ราเอนโดไฟต์ที่แยกจากใบประยงค์ *Aglaia odorata* Lour.

นางสาวสุวรรณา จันทสุบรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ENDOPHYTIC FUNGI ISOLATED FROM *Aglaia odorata* Lour. LEAVES

Miss Suwanna Chantasuban

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นางสาวสุวรรณา จันทสุบรรณ : ราเอนโดไฟต์ที่แยกจากใบประยงค์ *Aglaia odorata* Lour. (ENDOPHYTIC FUNGI ISOLATED FROM *Aglaia odorata* Lour. LEAVES) อาจารย์ที่ปรึกษา : ศ. ดร.โสภณ เรืองสำราญ, อาจารย์ที่ปรึกษาร่วม : รศ. ดร.ประกิตติ์สิน สีหนนทน์, 142 หน้า. ISBN 974-17-6098-1.

งานวิจัยนี้ทำการแยกสารออกฤทธิ์ทางชีวภาพที่สร้างจากราเอนโดไฟต์ที่แยกจากใบประยงค์ *Aglaia odorata* Lour. นำใบประยงค์จากจังหวัดขอนแก่น มหาสารคาม กรุงเทพมหานคร และปัตตานี มาคัดแยกราโดยผ่านวิธีการฆ่าเชื้อที่พื้นผิวนอกและวางบน potato dextrose agar สามารถแยกราได้ทั้งหมด 60 ไอโซเลต ทำการทดสอบฤทธิ์ของราเอนโดไฟต์โดยวิธี dual-culture agar diffusion พบว่าราเอนโดไฟต์ส่วนใหญ่ (67%) มีฤทธิ์ต้านจุลินทรีย์สายพันธุ์มาตราฐาน อาหารเพาะเชื้อราต่างชนิดกันส่งผลถึงฤทธิ์ต้านจุลินทรีย์ของราเอนโดไฟต์ ราเอนโดไฟต์ที่มีฤทธิ์ต้านจุลินทรีย์มีจำนวนสูงเมื่อเพาะเลี้ยงบน yeast extract sucrose agar พบว่าราเอนโดไฟต์ไอโซเลต Aopn12 เมื่อเพาะเลี้ยงบน malt extract agar มีฤทธิ์ต้านจุลินทรีย์ได้กว้าง การศึกษาทางสัณฐานวิทยาพบว่าราเอนโดไฟต์ไอโซเลต Aopn12 ไม่สร้างสปอร์ จึงจัดเป็น Mycelia sterilia และการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ ITS ของ rDNA พบว่ามีความใกล้เคียงทางวิวัฒนาการกับ Oat root associated fungus OOO45 5 เมื่อทำการแยกสารออกฤทธิ์ทางชีวภาพ โดยเลี้ยงในอาหารเหลว malt extract broth แยกส่วนสกัดเอธิลแอซิเตตจากเส้นใยของราเอนโดไฟต์ไอโซเลต Aopn12 ได้ของผสม 2 ชนิดคือ ไดกลีเซอไรด์ (ของผสม 1) และกรดคาร์บอกซิลิกโซ่ตรงยาว C_{16}-C_{18} (ของผสม 2) และสารบริสุทธิ์ 1 ชนิดคือ ของแข็งสีส้ม (สาร 1) ซึ่งอาจมีสูตรโมเลกุลเป็น $C_{23}H_{31}NO_5$ และหาสูตรโครงสร้างของสารเหล่านี้ โดยอาศัยคุณสมบัติทางกายภาพ เทคนิคแก๊สโครมาโทกราฟี และเทคนิคทางสเปกโทสโกปี นำสารบริสุทธิ์ที่แยกได้มาทดสอบฤทธิ์ทางชีวภาพในการยับยั้งจุลินทรีย์และทดสอบความเป็นพิษต่อเซลมะเร็งคน 5 ชนิด พบว่าของแข็งสีส้ม (สาร 1) แสดงฤทธิ์ต้านแบคทีเรีย *B. subtilis* อย่างอ่อนด้วยค่า MIC 500 μg/ml และมีฤทธิ์ยับยั้งเซลล์มะเร็ง SW620 (ลำไส้ใหญ่) BT474 (เต้านม) KATO-3 (กระเพาะอาหาร) HEP-G2 (ตับ) และ CHAGO (ปอด) ได้สูง โดยมีค่า IC_{50} เท่ากับ 4.5, 5.4, 6, 5.4 และ 3.3 μg/ml ตามลำดับ

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

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SUWANNA CHANTASUBAN : THESIS TITLE. ENDOPHYTIC FUNGI ISOLATED FROM *Aglaia odorata* Lour. LEAVES THESIS ADVISOR : PROF. SOPHON ROENGSUMRAN, THESIS COADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, 142 pp. ISBN 974-17-6098-1.

The purpose of this research was to isolate bioactive compounds from endophytic fungi from *Aglaia odorata* Lour. leaves, Thai medicinal plant. Plant samples were collected from 4 provinces; Bangkok, Khonkean, Maha Sarakham and Pattani. Fungal endophytes were isolated from leaves by surface sterilization method and placed on potato dextrose agar. The total of 60 fungal isolates were obtained and tested for the production of antimicrobial compounds. By dual-culture agar diffusion assay, most of them (67 %) exhibited antimicrobial activities against the standard strains. Effect of culture media, yeast extract sucrose agar was found to be the medium of choice for used in cultivation of endophytic fungus isolates on expression of antimicrobial activities. Aopn12 and malt extract agar were chosen for the further study of bioactive compounds because the malt extract agar blocks of the isolate were active against a large number of test microorganisms. Based on morphology, the fungus isolate Aopn12 was found as Mycelia sterilia. Based on nucleotide sequencing of ITS region, it was closely related to Oat root associated fungus OOO45 5. Isolation of mycelium ethyl acetate crude of the fungus isolate Aopn12 gave diglyceride (mixture 1), C_{16-18} long chain carboxylic acid (mixture 2) and an orange solid which molecular formula may probably be $C_{23}H_{31}NO_5$ (compound 1). These structures were established on basis of spectroscopic analysis and GC analysis. Antimicrobial activities and cytotoxicity of the pure compound were tested. An orange solid (compound 1) was found to exhibit weak activity against *B. subtilis* with the MIC value of 500 μg/ml, and exhibit high cytotoxic activity against SW620 (colon), BT474 (breast), KATO-3 (gastric), HEP-G2 (hepatoma) and CHAGO (lung) cell line with IC_{50} 4.5, 5.4, 6, 5.4 and 3.3 μg/ml, respectively.

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

LIST OF ABBREVIATIONS (continued)

mg	milligram
min	minute
ml	milliliter (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	microliter
δ	Chemical shift
λ_{max}	the wavelength at maximum absorption (UV)
ν_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

So far more than 4,000 fungal metabolites are described (Dreyfuss and Chapela, 1994) and 5,000-7,000 taxonomic species have been studied with respect to their chemistry (Hawksworth, 1991). In 1995 Hawksworth estimated the probable number of existing fungi to be 1.5 million with only 71,000 being described so far. Apparently, the majority of fungi inhabiting the world has not yet been described. The implicates fungi to represent an enormous source for natural products with diverse chemical structures and activities. Of special interest are creative fungal strains. Creativity in this sense is defined as the ability to produce compounds of interest for human activities (Dreyfuss and Chapela, 1994). Fungi have been surveyed in various sources such as soil, marine, fresh water, litter, dung and decaying remains of plants and animals. Living plant is an interesting source for screening of new microorganisms. Fungi from special source like plant may also produce novel compounds possessing biological activities.

Over the years, a great deal of scientific attention has been given to economically important plants suffering from disease distress. However, it is now known that plants serve as a reservoir for an untold number of microbes known as endophytes (Bacon and White, 2000). Some of these endophytes produce various useful bioactive molecules, which has encouraged a worldwide scientific effort to isolate and study them. While there is a myriad of epiphytic microbes associated with plants, the endophytic ones seem to be attracting more attention possibly because they developed closer biological associations with these. The types of biological associations that endophytic microbes may have developed with higher plants range from borderline pathogenic, to commensal, and to symbiotic. In any of these situations, it is obvious that the minimum contribution of the plant to the endophyte is one of providing nutrition. However, it is also possible that the plant provides, to the endophyte, compounds critical for the completion of its life cycle or essential for growth or self-defense. In addition, one of the least studied, yet imaginable, roles of endophytic fungi is to initiate the biological

degradation of the dead or dying host plant, which begins the critical processes of nutrient recycling. On still another, more molecular biological front, it is likely that mechanisms exist for the transfer of nucleic acids from plants to endophytes, or vice versa, since some of the same relatively rare bioorganic molecules made by specific higher plants can be produced by certain endophytes as well.

In this research, *Aglaia odorata* Lour. leaf (Meliaceae) was employed as a plant source for isolation of endophytic fungi because *A. odorada* has been used as Thai medicinal plant, with various pharmacological activities. The leaves are used for treatment of menorrhagia, syphilis, cough, fever and itch. (นันทวัน และ อรนุช, 2539). Odorine and odorinol obtained from this plant has cancer chemopreventive activity (Inad et al., 2001). This study may lead to selection of endophytic fungus isolates that are a potential source of novel bioactive compounds.

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

1. To isolate endophytic fungi found within healthy mature leaves of *Aglaia odorata* Lour. from Bankok, Khonkaen, Maha sarakham and Pattani province.

2. To screen endophytic fungi isolated for antibacterial and antifungal activities.

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

4. To extract, isolate and purify the bioactive compounds of a selected endophytic fungus.

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

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

CHAPTER II

LITERATURE REVIEW

2.1 Endophytic fungi

2.1.1 **Definition of endophyte** (Stone, 2000)

The biological and ecological diversity of endophytes is reflected in varying emphasis and herterogeneity of concepts among researchers concerned with studying them. Often the terms “endophyte” and “endophytic” are used with particular meaning by different workers and for particular groups of hosts and microbes. Workers investigating asymptomatic fungal infections of grasses caused by species of Clavicipitaceae and those investigating other microbes, such as endophytic bacteria, have adopted the term “endophyte” and “endophytic” and application of the same terms to different systems has contributed to some confusion and controversy. Contemporary application of the terms is not always consistent or accepted among all workers, although as commonly used the terms generally apply to microbes capable of symptomless occupation of apparently healthy plant tissue.

Important papers published during the past 20 years or so by various authors have stressed particular concepts of endophytism. These have varied in degree of inclusiveness depending on whether primarily organismal interactions or descriptive ecology of endophyte assemblages was emphasized, and on whether the research methodology was primarily histological or based on cultural isolations. The use of “endophyte” and “endophytic” in these varied contexts has contributed to a sense of ambiguity in the application of the terms.

Researchers with a primarily organismal focus have attempted to characterize endophytes in term of particular ecological or physiological attributes, often emphasizing aspects of host specificity and adaptation. Examples of studies that have focused attention on particular mechanisms of host recognition, attachment, and initial infection include studies on selective adhesion and germination behavior of the fungus

Discula umbrinella on leaves and of the fungus Hypoxylon fragiforme on Fagus sylvatica.

Alternatively, investigators whose primary interest is in descriptive ecology, biodiversity studies, or community analysis broadly include all microbes found inhabiting host tissue, often including ruderal saprobe and species familiar to plant pathologists as latent, quiescent, or opportunistic pathogens among lists of endophytes. Much of the early interest in endophyte infections emphasized their symtomless nature and invited comparison with mutualisms such as mycorrhizal associations. Over the past several years, recognition of the herterogeneity of endophyte - host interactions has gradually broadened so that mutualism is not commonly considered to be an assumed feature of endophyte interactions. Interactions other than mutualistic interactions in endophytes are recognized and anticipated.

Among the definitions proposed for the term endophyte are “fungi colonizing plant tissue without causing any immediate, overt negative effects”. This definition includes virtually the entire spectrum of symbiotic interactions in which fungi and plants participate: parasitism, commensalism, and mutualism. This definition, however, fails to include prokaryotic microbes, such as bacteria and blue-green algae, or endophytic vascular plants. A more inclusive definition of “endophyte” should stress the symptomless nature of the infection on the host without limiting the term to any particular group of organisms. Latent and quiescent pathogens are endophytes as are mutualistic microbes and benign commensals. Petrini considers the term endophyte to be purely topographical: “Endophytes colonize symtomlessly the living, internal tissues of their host, even though the endophyte may, after an incubation or latency period, cause disease”. This latter definition is broad enough to include virtually any microbe or vascular plant that colonizes the internal tissues of plant

2.1.2 Endophytic fungi

Endophytic fungi are to be found in almost all plants. These include trees, grass, algae, and herbaceous plants. Most endophytic fungi belong to the

Ascomycetes and Fungi imperfecti. Under normal circumstances they live within the host plant without causing any noticeable symptoms of disease (Konig et al., 1999) Inter- and intracellular colonization of plant tissue with endophytic fungi is shown in Figure 2.1

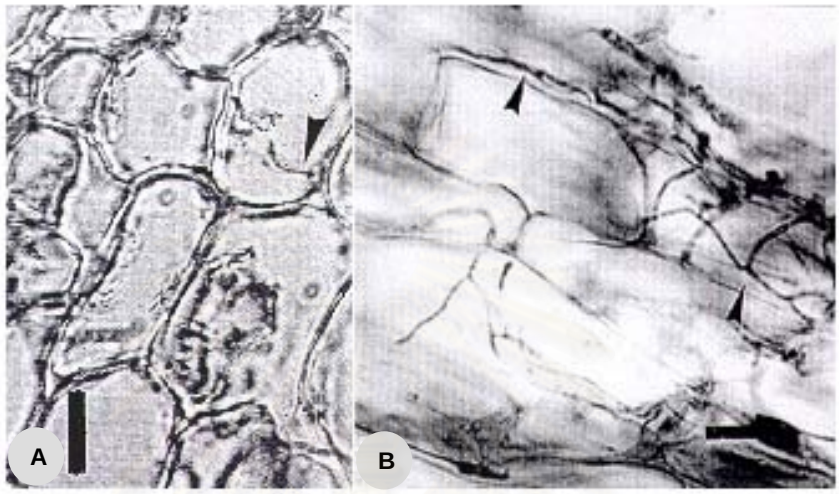


Figure 2.1 Inter- and intracellular colonization (arrows) of larch with *Cryptosporiopsis* sp. (A) and of barley with *Fusarium* sp. (B). Scale bars = 20 μm (Schulz et al., 1999)

Furthermore, the endophytic fungi are not considered as saprophytes since they are associated with living tissues, and may in some way contribute to the well being of the plant. That is, the plant is thought to provide nutrients to the microbe, while the microbe may produce factors that protect the host plant from attack by animals, insects or microbes (Yang et al., 1994). When the host is stressed, however, some endophytic fungi may become pathogenic. The delicate equilibrium between host and endophytic fungus seems to be controlled in part by chemical factors, for example, herbicidal natural products produced by the fungi versus antifungal metabolites biosynthesized by the host plant. Meanwhile, there are also documents demonstrating that many antitumor agents, such as taxol could be produced by endophytic fungi (Strobel et al., 1996; Wang et al., 2000). Thus, the endophytic fungi are expected to be a potential source for new natural bioactive agents.

2.2 Study of secondary metabolites from endophytic fungi

Over the years, a great deal of scientific attention has been given to economically important plants suffering from disease distress. However, it is now known that plants serve as a reservoir for an untold number of microbes known as endophytes (Bacon and White, 2000). Some of these endophytes produce various useful bioactive molecules, which has encouraged a worldwide scientific effort to isolate and study them. The study of plant-associated microbes, in general, may offer opportunities for discoveries relating to agriculture, industry, and medicine.

Strobel (2002) recently reviewed endophyte microbes isolated from various higher plants from a wide range of rain forests, such as endophyte producing taxol, endophytes producing antimycotics and endophytes with other important biological activities.

2.2.1 Endophytes producing taxol (Strobel, 2002)

Pestalotiopsis spp. are some of most commonly isolated endophytic fungi of rain forest plants. One of the most common species of *Pestalotiopsis* is *P. microspora.* Organisms virtually identical to the taxonomic description of *P. microspora* are numerous and have usually been isolated as leaf and stem pathogens of economically important tropical plants, such as the palms, pines, loquats, guavas, mangoes, and a large number of ornamental plants. Generally, this fungus is considered a relatively weak plant pathogen, which, at times, acts in a more severe manner to cause major plant loss. The widely held view that this is a relatively obscure fungal group of interest only to tropical pathologists should undoubtedly be revisited. It seems that this fungus and its close relatives are not as important as plant pathogens as they are in playing some role as endophytic fungi living in symbiotic relationships with plants in each of the world's temperate and tropical rain forests.

Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species. This compound is the world's first billion dollar anticancer drug

and is used to treat a number of other human tissue proliferating diseases as well. Its high cost makes it unavailable to many people in the world. Therefore, alternative sources are needed since organic synthesis, while having been accomplished, is not yet economically feasible. A few years ago, they reasoned that yew trees may support certain endophytic microbes that also make taxol. Thus, if a microbial source of the drug would be available, it would eliminate the need to harvest and extract the slow growing and relatively rare yew trees for this drug. The price for the drug would then be reduced, since taxol would conceivably be produced via fermentation in much the same way that penicillin is produced. After several years of effort, a taxol-producing endophytic fungus, *Taxomyces andreanae*, was discovered (Strobel et al., 1993; Stierle et al., 1993). They have now isolated taxol-producing fungi from virtually all yew species, and a myriad of trees in each of the major rain forests of the world (Li et al., 1996; Strobel et al., 1996). Efforts of several pharmaceutical companies are now underway to determine the feasibility of making microbial taxol a commercial reality.

The studies on *P. microspora* have led to a critical examination of other aspects of its biology. One extremely interesting feature of *Pestalotiopsis* spp. is the wide genetic and biological diversity. For instance, from one small cypress limb, they obtained 21 isolates of *P. microspora* (Li et al., 1996). Only two appeared to be identical in all cultural and biological respects. Of these isolates, nine isolates produced taxol. In another study, they examined the methylene chloride extract of 15 isolates of *P. microspora*, obtained from at least four continents, by thin layer chromatography and observed that no two chromatograms were identical. They have other indications that enormous variability must exist in this organism, arising through mutation, genetic crossing, or as yet unsubstantiated mechanisms such as genetic exchange with its hosts. It also appears that *P. microspora* is a microbial factory of bioactive secondary metabolites.

One such secondary metabolite is ambuic acid, an antifungal agent, which has recently been described from several isolates of *P. microspora* obtained in many of the world's rain forests (Li et al., 2001). Ambuic acid is only one of a number of

novel secondary products that have recently been isolated and described from *P. microspora*. Torreyanic acid, a selectively cytotoxic quinone dimer, was isolated from *P. microspora* originally found as an endophyte associated with the endangered tree *Torreya taxifolia* Arn. (Florida torreya) (Lee et al., 1996). Other products of this same fungus included pestaloside, an aromatic β-glucoside, and two pyrones, pestalopyrone and hydroxypestalopyrone (Lee et al., 1995). These products also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. micro*spora (endophytic on *Taxus brevifolia* Nutt.) include two new caryophyllene sesquiterpenes (pestalotiopsins A and B) (Pulici et al. 1996). Other novel sesquiterpenes produced by this fungus are 2-α-hydroxydimeninol and a highly functionalized humulane (Pulici et al., 1996). Variation in the amount and kinds of products found in this fungus is dependent upon the cultural conditions of the organism as well as the original plant source from which it was isolated.

2.2.2 Endophytes producing antimycotics

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. This fungus and related species occur as endophytes in many parts of the world. It was isolated as an endophyte from *Tripterygium wilfordii* Hook. f., a medicinal plant native to Eurasia (Strobel et al., 1999). On Petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens (*Candida albicans* and *Trichophyton* spp.). Since infections caused by such fungi are a growing health problem, especially among AIDS patients and those who are otherwise immunocompromised, new antimycotics are needed. A unique peptide antimycotic, termed cryptocandin, was isolated and characterized from *C. quercina*. This bioactive compound is related to known antimycotics, the echinocandins and the pneumocandins, and was also active against a number of plant pathogenic fungi, including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Cryptocandin is currently being considered for applications against several of the fungi that cause human diseases. Cryptocin, a tetramic acid, is also produced by *C. quercina*. This unusual compound

possesses potent activity against *Pyricularia oryzae* and a number of other plant pathogenic fungi (Li et al., 2000). It was ineffective against a general array of human pathogenic fungi. Nevertheless, with minimum inhibitory concentrations of 0.39 μg/mL against *P. oryzae*, this compound is being examined as a natural chemical control agent for rice blast. Given the general interest on the part of the public for industry to find safer and more environmentally compatible plant disease control agents, perhaps endophytic fungi do serve as a reservoir of untapped biologically based compounds that may also help agriculture.

2.2.3 Endophytes with other important biological activities

Biologically important compounds from endophytic fungi will be found only when assay systems will be devised to allow biologically guided fractionation of the culture extracts. Despite an obscure role to either the endophyte or the host–endophyte relationship in most cases, one or more bioactive compounds are still produced. Such is the case of subglutinols A and B, which are immunosuppressive compounds produced by *Fusarium subglutinans*, an endophyte of *Tripterygium wilfordii* (Lee et al., 1995). The compounds both have mean inhibitory concentration (IC_{50}) values of 0.1 μM in the mixed lymphocyte reaction assay. In the same assay system, cyclosporin is roughly as potent as the subglutinols. These compounds are being examined more thoroughly for their ability to serve as immunosuppressive agents.

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

2.3 Plant sample : *Aglaia odorata* Lour.

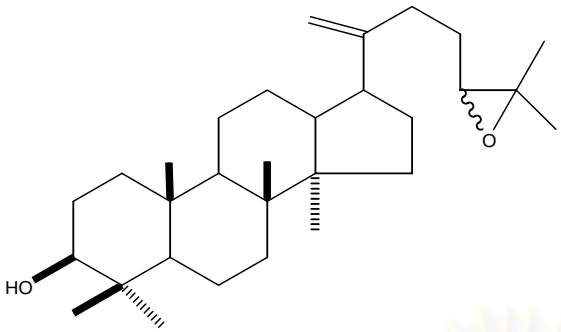
Aglaia, Lour is belongs to the family Meliaceae. Trees often large, bushes or shrubs, glabrous, pubescent or scaly. Leaves pinnate. Flowers in panicles usually bisexual very small, more or less globose and yellow, numerous. Calyx 5-lobed imbricate in bud. Petals 5 imbricate short. Stamen-tube sub-globose, 5-toothed or nearly entire; anthers usually 5, rarely 4 or 10 included or partially exsert. Disc minute or 0.

Ovary ovoid 1 to 3 celled with 1 or 2 ovules in each cell. Fruit 1 or 2 celled and seeded indehiscent with a thin pericarp. Seed with a pulpy integument. (Ridley,1967)

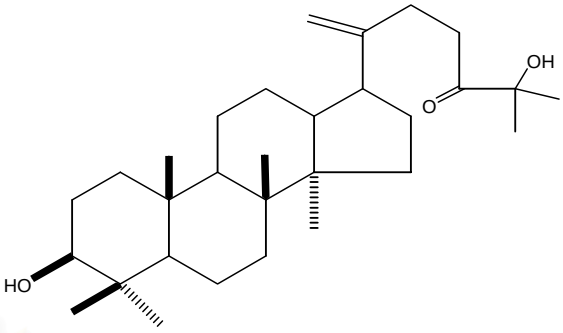
Aglaia odorata Lour. (Figure 2.2) is a small tree with deep green leaves and very small fragrant yellow flowers found in Thailand, Malaysia, China and The Philippines. In China, it is often cultivated by chinese for its flowers, which are put in their tea to flavour it (Ridley,1967). In Thailand, it is commonly known as Pra-yong or Hom-glai (ธงชัย และ นิวัตร, 2544). The sample plant is traditionally used as a herbal remedy for pectoral, stimulant, antipyretic, tonic and remedy for convulsion (Ponglux, 1987), and as an expectorant (Phaetthanesuan, 1972).

Figure 2.2 Leaves of *Aglaia odorata* Lour.

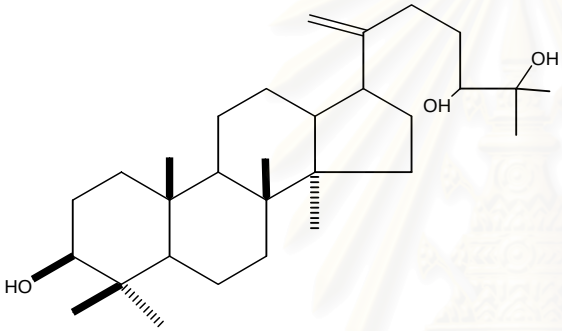
Phytochemical examination of A. odorata leaves have yeilded the tetracyclic triterpenes, aglaiol [1], aglaiondiol [2] and two isomers of aglaitriol [3] and namely myricyl alcohol and β-sitosterol [4] (Shiengthong et al., 1974), and the nitrogenous compounds, odorine [5] and odorinol [6] (Shiengthong et al., 1979). A. odorata twigs have found the flavonoid lupinofilin [7] and the benzofuran rocaglamide [8] (Janprasert et al., 1993). Several compounds were found from flowers of *A. odorata* Lour. such as ceryl alcohol, β-sitosterol and odoram [9] (Shiengthong and Techasauvapak, 1982). Structure of some compounds from *Aglaia odorata* Lour is shown in Figure 2.3.



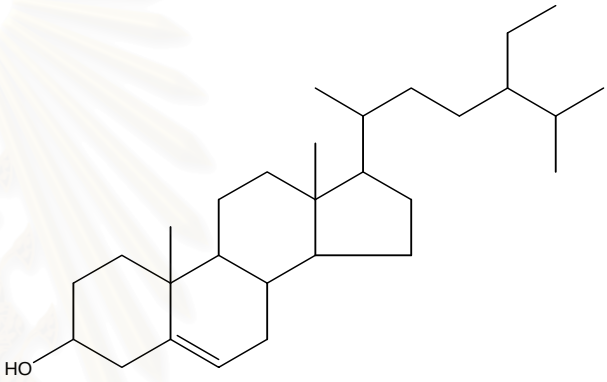
Aglaiol [1]



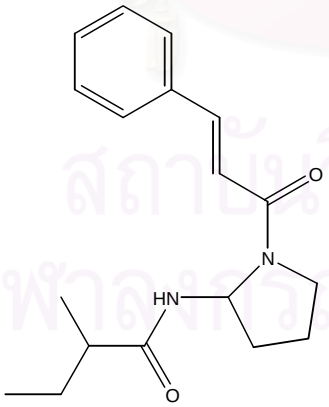
Aglaiondiol [2]



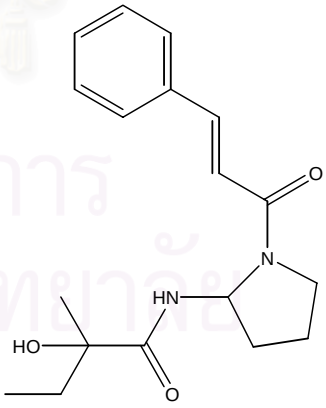
Aglaitriol [3]



β-sitosterol [4]

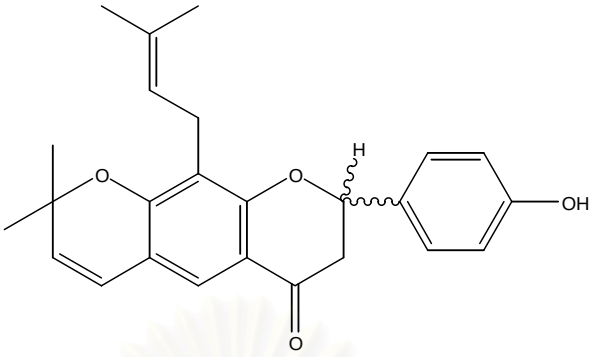


Odorine [5]

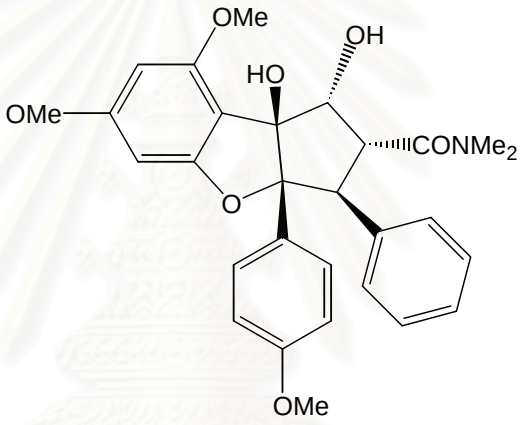


Odorinol [6]

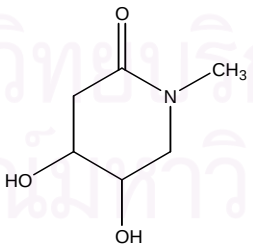
Figure 2.3 Structure of some compounds from *Aglaia odorata* Lour.



Lupinofilin [7]



Rocaglamide [8]



Odoram [9]

Figure 2.3 Structure of some compounds from *Aglaia odorata* Lour. (continued)

CHAPTER III

EXPERIMENTS

3.1 Plant samples collection

Healthy young Pra-yong (*Aglaia odorata* Lour.) leaves were collected from many provinces in Thailand including Chulalongkorn University, Bangkok; Amphoe Maung, Khonkaen Province ; Amphoe Maung, Mahasarakham Province and Amphoe Sai Buri, Pattani Province during April - July 2001. The leaf samples were kept in a plastic bag and stored in a refrigerator. The samples were processed within 48 hours of collection.

3.2 Culture media

Media were used for isolation was Potato Dextrose Agar (PDA). Malt Czapek Agar (MCA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud Glucose Agar (SGA) 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). Media for growing bacterial were Nutrient Agar and Broth. Sabouraud Glucose Agar and Broth were used for growing yeasts. The formula for media composition was shown in Appendix B.

3.3 Isolation of Endophytic fungi

Endophytic fungi were isolated by using the surface sterilization method. The leaf samples were washed in running tap water and dried in laminar air flow. From each leaf, the leaf was cut into 7 mm of diameter disks from the middle, rib, and lamina and leaf segments. The disks were surface sterilized by sequential immersion in 95% ethanol for 1 min, in Chlorox (5% available chlorine) for 3 min, in 95% ethanol for 30 sec, rinsed twice with sterile distilled water and allowed to surface-dry on sterile filter papers. This method of surface sterilization was modified from the method described by Blodgett et al., (2000)

The sterile leaf disks were placed in Petri dishes containing potato dextrose agar (PDA). The Petri dishes were incubated at room temperature (25-30°C) and examine periodically for fungal mycelium from leaves under stereomicroscope. Outgrowing mycelia were purified and transferred into Petri dishes containing PDA. They were incubated for 10 days at room temperature and purity was determined by colony morphology. Fungal isolates were used for further study.

3.4 Screening of endophytic fungi for their antimicrobial activities

Screening of endophytic fungi for their antimicrobial activities by dual-culture agar diffusion assay was modified from the method described Sriubolmas et al. (2001).

3.4.1 Test microorganisms for antimicrobial assay

The test microorganisms are listed in Table 3.1.

Table 3.1 Test microorganisms for antimicrobial assay

Type of 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

3.4.2 Cultivation of endophytic fungal isolates

Endophytic fungus isolates were grown on MCA, MEA, PDA, SGA and YES at room temperature. After cultivation for 10 days, they were photographed for characterize endophytic fungi. The mycelial cultures were then cut into 7 mm diameter of small cylinder by a flamed cork hole borer for antimicrobial activities test.

3.4.3 Preparation of bacterial and yeast tested inoculum

Test bacteria were grown in Nutrient Broth (NB) 37°C for 2-6 h, depending on the growth rate. The bacterial cultures were adjusted with nutrient broth to match with that of 0.5 McFarland standard (OD 0.1 at 625 nm).

C. albicans and *S. cerevisiae* were grown in Sabouraud Glucose Broth (SGB) at room temperature (25-30°C) for 2-6 h. The yeast cultures were adjusted with sabouraud glucose broth to match with that of 0.5 McFarland standard (OD 0.1 at 625 nm).

3.4.4 Testing for antimicrobial activities

A sterile cotton swab was dipped into the microbial suspension and presses lightly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab was then streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60°C each time to ensure an even distribution of the inoculum. The agar blocks, from section 3.4.2, were distributed evenly on the surface. The plates were left at room temperature for 1 h. Bacteria and yeast plates were incubated at 37°C and room temperature (25-30°C), respectively for 24 h. The inhibition zones around the agar blocks were measured in mm with a ruler.

3.5 Identification of selected endophytic fungus isolate Aopn12

Identification of the isolated endophytic fungus was done by both morphological characteristic and molecular identification.

3.5.1 Colonial morphology identification of endophytic fungus isolate Aopn12

Colony morphology of endophytic fungus isolate Aopn12 was determined by growing the fungus at room temperature (25-30°C) for 2 months on different media, such as MCA, MEA, PDA, SGA and YES. The fungal culture was examined for fruiting body production under a stereomicroscope. Wet mount of the

fungal culture with lactophenol cotton blue was examined for spore production under a light microscope.

3.5.2 Molecular Identification of endophytic fungus isolate Aopn12

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.

