สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกได้จากสารภี *Mammea siamensis*
บุนนาค *Mesua ferrea* และกระทิง *Calophyllum inophyllum*

นายพลวัต นิวเคลียร์

BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM

Mammea siamensis, Mesua ferrea, AND *Calophyllum inophyllum*

Mr. Paulwatt Nuclear

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

**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 2010**

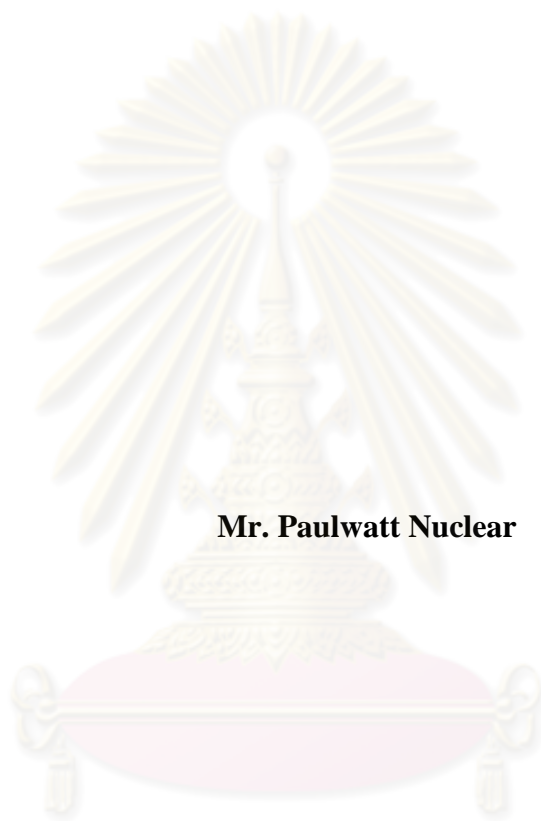Thesis Title   BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI
                             ISOLATED FROM *Mammea siamensis, Mesua ferrea*,
                             AND *Calophyllum inophyllum*
By   Mr. Paulwatt Nuclear
Field of study   Biotechnology
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พลวัต นิวเคลียร์ : สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกได้จากสารภี *Mammea siamensis* บุนนาค *Mesua ferrea* และกระทิง *Calophyllum inophyllum*. (BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Mammea siamensis*, *Mesua ferrea*, AND *Calophyllum inophyllum*). อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. ขนิษฐา พุดหอม, 166 หน้า.

ในงานวิจัยนี้สามารถคัดแยกราเอนโดไฟต์ที่สามารถผลิตสารออกฤทธิ์ทางชีวภาพได้ 4 ชนิดคือ MS1 (*Aspergillus terreus*), CI3 (*Colletotrichum gloeosporioides*), CI4 (*Phoma herbarum*) และ CI5 (*Ascomycete* sp.) จากพืชสกุล *Garcinia* 3 ชนิดคือ สารภี *Mammea siamensis*, บุนนาค *Mesua ferrea* และกระทิง *Calophyllum inophyllum* จากการนำส่วนสกัดเอธิลแอซิเทตของราเอนโดไฟต์ที่คัดเลือกทั้ง 4 ชนิดที่ทำการเพาะเลี้ยงในอาหารที่เหมาะสมมาทำการแยกให้บริสุทธิ์ พบว่ารา *Aspergillus terreus* (MS1) จากสารภีสามารถผลิตอนุพันธ์ butyrolactone 7 ชนิดเมื่อทำการเพาะเลี้ยงในอาหารเหลว corn steep broth ซึ่งเป็นสารชนิดใหม่ 2 ชนิดคือ aspernolide D (4) และ asperterone (5) และสารที่มีการรายงานมาก่อนอีก 5 ชนิดได้แก่ butyrolone I (1), II (2), aspernolide B (3), butyrolactone III (6) และ butyrolactone IV (7) นอกจากนี้ยังพบว่าราชนิดนี้ยังสามารถผลิตสารอีกกลุ่มหนึ่งคือ terrein (8) และให้ผลผลิตที่สูงมากเมื่อนำมาเพาะเลี้ยงในอาหารเหลว malt extract broth ซึ่งชี้ให้เห็นว่าอาหารที่ใช้ในการเพาะเลี้ยงมีผลต่อโครงสร้างของสารที่ราชนิดนี้ผลิตได้ ในกรณีของราเอนโดไฟต์ *C. gloeosporioides* (CI3), *P. herbarum* (CI4) และ *Ascomycete* sp. (CI5) พบว่าเมื่อนำมาทำการเพาะเลี้ยงบนอาหารแข็ง rice medium และนำส่วนสกัดเอธิลอะซีเตตมาทำการแยกให้ได้สารบริสุทธิ์ พบว่าสามารถแยกสารที่มีการรายงานมาก่อนได้ 15 ชนิด (9-23) การพิสูจน์ทราบโครงสร้างทางเคมีของสารที่แยกได้ทำได้โดยอาศัยวิธีการทางสเปกโทรสโกปี

เมื่อนำสารที่แยกได้จากราเอนโดไฟต์ *A. terreus* (MS1) มาทดสอบฤทธิ์ยับยั้งเซลล์มะเร็ง ฤทธิ์ยับยั้งเชื้อแบคทีเรีย และฤทธิ์ยับยั้งมาลาเรีย พบว่า เฉพาะสาร 5 และ 8 แสดงฤทธิ์ยับยั้งเซลล์มะเร็งโดยมีค่า IC_{50} อยู่ระหว่าง 2.70-5.63 μM ขณะที่สาร 1, 3-5 และ 6 มีฤทธิ์ยับยั้งเชื้อแบคทีเรียอย่างอ่อนถึงปานกลาง และพบว่ามีเฉพาะสาร 8 เท่านั้นที่แสดงฤทธิ์ยับยั้งมาลาเรียโดยมีค่า IC_{50} = 3.17 μM

สาขาวิชา .....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....พลวัต นิวเคลียร์.....

ปีการศึกษา .....2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

5073851123 : MAJOR BIOTECHNOLOGY

KEYWORDS : Fungal metabolites/ Cytotoxicity / Phosphodiesterases / Anti-bacteria

PAULWATT NUCLEAR: BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Mammea siamensis, Mesua ferrea*, AND *Calophyllum inophyllum* ADVISOR : ASST. PROF. KHANITHA PUDHOM, Ph.D., 166 pp.

In the present study, four endophytic fungal strains, *Aspergillus terreus* (MS1), *Colletotrichum gloeosporioides* (CI3), *Phoma herbarum* (CI4) and *Ascomycete* sp. (CI5) obtained from *M. siamensis, M. ferrea* and *C. inophyllum,* were selected for bioactive metabolite isolation. Investigation on chemical constituents of each fungus cultured on suitable medium led to the isolation of two novel butyrolactone derivatives, aspernolide D (**4**) and asperterone (**5**) from EtOAc extract of the fungus *A. terreus*, together with five known derivatives, butyrolactones I (**1**), II (**2**), aspernolide B (**3**), butyrolactones III (**6**) and IV (**7**). Furthermore, terrein (**8**) was found to be a major metabolite (relatively high yield) when cultured in malt extract broth. This indicated that cultured medium has much effect on chemical structure of metabolites produced. In the case of fungal strains CI3, CI4 and CI5, after ferment on solid rice medium, purification of their EtOAc extracts by chromatographic techniques led to the isolation of fiveteen known compounds (**9-23**). The structures of all isolated metabolites were established on the basis of spectroscopic data.

Further, compounds obtained from the fungus *A. terreus* were evaluated for their cytotoxicity against cancer cell lines, antimalarial and antibacterial activities. Only compounds **5** and **8** exhibited cytotoxicity on cancer cell lines tested with IC_{50} ranging of 2.70-5.63 μM, whereas compounds **1**, **3-5** and **6** displayed weakly to moderately antibacterial activity. In addition, it was found that compound **8** could inhibit potent antimalarial activity with IC_{50} value of 3.17 μM.

Field of Study:......Biotechnology.......... Student's Signature....Paulwatt Nuclear......

Academic Year:....2553.................... Advisor's Signature....Khanitha Pudhom....

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

acetone-d_6	Deuterated acetone
$^{\circ}$C	Degree Celsius
^{13}C NMR	Carbon-13 nuclear magnetic resonance
CDCl$_3$	Deuterated chloroform
CHCl$_3$	Chloroform
CH$_2$Cl$_2$	Methylene chloride
δ	Chemical shift
d	Doublet (for NMR spectral data)
dd	Doublet of doublets (for NMR spectral data)
DNA	Deoxyribonucleic acid
DEPT	Distortionless enhancement by polarization transfer
ε	Molar absorptivity
$et\ al$	And other
EtOAc	Ethyl acetate
ESI-TOF MS	Electrospray Ionization Time of Flight Mass
g	Gram
μg	Microgram
h	Hour
^{1}H-^{1}H COSY	Homonuclear (proton-proton) correlation spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance
HMBC	^{1}H-detected heteronuclear multiple bond correlation
HMQC	^{1}H-detected heteronuclear multiple quantum coherence
Hz	Hertz
IC$_{50}$	Inhibitory concentration required for 50% inhibition of growth
IR	Infrared
ITS	Internally transcribed spacers
J	Coupling constant
KBr	Potassium bromide
L	Liter (s)

λ_{max}	Maximum wavelength
μg	Microgram (s)
μL	Microliter (s)
μM	Micromolar
M	Molar
m	Multiplet (for NMR spectral data)
MEB	Malt Extract Broth
m.p.	Melting point
MeOH	Methanol
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
MHz	Megahertz
MW	Molecular weight
MS	Mass spectroscopy
m/z	Mass to charge ratio
nm	Nanometer
No.	Number
NMR	Nuclear magnetic resonance
NTP	Nucleotide triphosphate
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
$[M+H]^+$	Protonated molecule
$[M+Na]^+$	Pseudomolecular ion
ppm	Part per million
q	Quartet (for NMR spectral data)
rDNA	Ribosomal deoxyribonucleic acid
rpm	Round per minute

rRNA	Ribosomal ribonucleic acid
s	Singlet (for NMR spectral data)
sp.	Species
t	Triplet (for NMR spectral data)
TAE	*Tris*-HCl, acetate and EDTA
TE	*Tris*-HCl and EDTA
TLC	Thin layer chromatography
U	Unit
UV	Ultraviolet
V	Volt
v	Volume
v_{max}	Wave number at maximum absorption

CHAPTER I

INTRODUCTION

1.1 Needs for new drug discovery

During the past two decades the emergence and spread of antibiotic resistance, the accumulation of new pathogens such as avian influenza (bird flu), AIDs and severe acute respiratory syndrome (SARs), and the resurgence of the old diseases thought to have been controlled such as tuberculosis pose important public health problems for both the developed and developing nations. This emergence is the result of the changes in society, technology, the environment, and the microbes themselves. Important factors influencing emergence include changes in human behavior, change in technology and industry, changes in economic development and land use, increasing and rapid international travel and commerce, microbial adaptation and the breakdown of public health measures.

Malaria, the terrible health problem in third-world and some developing countries, is an instance for the spread of the disease caused by the parasite adaptation. One of the major obstacles in the fight against malaria is *Plasmodium* resistance to antimalarial drugs quickly. Resistance to Artemisinin, the best antimalarial drug, has recently been observed in clinical trials at the Cambodia-Thailand border. (WHO, Global Reports on Antimalarial Drug Efficacy and Drug Resistance: 2000-2010)

Not only do the infectious diseases continue to cause a large number of deaths, but cancer, a class of diseases which a group of cells displays uncontrolled growth through division, invasion and metastasis, have also threaten to increase in the near future relative to populations affected. The cancer can affect just about every organ in human body, more than 100 different types have thus been found. Additionally, it can affect people at all ages with the risk for most type increasing with age. As of 2007, it caused about 13% of all human deaths (7.6 million). Cancers are primarily an environmental disease with 90-95% of cases due to lifestyle and environmental factors and 5-10% due to genetics. Common environmental factors leading to cancer

death include tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation, stress, lack of physical activity, and environmental pollutants. These environmental factors cause abnormalities in the genetic material of cells. The cancer occurrence in both male and female are shown in Figure 1.1. (http://en.wikipedia.org/wiki/cancer)
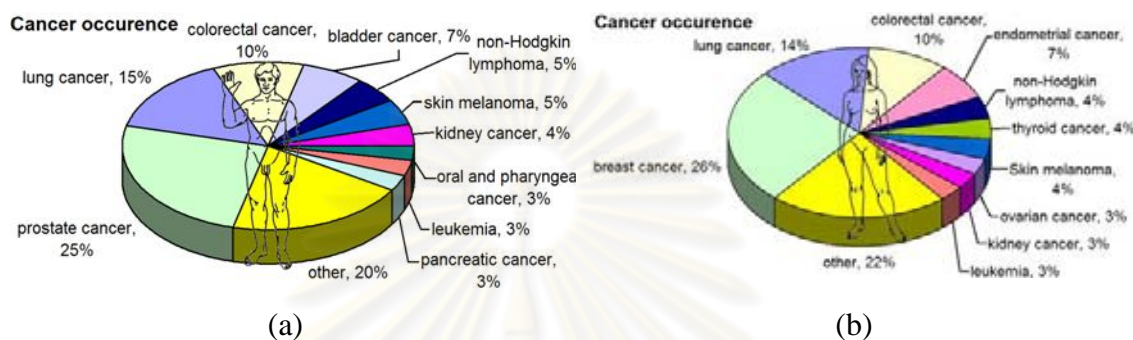
Figure 1.1 (a) cancer occurrence in male. (b) cancer occurrence in female.

As mentioned above, emergent and resurgent diseases in the changing world require the discovery and development of new drugs to combat them including new therapies needed for treating ancillary infections which are a consequence of a weakened immune system such as AIDS.

1.2 Role of natural products on drug discovery

Natural products are chemical compounds or substances produced by living organisms such as microorganisms, plants and animals. These products usually have some form of pharmacological or biological activity, and have been exploited for the treatment of human diseases for thousands of years. Additionally, these small molecules from nature still play a major role in drug treatment and drug discovery either as the drug or as a "precursor" in the synthesis or design of the agents, which have had an immense impact on modern medicines. Inspection of Figure 1.2 reveals that, overall, of the 1184 new chemical entities covering all diseases/countries/sources in the time-frame of 1981-2006, only 30% were synthetic in origin, while over 60% were naturally-derived (B, N and ND) or were modeled on a natural product parent (S* and NM), thus demonstrating the influence of natural product-derived compounds

on drug discovery and approval [1]. It should be noted, however, the numbers of approved drugs do not correlate with the "value" as measured by sales, since the best selling drug of all is atorvastatin, a hypocholesterolemic descended directly from a natural product, which sold over \$11 billion in 2004 and is at or above this level even today.
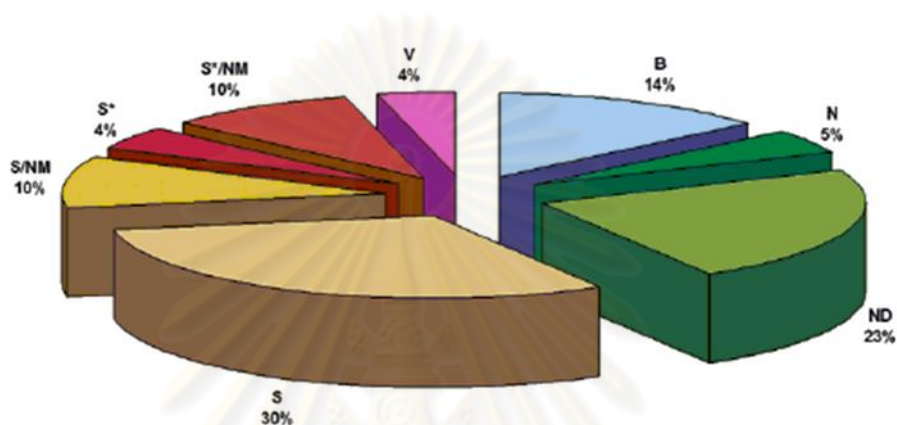
"B" biological, usually peptide or protein

"N" natural product

"ND" derived from natural products and semisynthetic modification

"NM" natural product mimic

"S" totally synthetic drug

"S*" made by synthesis, but pharmacophore is from a natural product

Figure 1.2 All new chemical entities, 01/1981-06/2006, by source ($N = 1184$)

Over the years, although the emerging approaches such as high-throughput screening, genetic engineering, combinatorial chemistry and biology, and computer-assisted de novo drug design continue to play an important role in the drug development process, a renewed interest in obtaining biologically active principles from natural sources has been observed. This might be because formulations based on natural products are their low or absent toxicity, their complete biodegradability, their availability from renewable sources, and, in most cases, their low cost when compared with those compounds obtained by total chemical synthesis. More

importantly, most biologically active natural product compounds are secondary metabolites with very complex structures. This has an advantage in that they are extremely novel compounds but this complexity also make the synthesis of lead compounds quite difficult, and many of today's medicines are obtained directly from a natural source. Therefore, the continuing and overwhelming contribution of natural products to the expansion of the chemotherapeutic armamentarium is clearly evident, and we can state that much of Nature's "treasure trove of small molecules' remained to be explored, particularly from the marine and microbial world.

1.3 Endophytic fungi, a source of biologically active secondary metabolites

Among all known producers of natural product molecules, microorganisms represent a rich source of biologically active metabolites that find a broad spectrum of utility in medicine (e.g. anticancer, antiparasitic and immunosuppressant functions), agriculture and industry. It has been estimated that less than 1% of bacterial species and less than 5% of fungal species are currently known, indicating that millions of microbial species remain to be discovered [2]. Another significant development in the field of microbe-derived natural products is the demonstration of the potential to bring up a variety of new metabolites from a single strain of a microorganism by systematic alteration f its cultivation parameters known as OSMAC (one strain many compounds) approach [3-5]. Of all microorganisms studied, Actinomycetes and fungi have been found to be the most prolific producers and continue to serve as excellent sources of secondary metabolites. Additionally, one kind of fungal species, known as "endophytic fungi", has received much attention over the last ten years as outstanding sources of metabolites with diverse structures and biological functions.

The term endophytic fungus refers to a fungal microorganism that spends a part or all of its whole life cycle inside living tissues of a host plant and establishes a relationship with its host that may range from symbiotic to slightly pathogenic (Figure 1.3) Moreover, it is thought to interact mutualistically with its host plant, so that the host plant provides nutrients to the endophyte, which in turn produces bioactive substances to enhance growth and competitiveness of the host in nature [6-7]. Consequently, endophytes are currently viewed as a wellspring of a wide array of new pharmacologically active metabolites that might prove suitable for specific medicinal

or agrochemical applications [8]. Current interest in natural products from endophytic fungi is also evident from the number of review articles that have appeared in the recent literature such as by Tan and Zou [6-7 and 9].
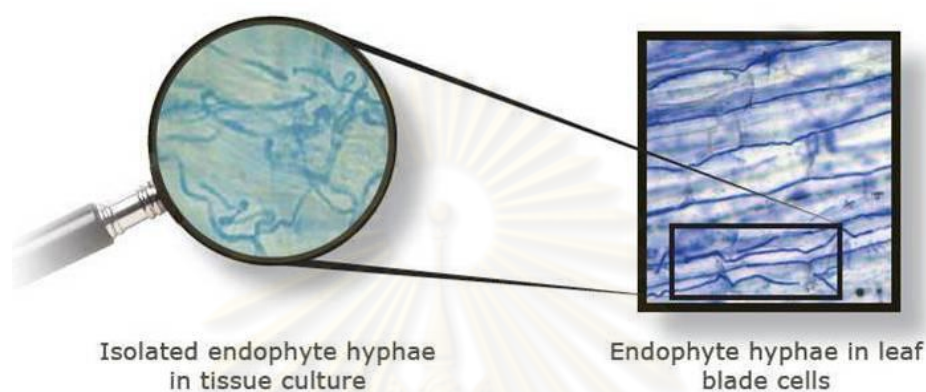
Figure 1.3 Endophyte hyphae in plant cells

Interestingly, very often such fungi may either mimic or produce some of the same compounds as the host plant that supports them. It has been believed that the reason might be related to a genetic recombination of such microbes with the host that occurred in evolutionary time [7]. One of the most important discoveries is that of the taxol-producing fungi [17-21]. Paclitaxel (**1**, commercial name Taxol, Figure 1.4) is the effective compound using in chemo-theraputic treatment which was first extracted in a very low yield from the Pacific Yew trees (*Taxus brevifolia*) [12]. It is an important natural product that has made an enormous impact on medicine. It interacts with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules ad thereby interrupts the cell division [10]. Its original target diseases were ovarian and breast cancers, but now it is utilized to treat a number of human tissue-proliferating diseases as well [11].

Plant cell culture, total synthesis and semi-synthesis could provide alternative sources of taxol, but practical and economical way to make in the large-scale production could not be accomplished [13-16]. Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also help to

preserve the world's diminishing biodiversity. Other advantages of using endophytic fungi as the chemical sources are not only reduce the burden on the environment as fungi can grow and cultivate in laboratories, but also decrease the chemical consumption and the cost of production, thereby reducing its market price.
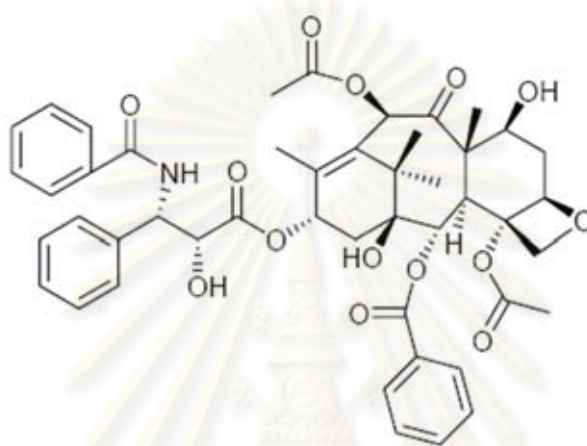
Figure 1.4 Paclitaxel (**1**, Taxol)

Furthermore, very recently a report appeared that another cytotoxic plant quinoline alkaloid, camptothecin (**2**, CPT), which is commercially exploited as an antineoplastic agent, was also produced by an edophytic fungus *Fusarium solani* INFU/Ca/KF/3, isolated from *Camptotheca acuminata*, a plant native to mainland China (Figure 1.5) [22]. This compound was first isolated by Wall et al. from the wood of *C. acuminate* [23]. Its potency as an antitumor agent is by virtue of a unique mechanism of action involving interferace with eukaryotic DNA [24-27]. CPT primarily targets the intranuclear enzyme DNA topoisomerase I, which is required for the swivelling and relaxation of DNA during molecular events, namely DNA replication and transcription [28].

More importantly, *F. solani* can actually be obtained from various sources but they do not produce CPT, it can be presumed that the inclusion of gene(s) responsible for the production of this metabolite into the fungal genome has been obtained from the plant host by means of horizontal gene transfer. It must thus be the difference in the genetic makeup of this INFU/Ca/KF/3 edophytic fungal strains and *F. solani*

isolated from other sources. Therefore the potential of using endophytes as effective alternative or novel source for therapeutic compounds has been recognized.
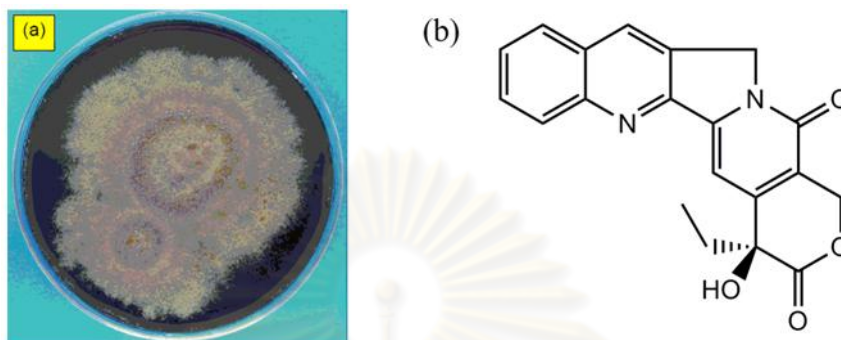
Figure 1.5 (a) *Fusarium solani* INFU/Ca/KF/3 strains
(b) camptothecin (**2**, CPT)

During the last ten years, several researchers have also reported a large number of structurally novel secondary metabolites with biological activity produced by endophytic fungi isolated from numerous species of terrestrial plants around the world. Presented in Table 1.1. are endophytic fungal strains, host plants from which these microbes have been isolated, their metabolites together with their reported biological activities. Culture conditions are included in the table, as it is known that the nature and yield of microbial metabolites are strongly influenced by cultivation parameters [3-5]. The structures of isolated metabolites are shown in Figure 1.6. Based on these data the potential of using endophytes as an effective alternative or novel source for therapeutic compouds has been recognized.

Table 1.1 Natural products from endophytic fungal strains

fungal strain	plant host	culture conditions	secondary metabolite(s)	biological activity	ref
Aspergillus fumigatus CY018	*Cynodon dactylon*	PDB; 25 ^{o}C; 5 days	9-deacetoxy-fumigaclavine (**3**)	cytotoxic	[29]
Aspergillus parasiticus RDWD1-2	*Sequoia sempervirens*	DIFCO mycologic al broth; 19 days	sequoiatone C (**4**) sequoiatone D (**5**) sequoiatone E (**6**)	toxic to brine shrimp	[30]
Chaetomiu-m chiversii CS-36-62	*Ephedra fasciculata*	PDB; 27 ^{o}C; 14 days	radicicol (**7**)	cytotoxic; Hsp90 inhibitor	[31]
Edenia sp.	*Petrea volubilis*	MEB; 15 days	preussomerin EG1 (**8**) palmarumycin CP$_{17}$ (**9**) palmarumycin CP$_{18}$ (**10**)	antileish-manial	[32]
Microspha-eropsis olivacea	*Pigerodendron uviferum*	rice medium; 25 ^{o}C; 30 days	graphislacone A (**11**) botrallin (**12**)	AchE inhibitor	[33]
Microspha-eropsis sp. NRRL 15684	*Buxus sempervirens*	SL medium; 24 ^{o}C; 13 days	lactone S 39163/F-I (**13**)	antiviral; antibiotic	[34]
Nodulispor -ium sp. MF5954	*Bontia daphnoides*	nutrient medium; 25 ^{o}C; 28 days	nodulisporic acid A$_1$ (**14**) nodulisporic acid A$_2$ (**15**)	insecticidal	[35]

Table 1.1 Natural Products from Endophytic Fungal Strains (continue)

fungal strain	plant host	culture conditions	secondary metabolite(s)	biological activity	ref
Paecilomyces sp. H-036 and W-001	*Taxus mairei*	PDA; 25 ^{o}C; 7 days	brefeldin A (**16**)	antifungal; antiviral; anticancer	[36]
Penicillium janczewskii	*Prumnopity andina*	PDB; 25 ^{o}C; 23 days	peniprequinolone (**17**) gliovictin (**18**)	nematicidal	[37]
Penicillium sp.	*Limonium tubiflorum*	rice medium; rt; 30 days	11α-methoxycurvularin (**19**) 11β-methoxycurvularin (**20**)	antitrypano-somal	[38]
Periconia sp. OBW-15	*Taxus cuspidata*	S-7 liquid medium; 25 ^{o}C; 2 days	periconicin A (**21**) periconicin B (**22**)	antimycotic	[39]
Phomopsis sp.	*Azadirachta indica*	PDB; 28 ^{o}C; 6 days	lactone (**23**)	antifungal	[40]
Preussia sp.	*Angelica sinensis*	wheat bran liquid medium; 24 ^{o}C; 8 days	spiropreussione A (**24**)	cytotoxic	[41]
Xylaria sp.	*Piper aduncun*	PDA; 25 ^{o}C; 10 days	9,15-dihydroxy presilphiperfolan-4-oic acid (**25**)	antifungal; cytotoxic	[42]
Xylaria sp.	unidentified plant	PDB; 28 ^{o}C; 8 days	xylopimarane (**26**)	cytotoxic	[43]

lactone S39163/F-1 (**13**)

nodulisporic acid A$_1$ (**14**) : R =

nodulisporic acid A$_2$ (**15**) : R =

brefeldin A (**16**)

peniprequinolone (**17**)

gliovictin (**18**)

11α -methoxycurvularin (**19**)
11β -methoxycurvularin (**20**)

palmarumycin CP$_{18}$ (**10**)   graphislacone A (**11**)   botrallin (**12**)
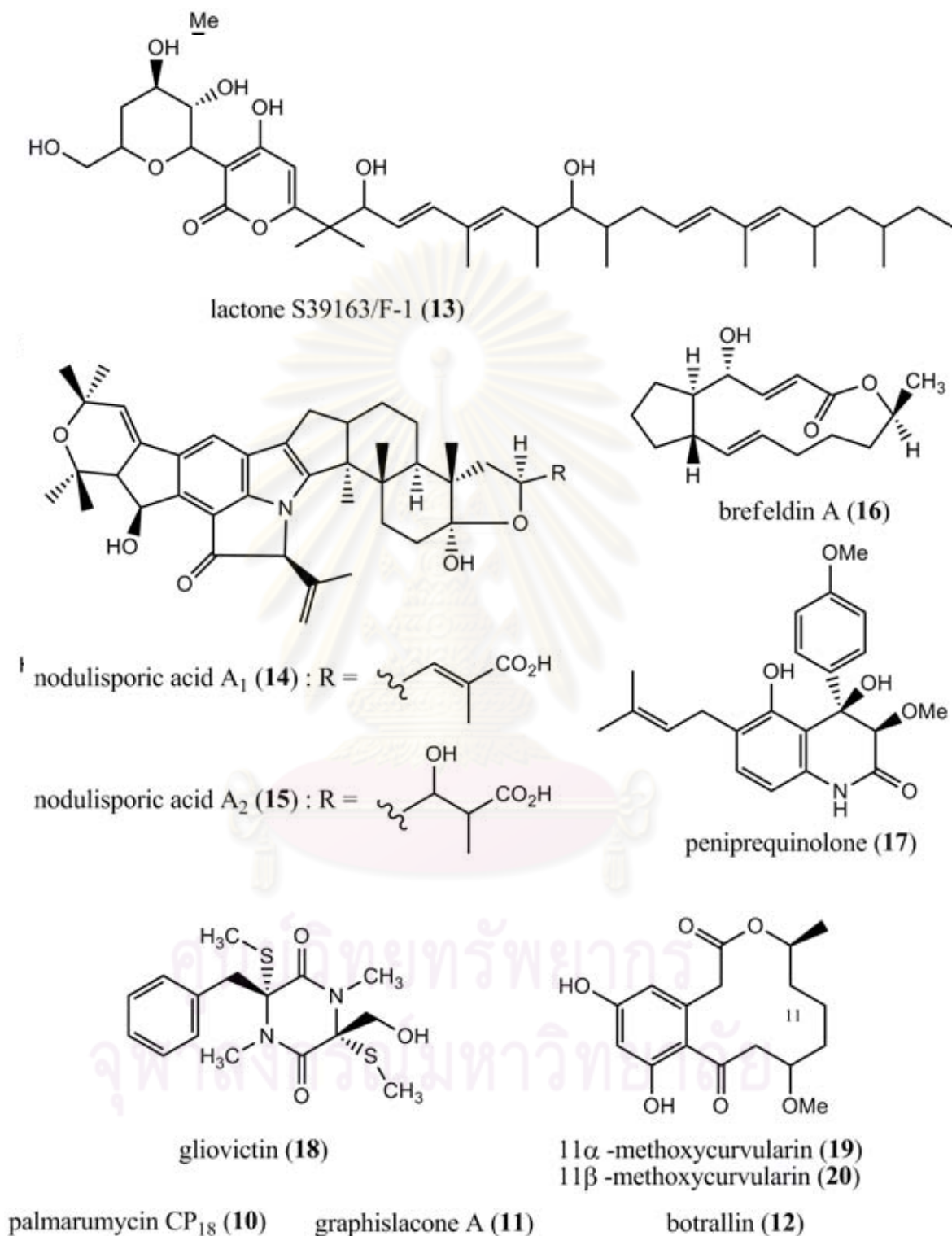
Figure 1.6 Structures of isoated metabolites from endophytic fungi

periconicin A (**21**) : R = CH₃
periconicin B (**22**) : R = CH₂OH

lactone (**23**)

spiropreussione A (**24**)

9,15-dihydroxy presilphiperfolan-
4-oic acid (**25**)

xylopimarane (**26**)
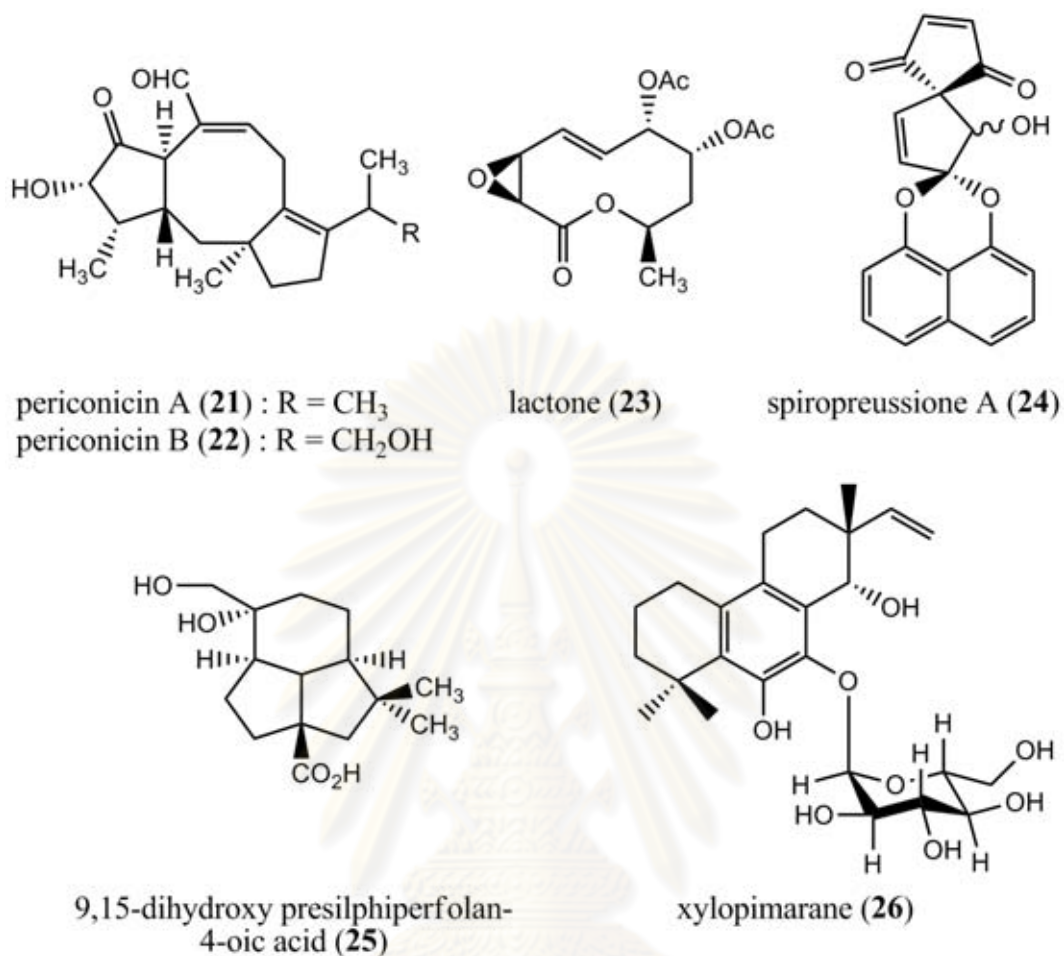
Figure 1.6 Structures of isolated metabolites from endophytic fungi (continue)

1.4 Thai medicinal plants, a potential source of bioactive compound-producing endophytes

As reviewed by Strobel et al., it is important to understand the methods and rationale used to provide the best opportunities to obtain an endophytic fungus that can produce the interesting compounds. One of the most important things is how to select the plant for endophyte isolation and natural product discovery, since the number of plant species in the world is so great. A specific rationale for the collection of each plant needs to be used, and one of them is plants growing in areas of great biodiversity, it follows, also have the prospect of housing endophytes with great biodiversity [11]. Thailand is also located in a tropical area, furnishing great biodiversity ranging from higher organisms (plants and animals) to microorganisms

incluing fungi. Thai medicinal plants must be a potential source of fungal endophyte. This can be evident from the number of published articles during the last five years. Examples of Thai medicinal plant-derived fungi leading the compounds with bioactivity are as follows.

    Phomopsis sp. BCC. 9789 is an endophytic fungal species from a wild banana (*Musa acuminata*) leaf collected at Doi Suthep Pui, Chiang Mai Province. It produces various oblongolides (**27-29**) having anti-HSV and cytotoxic activity (Figure 1.7)[44].
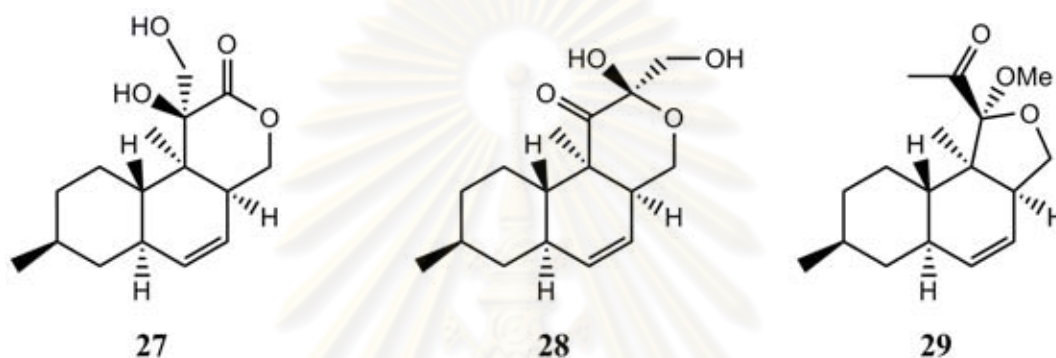
Figure 1.7 Oblongolides from *Phomopsis* sp. BCC. 9789

    Dothideopyrone derivatives (**30-31**) have been isolated from the extract of Dothideomycete sp. LRUB20, an endophytic fungus isolated from Leea rubra, a Thai medicinal plant collected from Pitsanulok province (Figure 1.8). They displayed moderate cytotoxicty on human cancer cell lines. Interestingly, the LRUB20 fungus also produced muconic acid (**32**), a biomarker in environments after expose to benzene and phenol (or derivatives), in relatively high yield (47.8 mg/L) [45].
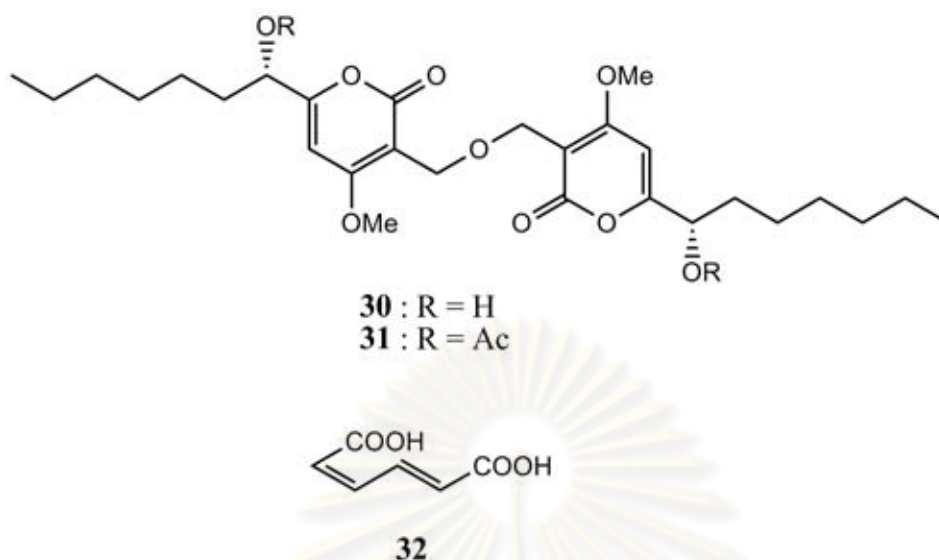
Figure 1.8 Dothideopyrones (**30**-**31**) and muconic acid (**32**)

Corynesidones A (**33**) and B (**34**), and corynether (**35**) were isolated from the culture fluid of *Corynespora cassiicola* L36, a fungus from the leaf of *Lindenbergia philippensis* collected from Kanchanaburi province (Figure 1.9). All compounds showed potent anti-oxidant activity, and corynesidone A also inhibited aromatase activity [46].

33 : R$_1$ = H; R$_2$ = H
34 : R$_1$ = OH; R$_2$ = COOH
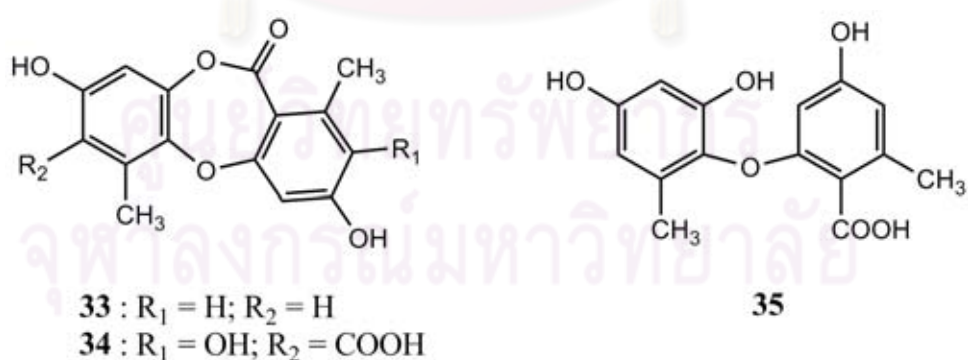
Figure 1.9 Corynesidones A (**33**) and B (**34**), and corynether (**35**)

Filamentous fungus *Diaporthe* sp., an endophytic fungus isolated from *Croton sublyratus* leaf (collected from Bangkok), was found to produce a highly functionalized compound, diaporthichalasin (**36**), and pycnidione (**37**) (Figure 1.10).

Diaporthichalasin exhibited significantly potent inhibition of CYP3A4, the most abundant enzyme in human liver microsome, metabolizing over 50% of drug biotransformed by this enzyme [47].
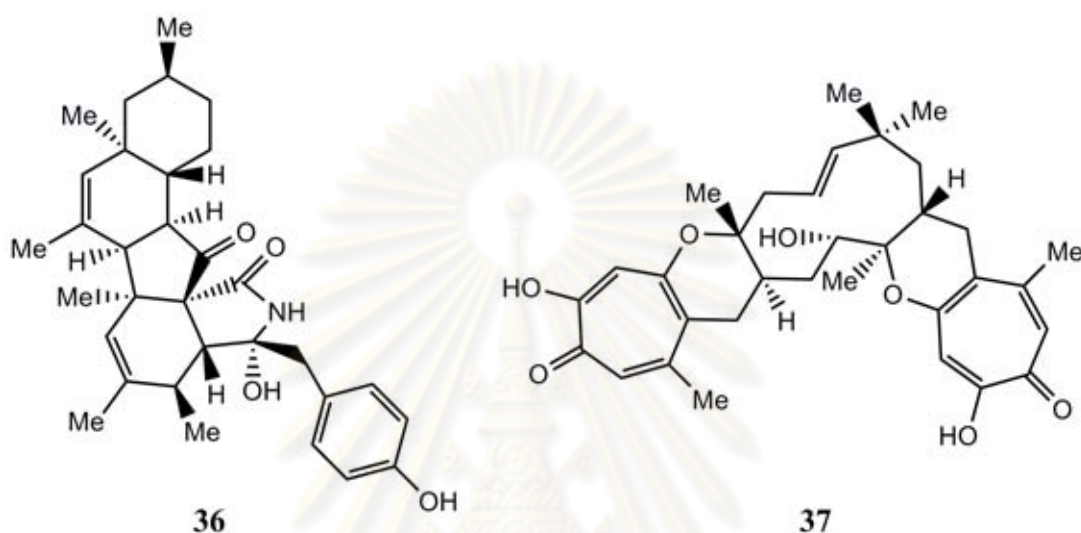
Figure 1.10 Diaporthichalasin (**36**) and pycnidione (**37**)

Monocerin (**38**) was obtained as a major product in relatively high yield (225 mg/L) from the broth extract of *Exerohilum rostratum*, a fungal species from root of *Stemona* sp. collected from Ayuttaya province, along with its derivative 11-hydroxymonocerin (**39**) (Figure 1.11). Both compounds displayed potent antiplasmodial activity against *Plasmodium falciparum*, while no cytotoxicity was observed [48].
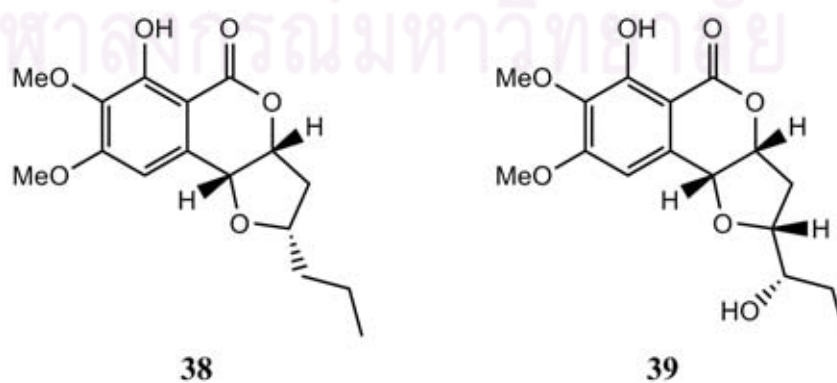
Figure 1.11 Monocerin (**38**) and 11-hydroxymonocerin (**39**)

1.5 Present Study: Aim and Scope

As mentioned in Section 1.4, Thai medicinal plants should be a potential source of endophytic fungi which could produce bioactive and chemically novel compounds due to its location and biodiversity, and more importantly, Thai medical plant-derived fungal species are still poorly investigated. Consequently, this motivated us to isolate and characterize the secondary metabolites of those fungi with the aim to find the fungal strains able to produce structurally novel and biologically active small molecules. In the present study, three plants from the family GUTTIFERAE, *Mammea siamensis*, *Mesua ferrea* and *Calophyllum inophyllum*, were selected to be the sources of fungal endophyte isolation, since they have been utilized in the formulations of Thai folk medicine for long time.

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Malpighales
Family:	Clusiaceae (Guttiferae)
Genus:	Mammea
Species:	*Mammea siamensis*

Figure 1.12 *Mammea siamensis*

The tree *Mammea siamensis* Kosterm is known in Thai as "Sarapee", a small, evergreen which grows up to 15 m tall and 10-30 cm in diameter; it is native to Myanmar, Thailand, Laos, Cambodia and Vietnam [49]. The flowers of this plant are

used for a heart tonic, reducing of fever and enhancement of appetite in Thai traditional medicine [50]. It is interesting to note that several phenylcoumarins have been isolated from the flowers of this plant [51].

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Malpighales
Family:	Clusiaceae (Guttiferae)
Genus:	Measua
Species:	*Measua ferrea*

Figure 1.13 *Measua ferrea*

Measua ferrea, called in Thai as "Bun-Nak", is a medium-sized or fairly large evergreen tree up to 36 m tall, indigenous to tropical Asia including Thailand. It has simple, narrow, oblong, dark green leaves 7-15 cm long, with a whitish underside; the emerging young leaves are red to yellowish pink and drooping. The flowers are 4-7.5 cm diameter, with four white petals and a center of numerous yellow stamens. The mixture of pounded kernels and seed oil of this plant is used for poulticing wounds. The seed oil is also used for treating itch and other skin eruptions, dandruff and against rheumatism. The stamens have been used as an active gradient in traditional medicine as a haemostatic, antidysenteric and antidiarrhoetic. Phytochemical investigation of different parts of the plant showed the occurrence of xanthones, coumarins, biflavones, cyclohexadione derivatives and essential oil [52].

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Malpighales
Family:	Clusiaceae (Guttiferae)
Genus:	Calophyllum
Species:	*Calophyllum inophyllum*

Figure 1.14 *Calophyllum inophyllum*

Calophyllum inophyllum Linn. is an evergreen shrub widely distributed in tropical areas, and called in Thai as "Krating" or "Sarapee-Talay". It usually reaches 8 to 20 m in height. This tree often grows in coastal regions as well as nearby lowland forests. The flower is 25 mm wide and occurs in racemose or paniculate inflorescences consisting of 4 to 15 flowers. The fruit (the ballnut) is a round, green drupe reaching 2 to 4 cm in diameter and having a single large seed. Active ingredients in the seed oil are believed to regenerate tissue, so is sought after by cosmetics and manufacturers as an ingredient in skin crèmes [53]. Studies on the chemical components revealed that C. inophyllum is rich in pyrocoumarins, xanthones, triterpenes and flavonoids. [54-59].

Most importantly, some reports also revealed that plants belonging to this family collected from Thailand, mostly the genus *Garcinia*, have proved to be an outstanding source of potential endophytic microbes leading structurally novel and biologically active metabolites. For instance, a fungicolous isolate of *Phomopsis* sp. from *Garcinia dulcis* leaves collected from Songkla province was found to produce three novel compounds (**40-42**), along with a number of known anitmycobacterial compounds (Figure 1.15) [60]. Another example is the isolation of cultured endophytic fungi *Botryosphaeria rhodina* from *G. mangostana* leaves collected from Suratthani province furnished a variety of structurally new metabolites (**43-47**) (Figure 1.16) [61]. Thus, it prompted us to investigate the secondary metabolism of endophytic fungi isolated from plants in the other genus of this family including the genus *Mammea*, *Mesua* and *Calophyllum*, as aforementioned, which are still rarely investigated. Moreover, the samples of each plant studied will be collected from several places as, quite commonly, it is known that the ecosystem have also influenced on the number, the kinds and the general metabolism of endophytic microorganisms, not only the plant category.
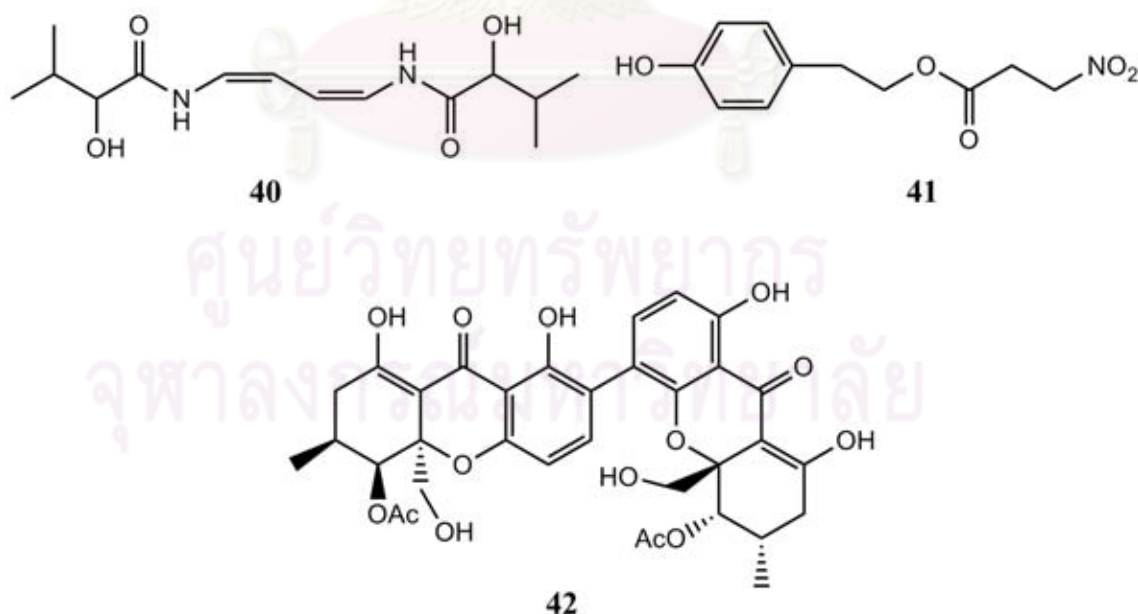
Figure 1.15 Isolated compounds (**40-42**) from *Phomopsis* sp.
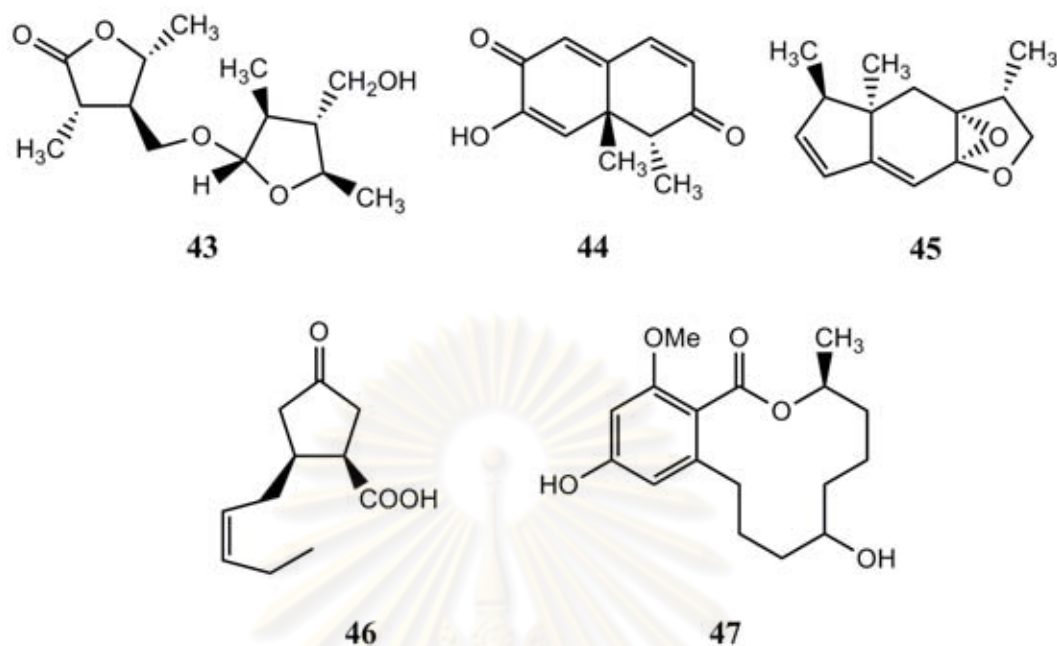
Figure 1.16 Isolated compounds (**43**-**47**) from *B. rhodina*

Therefore the objectives of the present study could be divided into the following three parts as follows:

1. To isolate and identify endophytic fungi from *Mammea siamensis*,

   Mesua ferrea, and *Calophyllum inophyllum* collected from various areas of Thailand.

2. To isolate and elucidate the structures of the isolated compounds from cultured endophytic fungi studied by spectroscopic techniques.

3. To evaluate the biological activities of the isolated compounds including antibacterial, anticancer and antimalarial assay.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

The solvents used for extraction, TLC and column chromatography (CC) in the present study including hexane, dichloromethane (CH_2Cl_2), acetone, ethyl acetate (EtOAc) and methanol (MeOH) were commercial grade and purified prior to use by distillation, except for benzene (AR grade). For separation by HPLC, the HPLC grade solvents were utilized. Deuterated solvents, chloroform-d ($CDCl_3$) and acetone-d_6, were used for NMR experiments.

2.2 Culture Media for endophytic fungi cultivation

Water agar (WA) was culture medium for isolation of endophytic fungi. Potato dextrose agar (PDA) was used for observing morphology of isolated endophytic fungi. In addition, malt extract broth (MEB), corn steep liquor broth (CSB), and solid rice medium (RM) were used for growing isolated endophytic fungi.

The formulae for each culture media were shown in Appendix A.

2.3 Plant sample Collection

Healthy leaves of *Mammea siamensis*, *Mesua ferrea*, and *Calophyllum inophyllum* were collected from Rayong, Bangkok, and Nakorn Pathom Provinces, Thailand, in May 2008. The fresh-cut ends of plant samples were wrapped with parafilms before they were placed in zip-lock plastic bags and stored less than 72 h in a refrigerator prior to isolation of endophytic fungi.

2.4 Isolation and Purification of Fungal endophyte

Healthy leaves of the collected plant samples were washed in tap water and air-dried. The cleaned leaf fragments were surface-sterilized as described by Schulz and co-workers [62] with some modifications. These fragments were sequentially immersed in 70% EtOH for 1 min, 6% NaOCl solution for 5 min, and sterile distilled water for 1 min (two times). Then, the surface-sterilized fragments were cut into small

pieces (ca. 5 mm in length) using a sterile blade and placed on sterile water agar plates for further incubation at 30°C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile pipette and transferred onto a potato dextrose agar plate (Figure 2.1). After incubation at 30 °C for 7-14 days, culture purity was determined from colony morphology.
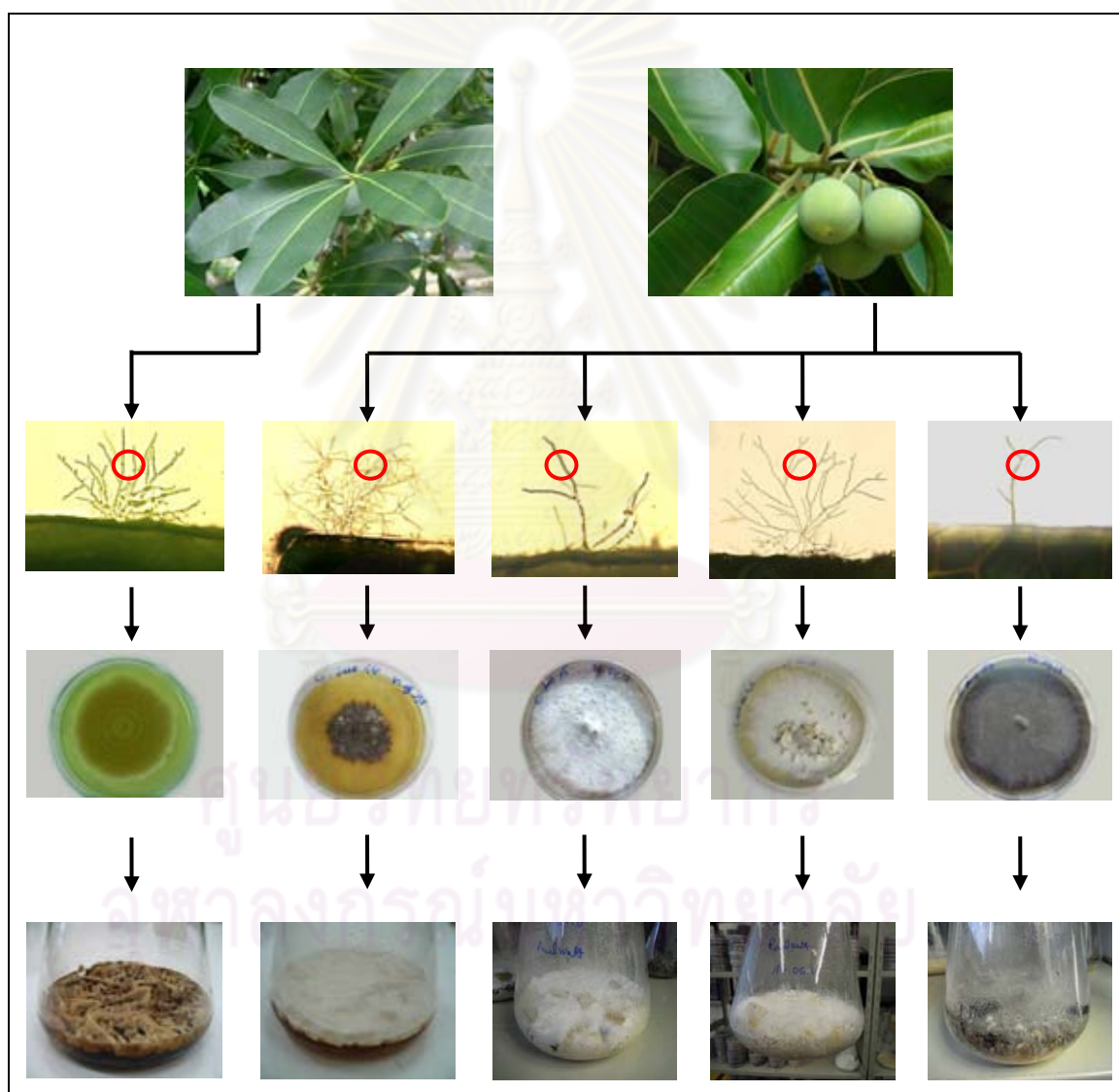
Figure 2.1 Isolation, purification and cultivation of fungal strains

2.5 Selection of endophytic fungal isolates

All isolated fungal strains, 24 isolates *M. siamensis* (MS1-MS24), 20 isolates from *M. ferrea* (MF1-MF20) and 12 isolates from *C. inophyllum* (CI1-CI12) were first grown on PDA plate at 30°C for 7 days. Each grown culture was further cultivated in three different media, namely, MEB, corn steep broth and rice medium by adding five pieces (5 x 5 mm^2) of the grown culture into 250 mL Erlenmeyer flasks (x 5) containing 100 mL of each medium and incubating at 30°C for 3 weeks under static conditions (Figure 2.1). Subsequently, fungal cultures were extracted with an appropriate solvent to yield their crude extracts which were further examined the metabolites by ^{1}H NMR spectroscopy. The extracts that show characteristic interesting signals were selected for large scale cultivation and isolation. Finally, four fungal strains, MS1 and CI3-CI5, were selected for the further study.

