สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกจากกระชายดำ *Kaempferia parviflora*

นางสาวนิรมล จันทร์คง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Kaempferia parviflora*

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นิรมล จันทร์คง : สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกจากกระชายดำ *Kaempferia parviflora*. (BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Kaempferia parviflora*) อาจารย์ที่ปรึกษา : ศ. ดร. โสภณ เรืองสำราญ, อ. ที่ปรึกษาร่วม : รศ. ดร. ประกิตติ์สิน สีหนนท์, 163 หน้า. ISBN 974-53-1576-1.

วัตถุประสงค์ของงานวิจัยนี้เพื่อแยกสารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกได้จากตัวอย่างใบกระชายดำ *Kaempferia parviflora* ที่สุ่มเก็บจาก 3 จังหวัดในประเทศไทยโดยใช้เทคนิคฆ่าเชื้อที่ผิวและได้ 36 ไอโซเลต จากการทดสอบฤทธิ์ต้านจุลินทรีย์โดยวิธี Dual culture agar diffusion technique ของราเอนโดไฟต์ที่แยกได้ พบว่าราเอนโดไฟต์สายพันธุ์ KPCH007 เมื่อเพาะเลี้ยงบนอาหาร มอลท์ เอกซ์แทรกซ์ มีฤทธิ์ยับยั้งจุลินทรีย์ทดสอบดีที่สุด เนื่องจากเป็นราเอนโดไฟต์ที่สามารถยับยั้งจุลินทรีย์ทดสอบได้ทั้งแบคทีเรียแกรมบวก *Bacillus subtilis* และ *Staphylococcus aureus* แบคทีเรียแกรมลบ *Pseudomonas aeruginosa* เมื่อทำการจัดจำแนกสายพันธุ์โดยศึกษาลักษณะทางสัณฐานวิทยา และการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ ITS ของ rDNA พบว่าราเอนโดไฟต์ ไอโซเลต KPCH007 คือ *Alternaria tenuissima* ทำการแยกสารสกัดด้วยเอธิลแอซิเตตจากน้ำเลี้ยงเชื้อของราเอนโดไฟต์ ไอโซเลต KPCH007 ที่เพาะเลี้ยงในอาหารเลี้ยงเชื้อเหลว มอลท์ เอกซ์แทรกซ์ ได้สารบริสุทธิ์ 1, สารบริสุทธิ์ 2 และ สารบริสุทธิ์ 3 ส่วนสารสกัดด้วยเอธิลแอซิเตตจากเส้นใยแยกได้ ของผสม 4, ของผสม 5 และ สารบริสุทธิ์ 6 เมื่อวิเคราะห์โครงสร้างของสารที่แยกได้โดยอาศัยคุณสมบัติทางกายภาพและเทคนิคทางสเปกโตรสโกปี พบว่า สารบริสุทธิ์ 1 คือ 1-methyl-2-pyrrolidone, สารบริสุทธิ์ 2 คือ dehydroaltenusin, สารบริสุทธิ์ 3 คือ 1,6-dihydroxy-3-methoxy-8-methyl-9*H*-xanthen-9-one และจากเส้นใย ของผสม 4 คือ triglycerides, ของผสม 5 คือ macrosporin and alternariol-9-methyl และสารบริสุทธิ์ 6 คือ Alternariol-9-methyl และหาสูตรโครงสร้างของสารเหล่านี้ โดยอาศัยคุณสมบัติทางกายภาพ เทคนิคแกสโครมาโทกราฟี และเทคนิคทางสเปกโทสโกปี นำสารบริสุทธิ์ที่แยกได้มาทดสอบฤทธิ์ทางชีวภาพในการยับยั้งจุลินทรีย์และทดสอบความเป็นพิษต่อเซลมะเร็งคน 5 ชนิด พบว่าสารบริสุทธิ์ 1 แสดงฤทธิ์ต้านแบคทีเรีย *B. subtilis, S. aureus, P. aeruginosa* และ *E. coli* ด้วยค่า MIC 31.25, 31.25, 62.50 และ 62.50 μg/ml, สารบริสุทธิ์ 5 แสดงฤทธิ์ต้านแบคทีเรีย *B. subtilis, S. aureus, P. aeruginosa* และ *E. coli* ด้วยค่า MIC 125, 250, 250 และ 250 μg/ml และพบว่าสาร บริสุทธิ์ 6 แสดงฤทธิ์ต้านแบคทีเรีย *B. subtilis, S. aureus, P. aeruginosa, E. coli* และ *C. albicans* ด้วยค่า MIC 62.50, 62.50, 125, 125 และ 250 μg/ml ตามลำดับ นอกจากนี้ สารบริสุทธิ์ 1 มีฤทธิ์ยับยั้งเซลล์มะเร็ง SW620 (ลำไส้ใหญ่) BT474 (เต้านม) KATO-3 (กระเพาะอาหาร) HEP-G2 (ตับ) และ CHAGO (ปอด) มีค่า IC_{50} เท่ากับ >10,>10,>10,>10,8.4 μg/ml สารบริสุทธิ์ 3 มีฤทธิ์ยับยั้งเซลล์มะเร็ง มีค่า IC_{50} เท่ากับ >10,7.4,>10,>10,>10 สารบริสุทธิ์ 5 มีฤทธิ์ยับยั้งเซลล์มะเร็งมีค่า IC_{50} เท่ากับ >10, 6.4, >10, 10,>10 μg/ml และ สารบริสุทธิ์ 6 มีฤทธิ์ยับยั้งเซลล์มะเร็งมีค่า IC_{50} 10, 6.4, 8.8, 7.9 และ 10 μg/ml ตามลำดับ

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ..... ลายมือชื่อนิสิต.....

ปีการศึกษา....2547.....…… ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEYWORLD: Endophytic fungi/ *Kaempferia parviflora* / *Alternaria tenuissima* / antimicrobial activity / cytotoxicity.

NIRAMON JANKONG : THESIS TITLE. (BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Kaempferia parviflora*)

THESIS ADVISOR : PROF. SOPHON ROENGSUMRAN, THESIS COADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, 163 pp. ISBN 974-53-1576-1.

The purpose of this research was to isolate bioactive compounds from endophytic fungi isolated from *Kaemperia parviflora* leaves were collected from 3 provinces in Thailand. The endophytic fungi were isolated using surface-sterillization technique and obtained 36 isolates. All isolates were examined antimicrobial activity using dual culture agar diffusion technique. Endophytic fungus isolate KPCH007 cultured on malt extract agar had the best antimicrobial activity because it can inhibited the growth of test gram positive *Bacillus subtilis*, *Staphylococcus aureus* and negative bacteria *Pseudomonas aeruginosa*. Based on morphology and nucleotide sequencing analysis of ITS regions of rDNA, endophytic fungus isolate KPCH007 was identified as *Alternaria tenuissima*. Isolation of culture broth EtOAc crude extract of endophytic fungus isolate KPCH007 cultivated in malt extract broth gave compound 1, compound 2 and compound 3. Isolation of mycelia EtOAc crude extract gave mixture 4, mixture 5 and compound 6. The structure elucidation of these compounds was achieved by analysis of spectroscopic data and physical properties. Compound 1 was identified as 1-methyl-2-pyrrolidone. Compound 2 was identified as dehydroaltenusin . Compound 3 was identified as 1,6-dihydroxy-3-methoxy-8-methyl-*9H*-xanthen-9-one. From the mycelia ; Mixture 4 was obtained as yellow liquid and it was identified as a mixture of triglycerides. Mixture 5 was a mixture of macrosporin and alternariol-9-methyl ether and Compound 6 was identified as alternariol-9-methyl ether . Antimicrobial activities and cytotoxicity of the pure compound were tested. For antimicrobial activities, Compound 1 was found to exhibit activity against *B. subtilis , S. aureus, P. aeruginosa* and *E. coli.* with the MIC value of 31.25, 31.25, 62.50 and 62.50 μg/ml. Mixture 5 was found to exhibit activity against all four bacteria with the MIC value of 125, 250, 250, and 250 μg/ml. Compound 6 was found to exhibit activity against all four bacteria with the MIC value of 62.50, 62.50, 125, 125 μg/ml and inhibited *C. albicans* at 250 μg/ml., respectively. In addition, compound 1 can exhibited cytotoxic activities against SW620 (colon), BT474 (breast), KATO-3 (gastric), HEP-G2 (hepatoma) and CHAGO (lung) cell line with IC_{50} >10, >10, >10, >10, 8.4 μg/ml, Compound 3 with IC_{50} >10, 7.4, >10, >10, >10 μg/ml, Mixture 5 with IC_{50} >10, 6.4, >10, 10, >10 μg/ml and Compound 6 with IC_{50} 10, 6.4, 8.8, 7.9 and 10 μg/ml, respectively.

Field of study Biotechnology Student's signature……

Acadamic Year 2004 Advisor's signature……

Co-advisor's signature……

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
cm^{-1}	reciprocated centimeter (unit of wave number)
^{13}C NMR	carbon-13 nuclear magnetic resonance
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	doublet doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
EI	Electron impact
EtOAc	Ethyl acetate
g	gravity (NMR)
h	hour
HMBC	Heteronuclear Multiple Bond Cerrelation
HMQC	Heteronuclear Multiple Quantum Correlation
^{1}H NMR	proton nuclear magnetic resonance
Hz	Hertz
IR	infared
ITS	internally transcribed spacers
J	coupling constant
m	multiplet (NMR)
m	medium (IR)
M+	molecular ion
MCA	Malt Czapek agar
MEA	Malt extract agar
MeOH	methanol
MHB	Mueller-Hinton broth
MHz	megahertz

mg	milligram
min	minute
ml	millilitre (s)
MS	mass spectroscopy
m/z	mass to change ratio
NA	Nutrient agar
NB	Nutrient broth
nm	namometer
No.	number
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PDA	Potato dextrose agar
ppm	part per million
PCR	poly chain reaction
q	quartet
SGA	Sabouraud glucose agar
s	singlet (NMR)
sp.	species
t	tripet (NMR)
TLC	thin layer chromatography
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet
YES	Yeast extract sucrose agar
μg	microgram
μl	microlitre
δ	Chemical shift
λ_{max}	the wavelength at maximum absorption (UV)
ν_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

The fungi are a very large and diverse group of organisms which have a unique life-style. Endophytes are microorganisms that live in the intercellular spaces of stem, petioles, root and leaves of plants causing no discernible manifestation of their presence and have typically gone noticed.The symbiosis between plant and endophyte was ascertained, namely, the former protects and feeds the latter which produces in return bioactive (plant growth regulatory, antibacterial, antifungal, antiviral, insecticidal, etc.) substances to enhance the growth and competitiveness of the host in nature. Accordingly, some endophytes could be reliable sources of materials of the agricultural and/or pharmaceutical potencial as exemplified by taxol, subglutinol A and B and peptide leucinostatin A (all could be produced by both endophytes and the hosts). Endophytic fungi constitute valuable sources of bioactive secondary metabolites for potential therapeutic use ; some of them have new structures (Strobel et al.,1999; Brady & Clardy 2000) and fungal endophytes were considered as biological control agents and potential genetic vectors in plant biotechnology. As research model systems for investigation of host parasite interactions and evolution in natural systems. (Bacon and White, 2000)

Recent interest has focused on endophytic microbes for their pharmaceutical potential. Fungal endophytes *Taxomyces andreanae* and *Pestalotiopsis microspora* , and several other fungi isolated from the bark of yew trees are potential new sources of the anticancer drug taxol (Strobel et al., 1993). The clavicipitaceous grass endophytes are known to produce indole derivatives and other products that are active as plant hormones, antifungal agents, hallucinogens, vasoconstrictors, etc. Many other endosymbiotic microbes have not been investigated for their pharmaceutical value. Since endosymbiotic microbes must interest biochemically with host tissues to obtain nutrients, overcome host defenses, and defend host tissues, it is likely that many endophytes produce secondary metabolites which perform key roles related to the

survival of the microbe and symbiotic unit. The endophytic habitat is a niche that continued exploration. (Bacon et al., 2000)

One thousand three hundred and eight fungal cultures were isolated from various parts of Thailand. Members of the Ascomycotina, Basidiomycotina, Deuteromycotina, and some Oomycetes have been isolated as endophytes. Endophytic fungi have been isolated from phanerogams in alpine, temperate and tropical regions, although the plants of the Coniferae, Ericaceae and Grmineae have been most intensively sampled (Clay, 1991; Petrini, 1986; Siegal *et al*., 1987). Endophytic fungi are probably an important resource for novel metabolites to antibacterial, antifungal, and cytotoxicity activities. Thus, this aimed investigation of bioactive compounds which produced by endophytic fungi isolated from *Kaempferia parviflora*.

In this research, *Kaempferia parviflora* have been used as a plant source of endophytic fungi because *Kaempferia parviflora* is regularly used as Thai medicinal plant including produced various flavoniods compounds. In addition, some compounds possessed antioxidant property and inhibited the growth of cancer cell lines.

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

1. Isolation of the endophytic fungi from *Keampferia parviflora*.
2. Screening of endophytic fungi by microbiological activities testing and thin layer chromatographic method.
3. Isolation and characterization of secondary metabolites of endophytic fungi from *Kaempferia parviflora* .
4. Evaluation of biological activities of the isolated compounds.

CHAPTER II

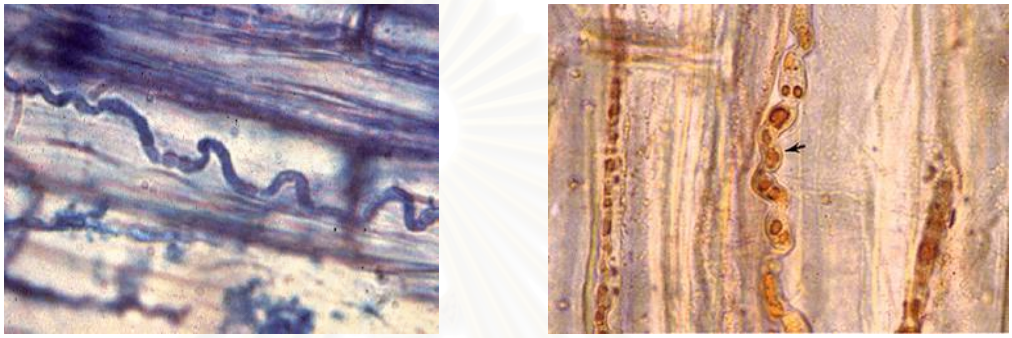
LITERATURE REVIEWS

2.1 Association of endophytic fungi and plant

Fungi are commonly isolated from the tissues of a plant, especially the leaves. Where the tissue is apparently healthy, the fungi may be either endophytes or latent pathogens. Pathogens, under some circumstances, will cause disease in which tissues are damaged. Endophytes, on the other hand, are contained within the plant without disease. Endophytic fungi may be mutualistic. Tissues remain entire and functional, and may have enhanced longevity and photosynthetic capacity. Both types of association are apparently asymptomatic and inconspicuous, and thus may be confused. Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in the living tissue of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture, and industry (Strobel et al., 2004).

Endophytic fungi colonize live into the plant tissues by penetration of fungi hyphae between plants cells or may also grow intracellularly as shown in Figure 2.1 and must obtain nutrient materials through this intimate contact with the host (Isaac, 1992). The relationship of fungi with plant ranges from mutualistic symbiosis, or commensalism to borderline latent pathogen. (Strobel and Long, 1998) Results of their interaction are 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.

Symbiotic associations between microorganisms and plants are shown in Figure 2.2 are ancient and fundamental, and many examples of complex and highly specific symbioses between plants and microbes have been described. Endophytic microbes are an intriguing group of organisms associated with various tissues and organs of landed and some aquatic plants and are the subject of increasing interest to mycologists, ecologists, and plant pathologists (Bacon and White, 2000).



(a) (b)

Figure 2.1 Endophytic fungi in leaves (a) and in clum of grass(b).

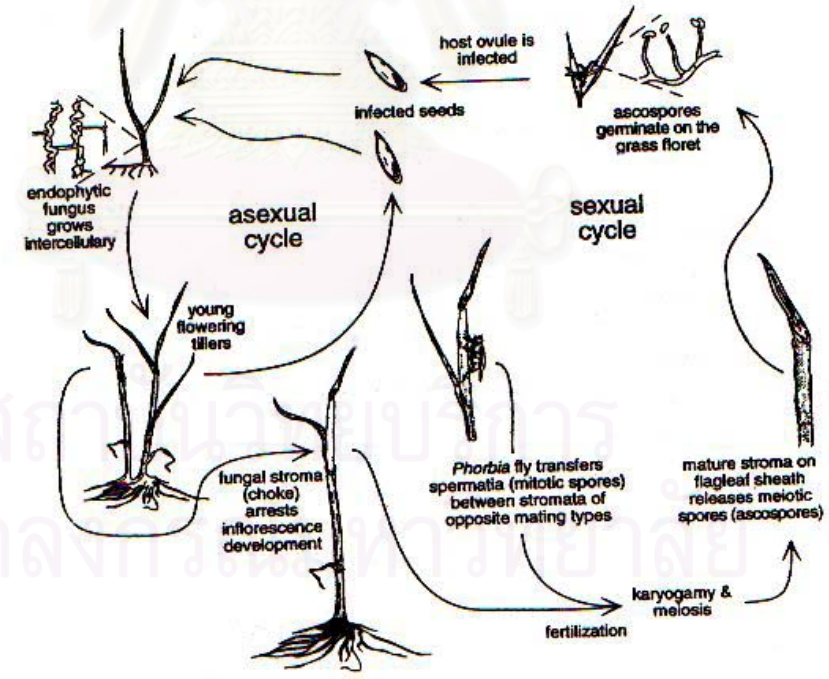


Figure 2.2 Alternative asexual and sexual life cycle of *Epichloe festucae* in symbiosis with *Festuca* sp. (Bush et al., 1997)

2.2 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 (Strobel et al., 2004).

Biodiversity of Fungi is essential for anyone collecting and/or monitoring any fungi. Fascinating and beautiful, fungi are vital components of nearly all ecosystems and impact human health and our economy in a myriad of ways. Standardized methods for documenting diversity and distribution have been lacking. An wealth of information, especially regrading sampling protocols, compiled by an international team of fungal biologists, make biodiversity of Fungi an incredible and fundamental resource for the study of organismal biodiversity. Chapters cover everything from what is a fungus, to maintaining and organizing a permanent study collection with associated databases; from protocols for sampling slime molds to insect associated fungi; from fungi growing on and in animals and plants to mushrooms and truffles. The chapters are arranged both ecologically and by sampling method rather than by taxonomic group for ease of use. The information presented here is intended for everyone interested in fungi, anyone who needs tools to study them in nature including naturalists, land managers,

ecologists, mycologists, and even citizen scientists and sophiscated amateurs (Strobel et al., 2004).

Almost all vascular plant species examined to date were found to harbor endophytic bacteria and/or fungi. Moreover, the colonization of endophytes in marine algae, mosses and ferns has also been recorded. As a matter of fact, endophytes are important components of microbial biodiversity. Commonly, several to hundreds of endophyte species can be isolated from a single plant, among them, at least one species showing host specificity. The environmental conditions under which the host is growing also affect the endophyte population, and the endophyte profile may be more diversified in tropical areas. Isolated 418 endophyte morphospecies (estimated 347 genetically distinct taxa) from 83 healthy leaves of *Heisteria concinna* and *Ourater lucens* in a lowland tropical forest of central Panama, and proposed that tropical endophytes themselves could be hyperdiverse with host preference and spatial heterogeneity. Moreover, genotypic diversity has been observed in single endophyte species originating from conifers, birch and grasses. Accordingly, endophytes are presumably ubiquitous in the plant kingdom with the population being dependent on host species and location (Tan and Zou, 2001).

2.3 Fungal endophyte Metabolites

The search for new products for the pharmaceutical and agrochemical industries was an on-going process that requires continual optimization. Previously, the screening of 10,000 natural products resulted in one commercial product. In the advent of combinatorial chemistry, this relationship changed. Presently, the screening of 100,000 structures per day from combinatorial chemistry together with the natural products screened yield less than one commercial product per year. Considering that 6 of 20 of the most commomly prescribed medications are of fungal origin and only about 5 % of the fungi have been described, fungi offer an enormous potential for new products (Schulz et al., 2002).

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 assist to helping solve not only human health, but plant and animal health problem also. Endophytic fungi reside in the tissue between living plant cells. The relationship that establish 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, 2001).

The industrial scientists screening for novel biologically active secondary metabolites are both interested in previously unknown activities for known metabolites, and in attaining a high proportion of novel structure from the culture extracts. A comparison of 135 isolated metabolites whose structures were determined shows that the proportion of novel structures produced by endophytes (51%) is considerably higher than that produced by soil isolated (38%). The metabolitic interaction of endophyte with its host may favour the synthesis of biologically active secondary metabolites. The biological activities and the metabolites produced are associated with the respective biotope and/or host (Schulz et al., 2002).

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, posses 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 disease such as AIDS require drugs that target them specifically, but new therapies are needed for treating ancillary infection 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. In addition, more drugs are need to efficiently treat parasitic protozoan and nematodal infections such as malaria, leishmaniasis, trypanomiasis, and filariasis. Malaria, by itself, is more effective in claiming lives each year than any other single infectious agent with the exception of AIDS and TB. However, the enteric diseases complex, and unfortunately, the victims are mostly children. 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).

Recently, fungal endophyte research has focused on screening of secondary metabolites that exhibit interesting biologically activity 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-dehydroxycytochalasin H), was a potent competitive inhibitor of HIV-1 viral protease (Dombrowski et al., 1992 ; Ondeyka et al., 1992). Echinocandin analogues, potent lipopeptide inhibitors of fungal B-1,3 glucan syntase, have been isolated from *Cryptosposiopsis spp*. (Noble et al., 1991)

2.3.1 Products of Endophytic fungi as Antibiotics

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

2.3.1.1 Antibacterial and Antifungal compounds

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

agents related to cryptocandin are also produced by *C.* cf. *quercina.* Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea.* Cryptocandin and its related compounds are currently being considered for use against a number of fungal-causing diseases of the skin and nails.

Pestalotiopsis microspora is a common rainforest endophyte. It turns out that enormous biochemical diversity does exist in this endophytic fungus, and many secondary metabolites are produced by various strains of this widely dispersed organism (Li et al., 1996, Strobel et al., 1996, Strobel, 2002a, Strobel, 2002b). One such secondary metabolite is ambuic acid, an antifungal agent, which has been recently described from several isolates of *P. microspora* found as representative isolates in many of the world's rainforests (Li et al., 2001). This compound as well as another endophyte product, therein, have been used as models to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 µg/disk) against *Bacillus subtilis, Salmonella gallinarum,* and *Staphylococcus aureus.* It also displays a moderate activity against the yeast *Candida tropicalis* (Horn et al., 2001).

Colletotrichum sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed antimicrobial activity as well. *A. annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. The *Colletotrichum* sp. found in *A. annua* produced a new indole derivative 6-isoprenylindole-3-acetic acid, not only metabolite with activity against human pathogenic fungi and bacteria but also metabolite that was fungistatic to plant pathogenic fungi (Lu et al., 2000).

2.3.1.2 Antiviral compounds

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

2.3.1.3 Volatile antibiotics from endophytic fungi

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

have usefulness in treating seeds, fruits, and other plant parts in storage and while being transported.

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

2.3.2 Products of Endophytic fungi with Anticancer Activities

Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes. Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia* (Wani et al., 1993; Suffness, 1995).

The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well. The presence of taxol in yew species prompted the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel taxol-producing endophytic fungus, *Taxomyces andreanae*, was discovered in *Taxus brevifolia* (Strobel et al., 1993). The most critical line of evidence for the presence of taxol in the culture fluids of this fungus was the electrospray mass spectrum of the putative taxol isolated from *T. andreanae.* In electrospray mass spectroscopy, taxol usually gives two peaks, one at *m*/*z* 854 which is $[M + H]^+$ and the other at *m*/*z* 876 which is $[M + Na]^+$. Fungal taxol had a mass spectrum identical to that of authentic taxol. Then, ^{14}C labeling studies showed the presence of fungal-derived taxol in the culture medium (Stierle, Strobel, and

Stierle, 1993). This early work set the stage for a more comprehensive examination of the ability of other *Taxus* species and many other plants to yield endophytes producing taxol.

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

2.3.3 Products of Endophytic fungi with Antioxidants Activities

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage system of Papua New Guinea (Strobel et al., 2002; Harper et al., 2003a). Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity based on its structural similarity to the flavonoids. Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution (Strobel et al., 2002). Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity (Harper et al., 2003). The proposed antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C-H bond and, to a lesser extent, through O-H abstraction. The antioxidant activity of pestacin is at least 1 order of magnitude more potent than that of trolox, a vitamin E derivative (Harper et al., 2003).

2.3.4 Products of Endophytic fungi with Insecticidal Activities

Bioinsecticides are only a small part of the insecticide field, but their market is increasing (Demain, 2000). Several endophytes are known to have anti-insect properties.

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

2.3.5 Products of Endophytic fungi with Antidiabetic Activities

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

2.3.6 Products of Endophytic fungi with Immunosuppressive Activities

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinols A and B (Lee et al., 1995a). Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) and thymocyte proliferation (TP) assays with an IC_{50} of 0.1 μM. In the same assay systems, the famed immunosuppressant drug cyclosporine A, also a fungal metabolite, was roughly as potent in the MLR assay and 10^4 more potent in the TP assay. Still, the lack of toxicity

associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants (Lee et al., 1995a).

The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi were summarized in Table A1 and Figure A1 (in Appendix A)

2.4 Botanical aspects of *Kaempferia parviflora*

Kaempferia parviflora **or Black Ginger** as shown in Figure 2.3, classified plant arranged in the Zingiberaceae family, which have many species widely distributed in Thailand. *Kaempferia parviflora* is very variable in the wild, with many pattern types and of course several forms with no pattern at all. The standard cultivated form of *Kaempferia parviflora* is much larger and is grown as a medicinal plant in thailand. Zingiberaceae is one of the largest families of the plant kingdom. It is important natural sources that provide useful many products for food, spicies, medicines, dyes, perfume and aesthetics to man. Black ginger is a short-lived herbaceous plant with dark-purple, ginger- like underground stems called rhizomes. Round- or oval-shaped leaves sprout from the rhizome. Bracts enclose flower clusters in whose midst comprise dark - purple flowers.

Back ginger rhizomes are Thai herbs believed to enhance libido, hence they are commonly referred to as Thai Ginseng. Proven therapeutic properties are to supplement energy, to increase sexuality, to treat male impotence, to fortify the heart, to counter bodily aches and pains, to alleviate pains of the joints, to use as a diuretic or carminative, and to purge various toxins from the body.

Figure 2.3 Picture of *Kaempferia parviflora*

2.4.1 Chemical constituents and Pharmacological studies

Nine flavonoids have been isolated from *Kaempferia parviflora* (5-Hydroxy-3,7-dimethoxyflavone (**1**, 0.2%), 5-hydroxy-7-methoxyflavone (**2**, 1.3%), 5-hydroxy-3,7,4′-trimethoxyflavone (**3**, 0.08%), 5-hydroxy-7,4′-dimethoxyflavone (**4**, 0.3%), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**5**, 1.5%), 3,5,7-trimethoxyflavone (**6**, 0.14%), 3,5,7,4′-tetramethoxyflavone (**7**, 0.6%), 5,7,4′-trimethoxyflavone (**8**, 1.6%) and 5,7,3′,4′-tetramethoxyflavone (**9**, 1.01%). Among these flavonoids, 5,7,4′-trimethoxyflavone (**8**) exhibited antiplasmodial, antifungal and antimycobacterial activities. Flavonoids **7** and **8** showed mild antifungal and antimycobacterial activities. These isolated flavonoids possessed no cytotoxicity against KB, BC and NCI-H187 cell lines, and this cytotoxic information suggests that the rhizomes of *K. parviflora* may be safe when using as an ingredient in traditional medicine. (Yenjai, C. et al, 2004)

Table 2.1 Show the compounds 1-9 that isolated from *Kaempferia parviflora.*

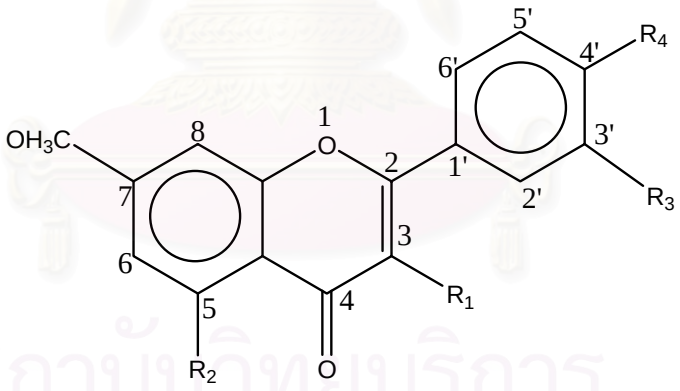


Table 2.1 (continued)

Compound	R_1	R_2	R_3	R_4
1	OCH_3	OH	H	H
2	H	OH	H	H
3	OCH_3	OH	H	OCH_3
4	H	OH	H	OCH_3
5	OCH_3	OH	OCH_3	OCH_3
6	OCH_3	OCH_3	H	H
7	OCH_3	OCH_3	H	OCH_3
8	H	OCH_3	H	OCH_3
9	H	OCH_3	OCH_3	OCH_3

2.4.2 Uses in traditional medicine

Presently, **Black Ginger** has been widely recognized as herb from consumers and Thai medical physician in believes of medical property. Even though, there still unofficial announcement from medical physician, from black ginger consumer's experience has reported that black ginger has medical property as strength nourishment, heart nourishment, heart shatter, articulation ache, stroke remedy, stuffy chest, mouth wound, better blood circulation, urine expulsion, stomachic remedy, and stomachache, etc. And the most remark as increase sexuality efficiency.

Among local people in the northeast of Thailand, the rhizomes of *K. parviflora* have been known as health-promoting herbs, and also frequently used for the treatment of colic disorder, peptic and duodenal ulcers. In Thailand, a tonic drink made from the rhizomes of *K. parviflora* is commercially available, and is believed to relieve impotent symptoms.

CHAPTER III

EXPERIMENTS

3.1 Instruments and equipments

3.1.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

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

3.1.2 Nuclear Magnetic Resonance Spectrometry (NMR)

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

3.1.3 Mass Spectrometry (MS)

The mass spectra were recorded on a Polaris Q Finnigan Instrument Mass Spectrometer and MALDI-TOF BRUKER Germany

3.1.4 UV-Vis spectrometry

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

3.1.5 Polarimetry

Specific optical rotation were recorded on a Perkin Elmer 341 in $CHCl_3$ and H_2O.

3.1.6 Electrothermometer

Melting point were measured on a Electrothermal 9100.

3.1.7 Gas Column 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. (Shimadsu Ltd, Kyoto) For analyzed fatty acid.

Prepare sample in methyl ester form to analyzed with GC method about 40.0 mg. First add 0.5 N NaOH in methanol 4 ml by reflux method for 15 minutes, add BF_3-methanol 4 ml for 3 minutes (change to fatty acid methyl ester form) and add Heptane 1 ml for 1 minutes. Then move the solution until it have same room temperature. Finally add saturated NaCl and used a solution that separate from all solution which on a top to analyzed with GC.

3.2 Chemicals

3.2.1 Solvents

All solvents used in this research such as hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), methanol (MeOH) and acetic acid (AcOH) were commercial grade and purified prior to use by distillation.

3.2.2 Other chemicals

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

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

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

4. TLC spots were visualized under ultraviolet light at wavelengths 254 and 365 nm, in iodine vapour, and under daylight after spraying with vanillin reagent (Dissolve

0.5 g vanillin in 95 ml ethanol and add 4.5 ml concentrated sulfuric acid) and heating until the colors developed.

3.3 Plant sample collection

Healthy leaves of *Kaempferia parviflora* trees were collected from Sriracha District, Chonburi Province, Patumthani Province and Bangkok Province Thailand, on November 29, 2002. Plant samples were kept in plastic bag and stored in a refrigerator. The samples must be processed before 48 hours after collection.

3.4 Culture media

As the media for isolation and cultivation of endophytic fungi, Potato dextrose agar (PDA), Malt extract agar (MEA), and Yeast extract sucrose agar (YES) were used for study macroscopic morphology and screening of endophytic fungus isolates for their antimicrobial activities. The medium for cultivation for study metabolites of bioactive compounds was Malt extract medium (agar and broth). Culture medium for growing tested bacterial was Nutrient medium (agar and broth). YMA medium (agar and broth) was used for growing tested yeasts.

3.5 Fungal isolation and culture of endophytic fungi

Endophytic fungi were isolated by using the surface sterilization method. Plant samples were cleaned with tap water and dried in laminar air flow. The leaf sections were then cut into 7 mm diameter disks from the middle, rib and lamina and were immersed in 95% ethanol for 1 minute, followed with Clorox (5 % available chlorine) for 4 minutes and then were transferred to 70% ethanol for 30 seconds before rinsing twice with sterile distilled water. Surface sterilization method was modified from the method described by Blodgett et al.,(2000)

Endophytic fungus isolates were grown on Malt extract agar (MEA), Potato dextrose agar (PDA) and Yeast extract sucrose agar (YES). After cultivation for 14 days at room temperature they were photographed for characterize endophytic fungus. The agar cultures were cut into 7 mm diameter disks by flamed cork hole borer for antimicrobial activities test.

3.6 Determination of antimicrobial activity

3.6.1 Test microorganisms used for antimicrobial activity

The test microorganisms used antimicrobial activity are listed in Table 3.1 (below).

Table 3.1 Test microorganisms for assay.

Type of tested microorganisms	Reference strains
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
Pathogen yeast	*Candida albicans* ATCC 10231
Brewing yeast	*Saccharomyces cerevisiae* TISTR 5169

3.6.2 Procedures

A. Preparation of tested bacterial inoculum

Bacteria were grown on Nutrient agar (NA) for 24 h at 37 °C. Selected fresh single colonies (4-5) were inoculated into 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 a 0.5 McFarland (OD 0.1 at 625 nm).

B. Preparation of tested yeast inoculum

Yeasts were grown on Yeast-malt extract agar (YMA) for 24 h at room temperature (25-30 °C). Selected fresh single colonies (4-5) were inoculated into 5 ml of Yeast-malt extract broth (YMB) and incubated at room temperature for 2-3 h, depending on the growth rate. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of a 0.5 McFarland (OD 0.1 at 625 nm).

C. Assay for antimicrobial activity

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

inoculated by streaking the swab across the entire surface. This was repeated twice, turning the plate 60 degree between each streaking. The surface of the medium was allowed to dry for 3-5 minutes. The agar blocks, from section 3.9.1 were distributed evenly on the surface. The plates were left at room temperature for 1 h. Bacterial and yeast plates were incubated at 37°C and room temperature, respectively for 24 h. Inhibition zones around the agar blocks were measured in mm with a ruler.

3.7 Identification of isolated endophytic fungi

3.7.1 Preparation specimen for light microscope

The isolated endophytes were grown on various kinds of media potato potato agar (PDA), malt extract agar (MEA) were yeast extract sucrose agar (YES). The culture were examined periodically until sporulation which were used for identification. Cultural characteristics (i.e. colony colour, texture and growth rate) on these media were recorded. Semipermanent slides were prepared by mounting fungal mycelium in lactophenol cotton blue onto slides and sealed with nail varnish. The slides were observed under light microscope.

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

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

3.7.3 Preparation specimen for scanning electron microscope

The cultured of endophytic fungi were cut into 1x1 cm and fixed in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodelate buffer (pH 7.2) for 2 hrs. The samples were then dehydrated under the serine concentration (70-95%) within 15 minutes. The sample were dried under critical point dried and coated with gold under sputter coater model. Changes of each fine immersed in absolute ethanol for 30 minutes for each twice and observed and photographed with a JSM-5410 LV scanning electron microscope.

3.8 Species Identification by molecular method

Species identification was performed by molecular technique based on internal transcribed space (ITS) region of rDNA (Figure 3.1) at the Asian Natural Environmental Science Center, the University of Tokyo, Japan.

3.8.1 DNA extraction

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

A. DNA extraction

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

3.8.2 ITS amplification

The ITS region of isolated endophytic fungus was amplified with the primers ITS1f (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1994), and ITS4 (White *et al*., 1990). Twenty microliters of reaction mixture contained 5 ng of template DNA, 0.2 mM of each dNTP, 1xPCR buffer, 1.5 mM Mg^{2+}, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 μM of the primer pair. The amplification reactions were performed in a thermal cycler (TP 3000; Takara Shuzo, Tokyo, Japan). Amplification was started at 94 ^{o}C for 9 min, followed by 38 cycles of a denaturing step at 94 ^{o}C for 1 min, an annealing step at 51 ^{o}C for 1 min, and an extension step at 72 ^{o}C for 1 min, and ended with an additional 5-min extension step at 72 ^{o}C. PCR product was kept at -30 ^{o}C for further study.

3.8.3 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 MRF. Ligation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Texas Red (Hitachi) in and SQ-5500E sequencer (Kanchanaprayudh *et. al*., 2003).

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

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

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

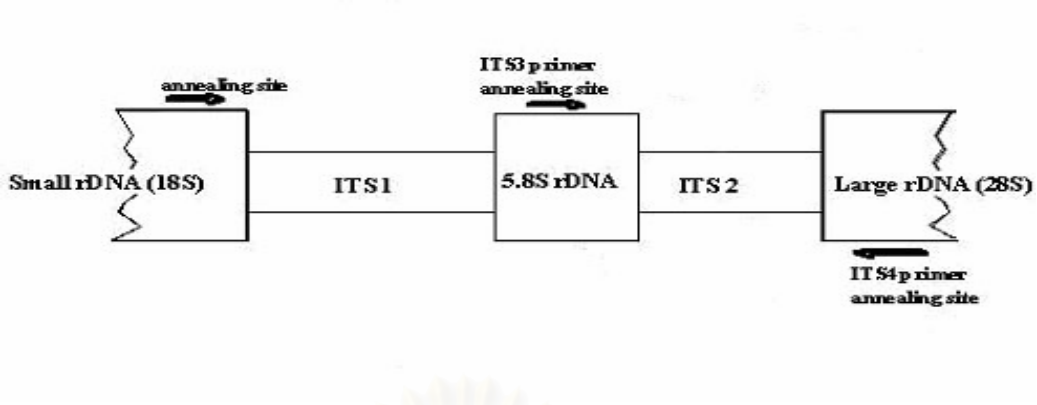


Figure 3.1 ITS regions of rDNA (Kanchanaprayudh *et. al*., 2003)

3.9 Tested of isolated fungal endophyte metabolites for antimicrobial activity

3.9.1 Endophyte fungal cultivation for secondary metabolize determination

Endophytic fungi which isolate in section 3.6.2(C) that antimicrobial activity were chosen for the study metabolites and cultivated on MEA at room temperature (25-30 C) for 1 week. The agar culture was then cut into 7 mm diameter disks using a flamed cork hole borer. Five pieces of agar culture were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract broth. The flasks were incubated at room temperature for 1 month. Several flasks of each isolates were prepared to obtain 0.5 L of MEB.

3.9.2 Secondary metabolite extraction

The culture broth was filtered twice through a filter paper (Whatman No.93). The culture broth was extract with ethyl acetate and evaporated by using a rotary evaporator at 37 C. The culture broth was dried and kept in a refrigerator at 4 C for bioassays.

The fungal mycelia were extracted with ethyl acetate. The extracted solvent was filtered through filter paper (Whatman No.93). The filtrate was evaporated by using a rotary evaporator at 37 C. The mycelium extracts were kept in a refrigerator at 4 C for bioassays.

3.10 Determination of metabolites profile of the extracts from endophytic fungi

A few milligrams of the culture broth extracts and mycelium extracts in the section 3.9.2 were dissolved with ethyl acetate for determination of metabolites profile of the extracts by using TLC technique that is described in later.

