สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ *Fusarium verticillioides* ที่แยกจาก เปล้าใหญ่ *Croton oblongifolius* จังหวัดกาญจนบุรี

นางสาว วันทนีย์ ทมมืด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI *Fusarium verticillioides* ISOLATED FROM *Croton oblongifolius* IN KANCHANABURI PROVINCE

Miss Wantanee Tommeurd

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

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By	Miss Wantanee Tommeurd
Field of study	Biotechnology
Thesis Advisor	Assistant Professor Surachai Pornpakakul, Ph.D.
Thesis Co-advisor	Associate Professor Prakitsin Sihanonth, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Deputy Dean for Administrative Affairs, Acting Dean, The Faculty of Science
(Associate Professor Tharapong Vitidsant, Ph.D.)

Thesis committee

……Chairman
(Professor Sophon Roengsumran, Ph.D.)

……Thesis Advisor
(Assistant Professor Surachai Pornpakakul, Ph.D.)

……Thesis Co-advisor
(Associate Professor Prakitsin Sihanonth, Ph.D.)

……Member
(Associate Professor Amorn Petsom, Ph.D.)

……Member
(Associate Professor Nattaya Ngamrojnavanich, Ph.D.)

วันทนีย์ ทมมีด: สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ *Fusarium verticillioides* ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* จังหวัดกาญจนบุรี (BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI *Fusarium verticillioides* ISOLATED FROM *Croton oblongifolius* IN KANCHANABURI PROVINCE) อาจารย์ที่ปรึกษา: ผศ.ดร. สุรชัย พรภคกุล, อาจารย์ที่ปรึกษาร่วม รศ.ดร. ประกิตติ์สิน สีหนนทน์ 193 หน้า. ISBN 974-53-1579-6.

วัตถุประสงค์ของงานวิจัยนี้เพื่อแยกสารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกได้จากใบเปล้าใหญ่ *Croton oblongifolius* ในจังหวัดกาญจนบุรีทำการแยกราเอนโดไฟต์โดยเทคนิคฆ่าเชื้อที่ผิวและได้ 72 ไอโซเลต ประกอบด้วย *Bipolaris* sp., *Fusarium* sp., *Phomopis* sp. และราในกลุ่ม Xylariaceae ทุกไอโซเลตทำการทดสอบการยับยั้งเชื้อจุลินทรีย์โดยวิธี Dual culture agar diffusion technique พบว่า ราเอนโดไฟต์ที่แยกได้ยับยั้งเฉพาะแบคทีเรีย 15% ยับยั้งเฉพาะยีสต์ 8% ยับยั้งทั้งแบคทีเรียและยีสต์ 9.7% และ ไม่ยับยั้งเชื้อทดสอบใดๆ 67% ทำการเลือกไอโซเลต KBLM02 เนื่องจากสามารถออกฤทธิ์ยับยั้ง *Bacillus subtilis*, *Staphylococcus aureus* และ *Candida albicans* โดยมีบริเวณยับยั้ง 10, 11 และ 14.5 มม. ตามลำดับ โดยอาศัยลักษณะทางสัณฐานวิทยาและวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ Internal Transcribed Spacer ของ rDNA ราเอนโดไฟต์ไอโซเลต KBLM02 คือ *Gibberella moniliformis* (Telemorph) หรือ *Fusarium verticillioides* (Anamorph) ทำการแยกไอโซเลต KBLM02 ในอาหารเหลว Sabouraud's Dextrose ด้วยการสกัด, วิธีโครมาโทกราฟีและการตกผลึก ได้ 4 สารประกอบและ 1 ของผสมโดยอาศัยสมบัติทางกายภาพและเทคนิคทางสเปกโทสโกปี ชื่อโครงสร้างของสารดังกล่าว ของผสมของ fusaric acid **1** และ dehydrofusaric acid **2**, dehydrofusaric acid **2**, 8-*O*-methylbostrycoidin **3**, 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol **4** และ bikaverin **5** นำสารบริสุทธิ์ที่แยกได้มาทดสอบฤทธิ์ทางชีวภาพในการยับยั้งจุลินทรีย์ทดสอบ พบว่า สารประกอบ **2**, **3**, **4**, **5** และของผสม **1** และ **2** ออกฤทธิ์ยับยั้ง *Bacillus subtilis* โดยมีค่า MIC เท่ากับ 3.95 (22.3), 7.81 (26.1), 250 (606.8), 125 (327.2) และ 15.62 μg/ml (μM) ตามลำดับ สารประกอบ **3**, **5** และของผสม **1** และ **2** ออกฤทธิ์ยับยั้ง *Staphylococcus aureus* โดยมีค่า MIC เท่ากับ 15.62 (52.2), 7.81 (20.4) และ 31.25 μg/ml (μM) ตามลำดับ สารประกอบ **4**, **5** และของผสม **1** และ **2** ออกฤทธิ์ยับยั้ง *Escherichia coli* โดยมีค่า MIC เท่ากับ 250 (606.8), 7.81 (20.4) และ 250 μg/ml (μM) ตามลำดับ สารประกอบ**2** และ **4** ออกฤทธิ์ยับยั้ง *Pseudomonas aeruginosa* โดยมีค่า MIC เท่ากับ 3.95 (22.3) และ 3.95 (9.59) μg/ml (μM) ตามลำดับ สารประกอบ **4** และของผสม **1** และ **2** ออกฤทธิ์ยับยั้ง *Candida albicans* โดยมีค่า MIC เท่ากับ 250 (606.8) และ 250 μg/ml (μM) ตามลำดับ นอกจากนี้สารประกอบ **3** มีฤทธิ์ยับยั้งเซลล์มะเร็ง HEP-G2 และ KATO-3 โดยมีค่า IC_{50} 9.5 (31.8) μg/ ml (μM)

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

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

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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WANTANEE TOMMEURD: BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI *Fusarium verticillioides* ISOLATED FROM *Croton oblongifolius* IN KANCHANABURI PROVINCE. THESIS ADVISOR: ASST. PROF. SURACHAI PORNPAKAKUL, Ph.D., THESIS COADVISOR: ASSOC. PROF. PRAKITSIN SRIHANONTH, Ph.D. 193 pp. ISBN 974-53-1579-6.

The purpose of this research was to isolate bioactive compounds from endophytic fungi isolated from *Croton oblongifolius* leaves in Kanchanaburi province. The endophytic fungi were isolated using surface-sterilization technique and obtained 72 isolates including *Bipolaris* sp., *Fusarium* sp., *Phomopis* sp. and members of Xylariaceae. All isolates were examined antimicrobial activity using dual culture agar diffusion technique. The results showed that 15% of the fungi inhibited only bacteria, 8% of the fungi inhibited only yeast, 9.7% of the fungi inhibited bacteria and yeast, and 67% of the fungi exhibited no activity. Isolate KBLM02 was selected for the study because it exhibited activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans* with inhibition clear zone 10, 11, and 14.5 mm, respectively. Based on morphology and nucleotide sequencing of ITS regions of rDNA, isolate KBLM02 was identified as *Gibberella moniliformis* (Telemorph) or *Fusarium verticillioides* (Anamorph). Sabouraud's Dextrose culture broth of the isolate KBLM02 was isolated by extraction, chromatographic technique and crystallization to give 4 compounds and a mixture. On the basis of physical properties and spectroscopic data, they were elucidated as a mixture of fusaric acid **1** and dehydrofusaric acid **2**, dehydrofusaric acid, 8-*O*-methylbostrycoidin **3**, 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol **4** and bikaverin **5**. Antimicrobial activities of these compounds were investigated. Compound **2**, **3**, **4**, **5** and a mixture of **1** and **2** showed activity against *Bacillus subtilis* with MIC 3.95 (22.3), 7.81 (26.1), 250 (606.8), 125 (327.2) and 15.62 μg/ml (μM), respectively. Compound **3**, **5** and a mixture of **1** and **2** showed activity against *Staphylococcus aureus* with MIC 15.62 (52.2), 7.81 (20.4) and 31.25 μg/ml (μM), respectively. Compound **4**, **5** and a mixture of **1** and **2** showed activity against *Escherichia coli* with MIC 250 (606.8), 7.81 (20.4) and 250 μg/ml (μM), respectively. Compound **2** and **4** showed activity against *Pseudomonas aeruginosa* with MIC 3.95 (22.3) and 3.95 (9.59) μg/ml (μM), respectively. Compound **4** and a mixture of **1** and **2** showed activity against *Candida albicans* with MIC 250 (606.8) and 250 μg/ml (μM), respectively. Additionally, compound **3** exhibited cytotoxic activity against HEP-G2 and KATO-3 with IC_{50} 9.5 (31.8) μg/ml (μM).

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

Academic year……2004……Advisor's signature……

Co-advisor's signature……

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

$[\alpha]_D^{20}$	= Specific rotation at 20° and Sodium D line (589 nm)
ATCC	= American Type Culture Collection, Maryland, U.S.A
br s	= broad singlet (for NMR spectral data)
^{o}C	= degree Celsius
^{13}C-NMR	= carbon-13 nuclear magnetic resonance
$CDCl_3$	= deuterated chloroform
CD_3OD	= deuterated methanol
$CHCl_3$	= chloroform
CH_2Cl_2	= dichloromethane
cm	= centimeter
COSY	= ^{1}H-^{1}H correlation spectroscopy
CFU	= Colony forming unit
δ	= chemical shift
d	= doublet (for NMR spectral data)
dd	= doublet of doublet (for NMR spectral data)
dt	= doublet of triplets (for NMR spectral data)
ε	= molar absorptivity
EIMS	= electron impact mass spectroscopy
eq	= equatorial
EtOAc	= ethyl acetate
g	= gram
HMBC	= Heteronuclear Multiple Bond Correlation
HSQC	= Heteronuclear Single Quantum Coherence
^{1}H-NMR	= Proton Nuclear Magnetic Resonance
Hz	= hertz
IR	= infrared spectroscopy
l	= liter
μl	= micro liter

LIST OF ABBREVIATIONS (CONTINUED)

λ_{max} = wavelength of maximum absorption

$[M+H]^+$ = protonated molecular ion

m = multiple (for NMR spectral data)

MEA = Malt extract agar

MHB = Mueller- Hinton broth

MeOH = methanol

MIC = Minimum inhibitory concentration

mg = milligram

μg = microgram

MHz = megahertz

ml = milliliter

mm = millimeter

ν_{max} = wave number at maximum absorption

NMR = nuclear magnetic resonance

No. = Number

ppm = part per million

PDA = Potato Dextrose Agar

q = quartet (for NMR spectral data)

s = singlet (for NMR spectral data)

SDA = Sabouraud's Dextrose Agar

SEM = scanning electron microscope

t = triplet (for NMR spectral data)

TLC = thin layer chromatography

UV = Ultraviolet

YES = Yeast Extract Agar

CHAPTER I

INTRODUCTION

The fungi are one of microorganisms that have highly diversity. They are found everywhere and affect our lives everyday, from mushrooms in the dish to industrially products to human disease. Because of their various effects, it is necessary to know some of fungal biology in order that we can control and exploit them for our own purpose.

Endophytic fungi are fungi that live almost their entire life cycle in the tissue of living plants with unapparent and asymptomatic infections. At first the endophyte research began with their demonstrable role in mediation interactions of herbivores with some grass host plants (Petrini 1991) and then, most of them have been carried out using plants from temperate and tropical regions.

Recent interest has focused on endophytic fungi for their pharmaceutical, medicinal and agricultural potential. For example, *Pestalotiopsis microspora* that was isolated from the bark of Himalayan yew tree are potential new sources of the anticancer drug taxol (Strobel et al., 1996), and *Colletotrichum sp.* from the Chinese herb, *Artemisia annua*, can produce growth hormones and fungicide for plants. In addition, the clavicipitaceous grass endophytes are known to produce indole derivatives and other compounds that are active as plant hormones, antifungal agents, hallucinogens, vasoconstrictors, etc. (Bacon and White 2000)

Croton oblongifolius was used in this research because it is one of the most interesting Thai medicinal plants. It is believed that all its parts can be used in the treatment of many ailments, for instance, the leaves can be used as a tonic, the flowers are used as a teniacide, the fruits are used to treat dysmenorrhea, and the seeds are used as a purgative, the bark is used to treat dyspepsia, and the roots are used to treat dysentery. (เสงี่ยม พงษ์บุญรอด, 2502)

Objectives

1. Isolation of endophytic fungi from *Croton oblongifolius* in Kanchanaburi Province.

2. Screening of isolated endophytic fungi for antimicrobial activities.

3. Identification of the endophytic fungi isolated from *Croton oblongifolius* in Kanchanaburi Province.

4. Extraction, isolation and purification of the bioactive compounds of a selected endophytic fungus

5. Elucidation of the structural formula of the isolated bioactive compounds.

6. Evaluation of the biological activity of the bioactive compounds obtained.

CHAPTER II

LITERATURE REVIEWS

2.1 Association of endophytic fungi and plant

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

The term endophyte was originally defined by De Bary in 1866. At the most basic level, it simply refers to the organism: “endo” is the Greek word meaning within and “phyte” is the Greek word for plant, so an endophyte is an organism which lives inside a plant (Figure 2.1). This is in contrast to epiphyte which refers to organisms living on the outer space of the plant. Since then endophyte has become deeply embedded in the literature and within the last decade, different authors have proposed of similar, but more complex definitions e.g. Carroll 1986, Petrini 1991 (Wilson, 1995). In 1986, Carroll restricted the use of this term to organisms that cause asymptomatic infections within plant issues, excluding pathogenic fungi and mutualists such as mycorrhizal fungi (Carroll, 1986 cited in Petrini, 1991). Petrini (1991) proposed an expansion of Carroll’s definition and incorporated to it, his concept of latent pathogens know to live symptomlessly inside the host tissues for only part of their life cycle. Accordingly to Wilson (1995), endophytes are fungi or bacteria which for all or part of their life cycle invade the tissue of living plants and cause unapparent and asymptomatic infections entirely within plant tissues. This definition also excludes mycorhizal fungi but includes latent pathogens as well as those fungi that are unable to induce disease symptoms. Hirsch and Braun (1992) define endophytes as fungi colonizing living plant tissues without causing any immediate, overt negative effects. The term endophyte has been controversial and mycologists have been cogitation upon its exact meaning for some time (for e.g. Wennstrom, 1994; Wilson 1995). Indeed, by using De Bary term which is based on

location rather than the taxonomy, mycologists open up the door to disagreements about the very nature of the relationship between the host and the fungus. At the very best, the term allows mycologists to work on such fungi and push the frontier of mycology forward.

Endophytic fungi have been isolated from a very wide range of host plant species. An individual plant may be host to the range of endophytic fungal symbionts simultaneously. Results of their interaction make plant increase the ability of disease resistance and survival from environment by producing bioactive compounds of plant growth promoting, antibacterial, antifungal and insecticidal to enhance the plant growth. Many grasses, especially the cool-season grasses, form specific mutualistic associations with clavicipitaceous fungi. These endophytic fungi produce mycelium throughout the aerial parts of their host and are transmitted through host seeds. The grass endophytes produce anti-herbivore compounds that deter insect pests and hence, the infected grasses perform better than the uninfected conspecifics (Clay, 1991; Bacon and White, 1994).

There exists another group of fungal endophytes that infects leaves and stems of non-grass plants. These belong to diverse taxa and cause discrete symtmoptess infections with very restricted hyphal growth within the host tissues. Southcott and Johnson (1997) presented the first micrograph on endophytes inside a tropical plant leaf (palm). The pattern of colonization was determined to be characteristic of the endophyte and the host. These endophytes are not seed-borne but are transmitted horizontally from plant to plant by way of spores (Saikkonen et al. 1998). Their contribution to host fitness, except in a few cases, is not as apparent as in grass endophytes. Some of these endophytes possess insectidal properties (Webber, 1981; Carroll, 1986; Johnson and Whitney, 1994).

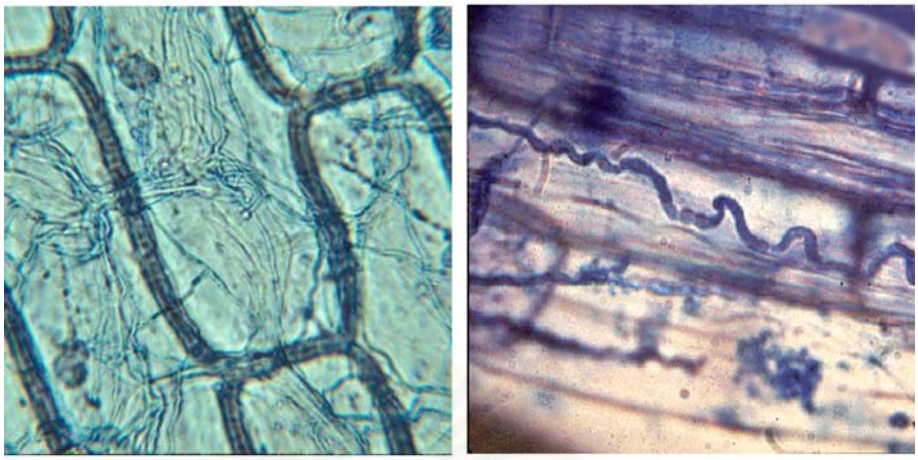


Figure 2.1 Vegetative growths in endophytic fungi of grasses. (A) A hi-power shot of the fungal endophytes within the leaf sheaths of Arizona fescue (B) Mycelium of fungal endophyte (*Neothyphodium coenophialum*) in tall festue leaf sheath. (from:www.mc.maricopa.edu/~dwilson/Images%20of%20Cells/Thumbnail Frame.htm)

2.2 Origin and evolution of endophytes

Some phytopathogens in the environment are of endophyte origins (Carroll, 1988). Many innocuous fungal endophytes are quiescent phytopathogens which may cause infectious symptoms when the host plant is aged and/or stressed. On the other hand, during the long co-evolution of the phytopathogen and its host plant, an endophytic mutant may result from balanced antagonism and/or gene mutation. Dual cultures of the host calli and endophytes demonstrated that both of the endophytes and the host calli excrete metabolites toxic to each others (Sieber et al. 1990; Peters et al. 1998). Further investigation led to the development of the hypothesis that the endophyte-host interaction could be a balanced pathogen-host antagonism (Schluz, 1990). Freeman and Rodriguez (1993) found that a naturally occurring nonpathogenic endophytic mutant developed from the mutation of a single locus in the genome of the wild-type *Colletotrichum magna*, a pathogen causing anthracnose in cucurbit plants. This mutant is able to grow systemically inside the host plant without pathogenic symptoms, but retaining wild-type levels of *in*

vitro sporulation, spore adhesion, appressoria formation, and infection and host specificity.

The *Acremonium* (asexual fungi now reclassified in the genus *Neotyphodium* Glenn, Bacon and Hanlin, 1996) endophytes, which usually inhabit tall fescue, perennial ryegrass (*Lolium perenne* L.), and many cool-season grasses, are considered mutualistis symbionts of the host grasses. The grass and the endophytic fungus are so intimately associated that they act 'as a whole', much like 'a single organism'. And, indeed, some of these endophytic *Neotyphodium* species can only spread by infecting seeds from the mother plants (Fig.2.2)

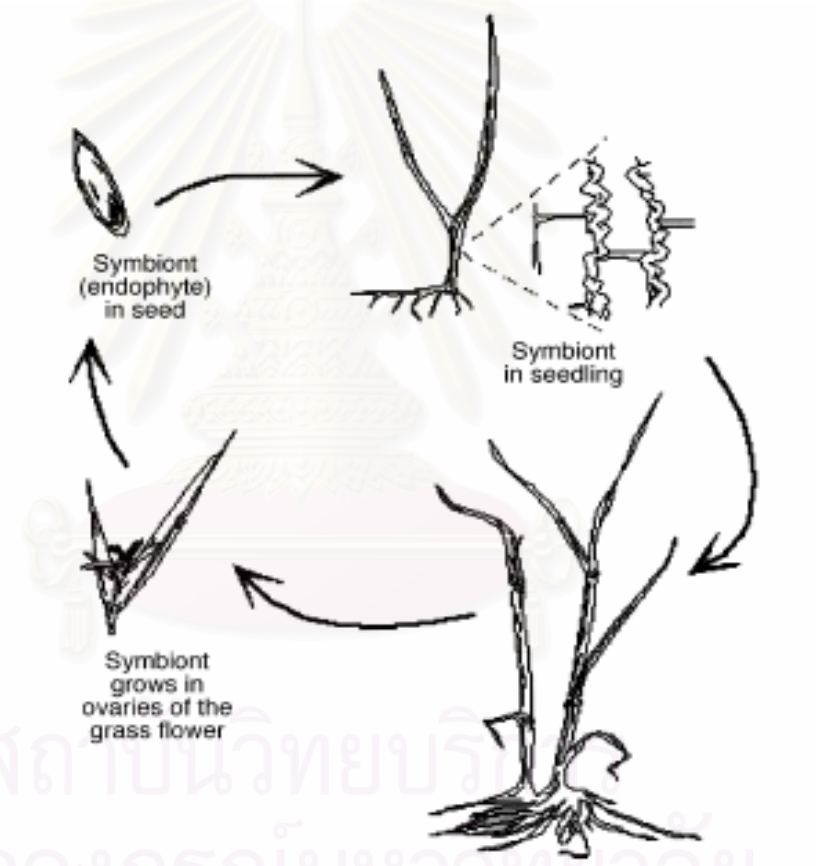


Figure 2.2 Schematic illustration of endophyte-grass symbiont (Tan et al. 2001)

2.3 Physiological and ecological roles of endophytes

Endophytes colonizing inside plants tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain functional metabolites

2.3.1 Growth promotion of the host plant

Endophyte-infected plants often grow faster than non-infected ones (Cheplick, Clay and Marks, 1989). This effect is at least in part due to the endophytes' production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances (Zou and Tan, 1999), and/or partly owing to the fact that endophytes could have enhanced the hosts' uptake of nutritional elements such as nitrogen (Reis et al. 2000) and phosphorus (Gasoni and Gurfinkel, 1997; Malinowski and Belesky; 1999).

2.3.2 Improvement of the hosts' ecological adaptability

Certain endophytes improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens and/or herbivores including some insects feeding on the host plant. Endophyte-infected grasses usually possess as increased tolerance to drought (Arachevaleta et al. 1989; Ravel et al. 1997) and aluminium toxicity (Malinowski and Belesky, 1999). Furthermore, some endophytes are able to provide the host plant with protection against some nematodes (Kimmons, Gwinn and Bernard, 1990; Hallmann and Sikora, 1996), mammal (Bacon et al. 1977), and insect herbivores (Prezler, Gaylord and Boecklen, 1996; Wilkinson et al. 2000) as well as bacterial and fungal pathogens (Christensen, 1996; Sturz et al. 1999). Some endophytes are capable of enhancing the hosts' allelopathic effects on other species co-growing nearby, usually being competitor(s) for the nutrition and the space (Sturz and Christie, 1996 and Sturz, Christie and Matheson, 1998). This could be the reason why some plants with special endophytes are usually competitive enough to become dominant species in successional fields (Clay and Holah, 1999).

2.4 Taxonomy of fungal endophytes

The leaf tissue functions as a niche for a wide variety of fungi. The foliar fungal endophytes belong mainly to ascomycetous and mitosporic fungi; very few basidiomycetes fungi occur as endophytes (Petrini, 1986). Different ecoligacal groups of fungi such as the dung fungi, saprotrophs ans leaf-spot fungi survive as endophytes, suggesting that this endophytic strategy is a survival mechanism for times when the preferred substrate or host plant is not available (Carroll, 1999). A single host species usually harbours different fungi as endophytes. In a study on endophytes of evergreen forest trees of Southern India, Suryanarayanan and Rajagopol (1998) isolated eighteen different fungi from the leaves of *Rhododendron arboretum*, sixteen from *Acacia dealbata*, twenty from *A. melanoxylon*, fourteen from *Michelia nilagirica*, and twelve from *Eucalyptus globulus*. Similarly, Brown et al. (1998) isolated twenty-five endophytes from *Musa acuminate* collected from Hong Kong and Australian. In another study, Suryanarayanan et al. (1998a) recovered twenty-eight and twenty-five endophytes, respectively, from the leaves of *Rhizophora apiculata* and *R. mucronata*.

2.4.1 Hyphomycetes as endophytes

Several hyphomycete genera invariably occur as endophytes in the leaves of angiosperms. Of these, some phylloplane fungi such as *Alternaria*, *Aureobasidium* and *Cladosporium* are routinely isolated as endophytes from a wide range of plant species growing in different habitats (Petrini, 1986; Johnson and Whitney, 1989; Bills, 1996; Pelaez et al 1998). These phylloplane fungi are capable of penrtrating the superficial layers of the leaf or may be localized in the substomatal chambers (Cabral et al. 1993), thus surviving the rigorous surface sterilization procedure used for isolating endophytes (Verhoeff, 1974; Suryanarayanan and Rajagopol, 1998). Though the host defense systems are apt to restrict the growth of phylloplane fungi that have penetrated the surface layers of the host leaf, such fungi may be protected, at least temporarily, from abiotic and biotic stress. Consequently, these endophytes can be termed opportunistic saprophytes (Cabral et al. 1993).

2.4.2 Coelomycetes as endophytes

Some members of coelamycetes such as species of *Cryptocline*, *Cryptosporiopsis*, *Phyllosticta* and *Phomopsis* are constantly associates with the internal

leaf issues of many plants (Johnson and Whitney, 1989; Stone et al. 1996). Bills and Polishook (1992) classify these fungi as 'almost exclusive' endophytes. *Phyllosticta* is the dominant endophyte in many hosts. Brown et al. (1998) also noted that *Phyllosticta* was among the most frequrntly isolated endophytes from *Musa* species. Needle leaves of several gymnosperms such as *Abies amabilis*, *A. concolor*, *A. grandis*, *A. magnifica*, *A.procera*, *Pseudotsuga menziesii*, *Taxus brevifolia* and *Tsuga mertensiana* growing in temperate regions also harbour *Phyllosticta* as one of the common endophytes (Carroll and Carroll, 1978).

2.4.3 Ascomycota as endophytes

Among the endophytic Ascomycota, the xylariaceous forms are often isolated from tropical hosts (Dreyfuss and Petrini, 1984). The enodophytic Xylariaceae is represented by *Hypoxylon* and *Rosellinia* (Petrini, 1986). Intriguingly, some coprophilous genera such as *Ascobolus, Podospora, Sordaria* and *Sporormiella* are frequently isolated as endophytes (Petrini, 1986; Fisher et al. 1986). Several of the tropical angiosperm hosts also harbour Sporamiella in their leaf tissue (Suryanarayanan et al. 1998a, 1999). In addition, other ascomycete genera such as *Chaetomium, Glomerella* and *Guignardia* are often encountered as endophytes

2.4.4 Sterile mycelia

The widespread occurrence of sterile mycelia as endophytes is the bane of endophyte research since these fungi cannot be categorized by conventional methods. They demand the use of molecular techniques for classifying them (Bills, 1996). A few methods have been suggested to induce sporulation in sterile form. These include incubating them under near-ultraviolet light or at low temperatures (Bills, 1996). Growing the sterile fungi on a disk of cellophane overlying a glycine-amended mediumhas induced sporulation in a few taxa (Rajagopol, 1998). Most of the sterile forms, however,
remain sterile. A simple method to characterize these forms is to group them into morphotypes based on culture characteristics such as colony morphology, growth rate, etc. (Bills and Polishook, 1994; Suryanarayanan et al. 1998a). Dubos et al. (1999) used a rDNA sequencing technique and concluded that several sterile endophytes isolated from *Vaccinium macrocarpus* belonged to the major cladeswithin thw ascomycetes.

Guo et al. (1999) employed this molecular approach to place ten sterile endophytes of *Livistonia chinensis*, into the genera *Diaporthe*, *Mycosphaerella*, *Xylaria* and *Beauveria*.

2.5 Ecology of endophytes

Investigation of the species composition of endophyte assemblages of several host plants have revealed that although many different fungi colonize a host, it is only one or a few fungal species that dominate the assemblage (Petrini, 1991). In general, a relatively short list of endophyte taxa makes up 85 to 95% of the isolates (Johnson and Whitney, 1989; Suryanarayanan et al. 1998a). The most frequently isolated endophytes are either coincidentally present in large numbers or are successful in niche exploitation.

Almost all vascular plant species examined to date were found to harbor endophytic bacteria and/or fungi, the colonization of endophytes in marine algae (Smith et al. 1989), mosses and ferns (Petrini, Fisher and Petrini, 1992; Raviraja, Sridhar and Barlocher, 1996) has also been recorded. Although most endophytes are generalists, some of them are specific to their hosts. Petrini and Carroll (1981) contend that fungal endophytes exhibit some degree of host specificity at least for families of host plants and that this specificity determines endophyte distribution more than the geographical location of the hosts. For example, *Camarosporium* was the dominant endophyte in the halophyte *Suaeda maritima* growing in south India (Suryanarayanan and Kumaresan, 2000) and also in *S. fruticosa* growing in Chesil beach, Dorset (Fisher and Petrini, 1987). It is known that many endophytic fungi exhibit some degree of tissue specificity. For example, the endophyte communities for beech leaves, twigs and roots differed considerably (Petrini, 1991).

It is well established fact that the composition of endophytes varies with the age of the leaf tissue. In many temperate trees, there exists a positive correlation between leaf age and endophytic species richness and infection density (Stone, 1987; Espinosa-Garcia and Langenheim, 1990). Johnson and Whitney (1992) showed that new buds of black spruce are virtually endophyte-free. The frequency of colonization by endophytes increased with needle age in this plant. Marked seasonal variation in the rate of colonization by endophytes has also been observed. Leaves of *Quercus garryana*

(Wilson and Carroll, 1994) and *Euterpe oleracea* (Rodrigues, 1994) harbour more endophytes during the wet season. However, Asai et al. (1998) reported that the frequency of occurrence of endophytes in needles of Japanese black pine decreases due to acid precipitation. It is not known how pollution affects the endophyte community of tropical plants.

2.6 Plant sample

2.6.1 Botanical aspects of *Croton oblongifolius*

Croton oblongifolius is a medium sized tree in the Euphobiaceae family. There are about 700 species in this family that are scattered around evergreen forests, deciduous forests and the groves. In Thailand, it is commonly called Plao Yai (Central) or Plao Luang (Northern). Its calyx and ovary are clothed with minute orbicular silvery scales. Leaves are 5.6-12.0 by 13.0-24.0 cm in size and their shape is oblong-lanceolate. Its flowers are pale yellowish green and solitary in the axial of minute bracts on long erect racemes. The male flowers are located in the upper part of the raceme and the females in the lower part. Furthermore, the male flowers are slender and have of 4.0 mm in length. The calyx is more than 6.0 mm long and segments are woolly. The twelve stamens are inflexed in bud and the length of the filaments is 3.0 mm. In female flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely ciliated margins. The diameter of the fruit is less than 1.3 cm, slightly 3-lobbed and clothed with small orbicular scales. In each fruit, the members of seeds are eight
which are 6.0 mm long rounded and quite smooth on the back (เต็ม สมิตินันท์, 2523; ลีนา ผู้พัฒนพงศ์, 2530). The leaf and flower of *Croton oblongifolius* are shown in Figure 2.3.

Croton oblongifolius is used as a traditional medicine for many applications such as for dysmenorrhea, dysenteries and as a purgative. Furthermore, it has been used as folk-medicine in conjunction with *Croton sublyratus* to treat gastric cancers. The chemical constituents of *Croton oblongifolius* were summarized in Appendix A.

Figure 2.3 Leaves and flowers of *Croton oblongifolius*

2.7 Study of secondary metabolites from the endophytic fungi

In the continual search by both pharmaceutical and agricultural industries for new products, natural selection has been found to be superior to combinatorial chemistry for discovering novel substances that have the potential to be developed into new industrial products. The fungi provide us with an enormous variety of strange and wonderful "secondary metabolites", some of which have profound biological activities that we can exploit. Secondary metabolites are those that are not essential for vegetative growth in pure culture. Secondary metabolism occurs as growth rate declines and during the stationary phase, and often is associated with differentiation and sporulation (Carlile et al., 2001).

Endophytic fungi are a potent source of novel chemistry and biology, helping not only plant health but also human and animal health. They reside in the tissue among living plant cells without causing apparent disease symptoms and growth in this habitat involves continual metabolic interaction between fungus and host. The relationship that establishes with the plant varies from symbiosis borderline on pathogenic. As a result, the opportunity to find new and interesting endophytic fungi among the myriad of plant is great.

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

Recently, fungal endophyte research has focused on screening of secondary metabolites that exhibit interesting biologically activities such as antibacterial, antifungal, antiviral, algicide, herbicidal, insectidal, antifeedant, antioxidant and anticancer drugs. Tan and Zou (2001) reviewed the diversity of metabolites that have been isolated from endophytic fungi emphasizing their potential ecological roles. These secondary metabolites of endophytes are synthesized via various metabolic pathways of diverse structural groups e.g. alkaloids, polyketides, isoprenoids, amino acid derivatives, steroids, xanthones, phenols, isocoumarines, pyrylene derivatives, quinones, furandiones, terpenoids, depsipeptides and cytochalasins (Schulz et al., 2002). The chemical compound, sources, biological activities of secondary metabolite of endophytic fungi were summarized in Appendix A.

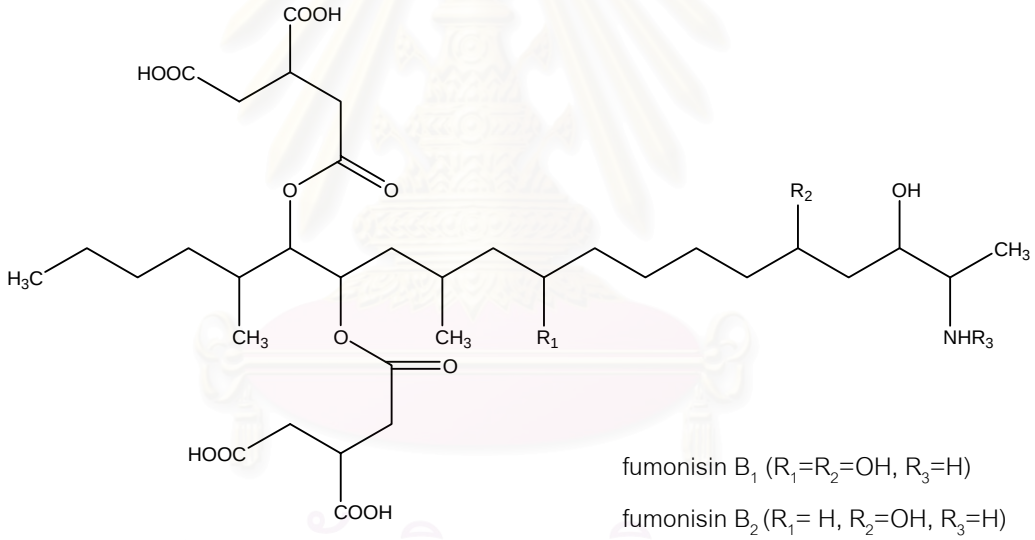
2.8 Metabolites from *Fusarium verticillioides*

Fusarium spp., associated with symptomatic and asymptomatic plants, may be a primary causal agent of disease, a secondary invader or an endophyte. The species *Fusarium verticillioides* (synonym = *F. moniliforme*, teleomorph *Gibberella moniliformis*) is often found in maize seeds, constituting an important source of inoculum in the field. It causes a widespread disease of maize ears known as "*Fusarium* kernel or ear rot" in which a powdery or cottony-pink mold growth develops on kernels damaged by corn insects or on individual infected kernels scattered over the ear (Shurtleff, 1984). In their study of the molecular systematics of the *Gibberella fujikuroi* complex, O'Donnell et al.

(1998) rejected the name *F. moniliforme* because it has always been applied to a multitude of phylogenetically distinct species (Booth, 1971; Nelson et al., 1983) and because it is a later synonym of *F. verticillioides.*

2.9.1 Fumonisins

Fumonisins are polyketide-derived secondary metabolites which are the most prominent toxin produced by *Fusarium verticillioides*. They are a group of economically important mycotoxins found in corn and corn-based products. At present, six fumonisins A-series and B-series) are known (Cawood et al., 1991). The structure is shown in Figure 2.4. Fumonisin B_1 which is the most abundant of the group was reported to be neurotoxic and carcinogenic in a number of animal species (Marasas et al., 1988; Bucci et al., 1996; Haschek et al., 1992; Gelderblom et al., 2001; Howard et al., 2001). Today FB_1 is classified as possibly carcinogenic to humans (class 2B) (IARC, 1993, 2002).



fumonisin B_1 (R_1=R_2=OH, R_3=H)

fumonisin B_2 (R_1= H, R_2=OH, R_3=H)

fumonisin B_3 (R_1=OH, R_2=H, R_3=H)

fumonisin B_4 (R_1=R_2= R_3=H)

fumonisin A_1 (R_1=R_2=OH, R_3=CH_3CO)

fumonisin A_2 (R_1= H, R_2=OH, R_3=CH_3CO)

Figure 2.4 Structures of Fumonisins

2.9.2 Fusarin C

Fusarin C was found in healthy and visibly Fusarium-infected corn kernels from rural households in South Africa (Gelderblom et al, 1984), and is found in extracts of some cultures of *F. verticillioides, F. oxysporum, F. sporotrichioides,* and *F. poae*.(Yoshizawa, Yamashita and Luo, 1994). Fusarin C in the presence of a liver microsomal activation system induces the formation of 6-thioguanine resistant mutants, micronuclei, sister chromatid exchange, DNA strand breaks, other chromosomal aberrations in V79 cells, and induces asynchrous replication of polyoma virus DNA (Bever et al, 2000). As a result, fusarin C has been suggested to be a possible etiological agent for the high incidence of human esophageal cancer in South Africa. The structure is shown in Figure 2.5.

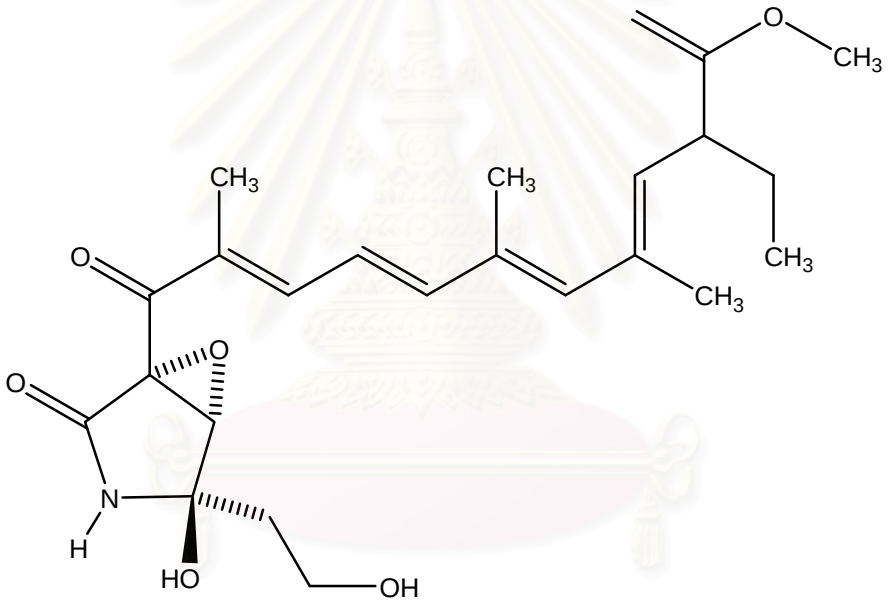


Figure 2.5 Structure of Fusarin C

2.9.3 Fusaric acid

Fusaric (5-butylpicolinic) acid is a *Fusarium* phytotoxin produced mainly by *Fusarium monoliforme* (Burmeister et al., 1985). Fusaric acid is a hypotensive agent (Hidaka et al., 1969) and has a low acute toxicity when compared to other *Fusarium* metabolites. The structure is shown in Figure 2.6.

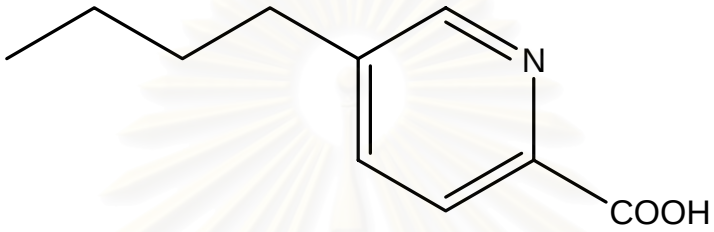


Figure 2.6 Structure of Fusaric acid

2.9.4 Other metabolites

Some volatiles can be emitted from *Fusarium* species (Borjesson et al., 1989). The volatiles produced by *Fusarium verticillioides* were studied to establish whether they could attract sap beetles. This fungus consistently produces a blend of five alcohols (ethanol, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol), acetaldehyde, and ethylacetate. The fungus also produced four phenolic compounds (the most abundant of which is ethylguaiacol), a series of unidentified sesquiterpene hydrocarbons, and an unidentified compound that is probably a 10-carbon ketone (Bartelt and Wicklow, 1999).

CHAPTER III

EXPERIMENTS

3.1 Materials and Instruments

3.1.1 Chromatographic techniques

- **Thin-layer chromatography (TLC)**

Technique : one dimension ascending

Absorbent : silica gel F_{254}coated on aluminum sheet Art.1.05554.0001 (E. Merck)

Layer thickness : 250 μm

Distance : 5 cm

Temperature : Laboratory temperature (25-30°C)

3.1.2 Spectroscopic techniques

3.1.2.1 Fourier Transform Infrared Spectrometry (FT-IR)

The FT-IR spectra were record on Perkin-Elmer Model 1760x Fourier Transform Infrared Spectrophometer. Solid samples were formally examined by incorporating the sample with potassium bromide (KBr) to form a pellete.

3.1.2.2 Nuclear Magnetic Resonance Spectrometry (NMR)

^{1}H-NMR,^{13}C-NMR, gCOSY, gHSQC, gHMBC and NOESY spectra were recorded on Varian Mercury+400 Spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated chloroform at δ = 7.26 ppm.

3.1.2.3 Optical rotation

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

3.1.2.4 Mass Spectrometry (MS)

The mass spectra were recorded on a Polaris Q Finnigan Instrument Mass Spectrometer in EI mode at 70 eV and MALDI-TOF Mass Spectrometer from BRUKER Germany.

3.1.2.5 Melting point

Melting points were examined using a Stuart scientific melting point SMP1 apparatus.

3.1.2.6 UV-VIS spectrometry

UV-Vis spectra were recorded on a Perkin Elmer Lambda 25 UV-Vis Spectrophotometer in MeOH.

3.1.2 Chemicals

3.1.2.1 Solvents

All solvent used in this research such as hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$) and Methanol (MeOH) were commercial grade and were purified prior to use by distillation. The reagent grades solvents were used for re-crystallization and TLC analysis.

3.1.2.2 Deuterated solvents

Deuterated solvents used in this research including CD_3OD, $CDCl_3$ and DMSO-d_6 were purchased from E.Merck.

3.1.2.3 Antibiotics

Penicillin G Sodium (FW 356.4) and Streptomycin Sulfate (FW 1457) were purchased from M&H Manufactering Co., Ltd.

Ketoconazole (FW 531.4) was purchased from M&H Manufactering Co., Ltd.

3.1.2.4 Other chemicals

- Merck's silica gel 60 Art. 1.09385.1000 (230-400 mesh ASTM) was used as adsorbent for column chromatography.

- Merck's silica gel $60GF_{254}$ Art. 1.07731.1000 was applied as adsorbent for preparative TLC.

- Merck's TLC aluminium sheet, silica gel $60F_{254}$ Art.1.05554.0001 procoated 25 sheets, 20x20 cm^2, layer 0.2 mm was used to identical fractions.

3.2 General procedure

- **Monitoring TLC chromatogram**

1. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm.
2. Visual detection in iodine vapour.
3. Visual detection under daylight after spraying with vanillin reagent (Dissolved 1 g vanillin in 95 ml ethanol and add 4 ml concentrated sulfuric acid) and heating until the colors developed.

3.3 Plant sample collection

Healthy mature leaves of *Croton oblongifolius* were collected from Sai Yoke District, Kanchanaburi province Thailand, on November, 2002. Plant samples were kept in plastic bag and stored in a refrigerator. The samples were processed in 48 hours after collection.

3.4 Isolation of endophytic fungi

Endophytic fungi were isolated using modified Petrini's method (Petrini, 1986). The leaves of *Croton oblongifolius* were cleaned with water, dried in laminar air flow and cut into small pieces (5 x 5 mm) followed by surface sterilization as follow: immersing in 95 % ethanol for 1 minutes, immersing in 12% sodium hypochlorite for 5 minutes and then immersing in 95 % ethanol for 30 seconds. Finally, they were washed in sterilized water twice, dried with tissue paper and placed on the surface of potato dextrose agar plates. Plates were then incubated at room temperature (25-30 ^{o}C) and examined for fungal germination everyday. Fungal endophytes germinating from the leaf pieces were transferred to fresh PDA medium plates by hyphal tip transfer. They were incubated for 7-14 days at room temperature (25-30 ^{o}C) and purity was determined by colony morphology. Fungal isolates with different morphology were collected for further study.

3.4.1 Culture media

Potato Dextrose Agar (PDA) was used for the endophytic fungi isolation. Observation morphology and determination of antimicrobial activities of isolated endophytic fungi were carried out by culturing in PDA, Malt Extract Agar (MEA), Malt Czapek Agar (MczA), Corn Meal Agar (CMA), Yeast Extract Sucrose Agar (YES), and Sabouraud's Dextrose Agar (SDA).

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

The media's formula are shown in appendix A.

3.5 Identification of endophytic fungi

3.5.1 Morphological identification of selected endophytic fungus

- **Microscopic feature**

The microscopic analyses were based on observations by light microscopy on an Olympus CH2 using a 40x dry objective. Specimens for light microscopy preparation were mounted in lectophenol-cotton blue for observation of spores and other characteristic

- **Macroscopic features**

Characters such as shape, size, color, type of stroma surface, and others were studied using stereomicroscope on a Leica MZ6.

3.5.2 Molecular Identification of selected endophytic fungus

Molecular identification was performed based on internal transcribed space (ITS) region of rDNA (Figure 3.1) at Macrogen, Inc. Seoul, South Korea.

A. DNA extraction

Genomic DNA was prepared from the mycelial sample by homogenization in 1.5 ml tubes with a FastPrep FP120 homogenizer (Savant, faxmingdale, NY, USA) and followed by extraction with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999). Fungal DNA extract was applied in CTAB buffer (2% CTAB, 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4M NaCl and 0.5% 2-mercaptoethanol) at 65 ^{o}C for 1 h, extracted with phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), then extracted twice with phenol-chloroform-isoamyl alcohol mixture (25:24:1, v/v/v). Fungal DNA was

precipitated by isopropanol and centrifuged at 8000 rpm for 5 min. The fungal DNA was dissolved in a solution of 10 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 (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1994), and ITS4 (White *et al.*, 1990). Twenty microlitres of reaction mixture containing of 5 ng of template DNA, 0.2 mM of each dNTP, 1xPCR buffer, 1.5 mM Mg^{2+}, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 µM of the primer pair. The amplification reactions were performed in a thermal cycler (TP 3000; Takara Shuzo, Tokyo, Japan). Amplification was started at 94 ^{o}C for 9 min, followed by 38 cycles of a denaturing step at 94 ^{o}C for 1 min, an annealing step at 51 ^{o}C for 1 min, and an extension step at 72 ^{o}C for 1 min, and ended with an additional 5-min extension step at 72 ^{o}C. PCR product was kept at -30 ^{o}C for further study.

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

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

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

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

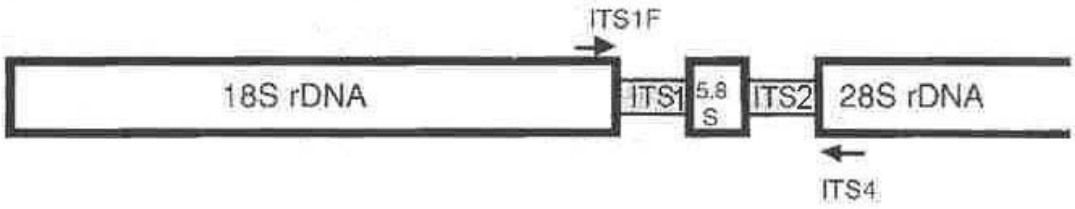


Figure 3.1 ITS regions of rDNA (Kanchanaprayudh *et. al.*, 2003)

3.6 Antimicrobial production of endophytic fungi

. . Antimicrobial production was tested by dual culture agar diffusion technique which was modified from the method described by Weaver, Angel and Botlomley (1994) and Joseph, Dave and Shah (1998).

The endophytic fungi were cultured in PDA at room temperature for 14 days. Cultures agar were removed by cut disks with flamed cork hole borer (8 mm diameter) and then placed on test plate. All plates were incubated at 37 ^{o}C overnight. Inhibition zones were measured in mm using a ruler.

3.6.1 Test microorganisms

The test microorganisms for antimicrobial activity assay are listed in Table 3.1.

Table 3.1 Tested microorganisms for antimicrobial activity assay

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

3.6.2 Procedures for evaluation of antimicrobial activity

A. Preparation of medium

Nutrient agar plates were poured into dishes of 90 mm internal diameter. If the plates were not required for immediate use they were stored in refrigerator and protected from desiccation.

B. Preparation of inoculum

From a pure culture of the pathogen, four or five colonies were taken with a wire loop and transferred onto 5 ml of nutrient broth. The inoculum was incubated of at 37°C for 6-8 hours. Turbidity was adjusted with nutrient broth to match the turbidity of Standard Mc.Farland No. 0.5 (OD 0.1 at 625 nm for bacteria and 620 nm for yeast)

C. Inoculation of the test plate

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

D. Application of antimicrobial by dual culture agar diffusion technique

Cultures agar were removed as a disk cut with flamed cork hole borer (8 mm diameter) then placed on test plate. All plates were incubated at 37°C overnight. Inhibition zones were measured in mm using a ruler.

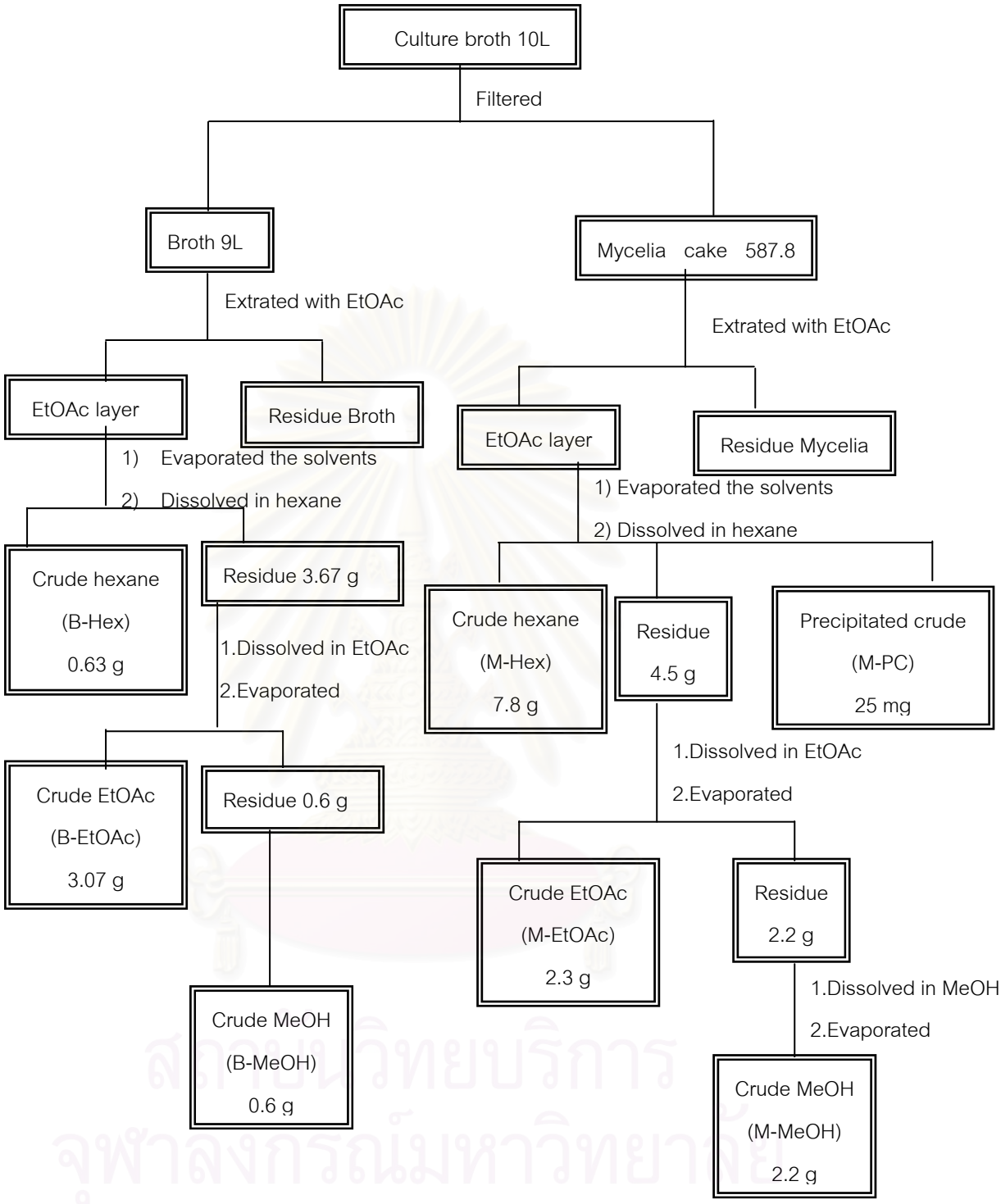
3.7 Cultivation and metabolite extraction of endophytic fungus isolate KBLM02

3.7.1 Cultivation

Fungal endophyte isolate KBLM02 was cultivated on SDA for 2 weeks. The agar culture was cut with flamed cork borer (diameter 8 mm). Five pieces of agar culture were transferred aseptically into 100-250 ml-Erlenmeyer flasks containing 100 ml of SDB, and then statically incubated at room temperature (25-30°C) for 30 days.

3.7.2 Metabolite extraction of endophytic fungus isolate KBLM02

The culture broth (9 L) was filtered through filter paper (Whatman No. 93). The filtrate was extracted with an equal volume of ethyl acetate (EtOAc) 5 times. The EtOAc layers were collected and dehydrated with anhydrous sodium sulfate then evaporated under reduced pressure at 35°C to yield 4.3 g of EtOAc extract (reddish brown liquid). The mycelium were blended and extracted with EtOAc (500 ml x 15) in ultrasonic bath to yield 12.3 g (brown liquid) of crude extract. The extraction of the culture broth and mycelium of the endophytic fungus isolate KBLM02 is shown in Scheme 3.1.



