สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* จังหวัดฉะเชิงเทรา

นายจตุพล เหลี่ยงสกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOLOGICALLY ACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* IN CHACHOENGSAO PROVINCE

Mr. Jatupol Liangsakul

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By	Mr. Jatupol Liangsakul
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Thesis Advisor	Assistant Professor Surachai Pornpakakul, Ph.D.
Co-Advisor	Associate Professor Prakitsin Sihanonth, Ph.D.

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

……..Dean of Faculty of Science
(Professor Piamsak Menasaveta, Ph.D.)

Thesis Committee

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

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

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

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

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

จตุพล เหลี่ยงสกุล : สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* จังหวัดฉะเชิงเทรา (BIOLOGICALLY ACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* IN CHACHOENGSAO PROVINCE) อาจารย์ที่ปรึกษา : ผศ.ดร. สุรชัย พรภคกุล อาจารย์ที่ปรึกษาร่วม : รศ.ดร.ประกิตต์สิน สีหนนทน์. 252 หน้า. ISBN 974-17-4723-3

ราเอนโดไฟต์ 84 สายพันธุ์ แยกได้จากใบ ก้านใบ กิ่ง และเปลือกของเปล้าใหญ่ จังหวัดฉะเชิงเทรา มีเพียง 7 สายพันธุ์เท่านั้น ที่สร้างสารเมแทบอไลต์ที่ต่อต้านจุลินทรีย์ที่ต่ำ ในการต่อต้านแบคทีเรียและยีสต์ สายพันธุ์ CsLm08 และ CsPm 09 เลือกมาทำการศึกษา โดยอาศัยลักษณะทางสัณฐานวิทยา สรีรวิทยา ลักษณะการเจริญบนอาหารเลี้ยงเชื้อ และการตรวจสอบลำดับเบสที่ประมวลรหัสของตำแหน่ง ITS พบว่า สายพันธุ์ CsLm08 และ CsPm 09 คือ *Lasiodiplodia theobromae* และ *Emericella variecolor* ตามลำดับ แยกส่วนสกัดเอธิลแอซิเตตจากเส้นใยของ *Lasiodiplodia theobromae* ได้ ergosta-5,22-dien-3-ol, กรดคาร์บอกซิลิกโซ่ตรงยาว C_{22}-C_{24} และ lasiodiplodin ส่วนสกัดเอธิลแอซิเตตจากน้ำหมักของ *Emericella variecolor* แยกได้ terrein และส่วนสกัดเอธิลแอซิเตตจากเส้นใยแยกสารได้ 5 ชนิด ประกอบด้วย stellatic acid และสารเมแทบอไลต์อนุพันธ์ของแซนโทน 4 ชนิด ประกอบด้วย shamixanthone, 14-methoxy-tajixanthone-25-acetate, tajixanthone hydrate และสารอนุพันธ์แซนโทนใหม่หนึ่งชนิด คือ 8-(3-hydroxy-2-methoxy-3-methylbutyl)-1,11-dihydroxy-2-isopropenyl-5-methyl-2,3-dihydro-1*H*-pyrano[3,2*a*]-xanthen-12-one หาสูตรโครงสร้างของสารเหล่านี้โดยอาศัยคุณสมบัติทางกายภาพและเทคนิคทางสเปกโตรสโกปี นำสารที่แยกได้มาทดสอบความเป็นพิษต่อเซลล์มะเร็งคน 5 ชนิด ประกอบด้วย HEP-G2 (ตับ), SW 620 (ลำไส้ใหญ่), CHAGO (ปอด), KATO-3 (กระเพาะอาหาร), และ BT 474 (เต้านม) พบว่า tajixanthone hydrate มีฤทธิ์การยับยั้งเซลล์มะเร็ง HEP-G2 (ตับ), SW 620 (ลำไส้ใหญ่), CHAGO (ปอด), KATO-3 (กระเพาะอาหาร) ได้สูงที่สุดโดยมีค่า IC_{50} เท่ากับ 16.4, 13.6, 11.6 และ 10.9 nM ตามลำดับ และ 14-methoxy-tajixanthone-25-acetate ยับยั้งเซลล์มะเร็ง BT 474 (เต้านม) ได้สูงที่สุดโดยมีค่า IC_{50} เท่ากับ 12.1 nM ส่วนสารอนุพันธุ์แซนโทนใหม่ พบว่ามีผลยับยั้งเซลล์มะเร็ง SW 620 (ลำไส้ใหญ่), CHAGO (ปอด), KATO-3 (กระเพาะอาหาร), และ BT 474 (เต้านม) ในระดับความเข้มข้นปานกลางโดยมีค่า IC_{50} เท่ากับ 19.2, 17.2, 20.0 และ 14.1 nM ตามลำดับ และเป็นครั้งแรกในการรายงานผลการทดสอบการยับยั้งมะเร็งของ lasiodiplodin ซึ่งเป็นสารประกอบหลักของ *Lasiodiplodia theobromae*.

ภาควิชา.....-..... ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีชีวภาพ.... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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JATUPOL LIANGSAKUL: BIOLOGICALLY ACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* IN CHACHOENGSAO PROVINCE. THESIS ADVISOR : ASST. PROF. SURACHAI PORNPAKAKUL, Ph.D. THESIS CO-ADVISOR : ASSOC. PROF. PAKITSIN SIHANONT, Ph.D.; 252 pp. ISBN.974-17-4723-3

Eighty-four isolates of endophytic fungi were isolated from leaves, petrioles, twigs, and barks of *Croton oblongifolius* in Chachoengsao Province. Only seven strains produced antimicrobial metabolites against bacteria and yeasts with lesser activity. The strains CsLm 08 and CsPm 09 were selected for further studied. Based on morphology, physiology, cultural characteristics and nucleotide sequence of ITS regions, the strains CsLm 08 and CsPm 09 were *Lasiodiplodia theobromae* and *Emericella variecolor*, respectively. Isolation of mycelium ethyl acetate crude of *Lasiodiplodia theobromae* gave ergosta-5,22-dien-3-ol, C_{22}-C_{24} long chain carboxylic acid, and lasiodiplodin. The ethyl acetate crude of fermentation broth of *Emericella variecolor* gave terrein and the ethyl acetate crude of mycelium gave five compounds including stellatic acid and four xanthone derivatives consisting of; shamixanthone, 14-methoxy-tajixanthone-25-acetate, tajixanthone hydrate and a novel xanthone derivative, 8-(3-Hydroxy-2-methoxy-3-methylbutyl)-1,11-dihydroxy-2-isopropenyl-5-methyl-2,3-dihydro-1*H*-pyrano[3,2*a*]-xanthen-12-one. These structures were estrablished on basis of physical properties and detail analyses of spectroscopic data. These compounds were tested for cytotoxicity against human cancer cell lines; HEP-G2 (hepatoma), CHAGO (lung), SW 620 (colon), KATO-3 (gastric) and BT 474 (breast). Tajixanthone exhibited highest activity against HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung) and KATO-3 (gastric) cancer cell lines with IC_{50} 16.4, 13.6, 11.6, 10.9 nM, respectively and 14-methoxy-tajixanthone-25-acetate exhibited highest activity against BT 474 with IC_{50} 12.1 nM. The novel compound exhibited against SW 620 (colon), KATO-3 (gastric) and BT 474 (breast) cancer cell lines in moderate level with IC_{50} 19.2, 17.2, 20.0 and 14.1 nM, respectively. Furthermore, this represents the first report of cytotoxicity test of lasiodiplodin which was the main product of *Lasiodiplodia theobromae*.

Department……..-……. Student's signature…….

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

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LIST OF ABBREVIATIONS

br	broad
^{o}C	degree Celsius
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CMA	corn meal agar
^{13}C NMR	carbon-13 nuclear magnetic resonance
cm^{-1}	reciprocal centimeter (unit of wave number)
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	double doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
EI	Electron impact
EtOAc	Ethyl acetate
g	gravity (NMR)
Hz	Hertz
^{1}H NMR	proton nuclear magnetic resonance
h	hour
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Multiple Quantum Correlation
IR	infrared
ITS	internally transcribed spacers
J	coupling constant
L	liter
m	multiplet (NMR)
m	medium (IR)
M^{+}	molecular ion
MczA	Malt Czapek agar
MeOH	methanol

LIST OF ABBREVIATIONS (continued)

MEA	malt extract agar
MHz	megahertz
min	minute
ml	milliliter (s)
mg	milligram
MS	mass spectroscopy
m/z	mass to charge ratio
nm	nanometer
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PDA	potato dextrose agar
ppm	part per million
PCR	poly chain reaction
PTLC	preparative thin layer chromatography
q	quartet
SDA	Sabouraud's dextrose agar
s	singlet
sp.	species
t	triplet
TLC	thin layer chromatography
TOCSY	Total Correlation spectroscopy
μl	microliter
μg	microgram
UV	ultraviolet
w	weak
YEA	yeast extract agar
δ	Chemical shift
ε	The reciprocating wavelength (IR)
λ_{max}	the wavelength at maximum absorption (UV)
ν_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

In the nature, microbes and plants are closely associated with the ecological system on the earth. The fungi are a very large and diverse group of organisms which have a unique life-style. Endophytic fungi live almost entirely within the leaves, stems, tissues and organs of apparently healthy host plants, and often without causing any visible signs of diseases. The majority of endophytic species which have been successfully identified were Ascomycetes and Deuteromycetes with a few Basidiomycetes and a very small number of Oomycetes (Isaac, 1992). Application of endophytes included potential biological control agents, sources of novel metabolites for medicine, plant protection, and industrial uses, and as research model systems for investigation of host parasite interactions and evolution in natural systems (Bacon and White, 2000).

The association of these microorganisms with higher plants ranged from mutualistic symbiosis or commensalism to borderline latent pathogenicity. Endophytes could be transmitted from one generation to next through the tissue of host seeds or vegetative propagules. Some grass endophytes appear to be transmitted horizontally, external to host tissues, with their aeril spores (Bacon et al., 2000).

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

endophytes are produced secondary metabolites to perform key roles related to the survival of the microbe and symbiotic unit. Thus, endophytic habitat is a niche that has been continued exploration (Bacon et. al., 2000).

Thailand locate in a tropical rainforest region with many varieties of plants that may support endophytes. Endophytic fungi had been known as the important resource for novel metabolites to antibacterial, antifungal, and cytotoxicity activities (Tan and Zou, 2001). For example, many endophytic fungi species have been found to produce taxol and other novel compounds (Strobel and Long, 1998).

In this research, bioactive compounds which produced by endophytic fungi isolated from (*Croton oblongifolius*) were investigated. Plao Yai had been used as a plant source for isolating endophytic fungi because Plao Yai is regularly used as Thai medicinal plant including produced various diterpenoid compounds (Roengsumran et al., 2002). Especially Plao Yai in from Chachoengsao Province had been isolated to give clerodane and labdane diterpenoids from bark and some compound have been shown significantly inhibit cancer cell lines (Bunyamanee, 2000).

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

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

2. Screening of endophytic fungi metabolites by microbiological activity test and thin layer chromatography method.

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

4. Characterization biological activities of the compounds isolated from endophytic fungi.

5. Evaluation of biological activity of the compounds.

CHAPTER II

LITERATURE REVIEWS

2.1 Association of endophytic fungi and plant

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

The term endophyte was originally defined by De Bary and referred to any organism occuring within plant tissues. Since it has become deeply embedded in the literature and within the last decade, different authors have proposed a range of similar, but more complex definitions e.g. Carroll 1986, Petrini 1991 (Wilson, 1995). In 1986, Carroll restricted the use of the term endophyte to organisms that cause asymtomatic infections within plant tissues, excluing pathogenic fungi and mutualists such as mycorrhizal fungi (Carroll, 1986 cited in Petrini, 1991). Petrini (1991) proposed an expansion of Carroll's definition and incorporated into it, his concept of latent pathogens known to live symtomlessly inside the host tissues for only part of their life cycle. Wilson (1995) described endophytes as fungi and other microorganisms such as bacteria that invade tissues of plants and cause unapparent and asymptomatic infection for all or part of their life cycle.

Modern usage of the term endophyte in mycology refers to those fungi which live almost entirely within the leaves and stems of apparently healthy host plants, doing so asymptomatically, causing no visible signs of infection. The term endophyte was originally defineby De Bary (1866) to distinguish those species which invade and reside within host tissues or cells from epiphytes, those fungi living on the outer surface of host plants. Parasitic antagonistic symbionts which cause visible disease symptoms are

more usually referred to as pathogens even though these may live almost entirely within host tissues. Additionally, although mycorrhizal fungi live both in and on host tissue such associations are not usually included within the endophyte category. Endophytes have been identified in a very wide range of host plant species, including representatives of most major taxonomic groups, including mosses, ferns and liverworts. It has been suggested that the majority of living plants are hosts to endophytic fungi (Petrini, 1986; Carroll, 1986, 1988). An individual plant may be host to a range of endophytic fungal symbionts simultaneously. In morphological terms the fungal hyphae growing in plant tissues are not concerned within host tissues. The majority of the endophytic species which have been successfully identified are Ascomycetes and Deuteromycetes with a few Basidiomycetes and a very small number of Oomycetes (Isaac, 1992).

Endophytic fungi colonized live into the plant tissues by penetration of fungal hypha between plants cells or may also grew intracellularly and must obtain nutrient materials through this intimate contact with the host (Isaac, 1992). The relationship of fungi with plant ranges from mutualistic symbiosis, or commensalism to borderline latent pathogen. Results of their interaction were increase capacity of a plant to resist disease and increase survival of plant from natural environment by producing bioactive compounds of plant growth promoting, antibacterial, antifungal and insecticidal to enhance the plant growth (Strobel and Long, 1998).

2.2 Study of secondary metabolites from the endophytic fungi

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

Microorganisms, in particular fungi, are important sources of secondary metabolites. Fungal secondary metabolites are a diverse group of compounds produced by a wide range of different fungi. Endophytic fungi are a potent source of novel chemistry and biology to assist to helping solve not only human health, but plant and animal health problem also. Endophytic fungi reside in the tissue between living plant cells. The relationship that establish with the plant varies from symbiosis borderline on pathogenic. As a result, the opportunity to find new and interesting endophytic fungi among the myriad of plants is great. Sometimes extremely unusual and valuable organic substances are produced by these endophytic fungi (Tan and Zou, 2001).

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

Tan and Zou (2001) recently reviewed the diversity of metabolites that have been isolated from endophytic fungi emphasizing their potential ecological role. These secondary metabolites of endophytes are synthesized via various metabolic pathways of diverse structural groups such as alkaloids, polyketides, isoprenoids, amino acid derivations, steroids, xanthones, phenols, isocoumarines, pyrylene derivatives, quinones, furandiones, terpenoids, depsipeptides and cytochalasines (Schulz et al., 2002).

2.2.1 Alkaloids from endophyte-infected grasses (Bush et al., 1997)

Fungi that live their entire life cycle within the aerial portions of grasses and sedges by forming a nonpathogenic, systemic and usually intercellular association are referred to as endophytic fungi orgrass endophytes. Grass/ endophyte symbiota are extremely intimate and perennial, and exhibit close matching of the host and symbiont life cycle. Each symbiotum exhibits one of three possible life strategies, resulting in pure vertical transmission of the endophyte, pure horizontal transmission of the endophyte, or mixture of the two life cycles. The sexual *Neotyphodium* species are restricted to pure vertical transmission, a highly efficient clonal propagation in flowering meristems, ovules, seeds, and ultimately, progeny seedlings of infected mother plants. In contrast, the pure horizontal transmission strategy of some *Epichloe* species relies on the production of contagious sexual spores. These spores can only be produced on a fungal structure (stroma) surrounding the grass flagleaf sheath, but as soon as stroma are produced, the inflorescence of the affected tiller ceases development (it seems likely that this is due to fungal products mimicking plant growth regulators). The resulting suppression of seed production is termed "choke disease". The most highly pathogen *Epichloe* species completely suppress host seed production, and because seeds cannot be a vehicle for their dissemination, the species are obligately sexual and horizontal transmitted. Many *Epichloe* species use a third, remarkably balanced stagely of mixed transmission. These fungi choke some flowering tillers and produce sexual spores but leave/other tiller (usually a majority) unaffected and fertile. Like the asexual endophytes they are transmitted in nearly all seeds produced by infected mother plants (Figure 2.1).

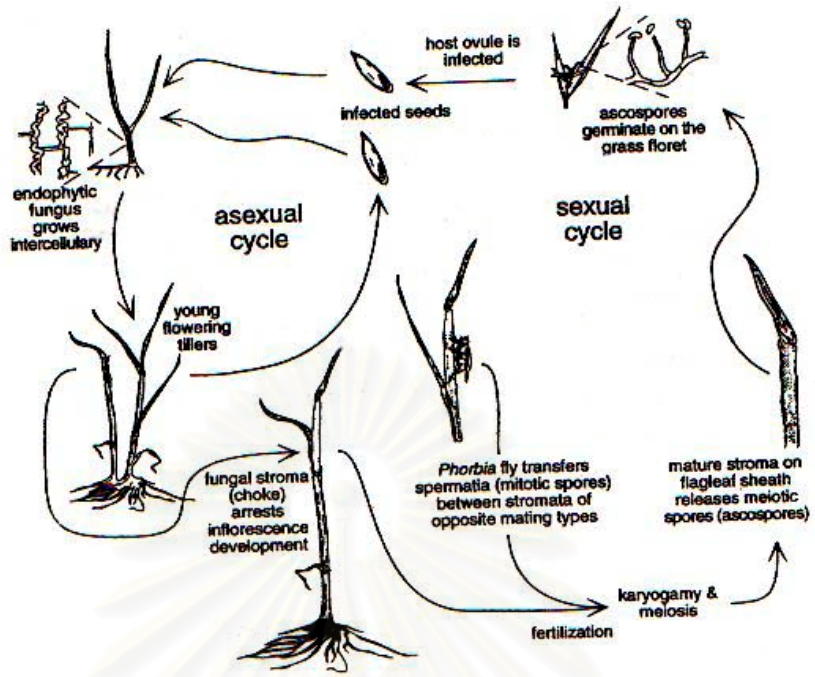


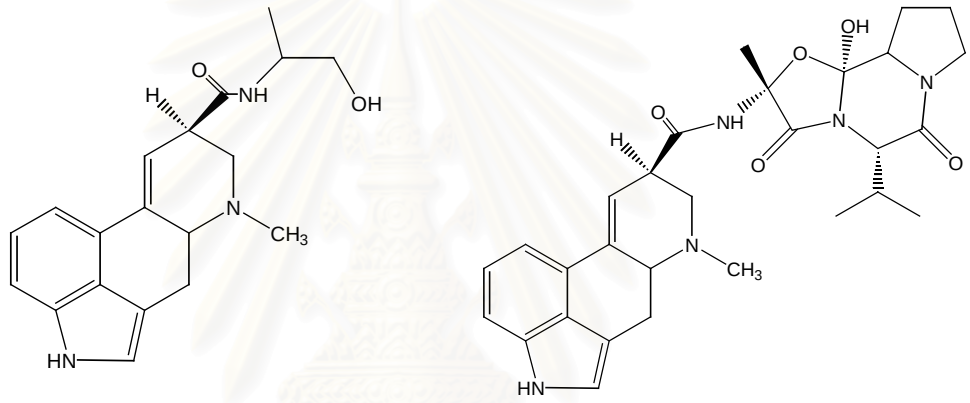
Figure 2.1 Alternative asexual and sexual life cycle of *Epichloe festucae* in symbiosis with *Festuca* sp. (Bush et al., 1997)

The search of bioprotective alkaloids in grass/endophyte symbiota are generally grouped as pyrrolizidines, ergot alkaloids, indole diterpenes, and pyrrolopyrazines.

2.2.1.1 Activities and biosynthesis of Ergot Alkaloids (Bacon and White, 2000)

Ergot alkaloids are derivatives of lysergic acid. Several grasses infected with different species of endophytes have been shown to contain these compound. Compounds of this class have been implicated strongly in the observed toxicity suffered by livestock consuming endophyte-infected grasses. Many of the symptoms reduced weight gain, elevated body temperature, restricted blood flow, reduced reproduction, and reduced milk production. Perennial ryegrass (*Lolium perenne*) infected with appropriate strains of the endophytic fungus *Acremonium lolii* is resistant to a number of insect species and ergot alkaloids have been implicated strongly in the observed toxicity to grazing livestock. Animals grazing endophyte-infected ryegrass may suffer from the neurotoxicity syndrome of ryegrass staggers, and also show reduced live-weight gains and serum prolactin levels.

All endophytic species, primarily *Balansia*, found growing in association with major forage grass have been shown to produce ergot alkaloids. Other fungi, such as *Atkinsonella* and *Myriogenospora*, have not been examined for this group of toxins. While most produce ergot alkaloids, there is a difference in the type of ergot alkaloids produced by each of the two groups of endophytes. Many species of *Balansia* and other members of the seedborne indirect group produce the clavine type of ergot alkaloid as the major class. Species of *Acremonium* and other members of the seedborne direct group produce the peptide types of ergot alkaloids. Hower, both types of alkaloids have been reported as minor constituents in each endophytic group.



Ergonovine Ergovaline

Figure 2.2 Ergot alkaloids (Ergonovine and Ergovaline)

The ergopeptide ergovaline, together with its 8-epimer ergovalinine ergot alkaloids (Figure 2.2) based on lysergic acid amide occur as epimeric pairs is the major ergot alkaloids present in tall fescue (*Festuca arundinacea*), perennial ryegrass (*Lolium perenne*) and *Hordeum* spp. infected with *Neotyphodium* endophytes. Other ergopeptides, clavine alkaloids, and lysergic acid amide derivatives, have also been identifid from *Neotyphodium*-infected grasses. Lysergic acid amide derivatives predominate in *Neotyphodium*-infected sleepygrass (*Stipa robusta*) and drunken horse grass (*A. inebriens*).

Endophyte ergopeptide alkaloids appear to be the major factor in fescue toxicosis. While direct experimental evidence of the activity of ergovaline has been limited by scarcity of the compound, the pattern of activity toward mammals appears to be similar to that of other ergot alkaloids. The characteristic symptoms of fescue toxicosis, including elevated body temperatures under external stress, vasoconstriction, and reduced prolactin levels, are all consistent with ergopeptide toxicity, although recent oral dosing experiments with ergovaline at in planta concentrations did not entirely reproduce the effects of feeding endophyte-infected plant meterial. Ergot alkaloids are also toxic to, or inhibit the feeding of, many insects.

Biosynthesis of ergot alkaloids is best understood because of previous research on ergot alkaloid biosynthesis in *Claviceps purpurea*. The mevalonic acid derivative dimethylallyl diphosphate and Trp are the precursor for DMAT synthesis to the ergolene class of alkaloids and, subsequently, the ergolene acids, including lysergic acid and the ergolene alcohols. The simple and complex derivatives of lysergic acid are major accumulation products in several grass/endophyte symbiota. Dimethylallydiphosphate :L-Trp dimethylallyltransferase (DMAT synthase) catalyzes the first pathway-specific and probably the limiting step in the formation of these alkaloids. Another well characterized step is the incorporation of lysergic acid into D-lysergyl peptide lactams, oligopeptide precursors of ergotamine, and related alkaloids. This step is carried out by a complex of two peptide synthase from *C. purpurea* and closely related enzyme complex is involved in ergovaline biosynthesis in *Epichloe* and *Neotyphodium* species (Figure 2.3).

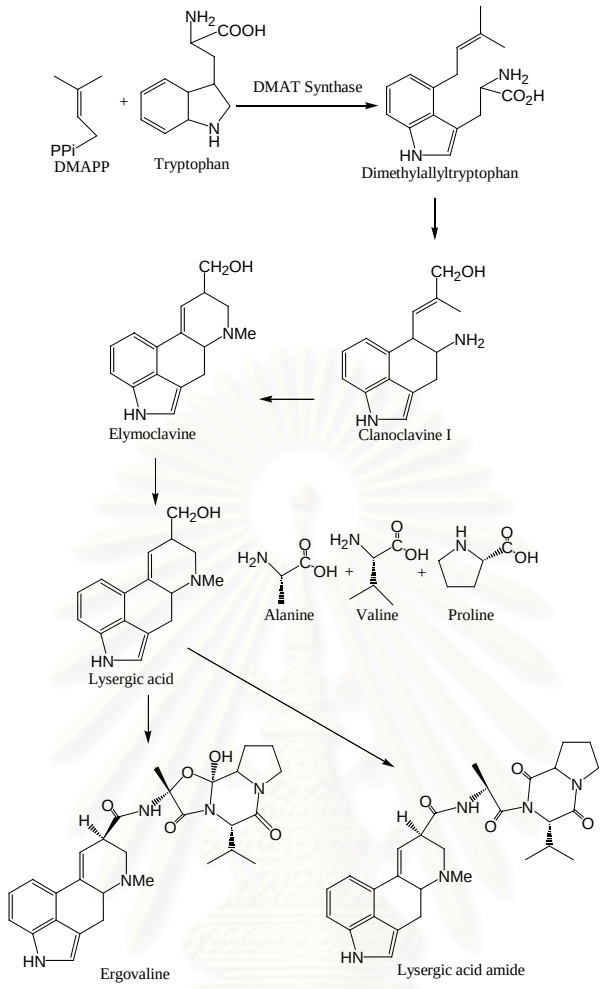
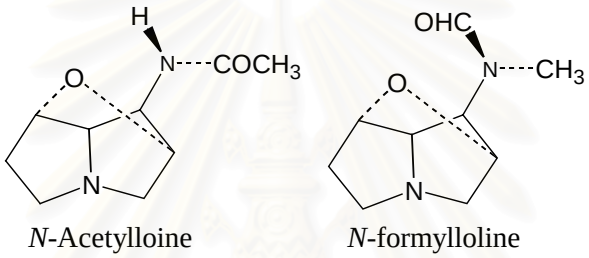


Figure 2.3 Biosynthesis pathway of Ergot alkaloids (Bacon and White 2000)

2.2.1.2 Activities and biosynthesis of lolines (Bacon and White, 2000)

Pyrrolizidine alkaloids of the loline group are common in *Festuca* and annual *Lolium* spp. infected with *Neotyphodium* endophytes. Lolines contain an amino nitrogen substitution on a usually saturated pyrrolizidine ring system with a stable cyclic ether structure. In endophyte-infected grasses they occur with or without methylation and acetylation of the amino nitrogen, with *N*-formylloline and *N*-actylloline commonly the major components (Figure2.4). Recently a novel ring-unsaturated variant, tentatively identified by gas chromatography-mass spectrometry (GC-MS) as 5,6-dehydro-*N*-acetylloline, has been found in endophyte-infected *Festuca argentina*. Loline apparently act both as metabolitic toxins and as feeding deterents, depending upon the species of insect. Lolines are found in root tissue at levels that are much lower than in shoot tissue ,

but may be sufficient to help protect against some insects. Lolines may be involved in vasoconstriction and vascular thickening, and the site of action may be α-2-adrenergic, D_2 dopamine, or serotonic receptors. Also, significant immunosuppressive effect has been note in feeding studies in mice. Some allelophathic properties that have long associated with tall fescue may be attributable to lolines. *N*-formylloline and *N*-acetylloline inhibit the rate but not amount of seed germination of several monocots and dicots. The amount of *N*-formylloline detected in soil below tall fescue plants infected with *N. coenophialum* is four times the level required to inhibit germination of *Lolium multiflorum*.



N-Acetylloine *N*-formylloline

Figure 2.4 Loline alkaloids (*N*-Acetylloline and *N*-Formylloline)

Loline biosynthesis is poorly understood and the proposed biosynthetic pathway is based on other pyrrolizidine alkaloid biosynthetic schemes. Spermidine is the likely precursor of the pyrrolizidine ring system of lolines, whereas homospermidine may be the precursor of most plant pyrrolizidines. Enzymatic steps from spermidine are expected to include N-methylation, oxidative deamination, and cyclization (Figure 2.5).

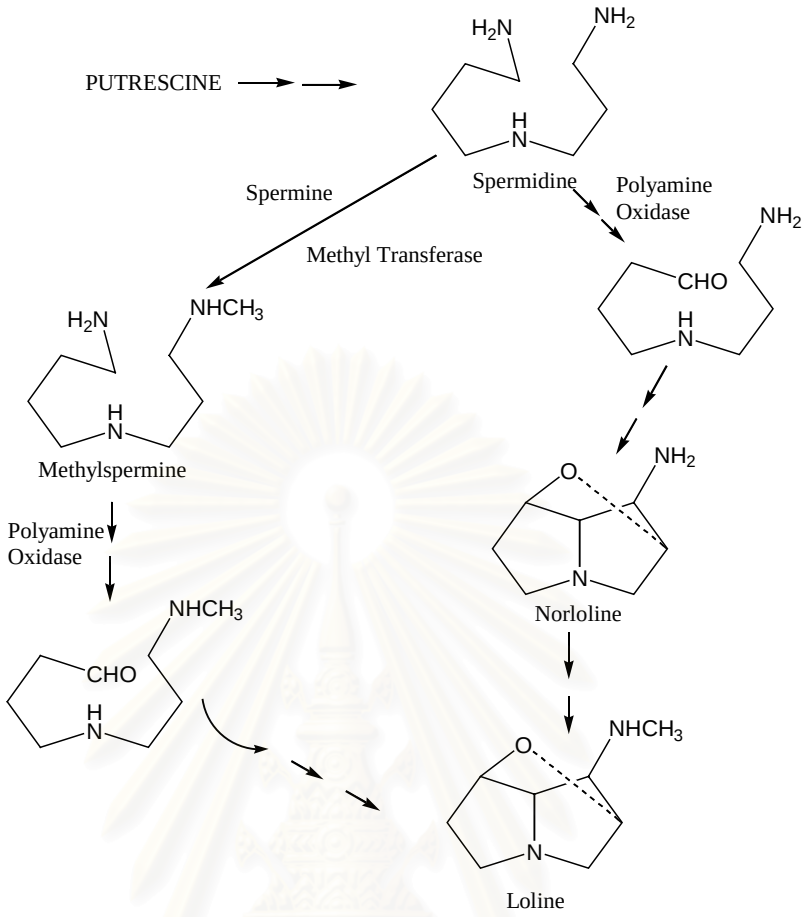


Figure 2.5 Biosynthesis pathway of Loline alkaloids (Bush *et al*., 1997)

2.2.1.3 Activities and biosynthesis of Lolitrems (Bacon and White, 2000)

The lolitrems are tremorgenic neurotoxins considered responsible for the ryegrass staggers toxicity syndrome experienced by livestock grazing endophyte-infected ryegrass. Lolitrems are complex indole diterpenes which seem to be produced particularly by endophyte-grass associations involving competent strains of the endophyte *Neotyphodium lolii*. It is structurally related to the penitrems, jantritrems, paxilline, paspaline, and related mycotoxins. Lolitrem B is highly tremorgenic when administered by the intravenous and intraperitoeal routes, inducing tremors lasting up to several days. More recently, an extensive range of minor lolitrems and related indole-diterpenoids have been isolated from *N. lolii*-infected perennial ryegrass seed. In addition to their tremorgenic effects, lolitrem B and related compounds exhibit other

toxicities to mammals, are toxic or feeding deterrent to insects, modulate calcium-activitied potassium channel activity, and inhibit acyl-CoA:cholesterol acyl transferase. Although lolitrem B has not been identified in fungi other than *Epichloe*/*Neotyphodium* spp., indole-diterpenoids paspaline, paxilline, and 13-deoxypaxilline identified in *N. lolii*-infected perennial ryegrass seed are also present in other fungal genera. Within the family Clavicipitaceae, paspaline occurs in *Clavicep paspali*, the agent responsible for paspalum staggers of cattle grazing ergotized *Paspalum dilatatum*. Paspaline is also found in *Penicillium*, *Emericella*, and *Albophoma* spp. and paxilline found in *Eupenicillium* spp. too.

The activity of lolitrem depends on a relatively plannar ring structure, but if the A-ring protudes from the plannar surface onto the α-face, activity is lost. Apparently, interence with the α-face prevents effective binding to the receptors. Indole diterpenes are potent inhibitors of high conductance potassium channels, but this effect does not account for complete symptomology of lolitrem poisoning.

Indole diterpenes biosynthetic shemes of likely intermediates from *N.lolii* and other indole-diterpene-producing fungi such as *Penicillium paxilli*. Geranylgeranylpyrophosphate and Trp are logical precursors to paxilline via emindol SB, paspaline, and dehydroxypaxilline. Intermediates in the conversion of paxilline to lolitrem B likely include α-paxitriol, lolitriol, and lolitrem E.

Many of these transfot\rmation operate in fungi in a more or less modular fashion in a metabolite grid, leading to a diversity of indole-diterpenoid metabolites. Approximately 70 metabolites of this class have so far been identified. Most of these transformations also appear to occur in *N.lolii* during the biosynthesis of the lolitrem neurotoxins, suggesting that the indole -diterpenoid biosynthetic pathway in all of these fungi has evolved from a common pathway similar to the simpler one that operates in *C.paspali* many of the lolitrems are analogs of simpler indole-diterpenoids found in other fungi, such as paspaline, paspaline B, paxilline and several tenpendoles (Figure 2.6).

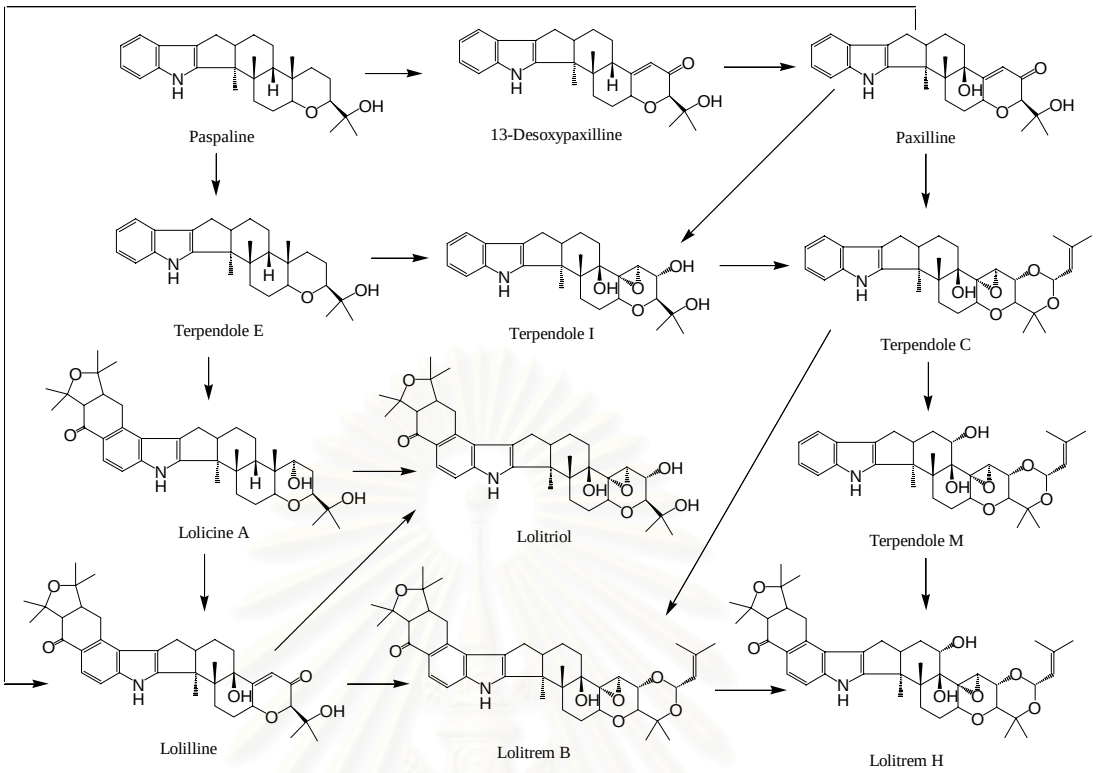


Figure 2.6 Biosynthesis pathway of Lolitrem alkaloids (Munday-Finch, 1997)

2.2.1.4 Activities and biosynthesis of Peramine (Bush et al., 1997)

Peramine is the only known pyrrolopyrazine alkaloid isolated from grass symbiota, which occurs in Epichloe and Neotyphodium-infected grass . It is a highly active feeding deterrent to the Argentine stem weevil (*Listronotus bonariensis*), a major pest of New Zealand pasturesbut has not shown toxicity to mammals or plants (Figure 2.7).