B. ITS amplification

The ITS region of isolated endophytic fungus was amplified with the primers ITS1f (Gardes and Bruns, 1994), and ITS4 (White *et al.*, 1990). One primer was labeled with Texas red fluorescent dye (Genset KK, Kyoto, Japan) at the 5'-end for analysis with a sequencer. The primer pairs comprised labeled ITS1f and ITS4, ITS4, ITS1f and labeled ITS4. Fragments amplified by primers ITS1f and ITS4 were designated as ITS_{1f-4}. Twenty microliters of reaction mixture contained 5 ng template DNA, 0.2 mM each dNTP, 1xPCR buffer, 1.5 mM $Mg2^{+}$, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 µM of the primer pair. The amplification

reactions were performed in a thermal cycler (TP 3000; Takara Shuzo, Tokyo, Japan). Amplification was started at 94°C for 9 min, followed by 38 cycles of a denaturing step at 94°C for 1 min, an annealing step at 51°C for 1 min, and an extension step at 72°C for 1 min, and ended with an additional 5-min extension step at 72°C. Two kinds of labeled ITS_{1f-4} were used in the following terminal-RFLP analysis (Zhou and Hogetsu, 2002). Three microliters ITS_{1f-4} was digested with 5U restriction endonuclease (*Alu*I or *Hin*fI) at 37°C for 8 h. After tenfold dilution, polymerase chain reaction (PCR) products of ITS and their restricted fragments were denatured at 94°C for 5 min and eletrophoresed on 6% Long Panger acrylamide gels (FMC Bioproducts, Pockland, ME, USA), with 6.1 M urea, and 1,2xTBE (0.1 M Tris (hydroxymethyl) aminomethane, 3.0 mM ethylene diaminetetraacetic acid (EDTA), and 0.1 M boric acid), in a sequencer (SQ-5500E; Hitachi, Tokyo, Japan) (Kanchanaprayudh et. al., 2003).

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

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

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

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

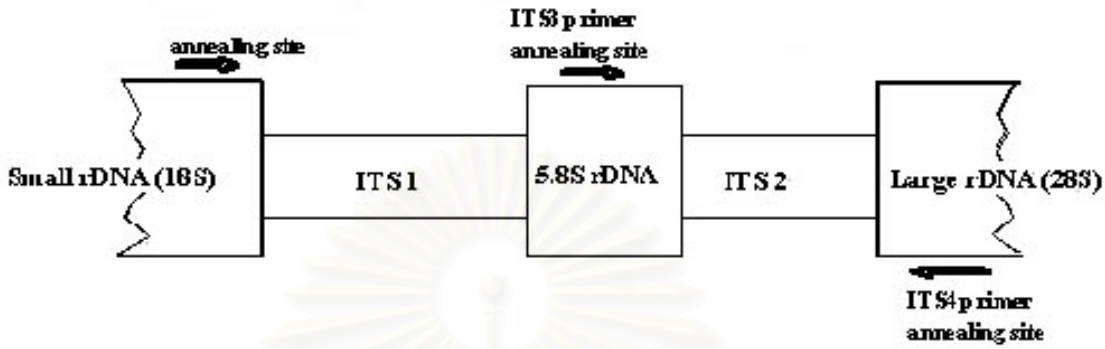


Figure 3.1 ITS regions of rDNA (Kanchanaprayudh et. al., 2003).

3.6 Fungal endophyte metabolites production of the isolate Aopn12

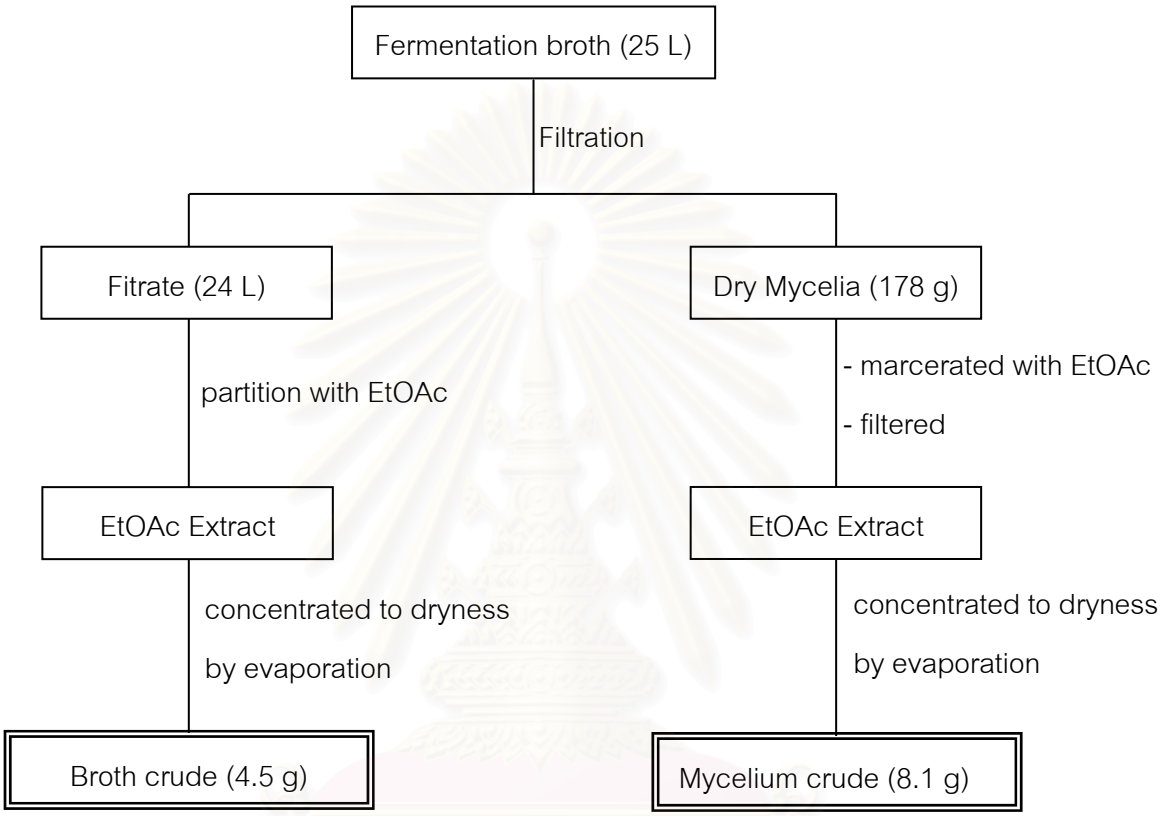
Fungal endophyte Aopn12 was grown on MEA at room temperature (25-30 °C) for 1 week. The agar culture was then cut into 7 mm diameter disks by a flamed cork hole borer. Five disks were inoculated into 250 Erlenmeyer flasks containing 100 ml of MEB medium. The cultures were incubated at room temperature (25-30 °C) for 21 days. Several flasks of culture were prepared to obtain 25 L of MEB.

3.6.1 Crude extraction of endophytic fungus Aopn12

The fermentation broth of isolate Aopn12 (25 L) was filtered through 4 layers of cotton gauze and exhaustively pressed. The filtrate was extracted with an equal volume of EtOAc 3 times. The solvent was then evaporated and removed using a rotary evaporator at 40 °C. The crude extract of culture broth was obtained as a mixture of brown solid and dark brown viscous liquid (4.5 g).

The fungal mycelium (178 g) was extracted 10 times with EtOAc and filtered through filter paper (Whatman no.4). The crude extract of fungal mycelia was obtained as a dark brown viscous liquid (8.1 g).

The extraction of the fermentation broth and mycelia of the endophytic fungus isolate Aopn12 is shown in Scheme 3.1



Scheme 3.1 Diagram of extraction of the fermentation broth and mycelia of the endophytic fungus isolate Aopn12

3.6.2 Isolation of fermentation broth of endophytic fungus isolate Aopn12

The ethyl acetate crude of the isolate Aopn12 fermentation broth (4.5 g) was subjected to colomn chromatography (silica gel, 120 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH. The results from the isolation of the broth crude were presented in Table 3.2

Table 3.2 Isolation of ethyl acetate crude of fermentation broth from the isolate Aopn12

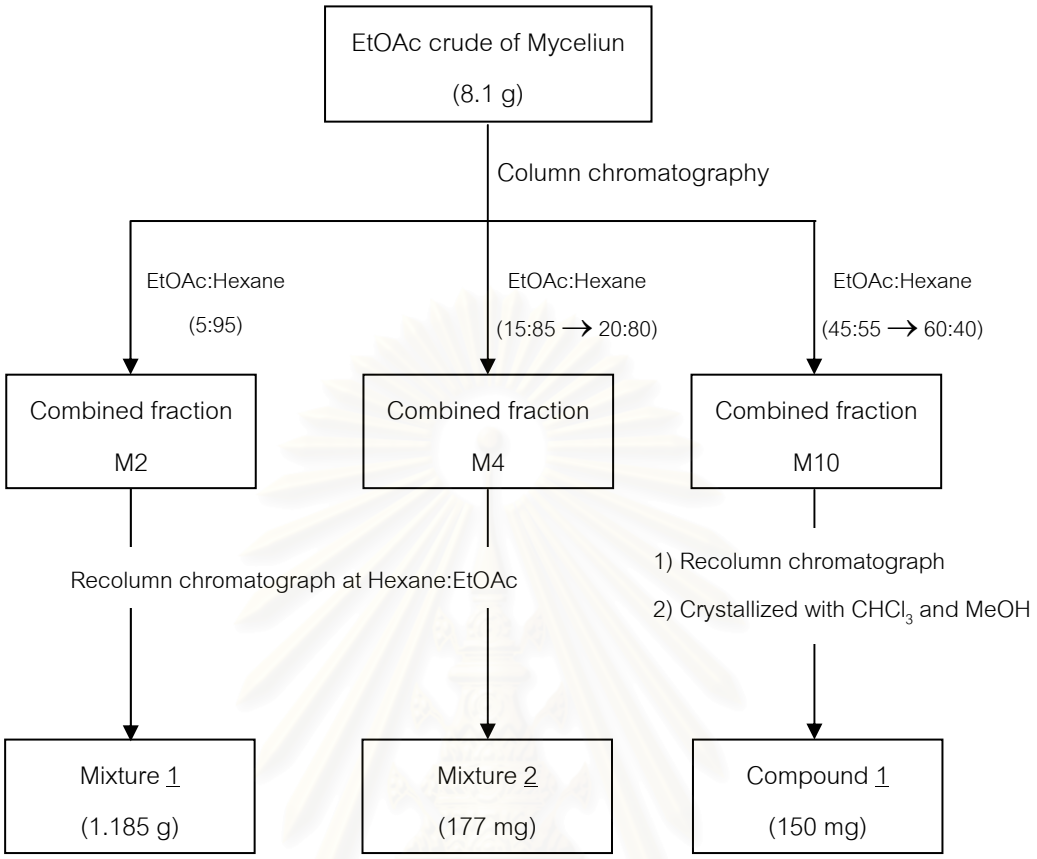
Combined fractions	Eluents	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
B1	0:100 → 5:95	1-3	Colourless viscous liquid	61
B2	5:95	4-6	Yellow viscous liquid	361
B3	10:90	7-11	Yellow viscous liquid	38
B4	10:90 → 15:85	12-21	Orange viscous liquid	117
B5	15:85 → 25:75	22-37	Orange brown viscous liquid	197
B6	25:75 → 35:65	38-59	Brown viscous liquid	212
B7	35:65	60-69	Dark brown viscous liquid	108
B8	35:65 → 45:55	70-90	Dark brown viscous liquid	122
B9	45:55 → 60:40	91-120	Brown viscous liquid	265
B10	65:35 → 85:15	121-154	Dark brown viscous liquid	374
B11	85:15 → 100:0	155-186	Dark brown viscous liquid	564
	MeOH:EtOAc			
B12	0:100 → 10:90	187-210	Dark brown viscous liquid	752
B13	15:85 → 20:80	211-225	Dark brown viscous liquid	319
B14	20:80 → 100:0	226-265	Black brown solid	982

3.6.3 Isolation of mycelium of endophytic fungus isolate Aopn12

The ethyl acetate crude of mycelium (8.1 g) was subjected to colomn chromatography (silica gel, 300 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH. The results from the isolation of mycelium crude were presented in Table 3.3 and scheme 3.2

Table 3.3 Isolation of ethyl acetate crude of mycelium from the isolate Aopn12

Combined fractions	Eluent	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
M1	0:100 → 5:95	1-25	Yellow viscous liquid	29
M2	5:95	26-39	Green yellow viscous liquid	4,600
M3	10:90 → 15:85	40-58	Orange viscous liquid	63
M4	15:85 → 20:80	59-95	White solid in yellow viscous liquid	1,060
M5	25:75	96-108	White solid in orange viscous liquid	161
M6	25:75 → 30:70	109-125	Orange viscous liquid	151
M7	30:70 → 35:65	126-159	Brown viscous liquid	132
M8	35:65 → 40:60	160-180	Dark brown viscous liquid	58
M9	40:60 → 45:55	181-226	Dark brown viscous liquid	114
M10	45:55 → 60:40	227-294	Orange solid in brown viscous liquid	301
M11	60:40 → 70:30	295-354	Dark brown viscous liquid	120
M12	70:30 → 100:0	355-464	Dark brown viscous liquid	148
	MeOH:EtOAc			
M13	0:100 → 15:85	465-526	Dark brown viscous liquid	591
M14	15:85 → 100:0	527-666	Dark brown viscous liquid and solid	572



Scheme 3.2 Diagram of isolation procedure of mycelium crude from the endophytic fungus isolate Aopn12

3.7 Purification and properties of metabolites from the endophytic fungus Aopn12

3.7.1 Purification and properties of mixture 1

The combined fraction M2 was obtained from column chromatography of the Aopn12 mycelium ethyl acetate crude extract using 5% ethyl acetate in hexane as eluent. The green yellow viscous liquid of the fraction M2 (4.6 g) was subjected to isolation by column chromatography using 4% ethyl acetate in hexane as eluent to afford a mixture 1 as a white solid in colorless viscous liquid (1.185 g).

FT-IR spectrum: ν_{max} 3200-3600 (b), 2918 (s), 2851 (s), 1734 (s), 1454 (m), 1236 (w) and 722 (w) cm^{-1}. (Figure D1 in appendix D)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz): δ 0.91, 1.30, 1.64, 2.04, 2.34, 3.70, 4.17, 4.32, 5.30 and 5.37 ppm. (Figure D2 in appendix D)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.): δ 14.2, 22.7, 24.9, 27.2, 27.3, 29.1, 29.2, 29.3, 29.4, 29.4, 29.5, 29.6, 29.7, 29.7, 29.7, 29.8, 32.0, 34.1, 62.1, 129.7, 130.1 and 173.3 ppm. (Figure D3 in appendix D)

Mixture 1 was analyzed fatty acid by gas chromatography. The retention time of standard methy esters (Std.-Me) of fatty acid is shown in Table 3.4

Table 3.4 Retention time of standard methyl ester (Std.-ME) of fatty acid

Std.-ME	Retantion time (min)
C14:0	8
C14:1 (Δ9)	9
C16:0	15
C16:1 (Δ9)	17
C18:0	28
C18:1 (Δ9)	32
C18:2 (Δ9,12)	39
C18:3 (Δ9, 12, 15)	51

3.7.2 Purification and properties of mixture 2

The combined fraction M4 was obtained from column chromatography of the Aopn12 mycelium ethyl acetate crude extract using EtOAc:hexane (15:85 → 20:80) as eluent. The white solid in yellow viscous liquid of the fraction M4 (1.06 g) was subjected to isolation by column chromatography using EtOAc:hexane (4:96) as eluent to afford a mixture 2 as a white solid (177 mg);

FT-IR spectrum (KBr): ν_{max} 2500-3300 (s), 2918 (s), 2850 (s), 1702 (s), 1464 (m), 1297 (w), 939 (w) and 723 (w) cm^{-1}.(Figure D5 in appendix D)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz): δ 0.90, 1.29, 1.67, 2.05, 2.38 and 5.38 ppm. (Figure D7 in appendix D)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ 14.2, 22.7, 24.7, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 32.0, 34.1 and 180.4 ppm. (Figure D7 in appendix D)

MS spectrum (EI 70 eV): *m/s* 284 and 256. (Figure D8 in appendix D)

3.7.3 Purification and properties of compound 1

The combined fraction M10 was obtained from column chromatography of the Aopn12 mycelium ethyl acetate crude extract using EtOAc:hexane (45:55 → 60:40) as eluent. The orange and brown solid of the fraction M10 (301 mg) was subjected to isolation by column chromatography using EtOAc:hexane (45:55) as eluent to afford a compound 1 as orange solid (150 mg);

m.p. 200-202 ^{o}C, λ_{max}: 398 nm in MeOH (Figure D9 in appendix D)

FT-IR spectrum (KBr): ν_{max} 3200-3500 (br), 2923 (s), 2868 (s), 1641 (s), 1415 (m), 1327 (w), 1047 (w) cm^{-1}. (Figure D10 in appendix D)

^{1}H-NMR spectrum (DMSO-d6, 400 MHz.): δ 0.85, 0.88, 1.10, 1.08, 1.23, 1.27, 1.32, 1.42, 1.52, 1.55, 1.58, 1.62, 1.70, 1.73, 1.85, 2.54, 2.88, 4.12, 5.58 and 7.14 ppm. (Figure D11 in appendix D)

^{13}C-NMR spectrum (DMSO-d6, 100 MHz.): δ 18.1, 18.2, 18.4, 21.9, 24.9, 25.0, 31.3, 32.1, 33.3, 34.0, 41.6, 43.3, 44.1, 45.7, 80.8, 115.1, 120.3, 125.1, 129.2, 130.7, 164.8, 166.7 and 185.7 ppm. (Figure D12 in appendix D)

LC-MS spectrum (*m/z*): 390. (Figure D19 and D20 in appendix D)

3.8 Fungal endophyte metabolites of isolate Aopn12 in different culture media

Fungal endophyte Aopn12 was grown on Malt Czapek Agar (MCA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud Glucose Agar (SGA) and Yeast Extract Sucrose agar (YES) at room temperature (25-30°C) for 1 week. The agar culture was then cut into 7 mm diameter disks by a flamed cork hole borer. Five disks were inoculated into 250 Erlenmeyer flasks containing 100 ml of MCB, MEB, PDB, SGB and YES broth. The cultures were incubated at room temperature (25-30°C) for 21 days. Several flasks of culture were prepared to obtain 2 L of individual culture broth.

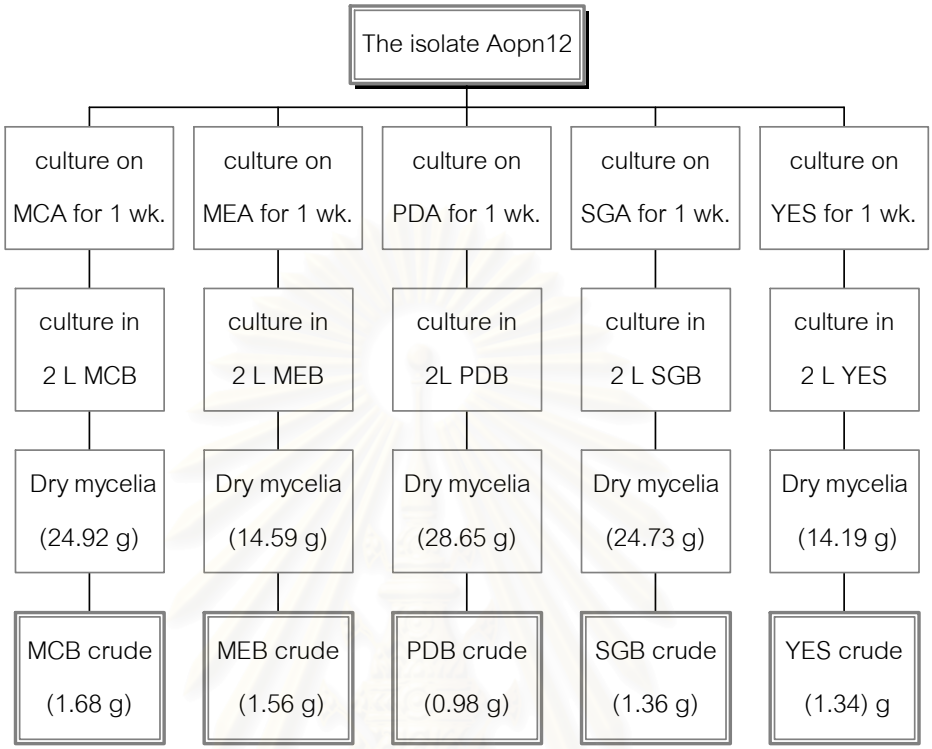
3.8.1 Extraction procedure of endophytic fungus Aopn12 mycelium

The fermentation broth of isolate Aopn12 (2 L) was filtered through 4 layers of cotton gauze and exhaustively pressed. The fungal mycelium was extracted 10 times with EtOAc and filtered through filter paper (Whatman no.4).

The extraction of mycelia of the endophytic fungus isolate Aopn12 is shown in Table 3.5 and Scheme 3.3.

Table 3.5 Appearance and dry weight of mycelium crude of the isolate Aopn12 from different culture media

Mycelium crude	Dry weight (g)	Appearance
MCB crude	1.68	Dark brown viscous liquid
MEB crude	1.56	Dark brown liquid and viscous liquid
PDA crude	0.98	Dark brown viscous liquid
SGB crude	1.36	Dark brown viscous liquid
YES crude	1.34	Dark brown viscous liquid



Scheme 3.3 Diagram of extraction of mycelia of the endophytic fungus isolate Aopn12 in 5 different culture media

3.8.2 Isolation of mycelium of the endophytic fungus Aopn12 cultured in Malt Czapek Broth (MCB)

The ethyl acetate crude of mycelium from the endophytic fungus isolate Aopn12 cultured in Malt czapek broth (MCB crude, 1.68 g) was subjected to colomn chromatography (silica gel, 120 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc and MeOH. The results from the isolation of MCB crude were presented in Table 3.6 and scheme 3.4

Table 3.6 Isolation of ethyl acetate crude of mycelium from the isolate Aopn12 cultured in Malt Czapek Broth (MCB)

Combined fractions	Eluent	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
MC1	0:100	1-6	Yellow viscous liquid	25
MC2	5:95	7-8	Yellow viscous liquid	276
MC3	5:95 → 10:90	9-11	Yellow solid in yellow viscous liquid	32
MC4	10:90 → 30:70	12-16	Yellow solid in yellow viscous liquid	204
MC5	30:70 → 40:60	17-19	Dark brown viscous liquid	22
MC6	40:50 → 40:50	20-24	Dark brown viscous liquid	36
MC7	60:40 → 80:20	25-29	Orange and black orange solid	64
MC8	80:20 → 100%MeOH	30-31	Brown viscous liquid	1,010

3.8.3 Isolation of mycelium of the endophytic fungus Aopn12 cultured in Malt Extract Broth (MEB)

The ethyl acetate crude of mycelium from the endophytic fungus isolate Aopn12 cultured in Malt extract broth (MEB crude, 1.56 g) was subjected to colomn chromatography (silica gel, 120 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc and MeOH. The results from the isolation of MEB crude were presented in Table 3.7 and scheme 3.5

Table 3.7 Isolation of ethyl acetate crude of mycelium from the isolate Aopn12 cultured in Malt Extract Broth (MEB)

Combined fractions	Eluent	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
ME1	0:100	1-2	Light yellow viscous liquid	21
ME2	5:95	3-5	Yellow viscous liquid	404
ME3	5:95	6-11	Yellow viscous liquid	6
ME4	5:95	12-15	White solid in yellow viscous liquid	46
ME5	10:90 → 20:80	16-22	Orange viscous liquid	84
ME6	30:70	23-25	Dark brown viscous liquid	9
ME7	35:65 → 40:60	26-29	Dark brown viscous liquid	17
ME8	40:60 → 80:20	30-50	Orange and black orange solid	172
ME9	80:20 → 100%MeOH	51-52	Dark brown viscous liquid	707

3.8.4 Isolation of mycelium of the endophytic fungus Aopn12 cultured in Potato Dextrose Broth (PDB)

The ethyl acetate crude of mycelium from the endophytic fungus isolate Aopn12 cultured in Potato Dextrose Broth (PDB crude, 0.98 g) was subjected to colomn chromatography (silica gel, 120 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc and MeOH. The results from the isolation of PDB crude were presented in Table 3.8 and scheme 3.6

Table 3.8 Isolation of ethyl acetate crude of mycelium from the isolate Aopn12 cultured in Potato Dextrose Broth (PDB)

Combined fractions	Eluent	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
PD1	0:100 → 1:99	1-3	Light yellow viscous liquid	16
PD2	2:98	4-9	Yellow viscous liquid	11
PD3	5:95	10-12	Yellow viscous liquid	21
PD4	5:95	13-20	Yellow solid in yellow viscous liquid	64
PD5	5:95 → 10:90	20-31	Yellow orange viscous liquid	153
PD6	20:80 → 25:75	32-36	Brown viscous liquid	13
PD7	25:75 → 40:60	37-48	Dark brown viscous liquid	41
PD8	45:55 → 60:40	49-68	Orange and black orange solid	97
PD9	60:40 → 100%MeOH	69-71	Dark brown viscous liquid	459

3.8.5 Isolation of mycelium of the endophytic fungus Aopn12 cultured in Sabouraud Glucose Broth (SGB)

The ethyl acetate crude of mycelium from the endophytic fungus isolate Aopn12 cultured in Sabouraud Glucose Broth (SGB crude, 1.36 g) was subjected to colomn chromatography (silica gel, 120 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc and MeOH. The results from the isolation of SGB crude were presented in Table 3.9 and scheme 3.7

Table 3.9 Isolation of ethyl acetate crude of mycelium from the isolate Aopn12 cultured in Sabouraud Glucose Broth (SGB)

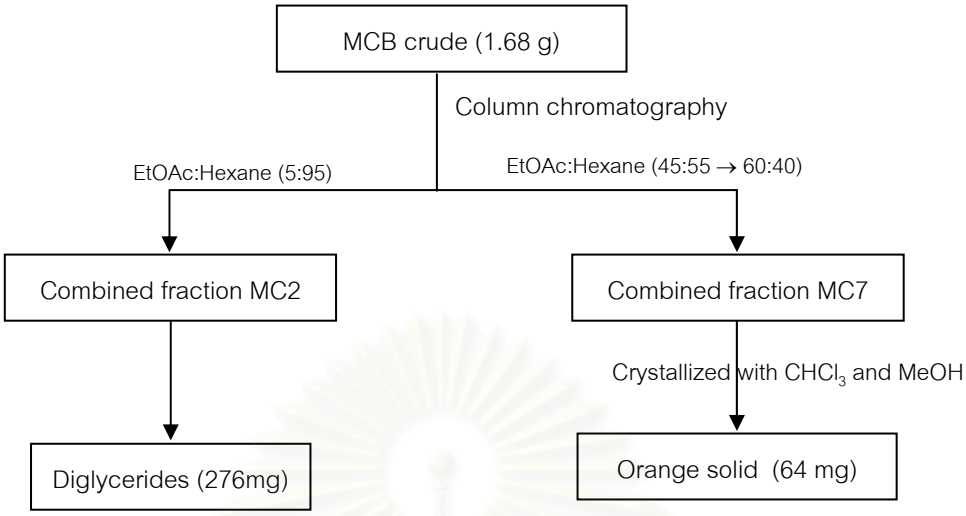
Combined fractions	Eluent	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
SG1	0:100	1-3	Light yellow viscous liquid	16
SG2	5:95	4-5	Yellow viscous liquid	205
SG3	5:95	6-8	Yellow viscous liquid	9
SG4	5:95	9-11	Light yellow solid in yellow viscous liquid	95
SG5	10:90 → 25:75	12-16	Brown orange viscous liquid	145
SG6	30:70 → 35:65	17-20	Brown viscous liquid	12
SG7	35:65	21-23	Dark brown viscous liquid	17
SG8	40:60 → 50:50	24-30	Dark brown viscous liquid	22
SG9	50:50 → 70:30	31-35	Orange and black orange solid	24
SG10	70:30 → 100%MeOH	36-38	Dark brown viscous liquid	737

3.8.6 Isolation of mycelium of the endophytic fungus Aopn12 cultured in Yeast Extract Sucrose broth (YES)

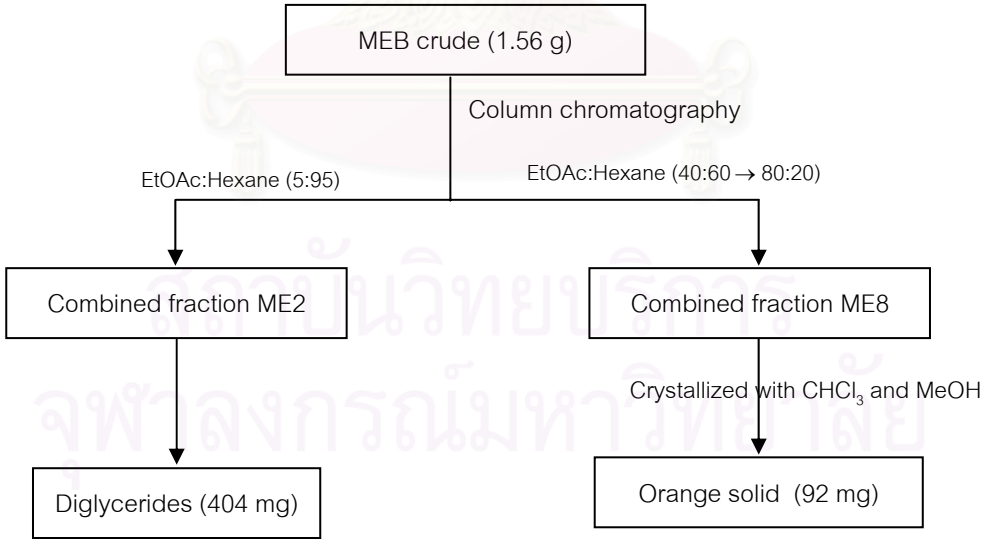
The ethyl acetate crude of mycelium from the endophytic fungus isolate Aopn12 cultured in Yeast Extract Sucrose broth (YES crude, 1.34 g) was subjected to colomn chromatography (silica gel, 120 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc and MeOH. The results from the isolation of YES crude were presented in Table 3.10 and scheme 3.8

Table 3.10 Isolation of ethyl acetate crude of mycelium from the isolate Aopn12 cultured in Yeast Extract Sucrose broth (YES)

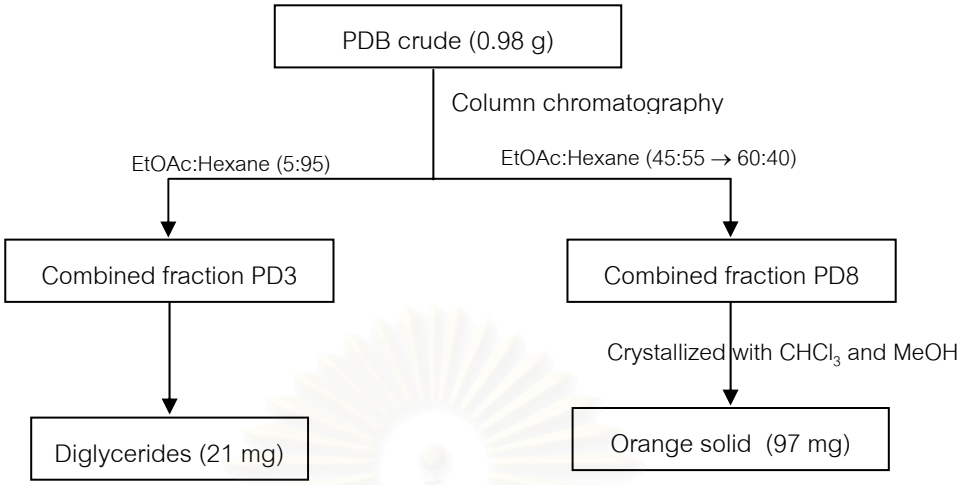
Combined fractions	Eluent	Fraction No.	Appearance	Weight (mg)
	EtOAc:Hexane			
YE1	0:100	1-2	Yellow viscous liquid	10
YE2	5:95	3-4	Orange yellow viscous liquid	207
YE3	5:95 → 8:92	5-9	Yellow viscous liquid	17
YE4	8:92 → 15:85	10-15	Yellow solid in yellow viscous liquid	155
YE5	15:85 → 30:70	16-20	Brown viscous liquid	25
YE6	30:70 → 35:65	21-24	Brown viscous liquid	33
YE7	40:60 → 70:30	25-28	Black orange solid	40
YE8	70:30 → 100%MeOH	29-30	Dark brown viscous liquid	790



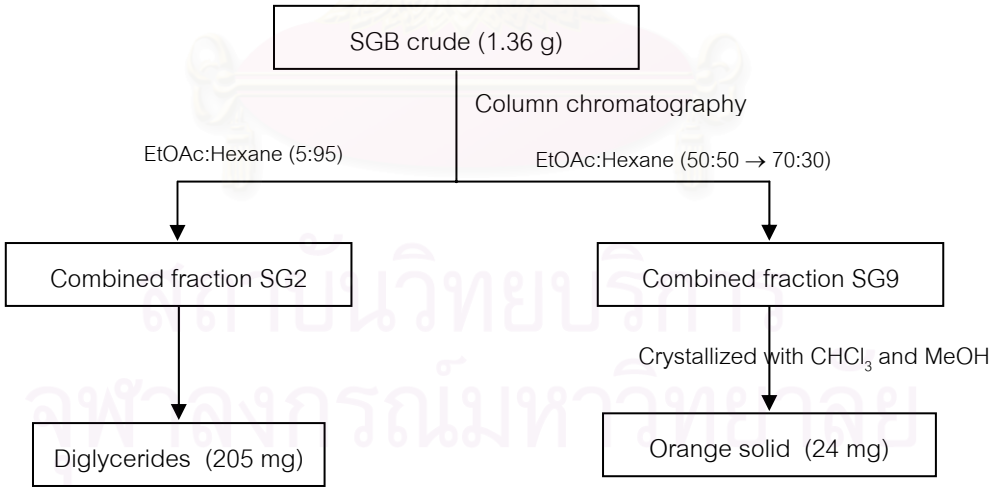
Scheme 3.4 Diagram of isolation of ethyl acetate crude of mycelium from endophytic fungus isolate Aopn12 cultured in Malt Czapek Broth (MCB)



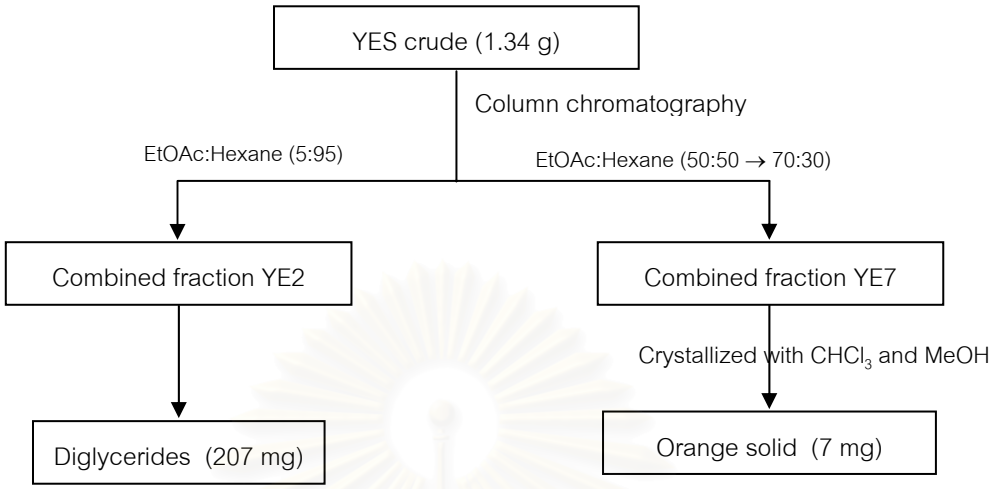
Scheme 3.5 Diagram of isolation of ethyl acetate crude of mycelium from endophytic fungus isolate Aopn12 cultured in Malt Extract Broth (MEB)



Scheme 3.6 Diagram of isolation of ethyl acetate crude of mycelium from endophytic fungus isolate Aopn12 cultured in Potato Dextrose Broth (PDB)



Scheme 3.7 Diagram of isolation of ethyl acetate crude of mycelium from endophytic fungus isolate Aopn12 cultured in Sabouraud Glucose Broth (SGB)



Scheme 3.8 Diagram of isolation of ethyl acetate crude of mycelium from endophytic fungus isolate Aopn12 cultured in Yeast Extract Sucrose broth (YES)

3.9 Instruments and equipments

1. UV-Vis spectrometry

UV-Vis spectra were recorded on a Perkin Elmer Lambda 25 UV-VIS spectrophotometer in $CHCl_3$ and MeOH

2. Fourier Transform Infrared Spectrophotometer (FT-IR)

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

3. Mass Spectrometry (MS)

The mass spectra were recorded on a Bruker DataAnalysis Esquire-LC.