Scheme 3.1 Diagram of method for culture broth and mycelia extraction of endophytic fungus isolate KBLM02 by EtOAc

3.8 Isolation of bioactive compounds from endophytic fungus isolate KBLM02

3.8.1 Isolation of bioactive compounds from crude broth EtOAc extract (B-EtOAc)

The B-EtOAc from endophytic fungus KBLM02 (3.07 g) was subjected to column chromatography (silica gel, 97.23 g), using wet packing loading method. Eluents of increasing polarity from hexane to MeOH were used. Fractions (50 ml each) were collected. Silica gel TLC analysis was used for monitoring each fraction. Fractions with the same TLC pattern were combined and evaporated. The results of the combined fractions are presented in Table 3.2.

Table 3.2 The results from separation of EtOAc crude from broth extract

Combined fractions	Fraction No.	Eluents	Appearance	Weight (mg)
BE01	1-13	50%EtOAc in Hexane	Yellow viscous liquid	600
BE02	14-19	50%EtOAc in Hexane	Yellow viscous liquid	200
BE03	20-22	50%EtOAc in Hexane	Colorless crystal in yellow viscous liquid	41.2
BE04	23-27	50%EtOAc in Hexane	Red brown viscous liquid	36.0
BE05	28-32	50-60%EtOAc in Hexane	Orange viscous liquid	40.3
BE06	33-43	70%EtOAc in Hexane	Colorless crystal in orange viscous liquid	58.0
BE07	44-53	80%EtOAc in Hexane	Orange brown viscous liquid	94.0
BE08	54-59	80%EtOAc in Hexane	Orange brown viscous liquid	52.0
BE09	60-68	80%EtOAc in Hexane	Orange viscous liquid	86.1
BE10	69-87	80%EtOAc in Hexane	Orange viscous liquid	30.6
BE11	88-90	80%EtOAc in Hexane	Orange viscous liquid	22.5
BE12	91-110	80%EtOAc in Hexane	Orange viscous liquid	55.3
BE13	111-121	80%EtOAc in Hexane	Yellow viscous liquid	78.6
BE14	123-137	80%EtOAc in Hexane	Yellow viscous liquid	149.1
BE15	138-151	90%EtOAc in Hexane	Yellow viscous liquid	96.2
BE16	152-159	90%EtOAc in Hexane	Yellow viscous liquid	34.1
BE17	160-203	100%EtOAc 5-10% MeOH in EtOAc	Reddish viscous liquid	198.4
BE18	204-209	20% MeOH in EtOAc	Brown viscous liquid	32.7
BE19	210-215	20% MeOH in EtOAc	Brown viscous liquid	58.1
BE20	216-223	20-30% MeOH in EtOAc	Brown viscous liquid	65.0
BE 21	224-230	30% MeOH in EtOAc	Brown viscous liquid	62.1
BE22	231-254	30-50% MeOH in EtOAc	Dark brown viscous liquid	132.8
BE24	255-267	50-70% MeOH in EtOAc	Dark brown viscous liquid	124.5

3.8.1.1 Isolation of mixture BE1

Mixture 1 was obtained from the elution of silica gel chromatography with 50% EtOAc in hexane and was purified by re-crystallization with MeOH and hexane to obtain a colorless needle crystal (30 mg). The isolation procedure is shown in scheme 3.2.

Mixture BE1 is a colorless needle crystal (30 mg);

UV λ_{max} (nm), MeOH (log ε): 216.9 (4.36) and 268.95 (3.98)(Figure 1 in Appendix C).

FT-IR spectrum (KBr): $\nu_{max}(cm^{-1})$ 3082 (b), 2853 (m), 1710 (s), 1584 (s), and 1022 (m). (Figure 2 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz): δ (ppm) 0.98(3H, t, *J*= 7.2 Hz), 1.41 (2H, dq, *J*= 7.2 and 14.8 Hz), 1.69 (2H, dt, *J*= 7.6 and 15.2 Hz), 2.46 (2H, q, *J*= 7.6 Hz), 2.77 (2H, t, *J* = 7.6 Hz), 2.89 (2H,t, *J*= 7.2 Hz), 5.02 (2H, dd, *J*= 7.6 and 15.0 Hz), 5.82 (1H, m), 7.81 (1H, d, *J*= 7.6 Hz), 8.21 (1H, d, *J* = 7.6 Hz), 8.60 (1H, s). (Figure 3 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ (ppm) 144.8(s), 144.6(s), 124.3(d), 138.7(d), 138.6(d), 143.2(s), 142.1(s), 147.8(d), 147.6(d), 32.7(t), 34.6(t), 32.8(t), 32.3(t), 22.2(t), 136.2(d), 13.7(q), 116.4 (t) and 165.0(s). (Figure 4 in Appendix C)

EI-MS spectrum (70 eV): *m/z* 180.1(28%), 178.1(50) and 135.2(100).(Figure 9 in Appendix C)

3.8.1.2 Purification of compound BE2

Compound BE2 was obtained from the elution of silica gel chromatography with 70% EtOAc in hexane and was purified by re-crystallization with MeOH and hexane to obtain a brown needle crystal (58 mg). The isolation procedure is shown in scheme 3.2. Compound BE2 has m.p. 122 ^{o}C.

Compound BE2 is a brown needle crystal (58 mg);

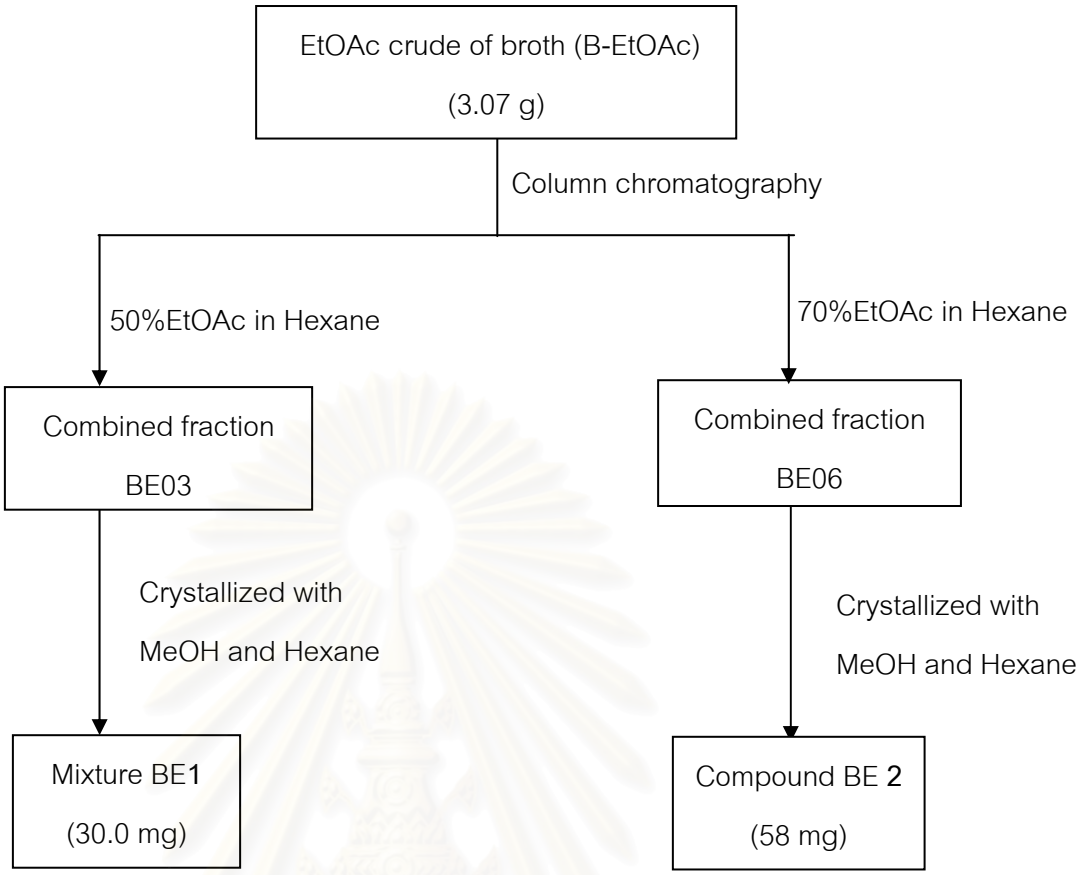
UV λ_{max} (nm), MeOH (log ε): 226.07 (3.93) and 267.05 (3.62) (Figure 10 in Appendix C).

FT-IR spectrum (KBr): ν_{max}(cm^{-1}) 3082 (b), and 1701 (s). (Figure 11 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz): δ (ppm) 8.68 (1H, s br), 8.22 (1H, d, *J*= 8 Hz), 7.82 (1H, d, *J*= 8 Hz), 5.79 (1H, m), 2.45 (2H, dt, *J*= 8 Hz), 2.87 (2H, t, *J*= 7.6 Hz), 5.02 (2H, m). (Figure 12 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ (ppm) 145.0(s), 142.1 (s), 147.8(d), 124.5(t), 138.8 (t), 136.3 (d), 34.6 (t), 32.3 (t), 116.4 (t), 165.3 (s). (Figure 13 in Appendix C)

The MALDI-TOF mass spectra (Figure 18 Appendix C) showed the [MH^{+}] ion peak at *m/z* 177.



Scheme 3.2 Isolation procedure of combined fractions BE03 and BE06

3.8.1.3 Purification of compound BE3

Compound BE3 was obtained from the elution of silica gel chromatography with 80% EtOAc in hexane and was washed with sodium bicarbonate, to remove fusaric acid and dehydrofusaric acid, to obtain the dark yellow solid (8 mg). The isolation procedure was shown in scheme 3.3. Compound BE3 had m.p. 122 ^{O}C.

Compound BE3 is a dark yellow solid (8 mg);

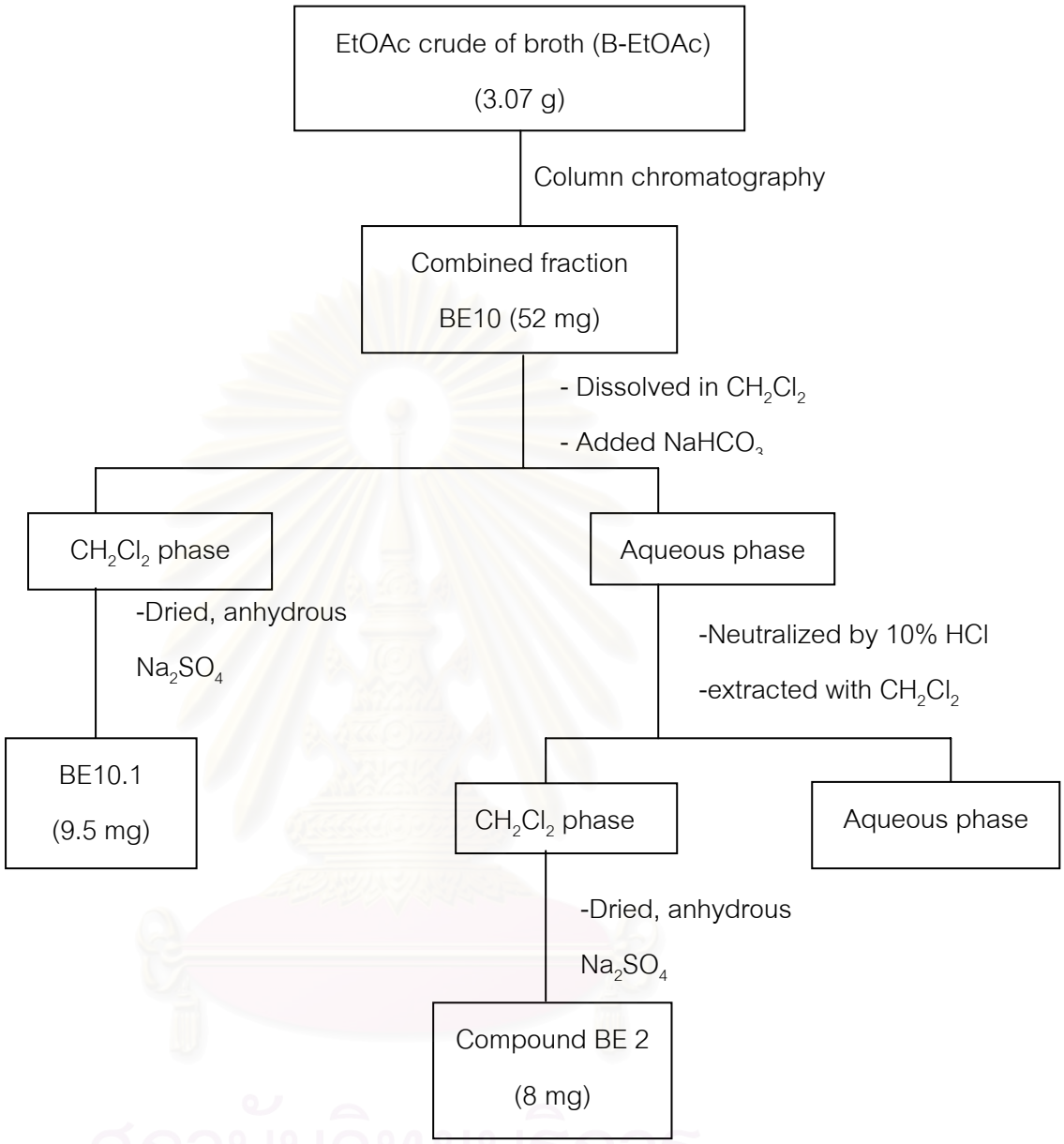
UV λ_{max} (nm), CH_2Cl_2 (log ε): 243.95 (4.55), 248 (4.55), and 313 (3.99) (Figure 19 in Appendix C)

FT-IR spectrum: ν_{max}(cm^{-1}) 3393 (br), 2948 (s), 2836 (s), 1651 (m), and 1025(s). (Figure 20 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.): δ (ppm) 9.45 (1H, s), 7.87 (1H, s), 6.89 (1H, s), 4.06 (3H, s), 4.06 (3H, s). (Figure 21 in Appendix C)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ (ppm) 150.0 (d), 164.1 (s), 117.1 (s), 149.2 (d), 155.5 (s), 104.1 (s), 156.3 (d), 179.5 (s), 189.2 (s), 115.6 (s), 111.0 (s), 125.7 (s), 137.7 (s), 25.1 (s), 56.5 (q), 57.1 (q). (Figure 22 in Appendix C)

EI-MS spectrum (70 eV): *m/z* 299 (82%) and 270 (100). (Figure 25 in Appendix C)



Scheme 3.3 Isolation procedure of combined fraction BE10

3.8.2 Isolation of bioactive compounds from crude hexane (M-Hex) of mycelium extract

The crude hexane of mycelium extract from endophytic fungal isolate KBLM02 (7.8 g) was subjected to column chromatography (silica gel, 200 g), using wet packing and dry loading method. Eluents of increasing polarity from hexane to MeOH were used. Fractions (50 ml each) were collected. Silica gel TLC analysis was used for pooling fractions. Fractions with the same TLC pattern were combined and evaporated. The results of the combined fractions were presented in Table 3.3.

Table 3.3 The results from separation of hexane crude from mycelia extract

Combined fractions	Fraction No.	Eluents	Appearance	Weight (mg)
MH01	1-22	100% Hexane	Colorless liquid	50
MH02	23-37	10% EtOAc in Hexane	Light yellow viscous liquid	3900
MH03	38-41	15% EtOAc in Hexane	Yellow viscous liquid	500
MH04	42-80	15-40% EtOAc in Hexane	Yellow viscous liquid	2500
MH05	81-101	45-50%EtOAc in Hexane	Orange viscous liquid	133.6
MH06	102-136	55-65%EtOAc in Hexane	Orange viscous liquid	174.8
MH07	137-141	65-70%EtOAc in Hexane	Orange viscous liquid	12.3
MH08	142-156	70-75%EtOAc in Hexane	Orange viscous liquid	14.4
MH09	157-190	75-85%EtOAc in Hexane	Yellow viscous liquid	169.7
MH10	191-204	85-90%EtOAc in Hexane	Yellow viscous liquid	14.3
MH11	205-220	90-100%EtOAc in Hexane	Yellow viscous liquid	39.5
MH12	221-225	5% MeOH in EtOAc	Orange viscous liquid	11.0
MH13	226-230	10% MeOH in EtOAc	Orange viscous liquid	16.9
MH14	231-236	10% MeOH in EtOAc	Orange viscous liquid	25.3
MH15	237-242	10% MeOH in EtOAc	Yellow solid	28.6
MH16	243-307	15-45% MeOH in EtOAc	Red viscous liquid	147.8
MH17	308-344	50-100% MeOH in EtOAc	Yellow liquid	12.1

3.8.2.1 Isolation of mixture MH1

Mixture MH1 was obtained from the elution of silica gel chromatography with 10%EtOAc in hexane and appeared as a light yellow viscous liquid (3.9 g).

FT-IR spectrum :ν_{max}(cm^{-1}) 3450 (br), 2927(s), 2850 (s), 1745 (s), 1456 (m), 1157 (m) and 721(w).(Figure 29 in Appendix C)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.): δ (ppm) 5.4 (m), 5.28 (m), 4.18 (dd, *J*= 4.0 and 8.0 Hz), 2.8 (t, *J*= 3.2 Hz), 2.38 (t, *J* = 3.2 Hz), 2.02 (m), 1.61 (s), 1.29 (m), 0.93 (t, *J*= 8.0 Hz) (Figure 27 in Appendix C).

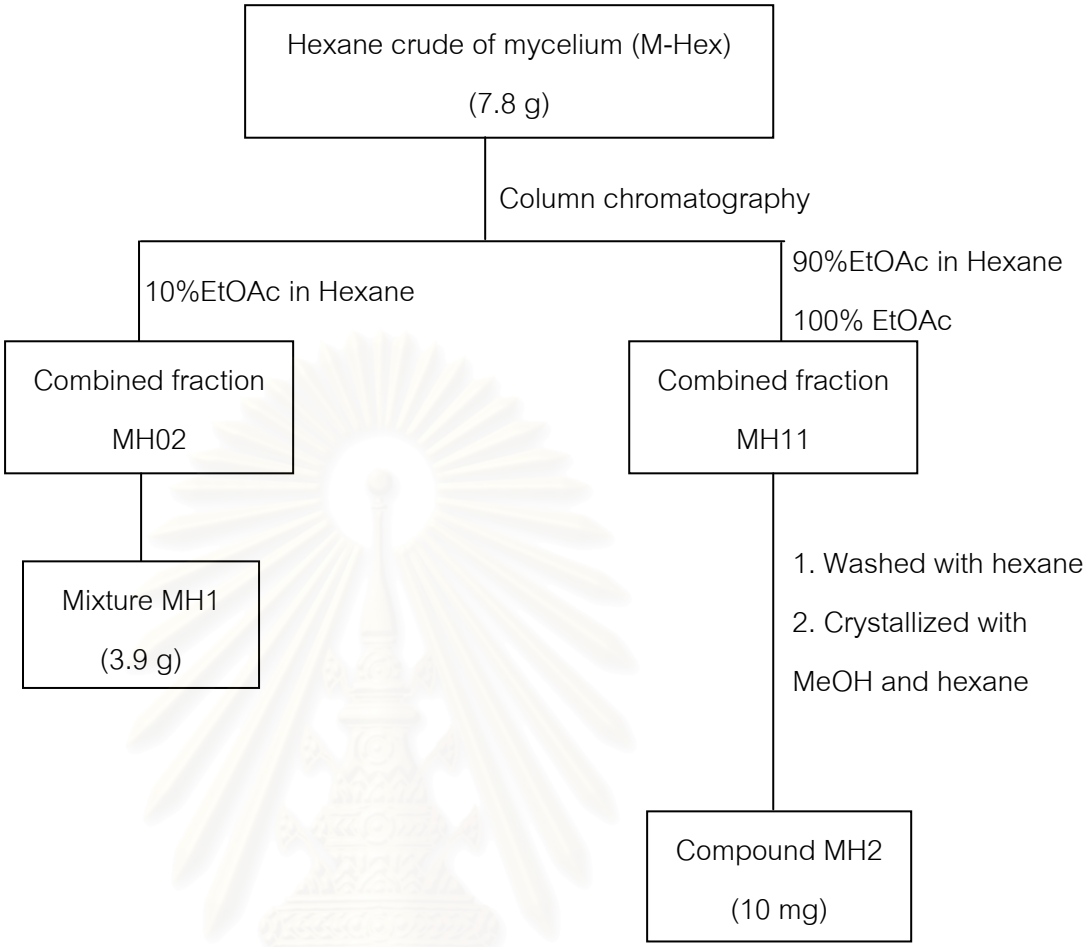
^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ (ppm) 173.2, 172.8, 130.0, 129.7, 68.9, 62.1, 34.2, 34.1, 31.9, 29.8, 29.73, 29.69, 29.65, 29.51, 29.4, 29.35, 29.3, 29.2, 29.1, 27.2, 27.19, 24.9, 22.7, 14.1. (Figure 28 in Appendix C)

3.8.2.2 Purification of compound MH2

Compound MH2 was obtained from the elution of silica gel chromatography with 90%EtOAc in hexane to 100% EtOAc and was purified by wash with hexane and re-crystallization with MeOH and hexane to obtain white amorphous solid (10 mg). The isolation procedure was shown in scheme 3.4.

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.): δ (ppm) 1.48(2H, s), 1.18(2H, s), 4.0(1H, m), 1.69(,m), 3.55(1H, d, *J*= 4.8 Hz), 5.28(1H, d, *J*= 5.2 Hz), 1.91(1H, s), 1.50(2H, s), 2.07(2H, d, *J*=6 Hz), 1.84 (1H, m), 1.39(1H, d, *J*= 6 Hz), 1.46(1H, m), 1.22 (2H, d, *J*=8 Hz), 1.23 (1H, d, *J*=8 Hz), 0.53(3H, s), 1.01(3H, s), 1.97(1H, s), 0.95(3H, d, *J*= 6.4 Hz), 5.12(1H, m), 1.78 (1H, d, *J*=6 Hz), 1.48(1H, s), 0.75 (3H, d, *J*=6.8 Hz), 0.79 (3H, d, *J*= 8 Hz), and 0.83 (3H, d, *J*= 6.8 Hz) (Figure 30 in Appendix C).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ (ppm) 33.1(t), 29.7 (t) ,67.7 (d), 39.4 (t), 76.0 (s), 73.6 (d), 117.5 (d), 144.0 (s), 43.4 (d), 37.1 (s), 22.1 (t), 39.2 (t), 43.8 (s), 54.7 (d), 22.8 (t), 55.9 (t), 12.3 (q), 18.6 (q), 40.4 (d), 21.1 (q), 135.4 (d), 132.2 (d), 42.8 (d), 32.9 (d), 19.6 (q), 19.97 (q), and 17.6 (q) (Figure 31 in Appendix C).



Scheme 3.4 Isolation procedure of combined fractions MH02 and MH11

3.8.3 Isolation of bioactive compounds from precipitated crude (M-PC) of mycelium extract

The precipitated crude (16 mg) of mycelium extracts was re-crystallized with hexane and CH_2Cl_2 to obtain dark red crystals. The procedure for separation of compound M1 from precipitated crude extract of mycelium extract is shown in scheme 3.5. Compound M1 had m.p. 318-320 ^{O}C.

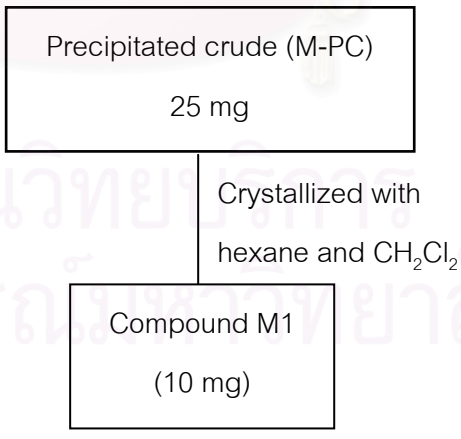
UV λ_{max} (nm), CH_2Cl_2 (log ε): 253.1 (4.36), and 274 (4.44)(Figure 37 in Appendix C).

FT-IR spectrum: $\nu_{max}(cm^{-1})$ 1663 (m), 1610 (m) 1249 (m) and 1143(m) (Figure 38 in Appendix C).

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.): δ (ppm) 2.79(3H, s), 3.86(3H, s), 3.89(3H, s), 6.86 (1H, s), 6.28 (1H, s), 6.73 (1H, s), 12.7 (1H, s) and14.25 (1H, s) (Figure 39 in Appendix C).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz): δ (ppm) 23.5(d), 56.0(d), 56.8(d), 98.8(d), 106.0(s), 112.4 (d), 116.0(s), 117.0(s), 117.8(d), 129.5(s), 144.0(s), 145.9(s), 154.8(s), 156.8(s), 158.5(s), 158.8(s), 164.0(s), 181.0(s),and 187.0(s). (Figure 40 in Appendix C)

EI-MS spectrum (70 eV): *m/z* 382(100%), 367 (89.2) and 339 (83.2). (Figure 45 in Appendix C)



Scheme 3.5 Isolation procedure of precipitated crude (M-PC) from mycelium extract

3.9 Biological activity test

3.9.1 Antimicrobial activity test

3.9.1.1 Antimicrobial activity of pure compounds

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

A. Preparation of pure compounds and antibiotic drug standards

1 mg of pure compounds and antibiotic drug standards were dissolved in 2 ml of 10% DMSO in sterile distilled water and kept in a refrigerator at 4 ^{o}C for bioassay. Antibacterial (Streptomycin and Penicillin G) and antifungal (Ketoconazole) agents were used as positive controls.

B. Preparation of bacterial inoculum

From a pure culture of the pathogen, four or five colonies were taken with a wire loop, then transferred onto 5 ml of Mueller-Hinton broth (MHB) and incubated at 37^{o}C for 6-8 hours. Turbidity was adjusted with MHB to match the turbidity of Standard Mc.Farland No. 0.5 (OD 0.1 at 625 nm).

C. Preparation of yeast inoculum

From a pure culture of the pathogen, four or five colonies were taken with a wire loop, then transferred onto 5 ml of Yeast Malt extract broth (YMB) and incubated at 37^{o}C for 6-8 hours. Turbidity was adjusted with YMB to match the turbidity of Standard Mc.Farland No. 0.5 (OD 0.1 at 620 nm).

D. Assay procedure

Solutions of pure compounds and antibiotic drug standards were diluted with Mueller-Hinton Broth (MHB) and YMB for assays of antibacterial and antifungal (yeast form) activity respectively. Fifty µl of pure compound was dispensed into each well in sterile

micro titer plates (96-well bottom wells). Fifty µl of the final adjusted microbial suspension was inoculated into each well (Final inoculums size of bacterial and yeast was approximately $2.5X10^5$ and $2.5X10^4$ CFU/ml, respectively). One hundred µl of medium only was used as the sterility control. A 100 µl volume of medium and microbial inoculums mixture acted as the growth control. Microbial microtitre plates were incubated at 37 ^oC and room temperature for bacteria and yeast, respectively.

E. Reading of microtitre plates assays

Antibacterial and antifungal (yeast form) activities were determined by measuring the turbidity of each well in the microtitre 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).

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

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

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

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi from *Croton oblongifolius* in Kanchanaburi province

Culture medium used for the isolation of endophytic fungi was potato dextrose agar (PDA). Seventy-two endophytic fungal isolates were isolated from healthy young leaves and mature leaves of *Croton oblongifolius*. All endophytic fungal isolates were selected for further study, as shown in Table 4.1.

Table 4.1 Endophytic fungi isolated from *Croton oblongifolius*

Plant sections	Amount of endophytic fungi	Code of endophytic fungi
Branch	9	KBLB01 to 09
Leaf margin	20	KBLM01 to 20
Leaf vein	20	KBLV01 to 20
Leaf mid-vein	23	KBLMV01 to 23
Total (isolates)	72	72

4.2 Characterization of endophytic fungi

Each fungus isolates was grown on PDA media, for 2-4 weeks at room temperature. Colonial morphology of 15 fungal isolates is shown in Figures 4.1-4.15, as examples. A total of 62 isolates of endophytic fungi were identified. Twenty two fungal isolates were identified as belonging to typical genera of endophytes including *Phomopsis* sp., *Fusarium* sp., *Bipolaris* sp., and members of xylariaceae. The remaining of 50 isolates of endophytic fungi did not produce conidia or sporulate on media and were therefore recorded as mycelia sterilia. The endophytic fungi appearances on PDA, MEA and CMA media are shown in Table 4.2-4.5 and a summary of identification of endophytic fungi compared to endophytic fungi isolated from different sources of *Croton oblongifolius* (Puriso, 2003; Liangsakul, 2003) is shown in Table 4.6.

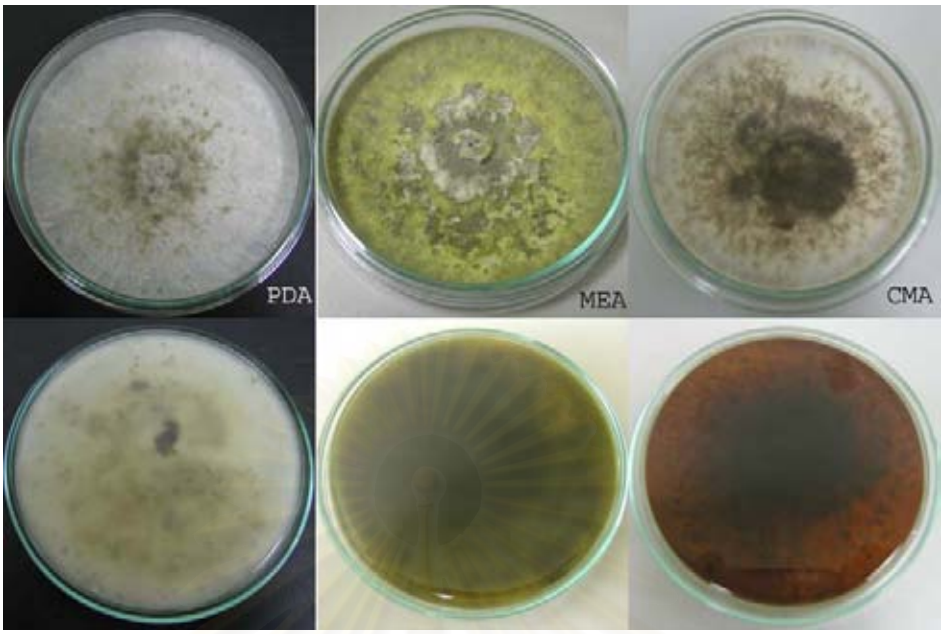


Figure 4.1 Colonial morphology characteristics of endophytic fungus isolate, KBLB04, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below).

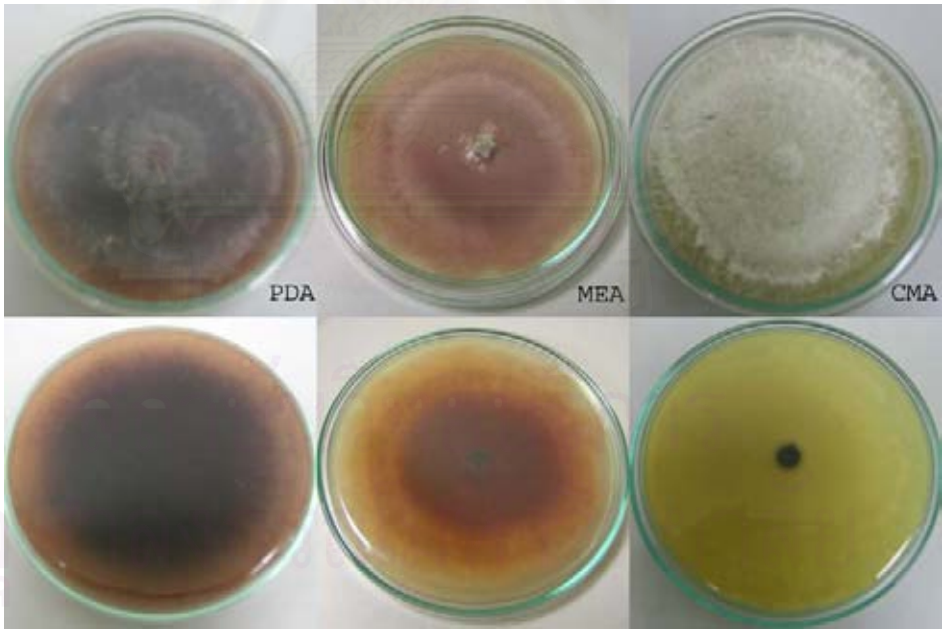


Figure 4.2 Colonial morphology characteristics of endophytic fungus isolate, KBLB07, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

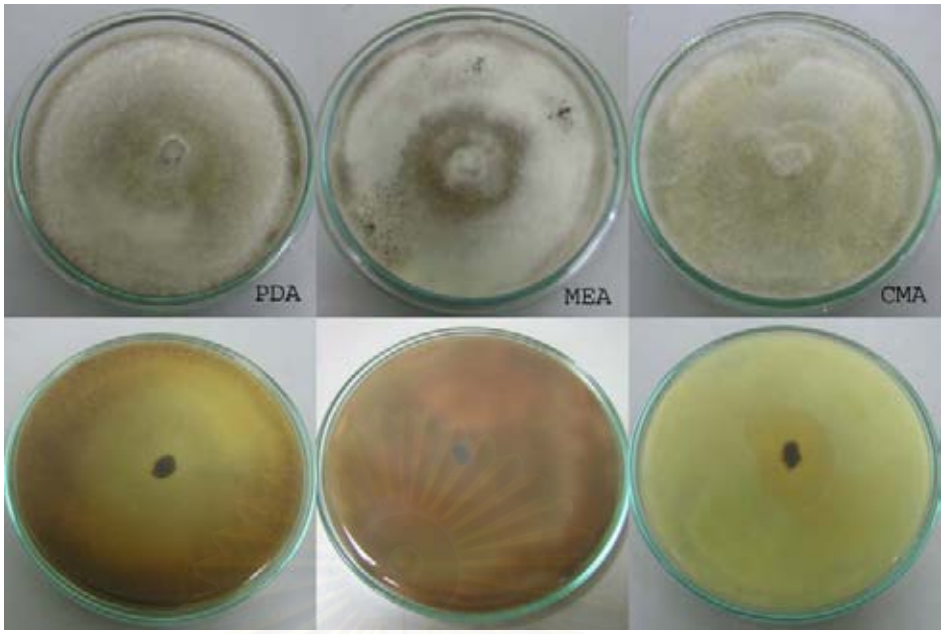


Figure 4.3 Colonial morphology characteristics of endophytic fungus isolate, KBLB08, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above) and bottom view (below).

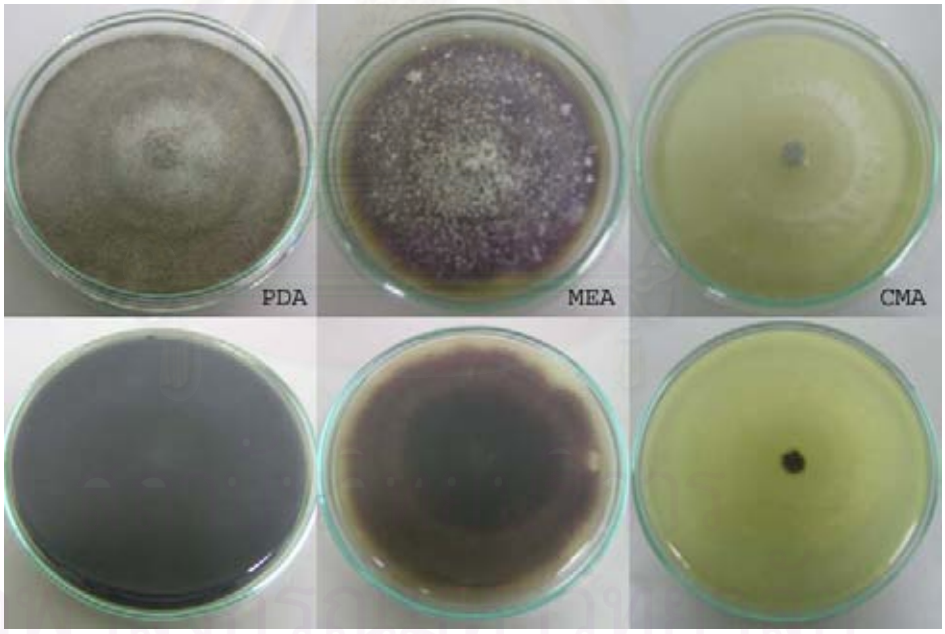


Figure 4.4 Colonial morphology characteristics of endophytic fungus isolate, KBLB09, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above) and bottom view (below).

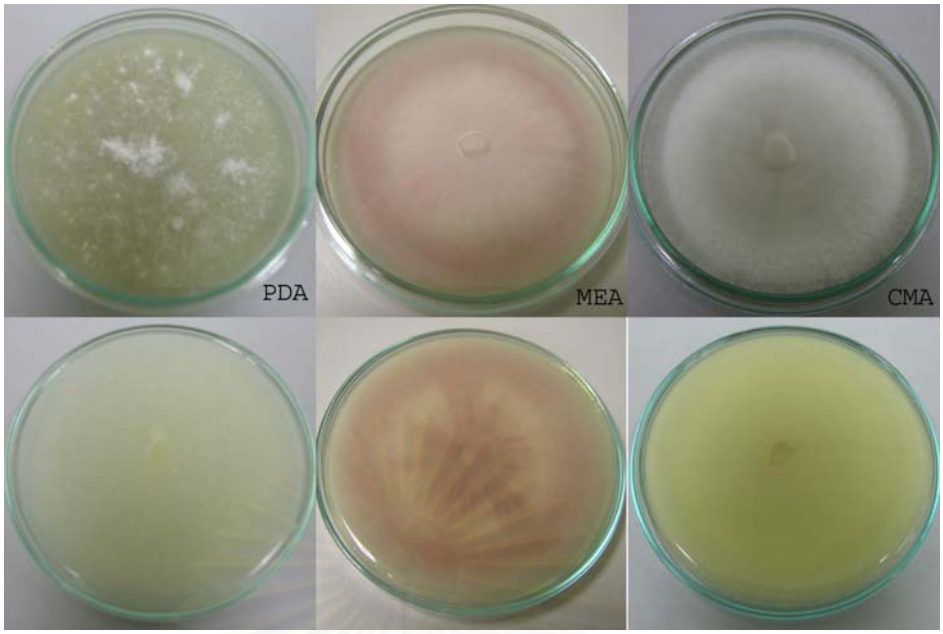


Figure 4.5 Colonial morphology characteristic of endophytic fungus isolate, KBLM02, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

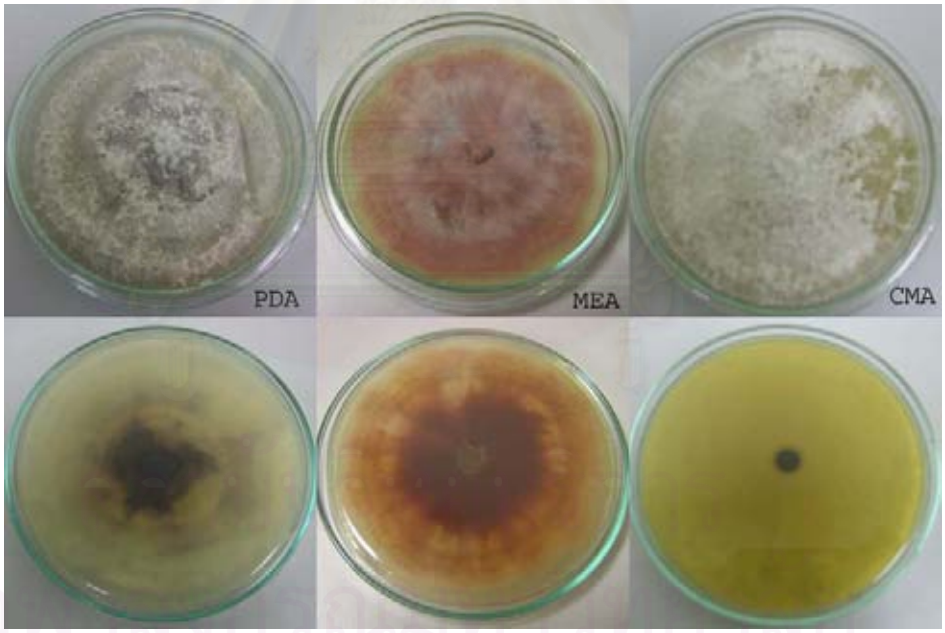


Figure 4.6 Colonial morphology characteristic of endophytic fungus isolate, KBLM06, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

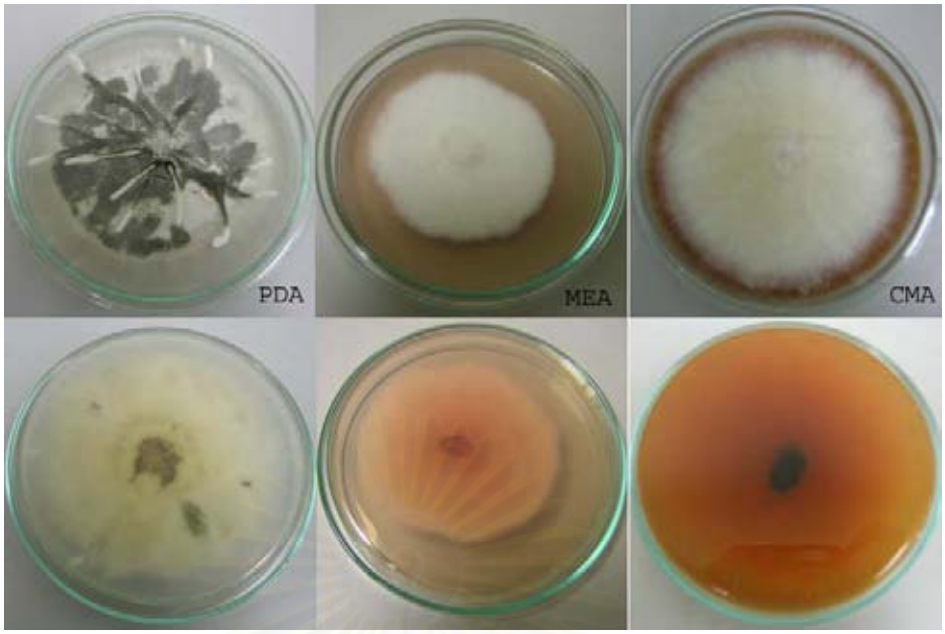


Figure 4.7 Colonial morphology characteristic of endophytic fungus isolate, KBLM13, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

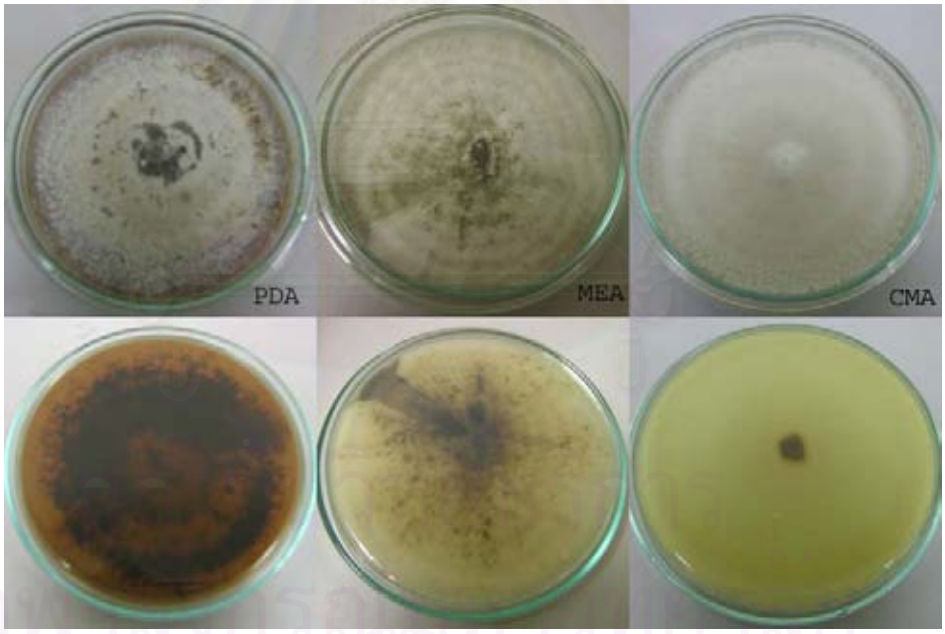


Figure 4.8 Colonial morphology characteristic of endophytic fungus isolate, KBLM15, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

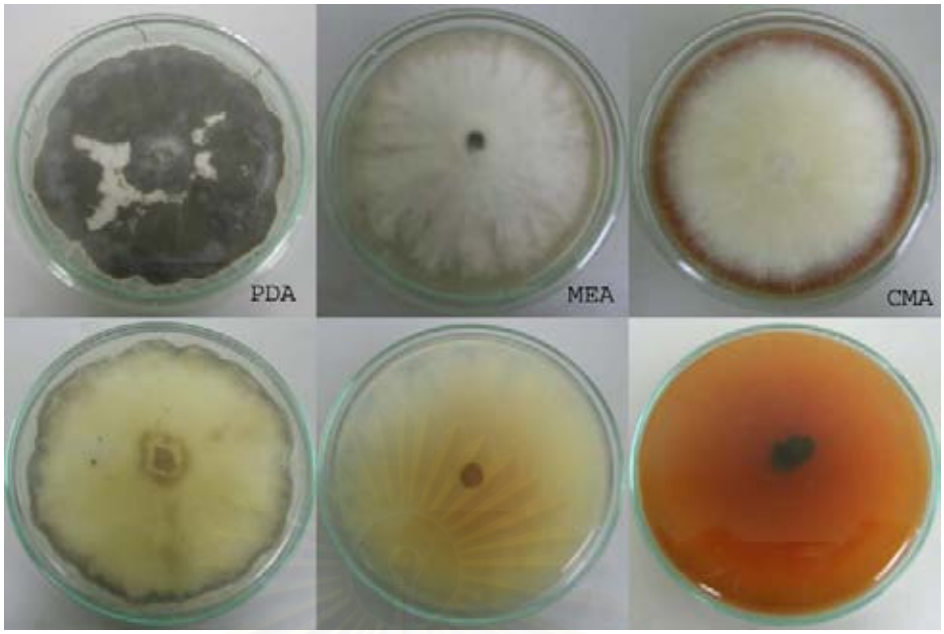


Figure 4.9 Colonial morphology characteristic of endophytic fungus isolate, KBLM16, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

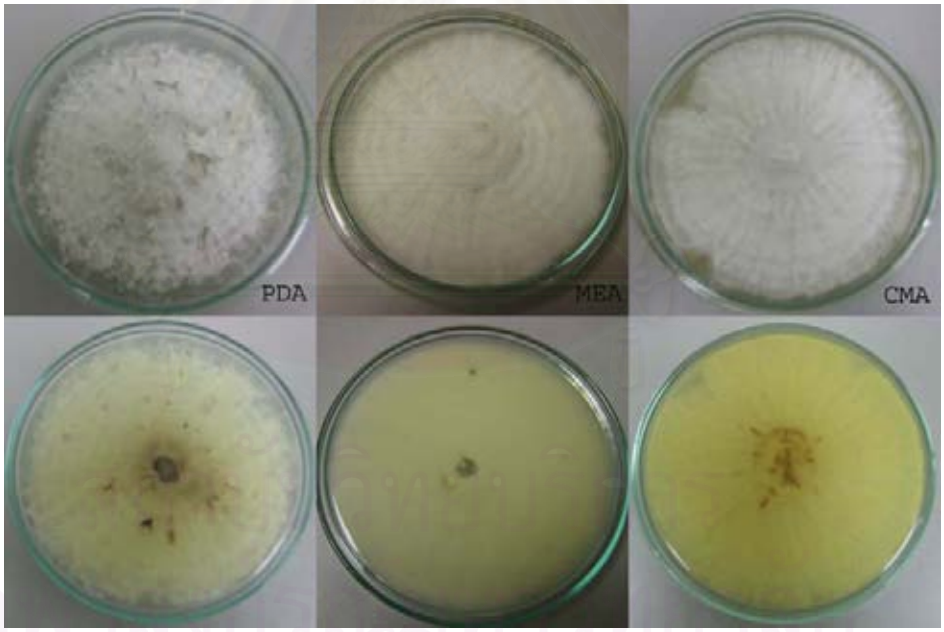


Figure 4.10 Colonial morphology characteristic of endophytic fungus isolate, KBLM20, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

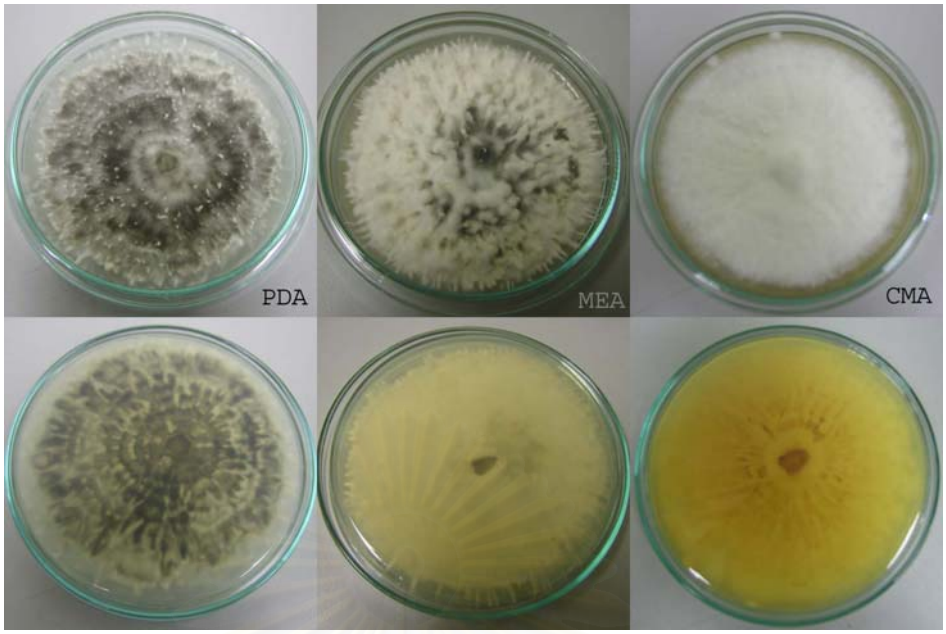


Figure 4.11 Colonial morphology characteristic of endophytic fungus isolate, KBLMV10, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

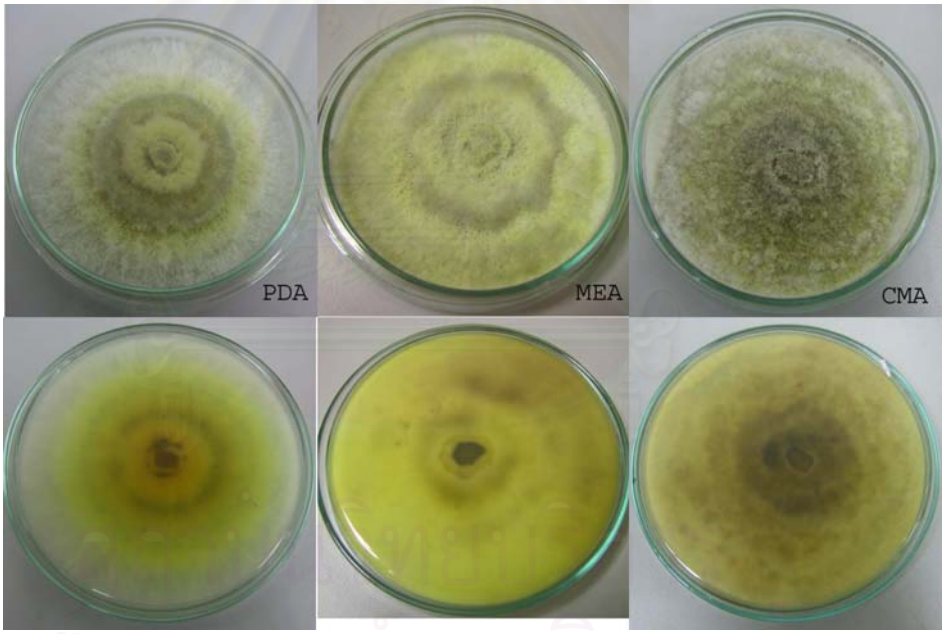


Figure 4.12 Colonial morphology characteristic of endophytic fungus isolate, KBLMV13, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

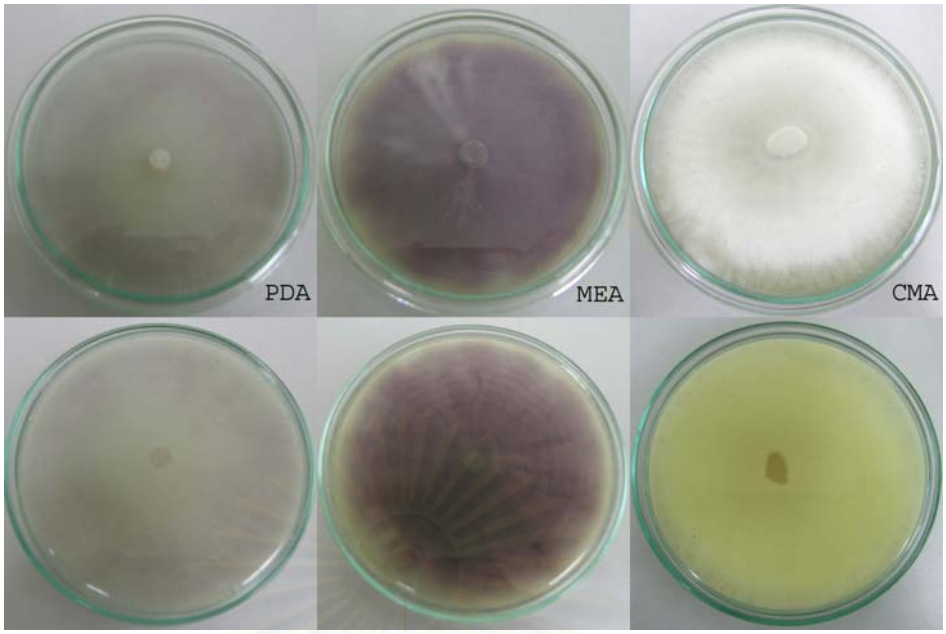


Figure 4.13 Colonial morphology characteristic of endophytic fungus isolate, KBLV6, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

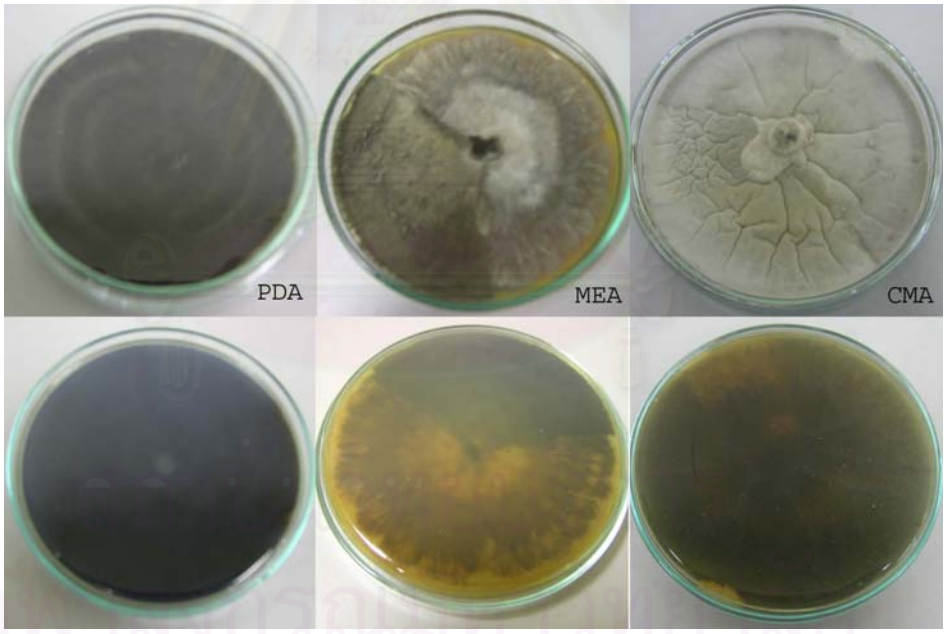


Figure 4.14 Colonial morphology characteristic of endophytic fungus isolate, KBLV18, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

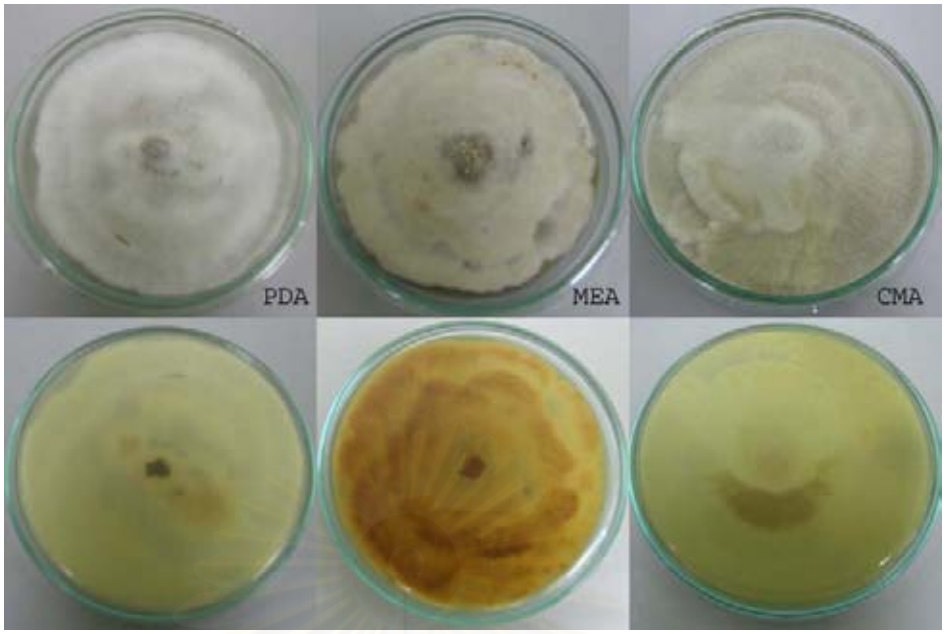


Figure 4.15 Colonial morphology characteristic of endophytic fungus isolate, KBLV19, on PDA, MEA, and CMA media incubated at room temperature for 2 weeks. Appearance on top view (above row) and bottom view (below row).