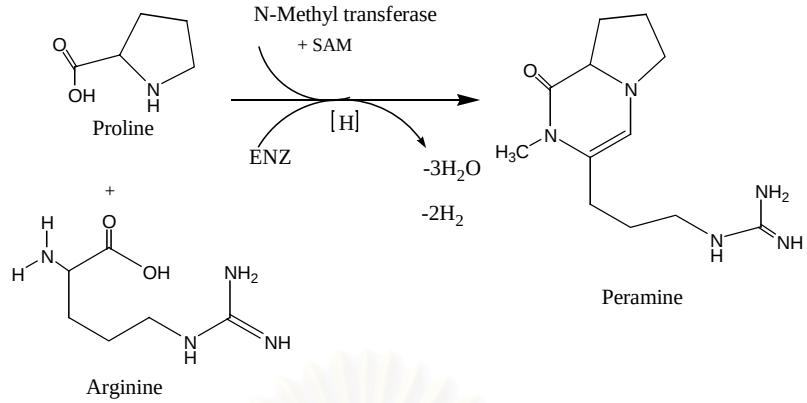


Figure 2.7 Biosynthesis of Peramine (Bacon and White 2000)

Peramine is the simplest of the alkaloids in these symbiota, and a biosynthetic scheme scheme are involving Pro, Arg, and a methyl donor. Such a scheme is consistent with possibility that a multidomain peptide synthase is involving.

2.2.2 Steroids

Along with ergosterol, 3β,5α,6β-trihydroxyergosta-7,22-diene, 3β-hydroxy ergo sta-5-ene, 3-oxaergosta-4,6,8(14),22-tetraene, 3α-hydroxy-5α,8α-epidioxy-ergosta-6,22-diene, 3α-hydroxy-5α,8α-epidioxy-ergosta-6,9(11),22-triene and 3-oxo-ergosta-4-ene, two new steroids, 3α-5α-dihydroxy-6α-acetoxy-ergosta-7,22-diene and 3α-5α-dihydroxy-6α-phenylacetyloxy-ergosta-7,22-diene were characterized from the liquid culture of an fungal endophyte *Collectotrichum* sp. of *Artemisia annua*. (Lu et al., 2000)

2.2.3 Terpenoids

Terpenoids have been isolated from some endophyte culture originating from a variety of host plants. Those identified so far are mainly sesqui- and diterpenes, some of which are partly degraded.

2.2.3.1 Sesquiterpenes

2α-Hydroxydimeninol, Humulene derivative and pestalotiopsins A-C are sesquiterpenes characterized from endophytic *Pestalotiopsis* spp. associated with *Taxus brevifolia*. In particular, the new sesquiterpene is a highly functionalized

humulene derivative, the first of fungal origin (Pulici et al., 1996 and 1997). Heptidic acid and hydroheptidic acid isolated from Phyllosticta sp., an endophytic fungus of *Abies balsamea*, have been shown to be toxic to spruce bud worm (*Choristoneura fumiferana*) larvae. (Calhoun et al., 1992). The new benzofuran-carrying normonoterpene derivatives 5-Hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl)benzofuran and 5-Hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl)benzofuran, toxic to spruce bud worm larvae and/ or cells, have been characterized from a culture of an unidentified endophytic fungus obtained from wintergreen *Gaultherna procumbens*. (Findlay et al., 1997).

2.2.3.2 Diterpenes

Two new insect toxins 9α-hydroxy-1,8(14),15-isopimaratrien-3,7,11-trione and 9α-hydroxy-1,8(14),15-isopimaratrien-3,11-dione, of a pimarane diterpene framework were isolated from the broth of an unidentified endophyte from a needle of the balsam fir *Abies balsamea*. (Findlay et al., 1995). Subglutinol A and B, immunosuppressive but noncytotoxic, were produced by *Fusarium subglutinans*, an endophytic fungus from the perennial twining vine *Tripterygium wilfordii*. (Lee et al., 1995). Guanasterpene, a novel diterpenoid produced by an unidentified fungus from the branch of *Daphnopsis americana* growing in Guanaste, Costa Rica, was shown to be antibacterial against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Singh et al., 2000).

Taxol (paclitaxel) originally characterized from the inner bark of the Pacific yew, *Taxus brevifolia*, is an efficacious anticancer diterpene found in extremely small quantities in slowly growing Taxus species. (Wani et al., 1971 and Georg et al., 1994 cited in Tan and Zou, 2001). Its unique mode of action, of perventing the depolymerization of tubulin during the processes of cell division, made it a huge success in both clinic and market. However, the source of Taxol is a frustrating problem all over the world owing to the difficulty and unacceptably low yield in its total synthesis. (Nicolaou et al., 1994 cited in Tan and Zou, 2001)

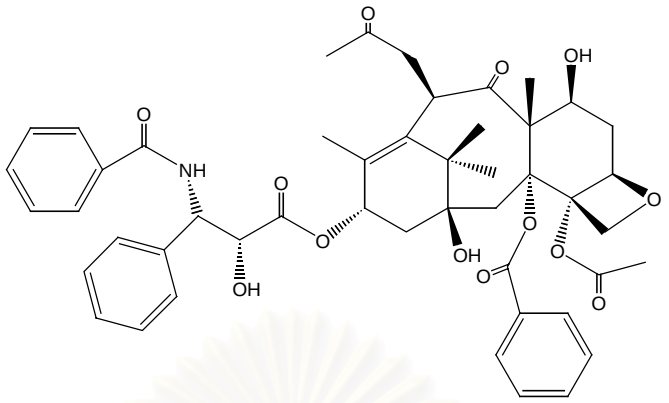


Figure 2.8 The structure of Taxol

It has been reported endophytic microbes *Taxomyces andreanae*, isolated from the Pacific yew tree, *Taxus brevifolia*, could produced taxol as well as. This is an interesting study as a model for learning the biological interaction of bioactive compounds between plant and endophytic fungi (Strobel et al., 1993, Stierle et al., 1993 and Sterle and Strobel et al., 1995).

Furthurmore, *Pestalotiopsis microspora* occurs as a range of strains in bald cypress, *Taxodium distichum*. This microorganism live as endophytes in the bark, phloem and xylem produced taxol. Thus, this report provides evidence of taxol production by fungi not associated with a plant that make taxol, and indicates that other plant species residing in certain damp environments may also be hosts to such taxol-producing endophytes (Li et al., 1996).

Pestalotiopsis microspora is an endophyte of the Himalayan yew, *Taxus wallachiana* produce taxol in liquid culture. Defining culture amendments to optimize taxol production by *P. microspora* is a critical step toward the realization of fungal taxol for treating human cancers. The lowering of phosphate and the addition of sodium benzoate in the medium increased taxol production. In addition, sterol biosynthesis inhibitors, such as terbuconazole and triadimefon, increased taxol yields (Li et al., 1998 and Metz et al., 2000).

Taxol producting endophytic fungus *Tubercularia sp.* strain TF5 isolated from the inner bark of Taxus mairei and culture in PDA medium. The fungal taxol, which was isolated from the organic extract of the TF5 culture, had strong cytotoxic activity towards KB and P388 cancer cells in vitro, tested by the MTT assay (Wang et al., 2000). An endophytic fungus, *Aspergillus niger*, isolated from the inner bark of a *Taxus chinensis* tree, was produced taxol in a *Taxus chinensis* cell suspension culture (Wang et al., 2001).

2.2.4 Isocoumarin derivatives

(*R*)-Mellein, an isocoumarin isolated from Pezicula spp. strongly fungicidal, herbicidal and algicidal (Schulz et al., 1995).

2.2.5 Quinones

Insecticidal rugulosin was characterized from *Hormonema dematioides*, an endophytic fungus of balsam fir. From cultures of an unidentified endophyte obtained from an eastern larch (*Larix laricina*) needle, 8,1',5'-trihydroxy-3',4'dihydro-1'*H*-[2,4'] binaphthalenyl-1,4,2'-trione was characterized as a toxin to spruce budworm larvae (Findlay et al., 1997). A highly hydroxylated quinone altersolanol A, characterized from phytopathogenic *Alternalia* spp., was reisolated from an endophytic Phoma sp. with its antibacterial activity enclosed (Yang et al., 1994).

2.2.6 Flavonoids

Tricin and related flavone glycosides, toxic to mosquito larvae, have been isolated from endophyte-infected blue grass (*Poa ampla*) (Ju et al., 1998).

2.2.7 Phenylpropanoids and lignans

Three new phenylpropanoids and lignan were characterized from stromata of Epichloe typhina on *Phleum pratense* (Koshino et al., 1988).

2.2.8 Peptides

Leucinostatin A, an oligopeptide with phytotoxic, anticancer and antifungal properties was isolated from culture of *Acremonium* sp., an endophytic fungus from *Taxus baccata* (Strobel et al., 1997). The cyclopeptides echinocandins A, B, D and H, were isolated from endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in *Pinus sylvestris* and *Fagus sylvatica* and shown to be antimicrobial. (Noble *et al*., 1991). Cryptocandin, a cyclopeptide with potent antifungal activities, is a metabolite of endophytic *Cryptosporiopsis* cf. *quercina* of redwood (Strobel et al., 1999).

2.2.9 Phenol and phenolic acids

From Collectotrichum gloeosporioides, an endophytic fungus of *Artemisia mongolica*, a new antimicrobial tridepside collectotric acid was characterized in our laboratory (Zou et al., 2000). Furthermore, two isomeric novel tridepsides cytonic acids A and B were reported as human cytomegalovirus (an ubiquitous opportunistic pathogen) protease inhibitors from the culture of the endophytic fungus *Cytonaema* sp. isolated from *Quercus* sp (Guo et al., 2000).

2.2.10 Aliphatic compounds

Phomodiol , metabolite of endophytic *Phomopsis* spp. present in the *Salix* spp. , may serve as potential markers for taxonomy of these fungi (Horn et al., 1994).

2.2.11 Chlorinated metabolites

Three chlorinated metabolites from endophytic fungi were reported including an insect-toxic heptelidic acid chlorohydrin were isolated from cultures of balsum fir needle endophyte *Phyllosticta* sp. strain 76 (Calhoun et. al., 1992). Two antimicrobial and algicidal compounds (-)-mycorrhizin A and (+)-cryptosporiopsin, were isolated from cultures of tree endophytes *Pezicula* sp. (-)-mycorrhizin A and (+)-cryptosporiopsin possess sesquiterpenes skeletons, the latter being partly degraded (Schulz et al. 1995).

2.2.12 Others metabolites

A new antifungal pentaketide was recently characterized from a *Fusarium* sp., an endophytic fungus living in the interior part of *Selaginella pallescens* stem (Brady et al., 2000). Sesquoiatones A-F and sesquoiamonascins A-D novel anticancer metabolites were isolated from an endophytic fungus Aspergillus parasiticus of redwood (Stierle, et. al. 1999, Stierle, et al., 2001 and Stierle, et al., 2003). The biological activities, sources, chemical compounds of secondary metabolites from fungal endophyte were summarized in Appendix A.

2.3 Fungal endophyte Metabolites

2.3.1 Metabolites of *Lasiodiplodia theobromae*

Lasiodiplodia theobromae (or the synonymous *Botryodiplodia theobromae*) is a fungus whose culture filtrate inhibits the growth of higher plants, and produces various organic metabolites, such as jasmonic acid, which has strong potato tuber inducing activity. Previous studies with *L. theobromae* IFO 31059 afforded some some biologically active compounds, such as jasmonic acid, mellein, theobroxide, 5-oxlasiodiplodin and 5-hydroxylasiodiplodins. However, the bioassay results showed that there were still active functions left uninvestigated which might yield biologically active compounds. As a result of successive studies, three previously unreported hydroxylasioplodins: (3*R*),(4*S*)-4-hydroxylasiodiplodin, (3*R*),(6*R*)-6-hydroxy-de-*O*-methyllasiodiplodin and (3*R*),(5*R*)-5-hydroxy-de-*O*-methyllasiodiplodin were obtained (Aldridge et al., 1967, Matsuura et al., 1998 and Nakamori et al., 1994).

2.3.2 Metabolites of *Emericella variecolor* (*Aspergillus variecolor*)

Xanthone and dibenzoxepin metabolites of a number of variant strains of *A. variecolor*, a biogenetically unrelated metabolite, $C_{12}H_{12}O_4$, was isolated from strain IMI 53749. Initially with arugosin C and minor amounts of arugosins A and B. In later cultures it was formed in decreasing quantities and finally production ceased altogether. At the same time the amount of arugosin C also decreased and was replaced by a mixture by a mixture of shamixanthone, epishamixanthone, 25-*O*-methylarugosin A, arugosin D, and sterigmatocystin. The biosynthesis of the major secondary metabolites

by examining the co-occurance of minor metabolites or derived from them on pathways. The minor metabolites of nine strains of *A.variecolor* in an attempt to throw light on the biogenesis of these and the relates compounds arugosins A-C. The metabolites from variant strains of *A. variecolor* are shown shamixanthone, tajixanthone, tajixanthone hydrate, variecoxanthones A-C, 25-*O*-methylarugosin A-D (Chexal, et al 1974 and 1975 and Holker et al., 1974).

Recently, increased interest in the chemistry of fungi isolated from the marine environment has been documented. Marine fungi are interesting orgnisms from an ecological point of view, because they are serious pathogens in the marine environment. Furthermore, since many can source. During their studies on the chemistry and biology of fungi, investigation of a marine strain of the fungus *Emericella variecolor*, isolated from a sponge collected in Venezuelan waters of the Caribbean Sea. *E. variecolor* (Berk and Br.) Thom and Raper. From different terrestrial strains of *A. variecolor* terrein, 2-methoxy-6(3,4-dihydroxyhepta-1,5-dienyl)benzyl alcohol, 4,7-dimethoxy-5-methylcoumarin and two sesterterpenoids (variecolin and astellatol) as well as numerous xanthones and meroterpenoids of mixed polyketide and terpenoid origins, have been isolated. Moreover in a terrestrial strain of *E. variecolor*, asteltoxin has been found (Malmstrom et al., 2002).

Aspergillus variecolor (*syn. A. stellatus*) has proved to be a rich source of secondary metabolites of mixed polyketide and terpenoid origins. These include large families of xanthones and meroterpenoids whose structures and biosyntheses have been subject to extensive studies. Astellatol is formed via cyclisation and rearrangement of geranylfarnesyl pyrophosphate, so confirming its sesterterpenoid origin (Simpson, et al., 1994).

The sesterterpenoids are the least common family of terpenoids, although they have been isolated from a wide range of sources: fungi, lichens, plants, marine oranisms and insects. It is noteworthy that another sesterterpenoid metabolite, stellatic acid, has also been isolated from A. stellatus. This metabolite is also present in the astellatol-

producing strain, but it is clearly formed by a different mode of initial cyclisation of geranylfanesyl pyrophosphate. The tricyclic carbocation is also implicated in retigeranic acid biosynthesis in which it is subsequently cyclised to form the pentacyclic skelaton (Qureshi et al., 1980 and Simpson, 1994).

2.4 Botanical aspects of *Croton oblongifolius*

Croton Oblongifolius (Plao Yai) classified plant arranged in the Euphorbiaceae family, which have many species widely distributed in Thailand. Plao Yai is scattered around all parts of evergreen forests, deciduous forests and the groves or brushwood. *Croton oblongifolius* is a medium sized medicinal plant. Its calyx and ovary are clothed with minute orbicular silvery scales. The magnitudes of leaves between 5.6-12.0 to 13.0-24.0 cm. The flat-edged leaf is oblong-lnaceolate. The flower are pale yellowish-green and solitary in the axials of minute bracts on long erect racemes. The male flowers locate in the upper part of the raceme and the female in the lower part. Male flowers are slender and have the length of pedicles of 4.0 mm. Calyx is more than 6.0 mm long and segments are ovate, obtuse and more than 2.5 mm long. Petals are 3.0 mm long, elliptic -lanceolate and woolly. The twelve stamens are inflexed in bud and the length of filaments are 3.0 mm. Infemals flowers, the pedicles are short and stout. Its sepals are more acute than in the male with densely ciliated margins. Diameter of fruit is less than 1.3 cm, slightly 3-lobed and clothed with small orbicular scales. In each fruit, the member of seeds are eight which are 6.0 mm long rounged and quite smooth on the back (เสงี่ยม 2502, เต็ม 2523 and ลีนา 2530). The picture of stem, leaves, flowers of *Croton oblongifolius* are shown in Figure 2.9.

Croton oblongifolius (Euphorbiaceae) has been used as a traditional medicine for many applications such as for dysmenorrhea, as a purgative, and to treat dyspepsia and dysenteria. Moreover, this plant had been used as folk-medicine in conjunction with *Croton sublyratus* to treat gastric cancers.

Figure 2.9 Leaves, flowers and stems of *Croton oblongifolius*

2.4.1 The Chemical constituents of *Croton oblongifolius*.

Roengsumran, et al., 1999 obtained crotocembraneic acid, neocrotocembraneic acid, neocembranal from hexane crude extract of stem bark of *C. oblongifolius* by silica gel column chromatography, eluted with hexane. A new cembranoid diterpene, neocrotocembranal inhibited platelet aggregation induced by thrombin, with an IC_{50} value of 47.12 μg/ml, and exhibited cytotoxicity against P-388 cells in vitro, with an IC_{50} value of 6.48 μg/ml.

Roengsumran et al., 2001 obtained three labdane diterpenoids, 2-acetoxy-3-hydroxy-labda-8(17),12(E)-14-triene, 3-acetoxy-2-hydroxy-labda-8(17),12(E)-14-triene and 2,3-dihydroxy-labda-8(17),12(E),14-triene from stem bark of Croton oblongifolius. Their structures estrablished for cytotoxicity against various human tumor cell lines.

Roengsumran et al., 2001 obtained a new furoclerodane, croblongifolin, together with one known clerodane, crovatin and one known labdane, nidorellol from the stem bark of *C. oblongifolius*. Croblongifolin showed a significant cytotoxicity against various human tumor cell lines including HEP-G2, SW620, CHAGO, KATO3 and BT474.

Roengsumran et al., 2004 obtained three new halimane-type diterpenoids, crotohalimaneic acid, crotohalimoneic acid and 12-benzoyloxycrotohalimaneic acid from the stem bark of *C. oblongifolius*. Crotohalimaneic acid and crotohalimoneic acid showed non-specific strong cytotoxicity against a panel of human tumor cell lines; whereas 12-benzoyloxycrotohalimaneic acid was inactive.

Roengsumran et al., 1998 and 1999 obtained cembranoid diterpenes, namely, crotocembraneic acid, neocrotocembraneic acid, poilaneic acid, as well as their synthetic derivatives including methyl crotocembraneate, crotocembranol, crotocembranal, methyl poilaneate, poilaneol and poilanal, were evaluated for their inhibitory activity against cAMP phosphodiesterase. Cembranoids with carboxylic acid functional groups showed higher inhibitory activity than those with other functionalities in the following order carboxylic acid, hydroxyl, methyl ester and aldehyde.

Roengsumran et al., 1999 obtained four new labdane diterpenoids, labda-7,12 (E),14-triene-17-al, labda-7,12(E),14-triene-17-ol, labda-7,12(E),14-triene-17-oic acid, from the stem bark of *C. oblongifolius*. The structure of these compounds were established by spectroscopic data and chemical transformation.

CHAPTER III

EXPERIMENTS

3.1 Instruments and equipments

3.1.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

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

3.1.2 Nuclear Magnetic Resonance Spectrometry (NMR)

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

3.1.3 Mass Spectrometry (MS)

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV.

3.1.4 UV-Vis spectrometry

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

3.1.5. Polarimetry

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

3.1.6. Electrothermometer

Melting point were measured on a Electrothermal 9100.

3.2 Chemicals

3.2.1 Solvents

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

3.2.2 Other chemicals

3.2.2.1 Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) was used as adsorbent for column chromatography.

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

3.2.2.3 Merck's silica gel $60GF_{254}$. 1.07731.1000 were applied as adsorbent for preparative TLC.

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

3.3 Culture media

Culture media used for culturvation of endophytic fungi were potato dextrose agar (PDA) and malt extract agar (MEA).

The medium for growing bacteria was nutrient agar (NA).

3.4 Sample collection

Leaves, twigs and barks of *Croton oblongifolius* trees were collected in June 22nd, 2002, Amphur Panomsarakam (N 13^{o} 37' 52.9'' E 101^{o} 18' 22.7), Chachoengsao Province, Thailand.

3.5 Fungal isolation and culture of endophytic fungi

Leaves, petriole of leaves, twigs and barks of *Croton oblongifolius* were cleaned with tap water and 70% ethanol to eliminate epiphyllous microorganisms from endophyte culture. Leaves were cut into 5x 5 mm^2 speciments and petrioles were cut

approximately 5 mm in length and barks cut into 1 x 1 cm^2 speciments. The cut speciments were surface sterilized using the protocol described by Schulz et al., 1993. The sample were immersed in sequence of 95% ethanol for 1 minutes, 10% sodium hypochlorite solution for 5 minutes, 95% ethanol for 30 seconds and then before being rinsed in sterile distilled water. For twig and bark sample were surface sterilized with saturated sodium hypochlorite for 5 min and rinsed twice with sterilize distilled water for 1 min. The sterilized speciments were placed on potato dextrose agar (PDA) medium in petri dishes and incubated at room temperature. Fungal colonies development eyes. were observed daily by morphological were subcultured to new media to obtain were cultured. The purity of isolated fungal mycelium were checked under light microscope.

3.6 Identification of endophytic fungi

3.6.1 Preparation specimen for light microscope

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

3.6.2 Preparation specimen for scanning electron microscope

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

3.7 Indentication of endophytic fungi using traditional techniques

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

3.7.1 DNA extraction

Genomic DNA was prepared from the mycelial sample by homogenization in 1.5 ml tubes with a FastPrep FP120 homogenizer (Savant, faxmingdale, NY, USA) and followed by extraction with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999). Fungal DNA extract was applied in CTAB buffer (2% CTAB, 0.1 M Tris-HCl (pH8.0), 20 mM EDTA (pH8.0), 1.4M NaCl and 0.5% 2-mercaptoethanol) at 65 ^{o}C for 1 h, extracted with phenol-chloroform-isoamyl alcohol (25:24:1,v/v), then extracted twice with phenol-chloroform-isoamyl alcohol mixture (25:24:1, v/v) . Fungal DNA was precipitated by isopropanol and centrifuged at 8000 rpm for 5 min. The fungal DNA was dissolved in a solution of 10 µl TE buffer (10mM Tris-HCl (pH8.0) and 1mM EDTA) and kept at -30 ^{o}C for further study.

3.7.2 ITS amplification

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

3.7.3 DNA Sequencing

ITS_{1f-4} regions were amplified from the representative sample of isolated endophytic fungus. Amplified ITS_{1f-4} fragments were cloned using pT7 Blue vectors (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* strain XL1-Blue MRF. Ligation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Texas Red (Hitachi) in and SQ-5500E sequencer (Kanchanaprayudh *et. al*., 2003).

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

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

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

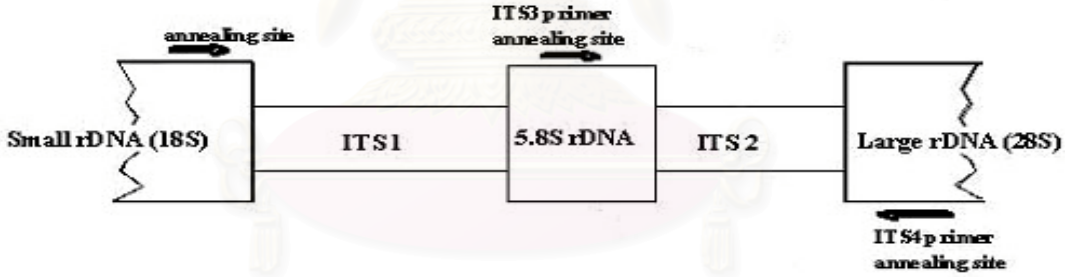


Figure 3.1 ITS regions of rDNA (Kanchanaprayudh *et. al*., 2003)

3.8 Metabolites production of endophytic fungi

Fifty five endophytic fungal isolates were selected from eighty four isolates endophytic fungi and cultured in 100 ml of potato dextrose broth in 250 ml flask. The endophytic fungi stock were cultured in PDA at room temperature for 7 days. The stock cultures were cut using ϕ 8 mm cork hole borrer, transferred into the potato dextrose broth (PDB) 100 ml of PDB in 250 ml flask and then placed statically at room temperature for 2 months. Methanol were added into cultured broth and then methanolic

fermentation broths were filtered though filter paper Whatman No.1. The filtrate were evaporated to remove methanol. The filtered broths were evaporated in *vacuo* and then lyophilized to dryness. The broth crudes were extracted with methanol (20 ml x 2). The methanol extracts were investigated chemical constituents by TLC. The TLC results were monitored by UV (254 and 365 nm), iodine vapor and vanillin/H_2SO_4 reagent.

3.9 Antimicrobial activity test

Microorganisms used in antimicrobial activity assay were *Bacillus subtilis* ATTC 6633, *Staphyllococcus aureus* ATTC 25923, *Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853, *Saccharomyces* cerevisiae, and *Candida albicans* ATTC 10231.

Broth crudes (5 mg) from (**3.8**) was dissolved in 1 ml of sterile water and shaked to mix. Microorganisms test were cultured in the PDB and NA medium for yeast and bacteria, respectively, for 18-24 h and determined terbidity compared with Standard McFarland No. 0.5. The microorganisms were swab on the layer of culture plate. Cork borer made 7 mm diameter hole on the agar, filled with 100 μl of the extract broth solution in sterile water in the each hole and incubated at 37 ^{o}C for bacteria and room temperature for yeast for 18-24 hr, followed by observation the clear zone.

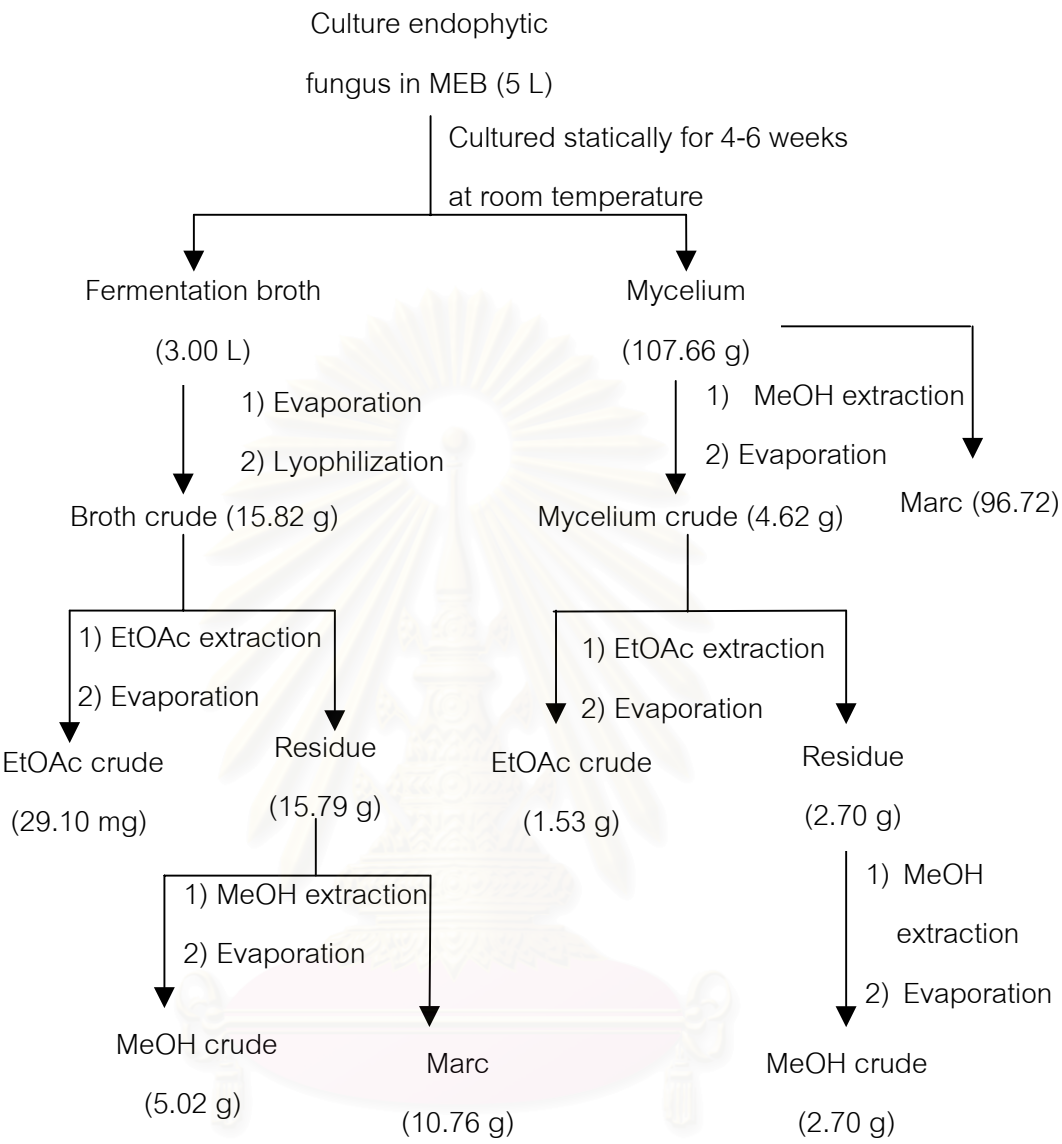
3.10 Fungal endophyte metabolites of strain CsLm 08

Stock culture of endophytic fungus strain CsLm 008 cultured in the plates at room temperature for 7 days were cut by ϕ 8 mm cork hole borrer and inoculated into 100 ml of PDB in 250 ml flask (x 50). The endophytic fungus were cultured atatically at room temperature for 2 months. Fermentation broth and mycelium were filtered through a filter paper Whatman no.1.

3.10.1 Extraction procedure of endophytic fungus strain CsLm 08

Endophytic fungi strain CsLm 08, cultured in Potato dextrose broth (5 L) for 2 months at room temperature, was filtered through filter paper Whatman No. 1 to obtain the fermentation broth (3 L) and mycelium (107.66 g of wet weight). Methanol (500 ml) was added into the fermentation broth for preservated and evaporated by rotary evaporator *in vacuo* to remove methanol and partial water. Then the fermentation broth was lyophilized to give a residue as a dark brown solid (15.82 g). The residue was extracted with ethyl acetate (500 ml x 10) in ultrasonic bath. The ethyl acetate extract was evaporated *in vacuo* to obtain a dark brown solid (29 mg). The remaining residue (15.79 g) was further extracted with methanol (500 ml x 10) in ultrasonic bath. After evaporating methanol extract (5.02 g) was obtained a residue (10.76 g). The extraction procedure and the results are shown in Scheme 3.1.

The mycelium (107.66 g of wet weight) was extracted with methanol (500 ml x 10) and then evaporated the solvent to give a dark brown solid (4.62 g). The dark brown solid was extracted with ethyl acetate (500 ml x 10) and followed by extraction with methanol (500 ml x 10). After removal of the solvent, (1.53 g) of ethyl acetate crude extract was obtained and the methanol crude residue (2.70 g) was remained. The extraction procedure was summarized in Scheme 3.1.



Scheme 3.1 Extraction of fermentation broth and mycelium of endophytic fungus strain CsLm 008

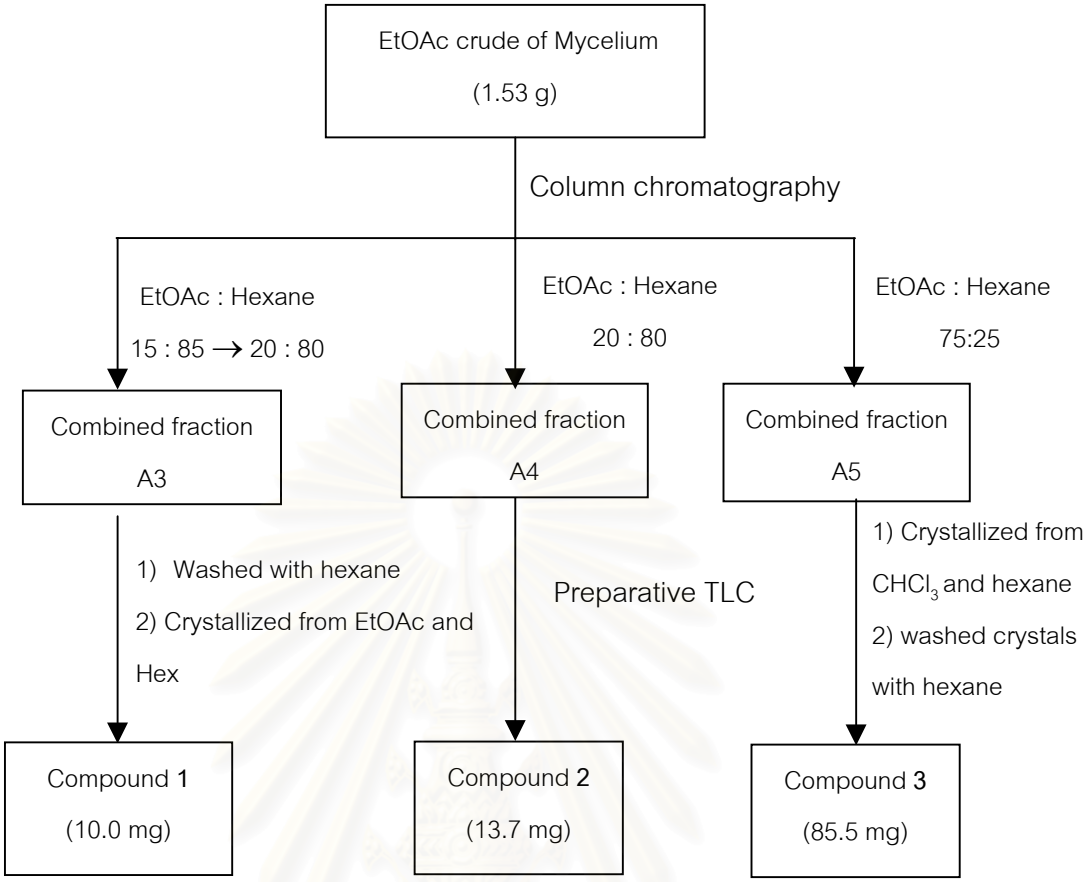
3.10.2 Isolation of metabolites of mycelium of endophytic fungi strain CsLm 08

The ethyl acetate crude from mycelium (1.53 g) was subjected to column chromatography (silica gel, 300 g) using eluents of increasing polarity from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH, MeOH.