4. Nuclear Magnetic Resonance Spectrometry (NMR)

^{1}H NMR, ^{13}C NMR, DEPT, gCOSY, gHMQC, gHMBC, NOESY and TOCSY spectra were recorded on an OxFord Model YM400 Spectrometer at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. Deuterated Solvent; Chloroform-*d* ($CDCl_3$) and Dimethy sulfoxide-D6 (DMSO-d6) were use in NMR experiments. Reference signals of residual protonated solvents at δ_H 7.29 ppm and δ_C 77.3 ppm for $CDCl_3$ and δ_H 2.54 ppm and δ_C 40.4 ppm for DMSO-d6

6. Melting point apparatus

Melting point were measured on a Electrothermal 9100.

7. Gas Chromatography (GC)

Mixture 1 was analyzed on 3 mm glass column of 15%DEGS on 60/80 mesh Chromasorb S AW at 180°C using Shimadzu 15A Gas Chromatograph. It was sent

for analyzed at the Instituent of Biotechnology and Genetic Engineering (IBGE): Chulalongkorn University, Bangkok, Thailand.

3.10 Chemicals

3.10.1 Solvents

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

3.10.2 Other Chemicals

1. Merck's silica gel Art 1.09385.1000 (230-400 mesh ASTM) was used as absorbent for column chromatography.

2. Merck's TLC aluminium sheets , silica gel 60 F_{254} precoated 25 sheets, 20x20 cm^2. Layer thickness 0.2 mm was used to identical fraction and was developed using a suitable solvent system.

3.11 Determination of biological activity

3.11.1 Antimicrobial activity test

3.11.1.1 Antimicrobial activity of the crude extract and pool fractions

Evaluation of the antimicrobial activity of the crude extracts and pool fractions was determined by the agar well diffusion method which was modified from the method described by Weaver, Angel and Botlomley (1994). 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*s ATCC 10231.

A. Preparation of samples

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

B. Preparation of bacterial and yeast inoculum

Bacterial and yeast inoculum were prepared to the same manner as described in section 3.4.3

C. Inoculation of the test plate

A sterile cotton swab was dipped into the microbial suspension and presses lightly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab was then streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60^{o}C each time to ensure an even distribution of the inoculum. The plates were left to dry for 3-5 minutes.

D. Application of the crude extract and pool fractions

Wells were made in the agar by removing disks cut (7 mm diameter) with a flamed cork hole borer. One hundred µl of the samples was pipetted into the agar wells. This was absorbed by the media surrounding the wells. Bacteria and yeast plates were incubated at 37^{o}C and room temperature, respectively for 24 h. The inhibition zone diameter was measured in mm with a ruler.

3.11.1.2 Antimicrobial activity of pure compounds

Evaluation of the antimicrobial activity of pure compounds was determined by the antimicrobial susceptibility test broth microdilution method. 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. albicans* ATCC 10231.

A. Preparation of pure compounds and antibiotic drug standards

Four milligrams 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°C for bioassay. Antibiotic drug standards (Tetracycline HCl and Amoxicillin) were used as positive control.

B. Preparation of bacterial and yeast inoculum

A bacterial and a yeast inoculum were prepared in the same manner as described in section 3.4.3. The final bacterial inoculum was diluted with NB to obtain a cell suspension containing approximately 10^6 CFU/ml. The final yeast inoculum was approximately 10^5 CFU/ml.

C. 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°C and room temperature for bacterial and yeast, respectively.

D. Reading of microtiter plates assays

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

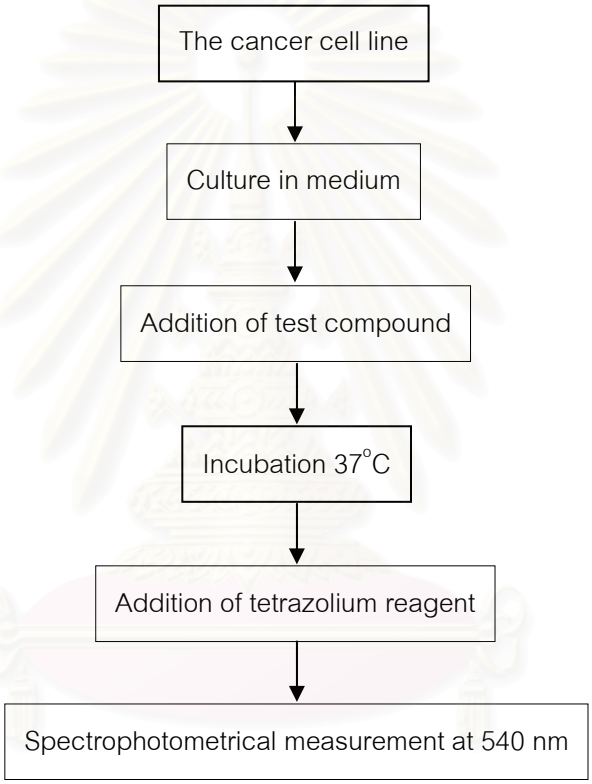
3.11.2 Cytotoxicity test

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) as shown in Scheme 3.9.

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 SW620 (colon), BT 474 (breast), KATO-III (gastric), HEP-G2 (hepatoma) and Chago (lung) and following the experimental method of bioassay of cyototoxic activity.



Scheme 3.9 MTT bioassay for cytotoxic activity

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

A total of 60 isolates of endophytic fungi were obtained from healthy *Aglaia odorata* leaves from 4 provinces during April-July 2001, as shown in Table 4.1. Fourteen isolates of endophytic fungi were obtained from *A. odorata* leaves collected at Chulalongkorn University, Bangkok. Four isolates of endophytic fungi were obtained from *A. odorata* leaves collected at Amphoe Maung, Khonkaen Province. Thirty isolates of endophytic fungi were obtained from *A. odorata* leaves collected at Amphoe Maung, Mahasarakham Province. Twelve isolates of endophytic fungi were obtained from *A. odorata* leaves collected at Amphoe Sai Buri, Pattani Province. Colony morphology of the 60 fungal isolates is shown in Figure 4.1. All endophytic fungal isolates were selected for further study.

Table 4.1 Number of endophytic fungi isolated from *Aglaia odorata* Lour. leaves

Sampling site	Isolated code	Number of isolate
Bangkok	Aobk	14
Khonkaen	Aokk	4
Mahasarakham	Aomh	30
Pattani	Aopn	12

Figure 4.1 Colony characteristic of endophytic fungi isolated from *A. odorata* leaves, on PDA after cultivation for 10 days at room temperature. Isolate numbers are shown in each pictures

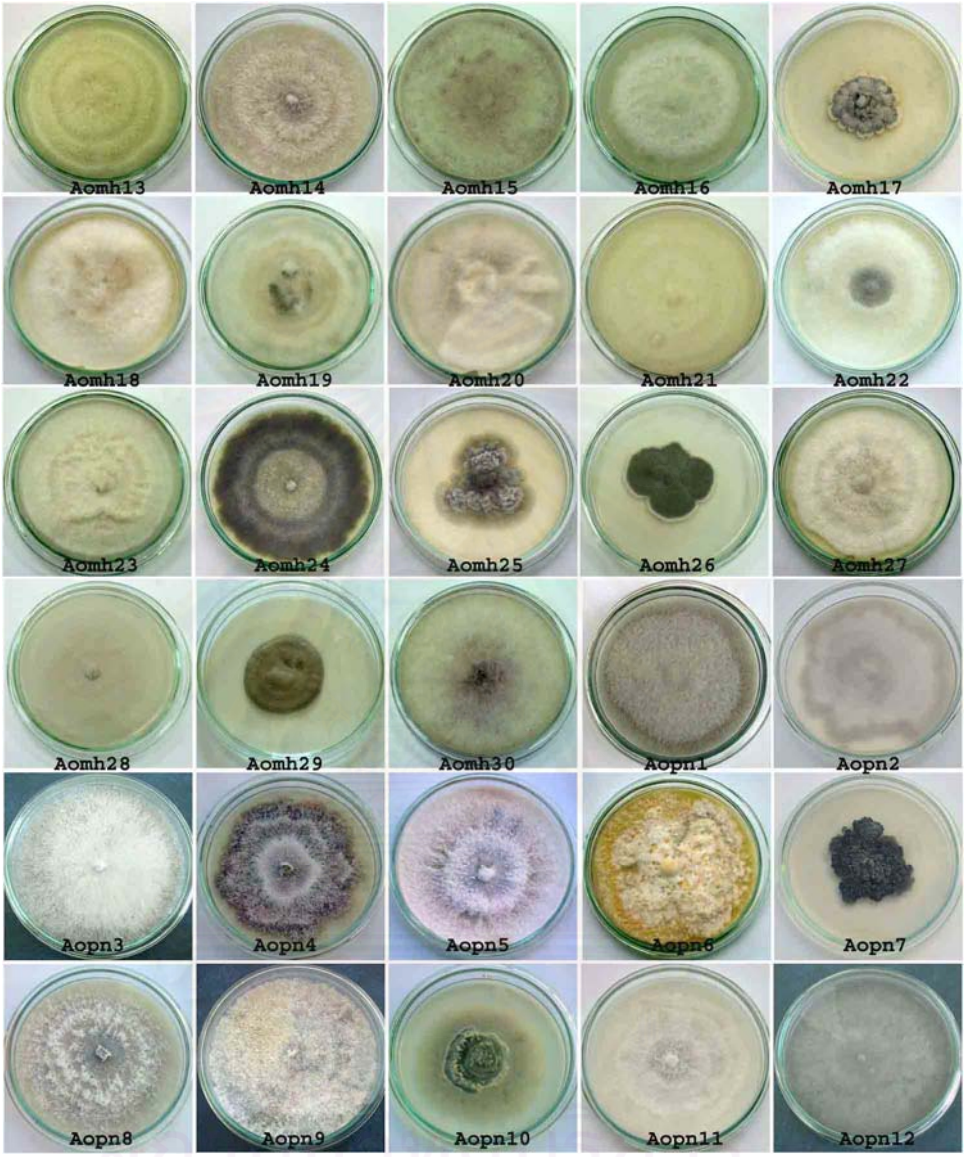


Figure 4.1 Colony characteristic of endophytic fungi isolated from *A. odorata* leaves, on PDA after cultivation for 10 days at room temperature. Isolate numbers are shown in each pictures (continued)

4.2 Antimicrobial activities of isolated endophytic fungi

The 60 isolated of endophytic fungi were grown on Malt Czapek Agar (MCA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouroud Glucose Agar (SGA) and Yeast Extract Sucrose agar (YES) at room temperature for 10 days. The inhibition zone around the agar blocks of endophytic fungus isolate was observed, as shown in Table C2 in appendix C. Figure 4.2 summarized the percent of active endophytic fungi isolates exhibiting antimicrobial activities per total endophytic fungus isolated.

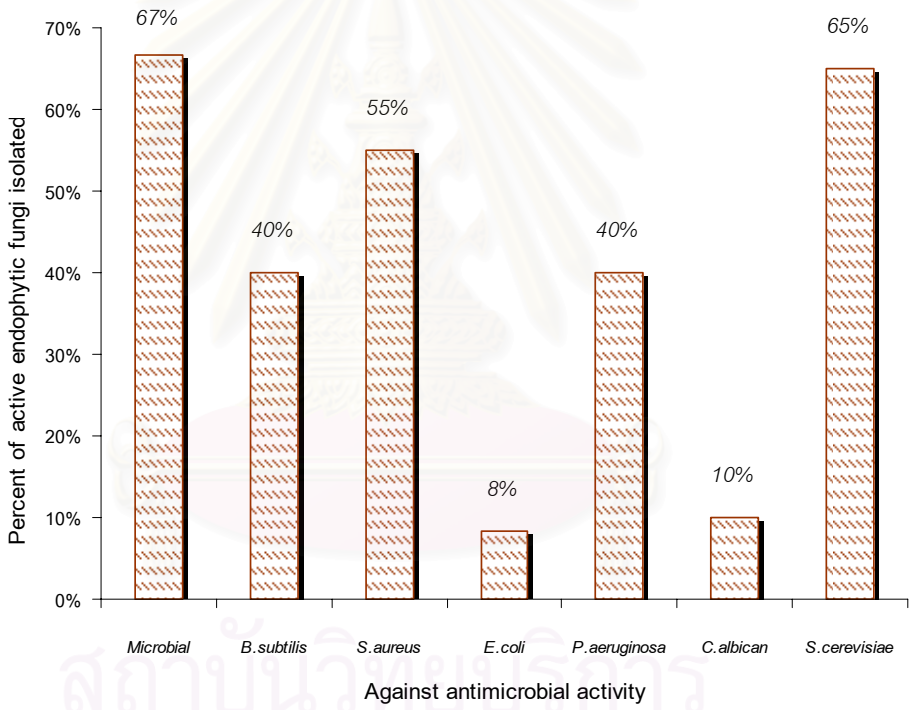


Figure 4.2 Percent of active endophytic fungi isolated exhibiting antimicrobial activities

By dual-culture agar diffusion assay, 40 isolates (67%) of endophytic fungus were found antimicrobial activities. The number of active endophytic fungus isolates exhibiting activities against bacteria *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomona aeruginosa* were 20 (33%), 18 (30%), 2 (3%) and 17 (28%) isolates, respectively. Anti-*Candida albicans* and anti-*Saccharomyces cerevisiae* activities were evident in 4 (7%) and 18 (30%) isolates, respectively. The high

percentage of active endophytic fungus isolates is consistent with the previous idea that fungal endophytes are untapped source of bioactive compounds (Strobel and David, 1998). Figure 4.3 demonstrated antimicrobial activities against *S. aureus* and *C. albican* of endophytic fungi isolates from dual-culture agar diffusion assay.

Figure 4.3 Dual-culture agar diffusion exhibit against *Staphylococcus aureus* and *Candida albican*s of activity of endophytic fungus isolated.

4.3 Effect of culture on antimicrobial activities of fungal endophyte isolated

The effects of different media (MCA, MEA, PDA, SGA and YES) on the type and intensity of antimicrobial activities produced by endophytic fungi could be observed, as shown in Table C2 in appendix C. Figure 4.4 and Table C1 in appendix C summarized the variation in number of fungal endophyte isolates that exhibited antimicrobial activities when they were cultured on different media.

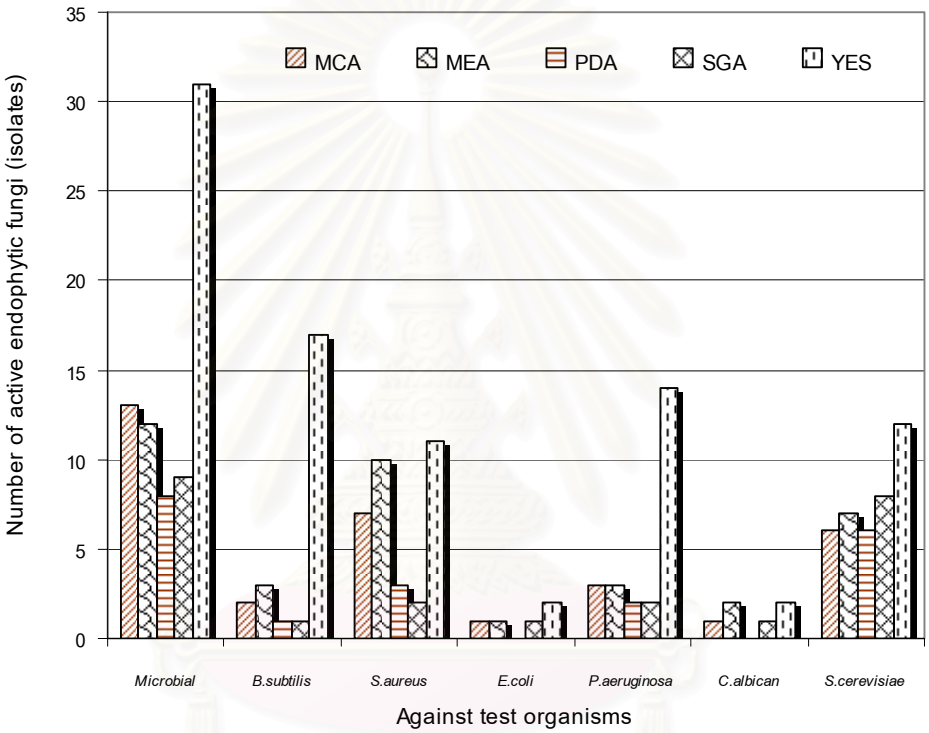


Figure 4.4 Number of active endophytic fungus isolates demonstrating activities against test microorganiams when culture on different media

By dual agar diffusion assay, the culture media was found to be the one of factor affecting expression of antimicrobial activity. The highest numbers of active isolates against all test organisms were found when they were grown on YES. Anti-*E. coli* and anti-*C. albicans* activities could not be observed in culture grown on PDA. There is no suitable culture medium that provides all of antimicrobial activities. Using numerous culture media probably will be increasing the opportunity to obtain more antimicrobial activities, but this has to be considered with the financial facility.

4.4 Selection of endophytic fungus isolates for study bioactive compounds

The number of endophytic fungi that exhibited antimicrobial activities were found to be high. From the total of 60 isolates grown on 5 culture media and test for 4 antibacterial and 2 antifungal activities, 40 isolates (67%) were found produced one or more antimicrobial activities, 12 isolates (20%) were found produced three or more antimicrobial activities, as shown in Table 4.2 and Figure 4.4. Colony morphology of the 12 active fungal isolates grown on 5 different media is shown in Figure 4.6 - 4.17.

Table 4.2 Inhibition zone (mm) measured from the agar block of the 12 active endophytic fungus

No.	Isolate	Culture medium	Test organisms and Inhibition zone (mm)					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
1	Aobk5	PDA	-	2.5	-	2.5	-	2.5
2	Aobk6	MEA	-	2	-	1	-	2.75
3	Aokk1	YES	1	-	-	1	-	1
4	Aokk2	YES	1.5	1.5	1.3	-	-	-
5	Aokk4	MEA	-	2	-	-	3	4
6	Aomh11	YES	2.5	4	-	3	-	5
7	Aomh16	YES	1.5	2	-	2	1	1
8	Aomh19	YES	1.5	-	-	1.5	-	5
9	Aomh24	YES	-	3	-	-	3	7
10	Aomh27	YES	2.2	2	-	2.5	-	-
11	Aopn2	MEA	2	1.5	-	-	-	10
12	Aopn12	MCA	4	7.5	2	-	5	6
		MEA	9	10	4	2	7	10
		PDA	2	2.5	-	-	-	3.5
		SGA	2	10	1.5	1	4.5	3
		YES	1.5	2	1	1	-	-

B.S. = *B. subtilis* ATCC 6633
S.A. = *S. aureus* ATCC 25923
E.C. = *E. coli* ATCC 25922
P.A. = *P. aeruginosa* ATCC 27853
C.A. = *C. albicans* ATCC 10231
S.C. = *S. cerevisiae* TISTR 5169

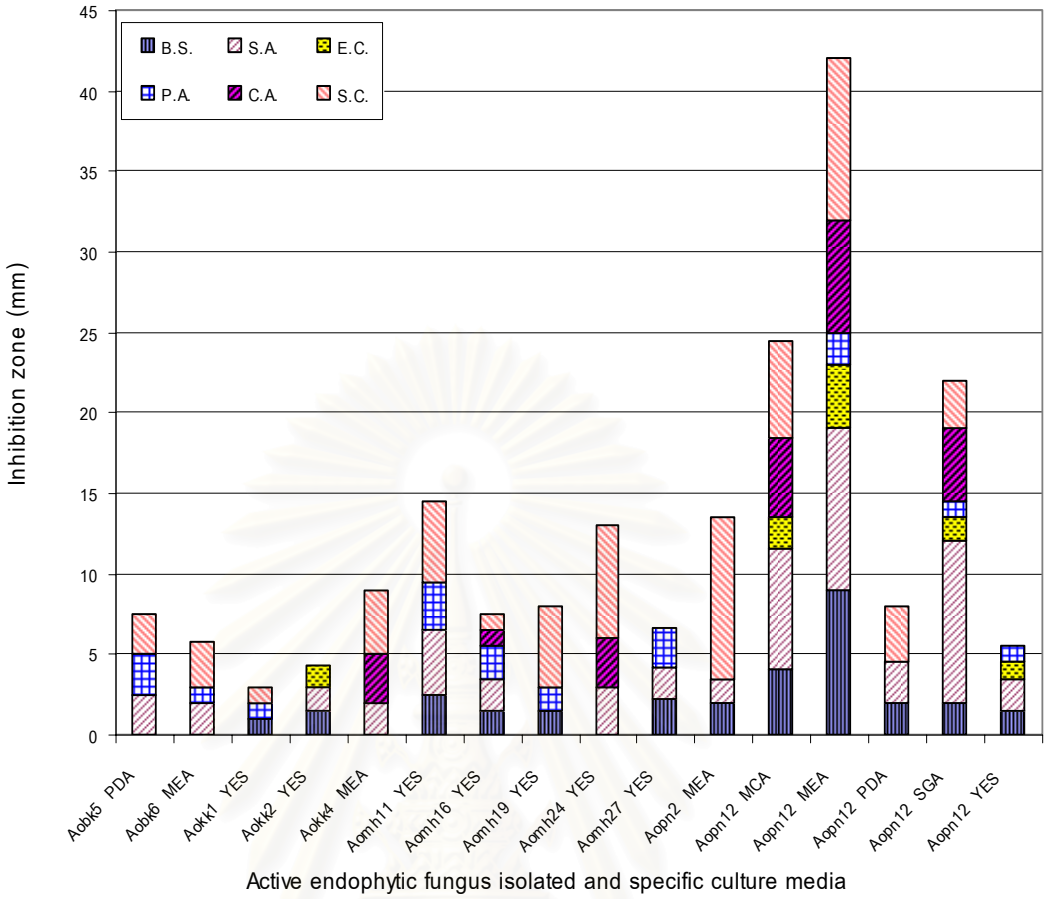


Figure 4.5 Inhibition zone (mm) measured from the agar block of the 12 active endophytic specific culture media

Fungal isolate Aopn12 and malt extract agar were chosen for study bioactive compounds. This is because the malt extract agar blocks of fungal isolate Aopn12 both were active a large number of test microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 10231 and *S. cerevisiae* TISTR 5169 and exhibited high antimicrobial activities with highest inhibition zone.

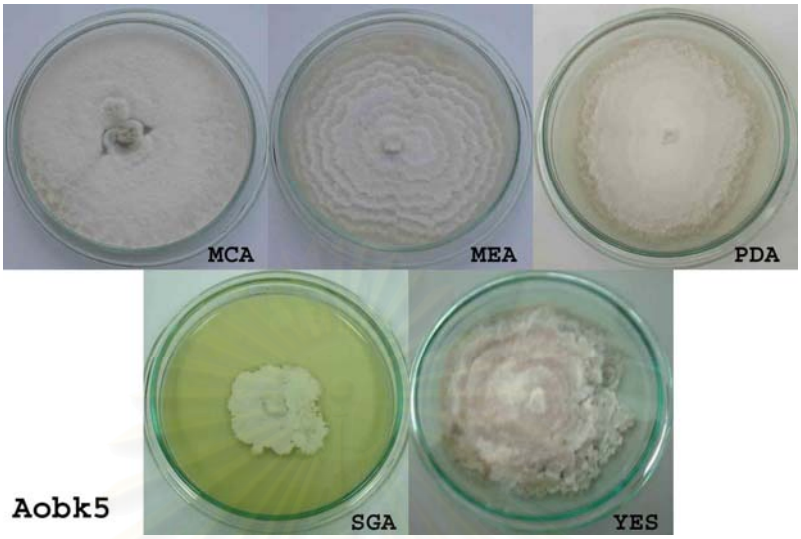


Figure 4.6 Colony characteristic of endophytic fungus isolate Aobk5 was grown on 5 different media at room temperature for 10 days

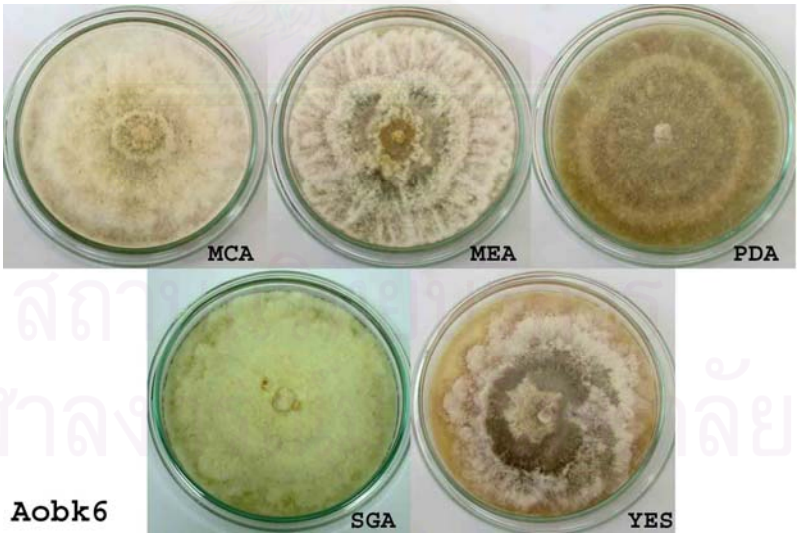


Figure 4.7 Colony characteristic of endophytic fungus isolate Aobk6 was grown on 5 different media at room temperature for 10 days

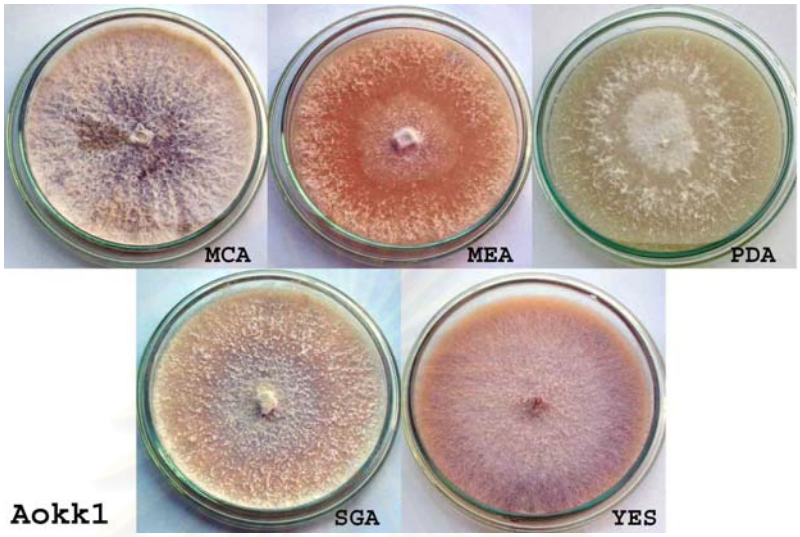


Figure 4.8 Colony characteristic of endophytic fungus isolate Aokk1 was grown on 5 different media at room temperature for 10 days

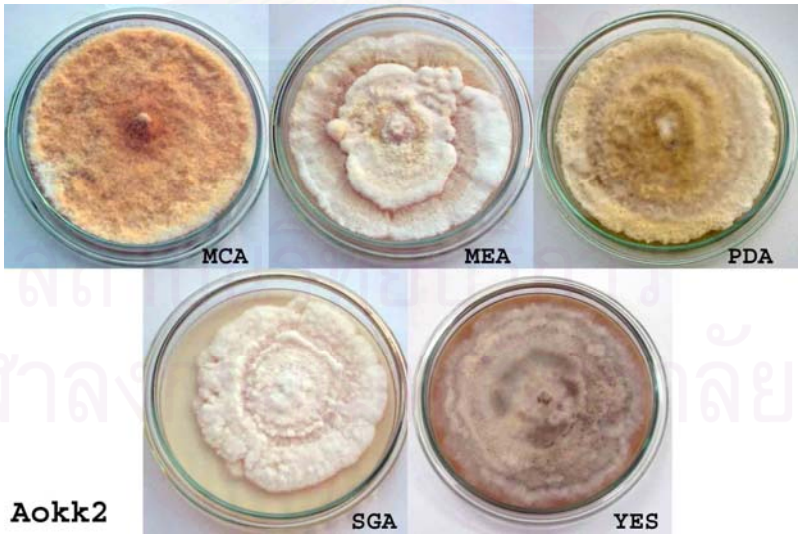


Figure 4.9 Colony characteristic of endophytic fungus isolate Aokk2 was grown on 5 different media at room temperature for 10 days

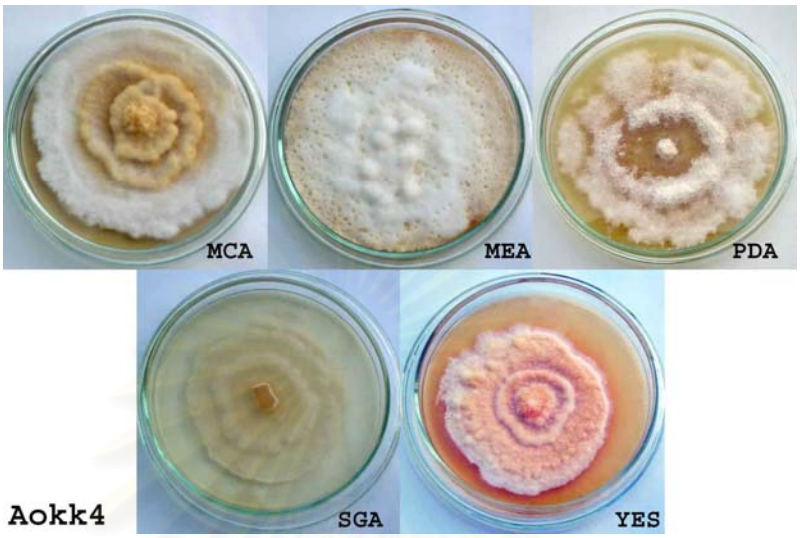


Figure 4.10 Colony characteristic of endophytic fungus isolate Aokk4 was grown on 5 different media at room temperature for 10 days

