สารทุติยภูมิของราเอนโดไฟต์จากใบกระท้อน *(Sandoricum koetjape)*

นางสาวศรีนวล ตันสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

SECONDARY METABOLITES OF ENDOPHYTIC FUNGI FROM SANTOL *(Sandoricum koetjape)* LEAVES

Miss Srinuan Tansuwan

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic year 2006

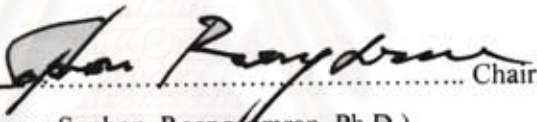
Thesis Title	SECONDARY METABOLITES OF ENDOPHYTIC FUNGI FROM SANTOL *(Sandoricum koetjape)* LEAVES
By	Miss Srinuan Tansuwan
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Surachai Pornpakakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Faculty of Science

(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE



..... Chairman

(Professor Sophon Roengsumran, Ph.D.)

Surachai Pornpakakul ..... Thesis Advisor

(Assistant Professor Surachai Pornpakakul, Ph.D.)

Prakitsin Sihanonth ..... Member

(Associate Professor Prakitsin Sihanonth, Ph.D.)

N. Ngamrojanavanich ..... Member

(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

Jittra Piapukiew ..... Member

(Jittra Piapukiew, Ph.D.)

S. Thienhirun ..... Member

(Surang Thienhirun, Ph.D.)

นางสาวศรีนวล ตันสุวรรณ : สารทุติยภูมิของราเอนโดไฟต์จากใบกระท้อน *(Sandoricum koetjape)*. [SECONDARY METABOLITES OF ENDOPHYTIC FUNGI FROM SANTOL *(Sandoricum koetjape)* LEAVES] อ. ที่ปรึกษา : ผศ. ดร.สุรชัย พรภคกุล, 159 หน้า.

ราเอนโดไฟต์จำนวน 213 ไอโซเลตแยกได้จากใบกระท้อน *Sandoricum koetjape* จาก 10 จังหวัด จากลักษณะทางสัณฐานวิทยาสามารถจำแนกราเหล่านั้นได้เป็น *Alternaria* sp. (2 ไอโซเลต), *Aspergillus* sp. (3 ไอโซเลต), *Cladosporium* sp. (5 ไอโซเลต), *Colletotrichum* sp. (6 ไอโซเลต), *Fusarium* sp. (8 ไอโซเลต), *Pestalotia* sp. (4 ไอโซเลต), *Phomopsis* sp. (13 ไอโซเลต), Xylariaceae (18 ไอโซเลต) และ Mycelia sterilia (155 ไอโซเลต) ราเอนโดไฟต์เหล่านี้มีจำนวน 54 ไอโซเลตที่ออกฤทธิ์ยับยั้งจุลินทรีย์ทดสอบ (อย่างน้อย 2 สายพันธุ์) โดยมีบริเวณการยับยั้ง 1-12 มิลลิเมตร และราเอนโดไฟต์มีฤทธิ์ยับยั้งแบคทีเรียแกรมบวก *Bacillus subtilis* และ *Staphylococcus aureus* ได้มากกว่าแบคทีเรียแกรมลบ *Escherichia coli* และ *Pseudomonas aeruginosa* คัดเลือกราเอนโดไฟต์ไอโซเลต PB-30 และ MK-22 เพื่อนำมาศึกษา จากลักษณะทางสัณฐานวิทยาและการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ ITS ของ DNA เชื้อรา พบว่า PB-30 และ MK-22 พิสูจน์เอกลักษณ์เป็น *Xylaria* sp. แยกสารประกอบทางเคมีของราเอนโดไฟต์ PB-30 ได้สารประกอบ 6 ชนิด ได้แก่ D-mannitol **1**, mellein **2**, 4-hydroxymellein **3**, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **4**, 7-hydroxy-8-methoxy-3,6-dimethyl-dibenzofuran-1,4-dione **5** และ 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **6** แยกสารประกอบจากส่วนสกัดเมธานอลจากเส้นใยของราเอนโดไฟต์ MK-22 ได้ integric acid **7** สารประกอบ **2-6** นำไปทดสอบฤทธิ์ต้านเชื้อมาลาเรีย *Plasmodium falciparum* (สายพันธุ์ K1) สารประกอบ **4** และ **5** มีฤทธิ์ยับยั้งเชื้อมาลาเรียที่ค่า IC_{50} เท่ากับ 1.83 และ 6.69 μM ตามลำดับ ขณะที่สารประกอบ **2**, **3** และ **6** ไม่มีฤทธิ์ยับยั้งเชื้อมาลาเรีย ทดสอบความเป็นพิษของสารประกอบ **2-7** ต่อเซลล์มะเร็งของมนุษย์ 5 ชนิด ได้แก่ BT474 (เต้านม) CHAGO (ปอด) HEP-G2 (ตับ) KATO-3 (กระเพาะอาหาร) และ SW620 (ลำไส้ใหญ่) สารประกอบ **2** และ **3** ไม่มีฤทธิ์ยับยั้งเซลล์มะเร็งทุกชนิด ขณะที่สารประกอบ **4-7** มีฤทธิ์ยับยั้งเซลล์มะเร็งทุกชนิด นอกจากนี้ สารประกอบ **4** และ **5** มีความเป็นพิษต่อเซลล์ปกติ (Vero cells; African green monkey kidney fibroblasts) ที่ค่า IC_{50} เท่ากับ 1.34 และ >181 μM ตามลำดับ

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

ปีการศึกษา ....2549..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

4573838223 : MAJOR Biotechnology

KEY WORD: *Sandoricum koetjape* / endophytic fungi / secondary metabolite / cytotoxicity

SRINUAN TANSUWAN : SECONDARY METABOLITES OF ENDOPHYTIC FUNGI FROM SANTOL *(Sandoricum koetjape)* LEAVES. THESIS ADVISOR : ASST. PROF. SURACHAI PORNPAKAKUL, Ph.D. 159 pp.

The endophytic fungi, 213 isolates were isolated from *Sandoricum koetjape* leaves collected in 10 provinces. Based on fungal morphology, those fungi were identified as *Alternaria* sp. (2 isolates), *Aspergillus* sp. (3 isolates), *Cladosporium* sp. (5 isolates), *Colletotrichum* sp. (6 isolates), *Fusarium* sp. (8 isolates), *Pestalotia* sp. (4 isolates), *Phomopsis* sp. (13 isolates), Xylariaceae (18 isolates), and Mycelia sterilia (155 isolates). Among these fungi, there are 54 isolates exhibited antimicrobial activity (at least against two microorganisms) with the inhibition zone of 1-12 mm and the endophytic fungi exhibited antimicrobial activity against gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, were more than against gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. The endophytes, PB-30 and MK-22, were selected for further studies. On the basis of fungal morphology and analysis of the DNA sequence of the ITS region, the fungi PB-30 and MK-22 were identified to *Xylaria* sp. Chemical investigation of the metabolites produced by the endophytic fungus PB-30 was carried out to afford 6 compounds including D-mannitol **1**, mellein **2**, 4-hydroxymellein **3**, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **4**, 7-hydroxy-8-methoxy-3,6-dimethyl-dibenzofuran-1,4-dione **5** and 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **6**. Isolation of metabolite from mycelia MeOH extract of the endophytic fungus MK-22 gave integric acid **7**. Compounds **2-6** were examined antiplasmodial activity against *Plasmodium falciparum* (K1 strain). Compounds **4** and **5** exhibited antiplasmodial activity with the IC_{50} values of 1.83 and 6.69 μM, respectively while compounds **2**, **3** and **6** were inactive. Cytotoxic activity of compound **2-7** were examined against 5 human cancer cell lines including, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon). Compound **2** and **3** were inactive while compound **4-7** exhibited cytotoxic activity against all cell lines. Moreover, compounds **4** and **5** exhibited cytotoxic activity against Vero cells (African green monkey kidney fibroblasts) with the IC_{50} values of 1.34 and >181 μM, respectively.

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

Academic year ...2006..... Advisor's signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and sincere gratitude to Assistant Professor Surachai Pornpakakul, my thesis advisor, who gave me the comments and knowledge, especially chemical compounds interpretation. I would like to thank Professor Sophon Roengsumran to concern about my work and gave some advice. I would like to thank Associate Professor Prakitsin Sihanonth for his suggestion on the method for identification of fungi.

Special thanks are to Associate Professor Amorn Petsom for help of cytotoxicity test and chemical explanation. I would like to thank Assistant Professor Nongnuj Muangsin for X-rays chrystallography analysis. Thanks also to Dr. Narongsak Chaichit, the Department of Physics, the Faculty of Science and Technology, Thammasart University, for his help to analyse the compound crystals.

I am gratitude Associate Professor Nattaya Ngamrojanavanich for her suggestion of thesis, Dr. Jittra Piapukiew for her help and advice for the molecular identification of fungi. The special thanks are extended to Dr. Surang Thienhirun for some advice of this thesis.

I would like to thank the Graduate school, Chulalongkorn University for financial supports. I also wish to thank the Program of Biotechnology and the Department of Chemistry, the Faculty of Science, Chulalongkorn University for providing facilities during my study. I also Miss Sunadda Yomyart for assistant of DNA analysis, Mrs. Songchan Puthong, Institute of Biotechnology and Genetic Engineering, for cytotoxicity test and the colleagues of Research Centre for Bioorganic Chemistry (RCBC) for their assistant and comments.

Finally, I am extremely grateful to my family for the lasting support throughout my studies.

CONTENTS

page

page

page

page

LIST OF TABLES

LIST OF FIGURE

Figure **page**

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
°C	degree Celsius
cm	centimeter
cm^{-1}	reciprocated centimeter (unit of wave number)
^{13}C NMR	carbon-13 nuclear magnetic resonance
conc.	concentration
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	doublet doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
ESI-TOF	Electrospray Ionization-Time of flight
g	gravitational accerelation
h	hour
HMBC	Heteronuclear Multiple Bond Cerrelation
^{1}H NMR	proton nuclear magnetic resonance
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
IR	infared
J	coupling constant
m	multiplet (NMR)
M^{+}	molecular ion
MHz	megahertz
mg	milligram
min	minute
mL	milliliter (s)
mm	millimeter
MS	mass spectroscopy
m/z	mass to change ratio
nm	namometer
No.	number
NOESY	Nuclear Overhauser Enhancement Spectroscopy

ppm	part per million
q	quartet (NMR)
R_f	rate of flow in chromatography
rpm	round per minute
s	singlet (NMR)
sec	second
sp.	species
t	tripet (NMR)
TLC	thin layer chromatography
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet
v/v	volume by volume
μg	microgram
μL	microliter
$[\alpha]_D^{20}$	specific rotation at 20°C and sodium D line (569nm)
δ	Chemical shift
λ_{max}	wavelength at maximum absorption (UV)
ν_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

Currently, the natural products are attended and studied in different ways especially the products such as medicine from plants, microorganisms or animals (Strobel *et al*., 2004). Investigating the secondary metabolites of microorganisms isolated from unusual or specialized ecological niches or specialized ecological niches many increase the chance of finding novel, bioactive compounds (Stierle, Stierle and Bugni, 2001). The products from plants are the major source of compounds for the long periods. Nowadays scientist interested on the new origin of compounds which have some biological activities to apply for the human life. These are extending to the microorganisms which are relative or living in plants. Strobel and Daisy (2003) reported that nearly 300,000 plant species are being on earth and each plant is the host of microorganisms called “endophytes” which is focus on the fungi. These endophytes produce various useful bioactive molecules which some compounds show the powerful of antimicrobial, antiviral, anticancer, antioxidant etc.

Fungi are heterotrophs microorganisms and have been important in ecosystems. They are very versatile and play several roles such as saprotrophs, parasites, mutualistic symbionts etc. (Dix and Webster, 1995). The enormous varieties of fungi give the secondary metabolites (Carlile and Watkinson, 1994). Dreyfuss and Chapela (1994) described the fungal metabolites more than 4,000. This is indicated, the opportunity to find the novel metabolites for endophytic fungi.

Some Thai medicinal plants have various pharmacological activities, therefore, it is interesting to investigate the interested compounds from local plants. In this research, *Sandoricum koetjape* leaves were used as a source for isolation of endophytic fungi and some secondary metabolites were investigated on biological activities.

Objectives

1. To isolate and identify the endophytic fungi from leaves of santol, *Sandoricum koetjape*
2. To investigate secondary metabolites produced by the endophytic fungi of santol

3. To isolate and characterize the metabolites of the selected endophytic fungal isolates
4. To evaluate the biological activity of the isolated secondary metabolites

CHAPTER II

LITERATURE REVIEW

2.1 Introduction kingdom Fungi

2.1.1 Definition and key role

The word Latin "fungus" (pl. fungi) is mushroom from the Greek "sphonggis", a spong, have been used in English from the early sixteenth century (Ainsworth, 1976; Alexopoulos and Mims, 1979) and difficult to give a limited definition of a fungus (Webster, 1980; Ross, 1979). Burnett (1968) suggested the fungi features, "*hetrotrophic organisms and the vast majority are constructed of more or less microscophic, cylindrical filaments, or hyphae, with well defined cell wall".* They also are eukaryotic, spore-baring, lack of chlorophylls, sexual and asexual reproduction, branch somatic structure or surrounded, cell walls containing chitin and/or cellulose (Alexopoulos and Mims, 1979).

After the 1660s, when fungi were first described by Robert Hooke, these microorganisms were collected and investigated by botanists (Atlas, 1995; Alcomo, 2003). Hawksworth (2001) was estimated the species of fungi that there are 1.5 million and about 74,000-120,000 species have been described. The fungi are an important microorganisms and high biodiversity in the ecological system (Sangan, 1990; Hawsworth, 2002). The main function of the fungi is key role decomposers of organic materials in the global carbon, nitrogen and other nutrients cycles (Griffin, 1981). Some of fungi are useful in industrial processes involving fermentation (Prescott, Harley and Klein 2005). They have an enormous potential for human being to produce some drugs, organic acids, vitamin preparations and antibiotics (Alexopoulos and Mims, 1979). However, the fungi are the eukaryotic cell, like animals and plants, then they are choose to be a research tools in the study of biological processes (Moore and Frazer, 2002) because they have short generation times and easy to handle in a large number (Zhan and McDonald, 2005).

2.1.2 Classification and Identification

Carl Linnaeus, a scientist in the eighteen century, was defined classification system for organism based on anatomy characteristics (Atlas, 1995; Prescott *et al*., 2005). In 1887, de Bary was established the fungal life cycles and morphology by the fungi characters, for example, shapes of thallus, form, color and size of spore (Alexopoulos

and Mims, 1996). In the older classification schemes, the fungi were classified within the green plants. Later, the mycologists were moved fungi in the kingdom Protista (Talaro and Talaro, 1996).

In 1969, Whittaker devised a five-kingdom classification system in which the fungi are assigned to their own kingdom based on extracellular digestion and absorption. (Moore-Landecker, 1982).

Consequently, taxonomy of fungi is more difficult to be grouping. Hence, the taxonomists have been classified the fungi taxa are significance of the suffixes in Table 2.1 (Griffin, 1981).

Table 2.1 Suffixes of fungal taxa.

Rank	**Suffix**
Division	– mycota
Subdivision	– mycotina
Class	– mycetes
Subclass	– mycetidae
Order	– ales

Source: Griffin (1981)

In 1973, Ainsworth was classified kingdom Fungi into two divisions. First is Myxomycota; plasmodium or pseudoplasmodium persent and the other is Eumycota; plasmodium or pseudoplasmodium absent, assimilative phase typically filamentous (Webster, 1980). The major fungal, division Eumycota, can be divided in five subdivisions (Table 2.2); four natural and one artificial (Burnett, 2003).

The keys to the taxa of fungi by Ainsworth (1973) were nutrition, thallus, cell wall, nuclear status, life cycle sexuality, sporocarps, habitat and distribution. Many characteristics are used in classifying and identifying microorganisms. These characteristics have been divided into two groups: classical and molecular (Prescott *et al.*, 2005).

Table 2.2 Classification of division Eumycota.

Subdivision	Characteristics
Chytridiomycotina	Motile phase, possibly polyphyletic
Zygomycotina	A small group, “pin moulds” and their allies, almost certainly polyphyletic
Ascomycotina	The largest group, many common “molds”, mildews and many of the yeasts; the fungal component of lichens
Basidiomycotina	Heterogenous group; mushrooms and toadstools, bracket fungi (conks), jelly fungi, earth stars, truffles, stinkhorns, rust and smut
Fungi Anamorphici (Fungi Imperfecti, Deuteromycotina)	Not found the sexual reproduction or mitosporic fungi

Source: Burnett (2003)

The classical characteristics are morphological, physiological, biological, ecological and genetic characteristics (Prescott *et al.*, 2005). The molecular characteristics approaches to taxonomy are through the ribosomal nucleotide acid sequence, nucleic acid reassociation and hybridization, protein composition (e.g., enzyme, antigenic properties), phylogenetic trees etc. (Guarro, Gene and Stchigel, 1999; Prescott *et al.*, 2005).

2.1.3 Community and ecology

Some of fungi may live as saprotrophs which obtain the energy from nonliving organic materials, dead plants or animals, their parts, or their wastes (Moore-Landecker, 1982; Garraway and Evans, 1984). When the fungi are associated with other organisms, in which called “symbiosis” and the partners are “symbionts” (Moore-Landecker, 1982). Sometimes symbiosis has been comprised to mutualistic, both partners benefiting (Carlile and Watkinson, 1994), or parasitic that one partner is absorbed the nutrients from host organisms (Garraway and Evans, 1984).

The fungi diversity is spreading in several habitats such as terrestrial, aquatic, animals and plants (Moore and Frazer, 2002). The plant habitats of fungi include living vascular plants, dead vascular plants and nonvascular plants (Hawksworth and Mueller, 2005).

2.2 Secondary metabolites of fungi

2.2.1 Definition and classification

The metabolites were produced during the microorganisms stationary phase or the reduced growth phase are called secondary metabolites (Brock and Madigan, 1991; Martin and Gutierrez, 1992; Carlile and Watkinson, 1994). Bu'Lock, in 1965, described the two phases of secondary metabolites production, *trophophase*—the period of exponential growth and *idiophase*—the period of growth limitation and produced secondary metabolites also called production phase (Garraway and Evans, 1984; Brock and Madigan, 1991; Demain, 1996).

Secondary metabolites or idiolites are produced naturally by many fungi and not essential for the vegetative growth (Singh, 1999; Demian, 1994) but believed that have some relationship to the metabolism processes in the cell (Guarro *et al*., 1999). More recently, fungi can produced the secondary metabolites as a group of similarity structure and easily demonstrated in batch culture (Waitese *et al*., 2001). The crucial compounds with antibiotic activity have been the member of secondary metabolites (Brakhage and Caruso, 2004).

During the 1920s and 1930s, the organic chemists observed the filamentous fungi have been the endless organic compounds (Rose, 1978). More than 200 fungal secondary metabolites were studied since 1922 by Harold Raistrick, the leadership who was characterized the mould metabolites (Keller, Turner and Bennett, 2005).

The molecules of secondary metabolites are divert and found in many classes of organic compounds: aminocyclitols, amino sugars, quinines, coumarins, epoxides, glutarimides, indole derivatives, lactones, macrolides, naphtalenes, nucleosides, peptides, phenazines, polyacetylenes, polyenes, pyrroles, quinolines, terpenoids and tetracyclines (Martin and Gutierrez, 1992). Keller *et al*. (2005) classified the fungal secondary metabolites into four groups which were according to the enzyme classes involved in their biosynthesis: polyketides, alkaloids, terpenes and peptides. The examples of fungal species that produced secondary metabolites were showed in Table 2.3.

Table 2.3 The species of fungal and their secondary metabolites.

Secondary metabolites	**Fungal species**
Polyketides	
- Aflatoxin	*Aspergillus flavus, A. parasiticus*
- Griseofulvin	*Penicillium griseofulvum*
- 6-Methylsalicylic acid	*P. patulum*
- Patulin	*A. expansum*
Alkaloids	
- Elymoclavine	*Claviceps fusiformis*
- Lysergic acid	*C. paspali*
- Lolitrem	*Acremonium lolii*
- Brevianamides A and E	*P. brevicomactum*
Terpenes	
- Aristolochene	*A. terreus, P. roquefortii*
- Carotenoid	*Neurospora crassa*
- Gibberellin	*Gibberella fujikuroi*
- Paxilline	*P. paxilli*
Peptides	
- Alamethicin	*Trichoderma viridae*
- Penicillin	*P. notatum*
- Ferrichrome	*A. quadricinctus*
- Enniantin	*Fusarium oxysporum*

Source: Martin and Gutierrez (1992); D'Mello and Macdonald (1997); Keller *et al*. (2005)

2.2.2 Investigation and advantages

After, Alexander Fleming discovered 'penicillin' from *Penicillium notatum* in 1929, the fungal metabolites were focus on the bioactive compounds and investigated for secondary metabolites production in the industrial scale. Many of fungal secondary metabolites are showing antibiotic activity against microorganism (Che *et al*., 2004) antiviral (Dai *et al*., 2001), antitumor (He *et al*., 2006), fungicide agents (Brakhage and Caruso, 2004). Singh (1999) showed some medicines from fungi and mode of actions in Table 2.4.

Table 2.4 Some medicines and mode of action.

Compound	Mode of action
Ranitidine	H2-antagonist
Omeprazole	proton pump inhibitor
Enalapril	ACE inhibitor
Nifedipine	calcium antagonist
Fluoxetine	antidepressant
Acyclovir	antiviral
Lovastatin, Pravastatin, Simvastatin	hypolipidaemic

Source: modified from Singh (1999)

Many researches of fungal metabolites have been investigated to cytotoxic, mutagenic, teratogenic, immunosuppressive, enzyme inhibitory, etc. Between 1993 and 2001, 1,500 compounds were found and more than half of these had antibacterial, antifungal or antitumor activity (Keller *et al*., 2005).

At present, the fungal secondary metabolites have been used to pharmacological activity such as anti-inflammatory, immunoregulation and antiatherosclerotic action (Hashimoto, 2001; Singh *et al*., 2004). In addition, the metabolites also play a role in the ecological interactions which were relationship between plant-herbivore, insect-insect and plant-plant (Mann, 1978). Secondary metabolites called “phytotoxins” that had some member in polyketides, peptides and terpene (Strobel *et al*., 1991). These compounds were significant role in plant pathogenic fungi (Scheffer, 1991; Evidente *et al*., 2005) and used to a weapons directed against plants (Demain, 1996). Moreover, fungal secondary metabolites have been act as mycotoxin (D’Mello and Macdonald, 1997), plant growth stimulators (Kimura *et al*., 1992), sexual hormones (Gooday, 1987), anti-infective agents (Jayasuriya *et al*., 2003), metal transport agents, effectors of differentiation and sporulation agents (Demain, 1996).

2.3 Endophytic fungi

2.3.1 Definition

The fungi are found inside the host plant for most of their life cycle and capable of symptomless in healthy plant tissues, called “endophytic fungi” (Pereira, Azevedo and Petrini, 1993; Stone, Polishook and White, 2004). This term, in 1966, was commenced by De Bary (Isaac, 1992). These fungus are associated with roots, stems,

leaves, flowers and seeds (Rodriguez, Redman and Henson, 2005) and also a mutualistic relationships with host plant (Carroll, 1988; Saikkonen *et al*., 1998). However, endophytic fungi can be a pathogens and saprophytes (Fisher and Petrini, 1992; Huang *et al*., 2001). All kind of plants include trees, grass, algae and herbs can be found the endophytic fungi (Huang *et al*., 2001) and isolated a lot of endophyte species from a single plant (Dix and Webster, 1995; Tan and Zou, 2001). Most of these endophytes are Ascomycetes and Fungi imperfecti's member (König *et al*., 1999; Davis *et al*., 2003).

2.3.2 A rich bioactive source

Endophytic fungi, in many ways, have been inquired to novel secondary metabolites after they were found in 1904 by Darnel (Tan and Zou, 2001). Some of endophytic fungi was prolific the bioactive compounds and which a feasibility source of novel metabolites for medicinal and agricultural industrial (Tan and Zou, 2001; Strobel *et al*., 2004). This is an attractive reasons of endophytic fungi studying in the past few decades (Faeth, 2002).

Tan and Zou (2001) inspected group of the secondary metabolites from endophytes as alkaloids, indole derivatives, pyrrolizidines, steroids, terpenoids, isocoumarin derivatives, quinines, flavonoids, phenylpropanoids, peptides, phenol and phenolic acids and aliphatic compound. More recently, they reported the phytohormones from endophytic fungus, *Collectotrichum gloeosporioides*, which induced the growth of host callus.

2.3.2.1 Endophytes products as antibiotics

Some compounds of these endophytes have been inhibit or destroy an organisms such as bacteria, fungi, viruses and protozoans which affect humans and animals (Strobel and Daisy, 2003).

The phytopathogen, *Crinipellis perniciosa*, causal agent of Witche's Broom disease were inhibited by endophytic fungal community of cacao (Rubini *et al*., 2005) Volatile antimicrobials from endophytic fungus, *Muscodor albus* which isolated from small limbs of *Cinnamomum zeylanicum*, cinnamon tree (Strobel *et al*., 2001).

2.3.2.2 Endophytes products as anticancer and antitumor agents

The common endophytic fungi found in the world's yews are *Psetalotiopsis* spp. such as *P. microspora* that produced paclitaxel, an anticancer agents (Strobel *et al*., 1996). Podophyllotoxin, a precursor to anticancer drugs, was produced by

endophytic fungal isolated from *Podophyllum peltatum* (Eyberger, Dondapati and Porter, 2006).

For example of antitumor agent was investigated the fungi from three kinds of pharmaceutical plants and found the fermentation broths displayed cytotoxicity activity on HL-60 cells (Huang *et al*., 2001). Sequoiatones A and B, antitumor metabolites isolated from the fungus *Aspergillus parasiticus*, a redwood endophyte (Stierle, Stierle and Bugni, 1999).

2.3.2.3 Endophytes products as insecticide agents

The bioinsecticidal activity have been found in endophytic fungi such as an endophyte, *Muscodor vitigenus* from *Paullinia paullinioides*. This fungi was presented naphthalene, an insect repellent (Daisy *et al*., 2002). Another endophytic fungus, *Phomopsis phaseoli* isolated from leaf of the tropical tree and produced 3-hydroxypropionic acid that was a nematicide metabolites (Schwarz, 2004).

2.3.2.4 Endophytes products as antioxidants agents

The unnamed endophytic fungi from *Podophyllum hexandrum*, was possessed the metabolite podophyllotoxin that fined application as antioxidant (Puri, *et al*., 2006). Secondary metabolite, Graphislactone A, from cultures of endophyte, *Cephalosporium* sp. IFB-E001 which isolated from *Trachelospermum jasminoides* were assayed for *in vitro* antioxidant activity and free radical-scavenging agents (Song *et al.*, 2005).

2.3.2.5 Endophytes products as another bioactive agents

The fungus neurotoxin, lolitrem B is an indole diterpene produced by the endophyte *Neotyphodium lolii*, was inhibited *hSlo* –large conductance calcium activated potassium channels. This is a new tool for studying the functional properties of human BK channel expressed in human embryonic kidney cells (Dalziel, Finch and Dunlop, 2005). Cytochalasin F, an inhibitor of photosynthesis, was extracted from *Geniculosporium* sp. that isolated *Teucrium scorodonia* (König *et al*., 1999).

2.3.3 Identification of endophytic fungi

The endophytic fungi are classifying and identifying base on morphological characteristics (Cohen, 2005). Many endophytes are hard to investigation and report under the microscopy because of these fungi rarely produced reproductive structures and some of these are poorly differentiated filamentous or mycelium (Isaac, 1992;

Strange, 2003; Worapong *et al*., 2001). Consequently, the molecular characteristics such as sequence analysis of rDNA were helpful for investigate in taxonomy (Strobel *et al*., 2004; Cohen, 2005). The rDNA are easily to amplifiy by PCR because it has a single nuclear copy (Worapong *et al*., 2001).

The internal transcribed spacer (ITS) region is the most widely sequenced rDNA region in fungi for molecular systematics. This is contains two variable non-coding regions which was variation among individual rDNA repeats. In fungi, the ITS region is often between 600-800 bp and can amplified with PCR (polymerase chain reaction) and universal primers (Gardes and Bruns, 1993). DNA sequences were established by using specific primers and PCR and then exanimate as relationships among the organism (Rodriguez *et al*., 2004).

2.4 *Sandoricum koetjape*

2.4.1 Species identify

Family name : Meliaceae

Synonymes : *Melia koetjape* Burm. f. (1768), *S. indicum* Cav. (1789), *S. nervosum* Blume (1825)

Common name :

Brunei: *klampu*;

Cambodia: *kôm piing riëch*;

English: *santol, kechapi, sentol*;

France: *faux mangoustan*;

Guam: *santor, wild mangosteen*;

India: *sayai, sevai, sevamanu, visayan*;

Indonesia: *kacapi, ketuat, sentul, ketjapi, sentool*;

Lous: *toongz*;

Malaya : *seatieh, sentol, setol, sentul, setui, kechapi, ketapi, entor*;

Mianmar: *thitto*;

Philippines: *santor, katul*;

Thailand: s*aton, satown, katon, ka-thon, matong, mat*;

Vietnam: *sâu, sau chua, sau tia, sau do*.

2.4.2 Origin and distribution

The native of santol have been in Indochina and the Southeast Asia. They are distribute in the nature and in the warm climate of Asia such as Indonesia, Malaysia,

Philippines, Thailand and Vietnam (Ismail, Ito, and Mukainaka, 2003; Rasadah *et al*., 2004).

2.4.3 Description

Morton (1987) was descriped this plant, "a fast-growing, straight-trunked, pale-barked tree 50 to 150 ft (15-45 m) tall, branched close to the ground and buttressed when old. Young branchlets are densely brown-hairy. The evergreen, or very briefly deciduous, spirally-arranged leaves are compound, with 3 leaflets, elliptic to oblong-ovate, 4 to 10 in (20-25 cm) long, blunt at the base and pointed at the apex. The greenish, yellowish, or pinkish-yellow, 5-petalled flowers, about 3/8 in (1 cm) long are borne on the young branchlets in loose, stalked panicles 6 to 12 in (15-30 cm) in length. The fruit (technically a capsule) is globose or oblate, with wrinkles extending a short distance from the base; 1 1/2 to 3 in (4-7.5 cm) wide; yellowish to golden, sometimes blushed with pink. The downy rind may be thin or thick and contains a thin, milky juice. It is edible, as is the white, translucent, juicy pulp (aril), sweet, subacid or sour, surrounding the 3 to 5 brown, inedible seeds which are up to 3/4 in (2 cm) long, tightly clinging or sometimes free from the pulp."

2.4.4 Varieties

Two general types of santol are classified: the yellow (*S. indicum* or *S. nervosum*); and the red (*S. koetjape*).

Table 2.5 The characteristics of santol varities.

Characters	**Varieties**	
	yellow	**red**
Leaflets		
- color (old)	yellow	red
- long	6 in (15 cm)	12 in (30 cm)
Flower		
- color	pinkish-yellow	greenish or ivory
- panicle	6 in (15 cm)	12 in (30 cm)
Fruit	thin	thick
- rind	thin	thick
- pulp	1/4 to 1/2 in (0.6-1.25 cm)	1/2 in (1.25 cm)
- taste	sweet	sour

Source: Morton (1987)

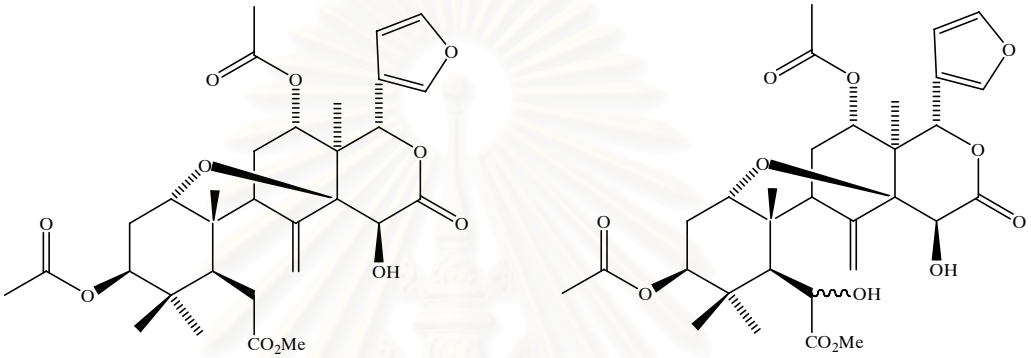
2.4.5 Medicinal used

The preserved pulp is used as an astringent (Morton, 1987). The fresh leaves are applied as poultice to the skin that are induces sweat and perspiration and as

a decoction is used to bathe. The bark is used as a folk medicines such as a tonic after childbirth (Burkill, 1966), against colic and leucodiarrhea (Perry, 1980).

2.4.6 Compounds

Many compounds from *S. koetjape* were investigated and have been reported in many research. Sandoricin and 6-hydroxysandoricin, the seed extract, were effective antifeedants utilizing larvae of fall armyworm, *Spodoptera frugiperda*, and European corn borer, *Ostrina nubilalis* (Powell *et al*., 1991).



sandoricin 6-hydroxysandoricin

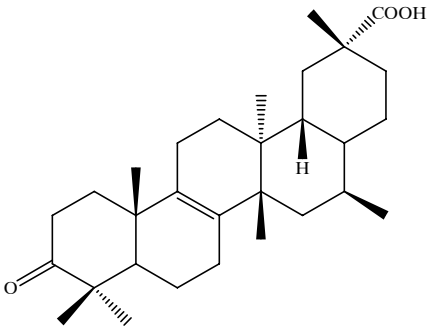
Figure 2.1 Structure of sandoricin and 6-hydroxysandoricin

The Et_2O extract of stem gave a secotriterpene, koetjapic acid and two triterpene, 3-oxo-olean-12-en-29-oic acid, katonic acid. Two triterpene were exhibited cytotoxic activity against cultured P-388 cells that was showed ED_{50} values of 0.61 and 0.11 μg/mL, respectively (Kaneda *et al.*, 1992).

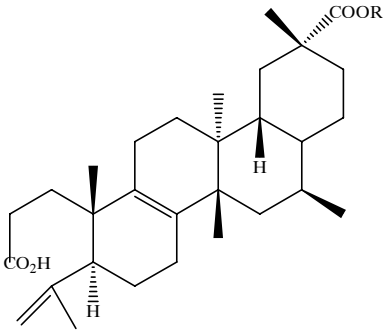
Crude methyl ethyl ketone extracted from stem bark and wood showed the DNA polymerase β inhibition. Isolation of three active compounds, katonic acid, koetjapic acid and 3-oxo-olean-12-en-29-oic acid showed IC50 values of 36, 20 and 22 μM, respectively (Sun *et al*., 1999).

Bryononic acid and two secomultiflorane-type triterpenoids, secobryononic acid and secoisobryononic acid were extracted from dried stem bark with petrol for a week and then separated using column chromatography (Kosela *et al*., 1995).

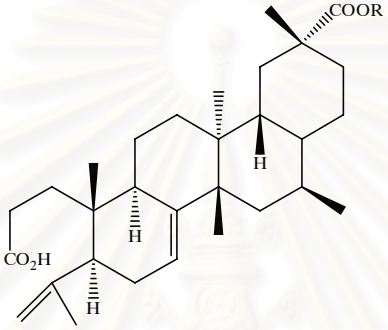
Rasadah *et al*. (2004) reported the anti-inflammatory activity of stem extracts. Katonic acid and 3-oxo-olean-12-en-29-oic acid indicated that were mouse ear inflammation.



bryononic acid

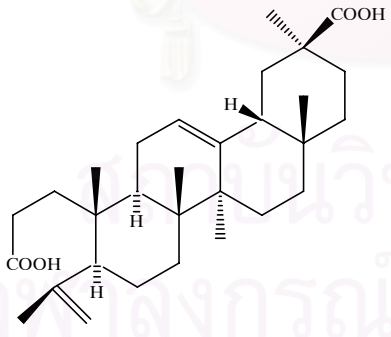


secobryononic acid

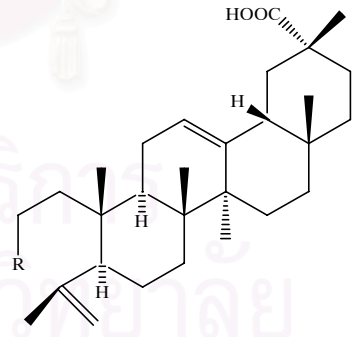


secoisobryononic acid

Figure 2.2 Structure of bryononic acid, secobryononic acid and secoisobryononic acid



koetjapic acid



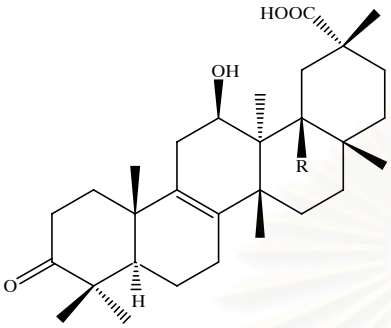
1 R= =O

2 R= ···OH

Figure 2.3 Structure of koetjapic acid, 3-oxo-olean-12-en-29-oic acid (**1**) and katonic acid (**2**).

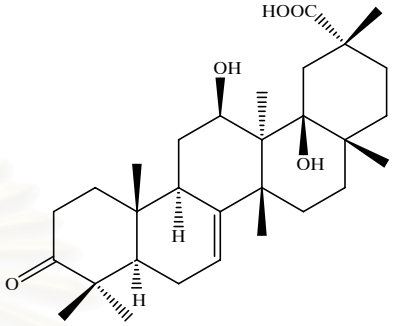
Three of 12 *β*-hydroxymultiflorane triterpenoid acids (sandorinic acid A-C) and five triterpenes: koetjapic acid, katonic acid, 3-oxo-olean-12-en-29-oic acid, 20-

epikoetjapic acid and 3- epikatonic acid. All compounds were extracted by MeOH and apprised against tumor cell lines (Tanaka *et al*., 2001). Moreover, koetjapic acid and 3-oxo-olean-12-en-29-oic acid showed ichthyotoxic activity to killifish, *Oryzias latipes* (Ismail, Ito, Mukainaka *et al*., 2003).

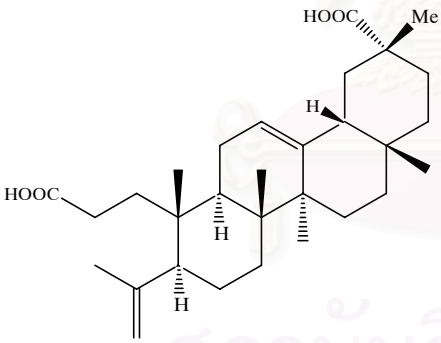


3 R= OH

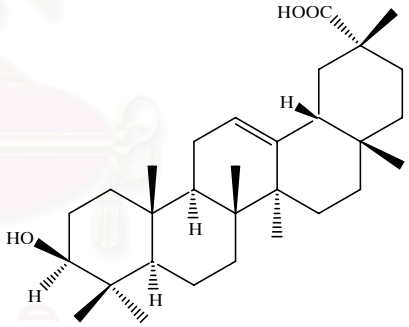
4 R= H



sandorinic acid B



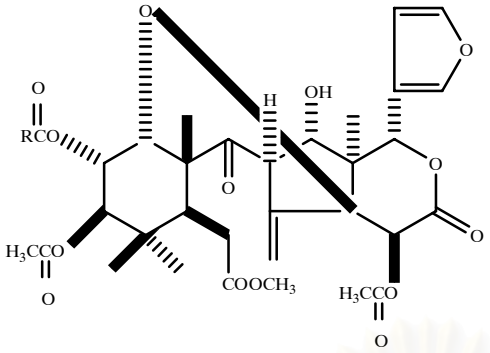
20-epikoetjapic acid



3-epikatonic acid

Figure 2.4 Structure of sandorinic acid A (**3**), sandorinic acid B, sandorinic acid C (**4**), 20-epikoetjapic acid and 3-epikatonic acid.

Ismail, Ito, Hatano *et al*. (2003) reported the modified limonoids, sandrapins A-C and in 2004, this researcher investigated trijugin-type limonoids, sandrapins D and E.



5 R= —CH_3

6 R= —CH(—CH_3)—CH_2—CH_3

7 R= —CH(—CH_3)—CH_3

8 R= —C(—CH_3)=C(—H)—CH_3

9 R= —C(—CH_3)=CH_2

Figure 2.5 Structure of sandrapins A-E (**5-9**).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant samples

Healthy and mature leaves of *Sandoricum koetjape* were carefully collected from 10 provinces; Bangkok, Chiang Mai, Khonkaen, Mahasarakham, Pathumthani, Pattani, Phangnga, Phayao, Prachinburi, and Samutsongkhram. Fresh specimens were kept in a plastic bag and then immediately brought to the laboratory and processed with in 24 h after collection.

3.1.2 Culture media for endophytic fungi cultivation

The formulae for culture media was shown in Appendix A. Malt extract agar and broth (MEA and MEB) were culture medium for isolation and cultivation of endophytic fungi and plant pathogenic fungi. Corn meal agar (CMA), Potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA) and V8-juice agar were used for the endophytic fungi morphology observation. The culture media for bacteria were Nutrient agar (NA) and Mueller Hinton agar (MHA). Yeast-malt extract agar (YMA) was used for growing yeasts.

3.1.3 Equipments

3.1.3.1 UV-VIS spectrometer

UV-VIS spectra were measured in MeOH and recorded on a Varian cary 50 probe UV-VIS spectrophotometer.

3.1.3.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

FT-IR spectra were recorded on a Nicolet Impact 410 FT-IR. Potassium bromide (KBr) was used to form a pellet with the solid samples. The liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

3.1.3.3 Mass spectrometer (MS)

HRESIMS were performed on a Micromass LCT (LC/MS) at National Center for Genetic Engineering and Biotechnology (BIOTECH), National Science and Technology Development Agency Building (NSTDA).

3.1.3.4 Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H and ^{13}C NMR data were performed on Varian Model Mercury +400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Deuterated solvents, chloroform-*d* ($CDCl_3$) and deuterium oxide (D_2O), were used for NMR experiments and chemical shifts (δ) were referenced the signals of residual solvents at 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C) for $CDCl_3$ and at 4.79 ppm for D_2O.

3.1.3.5 Optical rotation

The optical rotations were measured on a Perkin-Elmer Model 341 Polarimeter, using a sodium lamp at wavelength 589 nm.

3.1.3.6 Melting point

Melting points were examined using a Electrothermal Mel-Temp® melting point apparatus.

3.1.3.7 X-ray Diffractometer

Crystal data were obtained with a BRUKER SMART CCD Diffractometer at the Department of Physics, the Faculty of Science and Technology, Thammasart University.

3.1.4 Chemicals used in this experiments

3.1.4.1 Solvent

The solvents used for column chromatography were commercial grade and were distilled prior to use.

The deuterated solvents for NMR experiments including $CDCl_3$ and D_2O were purchased from Merck.

3.1.4.2 Other

-Silica gel 60 (0.040-0.063 mm) (Merck)

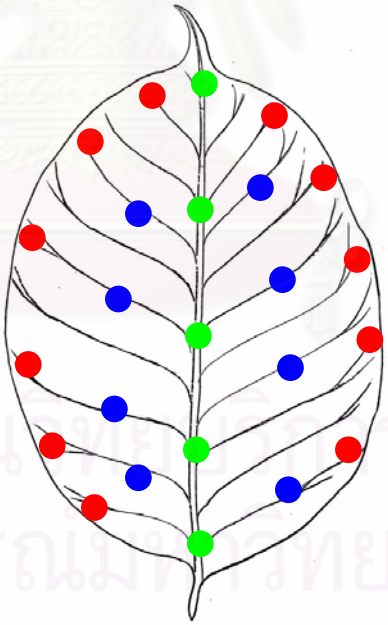
-TLC aluminium sheets, silica gel 60 F_{254} (Merck)

-Clorox®(5.25% NaOCl)

3.2 Methods

3.2.1 Isolation endophytic fungi

Endophytic fungi were isolated using the surface sterilization which was modified from the method described by Petrini (1982). Plant leaves were washed in running tap water and dried in laminar air flow. The leaves were cut into a diameter 8 mm from the midrib and lateral vein (Figure 3.1). The sample were surface steriled respectively in 90% EtOH for 30 sec, followed with a solution of 10% Clorox® for 1 min and then were transferred to 90% EtOH for 30 sec before rinsing twice with sterile distilled water. The surface sterilized samples were dried on sterile filter papers and put on MEA. All Petri dishes were incubated at room temperature and examined the fungal growth every day under a stereomicroscope. The fungal hyphal tips were transfered to



Green = midrib

Blue = lateral vein

Red = lateral vein (leaf margin)

Figure 3.1 Sampling position for the isolation of endophytic fungi.

3.2.2 Identification and classification of endophytic fungi

Endophytic fungi were characterized on the basis of morphological identification, microscopic (e.g., spores, mycelia) and macroscopic features (e.g., shape, size, color, margin, pigment) observed by compound microscopy and stereomicroscopy. Nomenclatures of the fungi were followed Barron (1977), Von Arx (1981), Ellis and Ellis (1985) and Barnett and Hunter (1998).

3.2.3 Metabolite production of the endophytic fungi

3.2.3.1 Fungal cultivation

The inocula were prepared by introducing the 7 to 14-day-old plate cultures of each endophyte. The agar culture was cut into 8 mm diameter disks by a flamed cork borer. Five disks were inoculated into 250 mL flask containing 100 mL of MEB and cultured under static condition at room temperature for 5 weeks. All endophytic fungi were cultured at this condition.

3.2.3.2 TLC analysis of metabolites

Culture broth of each endophyte isolate was filtered through filter paper (Whatman No. 1) and the mycelia were frozen at -20°C. The filtrates, exhibited biological activities, were extracted with EtOAc and mycelia were extracted with MeOH. The solvent was evaporated under reduced pressure. Both extracts were analyzed by TLC visualized using UV light, iodine vapor and vanillin/H_2SO_4 reagent.

3.2.3.3 Investigation of the antimicrobial metabolite production of the endophytic fungi

The metabolites were determined the antimicrobial by the agar well diffusion method (National Committee for Clinical Laboratory Standards; NNCLS, 2003 and 2004).

3.2.3.3.1 Preparation of samples

Ten mg of each culture broth extract and mycelium extract were dissolved in 1 mL of a solution of 10% DMSO in sterile distilled water.

3.2.3.3.2 Preparation of bacterial inoculum

The test bacteria were *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus*

aureus ATCC 25923. Bacteria were grown on NA and MHA at 37ºC for 24 h. Selected single colonies were inoculated into NB (5 mL) and MHB (5 mL) and incubated at 37ºC for 2-6 h, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with NB and MHB to match the turbidity of a 0.5 McFarland (OD 0.08-0.1 at 625 nm).

3.2.3.3.3 Preparation of yeast inoculum

Candida albicans ATCC 10231 was grown on YMA at room temperature for 24 h. Selected single colonies were inoculated into YMB (5 mL) and incubated at room temperature for 2-3 h, depending on the growth rate. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of a 0.5 McFarland (OD 0.08-0.1 at 625 nm).

3.2.3.3.4 Inoculation of the test plate

A sterile cotton applicator was dipped into the microbial inoculum suspension. The agar was inoculated by streaking and swabbing the microorganism across the entire surface twice. The surface of the medium was allowed to dry for 3-5 min.

3.2.3.3.5 Application of culture broth extracts and mycelia extracts

The flamed cork borer was made a hole by removing disks cut (8 mm diameter). 100 μL of culture broth extract and mycelia extracts was pipetted into the agar wells. Bacteria and yeast plates were incubated at 37ºC and room temperature, respectively.

3.2.3.3.6 Preparation of antibiotic drug stock solution

Antibiotic drug standards were dissolved in 1 mL of 10% DMSO in sterile distilled water and kept in a refrigerator at 4ºC prior to the bioassay. Streptomycin was used as a positive control for antibacterial and ketoconazole was used as a positive control for antifungal (yeast form).

3.2.4 Identification of the selected endophytic fungi

The selected endophytic fungi were characterized based on morphological identification in section 3.2.2 and molecular identification. Cultivation of selected endophytic fungi in MEB 100 mL for a few weeks and filtered through filter paper (Whatman No. 1). Mycelia of endophytic fungi from cultured broth were filtrated and

kept at 4ºC for genomic DNA extraction as modified from Zhou, Miwa and Hogetsu (1999).

3.2.4.1 DNA extraction

The mycelia were homogenized in 1,000 μL of a solution of washing buffer (0.1 M Tris-HCl (pH 8.0), 2% 2-mercaptoethanol, 1% polyvinylpyrrolidone and 0.05 M ascorbic acid) with a pestle in a mortar. Then the sample was transferred to 1.5 mL microcentrifuged tube and centrifuged a mixture of the sample and the washing buffer at 15,000 g for 3 min. After removal of the supernatant, the pellet was washed 4-5 times using the washing buffer and centrifugation at 15,000 g for 3 min. DNA was then extracted from the washed pellet by adding 700 μL of cetyltrimethylammonium bromide (CTAB) lysis buffer and incubation in water bath 65ºC for 1 h followed by extracted with chloroform : isoamyl alcohol (24:1, v/v) twice. Fungal DNA was precipitated in ice bath with isopropanol and centrifuged at 4ºC, 8000 rpm for 10 min. After removal of the supernatant, 80% cool ethanol was added to wash the fungal DNA. TE buffer 100 μL was added to dissolve the fungal DNA and kept at -20ºC. The genomic DNA were checked by 1.2% agarose gel (ISC Bioexpress) electrophoresis.