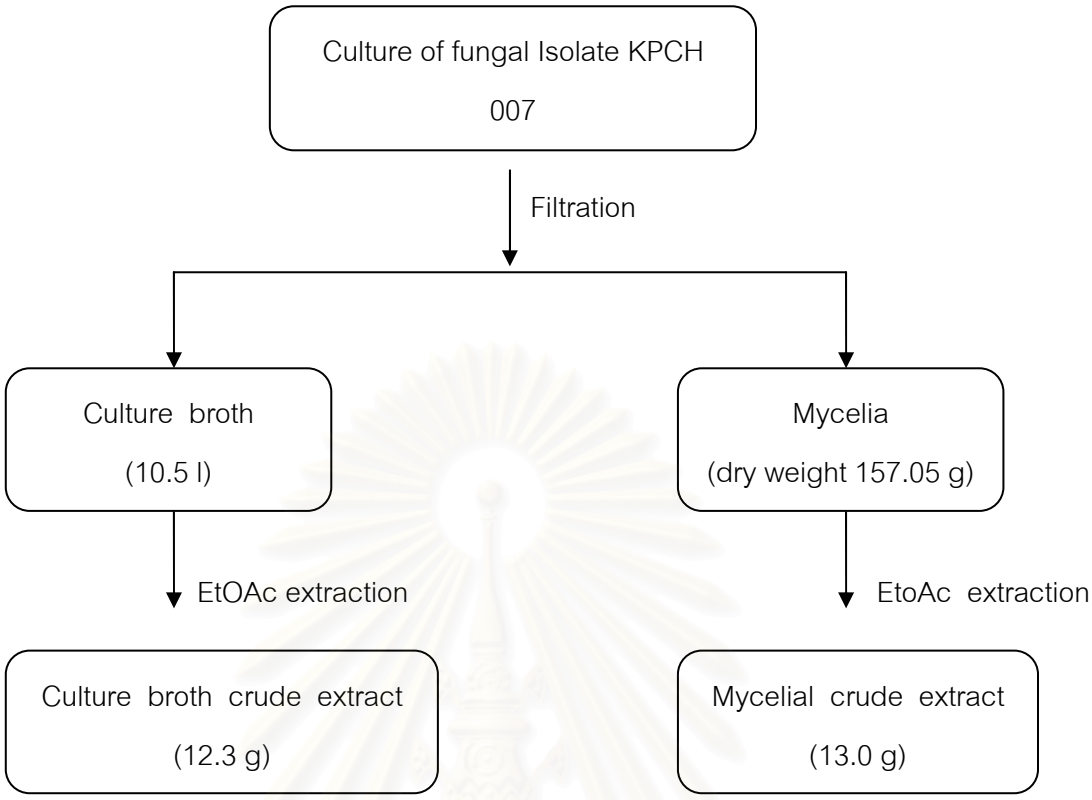
Analytical thin-layer chromatography (TLC)

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

3.11 Extraction procedure of endophytic fungus strain KPCH 007

Endophytic fungi strain KPCH 007 was chosen for study metabolites, The fungi were cultured in malt extract broth (15 L) for 1 months at room temperature and then filtered on through filter paper Whatman No.93. The filtrate was evaporated and partitioned with an equal volume of EtOAc 10 times. The EtOAc layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C .The crude EtOAc extract was obtained as a mixture of brown solid and dark brown viscous liquid (12.30 g).

Mycelia were weighted 822 g and extracted with 5 liters EtOAc and filtered through filter paper (Whatman No. 93). The EtOAc layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C. The crude EtOAc extract of mycelia cake was obtained as a dark brown viscous liquid (13.0g) .The extraction of the cultivation broth and mycelia of the endophytic fungus isolate KPCH 007 is shown in Scheme 3.1.



Scheme 3.1 Extraction diagram of culture broth and mycelia of endophytic fungus isolate KPCH 007

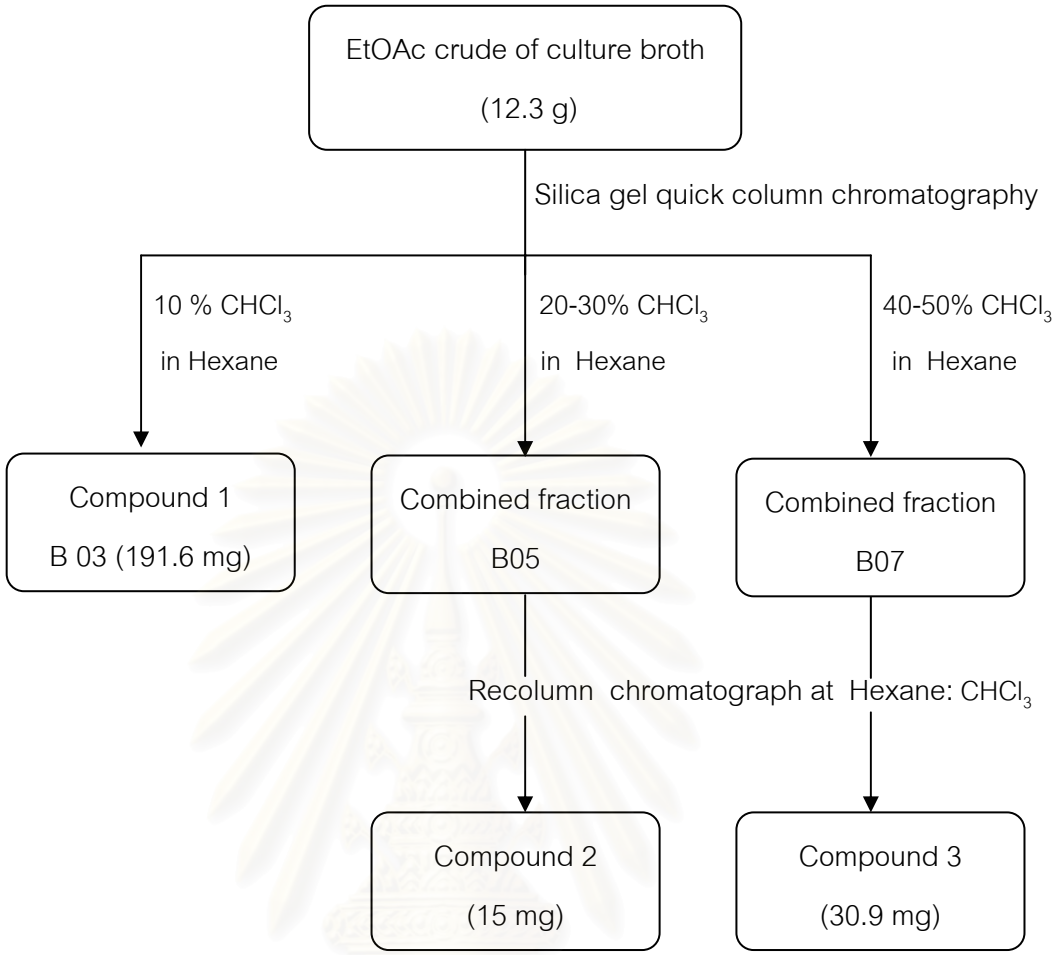
3.12 Isolation of bioactive compounds of isolated fungal endophytic fungus KPCH 007

3.12.1 Isolation of bioactive compounds from EtOAc crude of broth culture extract

The crude EtOAc from endophytic fungus isolate KPCH 007 culture broth extract (12.30 g) was fractionated by chromatography on a silica gel quick column (silica gel 290 g). Elution systems were Hexane, Hexane -$CHCl_3$ gradients, $CHCl_3$, $CHCl_3$-MeOH gradients, and MeOH. Each fraction (250 ml) was collected and examined. The fractions were combined by silica gel TLC. Fractions with the same TLC pattern were combined and dried. The results from the separation and purification of culture broth EtOAc extract crude are shown in Table 3.2 and Scheme 3.2.

Table 3.2 Characteristics of separation fraction of EtOAc crude culture broth extract of endophytic fungal isolate KPCH 007

Combined fraction	Fraction No.	Eluents	Characteristic	Weight (g)
B01	1-25	100% Hexane	Colourless viscous liquid	0.040
B02	26-33	5% $CHCl_3$ in Hexane	Yellow viscous liquid	0.009
B03	34-39	10% $CHCl_3$ in Hexane	Yellow viscous liquid with brown liquid	0.192
B04	40-43	10-20% $CHCl_3$ in Hexane	Yellow viscous liquid	0.074
B05	44-48	20-30% $CHCl_3$ in Hexane	Yellow viscous liquid with yellow solid	0.077
B06	49-57	30-40% $CHCl_3$ in Hexane	Yellow viscous liquid	0.190
B07	58-62	40-50% $CHCl_3$ in Hexane	Orange viscous liquid	0.092
B08	63-68	50-60% $CHCl_3$ in Hexane	Orange viscous liquid	0.150
B09	69-73	60-70% $CHCl_3$ in Hexane	Orange viscous liquid	0.200
B10	74-79	70-80% $CHCl_3$ in Hexane	Red brown viscous liquid	0.410
B11	80-87	80-90% $CHCl_3$ in Hexane	Red brown viscous liquid	0.880
B12	88-89	100 $CHCl_3$	Red brown viscous liquid	0.100
B13	90-97	5-20% MeOH in $CHCl_3$	Red brown viscous liquid	0.330
B14	98-101	30% MeOH in $CHCl_3$-100% MeOH	Black brown viscous liquid	0.040
B15	102-128	50% MeOH in $CHCl_3$-100% MeOH	Black brown viscous liquid	0.080



Scheme 3.2 Compound extraction diagram of procedure for EtOAc crude of culture broth extract of endophytic fungal isolate KPCH 007

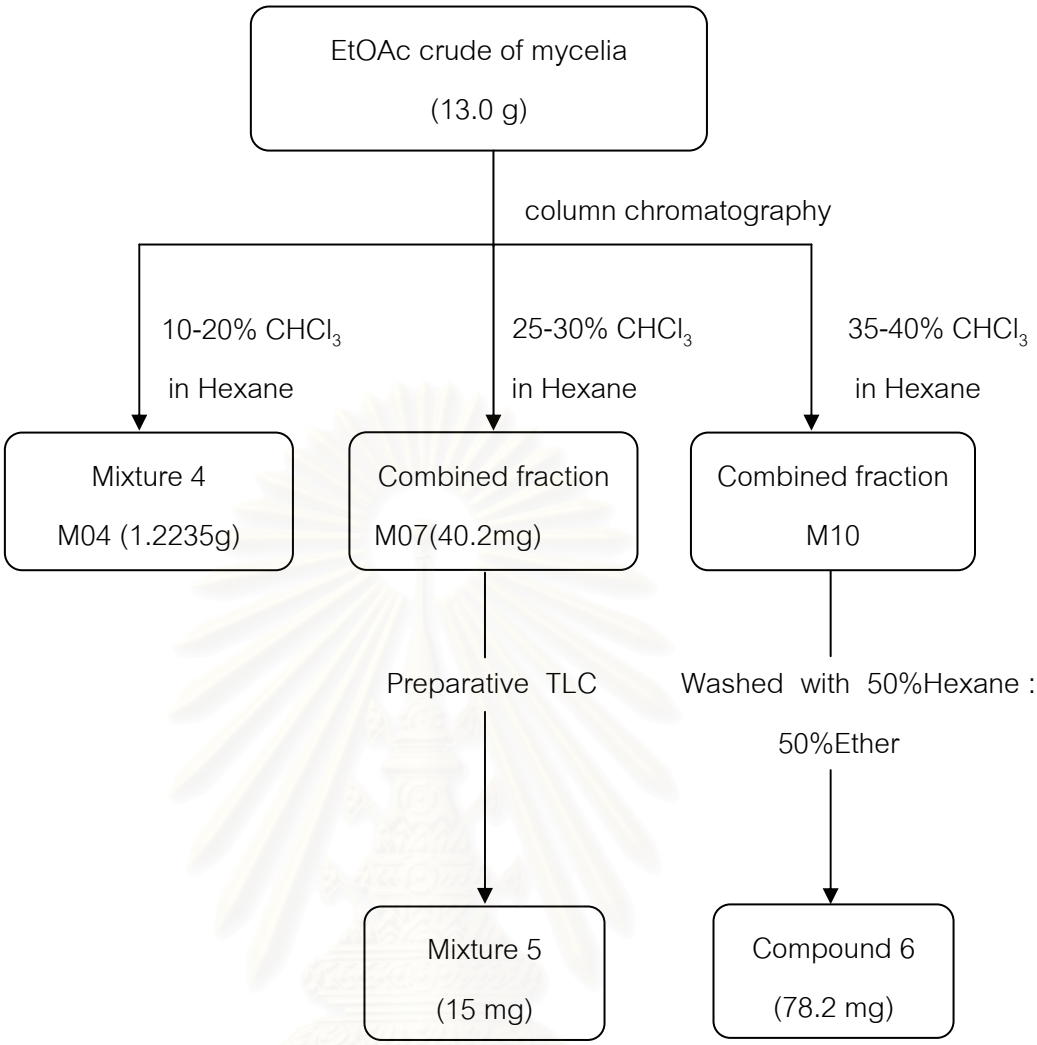
3.12.2 Isolation of bioactive compounds from EtOAc crude of mycelial extract

The crude EtOAc from endophytic fungus isolate KPCH 007 mycelia extract (13.0 g) was subjected to column chromatography (silica gel, 100 g), using wet packing and dry loading method. Eluents of increasing polarity from hexane to MeOH were used. Gradient elution with hexane, hexane and $CHCl_3$ mixtures, $CHCl_3$, $CHCl_3$ and MeOH mixtures, and MeOH were used. Fractions (25 ml each) were collected and examined. Fraction combination was by TLC on Silica gel plates with hexane, hexane and $CHCl_3$ mixtures, $CHCl_3$, $CHCl_3$ and MeOH mixtures, MeOH as the developing solvent. Fractions with the same TLC pattern were pooled and dried. The results from the isolation of

EtOAc crude of mycelial extract of endophytic fungal isolate KPCH 007 are shown in Table 3.3 and Scheme 3.3.

Table 3.3 Characteristics of separation fraction of EtOAc crude of mycelial extract of endophytic fungal isolate KPCH 007

Combined fraction	Fraction No.	Eluents	Characteristic	Weight (g)
M01	1-5	100% Hexane	Colourless viscous liquid	0.076
M02	6-10	2-5% $CHCl_3$ in Hexane	Green yellow viscous liquid	0.016
M03	11-17	5-10% $CHCl_3$ in Hexane	Yellow viscous liquid	0.009
M04	18-27	10-20% $CHCl_3$ in Hexane	Yellow viscous liquid	1.224
M05	28-29	10-20% $CHCl_3$in Hexane	Yellow viscous liquid	0.274
M06	30-35	20-25% $CHCl_3$ in Hexane	Yellow viscous liquid	0.950
M07	36-41	25-30% $CHCl_3$ in Hexane	Yellow viscous liquid with yellow solid	0.063
M08	42-44	25-30% $CHCl_3$ in Hexane	Orange viscous liquid	0.034
M09	45-49	30-35% $CHCl_3$ in Hexane	Orange viscous liquid	0.070
M10	50-58	40% $CHCl_3$ in Hexane	Orange viscous liquid	0.119
M11	59-66	40-50% $CHCl_3$ in Hexane	Orange brown viscous liquid	0.187
M12	67-76	50-60% $CHCl_3$ in Hexane	Orange brown viscous liquid	0.290
M13	77-95	60-70% $CHCl_3$ in Hexane	Red brown viscous liquid	0.980
M14	96-101	70-80% $CHCl_3$ in Hexane	Red brown viscous liquid	0.290
M15	102-109	80-100% $CHCl_3$ in Hexane	Red brown viscous liquid	0.430
M16	110-127	5-10% MeOH in $CHCl_3$	brown viscous liquid	0.560
M17	128-140	10-15% MeOH in $CHCl_3$	Dark brown viscous liquid	1.900
M18	141-150	15-20% MeOH in $CHCl_3$	Black brown solid	1.190



Scheme 3.3 Compound extraction diagram of procedure for EtOAc crude of mycelial extract of endophytic fungal isolate KPCH 007

3.13 Purification and properties of pure compounds from endophytic fungus isolated KPCH 007

3.13.1 Purification and characterization of compound 1

The combined fraction B03 was obtained from column chromatography of the KPCH007 culture broth ethyl acetate crude extract using Hexane:$CHCl_3$(95:5 → 90:10) as eluent. The yellow viscous liquid with brown liquid were further washed with hexane and ether mixtures to afford compound 1 as brown liquid (191.6 mg).

UVλ_{max}(nm): $CDCl_3$(ε) : 256(106) nm . (Figure C5 in appendix C)

FT-IR spectrum (NaCl): ν_{max} (cm^{-1}) : 2879 and 2941(m), 1688(s), 1505(w), 1411(w), 1302(m), 1112(w). (Figure C1 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.) δ (ppm) : 1.87 (2H, m, 4-H), 2.20 (2H, t, 3-H), 2.68 (3H, s, 6-H), 3.25 (2H, t, 5-H). (Figure C2 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.) δ (ppm): 17.47(t), 29.43(q), 30.55(t), 49.37(t), 175.18(s). (Figure C3 in Appendix C)

MALDI-TOF MS spectrum (*m/z*): $[M+H]^+$ 100.93. (Figure C4 in Appendix C)

3.13.2 Purification and characterization of compound 2

The combined fraction B05 was obtained from column chromatography of the KPCH007 culture broth ethyl acetate crude extract using Hexane:$CHCl_3$(80:20 $\rightarrow$ 70:30) as eluent. The yellow viscous liquid with yellow solid of the fraction B05(77.3mg) was subjected to isolation by column chromatography using $CHCl_3$ in hexane as eluent to afford a compound 2 as a yellow needles (13mg).

m.p. 189-190 ^{o}C, UVλ_{max}(nm): $CDCl_3$(ε) : 260(2989) nm . (Figure C11 in appendix C)

FT-IR spectrum (KBr): ν_{max} (cm^{-1}): 3400(br), 2925 and 2851(w), 1657(s), 1372(m), 1030(m), 754(m). (Figure C6 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.) δ (ppm): 1.78 (3H, s, 4a-Me), 3.98 (3H, s, 9-OMe), 6.32 (1H, s, 4-H), 6.68 (1H, s, 8-H), 6.75 (1H, s, 1-H), 6.80 (1H, s, 10-H), 11.32 (1H, s). (Figure C 7 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.) δ (ppm) : 30, 56, 79.8, 100, 103.8, 104.5, 116.5, 121, 135.5, 146, 153.5, 165, 166.8, 168, 181.(Figure C8 in Appendix C)

EI-MS spectrum (*m/z*): 288. (Figure C10 in Appendix C)

3.13.3 Purification and characterization of compound 3

The combined fraction B07 was obtained from column chromatography of the KPCH007 culture broth ethyl acetate crude extract using Hexane:$CHCl_3$(60:40 $\rightarrow$ 50:50) as eluent. The white viscous liquid of the fraction B07 (91.7mg) was subjected to isolation by column chromatography using $CHCl_3$ in hexane as eluent to afford a compound 3 as a white solid (11mg).

m.p. 212 ^{o}C, UVλ_{max}(nm): $CDCl_3$(ε) : 338(4568) nm . (Figure C16 in appendix C)

FT-IR spectrum (KBr): ν_{max} (cm^{-1}): 3322(m), 1660 and 1618(s), 1278(m), 1170(m), 1081(w). (Figure C12 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.): δ (ppm) : 1.32 (2H,s), 2.79 (3H,s), 3.95 (3H,s),6.57 (1H,d,*J*=2Hz),6.64 (1H,d,*J*=2.8Hz), 6.73 (1H,d,*J*=2Hz), 7.30 (1H,d,*J*=2Hz). (Figure C13 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.): δ (ppm) : 24.3, 29.3, 54.94, 98.6, 101.4, 103.5, 110, 117.3, 138.2, 164, 164.9, 166.5 . (Figure C14 in Appendix C)

EI-MS spectrum (*m/z*): 272 (Figure C15 in Appendix C)

3.13.4 Purification and characterization of mixture 4

The combined fraction M04 was obtained from column chromatography of the KPCH007 mycelium ethyl acetate crude extract using Hexane:$CHCl_3$ (90:10) as eluent. The yellow viscous liquid were furture washed with hexane and ether mixtures to afford mixture 4 as yellow liquid (1.224 g).

FT-IR spectrum (NaCl) : ν_{max} (cm^{-1}): 3474(br), 2851 and 2925(s), 1738(s), 1462(m), 1158(m), 726(w). (Figure C18 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz): δ (ppm) : 0.85, 1.22, 1.61, 2.10, 2.38, 2.80, 4.16, 4.30, 5.38 . (Figure C19 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.): δ (ppm) : 14.08, 14.14, 14.25, 22.6, 22.7, 24.8, 25.6, 25.7, 27.2, 29, 29.8, 31.6, 31.9, 34, 34.2, 62, 68.9, 77.1, 127.9, 129.7, 130, 130.2 . (Figure C20 in Appendix C)

3.13.5 Purification and characterization of mixture 5

The combined fraction M07 was obtained from column chromatography of the KPCH007 mycelium ethyl acetate crude extract using Hexane:$CHCl_3$(75:25 $\rightarrow$ 70:30) as eluent. The yellow viscous liquid with yellow solid of the fraction M07(40.2mg) washed using 50%Hexane : 50%Ether as eluent and separate by preparative thin layer chromatography to afford a mixture 5 as a yellow solid (15 mg).

m.p. 264-266 ^{o}C, UVλ_{max}(nm): $CDCl_3$(ε) : 286(8171) nm . (Figure C25 in appendix C)

FT-IR spectrum (KBr): ν_{max} (cm^{-1}): 3392(br), 2918 and 2840(w), 1750(w), 1567(s), 1330(s), 1225(m), 1162(m) . (Figure C21 in Appendix C)

^{1}H-NMR spectrum (DMSO-d6, 400 MHz.): δ (ppm) : 2.28 (s), 2.74 (s), 3.92 (s), 3.93 (s), 6.30 (d), 6.66 (d), 7.23 (d), 6.828 (d, J = 2.4 H_z), 7.153 (d, J = 2.4 H_z), 7.55 (s), 6.74 (d), 7.91 (s). (Figure C22 in Appendix C)

^{13}C-NMR spectrum (DMSO-d6, 100 MHz.): δ (ppm) : 186.98, 181, 166.64, 166.39, 165, 164.6, 162.05, 159.05, 153.10, 138.96, 138.26, 135.47, 133.50, 132.53, 130.59, 125.33, 118.08, 116.46, 111.49, 110.67, 109.27, 107.57, 106.15, 103.90, 102.10, 99.64, 56.77, 56.71, 25.51, 16.65. (Figure C23 in Appendix C)

MALDI-TOF MS spectrum (*m/z*): $[M+H]^{+}$ 285. (Figure C24 in Appendix C)

3.13.6 Purification and characterization of compound 6

The combined fraction M10 was obtained from column chromatography of the KPCH007 mycelium ethyl acetate crude extract using Hexane:$CHCl_3$(65:35 $\rightarrow$ 60:40) as eluent. The orange viscous liquid of the fraction M10(118.9mg) washed using 50%Hexane : 50%Ether as eluent to afford a compound 6 as a brown solid (78.2mg).

m.p. 276-277 ^{o}C, UVλ_{max}(nm): $CDCl_3$(ε) : 337(22467) nm . (Figure C30 in appendix C)

FT-IR spectrum (KBr): ν_{max} (cm^{-1}): 3357(br), 1610 and 1660(s), 1462(m), 1228(m), 1158(m), 1096(w). (Figure C26 in Appendix C)

^{1}H-NMR spectrum (DMSO-d6, 400 MHz.) δ (ppm) : 2.72 (3H, s, 1-Me), 3.90 (3H, s, 9-OMe), 6.61 (1H, d, *J*= 2.4 Hz, 8-H), 6.64 (1H, s, 4-H), 6.72 (1H, s, 2-H), 7.20 (1H, s, 10-H), 10.41(1H, s, 3-OH), 11.82 (1H, s, 7-OH). (Figure C27 in Appendix C)

^{13}C-NMR spectrum (DMSO-d6, 100 MHz.) δ (ppm) : 25.48(q), 56.29(q), 98.89(s), 99.60(d), 102.28(d), 103.84(d), 109.24(s), 118.06(d), 138.22(s), 138.92(s), 153.07(s), 159.03(s), 164.58(s), 165.14(s), 166.60(s). (Figure C28 in Appendix C)

MALDI-TOF MS spectrum (*m/z*): $[M+H]^+$ 273.04. (Figure C29 in Appendix C)

3.14 Biological activity test of each fraction

3.14.1 Antimicrobial activity test

3.14.1.1 Antimicrobial activity of pool fractions

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

3.14.1.2 Antimicrobial activity of pure compounds

Evaluation of the antimicrobial activity of pure compounds were determined by the antimicrobial susceptibility test broth microdilution method (Woods and Washington, 1995). Antimicrobial activity was performed against *B. subtilis* ATCC 6633*, S. aureus* ATCC 25923*, E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169, and *C. albican* ATCC 10231.

A. Preparation of pure compounds and antibiotic drug standards

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

B. Preparation of tested bacterial inoculum

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

C. Preparation of tested yeast inoculum

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

D. Assay procedure

Solutions of pure compounds and antibiotic drug standards were diluted with Mueller-Hinton Broth (MHB) and YMB for assays of antibacterial and antifungal (yeast form) activity respectively. Fifty µl of pure compound was dispensed into each well in sterile microtiter plates (96-well bottom wells). Fifty µl of the final adjusted microbial suspension was inoculated into each well (Final inoculum size of bacterial and yeast was approximately $2.5X10^5$ and $2.5X10^4$ CFU/ml, respectively). One hundred µl of medium only was as the sterility control. A 100 µl volume of medium and microbial

inoculum mixture acted as the growth control. Microbial microtiter plates were incubated at 37 ^{o}C and room temperature for bacterial and yeast, respectively.

E. Reading of microtiter plates assays

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

For filamentous fungi results were determined using the by agar well diffusion method (Weaver, Angel and Botlomley, 1994) in the same manner as described in section 3.7. Antimicrobial activity tests were performed against *Collectotichum glocospriodes*, *Alternaria brassicicola, Fusarium oxysporum,* and *Phytophthora palunivora*.

3.14.2 Cytotoxicity test

Cytotoxicity test were carried out at the Institute of Biotechnology and Genetic Engineering. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric 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 ^{o}C, 5% CO_2, 100% relative humidity,100 µl of culture medium, culture medium containing the sample was dispensed into the appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) “background” determinations. Culture plates were then incubated for 4

days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ ml PBS was sterilized and filtered through 0.45-µl filter units. MTT working solutions were prepared just prior to culture application by dilution of MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 µl) was added to each culture well, resulting in 50 µl MTT/ 250 µl total medium volumes; and cultures were incubated at 37 ^{o}C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 µl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 µl of DMSO using a pipette. Following formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

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

Samples were also tested for cytotoxic activity towards 5 cell lines, which contain HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric) and BT 474 (breast) following the experimental method of bioassay of cyototoxic activity.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Plant samples

Healthy young and mature leaves of *Kaempferia parviflora* were collected from Sriracha District, Chonburi Province, Patumthani Province and Bangkok Province Thailand, on November 29, 2002. Plant samples were kept in a plastic bag. Fresh specimens were processed with 24 hours after collection.

4.2 Isolation of endophytic fungi

Culture medium used for the isolation of endophytic fungi was malt extract agar (MEA). Thirty endophytic fungal isolates were isolated from healthy young leaves and mature leaves of *Kaempferia parviflora*. All endophytic fungal isolates were selected for further study, as shown in Table 4.1. Characteristics of colony and identification of endophytic fungi from *Kaempferia parviflora,* as shown in Table 4.2

Table 4.1 Number and isolate endophytic fungi

Plant	Number of isolated endophytic fungi	Endophytic fungi isolates code
Chonburi Province	18	KPCH 001 to 018
Bangkok Province	6	KPBK 001 to 006
Patumthani Province	12	KPPT 001 to 012
Total (isolates)	36	36

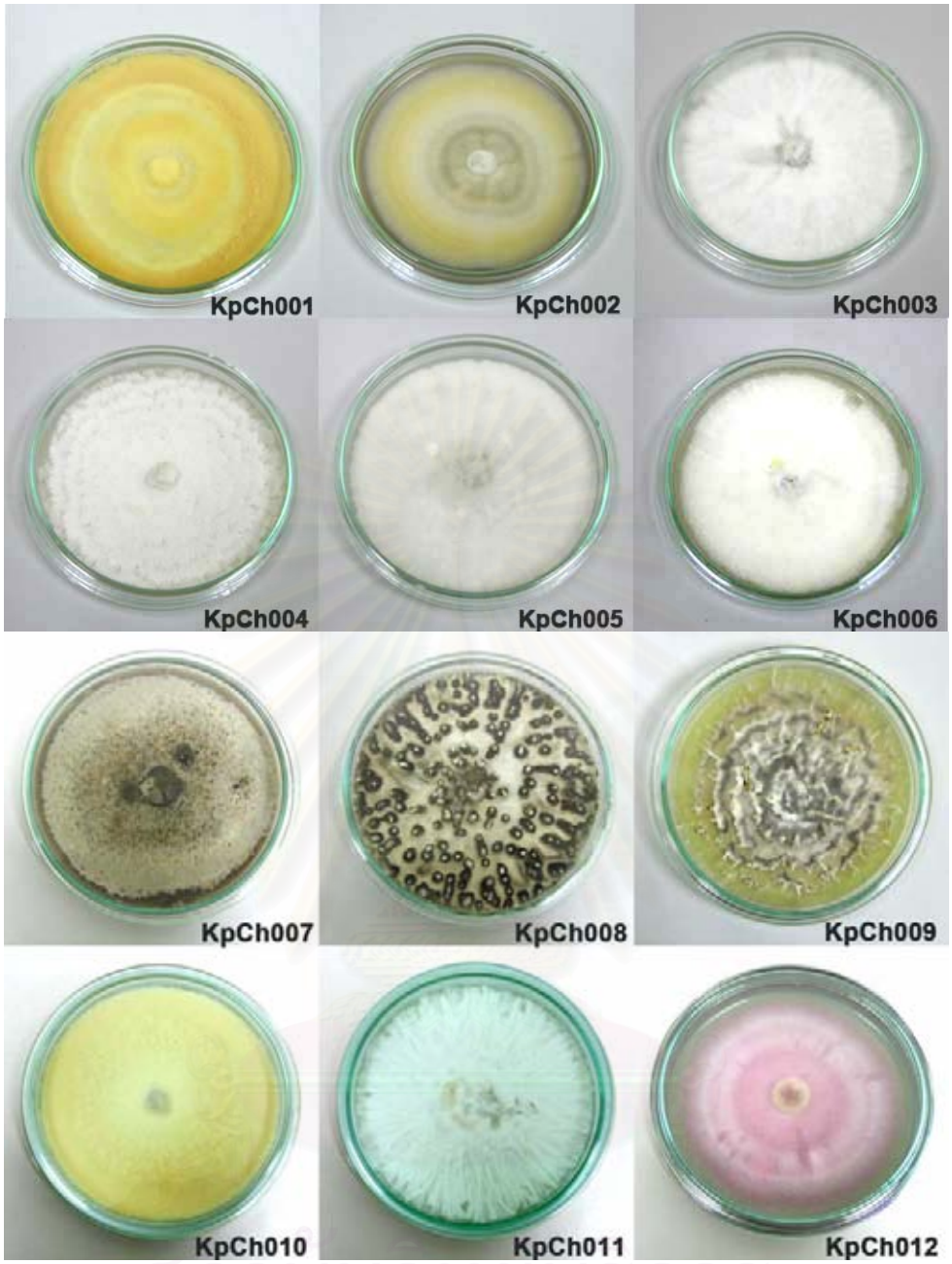


Figure 4.1 Colony characteristic of endophytic fungi isolated from *Kaempferia parviflora* leaves, on MEA after cultivation for 28 days at room temperature. Isolate numbers are shown in each pictures.

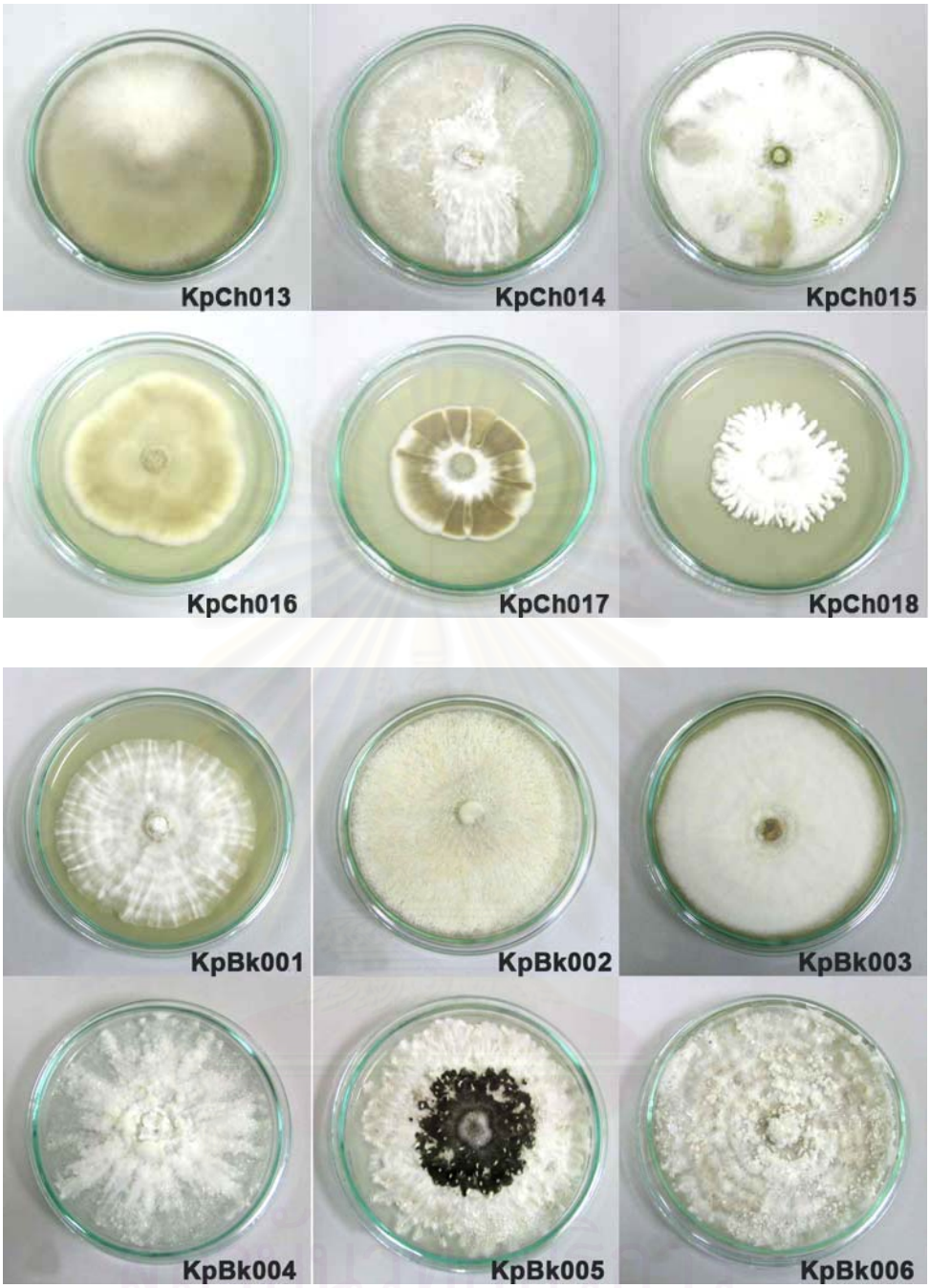


Figure 4.1 Colony characteristic of endophytic fungi isolated from *Kaempferia parviflora* leaves, on MEA after cultivation for 28 days at room temperature. Isolate numbers are shown in each pictures. (continued)

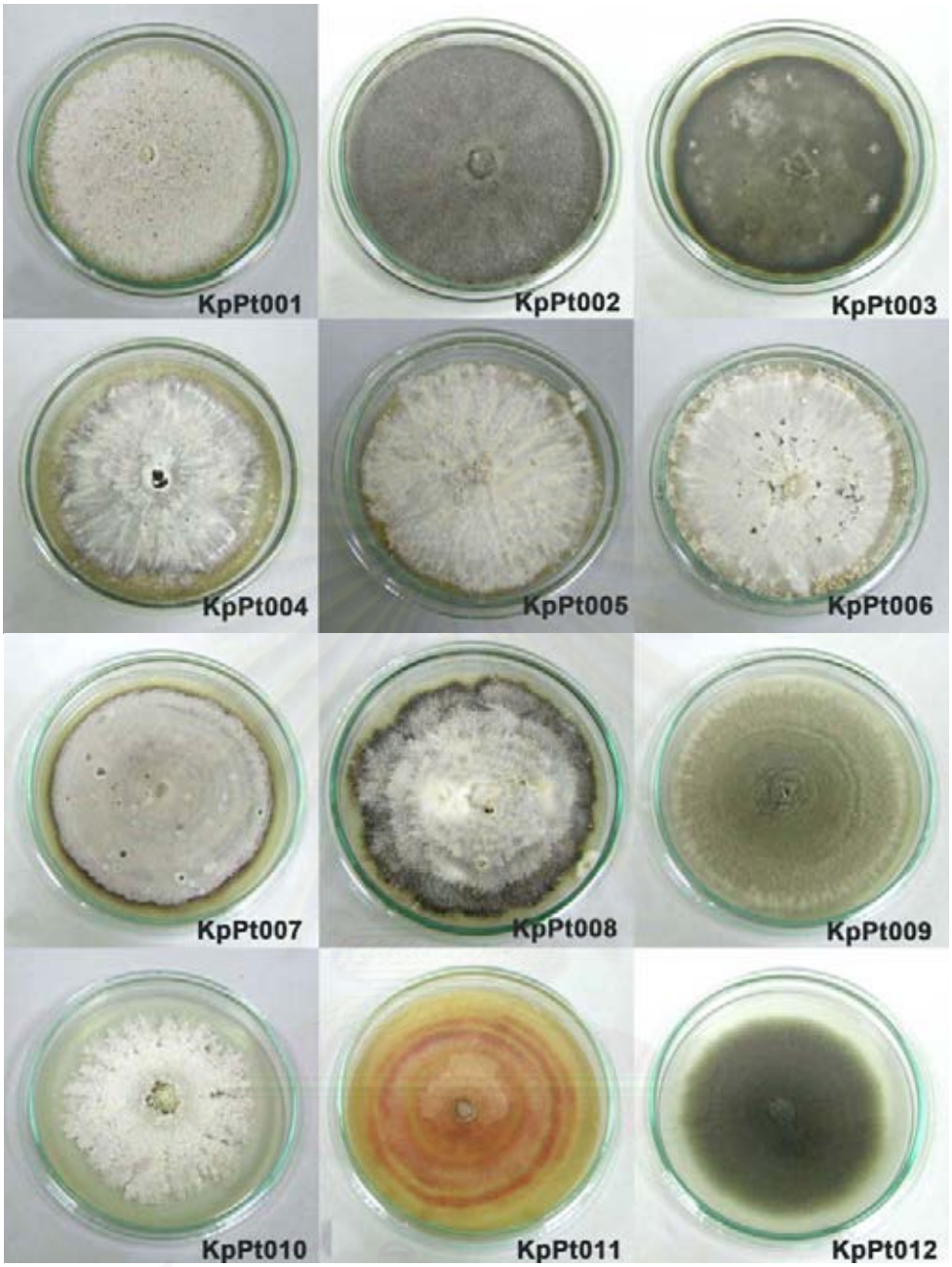


Figure 4.1 Colony characteristic of endophytic fungi isolated from *Kaempferia parviflora* leaves, on MEA after cultivation for 28 days at room temperature. Isolate numbers are shown in each pictures. (continued)

Table 4.2 Characteristics of colony and identification of endophytic fungi from Kaempferia parviflora.

Isolates	Endophytic fungi characteristics on the MEA medium			Fungal species
	Colony characteristic	Colony color	Color pigment production on the media	
KPCH001	Cottony	yellow	yellow	*Fusarium* sp.
KPCH002	Absence of elevation	brown	not produce	Mycelia sterilia
KPCH003	Cottony	white	not produce	Mycelia sterilia
KPCH004	Absence of elevation	white	not produce	Mycelia sterilia
KPCH005	Absence of elevation	white	not produce	Mycelia sterilia
KPCH006	Absence of elevation	white	not produce	Mycelia sterilia
KPCH007	Absence of elevation	dark brown	dark brown	*Alternaria* sp.
KPCH008	Stroma	Black and white	not produce	Xylariaceae
KPCH009	Stroma	Black and white	not produce	Xylariaceae
KPCH010	Absence of elevation	yellow	yellow	*Fusarium* sp.
KPCH011	Absence of elevation	white	not produce	Xylariaceae
KPCH012	Absence of elevation	pink	not produce	*Fusarium* sp.
KPCH013	Absence of elevation	brown	not produce	Mycelia sterilia
KPCH014	Cottony	brown	not produce	Mycelia sterilia
KPCH015	Absence of elevation	white	dark green	Mycelia sterilia
KPCH016	Absence of elevation	brown	not produce	Mycelia sterilia
KPCH017	Absence of elevation	dark brown	not produce	*Aspergillus* sp.
KPCH018	Absence of elevation	white	not produce	Mycelia sterilia
KPBK001	Stroma	white	not produce	Xylariaceae
KPBK002	Absence of elevation	white and yellow	not produce	Mycelia sterilia
KPBK003	Absence of elevation	white	not produce	Mycelia sterilia
KPBK004	Cottony	white	not produce	Mycelia sterilia
KPBK005	Cottony	black and white	not produce	Xylariaceae

Table 4.2 (continues)

KPBK006	Cottony	white	not produce	Mycelia sterilia
KPPT001	Absence of elevation	white	not produce	Mycelia sterilia
KPPT002	Absence of elevation	black	not produce	Mycelia sterilia
KPPT003	Absence of elevation	black	not produce	Mycelia sterilia
KPPT004	Absence of elevation	black and white	not produce	Mycelia sterilia
KPPT005	Absence of elevation	white	not produce	Mycelia sterilia
KPPT006	Absence of elevation	white	not produce	Mycelia sterilia
KPPT007	Absence of elevation	brown and white	not produce	*Phomopsis* sp.
KPPT008	Absence of elevation	black and white	not produce	*Phomopsis* sp.
KPPT009	Absence of elevation	brown and gray	not produce	Mycelia sterilia
KPPT010	Absence of elevation	white	not produce	Mycelia sterilia
KPPT011	Absence of elevation	Red and orange	orange	*Fusarium* sp.
KPPT012	Absence of elevation	black	not produce	Mycelia sterilia

4.3 Enumeration of test microorganisms

Viable counts of bacteria and yeast were performed for standardized inoculated whose turbidity matched a 0.5 McFarland standard. The CFU/ml values are shown in Table 4.3

Table 4.3 Viable counts of bacteria and yeast

Tests microorganism	Quantity (Colony forming unit)
Bacillus subtilis ATCC 6633	3.2×10^6
Escherichia coli ATCC25922	2.1×10^7
Pseudomonas aeruginosa ATCC 27853	2.2×10^7
Staphylococcus aureus ATCC 25923	1.8×10^7
Candida albicans ATCC 10231	3.5×10^5

4.4 Determination antimicrobial activity of isolated endophytic fungi

An antimicrobial activity assays of isolated endophytic fungi were performed by growing on four media potato dextrose agar, malt extract agar and yeast extract agar and determined by using dual culture agar diffusion technique. The antimicrobial activities of the isolates were determined antimicrobial activities by fungal disk dual culture method against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231.