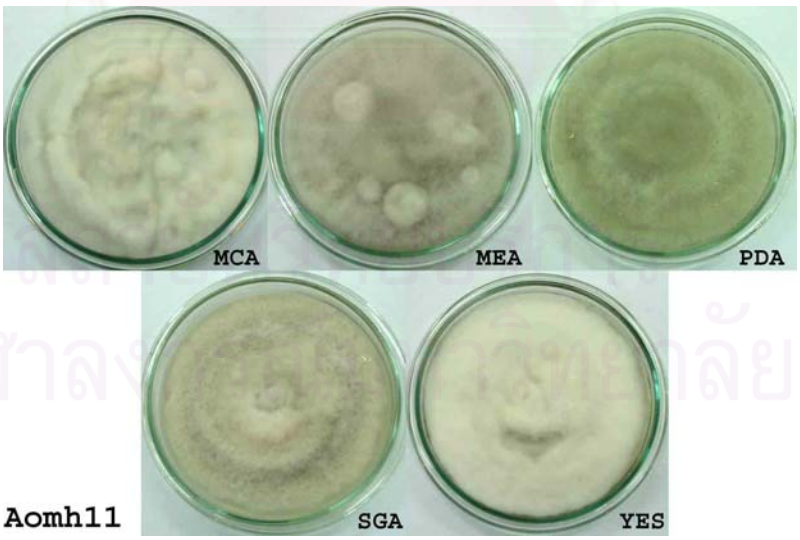


Figure 4.11 Colony characteristic of endophytic fungus isolate Aomh11 was grown on 5 different media at room temperature for 10 days

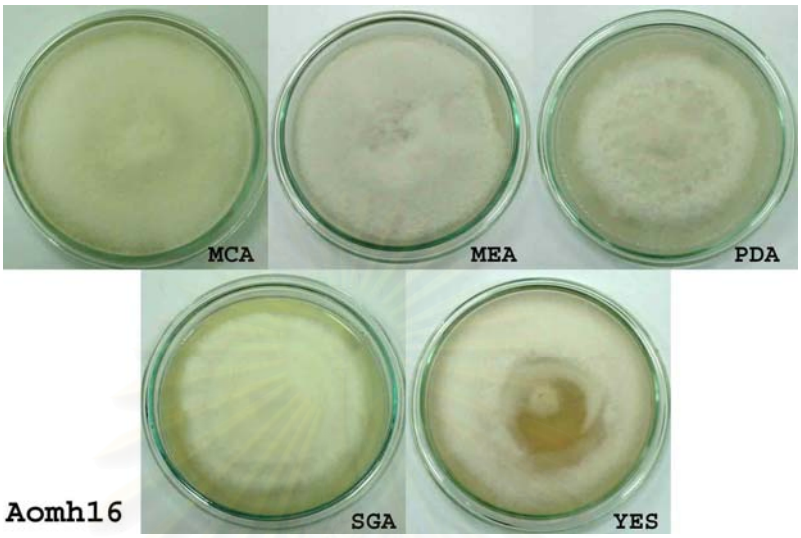


Figure 4.12 Colony characteristic of endophytic fungus isolate Aomh16 was grown on 5 different media at room temperature for 10 days

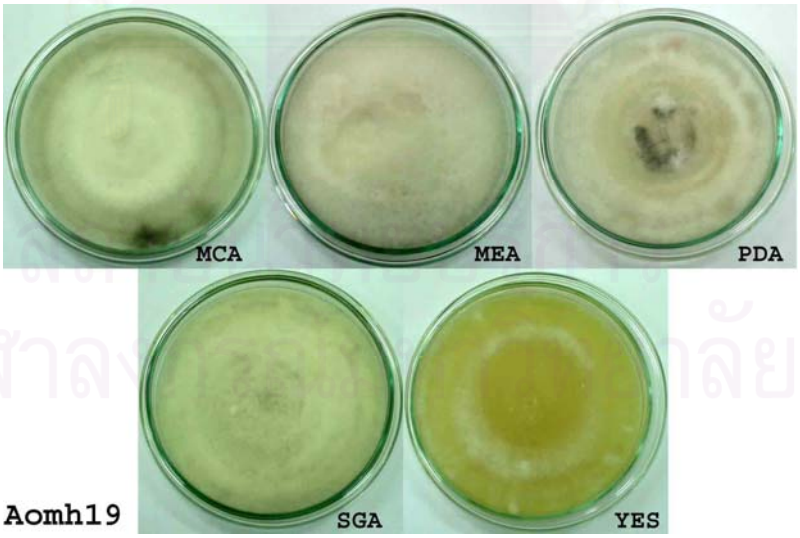


Figure 4.13 Colony characteristic of endophytic fungus isolate Aomh19 was grown on 5 different media at room temperature for 10 days

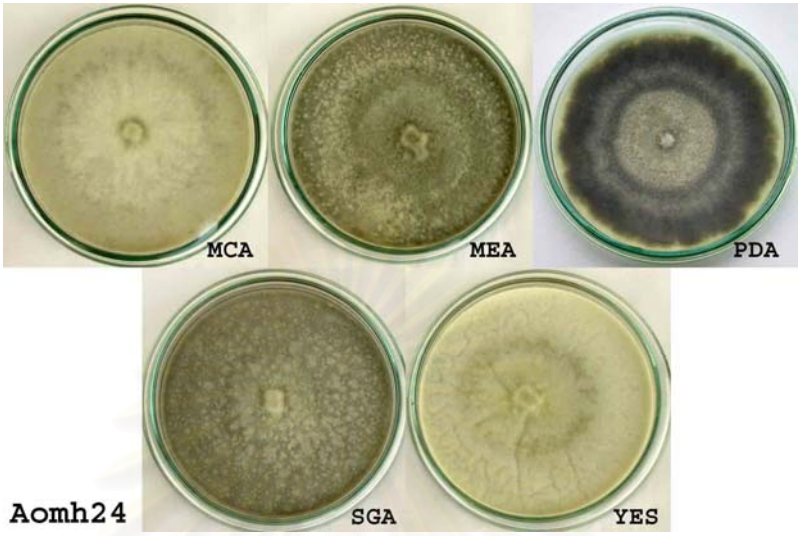


Figure 4.14 Colony characteristic of endophytic fungus isolate Aomh24 was grown on 5 different media at room temperature for 10 days

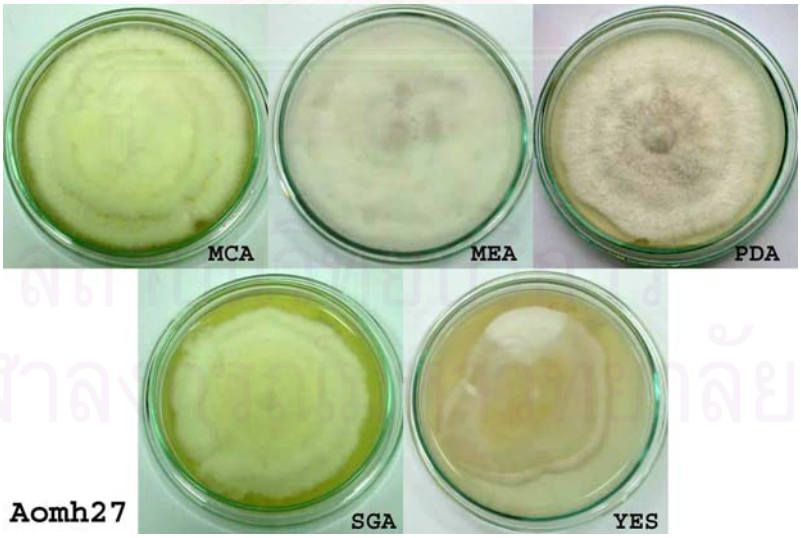


Figure 4.15 Colony characteristic of endophytic fungus isolate Aomh27 was grown on 5 different media at room temperature for 10 days

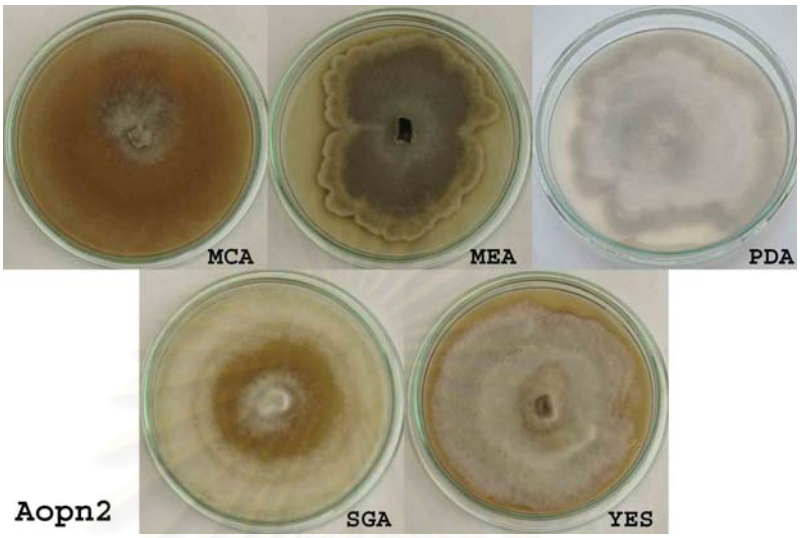


Figure 4.16 Colony characteristic of endophytic fungus isolate Aopn2 was grown on 5 different media at room temperature for 10 days

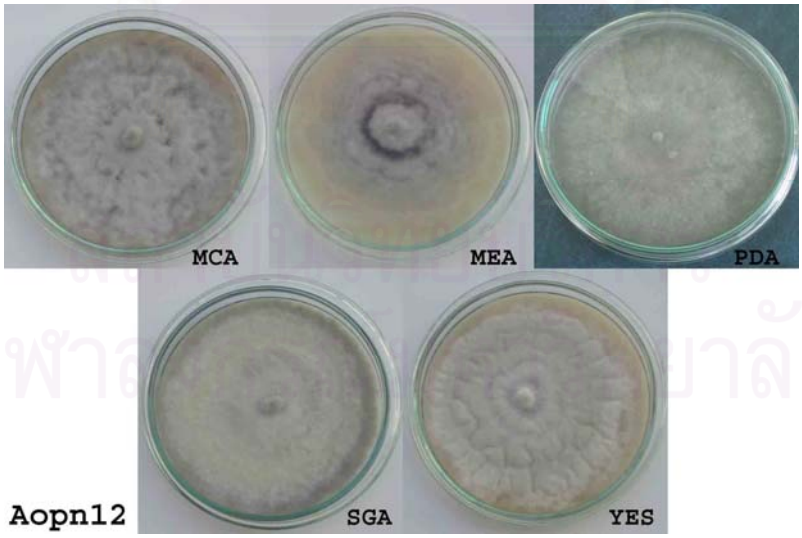


Figure 4.17 Colony characteristic of endophytic fungus isolate Aopn12 was grown on 5 different media at room temperature for 10 days

4.5 Identification of fungal endophyte Aopn12

Fungal isolate Aopn12 was chosen for bioactive compounds. This was because the malt extract agar blocks of fungal isolate Aopn12 were active against a large number of test microorganisms such as *B. subtilis* , *S. aureus, E. coli, P . aeruginosa, C. albicans* and *S. cerevisiae.*

4.5.1 Morphological identification

Fungal isolate Aopn12 did not produce conidium or spore except chlamydospores on common mycological media including Malt Czapek Agar, Malt Extract Agar, Potato Dextrose Agar, Sabouraud Glucose Agar and Yeast Extract Sucrose Agar. Therefore, Isolate Aopn12 was classified as Mycelia sterilia. Colony characteristic of the isolate Aopn12 is shown in Figure 4.17 and mycelia characteristics of fungus isolate Aopn12 is shown in Figure 4.18.

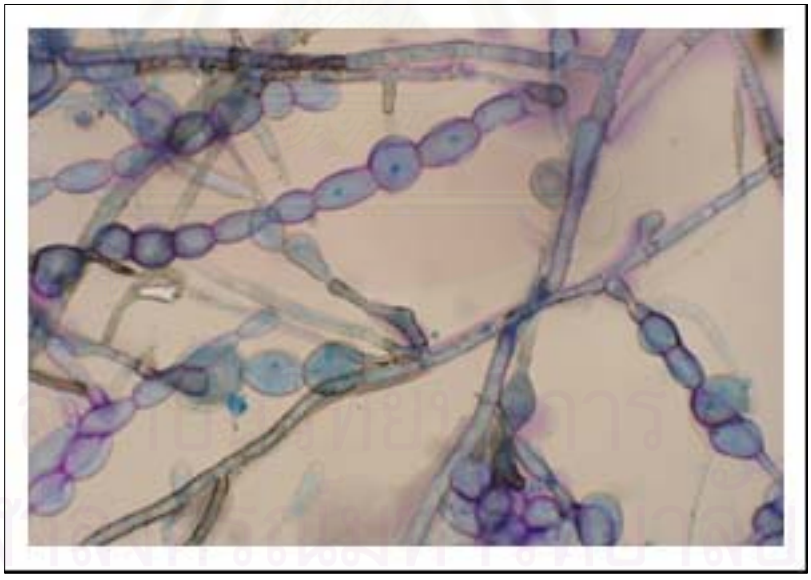


Figure 4.18 Mycelia characteristics of endophytic fungus isolate Aopn12 with formation chlamydospores

4.5.2 Molecular identification

The rDNA ITS region of fungus isolate Aopn12 was amplified with the conserved fungal primer ITS1f and ITS4. Isolate Aopn12 produced a single ITS band. The length of corresponding fragment was 684 bp., as shown in Figure 4.19, containing a part of the 18S; ITS1, 5.8S and 28S rDNA

```
      1
5'    CTTGGTCATT   TAGAGGAAGT   AAAAGTCGTA   ACAAGGTTTC
      CGTAGTGAAC   CTGCGGAAGG   ATCATTAGAA   ATGGCGGGGG
      AAGGTTCGCC   CTTCTCCCCA   AATCTCTATT   CTGTGCACTG
      TTTTTGTGTG   CTGGGCTAGC   TTTGGCTGGG   CCAGCATGGG
      GTGGAGCTTG   GAGATCGTTT   GCATGAATTT   TCCCCCGGGA
      AAGTTCCGCT   GGAAACTTTT   GACATCTCCG   CCGAAATTTA
      ATACTATTAC   TGACTGACCA   TGAAAAAGTT   AATATAACAA
      CTTTCAGCAA   TGGATCTCTC   GGCTTTCGTA   TCGATGAAGA
      ACGCAGCGAA   TCGCGATATG   TAGTGTGATC   TGCCTCTAGT
      GAATCATCAA   ATCTTTGAAC   GCATCTTGCA   CCCTTTGGTA
      TTCCAAAGGG   TACGCCTGTT   TCAGTATCAT   TATAACCTTT
      CTCCCCAAGG   CTTTTGGCCA   AGGAGGGGAA   GAGCATAACC
      GGTGGCCCAG   CGGTCTCCCA   TCAGGGGGAT   TGTCTCGGCT
      GGAATTACTG   TTGCAATGGT   GGGCCAAATG   GTCTCCCGCA
      CTTGATAAAT   GTACTTTTAG   AAAGAGCGGG   GCCGGTCTGC
      CGAAAGTTGC   AACTAACCCT   TTACCTTTGA   TCTGAAATCA
      GGCGGGATTA   CCCGCTGAAC   TTAAGCATAT   CAATAAGCGG
      AGGA  3'
         684
```

Figure 4.19 Nucleotide sequences of partial 18s region, complete ITS region of the isolate Aopn12

A blast search was performed to find a similar sequence to ITS region of fungal isolate Aopn12 in the Genbank DNA database. The ITS region of this isolate was similar to 92.135% identity of Oat root associated fungus OOO45 5, as shown in Figure 4.20. The reference was reported by Carter et al., 1999.

```
>>AJ246163|AJ246163.1 oat root associated fungus 00045 5 (690 nt)
initn: 1695 init1: 1345 opt: 2614 Z-score: 1626.8 bits: 311.4
E(): 2.3e-82 banded Smith-Waterman score: 2614; 92.135% identity
(93.031% ungapped) in 623 nt overlap (1-618:69-690)

                                                  10        20        30
                                          CTTGGTCATTTAGAGGAAGTAAAAGTCGTA
                                          :: : : ::::::::::::::::::::::
AJ2461   CACCCGAACCTGGAAGAGAACTATGGCAAACTAGATTATTTAGAGGAAGTAAAAGTCGTA
          40        50        60        70        80        90

                 40        50        60        70        80
          ACAAGGTTTCCGTA-GTGAACCTGCGGAAGGATCATTAGAAATGGCGGGGGAAGGTTCGC
          :::::::::::::: :::::::::::::::::::::::::::::::::::::::: ::::
AJ2461   ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGAAATGGCGGGGGAAGGCTCGC
          100       110       120       130       140       150

         90        100       110       120       130       140
          CCTTCTCCCC-AAATCTCTATTCTGTGCACTGTTTTTGTGTGCTGGGCTAGCTTTGGCTG
          :::::::::: :::::::: :::::::::::::: :::::::::::::: :: :::::
AJ2461   CCTTCTCCCCAAAATCTCTTTTCTGTGCACTGTTTCTGTGTGCTGGGCTAGTTTCGGCTG
         160       170       180       190       200       210

         150       160       170       180       190        200
          GGCCAGCATGGGGTGGAGCTTGGAGATCGTTTGCATGAATTTTCCCCC--GGGAAAGTTC
           :::::::: :::: :::: ::: : :: :::::::::::::::: :::: :::: : :::
AJ2461   GGCCAGCACGGGGCGGAGTTTGAAAATTGTTTGCATGAATTTTTCCCCTGGGGAGATTTC
         220       230       240       250       260       270

           210       220       230       240        250       260
          CGCTGGAAACTTTTGACATCTCCGCCGAAATTTAATACTATTACTGACTGACCATGAAAA
          ::::: :::::::::::::::::::::: :::::::::::::::::::::::::::::
AJ2461   CGCTGAAAACTTTTGACATCTCCGCCGAATTTTAATACTATTACTGACTGACCATGAAAA
         280       290       300       310       320       330

           270       280       290       300       310       320
          AGTTAATATAACAACTTTCAGCAATGGATCTCTCGGCTTTCGTATCGATGAAGAACGCAG
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AJ2461   AGTTAATATAACAACTTTCAGCAATGGATCTCTCGGCTTTCGTATCGATGAAGAACGCAG
         340       350       360       370       380       390

           330       340       350       360       370       380
          CGAATCGCGATATGTAGTGTGATCTGCCTCTAGTGAATCATCAAATCTTTGAACGCATCT
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AJ2461   CGAATCGCGATATGTAGTGTGATCTGCCTCTAGTGAATCATCAAATCTTTGAACGCATCT
         400       410       420       430       440       450

          390       400       410       420       430       440
          TGCACCCTTTGGTATTCCAAAGGGTACGCCTGTTTCAGTATCATTATAACCTTTCTCCCC
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AJ2461   TGCACCCTTTGGTATTCCAAAGGGTACGCCTGTTTCAGTATCATTATAACCTTTCTCCCC
         460       470       480       490       500       510
```