3.2.4.2 Internal transcribed spacer region

The ITS region was amplified in a total volume of 50 μL comprising of approx. 100 ng genomic DNA, 1x PCR Master Mix (Fermentas), and the primer ITS1f (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). The PCR amplification was performed in a thermocycler (T Gradient 96, Biometra) with 94ºC for 5 min, followed by 38 cycles of 94ºC for 1 min, 51ºC for 1 min and 72ºC for 1 min, with a final extension of 72ºC for 5 min. PCR product was purified and subcloned with PCR-Script™ Amp Cloning Kit (Stratagene) following the manufacturer's protocol.

3.2.4.3 DNA Sequencing

The DNA sequences were analyzed at Macrogen (Seoul, South Korea) using the same primers as for amplification.

3.2.5 Cultivation and chemical investigation of the selected endophytic fungi

3.2.5.1 Cultivation and chemical investigation of the endophytic fungus PB-30

The agar culture of the endophytic fungus PB-30 grown on MEA at room temperature for 7-14 days was cut into 8 mm diameter disks by a flamed cork

borer and then inoculated five disks into 250 mL Erlenmeyer flask containing 100 mL of MEB. After cultivation under static condition at room temperature for 5 weeks total of cultured media volume (10.5 L) were filtered through filter paper (Whatman No. 1). The filtrate was partitioned with an equal volume of hexane (x3) and EtOAc (x3), respectively and then the filtrate was concentrated by a rotary evaporation under reduced pressure at 30°C to give a viscous liquid (200 mL). The viscous liquid was extracted with 500 mL of MeOH (x3). The combined MeOH extracts were evaporated under reduced pressure to give MeOH crude as brown viscous oil (7.57 g). The hexane extract and EtOAc extract were evaporated to give hexane crude as brown viscous oil (515 mg) and EtOAc crude as dark brown viscous (10.69 g).

Fungal mycelia (371 g of wet weight) were ground using blender and then extracted with 500 mL of hexane (x5), 500 mL of EtOAc (x5) and 500 mL of MeOH (x5), respectively. After evaporation of the solvents under reduced pressure the hexane crude, the EtOAc crude and the MeOH crude were obtained as a brown viscous oil (26 mg), as a brown viscous residue (172 mg) and as a brown viscous oil (510 mg), respectively. Extraction of mycelia and broth of endophytic fungus isolate PB-30 were showed in Figure 3.2. All of the crude was kept in a refrigerator at 4°C before further studied.

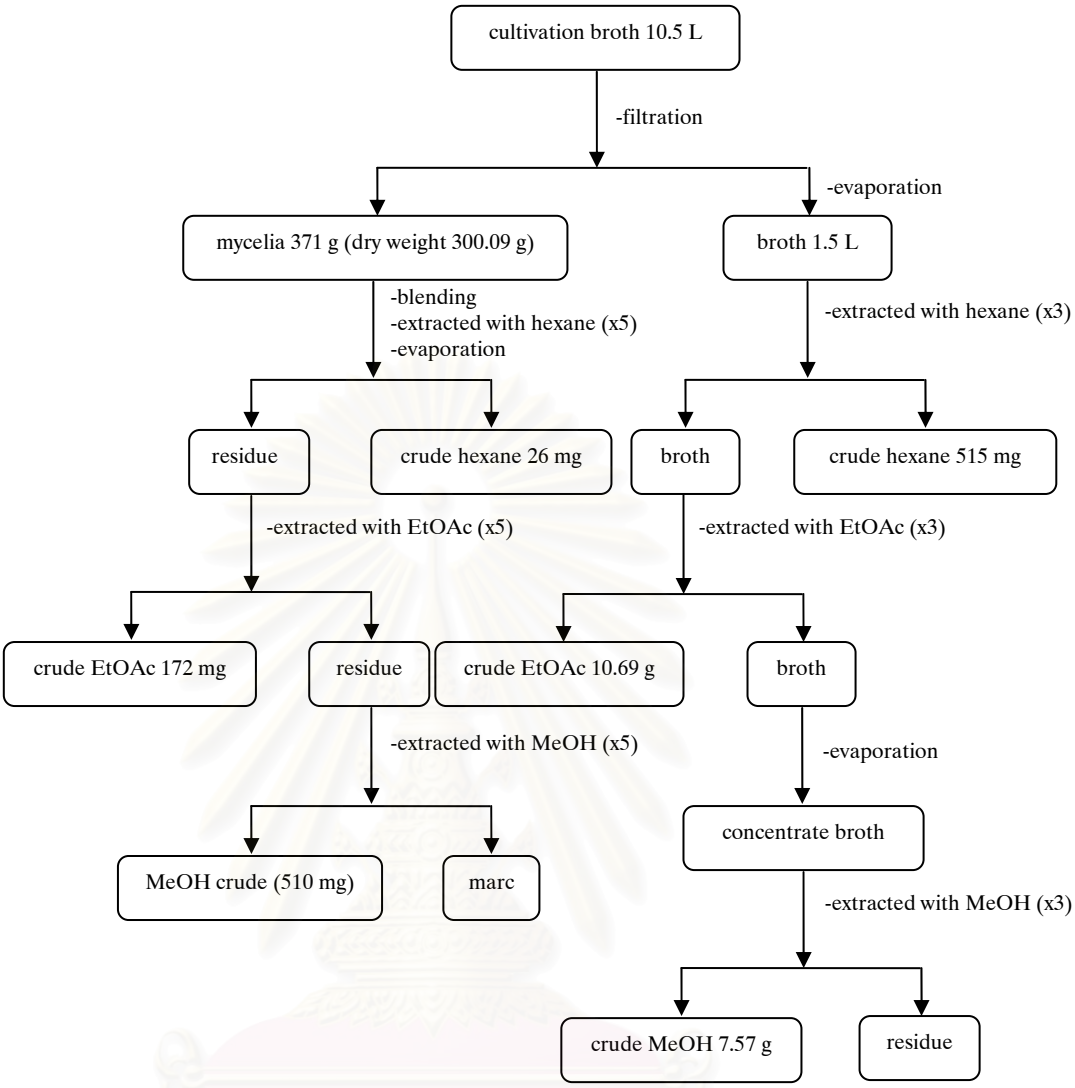


Figure 3.2 Extraction of mycelia and broth of endophytic fungus isolate PB-30.

3.2.5.1.1 Chemical investigation of the mycelia MeOH extracted crude

The MeOH crude (510 mg) was subjected to a column chromatography [silica gel 60 (80 g); column diameter 3 cm] eluted with hexane, CH_2Cl_2, EtOAc and MeOH in a stepwise fashion and 20 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine and vanillin/H_2SO_4 reagent to give 18 combined fractions (Table 3.1).

Table 3.1 The combined fractions obtained from the mycelia MeOH extracted crude

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
MM-1	1-8	hexane:EtOAc (50:50)	yellow viscous liquid	32.5
MM-2	9-15	hexane:EtOAc (50:50)	yellow viscous liquid	21.1
MM-3	16-21	hexane:EtOAc (50:50)	yellow viscous liquid	17.2
MM-4	22-26	hexane:EtOAc (40:60)	yellow viscous liquid	13.2
MM-5	27-37	hexane:EtOAc (30:70), hexane:EtOAc (20:80)	yellow viscous liquid	18.4
MM-6	38-51	hexane:EtOAc (10:90), EtOAc (100)	yellow viscous liquid	23.5
MM-7	52-61	EtOAc (100), EtOAc:MeOH (90:10)	yellow viscous liquid	16.9
MM-8	62-64	EtOAc:MeOH (80:20)	brown viscous liquid	17.7
MM-9	65-69	EtOAc:MeOH (80:20)	white solid and brown viscous liquid	38.3
MM-10	70-85	EtOAc:MeOH (70:30)	brown viscous liquid	24.7
MM-11	86-89	EtOAc:MeOH (60:40)	brown viscous liquid	15.8
MM-12	90-100	EtOAc:MeOH (55:45)	brown viscous liquid	13.4
MM-13	101-116	EtOAc:MeOH (50:50), EtOAc:MeOH (45:55)	brown viscous liquid	19.3
MM-14	117-128	EtOAc:MeOH (40:60)	brown viscous liquid	18.7
MM-15	129-137	EtOAc:MeOH (30:70), EtOAc:MeOH (20:80)	brown viscous liquid	10.3
MM-16	138-149	EtOAc:MeOH (10:90), MeOH (100)	gray brown viscous liquid	18.0
MM-17	150-159	MeOH (100)	gray brown viscous liquid	16.5
MM-18	160-165	MeOH (100)	gray brown viscous liquid	28.3

Fraction MM-9 (38.3 mg) eluted by EtOAc:MeOH (80:20) was a mixture of white solid and brown viscous liquid. Washing the white solid by the 50% EtOAc in MeOH and gave compound **1** (20.3 mg). This compound was characterized by NMR, MS, IR, UV, optical rotation and melting point.

3.2.5.1.2 Chemical investigation of the broth hexane extracted crude

The hexane extracted crude (515 mg) was subjected to a column chromatography [silica gel 60 (80 g); column diameter 3 cm] eluted with hexane, hexane in EtOAc, EtOAc, EtOAc in MeOH and MeOH in a stepwise fashion and 20 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine and vanillin/H_2SO_4 reagent to give 8 combined fractions (Table 3.2).

Table 3.2 The combined fractions obtained from the broth hexane extracted crude.

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BH-1	1-36	hexane (100), hexane:EtOAc (90:10)	yellow viscous liquid	55.50
BH-2	37-62	hexane:EtOAc (85:15), hexane:EtOAc (80:20), hexane:EtOAc (75:25)	yellow viscous liquid	36.66
BH-3	63-118	hexane:EtOAc (70:30), hexane:EtOAc (65:35)	white solid and yellow viscous liquid	105.87
BH-4	119-211	hexane:EtOAc (60:40), hexane:EtOAc (50:50), hexane:EtOAc (45:55)	yellow viscous liquid	26.97
BH-5	212-239	hexane:EtOAc (40:60), hexane:EtOAc (35:65), hexane:EtOAc (30:70), hexane:EtOAc (20:80)	brown viscous liquid	69.96
BH-6	240-274	hexane:EtOAc (15:85), hexane:EtOAc (10:80)	brown viscous liquid	35.91
BH-7	275-309	hexane:EtOAc (5:95), EtOAc (100), EtOAc:MeOH (90:10)	brown viscous liquid	27.77
BH-8	310-357	EtOAc:MeOH (80:20), EtOAc:MeOH (50:50), MeOH (100)	brown viscous liquid	76.99

Fraction BH-1 and BH-3 were showed a large bright blue spot on TLC developed by 2% MeOH in CH_2Cl_2 as mobile phase. The mixture BH-1 was re-chrystallisation by heaxane:EtOAc (60:40) to give compound **2** (16.35 mg) as clear viscous liquid. Fraction BH-3 (105.87 mg) was re-chrystallisation by hexane:EtOAc (20:80) to give compound **3** (46.77 mg) as white solid. Compound **2** and **3** was characterized by NMR, MS, IR, UV, optical rotation and melting point.

3.2.5.1.3 Chemical investigation of the broth EtOAc extracted crude

The EtOAc extracted crude (10.69 g) was subjected to a column chromatography [silica gel 60 (250 g); column diameter 5 cm] eluted with hexane, hexane in EtOAc, EtOAc, EtOAc in MeOH and MeOH in a stepwise fashion and 50 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine and vanillin/H_2SO_4 reagent to give 46 combined fractions (Table 3.3).

Table 3.3 The combined fractions obtained from the broth EtOAc extracted crude

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE-1	1-4	hexane:CH_2Cl_2 (50:50)	yellow viscous liquid	0.25
BE-2	5-8	hexane:CH_2Cl_2 (50:50)	yellow viscous liquid	0.81
BE-3	9-12	hexane:CH_2Cl_2 (45:55)	yellow viscous liquid	0.34
BE-4	13-24	hexane:CH_2Cl_2 (45:55)	yellow viscous liquid	1.35
BE-5	25-32	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	1.43
BE-6	33-39	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	5.79
BE-7	40-44	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	60.59
BE-8	45-48	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	50.60
BE-9	48-51	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	52.79
BE-10	52-57	hexane:CH_2Cl_2 (35:65)	yellow viscous liquid	83.73
BE-11	58-59	hexane:CH_2Cl_2 (30:70)	yellow viscous liquid	30.28
BE-12	60-61	hexane:CH_2Cl_2 (30:70)	yellow viscous liquid	70.12
BE-13	62-63	hexane:CH_2Cl_2 (30:70)	yellow viscous liquid	35.15
BE-14	64-82	hexane:CH_2Cl_2 (30:70)	yellow solid and yellow viscous liquid	88.81

Table 3.3 (continued)

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE-15	83-112	hexane:CH_2Cl_2 (30:70)	red solid and dark red viscous liquid	24.34
BE-16	113-125	hexane:CH_2Cl_2 (30:70)	dark red viscous liquid	10.51
BE-17	126-141	hexane:CH_2Cl_2 (20:80), hexane:CH_2Cl_2 (10:90), CH_2Cl_2 (100)	dark red viscous liquid	4.32
BE-18	142-151	CH_2Cl_2:MeOH (95:5)	dark red viscous liquid	25.59
BE-19	152-157	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	3.20
BE-20	158-159	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	11.30
BE-21	160-162	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	9.88
BE-22	163-165	CH_2Cl_2:MeOH (90:10)	white solid and dark red viscous liquid	12.40
BE-23	166-171	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	25.48
BE-24	172	CH_2Cl_2:MeOH (90:10)	red solid and dark red viscous liquid	57.94
BE-25	173	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	246.90
BE-26	174-175	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	252.11
BE-27	176	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	353.80
BE-28	177-179	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	272.60
BE-29	180-185	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	388.70
BE-30	186-200	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	579.50
BE-31	201-211	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	51.96
BE-32	212-217	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	175.60
BE-33	218-226	CH_2Cl_2:MeOH (85:15)	brown viscous liquid	16.10
BE-34	227-232	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	41.10
BE-35	233-248	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	362.80
BE-36	249-259	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	651.30
BE-37	260-267	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	166.60
BE-38	268-273	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	34.10
BE-39	274-336	CH_2Cl_2:MeOH (80:20)	gray brown viscous liquid	372.10

Table 3.3 (continued)

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE-40	337-346	CH_2Cl_2:MeOH (75:25)	gray brown viscous liquid	846.80
BE-41	347-368	CH_2Cl_2:MeOH (75:25)	gray brown viscous liquid	480.90
BE-42	369-376	CH_2Cl_2:MeOH (75:25)	gray brown viscous liquid	28.00
BE-43	377-421	CH_2Cl_2:MeOH (70:30)	gray brown viscous liquid	313.70
BE-44	422-437	CH_2Cl_2:MeOH (65:35)	gray brown viscous liquid	245.40
BE-45	438-448	CH_2Cl_2:MeOH (60:40)	black viscous liquid	244.80
BE-46	449-462	MeOH 100	black viscous liquid	293.00

BE-14 was purified by re-crystallization with hexane:$CHCl_3$ to obtain a compound **4** as yellow crystals (75.90 mg). BE-15 was purified by re-crystallization with hexane:$CHCl_3$:MeOH to obtain a compound **5** as a red amorphous solid (15.90 mg). BE-24 was purified by re-crystallization with hexane:$CHCl_3$:MeOH to obtain a compound **6** as red crystals (45.20 mg). Compound **4**, **5** and **6** was characterized by NMR, MS, IR, UV, optical rotation and melting point. The crystals of compound **4** and **6** were analyzed by X-ray diffractometer.

3.2.5.2 Cultivation and chemical investigation of the endophytic fungus MK-22

The agar culture of the endophytic fungus MK-22 grown on MEA at room temperature for 7-14 days was cut into 8 mm diameter disks by a flamed cork borer and then inoculated five disks into 250 mL Erlenmeyer flask containing 100 mL of MEB. After cultivation under static condition at room temperature for 5 weeks, the cultured media (10 L) were filtered through filter paper (Whatman No. 1). Fungal mycelia (817 g of wet weight) were dried, ground using blender and then extracted with 1L of MeOH (x5). The green extract was evaporated of under reduced pressure to 500 mL and kept in a refrigerator at 4°C. The amorphous solid was precipitated in the extract. After filtration of the solid, it was washed with EtOAc:MeOH (50:50) and crystallized from MeOH to give compound **7** as white solid (15.12 mg).

3.2.6 Bioassay of the isolated metabolites

3.2.6.1 Antimicrobial activity

3.2.6.1.1 Disk diffusion method

The disk diffusion method NNCLS (2003 and 2004) was used to determine the antimicrobial activity. Test microorganisms were as same as in the section 3.2.3.3. Plant pathogenic fungi; *Alternaria brasiicola, Collectotrichum gloeosporiodides,* and *Fusarium oxysporum*, were also used to evaluate the antimicrobial activity.

1). Preparation of samples

The isolated metabolites were weighed and dissolved in 1 mL of a solution of 10% DMSO in sterile distilled water.

2). Preparation of bacterial inoculum

Bacterial inoculum was prepared as the same manner described in 3.2.3.3.2.

3). Preparation of yeast inoculum

Yeast inoculum was prepared as the same manner described in 3.2.3.3.3.

4). Inoculation of the test plate

Bacteria and yeast were inoculated in the same manner as described in 3.2.3.3.4. Pure culture of each plant pathogenic fungi was cut into 8 mm diameter by a flamed cork borer. Pieces of fungi were placed on a new fresh Petri dish agar and incubated in room temperature for 2-3 days.

5). Application of secondary metabolites

Ten μL of the solution of the isolated metabolites was pipetted into the sterile paper disks (Whatman) 6 mm diameter which allowed to dry in the sterile Petri dish. Then the paper disks were placed on the test plate. Bacteria plates were incubated at 37^{o}C while yeast and plant pathogenic fungi plates were incubated at room temperature.

6). Preparation of antibiotic drug standards

Antibiotic drug standards were prepared in the same manner in 3.2.3.3.5.6. Antifungal (plant pathogenic) were used I prodione as positive controls.

3.2.6.1.2 Microbroth dilution method

This method was modified from the method described by NNCLS (2003 and 2004). Test microorganisms were obtained as same as in the section 3.2.3.3.

1). Preparation of samples

Weighted the secondary metabolites and dissolved in 1 mL of 10% DMSO in sterile distilled water. Each of metabolites was diluted to 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6. The samples were prepared in 7 concentrations.

2). Preparation of bacterial inoculum

Bacterial inoculum was prepared as the same manner described in 3.2.3.3.2 with approximately 10^8 CFU/mL cell suspension. The final inoculum was diluted with MHB to obtain a cell suspension containing approximately 10^7 CFU/mL. The accurate concentration of the inoculum was performed by colony counts.

3). Preparation of yeast inoculum

Yeast inoculum was prepared as the same manner described in 3.2.3.3.3 with approximately 10^8 CFU/mL cell suspension. The final inoculum was diluted with YMB to obtain a cell suspension containing approximately 10^7 CFU/mL. The accurate concentration of the inoculum was performed by colony counts.

4). Application of secondary metabolites

The final desired inoculum concentration was $5x10^5$ CFU/mL. Fifty μL of each isolated metabolite was dispensed into each well in sterile microtiter plates (96-well bottom wells). Fifty μL of microbial suspension was inoculated into each well. One hundred μl of medium only was used as the control. A mixture of 100 μL of medium and microbial inoculum was used as the growth control. Microbial

microtiter plates were incubated at 37°C and room temperature for bacterial and yeast, respectively.

5). Preparation of antibiotic drug solution

The antibiotic drug solution was prepared as the same manner described in 3.2.3.3.6.

6). Reading of microtiter plates assays

The activites were determined by measuring the turbidity each well by the Sunrise microplate reader (TECAN A-5082) after incubation. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibitory concentration (MIC).

3.2.6.2 Cytotoxicity test

Cytotoxic activity against 5 human tumor cell lines was carried out at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. The bioassay for *in vitro* cytotoxic activity toward five cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) were performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (Carmichael *et al*., 1987).

3.2.6.3 Antiplasmodial activity

The antiplasmodial activity was tested at Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTECH). The method was ascribed by Desjardins *et al.* (1979).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

The healthy and mature leaves of *Sandoricum koetjape* collected from 10 provinces were isolated using surface sterilization (Petrini 1982) to give two hundred and thirteen endophytic fungi isolates as shown in Table 4.1.

Table 4.1 A number of endophytic fungi isolated from leaves of *Sandoricum koetjape*.

Province	A number of endophytic fungi isolated from leaf section			Total
	midrib	lateral vein	lateral vein (leaf margin)	
Bangkok	8	3	6	17
Chiang Mai	12	5	7	24
Khonkaen	4	5	7	16
Mahasarakham	10	7	7	24
Pathumthani	10	6	-	16
Pattani	18	11	8	37
Phangnga	8	6	8	22
Phayao	15	4	2	21
Prachinburi	13	6	2	21
Samutsongkhram	9	3	3	15
Total	**107**	**56**	**50**	**213**

Surface sterilization were performed by soaking in 90% EtOH for 30 sec, in a solution of 10% Clorox® for 1 min, in 90% EtOH for 30 sec and rinsing twice with sterile distilled water.

4.2 Identification and classification of endophytic fungi

Each fungal isolate was grown on MEA, for 7 to 14 days at room temperature. A total of 213 isolates of endophytic fungi were classified using colony morphology such as sexual state, spores (sexual and asexual). Fifty eight fungal isolates were identified as *Alternaria* sp. (2 isolates), *Aspergillus* sp. (3 isolates), *Cladosporium* sp. (5 isolates),

Colletotrichum sp. (6 isolates), *Fusarium* sp. (8 isolates), *Pestalotia* sp. (4 isolates), *Phomopsis* sp. (13 isolates) and the fungi in Xylariaceae family (18 isolates). Other isolates of endophytic fungi were classified as mycelia sterilia due to no conidia or sporulate produced by those fungi. The results of the identification of the endophytic fungi from 10 provinces were shown in Table 4.2.

Table 4.2 Genera of isolated endophytic fungi from leaves of *Sandoricum koetjape*.

Province	**Genera of isolated endophytic fungi from leaves of *Sandoricum koetjape***									**Total**
	***Alternaria* sp.**	***Aspergillus* sp.**	***Cladosporium* sp.**	***Colletotrichum* sp.**	***Fusarium* sp.**	***Pestalotia* sp.**	***Phomopsis* sp.**	**Xylariaceae**	**Mycelia sterilia**	
Bangkok	-	-	1	1	1	-	2	2	10	17
Chiang Mai	-	-	-	2	1	-	1	3	17	24
Khonkaen	-	1	-	-	1	-	1	-	13	16
Mahasarakham	-	-	1	1	1	1	1	1	18	24
Pathumthani	1	1	1	-	-	-	-	2	11	16
Pattani	-	-	1	1	1	1	2	4	27	37
Phangnga	-	-	1	1	2	-	1	-	17	22
Phayao	-	1	-	-	1	1	2	2	14	21
Prachinburi	1	-	-	-	1	-	1	2	16	21
Samutsongkhram	-	-	-	-	-	1	1	2	12	15
Total	**2**	**3**	**5**	**6**	**8**	**4**	**12**	**18**	**155**	**213**

4.3 Screening the secondary metabolite production of the endophytic fungi

4.3.1 Investigation of the antimicrobial metabolite production of the endophytic fungi

The metabolites in culture broth produced by 213 isolates of the endophytic fungi were examined the antimicrobial activity against 5 microorganisms (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231) using an agar diffusion method (NNCLS, 2003 and 2004). The percentage of endophytic fungi isolates exhibiting antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231 were 75.93%, 68.52%, 9.26%, 9.26% and 40.74%, respectively. The culture broth of 54 isolates (25.35% of a total isolates) were exhibited antimicrobial activity against at least two microorganisms with the inhibition zone of 1-12 mm and the endophytic fungi exhibited antimicrobial activity against gram-positive bacteria, *B. subtilis* and *S. aureus*, were more than against gram-negative bacteria, *E. coli* and *P. aeruginosa*.

Table 4.3 The endophytic fungi produced antimicrobial metabolites.

Province	**Number of endophytic fungi**	**Number of endophytic fungi which exhibited antimicrobial activities (at least two microorganism)**
Bangkok	17	3
Chiang Mai	24	11
Khonkaen	16	7
Mahasarakham	24	5
Pathumthani	16	1
Pattani	37	7
Phangnga	22	3
Phayao	21	4
Prachinburi	21	7
Samutsongkhram	15	6
Total	**213**	**54**

Table 4.4 Number of endophytic fungi produced antimicrobial metabolites against 5 microorganisms.

Province	**Number of endophytic fungi which against tested microorganisms**				
	Bacillus subtilis	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
Bangkok	2	2	-	-	1
Chiang Mai	10		1	-	6
Khonkaen	4	3	2	3	5
Mahasarakham	4	2	-	-	1
Pathumthani	1	1	-	-	-
Pattani	5	5	1	-	4
Phangnga	2	3	-	-	-
Phayao	3	3	-	-	1
Prachinburi	6	5	-	2	1
Samutsongkhram	4	3	1	-	3
Total	**41**	**37**	**5**	**5**	**22**

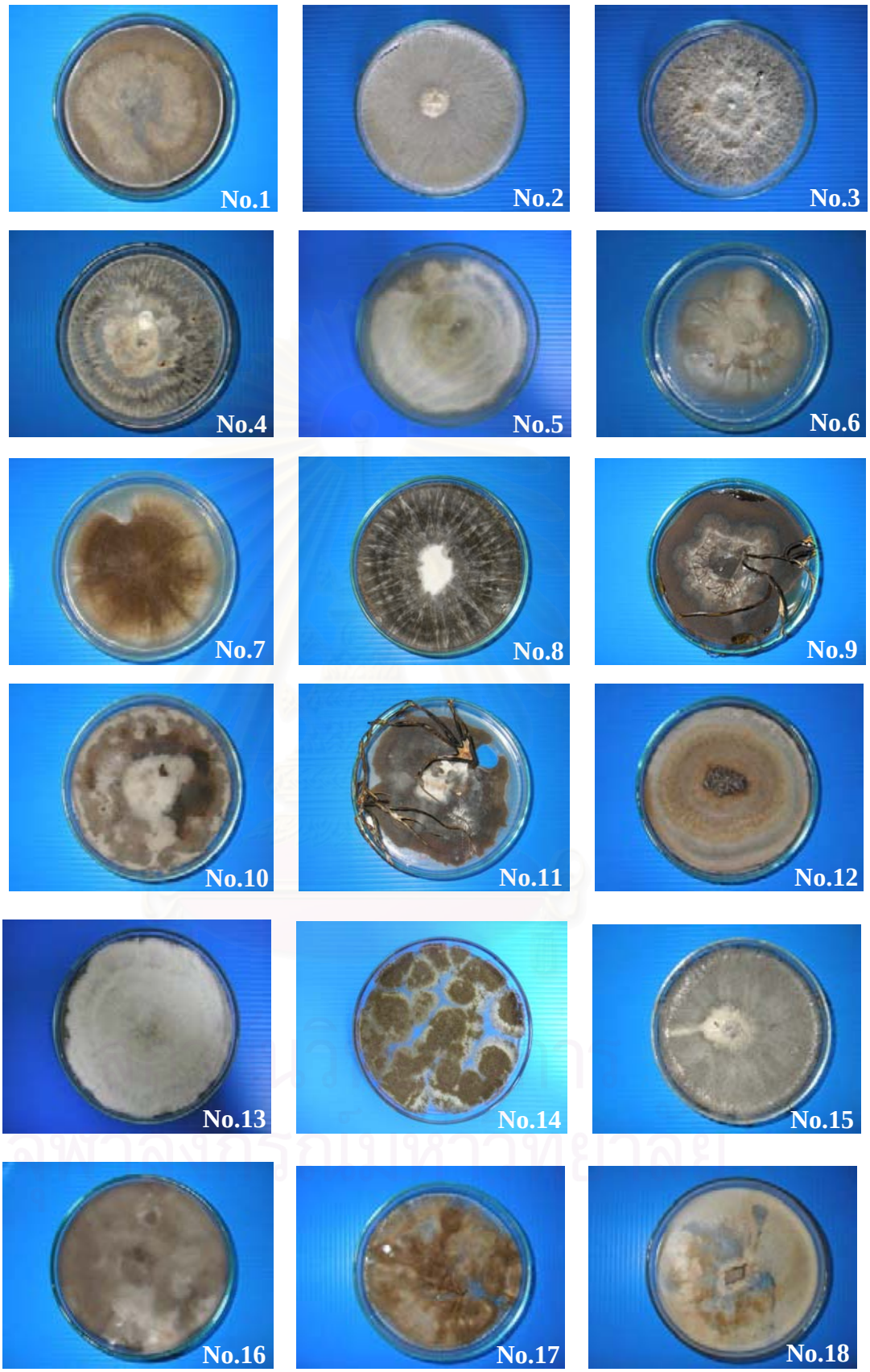


Figure 4.1 Colony of endophytic fungal were grown on PDA for 1-2 weeks at room temperature.

Figure 4.1 (continued)

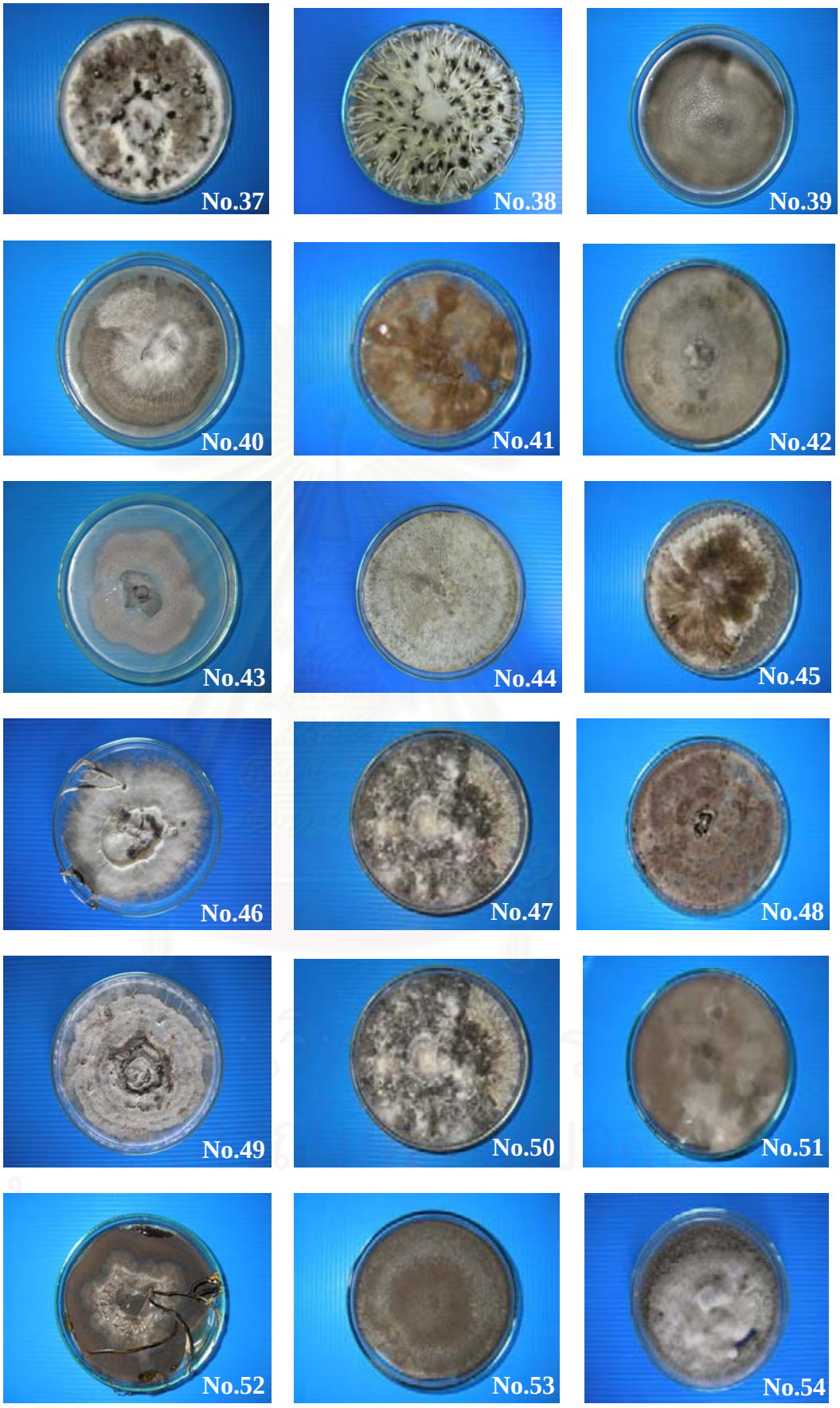


Figure 4.1 (continued)

Table 4.5 Morphological identification of endophytic fungi from endophytic fungi from leaves of *Sandoricum koetjape*.

No.	Endophytic fungi	Species
1	BK-3	Mycelia sterilia
2	BK-7	Mycelia sterilia
3	BK-8	*Phomopsis* sp.
4	BK-15	*Phomopsis* sp.
5	BK-18	Mycelia sterilia
6	CM-2	Mycelia sterilia
7	CM-4	Mycelia sterilia
8	CM-9	Xylariaceae
9	CM-10	Xylariaceae
10	CM-16	Mycelia sterilia
11	CM-24	Xylariaceae
12	CM-35	Mycelia sterilia
13	CM-52	Xylariaceae
14	KK-3	*Aspergillus* sp.
15	KK-9	Mycelia sterilia
16	KK-11	Mycelia sterilia
17	KK-17	Mycelia sterilia
18	KK-24	Mycelia sterilia
19	MK-4	Mycelia sterilia
20	MK-19	Mycelia sterilia
21	MK-20	Mycelia sterilia
22	MK-22	Xylariaceae
23	PB-9	Mycelia sterilia
24	PB-30	Xylariaceae
25	PB-33	Mycelia sterilia
26	PB-41	*Phomopsis* sp.
27	PB-60	Mycelia sterilia
28	PB-65	*Aspergillus* sp.
29	PNG-13	*Fusarium* sp.
30	PNG-23	*Phomopsis* sp.

Table 4.5 (continued)

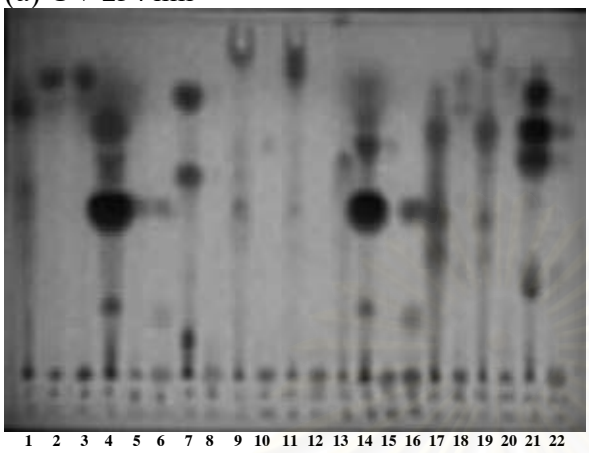
No.	Endophytic fungi	Species
31	PTA-7	Mycelia sterilia
32	PTA-10	Xylariaceae
33	PTA-11	Xylariaceae
34	PTA-16	Mycelia sterilia
35	PTA-20	Mycelia sterilia
36	PTA-23	Mycelia sterilia
37	PTA-32	*Phomopsis* sp.
38	PTA-39	Mycelia sterilia
39	PTA-40	Xylariaceae
40	PTU-5	Xylariaceae
41	PTU-10	*Alternaria* sp.
42	PTU-12	Xylariaceae
43	PTU-18	Mycelia sterilia
44	PTU-22	*Aspergillus* sp.
45	PTU-32	*Alternaria* sp.
46	PY-8	*Fusarium* sp.
47	PY-11	*Phomopsis* sp.
48	PY-19	Mycelia sterilia
49	PY-24	Xylariaceae
50	PY-25	Xylariaceae
51	PY-27	Mycelia sterilia
52	PY-43	Xylariaceae
53	SMK-1	Mycelia sterilia
54	SMK-8	Mycelia sterilia

4.3.2 Extraction and TLC analysis of the metabolites

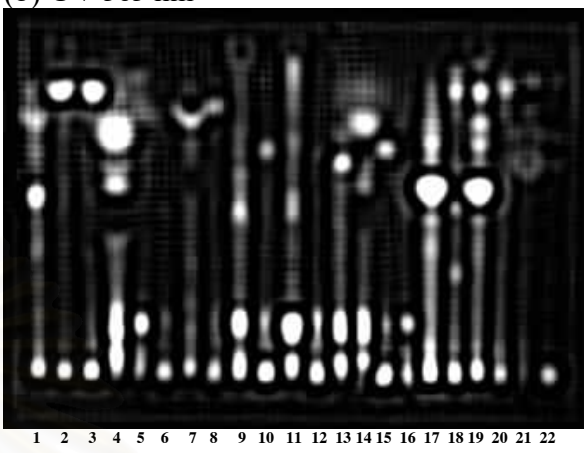
Fifty-four endophytic fungi were selected to study and culture in MEB for 5 weeks at static condition. Culture broth and mycelia of each endophytic fungus were filtered through filter paper (Whatman No. 1). The filtrates were extracted with EtOAc and mycelia were extracted with MeOH. The solvent was evaporated under reduced pressure. Both extracts of each fungus were analyzed by TLC and monitored by

UV, iodine and vanillin/H_2SO_4 reagent. The results of some metabolites have been shown in Figure 4.2.

(a) UV 254 nm



(b) UV 365 nm



TLC: silica gel; Mobile phase:15% $CHCl_3$ in hexane

1 = KK-3 (broth)
2 and 3 = KK-3 (mycelia)
4 = PB-30 (broth)
5 and 6 = PB-30 (mycelia)
7 = PY-4 (broth)
8 = PY-4 (mycelia)
9 = PTU-5 (broth)
10 = PTU-5 (mycelia)
11 = MK-22 (broth)
12 = MK-22 (mycelia)
13 = PY-25 (broth)
14 = PY-25 (mycelia)
15 = PTA-40 (broth)
16 = PTA-40 (mycelia)
17 = PB- 63 (broth)
18 = PB-63 (mycelia)
19 = CM-15 (broth)
20 = CM-15 (mycelia)
21 = PB-68 (broth)
22 = PB-68 (mycelia)

CM = Chiang Mai province
KK = Khonkaen province
MK = Mahasarakham province
PTU = Pathumthani province
PTA Pattani province
PY = Phayao province
PB = Prachinburi province

Figure 4.2 TLC profiles of the metabolites in the EtOAc extract of some endophytic fungi.

All of the selected endophytic fungi were cultured and extracted the culture broth with EtOAc. The extracted crude were primary screening by examined the antimicrobial activity against 5 standard microorganisms the result were showed in Table 4.5

The fungal isolate PB-30 was chosen for further study because the culture broth extracts was active against the test microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Table 4.5). The metabolite profiles of this fungi show in Figure 4.1 that indicated the large spots were detected under UV light at 254 nm and 365 nm in column No. 4.

The other selected endophytic fungal isolate was MK-22. This fungi was active against the test microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Table 4.5). More recently, this fungus could be against the plant pathogenic fungi such as *Alternaria brasiicola, Collectotrichum gloeosporiodides* and *Fusarium oxysporum*.

4.4 Identification of selected endophytic fungi

On the basis of antimicrobial activity and TLC profiles the endophytic fungi isolates PB-30 and MK-22 were selected for further studies.

Figure 4.3 Colony of endophytic fungi isolate PB-30 was grown on PDA (a) and MEB (b) for 2 weeks at room temperature.

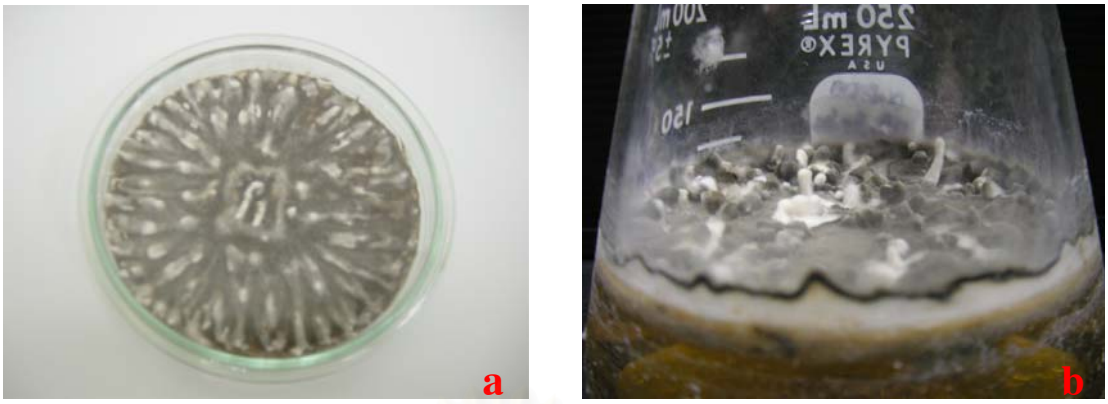


Figure 4.4 Colony of endophytic fungi isolate MK-22 was grown on PDA (a) and MEB (b) for 2 weeks at room temperature.

4.4.1 Identification of the endophytic fungus PB-30

Fungal isolate PB-30 was produced several stroma (Figure 4.3) which was the characteristic of xylariaceous fungi. From colony morphology of PB-30 it indicated the stroma shape as stipitate which were the characteristic of xylariaceous fungus. When characterized this fungi contemplate in the dichotomous and synoptic keys to xylariaceous genera (Fournier and Magni, 2002), this fungi was classified in genera *Xylaria*.

The xylariaceouse fungi are difficult to identified because they were seldom form the teleomorph in culture (Rodrigues and Petrini, 1997). PB-30 was identified based on fungal morphology and analysis of the DNA sequence of the ITS region. Total DNA was extracted from fungal mycelium grown in MAB followed in 3.2.4.1. Primers ITS1f and ITS4 were used to amplify the ITS1-5.8S-ITS2 region from total DNA extracted. The thermal cycle program was as follows: 94ºC for 5 min, followed by 38 cycles of 94ºC for 1 min, 51ºC for 1 min and 72ºC for 1 min, with a final extension of 72ºC for 5 min. The amplified DNA was purified and directly subjected to sequencing by Macrogen (Seoul, South Korea) using the same primers as for amplification. BLASTN 2.2.15 (Altschul *et al*., 1997) was used to search for similar sequences in the GenBank.

The ITS fragment length of fungal isolate PB-30 was 579 bp fragment as shown in Figure 4.5. A blast search was performed to find a similar sequence to ITS region of fungal isolate PB-30 in the GenBank DNA database. The ITS region of isolate PB-30 was similar to 91% identity of *Xylaria* sp. Alignment data of ITS region of isolates PB-30 was showed in Appendix C.

```
5′TTAAGTTCAG CGGGTATTCC TACCTGATCC GAGGTCAACC TTGATAAATT
  AGGGGTTTTA CGGCAGGGGA CCGGTCCAAC TAATAGGCGA GATAATATTT
  ACTACGTCTA GAGTGTGAAC CGACTCCGCC ACTAATTTTA AGGGGCTACC
  GCCATACGGT AGGCCCCCAA CGCTAAGCAA CAGAAGGCTT AAGGGTTGAA
  ATGACGCTCG AACAGGCATG CCCACTAGAA TACTAATGGG CGCAATGTGC
  GTTCAAAGAT TCGATGATTC ACTGAATTCT GCAATTCACA TTACTTATCG
  CATTTCGCTG CGTTCTTCAT CGATGCCAGA ACCAAGAGAT CCGTTGTTGA
  AAGTTTTAAC TTATTTAGTT GTAATTCAGA TATCCAGTAA TTAAACAGAG
  TTTAATGGGG CGCCGGCGGG CTTACCCGTG CCTACCGGGT AGGCACTTAC
  AGGTAAGTGC ACTACAGGGT AGGTACGACC CGCCGAGGCA ACGTTAGGTA
  TGTTCACATG GGGTTTGGGA GTTATAAACT CTTTAATGAT CCCTCCGCTG
  GTTCACCAAC GGAGACCTTG TTACGACTT 3′
```

Figure 4.5 Nucleotide sequences of partial 18S region, complete ITS region of the isolate PB-30.

4.4.2 Identification of the endophytic fungus MK-22

Fungal isolate MK-22 was produced several stroma (Figure 4.4) which indicated the stroma shape as stipitate and characterised of xylariaceous fungus. This fungi was classified in genera *Xylaria*. When characterized this fungi contemplate in the dichotomous and synoptic keys to xylariaceous genera (Fournier and Magni, 2002).

MK-22 was identified based on fungal morphology and analysis of the DNA sequence of the ITS region. Total DNA was extracted from fungal mycelium grown in MAB followed in 3.2.4.1. Primers ITS1f and ITS4 were used to amplify the ITS1-5.8S-ITS2 region from total DNA extracted. The thermal cycle program was same as PB-30. The amplified DNA was purified and directly subjected to sequencing by Macrogen (Seoul, South Korea) using the same primers as for amplification. BLASTN 2.2.15 (Altschul *et al*., 1997) was used to search for similar sequences in the GenBank.

Sequence analysed of the rDNA, partial 18S ITS region of the isolates MK-22 was 578 bp fragment as shown in Figure 4.6. The ITS1-5.8S-ITS2 sequences of MK-22 also showed the highest homology to those of *Xylaria arbuscula* with the sequence identity of 92%. These results suggested that MK-22 should tentatively be *Xylaria* sp., a fungus in family Xylariaceae. Alignment data of ITS region of isolates MK-22 was showed in Appendix C.

```
5′TTAAGTTCAG CGGGTATTCC TACCTGATCC GAGGTCAACC TTGAAAAATT
```

AGGGGGTTTT ACGGCAAGAG ACCGGCCTAA CCACAGACGA GATAGAAGCT
ACTACGTCTA GAGTGCGAAC CGACTCCGCC AAACTTTAGG GAGCTACAGA
GGACTGTAGG CTCCCAACAC TAAGCAACAG GGGCTTAAGG GTTGAAATGA
CGCTCGAATA GGCATGCCCA CTAGAATACT AATGGGCGCA ATGTGCGTTC
AAAGATTCGA TGATTCACTG AATTCTGCAA TTCACATTAC TTATCGCATT
TCGCTGCGTT CTTCATCGAT GCCAGAACCA AGAGATCCGT TGTTGAAAGT
TTTAACTTAT TTAGTTGTAA AATCAGAATA ACATATAATA AACAGTGTTT
TAACGGGCCA CTGGCAGGCG AACCCGTGAC TACCAGGTAG TCGCCTACAG
GGTAGGCGAC CACAGGGTAG ACACGACCTG CCGAGGCAAC AGAAGGTAAG
TTCACATGGG TTTGGGAGTT ACGAAAACTC TTTAATGATC CCTCCGCTGG
TTCACCAACG GAGACCTTGT TACGACTT 3′

Figure 4.6 Nucleotide sequences of partial 18S region, complete ITS region of the isolate MK-22.

The nucleotide sequence data of PB-30 and MK-22 were submitted in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB 285482 and AB 285483, respectively.

4.5 Growth profile and biological activity test of culture broth

4.5.1 Growth profile and biological activity test of PB-30 culture broth

The endophytic fungi PB-30 was culture into 250 mL flask containing 100 mL of MEB and culture under static condition at room temperature for 20 weeks. The culture broth were filtered through filter paper (Whatman No. 1) and mycelia were measured the cell mass. The filtrates were exhibited biological activities detected pH and reducing sugar (Figure 4.7). After then extracted by EtOAc and examined the antimicrobial activity against 5 microorganisms (Table 4.6).