The antimicrobial activities of isolated endophytic fungi which were active against at least one tested microorganisms are shown in Table 4.4 and Figure 4.3. The results showed that endophytic fungal 15 isolates (41.67 % of total 36 isolates) had the antimicrobial activities against at least one tested microorganisms. They were active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 at 36.1%, 13.9%, 8.3%, 16.7%, and 11.1%, respectively (Table 4.5 and Figure 4.4). This indicated that *B. subtilis* ATCC 6633, the gram positive rod bacterium, was more sensitive to isolated endophytic fungi than other tested microorganisms.

4.5 Identification of isolated endophytic fungi

From the method of preparation of the fungal mycelia on glass slides by Scoth tape preparation with the unfrosted, The preparation was made by placing a drop of lactophenol aniline stain on a microscope slide that have a mycelia of some part fungal are shown in Figure 4.2.

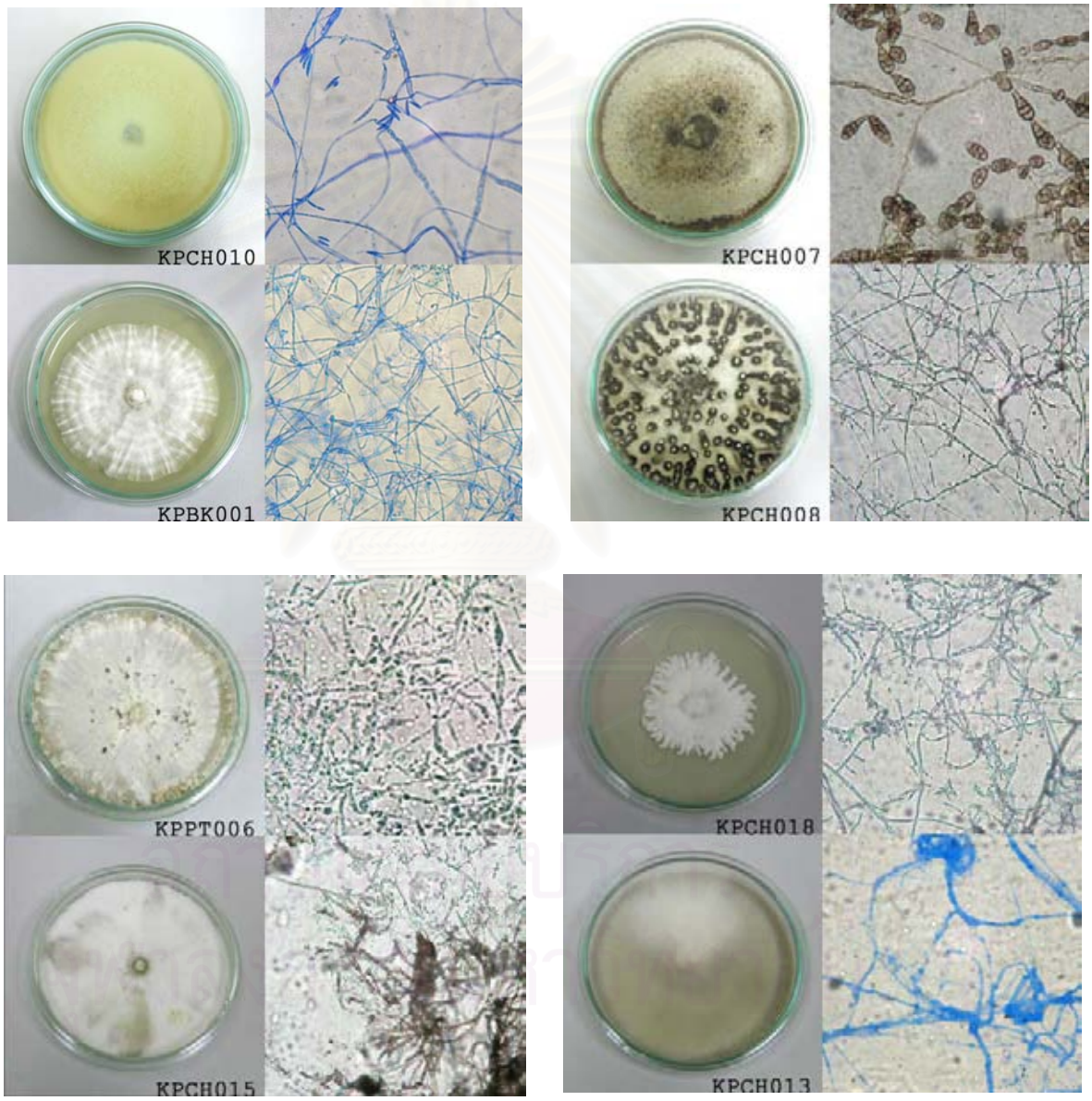


Figure 4.2 Picture of the fungal mycelia on glass slides by Scoth tape preparation

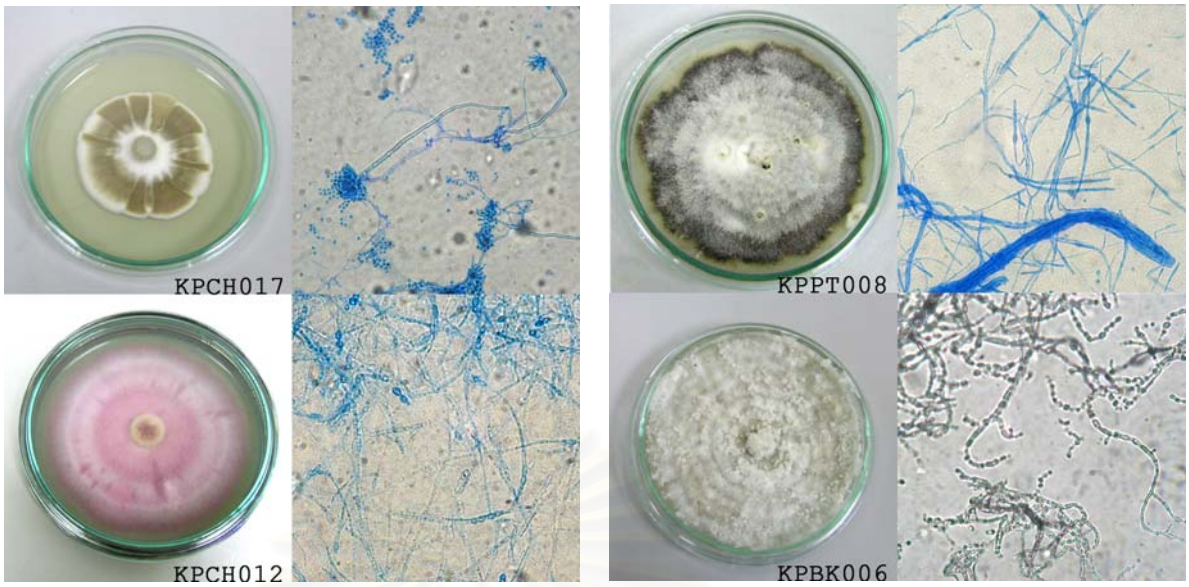


Figure 4.2 Picture of the fungal mycelia on glass slides by Scoth tape preparation (continues)

Table 4.4 The antimicrobial activities of endophytic fungi isolated from *K. parviflora*.

No. of endophytic fungal	Media	Antimicrobial activity				
		BS	EC	PA	SA	CA
KPCH001	PDA	-	-	-	-	-
	MEA	++	-	+	++	-
	YES	+	-	+	+	-
KPCH002	PDA	-	-	-	-	-
	MEA	+	-	-	+	-
	YES	+	-	-	+	-
KPCH003	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH004	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-

Table4.4 (continues)

KPCH005	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH006	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH007	PDA	-	-	-	-	-
	MEA	+	-	++	+	-
	YES	+	-	+	+	-
KPCH008	PDA	-	-	++	-	+
	MEA	-	-	++	-	+
	YES	-	-	-	-	-
KPCH009	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	+	-	-	+	+
KPCH010	PDA	-	-	-	-	-
	MEA	+	-	-	-	-
	YES	+	-	-	-	-
KPCH011	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH012	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH013	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH014	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH015	PDA	-	-	-	-	-
	MEA	+	+	-	-	-
	YES	-	-	-	-	-

Table4.4 (continues)

KPCH016	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH017	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPCH018	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPBK001	PDA	-	-	-	-	-
	MEA	++	-	-	++	+
	YES	+	-	-	+	+
KPBK002	PDA	-	-	-	-	-
	MEA	+	-	-	-	+
	YES	++	-	-	-	+
KPBK003	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPBK004	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPBK005	PDA	+	-	-	-	-
	MEA	+	-	-	-	-
	YES	-	-	-	-	-
KPBK006	PDA	-	-	-	-	-
	MEA	++	+	-	-	-
	YES	+	+	-	-	-
KPPT001	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT002	PDA	-	-	-	-	-
	MEA	++	+	+	-	-
	YES	-	-	-	-	-

Table4.4 (continues)

KPPT003	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT004	PDA	-	-	-	-	-
	MEA	-	-	+	-	-
	YES	-	-	+	-	-
KPPT005	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT006	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT007	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT008	PDA	-	-	-	-	-
	MEA	+	-	+	-	-
	YES	+	-	+	-	-
KPPT009	PDA	-	-	-	-	-
	MEA	+	-	-	-	-
	YES	+	-	-	-	-
KPPT010	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT011	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-
KPPT012	PDA	-	-	-	-	-
	MEA	-	-	-	-	-
	YES	-	-	-	-	-

Activities were classified according to the diameter of inhibition zones around the point of application of the sample

++ ; more than 10 mm ; + less than 10 mm ; - not inhibition

BS ; *Bacillus subtilis* ; EC ; *Escherichia coli* ; PA ; *Pseudomonas aeruginosa*

SA ; *Staphylococcus aureus* ; CA; *Candida albicans*

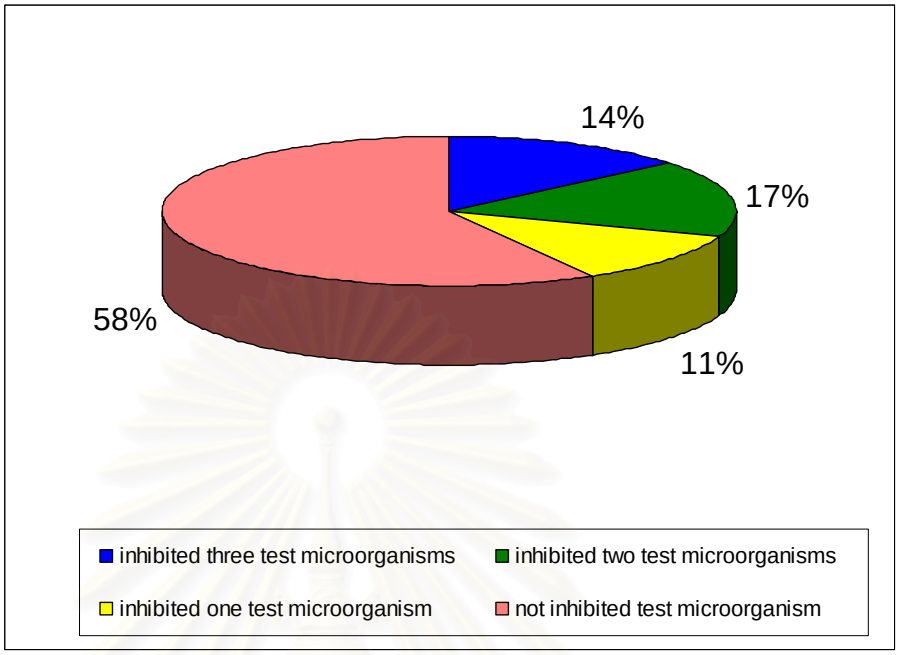


Figure 4.3 Summerize percentage of antimicrobial activities of isolated endophytic fungi against testes microorganism

Table 4.5 The amount of active endophytic fungal isolates

Tested microorganisms		Number of active isolates (isolates)	Percent of active isolate (%)
Gram positive bacteria	*B. subtilis* ATCC 6633	13	36.1
	S. aureus ATCC 25923	5	13.9
Gram negative bacteria	*E. coli* ATCC 25922	3	8.3
	P. aeruginosa ATCC 27853	6	16.7
Yeast	*C. albicans* ATCC 10231	4	11.1

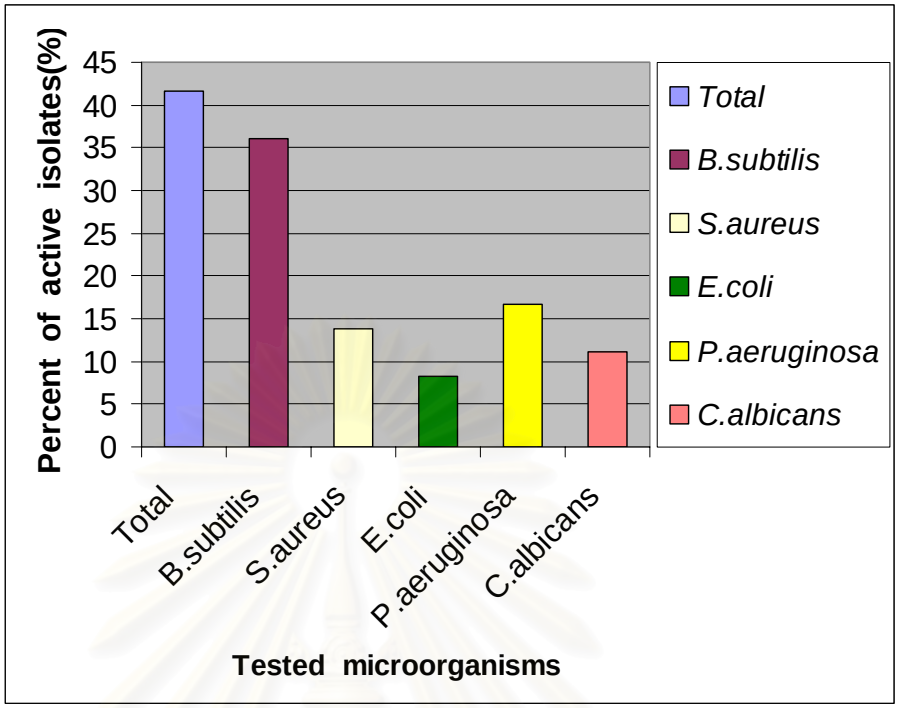


Figure 4.4 The histrogram showed a summery of the dual culture agar diffusion technique assay results for the antimicrobial activity of endophytic fungi

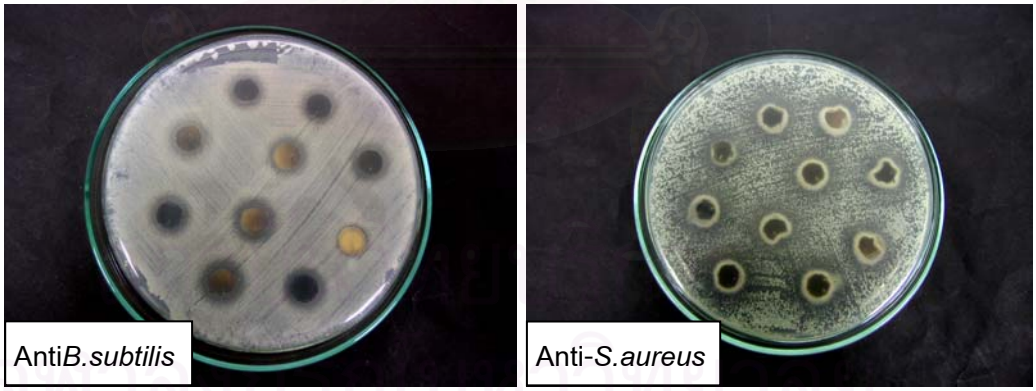


Figure 4.5 Dual-culture agar diffusion exhibit against *Bacillus subtilis* and *Staphylococcus aureus* of activity of endophytic fungus isolated.

4.6 Identification of fungal endophyte KPCH 007

Fungal isolated KPCH 007 was chosen for further study for bioactive compounds. Because KPCH 007 cultured on MEA was best activity that active against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 and the culture broth extracts were active against *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 .

4.6.1 Morphology identification

Fungal isolate KPCH 007 was identified as *Alternaria tenuissima*. The fungus was grown on MEA for a week at room temperature ; mycelium composed of hyaline, septate, branched hyphae and dark violet pigment. Colony morphology of isolate KPCH 007 that cultured on six media ; potato dextrose agar(PDA), yeast extract sucrose agar(YES), sabouroad glucose agar(SGA),potato carrot agar(PCA), malt extract agar(MEA) and corn meal agar(CMA) are shown in Figure 4.6. Colony morphology and slide culture of isolate KPCH 007 that cultured on MEA is shown in Figure 4.7 and Figure 4.8 is shown scanning electron microscope.

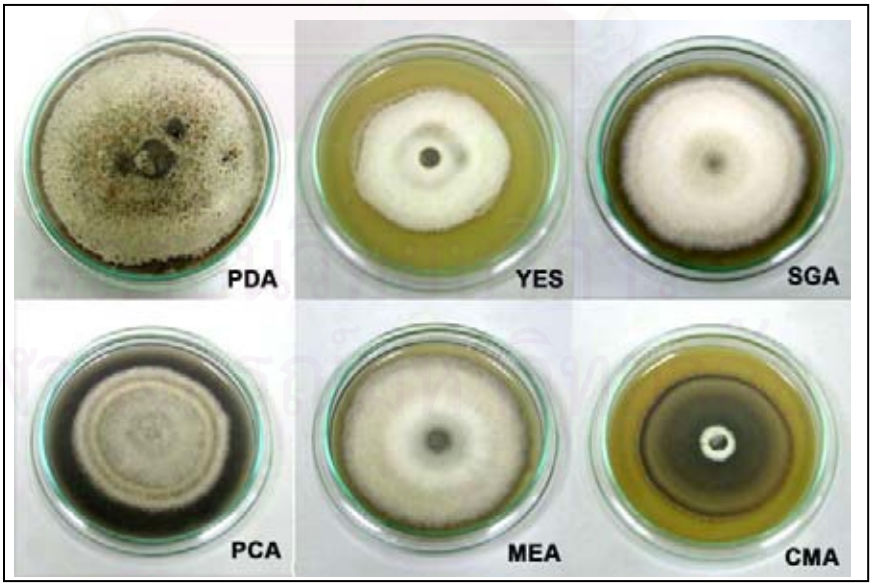


Figure 4.6 Colony characteristic of endophytic fungal isolate 007 was grown on PDA, YES, SGA, PCA, MEA and CMA media at room temperature and cultivated for 28 days.

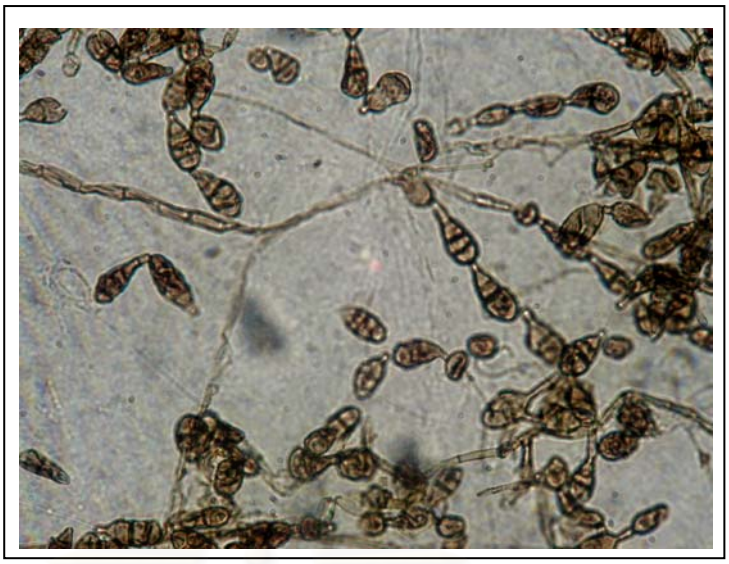


Figure 4.7 Colony morphology from slide culture of isolate KPCH 007 which grew on MEA.

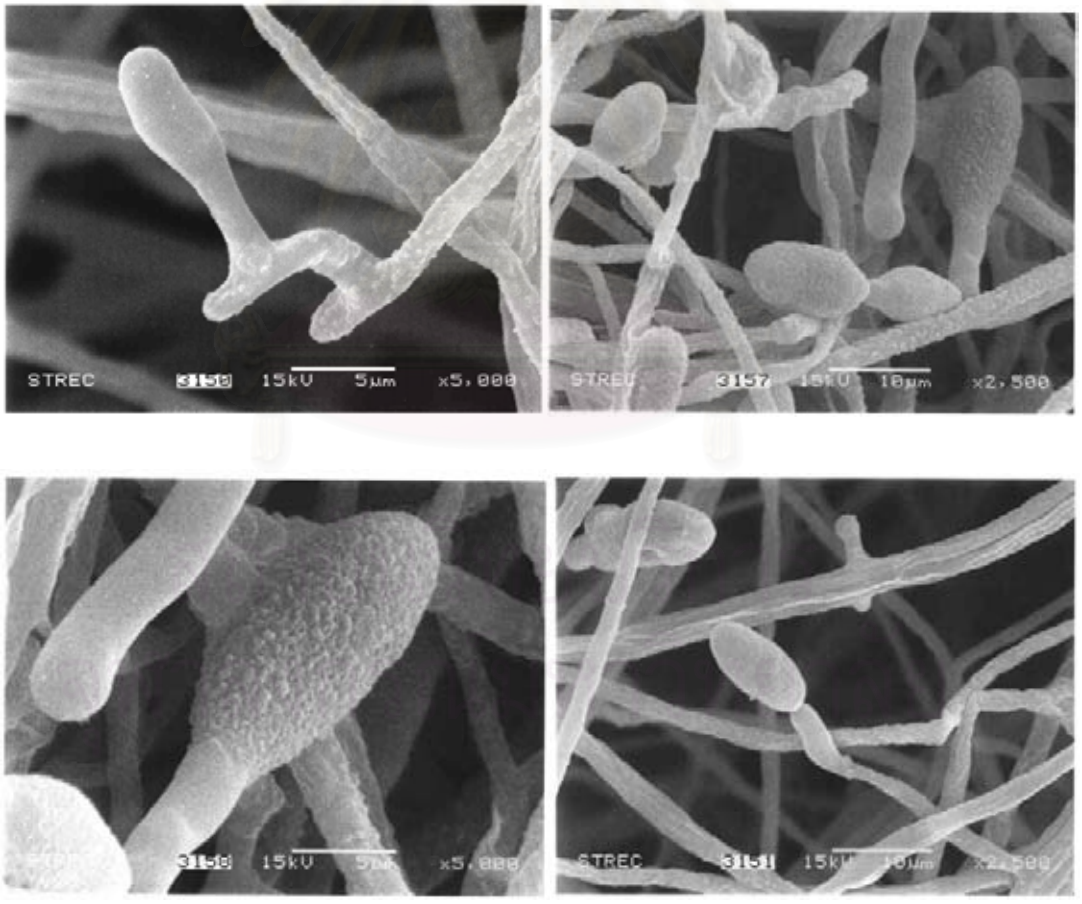


Figure 4.8 Scanning electron micrograpе of endophytic fungal isolate KPCH 007 as shown conidia and conidiophore.

4.6.2 Molecular identification

Endophytic fungus isolate KpCh 007 was sent for identification by molecular methods at the Asian Natural Environment Science Center, the University of Tokyo, Japan.

Sequencing of the nucleotide sequences of partial 18S. ITS region of the isolate, KpCh007 resulted in a 608 bp fragment, as shown in Figure 4.9

Classical identification of fungi is based on observe characteristics. Assignment of morphological species can be based on colony surface texture, hyphal pigments, exudates, margin shapes, growth rates, and sporulating structures (Redlin and Carris, 1985). Fungal isolate KpCh 007 was identified as belonging to the genera *Alternaria.*

```
     1
5'CTTGGTCATT   TAGAGGAAGT   AAAAGTCGTA   ACAAGGTCTC
  CGTAGTGAAC   CTGCGGAGGG   ATCATTACAC   AAATATGAAG
  GCGGGCTGGA   ATCTCTCGGG   GTTACAGCCT   TGCTGAATTA
  TTCACCCTTG   TCTTTTGCGT   ACTTCTTGTT   TCCTTGGTGG
  GTTCGCCCAC   CACTAGGACA   AACATAAACC   TTTTGTAATT
  GCAATCAGCG   TCAGTAACAA   ATTAATAATT   ACAACTTTCA
  ACAACGGATC   TCTTGGTTCT   GGCATCGATG   AAGAACGCAG
  CGAAATGCGA   TAAGTAGTGT   GAATTGCAGA   ATTCAGTGAA
  TCATCGAATC   TTTGAACGCA   CATTGCGCCC   TTTGGTATTC
  CAAAGGGCAT   GCCTGTTCGA   GCGTCATTTG   TACCCTCAAG
  CTTTGCTTGG   TGTTGGGCGT   CTTGTCTCTA   GCTTTGCTGG
  AGACTCGCCT   TAAAGTAATT   GGCAGCCGGC   CTACTGGTTT
  CGGAGCGCAG   CACAAGTCGC   ACTCTCTATC   AGCAAAGGTC
  TAGCATCCAT   TAAGCCTTTT   TTTCAACTTT   TGACCTCGGA
  TCAGGTAGGG   ATACCCGCTG   AACTTAAGCA   TATCAATAAG
  CGGAGGA  3'
       608
```

Figure 4.9 Nucleotide sequences of partial 18S region, complete ITS region of the isolate KpCh 007.

A blast search was performed to find a similar sequence to ITS region of fungal isolate KPCH007 in the Genbank DNA database. The ITS region of this isolate was similar to 99.507% identity of *Alternaria tenuissima*, Alignment data of ITS region of isolate KPCH 007 as shown in Figure 4.10. The reference was reported by Buzina et al., 2003.

>>AY154711|AY154711.1 Alternaria tenuissima strain IA285 (1488 nt) initn: 2806 init1: 2806 opt: 3001 Z-score: 3010.7 expect() 5.9e-160 99.507% identity in 608 nt overlap (1-607:311-918)

```
                10         20         30         40         50
        ....|....| ....|....| ....|....| ....|....|....|....|
AY1547  CGACCACCTC AAGCCGGAAA GTTCGTCAAA CTCGGTCATT AGAGGAAGT
007     ---------- ---------- ---------- CTTGGTCATT AGAGGAAGT
                 60         70         80         90      100
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  AAAAGTCGTA ACAAGGTCTC CGTAGGTGAA CCTGCGGAGG ATCATTACA
007     AAAAGTCGTA ACAAGGTCTC CGTA-GTGAA CCTGCGGAGG ATCATTACA

                110        120        130        140      150
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  CAAATATGAA GGCGGGCTGG AACCTCTCGG GGTTACAGCC TGCTGAATT
007     CAAATATGAA GGCGGGCTGG AATCTCTCGG GGTTACAGCC TGCTGAATT
                160        170        180        190      200
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  ATTCACCCTT GTCTTTTGCG TACTTCTTGT TTCCTTGGTG GTTCGCCCA
007     ATTCACCCTT GTCTTTTGCG TACTTCTTGT TTCCTTGGTG GTTCGCCCA
                210        220        230        240      250
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  CCACTAGGAC AAACATAAAC CTTTTGTAAT TGCAATCAGC TCAGTAACA
007     CCACTAGGAC AAACATAAAC CTTTTGTAAT TGCAATCAGC TCAGTAACA
                260        270        280        290      300
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  AATTAATAAT TACAACTTTC AACAACGGAT CTCTTGGTTC GGCATCGAT
007     AATTAATAAT TACAACTTTC AACAACGGAT CTCTTGGTTC GGCATCGAT
                310        320        330        340      350
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  GAAGAACGCA GCGAAATGCG ATAAGTAGTG TGAATTGCAG ATTCAGTGA
007     GAAGAACGCA GCGAAATGCG ATAAGTAGTG TGAATTGCAG ATTCAGTGA
                360        370        380        390      400
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  ATCATCGAAT CTTTGAACGC ACATTGCGCC CTTTGGTATT CAAAGGGCA
007     ATCATCGAAT CTTTGAACGC ACATTGCGCC CTTTGGTATT CAAAGGGCA
                410        420        430        440      450
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  TGCCTGTTCG AGCGTCATTT GTACCCTCAA GCTTTGCTTG TGTTGGGCG
007     TGCCTGTTCG AGCGTCATTT GTACCCTCAA GCTTTGCTTG TGTTGGGCG
                460        470        480        490      500
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  TCTTGTCTCT AGCTTTGCTG GAGACTCGCC TTAAAGTAAT GGCAGCCGG
007     TCTTGTCTCT AGCTTTGCTG GAGACTCGCC TTAAAGTAAT GGCAGCCGG
                510        520        530        540      550
        ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547  CCTACTGGTT TCGGAGCGCA GCACAAGTCG CACTCTCTAT AGCAAAGGT
007     CCTACTGGTT TCGGAGCGCA GCACAAGTCG CACTCTCTAT AGCAAAGGT
```

```
                560        570        580        590      600
          ....|....| ....|....| ....|....| ....|....|....|....|
AY1547    CTAGCATCCA TTAAGCCTTT TTTTCAACTT TTGACCTCGG ATCAGGTAG
007       CTAGCATCCA TTAAGCCTTT TTTTCAACTT TTGACCTCGG TCAGGTAGG

                610        620        630        640      650
          ....|....| ....|....| ....|....| ....|....| ...|....|
AY1547    GATACCCGCT GAACTTAAGC ATATCAATAA GCGGAGGAAA GAAACCAAC
007       GATACCCGCT GAACTTAAGC ATATCAATAA GCGGAGGA-- ---------

                660        670        680        690      700
          ....|....| ....|....| ....|....| ....|....|....|....|
AY1547    AGGGATTGCC CTAGTAACGG CGAGTGAAGC GGCAACAGC CAAATTTGAA
007       ---------- ---------- ---------- ---------- ---------

                710        720
          ....|....| ....|....|
AY1547    ATCTGGCTCT TTTAGAGTCC
007       ---------- ----------
```

Figure 4.10 Alignment data of ITS region of isolate KPCH 007 and 1 reference taxa.

4.7 Chemical constituents of endophytic fungus isolate KPCH 007 metabolites

Fungal isolate KPCH 007 was cultivated in malt extract broth totalling 15 l to yield 13 g of crude EtOAc mycelia extracts and 12.3 g of crude EtOAc from cultivation broth. Isolation of broth ethyl acetate crude extract by column chromatography gave three compounds. Isolation of mycelia ethyl acetate crude extract by column chromatography gave two mixture and one compound.

4.7.1 Structure elucidation of compound 1

Compound 1 was obtained from the combined fraction B03 of culture broth ethyl acetate crude extract, eluted with Hexane:$CHCl_3$ (95:5 → 90:10). Compound 1 was brown liquid. The structure of compound 1 was elucidated using spectroscopic techniques. The IR spectrum of compound 1 is shown in Appendix C Figure C1 and the absorption peaks were assigned as table 4.6 and indicated important absorption band at 3529 cm^{-1}(O-H stretching vibration of alcohol) and strong absorption band at 1688 cm^{-1} (C=O vibration of carbonyl group) due to the carbonyl stretching.

Table 4.6 The IR absorption bands assignment of compound 1

Wave number (cm^{-1})	Intensity	Tentative assignment
2941, 2879	Medium	C-H stretching vibration
1688	Strong	C=O vibration of carbonyl group
1505	Weak	C=C vibration of double bond
1411	Weak	C=C vibration of double bond
1302	Medium	C=C vibration of double bond

The ^{1}H-NMR spectrum (Figure C2 in Appendix C) of compound 1 possessed one methyl groups at 2.68 ppm and three methylene groups at 1.87, 2.20 and 2.25 ppm.

The ^{13}C-NMR spectrum (Figure C3 in Appendix C) of compound 1 showed 5 signals, which the carbonyl group corresponded to the signal at 175.18 ppm.

MALDI-TOF MS spectrum (*m/z*): $[M+H]^+$ (Figure C4 in Appendix C) show ion peak at *m/z* 100.93. The mass spectrum indicated that it possesses the molecular weight 99. If it is assumed that this compound contains protons, carbons, oxygen and nitrogen, then the molecular formular of C_5H_9NO can be established.

The information from 2D-NMR techniques, including HSQC correlation (Table4.7 and Table 4.8) 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)
175.18(s)	-
49.37(t)	3.25 (2H, t, 5-H)
30.55(t)	2.20 (2H, t, 3-H)
29.43(q)	2.68 (3H, s, 6-H)
17.47(t)	1.87 (2H, m, 4-H)

Table 4.8 The HSQC spectral data of compound 1

Position	δ_C	δ_H
1	29.43(q)	2.68 (3H, s, 6-H)
2	175.18(s)	-
3	30.55(t)	2.20 (2H, t, 3-H)
4	17.47(t)	1.87 (2H, m, 4-H)
5	49.37(t)	3.25 (2H, t, 5-H)

Compound 1 showed spectral data identical to that of N-methyl-γ-pyridon (1-methyl-pyrrolidin-2-one), which was reported in the literature (Roberto et al.,1989 and Kelleher et al.,2002) The ^{1}H-NMR and ^{13}C-NMR signals of compound 1 and N-methyl-γ-pyridon are presented in Table 4.9

Table 4.9 The ^{1}H-NMR and ^{13}C-NMR spectral data of compound 1 (in $CDCl_3$) and N-methyl-γ-pyridon (in Acetone- d_6)

Position	δ_H (ppm)		δ_C (ppm)	
	Compound1	NMP	Compound1	NMP
1	2.68 (3H, s, 6-H)	2.8 (3H)	29.43(q)	25
2	-	-	175.18(s)	171
3	2.20 (2H, t, 3-H)	2.25 (2H, t)	30.55(t)	*
4	1.87 (2H, m, 4-H)	1.8 (2H, m)	17.47(t)	*
5	3.25 (2H, t, 5-H)	3.35 (2H, t)	49.37(t)	*

After elucidation of compound 1 by 2D NMR technigue, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of compound 1 and N-methyl-γ-pyridon were compared signal by signal. This result indicated that the structure of compound 1 is identical to N-methyl-γ-pyridon . The structure was presented in Figure 4.11

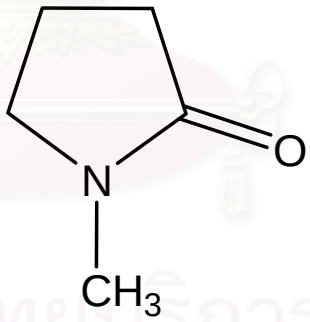


Figure 4.11 The structure of compound 1.

4.7.2 Structure elucidation of compound 2

Compound 2 was obtained from the combined fraction B05 of culture broth ethyl acetate crude extract, eluted with Hexane:$CHCl_3$ (80:20 → 70:30). Compound 2 was an orange solid, m.p. 189-190 °C. The structure of compound 2 was elucidated using spectroscopic techniques. The IR spectrum of compound 2 is shown in Appendix C Figure C6 and the absorption peaks were assigned as table 4.10 and indicated important absorption band at 3400 cm^{-1}(O-H stretching vibration of alcohol) and strong absorption band at 1657 cm^{-1} (C=O vibration of carbonyl group) due to the carbonyl stretching.

Table 4.10 The IR absorption bands assignment of compound 2

Wave number (cm^{-1})	Intensity	Tentative assignment
3400	Broad	O-H stretching vibration of alcohol
2925, 2851	Weak	C-H stretching vibration
1657	Strong	C=O vibration of carbonyl group
1372	Medium	C=C vibration of double bond
1030	Medium	C-O stretching vibration
754	Medium	C-H rocking vibration of $-(CH_2)_n-$

The ^{1}H-NMR spectrum (Figure C 7 in Appendix C) of compound 2 possessed two methyl groups at 1.78 (3H, s, 4a-Me) and 3.98 (3H, s, 9-OMe) ppm, Four methine groups at 6.32 (1H, s, 4-H), 6.68 (1H, s, 8-H), 6.75 (1H, s, 1-H) and 6.80 (1H, s, 10-H) ppm.

The ^{13}C-NMR spectrum (Figure C8 in Appendix C) of compound 2 showed 15 signals, which the carbonyl group corresponded to the signal at 181 ppm.

EI-MS spectrum (*m/z*): (Figure C10 in Appendix C) show ion peak at *m/z* 288. The mass spectrum indicated that it possesses the molecular weight

288. If it is assumed that this compound contains protons, carbons and oxygen, then the molecular formular of $C_{15}H_{12}O_6$ can be established.

The information from 2D-NMR techniques, including HSQC correlation (Table 4.11 and Table 4.12), HMBC correlation (Table 4.12, Figure 4.12), and NOESY correlation (Table 4.12, Figure 4.13) were used to assist the interpretation of compound 2 structure.