2.6 Identification and taxonomy of selected endophytic fungi

2.6.1 Morphological identification

Endophytic fungi were cultured on PDA. Microscorpic morphology of each isolate including colony characteristics, mycelia, spores, and others were examined by light microscopy.

2.6.2 Molecular identification

All fungal strains selected, MS1, CI2, CI3, CI4, and CI5, were identified using a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region. This was carried out by Mr. Mustapha El Amrani and Prof. Dr. Peter Proksch, Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany.

2.6.2.1 DNA isolation

Fungal DNA isolation and purification was performed using DNeasy$^{®}$ Plant Mini Kit (QIAgen). The lyophilized fungal mycelia were pulverized and disrupted with the help of glass beads. Then cell lysis were carried out by addition of lysis Buffer AP-1 and RNAse-A solution followed by incubation of the mixture at 65 $^{\circ}$C.

The remaining detergent, protein and polysaccharide were precipitated by addition of Buffer AP-2 to the lysate. The lysate was then applied to the Qiashredder^{TM} Mini Spin Column and centrifuged to remove the cell debris and other remaining precipitates. Subsequently, the lysate was transferred to a new tube.

An adequate volume of ethanolic Buffer AP3/E was added to the lysate and the mixture was then applied to DNeasy Mini Spin Column. After centrifugation, the filtrate was discarded. The column was washed by addition of ethanolic Buffer AW followed by centrifugation. Another portion of Buffer AW was added to the column and centrifuged at maximum speed to dry the membrane in the column from residual ethanol.

Fungal DNA incorporated into the membrane was eluted by the addition of Buffer AE directly to the membrane in the DNeasy column. The column was then incubated at room temperature for 5 minutes and then centrifuged to collect the filtrate, which was the fungal DNA dissolved in Buffer AE.

2.6.2.2 DNA amplification

The ITS1-5.8S-ITS2 ribosomal RNA gene region of fungal genomic DNA was amplified by Polymerase Chain Reaction (PCR). The PCR was carried out using HotStarTag Master Mix Kit (QIAgen). The Master Mix contains HotStarTag$^{®}$ DNA Polymerase, PCR buffer (with $MgCl_2$) and dNTPs.

ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) and ITS 4 (with base sequences TCCTCCGCTTATTGATATGC) (Invitrogen), as primers, were mixed with HotstarTaq Master Mix Kit and DNA template. Thus, each PCR reaction mixture contained 5-10 ng of genomic DNA, 1 μM each of the primers ITS 1 and ITS 4, and 1 U of Hot start Taq-Polymerase (Invitrogen) in a total volume of 50 μL. The mixture was then applied to the thermal cycler (BioRad) using the programmed PCR cycle as outlined below:

- Initial activation step in 95 ºC for 15 minutes to activate HotStarTaq$^{®}$ DNA Polymerase
- Cycling steps which were repeated 35 times:

    denaturing: 1 minute at 95 ºC,

    annealing: 1 minute at 56 ºC,

extension: 1 minute at 72 ºC

- Final extension for 10 minutes in 72 ºC

2.6.2.3 Purification of PCR products and DNA sequencing

The PCR product was purified using 2% Agarose-Gel-Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 500 bp stained DNA fragment was then excised from the agarose gel. The next step of PCR product purification was performed using Perfectprep® Gel Cleanup Kit (Eppendorf). The binding buffer was mixed to the PCR product and incubated at 50º C for 10 minutes in an eppendorf thermomixer at 1000 rpm. The mixture was mixed with a volume of isopropanol and then centrifuged. The filtrate was discarded and the column was washed with wash buffer twice followed by centrifugation.

Amplified fungal DNA (PCR product), which was incorporated into the column, was eluted by addition of elution buffer or molecular biology grade water to the centre of the column. The column was then centrifuged to collect the filtrate, which was the fungal DNA dissolved in elution buffer. The amplified fungal DNA was then submitted for sequencing by a commercial service and the base sequence was compared with publicly available databases such as GenBank with the help of Blast-Algorithmus.

2.6.3 Taxonomy

Based on morphological characteristics and DNA sequencing, taxonomy of each fungal strain was identified.

2.7 Large scale cultivation and extraction of selected endophytic fungi

2.7.1 Cultivation and extraction of fungal strain MS1

The fungus MS1 was first grown on PDA plate at 30°C for 7 days. Five pieces (5x5 mm^2) of the grown culture were cut and inoculated into 1,000 mL Erlenmeyer flasks (x25) containing 200 mL of malt extract broth (MEB), and of corn steep broth medium (CSB) at 30°C for 3 weeks under static conditions.

Fungal mycelia were further separated from culture medium by filtration through filter paper (Whatman No. 1) and marcerated in a 1:1 mixture of CH_2Cl_2 and MeOH for 2 days (x 3). The extract was pooled and concentrated under reduced pressure, followed by suspension in H_2O (1:1 v/v) and extraction with hexane and EtOAc, respectively (each for three times). The hexane extract was discarded, while the resulting EtOAc extract was concentrated under reduced pressure to yield EtOAc crude extract.

For culture medium, it was directly extracted with EtOAc for three times and the combined organic layer was taken to dryness by evaporation. The procedure of the extraction is described in Scheme 2.1.

MEB   CSB

— filter

mycelium   culture medium

— soaked in   — extract with EtOAc
CH_2Cl_2:MeOH   — evaporate
(1:1)

CH_2Cl_2-MeOH extract   EtOAc extract (broth)

— evaporate

residue

— suspend in H_2O
— extract with hexane

hexane phase   residue

— extract with EtOAc
— evaporate

EtOAc extract (mycelium)
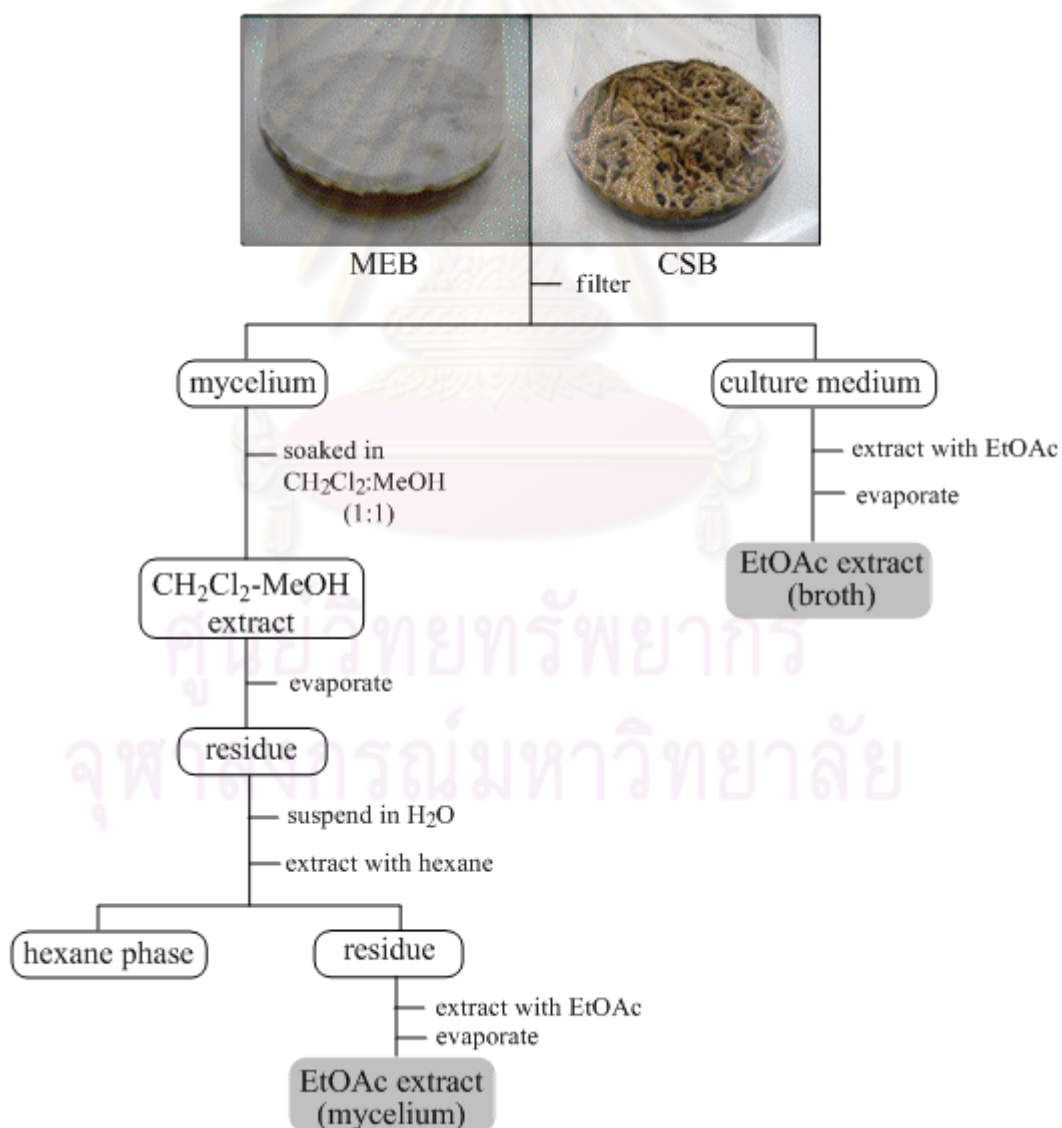
Scheme 2.1 Extraction of mycelium and culture media of fungus MS1

2.7.2 Cultivation and extraction of fungal strain CI3-CI5

All three fungal strains, CI3-CI5, were grown on PDA plate at $25°C$ for 7 days. Five pieces (20x20 mm^2) of the grown culture were cut and inoculated into 1,000 mL Erlenmeyer flasks (x10) containing 110 gram of rice medium (rice 90 g/ deionized water 100 mL), then left at $25°C$ for 3 weeks under static condition.

To the solid culture flasks of each endophytic fungus, 250 mL of EtOAc was added and left overnight. Culture media was then cut in pieces to allow complete extraction, followed by filtration and repeated extraction with EtOAc twice. The combined EtOAc layers were washed with distilled water and concentrated under reduced pressure to yield the crude extract. All dried crude extracts obtained were examined by HPLC-Library analysis for preliminarily screening of interesting compounds. The procedure of the extraction is summarized in Scheme 2.2.
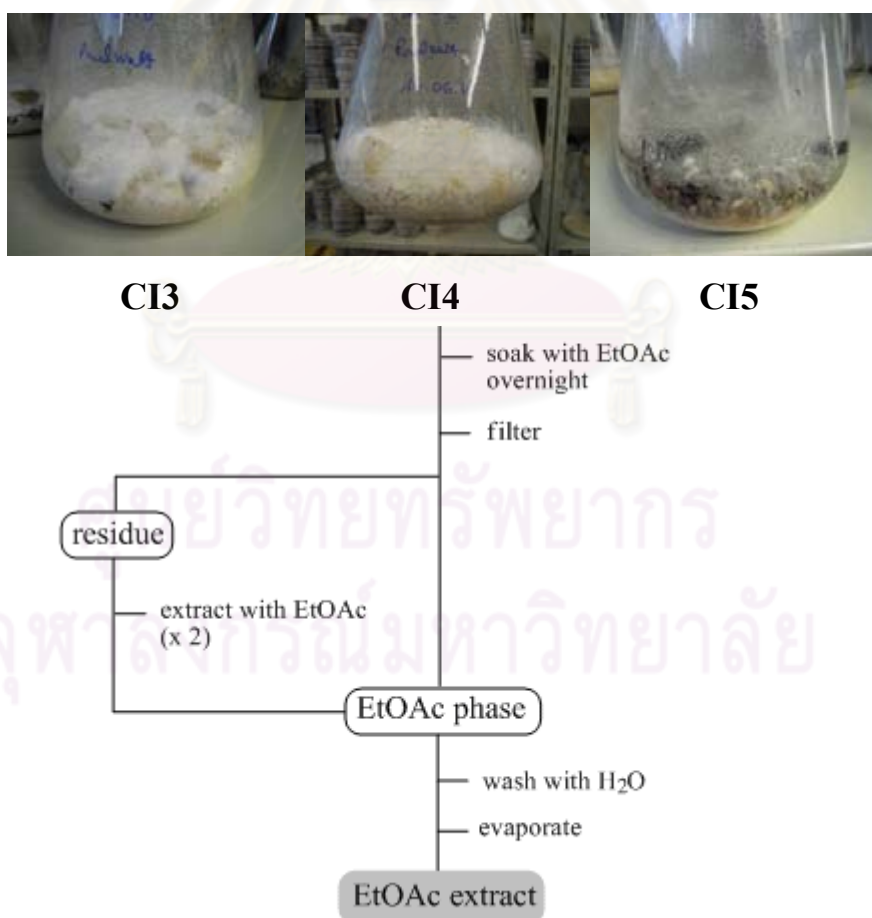
Scheme 2.2 Extraction procedure for solid culture of fungal strains CI3-CI5

2.8 Isolation of secondary metabolites from crude extracts of selected endophytic fungi

2.8.1 Isolation of secondary metabolites from the extract of fungus MS1 cultured on CSB medium

The EtOAc crude extract from culture medium (12.60 g) was first fractionated by Sephadex LH20 column chromatography eluting with MeOH to yield six fractions (AT1-AT6). Fraction AT3 was chromatographed on a silica gel column, eluted with acetone-benzene (1:3), to give butyrolactone I (**1**, 2.76 g), butyrolactone II (**2**, 1.64 g), aspernolide B (**3**, 346.0 mg), and ten subfractions (AT3.1 to AT3.10). Subfraction AT3.1 was subjected to flash silica gel column chromatography (acetone-benzene, 1:9) and further purified by a silica gel column (hexane-acetone, 3:1) to afford **4** (8.2 mg), whereas purification of subfraction AT3.7 by flash column chromatography, eluted with MeOH-CHCl$_3$ (1:30), gave **5** (16.2 mg) and butyrolactone III (**6**, 45.5 mg). Fraction AT3.8 was rechromatographed on a silica gel column (acetone-benzene, 1:2) followed by preparative TLC (MeOH-CH$_2$Cl$_2$, 5:95) to yield butyrolactone IV (**7**, 9.2 mg). The isolation procedure is summarized in **Scheme 2.3**.

2.8.2 Isolation of secondary metabolites from the extract of fungus MS1 cultured on MEB medium

The 21.0 g of EtOAc crude broth extract was passed through a Sephadex LH-20 column, eluted with MeOH to afford four fractions (AM1-AM4). EtOAc was added to subfraction AM3 (14.0 g), causing white solid precipitated out. Filter those precipitated compound and recrystallize with MeOH to get a white crystalline product, terrein (**8**) in relatively high yield (13.8 g).

EtOAc crude extract of MS1 (12.6 g)

Sephadex LH20,
MeOH
(6 fractions)

AT 3 (8.5 g)

SiO$_2$, CC
Acetone : Benzene (1 :3)
(10 fractions)

Butyrolactone I (1, 2.76 g)   **Aspernolide B (3**, 346 mg)

Butyrolactone II (2, 1.64 g)

AT3.1 (50 mg)

Flash SiO$_2$, CC
Acetone : Benzene (1:9)
and SiO$_2$, CC
Acetone : Hexane (1:3)

AT3.8 (60 mg)

Compound 4 (8.2 mg)

AT3.7 (170 mg)
Flash SiO$_2$, CC
MeOH : CHCl$_3$ (1:30)

SiO$_2$, CC
Acetone : Benzene (1:2)
and preparative TLC
MeOH : CH$_2$Cl$_2$ (5:95)

Compound 5 (16.2 mg)   **Butyrolactone III**
(6, 45.5 mg)

Butyrolactone IV (7, 9.2 mg)

Scheme 2.3 Isolation of a broth extract of MS1 cultured on CSB medium

2.8.3 Isolation of secondary metabolites from the extract of fungus CI3 cultured on rice medium

The extract of culture medium was initially fractionated by vacuum liquid chromatography (VLC) with standard gradient to yield 17 fractions (GC1-GC17), while fraction GC17 was found to be pure enough and identified as homodestruxin B (**9**, 30mg). Fraction GC4 was further chromatographed over Sephadex LH-20 using MeOH as eluent to give three pure compounds, alternariol (**10**, 1 mg), deoxyterphenylin (**11**, 3 mg) and zearalenone (**12**, 2 mg). Further separation of fraction GC8 by Sephadex LH-20 column chromatography, eluted with MeOH, led to the isolation of indole-3-carbaldehyde (**13**, 6 mg), *N,N*-dimethyltryptophane methyl ester (**14**, 12 mg) and five subfractions (GC8.1-GC8.5). Subfraction GC8.2 was subjected to Sephadex LH-20 column chromatography under the same condition again to afford dihomo-3´-hydroxymethyl-dihydroinfectopyron (**15**, 7 mg).

Fraction GC10 was also rechromatographed on a Sephadex LH-20 column using MeOH as eluent to yield malformin A (**16**, 1 mg), tenuazonic acid (**17**, 8 mg), adenosine (**18**, 10 mg) and other ten subfractions which were still mixtures. The isolation procedure of crude extract of fungus CI3 is summarized in Scheme 2.4.

2.8.4 Isolation of secondary metabolites from the extract of fungus CI4 cultured on rice medium

Similarly, the extract of culture broth (9.4 g) was first separated by vacuum liquid chromatography (VLC) with standard gradient to give 11 fractions (PH1-PH11) and a pure compound, Sumiki's acid (**19**, 3 mg). Further separation of PH7 by subjection to SiO_2 column chromatography using a mixture of CH_2Cl_2-MeOH (9:1) as eluent led to the isolation of 5-carboxymellein (**20**, 2 mg) and cytochalasin D (**21** 10 mg). The isolation procedure is summarized in Scheme 2.5.

EtOAc crude extract of fungus CI3 (2.5 g)

VLC, standard gradient
(17 fractions)

| Hexane : EtOAc (2 :3) | CH$_2$Cl$_2$: MeOH (9:1) | CH$_2$Cl$_2$: MeOH (7:3) | 100%MeOH |

GC4 (128 mg) **GC8** (317 mg) **GC10** (163 mg)

Homodestruxin B (9, 30 mg)

Sephadex LH20, MeOH

Sephadex LH20, MeOH
(5 fractions)

Sephadex LH20, MeOH
(14 fractions)

Alternariol (10, 1 mg)

Deoxyterphylin (11, 3 mg)

Malformin A (16, 1 mg)

Adenosine (18, 10 mg)

Zearalenone (12, 2 mg)

Tenuazonic acid (17, 8 mg)

GC8.2 (60 mg)

Indole-3-carbaldehyde (13, 6 mg)

N,N-dimethyl tryptophane methyl ester (14, 12 mg)

Sephadex LH20
MeOH

Dihomo-3´-hydroxymethyl-dihydroinfectopyron (15, 7 mg)

Scheme 2.4   Isolation of a crude extract of fungus CI3 cultured on rice medium

EtOAc crude extract of fungus CI4 (9.4 g)

VLC, standard gradient
(11 fractions)

CH$_2$Cl$_2$: MeOH
(7:3)

Sumiki's acid (19, 3 mg)

PH7 (91 mg)

SiO$_2$, CC
CH$_2$Cl$_2$: MeOH (9 :1)

5-carboxymellein (20, 2 mg)   **Cytochalasin D (21**, 10 mg)
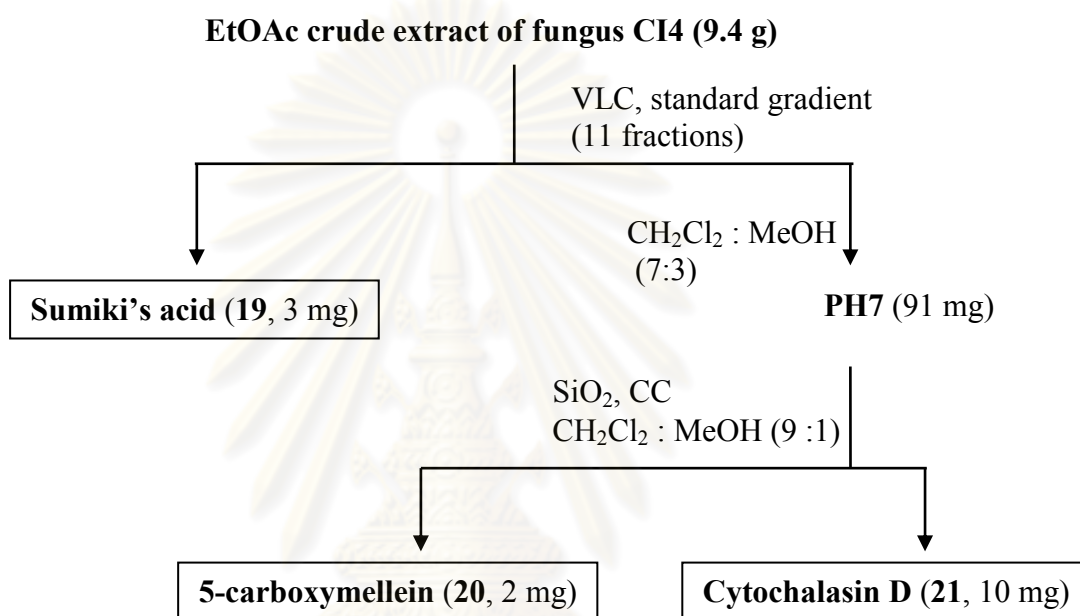
Scheme 2.5 Isolation of a crude extract of CI4 cultured on rice medium

2.8.5 Isolation of secondary metabolites from the extract of fungus CI5 cultured on rice medium

The EtOAc extract of culture medium of fungus CI5 (3.1 g) was also fractionated by vacuum liquid chromatography (VLC) with standard gradient to give ten fractions (AS1-AS10). Fraction AS1 was purified by a Sephadex LH-20 column chromatography using MeOH as eluent to afford 10 mg of alternariol (**10**), while the purification of fraction AS7 in the similar manner gave three pure compounds, tenuazonic acid (**17**, 90 mg), 2(4-bromophenyl) acetic acid (**22**, 10 mg) and citreodrimene F (**23**, 3 mg). The isolation procedure is summarized in Scheme 2.6.

EtOAc crude extract of fungus CI5 (3.1 g)

VLC, standard gradient
(10 fractions)

Hexane : EtOAc
(1 :1)

CH$_2$Cl$_2$: MeOH
(1:1)

AS1 (1.9 g)

AS6 (462 mg)

Sephadex LH20,
MeOH

Sephadex LH20,
MeOH

Alternariol (**10**, 10 mg)

Tenuazonic acid (**17**, 90 mg)

Citreodrimene F (**23**, 3 mg)

2(4-bromophenyl) acetic acid (**22**, 10 mg)
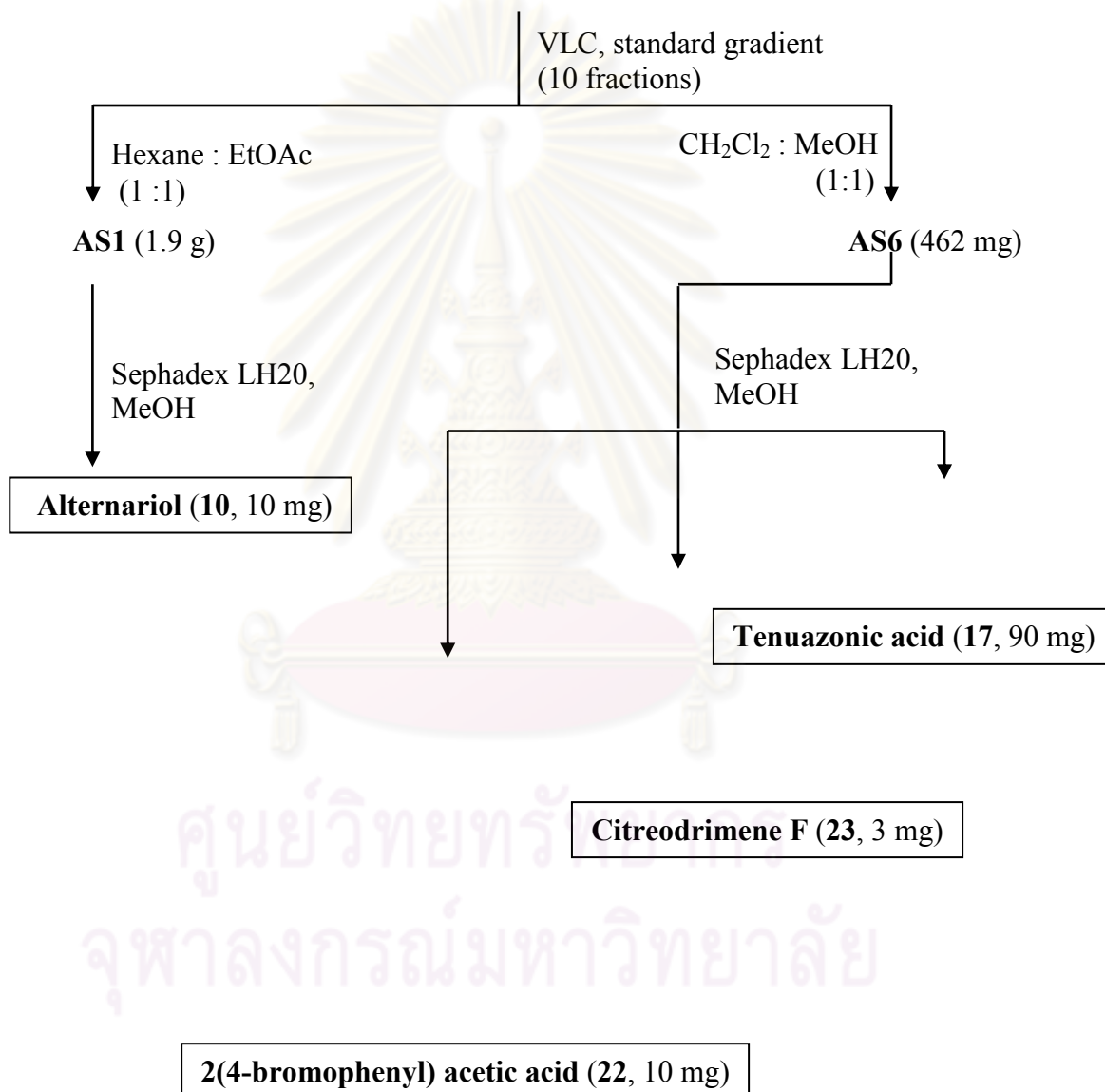
Scheme 2.6 Isolation of a crude extract of fungus CI5 cultured on rice medium

2.9 Chromatographic methods

2.9.1 Thin layer chromatography (TLC)

TLC was carried out on a siligal gel F254 coated on aluminium sheet (Merck) for monitoring of fractions. Detection was visualized under ultraviolet light at wavelengths of 254 and 356 nm and dipped with $(NH_4)_6Mo_7O_{24}$ solution in 5% H_2SO_4/EtOH or sprayed with anisaldehyde reagent.

2.9.2 Vacuum liquid chromatography (VLC)

VLC apparatus consists of a 500 cm sintered glass filter funnel with an inner diameter of 12 cm. Silica gel 60 was packed to a hard cake at a height of 5-10 cm under applied vacuum. The crude extract was adsorbed onto a small amount of silica gel using volatile solvents. The resulting mixture was then packed onto the top of the column. Using step gradient of increasing polarity as eluent (from hexane-EtOAc to CH_2Cl_2-MeOH), successive fractions were collected. The flow was produced by vacuum and the column was allowed to run dry after each fraction collected.

2.9.3 Column chromatography

Column chromatography (CC) was performed using Sephadex LH-20 (Pharmacia Code No. 17-0090-01) and Silica gel 60H (Merck code No. 7734 and No. 9385) as packing materials.

2.9.4 High pressure liquid chromatography (HPLC)

    2.9.4.1 Analytical high pressure liquid chromatography

Analytical HPLC was used to identify the distribution of peaks from extracts or fractions. The solvent gradient used started with MeOH:nanopure H_2O (10:90), which was adjusted to pH 2 with phosphoric acid, and reached to 100% MeOH in 35 minutes. The autosampler injected 20 mL of each sample. All peaks were detected by UV-Vis photodiode array detector. LC/UV system specifications are described as follows:

Pump	Dionex P580A LPG
Detector	Dionex Photodiode Array Detector UVD 340S
Column thermostat	STH 585
Autosampler	ASI-100T
HPLC program	Chromeleon (V.6.3)
Column	Knauer (125 x 4 mm, ID), pre-packed with Eurosphere 100-5 C18, with integrated pre-column

2.9.4.2 Semi-preparative high pressure liquid chromatography

Semi-preparative HPLC was used for purification of metabolites from fractions. The most appropriate solvent system must be determined before running the HPLC separation. The mobile phase combination was MeOH and nanopure H_2O with or without 0.01% TFA or 0.1% formic acid, pumped in gradient or isocratic manner depending on the retention time of compounds at a rate of 5 mL/min. Each injection consisted of 1-3 mg of fraction dissolved in 1mL of the solvent system. Detection was performed by the online UV detector and the eluted peaks were collected separately in Erlenmeyer flasks. Semi-preparative HPLC system specifications are described as follows:

Pump	Merck Hitachi L-7100
Detector	Merck Hitachi UV detector L-7400
Column	Knauer (300 x 8 mm, ID), pre-packed with Eurosphere 100-10 C18, with integrated pre-column

2.9.4.3 Preparative high pressure liquid chromatography

Preparative HPLC was used for purification of metabolites in higher amounts from fractions. Similarly, the most appropriate solvent system must be determined before running the HPLC separation. The mobile phase combination was MeOH or acetonitrile and nanopure H_2O with or without 0.01% TFA or 0.1% formic acid, pumped in gradient or isocratic manner depending on the retention time of compounds at a rate of 20 mL/min. Each injection consisted of 20-80 mg of fraction dissolved in 400 mL of the solvent system. Detection was performed by the online UV detector

and the eluted peaks were collected separately in Erlenmeyer flasks. Preparative HPLC system specifications are described as follows:

Pump	Varian, PrepStar 218
Detector	Varian, ProStar 320 UV-Vis detector
HPLC program	Varian Star (V. 6)
Column	Varian Dynamas (250 x 21.4 mm, ID), pre-packed with Microsorb 60-8 C18, with integrated pre-column

2.10 Structure elucidation of isolated secondary metabolites

Structures of isolated compounds were elucidated by the interpretation of NMR spectra. Additional spectroscopic techniques such as MS, UV-vis and IR were also employed for the structural elucidation. Melting points and optical rotation properties of the compounds were also measured.

2.10.1 Melting point

Melting points of the compounds isolated as crystals or powder were taken on an Electrothermal IA9100 digital melting point apparatus.

2.10.2 Optical rotation

Optical rotation of each compound containing chiral center was measured on a Perkin-Elmer 341 polarimeter or a Perkin–Elmer-241 MC polarimeter using a sodium lamp at wavelength 589 nm, equipped with a 1 mL cell (cell length 1.00 cm).

2.10.3 Ultraviolet-visible measurement (UV-vis)

UV-vis spectra were recorded on a Perkin Elmer Lambda 25 UV-vis spectrophotometer. Samples were prepared in a solution of MeOH or $CHCl_3$.

2.10.4 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were recorded on a Bruker vector22 Fourier transform infrared spectrophotometer. Samples were formally examined by incorporating them with potassium bromide (KBr) to form a pellet.

2.10.5 Mass Spectrometry (MS)

ESI-MS was conducted on a Finnigan LCQ-Deca mass spectrometer and HRESI-MS spectra were obtained on a Micromass Qtof 2 mass spectrometer.

2.10.6 Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded on a Varian YH400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR), a Bruker ARX-500 (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) and AVANCE DMX-600 (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR) NMR spectrometers, by using tetramethylsilane (TMS) or residual solvent signals as the internal standard. Samples were dissolved in different deuterated solvents depending on their solubility.

2.11 Determination of absolute stereochemistry by Mosher reaction

The reaction was performed according a modified Mosher ester procedure described by Ohtani and co-workers [63].

Determination of absolute configuration at C-8" of compound 5

Reaction with (*R*)-(-)-α-(trifluoromethyl) phenylacetyl chloride [(*R*)-MTPA Cl]

To a solution of **5** (3.0 mg) in pyridine (0.3 mL) was added (*R*)-MTPA Cl (20 μL). The reaction mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc and washed with H_2O and 1M $NaHCO_3$, and the organic layer was concentrated in vacuo. The residue was purified by a short silica gel column (MeOH-CH_2Cl_2) to obtain the (*S*)-MTPA ester **5a** (1.8 mg).

^{1}H NMR (CDCl$_3$, 400 MHz) δ 7.56 (2H, d, J = 8.8 Hz, H-2', H-6'), 6.86 (2H, d, J = 8.8 Hz, H-3', H-5'), 6.60 (s, H-2"), 6.50 (1H, d, J = 8.4 Hz, H-6"), 6.45 (1H, d, J = 8.4 Hz, H-5"), 4.46 (1H, t, J = 8.4 Hz, H-8"), 3.72 (s, 5-OMe), 3.50 (1H, d, J = 14.4 Hz, H-6a), 3.42 (1H, d, J = 14.4 Hz, H-6b), 2.94 (2H, m, H$_2$-7"), 1.24 (3H, s, Me-10"), 1.20 (3H, s, Me-11").

Reaction with (*S*)-(-)-α-(trifluoromethyl) phenylacetyl chloride [(*S*)-MTPA Cl]

Similarly, the reaction mixture of **5** (3.0 mg), (*S*)-MTPA Cl (20 μL) and pyridine (0.3 mL) was processed as described above for **5a** to afford **5b** (1.5 mg).

^{1}H NMR (CDCl$_3$, 400 MHz) δ 7.58 (2H, d, *J* = 9.2 Hz, H-2', H-6'), 6.99 (2H, d, *J* = 9.2 Hz, H-3', H-5'), 6.75 (s, H-2"), 6.47 (1H, d, *J* = 8.4 Hz, H-6"), 6.40 (1H, d, *J* = 8.4 Hz, H-5"), 4.51 (1H, t, *J* = 9.6 Hz, H-8"), 3.74 (s, 5-OMe), 3.62 (1H, d, *J* = 14.8 Hz, H-6a), 3.58 (1H, d, *J* = 14.8 Hz, H-6b), 3.00 (2H, m, H$_2$-7"), 1.22 (3H, s, Me-10"), 1.19 (3H, s, Me-11").

2.12 Synthesis of butyrolactone derivatives from butyrolactone I (1)
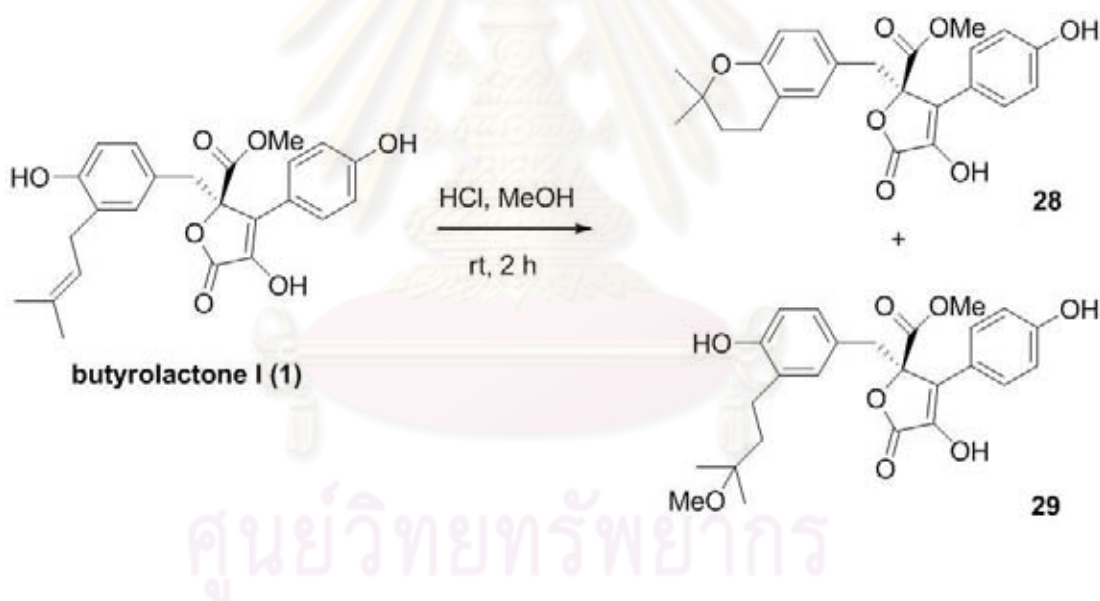
To a stirring solution of 0.3 mL of conc. HCl in MeOH (10 mL), 100 mg butyrolactone I (**1**) was added and the mixture was stirred at room temperature for 2 h or until complete conversion of 1 as indicated by TLC. Then, the solvent was removed under reduced pressure. The resulting residue was purified by SiO$_2$ column chromatography using gradient elution of MeOH-CHCl3 (0:1 to 2:8) to afford **24** (50 mg) and **25** (7 mg).

2.13 Evaluation of biological activities

The pure compounds were evaluated for their antibacterial, anticancer, and phosphodiesterases inhibitory activities.

2.13.1 Anti-bacterial activity assay

A total of 9 strains of gram-positive and gram-negative bacteria (**Table 2.1**) were selected for *in vitro* antimicrobial assay. The test was performed by using microdilution assays as follows:

Table 2.1 Gram-positive and gram-negative bacteria tested

Gram-positive bacteria	Gram-negative bacteria
1. *Enterococcus faecalis* ATCC 29212	1. *Escherichia coli* ATCC 35218
2. *Bacillus subtilis* ATCC 6633	2. *Pseudomonas aeruginosa* ATCC 27853
3. *Staphylococcus aureus* ATCC 25923	3. *Proteus vulgaris* ATCC 13315
4. *Staphylococcus epidermidis* ATCC 12228	4. *Salmonella typhimurium* ATCC 13311
5. *Staphylococcus hominis* ATCC 27844	

2.13.1.1 Preparation of bacterial inocula

Bacteria were grown on Mueller Hinton agar (MHA) for 24 h at 37°C. Selected fresh single colonies were inoculated into 10 mL of Mueller Hinton broth (MHB) and incubated in shaking incubator for 2-3 h at 37°C. The turbidity of the bacterial suspension was adjusted with sterile normal saline solution to match the turbidity of 0.5 McFarland standard (OD 0.1 at 625 nm). Then the suspension was diluted 1:100 with Mueller Hinton broth (MHB) to contain 1×10^6 CFU/mL.

2.13.1.2 Determination of minimum inhibitory concentration (MIC)

Solution of a test compound in DMSO (25.6 mg/mL) was diluted with MHB. The test compound was tested at the concentration ranges of 0.5 to 256 μg/mL. MIC is defined as the lowest concentration that inhibits growth of test microorganisms.

A 50 μL volume of MHB containing the test compound was dispensed into each well of microtiter plates (96-flat-bottom wells) for the evaluation of antibacterial activities. Sterile compound-free medium containing the corresponding amount of DMSO was dispensed in the growth control wells. The final adjusted bacterial suspensions were inoculated into each well with volume of 50 μL. Compound-free MHB in volumes of 100 µL were used as the sterility control. The experiments were done in duplicate. After incubation at 37°C for 24 h, a 20 μL of p-iodonitrotetrazolium (INT) solution (1mg/mL) was added into each well. The antibacterial assay plates were further incubated for 1 h. Growth in each well was indicated by a color change from colorless to violet. Compounds that inhibit microbial growth would prevent the development of a violet color. The well that shows no change in color indicates antimicrobial activity of the test compound.

2.13.2 Anticancer activity assay

Cytotoxic test was carried out at the institute of Biotechnology and Genetic Engineering, Chulalongkorn University. Bioassay of cytotoxicity activity was performed *in vitro* by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltrazolium bromide) colorimetric method against hepato carcinoma (Hep-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), gastric carcinoma (KATO-3) and breast carcinoma (BT-474). In Principle, the viable cell number/well was directly proportional to the production of formazan, followed by solubilization, and could be measured spectrophotometrically.

The human cancer cell line was harvested from exponential-phase maintenance cultures (T-25 cm^2 flask), counted by trypan blue exclusion, seed cells in a 96-well culture plates at a density of 1×10^5 cells/well in 200 μL of culture medium without compounds to be tested. Cells were cultured in a 5% CO_2 incubator at 37 $^{\circ}$C, 100% relative humidity for 24 h. Culture medium containing the sample was dispensed into the appropriate wells (control cells group, N = 3; each sample treatment

group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 3) and medium/DMSO blank (N = 3) "background" determination. Culture plates were then incubated for 3 days prior to the addition of tetrazolium reagent. MTT stock solution in a concentration of 5 mg/ml in PBS was sterilized by filtering through 0.45 μL filter units. MTT working solution was prepared just prior to culture application by dilution of MTT stock 1:5 (V/V) in prepared standard culture medium. The freshly prepared MTT reagent in a volume of 10 μL was added into each well and mixed gently for 1 minute on an orbital shaker. The cells were further incubated for 4 h at 37ºC in a 5% CO_2 incubator. After incubation, the formazan produced in the cells will capture as dark crystals in the bottom of the wells. All of the culture medium supernatant was removed from wells and 150 μL of DMSO was added to dissolve the resulting formazan. Samples in the culture plate were mixed for 5 minutes on an orbital shaker. Subsequently, 25 μL of 0.1 M Glycine pH 10.5 was added and the culture plate was shaken for 5 minutes. Following formazan solubilization, the absorbance was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00). The concentration of sample reduced cell survival by 50% was determined from cell survival curves.

Percentage of cell survival is expressed as:

$$\% \text{ cell survival} = \frac{\text{absorbance of treated cells} \times 100}{\text{absorbance of control cells}}$$

The compounds, which could reduce more than 50% of cell survival at a screening dose (1 mg/mL), would be identified to have cytotoxicity on those tumor cell lines. Thereafter, the compounds were retested their toxicity against cancer cells using the same procedure as mentioned in the screening stage, by five concentrations of 10.0, 5.0, 2.5, 1.25, 0.625 mg/mL of each compound in three replicates. The half maximal inhibitory concentrations (IC_{50}) were calculated from cell survival curves.

2.13.3 Antimalarial activity assay

Testing for antimalarial activity was conducted by Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The assay had been done by means of the following processes.

Plasmodium falciparum (K1, multidrug resistant strain) was cultured continuously according to the method of Tragen and Jensen. The quantitative assessment of the antimalarial activity in vitro was performed in accordance with the microculture radioisotope technique based upon the method described by Desjardins, *et al*. A standard antimalarial compound, dihydroartemisinin (IC$_{50}$ value of 0.004 μM), was used as the positive control for the assay.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation of endophytic fungi from *M. siamensis*, *M. ferrea*, and *C. inophyllum*

Based on different morphology of fungi, a total of 56 pure isolates of endophytic fungi were obtained from leaves of *M. siamensis*, *M. ferrea* and *C. inophyllum*, which were collected from three different places including Rayong, Bangkok and Nakorn Pathom. Isolated fungi are given as code "MS" for the fungi isolated from *M. siamensis*, while "MF" and "CI" were isolated from *M. ferrea* and *C. inophyllum*, respectively. According to Table 3.1, it was noticed that not only do the plant category affect the number and species of endophytic fungi isolated, but the environment in which each plant grows, also have much influenced on species of those fungal strains.

Table 3.1 Isolated endophytic fungi from *M. siamensis, M. ferrea,* and *C. inophyllum* collected from Rayong, Bangkok and Nakorn Pathom province.

Plant sample	Strain code from each province			Total
	Rayong	**Bangkok**	**Nakorn Pathom**	
M. siamensis	MS1, MS2, MS3, MS4, MS5, MS6, MS7, MS8, MS9, MS10, MS11, MS12	MS13, MS14, MS15, MS16, MS17	MS18, MS19, MS20, MS21, MS22, MS23, MS24	24
M. ferrea	MF1, MF2, MF3, MF4, MF5, MF6, MF7, MF8	MF9, MF10, MF11, MF12, MF13	MF14, MF15, MF16, MF17, MF18, MF19, MF20	20
C. inophyllum	CI1, CI2, CI3, CI4, CI5,	CI6, CI7, CI8	CI9, CI10, CI11, CI12	12
Total	25	13	18	**56**

After isolation of endophytic fungi, all pure strains were placed on potato dextrose agar (PDA) until grown fully on petri dish show in Figures 3.1, 3.2 and 3.3). Their morphological characteristics are including colony, color, produced pigment and sporulation. The characteristics of each strain are shown in Tables 3.2, 3.3 and 3.4.
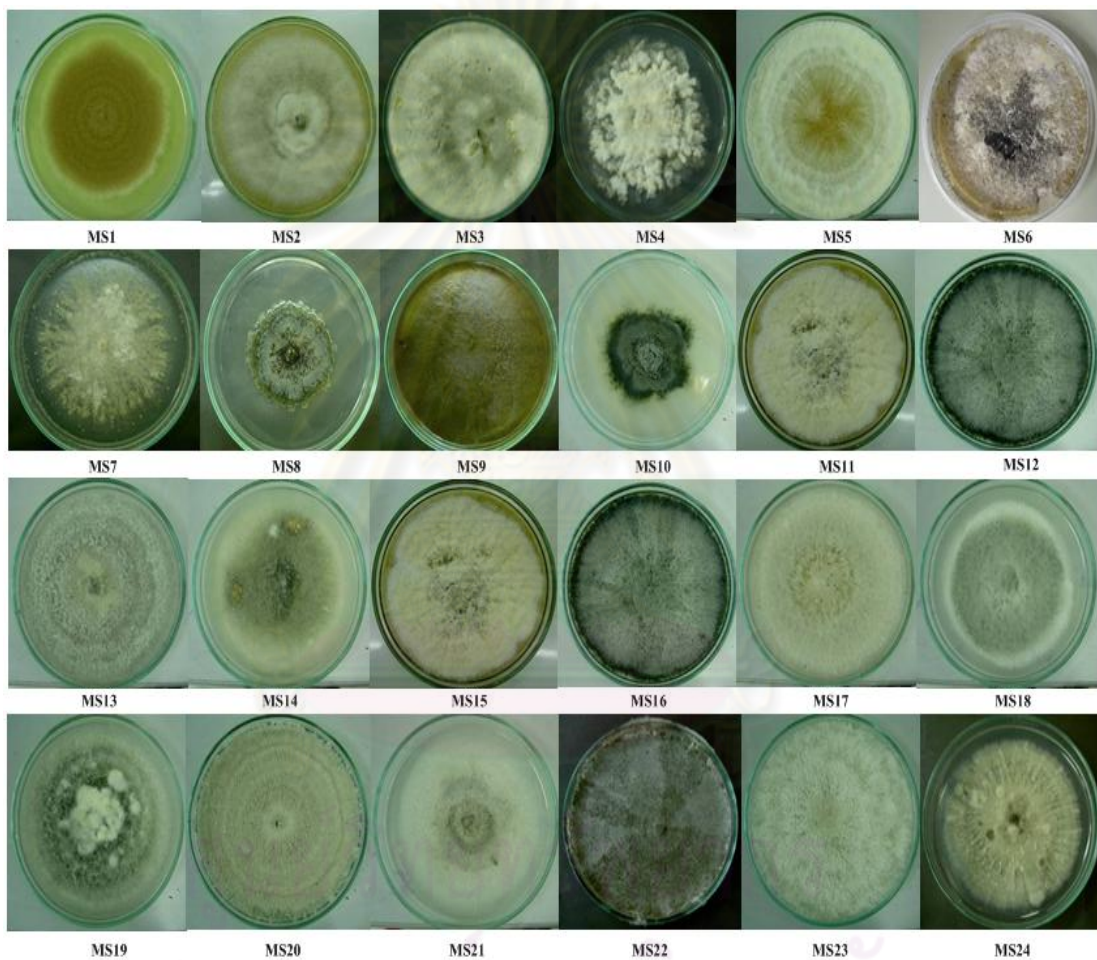
Figure 3.1 Colony morphology of each fungal strain isolated from *M. siamensis* on potato dextrose agar (PDA).

Table 3.2 Characteristics of each fungal strain isolated from *M. siamensis* on potato dextrose agar (PDA).

Strain code	Characteristics		
	Colony	**Color**	**Produced pigment**
MS1	flat	yellow and brown	yellow
MS2	flat	white	yellow
MS3	cottony	white	yellow
MS4	rough	white	not produce
MS5	cottony	white and yellow	not produce
MS6	cottony	white and grey	yellow
MS7	flat	white and yellow	not produce
MS8	cottony	green and grey	not produce
MS9	flat	green	green
MS10	cottony	green and grey	not produce
MS11	cottony	white	yellow
MS12	flat	white and yellow	yellow
MS13	flat	gray	not produce
MS14	rough	white and grey	not produce
MS15	rough	white	yellow
MS16	flat	dark grey	black
MS17	flat	white	yellow
MS18	flat	white and grey	not produce
MS19	cottony	white and grey	not produce
MS20	flat	grey	not produce
MS21	flat	white	not produce
MS22	flat	dark green	not produce
MS23	cottony	white	yellow
MS24	flat	white	not produce
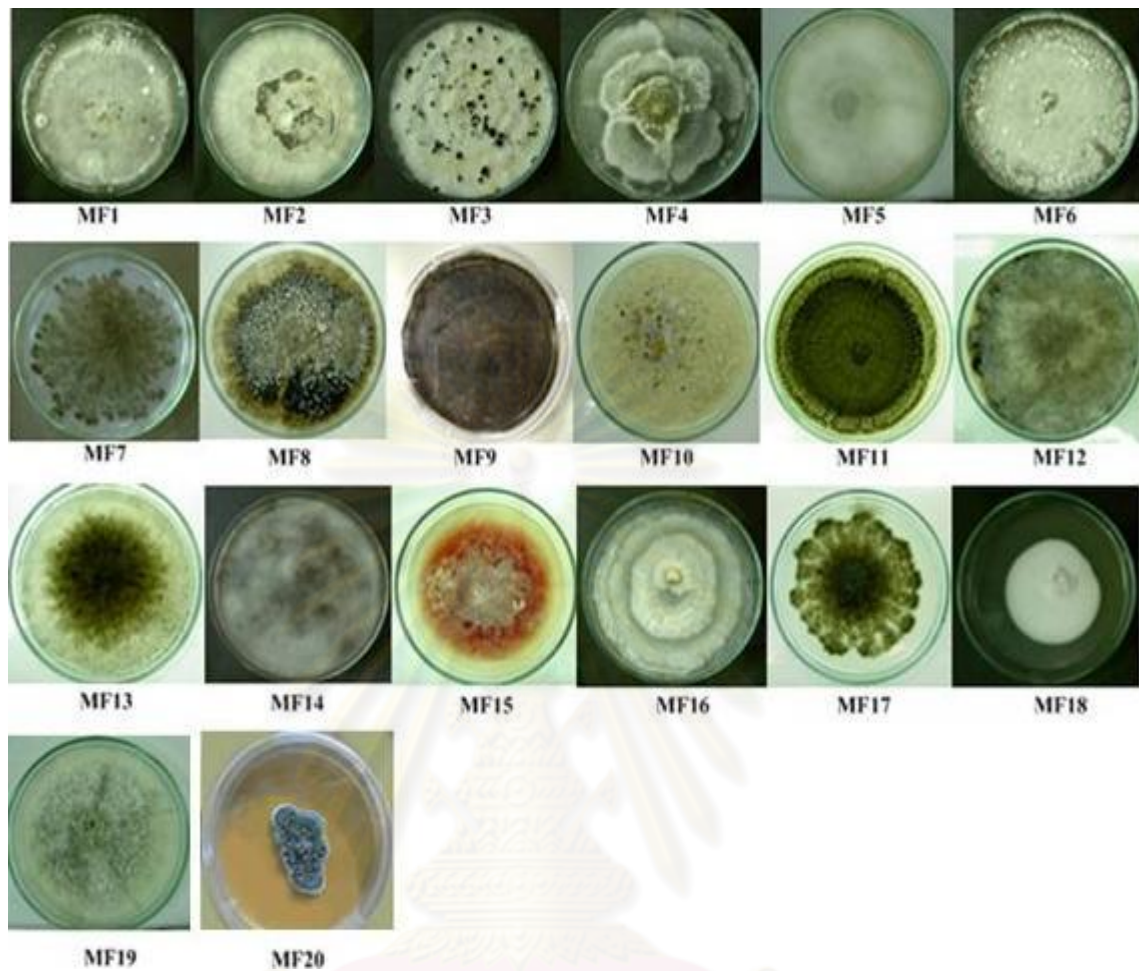
Figure 3.2 Colony morphology of each fungal strain isolated from *M. ferrea* on potato dextrose agar (PDA).

Table 3.3 Characteristics of each fungal strain isolated from *M. ferrea* on potato dextrose agar (PDA).

Strain code	Characteristics		
	Colony	**Color**	**Produced pigment**
MF1	flat	white	not produce
MF2	flat	white	not produce
MF3	cottony	white	black
MF4	like as flower	white	yellow
MF5	cottony	white	yellow
MF6	cottony	white	not produce
MF7	cottony	white and grey	not produce
MF8	flat	dark green	not produce
MF9	flat	dark green	not produce
MF10	flat	black	not produce
MF11	flat	cream	not produce
MF12	flat	dark green	not produce
MF13	flat	grey	brown
MF14	flat	dark grey	not produce
MF15	flat	dark green	not produce
MF16	cottony	white and grey	dark brown
MF17	flat	red	red
MF18	cottony	white	not produce
MF19	flat	dark green	not produce
MF20	flat	white	not produce
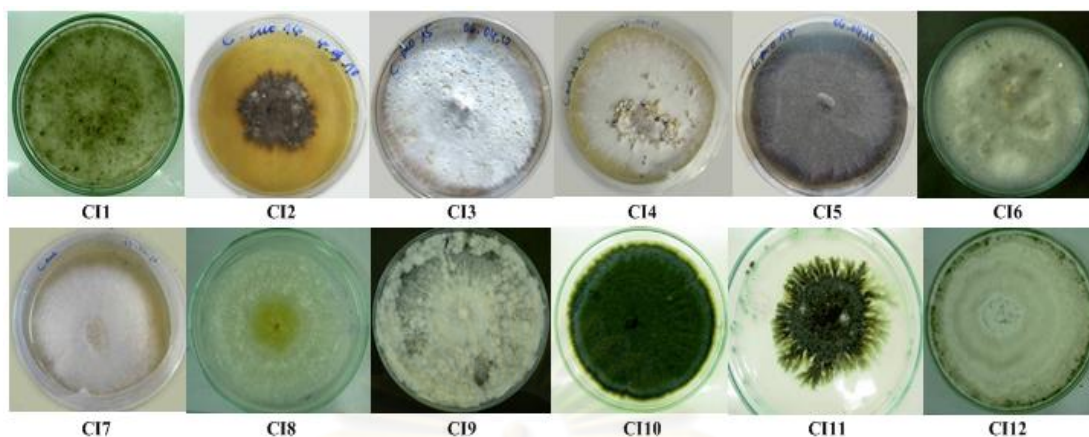
Figure 3.3 Colony morphology of each fungal strain isolated from *C. inophyllum* on potato dextrose agar (PDA).

Table 3.4 Characteristics of each fungal strain isolated from *C. inophyllum* on potato dextrose agar (PDA).

| Strain code | Characteristics | | |
	Colony	Color	Produced pigment
CI1	flat	dark green	not produce
CI2	flat	dark grey	yellow
CI3	flat	white	not produce
CI4	flat	white	yellow
CI5	flat	black	dark brown
CI6	cottony	white	not produce
CI7	cottony	white	not produce
CI8	flat	white clear	yellow
CI9	rough	white	not produce
CI10	flat	dark green	not produce
CI11	flat	dark green	not produce
CI12	flat	grey	dark brown

3.2 Selection of endophytic fungal isolates for large scale cultivation

Commonly, it is known that the source of medium has also played an important role for the metabolite production of each fungal strain. Herein, cultivation of each isolated endophyte on three kinds of medium, including MEB (malt extract broth), CSB (corn steep liquor broth) and solid rice medium were carried out. After cultivation for 21 days, followed by extraction according to Schemes 2.1 and 2.2, the EtOAc crude extracts of each fungal strain cultured on each medium were subjected to the analysis by ^{1}H NMR spectroscopy. Consequently, fungal strain MS1 grown on both CSB and MEB, and strains CI3-CI5 grown on solid rice medium, were selected to cultivate in large scale (5-10 L) for isolating bioactive metabolites the further step, due to the signals of various functionalities including aromatic (6-8 ppm), olefinic (5-6 ppm) and oxygenated (3-4 ppm) protons as shown in Figures 3.4 and 3.5.

(a)

(b)
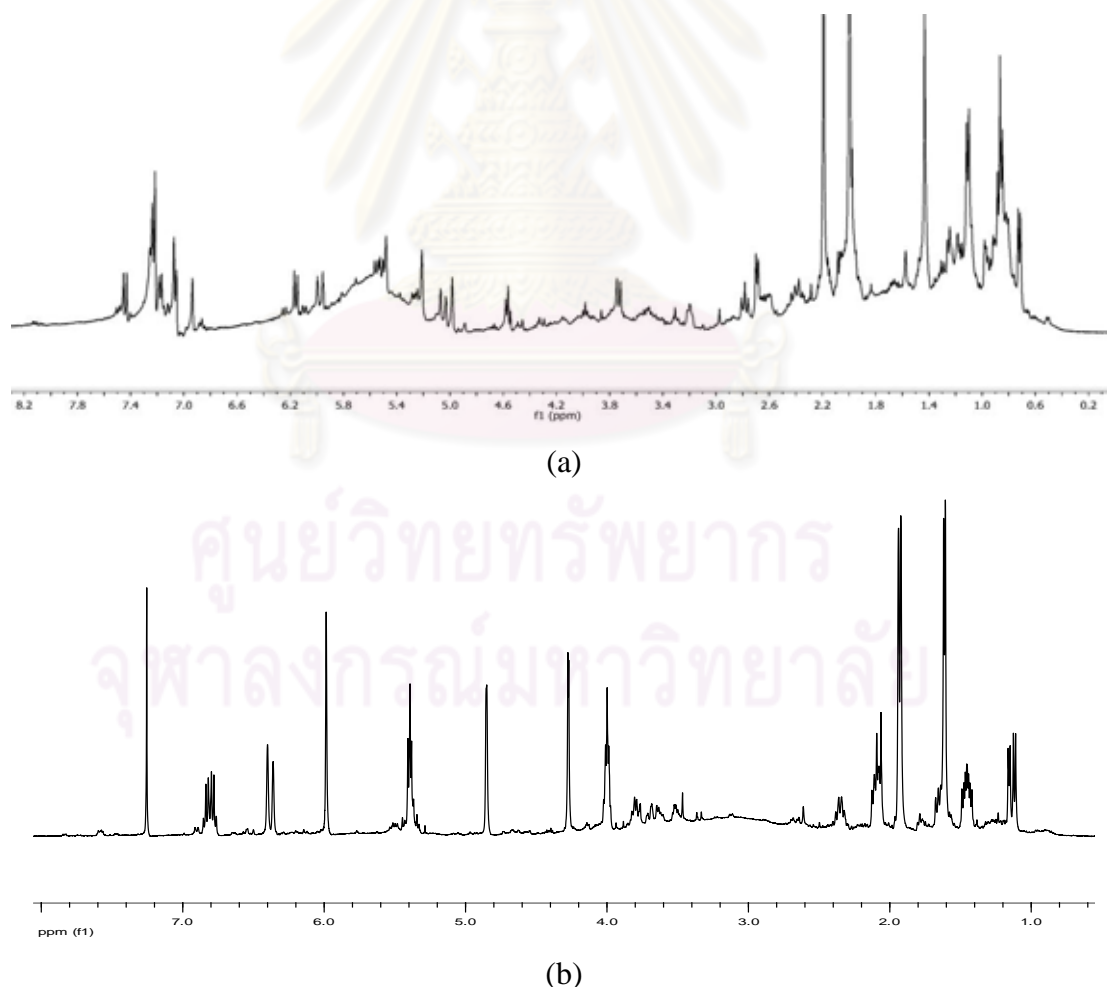
Figure 3.4 ^{1}H NMR spectrum of EtOAc extract of fungus MS1 on CSB (a) and MEB (b)

(c)

(d)

(e)
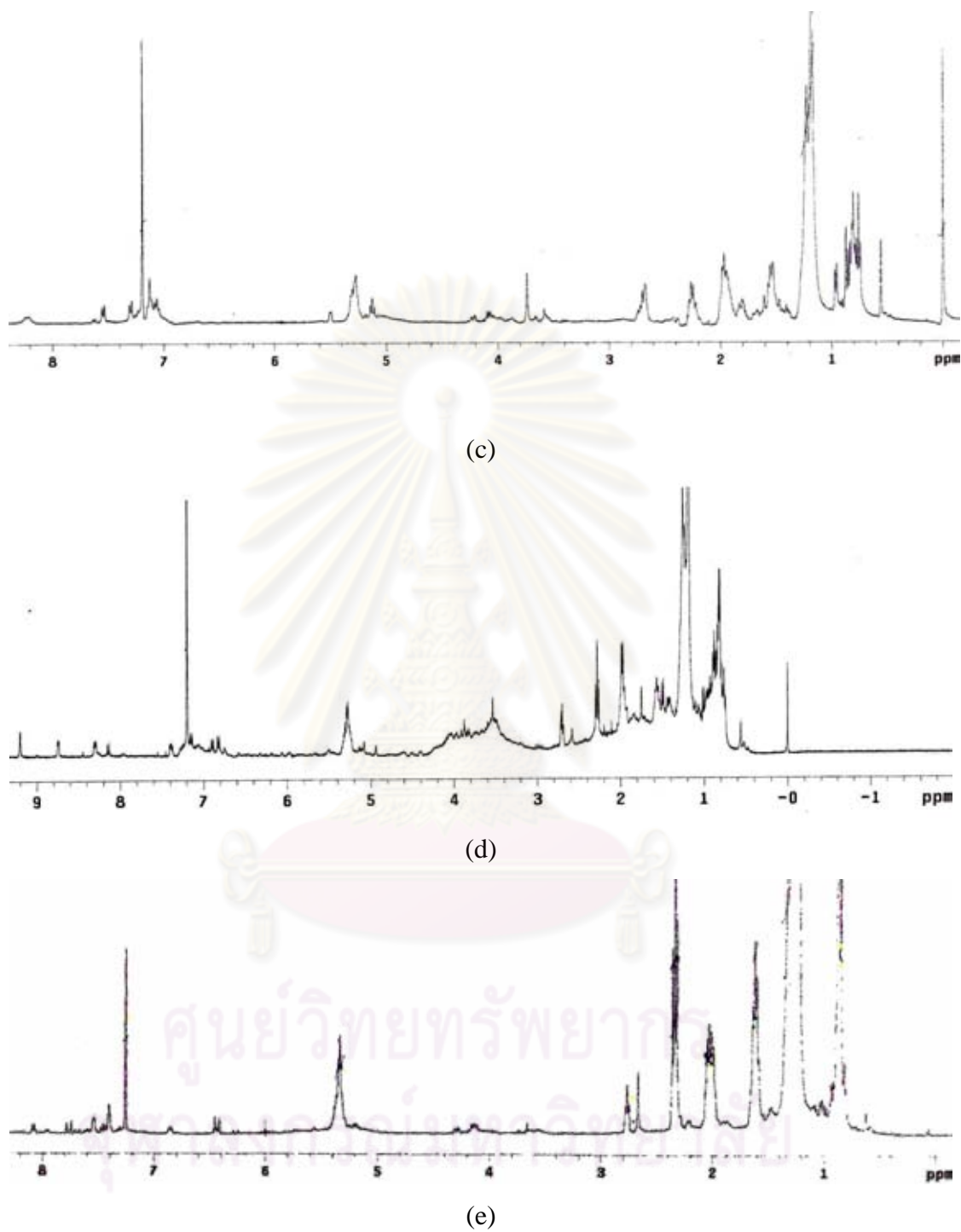
Figure 3.5 ^{1}H MNR spectrum of EtOAc extracts of isolates CI3 (c), CI4 (d) and CI5 (e) each grown on solid rice medium

3.3 Identification and taxonomy of selected endophytic fungi

Identification and taxonomy of five fungal isolates, MS1, CI3, CI4, and CI5, was performed on the basis of morphological characteristics and sequencing of the PCR product amplified from chromosomal DNA of each isolate.