The results from the separation and purification of ethyl actate crude was presented in Table 3.1 and Scheme 3.2

Table 3.1 Isolation of ethyl acetate crude of mycelium from the strain CsLm 08

Combined fraction	Eluents (%)	Fraction no.	Appearance	Weight (mg)
	EtOAc : Hex			
A1	5 : 95 →15 : 85	1-51	Yellow liquid	150
A2	15 : 85	52-60	Orange liquid	13
A3	15 : 85 → 20 : 80	61-63	Yellow solid	13
A4	20 : 80	64-75	Yellow solid	40
A5	20 : 80	76-85	Yellow solid	145
A6	20 : 80 → 25 : 75	86-98	Yellow solid	64
A7	25 : 75 → 30 : 70	99-138	yellow solid	155
A8	30 : 70 → 70 : 30	139-146	Light brown liquid	58
A9	70 : 30 → 80 : 20	147-166	Brown liquid	90
A10	EtOAc : Hex (10 : 90) → MeOH : EtOAc (40 : 60)	167-173	Brown liquid	98
A11	MeOH : EtOAc (40 : 60)	174-185	Brown oil	312
A12	MeOH : EtOAc (40 : 60) → MeOH : EtOAc (75 : 25)	186-189	Light yellow liquid	465
A 13	MeOH : EtOAc (100 : 0)	196-240	Light yellow liquid	-



Scheme 3.2 Isolation procedure of ethyl acetate crude from mycelium of endophytic fungus strain CsLm 08

The yellow solid of the combined fraction A3 (13 mg) was washed with hexane (10 ml x 3), and followed by crystallization from hexane and ethyl acetate to afford compound **1** as a white solid (10 mg);

m.p. 149-150 ^{o}C; $[\alpha]_D^{20}$ -24 ($CHCl_3$, *c* 0.1); λ_{max} ($CHCl_3$) (ε) 240 (2388) nm;

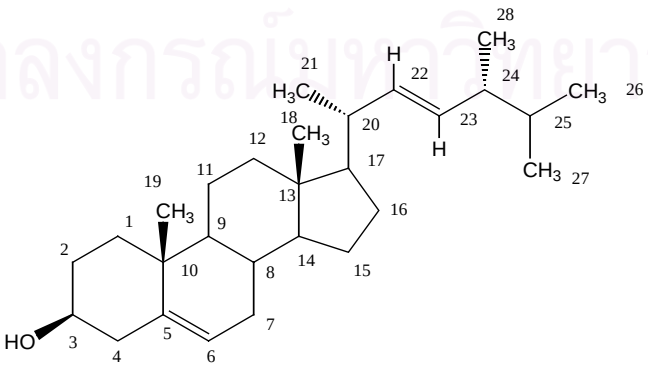
[Lit. (Kavanau, 1965); m.p. 149-151 ^{o}C; $[\alpha]_D$: -66 ($CHCl_3$, *c* 1.47)];

ν_{max} (KBr) 3431 (br.m), 2949 and 2863 (s), 1660 (w), 1458 and 1372 (m), 1049 (m) and 964 (m) cm^{-1};

MS (EI 70 eV) *m/z* 398 [M^+, 29%], 300 (25), 271 (28), 255 (35), 213 (16), 159 (23), 145 (28), 133 (27), 105 (30), 91 (48), 79 (50), 69 (68) and 55 (100);

δ_H ($CDCl_3$, 400 MHz) 5.38 (1H, d, *J* = 4.8 Hz, 6-H), 5.15-5.27 (2H, qt, *J* = 7.2 and 13.2 Hz, 22-H and 23-H), 3.55 (1H, m, 3-H), 2.32 (2H, m, 7 and 11-H), 2.28 (2H, m, 7 and 11-H), 2.04 (1H, m, 20-H), 2.01 (1H, m, 12-H), 2.00 (1H, m, 2-H), 1.88 (1H, m, 4-H), 1.87 (1H, m, 24-H), 1.85 (1H, m, 15-H), 1.69 (1H, m, 16-H), 1.57 (1H, m, 1-H), 1.55 (1H, m, 2-H), 1.51 (1H, m, 25-H), 1.48 (1H, m, 8-H), 1.24 (1H, m, 16-H), 1.23 (1H, m, 12-H), 1.13 (1H, m, 17-H), 1.10 (1H, m, 4-H), 1.05 (1H, m, 1 and 19-H), 1.04 (3H, s, 21-H), 1.00 (3H, m, 14-H), 0.94 (1H, m, 9-H), 0.93 (3H, d, *J* = 7.2 Hz, 28-H), 0.86 (1H, d, *J* = 6.4 Hz, 26-H), 0.85 (3H, d, J = 6.8 Hz, 27-H), 0.72 (3H, s, 18-H) ppm;

δ_C ($CDCl_3$, 100 MHz) 140.75 (5-C), 135.85 (22-CH), 131.73 (23-CH), 121.75 (6-CH), 71.84 (3-CH), 56.85 (14-CH), 56.01 (17-CH), 50.15 (9-CH), 42.82 (24-CH, 13-C), 42.29 (7 and 11-CH_2), 40.19 (20-CH), 39.68 (12-CH_2), 37.26 (4-CH_2), 36.53 (10 and 13-C), 33.11 (25-CH), 31.91 (2-CH), 31.65 (15-CH_2), 28.57 (16-CH_2), 24.30 (1-CH_2), 21.08 (8-CH_2), 20.98 (19-CH_2), 19.98 (26-CH_3), 19.66 (27-CH_3), 19.42 (21-CH_3), 17.64 (28-CH_3) and 12.09 (18-CH_3) ppm.



Compound **1**

The yellow solid of the fraction A4 (40 mg) was isolated by preparative thin layer chromatography using 20% ethyl acetate in hexane as eluent to give a yellow solid (R_f = 0.48) and the solid was further washed with hexane to afford compound **2** as a amorphous white solid (14 mg);

m.p. 84.0-86.0 ^{o}C, λ_{max} ($CHCl_3$) (ε) 242 (1251) nm;

ν_{max} (KBr) 3443 (br.s), 2918 (s), 2851 (s), 1715 (s), 1559 (m), 1462 (m), 1411 (m), 1372 (w), 968 (w), 894 (w) and 711 (w) cm^{-1};

MS (EI 70 eV) *m/z* 372 [$C_{26}H_{52}O_2^+$, 12], 368[$C_{24}H_{48}O_2^+$, 34], 340 (15), 325 (10), 185 (11), 129 (26), 97 (28), 83 (51), 73 (76), 57 (80) and 55 (100);

δ_H ($CDCl_3$, 400 MHz) 0.9 (3H, t) 1.25(m), 1.68 (s) and 2.38 (m) ppm;

δ_C ($CDCl_3$, 100 MHz) 14.0 (CH_3), 22.5 (CH_2), 24.9 (18xCH_2), 29.5 (CH_2), 32.0 (CH_2) 34.0 (CH_2) and 179.5 (1-COOH) ppm.

$CH_3(CH_2)_{22-24}COOH$

Compound **2**

Compound **3** was obtained in the combined fraction A5 (144 mg) from column chromatography using EtOAc : Hex (20 : 80 → 25 : 75) as eluents. The yellow solid of combined fraction A5 was crystallized from chloroform-hexane to afford compound **3** as off-white crystals (85.5 mg);

m.p. 185-186°C; $[\alpha]_D^{20}$ +6 ($CHCl_3$, *c* 0.1) ; λ_{max}($CHCl_3$) (ε) 282 (2570) and 240 (5840) nm;

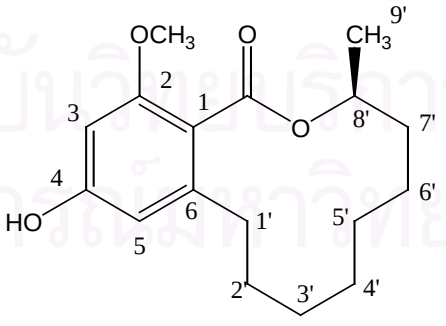
[Lit. (Lee et.al., 1982); m.p. 183-184°C; $[\alpha]_D^{20}$: +5.9 ($CHCl_3$, *c* 1.0)];

ν_{max} (KBr) 3388 (br.m), 2925 (m), 2844 (m), 1692 (s), 1598 (s), 1466 (m), 1427 (m), 1341 (m), 1264 (m), 1197 (m), 1162 (m), 1092 (m) and 847 (m) cm^{-1};

EI-MS *m/z* 292 [M^+, 58 %], 275 (8), 205 (10), 191 (25), 182 (100), 177 (62), 164 (25), 138 (70), 121 (15), 91 (12), 77 (22), 69 (29) and 55 (16);

δ_H ($CDCl_3$, 100 MHz) 6.20 (1H, s, br.s, 3- and 5-H), 5.28 (1H, m, 8'-H), 3.68 (3H, s, OCH_3), 2.62 (1H, m, 1'-H), 2.45 (1H, m, 1'-H), 1.93 (1H, m, 7'-H), 1.66 (1H, m, 7'-H), 1.62 (1H, m, 2'-H), 1.56-1.65 (2H, m, 3'-H), 1.38-1.46 (2H, m, 4'-H), 1.45 (1H, m, 6'-H), 1.40 (1H, m, 2'-H), 1.35 (1H, m, 5'-H), 1.32 (3H, d, 6.4 Hz, 9'-H), 1.28 (2H, m, 6'-H) and 1.26 (2H, m, 5'-H) ppm;

δ_C ($CDCl_3$, 100 MHz) 169.48 (C=O), 157.91 (2-C), 157.80 (4-C), 142.90 (6-C), 116.80 (1-C), 108.36 (5-CH), 96.90 (3-CH), 72.53 (8-CH), 55.67 (OMe), 32.19 (2'-CH_2), 30.28 (1'-CH_2), 29.99 (3'-CH_2), 26.27 (4'-CH_2), 25.37 (5'-CH_2), 24.01 (6'-CH_2), 21.21 (7'-CH_2) and 19.42 (9'-CH_3) ppm.



Compound **3**

3.11 Fungal endophyte metabolites of strain CsPm 09

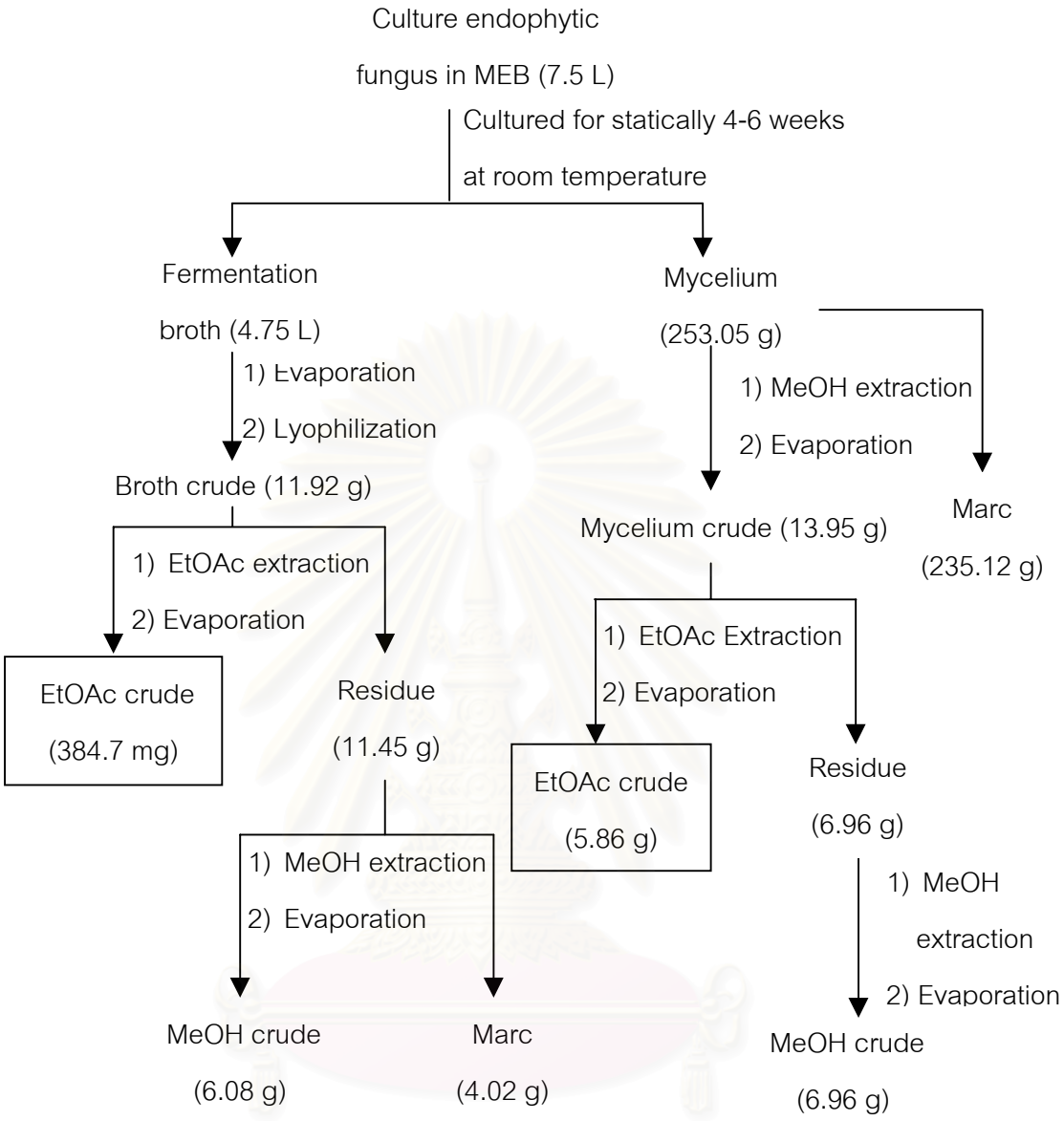
Culture of endophytic fungus strain CsPm 09 in the plates at room temperature for 7 days were cut by ϕ mm 8 cork hole borer and inoculated into 100 ml of MEB in 250 ml flask (x75). The endophytic fungus was cultured at room temperature for 6 weeks. Fermentation broth and mycelium were filtered through filter paper Whatman no.1.

3.11.1 Extraction procedure of metabolites of endophytic fungus strain CsPm 09

To the fermentation broth, methanol (500 ml) was added for preservation and then the solvents were evaporated by rotary evaporator *in vacuo* to give a residue as a dark reddish solid (11.92 g). The residue was extracted with ethyl acetate (500 ml x 10) in ultrasonic bath. The ethyl acetate extract was evaporated *in vacuo* to obtain a dark brown solid (385 mg). The remaining residue (11.45 g) was further extracted with methanol (500 ml x 10) in ultrasonic bath. After evaporating methanol extract (6.08 g) a viscous residue (4.02 g) was obtained. The isolation procedure was shown in Scheme 3.3.

3.11.2 Isolation of fermentation broth of endophytic fungus strain CsPm 09

The mycelium (253 g of wet weight) was extracted with methanol (500 ml x 10). The methanol extract was evaporated to give a myceliuml crude as a dark reddish solid (13.95 g). The dark reddish solid was extracted with ethyl acetate (500 ml x 10). After removal of the solvent, a 5.86 g of ethyl acetate crude was obtained and the mycelium crude (6.96 g) was remained. The extraction procedure was shown in Scheme 3.3.



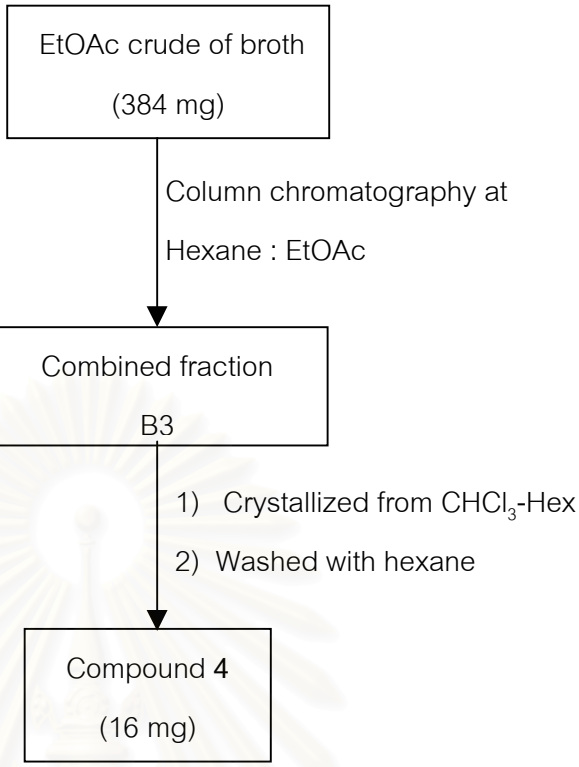
Scheme 3.3 Extraction procedure of fermentation broth and mycelium of endophytic fungus strain CsPm 09

The ethyl acetate crude of the strain CsPm 09 fermentation broth (384.7 mg) was subjected to column chromatography (silica gel, 100 g) and the results were shown in Table 3.2.

The results of isolation was illustrated in Table 3.2 and Scheme 3.4.

Table 3.2 Isolation of ethyl acetate crude of fermentation broth from the strain CsPm 09

Combined fractions	Eluents	Fraction No.	Appearance	Weight (mg)
	EtOAc : Hex			
B1	5 : 95 → 50 : 50	1- 143	Brown liquid	26
B2	50 : 50 → 60 : 40	144-165	Yellow solid	67
B3	60 : 40 → 80 : 20	166-185	light brown solid	45
B4	EtOAc : Hex (80 : 20) → MeOH : EtOAc (30 : 70)	186-242	light brown liquid	80
B5	MeOH : EtOAc (30 : 70)	243-345	light brown liquid	170
B6	MeOH : EtOAc (30 : 70) → AcOH : MeOH (1 : 99)	346-382	light brown liquid	24



Scheme 3.4 Isolation procedure of ethyl acetate crude from fermentation broth of endophytic fungus strain CsPm 09

The combined fraction B3 (45 mg) from column chromatography of the crude of fermentation broth CsPm 09 using EtOAc : Hex (60 : 40 → 80 : 20) as eluents. The combined fraction B3 was crystallized from $CHCl_3$-Hex and then washed the crystals with hexane to afford a white solid (16 mg). Recrystallization of compound **4** from $CHCl_3$-Hex gave colorless crystals (16 mg);

m.p. 128-130 ^{o}C; $[\alpha]_D^{20}$ + 69 ($CHCl_3$, c 0.1); λ_{max} ($CHCl_3$) (ε) 277 (11580) and 235 (12936) nm;

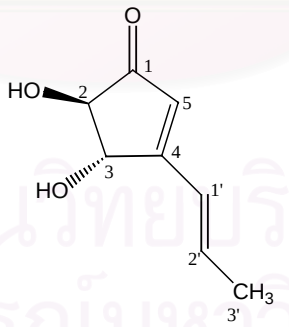
[Lit (Dunn et.al., 1975); $[\alpha]_D^{20}$ +155 (c H_2O); m.p. 121-122 ^{o}C; λ_{max} (EtOH) (ε) 273 (30000) and 345 (125) nm];

ν_{max} (KBr) 3396 (br), 2914 (w), 2848 (w), 1785 (w), 1692 (w), 1629 (s), 1563 (s), 1415 (m), 1330 (m), 1193 (m), 1112 (m), 1084 (m), 960 (m) and 863 (m) cm^{-1};

MS (EI 70 eV) *m/z* 155 [$(M+H)^+$, 78 %], 139 (100), 121 (43), 109 (28), 95 (25), 79 (50), 67 (16) and 65 (17);

δ_H($CDCl_3$, 400 MHz) 6.73 (1H, dq, *J* = 14 and 6.8 Hz, 2'-H), 6.33 (1H, d, 15.6 Hz, 1'-H), 5.95 (1H, s, 2-H), 4.82 (1H, d, 2.4 Hz, 3-H), 4.20 (1H, d, 2.8 Hz, 2-H) and 1.88 (3H, d, 1.2 Hz, 3'-H) ppm;

δ_C ($CDCl_3$, 100 MHz) 202.68 (1-C=O), 168.34 (4-C), 141.57 (2'-CH), 125.07 (1'-CH), 124.94 (5-CH), 81.90 (2-CH), 76.86 (3-CH) and 19.61 (3'-CH_3) ppm.



Compound **4**

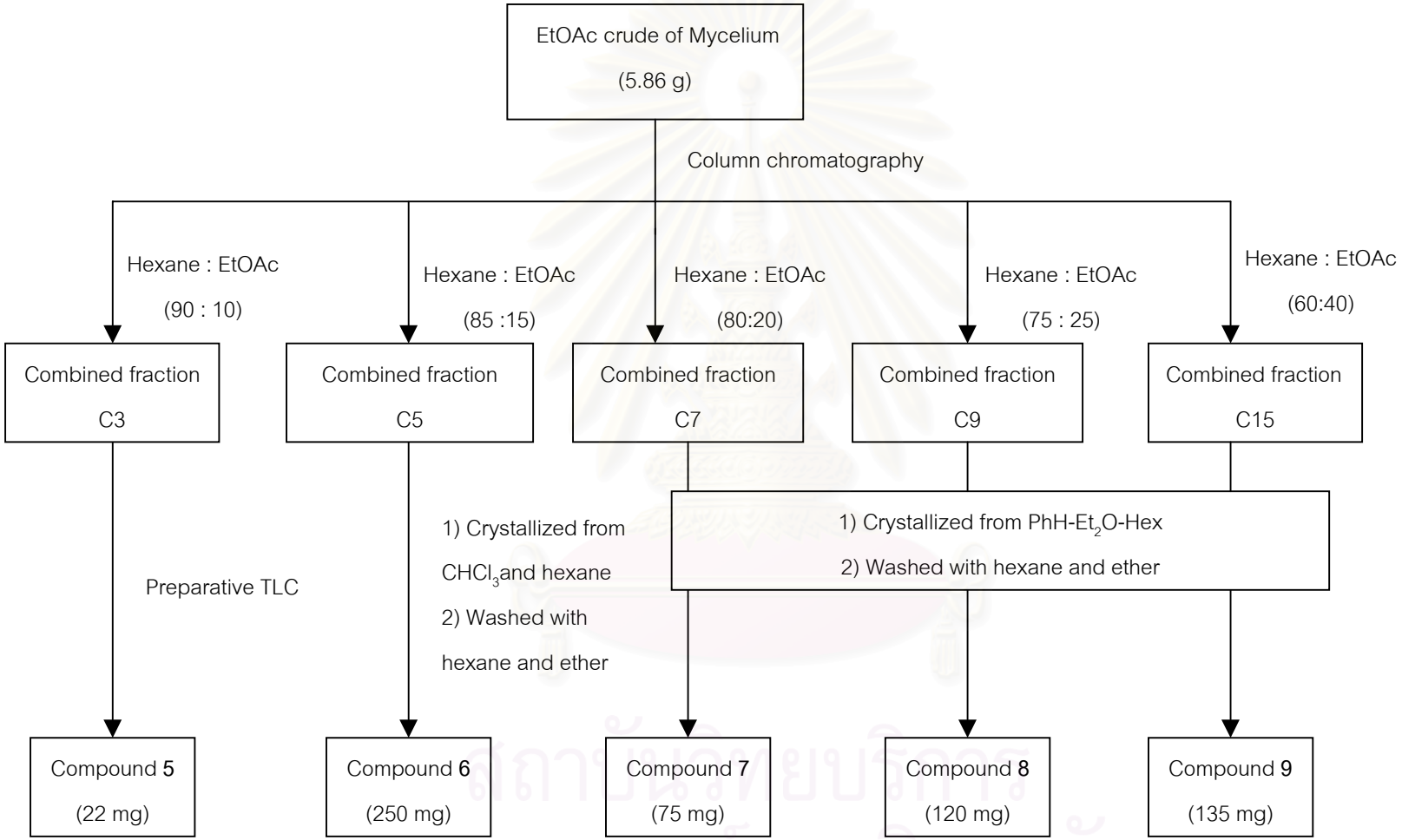
3.11.3 Isolation of mycelium of endophytic fungus strain CsPm 09

The ethyl acetate crude of mycelium (5.86 g) was subjected to column chromatography (silica gel, 300 g) using eluents of increasing polarity of solvents; hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH.

The results from the isolation of ethyl actate crude were presented in Table 3.3 and Scheme 3.5.

Table 3.3 Isolation of ethyl acetate crude of mycelium from the strain CsLm 09

Combined fractions	Eluents	Fraction No.	Appearance	weight (mg)
	EtOAc : Hexane			
C1	5 : 95 → 10 : 90	1-32	Yellow liquid	32
C2	10 : 90	33-44	Orange liquid	139
C3	10 : 90	45-57	Orange solid	131
C4	10 : 90 → 12.5 : 87.5	58-95	Orange solid	164
C5	12.5 : 87.5 → 15 : 85	96-140	Orange solid	533
C6	15 : 85 → 17.5 : 82.5	141-165	Orange and red solids	146
C7	17.5 : 82.5 → 20 : 80	166-195	Orange solid	155
C8	20 : 80 → 22.5 : 77.5	196-235	Orange liquid	194
C9	22.5 : 77.5 → 25 : 75	236-250	Orange solid	343
C10	25 : 75	251-262	Orange and red solids	43
C11	25 : 75 → 27.5 : 72.5	263-310	Orange solid	74
C12	30 : 70	311-340	Orange and red solids	69
C13	30 : 70 → 35 : 65	341-376	Red solids	136
C14	35 : 65 → 40 : 60	377-385	Light yellow solid	52
C15	40 : 60	386-420	Orange solid	281
C16	40 : 60 → 45 : 55	421-428	Orange solid	30
C17	45 : 55 → 50 : 50	429-470	Dark brown solid	90
C18	50 : 50 → 70 : 30	471-520	Dark brown solid	228
C19	EtOAc : Hexane (70 : 30) → 100 : 0	521-600	Black solid	371
C20	AcOH : MeOH (1: 99)	601-640	Black solid	486



Scheme 3.5 Isolation procedure of ethyl acetate crude from mycelium of endophytic fungus strain CsPm 09

The combined fraction C3 was obtained from column chromatography of the EtOAc crude of mycelium CsPm 09 using 10 % ethyl acetate in hexane as eluent. The orange solid of the fraction C3 (130 mg) was subjected to isolation by preparative thin layer chromatography using 10 % EtOAc in hexane as mobile phase to give a yellow solid (R_f 0.40) and then followed by crystallization from $CHCl_3$ and hexane to afford compound **5** as yellow needle crystals (22 mg);

m.p. 139-140 °C; $[\alpha]_D^{20}$ + 16 ($CHCl_3$, c 0.1); λ_{max} ($CHCl_3$) (ε) 240 (19325) 259 (8932), 270 (11368), 278 (12992), 300 (3654) and 397 (2030), nm;

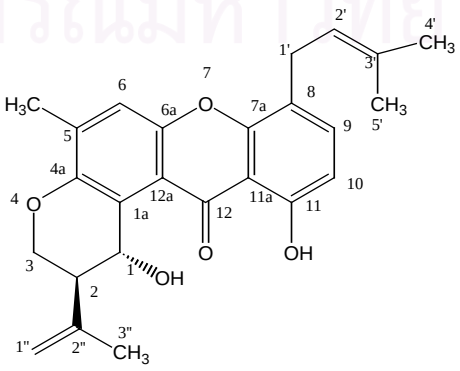
[(Lit (Chexal et al., 1974); m.p. 154-156 °C; $[\alpha]_D^{20}$: +25.2 ($CHCl_3$, c 0.33)];

ν_{max} (KBr) 3447 (br.s), 2921 (w), 2851 (w), 1734 (m), 1645 (m), 1567 (m), 1478 (m), 1419 (m), 1240, 1116, 1022 (w) and 820 (w) cm^{-1};

MS (EI 70 eV) *m/z* 406 [M^+, 20 %], 388 (11), 375 (13), 363 (25), 337 (53), 310 (34), 295 (100), 279 (12), 255 (15), 242 (20) and 67 (24);

δ_H ($CDCl_3$, 400 MHz) 12.63 (1H, s, OH), 7.46 (1H, d, 8.0 Hz, 9-H), 7.32 (1H, s, 6-H), 6.77 (1H, d, 8.4 Hz, 10-H), 5.12 (1H, br.s, OH), 5.43 (1H, s, 1-H), 5.34 (1H, dd, 7.2 and 7.6 Hz, 2'-H), 4.83 (1H, s, 1''-H), 4.61 (1H, s, 1''-H), 4.45 (1H, dd, 2.8 and 11.2 Hz, 3- H), 4.37 (1H, dd, 2.8 and 10.8 Hz, 3-H), 3.52 (2H, dd, 6 and 5.2 Hz, 1'-H), 2.76 (1H, s, 2-H), 2.38 (3H, s, *CH_3*-C5), 1.88 (3H, s, 3''-H), 1.82 (3H, s, 5'-H) and 1.78 (3H, s, 4'-H) ppm;

δ_C ($CDCl_3$, 100 MHz) 184.51 (12-CO), 159.72 (11-C), 152.80 (7a-C), 149.45 (4a-C), 142.61 (2''-C), 138.38 (5-C), 136.56 (9-CH), 133.33 (3'-C), 121.68 (2'-CH), 120.92 (1a-C), 119.38 (6-CH), 118.95 (8-C), 116.89 (12a-CH_2), 112.30 (1''-CH), 109.73 (10-CH), 109.24 (11a -C), 64.56 (3-CH_2), 63.22 (1-CH), 44.93 (2-CH), 27.52 (1'-CH_2), 25.82 (5'-CH_3), 22.60 (3''-CH_3), 17.96 (4'-CH_3) and 17.49 (*CH_3*-C5) ppm.



Compound **5**

The combined fraction C5 was obtained from column chromatography using EtOAc : Hex (12.5 : 87.5 → 15 : 85) as eluents. The orange solid (533.5 mg) of fraction C5 crystallized from chloroform and hexane and washed the crystals with hexane and ether to afford compound **6** as a white solid (250 mg);

m.p. 228-230 °C, $[\alpha]_D^{25}$ +8 ($CHCl_3$, c 0.3); λ_{max} ($CHCl_3$) (ε) 240 (15540) nm;

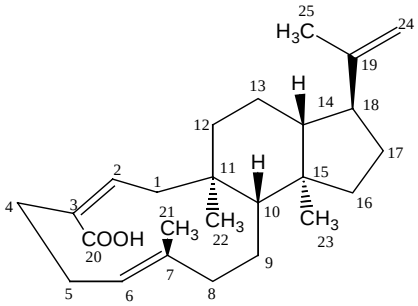
[Lit (Quereshi et. al., 1980), m.p. 228-230 °C; $[\alpha]_D^{25}$ +13.5 ($CHCl_3$, c 0.3); λ_{max} (ethanol) (ε) : 223 (8500) nm];

ν_{max} (KBr) 3435 (br.s), 2945 (w), 2855 (w), 1745 (m), 1649 (m), 1563 (m), 1411 (m), 1264 (w) and 1022 (w) cm^{-1};

MS (EI 70 eV) *m/z* 370 [M^+, 53 %], 355 (23), 327 (32), 257 (12), 246 (15), 203 (29), 189 (100), 175 (24), 161 (40), 147 (52), 135 (40), 121 (45), 107 (60), 95 (50), 93 (48), 81 (42), 67 (28) and 55 (24);

δ_H ($CDCl_3$, 400 MHz) 5.94 (1H, d, 4.4 Hz, 2-H), 4.97 (1H, dd, 4.8 and 4.8 Hz, 6-H), 4.75 (s, 1H, H-24), 4.73 (1H, s, 24-H), 2.89 (1H, d, 12.0 Hz, 4-H), 2.71 (1H, dd, 6.8 and 7.6 Hz, 1-H), 2.31 (1H, m, 5-H), 2.26 (1H, m, 18-H), 2.11 (1H, m, 5-H), 2.09 (1H, m, 1-H), 2.06 (1H, m, 8-H), 1.97 (1H, m, 8-H), 1.90 (1H, m, 16-H), 1.87 (1H, m, 4-H), 1.75 (3H, s, 25-H), 1.57 (1H, m, 13-H), 1.54 (1H, m, 16-H), 1.50 (1H, m, 9-H), 1.48 (1H, m, 17-H), 1.46 (1H, m, 12-H), 1.32 (3H, s, 21-H), 1.31 (1H, m, 13-H), 1.30 (1H, m, 17-H), 1.28 (1H, m, 12-H), 1.26 (1H, m, 10-H), 1.20 (1H, m, 12-H), 1.17 (1H, m, 14-H), 0.90 (s, 3H, 22-H) and 0.84 (3H, s, 23-H) ppm;

δ_C ($CDCl_3$, 100 MHz) 173.19 (20-CO), 150.16 (2-CH), 148.12 (19-C), 138.74 (7C), 125.28 (3-C), 123.68 (6-CH), 109.61 (24-CH_2), 54.43 (14-CH), 49.63 (10-CH), 47.59 (18-CH), 45.85 (15-C), 42.64 (1-CH_2), 41.15 (13-CH_2), 40.37 (8-CH_2), 39.17 (12-CH_2), 37.92 (11-C), 34.85 (4-CH_2), 27.69 (16-CH_2), 27.35 (5-CH_2), 24.13 (22-CH_3), 22.27 (9-CH_2), 21.03 (17-CH_2), 19.98 (25-CH_3), 15.98 (21-CH_3) and 15.57 (23-CH_3) ppm.



Compound **6**

From column chromatography using EtOAc : Hex (17.5 : 82.5 → 20 : 80) as eluents, compound **7** was obtained in the combined fraction C7 (154.8 mg). The orange solid of the combined fraction C7 was crystallized from benzene, ether and hexane. Then the crystals were filtered and washed with hexane to afford compound **7** as yellow needle crystals (75 mg);

m.p. 219-220 ^{o}C; $[\alpha]_D^{20}$ -38 ($CHCl_3$, c 0.1); λ_{max} ($CHCl_3$) (ε) 387 (6916), 295 (9880), 274 (15413), 267 (30628), 253 (31616) and 237 (57304) nm;

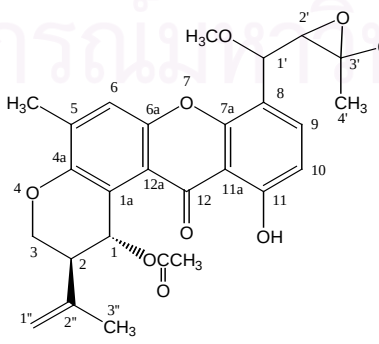
[Lit (Chexel et al., 1975); m.p. 198-200 ^{o}C; $[\alpha]_D$ -19.6 ($CHCl_3$, c 1.0)];

ν_{max} (KBr) 3447 (br.s), 2921 (w), 1746 (m), 1637 (m), 1559 (m), 1470 (w), 1423 (w), 1369 (w), 1236 (m), 1077 (w), 1018 (w) and 828 (w) cm^{-1};

MS (EI 70 eV) *m/z* 494 [M^+, 8 %], 451 (6), 434 (16), 423 (16), 363 (100), 347 (12), 333 (14), 307 (10) and 293 (8);

δ_H ($CDCl_3$, 400 MHz) 13.14 (1H, s, OH), 7.69 (1H, d, 8.4 Hz, 9-H), 7.29(1H, s, 6-H), 6.93 (1H, s, 1-H), 6.86 (1H, d, 8.4 Hz, 10-H), 4.84 (1H, s, 1''-H), 4.79 (1H, s, 1''-H), 4.66 (1H, d, 8.0 Hz, 1'-H), 4.58 (1H, d, 11.2 Hz, , 3-H), 4.34 (1H, dd, 3.2 and 11.2 Hz, 3-H), 3.37 (3H, s, OCH_3), 3.20 (1H, d, 8.0 Hz, 2'-H), 2.75 (1H, s, 2-H), 2.38 (3H, s, 5-CH_3), 2.11 (3H, s, C*H*$_3$COO), 1.92 (3H s, 3''-H), 1.34 (3H, s, 5'-H) and 1.26 (3H, s, 4'-H) ppm;

δ_c ($CDCl_3$, 100 MHz) 183.16 (12-CO), 170.01 (CH_3*C*OO), 162.21 (11-C), 152.54 (7a-C), 151.58 (6a-C), 150.33 (4a-C), 141.44 (2''-C), 137.96 (5-C), 135.10 (9-CH), 120.30 (6-CH), 116.21 (12a-C), 115.49 (8-C), 114.90 (1a-C), 112.80(1''-CH_2), 110.82 (10-CH), 109.11 (11a-C), 76.08 (1'-CH), 66.69 (2'-CH), 65.49 (1-CH), 63.77 (3-CH_2), 57.82 (3'-C), 56.76 (OCH_3), 42.44 (2-CH), 24.82 (4'-CH_3), 22.42 (3''-CH_3), 21.26 (*C*H_3COO), 19.84 (5'-CH_3) and 17.39 (*C*H_3-C5) ppm.