Table 4.11 The HSQC spectral data of compound 2

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
181	-
168	-
166.8	-
165	-
153.5	-
146	-
135.5	-
121	6.75 (1H, s, 1-H)
116.5	6.32 (1H, s, 4-H)
104.5	6.80 (1H, s, 10-H)
103.8	6.68 (1H, s, 8-H)
100	-
79.8	-
56	3.98 (3H, s, 9-OMe)
30	1.78 (3H, s, 4a-Me)

Table 4.12 The HSQC, HMBC and COSY spectral data of compound 2

Position	δ_C	δ_H	HMBC	NOESY
1	121	6.75 (1H, s, 1-H)	C-3 , C-10a	-
1a	153.5	-	-	-
2	181	-	-	-
3	146	-	-	-
4	116.5	6.32 (1H, s, 4-H)	C-4a-Me , C-2 , C-1a, C-3a ,C-10a	H-4a-Me
4a	79.8	-	-	-
6	168	-	-	-
6a	100	-	-	-
7	165	-	-	-
8	103.8	6.68 (1H, s, 8-H)	C-9 , C-7 , C-10 , C-6a	H-9-OMe
9	166.8	-	-	-
10	104.5	6.80 (1H, s, 10-H)	C-9 , C-1a , C-8 , C-6a	H-9-OMe
10a	135.5	-	-	-
4a-Me	30	1.78 (3H, s, 4a-Me)	C-1a , C-4	H-4
9-OMe	56	3.98 (3H, s, 9-OMe)	C-9	H-10, H-8

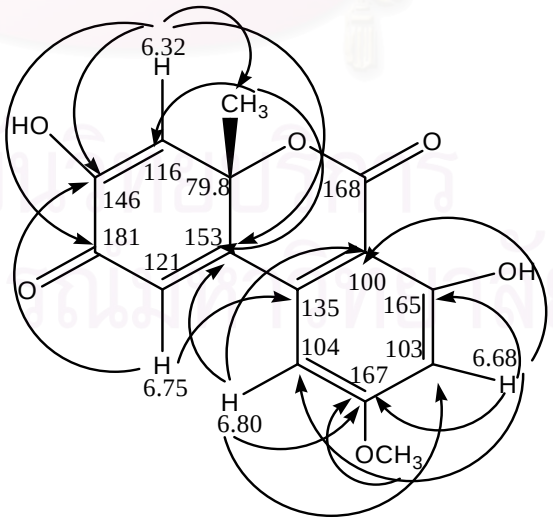


Figure 4.12 HMBC correlation of compound 2

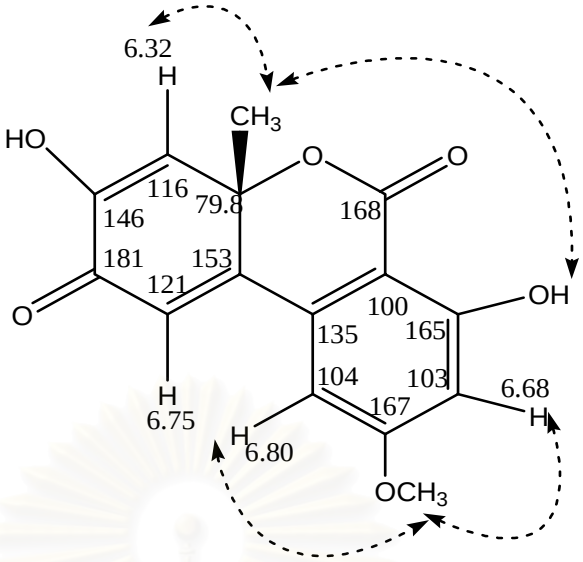


Figure 4.13 NOESY correlation of compound 2

Compound 2 showed spectral data identical to that of dehydroaltenusin, (γ-lactone derivative of β-resorcylic acid monomethyl ether), which was reported in the literature (Kamisuki et al., 2004) The ^{1}H-NMR and ^{13}C-NMR signals of compound 2 dehydroaltenusin are presented in Table 4.13

Table 4.13 The ^{1}H-NMR and ^{13}C-NMR spectral data of compound 2 (in $CDCl_3$) and dehydroaltenusin (in $CDCl_3$)

Position	δ_H (ppm)		δ_C (ppm)	
	Compound2	dehydroaltenusin	Compound2	dehydroaltenusin
1	6.75 (1H, s, 1-H)	6.69 (1H, s)	121	120.7
1a	-	-	153.5	152.9
2	-	-	181	180.6
3	-	-	146	145.9
4	6.32 (1H, s, 4-H)	6.28 (1H, s)	116.5	116.1

Table4.13 (continues)

4a	-	-	79.8	79.1
6	-	-	168	167.2
6a	-	-	100	99.8
7	-	-	165	164.5
8	6.68 (1H, d, 8-H)	6.63 (1H,d,*J*=2.4Hz)	103.8	103.6
9	-	-	166.8	166.2
10	6.80 (1H, d,10-H)	6.73 (1H,d,*J*=2.4Hz)	104.5	104.3
10a	-	-	135.5	134.9
4a-Me	1.78 (3H, s, 4a-Me)	1.73 (3H, s, Me)	30	29.6
9-OMe	3.98 (3H, s, 9-OMe)	3.91 (3H, s, OMe)	56	56.0

After elucidation of compound 2 by 2D NMR technigue, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of compound 2 and dehydroaltenusin were compared signal by signal. This result indicated that the structure of compound 2 is identical dehydroaltenusin. The structure was presented in Figure 4.14

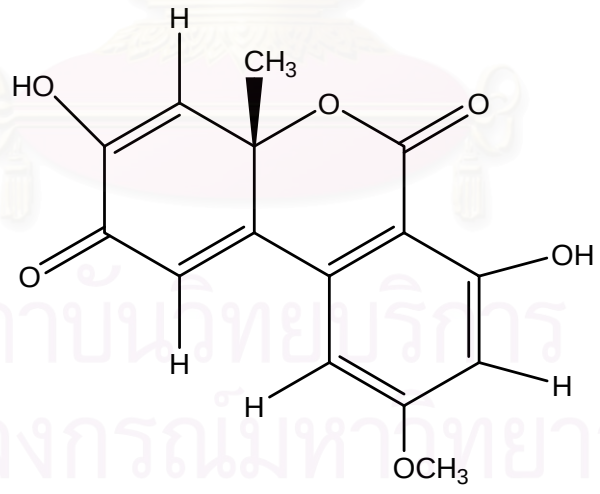


Figure 4.14 The structure of compound 2.

4.7.3 Structure elucidation of compound 3

Compound 3 was obtained from the combined fraction B07 of culture broth ethyl acetate crude extract, eluted with Hexane:$CHCl_3$ (60:40 → 50:50). Compound 3 was an white solid, m.p. 210-212 ^{o}C. The structure of compound 3 was elucidated using spectroscopic techniques. The IR spectrum of compound 3 is shown in Appendix C Figure C12 and the absorption peaks were assigned as table 4.14 and indicated important absorption band at 3322 cm^{-1}(O-H stretching vibration of alcohol) and strong absorption band at 1660 and 1618 cm^{-1} (C=O vibration of carbonyl group) due to the carbonyl stretching.

Table 4.14 The IR absorption bands assignment of compound 3

Wave number (cm^{-1})	Intensity	Tentative assignment
3322	Broad	O-H stretching vibration of alcohol
1660 , 1618	Strong	C=O vibration of carbonyl group
1287	Medium	C=C vibration of double bond
1170	Medium	C-O stretching vibration
1081	Weak	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure C13 in Appendix C) of compound 3 possessed two methyl group at 2.79(s) and 3.95(s) ppm. Four methine groups at 6.57(d), 6.64(d), 6.73(d) and 7.30(d) ppm.

The ^{13}C-NMR spectrum (Figure C14 in Appendix C) of compound 3 showed 15 signals which the carbonyl group, methoxy group corresponded to the signal at 54.94 ppm and methyl group corresponded to the signal at 24.3 ppm.

The EI-MS spectrum (*m/z*) (Figure C15 in Appendix C) showed the ion peak at *m/z* 272. The mass spectrum indicated that it possesses the molecular weight 272. If it is assumed that this compound contains protons, carbons and oxygen, then the molecular formular of $C_{15}H_{12}O_5$ can be established.

The information from 2D-NMR techniques, including HSQC correlation (Table 4.15 and Table 4.16), HMBC correlation (Table 4.16, Figure 4.15) were used to assist the interpretation of compound 3 structure.

Table 4.15 The HSQC spectral data of compound 3

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
166.5	-
164.9	-
164	-
138.2	-
117.3	6.736.57 (1H,d,*J*=2Hz)
110	-
103.5	7.306.57 (1H,d,*J*=2Hz)
101.4	6.64 (1H,d,*J*=2.8Hz)
98.6	6.576.57 (1H,d,*J*=2Hz)
54.94	3.95 (3H,s)
29.3	1.32 (2H,s)
24.3	2.79 (3H,s)

Table 4.16 The HSQC, HMBC and COSY spectral data of compound 3

Position	δ_C	δ_H	HMBC
1	*	-	-
1a	101.4	6.64 (1H,d,*J*=2.8Hz)	H-8a,H-6
2	98.6	6.57 (1H,d,*J*=2Hz)	H-2,H-3,H-1a
3	166.5	-	-
4	*	-	-
4a	164.0	-	-
5	117.3	6.73 (1H,d,*J*=2Hz)	H-3
5a	*	-	-
6	164.9	-	-
7	103.5	7.30 (1H,d,*J*=2Hz)	H-8a
8	138.2	-	-
8a	110	-	-
9	*	-	-
3-OMe	54.94	3.95 (3H,s)	H-2,H-3
8-Me	24.3	2.79 (3H,s)	H-8,H-8a,H-7,H-5

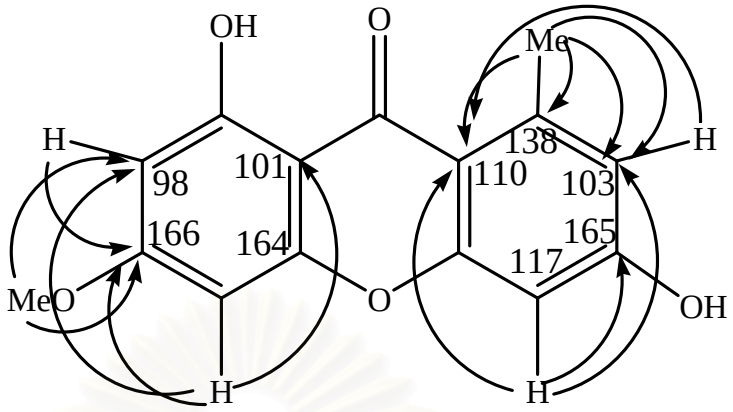


Figure 4.15 HMBC correlation of compound 3

Compound 2 showed spectral data identical to that of 1,6-dihydroxy-3-methoxy-8-methyl-*9H*-xanthen-9-one. After elucidation of compound 3 by 2D NMR technigue, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of compound 3 and this result indicated that the structure of compound 3 is identical 1,6-dihydroxy-3-methoxy-8-methyl-*9H*-xanthen-9-one. The structure was presented in Figure 4.16

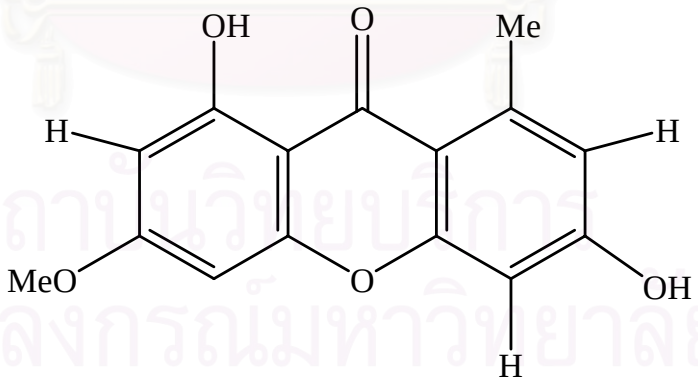


Figure 4.16 The structure of compound 3

4.7.4 Structure elucidation of mixture 4

Mixture 4 was obtained from the combined fraction M04 of mycelium ethyl acetate crude extract, eluted with Hexane:$CHCl_3$(90:10) . Mixture 4 as yellow liquid (1.2235g). Chemical structures of these mixture were determined by analyzes of GC; Gas chromatography, as well as by comparison their spectral data with those of published values.

The IR spectrum of mixture 4 is shown in Appendix C Figure C18 and the absorption peaks were assigned as table 4.17 and indicated important absorption band at 3474 cm^{-1} (O-H stretching vibration of alcohol),strong absorption band at 2925 and 2851 cm^{-1} (C-H stretching vibration of CH_3,CH_2) and strong absorption band at 1738 cm^{-1} (C=O stretching vibration of carbonyl group) due to the carbonyl stretching.

Table 4.17 The IR absorption bands assignment of mixture 4

Wave number (cm^{-1})	Intensity	Tentative assignment
3474	Broad	O-H stretching vibration of alcohol
2925, 2851	Sharp, Strong	C-H stretching vibration of CH_3,CH_2
1738	Sharp, Strong	C=O stretching vibration of carbonyl group
1462	Sharp, Medium	C=C stretching vibration of olefinic
1158	Medium	C-O stretching vibration
726	Weak	C-H rocking vibration of $-(CH_2)_n-$

The ^{1}H-NMR spectrum (Figure C19 in Appendix C) of mixture 4 possessed methyl, methylene and methine groups.

The ^{13}C-NMR spectrum (Figure C20 in Appendix C) of mixture 4 showed 22 signals, which the carboxylic group and methyl group.

The structure of mixture 4 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 and the chemical structure and chemical name of fatty acids in mixture 4 (Table 4.18 and Table 4.19) (Figure C24 in appendix C).

Table 4.18 GC Retention time of mixture 4 and standard methyl esters (Std.-ME) of those fatty acids.

Fatty acid	Retention time(min)		Fatty acids composition (%) of total fatty acids
	Std.-ME	Mixture M 4	
C14:0	5.697	5.382	0.59
C16:0	10.295	9.768	66.71
C16:1	11.292	11.442	0.54
C18:0	18.999	18.003	14.09
C18:1	21.601	20.538	16.04
Other	-	-	2.03

Table 4.19 The chemical structure and chemical name of fatty acids in mixture 4 (Ritter, 1996)

Systematic Name	Common name	Symbol	Structure
SATURATED			
Tetradecanoic acid	Myristic acid	C14:0	$CH_3(CH_2)_{12}COOH$
Hexadecanoic acid	Palmitic acid	C16:0	$CH_3(CH_2)_{14}COOH$
Octadecanoic acid	Stearic acid	C18:0	$CH_3(CH_2)_{16}COOH$
UNSATURATED			
9-Hexadecenoic acid	Palmitoleic acid	C16:1	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
9-Octadecenoic acid	Oleic acid	C18:1	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$

The GC data revealed that mixture 4 consist of three saturated fatty acids including, myristic acid, palmitic acid, and stearic acid and two unsaturated fatty acids including, palmitoleic acid, oleic acid .

4.7.5 Structure elucidation of mixture 5

Mixture 5 was obtained from the combined fraction M07 of of mycelium ethyl acetate crude extract, eluted with Hexane:$CHCl_3$(75:25 → 70:30). Mixture 5 was an yellow solid, m.p. 263-265 ^{o}C. The structure of mixture 5 was elucidated using spectroscopic techniques. The IR spectrum of mixture 5 is shown in Appendix C Figure C21 and the absorption peaks were assigned as table 4.20 and indicated important absorption band at 3392 cm^{-1}(OH stretching) and strong absorption band at 1567 cm^{-1} (C=C vibration of double bond) due to the carbonyl stretching.

Table 4.20 The IR absorption bands assignment of mixture 5

Wave number (cm^{-1})	Intensity	Tentative assignment
3392	Broad	OH stretching vibration
2918, 2840	Weak	C-H stretching vibration
1750	weak	C=O stretching vibration of carbonyl group
1567	Strong	C=C vibration of double bond
1330	Strong	C=C stretching vibration
1225	Medium	C-O stretching vibration
1162	Medium	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure C22 in Appendix C) of mixture 5 possessed two methyl groups at 2.282 and 2.743, two methoxy groups at 56.77 and 56.31 ,eight methine groups at 7.915, 6.736, 7.551, 7.153(d, J = 2.4 H_z), 6.828(d, J = 2.4 H_z), 7.23, 6.66 and 6.30 ppm.

The ^{13}C-NMR spectrum (Figure C23 in Appendix C) of mixture 5 showed 30 signals, which the carbonyl group corresponded to the signal at 181 and 186.98 ppm.

MALDI-TOF MS spectrum (*m/z*): $[M+H]^{+}$ (Figure C24 in Appendix C) showed the ion peak at 285. The mass spectrum indicated that it possesses the

molecular weight 284. If it is assumed that this compound contains protons, carbons and oxygens.

The information from 2D-NMR techniques, including HSQC correlation (Table 4.21 and Table 4.22), HMBC correlation (Table 4.2/, Figure 4.17), COSY correlation and NOESY correlation (Table 4.22, Figure 4.18) were used to assist the interpretation of mixture 5 structure.

Table 4.21 The HSQC spectral data of mixture 5

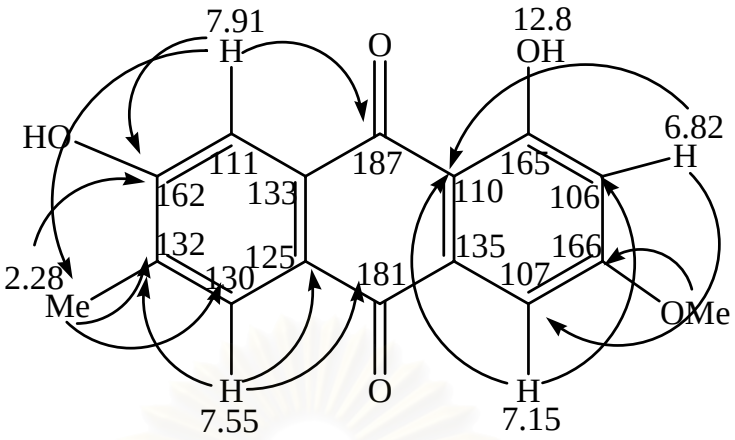
^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
186.98	-
181	-
166.39	-
165	-
162.05	-
135.47	-
133.50	-
132.53	-
130.59	7.915 (s)
125.33	-
111.49	7.551 (s)
110.67	-
107.57	7.153 (d, *J* = 2.4 Hz)
106.15	6.828 (d, *J* = 2.4 Hz)
56.31	3.92 (s)
16.65	2.282 (s)

Table 4.21 (continues)

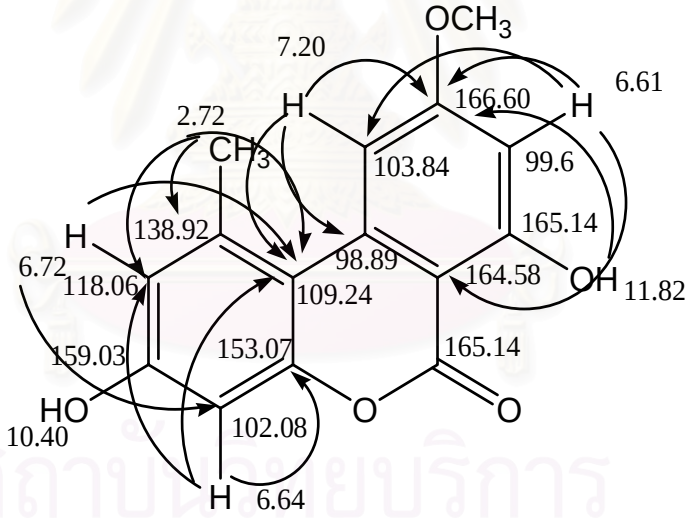
^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
166.6(s)	-
165.14(s)	-
164.58(s)	-
159.03(s)	10.41 (1H, s, 3-OH)
153.07(s)	-
138.92(s)	-
138.22(s)	11.82 (1H, s, 7-OH)
118.06(d)	6.72 (1H, s, 2-H)
109.24(s)	-
103.84(d)	7.20 (1H, s, 10-H)
102.08(d)	6.64 (1H, s, 4-H)
99.60(d)	6.61 (1H, d, *J*=2.4Hz, 8-H)
98.89(s)	-
56.29(q)	3.90 (3H, s, 9-OMe)
25.48(q)	2.72 (3H, s, 1-Me)

Table 4.22 The HSQC, HMBC and COSY spectral data of mixture 5

Position	δ_C	δ_H	HMBC	NOESY
1	111.49	7.551 (s)	C-4a, C-3, C-10	-
1a	133.50	-	-	-
2	162.05	-	-	-
3	132.53	-	-	-
4	130.59	7.915 (s)	C-9, C-2, C-3Me	-
4a	125.33	-	-	-
5	107.57	7.153 (d, *J* = 2.4 Hz)	C-7, C-8a	H-6-OMe
5a	135.47	-	-	-
6	166.39	-	-	-
7	106.1	6.828 (d, *J* = 2.4 Hz)	C-8a, C-5	-
8	165	-	-	-
8a	110.67	-	-	-
9	186.98	-	-	-
10	181	-	-	-
3-Me	16.65	2.282 (s)	C-4, C-3, C-2	-
6-OMe	56.31	3.92 (s)	C-6	H-5

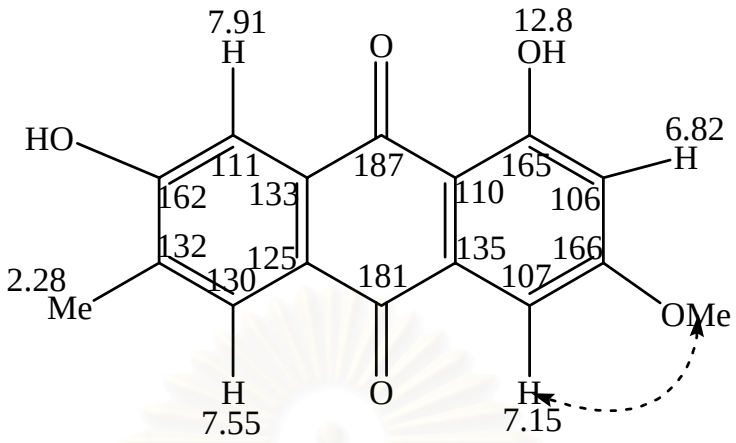


Macrosporin

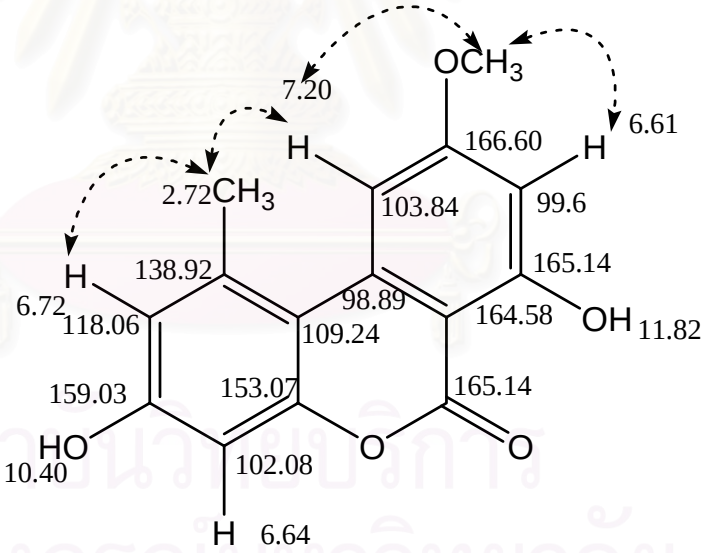


Alternariol-9-methyl ether

Figure 4.17 HMBC correlation of mixture 5



Macrosporin



Alternariol-9-methyl ether

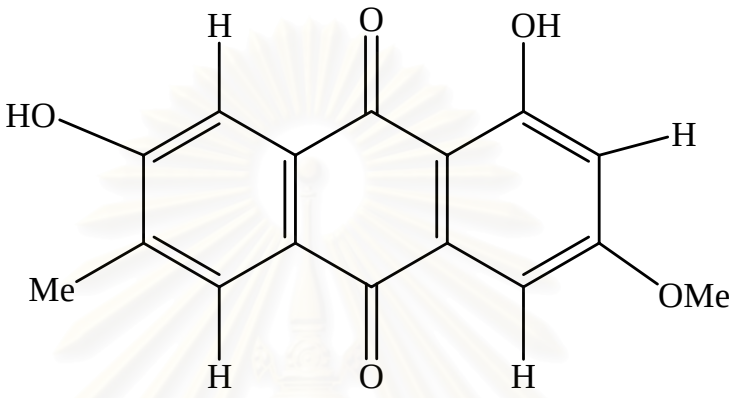
Figure 4.18 NOESY correlation of mixture 5

Mixture 5 showed spectral data identical to that of macrosporin (1,7-dihydroxy-3-methoxy-6-methyl-anthraquinone), which was reported in the literature (Suemitsu, R. et al., 1989) and alternariol-9-methyl ether (compound 6), (3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo-[*b,d*]pyran-6-one), which was reported in the literature (An et al., 1989). The ^{1}H-NMR and ^{13}C-NMR signals of mixture 5, macrosporin are presented in Table 4.23

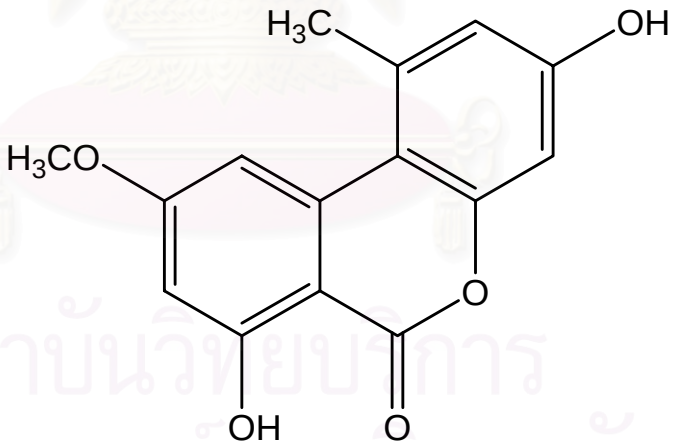
Table 4.23 The ^{1}H-NMR and ^{13}C-NMR spectral data of mixture 5 (in $CDCl_3$) and macrosporin

Position	δ_H (ppm)		δ_C (ppm)	
	Mixture 5	Macrosporin	Mixture 5	Macrosporin
1	7.551 (s)	7.51 (s)	111.49	111.6
1a	-	-	133.50	134.6
2	-	-	162.05	162.3
3	-	-	132.53	133.0
4	7.915 (s)	8.10 (s)	130.59	131.1
4a	-	-	125.33	126.9
5	7.153 (d, J = 2.4 H_z)	7.27 (d, J=2.2Hz)	107.57	108.0
5a	-	-	135.47	136.5
6	-	-	166.39	167.4
7	6.828 (d, J = 2.4 H_z)	6.70 (d, J=2.2Hz)	106.1	106.1
8	-	-	165	166.4
8a	-	-	110.67	111.5
9	-	-	186.98	187.9
10	-	-	181	181.2
3-Me	2.282 (s)	2.32 (s)	16.65	16.4
6-OMe	3.92 (s)	3.91 (s)	56.31	56.4

After elucidation of mixture 5 by 2D NMR technigue, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of mixture 5, macrosporin and alternariol-9-methyl ether were compared signal by signal. This result indicated that the structure of mixture 5 is identical to macrosporin and alternariol-9-methyl ether (compound 6). The structure was presented in Figure 4.19



Macrosporin



Alternariol-9-methyl ether

Figure 4.19 The structure of mixture 5

4.7.6 Structure elucidation of compound 6

Compound 6 was obtained from the combined fraction M10 mycelium ethyl acetate crude extract, eluted with Hexane:$CHCl_3$(65:35 → 60:40).Compound 6 was a brown solid, m.p. 272-275 ^{o}C. The structure of compound 6 was elucidated using spectroscopic techniques. The IR spectrum of compound 6 is shown in Appendix C Figure C26 and the absorption peaks were assigned as table 4.24 and indicated important absorption band at 3357 cm^{-1} (O-H stretching vibration of alcohol) and strong absorption band at 1660 and 1610 cm^{-1} (C=O vibration of carbonyl group) due to the carbonyl stretching.

Table 4.24 The IR absorption bands assignment of compound 6

Wave number (cm^{-1})	Intensity	Tentative assignment
3357	Broad	O-H stretching vibration of alcohol
1660, 1610	Sharp, Strong	C=O stretching vibration of carbonyl group
1462	Sharp, Strong	C=C stretching vibration of aromatic ring
1228	Medium	C-O stretching vibration
1158	Medium	C-O stretching vibration
1096	Weak	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure C27 in Appendix C) of compound 6 possessed two methyl groups at 2.72 and 3.90 ppm, Four methine groups at 6.61, 6.64, 6.72 and 7.20 ppm.

The ^{13}C-NMR spectrum (Figure C28 in Appendix C) of compound 6 showed 15 signals, which the carbonyl group corresponded to the signal at 165.14 ppm.

MALDI-TOF MS spectrum (*m/z*): $[M+H]^{+}$ (Figure C29 in Appendix C) showed the ion peak at *m/z* 273.04. The mass spectrum indicated that it possesses the molecular weight 272. If it is assumed that this compound contains protons, carbons and oxygens, then the molecular formular of $C_{15}H_{12}O_5$ can be established.

The information from 2D-NMR techniques, including HSQC correlation (Table 4.25 and Table 4.26), HMBC correlation (Table 4.26, Figure 4.20), and NOESY correlation (Table4.26, Figure 4.21) were used to assist the interpretation of compound 6 structure.

Table 4.25 The HSQC spectral data of compound 6

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
166.6(s)	-
165.14(s)	-
164.58(s)	-
159.03(s)	10.41 (1H, s, 3-OH)
153.07(s)	-
138.92(s)	-
138.22(s)	11.82 (1H, s, 7-OH)
118.06(d)	6.72 (1H, s, 2-H)
109.24(s)	-
103.84(d)	7.20 (1H, s, 10-H)
102.08(d)	6.64 (1H, s, 4-H)
99.60(d)	6.61 (1H, d, *J*=2.4Hz, 8-H)
98.89(s)	-
56.29(q)	3.90 (3H, s, 9-OMe)
25.48(q)	2.72 (3H, s, 1-Me)

Table 4.26 The HSQC, HMBC and COSY spectral data of compound 6

Position	δ_C	δ_H	HMBC	NOESY
1	138.92(s)	-	-	-
1a	109.24(s)	-	-	-
2	118.06(d)	6.72 (1H, s, 2-H)	C-1a , C-4	H-1-Me
3	159.03(s)	10.41 (1H, s, 3-OH)	-	-
4	102.08(d)	6.64 (1H, s, 4-H)	C-2 , C-1a , C-4a	-
4a	153.07(s)	-	-	-
6(C=O)	165.14(s)	-	-	-
6a	164.58(s)	-	-	-
7	138.22(s)	11.82 (1H, s, 7-OH)	C-9	-
8	99.60(d)	6.61 (1H, d, *J*=Hz, 8-H)	C-6a,C-9,C-10,C-10a	H-9-OMe
9	166.6(s)	-	-	-
10	103.84(d)	7.20 (1H, s, 10-H)	C-1a , C-10a , C-9a	H-9-OMe,H-1-Me
10a	98.89(s)	-	-	-
1-Me	25.48(q)	2.72 (3H, s, 1-Me)	C-1 , C-2 , C-1a	H-10 , H-2
9-OMe	56.29(q)	3.90 (3H, s, 9-OMe)	-	H-10 , H-8

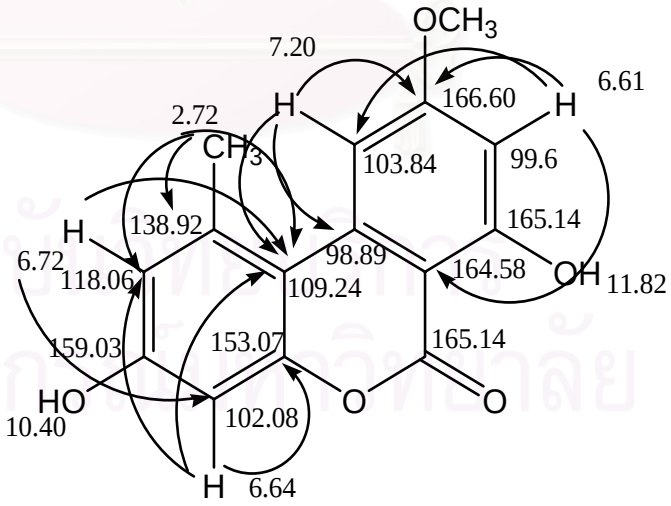


Figure 4.20 HMBC correlation of compound 6

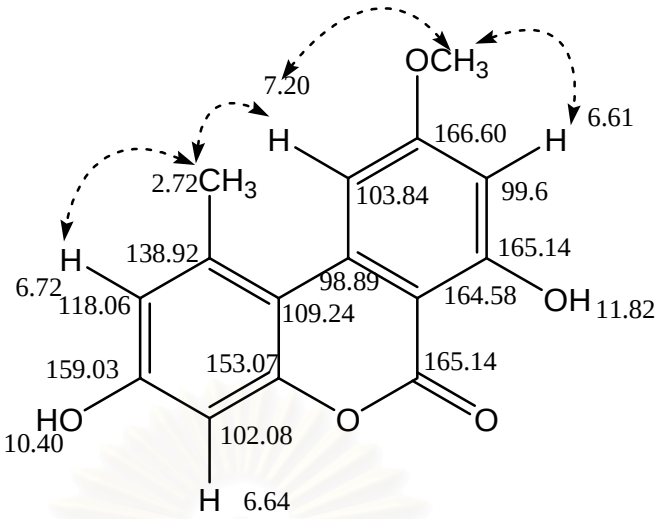


Figure 4.21 NOESY correlation of compound 6

Compound 6 showed spectral data identical to that of alternariol-9-methyl ether, (3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo-[*b,d*]pyran-6-one), which was reported in the literature (An et al., 1989) The ^{1}H-NMR and ^{13}C-NMR signals of compound 6 and alternariol-9-methyl ether are presented in Table 4.27

Table 4.27 The ^{1}H-NMR and ^{13}C-NMR spectral data of compound 6 (in DMSO) and alternariol-9-methyl ether (in $CDCl_3$ ^{1}H-NMR, 200 MH_z and ^{13}C-NMR, 50.3 MH_z)

Position	δ_H (ppm)		δ_C (ppm)	
	Compound6	AME	Compound6	AME
1	-	-	138.92(s)	138.2
1a	-	-	109.24(s)	109.6(s)
2	6.72 (1H, s, 2-H)	6.5 (2H, d, *J*=2 Hz, 2-H)	118.06(d)	117.5(d)
3	10.41 (1H, s, 3-OH)	9.6 (OH)	159.03(s)	157.9(s)
4	6.64 (1H, s, 4-H)	*	102.08(d)	101.8(d)

Table4.27 (continues)

4a	-	-	153.07(s)	152.8(s)
6(C=O)	-	-	165.14(s)	164.3(s)
6a	-	-	164.58(s)	161.3(s)
7	11.82 (1H, s, 7-OH)	12.0 (OH)	138.22(s)	138.1(s)
8	6.61 (1H, d, *J*=2.4Hz, 8-H)	6.3 (1H, d, *J*=2 Hz, 8-H)	99.60(d)	101.8(d)
9	-	-	166.6(s)	165.5(s)
10	7.20 (1H, s, 10-H)	7.1 (d, *J*=2 Hz, 10-H)	103.84(d)	104(d)
10a	-	-	98.89(s)	98.5(s)
1-Me	2.72 (3H, s, 1-Me)	2.62 (3H, s, CH_3)	25.48(q)	25.23(q)
9-OMe	3.90 (3H, s, 9-OMe)	3.79 (3H, s, OCH_3)	56.29(q)	55.4(s)

After elucidation of compound 6 by 2D NMR technigue, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of compound 6 and alternariol-9-methyl ether were compared signal by signal. This result indicated that the structure of compound 6 is identical to alternariol-9-methyl ether. The structure was presented in Figure 4.22

Figure 4.22 The structure of compound 6

4.8 Biological activity

4.8.1 Antimicrobial activity of the culture broth of endophytic fungus isolate KPCH007

The antimicrobial activity of the culture broth of endophytic fungus isolates KPCH 007 was evaluated by the agar well diffusion method. Aliquote of 100 ul of culture filtrate was pipetted into the agar wells. The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganism. Results indicated that culture broth was active against *B. subtilis* ATCC 6633 that shown in Figure 4.23

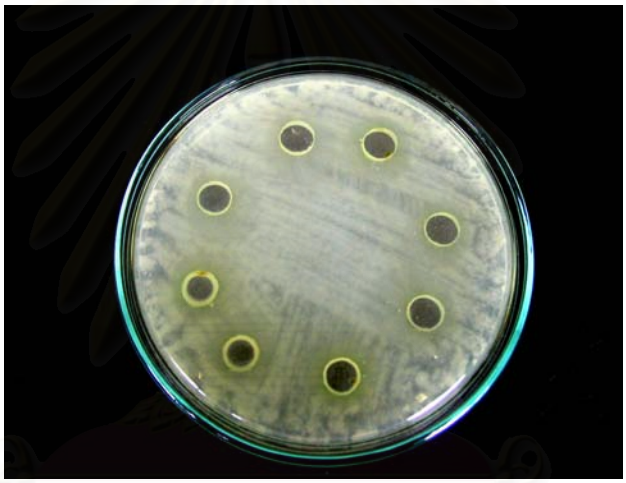


Figure 4.23 Antimicrobial activities of EtoAc of culture broth of endophytic fungal isolate KPCH007 against *B. subtilis* ATCC 6633.

4.8.2 Antimicrobial activity of the crude extracts from endophytic fungus isolate KPCH 007

The antimicrobial activity of the crude extracts from endophytic fungus isolate KPCH007 was evaluated by the disc diffusion method. Both crude extract were examined at a concentration of 5 and 10 mg/ml and dissolved in 10% DMSO in sterile distilled water and drop solution of both crude extract on paper disc (0.5 and 1 mg/disc, diameter 6 mm.). The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganisms, including the bacterial strains *B. subtilis, S. aureus, E.coli, P. aeruginosa*, and fungi, yeast form strains *C. albican*s and *S.*

cerevisiae. Antimicrobial activity of the crude extracts is shown in Figure 4.24 and Table 4.28

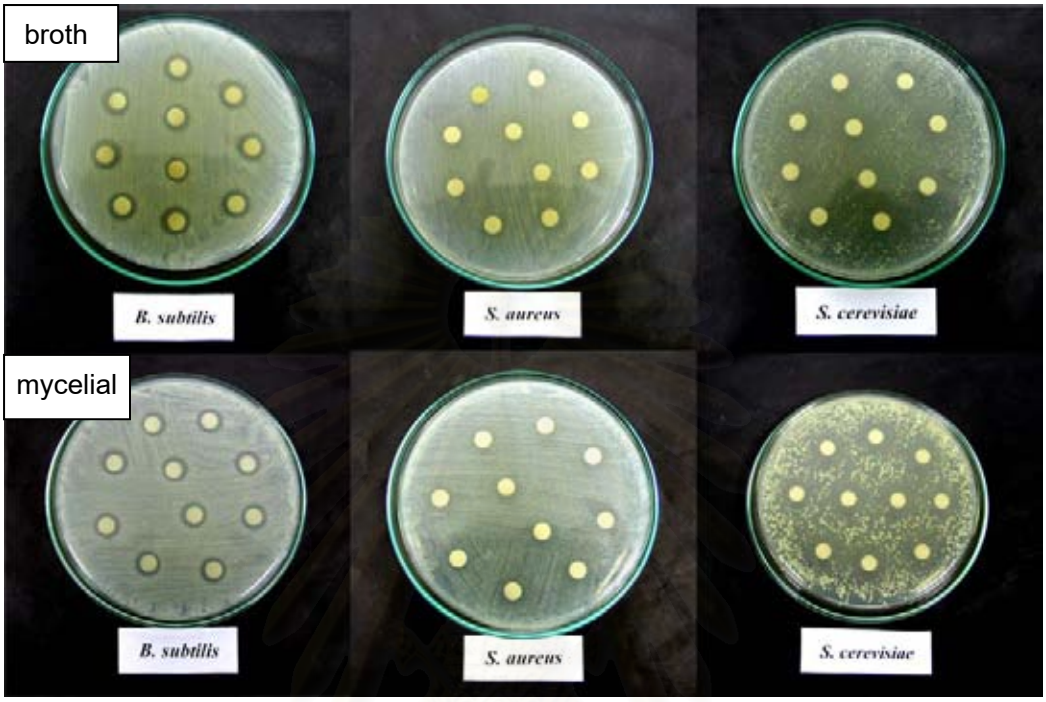


Figure 4.24 Antimicrobial activities of EtoAc crude of culture broth and mycelial extract of endophytic fungal isolate KPCH007 against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 and *S. cerevisiae* .

Table 4.28 Antimicrobial activities of the crude extracts from endophytic fungus isolate KPCH 007

Test microorganisms	Crude extract			
	Culture broth		Mycelium	
	5 mg/ml	10 mg/ml	5 mg/ml	10 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

+++ = Inhibition zone more than 10 -15 mm

++ = Inhibition zone more than 5 -10 mm

+ = less than 5 mm

- = No inhibition

4.8.3 Antimicrobial activities of the fractions from crude extracts

The animicrobial activity of the fractions from crude extracts was evaluated by the agar well diffusion method. The fractions were examined at a concentration of 1000 μg/ml (0.1 mg/well ; 8 mm diameter). The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganisms, including the bacterial strains *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and fungi, yeast form strains *C. albican* ATCC 10231. Antimicrobial activity of the fractions from crude extracts is shown in Table 4.29 and Table 4.30

Table 4.29 Antimicrobial activity of the fractions of EtOAc crude from culture broth extract

Combined fraction	Inhibition zone diameter (mm)				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
B01	-	-	-	-	-
B02	-	-	-	-	-
B03	++	++	+	+	-
B04	+	+	-	+	-
B05	+	+	-	+	-
B06	+	+	-	-	-
B07	+	+	-	+	-
B08	+	-	-	-	-
B09	+	+	-	-	-
B10	-	-	-	-	-
B11	+	-	-	-	-
B12	+	-	-	-	-
B13	-	-	-	-	-
B14	ND	ND	ND	ND	ND
B15	ND	ND	ND	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 10 mm; +, less than 10 mm; -, no inhibition; ND, not determined.

Table 4.30 Antimicrobial activity of the fractions of EtOAc crude from mycelia extract

Combined fraction	Inhibition zone diameter (mm) of tested microorganism				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
M01	-	-	-	-	-
M02	-	-	-	-	-
M03	-	-	-	-	-
M04	-	-	-	-	-
M05	-	-	-	-	-
M06	+	-	-	-	-
M07	+	+	-	-	-
M08	-	-	-	-	-
M09	+	-	-	-	-
M10	++	++	+	+	-
M11	-	-	-	-	-
M12	-	-	-	-	-
M13	+	-	-	+	-
M14	+	-	-	-	-
M15	+	-	-	-	-
M16	ND	ND	ND	ND	ND
M17	ND	ND	ND	ND	ND
M18	ND	ND	ND	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 10 mm; +, less than 10 mm; -, no inhibition; ND, not determined.