Figure 4.20 Alignment data of ITS region of isolate Aopn12 and 1 reference taxa.

```
          450         460       470       480       490       500
         AAGGC-TTTTGGCCAAGGAGGGGAAGAGCATAACCGGTGGCCCAGCGGTCTCCCATCAGG
            :: :: ::::::::: :: : :::::::::::::::::::::: ::::::::::::
AJ2461   AAAGCTTTTTGGCCATGGGAAGAAAGAGCATAACCGGTGGCCCAGCAATCTCCCATCAGG
        520       530       540       550       560       570

          510       520       530       540       550       560
         GGGATTGTCTCGGCTGGAATTACTGTTGCAATGGTGGGCCAAATGGTCTCCCGCACTTGA
          ::::: :::::::::::::::: :::::::::::::::::: ::::: :::: :::::
AJ2461   GGGATCGTCTCGGCTGGAATTAGTGTTGCAATGGTGGGCCAACAGGTCT-CCGCTCTTGA
        580       590       600       610       620       630

          570       580       590       600       610       620
         TAAATGTACTTTTAGAAAGAGCGGGGCCGGTCTGCCGAAAGTTGCAACTAACCCTTTACC
         :::::: : ::::: ::::::  :::  ::::  :::::::::: :::::: :
AJ2461   TAAATGAATTTTTATAAAGAGTAGGGATGGTCAACCGAAAGTTGGAACTAAAC
         640       650       660       670       680       690

          630       640       650       660       670       680
         TTTGATCTGAAATCAGGCGGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

Figure 4.20 Alignment data of ITS region of isolate Aopn12 and 1 reference taxa (continued)

Classical identification of fungi is based on observe characteristics. Assignment of morphological species can be based on colony surface texture, hyphal pigment, exudates, margin shapes, growth rate, and sporulating structure (Redlin and Carris, 1985), Fungal isolate Aopn12 limited in spore formation and was identified as Mycelia sterilia.

Molecular method of identification was also performed. The nucleotide sequence of the ITS region of rDNA is conserved. It can be used to delineate species relationship and separated taxonomy from class to species (Mitchell et al., 1995). The nucleotide sequence of the ITS region of fungal isolate Aopn12 was similar to 92.135% identity of Oat root associated fungus OOO45 5 reported by Carter et al., 1999.

Carter et al. (1999) isolated the Oat root associated fungus OOO45 5 from the root of oat plants. They found that the ITS1 and ITS2 sequences of Oat root associated fungus OOO45 5 did not have close sequence matches in the database (defined as > 75% identity).

4.6 Chemical constituents of endophytic fungus isolate Aopn12 metabolites

Endophytic fungus strain Aopn12, isolated from mature leave of *Aglaia odorata* Lour., was cultivated statically in MEB for 3 weeks. The broth and mycelium were separated and investigated chemical constituents of theirs metabolites (Scheme 3.1 and 3.2). Isolation of mycelium ethyl acetate crude extract by column chromatography gave two mixtures and one compound.

4.6.1 Structure elucidation of mixture 1

Mixture 1 was obtained as a white solid in colorless viscous liquid from the combined fraction M2 of mycelium ethyl acetate crude extract, eluted with 5% ethyl acetate in hexane.

The IR spectrum of mixture 1 (Figure D1 in appendix D) showed important absorption bands at 3200-3600 cm^{-1} (O-H stretching vibration), 2918 and 2851 cm^{-1} (CH stretching vibration), 1734 cm^{-1} (C=O vibration), and 1236 cm^{-1} (C-O stretching vibration). The IR data certainly supported the assignment of mixture 1 as shown in Table 4.3.

Table 4.3 The IR absorption band assignments of mixture 1

Wave number (cm^{-1})	Peak intensity	Tentative assignment
3600-3200	Broad	O-H stretching vibration
2918, 2851	Strong	C-H stretching vibration
1734	Strong	C=O stretching vibration
1454	Medium	C-H bending vibration
1236	Weak	C-O stretching vibration
723	Weak	C-H rocking mode of $-(-CH_2)_n-$

The ^{1}H-NMR spectrum (Figure D2 in appendix D) indicated that it possesses a methyl proton at δ 0.91 ppm, a methylene proton attached to a carbonyl proton at δ 2.34 ppm and four olefinic protons at δ 4.17, 4.32, 5.30 and 5.37 ppm.

The ^{13}C-NMR spectrum (Figure D3 in appendix D) indicated that it posseses one methyl carbon signal at δ 14.2 ppm, eighteen methylene carbon signals at 22.7, 24.9, 27.2, 27.3, 29.1, 29.2, 29.3, 29.4, 29.4, 29.5, 29.6, 29.7, 29.7, 29.7, 29.8, 32.0, 34.1 and 34.2 ppm, two olefinic carbon signals at δ 129.7 and 130.0 ppm, and the carbon signal at δ 173.3 ppm should be the carbonyl group.

The structure of mixture 1 was established on the base of spectroscopic analysis and based on retention time of standard methyl ester of fatty acid by gas chromatography. The relative percentages of fatty acids were determined by the area of the peaks in the chromatograms. Chromatogram of mixture 1 is shown in Figure D4 (in appendix D) and fatty acid composition of mixture 1 is presented in Table 4.4.

Table 4.4 Relative fatty acid composition of mixture 1

Symbol	% composition of total fatty acids	Total weight of fatty acid (g)	Weight (mg) / g dry weight mycelia
C14:0	0.90	1.07	6.0
C16:0	38.11	45.16	253.7
C16:1	0.64	0.76	4.3
C18:0	19.26	22.82	128.2
C18:1	28.32	33.56	188.5
C18:2	1.22	1.15	8.1

From GC data, mixture 1 was a mixture of diglycerides that contains 6 type of fatty acids including three saturated fatty acids; myristic , palmitic and stearic acids, and three unsaturated fatty acids; palmitoic , oleic and linoleic acids. Fatty acids range from C14 to C18 in chain length. C16:0 palmitic acid and C18:0 stearic acid are the major saturated fatty acid and C18:1 oleic acid is the major unsaturated fatty acid. Diglycerides are fat and oil that are ester of glycerol with one or more fatty acids and contain two fatty acids linked to the glycerol. Natural oils and fats usually contain several different mixed glycerides. The chemical structure and name of fatty acids in mixture 1 is shown in Table 4.5.

Table 4.5 The chemical structure and chemical name of fatty acids in mixture 1 (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	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
9,12-Octadecenoic acid	Linoleic acid	C18:2	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$

4.6.2 Structure elucidation of mixture 2

Mixture 2 was obtained from the combined fraction M4 of mycelium ethyl acetate crude extract. This mixture 2 was an amorphous white solid (177 mg). The IR spectrum of mixture 2 is depicted as shown in Figure D5 (in appendix D) and revealed the presence of carboxylic group according to the broad absorption band between 2500 to 3300 cm^{-1} and the strong absorption band at 1702 cm^{-1} due to the carboxylic acid carbonyl stretching. The tentative IR absorption band assignment of mixture 2 was presented as summarized in Table 4.6.

Table 4.6 The IR absorption band assignment of mixture 2

Wave number (cm^{-1})	Peak intensity	Tentative assignment
3300 - 2500	Broad	O-H stretching vibration of carboxylic acid
2918, 2850	Strong	C-H stretching vibration of $–CH_3$, $-CH_2-$
1702	Strong	C=O stretching vibration of carboxylic acid
1464	Medium	C-H bending vibration of $–CH_3$, $-CH_2-$
1297	Weak	C-O stretching vibration of carboxylic acid
939,723	Weak	C-H rocking mode of $–(-CH_2)_n-$

The ^{1}H-NMR spectrum (Figure D6 in appendix D) of mixture 2 showed the proton signal of a methylene proton attached to a carboxyl group at δ 2.38 ppm and a methyl proton at δ 0.95 ppm.

The ^{13}C-NMR spectrum (Figure D7 in appendix D) of mixture 2 showed 12 signals, ten signals of methylene carbons appeared at δ 22.7, 24.7, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 32.0 and 34.1 ppm and a signal of methyl carbon appeared at δ 14.2 ppm. The carbon signal at δ 180.4 ppm should be the carbonyl group of carboxylic acid.

The EI mass spectrum (Figure D8 in appendix D) of mixture 2 displayed the molecular ions at *m/z* 256 and 284. They separated by 28 mass units (-CH_2-CH_2-) and showed the fragmentation pattern of long chain hydrocarbons.

From all data, they can be conclude that the structure of mixture 2 was a mixture of long chain carboxylic acid $C_{16\text{-}18}$ or mixed by C16:0; Palmitic acid and C18:0; Stearic acid. The chemical structure was shown in Figure 4.21

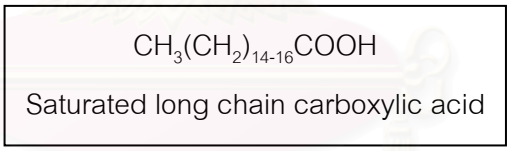


Figure 4.21 The chemical structure and chemical name of mixture 2

4.6.3 Structure elucidation of compound 1

Compound 1 was obtained from the combined fraction M10 of mycelium ethyl acetate crude extract, eluted with EtOAc:hexane (45:55 → 60:40). Compound 1 was an orange solid, m.p. 200-202 °C. The structure of compound 1 was elucidated using spectroscopic techniques. The IR spectrum of compound 1 is shown in Figure D10 (in appendix D) and the absorption peaks were assigned as summarized in Table 4.7. Its IR spectrum indicated important absorption bands at 3200-3500 cm^{-1} (O-H stretching vibration of alcohol), 2929 and 2868 cm^{-1} (C-H stretching vibration), 1641 cm^{-1}

(C=O stretching vibration of carbonyl group), 1415 cm^{-1} (C=C stretching vibration of double bond).

Table 4.7 The IR absorption band assignment of compound 1

Wavenumber (cm^{-1})	Intensity	Vibration
3200-3500	Broad	O-H stretching vibration of alcohol
2939, 2868	Medium	C-H stretching vibration of $-CH_3$, $-CH_2$
