E., Li, J.Y. and Sidhu, R.S. 2001. *Stegolerium kukenani* gen. et sp. nov. an endophytic, taxol producing fungus from the Roraima and Kukenan tepuis of Venezuela. Mycotaxon LXXVIII: 353-361.

Strobel, G., Ford, E., Worapong, J., Harper, J.K., Arif, A.M., Grant, D.M., Fung, P.C.W. and Chau, R.M.W. 2002. Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. Phytochemistry 60: 179-183.

Strobel, G. 2003. Endophytes as sources of bioactive products. Microbes and Infection 5: 535-544.

Strobel, G., Daisy B., Castillo, U.,and Harper J. 2004. Natural Products from Endophytic Microorganisms. J. Nat. Prod. 67(2): 257 –268.

Suske, J., and Acker, G. 1989. Identification of endophytic hyphae of *Lophodermium piceae* in tissue of green, symptomless Norway spruce needles by immunoelectron microscopy. Can. J. Bot. 67: 1768-1774.

Suzuki, Y,. Unno, T,. Ushitani, M,. Hayashi, K, and Kakuda, T. Antiobesity activity of extracts from Lagerstroemia speciosa L. leaves on female KK-Ay mice. J Nutr Sci Vitaminol (Tokyo), 45(6): 791-5.

Tan, R.X., Liu, J.Y., Song, Y.C., Zhang, Z., Wamg, L., Guo, Z.J., and Zou, W.X. 2004. *Aspergillus fumigatus* CY018, an endophytic fungus in *Cynodon dactylon* as a versatile producer of new and bioactive metabolites. Journal of Biotechnology 114(3): 279-287.

Tscherter, H. and Dreyfuss, M.M. 1982. New metabolites. Processes for their production and their use. International Patent Application PCT/EP8/00121:

Tscherter, H., Hofmann, H., Ewald, R. and Dreyfuss, M.M. 1988. Antibiotic lactone compound. U.S. Patent 4,753,959.

Unno, T., Sugimoto, A. and Kakuda T. 2004. Xanthine oxidase inhibitors from the leaves of Lagerstroemia speciosa (L.) Pers. J Ethnopharmacol, 93(2-3): 391-5.

Wagenaar, M.M., Corwin, J., Strobel, G. and Clardy, J. 2000. Three new cytochalasins produced by an endophytic fungus in the Genus *Rhinocladiella*. J. Nat. Prod. 63: 1692-1695.

Wagenaar, M.M. and Clardy, J. 2001. Dicerandrols, new antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an Endangered Mint. J. Nat. Prod. 64: 1006-1009.

Wang, C., Wu, J. and Mei, X. 2001. Enhancement of taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. Appl. Microbiol. Biotechnol. 55: 404-410.

Wang, J., Huang, Y., Fang, M., Zhang, Y., Zheng, Z., Zhao, Y. and Su, W. 2002. Brefeldin A, a cytotoxin produced by *Paecilomyces* sp. and *Aspergillus clavatus* isolated from *Taxus mairei* and *Torreya grandis*. FEMS Immunology and Medical Microbiology 34: 51-57.

Wilson, A.D.1996. Resoures and testing of endophyte-infected germplasm in national grass repository collections. In S. C. Redlin, and L.M. Carris (eds.), Endohytic fungi in grasses and Woody plants 2nd ed., pp. 179-195. St. Paul, MN: The American Phytopathological Society.

White, J.F.,Jr., and Morgan-Jones,G. 1996. Morphological and physiological adaptations of Balansieae and trend in the evolution of grass endophytes. In S.C. Redin, and L.M. Carris (eds.), Endophytic fungi in grasses and Woody plants 2nd ed., pp.133-154. St. Paul, MN: The American Phytopathological Society.

Wolock-Medej, C., and Clay, K. 1991. Avain seed preference and weight loss experiment: the role of fungal-infected fescue seeds. Oecologia 88: 296-302.

Yang, X., Strobel, G., Stierle, A., Hess, W.M., Lee, J. and Clardy, J. 1994. A fungal endophyte-tree relationship: *Phoma* sp. in *Taxus wallachiana*. Plant Science 102: 1-9.

Yue, Q., Miller, C.J., White, J.F. and Richardson, M.D. 2000. Isolation and characterization of fungal inhibitors from *Epichloe festucae*. J. Agric. Food Chem. 48: 4687-4692.

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

Zou, W.X., Meng, J.C., Lu, H., Chen, G.X., Shi, G.X., Zhang, T.Y. and Tan, R.X. 2000. Metabolites of *Collectotrichum gloeosporioides*, and endophytic fungus in *Artemisia mongolica*. J.Nat.Prod. 63: 1529-1530.

APPENDICES

APPENDIX A

MEDIA

1. Malt czapek agar (MCzA)

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

Czapek stock solution A

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

Keep in a refrigerator.

Czapek stock solution B

K_2HPO_4	20.0	g
Distilled water	1000	ml

Keep in a refrigerator.

2. Malt extract agar (MEA)

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

APPENDIX B

Method of characterization of the endophytic fungi studies

Morphological identification of isolated fungi

Classical identification of fungi is based on observation of characteristics. Assignment of morphological species can be based on colony surface texture, hyphal pigments, exudates, margin shapes, growth rates, and sporulating structures.

Macroscopic features

Characters such as shape, size, color, type of stoma surface, and others

Microscopical features

The microscopic analyses were based on observations by light microscopy. Specimens for light microscopy were mounted in lacto phenol-cotton blue and lacto phenol aniline blue for observations of spores and other characteristics, and then identified.

■ Preparation of the specimens for light microscope

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

- **Preparation of the fungal mycelia on glass slides by teasing mycelia**

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

- **Preparation of the fungal mycelia on glass slides by Scotch tape preparation**

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

- **Preparation of slide culture**

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

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

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

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

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

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

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

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

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

- **Preparation of the specimens for scanning electron microscope**

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

The cultures of endophytic fungi isolate LSS06 were cut into 1x1 cm and fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodelate buffer (pH7.2) for 2 h. The samples were then dehydrated under the serine concentration (70-95%) within 15 minutes. The samples were dried under critical point dried and coated with gold under sputter coater model. Changes of each fine immersed in absolute ethanol for 30 minutes for each twice and observed and photographed with a JSM-5410 LV scanning electron microscope.

Molecular identification of isolated fungi

Since the distinguishing morphological characteristics of a fungus are frequently too limited to allow its identification, physiological and biochemical techniques are applied. However for the poorly differentiated filamentous fungi, these methods are laborious, time consuming, and somewhat variable and provide insufficient taxonomic resolution. In contrast, molecular methods are universally applicable. The procedures were listed as below.

DNA extraction

Genomic DNA was prepared from the fresh mycelium by homoginization in 1.5 ml tubes with a FastPrep FP 120 homogenizer (Savant, faxmingdale, NY, USA) and followed by extraction with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999).Fungal DNA extract was applied in CTAB buffer (2% CTAB,0.1 M Tris-HCl (pH8.0),20mM EDTA (pH8.0), 1.4M NaCl and 0.5% 2-mercaptoethanol) at 65°C for 1 hour, extracted with chloroform-isoamyl alcohol (25:24:1,v/v), then extracted with phenolchloroform-isoamyl alcohol mixture (24:1,v/v) twice. Fungal DNA was precipitated with isopropanol and centrifuged at 8000 rpm for 5 min. Fungal DNA was dissolved in 100 μl TE buffer (10mM Tris-HCl (pH8.0) and 1mM EDTA) and kept at -30 °C for further study.

ITS amplification

The ITS region of isolated endophytic fungus was amplified with the primers ITS1f (Gardes and Burns,1993), and ITS4 (White et al.,1996). Twenty micrometers of reaction mixture contained 5 ng template DNA, 0.2 mM each dNTP,1xPCR buffer, 1.5mM $Mg2^{+}$, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 μM of primer pair. The amplification reactions were performed in a thermal cycle (TP 3000;Takara Shuzo, Tokyo,Japan). Amplification was started at 94°C for 9 min,followed by 38 cycles of denaturing step at 94°C for 1 min, an annealing step at 51°C for 1 min, and an extension step at 72°C for 1 min, and ended with an additional 5-min extension step at 72°C (Kanchanaprayudh et al.,2003).

DNA sequencing

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

ITS_{1f-4} sequences were automatically aligned with fungi ITS sequences obtained from Genbank DNA database (http:// www.ddbj.nig.ac.jp)

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

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

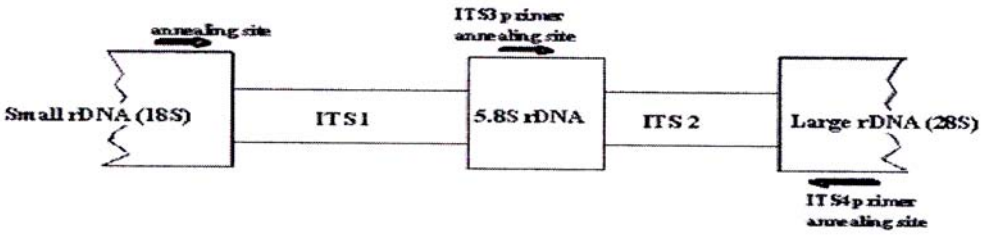


Figure B1 ITS regions of rDNA (Kanchanaprayudth et al., 2003)

Cytotoxic test

Cytotoxic test were carried out at the Institute of Biotechnology and Genetic Engineering. Chulalongkorn University. Bioassay of cytotoxic activity againts human tumor cell culture in *vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltetrazolium bromide) calorimetric method (Carmichael et al., 1987). In Principle, the viable cell number/well was directly proportional to the production of formazan, followed by solubilization, and could be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed into replicate 96-well culture plates in 100-μl volumes using a repeating pipette. Following a 24-h incubation at 37°C,5% CO_2, 100% relative humidy,100 μl of culture medium, culture medium containing the sample was dispensed into the appropriate wells (control group,N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) " background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was sterilized and filtered through 0.45-μl filter units. MTT working solutions were prepared just prior to culture application by dilution of MTT stock solution 1:5 (V/V) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well, resulting in 50 μl MTT/250 μl total medium volumes; and cultures were incubated at 37°C for 4 to 24 h depending upon individual cell line

requirements. Following incubated cell monolayer and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following formazan solubilization, the absorbance of each well was measured using a micro culture plate reader at 540 nm (single wavelength, calibration factor = 1.00) as shown in scheme 3.4

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

Sample were also tested for cytotoxic activity towards 5 cell lines, including HEP-G2 (hepatoma), SW 620 (colon), Chaco (lung), Kato-3 (gastric), and BT474 (breast) following the experimental method of bioassay of cytotoxic activity.

APPENDIX C

Table C.1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
1	Asperfumoid	*Aspergillus fumigatus* CY018	*Cynodon dactylon*	antifungal	Tan et al., 2004
2	Asperfumin				
3	Spiroquinazoline	*Eupenicillium* sp.	*Murraya paniculata*	-	Barros et al., 2004
4	Alantrypinone				
5	Alanditrypinone				
6	Alantryphenone				
7	Alantrypinene B				
8	Alantryleunone				
9	Taxol	*Taxomyces andreanae*	*Taxus brevifolia*	Anticancer	Strobel *et al*., 2003, Stierle and Strobel,1995, Stierle *et al*., 1993, Strobel and Stierle, 1993

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

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
10	1,3,5,7 cyclooctatetraene or [8]annulene	*Gliocladium* sp.	*Eucryphia cordifolia*	Antimicrobial	Stinson *et al*., 2003
11	Lactones 1893 A	Endophytic fungus No. 1893	*Kandelia candel*	Cytotoxic	Chen *et al*., 2003
12	Lactones 1893 B				
13	Pestacin	*Pestalotiopsis microspora*	Rainforest	Antioxidant and antimycotic	Harper *et al*., 2003
14	7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one	*Geotrichum* sp.	*Crassocephalum crepidioides*	Antimalarial, antituberculous and antifungal	Kongsaeree *et al*., 2003
15	7-Butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one				
16	7-Butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one				

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

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

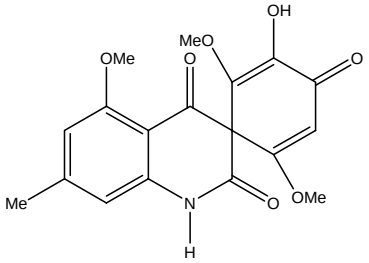
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
37	3beta,5alpha-Dihydroxy-6beta-acetoxy-ergosta-7,22-diene	*Collectotrichum* sp.	*Artemisia annua*	Antibactirial and antifungal	Lu *et al*., 2000
38	3beta,5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7,22-diene				
39	Indole-3-acetic acid (IAA)	*Epichloe/Neotyphodium* spp.	Grasses	Antifungal	Yue *et al*., 2000
40	Indole-3-ethanol (IEtOH)				
41	Methylindole-3-carboxylate				
42	Indole-3-carboxaldehyde				
43	Diacetamide				
44	Cyclonerodiol				
45	Colletotric acid	*Colletotrichum gloeosporioides*	*Artemisia mongolica*	Antimicrobial	Zou *et al*., 2000

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
46	CR377, pentaketide	*Fusarium* sp.	*Selaginella pallescens*	Antifungal	Brady and Clardy, 2000
47	Cytochalasin 1	*Rhinocladiella* sp.	*Tripterygium wilfordii*	Cytotoxic	Wagenaar *et al.*, 2000
48	Cytochalasin 2				
49	Cytochalasin 3				
50	Cytochalasin E				
51	Cryptocandin	*Cryptosporiopsis* cf. *quercina*	*Tripterigeum wilfordii*	Antimycotic	Strobel *et al.*, 1999
52	Geniculol	*Geniculosporium* sp.	*Teucrium scorodania*	Antialgal	Konig *et al.*, 1999
53	Cytochalasin F				
54	Sequoiatones A	*Aspergillus parasiticus*	*Sequoia sempervirens*	Antitumor	Stierle *et al.*, 1999
55	Sequoiatones B				
56	Terpendole M	*Neotyphodium lolii*	*Lolium perenne*	neurotoxins	Gatenby *et al.*, 1999
57	Tricin (1)	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al.*, 1998
58	7-O-(B-D-glucopyranosyl) tricin				
59	Isoorientin (3)				

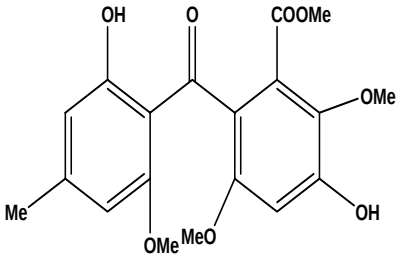
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
60	7-o-[α-L-Rhamnopyranosyl(1-6)-β-D-glucopy-ranosyl]tricin	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998
61	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxic	Berny *et al*., 1997
62	Leucinostatin A	*Acremoium* sp.	*Taxus baccata*	Antifungal and anticacer	Strobel *et al*., 1997
63 64 65 66	Oreganic acid (1) Trimethyester (2) Desulfated analog (3) Desulfated analog (4)	Endophytic fungus (MF 6046)	*Berberis oregana*	Anticancer	Jayasuriya *et al*., 1996
67 68	Pestalotiopsins A Pestalotiopsins B	*Pestalotiopsis* sp.	*Taxus brevifolia*	-	Pulici *et al*., 1996
69 70	(R)-mellein (-)-mycorrhizin A	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
71	2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995
72	(+)-cryptosporiopsin				
73	4-epi-ethiosolide				
74	Altersolanol A	*Phoma* sp.	*Taxus wallachiana*	Antibacterial	Yang *et al*., 1994
75	2-hydroxy-6-methylbenzoic acid				
76	Preussomerin D	*Hormonema dematioides*	Conifer wood	Antifungal	Polishook *et al*., 1993
77	Lolitrem C	*Acremonium lolii*	*Lolium perenne*	Neurotoxic and insect antifeedant	Rowan *et al*., 1993
78	Peramine R=H				
79	Diacetylperamine R=Ac				
80	Paxilline				
81	Loline alkaloid				
82	Ergovaline				

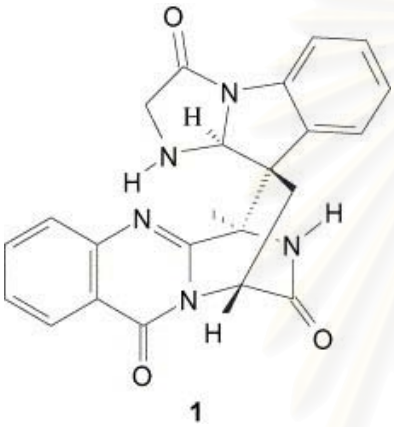
No.	Compounds	Endophytic fungi	Host plants	cal activities	References
83	Lysergic acid	*Acremonium coenophialun*	*Festuca arundinacea*	Toxin	Garner *et al.*, 1993
84	Isolysergic acid				
85	Pospalic acid				
86	Lysergol				
87	Lysergic acid amide				
88	Lysergic acid diethyl-amide				
89	Lycergic acid-2-propanolamide or (Ergonovine)				
90	Rhizoctonic acid	*Rhizoctonia* sp.	*Cynodon dactylon*	Anti-Helicobacter pylori	Tan et al., 2004
91	Monomethylsulochrin				
92	Ergosterol				
93	3β,5α,6β-trihydroxyergosta-7,22-diene				



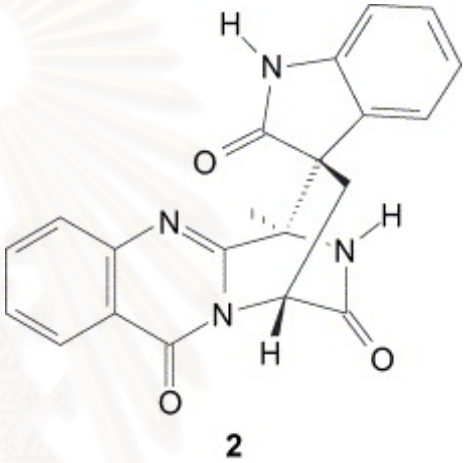
[1] Asperfumoid



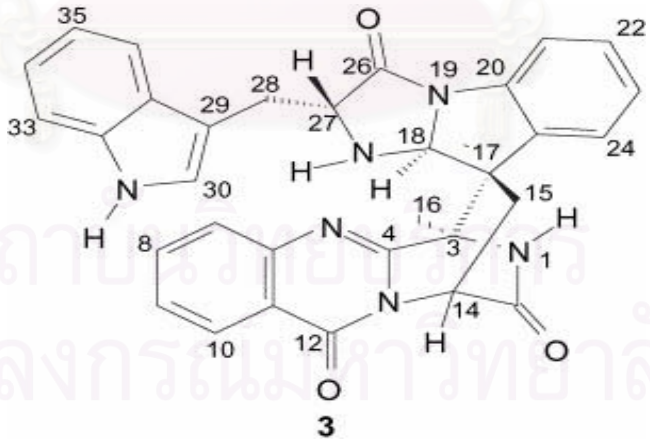
[2] Asperfumin



[3] Spiroquinazoline

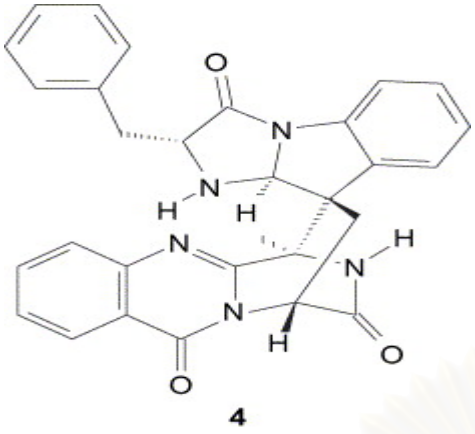


[4] Alantrypinone



[5] Alanditrypinone

Figure C.1 Structure of secondary metabolites of endophytic fungi



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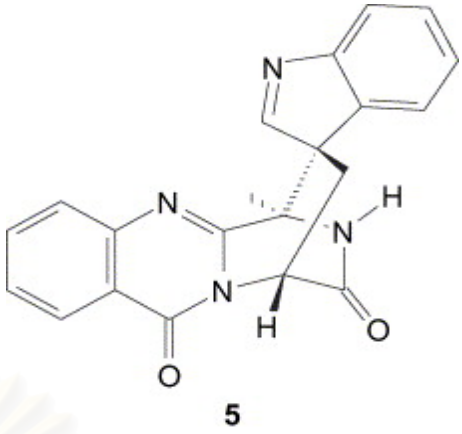
[6] Alantryphenone



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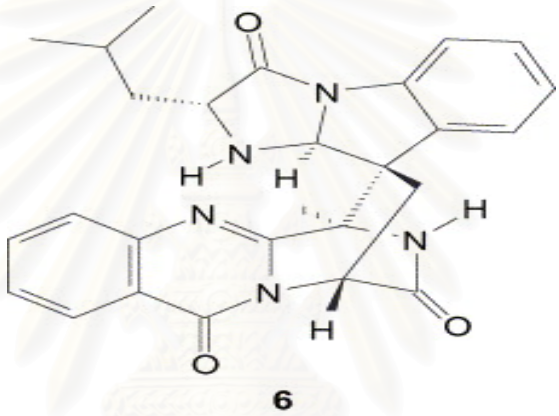
[7] Alantrypinene B



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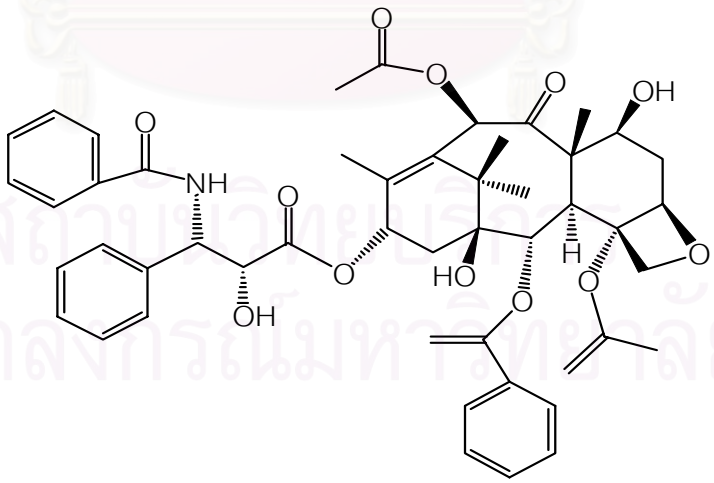
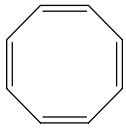
[8] Alantryleunone