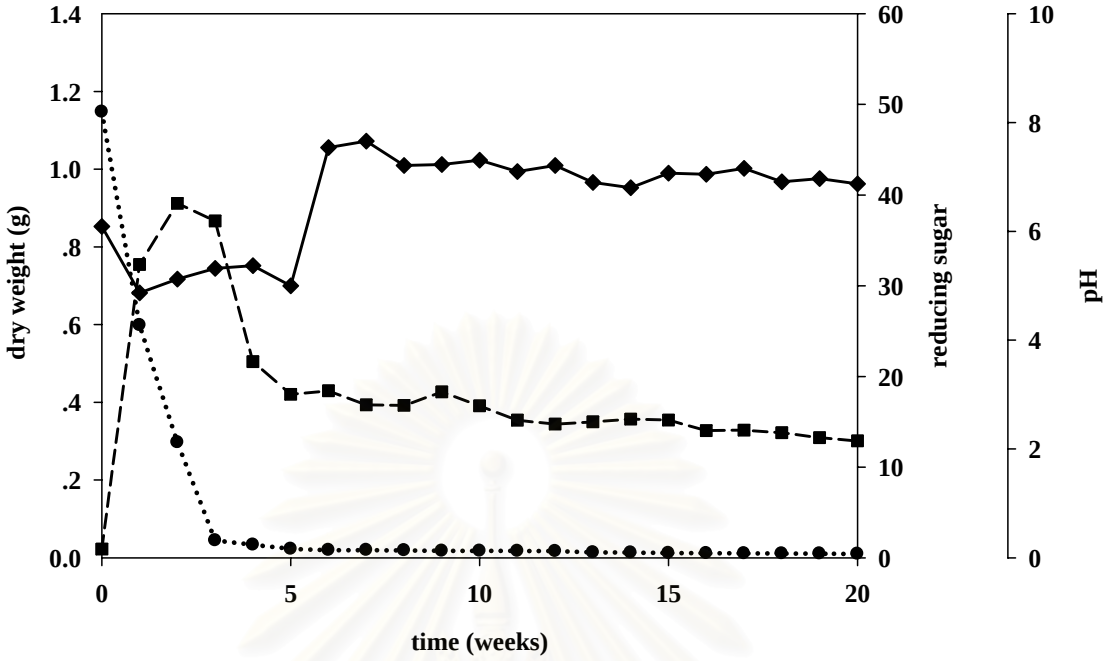


Figure 4.7 Growth profile of PB-30 culture extracted crude, (■) dry weight; (●) reducing sugar; (◆) pH

Table 4.6 Biological activity test of PB-30 culture extracted crude against tested

PB-30 culture extracted crude (weeks)	Clear zone (mm) against tested microorganisms				
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
1	9.0	9.5	-	-	-
2	9.5	9.0	8.0	-	-
3	13.0	16.5	10.0	-	-
4	15.5	18.5	10.0	-	-
5	18.5	22.5	11.5	-	-
6	11.5	12.5	10.0	-	-
7	8.0	8.0	7.5	-	-
8	8.0	8.5	-	-	-
9	10.0	11.5	-	-	-
10	8.0	8.5	-	-	-
11	8.0	8.0	-	-	-
12	7.0	-	-	-	-

- inactive

Table 4.6 (continued)

PB-30 culture extracted crude (weeks)	Clear zone (mm) against tested microorganisms				
	Bacillus subtilis	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
13	7.5	-	-	-	-
14	7.0	-	-	-	-
15	7.0	-	-	-	-
16	9.0	-	-	-	-
17	8.0	-	-	-	-
18	8.5	-	-	-	-
19	8.0	-	-	-	-
20	8.0	-	-	-	-

- inactive

4.5.2 Growth profile and biological activity test of MK-22 culture broth

The endophytic fungi MK-22 was culture into 250 mL flask containing 100 mL of MEB and culture under static condition at room temperature for 20 weeks. The culture broth were filtered through filter paper (Whatman No. 1) and mycelia were measured the cell mass. The filtrates were exhibited biological activities detected pH and reducing sugar (Figure 4.8). After then extracted by EtOAc and examined the antimicrobial activity against 5 microorganisms (Table 4.7).

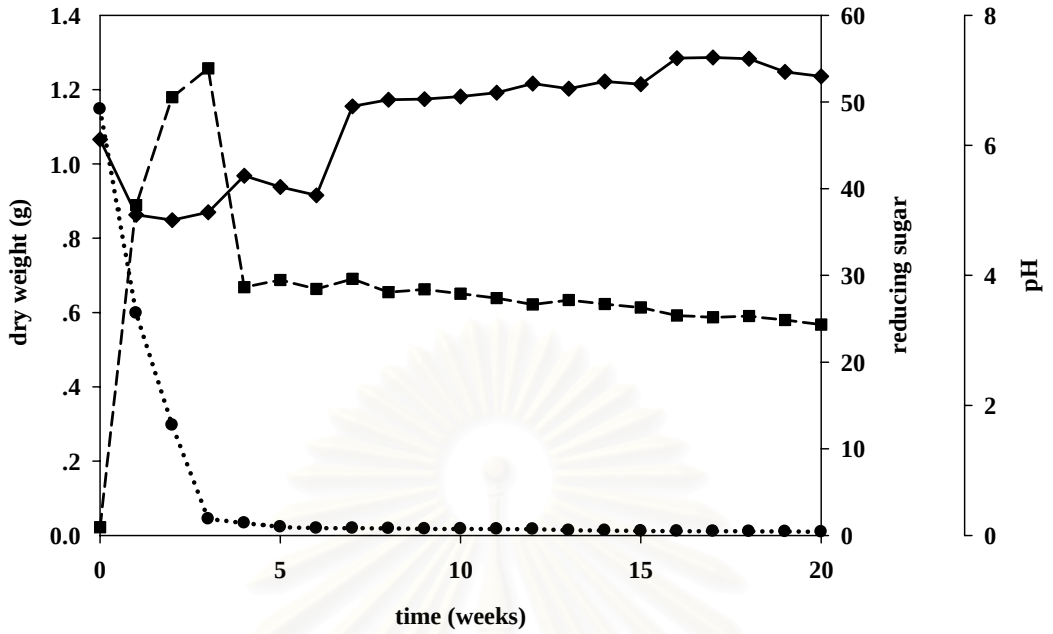


Figure 4.8 Growth profile MK-22 culture extracted crude, (■) dry weight; (●) reducing sugar; (◆) pH

Table 4.7 Biological activity test of MK-22 culture extracted crude against tested microorganisms.

MK-22 culture extracted crude (week)	**Clear zone (mm) against tested microorganisms**				
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
1	-	-	-	-	-
2	7.0	7.0	-	-	-
3	8.0	8.0	-	-	-
4	10.0	12.0	8.0	-	-
5	12.0	12.5	9.0	-	-
6	12.0	10.0	7.0	-	-
7	12.0	11.0	-	-	-
8	10.5	10.5	-	-	-
9	8.5	8.0	-	-	-
10	8.0	8.0	-	-	-
11	7.0	-	-	-	-

- inactive

Table 4.7 (continued)

MK-22 culture extracted crude (week)	Clear zone (mm) against tested microorganisms				
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
12	7.0	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	-	-	-	-	-

- inactive

4.6 Chemical investigation of the metabolites produced by the fungi PB-30 and MK-22

4.6.1 Cultivation and chemical investigation of the metabolites produced by the endophytic fungus PB-30

The endophytic fungus PB-30 was cultivated in malt extract broth under static condition at room temperature for 5 weeks and then the whole culture (10.5 L) was filtered through filter paper (Whatman no. 1). After the filtrate was concentrated under reduced pressure to 500 mL the concentrated filtrate was partitioned with an equal volume of hexane, EtOAc and MeOH, respectively followed by removal of the solvent to yield 515 mg, 10.69 g and 7.57 g of the extracts, respectively.

Fungal mycelia were extracted with hexane, EtOAc and MeOH to give the hexane extract as brown viscous oil (26 mg), the EtOAc extract as brown viscous residue (172 mg) and the MeOH extract as brown viscous oil (510 mg), respectively. Extraction diagram of mycelia and broth of endophytic fungus PB-30 was shown in Figure 3.2.

4.6.1.1 Chemical investigation of MeOH crude extracted from mycelia of the fungus PB-30

The MeOH crude obtained from mycelia was isolated by silica gel column chromatography eluted with increasing polarity from hexane, EtOAc in hexane,

EtOAc, MeOH in EtOAc and MeOH. The similar fractions were combined on the basis of TLC profile to give 18 combined fractions as shown in Table 4.8.

Table 4.8 The combined fractions obtained from the mycelia MeOH extracted crude

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
MM-1	1-8	hexane:EtOAc (50:50)	yellow viscous liquid	32.5
MM-2	9-15	hexane:EtOAc (50:50)	yellow viscous liquid	21.1
MM-3	16-21	hexane:EtOAc (50:50)	yellow viscous liquid	17.2
MM-4	22-26	hexane:EtOAc (40:60)	yellow viscous liquid	13.2
MM-5	27-37	hexane:EtOAc (30:70), hexane:EtOAc (20:80)	yellow viscous liquid	18.4
MM-6	38-51	hexane:EtOAc (10:90), EtOAc (100)	yellow viscous liquid	23.5
MM-7	52-61	EtOAc (100), EtOAc:MeOH (90:10)	yellow viscous liquid	16.9
MM-8	62-64	EtOAc:MeOH (80:20)	brown viscous liquid	17.7
MM-9	65-69	EtOAc:MeOH (80:20)	white solid and brown viscous liquid	38.3
MM-10	70-85	EtOAc:MeOH (70:30)	brown viscous liquid	24.7
MM-11	86-89	EtOAc:MeOH (60:40)	brown viscous liquid	15.8
MM-12	90-100	EtOAc:MeOH (55:45)	brown viscous liquid	13.4
MM-13	101-116	EtOAc:MeOH (50:50), EtOAc:MeOH (45:55)	brown viscous liquid	19.3
MM-14	117-128	EtOAc:MeOH (40:60)	brown viscous liquid	18.7
MM-15	129-137	EtOAc:MeOH (30:70), EtOAc:MeOH (20:80)	brown viscous liquid	10.3
MM-16	138-149	EtOAc:MeOH (10:90), MeOH (100)	gray brown viscous liquid	18.0
MM-17	150-159	MeOH (100)	gray brown viscous liquid	16.5
MM-18	160-165	MeOH (100)	gray brown viscous liquid	28.3

Fraction MM-9 appearances the white solid and brown viscous liquid and showed the interest spot in TLC. The white solid was washed by 50% EtOAc

in MeOH and gave compound **1** (20.3 mg, 3.98% of total MeOH crude extracted). This compound was characterized by NMR, MS, IR, UV, optical rotation and melting point.

4.6.1.1.1 Structure elucidation of compound 1

Compound **1** was as amorphous white solid was dissolved in H_2O;

m.p. 168-170 ºC;

FT-IR spectrum (KBr) (Figure D1-1) ν_{max} (cm^{-1}): 3387, 2933, 1083 and 1019;

^{1}H-NMR spectrum (D_2O, 400 MHz) (Figure D 1-2) 3.65 (2H, m), 3.76 (2H, t, J = 8), 3.81 (2H, dd, J = 2.8, 4) and 3.87 (2H, dd, J = 2.8, 8) ppm;

^{13}C-NMR spectrum (D_2O, 100 MHz) (Figure D 1-3) 70.67 (C-2 and C-5), 69.10 (C-3 and C-4), and 63.11 (C-1 and C-6) ppm;

ESI spectrum (Figure D 1-7) $[M+Na]^+$ at m/z = 205.1 and the molecular formula of compound **1** was $C_6H_{14}O_6$.

The IR spectrum of compound **1** were indicate the broad absorption of O─H stretching vibration at 3387 cm^{-1}, the signal absorption of the C─H stretching vibration (for symmetric stretching) at 2933 cm^{-1} and the absorption of C─O stretching vibration at 1083, 1019 cm^{-1}.

The ^{1}H NMR spectrum of compound **1** displayed the signals of methylene protons of the oxygenated carbons at δ 3.65 1nd 3.87 ppm, and two protons of the oxygenated methine carbons at δ 3.76 and 3.81 ppm.

The ^{13}C NMR spectrum of compound **1** revealed 3 carbons compring of methylene carbon at 63.11 ppm and two methine carbons at δ 69.10 and 70.67 ppm.

In comparison with ^{1}H and ^{13}C NMR spectral data of compound **1** and D-mannitol (Hakiwara *et al*., 2005) (Table 4.9).

Table 4.9 ^{13}C NMR data for compound **1** and D-mannitol.

No.	Compound 1[a]		D-mannitol[b] (Hakiwara *et al*., 2005)	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1,6	3.87 (2H, dd, *J* = 2.8, 8), 3.65 (2H, m)	63.11	3.84 (2H, dd, *J* = 3.3, 11.8), 3.65 (2H, dd, *J* = 6.1, 11.9)	63.58
2,5	3.76 (2H, t, *J* = 8)	70.67	3.76 (2H, t, *J* = 8.6)	71.19
3,4	3.81 (2H, dd, *J* = 2.8, 4)	69.10	3.73 (2H, m)	69.63

[a] δ_H (D_2O, 400 MHz); δ_C (D_2O, 100 MHz)

[b] δ_H (D_2O, 500 MHz); δ_C (D_2O, 125 MHz)

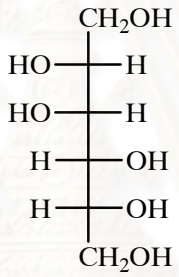


Figure 4.9 Structure of compound **1.**

4.6.1.2 Chemical investigation of hexane crude extracted from culture broth of the fungus PB-30

The hexane crude (515 mg) was separated by silica gel column chromatography eluented with increasing polarity from hexane, hexane in EtOAc, EtOAc, EtOAc in MeOH and MeOH. The similar fractions were combined on the basis of TLC profile to give 8 combined fractions as shown in Table 4.10.

Table 4.10 The combined fractions obtained from the broth hexane extracted crude

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BH-1	1-36	hexane (100), hexane:EtOAc (90:10)	yellow viscous liquid	55.50
BH-2	37-62	hexane:EtOAc (85:15), hexane:EtOAc (80:20), hexane:EtOAc (75:25)	yellow viscous liquid	36.66
BH-3	63-118	hexane:EtOAc (70:30), hexane:EtOAc (65:35)	white solid and yellow viscous liquid	105.87
BH-4	119-211	hexane:EtOAc (60:40), hexane:EtOAc (50:50), hexane:EtOAc (45:55)	yellow viscous liquid	26.97
BH-5	212-239	hexane:EtOAc (40:60), hexane:EtOAc (35:65), hexane:EtOAc (30:70), hexane:EtOAc (20:80)	brown viscous liquid	69.96
BH-6	240-274	hexane:EtOAc (15:85), hexane:EtOAc (10:80)	brown viscous liquid	35.91
BH-7	275-309	hexane:EtOAc (5:95), EtOAc (100), EtOAc:MeOH (90:10)	brown viscous liquid	27.77
BH-8	310-357	EtOAc:MeOH (80:20), EtOAc:MeOH (50:50), MeOH (100)	brown viscous liquid	76.99

Fraction BH-1 and BH-3 were showed a large bright blue spot on TLC which developed in mobile phase as 2% MeOH in CH_2Cl_2. BH-1 was eluted by hexane (100%), hexane:EtOAc (95:5) and hexane:EtOAc (90:10), respectively and gave yellow viscous liquid. The compound **2** (16.35 mg, 3.20% of total hexane crude extract), clear viscous liquid, was purified from mixture BH-1 by heaxane:EtOAc (60:40). Fraction BH-3 (105.87 mg), white solid and yellow viscous liquid, was eluted by hexane:EtOAc (70:30) and hexane:EtOAc (65:35). The white solid was purified by hexane:EtOAc (20:80) and gave compound **3** (46.77 mg, 9.15% of total hexane crude extract). Compound **2** and **3** was characterized by NMR, MS, IR, UV, optical rotation and melting point.

4.6.1.2.1 Structure elucidation of compound 2

Compound **2** was obtained from the elution of column chromatography with 100% hexane to 90% hexane in EtOAc and further purified by 60% hexane in EtOAc to give compound **2** as a white solid and dissolved in hexane, EtOAc, CH_2Cl_2 and MeOH;

$[\alpha]_D^{20}$ +103(*c* 0.025, MeOH);

λ_{max} (MeOH) (log ε) 247.93 (3.08) nm;

FT-IR spectrum (KBr) (Figure D 2-1) ν_{max} (cm^{-1}): 3100, 3058, 2975, 2917, 1671, 1619, 1579, 1460, 1378, 1290, 1119, 1049 and 951;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure D 2-2) 1.52 (3H, d, *J* = 6.4, Me-3), 2.92 (2H, d, *J* = 6.88, H-4), 4.72 (1H, dd, *J* = 6.9 Hz, H-3), 6.68 (1H, d, *J* = 7.2 Hz, H-5), 6.87 (1H, d, *J* = 8.8 Hz, H-7), 7.39 (1H, dd, *J* = 8.0 and 7.6 Hz, H-6) and 11.01 (1H, s, OH-8) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (FigureD 2-3) 20.72 (3-Me), 34.57 (C-4), 76.07 (C-3), 108.27 (C-8a), 116.19 (C-7), 117.88 (C-5), 136.11 (C-6), 139.37 (C-4a), 162.016 (C-8) and 169.92 (C-1) ppm;

R_f value 0.46 developed on silica gel TLC using 50% MeOH in CH_2Cl_2 as the mobile phase and the spot was visualized with UV lamp (365 nm).

The IR spectrum of compound **2** were exhibited absorption band at ~3100 cm^{-1} was showed the O─H stretching vibration, the absorption band at 3058 cm^{-1} was showed the =C─H stretching vibration, the signal absorption band at 2975 cm^{-1} of the ─C─H stretching vibration (for asymmetric stretching of methyl group), the signal absorption band at 2917 cm^{-1} of the ─C─H stretching vibration (for symmetric stretching of methyl group), the strong absorption band at 1671 cm^{-1} of the C=O stretching vibration of carbonyl group, the absorption bands at 1619, 1579, 1460, 1378, 1290, 1119 cm^{-1} of the C=C stretching vibration and the absorption bands at 1049, 951 cm^{-1} of C─O stretching vibration.

The ^{1}H NMR spectrum of compound **2** indicated that it one methyl group at δ 1.52 ppm, one methylene group at δ 2.92 ppm, four methines at δ 4.72, 6.68, 6.87 and 7.39 ppm and one aromatic proton at δ 11.01 ppm.

The ^{13}C NMR spectrum of compound **2** displayed 10 carbon resonances. The signal at δ 169.92 and 162.16 ppm could be ascribed to carbonyl

group of ketone and an aromatic carbon bound to hydroxy group, respectively. The resonance at δ 139.37 and 108.27 ppm were ascribed to aromatic carbons. Three signals of quaternary carbon at δ 136.11, 117.88, and 116.19 ppm. One signal of a methine carbon of at δ 76.07 ppm, a signal of methylene carbon at δ 34.57 ppm and a methyl carbon at 20.72 ppm. In comparison of ^{1}H and ^{13}C NMR data of compound **2** with mellein reported in literatures (Table 4.11) compound **2** was identified as HMBC correlation of compound **2** revealed that the structure of compound **2** was **8-dihydroxy-3-methyl-isochroman-1-one** or **mellein.**

Table 4.11 ^{1}H and ^{13}C NMR data for compound **2** and mellein.

No.	**compound 2**		**mellein** (Cloe *et al*., 1971)	**R-(—)-mellein** (Edwards *et al*., 1999)	
	δ_C	**δ_H (mult., *J* in Hz)**	**δ_H (mult., *J* in Hz)[a]**	**δ_C**	**δ_H (mult., *J* inHz)**
1	169.92			169.96	
2					
3	76.07	4.72 (1H, dd, *J*=6.9)	4.50 (1H, sextet, *J*=3)	76.10	4.75 (1H, m)
3-Me	20.72	1.52 (3H, d, *J*=6.4)	1.22 (3H, d, *J*=3)	20.75	1.53 (3H, d, *J*=6.2)
4	34.57	2.92 (2H, d, *J*=6.8)	2.83 (2H, d, *J*=3)	34.58	2.93 (2H, d, *J*=7.3)
4a	139.37			136.15	
5	117.88	6.68 (1H, d, *J*=7.2)	7.22 (1H, t, *J*=4)	117.91	7.44 (1H, t, *J*=7.7)
6	136.11	7.39 (1H, dd, *J*=8.0 and 7.6)	6.60 (1H, d, *J*=4)	139.39	6.60 (1H, d, *J*=7.9)
7	116.19	6.87 (1H, d, *J*=8.8)	6.46 (1H, d, *J*=4)	116.20	6.89 (1H, d, *J*=7.9)
8	162.16			162.15	
8-OH		11.01 (1H, s)	11.03 (1H, s)		11.03 (1H, s)
8a	108.27			108.26	

[a] Not showed the NMR frequency.

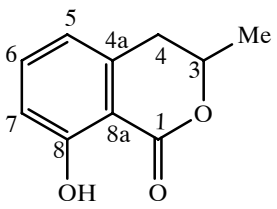


Figure 4.10 Structure of compound **2.**

4.6.1.2.1 Structure elucidation of compound 3

Compound **3** was obtained from the elution of column chromatography with 90% CH_2Cl_2 in MeOH and re-crystallization with $CHCl_3$ and hexane to obtain a white powder (92.50 mg). Compound **3** is soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 95-97°C;

$[\alpha]_D^{20}$ +46 - +47 (c = 0.075, $CHCl_3$);

λ_{max} (MeOH) (log ε) 219 (4.86) nm;

FT-IR spectrum (NaCl) (Figure D 3-1) ν_{max} (cm^{-1}): 3395, 3069, 2995, 2917, 2821, -3200, 1673, 1582, 1478, 1413, 1295, 1195, 1091, 1021, 960, 917, 821, 747, 673, 591 and 482;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure D 3-2) 1.59 (3H, s, Me-3), 3.49 (1H, s, OH-4), 4.59 (1H, s, H-4), 4.65 (1H, d, *J* = 8.58 Hz, H-3), 6.93 (1H, d, *J* = 7.0 Hz, H-5), 7.04 (1H, d, *J* = 8.6 Hz, H-7), 7.52 (1H, dd, *J* = 7.8 Hz, H-6) and 11.03 (1H, s, OH-8) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure D 3-3) 16.02 (OMe), 67.23 (C-4), 78.27 (C-3), 106.58 (C-8a), 118.34 (C-7), 118.50 (C-5), 136.81 (C-6), 140.57 (C-4a), 162.07 (C-8) and 169.25 (C-1) ppm;

ESI-TOF (Figure D 3-9) *m/z* 195.0657 $[M+H]^+$ *calc.* for $C_{10}H_{10}O_4$ 195.1953;

R_f value 0.54 developed on silica gel TLC using 2% MeOH in CH_2Cl_2 as the mobile phase and the spot was visualized with UV lamp (365 nm).

The IR spectrum of compound **3** were indicate the absorption band at 3700-3100 cm^{-1} was broad and showed the O─H stretching vibration, the signals absorption band at 3069 cm^{-1} showed the =C─H stretching vibration, the absorption band at 2995 cm^{-1} showed the ─C─H stretching vibration (for asymmetric stretching of methyl group), the absorption broad band at ~2900 cm^{-1} showed the ─C─H stretching vibration (for symmetric stretching of methyl group), the strong absorption band at 1673 $cm^{-1}$of the C=O stretching vibration of carbonyl group, the absorption bands at 1478, 1413, 1295, 1195 $cm^{-1}$of the C=C stretching vibration and the absorption bands at 1091, 1021 $cm^{-1}$of C─O stretching vibration.

The ^{1}H NMR spectrum of compound **3** indicated that it one methyl group at δ 1.59 ppm, one proton attached with oxygen group at δ 3.49 ppm,

five methines at δ 4.59, 4.65, 6.93, 7.04 and 7.52 ppm and one aromatic proton at δ 11.03 ppm.

The ^{13}C NMR spectrum of compound **3** displayed 10 carbon resonances. The signals at δ 169.25 and 162.07 ppm could be ascribed to carbonyl group of ketone and an aromatic carbon bound to hydroxy group, respectively. The resonances at δ 140.57 and 106.58 ppm were ascribed to aromatic carbons. Three signals of quaternary carbon at δ 136.81, 118.50, and 118.34 ppm. One signal of methine carbon appeared at δ 78.27 ppm, a methylene carbon of hydroxy group at δ 67.23 ppm and a methyl carbon at δ 16.02 ppm.

From spectroscopic data analysis of compound **3** was indicated to **4,8-dihydroxy-3-methyl-3,4-dihydroisochroman-1-one** or **4-hydroxymellein**, an isocoumarin metabolite. This metabolite was isolated from microorganism such as *Aspergillus oniki* (Sasaki *et al.,* 1970), *Aspergillus ochraceus* (Cole *et al*., 1971; Moore *et al*., 1972), *Cercospora taiwanensis* (Camarda *et al*., 1976), *Cercospora* sp. (Assante *et al*., 1977), *Lasiodiplodia theobromae* (Devys *et al*., 1980), *Microsphaeropsis* sp. (Höller *et al*., 1999) and endophytic fungi including *Xylaria longiana* (Rehm.) (Edwards *et al.*, 1999) and *Diplodia corticola* (Evidente *et al*., 2006). More recently, this metabolite had found in plant, the stem bark of *Uvaria hamiltonii* (Asha, *et al*., 2004).

Table 4.12 ^{1}H and ^{13}C NMR data for compound **3** and 4-hydroxymellein.

No.	**compound 3**		**4-hydroxymellein (Cloe *et al*., 1971)**	**4-hydroxymellein (Devys *et al*., 1980)**
	δ_C	**δ_H (mult., *J* in Hz)**	**δ_H (mult., *J* in Hz)[a]**	**δ_H (mult., *J* in Hz)[b]**
1	169.25			
2				
3	78.27	4.65 (1H, d, J=8.53)	4.40 (1H, s)	4.56 (1H, dd, J=6, $J_{3,4}$=2)
3-Me	16.02	1.59 (3H, d, J=7.0)	1.22 (3H, d, J=3)	1.55 (3H, d, J=6)
4	67.23	4.59 (1H, s)	4.45 (1H, d, J=2)	4.50 (1H, d, J=2)
4-OH		3.49 (1H, s)	2.61 (1H, d, J=3)	3.26 (1H, s large)
4a	140.57			
5	118.50	6.93 (1H, d, J=7.0)	6.85(1H, d, J=4)	6.95 (1H, d, J=4)
6	136.81	7.52 (1H, dd, J=7.8)	7.42 (1H, t, J=4)	7.52 (1H, dd, J=4)

No.	compound 3		4-hydroxymellein (Cloe *et al*., 1971)	4-hydroxymellein (Devys *et al*., 1980)
	δ_C	δ_H (mult., *J* in Hz)	δ_H (mult., *J* in Hz)[a]	δ_H (mult., *J* in Hz)[b]
7	118.34	7.04 (1H, d, *J*=8.6)	6.82 (1H, d, *J*=4)	6.88 (1H, d, *J*=3.5)
8	162.07			
8-OH		11.03 (1H, s)	11.03 (1H, s)	11 (1H, s)
8a	106.58			

[a,b] Not showed the NMR frequency.

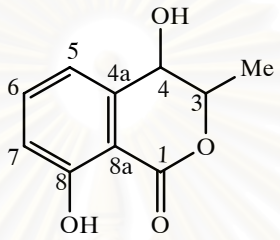


Figure 4.11 Structure of compound **3.**

4.6.1.3 Chemical investigation of EtOAc crude extracted from culture broth of the fungus PB-30

EtOAc crude extract (10.69 g) was separated by silica gel column chromatography eluted with hexane, hexane in CH_2Cl_2, hexane in acetone, CH_2Cl_2, CH_2Cl_2 in MeOH and MeOH in stepwise fashion. The similar fractions were combined on the basis of TLC profile to give 46 combined fractions as shown in Table 4.13.

Table 4.13 The combined fractions obtained from the broth EtOAc extracted crude

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE-1	1-4	hexane:CH_2Cl_2 (50:50)	yellow viscous liquid	0.25
BE-2	5-8	hexane:CH_2Cl_2 (50:50)	yellow viscous liquid	0.81
BE-3	9-12	hexane:CH_2Cl_2 (45:55)	yellow viscous liquid	0.34
BE-4	13-24	hexane:CH_2Cl_2 (45:55)	yellow viscous liquid	1.35
BE-5	25-32	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	1.43
BE-6	33-39	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	5.79
BE-7	40-44	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	60.59
BE-8	45-48	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	50.60
BE-9	48-51	hexane:CH_2Cl_2 (40:60)	yellow viscous liquid	52.79
BE-10	52-57	hexane:CH_2Cl_2 (35:65)	yellow viscous liquid	83.73

Table 4.13 (continued)

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE-11	58-59	hexane:CH_2Cl_2 (30:70)	yellow viscous liquid	30.28
BE-12	60-61	hexane:CH_2Cl_2 (30:70)	yellow viscous liquid	70.12
BE-13	62-63	hexane:CH_2Cl_2 (30:70)	yellow viscous liquid	35.15
BE-14	64-82	hexane:CH_2Cl_2 (30:70)	yellow solid and yellow viscous liquid	88.81
BE-15	83-112	hexane:CH_2Cl_2 (30:70)	red solid and dark red viscous liquid	24.34
BE-16	113-125	hexane:CH_2Cl_2 (30:70)	dark red viscous liquid	10.51
BE-17	126-141	hexane:CH_2Cl_2 (20:80), hexane:CH_2Cl_2 (10:90), CH_2Cl_2 (100)	dark red viscous liquid	4.32
BE-18	142-151	CH_2Cl_2:MeOH (95:5)	dark red viscous liquid	25.59
BE-19	152-157	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	3.20
BE-20	158-159	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	11.30
BE-21	160-162	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	9.88
BE-22	163-165	CH_2Cl_2:MeOH (90:10)	white solid and dark red viscous liquid	12.40
BE-23	166-171	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	25.48
BE-24	172	CH_2Cl_2:MeOH (90:10)	red solid and dark red viscous liquid	57.94
BE-25	173	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	246.90
BE-26	174-175	CH_2Cl_2:MeOH (90:10)	dark red viscous liquid	252.11
BE-27	176	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	353.80
BE-28	177-179	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	272.60
BE-29	180-185	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	388.70
BE-30	186-200	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	579.50
BE-31	201-211	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	51.96
BE-32	212-217	CH_2Cl_2:MeOH (90:10)	brown viscous liquid	175.60
BE-33	218-226	CH_2Cl_2:MeOH (85:15)	brown viscous liquid	16.10
BE-34	227-232	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	41.10
BE-35	233-248	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	362.80

Table 4.13 (continued)

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE-36	249-259	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	651.30
BE-37	260-267	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	166.60
BE-38	268-273	CH_2Cl_2:MeOH (80:20)	brown viscous liquid	34.10
BE-39	274-336	CH_2Cl_2:MeOH (80:20)	gray brown viscous liquid	372.10
BE-40	337-346	CH_2Cl_2:MeOH (75:25)	gray brown viscous liquid	846.80
BE-41	347-368	CH_2Cl_2:MeOH (75:25)	gray brown viscous liquid	480.90
BE-42	369-376	CH_2Cl_2:MeOH (75:25)	gray brown viscous liquid	28.00
BE-43	377-421	CH_2Cl_2:MeOH (70:30)	gray brown viscous liquid	313.70
BE-44	422-437	CH_2Cl_2:MeOH (65:35)	gray brown viscous liquid	245.40
BE-45	438-448	CH_2Cl_2:MeOH (60:40)	black viscous liquid	244.80
BE-46	449-462	MeOH 100	black viscous liquid	293.00

Fraction BE-14 was showed a yellow spot on TLC which was developed by 20% hexane in CH_2Cl_2. All of three fractions were exhibited a single spot and. Fraction BE-15 and 24 were showed a purple red spot which were developed by 20% hexane in CH_2Cl_2 and 10% MeOH in CH_2Cl_2, respectively. BE-14 was purified by re-crystallization with hexane:$CHCl_3$ to obtain a compound **4** which is a yellow crystal (75.90 mg, 0.007% of total EtOAc crude extract). BE-15 was purified by re-crystallization with hexane:$CHCl_3$:MeOH to obtain a compound **5**, which is a red amorphous solid (15.90 mg, 0.001% of total EtOAc crude extract). BE-24 was purified by re-crystallization with hexane:$CHCl_3$:MeOH to obtain a compound **6**, which is a red crystal (45.20 mg, 0.004% of total EtOAc crude extract). Compound **4**, **5** and **6** were characterized by NMR, MS, IR, UV, optical rotation and melting point. The crystals of compound **4** and **6** were catalysed by X-ray diffractometer.

4.6.1.3.1 Structure elucidation of compound 4

Compound **4** was obtained from the elution of column chromatography with 30% hexane in CH_2Cl_2 and re-crystallization with hexane-$CHCl_3$ to obtain a yellow crystal (75.90 mg). Compound **4** was as white power and soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 133-135 ^oC;

λ_{max} (MeOH) (log ε) 273 (4.16) nm;

FT-IR spectrum (NaCl) (Figure D 4-1) ν_{max} (cm^{-1}): 1680, 1643, 1588, 1439, 1363, 1363, 1299, 1232, 1073, 1043, 991, 927, 866, 832, 784, 729, 698, 625, 573, 497 and 457;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure D 4-2) 2.14 (3H, s, Me-3), 3.80 (3H, s, OMe) and 5.97 (1H, s, H-6) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure D 4-3) 13.4 (Me-3), 56.6 (OMe), 106.6 (C-6), 140.1 (C-2), 141.5 (C-3), 158.8 (C-5), 179.1 (C-1) and 179.6 (C-4) ppm;

ESI-TOF spectrum (Figure D 4-1) *m/z* 187.0156 $[M+H]^+$ *calc.* for $C_8H_8O_3Cl$ 187.0162;

R_f value 0.46 on TLC plate using 20% hexane in CH_2Cl_2 as the mobile phase.

The IR spectrum of compound **4** were indicate the signal absorption band at 3061 cm^{-1} of the =C─H stretching vibration, the signal absorption band at 2942 $cm^{-1}$of the ─C─H stretching vibration (for asymmetric stretching of methyl group), the absorption band at 2853 cm^{-1} of the ─C─H stretching vibration (for symmetric stretching of methyl group), the absorption bands at 1680, 1643 $cm^{-1}$of the C=O stretching vibration of two carbonyl groups, the absorption band at 1591 $cm^{-1}$of the C=C stretching vibration, the absorption bands at 1235 $cm^{-1}$of C─O stretching vibration and the absorption bands at 1079, 997 $cm^{-1}$of the C─O stretching vibration.

The ^{1}H NMR spectrum of compound **4** indicated that it one methyl group at δ 2.14 ppm, one methoxy group at δ 3.80 ppm and one aromatic proton at δ 5.97 ppm.

The ^{13}C NMR spectrum of compound **4** displayed 8 carbon resonances. Two signals at δ 179.6 and 179.1 ppm could be ascribed to carbonyl group of ketone. The signals at δ 158.8 and 141.5 ppm were aromatic carbon attached with methoxy group and methyl group, respectively. The resonance at δ 140.1 ppm was ascribed to aromatic carbon attached with hetero atom, which was a chlorine atom because of HRESIMS = 187.0156. A signal at δ 106.7 ppm was a methine carbon. The resonances at δ 56.6 and 13.5 ppm were methoxy carbon and methyl carbon, respectively.

The structure of compound **4** was established by gHMBC correlations (Figure 4.12) and this structure was confirmed by X-ray crystallography analysis (Figure 4.13).

Table 4.14 ^{1}H and ^{13}C NMR data for compound **4**.

no.	δ_H (mult., *J* in Hz)	δ_C	gHMBC
1		179.1	
2		140.1	
3		141.5	
3-Me	2.14 (3H, s)	13.5	C2, C3, C4
4		179.6	
5		158.8	
5-OMe	3.80 (3H, s)	56.6	C5
6	5.97 (1H, s)	106.7	C1, C2, C4, C5

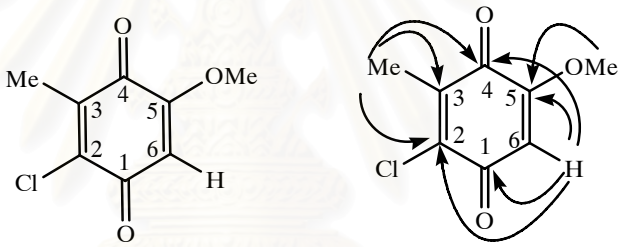


Figure 4.12 Structure of compound **4** and gHMBC correlation.

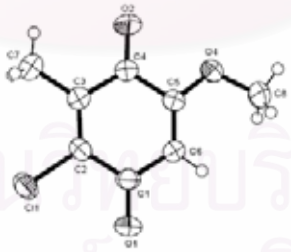


Figure 4.13 ORTEP view for compound **4**.

NMR data of compound **4** was signified to **2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione** which was an organohalogen. In general, organohalogens were found in the ocean living organisms more than in terrestrial living organisms (Gribble, 2003). Some new halogenate natural products were produced by fungi genus *Xylaria* (Davis, 2005; Davis *et al*., 2005).

A literature survey revealed that compound **4** is a new compound.

4.6.1.3.2 Structure elucidation of compound 5

Compound **5** was obtained from the elution of column chromatography with 30% hexane in CH_2Cl_2 and re-crystallization with hexane:$CHCl_3$:MeOH to obtain a red amorphous solid (15.90 mg). It was soluble in MeOH and slightly soluble in $CHCl_3$, CH_2Cl_2, hexane;

m.p. 132-134°C;

λ_{max} (MeOH) (log ε) 253 (3.53), 209 (3.73) nm;

FT-IR spectrum (NaCl) (Figure D 5-1) ν_{max} (cm^{-1}): 1646, 1601, 1561, 1463, 1424, 1357, 1277, 1235, 1131, 1073, 988 and 942;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure D 5-2) 2.16 (3H, d, J = 1.6 Hz, Me-3), 2.45 (3H, s, Me-6), 3.49 (1H, s, OH-7), 4.00 (3H, s, OMe), 6.53 (1H, d, J = 1.6 Hz, H-2) and 7.31 (1H, s, H-9) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure D 5-3) 8.6 (Me-6), 15.7 (Me-3), 56.5 (OMe-8), 99.2 (C-9), 108.5 (C-6), 113.4 (C-9a), 123.2 (C-1a), 133.1 (C-2), 145.4 (C-3), 146.4 (C-7), 146.7 (C-8), 150.8 (C-4a), 152.0 (C-5a), 176.5 (C-4) and 184.5 (C-1) ppm;

ESI-TOF (Figure D 5-6) *m/z* 273.0741 $[M+H]^+$ *calc.* for $C_{15}H_{13}O_5$ 273.0763;

R_f value 0.48 on TLC plate using 20% hexane in CH_2Cl_2 as the mobile phase.

The IR spectrum were exhibited absorption band at 3500-3300 cm^{-1} was broad and showed the O─H stretching vibration, the absorption band at 3008 cm^{-1} showed the =C─H stretching vibration, the absorption band at 2923 cm^{-1} showed the ─C─H stretching vibration (for asymmetric stretching of methyl group), the absorption band at 2853 cm^{-1} showed the ─C─H stretching vibration (for symmetric stretching of methyl group), the absorption band at 1658 $cm^{-1}$of the C=O stretching vibration of carbonyl group, the absorption bands at 1616, 1567, 1466, 1424, 1366 cm^{-1} of the C=C stretching vibration, the absorption band at 1280 cm^{-1} of the C─O stretching vibration, the absorption bands at 1131, 1082 cm^{-1} of the C=C stretching vibration and the absorption bands at 939, 878, 838, 796, 750, 668, 558, 518, 457 cm^{-1} of the C─H bending vibration.

The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **5** displayed two methyl groups at δ 2.16 (d, J = 1.6 Hz) ppm and at δ 2.45 (s) ppm, a

hydroxyl proton signal at δ 3.49 ppm, a methoxy signal at δ 4.00 ppm and an aromatic proton at δ 7.31 ppm.

The ^{13}C NMR spectrum and HSQC experiment indicated two methyl carbon signals, a methoxy carbon signal, two methine sp^2-carbon signals, eight quaternary sp^2-carbon signals and two carbonyl carbons. The connectivities of **5** were established by HMBC correlations, shown in Figure 4.22. HMBC correlations from the methyl group (δ_H 2.16) of the quinone ring to the quaternary C-3, the methine C-2 and carbonyl carbon C-4, it suggested that this methyl group was located at C-3. The HMBC correlations of the other methyl group (δ_H 2.45), correlated with the sp^2-hybridized carbons C-5a, C-6 and C-7, and revealed that this methyl group was located at C-6. Due to the correlation of methoxy group to C-8, the methoxy group was located at C-8. The HMBC correlations from H-9 to the sp^2-hybridized carbons C-1a, C-5a and C-7 and from H-2 to C-1a suggested that C-1a of the quinone ring was connected to C-9a of the aromatic ring and C-9 was adjacent with C-9a and C-8. Since seven of the ten unsaturations were accounted for, it was implied that **5** should contain three rings. The chemical shift of C-4a (150.8) and C-5a (152.0) suggested that both carbons were oxygenated sp^2-carbons and C-4a must attach to C-5a through an oxygen bridge, thus forming a ring and accounting for the one remaining degree of unsaturation. On the basis of the spectroscopic data, compound **5** was identified as **7-hydroxy-8-methoxy-3,6-dimethyl-dibenzofuran-1,4-dione**.

Table 4.15 ^{1}H and ^{13}C NMR data for compound **5**.

No.	δ_H (mult., *J* in Hz)	δ_C	gHMBC
1		184.5	
1a		123.2	
2	6.53 (1H, d, J = 1.6)	133.1	C3, C1a, C3-Me, C4
3		145.4	
3-Me	2.16 (3H, d, J = 1.6)	15.7	C2, C3, C4
4		176.5	
4a		150.8	
5a		146.4	
6		108.5	

Table 4.15 (continued)

No.	δ_H (mult., *J* in Hz)	δ_C	gHMBC
6-Me	2.45 (3H, s)	8.6	C5a, C6, C7
7		152.0	
7-OH	3.49 (1H, s)		
8		146.7	
9	7.31 (1H, s)	99.2	C1a, C5a, C7
9a		113.4	
8-OMe	4.00 (3H, s)	56.5	C8

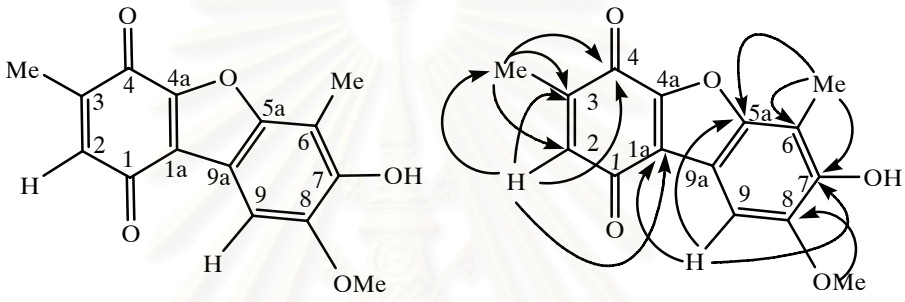


Figure 4.14 Structure of compound **5** and gHMBC correlation.

A literature survey revealed that compound **5** is a new compound.

4.6.1.3.3 Structure elucidation of compound 6

Compound **6** was obtained from the elution of column chromatography with 10% MeOH in CH_2Cl_2 and re-crystallization with hexane:$CHCl_3$:MeOH to obtain a red crystal (45.20 mg). Compound **6** was as white power, soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 149-151 ^oC;

λ_{max} (MeOH) (log ε) 278 (4.12), 206 (3.87) nm;

FT-IR spectrum (NaCl) (FigureD 6-1) ν_{max} (cm^{-1}): 3600, 3552, 3473, 3391, 3078, 2986, 2939, 1643,1613, 1521, 1456, 1395, 1317, 1226, 1065, 1021, 939, 852, 778, 595 and 447;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure D 6-2) 1.94 (3H, s, Me-3), 3.86 (3H, s, OMe), 5.84 (1H, s, H-6) and 7.27 (1H, s, OH) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure D 6-3) 7.8 (Me-3), 56.8 (OMe), 102.1 (C-6), 114.8 (C-3), 151.6 (C-2), 161.2 (C-5), 182.0 (C-4) and 184.5 (C-1) ppm;

ESI-TOF (Figure D 6-8) *m/z* 169.0549 $[M+H]^+$ *calc.* for $C_8H_9O_4$ 169.0549;

R_f value 0.63 on TLC plate using 10% MeOH in CH_2Cl_2 as the mobile phase.

The important IR absorption bands at 3552 and 3473 cm^{-1} were showed the O─H stretching vibration, the signals absorption band at 3078 cm^{-1} showed the =C─H stretching vibration, the absorption band at 2939 cm^{-1} showed the ─C─H stretching vibration (for asymmetric stretching of methyl group), the absorption broad band at 2847 cm^{-1} showed the ─C─H stretching vibration (for symmetric stretching of methyl group), the strong absorption bands at 1643 and 1613 $cm^{-1}$of the C=O stretching vibration of carbonyl group, the absorption bands at 1521, 1456, 1395, 1317 $cm^{-1}$of the C=C stretching vibration and the absorption bands at 1226, 1065 $cm^{-1}$of C─O stretching vibration.

The ^{1}H NMR spectrum of compound **6** indicated that it one methyl group at δ 1.94 ppm, one methoxy group at δ 3.86 ppm, one aromatic proton at δ 5.84 ppm and one hydroxy group at δ 7.27 ppm.

The ^{13}C NMR spectrum of compound **6** displayed 8 carbon resonances. Two signals at δ 184.5 and 182.0 ppm could be ascribed to carbonyl group of ketone. The signals at δ 161.2 and 114.8 ppm were aromatic carbon attached with methoxy group and methyl group, respectively. A signal at δ 102.1 ppm was a methine carbon. The resonances at δ 56.8 and 7.8 ppm were methoxy carbon and methyl carbon, respectively.

The NMR data (Table 4.15) of **6** were similar to **4** (Table 4.13), except the chlorine atom of **4** was replaced with an hydroxyl group. HMBC correlations of **6** led to establishment of the structure as shown in Figure 4.23 and this structure was confirmed by X-ray crystallographic analysis in Figure 4.24. The structure of compound **6** was identified as **2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione** Compound **6** is a known compound, which has been synthesized by reacting 2,5-dihydroxy-3-methylbenzoquinone with boiling MeOH (Kiuchi, Takashima and Tsuda, 1998). This is the first report of compound **6** found as a natural product.

The structure of compound **6** was identified **as 2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione** using gHMBC correlations (Figure 4.15) and this structure was confirmed by X-ray crystallography analysis (Figure 4.16).

Table 4.16 ^{1}H and ^{13}C NMR data for compound **6**.

No.	δ_H (mult., *J* in Hz)	δ_C	gHMBC
1		184.5	
2		151.6	
2-OH	7.27 (1H, s)		C1, C2
3		114.8	
3-Me	1.94 (3H, s)	7.8	C2, C3,C4
4		182.0	
5		161.2	
5-OMe	3.86 (3H, s)	56.8	C5
6	5.84 (1H, s)	102.1	C2, C4, C5

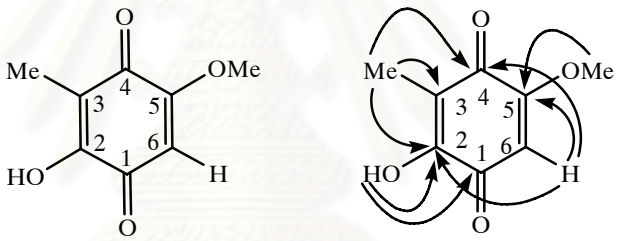


Figure 4.15 Structure of compound **6** and gHMBC correlation.

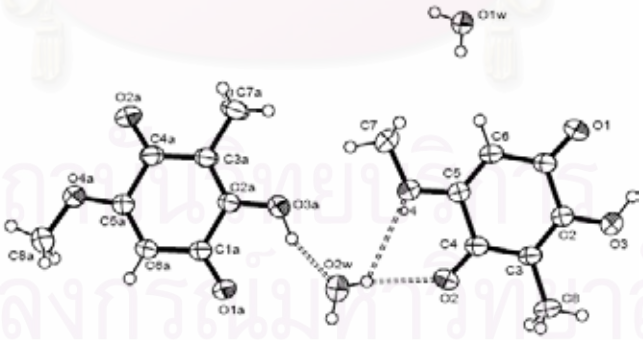


Figure 4.16 ORTEP view for compound **6**.

4.6.2 Cultivation and chemical investigation of the metabolites produced by the endophytic fungus MK-22

This selected endophytic fungi was cultivated in malt extract broth total 10 L. Mycelia from culture was 817.17 g and then dry at 35°C in hot air oven, over night (dry weight = 668.59 g). Dry cells were divided using blender and extracted with 1L of

MeOH (x5). The green extract was evaporated of under reduced pressure to 500 mL and kept in a refrigerator at 4°C. The amorphous solid was found in green extract. Washing the solid by EtOAc:MeOH (50:50) and gave the compound **7**.

4.6.2.1 Structure elucidation of compound 7

Compound **7** was found in MeOH crude extracted from mycelia when kept in a refrigerator at 4°C. Washing the solid by EtOAc:MeOH (50:50) and gave the compound 7 as amorphous solid and soluble in $CHCl_3$;

λ_{max} (MeOH) (log ε) 249 (3.54) nm;

FT-IR spectrum (NaCl) (Figure D 7-1) ν_{max} (cm^{-1}): 3439, 2957, 2923, 2863, 1716, 1686, 1646, 1558, 1439, 1393, 1241, 1168, 1085, 1003, 915, 863 and 774;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure D 7-2) 0.87 (3H, t, J = 7.2 Hz, Me-8´), 1.00 (3H, d, J = 6.8 Hz, Me-9´), 1.23 (2H, m, H-7´), 1.29 (2H, m, H-6´), 1.34 (1H, m, H-5´), 1.40 (1H, m, H-5´), 1.52 (3H, s, H-14), 1.78 (1H, br.t, J = 14.4 Hz, H-2), 1.84 (3H, s, H-10´), 1.92 (1H, m, H-3), 2.17 (1H, dd, J = 4.4 and 13.2 Hz, H-6), 2.20 (1H, dd, J = 2.4 and 13.2 Hz, H-2), 2.32 (1H, m, H-6), 2.36 (1H, m, H-3), 2.46 (1H, dd, J = 2.8 and 12.8 Hz, H-4), 2.50 (1H, d, J = 10.4 Hz, H-4´), 3.74 (1H, dd, J = 4.0 and 14.4 Hz, H-7), 5.52 (1H, s, H-1), 6.14 (1H, s, H-9), 6.25 (1H,s, H-12), 6.40 (1H, s, H-12) and 6.55 (1H, dd, J = 10.4 Hz, H-3´) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure D 7-3) 12.65 (Me-10´), 14.08 (Me-8´), 19.53 (Me-14), 19.99 (Me-9´), 20.09 (C-3), 22.78 (C-6´), 29.66 (C-7´), 29.74 (C-2), 33.34 (C-4´), 36.53 (C-5´), 38.26 (C-5), 43.08 (C-7), 43.12 (C-6), 53.29 (C-4), 72.67 (C-1), 125.84 (C-2´), 129.62 (C-9), 136.63 (C-12), 147.67 (C-11) 149.77 (C-3´), 159.02 (C-10), 166.86 (C-1´), 177.90 (C-15), 193.25(C-13) and 196.96 (C-8) ppm;

ESI-TOF (Figure D 7-9) m/z = 453.2624 $[M+Na]^+$ *calc.* for $C_{25}H_{34}O_6$;

R_f value 0.62 on TLC plate using 5% MeOH in CH_2Cl_2 as the mobile phase.