1641	Strong	C=O stretching vibration of carbonyl group
1415	Medium	C=C stretching vibration of double bond
1327	Weak	C-H bending vibration of CH_2 and CH_3
1047	Weak	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure D11 in appendix D) of compound 1 indicated that it possesses four methyl groups at 0.85, 0.88, 1.10 and 1.28 ppm.

The ^{13}C-NMR spectrum (Figure D12 in appendix D) of compound 1 showed 23 signals, which two carbonyl groups corresponded to the signals at 185.7 and 166.6 ppm. Five signals of olefinic carbon appeared at δ 130.7, 129.2, 125.1, 120.3 and 115.1 ppm.

DEPT experiments (Figure D13 in appendix D) of compound 1 showed six methylene carbons at 41.6, 32.1, 31.3, 24.9, 18.4 and 18.2 ppm, four methyl carbons at 34.0, 25.0, 21.9 and 18.1 ppm, and one methine carbon at 45.7 ppm.; which indicated that the carbon signals at 44.1, 43.1 and 33.3 ppm were quaternary.

The LC-MS spectrum (Figure D20 in appendix D) showed the $[M+H]^+$ ion peak at m/z 390. The mass spectrum indicated that it possesses the molecular weight 389.

The information from 2D-NMR techniques; HMQC correlation (Table 4.8, Figure D14 in appendix D) and HMBC, COSY, NOESY correlation (Table 4.9, Figure 4.23-4.25 and Figure D15-D17 in appendix D) were used to assist the interpretation of the compound 1 structure, then compound 1 was an orange solid which molecular formula may probably be $C_{23}H_{31}NO_5$ as shown in Figure 4.22.

Table 4.8 HMQC spectra data of compound 1

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
185.7, 166.6, 164.8, 130.7	-
129.2	7.14 (1H, s)
125.1, 120.3	-
115.1	5.58 (1H, s)
80.8	-
45.7	1.70 (1H, d, *J* = 12)
44.1, 43.1	-
41.6	1.32 (1H, d, *J* = 13.2), 1.08 (1H, br t, *J* = 13.2)
34.0	0.88 (3H, s)
33.3	-
32.1	1.27 (1H, d, *J* = 14.8), 1.55 (1H, d, *J* = 14.8)
31.3	1.85 (1H, br t, *J* = 12.4) 1.73 (1H, d, *J* = 14.8)
25.0	1.23 (3H, s)
24.9	2.88 (1H, d, *J* = 16.8), 2.54 (1H, d, *J* = 18.4)
21.9	0.85 (3H, s)
18.4	1.58 (1H, m), 1.52 (1H, m)
18.2	1.62 (1H, m), 1.42 (1H, m)
18.1	1.10 (3H, s)
OH	4.18 (1H, s)

Table 4.9 The correlation of gHMQC, gHMBC, COSY and NOESY of compound 1

Position	δC	δH	HMBC (H to C)	COSY	NOESY
1	32.1	1.27 (1H, d, *J* = 14.8), 1.55 (1H, d, *J* = 14.8)	C-2, C-3, C-5 -	H-1 (1.55) H-1 (1.27)	- H-23 (1.10)
2	18.2	1.62 (1H, m), 1.42 (1H, m)	C-10, C-23 C-23	H-2 (1.42) H-2 (1.62), H-3(1.08)	- -
3	41.6	1.32 (1H, d, *J* =13.2), 1.08 (1H, br t, *J* = 13.2)	C-4, C-5, C-7, C-21 C-20, C-21	H-3 (1.08) H-2 (1.62), H-3 (1.32)	H-21(0.85),H-22(0.88)
4	33.3	-	-	-	-
5	45.7	1.70 (1H, d, *J* = 12)	C-4, C-7, C-9, C-10, C-21, C-23	-	H-20 (0.88)
6	18.4	1.58 (1H, m), 1.52 (1H, m)	C-7 -	- -	H-22(1.23),H-20(0.88) -
7	31.3	1.85 (1H, br t, *J* = 12.4) 1.74 (1H, d, *J* = 14.8)	C-5, C-8, C-22 C-5, C-8	H-1 (1.55), H-7 (1.74) H-7 (1.85)	- H-22 (1.23)
8	44.1	-	-	-	-
9	80.8	4.18 (1H, s)	C-8, C-22	-	-
10	43.1	-	-	-	-
11	24.9	2.88 (1H, d, *J* = 16.8), 2.54 (1H, d, *J* = 18.4)	C-9, C-12, C-13, C-16 C-8, C-9, C-12, C-13, C-16	H-11 (2.54) H-11 (2.88)	H-22(1.23),H-23(1.10) -
12	130.7	-	-	-	-
13	120.3	-	-	-	-
14	115.1	5.58 (1H, s)	C-8, C-11, C-12, C-13, C-17, C-18, C-19-	-	-
15	164.8	-		-	-
16	129.2	7.14 (1H, s)	C-12, C-13, C-17,C-18, C-19	-	-
17	125.1	-	-	-	-
18	166.6	-	-	-	-
19	185.7	-	-	-	-
20	34.0	0.88 (3H, s)	C-3, C-4, C-5, C-6, C-21	-	H-3 (1.32),H-5 (1.70), H-6(1.58), H-21 (0.85)
21	21.9	0.85 (3H, s)	C-3, C-4, C-5	-	H-3 (1.32),H-20 (0.88)
22	25.0	1.23 (3H, s)	C-7, C-8, C-9, C-15	-	H-7 (1.74)
23	18.1	1.10 (3H, s)	C-1, C-5, C-9,C-10, C-11	-	H-6 (1.58), H-7 (1.74), H-11 (2.88)

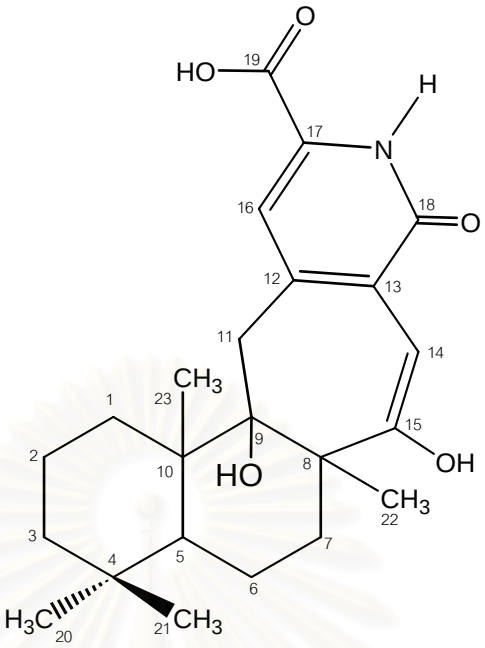


Figure 4.22 The chemical structure of compound 1

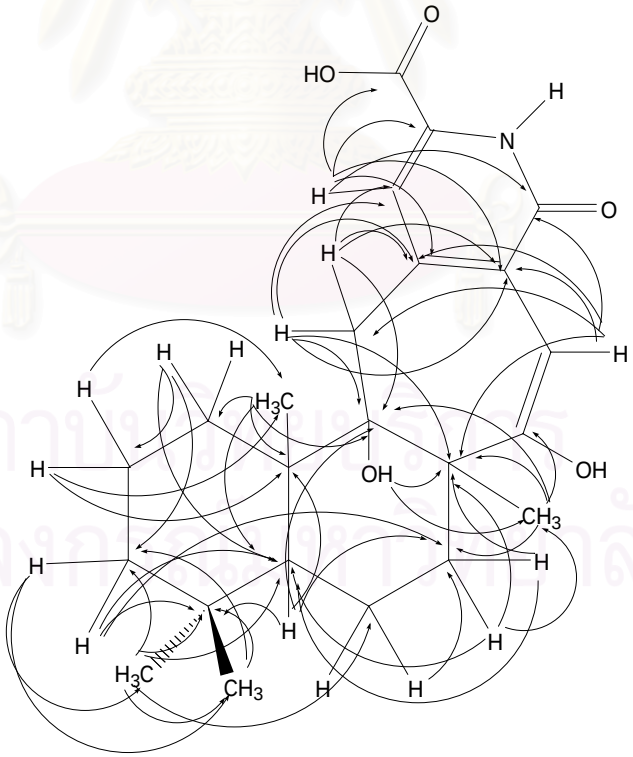


Figure 4.23 The gHMBC correlation of compound 1

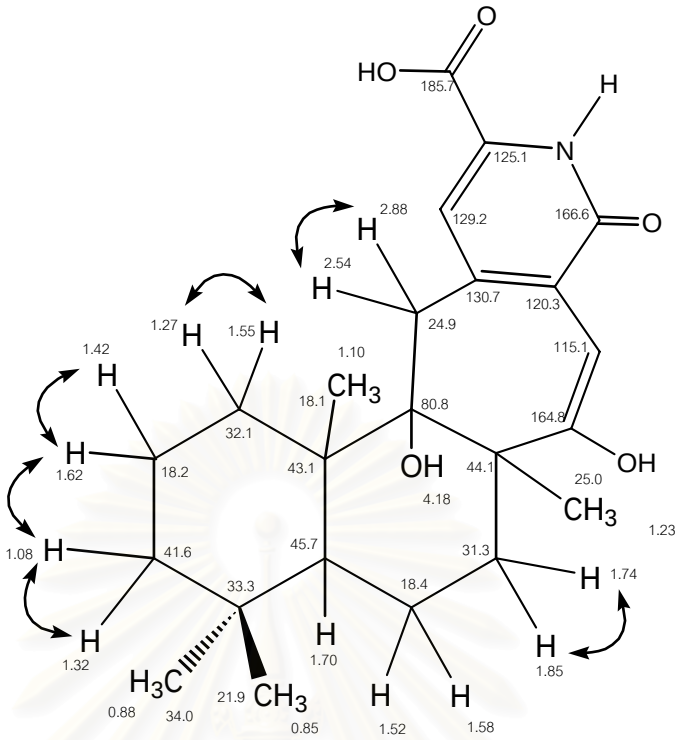


Figure 4.24 The COSY correlation of compound 1

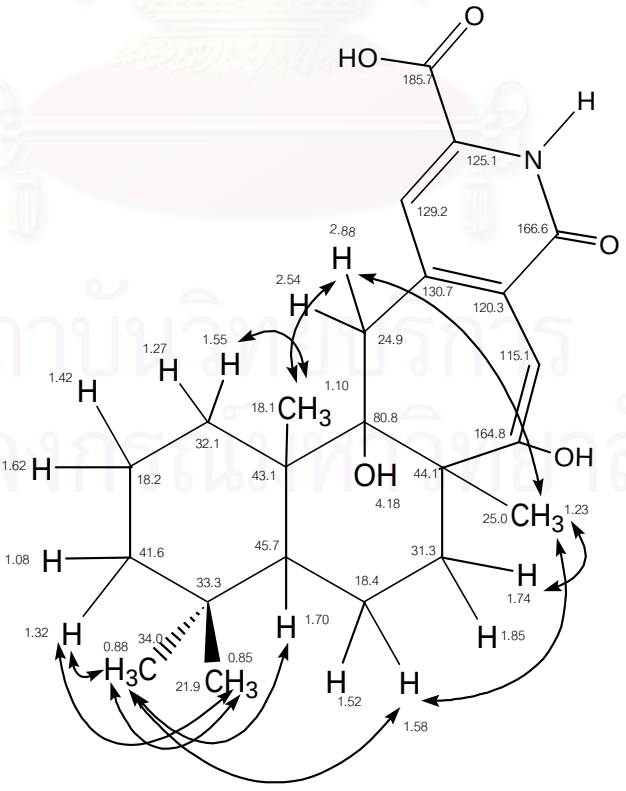


Figure 4.25 The NOESY correlation of compound 1

4.7 Metabolites of endophytic fungus isolate Aopn12 cultured in different media.

In comparison of metabolites from mycelium of the fungal isolate Aopn12 cultivated in 5 different culture mediums; MCB, MEB, PDB, SGB and YES broth. There were one mixture and one compound, as shown in Table 4.10.

Table 4.10 Metabolites from mycelium crude of the isolate Aopn12 cultured in MCB, MEB, PDB, SGB, YES broth

Mycelium from culture medium	mg metabolites weight/ g dry weight mycelium	
	Mixture 1 (diglycerides)	Compound 1 (orange solid)
MCB	10.8	2.6
MEB	27.7	6.3
PDB	0.7	3.4
SGB	8.6	1.0
YEB	14.8	0.5

There were one mixture and one compound from mycelium ethyl acetate crude of fungal isolate Aopn12. Mixture 1, a mixture of fatty acids was found in mycelia from every media. Diglycerides content in mycelia cultured on MCB, MEB, PDB, SGB and YES broth studied ranged from 0.7 to 27.7 mg/g dry weight. The differences are due to the nutrient source in the culture media. Diglycerides are component of lipid that is important fungal components both in terms of structure and membrane constitution. The major factors influencing the extent of lipid production are the nature and proportion of carbon (C) and nitrogen (N) as nutrient sources in the medium (Pupin et al., 2000).

Compound 1, an orange solid was also found in mycelia from every culture media, with maximum amount in MEA, so compound 1 may be used as chemotaxonomy of fungal isolate Aopn12.

4.8 Biological activities

4.8.1 Antimicrobial activity of the crude extract and pool fractions

The antimicrobial activity of the crude extracts and pool fraction from endophytic fungus isolate Aopn12 was evaluated by the agar well diffusion method. The fractions were examined at a concentration of 10 mg/ml (1 mg/well; 7 mm diameter). 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 Table 4.11 and antimicrobial activity of the fractions from crude extracts is shown in Table 4.12 - 4.13.

Table 4.11 Antimicrobial activity of the crude extracts from endophytic fungus isolate Aopn12

Test microoraganisms	The EtOAc crude extract	
	Mycelia	Culture broth
B. subtilis ATCC 6633	++	+
S. aureus ATCC 25923	-	-
E. coli ATCC 25922	-	-
P. aeruginosa ATCC 25753	-	-
C. albicans ATCC 10231	-	-
S. cerevisiae TISTR 5169	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.12 Antimicrobial activity of the fractions of EtOAc crude from mycelia extract

Combined fractions	Test microorganisms					
	Gram positive bacteria		Gram negative bacteria		Yeast	
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. cerevisiae*	*C. albicans*
M1	-	-	-	-	-	-
M2	-	-	-	-	-	-
M3	+	-	-	-	-	-
M4	-	-	-	-	-	-
M5	+	-	-	-	-	-
M6	+	-	-	-	-	-
M7	+	-	-	-	-	-
M8	+	-	-	+	-	-
M9	+	-	-	-	-	-
M10	+	-	-	-	-	-
M11	+	-	-	-	-	-
M12	+	-	-	-	-	-
M13	+	-	-	-	-	-
M14	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.13 Antimicrobial activity of the fractions of EtOAc crude from culture broth extract

Combined fractions	Test microorganisms					
	Gram positive bacteria		Gram negative bacteria		Yeast	
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. cerevisiae*	*C. albicans*
B1	-	-	-	-	-	-
B2	-	-	-	-	-	-
B3	-	-	-	-	-	-
B4	-	-	-	-	-	-
B5	-	-	-	-	-	-
B6	+	-	-	++	-	-
B7	+	-	+	+	-	-
B8	+	-	-	+	-	-
B9	+	-	-	+	-	-
B10	-	-	-	-	-	-
B11	-	-	-	+	-	-
B12	-	-	-	-	-	-
B13	-	-	-	-	-	-
B14	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

4.8.2 Biological activities of pure compound

4.8.2.1 Antimicrobial activity of pure compound

All the three metabolites isolated from mycelium of the endophytic fungus Aopn12 were subjected to antimicrobial assay. It was found that compound 1 was active against *B. subtilis* ATCC 6633 with concentration MIC 500 µg/ml. Mixture 1 and mixture 2 showed no antimicrobial activity against 6 test microorganism as shown in Table 4.14.

Table 4.14 Broth microdilution method for antimicrobial activity of pure compound

Compounds	Test microorganisms and MIC (µg/ml)					
	Gram positive bacteria		Gram negative bacteria		Yeast	
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. cerevisiae*	*C. albicans*
Mixture 1	-	-	-	-	-	-
Mixture 2	-	-	-	-	-	-
Compound 1	500	-	-	-	-	-
Tetracycline HCl	31.3	0.8	1.0	ND	ND	ND
Amoxicillin	-	0.8	1.0	ND	ND	ND

MIC was the minimum inhibitory concentration

- = inactive, **ND = not done**

4.8.2.2 Cytotoxic activity of pure compound

The *in vitro* activity of a compound from fungal isolate Aopn12 was tested against 5 cell lines including, HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) and is reported in Table 4.15.

Table 4.15 Cytotoxic activities against cell line of pure compound from endophytic fungus isolate Aopn12

Compounds	IC_{50} (µg/ml)				
	SW620 (colon)	BT474 (breast)	KATO-3 (gastric)	HEP-G2 (hepatoma)	CHAGO (lung)
Compound 1	4.5	5.4	6	5.4	4.3
Adriblastina	5.8	4.8	6.4	6.6	4.2

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

The result showed that the compound 1 exhibited high cytotoxic activities against 5 tumor cell lines; HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) with IC_{50} 5.4, 4.5, 4.3, 6, and 5.4 µg/ml, respectively.

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CHAPTER V

CONCLUSION