4.8.4 Antimicrobial activity of pure compounds

The animicrobial activity of pure compounds was evaluated by the antimicrobial susceptibility test, broth microdilution method. The pure compound was examined at a concentration of 31.25-1,000 µg/ml . Antimicrobial activity tests were performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169, and *C. albicans* ATCC 10231. The lowest concentration of pure compound showing complete inhibition of growth is recorded as the minimal inhibitory concentration (MIC) in Table 4.31

For filamentous fungi results were determined using the agar well diffusion method. The pure compound was examined at a concentration of 100 µg/well. Antimicrobial activity was tested against *Collectotichum glocospriodes*, *Alternaria brassicicola, Fusarium oxysporum,* and *Phytophthora palunivora*. Antimicrobial activity of isolated compounds is shown in Table 4.32

Table 4.31 Broth microdilution method for antimicrobial activity of pure compounds

Compounds	Testes microorganisms and MIC (µg/ml)					
	Gram positive bacteria		Gram negative bacteria		Yeasts	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231
Compound 1	31.25	31.25	62.50	62.50	ND	-
Mixture 5	125	250	250	250	ND	-
Compound 6	62.5	62.5	125	125	ND	250
Tetracycline HCl	31.3	0.8	1.0	ND	ND	ND
Amoxicillin	-	0.8	1.0	ND	ND	ND
Penicillin G	7.81	7.81	ND	ND	ND	ND
Streptomycin	ND	ND	0.98	3.90	ND	ND
Ketokonazole	ND	ND	ND	ND	ND	31.25

- = Inactive

ND = not determined

Table 4.32 Agar well diffusion method for antimicrobial activity of pure compounds

Compounds	Concentration (µg/well)	Test microorganisms and inhibiton zones (mm)			
		Filamentous fungi (Plant phatogenic fungi)			
		A . brassicicola	*C. glocospriodes*	*F. oxysporum*	*P. palunivora*
compound1	100	-	-	-	-
Mixture 5	100	-	-	-	-
compound6	100	-	-	-	-

- = Inactive, + = active, (+) = Static, ND = not determined

4.8.5 Cytotoxic activity

The *in vitro* cytotoxic activity of some compounds from fungal isolate 007 was tested against 5 cell lines including, HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) is reported in Table 4.33 as the minimum concentration of 50% inhibitory activity (IC_{50})

Table 4.33 Cytotoxic activities against cell line of pure compounds from endophytic fungus isolate 007

Compounds	IC_{50} (µg/ml)				
	HEP-G2 (hepatoma)	SW620 (colon)	CHAGO (lung)	KATO-III (gastric)	BT474 (breast)
Compound1	>10	>10	>10	>10	8.4
Compound3	>10	7.4	>10	>10	>10
Mixture 5	>10	6.4	>10	10	>10
Compound6	10	6.4	8.8	7.9	10
Adriblastima	6.6	5.8	4.2	6.4	4.8
Doxoroabicin	0.7	0.09	0.7	>10	>10

IC_{50} was the minimum concentration of 50% inhibitory activity.

- = Inactive, ND = not determined

From the data in Table 4.28, the compound 1, compound 3, mixture 5 and compound 6 showed activtity on cytotoxicity against 5 tumor cell lines.

4.9 Discussion

There is a need to search for new bioactive agents because health problems, which caused by various cancers and drug-resistant pathogens, are still a global problem. Endophytic fungi are a novel source of efficient medicinal compounds. In this research, endophytic fungi were isolated from one of Thai medicinal plants, *Kaempferia parviflora*.

Compound 1 (191.6 mg), was identified as 1-methyl-pyrrolidin-2-one which was reported in the literature (Kelleher et al., 2002), suggested as a skin penetration enhancer for used in transdermal therapy in humans. In addition , it was an industrial solvent used extensively in chemical processing as an intermediate in the pharmaceutical industry and a vehicle in the cosmetics industry.

Compound 2 (13 mg), was identified as dehydroaltenusin which was report in the literature(Kamisuki et al., 2004), First discovered from mycelium extracts of *Alternaria tennuis* and *A.kikuchiana,* then this compound has been found from a variety of fungi. In addition to, it was a natural enzyme inhibitor of mammalian DNA polymerase α in vitro but did not influence the activities of the other replicative DNA polymerase and induced apoptosis of the cells.

Compound 3 (11 mg), was identified as 1,6-dihydroxy-3-methoxy-8-methyl-*9H*-xanthen-9-one which was a xanthones derivative and this compounds were known from natural sources although not from higher plants.

Mixture 4 (1.22 g), was identified as triglyceride that produced from mycelia. The structure of mixture 4 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 and the chemical structure and chemical name of fatty acids.

Mixture 5 (15 mg), was identified as macrosporin and alternariol-9-methyl ether which was report in the literature(Suemitsu et al., 1989), Macrosporin was a ,metabolite pigment of the important phytopathogenic fungi from *Alternaria porri, Alternaria solani, Alternaria bataticola*, whose host that are stone-leek, potato

and sweet potato.In addition to, it had been isolated from several fungi, isolated from ATCC12831 strain of *Dactylaria lutea.*

Compound 6 (72.8 mg), was identified as alternariol-9-methyl ether (AME) which was report in the literature(An et al., 1989), it was an *Alternaria* toxin that isolated from the media of *Alternaria alternate* and it appeared the mutagenic action of AME exhibited some selectivity toward specific genomic regions or DNA sequences. In addition to, as a strong mutagen in *Escherichia coli* strain ND-160 and weekly mutagenic to *Salmonella typhimurium* strain TA98.

CHAPTER V

CONCLUSION

Endophytic fungi were isolated by using the surface sterilization method. Thirty six endophytic fungi were isolated from mature leaves of the Thai medicinal plant, *Kaempferia parviflora*. Plant samples were collected from 3 provinces in Thailand; Bangkok, Chonburi and Patumthani.

Forty one percent of endophytic fungus isolates were found to have antimicrobial activities. By dual-culture agar diffusion assay, the culture media was found to be the one of factor affecting expression of antimicrobial activity. MEA was found to be the medium of choice for expression of antimicrobial activities.

Fungal isolate KPCH007 and malt extract broth was chosen for further study for bioactive compound because the malt extract agar blocks of the isolate KPCH007 were active against a number of test microorganisms such as *B. subtilis, S. aureus* and *P. aeruginosa.* Based on colony characteristic, microscopic characteristic and nucleotide sequencing analysis of the ITS region of this isolate was similar to 99.507% identity of *Alternaria tenuissima.*

In the present investigation, six metabolites from broth and mycelia of the fungal isolate KPCH007 comprising three compounds from broth and two compounds and one mixture from mycelia were isolated by using silica gel column chromatography. Compound 1 was 1-methyl-2-pyrrolidone. Compound 2 was dehydroaltenusin. Compound 3 was 1,6-dihydroxy-3-methoxy-8-methyl-9*H*-xanthen-9-one . From the mycelia ; Mixture 4 was a mixture of triglycerides. Mixture 5 was a mixture of macrosporin and alternariol-9-methyl ether and Compound 6 was alternariol-9-methyl ether. There structures were established on the basis of spectroscopic analysis including the ^{1}H-NMR, ^{13}C-NMR, IR, MS, UV and 2D NMR including gHMQC, gCOSY, gHMBC, NOESY and TOCSY spectra, and GC analysis.

For Antimicrobial activities, Compound 1 was found to exhibit activity against *B. subtilis , S. aureus, P. aeruginosa* and *E. coli.* with the MIC value of 31.25, 31.25, 62.50 and 62.50 μg/ml. Mixture 5 was found to exhibit activity against all four bacteria with the MIC value of 125, 250, 250, and 250 μg/ml. Compound 6 was found to exhibit activity against all four bacteria with the MIC value of 62.50, 62.50, 125, 125 μg/ml and inhibited *C. albicans* at 250 μg/ml., respectively. In addition, compound 1 can exhibited cytotoxic activities against SW620 (colon), BT474 (breast), KATO-3 (gastric), HEP-G2 (hepatoma) and CHAGO (lung) cell line with IC_{50} >10, >10, >10, >10, 8.4 μg/ml, Compound 3 with IC_{50} >10, 7.4, >10, >10, >10 μg/ml, Mixture 5 with IC_{50} >10, 6.4, >10, 10, >10 μg/ml and Compound 6 with IC_{50} 10, 6.4, 8.8, 7.9 and 10 μg/ml, respectively.

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APPENDICES

APPENDIX A

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi

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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

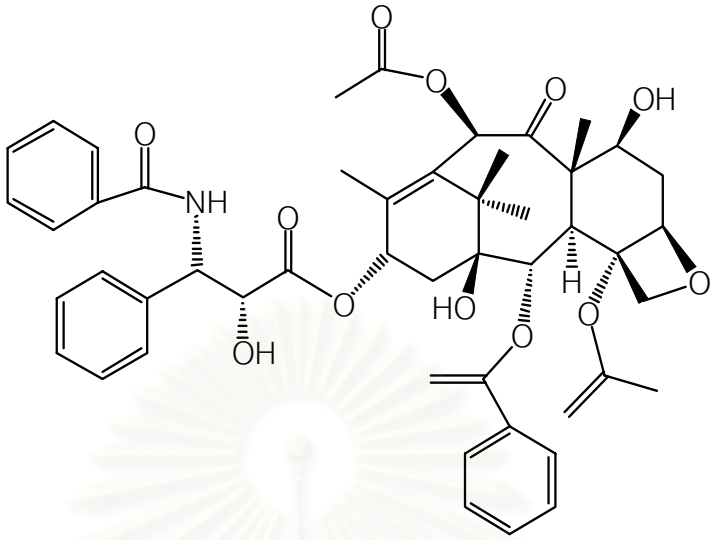
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
52	7-O-[α-L-Rhamnopyranosyl(1-6)-β-D-glucopy-ranosyl]tricin	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998
53	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxic	Berny *et al*., 1997
54	Leucinostatin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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

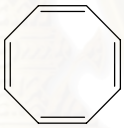
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
63	2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995
64	(+)-cryptosporiopsin				
65	4-epi-ethiosolide				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

Table A1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi (continued)

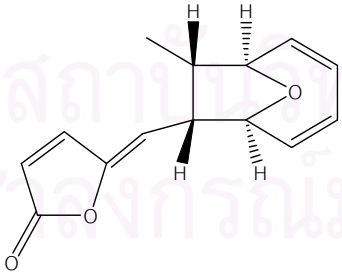
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
75	Lysergic acid	*Acremonium coenophialun*	*Festuca arundinacea*	Toxin	Garner *et al*., 1993
76	Isolysergic acid				
77	Pospalic acid				
78	Lysergol				
79	Lysergic acid amide				
80	Lysergic acid diethyl-amide				
81	Lycergic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



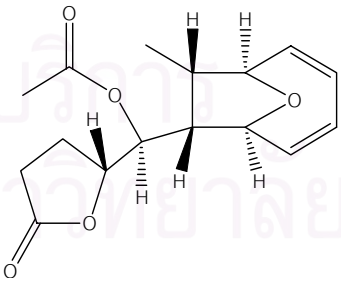
[1] Taxol



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

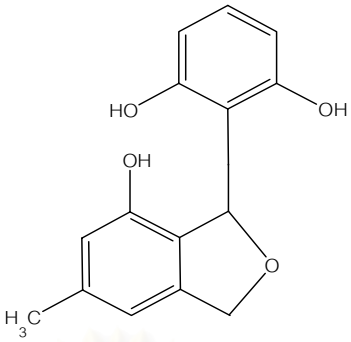


[3] Lactones 1893 A

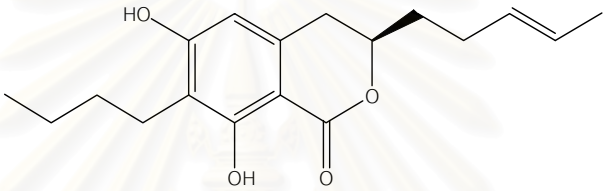


[4] Lactones 1893 B

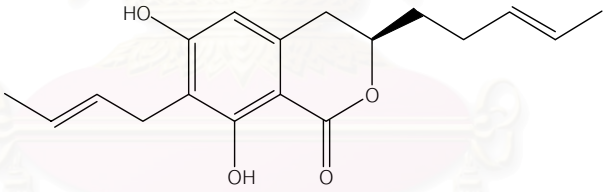
Figure A1 Structure of secondary metabolites of endophytic fungi



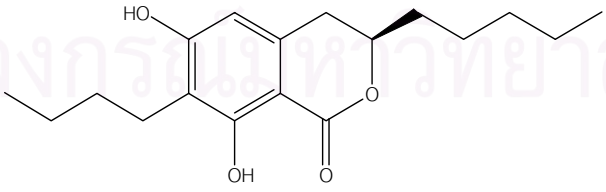
[5] Pestacin



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



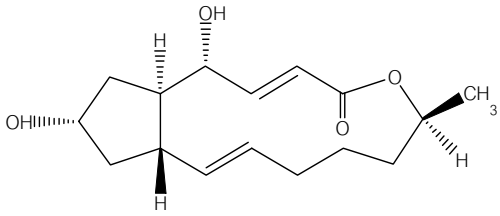
[7] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



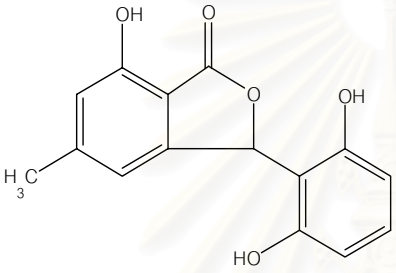
[8] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

Dihydroisocumarins [6-8]

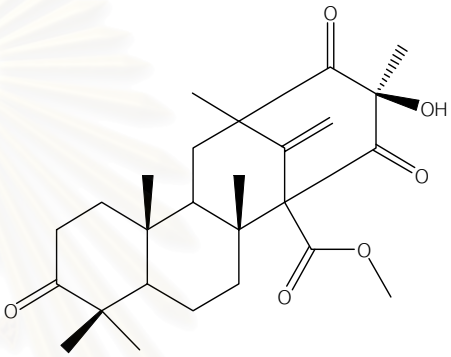
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



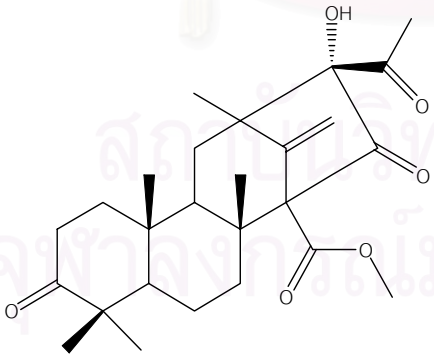
[9] Brefeldin A



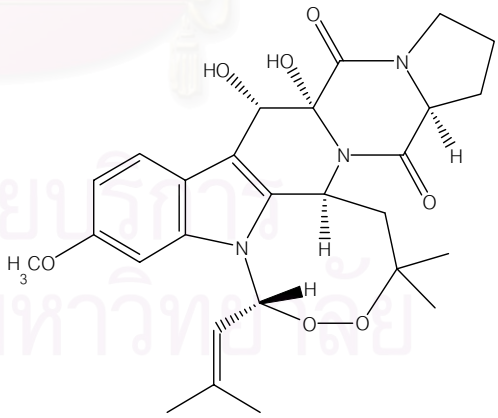
[10] Isopestacin



[11] Preaustinoid A

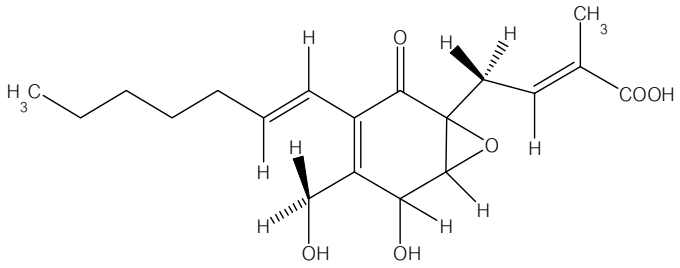


[12] Preaustinoid B

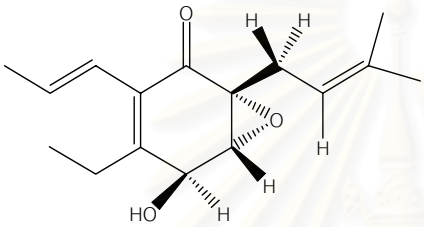


[13] Alkaloid verruculogen

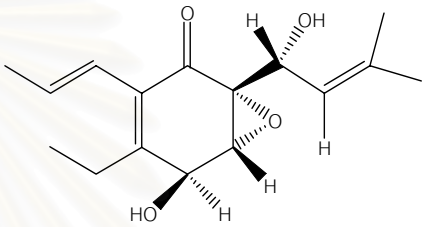
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



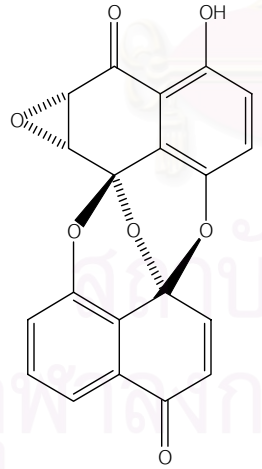
[14] Ambuic acid



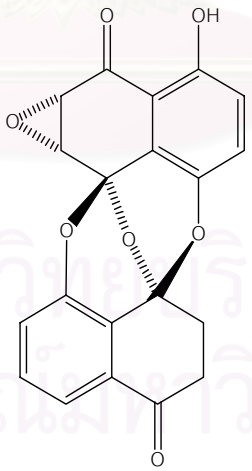
[15] Jesterone



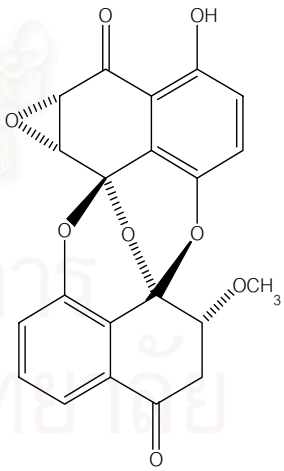
[16] Hydroxy-jesterone



[17] Preussomerin G

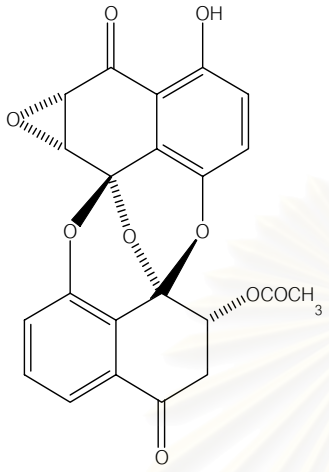


[18] Preussomerin H

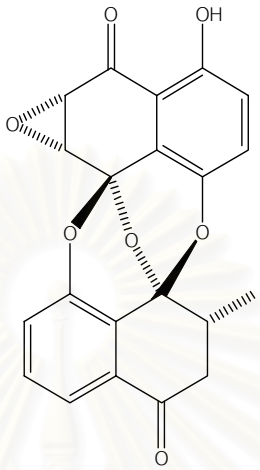


[19] Preussomerin I

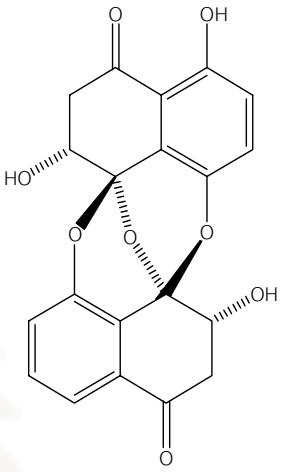
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



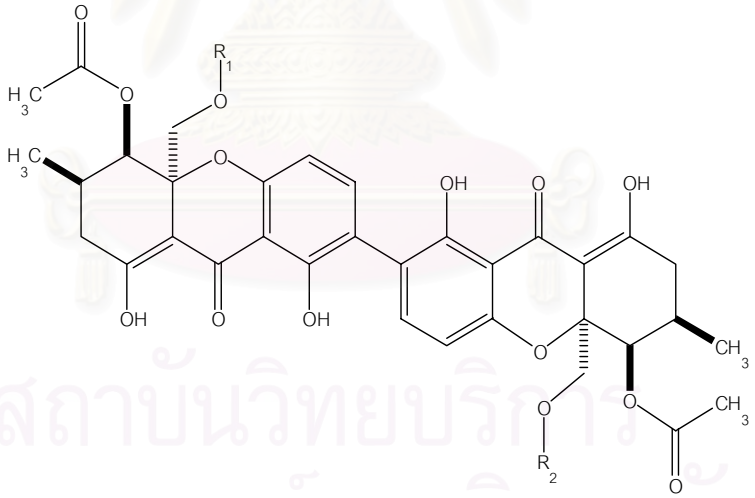
[20] Preussomerin J



[21] Preussomerin K



[22] Preussomerin L

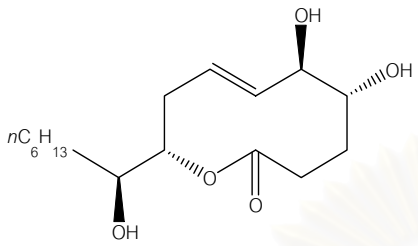


[23] Dicerandrols A, R1=R2=H

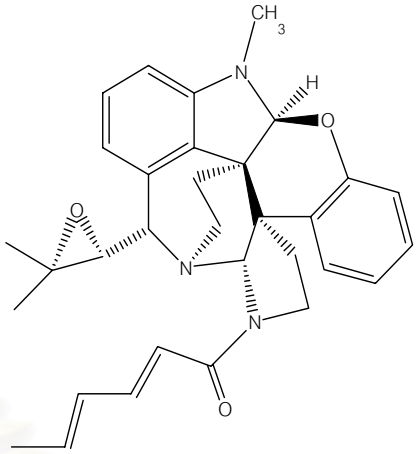
[24] Dicerandrols B, R1=Ac, R2=H

[25] Dicerandrols C, R1=R2=Ac

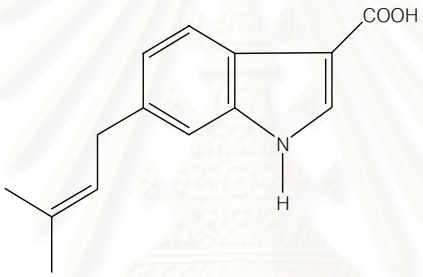
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



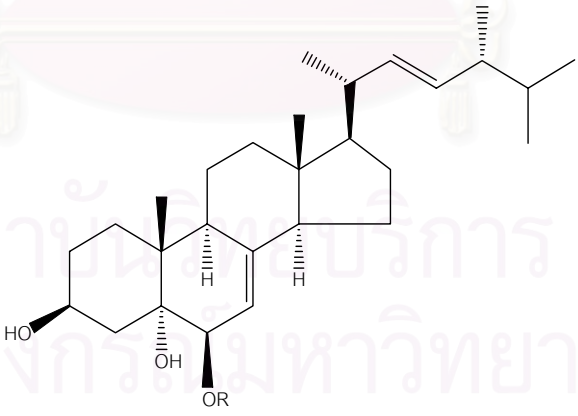
[26] Microcarpalide



[27] Nomofungin



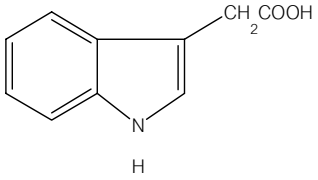
[28] Isoprenylindole-3-carboxylic acid



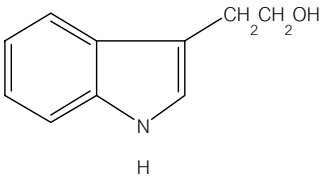
[29] 3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7, 22-diene, R=$COCH_3$

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

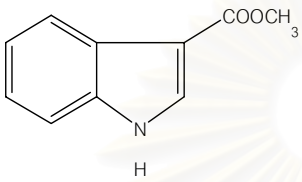
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



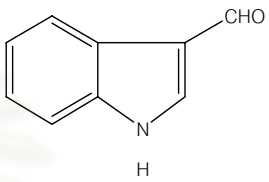
[31] Indole-3-acetic acid (IAA)



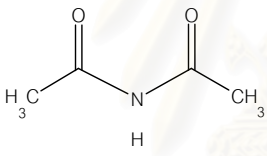
[32] Indole-3-ethanol (IEtOH)



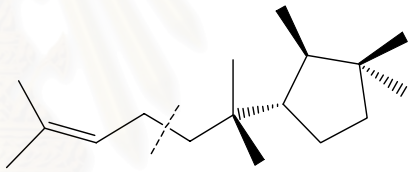
[33] Methylindole-3-carboxylate



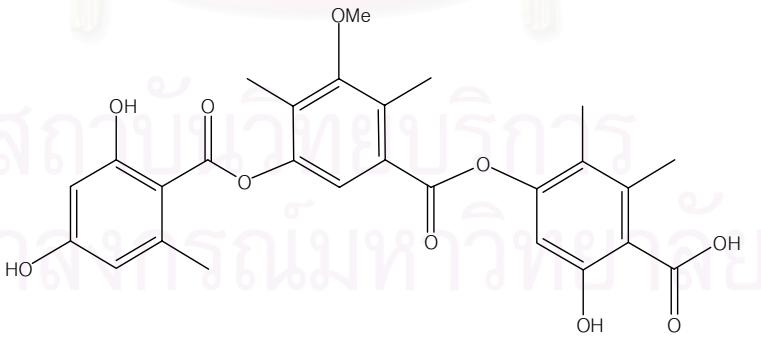
[34] Indole-3-carboxaldehyde



[35] Diacetamide

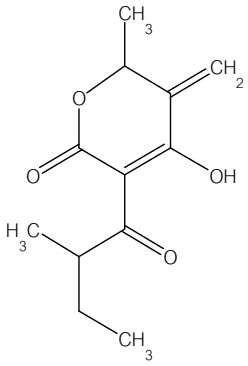


[36] Cyclonerodiol

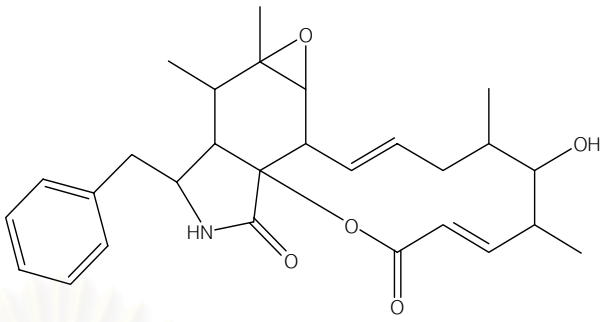


[37] Colletotric acid

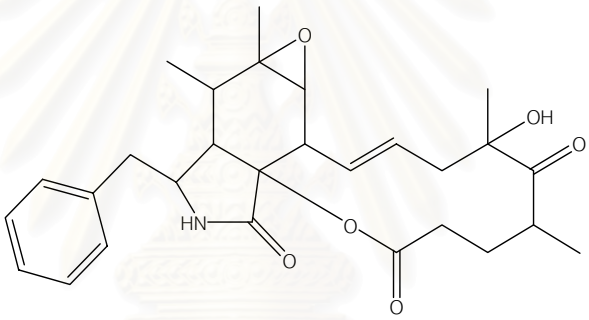
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



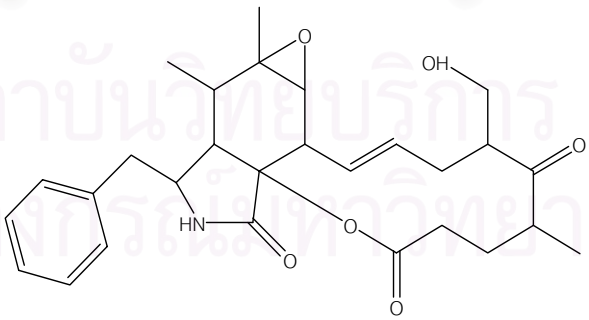
[38] CR377, pentaketide



[39] Cytochalasin 1

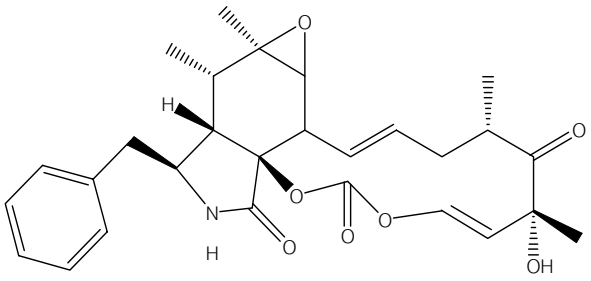


[40] Cytochalasin 2

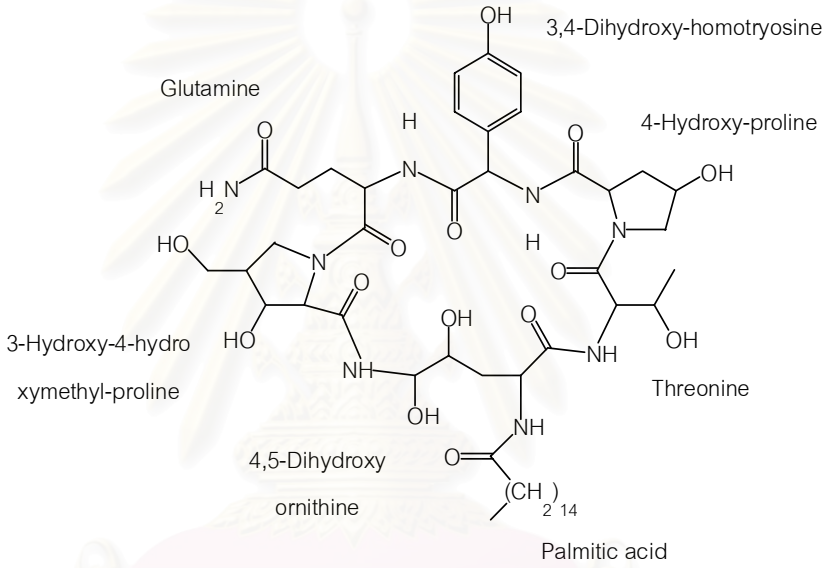


[41] Cytochalasin 3

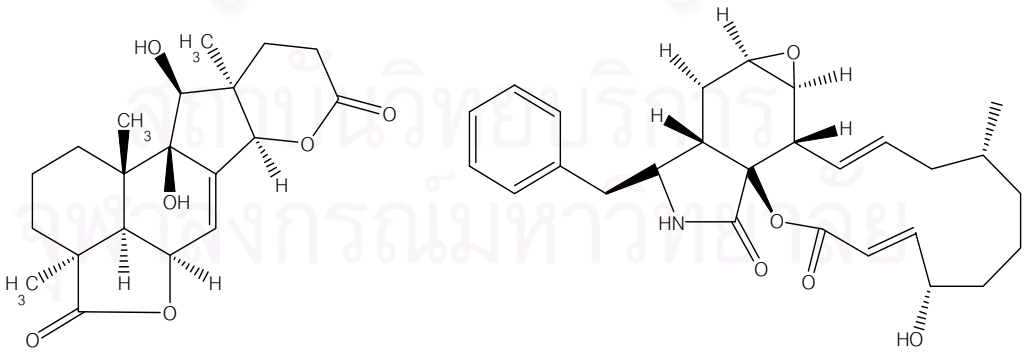
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



[42] Cytochanlasin E



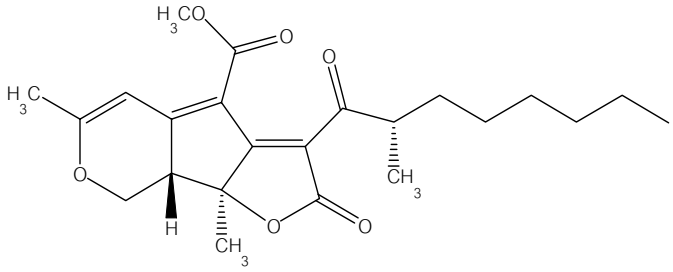
[43] Cryptocandin



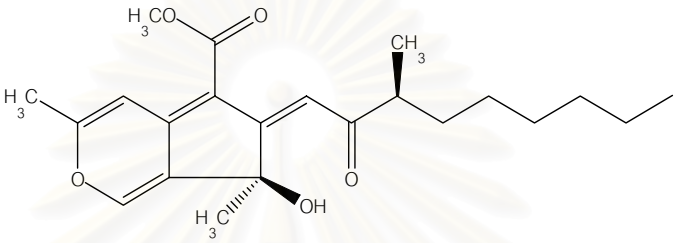
[44] Geniculol

[45] Cytochalasin F

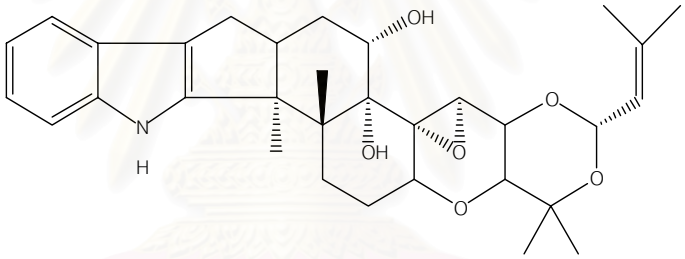
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



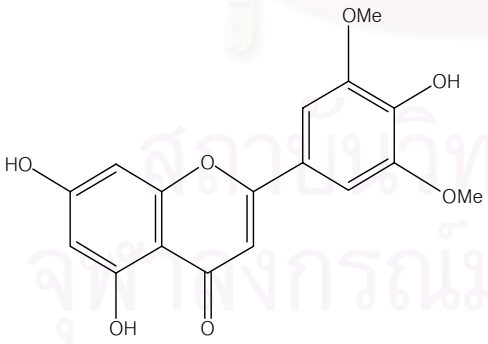
[46] Sequoiatones A



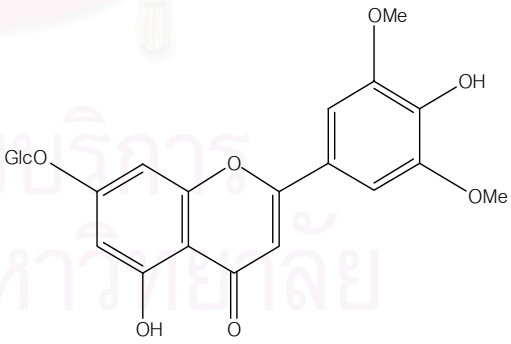
[47] Sequoiatones B



[48] Terpendole M

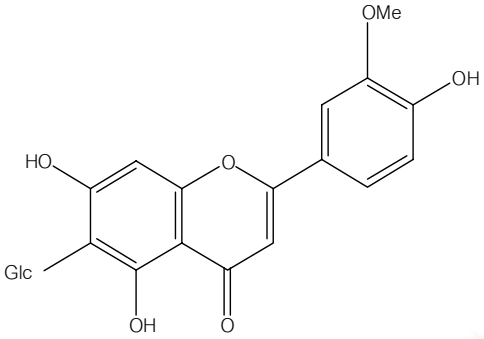


[49] Tricin

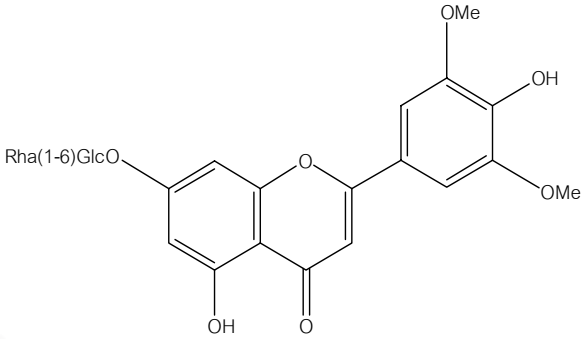


[50] 7-O-(B-D-glucopyranosyl)tricin

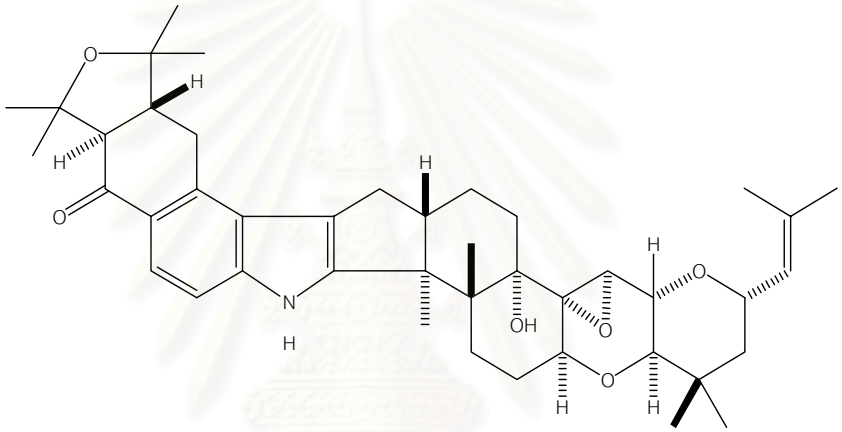
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



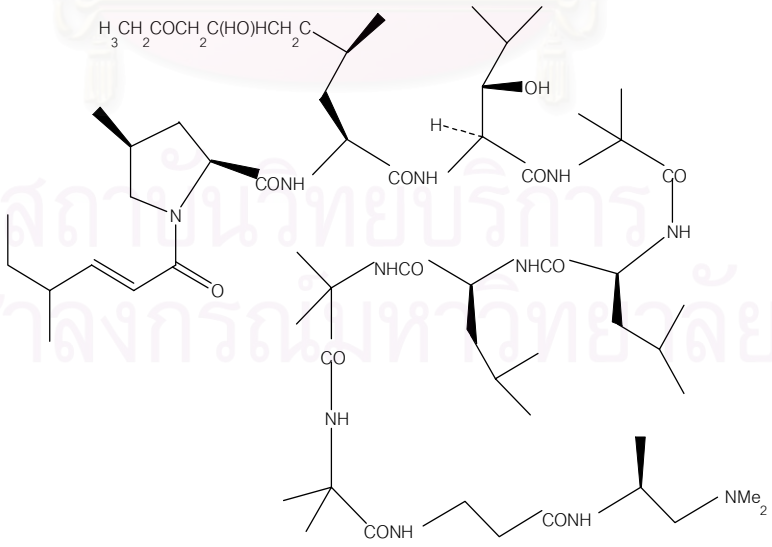
[51] Isoorientin



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

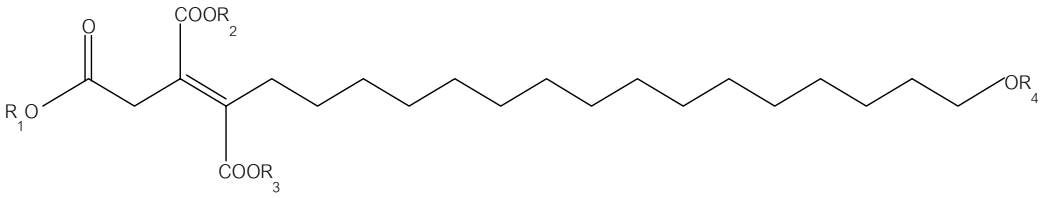


[53] Lolitrem B



[54] Leucinostatin A

Figure A1 Structure of secondary metabolites of endophytic fungi (continued)

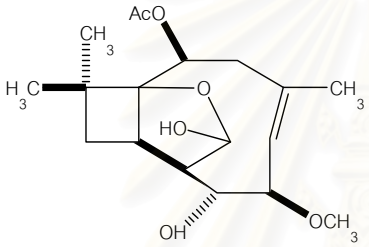


[55] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H

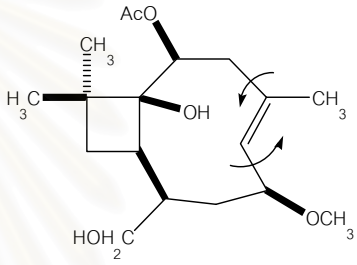
[56] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H

[57] 3: Desulfated analog, R1=R2=R3=CH3, R4=H

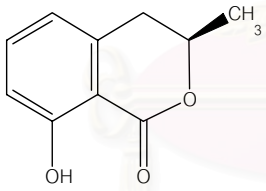
[58] 4: Desulfated analog, R1=R2=R3=R4=H



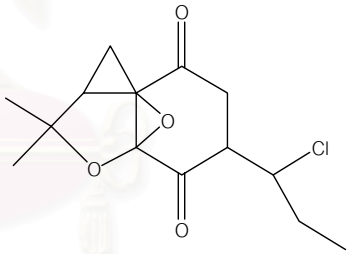
[59] Pestalotiopsins A



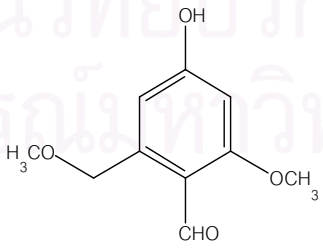
[60] Pestalotiopsins B



[61] (R)-mellein

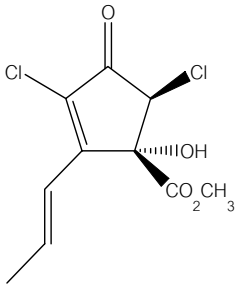


[62] (-)-mycorrhizin A

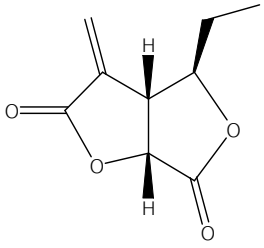


[63] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

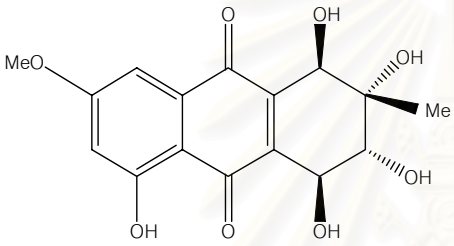
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