Table 4.2 Characteristics of colony and identification of endophytic fungi from branch in Kanchanaburi province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Color pigment production on the media	
KBLB01	Cottony	White	Yellow	Mycelia sterilia
KBLB02	Cottony	Light pink	Dark red	Mycelia sterilia
KBLB03	Powdery	white	not produce	*Phomopsis* sp.
KBLB04	Powdery	White and green	not produce	Mycelia sterilia
KBLB05	Cottony	Pink	not produce	Mycelia sterilia
KBLB06	Absence of elevation	yellow	not produce	Mycelia sterilia
KBLB07	Absence of elevation	Dark violet	orange	*Fusarium* sp.
KBLB08	Cottony	white	pink	Mycelia sterilia
KBLB09	Absence of elevation	Dark brown	not produce	Mycelia sterilia

Table 4.3 Characteristics of colony and identification of endophytic fungi from leaves margin in Kanchanaburi province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Color pigment production on the media	
KBLM01	Powdery	White and yellow	not produce	Mycelia sterilia
KBLM02	Powdery	Light violet and white	not produce	*Fusarium* sp.
KBLM03	Cottony	Black	not produce	Mycelia sterilia
KBLM04	Cottony	Grey	not produce	Mycelia sterilia
KBLM05	Cottony	Dark grey	not produce	Mycelia sterilia
KBLM06	Cottony	Violet and white	not produce	*Fusarium* sp
KBLM07	Cottony,stroma	White	not produce	Xylariaceae
KBLM08	Cottony	Brown	not produce	Mycelia sterilia
KBLM09	Powdery	White	not produce	Mycelia sterilia
KBLM10	Cottony	Dark green	not produce	Mycelia sterilia
KBLM11	Cottony	White and black	not produce	Xylariaceae
KBLM12	Cottony	White and light brown	not produce	Mycelia sterilia
KBLM13	Stroma	White and black	Red	Xylariaceae
KBLM14	Powdery	Green	not produce	Mycelia sterilia
KBLM15	Absence of elevation	White and brown	Brown	Mycelia sterilia
KBLM16	Absence of elevation	White and black	Red	Xylariaceae
KBLM17	Cottony	Black	not produce	Mycelia sterilia
KBLM18	Cottony	Grey and yellow	not produce	Mycelia sterilia
KBLM19	Cottony	Brown	not produce	Mycelia sterilia
KBLM20	Cottony, stroma	White	not produce	Xylariaceae

Table 4.4 Characteristics of colony and identification of endophytic fungi from leaves vein in Kanchanaburi province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Color pigment production on the media	
KBLV01	Powdery	White	not produce	*Phomopsis* sp.
KBLV02	Powdery	Dark green	not produce	Mycelia sterilia
KBLV03	Powdery	Grey	Light brown	Mycelia sterilia
KBLV04	Powdery	White	not produce	Mycelia sterilia
KBLV05	Powdery, stroma	White and yellow	not produce	Xylariaceae
KBLV06	Absence of elevation	Violet	not produce	*Fusarium* sp
KBLV07	Powdery,stroma	White and grey	not produce	Xylariaceae
KBLV08	Cottony	White	not produce	Mycelia sterilia
KBLV09	Powdery	Brown	not produce	Mycelia sterilia
KBLV10	Cottony, stroma	White and black	Yellow	Xylariaceae
KBLV11	Cottony	Black	not produce	Mycelia sterilia
KBLV12	Cottony	Grey	Brown	Mycelia sterilia
KBLV13	Absence of elevation	White	not produce	*Phomopsis* sp.
KBLV14	Powdery	Grey and black	Light brown	Mycelia sterilia
KBLV15	Powdery	White	Light brown	Mycelia sterilia
KBLV16	Powdery	White	not produce	Mycelia sterilia
KBLV17	Powdery	White and brown	not produce	Mycelia sterilia
KBLV18	Absence of elevation	Black	not produce	*Bipolaris* sp.
KBLV19	Cottony	White	not produce	Mycelia sterilia
KBLV20	Powdery	White and brown	not produce	Mycelia sterilia

Table 4.5 Characteristics of colony and identification of endophytic fungi from leaves mid-vein in Kanchanaburi province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Color pigment production on the media	
KBLMV01	Powdery	Brown	Not produce	Mycelia sterilia
KBLMV02	Cottony	White and green	Not produce	Mycelia sterilia
KBLMV03	Powdery	Brown	Not produce	*Phomopsis* sp.
KBLMV04	Cottony	White	Not produce	Mycelia sterilia
KBLMV05	Absence of elevation	Grey	Not produce	Mycelia sterilia
KBLMV06	Powdery	Yellow and black	Not produce	Mycelia sterilia
KBLMV07	Powdery	Black	Not produce	Mycelia sterilia
KBLMV08	Cottony	White	Not produce	Mycelia sterilia
KBLMV09	Powdery	White	Not produce	*Phomopsis* sp.
KBLMV10	Powdery, Stroma	White and black	Not produce	Xylariaceae
KBLMV11	Cottony	Black	Not produce	Mycelia sterilia
KBLMV12	Cottony	Brown	Yellow	Mycelia sterilia
KBLMV13	Cottony	White and yellow	Not produce	Mycelia sterilia
KBLMV14	Powdery	White	Not produce	Mycelia sterilia
KBLMV15	Absence of elevation	White	Not produce	Mycelia sterilia
KBLMV16	Powdery, stroma	Black	Not produce	Xylariaceae
KBLMV17	Cottony	White	Not produce	Mycelia sterilia
KBLMV18	Powdery, Stroma	White and black	Not produce	Xylariaceae
KBLMV19	Cottony	White	Not produce	Mycelia sterilia
KBLMV20	Powdery	Brown	Not produce	*Phomopsis* sp.
KBLMV21	Powdery	Dark green	not produce	Mycelia sterilia
KBLMV22	Powdery	White	not produce	Mycelia sterilia
KBLMV23	Absence of elevation	White and brown	Brown	Mycelia sterilia

Table 4.6 A summary of identification of endophytic fungi isolated from *Croton oblongogolius* in Kanchanaburi province, Prachuap Khiri Khan Province and Chachoengsoa Province.

Species	Source of *Croton oblongifolius* for isolating endophytic fungi		
	Kanchanburi Province	Prachuap Khiri Khan Province[a]	Chachoengsoa Province[b]
Bipolaris sp.	1	0	0
Cladosporium sp.	0	1	2
Colletotrichum sp.	0	5	0
Emericella sp.	0	0	1
Fusarium sp.	4	11	0
Lasiodiplodia sp.	0	0	1
Pestalotia sp.	0	1	0
Phomopsis sp.	6	2	21
Tetraploa sp.	0	0	1
Xylariaceae	11	1	0
Coenomycetes	0	0	17
Hyphomycetes	0	7	0
Mycelia sterilia	50	34	41
Total (isolates)	72	62	84

[a]Puriso, 2003

[b]Liangsakul, 2003

4.3 Determination of antimicrobial activities

The isolated endophytic fungi cultured on six media, including Potato Dextrose Agar (PDA), Sabouraud's Dextrose Agar (SDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), and Corn Meal Agar (CMA) were determined antimicrobial activities by dual culture agar diffusion technique. The antimicrobial activities of the various isolates are shown in Table 4.7.

The antimicrobial activities of isolated endophytic fungi which were active against at least one tested microorganisms are shown in Table 4.9. The results showed that endophytic fungal 24 isolates (33.3 % of total 72 isolates) had the antimicrobial activities against at least one tested microorganisms. They were active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 at 16.7%, 6.9%, 6.9%, 2.8%, and 19.4%, respectively (Table 4.8 and Figure 4.16). These indicated that *C. albicans* ATCC 10231 was more sensitive to the isolated endophytic fungi than other tested microorganisms.

Moreover, the results displayed that MEA gave the inhibition against all tested microorganisms and the highest number of active isolates against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 while SDA gave the highest number of active isolate against *B. subtilis* ATCC 6633. MEA and SDA gave equally highest number of active isolate against *C. albicans* ATCC 10231. These suggested that culture medium had effect on antimicrobial activities of isolated endophytic fungi as showed in Figure 4.17. This appears in harmony with other investigations in which the composition of growth medium affected bioactive metabolites production.

Table 4.7 Antimicrobial activities* of endophytic fungi isolated from *C. oblongifolius* leaves.

Code of Isolated Endophytic fungi	Culture medium	Inhibition zone diameters (mm)* / Tested microorganisms				
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
KBLM02	PDA	9	-	-	-	-
	SDA	10	11	-	-	14.5
	MEA	-	-	-	-	11
	CMA	9	-	-	-	-
KBLM06	SDA	-	-	-	-	10
	YES	-	-	-	-	9
KBLM11	MEA	-	-	-	-	10
KBLM12	PDA	14	-	-	-	-
	SDA	10	-	-	-	-
	MEA	14.5	-	-	-	-
KBLM13	PDA	10	10.5	-	-	-
	SDA	9	-	11.5	-	12
	MEA	-	-	8.5	-	10
	YES	-	10	-	-	-
KBLM14	PDA	-	-	-	-	15
	MEA	-	-	11	-	-
KBLM20	MCzA	-	-	-	-	10
KBLV01	MEA	9	-	-	-	-
	MCzA	9	-	-	-	11
	CMA	-	-	-	-	-
KBLV02	SDA	-	-	-	-	15
KBLV05	SDA	-	-	10	-	-
KBLV07	PDA	11	-	-	-	-
KBLV10	SDA	9	-	-	-	-

- = No inhibition zone

* Activity was determined by dual agar diffusion techniques

Table 4.7 (continued)

Code of Isolated Endophytic fungi	Culture medium	Inhibition zone diameters (mm)* / Tested microorganisms				
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
KBLV14	MEA	15	-	-	14	13
	MCzA	-	-	-	-	11
KBLV17	MEA	-	10	-	-	-
KBLB01	PDA	10.5	-	-	-	-
	MCzA	-	-	-	-	11
KBLB05	PDA	-	-	9	-	10.5
	SDA	9	-	-	-	9
	MEA	10	11	-	-	11
	MCzA	12	-	-	-	-
KBLMV03	MCzA	-	-	-	-	13.5
KBLMV05	PDA	17	-	-	-	-
	SDA	12.5	-	-	-	-
	MEA	10	-	-	-	-
	CMA	11	-	-	-	-
KBLMV07	SDA	-	9	-	-	-
KBLMV08	MEA	-	-	-	-	9
KBLMV11	SDA	-	-	-	-	11
	MCzA	-	-	13	-	-
KBLMV18	YES	9	-	-	-	-
KBLMV20	MEA	-	-	-	11	-
KBLMV22	SDA	10	-	-	-	-

- = No inhibition zone

* Activity was determined by dual agar diffusion techniques

Table 4.8 The percentage of endophytic fungi exhibiting antimicrobial against tested microorganisms

Tested microorganisms		Number of active isolates (isolates)	Percent of active isolate (%)
Gram positive bacteria	*B. subtilis* ATCC 6633	12	16.7
	S. aureus ATCC 25923	5	6.9
Gram negative bacteria	*E. coli* ATCC 25922	5	6.9
	P. aeruginosa ATCC 27853	2	2.8
Yeast	*C. albicans* ATCC 10231	14	19.4

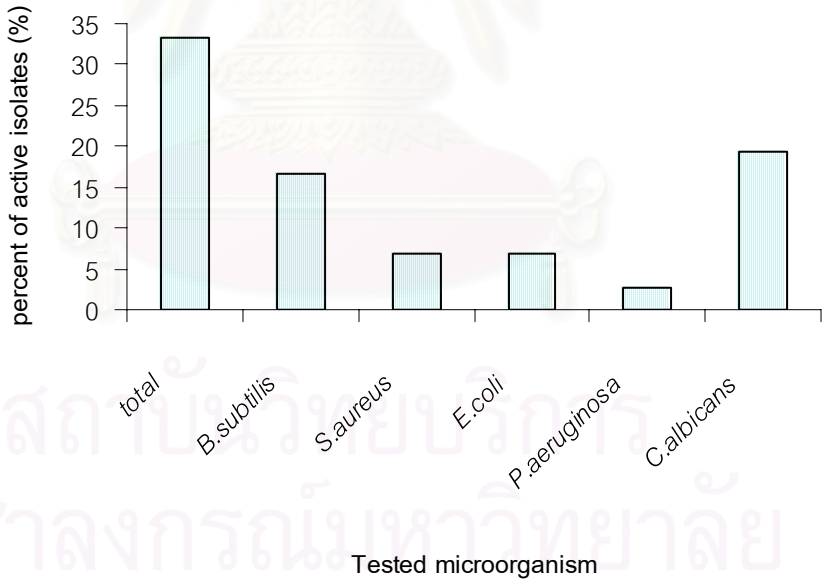


Figure 4.16 The percentage of active endophytic fungal isolates

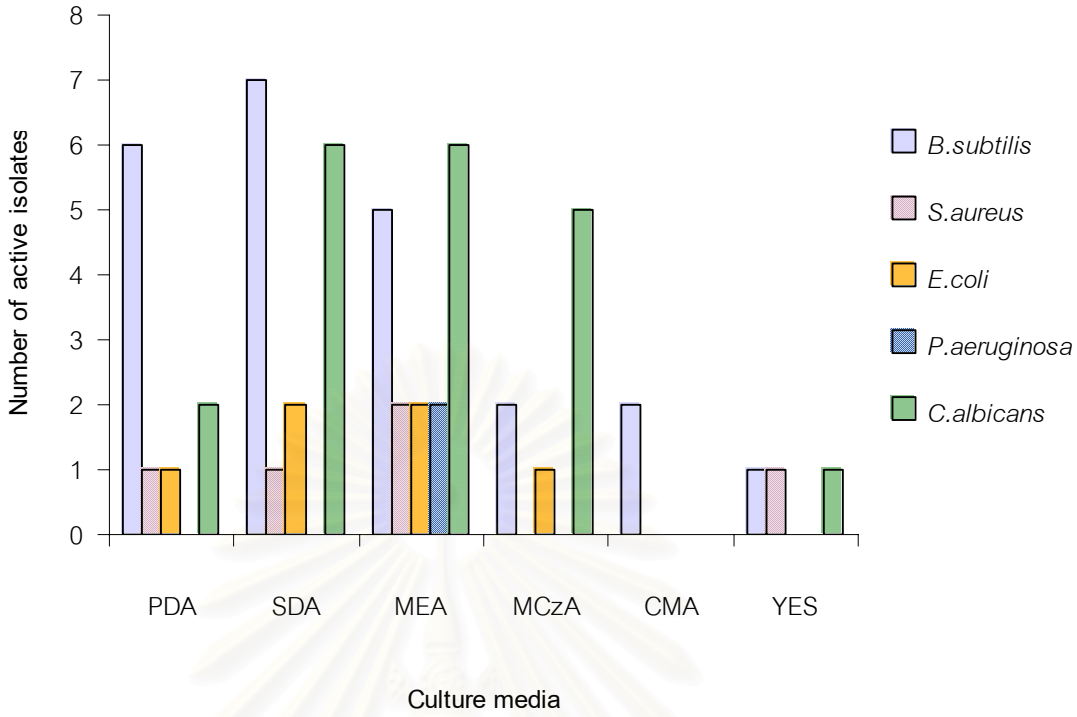


Figure 4.17 The effect of the culture medium on antimicrobial activities of the isolated endophytic fungi.

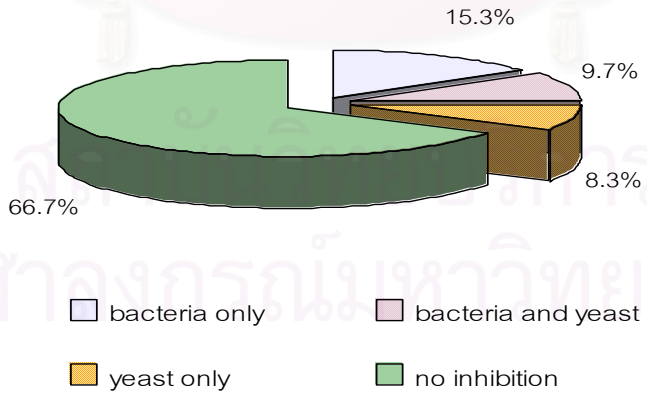


Figure 4.18 Percentage of antimicrobial activities of isolated endophytic fungi on microorganisms

Table 4.9 A summary of antimicrobial activities against group of tested microorganism by the isolated endophytic fungi

Antimicrobial activities against group of tested microorganisms	Isolated endophytic fungi	Total	%
Bacteria only	KBLM12, KBLV01, KBLV05, KBLV07, KBLV10, KBLV17, KBLMV05, KBLMV07, KBLMV18, KBLMV20, KBLMV22	11	15.3
Bacteria and yeast	KBLM02, KBLM13, KBLM14, KBLV14, KBLB01, KBLB05, KBLMV11	7	9.7
Yeast only	KBLM06, KBLM11, KBLM20, KBLV02, KBLMV03, KBLMV08	6	8.3
No antimicrobial activity	KBLM01, KBLM03, KBLM04, KBLM05, KBLM07, KBLM08, KBLM09, KBLM10, KBLM15, KBLM16, KBLM17, KBLM18, KBLM19, KBLV03, KBLV04, KBLV06, KBLV08, KBLV09, KBLV11, KBLV12, KBLV13, KBLV15, KBLV16, KBLV18, KBLV19, KBLV20, KBLB02, KBLB03, KBLB04, KBLB06, KBLB07, KBLB08, KBLB09, KBLMV01, KBLMV02, KBLMV04, KBLMV06, KBLMV09, KBLMV10, KBLMV12, KBLMV13, KBLMV14, KBLMV15, KBLMV16, KBLMV17, KBLMV19, KBLMV21, KBLMV23	48	66.7

As summarized in Table 4.9 and Figure 4.18, antimicrobial activities of the isolated endophytic fungi were classified into 4 types as bacterial inhibition only (15.3%), yeast inhibition only (8.3 %), bacterial and yeast inhibition (9.7%) and no antimicrobial activity against tested microorganisms (66.7 %).

4.4 Taxonomy of endophytic fungi

Description of genus BIPOLARIS (Shoemaker,1959)

Mycelium brown,gray or black. Conidiophores straight or flexuous, multiseptate, usually simple, smooth, macronematous, mononematous, often geniculate, sometimes nodoes, cylindrical. Conidiogenous cells cylindrical, integrated, terminal or intercalary,proliferating sympodially, cicatrized. Conidia acropleurogenous, fusoid, obpyriform, navicular, oblong, cylindrical, obclavate, clavate, ovoid, solitary, curved to straight, mostly smooth, rarely echinulate to rought-walled, 2-or more distoseptate, septa sometimes thickened and dark, pale brown, olivaceous brown, reddish brown or dark brown, germinating principally from one or both polar cells with the basal germ tube originating close to hilum and growing semiaxially, hilum asscociated with a slightly protruding, truncate section of the wall, and often visible as two dark lenticular spots in optical section section arranged close together and seperated by a small obscure narrow canal, or rarely protuberant, first conidial septum median to submedian, second septum delimiting the basal cells, the third septum distal, conidiogenous nodes rough to smooth.

Description of the genus FUSARIUM (Subramanian, 1971)

Mycelium composed of hyaline, septate, branched hyphae. Conidial masses typically formed in sporodochia or in pioonates or sometimes scattered in the mycelium. Conidiophores simple or branched once or repeatedly, terminating in phialides which are sometimes formed verticillately, hyaline, septate. Phialides variable in size and shape, but mostly subulate. Conidia of two types: microconidia and macroconidia. Microconidia usually 1-celled, variable in shape, hyaline, either produced singly at the tips of phialides, or else abstracted in succession at the tips of phialides to form simple chains. Macroconidia usually 3-many-septate, fusiform to falcate, dorsiventral, straight or curved

variously, often with a distinct pedicellate base, with the apical part obtuse to broadly rounded to caudate or acuminate, produced singly at the tips of phialides. Chlamydospores usually present , globose, ovoid or pear-shaped, 1-2 celled or in chain, or sometimes in cultures, terminal or intercalary, brownish in colour or becoming tinged with the colour of the stroma. Sclerotia spherical, solid, occurring singly, or in groups, or absent. Sclerotial stromata occur in many groups erumpent, hemispherical, smooth or rough and cauliflower-like, or erect, stiboid, sometimes with antler-like branching, sessile or stalked: remaining sterile or serving as a stroma for sporodochia. Conidial masses pale or brightly coloures (orange, salmon or ochre), slimy.

Description of the genus PHOMOPSIS (Sutton, 1980)

Mycelium immersed, branched, septate, hyaline to pale brown. Conidiomata eustromatic, immersed, brown to dark brown, septate or aggregated and confluent, globose, ampulliform or applanate, unilocular, multiocular or convoluted, thick-walled; walls of brown, thin-or thick-walled textura angularis, often somewhat darker in the upper rehion, lined by a layer of smaller-called tissue. Ostiole single, or several in complex conidiomata, circular, often papillate. Conidiophores branched and septate at the base and above, occasionally short and only 1-2 septate, more frequenily multiseptate and filiform, hyaline formed from the inner cells of the iocular walls. Conidiogenous cells enteroblastic, phialidic, determinate, integrated, rarely discrete, hyaline, cylindrical, apertures apical on long or short lateral and main branches of the conidiophores, collarette, channel and periclinal thickening minute. Conidia of two basic types, but in some species with intermediates between the two: α-conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end) but sometimes with more guttules, aseptate; β-conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate.

Figure 4.19 *Bipolaris* sp. A: culture on PDA (14 days). B: conidia (X100).

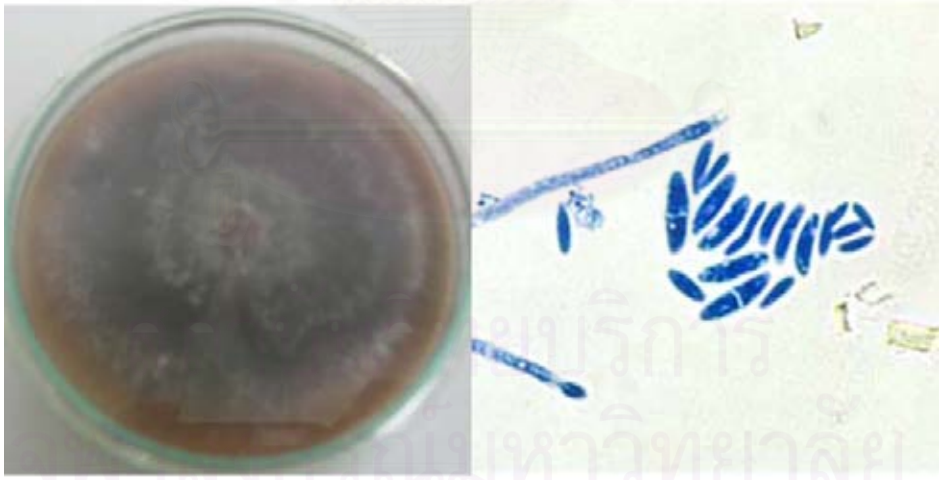


Figure 4.20 Fusarium sp. A: culture on PDA (14 days) B: conidia (x100)

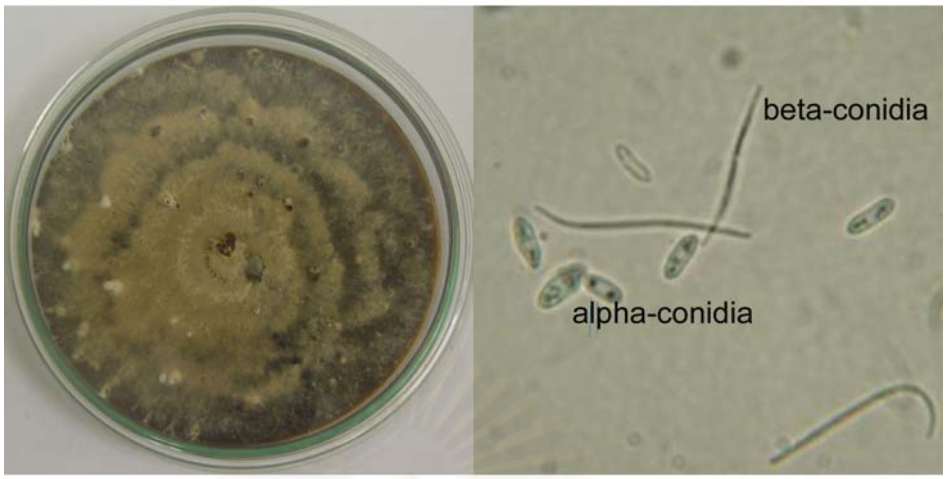


Figure 4.21 *Phomopsis* sp. A.culture on PDA (14 days) B: Alpha and beta conidia (x100)

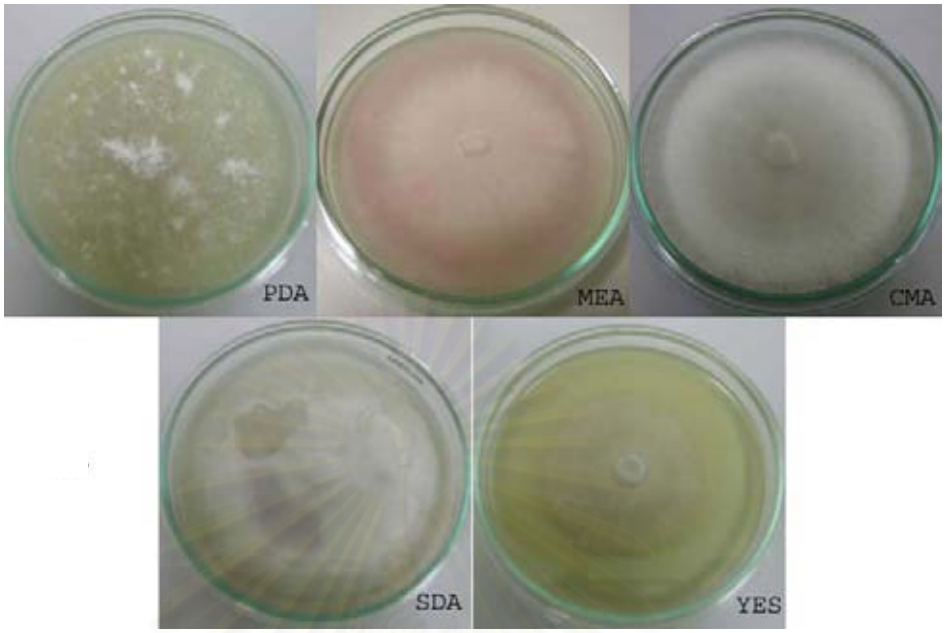
4.5 Identification of fungal endophyte KBLM02

Fungal isolate KBLM02 was chosen for further study for bioactive compounds because this isolate was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231 with inhibition zone 10, 11 and 14.5 mm, respectively.

4.5.1 Morphology identification

Fungal isolate KBLM02 was identified as *Fusarium* sp. Descriptions of the genus *Fusarium* is described in section 4.4. The fungus was grown on Sabouraud's Dextrose agar (SDA) for 14 days at room temperature. Colony morphology, scanning electron micrograph and light micrograph of isolate KBLM02 are shown in Figures 4.22, 4.23 and 4.24, respectively.

A. Appearance on top



B. Appearance on bottom

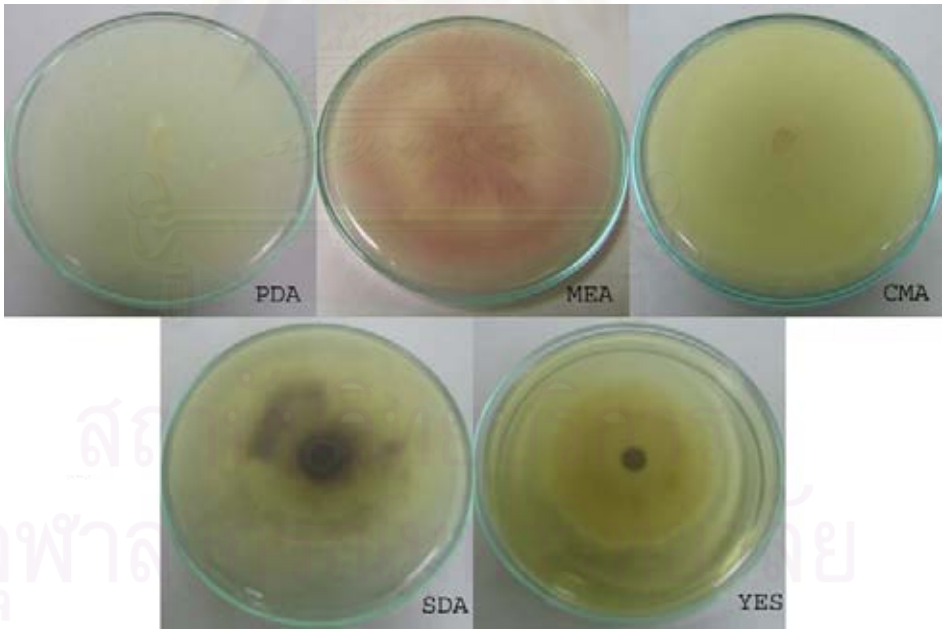


Figure 4.22 Colony characteristic of 14-day-old-endophytic fungal isolate KBLM02 grew on Potato Dextrose Agar, Malt Extract Agar, Corn Meal Agar, Sabouraud's Dextrose Agar, and Yeast Extract Sucrose Agar incubated at room temperature.

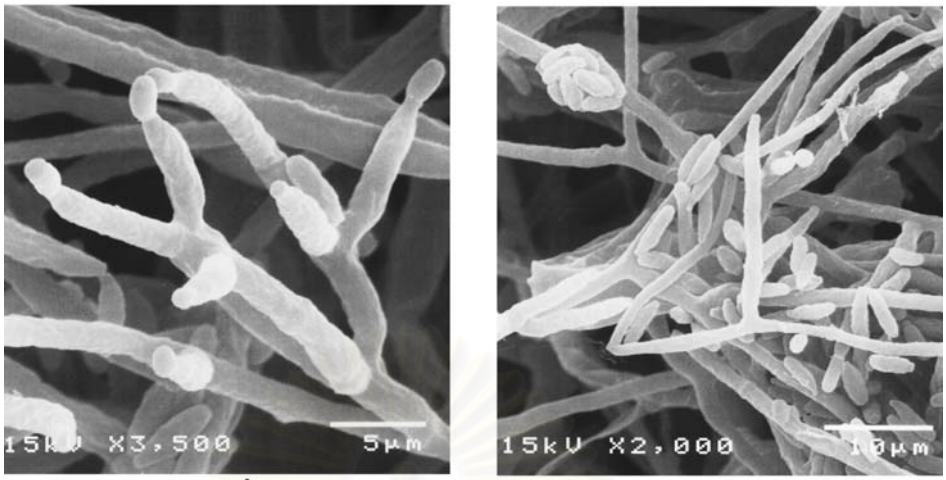


Figure 4.23 Scanning Electron Micrograph characteristic of 1 month old endophytic fungal isolate KBLM02. A. Conidiophores and young conidia (Bar=5 µm) B. Mature conidia (Bar=10 µm).

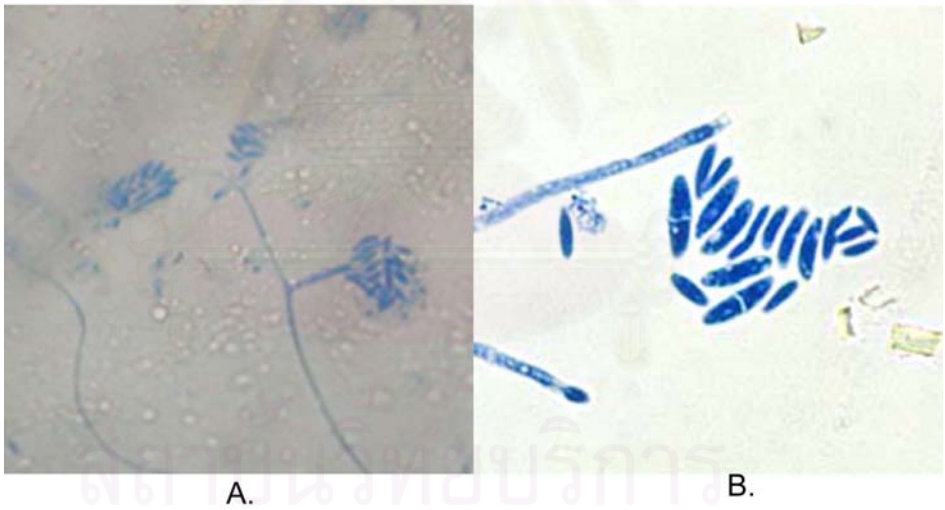


Figure 4.24 Light micrograph of 1 month old isolate KBLM02 culture in Sabouraud's Dextrose agar A. conidiophores and conidia (x 40) B. conidia (x100).

4.5.2 Molecular identification of endophytic fungi isolate KBLM02

The rDNA ITS region of isolate KBLM02 was amplified with the conserved fungal primer ITS_1 and ITS_4. Isolate KBLM02 produced a single ITS band.

The length of corresponding fragment was 573 bp.,containing a part of the 18S, ITS_1, 5.8 and 28 rDNA, as shown in Figure 4.25.

```
    1
5′GAGGAAGTAA    AAGTCGTAAC    AAGGTCTCCG    TTGGTGAACC
  AGCGGAGGGA    TCATTACCGA    GTTTACAACT    CCCAAACCCC
  TGTGAACATA    CCAATTGTTG    CCTCGGCGGA    TCAGCCCGCT
  CCCGGTAAAA    CGGGACGGCC    CGCCAGAGGA    CCCCTAAACT
  CTGTTTCTAT    ATGTAACTTC    TGAGTAAAAC    CATAAATAAA
  TCAAAACTTT    CAACAACGGA    TCTCTTGGTT    CTGGCATCGA
  TGAAGAACGC    AGCAAAATGC    GATAAGTAAT    GTGAATTGCA
  GAATTCAGTG    AATCATCGAA    TCTTTGAACG    CACATTGCGC
  CCGCCAGTAT    TCTGGCGGGC    ATGCCTGTTC    GAGCGTCATT
  TCAACCCTCA    AGCCCAGCTT    GGTGTTGGGA    CTCGCGAGTC
  AAATCGCGTT    CCCCAAATTG    ATTGGCGGTC    ACGTCGAGCT
  TCCATAGCGT    AGTAGTAAAA    CCCTCGTTAC    TGGTAATCGT
  CGCGGCCACG    CCGTTAAACC    CCAACTTCTG    AATGTTGACC
  TCGGATCAGG    TAGGAATACC    CGCTGAACTT    AAGCATATCA
  ATAAGCCGGA    GGA 3′
                  573
```

Figure 4.25 Nucleotide sequences of partial 18S region, complete ITS region of the isolate KBLM02, containing a partial of the 18S, ITS1, 5.8S and 28S rDNA.

A blast search was performed to find a similar sequence to ITS region of fungal isolate LSS6 in the Genbank DNA database, available from: http://www.ddbj.nig.ac.jp . The results revealed that ITS region of endophytic fungal isolate KBLM02 had 99.446% identity (99.815% ungapped) of *Gibberella moniliformis*, as showed in Figure 4.26.

>>AY533376|AY533376.1 Gibberella moniliformis small subu (573 nt)
 initn: 2661 init1: 2661 opt: 2671 Z-score: 1740.0 bits: 331.8 E(): 9.7e-89
banded Smith-Waterman score: 2671; 99.446% identity (99.815% ungapped) in 542 nt overlap (8-547:32-573)

```
                                        10          20        30
                                AGNNTCGTGGTGA--CAGCGGAGGGATCATTACCGAG
                                      ::::::  ::::::::::::::::::::
AY5333 AGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAG
              10        20        30        40        50        60

          40        50        60        70        80        90
       TTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCCGCTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333 TTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCCGCTC
              70        80        90       100       110       120

        100       110       120       130       140       150
       CCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333 CCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCT
             130       140       150       160       170       180

         160       170       180       190       200       210
       GAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333 GAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
             190       200       210       220       230       240

         220       230       240       250       260       270
       GAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333 GAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
             250       260       270       280       290       300

         280       290       300       310       320       330
       CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333 CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT
             310       320       330       340       350       360

         340       350       360       370       380       390
       CAACCCTCAAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAATTGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333 CAACCCTCAAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAATTGA
             370       380       390       400       410       420
```

```
         400       410       420       430       440       450
        TTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333  TTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTC
              430       440       450       460       470       480

          460       470       480       490       500       510
        GCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY5333  GCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCC
              490       500       510       520       530       540

          520       530       540
        GCTGAACTTAAGCATATCAATAGGCCGGAGGAAN
        :::::::::::::::::::: :::::::::
AY5333  GCTGAACTTAAGCATATCAATAAGCCGGAGGA
              550       560       570
```

Figure 4.26 Alignment data of ITS region of isolate KBLM02 and 1 reference taxa

Classical identification of fungi is based on observing characteristics. Assignment of morphological species can be based on colony surface texture, hyphal pigments, exudates, margin shapes, growth rates, and sporulating structures. Fungal isolates KBLM02 was identified as belonging to the genera *Fusarium*.

A molecular method of identification was also performed. The nucleotide sequence of the ITS region of rDNA is conserved. It can be based to delineate species relationships and separated taxonomy from class to species (Mitchell et al., 1995). The nucleotide sequence of the ITS region of fungal isolate KBLM02 was similar to 99.446% identity of *Gibberella moniliformis* (anamorph, *Fusarium verticillioides*) reported by Buzina et al., 2004.

The genus *Fusarium,* which is well known, is the anamorph in imperfect fungi or deuteromycetes while the genus *Gibberella* is a well known telemorph in ascomycetes. In this research, only the anamorph was studied because the sexual stage of fungal isolate KBLM02 did not appear on microbiological media.

4.6 Cultivation and extraction of endophytic fungi isolate KBLM02

Fungal isolate KBLM02 was cultivated in Sabouraud's Dextrose broth totalling 9 L to yield 4.3 g of crude EtOAC from culture broth, and 12.3 g of crude EtOAc from mycelium.

4.7 Isolation and purification of bioactive compounds in crude culture broth and crude mycelium

Three compounds were isolated from the culture broth and three compounds from crude mycelium of endophytic fungus isolated KBLM02. Mixture BE1 (30 mg, 0.7 % yield of crude culture broth) from fraction BE03 were obtained from crystallization as a colorless needle crystal, compound BE2 (58 mg, 1.35% yield of crude culture broth) from fraction BE06 were obtained from crystallization as the brown needle crystal and compound BE3 (8 mg, 0.19 % yield of crude culture broth) were obtained as the dark yellow solid by washing fraction BE10 with sodium bicarbonate to remove fusaric acid and dehydrofusaric acid. Mixture MH1 (3.9 g, 31.7 % yield of crude mycelium) from fraction MH02 were obtained as light yellow viscous liquid, compound MH2 (10 mg, 0.08 % yield of crude mycelium) from fraction MH11 were obtained from crystallization as a white amorphous solid and compound M1 (10 mg, 0.08 % yield of crude mycelium) were obtained from crystallization as the dark red crystals. Chemical structures of these compounds were determined by analyzes of spectroscopic data, including IR, UV, NMR and Mass spectra, as well as by comparison their spectral data with those of published values.

4.8 Structure elucidation of the pure compounds from endophytic fungus isolate KBLM02

4.8.1 Structure elucidation of mixture BE1

Mixture BE1 was a colorless needle crystal. The structure of mixture BE1 was elucidated by using spectroscopic techniques.

The IR spectra of mixture BE1 is shown in Appendix C Figure 5 and the absorption peaks were assigned as Table 4.10. Its IR spectrum indicated important absorption band at 3082 cm^{-1}(=CH_2 asymmetric stretching), 1710 cm^{-1} (C=O stretching in acid), and 1022 cm^{-1} (C-O stretching).

Table 4.10 The IR absorption band assignment of mixture BE1

Wave number (cm^{-1})	Intensity	Assignment
3082	Broad, Weak	$=CH_2$ asymmetric stretching
2853	Weak	C-H stretching in $-CH_2$, $-CH_3$
1710	Strong	C=O stretching in acid
1584	Medium	C=C stretching in aromatic ring
1022	Medium	C-O stretching

The ^{1}H-NMR spectrums (Figure 3 in Appendix C) of mixture BE1 possessed a methyl proton at 0.97 ppm, three aliphatic methylene protons at 1.40, 1.70 and 2.89 ppm, two benzylic protons at 2.48 and 2.78 ppm, two vinylic protons at 5.02 and 5.82 ppm and three aromatic protons at 7.80, 8.20 and 8.60 ppm.

The ^{13}C-NMR spectrum (Figure 4 in Appendix C) of mixture BE1 showed 18 signals, which the carbonyl group of carboxylic acid corresponded to the signal at 165.0 ppm.

The EI-MS mass spectrum (Figure 9 in Appendix C) showed the MH^+ ion peak at *m/z* 178 and 180 indicated a compound with an odd number of nitrogen atoms. It is assumed that this mixture contains carbons, protons, oxygen, and nitrogen, and then the molecular formula is $C_{10}H_{13}O_2N$ and $C_{10}H_{11}O_2N$. The first molecular formula, $C_{10}H_{13}O_2N$, indicated five degree of unsaturation, so mixture 1 consist of one aromatic ring and one carbonyl and the others, $C_{10}H_{11}O_2N$, indicated six degree of unsaturation, so mixture 1 consist of one aromatic ring and one carbonyl in addition to one double bond.

The information from 2D-NMR techniques, including HSQC (Table 4.11, Figure 5 in Appendix C), HMBC (Table 4.11, Figure 4.27 and Figure 6 in Appendix B), COSY (Table 4.11, Figure 7 in Appendix C) and NOESY (Figure 8 in Appendix B) were used to assist the interpretation of mixture BE1 structure.

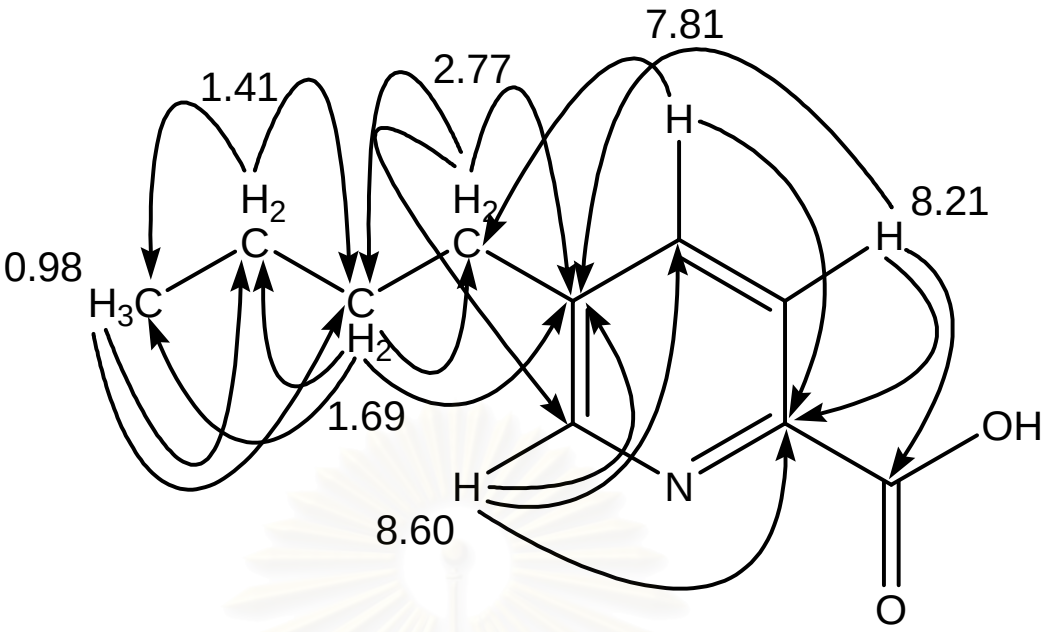
Table 4.11 HSQC, HMBC and COSY spectral data of mixture BE1:

For Fusaric acid

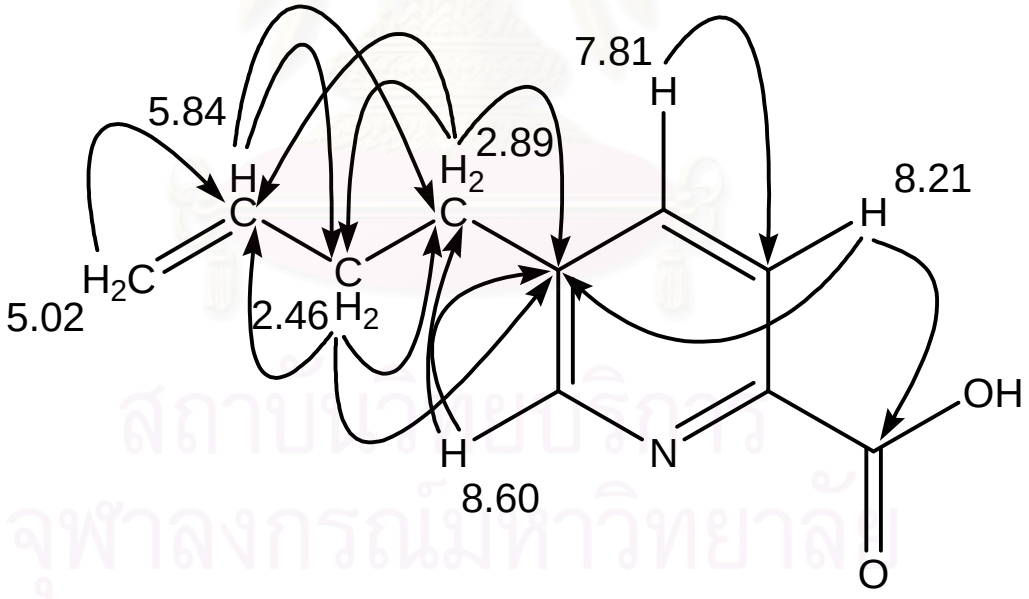
Position	δ_C	δ_H	HMBC (H to C)	COSY
1	-	-	-	-
	144.8 (s)	-	-	-
2	124.3 (d)	8.21(1H, d, *J*=7.6 Hz)	C-2, C-5, C-11	H-4 (7.81)
3	138.7 (d)	7.81(1H, d, *J*=7.6 Hz)	C-2, C-7	H-3 (8.21), H-6 (8.60)
4	143.2 (s)	-	-	-
5	147.8 (d)	8.60(1H, s)	C-2, C-4, C-5	H-4 (7.81)
6	32.7 (t)	2.77(2H, t, *J*=7.6 Hz)	C-5, C-6, C-8	H-8 (1.69)
7	32.8 (t)	1.69(2H, t, *J*=7.6,15.2 Hz)	C-5, C-7, C-9, C-10	H-7 (2.77), H-9 (1.41)
8	22.2 (t)	1.41(2H, dq, *J*=7.2,14.8 Hz)	C-8, C-10	H-10 (0.98), H-8 (1.69)
9	13.7 (q)	0.98(3H, t, *J*=7.2)	C-8, C-9	H-9 (1.41)
10	165.0 (s)	-	-	-
11				

For Dehydrofusaric acid

Position	δ_C	δ_H	HMBC (H to C)	COSY
1	-	-	-	-
2	144.6 (s)	-	-	-
3	124.3 (d)	8.21 (1H, d, *J*=7.6 Hz)	C-5, C-11	H-4 (7.81)
4	138.6 (d)	7.81 (1H, d, *J*=7.6 Hz)	C-3	H-3 (8.21), H-6 (8.60)
5	142.1 (s)	-	-	-
6	147.6 (d)	8.60 (1H, s)	C-5, C-7	H-4 (7.81)
7	34.6 (t)	2.89 (2H, t, *J*=7.2 Hz)	C-5, C-8, C-9	H-8 (2.46)
8	32.3 (t)	2.46 (2H, q, *J*=7.6 Hz)	C-5, C-7, C-9	H-7 (2.89)
9	136.2 (d)	5.84 (1H, m)	C-7, C-8	H-10 (5.02)
10	116.4 (t)	5.02 (2H, dd, *J*=7.6 Hz)	C-9	H-9 (5.84)
11	165.0 (s)	-	-	-



Fusaric acid



9,10-dehydrofusaric acid

Figure 4.27 HMBC of mixture BE1

Mixture BE1 showed spectral data identical to that of fusaric acid and 9,10-dehydrofusaric acid, which was reported in the literature (Abraham and Hassen, 1992). The ^{1}H-NMR and ^{13}C-NMR signal of mixture BE1, fusaric acid and 9,10-dehydrofusaric acid are presented in Table 4.12 and 4.13.