Compound **7**

From column chromatography using EtOAc : Hex (22.5 : 77.5 → 25 : 75) as eluents, compound **8** was obtained in the combine fraction C9 (343.5 mg). The orange solid of the combined fraction C9 was crystallized from benzene, ether and hexane then filtered and washed with hexane to afford compound **8** as yellow needle crystals (120 mg);

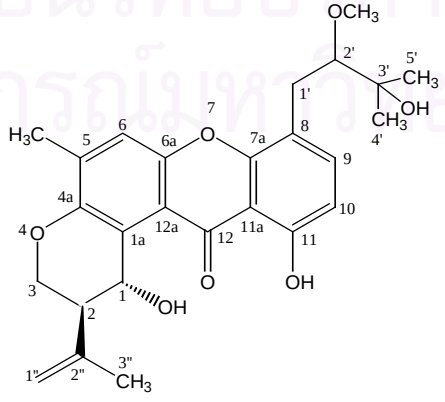
m.p. 197-198 ^{o}C; $[\alpha]_D^{20}$ -105 ($CHCl_3$, c 0.1); λ_{max}($CHCl_3$) (ε): 399 (10170), 300 (18527), 278 (72640), 268 (61744), 258 (49032) and 238 (99880) nm;

ν_{max} (KBr) 3447 (br.s), 2968 and 2925 (w), 1766 (w), 1641 and 1567 (m), 1466 and 1427 (m), 1345, 1240, 1190, 1092 and 1049 (w), 820 and 765 (w) cm^{-1};

MS (EI 70 eV) *m/z* 454 [M^+, 11 %], 424 (10), 385 (10), 363 (11), 333 (28), 255 (11) and 73 (100);

δ_H($CDCl_3$, 400 MHz) 12.64 (1H, s, C_{11}-OH), 7.60 (1H, d, 8.8 Hz, 9-H), 7.26 (1H, d, 16.8 Hz, 6-H), 6.79 (1H, d, 8.0 Hz, 10-H), 5.43 (1H, s, 1-H), 4.82 (1H, s, 1''-H), 4.59 (1H, s, 1''-H), 4.44 (1H, dd, 2.4 and 2.8 Hz, 3-H), 4.37 (1H, dd, 2.8 and 3.2 Hz, 3-H), 3.79 (1H, d, 10.4 Hz, 2''-H), 3.32 (1H, s, 2-H), 3.15 (1H, dd, 1.6 and 1.6 Hz, 1'-H), 2.74 (1H, ddd, 3.6, 3.6 and 2.0 Hz, 2-H), 2.68 (1H, s, 1'-H), 2.48 (1H, s, $C_{3'}$-OH), 2.37 (3H, s, 5-CH_3), 1.87 (3H, s, 3''-H), 1.68 (1H, s, C_1-OH), 1.35 (3H, s, 5'-H) and 1.28 (3H, s, 4'-H);

δ_C ($CDCl_3$, 100 MHz) 184.48 (12-CO), 160.25 (11-C), 153.04 (7a-C), 152.10 (6a-C), 149.51(4a-C), 142.59 (2''-C), 138.39 (5-C), 138.26 (9-CH), 121.11 (1a-C), 119.12 (6-CH), 116.90 (8-C), 116.86 (12a-C), 112.31 (1''-CH_2), 109.93 (10-CH), 109.24 (11a-C), 77.04 (3''-C), 76.51 (2''-CH_2), 64.59 (3-CH_2), 63.22 (1-CH), 49.31 (2''-OCH_3), 44.93 (2-CH), 31.26 (1'-C), 22.57(q, 3''-CH_3), 20.93 (5'-CH_3), 19.29 (4'-CH_3) and 17.46 (*C*H_3-C5).



Compound **8**

From column chromatography using 40 % ethyl acetate in hexane as eluents, compound **9** was obtained in the combined fraction C15. The orange solid was crystallized from benzene, ether and hexane. Then the crystals were filtered and washed with hexane and ether to afford compound **9** as yellow needle crystals;

m.p. 194-195 ^{o}C $[\alpha]_D^{20}$ -76 ($CHCl_3$, c 0.23); λ_{max} ($CHCl_3$) (ε) 237 (45760), 257 (17248), 268 (29920), 278 (24640), 300 (6160) and 399 (2640) nm;

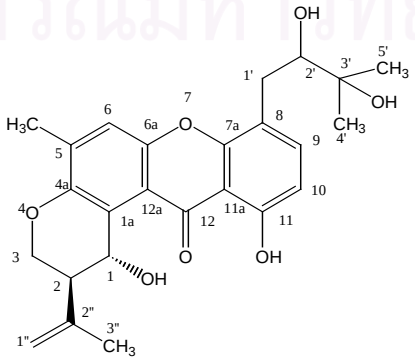
[Lit (Holker et al., 1974) $[\alpha]_D^{20}$: -71.5 ($CHCl_3$, c 2.3); m.p. 195-196 ^{o}C UV ($CHCl_3$) λ_{max} (ε) 241 (3020), 258 (2951), 270 (38018), 274 (40738), 297 (12882) and 393 (8710) nm];

ν_{max} (KBr) 3486 (br), 3073 (w), 2976 (w), 2883 (w), 1797 (w), 1738 (w), 1645 (s), 1571 (s), 1474 (m), 1345 (m), 1244 (m), 1046 (s), 1026 (s), 898 (m) and 820 (m) cm^{-1};

MS (EI 70 eV) *m/z* 440 [M^+, 14 %], 409 (14), 398 (8), 371 (44), 333 (100), 283 (46), 271 (22), 255 (56), 242 (8), 225 (56) and 59 (39);

δ_H($CDCl_3$, 400 MHz) 12.57 (1H, s, C_{11}-OH), 7.52 (1H, d, 8.4 Hz, 9-H), 7.22 (1H, s, 6-H), 6.75 (1H, d, 8.4 Hz, 10-H), 5.37 (1H, s, 1-H), 5.01 (1H, s, C_1-OH) 4.80 (1H, s, 1''-H), 4.56 (1H, s, 1''-H), 4.44 (1H, dd, 2.4 and 2.0 Hz, 3-H), 4.34 (1H, dd, 2.8 and 2.8 Hz, 3-H), 3.74 (1H, d, 10.8 Hz, 2'-H), 3.22 (1H, dd, 1.2 and 1.2 Hz, 1'-H), 2.72 (1H, s.br, 2-H), 2.64 (1H, dd, 10.8 and 10.8 Hz, 1'-H), 2.47 (1H, s, $C_{3'}$-OH), 2.40 (1H, s, 3'-H), 2.34 (3H, s, CH_3-C5), 1.85 (3H, s, 3''-H), 1.42 (3H, s, 4'-H) and 1.36 (3H, s, 5'-H) ppm;

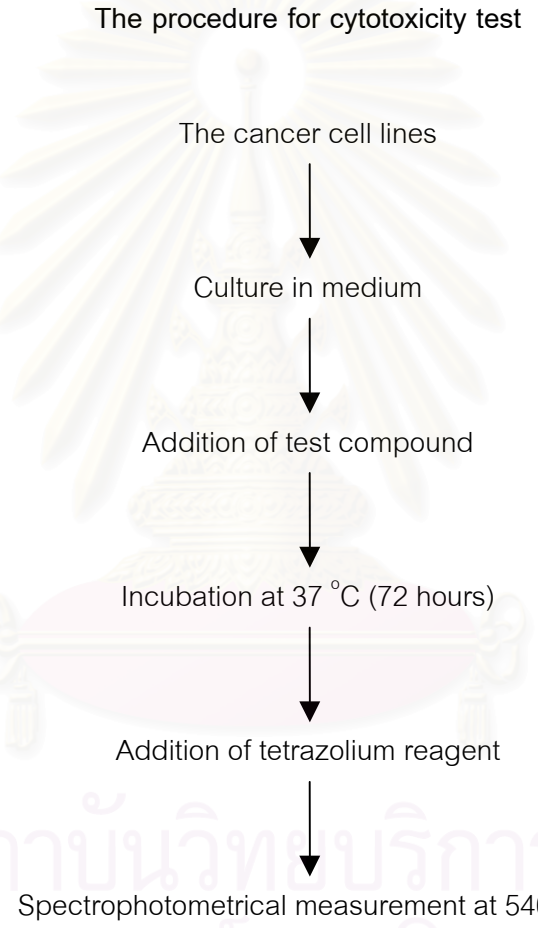
δ_C ($CDCl_3$, 100 MHz) 184.30 (12-CO), 160.32 (11-C), 153.10 (7a-C), 151.96 (6a-C), 149.54 (4a-C), 142.47 (2''-C), 138.57 (5-CH), 138.27 (9-C), 120.82 (1a-C), 119.16 (6-CH), 116.80 (12a-C), 116.26 (8-C), 112.34 (1''-C), 109.93 (10-CH), 109.21 (11a-C), 77.70 (2'-CH), 72.90 (3'-C), 64.49 (3-CH_2), 63.16 (1-CH), 44.80 (2-CH), 32.00 (1'-CH_2), 26.53 (5'-CH_3), 23.59 (4'-CH_3), 22.58 (3''-CH_3) and 17.45 (CH_3-C5) ppm.



Compound **9**

3.12 Cytotoxicity Test

The bioassay of cytotoxic activity against 5 tumor cell cultures *in vitro*, including Hep-G2 (hepatoma), Chago (lung), SW 620 (colon), Kato-3 (gastric) and BT 475 (breast) was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry assay and survival of the cell lines was detected by spectro photometer at 540 nm. The procedure of cytotoxic activity test was shown in Scheme 3.6.



Scheme 3.6 MTT bioassay for cytotoxic activity

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi from *Croton oblongifolius* in Chachoengsao Province

Endophytic fungi were isolated from mature and young leaves, mature and young patriole leaves, twigs and barks of *Croton oblongifolius* collected in Chachoengsao Province to give 84 isolates including 14 isolates from mature leaves, 20 isolates from young leaves, 16 isolates from mature patriole, 18 isolates from young patriole, 12 isolates from twig and 5 isolates from barks (see Table 4.1-4.6). Endophytic fungi were identified by microscopic method, it was found that there are 41 isolates of Mycelia sterilia, 17 isolates of Coelomycetes, 21 isolates of *Phomopsis* sp., 2 isolates of Cladosporium sp., 1 isolate of *Emericella variecolor*, 1 isolate of *Lasiodiplodia theobromae* and 1 isolate of *Tetraploa* sp.

Table 4.1 Characteristics of colony and identification of endophytic fungi from mature leaves in Chachoengsao province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Color pigment production on the media	
CsLm01	Absence of elevation	white	not produce	*Phomopsis* sp.
CsLm02	Absence of elevation	white	not produce	*Phomopsis* sp.
CsLm03	Absence of elevation	grey	not produce	*Phomopsis* sp.
CsLm04	Powdery	white	not produce	*Phomopsis* sp.
CsLm05	Absence of elevation	white	not produce	Mycelia sterilia
CsLm06	Absence of elevation	yellow	not produce	Mycelia sterilia
CsLm07	Absence of elevation	brown	not produce	Mycelia sterilia
CsLm08	Cottony	white	pink	Mycelia sterilia
CsLm09	Absence of elevation	Dark brown	not produce	Mycelia sterilia
CsLm10	Powdery	black	not produce	*Cladosporium* sp.
CsLm11	Powdery	white	not produce	*Phomopsis* sp.
CsLm12	Powdery	yellow	not produce	*Phomopsis* sp.
CsLm13	Powdery	orange and dark	yellow	*Phomopsis* sp.
CsLm14	Powdery	grey	not produce	Mycelia sterilia

Note Cs = Chachoengsoa

Lm = mature leaves

Table 4.2 Characteristics of colony and identification of endophytic fungi from young leaves in Chachoengsao province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Pigment Color production on the media	
CsLy01	Powdery	white	yellow and green	*Phomopsis* sp.
CsLy02	Absence of elevation	grey	not produce	*Phomopsis* sp.
CsLy03	Powdery	white	not produce	*Phomopsis* sp.
CsLy04	Absence of elevation	grey	not produce	Mycelia sterilia
CsLy05	Absence of elevation	dark blown	not produce	Mycelia sterilia
CsLy06	Cottony	white	not produce	*Phomopsis* sp.
CsLy07	Absence of elevation	blown	not produce	Mycelia sterilia
CsLy08	Absence of elevation	grey	not produce	Coelomycetes
CsLy09	Absence of elevation	grey	not produce	Mycelia sterilia
CsLy10	Cottony	black	not produce	Mycelia sterilia
CsLy11	Cottony	black	not produce	*Cladosporium* sp.
CsLy12	Absence of elevation	grey	not produce	Coelomycetes
CsLy13	Absence of elevation	grey	not produce	Coelomycetes
CsLy14	Absence of elevation	yellow	grey	Mycelia sterilia
CsLy15	Cottony	white	not produce	Coelomycetes
CsLy16	Absence of elevation	grey	not produce	Coelomycetes
CsLy17	Absence of elevation	grey	not produce	Coelomycetes
CsLy18	Absence of elevation	dark blown	not produce	Coelomycetes
CsLy19	Absence of elevation	grey	not produce	Coelomycetes
CsLy20	Absence of elevation	dark	not produce	Mycelia sterilia

Note Cs = Chachoengsoa

Ly = young leaves

Table 4.3 Characteristics of colony and identification of endophytic fungi from mature patriole leaves in Chachoengsao province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characterictic	Color of colony	Pigment color production on the media	
CsPm01	Powdery	grey	blown	*Phomopsis* sp.
CsPm02	Powdery	grey	not produce	*Phomopsis* sp.
CsPm03	Powdery	yellow	not produce	Mycelia sterilia
CsPm04	Absence of elevation	yellow	yellow	*Phomopsis* sp.
CsPm05	Absence of elevation	white	not produce	Mycelia sterilia
CsPm06	Absence of elevation	yellow	not produce	Mycelia sterilia
CsPm07	Absence of elevation	yellow and white	not produce	Mycelia sterilia
CsPm08	Cottony	white	grey	Coelomycetes
CsPm09	Absence of elevation	green	not produce	*Aspergillus* sp.
CsPm10	Absence of elevation	white	blown	Coelomycetes
CsPm11	Powdery	yellow	yellow	*Phomopsis* sp.
CsPm12	Powdery	grey	yellow	*Phomopsis* sp.
CsPm13	Powdery	orange	light orange	*Phomopsis* sp.
CsPm14	Powdery	white	not produce	Coelomycetes
CsPm15	Absence of elevation	grey	not produce	Coelomycetes
CsPm16	Absence of elevation	grey	not produce	Mycelia sterilia

Note Cs = Chachoengsoa
Pm = mature patriole of leaves

Table 4.4 Characteristics of colony and identification of endophytic fungi from young patriole leaves in Chachoengsao province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Pigment color production on the media	
CsPy01	Absence of elevation	white	not produce	*Phomopsis* sp.
CsPy02	Absence of elevation	grey	not produce	Coelomycetes
CsPy03	Absence of elevation	white	not produce	*Phomopsis* sp.
CsPy04	Powdery	white	not produce	*Phomopsis* sp.
CsPy05	Absence of elevation	white	not produce	Mycelia sterilia
CsPy06	Cottony	brown	not produce	Mycelia sterilia
CsPy07	Absence of elevation	brown	not produce	Mycelia sterilia
CsPy08	Cottony	green and blue	not produce	*Tetraploa* sp.
CsPy09	Absence of elevation	white	not produce	Coelomycetes
CsPy10	Powdery	White and grey	yellow	*Phomopsis* sp.
CsPy11	Powdery	White and grey	grey	Coelomycetes
CsPy12	Absence of elevation	grey	grey	Coelomycetes
CsPy13	Absence of elevation	white	not produce	Mycelia sterilia
CsPy14	Absence of elevation	Red brown	not produce	Mycelia sterilia
CsPy15	Cottony	light brown	not produce	Mycelia sterilia
CsPy16	Absence of elevation	dark grey	not produce	Mycelia sterilia
CsPy17	Cottony	white	yellow	Mycelia sterilia
CsPy18	Cottony	yellow	yellow	Mycelia sterilia

Note Cs = Chachoengsoa
Py = young patriole of leaves

Table 4.5 Characteristics of colony and identification of endophytic fungi from twig in Chachoengsao province

Isolates	Endophytic fungi characteristics on the PDA medium			Fungal species
	Colony characteristic	Colony color	Pigment color production on the media	
CsTw01	Powdery	white	not produce	Mycelia sterilia
CsTw02	Absence of elevation	white	not produce	Mycelia sterilia
CsTw03	Absence of elevation	white	not produce	Coelomycetes
CsTw04	Powdery	white	not produce	Mycelia sterilia
CsTw05	Cottony	white	grey	Mycelia sterilia
CsTw06	Cottony	dark	not produce	Mycelia sterilia
CsTw07	Cottony	dark	not produce	Mycelia sterilia
CsTw08	Powdery	grey	yellow	Coelomycetes
CsTw09	Absence of elevation	white	not produce	Mycelia sterilia
CsTw10	Absence of elevation	white	not produce	Mycelia sterilia
CsTw11	Absence of elevation	yellow	not produce	*Phomopsis* sp.
CsTw12	Absence of elevation	grey	not produce	Mycelia sterilia

Note Cs = Chachoengsoa
Tw = Twig

Table 4.6 Characteristics of colony and identification of endophytic fungi from bark in Chachoengsao province

Isolates	Endophytic fungi characteristics of on the PDA medium			Fungal species
	Colony characteristic	Colony color	Pigment color production on the media	
CsBa01	Cottony	black	Not produce	Mycelia sterilia
CsBa02	Cottony	black	Not produce	Mycelia sterilia
CsBa03	Cottony	black	Not produce	Mycelia sterilia
CsBa04	Absence of elevation	black	Not produce	Mycelia sterilia
CsBa05	Cottony	black	Not produce	Mycelia sterilia

Note Cs = Chachoengsoa
Ba = bark

4.2 Detailed characters of isolated endophytic fungal species

Cladosporium

Colonies characters were effuse or occasionally punctiform, often olivaceous but also sometimes grey, buff, brown or dark blackish brown, velvety, floccose or hairy. Mycelium immersed and often also superficial. Stroma sometimes present. Setae and hyphopodia absent. Conidiophores macronematous or semimacronematous and sometimes also micronematous; macronematous conidiophores straight or flexuous, mostly unbranched or with branched restricted to the apical region forming a stipe and head, olivaceous brown or brown, smooth or verrucose. Ramo-conidia often present. Conidiogenous cells polyblastic, more or less cylindrical, cicatrized, scars usually prominent. Conidia catenate as a rule but sometimes solitary especially in species with large conidia, often in branched chains, acropleurogenous, simple, cylindrical, doliiform, ellipsoidal, fusiform, ovoid, spherical, often with a distinctly protuberant scar at each end or just at the base, pale to dark olivaceous brown or brown, smooth, verruculose or echinulate, with 0-3 or occasionally more septa (Ellis, 1990) and (see Figure 4.1).

Phomopsis

Mycelium immersed, branched, septate, hyaline to pale brown. Conidiomata eustromatic, immersed, brown to dark brown, septate or aggregated and confluent, globose, ampulliform or applanate, unilocular, multiocular or convoluted, thick-walled; walls of brown, thin-or thick-walled textura angularis, often somewhat darker in the upper rehion, lined by a layer of smaller-called tissue. Ostiole single, or several in complex conidiomata, circular, often papillate. Conidiophores branched and septate at the base and above, occasionally short and only 1-2 septate, more frequenily multiseptate and filiform, hyaline formed from the inner cells of the iocular walls. Conidiogenous cells enteroblastic, phialidic, determinate, integrated, rarely discrete, hyaline, cylindrical, apertures apical on long or short lateral and main branches of the conidiophores, collarette, channel and periclinal thickening minute. Conidia of two basic types, but in some species with intermediates between the two: α-conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end) but sometimes with more guttules, aseptate; β-conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate (Ellis, 1990) and (see Figure 4.2).

Tetraploa

Colonies effuse, brown or dark greyish brown. Mycelium superficial. Conidiophores micronematous, branched and anastomosing to form a network, flexuous, hyaline to pale yellowish brown, often verruculose. Conidiogenous cells monoblastic or occasionally polyblastic, integrated, intercalary, determinate, cylindrical. Conidia solitary, dry, pleurogenous, appendaged, brown, verruculose or verrucose, muriform; in mature conidia there are shallow furrows between 4 (or rarely 3) columns of cells which develop independently, tend to diverge from one another apically and terminate each in a septate setiform appendage (Ellis, 1990) and (see Figure 4.3).

Emericella variecolor

The combination *Aspergillus variecolor*. The type material was re-examined by Patouillard, but neither Berkeley nor Patouillard reported a conidial state.

Aspergillus, as accepted here, is a form genus and a new combination in *Aspergillus* for the conidial state of *Emericella variecolor* is not necessary since *Aspergillus stellatus* Curzi is available, although Curzi used the name for a fungus producing asci, ascospores and conidia.

Accordingly, *Aspergillus stellatus* Curzi is accepted as the correct name for conidial *Emericella variecolor*. *Emericella medias* Chaudhuri & Mathur (1938) is a synonym of *E. variecolor*.

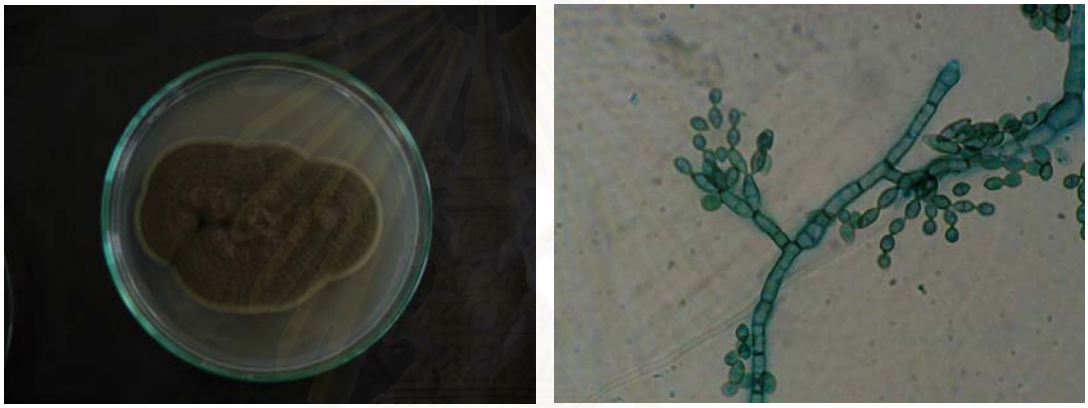
Colonies on Czapek's solution-agar with submerged vegetable mycelium, spreading slowly, producing green heads freely in the centre of the colony, less freely in the outer areas; colour on reverse shades of purple-red. Conidiophores arising directly from submerged hyphae, straight, smooth-walled, cinnamon-brown, mostly 140-200 μ long, 3-5 μ in diameter, broadening gradually above into a hemispherical vesicle about 8-10 μ in diameter. Phialides borne on metulae. Metulae 7-8 x 3-4 μ; phialides 8-9 x 2.5-3 μ. Conidia globose, rugulose, 3-3.5 μ. Conidial heads green, columnar, relatively long, mostly 100-200 μ, occasionally up to 300 μ long, 30-40 μ wide. Hulle cells abundant and similar to those of Aspergillus nidulans.

Perfect state: the earliest name for the perfect state is *Emericella variecolor* Berk. & Br. 1857.

Ascocarps grey, produced in clusters in colony centre and at the margin in some strains, with smaller ascocarps scattered along the intervening thinner areas of the colony, in other strains producing large perithecia abundantly thoughout the colony. Ascocarps when clustered 300-400 μ in diameter, surrounded by a felt of hyphae and hulle cells forming false stalks, giving the structures a pyriform appearance; scattered

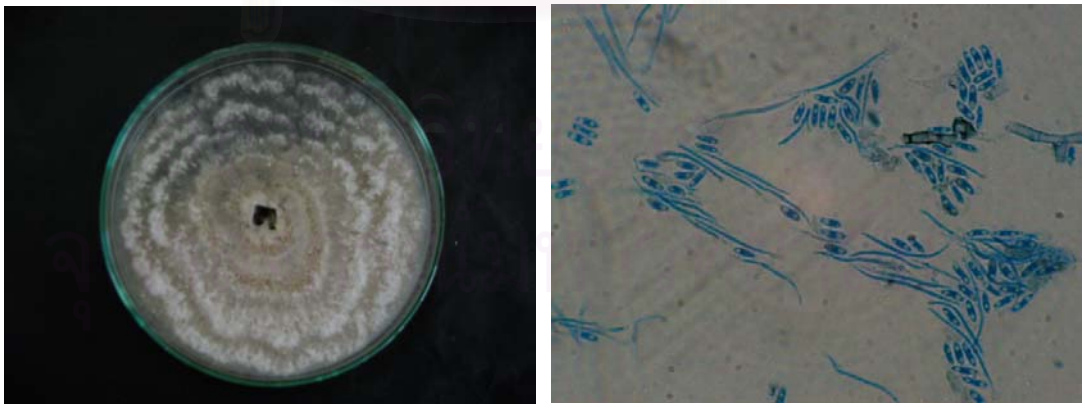
ascocarps much smaller and with envelope of supporting cells often much reduced in mass.

Ascocarp walls when freed from enveloping cells purple-red, brittle, consisting of a single layer of cells. Asci ripening quickly and breaking down to leave the cavity filled with ascospores. Ascospores purple-red, with spore bodies lenticular, 3.6-4 x 2.8-3 μ, with two prominent equatorial crests, up to 3.5 μ in width, pleated and cut to give a stellate appearance to the ascospores (Ellis, 1990) and (see Figure 4.5, 4.6 and 4.7).



(a) (b)

Figure 4.1 *Cladosporium* sp. (a) culture on MEA (7-10 days) (b) conidia (100x)



(a) (b)

Figure 4.2 *Phomopsis* sp. (a) culture on MEA (7-10 days) (b) α and β conidia (100x)

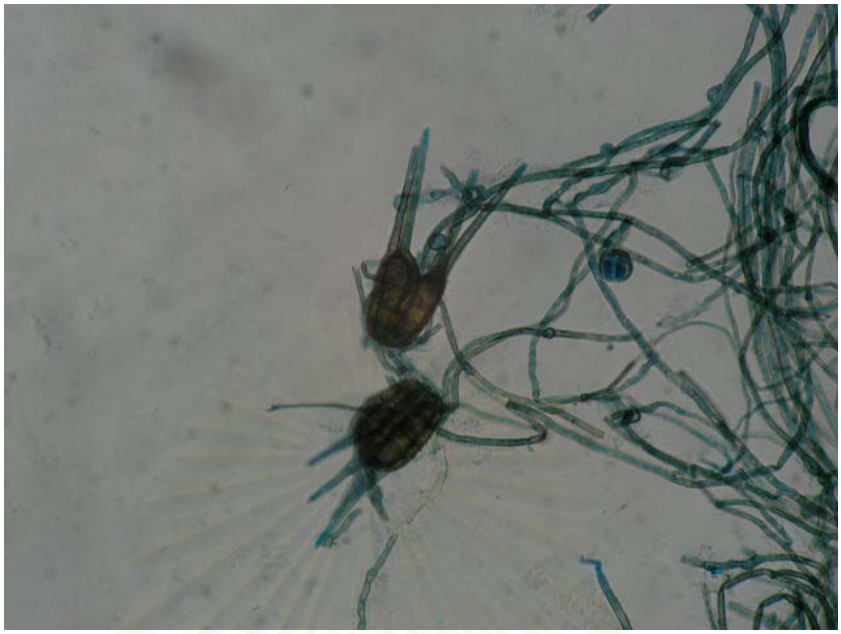
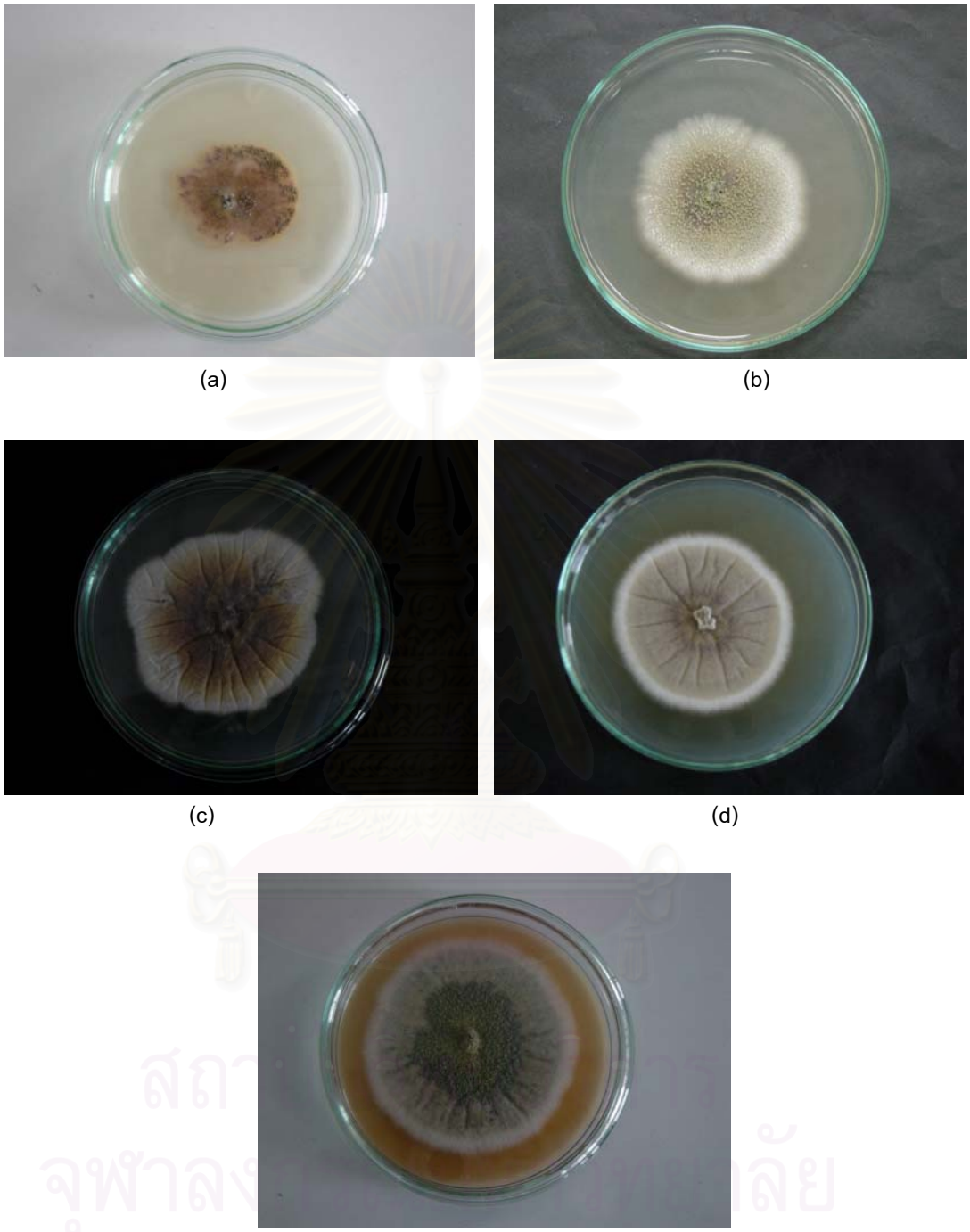


Figure 4.3 Conidia of *Tetraploa* sp. (40x)

(a) (b)

Figure 4.4 *Lasiodiplodia theobromae* (a) Culture on MEA (b) mycelia (40x)

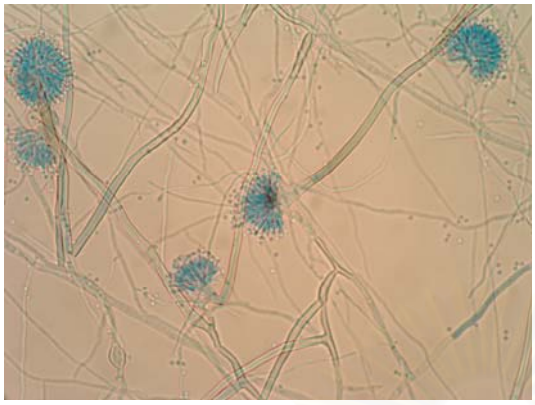


(a) (b)

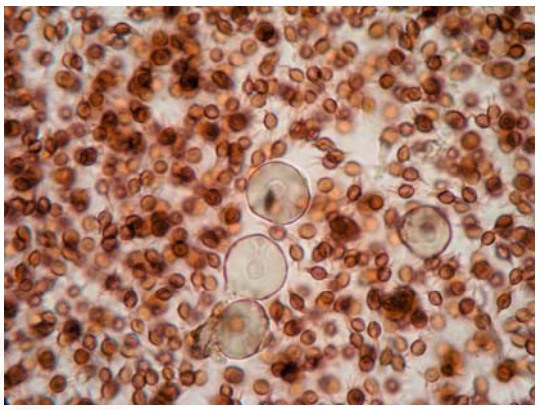
(c) (d)

(e)

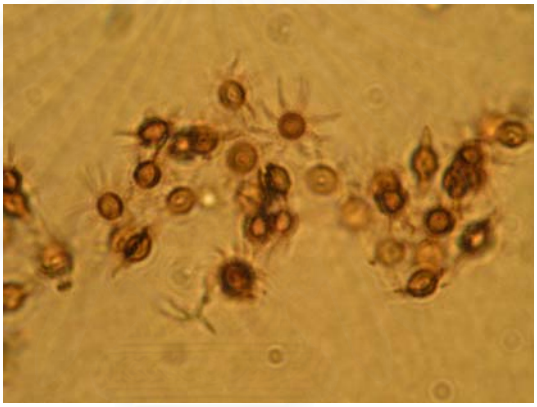
Figure 4.5 *Emericella variecolor* (a) culture on PDA (b) culture on MEA (c) culture on SBA (d) culture on YEA (e) culture on MCzA



(a)

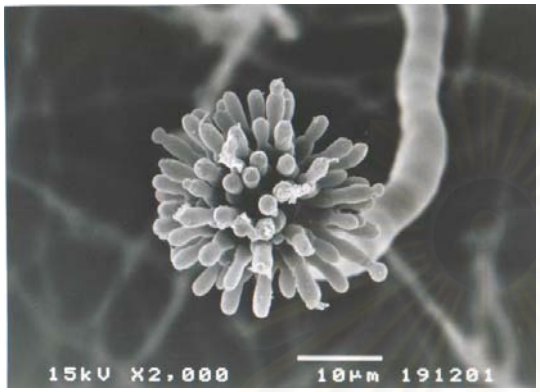


(b)

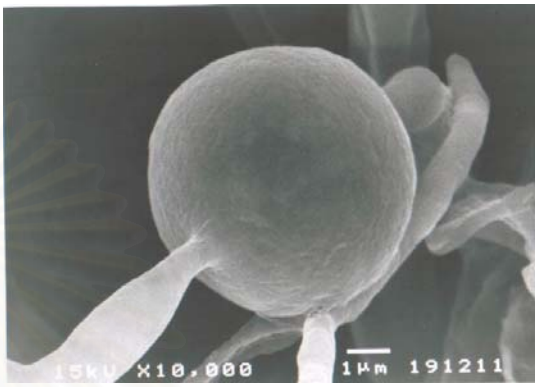


(c)

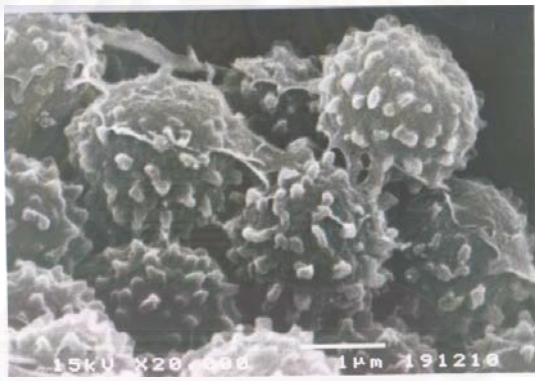
Figure 4.6 Slide culture of *Emericella variecolor* (a) conidia and conidiophores (40x) (b) ascus and ascospores (40x) (c) spike of sexual spores (100x)



(a)



(b)



(c)

Figure 4.7 Scanning Electron Microscope of *Emericella variecolor* (a) conidia and coniospore (Bar = 10 µm) (b) ascus (Bar = 1 µm) (c) spike of sexual spore (Bar =1 µm)

4.3 Antimicrobial activities test

Fifty-five isolates of endophytic fungi, were investigated antimicrobial activity using agar diffusion method and their capability to produce secondary metabolites of potential therapeutic interest.

The results as shown in Table 4.7-4.12 that only seven isolates, including isolates CsLy 02, CsLy 03, CsLm 03, CsLm 05, CsLm 08, CsPm 06 and CsPm 09 exhibited antimicrobial activity . The isolate CsLy 02 exhibited antimicrobial against *C. albicans* with 8 mm of inhibition zone. The isolate CsLy 03 exhibited antimicrobial against *C. albicans* with 8 mm of inhibition zone. Isolate CsLm 03 exhibited antimicrobial against *C. albicans* with 8 mm of inhibition zone. The isolate CsLm 05 exhibited antimicrobial against *S. aureus* with 8 mm of inhibition zone.The isolate CsLm 08 exhibited antimicrobial against B. *subtilis*, *S. aureus* and *C. albicans* with inhibition zone of 22, 8 and 8 mm diameter, respectively. The isolate CsPm 09 exhibited antimicrobial against *B. subtilis* with 12 mm of inhibition zone.