The IR important absorption bands at 3439 cm^{-1} was broad and showed the O─H stretching vibration, the signals absorption band at 2957 cm^{-1} showed the =C─H stretching vibration, the absorption band at 2923 cm^{-1} showed the ─C─H stretching vibration (for asymmetric stretching of methyl group), the absorption broad band at 2863 cm^{-1} showed the ─C─H stretching vibration (for symmetric stretching of methyl group), the strong absorption bands at 1716, 1686, 1646 cm^{-1} of the C=O stretching vibration of carbonyl group, the absorption bands at 1558, 1439, 1393 $cm^{-1}$of

the C=C stretching vibration, the absorption bands at 1241 $cm^{-1}$of C─O stretching vibration and the absorption bands at 1168, 1085, 1003, 915, 863, 774 cm^{-1} of the C─H bending vibration.

The ^{1}H NMR spectrum of compound **7** indicated that it four methyl groups at δ 0.87, 1.00, 1.52 and 1.84 ppm, one hydroxy group at δ 9.58 ppm.

The ^{13}C NMR spectrum of compound **7** displayed 25 carbon resonances. Four signals at δ 196.96, 193.25, 177.90 and 166.86 ppm would attributed to carbonyl group of ketone. The resonances at δ 19.99, 19.53, 14.08 and 12.65 ppm were methyl carbon.

The structure of compound 7 was established by gHMBC correlations (Figure 4.15).

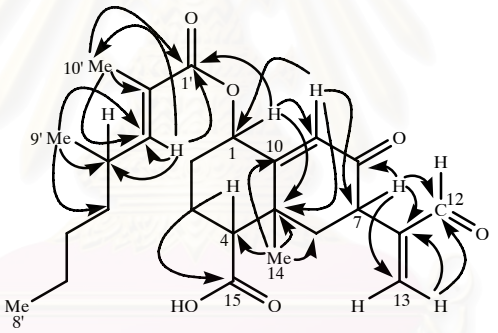


Figure 4.17 Structure of compound **7** and gHMBC correlation.

NMR data of compound **7** was identified as **4-(2,4-dimethyl-oct-2-enoyloxy)-7-(1-formyl-vinyl)-8a-methyl-6-oxo-1,2,3,4,6,7,8,8a-octahydro-naphthalene-1-carboxylic acid** named **integric acid**. This compound was found in 1999 by Singh *et al*. from *Xylaria* sp. and inhibited an enzyme in virus replication, HIV-1 integrase.

Table 4.17 ^{1}H and ^{13}C NMR data for compound **7** and integric acid.

No.	compound 7[a]		integric acid[b] (Singh *et al*., 1999)		
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	gHMBC (H ⟶ C)
1	5.25 (1H, s)	72.67	5.25 (1H, t, *J* = 2.8)	72.65	H9
2	2.20 (1H, dd, 2.4 and 13.2) 1.78 (1H, br.t, 14.4)	29.74	β = 2.15 (2H, m) α = 1.70 (2H, m)	29.97	
3	1.92 (2H, m)	20.09	β = 1.90 (2H, m) α = 2.30 (2H, m)	20.12	H1, H2β, H4
4	2.46 (1H, dd, 2.8 and 12.8)	53.29	2.46 (1H, dd, 13.2 and 3.2)	53.31	H3α, H14
5		38.26		38.28	H1, H3α, H4, H6α, H9, H14
6	2.32 (1H, m) 2.17 (1H, dd, 4.4 and 13.2)	43.12	β = 2.12 (2H, dd, 13.2, 4) α = 2.26 (2H, t, 13.6)	43.18	H4, H7, H14
8		196.96		196.85	H6αβ, H7
9	6.14 (1H, s)	129.62	6.10 (1H, s)	129.60	H1
10		159.02		158.90	H1, H6αβ, H14
11		147.67		147.66	H7, H13
12	6.25 (1H, s) 6.40 (1H, s)	136.63	6.35 (2H, s) 6.25 (2H, s)	136.47	H7
13		193.25		193.13	H12
14	1.52 (3H, s)	19.53	1.50 (3H, s)	19.53	H6αβ
15		177.90		177.87	H4

No.	compound 7[a]		integric acid[b] (Singh *et al*., 1999)		
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	gHMBC (H ⟶ C)
1´		166.86		166.78	H1, H3´, H10´
2´		125.84		125.83	H3´, H4´ H10´
3´	6.55 (1H, d, 10.4)	149.77	6.54 (1H, dq, 10, 1.2)	149.69	H4´, H9´ H10´
4´	2.5 (1H, m)	33.34	2.50 (1H, m)	33.34	H3´, H9´
5´	1.40 (1H , m) 1.34 (1H, m)	36.53	1.3 (2H, m) 1.4 (2H, m)	36.53	H3´, H9´
6´	1.29 (2H, m)	22.78	1.3 (2H, m)	22.78	H8´
7´	1.23 (2H, m)	29.66	1.2 (2H, m)	29.66	H8´
8´	0.87 (3H, t, 7.2)	14.08	0.87 (3H, t, 7.2)	14.08	
9´	1.00 (3H, d, 6.8)	19.99	1.00 (3H, d, 6.6)	19.98	H3´, H4´
10´	1.84 (3H, s)	12.65	1.80 (3H, d, 1.6)	12.65	H3´

4.7 Biological activity test of secondary metabolites

4.7.1 Antimicrobial activity test

4.7.1.1 Disk diffusion method

Compound **2-7** were evaluated the animicrobial activity against 5 microorganisms by the disk diffusion method (modified from NCCLS, 2003 and 2004). The result was showed the diameter of inhibition zone (mm) in Table 4.18.

Table 4.18 Antimicrobial activities of pure compounds.

Compounds (conc; μg/10 μL /disk)	**Inhibition zone (mm)**				
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***
2 (7.0)	-	-	-	-	-
3 (7.0)	-	-	-	-	-
4 (16.0)	13	15	9	-	-
5 (4.0)	7	9	-	-	-
6 (25.0)	7	9	-	-	-
7 (27.0)	11	-	-	-	-
Streptomycin (1.0)	13	15	13	11	ND
Ketoconazole (1.0)	ND	ND	ND	ND	10
MeOH+ 10%Tween 80	-	-	-	-	-

- inactive

ND = not determined

4.7.1.2 Broth microdilution method

Compound **4**, **5**, **6** and **7** were further determined minimum inhibition concentration (MIC) using broth microdilution method (modified from NCCLS, 2003 and 2004).

The concentration of all compound were investigated as 3mg/mL and then dilution two-fold to 0.046875 mg/mL (last concentration). MIC result were exhibited the lowest concentration of pure compound showing complete inhibition of growth (Table 4.19).

Table 4.19 Concentration of pure compounds for broth microdilution method.

Test microorganism	MIC (mg/mL)			
	Compound 4	**Compound 5**	**Compound 6**	**Compound 7**
Bacillus subtilis	0.375	0.375	1.5	1.5
Straphylococcus aureus	0.375	0.375	1.5	1.5
Escherichia coli	3.0	-	-	-

- inactive

4.7.2 Cytotoxicity test

Compound **2**-**7** were tested against 5 cell lines (Table 4.20) and the results were expressed as the minimum concentration of 50% inhibitory activity, IC_{50} (μM). Doxorubicin hydrochloride, a chemotherapeutic substance in clinical use, was used as a positive control.

Compound **4**, **5**, **6** and **7** gave the positive for all cell lines. Cytotoxic activity against Vero cells (African green monkey kidney fibroblasts) was found that compound **4** and **5** exhibited cytotoxicity with the IC_{50} values of 1.34 μM and 181 μM, respectively.

Table 4.20 Cytotoxic activities against cell line of pure compounds.

Compounds	IC_{50} (μM)				
	BT474 (breast)	**CHAGO (lung)**	**HEP-G2 (hepatoma)**	**KATO-3 (gastric)**	**SW620 (colon)**
2	inactive	inactive	inactive	inactive	Inactive
3	inactive	inactive	inactive	inactive	Inactive
4	24.1	33.4	6.4	5.3	31.3
5	2.3	24.6	<0.0036	<0.0036	16.4
6	>59.17	>59.17	<0.005	>59.17	35.6
7	1.04	>23.2	0.02	<0.02	9.9
Doxorubicin hydrochloride	1.05	0.17	0.95	3.00	1.21

4.7.3 Antiplasmodial activity

The microculture radioisotope technique was used for antiplasmodial activity against *Plasmodium falciparum*, K1 strain. Each tested compounds, **2**-**6**, was dissolved in DMSO to a concentration of 10 μg/mL. The results were investigated the percentage of inhibition ≥ 50% and diluted to find IC_{50} value. Compounds **2**, **3** and **6** were inactive and compounds **4** and **5** exhibited antiplasmodial activity with IC_{50} 1.83 μM and 6.69 μM, respectively. Dihydroartimistinine used as positive control showed IC_{50} value of 3.3 nM.

CHAPTER V

CONCLUSION

The endophytic fungi from medicinal plant, santol (*Sandoricum koetjape*) leaves. Plant samples were collected from 10 provinces; Bangkok, Chiang Mai, Khonkaen, Mahasarakham, Pathumthani, Pattani, Phangnga, Phayao, Prachinburi, and Samutsongkhram. The total of 231 endophytic fungi isolates were characterized on the basis of morphological identification, microscopic and macroscopic features. Fifty eight fungal isolates were identified as *Alternaria* sp. (2 isolates), *Aspergillus* sp. (3 isolates), *Cladosporium* sp. (5 isolates), *Colletotrichum* sp. (6 isolates), *Fusarium* sp. (8 isolates), *Pestalotia* sp. (4 isolates), *Phomopsis* sp. (13 isolates) and the fungi in Xylariaceae family (18 isolates). Other isolates of endophytic fungi were classified as mycelia sterilia.

The metabolites in culture broth produced by 213 isolates of the endophytic fungi were examined the antimicrobial activity against 5 microorganisms using an agar diffusion method. The broth of 54 isolates, 25.35% of a total isolates, were exhibited antimicrobial activity at least against two test microorganisms. The number of active endophytic fungi isolates exhibiting activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231 were 41 (75.93%), 37 (68.52%), 5 (9.26%), 5 (9.26%) and 22 (40.74%) isolates, respectively.

Endophytic fungi, PB-30 and MK-22 were selected for the further study because it exhibited activity against *E. coli* ATCC 25922, *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923. Based on morphology, the fungi isolate PB-30 and MK-22 were found as the family Xylariaceae. Sequence analysed of the rDNA, partial 18S ITS region of the isolates MK-22 and PB-30 were 578 and 579 bp, respectively.The sequence results suggested that MK-22 and PB-30 should tentatively be *Xylaria* sp., a fungus in family Xylariaceae.

Isolation secondary metabolites from mycelia MeOH extracted, broth EtOAc extracted and broth hexane extracted of PB-30 which was found 5 compounds; D-mannitol **1**, mellein **2**, 4-hydroxymellein **3**, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **4**, 7-hydroxy-8-methoxy-3,6-dimethyl-dibenzofuran-1,4-dione **5** and 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **6**. Endophytic fungi, MK-22

was isolated one compound, integric acid **7**, from mycelia MeOH extracted. Compound **2**-**6** were examined for antiplasmodial activity against *Plasmodium falciparum* (K1 strain). Compound **4** and **5** exhibited antiplasmodial activity with IC_{50} values of 1.83 μM and 6.69 μM, respectively while compound **2**, **3** and **6** were inactive. Cytotoxic activity of compound **2**-**7** were tested against 5 cell lines including, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon). Compound **2** and **3** were showed the negative result and compound **4**, **5**, **6** and **7** gave the positive for all cell lines. Cytotoxic activity against Vero cells (African green monkey kidney fibroblasts) was found that compound **4** and **5** exhibited cytotoxicity with the IC_{50} values of 1.34 μM and 181 μM, respectively.

REFERENCES

Ainsworth, G. C. 1973. Introduction and keys to higher taxa. In G. C. Ainsworth, F. K. Sparrow and A. S. Sussman (eds.), **The fungi an advanced treatise volume IVA. a taxonomic review with keys: Ascomycetes and fungi imperfecti,** pp. 1-7. New York: Academic Press.

———. 1976. **Introduction to the history of mycology.** Cambridge: Cambridge University Press.

Alcano, I. E. 2003. **Microbes and society: An introduction to microbiology.** Boston: Jones and Bartlett.

Alexopoulos, C. J. and Mims, C. W. 1979. **Introduction mycology.** New York: John Wiley.

Altschul, S. F. *et.al*. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. **Nucleic Acids Res.** 25: 3389-3402.

Anderson, J. B. *et.al*. 2003. Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cereviseae*. **Genetics** 163: 1278-1298.

Asha, K. N., Chowdhury, R., Hasan, C. M. and Rashid, M. A. 2004. Steroids and polyketides from *Uvaria hamiltonii* stem bark. **Acta Pharm.** 54: 57-63.

Assante, G., Locci, R., Camarda, L., Merlini, L. and Nasini, G. 1977. Screening of the genus *Cercospora* for secondary metabolites. **Phytochemistry** 16: 243-247.

Atlas, R. M. 1995. **Microorganisms in our world.** St. Louis: Mosby-Year Book.

Barnett, H. L. and Hunter, B. B. 1998. **Illustrated genera of imperfect fungi.** Minnesota: APS Press.

Barron, G. L. 1977. **General of hyphomycetes from soil.** New York: Noble.

Brakhage, A. A. and Caruso, M. L. 2004. Biotechnical genetics of antibiotic biosynthesis. In U. Kück (ed.), **The mycota a comprehensive treatise on fungi as experimental systems for basic and applied research: Genetics and biotechnology II,** pp. 317-353. Berlin: Springer-Verlag.

Brock, T. D. and Madigan, M. T. 1991. **Biology of microorganisms.** Englewood Cliffs: Prentice-Hall.

Burkill, I. H. 1966. **Dictionary of the economic products of the Malay Peninsula, vol. II.** Kuala Lumpur, Ministry of Agriculture and Cooperatives.

Burnett, J. H. 1968. **Fundamentals of mycology.** London: William Clowes.

———. 2003. **Fungal populations and species.** Oxford: Oxford University Press.

Camarda, L., Merlini, L. and Nasini, G. 1976. Metabolites of *Cercospora*. taiwapyrone, an α-pyrone of unusual structure from *Cercospora taiwanensis*. **Phytochemistry** 15: 537-539.

Carlile, M. J. and Watkinson, S. C. 1994. **The fungi**. San Diego: Academic Press.

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. **Cancer Res.** 47: 936-942.

Carroll, G. 1988. Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. **Ecology** 69: 2-9.

Che, Y., Gloer, J. B., Scott, J. A. and Malloch, D. 2004. Communiols A-D: New mono- and bis-tetrahydrofuran derivatives from the coprophilous fungus *Podospora communis*. **Tetrahedron Lett.** 45: 6891-6894.

Cohen, S. D. 2005. A protocol for direct sequencing of multiple gene specific PCR products from *Discula umbrinella*, a fungal endophyte, utilizing bufferless precast electrophoresis. **J. Microbiol. Methods** 61: 131-135.

Cole, R. J., Moore, J. H., Davis, N. D., Kirksey, J. W. and Diener, U. L. 1971. 4-Hydroxymellein: A new metabolite of *Aspergillus ochraceus*. **J. Agr. Food Chem.** 19: 909-911.

Dai, J.-R. *et.al.* 2001. Circumdatin G, a new alkaloid from the fungus Aspergillus ochraceus. **J. Nat. Prod.** 64: 125-126.

Daisy, B. H. *et.al.* 2002. Napthalene, an insect repellent, is produced by *Muscodor vitigenus*, a novel endophytic fungus. **Microbiology** 148: 3737-3741.

Dalziel, J. E., Finch, S. C. and Dunlop, J. 2005. The fungal neurotoxin lolitrem B inhibits the function of human large conductance calcium-activated potassium channels. **Toxicol. Lett.** 155: 421-426.

Davis, E. C., Franklin, J. B., Shaw, A. J. and Vilgalys, R. 2003. Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: Phylogenetics, distribution, and symbiosis. **Am. J. Bot.** 90: 1661-1667.

Davis, R. A. 2005. Isolation and structure elucidation of the new fungal metabolite (−)-xylariamide A. **J. Nat. Prod.** 68: 769-772.

Davis, R. A., Watters, D. and Healy, P. C. 2005. The isolation and synthesis of 3-chloro-4-hydroxy-phenylacetamide produced by a plant-associated microfungus of the genus *Xylaria*. **Tetrahedron Lett.** 46: 919-921.

Demain, A. L. 1996. Fungal secondary metabolism: Regulation and functions. In B. C. Sutton (ed.), **A century of mycology,** pp. 233-354. Cambridge: Biddles.

Desjardins, R. E., Canfield, C. J., Haynes, J. D. and Chulay, J. D. 1979. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. **Antimicrob. Agents Chemother.** 16: 710-718.

Devys, M., Bousquet, J.-F., Kollmann, A. and Barbier, M. 1980. Dihydroisocoumarines et acide mycophenolique du milieu de culture du champignon phytopathogene *Septoria nodorum*. **Phytochemistry** 19: 2221-2222.

Dix, N. J. and Webster, J. 1995. **Fungal Ecology.** London: Chapman & Hall.

D'Mello, J. P. F. and Macdonald, A. M. C. 1997. Mycotoxins. **Anim. Feed Sci. Tech.** 69: 155-166.

Dreyfuss, M. M. and Chapela, I. H. 1994. Potential of fungi in the discovery of novel, low molecular weight pharmaceuticala. In V. P. Gullo (ed.), **The discovery of natural products with therapeutic potential.** pp. 49-80. Boston: Butterworth-Heinemann.

Edwards, R. L. *et.al.* 1999. Metabolites of the higher fungi. Part 31. longianone, a $C_7H_6O_4$ spiro bicyclic lactone from the fungus Xylaria longiana (Rehm.). **J. Chem. Soc., Perkin Trans. 1.** 715-719.

Ellis, M. B. and Ellis, J. P. 1985. **Microfun on land plants an identification handbook.** Beckenham: Croom Helm.

Evidente, A. *et.al.* 2006. Diplobifuranylones A and B, 5´-monosubstituted tetrahydro-2*H*-bifuranyl-5-ones produced by *Diplodia corticola*, a fungus pathogen of cork oak. **J. Nat. Prod.** 69: 671-674.

Evidente, A., Andolfi, A., Maddau, L., Franceschini, A. and Marras, F. 2005. Biscopyran, a phytotoxic hexasubstituted pyranopyran produced by *Biscogniauxia mediterranea*, a fungus pathogen of cork oak. **J. Nat. Prod.** 68: 568-571.

Eyberger, A. L., Dondapati, R. and Porter, J. R. 2006. Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. **J. Nat. Prod.** 69: 1121-1124.

Feath, S. H. 2002. Are endophytic fungi defensive plant mutualists?. **Oiokos** 98: 25-36.

Fisher, P. J. and Petrini, O. 1992. Fungal saprobes and pathoens as endophytes of rice (*Oryza sativa* L.). **New Phytol.** 20: 137-143.

Fournier, J. and Magni, J. -F. 2002. **Pyrenomycetes from southwestern France**[Online]. Available from: http://www.pyrenomycetes.free.fr/index.htm [2006, August 14]

Franklin, T. J., Snow, G. A., Barrett-Bee, K. L. and Nolan, R. D. 1989. **Biochemistry of antimicrobial action.** New York: Chapman and Hall.

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

Garg, V. P.and Verma, S. C. L. 1967. Chemical examination of *Clerodendron serratum* isolation and characterization of D-mannitol. **J. Pharm. Sci**. 56: 639-640.

Garraway, M. O. and Evans, R. C. 1984. **Fungal nutrition & physiology.** New York: John Wiley.

Gooday, G. M. 1987. Hormones and sexuality in fungi. In J. W. Bennett and A. Ciegler (eds.), **Secondary metabolism and differentiation in fungi,** pp. 239-266. New York: Mercel Dekker.

Gribble, G. W. 2003. The diversity of naturally produced organohalogens. **Chemosphere** 52: 289-297.

Griffin, D. H. 1981. Fungal physiology. New York: John Wiley.

Guarro, J., Gene, J. and Stchigel, A. M. 1999. Developments in fungal taxonomy. **Clin. Microbiol. Rev.** 12: 454-500.

Hagiwara, S. -Y. *et.al.* 2005. A phytochemical in the edible tamogi-take mushroom (*Pleurotus cornucopiae*), D-mannitol, inhibits ACE activity and lowers the blood pressure of spontaneously hypertensive rats. **Biosci. Biotechnol. Biochem.** 69: 1603-1605.

Hashimoto, S. 2001. Novel bioactive compounds from fungi. In B. H. Nga, H. M. Tan and K. Suzuki (eds.), **Microbial diversity in Asia technology and prospects.** pp. 199-207. New Jersey: World Scientific.

Hawsworth, D. L. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. **Mycol. Res.** 105 : 1422-1432.

———. 2002. Why study tropical fungi? In R. Watling, J. P. Frankland, A. M. Ainsworth, S. Isaac and C. H. Robinson (eds.), **Tropical mycology: volume 2, Micromycetes,** pp. 1-12. Oxon: CABI.

Hawsworth, D. L. and Mueller, G. M. 2005. Fungal communities: their diversity and distribution. In J. Dighton, J. F. White and P. Oudemans (eds.), **The fungal community its organization and role in the ecosystem,** pp. 27-37. Boca Raton: CRC Press.

He, H. *et.al.* 2006. Culicinin D, an antitumor peptaibol produced by the fungus *Culicinomyces clavisporus*, strain *LL*-121252. **J. Nat. Prod.** 69: 736-741.

Höller, U., König, G. M. and Wright, A. D. 1999. Three new metabolites from marine-derived fungi of the genera *Coniothyrium* and *Microsphaeropsis*. **J. Nat. Prod.** 62: 114-118.

Huang, Y., Wang, J., Li, G., Zheng, Z. and Su, W. 2001. Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxas mairei*, *Cephalataxus fortunei* and *Torreya grandis*. **FEMS Immuno. Med. Micro.** 31: 163-167.

Isaac, S. 1992. **Fungal-plant interactions.** Cambridge: The University Printing House.

Ismail, I. *et.al.* 2003. Ichthyotoxic and anticarcinogenic effects of triterpenoids from *Sandoricum koetjape* bark. **Biol. Pharm. Bull.** 26: 1351-1353.

Ismail, I. S., Ito, H., Hatano, T., Taniguchi, S. and Yoshida, T. 2003. Modified limonoids from the leaves of *Sandoricum koetjape*. **Phytochemistry** 64: 1345-1349.

———. 2004. Two new analogues of trijugin-type limonoids from the leaves of *Sandoricum koetjape*. **Chem. Pharm. Bull.** 52: 1145-1147.

Jayasuriya, H. *et.al.* 2003. Isolation, structure, and HIV-1 integrase inhibitory activity of cytosporic acid, a fungal metabolite produced by a *Cytospora* sp. **J. Nat. Prod.** 66: 551-553.

Kaneda, N., Pezzuto, J. M., Kinghorn, A. D. and Farnsworth, N. R. 1992. Plant anticancer agents, L. cytotoxic triterpenes from *Sandoricum koetjape* stems. **J. Nat. Prod.** 55: 654-659.

Keller, N. P., Turner, G. and Bennett, J. 2005. Fungal secondary metabolism-from biochemistry to genomics. **Nat. Rev. Microbiol.** 3: 937-947.

Kern, F. *et.al.* 1997. Mellein, a trail pheromone component of the ant *Lasius fuliginosus*. **J. Chem. Ecol.** 23: 779-792.

Kimura, Y., Mizumo, T., Nakajima, H. and Hamasaki, T. 1992. Altechromones A and B, new plant growth regulators produced by the fungus *Alternaria* sp. **Biosci. Biotechnol. Biochem.** 56: 1664-1665.

Kiuchi, F., Takashima, H. and Tsuda Y. 1998. Dimerization of 2,5-dihydroxybenzoquinones in water. **Chem. Pharm. Bull.** 46: 1129-1234.

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

Kosela, S., Yulizar, Y., Chairul, Tori, M. and Asakawa, Y. 1995. Secomultiflorane-type triterpenoid acids from stem bark of *Sandoricum koetjape*. **Phytochemistry** 38: 691-694.

Mann, J. 1978. Secondary metabolism and ecology. In J. Mann (ed.), **Secondary metabolism,** pp. 279-304. Oxford: Clarendon Press.

Martin, K. F. and Gutierrez, S. 1992. Molecular genetics of fungal secondary metabolite. In J. R. Kinghorn and G. Turner (eds.), **Applied molecular genetics of filamentous fungi,** pp. 214-252. London: Blackie Academic & Professional.

Moore, D. and Frazer, L. A. N. 2002. **Essential fungal genetics.** New York: Springer-Verleg.

Moore, J. H., Davis, N. D. and Diener, U. L. 1972. Mellein and 4-hydroxymellein production by *Aspergillus ochraceus* Wilhelm. **Appl. Microbiol.** 23: 1067-1072.

Moore-Landecker, E. 1982. **Fundaments of the fungi.** Englewood Cliffs: Prentice-Hall.

Morton, J. 1987. Santol. **Fruits of warm climates**[Online]. Available from: http://www.hort.purdue.edu/newcrop/morton/santol.html [2005, June 5]

National Committee for Clinical Laboratory Standards. 2003. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 6th ed.; Approved Standard. NCCLS Publication No. M7-A6. Wayne, PA.: National Committee for Clinical Laboratory Standards. Cited in G. A. Pankuch et.al. Activity of retapamulin against *Streptococcus pyogenes* and *Staphylococcus aureus* evaluated by agar dilution, microdilution, E-Test, and disk diffusion methodologies. **Antimicrob. Agents Chemother.** 50: 1727-1730, 2006.

———. 2004. Performance Standards for Antimicrobial Susceptibility Testing, 14th Informational Supplement. NCCLS Publication No. M100-S14. Wayne, PA.: National Committee for Clinical Laboratory Standards. Cited in G. A. Pankuch *et.al.* Activity of retapamulin against *Streptococcus pyogenes* and *Staphylococcus aureus* evaluated by agar dilution, microdilution, E-Test, and disk diffusion methodologies. **Antimicrob. Agents Chemother.** 50: 1727-1730, 2006.

Pereira, J. O., Azevedo, J. L. and Petrini, O. 1993. Endophytic fungi of Stylosanthes: A first report. **Mycologia** 85: 362-364.

Perry, L. M. 1980. Medicinal plants of east and Southeast Asia. Cambridge: The MIT press. Cited in I. S. Ismail *et.al.* Ichthyotoxic and anticarcinogenic effects of triterpenoids from *Sandoricum koetjape* bark. **Biol. Pharm. Bull.** 26: 1351-1353, 2003.

Petrini, O. 1982. Endophytic fungi in evergreen shrubs in western Oregon: A preliminary study. **Can. J. Bot.** 60: 789-796.

Powell, R. G., Mikolajczak, K. L. and Zilkowski, B. W. 1991. Limonoid antifeedants from seed of *Sandoricum koetjape*. **J. Nat. Prod.** 54: 241-246.

Prescott, L. M., Harley, J. P. and Klein, D. A. 2005. **Microbiology.** Singapore: McGraw-Hill.

Puri, S. C. *et.al.* 2006. The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. **J. Biotechnol** 122: 494-510.

Rasadah, M. A., Khozirah, S., Aznie, A. A. and Nik, M. M. 2004. Anti-inflammatory agents from *Sandoricum koetjape* Merr. **Phytomedicine** 11: 261-263.

Rodrigues, K. F. and Petrini, O. 1997. Biodiversity of endophytic fungi in tropical regions. In K. D. Hyde (ed.), **Biodiversity of tropical microfungi,** pp. 57-69. Hong Kong: United League & Graphic Printing.

Rodriguez, R. J., Cullen, D., Kurtzman, C. P., Khachatourians, G. G. and Hegedus, D. D. 2004. Molecular methods for discriminating taxa, monitoring species, and assessing fungal diversity. In G. M. Mueller, G. F. Bill and M. S. Foster (eds.), **Biodiversity of fungi inventory and monitoring methods**, pp. 77-102. Hong Kong: Elsevier Academic Press.

Rodriguez, R. J., Redman, R. S. and Henson, J. M. 2005. Symbiotic lifestyle expression by fungal endophytes and the adaptation of plants to stress: unraveling the complexities of intimacy. In J. Dighton, J. F. White and P. Oudemans (eds.), **The fungal community its organization and role in the ecosystem**, pp. 683-695. New York: CRC Press.

Rogers, J. D. 1993. Teleomorph, anamorph, and holomorph considerations in the Xylariaceae. In D. R. Reynolds and J. W. Taylor (eds.), **The fungal holomorph: mitotic and pleomorphic speciation in fungal systematics,** pp. 179-182. Wallingford: CAB International.

Rose, A. H. 1978. Production and industrial importance of primary products of microbial metabolism. In A. H. Rose (ed.), **Economic microbiology volume 2 primary produces of metabolism,** pp. 1-29. London: Academic Press.

Ross, I. K. 1979. **Biology of the fungi.** New York: McGraw-Hill.

Rubini, M. R. *et.al.* 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of Crinipellis perniciosa, causal agent of Witches's Broom Disease. **Int. J. Biol. Sci.** 1: 24-33.

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

Sangan, D. 1990. **Biospheres metamorphosis of planet earth.** New York: McGraw-Hill.

Sasaki, M. *et.al.* 1970. Studies on the compounds produced by molds part VII isolation of isocoumarin compounds. **Agr. Biol. Chem.** 34: 1296-1300.

Scheffer, R. P. 1991. Role of toxins in evolution and ecology of plant pathogenic fungi. **Experientia** 47: 804-811.

Schwarz, M., Kopcke, B., Weber, R. W. S., Sterner, O. and Anke, H. 2004. 3-hydroxypropionic acid as a nematicidal principle in endophytic fungi. **Phytochemistry** 65: 2239-2245.

Singh, J. 1999. Ethnomycology and folk remedies: fact and fiction. In J. Singh and K. R. Aneja (eds.), **From ethnomycology to fungal biotechnology,** pp. 11-17. New York: Plenum Press.

Singh, S. B. *et.al.* 1999. Structure and absolute stereochemistry of HIV-I integrase inhibitor integric acid a novel eremophilane sesquiterpenoid produced by a *Xylaria* sp. **Tetrahedron Lett.** 40: 8775-8779.

Singh, S. B. *et.al.* 2004. Aspercyclide A-C, three novel fungal metabolites from *Aspergillus* sp. as inhibitors of high-affinity IgE receptor. **Tetrahedron Lett.** 45: 7605-7608.

Skehan, P. *et.al.* 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. **J. Natl. Cancer Inst.** 82: 1107-1112.

Song, Y. C., Huang, W. Y., Sun, C., Wang, F. W. and Tan, R. X. 2005. Characterization of graphislactone A as the antioxidant and free radical-scavenging substance from the culture of *Cephalosporium* sp. IFB-E001, an endophytic fungus in *Trachelospermum jasminoides*. **Biol. Pharm. Bull.** 28: 506-509.

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

———. 2001. Sequoiatones C-F, Constituents of the redwood endophyte *Aspergillus parasiticus*. **J. Nat. Prod.** 64: 1350-1353.

Stone, J. K., Polishook, J. D. and White Jr., J. F. 2004. Endophytic fungi. In G. M. Mueller, G. F. Bills and M. S. Foster (eds.), **Biodiversity of fungi inventory and monitoring methods,** pp. 241-270. Hong Kong: Elsevier Academic Press.

Strobel, G. A., Dirske, E., Sears, J. and Markworth, C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. **Microbiology** 147: 2943-2950.

Strange, R. N. 2003. **Introduction to plant pathology.** Chichester: John Wiley&Sons.

Strobel, G. and Daisy, B. 2003. Bioprospecting for microbial endophytes and their natural products. **Microbiol. Mol. Biol. Rev.** 67 : 491-502.

Strobel, G., Daisy, B., Castillo, U. and Harper, J. 2004. Natural products from endophytic microorganisms. **J. Nat. Prod.** 67: 257-268.

Strobel, G., Kenfeild, D., Bunkers, G., Sugawara, F. and Clardy, J. 1991. Phytotoxin as potential herbicides. **Experientia** 47: 819-826.

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

Sun, D. A., Starck, S. R., Locke, E. P. and Hecht, S. M. 1999. DNA polymerase β inhibitors from *Sandoricum koetjape*. **J. Nat. Prod.** 62: 1110-1113.

Talaro, K. and Talaro, A. 1996. **Foundations in microbiology.** Dubuque: Times Mirror Higher Education Group.

Tan, R. X. and Zou, W. X. 2001. Endophytes: A rich source of functional metabolites. **Nat. Prod. Rep.** 18: 448-459.

Tanaka, T. *et.al.* 2001. New multiflorane-type triterpenoid acids from *Sandoricum indicum.* **J. Nat. Prod.** 64: 1243-1245.

Tortora, G. J., Funke, B. R. and Case, C. L. 1992. **Microbiology an introduction.** Redwood: The Benjamin/Cummings.

Trager, W. and Jensen, J. B. 1976. Human malaria parasites in continuous culture. **Science** 193(4254): 673-675.

Von Arx, J. A. 1981. **The genera of fungi sporulation in pure culture.** Hirschberg2: Strauss & Cramer.

Waitese, M. J., Morgan, N. L., Rockey, J. S. and Higton, G. 2001. **Industrial microbiology: An introduction.** Wiltshier: Blackwell Science.

Webster, J. 1980. **Introduction to fungi.** Oxford: Alden Press.

Whalley, A. J. S. and Edwards, R. L. 1999. **The xylariaceae: A case study in biological and chemical diversity**[Online]. Available from: http://www.iupac.org/symposia/proceedings/phuket97/whalley.pdf [2005, May 3]

White, T. J, Bruns, T. D., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (eds.), **PCR protocols: a guide to methods and applications,** pp. 315-322. New York: Academic Press.

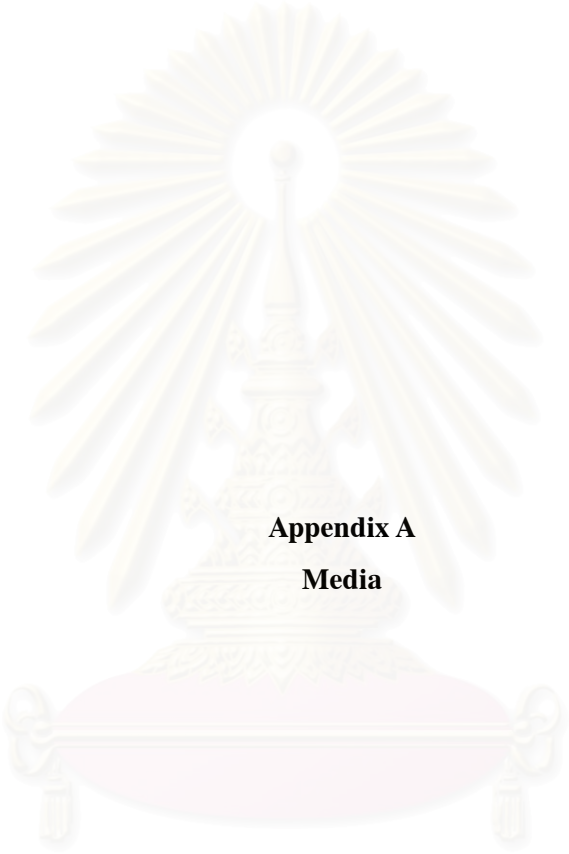
Wilson, M., McNab, R. and Henderson, B. 2002. **Bacterial disease mechanisms.** Cambridge: Cambridge University Press.

Worapong, J. *et.al.* 2001. *Muscodor albus*. anam. gen. et sp. nov., an endophyte from *Cinnamomium zeylanicum*. **Mycotaxon** 79: 67-79.

Zhan, J. and McDonald, B. A. 2005. Analytical and experimental methods for estimating population genetic structure of fungi. In J. Dighton, J. F. White and P. Oudemans (eds.), **The fungal community its organization and role in the ecosystem,** pp. 241-263. Boca Raton: CRC Press.

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

APPENDICES



Appendix A
Media

All of media were add distilled H_2O to a final volume of 1 L and steriled in the autoclave at 121°C for 15 min. The pH was adjusted with NaOH or HCl before addition of agar and sterilization. The broth media didn't add the agar in the formula.

1. Corn meal agar (CMA)

Suspened 17.0 g in 1 L distilled H_2O

2. Malt extract agar (MEA)

Malt extracts	20.0	g
Peptone	1.0	g
Glucose	20.0	g
Agar	15.0	g

3. Mueller Hinton agar (MHA)

Suspened 21.0 g in 1 L distilled H_2O

4. Nutrient agar (NA)

Peptone	5.0	g
Beef extract	3.0	g
Agar	15.0	g

5. Potato dextrose agar (PDA)

Potato (pelled and diced)	200.0	g
Glucose	20.0	g
Agar	15.0	g

Boil the potatoes for one hour in a litre of water. Filter, add the glucose and agar make up the filtrate to 1 L.

6. Sabouraud's dextrose agar (SDA)

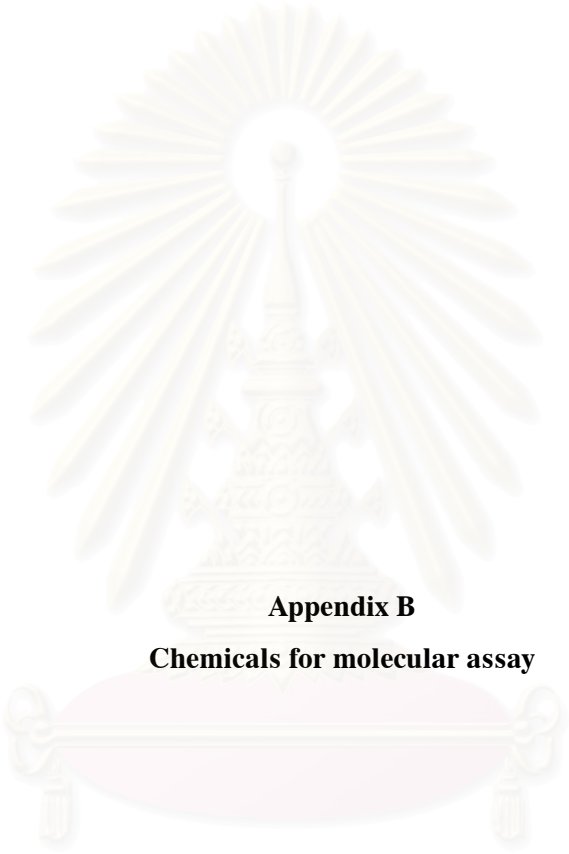
Peptone	10.0	g
Glucose	40.0	g
Agar	15.0	g

7. V8-juice agar

V8 Vegetable juice	200.0	mL
Calcium carbonate	4.0	g
Agar	15.0	g

8. Yeast-malt extract agar (YMA)

Malt extracts	3.0	g
Peptone	5.0	g
Yeast extracts	3.0	g
Glucose	10.0	g
Agar	15.0	g



Appendix B

Chemicals for molecular assay

1. Washing buffer

PVP (polyvinyl pyrrolidone)	1%	
Ascorbic acid	0.05	M
Tris-HCL (pH 8.0)	0.1	M
2-mercaptoethanol	2%	

2. 2x CTAB lysis buffer

cTAB	2%	
Tris-HCL (pH 8.0)	0.1	M
EDTA (pH 8.0)	20.0	mM
NaCl	1.4	M
2-mercaptoethanol	0.5%	

3. Chloroform : isoamyl alcohol (24:1 v/v)

Chloroform	192.0	mL
Isoamyl alcohol	8.0	mL
Final volume	200.0	mL

4. 20% Polyethylene glycol (PEG)

polyethylene glycol	20%	
NaCl	2.5	M

Autoclave 121°C, 20 min

5. TE buffer

Tris-HCl	10.0	mM
EDTA	1.0	mM

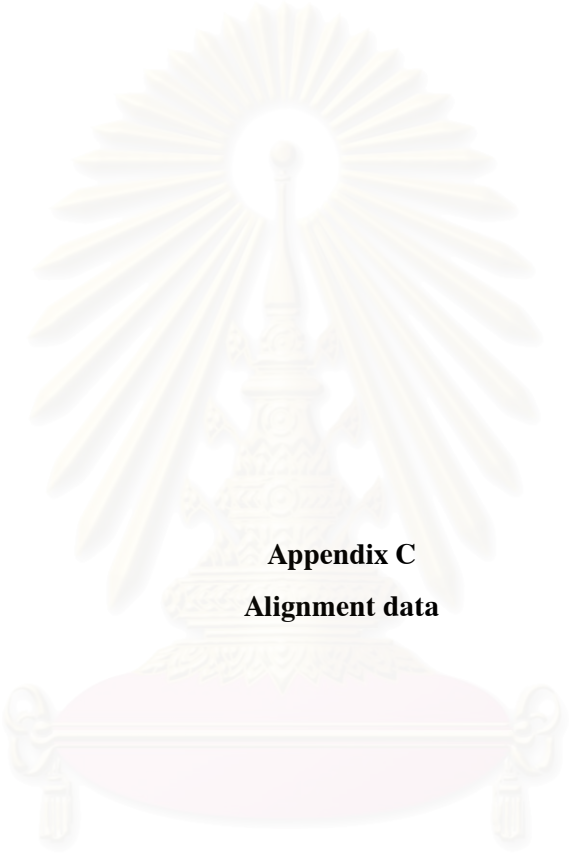
6. TBE buffer

Tris-base	54.0	g
EDTA	4.65	g
Boric acid	27.5	g

Add deionized H_2O to a final volume of 500 mL

7. 6x loading dye

Bromophenol blue	25.0	mg
Glycerol	4.0	mL



Appendix C

Alignment data

Sequences producing significant alignments:	Score (Bits)	E Value
gi|94450436|gb|DQ480344.1| Xylaria sp. NR-2006-A59 18S riboso...	690	0.0
gi|37223427|gb|AY315404.1| Xylaria sp. F19 internal transcrib...	648	0.0
gi|111379299|gb|DQ780445.1| Xylaria sp. IP-93 18S ribosomal R...	644	0.0
gi|111379302|gb|DQ780448.1| Xylaria sp. IP-39 18S ribosomal R...	638	5e-180
gi|8809737|gb|AF163038.1|AF163038 Xylaria longipes CBS 148.73...	638	5e-180
gi|113926787|emb|AM084368.1| Xylaria enteroleuca 18S rRNA gen...	618	5e-174
gi|37223428|gb|AY315405.1| Xylaria sp. F4 internal transcribe...	609	5e-171
gi|49073061|gb|AY572970.1| Podosordaria tulasnei 18S ribosoma...	589	4e-165
gi|94537188|gb|DQ485962.1| Fungal endophyte sp. MS29 18S ribo...	587	2e-164
gi|16303235|dbj|AB041994.1| Unidentified white mycelium 1 gen...	573	3e-160
gi|111379294|gb|DQ780440.1| Xylaria sp. IP-31 18S ribosomal R...	571	1e-159
gi|8809732|gb|AF163033.1|AF163033 Xylaria enteroleuca CBS 651...	571	1e-159
gi|8809725|gb|AF163026.1|AF163026 Xylaria acuta ATCC 56487 in...	555	6e-155
gi|111379300|gb|DQ780446.1| Xylaria sp. IP-29 18S ribosomal R...	553	2e-154
gi|111379298|gb|DQ780444.1| Xylaria sp. IP-94 18S ribosomal R...	547	1e-152
gi|28974195|gb|AY183369.1| Xylaria arbuscula 18S ribosomal RN...	541	9e-151
gi|8809730|gb|AF163031.1|AF163031 Xylaria cornu-damae CBS 724...	539	4e-150
gi|55818542|gb|AY787732.1| Xylaria sp. olrim973 18S ribosomal...	533	2e-148
gi|111379293|gb|DQ780439.1| Xylaria sp. IP-30 18S ribosomal R...	529	3e-147
gi|92430225|gb|DQ491487.1| Xylaria hypoxylon isolate AFTOL-ID...	529	3e-147
gi|37223425|gb|AY315402.1| Xylariaceae sp. F13 internal trans...	517	1e-143
gi|8809738|gb|AF163039.1|AF163039 Xylaria longipes SFC 960725...	517	1e-143
gi|8809727|gb|AF163028.1|AF163028 Xylaria arbuscula CBS 454.6...	509	3e-141
gi|37896677|gb|AY427785.1| Tubercularia sp. TF5 18S ribosomal...	502	8e-139
gi|8809728|gb|AF163029.1|AF163029 Xylaria arbuscula CBS 452.6...	502	8e-139
gi|51872588|gb|AY699645.1| Fungal sp. R10 18S ribosomal RNA g...	498	1e-137
gi|117557501|emb|AM403716.1| Xylariaceae sp. MS1 partial 18S ...	492	7e-136
gi|51872589|gb|AY699646.1| Fungal sp. R11 18S ribosomal RNA g...	492	7e-136
gi|51872592|gb|AY699649.1| Fungal sp. R14 18S ribosomal RNA g...	490	3e-135
gi|51872618|gb|AY699675.1| Fungal sp. R16 18S ribosomal RNA g...	488	1e-134
gi|92430231|gb|DQ491493.1| Xylaria acuta isolate AFTOL-ID 63 ...	484	2e-133
gi|94450447|gb|DQ480355.1| Xylariales sp. NR-2006-D50 18S rib...	470	3e-129
gi|11967310|gb|AF201711.1|AF201711 Xylaria hypoxylon 18S ribo...	468	1e-128
gi|8809739|gb|AF163040.1|AF163040 Xylaria mali CBS 385.35 int...	468	1e-128
gi|115335014|gb|DQ914422.1| Soil fungal sp. ANG34 18S ribosom...	462	7e-127
gi|113928037|dbj|AB255244.1| Xylaria sp. JP10 genes for 18S r...	462	7e-127
gi|37223419|gb|AY315396.1| Xylaria sp. F23 internal transcrib...	462	7e-127
gi|37223418|gb|AY315395.1| Xylaria sp. F20 internal transcrib...	462	7e-127
gi|62511261|gb|AY862572.1| Rosellinia mirabilis 18S ribosomal...	458	1e-125
gi|57634701|gb|AY843084.1| Fungal sp. TRN220 internal transcr...	458	1e-125
gi|37595928|gb|AY327478.1| Xylaria hypoxylon strain GB6391 18...	448	1e-122
gi|94450444|gb|DQ480352.1| Xylaria sp. NR-2006-D14 18S riboso...	446	4e-122
gi|20531546|gb|AF502739.1| Leaf litter ascomycete strain its2...	444	2e-121

Figure C1 Alignment data of ITS region of isolate PB-30.