3.3.1 Identification of selected endophytic fungi

3.3.1.1 Fungal strain MS1

The fungus MS1 was obtained from the leaves of *M. siamensis* collected from Rayong province, Thailand, in May 2008. The fungus on PDA agar showed typical suede-like cinnamon-buff to sand brown colonies, while reverse was yellow to deep dirty brown. Its conidial heads are compact, columnar and biseriate and conidiophores are hyaline and smooth-walled, and conidia are globose to ellipsoidal, hyaline to slightly yellow and smooth-walled (Figures 3.6a and 3.6b). The above information is related to the characteristics of a strain in the genus *Aspergillus*. Sequencing of the PCR product amplified from chromosomal DNA of isolate MS1 resulted in a 538 bp fragment. This comprised partial ITS1, complete 5.8S-ITS2 sequences, and partial of the 28S sequence of rRNA gene, as shown in Figure 3.6c. The ITS1-5.8S-ITS2 sequences of MS1 fungus was perfectly matched 100% homology with that of *Aspergillus terreus* (GenBank Accession number HQ636423).

obverse   reverse

(a)

Figure 3.6 Fungal strain MS1 (a) colony morphology on PDA (b) conidia (c) DNA sequence of the ITS1-5.8S-ITS2 rRNA gene

(b)
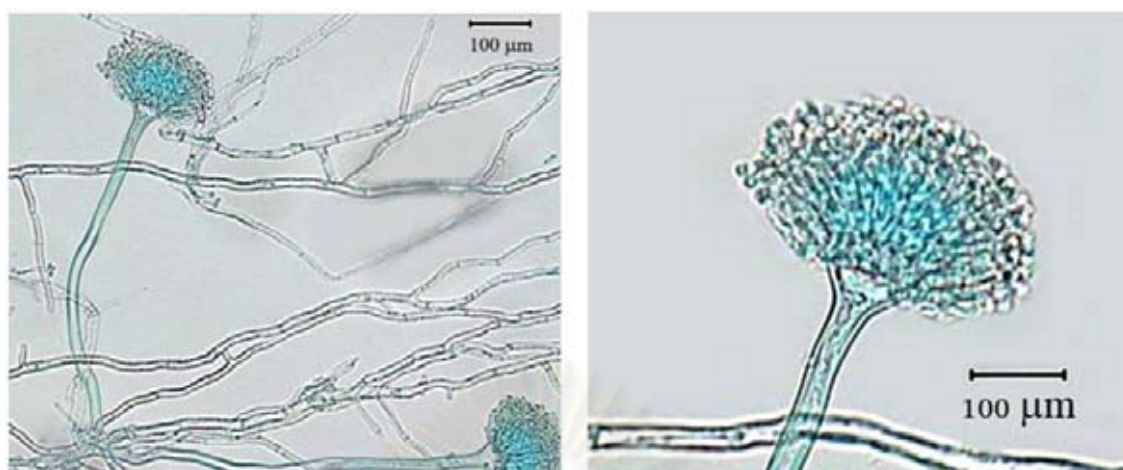
			ITS1	
10	20	30	40	50
TCTTTATGGC	CAACCTCCCA	CCCGTGACTA	TTGTACCTTG	TTGCTTCGGC
GGGCCCGCCA	GCGTTGCTGG	CCGCCGGGGG	GCGACTCGCC	CCCGGGCCCG
TGCCCGCCGG	AGACCCCAAC	ATGAACCCTG	TTCTGAAAGC	TTGCAGTCTG
AGTGTGATTC	TTTGCAATCA	GTTAA**AACTT**	**TCAACAATGG**	**ATCTCTTGGT**
TCCGGCATCG	**ATGAAGAACG**	**CAGCGAAATG**	**CGATAACTAA**	**TGTGAATTGC**
AGAATTCAGT	**GAATCATCGA**	**GTCTTTGAAC**	**GCACATTGCG**	**CCCCCTGGTA**
TTCCGGGGGG	**CATGCCTGTC**	**CGAGCGTCAT**	**T**GCTGCCCTC	AAGCCCGGCT
TGTGTGTTGG	GCCCTCGTCC	CCCGGCTCCC	GGGGGACGGG	CCCGAAAGGC
AGCGGCGGCA	CCGCGTCCGG	TCCTCGAGCG	TATGGGGCTT	CGTCTTCCGC
TCCGTAGGCC	CGGCCGGCGC	CCGCCGACGC	ATTTATTTGC	AACTTGTTTTT
TTTCCAGGTT	GACCTCGGAT	CAGGTAGGGA	TACCCGCT	

ITS1 5.8S

5.8S ITS2

ITS2 28S

(c)

Figure 3.6 Fungal strain MS1 (a) colony morphology on PDA (b) conidia (c) DNA
sequence of the ITS1-5.8S-ITS2 rRNA gene (continue)

3.3.1.2 Fungal strain CI3

The fungus CI3 was isolated from the leaves of *C. inophyllum* collected from Nakhon Pathom province, Thailand, in May 2008. It displayed white colonies and did not produce any pigments when grown on PDA (Figure 3.7a). Sequencing of the PCR product amplified from chromosomal DNA of isolate CI3 resulted in a 536 bp fragment. This comprised partial ITS1, complete 5.8S-ITS2 sequences, and partial of the 28S sequence of rRNA gene, as shown in Figure 3.7b. The ITS1-5.8S-ITS2 sequences of CI3 fungus was 99% homology with that of *Glomerella cingulata* (GenBank Accession number HQ636422).

(a)

```
         10              20          ITS1 30       40              50
ACGCTCTTAA  CCCTTTGTGA  ACATACCTAT  AACTGTTGCT  TCGGCGGGTA
GGGTCTCCGT  GACCCTCCCG  GCCTCCCGCC  CCCGGGCGGG  TCGGCGCCCG
CCGGAGGATA  ACCAAACTCT  GATTTAACGA  CGTTTCTTCT  GAGTGGTACA
   ITS1         5.8S
AGCAAATAAT  CAAAACTTTT  AACAACGGAT  CTCTTGGTTC  TGGCATCGAT
GAAGAACGCA  GCGAAATGCG  ATAAGTAATG  TGAATTGCAG  AATTCAGTGA
ATCATCGAAT  CTTTGAACGC  ACATTGCGCC  CGCCAGCATT  CTGGCGGGCA
         5.8S        ITS2
TGCCTGTTCG  AGCGTCATTT  CAACCCTCAA  GCTCTGCTTG  GTGTTGGGGC
CCTACAGCTG  ATGTAGGCCC  TCAAAGGTAG  TGGCGGACCC  TCCCGGAGCC
TCCTTTGCGT  AGTAACTTTA  CGTCTCGCAC  TGGGATCCGG  AGGGACTCTT
                           ITS2        28S
GCCGTAAAAC  CCCCCAATTT  TCCAAAGGTT  GACCTCGGAT  CAGGTAGGAA
TACCCGCTGA  ACTTAAGCAT  ATCAATAAGC  GGAGGA
```

(b)

Figure 3.7 Fungal strain CI3 (a) colony morphology on PDA (b) DNA sequence of the ITS1-5.8S-ITS2 rRNA gene

3.3.1.3 Fungal strain CI4

The fungus CI4 was isolated from the leaves of *C. inophyllum* collected from Nakhon Pathom province, Thailand, in May 2008. The surface colony color of this fungus on PDA medium was initially white becoming olive gray, with a tint of pink, and it produced a dark brown diffusible pigment on the reverse (Figure 3.8a). Sequencing of the PCR product amplified from chromosomal DNA of isolate CI4 resulted in a 499 bp fragment. This comprised partial ITS1, complete 5.8S-ITS2 sequences, and partial of the 28S sequence of rRNA gene, as shown in Figure 3.8b. The ITS1-5.8S-ITS2 sequences of CI4 fungus was 99% homology with that of *Phoma herbarum* (GenBank Accession number HQ636420).
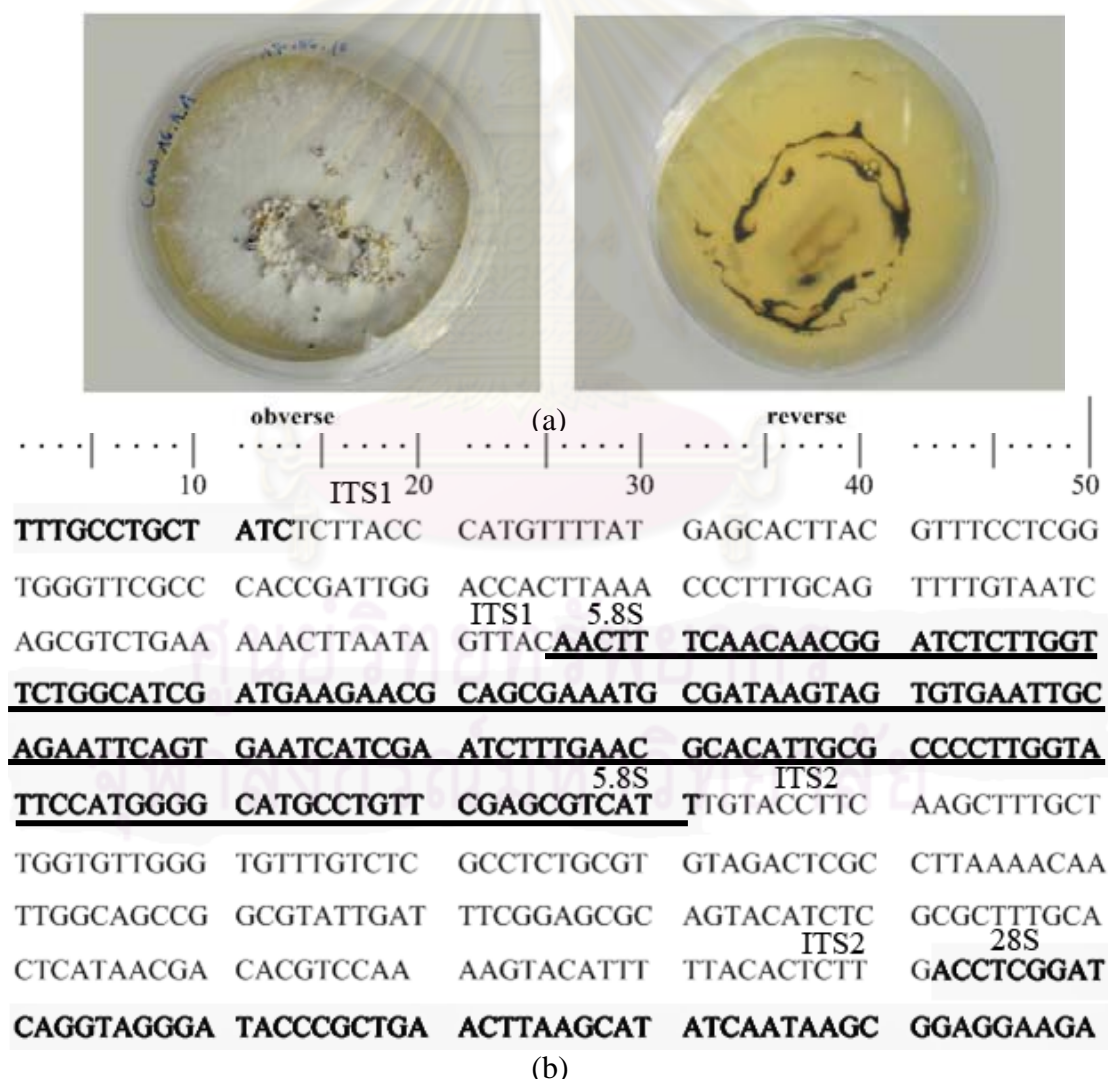
Figure 3.8 Fungal strain CI4 (a) colony morphology on PDA (b) DNA sequence of the ITS1-5.8S-ITS2 rRNA gene

3.3.1.4 Fungal Strain CI5

The fungus CI5 was isolated from the leaves of *C. inophyllum* collected from Nakhon Pathom province, Thailand, in May 2008. It showed flat, grey colonies and the reverse was dark brown when cultured on PDA agar as shown in Figure 3.9a. Sequencing of the PCR product amplified from chromosomal DNA of isolate CI5 resulted in a 511 bp fragment. This comprised partial ITS1, complete 5.8S-ITS2 sequences, and partial of the 28S sequence of rRNA gene, as shown in Figure 3.9b. The ITS1-5.8S-ITS2 sequences of CI5 fungus was 99% homology with that of *Ascomycete* sp. (GenBank Accession number HQ636419).

(a)

```
         obverse                                    reverse
. . . .|. . . .|  . . . .|. . . .|  . . . .|. . . .|  . . . .|. . . .  . . . .|. . . .|
       10                 20          ITS1 30              40                 50
TATGAAGGCT  TCGGCTGGAT  TATTTATTTC  ACCCTTGTCT  TTTGCGCACT
TGTTGTTTCC  TGGGCGGGTT  CGCCCGCCAC  CAGGACCACA  CCATAAACCT
                                                          ITS1
TTTTTGTTAA  TGCAGTCAGC  GTCAGTACAA  CAAATGTAAA  TCATTTACAA
        5.8S
CTTTCAACAA  CGGATCTCTT  GGTTCTGGCA  TCGATGAAGA  ACGCAGCGAA
ATGCGATACG  TAGTGTGAAT  TGCAGAATTC  AGTGAATCAT  CGAATCTTTG
AACGCACATT  GCGCCCTTTG  GTATTCCAAA  GGGCATGCCT  GTTCGAGCGT
5.8S    ITS2
CATTTGTACC  CTCAAGCTTT  GCTTGGTGTT  GGGCGTTTTG  TCTTTGCTCG
CCAAAGACTC  GCCTTAAAAC  GATTGGCAGC  CGACCTCTTG  GTTTCGCAGC
GCAGCACAAT  TTTGCGCTTG  CAAATCAGCA  AGTTGGCACT  CCATCAAGAA
        ITS2                28S
CATTTTCTTA  CGTTTGACCT  CGGATCAGGT  AGGGATACCC  GCTGAACTTA
AGCATATCAA  T
```

(b)

Figure 3.9 Fungal strain CI5 (a) colony morphology on PDA (b) DNA sequence of the ITS1-5.8S-ITS2 rRNA gene

3.3.2 Taxonomy of selected endophytic fungi

The taxonomy classification of each fungal isolate selected on the basis of morphological characteristics and DNA sequence analysis as mentioned above, could be summarized in Table 3.5.

Table 3.5. Taxonomy of fungal strains MS1, CI3, CI4, and CI5

	MS1	CI3	CI4	CI5
Kingdom	Fungi	Fungi	Fungi	Fungi
Phylum	Ascomycota	Ascomycota	Ascomycota	Ascomycota
Class	Eurotiomycetes	Sordariomycetes	Dothideomycetes	Not assigned
Order	Eurotiales	Not assigned	Pleosporales	Not assigned
Family	Trichocomaceae	Glomerellaceae	Not assigned	Not assigned
Genus	Aspergillus	Glomerella	Phoma	Ascomycete
Species	*A. terreus*	*G. cingulata*	*P. herbarum*	Not assigned

3.4 Secondary metabolites from selected fungi

3.4.1 Secondary metabolites from the fungus *Aspergillus terreus* (MS1)

Chromatographic separation of EtOAc crude extract of culture broth of *A. terreus* on CSB medium led to the isolataion of butyrolactones I (**1**), II (**2**) and aspernolide B (**3**) as major constituents. In addition, compounds **4** and **5**, as well as butyrolactones III (**6**) and IV (**7**) were isolated as minor metabolites after fractionation and purification by Sephadex LH20 and silica gel column chromatography. It was further revealed that compounds **4** and **5** were novel derivatives of butyrolactone I and thus named as aspernolide D and asperterone, respectively. Interestingly, it was found that this fungus produced absolutely different structural compound, namely terrein (**8**), in relatively high yield (13.8 g/5L) when cultured on MEB medium. This suggested that the medium source has much effect on the metabolite production of this fungus. The structures of isolated compounds are shown in Figure 3.10.

butyrolactone I (**1**)

butyrolactone II (**2**)

aspernolide B (**3**)

aspernolide D (**4**)

asperterone (**5**)

butyrolactone III (**6**)

butyrolactone IV (**7**)

terrein (**8**)
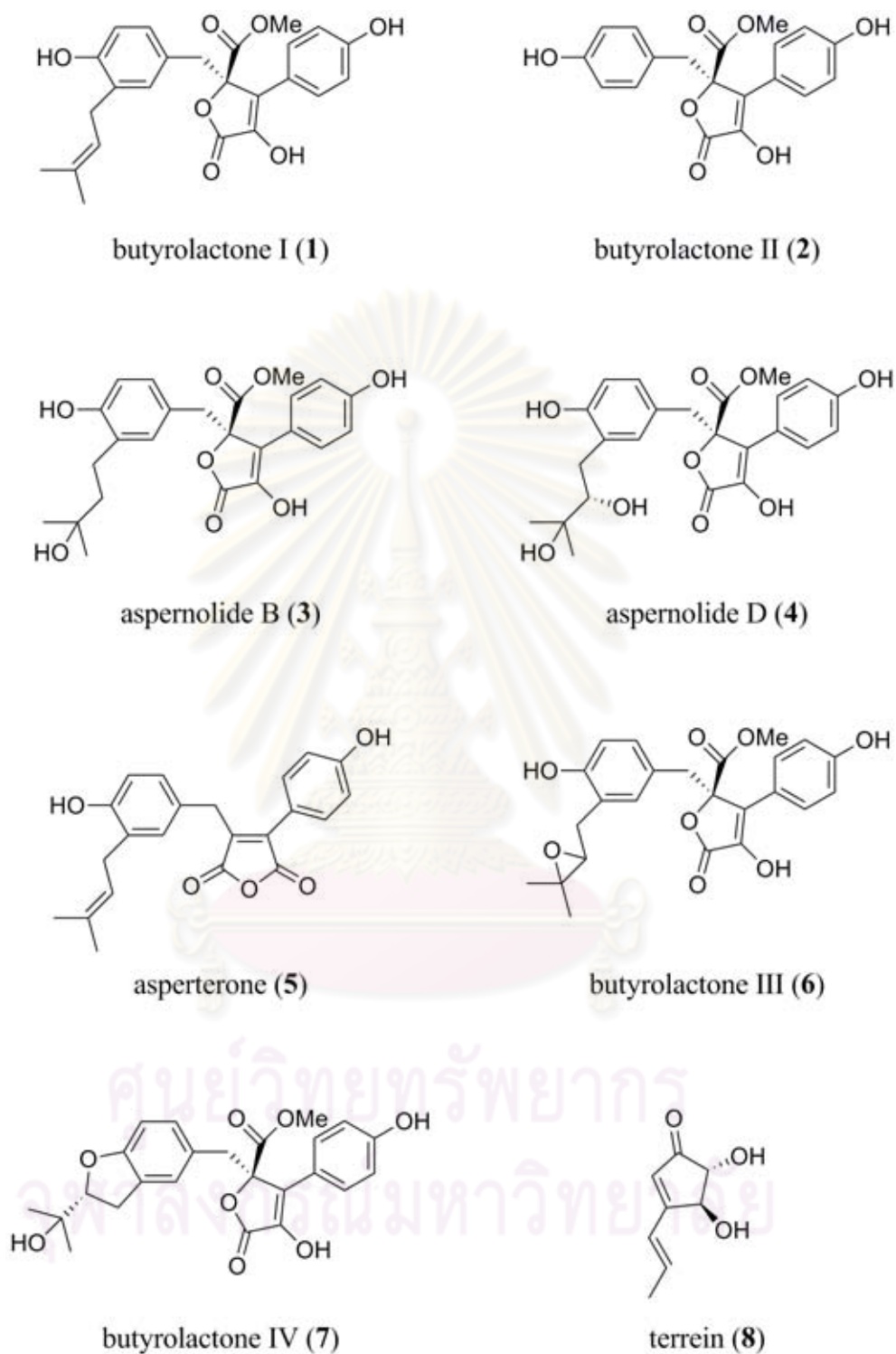
Figure 3.10 Chemical structures of secondary metabolites of *A. terreus*

3.4.1.1 Structural elucidation of compound 1
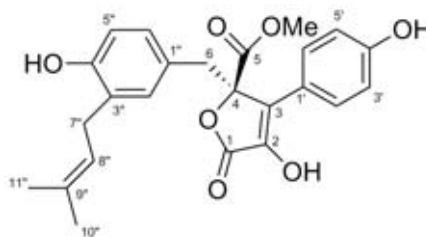
Figure 3.11 Structure of compound **1**

molecular formula	$C_{24}H_{24}O_7$
appearance	colorless solid
m.p.	92-94 ^{o}C
$[\alpha]_{20}^D$	+83 (*c* 0.2, MeOH)
IR (KBr) ν_{max}	3343, 1739, 1604, 1517 cm^{-1}
^{1}H and ^{13}C NMR	see Table 3.6

Compound **1**, isolated as a colorless solid, had the molecular formula $C_{24}H_{24}O_7$ as established by NMR data, implying 13 degree of unsaturations. The ^{1}H NMR spectrum revealed typical signals of two tertiary methyls (δ_H 1.56 and 1.60), a methoxy group (δ_H 3.68), an olefinic proton (δ_H 5.01, t, *J* = 7.2 Hz), three aromatic protons of a 1,2,4-trisubstituted phenol [δ_H 6.42 (s br), 6.44 (d, *J* = 8.4 Hz), 6.50 (dd, *J* = 8.4, 2.0 Hz)] and additional two protons of a 1,4-disubstituted phenolic moiety (δ_H 6.81 (d, *J* = 8.8 Hz), 7.52 (d, *J* = 8.8 Hz)]. Analysis of ^{13}C NMR and HSQC data suggested the presence of 10 aromatic carbon resonances for two aromatic rings, two ester carbonyls, four olefinic carbons (one oxygenated), two methylenes, an oxygenated quaternary carbon and a methoxy group as described in Table 3.6. The above data indicated that 12 of the 13 units of unsaturation came from two aromatic ring, two carbonyls and two carbon-carbon double bonds. Therefore compound **1** must have an aliphatic ring in addition to two aromatic rings. Its structure was corroborated mainly by analysis of HMBC correlations due to ^{1}H-^{1}H COSY correlations observed as shown in Figure 3.12. Protons of a pair of doublet of

methylene [δ_H 3.39, 3.42 (J = 14.8 Hz); δ_C 38.8), exhibiting HMBC correlations to C-1″, C-3″ and C-6″, and to C-4, was assigned as H$_2$-6, whereas protons of another methylene [δ_H 3.04 (d, J = 6.8 Hz); δ_C 29.5], was identified as H$_2$-7″ by strong HMBC correlations to C-2″, C-3″, C-4″ and C-8″. This also confirmed the connectivity of C-7″–C-11″ sidechain, an isopentenyl fragment, and C-3″. Similarly, observed HMBC correlations of H-2′/C-3 clarified the attachment of a 1,4-disubstituted phenol ring to C-3. Furthermore, comparison of NMR data of **1** with those previously reported by Cazar et al. [64] confirmed that compound **1** was butyrolactone I as shown in Table 3.6.
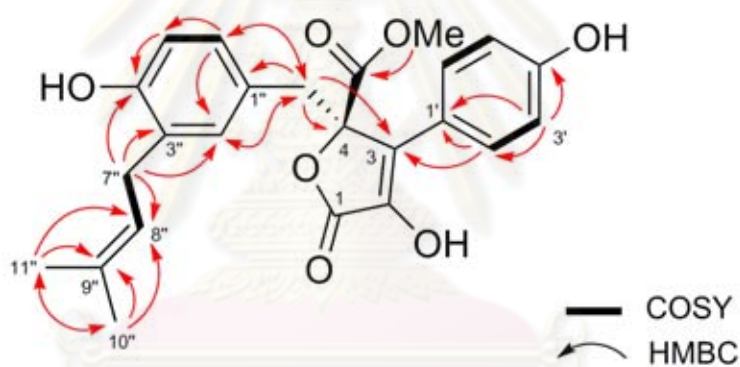
Figure 3.12 Key ^{1}H-^{1}H COSY and HMBC correlations of **1**

Table 3.6 NMR spectroscopic data (CDCl$_3$) of compound **1** and butyrolactone I

position	butyrolactone I		compound **1**	
	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C, mult
1		169.6		169.5, qC
2		137.4		137.4, qC
3		128.5		128.1, qC
4		86.2		86.3, qC
5		169.9		170.1, qC
6	3.58 d (14.7)	38.7	3.42 d (14.8)	38.8, CH$_2$
	3.52 d (14.2)		3.39 d (14.8)	
1'		122.1		122.6, qC
2', 6'	7.65 d (8.8)	129.1	7.52 d (8.8)	129.9, CH
3', 5'	6.90 d (8.8)	116.1	6.81 d (8.8)	116.3, CH
4'		157.0		156.7, qC
1"		124.6		124.9, qC
2"	6.51 d (2.0)	131.9	6.42 s br	132.2, CH
3"		126.8		126.7, qC
4"		153.2		153.6, qC
5"	6.52 d (7.8)	115.1	6.44 d (8.4)	115.5, CH
6"	6.58 dd (7.8, 2.0)	129.6	6.50 dd (8.4, 2.0)	129.6, CH
7"	3.12 d (7.3)	28.4	3.04 d (6.8)	29.5, CH$_2$
8"	5.08 t br (7.3)	121.5	5.01 t (7.2)	121.8, CH
9"		134.1		134.9, qC
10"	1.65 s	25.7	1.56 s	26.0, CH$_3$
11"	1.70 s	17.2	1.60 s	18.1, CH$_3$
5-OMe	3.80 s	53.6	3.68 s	53.9, CH$_3$

3.4.1.2 Structural elucidation of compound 2
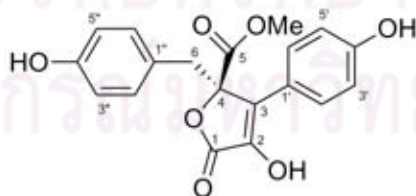
Figure 3.13 Structure of compound **2**

molecular formula	$C_{19}H_{16}O_7$
appearance	light yellow gum
$[\alpha]_{20}^{D}$	+82 (*c* 0.1, MeOH)
IR (KBr) ν_{max}	3356, 1739, 1604,
	1510, 1203 cm^{-1}
^{1}H and ^{13}C NMR	see Table 3.7

Compound **2** was obtained as a light yellow gum. Its molecular formula was established as $C_{19}H_{16}O_7$ according to NMR data, indicating 12 degrees of unsaturation. The ^{1}H-NMR spectrum (Table 3.7) displayed two pairs of doublets A_2B_2 system at δ_H 6.97 (d, *J* = 8.8 Hz) and 7.63 (d, *J* = 9.2 Hz), and at δ_H 6.58 (d, *J* = 8.8 Hz) and 6.67 (d, *J* = 8.8 Hz), indicating the existence of two para-disubstituted phenyl groups. In addition, the presence of a methylene and a methoxy group was inferred from the singlet signal at δ_H 3.48 and 3.78, respectively. Comparison of ^{1}H and ^{13}C NMR data of **2** with those of butyrolactone I (**1**) revealed that they possessed the same core structure. The marked difference was the absence of the isopentenyl side chain in compound **2**, and the presence of an additional para-disubstituted benzene ring. This suggested that the isopentenyl group in **1** was replaced by hydrogen atom in **2**. The structure of **2** was further confirmed by its ^{1}H-^{1}H COSY and HMBC correlations (Figure 3.14), and by comparing its NMR data to those previously reported [65] as shown in Table 3.7.
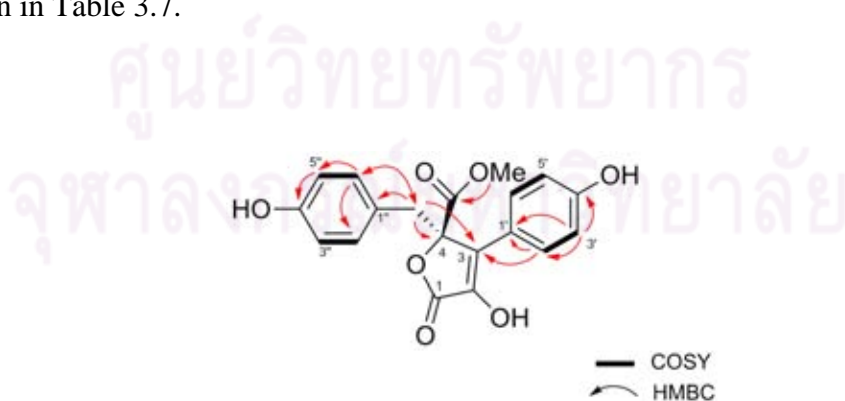
Figure 3.14 Key ^{1}H-^{1}H COSY and HMBC correlations of **2**

Table 3.7 NMR spectroscopic data of compound **2** and butyrolactone II

| position | butyrolactone II[a] | compound **2**[b] | |
	δ_C	δ_H, mult (J in Hz)	δ_C, mult
1	168.1		167.8, qC
2	138.3		138.2, qC
3	127.7		127.3, qC
4	84.9		85.0, qC
5	169.9		169.9, qC
6	38.2	3.48 s	38.2, CH$_2$
1'	121.2		121.8, qC
2', 6'	129.0	7.63 d (9.2)	115.7, CH
3', 5'	116.0	6.97 d (8.8)	129.2, CH
4'	158.1		158.1, qC
1"	123.4		123.9, qC
2", 6"	131.3	6.67 d (8.8)	131.4, CH
3", 5"	114.8	6.58 d (8.8)	114.6, CH
4"	156.4		156.5, qC
5-OMe	53.5	3.78 s	52.9, CH$_3$

[a] recorded in DMSO-d_6; [b] recorded in CDCl$_3$

3.4.1.3 Structural elucidation of compound 3
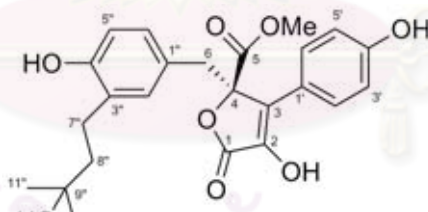
Figure 3.15 Structure of compound **3**

molecular formula	C$_{24}$H$_{26}$O$_8$
appearance	light yellow gum
$[\alpha]_{20}^D$	+113 (*c* 0.1, MeOH)
IR (KBr) ν_{max}	3360, 2987, 1756, 1604, 1517, 1204 cm^{-1}
^{1}H and ^{13}C NMR	see Table 3.8

Compound **3**, isolated as a colorless gum, had the molecular formula $C_{24}H_{26}O_8$ by analysis of NMR data. The NMR data of **3** (Table 3.8) also displayed characteristic signals associated with a γ-lactone in butyrolactone-type series, including a 1,4-disubstituted phenyl ring [δ_H 7.58 (d, J = 8.8 Hz), 6.94 (d, J = 8.8 Hz); δ_C 121.9 qC, 117.6 CH (x2), 131.1 CH (x2), 159.9 qC], an unsymmetrical trisubstituted phenyl ring [δ_H 6.49 (s br), 6.41 (d, J = 8.4 Hz), 6.52 (dd, J = 8.4, 1.6 Hz); δ_C 129.3 qC, 123.7 qC, 133.4 CH, 118.1 CH, 154.9 qC], a methoxycarbonyl group [δ_H 3.76 s; δ_C 54.7 CH_3, 169.6 qC], and four quaternary carbons of γ-lactone ring [δ_C 86.9, 126.0, 140.1, 169.6]. Moreover, the NMR data of **3** was closely related to those of butyrolactone I (**1**), with the only difference being the presence of a hydroxyl group at C-9" and an additional methylene group (C-8") in **3** instead of the carbon-carbon double bond of **1**. This was confirmed by ^{1}H-^{1}H COSY correlation of H_2-7"/ H_2-8", and by observed HMBC correlations of H_3-10"/C-9", H_3-10"/C-8", H_3-11"/C-9" and H_3-11"/C-8". Comparison of NMR data of **3** with those reported by Parvatkar et al, depicted in Table 3.8, also clarified that compound **3** was aspernolide B and its structure is shown in Figure 3.16. Aspernolide B was first reported in 2009 and isolated from the same fungus *A. terreus*; however, from different fungal sources. That fungal was obtained from a soft coral *Sinularia kavarattiensis* [66], while the source of our fungus was a terrestrial plant *Mammea siamensis*.
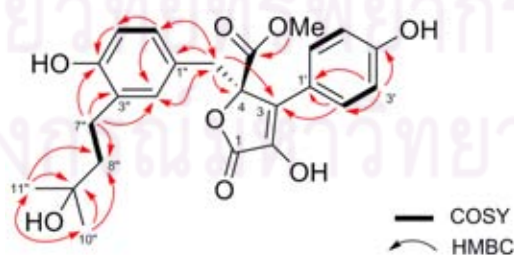
Figure 3.16 Key ^{1}H-^{1}H COSY and HMBC correlations of **3**

Table 3.8 NMR spectroscopic data of compound **3** and aspernolide B

position	aspernolide B[a]		compound **3**[b]	
	δ_H, mult (J in Hz)	δ_C	δ_H, mult (J in Hz)	δ_C, mult
1		169.2		169.6, qC
2		137.9		140.1, qC
3		128.3		126.0, qC
4		85.6		86.9, qC
5		170.1		171.9, qC
6	3.46 s	38.3	3.42 d (2.4)	34.2, CH_2
1'		121.6		121.9, qC
2', 6'	7.55 d (8.7)	129.1	7.58 d (8.8)	131.1, CH
3', 5'	6.87 d (8.7)	115.6	6.94 d (8.8)	117.6, CH
4'		157.5		159.9, qC
1"		128.4		129.3, qC
2"	6.41 d (1.8)	131.7	6.49 s br	133.4, CH
3"		124.0		123.7, qC
4"		153.2		154.9, qC
5"	6.52 d (8.7)	114.6	6.41 d (8.4)	118.1, CH
6"	6.55 dd (8.7, 1.8)	128.6	6.52 dd (8.4, 1.6)	130.9, CH
7"	2.37 m	24.2	2.54 m, 2.01 m	23.8, CH_2
8"	1.53 t (7.8)	43.2	1.68 (6.8)	40.2, CH
9"		70.8		75.6, qC
10"	1.20 s	28.4	1.20 s	27.9, CH_3
11"	1.19 s	28.5	1.19 s	28.0, CH_3
5-OMe	3.76 s	53.5	3.76 s	54.7, CH_3

[a] recorded in $CDCl_3$ + 2 drops of CD_3OD; [b] recorded in acetone-d_6

3.4.1.4 Structural elucidation of compound 4

Figure 3.17 Structure of compound **4**

molecular formula	$C_{24}H_{26}O_8$
appearance	light yellow gum
$[\alpha]_{20}^{D}$	+ 40 (c 0.1, MeOH)
UV (MeOH) λ_{max} (log ε)	290 (4.14) nm
IR (KBr) ν_{max}	3443, 2956, 1697, 1633, 1620, 1513, 1439, 1262, 1108, 939 cm^{-1}
HRESIMS (m/z)	481.1472 [M + Na]$^+$, calcd 481.1475
^{1}H and ^{13}C NMR	see Table 3.9

Compound **4** was isolated as a light yellow gum and its molecular formula was assigned to be $C_{24}H_{26}O_9$ by HRESIMS analysis (m/z 481.1472 [M + Na]$^+$, calcd 481.1475), indicating 12 degrees of unsaturation. IR absorptions at 3443 and 1697 cm^{-1} implied the presence of hydroxyl and carbonyl groups, respectively. The ^{1}H NMR spectrum (Table 1) showed the signals of two methyls at δ_H 1.12 and 1.27, a methoxy group at δ_H 3.67, and of A_2B_2 system at δ_H 6.90 (d, J = 8.8 Hz) and 7.60 (d, J = 8.8 Hz), indicating the presence of a para-disubstituted benzene ring. Another three aromatic signals at δ_H 6.47 (d, J = 8.4 Hz), 6.54 (d, J = 8.4 Hz), and 6.64 (s) were suggestive of the existence of an additional unsymmetrical trisubstituted benzene ring in the molecule. Analysis of ^{13}C NMR spectrum and the information from its 2D NMR studies (^{1}H-^{1}H COSY, HSQC, and HMBC) revealed the presence of 10 aromatic carbon resonances for two benzene moieties, an ester carbonyl (δ_C 169.4), tetrasubstituted olefinic carbons (δ_C 128.2, 137.4), two methylenes [δ_H 2.97 m, δ_C 30.5; δ_H 3.47 (d, J = 14.4 Hz), 3.54 (d, J = 14.4 Hz), δ_C 38.8], two oxygenated quaternary carbons (δ_C 72.4, 86.0), an oxygenated methine [δ_H 4.49 (t, J = 8.4 Hz), δ_C 89.1], and a carbomethoxy group (δ_H 3.76 s, δ_C 53.6, 169.8). The ^{1}H and ^{13}C NMR data of **4** were closely related to those of butyrolactone I (**1**), but with differences evident in the region of side chain unit (C-7" to C-11"). Observed HMBC correlations from Me-10" to C-8" and C-9", from Me-11" to C-8" and C-9", and from H$_2$-7" to C-8" (Figure 3.18) were indicative of the presence of dihydroxyl group at C-8" and C-9" in **4** instead of the $\Delta^{8",9"}$ double bond for butyrolactone I (**1**).

The absolute stereochemistry at C-8" of **4** was assigned by application of the modified Mosher's method [65-66]. The difference in chemical shift values ($\Delta\delta = \delta_S$ -

δ_R) for its MTPA esters **4a** and **4b**, indicated the *S*-configuration at C-8" (Figure 3.19). Thus, this compound was determined to be new and was named as aspernolide D.
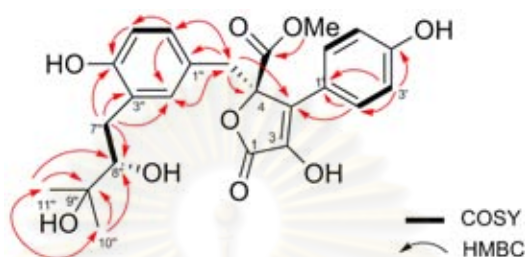
Figure 3.18 Key ^{1}H-^{1}H COSY and HMBC correlations of **4**
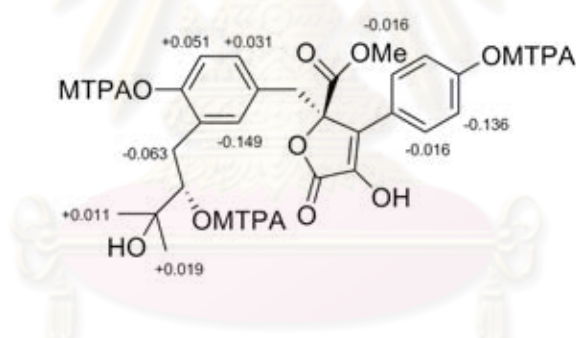
Figure 3.19 $\Delta\delta$ values of (δ_S - δ_R) of the MTPA esters of **4a** and **4b**

As the co-metabolite butyrolactone I (**1**) was determined to be 4*R*-confiugration by comparison of specific rotation data, $[\alpha]_D$ +84.32, with the previously reported result [67]. Therefore, the stereochemistry at C-4 of **1** could also be deduced to be 4*R* based on the biosynthetic grounds and similarity of the specific rotation, $[\alpha]_D$ +40. The complete structure and stereochemistry of **4** was assigned as shown in Figure 3.14.

Table 3.9 NMR spectroscopic data (CDCl$_3$) of compound **4**

position	compound **4**		
	δ_H, mult (J in Hz)	δ_C, mult	HMBC correlations
1		169.4, qC	
2		137.4, qC	
3		128.2, qC	
4		86.0, qC	
5		169.8, qC	
6	3.54 d (14.4)	38.8, CH$_2$	2, 3, 4, 1", 2", 6"
	3.47 d (14.4)		2, 3, 4, 1", 2", 6"
1'		122.1, qC	
2', 6'	7.60 d (8.8)	129.5, CH	3, 1', 3', 6' (2')
3', 5'	6.90 d (8.8)	116.1, CH	1', 2', 4', 5' (3')
4'		156.8, qC	
1"		124.7, qC	
2"	6.64 s	126.9, CH	6, 6", 7"
3"		126.7, qC	
4"		158.7, qC	
5"	6.46 d (8.4)	108.5, CH	4"
6"	6.54 d (8.4)	130.1, CH	6, 2", 4"
7"	2.97 m	30.5, CH$_2$	4", 2", 3", 8"
8"	4.49 t (8.4)	89.1, CH	9", 10", 11"
9"		72.4, qC	
10"	1.27 s	25.8, CH$_3$	8", 9", 11"
11"	1.14 s	23.9, CH$_3$	8", 9", 10"
5-OMe	3.76 s	53.6, CH$_3$	5

Aspernolide D (**4**) should be originated from butyrolactone I (**3**), and the most plausible biosynthetic pathway for the formation of compound **1** from butyrolactone I is the epoxidation of $\Delta^{7",8"}$ double bond to give the another co-metabolite, butyrolactone III, followed by ring-opening with water as shown in Scheme 3.1.
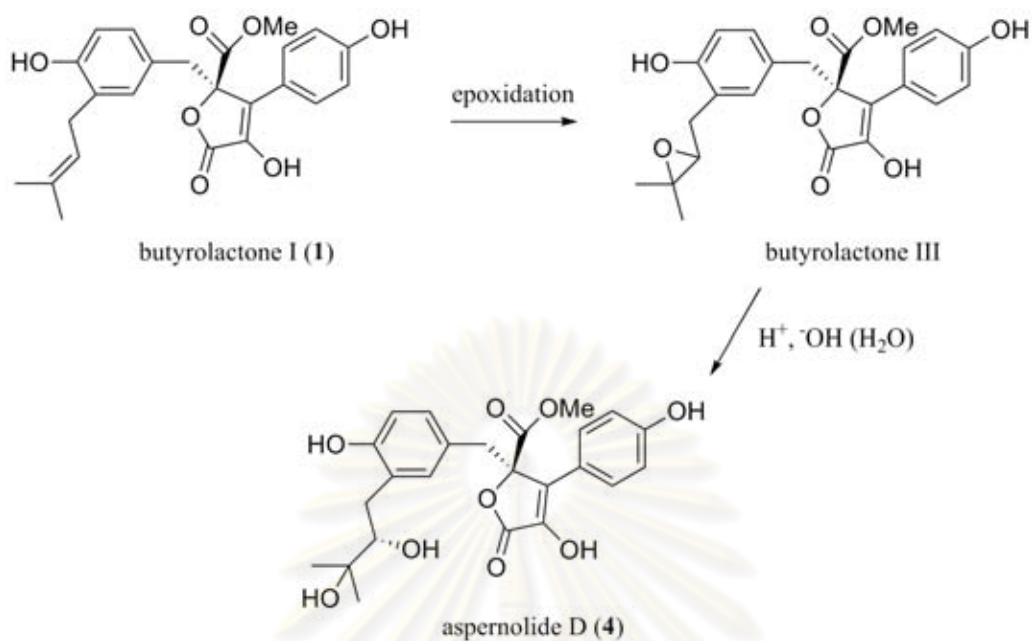
Scheme 3.1 Proposed biosynthetic pathway of compound **4**

3.4.1.5 Structural elucidation of compound 5
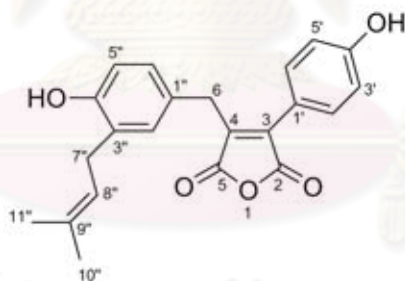
Figure 3.20 Structure of compound **5**

molecular formula	$C_{22}H_{20}O_5$
appearance	yellow gum
UV (MeOH) λ_{max} (log ε)	205 (3.97) nm
IR (KBr) ν_{max}	3443, 2926, 1730, 1660, 1604, 1511, 1439, 1238, 1170, 1114 cm^{-1}
HRESIMS (*m/z*)	387.1210 [M + Na]$^+$, calcd 387.1208
^{1}H and ^{13}C NMR	see Table 3.10

Compound **5** was isolated as a yellow gum. The molecular formula $C_{22}H_{20}O_5$ was established on the basis of the $[M + Na]^+$ peak at *m/z* 387.1210 (calcd 387.1208) in the HRESIMS, implying 13 degrees of unsaturation. The NMR spectrum of **5** (Table 3.10) displayed a similar signal pattern to those of butyrolactone I (**1**) for a para-disubstituted benzene ring, a trisubstituted benzene ring with isopentenyl unit at C-3″, except for the absence of carbomethoxy moiety and the presence of an additional ester carbonyl group. The existence of the furan-3-en-2,5-dione at the central core was corroborated by HMBC correlations from H-2′ to C-3, from H_2-6 to both ester carbonyl carbons (C-2 and C-5) at δ_C 165.5 and 166.4 (Figure 3.21) and from H_2-6 to $\Delta^{3,4}$ olefinic carbons which were considerably deshielded to resonate at δ_C 140.2 and 137.9, respectively (Figure 3.22). This suggested that each of these olefinic carbons should be connected to the ester carbonyls. In addition, two aromatic rings, isopentenyl group, two carbonyls, and a double bond account for a total of 12 unsaturation units, two ester groups must thus be connected together through an oxygen atom to form furandione ring, consistent with another unsaturation remaining. Therefore, the structure of **5** was established as shown and it was found to be new and given name as asperterone. It was further revealed that this compound was sensitive to acidic condition, since it gradually decomposed in $CDCl_3$, when left in NMR tube.
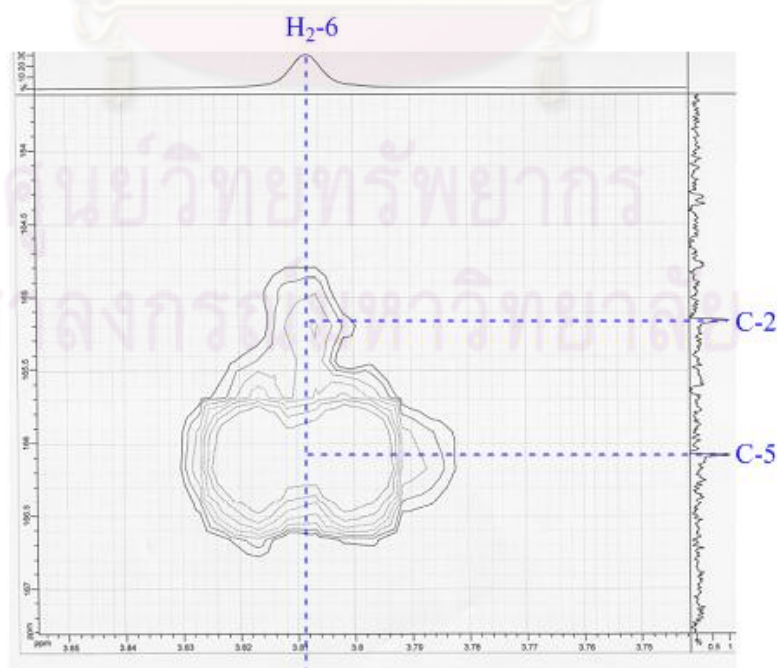
Figure 3.21 Key HMBC correlations from H_2-6 to both carbonyl carbons of **5**
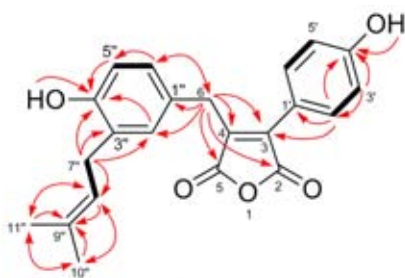
Figure 3.22 Key ^{1}H-^{1}H COSY and HMBC correlations of **5**

Table 3.10 NMR Spectroscopic Data (acetone-d_6) of compound **5**

position	compound **5**		
	δ_H, mult (J in Hz)	δ_C, mult	HMBC correlations
2		165.1, qC	
3		139.7, qC	
4		137.6, qC	
5		166.1, qC	
6	3.94 s	28.8, CH$_2$	2, 3 , 4 , 1", 2",6"
1'		118.7, qC	
2', 6'	7.64 d (8.7)	131.1, CH	3, 1', 4', 6' (2')
3', 5'	6.99 d (8.7)	115.3, CH	2', 4', 5' (3')
4'		159.5, qC	
1"		126.5, qC	
2"	7.00 s	129.1, CH	4"
3"		127.8, qC	
4"		153.4, qC	
5"	6.75 d (8.2)	114.7, CH	4"
6"	6.92 d (8.2)	126.1, CH	6, 5"
7"	3.26 d (7.4)	27.5, CH$_2$	2", 3", 4", 8"
8"	5.27 t (7.4)	122.1, CH	9", 10", 11"
9"		131.2, qC	
10"	1.69 s	24.5, CH$_3$	8", 9", 11"
11"	1.66 s	16.4, CH$_3$	8", 9", 10"
4'-OH	9.15 s		4', 6'
4"-OH	8.20 s		4"

3.4.1.6 Structural elucidation of compound 6
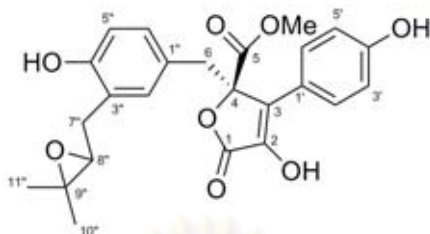
Figure 3.23 Structue of compound **6**

molecular formula	$C_{24}H_{24}O_8$
appearance	light yellow gum
$[\alpha]_{20}^D$	+ 84 (*c* 0.1, MeOH)
IR (KBr) ν_{max}	3308, 1739, 1500, 1378, 1252 cm^{-1}
^{1}H and ^{13}C NMR	see Table 3.11

Compound **6** was obtained as a light yellow gum, and its molecular formula was deduced as $C_{24}H_{24}O_8$ from NMR data. The ^{1}H and ^{13}C NMR (Table 3.11), as well as the 2D NMR spectra, suggested that compound **6** is also a butyrolactone analog possessing a para-disubstituted and a trisubstituted benzene ring. Compared to compound **4**, which has the same carbon resonances for C-7"–C-11" side chain, revealed that the obvious difference between two compounds was the upfield shift of two oxygenated carbons at C-8" (δ_C 69.6) and C-9" (δ_C 59.9) in **6**. This implied the existence of an epoxide ring between C-8" and C-9" in place of dihydroxyl group in **4**. In addition, its molecular formula $C_{24}H_{24}O_8$ required 13 degrees of unsaturation, while 12 units were present in **4**. Therefore, compound **6** must possess an additional ring. COSY correlation between C-7" and C-8", and HMBC correlations from H$_3$-10" and H$_3$-11" to C-8" and C-9", also supported the present of epoxide ring at this position (Figure 3.24). Compound **6** was determined to be butyrolactone III, which has been aforementioned as intermediate of a new compound, aspernolide D (**4**). As shown in Table 3.11, the NMR data of **6** are also in fully agreement with those previously reported by Cazar and co-workers [64].

Figure 3.24 Key ^{1}H-^{1}H COSY and HMBC correlations of **6**

Table 3.11 NMR spectroscopic data (CDCl$_3$) of compound **6** and butyrolactone III

position	butyrolactone III		compound **6**	
	δ_H, mult (J in Hz)	δ_C	δ_H, mult (J in Hz)	δ_C, mult
1		168.1		169.3, qC
2		138.3		137.4, qC
3		127.3		129.1, qC
4		84.9		85.9, qC
5		170.0		169.7, qC
6	3.53 d (14.2)	38.2	3.46 d (14.4)	38.6, CH$_2$
	3.48 d (14.2)		3.37 d (14.4)	
1'		121.2		118.1, qC
2', 6'	7.62 d (8.8)	129.0	7.52 d (8.8)	129.5, CH
3', 5'	6.91 d (8.8)	116.1	6.84 d (8.8)	116.0, CH
4'		158.1		156.6, qC
1"		123.4		122.3, qC
2"	6.51 d (2.2)	131.4	6.52 s br	132.1, CH
3"		124.8		124.7, qC
4"		156.5		156.6, qC
5"	6.50 d (8.1)	114.8	6.84 d (8.8)	116.0, CH
6"	6.57 dd (8.0, 2.0)	128.6	6.52 s br	139.1, CH
7"	2.37 m	26.6	2.80 dd (17.2, 4.8)	31.0, CH$_2$
			2.55 dd (17.2, 4.8)	
8"	2.41 m	65.4	3.69 t (5.2)	69.6, CH$_2$
9"		58.3		59.9, qC
10"	1.24 s	23.3	1.17 s	24.5, CH$_3$
11"	1.25 s	23.3	1.24 s	22.4, CH$_3$
5-OMe	3.78 s	53.5	3.70 s	54.0, CH$_3$

Butyrolactone III has been previously reported as synthetic derivative from butyrolactone I. To the best of our knowledge, this is the first report of butyrolactone III from a natural source, since it has been previously reported as synthetic derivative from the epoxidation of butyrolactone I. Moreover, discovery of the production of butyrolactone I (**1**), aspernolide D (**4**) and butyrolactone III (**6**) from the same fungus *A. terreus* also supported our biosynthetic pathway of **4** as proposed in Scheme 3.1.

3.4.1.7 Structural elucidation of compound 7
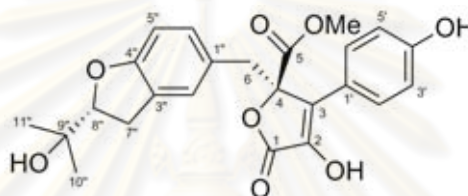
Figure 3.25 Structure of compound **7**

molecular formula	$C_{24}H_{24}O_8$
appearance	light yellow gum
$[\alpha]_{20}^{D}$	+ 80 (*c* 0.1, MeOH)
IR (KBr) ν_{max}	3295, 1730, 1613, 1439, 1269, 1182 cm^{-1}
^{1}H and ^{13}C NMR	see Table 3.12

Compound **7**, a light yellow gum, had the same molecular formula ($C_{24}H_{24}O_8$) as that of butyrolactone III (**6**). The NMR data of **7** (Table 3.12), indicating a para-disubstituted and a trisubstituted benzene ring, and a carbomethoxy group, were very similar to those of **6**. However, the resonances of side chain, a isopentyl group attached to C-3", were slightly different, particularly those of oxygenated C-8" and C-9". A strong HMBC correlation of H-8" to C-4" of a trisubstituted benzene ring indicated the connectivity of C-8" to C-4" through oxygen atom to form a furan ring. Additionally, the presence of a hydroxyl group at C-9" was clarified by its downshifted chemical shift (δ_C 72.4). Thus, compound **7** was determined as

butyrolactone IV, and this could be confirmed by comparison of its NMR data with those previously reported by Rao and co-workers [65] as shown in Table 3.12.
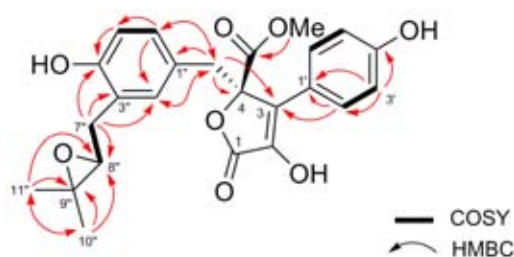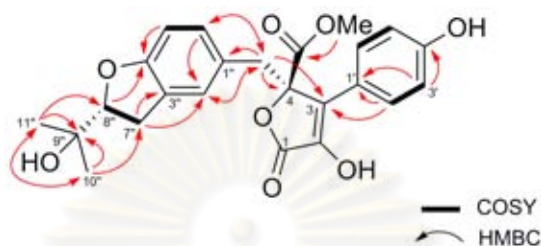

Figure 3.26 Key ^{1}H-^{1}H COSY and HMBC correlations of **7**

Table 3.12 NMR spectroscopic data of compound **7** and butyrolactone IV

position	butyrolactone IV[a]		compound **7**[b]	
	δ_H, mult (J in Hz)	δ_C	δ_H, mult (J in Hz)	δ_C, mult
1		167.9		168, qC
2		138.1		137.6, qC
3		127.6		127.5, qC
4		84.8		86.0, qC
5		169.7		169.9, qC
6	3.4 s	38.3	3.53 d (14.2)	38.2, CH$_2$
			3.46 d (14.2)	
1'		121.0		122, qC
2', 6'	7.5 d (8.6)	128.8	7.59 d (8.6)	128.6, CH
3', 5'	6.9 d (8.6)	115.8	6.85 d (8.6)	116, CH
4'		157.9		157, qC
1"		124.4		124.5, qC
2"	6.5 s	129.4	6.65 s	129.5, CH
3"		126.9		127, qC
4"		158.8		158.8, qC
5"	6.6 d (9.0)	107.8	6.47 d (8.6)	108.2, CH
6"	6.6 d (9.0)	126.6	6.55 d (8.6)	127, CH
7"	3.0 m	29.8	2.99 m	29.7, CH$_2$
8"	4.5 t (7.6)	89.0	4.49 t (8.4)	89.1, CH
9"		70.0		72.5, qC
10"	1.1 s	24.8	1.23 s	24.7, CH$_3$
11"	1.1 s	25.9	1.14 s	25.8, CH$_3$
5-OMe	3.8 s	53.4	3.74 s	53.7, CH$_3$

[a] recorded in DMSO-d_6; [b] recorded in CDCl$_3$

3.4.1.8 Structural elucidation of compound 8
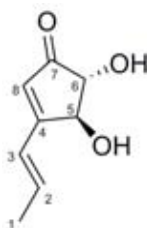
Figure 3.27 Structure of compound **8**

molecular formula	$C_8H_{10}O_3$
appearance	coloress needles
$[\alpha]_{20}^{D}$	+ 59 (*c* 0.18, MeOH)
IR (KBr) ν_{max}	3204, 2892, 1696,
	1630, 1362, 119 cm^{-1}
^{1}H and ^{13}C NMR	see Table 3.13

Compound **8** was isolated as coloress needles in relatively high yield when the fungus *A. terreus* was cultured in MEB medium. It had a molecular formula $C_8H_{10}O_3$ based on NMR data (Table 3.13). Analysis of 1D and 2D NMR data of **8** indicated the presence of a 1- propenyl group [δ_H 1.89 (d, *J* = 6.8 Hz), 6.79 (m), 6.42 (d, *J* = 15.6 Hz); δ_C 18.5 CH_3, 139.2 CH, 125.5 CH], a ketone (δ_C 202.7), a diol moiety [δ_H 4.73 (d, *J* = 4.4 Hz), 4.07 (s br); δ_C 76.9 CH, 81.4 CH] and a carbon-carbon double bond [δ_H 5.94 (s); δ_C 124.9 CH, 168.3 qC]. Based on ^{1}H-^{1}H COSY and HMBC correlations described in Figure 3.28, the structure of **8** was assigned as shown and it was further found that compound **8** was terrein, a well-known metabolite of various fungal strains including *A. terreus*, and *Penicillium* and *Phoma* genera [68-70].
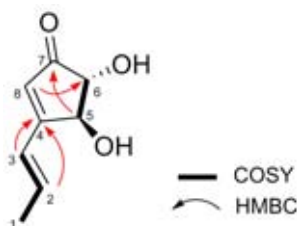
Figure 3.28 Key ^{1}H-^{1}H COSY and HMBC correlations of **8**

Table 3.13 NMR spectroscopic data (acetone-d_6) of compound **8**

position	compound **8**		
	δ_H, mult (J in Hz)	δ_C, mult	HMBC correlations
1	1.89 d (6.8)	18.5, CH_3	2, 3, 4
2	6.79 m	139.2, CH	1, 4
3	6.42 d (15.6)	125.5, CH	4, 5, 8
4		168.3, qC	
5	4.73 d (4.4)	76.9, CH	4, 6
6	4.07 s br	81.4, CH	
7		202.7, qC	
8	5.94 s	124.9, CH	3, 4, 5, 6, 7
5-OH	4.93 d (6.8)		4, 5
6-OH	4.61 d (4.0)		6

3.4.1.9 Synthetic analogs of compound 1 (butyrolactone I)

To extend the structurally array of butyrolactone compound series for looking at their structure-activity relationship, butyrolactone I (**1**), one of major constituent of *A. terreus*, was selected to be a starting material. Acid-catalyzed cyclization of 4"-OH and a $\Delta^{8",9"}$ double bond was carried out. On heating of **1** in the presence of 2% conc. HCl in MeOH, the desired product **24** was obtained in 70.0% yield, along with aspernolide C (**25**) as a minor product (9.3% yield) as shown in Scheme 3.2.
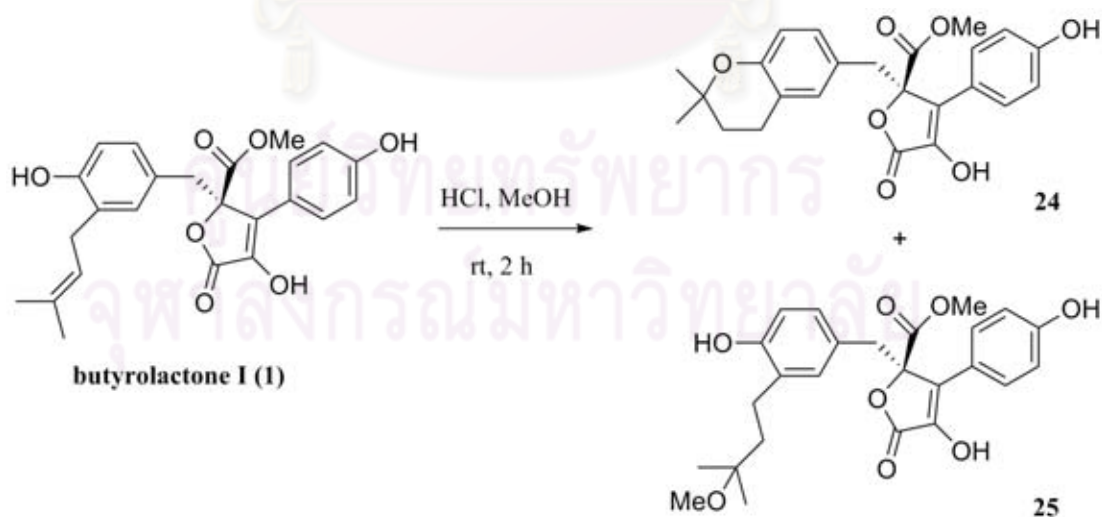
Scheme 3.2 Acid-catalyzed reaction of butyrolactone I (**1**)

According to a comparison of NMR spectra of butyrolactone I (**1**) and aspernolides A (**24**) and C (**25**), an olefinic proton signal of $\Delta^{8'',9''}$ double bond of **1** (δ_H 5.01) was absent and additional oxygenated quaternary carbon at δ_C 74.0 was observed in **24**, whereas an additional methoxy signal (δ_H 3.22) appeared in **25** (Table 3.14). The NMR data of both synthetic compounds were also in fully agreement with those previously reported by Parvatkar et al. [66].