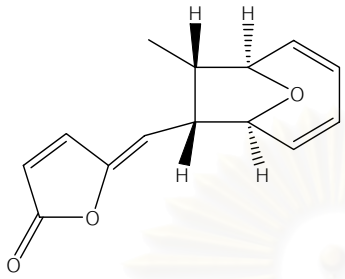


[9] Taxol

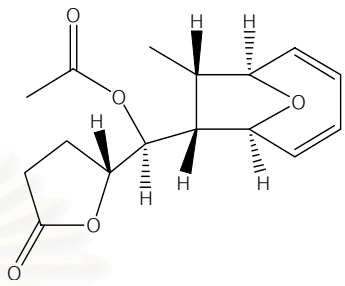
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



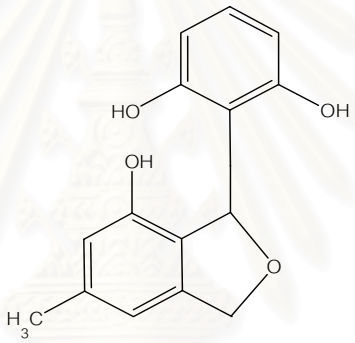
[10] 1,3,5,7 cyclooctatetraene or (8)-annulene



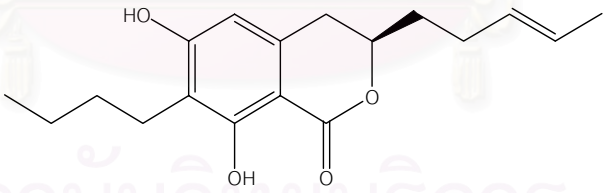
[11] Lactones 1893 A



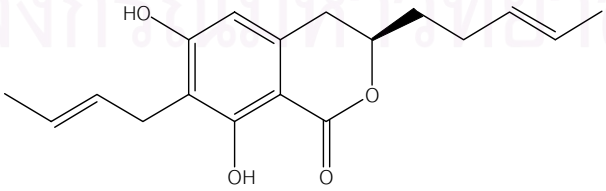
[12] Lactones 1893 B



[13] Pestacin

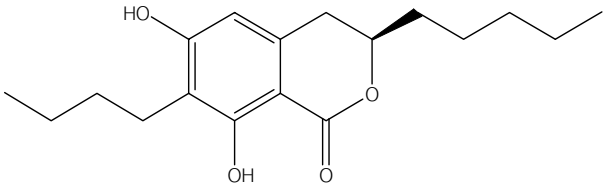


[14] 7-Butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



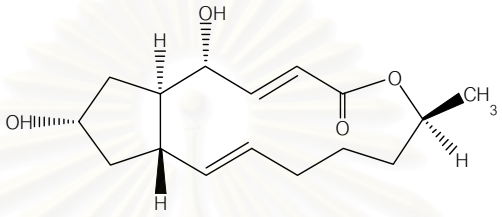
[15] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one

Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)

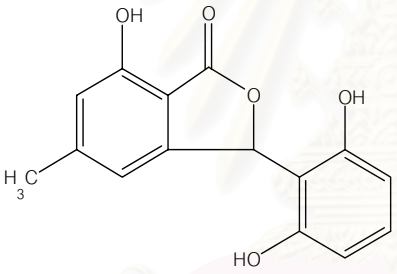


[16] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

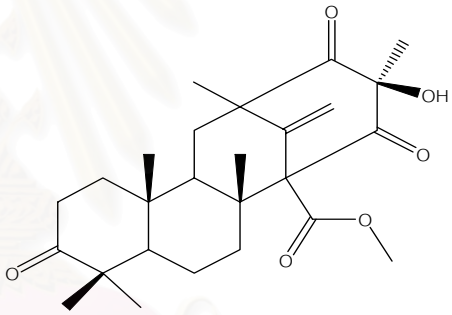
Dihydroisocumarins [6-8]



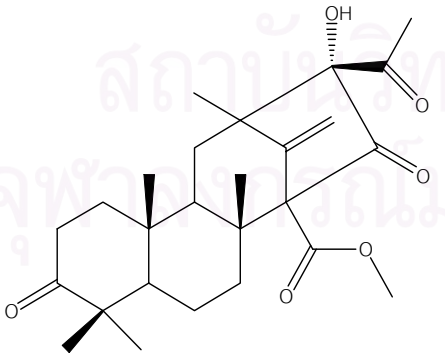
[17] Brefeldin A



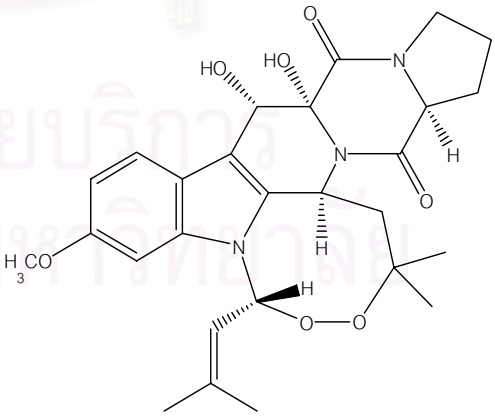
[18] Isopestacin



[19] Preaustinoid A

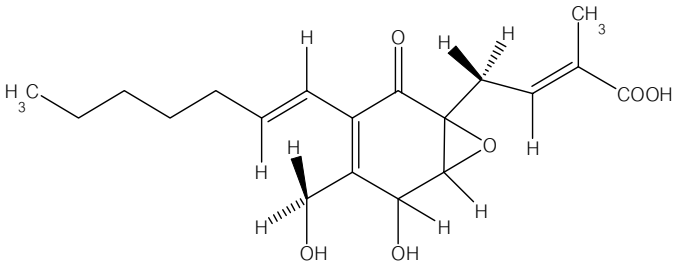


[20] Preaustinoid B

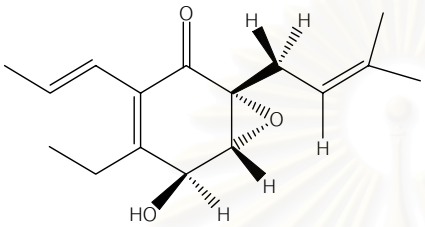


[21] Alkaloid verruculogen

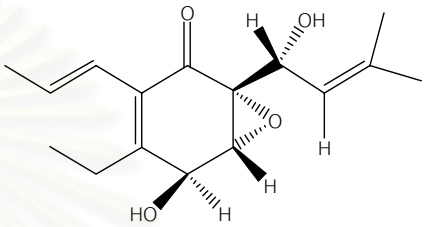
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



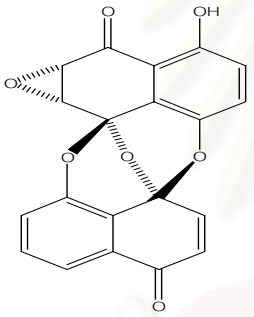
[22] Ambuic acid



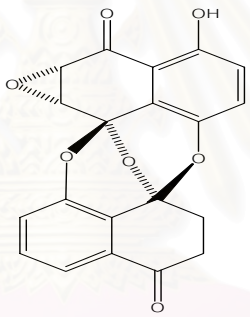
[23] Jesterone



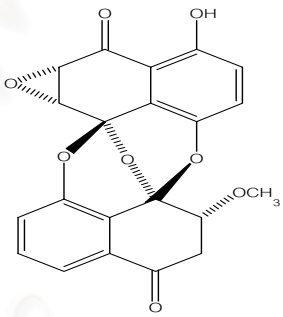
[24] Hydroxy-jesterone



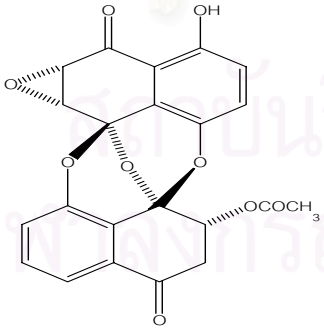
[25] Preussomerin G



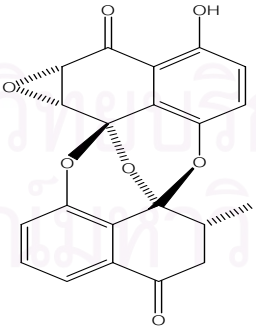
[26] Preussomerin H



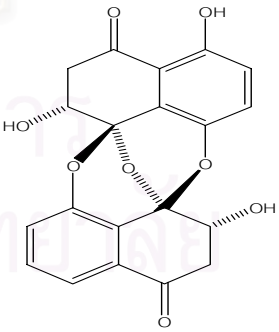
[27] Preussomerin I



[28] Preussomerin J

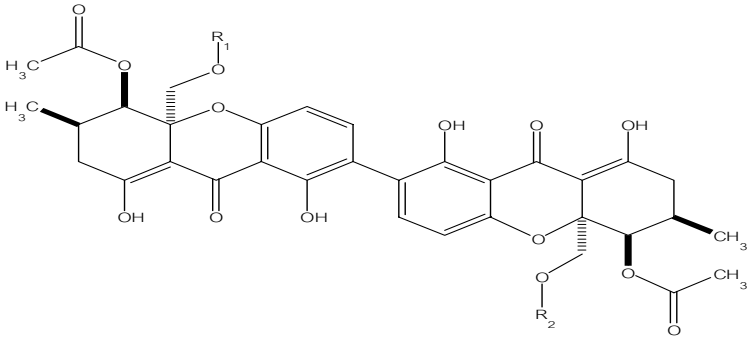


[29] Preussomerin K



[30] Preussomerin L

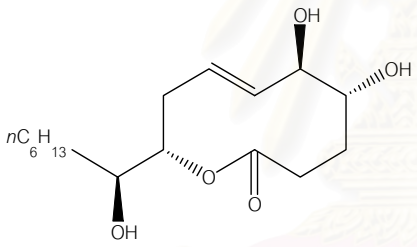
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



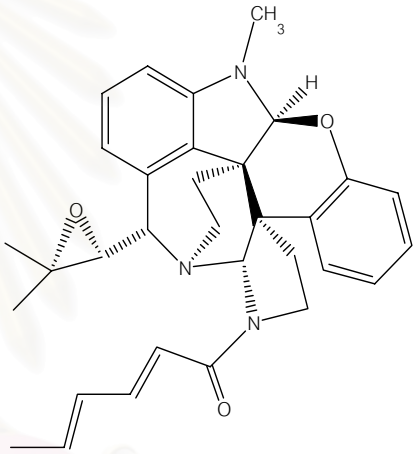
[31] Dicerandrols A, R1=R2=H

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

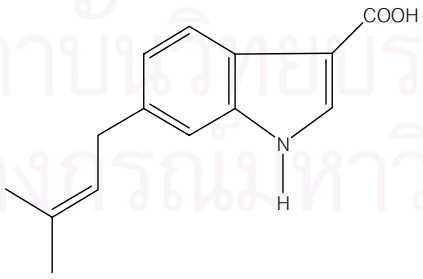
[33] Dicerandrols C, R1=R2=Ac



[34] Microcarpalide

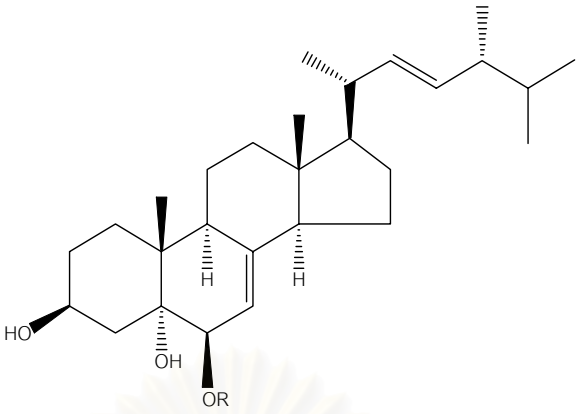


[35] Nomofungin



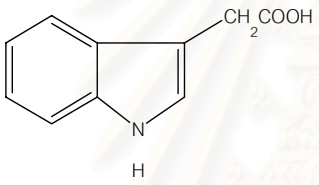
[36] Isoprenylindole-3-carboxylic acid

Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)

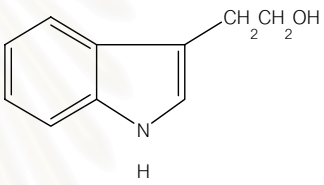


[37] 3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7, 22-diene, R=$COCH_3$

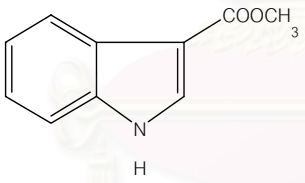
[38] 3beta, 5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7, 22-diene, R=$COCH_2C_6H_5$



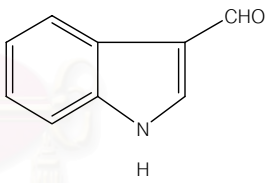
[39] Indole-3-acetic acid (IAA)



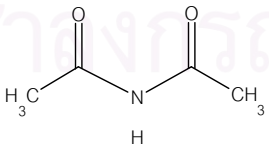
[40] Indole-3-ethanol (IEtOH)



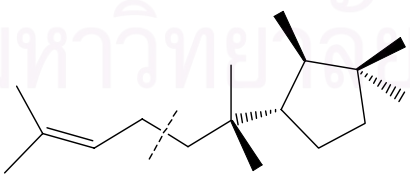
[41] Methylindole-3-carboxylate



[42] Indole-3-carboxaldehyde

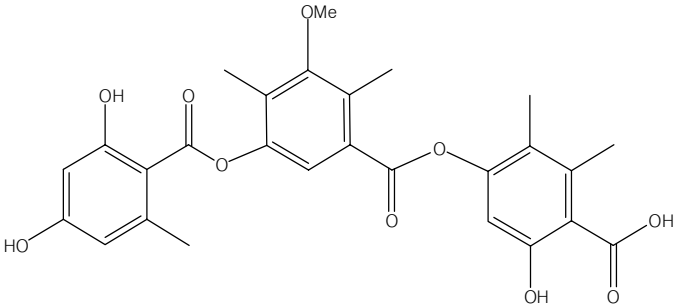


[43] Diacetamide

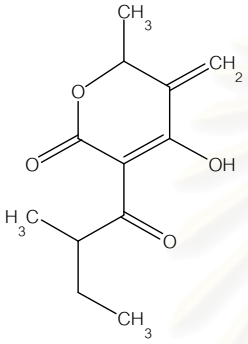


[44] Cyclonerodiol

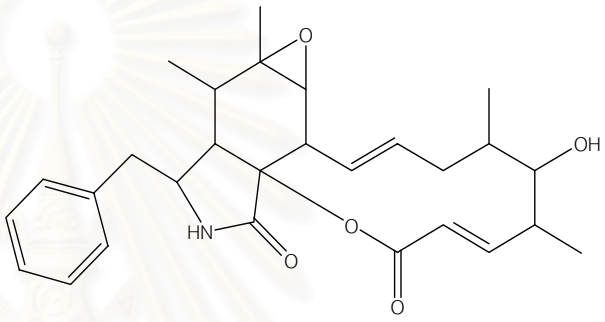
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



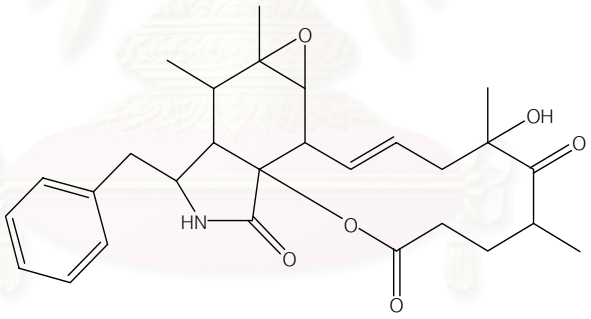
[45] Colletotric acid



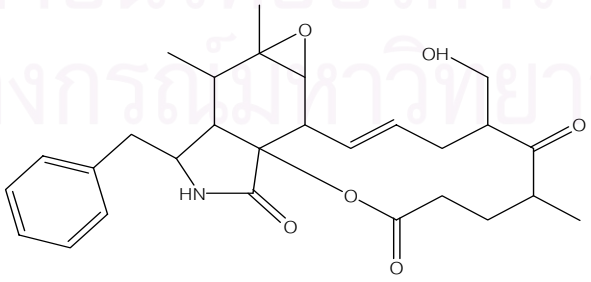
[46] CR377, pentaketide



[47] Cytochalasin 1

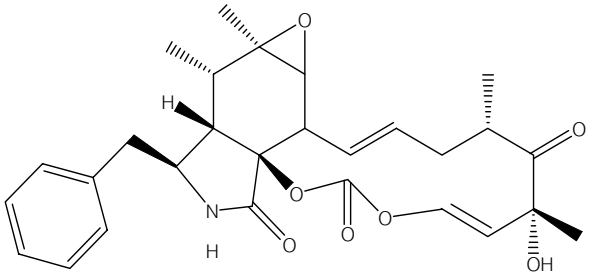


[48] Cytochalasin 2

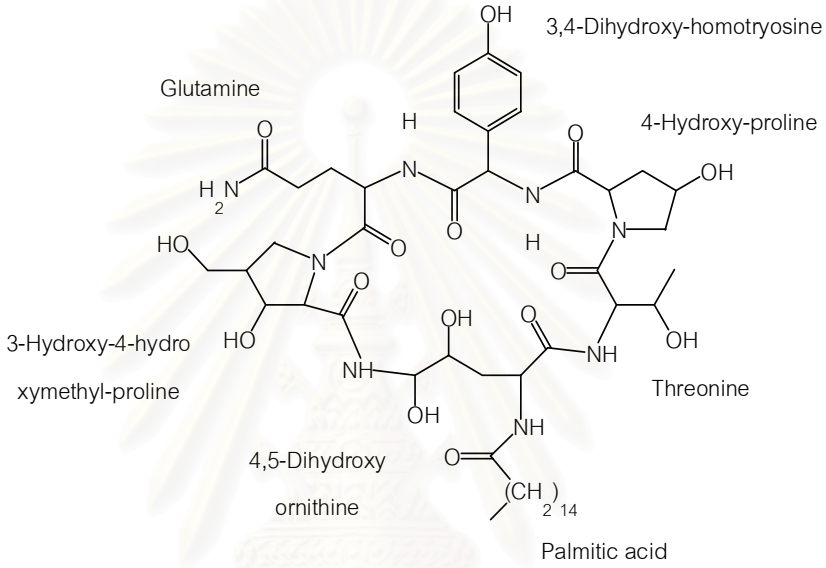


[49] Cytochalasin 3

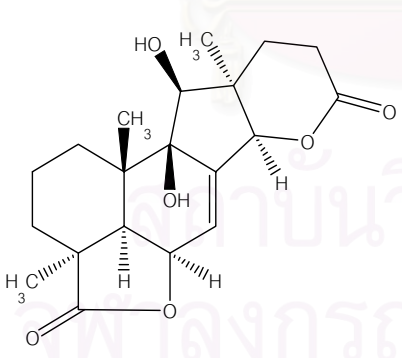
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



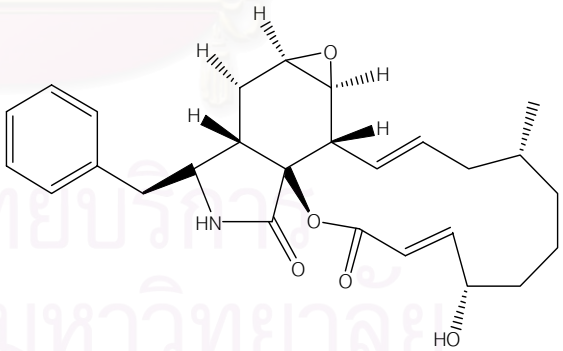
[50] Cytochanlasin E



[51] Cryptocandin

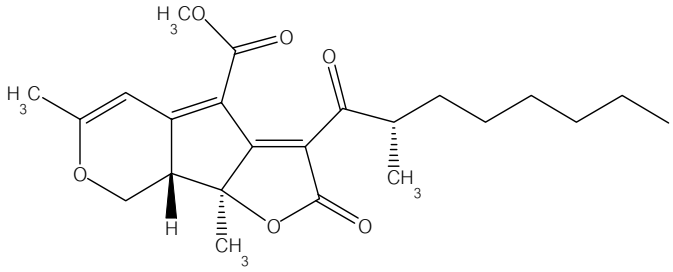


[52] Geniculol

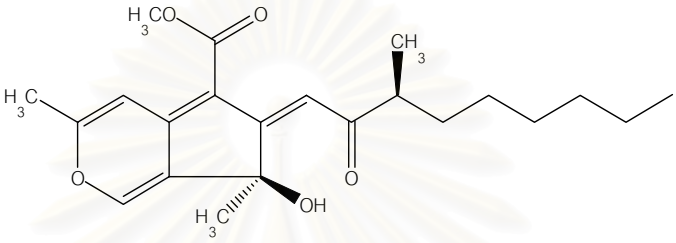


[53] Cytochalasin F

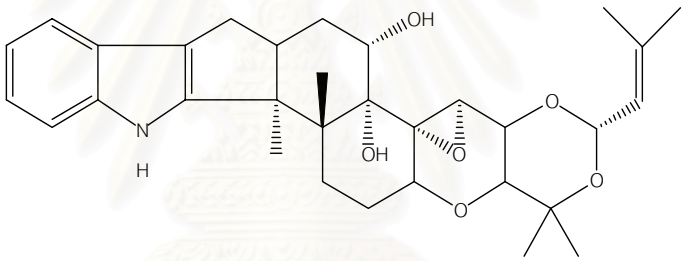
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