Endophytic fungi were isolated by using the surface sterilization method. Sixty endophytic fungi were isolated from mature leaves of the Thai medicinal plant, *Aglaia odorata* Lour. Plant samples were collected from 4 provinces in Thailand; Bangkok, Khonkean, Maha sarakham and Pattani.

Sixty seven 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. YES was found to be the medium of choice for expression of antimicrobial activities.

Fungal isolate Aopn12 and malt extract broth was chosen for further study for bioactive compound because the malt extract agar blocks of the isolate Aopn12 were active against a large number of test microorganisms such as *B. subtilis, S. aureus, E. coli, P. aeruginosa, C. albicans* and *S. cerevisiae.* Based on morphology, the fungus isolate Aopn12 was found as Mycelia sterilia. Based on nucleotide sequencing of ITS region, it was closely related to Oat root associated fungus OOO45 5.

In the present investigation, three metabolites from mycelia of the fungal isolate Aopn12 were isolated by using silica gel column chromatography. There were two mixtures and one compound. Mixture 1 was a mixture of diglyceride. Mixture 2 was a mixture of long chain carboxylic acid $C_{16\text{-}18}$, Palmitic and Stearic acids. Compound 1 was an orange solid which molecular formula may probably be $C_{23}H_{31}NO_5$. This structure were established on 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.

In comparison of metabolites from mycelia of the fungal isolate Aopn12 cultivated in 5 different culture broths; MCB, MEB, PDB, SGB and YES. There were one mixture and one compound. Mixture 1, a mixture of diglycerides was found in mycelia from every media, with maximum amount in MEB. Compound 1, an orange solid was

also found in mycelia from every culture media, with maximum amount in MEA, so compound 1 may be used as chemotaxonomy of fungal isolate Aopn12.

Antimicrobial activity and cytotoxicity of the pure compound were tested. Compound 1 was found to exhibited weak activity against *B. subtilis* with the MIC value of 500 μg/ml, and exhibited high cytotoxic activities against SW620 (colon), BT474 (breast), KATO-3 (gastric), HEP-G2 (hepatoma) and CHAGO (lung) cell line with IC_{50} 4.5, 5.4, 6, 5.4 and 3.3 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)

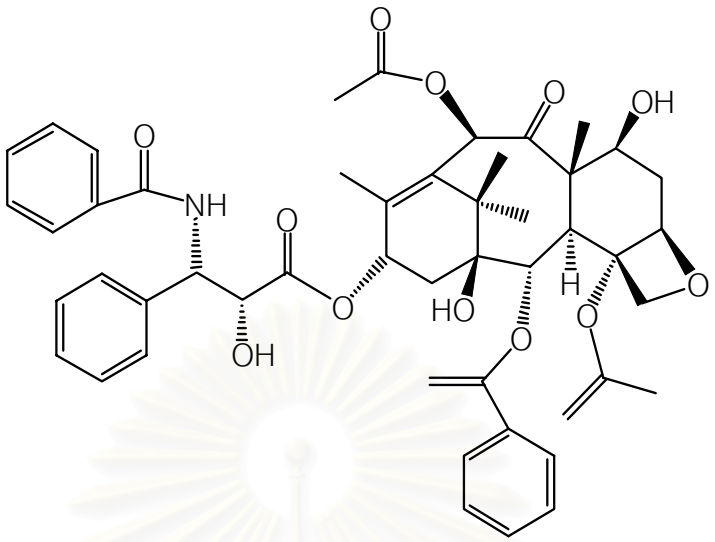
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
52	7-O-[α-L-Rhamnopyranosyl(1-6)-β-D-glucopy-ranosyl]tricin	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998
53	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxic	Berny *et al*., 1997
54	Leucinostatin 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)				



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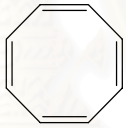
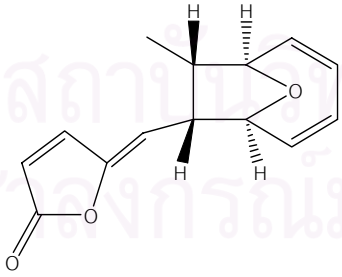
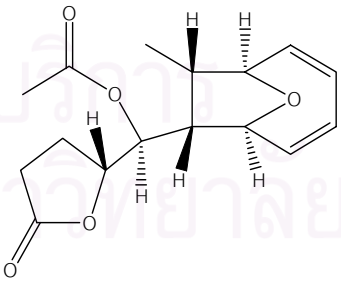


[2] 1,3,5,7 cyclooctatetraene or (8)-annulene

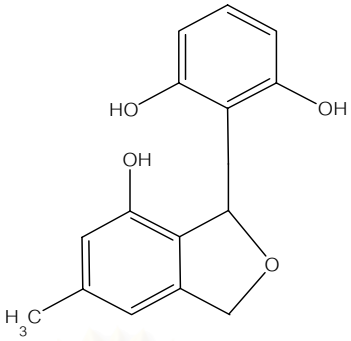


[3] Lactones 1893 A

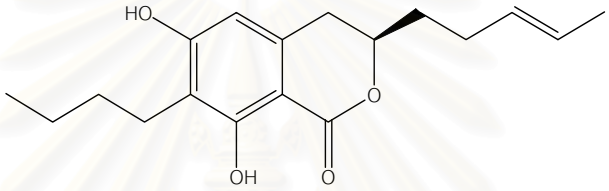


[4] Lactones 1893 B

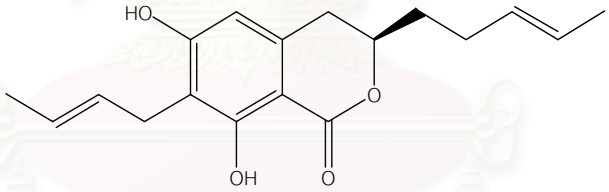
Figure A1 Structure of secondary metabolites of endophytic fungi



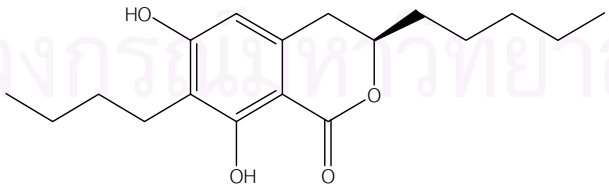
[5] Pestacin



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



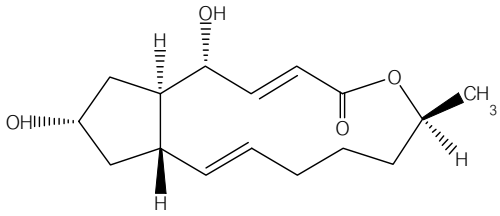
[7] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



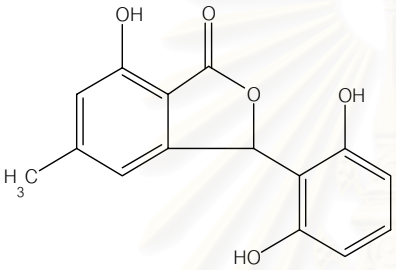
[8] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

Dihydroisocumarins [6-8]

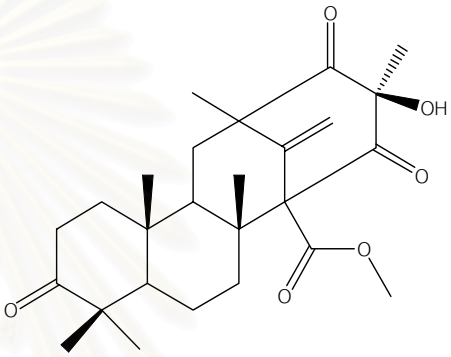
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



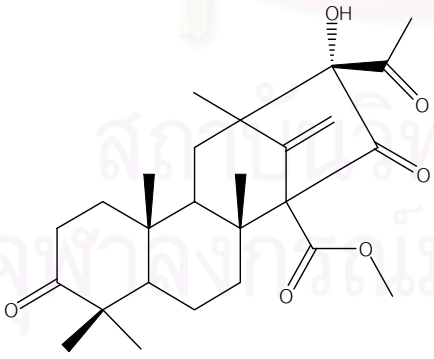
[9] Brefeldin A



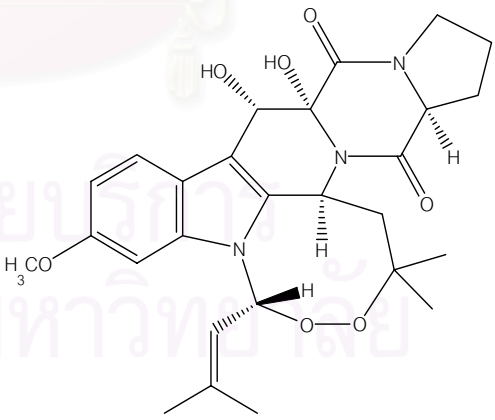
[10] Isopestacin



[11] Preaustinoid A

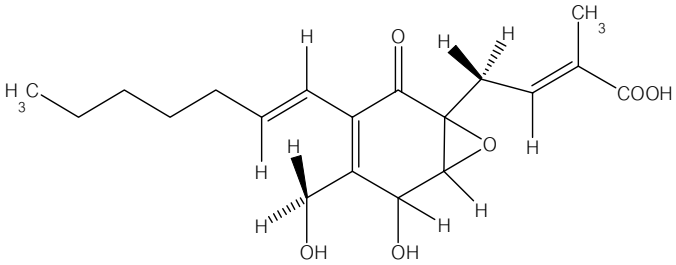


[12] Preaustinoid B

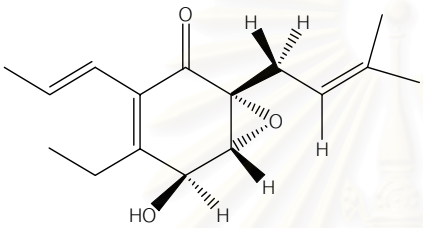


[13] Alkaloid verruculogen

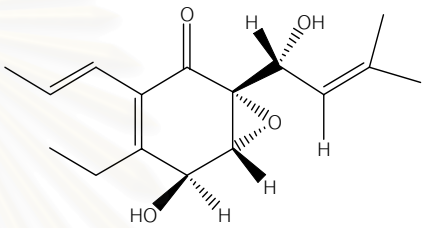
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



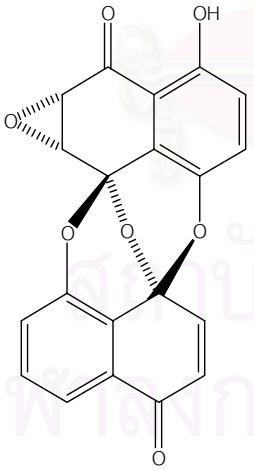
[14] Ambuic acid



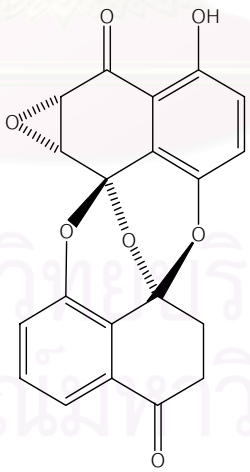
[15] Jesterone



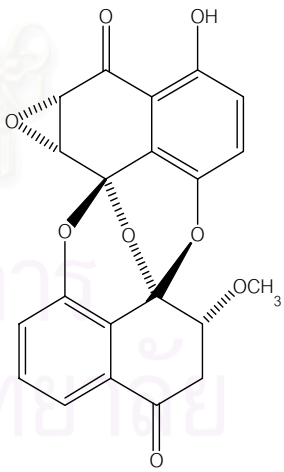
[16] Hydroxy-jesterone



[17] Preussomerin G

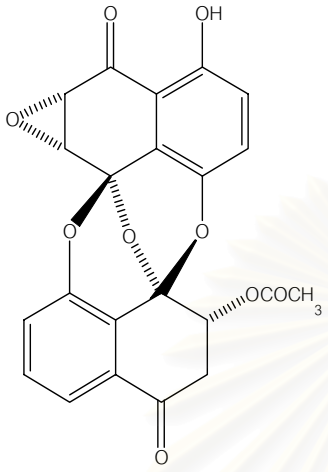


[18] Preussomerin H

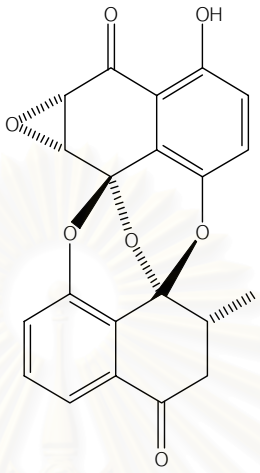


[19] Preussomerin I

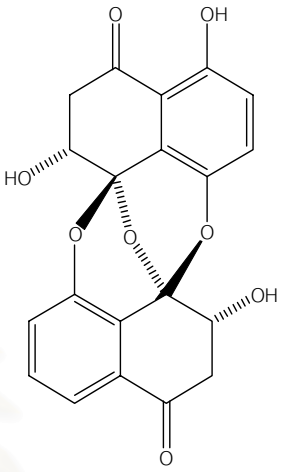
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



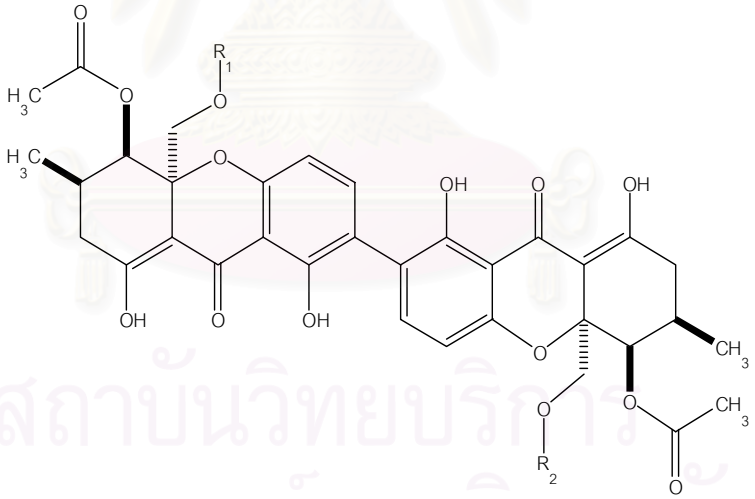
[20] Preussomerin J



[21] Preussomerin K



[22] Preussomerin L

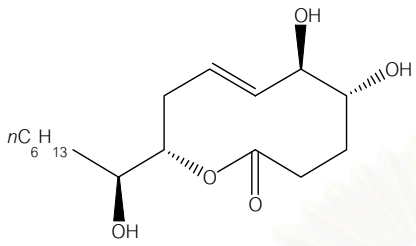


[23] Dicerandrols A, R1=R2=H

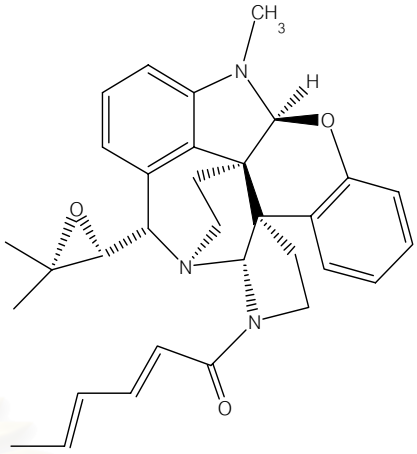
[24] Dicerandrols B, R1=Ac, R2=H

[25] Dicerandrols C, R1=R2=Ac

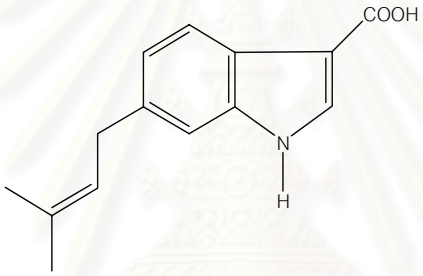
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



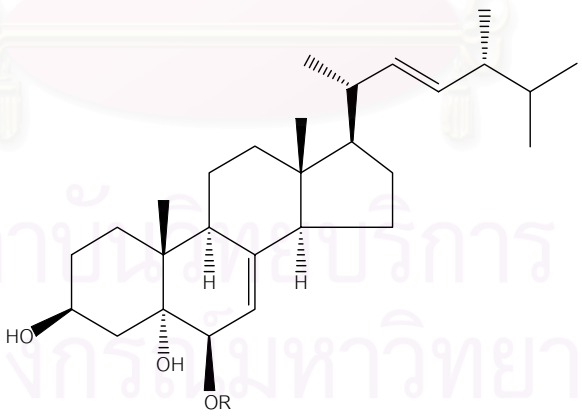
[26] Microcarpalide



[27] Nomofungin



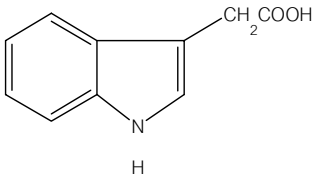
[28] Isoprenylindole-3-carboxylic acid



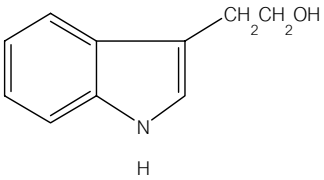
[29] 3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7, 22-diene, R=$COCH_3$

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

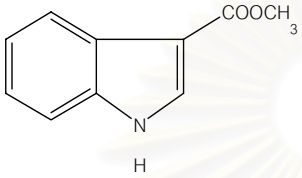
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



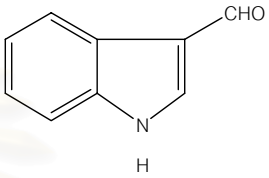
[31] Indole-3-acetic acid (IAA)



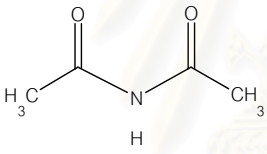
[32] Indole-3-ethanol (IEtOH)



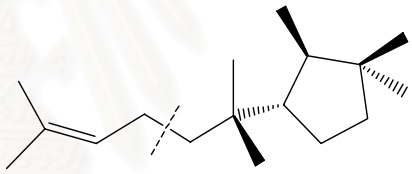
[33] Methylindole-3-carboxylate



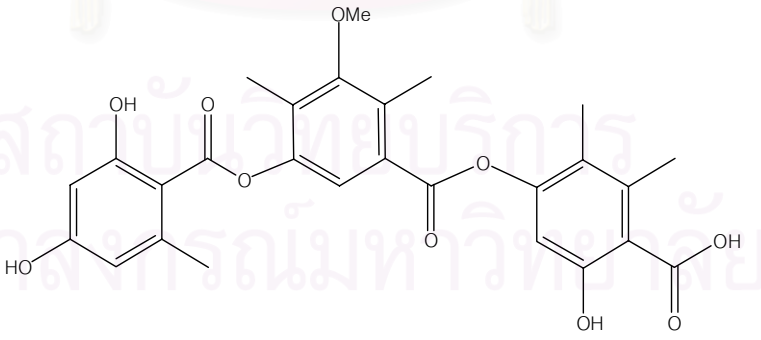
[34] Indole-3-carboxaldehyde



[35] Diacetamide

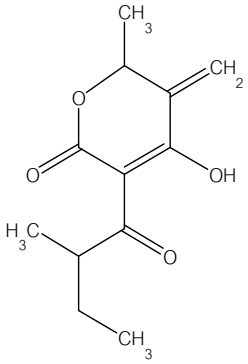


[36] Cyclonerodiol

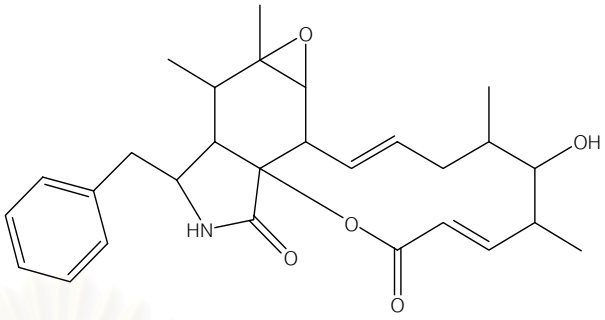


[37] Colletotric acid

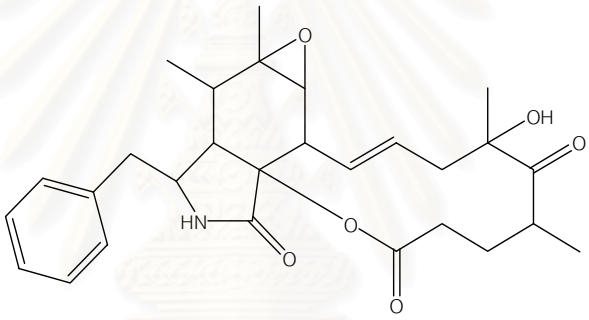
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



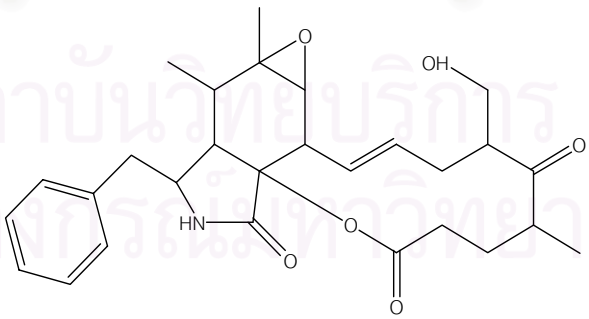
[38] CR377, pentaketide



[39] Cytochalasin 1

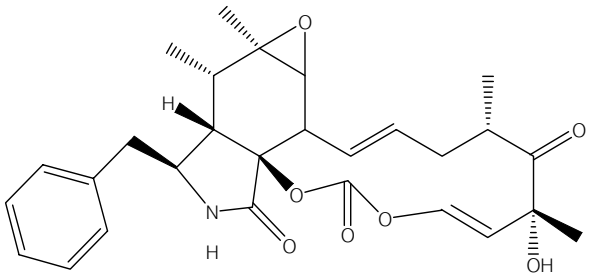


[40] Cytochalasin 2

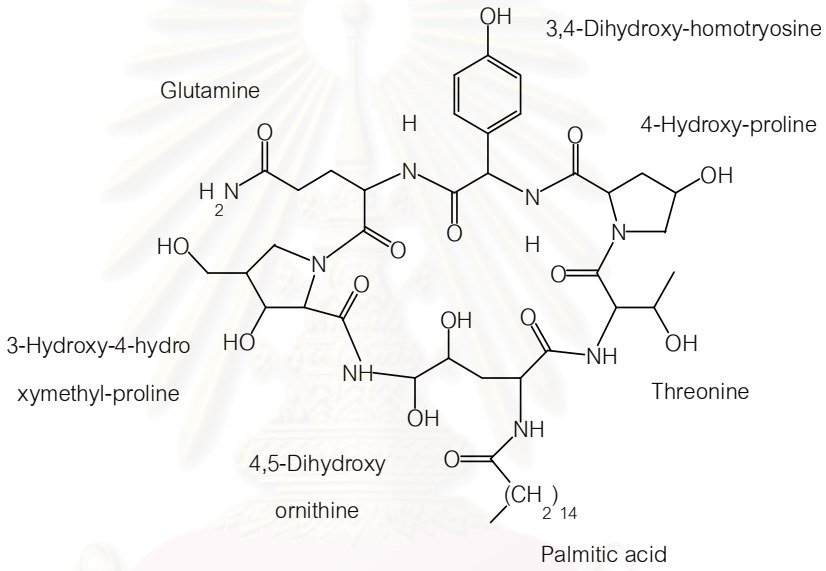


[41] Cytochalasin 3

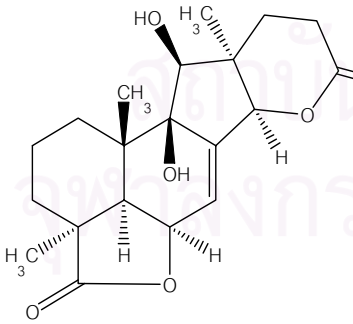
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



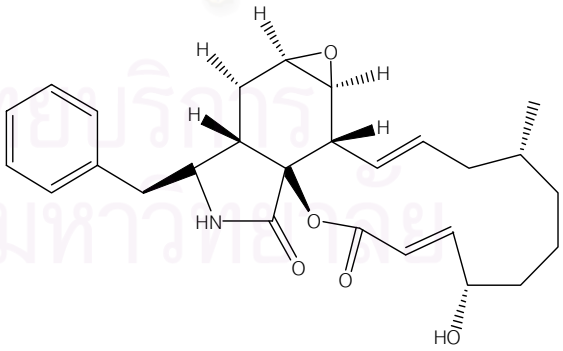
[42] Cytochanlasin E



[43] Cryptocandin

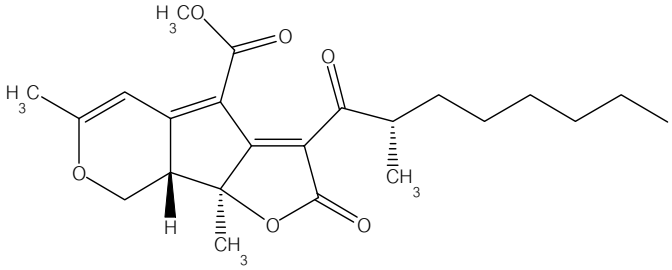


[44] Geniculol

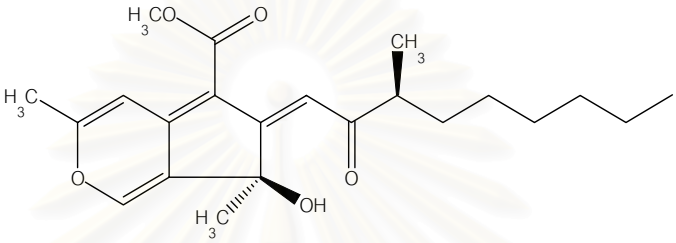


[45] Cytochalasin F

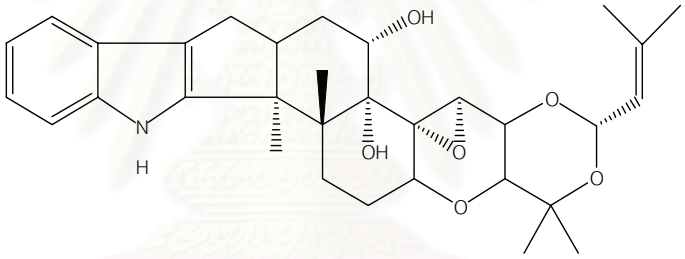
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



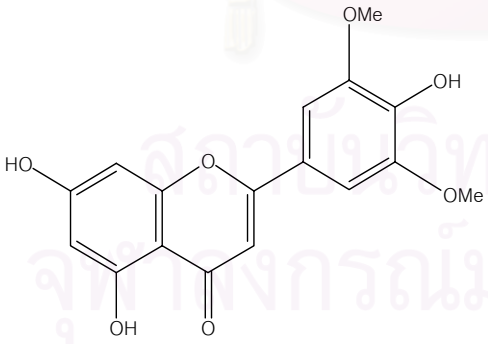
[46] Sequoiatones A



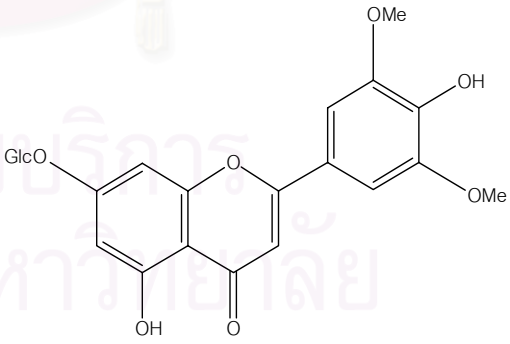
[47] Sequoiatones B



[48] Terpendole M

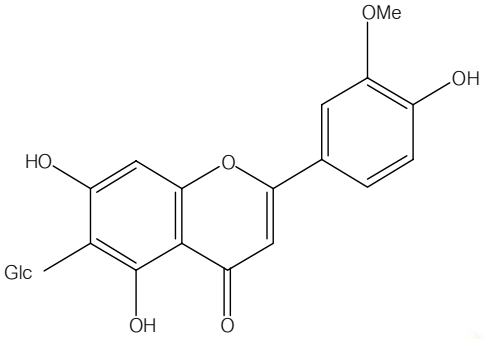


[49] Tricin

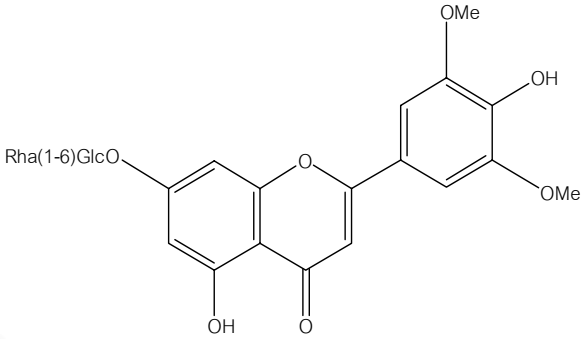


[50] 7-O-(B-D-glucopyranosyl)tricin

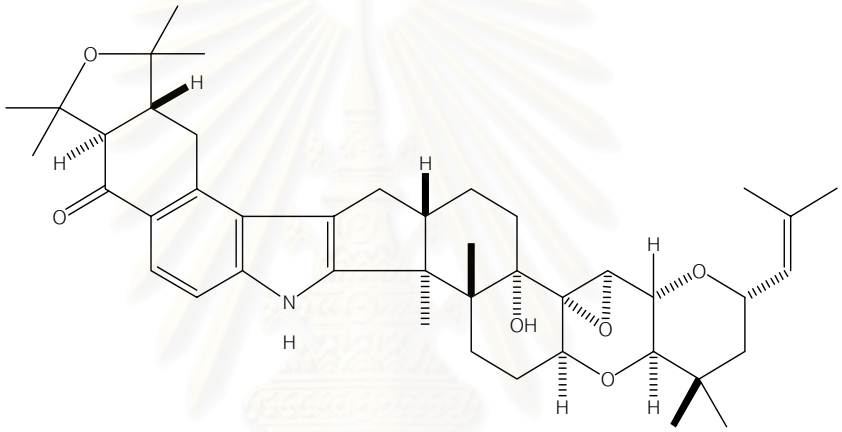
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



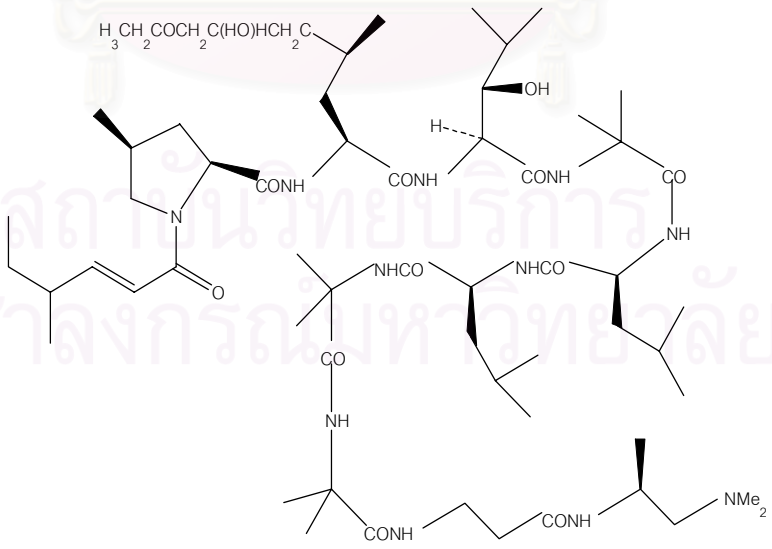
[51] Isoorientin



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

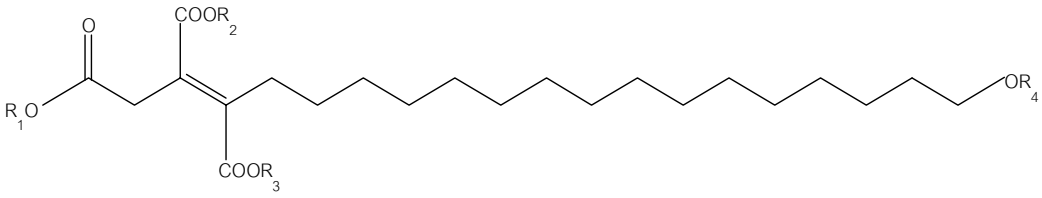


[53] Lolitrem B



[54] Leucinostatin A

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

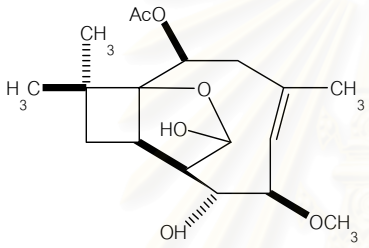


[55] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H

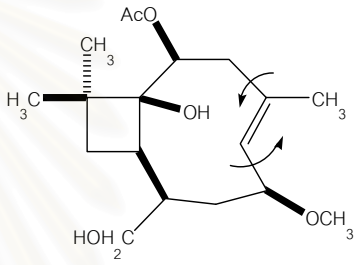
[56] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H

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

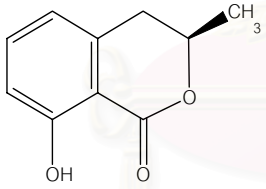
[58] 4: Desulfated analog, R1=R2=R3=R4=H



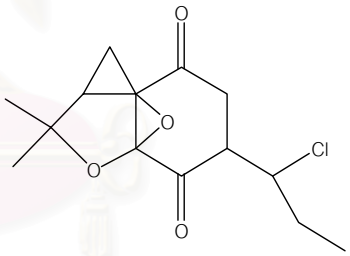
[59] Pestalotiopsins A



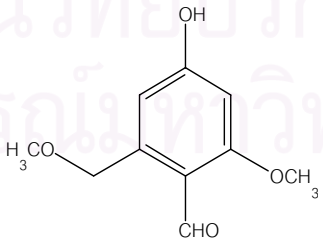
[60] Pestalotiopsins B



[61] (R)-mellein

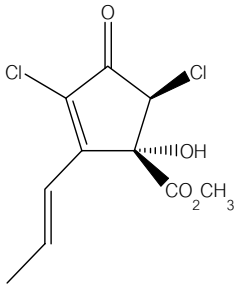


[62] (-)-mycorrhizin A

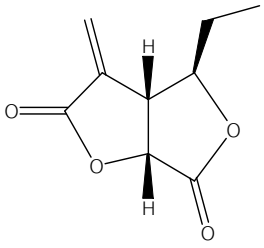


[63] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

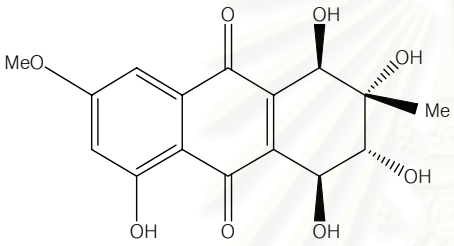
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



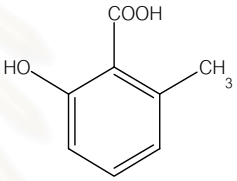
[64] (+)-cryptosporiopsin



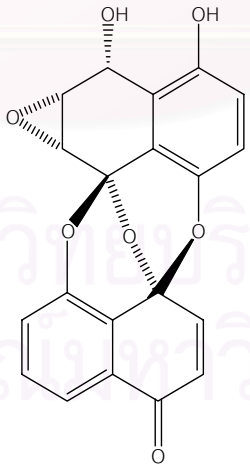
[65] 4-epi-ethiosolide



[66] Altersolanol A

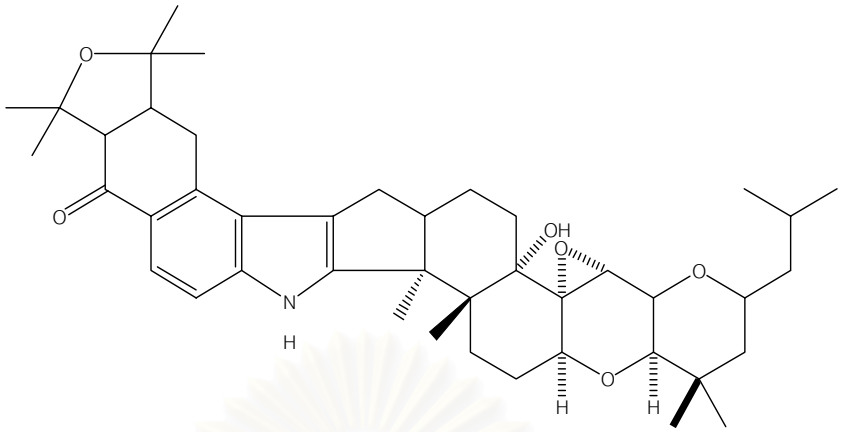


[67] 2-hydroxy-6-methyl benzoic acid

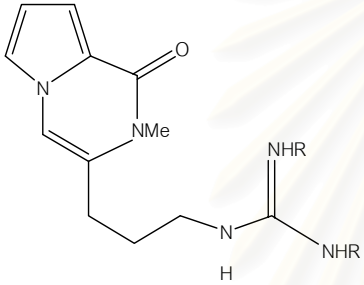


[68] Preussomerin D

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

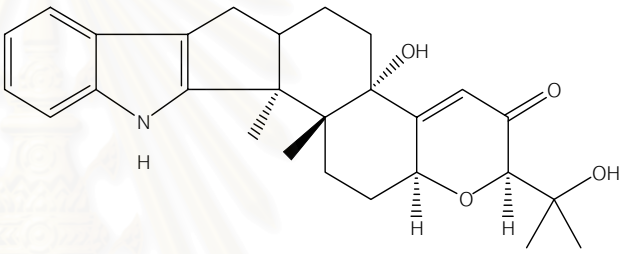


[69] Lolitrem C

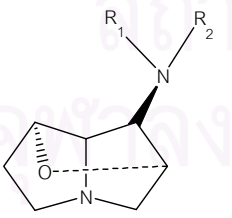


[70] Peramine R=H

[71] Diacetylperamine R=Ac

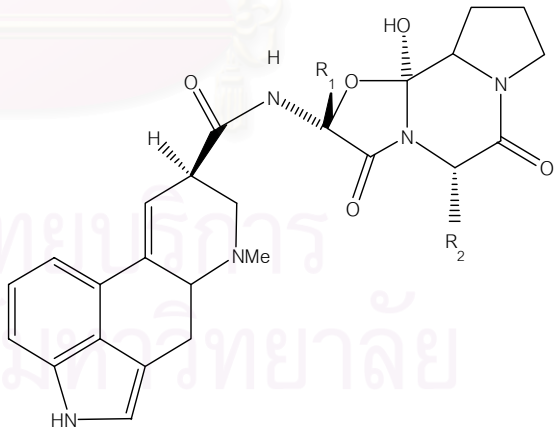


[72] Paxilline



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

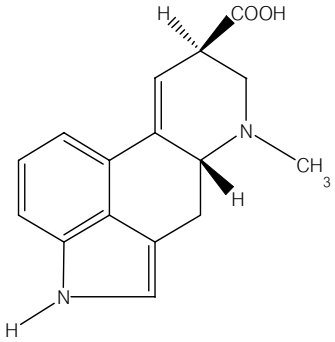
R_2= H, HCO, Ac



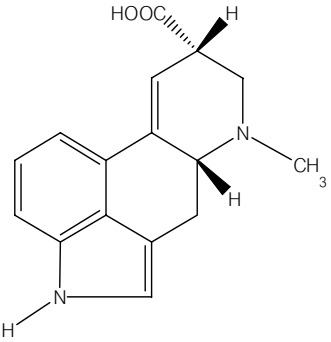
[74] Ergopeptine alkaloids

Ergovaline R_1=Me, R_2=i-Pr

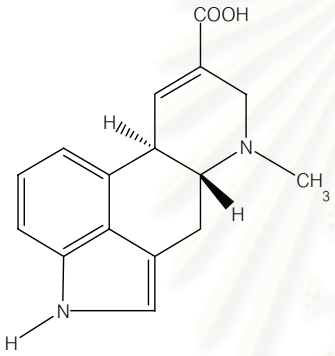
Figure A1 Structure of secondary metabolites of endophytic fungi (continued)



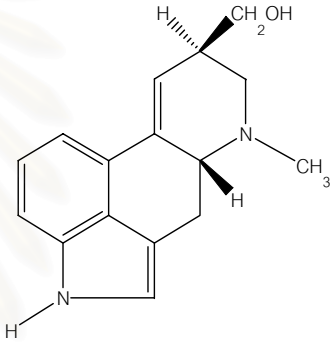
[75] Lysergic acid



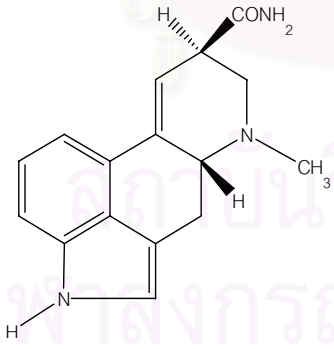
[76] Isolysergic acid



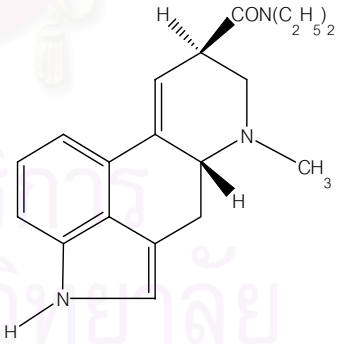
[77] Paspalic acid



[78] Lysergol

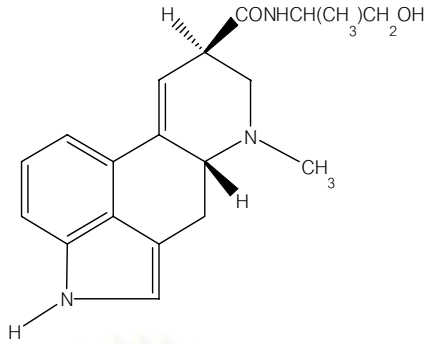


[79] Lysergic acid amide



[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°C for 15 minutes.

1. Nutrient Agar (NA)