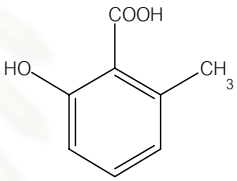
[64] (+)-cryptosporiopsin



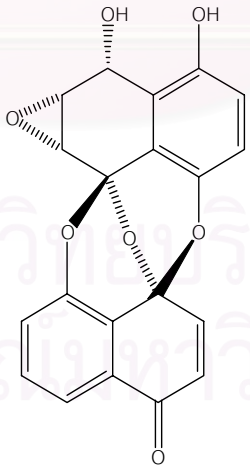
[65] 4-epi-ethiosolide



[66] Altersolanol A

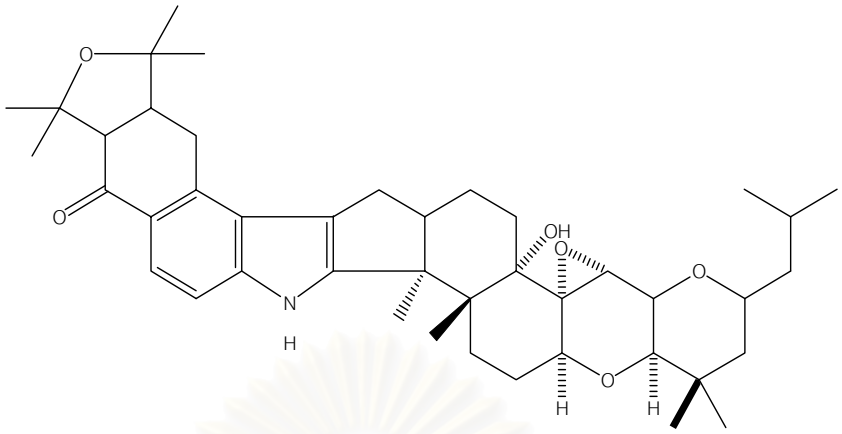


[67] 2-hydroxy-6-methyl benzoic acid

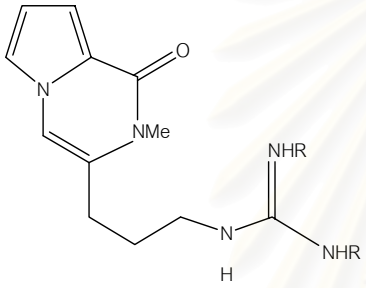


[68] Preussomerin D

Figure A1 Structure of secondary metabolites of endophytic fungi (continued)

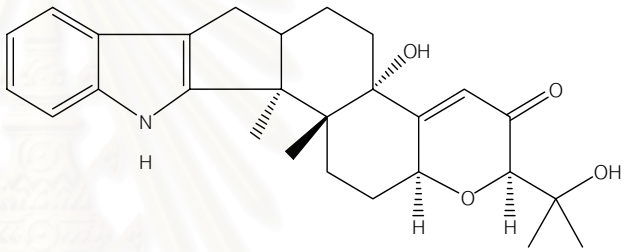


[69] Lolitrem C

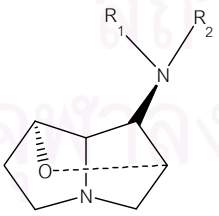


[70] Peramine R=H

[71] Diacetylperamine R=Ac

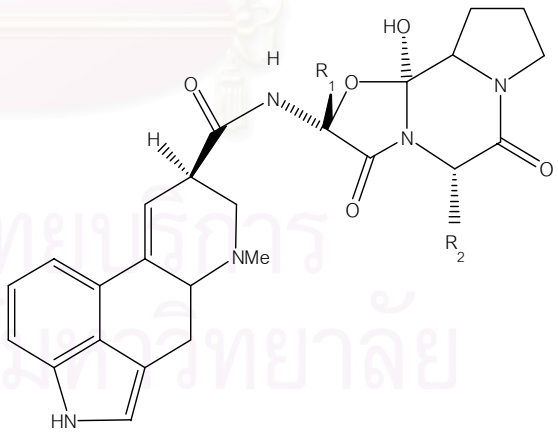


[72] Paxilline



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

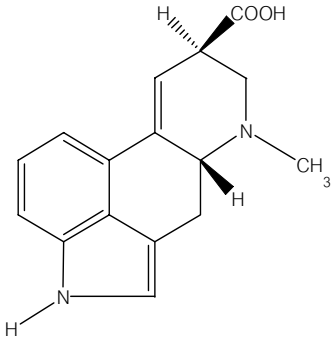
R_2= H, HCO, Ac



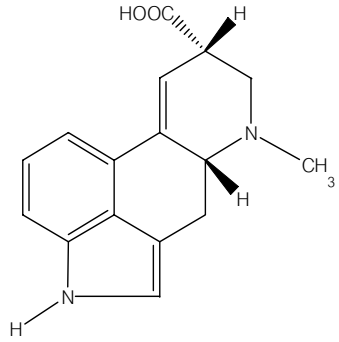
[74] Ergopeptine alkaloids

Ergovaline R_1=Me, R_2=i-Pr

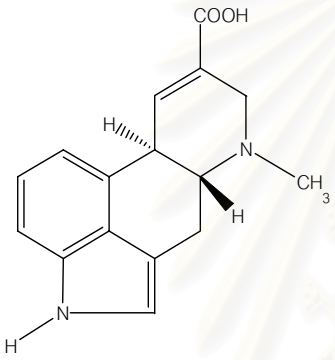
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



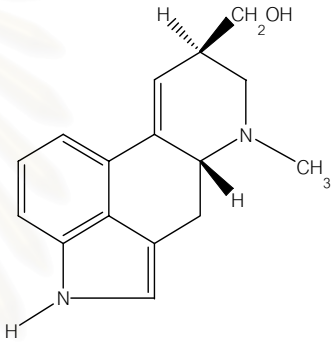
[75] Lysergic acid



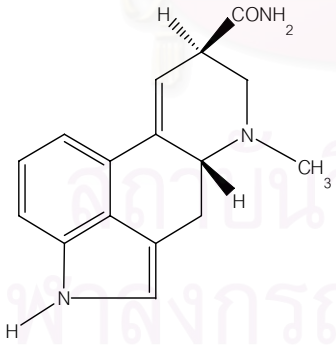
[76] Isolysergic acid



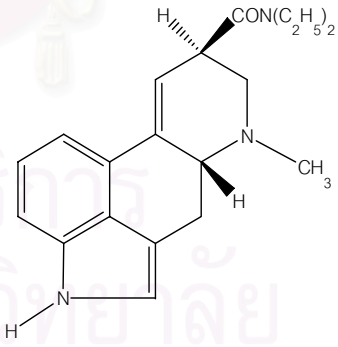
[77] Paspalic acid



[78] Lysergol

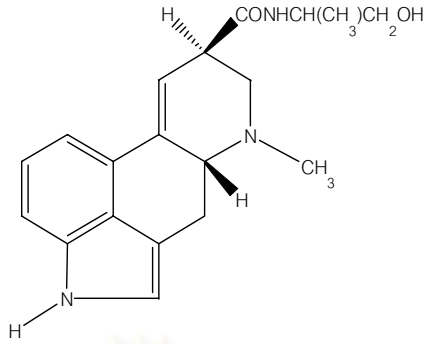


[79] Lysergic acid amide



[80] Lysergic acid diethyl amide

Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



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

Figure A1 Structure of secondary metabolites of endophytic fungi (continued)

APPENDIX B

MEDIA

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

1. Potato Dextrose Agar (PDA)

Potato, (pelled and diced)	200	g
D-glucose	20.0	g
Agar	15.0	g
Distilled water	1,000	ml