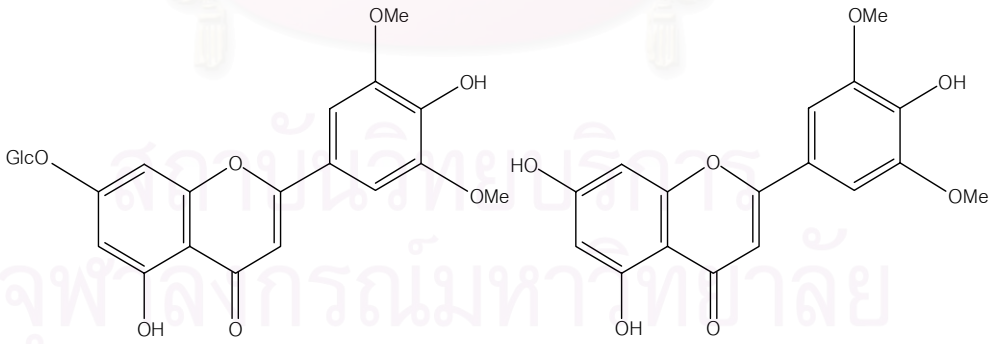
[54] Sequoiatones A



[55] Sequoiatones B



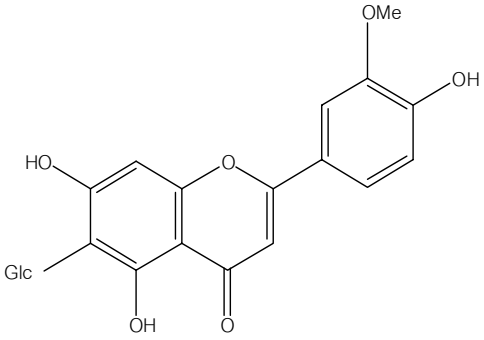
[56] Terpendole M



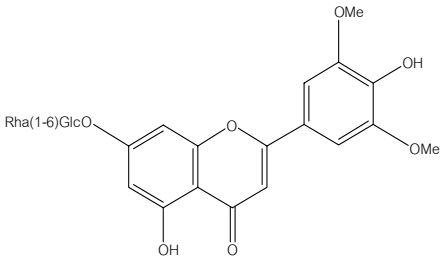
[57] Tricin

[58] 7-O-(B-D-glucopyranosyl)tricin

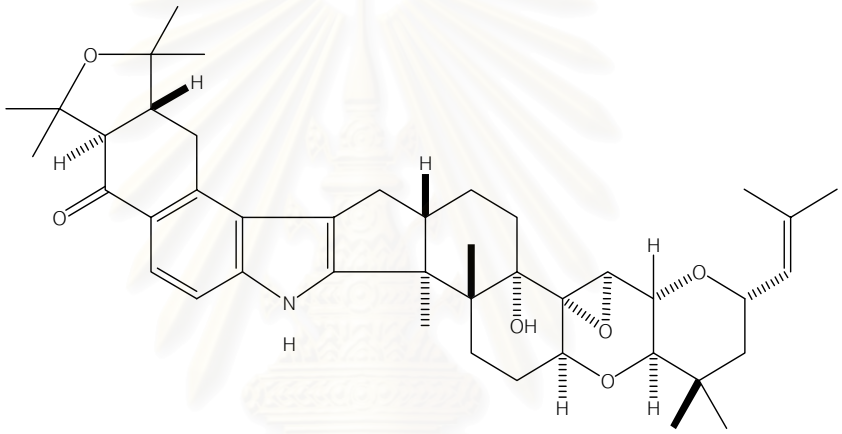
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



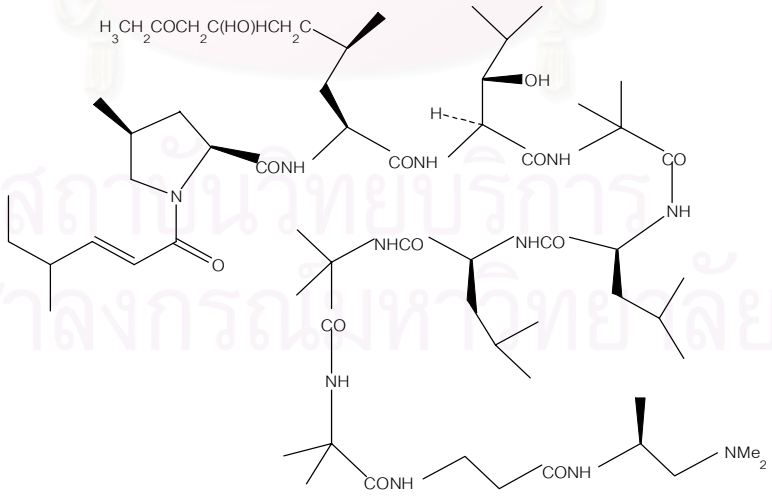
[59] Isoorientin



[60] 7-**o**-[**α**-L-Rhamnopyranosyl(1-6)-**β**-D-glucopy-ranosyl]tricin

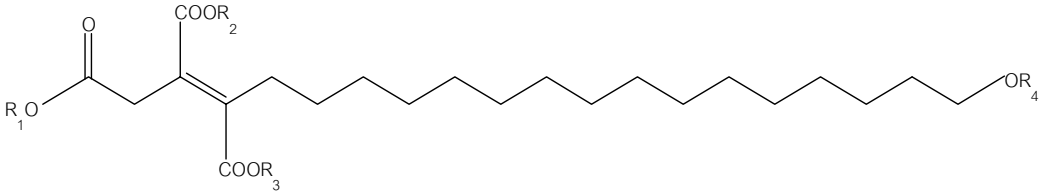


[61] Lolitrem B



[62] Leucinostatin A

Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)

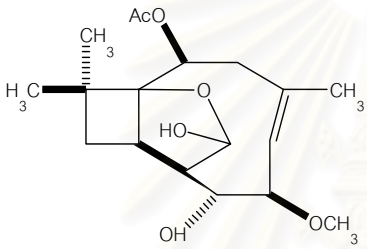


[63] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H

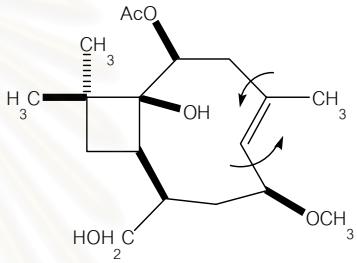
[64] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H

[65] 3: Desulfated analog, R1=R2=R3=CH3, R4=H

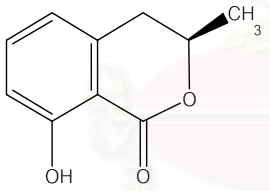
[66] 4: Desulfated analog, R1=R2=R3=R4=H



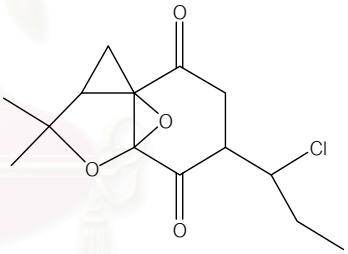
[67] Pestalotiopsins A



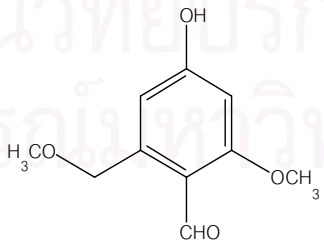
[68] Pestalotiopsins B



[69] (R)-mellein

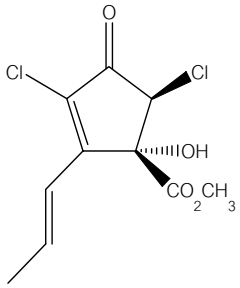


[70] (-)-mycorrhizin A

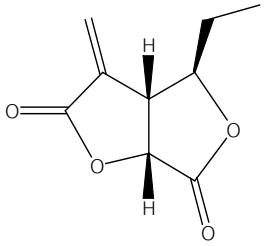


[71] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

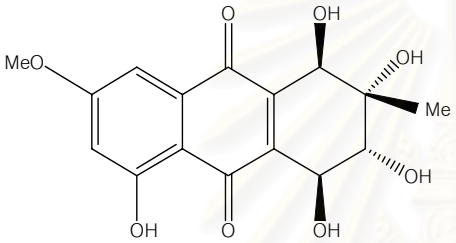
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



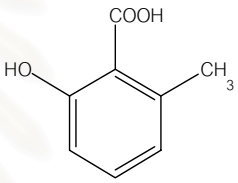
[72] (+)-cryptosporiopsin



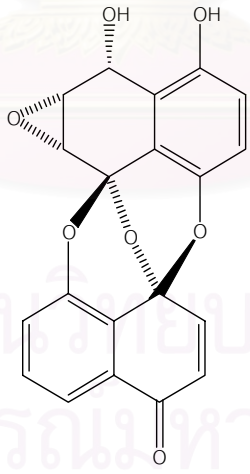
[73] 4-epi-ethiosolide



[74] Altersolanol A

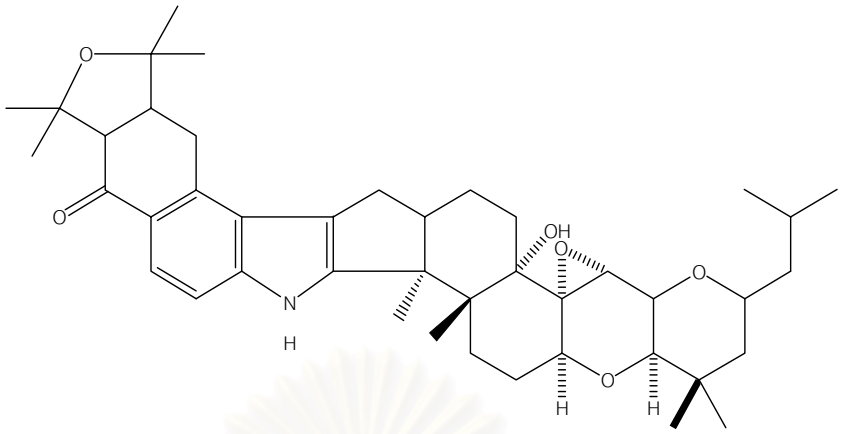


[75] 2-hydroxy-6-methyl benzoic acid

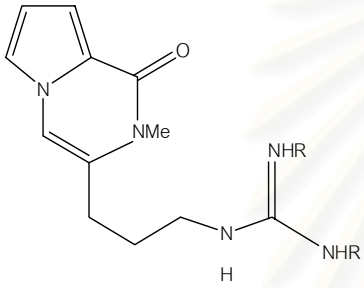


[76] Preussomerin D

Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)

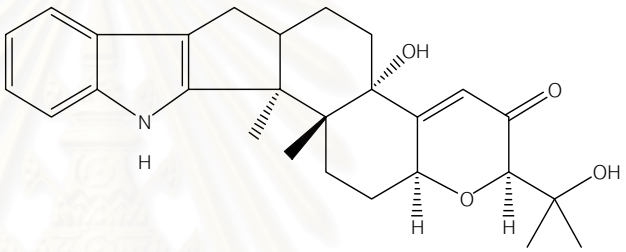


[77] Lolitrem C

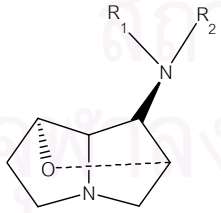


[78] Peramine R=H

[79] Diacetylperamine R=Ac

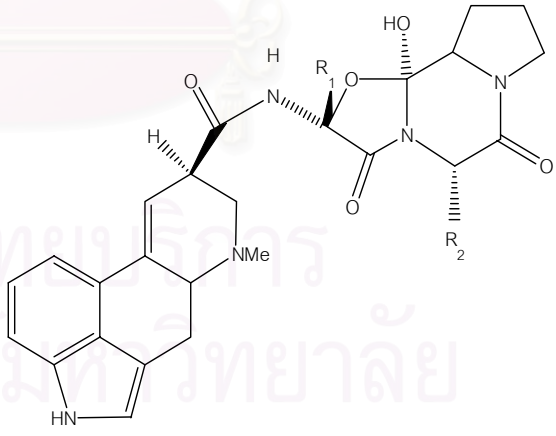


[80] Paxilline



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

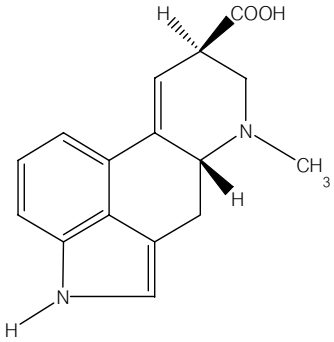
R_2= H, HCO, Ac



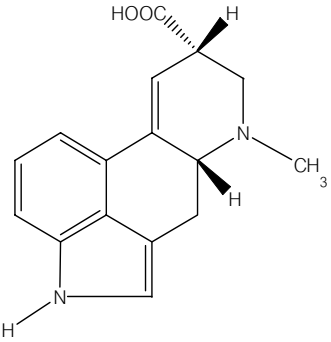
[82] Ergopeptine alkaloids

Ergovaline R_1=Me, R_2=i-Pr

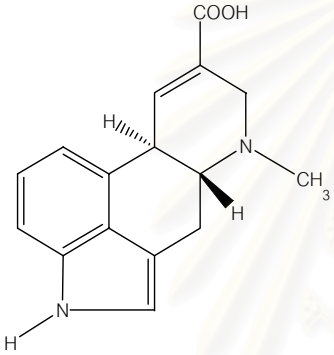
Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)



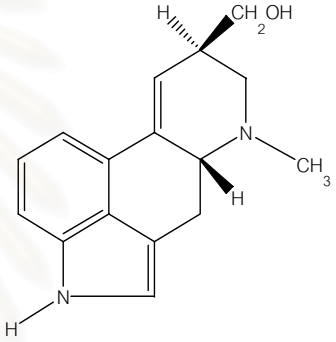
[83] Lysergic acid



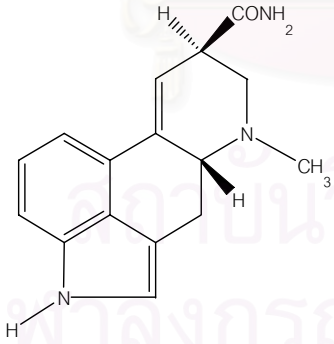
[84] Isolysergic acid



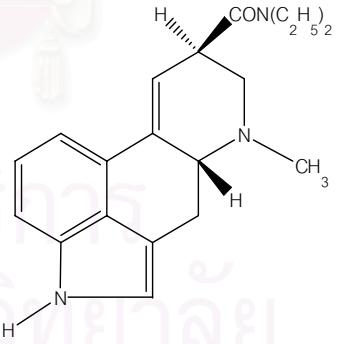
[85] Paspalic acid



[86] Lysergol

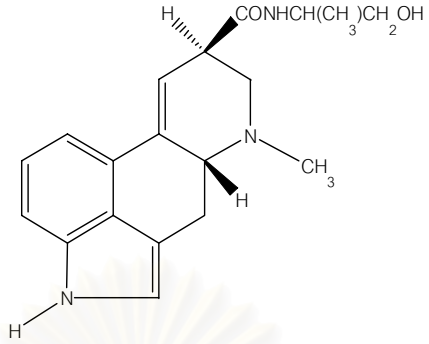


[87] Lysergic acid amide



[88] Lysergic acid diethyl amide

Figure C.1 Structure of secondary metabolites of endophytic fungi **(continued)**



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

Figure C.1 Structure of secondary metabolites of endophytic fungi (continued)

APPENDIX D

Table D.1 The raw data of mycelium dry weight

Date	Mycelium dry weight (g)			
	Repeat1	Repeat2	Repeat3	average
0	0.0000	0.0000	0.0000	0.0000
2	0.2419	0.2271	0.2772	0.2487
4	0.3308	0.3189	3.3486	0.3328
6	0.4650	0.4436	0.4658	0.4581
8	0.5371	0.5272	0.5513	0.5385
10	0.6142	0.6018	0.6848	0.6336
12	0.5616	0.6641	0.6017	0.6091
14	0.6267	0.6177	0.6604	0.6349
16	0.6459	0.5892	0.6175	0.6175
18	0.4957	0.6245	0.5283	0.5495
20	0.4232	0.4592	0.4475	0.4433
22	0.4085	0.4210	0.4729	0.4413
24	0.3892	0.3771	0.3216	0.3626
26	0.3900	0.3618	0.3747	0.3755
28	0.3395	0.3626	0.3245	0.3422
30	0.3522	0.3496	0.3711	0.3576
32	0.3535	0.3217	0.3141	0.3298
34	0.3315	0.3476	0.3674	0.3488
36	0.3007	0.3224	0.3069	0.3100
38	0.3110	0.3211	0.3048	0.3123
40	0.3057	0.3278	0.3116	0.3150