Table 4.7 Antimicrobial activity of methanol crude of endophytic fungi isolated from young leaves of *Croton oblongifolius*

Isolates	Growth inhibition					
	Gram possitive bacterias		Gram negative bacterias		Yeasts	
	B. subtilis ATTC 6633	*S. aureus* ATTC 25923	*E. coli* ATTC 25922	*P. aeruginosa* ATTC 27853	*S. cerevisiae* (Sage yeast)	*C. albicans* ATTC 10231
CsLy 001	-	-	-	-	-	-
CsLy 002	-	-	-	-	-	(+)
CsLy 003	-	-	-	-	-	(+)
CsLy 004	-	-	-	-	-	-
CsLy 005	-	-	-	-	-	-
CsLy 006	-	-	-	-	-	-
CsLy 007	-	-	-	-	-	-
CsLy 008	-	-	-	-	-	-
CsLy 009	ND	ND	ND	ND	ND	ND
CsLy 010	-	-	-	-	-	-
CsLy 011	-	-	-	-	-	-
CsLy 012	-	-	-	-	-	-
CsLy 013	-	-	-	-	-	-
CsLy 014	ND	ND	ND	ND	ND	ND
CsLy 015	ND	ND	ND	ND	ND	ND
CsLy 016	ND	ND	ND	ND	ND	ND
CsLy 017	ND	ND	ND	ND	ND	ND
CsLy 018	ND	ND	ND	ND	ND	ND
CsLy 019	ND	ND	ND	ND	ND	ND
CsLy 020	ND	ND	ND	ND	ND	ND

Notes

ND not determined

- not have growth inhibit for microbial test

\+ inhibition zone range 8-15 mm

++ inhibition zone range more 16 mm

(+) inhibition zone less than normal range 8-15 mm

(++) inhibition zone less than normal range more 16 mm

Table 4.8 Antimicrobial activity of methanol crude of endophytic fungi isolated from mature leaves of *Croton oblongifolius*

Isolates	Growth inhibition					
	Gram possitive bacterias		Gram negative bacterias		Yeasts	
	B. subtilis ATTC 6633	*S. aureus* ATTC 25923	*E. coli* ATTC 25922	*P. aeruginosa* ATTC 27853	*S. cerevisiae* (Sage yeast)	*C. albicans* ATTC 10231
CsLm 001	-	-	-	-	-	-
CsLm 002	-	-	-	-	-	-
CsLm 003	-	-	-	-	-	(+)
CsLm 004	-	-	-	-	-	-
CsLm 005	-	(+)	-	-	-	-
CsLm 006	-	-	-	-	-	-
CsLm 007	-	-	-	-	-	-
CsLm 008	++	(+)	-	-	-	(+)
CsLm 009	-	-	-	-	-	-
CsLm 010	-	-	-	-	-	-
CsLm 011	ND	ND	ND	ND	ND	ND
CsLm 012	ND	ND	ND	ND	ND	ND
CsLm 013	ND	ND	ND	ND	ND	ND
CsLm 014	ND	ND	ND	ND	ND	ND

Notes

ND not determined

- not have growth inhibit for microbial test

\+ inhibition zone range 8-15 mm

++ inhibition zone range more 16 mm

(+) inhibition zone less than normal range 8-15 mm

(++) inhibition zone less than normal range more 16 mm

Table 4.9 Antimicrobial activity of methanol crude of endophytic fungi isolated from young petrioles of *Croton oblongifolius*

Isolates	Microbial test					
	Gram possitive bacterias		Gram negative bacterias		Yeasts	
	B. subtilis ATTC 6633	*S. aureus* ATTC 25923	*E. coli* ATTC 25922	*P. aeruginosa* ATTC 27853	*S. cerevisiae* (Sage yeast)	*C. albicans* ATTC 10231
CsPy 001	-	-	-	-	-	-
CsPy 002	-	-	-	-	-	-
CsPy 003	-	-	-	-	-	-
CsPy 004	-	-	-	-	-	-
CsPy 005	-	-	-	-	-	-
CsPy 006	-	-	-	-	-	-
CsPy 007	ND	ND	ND	ND	ND	ND
CsPy 008	ND	ND	ND	ND	ND	ND
CsPy 009	ND	ND	ND	ND	ND	ND
CsPy 010	ND	ND	ND	ND	ND	ND
CsPy 011	ND	ND	ND	ND	ND	ND
CsPy 012	ND	ND	ND	ND	ND	ND
CsPy 013	ND	ND	ND	ND	ND	ND
CsPy 014	ND	ND	ND	ND	ND	ND
CsPy 015	-	-	-	-	-	-
CsPy 016	ND	ND	ND	ND	ND	ND
CsPy 017	-	-	-	-	-	-
CsPy 018	-	-	-	-	-	-

Notes

ND not determined

- not have growth inhibit for microbial test

\+ inhibition zone range 8-15 mm

++ inhibition zone range more 16 mm

(+) inhibition zone less than normal range 8-15 mm

(++) inhibition zone less than normal range more 16 mm

Table 4.10 Antimicrobial activity of methanol crude of endophytic fungi isolated from mature petrioles of *Croton oblongifolius*

Isolates	Growth inhibition					
	Gram possitive bacterias		Gram negative bacterias		Yeasts	
	B. subtilis ATTC 6633	*S. aureus* ATTC 25923	*E. coli* ATTC 25922	*P. aeruginosa* ATTC 27853	*S. cerevisiae* (Sage yeast)	*C. albicans* ATTC 10231
CsPm 001	-	-	-	-	-	-
CsPm 002	-	-	-	-	-	-
CsPm 003	ND	ND	ND	ND	ND	ND
CsPm 004	-	-	-	-	-	-
CsPm 005	-	-	-	-	-	-
CsPm 006	-	-	-	-	-	(+)
CsPm 007	-	-	-	-	-	-
CsPm 008	-	-	-	-	-	-
CsPm 009	+	-	-	-	-	-
CsPm 010	-	-	-	-	-	-
CsPm 011	ND	ND	ND	ND	ND	ND
CsPm 012	ND	ND	ND	ND	ND	ND
CsPm 013	ND	ND	ND	ND	ND	ND
CsPm 014	ND	ND	ND	ND	ND	ND
CsPm 015	ND	ND	ND	ND	ND	ND
CsPm 016	ND	ND	ND	ND	ND	ND

Notes

ND not determined

- not have growth inhibit for microbial test

\+ inhibition zone range 8-15 mm

++ inhibition zone range more 16 mm

(+) inhibition zone less than normal range 8-15 mm

(++) inhibition zone less than normal range more 16 mm

Table 4.11 Antimicrobial activity of methanol crude of endophytic fungi isolated from twigs of *Croton oblongifolius*

Isolates	Microbial test					
	Gram possitive bacterias		Gram negative bacterias		Yeasts	
	B. subtilis ATTC 6633	*S. aureus* ATTC 25923	*E. coli* ATTC 25922	*P. aeruginosa* ATTC 27853	*S. cerevisiae* (Sage yeast)	*C. albicans* ATTC 10231
CsTw 001	-	-	-	-	-	-
CsTw 002	-	-	-	-	-	-
CsTw 003	-	-	-	-	-	-
CsTw 004	-	-	-	-	-	-
CsTw 005	-	-	-	-	-	-
CsTw 006	-	-	-	-	-	-
CsTw 007	-	-	-	-	-	-
CsTw 008	ND	ND	ND	ND	ND	ND
CsTw 009	ND	ND	ND	ND	ND	ND
CsTw 010	ND	ND	ND	ND	ND	ND
CsTw 011	-	+	-	-	-	-
CsTw 012	-	-	-	-	-	-

Notes

ND not determined

- not have growth inhibit for microbial test

+ inhibition zone range 8-15 mm

++ inhibition zone range more 16 mm

(+) inhibition zone less than normal range 8-15 mm

(++) inhibition zone less than normal range more 16 mm

Table 4.12 Antimicrobial activity of methanol crude of endophytic fungi isolated from barks of *Croton oblongifolius*

Isolates	Growth inhibition					
	Gram possitive bacterias		Gram negative bacterias		Yeasts	
	B. subtilis ATTC 6633	*S. aureus* ATTC 25923	*E. coli* ATTC 25922	*P. aeruginosa* ATTC 27853	*S. cerevisiae* (Sage yeast)	*C. albicans* ATTC 10231
CsBa 001	-	-	-	-	-	-
CsBa 002	ND	ND	ND	ND	ND	ND
CsBa 003	ND	ND	ND	ND	ND	ND
CsBa 004	ND	ND	ND	ND	ND	ND
CsBa 005	ND	ND	ND	ND	ND	ND

Notes

ND not determined

- not have growth inhibit for microbial test

\+ inhibition zone range 8-15 mm

++ inhibition zone range more 16 mm

(+) inhibition zone less than normal range 8-15 mm

(++) inhibition zone less than normal range more 16 mm

4.4 Investigation of metabolites

Due to only a few endophytic fungi showed a little of antimicrobial activity, production of metabolites of the fungi were investigated. Fifty isolates of fungi were cultured in potato dextrose broth (100 ml) in 250 ml flask. The endophytic fungi stock, cultured in PDA at room temperature for 7 days, were cut using cork hole borrer (ϕ 8 mm) and inoculated into the potato dextrose broth (PDB), followed by place statically at room temperature for 2 months. Methanol was added into each cultured broth and soaked for 30 min. Then the methanolic broths were filtered though filter paper Whatman no.1. Filtrates were evaporated off methanol and followed by lyophilization to dryness. Each broth crude was extracted with methanol (20 ml). The methanol extracts of each broth were investigated their chemical constituents by TLC. The TLC results were monitored by UV (254 and 365 nm), iodine vapor and vanillin/H_2SO_4 reagent. It was found that only two isolates of endophytic fungi including isolates CsLm 08 and CsPm 09 produced compounds while other produced more less metabolites. Thus, two isolates were selected for investigation of their metabolites.

4.5 Identifiication by ITS region

4.5.1 Endophytic fungus strain CsLm 08

Endophytic fungus strain CsLm08 was sent for identification by molecular methods at the Asian Natural Environmental Science Center, the University of Tokyo, Japan.

Sequencing of the nucleotide sequences of partial 18S, ITS1-5.8S-ITS2 region of strain CsLm08 resulted in a 583 bp fragment, as shown in Figure 4.8.

```
cttggtcatt  tagaggaagt  aaaagtcgta  acaaggtttc
cgtagtgaac  ctgcggaagg  atcattaccg  agttttcggg
Cttcggctcg  actctcccac  cctttgtgaa  cgtacctctg
ttgctttggc  ggctccggcc  gcaaaggaac  ctccaaactc
cagtcagtaa  acgcagacgt  ctgataaaca  agttaataaa
ctaaaacttt  caacaacgga  tctcttggtt  ctggcatcga
tgaagaacgc  agcgaaatgc  gataagtaat  gtgaattgca
gaattcagtg  aatcatcgaa  tctttgaacg  cacattgcgc
cccttggtat  tccgggggggc  atgcctgttc  gagcgtcatt
acaaccctca  agctctgctt  ggaattgggc  accgtcctca
ctgcggacgc  gcctcaaaga  cctcggcggt  ggctgttcag
ccctcaagcg  tagtagaata  cacctcgctt  tggagtggtt
ggcgtcgccc  gccggacgaa  ccttctgaac  ttttctcaag
gttgacctcg  gatcaggtag  ggatacccgc  tgaacttaag
catatcaata  agcggagga
```

Figure 4.8 Nucleotide sequences of partial 18S region, complete ITS1-5.8S-ITS2 region of the strain CsLm08

>>AY160201|AY160201.1 *Lasiodiplodia theobromae* isolate L (512 nt) initn: 2497 init1: 2497 opt: 2497 Z-score: 2592.2 expect() 2.5e-136 98.633% identity in 512 nt overlap (59-570:1-512)

```
               30        40        50        60        70        80
       TAACAAGGTTTCCGTAGTGAACCTGCGGAAGGATCATTACCGAGTTTTCGGGCTTCGGCT
                                  ::::::::::::::: ::::::::::::
AY1602                            GGATCATTACCGAGTTCTCGGGCTTCGGCT
                                          10        20        30

       90       100       110       120       130       140
       CGACTCTCCCACCCTTTGTGAACGTACCTCTGTTGCTTTGGCGGCTCCGGCCGCAAAGGA
       :::::::::::::::::::::::::::::::::::::::::::::::::::: :: :
AY1602 CGACTCTCCCACCCTTTGTGAACGTACCTCTGTTGCTTTGGCGGCTCCGGCCGCCAAAGG
              40        50        60        70        80        90

      150       160       170       180       190       200
       ACCTCCAAACTCCAGTCAGTAAACGCAGACGTCTGATAAACAAGTTAATAAACTAAAACT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY1602 ACCTCCAAACTCCAGTCAGTAAACGCAGACGTCTGATAAACAAGTTAATAAACTAAAACT
              100       110       120       130       140       150

      210       220       230       240       250       260
       TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY1602 TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
              160       170       180       190       200       210

      270       280       290       300       310       320
       ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGT
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY1602 ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGT
              220       230       240       250       260       270

      330       340       350       360       370       380
       ATTCCGGGGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGAATTGG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY1602 ATTCCGGGGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGAATTGG
              280       290       300       310       320       330

      390       400       410       420       430       440
       GCACCGTCCTCACTGCGGACGCGCCTCAAAGACCTCGGCGGTGGCTGTTCAGCCCTCAAG
       :::::::::: :::::::::::::::::::::::::::::::: ::::::::::::
AY1602 GCACCGTCCTCGCTGCGGACGCGCCTCAAAGACCTCGGCGGTGGCTCTTCAGCCCTCAAG
              340       350       360       370       380       390

      450       460       470       480       490       500
       CGTAGTAGAATACACCTCGCTTTGGAGTGGTTGGCGTCGCCCGCCGGACGAACCTTCTGA
       :::::::::::::::::::::::::: :::::::::::::::::::::::::::::::
AY1602 CGTAGTAGAATACACCTCGCTTTGGAGCGGTTGGCGTCGCCCGCCGGACGAACCTTCTGA
              400       410       420       430       440       450

      510       520       530       540       550       560
       ACTTTTCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AY1602 ACTTTTCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAA
              460       470       480       490       500       510

      570
       TAAGCGGAGGA
       ::
AY1602 TA
```

Figure 4.9 Alignment data of partial 18S region, complete ITS1-5.8S-ITS2 region of the strain CsLm 08 and *Lasiodiplodia theobromae*

From results of nucleotide sequences on ITS regions of endophytic fungus strain CsLm 08 compared with nucleotide sequences on ITS regions were recorded and collected in GenBank database, website http://www.ddbj.nig.ac.th. found that

nucleotide sequences on ITS regions of endophytic fungus strain CsLm 08 similarly with *Lasiodiplodia theobromae* 98.633% identity was shown in Figure 4.9.

4.5.2 Endophytic fungi strain CsPm 09

Endophytic fungus strain CsPm09 was sent for identification by molecular methods at the Asian Natural Environmental Science Center, the University of Tokyo, Japan.

Sequencing of the nucleotide sequences of partial 18S, ITS1-5.8S-ITS2 region of strain CsPm 09 resulted in a 583 bp fragment as shown in Figure 4.10.

```
AAAAGGTTCG  TAGTGACCTG  CGGAGGATCA  TTACCGAGTG
AGGGCTGCCT  CCGGGCGCCC  AACCTCCCAC  CCGTGAATAC
CTAACACTGT  TGCTTCGGCG  GGGAGCCCTC  TCGGGGGCGA
GCCGCCGGAG  ACCACTGAAC  TTCATGCCTG  TAGTGATGAG
TCTGAGCCTA  AATGAAAATT  TAGTCAAAAC  TTTCAACAAT
GGATCTCTTG  GTTCCGGCAT  CGATGAAGAA  CGCAGCGAAC
TGCGATAAGT  AATGTGAATT  GCAGAATTCA  GTGAATCATC
GAGTCTTTGA  ACGCACATTG  CGCCCCCTGG  CATTCCGGGG
GGCATGCCTG  TCCGAGCGTC  ATTGCTGCCC  TTCAAGCCCG
GCTTGTGTGT  TGGGTCGTCG  TCCCCCCCGG  GGGACGGGCC
CGAAAGGCAG  CGGCGGCACC  GTGTCCGGTC  CTCGAGCGTA
TGGGGCTTTG  TCACCCGCTC  GATTAGGGCC  GGNCGGGNGC
CANCCGGCNT  CTCCAACCTT  ATTTTTCTCA  GTTGACCTCT
GATCANGTAG  GATACCCNCT  NAANTTANAT  ATCAANANAN
 AANTNTNN
```

Figure 4.10 Nucleotide sequences of partial 18S region, complete ITS1-5.8S-ITS2 region of the strain CsPm09

>>AJ000932|AJ000932.1 *Emericella variecolor* 5.8S rRNA ge (563 nt) initn: 2564 init1: 2016 opt: 2591 Z-score: 2624.7 bits: 495.6 E(): 3.8e-137 banded Smith-Waterman score: 2591; 96.064% identity (99.261% ungapped) in 559 nt overlap (7-561:1-559)

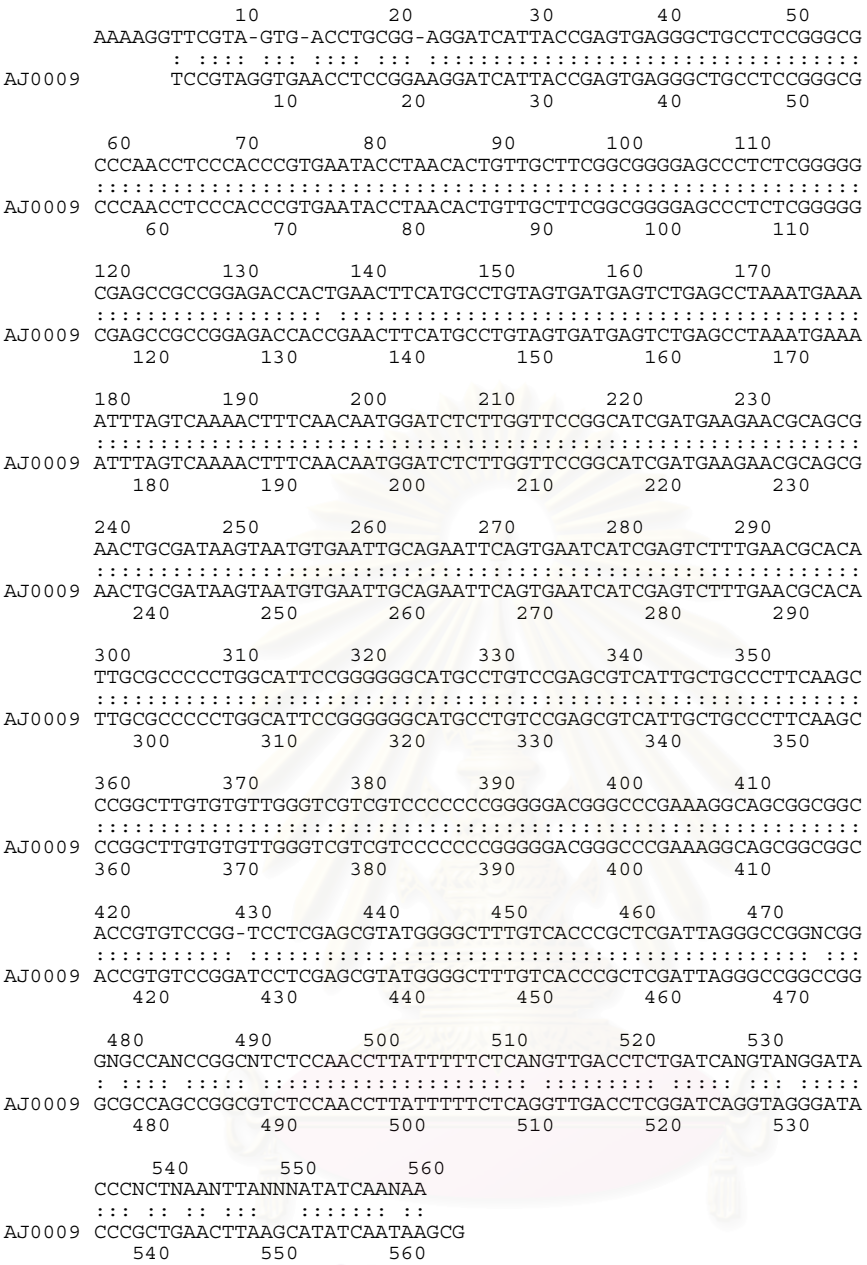


Figure 4.11 Alignment data of partial 18S region, complete ITS1-5.8S-ITS2 region of the strain CsPm 09 and *Emericella variecolor*

From results of nucleotide sequences on ITS regions of endophytic fungus strain CsLm 09 compared with nucleotide sequences on ITS regions were recorded and collected in GenBank database, website http://www.ddbj.nig.ac.jp found that nucleotide sequences on ITS regions of endophytic fungus strain CsLm 09 similarly with *Emericella variecolor* 96.064% identity (99.261% ungapped) was shown in Figure 4.11.

4.6 Chemical constituents of endophytic fungus strain CsLm 08 metabolites

Endophytic fungus strain CsLm 08, isolated from mature leaves of *Croton oblongifolius*, was cultured statically in PDB (5L) for 2 months. The broth and mycelium were separated and investigated chemical constituents of its metabolites (see scheme 3.1 and 3.2). Isolation of mycelium EtOAc crude by column chromatography gave 3 compounds.

Compound **1** was obtained as a white solid m.p. 149-150 ^{o}C and M^{+} at *m/z* 398, ($C_{28}H_{46}O$) from the combined fraction A3 was washed with hexane (10 ml x 3) and crystallization from EtOAc-Hex. The structure of compound **1** was established on the basis of spectroscopic analysis and physical properties especially $[\alpha]_D$ as ergosta-5,22-dien-3-ol (Figure 4.12).

The IR spectrum of compound **1** is shown the absorption peaks which are assigned as shown in Table 4.13 and indicated that compound **1** contained hydroxy and olefin moieties (see Table 4.13).

Table 4.13 The IR sbsorption bands assignment of compound **1**

Wave number (cm^{-1})	Intensity	Tentative assignment
3431	Broad, Medium	O-H stretching vibration of alcohol
2949 and 2863	Sharp, Strong	C-H stretching vibration of CH_2, CH_3
1660	Weak	C=C stretching vibration of olefin
1458 and 1372	Medium	CH bending vibration
1049	Medium	C-O stretching vibration
964	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum of compound **1** showed signals of angular methyl, methylene and methine groups of steroids at 0.72-2.40. The proton on carbon attached by hydroxy group was shown as the multiplet at 3.55 ppm which the multiplet signal at 5.21 ppm was the disubstituted vinyl protons (-CH=CH-). The most downfield signal at 5.38 ppm was the signals of trisubstituted vinyl protons (-CH=C-).

The ^{13}C-NMR data showed the carbon signals at 20.98, 19.98, 19.66, 19.43, 17.64 and 12.09 ppm which assigned as CH_3, CH_2 and CH. The olefinic carbon signals observed at 121.75, 131.73, 135.85 and 140.75 ppm, while the signal at 71.84 ppm exhibited the C_{sp3} attached to OH group. The ^{13}C and ^{1}H chemical shifts of compound **1** was shown in Table 4.14.

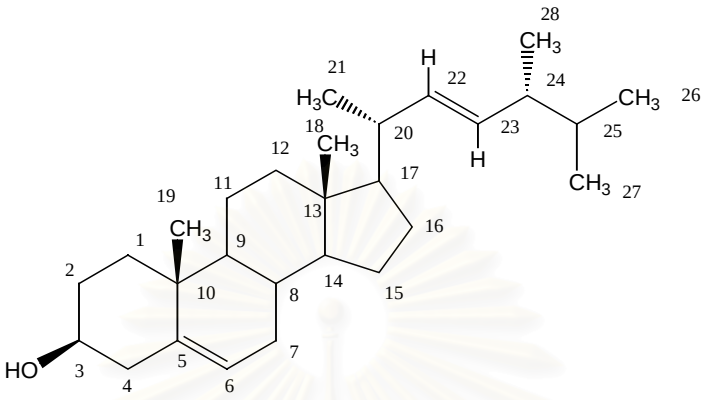
Table 4.14 ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **1**

Position	Compound **1**	
	δ_C	δ_H
1	24.30 (t)	1.05 (m) 1.57 (m)
2	31.91 (t)	1.55 (m) 2.00 (m)
3	71.85 (d)	3.55 (m)
4	37.26 (t)	1.10 (m) 1.88 (m)
5	140.75 (s)	-
6	121.76 (d)	5.38 (d, 4.8 Hz)
7	42.29 (t)	2.28 (m) 2.32 (m)
8	21.08 (d)	1.48 (m)
9	50.16 (d)	0.94 (m)
10	36.53 (s)	-

Table 4.14 (continued) ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **1**

Position	Compound **1**	
	δ_C	δ_H
11	42.29 (t)	2.28(m) 2.32 (m)
12	36.53 (t)	1.23 (m) 2.01 (m)
13	36.53 (s)	-
14	56.85 (d)	1.00 (m)
15	31.65 (t)	1.58 (m) 1.85 (m)
16	28.57 (t)	1.24 (m) 1.69 (m)
17	56.01 (d)	1.13 (m)
18	12.09	0.72 (s)
19	20.98 (q)	1.05 (s)
20	40.19 (d)	2.04 (m)
21	19.43 (q)	1.04 (s)
22	135.86 (d)	5.15-5.27 (m)
23	131.73 (d)	5.15-5.27 (m)
24	42.82 (d)	1.87 (m)
25	31.11 (d)	1.51 (m)
26	19.98 (q)	0.86 (d, 7.2 Hz)
27	19.66 (q)	0.85 (d, 6.4 Hz)
28	19.98 (q)	0.93 (s)

Mass spectrum and fragmentation pattern indicated that compound **1** was corresponded to ergosta-5,22-dien-3-ol with M^+ ion at *m/z* 398. Thus, the chemical structure of compound **1** was shown in Figure 4.12.



Ergosta-5,22-dien-3-ol

Figure 4.12 The chemical structure of compound **1**

Compound **2** was obtained as a amorphous white solid from the combined fraction A4 of mycelium ethyl acetate crude extract (m.p. 84-86 ^{o}C). MS spectrum showed M^+ ion peak of a C_{24} long chain carboxylic acid at *m/z* 368, M^+ ion peak of a C_{26} long chain carboxylic acid at *m/z* 396 in EIMS. ^{1}H and ^{13}C-NMR data indicated the NMR spectroscopic pattern of long chain carboxylic acid; methyl carbons at 14.0 ppm, methylene carbon at 22.5, 29.5, 32.0 and 34.0 ppm and the COOH at 179.5 ppm.

The IR (Table 4.15) and 2D-NMR data certainly supported the assignments of compound **2**.

Table 4.15 The IR absorption bands assignment of compound **2**

Wave number (cm^{-1})	Intensity	Tentative assignment
3443	Broad, Strong	O-H stretching vibration of carboxylic acid
2918 and 2851	Sharp, Strong	CH stretching vibration of CH_2, CH_3
1715	Strong	C=O stretching vibration of carbonyl group
1559, 1462, 1411	Medium	C-H bending vibration of CH_2, CH_3
1372	Weak	C-O stretching vibration of carboxylic acid
968, 894, 711	Weak	C-H out of plane bending vibration

From 2D-NMR including gCOSY, gHMBC and mass spectrum showed a mlecular ion at m/z 368 and 372 and fragmentation pattern of mass spectrum indicated that the structure of compound **2** was long chain carboxylic acid $C_{22\text{-}24}$. The chemical structure of compound was shown in Figure 4.13.

$CH_3(CH_2)_{22\text{-}24}COOH$

Long chain carboxylic acid

Figure 4.13 The Chemical structure and chemical name of compound **2**

Compound **3** was obtained as off-white crystals, m.p. 185-186 ^{o}C and *m/z* 292, ($C_{17}H_{24}O_4$) from the combined fraction A5 (144 mg) after crystallization from $CHCl_3$-Hex. The structure of compound **3** was established on the basis of spectroscopic analysis as lasiodiplodin (Figure 4.14).

IR spectrum of compound **3** showed the absorption peak of OH stretching vibration at 3388 cm^{-1}, of CH stretching vibration at 2925 and 2844 cm^{-1}, of C=O stretching vibration at 1692 cm^{-1}, of C=C stretching vibration of aromatic ring at 1598 cm^{-1}, of C-H bending vibration at 1466 and 1427 cm^{-1}, of C-O stretching vibration at 1341, 1264, 1197, 1162 and 1092 cm^{-1} and of CH out of plane bending vibration at 847 cm^{-1} (see Table 4.16).

Table 4.16 The IR absorption bands assignment of compound **3**

Wave number (cm^{-1})	Intensity	Tentative assignment
3388	Broad, Medium	O-H stretching vibration
2925 and 2844	Medium	CH stretching vibration
1692	Sharp, Strong	C=O stretching vibration
1598	Sharp, Strong	C=C stretching vibration
1466 and 1427	Medium	C-H bending vibration
1341, 1264 and 1197	Medium	C-O stretching vibration
1162 and 1092	Weak	C-O stretching vibration
847	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum indicated one broad singlet signal of aromatic protons at δ 6.20 ppm, a methoxy signal at δ 3.68 ppm, a doublet signal of methyl protons at δ 1.32 ppm (J = 6.4 Hz), a multiplet signal of 8'-H at δ 5.28 ppm, two multiplet signals of 7'-H_2 at δ 1.93 and 1.66 ppm and two multiplet signals of 1'-H_2 at δ 2.45 and 2.62 ppm.

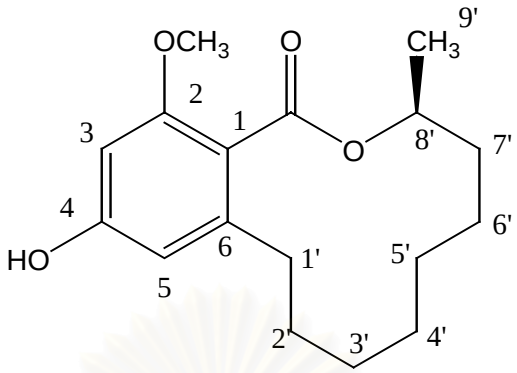
The gHMBC (Figure 4.15), NOESY (Figure 4.16) and TOCSY (Figure 4.17) correlations could assigned the structure of compound **3** similar to a known compound, lasiodiplodin (Figure 4.14). The long range correlation of gHMBC as shown in Figure 4.15 were observed between the following proton and carbon signals; OMe (2-C, 3-C and 4C); 3-H (2-C, 4C and CO); 5-H (3-C, 4-C, 1'-C and 3'C) and Me (6'C, 8'-C and CO).

^{1}H and ^{13}C-NMR data of compound **3** were shown in Table 4.17 in comparison with lasiodiplodin. The structure of compound **3** was also confirmed by comparison with specific optical rotation of lasiodiplodin ($[\alpha]_D$ +6 ($CHCl_3$, c 0.1), lit. (Lee et al., 1982); ($[\alpha]_D$ +5.9 ($CHCl_3$, c 0.1).

The result showed that compound **3** was the same as lasiodiplodin.

Table 4.17 Comparison of ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **3** and Lasiodiplodin (Lee et. al., 1982)

Position	Compound **3**		Lasiodiplodin	
	δ_C	δ_H	δ_C (62.89 MHz)	δ_H (250 MHz)
1	116.80 (s)	-	117.40 (s)	-
2	157.91 (s)	-	158.07 (s)	-
3	96.90 (d)	6.20 (s, br)	97.17 (d)	6.24 (d, 1.5 Hz)
4	157.80 (s)	-	157.87 (s)	-
5	108.36 (d)	6.20 (s, br)	108.49 (d)	6.22 (d, 1.5 Hz)
6	142.90 (s)		143.07 (s)	-
1'	30.28 (t)	2.45 (m) 2.62 (m)	32.46 (t)	2.46 (m) 2.64 (m)
2'	32.19 (t)	1.40 (m) 1.62 (m)	30.46 (t)	1.56-1.74 (m)
3'	29.99 (t)	1.56-1.65 (m)	30.08 (t)	1.26-1.47 (m)
4'	26.27 (t)	1.38-1.46 (m)	26.47 (t)	1.26-1.47 (m)
5'	25.37 (t)	1.35 (m) 1.26 (m)	25.55 (t)	1.26-1.47 (m)
6'	24.01 (t)	1.28 (m) 1.45 (m)	24.26 (t)	1.26-1.47 (m)
7'	21.21 (t)	1.66 (m) 1.93 (m)	21.47 (t)	1.67 (m) 1.92 (m)
8'	72.53 (d)	5.28 (m)	72.49 (d)	5.29 (m)
9'	19.42 (q)	1.32 (d)	19.43 (q)	1.38 (d)
C=O	169.48 (s)	-	169.48 (s)	-
OMe	55.67 (q)	3.68 (s)	55.87 (q)	3.71 (s)



Lasiodiplodin

Figure 4.14 The chemical structure of compound **3**

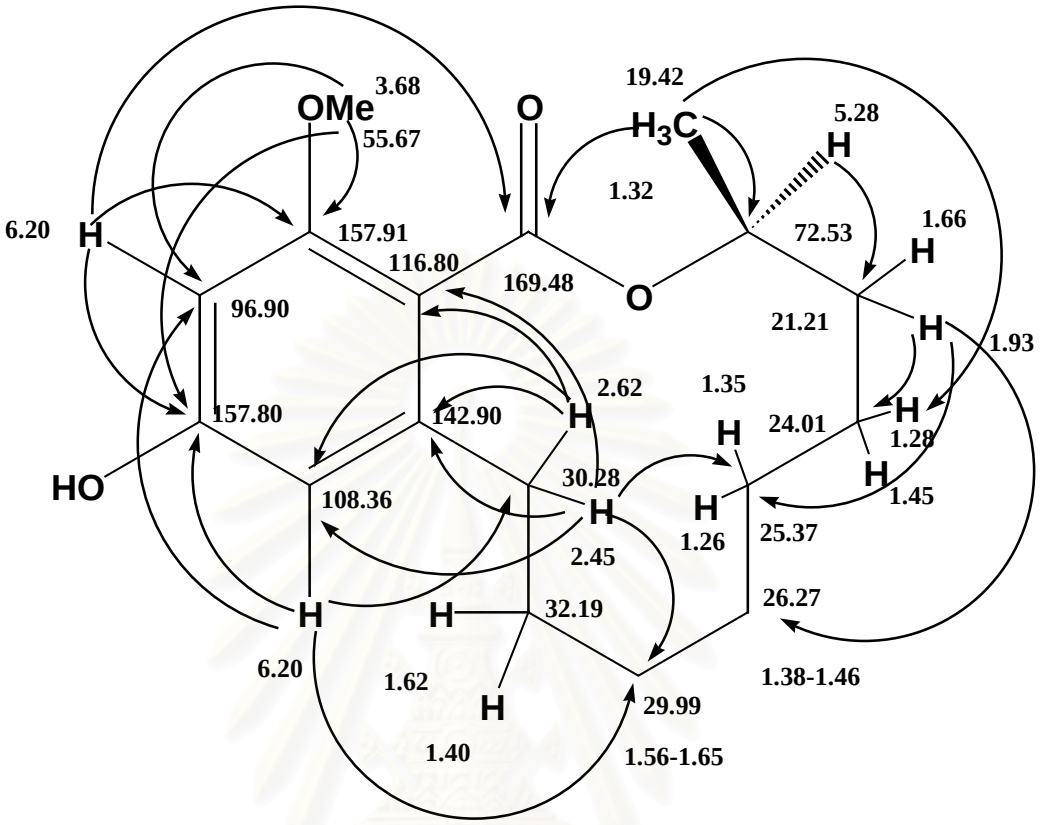


Figure 4.15 The gHMBC correlation of compound 3

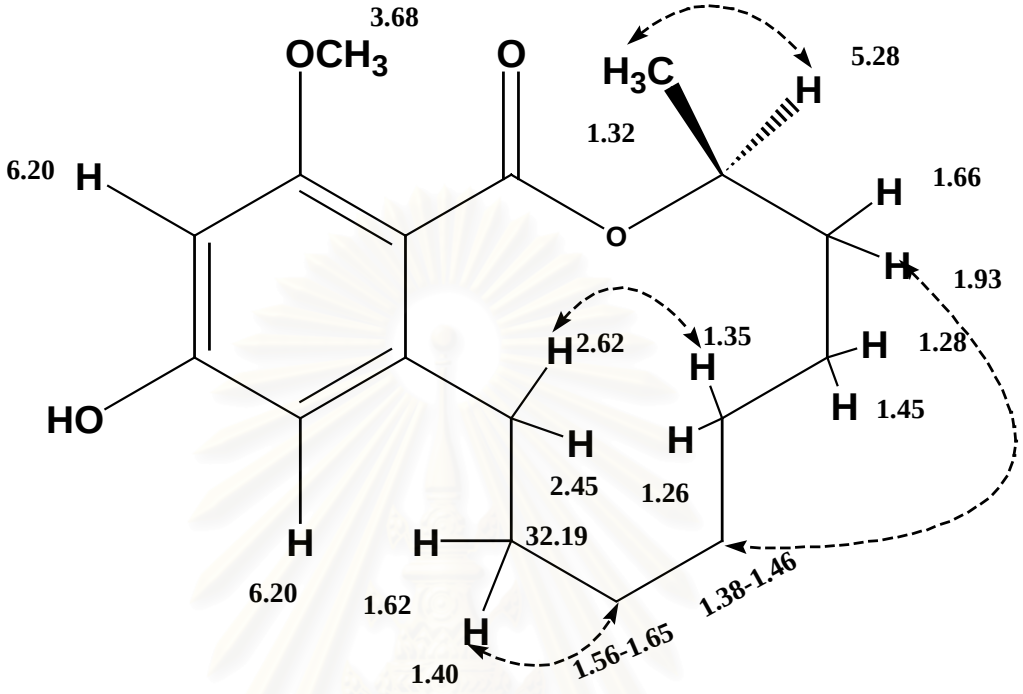


Figure 4.16 The NOESY correlation of compound 3

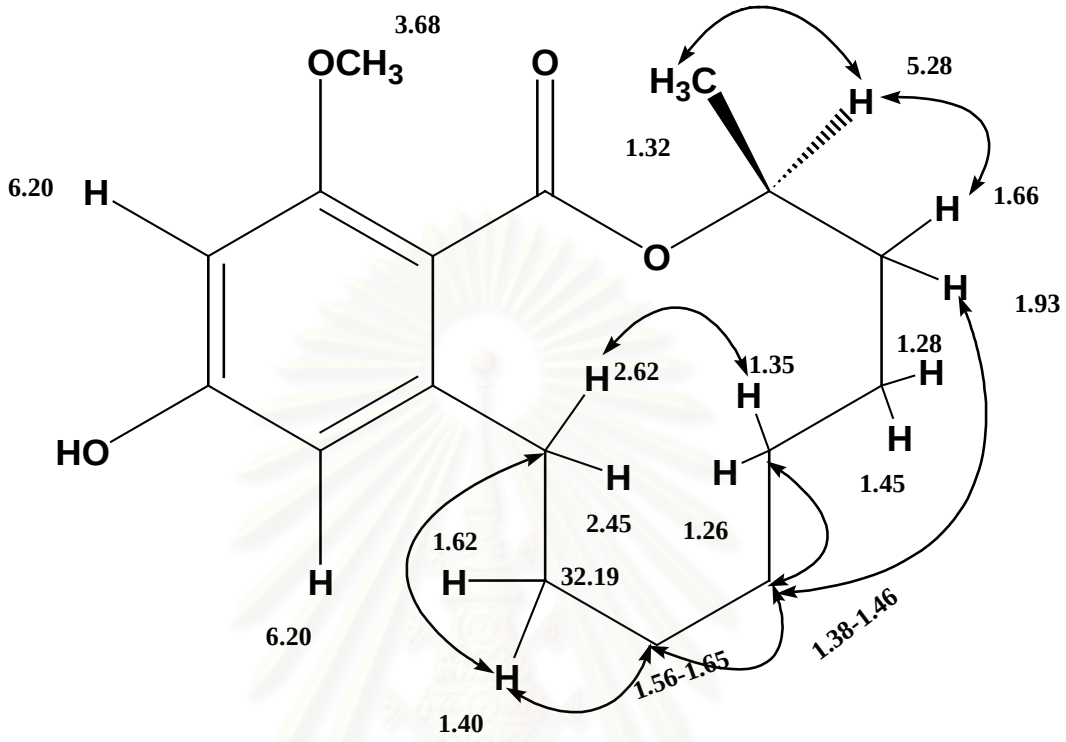


Figure 4.17 The TOCSY correlation of compound 3

4.7 Chemical constituents of endophytic fungus strain CsPm 09 metabolites

4.7.1 Chemical constituents of the strain CsPm 09 in fermentation broth

Endophytic fungus strain CsPm 09 was isolated from mature petriole leave and identified as *Emericella variecolor* by molecular method using ITS region. After culture of strain CsPm 09 in MEB (7.5 L) at room temperature for 4-6 weeks, fermentation broth and mycelium were separated by filtration through filter paper Whatman no.1 and then investigated chemical constituents in broth and mycelium according to scheme 3.3, 3.4 and 3.5.