2. Malt Extract Agar (MEA)

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

3. Yeast Extract Sucrose Agar (YES)

Yeast extract	20	g
Sucrose	15	g
Agar	20	g
Distilled water	1000	ml

4. Nutrient Agar (NA)

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

5. Yeast-malt extract Agar (YMA)

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

6. Sabouroud Glucose Agar (SGA)

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

7. Carrot Agar (CA)

carrot	20.0	g
Distilled water	1000	ml
Agar	20.0	g

8. Potato Carrot Agar (PCA)

Potato	10.0	g
Carrot	10.0	g
Dextrose	20.0	g
Distilled water	1000	ml
Agar	20.0	g

9. Water Agar (WA)

Distilled water	1000	ml
Agar	20.0	g

APPENDIX C

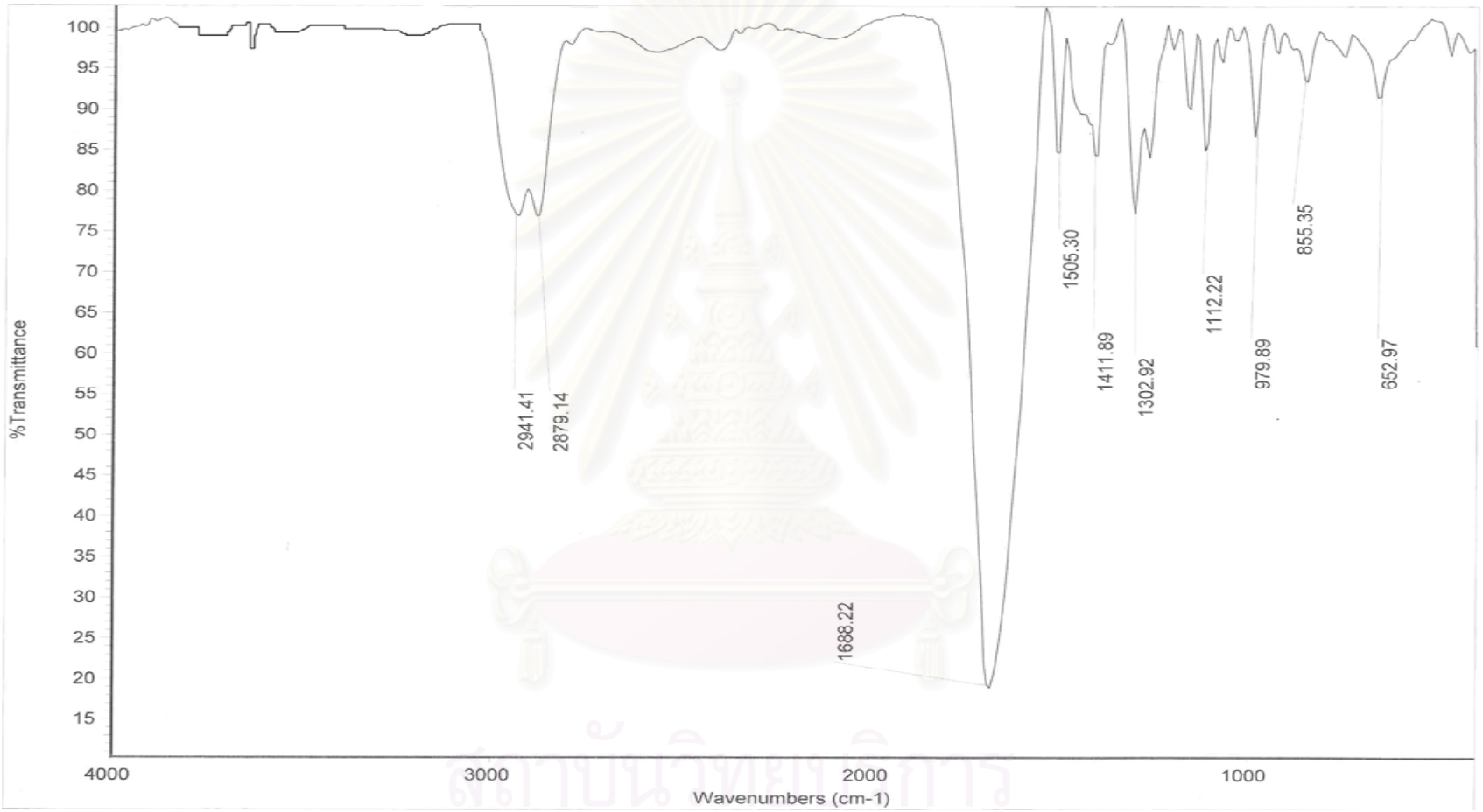


Figure C1 IR spectrum of compound 1

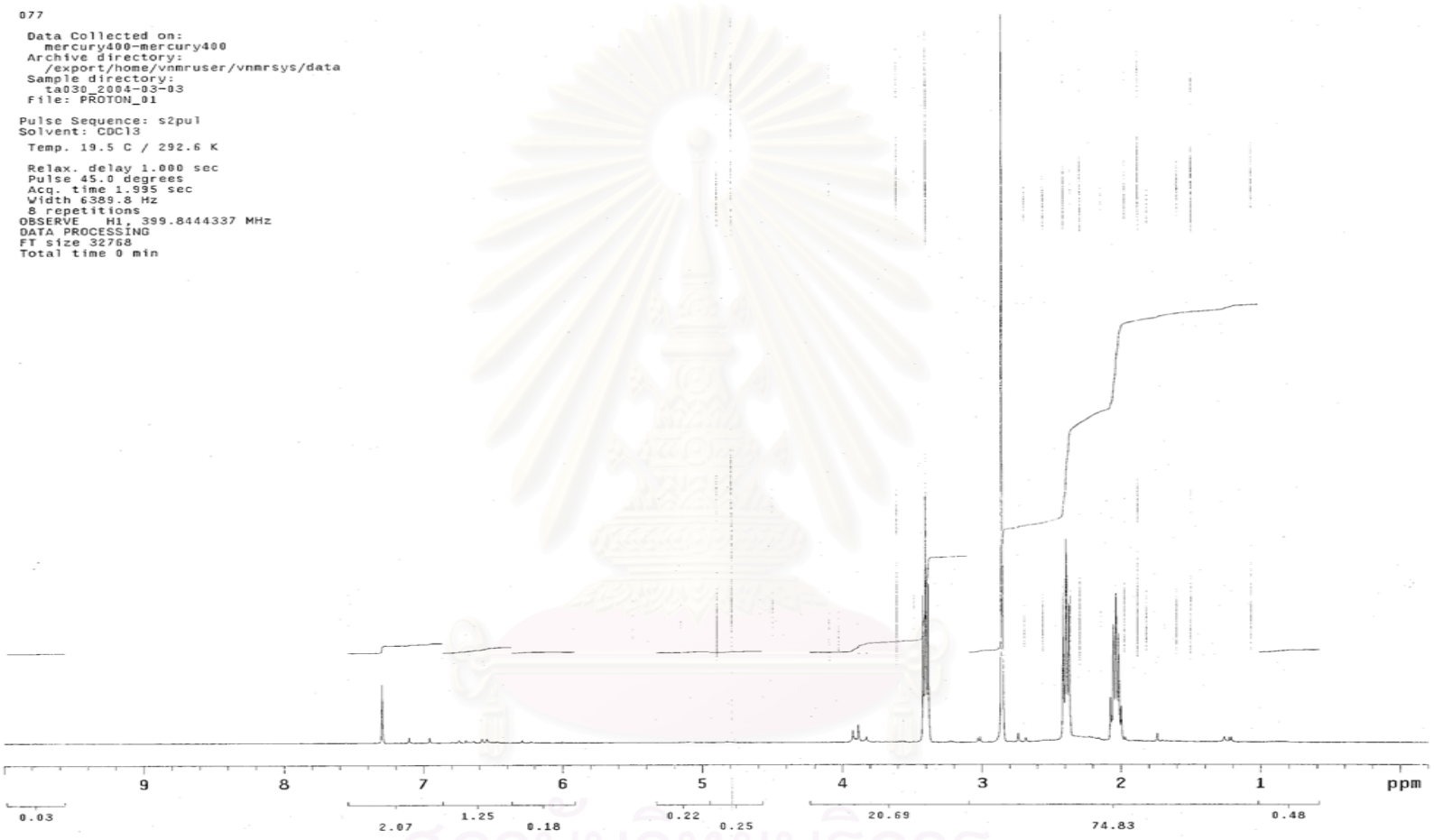


Figure C2 ^{1}H-NMR spectrum of compound 1

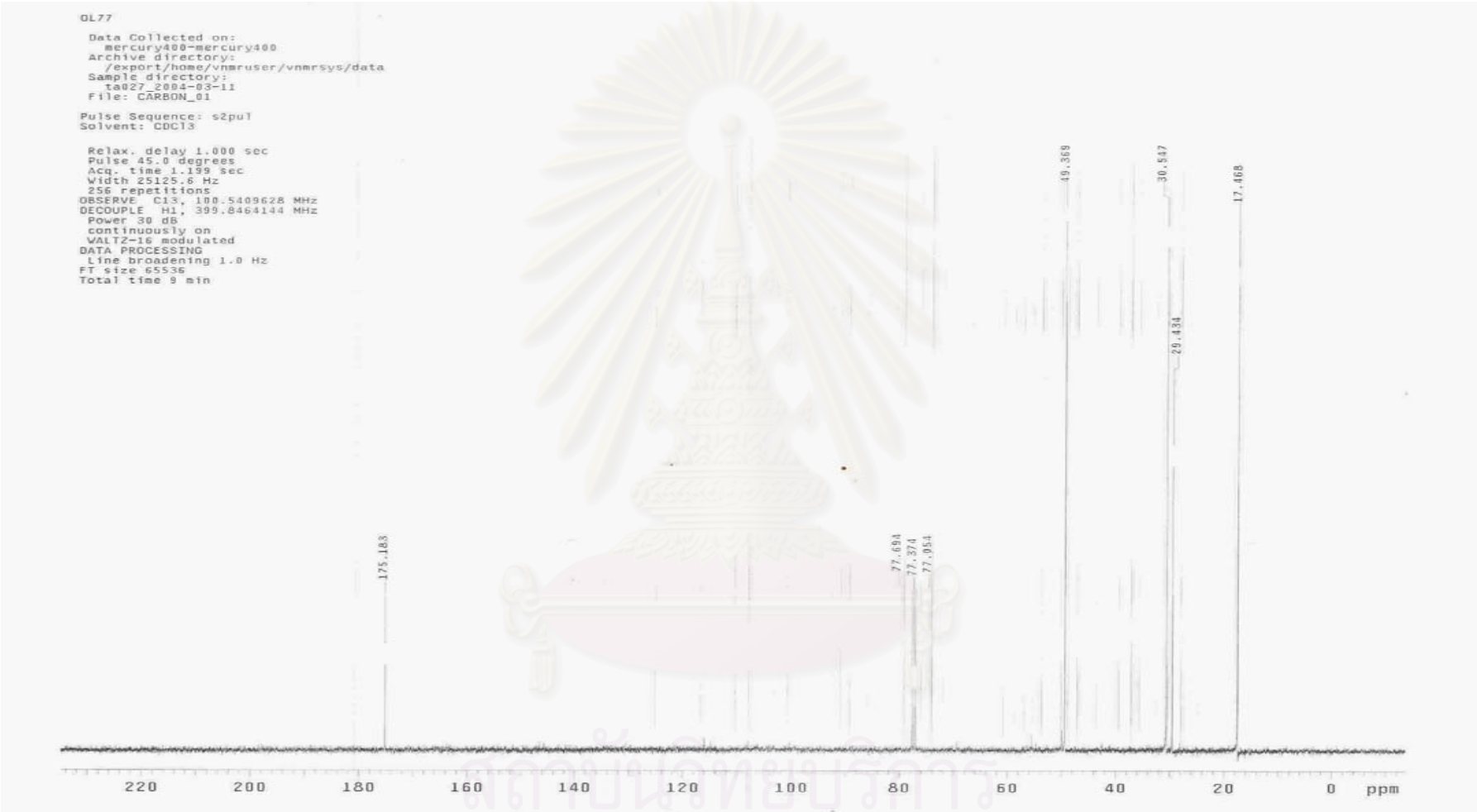


Figure C3 ^{13}C-NMR spectrum of compound 1

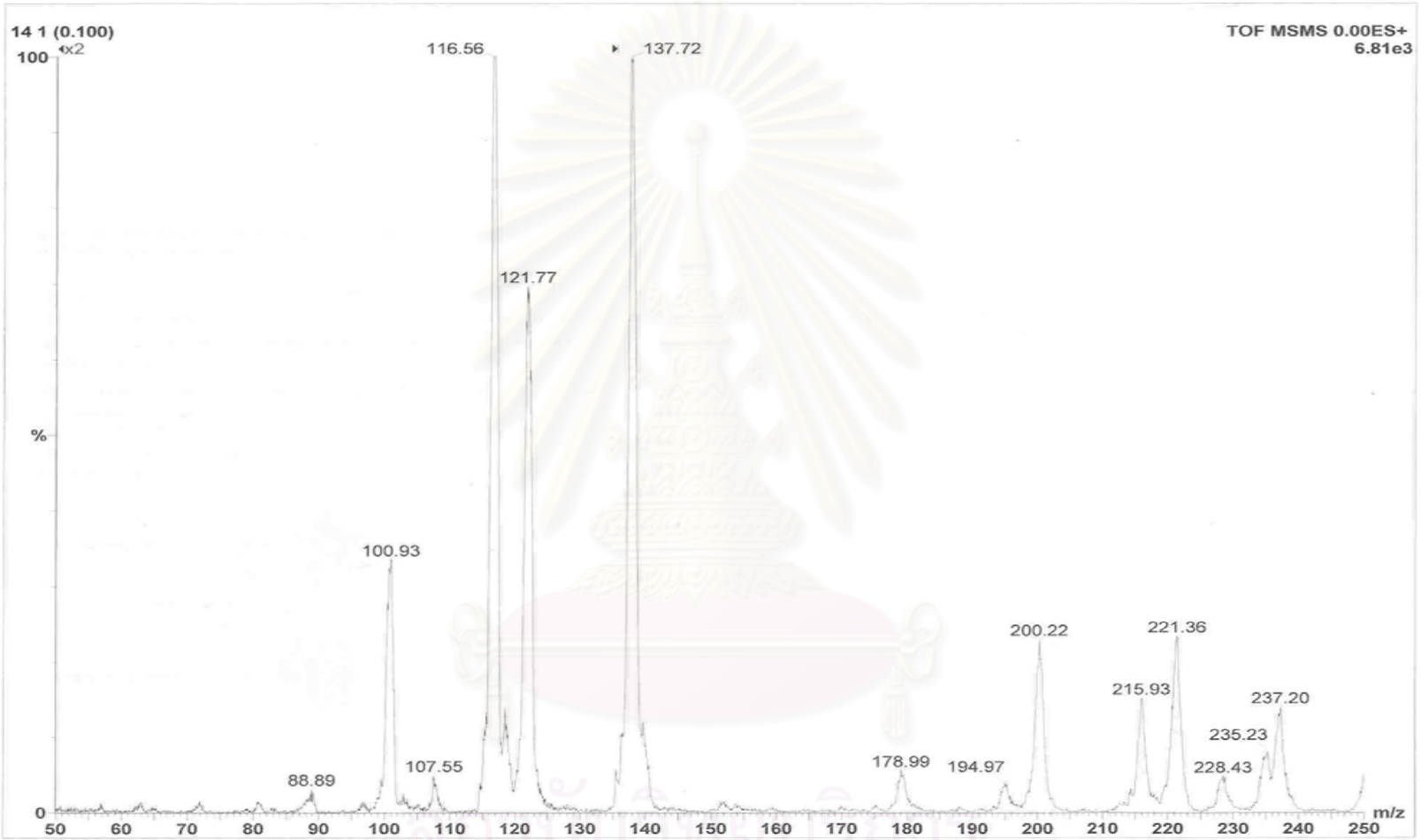


Figure C4 Mass spectrum of compound 1

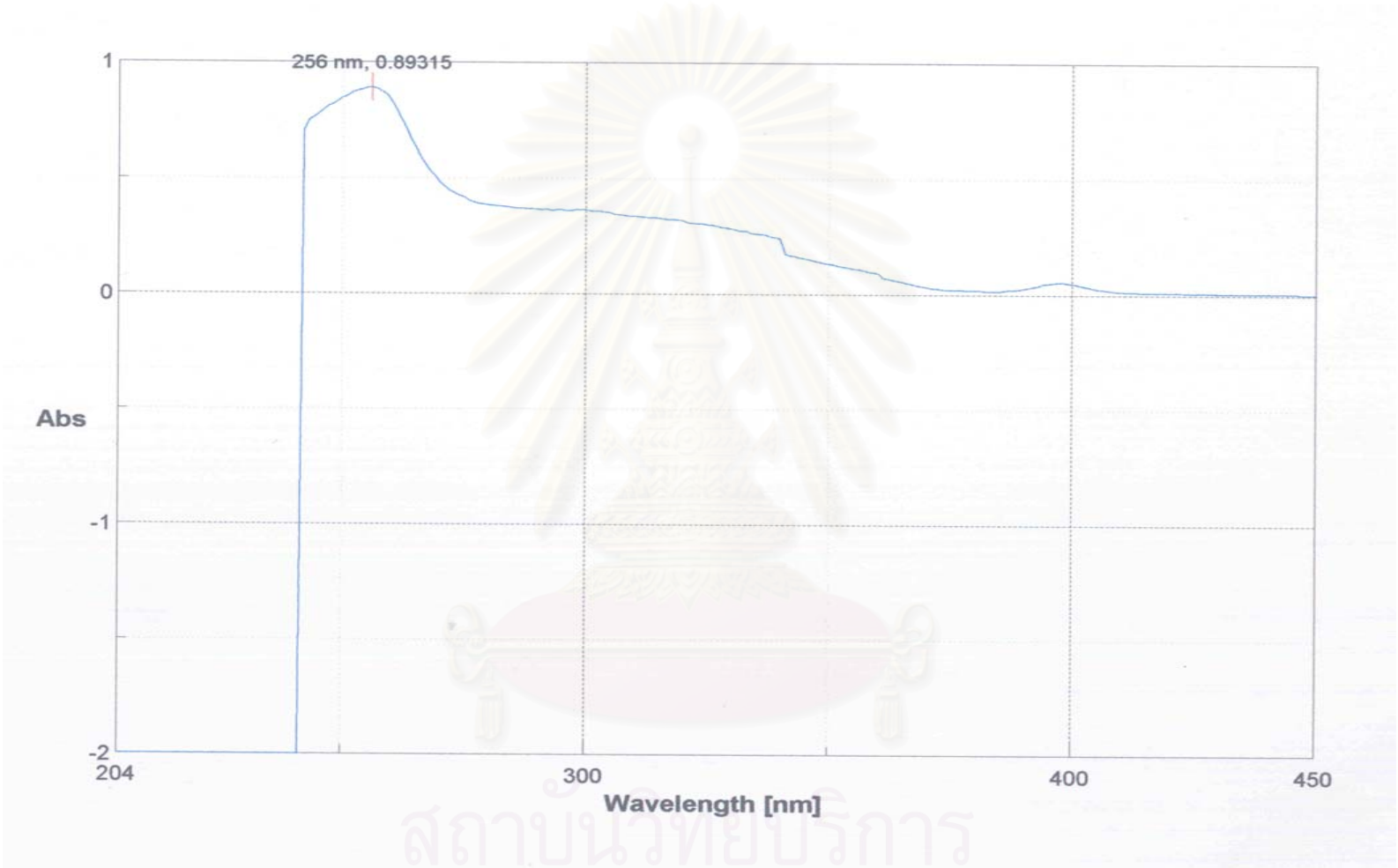


Figure C5 UV spectrum of compound 1

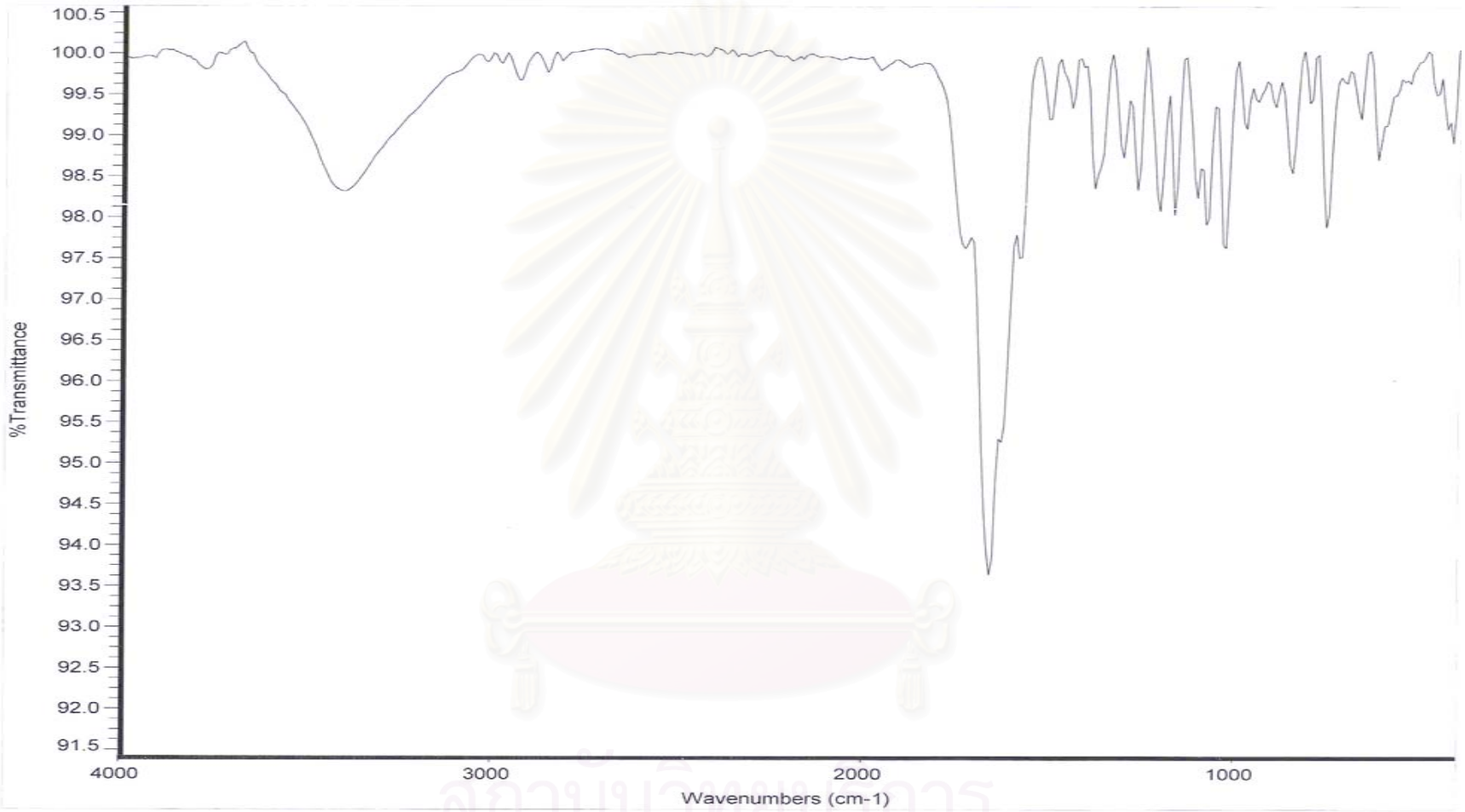


Figure C6 IR spectrum of compound 2

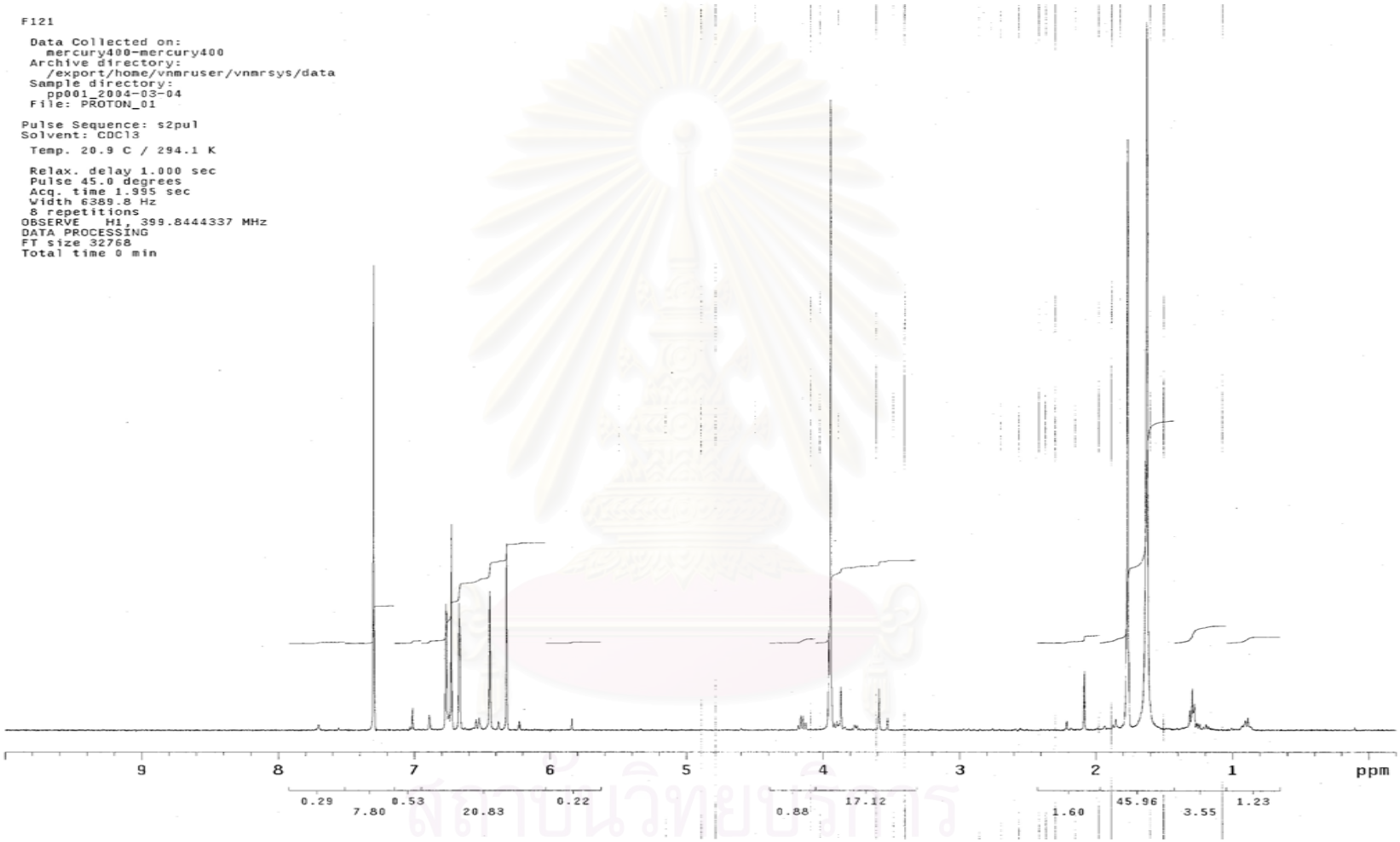


Figure C7 ^{1}H-NMR spectrum of compound 2

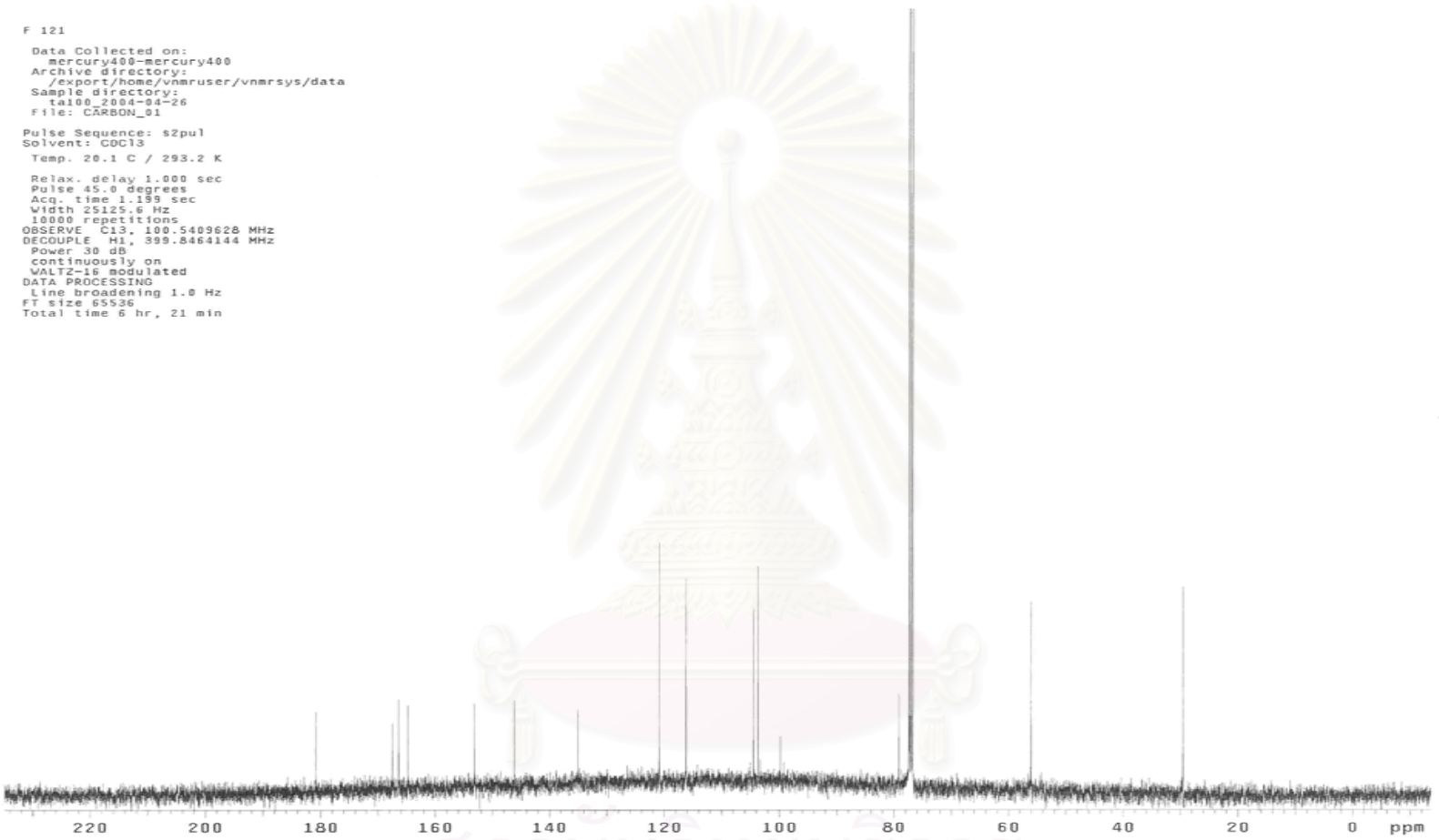


Figure C8 ^{13}C-NMR spectrum of compound 2

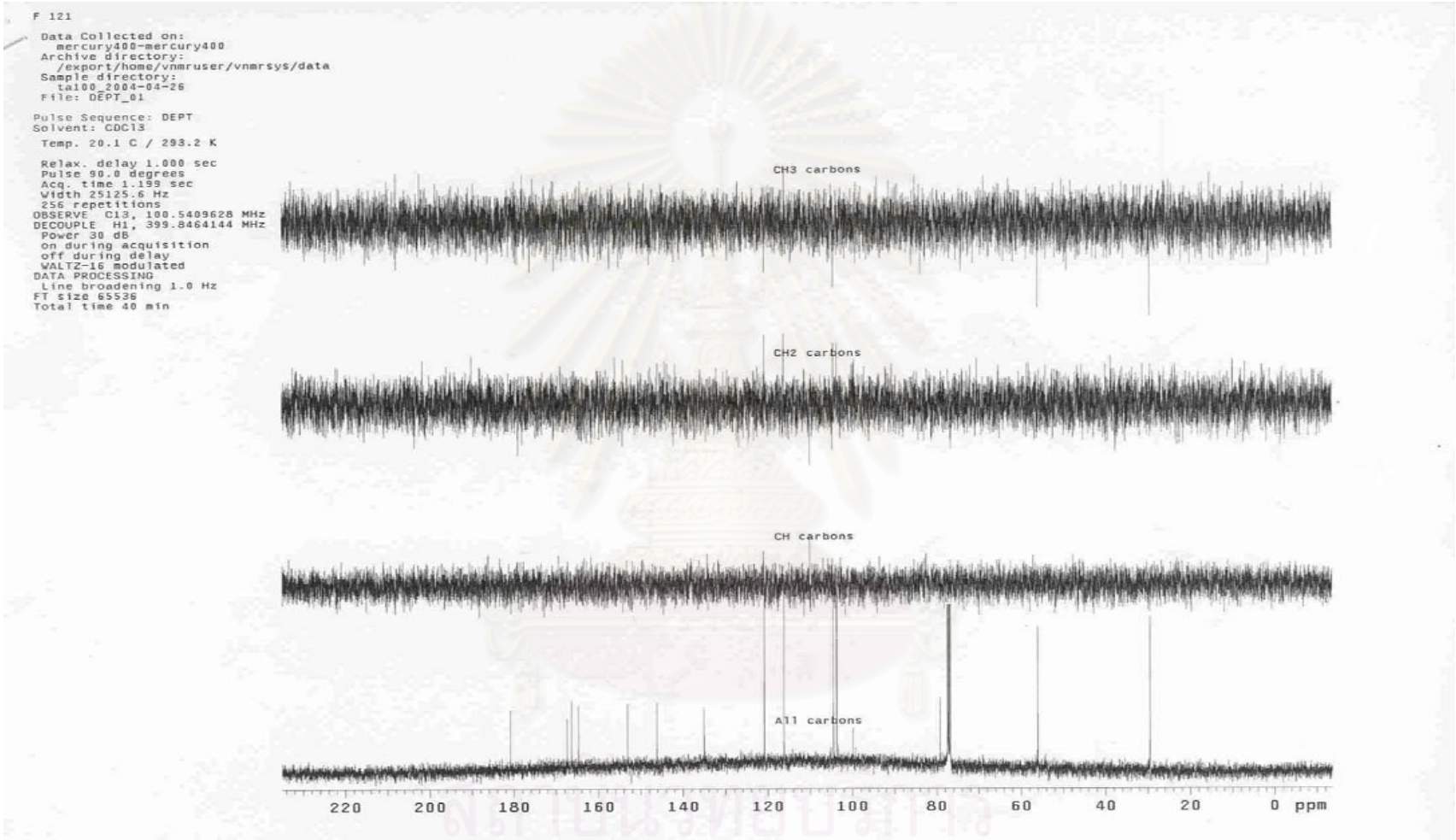


Figure C9 DEPT spectrum of compound 2

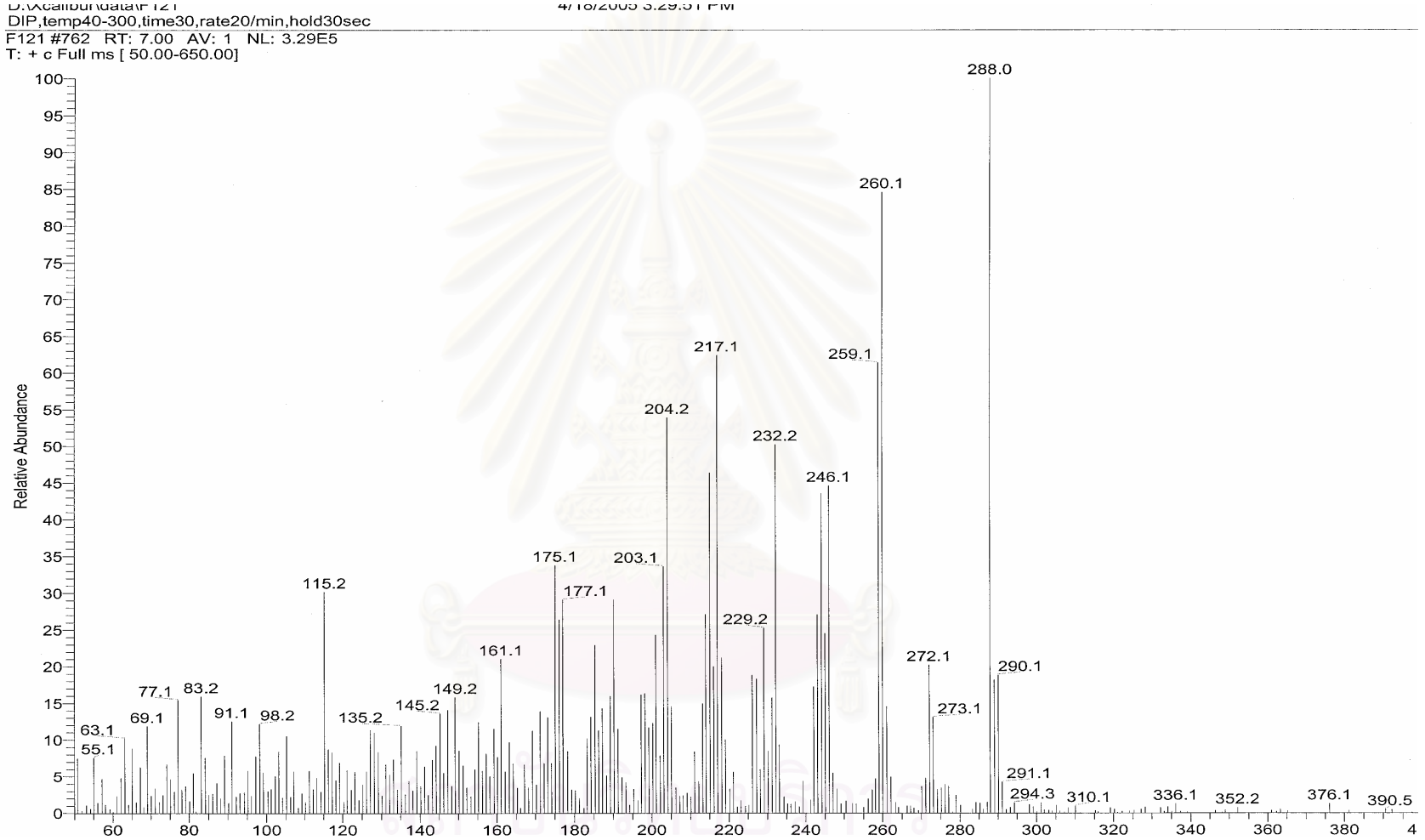


Figure C10 Mass spectrum of compound 2

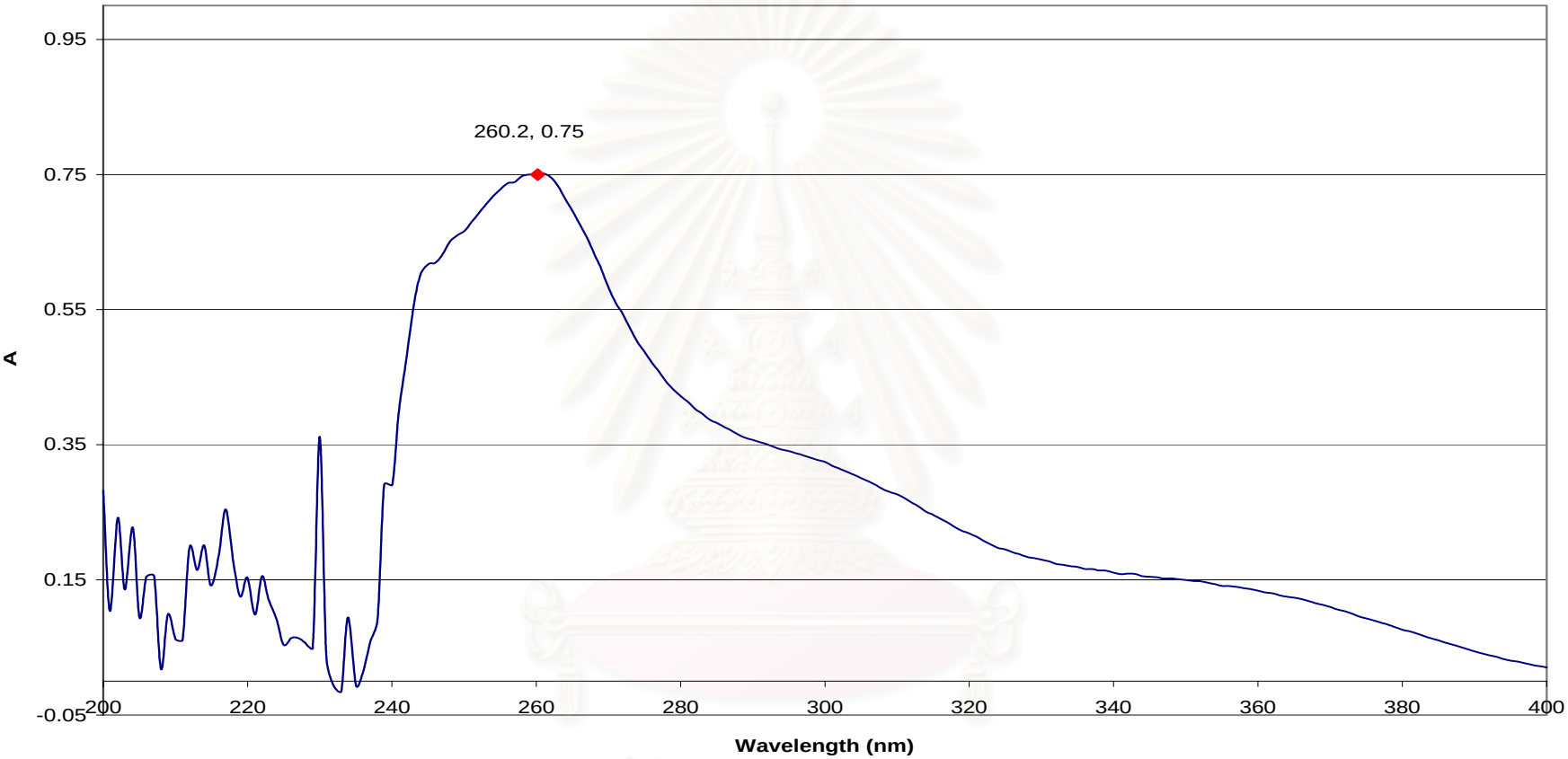


Figure C11 UV spectrum of compound 2

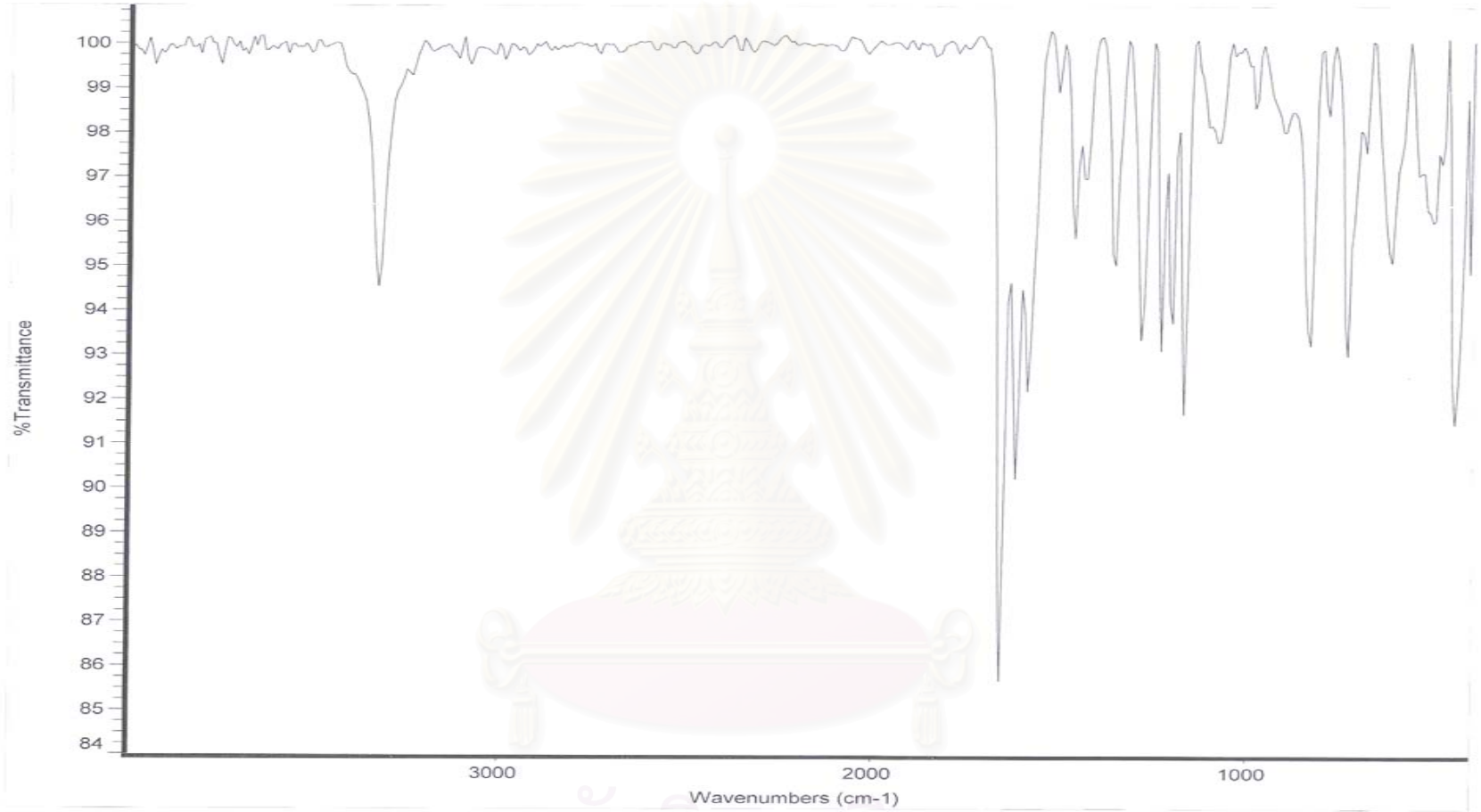


Figure C12 IR spectrum of compound 3

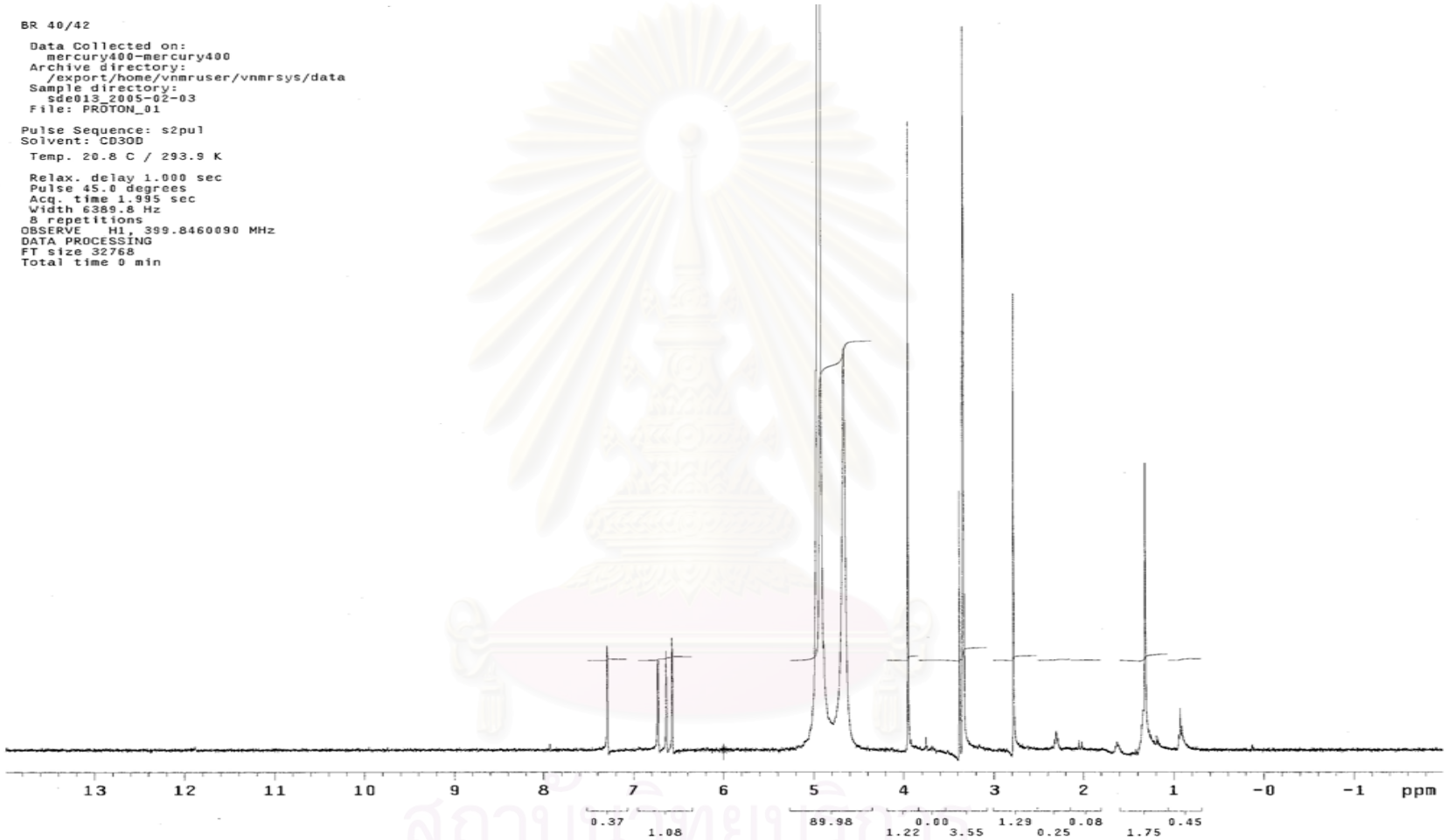


Figure C13 ^{1}H spectrum of compound 3