Beef extract	3	g
Peptone	4	g
Agar	15	g
Distilled water	1,000	ml

2. Malt Czapek Agar (MCA)

Sucrose	30	g
Malt extract	40	g
Sodium nitrate	2	g
Potassium chloride	0.5	g
Magnesium sulphate	0.5	g
Ferrous sulphate	0.01	g
Dipotassium phosphate	1	g
Agar	20	g
Distilled water	1,000	ml

3. Malt Extract Agar (MEA)

Malt extract	20	g
Glucose	20	g
Peptone	1	g
Agar	15	g
Distilled water	1,000	ml

4. Potato Dextrose Agar (PDA)

Potato,(pelled and diced)	200	g
D-glucose	20	g
Agar	15	g
Distilled water to	1,000	ml

Boil 200 g of peeled, diced potatoes for one hour in a litre of water. Filter, and make up the filtrate to one litre. Add the glucose and agar and dissolve by steaming.

5. Sabouroud Glucose Agar (SGA)

Glucose	40	g
Peptone	10	g
Agar	15	g
Distilled water	1,000	ml

6. Yeast Extract Sucrose Agar (YES)

Yeast extract	20	g
Sucrose	15	g
Agar	20	g
Distilled water	1,000	ml

APPENDIX C

Antimicrobial activities of endophytic fungus isolates

Table C1 A summary of number of active endophytic fungus isolates demonstrating activities against test microorganisms when cultured on different media

Antimicrobial activity	Total number of active isolates	Number of active isolates on culture medium				
		MCA	MEA	PDA	SGA	YES
Anti-Microbial	40	13	12	8	9	31
anti-*B. subtilis*	20	2	3	1	1	17
anti-*S. aureus*	18	7	10	3	2	11
anti-*E. coli*	2	1	1	-	1	2
anti-*P. aeruginosa*	17	3	3	2	2	14
anti-*C. albicans*	4	1	2	-	1	2
anti-*S. cerevisiae*	18	6	7	6	8	12

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
1	Aobk1	MCA	-	1.5	-	-	-	-
		MEA	-	3	-	-	-	-
		PDA	-	1	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	3	-	-	-	-
2	Aobk2	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	1.5	-	-
3	Aobk3	MCA	-	1	-	-	-	-
		MEA	-	1	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
4	Aobk4	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
5	Aobk5	MCA	-	-	-	1	-	1.5
		MEA	-	-	-	-	-	-
		PDA	-	2.5	-	2.5	-	2.5
		SGA	-	-	-	-	-	3
		YES	-	-	-	-	-	-
6	Aobk6	MCA	-	1	-	-	-	2
		MEA	-	2	-	1	-	2.75
		PDA	-	-	-	-	-	1.25
		SGA	-	-	-	-	-	3.5
		YES	-	-	-	-	-	2.5
7	Aobk7	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	1	-	-
		YES	-	-	-	-	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
8	Aobk8	MCA	-	-	-	-	-	-
		MEA	-	-	-	1.5	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	1	-	-
9	Aobk9	MCA	-	-	-	-	-	1
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	1
		SGA	-	-	-	-	-	-
		YES	1	-	-	-	-	-
10	Aobk10	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
11	Aobk11	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1	-	-	1	-	-
12	Aobk12	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	4.5
13	Aobk13	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
14	Aobk14	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
15	Aokk1	MCA	-	-	-	1.5	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	1	-	-
		SGA	-	-	-	-	-	-
		YES	1	-	-	1	-	1
16	Aokk2	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.5	1.5	1.3	-	-	-
17	Aokk3	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
18	Aokk4	MCA	-	-	-	-	-	1
		MEA	-	2	-	-	3	4
		PDA	-	-	-	-	-	-
		SGA	-	1	-	-	-	1
		YES	-	-	-	-	-	1.5
19	Aomh1	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
20	Aomh2	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	2
21	Aomh3	MCA	-	-	-	1	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	1	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
22	Aomh4	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
23	Aomh5	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
24	Aomh6	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.75	-	-	2	-	-
25	Aomh7	MCA	-	-	-	-	-	-
		MEA	-	2.5	-	-	-	1.5
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
26	Aomh8	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.5	-	-	-	-	-
27	Aomh9	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
28	Aomh10	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1	-	-	-	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
29	Aomh11	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	2.5	4	-	3	-	5
30	Aomh12	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
31	Aomh13	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.5	2	-	-	-	-
32	Aomh14	MCA	-	-	-	-	-	-
		MEA	-	1	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	1
		YES	-	-	-	-	-	-
33	Aomh15	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	6
34	Aomh16	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.5	2	-	2	1	1
35	Aomh17	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
36	Aomh18	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
37	Aomh19	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.5	-	-	1.5	-	5
38	Aomh20	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
39	Aomh21	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
40	Aomh22	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	4.5
41	Aomh23	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1	-	-	1.5	-	-
42	Aomh24	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	3.5
		SGA	-	-	-	-	-	-
		YES	-	3	-	-	3	7

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
43	Aomh25	MCA	-	1	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
44	Aomh26	MCA	-	1	-	-	-	-
		MEA	-	2	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	1	-	-	-	-
45	Aomh27	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
		YES	2.2	2	-	2.5	-	-
46	Aomh28	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	3.5	-	-	2.5	-	-
47	Aomh29	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	2	-	-	-	-
48	Aomh30	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	3	-	-	2.5	-	-
49	Aopn1	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	1.5	1	-	-	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
50	Aopn2	MCA	-	-	-	-	-	-
		MEA	2	1.5	-	-	-	10
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	2
		YES	-	-	-	-	-	-
51	Aopn3	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
52	Aopn4	MCA	1	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
53	Aopn5	MCA	-	3	-	-	-	7
		MEA	-	4	-	-	-	8
		PDA	-	-	-	-	-	5
		SGA	-	-	-	-	-	4
		YES	-	-	-	-	-	-
54	Aopn6	MCA	-	-	-	-	-	-
		MEA	1	-	-	-	-	2
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	2
		YES	-	-	-	-	-	1
55	Aopn7	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
56	Aopn8	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-

Table C2 Inhibition zone (mm) measured from the agar block of endophytic fungus culture (continued)

No.	Isolate	Culture medium	Test organisms					
			B.S.	S.A.	E.C.	P.A.	C.A.	S.C.
57	Aopn9	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
58	Aopn10	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
59	Aopn11	MCA	-	-	-	-	-	-
		MEA	-	-	-	-	-	-
		PDA	-	-	-	-	-	-
		SGA	-	-	-	-	-	-
		YES	-	-	-	-	-	-
60	Aopn12	MCA	4	7.5	2	-	5	6
		MEA	9	10	4	2	7	10
		PDA	2	2.5	-	-	-	3.5
		SGA	2	10	1.5	1	4.5	3
		YES	1.5	2	1	1	-	-

B.S. = *Bacillus subtilis* ATCC 6633

S.A. = *Staphylococcus aureus* ATCC 25923

E.C. = *Escherichia coli* ATCC 25922

P.A. = *Pseudomonas aeruginosa* ATCC 27853

C.A. = *Candida albicans* ATCC 10231

S.C. = *Saccharomyces cerevisiae* TISTR 5169

APPENDIX D

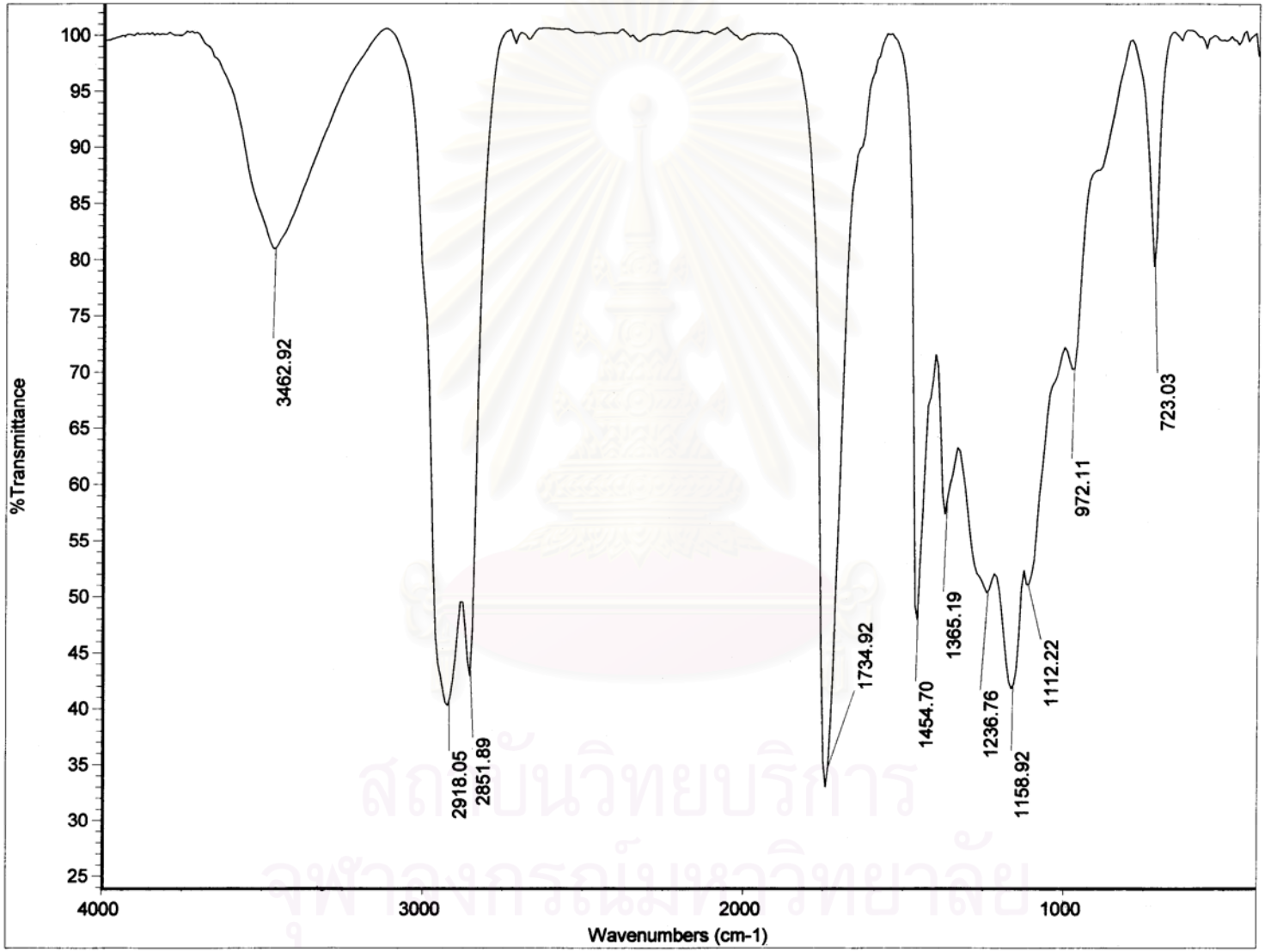


Figure D1 IR spectrum of mixture 1

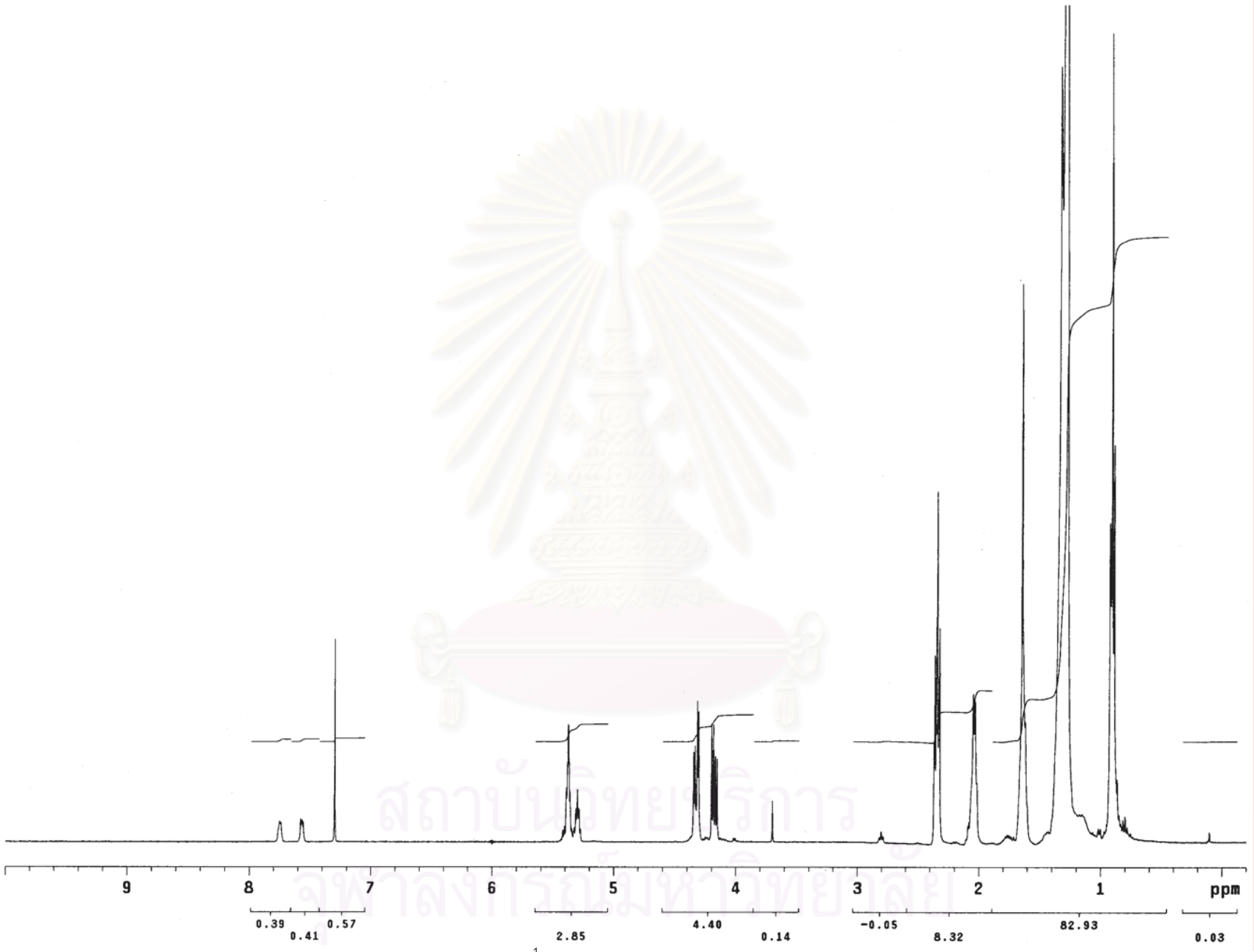


Figure D2 ^{1}H-NMR spectrum of mixture 1

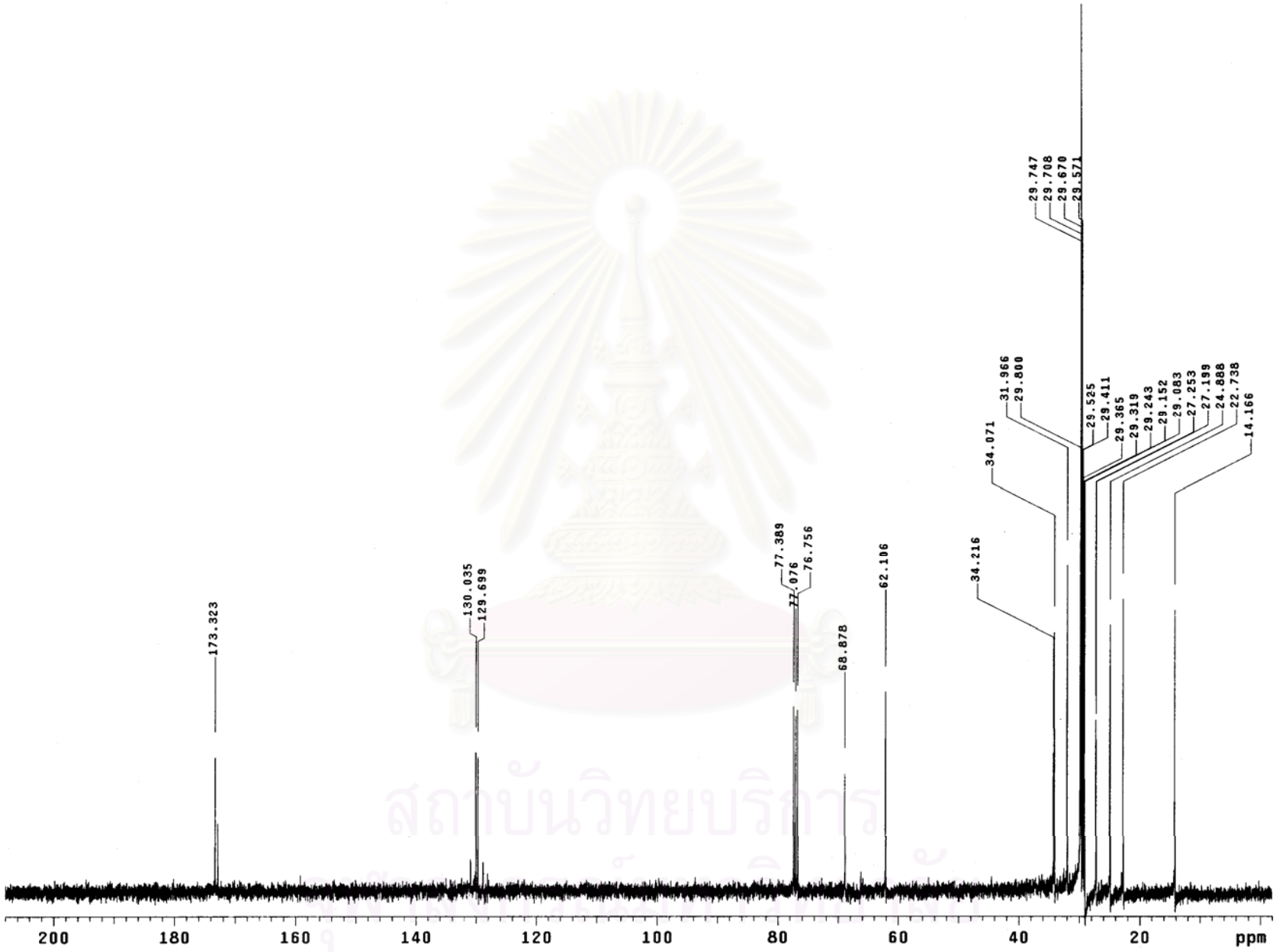
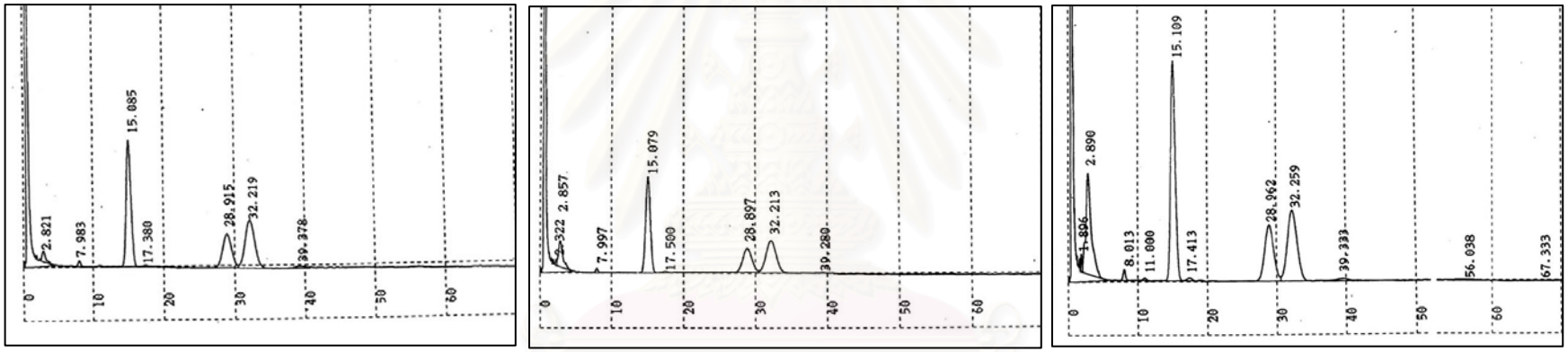


Figure D3 ^{13}C-NMR spectrum of mixture 1



Experiment 1st Experiment 2nd Experiment 3rd

Figure D4 GC spectrum of mixture 1 (experiment 1st , 2nd and 3rd)

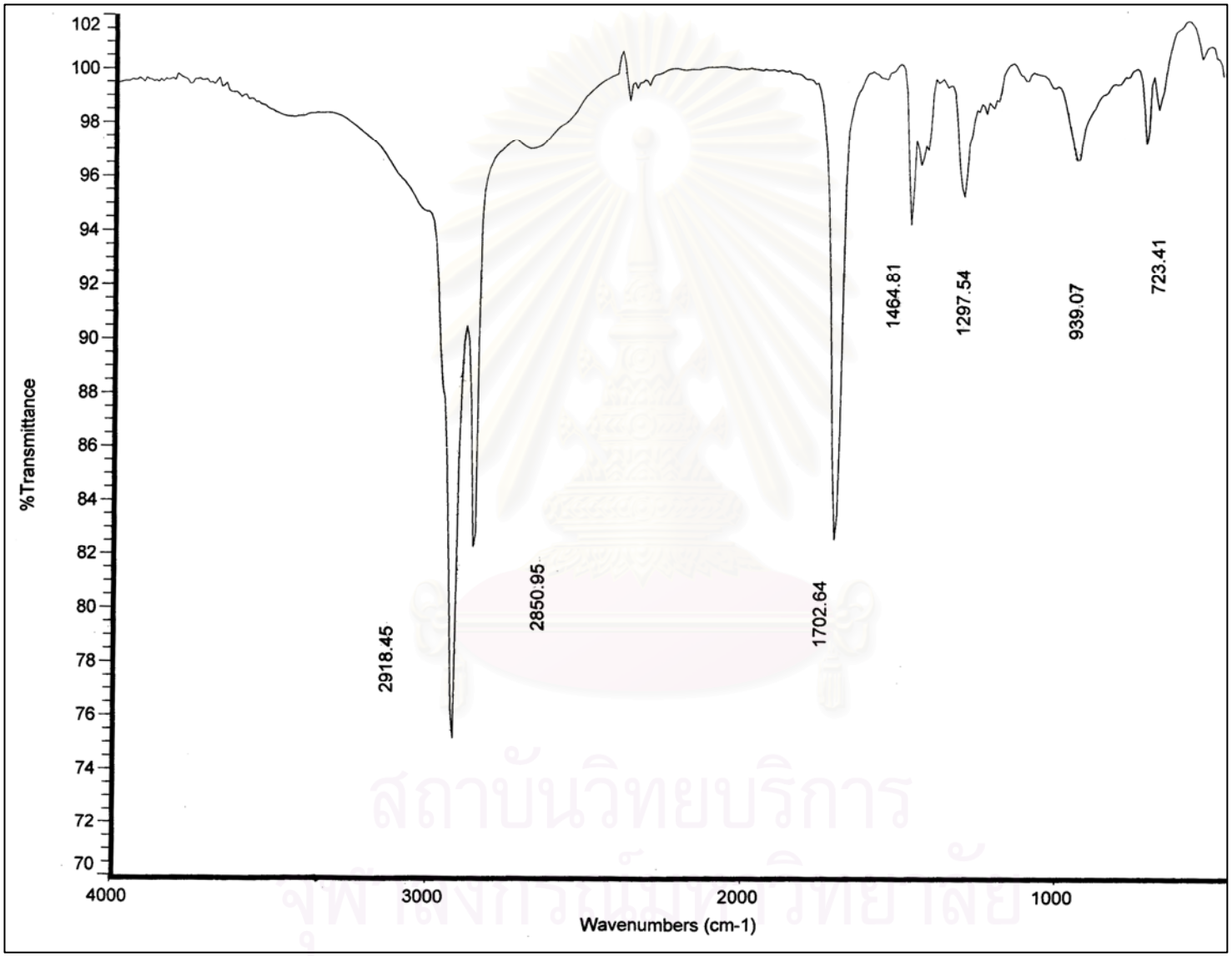


Figure D5 IR spectrum of mixture 2

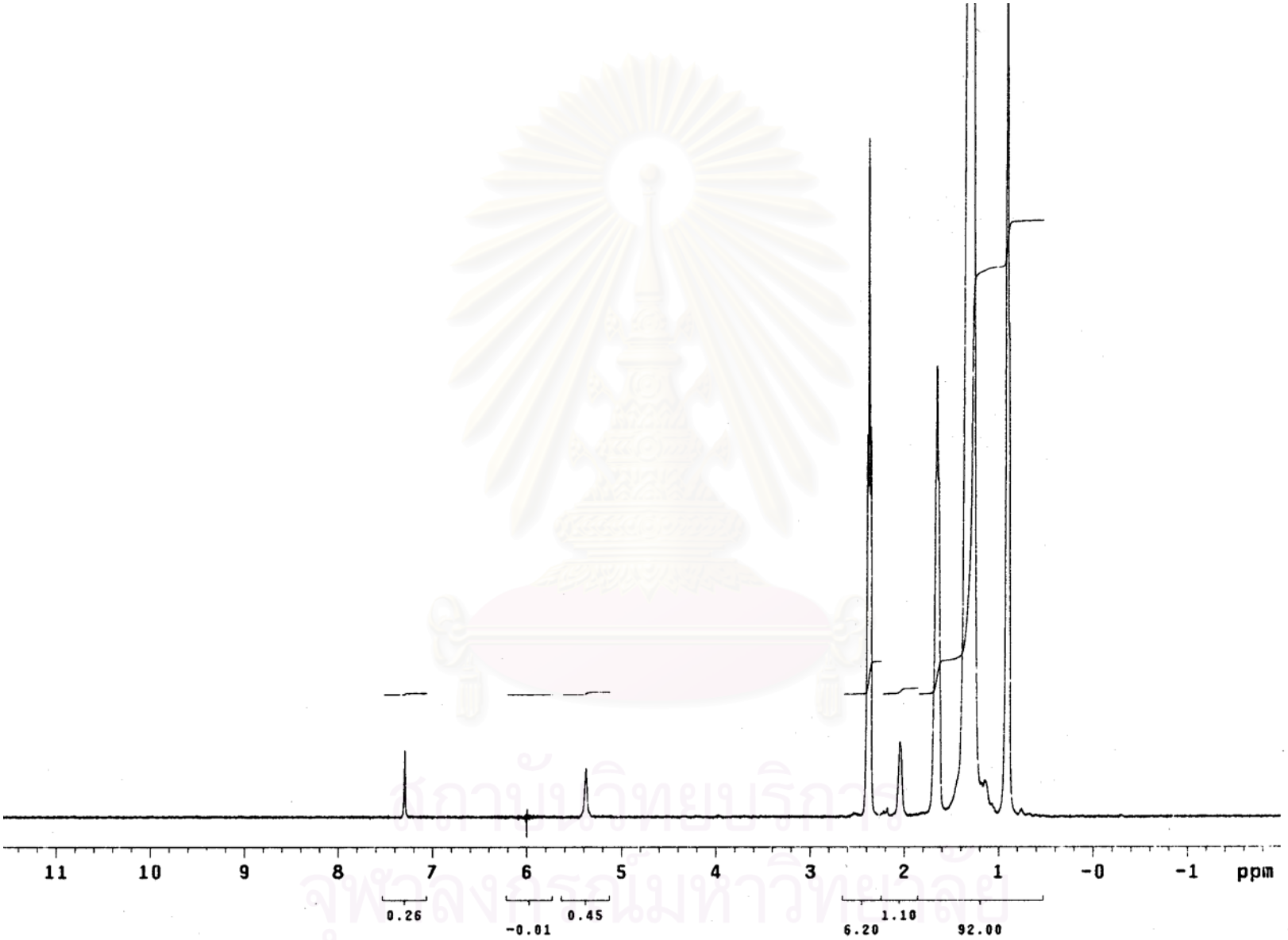


Figure D6 ^{1}H-NMR spectrum of mixture 2

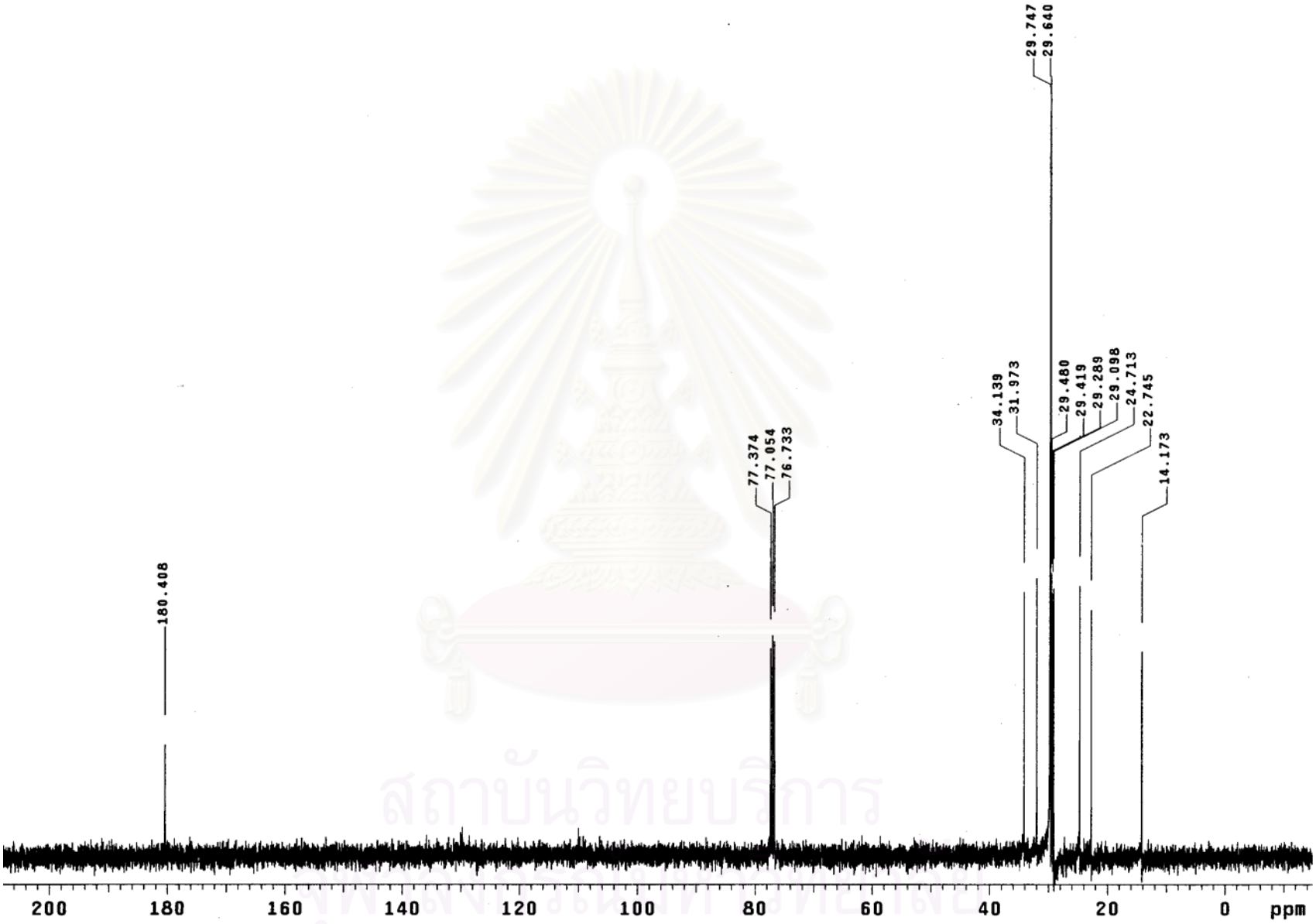


Figure D7 ^{13}C-NMR spectrum of mixture 2

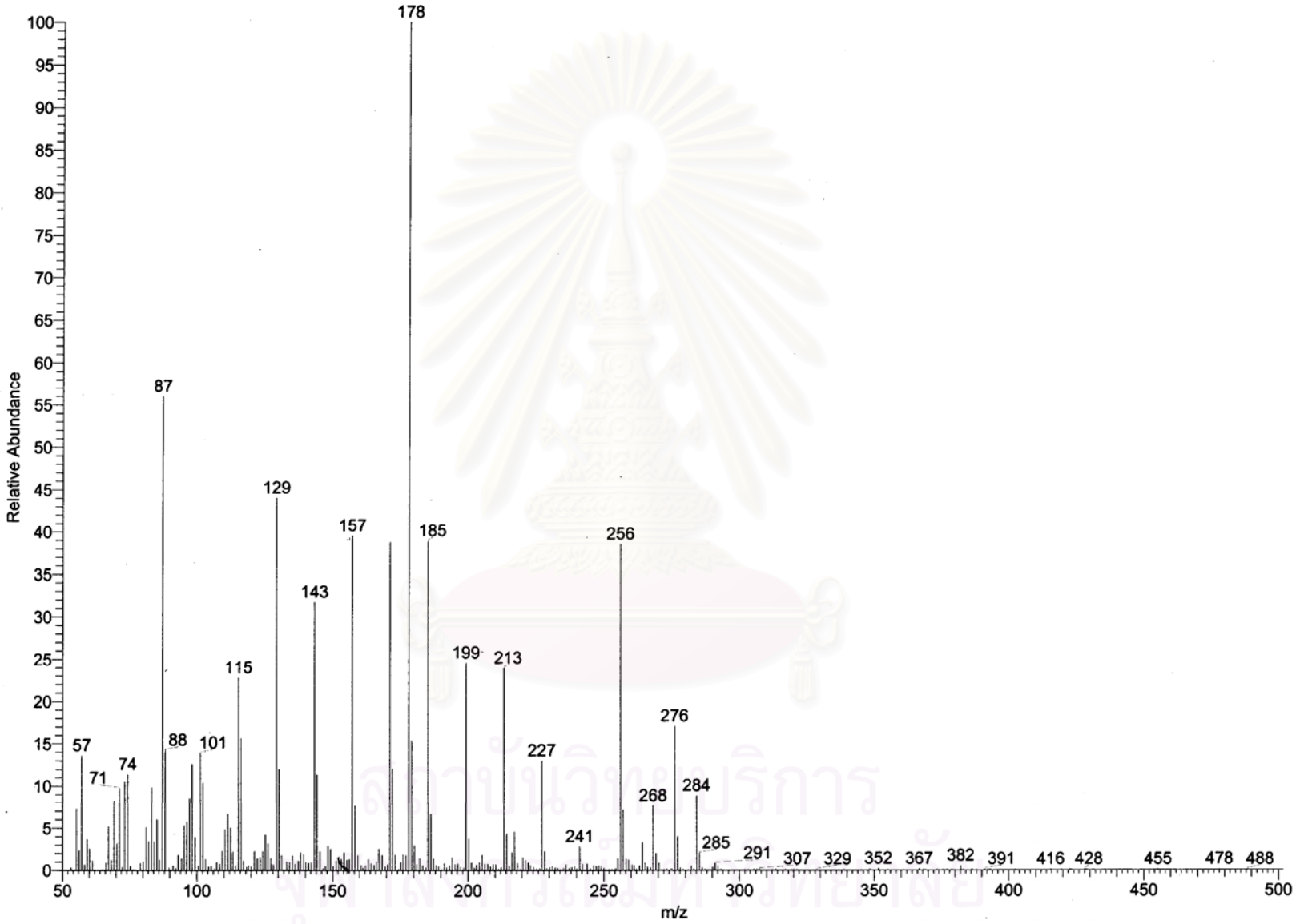


Figure D8 MS spectrum of mixture 2

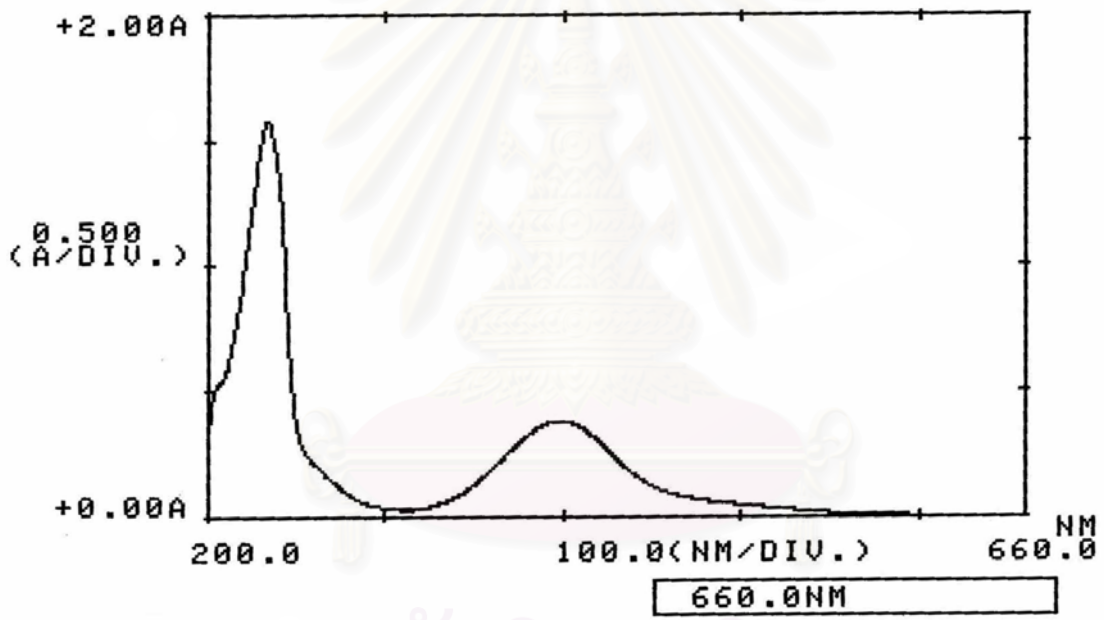


Figure D9 UV spectrum of compound 1

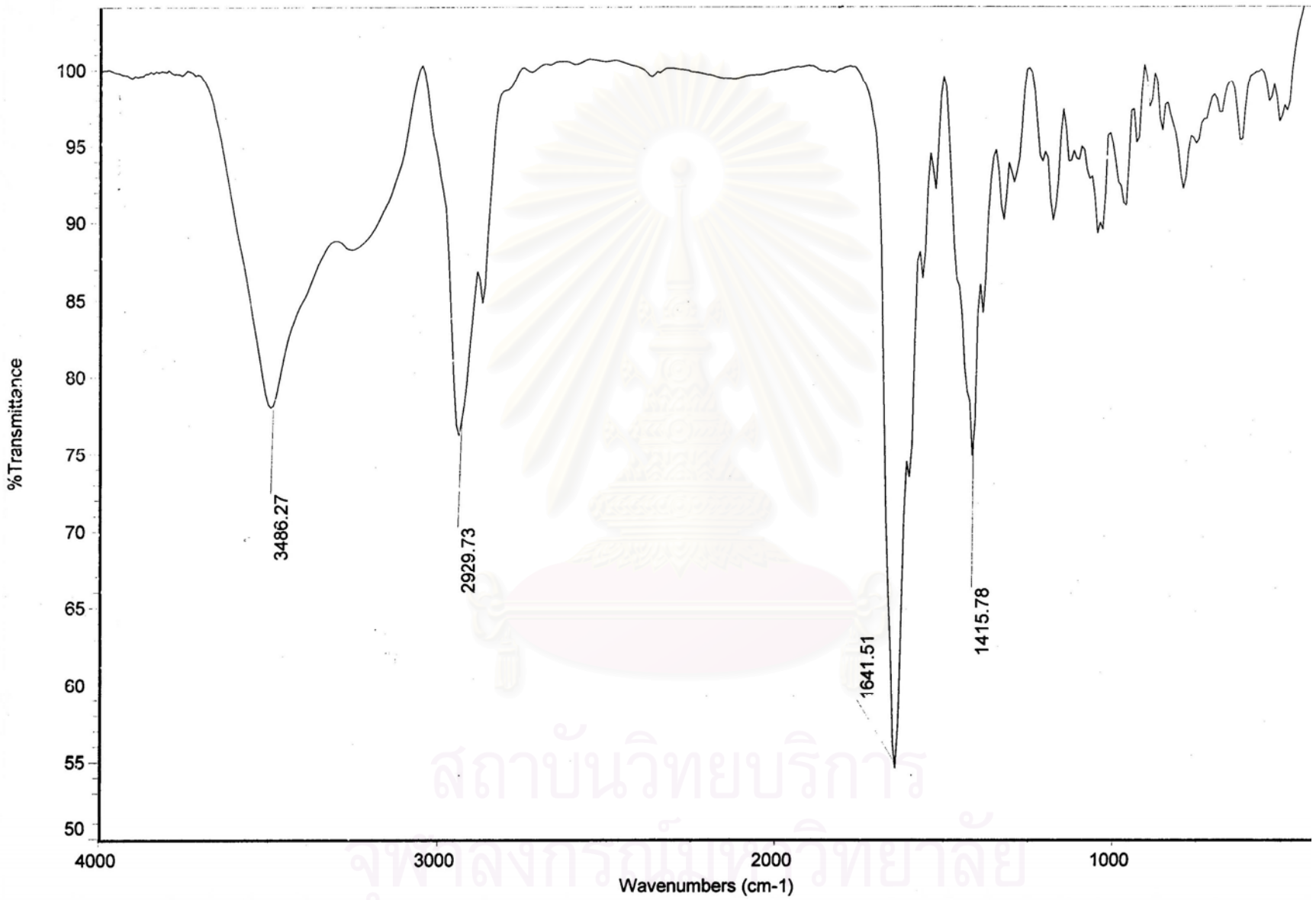


Figure D10 IR spectrum of compound 1

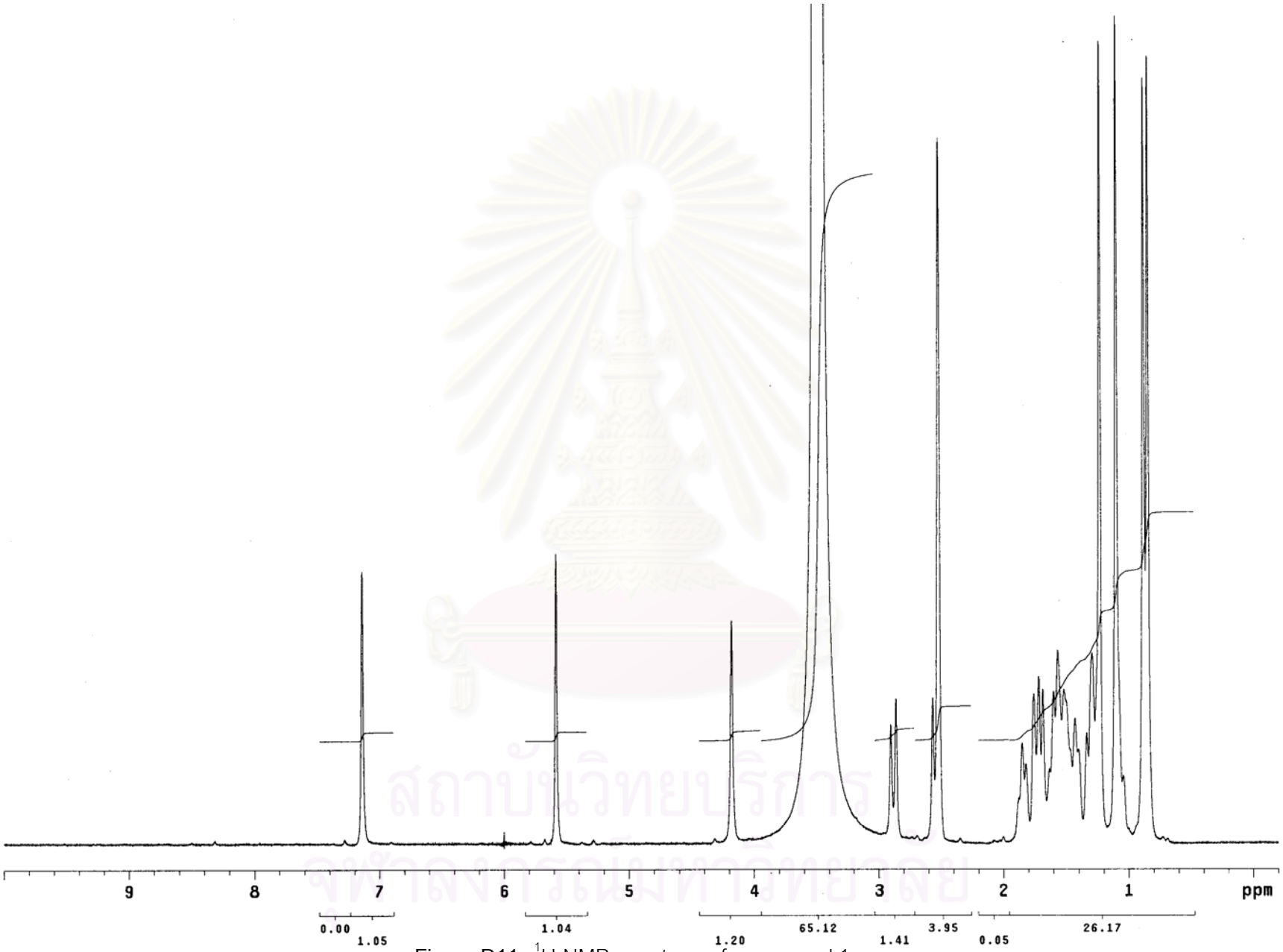


Figure D11 ^{1}H-NMR spectrum of compound 1

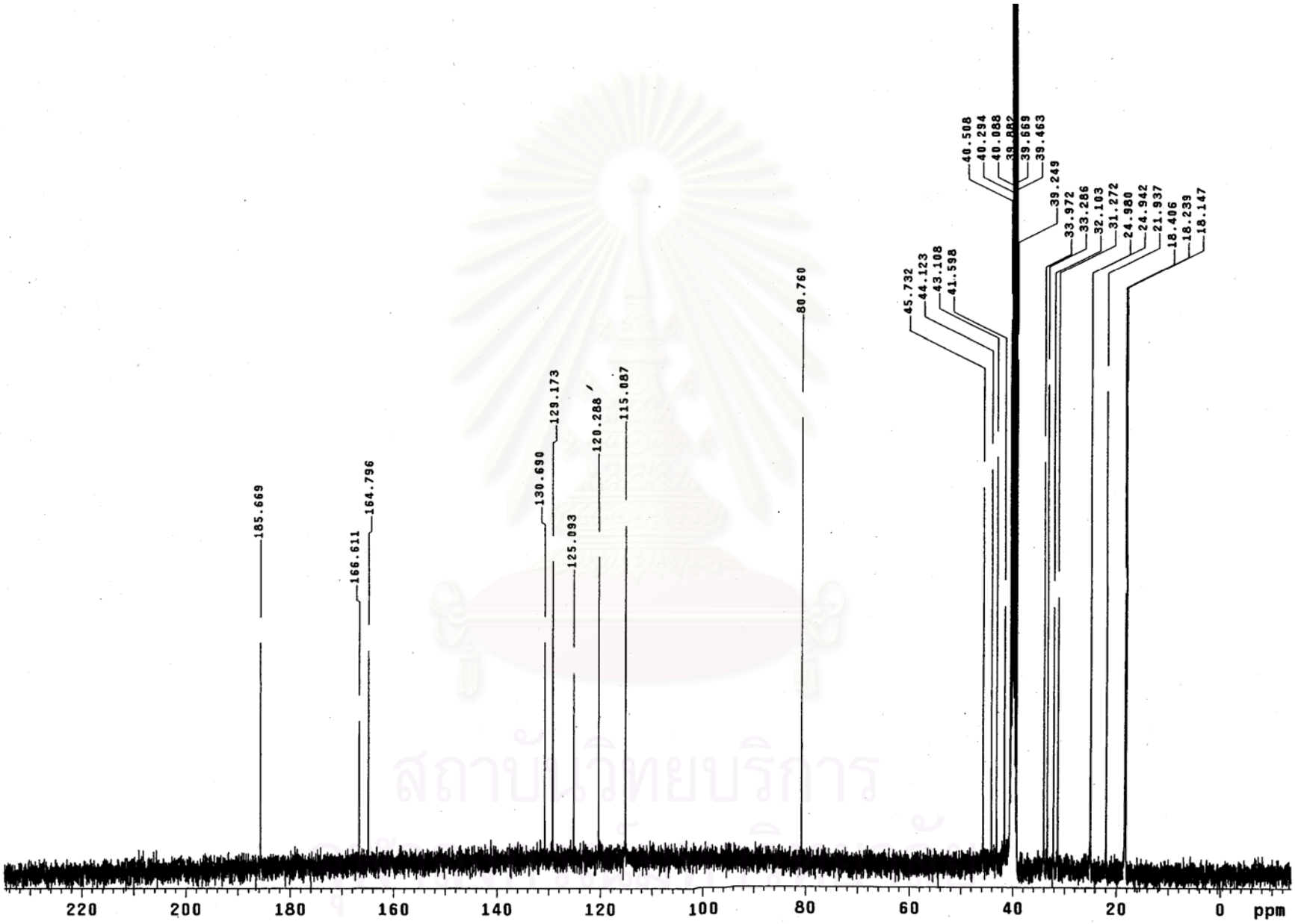


Figure D12 ^{13}C-NMR spectrum of compound 1

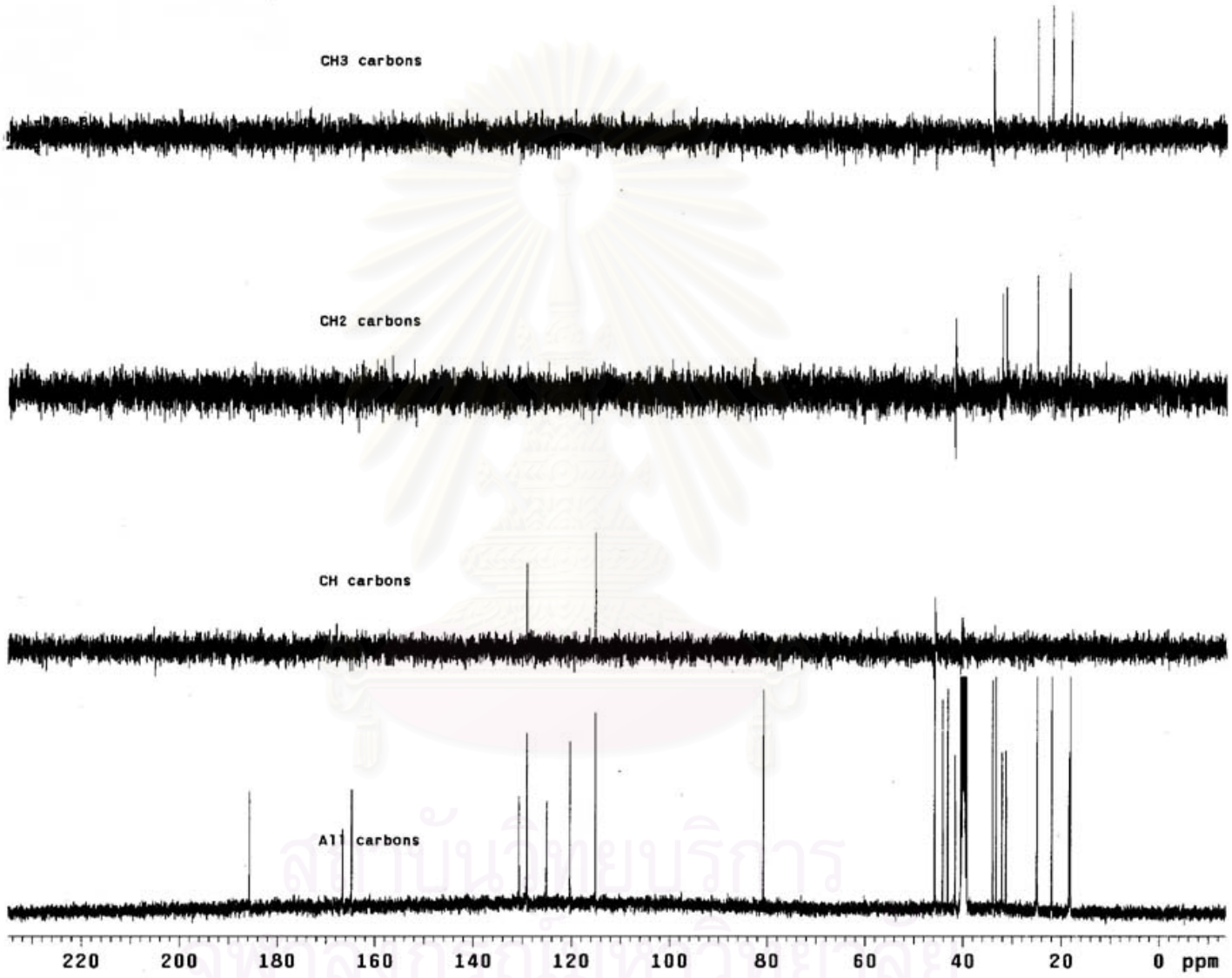


Figure D13. DEPT spectrum of compound 1

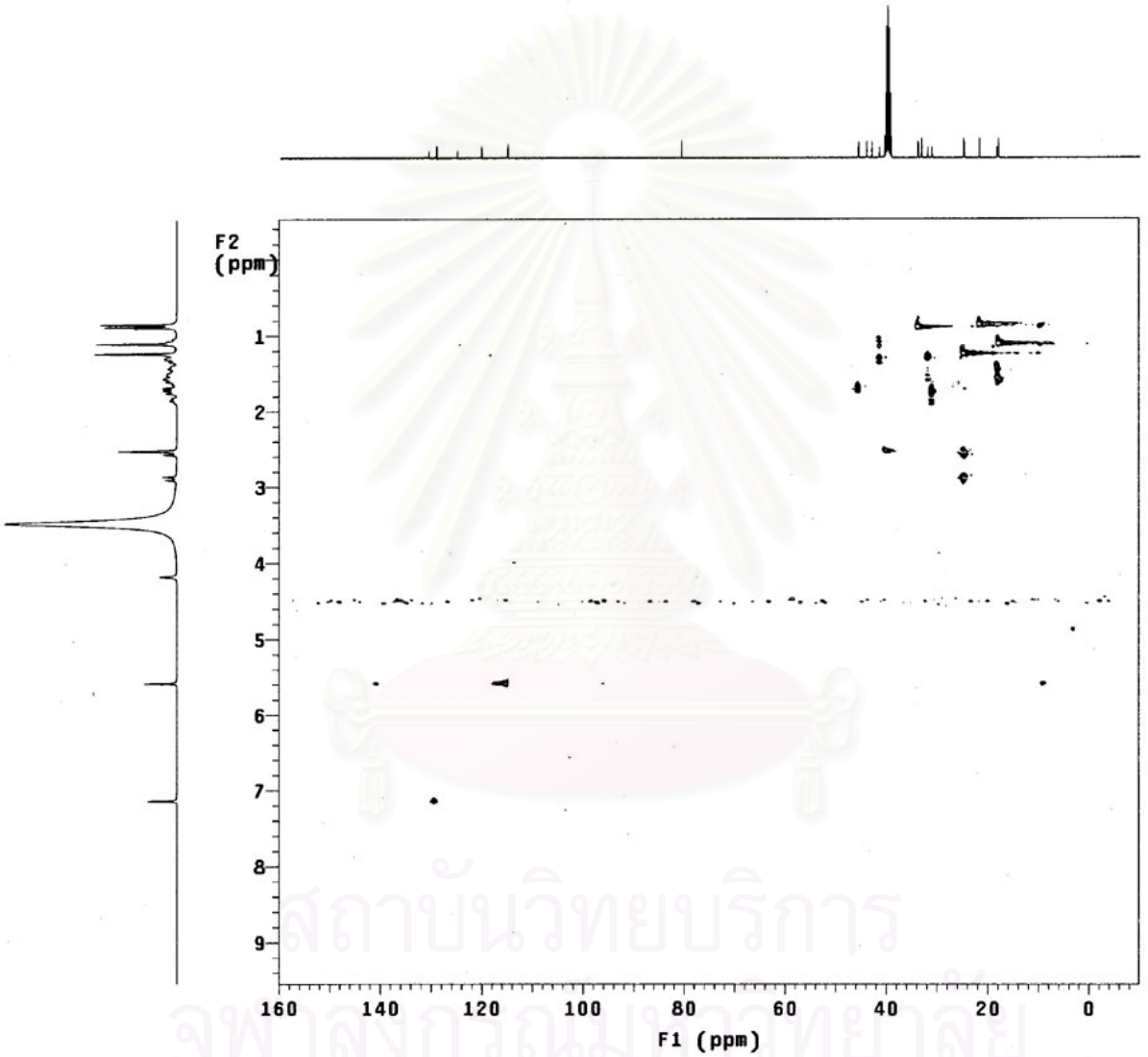


Figure D14 HMQC spectrum of compound 1

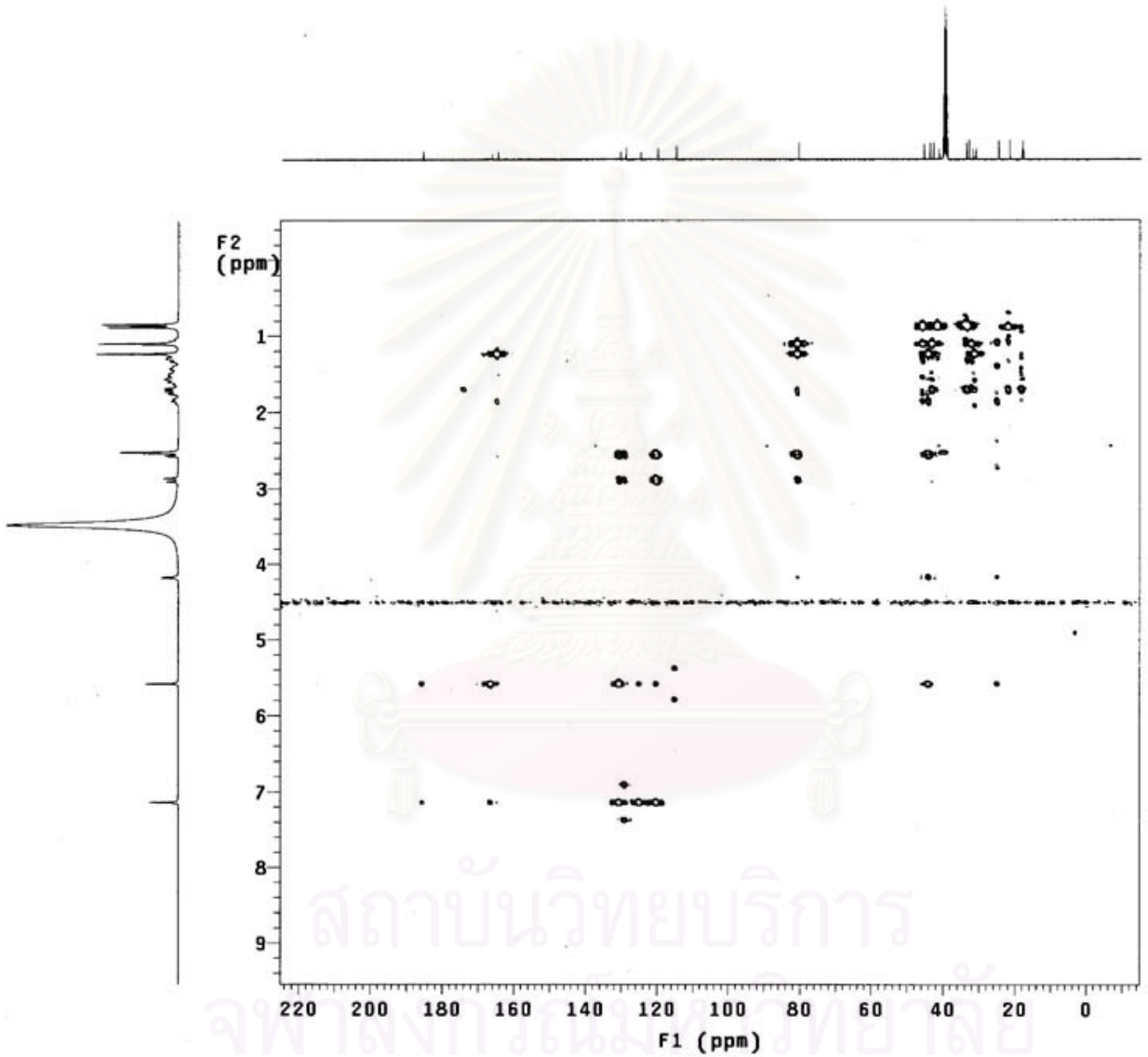


Figure D15 HMBC spectrum of compound 1

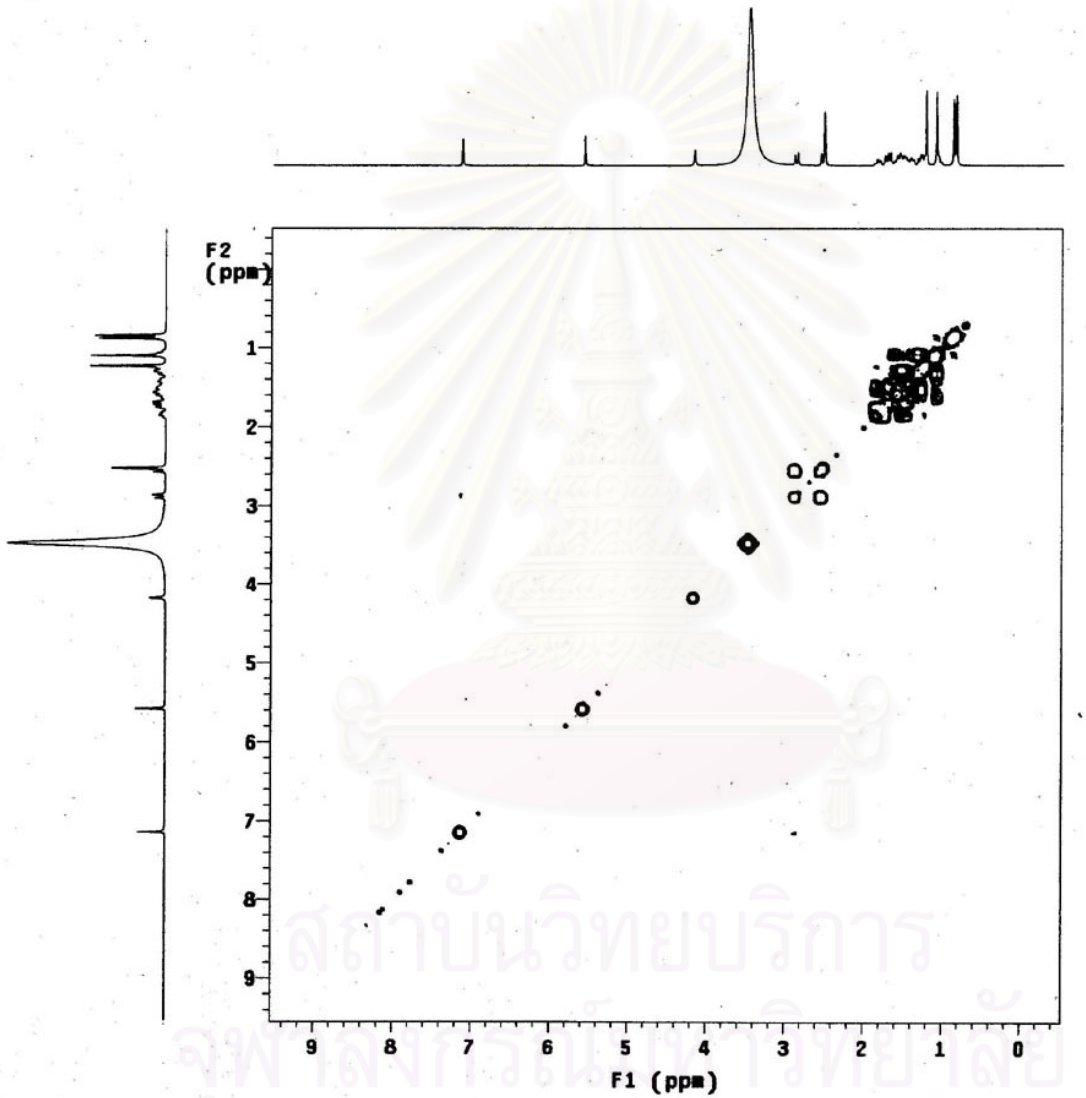


Figure D16 COSY spectrum of compound 1

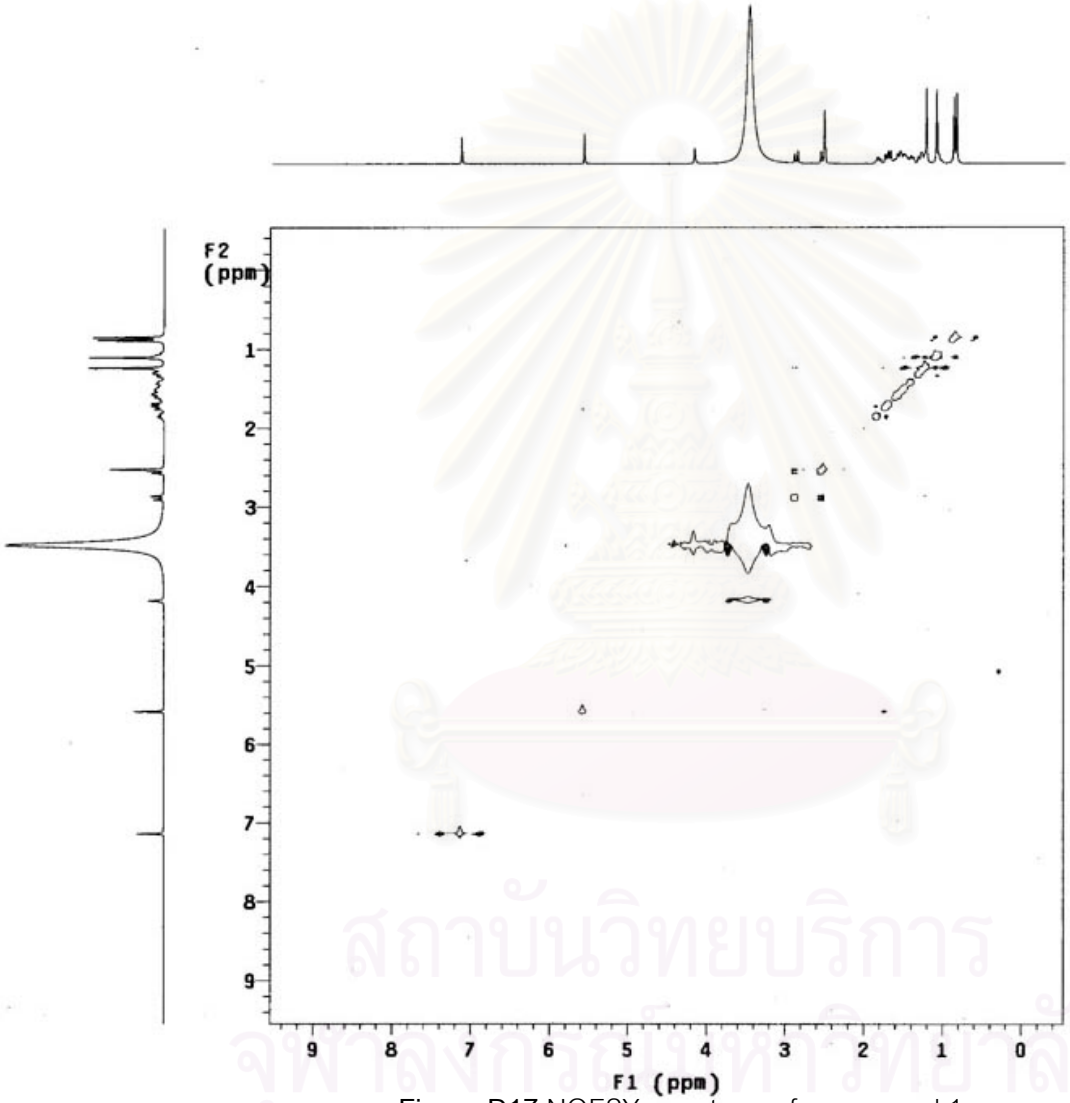


Figure D17 NOESY spectrum of compound 1

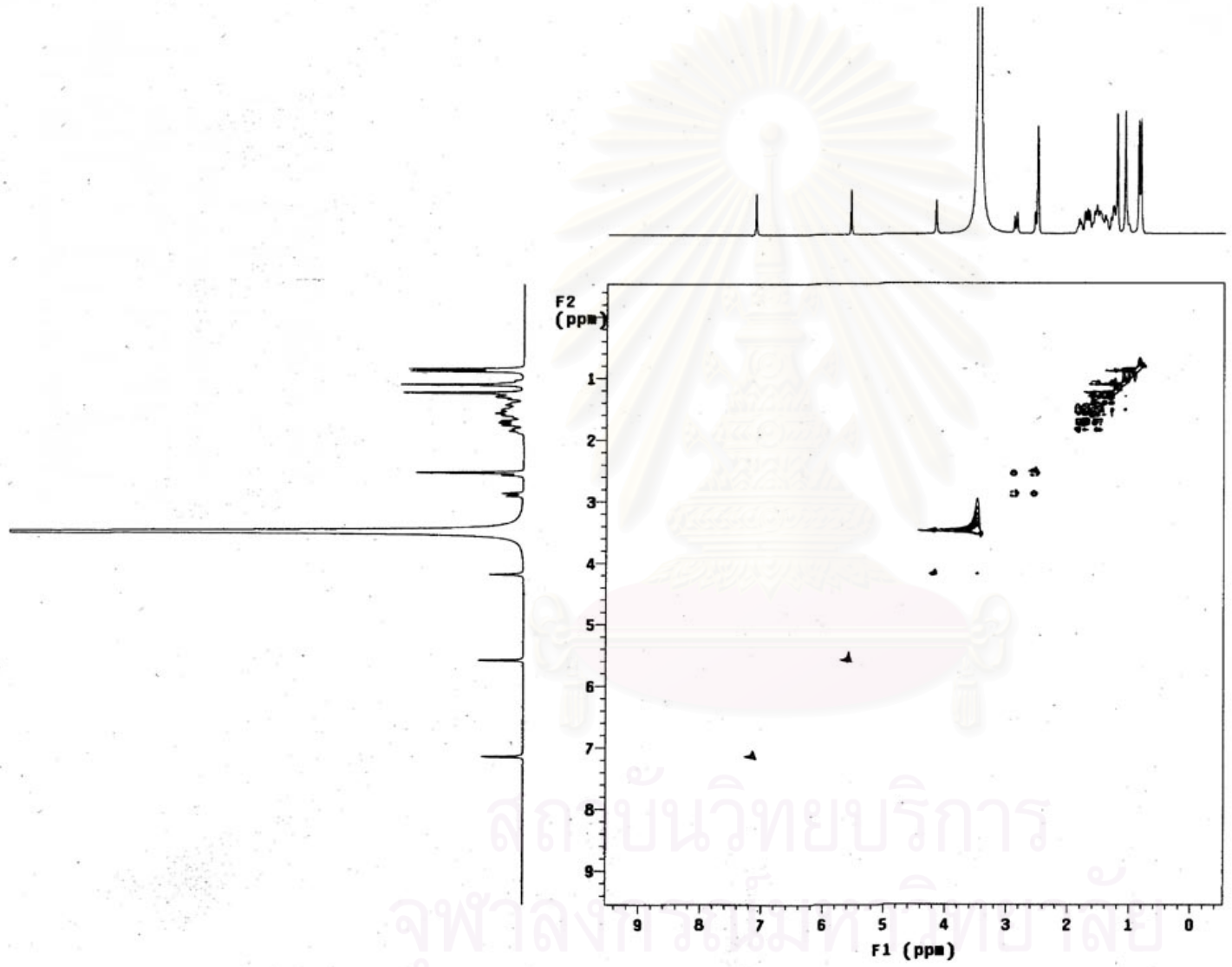


Figure D18 TOCSY spectrum of compound 1

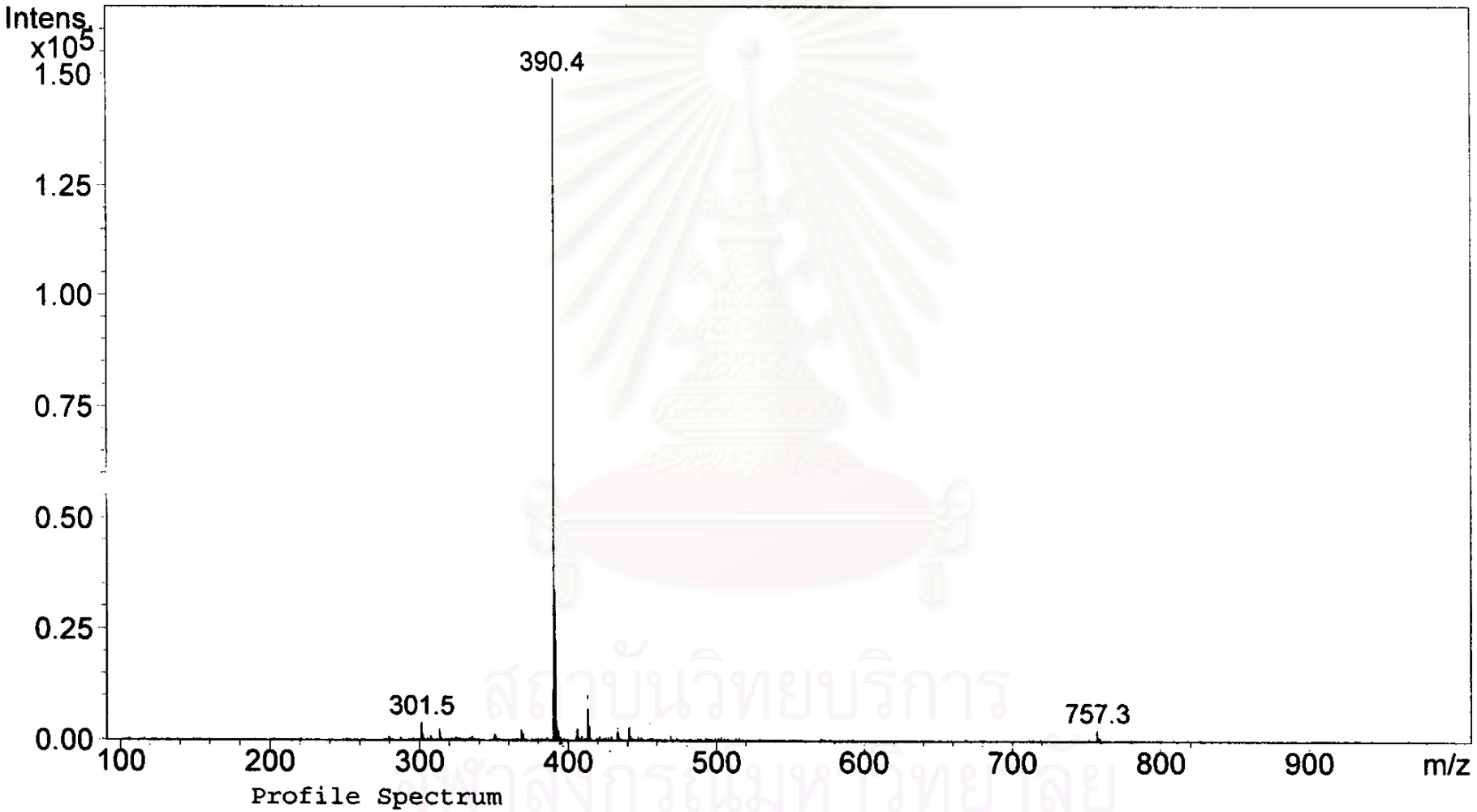


Figure D19 LC-MS spectrum of compound 1

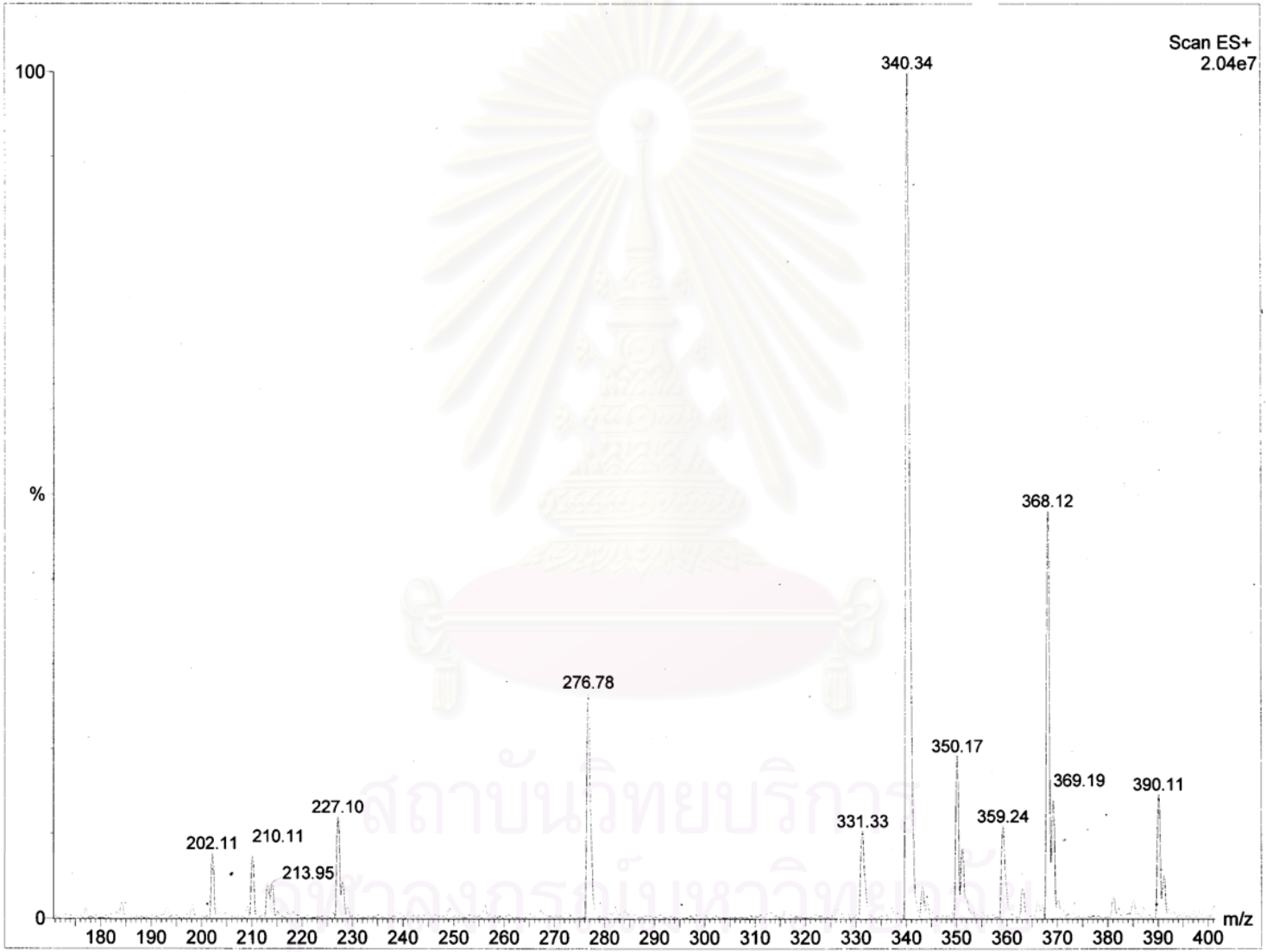


Figure D20 LC-MS spectrum of compound 1

BIOGRAPHY

Miss Suwanna Chantasuban was born on September 14, 1973 in Pattani province, Thailand. He graduated with a Bachelor Degree of Science in Microbiology from the Faculty of Science, Prince of Songkhla University, Thailand in 1995. She has been studying for a Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand since 2001.