Sequences producing significant alignments:		Score (Bits)	E Value
gi|37595927|gb|AY327477.1|	Xylaria hypoxylon strain ATCC 4276...	434	2e-118
gi|37223412|gb|AY315389.1|	Xylaria sp. F6 internal transcribe...	434	2e-118
gi|37223410|gb|AY315387.1|	Xylaria sp. F2 internal transcribe...	434	2e-118
gi|111379301|gb|DQ780447.1|	Xylaria sp. IP-38 18S ribosomal R...	430	2e-117
gi|10732532|gb|AF153731.1|AF153731	Xylaria sp. MS339 internal...	430	2e-117
gi|111379295|gb|DQ780441.1|	Xylaria sp. IP-32 18S ribosomal R...	426	4e-116
gi|25990268|gb|AY063300.1|	Fungal endophyte WMS4 internal tra...	426	4e-116
gi|25990267|gb|AY063299.1|	Fungal endophyte WMS3 internal tra...	426	4e-116
gi|25990266|gb|AY063298.1|	Fungal endophyte WMS2 internal tra...	426	4e-116
gi|37223415|gb|AY315392.1|	Xylaria sp. F14 internal transcrib...	426	4e-116
gi|37223413|gb|AY315390.1|	Xylaria sp. F8 internal transcribe...	426	4e-116
gi|37223409|gb|AY315386.1|	Xylaria sp. F1 internal transcribe...	426	4e-116
gi|10732545|gb|AF153744.1|AF153744	Xylaria sp. MS1083 interna...	424	1e-115
gi|10732525|gb|AF153724.1|AF153724	Xylaria sp. MS1066 interna...	424	1e-115
gi|111379303|gb|DQ780449.1|	Xylaria sp. IP-40 18S ribosomal R...	422	6e-115
gi|111379296|gb|DQ780442.1|	Xylaria sp. IP-33 18S ribosomal R...	422	6e-115
gi|10732533|gb|AF153732.1|AF153732	Xylaria sp. MS366 internal...	422	6e-115
gi|72199353|gb|DQ139271.1|	Xylaria sp. BCC 1067 internal tran...	420	2e-114
gi|25989356|gb|AY063302.1|	Fungal endophyte WMS6 internal tra...	418	9e-114
gi|25989355|gb|AY063301.1|	Fungal endophyte WMS5 internal tra...	418	9e-114
gi|10732546|gb|AF153745.1|AF153745	Xylaria sp. MS1092 interna...	418	9e-114
gi|94450449|gb|DQ480357.1|	Xylariales sp. NR-2006-D55 interna...	406	3e-110
gi|71025071|gb|DQ068350.1|	Fungal sp. V-I7 internal transcrib...	406	3e-110
gi|62526456|gb|AY971716.1|	Fungal sp. 25.28 18S ribosomal RNA...	406	3e-110
gi|52222399|gb|AY601896.1|	Fungal endophyte sp. J48 internal ...	402	5e-109
gi|71025074|gb|DQ068353.1|	Fungal sp. V-E9 internal transcrib...	398	8e-108
gi|20531550|gb|AF502743.1|	Leaf litter ascomycete strain its2...	398	8e-108
gi|11066006|gb|AF194027.1|AF194027	Xylaria hypoxylon 5.8S rib...	398	8e-108
gi|18157728|gb|AF432179.1|AF432179	Arthroxylaria elegans stra...	394	1e-106
gi|113928089|dbj|AB255296.1|	Xylaria sp. JP198 genes for 18S ...	391	2e-105
gi|113928074|dbj|AB255281.1|	Xylaria sp. JP123 genes for 18S ...	391	2e-105
gi|70724177|gb|DQ094165.1|	Fungal sp. YX-41 internal transcri...	391	2e-105
gi|70724175|gb|DQ094163.1|	Fungal sp. YX-27 internal transcri...	391	2e-105
gi|10732534|gb|AF153733.1|AF153733	Xylaria sp. MS370 internal...	391	2e-105
gi|10732526|gb|AF153725.1|AF153725	Xylaria sp. MS358 internal...	391	2e-105
gi|111035821|emb|AJ972672.1|	Rosellinia necatrix 18S rRNA gen...	385	1e-103
gi|48429309|gb|AY618235.1|	Hypoxylon fragiforme internal tran...	385	1e-103
gi|52547808|gb|AY616690.1|	Hypoxylon fragiforme isolate agrD4...	385	1e-103
gi|52547807|gb|AY616689.1|	Hypoxylon fragiforme isolate agrD4...	385	1e-103
gi|62822657|gb|AY974309.1|	Fungal sp. VLam1 internal transcri...	385	1e-103
gi|11967308|gb|AF201709.1|AF201709	Hypoxylon fragiforme 18S r...	385	1e-103
gi|4519362|dbj|AB017660.1|	Rosellinia arcuata DNA for 18S rRN...	385	1e-103
gi|4519360|dbj|AB017658.1|	Rosellinia necatrix DNA for 18S rR...	385	1e-103
gi|4519359|dbj|AB017657.1|	Rosellinia necatrix DNA for 18S rR...	385	1e-103

Figure C1 (continued)

Sequences producing significant alignments:	Score (Bits)	E Value
gi|12038852|emb|AJ390403.1|HAN390403 Hypoxylon fragiforme 18S...	385	1e-103
gi|12038851|emb|AJ390402.1|HAN390402 Hypoxylon fragiforme 18S...	385	1e-103
gi|12038850|emb|AJ390401.1|HAN390401 Hypoxylon fragiforme 18S...	385	1e-103
gi|95136090|emb|AM262409.1| Creosphaeria sassafras 5.8S rRNA ...	383	5e-103
gi|12038874|emb|AJ390425.1|HAN390425 Creosphaeria sassafras 1...	383	5e-103
gi|37223430|gb|AY315407.1| Xylariaceae sp. F9 internal transc...	379	8e-102
gi|52547802|gb|AY616684.1| Daldinia eschscholzii isolate agtS...	377	3e-101
gi|25989364|gb|AY063313.1| Fungal endophyte WMS17 internal tr...	377	3e-101
gi|25989365|gb|AY063312.1| Fungal endophyte WMS16 internal tr...	377	3e-101
gi|25989362|gb|AY063311.1| Fungal endophyte WMS15 internal tr...	377	3e-101
gi|25989367|gb|AY063308.1| Fungal endophyte WMS12 internal tr...	377	3e-101
gi|25989358|gb|AY063304.1| Fungal endophyte WMS8 internal tra...	377	3e-101
gi|10732539|gb|AF153738.1|AF153738 Unidentified xylarialean s...	377	3e-101

> gi|94450436|gb|DQ480344.1| *Xylaria* sp. NR-2006-A59 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
Length=565

Score = 690 bits (348), Expect = 0.0
Identities = 524/573 (91%), Gaps = 11/573 (1%)
Strand=Plus/Minus

```
Query  4    AGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACCTTGATAAATTAGGGGTTTTACGG  63
            |||||||||||||||||||||||||||||||||||||| | ||||||||||||||||||
Sbjct  565  AGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACCTTTAAAAATTAGGGGTTTTACGG  506

Query  64   CAGGGGACCGGTCCAACTAATAGGCGAGATAATATTTACTACGTCTAGAGTGTGAACCGA  123
            || ||||||||||||||| |||||||||||||| || ||||||||||||||||||||||||
Sbjct  505  CAAGGGACCGGTCCAACTGATAGGCGAGATAAAATCTACTACGTCTAGAGTGTGAACCGA  446

Query  124  CTCCGCCACTAATTTTAAGGGGCTACCGCCATACGGTAGGCCCCCAACGCTAAGCAACAG  183
            |||||||||||| ||| ||| ||||| |   | | |||||| |||||| ||||||||||||
Sbjct  445  CTCCGCCACTAACTTTGAGGAGCTACAG---TGCCGTAGGCTCCCAACACTAAGCAACAG  389

Query  184  AAGGCTTAAGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATACTAATGGGCGC  243
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  A-GGCTTAAGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATACTAATGGGCGC  330

Query  244  AATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  329  AATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT  270
```

Figure C1 (continued)

```
Query  304  TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTTA  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTTA  210

Query  364  TTTAGTTGTA-ATTCAGATATCCAGTAATTAAACAGAGTTTAATGGGGCGCCGGCGGGCT  422
            ||||||| ||  |||||| || || | | ||||||||||||  |||| ||||||| ||||
Sbjct  209  TTTAGTTATAGGTTCAGA-ATTCAAT-AGTAAACAGAGTTTCGTGGGCCGCCGGCAGGCT  152

Query  423  TACCCGTGCCTACCGGGTAGGCACTTACAGGTAAGTGCACTACAGGGTAGGTACGACCCG  482
            |||||| | |||||||||| |  | ||||||  || |  |||| |||||||  ||||| |
Sbjct  151  TACCCGCGTCTACCGGGTACG-TCCTACAGGGTAGGGGGCTACTGGGTAGGCGCGACCTG  93

Query  483  CCGAGGCAACGTTAGGTATGTTCACATGGGGTTTGGGAGTTATA-AACTCTTTAATGATC  541
            |||||||||||| ||||||||||||||||  |||||||||||| ||||||||||||||||
Sbjct  92   CCGAGGCAACGTAAGGTATGTTCACATGGG--TTGGGAGTTATAGAACTCTTTAATGATC  35

Query  542  CCTCCGCTGGTTCACCAACGGAGACCTTGTTAC  574
            ||||||||||||||||||||||||||||||||
Sbjct  34   CCTCCGCTGGTTCACCAACGGAGACCTTGTTAC  2
```

Figure C1 (continued)

Sequences producing significant alignments:	Score (Bits)	E Value
gi|28974195|gb|AY183369.1| Xylaria arbuscula 18S ribosomal RN...	777	0.0
gi|92430225|gb|DQ491487.1| Xylaria hypoxylon isolate AFTOL-ID...	755	0.0
gi|8809739|gb|AF163040.1|AF163040 Xylaria mali CBS 385.35 int...	741	0.0
gi|52222399|gb|AY601896.1| Fungal endophyte sp. J48 internal ...	722	0.0
gi|8809728|gb|AF163029.1|AF163029 Xylaria arbuscula CBS 452.6...	714	0.0
gi|8809727|gb|AF163028.1|AF163028 Xylaria arbuscula CBS 454.6...	688	0.0
gi|8809734|gb|AF163035.1|AF163035 Xylaria hypoxylon CBS 499.8...	686	0.0
gi|11066006|gb|AF194027.1|AF194027 Xylaria hypoxylon 5.8S rib...	648	0.0
gi|18076648|emb|AJ309350.1|XHY309350 Xylaria hypoxylon 18S rR...	646	0.0
gi|10732527|gb|AF153726.1|AF153726 Xylaria sp. MS259 internal...	577	2e-161
gi|111379302|gb|DQ780448.1| Xylaria sp. IP-39 18S ribosomal R...	527	1e-146
gi|8809737|gb|AF163038.1|AF163038 Xylaria longipes CBS 148.73...	527	1e-146
gi|94450436|gb|DQ480344.1| Xylaria sp. NR-2006-A59 18S riboso...	523	2e-145
gi|113928074|dbj|AB255281.1| Xylaria sp. JP123 genes for 18S ...	513	2e-142
gi|70724177|gb|DQ094165.1| Fungal sp. YX-41 internal transcri...	513	2e-142
gi|70724175|gb|DQ094163.1| Fungal sp. YX-27 internal transcri...	505	5e-140
gi|8809726|gb|AF163027.1|AF163027 Xylaria apiculata CBS 365.8...	504	2e-139
gi|117557501|emb|AM403716.1| Xylariaceae sp. MS1 partial 18S ...	502	8e-139
gi|49073061|gb|AY572970.1| Podosordaria tulasnei 18S ribosoma...	500	3e-138
gi|20531546|gb|AF502739.1| Leaf litter ascomycete strain its2...	498	1e-137
gi|37223425|gb|AY315402.1| Xylariaceae sp. F13 internal trans...	496	5e-137
gi|16303235|dbj|AB041994.1| Unidentified white mycelium 1 gen...	496	5e-137
gi|51872588|gb|AY699645.1| Fungal sp. R10 18S ribosomal RNA g...	488	1e-134
gi|37223427|gb|AY315404.1| Xylaria sp. F19 internal transcrib...	488	1e-134
gi|111379299|gb|DQ780445.1| Xylaria sp. IP-93 18S ribosomal R...	484	2e-133
gi|93116032|gb|DQ022415.2| Xylariaceae sp. YX-28 18S ribosoma...	484	2e-133
gi|37223428|gb|AY315405.1| Xylaria sp. F4 internal transcribe...	484	2e-133
gi|113926787|emb|AM084368.1| Xylaria enteroleuca 18S rRNA gen...	480	3e-132
gi|94537188|gb|DQ485962.1| Fungal endophyte sp. MS29 18S ribo...	480	3e-132
gi|51872592|gb|AY699649.1| Fungal sp. R14 18S ribosomal RNA g...	480	3e-132
gi|52222400|gb|AY601897.1| Fungal endophyte sp. J26 internal ...	480	3e-132
gi|111379300|gb|DQ780446.1| Xylaria sp. IP-29 18S ribosomal R...	476	4e-131
gi|111379294|gb|DQ780440.1| Xylaria sp. IP-31 18S ribosomal R...	474	2e-130
gi|11967310|gb|AF201711.1|AF201711 Xylaria hypoxylon 18S ribo...	474	2e-130
gi|8809732|gb|AF163033.1|AF163033 Xylaria enteroleuca CBS 651...	474	2e-130
gi|71025071|gb|DQ068350.1| Fungal sp. V-I7 internal transcrib...	472	7e-130
gi|62526456|gb|AY971716.1| Fungal sp. 25.28 18S ribosomal RNA...	472	7e-130
gi|51872589|gb|AY699646.1| Fungal sp. R11 18S ribosomal RNA g...	468	1e-128
gi|37223419|gb|AY315396.1| Xylaria sp. F23 internal transcrib...	468	1e-128
gi|37223418|gb|AY315395.1| Xylaria sp. F20 internal transcrib...	468	1e-128
gi|20531550|gb|AF502743.1| Leaf litter ascomycete strain its2...	468	1e-128
gi|71025074|gb|DQ068353.1| Fungal sp. V-E9 internal transcrib...	464	2e-127
gi|8809730|gb|AF163031.1|AF163031 Xylaria cornu-damae CBS 724...	464	2e-127

Figure C2 Alignment data of ITS region of isolate MK-22.

Sequences producing significant alignments:	Score (Bits)	E Value
gi|94450449|gb|DQ480357.1| Xylariales sp. NR-2006-D55 interna...	460	3e-126
gi|113928037|dbj|AB255244.1| Xylaria sp. JP10 genes for 18S r...	444	2e-121
gi|37595927|gb|AY327477.1| Xylaria hypoxylon strain ATCC 4276...	440	2e-120
gi|10732526|gb|AF153725.1|AF153725 Xylaria sp. MS358 internal...	440	2e-120
gi|57634701|gb|AY843084.1| Fungal sp. TRN220 internal transcr...	440	2e-120
gi|111379293|gb|DQ780439.1| Xylaria sp. IP-30 18S ribosomal R...	432	6e-118
gi|111379301|gb|DQ780447.1| Xylaria sp. IP-38 18S ribosomal R...	428	9e-117
gi|94450444|gb|DQ480352.1| Xylaria sp. NR-2006-D14 18S riboso...	428	9e-117
gi|10732532|gb|AF153731.1|AF153731 Xylaria sp. MS339 internal...	428	9e-117
gi|8809738|gb|AF163039.1|AF163039 Xylaria longipes SFC 960725...	428	9e-117
gi|8809729|gb|AF163030.1|AF163030 Xylaria castorea ATCC 76020...	428	9e-117
gi|10732534|gb|AF153733.1|AF153733 Xylaria sp. MS370 internal...	426	4e-116
gi|55818542|gb|AY787732.1| Xylaria sp. olrim973 18S ribosomal...	424	1e-115
gi|37595926|gb|AY327476.1| Xylaria hypoxylon strain F127076 1...	422	6e-115
gi|111379296|gb|DQ780442.1| Xylaria sp. IP-33 18S ribosomal R...	420	2e-114
gi|10732533|gb|AF153732.1|AF153732 Xylaria sp. MS366 internal...	420	2e-114
gi|10732528|gb|AF153727.1|AF153727 Xylaria sp. MS61 internal ...	420	2e-114
gi|111379297|gb|DQ780443.1| Xylaria sp. IP-34 18S ribosomal R...	418	9e-114
gi|111379303|gb|DQ780449.1| Xylaria sp. IP-40 18S ribosomal R...	416	4e-113
gi|25989357|gb|AY063303.1| Fungal endophyte WMS7 internal tra...	416	4e-113
gi|92430231|gb|DQ491493.1| Xylaria acuta isolate AFTOL-ID 63 ...	412	6e-112
gi|37223415|gb|AY315392.1| Xylaria sp. F14 internal transcrib...	412	6e-112
gi|37223413|gb|AY315390.1| Xylaria sp. F8 internal transcribe...	412	6e-112
gi|37223409|gb|AY315386.1| Xylaria sp. F1 internal transcribe...	412	6e-112
gi|37595928|gb|AY327478.1| Xylaria hypoxylon strain GB6391 18...	408	9e-111
gi|72199353|gb|DQ139271.1| Xylaria sp. BCC 1067 internal tran...	406	3e-110
gi|10732525|gb|AF153724.1|AF153724 Xylaria sp. MS1066 interna...	406	3e-110
gi|10732545|gb|AF153744.1|AF153744 Xylaria sp. MS1083 interna...	404	1e-109
gi|111379295|gb|DQ780441.1| Xylaria sp. IP-32 18S ribosomal R...	402	5e-109
gi|25989356|gb|AY063302.1| Fungal endophyte WMS6 internal tra...	402	5e-109
gi|25990268|gb|AY063300.1| Fungal endophyte WMS4 internal tra...	402	5e-109
gi|25990267|gb|AY063299.1| Fungal endophyte WMS3 internal tra...	402	5e-109
gi|25990266|gb|AY063298.1| Fungal endophyte WMS2 internal tra...	402	5e-109
gi|37223430|gb|AY315407.1| Xylariaceae sp. F9 internal transc...	402	5e-109
gi|37223412|gb|AY315389.1| Xylaria sp. F6 internal transcribe...	402	5e-109
gi|37223410|gb|AY315387.1| Xylaria sp. F2 internal transcribe...	402	5e-109
gi|18157728|gb|AF432179.1|AF432179 Arthroxylaria elegans stra...	402	5e-109
gi|111035821|emb|AJ972672.1| Rosellinia necatrix 18S rRNA gen...	398	8e-108
gi|4519362|dbj|AB017660.1| Rosellinia arcuata DNA for 18S rRN...	398	8e-108
gi|4519360|dbj|AB017658.1| Rosellinia necatrix DNA for 18S rR...	398	8e-108
gi|4519359|dbj|AB017657.1| Rosellinia necatrix DNA for 18S rR...	398	8e-108
gi|48429309|gb|AY618235.1| Hypoxylon fragiforme internal tran...	396	3e-107
gi|52547808|gb|AY616690.1| Hypoxylon fragiforme isolate agrD4...	396	3e-107

Figure C2 (continued)

Sequences producing significant alignments:	Score (Bits)	E Value
gi|52547807|gb|AY616689.1| Hypoxylon fragiforme isolate agrD4...	396	3e-107
gi|62822657|gb|AY974309.1| Fungal sp. VLam1 internal transcri...	396	3e-107
gi|11967308|gb|AF201709.1|AF201709 Hypoxylon fragiforme 18S r...	396	3e-107
gi|12038852|emb|AJ390403.1|HAN390403 Hypoxylon fragiforme 18S...	396	3e-107
gi|12038851|emb|AJ390402.1|HAN390402 Hypoxylon fragiforme 18S...	396	3e-107
gi|12038850|emb|AJ390401.1|HAN390401 Hypoxylon fragiforme 18S...	396	3e-107
gi|94450447|gb|DQ480355.1| Xylariales sp. NR-2006-D50 18S rib...	394	1e-106
gi|52547823|gb|AY616705.1| Hypoxylon macrocarpum isolate agtS...	392	5e-106
gi|62632882|gb|AY971620.1| Fungal sp. S7.4.7 internal transcr...	392	5e-106
gi|62632879|gb|AY971617.1| Fungal sp. S6.1.6 internal transcr...	392	5e-106
gi|62632878|gb|AY971616.1| Fungal sp. S1.4.3 internal transcr...	392	5e-106
gi|62632877|gb|AY971615.1| Fungal sp. S7.4.4 internal transcr...	392	5e-106
gi|62632876|gb|AY971614.1| Fungal sp. S7.3.8 internal transcr...	392	5e-106
gi|62632875|gb|AY971613.1| Fungal sp. S6.3.10 internal transc...	392	5e-106

> gi|28974195|gb|AY183369.1| Xylaria arbuscula 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Length=576

Score = 777 bits (392), Expect = 0.0

Identities = 537/579 (92%), Gaps = 6/579 (1%)

Strand=Plus/Minus

```
Query  3    AAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACCTTGAAAAATTAGGGGGTTTTAC  62
            |||||||||||||||||||||||||||||||||||||||| ||||||| ||||||||||
Sbjct  576  AAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACCTTTAAAAATTCGGGGGTTTTAC  517

Query  63   GGCAAGAGACCGGCCTAACCACAGACGAGATAGAAGCTACTACGTCTAGAGTGCGAACCG  122
            |||| | ||||||||| || |||||||||||||| |||||||||||||||| ||||||
Sbjct  516  GGCAGGGGACCGGCCTCACTACAGACGAGATAGAATCTACTACGTCTAGAGTGTGAACCG  457

Query  123  ACTCCGCCA--AACTTTAGGGAGCTACAGAGGACTGTAGGCTCCCAACACTAAGCAACAG  180
            |||||||||  |||||| |||||||||||| | ||||||||||||||| ||||||||||
Sbjct  456  ACTCCGCCACTAACTTTGGGGAGCTACAGAAGGCTGTAGGCTCCCAACGCTAAGCAACAG  397

Query  181  GGGCTTAAGGGTTGAAATGACGCTCGAATAGGCATGCCCACTAGAATACTAATGGGCGCA  240
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  396  GGGCTTAAGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATACTAATGGGCGCA  337

Query  241  ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATT  277

Query  301  TCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTTAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  TCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTTAT  217

Query  361  TTAGTTGTAAAATCAGAATAACATATAATAAACAGTGTTTTAACGGGCCACTGGCAGGCG  420
            |||||| || | |||||||||||||| ||| ||||| | ||| | ||||||| ||||||||
Sbjct  216  TTAGTTATAGATTCAGAATAACATAGAATTAACAGAG-TTTCATGGGCCACCGGCAGGCG  158
```

Figure C2 (continued)

```
Query  421  AACCCGTGACTACCAGGTAGTCGCCTACAGGGTAGGCGACCACAGGGTAGACACGACCTG  480
             ||||||| ||||||||||   |||||||||||||    |||||||| ||||||||||
Sbjct  157  TACCCGTGTCTACCAGGTAGGGTCCTACAGGGTAGGACCTCACAGGGTAAACACGACCTG  98

Query  481  CCGAGGCAACAGAAGGTAAGTTCACATGGGTTTGGGAGTTACGA-AAACTCTTTAATGAT  539
             |||||||||||||||||||||||||||||||||||||||||   |  |||||||||||||
Sbjct  97   CCGAGGCAACAGAAGGTAAGTTCACATGGGTTTGGGAGTTGTAATTAACTCTTTAATGAT  38

Query  540  CCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTT  578
             |||||||||| |||||||| ||||||||||||||||||
Sbjct  37   CCCTCCGCTGG-TCACCAAC-GAGACCTTGTTACGACTT  1
```

Figure C2 (continued)

Appendix D

Chemical data

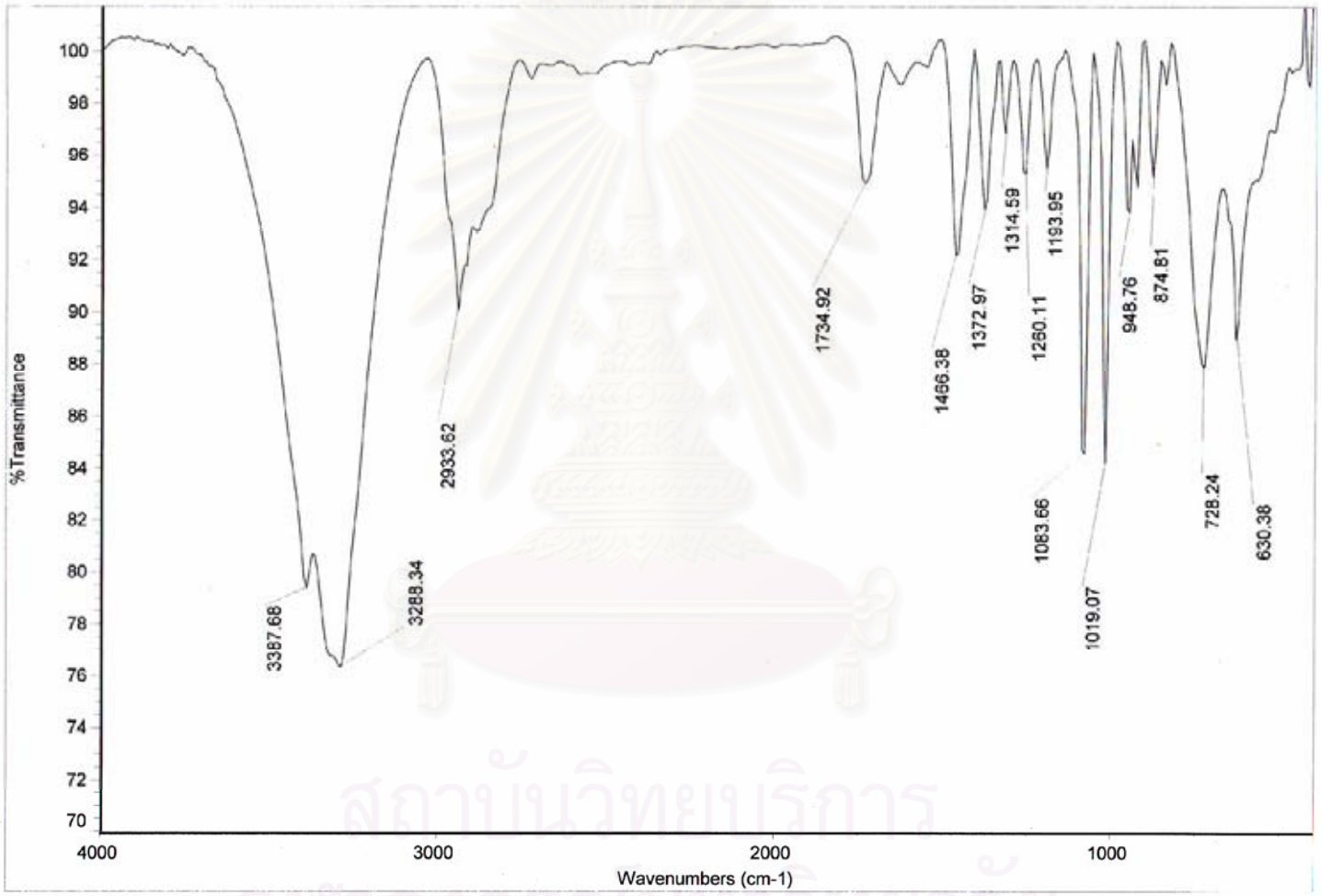


Figure D 1-1 The IR spectrum of compound **1**

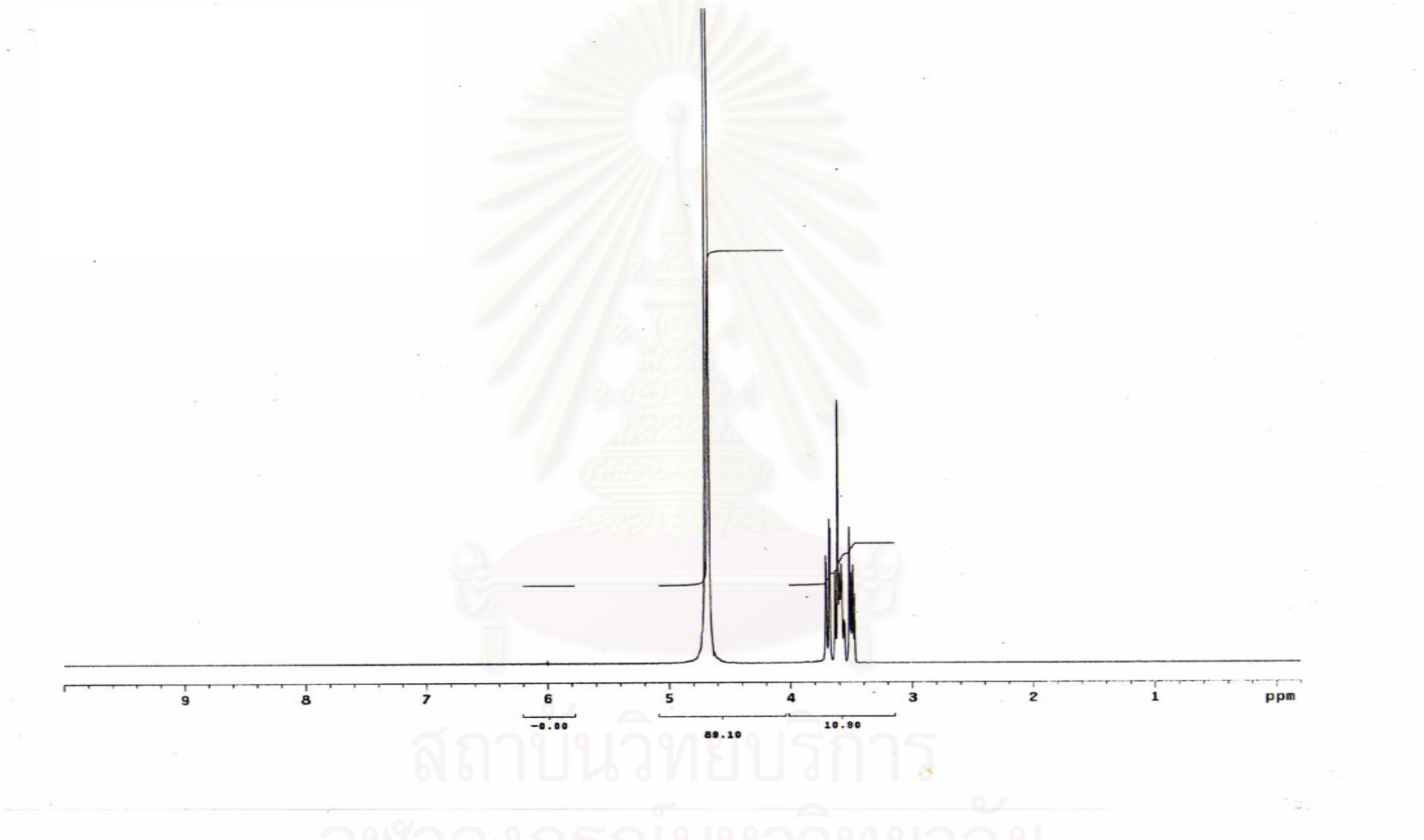


Figure D 1-2 ^{1}H NMR spectrum of compound **1**

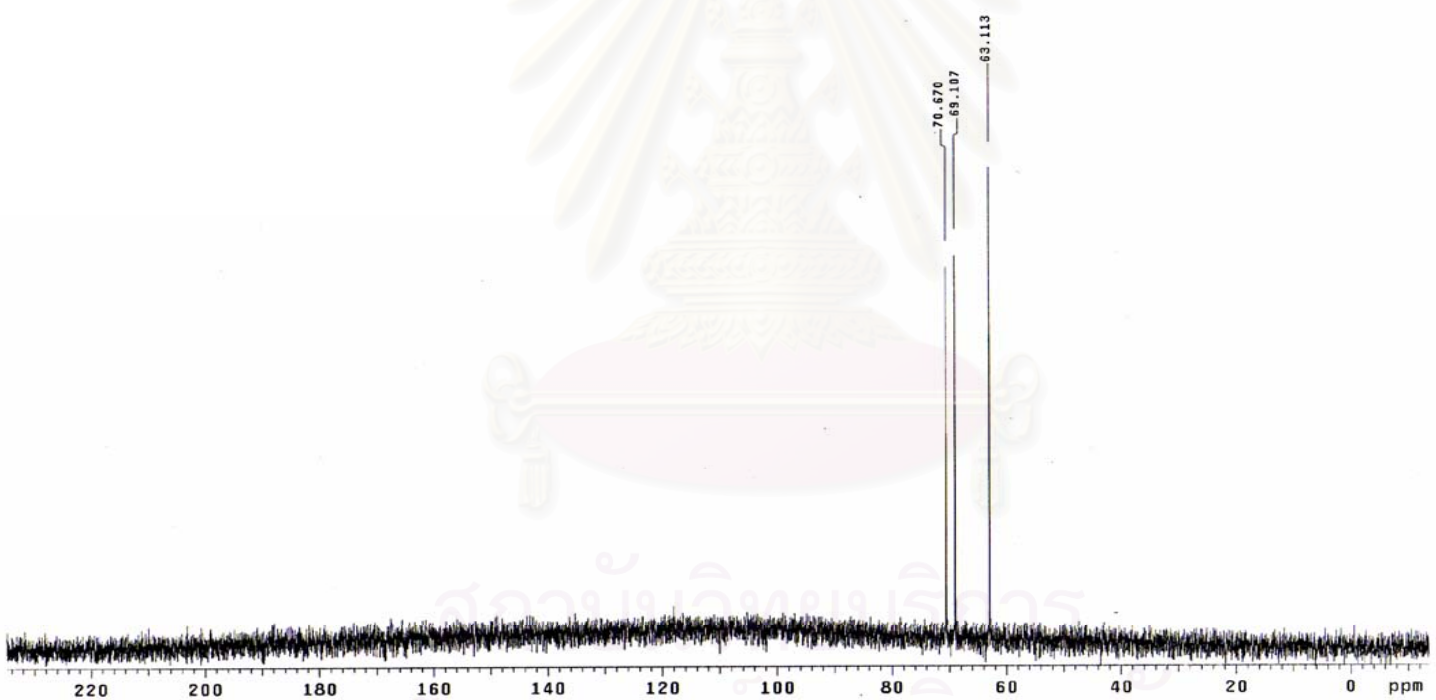


Figure D 1-3 ^{13}C NMR spectrum of compound **1**

106

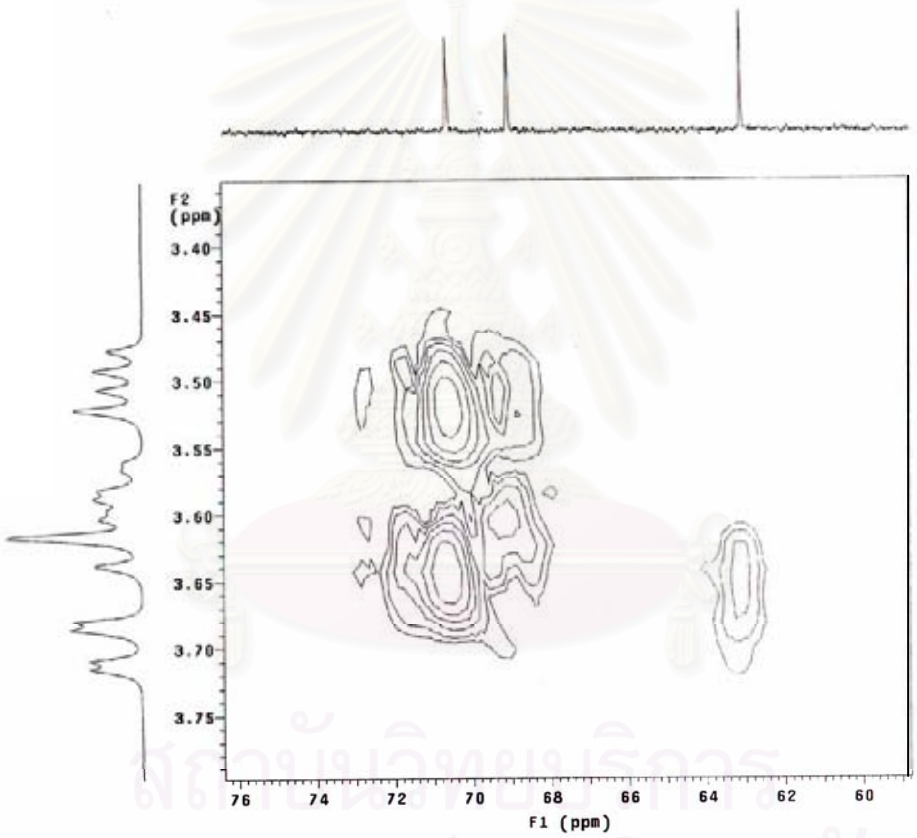


Figure D 1-4 HMBC spectrum of compound **1**

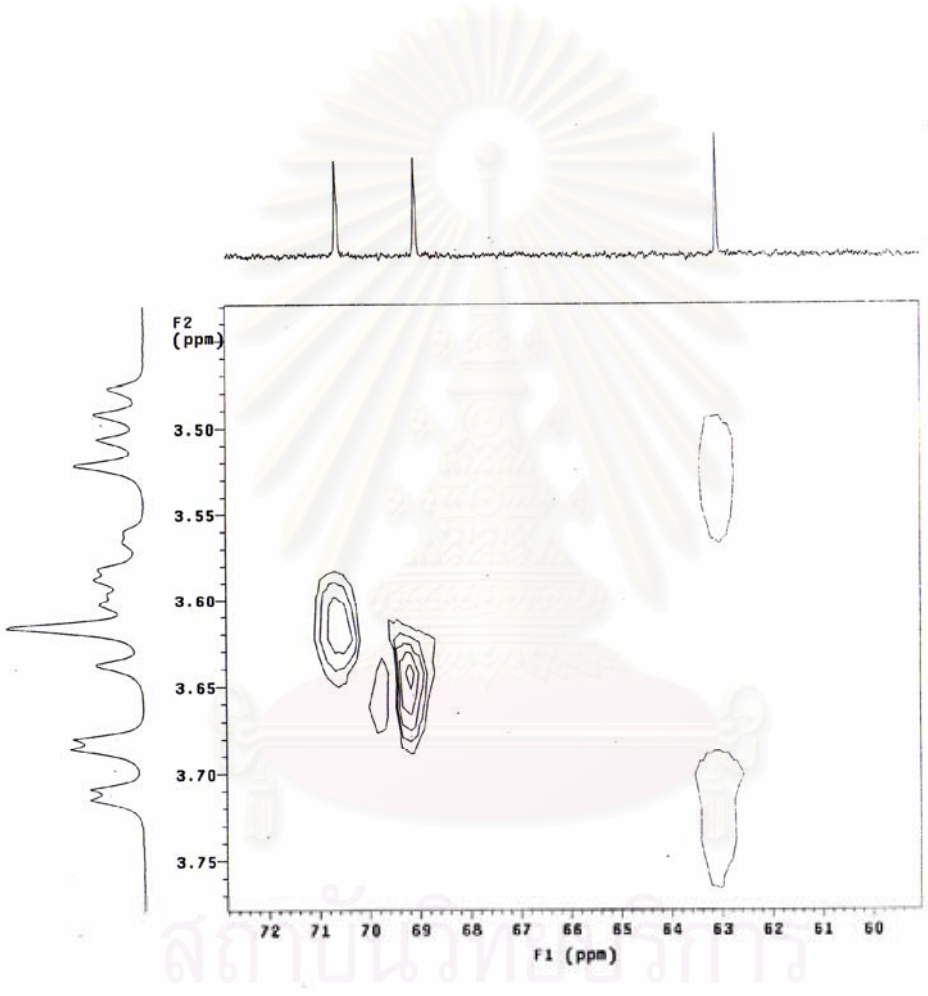


Figure D 1-5 HSQC spectrum of compound **1**

108

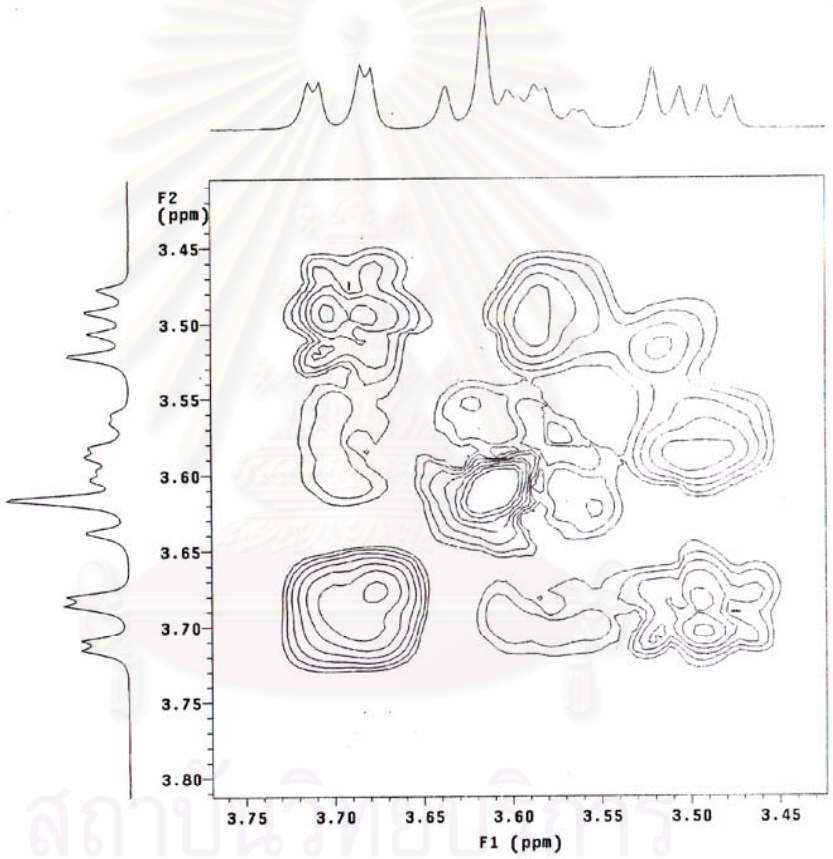


Figure D 1-6 COSY spectrum of compound **1**

Profile MS Report

Analysis Info:

File: C:\HPCHEM\1\DATA\SC\CU000004.D\4231.SCS
Printed: Thu, 5.8.2004, 10:56:08
Date acquired: Thu, 5.8.2004, 10:44:00
Instrument: ESQUIRE
Operator : SC
Task : SC
Method : KKTM.MS
Sample : fraction
Description: fraction 65-69 in MeOH

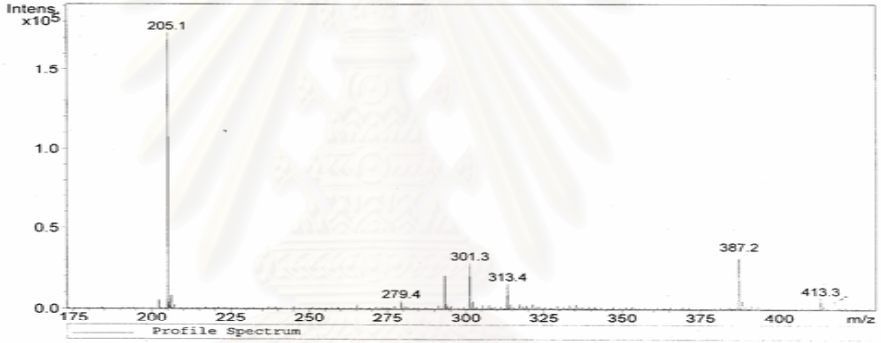
Sample Info:

Sample Name: fraction
Remark : None

Acquisition Parameter:

Source : ESI
Polarity : Positive
Mode : Std/Enhanced
CapExit : 100.0 Volt
Skimmer 1 :
Scan Range: 100.00 - 1000.00 m/z
Cut-Off : 40 m/z
Accum.time: 20976 µs
Summation : 8 Spectra
MS/MS :

Profile Spectrum, No.: 1, Time: 0 min



MS Peak List (Profile Spectrum):

Mass	Intensity	Width	Mass	Intensity	Width	Mass	Intensity	Width
202.3	5525	0.25	293.3	20777	0.13	387.2	34355	0.19
205.1	173222	0.13	293.8	3300	0.13	388.2	5441	0.19
205.5	4232	0.13	301.3	28498	0.13	413.3	7098	0.19
206.1	8205	0.19	302.3	5113	0.25			
279.4	5085	0.19	313.4	15752	0.19			