APPENDIX E

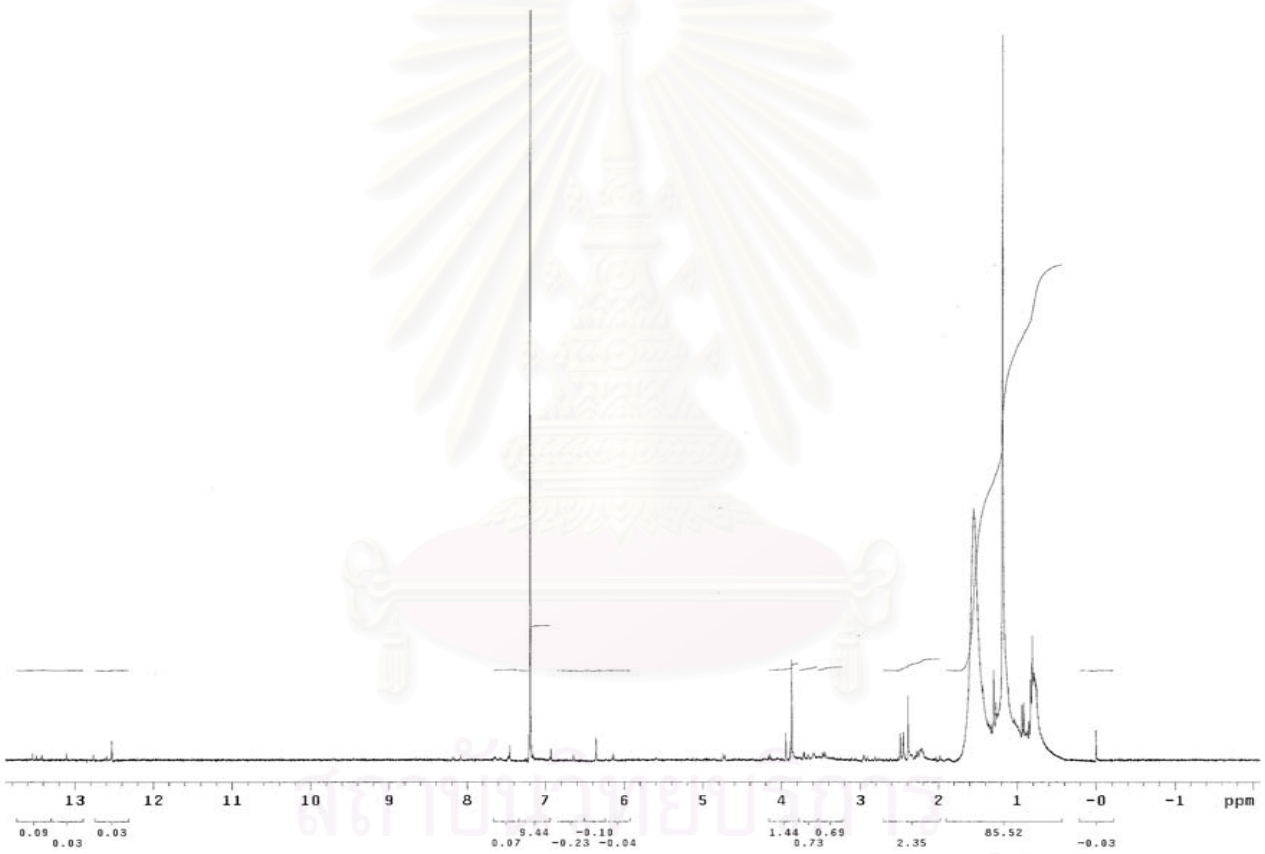


Figure E.1 ^{1}H-NMR spectrum of crude culture broth at date 6

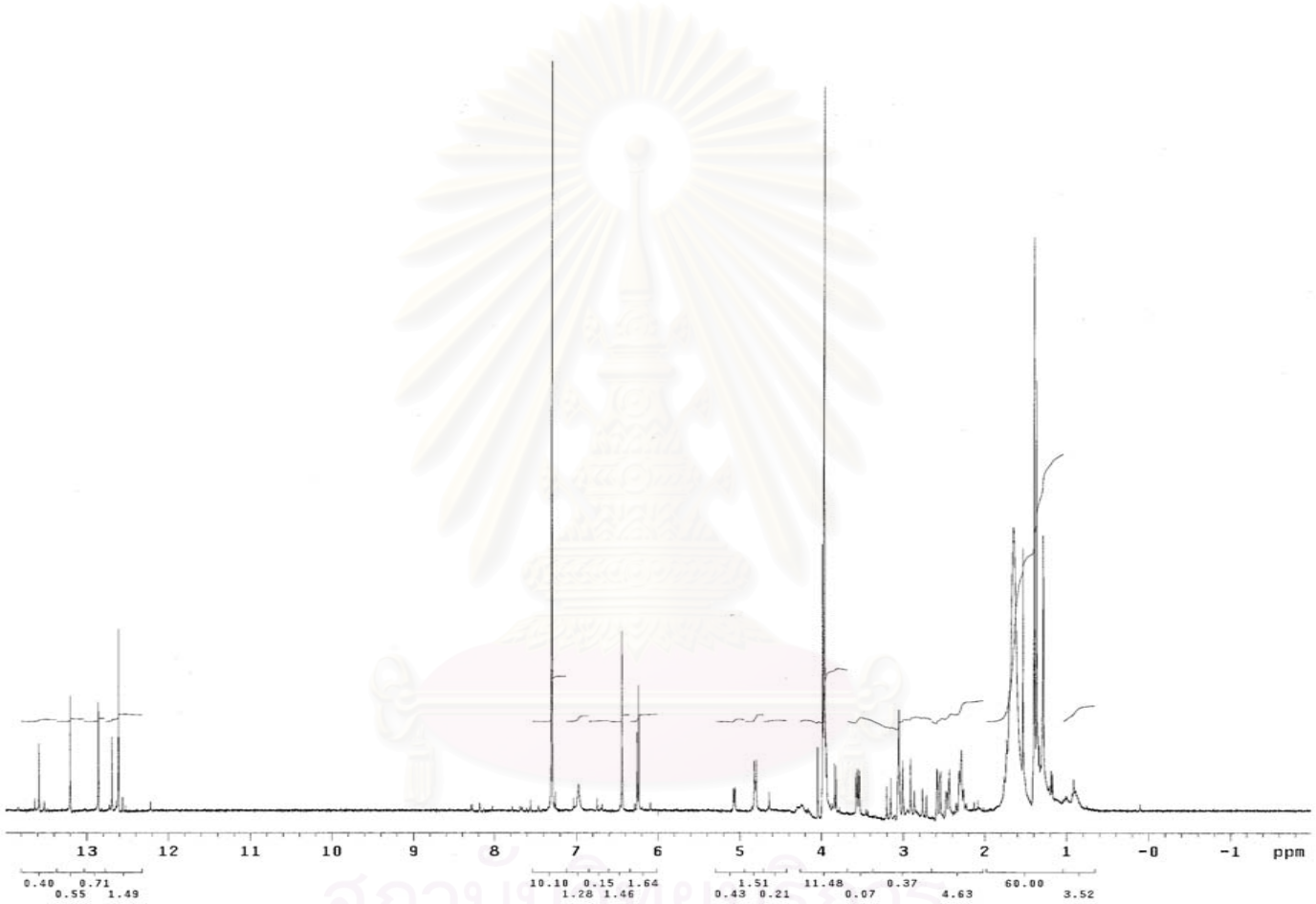


Figure E.2 ^{1}H-NMR spectrum of crude culture broth at date 14

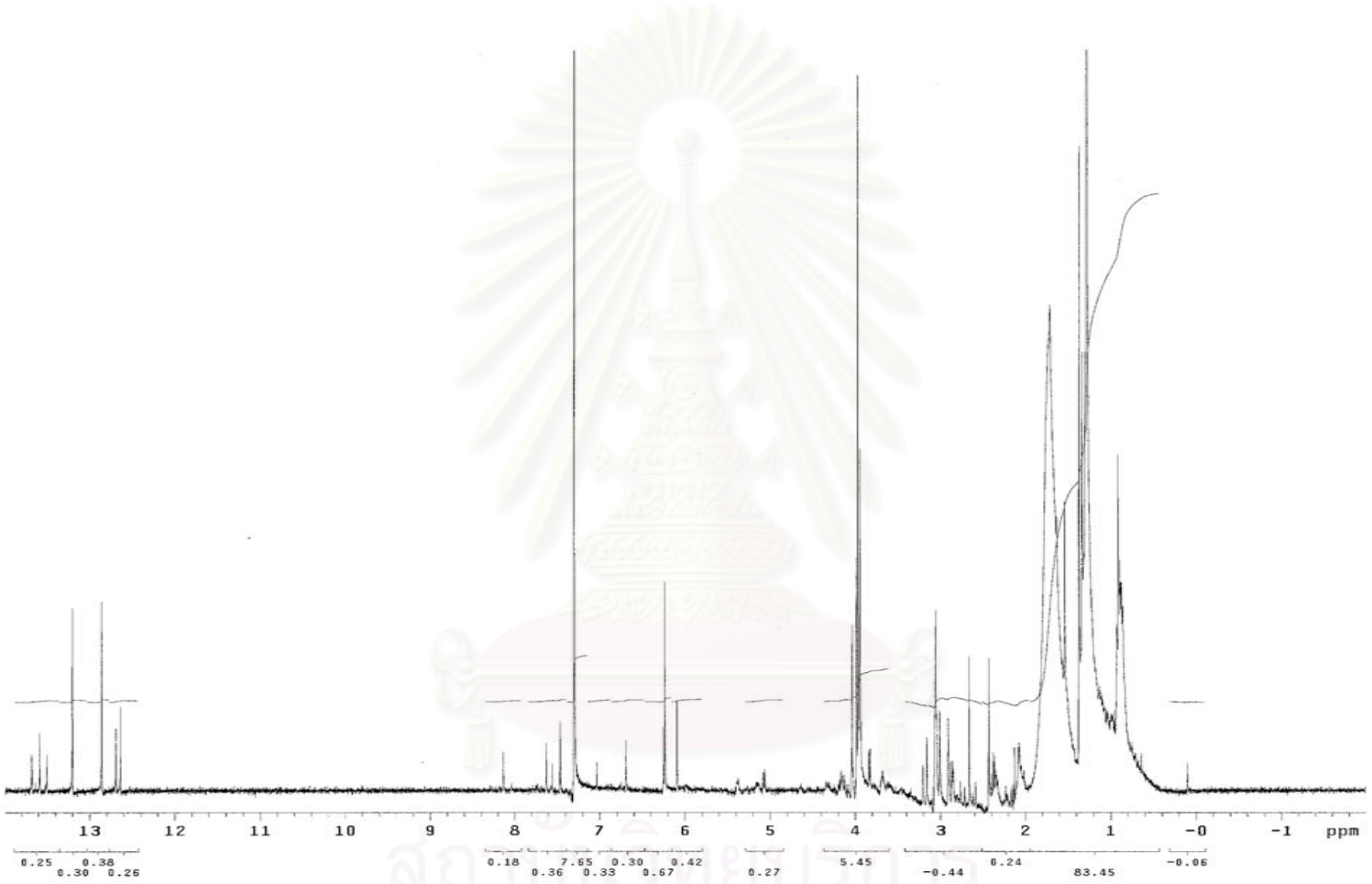


Figure E.3 ^{1}H-NMR spectrum of crude culture broth at date 28

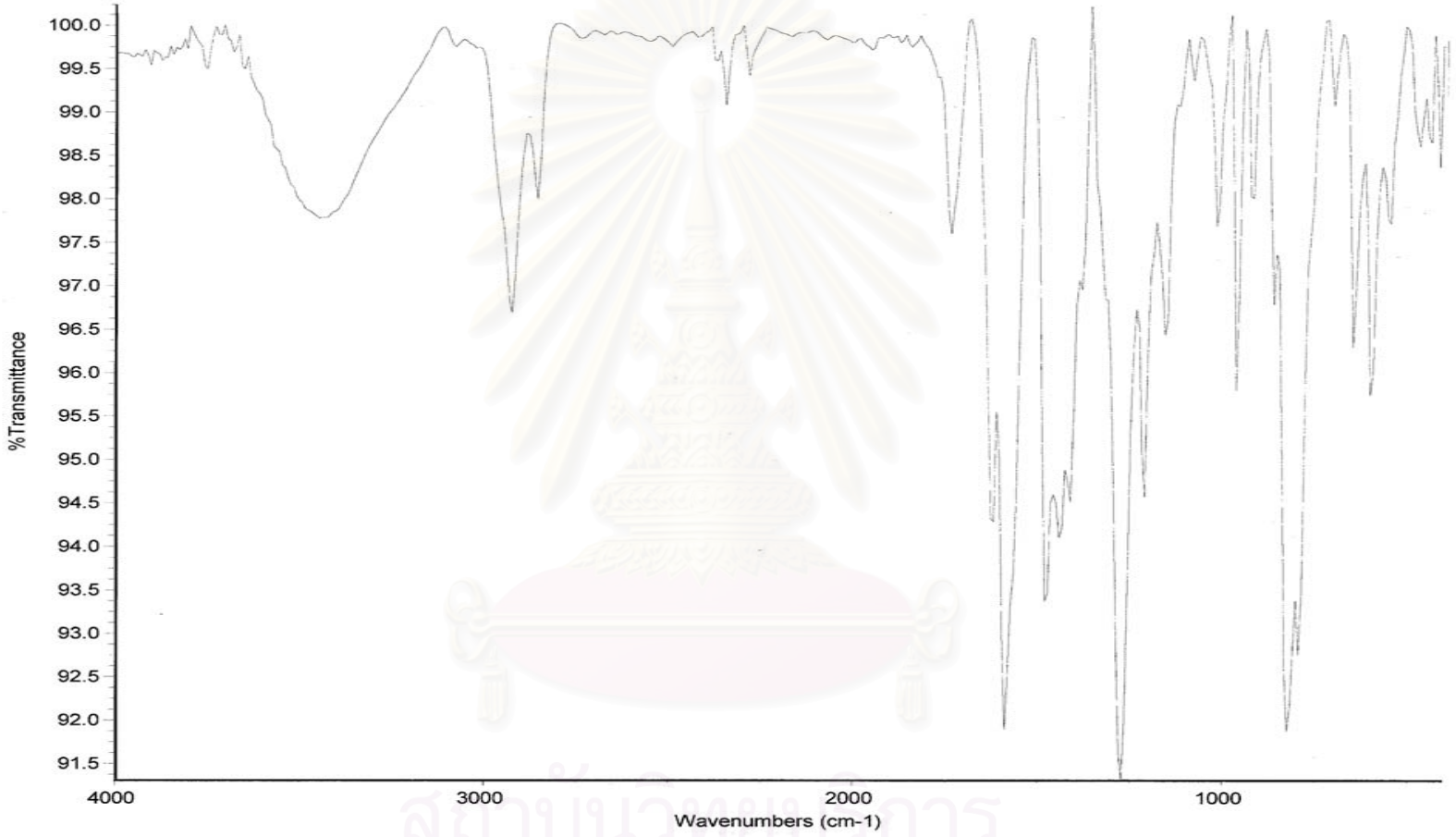


Figure E.4 IR spectrum of compound C1

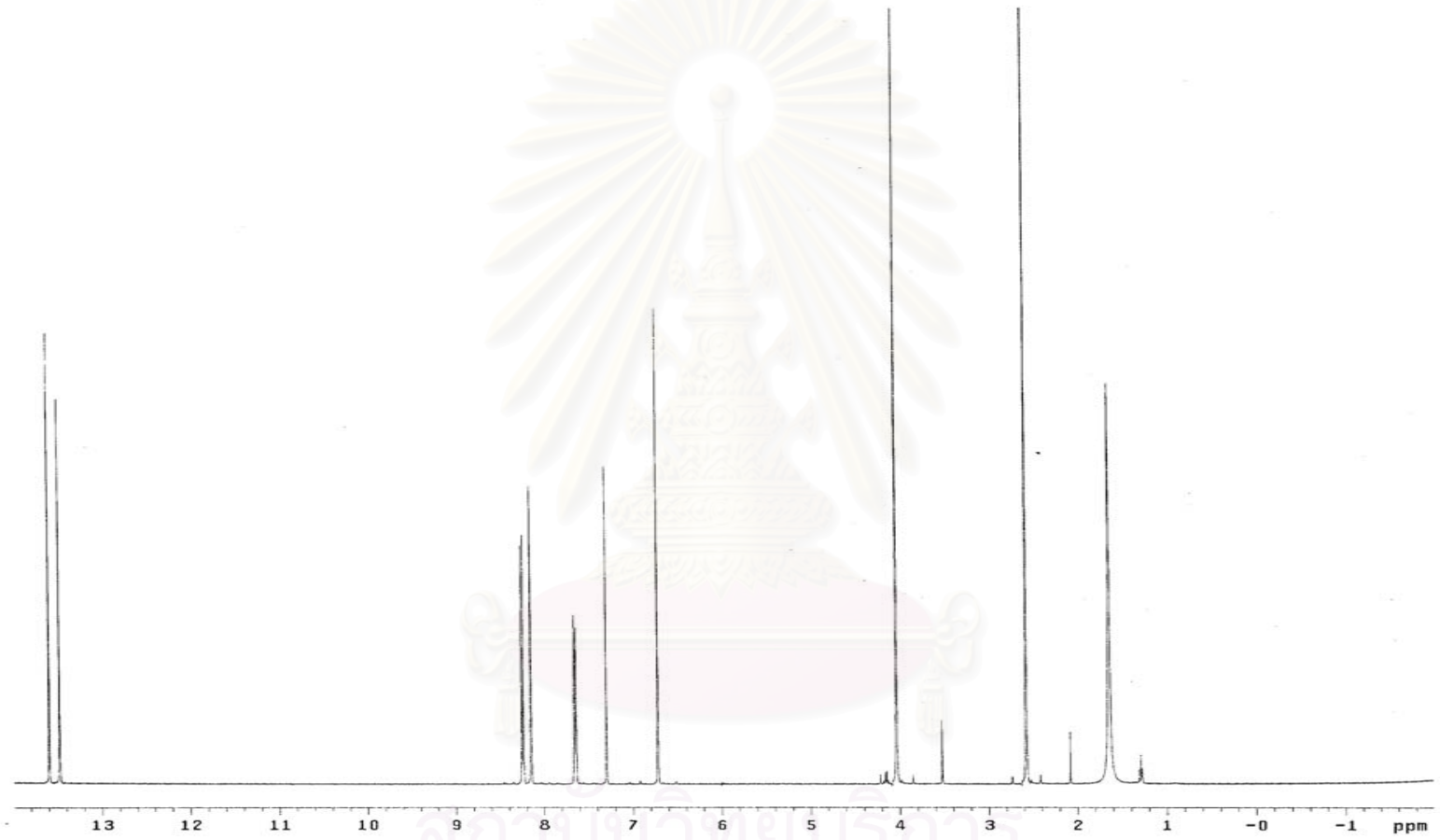


Figure E.5 ^{1}H-NMR spectrum of compoumd C1

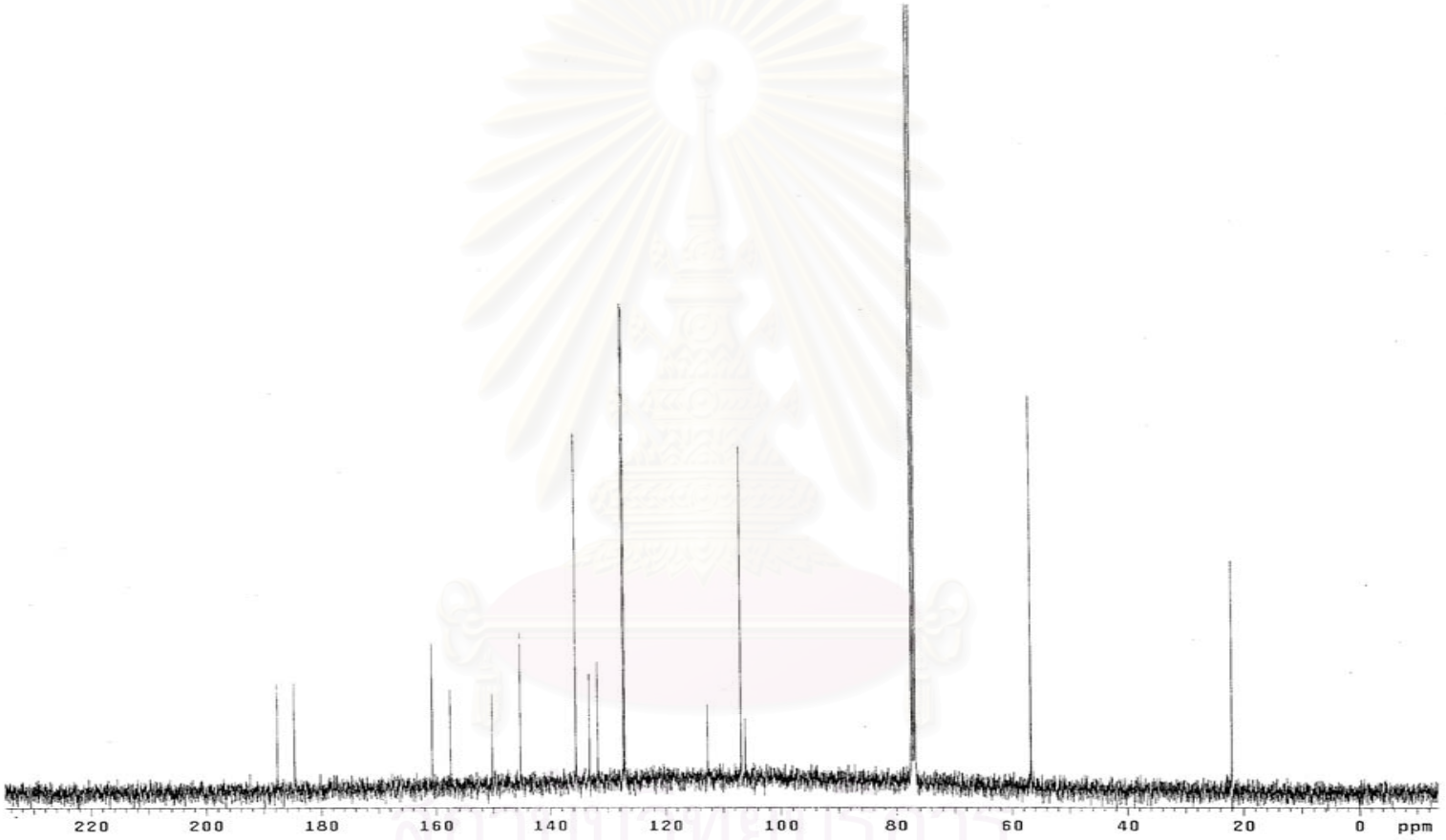


Figure E.6 ^{13}C-NMR spectrum of compound C1

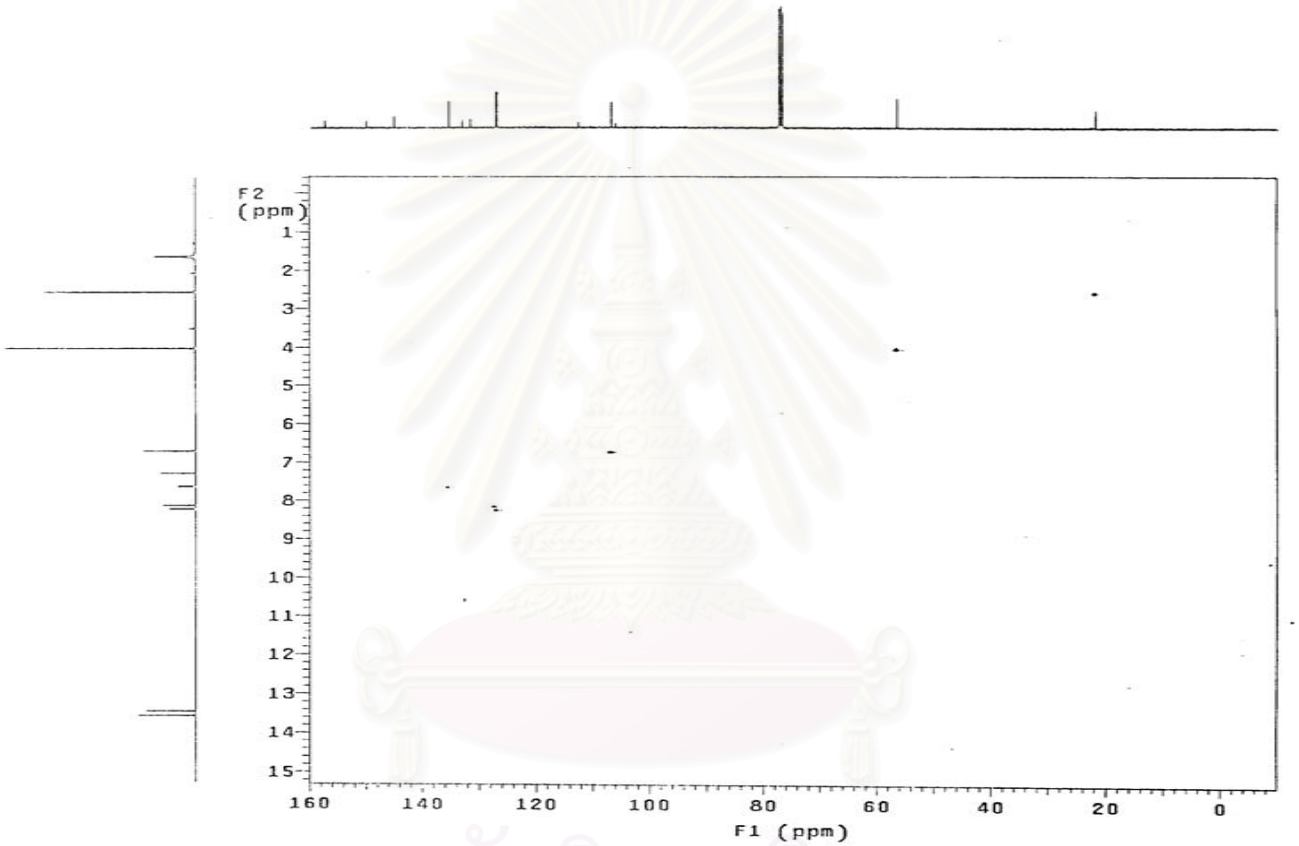


Figure E.7 HSQC spectrum of compound C1

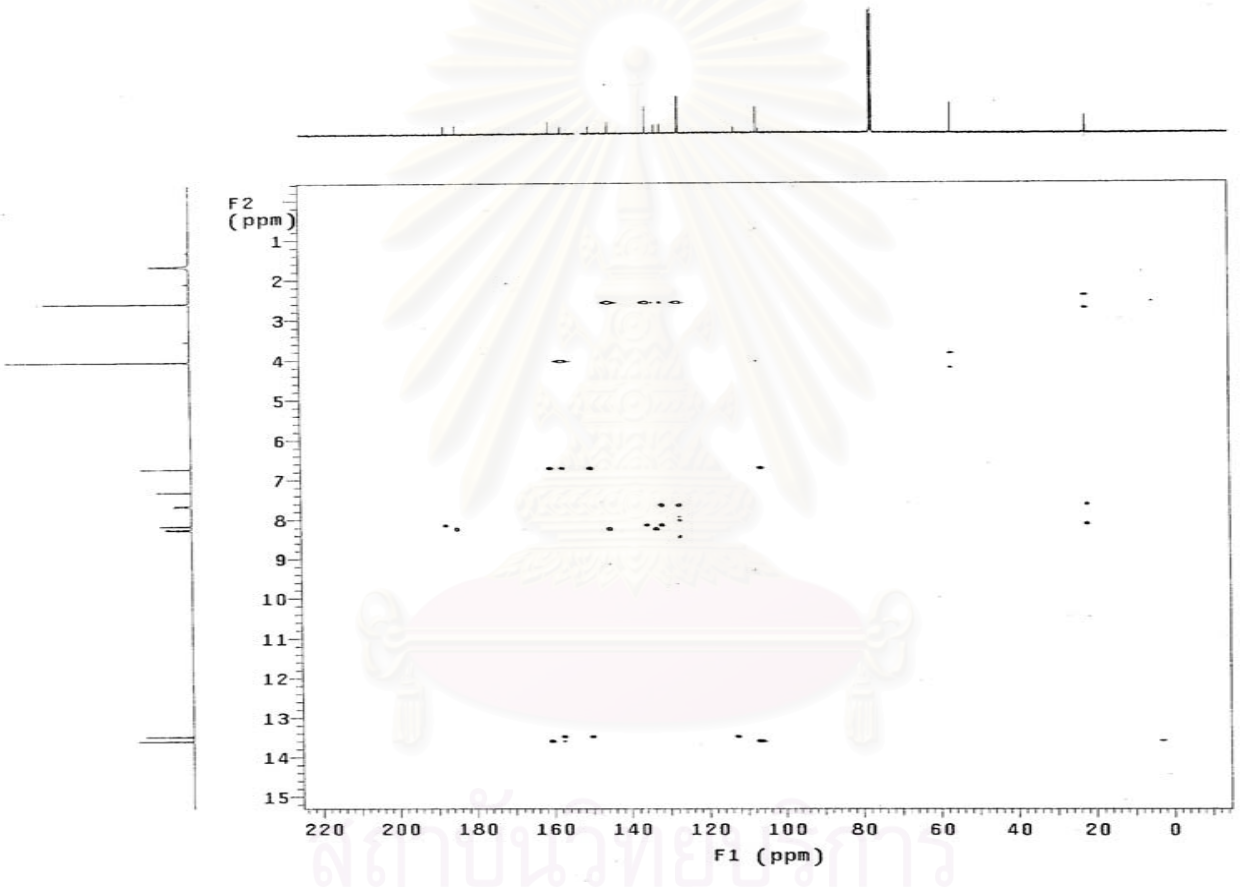


Figure E.8 HMBC spectrum of compound C1

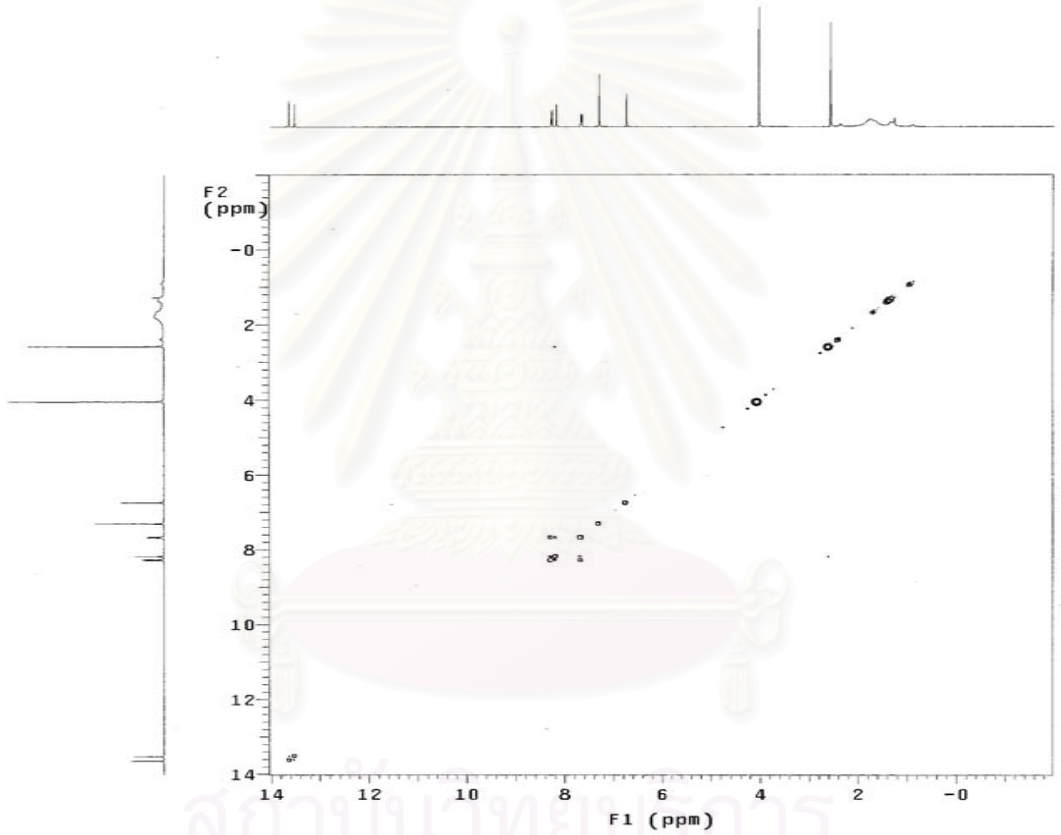


Figure E.9 COSY spectrum of compound C1

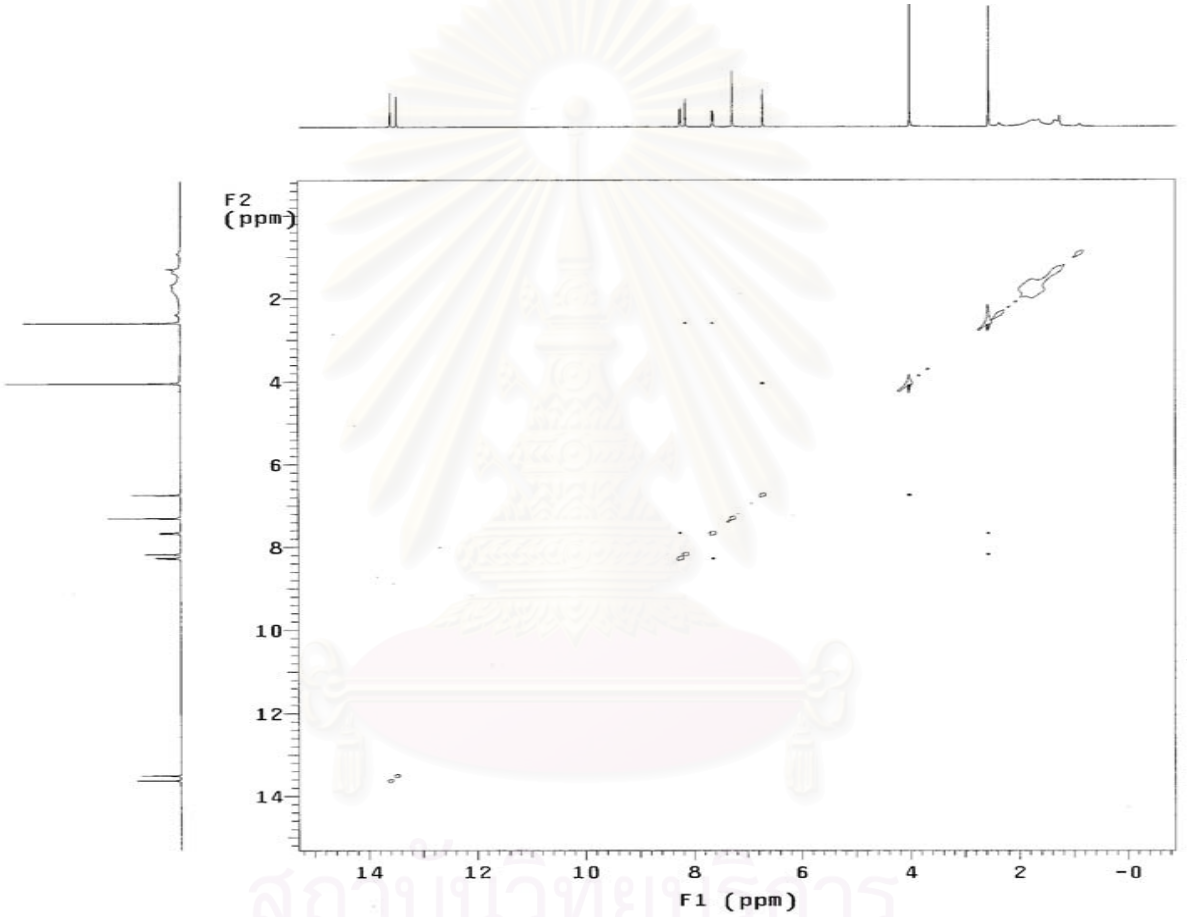


Figure E.10 NOESY spectrum of compound C1

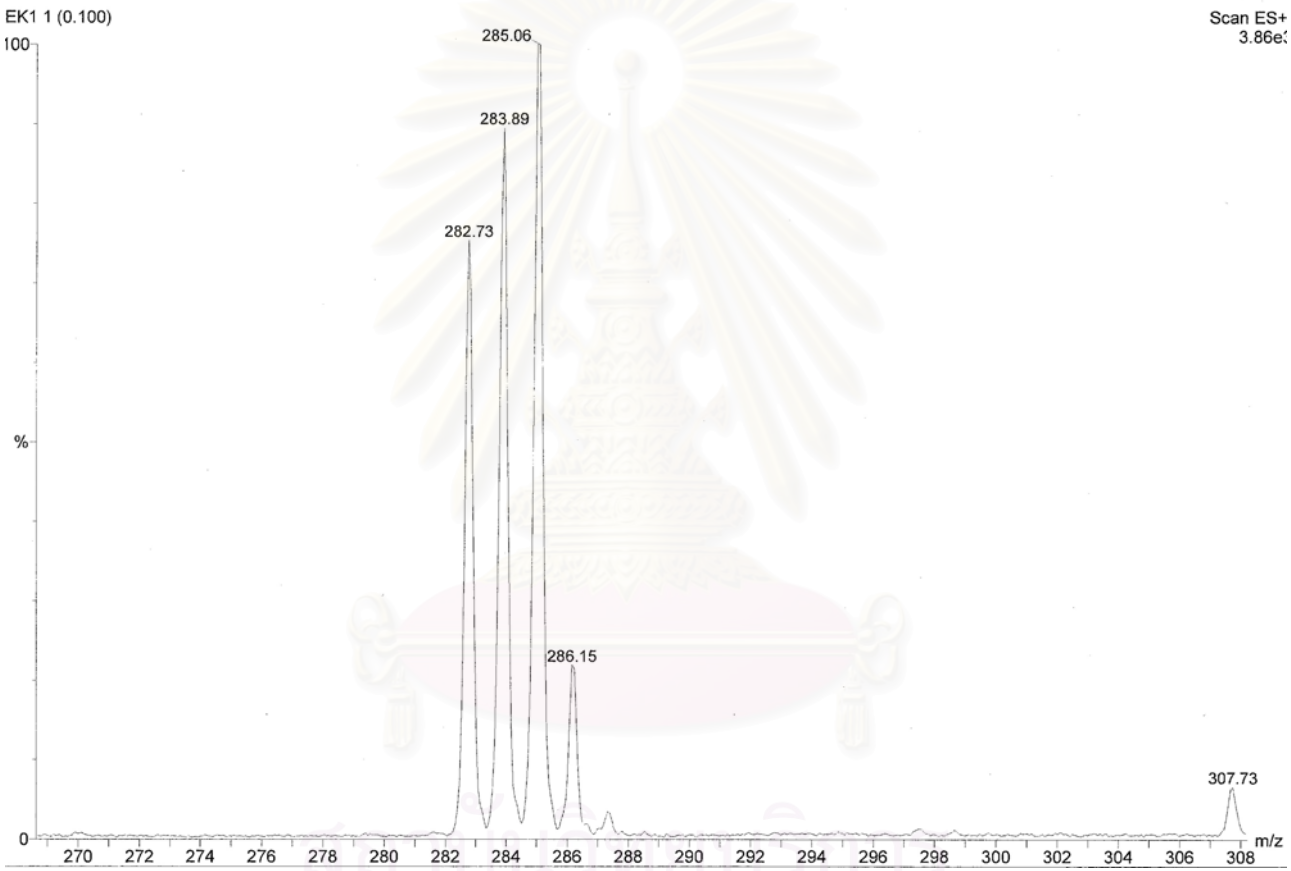


Figure E.11 MS spectrum of compound C1

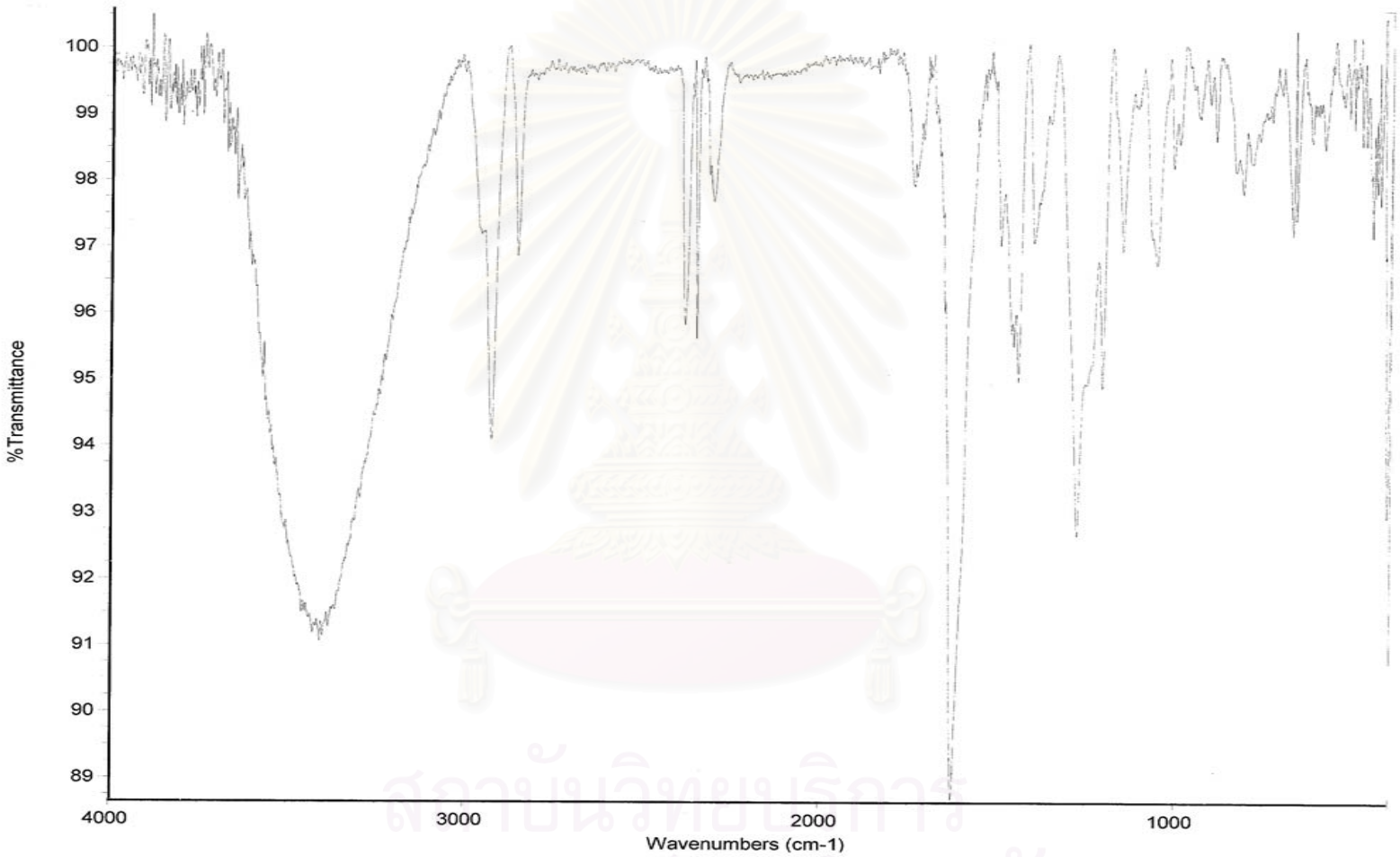


Figure E.12 IR spectrum of compound C2

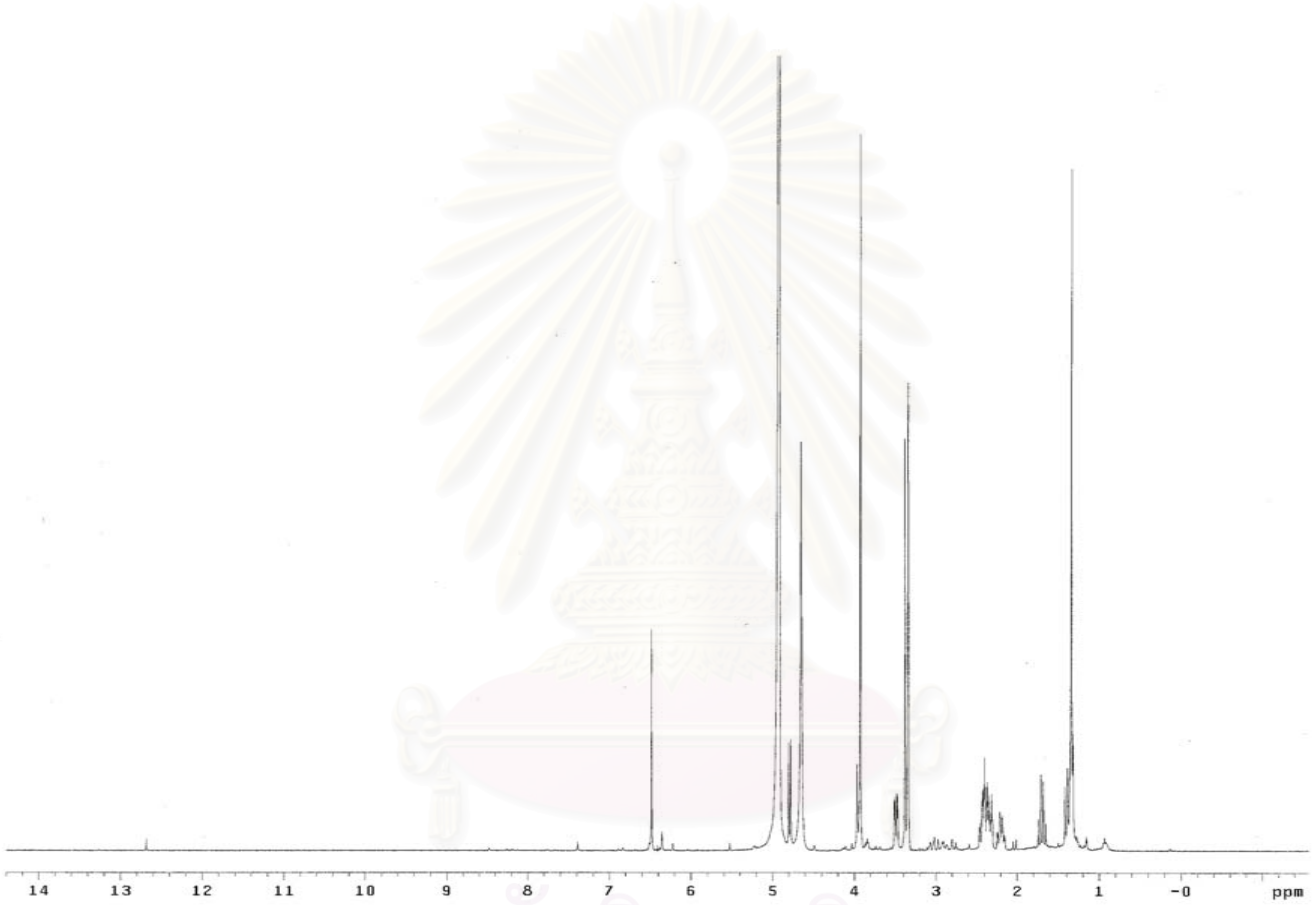


Figure E.13 ^{1}H-NMR spectrum of compoumd C2

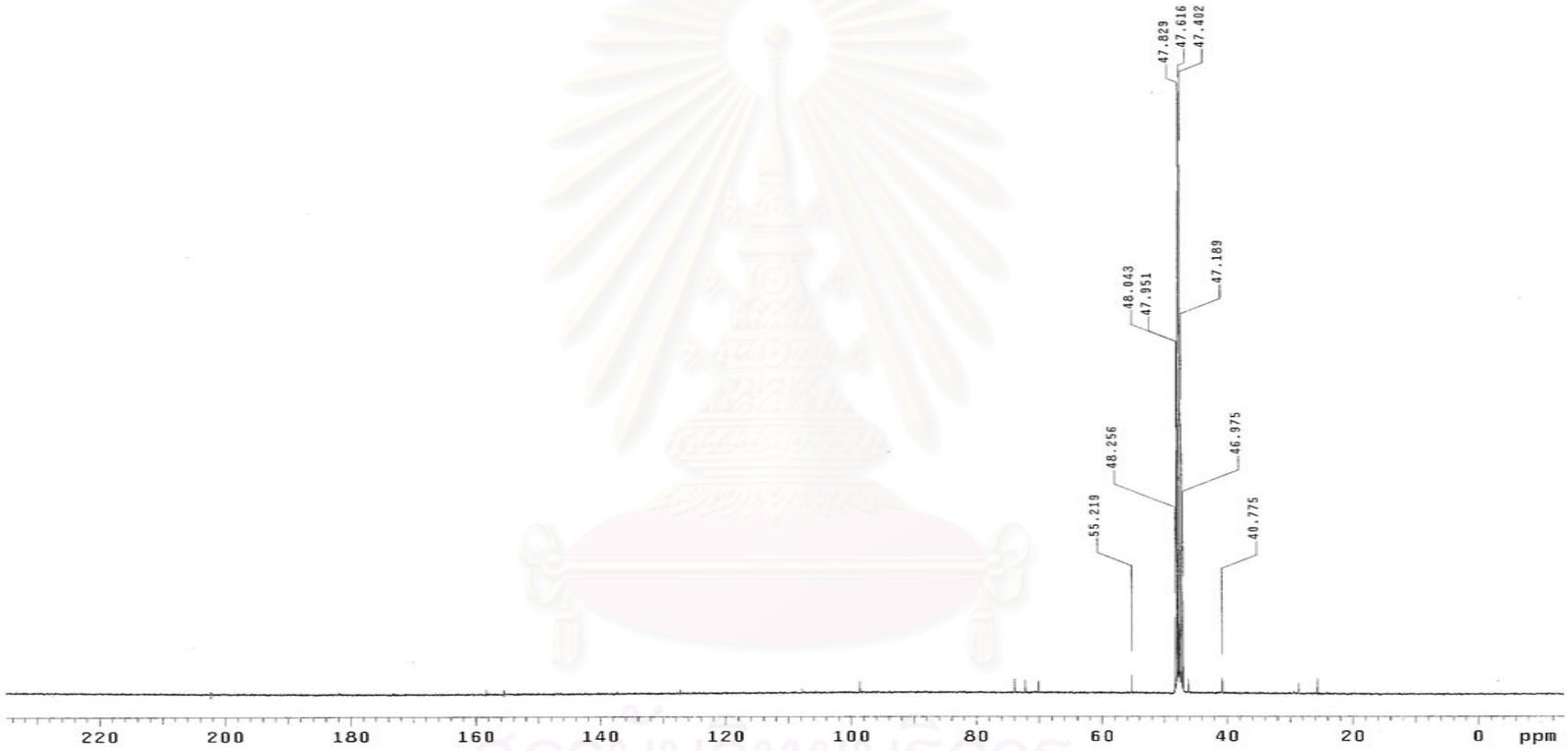


Figure E.14 ^{13}C-NMR spectrum of compound C2

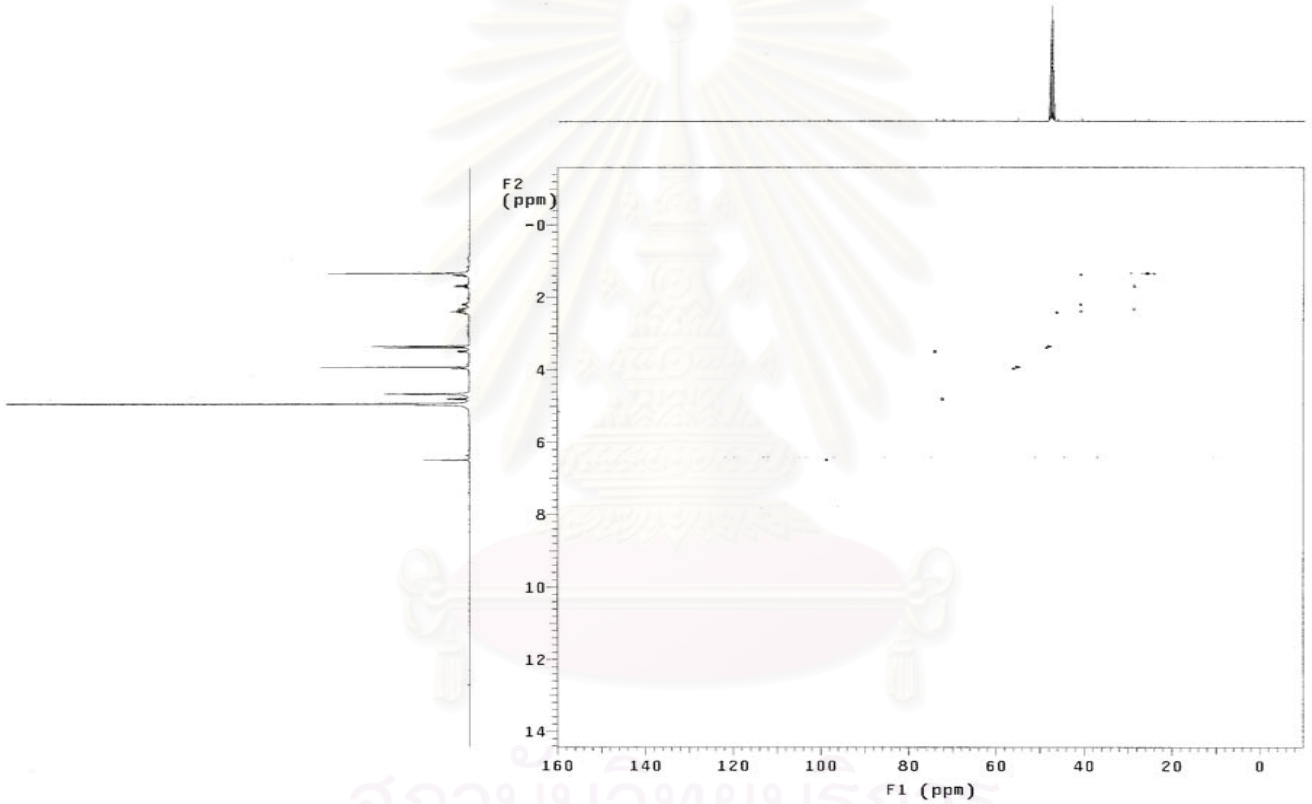


Figure E.15 HSQC spectrum of compound C2

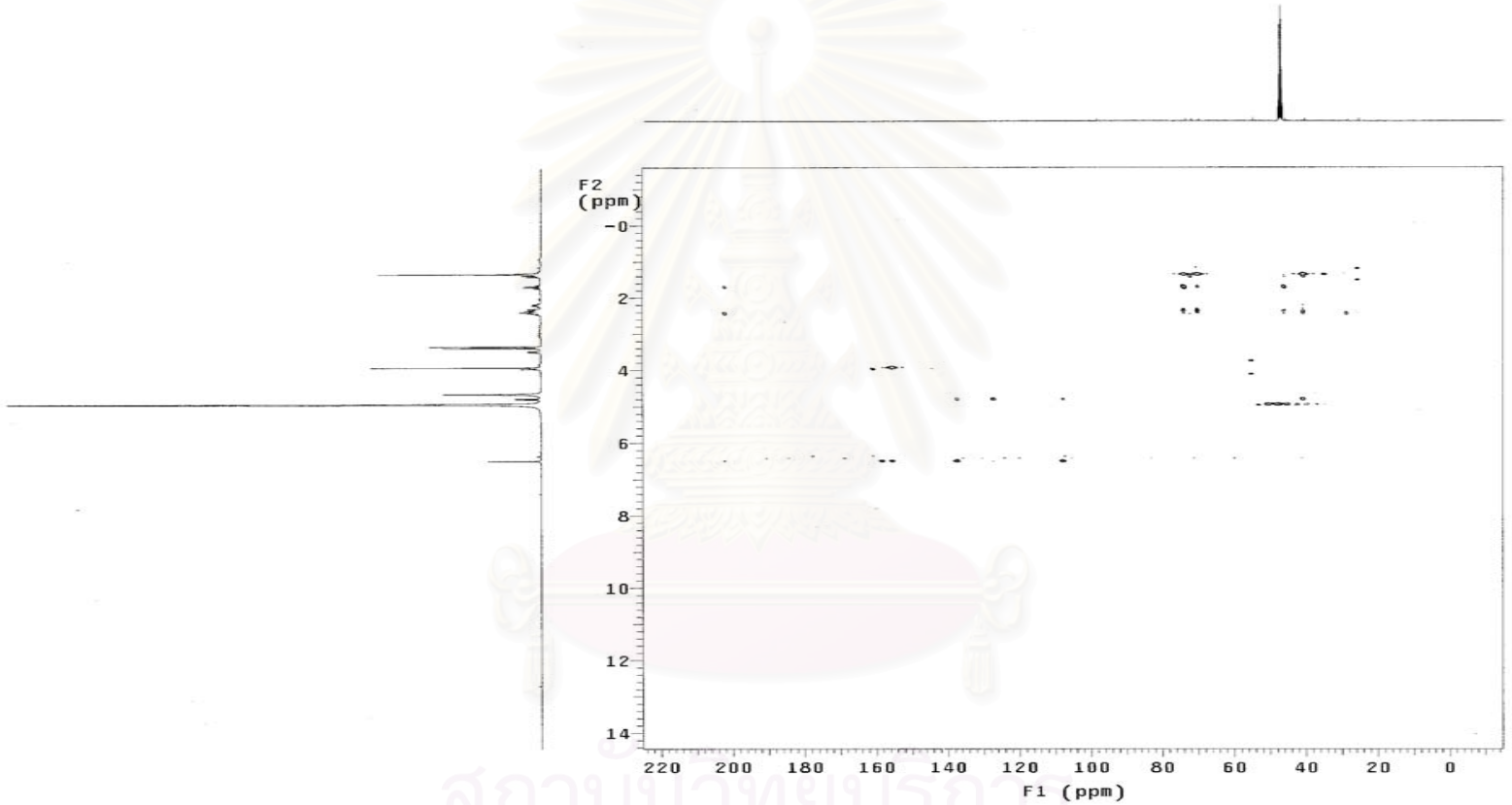


Figure E.16 HMBC spectrum of compound C2

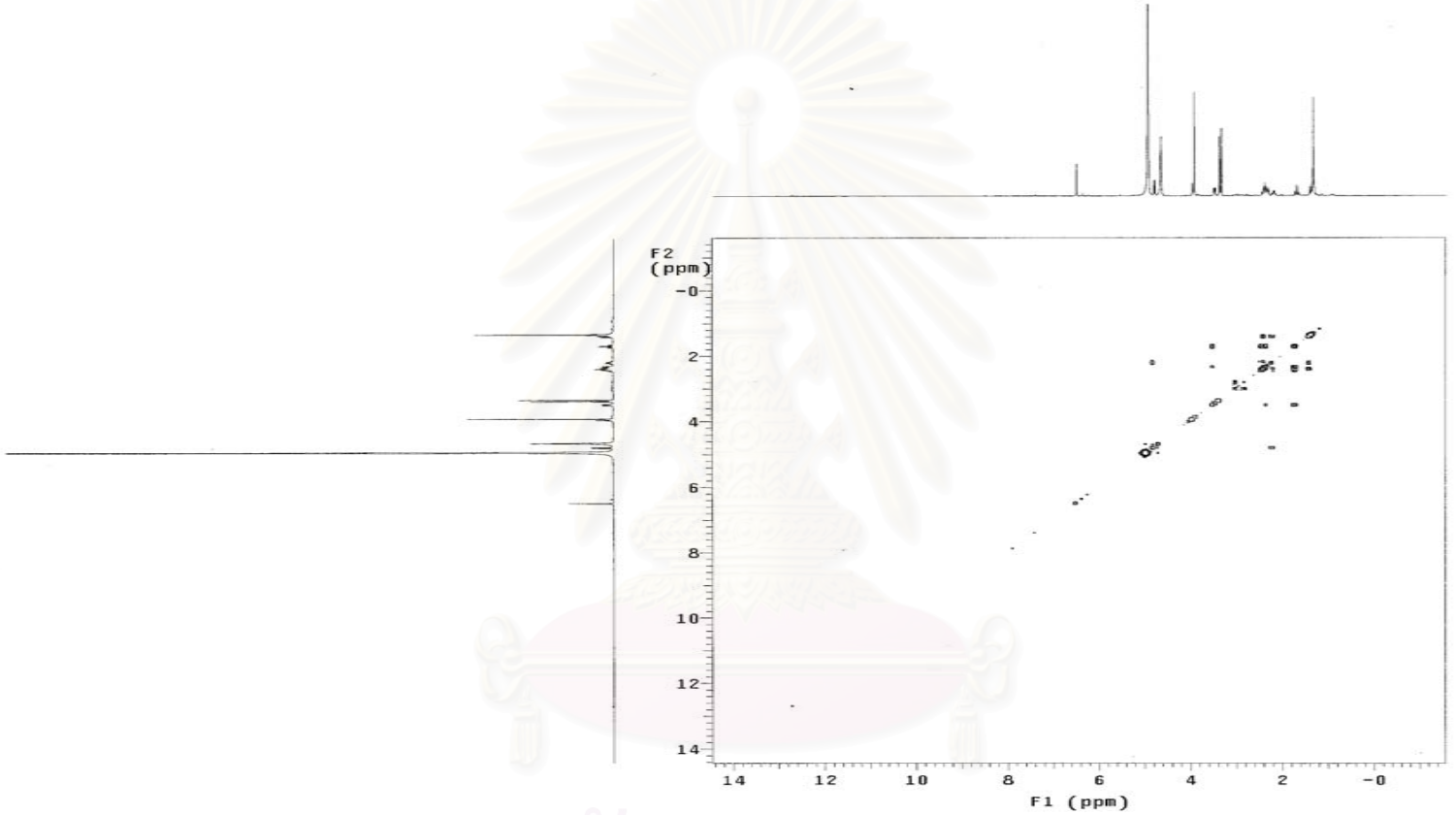


Figure E.17 COSY spectrum of compound C2

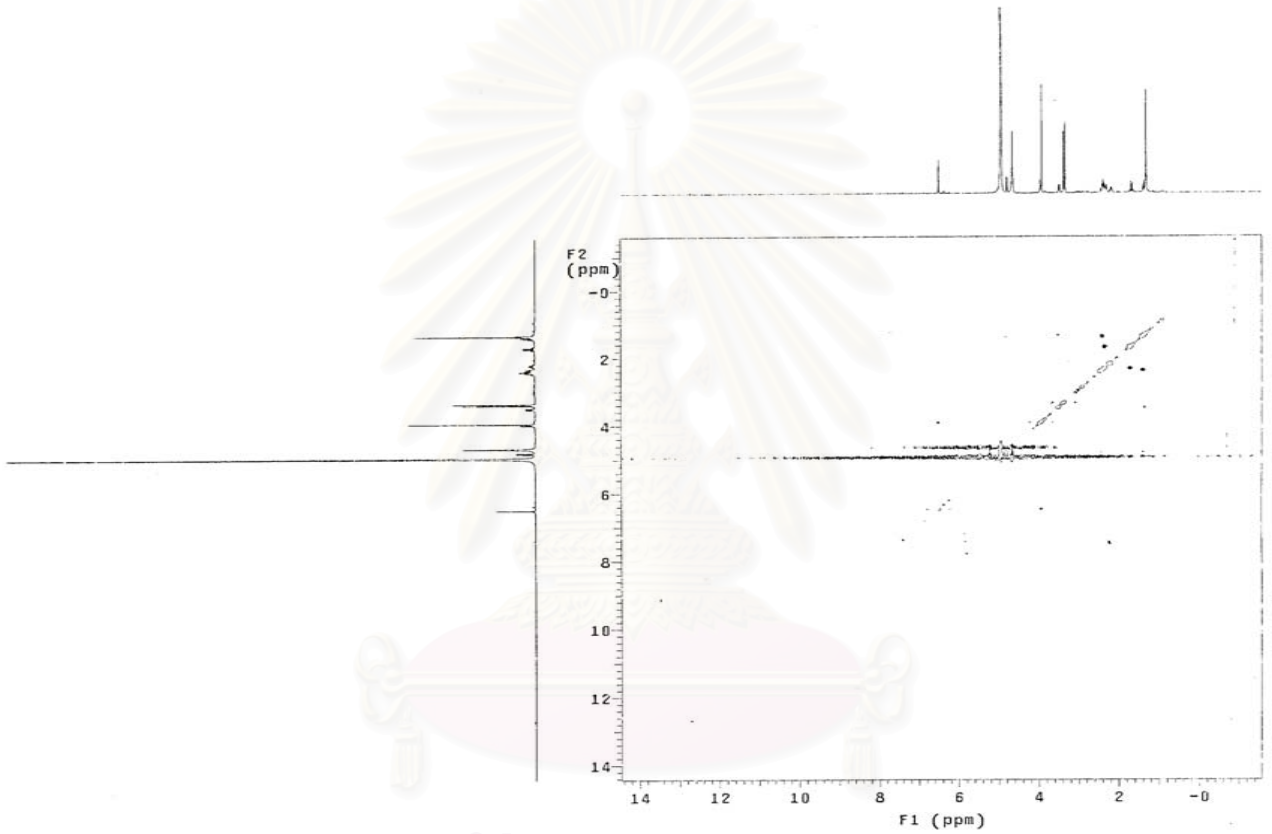