Table 3.14 NMR spectroscopic data (CDCl$_3$) of compounds **24** and **25**

position	compound **24**		compound **25**	
	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult
1		169.1, qC		169.2, qC
2		136.9, qC		137.2, qC
3		128.1, qC		127.9, qC
4		85.8, qC		86.1, qC
5		169.5, qC		169.8, qC
6	3.44 d (14.4)	38.3, CH$_2$	3.47 d (14.8)	38.5, CH$_2$
	3.54 d (14.4)		3.53 d (14.8)	
1'		121.7, qC		122.4, qC
2', 6'	7.61 d (8.8)	129.2, CH	7.60 d (8.8)	129.7, CH
3', 5'	6.92 d (8.8)	115.7, CH	6.91 d (8.8)	116.0, CH
4'		156.5, qC		156.6, qC
1"		123.3, qC		128.5, qC
2"	6.57 s br	131.2, CH	6.48 s br	132.0, CH
3"		120.1, qC		124.1, qC
4"		152.6, qC		153.4, qC
5"	6.51 s br	116.3, CH	6.56 s br	115.8, CH
6"	6.51 s br	128.8, CH	6.56 s br	129.2, CH
7"	2.57 m	21.8, CH$_2$	2.45 t (6.8)	29.7, CH$_2$
8"	1.71 t (6.4)	32.3, CH$_2$	1.61 m	41.3, CH$_2$
9"		74.0, qC		75.8, qC
10"	1.26 s	26.4, CH$_3$	1.15 s	24.3, CH$_3$
11"	1.26 s	26.3, CH$_3$	1.14 s	24.3, CH$_3$
5-OMe	3.78 s	53.2, CH$_3$	3.79 s	53.6, CH$_3$
9-OMe			3.22 s	49.3, CH$_3$

3.4.2 Isolation of metabolites from fungal strains CI3, CI4 and CI5

The work from this part was performed at "Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Germany" under the supervision of Prof. Dr. Peter Proksch. Isolation of secondary metabolites of three fungal strains, *Colletotrichum gloeosporioides* (CI3), *Phoma herbarum* (CI4) and *Ascomycete* sp. (CI5) was carried out mostly by column chromatography over Sephadex LH20 and by semi-preparative or preparative HPLC. For the structural elucidation of isolated compounds, it was first screened by determination of their maximum UV absorption and molecular weight with ESI-MS technique. Subsequently, comparison of the compound data obtained with those in library source of the laboratory would let us know immediately that those compounds would be new or known. In the case of compounds isolated in enough amounts, they would further be subjected to ^{1}H and ^{13}C NMR measurement.

3.4.2.1 Secondary metabolites from the fungus *Colletotrichum gloeosporioides* (CI3)

The fungus *C. gloeosporioides* obtained from *C. inophyllum* was grown on solid rice medium. The combined EtOAc extract from whole culture were fractionated by vacuum liquid chromatography and column chromatography on Sephadex LH-20, respectively, as well as by semi-preparative HPLC, to afford 11 known metabolites, homodestruxin B (**9**), alternariol (**10**), deoxyterphenylin (**11**), zearalenone (**12**), indole-3-carbaldehyde (**13**), *N,N*-dimethyltryptophane (**14**), dihomo-3´-hydroxy-methyl-dihydroinfectopyron (**15**), malformin A (**16**), tenuazonic acid (**17**) and adenosine (**18**). The structures of isolated compounds are shown in Figure 3.29.

3.4.2.2 Secondary metabolites from the fungus *Phoma herbarum* (CI4)

Similarly, the EtOAc extract of whole culture of *P. herbarum* grown on solid rice medium was fractionated and purified mainly by column chromatography on Sephadex LH-20 and SiO_2 to yield Sumiki's acid (**19**), 5-carboxymellein (**20**) and cytochalasin D (**21**). The structures of isolated metabolites are displayed in Figure 3.30.

homodestruxin B (**9**)

alternariol (**10**)

deoxyterphenylin (**11**)

zearalenone (**12**)

indole-3-carbaldehyde (**13**)

N,N-dimethyltryptophane (**14**)

dihomo-3'-hydroxymethyl-
dihydroinfectopyron (**15**)

malformin A (**16**)

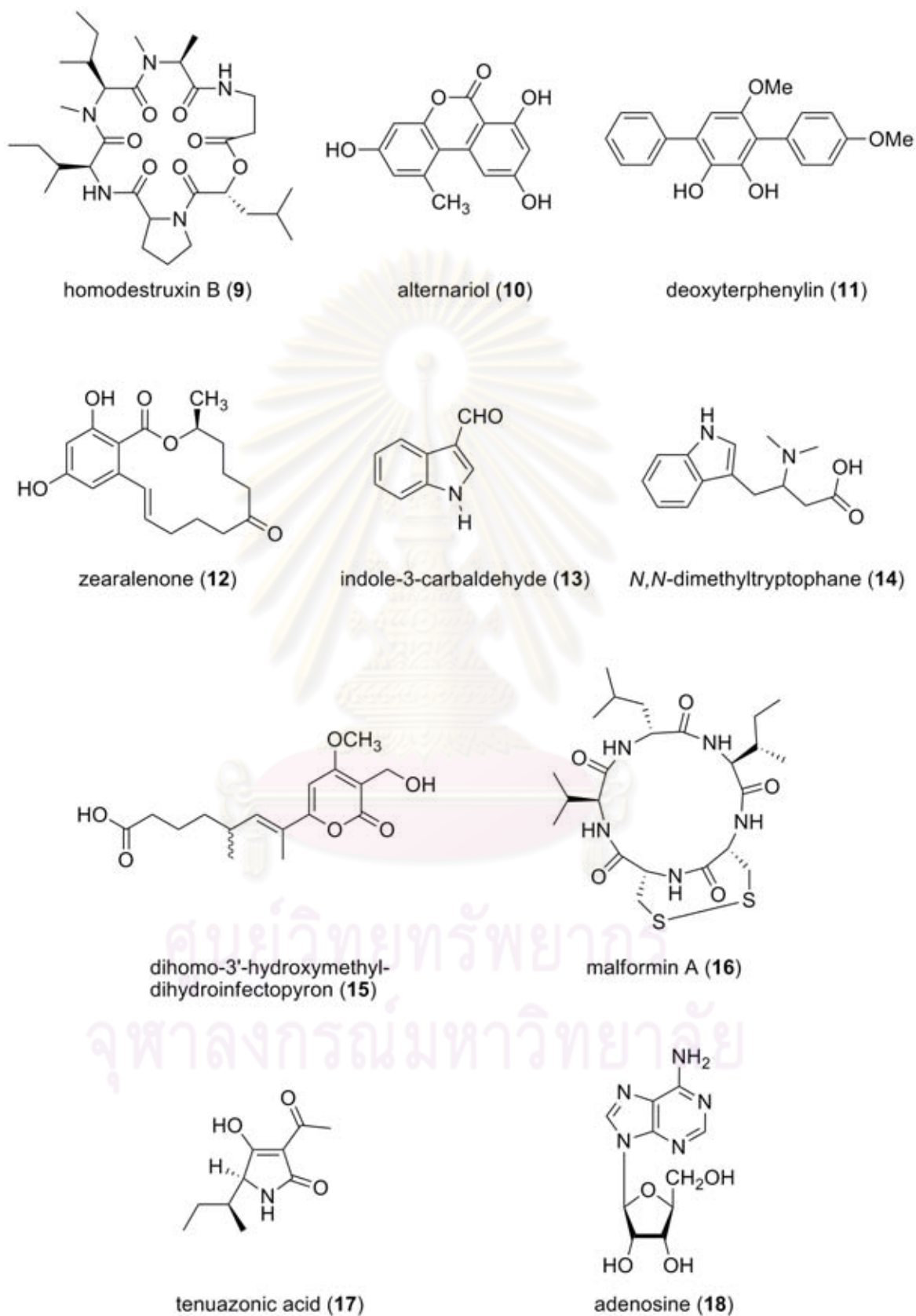tenuazonic acid (**17**)

adenosine (**18**)

Figure 3.29 Structures of isolated metabolites from *C. gloeosporioides* (CI3)
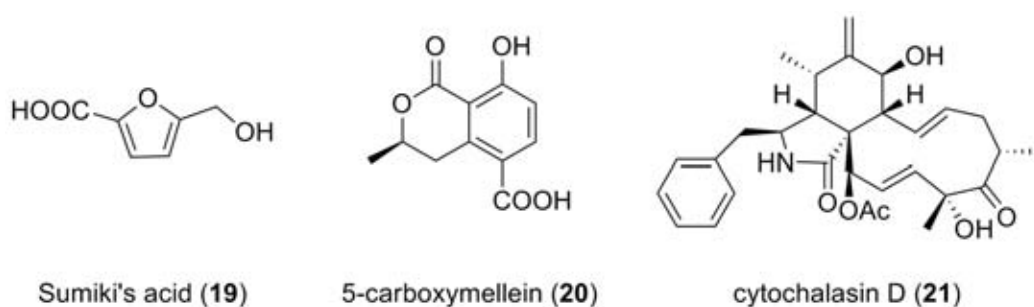
Figure 3.30 Structures of isolated metabolites from *P. herbarum* (CI4)

3.4.2.3 Secondary metabolites from the fungus *Ascomycete* sp. (CI5)

In the same way, the fungus *Ascomycete* sp. (CI5) was fermented on solid rice medium, then fractionation by vacuum liquid chromatography followed column chromatography on Sephadex LH-20 of its EtOAc extract led to the isolation of 2(4-bromophenyl) acetic acid (**22**) and citreodrimene F (**23**), along with alternariol (**10**) and tenuazonic acid (**17**), which were also produced by *C. gloeosporioides* (CI3) as abovementioned. The structures of isolated metabolites are depicted in Figure 3.31.
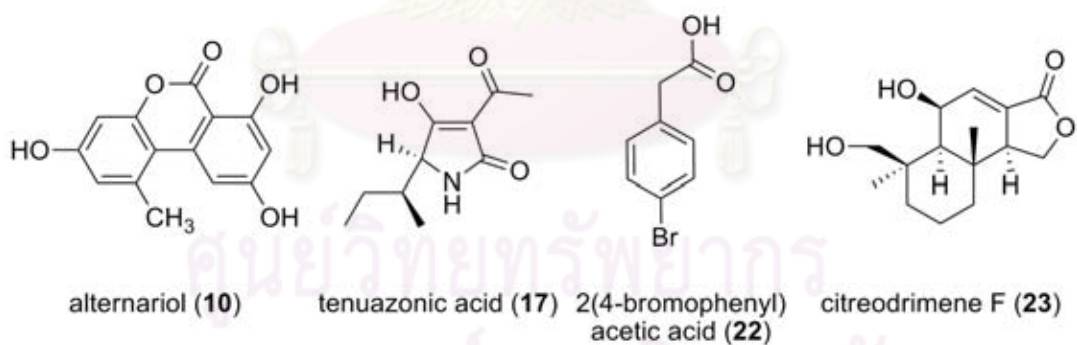

Figure 3.31 Structures of isolated metabolites from *Ascomycete* sp. (CI5)

3.4.2.4 Structural elucidation of isolated compounds from fungal strains CI3, CI4 and CI5

3.4.2.4.1 Structural elucidation of compound 9

Compound **9** was isolated as a white powder from the extract of *C. gloeosporioides* (CI3). This metabolite was determined as homodetruxin B, since its ESIMS showing a molecular ion at *m/z* 608.7 {M + H]$^+$ and UV maximum absorption at 210.8 nm, were identical to those data in library source. The structure of **9** and its UV spectrum and ESIMS are shown in Figure 3.32. Homodextruxin B was first obtained from cultures of *Alternaria brassicae*, a plant pathogenic fungus [71].

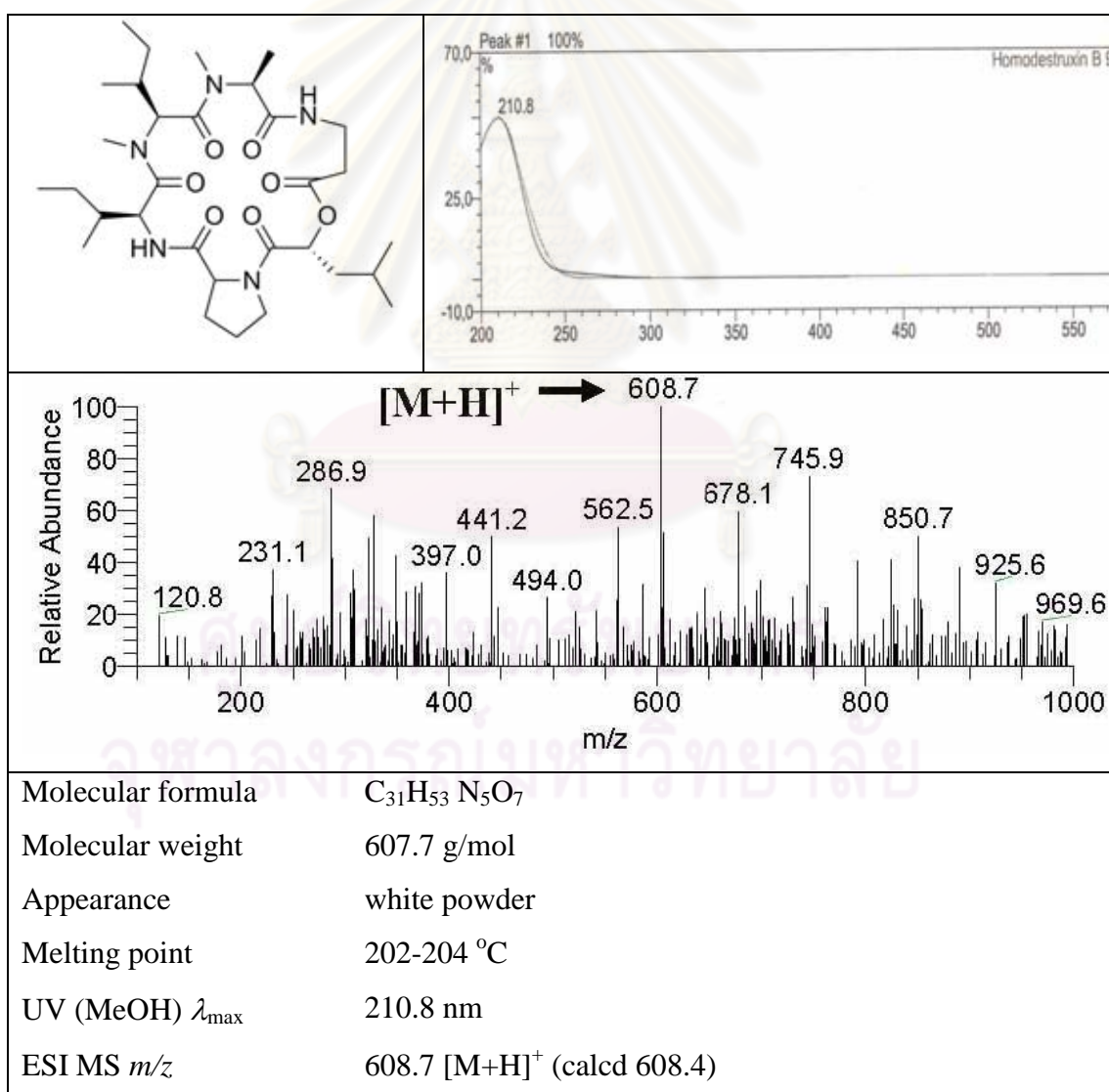| Molecular formula	$C_{31}H_{53}N_5O_7$
Molecular weight	607.7 g/mol
Appearance	white powder
Melting point	202-204 $^{\circ}$C
UV (MeOH) λ_{max}	210.8 nm
ESI MS *m/z*	608.7 [M+H]$^+$ (calcd 608.4)

Figure 3.32 Structure, UV spectrum and ESIMS of compound **9**

3.4.2.4.2 Structural elucidation of compound 10

Compound **10** was obtained from the extracts of both *C. gloeosporioides* (CI3) and *Ascomycete* sp. (CI5). Based on its ESIMS at *m/z* 259.2 [M + H]$^+$ and UV maximum absorption at 256.0 nm, matching with those data in library source, compound **10** was assigned as alternariol. Its structure, UV spectrum and ESIMS are shown in Figure 3.33. Moreover, the NMR data of **10**, described in Table 3.15, were also comparable to those previously reported [72]. Alternariol is a common metabolite of the fungi in *Altenaria* sp. [73-78].
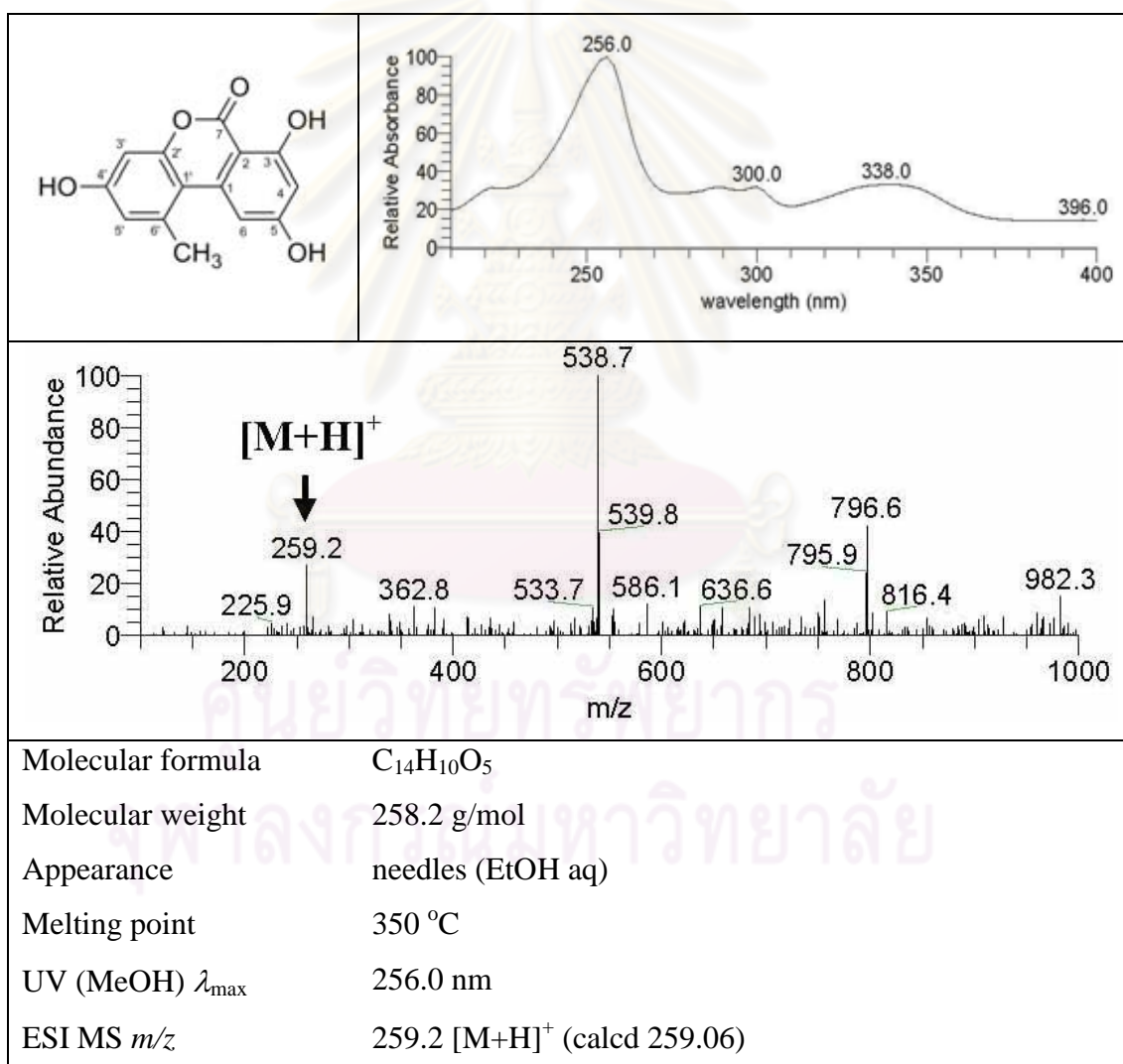

Molecular formula	$C_{14}H_{10}O_5$
Molecular weight	258.2 g/mol
Appearance	needles (EtOH aq)
Melting point	350 ^{o}C
UV (MeOH) λ_{max}	256.0 nm
ESI MS *m/z*	259.2 [M+H]$^+$ (calcd 259.06)

Figure 3.33 Structure, UV spectrum and ESIMS of compound **10**

Table 3.15 NMR spectroscopic data (MeOD) of compound **10**

position	compound **10**	
	δ_H, mult (*J* in Hz)	δ_C, mult
1		137.0, qC
2		105.0, qC
3		163.0, qC
4	6.2 s	101.0, qC
5		165.0, qC
6	6.6 s	105.0, qC
7		168.0, qC
1'		112.0, qC
2'		152.0, qC
3'	6.4 s	106.0, CH
4'		157.0, qC
5'	6.4 s	112.0, CH
6'		138.0, qC
6'-Me	2.2 s	18.0, CH_3

3.4.2.4.3 Structural elucidation of compound 11

Compound **11**, a white powder, was isolated from the extract of *C. gloeosporioides* (CI3). Its ESIMS at *m/z* 323.0 [M + H]$^+$ and λ_{max} in UV spectrum at 202.5 nm was found to be associated with a compound in library source, deoxyterphenylin. The ^{1}H NMR spectrum of **11** (Table 3.16) were also recorded and it related to that previously reported [79]. Structure, UV spectrum and ESIMS of compound **11** are shown in Figure 3.34.

Table 3.16 ^{1}H NMR spectroscopic data (MeOD) of compound **11**

Position	δ_H, mult (*J* in Hz)
2, 6	7.3 d (7.3)
3, 5	7.2 t (7.9)
4	7.1 t (7.6)
6'	6.3 s
2", 6"	7.2 d (8.8)
3", 5"	6.7 d (8.8)
5'-OMe	3.7 s
4"-OMe	3.7 s

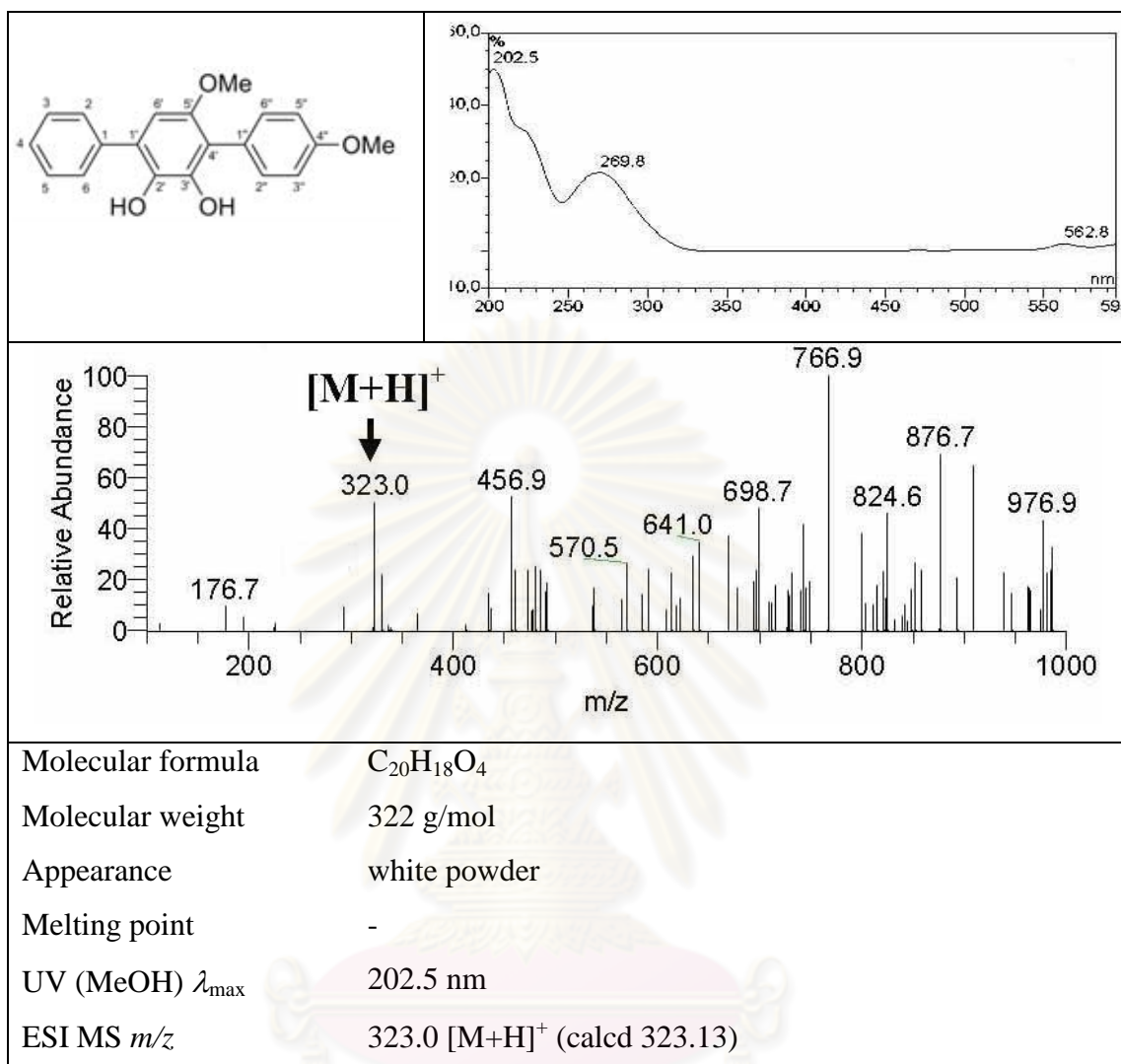| Molecular formula | $C_{20}H_{18}O_4$ |
Molecular weight	322 g/mol
Appearance	white powder
Melting point	-
UV (MeOH) λ_{max}	202.5 nm
ESI MS m/z	323.0 $[M+H]^+$ (calcd 323.13)

Figure 3.34 Structure, UV spectrum and ESIMS of compound **11**

3.4.2.4.4 Structural elucidation of compound 12

Compound **12** was isolated as a powder from the extract of *C. gloeosporioides* (CI3). It was identified as zearalenone owing to its UV maximum absorption at 202.5 nm and mass spectrum (ESIMS) displaying a molecular ion at m/z 323.0 $\{M + H\}^+$ corresponding to a specific pattern of the compound in database. Structure, UV spectrum and ESIMS of compound **12** are depicted in Figure 3.35.
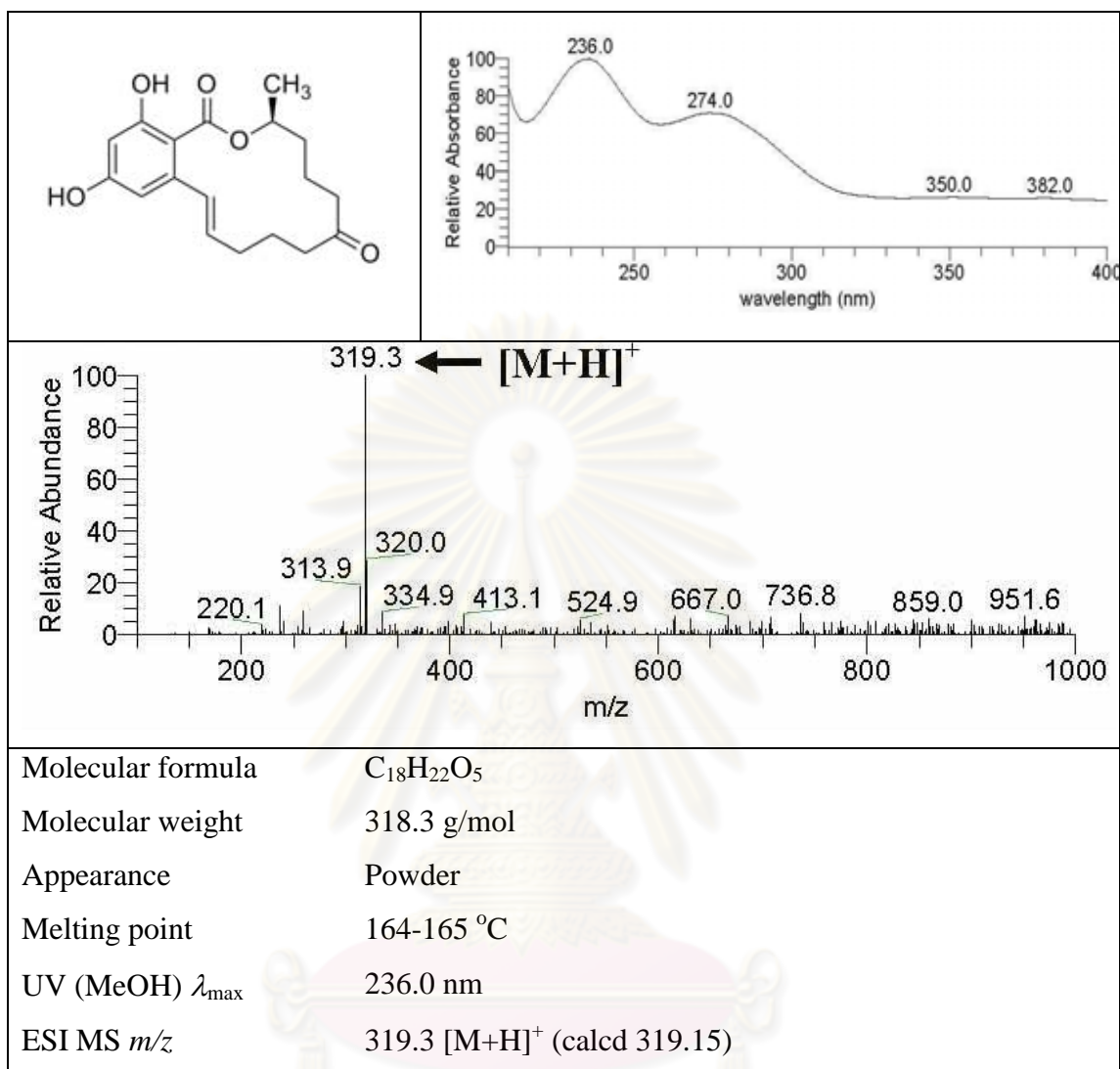
Figure 3.35 Structure, UV spectrum and ESIMS of compound **12**

Molecular formula	$C_{18}H_{22}O_5$
Molecular weight	318.3 g/mol
Appearance	Powder
Melting point	164-165 ^{o}C
UV (MeOH) λ_{max}	236.0 nm
ESI MS m/z	319.3 [M+H]$^+$ (calcd 319.15)

3.4.2.4.5 Structural elucidation of compound 13

Compound **13** was also obtained from the extract of *C. gloeosporioides* (CI3) as crystals. Comparison of its measured UV (λ_{max} 236.0 nm) and mass (ESIMS, m/z 319.3 [M + H]$^+$) spectrum to those in database revealed that compound **13** was indole-3-carbaldehyde as shown in Figure 3.36. Furthermore, its ^{1}H NMR data (Table 3.17) were associated with those previously reported [80].
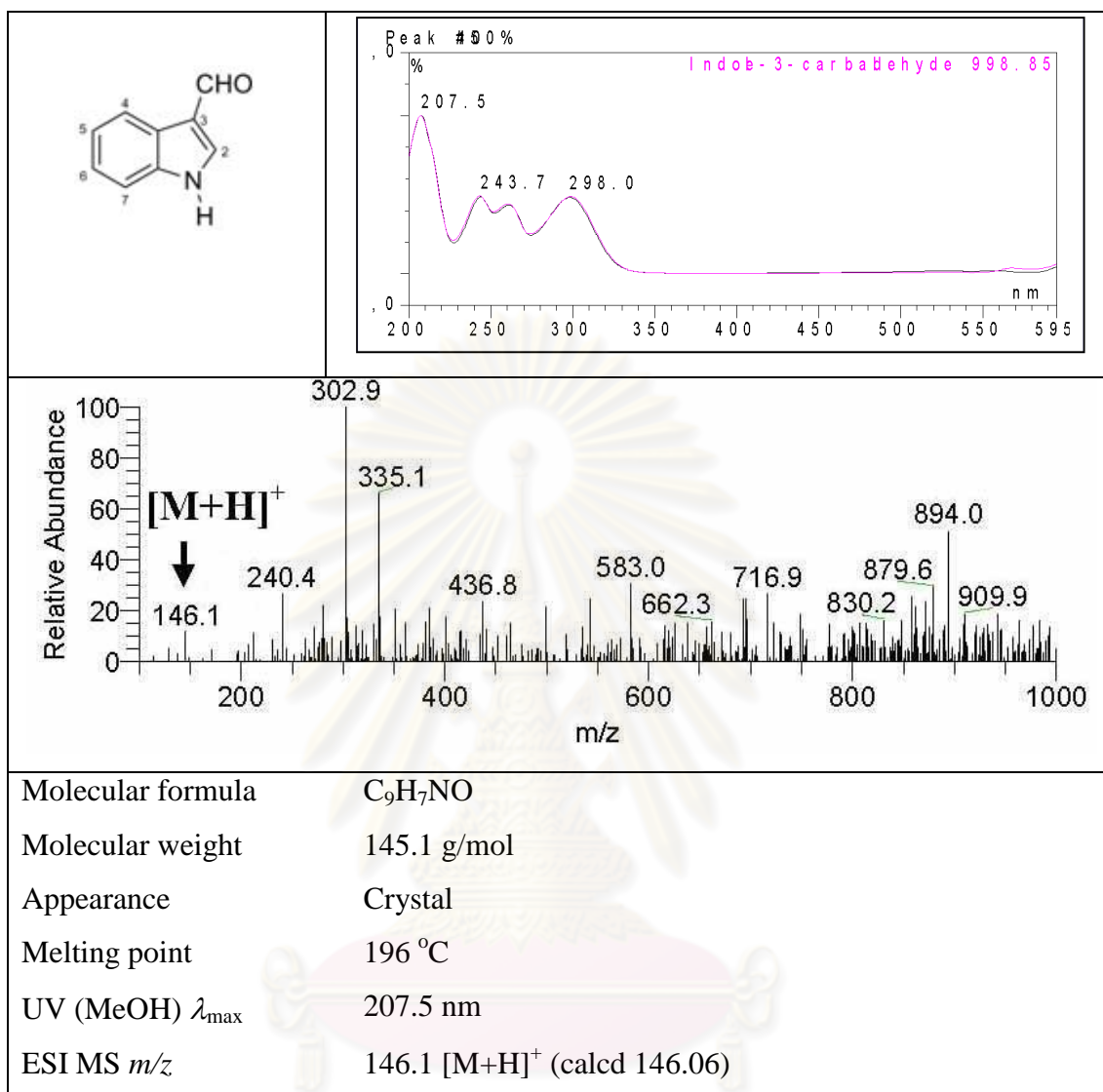
Figure 3.36 Structure, UV spectrum and ESIMS of compound **13**

Table 3.17 ^{1}H NMR spectroscopic data (CD$_3$OD) of compound **13**

Position	δ_H, mult (J in Hz)
2	7.9 s
4	8.0 d (8.4)
5	7.1 m
6	7.2 m
7	7.3 d (8.6)
3-CHO	9.8 s

3.4.2.4.6 Structural elucidation of compound 14

Also, compound **14**, small prisms, was isolated from the extract of *C. gloeosporioides* (CI3). Since molecular ion at *m/z* 247.3 [M +H]$^+$ in ESIMS spectrum and λ_{max} at 217.7 nm in UV spectrum of compound **14** matched with those of a compound in library source, it was thus determined to be *N,N*-dimethyltryptophane methyl ester. Structure, UV spectrum and ESIMS of compound **14** are shown in Figure 3.37.
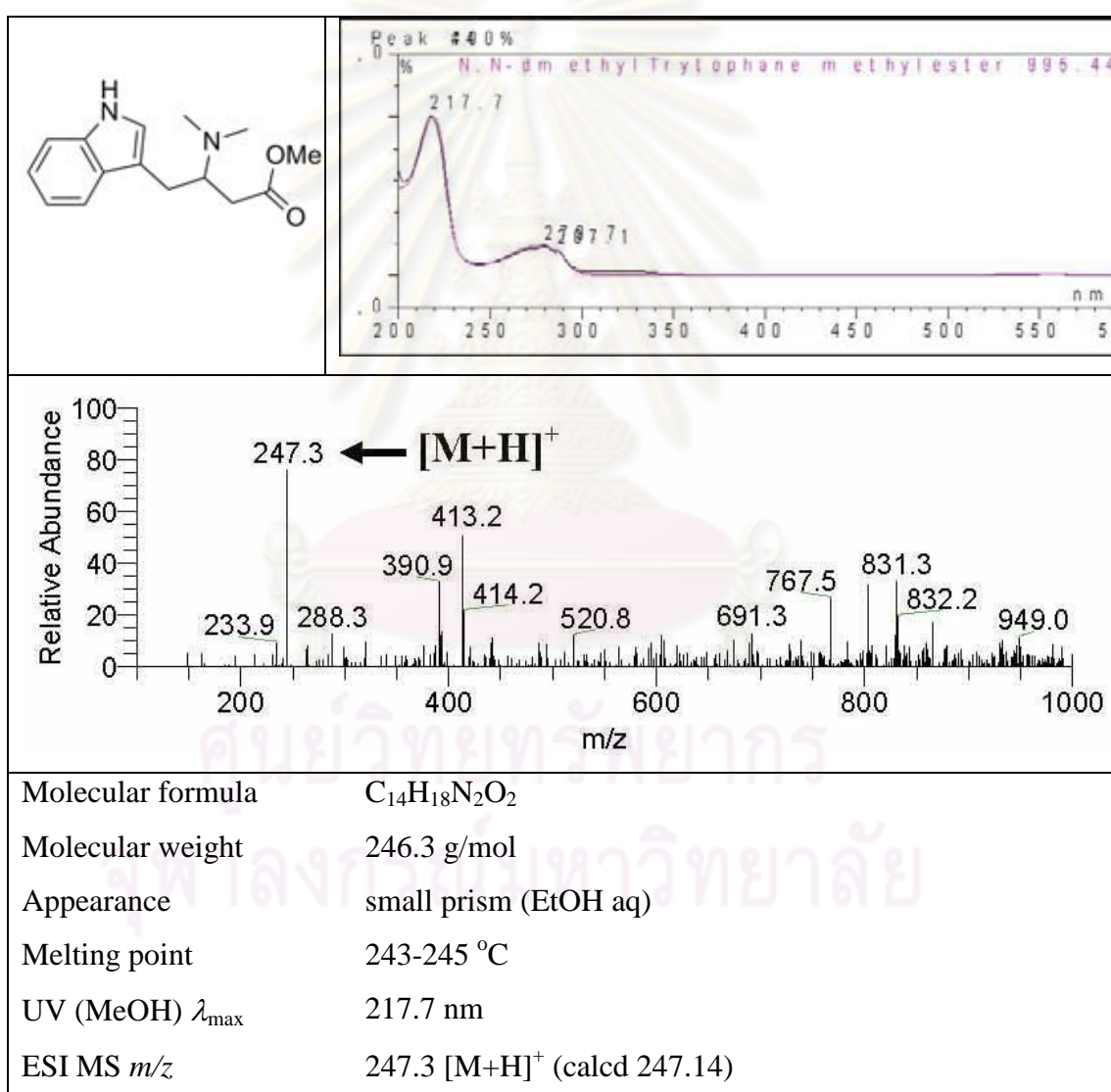

Molecular formula	$C_{14}H_{18}N_2O_2$
Molecular weight	246.3 g/mol
Appearance	small prism (EtOH aq)
Melting point	243-245 ^{o}C
UV (MeOH) λ_{max}	217.7 nm
ESI MS *m/z*	247.3 [M+H]$^+$ (calcd 247.14)

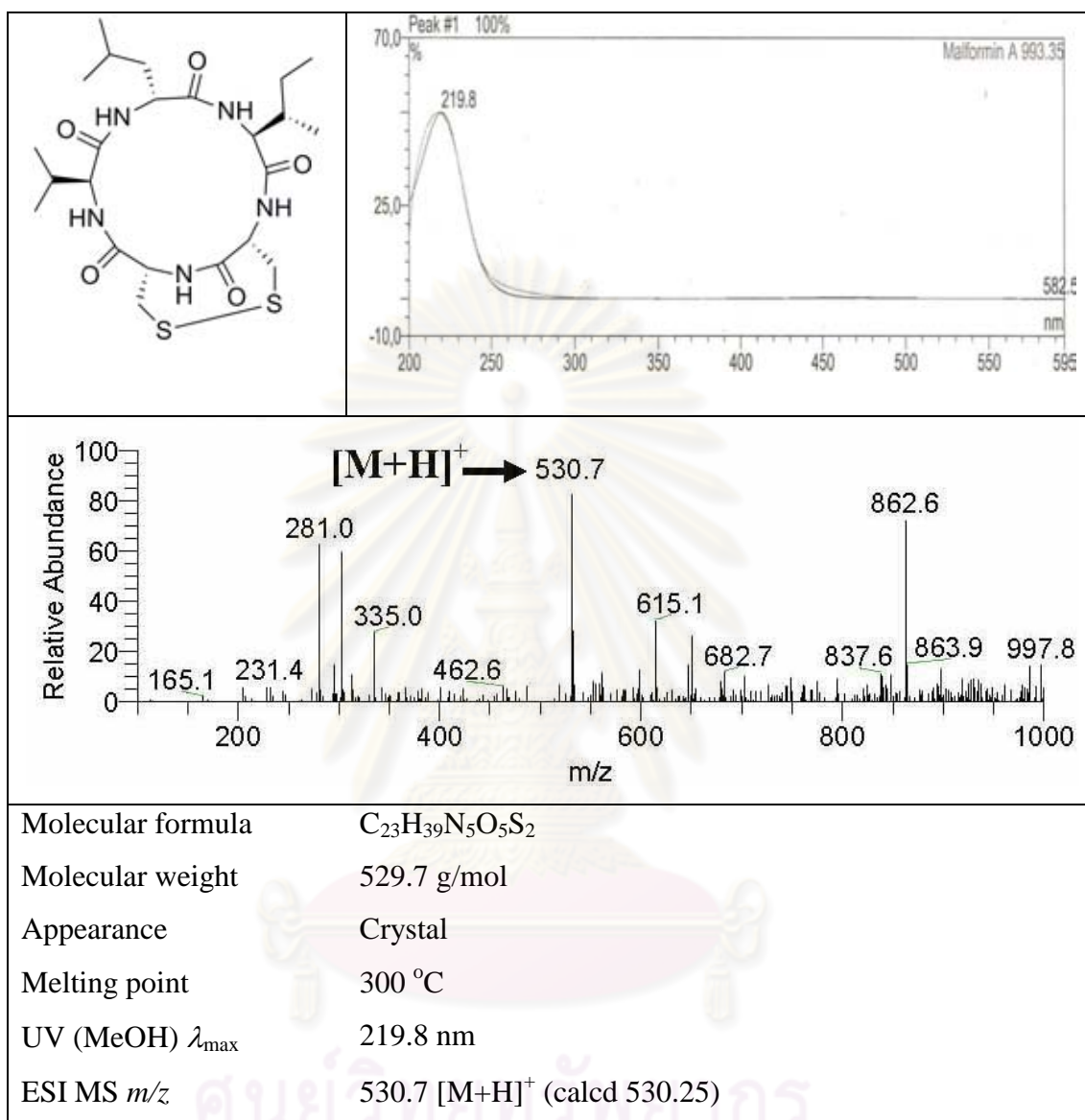**Figure 3.37** Structure, UV spectrum and ESIMS of compound **14**

3.4.2.4.7 Structural elucidation of compound 15

Compound **15**, a yellow-green powder, was also obtained from the extract of *C. gloeosporioides* (CI3). On the basis of its ESIMS showing *m/z* 311.0 [M + H]$^+$ and UV maximum absorption at 229.1 nm, corresponding to those patterns in database, compound **15** was identified as dihomo-3′-hydroxymethyl-dihydroinfectopyron. Its structure, measured UV spectrum and ESIMS are depicted in Figure 3.38. Furthermore, the NMR data of **15** comparable to those previously reported [81] (Table 3.17) also confirmed the above identification.

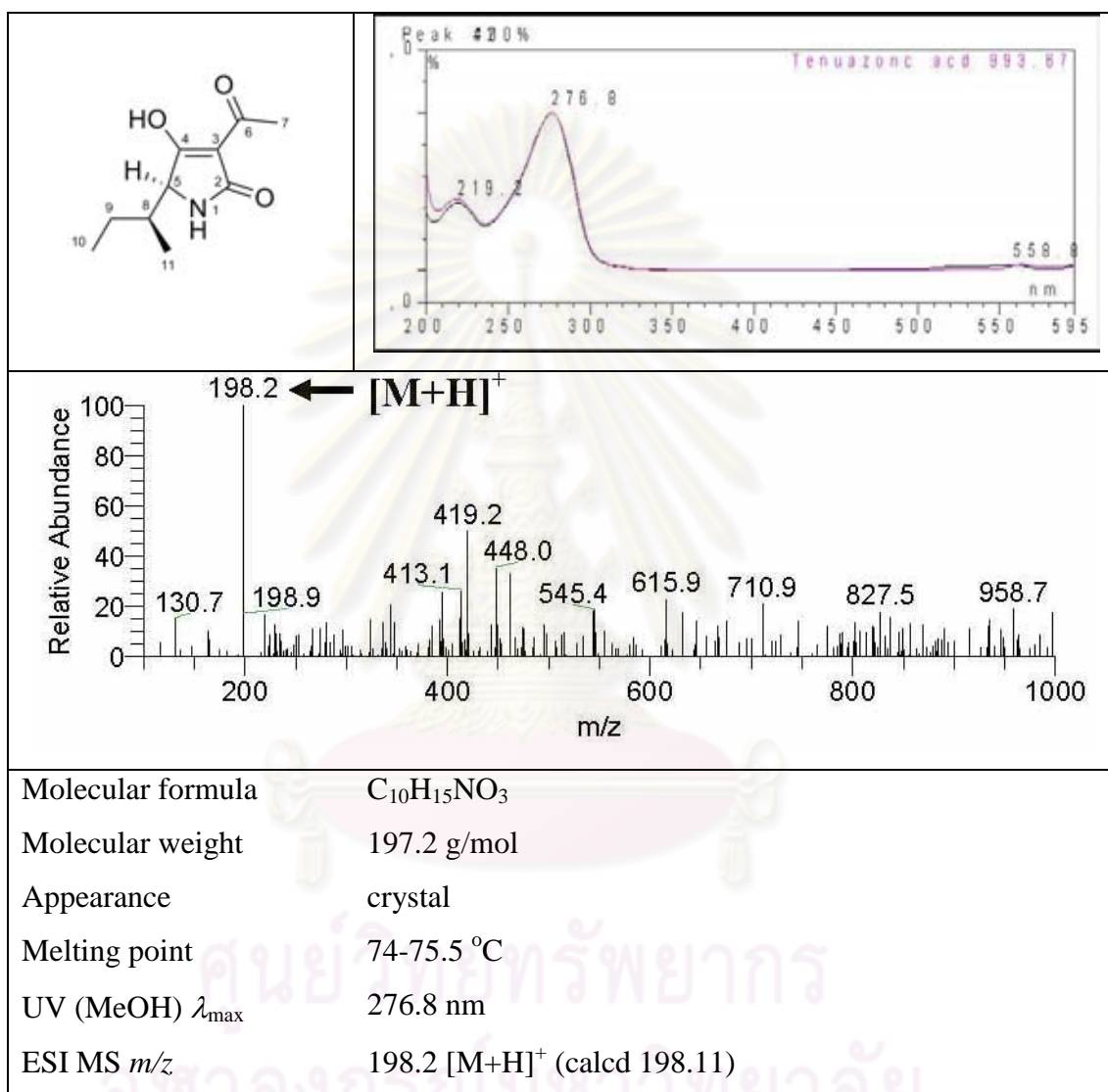| Molecular formula | $C_{16}H_{22}O_6$ |
Molecular weight	310.3 g/mol
Appearance	yellow-green powder
Melting point	-
UV (MeOH) λ_{max}	229.1 nm
ESI MS *m/z*	311.0 [M+H]$^+$ (calcd 311.15)
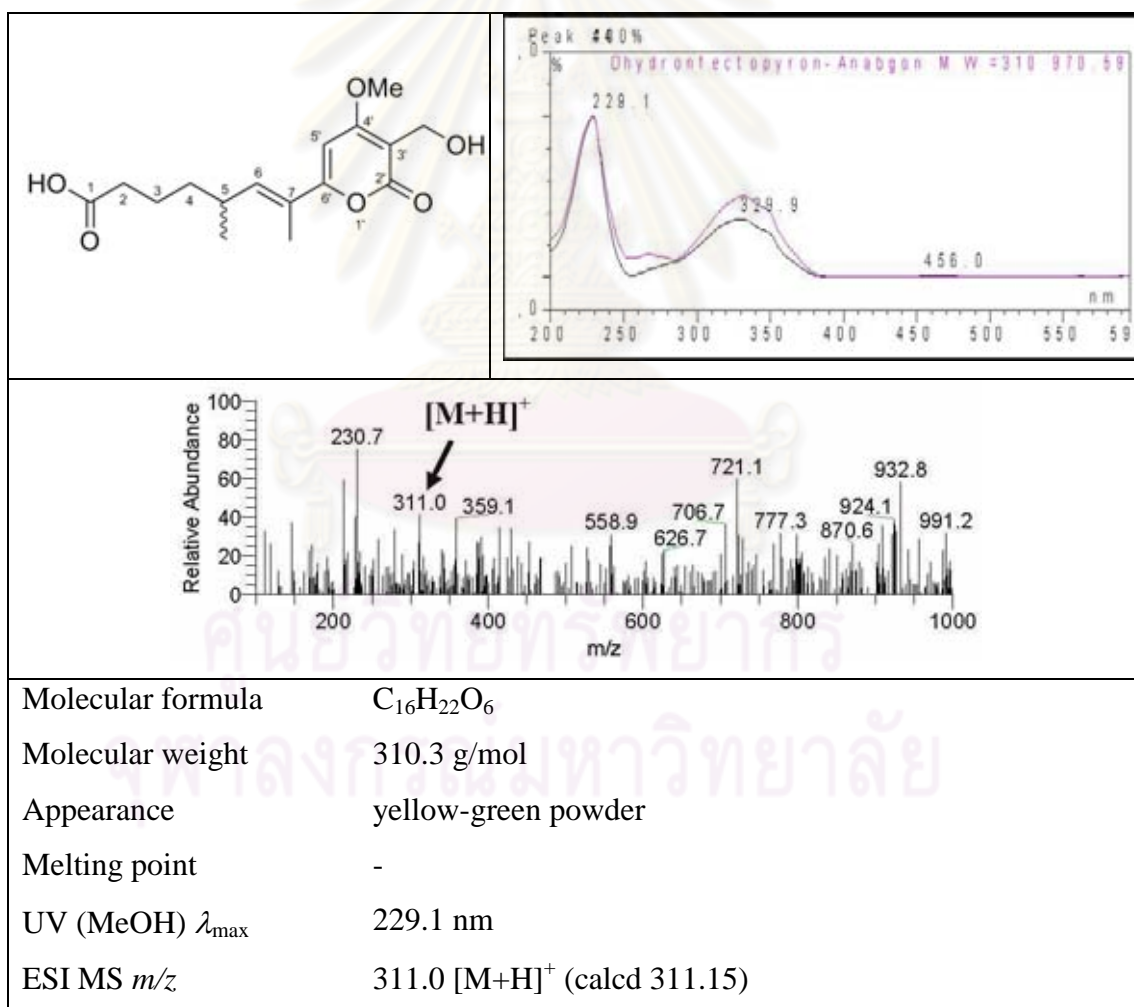
Figure 3.38 Structure, UV spectrum and ESIMS of compound **15**

Table 3.18 NMR spectroscopic data (MeOD) of compound **15**

| position | compound **15** | |
	δ_H, mult (J in Hz)	δ_C, mult
1		176.0, qC
2	2.2 m	36.0, CH$_2$
3	1.6 m	22.3, CH$_2$
4	1.2 m	37.0, CH$_2$
5	2.3 m	32.0, CH
6	5.3 dd (10.0, 1.2)	131.0, CH
7		126.0, qC
2'		164.0, qC
3'		101.0, qC
4'		164.8, qC
5'	6.4 s	100.0, CH
6'		158.0, qC
3'-CH$_2$OH	4.2 s	53.0, CH$_2$
4-OMe	3.5 s	54.0, CH$_3$
5-Me	1.0 s	22.3, CH$_3$
7-Me	1.0 s	10.2, CH$_3$

3.4.2.4.8 Structural elucidation of compound 16

Compound **16** was isolated as crystals from the extract of *C. gloeosporioides* (CI3). This compound was assigned as malformin A, because ESIMS showing a molecular ion at *m/z* 530.7 [M+H]$^+$ and UV spectrum giving a λ_{max} at 219.8 nm, were identical to the patterns of a compound in library source. Structure, measured UV spectrum and ESIMS of compound **16** are shown in Figure 3.39.

Figure 3.39 Structure, UV spectrum and ESIMS of compound **16**

Molecular formula	$C_{23}H_{39}N_5O_5S_2$
Molecular weight	529.7 g/mol
Appearance	Crystal
Melting point	300 °C
UV (MeOH) λ_{max}	219.8 nm
ESI MS m/z	530.7 $[M+H]^+$ (calcd 530.25)

3.4.2.4.9 Structural elucidation of compound 17

Compound **17** was isolated as crystals from the extracts of both *C. gloeosporioides* (CI3) and *Ascomycete* sp. (CI5). Comparison of ESIMS and UV spectra displaying a molecular ion at m/z 198.2 $[M+H]^+$ and λ_{max} at 276.8 nm, respectively, to a database led to the identification of compound **17** as tenuazonic acid. Its NMR data shown in Table 3.18 were also in fully agreement with those

previously reported [82]. Structure, UV spectrum and ESIMS of compound **17** are depicted in Figure 3.40.

Molecular formula	$C_{10}H_{15}NO_3$
Molecular weight	197.2 g/mol
Appearance	crystal
Melting point	74-75.5 $^{\circ}$C
UV (MeOH) λ_{max}	276.8 nm
ESI MS m/z	198.2 [M+H]$^{+}$ (calcd 198.11)

Figure 3.40 Structure, UV spectrum and ESIMS of compound **17**

Table 3.19 NMR spectroscopic data (MeOD) of compound **17**

| position | compound **17** | |
	δ_H, mult (J in Hz)	δ_C, mult
2		174.0, qC
3		101.0, qC
4		187.0, qC
5	3.7 d (3.0)	60.0, CH
6		198.0, qC
7	2.3 s	26.7, CH$_3$
8	1.7 m	35.0, CH
9	1.1 m	26.7, CH$_2$
10	0.9 t (7.2)	101.0, qC
11	1.0 d (6.9)	12.0, CH$_3$
1-NH	8.0 s br	17.0, CH$_3$
4-OH	15.0 s br	

3.4.2.4.10 Structural elucidation of compound 18

Compound **18**, a secondary metabolite of *C. gloeosporioides* (CI3), was obtained as white crystals. It was elucidated as adenosine by ESIMS at *m/z* 262.8 [M + H]$^+$ and λ_{max} at 258.0 nm, corresponding to those in database. In addition, the NMR data of **18** (Table 3.19) were virtually identical to those previously reported in Antibase 2002. Its structure, UV spectrum and ESIMS are displayed in Figure 3.41.