End of report

Figure D 1-7 The profile mass spectrum of compound **1**

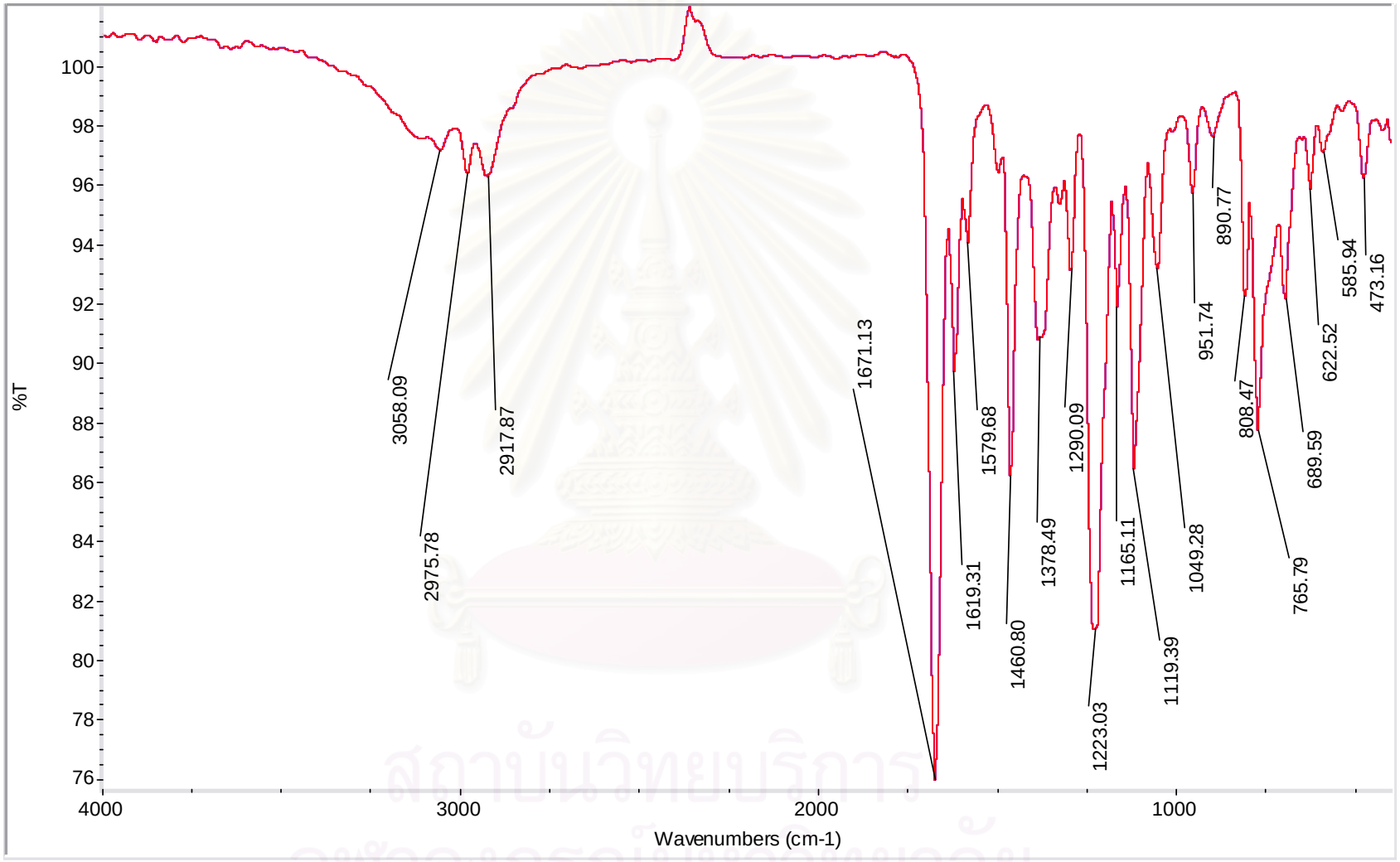


Figure D 2-1 The IR spectrum of compound **2**

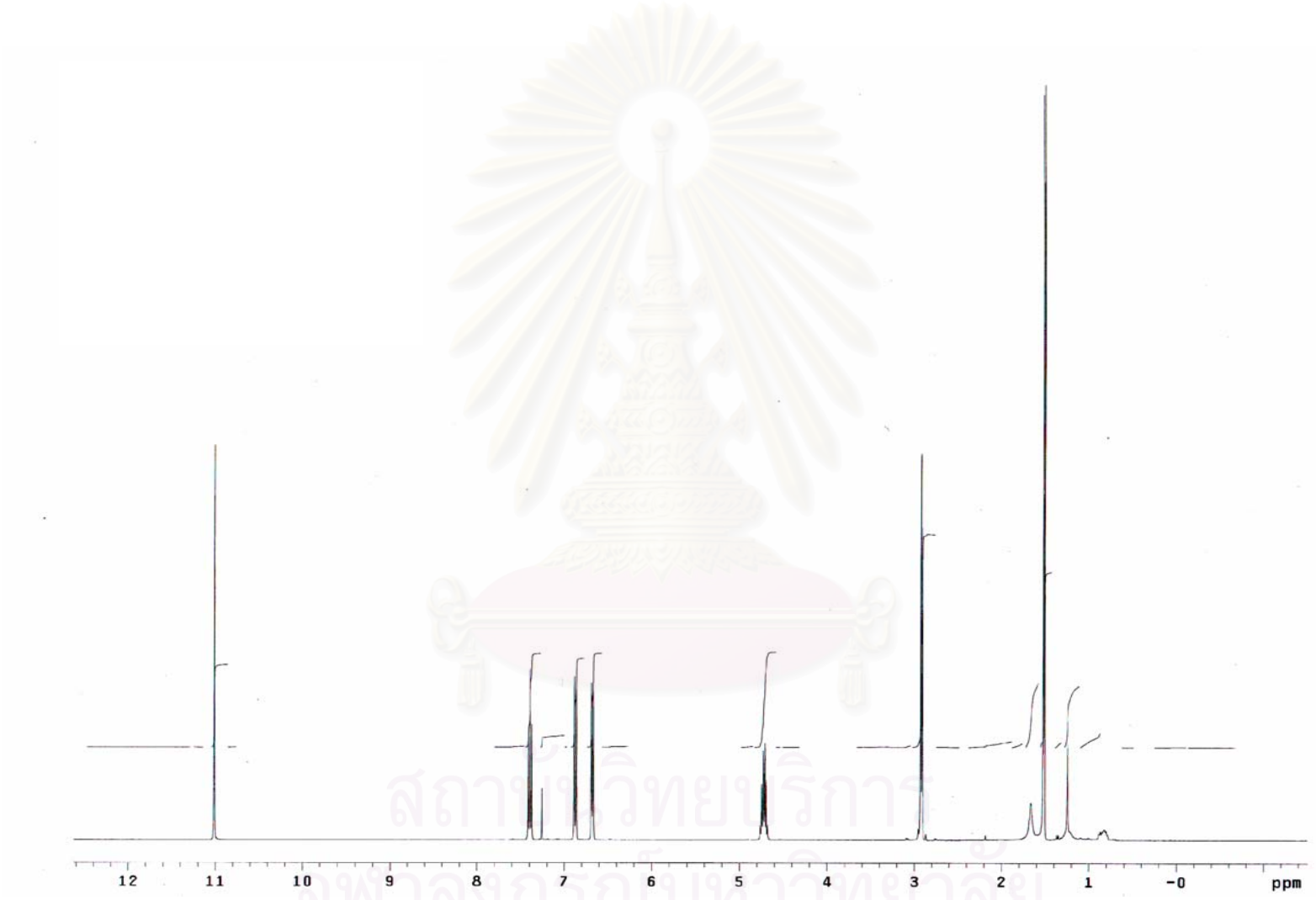


Figure D 2-2 ^{1}H NMR spectrum of compound **2**

112

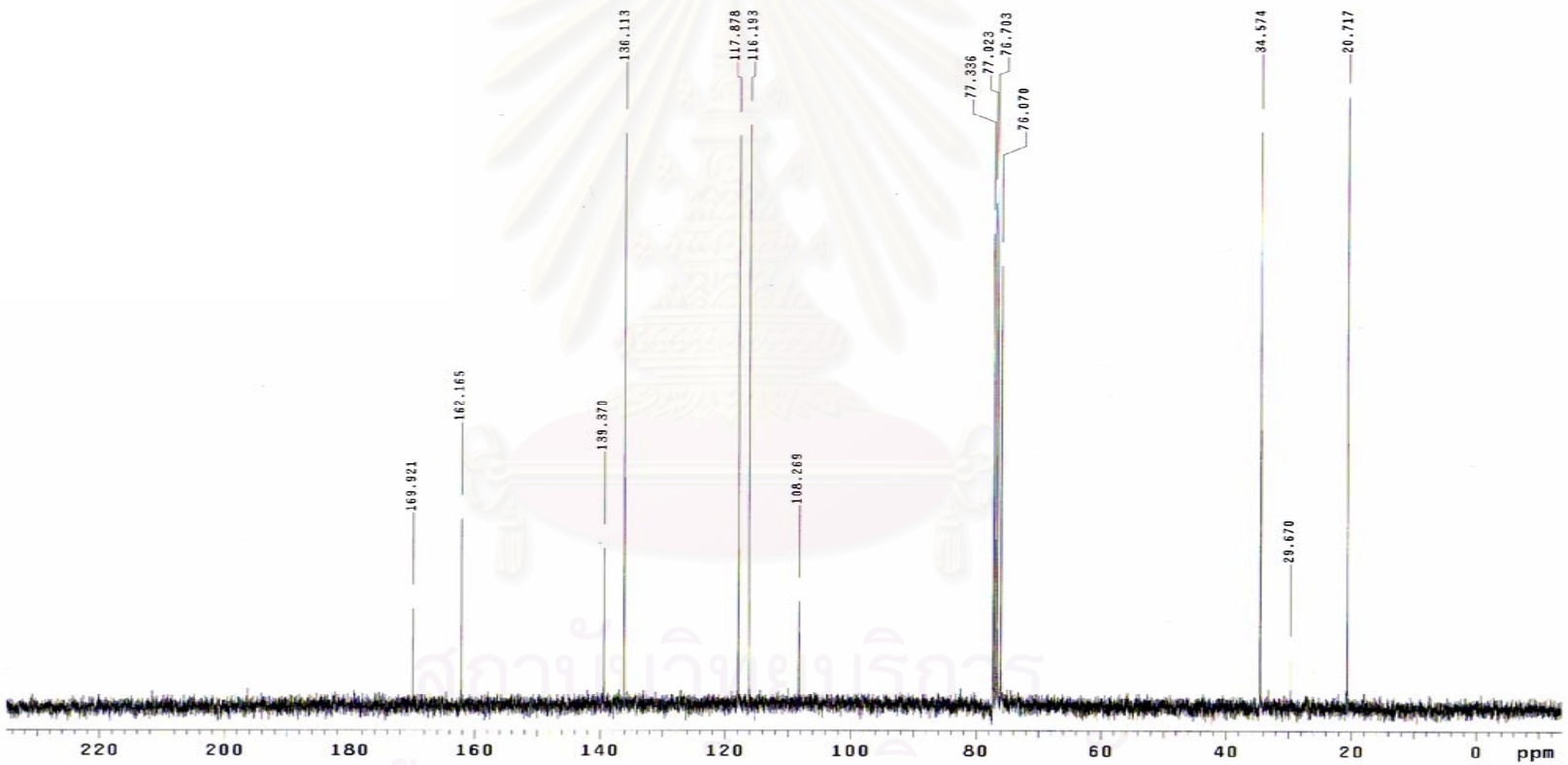


Figure D 2-3 ^{13}C NMR spectrum of compound **2**

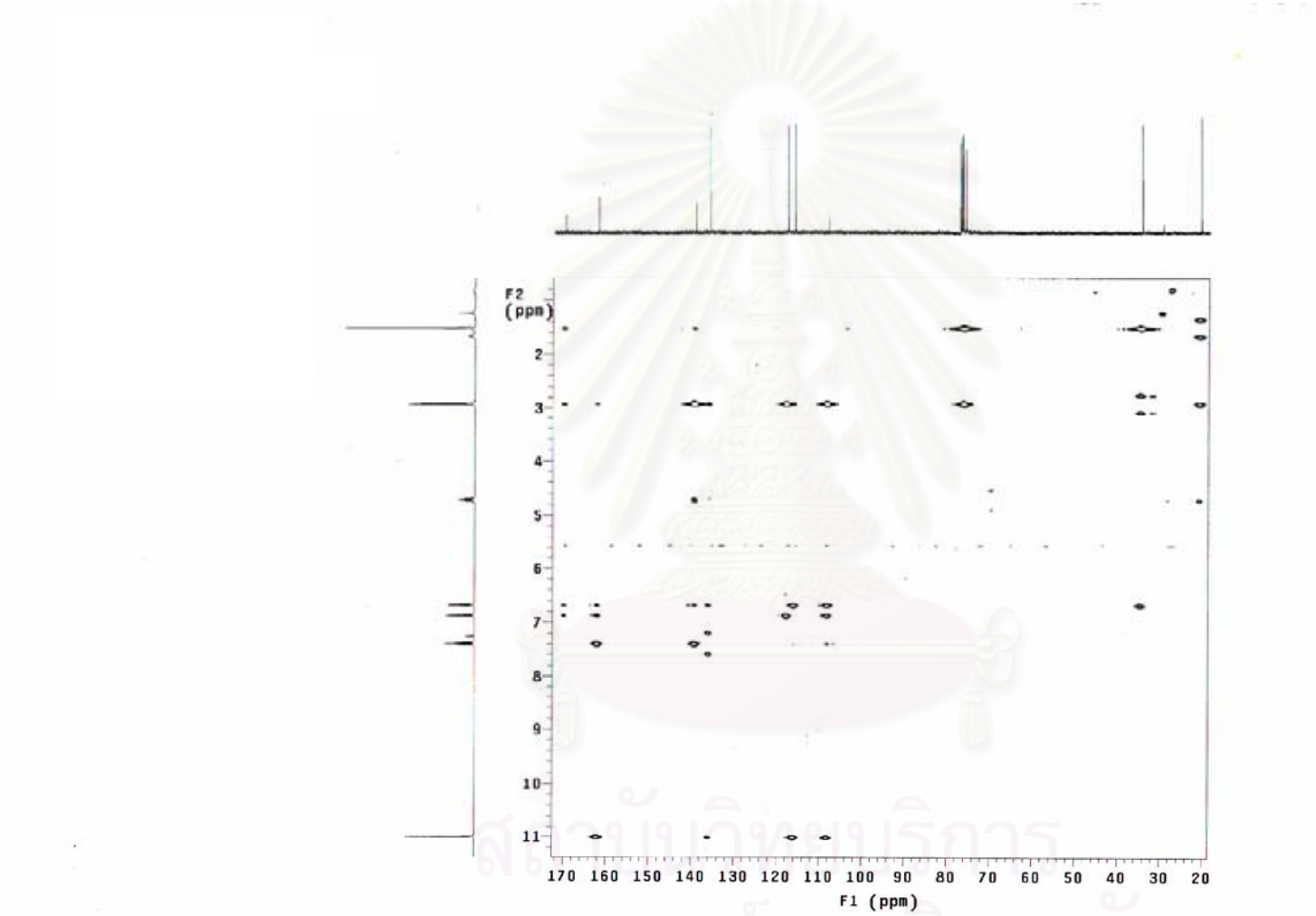


Figure D 2-4 HMBC spectrum of compound **2**

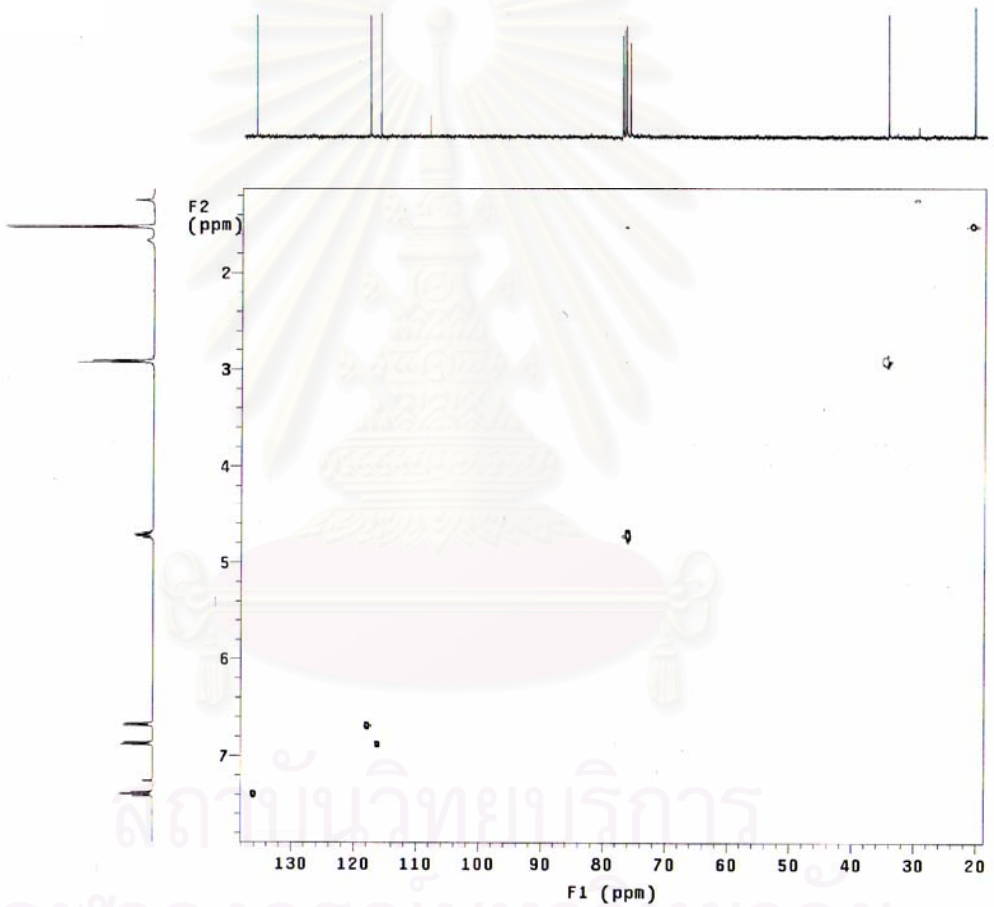


Figure D 2-5 HSQC spectrum of compound **2**

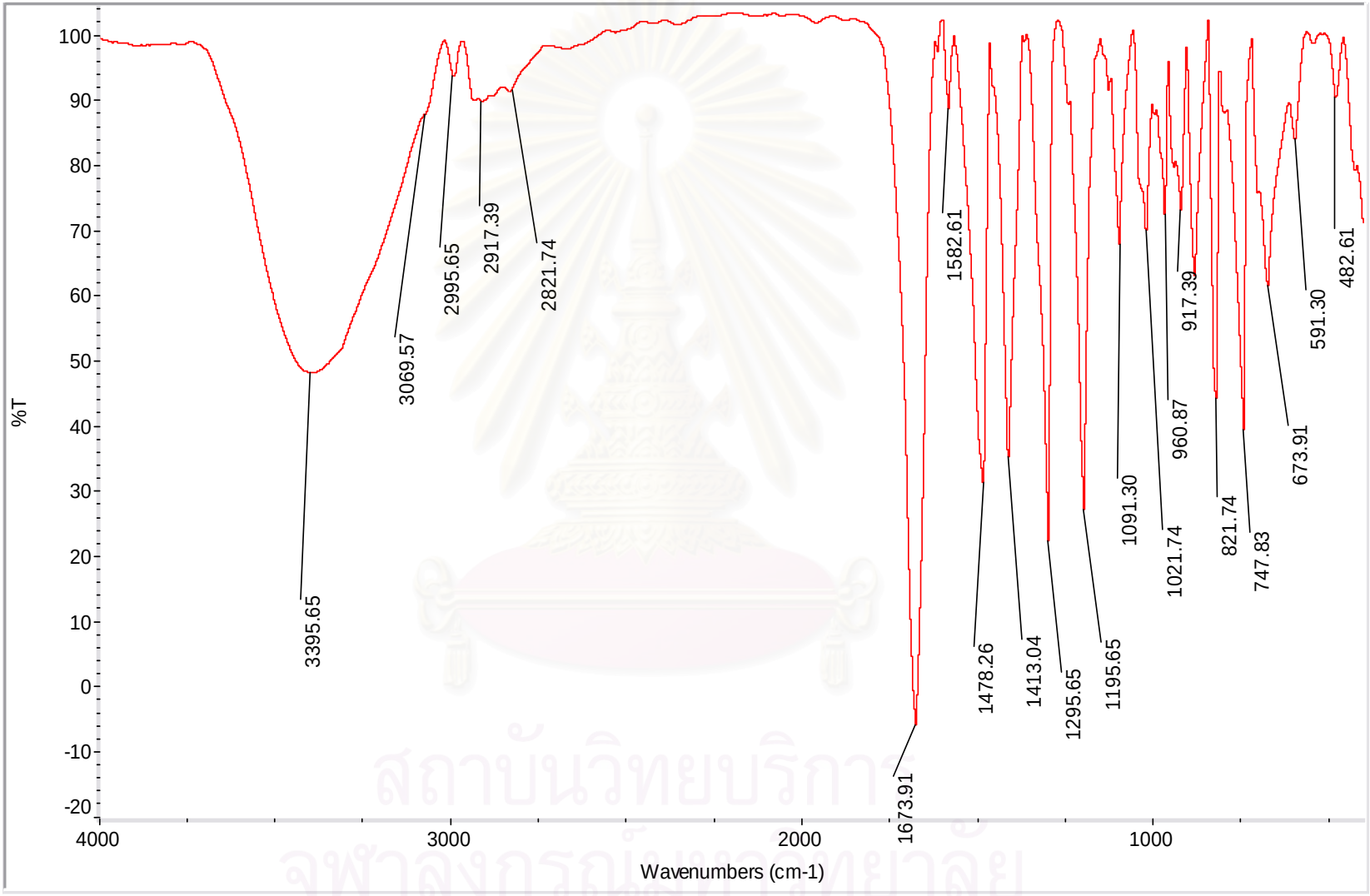


Figure D 3-1 The IR spectrum of compound **3**

116

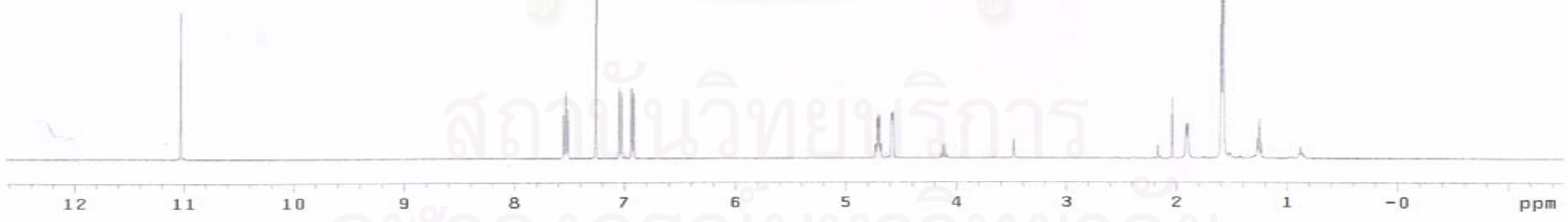


Figure D 3-2 ^{1}H NMR spectrum of compound **3**

117

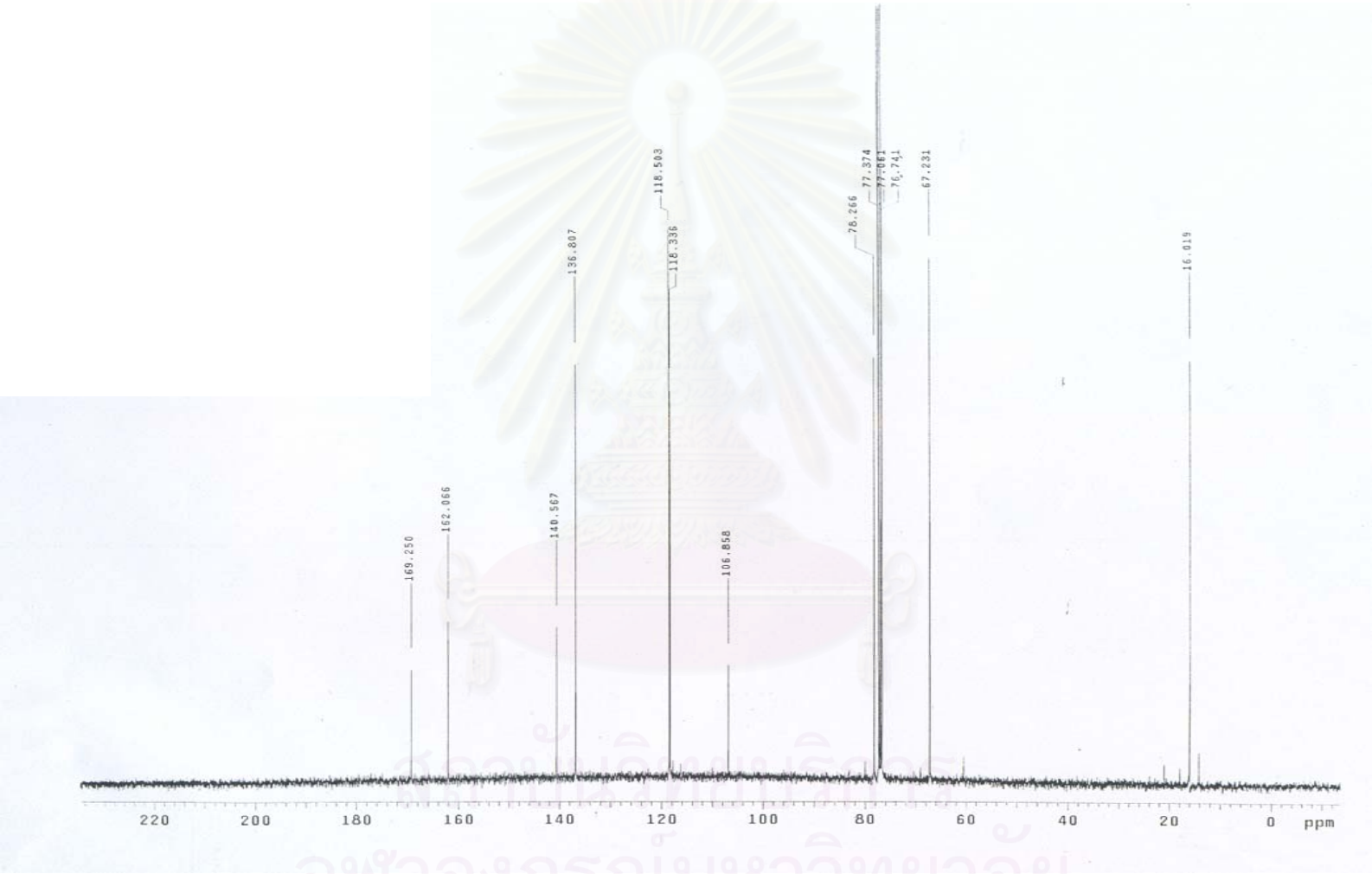


Figure D 3-3 ^{13}C NMR spectrum of compound **3**

118

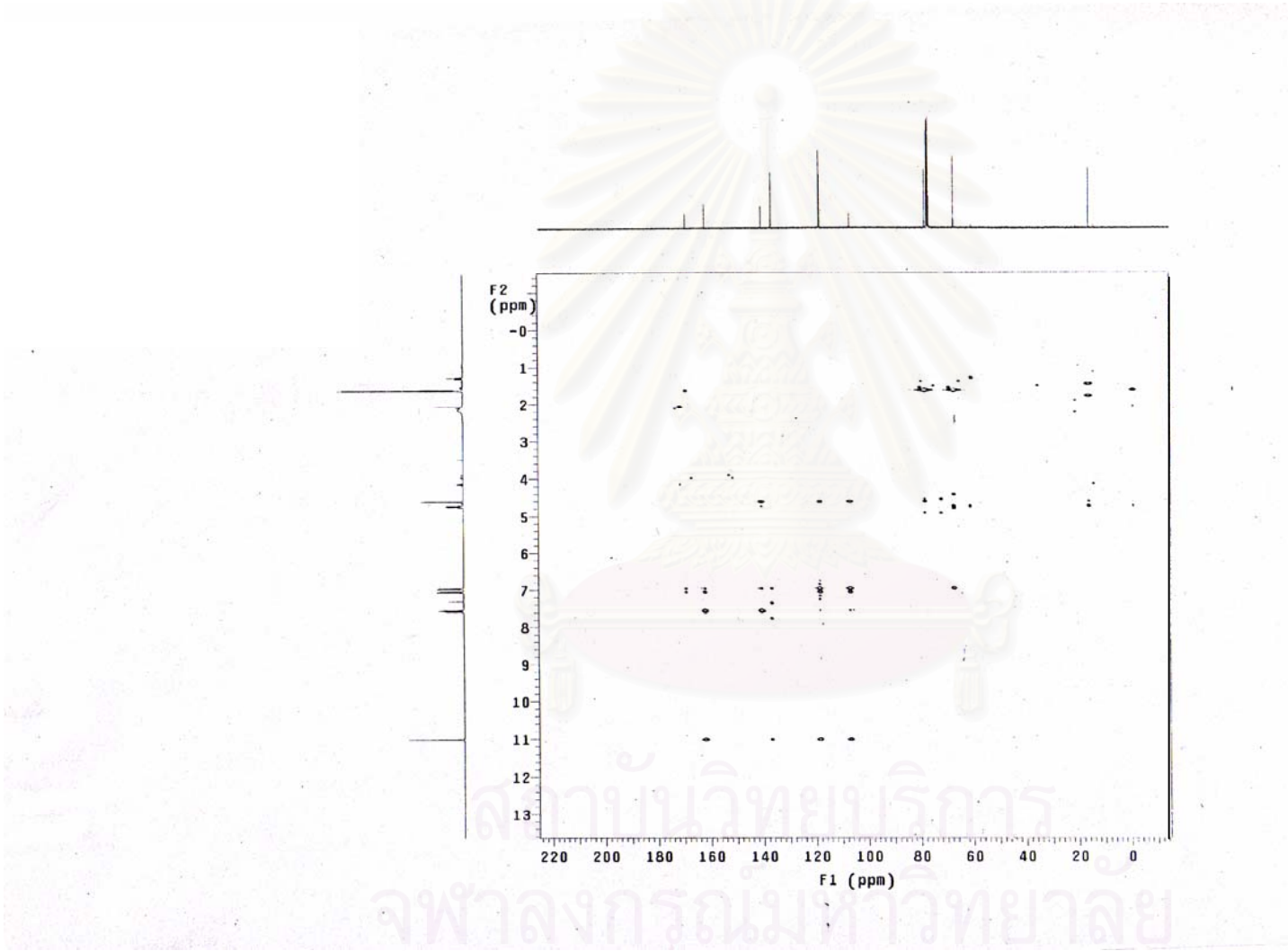


Figure D 3-4 HMBC spectrum of compound **3**

119

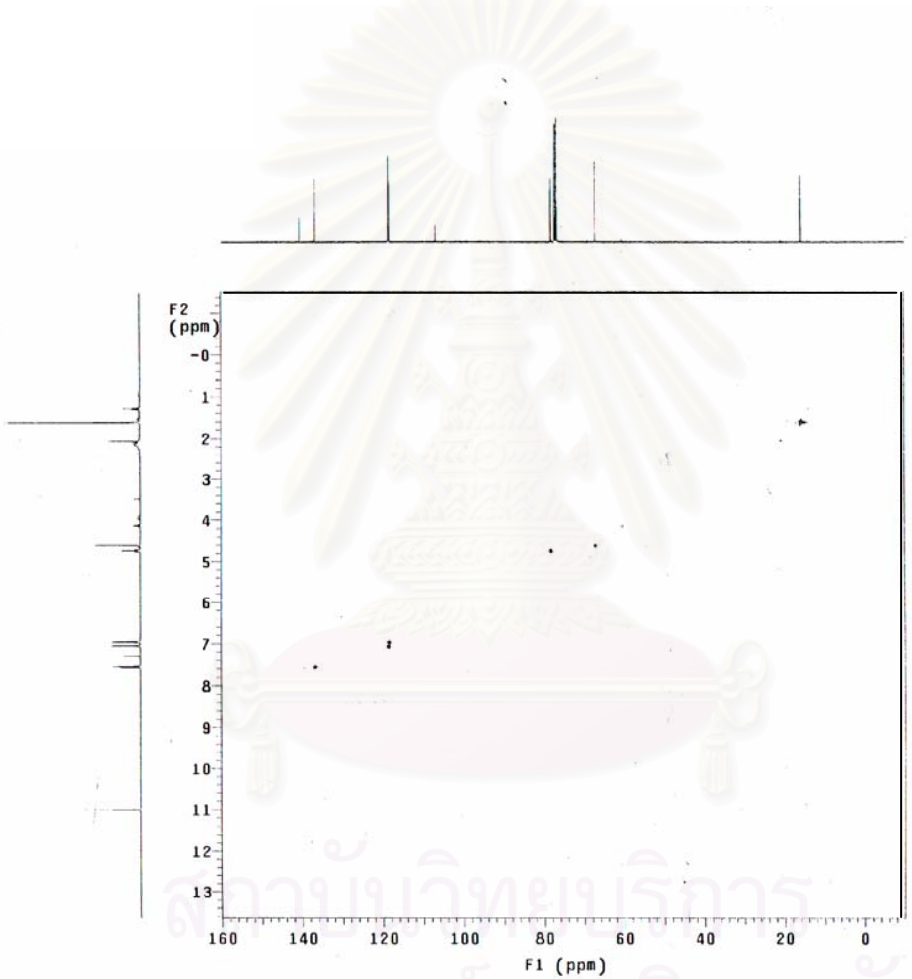


Figure D 3-5 HSQC spectrum of compound **3**

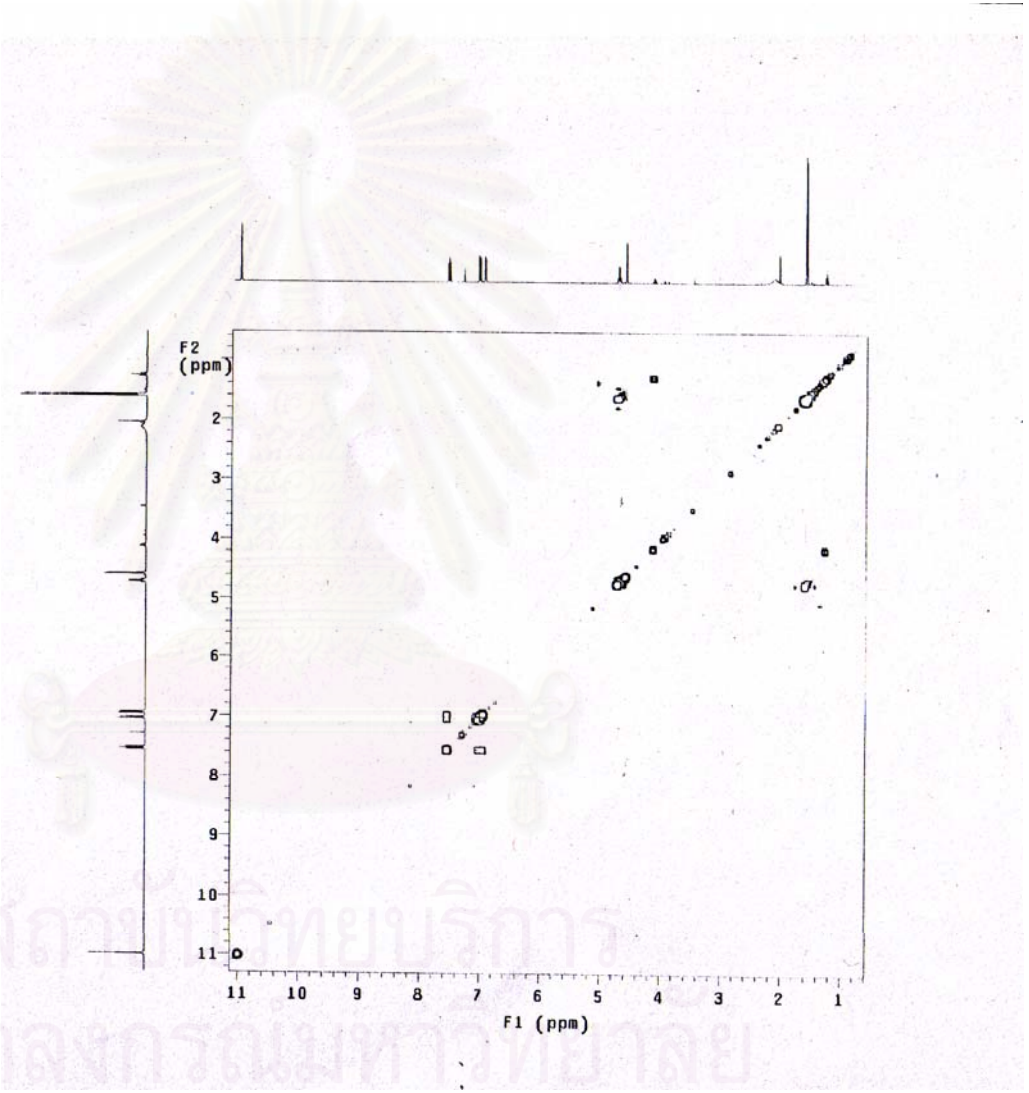


Figure D 3-6 COSY spectrum of compound **3**

121

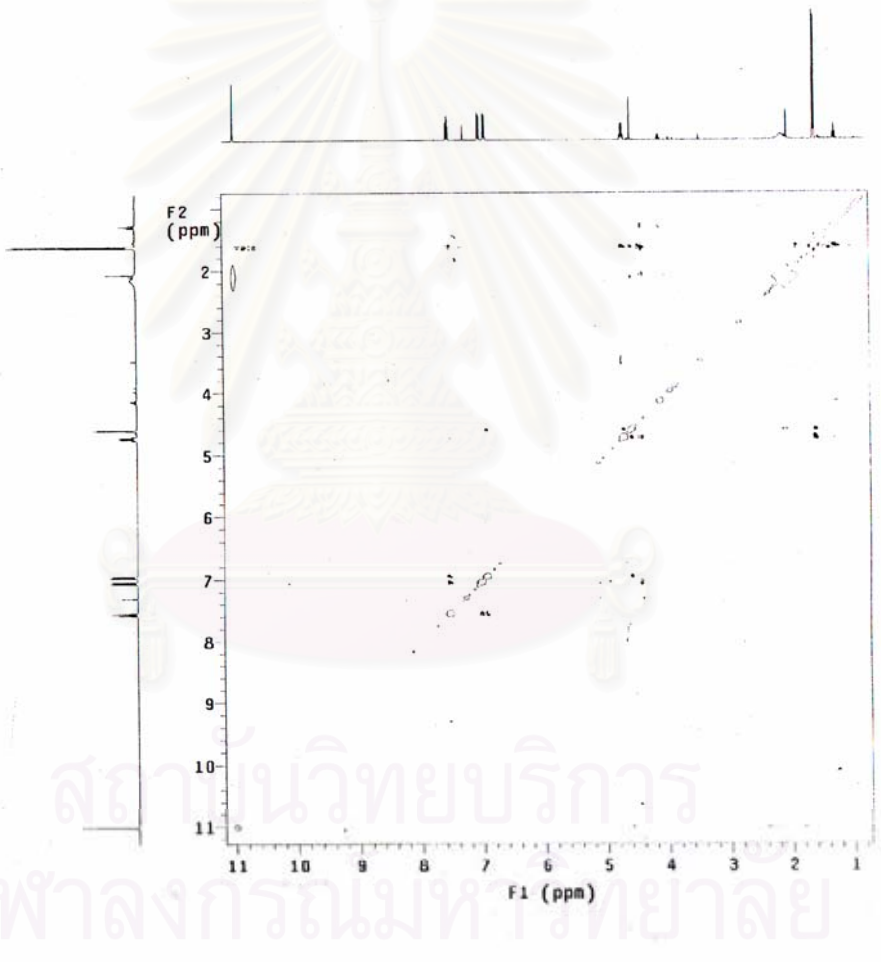


Figure D 3-7 NOESY spectrum of compound **3**

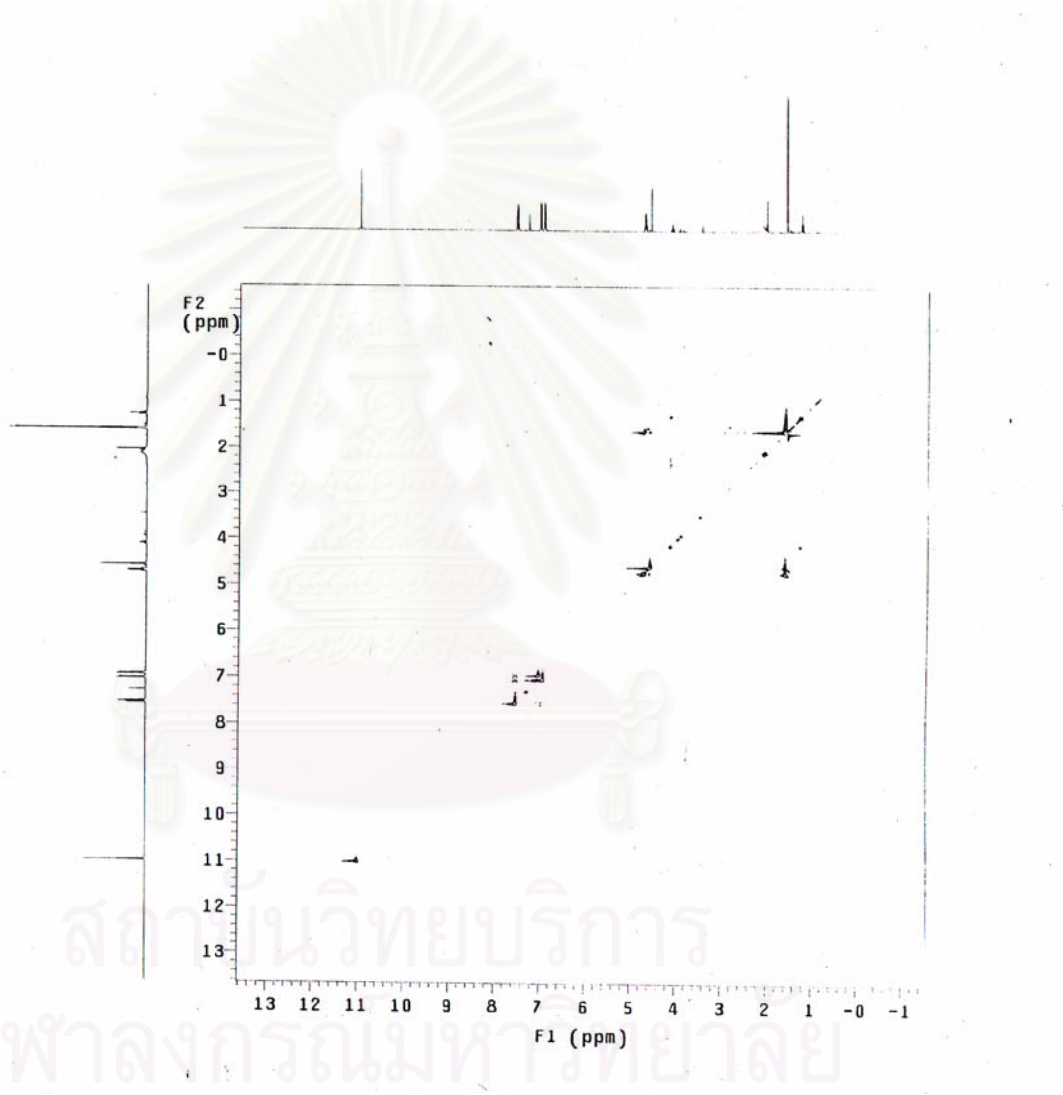


Figure D 3-8 TOCSY spectrum of compound **3**

123

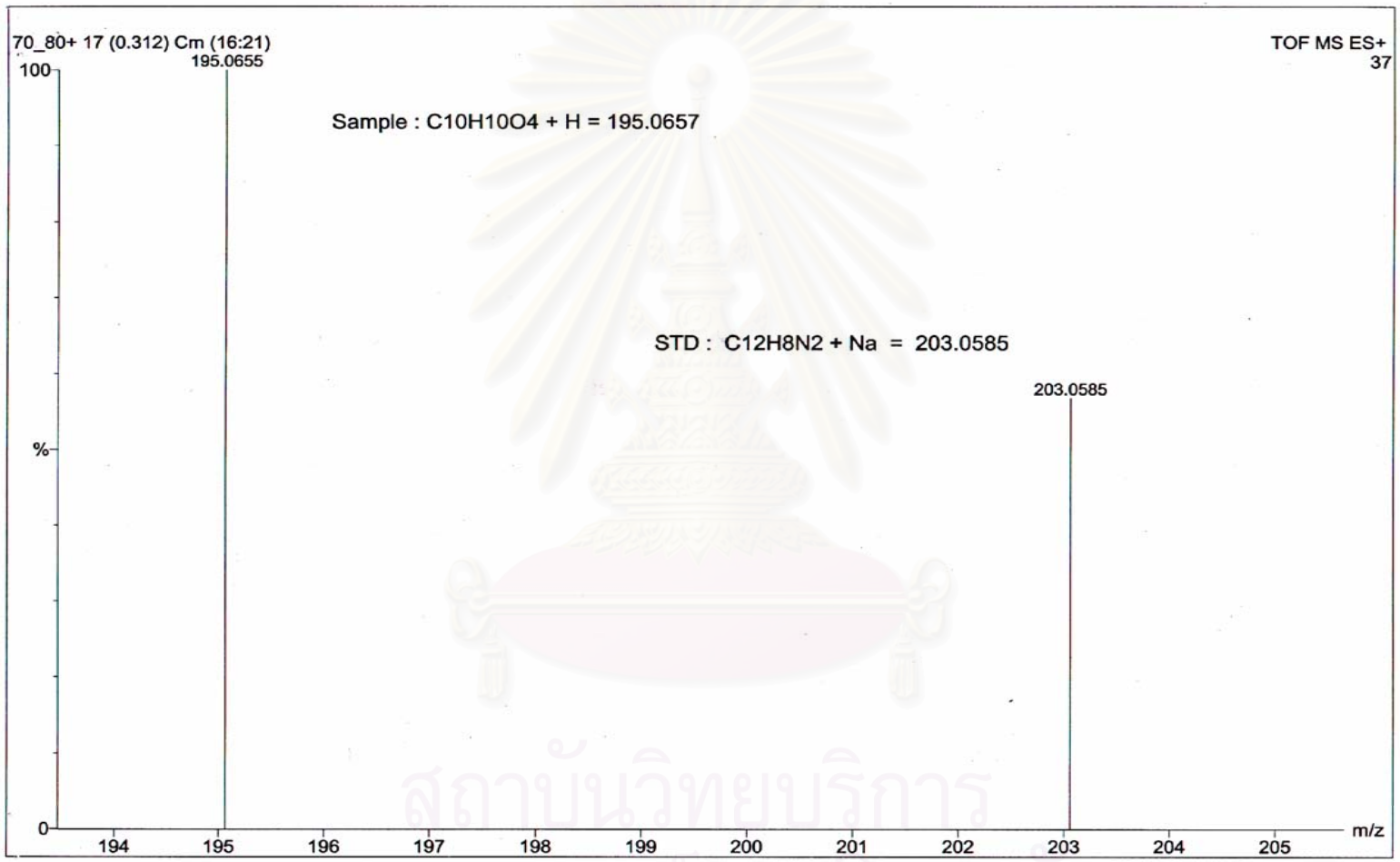


Figure D 3-9 The profile mass spectrum of compound **3**

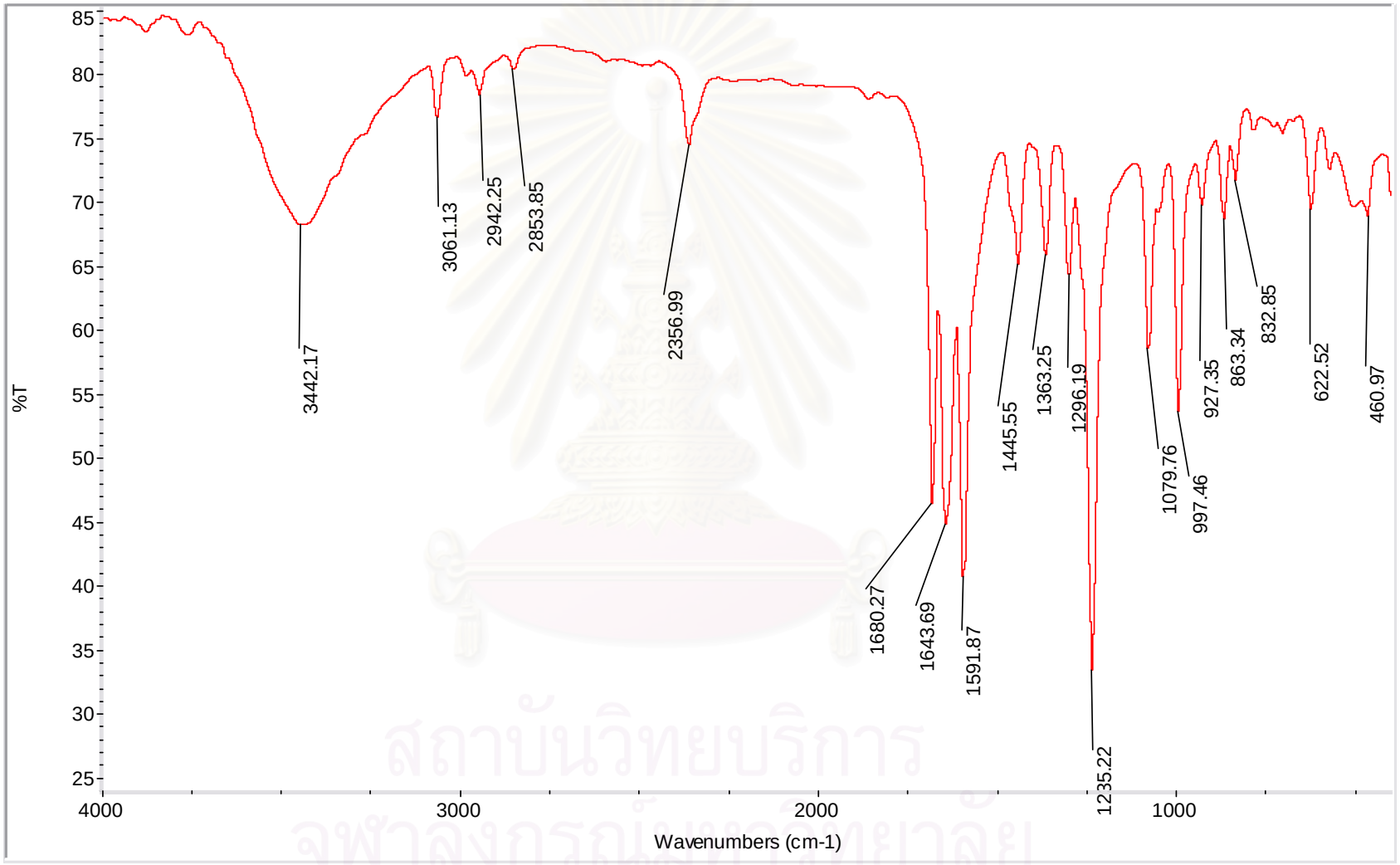


Figure D 4-1 The IR spectrum of compound **4**

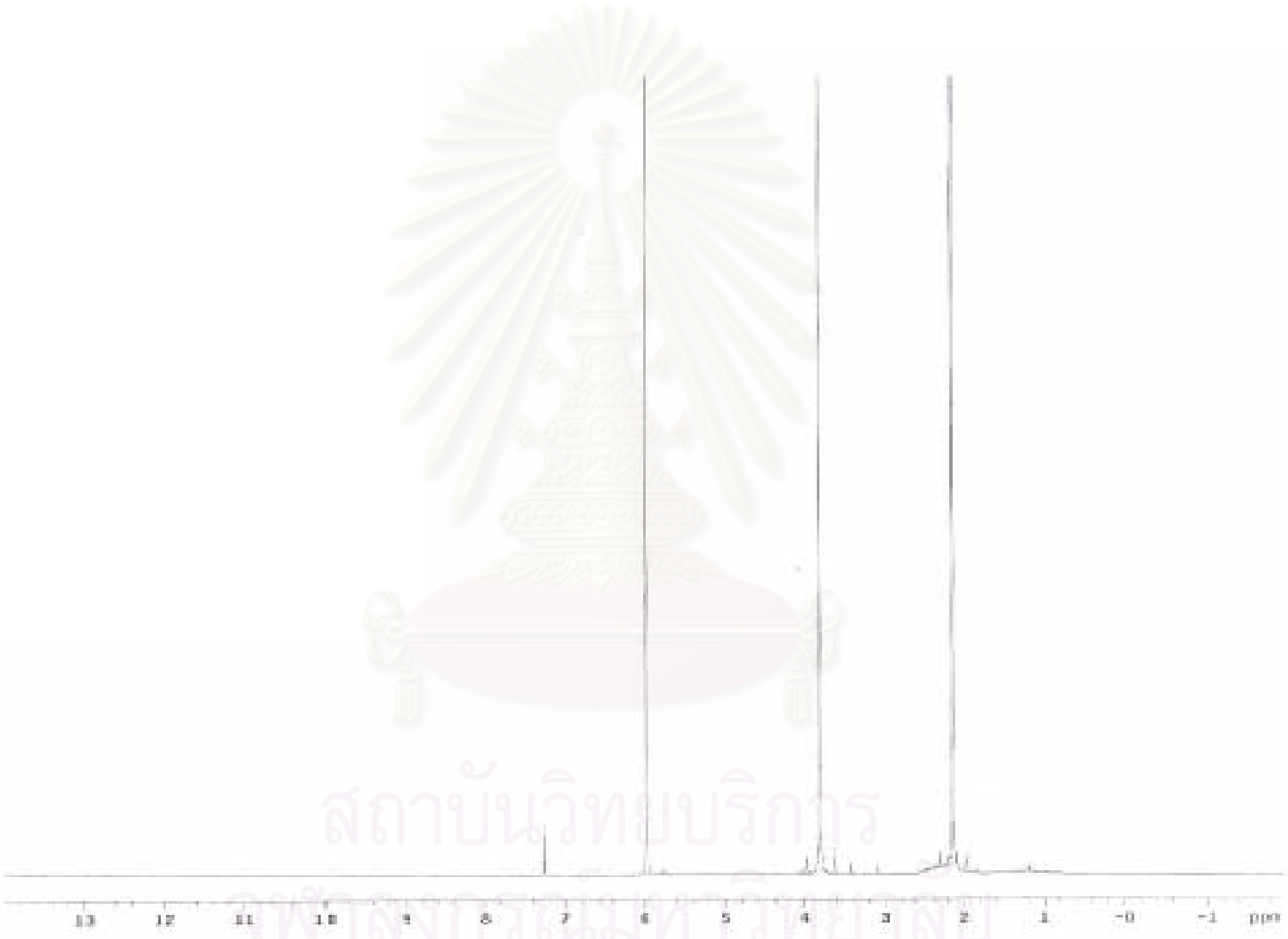


Figure D 4-2 ^{1}H NMR spectrum of compound **4**

126

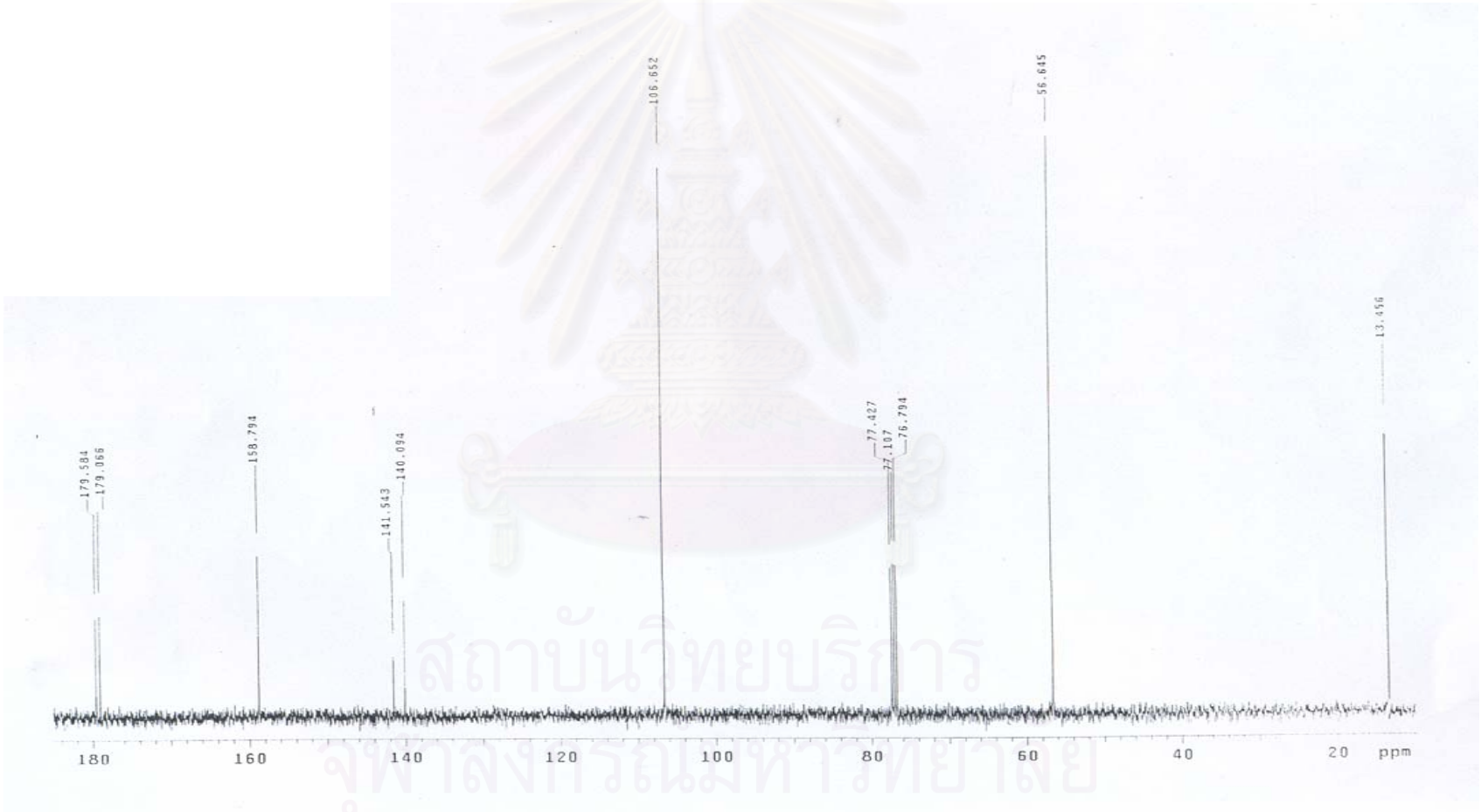


Figure D 4-3 ^{13}C NMR spectrum of compound **4**

127

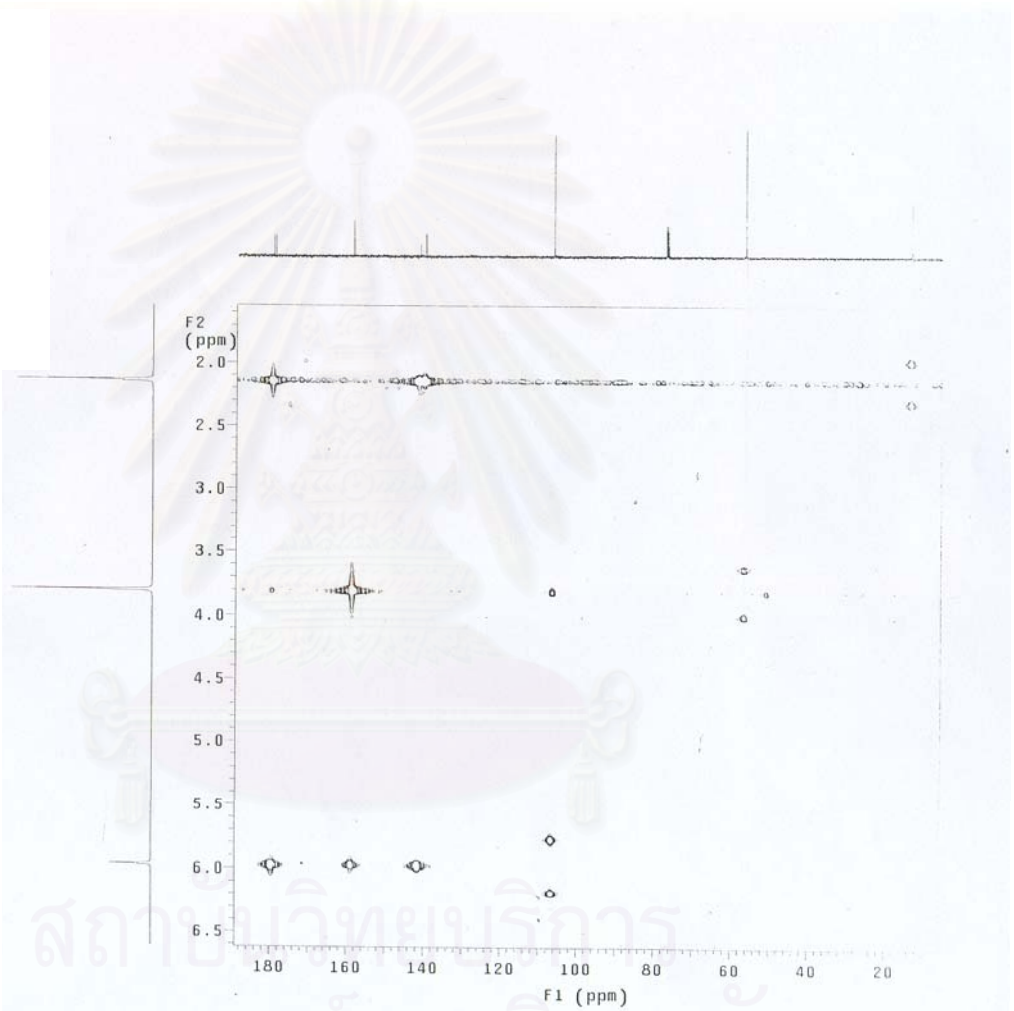


Figure D 4-4 HMBC spectrum of compound **4**

128

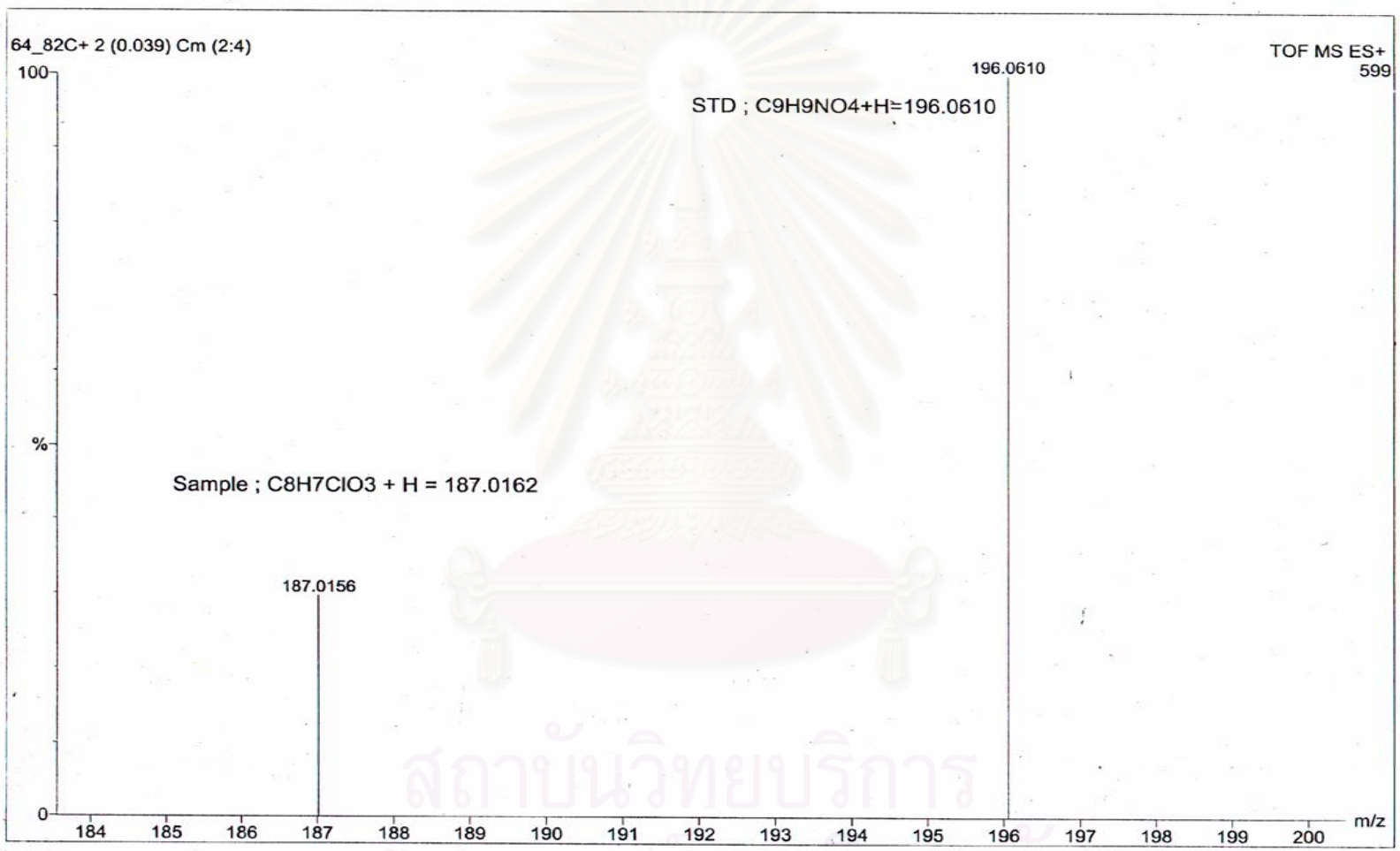


Figure D 4-5 The profile mass spectrum of compound **4**

129

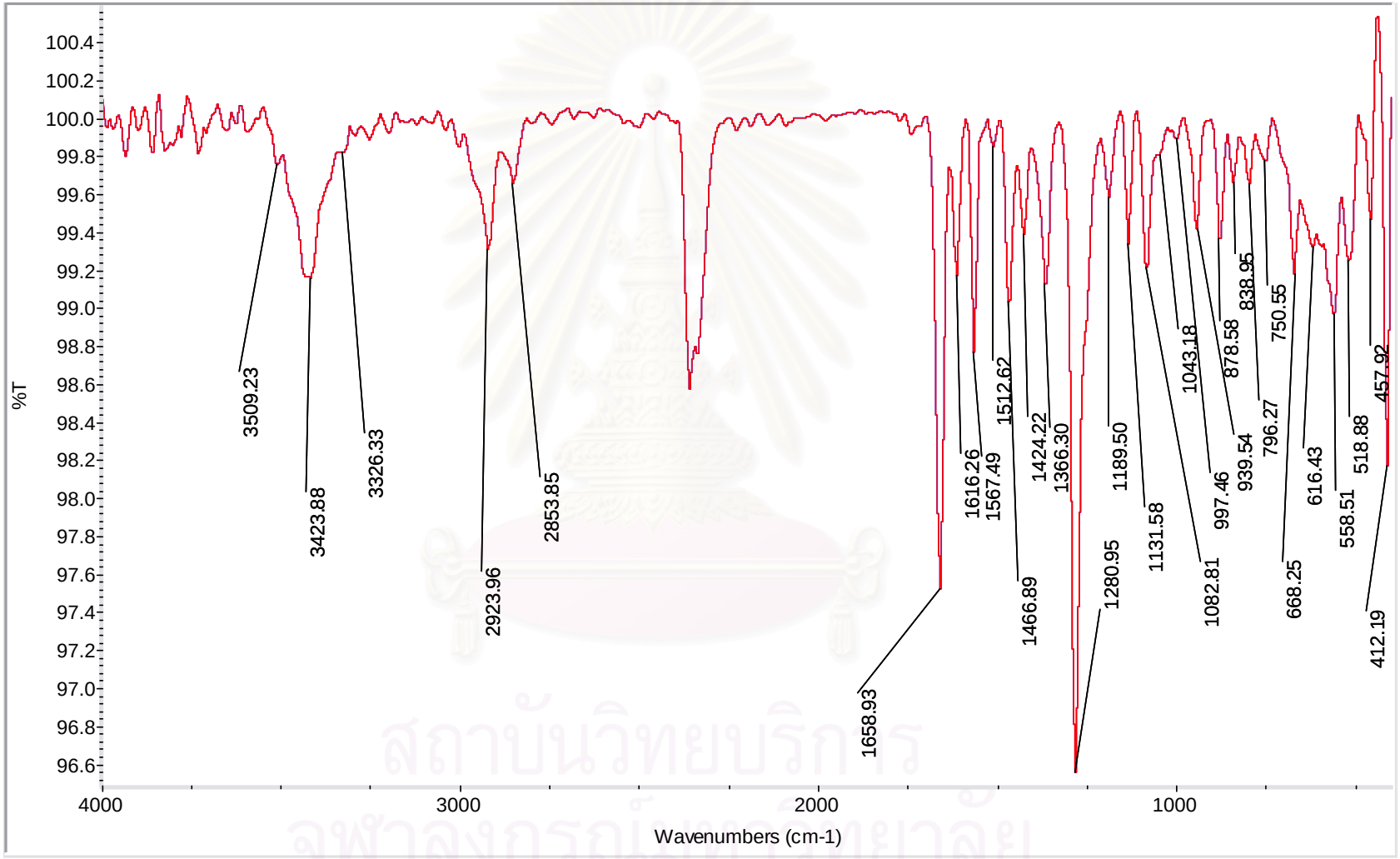


Figure D 5-1 The IR spectrum of compound **5**

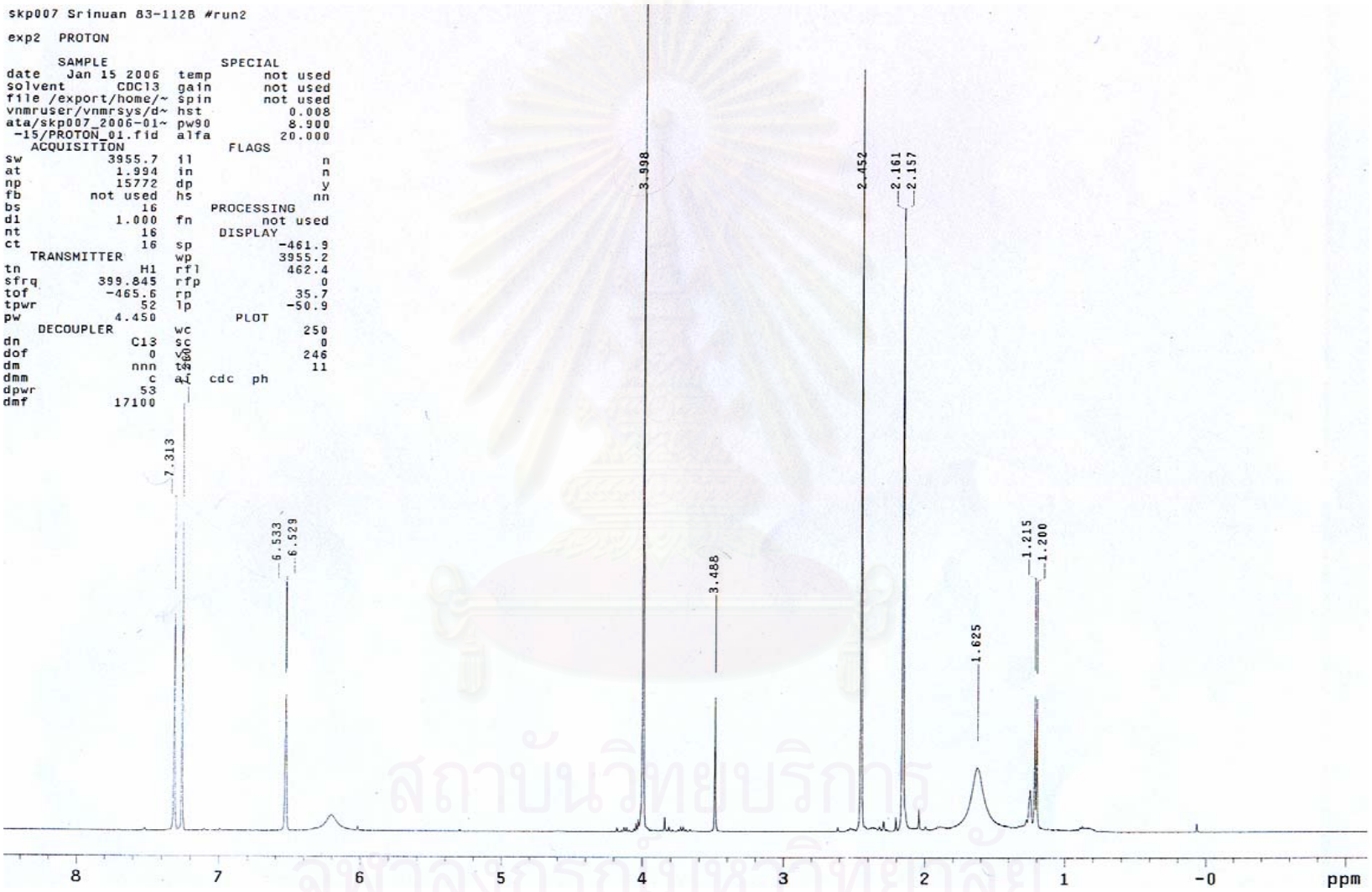


Figure D 5-2 ^{1}H NMR spectrum of compound **5**

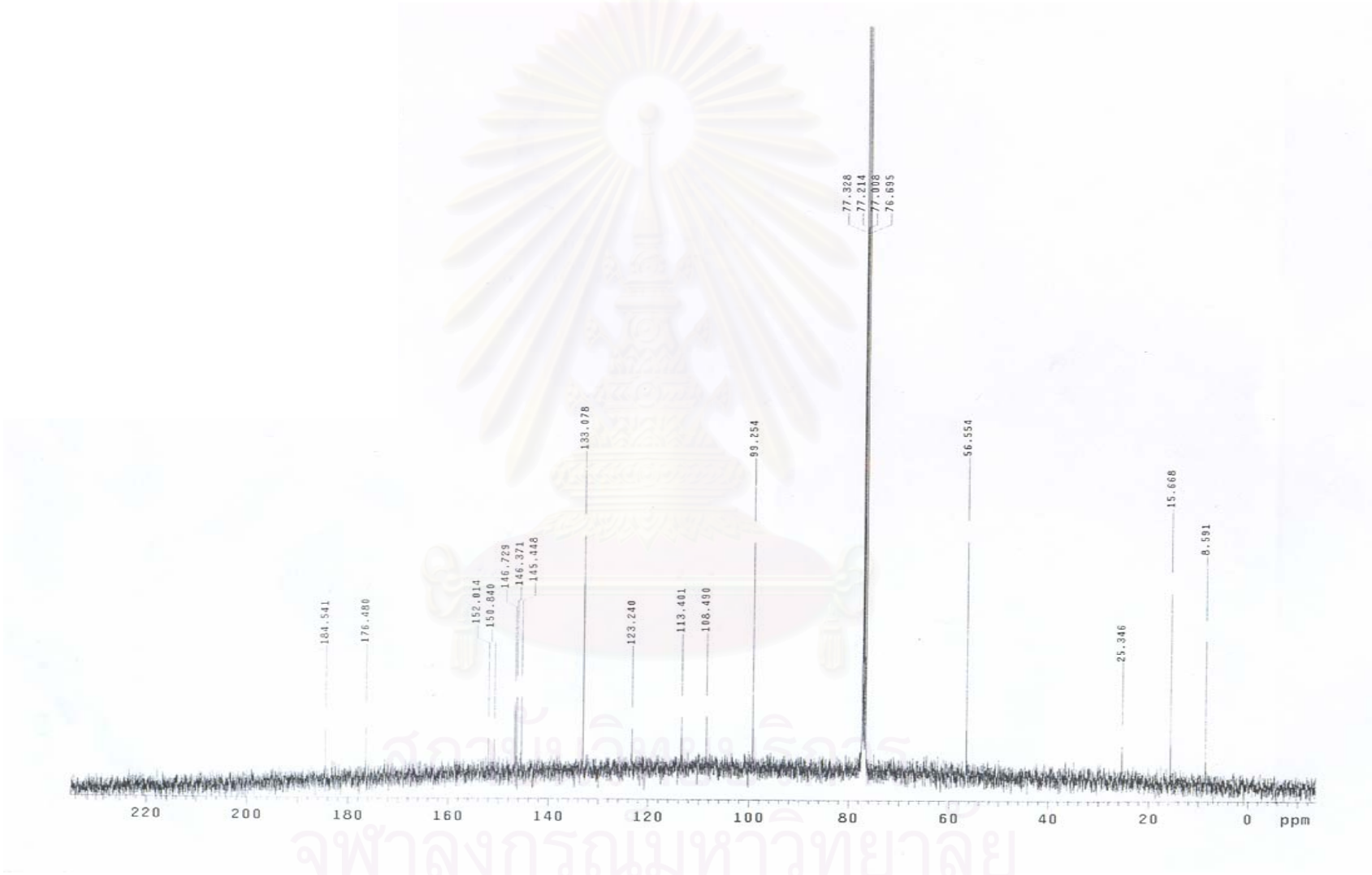


Figure D 5-3 ^{13}C NMR spectrum of compound **5**

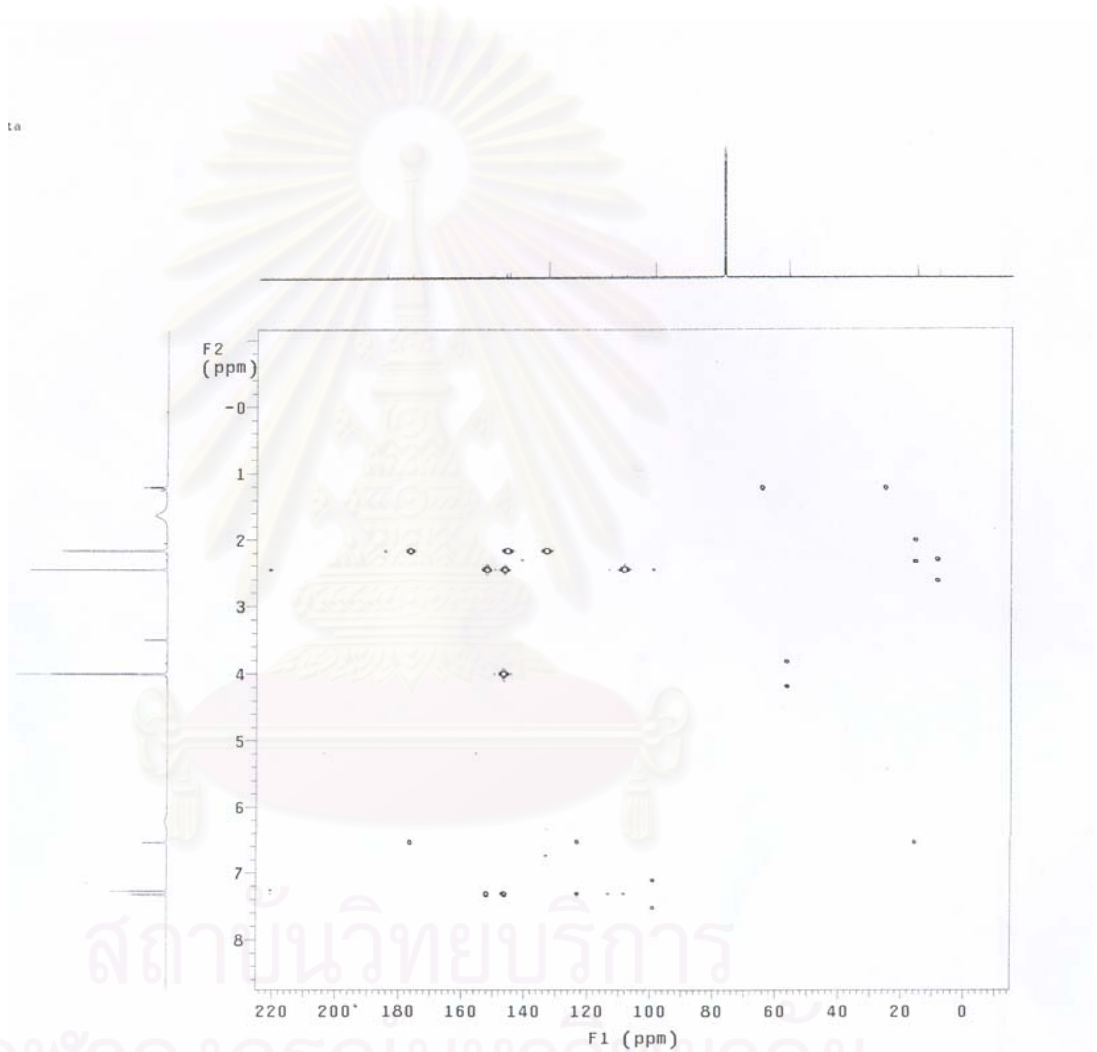


Figure D 5-4 HMBC spectrum of compound **5**

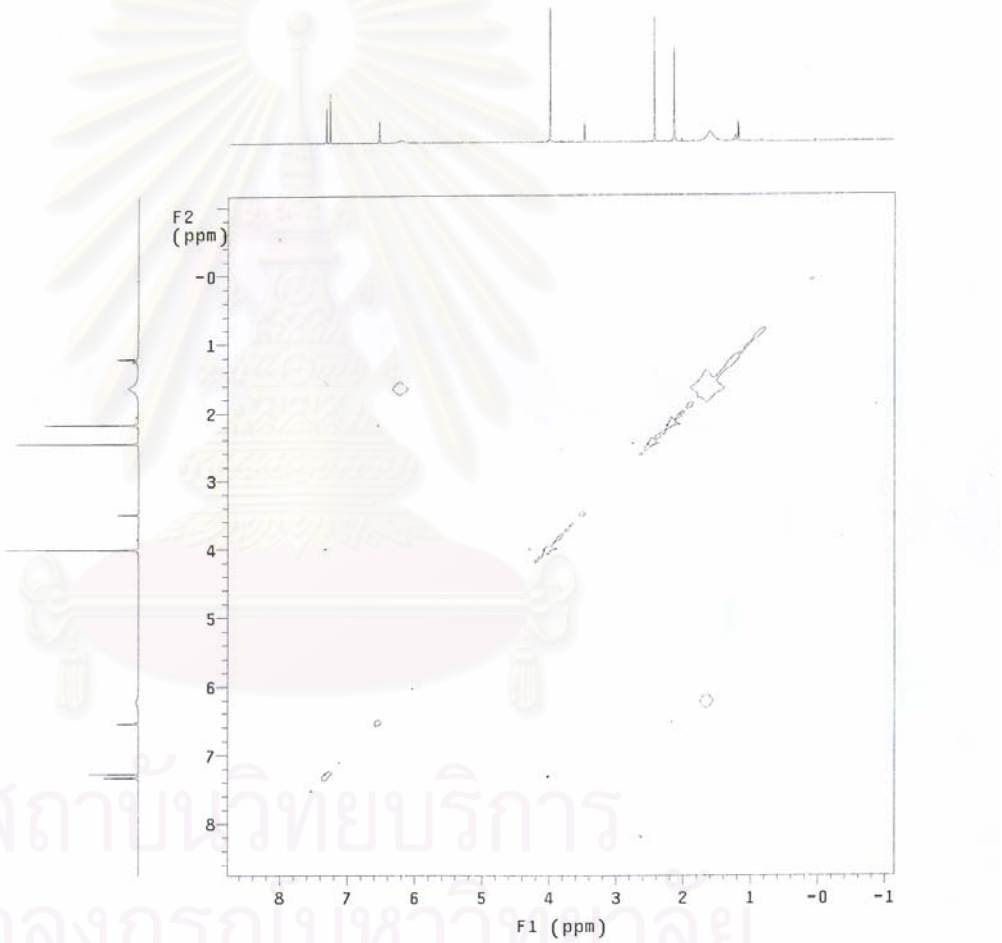


Figure D 5-5 NOESY spectrum of compound **5**

134

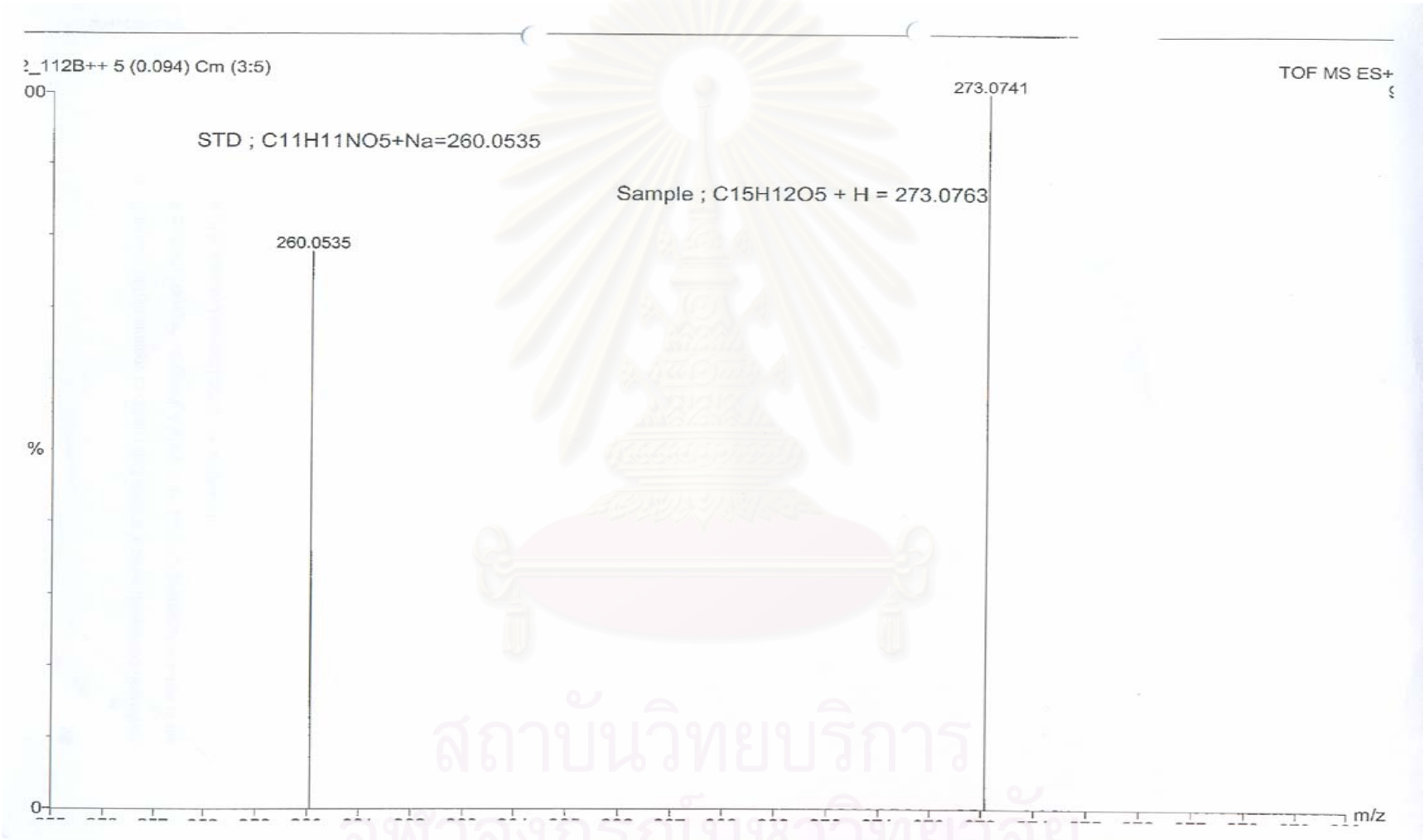


Figure D 5-6 The profile mass spectrum of compound **5**

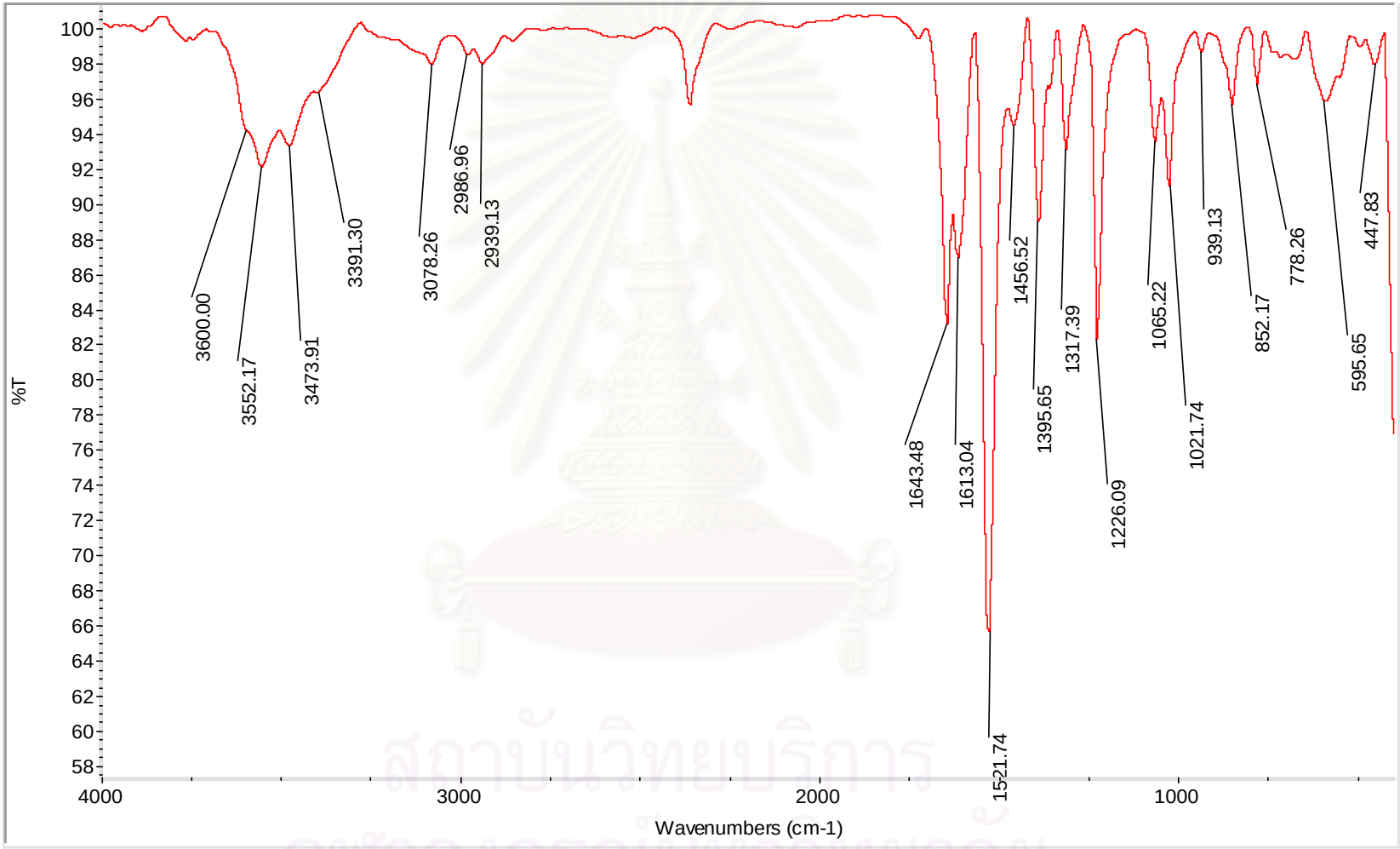


Figure D 6-1 The IR spectrum of compound **6**

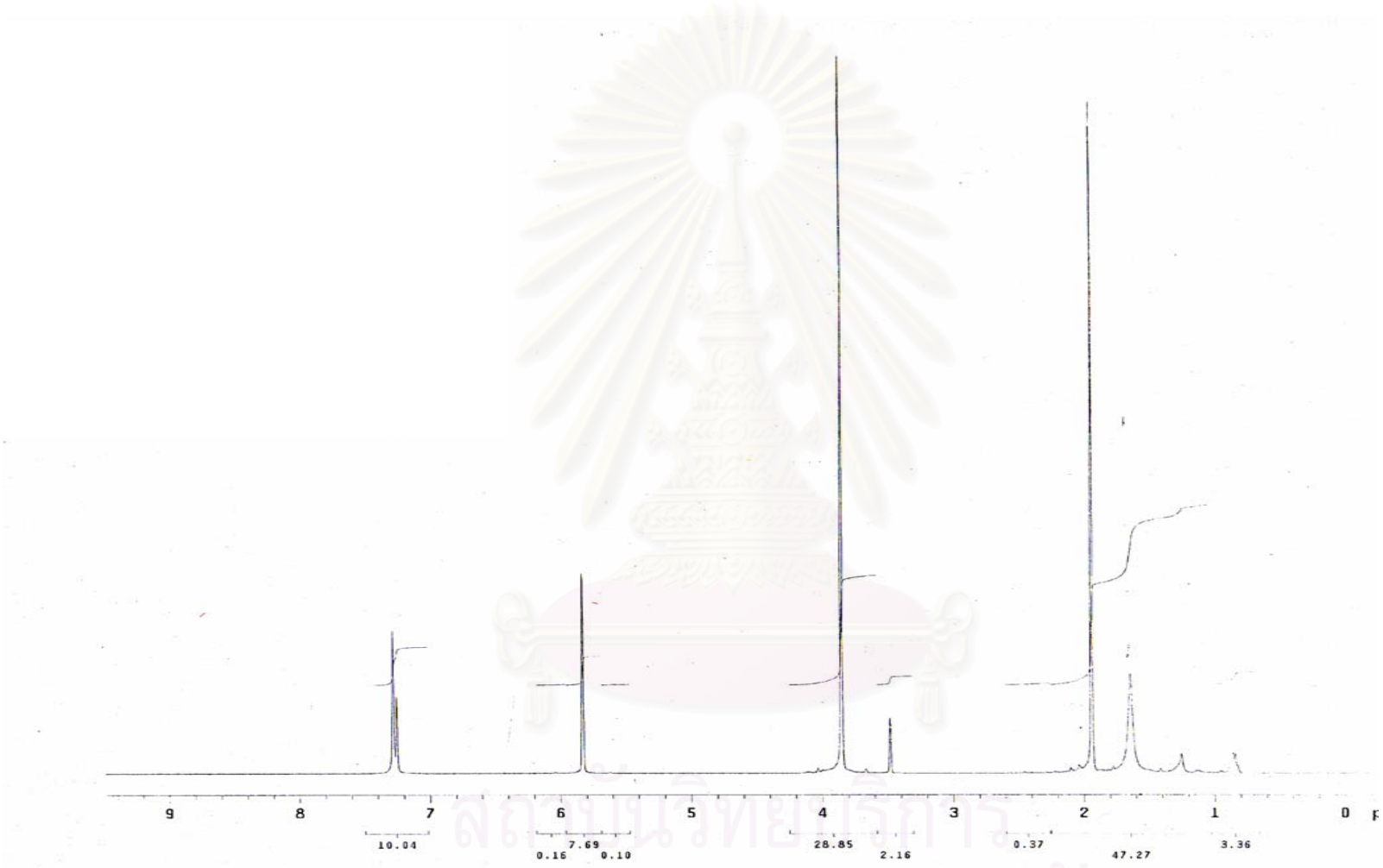


Figure D 6-2 ^{1}H NMR spectrum of compound **6**

137

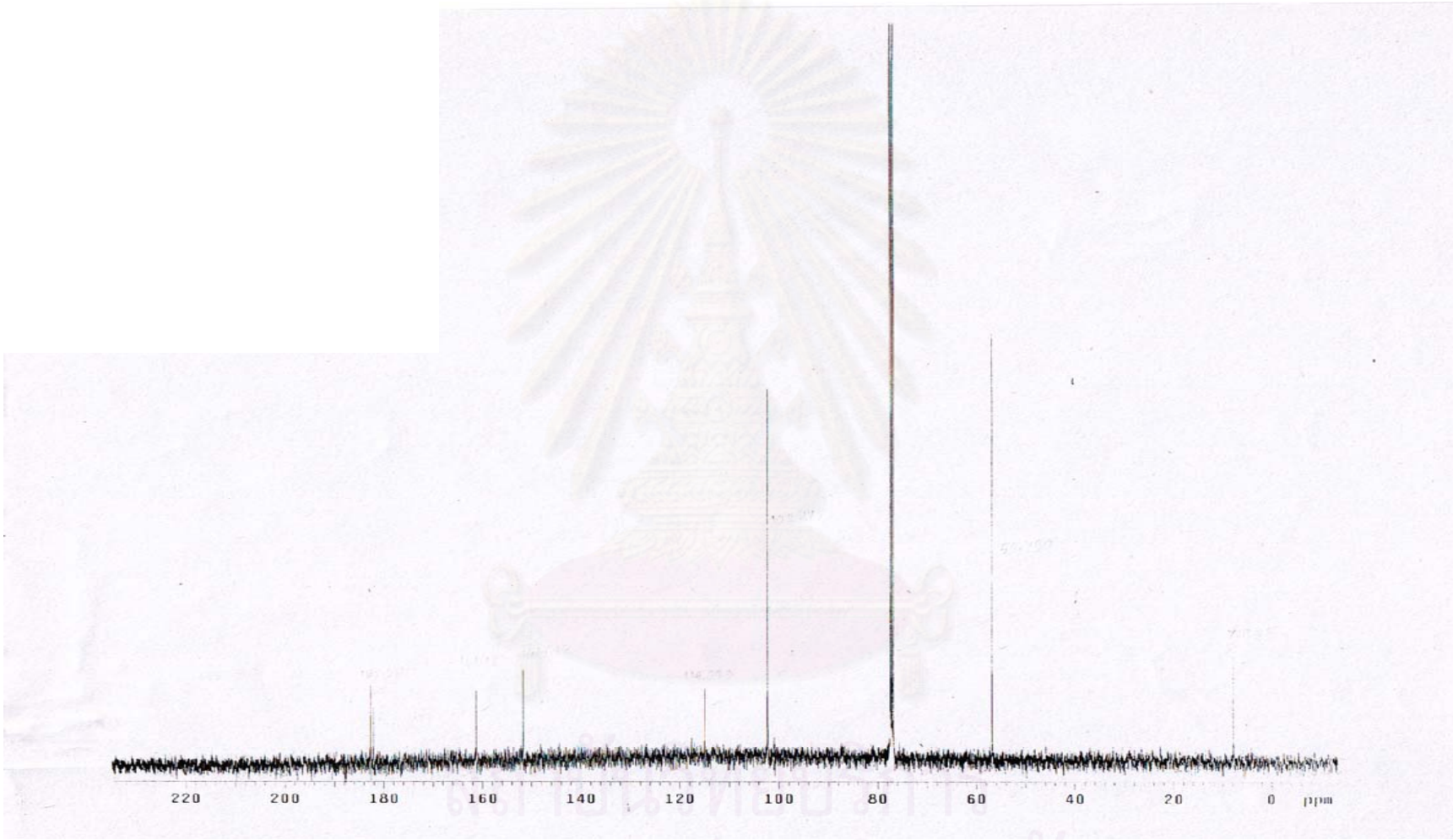


Figure D 6-3 ^{13}C NMR spectrum of compound **6**

138

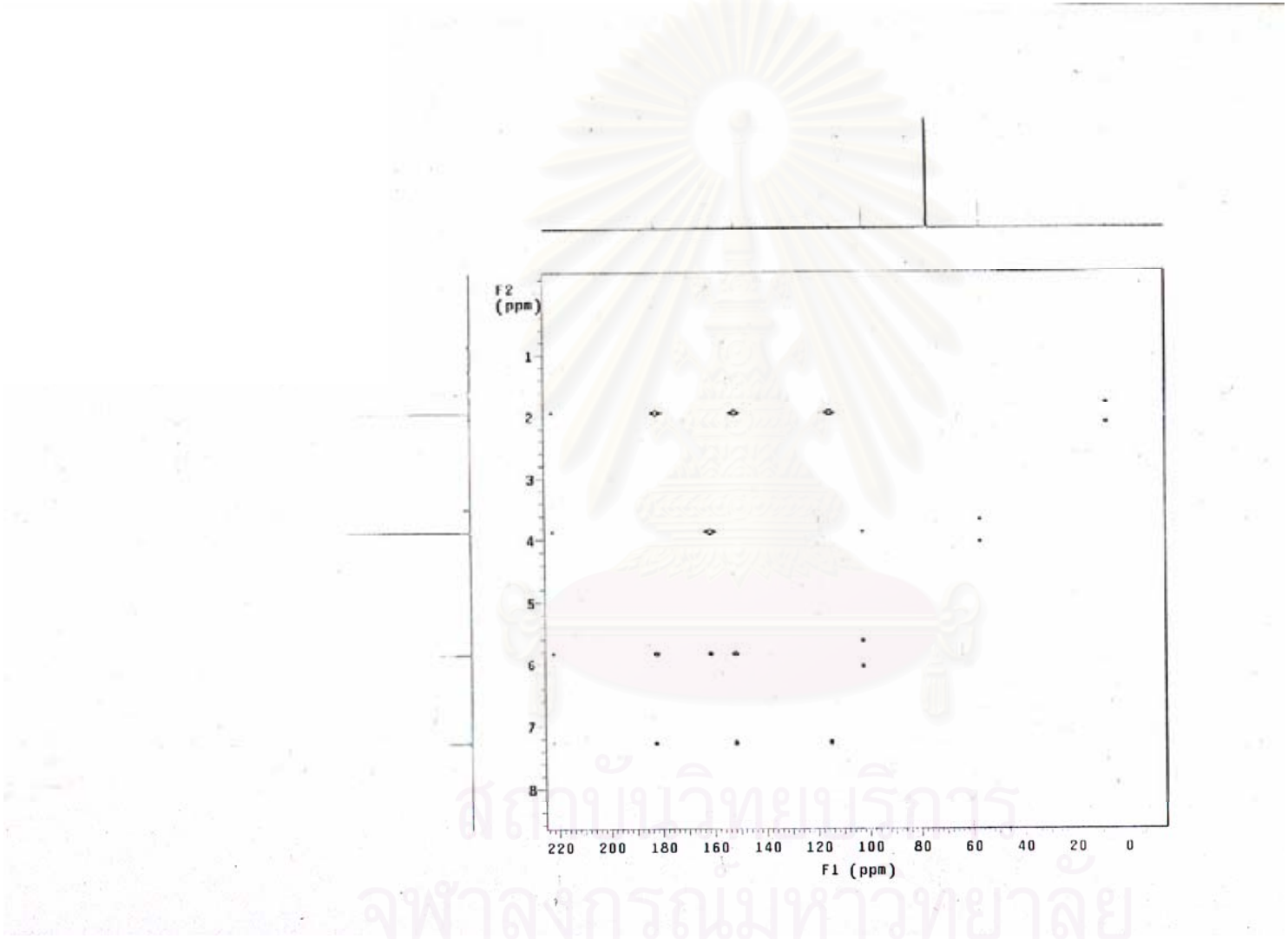


Figure D 6-4 HMBC spectrum of compound **6**

139

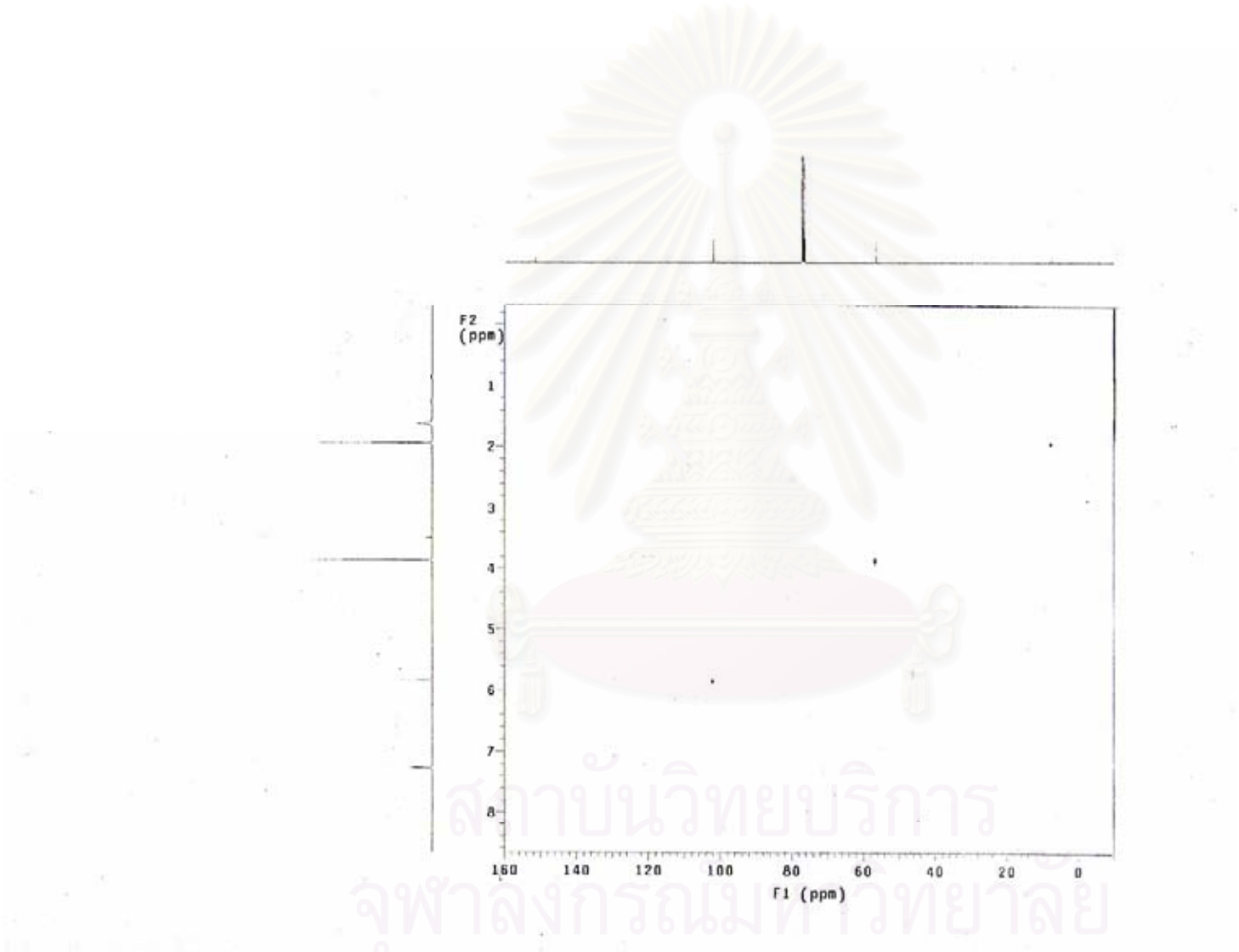


Figure D 6-5 HSQC spectrum of compound **6**

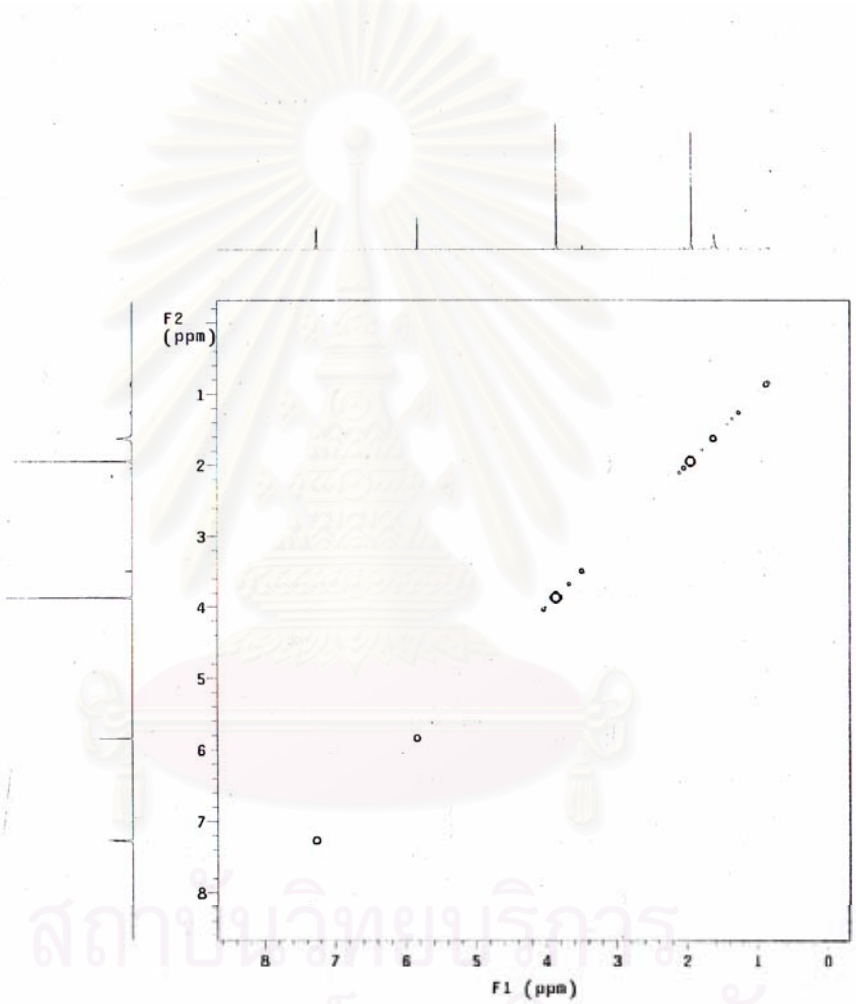


Figure D 6-6 COSY spectrum of compound **6**

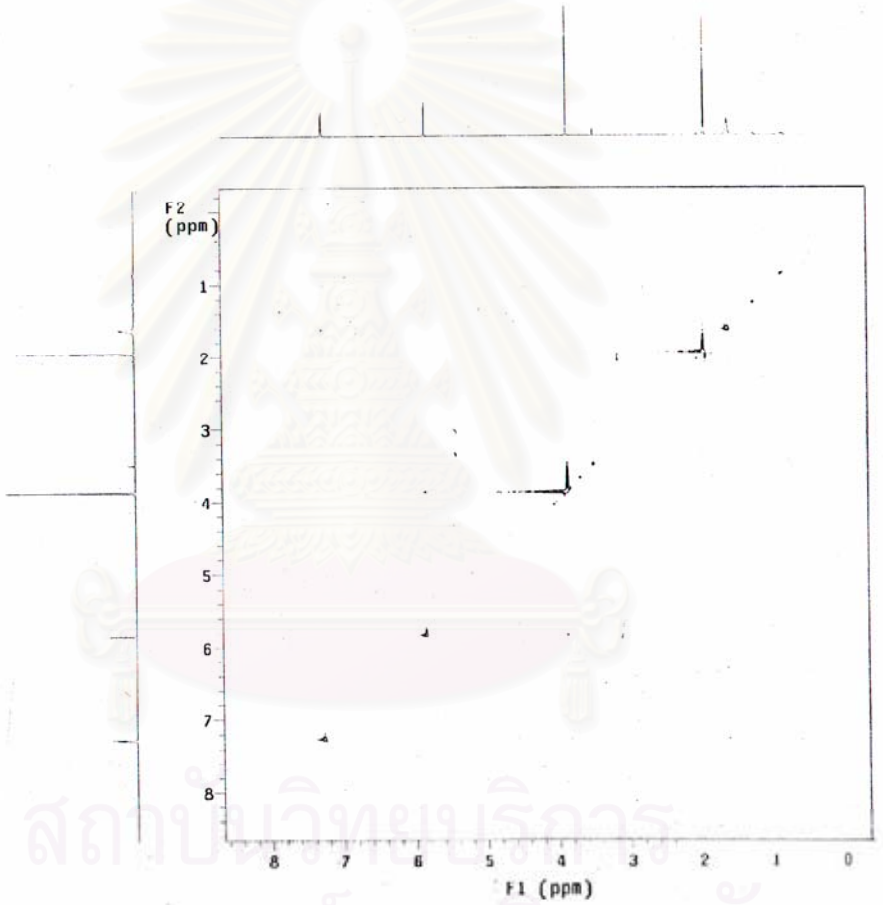


Figure D 6-7 TOCSY spectrum of compound **6**

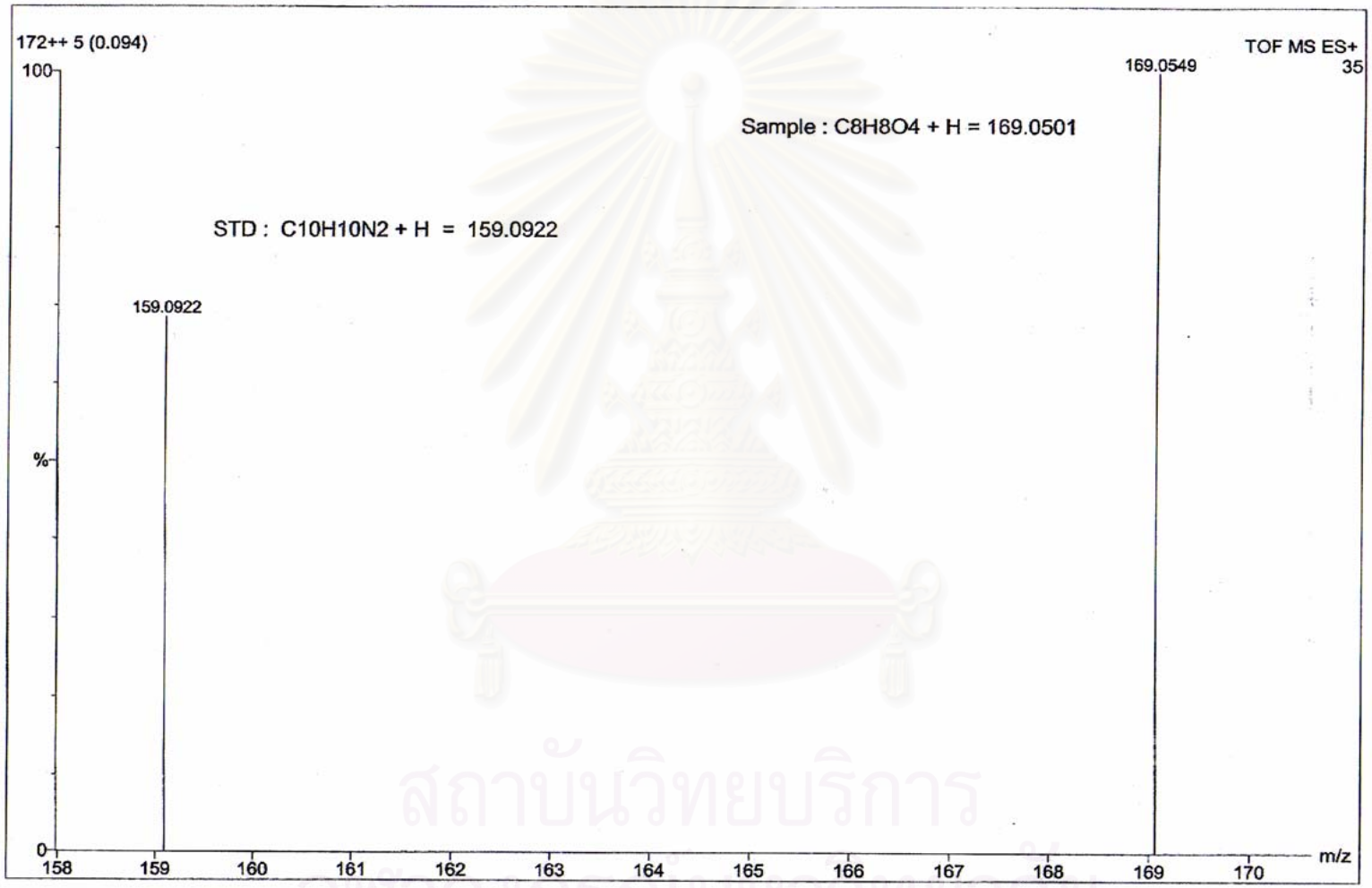