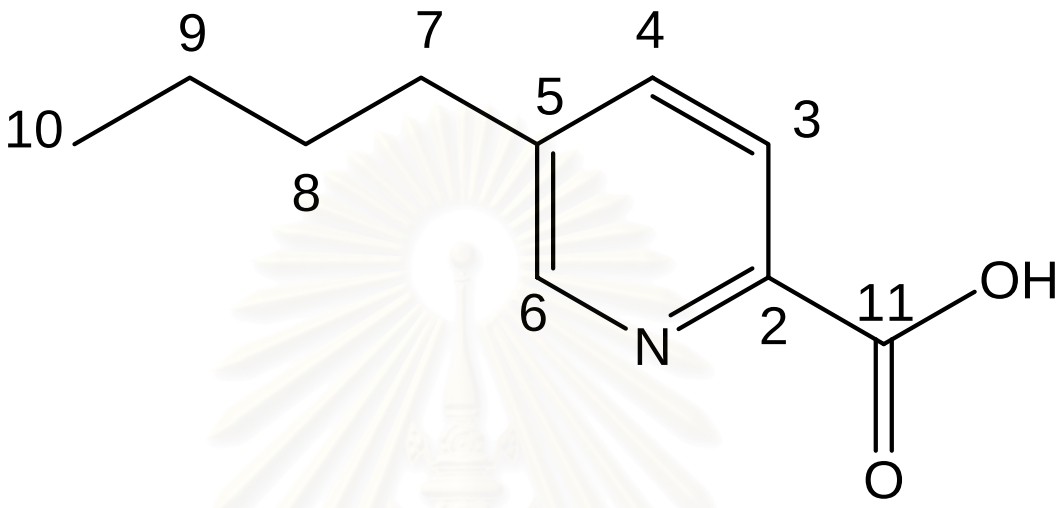
Table 4.12 ^{1}H-NMR spectral data of mixture BE1, fusaric acid and 9,10-dehydrofusaric acid in $CDCl_3$

Mixture BE1	Fusaric acid	9,10-dehydrofusaric acid
8.21 (1H, d, *J* =7.6 Hz)	8.19 (d, *J* = 5 Hz) (H-3)	8.19 (d, *J* =5 Hz) (H-3)
8.60 (1H, s)	8.65 (s br) (H-6)	8.65 (s br) (H-6)
7.81 (1H, d, *J* =7.6 Hz)	7.80 (d br, *J* = 5 Hz) (H-4)	7.80 (d br, *J* =5 Hz) (H-4)
5.84 (1H, m)	-	5.82 (m, *J* = 8 Hz) (H-9)
5.02 (2H,dd, *J* =7.6, 15.0 Hz)	-	5.02 (m) (H-10)
2.89 (2H, t, *J* =7.2 Hz)	-	2.85 (dt, *J* = 8 Hz) (H-7)
2.77 (2H, t, *J* =7.6 Hz)	2.74 (t, *J* = 8 Hz) (H-7)	-
2.46 (2H, q, *J* =7.6 Hz)	-	2.44 (t, *J* = 8 Hz) (H-8)
1.69 (2H, dt, *J* =7.6, 15.2 Hz)	1.66 (tt, *J* = 8 Hz) (H-8)	-
1.41(2H, dq, *J* =7.2, 14.8 Hz)	1.37 (tq, *J* = 9 Hz) (H-9)	-
0.98 (3H, t, *J* =7.2 Hz)	0.94 (t, *J* = 7 Hz) (H-10)	

Table 4.13 ^{13}C-NMR spectral data of mixture BE1, fusaric acid and 9,10-dehydrofusaric acid in $CDCl_3$

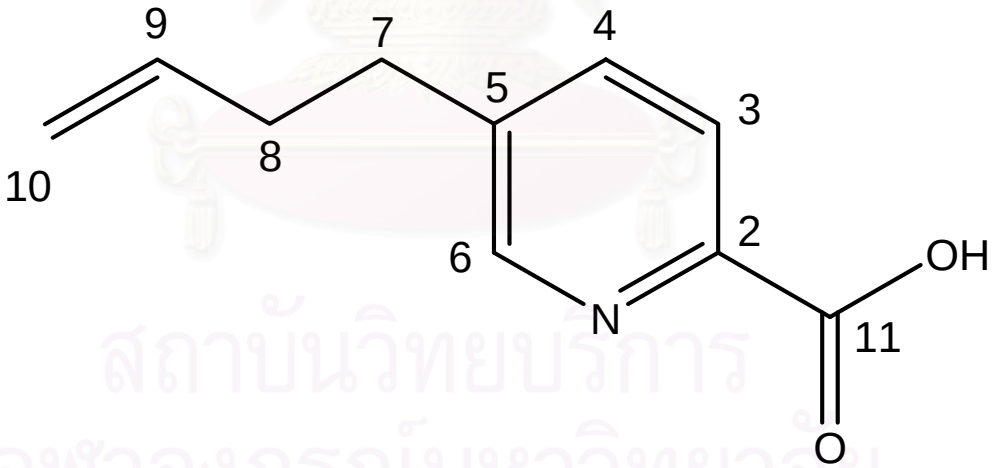
Mixture BE1	Fusaric acid	9,10-dehydrofusaric acid
165.0 (s)	165.3 (s) (C-11)	165.3 (s) (C-11)
147.8 (d)	148.1 (d) (C-6)	-
147.6 (d)	-	148.3 (d) (C-6)
144.8 (s)	144.8 (s) (C-6)	-
144.6 (s)	-	145.0 (s) (C-2)
143.2(s)	142.0 (s) (C-5)	-
142.1(s)	-	141.9 (s) (C-5)
138.7 (d)	138.1 (d) (C-4)	-
138.6 (d)	-	138.2 (d) (C-4)
136.2 (d)	-	136.3 (d) (C-9)
124.3 (d)	124.7 (d) (C-3)	124.7 (d) (C-3)
116.4 (t)	-	116.2 (t) (C-10)
32.7 (t)	32.2 (t) (C-7)	-
34.6 (t)	-	34.4 (t) (C-7)
32.8 (t)	32.7 (t) (C-8)	-
32.3 (t)	-	32.8 (t) (C-8)
22.2 (t)	22.1 (t) (C-9)	-
13.7 (q)	13.6 (q) (C-10)	-

From all of the data, it could be concluded that mixture BE1 was a mixture of fusaric acid and 9,10-dehydrofusaric acid. The structure of this mixture is presented in Figure 4.28.



Fusaric acid

(5-butylpicolinic acid)



9,10-dehydrofusaric acid

Figure 4.28 Structure of mixture 1

4.7.2 Structure elucidation of compound BE2

Compound BE2 was the orange crystal, m.p. 122°C. The structure of compound was elucidated by using spectroscopic techniques.

The IR spectra of compound BE2 is shown in Appendix C Figure 11 and the absorption peaks were assigned as Table 4.14. Its IR spectrum indicated important absorption band at 3300-2500 cm^{-1} (O-H stretching vibration) and 1701 cm^{-1} (C=O stretching in carbonyl compound).

Table 4.14 The IR absorption band assignment of compound BE2

Wave number (cm^{-1})	Intensity	Assignment
3300-2500	Weak	O-H stretching vibration
1701	Strong	C=O stretching in carbonyl compound

The ^{1}H-NMR spectrums (Figure 12 in Appendix C) of compound BE2 possessed one aliphatic methylene proton at 2.45 ppm, one benzylic proton at 2.87 ppm, two vinylic protons at 5.02 and 5.79 ppm and three aromatic protons at 7.82, 8.22 and 8.68 ppm.

The ^{13}C-NMR spectrum (Figure 13 in Appendix C) of compound BE2 showed 10 signals, which the carbonyl group of carboxylic acid corresponded to the signal ,165.7 ppm.

The MALDI-TOF mass spectra (Figure 18 Appendix C) showed the $[M^{+}]$ ion peak at *m/z* 177 indicated a compound with an odd number of nitrogen atoms. It is assumed that this mixture contains carbons, protons, oxygen, and nitrogen, and then the molecular formula is $C_{10}H_{11}O_2N$.

The information from 2D-NMR techniques, including HSQC (Table 4.15, Figure 14 in Appendix C), HMBC (Table 4.15, Figure 15 in Appexdix C), and NOESY (Figure 4.29, Figure 17 in Appendix C) were used to assist the interpretation of compound BE2 structure.

Table 4.15 HSQC, HMBC and NOESY spectral data of compound BE2

Position	δ_C	δ_H	HMBC (H to C)	NOESY
1	-	-	-	-
2	145.0 (s)	-	-	-
3	124.5 (d)	8.22 (d, *J*=8 Hz)	C-5, C-11, C-2	H-4 (7.82)
4	138.8 (d)	7.82 (d, *J*= 8 Hz)	C-6, C-2, C-7	H-3 (8.22), H-7 (2.45)
5	142.1 (s)	-	-	-
6	147.8 (d)	8.68 (s)	C-2,C-5, C-7 C-4	H-8 (2.87)
7	34.6 (t)	2.45 (dt, *J*= 8 Hz)	C-6, C-5, C-8, C-9, C-4	H-8 (2.87), H-4 (7.82), H-6 (8.68)
8	32.3 (t)	2.87 (t, *J*=7.6 Hz)	C-5, C-10, C-7, C-9	H-6 (8.68)
9	136.3 (d)	5.79 (m)	C-10, C-7, C-8	H-10 (5.02)
10	116.4 (t)	5.02 (m)	C-8, C-9	H-9 (5.79)
11	165.3 (s)	-	-	-

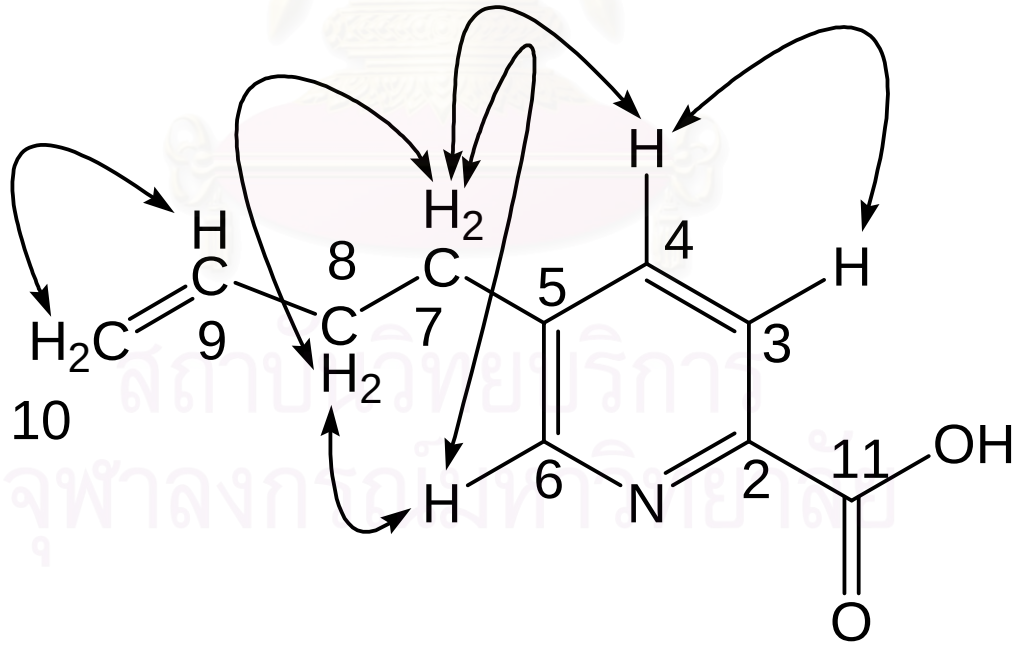


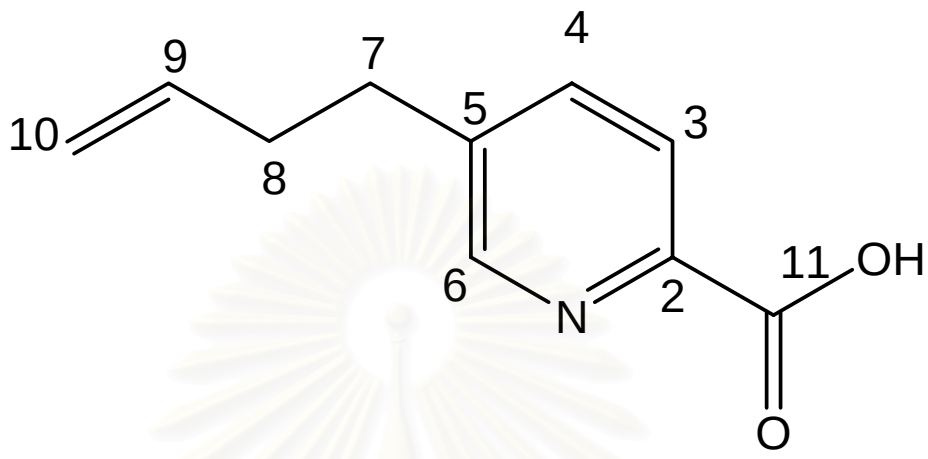
Figure 4.29 NOESY correlation of compound BE2

Compound BE2 showed spectral data identical to that of 9,10-dehydrofusaric acid, which was reported in the literature (Abraham and Hassen, 1992). The ^{1}H-NMR and ^{13}C-NMR signal of compound BE2 and 9,10-dehydrofusaric acid are presented in Table 4.16.

Table 4.16 ^{1}H-NMR and ^{13}C-NMR spectral data of compound and 9,10-dehydrofusaric acid in $CDCl_3$

Position	^{1}H-NMR chemical shifts (ppm)		^{13}C-NMR chemical shifts (ppm)	
	Compound BE2	9,10-dehydrofusaric acid	Compound BE2	9,10-dehydrofusaric acid
1	-	-	-	-
2	-	-	145.0 (s)	145.0 (s)
3	8.20 (d, *J* = 5 Hz)	8.19 (d, *J*=5 Hz)	124.7 (d)	124.7 (d)
4	7.80 (d, *J* = 5 Hz)	7.8 (d br)	138.8 (d)	138.2 (d)
5	-	-	142.0 (s)	141.9 (s)
6	8.68 (s)	8.65 (s br)	148.1 (d)	148.3 (d)
7	2.90 (t, *J* = 8 Hz)	2.85 (t, *J* =8 Hz)	31.7 (t)	32.3 (t)
8	2.48 (dt, *J* = 8 Hz)	2.44 (dt, *J* = 8 Hz)	34.4 (t)	32.8 (t)
9	5.90 (m)	5.82 (m, *J* = 7 Hz)	136.7 (d)	136.3 (d)
10	5.0 (d, *J* = 8 Hz)	5.02 (m)	115.1 (t)	116.2 (t)
11	-	-	165.7 (s)	165.3 (s)

From all of the data, it could be concluded that compound BE2 was 9,10-dehydrofusaric acid. The structure is presented in Figure 4.30.



9,10-dehydrofusaric acid

Figure 4.30 Structure of compound BE2

4.8.3 Structure elucidation of compound BE3

Compound BE3 was the dark yellow solid, m.p. 213-214°C. The structure of compound was elucidated by using spectroscopic techniques.

The IR spectrum of compound BE3 is shown in Appendix C Figure 20 and the absorption peaks were assigned as Table 4.17. Its IR spectrum indicated important absorption band at 3300-3600 cm^{-1} (O-H stretching vibration), 2948 cm^{-1} and 2836 cm^{-1} (C-H stretching vibration), 1651 cm^{-1} (C=C stretching vibration), and 1025 cm^{-1} (C-O stretching vibration).

Table 4.17 The IR absorption band assignment of compound BE3

Wave number (cm^{-1})	Intensity	Assignment
3300-3600	Broad	O-H stretching vibration
2948, 2836	Strong	C-H stretching vibration
1651	Medium	C=C stretching vibration
1025	Strong	C-O stretching vibration

The ^{1}H-NMR spectrums (Figure 21 in Appendix C) of compound BE3 possessed one methyl proton at 2.76 ppm, two methoxy protons at 4.06 ppm, one hydroxyl at 13.27 ppm and three aromatic protons at 6.89, 7.87 and 9.45 ppm.

The ^{13}C-NMR spectrum (Figure 22 in Appendix C) of compound BE3 showed 16 signals, which the carbonyl groups corresponded to the signal, 189.2 and 179.5 ppm.

The EI-MS mass spectrum (Figure 25 in Appendix C) showed the $[M]^{+}$ ion peak at *m/z* 299 indicated a compound with an odd number of nitrogen atoms. It is assumed that this mixture contains carbons, protons, oxygen, and nitrogen, and then the molecular formula is $C_{16}H_{13}O_5N$.

The information from 2D-NMR techniques, including HSQC (Table 4.18, Figure 23 in Appendix C), HMBC (Table 4.18, Figure 4.31 and Figure 24 in Appendix C), were used to assist the interpretation of compound BE3 structure.

Table 4.18 HSQC and HMBC spectral data of compound BE3

Position	δ_C	δ_H	HMBC (H to C)
1	150.0 (d)	9.45 (1H, s)	C-9, C-3, C-13, C-14
2	-	-	-
3	164.1 (s)	-	-
4	117.1 (s)	7.87 (1H, s)	C-10, C-3, C-13, C-15
5	149.2 (d)	-	-
6	155.5 (s)	-	-
7	104.1 (s)	6.89 (1H, s)	C-8, C-6, C-5, C-12
8	156.3 (s)	-	-
9	179.5 (s)	-	-
10	189.2 (s)	-	-
11	115.6 (s)	-	-
12	111.0 (s)	-	-
13	125.7 (s)	-	-
14	137.7 (s)	-	-
15	25.1 (s)	2.76 (s)	C-3, C-4
6-OCH_3	56.5 (q)	4.06 (3H, s)	C-6
8-OCH_3	57.2 (q)	4.06 (3H, s)	C-8
OH	-	13.20 (s)	C-6, C-5, C-11

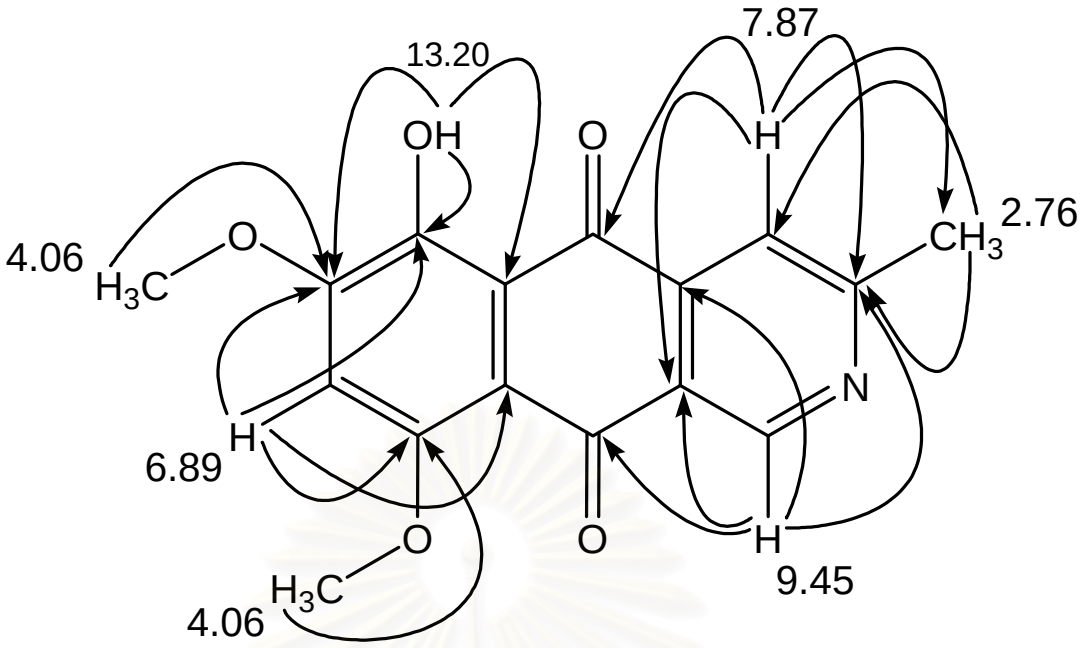


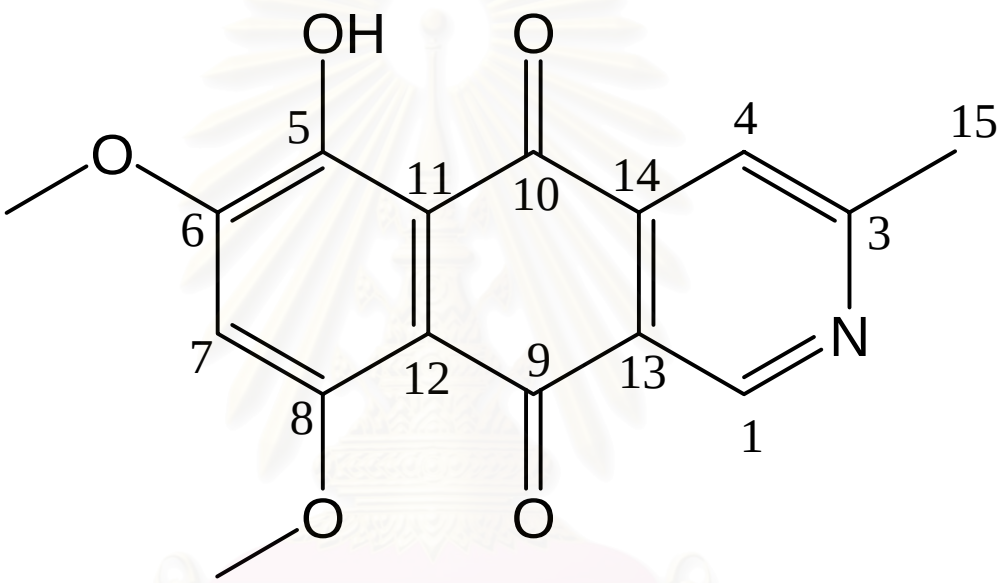
Figure 4.31 HMBC correlations of compound BE3

Compound BE3 showed spectral data identical to that of 8-*O*-methylbostrycoidin, which is reported in the literature (Steyn and Wessels, 1979). The ^{1}H-NMR and ^{13}C-NMR signals of compound BE3 and 8-*O*-methylbostrycoidin are presented in Table 4.19.

Table 4.19 ^{1}H-NMR and ^{13}C-NMR spectral data of compound BE3 and 8-*O*-methylbostrycoidin in $CDCl_3$

Position	^{1}H-NMR chemical shifts (ppm)		^{13}C-NMR chemical shifts (ppm)	
	Compound BE3	8-*O*-methylbostrycoidin	Compound BE3	8-*O*-methylbostrycoidin
1	9.45 (s)	9.44 (s)	150.0 (d)	149.6 (d)
2	-	-	-	-
3	-	-	164.1 (s)	163.9 (s)
4	7.87 (s)	7.85 (s)	117.1 (d)	116.9 (d)
5	-	-	149.2 (d)	148.9 (s)
6	-	-	155.5 (s)	155.1 (s)
7	6.89 (s)	6.86 (s)	104.1 (s)	104.2 (d)
8	-	-	156.3 (s)	156.0 (s)
9	-	-	179.5 (s)	179.0 (s)
10	-	-	189.2 (s)	188.7 (s)
11	-	-	115.6 (s)	115.4 (s)
12	-	-	111.0 (s)	110.9 (s)
13	-	-	125.7 (s)	125.5 (s)
14	-	-	137.7 (s)	137.4 (s)
15	2.76 (s)	2.76 (s)	25.1 (q)	25.1 (q)
6-OCH_3	4.06 (s)	4.05 (s)	56.5 (q)	56.4 (q)
8-OCH_3	4.06 (s)	4.05 (s)	57.2 (q)	57.0 (q)
OH	13.20 (s)	13.19 (s)	-	-

After elucidation of compound BE3 by 2D NMR technique, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of compound BE3 and 8-*O*-methylbostrycoidin were compared signal by signal. This result indicated that the structure of compound BE3 is identical to 8-*O*-methylbostrycoidin. Thus, it could be concluded that compound BE3 was 8-*O*-methylbostrycoidin. The structure is presented in Figure 4.32.



8-*O*-methylbostrycoidin

Figure 4.32 Structure of compound BE3

4.7.4 Structure elucidation of mixture MH1

Mixture MH1 was a light yellow viscous liquid. The structure of mixture MH1 was elucidated by using spectroscopic techniques.

The IR spectra of mixture MH1 is shown in Appendix C Figure 29 and the absorption peaks were assigned as Table 4.20. Its IR spectrum indicated important absorption band at 3450 cm^{-1} (O-H stretching vibration of hydroxyl group), 2927 and 2850 cm^{-1} (C-H stretching vibration), 1456 cm^{-1} (C-H bending vibration), and 721 cm^{-1}(C-H rocking mode of-$(CH_2)_n$-).

Table 4.20 The IR absorption band assignment of mixture BH1

Wave number (cm^{-1})	Intensity	Assignment
3600-3200	Broad	-OH stretching vibration of hydroxyl group
2927, 2850	Strong	C-H stretching vibration
1456	Medium	C-H bending vibration
721	Weak	C-H rocking mode of-$(CH_2)_n$-

The ^{1}H-NMR spectrums (Figure 27 in Appendix C) of mixture BE1 possessed a methyl proton at 0.93 ppm, a methylene proton attached to a carbonyl proton (-CH_2-COOH) at 2.38 ppm and four olefinic protons at 4.18, 4.25, 5.28 and 5.4.

The ^{13}C-NMR spectrum (Figure 28 in Appendix C) of mixture MH1 indicated the NMR spectroscopic pattern of long chain carboxylic acid: one methyl carbon signal at 14.1 ppm, eighteen methylene carbon signals at 34.2, 34.1, 31.9, 29.8, 29.73, 29.69, 29.65, 29.51, 29.4, 29.35, 29.3, 29.2, 29.1, 27.2, 27.19, 24.9, 22.7 and 14.1 ppm. Two olefinic carbon signals at 130.0 and 129.7 ppm, and the carbon signal at 173.2 and 172.8 ppm should be the carbonyl group of carboxylic acid.

The structure of mixture 1 was established on base of spectroscopic analysis and based on retention time of standard methyl ester of fatty acid by gas chromatography (GC). The relative percentages of fatty acids were determined by the area of the peaks in the chromatograms. Chromatogram of mixture MH1 was shown in Figure 26, appendix C. The result of comparison their spectral data with those of published values was shown in Table 4.21 and 4.22.

Table 4.21 GC Retention time of mixture MH1 and standard methyl esters (Std.-ME) of those fatty acids.

Fatty acid	Retention time (min)	
	Std.-ME	Mixture MH1
C14:0	5.697	5.314
C16:0	10.295	9.653
C16:1	11.292	11.330
C18:0	18.999	17.996
C18:1	21.558	20.470
C18:2	27.005	25.488
C18:3	34.258	33.587

Table 4.22 The chemical structure and the name of fatty acids composition of mixture MH1

Systematic name	Common name	Symbol	Structure	Relative fatty acids composition (%) of total fatty acids	Total weight of fatty acid (mg)
SATURATED					
Tetradecanoic acid	myristic acid	C14:0	$CH_3(CH_2)_{12}COOH$	0.87	33.9
Hexadecanoic acid	palmitic acid				
Octadecanoic acid	stearic acid	C16:0	$CH_3(CH_2)_{14}COOH$	33.08	1290.0
		C18:0	$CH_3(CH_2)_{18}COOH$	15.12	590.0
UNSATURATED					
9-hexadecenoic acid	palmitoleic acid	C16:1	$CH_3(CH_2)_5CH=CH-(CH_2)_7COOH$	0.65	25.3
9-octadecenoic acid	oleic acid	C18:1	$CH_3(CH_2)_7CH=CH-(CH_2)_7-COOH$	45.4	176.9
9,12-octadece-noic acid	linoleic acid	C18:2	$CH_3(CH_2)_4(CH=CHCH_2)_2-(CH_2)_6COOH$	2.88	112.3
9,12,15-octadeca dienonic acid	α-linoleic acid	α-C18:3	$CH_3(CH_2)_7[(CH=CH(CH_2)]_3-COOH$	1.70	66.3

From all data, mixture MH1 should be diglyceride which composed of three saturated fatty acids, including myristic acid, palmitic acid, and stearic acid and three unsaturated fatty acids which were palmitoleic acid, oleic acid, and linoleic acid. The major components of saturated and unsaturated fatty acid in mixture MH1 were palmitic acid and oleic acid, respectively.

4.7.5 Structure elucidation of compound MH2

Compound MH2 was the white amorphous solid. The structure of compound was elucidated by using spectroscopic techniques.

The ^{1}H-NMR spectrums (Figure 30 in Appendix C) of compound MH2 possessed 0.53, 0.78, 0.79, 0.83, 0.95 , 1.01 , 1.48, 1.18, 1.69, 1.50, 2.07, 1.39, 1.46, 1.22, 4.00, 3.55, 5.30, 1.91, 1.81, 1.23, 1.97, 5.12, 5.12, 1.78, and 1.48 ppm.

The ^{13}C-NMR spectrum (Figure 31 in Appendix C) of compound MH2 showed 28 signals.

The EI-MS mass spectrum (Figure 36 in Appendix C) showed the $[M]^{+}$ ion peak at *m/z* 412. It is assumed that this mixture contains carbons, protons, and oxygen and then the molecular formula is $C_{28}H_{44}O_{2}$.

The information from 2D-NMR techniques, including HSQC (Table 4.26, Figure 32 in Appendix C), HMBC (Table 4.27, Figure 4.36 and Figure 33 in Appendix C), were used to assist the interpretation of compound MH2 structure.

Table 4.23 HSQC, HMBC and COSY spectral data of compound MH2

Position	δ_C	δ_H	HMBC (H to C)	COSY	NOESY
1	33.1 (d)	1.48 (2H, s)	-	-	H-2
2	29.7 (t)	1.18 (2H, s)	-	-	H-1
3	67.7 (d)	4.0 (1H, m)	-	H-4	-
4	39.4 (d)	1.69 (1H, m), 2.07 (1H, s)	C-10, C-3	H-4, H-3	-
5	76.0 (s)	-	-	-	-
6	73.6 (d)	3.55 (1H, d, *J*=4.8 Hz)	C-7, C-8	H-7	-
7	117.5 (d)	5.28 (1H, d, *J*=5.2 Hz)	C-5, C-14,C-13	H-6	-
8	144.0 (s)	-	-	-	-
9	43.4 (d)	1.91 (1H, s)	C-8	-	-
10	37.1 (s)	-	-	-	-
11	22.1 (d)	1.50 (2H, s)	-	-	-
12	39.2 (t)	2.07 (2H, d)	C-22	H-17	-
13	43.8 (s)	-	-	-	-
14	54.7 (d)	1.84 (1H, m)	C-8	-	-
15	22.8	1.39 (1H, d, *J*=6 Hz), 1.46 (1H, m)	-	-	-
16	27.9 (t)	1.22 (2H, d, *J*=8 Hz)	-	-	-
17	55.9 (d)	1.23 (1H, d, *J*=8 Hz)	-	H-20	-
18	12.3 (d)	0.53 (3H, s)	C-17,C-14,C-13,C-12	-	H-20
19	18.6 (d)	1.01 (3H, s)	C-9,C-10,C-5,C-1	-	-
20	40.4 (d)	1.97 (1H, s)	-	H-17, H-21	H-18, H-21
21	21.1 (q)	0.95 (3H, d, *J*=6.4 Hz)	C-20,C-22,C-17	H-20	H-20
22	135.4 (d)	5.12 (1H, m)	-	-	-
23	132.2 (d)	5.12 (1H, m)	C-20	-	-
24	42.8 (t)	1.78 (1H, d, *J*=6 Hz)	C-22, C-23, C-25	H-28	-
25	33.0 (d)	1.48 (1H, s)	-	-	-
26	19.6 (q)	0.75 (3H, d, *J*=6.8 Hz)	C-24,C-27,C-25	-	-
27	19.97 (q)	0.79 (3H, d, *J*=8 Hz)	C-24,C-25	-	-
28	17.6 (q)	0.83 (3H, d, *J*=6.8 Hz)	C-23, C-24,C-25	H-24	-

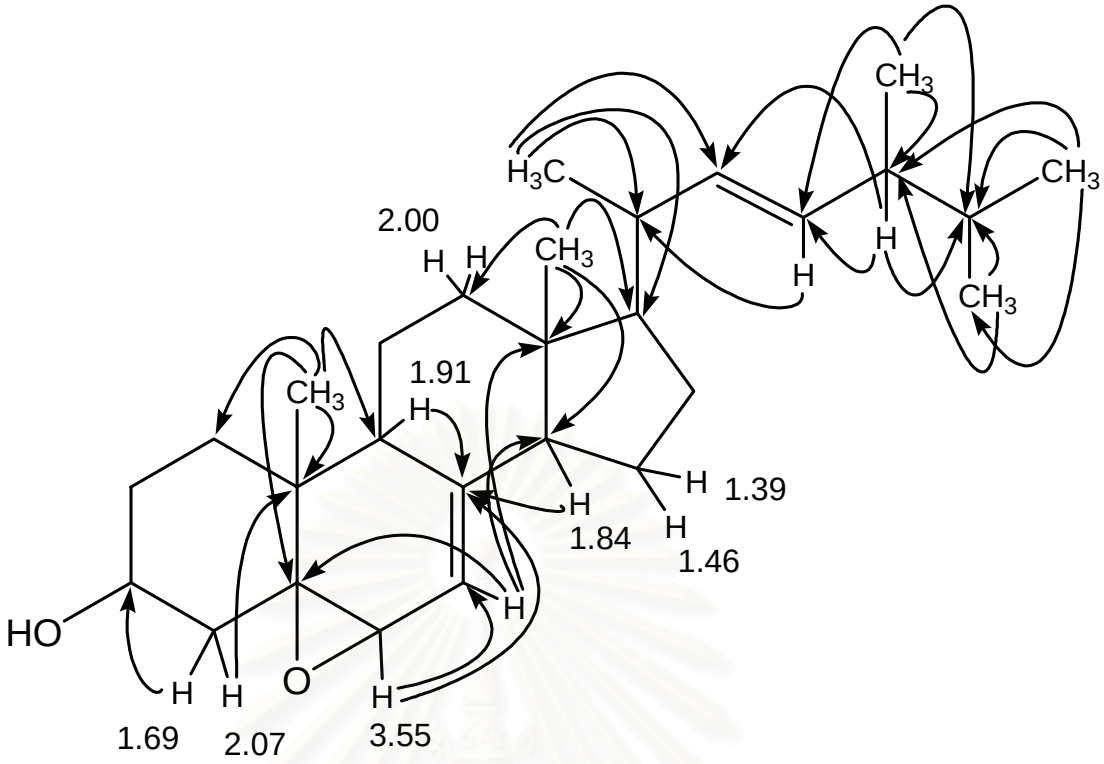


Figure 4.33 HMBC of compound MH2

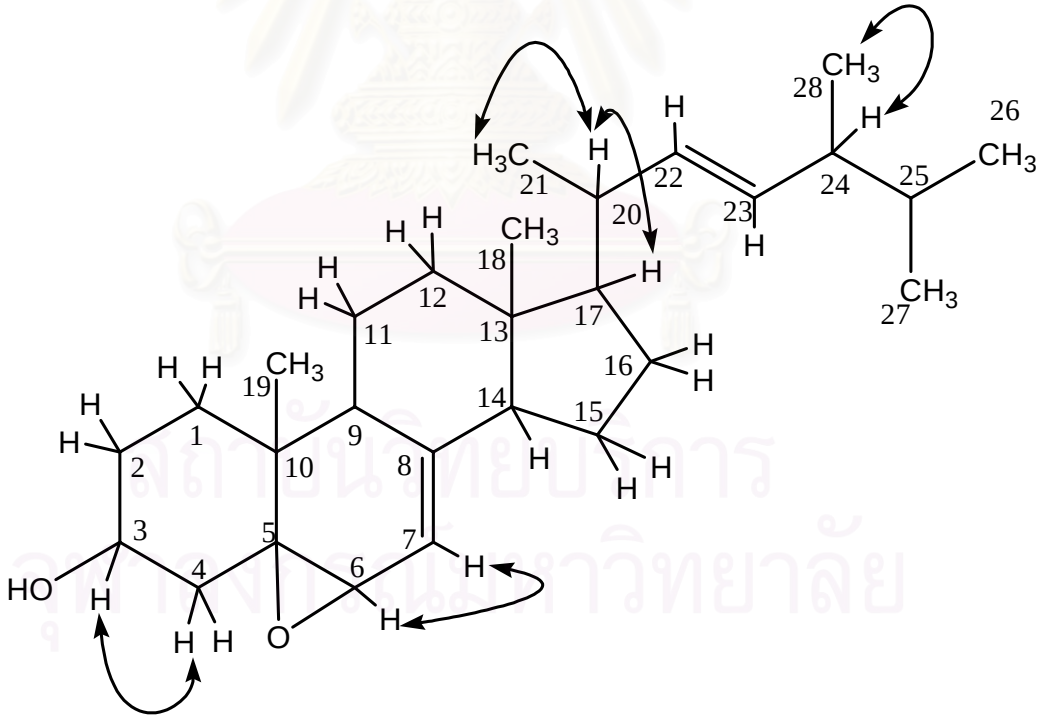


Figure 4.34 COSY of compound MH2

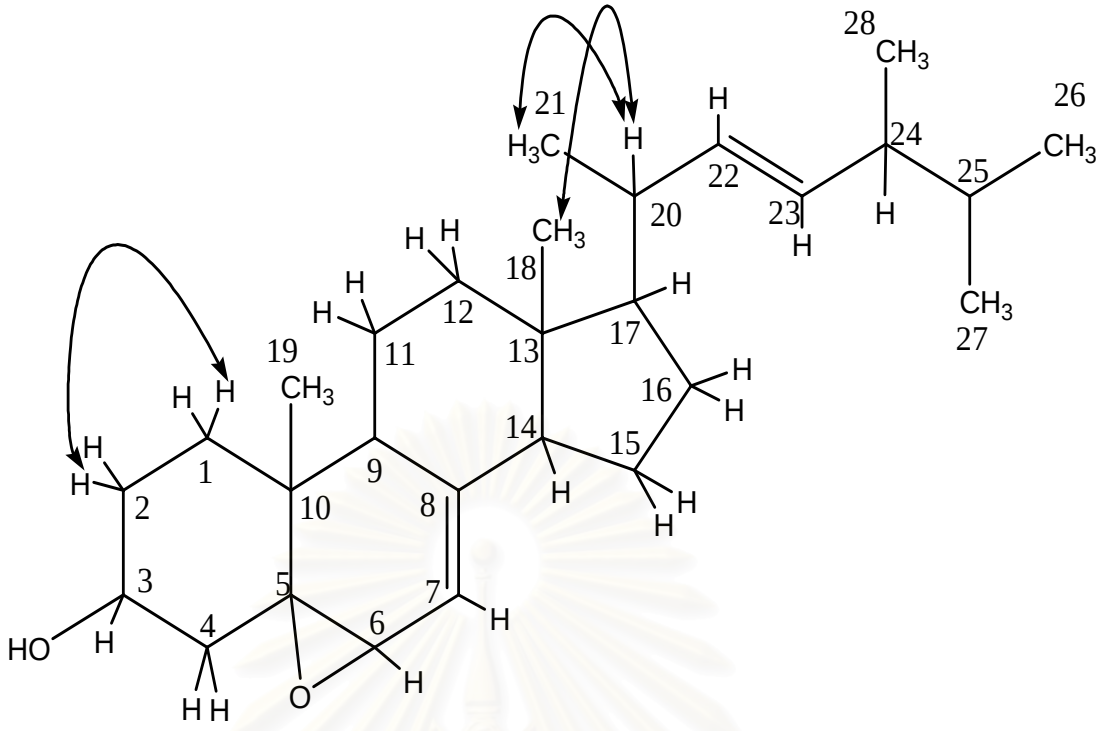


Figure 4.35 NOESY of compound MH2

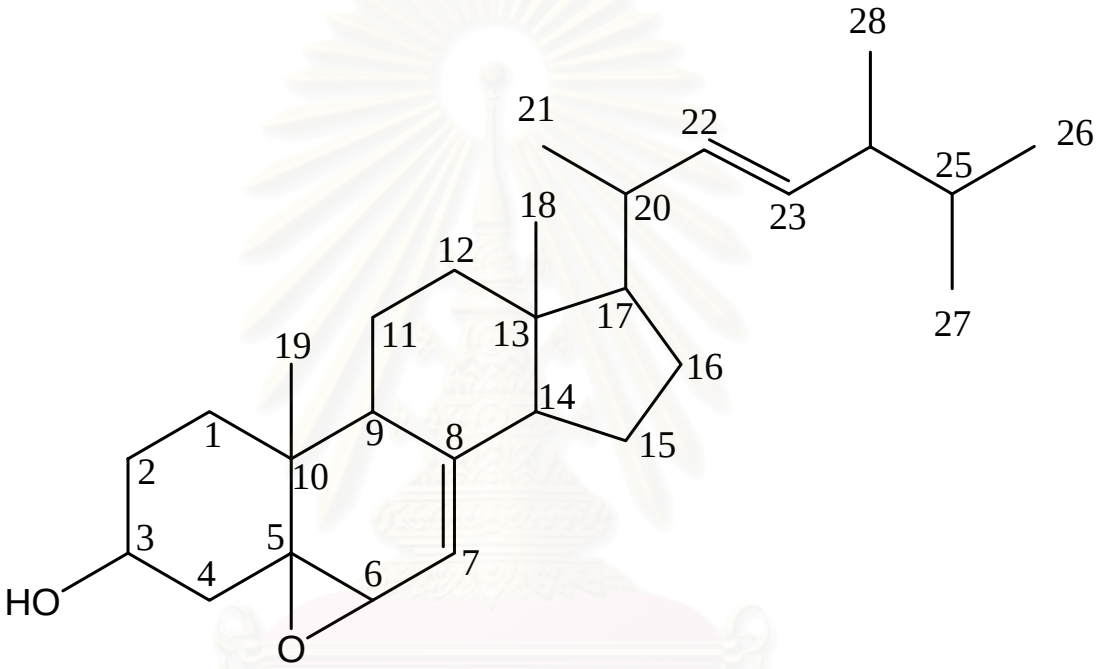
Compound MH2 showed spectral data identical to that of 4α,5-epoxy-7,22(*E*)-ergostadien-3β -ol which was reported in the literature (Goldstein, 1996). The ^{1}H-NMR signal of compound MH2 and 4α,5-epoxy-7,22(*E*)- ergostadien-3β-ol are presented in Table 4.24.

Table 4.24 ^{1}H-NMR spectral data of compound MH2 and 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol in $CDCl_3$

Position	^{1}H -NMR chemical shifts (ppm)	
	Compound MH2	4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol (500 Hz)
1	1.48 (2H, s)	*
2	1.18 (2H, s)	*
3	4.0 (1H, m)	3.88 (1H,m)
4	1.69 (1H, m), 2.07 (1H, s)	2.90 (1H, d, *J* = 4.0 Hz)
5	-	-
6	3.55 (1H, d, *J*=4.8 Hz)	2.68-2.65 (1H, dd)
7	5.28 (1H, d, *J*=5.2 Hz)	5.17-5.09 (1H,m)
8	-	-
9	1.91 (1H, s)	*
10	-	-
11	1.50 (2H, s)	*
12	2.00 (2H, s)	*
13	-	-
14	1.84 (1H, m)	*
15	1.39 (1H, d, *J* = 6 Hz), 1.46 (1H, m)	*
16	1.22 (2H, d, *J* = 8 Hz)	*
17	1.23 (1H, d, *J* = 8 Hz)	*
18	0.53 (3H, s)	0.59 (3H, s)
19	1.01 (3H, s)	1.03 (3H, s)
20	1.97 (1H, s)	*
21	0.95 (3H, d, *J* = 6.4 Hz)	0.95 (3H, d, *J* = 6.9 Hz)
22	5.12 (1H, m)	5.17-5.09 (1H, m)
23	5.12 (1H, m)	5.17-5.09 (1H, m)
24	1.78 (1H, d, *J* = 6 Hz)	*
25	1.48 (1H, s)	*
26	0.75 (3H, d, *J* = 6.8 Hz)	0.76 (3H, t, *J* = 7.1 Hz)
27	0.79 (3H, d, *J* =8 Hz)	0.76 (3H, t, *J* = 7.1 Hz)
28	0.83 (3H, d, *J* = 6.8 Hz)	0.85 (3H, d, *J* = 6.9 Hz)

* data not shown

After elucidation of compound MH2 by 2D NMR technique, the chemical shift on ^{13}C-NMR spectrum of compound MH2 and 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol was compared signal by signal. This result indicated that the structure of compound MH2 is identical to 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol. Thus, it could be concluded that compound MH2 was 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol. The structure is presented in Figure 4.39.



4α,5-epoxy-7,22(*E*)-ergostadien-3β -ol

Figure 4.36 Structure of compound MH2

4.7.6 Structure elucidation of compound M1

Compound M1 was the dark red crystal, m.p. 213-214°C. The structure of compound was elucidated by using spectroscopic techniques.

The IR spectrum of compound M1 is shown in Appendix C Figure 38 and the absorption peaks were assigned as Table 4.29. Its IR spectrum indicated important absorption band at 1663 and 1610 cm^{-1} (C=C stretching vibration in aromatic compound) and 1249 and 1143 cm^{-1} (C-O stretching vibration).

Table 4.25 The IR absorption band assignment of compound M1

Wave number (cm^{-1})	Intensity	Assignment
1600-1500	Medium	C=C stretching vibration in aromatic compound
1280-1000	Medium	C-O stretching vibration

The ^{1}H-NMR spectrums (Figure 39 in Appendix C) of compound M1 possessed a methyl proton at 2.79 ppm, two methoxy protons at 3.86 and 3.89 ppm, two hydroxyl protons at 12.7 and 14.25 ppm and three aromatic protons at 6.28, 6.73 and 6.86 ppm.

The ^{13}C-NMR spectrum (Figure 40 in Appendix C) of compound M1 showed 20 signals, which the carbonyl groups corresponded to the signal, 187.0 and 181.0 ppm, one methyl carbon at 23.5 ppm, two methoxy carbon at 56.0 and 56.8 ppm, three methine carbon at 117.8, 112.4 and 98.8 ppm.

The EI-MS mass spectrum (Figure 45 in Appendix C) showed the $[M]^{+}$ ion peak at *m/z* 382. It is assumed that this mixture contains carbons, protons, and oxygen and then the molecular formula is $C_{20}H_{14}O_{8}$.

The information from 2D-NMR techniques, including HSQC (Table 4.26, Figure 41 in Appendix C), HMBC (Table 4.26, Figure 4.37 and Figure 42 in Appendix C), COSY (Table 4.26, Figure 4.38 and Figure 43 in Appendix C) and NOESY (Figure 4.39, Figure 44 in Appendix B) were used to assist the interpretation of compound M1 structure.

Table 4.26 HSQC, HMBC, COSY and NOESY spectral data of compound M1

Position	δ_C	δ_H	HMBC (H to C)	COSY	NOESY
1	116(s)	-	-	-	-
2	117.8(d)	6.73 (1H, s)	1-CH_3, C-1	H-4, 1-CH_3	1-CH_3
3	164(s)	-	-	-	-
4	98.8(d)	6.86 (1H, s)	C-2, C-4a	H-2	3-OCH_3
5	-	-	-	-	-
6	129.5(s)	-	-	-	-
7	181.0 (s)	-	-	-	-
8	158.8 (s)	-	-	-	-
9	112.4 (d)	6.28 (1H, s)	C-8,C-7,C-11a	-	8-OCH_3
10	187.0 (s)	-	-	-	-
11	154.8 (s)	-	-	-	-
12	187.0 (s)	-	-	-	-
1a	144.0 (s)	-	-	-	-
4a	158.5 (s)	-	-	-	-
5a	156.8 (s)	-	-	-	-
6a	145.9 (s)	-	-	-	-
11a	106.0 (s)	-	-	-	-
12a	117.0 (s)	-	-	-	-
1-CH_3	23.5 (q)	2.79 (3H, s)	C-2	C-2	H-2
3-OCH_3	56.0 (q)	3.86 (3H, s)	C-3	-	H-4
8-OCH_3	56.8 (q)	3.89 (3H, s)	C-8	-	H-9
6-OH	-	12.7 (1H, s)	-	-	-
11-OH	-	14.25 (1H, s)	C-11a, C-12a	-	-

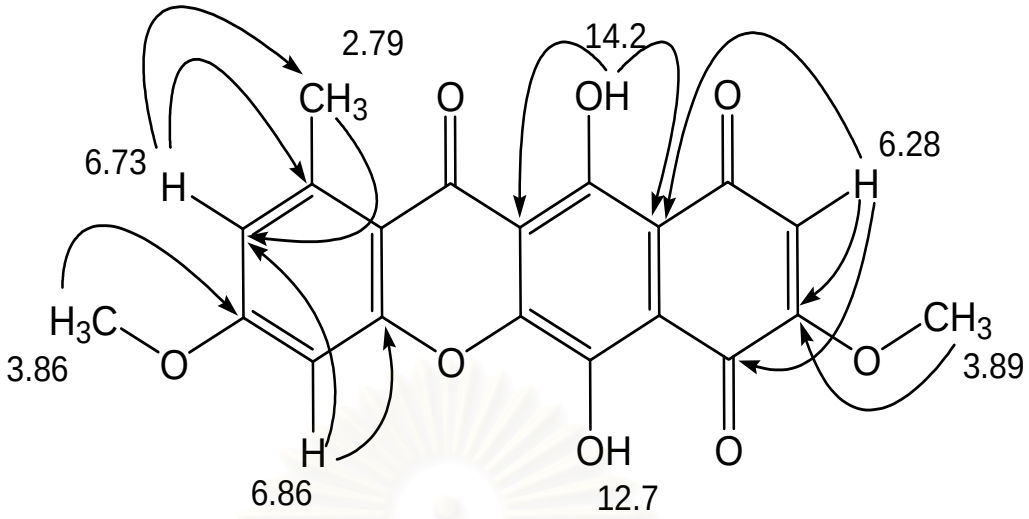


Figure 4.37 HMBC of compound M1

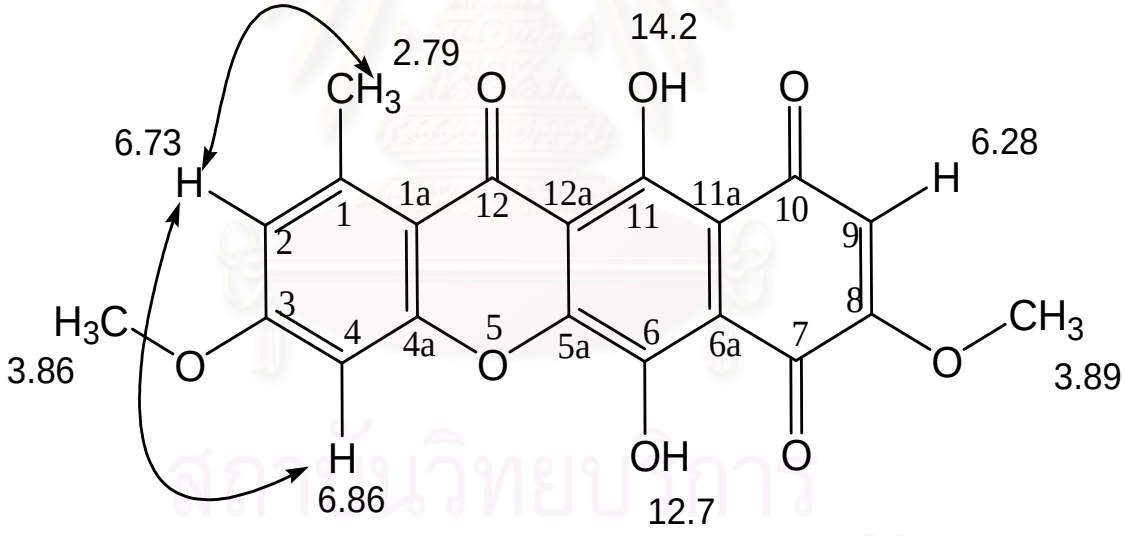


Figure 4.38 COSY of compound M1

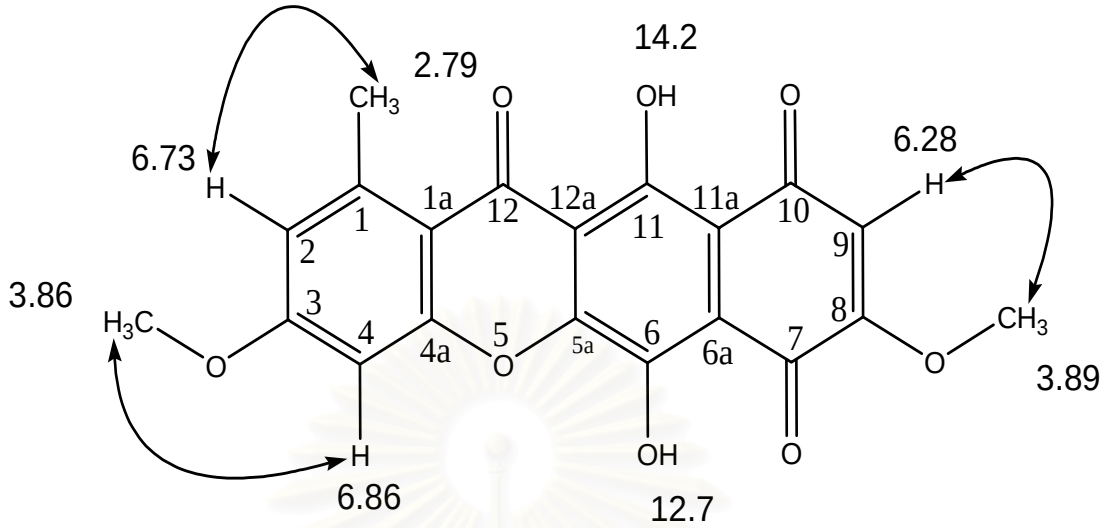


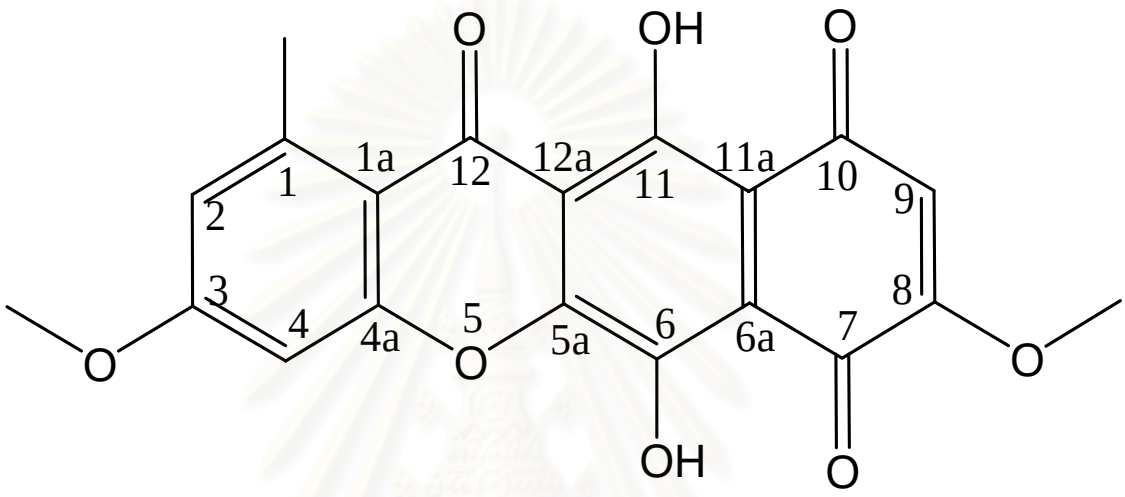
Figure 4.39 NOESY of compound M1

Compound M1 showed spectral data identical to that of bikaverin (7,10-dihydro-6,11-dihydroxy-3,8-dimethoxy-1-methyl-12*H*-benzo[*b*]xanthene-7,10,12-trione) which was reported in the literature (Katagiri, Nakana and Kato, 1981). The ^{1}H-NMR signal of compound M1 and bikaverin are presented in Table 4.27.