Table 3.20 NMR spectroscopic data (DMSO-d_6) of compound **18**

| position | compound **18** | |
	δ_H, mult (J in Hz)	δ_C, mult
2	8.3 s	152.4, CH
4		149.1, qC
5		119.4, qC
6		156.2, qC
8	8.1 s	139.9, CH
1'	5.9 d (6.0)	87.9, CH
2'	4.6 t (10.0)	70.7, CH
3'	4.0 dd (10.0, 8.0)	73.4, CH
4'	3.9 m	85.9, CH
5'	3.7 t (12.0)	61.7, CH$_2$
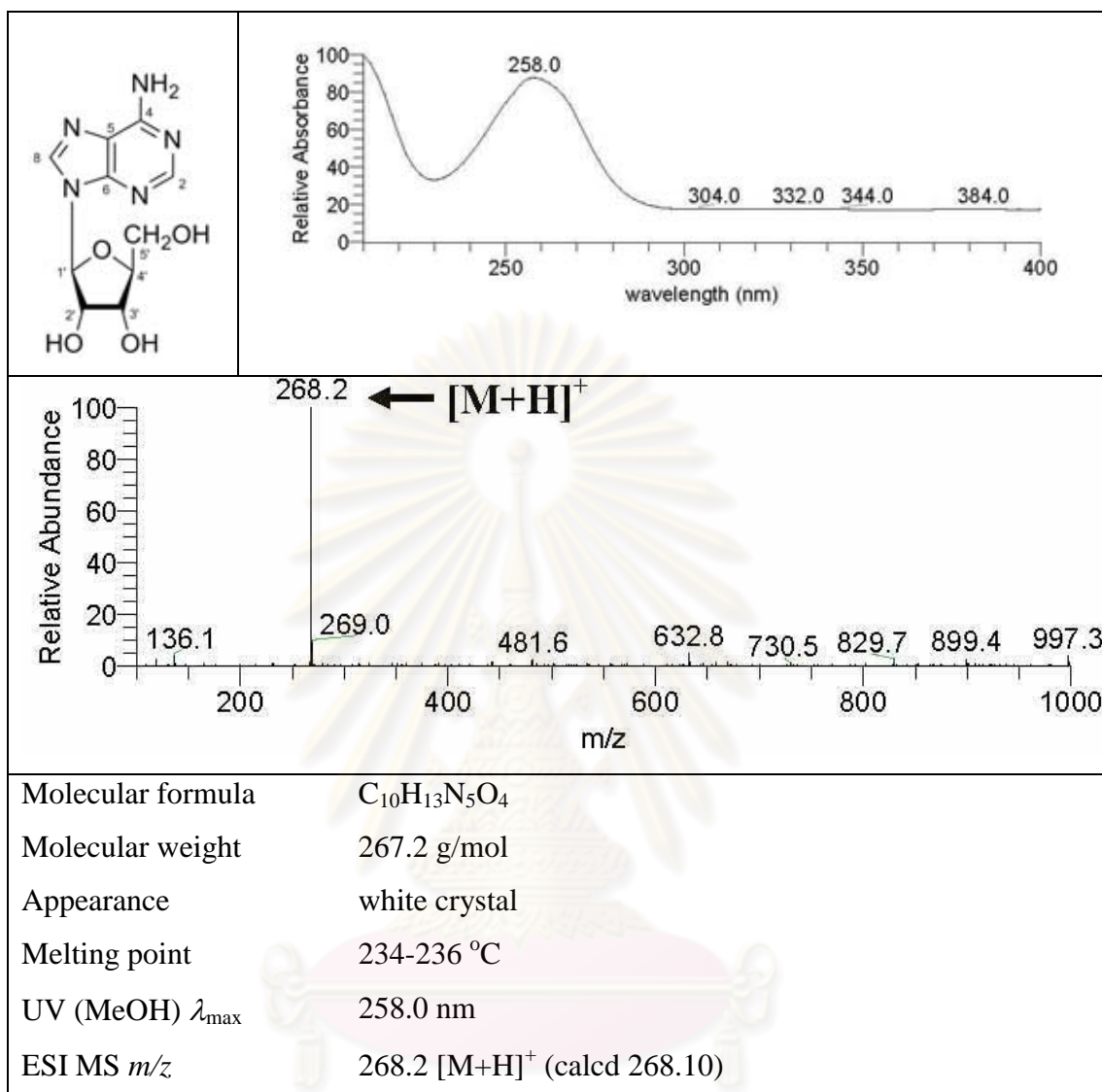
Figure 3.41 Structure, UV spectrum and ESIMS of compound **18**

3.4.2.4.11 Structural elucidation of compound 19

Compound **19** was obtained as crystals from the extract of *P. herbarum* (CI4). ESIMS exhibiting a molecular ion at *m/z* 307.2 [2M + Na]$^+$ and UV spectrum giving λ_{max} at 256.9 nm, relating to those in library source, led to the identification of compound **19** as Sumiki's acid. Its structure, UV spectrum and ESIMS are shown in Figure 3.42.
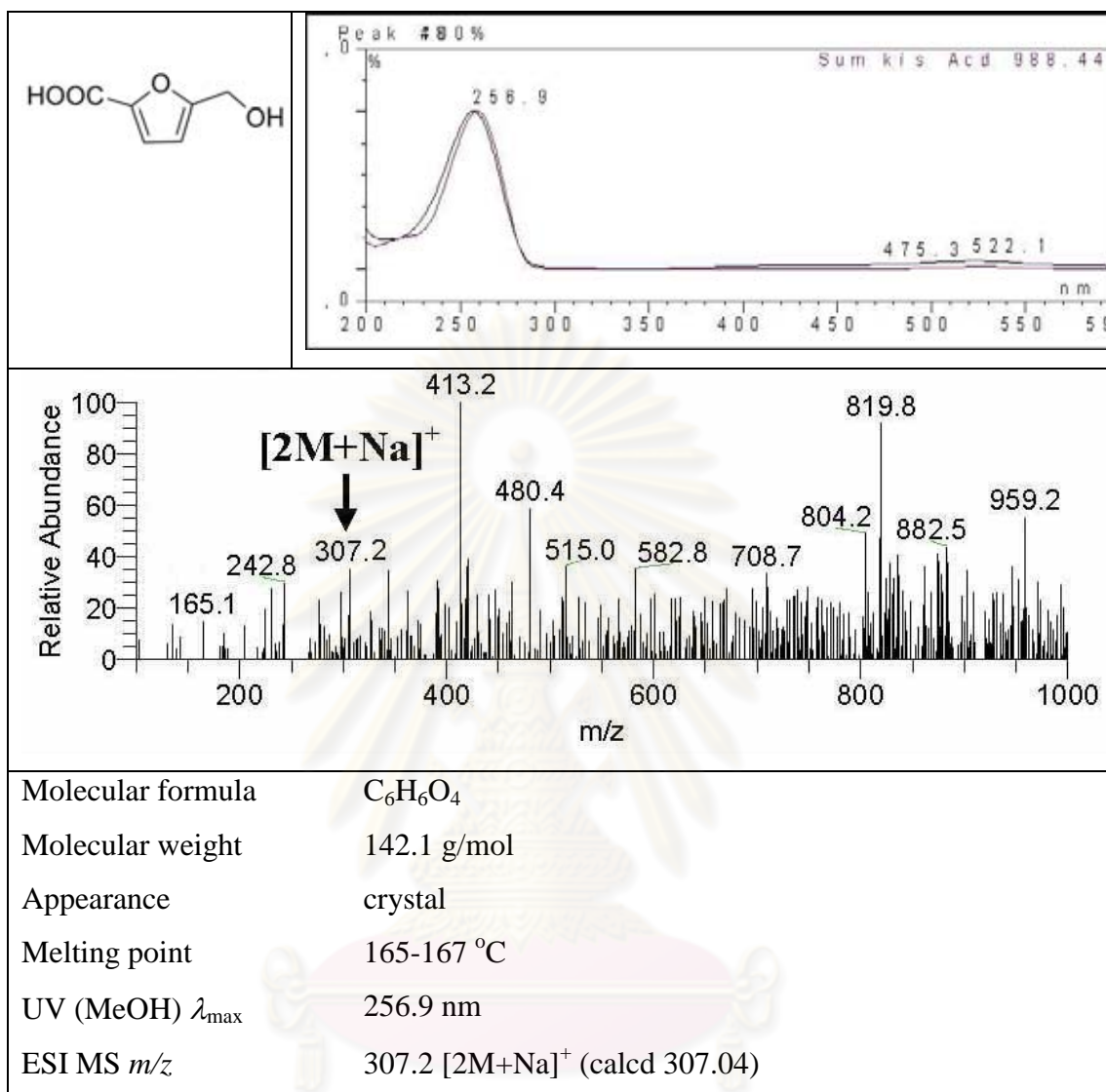
Molecular formula	$C_6H_6O_4$
Molecular weight	142.1 g/mol
Appearance	crystal
Melting point	165-167 °C
UV (MeOH) λ_{max}	256.9 nm
ESI MS m/z	307.2 [2M+Na]$^+$ (calcd 307.04)

Figure 3.42 Structure, UV spectrum and ESIMS of compound **19**

3.4.2.4.12 Structural elucidation of compound 20

Compound **20**, white crystals, was also isolated from the extract of *P. herbarum* (CI4). It was discovered that a molecular ion at m/z 223.1 [M +H]$^+$ in ESIMS spectrum and λ_{max} at 228.7 nm in UV spectrum of compound **20** matched with those in a database. Therefore compound **20** was determined to be 5-carboxymellein. This was also confirmed by its ^{1}H NMR data (Table 3.20), which were very similar to those reported in literature [82]. Structure, UV spectrum and ESIMS of compound **20** are shown in Figure 3.43.
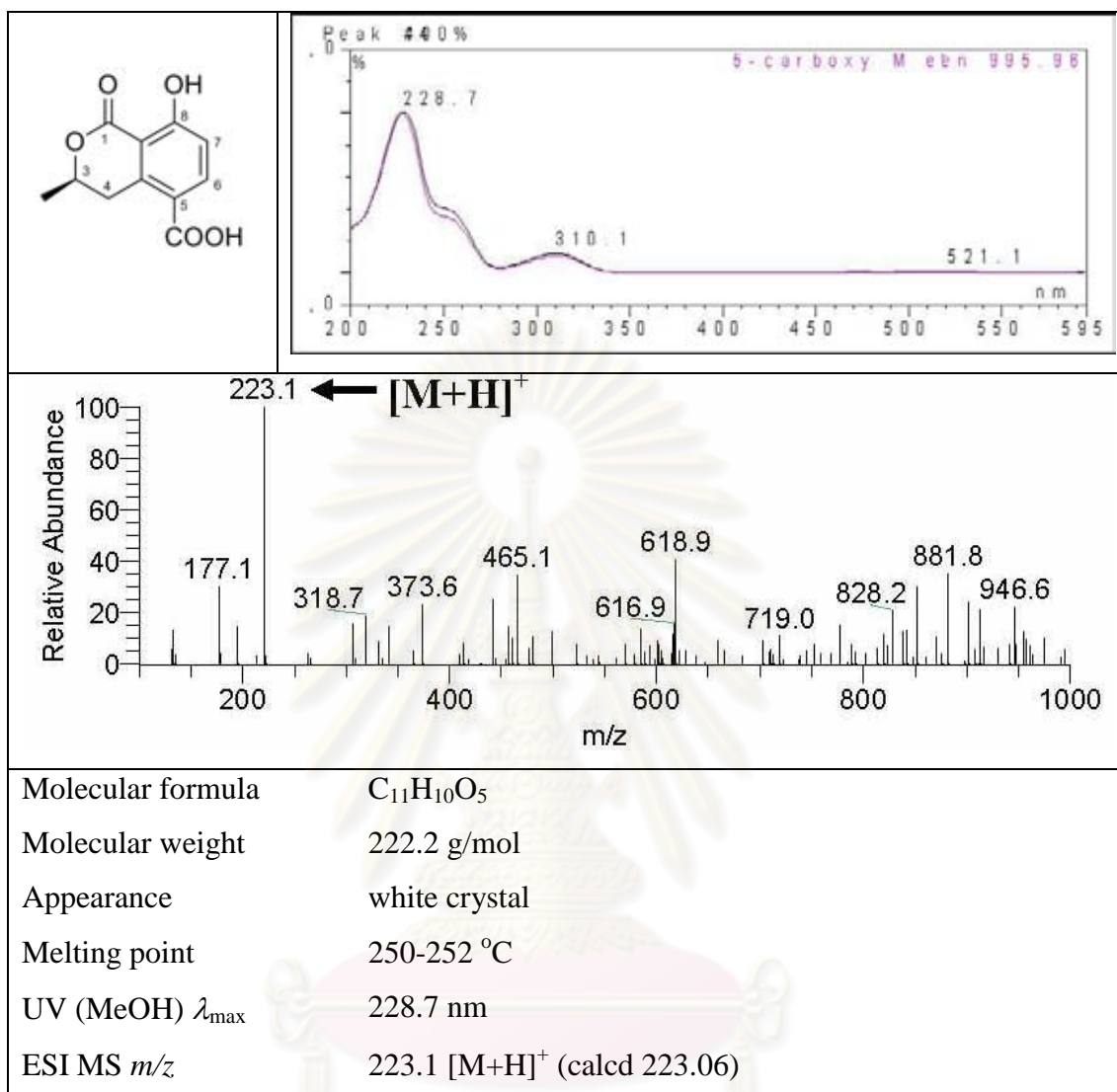
Figure 3.43 Structure, UV spectrum and ESIMS of compound **20**

Table 3.21 ^{1}H NMR spectroscopic data (MeOD) of compound **20**

postition	δ_H, mult (J in Hz)
3	4.5 m
4	3.1 m
6	7.9 d (8.4)
7	6.8 d (8.4)
3-Me	1.4 d (6.3)

3.4.2.4.13 Structural elucidation of compound 21

Compound **21**, isolated as white crystals, was found to be one of secondary metabolites produced by *Ascomycete* sp. (CI5). This compound was assigned as cytochalasin D, based on a molecular ion at *m/z* 508.6 [M + H]$^+$ and λ_{max} at 220.0 nm, corresponding to those in database. Additionally, comparison of its ^{1}H and ^{13}C NMR data (Table 3.21) with those reported in literature confirmed the above identification. Structure, UV spectrum and ESIMS of compound **21** are depicted in Figure 3.44. Generally, cytochalasin D is an alkaloid produced by various fungal strains in *Xylaria* genera. It was reported as a potent inhibitor of actin polymerization [83].

Molecular formula	$C_{30}H_{37}NO_6$
Molecular weight	507.6 g/mol
Appearance	white crystal
Melting point	268-271 ^{o}C
UV (MeOH) λ_{max}	220.0 nm
ESI MS *m/z*	508.6 [M+H]$^+$ (calcd 508.27)
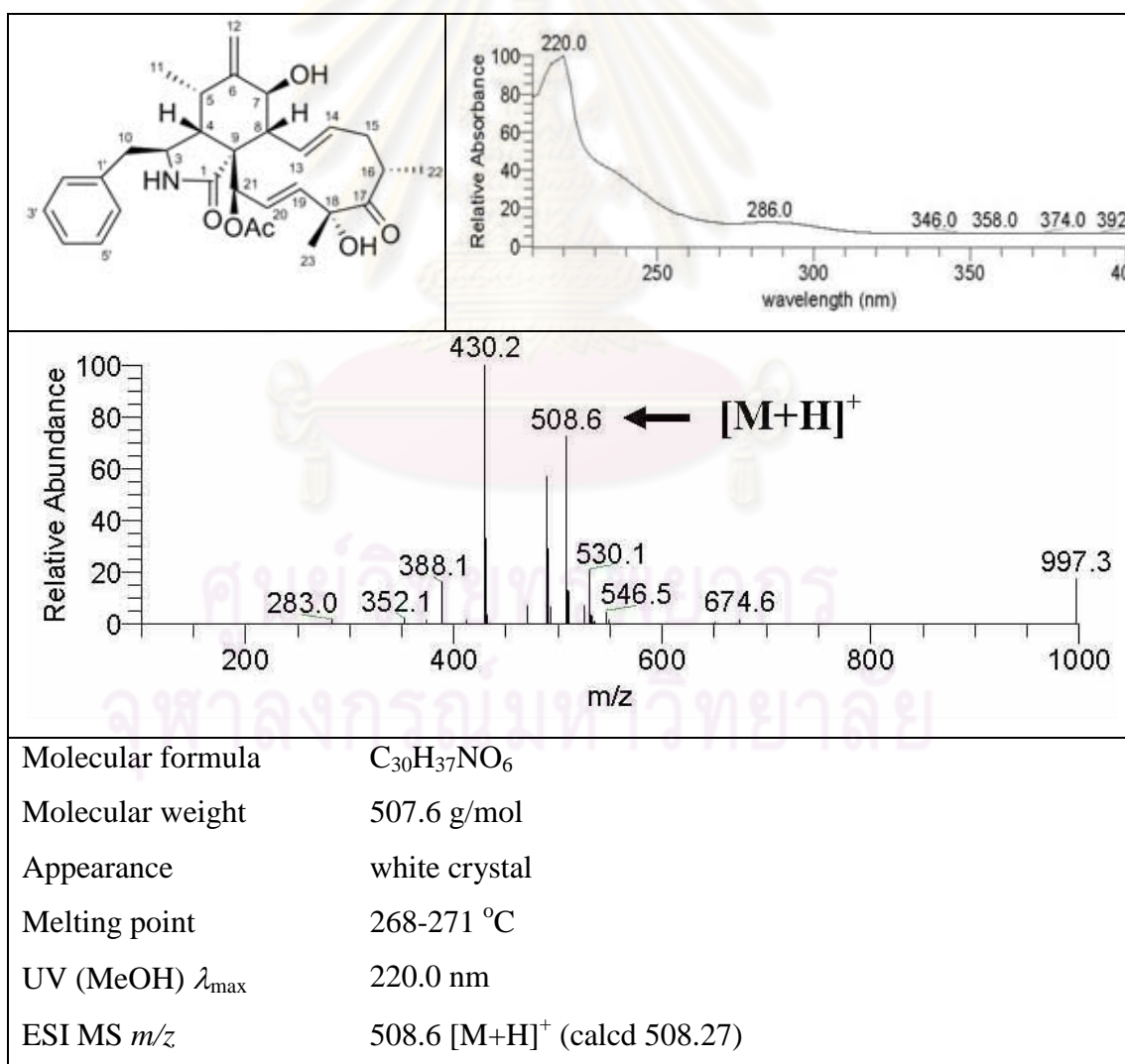
Figure 3.44 Structure, UV spectrum and ESIMS of compound **21**

Table 3.22 NMR spectroscopic data (DMSO-d_6) of compound **21**

| position | compound **21** | |
	δ_H, mult (J in Hz)	δ_C, mult
1		173.6, qC
2 (NH)	8.1 s br	
3	3.5 m	52.0, CH
4	2.4 m	50.0, CH
5	2.7 m	31.5, CH
6		150.7, qC
7	4.5 d (10.8)	70.2, CH
8	3.3 m	47.2, CH
9		53.4, qC
10	2.3 t (4.4)	45.8, CH$_2$
11	0.4 d (6.4)	12.8, CH$_3$
12	4.9 s	111.5, CH$_2$
	4.7 s	
13	5.4 m	131.4, CH
14	5.1 m	131.9, CH
15	2.7 m	37.6, CH$_2$
	1.9 dd (12.4, 4.4)	
16	2.2 m	43.4, CH
17		210.0, qC
18		77.5, qC
19	5.0 m	128.1, CH
20	5.8 dd (15.2, 1.2)	132.2, CH
21	5.2 m	76.4, CH
22	1.0 d (6.4)	19.3, CH$_3$
23	1.4 s	24.6, CH$_3$
1'		137.1, qC
2', 6'	7.2 m	130.6, CH
3', 5'	7.2 m	129.4, CH
4'	7.2 m	126.8, CH
21-OAc		170.0, qC
	2.3 s	20.5, CH$_3$

3.4.2.4.14 Structural elucidation of compound 22

Compound **22** was obtained as crystals from the extract of *Ascomycete* sp. (CI5). In the same way, the structure of this compound was elucidated as 2(4-bromophenyl) acetic acid by ESIMS at m/z 216.0 [M + H]$^+$ and UV maximum absorption at 216.3 nm, associating with those patterns in database. Structure, UV spectrum and ESIMS of compound **22** are displayed in Figure 3.45.

Molecular formula	$C_8H_7BrO_2$
Molecular weight	215.0 g/mol
Appearance	crystal
Melting point	21.5 °C
UV (MeOH) λ_{max}	216.3 nm
ESI MS m/z	216.0 $[M+H]^+$ (calcd 216.05)
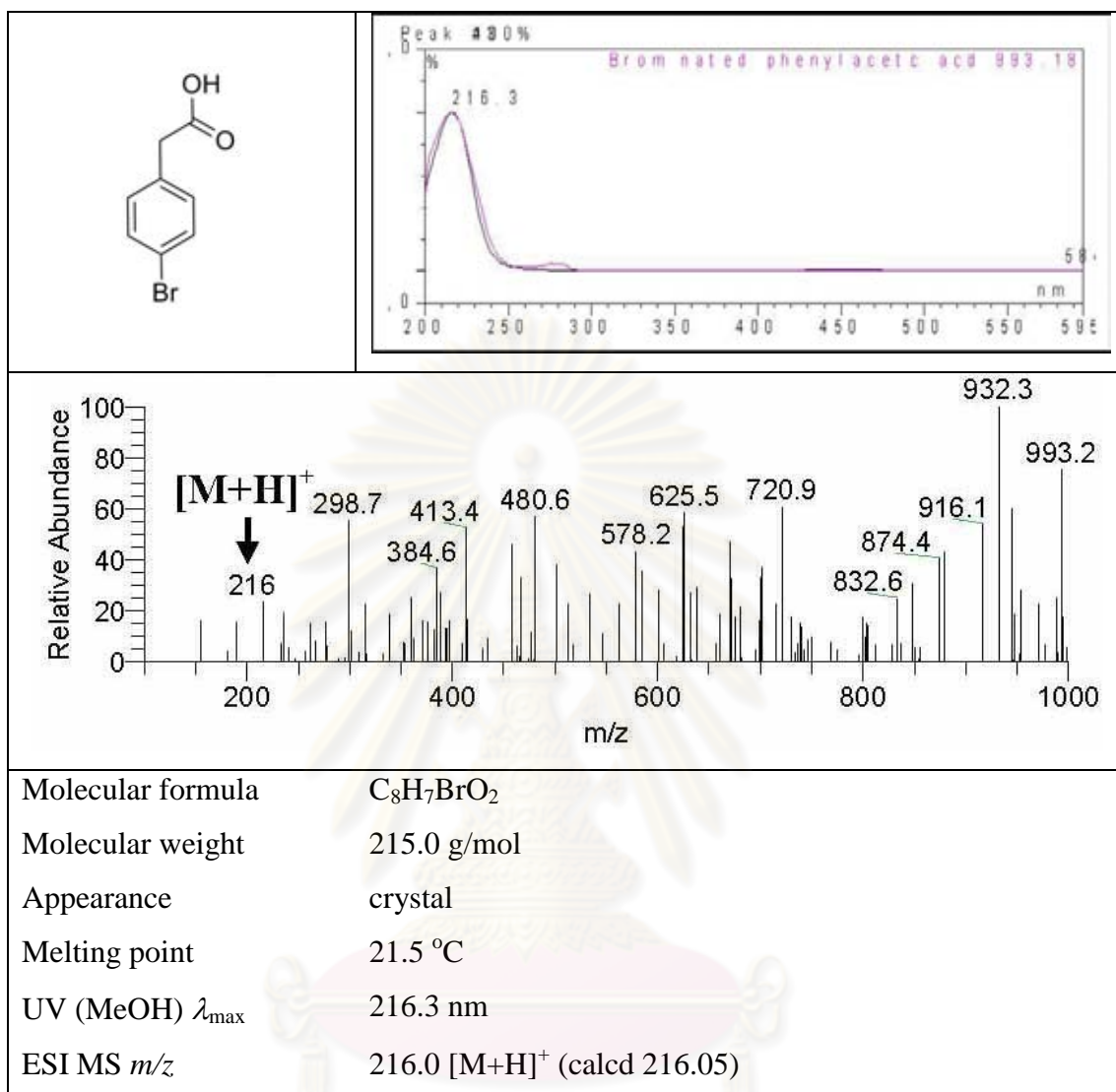
Figure 3.45 Structure, UV spectrum and ESIMS of compound **22**

3.4.2.4.15 Structural elucidation of compound 23

Compound **23** was also obtained from the extract of *Ascomycete* sp. (CI5) as a pale yellow solid. Its structure was elucidated from the results of measured ESIMS (m/z 267.0 $[M + H]^+$) and UV spectrum (λ_{max} 222.5 nm), matching with a database, it was thus identified as citreodrimene F. The structure, ESIMS and UV spectrum of this compound are shown in Figure 3.46.

Molecular formula	$C_{15}H_{22}O_4$
Molecular weight	266.3 g/mol
Appearance	pale yellow solid
Melting point	n.d.
UV (MeOH) λ_{max}	222.5 nm
ESI MS m/z	267.0 $[M+H]^+$ (calcd 267.16)
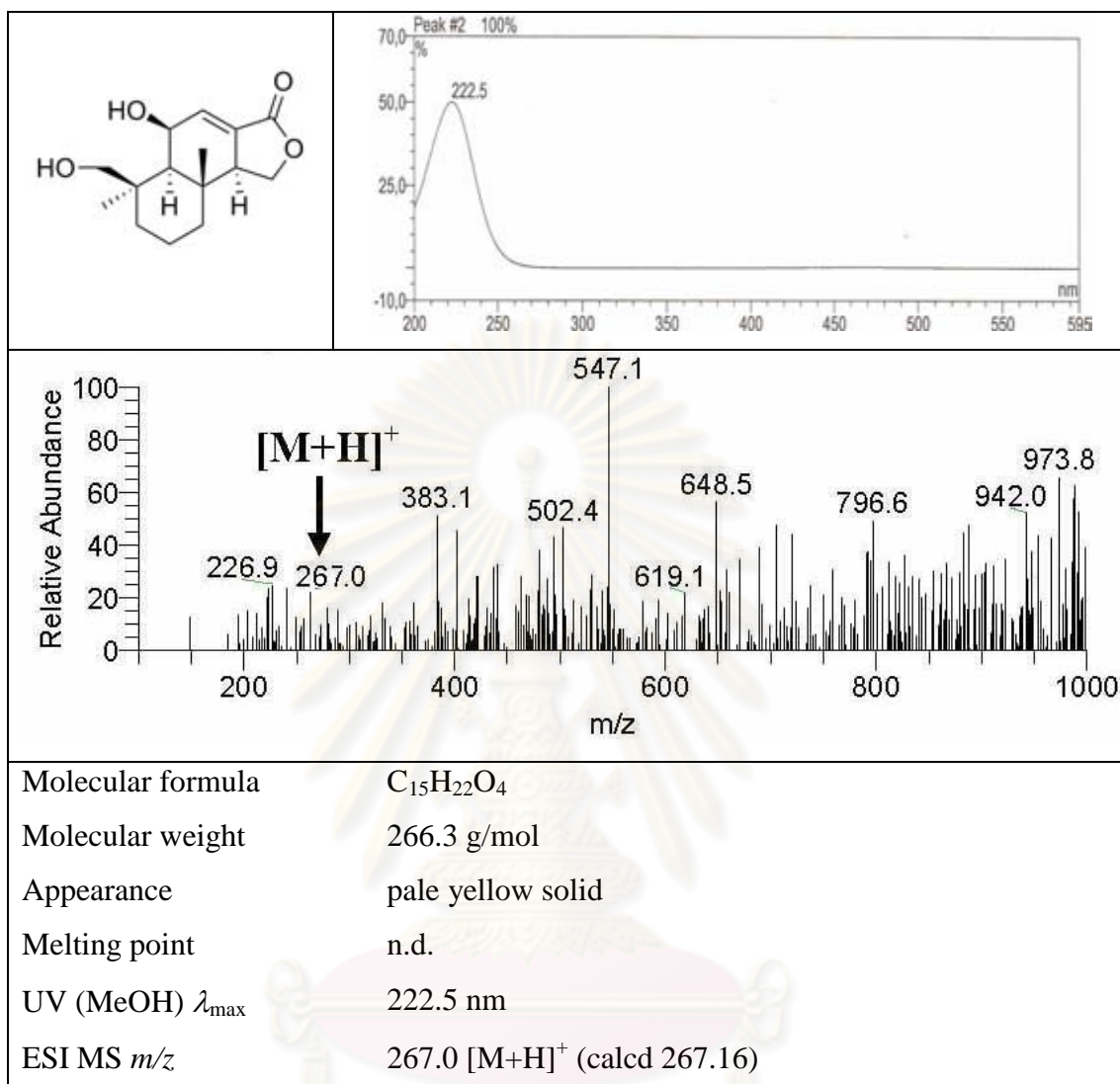
Figure 3.46 Structure, UV spectrum and ESIMS of compound **23**

3.5 Biological activity of isolated metabolites

Secondary metabolites isolated from the fungus *A. terreus* were tested for their biological activities including cytotoxicity on tumor cell lines, antibacterial and antimalarial activities.

3.5.1 Cytotoxicity

A total of eight isolated compounds from *A. terreus* were determined for their cytotoxic effect against three human tumor cell lines, colon carcinoma (SW-620), hepato carcinoma (HEP-G2) and lung carcinoma (CHAGO), by using MTT colorimetric method [84]. Results are presented in Table 3.22. Only asperterone (**5**) and terrein (**8**) exhibited cytotoxicity on all cell lines tested with IC_{50} values ranging from 2.70 to 5.63 μM, whereas other compounds did not show any detectable activity at a dose of 10 μM.

Table 3.23 Cytotoxicity of compounds **1**-**5, 8** and **24**-**25** on human tumor cell lines.

Compound	IC_{50} (μM)				
	BT-474	KATO-3	SW-620	HEP-G2	CHAGO
Butyrolactone I (**1**)	I	I	I	I	I
Butyrolactone II (**2**)	I	I	I	I	I
Aspernolide B (**3**)	I	I	I	I	I
Aspernolide D (**4**)	I	I	I	I	I
Asperterone (**5**)	I	I	2.70	3.81	5.63
Terrein (**8**)	I	I	2.80	5.56	3.51
Aspernolide A (**24**)	I	I	I	I	I
Aspernolide C (**25**)	I	I	I	I	I
Doxorubicin	0.91	0.98	0.14	0.16	0.81

BT-474: breast, KATO-3: gastric, SW-620: colon, HEP-G2: hepatic, CHAGO: lung cell lines.
I = inactive (determined by cell survival > 50% at a screening dose of 10 μM)

3.5.2 Antibacterial activity

Compounds **1**-**5, 8** and two synthetic butyrolactone derivatives (**24**-**25**) were further determined for their minimum inhibitory concentrations (MICs) against five Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus epidermidis,* and *Enterococcus faecalis*), and four Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Salmonella typhimurium*). Their MIC values are summarized in Table 3.23.

Table 3.24 MIC values of compounds **1**-**5, 8** and **24**-**25**

Compound	MIC (μg/mL)								
	Gram-positive bacteria					Gram-negative bacteria			
	BS	SA	SE	SH	EF	ST	EC	PA	PV
Butyrolactone I (**1**)	64	64	64	64	64	256	I	I	I
Butyrolactone II (**2**)	I	I	I	I	I	I	I	I	I
Aspernolide B (**3**)	256	256	64	256	I	I	I	I	I
Aspernolide D (**4**)	I	I	I	256	I	I	I	I	I
Asperterone (**5**)	I	256	I	I	I	I	I	I	I
Terrein (**8**)	I	I	I	256	I	I	128	256	256
Aspernolide A (**24**)	I	I	128	64	I	I	I	I	I
Aspernolide C (**25**)	I	I	256	256	I	I	I	I	I
Vancomycin	0.5 <	0.5 <	0.5 <	0.5 <	0.5 <	I	I	I	I
Gentamycin	I	I	I	I	I	0.5 <	0.5 <	0.5 <	0.5 <

BS : *B. subtilis*, SA : *S. aureus*, SE : *S. epidermidis*, SH : *S. hominis*, EF : *E. faecalis*, ST : *S. typhi*, EC : *E. coli*, PA : *P. aeruginosa*, and PV : *P. vulgaris*

I = inactive > 256 μg/mL

Butyrolactone I (**1**) was found to inhibit all Gram-positive bacteria tested with the same MIC value of 64 μg/mL and showed weak activity on only one Gram-negative strains, *S. typhimurium* (MIC 256 μg/mL). Aspernolide B (**3**) also displayed weak to moderate activity on all Gram-positive bacteria tested, except for *E. faecalis*, while aspernolide D (**4**) and asperterone (**5**) were active on *S. hominis* and *S. aureus* at a dose of 256 μg/mL, respectively. Both synthetic products, **24** and **25**, exhibited antibacterial activity on two gram-positive strains in the genus *Staphylococcus, S. epidermidis* and *S. hominis*; however, cyclized product **24** showed better activity. Terrein (**8**), only a compound was shown to be active against three out of four Gram-negative strains tested, *E. coli*, *P. aeruginosa* and *P. vulgaris*.

3.5.3 Antimalarial activity

The isolated compounds were evaluated for their antimalarial activity against a multidrug-resistant parasite, *Plasmodium falciparum* K1 strains by using microculture radioisotope technique [85]. Results are presented in **Table 3.24**. Only terrein (**8**) exhibited antimalarial activity with IC_{50} value of 3.17 μM. However, it could not be comparable to dihydroartemisinin, a standard drug (IC_{50}, 0.004 μM).

Table 3.25 Antimalarial effect of compounds **1-5** and **8**

Compound	IC_{50} μM
	P. falciparum (K1 strain)
Butyrolactone I (**1**)	I
Butyrolactone II (**2**)	I
Aspernolide B (**3**)	I
Aspernolide D (**4**)	I
Asperterone (**5**)	I
Terrein (**8**)	3.17
Aspernolide A (**24**)	I
Aspernolide C (**25**)	I
Dihydroartemisinin	0.004

I = inactive

CHAPTER IV

CONCLUSION

The goals of this research are to investigate and structural elucidate bioactive compounds from endophytic fungi isolated from three plants belonging to the genus Garcinia, *Mammea siamensis, Mesua ferrea* and *Calophyllum inophyllum.* Based on analysis of chemical constituents of EtOAc extracts of each fungus by ^{1}H NMR spectroscopy, four fungal strains were selected and identified as *Aspergillus terreus* (MS1), *Colletotrichum gloeosporioides* (CI3), *Phoma herbarum* (CI4) and *Ascomycete* sp. (CI5). Purification of EtOAc extract of *A. terreus* cultured on corn steep liquor broth by chromatographic technique led to the isolation of two new butyrolactone derivatives, aspernolide D (**4**) and asperterone (**5**), along with five known derivatives, butyrolactones I (**1**), II (**2**), aspernolide B (**3**), butyrolactones III (**6**) and IV (**7**). Moreover, *A. terreus* also gave terrein as a major product in relatively high yield when fermented in malt extract broth. This indicated that cultured medium has much effect on the production of secondary metabolites of the fungus *A. terreus*. Furthermore, other three fungi selected, CI3, CI4 and CI5 gave additional 15 known metabolites, homodestruxin B (**9**), alternariol (**10**), deoxyterphenylin (**11**), zearalenone (**12**), indole-3-carbaldehyde (**13**), *N,N*-dimethyltryptophane (**14**), dihomo-3´-hydroxy-methyl-dihydroinfectopyron (**15**), malformin A (**16**), tenuazonic acid (**17**), adenosine (**18**), Sumiki's acid (**19**), 5-carboxymellein (**20**), cytochalasin D (**21**), 2(4-bromophenyl) acetic acid (**22**) and citreodrimene F (**23**).

Metabolites obtained from *A. terreus* (**1**-**8**) and two synthetic derivatives (**24**-**25**) were evaluated for their biological activities including cytotoxicity against three human tumor cell lines (hepato carcinoma (HEP-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), anti-bacterial activity against five Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus epidermidis,* and *Enterococcus faecalis*), and four Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Salmonella typhimurium*) and antimalarial activity against *Plasmodium falciparum* (multidrug resistant K1 strains). Only compounds **5** and **8** exhibited cytotoxicity on cancer cell

lines tested with IC$_{50}$ ranging of 2.70-5.63 μM, whereas compounds **1**, **3-5** and **6** displayed weakly to moderately antibacterial activity. In addition, it was found that only terrein (**8**) was shown to be active on the malarial parasite *P. falciparium* K1 tested with IC$_{50}$ value of 3.17 μM.

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APPENDICES

APPENDIX A

MEDIA

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

Potato dextrose agar (PDA)

Potato dextrose broth	240.0 g
Agar	15.0 g
Distilled water	1000 ml

Malt extract broth (MEB)

Malt extract	15.0 g
Peptone	1.0 g
Glucose	20.0 g
Distilled water	1000 ml

Corn steep broth (CSB)

Corn steep liquor	1.0 g
Mannitol	10.0 g
Maltose	10.0 g
Glucose	5.0 g
Monosodium glutamate	10.0 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.3 g
Yeast extract	3.0 g
Distilled water	1000 ml

Rice medium (RM)

Rice	90.0 g
Deionized water	100 ml

Water agar (WA)

Agar	15.0 g
Distilled water	1000 ml

APPENDIX B

NMR spectra and IR
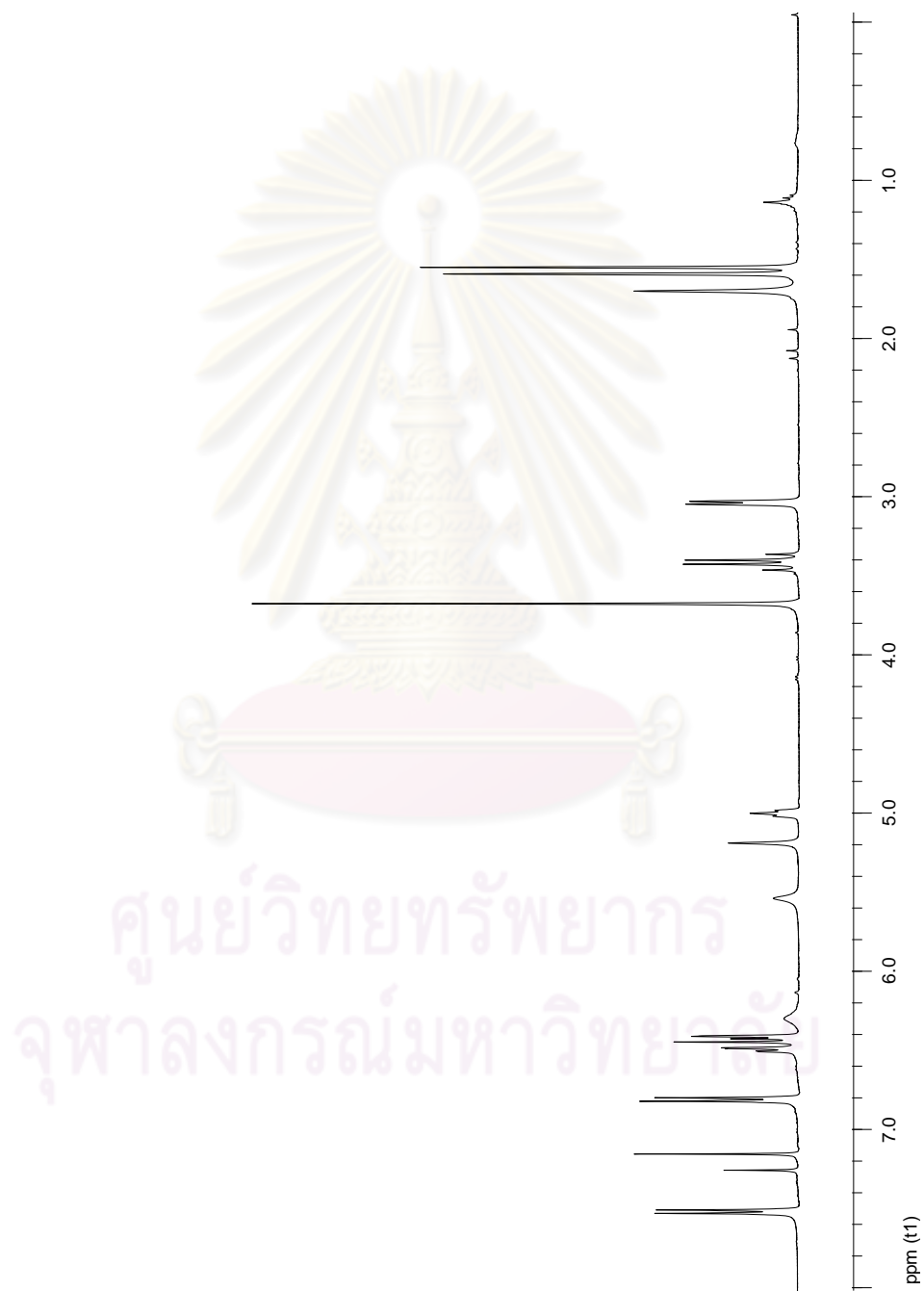
Figure S-1 ^{1}H NMR(400 MHz) spectrum of compound **1** (CDCl$_3$)

ppm (t1)
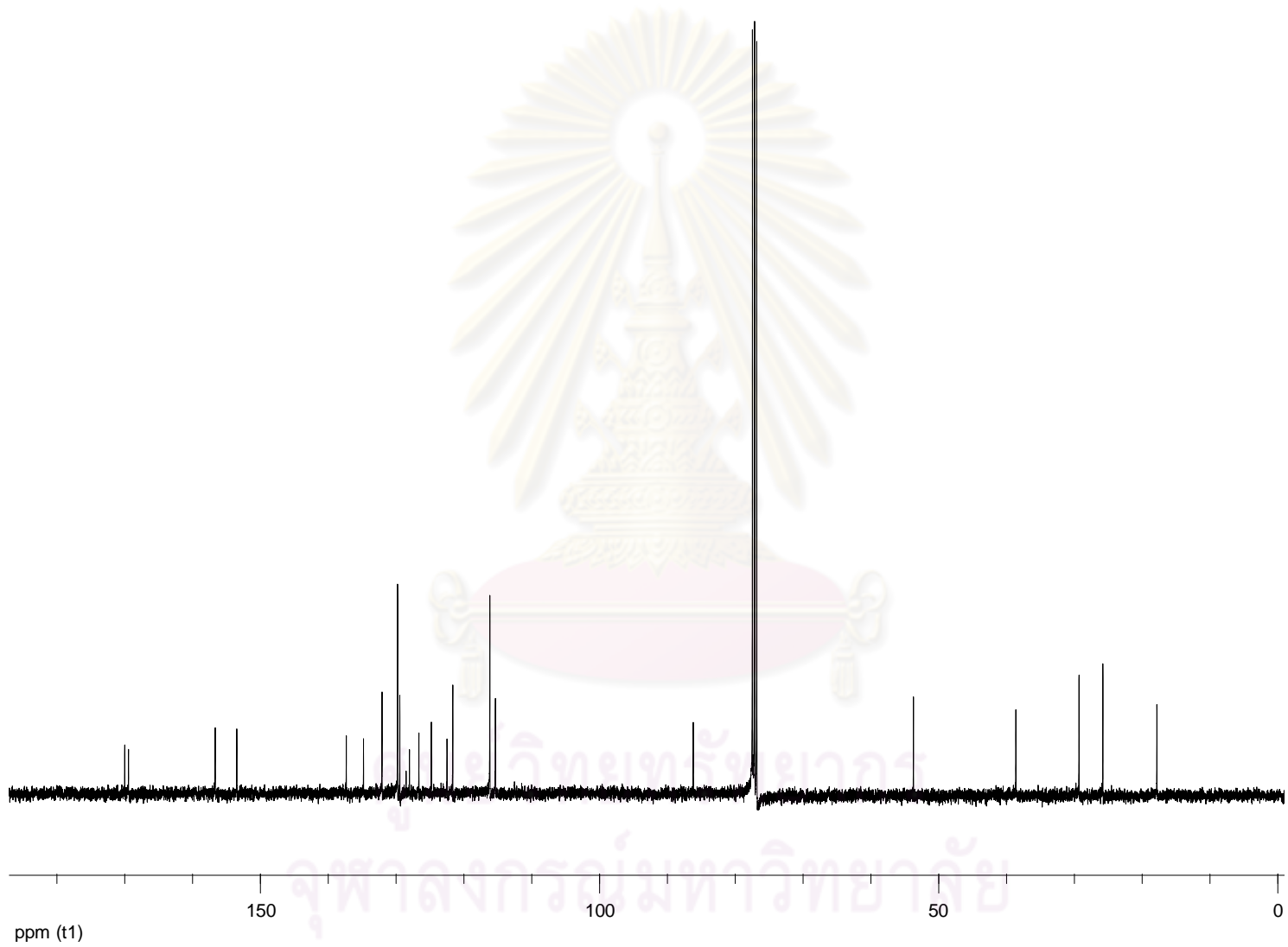
Figure S-2 ^{13}C NMR(100 MHz) spectrum of compound **1** (CDCl$_3$)

116
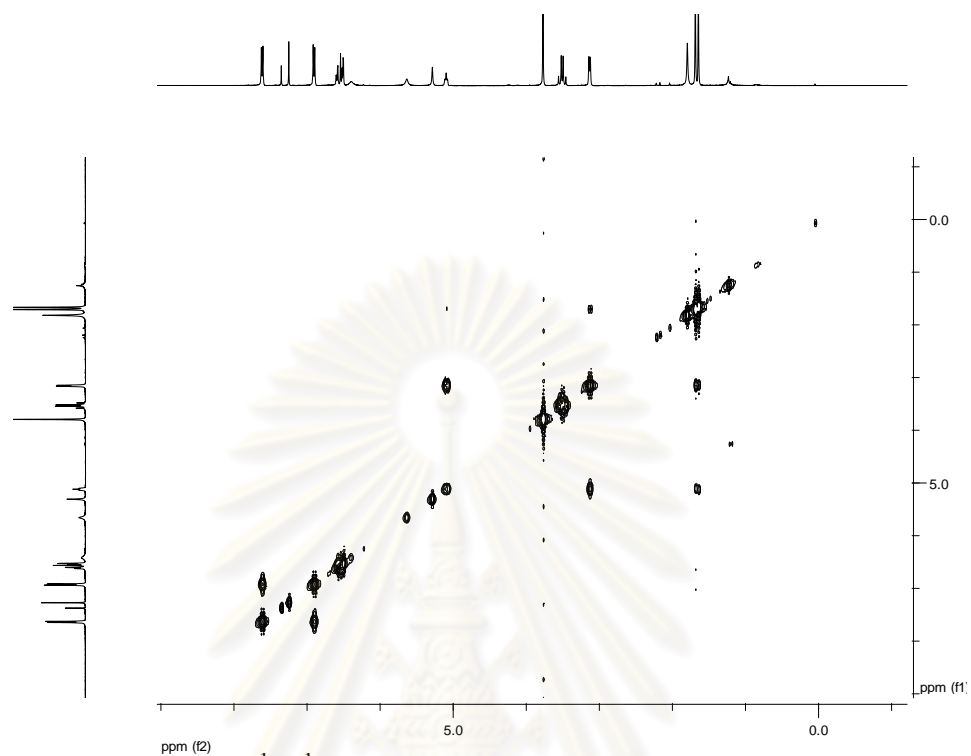
Figure S-3 ^{1}H-^{1}H COSY spectrum of compound **1** (CDCl$_3$)
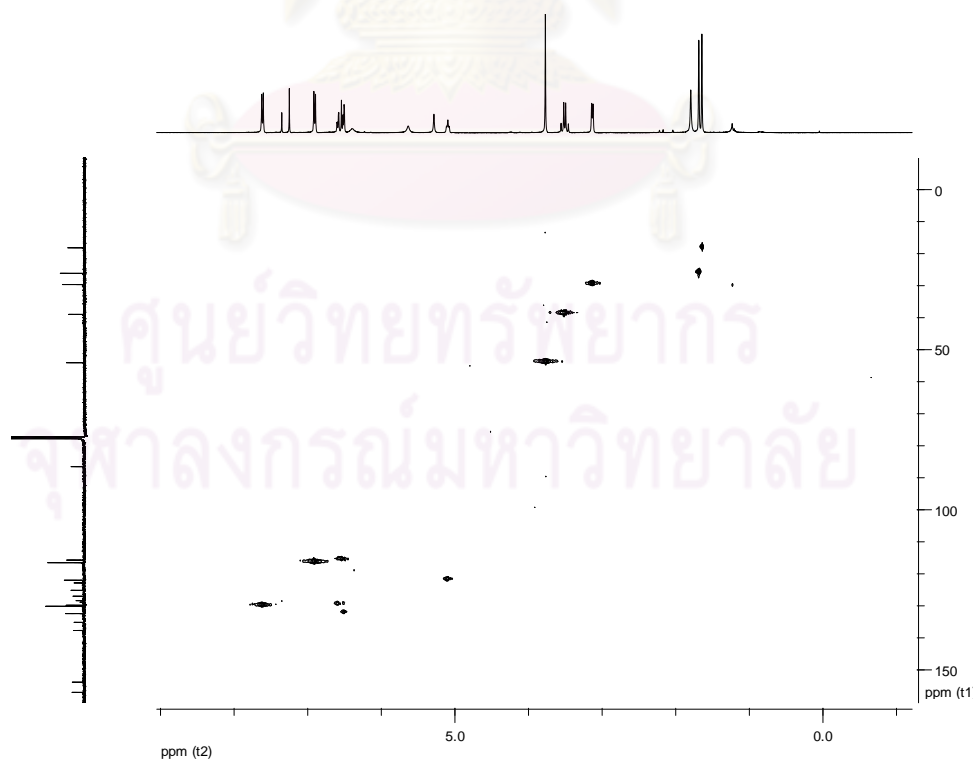
Figure S-4 HSQC spectrum of compound **1** (CDCl$_3$)
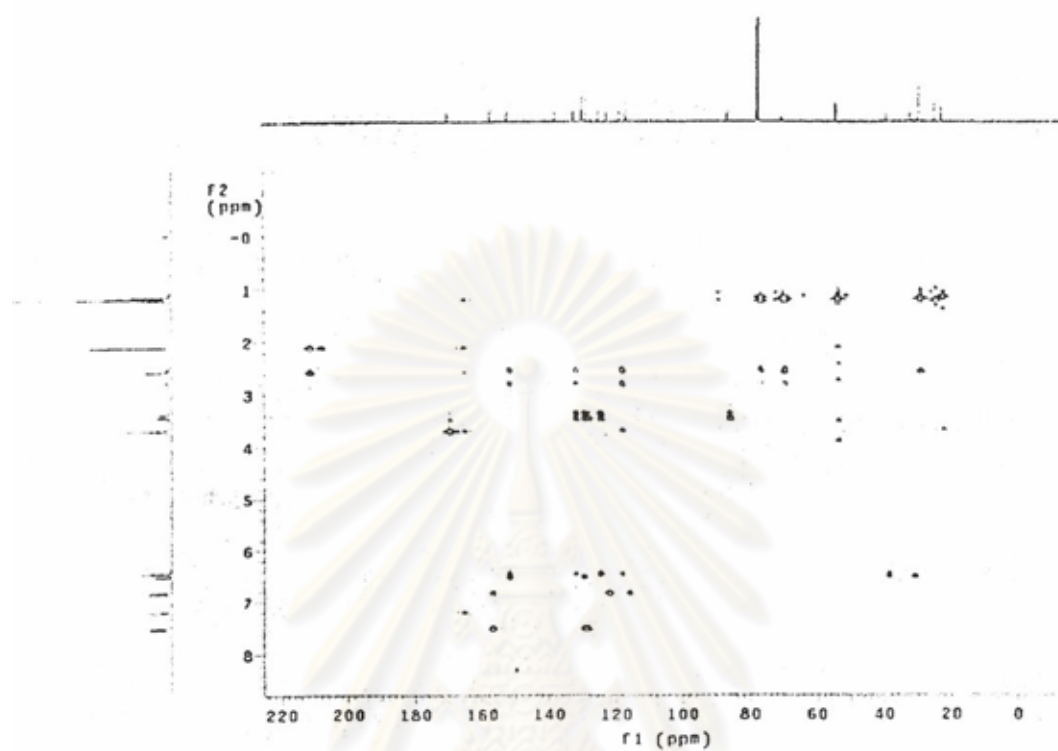
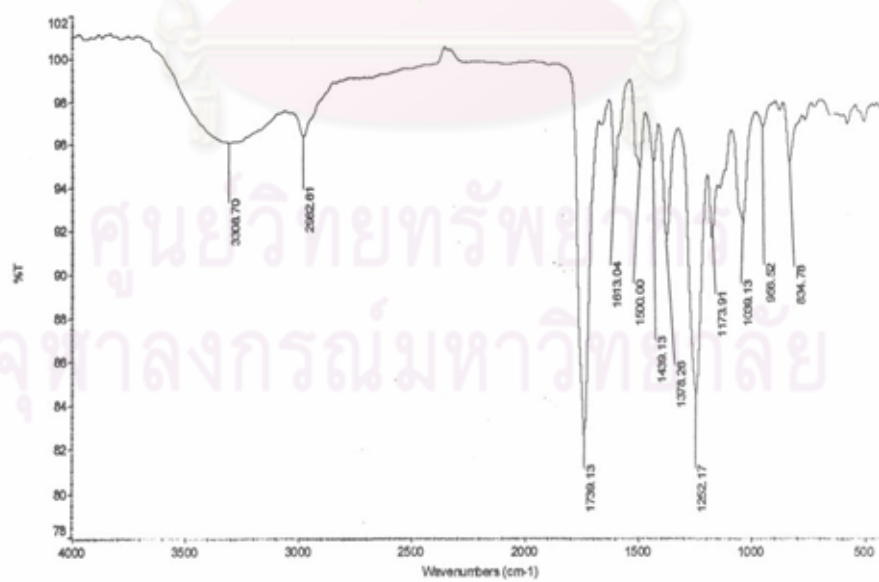**Figure S-5** HMBC spectrum of compound **1** (CDCl₃)
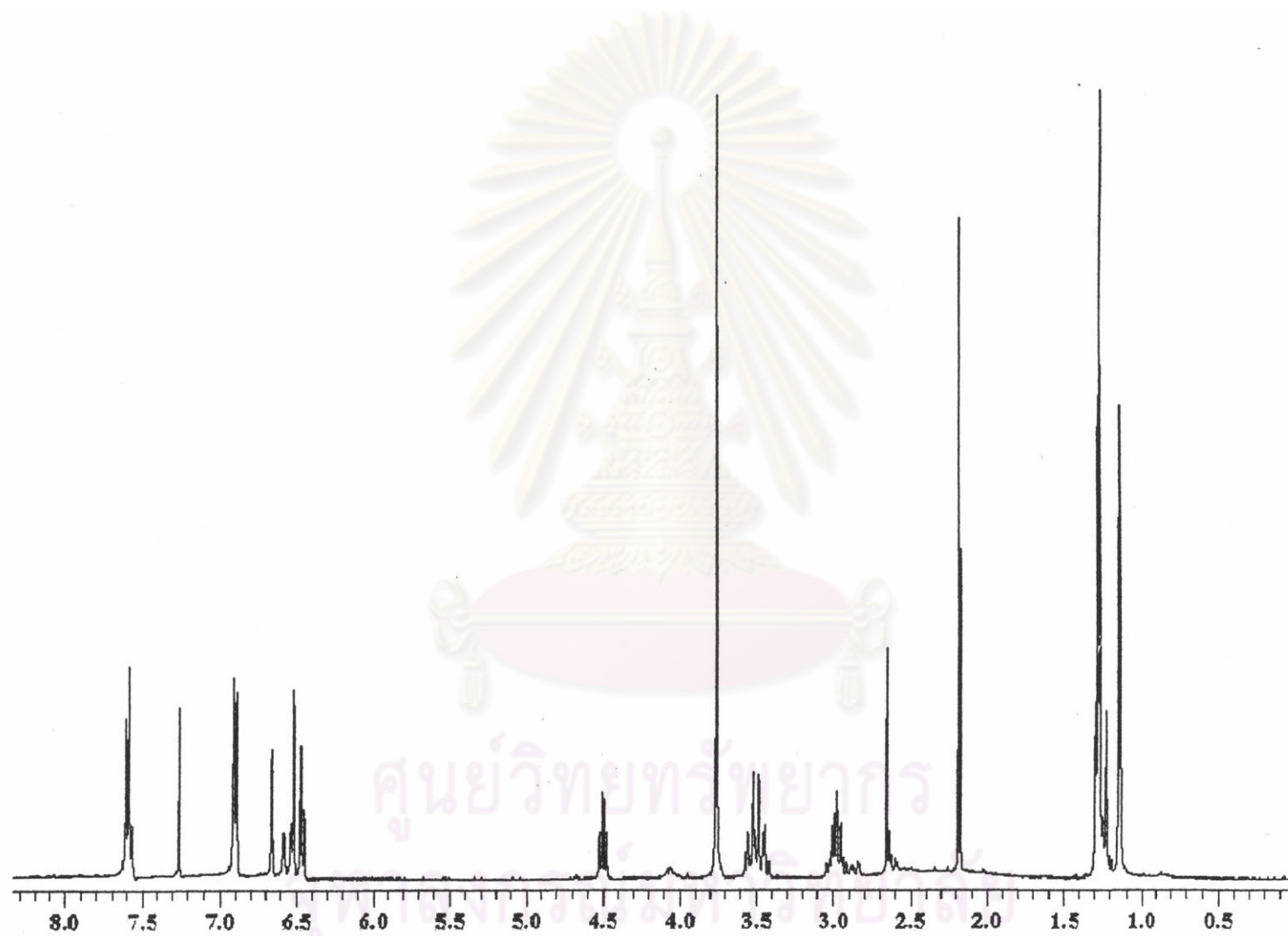