Figure E.18 NOESY spectrum of compound C2

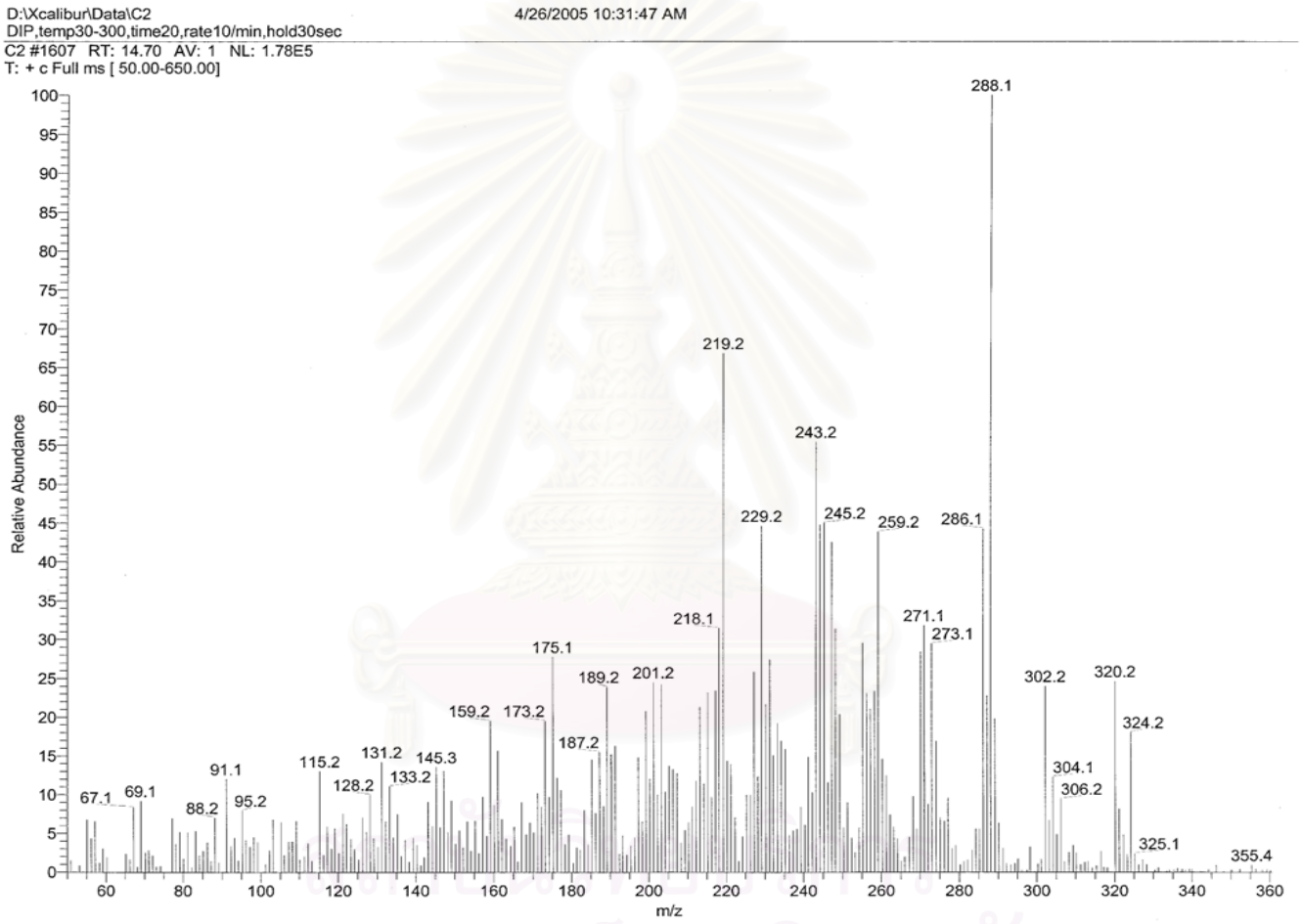


Figure E.19 MS spectrum of compound C2

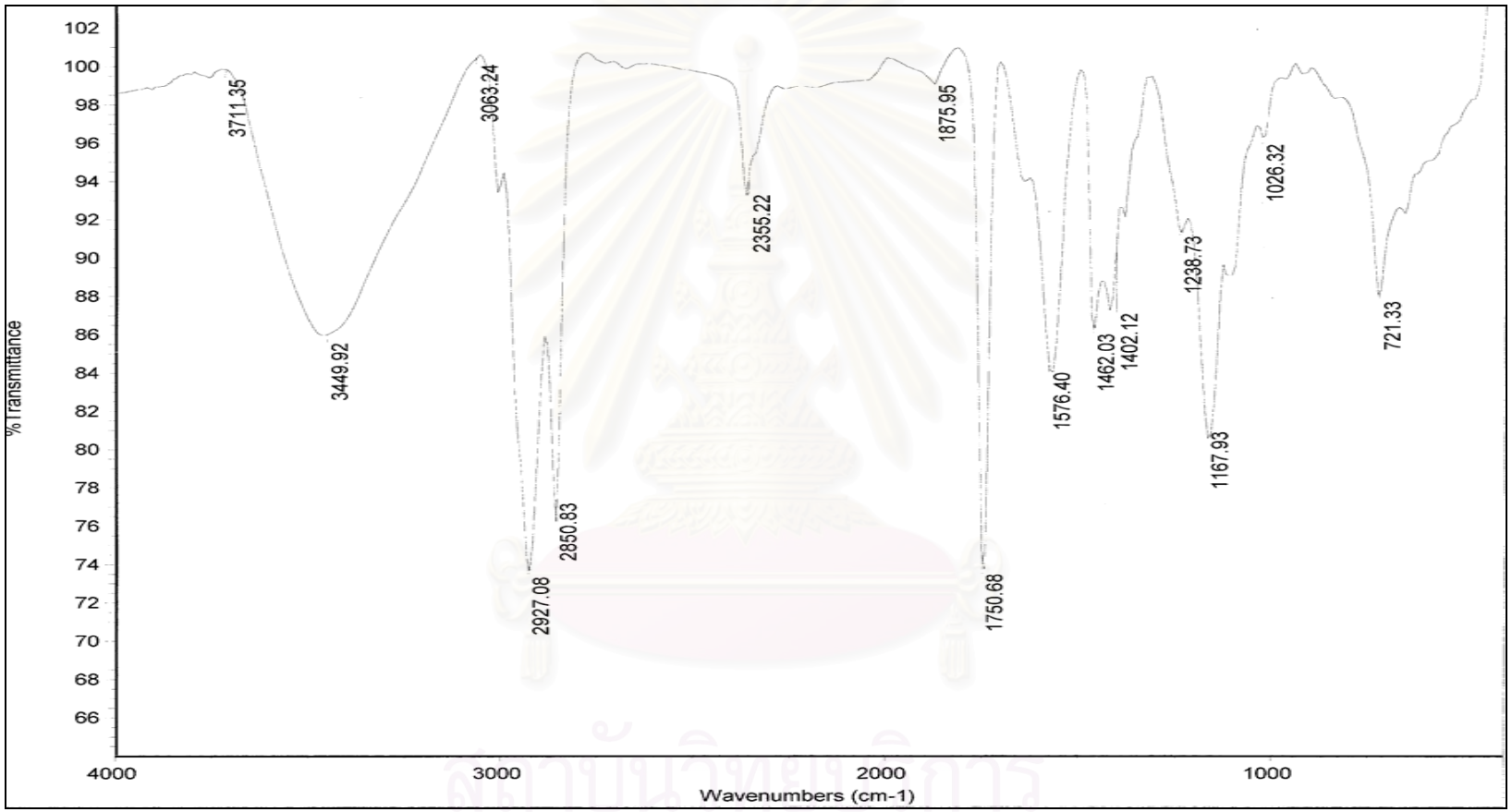


Figure E.20 IR spectrum of mixture1

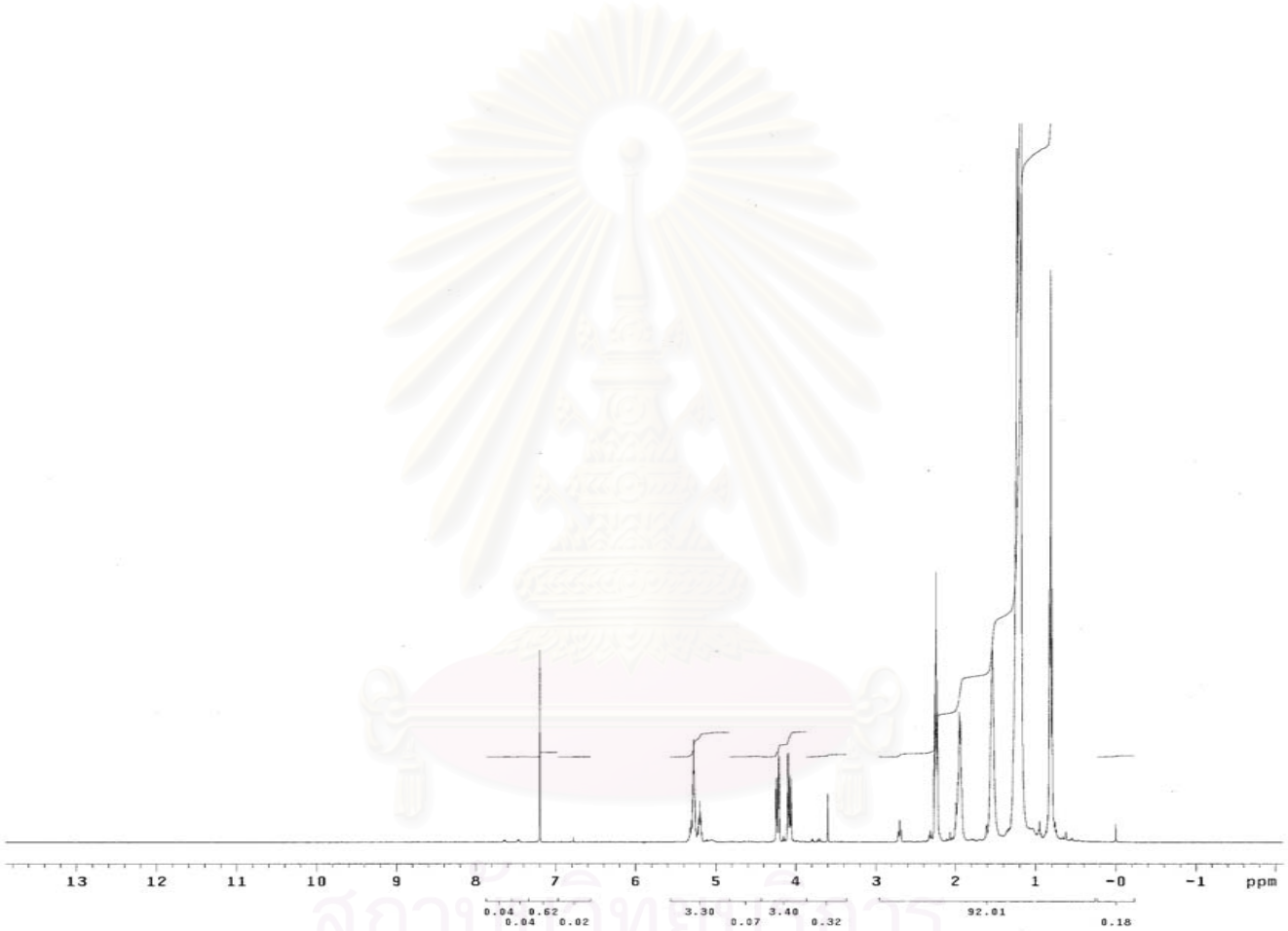


Figure E.21 ^{1}H-NMR spectrum of mixture1

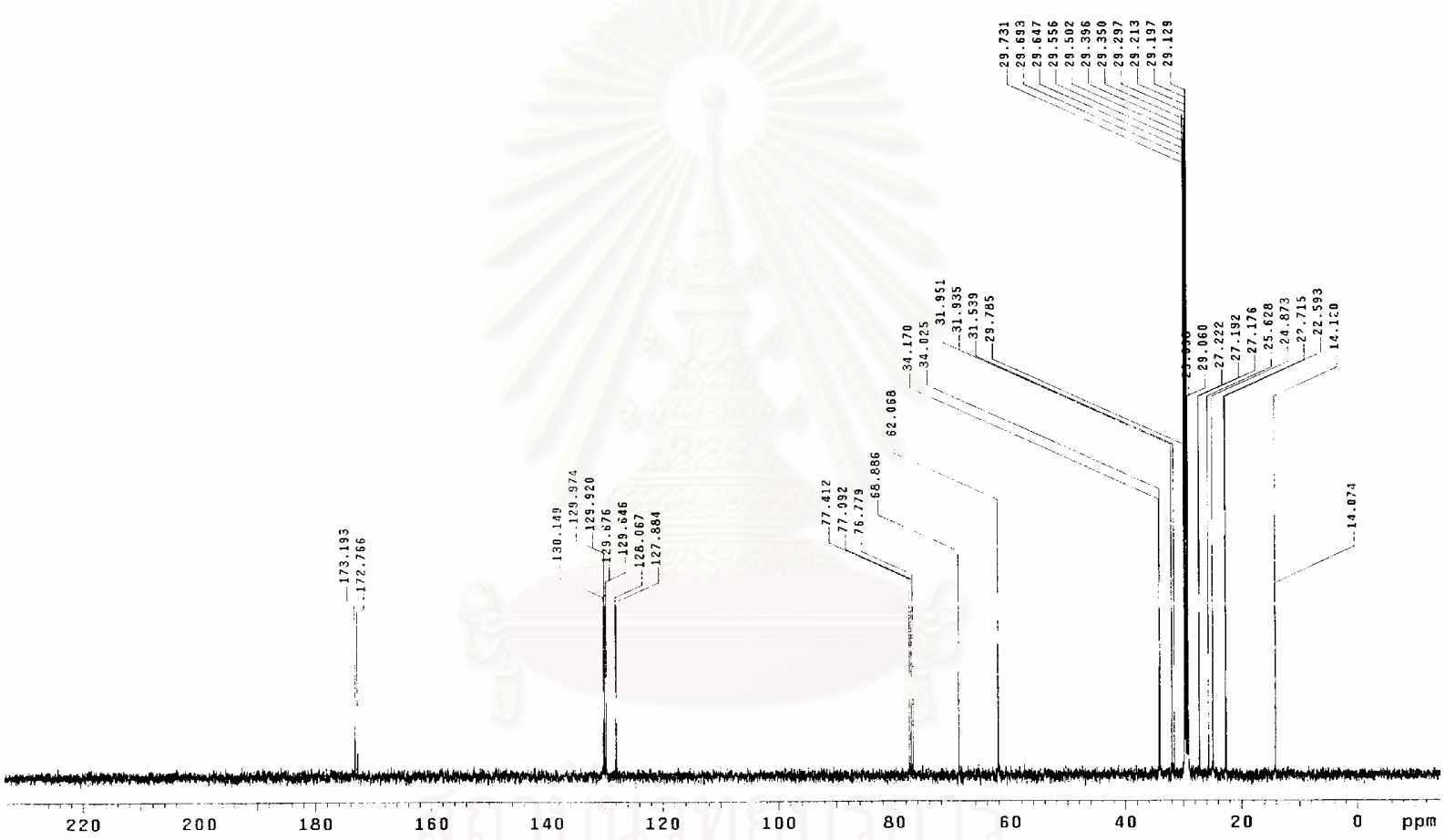


Figure E.22 ^{13}C-NMR spectrum of mixture1

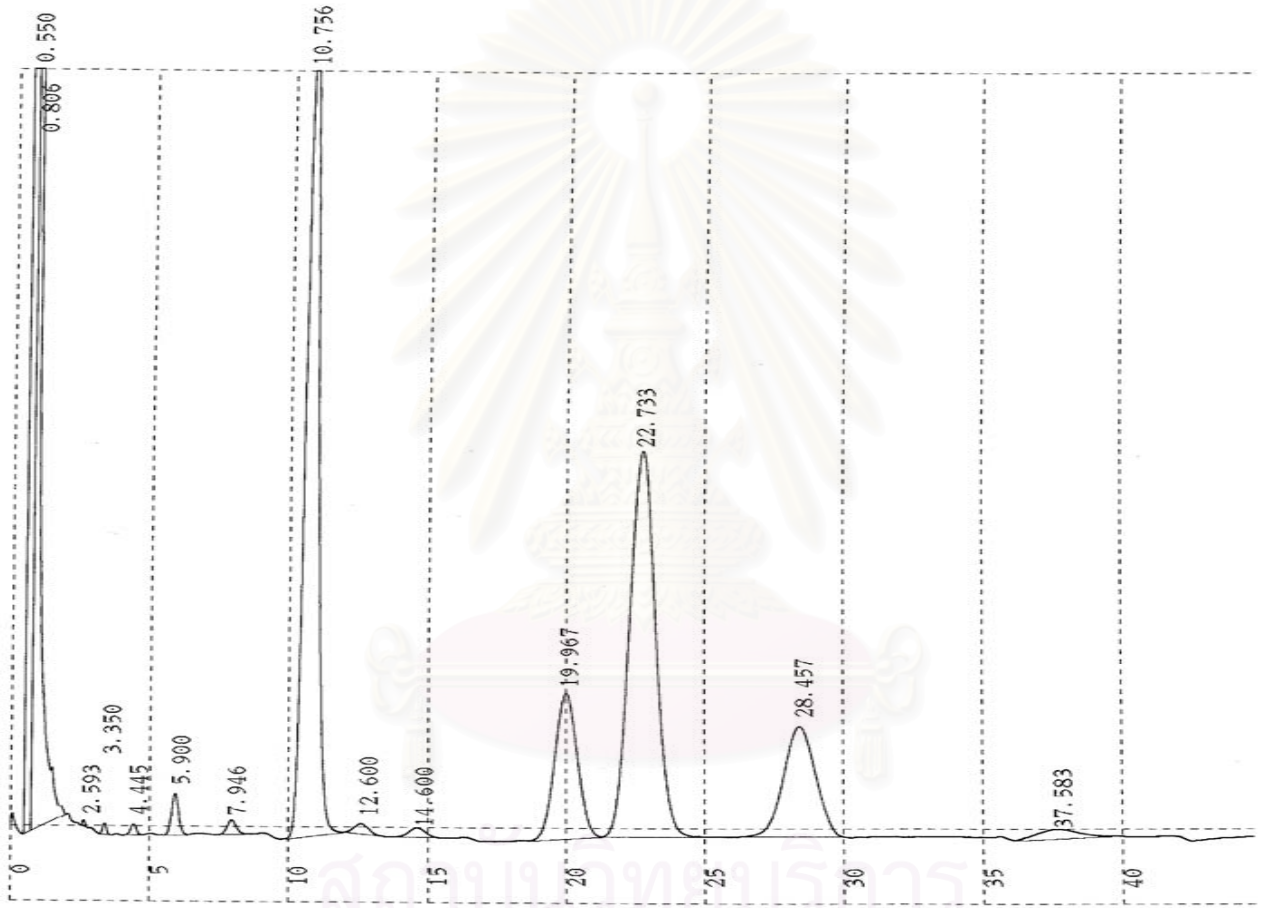


Figure E.23 GC spectrum of mixture1

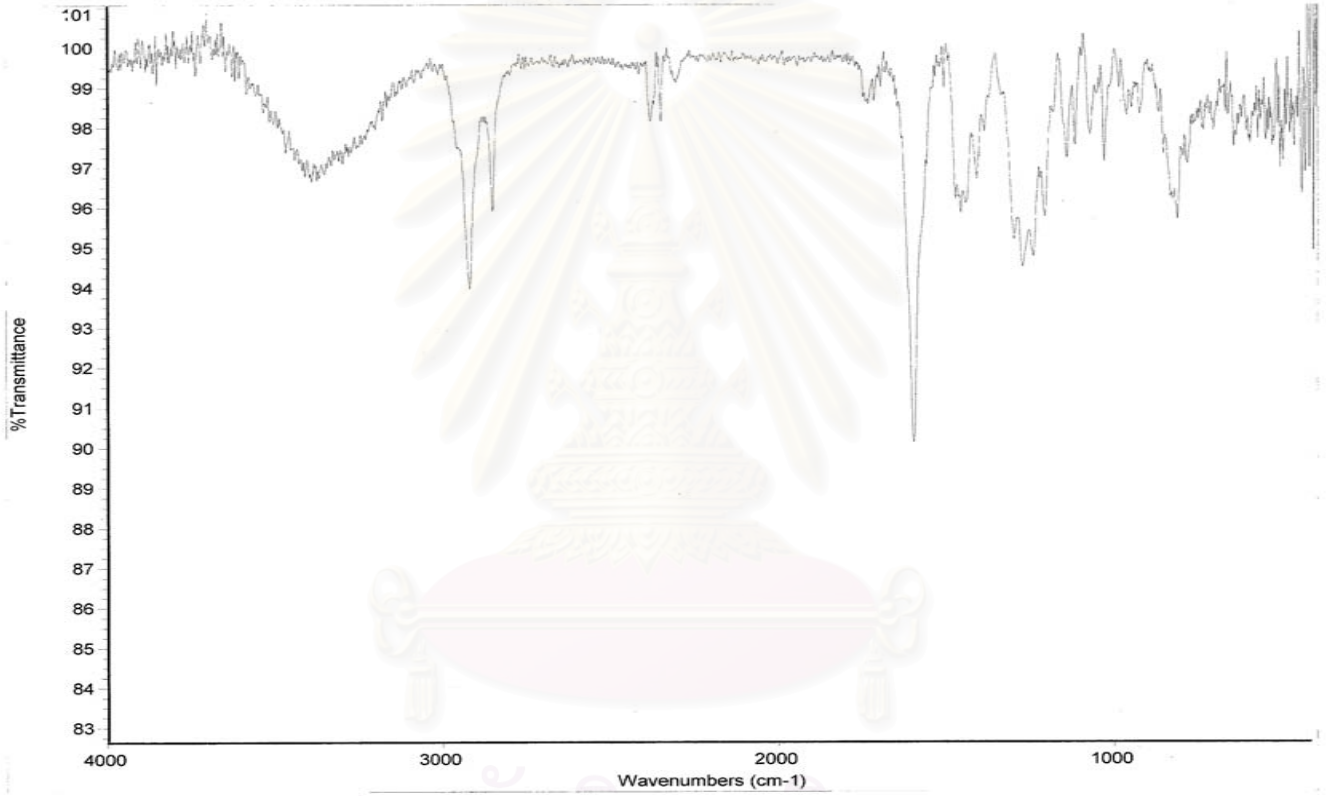


Figure E.24 IR spectrum of mixture 2

Figure E.25 ^{1}H-NMR spectrum of mixture 2

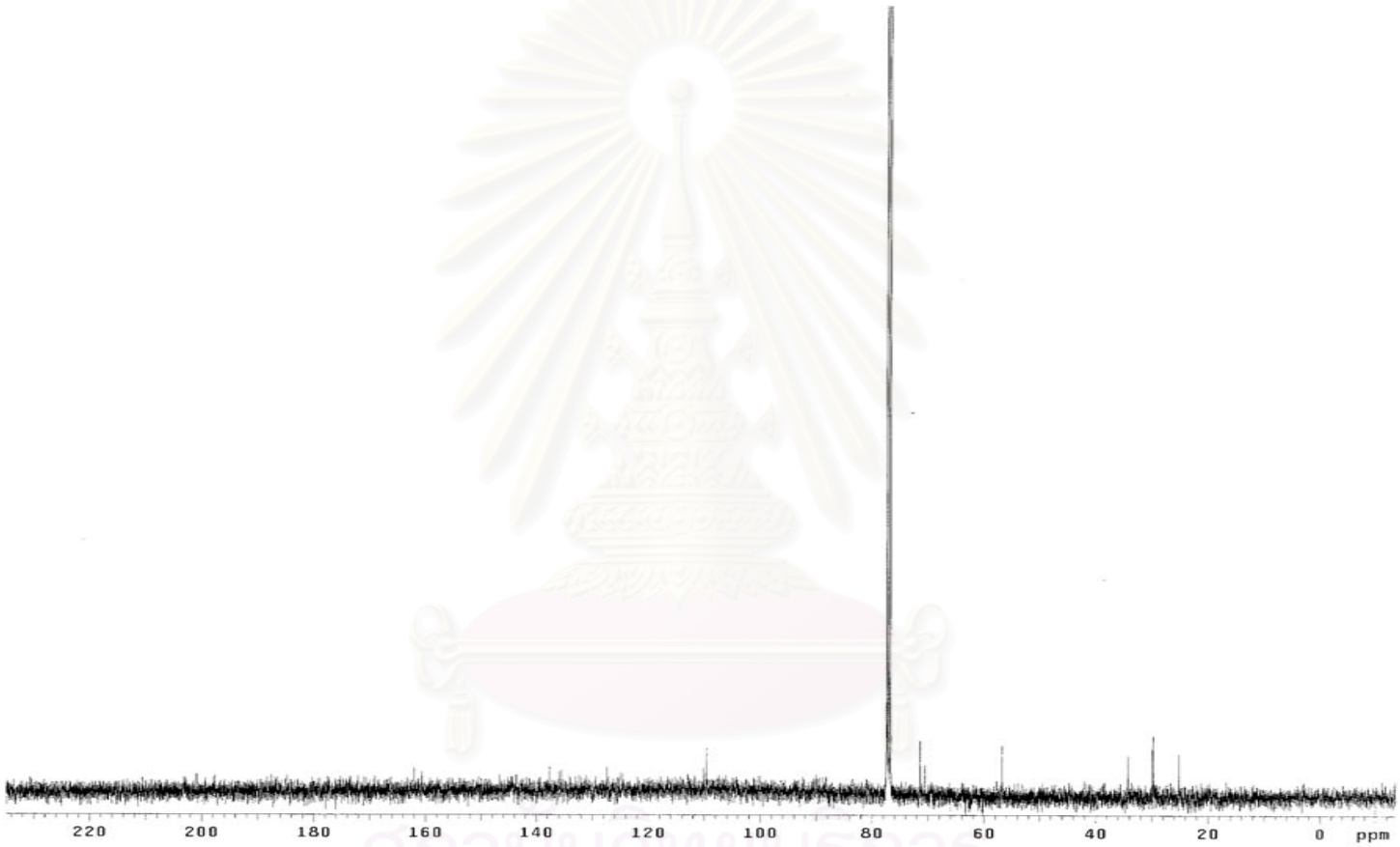


Figure E.26 ^{13}C-NMR spectrum of mixture 2

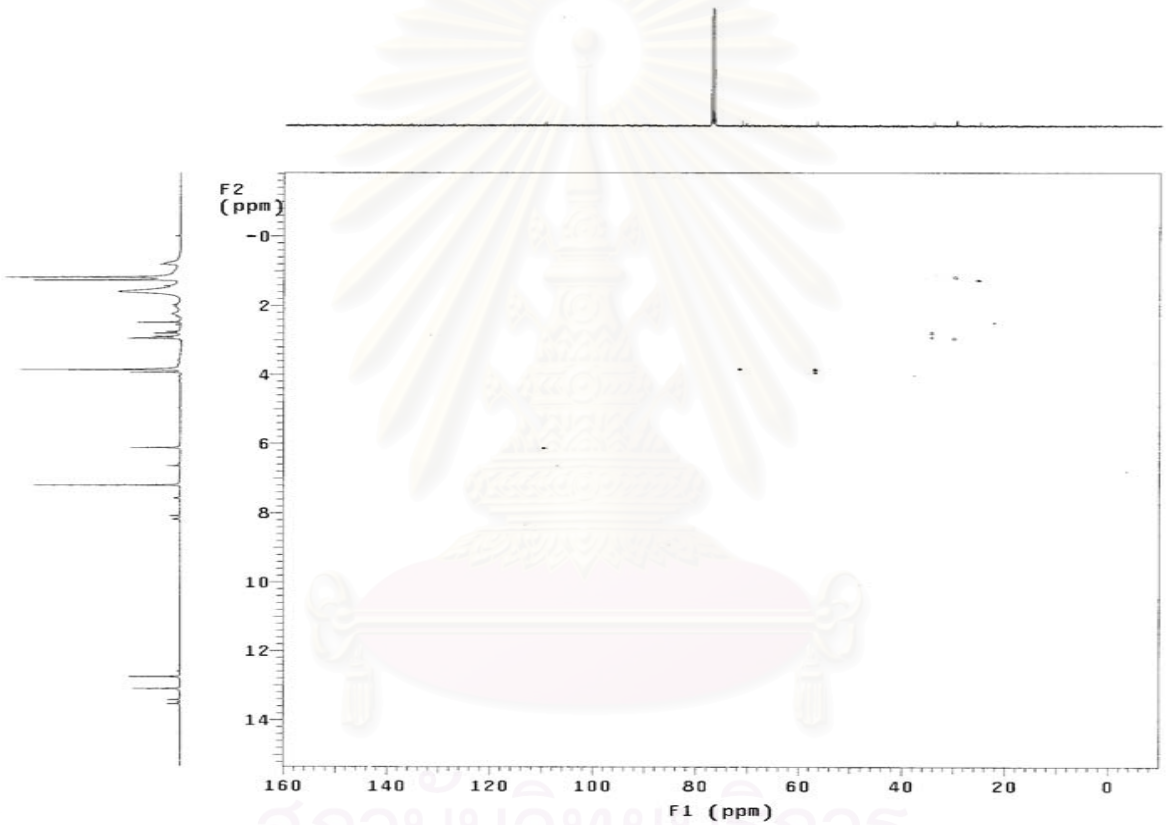


Figure E.27 HSQC spectrum of mixture 2

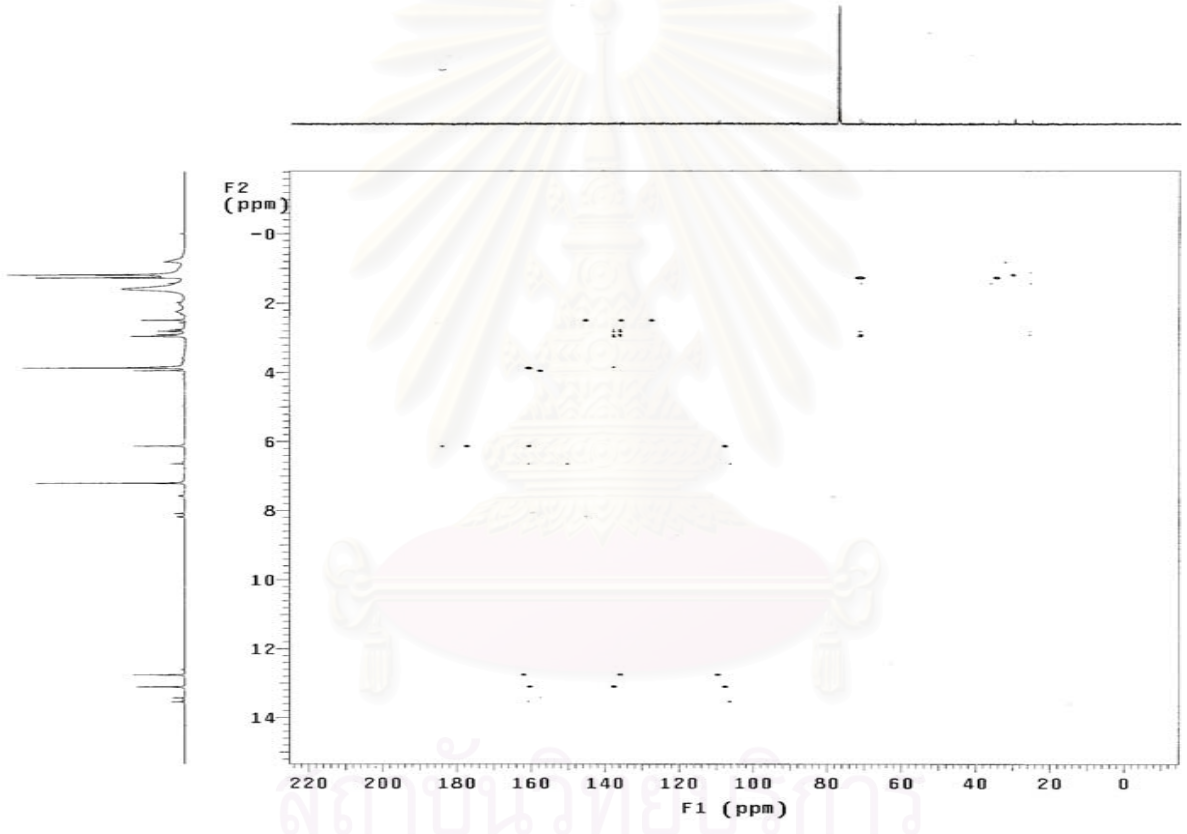


Figure E.28 HMBC spectrum of mixture 2

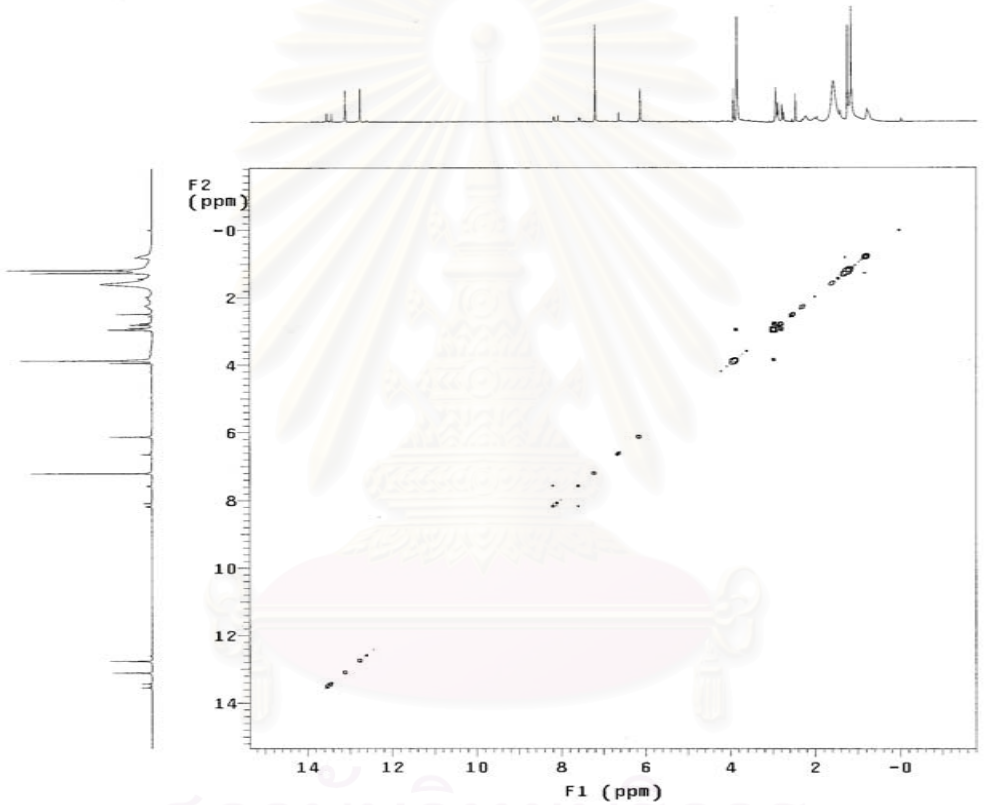


Figure E.29 COSY spectrum of mixture 2

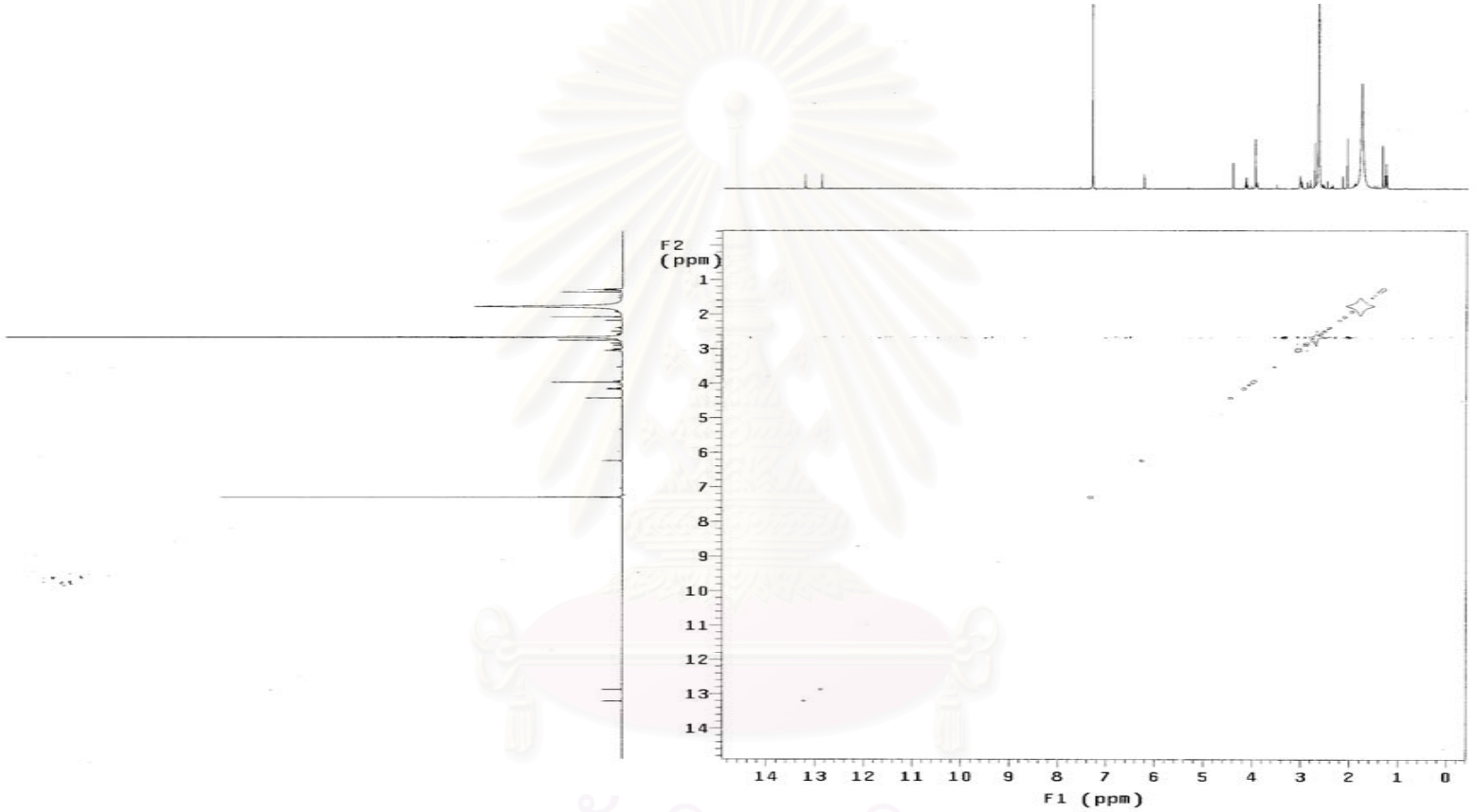


Figure E.30 NOESY spectrum of mixture 2

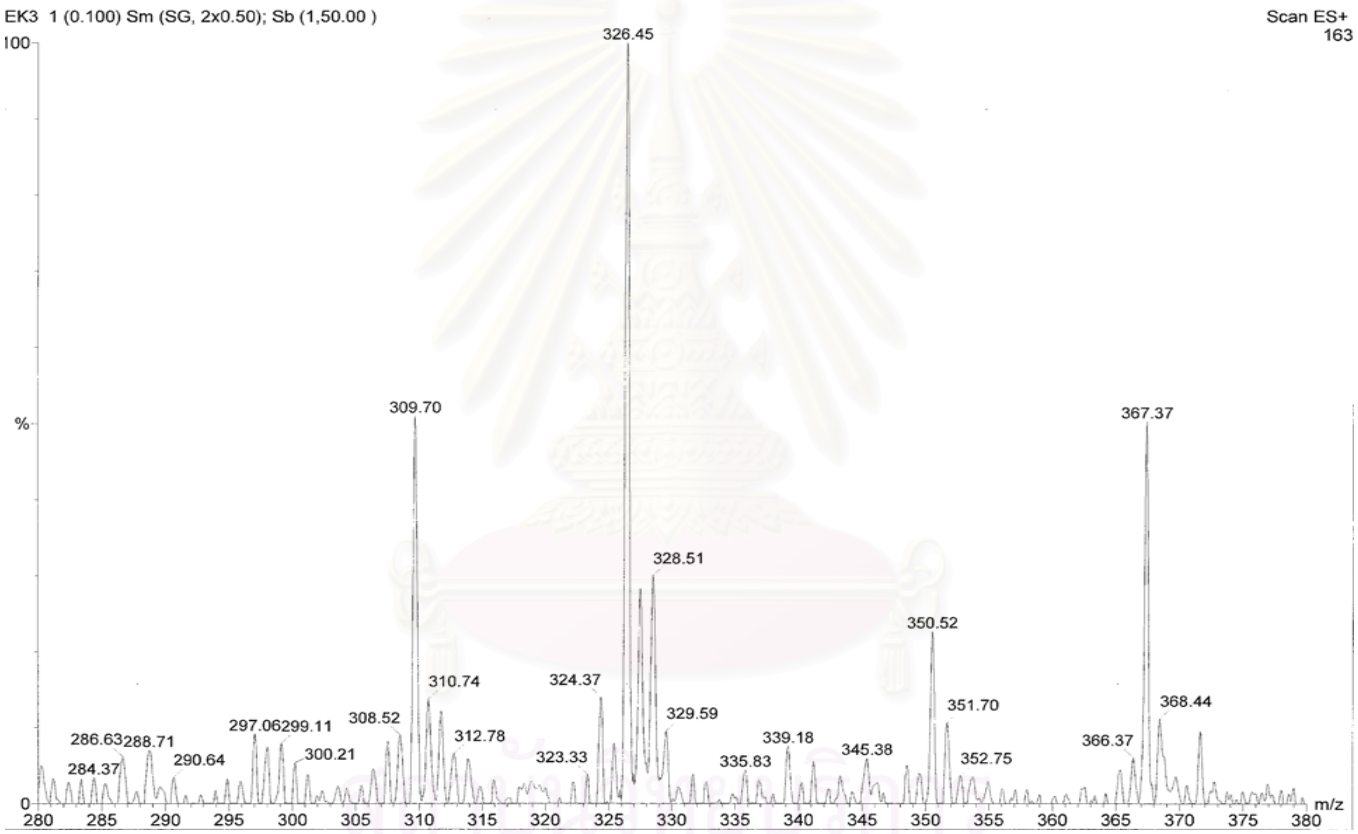


Figure E.31 MS spectrum of mixture 2

APPENDIX F

Table F.1 Crystal data and structure refinement for Compound 1

Identification code	Compound 1	
Empirical formula	$C_{40}\ H_{40}\ O_{10}$	
Formula weight	680.75	
Temperature	293(2) K	
Wavelength	0.71073 Å	
Crystal system, space group	monoclinic, $P_{2/m}$	
Unit cell dimensions	a = 8.6758(2) Å	alpha = 90 deg.
	b = 7.9050(2) Å	beta = 97.0990(10) deg.
	c = 18.6415(5) Å	gamma = 90 deg.
Volume	1268.67(6) $Å^3$	
Z, Calculated density	2, 1.782 Mg/m^3	
Absorption coefficient	0.128 mm^{-1}	