Table 4.27 ^{1}H-NMR spectral data of compound M1 and bikaverin in $CDCl_3$

Position	^{1}H-NMR chemical shifts (ppm)	
	Compound M1	Bikaverin
1	-	-
2	6.73 (1H, s)	6.68-6.85 (2H, m)
3	-	-
4	6.86 (1H, s)	6.68-6.85 (2H, m)
5	-	-
6	-	-
7	-	-
8	-	-
9	6.28 (1H, s)	6.28 (1H, s)
10	-	-
11	-	-
12	-	-
1a	-	-
4a	-	-
5a	-	-
6a	-	-
11a	-	-
12a	-	-
1-CH_3	2.79 (3H, s)	2.84 (3H, s)
3-OCH_3	3.86 (3H,s)	3.88 (3H, s)
8-OCH_3	3.89 (3H, s)	3.90 (3H, s)
6-OH	12.7 (1H, s)	12.6 (1H, s)
11-OH	14.25 (1H, s)	14.14 (1H, s)

After elucidation of compound M1 by 2D NMR technique, the chemical shift on ^{1}H-NMR spectrum of compound M1 and bikaverin was compared signal by signal. This result indicated that the structure of compound M1 is identical to bikaverin. Thus, it could be concluded that compound M1 was bikaverin. The structure is presented in Figure 4.40.



Bikaverin

(7,10-dihydro-6,11-dihydroxy-3,8-dimethoxy-1-methyl-12*H*-benzo[*b*]xanthene-7,10,12-trione)

Figure 4.40 Structure of compound M1

4.8 Biological activities

4.8.1 Antimicrobial activity of the culture broth of endophytic fungus isolate KBLM02

The antimicrobial activity of the culture broth of endophytic fungus isolates KBLM02 was evaluated by the agar well diffusion method. Aliquote of 100 μl of culture filtrate was pipetted into the agar wells. The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganism. Results indicated that culture broth was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25932, and *C. albicans* ATCC 10231.

4.8.2 Antimicrobial activity of the crude extracts from endophytic fungus isolate KBLM02

The antimicrobial activity of the crude extracts from endophytic fungus isolate KBLM02 were evaluated by the disc diffusion method. The crude extracts were examined at 0.5mg /disc (6 mm diameter). The antimicrobial activities were calculated from the inhibition zones (mm) of test microorganisms, including the bacterial strains *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and fungi, yeast form strains *C. albicans* ATCC 10231. Antimicrobial activity of each fraction from mycelium crude extract was shown in Table 4.28.

Table 4.28 Antimicrobial activities of the mycelium crude extracts from endophytic fungus isolate KBLM02

Test microorganisms	Inhibition of mycelium crude extracts against tested microorganisms		
	Crude hexane	Crude EtOAc	Crude MeOH
B. subtilis	+	++	+
S. aureus	-	-	+
E. coli	-	++	-
P. aureuginosa	++	+	-
C. albicans	-	-	-

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

+++ = Inhibition zone among 19 –28 mm

++ = Inhibition zone among 8 –18 mm

+ = less than 8 mm

- = No inhibition

4.8.3 Antimicrobial activities of pure compounds

The antimicrobial activities of pure compounds were evaluated by the antimicrobial susceptibility test, broth microdilution method. The pure compound was examined at concentration of 3.95-1,000 μg/ml (two-fold dilution). Antimicrobial activities tests were performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATTC 10231. The lowest concentration of pure compound showing complete inhibition of growth is recorded as the minimal inhibitory concentration (MIC). Antimicrobial activities of pure compounds are shown in Table 4.29.

Table 4.29 Broth microdilution method for antimicrobial activities of pure compounds

Compound	Test microorganisms and MIC (μg/ ml), (μM)				
	Gram positive bacteria		Gram negative bacteria		Yeasts
	B. subtils ATCC6633	*S. aureus* ATCC 25923	*E.coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
Mixture BE1	15.62	31.25	250	-	250
Compound BE2	3.95 (22.3)	-	-	3.95 (22.3)	-
Compound BE3	7.81 (26.1)	15.62 (52.2)	-	-	-
Compound M1	125 (327.2)	7.81 (20.4)	7.81 (20.4)	-	-
Compound MH2	250 (606.8)	-	250 (606.8)	3.95 (9.59)	250 (606.8)
Streptomycin	0	0	1.95 (1.34)	15.62 (10.7)	0
Penicillin G	15.6 (43.8)	15.6 (43.8)	0	0	0
Ketoconazole	0	0	0	0	125 (235.2)

- = inactive

4.8.4 Cytotoxic activity

The *in vitro* activity of pure compounds from fungal isolate KBLM02 was tested against 5 cell lines including, HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) and is reported in Table 4.35.

Table 4.30 Cytotoxic activities against cell line of pure compound from endophytic fungus isolate KBLM02

Compounds	IC_{50} (μg/ml) (μM)				
	HEP-G2 (hepatoma)	SW620 (colon)	CHAGO (lung)	KATO-3 (gestric)	BT474 (breast)
Compound BE3	9.5 (31.8)	>10	>10	9.5 (31.8)	>10
Doxorubicin	0.7(1.21)	0.09(0.15)	0.7(1.21)	>10	>10

There is a need to search for new bioactive agents because health problems, which caused by various cancers and drug-resistant pathogens, are still a global problem. Endophytic fungi are a novel source of efficient medicinal compounds. In this research, endophytic fungi were isolated from one of Thai medicinal plants, *Croton oblongifolius*. Five bioactive compounds were obtained from cultures of endophytic fungal isolate KBLM02, mixture BE1 which was a mixture of fusaric acid and 9,10-dehydrofusaric acid, compound BE2 which was 9,10-dehydrofusaric acid, compound BE3 which was 8-*O*-Methylbodtricoidin, compound MH2 which was 4α,5-epoxy-7,22(*E*)- ergostadien-3β-ol, and compound M1 which was bikaverin.

The pure compounds showed antimicrobial activities. Mixture of fusaric acid and 9,10-dehydrofusaric acid (mixture BE1) exhibited a broad spectrum of activity against test microorganisms and no activity on cytotoxicity against cell lines. 9,10-dehydrofusaric acid (compound BE2) showed activity against some gram positive and gram negative bacteria and no activity on cytotoxicity against cell lines. 8-*O*-Methylbostrycoidin was active against only gram positive bacteria and showed cytotoxic activity on HEP-G2 and KATO-3. 4α,5-epoxy-7,22(*E*)- ergostadien-3β-ol (compound MH2) also exhibited a broad spectrum of activity against test microorganisms. In addition, bikaverin (compound M1) showed activity against gram positive bacteria and some gram negative bacteria.

Fusaric acid (5-butylpicolinic acid) was first discovered during the laboratory culture of *Fusarium hetesporum* by Yubuta et al. (1937). Fusaric acid is a fungal toxin with low to moderate toxicity synthesized by some Fusarium species such as *F. moniliforme* (sexual stage *Gibberella fujikuroi*), *F. crookwellense*, *F. subglutinanas*, *F. sambucinum*, *F. napiforme*, *F. heterosporum*, *F. oxysporum*, *F. solani*, and *F. proliferatum* (Wang and Ng, 1999; Bacon et al., 1996). Fusaric acid is a class of alkaloid natural products with important biological activities (Song and Yee, 2001). The *Fusarium* spp. mycotoxin, fusaric acid was tested for antimicrobial activity against *Ruminococcus albus* and *Methanobrevibacter ruminantium*. The growth of both organisms was inhibited by fusaric acid as low as 15 mg/ml (May, Wu, and Blake, 2000). Furthermore, the *in vitro* activity of fusaric acid exhibited activity against *Mycobacterium leprae* (Wang and Ng, 1999).

8-*O*-Methylbodtrycoidin is the major pigment from cultures of *Fusarium moniliforme* Sheldon. It was tested for cytotoxicity to cultured mammalian cell lines and found that 8-*O*-Methylbodtrycoidin was cytotoxic to rat hepatoma (RH) and Chinese hamster ovary (CHO) fibroblast cells at 5 µg/ml (LC_{50}) and 10 µg/ml (LC_{100}), respectively. (Vesonder, Gasdorf and Peterson, 1993)

Bikaverin is the wine-red pigment in benzoxanthentrione group isolated from *Gibberella fujikuroi* (=*Fusarium moniliforme*). It's a mycotoxin with antiprotozoal and antifungal activities (Balan et al 1970; Cornforth et al 1971; Kjær et al 1971). Besides, it's an uncoupler of oxidative phosphorylation of tumor cells and of isolated rat liver mitochondria and it's also an efficient haemolytic agent (Kovac, Bohmerova and Fuska, 1978).

CHAPTER V

CONCLUSION

Seventy two endophytic fungal isolates were isolated from surface-sterilized leaves of *Croton oblongifolius* collected from Sai Yoke District, Kanchanaburi Province.

The isolated endophytic fungi cultured on six different media, including Potatoes Dextrose Agar (PDA), Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Yeast Extract Sucrose agar (YES), Corn Meal agar (CMA) and Sabouraud's Dextrose Agar (SDA) were determined for their antimicrobial activities by fungal disk dual culture method.

A total of 72 isolates (100%) of endophytic fungi were identified. Twenty two isolates (30.5%) were identified as belonging to typical genera of endophytes such as *Bipolaris* sp., *Fusarium* sp., *Phomopsis* sp., and members of xylariaceae. The remaining 50 isolates (69.5%) of endophytic fungi were mycelia sterilia.

Fungal isolate KBLM02 cultured in Sabouraud's Dextrose agar exhibited antimicrobial activity against a large number of test microorganisms and the highest inhibition zone against *C. albicans*. Fermentation broth of this fungal was active against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. Fungal isolate KBLM02 was identified as *Fusarium verticillioides* and *Gibberella moniliformis* based on morphological features and nucleotide sequencing of ITS region, respectively.

The growth medium had shown the effect on antimicrobial activities of isolated endophytic fungi. MEA showed the highest number of active isolates in every test microorganisms except *B. subtilis* and *C. albicans* which SDA gave the highest activity against them.

With chromatographic and crystallization techniques, crude culture broth and mycelium were isolated by extraction, chromatographic technique and crystallization. Five pure compounds were elucidated by using their physical properties and spectroscopic techniques. Mixture BE1 was a mixture of fusaric acid **1** and 9,10-dehydrofusaric acid, compound BE2 was 9,10-dehydrofusaric acid **2**, compound BE3 was 8-*O*-methylbostrycoidin **3**, compound MH2 was 4α,5-epoxy-7,22(*E*)-ergostadien-3β-ol **4** and compound M1 was bikaverin **5**.

Antimicrobial activities and cytotoxicity of the pure compounds were tested. It was found that Compound **2**, **3**, **4**, **5** and a mixture of **1** and **2** showed activity against *Bacillus subtilis* with MIC 3.95 (22.3), 7.81 (26.1), 250 (606.8), 125 (327.2) and 15.62 μg/ml (μM), respectively. Compound **3**, **5** and a mixture of **1** and **2** showed activity against *Staphylococcus aureus* with MIC 15.62 (52.2), 7.81 (20.4) and 31.25 μg/ml (μM), respectively. Compound **4**, **5** and a mixture of **1** and **2** showed activity against *Escherichia coli* with MIC 250 (606.8), 7.81 (20.4) and 250 μg/ml (μM), respectively. Compound **2** and **4** showed activity against *Pseudomonas aeruginosa* with MIC 3.95 (22.3) and 3.95 (9.59) μg/ml (μM), respectively. Compound **4** and a mixture of **1** and **2** showed activity against *Candida albicans* with MIC 250 (606.8) and 250 μg/ml (μM), respectively. Additionally, compound **3** exhibited cytotoxic activity against HEP-G2 and KATO-3 with IC_{50} 9.5 (31.8) μg/ml (μM).

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APPENDICES

Appendix A

Table 1 Source and biological activities of secondary metabolites of endophytic fungi

NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
1	Peramine	Pyrrolopyrazine alkaloid	*Neotyphodium coenophialum* *N. lolli* *Epichloe festucae* *E. typhina*	Tall fescue, ryegrass	Insect Toxic	Schardl and Phillips,1997
2	Ergobalansine	Ergot alkaloid	*Neotyphodium* spp. *Clavicaeps purpurea*	*Festuca* spp.	Neurotoxic	Powell et al., 1992
3	Ergotamine					
4	Ergosine					
5	β-ergosine					
6	Ergovaline					
7	Ergostine					
8	Ergoptine					
9	β-ergoptine					
10	Ergonine					
11	Ergocristine					
12	α-ergocrptine					

Table 1 (continued)

NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
13	β-ergocryptine	Ergot alkaloid	*Neotyphodium* spp. *Clavicaeps purpurea*	*Festuca* spp.	Neurotoxic	Powell et al., 1992
14	Ergocornine					
15	Ergonovine					
16	Lysergamind					
17	8-hydroxylsergamind					
18	Isolysergamide					
19	Phomopsichalasin	Cyclochalasan	*Phomasis* sp.	*Salix gracilostyla* var. *melanostachys*	Antibacterial and Antifungal	Horn et al., 1995
20	Cryptocin	Tetramic acid	*Cryptosporiopsis* cf. *quercina*	*Tripterygium wilfordii*	Antimycotic	Li et al., 2000
21	Lolitrem N	Indole diterpene alkaloid	*Neotyphodium lolli*	*Lolium perenne*	Neurotoxic	Munday-Finch et al.,1998
22	Lolitriol					
23	Lolicine A					
24	Lolicine B					

Table 1 (continued)

NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
25	Loline	Saturated aminopyrolizidine alkaloids	*Neotyphodium coenophialum* *N. unicinatum*	*Festuca arundinacea* *F. pratensis*	Insecticide	Schardl and Phillips,1997
26	Norloline					
27	*N*-methylloline					
28	*N*-formylnorloline					
29	*N*-acetylnorloline					
30	3β-hydroxyergosta-5-ene	Ergosterol	*Collectotrichum* sp.	*Artemisia annua*	Antifungal	Lu et al., 2000
31	3-oxoergosta-4,6,8(14),22-tetraene					
32	3β,5α-dihydroxy-6β-acetoxyergosta-7,22-diene					
33	3β,5α-dihydroxy-6β-phenylacetoxyergosta-7,22-diene					

Table 1 (continued)

NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
34 35	Heptelidic acid Hydroheptelidic acid	Sesquiterpene	*Phyllostica* sp.	*Abies balsamea*	Toxic to spruce bud worm	Calhoun et al., 1992
36 37	Subglutinol A Subglutinol B	Diterpenes	*Fusarium subglutinans*	*Tripterygium wilfordii*	Immonosuppressive	Lee et al.,1995
38	Taxol	Diterpenes	*Taxomyces andrenea*	*Taxus brevifolia*	Anticancer	Strobel et al., 2003, Stierle and Strobel ,1995
			Stegolerium kukenani	*Stegolepis guianensis*	Anticancer	Strobel et al.,2001
			Aspergillus niger	*Taxus chinensis*		Wang et al., 2001
			Tubercularia sp.	*Taxus mairai*		Strobel et al., 2003 Wang et al., 2000
			Pestalotiopsis microspora	*Taxus wallachina* *Taxodium distichum*		Strobel et al., 2003, Li et al., 1998 Li et al., 1996
			Periconia sp.	*Torreya grandifolia*		Li et al., 1998
			Pestalotiopsis guepinii	*Wollemia nobilis*		Strobel et al., 1997

Table 1 (continued)

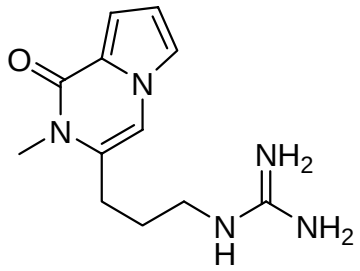
NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
39	Leucinostatin A	Oligopeptide	*Acremonium* sp.	Taxus baccata	Phytotoxic, Anticancer, Antifungal	Strobel et al., 1997
40	Cryptocandin	Cyclopeptide	*Cryptosporiopsis* cf. *quercina*	Red wood	Antifungal	Strobel et al., 1999
41 42	Cytonic acids A Cytonic acid B	Tridepsides	*Cytonaema* sp.	*Quercus* sp.	Antiviral	Guo et al.,2000
43 44	Sequoiatone A Sequoiatone B	Ester	*Aspergillus parasiticus*	Red wood	Antitumor	Stierle et al., 1999
45 46 47	Dicerandrol A Dicerandrol B Dicerandrol C	2,2'-dimeric tetrahydroxanthones	*Phomopsis longicolla*	*Dicerandra frutescens*	Cytotoxic, Antibacterial	Wagenaar and Clardy, 2001

Table 1 (continued)

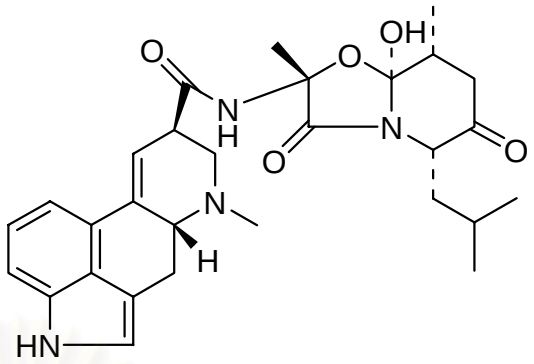
NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
48	Ambuic acid	Cyclohexenone	*Pestalotiopsis microspora*	Taxus spp.	Antifungal	Li et al., 2001
49	CR377	Pentaketide	*Fusarium* sp.	*Selaginella pallescens*	Antifungal	Brandy and Clardy, 2000
50	Colletotric acid	Tridepside	*Collectrichum gloeosporiodes*	*Artemisia mongolica*	Antimicrobial	Zou et al., 2000
51	Pestacin	Isobenzofuran	*Pestalotiopsis microspora*	*Terminalia morobensis*	Antimicrobial, Antioxidant	Harper et al., 2003
52	Isopestacin	Isobenzofuranone	*Pestalotiosis microspora*	*Terminalia morobensis*	Antimicrobial, Antioxidant	Strobel et al., 2002
53	Naphthalene	Benzene	*Muscudor vitigenus*	*Paullina paullinioides*	Insect repellant	Daisy et al., 2002
54	Fusaricide	Pyridine alkaloid	*Fusarium* sp.	*Oxydendron arborcum*	Cytotoxic	Kimberly et al., 1996
55	Torreyanic acid	Quinone dimer	*Pestalotiopsis microspora*	*Torreya taxifolia*	Cytotoxic	Lee et al., 1996
56	Cytosporone D Cytosporone E	Octaketide	*Cytospora sp.* *Diaporthe sp.*		Antimicrobial, Antifungal	Brady et al., 2004

Table 1 (continued)

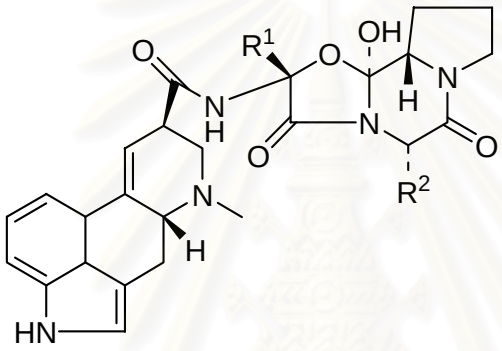
NO.	Compounds	Types	Endophytic fungi	Host plants	Activities	References
57	3-hydroxypropionic acid		*Phomopsis phaseoli* *Melanconium betulinum*	Broaded leaves tree *Betula pendula* *B. pubescens*	Nematocide	Schwarz et al., 2004
58	Spiroquinazoline alkaloids alanditrypinone Alantryphenone Alantrypinene Alantryleunone	Quinazoline alkaloids	*Eupenicillium* spp.	*Murraya paniculata* (Rutaceae)		Barros et al., 2005
59	Asperfumoid Asperfumin		*Aspergillus fumigatus* CY018	*Cynodon dactylon*.	Antifungal	Liu et al., 2004



1. Paramine



2. Ergobalansine



3. Ergotamine

4. Ergosine R^1= Me, R^2 = Bu^i

5. b-ergosine R^1= Me, R^2= *sec* Bu

6. Ergovoline R^1= Me, R^2= Pr^i

7. Ergostine R^1 = Et, R^2 = $PhCH_2$

8. Ergoptine R^1 =Et, R^2 = Bu^i

9. β-ergoptine R^1=Et, R^2 = *sec*-Bu

10. Ergonine R^1= Et, R^2 = Pr^i

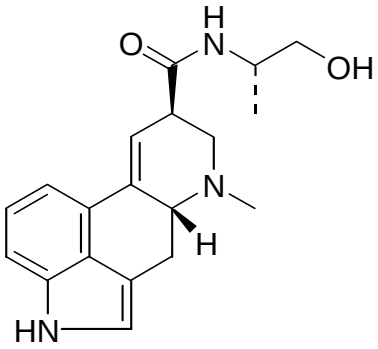
11. Ergocristine R^1=Pr^i, R^2 = $PhCH_2$

12. α-ergocryptine R^1= Pr^i, R^2= Bu^i

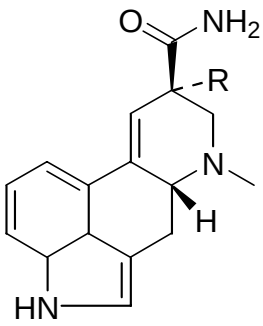
13. β-ergocryptine R^1= Pr^i, R^2= *sec* Bu

14. Ergocornine R^1=R^2=Pr^i

Figure 1 Structure of secondary metabolites of endophytic fungi

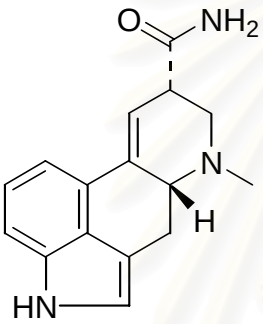


15. Ergonovine

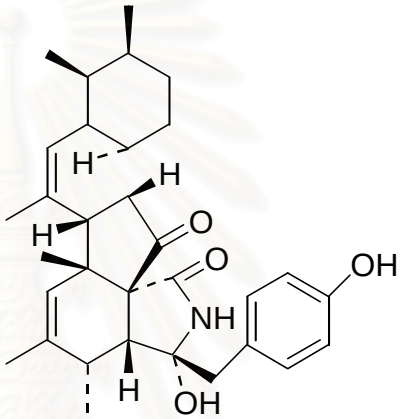


16. Lysergamind R=H

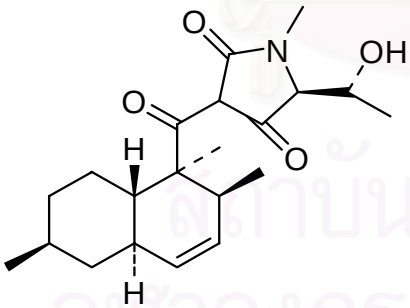
17. 8-hydroxylysergamind R=OH



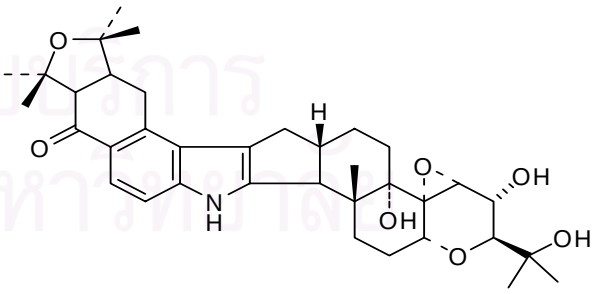
18.Isolysergamind



19. Phomopsichalasin



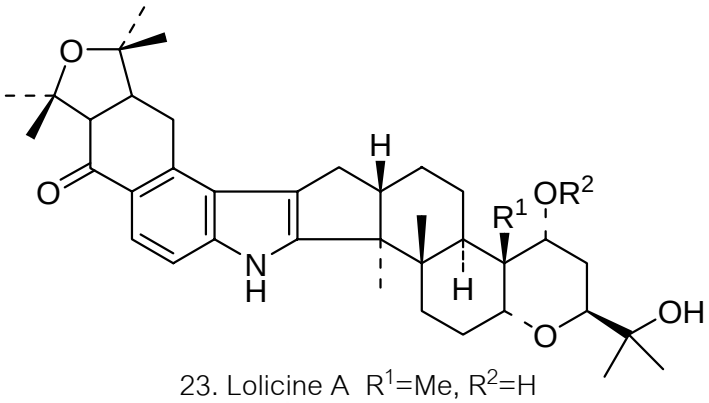
20. Cryptocin



21. Lolitrem N 31 beta,35 alpha

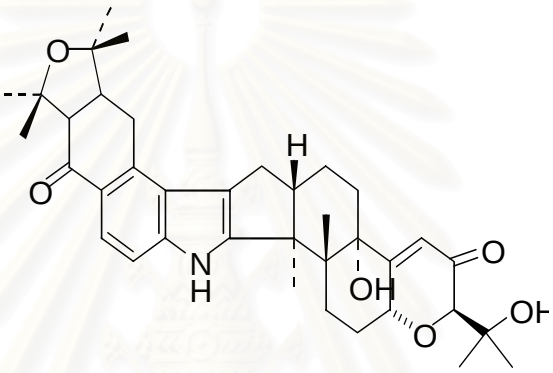
22. Lolitriol 31alpha,35 beta

Figure 1 (continued)

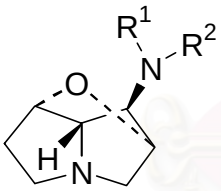


23. Lolicine A R^1=Me, R^2=H

24. Lolicine B R^1 = CHO, R^2 =H



25. Loliline

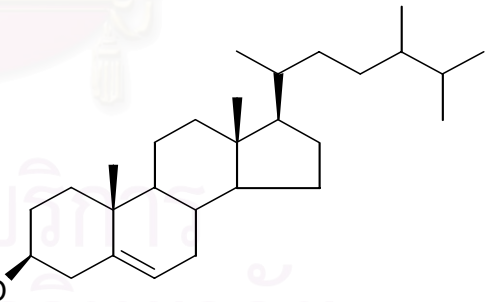


26. Norloline R^1=R^2=H

27. *N*-methylloline R^1=R^2=Me

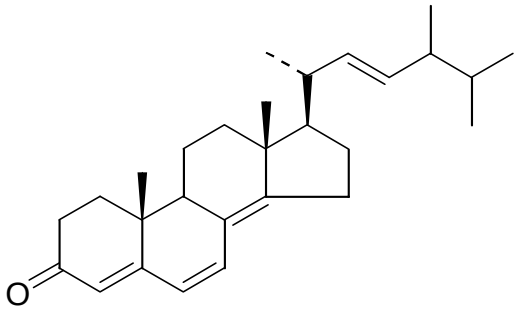
28. *N*-formylnorloline R^1=H, R^2 =CHO

29. *N*-acetylnorloline R^1=H, R^2=Ac

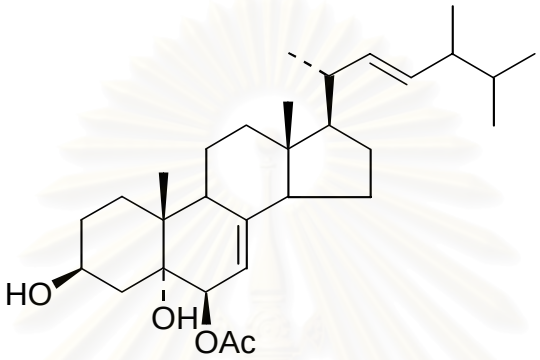


30. 3β-hydroxyergosta-5-ene

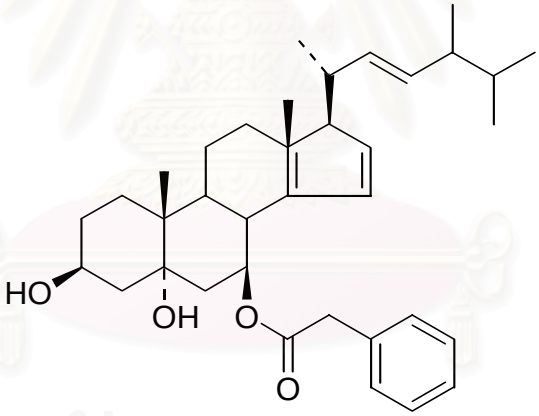
Figure 1 (continued)



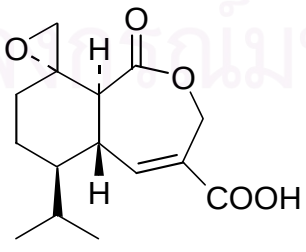
31. 3-oxoergosta-4,6,8(14),22-tetraene



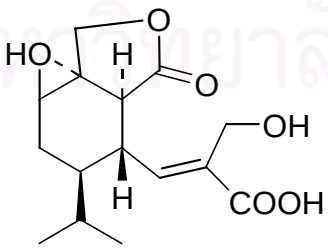
32. 3β,5α-dihydroxy-6β-acetoxyergosta-7,22-diene



33. 3β,5α-dihydroxy-6β-phynylacetoxyergosta-7,22-diene

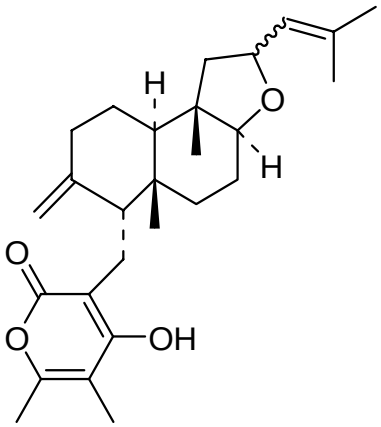


34. Heptelidic acid



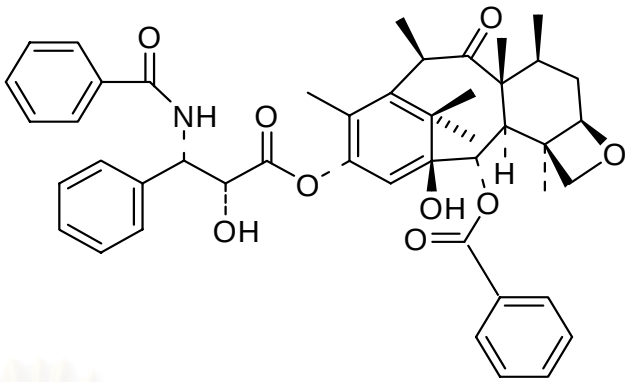
35. hydroheptelidic acid

Figure 1 (continued)

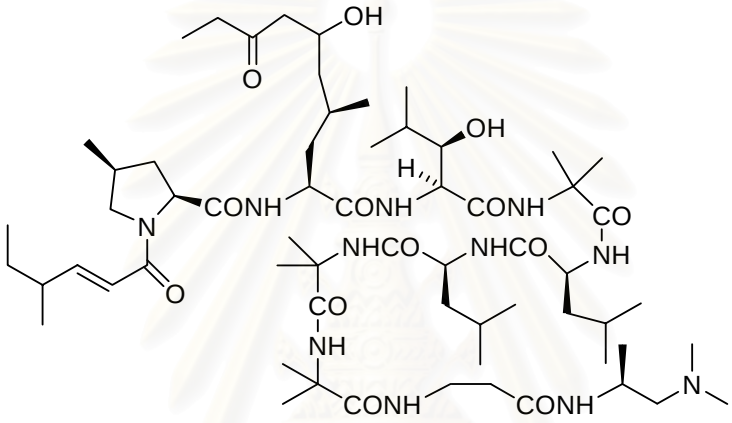


36. Sublutinol A 12 *S*

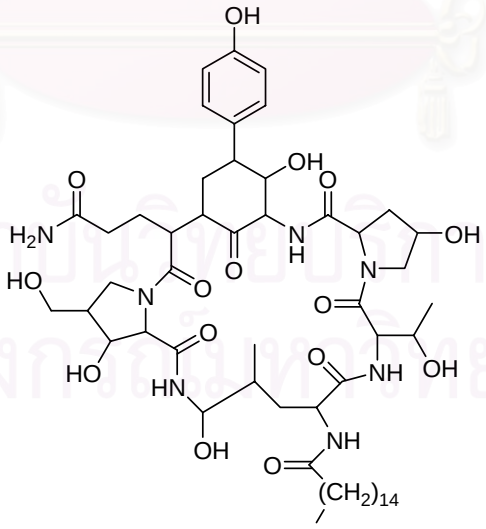
37. Sublutinol B 12 *R*



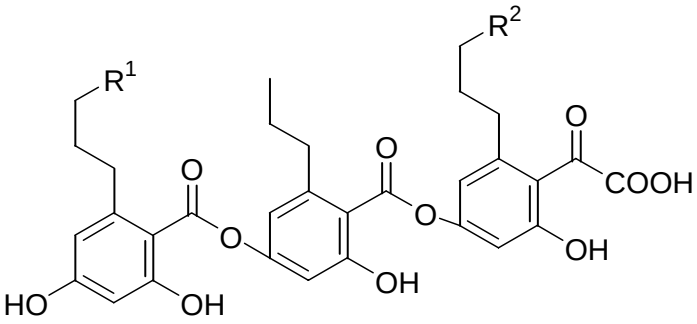
38. Taxol



39. Leucinostatin

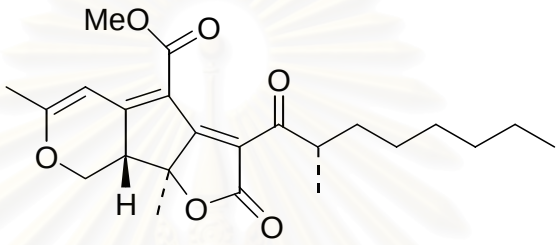


40. Cryptocandin

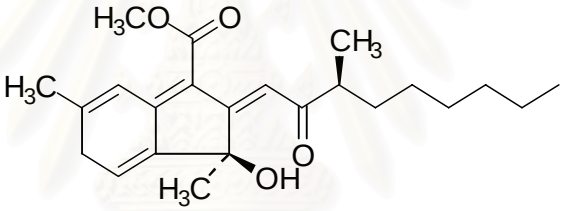


41. Cytonic acid A R^1= Et, R^2 = H

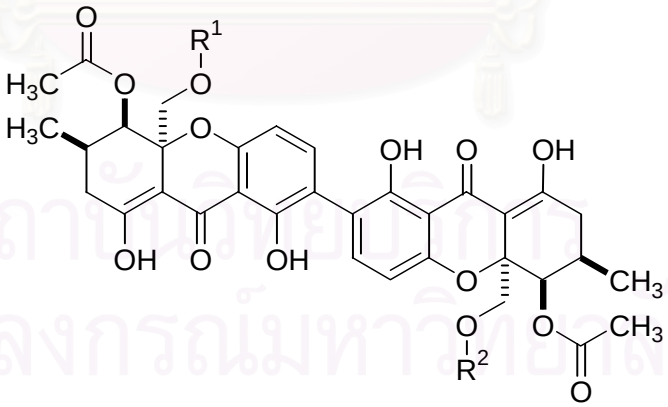
42. Cytonic acid B R^1= H, R^2 = Et



43. Sequoiatone A



44. Sequoiatone B

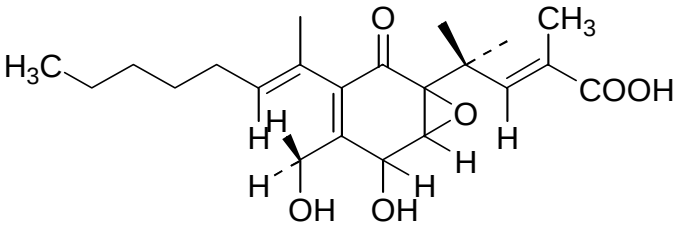


45. Dicerdrols A R_1 =R_2 = H

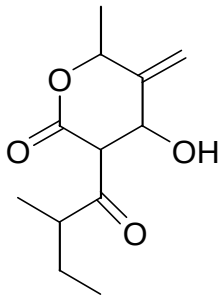
46. Dicerdrols B R_1 = Ac R_2 = H

47. Dicerdrols C R_1 = R_2 = Ac

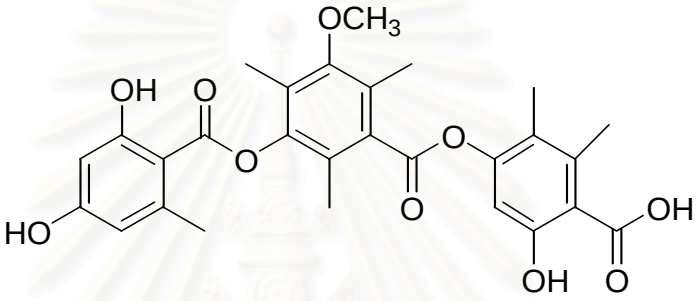
Figure 1 (continued)



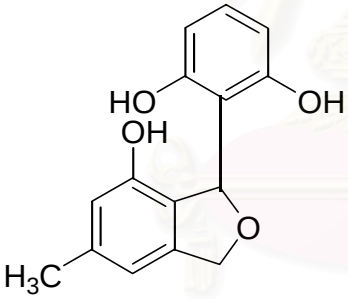
48. Ambuic acid



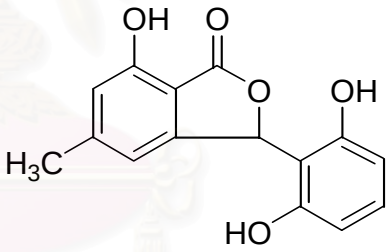
49. CR 377



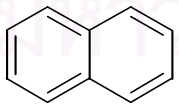
50. Colletoria acid



51. Pestacin

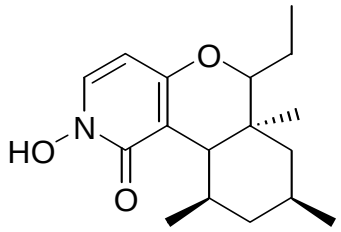


52. Isopestacin

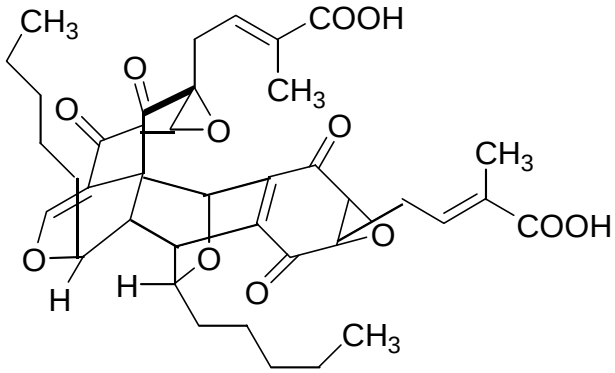


53.Naphthalene

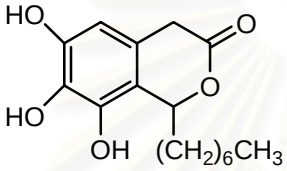
Figure 1 (continued)

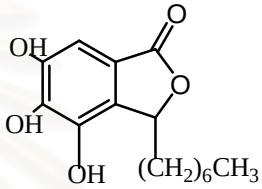


54. Fusaricide



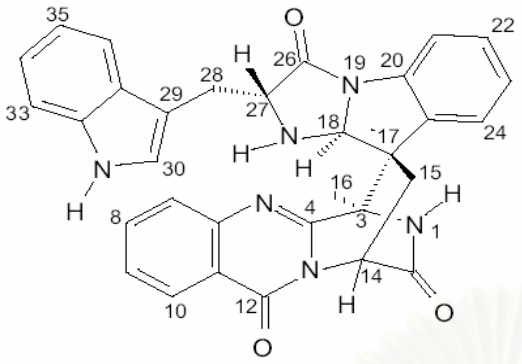
55. Torreyanic acid

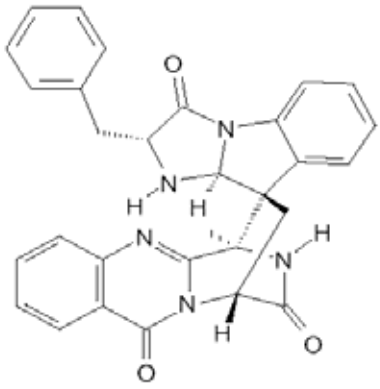


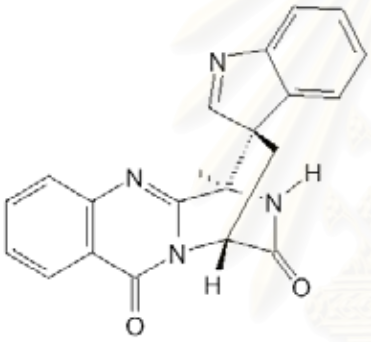


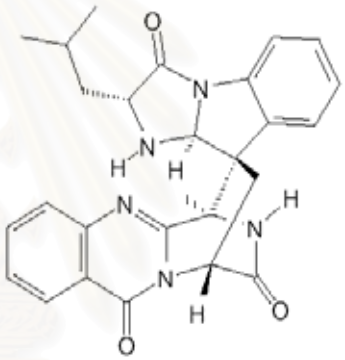
56. Cytosporone D and E

Figure 1 (continued)





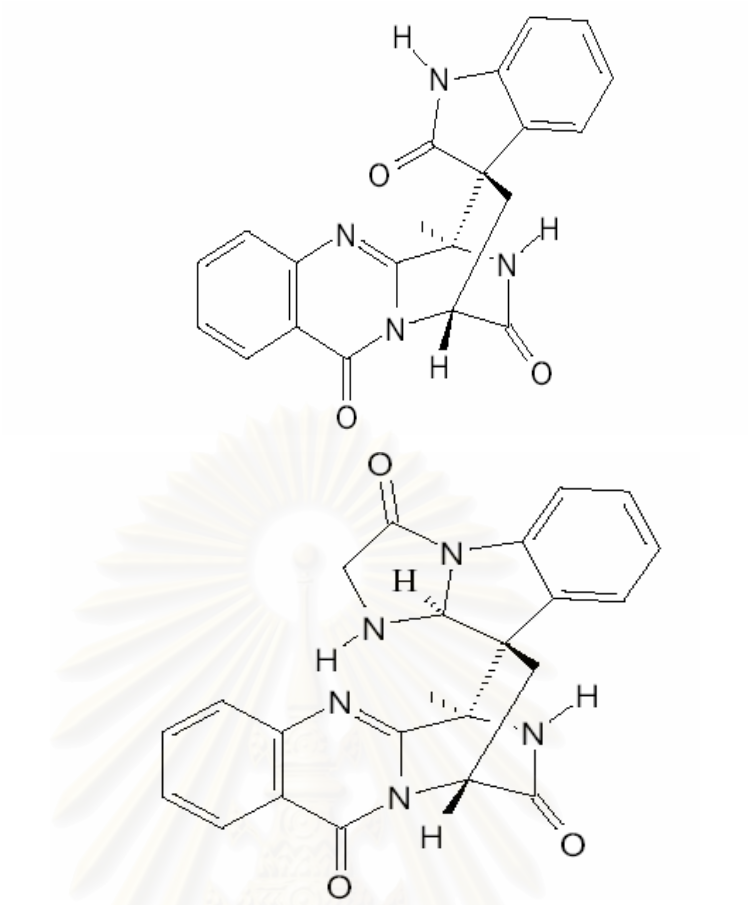




58. Alanditrypinone , Alantryphenone

Alantrypinene and Alantryleunone

Figure 1 (continued)



59. Asperfumoid and Asperfumin

Figure 1 (continued)

Table 2 Chemical constituents of *Croton oblongifolius*

Compounds	Plants parts	Reference
<u>Diterpenes</u>		
1. **Labdane Diterpenes**		
- Labda-7,12 (*E*),14-triene	Stem bark	Roengsumran et al., 1999a
- Labda-7,12 (*E*),14-triene-17-al	Stem bark	Roengsumran et al., 1999a
- Labda-7,12 (*E*),14-triene-17-ol	Stem bark	Roengsumran et al., 1999a
- Labda-7,12 (*E*),14-triene-17-oic acid	Stem bark	Roengsumran et al., 1999a
- *ent*-8(17),12*E*,14-labdatrien-18-oic acid	Stem bark	Pattamadilok, 1998
- 12,15-epoxy-8(17),12,14-labdatriene	Stem bark	Pattamadilok, 1998
- labda-7,13(Z)-diene-17,12-olide	Stem bark	Baiagern, 1999
- labda-7,13(Z)-diene-17,12-olide-16-ol	Stem bark	Baiagern, 1999
- 2-acetoxy-labda-8(17),12(*E*),14-triene-3-ol	Stem bark	Kuptiyanuwat, 1999; Roengsumran et al., 2001
- 3- acetoxy-labda-8(17),12(*E*),14-triene-2-ol	Stem bark	Kuptiyanuwat, 1999; Roengsumran et al., 2001
- Labda-8(17), 12 (*E*),14-triene-2,3-diol	Stem bark	Kuptiyanuwat, 1999; Roengsumran et al., 2001
- 12 (*E*),14-labdadiene-7,8-diol	Stem bark	Boontha, 2000
- 6-acetoxy-12 (*E*),14- labdadiene-7,8-diol	Stem bark	Boontha, 2000
- 12 (*E*), 14-labdadiene-6,7,8-triol	Stem bark	Boontha, 2000
- nidorello	Stem bark	Roengsumran et al., 200

Table 2 (continued)

Compounds	Plants parts	Reference
- (5*S*,8*S*,9*S*,10*R*,13*S*)-8,13-epoxylabda-1,14-diene-3-one	Stem bark	Permpanya, 2003
- (5*S*,8*S*,9*S*,10*R*,12*S*,13*S*)- 8,13-epoxy-12-hydroxy-labda-1,14-diene-3-one	Stem bark	Permpanya, 2003
2. **Clerodane Diterpenes**		
- (-)-hardwickiic acid	Root bark, wood	Aiyar and Seshadri, 1972b
	Stem bark	Aiyar and Seshadri, 1972a
		Surachethapan, 1996; Baiagern, 1999:
		Sirimongkhon, 2000; Sriyangnok, 2000
- 11-dehydro-(-)-hardwickiic acid	Stem bark	Aiyar and Seshadri, 1972a
	Root bark, wood	Aiyar and Seshadri, 1972b
- (-)-20-benxyloxyhardwickiic acid	Stem bark	Baiagern, 1999
- methyl-15,15-epoxy-12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate	Stem bark	Tanwattanakun, 1999
- crovatin	Stem bark	Siriwat, 1999
- croblongifolin	Stem bark	Roengsumran et al., 2002

Table 2 (continued)

Compounds	Plants parts	Reference
3. Pimarane Diterpenes		
- oblongiloliol	Stem bark	Rao et al., 1968
	Root bark, wood	Aiyar and Seshadri, 1972b
- 19-deoxyoblongiloliol	Stem bark	Rao et al., 1968
	Root bark, wood	Aiyar and Seshadri, 1972b
- 3-deoxyoblongiloliol	Stem bark	Aiyar and Seshadri, 1971a
	Root bark, wood	Aiyar and Seshadri, 1972b
- oblongifolic acid	Stem bark	Aiyar and Seshadri, 1970
	Root bark, wood	Aiyar and Seshadri, 1972b
- *ent*-isopimara-7,15-dien	Stem bark	Aiyar and Seshadri, 1971b
	Root bark, wood	Aiyar and Seshadri, 1972b
- *ent*- isopimara-7,15-dien-19-aldehyde	Stem bark	Aiyar and Seshadri, 1971b
	Root bark, wood	Aiyar and Seshadri, 1972b
- 19-hydroxy-*ent*-isopimara-7,15-dien	Stem bark	Aiyar and Seshadri, 1971b
- (-)-pimara-9(11),15-dien-19-oic acid (acanthoic acid)	Stem bark	Tanwattanakun, 1999
- (-)-pimara-9(11),15-dien-19-ol	Stem bark	Tanwattanakun, 1999

Table 2 (continued)

Compounds	Plants parts	Reference
4. Kaurane Diterpene		
- *ent*-kaur-16-en-19-oic acid	Stem bark	Pattamadilok, 1998; Sirimongkhon, 2000
5. Cembrane Diterpenes		
- Crotocembraneic acid	Stem bark	Surachethapan, 1996; Roengsumran et al.,1998
- Neocrotocembraneic acid	Leaves	Achayindee, 1996;
	Stem bark	Roengsumran et al., 1998
- Neocrotocembranal	Stem bark	Roengsumran et al., 1999b
- Poilaneic acid	Stem bark	Boontha, 2000
- (2E,7E,11E) 1-isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca-2,7,11-triene-12-carboxylic acid	Stem bark	Tanwattanakun, 1999
6. Cleistanthane Diterpene		
- 3,4-seco-cleistantha-4(18),13(17),15-trien-3-oic acid	Stem bark	Siriwat, 1999;
7. Trachylobane Diterpene		Sriyangnok, 2000
- Trachyloban-19-oic acid	Stem bark	Boontha, 2000

Table 2 (continued)

Compounds	Plants parts	Reference
8. **Abeitane Diterpene**		
- Abeita-7,13-dien-3-one	Stem bark	Sriyangnok, 2000
<u>Triterpenes</u>		
- Acetyl aleuritolic acid	Stem bark	Aiyar, et al., 1971c
<u>Steroids</u>		
- β-sitosterol	Stem bark	Roa, et al., 1968
	Wood	Chaicharoenpong, 1996
	Leaves	Achayindee, 1996
	Wood	Chaicharoenpong, 1996
- campeserol	Stem bark	Pattamadilok, 1998
	Wood	Chaicharoenpong, 1996
- stigmasterol	Leaves	Achayindee, 1996
	Stem bark	Pattamadilok, 1998

Table 2 (continued)

Compounds	Plants parts	Reference
Steroid Glucosides		
- β-sitosteryl-3-O- β—D-glucopyranoside	Wood	Chaicharoenpong, 1996
	Stem bark	Surachethapan, 1996
- campesteryl-3- O- β—D-glucopyranoside	Wood	Chaicharoenpong, 1996
	Stem bark	Surachethapan, 1996
- stigmasteryl-3- O- β—D-glucopyranoside	Wood	Chaicharoenpong, 1996
-	Stem bark	Surachethapan, 1996
Coumarin		
- 7-hydroxy-6-methoxycoumarin (Scopoletin)	Wood	Chaicharoenpong, 1996
miscellaneous		
- mixture of long chain aliphatic hydrocarbons	Wood	Chaicharoenpong, 1996
(C_{27}-C_{33})	Leaves	Achayindee, 1996
- mixture of long chain aliphatic carboxylic acid(C_{18}, C_{22}-C_{34})	Wood	Chaicharoenpong, 1996
- mixture of long chain alcohols (C_{28}-C_{29}, C_{31}-C_{32}, C_{34})	Leaves	Achayindee, 1996
- 6,10,14-trimethyl-2-pentadecanone	Leaves	Achayindee, 1996
- potassium chloride	Leaves	Achayindee, 1996

APPENDIX B

MEDIA

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

1. Malt extract agar (MEA)

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

2. Yeast-malt extract agar (YMA)

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

3. Nutrient agar (NA)

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

4. Potato dextrose agar (PDA)

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

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

5. Sabouraud's dextrose agar (SDA)

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

6. Yeast extract sucrose agar (YES)

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

7. Corn Meal Agar (CMA)

Corn meal	20.0	g
Peptone	20.0	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water	1000	ml