Figure S-6 IR spectrum of compound **1** (KBr)
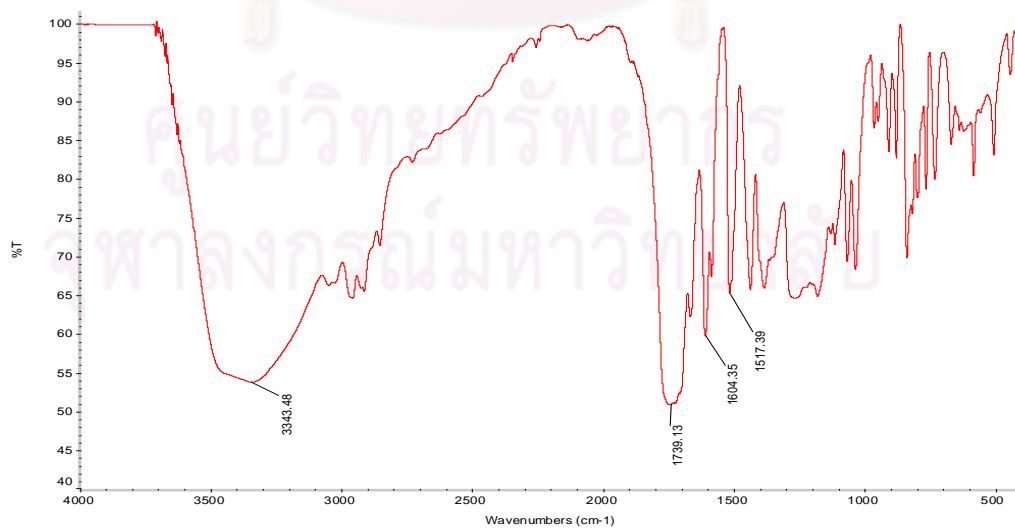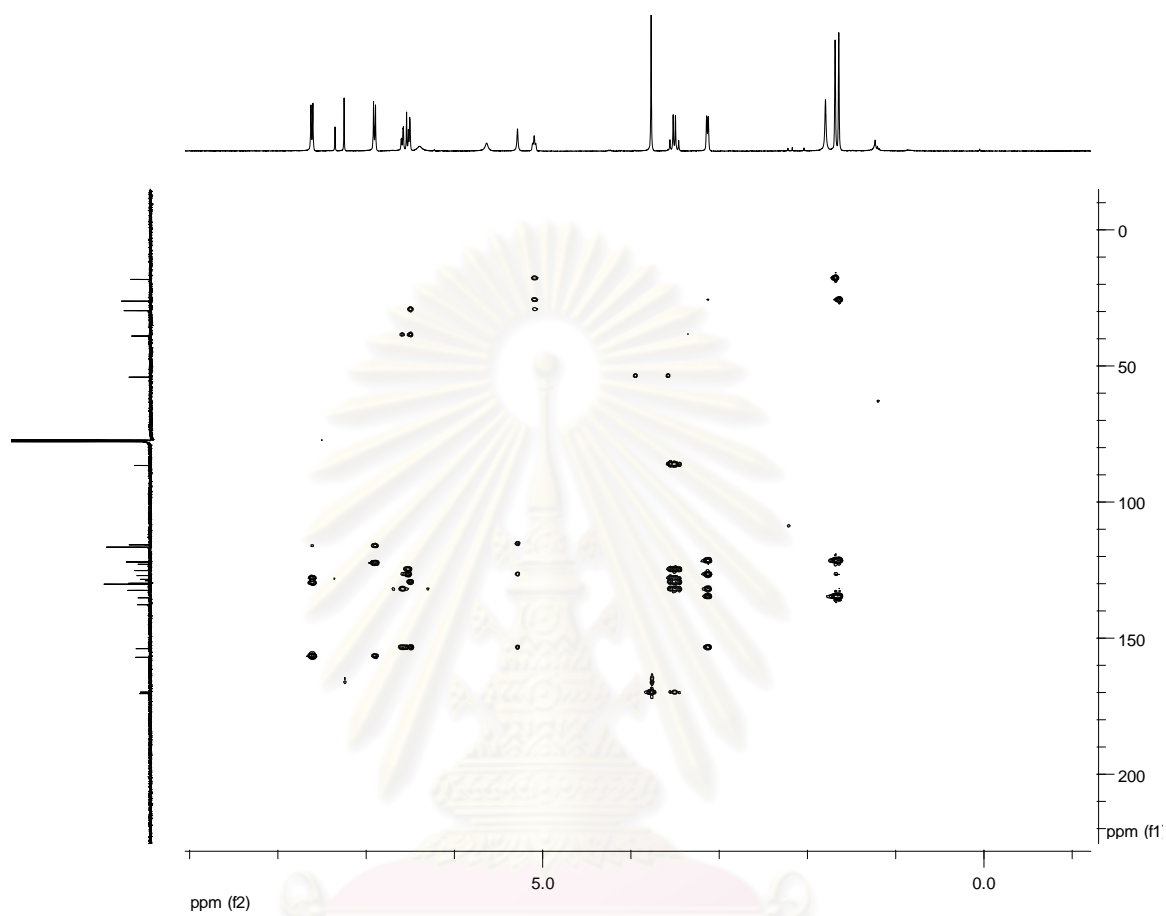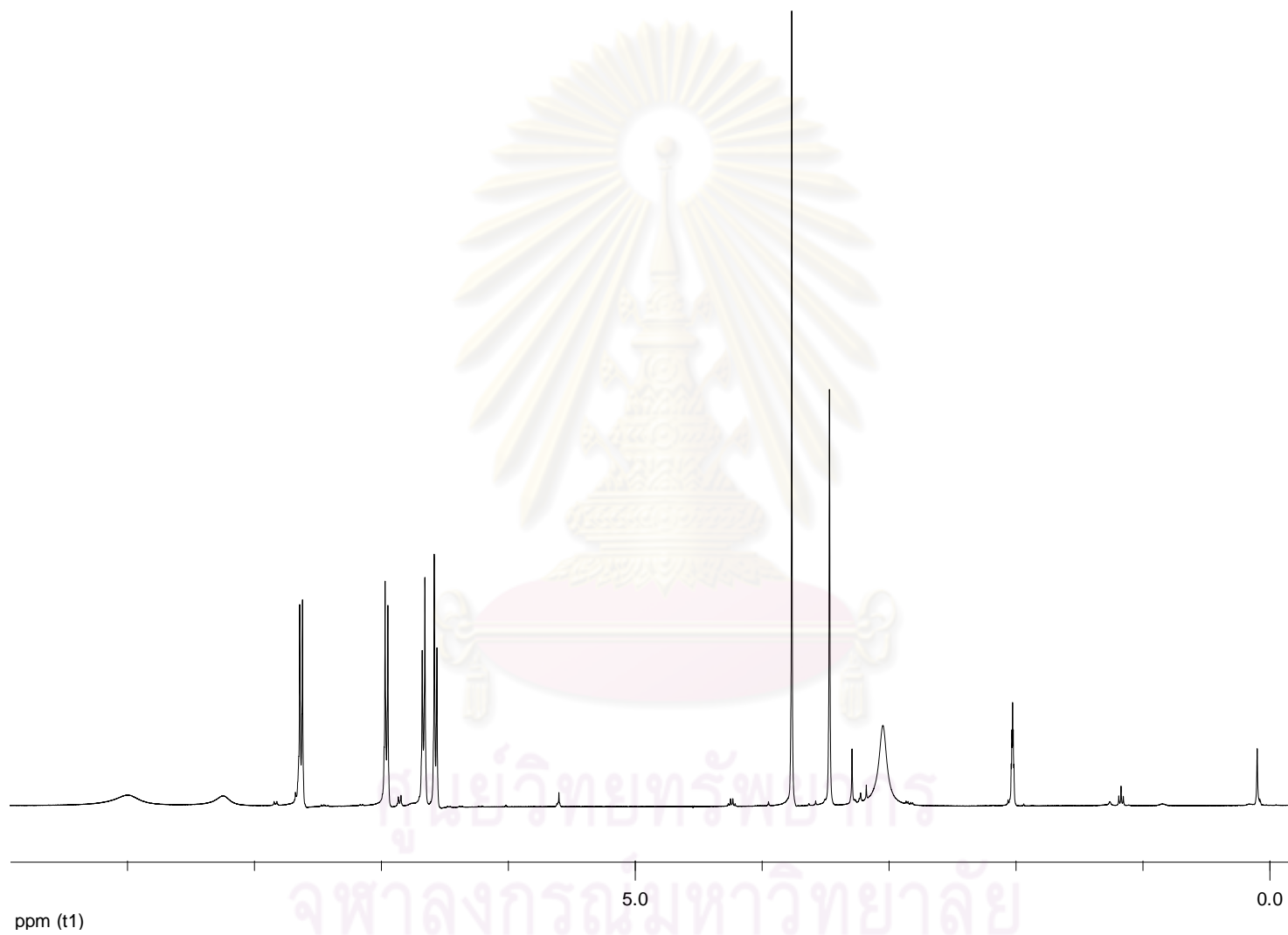
Figure S-7 ^{1}H NMR(400 MHz) spectrum of compound **2** (acetone-*d₆*)
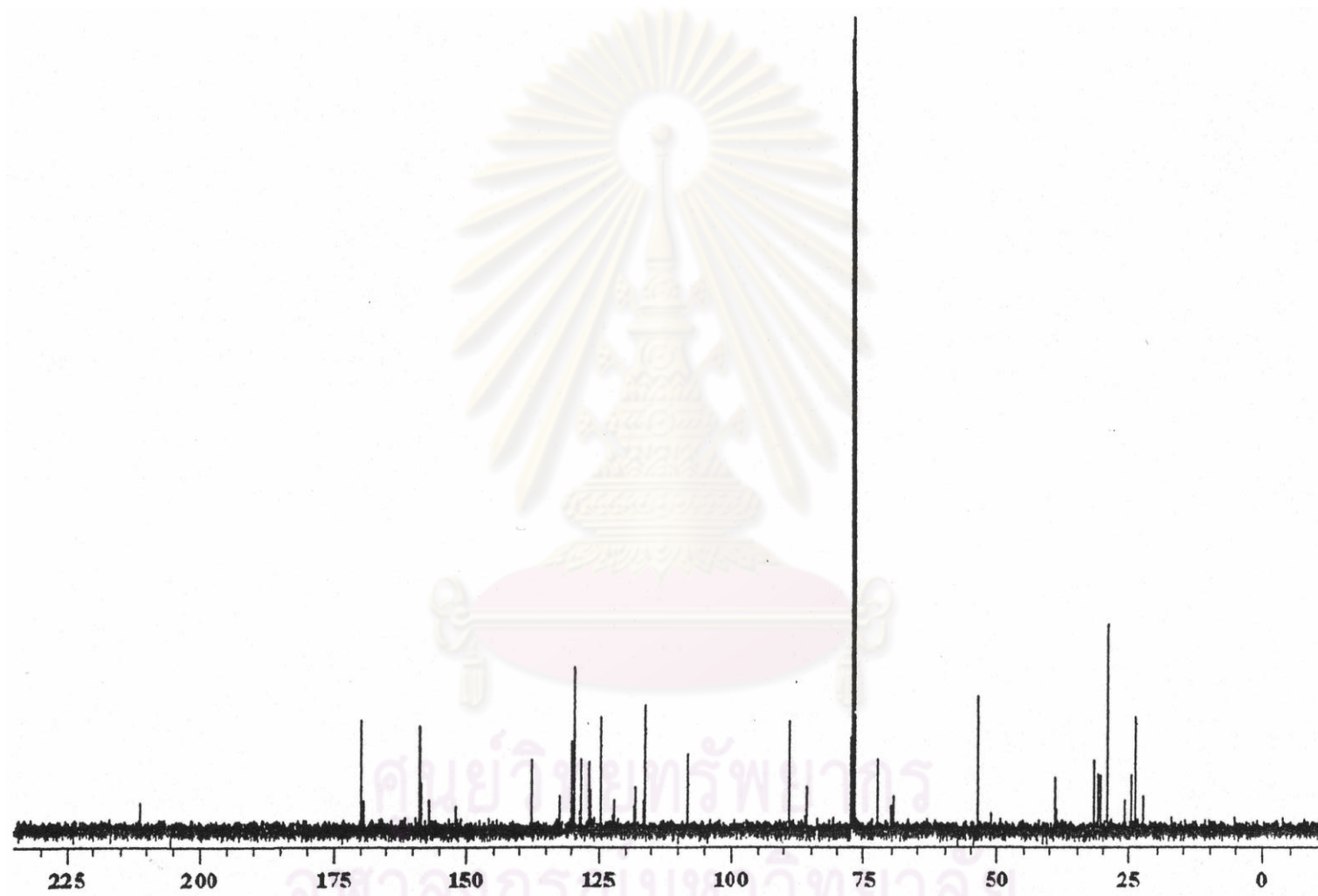
ppm (t1)

5.0

0.0
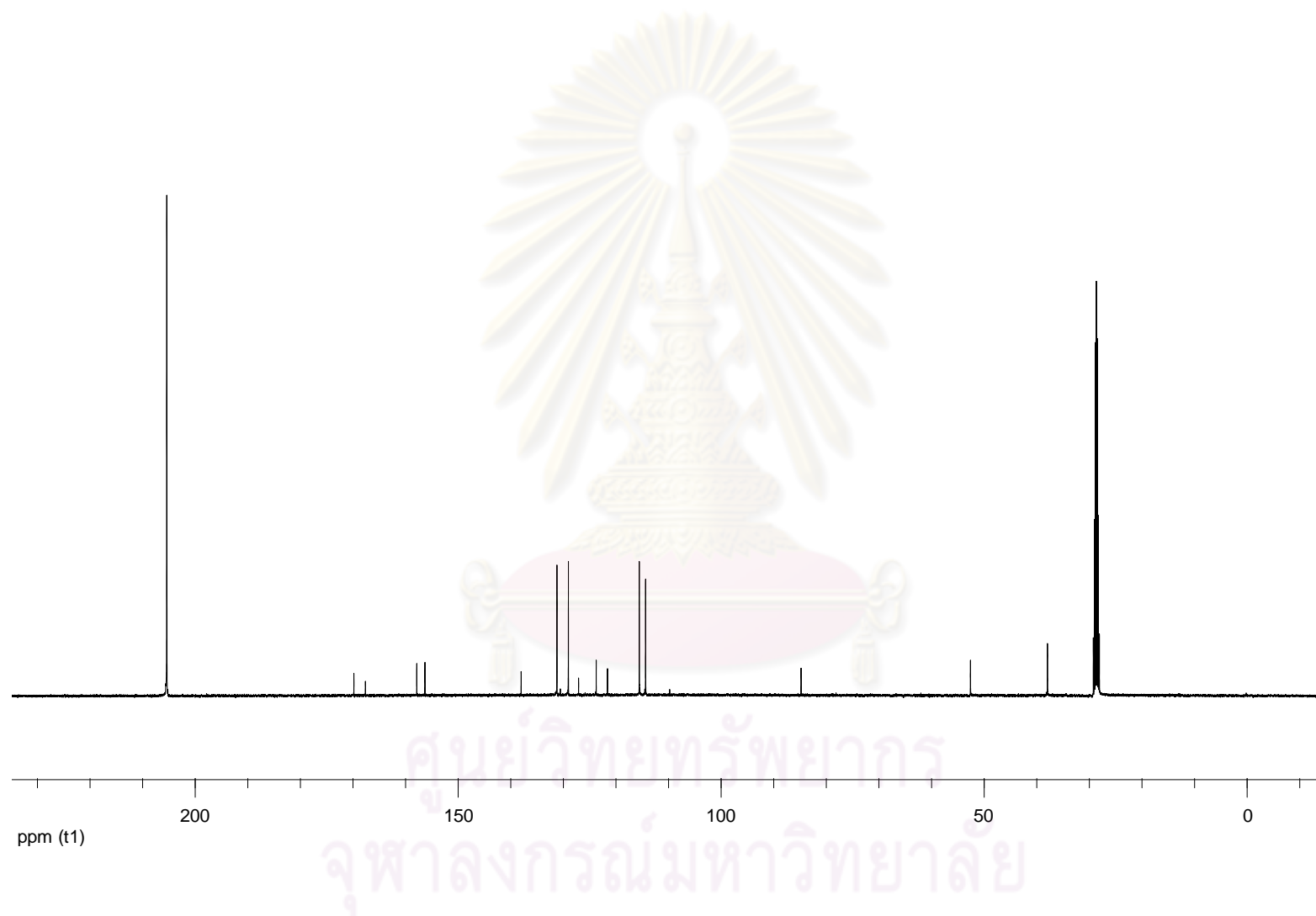
Figure S-8 ^{13}C NMR(100 MHz) spectrum of compound **2** (acetone-*d₆*)
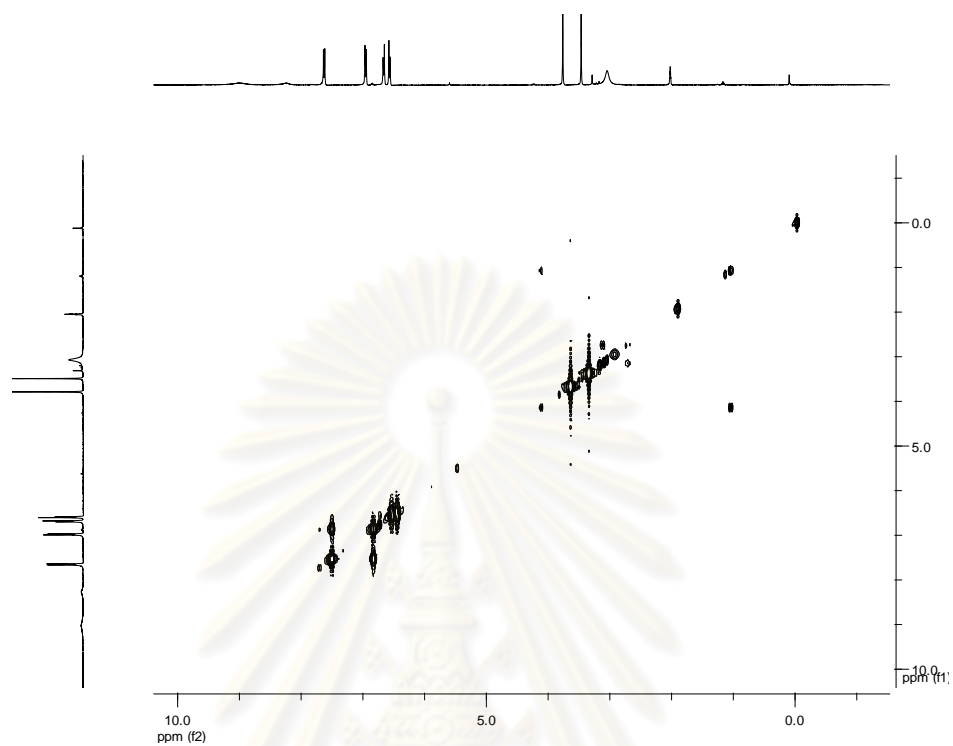
Figure S-9 COSY spectrum of compound **2** (acetone-*d₆*)
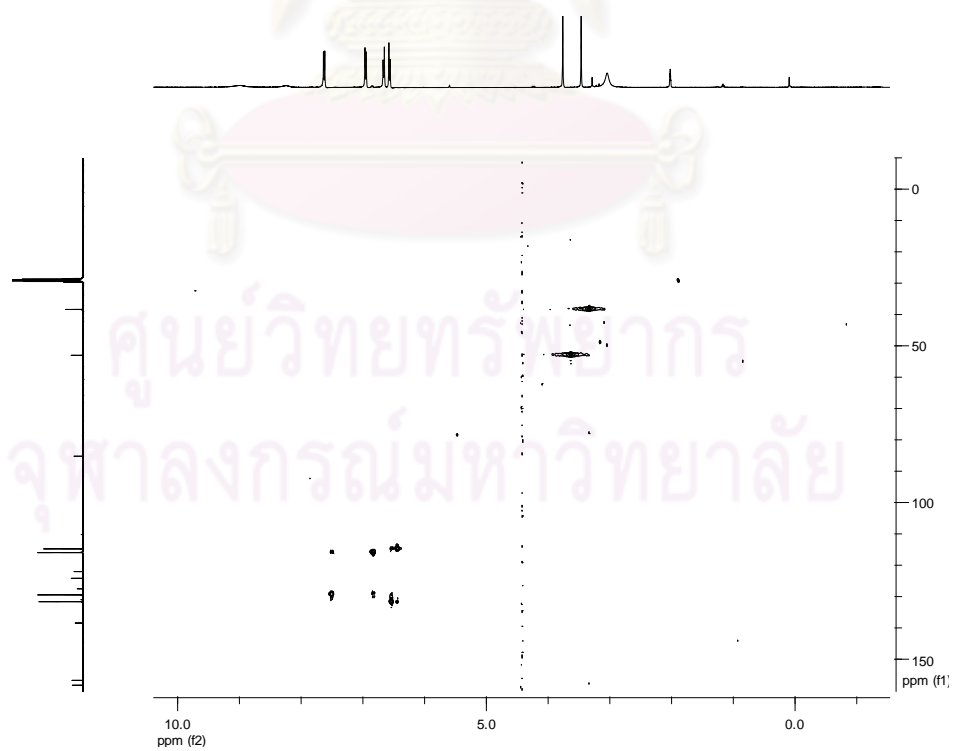
Figure S-10 HSQC spectrum of compound **2** (acetone-*d₆*)
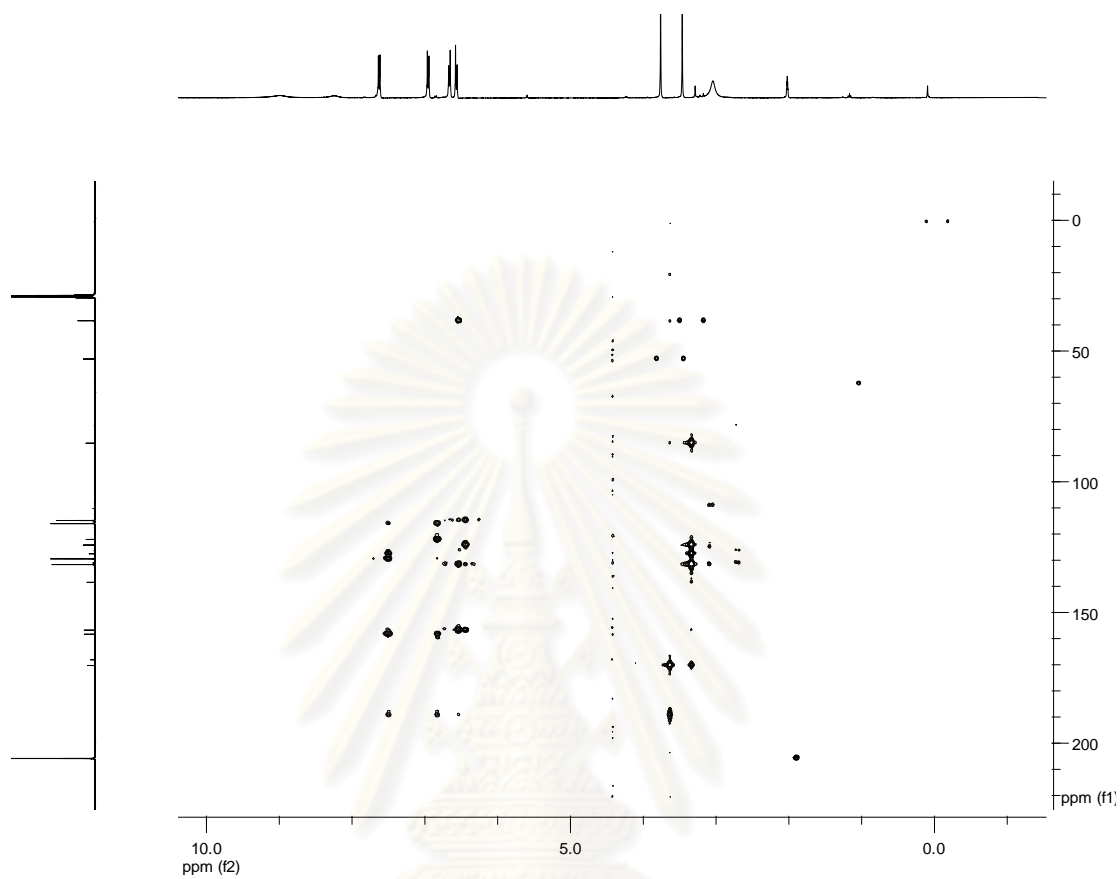
Figure S-11 HMBC spectrum of compound **2** (acetone-*d₆*)
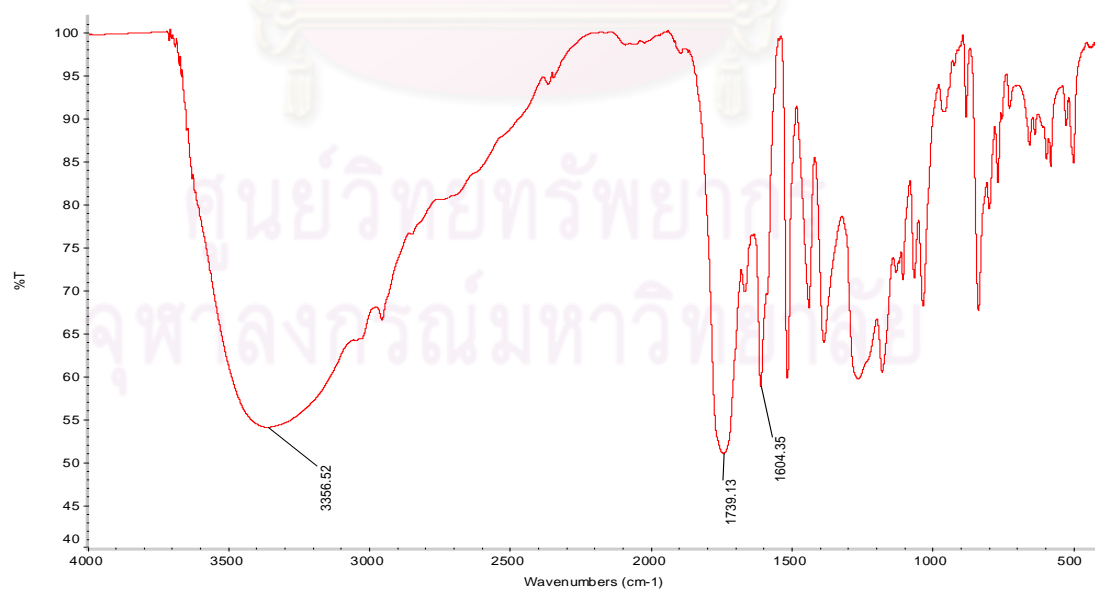
Figure S-12 IR spectrum of compound **2** (KBr)
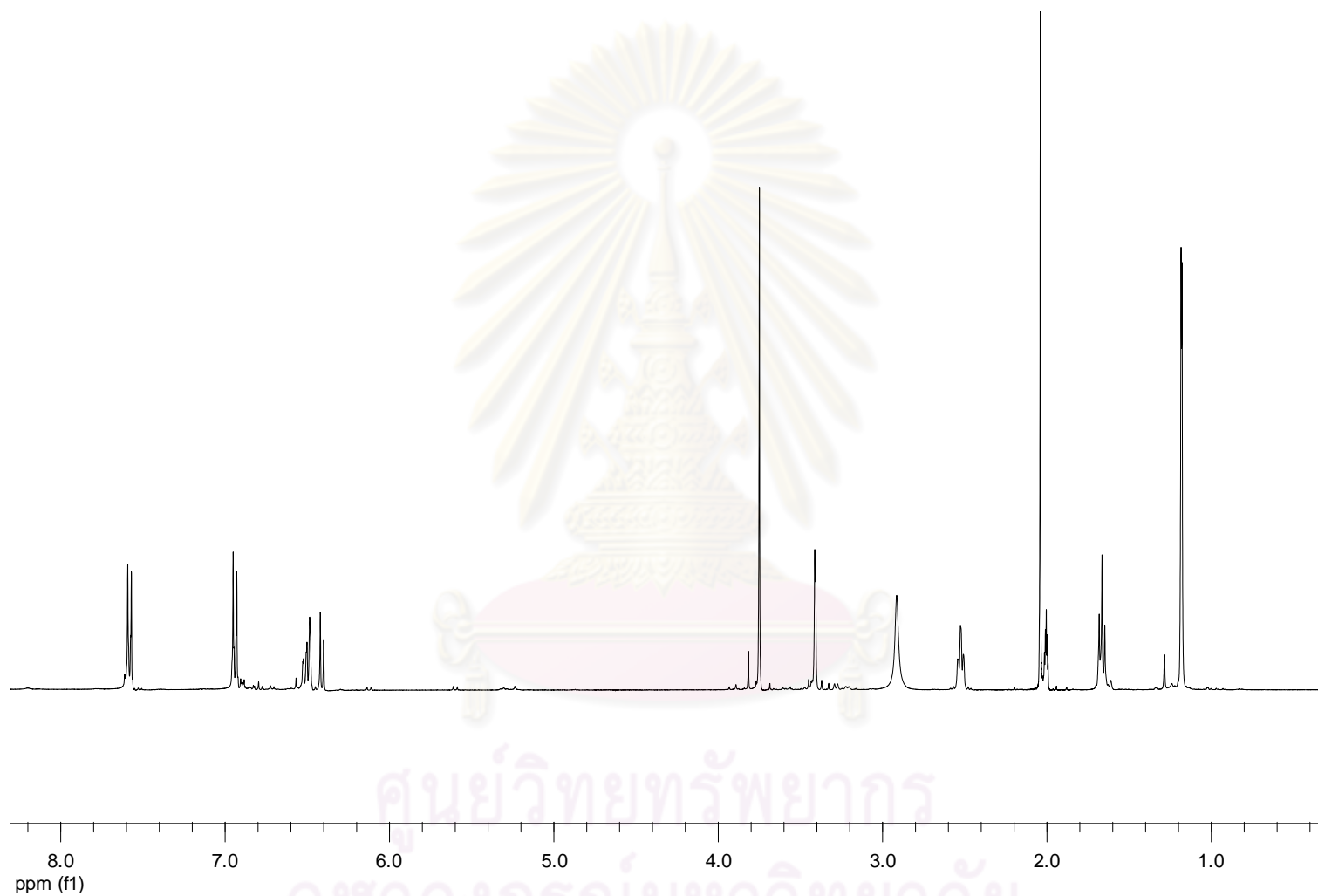
Figure S-13 ^{1}H NMR(400 MHz) spectrum of compound **3** (acetone-d_6)

Figure S-14 ^{13}C NMR(100 MHz) spectrum of compound **3** (acetone-d_6)

ppm (f1)
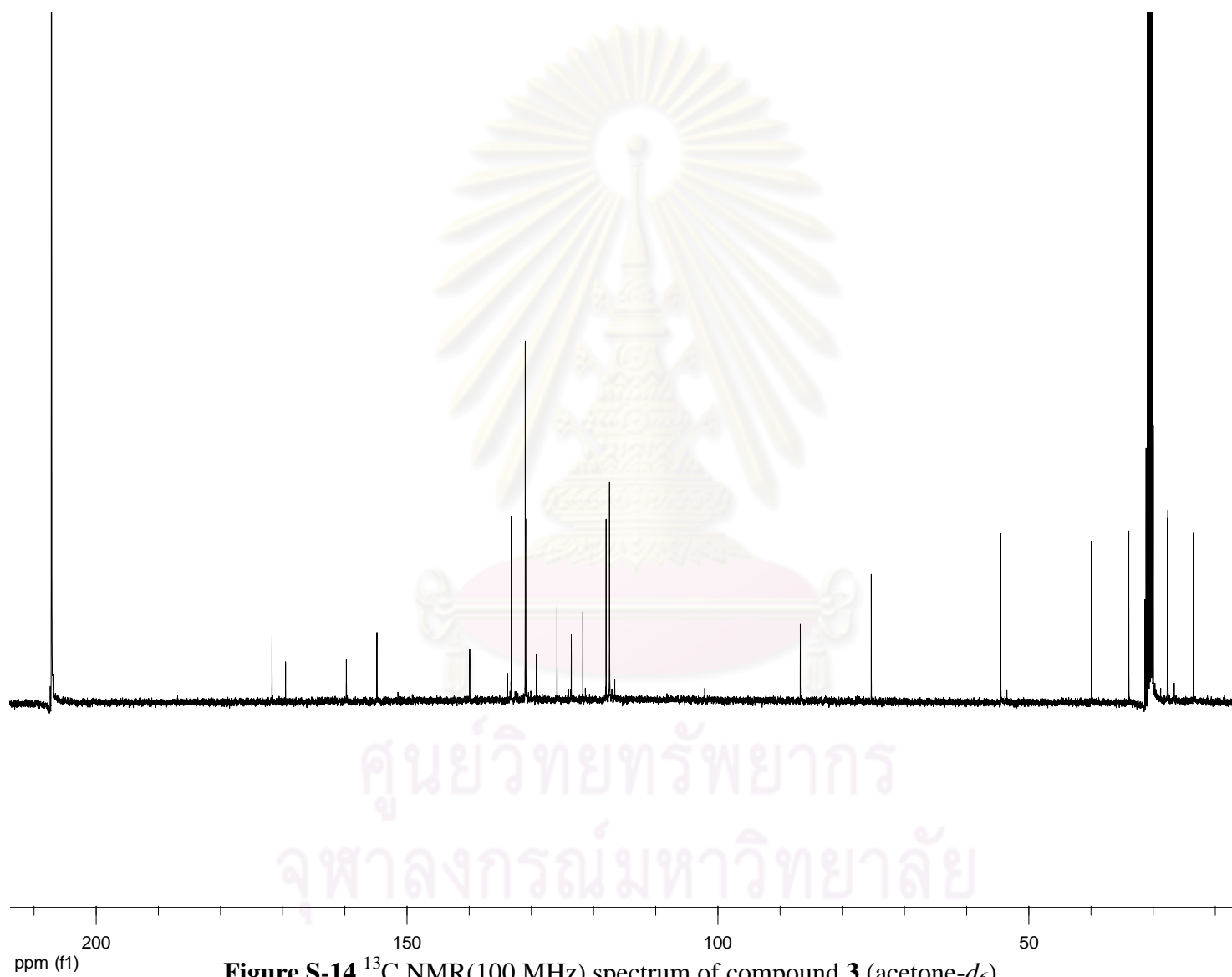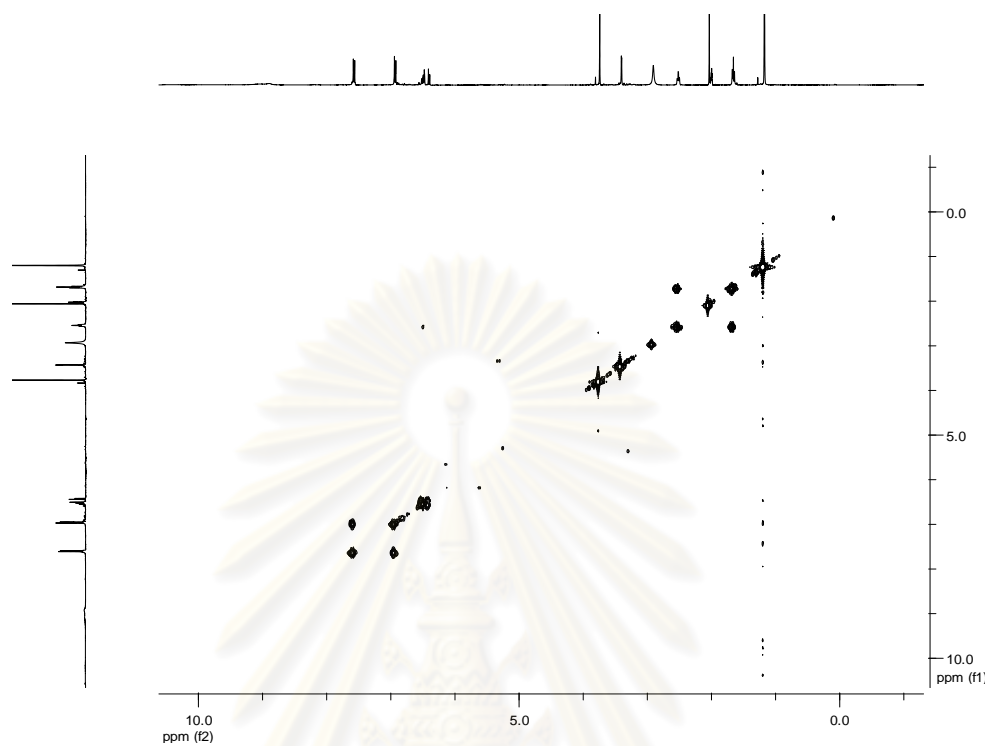
Figure S-15 COSY spectrum of compound **3** (acetone-*d₆*)
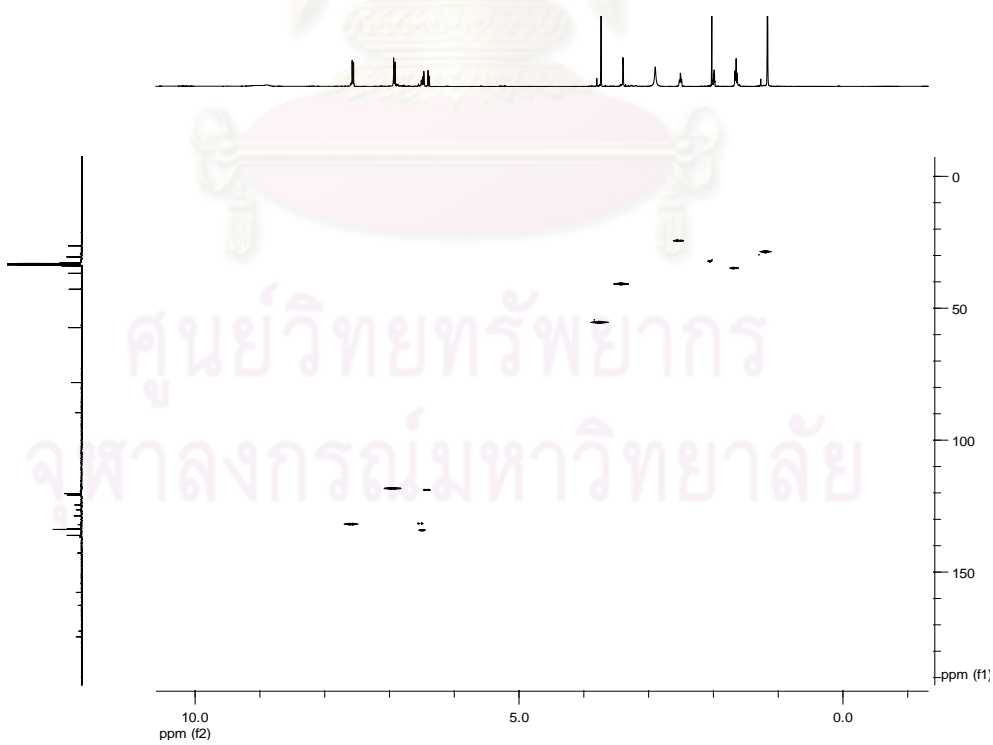
Figure S-16 HSQC spectrum of compound **3** (acetone-*d₆*)
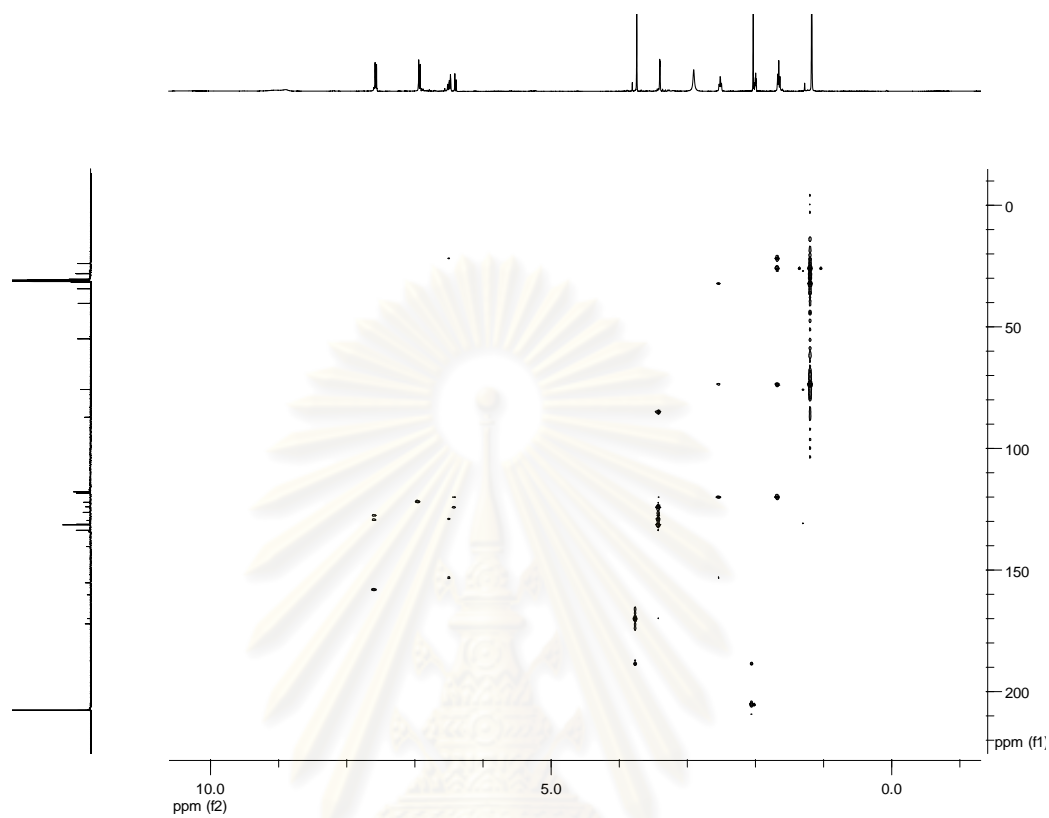
Figure S-17 HMBC spectrum of compound **3** (acetone-d_6)
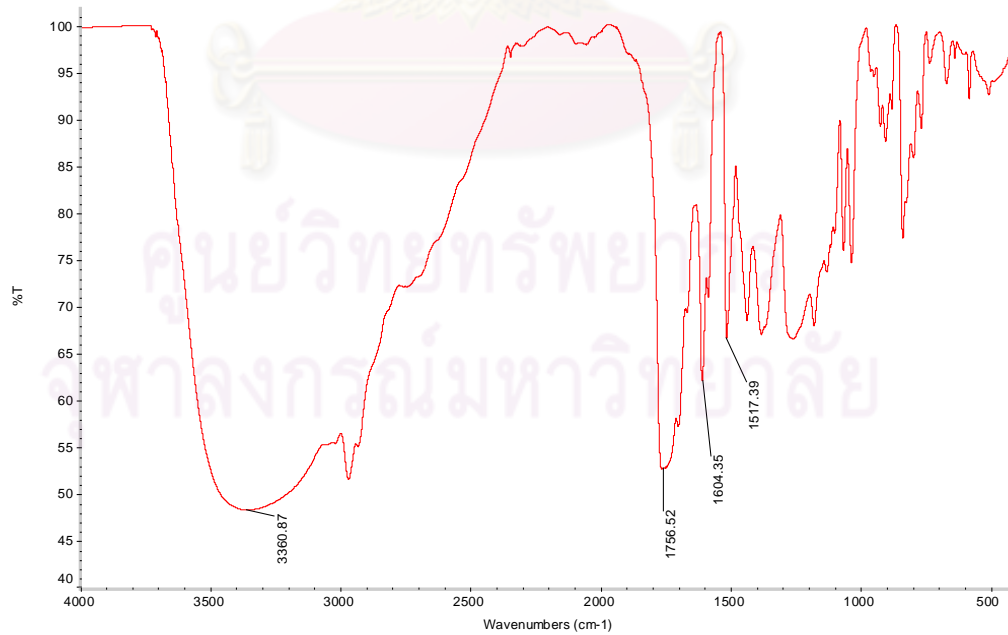
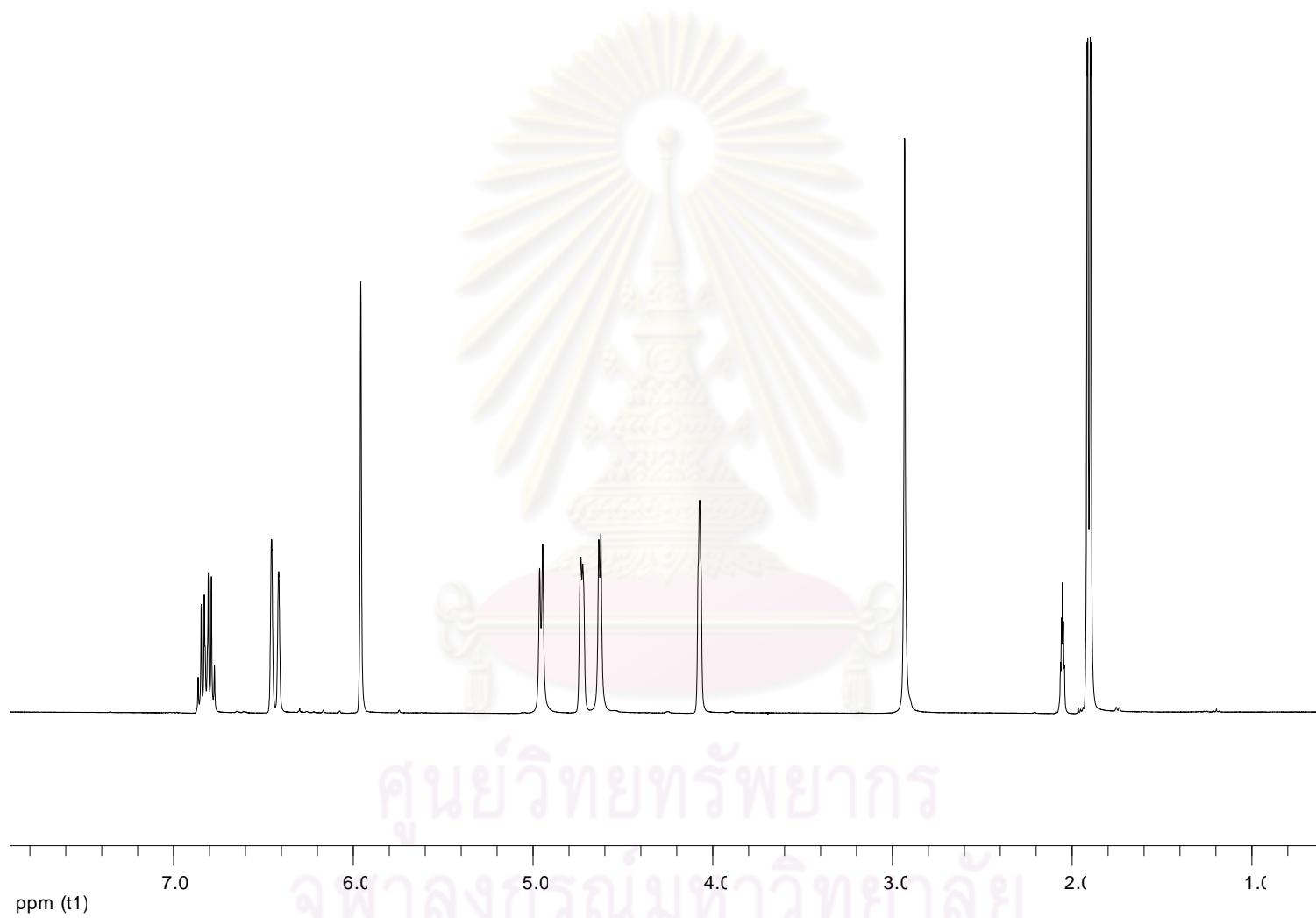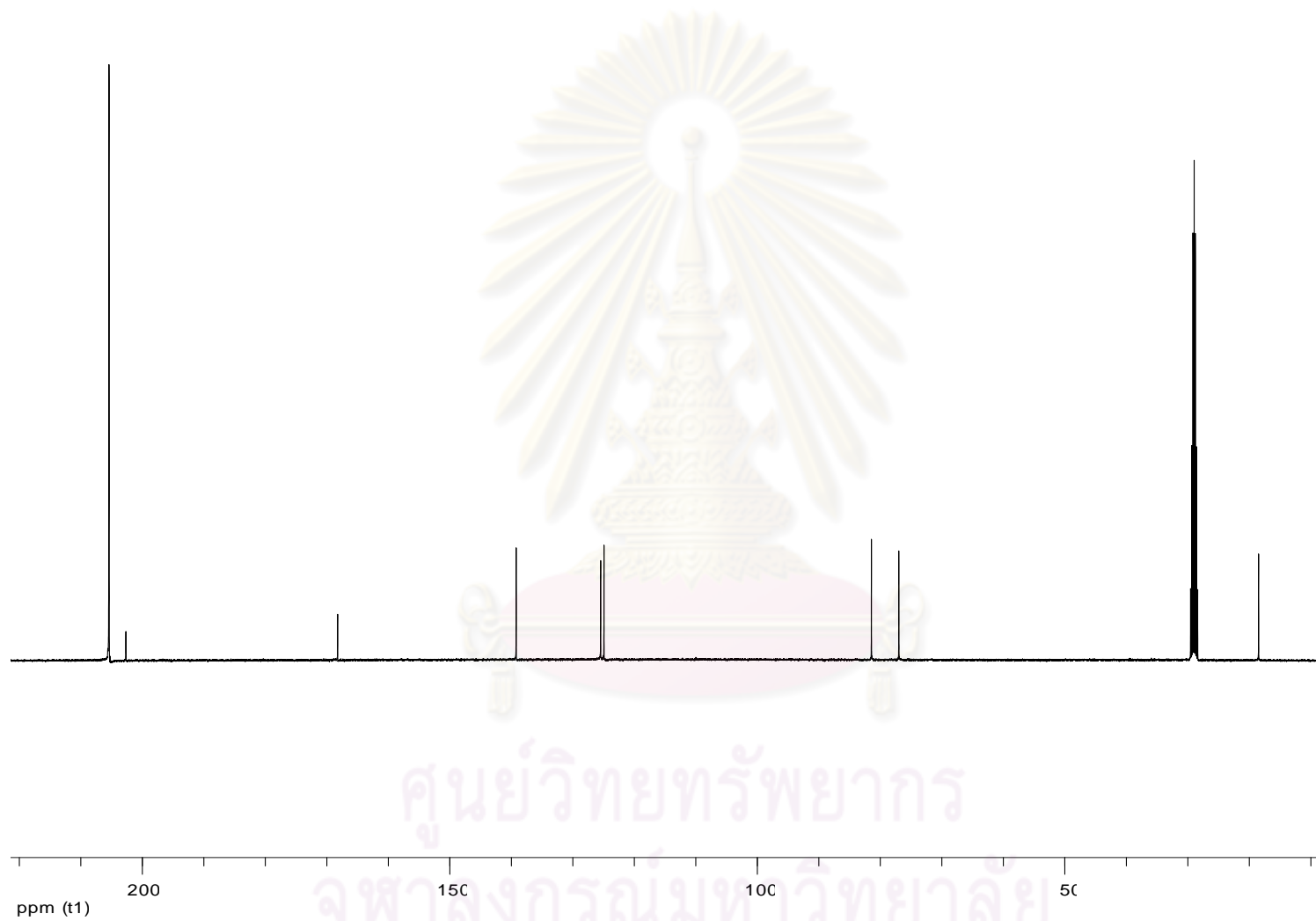**Figure S-18** IR spectrum of compound **3** (KBr)
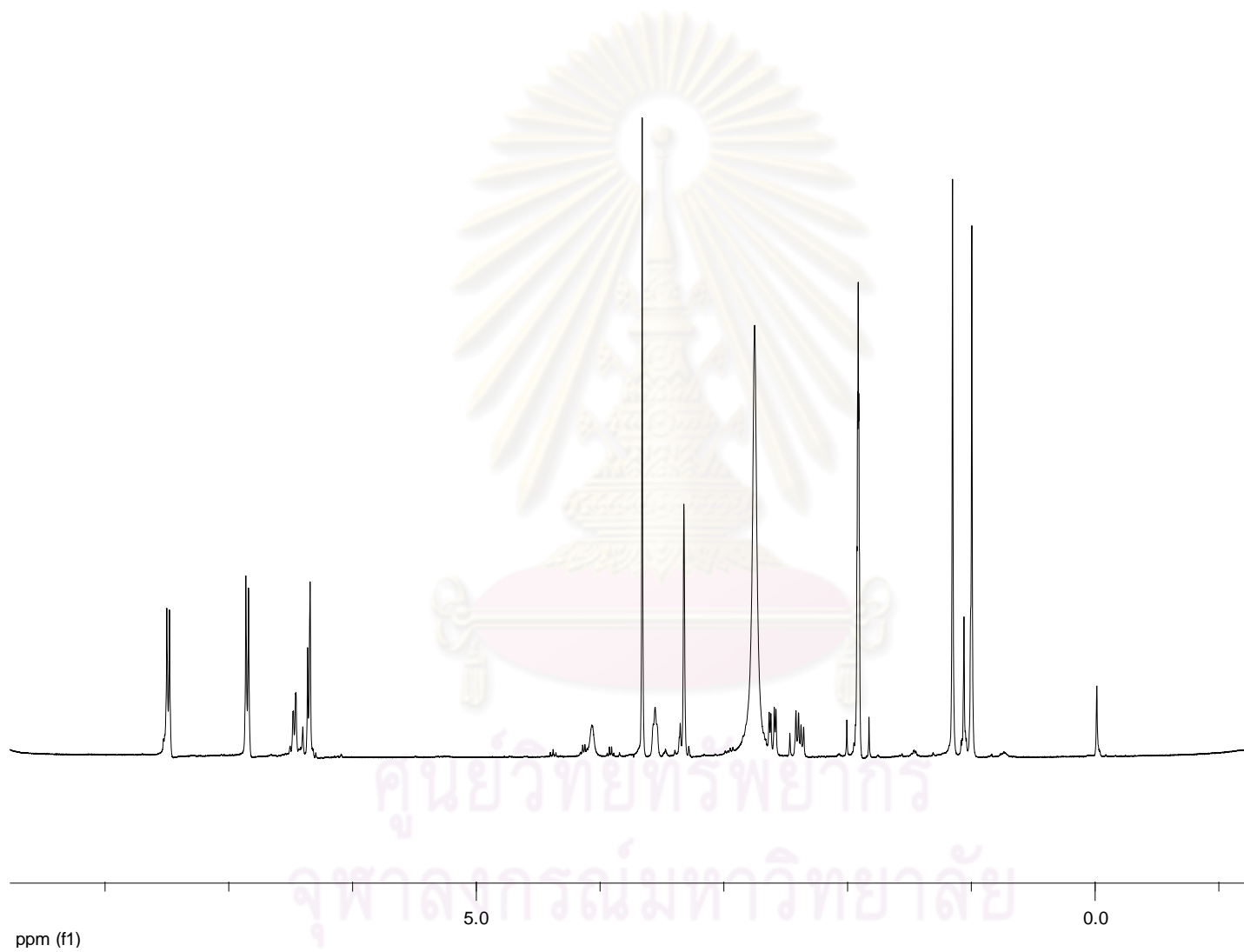
Figure S-19 ^{1}H NMR(400 MHz) spectrum of compound **4** (acetone-d_6)

Figure S-20 ^{13}C NMR(100 MHz) spectrum of compound **4** (acetone-d_6)

ppm (f1)   200   150   100   50   0
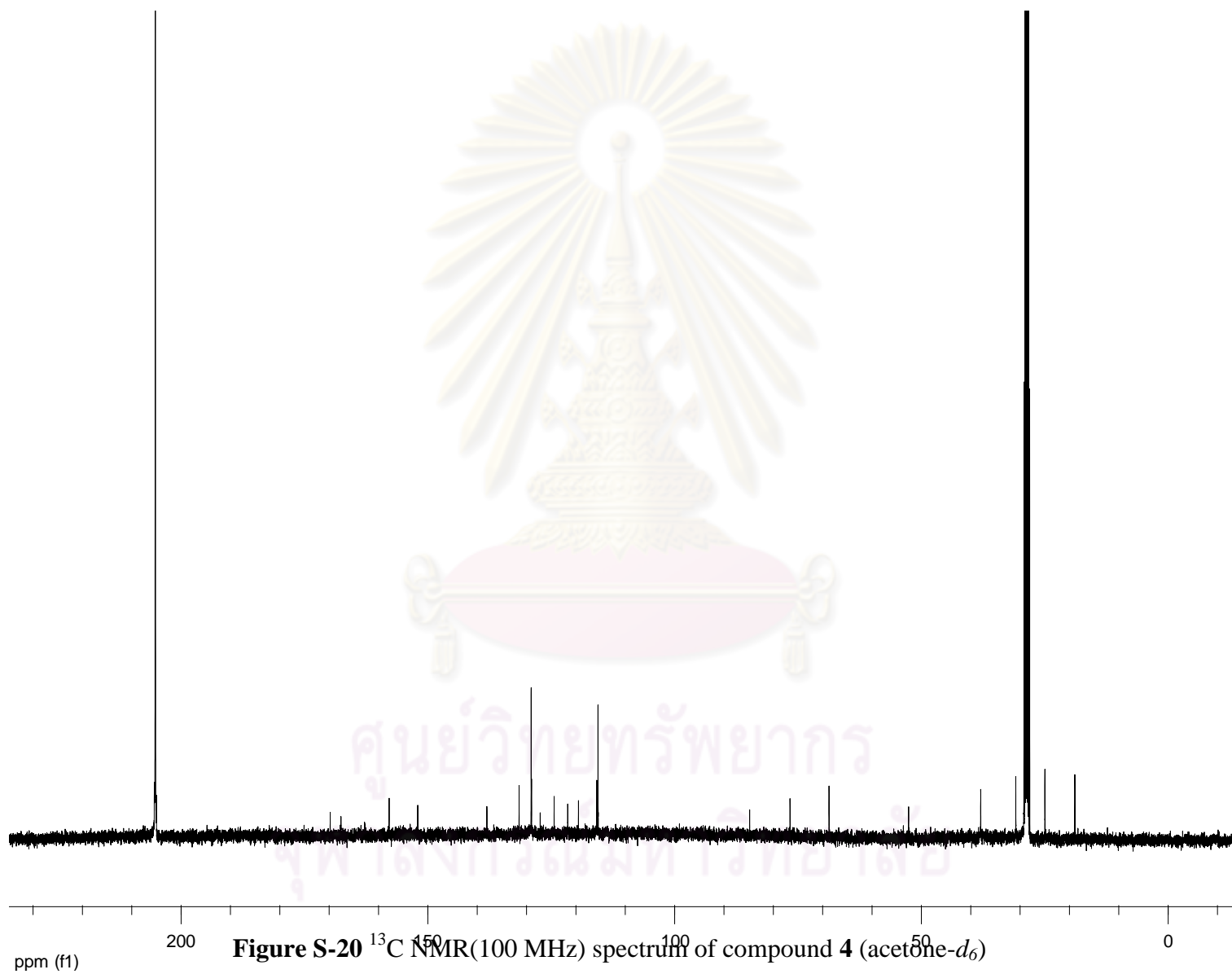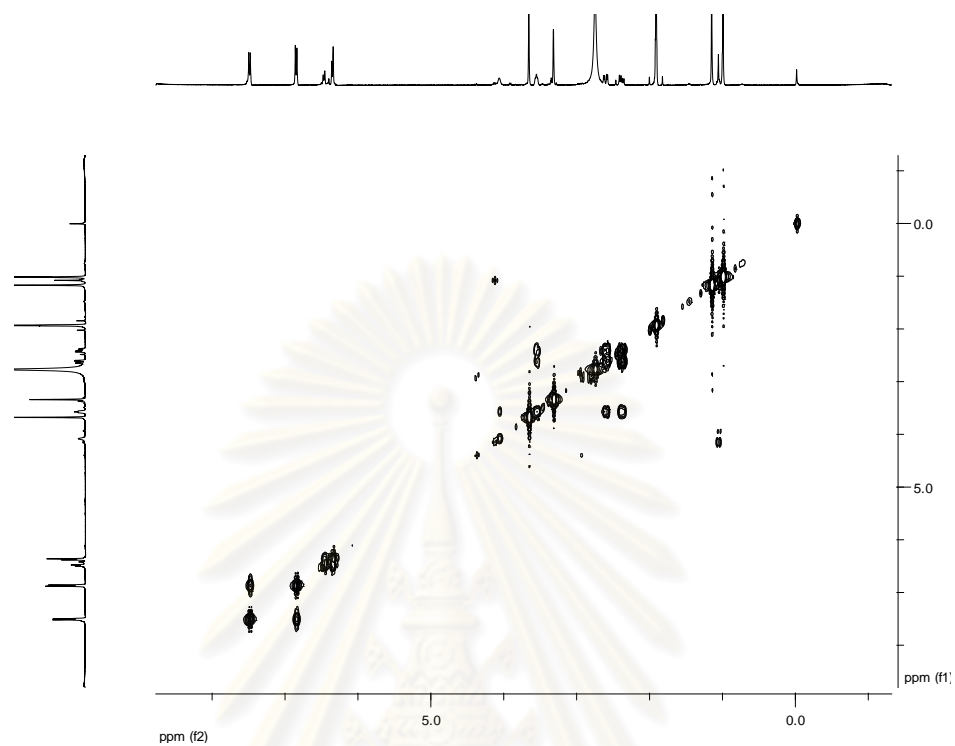
Figure S-21 COSY spectrum of compound **4** (acetone-*d₆*)
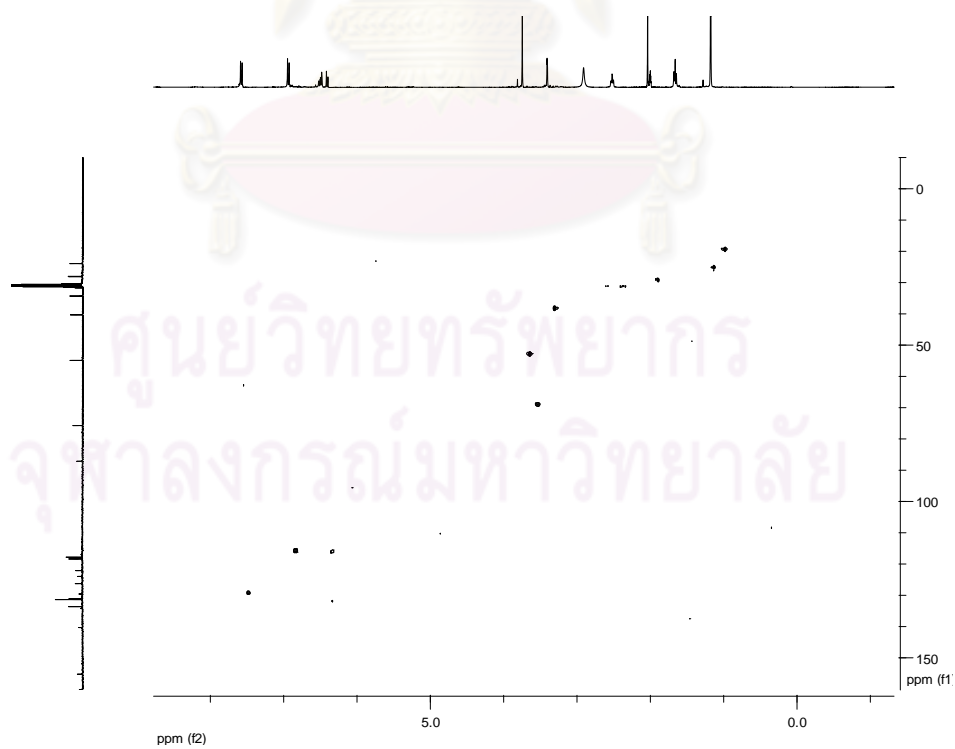
Figure S-22 HSQC spectrum of compound **4** (acetone-*d₆*)
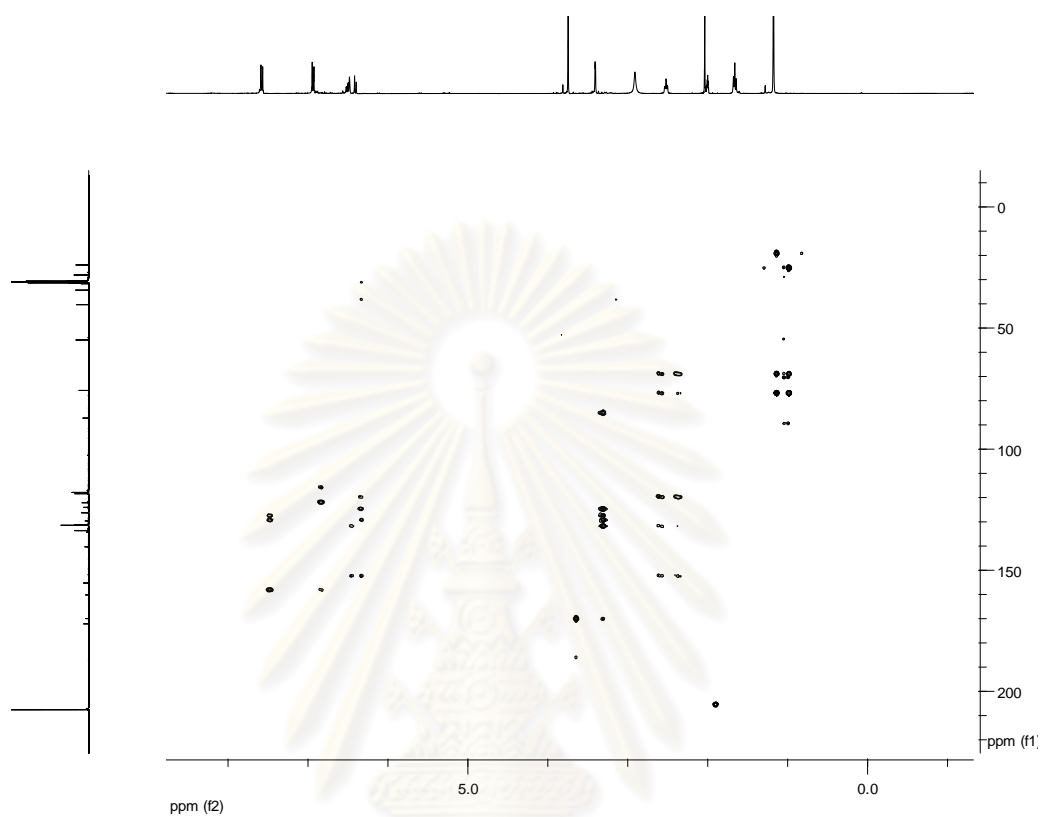
Figure S-23 HMBC spectrum of compound **4** (acetone-*d₆*)
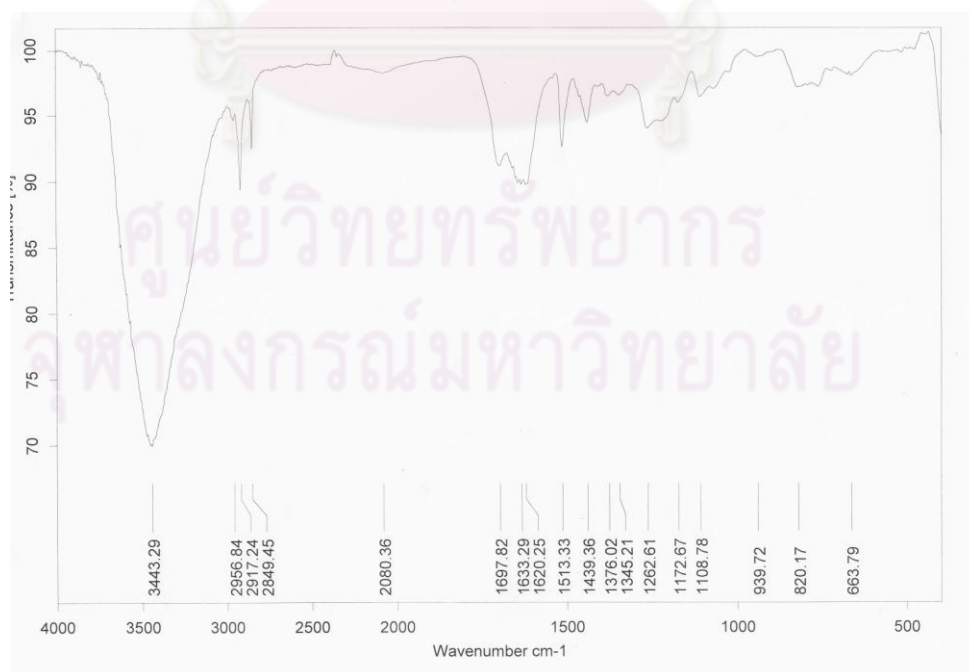
Figure S-24 IR spectrum of compound **4** (KBr)
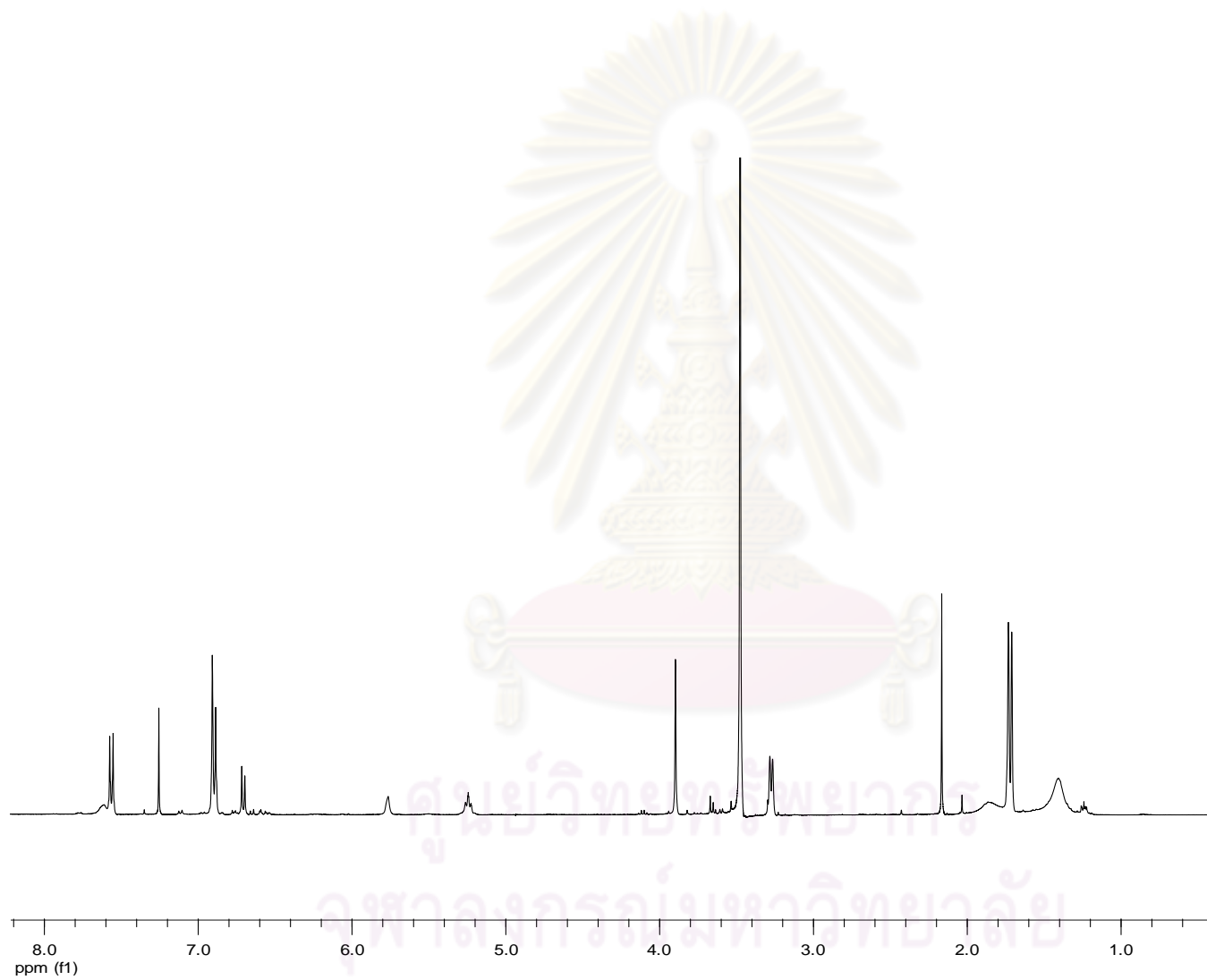
Figure S-25 ^{1}H NMR(400 MHz) spectrum of compound **5** (CDCl$_3$)
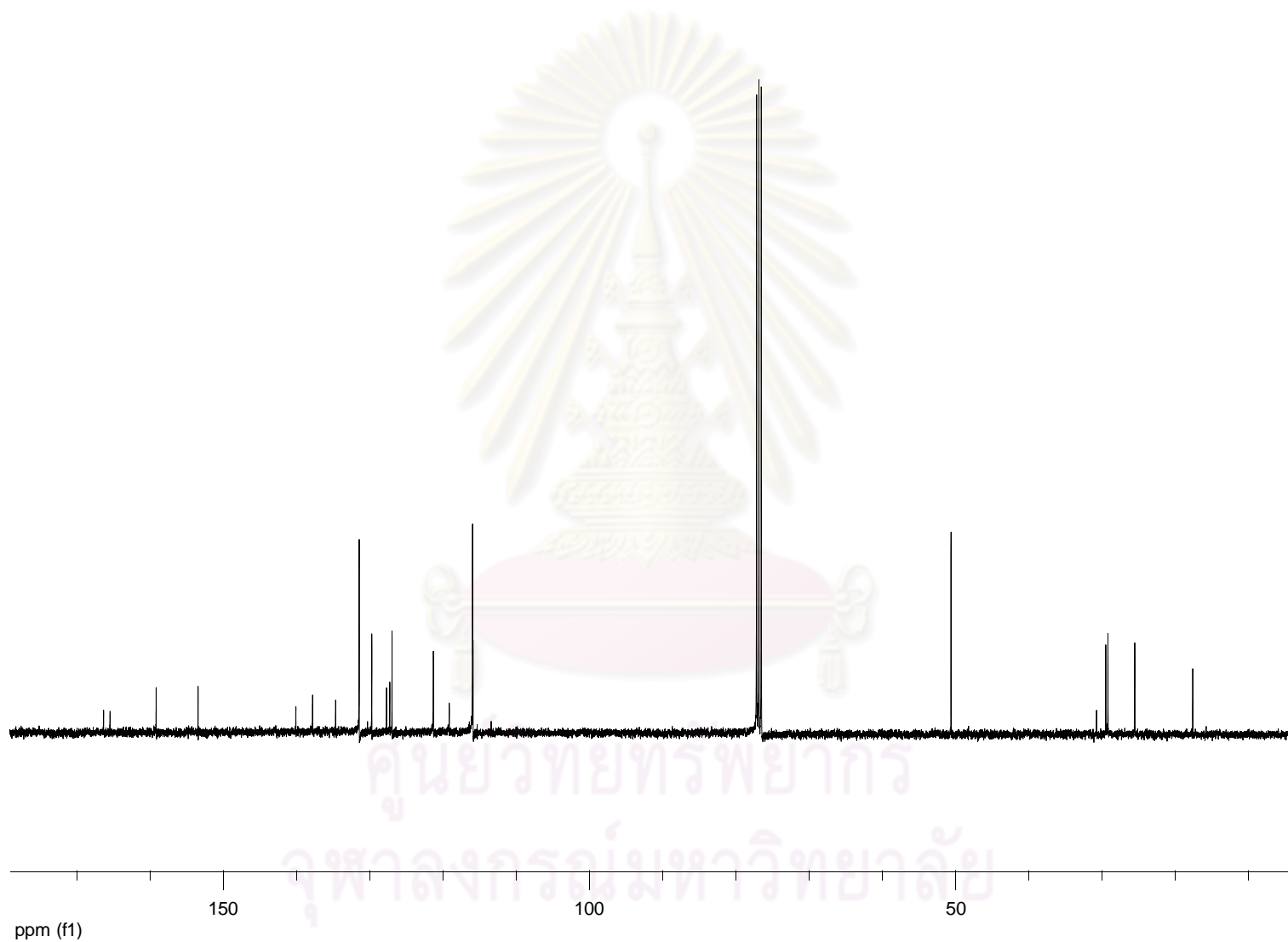
Figure S-26 ^{13}C NMR(100 MHz) spectrum of compound **5** (CDCl$_3$)