F(000)	720	
Theta range for data collection	2.37 to 30.42 deg.	
Limiting indices	-11<=h<=12, -9<=k<=11, -25<=l<=26	
Reflections collected / unique	8920 / 3576 [R(int) = 0.0205]	
Completeness to theta = 30.42	93.0 %	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	3576 / 0 / 198	
Goodness-of-fit on F^2	1.056	
Final R indices [I>2sigma(I)]	R1 = 0.0497, wR2 = 0.1504	
R indices (all data)	R1 = 0.0680, wR2 = 0.1639	
Largest diff. peak and hole	0.323 and -0.237 $e.Å^{-3}$	

Table F.2 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$ x 10^3) for Compound 1

	x	y	z	U(eq)
C(1)	2745(2)	-4492(2)	1518(1)	53(1)
C(2)	2667(2)	-2161(2)	704(1)	39(1)
C(3)	3579(2)	-2958(2)	252(1)	42(1)
C(4)	3927(1)	-2147(2)	-378(1)	40(1)
C(5)	3340(1)	-534(2)	-569(1)	36(1)
C(6)	2392(1)	295(2)	-97(1)	34(1)
C(7)	2071(1)	-500(2)	533(1)	37(1)
C(8)	1751(1)	1977(2)	-275(1)	36(1)
C(9)	2080(1)	2782(2)	-958(1)	36(1)
C(10)	3032(1)	1969(2)	-1411(1)	38(1)
C(11)	3698(2)	279(2)	-1224(1)	39(1)
C(12)	3337(2)	2782(2)	-2044(1)	45(1)
C(13)	2680(2)	4339(2)	-2232(1)	47(1)
C(14)	1701(2)	5144(2)	-1793(1)	44(1)
C(15)	1431(2)	4363(2)	-1152(1)	41(1)
C(18)	962(2)	6821(2)	-2018(1)	60(1)
O(2)	2267(1)	-2795(1)	1325(1)	50(1)
O(3)	4546(1)	-431(1)	-1635(1)	54(1)
O(4)	1186(1)	200(1)	998(1)	48(1)
O(5)	941(1)	2721(1)	137(1)	46(1)
O(6)	4857(1)	-2973(1)	-792(1)	53(1)

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

Table F.3 Bond lengths [Å] and angles [deg] for Compound 1.

Bond lenghts	(Å)
C(1)-O(2)	1.4366(17)
C(2)-O(2)	1.3457(16)
C(2)-C(3)	1.3777(18)
C(2)-C(7)	1.4326(18)
C(3)-C(4)	1.4025(19)
C(4)-O(6)	1.3527(15)