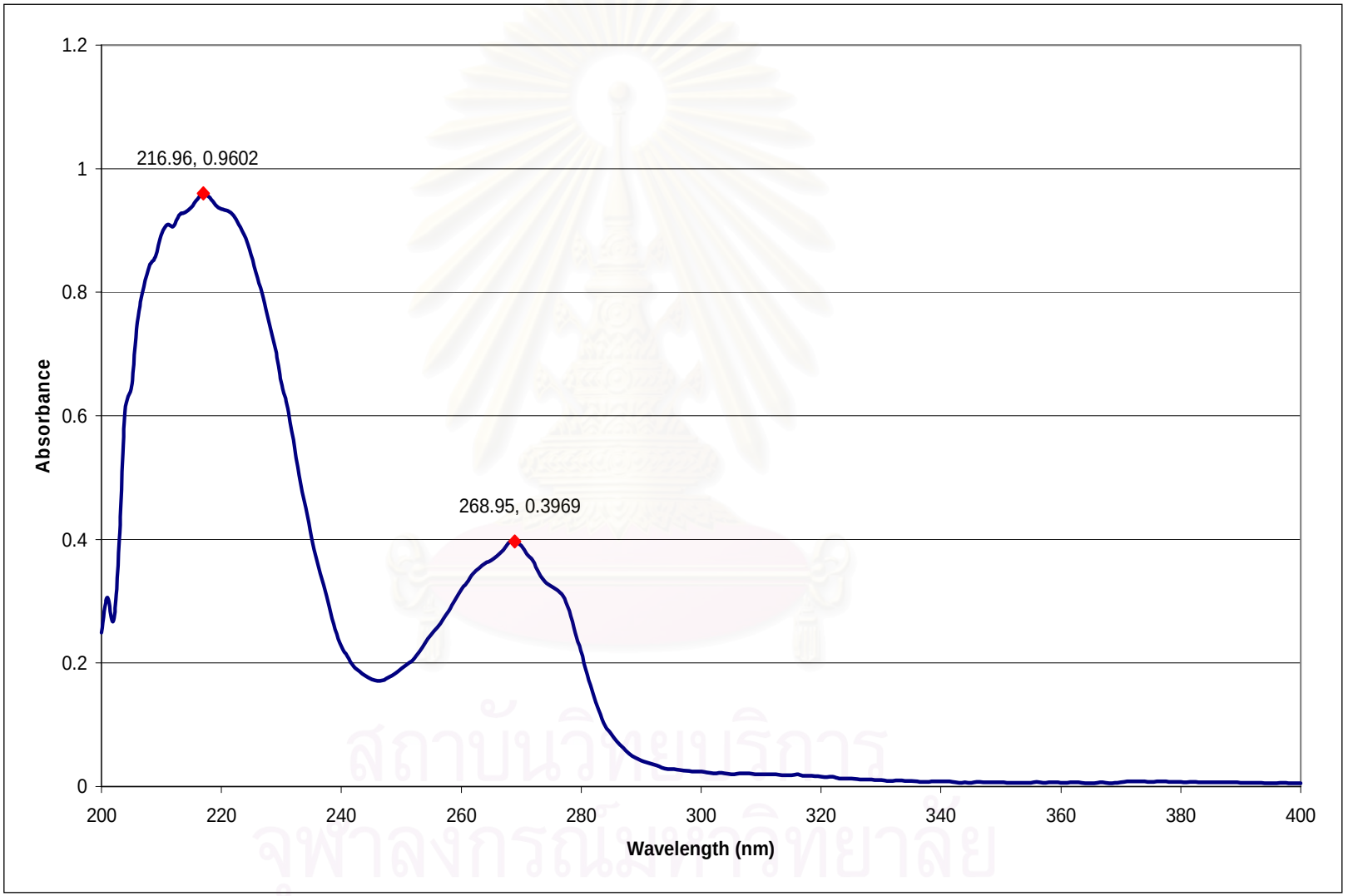


Figure 1 UV spectrum of mixture BE1

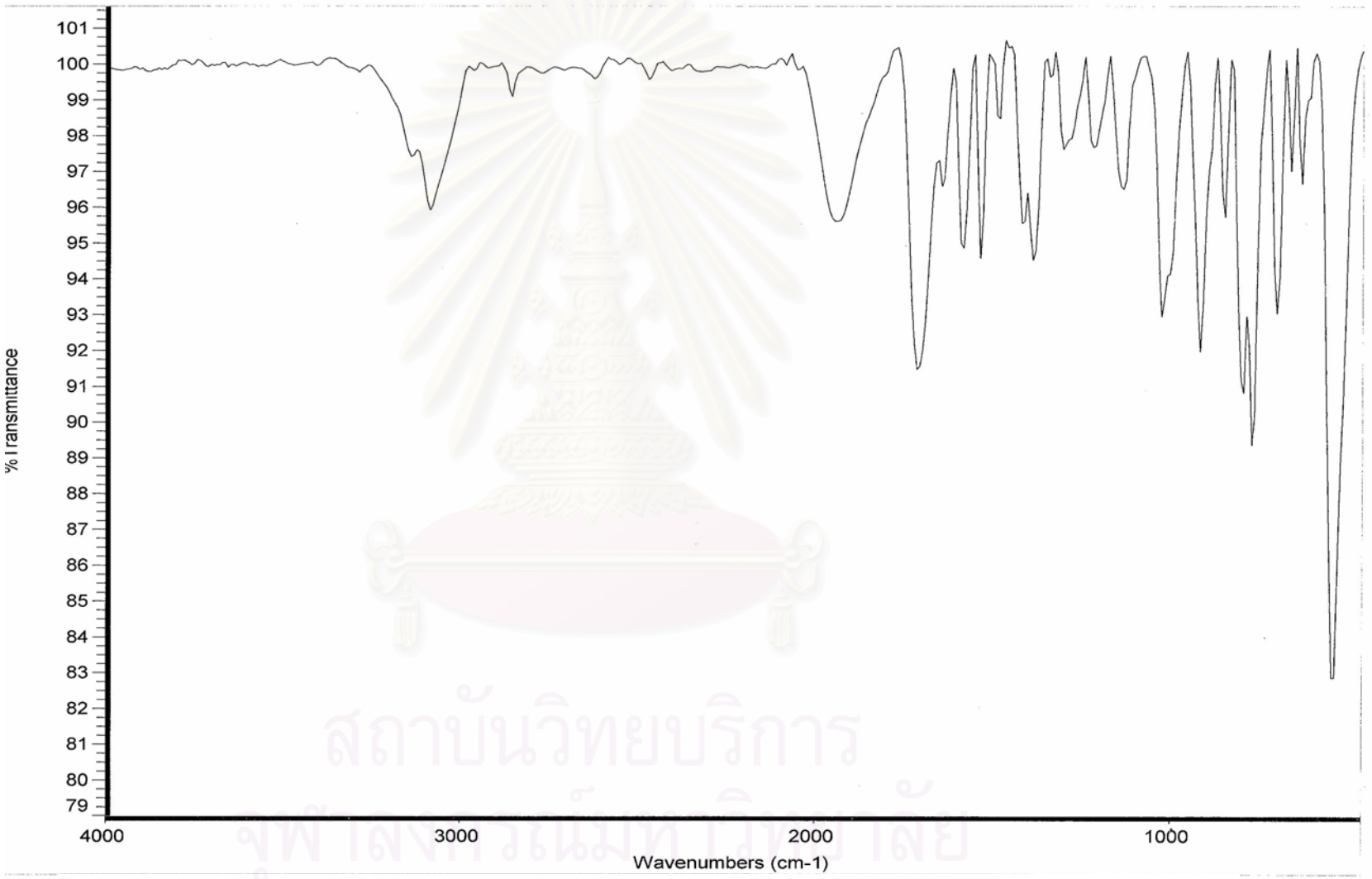


Figure 2 IR spectrum of mixture BE1

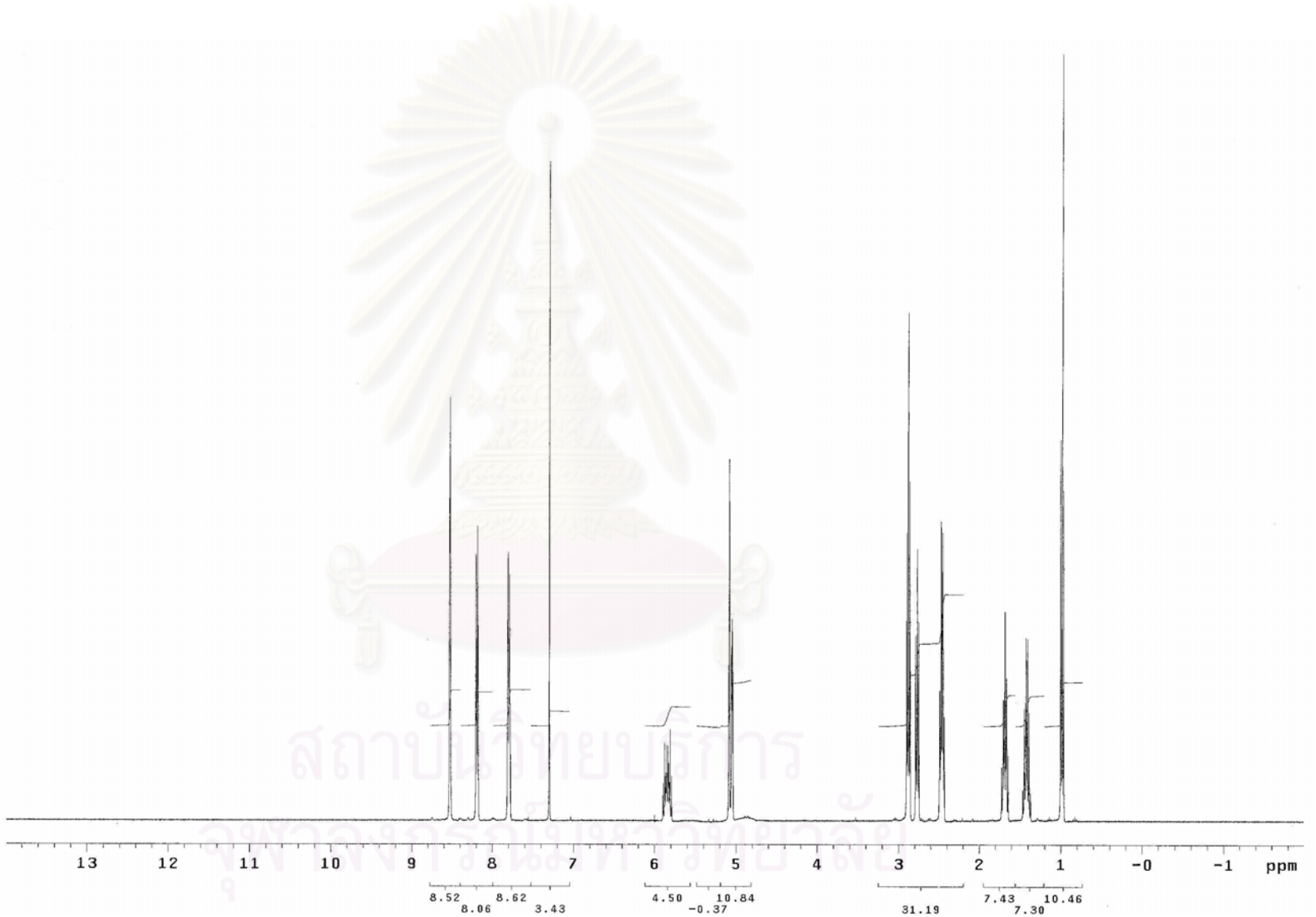


Figure 3 [1]H-NMR spectrum of mixture BE 1

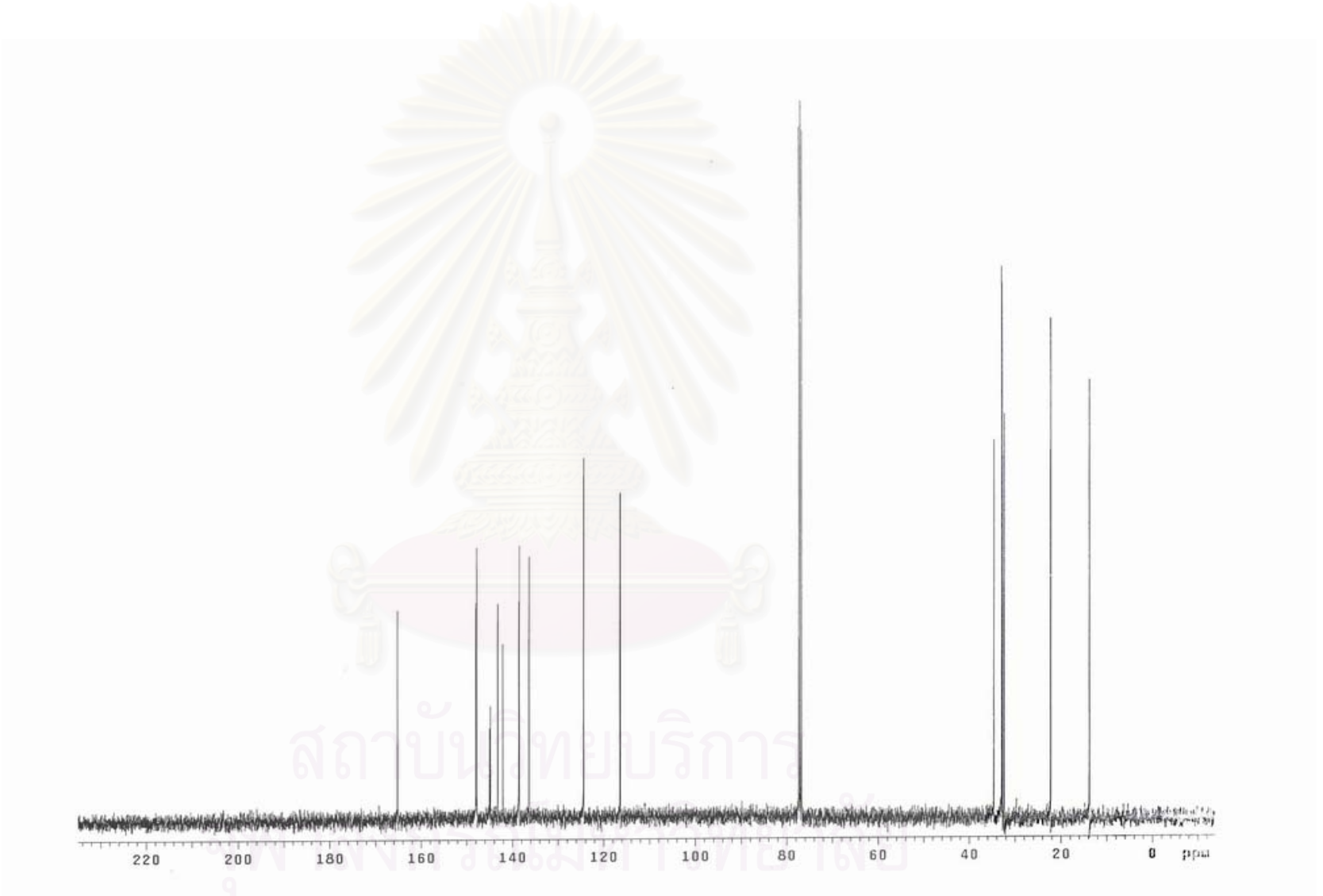


Figure 4 ^{13}C-NMR spectrum of mixture BE 1

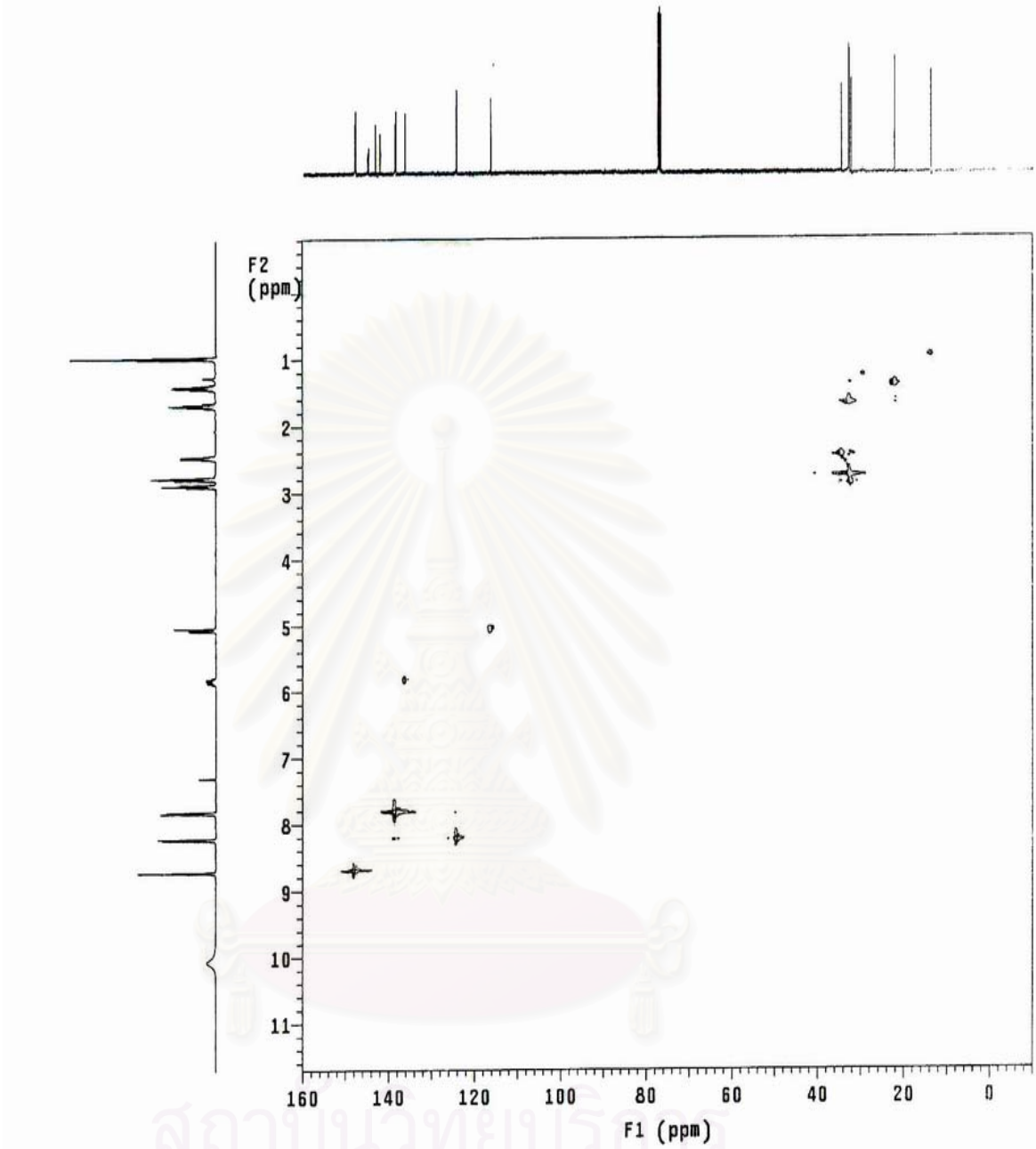


Figure 5 HSQC spectrum of mixture BE1

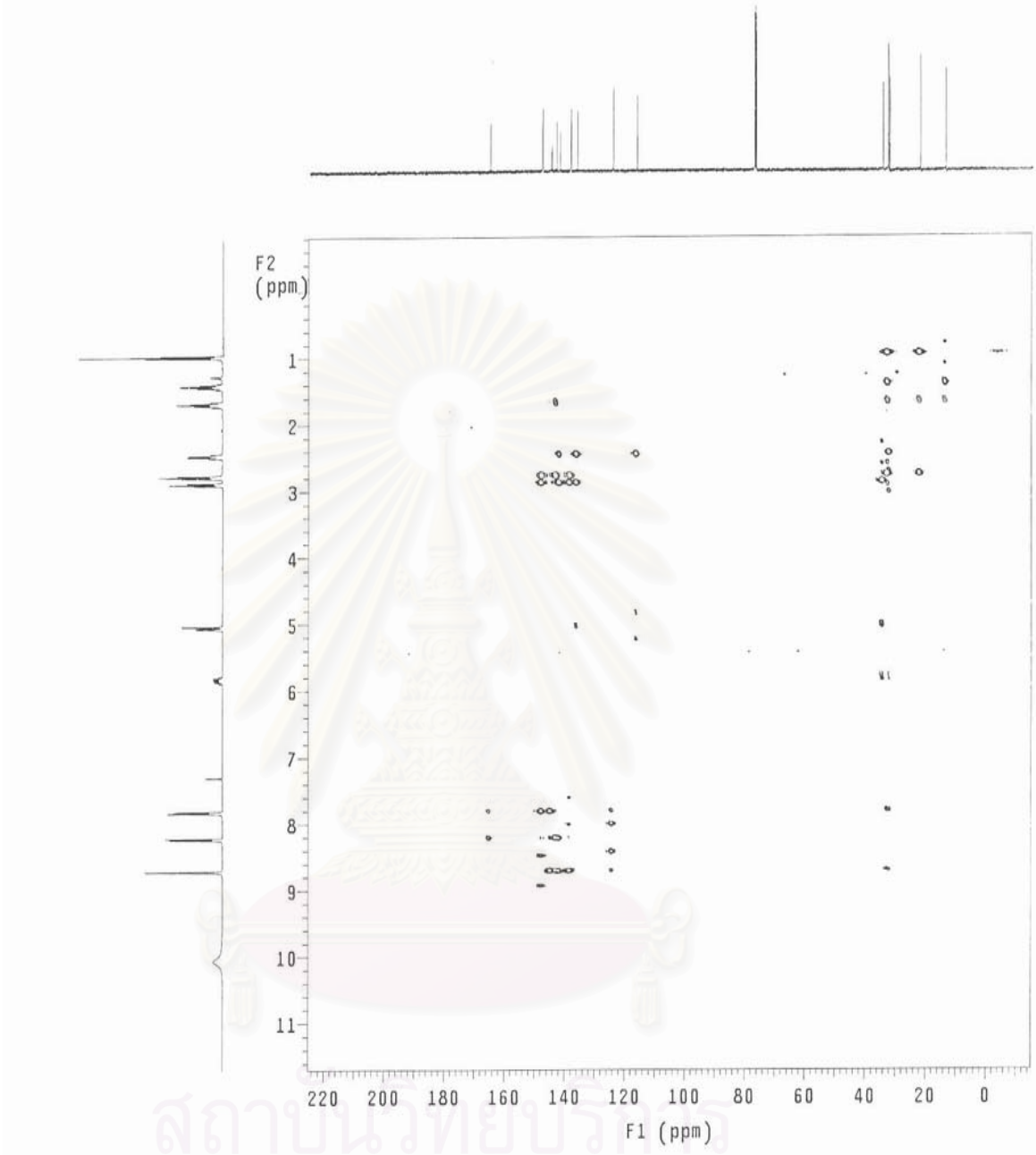


Figure 6 HMBC spectrum of mixture BE1

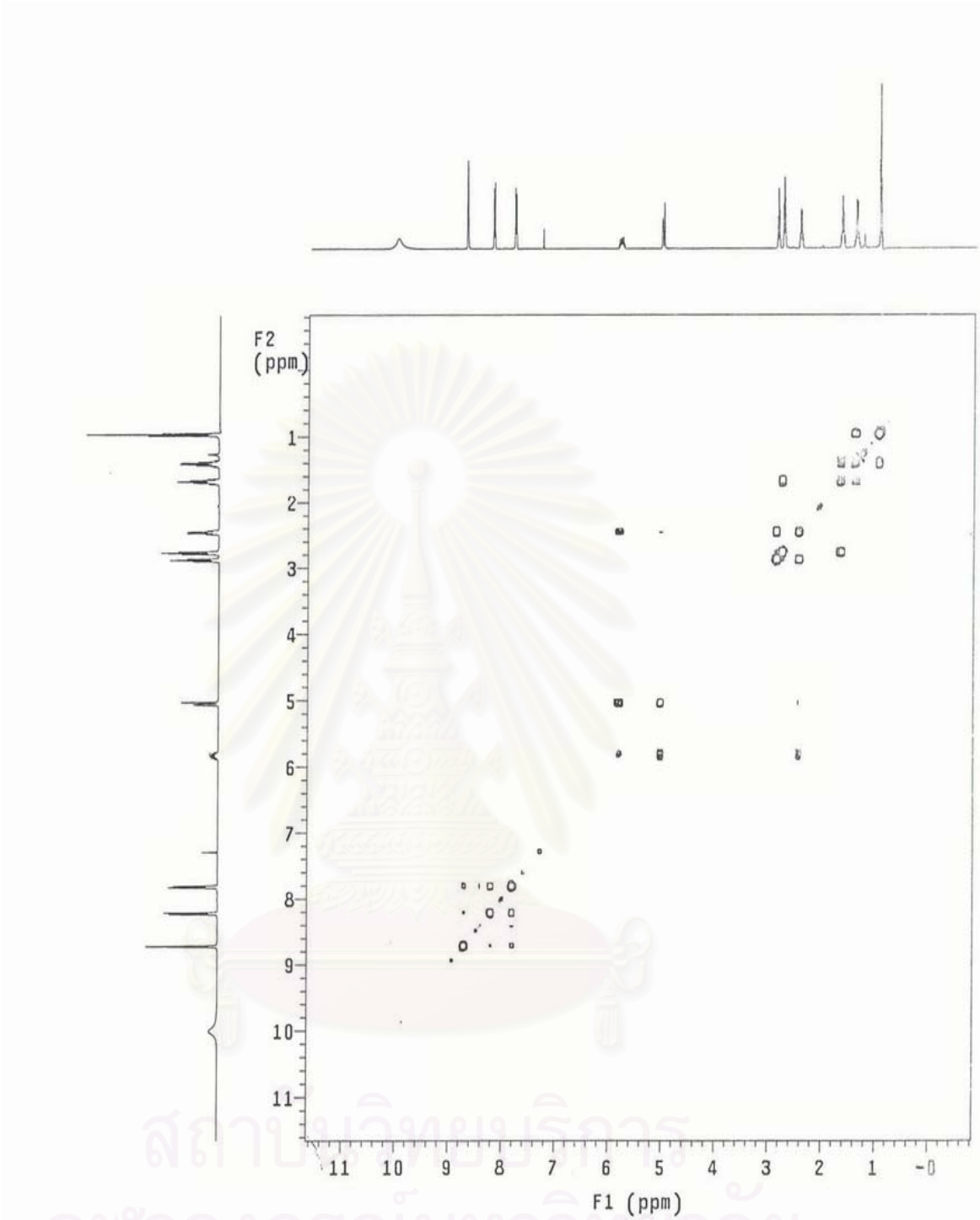


Figure 7 COSY spectrum of mixture BE1

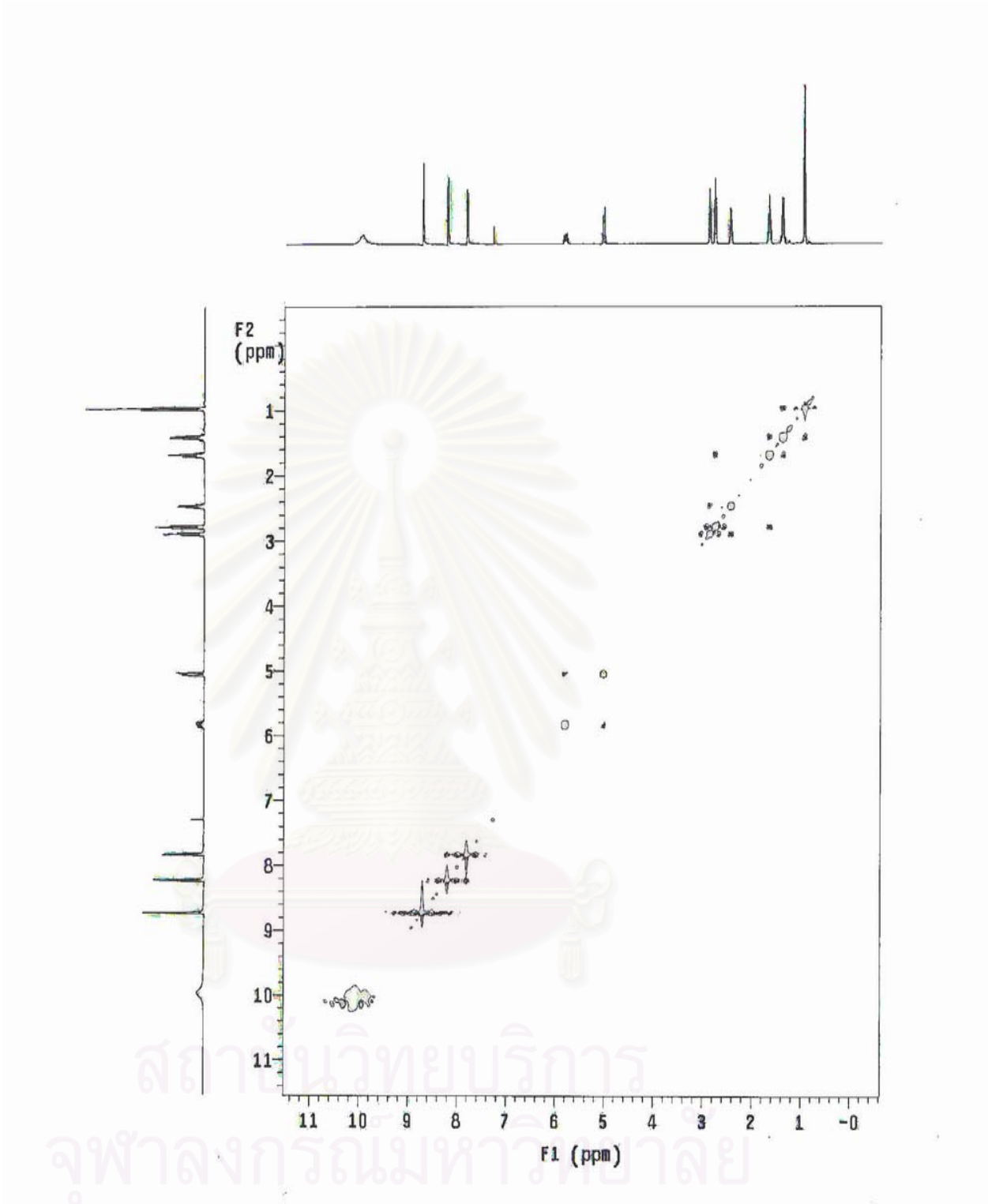


Figure 8 NOESY spectrum of mixture BE1

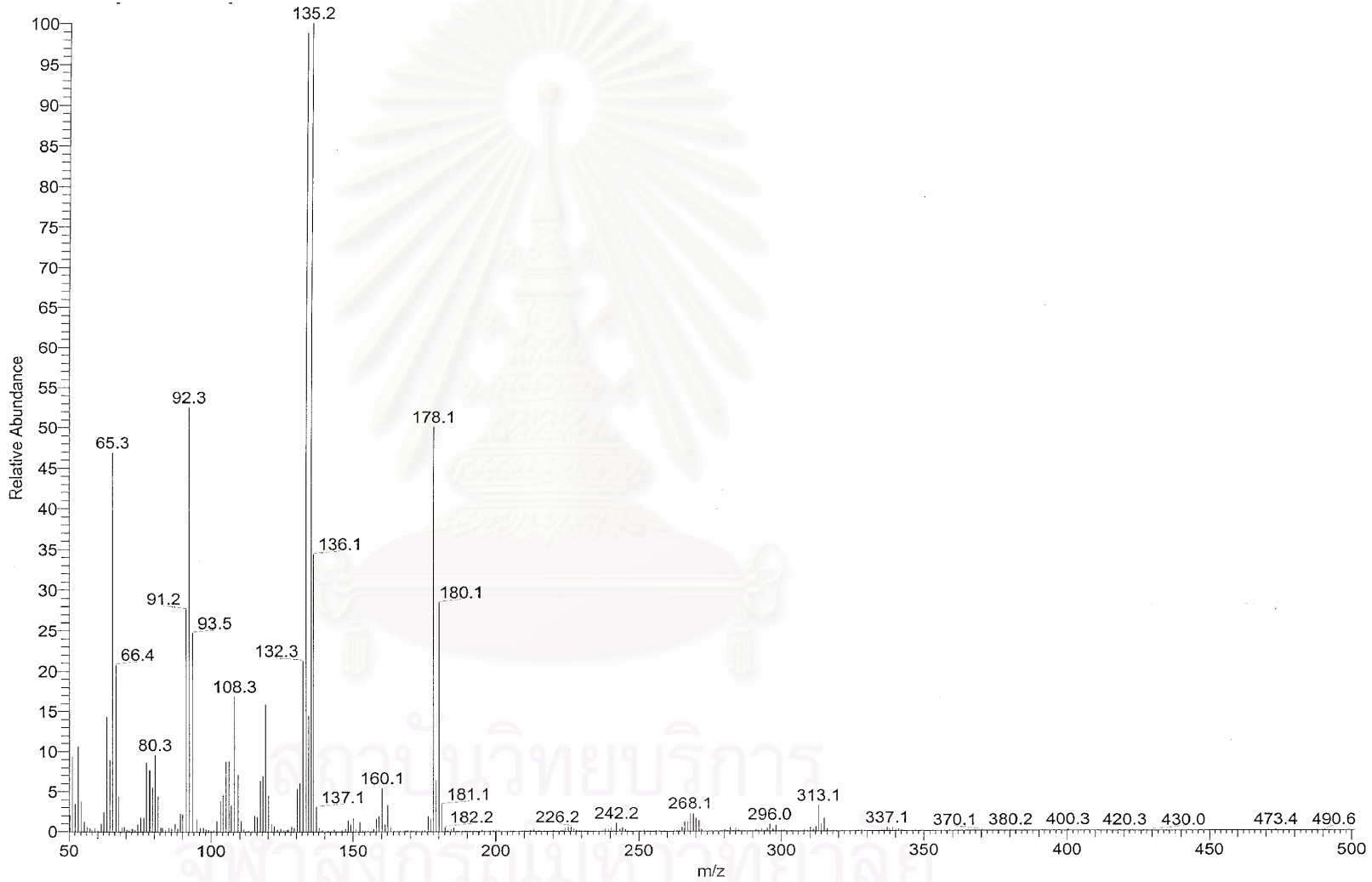


Figure 9 EI-MS spectrum of mixture BE1

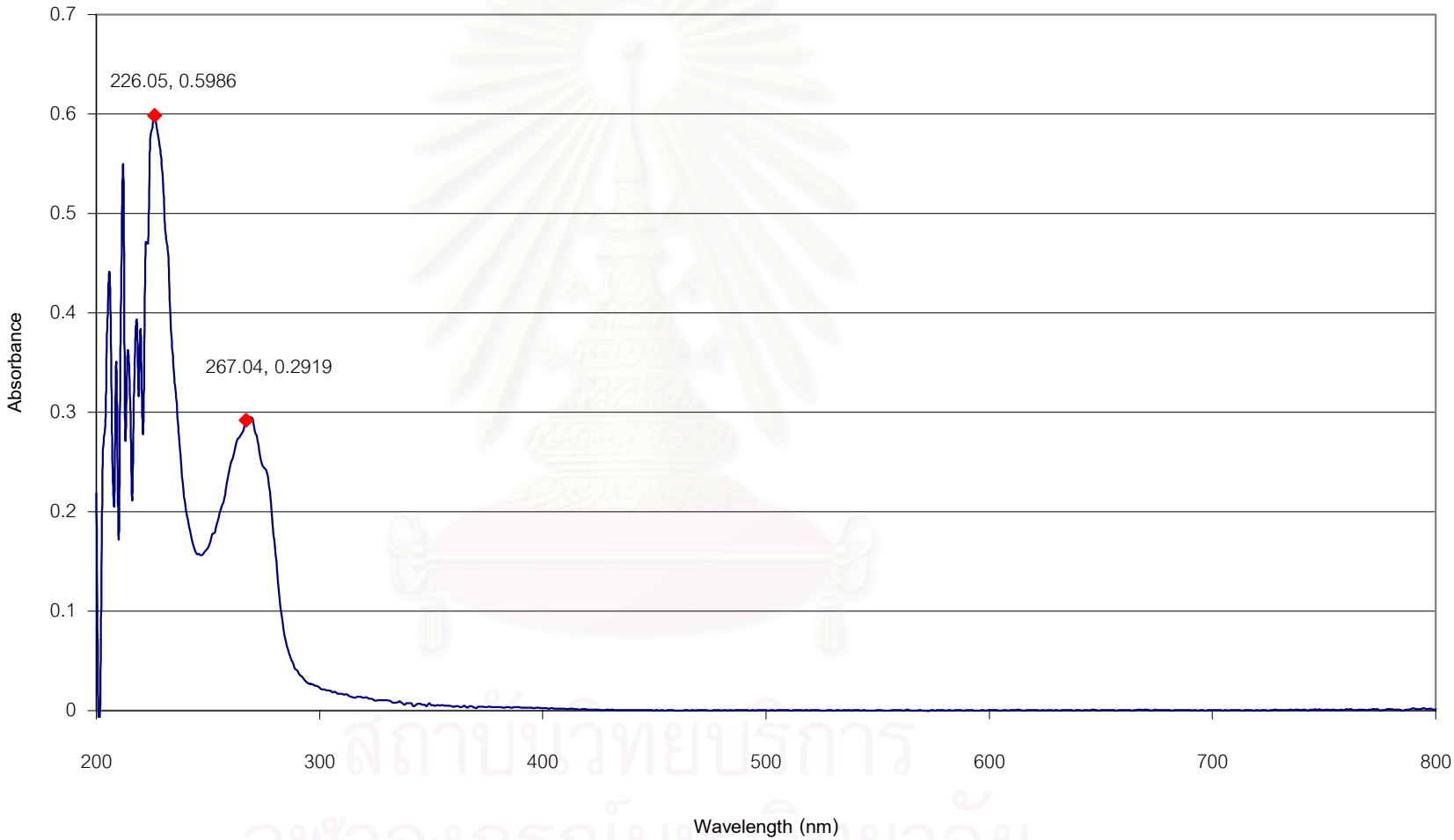


Figure 10 UV spectrum of compound BE2

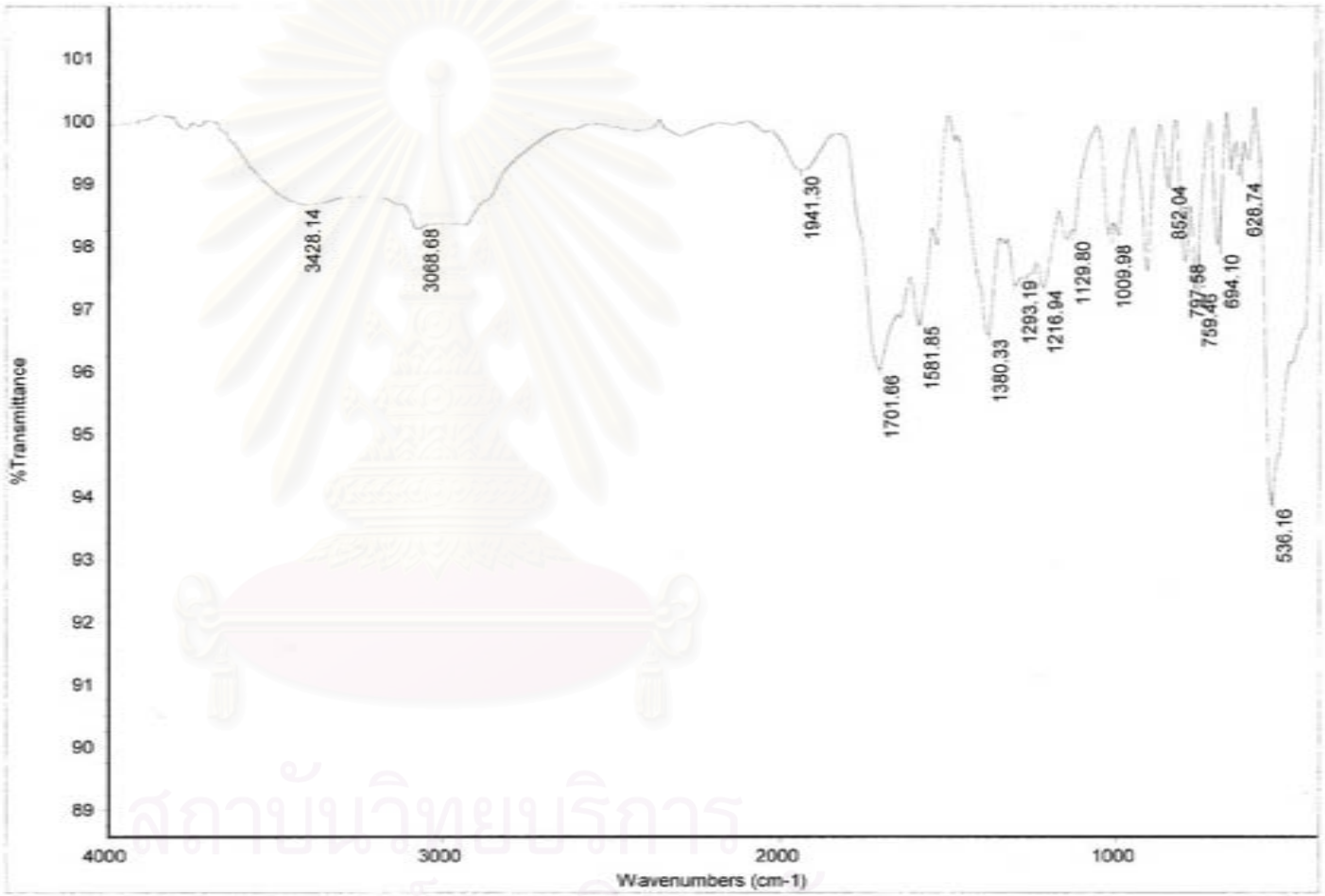


Figure 11 IR spectrum of compound BE2

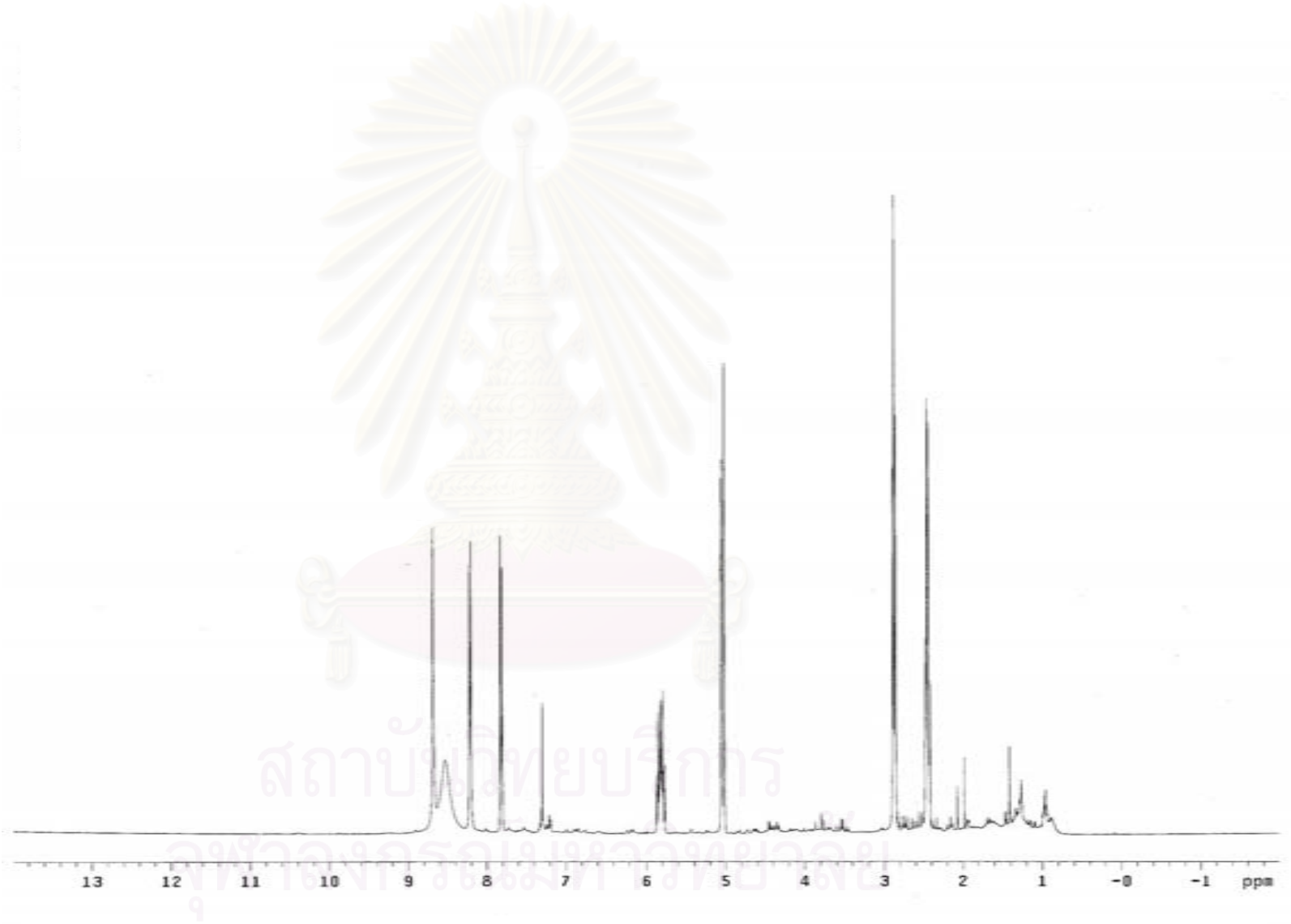


Figure 12 ^{1}H-NMR spectrum of compound BE2

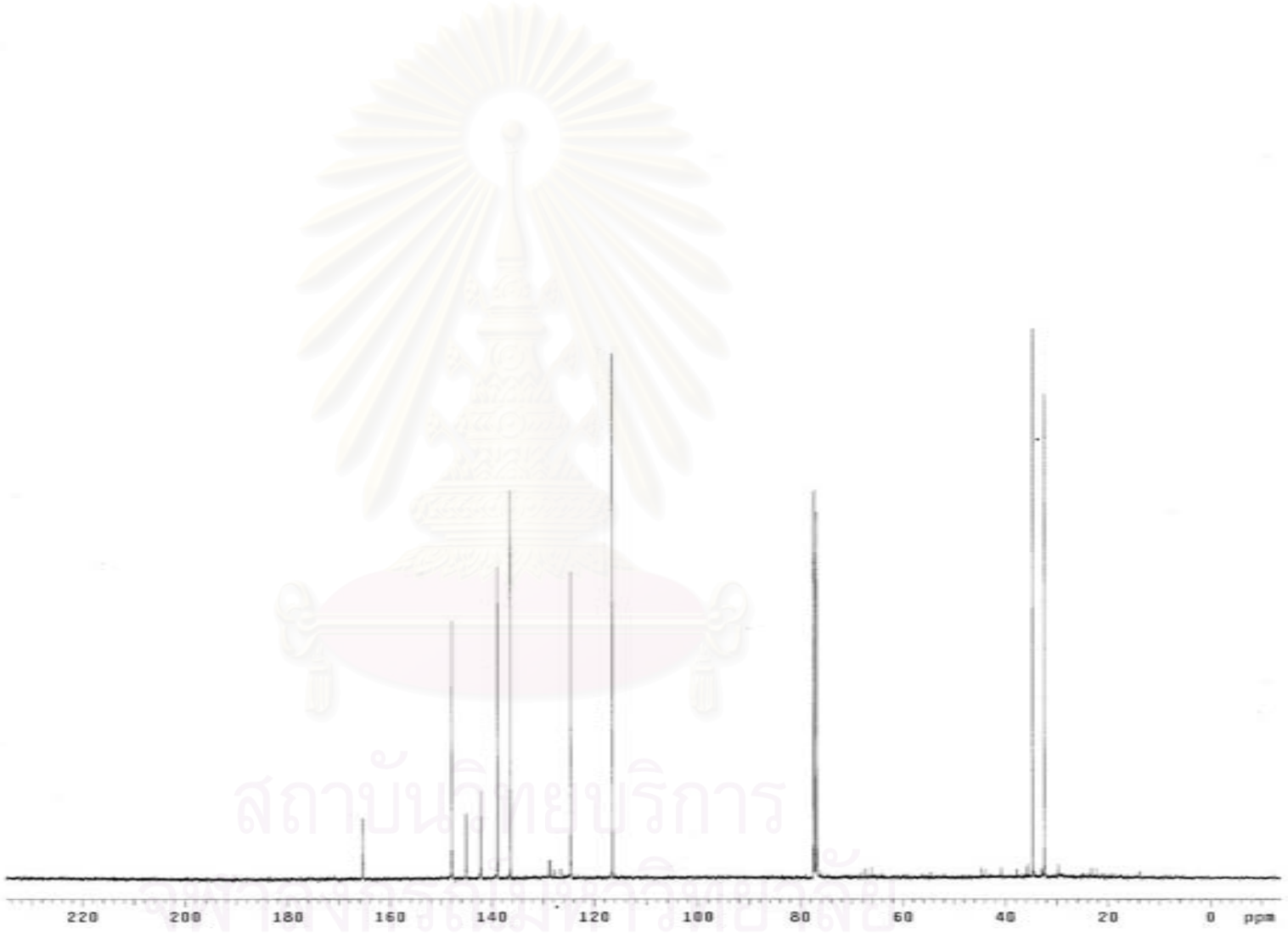


Figure 13 ^{13}C-NMR spectrum of compound BE2

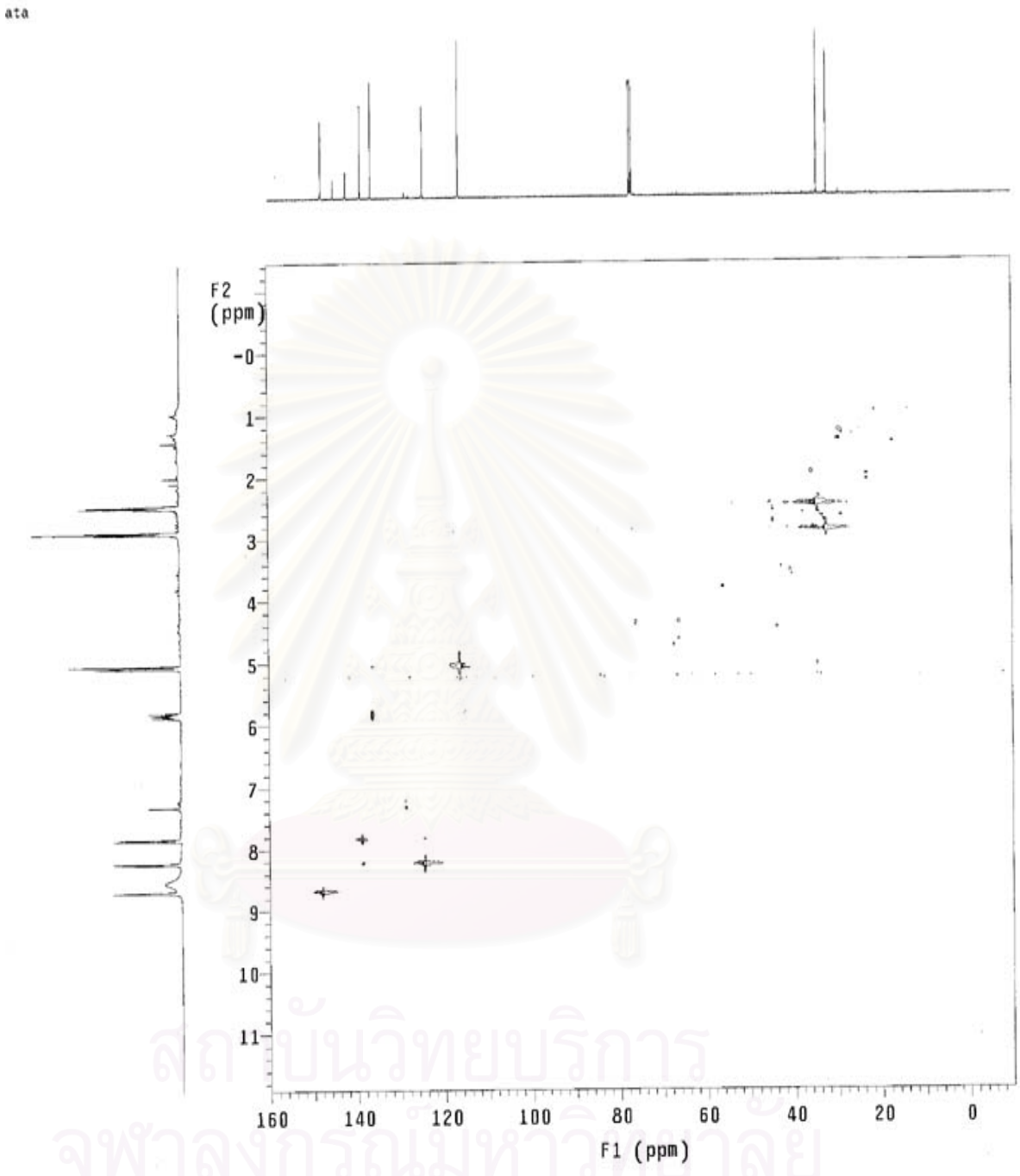


Figure 14 HSQC spectrum of compound BE2

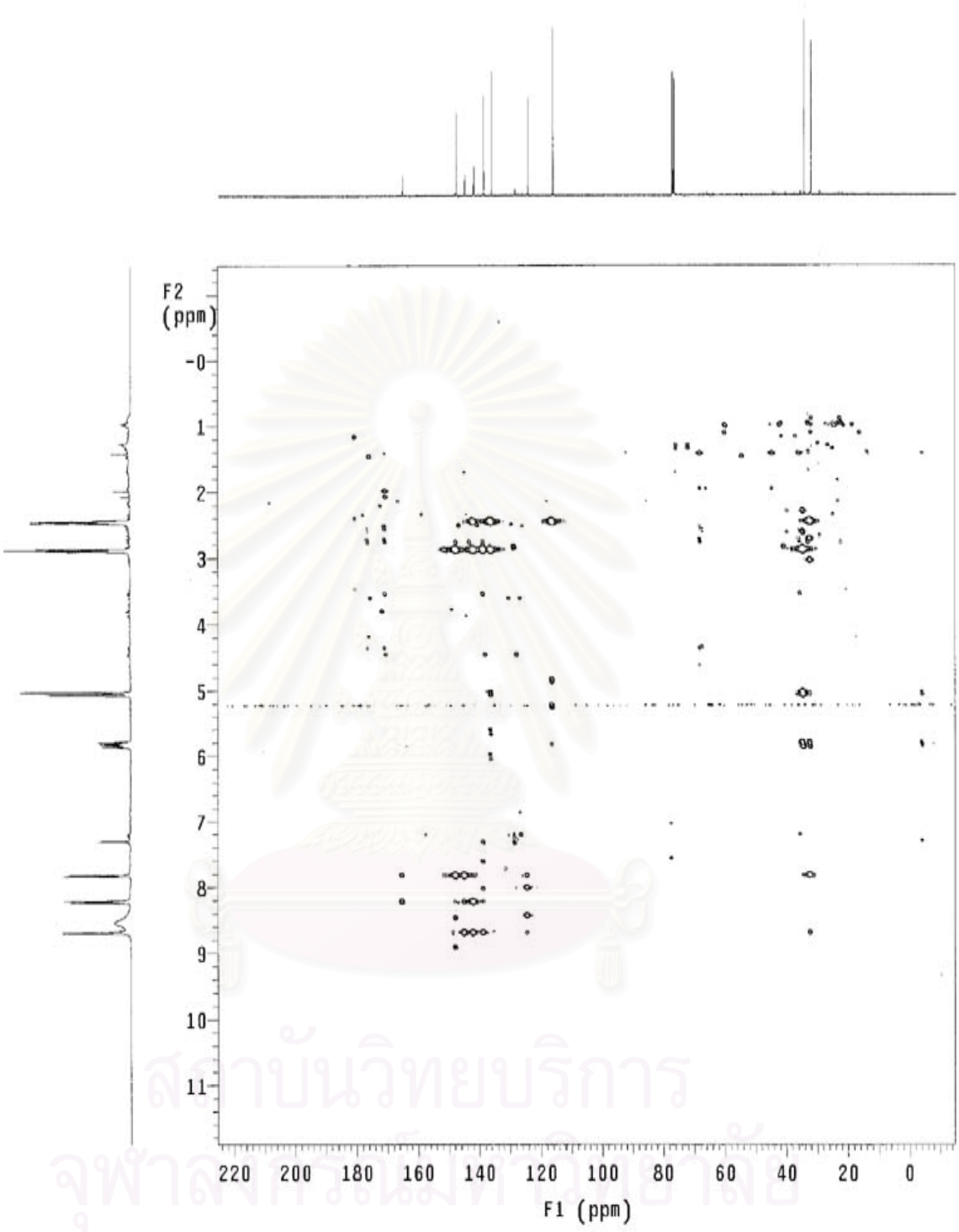


Figure 15 HMBC spectrum of compound BE2