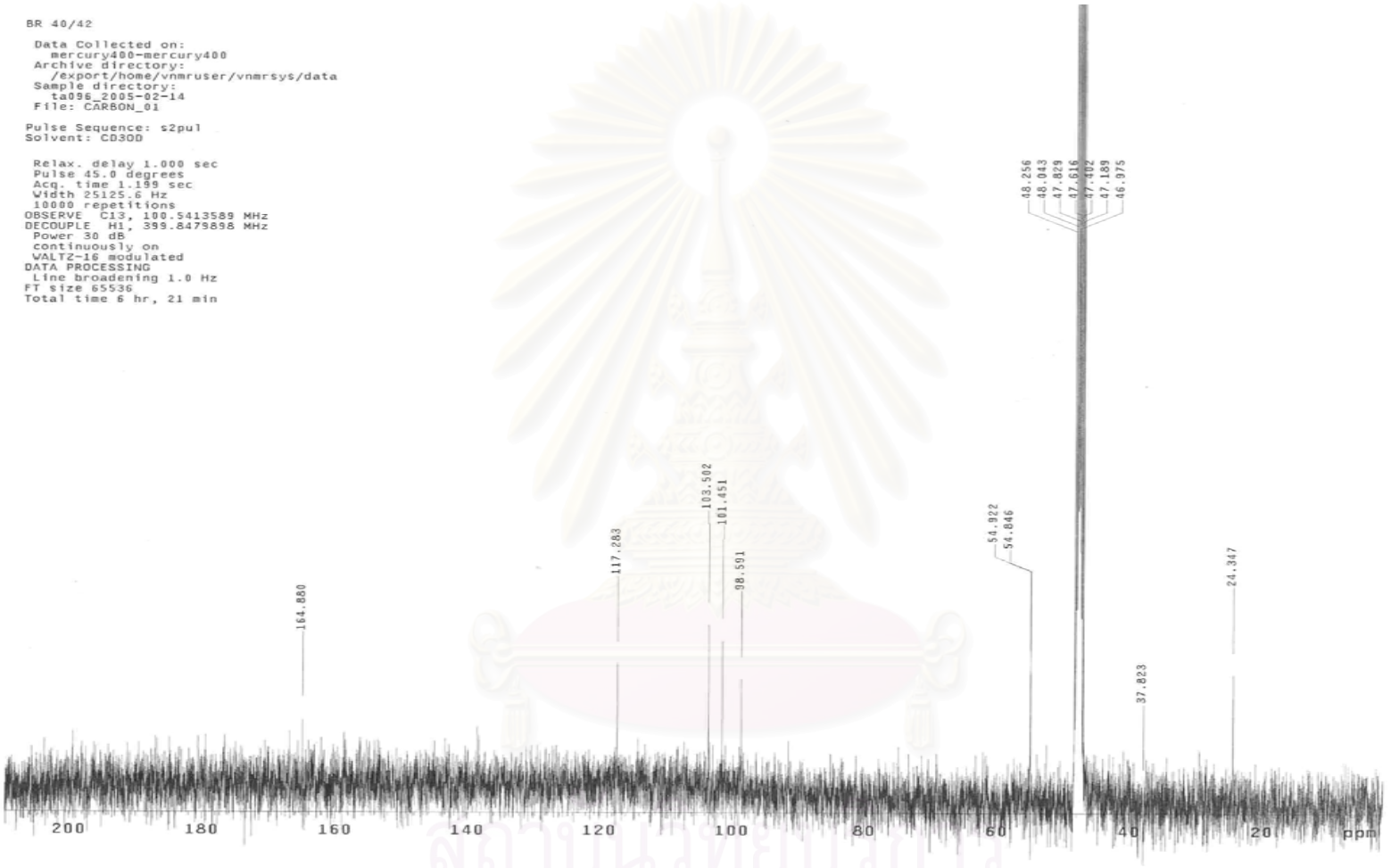


Figure D14 ^{13}C spectrum of compound 3

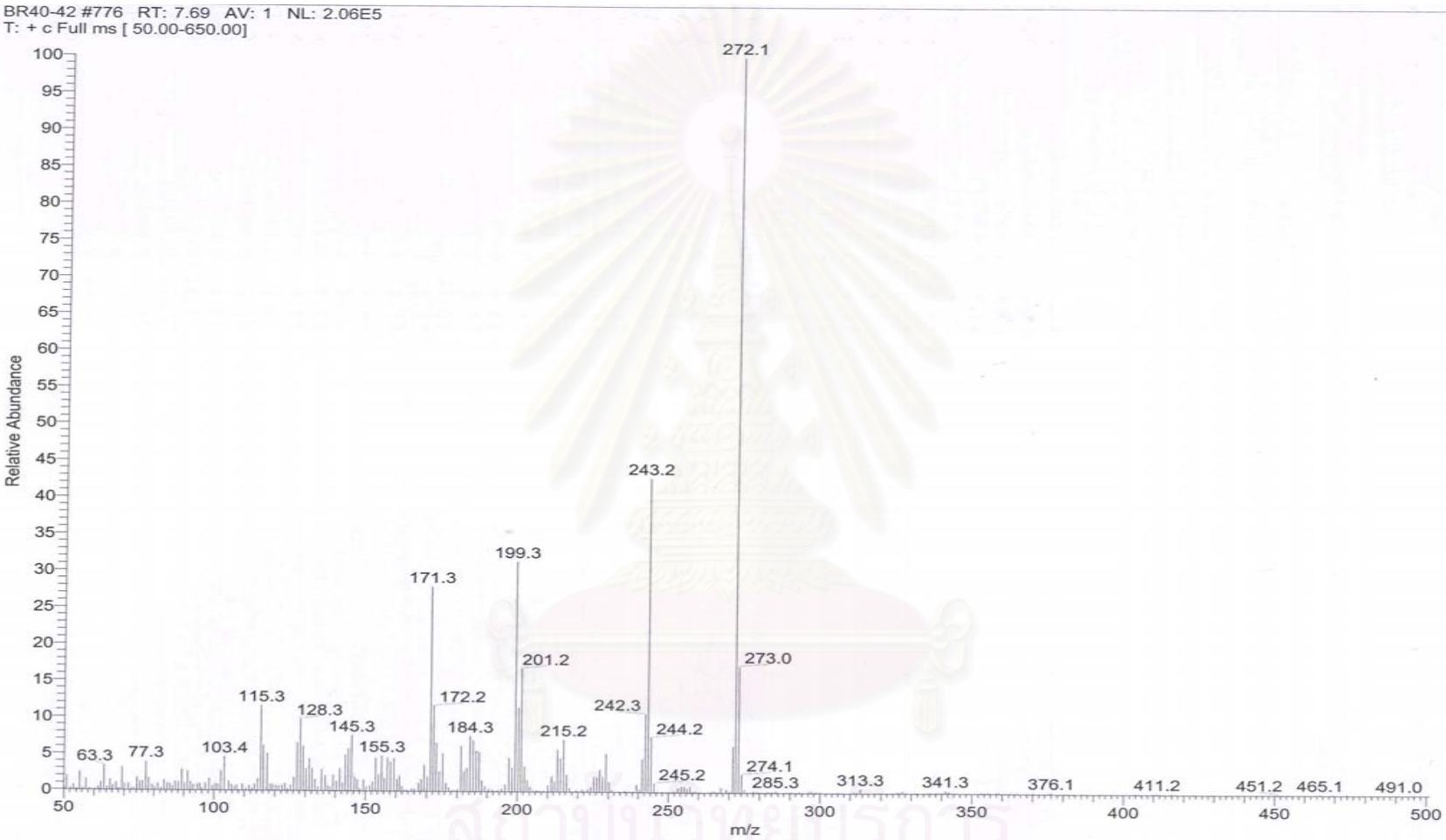


Figure C15 Mass spectrum of compound 3

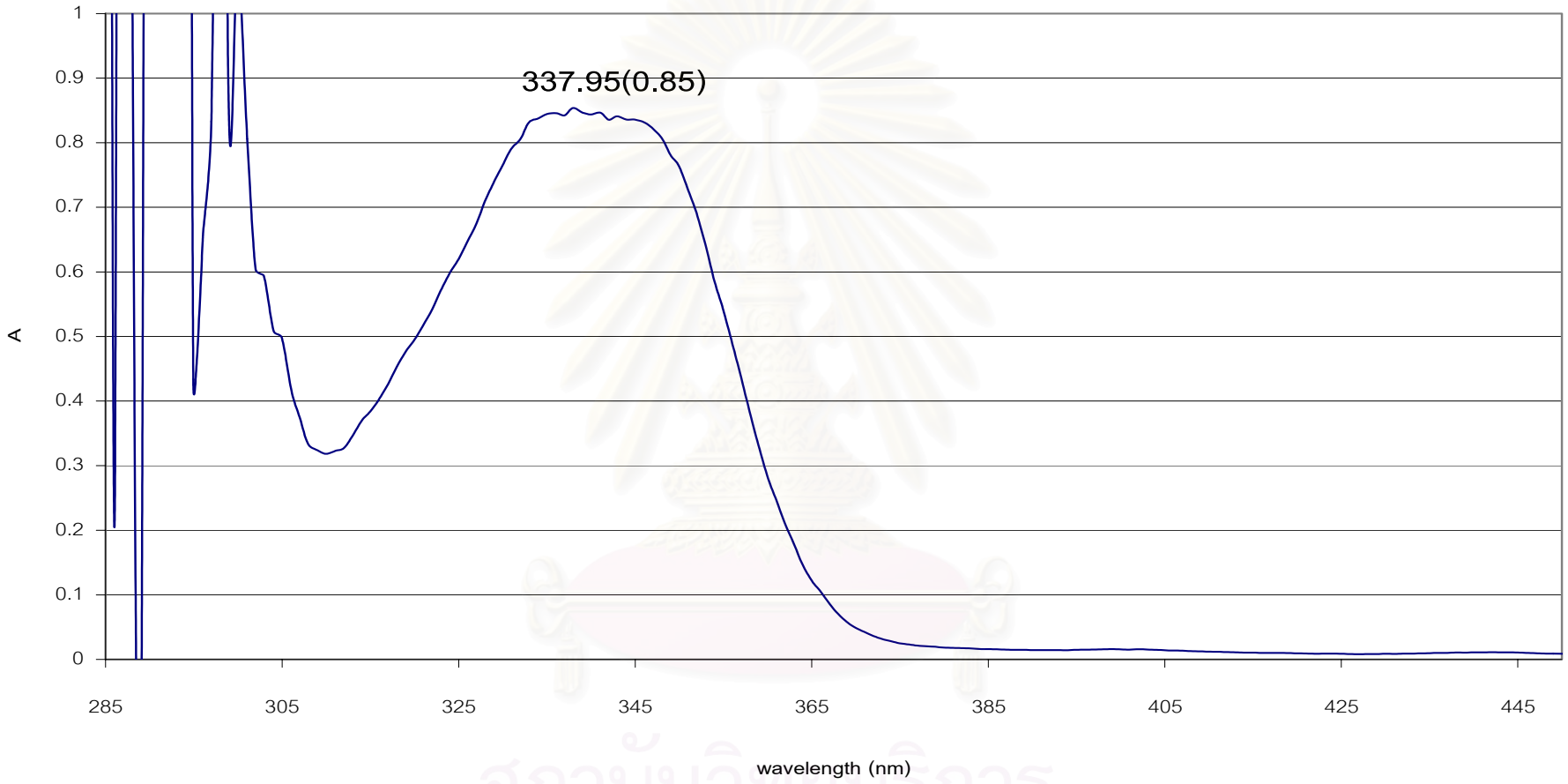


Figure C16 UV spectrum of compound 3

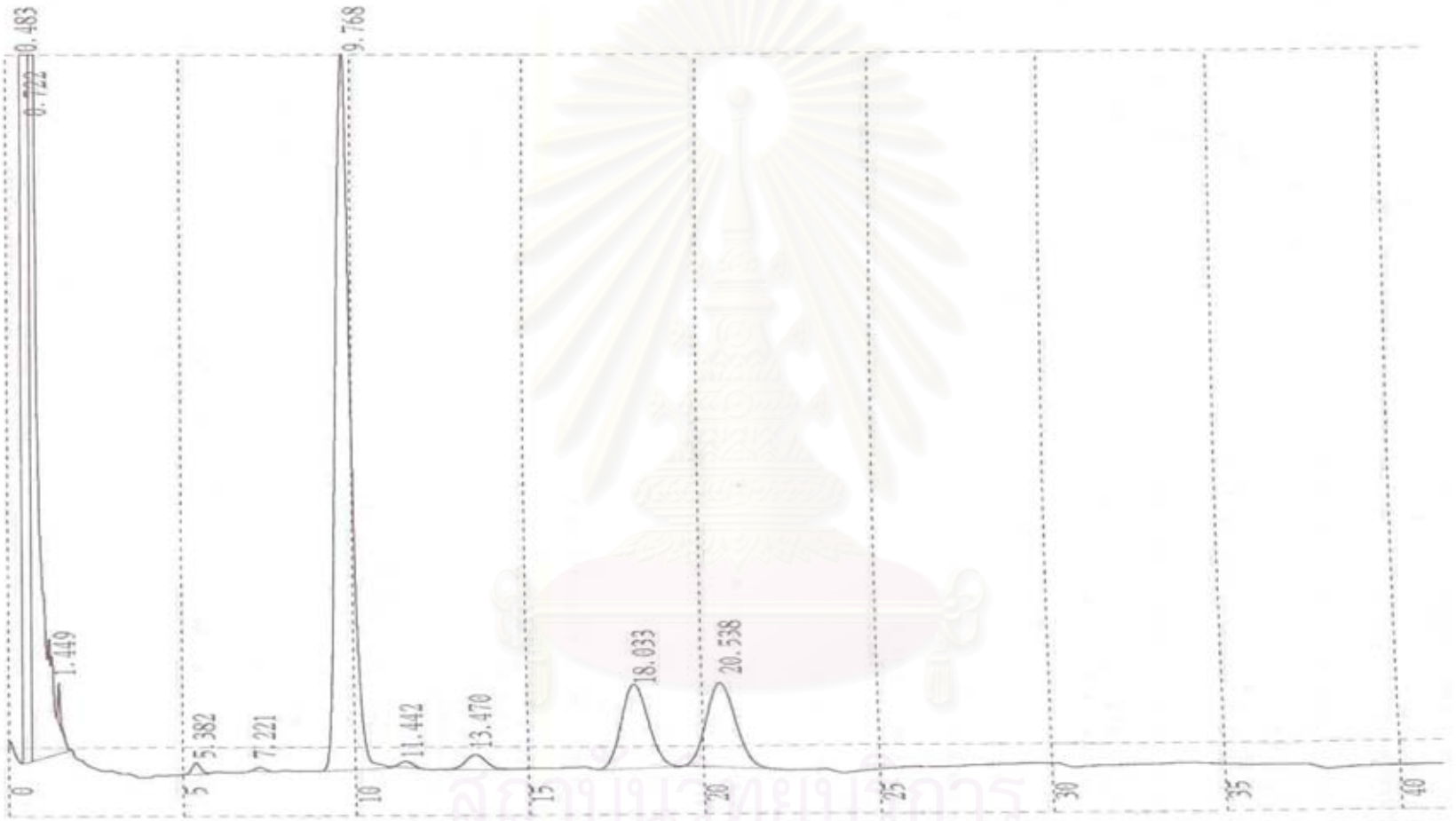


Figure C17 GC spectrum of Mixture 4

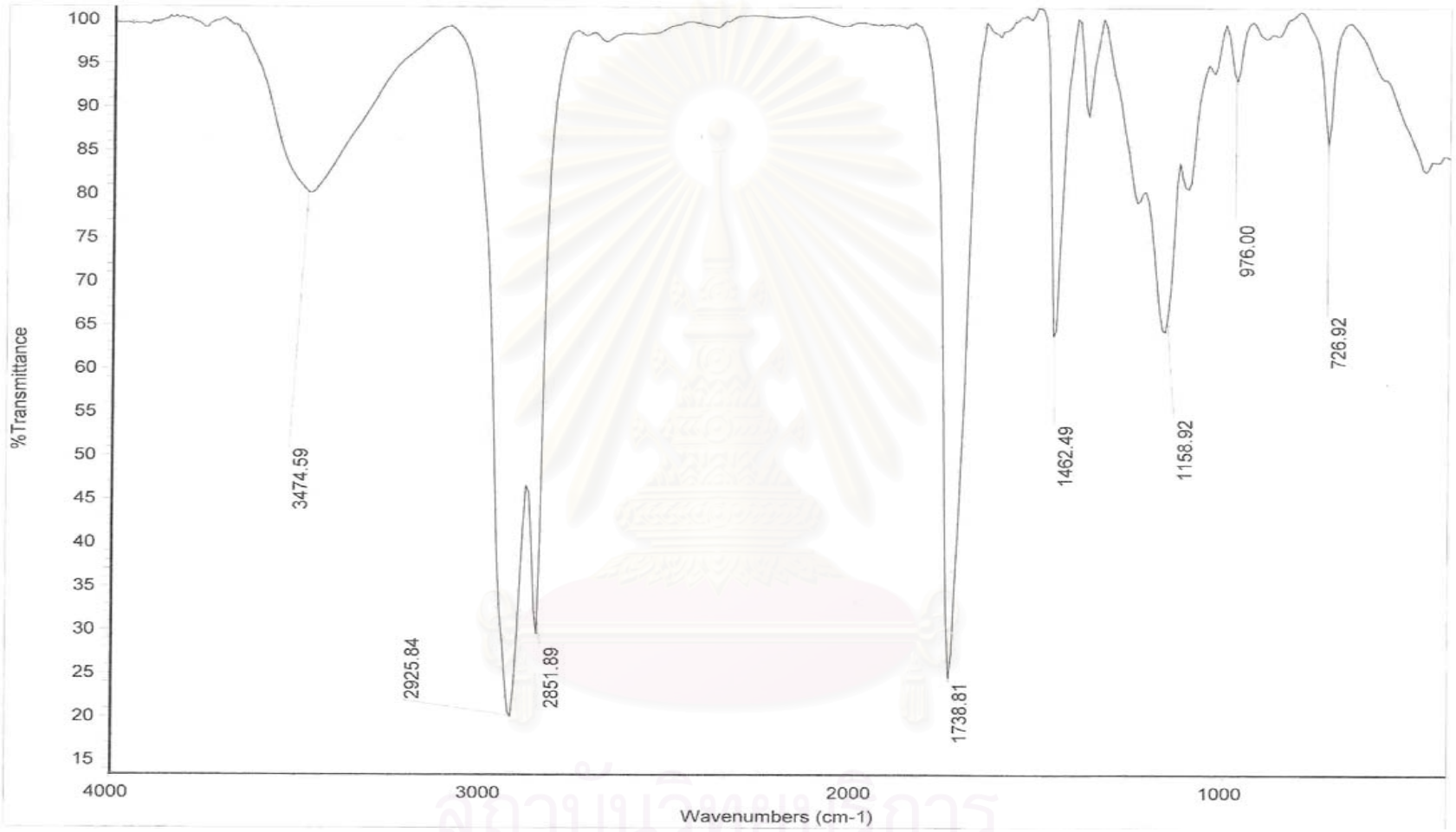


Figure C18 IR spectrum of mixture 4

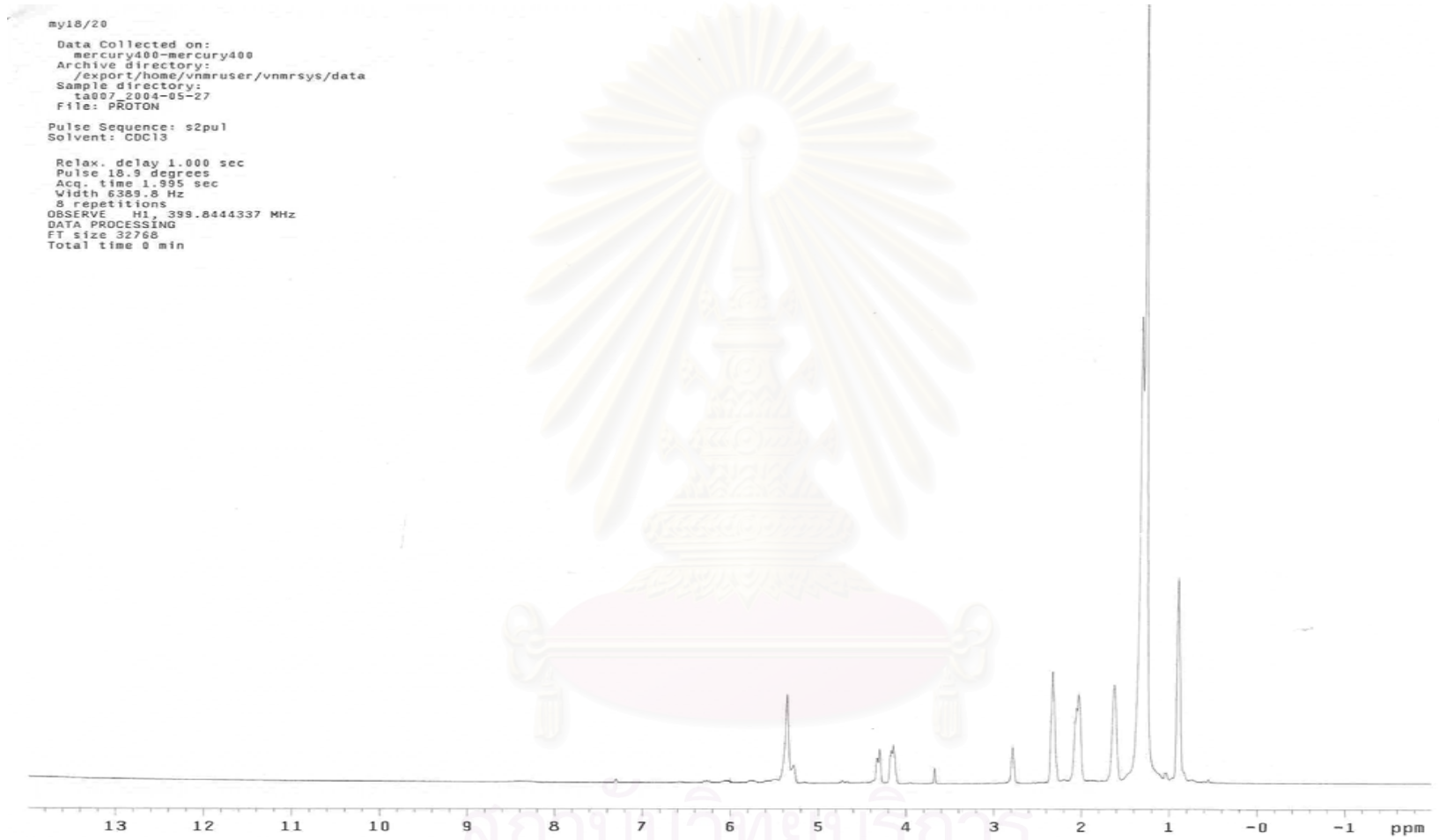


Figure C19 ^{1}H-NMR spectrum of mixture 4

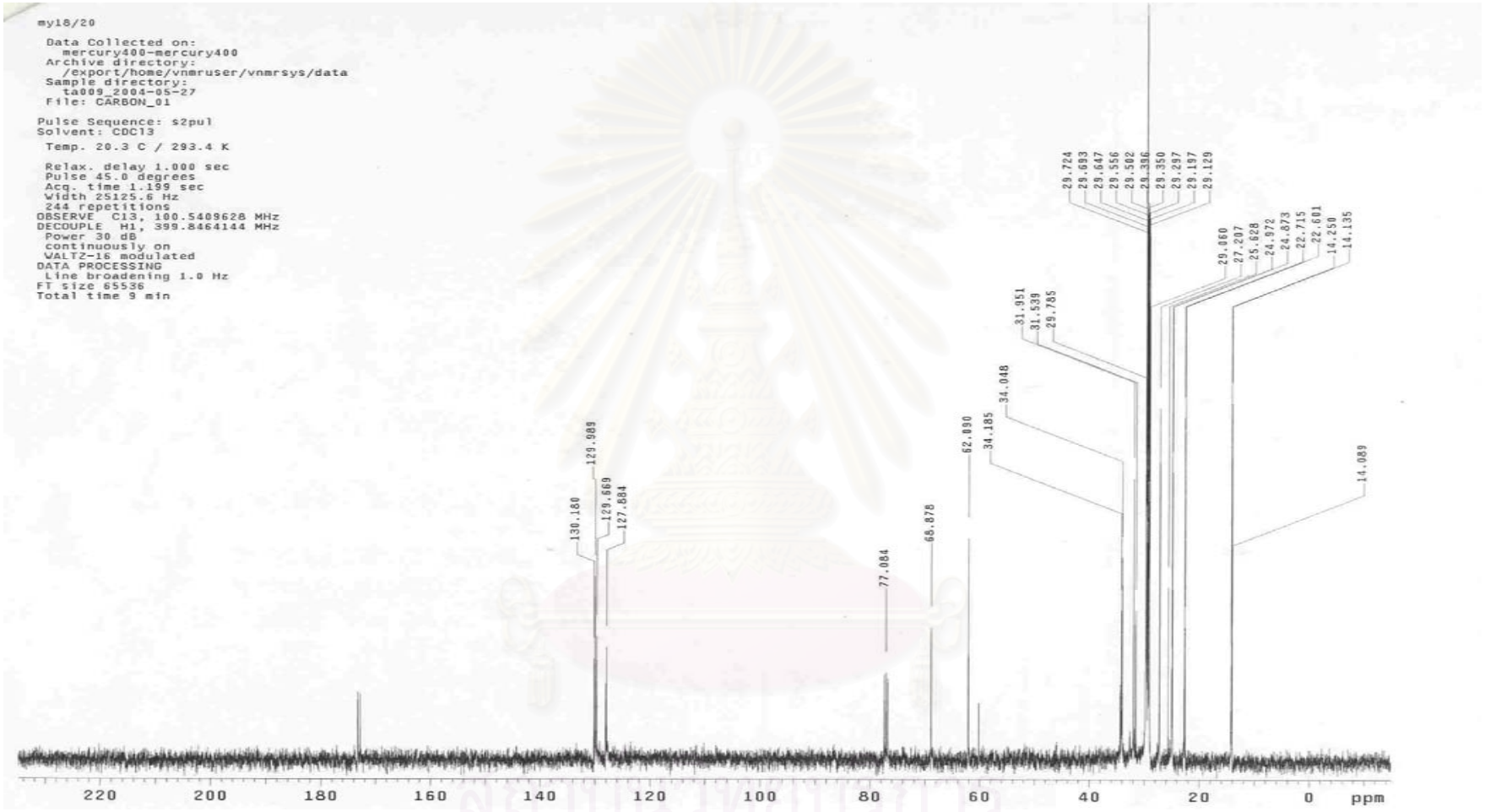


Figure C20 ^{13}C-NMR spectrum of mixture 4

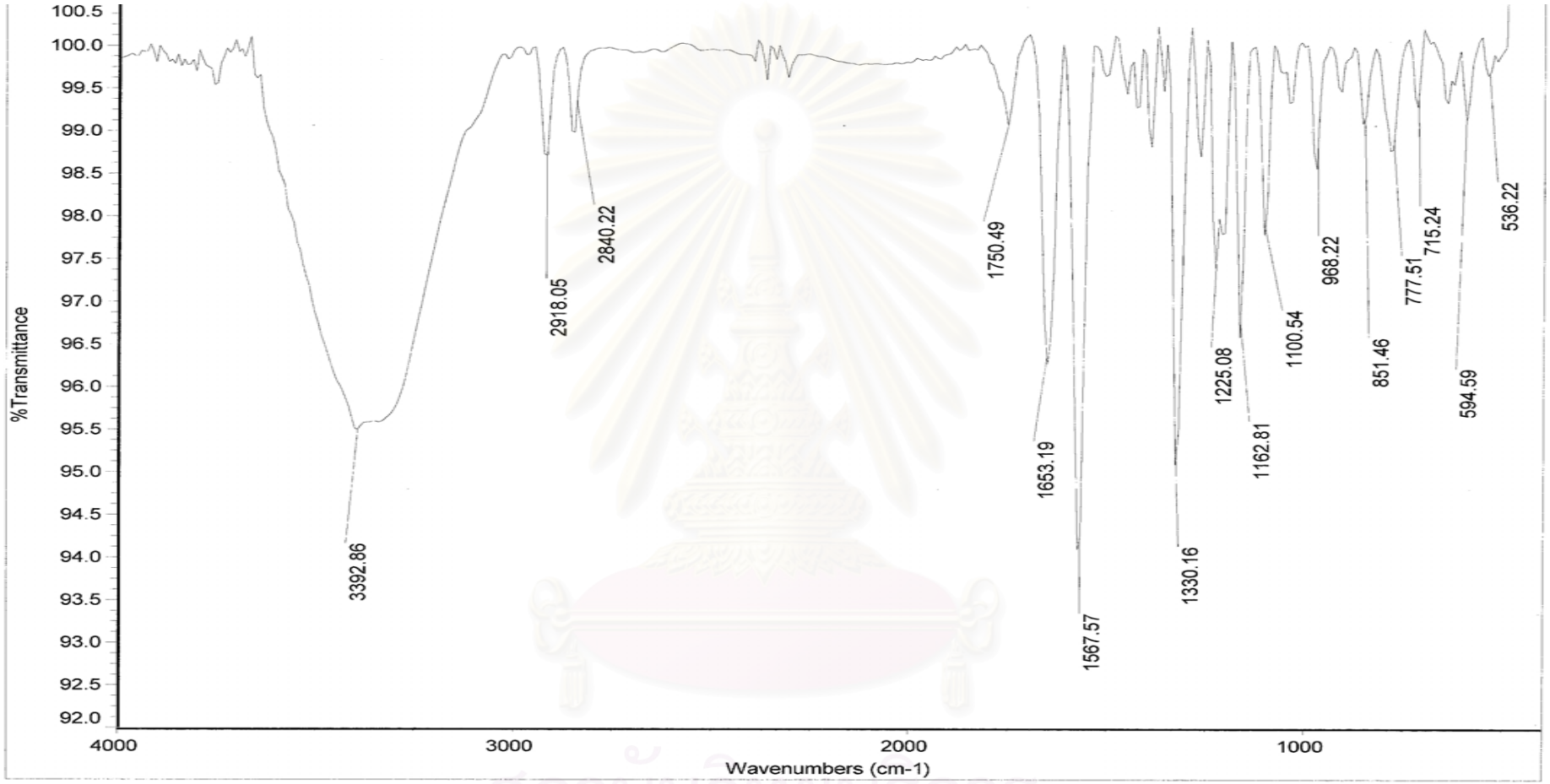


Figure C21 IR spectrum of mixture 5

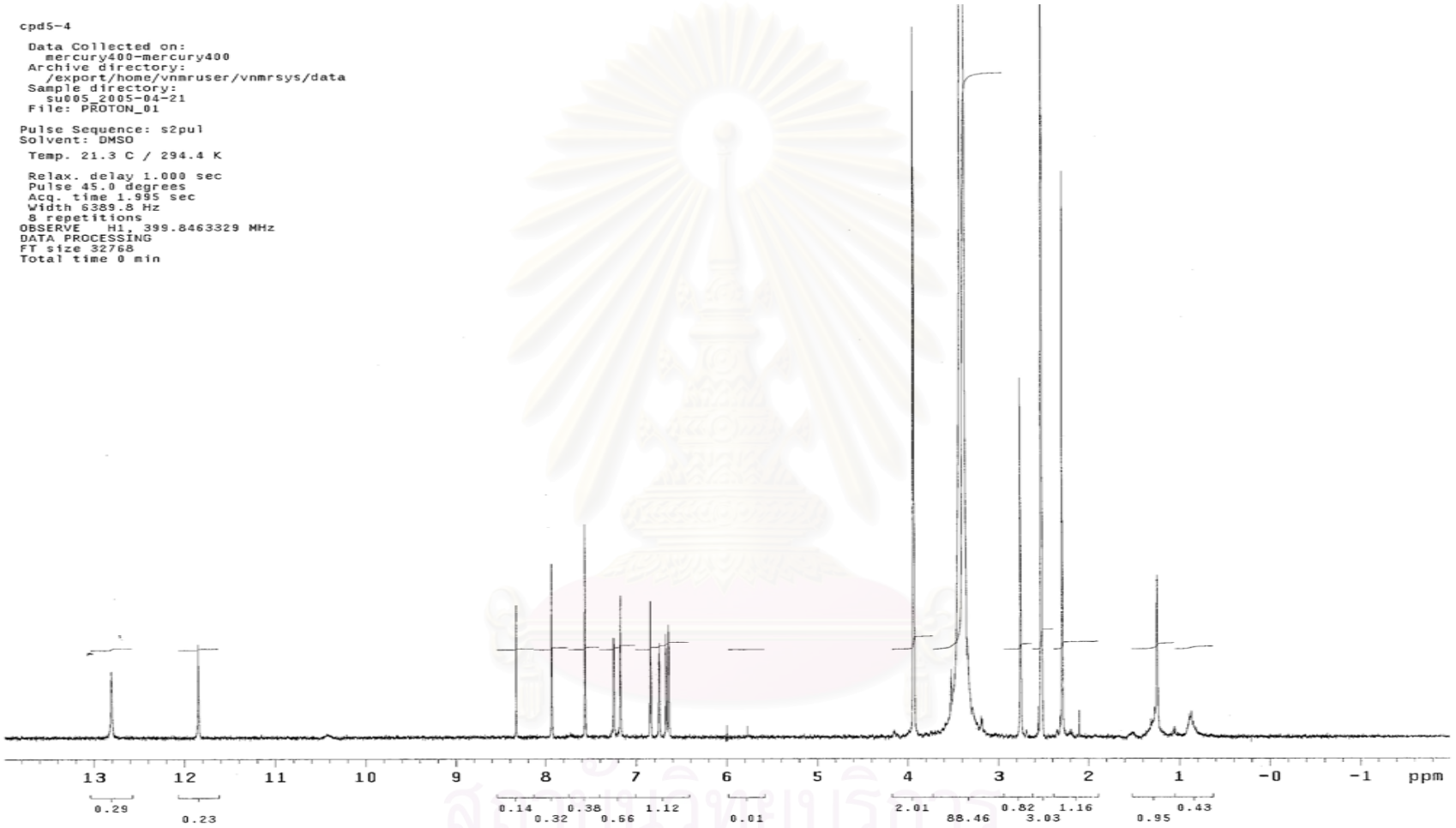


Figure C22 ^{1}H spectrum of mixture 5

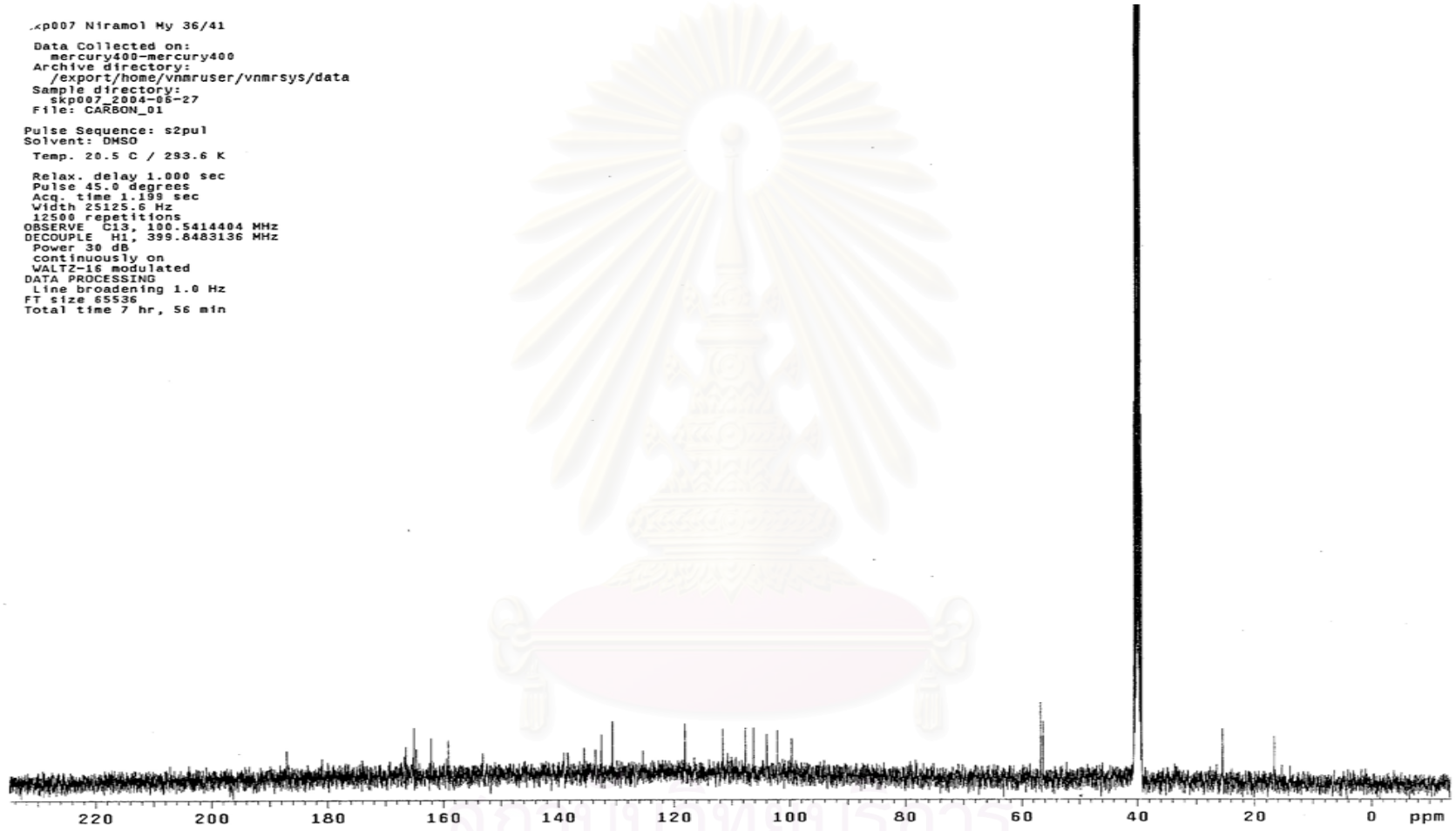


Figure C23 ^{13}C spectrum of mixture 5

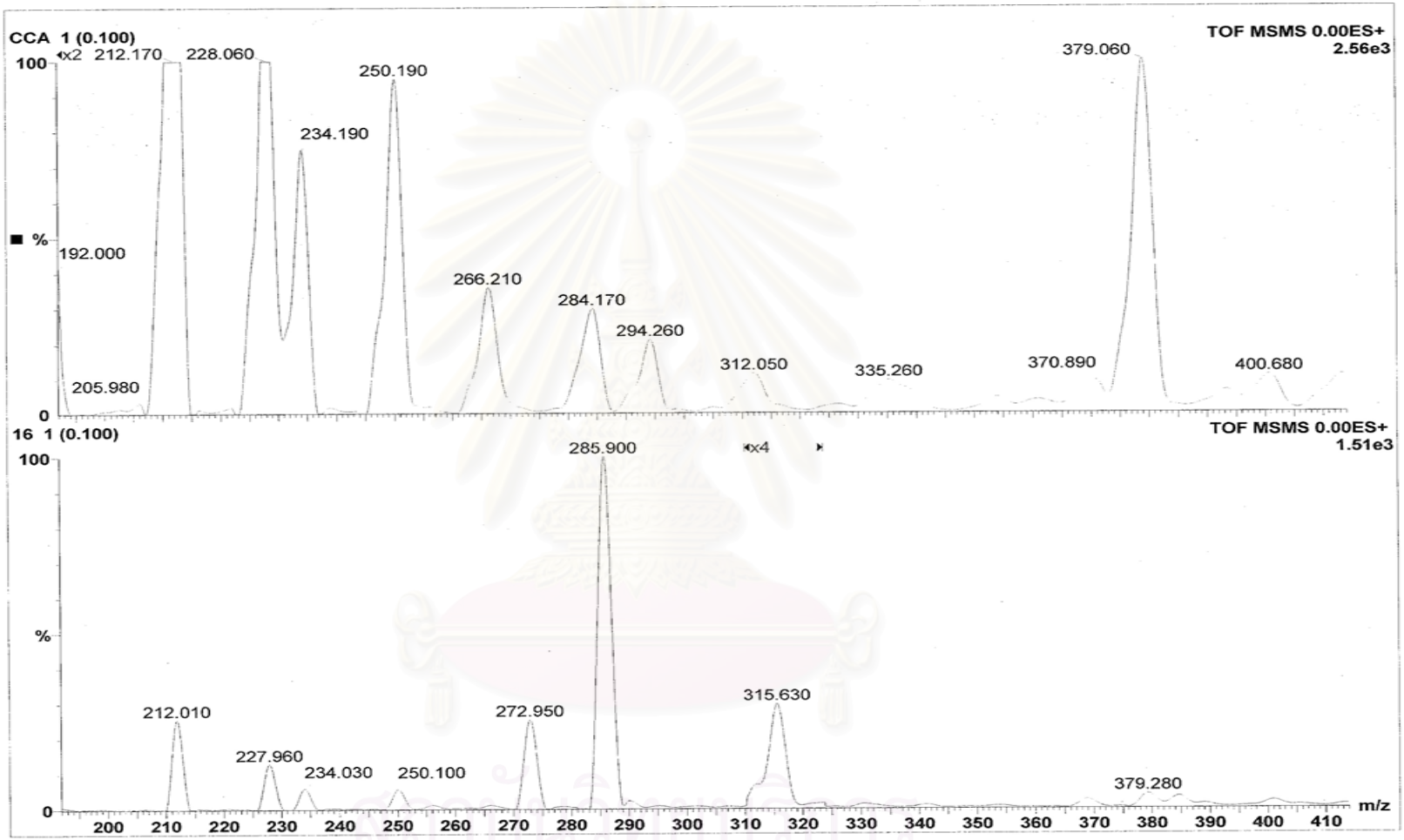


Figure C24 mass spectrum of mixture 5

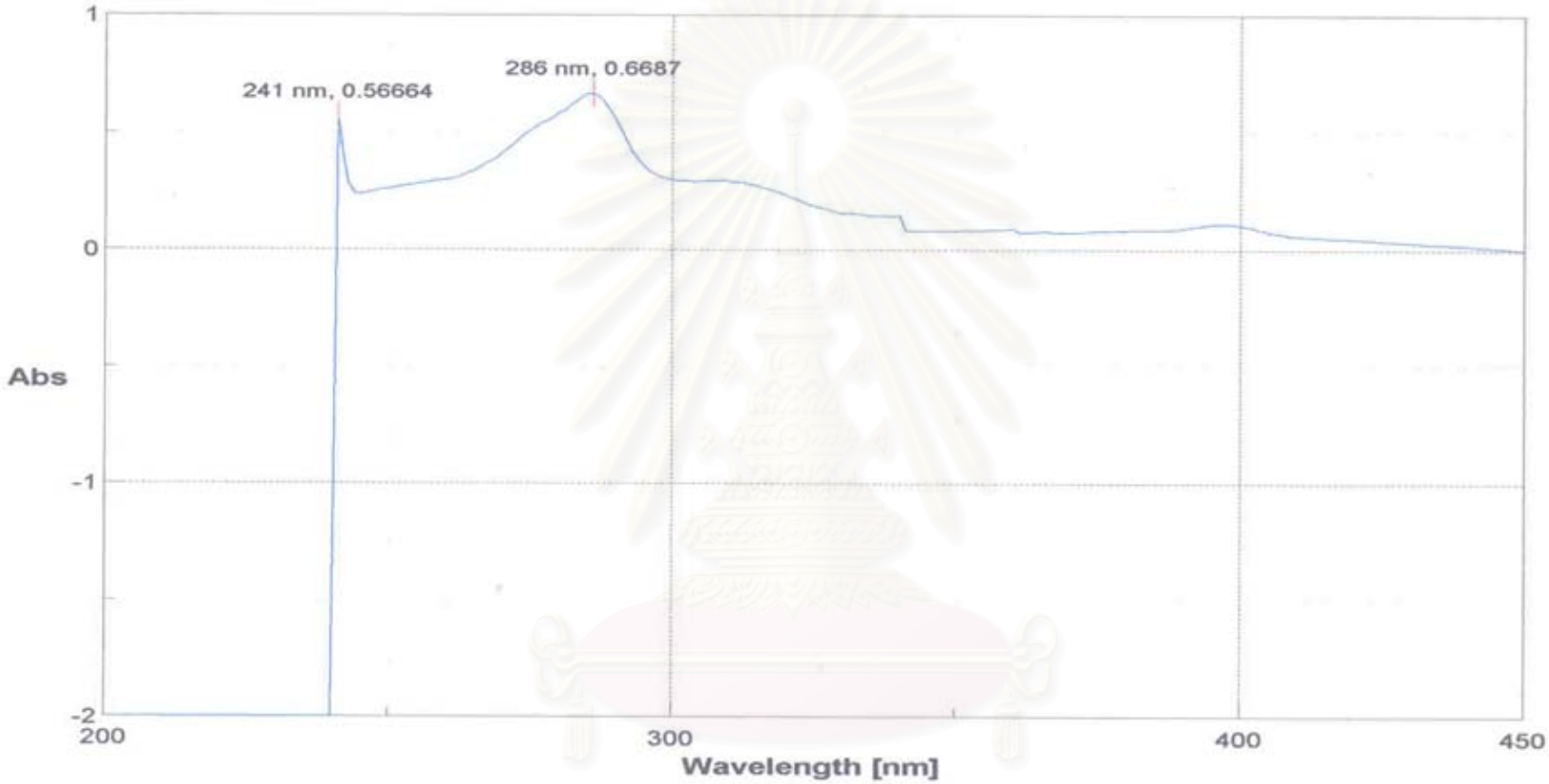


Figure C25 UV spectrum of mixture 5

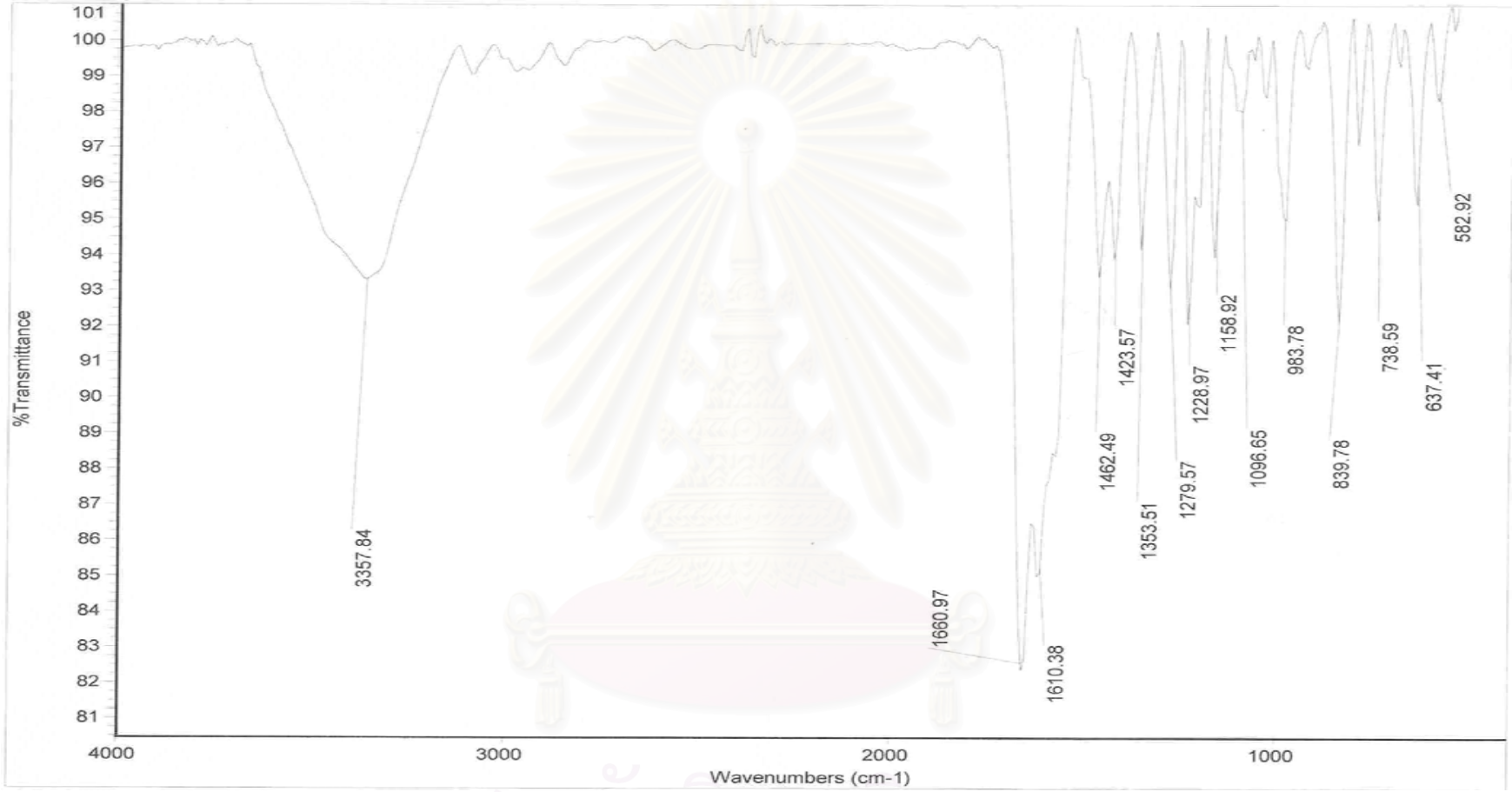


Figure C27 IR spectrum of compound 6

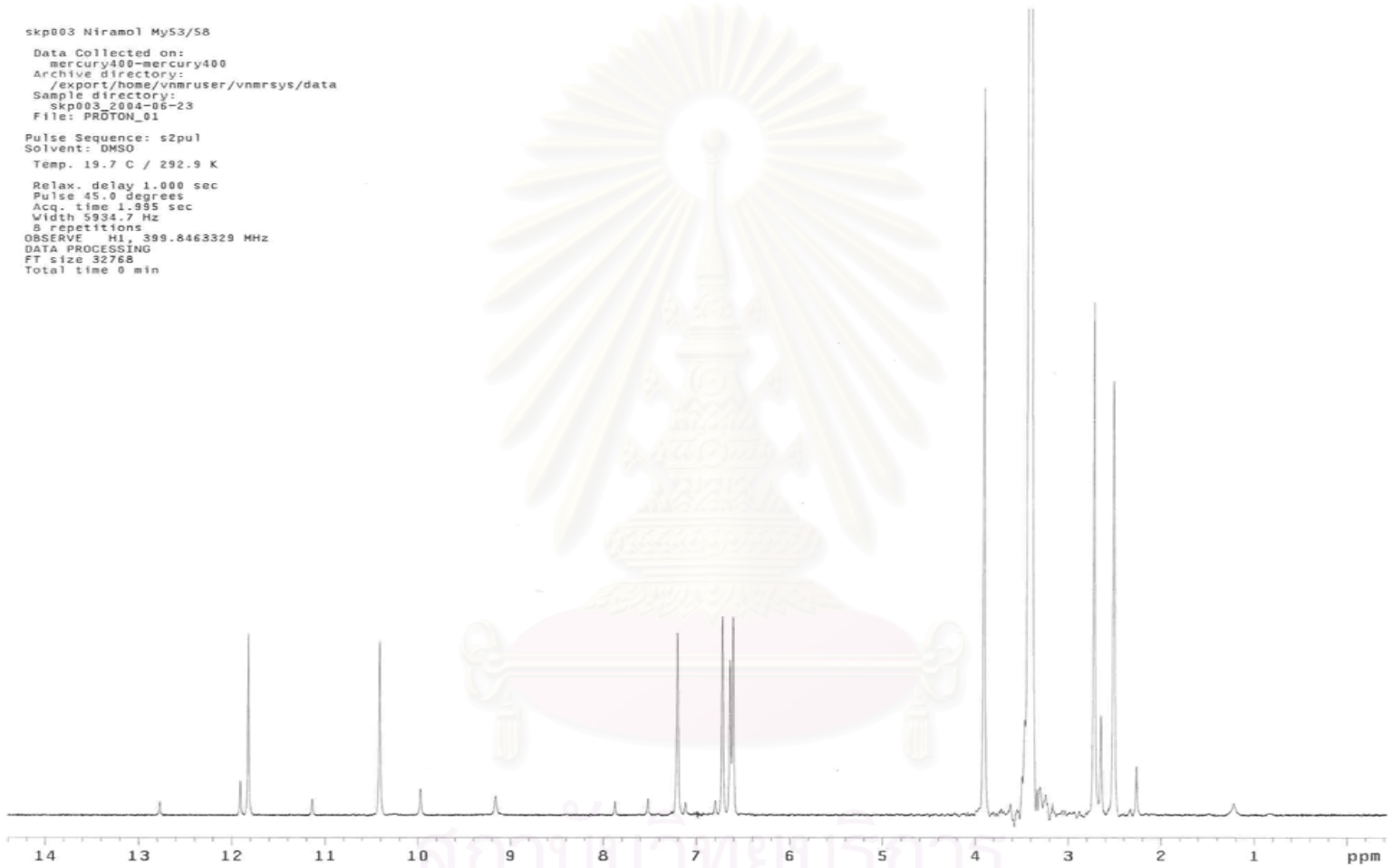


Figure C28 ^{1}H-NMR spectrum of compound 6

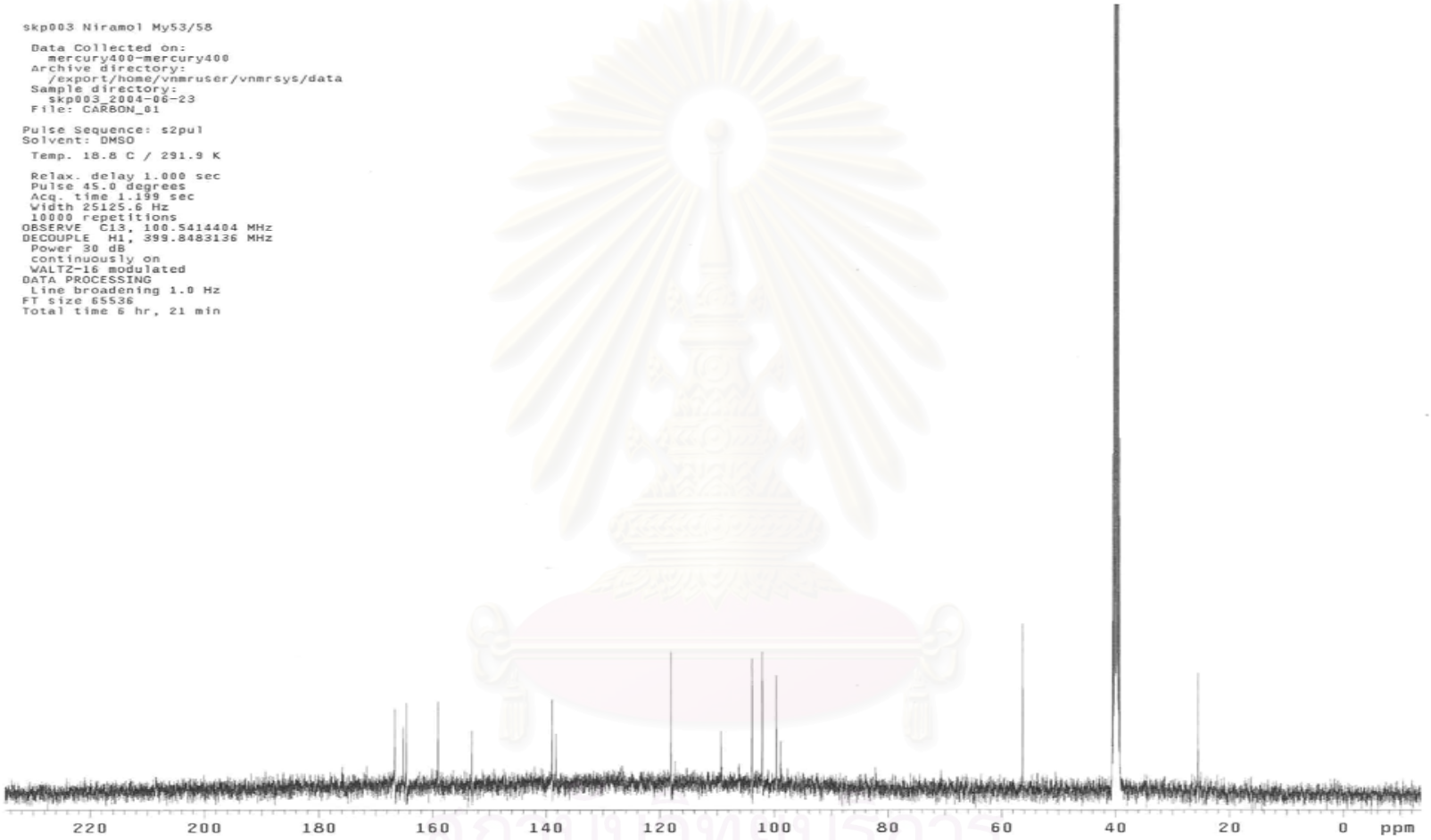


Figure C29 ^{13}C-NMR spectrum of compound 6

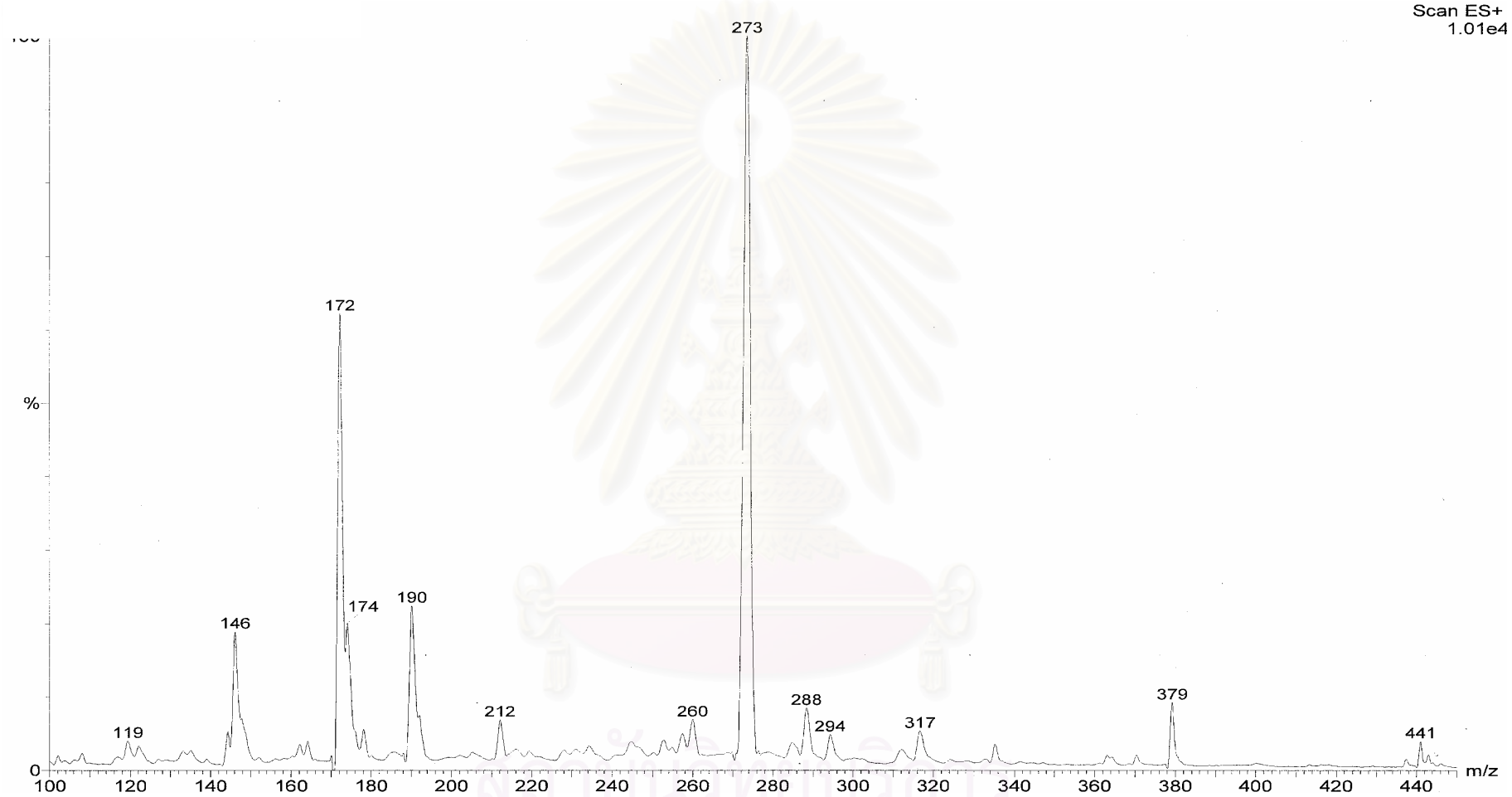


Figure C30 Mass spectrum of compound 6

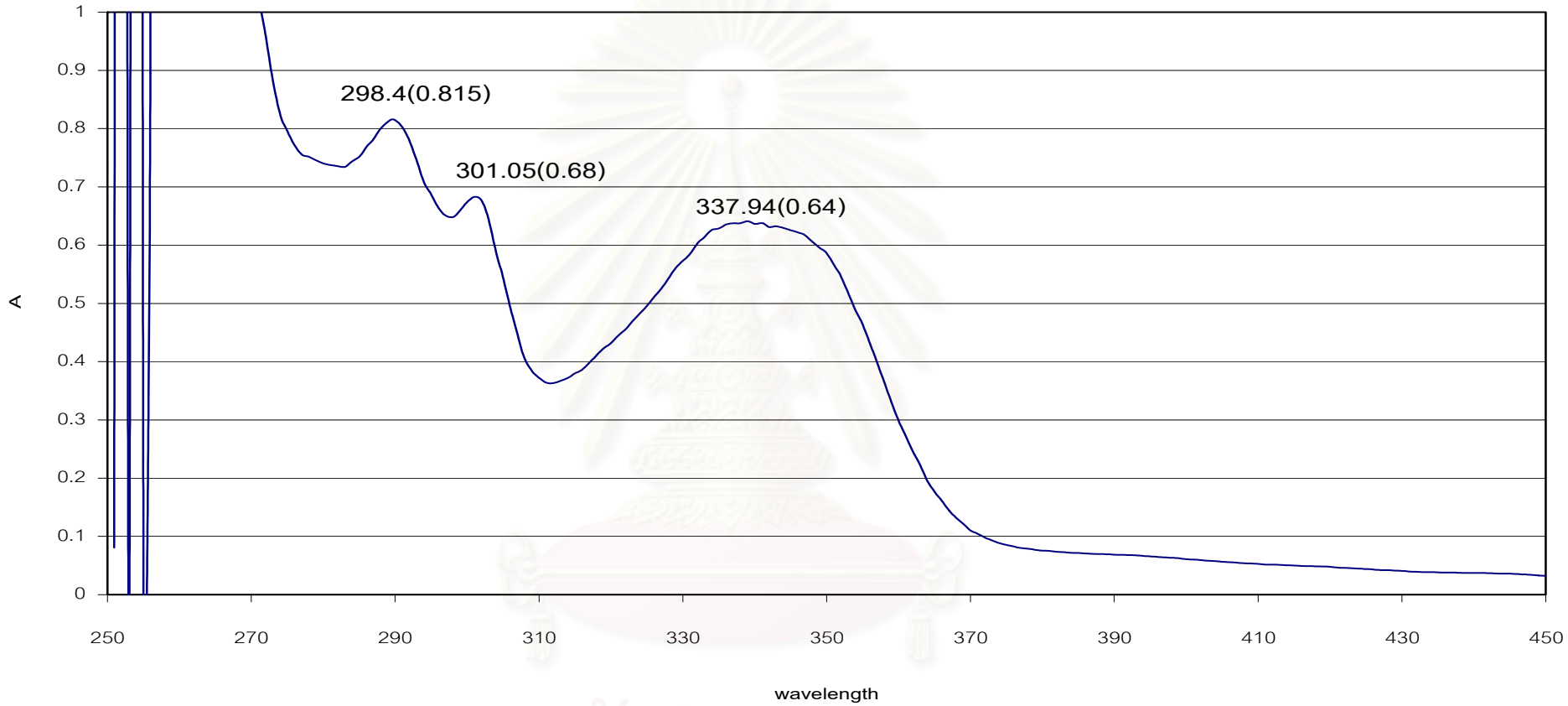


Figure C31 UV spectrum of compound 6

BIOGRAPHY

Miss Niramon Jankong was born on June 30, 1979 in Nakornsritammarat province, Thailand. She, graduated with Bachelor Degree of Science in General Science Department, from the faculty of Science, Kasetsart University, Thailand in 2001. She had been studying for a Master Degree of Science in Biotechnology. Faculty of Science, Chulalongkorn University, Thailand since 2002.