C(4)-C(5)	1.4024(18)
C(5)-C(6)	1.4343(16)
C(5)-C(11)	1.4473(18)
C(6)-C(7)	1.3902(18)
C(6)-C(8)	1.4637(17)
C(7)-O(4)	1.3462(14)
C(8)-O(5)	1.2490(14)
C(8)-C(9)	1.4816(17)
C(9)-C(15)	1.4001(19)
C(9)-C(10)	1.4073(17)
C(10)-C(12)	1.3987(18)
C(10)-C(11)	1.4801(19)
C(11)-O(3)	1.2578(15)
C(12)-C(13)	1.384(2)
C(13)-C(14)	1.403(2)
C(14)-C(15)	1.3900(19)
C(14)-C(18)	1.509(2)

Table F.4 Bond angles [deg] for Compound 1.

Bond angles	(Å)	Bond angles	(Å)
O(2)-C(2)-C(3)	125.77(12)	O(5)-C(8)-C(9)	120.55(11)
O(2)-C(2)-C(7)	114.10(11)	C(6)-C(8)-C(9)	118.43(10)
C(3)-C(2)-C(7)	120.12(12)	C(15)-C(9)-C(10)	119.92(11)
C(2)-C(3)-C(4)	120.13(12)	C(15)-C(9)-C(8)	119.46(11)
O(6)-C(4)-C(5)	121.15(12)	C(10)-C(9)-C(8)	120.62(11)
O(6)-C(4)-C(3)	117.57(12)	C(12)-C(10)-C(9)	118.92(12)
C(5)-C(4)-C(3)	121.27(11)	C(12)-C(10)-C(11)	120.28(11)
C(4)-C(5)-C(6)	118.52(12)	C(9)-C(10)-C(11)	120.80(11)
C(4)-C(5)-C(11)	120.57(11)	O(3)-C(11)-C(5)	121.27(12)
C(6)-C(5)-C(11)	120.91(12)	O(3)-C(11)-C(10)	119.99(12)
C(7)-C(6)-C(5)	120.08(11)	C(5)-C(11)-C(10)	118.73(10)
C(7)-C(6)-C(8)	119.45(10)	C(13)-C(12)-C(10)	120.52(13)
C(5)-C(6)-C(8)	120.46(11)	C(12)-C(13)-C(14)	121.04(12)
O(4)-C(7)-C(6)	123.25(12)	C(15)-C(14)-C(13)	118.61(13)
O(4)-C(7)-C(2)	116.89(11)	C(15)-C(14)-C(18)	121.18(13)
C(6)-C(7)-C(2)	119.85(11)	C(13)-C(14)-C(18)	120.21(13)
O(5)-C(8)-C(6)	121.02(11)	C(14)-C(15)-C(9)	120.95(12)
		C(2)-O(2)-C(1)	117.77(11)

Table F.5 Anisotropic displacement parameters ($Å^2 \times 10^3$) for Compound 1.

The anisotropic displacement factor exponent takes the form:

-2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