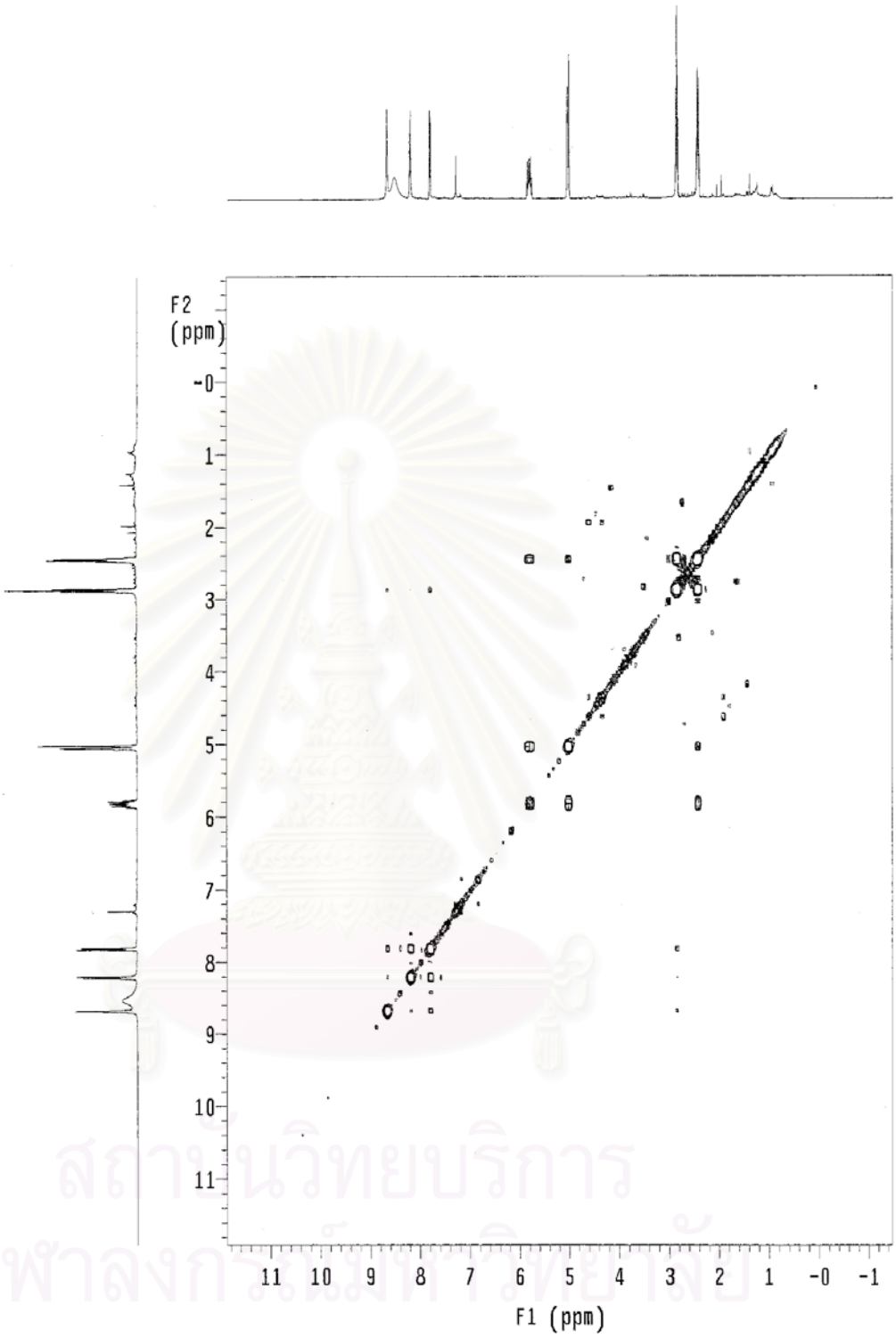


Figure 16 COSY spectrum of compound BE2

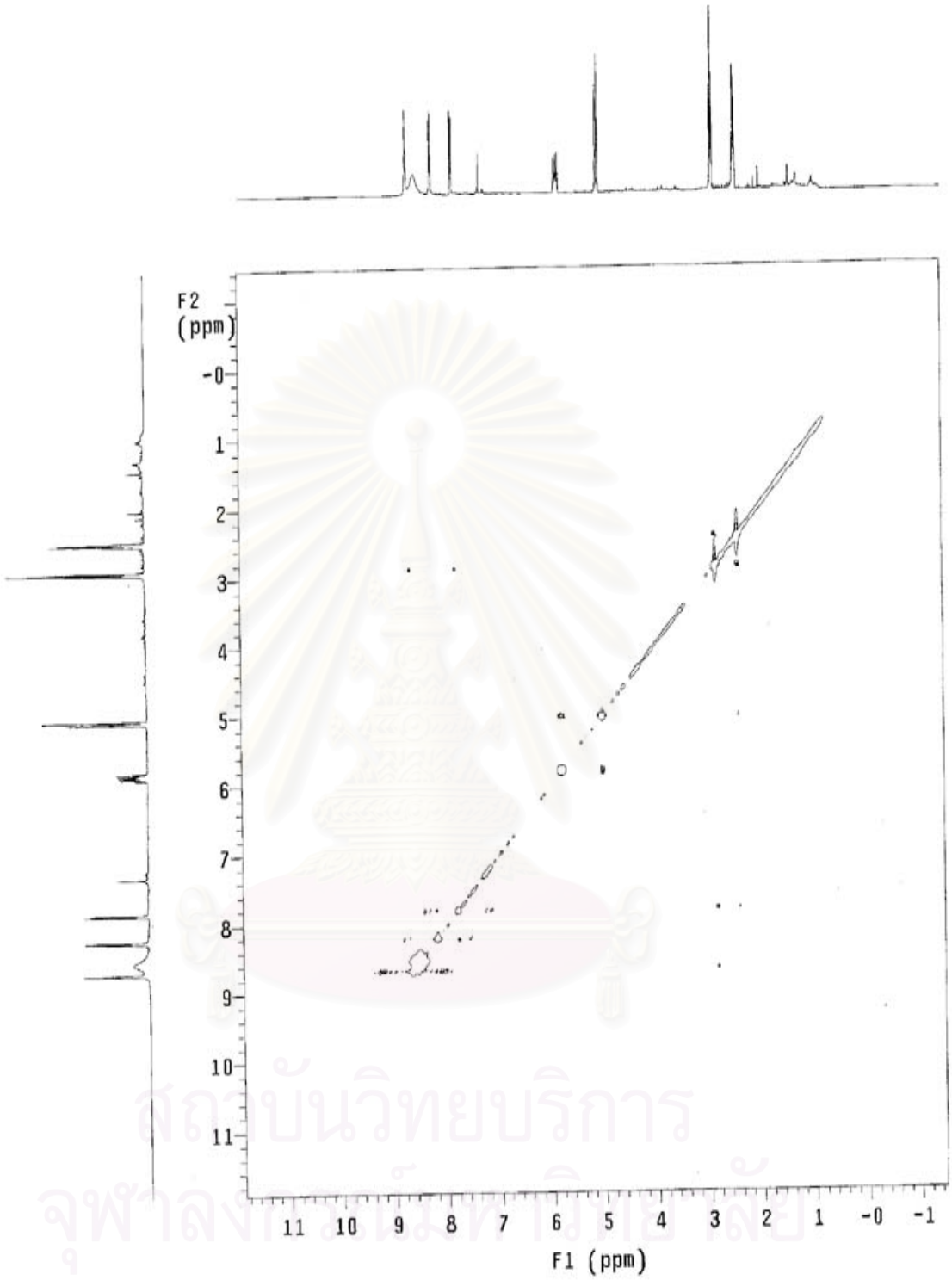


Figure 17 NOESY spectrum of compound BE2

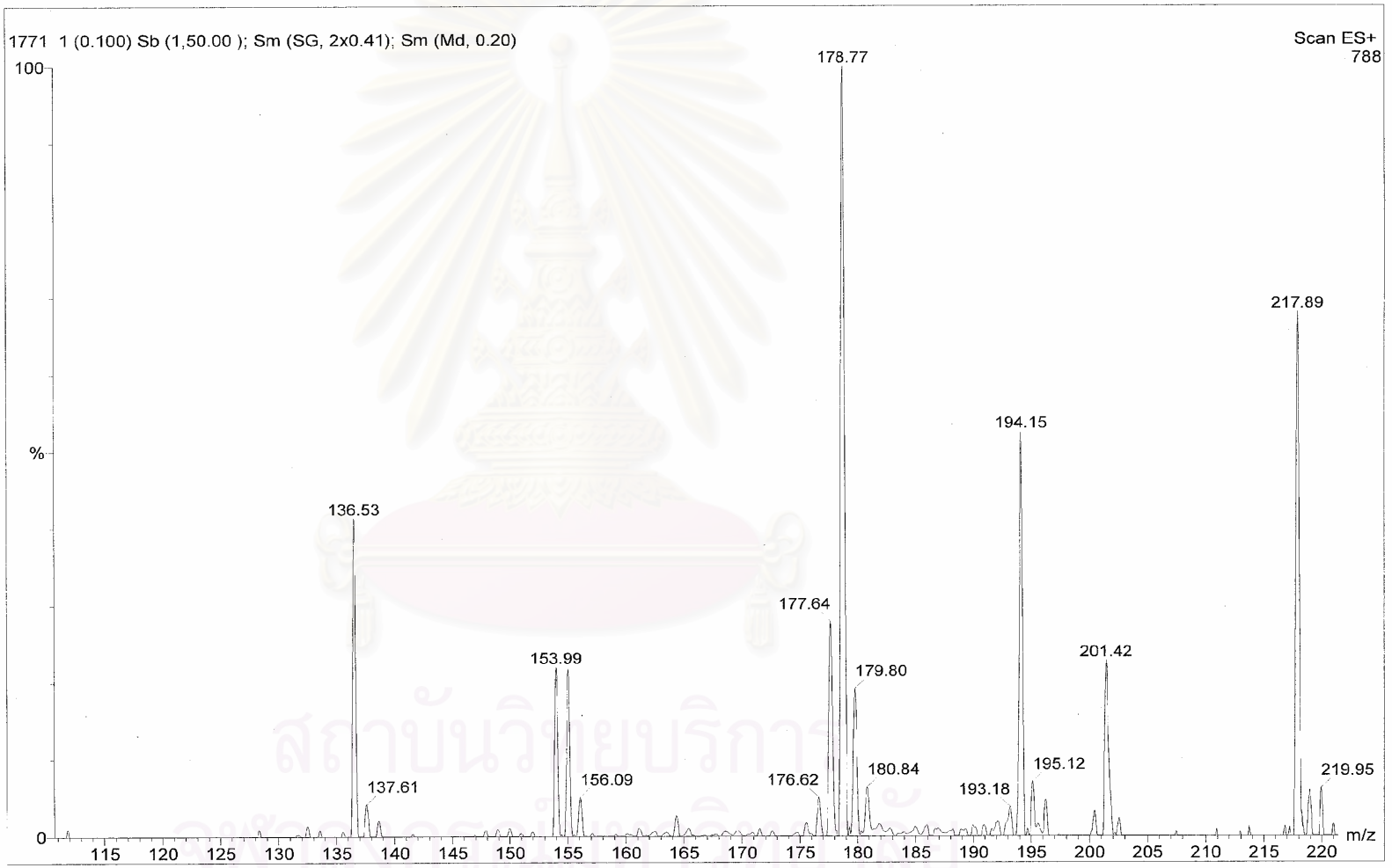


Figure 18 MALDI-TOF-MS spectrum of compound BE 2

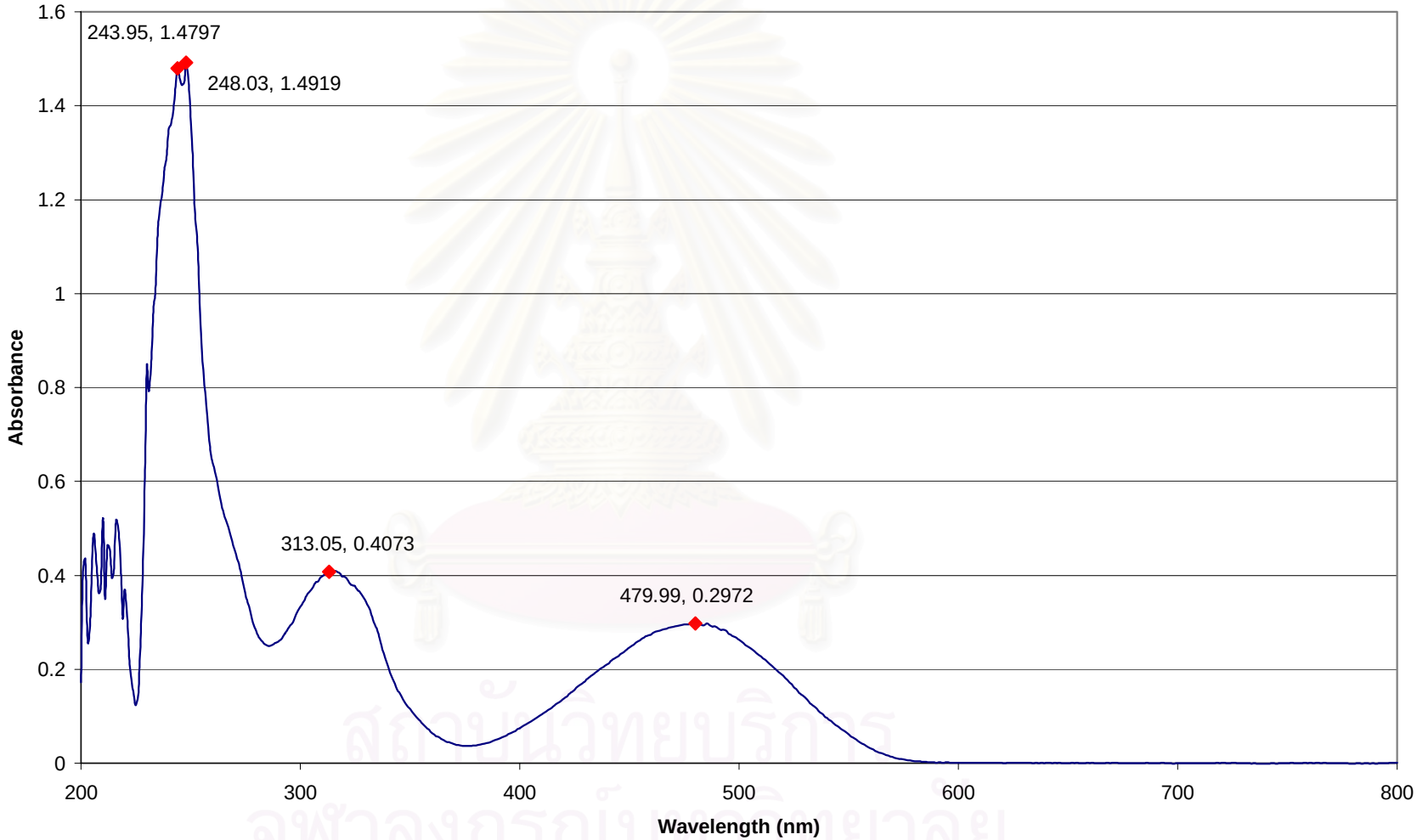


Figure 19 UV spectrum of compound BE3

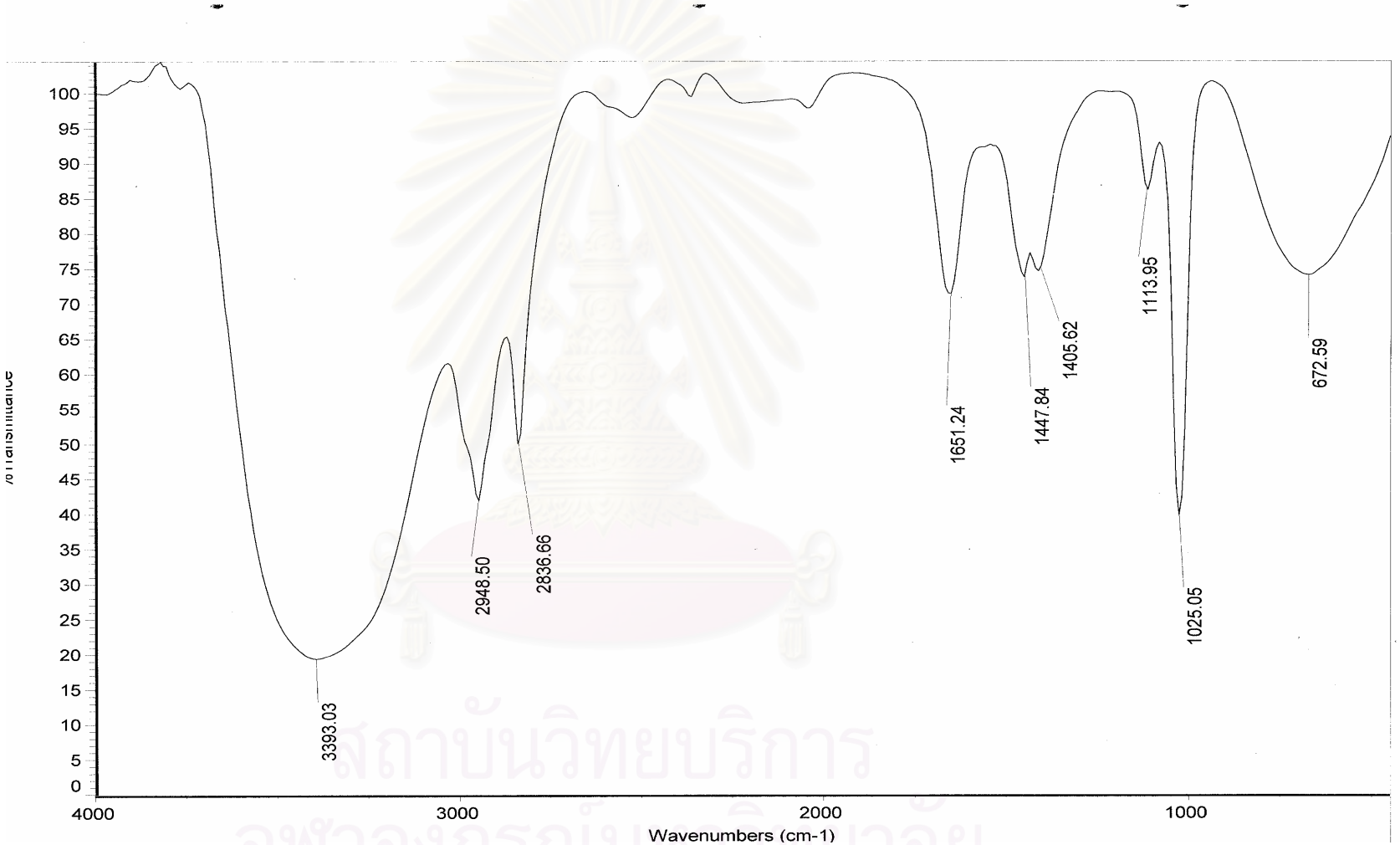


Figure 20 IR spectrums of compound BE3

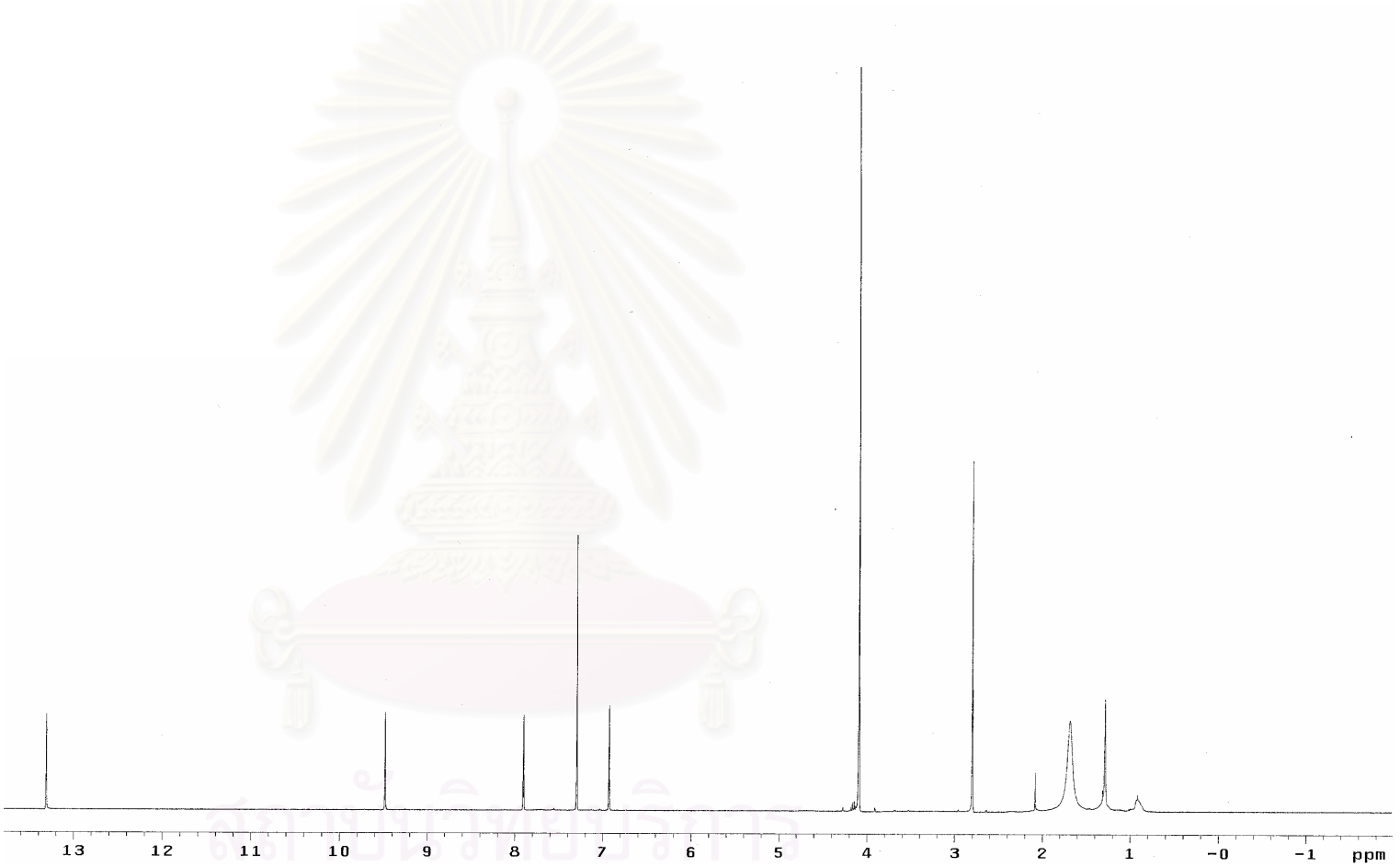


Figure 21 ^{1}H-NMR spectrum of compound BE 3

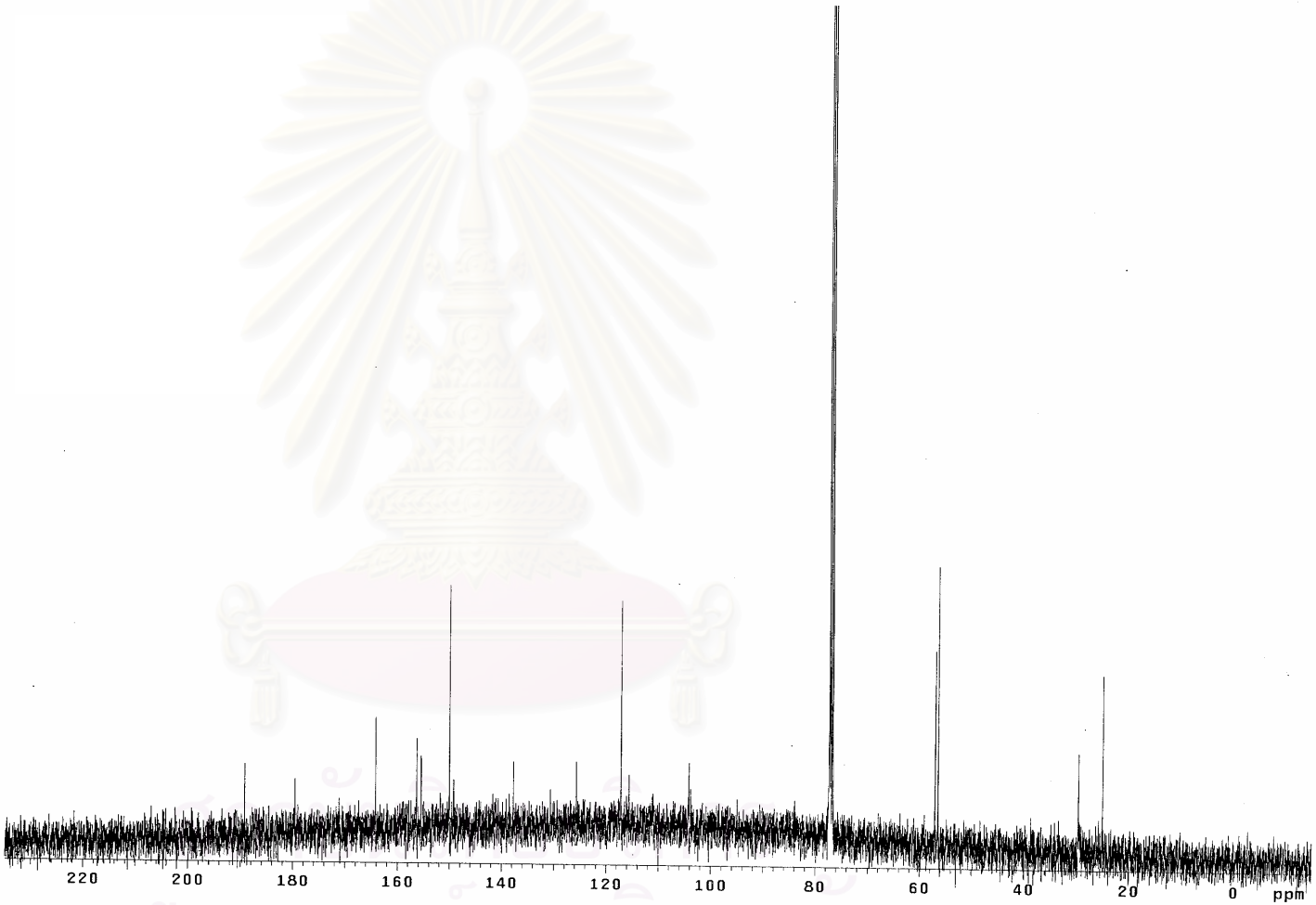


Figure 23 ^{13}C-NMR spectrum of compound BE3

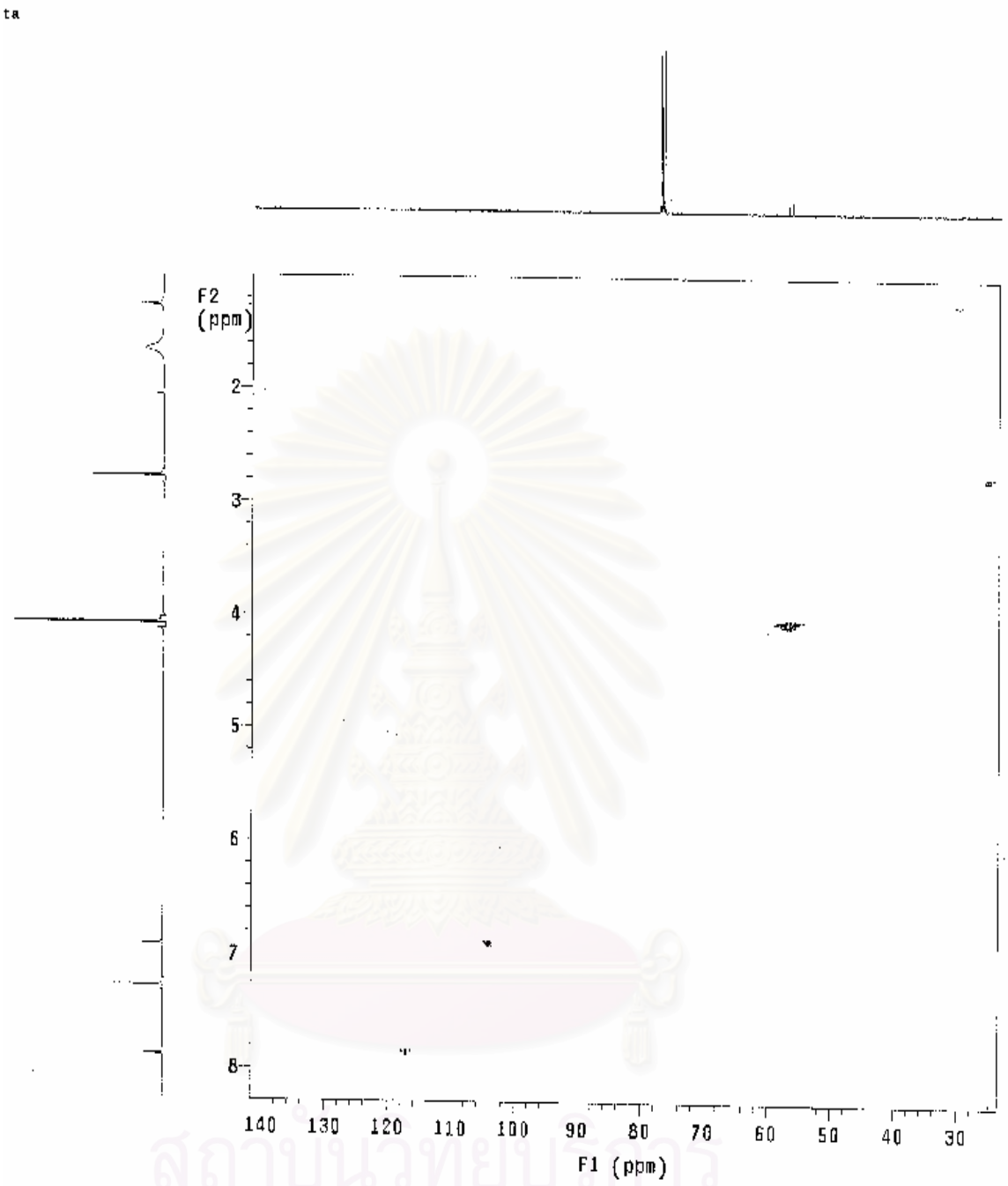


Figure 23 HSQC spectrum of compound BE3

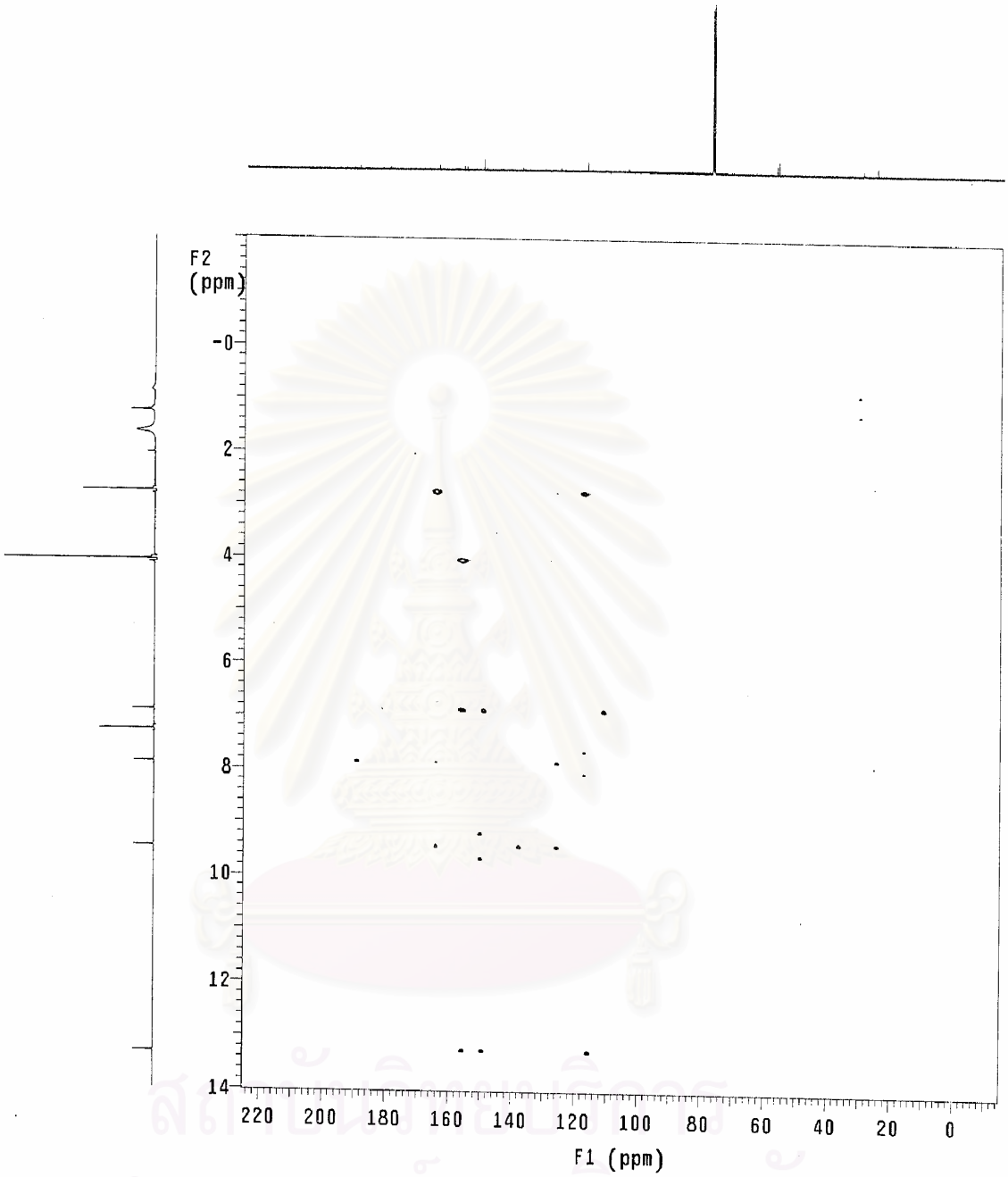


Figure 24 HMBC spectrums of compound BE3

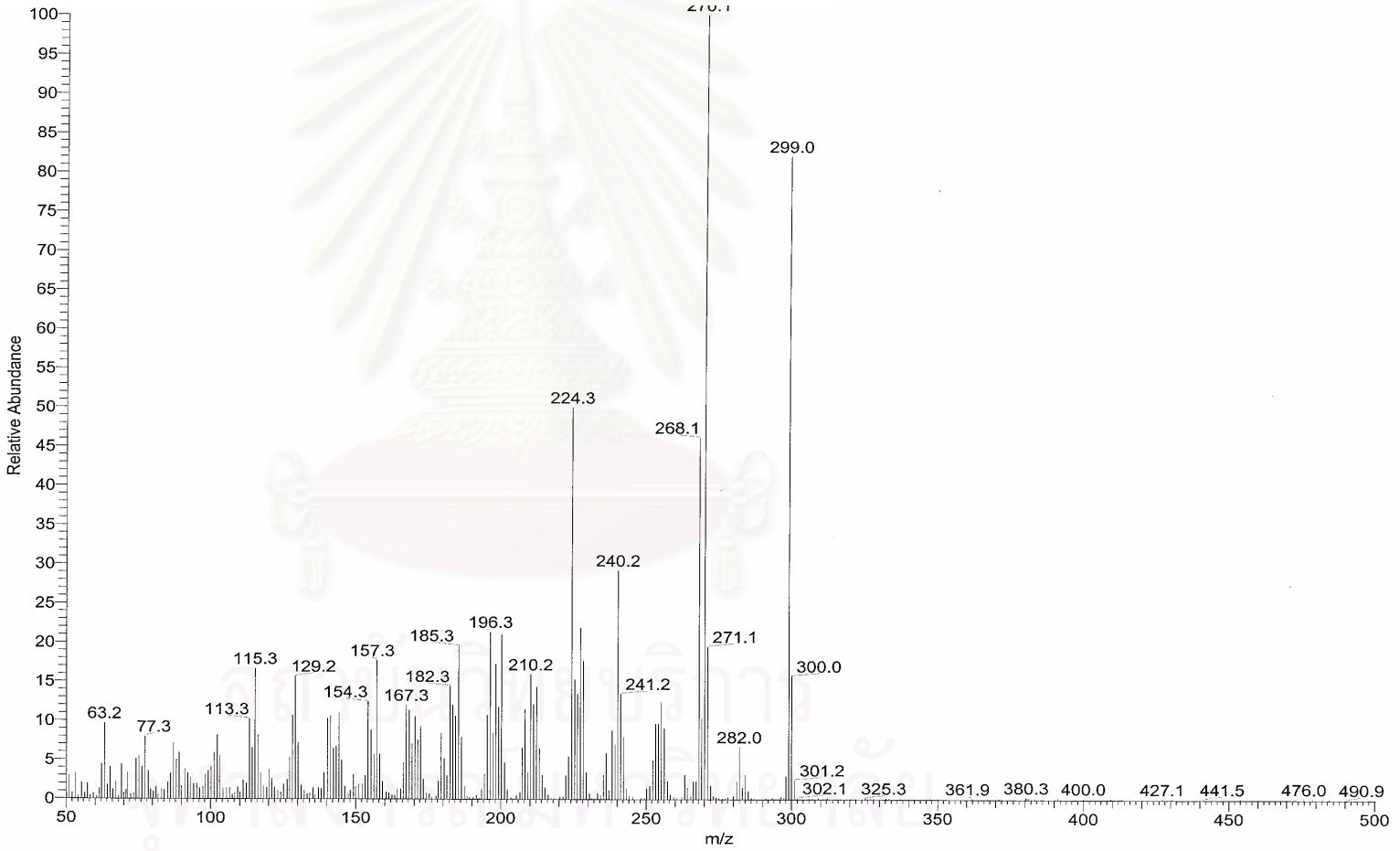
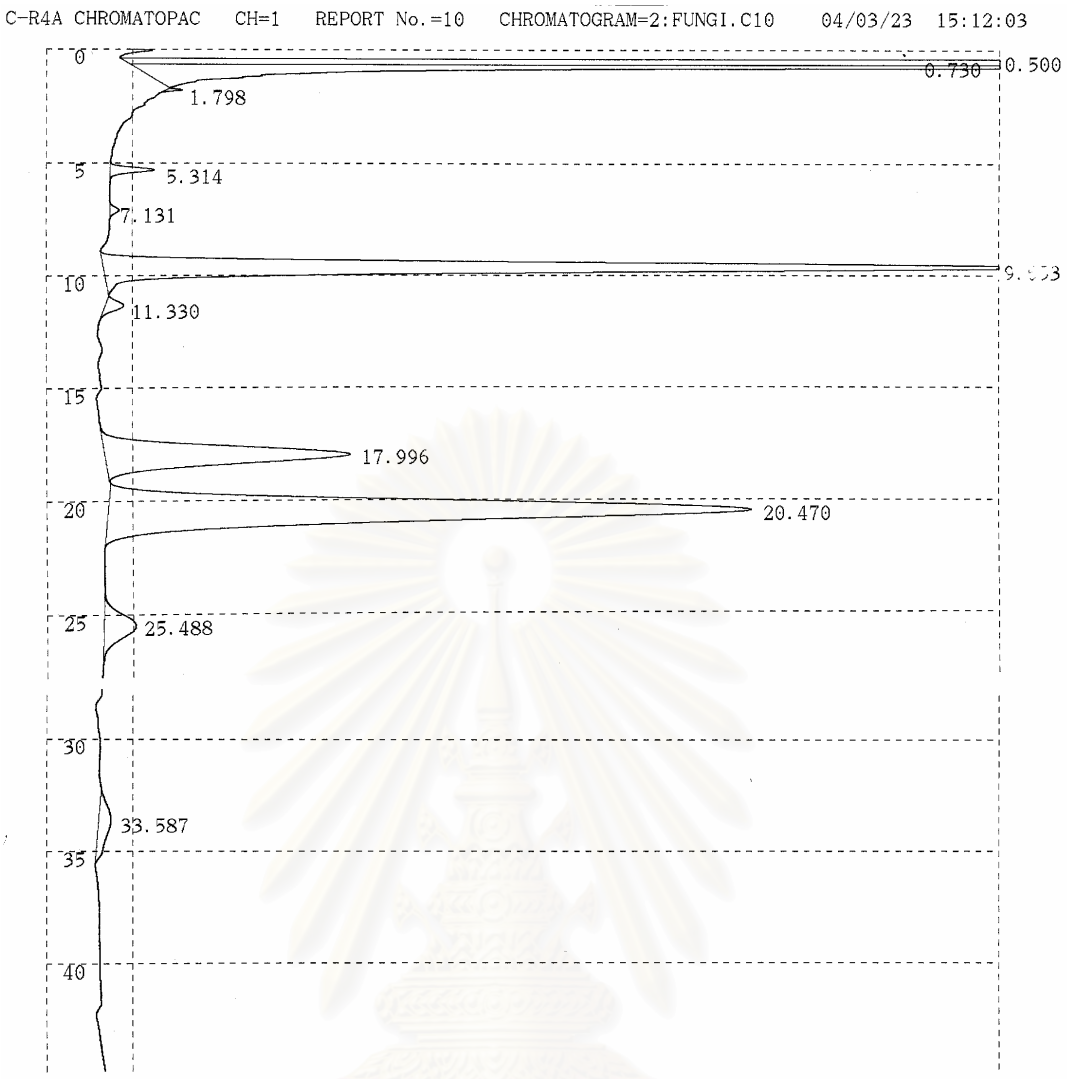


Figure 25 EI-MS spectrum of compound BE3



** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.5	8219622	1205766	E			
	2	0.73	434142	134771	V			
	3	1.798	394	71				
	4	5.314	3417	206				
	5	7.131	832	41				
	6	9.653	129735	4371				
	7	11.33	2542	84				
	8	17.996	59249	1132				
	9	20.47	178086	2971				
	10	25.488	11301	151				
	11	33.587	6670	56				
		TOTAL	9045986	1349620			0	