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Figure S-27 COSY spectrum of compound **5** (CDCl$_3$)

Figure S-28 HSQC spectrum of compound **5** (CDCl$_3$)
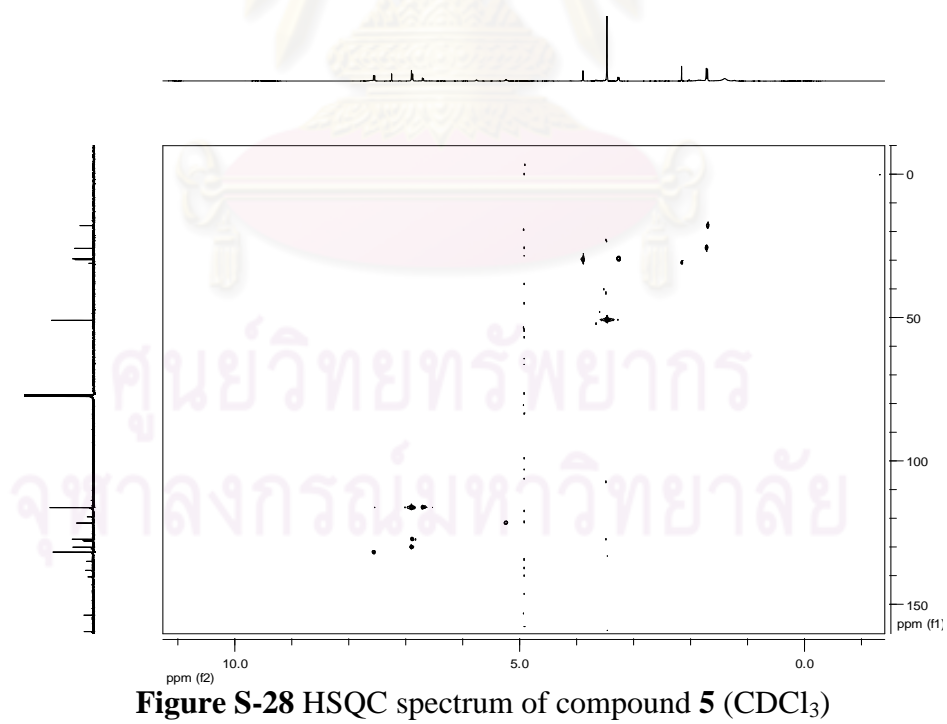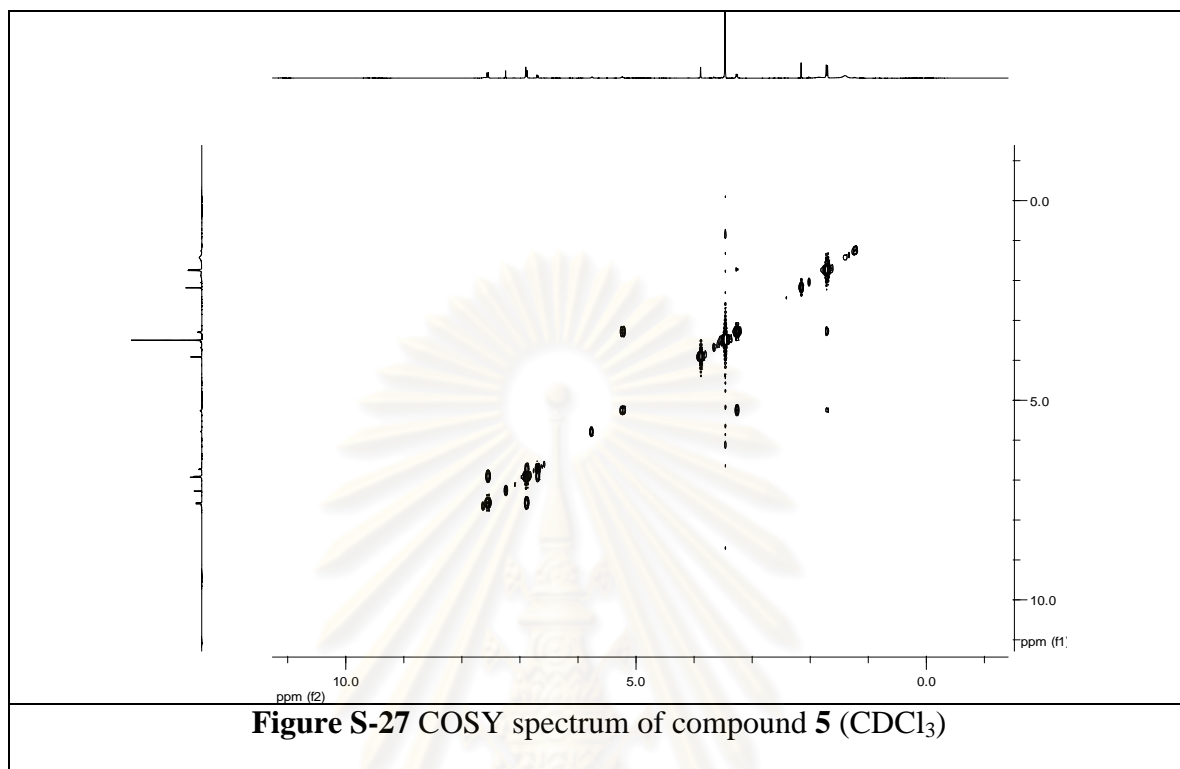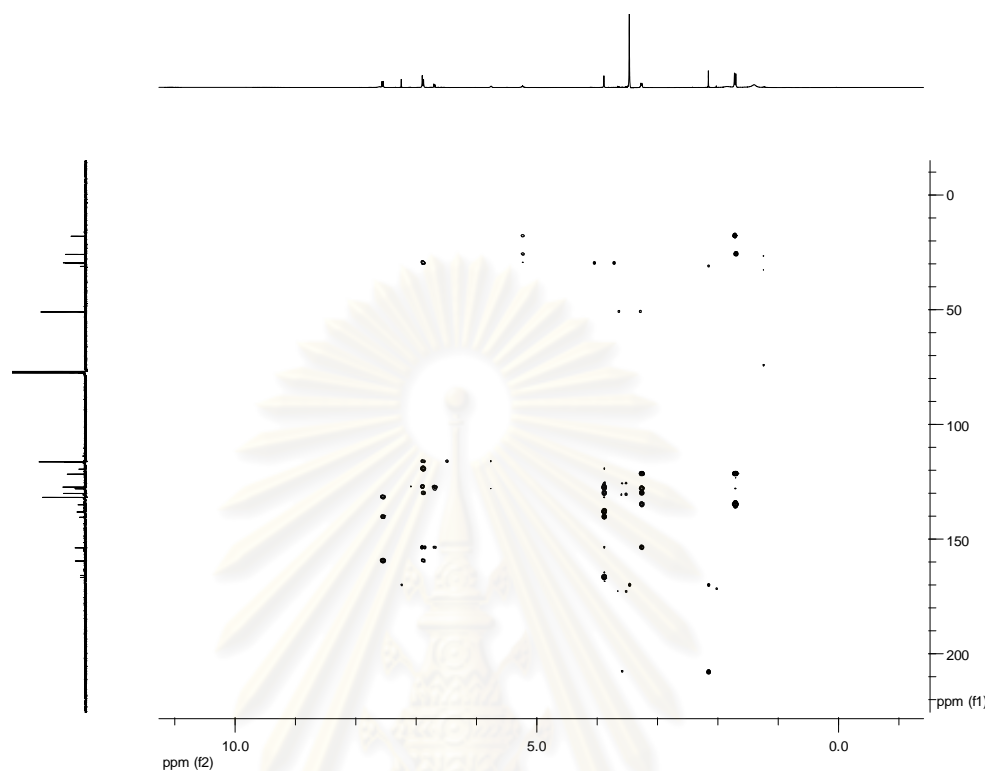
Figure S-29 HMBC spectrum of compound **5** (CDCl₃)
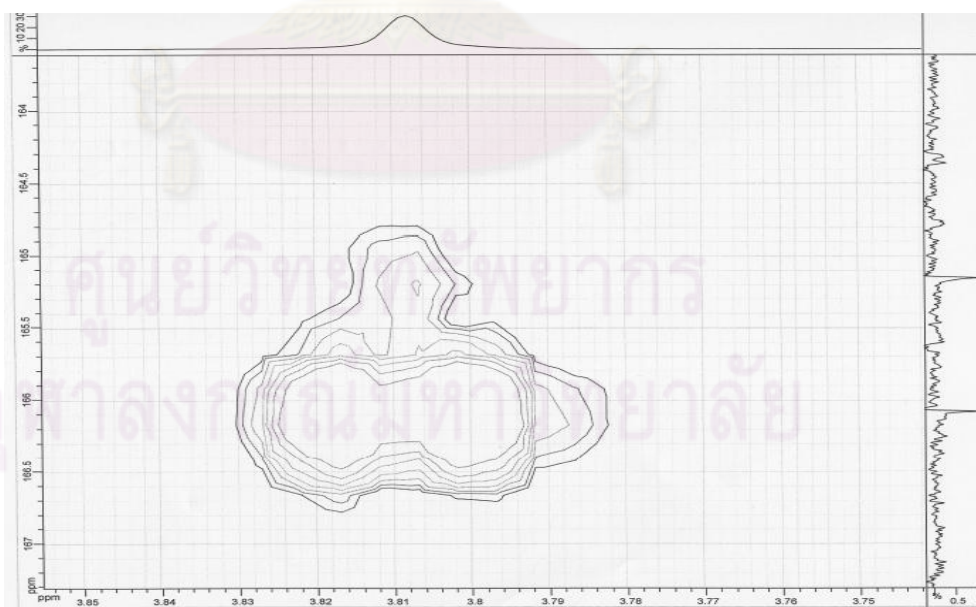
Figure S-30 HMBC spectrum (expansion) of compound **5** with correlations between
H2-6 and two carbonyls at C-2 and C-5
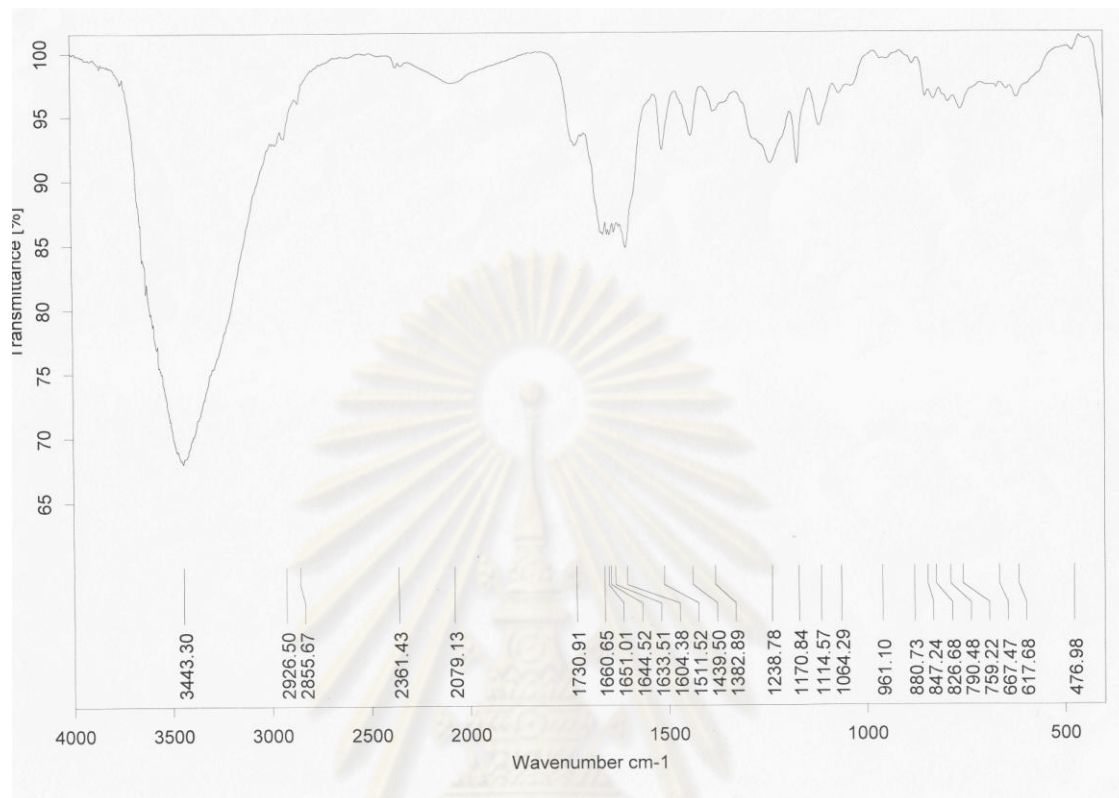
Figure S-31 IR spectrum of compound **5** (KBr)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย
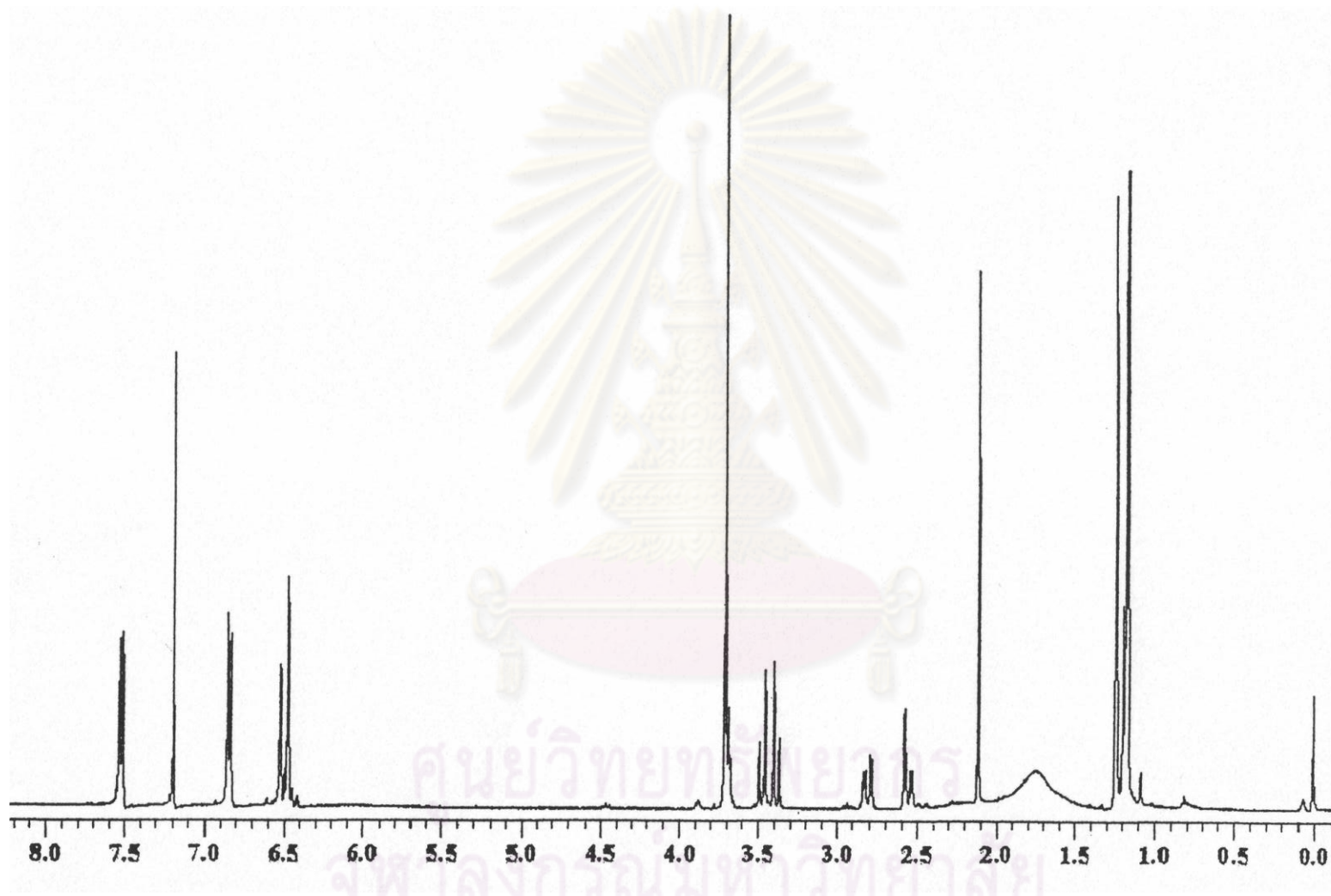
Figure S-32 ^{1}H NMR(400 MHz) spectrum of compound **6** (CDCl$_3$)
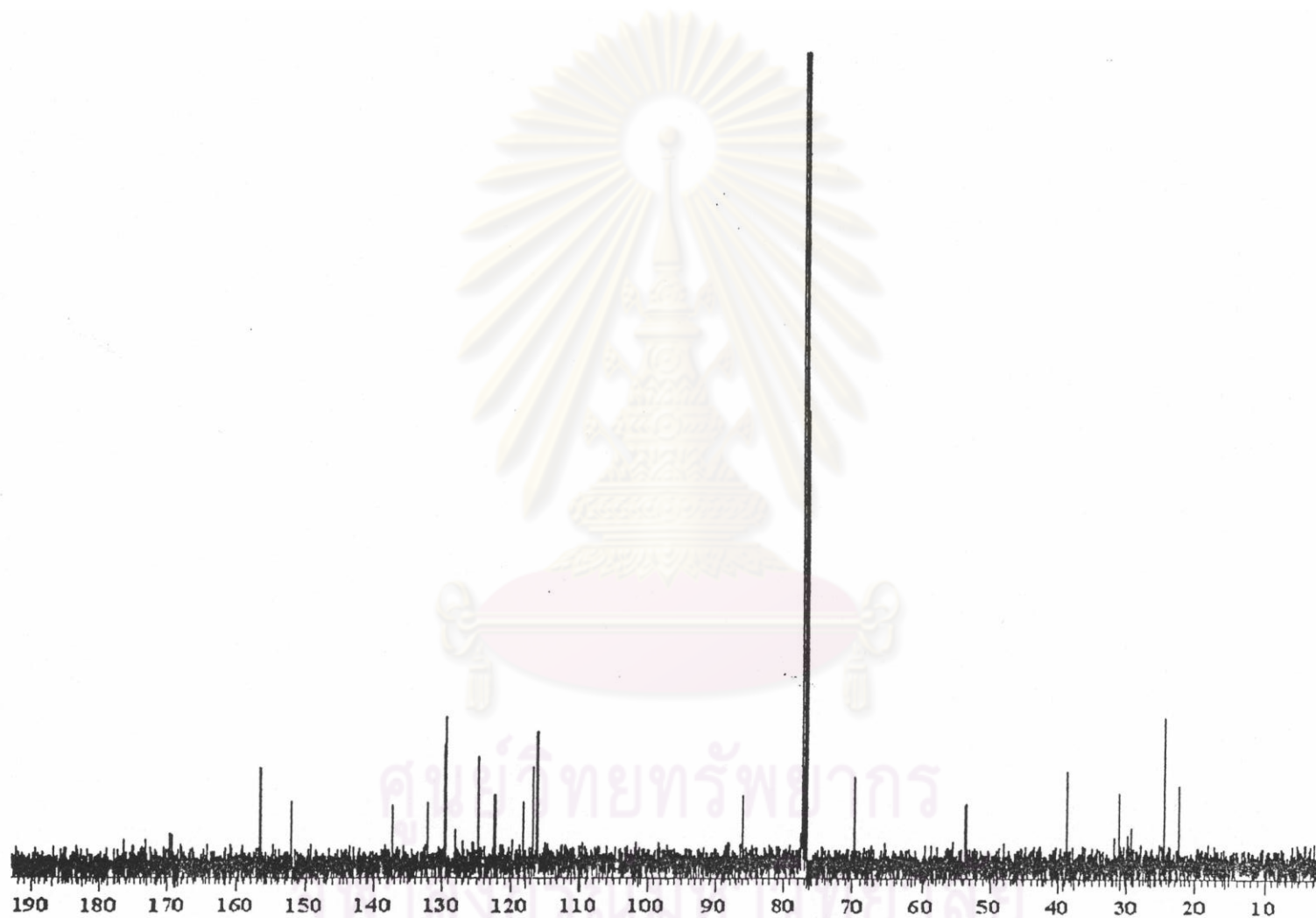
Figure S-33 ^{13}C NMR(100 MHz) spectrum of compound **6** (CDCl$_3$)
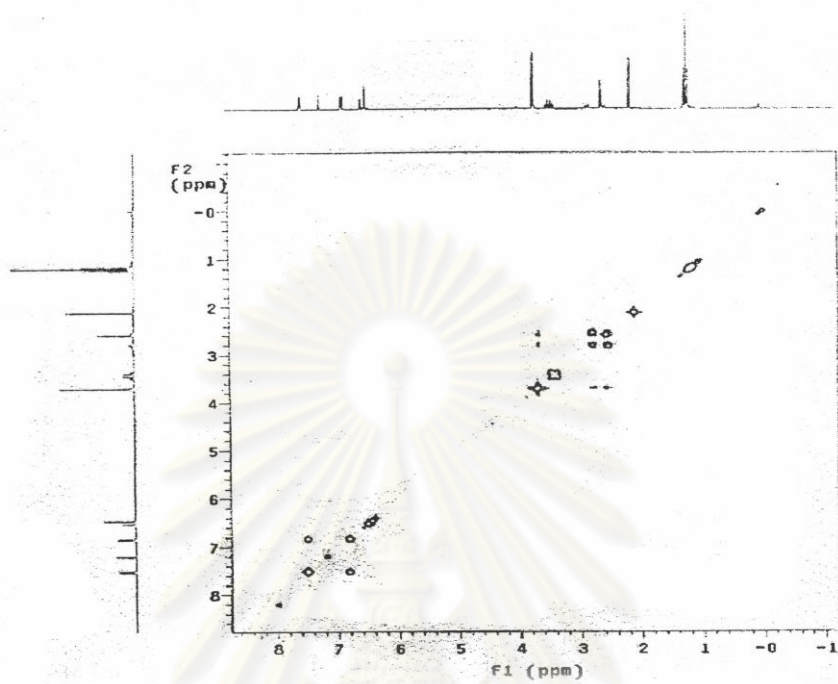
Figure S-34 COSY spectrum of compound **6** (CDCl₃)
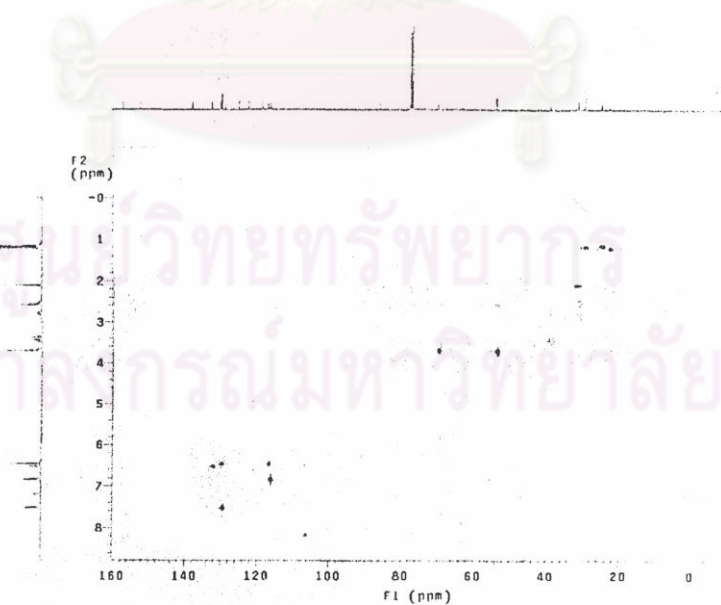
Figure S-35 HSQC spectrum of compound **6** (CDCl₃)

Figure S-36 HMBC spectrum of compound **6** (CDCl$_3$)

Figure S-37 IR spectrum of compound **6** (CDCl$_3$)

Figure S-38 ^{1}H NMR(400 MHz) spectrum of compound **7** (CDCl$_3$)

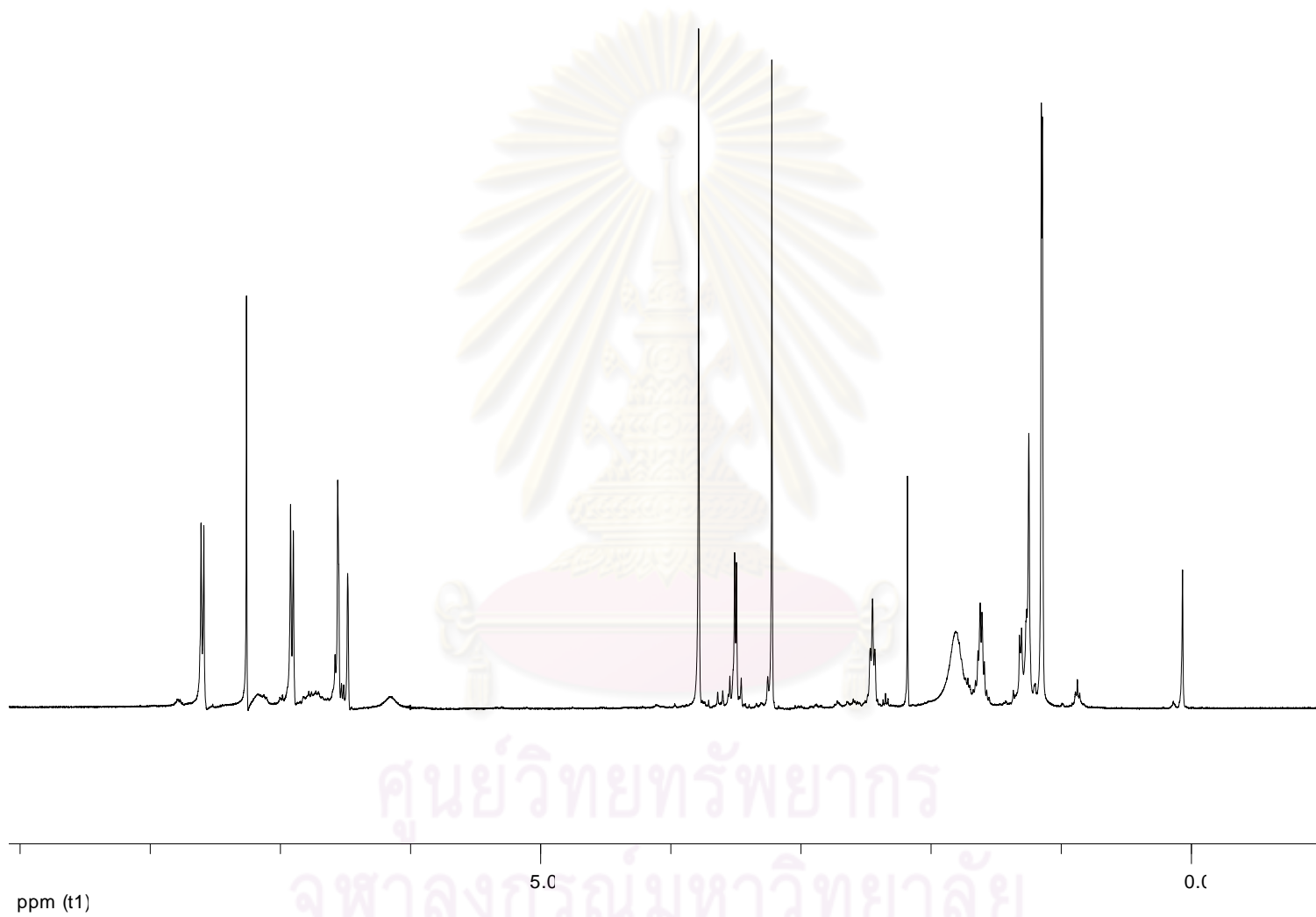**Figure S-39** ^{13}C NMR(100 MHz) spectrum of compound **7** (CDCl$_3$)
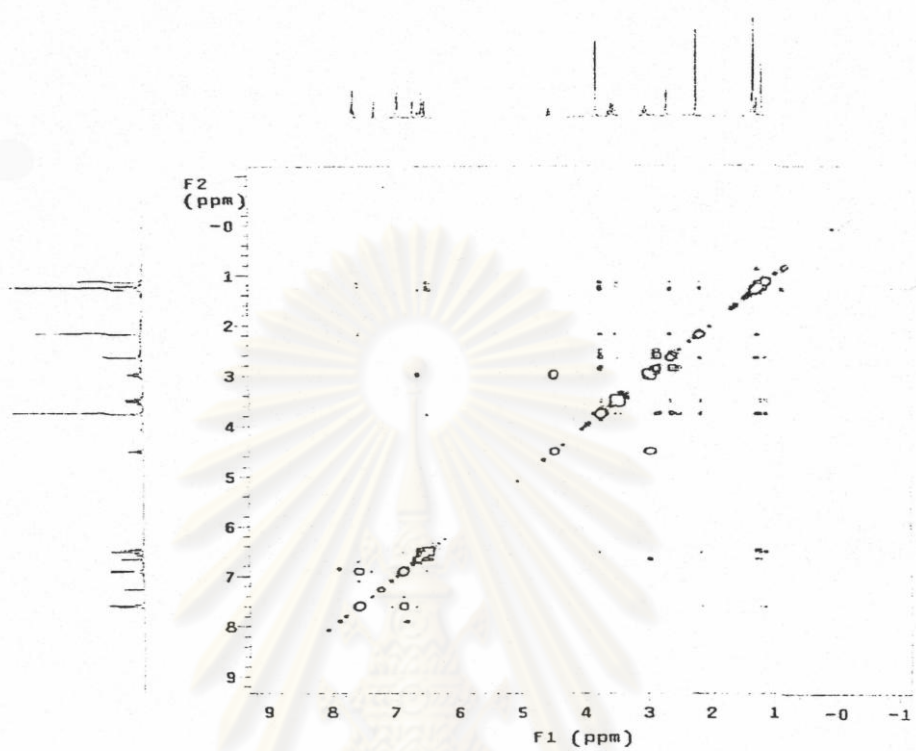
Figure S-40 COSY spectrum of compound **7** (CDCl$_3$)
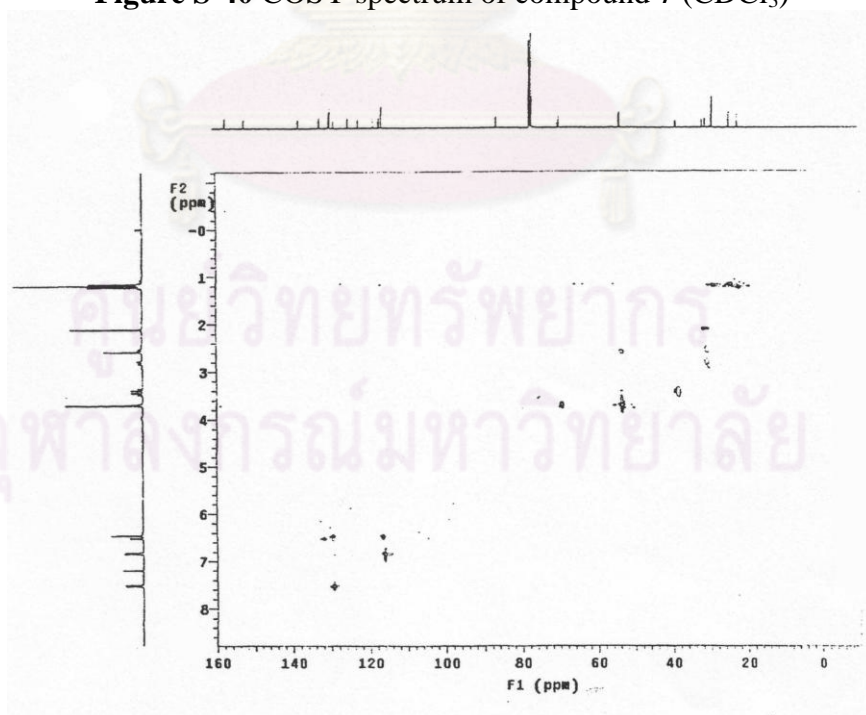
Figure S-41 HSQC spectrum of compound **7** (CDCl$_3$)
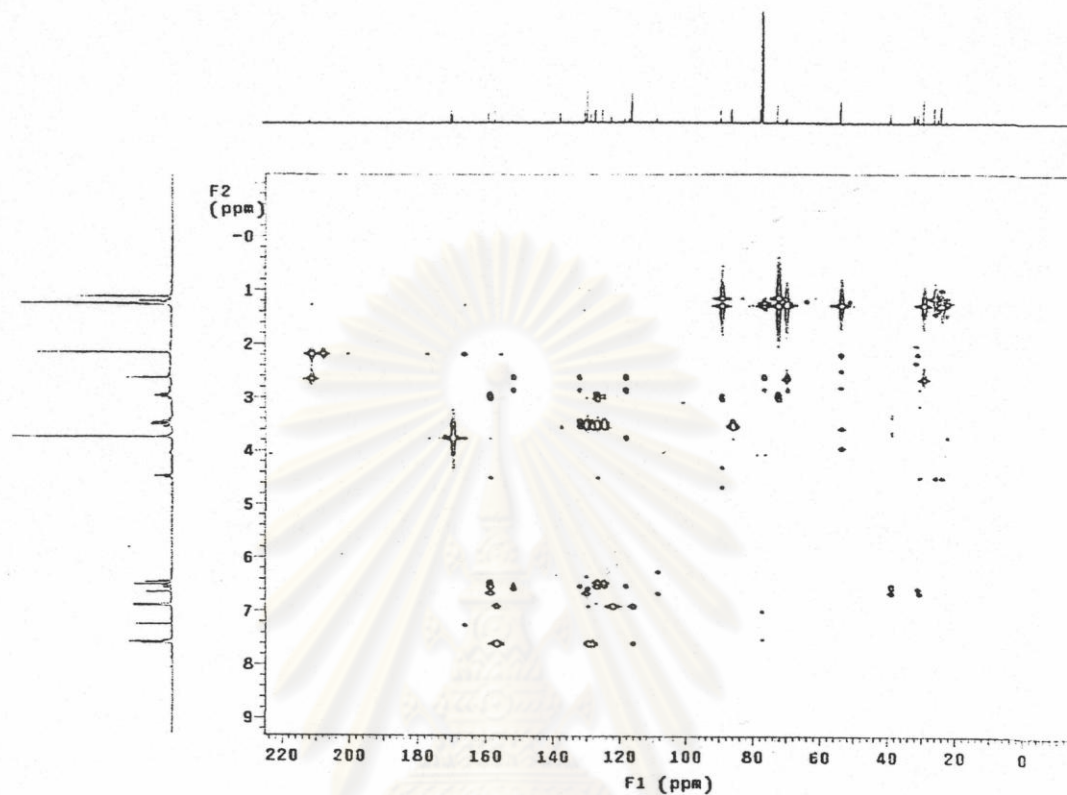
Figure S-42 HMBC spectrum of compound **7** (CDCl$_3$)
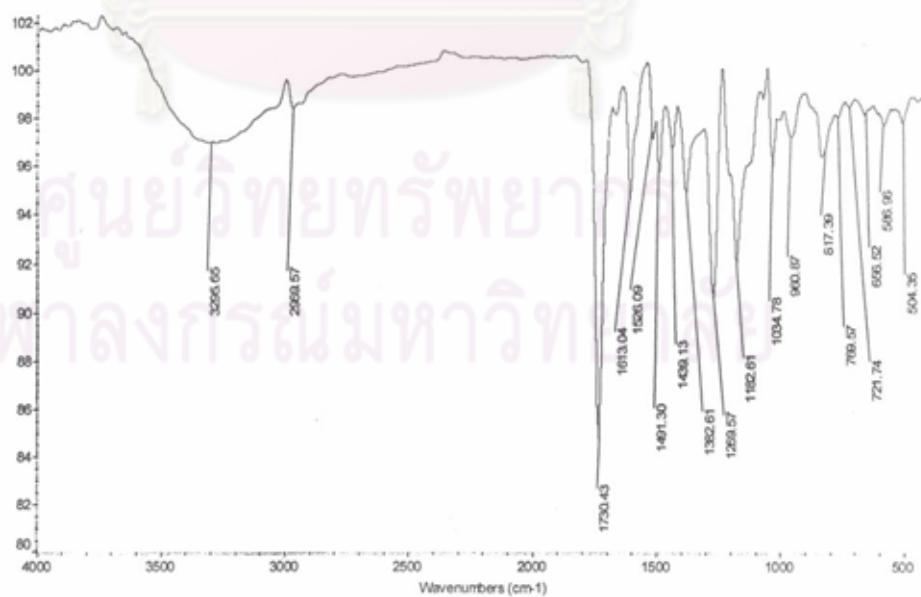
Figure S-43 IR spectrum of compound **7** (CDCl$_3$)

Figure S-44 ^{1}H NMR(400 MHz) spectrum of compound **8** (acetone-d_6)

Figure S-45 ^{13}C NMR(100 MHz) spectrum of compound **8** (acetone-d_6)

Figure S-46 COSY spectrum of compound **8** (acetone-*d6*)

Figure S-47 HSQC spectrum of compound **8** (acetone-*d6*)
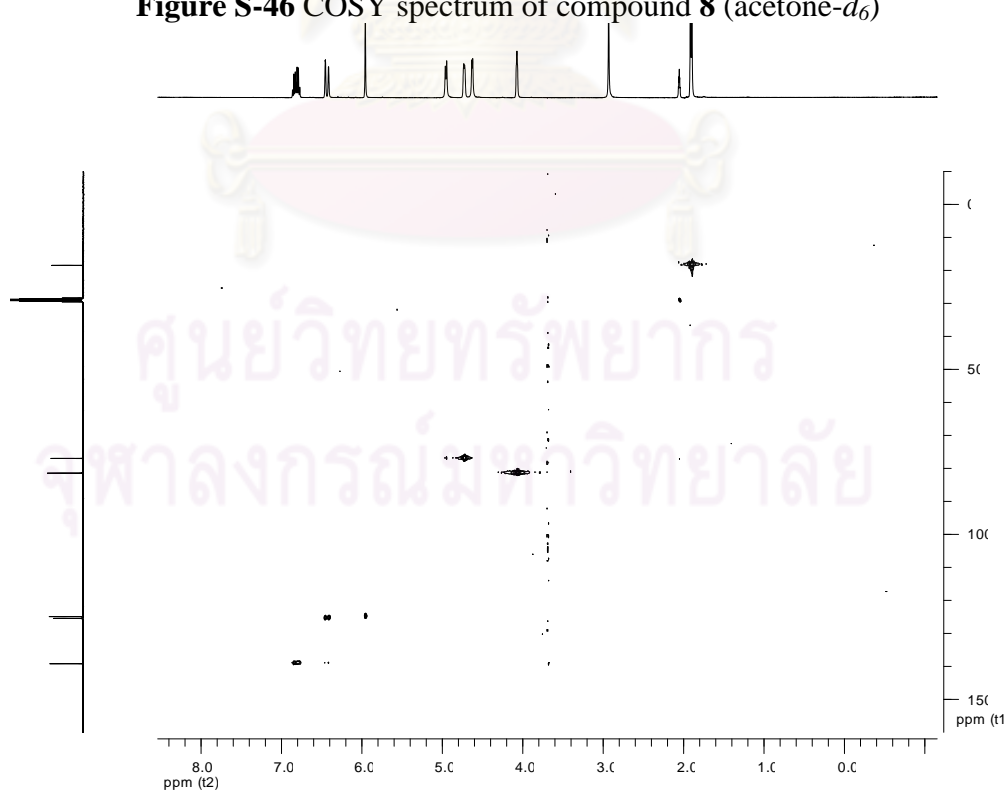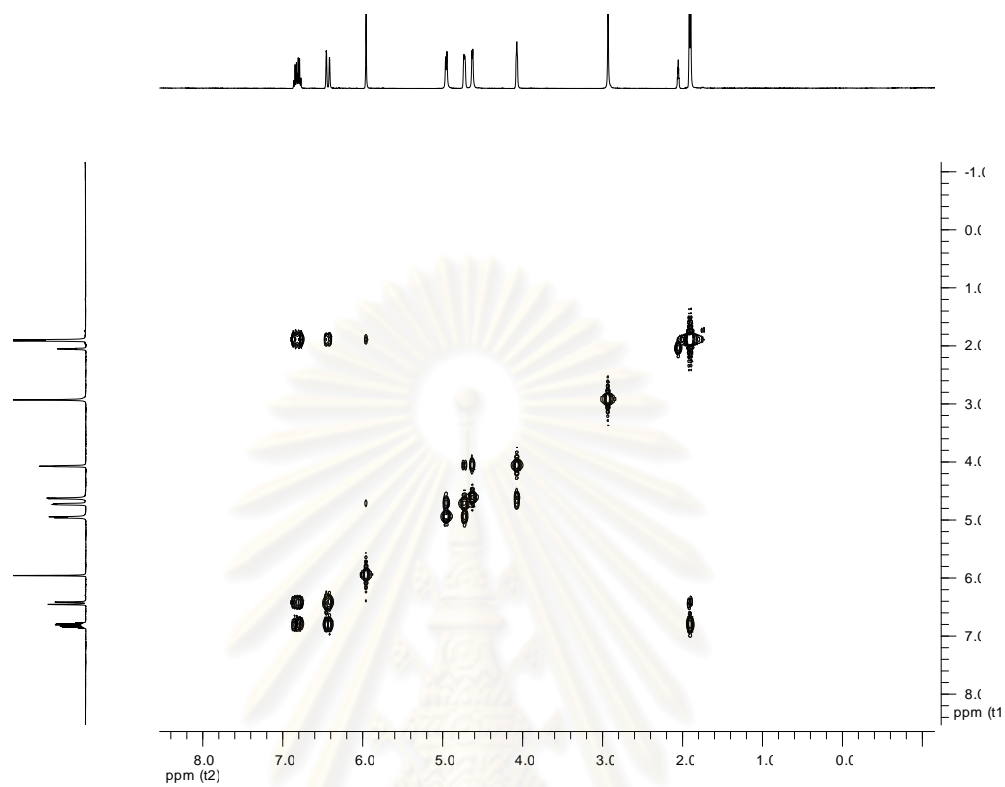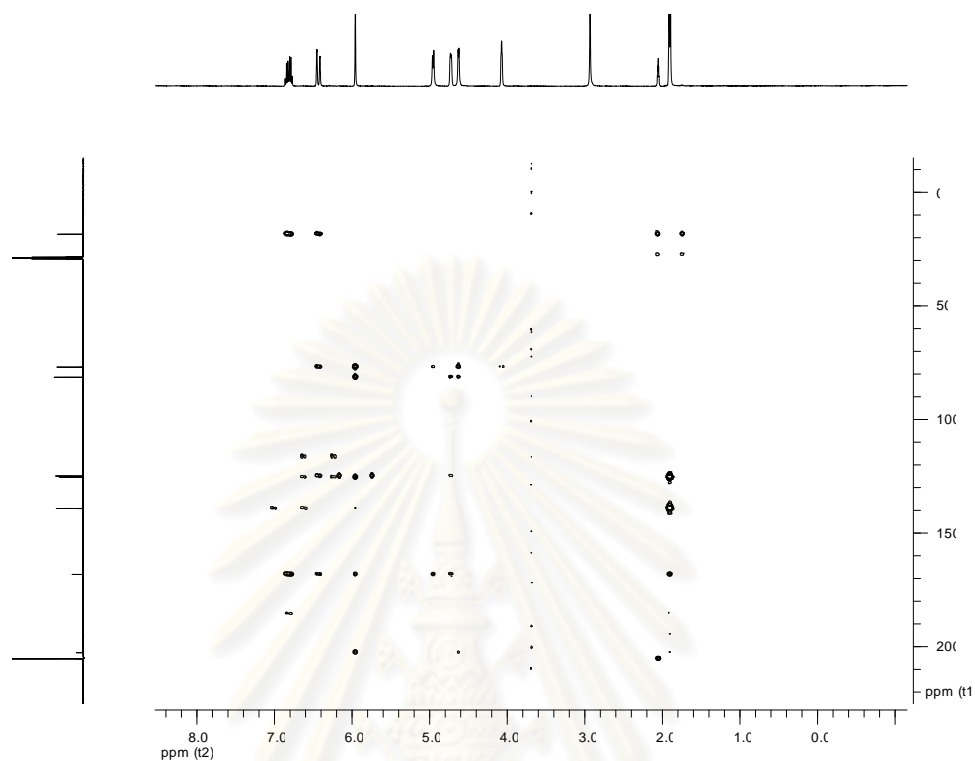
Figure S-48 HMBC spectrum of compound **8** (acetone-*d₆*)
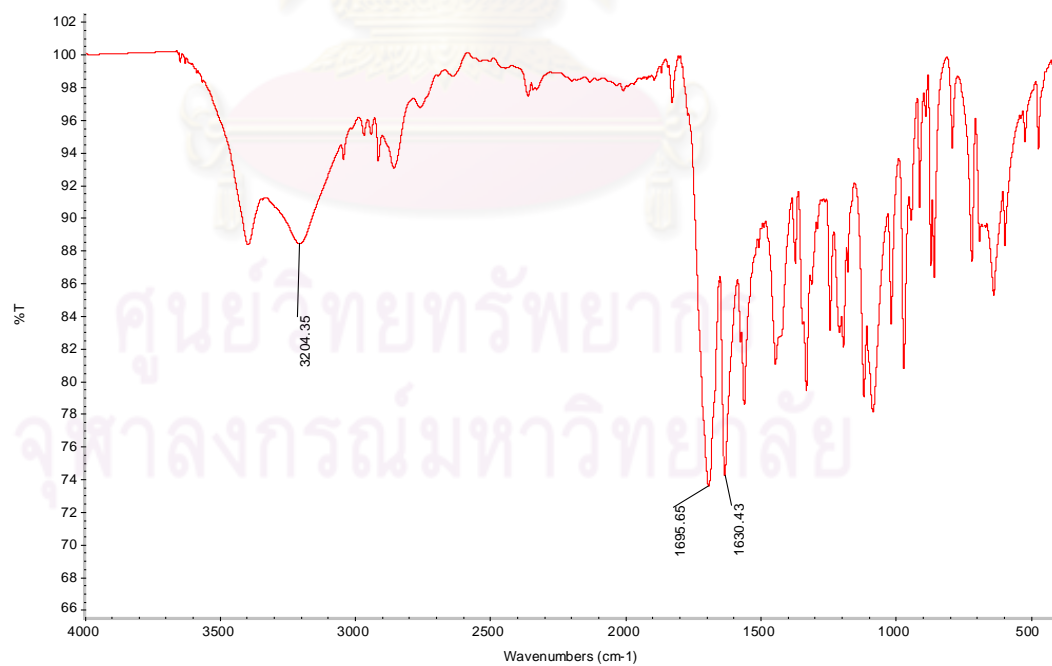
Figure S-49 IR spectrum of compound **8** (KBr)

Figure S-50 ^{1}H NMR(400 MHz) spectrum of compound **24** (CDCl$_3$)
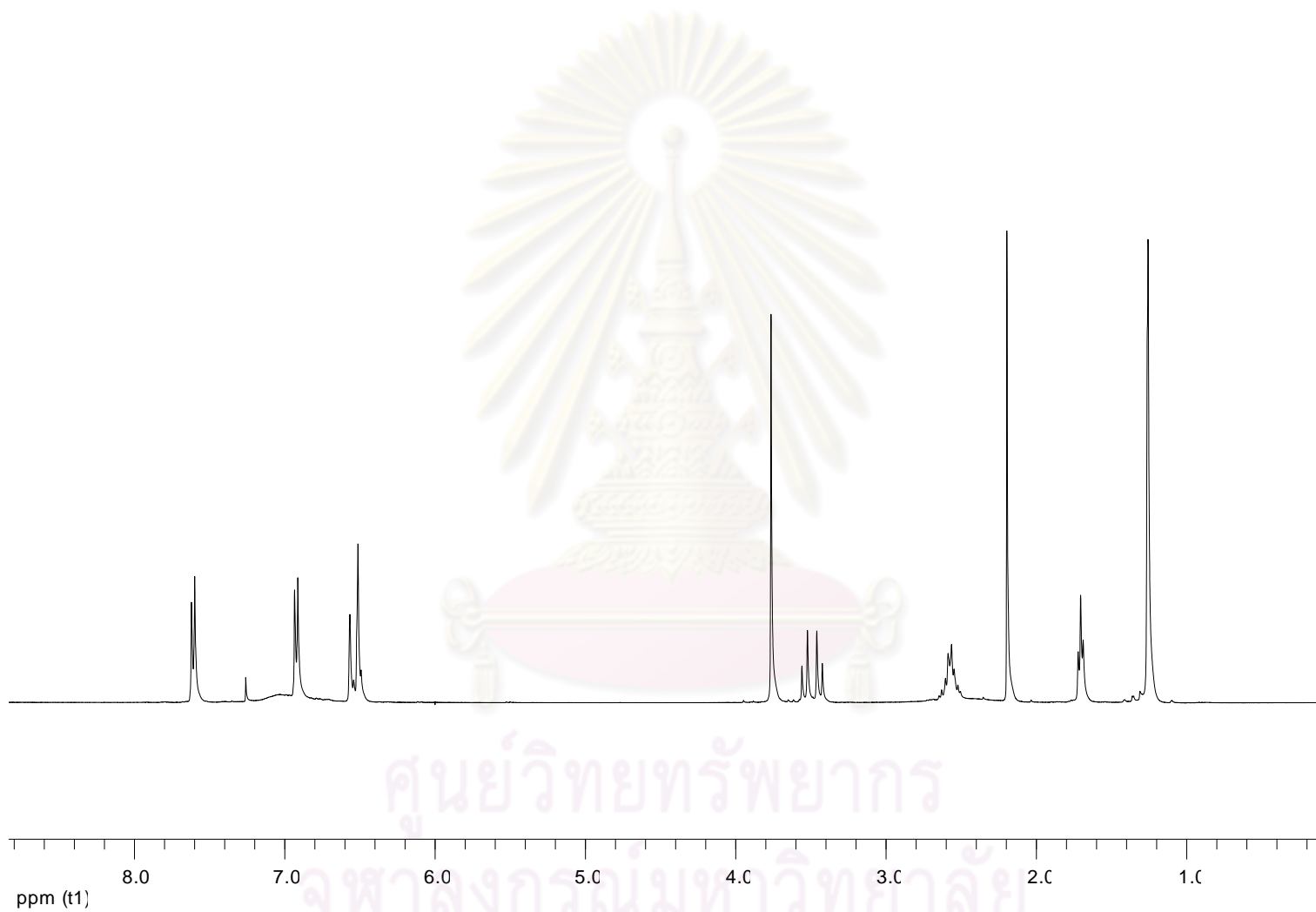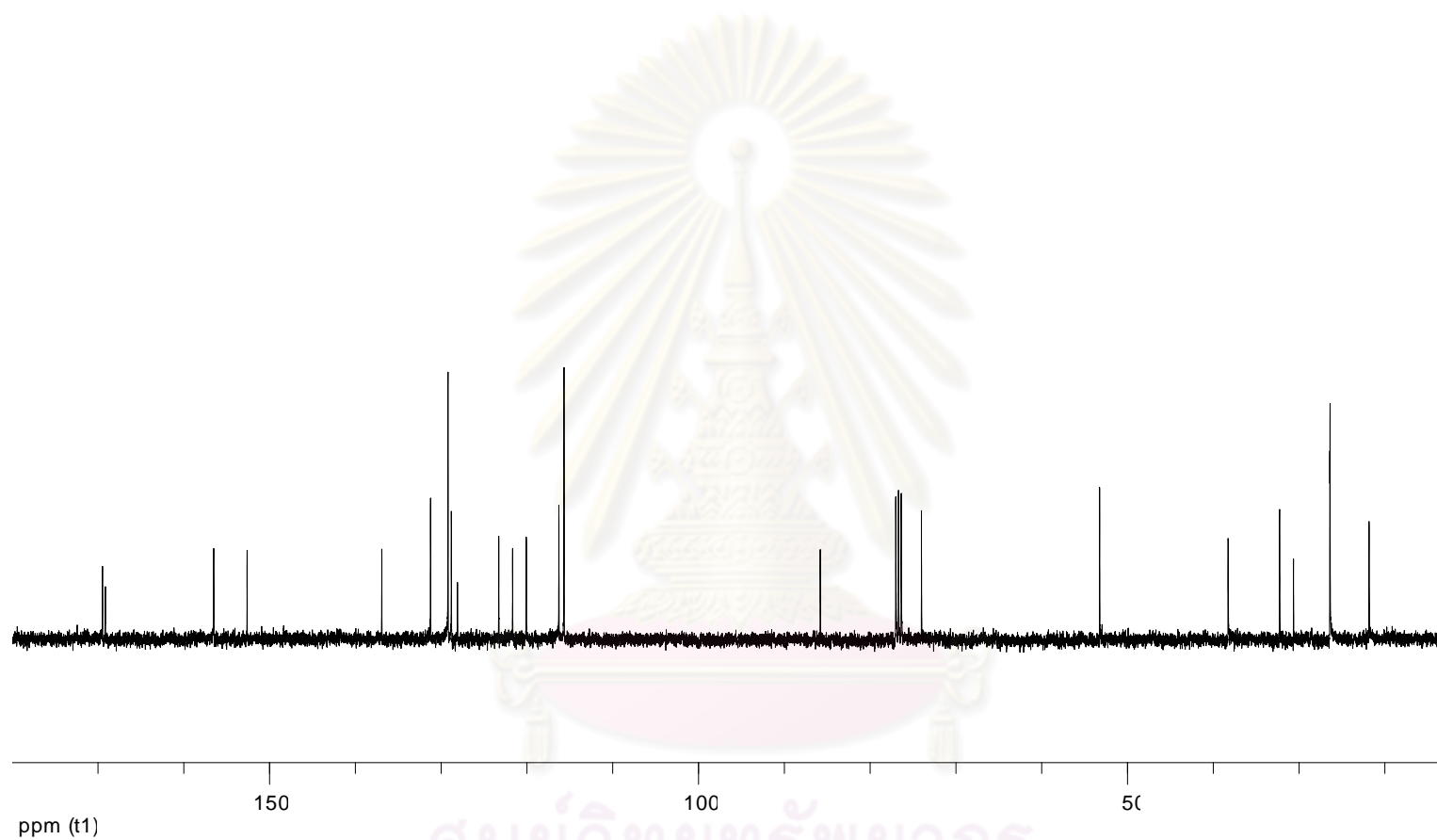
Figure S-51 ^{13}C NMR(100 MHz) spectrum of compound **24** (CDCl$_3$)