C(1)	62(1)	41(1)	55(1)	5(1)	7(1)	7(1)
C(2)	38(1)	40(1)	37(1)	-3(1)	2(1)	3(1)
C(3)	40(1)	37(1)	47(1)	-6(1)	3(1)	6(1)
C(4)	33(1)	42(1)	44(1)	-13(1)	5(1)	5(1)
C(5)	31(1)	41(1)	35(1)	-9(1)	4(1)	2(1)
C(6)	31(1)	39(1)	33(1)	-7(1)	3(1)	3(1)
C(7)	36(1)	41(1)	33(1)	-6(1)	4(1)	5(1)
C(8)	32(1)	40(1)	34(1)	-5(1)	4(1)	3(1)
C(9)	31(1)	42(1)	34(1)	-4(1)	3(1)	-1(1)
C(10)	32(1)	45(1)	35(1)	-7(1)	4(1)	-3(1)
C(11)	33(1)	48(1)	38(1)	-10(1)	6(1)	0(1)
C(12)	43(1)	55(1)	38(1)	-7(1)	10(1)	-6(1)
C(13)	49(1)	56(1)	38(1)	2(1)	7(1)	-9(1)
C(14)	41(1)	47(1)	44(1)	2(1)	2(1)	-6(1)
C(15)	37(1)	44(1)	42(1)	-1(1)	5(1)	0(1)
C(18)	65(1)	54(1)	62(1)	15(1)	9(1)	1(1)
O(2)	62(1)	44(1)	44(1)	4(1)	13(1)	11(1)
O(3)	55(1)	61(1)	51(1)	-9(1)	24(1)	8(1)
O(4)	57(1)	50(1)	38(1)	0(1)	17(1)	15(1)
O(5)	53(1)	46(1)	43(1)	-1(1)	17(1)	13(1)
O(6)	53(1)	49(1)	61(1)	-11(1)	21(1)	13(1)

Table F.6 Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for Compound 1.

	x	y	z	U(eq)
H(1A)	2383	-4791	1967	79
H(1B)	3858	-4560	1569	79
H(1C)	2313	-5257	1147	79
H(3)	3965	-4036	365	50
H(12)	3987	2272	-2342	54
H(13)	2890	4861	-2657	57
H(15)	811	4898	-848	49
H(18A)	1278	7162	-2472	90
H(18B)	1285	7658	-1657	90
H(18C)	-148	6710	-2067	90
H(4O)	900(30)	1310(30)	790(14)	101(8)
H(6O)	4830(20)	-2170(30)	-1203(12)	73(6)

BIOGRAPHY

Miss Watsaya Wisetsakdakorn was born on September 1, 1981 in Bangkok, Thailand. She graduated with Bachelor Degree of Science in General Science from the Faculty of Science, Kasetsart University, Thailand in 2002. She has been studying for a Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand since 2002.