Figure D 6-8 The profile mass spectrum of compound **6**

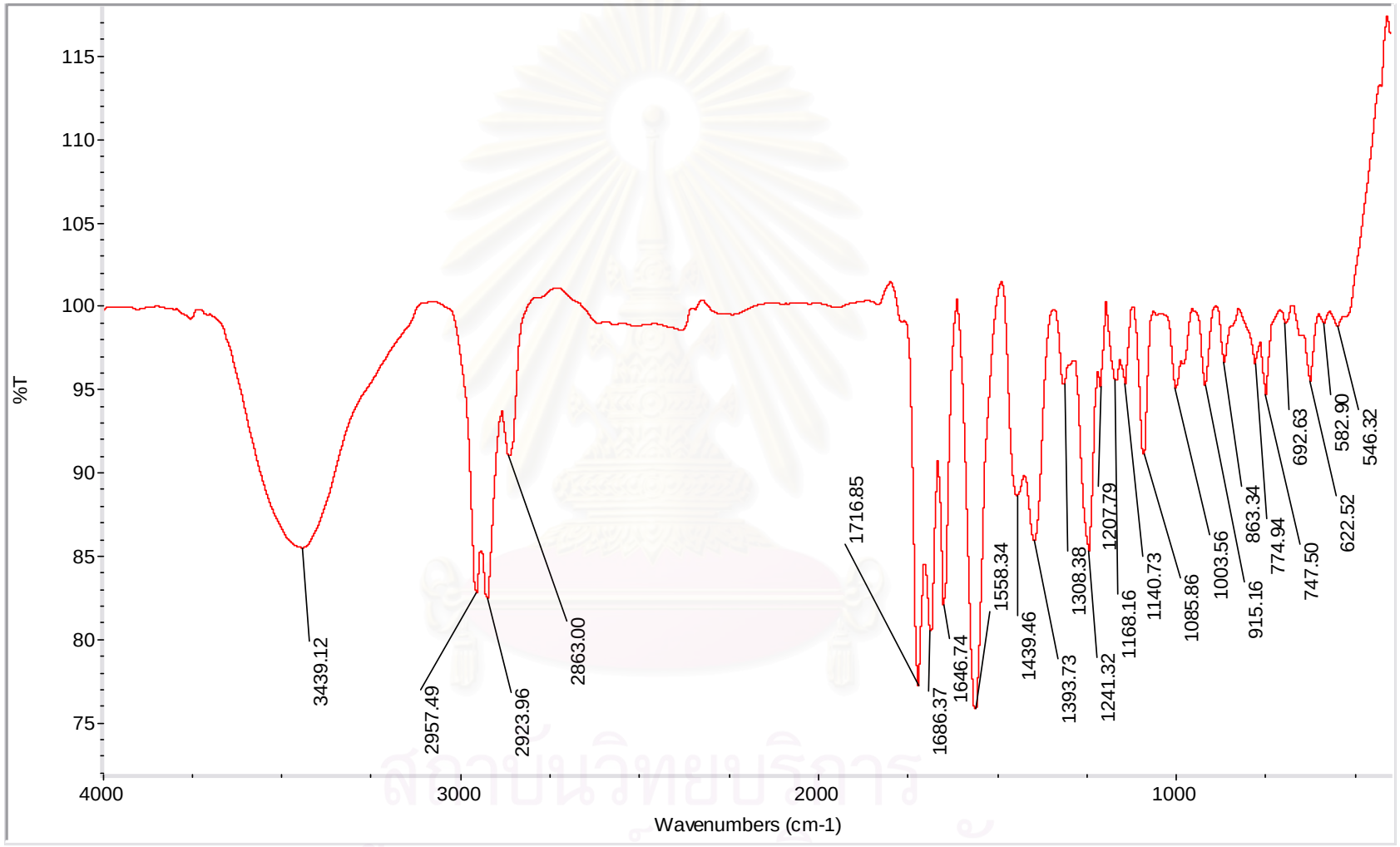


Figure D 7-1 The IR spectrum of compound **7**

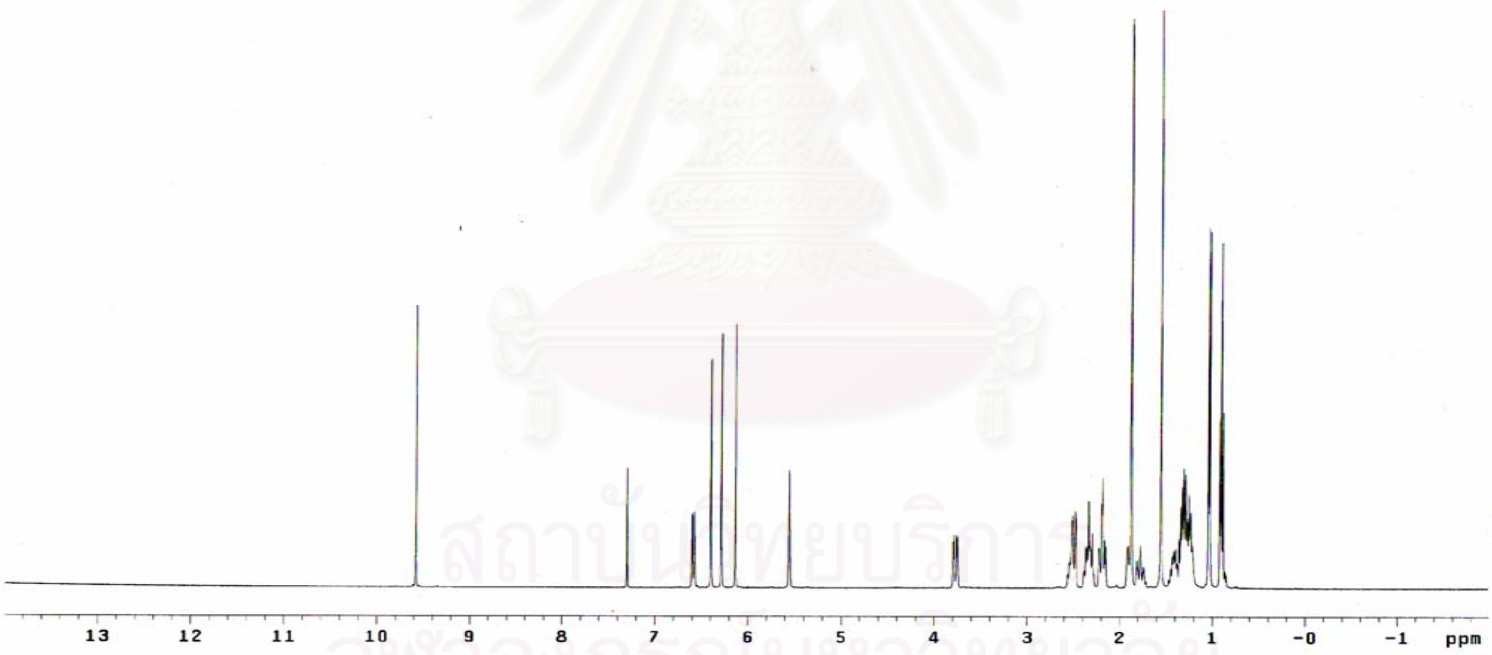


Figure D 7-2 ^{1}H NMR spectrum of compound **7**

145

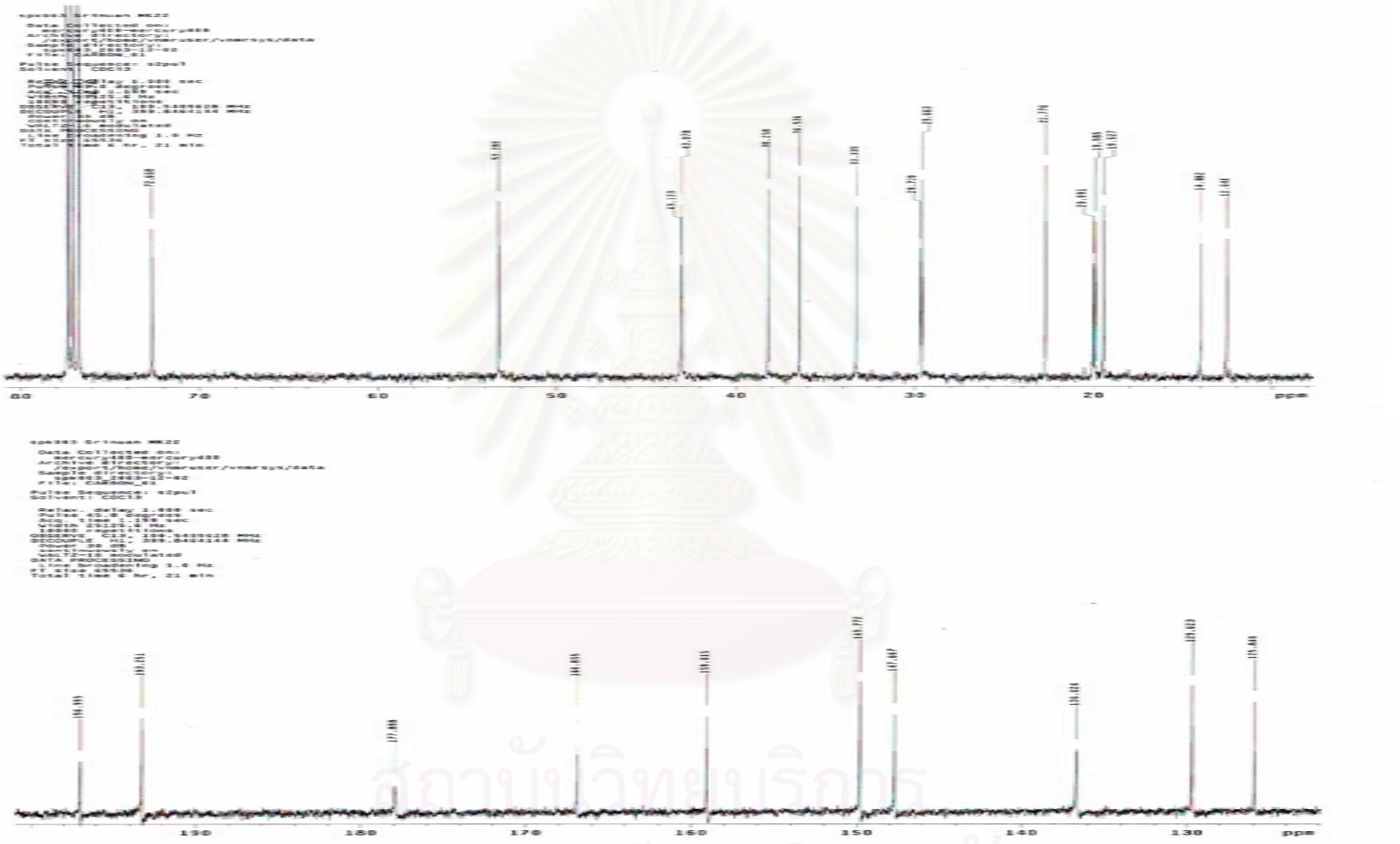


Figure D 7-3 ^{13}C NMR spectrum of compound **7**

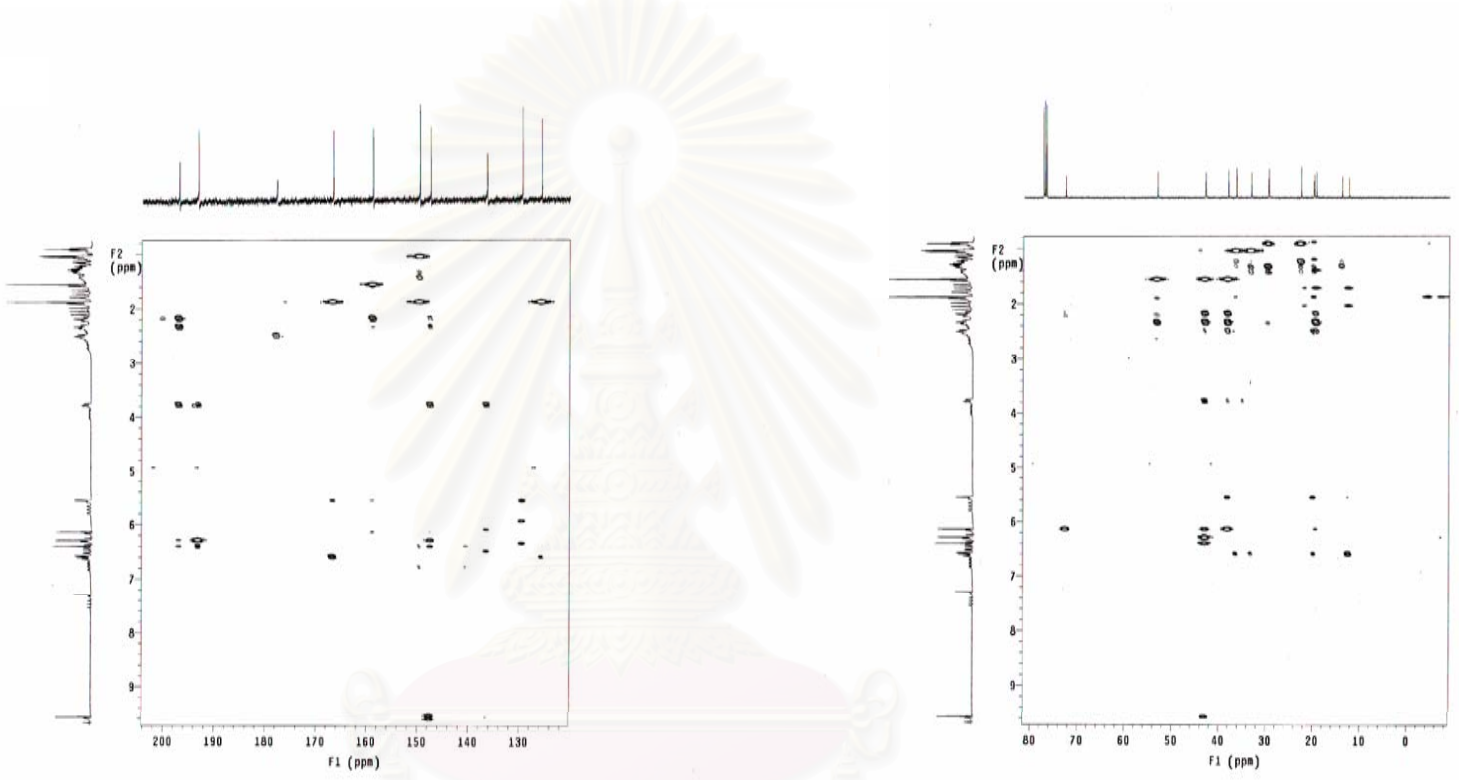


Figure D 7-4 HMBC spectrum of compound **7**

147

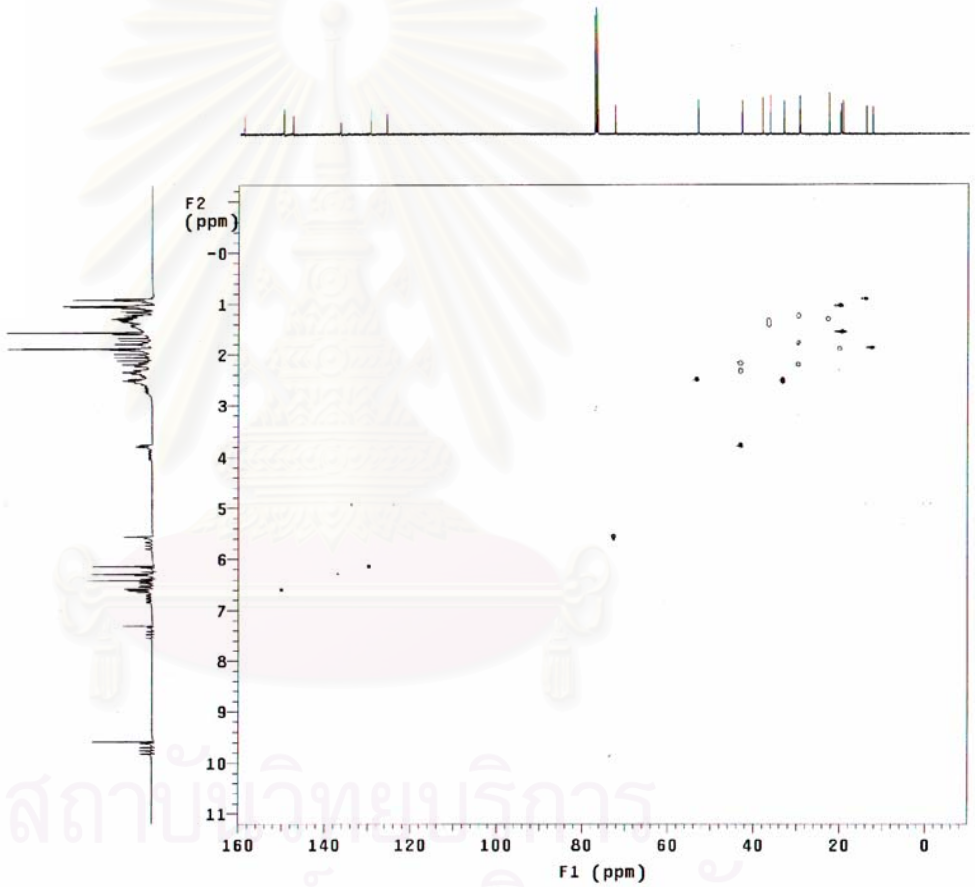


Figure D 7-5 HSQC spectrum of compound **7**

148

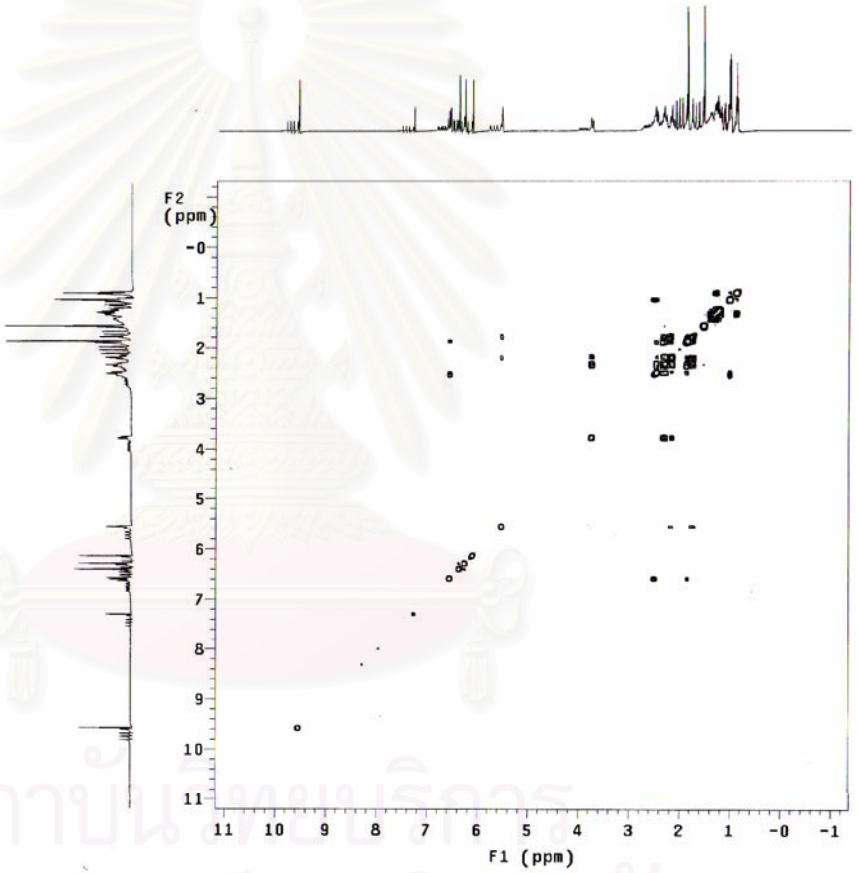


Figure D 7-6 COSY spectrum of compound **7**

149

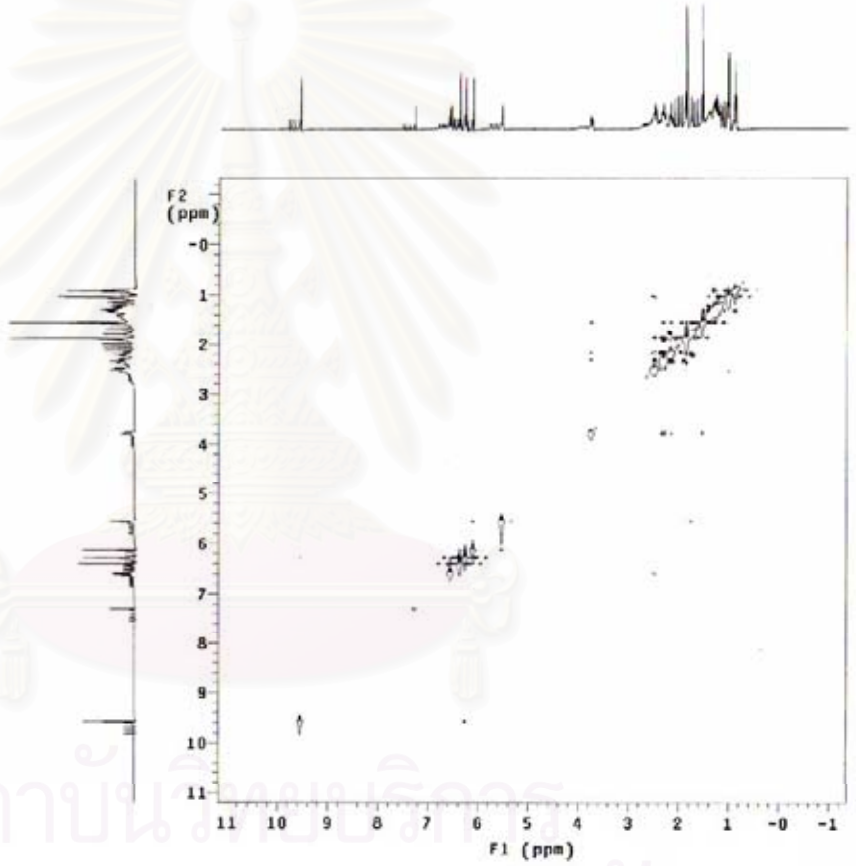


Figure D 7-7 NOESY spectrum of compound **7**

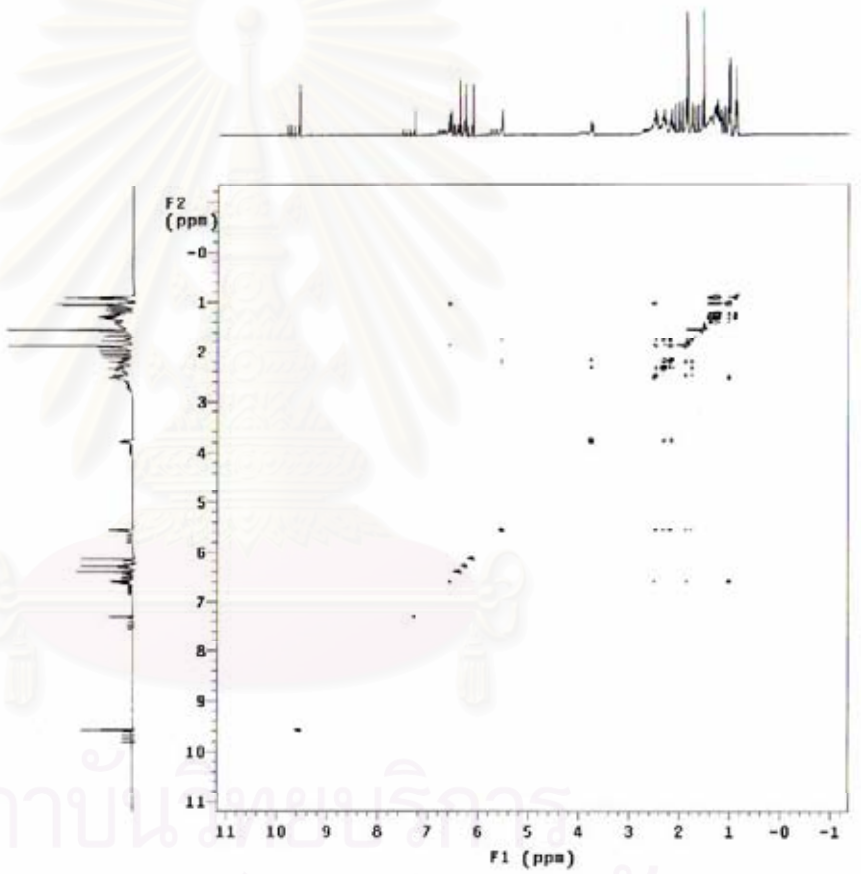


Figure D 7-8 TOCSY spectrum of compound **7**

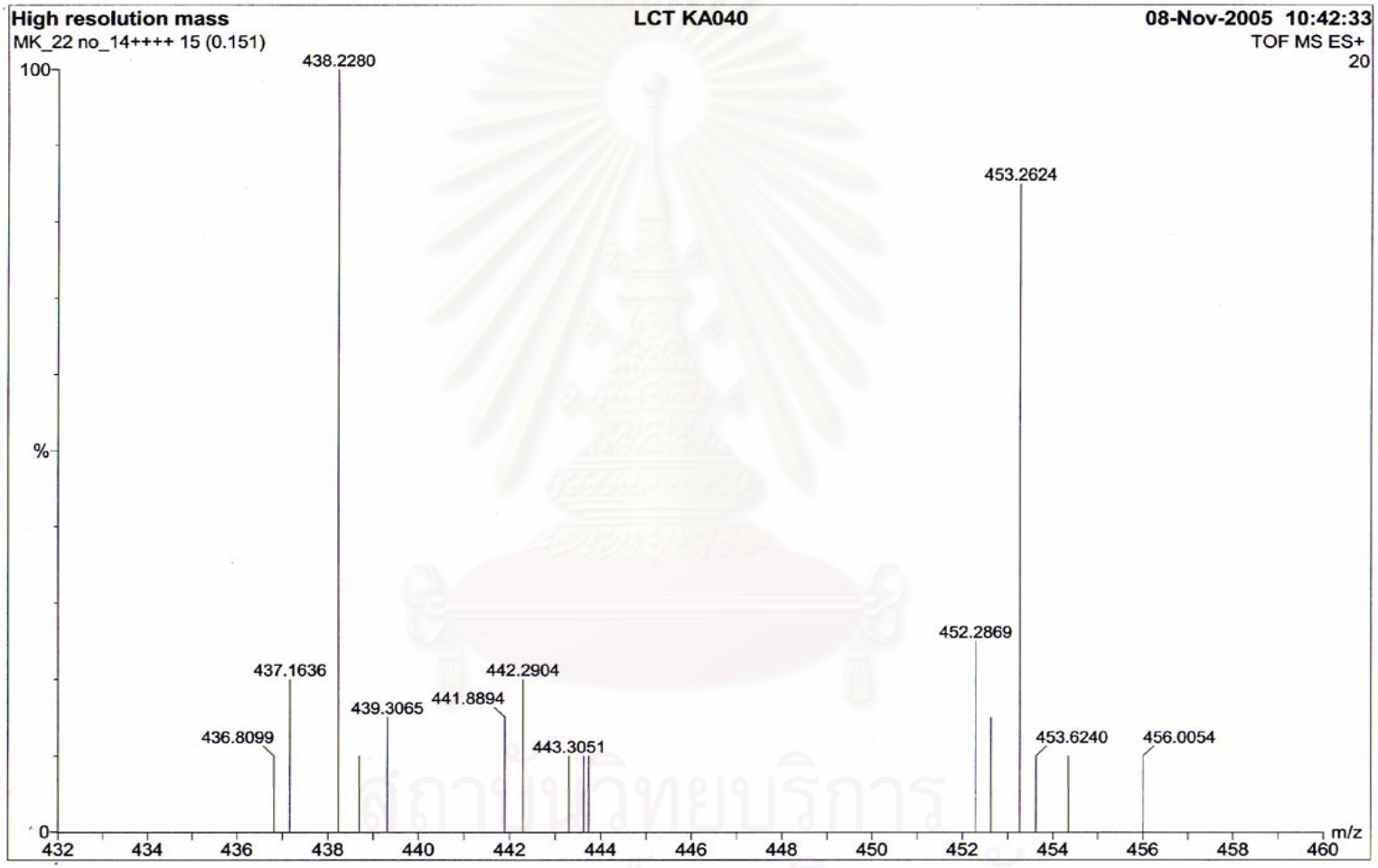
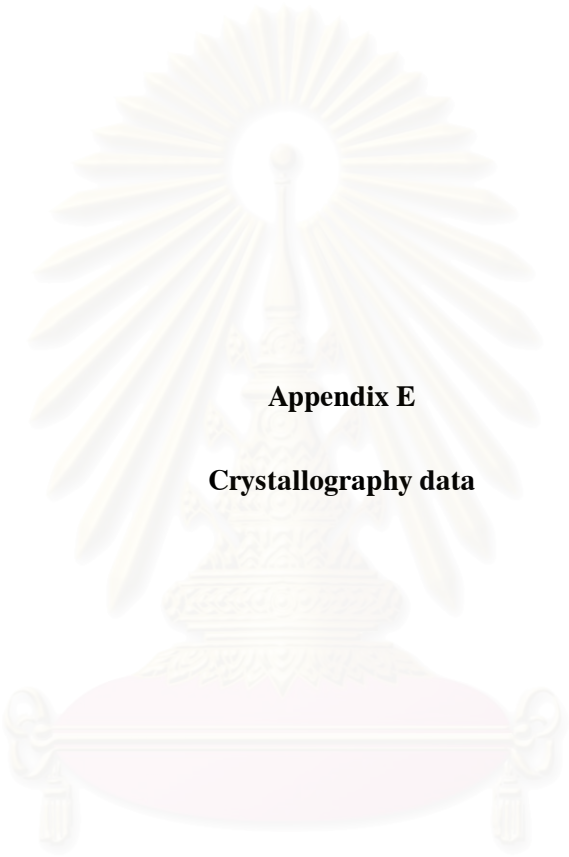


Figure D 7-9 The profile mass spectrum of compound **7**



Appendix E

Crystallography data

Table E1. Crystal data and structure refinement for compound **4** (2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione)

Empirical formula	$C_8H_7ClO_3$
Formula weight	186.59
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system, space group	triclinic, *P(-1)*
Unit cell dimensions	*a* = 7.3112(9) Å alpha = 112.137(2) deg.
	b = 7.3839(9) Å beta = 97.002(2) deg.
	c = 8.5878(10) Å gamma = 103.759(2) deg.
Volume	405.42(8) $Å^3$
Z, Calculated density	2, 1.528 Mg/m^3
Absorption coefficient	0.430 mm^{-1}
F(000)	192
Theta range for data collection	2.63 to 28.32 deg.
Limiting indice	-9<=h<=9, -8<=k<=9, -11<=l<=11
Reflections collected / unique	4793 / 1913 [R_{int} = 0.0212]
Completeness to theta	28.32 95.0 %
Refinement method	full-matrix least-squares on F^2
Data / restraints / parameters	1913 / 0 / 137
Goodness-of-fit on F^2	1.161
Final *R* indices [*I*>2sigma(*I*)]	R_1 = 0.0498, wR_2 = 0.1174
R indices (all data)	R_1 = 0.0634, wR_2 = 0.1244
Largest diff. peak and hole	0.232 and -0.312 e. $Å^{-3}$

Table E2 Bond lengths [A] and angles [deg] for compound **4** (2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione)

C(7) ─C(3)	1.496 (3)
Cl(1) ─C(2)	1.726 (19)
C(1) ─O(1)	1.222 (2)
C(1) ─C(6)	1.446 (3)
C(1) ─C(2)	1.491 (3)
C(2) ─C(3)	1.334 (3)
C(3) ─C(4)	1.487 (3)
C(4) ─O(4)	1.204 (2)
C(4) ─C(5)	1.503 (3)
C(5) ─O(2)	1.322 (2)
C(5) ─C(6)	1.341 (3)
C(8) ─O(2)	1.441 (3)
O(1) ─C(1) ─C(6)	121.20 (2)
O(1) ─C(1) ─C(2)	121.04 (18)
C(6) ─C(1) ─C(2)	117.77 (17)
C(6) ─C(1) ─C(2)	123.49 (17)
C(3) ─C(2) ─Cl(1)	121.29 (16)
C(1) ─C(2) ─Cl(1)	115.22 (14)
C(2) ─C(3) ─C(4)	118.30 (18)
C(2) ─C(3) ─C(7)	124.40 (2)
C(4) ─C(3) ─C(7)	117.30 (19)
O(4) ─C(4) ─C(3)	121.60 (2)
O(4) ─C(4) ─C(5)	120.00 (2)
C(3) ─C(4) ─C(5)	118.34 (17)
O(2) ─C(5) ─C(6)	127.50 (2)
O(2) ─C(5) ─C(4)	111.46 (17)
C(6) ─C(5) ─C(4)	121.01 (19)
C(5) ─C(6) ─C(1)	121.10 (2)
C(5) ─O(2) ─C(8)	116.68 (18)

Symmetry transformations used to generate equivalent atoms:

Table E3. Crystal data and structure refinement for compound **6** (2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione)

Empirical formula	$C_{16}\ H_20\ O_{10}$
Formula weight	372.32
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system, space group	triclinic, *P*(-1)