Isolation of broth ethyl acetate crude by column chromatography and crystallization gave compound **4** as colorless crystals m.p. 128-130 ^{o}C, which showed the $[M+H]^{+}$ ion at *m/z* 155 in EIMS. The purified compound **4** was subjected to spectroscopic analysis.

IR spectrum of compound **4** showed that the absorption peaks assigned as in Table 4.18 were a OH stretching vibration at 3396, CH stretching vibration at 2914 and 2848, C=O stretching vibration at 1785 and 1692, C=C stretching vibration at 1629 and 1563, CH bending vibration at 1415 and 1330, 1193, CO stretching at 1112 and 1084, CH out of plane bending vibration at 960 and 863 cm^{-1} (see Table 4.18).

Table 4.18 The IR absorption band assignment of compound **4**

Wave number (cm^{-1})	Intensity	Tentative assignment
3396	Broad, Strong	O-H stretching vibration of alcohol
2914, 2848	Weak	CH stretching vibration of CH_2, CH_3
1785, 1692	Weak	C=O stretching vibration of carbonyl group
1629, 1563	Strong	C=C stretching vibration of olefin
1415, 1330	Strong	C-H bending vibration of CH_2, CH_3
1193, 1112, 1084	Medium	C-O stretching of alcohol
960, 863	Weak	C-H out of plane bending vibration

From ^{13}C-NMR and ^{1}H-NMR data, chemical shifts of compound **4** were very similar to terrein (Dunn et al., 1975) and the small concoupling constant of 2-H and 3-H (*J* = 2.8 and 2.4 Hz, respectively) and the large coupling constant of of 1'H, and 2'-H (*J* = 15.6 Hz and 14 and 6.8 Hz, respectively)indicated the same configuration as terrein (see Table 4.19).

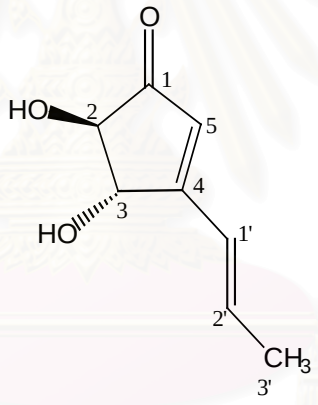
Table 4.19 Comparison of ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **4** and Terrein (Dunn et. al., 1975)

Position	Compound **4**		Terrein (D_2O)	
	δ_C	δ_H	δ_C	δ_H
1	202.68 (s)	-	204.9 (s)	-
2	81.90 (d)	4.20 (d)	81.3 (d)	5.08 (d)
3	76.86 (d)	4.82 (d)	77.0 (d)	4.50 (d)
4	168.34 (s)	-	171.4 (s)	-
5	124.94 (d)	5.95 (s)	125.3 (d)	6.38 (s)
1'	125.07 (d)	6.33 (d)	125.3 (d)	6.71 (d)
2'	141.57 (dq)	6.73 (octet)	143.9 (d)	7.09 (octet)
3'	19.61 (q)	1.88 (d)	19.7 (q)	2.17 (d)

From 2D-NMR including gHMBC, NOESY as presented in the Table 4.20 and Figure 4.19 and 4.20 was used to confirm the connectively of compound **4**. The molecular ion with 155 [M^++H] and showed the chemical structure of compound **4** assigned was Terrein in Figure 4.18.

Table 4.20 The correlation of gHMBC and NOESY of compound **4**

Position	^{13}C-NMR	^{1}H-NMR	gHMBC	NOESY
1	202.68 (s)	-	-	-
2	81.90 (d)	4.20 (d)	C-1, C-3	-
3	76.86 (d)	4.82 (d)	C-2, C-4, C-5, C1'	-
4	168.34 (s)	-	-	-
5	124.94 (d)	5.95 (s)	C-2, C-3, C-4, C-5, C-1'	H-1'
1'	125.07 (d)	6.33 (d)	C-2', C-3'	5-H
2'	141.57 (d)	6.73 (octet)	C-1', C-3'	-
3'	19.61 (q)	1.88 (d)	C-4, C-1', C-2',	-



Terrein

Figure 4.18 The chemical structure of compound **4**

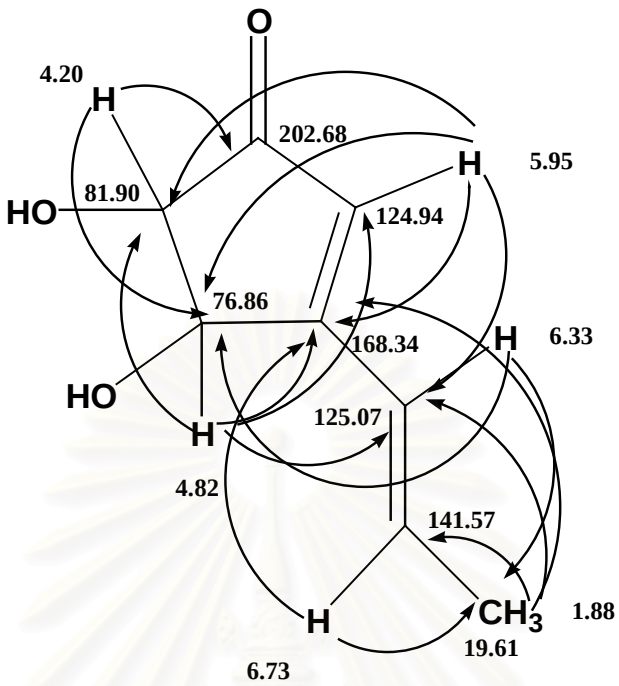


Figure 4.19 The gHMBC correlation of compound 4

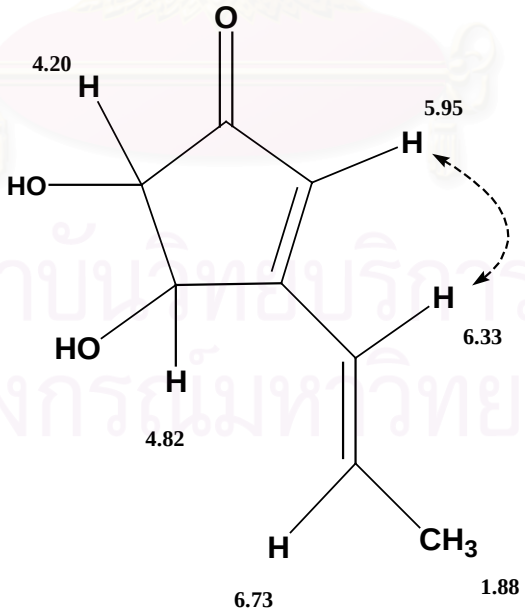


Figure 4.20 The NOESY correlation of compound 4

4.7.2 Chemical constituents of the strain CsPm 09 in mycelium

The methanolic extract crude of the strain CsPm 09, *Emericella variecolor*, was extracted with EtOAc to obtain the EtOAc extract crude after evaporation as a dark red viscous residue. The EtOAc crude was subjected to silica gel column chromatography and then obtained five compounds (see scheme 3.5) including compound **5**, **6**, **7**, **8** and **9**.

Isolation of mycelium EtOAc extract crude by column chromatography and crystallization from $CHCl_3$-Hex gave compound **5** as yellow needle crystals, m.p. 139-140 ^{o}C, which showed the M^+ ion at *m/z* 406 in EIMS and on the basis of NMR analysis assigned the structure of Shamixanthone.

IR spectrum of compound **5** showed that the absorption peaks, presented in Table 4.21, were a OH stretching vibration at 3447, CH stretching vibration at 2921 and 2851, C=O stretching vibration at 1734, C=C stretching vibration at 1645, 1567 and 1478, C-H bending vibration at 1419, C-O stretching vibration at 1240, 1116, 1022 , C-H out of plane bending vibration at 820 cm^{-1} (see Table 4.21).

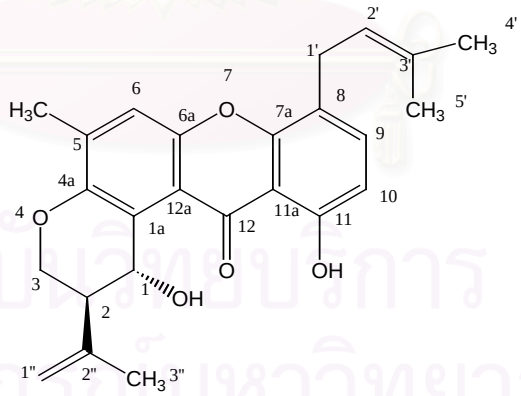
Table 4.21 The IR absorption bands assignment of compound **5**

Wave number (cm^{-1})	Intensity	Tentative assignment
3447	Broad, Strong	O-H stretching vibration of alcohol
2921, 2851	Weak	stretching vibration of CH_2, CH_3
1734	Weak	C=O stretching vibration of carbonyl group
1645, 1567, 1478	Medium	C=C stretching vibration of aromatic of olefinic
1240, 1116, 1022	Weak	C-O stretching vibration
820	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum of compound **5** indicated three aromatic protons at δ 6.77, 7.32 and 7.46 ppm, three olefinic proton at δ 4.61, 4.83 and 5.34 ppm, four methyl group at δ 1.78, 1.82, 1.88 and 2.38 ppm and a hydroxy proton of phenolic compound at δ 12.63 ppm.

The ^{13}C-NMR spectrum showed 25 signals, eleven signals of aromatic carbons appeared at δ 109.24, 109.76, 116.95, 118.95, 119.38, 120.92, 138.38, 136.56, 149.45, 152.26, 152.86 ppm and a phenyl carbon appeared at δ 159.72 ppm, four olefinic carbons at δ 112.30, 121.52, 133.33 and 142.61ppm, four methyl carbons appeared at δ 17.49, 17.96, 22.60 and 25.82 ppm and other sp^{3} carbons at δ 27.52, 44.93, 63.22 and 64.56 ppm. The carbonyl group appeared at δ 184.51 ppm.

From 2D-NMR including gHMBC (Figure 4.22), and NOESY (Figure 4.23) correlation in the Table 4.23. and assigned the chemical structure of compound **5** was assigned as Shamixanthone shown in Figure 4.23. The ^{13}C-NMR chemical shifts of compound **5** and Shamixanthone were compared in Table 4.22.



Shamixanthone

Figure 4.21 The chemical structure of compound **5**

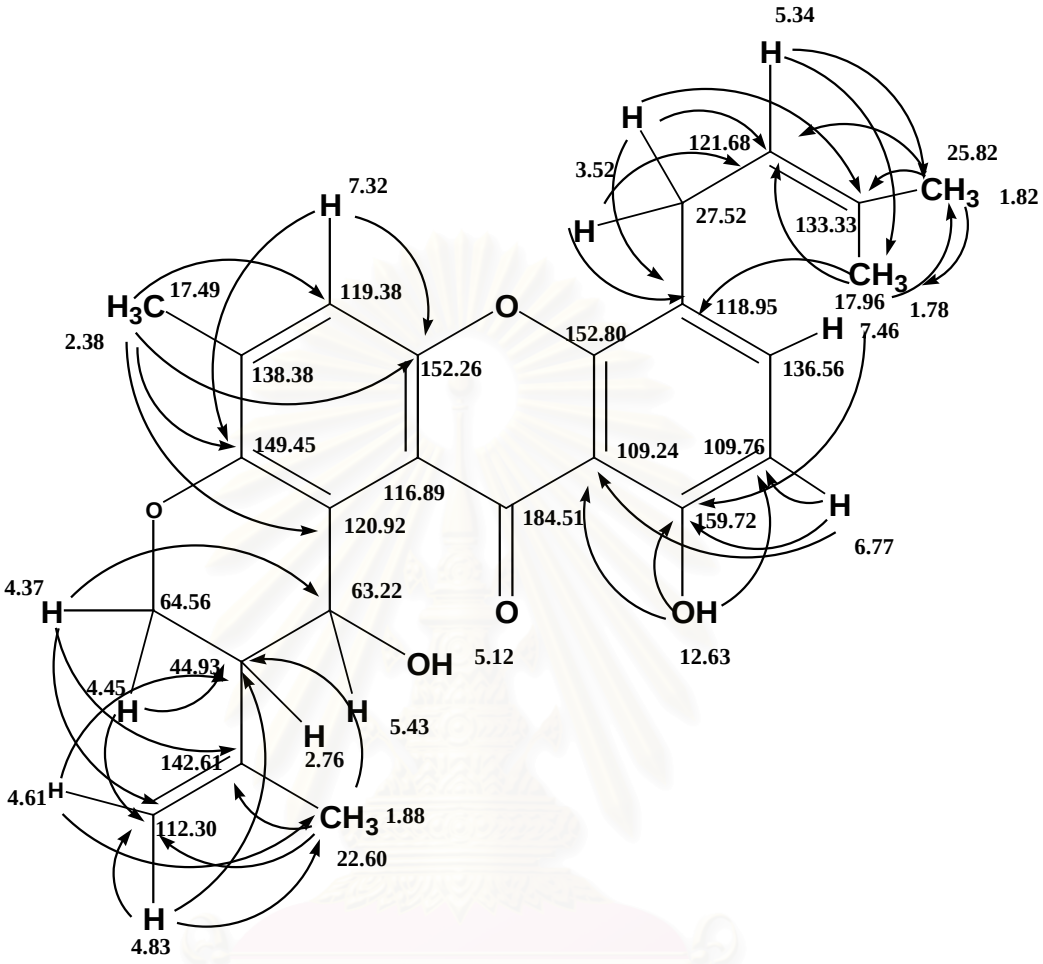


Figure 4.22 The gHMBC correlation of compound 5

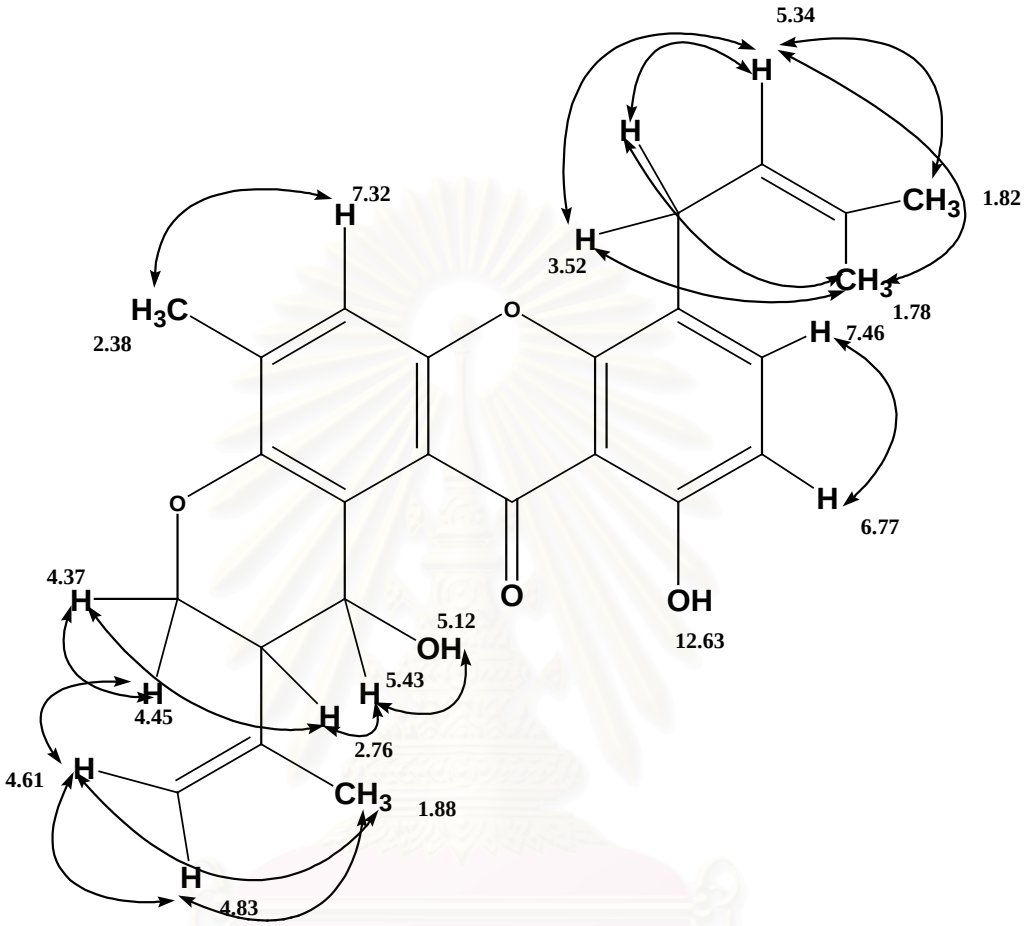


Figure 4.23 The NOESY correlation of compound 5

Table 4.22 Comparison of ^{13}C-NMR chemical shifts of compound **5** and Shamixanthone

Position	Compound 5	Shamixanthone
	δ_C	δ_C
1	63.22 (d)	63.0 (d)
1a	120.92 (s)	120.5 (s)
2	44.93 (d)	44.9 (d)
3	64.56 (t)	64.4 (t)
4a	149.45 (s)	148.8 (s)
5	138.38 (s)	137.6 (s)
6	119.38 (d)	118.7(d)
6a	152.26 (s)	151.5 (s)
7a	152.80 (s)	159.2 (s)
8	118.95 (s)	118.4 (s)
9	136.56 (d)	135.8 (d)
10	109.73 (d)	109.3 (d)
11	159.72 (s)	152.1 (s)
11a	109.24 (s)	108.7 (s)
12	184.51 (s)	183.6 (s)
12a	116.89 (s)	116.4 (s)
1’	27.52 (t)	27.4 (t)
2’	121.68 (d)	121.3 (d)
3’	133.33 (s)	132.6 (s)
4’	17.96 (q)	17.9 (q)
5’	25.82 (q)	25.7 (q)
1’’	112.30 (t)	111.8 (t)
2’’	142.61 (s)	142.1 (s)
3’’	22.60 (q)	22.5 (q)
CH_3-C5	17.49 (q)	17.3 (q)

Table 4.23 The correlation of gHMBC and NOESY of compound **5**

Position	^{13}C-NMR	^{1}H-NMR	g-HMBC	g-NOESY
1	63.22 (d)	5.43 (s)	-	H-2, H-1'', 1-OH
1a	120.92 (s)	-	-	-
2	44.93 (d)	2.76 (s)	-	1-OH, H-3
3	64.56 (t)	4.37 (dd)	C-1, C-1'', C-2''	H-2, H-3, H-1''
		4.45 (dd)	C-2, C-4a, C-1''	H-2, H-3, H-1''
4a	149.45 (s)	-	-	-
5	116.89 (s)	-	-	-
6	119.38 (d)	7.32 (s)	C-4a, C-6a, C-12a	CH_3-C5
6a	152.26 (s)	-	-	-
7a	152.80 (s)	-	-	-
8	118.95 (s)	-	-	-
9	136.56 (d)	7.46 (d)	C-7a	H-10
10	109.73 (d)	6.77 (d)	C-8, C-10, C-11a	H-9
11	159.72 (s)	-	-	-
11a	109.24 (s)	-	-	-
12	184.51 (s)	-	-	-
12a	116.95 (s)	-	-	-
1'	27.52 (t)	3.52 (dd)	C-2, C-6, C7a, C-9, C3'	-
2'	121.68 (d)	5.34 (dd)	C-4', C-5'	H-1'
3'	133.33 (s)	-	-	-
4'	17.96 (q)	1.82 (s)	C-2', C-3', C-4'	H-4'
5'	25.82 (q)	1.78 (s)	C-8, C-2', C-3', C-5'	H-5'
1''	112.30 (t)	4.61 (s)	C-2, C-3''	H-1, H-3, H-1''
		4.83 (s)	C-2, C1'', C-3''	H-1'', H-3''
2''	142.61 (s)	-	-	-
3''	22.60 (q)	1.88 (s)	C-2, C-1'', C-2''	1-OH, H-1''
CH_3-C5	17.49 (q)	2.38	-	H-6
OH-C1	-	5.12 (s)	C-2, C-1''	H-1, H-1''
OH-C11	-	12.63	C-10, C-11, C-11a	-

Isolation of mycelium EtOAc extract crude by column chromatography and crystallization gave compound **6** as a white solid m.p. 228-230 ^{o}C, which showed the M^{+} ion at 370 in EIMS and on the basis of NMR analysis assigned the structure of stellatic acid.

The IR spectrum of compound **6** showed that the absorption peaks assigned as in Table 4.24 and indicated absorption bands were a OH stretching vibration at 3435 cm^{-1}, CH stretching vibration at 2945 and 2855 cm^{-1}, C=O stretching vibration at 1745 cm^{-1}, C=C stretching vibration at 1649 and 1563 cm^{-1}, C-H bending vibration at 1411 cm^{-1}, C-O stretching vibration at 1264 and 1022 cm^{-1}, C-H out of plane bending vibration at 656 cm^{-1}.

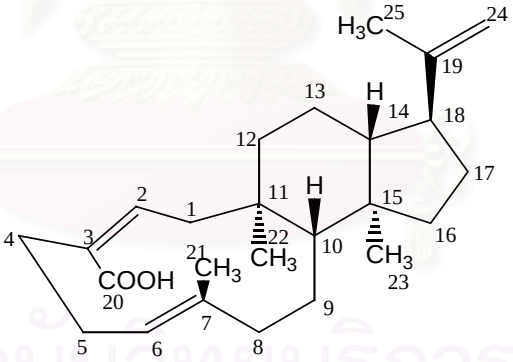
Table 4.24 The IR absorption bands assignment of compound **6**

Wave number (cm^{-1})	Intensity	Tentative assignment
3435	Broad, Strong	O-H stretching vibration of carboxylic acid
2945, 2855	Weak	stretching vibration of CH_2, CH_3
1745	Weak	C=O stretching vibration of carbonyl
1649, 1563	Medium	C=C stretching vibration of olefin
1411	Medium	C-H bending vibration of CH_2, CH_3
1264, 1022	Weak	C-O stretching vibration of carbonyl group
656	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum of compound **6** indicated that it possesses 3 olefinic protons (δ 4.73, 4.75 and 5.94 ppm) and four methyl group (δ 1.32, 0.90, 0.84 and 1.75 ppm).

The ^{13}C-NMR and DEPT spectrum showed 25 signals including six signals of olefinic carbons (δ 109.61, 123.68, 125.28, 139.74, 148.12 and 150.16 ppm), a acyl carbon at δ 173.19 ppm, four methyl signals (δ 15.57, 15.98, 19.98, 24.13 ppm), ten C_{sp3} methylene signals (δ 22.27, 22.35, 27.35, 34.85, 39.17, 40.37, 41.15, 42.64, 54.43 ppm), two methine signals (δ 47.59 and 49.63 ppm) and two quarternary carbons (δ 37.92 and 45.85 ppm).

The mass spectrum showed the molecular ion of compound **6** at *m/z* 370. On the basis of spectroscopic data including ^{1}H, ^{13}C, gHSQC, gHMBC, gCOSY, NOESY and TOCSY, the chemical structure of compound **6** was assigned as a known compound, stellatic acid (Figure 4.27). The long range correlations of gHMBC and NOESY was observed as shown in Figure 4.25 and Figure 4.26, respectively. The specific optical rotation of compound **6**, $[\alpha]_D^{25}$ +8 ($CHCl_3$, c 0.3), was corresponded to the previous report [Lit (Quereshi et. al., 1980) $[\alpha]_D^{25}$ +13.5 ($CHCl_3$, c 0.3)]



Stellatic acid

Figure 4.24 The chemical structure of compound **6**

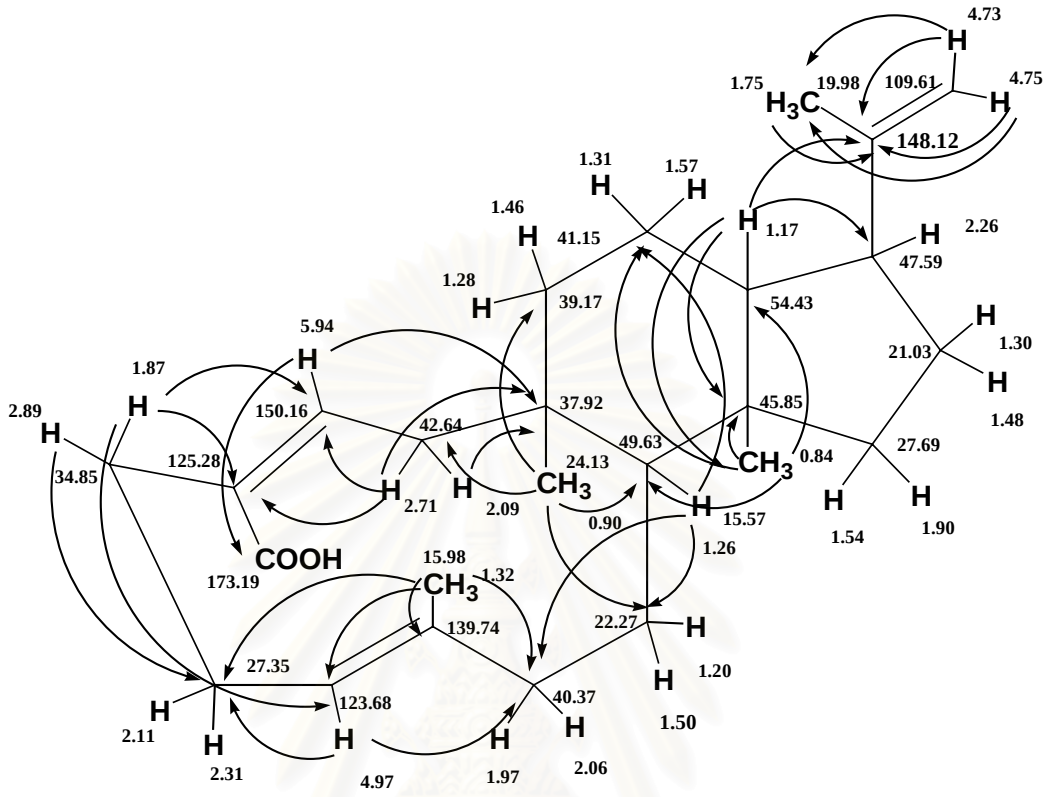


Figure 4.25 The gHMBC correlation of compound 6

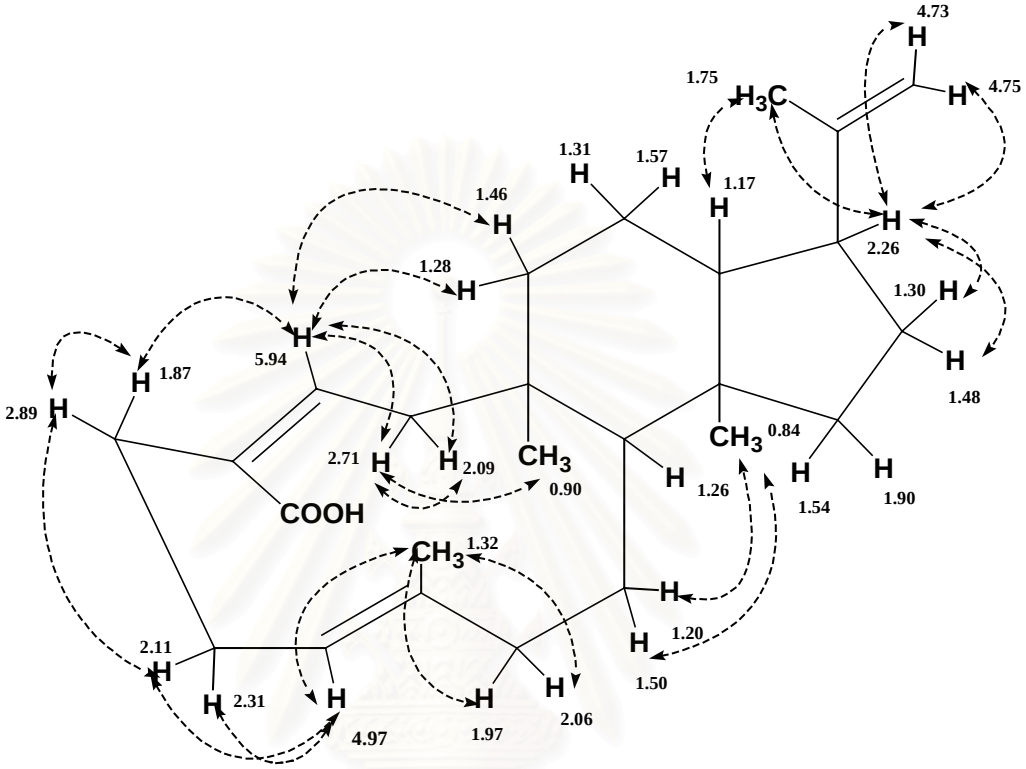


Figure 4.26 The NOESY correlation of compound 6

Table 4.25 ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **6**

Position	Compound **6**	
	δ_C	δ_H
1	42.64 (t)	2.09 (m) 2.71 (dd)
2	150.16 (d)	5.94 (d)
3	125.28 (s)	-
4	34.85 (t)	1.87 (m) 2.89 (d)
5	27.35 (t)	2.11 (m) 2.31 (m)
6	123.68 (d)	4.97 (dd)
7	139.74 (s)	1.32 (s)
8	40.37 (t)	1.97 (m) 2.06 (m)
9	22.27 (t)	1.20 (m) 1.50 (m)
10	49.63 (d)	1.26 (m)
11	37.92 (s)	-
12	39.17 (t)	1.28 (m) 1.46 (m)
13	41.15 (t)	1.31 (m) 1.57 (m)
14	54.43 (t)	1.17 (m)
15	45.85 (s)	-
16	27.69 (t)	1.54 (m) 1.90 (m)
17	21.03 (t)	1.30 (m) 1.48 (m)
18	47.59 (d)	2.26 (m)

Table 4.25 (continued) ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **6**

Position	Compound **6**	
	δ_C	δ_H
19	148.12 (s)	-
20	173.19 (s)	-
21	15.98 (q)	-
22	24.13 (q)	0.90 (s)
23	15.57 (q)	0.84 (s)
24	109.61 (t)	4.73 (m) 4.75 (m)
25	19.98 (q)	1.75 (s)

Table 4.26 Comparison of ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **6** and Stellatic acid

Position	Compound **6**		Stellatic acid	
	δ_C	δ_H	δ_C	δ_H
2	150.16 (d)	-	150.0 (d)	-
3	125.28 (s)	-	125.3 (s)	-
6	123.68 (d)	-	123.7 (d)	-
7	139.74 (s)	1.32 (s)	139.6 (s)	1.31 (s)
19	148.12 (s)	-	148.0 (s)	-
20	173.19 (s)	-	173.1 (s)	-
22	-	0.90 (s)	-	0.88 (s)
23	-	0.84 (s)	-	0.82 (s)
24	109.61 (t)	-	109.6 (t)	-
25	-	1.75 (s)	-	1.72 (s)

Isolation of mycelium EtOAc extract crude by column chromatography and crystallization gave compound **7** as yellow needle crystals, m.p. 219-220 ^{o}C, which showed the M^{+} ion *m/z* at 494 in EIMS and on the basis of NMR analysis assigned the structure of 14-methoxy-tajixanthone-25-acetate.

IR spectrum of compound **7** showed that the absorption peaks were assigned as in Table 4.27 and indicated absorption bands were a OH stretching at 3447 cm^{-1}, C-H stretching vibration at 2921 cm^{-1}, C=O stretching vibration at 1746 cm^{-1}, C=C stretching vibration at 1637 and 1559 cm^{-1}, CH bending vibration at 1470, 1423 and 1369 cm^{-1}, CO stretching vibration at 1236, 1077 and 1018 cm^{-1}, C-H out of plane bending vibration at 828 cm^{-1}.