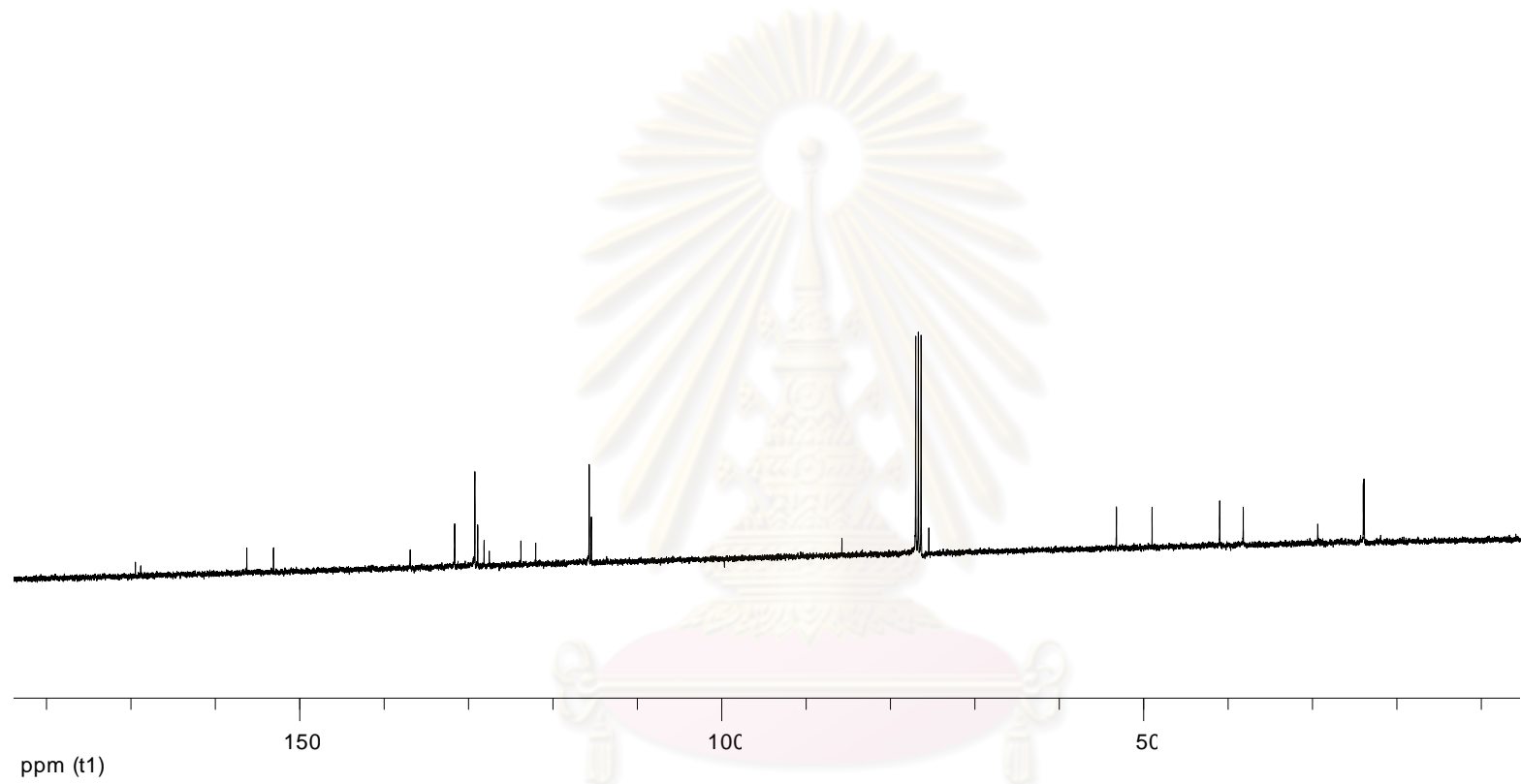**Figure S-52** ^{1}H NMR(400 MHz) spectrum of compound **25** (CDCl$_3$)

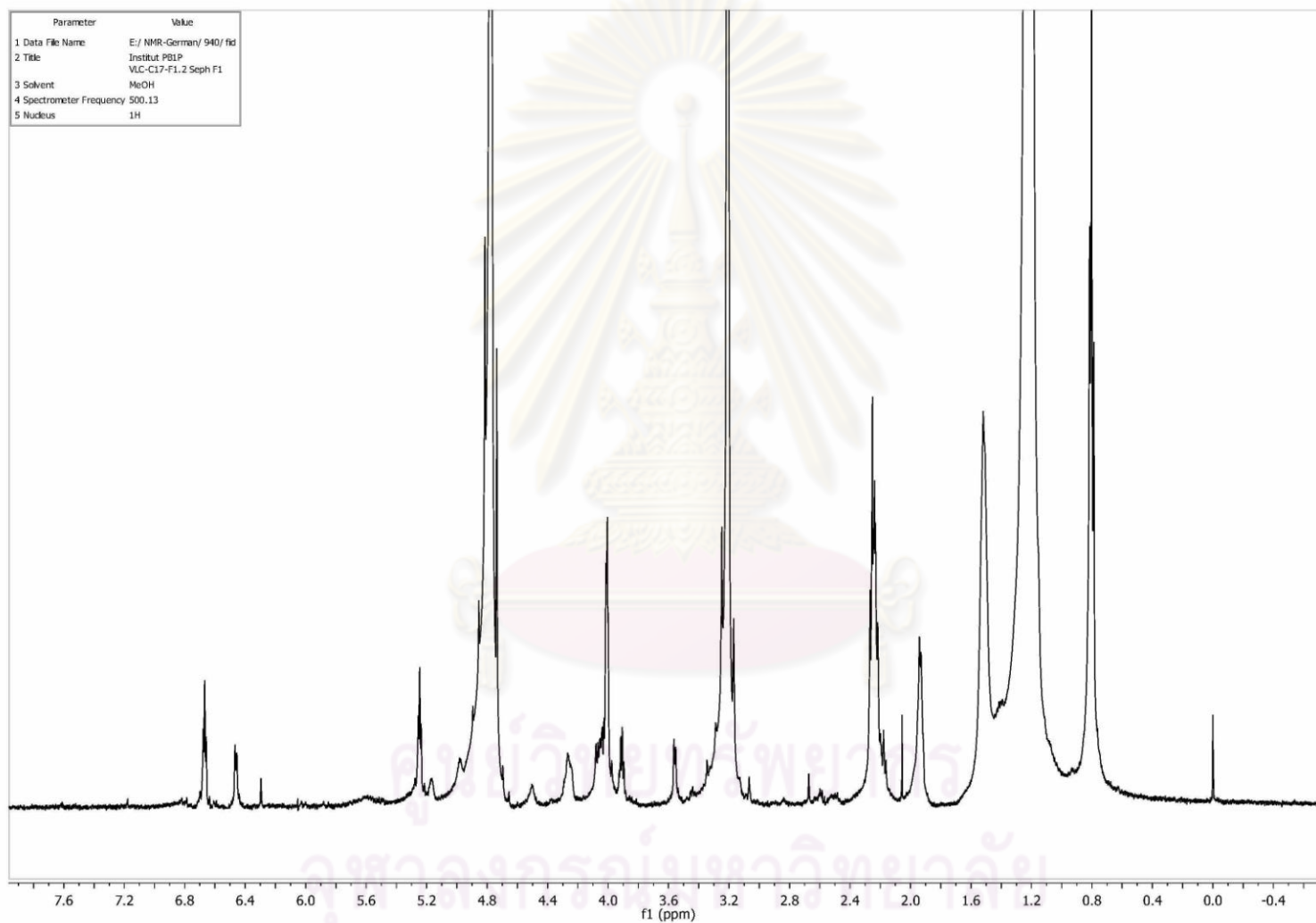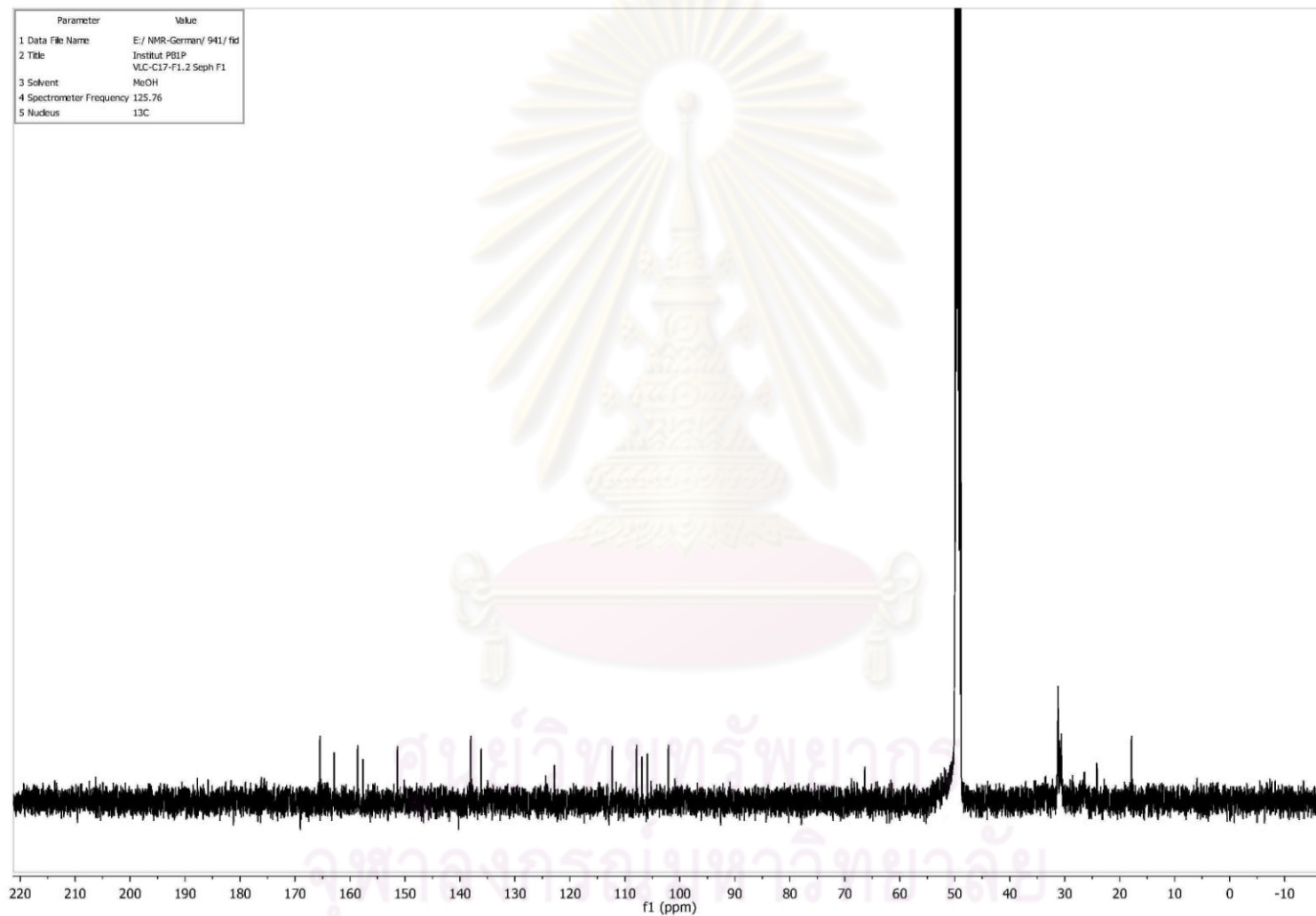**Figure S-53** ^{13}C NMR(100 MHz) spectrum of compound **25** (CDCl$_3$)

	Parameter	Value
1	Data File Name	E:/ NMR-German/ 940/ fid
2	Title	Institut PB1P
		VLC-C17-F1.2 Seph F1
3	Solvent	MeOH
4	Spectrometer Frequency	500.13
5	Nucleus	1H

Figure S-54 ^{1}H NMR(500 MHz) spectrum of compound **10** (MeOD)

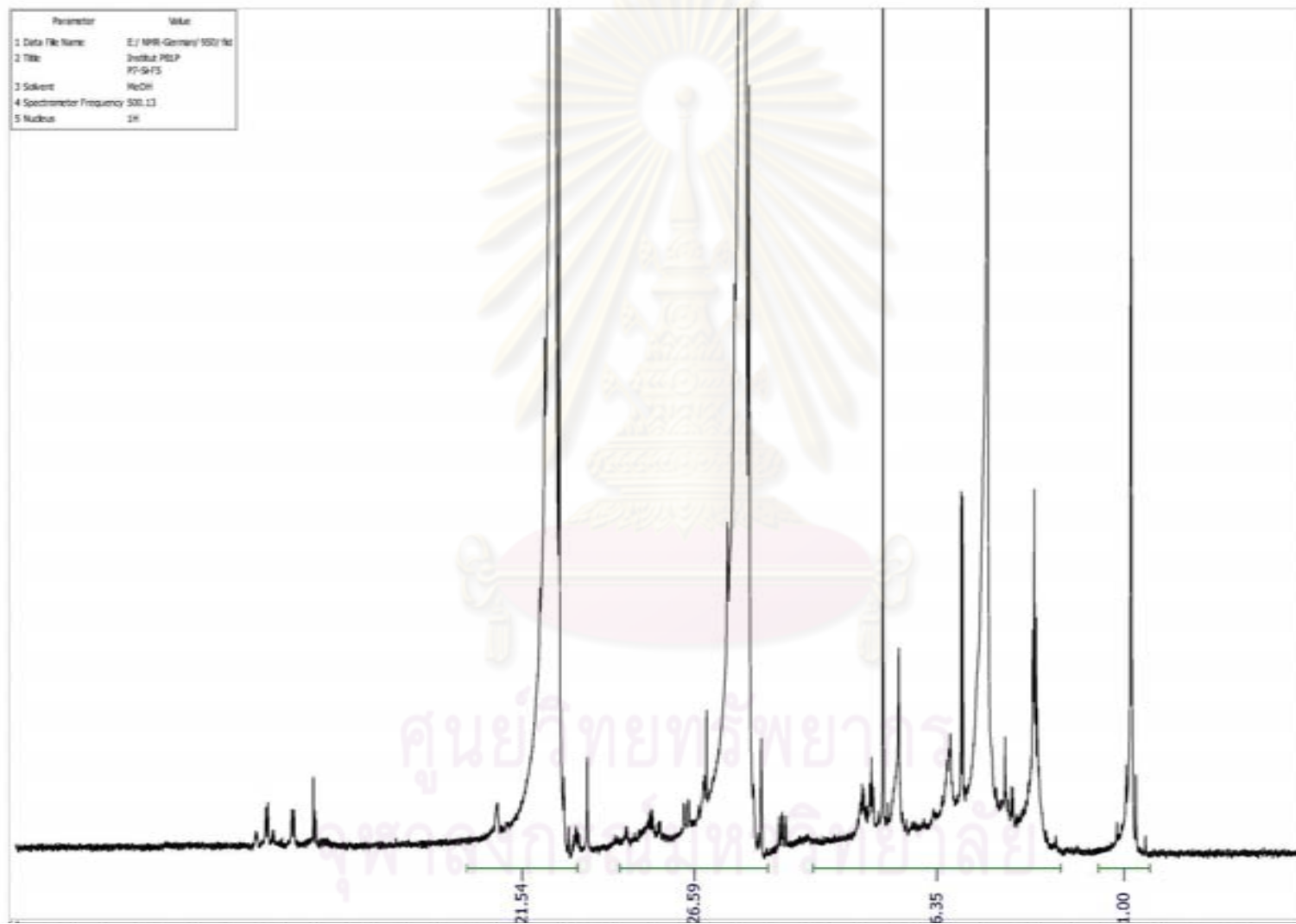| Parameter	Value
1 Data File Name	E:/ NMR-German/ 941/ fid
2 Title	Institut PB1P
	VLC-C17-F1.2 Seph F1
3 Solvent	MeOH
4 Spectrometer Frequency	125.76
5 Nucleus	13C

Figure S-55 ^{13}C NMR(100 MHz) spectrum of compound **10** (MeOD)

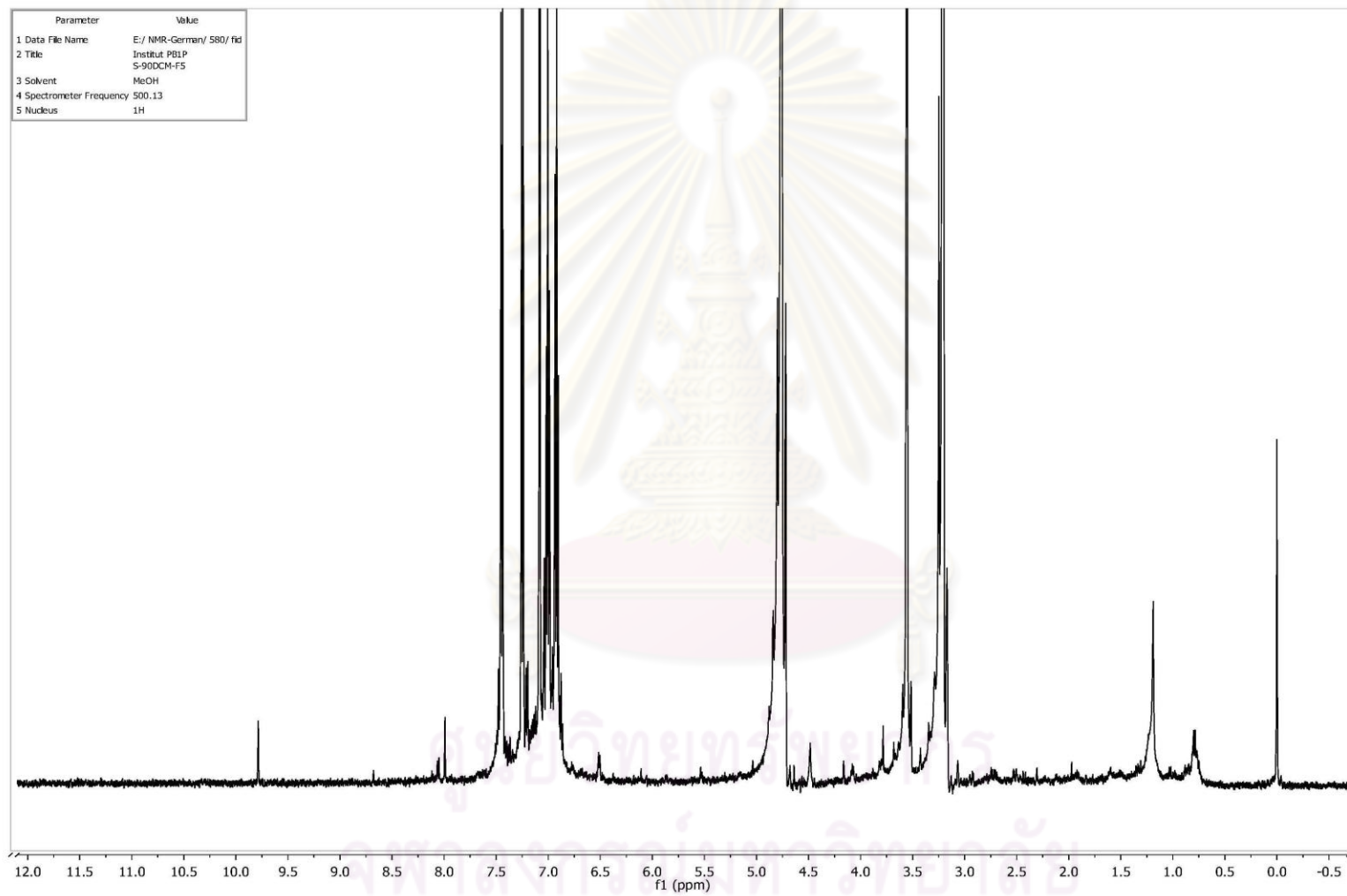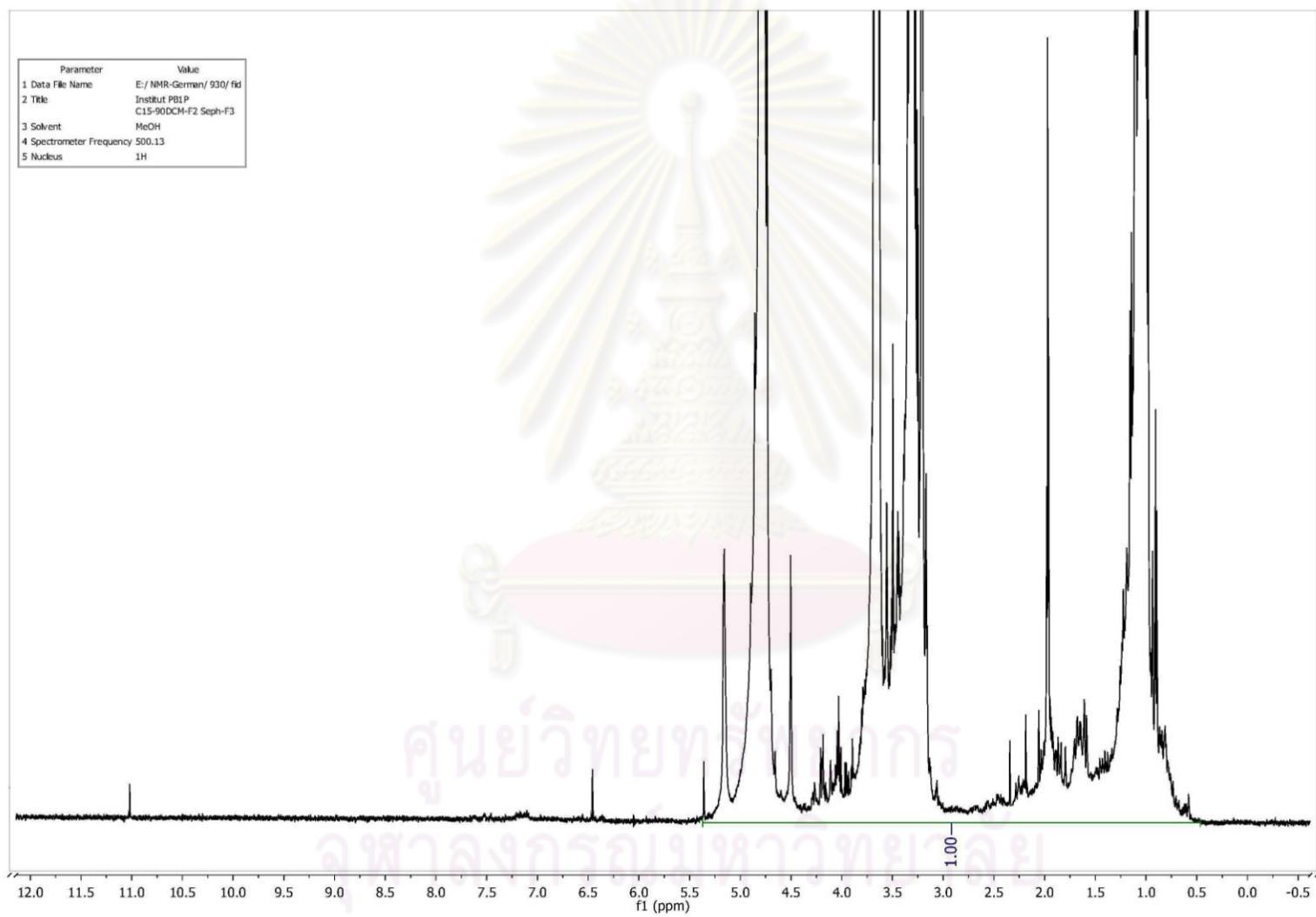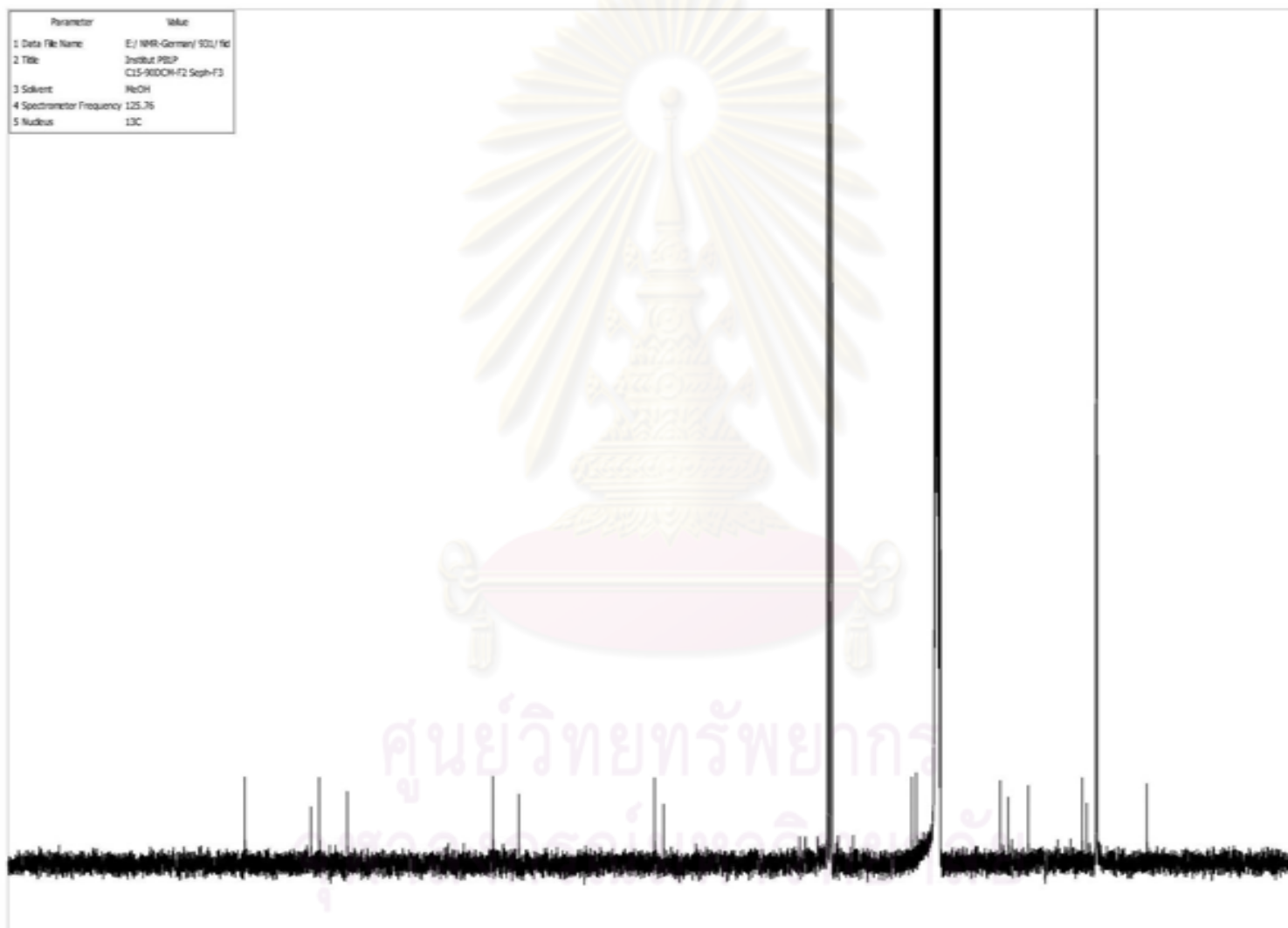**Figure S-56** ^{1}H NMR(500 MHz) spectrum of compound **11** (MeOD)

	Parameter	Value
1	Data File Name	E:/ NMR-German/ 580/ fid
2	Title	Institut PB1P
		S-90DCM-F5
3	Solvent	MeOH
4	Spectrometer Frequency	500.13
5	Nucleus	1H

Figure S-57 ^{1}H NMR(500 MHz) spectrum of compound **13** (MeOD)

Figure S-58 ^{1}H NMR(500 MHz) spectrum of compound **15** (MeOD)

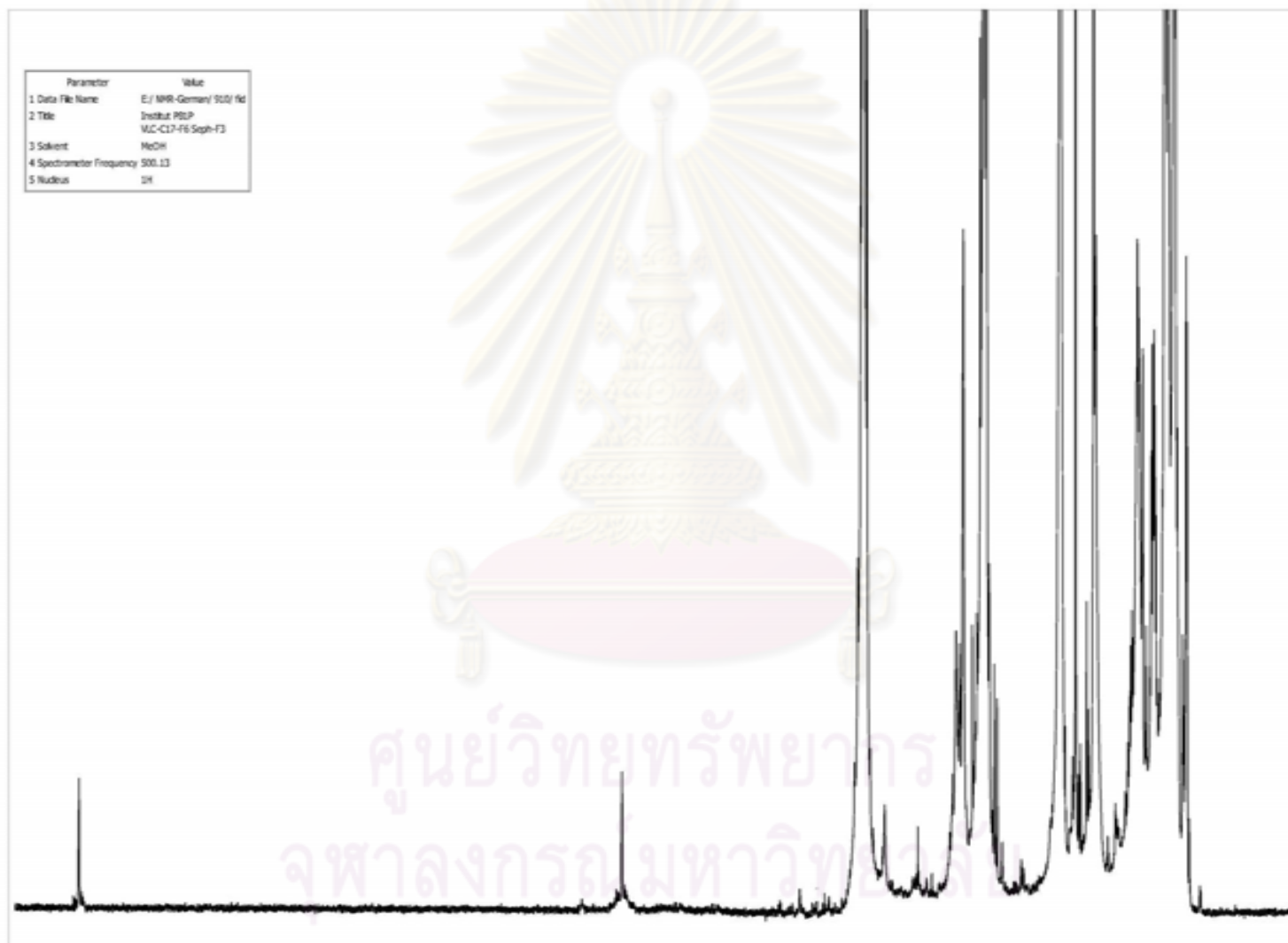| Parameter	Value
1 Data File Name	E:/ NMR-German/ 931/ fid
2 Title	Institut PBLP
	C15-90DCM-f2 Seph-F3
3 Solvent	MeOH
4 Spectrometer Frequency	125.76
5 Nucleus	13C

Figure S-59 ^{13}C NMR(100 MHz) spectrum of compound **15** (MeOD)

Parameter	Value
1 Data File Name	E:/ NMR-German/ 910/ fid
2 Title	Institut PBLP
	VLC-C17-F6 Seph-F3
3 Solvent	MeOH
4 Spectrometer Frequency	500.13
5 Nucleus	1H

Figure S-60 ^{1}H NMR(500 MHz) spectrum of compound **17** (MeOD)

	Parameter	Value
1	Data File Name	E:/ NMR-German/ 911/ fid
2	Title	Institut PB1P
		VLC-C17-F6 Seph-F3
3	Solvent	MeOH
4	Spectrometer Frequency	125.76
5	Nucleus	13C
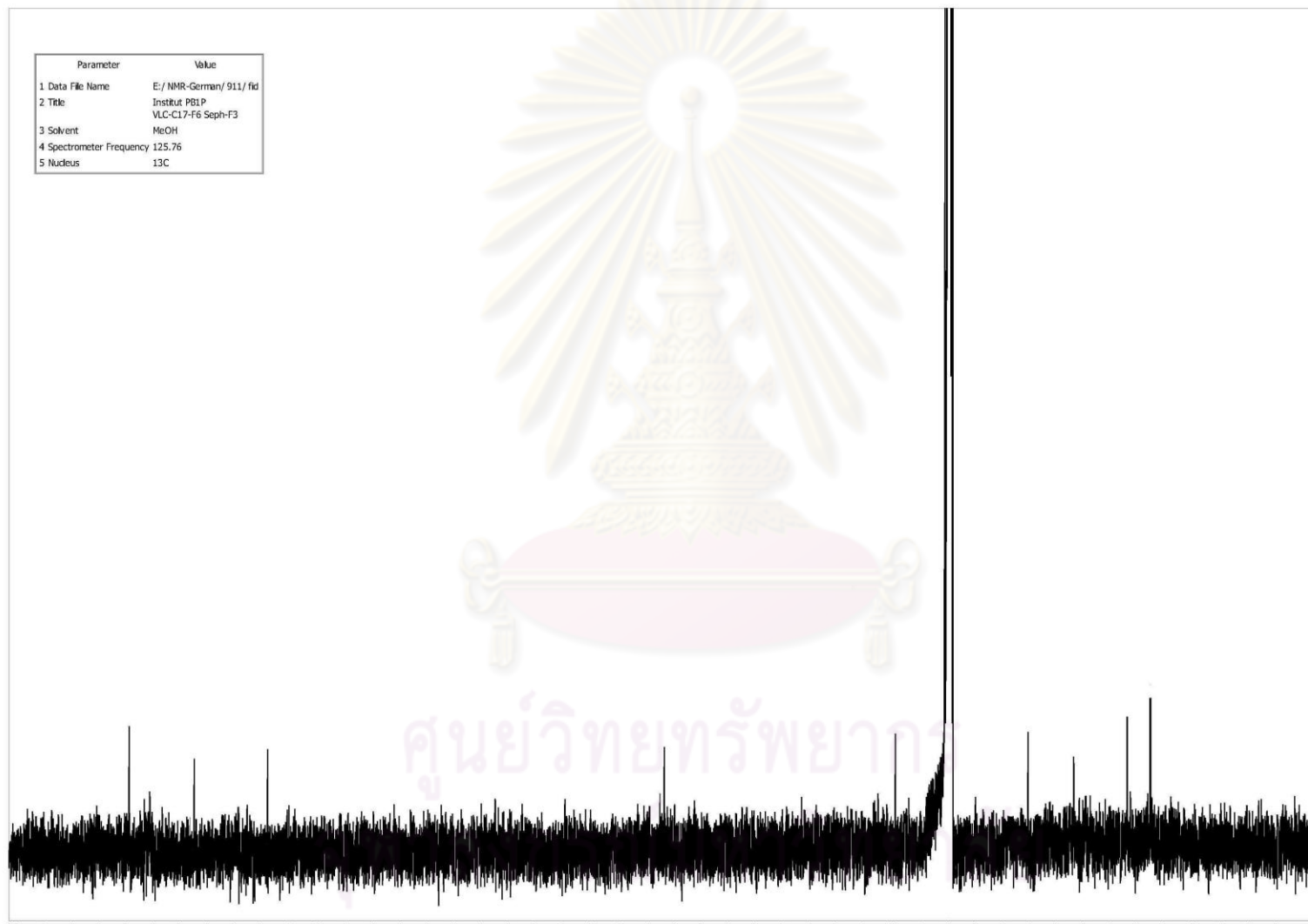
Figure S-61 ^{13}C NMR(100 MHz) spectrum of compound **17** (MeOD)
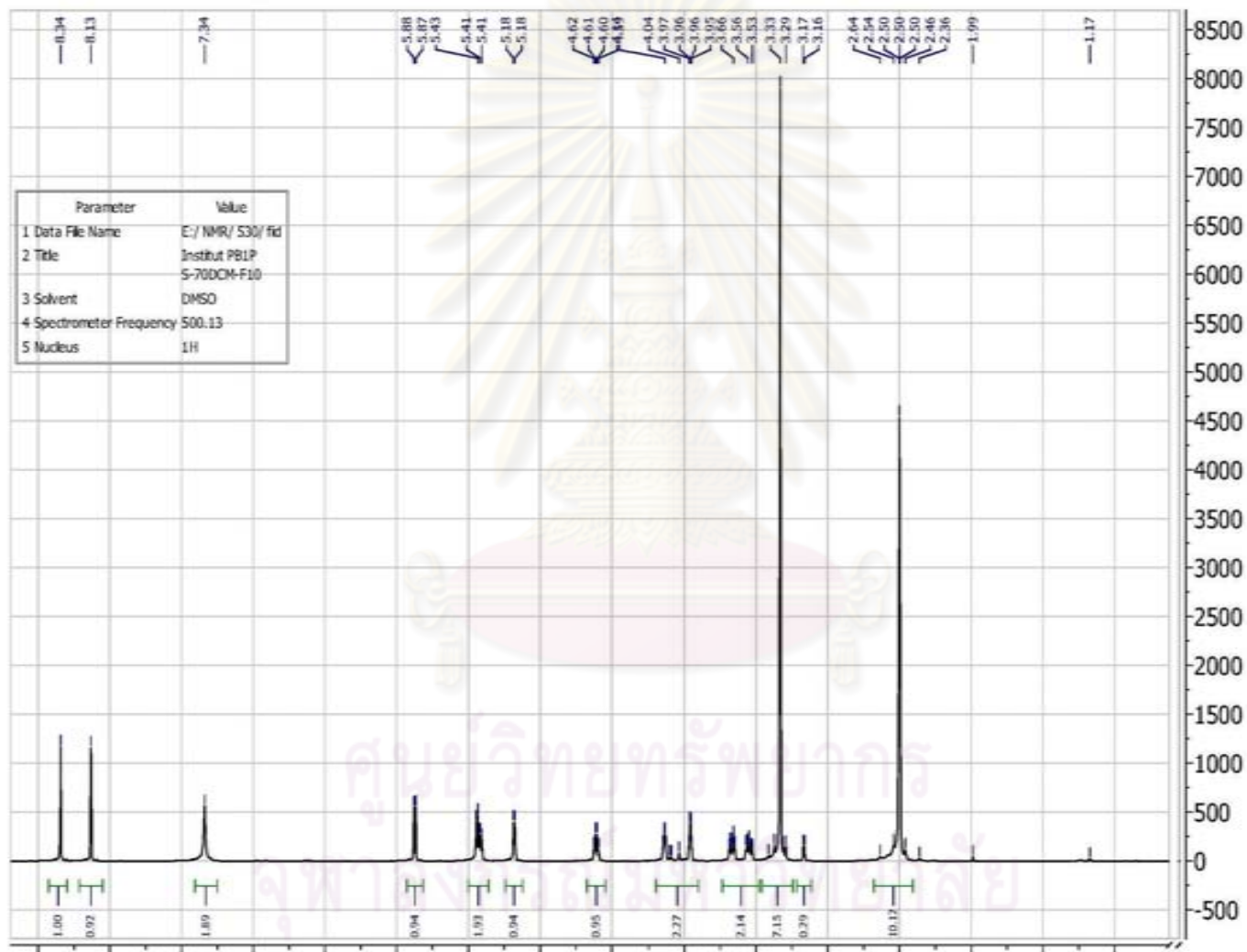
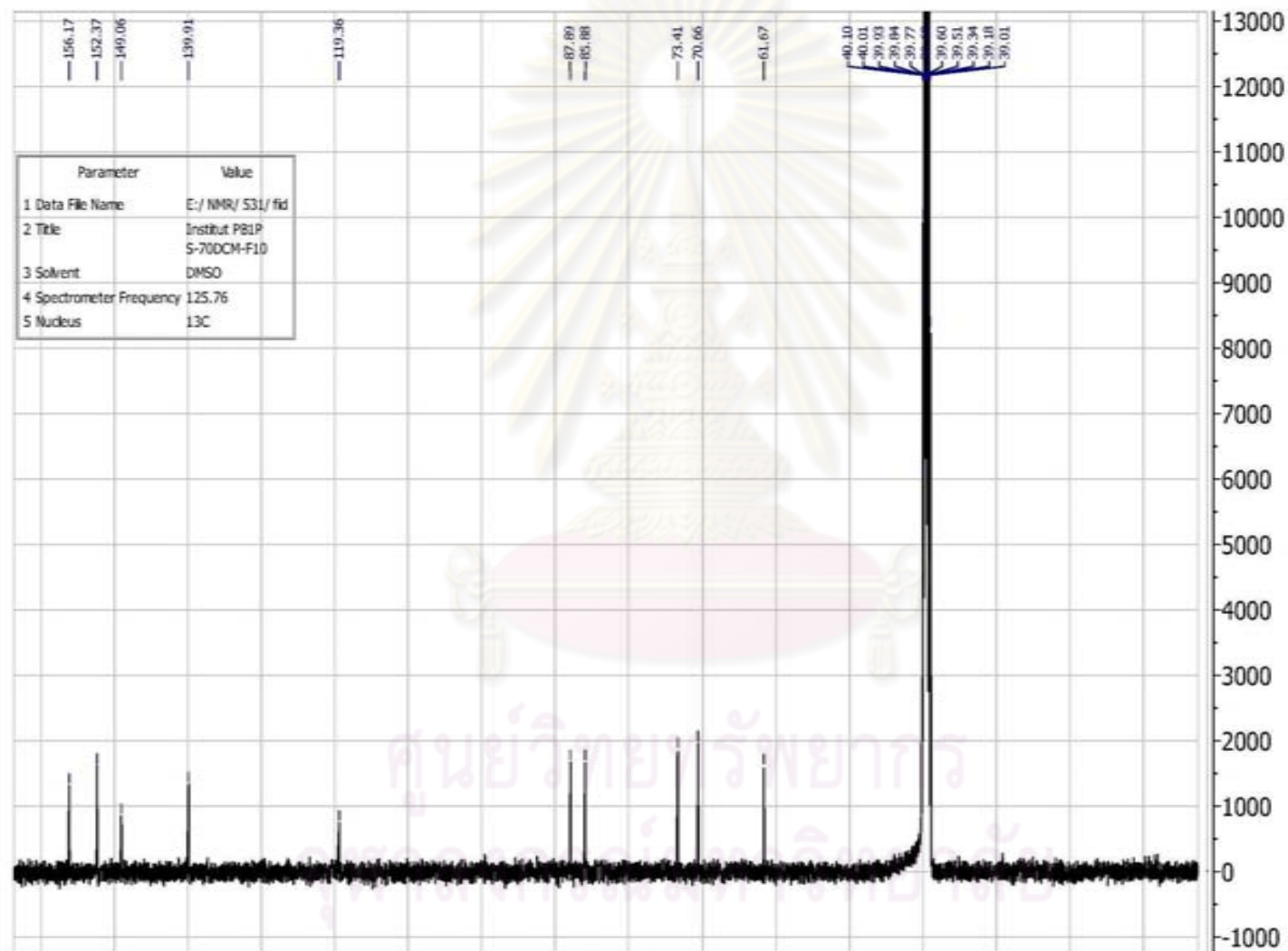**Figure S-62** ^{1}H NMR(500 MHz) spectrum of compound **18** (DMSO-d_6)

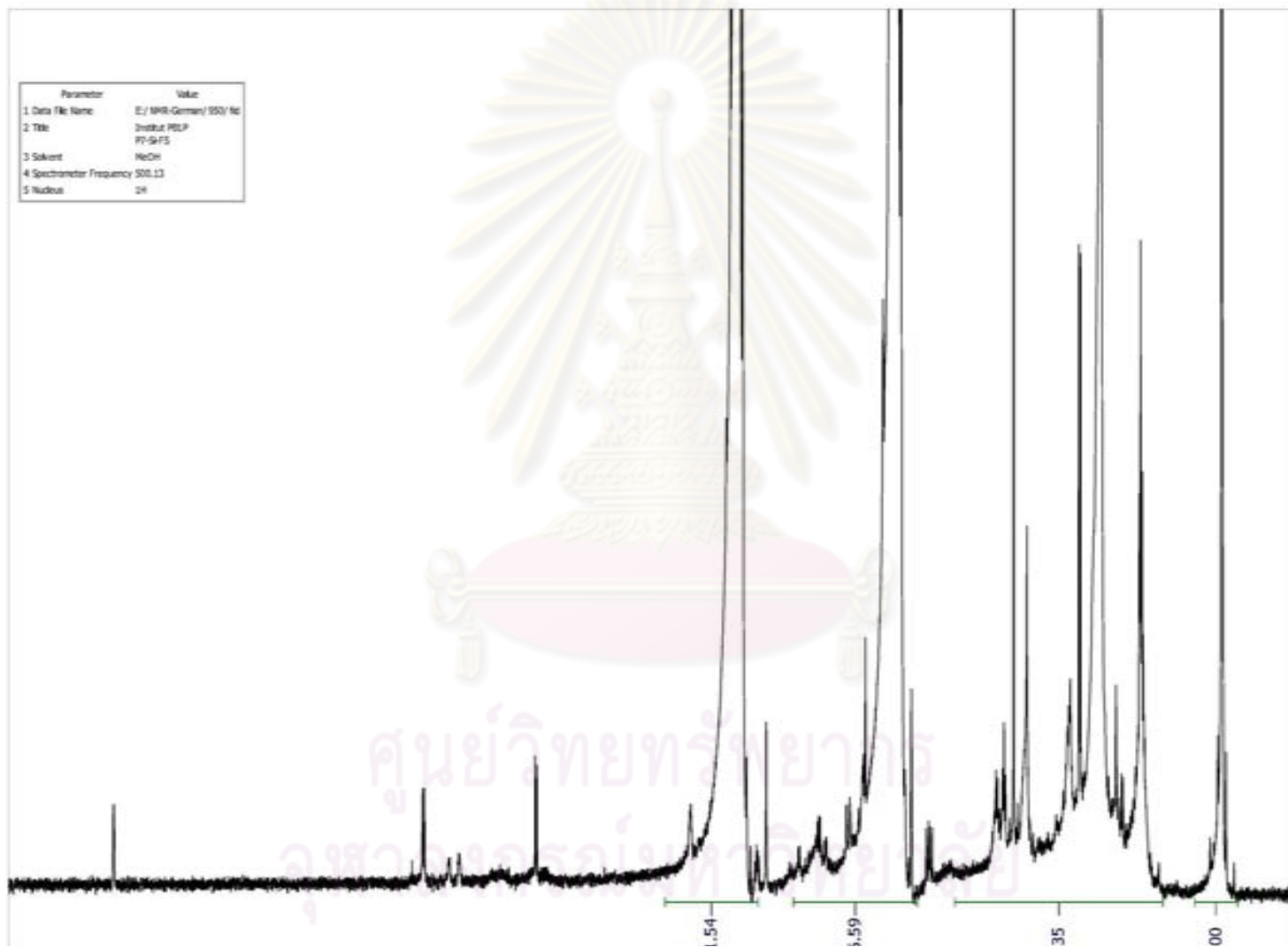**Figure S-63** ^{13}C NMR(100 MHz) spectrum of compound **18** (DMSO-d_6)

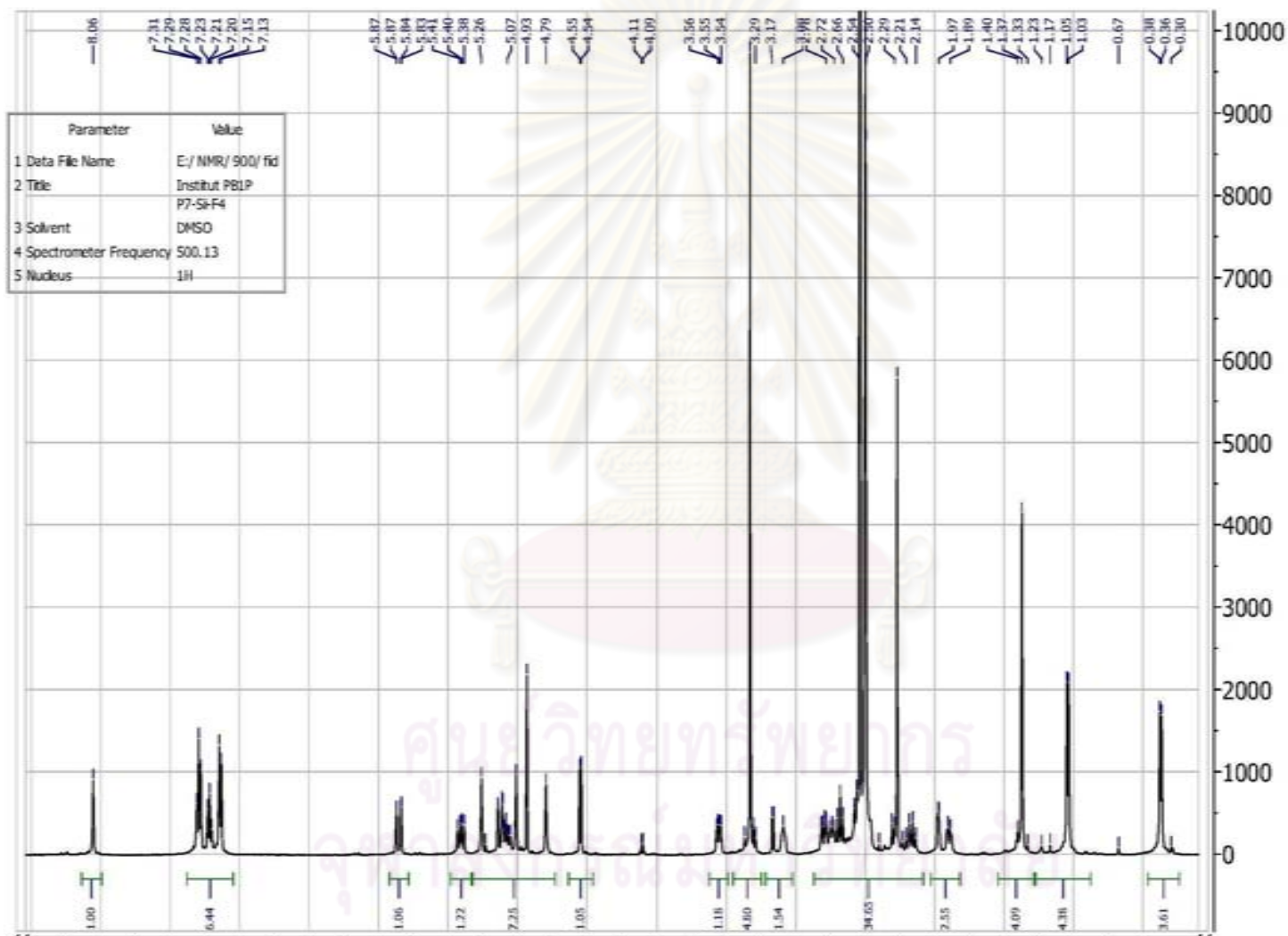**Figure S-64** ^{1}H NMR(500 MHz) spectrum of compound **20** (MeOD)

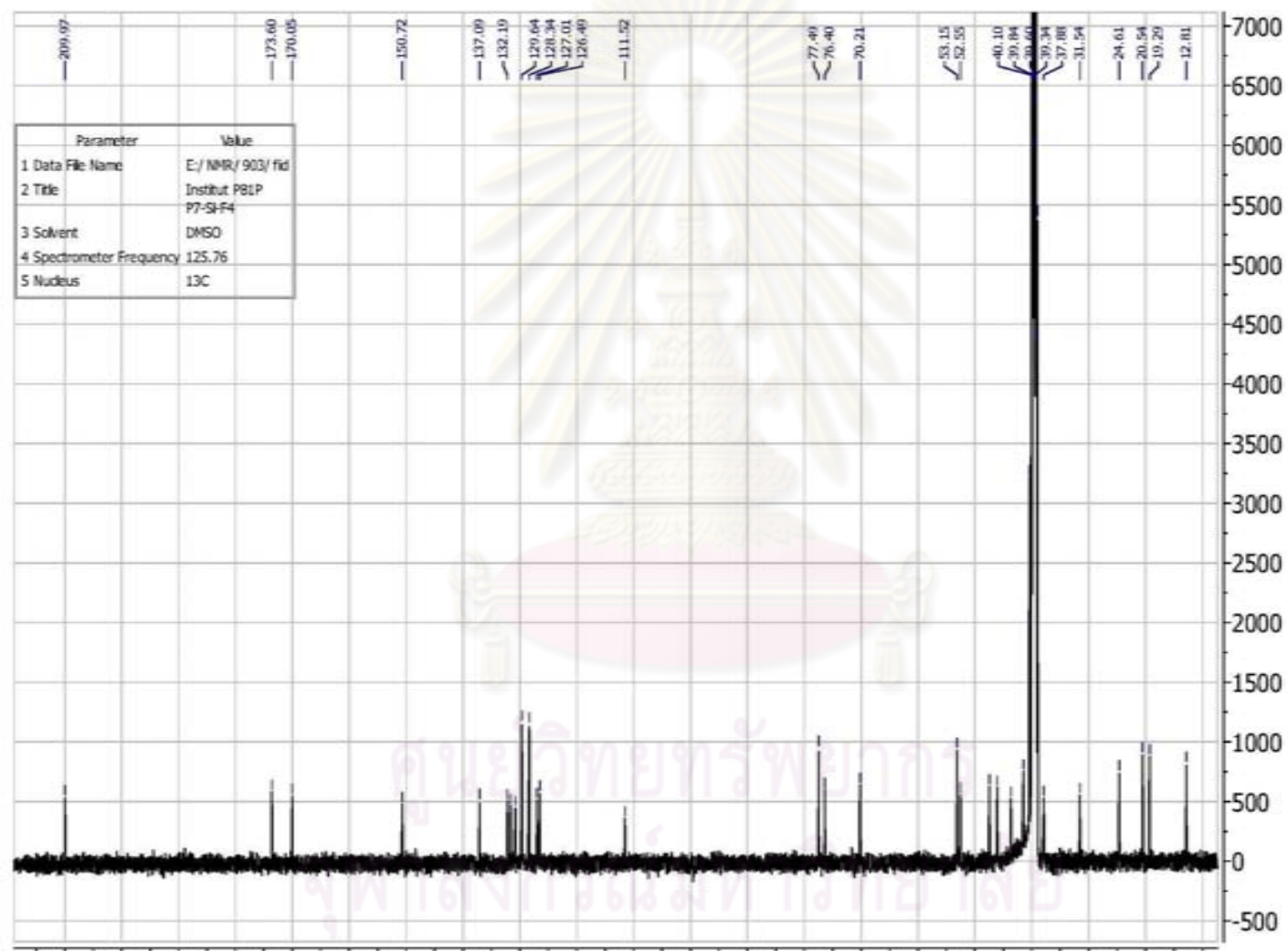**Figure S-65** ^{1}H NMR(500 MHz) spectrum of compound **21** (DMSO-d_6)

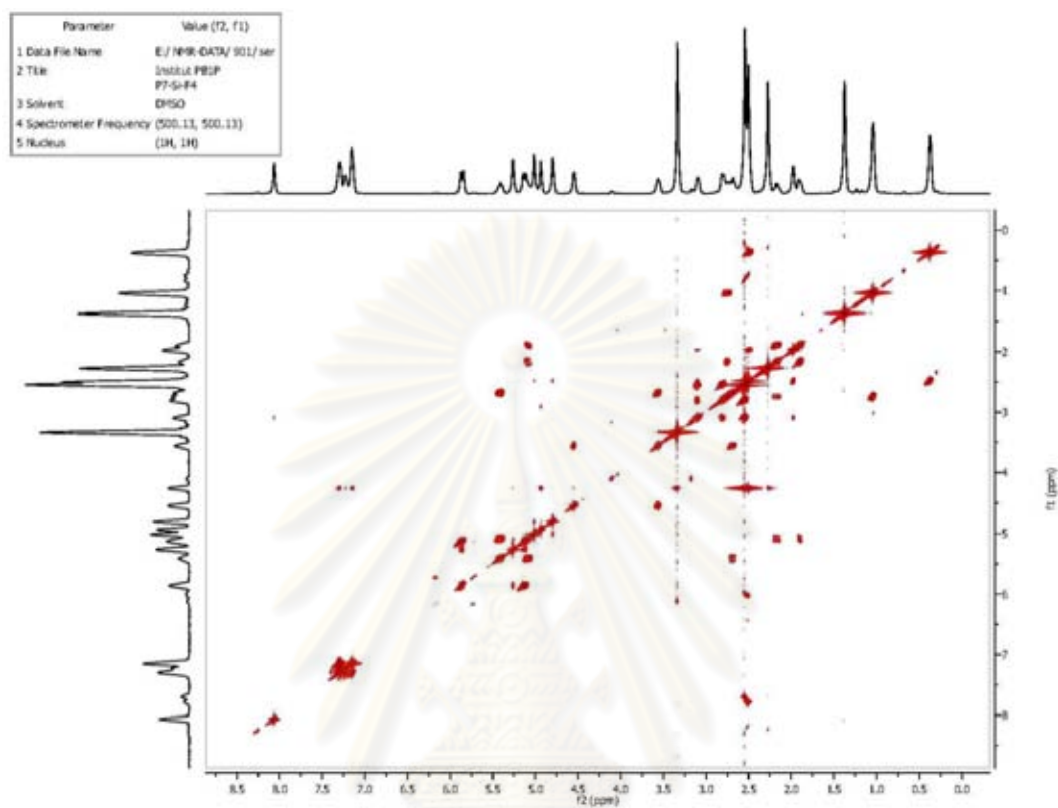**Figure S-66** ^{13}C NMR(100 MHz) spectrum of compound **21** (DMSO-d_6)

Parameter	Value (f2, f1)
1 Data File Name	E:/ NMR-DATA/ 901/ ser
2 Title	Institut PBSP
	P7-Si-F4
3 Solvent	DMSO
4 Spectrometer Frequency (500.13, 500.13)	
5 Nucleus	(1H, 1H)

Figure S-34 COSY spectrum of compound **21** (DMSO-d_6)

VITAE

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