Unit cell dimensions	*a* = 7.5959(2) Å alpha = 95.0380(10) deg.
	b = 9.2871(3) Å beta = 93.2420(10) deg.
	c = 12.5007(3) Å gamma = 95.787(2) deg.
Volume	872.06(4) $Å^3$
Z, Calculated density	2, 1.418 Mg/m^3
Absorption coefficient	0.120 mm^{-1}
F(000)	392
Theta range for data collection	1.64 to 30.47 deg.
Limiting indice	-10<=h<=10, -8<=k<=13, -17<=l<=17
Reflections collected / unique	6433 / 4689 [R_{int} = 0.0179]
Completeness to theta	30.47 88.3 %
Refinement method	full-matrix least-squares on F^2
Data / restraints / parameters	4689 / 0 / 303
Goodness-of-fit on F^2	1.018
Final *R* indices [*I*>2sigma(*I*)]	R_1 = 0.0536, wR_2 = 0.1396
R indices (all data)	R_1 = 0.0828, wR_2 = 0.1615
Largest diff. peak and hole	0.321 and -0.243 e. $Å^{-3}$

Table E4 Bond lengths [A] and angles [deg] for compound **6** (2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione)

C(001)─O(3)	1.333(2)
C(001)-C(002)	1.350(2)
C(001)-C(007)	1.499(2)
C(002)-C(009)	1.455(3)
C(002)-C(015)	1.503(2)
C(003)-O(9)	1.325(2)
C(003)-C(005)	1.355(2)
C(003)-C(006)	1.505(2)
C(005)-C(010)	1.449(2)
C(005)-C(014)	1.505(2)
C(006)-O(8)	1.219(2)
C(006)-C(19)	1.447(2)
C(007)-O(6)	1.228(2)
C(007)-C(18)	1.444(2)
C(008)-O(1)	1.329(2)
C(008)-C(18)	1.349(2)
C(008)-C(009)	1.518(2)
C(009)-O(11)	1.228(2)
C(010)-O(10)	1.225(2)
C(010)-C(013)	1.515(2)
C(013)-O(2)	1.336(2)
C(013)-C(19)	1.342(2)
C(017)-O(1)	1.447(2)
C(018)-O(2)	1.442(2)
O(3)-C(001)-C(002)	121.45(15)
O(3)-C(001)-C(007)	116.37(14)
C(002)-C(001)-C(007)	122.18(16)
C(001)-C(002)-C(009)	119.07(14)
C(001)-C(002)-C(015)	122.33(18)
C(009)-C(002)-C(015)	118.60(16)

Table E4 (Continued)

O(9)-C(003)-C(005)	120.84(15)
O(9)-C(003)-C(006)	117.25(14)
C(005)-C(003)-C(006)	121.89(15)
C(003)-C(005)-C(010)	119.14(14)
C(003)-C(005)-C(014)	122.34(16)
C(010)-C(005)-C(014)	118.51(15)
O(8)-C(006)-C(19)	123.25(15)
O(8)-C(006)-C(003)	118.24(16)
C(19)-C(006)-C(003)	118.51(14)
O(6)-C(007)-C(18)	123.16(15)
O(6)-C(007)-C(001)	117.74(15)
C(18)-C(007)-C(001)	119.09(14)
O(1)-C(008)-C(18)	126.96(15)
O(1)-C(008)-C(009)	111.43(14)
C(18)-C(008)-C(009)	121.61(15)
O(11)-C(009)-C(002)	122.81(15)
O(11)-C(009)-C(008)	118.70(16)
C(002)-C(009)-C(008)	118.49(14)
O(10)-C(010)-C(005)	122.48(14)
O(10)-C(010)-C(013	118.63(15)
C(005)-C(010)-C(013)	118.89(14)
O(2)-C(013)-C(19)	127.57(15)
O(2)-C(013)-C(010)	111.19 (13)
C(19)-C(013)-C(010)	121.24 (15)
C(19)-C(013)-C(010)	119.49 (15)
C(013)-C(19)-C(006)	120.16 (15)
C(008)-O(1)-C(017)	116.86 (14)
C(013)-O(2)-C(018)	116.81 (14)

Symmetry transformations used to generate equivalent atoms:

BIOGRAPHY

Ms. Srinuan Tansuwan was born on February 6, 1969 in Bangkok, Thailand. She was graduated with a Bachelor Degree (Biotechnology) from the Faculty of Biotechnology, Rangsit University in 1992. In 1997 she was graduated with a Master Degree (Biotechnology) from the Graduate School, Kasetsart University. She has been studying for a Degree of Dortoral Phylosophy of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2002. At present she is an instructor of Biology and Applied Biology Program, Faculty of Science and Technology, Rajabhat Nakornrachasema University.