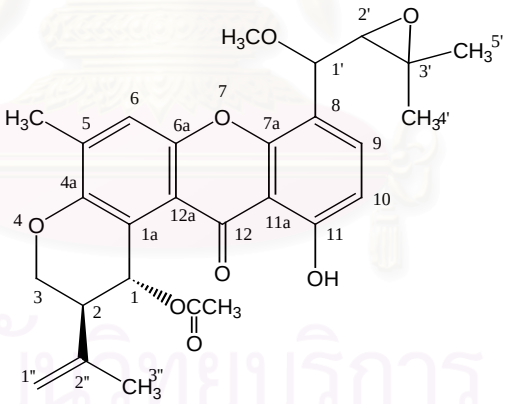
Table 4.27 The IR absorption bands assignment of compound **7**

Wave number (cm^{-1})	Intensity	Tentative assignment
3447	Broad, Strong	O-H stretching of alcohol
2921	Weak	C-H stretching vibration of CH_2, CH_3
1746	Medium	C=O stretching vibration of carbonyl group
1637 and 1559	Medium	C=C stretching vibration of aromatic ring
1470, 1423 and 1369	Weak	bending vibration of CH_2, CH_3
1236,1077 and 1018	Medium	C-O stretching vibration
828	Medium	C-H out of plane bending vibration

The ^{1}H-NMR data of compound **7** (Figure 4.27) indicated that it possesses four methyl groups at δ 1.26, 1.34, 1.92 and 2.38 ppm, a methoxy group at δ 3.37 ppm and a acetoxy group at δ 2.11 ppm, three aromatic proton at δ 6.86, 7.29 and 7.69 ppm, two olefinic proton at δ 4.79 and 4.84 ppm and a hydroxy group at δ 13.14 ppm.

The ^{13}C-NMR data of compound **7** (Figure 4.27) showed 28 signals consisting of a carbonyl carbon appeared at δ 183.16 ppm, an acetoxy carbon at δ 170.01 ppm, methyl carbons appeared at δ 19.84, 24.82, 17.39 and 22.42 ppm, a methoxy carbon at δ 56.76 ppm and a methyl of acetoxy at δ 21.26 ppm, aromatic carbons appeared at δ 109.11, 110.82, 114.90, 115.49, 116.21, 120.30, 135.10, 137.96, 150.33, 151.58, 152.49 and 162.61 ppm. Two signals of olefinic carbons at δ 112.80 and 141.44 ppm and five sp^3 carbons at δ 42.44, 63.77, 65.49, 66.69 and 76.08 ppm.

The NMR spectroscopic data (^{1}H, ^{13}C, gHMBC, gCOSY and NOESY) and molecular ion with m/z 494 could assign the structure of compound **7** as a known compound, 14-methoxy tajixanthone 25-actate. The gHMBC and NOESY correlations were illustrated in Figure 4.28 and 4.29. The ^{1}H and ^{13}C data and $[\alpha]_D$ of compound **7** were compared with 14-methoxy tajixanthone 25-actate. It was found that the NMR data were identical.



14-methoxy tajixanthone 25-actate

Figure 4.27 The chemical structure of compound **7**

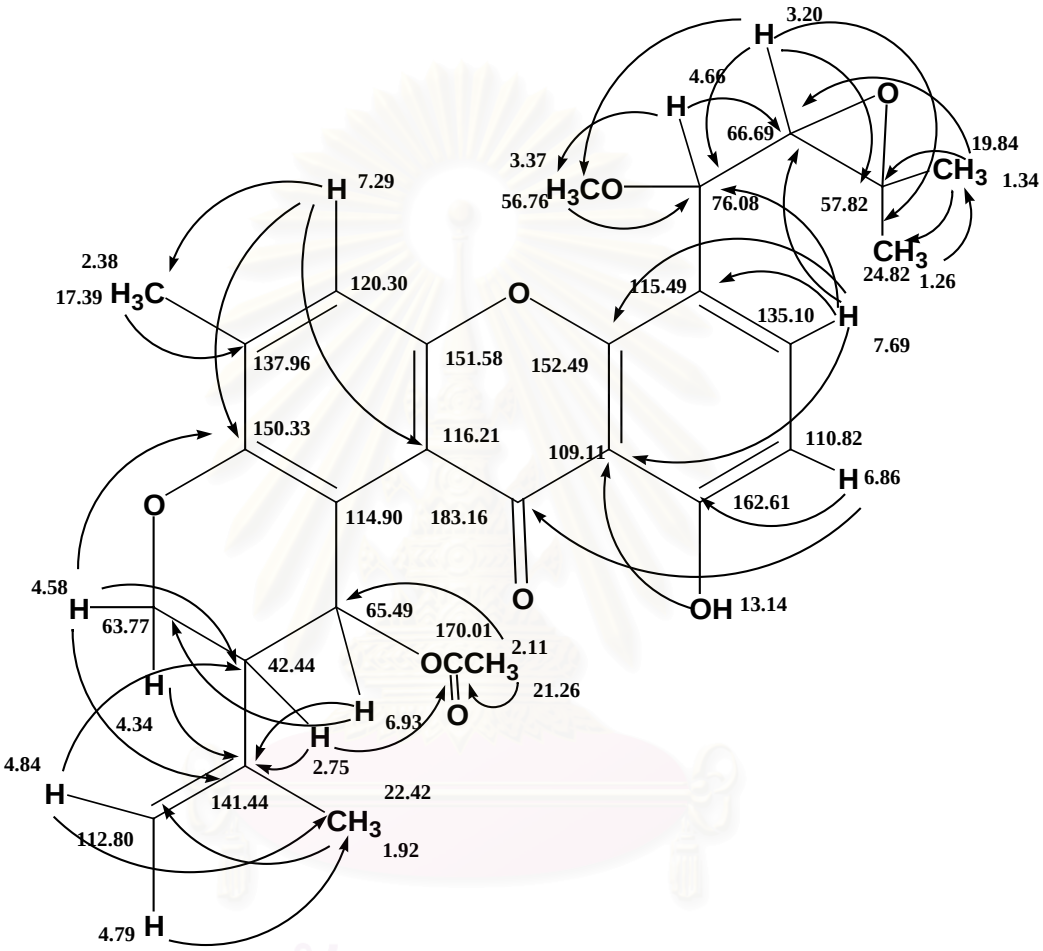


Figure 4.28 The gHMBC correlation of compound 7

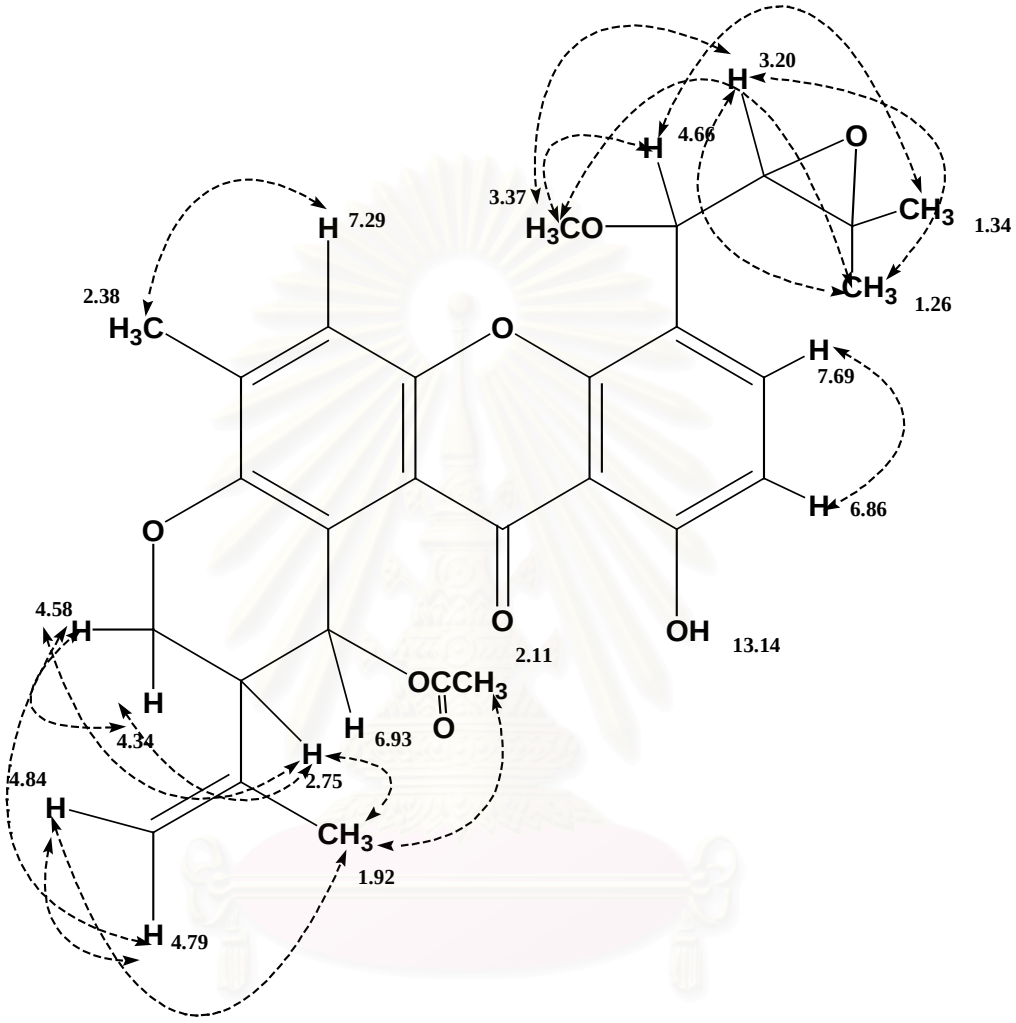


Figure 4.29 The NOESY correlation of compound 7

Table 4.28 ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **7**

Position	Compound 7	
	δ_C	δ_H
1	65.49 (d)	6.93 (s)
1a	114.90 (s)	-
2	42.44 (d)	2.75 (s)
3	63.77 (t)	4.34 (dd) 4.58 (dd)
4a	150.33 (s)	-
5	137.96 (s)	-
6	120.30 (d)	7.29 (s)
6a	151.58 (s)	-
7a	152.54 (s)	-
8	115.49 (s)	-
9	135.10 (d)	7.69 (d)
10	110.82 (d)	6.86 (d)
11	162.21 (s)	-
11a	109.11 (s)	-
12	183.16 (s)	-
12a	116.21 (s)	-
1’	76.08 (d)	4.66 (d)
2’	66.69 (d)	3.20 (d)
3’	57.82 (s)	-
4’	24.82 (q)	1.26 (s)
5’	19.84 (q)	1.34 (s)
1”	112.80 (t)	4.79 (s) 4.84 (s)
2”	141.44 (s)	-
3”	22.42 (q)	1.92 (s)

Table 4.28 (continued) ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **7**

Position	Compound 7	
	δ_C	δ_H
Me-C5	17.39 (q)	2.38 (s)
OH-C11	-	13.14 (s)
OCH_3-C1	56.77 (q)	3.37 (s)
$\underline{OC}=O\underline{C}H_3$-C-1	170.01 (s)	-
$OC=O\underline{CH_3}$-C1	21.27 (q)	2.11 (s)

Table 4.29 The correlation of gHMBC and NOESY of compound **7**

Position	^{13}C-NMR	^{1}H-NMR	g-HMBC	NOESY
1	65.49 (d)	6.93 (s)	C-1, C-1a, C-2, C-3, C-4a, C-12a, C-2', OC=OCH$_3$,	H-2, H-1'', H-3''
1a	114.90 (s)	-	-	-
2	42.44 (d)	2.75 (s)	C-1, C-1a, C-1'', C-2'', C-3''	H-1, H-3, H-3''
3	63.77 (t)	4.34 (dd)	C-1, C-2 , C-3, C-2''	H-2, H-3
		4.58 (dd)	C-1, C-2, C-4a, C-1'', C-2''	H-2, H-3, H-1''
4a	150.33 (s)	-	-	-
5	137.96 (s)	-	-	-
6	120.30 (d)	7.29 (s)	C-4a, C-6a, C-12, C-12a, Me-C5	CH$_3$-C5
6a	151.58 (s)	-	-	-
7a	152.54 (s)	-	-	-
8	115.49 (s)	-	-	-
9	135.10 (d)	7.69 (d)	C7a, C-11a, C-1'	H-10
10	110.82 (d)	6.86 (d)	C-7a, C-8, C-11, C-11a, C-12	H-9
11	162.21 (s)	-	-	-
11a	109.11 (s)	-	-	-
12	183.16 (s)	-	-	-
12a	116.21 (s)	-	-	-
1'	76.08 (d)	4.66 (d)	C-7a, C-8, C-9, C-2', C-3', OCH$_3$-C1'	H-2', H-5', OCH$_3$-C1
2'	66.69 (d)	3.20 (d)	C-1'. C3', C-5', OCH$_3$-C1'	H-1', H-4', OCH$_3$-C1
3'	57.82 (s)	-	-	-
4'	24.82 (q)	1.26 (s)	C-1', C-2', C-3', C-4'	H-2', H-5', OCH$_3$-C1
5'	19.84 (q)	1.34 (s)	C-2', C-3', C-5'	H-1', H-4'
1''	112.80 (t)	4.79 (s)	C-2, C-1'', C-2'', C3''	H-1, H-3, H-1''
		4.84 (s)	C-2, C-2'', C-3''	H-1'', H-3''
2''	141.44 (s)	-	-	-
3''	22.42 (q)	1.92 (s)	C-1, C-2, C1'', C-2'', OC=OCH$_3$	H-1, H-2, H-1'', OC=OCH$_3$
OH-C11	-	13.14 (s)	C-9, C-10, C-11, C-11a	-
OCH$_3$-C1	56.77 (q)	3.37 (s)	C-1', C-2'	H-1', H-2'
OC=OCH$_3$	170.01 (s)	-	-	-

Table 4.29 (continued) The correlation of gHMBC and NOESY of compound **7**

Position	^{13}C-NMR	^{1}H-NMR	g-HMBC	NOESY
OC=O$\underline{CH_3}$	21.27 (q)	2.11 (s)	C-1, C-3'', $\underline{OC=O}CH_3$	H-3'', CH_3-C5
CH_3-C5	17.39	2.38	C-4a, C-5, C-6	H-6, O=$COCH_3$

Isolation of mycelium EtOAc extract crude by column chromatography and crystallization gave compound **8** as yellow needle crystals m.p. 197-198 ^{o}C, which showed the M^{+} ion at *m/z* 424 in EIMS and on the basis of NMR analysis assigned the structure of novel compound, 8-(3-Hydroxy-2-methoxy-3-methylbutyl)-1,11-dihydroxy-2-isopropenyl-5-methyl-2,3-dihydro-1*H*-pyrano[3,2*a*]-xanthen-12-one.

IR spectrum of compound **8** showed that the absorption peaks were assigned as in Table 4.30 and indicated absorption bands were a O-H stretching vibration at 3447 cm^{-1}, C-H stretching vibration at 2968 and 2925 cm^{-1}, C=O stretching vibration at 1766 cm^{-1}, C=C stretching vibration at 1641 and 1567 cm^{-1}, CH bending vibration at 1466 and 1427 cm^{-1}, C-O stretching vibration at 1345, 1240, 1190, 1092 and 1049 cm^{-1}, C-H out of plane bending vibration at 820 and 765 cm^{-1}.

Table 4.30 The IR absorption bands assignment of compound **8**

Wave number (cm^{-1})	Intensity	Tentative assignment
3447	Broad,	O-H stretching vibration of alcohol
2968, 2925	Strong	C-H stretching vibration of CH_2, CH_3
1766	Weak	C=O stretching vibration of carbonyl
1641, 1567	Weak	C=C stretching vibration of olefinic
1466, 1427	Medium	bending vibration of parafinic
1345, 1240, 1092 and 1049	Medium	C-O stretching vibration
820, 765	Weak	C-H out of plane bending vibration

The ^{1}H-NMR spectrum of compound **8** indicated that it possesses three aromatic protons δ 6.79, 7.26 and 7.60 ppm, two olefinic proton (δ 4.59 and 4.82 ppm), two methylene protons at δ 2.68 with 3.15 ppm and δ 4.37 with 4.44 ppm, three methine protons at δ 2.75, 3.79 and 5.43 ppm, four methyl protons at δ 1.28, 1.35, 1.87 and 2.37 ppm and a methoxy proton at δ 3.32 ppm. Two proton of alcohol at δ 1.68 and 2.48 ppm, a hydroxy proton at δ 12.64 ppm.

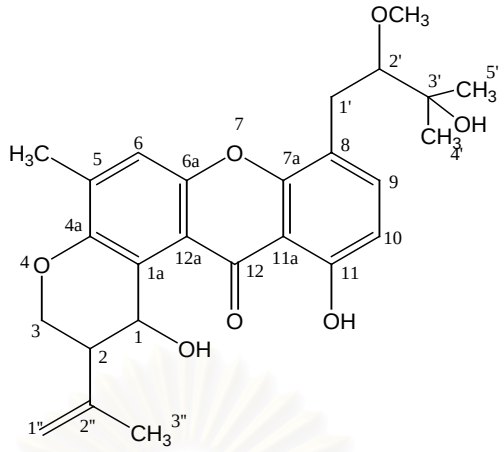
The ^{13}C-NMR and DEPT data could indicated the 26 carbon signals consisted of eleven signals of aromatic carbons appeared at δ 109.24, 109.93, 116.86, 116.90, 119.12, 121.11, 138.26, 138.39, 149.51, 152.10, 153.04 ppm and aromatic carbon attached to OH group at 160.25 ppm. Two olefinic carbons signals at δ 112.31 and 142.59 ppm. Two methylene carbon signals at δ 31.28 and 64.59 ppm. Three methine carbon signals at δ 44.93, 63.22 and 76.51 ppm and a quarternary carbon at δ 77.04 ppm. Four methyl carbons signals at δ 17.46, 19.29, 20.93 and 22.57 ppm, and a methoxy carbon at δ 49.31 ppm. The signal at δ 184.48 ppm.

Compound **8** showed a molecular ion at *m/z* 454 ($C_{26}H_{31}O_7$) that indicated DBE of 10. The information from 2D-NMR techniques, including gHSQC correlations, gCOSY correlations, NOESY correlations, gHSQC orrelations were used to assist the interpretion the structure of compound **8** as a novel compound, 8-(3-Hydroxy-2-methoxy-3-methylbutyl)-1,11-dihydroxy-2-isopropenyl-5-methyl-2,3-dihydro-1*H*-pyrano[3,2*a*]-xanthen-12-one (Figure 4.30).

The gHMBC correlation (Figure 4.31) showed that a proton of aromatic ring at δ 6.79 ppm was coupled with the carbons at δ 109.24, 109.93, and 160.25 ppm. The proton at 7.60 ppm coupled with the carbons at 31.26, 153.04 and 160.25 ppm, a hydroxy proton at δ 12.64 ppm coupled with the carbons at δ 109.24, 109.93 and 160.25 ppm; a proton of methyl at 1.28 ppm coupled with the carbons at δ 20.93, 76.51 and 77.04 ppm; a proton of at 1.35 ppm coupled with the carbons at δ 19.29, 76.51 and 77.04 ppm; a methyl group at 2.68 ppm coupled with the carbons at δ 76.51, 77.04, 116.90, 138.26 and 153.04 ppm; a proton at 3.15 ppm coupled with the carbons at δ

49.31, 76.51, 77.04, 116.90, 138.26 and 153.04 ppm; a proton at 3.79 ppm coupled with a carbon at δ 116.90 ppm; a proton of methoxy at δ 3.32 ppm coupled with the carbons at δ 76.51 and 77.04 ppm; a hydroxy proton at 2.48 ppm coupled with a carbons at 19.29 ppm; a proton of aromatic ring at δ 7.26 ppm was coupled with the carbons at δ 17.46, 116.86, 149.51 and 152.10 ppm; a proton of methyl at 2.37 ppm was coupled with the carbons at δ 116.86, 119.12 and 121.11 ppm; a proton at δ 4.37 ppm was coupled with the carbons at δ 63.22, 112.31, 138.39, 142.59 and 149.51 ppm; a proton at δ 4.44 ppm was coupled with the carbons at 63.22, 44.93, 142.59 and 149.51 ppm; the proton at δ 4.59 and 4.82 ppm was coupled with the carbons at δ 22.57, 44.93, 112.31 and 142.59 ppm; a proton of methyl at δ 1.87 ppm was coupled with the carbons at δ 44.93, 112.31 and 142.59 ppm; a proton at δ 2.74 ppm was coupled with the carbons at δ 116.86 and 138.39 ppm; a proton 12.64 ppm was coupled with the carbons at δ 109.24, 109.93 and 160.25 ppm.

The NOESY correlation (Figure 4.32) showed that a proton at δ 7.60 ppm was coupled with proton at 2.68 ppm; the protons at δ 1.28 and 1.35 ppm was coupled with the protons at δ 2.68, 3.15, 3.32 and 3.79 ppm; a hydroxy proton at δ 2.48 ppm was coupled with protons at δ 1.28 and 1.35 ppm; a proton at δ 2.68 ppm was coupled with the protons at δ 1.28, 1.35, 3.15 and 3.79 ppm; a proton at δ 3.15 ppm was coupled with the protons at δ 3.32 and 3.79 ppm; a proton at 3.32 ppm was coupled with the protons at δ 1.28, 1.35, 2.68, 3.15 and 3.79 ppm; a proton at 3.79 ppm was coupled with the protons at δ 1.28, 1.35, 2.68, 3.15 and 3.32 ppm; a proton at δ 1.68 ppm was coupled with the protons at δ 2.74, 4.82 and 5.43 ppm; a proton at δ 1.87 ppm was coupled with the protons at δ 2.74, 4.37, 4.44, and 5.43 ppm; the protons at δ 4.37 and 4.44 ppm was coupled with the protons at δ 2.74 and 4.59 ppm; a proton at 4.59 ppm was coupled with the protons at δ 4.37, 4.82 and 5.43 ppm; a proton at δ 4.82 ppm was coupled with a protons at δ 4.44 ppm; a proton at δ 5.43 ppm was coupled with a proton at δ 4.59 ppm.



8-(3-Hydroxy-2-methoxy-3-methylbutyl)-1,11-dihydroxy-

2-isopropenyl-5-methyl-2,3-dihydro-1*H*-pyrano[3,2*a*]-xanthen-12-one

Figure 4.30 The chemical structure of compound **8**

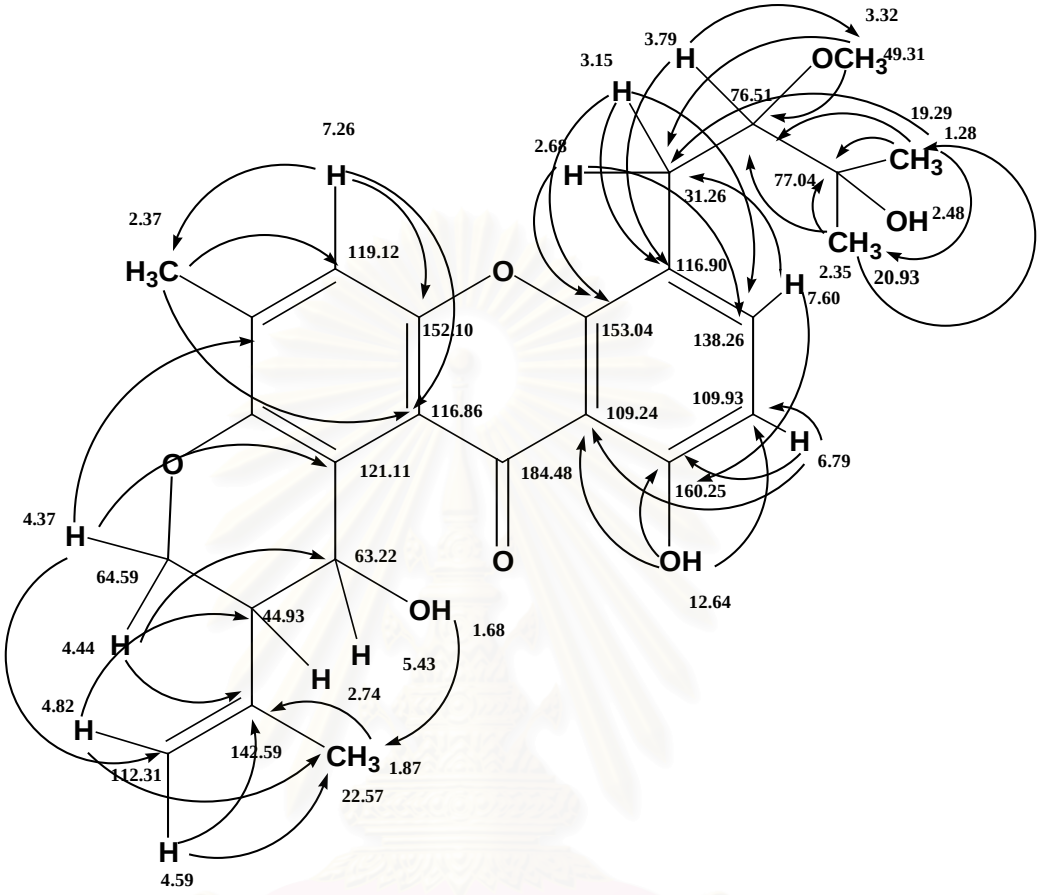


Figure 4.31 The gHMBC correlation of compound 8

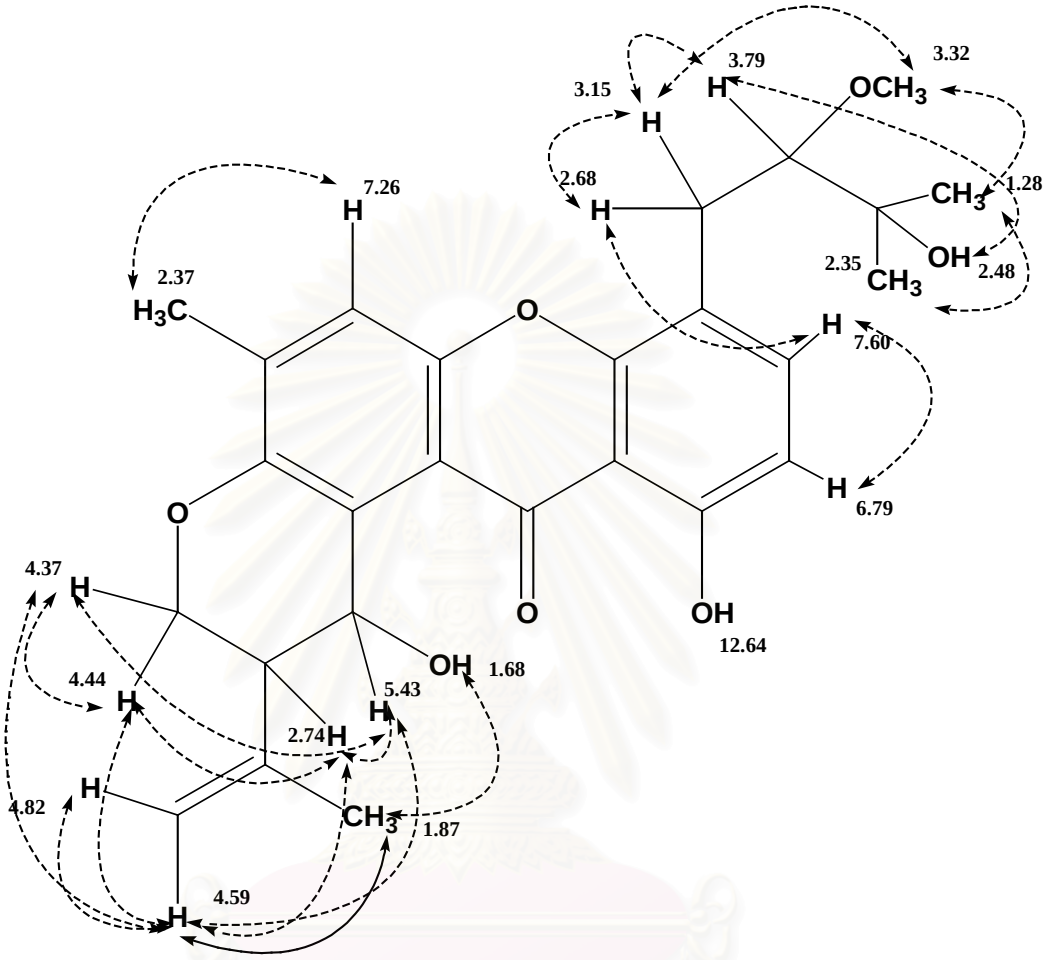


Figure 4.32 The NOESY correlation of compound 8

Table 4.31 ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **8**

Position	Compound **8**	
	δ_C	δ_H
1	63.22	5.43 (s)
1a	121.11	-
2	44.93	2.74 (ddd, *J* = 3.6, 3.6, 2.0 Hz)
3	64.59	4.37 (dd, *J* = 2.8 and 3.2 Hz) 4.44 (dd, *J* = 2.4 and 2.8 Hz)
4a	149.51	-
5	138.39	-
6	119.12	7.26 (d, *J* = 16.8 Hz)
6a	152.10	-
7a	153.04	-
8	116.90	-
9	138.26	7.60 (d *J* = 8.8 Hz)
10	109.93	6.79 (d *J* = 8.0 Hz)
11	160.25	-
11a	109.24	-
12	184.48	-
12a	116.86	-
1’	31.26	2.68 (s), 3.15 (dd *J* = 1.6 and 1.6 Hz)
2’	76.51	3.79 (d *J* = 10.4 Hz)
3’	77.04	-
4’	19.29	1.35 (s)
5'	20.93	1.28 (s)
1''	112.31	4.59 (s) 4.82 (s)
2''	142.59	-

Table 4.31 (continued) ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **8**

Position	Compound 8	
	δ_C	δ_H
3''	22.57	1.87 (s)
CH_3-C5	17.46 (q)	2.37 (s)
1-OH	-	1.68 (s)
3-OH	-	2.48 (s)
11-OH	-	12.64 (s)
2'-OCH_3	49.31 (q)	3.32 (s)

Table 4.32 The correlation of gHMBC and NOESY of compound **8**

Position	^{13}C-NMR	^{1}H-NMR	g-HMBC	NOESY
1	63.22	5.43 (s)	-	H-2, H-1''
1a	121.11	-	-	-
2	44.93	2.74 (ddd)	C-5, C-12a	H-1, H-3,H-1'',
3	64.59	4.37 (dd)	C-1, C-4a, C-5, C-1'', C-2''	H-2, H-3, H-1''
		4.44 (dd)	C-1, C-2, C4a, C-2''	H-2, H-3, H-1''
4a	149.51	-	-	-
5	138.39	-	-	-
6	119.12	7.26 (d)	C-4a, C-6a, C-12a, CH_3-C5	CH_3-C5
6a	152.10	-	-	-
7a	153.04	-	-	-
8	116.90	-	-	-
9	138.26	7.60 (d)	C-7a, C-11, C-1'	H-10, H-1'
10	109.93	6.79 (d)	C-10, C-11, C-11a	H-9
11	160.25	-	-	-
11a	109.24	-	-	-
12	184.48	-	-	-
12a	116.86	-	-	-
1'	31.26	2.68 (s)	C-7a, C-8, C-9, C-2', C-3'	H-1b', H-2', H-4', H-5'
		3.15 (dd)	C-7a, C8, C-9, C-2', OCH_3-C2'	H-2, H-1', H-2', H-4', H-5', 2'-OCH_3
2'	76.51	3.79 (d)	C-8	H-1a' , H-1b', H-4', H-5', OCH_3
3'	77.04	-	-	-
4'	19.29	1.35 (s)	C-2', C-3', C-4'	H-1a', H-1b', H-2', H-4', OCH_3
5'	20.93	1.28 (s)	C-2', C-3', C-5'	H-1',H-2', H-5', OCH_3
1''	112.31	4.59 (s)	C-2, C-1'', C-2'', C-3''	H-3, H-1''
		4.82 (s)	C-2, C-1'', C-2'', C-3''	H-3, H-3''
2''	142.59	-	-	-
3''	22.57	1.87 (s)	C-2, C-1'', C-2''	H-1, H-2, H-3, H-1b'', 1-OH
CH_3-C5	17.47	2.37 (s)	C-1a, C-6, C-12a	H-6
1-OH	-	1.68 (s)	C-3''	H-1, H-2
3-OH	-	2.48 (s)	C-4'	H-1', H-2', H-4', H-5'
11-OH	-	12.64 (s)	C-10, C-11, C-11a	-
OCH_3	49.31	3.32	C-2', C-3'	H-1', H-4', H-5'

Isolation of mycelium EtOAc extract crude by column chromatography and crystallization gave compound **9** as yellow needle crystals m.p. 194-195 ^{o}C, which showed the M^{+} ion at *m/z* 440 in EIMS and on the basis of NMR analysis assigned the structure of tajixanthone hydrate.

The IR spectrum of compound **9** showed that absorption peaks were assigned as summarized in Table 4.33 and indicated absorption bands were a O-H stretching vibration at 3486 cm^{-1}, C-H stretching vibration of aromatic ring at 3073 cm^{-1}, (C-H stretching vibration at 2976, 2883 and 1797 cm^{-1}, C=O stretching vibration at 1738 cm^{-1}, C=C stretching vibration at 1645 and 1571 cm^{-1}, CH bending vibration at 1474 cm^{-1}, C-O stretching vibration at 1345, 1244, 1046 and 1026 cm^{-1}, C-H out of plane bending vibration at 898, 820, 750 cm^{-1}.

Table 4.33 The IR absorption bands assignment of compound **9**

Wave number (cm^{-1})	Intensity	Tentative assignment
3486	Broad, Strong	O-H stretching vibration of alcohol
3073	Weak	C-H stretching vibration of aromatic
2976, 2883	Weak	ring
1797, 1738	Weak	C-H stretching vibration of CH_2, CH_3
1645, 1571	Medium	C=O stretching vibration
1474	Strong	C=C stretching vibration of olefinic
1345,1244	Medium	bending vibration of parafinic
1046, 1026	Strong	C-O stretching vibration
898, 820, 750	Medium	C-O stretching vibration
		C-H out of plane bending vibration

The ^{1}H-NMR spectrum of compound **9** indicated that it possesses one phenolic hydroxy proton (δ 12.57 ppm), three aromatic protons (δ 7.52, 7.22 and 6.75 ppm), two olefinic protons (4.56 and 4.80 ppm) two methylene protons (δ 2.64 ppm with 3.22 ppm and δ 4.34 ppm with 4.44 ppm) three methine protons (δ 2.72, 3.74 and 5.37 ppm) and four methyl groups (δ 1.36, 1.42, 1.85 and 2.34 ppm) and three hydroxy proton of alcohol (δ 2.40, 2.47 and 5.01 ppm).

The ^{13}C-NMR spectrum showed 25 signals indicated that the phenolic hydroxy carbon appeared at δ 160.31 ppm, the aromatic carbons appeared at δ 109.21, 109.92, 116.26, 116.79, 119.15, 120.81, 138.28, 138.56, 149.54, 151.95 and 153.10 ppm, two signals of olefinic carbons at δ 112.33 and 142.47 ppm, the signal at δ 184.29 ppm should be the carbonyl group and the sp^3 carbon signals at δ 17.44, 22.57, 23.59, 26.52, 32.00, 44.80, 63.16, 64.49, 72.90 and 77.70 ppm.

Table 4.34 Comparison of ^{13}C-NMR chemical shifts of compound **9** and Tajixanthone hydrate

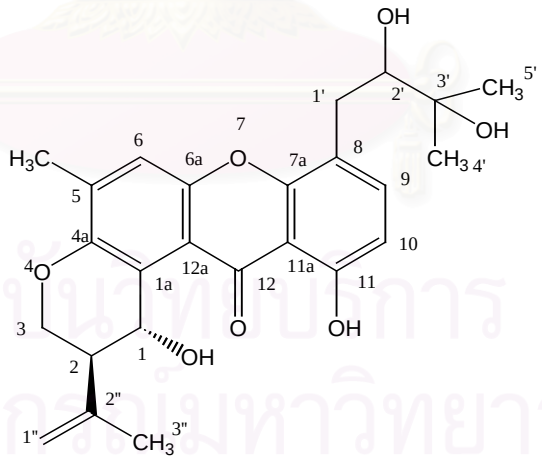
Position	Compound **9**	Tajixanthone hydrate
	δ_c	δ_c
1	63.16 (d)	62.9 (d)
1a	120.81 (s)	120.4 (s)
2	44.80 (d)	44.6 (d)
3	64.49 (t)	64.3 (t)
4a	149.54 (s)	149.0 (s)
5	138.56 (s)	137.9 (s)
6	119.15 (d)	118.7 (d)
6a	151.95 (s)	151.3 (s)
7a	153.10 (s)	159.5 (s)
8	116.26 (s)	115.9 (s)
9	138.28 (d)	137.7 (d)
10	109.92 (d)	109.5 (d)
11	160.31 (s)	152.4 (s)
11a	109.21 (s)	108.7 (s)
12	184.29 (s)	183.5 (s)
12a	116.79 (s)	116.3 (s)
1’	32.00 (t)	31.9 (t)
2’	77.70 (d)	77.4 (d)
3’	72.90 (s)	72.6 (s)
4’	23.59 (q)	23.6 (q)
5’	26.52 (q)	26.3 (q)
1’’	112.33 (t)	111.8 (t)
2’’	142.47 (s)	141.9 (s)

Table 4.34 (continued) Comparison of ^{13}C-NMR chemical shifts of compound **9** and Tajixanthone hydrate

Position	Compound **9**	Tajixanthone hydrate
	δ_C	δ_C
3''	22.57 (q)	22.4 (q)
CH_3-C5	17.44 (q)	17.3 (q)
OH-C11	-	-
OH-C2'	-	-
OH-C3'	-	-

Two-dimensional NMR techniques were used to assist the structure assignment and The protons directly attached to carbon in compound **9** were assigned by gHMBC and NOESY spectra assigned in Figure 4.34 and 4.35, respectively.