Figure 26 Result of fatty acid composition in mixture MH1 detected by GC

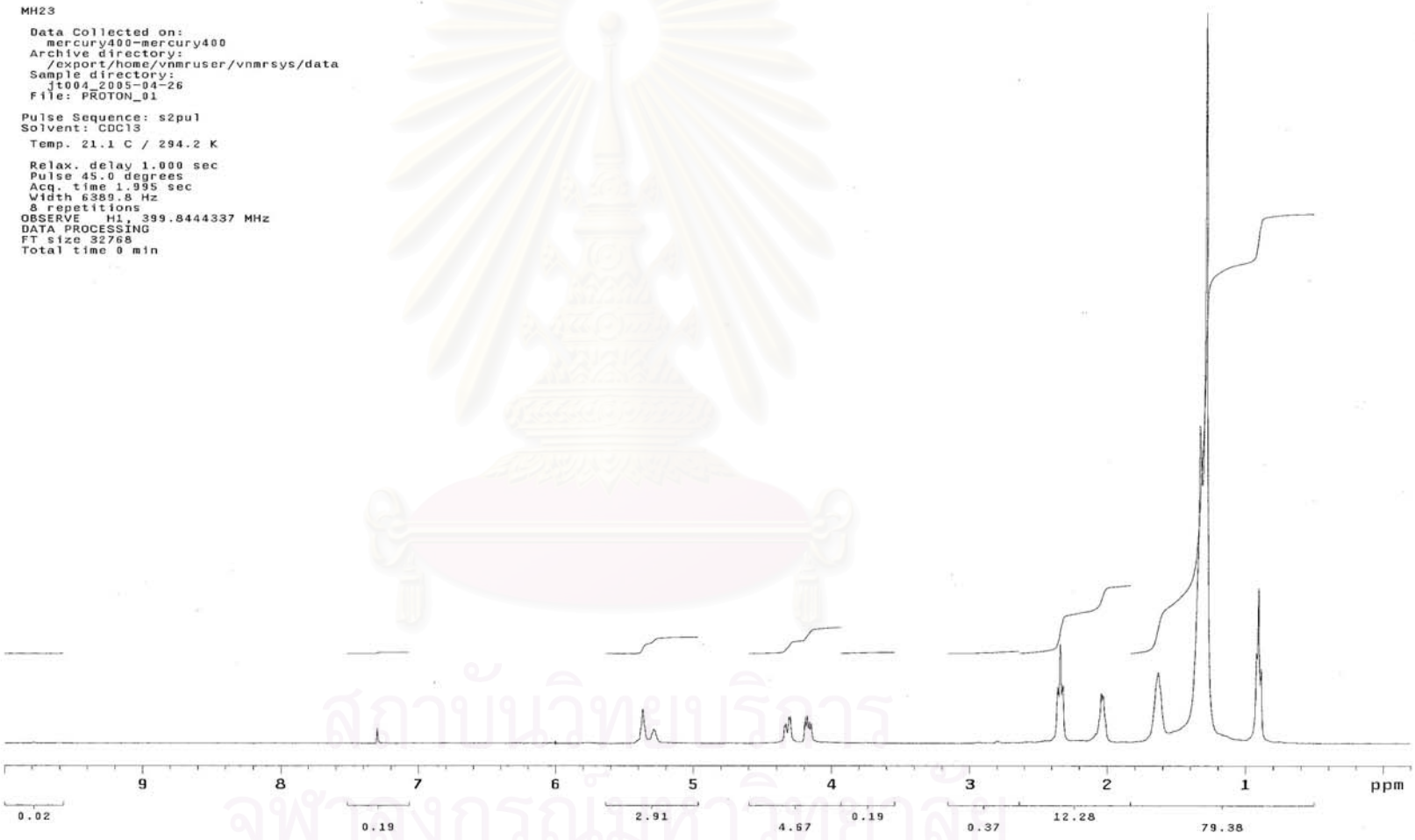


Figure 27 ^{1}H-NMR spectrum of mixture MH 1

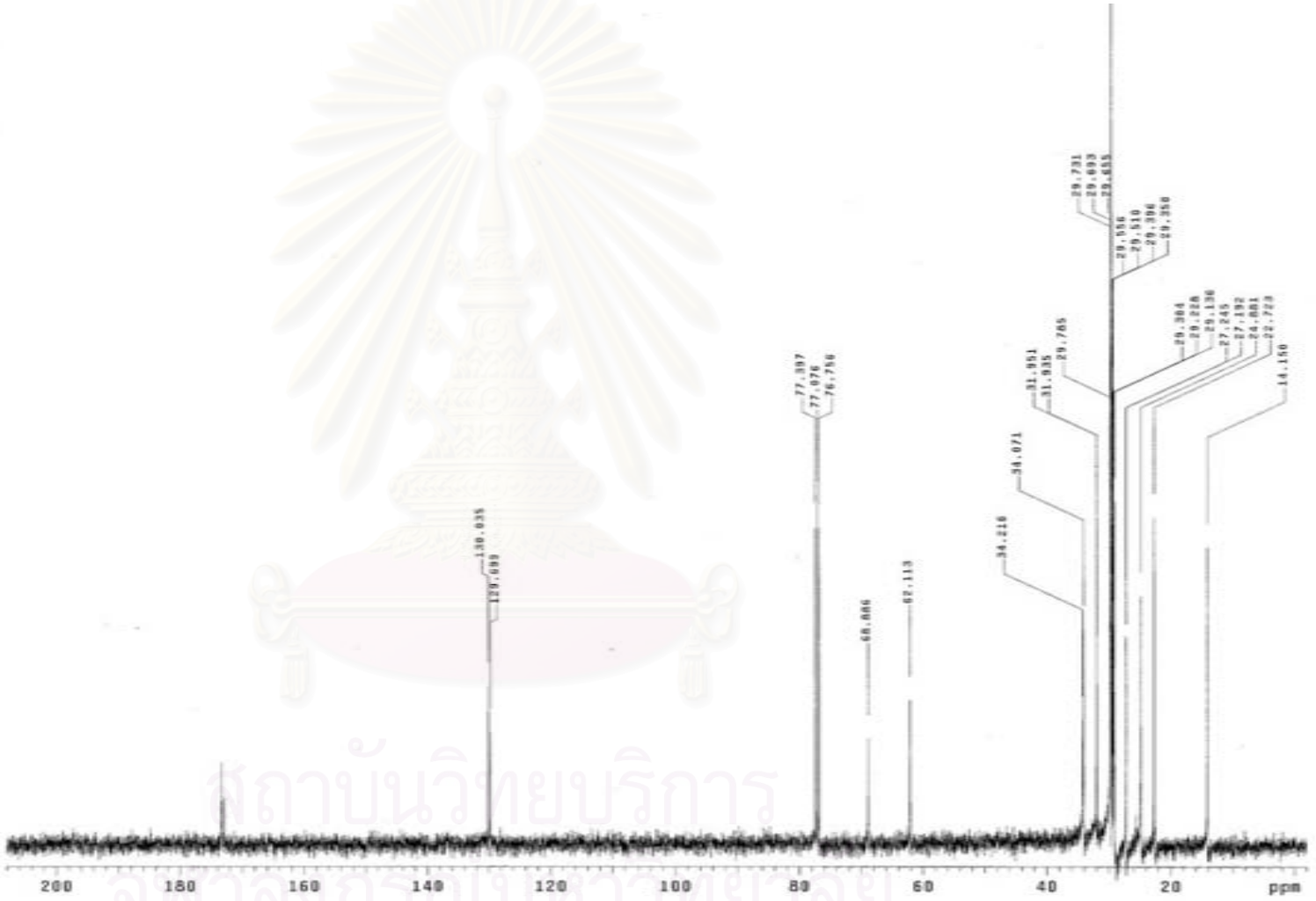


Figure 28 ^{13}C-NMR spectrum of mixture MH1

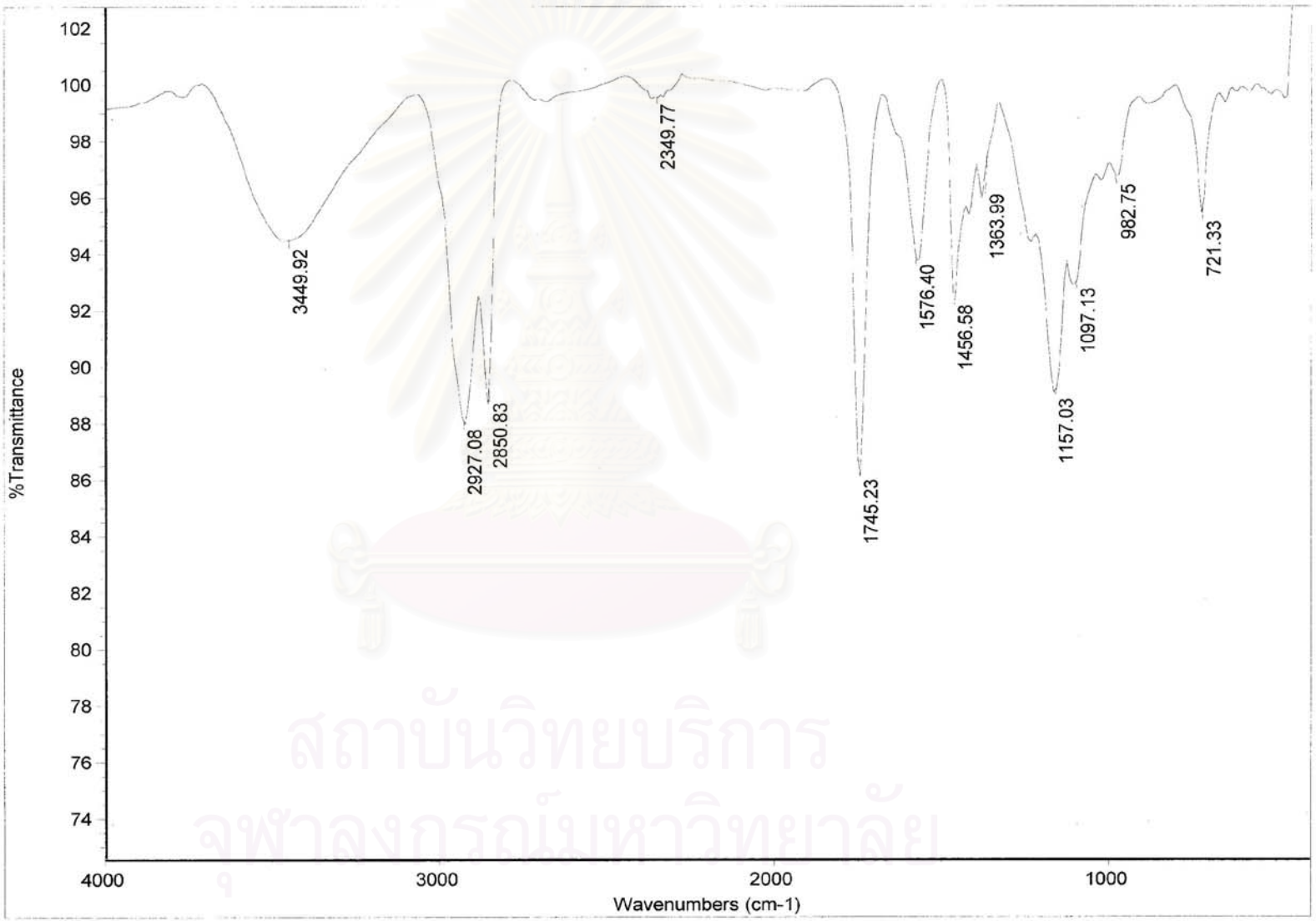


Figure 29 IR spectrum of mixture MH1

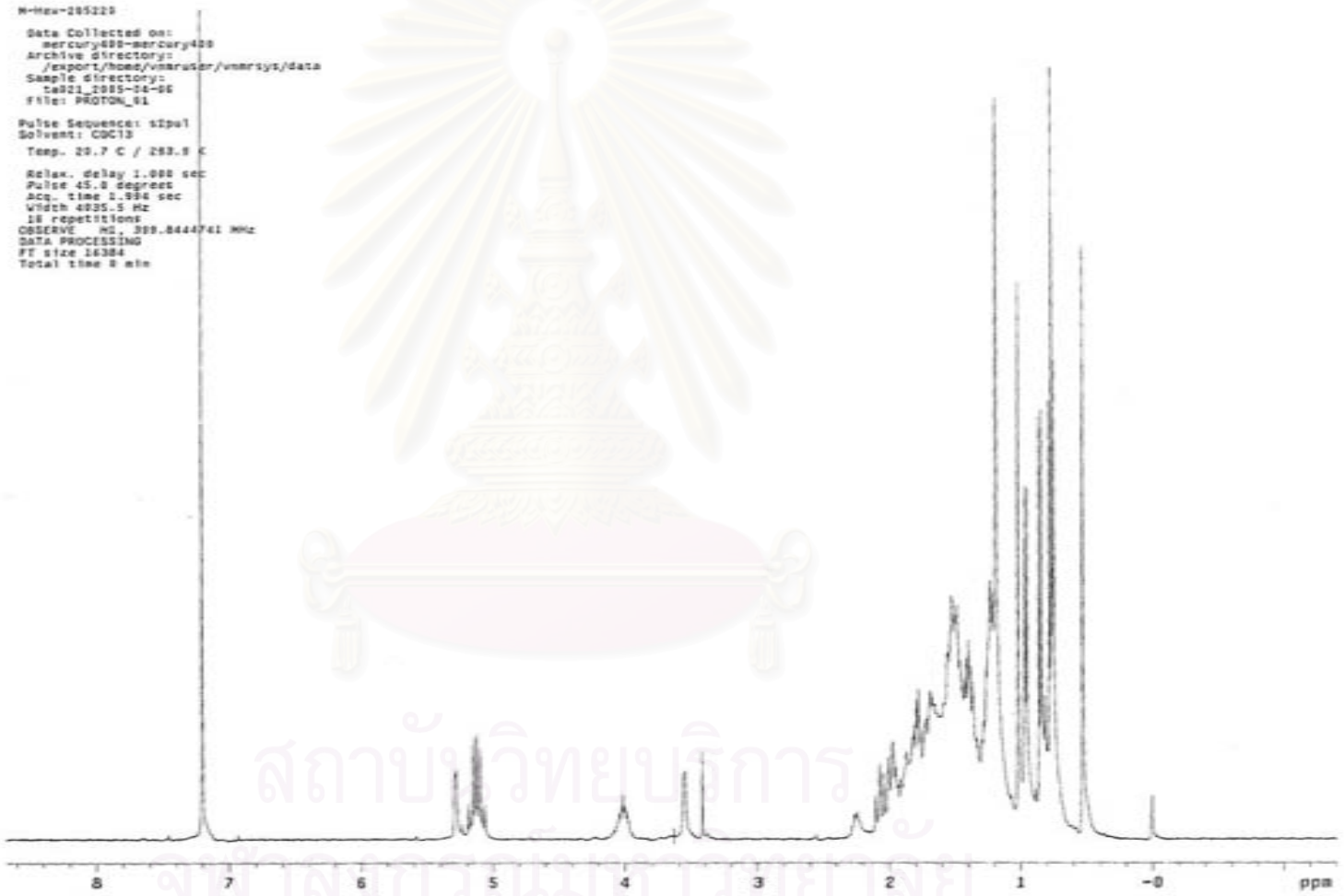


Figure 30 ^{1}H-NMR spectrum of compound MH 2

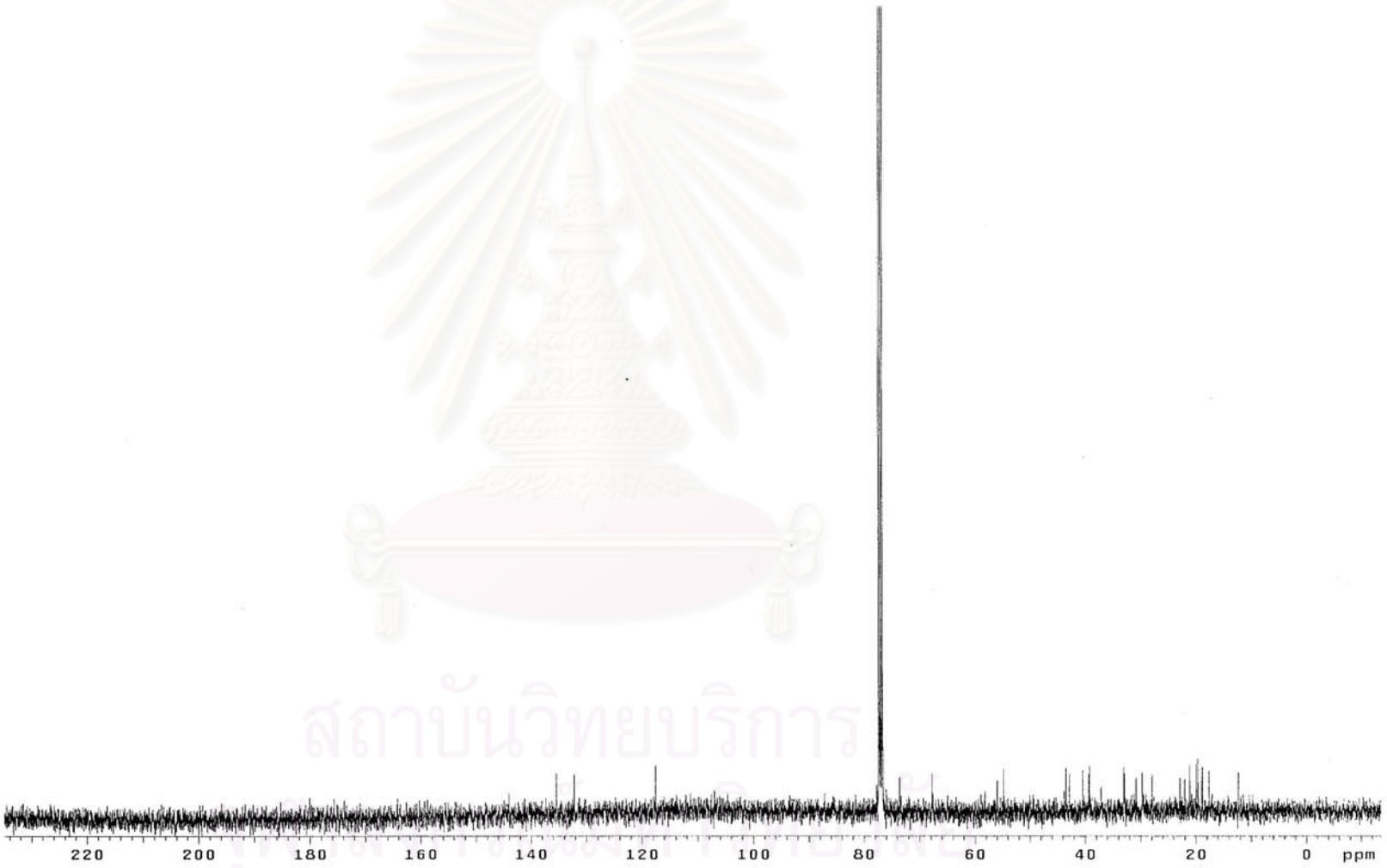


Figure 31 ^{13}C-NMR spectrum of compound MH2

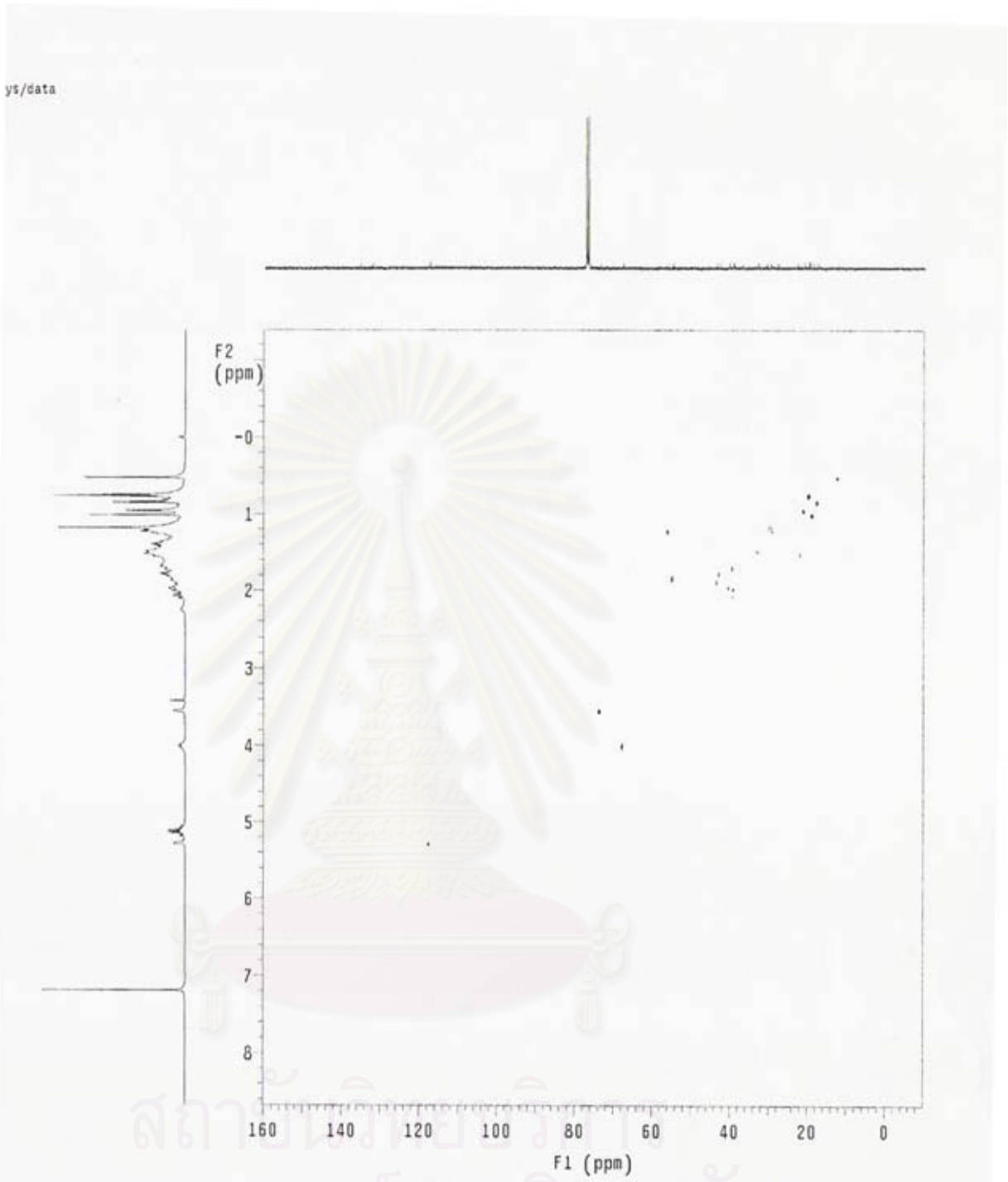


Figure 32 HSQC spectrums of compound MH2

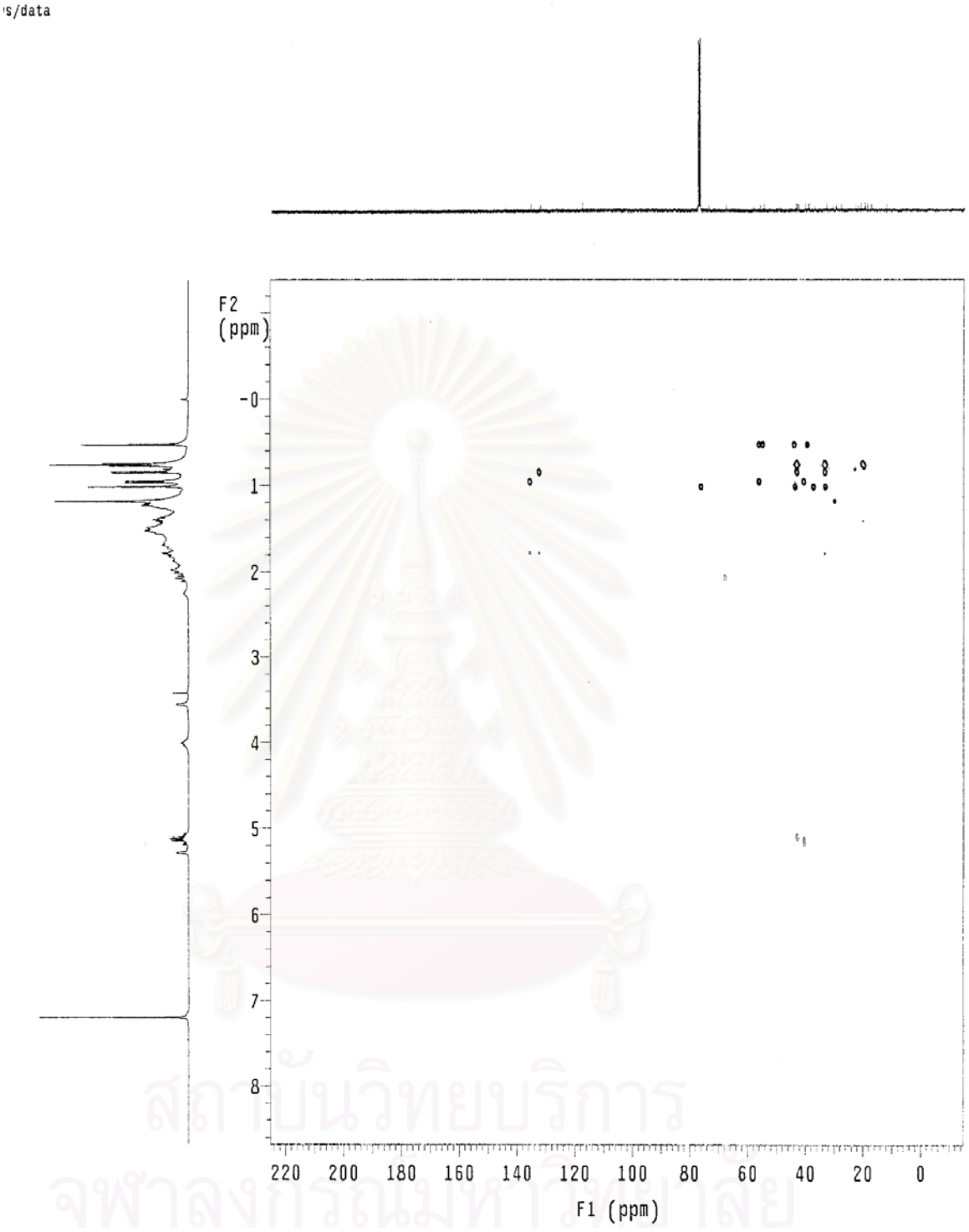


Figure 33 HMBC spectrums of compound MH2

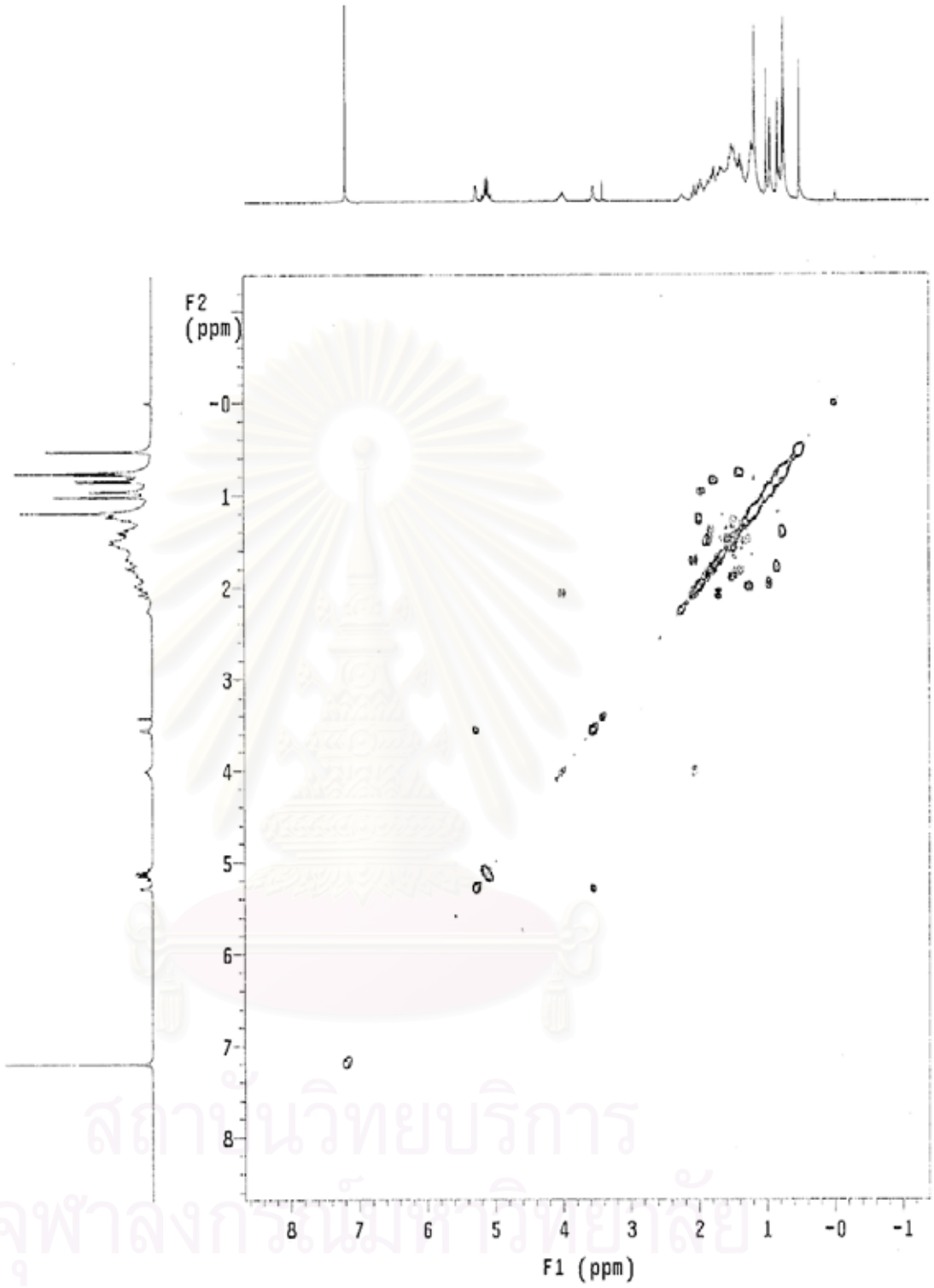


Figure 34 COSY spectrums of compound MH2

Figure 35 NOESY spectrums of compound MH2

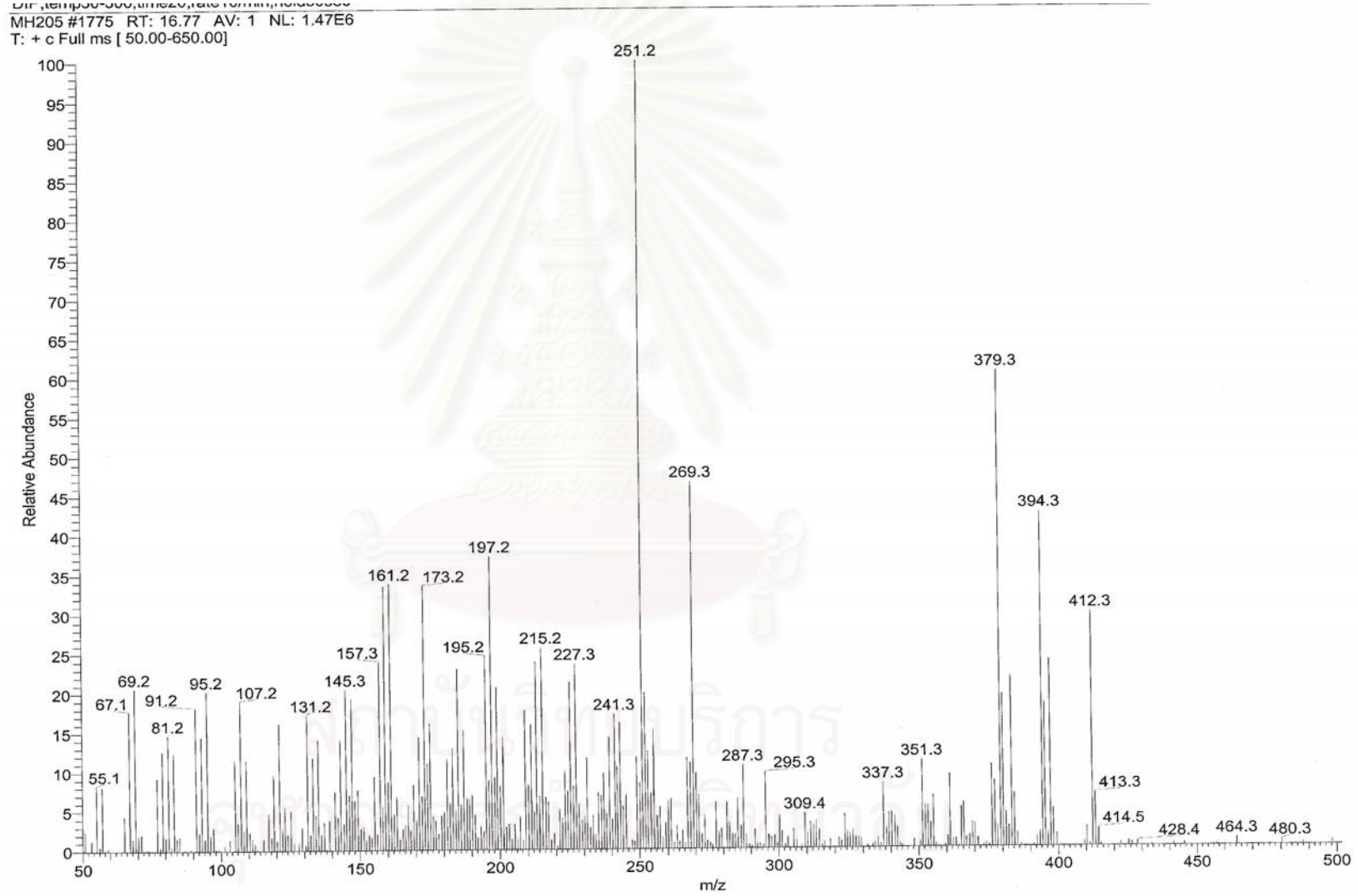


Figure 36 EI-MS spectrum of compound MH2

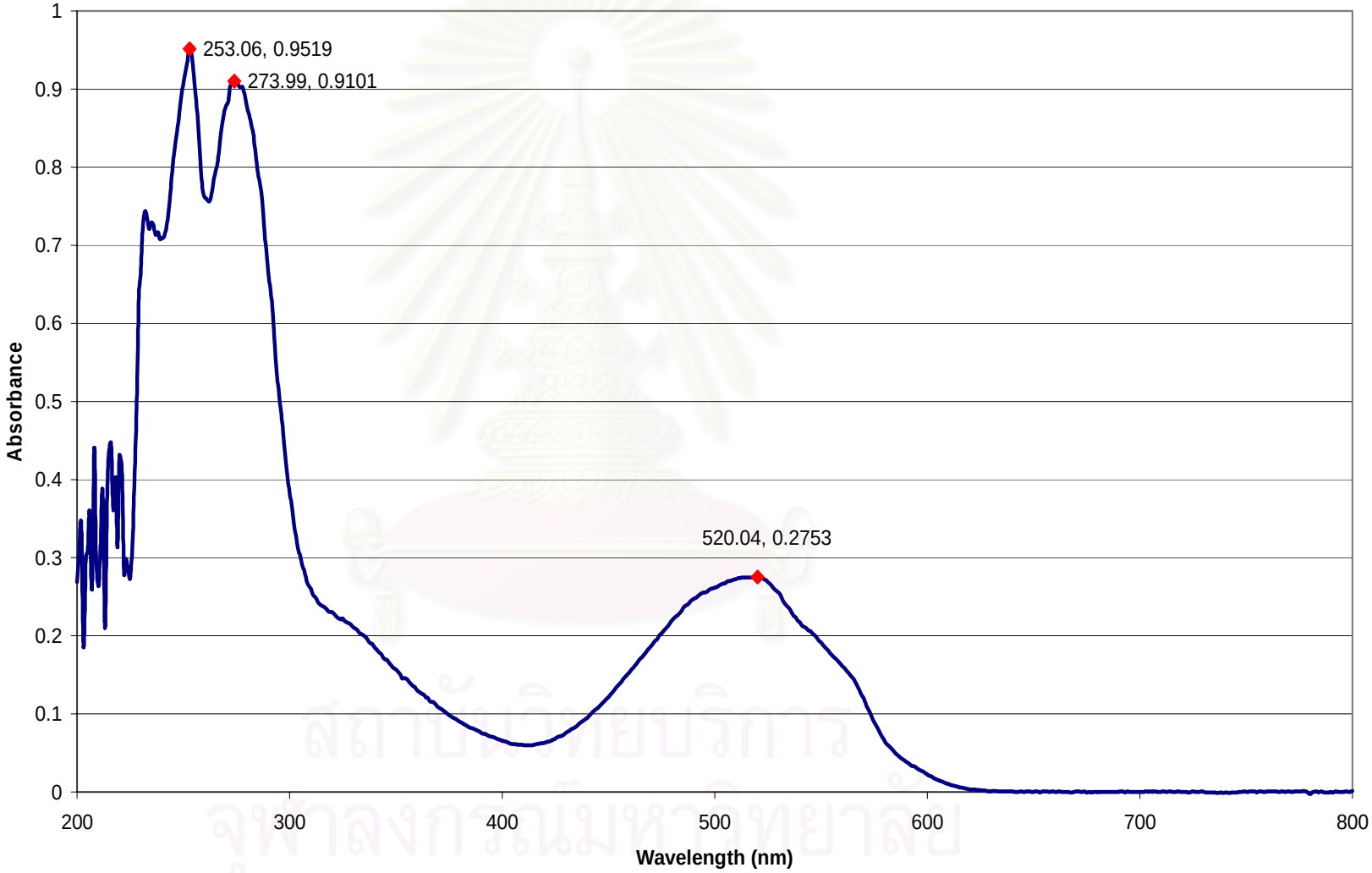


Figure 37 UV spectrum of compound MH2

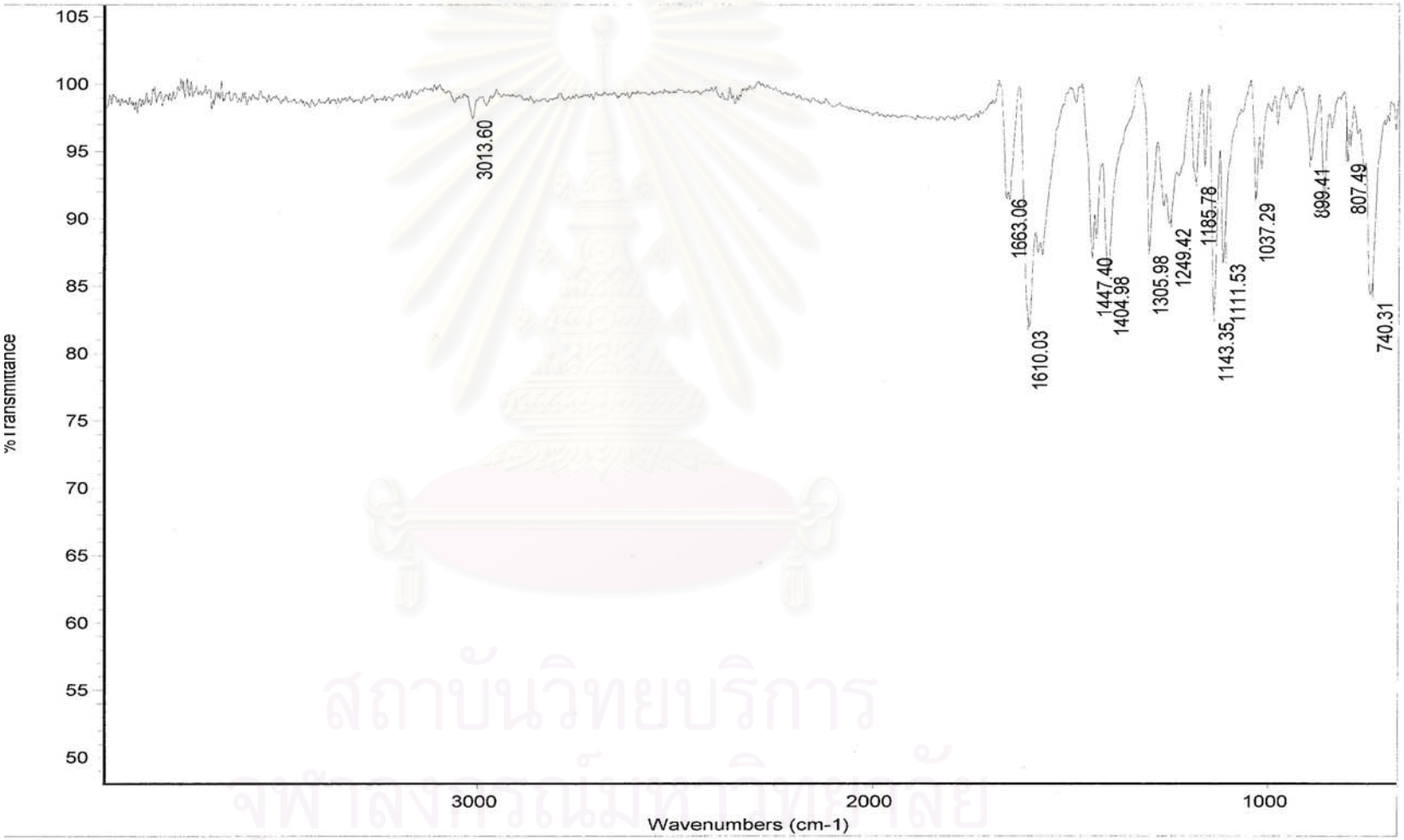


Figure 38 IR spectrums of compound M1

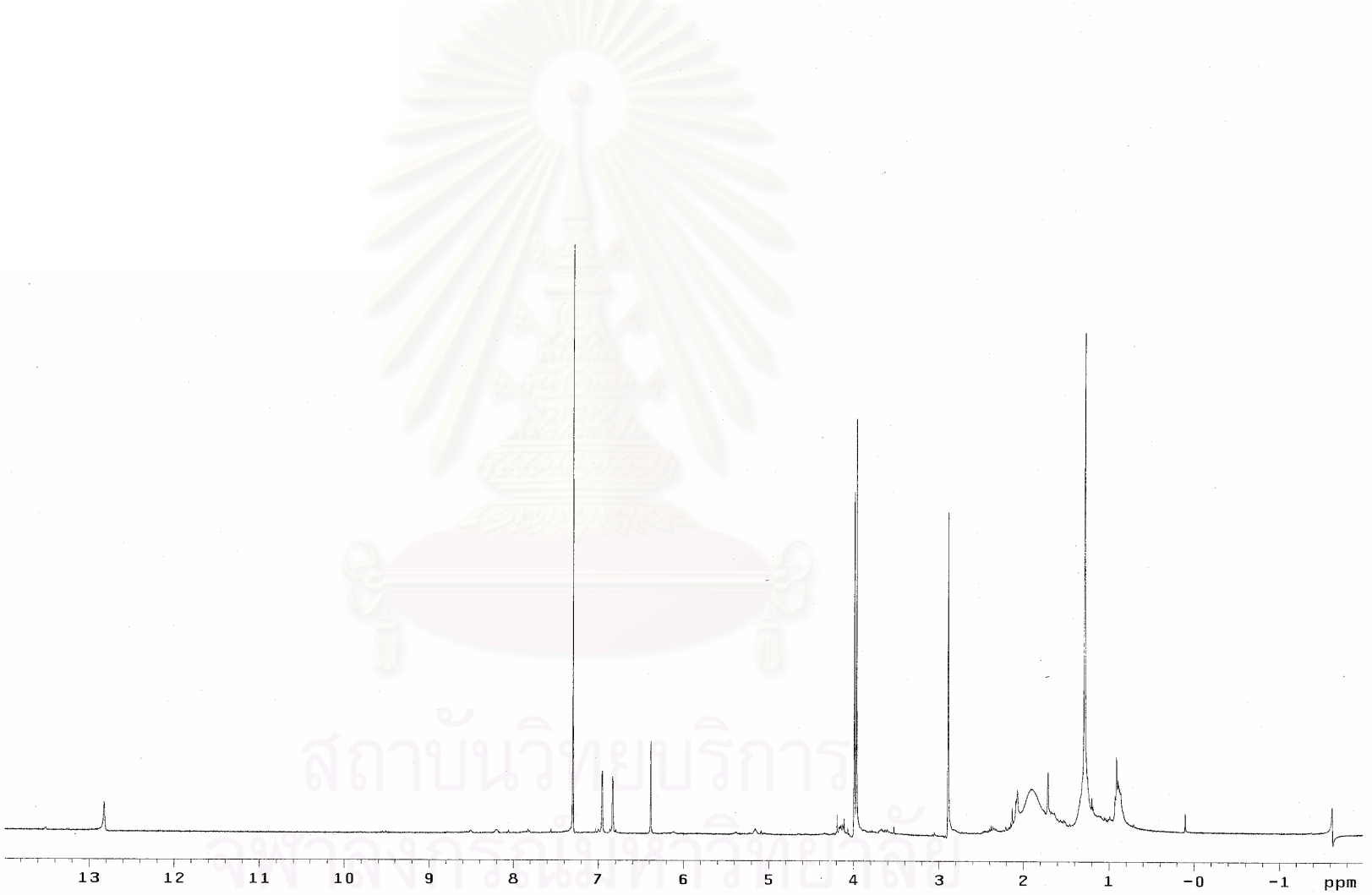


Figure 39 ^{1}H-NMR spectrum of mixture M1

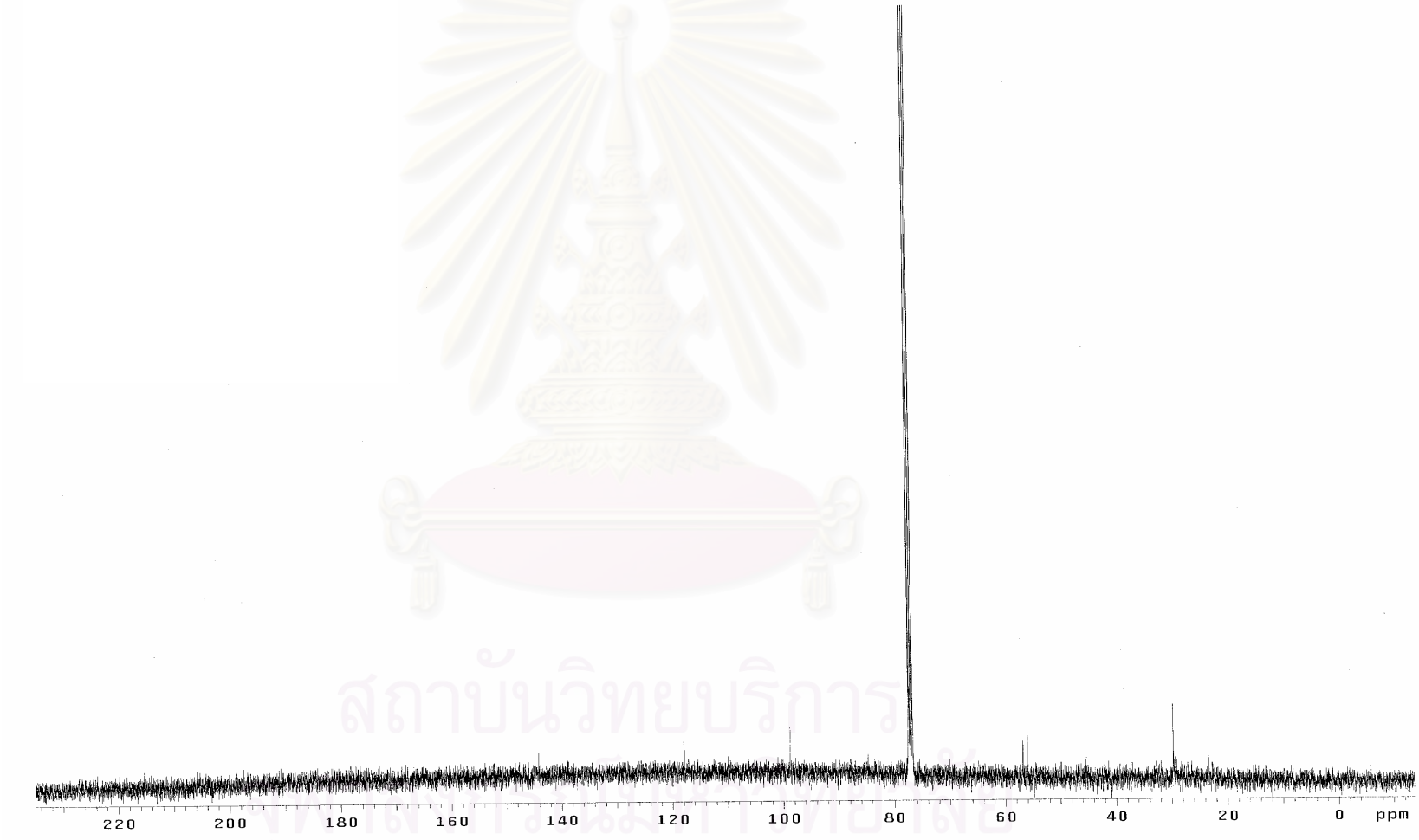


Figure 40 ^{13}C-NMR spectrum of mixture M1

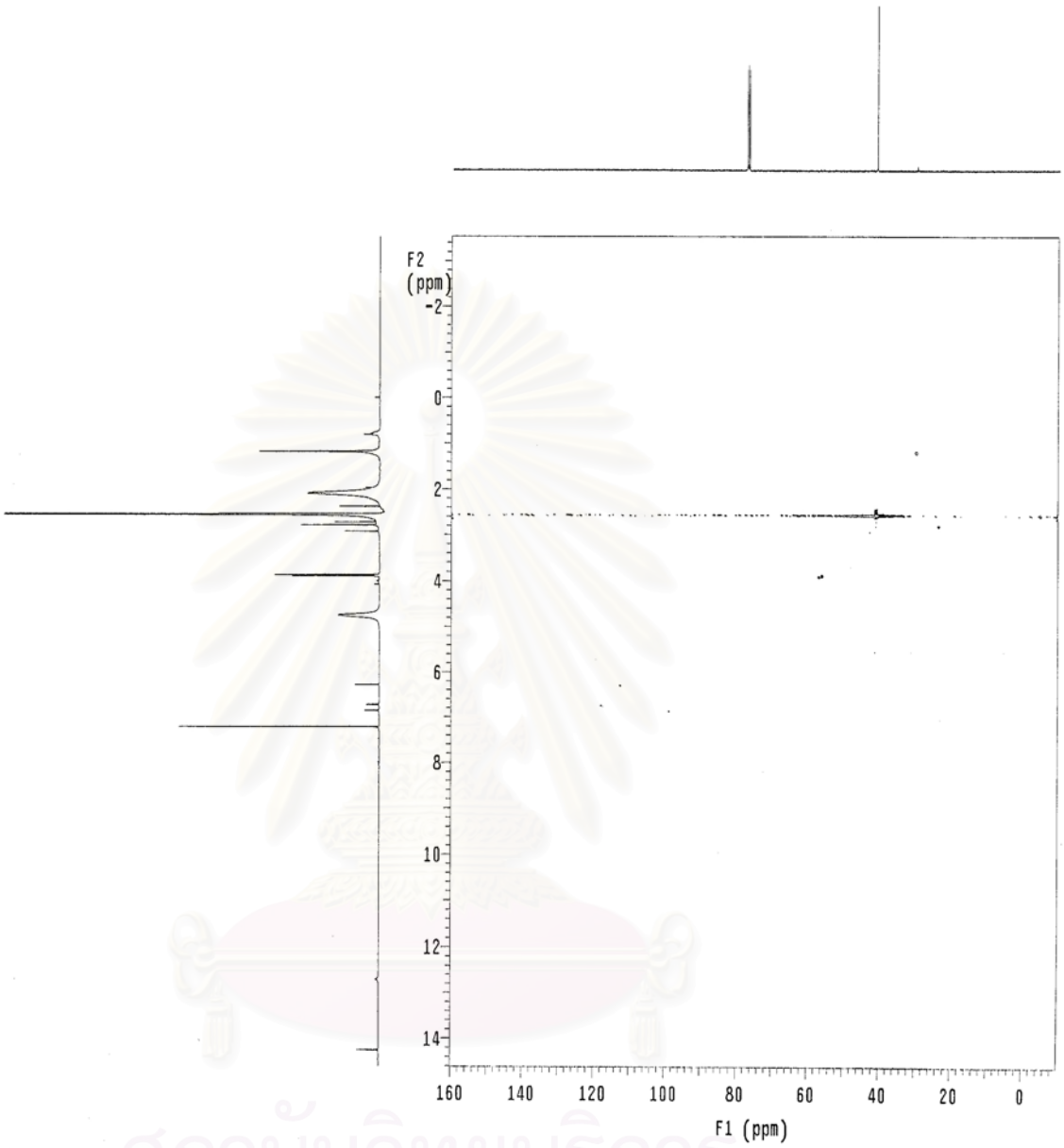


Figure 41 HSQC spectrums of compound M1

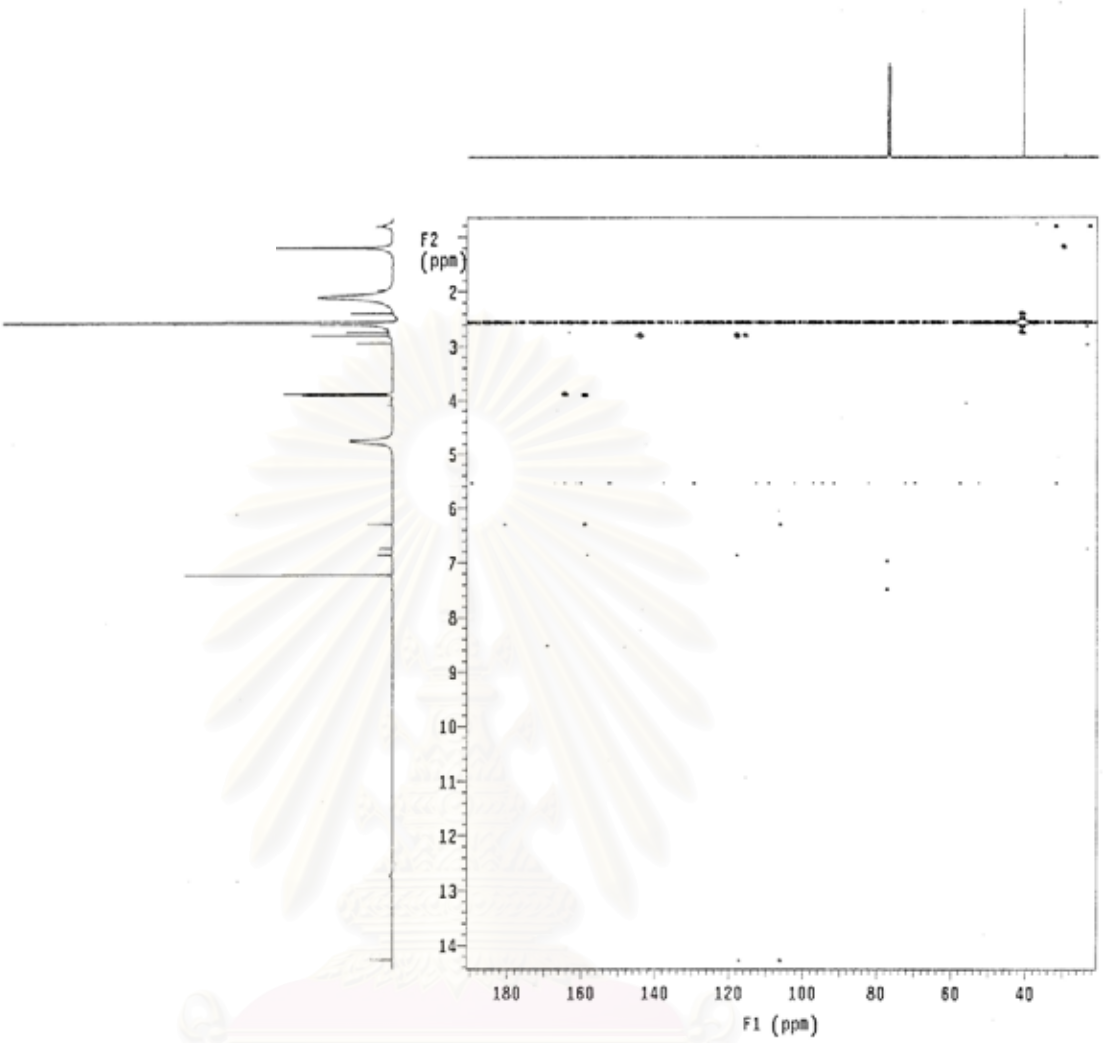


Figure 42 HMBC spectrums of compound M1

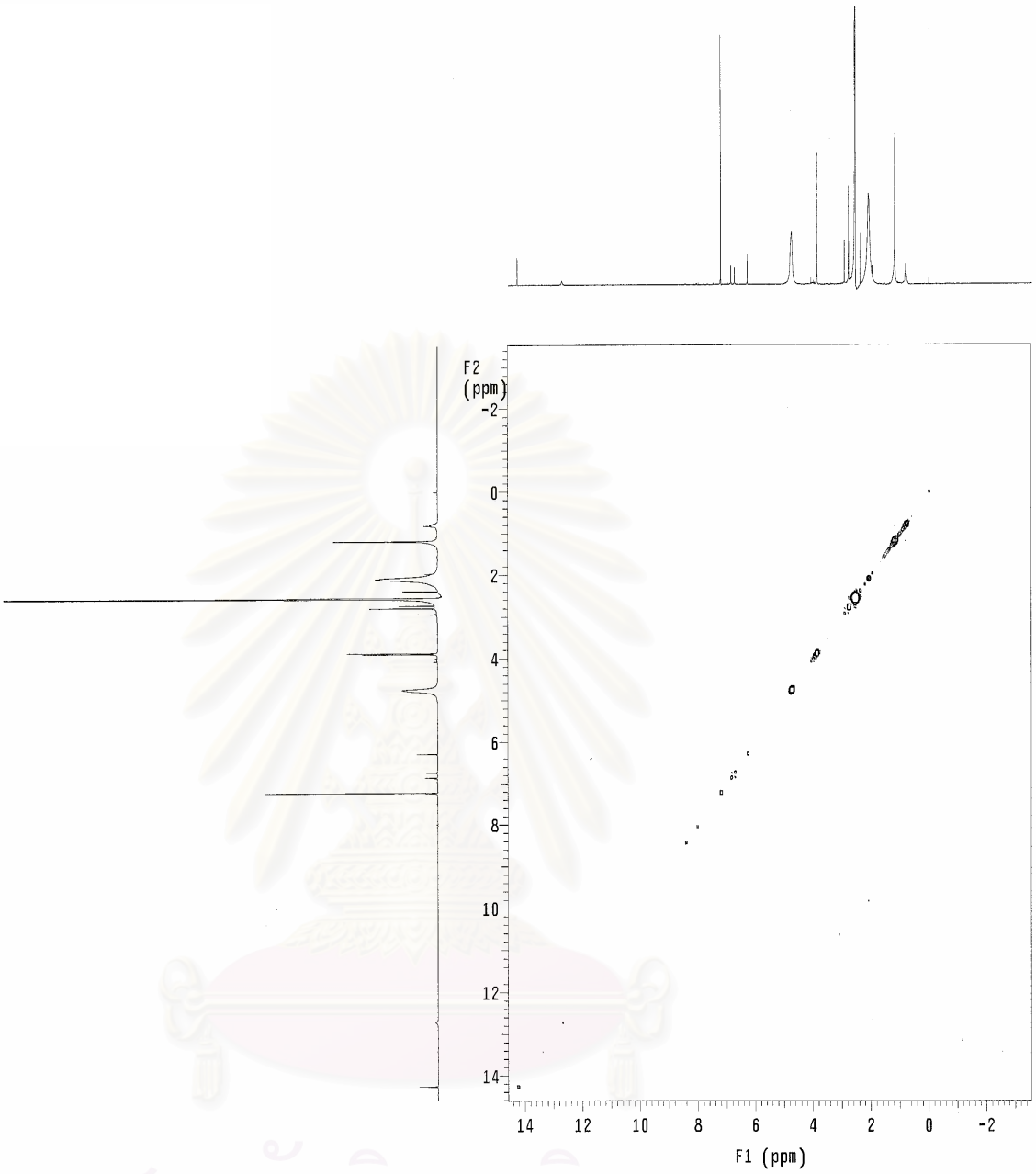


Figure 43 COSY spectrums of compound M1

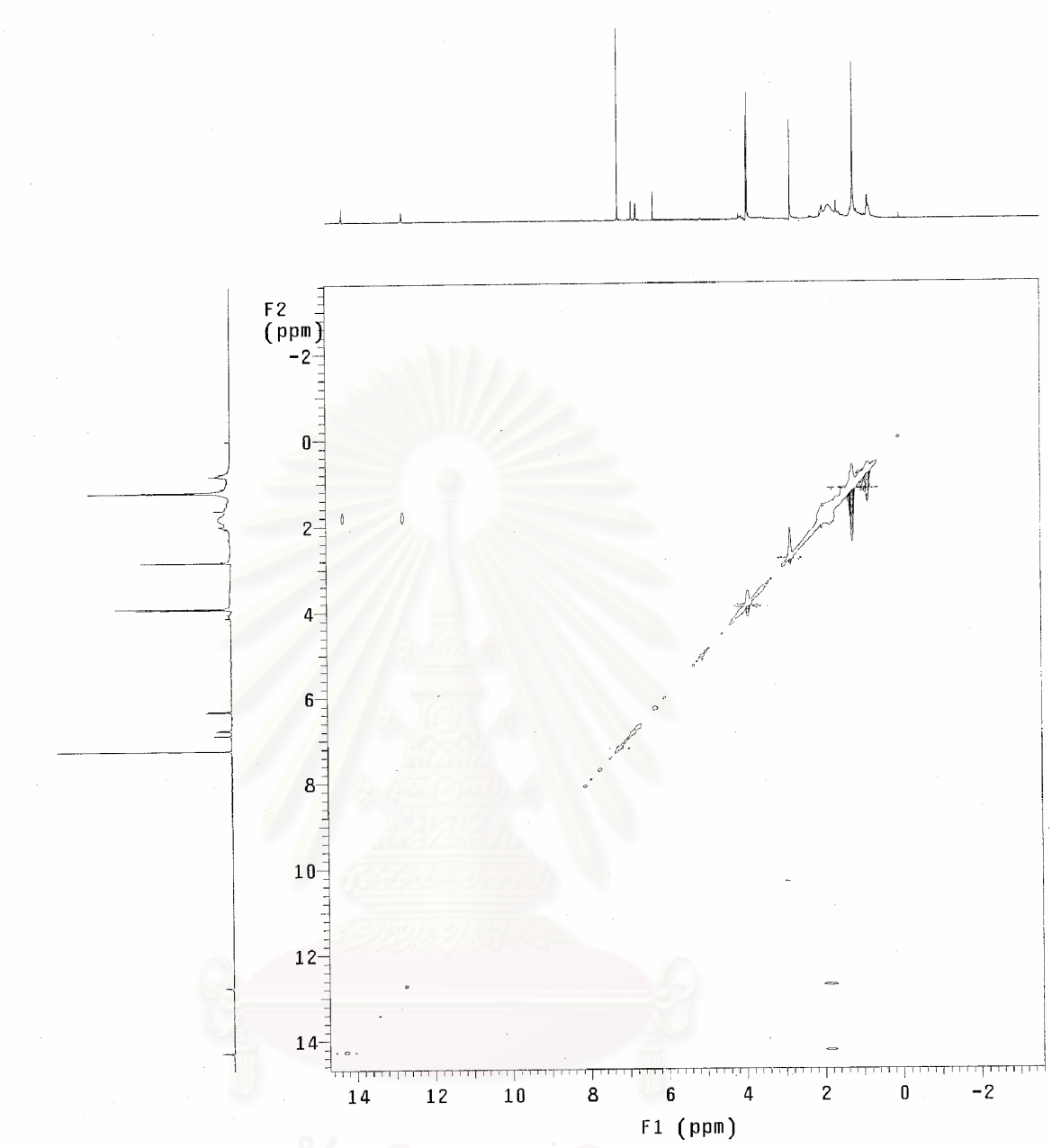


Figure 44 NOESY spectrums of compound M1

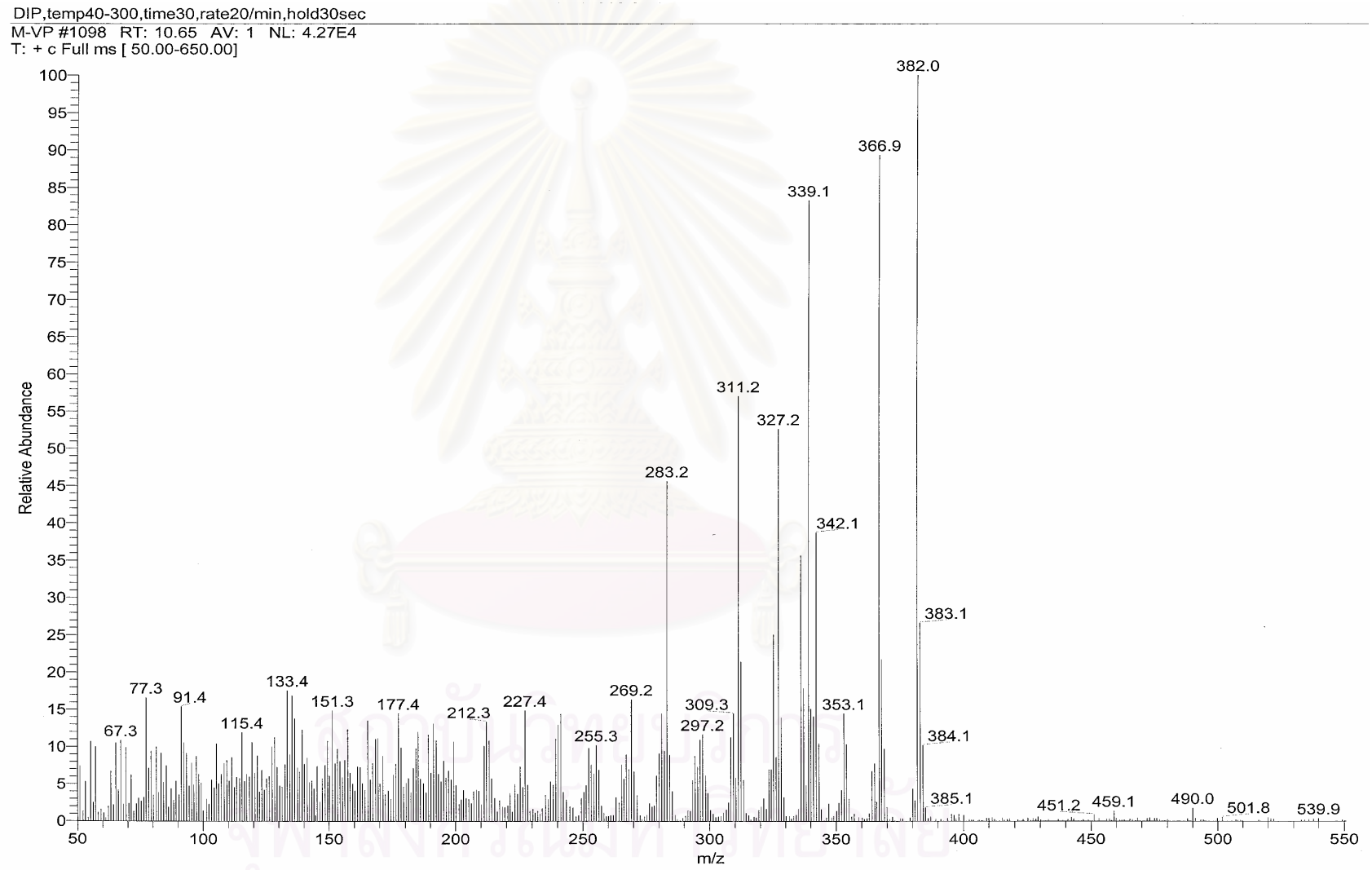


Figure 45 EI-MS spectrums of compound M1

BIOGRAPHY

Miss Wantanee Tommeurd was born on February 20, 1980 in Chonburi province, Thailand. She graduated with a Bachelor Degree of Science in Biochemistry Department from the Faculty of Science, Kasetsart University, Thailand in 2001. She had been studying for a Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand since 2002.