^{13}C-NMR data of compound **9** and tajixanthone hydrate were compare in Table 4.34 and the data were similar to each other.



Tajixanthone hydrate

Figure 4.33 The chemical structure of compound **9**

Table 4.35 The correlation of gHMBC and NOESY of compound **9**

Position	^{13}C-NMR	^{1}H-NMR	g-HMBC	NOESY
1	63.16 (d)	5.37 (s)	C1a,C-2, C-3, C-4a	H-2, H-1'', 1-OH
1a	120.81 (s)	-	-	-
2	44.80 (d)	2.72 (br)	C-1, C-5, C-12a, C-1''	H-1, H-3a
3	64.49 (t)	4.34 (dd)	C-1, C1'', C-2''	H-3, H-1''
		4.44 (dd)	C-1, C-2, C-4a, C-2''	H-3, H-1''
4a	149.54 (s)	-	-	-
5	138.56 (s)	-	-	-
6	119.15 (d)	7.22 (s)	C-1a, C-4a, C-5, C-6a, C-12a, CH_3-C5	CH_3-C5
6a	151.95 (s)	-	-	-
7a	153.10 (s)	-	-	-
8	116.26 (s)	-	-	-
9	138.28 (d)	7.52 (d)	C7a, C-9, C-10, C-11, C-11a, C-1'	H-10
10	109.92 (d)	6.75 (d)	C-7a, C-9, C-11, C-11a	H-9
11	160.31 (s)	-	-	-
11a	109.21 (s)	-	-	-
12	184.29 (s)	-	-	-
12a	116.79 (s)	-	-	-
1'	32.00 (t)	2.64 (dd)	C-1a, C-7a, C-8, C-9, C-2', C-3'	H-1b'
		3.22 (dd)	C-1a, C-7a, C-8, C-9, C-2', C-3'	H-1', H-2'
2'	77.70 (d)	3.74 (d)	-	H-1a', H-5'
3'	72.90 (s)	-	-	-
4'	23.59 (q)	1.42 (s)	C-2', C-3', C-4'	H-4', H-1', H-2'
5'	26.52 (q)	1.36 (s)	C-2', C-3', C-5'	H-5', H-1'
1''	112.33 (t)	4.56 (s)	C-2, C-1'', C-2'', C-3''	H-3, H-1'', 1-OH
		4.80 (s)	C-2, C-1'', C-2'', C-3''	H-1'', 1-OH
2''	142.47 (s)	-	-	-
3''	22.57 (q)	1.85 (s)	C-2, C-1'', C-2''	CH_3-C5, 1-OH

Table 4.35 (continued) The correlation of gHMBC and NOESY of compound **9**

Position	^{13}C-NMR	^{1}H-NMR	g-HMBC	NOESY
CH_3-C5	17.44 (q)	2.34 (s)	C-1a, C-4a, C-5, C-6, C-12a	H-6, 1-OH
OH-1	-	5.01 (s)	C-1, C-2, C-1''	H-1, H-1'', CH_3-C5
OH-C11	-	12.57 (s)	C-9, C-10, C-11, C-11a	-
OH-C2'	-	2.40 (s)	C-2', C-3'	H-1'
OH-C3'	-	2.47 (s)	C-1'	H-1'

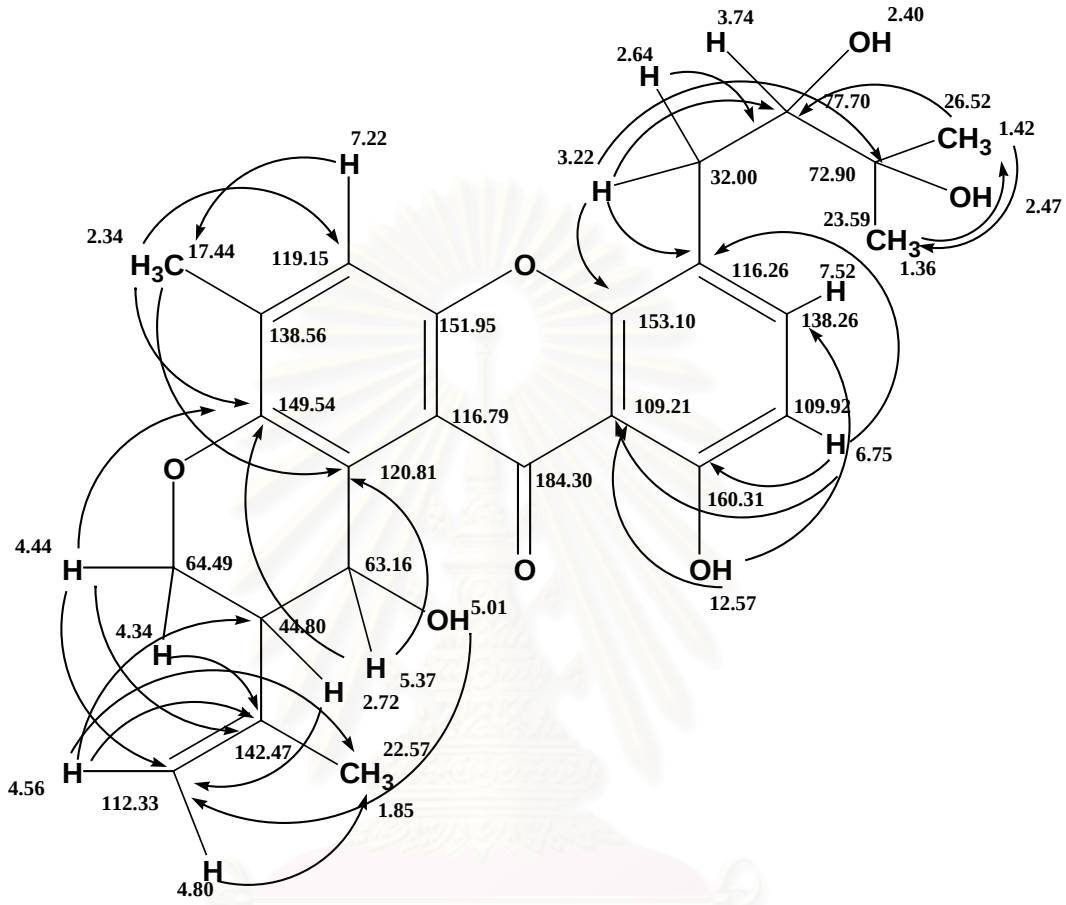


Figure 4.34 The gHMBC correlation of compound 9

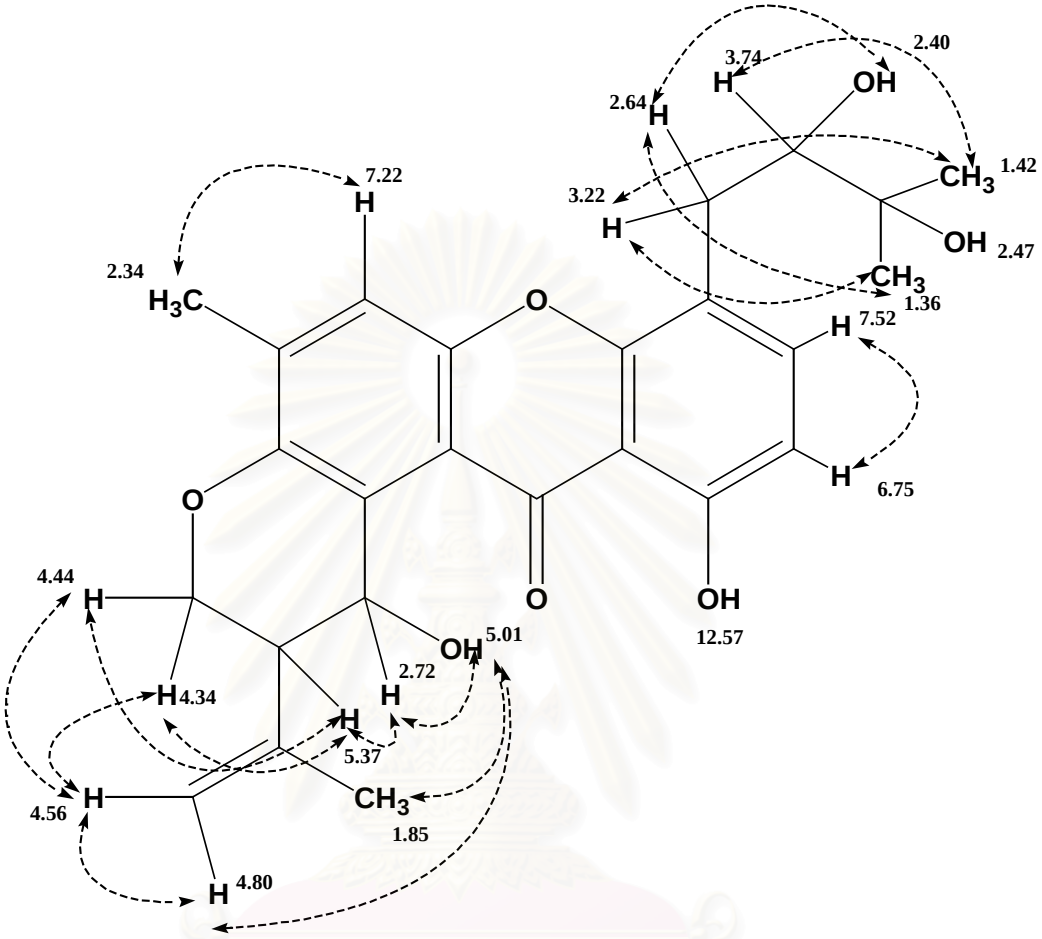


Figure 4.35 The NOESY correlation of compound 9

Table 4.36 ^{13}C-NMR data of compound **5**, **7**, **8** and **9**

Position	Chemical shift of ^{13}C-NMR			
	Compound 5	Compound 7	Compound 8	Compound 9
1	63.22 (d)	65.49 (d)	63.22 (d)	63.16 (d)
1a	120.92 (s)	114.90 (s)	121.11 (s)	120.81 (s)
2	44.93 (d)	42.44 (d)	44.93 (d)	44.80 (d)
3	64.56 (t)	63.77 (t)	64.59 (t)	64.49 (t)
4a	149.45 (s)	150.33 (s)	149.51 (s)	149.54 (s)
5	138.38 (s)	137.96 (s)	138.39 (s)	138.56 (s)
6	119.38 (d)	120.30 (d)	119.12 (d)	119.15 (d)
6a	152.26 (s)	151.58 (s)	152.10 (s)	151.95 (s)
7a	152.80 (s)	152.54 (s)	153.04 (s)	153.10 (s)
8	118.95 (s)	115.49 (s)	116.90 (s)	116.26 (s)
9	136.56 (d)	135.10 (d)	138.26 (d)	138.28 (d)
10	109.73 (d)	110.82 (d)	109.93 (d)	109.92 (d)
11	159.72 (s)	162.21 (s)	160.25 (s)	160.31 (s)
11a	109.24 (s)	109.11 (s)	109.24 (s)	109.21 (s)
12	184.51 (s)	183.16 (s)	184.48 (s)	184.29 (s)
12a	116.89 (s)	116.21 (s)	116.86 (s)	116.79 (s)
1’	27.52 (t)	76.08 (d)	31.26 (t)	32.00 (t)
2’	121.68 (d)	66.69 (d)	76.51 (d)	77.70 (d)
3’	133.33 (s)	57.82 (s)	77.04 (s)	72.90 (s)
4’	17.96 (q)	19.84 (q)	19.29 (q)	23.59 (q)
5’	25.82 (q)	24.82 (q)	20.93 (q)	26.52 (q)
1’’	112.30 (t)	112.80 (t)	112.31 (t)	112.33 (t)
2’’	142.61 (s)	141.44 (s)	142.59 (s)	142.47 (s)
3’’	22.60 (q)	22.42 (q)	22.57 (q)	22.57 (q)
CH_3-C5	17.49 (q)	17.39 (q)	17.37 (q)	17.44 (q)
OCH_3-C1’	-	56.77 (q)	-	-

Table 4.36 (continued) ^{13}C-NMR data of compound **5**, **7**, **8** and **9**

Position	Chemical shift of ^{13}C-NMR			
	Compound 5	Compound 7	Compound 8	Compound 9
OCH_3-C2'	-	-	49.31 (q)	-
OC=***O***CH_3- C1	-	170.01 (s)	-	-
OC=O***CH_3***-C1	-	21.27 (q)	-	-

Table 4.37 ^{1}H-NMR data of compound **5, 7, 8, 9**

Position	Chemical shift of ^{1}H-NMR			
	Compound 5	Compound 7	Compound 8	Compound 9
1	5.43 (s)	6.93 (s)	5.43 (s)	5.37 (s)
2	2.76 (s)	2.75 (s)	2.74 (ddd)	2.72 (br)
3a	4.37 (dd)	4.34 (dd)	4.37 (dd)	4.34 (dd)
3b	4.45 (dd)	4.58 (dd)	4.44 (dd)	4.44 (dd)
6	7.32 (s)	7.29 (s)	7.26 (d)	7.22 (s)
9	7.46 (d)	7.69 (d)	7.60 (d)	7.52 (d)
10	6.77 (d)	6.86 (d)	6.79 (d)	6.75 (d)
1a'	3.52 (dd)	4.66 (d)	2.68 (s)	2.64 (dd)
1b'	3.52 (dd)	-	3.15 (dd)	3.22 (dd)
2'	5.34 (dd)	3.20 (d)	3.79 (d)	3.74 (d)
4'	1.78 (s)	1.26 (s)	1.28 (s)	1.36 (s)
5'	1.82 (s)	1.34 (s)	1.35 (s)	1.42 (s)
1a''	4.61 (s)	4.79 (s)	4.59 (s)	4.56 (s)
1b''	4.83 (s)	4.84 (s)	4.82 (s)	4.80 (s)
3''	1.88 (s)	1.92 (s)	1.87 (s)	1.85 (s)
CH_3-C5	2.38 (s)	2.38 (s)	2.37 (s)	2.34 (s)
OH-C1	5.12 (s)	-	1.68 (s)	5.01 (s)
OC=OCH_3-C1	-	2.11 (s)	-	-
OH-C11	12.63 (s)	13.14 (s)	12.64 (s)	12.57 (s)
OH-C1'	5.12	-	1.68	5.01
OH-C2'	-	-	-	2.40 (s)
OH-C3'	-	-	2.48 (s)	2.47 (s)
OCH_3-C1'	-	3.37 (s)	-	-
OCH_3-C2'	-	-	3.32 (s)	-

4.8 Cytotoxic activity test against cancer cell lines

In *vitro* cytotoxiv activity of compound **2-9** against five cell lines, including HEP-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) cancer was reported in Table 4.38.

Table 4.38 Cytotoxic activity against cell lines of compound **2-9**

Compound	IC_{50} μg/ml (nM)				
	HEP-G2 (hepatoma)	SW 620 (colon)	CHAGO (lung)	KATO-3 (gastric)	BT474 (breast)
2	>10	>10	>10	>10	>10
3	9.2 (31.5)	5.6 (19.2)	5.9 (20.2)	5.9 (20.2)	5.2 (17.8)
4	10	>10	8.1 (52.6)	7.8 (50.6)	4.4 (28.6)
5	>10	8.7 (21.4)	>10	6.1 (15.0)	5.1 (12.5)
6	>10	-	>10	-	-
7	8.7 (17.6)	7.1 (14.4)	>10	5.7 (11.5)	6.0 (12.1)
8	>10	8.7 (19.2)	7.8 (17.2)	9.1 (20.0)	6.4 (14.1)
9	7.2 (16.4)	6.0 (13.6)	5.1 (11.6)	4.8 (10.9)	5.4 (12.3)

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

The results showed that a mixture of long chain carboxylic acid C_{24-26} **2** and stellatic acid **6** were inactive against cancer cell lines. Lasiodiplodin **3** exhibited cytotoxic activity against HEP-G2, SW 620, CHAGO, KATO-3 and BT 474 with IC_{50} 31.5, 19.2, 20.2, 20.2 and 17.8, respectively. Xanthone derivatives **5**, **7**, **8** and **9** seem to inhibit selectively against beast cancer cell. Shamixanthone **5** exhibited cytotoxic activity against SW 620, KATO-3 and BT 474 cell lines with IC_{50} 21.4, 15.0 and 12.5 nM, respectively. 14-methoxy-tajixanthone-25-acetate **7** exhibited high cytotoxic activity against KATO-3 (gastric) and BT 474 (breast) cell lines with IC_{50} 11.5 and 12.1 nM, respectively which other cell lines were lesser activity. The most active compound compared with other was tajixanthone hydrate **9** which exhibited cytotoxic activity against five cell lines with IC_{50} 16.4, 13.6, 11.6, 10.9, and 12.3 nM, respectively. Furthermore, a novel compound **8** inhibit HEP-G2, SW 620, CHAGO, KATO-3 and BT 474 with lowest concentration (IC_{50} 14.1 nM) against BT 474 cell line, with moderate concentration (IC_{50} 19.2, 17.2 and 20.0 nM against SW 620, CHAGO and KATO-3 cell lines, respectively and with high concentration against HEP-G2 cell lines.

CHAPTER V

CONCLUSION

The objectives of this research were the isolation of endophytic fungi from *Croton oblongifolius* obtained from Chachoengsao province, antimicrobial activity test, identification of the fungi based on their morphology, physiology, cultural characteristics and ITS region's base sequence. Moreover, the fungal metabolites were determined using specroscopic data. The isolated compounds were tested for their cytotoxicity against cancer cell lines including HEP G-2 (hepatoma), SW 20 (colon), CHAGO (lung), KATO-3 (gastric) and BT474 (breast) cancers.

Endophytic fungi were isolated from leaves, petrioles, twigs and barks of *Croton oblongifolius* using surface sterilize technique. Leaves, petrioles and twigs were surface sterilized with 95 % ethanol for 1 minutes, 10 % sodium hypochlorite solution for 5 minutes, 95 % ethanol for 30 seconds. For twigs and bark were surface sterilized with saturated sodium hypochlorite for 5 min and washed 2 times in sterilized water for 1 min. (Schulz et al., 1993). The 84 isolates of endophytic fungi were found to be mycelia sterilia (41 isolates), Coelomycetes (17 isolates), *Phomopsis sp.* (21 isolates), *Cladosporium sp.* (2 isolates), *Tetraploa sp*, (1 isolate), *Emericella variecolor* (1 isolate), *Lasiodiplodia theobromae* (1 isolate). The endophytic fungi were tested for the inhibition of bacteria and yeasts by agar diffusion method.

The selected fungi were identified by slide culture method and strain CsLm 08 and CsPm 09 were identified by ITS_{1f-4} base pair regions. ITS_{1f-4} regions were amplified from the representative sample of isolated endophytic fungi. Amplified ITS_{1f-4} fragments were cloned using pT7 Blue vectors (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* strain XL1-Blue MRF. Plasmid DNA was extracted from possitive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Texas Red (Hitachi) in and SQ-5500E sequencer (Kanchanaprayudh et. al., 2003). ITS_{1f-4} sequences were automatically

aligned with fungi ITS sequences obtained from GenBank DNA database (http://www.ddbj.nig.ac.jp). ITS sequences of CsLm08 found that base pair sequences were 98.633 percent similar with ITS sequences of *Lasiodiplodia theobromae*. Nucleotide sequences of ITS regions of CsPm09 were similar 99.261 percent with nucleotide sequences of *Emericella variecolor* ITS regions.

In the present investigation, three metabolites from mycelia of *Lasiodiplodia theobromae* were isolated by using silica gel column chromatography. They were identified as Ergosta-5,22-dien-3-ol **1**, mixture of long chain carboxylic acid $C_{24\text{-}26}$ **2** and lasiodiplodin **3**. Metabolites of *Emericella variecolor* obtained from fermentation broth was mainly terrein **4** and metabolites from mycelia were stellatic acid **6** and four xanthones derivatives consisting of shamixanthone **5**, 14-methoxy-tajixanthone-25-acetate **7**, tajixanthone hydrate **9** and a novel xanthone, 8-(3-hydroxy-2-methoxy-3-methyl-butyl)-1,11-dihydroxy-2-isopropenyl-5-methyl-2,3-dihydro-1*H*-pyrano[3,2*a*]-xanthen-12-one **8**. These structures were estrablished on basis of physical properties and detail analyses of spectroscopic data including the ^{1}H-NMR, ^{13}C-NMR, MS, UV and 2D NMR including gHSQC, gCOSY, gHMBC, NOESY and TOCSY spectra.

The bioassay of cytotoxic activity against five tumor cell lines *in vitro*, which were Hep-G2 (hepatoma), SW 620 (colon), CHAGO (lung), KATO-3 (gastric) and BT 475 (breast) found that a mixture of long chain carboxylic acid and stellatic acid **6** were inactive against cancer cell lines. Lasiodiplodin **3** exhibited cytotoxic activity against HEP-G2, SW 620, CHAGO, KATO-3 and BT 474 with IC_{50} 31.5, 19.2, 20.2, 20.2 and 17.8, respectively. Xanthone derivatives **5**, **7**, **8** and **9** seem to inhibit selectively against beast cancer cell. Compound **5** exhibited cytotoxic activity against SW 620 (colon), KATO-3 (gastric) and BT 474 (breast) cell lines with IC_{50} 21.4, 15.0 and 12.5 nM, respectively. Compound **7** exhibited high cytotoxic activity against KATO-3 (gastric) and BT 474 (breast) cell lines with IC_{50} 11.5 and 12.1 nM, respectively which other cell lines were lesser activity. The most active compound compared with other was tajixanthone hydrate **9** which exhibited cytotoxic activity against five cell lines with IC_{50} 16.4, 13.6, 11.6, 10.9, and 12.3 nM, respectively. Furthermore, a novel compound **8** exhibited

cytotoxic activity against BT 474 cell line with lowest concentration (IC_{50} 14.1 nM), against SW 620 (colon), CHAGO (lung) and KATO-3 (gastric) cancer cell lines with moderate concentration (IC_{50} 19.2, 17.2 and 20.0 nM), respectively and with high concentration against HEP-G2 cell lines.

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APPENDICES

Appendix A

Table 1

No.	Compound	Endophytic fungi	Host plants	Activities	References
1.	1,3-*O*-di-trans-p-coumaroylglycerol	*Epichole typhina*	*Phleum pratense*	-	Koshino et al.,
2.	1,2-*O*-di-trans-p-coumaroylglycerol			-	1989
3.	Chokorin			-	
4.	Trans-p-coumaric acid			-	
5.	Cis-p-coumaric acid			Antifungal	
6.	p-hydroxybenzoic acid			Antifungal	
7.	p-hydroxyphenylacetic acid			Antifungal	
8.	tyrosol			Antifungal	
9.	1(10→6)abeo-ergosta-5,7,9,22-tetraen-3α-ol	*Epichloe typhina*	*Phleum pratense*	-	Koshino et al., 1989

Table1. (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
10.	L-671,329	*Cryptosporiopsis* sp. and *Pezicula* sp.	*Pinus sylvestris* and *Fagus sylvatica*	Antifungall	Noble et al., 1991
11. 12. 13. 14.	Heptedic acid Heptedic acid chlorohydrin Hydroxyheptedic acid Rugulosin	*Phyllostica* sp. *Hormonema dematioides*	*Abies balsamea* and *Picea rubens*	Toxic to spruce budworm	Calhoun et al., 1992
15. 16.	3-hydroxy-9-oxo-4-tetradecyl-5-oxa-1-azabicyclo[4.3.0.]nonane-2-methanol 3-hydroxy-9-oxo-4-(4E-tetradecenyl)-5-oxa-1-azabicyclo [4.3.0.]nonane-2-methanol	*Epichloe typhina*	*Phleum pratense*	Against *Cladosporium herbarum*	Koshino et al., 1992

Table 1.(continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
17.	Taxol	*Taxomyces andreanae* *Pestalotiopsis microspora* *Pestalotiopsis microspora* *Tubercularia sp*. strain TF5 *Aspergillus niger*	*Taxus brevifolia* *Taxodium distichum* *Taxus wallachiana* *Taxus mairei* *Taxus chinensis*	Anticancer and antifungal	Stierle et al., 1993. Strobel et al.,1993 Stierle et al., 1995. Li et al., 1996 Li et al., 1998 Wang et al., 2000 Metz et al., 2000 Wang et al., 2001
18. 19.	Ergobalansine Ergobalansinine	*Balansia obtecta* *Balansia cyperi*	*Cenchrus echinatus*	- -	Powell et al., 1990
20. 21.	Paspalitrem A Paspalitrem C	*Phomopsis sp.*	*Cavendishia pubescens*	Causative agent	Bill et al., 1992. Tan and Zou, 2001

Table (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
22. 23. 24. 25. 26. 27. 28.	Paxilline 7α-hydroxypaxilline 7α-hydroxy-13-desoxy paxilline 10β-hydroxy13-desoxypaxilline 5α-ergosta-7,22-dien-3β-ol Tryptophol β-hydroxyphenylalanyl-propyl diketopiperrazine	*Acremonium lolii* and *Penicillium paxilli*	*Lolium perenne*	Tremorgenic mycotoxin	Mantle et al., 1994.
29. 30. 31. 32.	Lysergic acid amide Ergonovine Isolysergic acid amide Ergonovinine	*Acremonium* sp.	*Achnatherum inebriens*	Drunken horse grass	Garner et al., 1993 Miles et al., 1996

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
33.	Lolicine A	*Acremonium lolii*	*Lolium perenne*	Tremogenic	Munday-Finch et al.,
34.	Lolicine A 11-*O*-propionate	*Neotyphodium lolii*		mycotoxin	1996
35.	Lolicine B				Berny et al., 1997
36.	Lolicine B 11-*O*-propionate				Munday-Finch et al.,
37.	Lolitrem B				1998
38.	Lolitrem F				Gaterby., et al., 1998
39.	lolitrem H				
40.	31-epi-Lolitrem B				
41.	31-epi-Lolitrem F				
42.	Lolitriol				
43.	Lolitriol 10-O-propionate				
44.	Lolitrem N				
45.	Lolitrem N 10-O-propionate				
46.	31-epi-Lolitrem N				
47.	31-epi-Lolitrem F 10-O-acetate				

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
48.	Paspaline B	*Acremonium lolii*	*Lolium perenne*	Tremogenic	Munday-Finch et al.,
49.	Terpendole E			mycotoxin	1996
50.	Terpendole F				Berny et al., 1997
51.	Terpendole G				Munday-Finch et al.,
52.	Terpendole M				1998
53.	Terpendole C				Gaterby., et al., 1998
54.	Paspaline				
55.	13-Desoxypaxilline				
56.	Paspalinine				
57.	14α-Hydroxypaspalinine				
58.	lolitrem H				
59.	Paspalicine				
60.	Paspalinine				
61.	α-Paxitriol				

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
62. 63. 64. 65. 66. 67.	Lolinine Loline *N*-formylloline *N*-methylloline *N*-Acetylnorloline 5,6-dehydro-*N*-acetylloline	*Acremonium* sp. *Neotyphodium* spp. and *Epichloe* spp. *Neotyphodium uncinatum*	*Festuca argentina* *Argyreia mollis* *Festuca pratensis*	Toxic to livestock	Casabuono et al.,1997 Tofern et al., 1999 Blankenship et al., 2001
68. 69. 70. 71. 72. 73. 74. 75. 76.	Ergine Aci-ergovaline Didehydroergovaline Ergobalansine Ergotamine Ergosine β-Ergosine Ergovaline Ergostine	*Neotyphodium coenophialum*	*Festuca arundinacea*	Drunken horse grass	Sherby et al.,1997 Tan and Zou, 2001

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
77. 78. 79. 80. 81. 82. 83.	Ergoptine β-Ergoptine Ergonine Ergocristine α -Ergocryptine β- Ergocryptine Ergocornine	*Neotyphodium coenophialum*	*Festuca arundinacea*	Drunken horse grass	Sherby et al.,1997 Tan and Zou, 2001
84. 85.	α-ergocryptine α-ergocryptinine	*Claviceps zizaniae*	*Zizinia aquqtica* and *Z.palustris*	Drunken horse grass	Kantorova et al.,2002
86.	Peramine	*Acremonium lolii*	*Lolium perenne*	Resistant to argentine stem weevil	
87.	Phomodiol	*Phomopsis* spp.	*Salix* spp.	Antifungal	Horn et al.,1994.
88. 89.	Altersolanol A 2-hydroxy-6-methylbenzoic acid	*Phoma* sp.	*Taxus wallachiana*	Antibacterial	Yang et al., 1994 Strobel et al., 1998

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
90. 91. 92.	Pestalopyrone Hydroxypestalopyrone Pestaloside	*Pestalotiopsis microspora*	*Pestalotiopsis microspora*	Antifungal	Lee et al., 1995
93. 94. 95. 96. 97.	(R)-mellein (-)-mycorrhizin 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde (+)-cryptosporiopsin 4-epi-ethisolide	*Pezicula* sp.	*Fagus sylvatica*	Antifungal, algicidal and antibacterial	Schulz et al., 1995
98.	Phomopsichalasin	*Phomposis* sp.	*Salix gracilostyla* var. *melanostachys*	Antifungal	Horn et al., 1995
99	(3R,4S,4αR)-4,8-Dihydroxy-3-methyl-3,4,4α,5-tetrahydro-1H-2-benzopyran-1-one	*Conoplea elegantula*	*Picea mariana*	Toxic to spruce budworm cells	Findlay et al., 1995

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
100.	(3R,4R,4αR)-4,8-Dihydroxy-3-methyl-3,4,4α,5-tetrahydro-1H-2-benzopyran-1-one	*Conoplea elegantula*	*Picea mariana*	Toxic to spruce budworm cells	Findlay et al., 1995
101.	(3R,4αS,8S,8αR)-8-Hydroxy-3-methyl-3,4,4α,5,6,7,8,8α-octahydro-1H-2- benzopyran-1-one				
102.	(3R,4S,6R)-3,4,4α,5,6,7-Hexahydro-4,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one				
103.	Ramulosin				
104.	(3R,6R)-3,4,4α,5,6,7-Hexahydroxy-6,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one				
105.	(3R,4R, 4αR,6R)-4-Dihydroxy-6,7-epoxy-3,4,4α-5,6,7,hexahydro-1H-2-benzopyran-1-one				
106.	(3R,4S)-3,4-Dihydro-4,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one				

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
107.	4-Hydroxy-3-methyl-2-oxabicyclo [3.3.1] non-6-one	*Conoplea elegantula*	*Picea mariana*	Toxic to spruce budworm cells	Findlay et al., 1995
108. 109.	9α-hydroxy-1,8(14),15-isopimaratrien-3,7,11-trione 9α-hydroxy-1,8(14),15-isopimaratrien-3,11-dione	*Hormononema dermatoides* and *Phyllosticta* sp.	*Abies balsamea*	Toxic to spruce budworm cells and larvae	Findlay et al., 1995
110. 111.	Subglutinol A Subglutinol B	*Fusarium subglutinans*	*Tripterygium wilfordii*	Immunosuppressive agents	Lee et al., 1995
112.	Torreyanic acid	*Pestalotiopsis microspora*	*Torreya taxifolia*	Cytotoxic	Lee et al., 1996.
113.	L-755,807	*Microsphaeopsis* sp.	*Prosopsis glandulosa*	Bradykitin binding inhibitor	Lam et al., 1996
114.	Oreganic acid	Unidentified endophytic fungus (MF 6046)	*Berberis oregana*	FPTase inhibitor	Jayasuriya et al., 1996
115.	2α-Hydroxydimeninol	*Pestalotiopsis* sp.	*Taxus brevifolia*	No data	Pulici et al., 1996

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
116.	Fusariside	*Fusarium* sp.	*Oxydendron arboreum*	Antiviral Antifungal Antitumor	McBrien et al., 1996
117.	Khafrefungin	No data	No data	Antifungal Inhibit the synthesis of sphingolipids	Mandala et al., 1997
118. 119.	5-Hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl)benzofuran 5-Hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl)benzofuran	Endophyte strain #4GP4C2	*Gaultheria procumbens*	Toxicity to spruce budworm	Findlay et al., 1997
120. 121. 122. 123.	Pestalotiopsin A Pestalotiopsin B Pestalotiopsin C Humulene derivative	*Pestalotiopsis* spp.	*Taxus brevifolia*	No data	Pulici et al., 1997

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
124.	Leucinostatin A	*Acremonium* sp.	*Taxus baccata*	Antifungal and anticancer	Strobel et al., 1997
125.	Oxysporidinone	*Fusarium oxysporum*	Unidentified	Antifungal	Brienholt et al., 1997
126. 127. 128. 129.	Tricin Tricin-7-O-β-D-glucopyranoside Isoorientin Tricin-7-O-[α-L-rhamnopyranosyl (1-6)- β-D-glucopyranoside	*Neotyphodium typhnium*	*Poa ampla*	Against mosquito larvae	Ju et al., 1998
130. 131.	Geniculol Cytochalasin F	*Geniculosporium* sp.	*Teucrium scorodonia*	Inhibitor of photosynthesis	Konig et al.,1999
132.	Herbarulide	*Pleospora herbarum*	*Medicago lupulina*	No data	Krohn et al., 1999
133.	Cryptocandin	*Cryptosporiopsis* cf. *querina*	*Pezicula cinnamomea*	Antioomycotic	Strobel et al., 1999

Table 1(continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
134. 135. 136. 137. 138. 139. 140. 141. 142. 143.	Sequoiatone A Sequoiatone B Sequoiatone C Sequoiatone D Sequoiatone E Sequoiatone F Sequoiamonascin A Sequoiamonascin B Sequoiamonascin C Sequoiamonascin D	*Aspergillus parasiticus*	*Sequoia sempervirens*	Antifungal , Antitumor and Cytotoxic to brine shrimp	Stierle et al., 1999 Stierle et al., 2001 Stierle et al., 2003
144. 145.	Cytoskyrin A Cytoskyrin B	*Cytospora* sp.	*Conocarpus erecta*	No data	Brady et al., 2000
146.	CR377	*Fusarium* sp.	*Selaginella pallescens*	Against Candida albicans	Brady and Clardy., 2000

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
147.	Cryptocin	*Cryptosporiopsis* cf. *querina*	*Tripterygium wilfordii*	Antifungal	Li et al., 2000
148.	Collectotric acid	*Collectotrichum gloeosporioides*	*Artemisia annua*	Antibacterial	Zou et al., 2000
149. 150. 151. 152. 153.	22-Oxa-[12]-cytochalasin 1 22-Oxa-[12]-cytochalasin 2 22-Oxa-[12]-cytochalasin 3 22-Oxa-[12]-cytochalasin 4 Cytochalasin E	*Rhinocladiella sp.*	*Tripterygium wilfordii*	Cytotoxic	Wagenaar et al., 2000
154. 155.	Cytonic acid A Cytochalasin B	*Cytonaema* sp.	*Quercus* sp.	Inhibitor of hCMV protease	Guo et al., 2000

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
156.	Indole-3-acetic acid	*Epichloe festucae* and *Neotyphodium tembladerae*	*Festuca rubra* *Poa ampla*	Antifungal	Yue et al., 2000
157.	Indole-3-ethanol				
158.	Methyindole-3-carboxylate				
159.	Indole-3-carboxaldehyde				
160.	N,N'-diacetamide				
161.	Cyclonerodiol				
162.	Ergosterol	*Collectotrichum* sp.	*Artemisia annua*	No data	Lu et al., 2000
163.	3β-5α,6β-trihydroxyergosta-7,22-diene			No data Antifungal	
164.	3β-hydroxy-ergosta-5-ene			Antifungal	
165.	3-oxo-ergosta-4,6,8(14),22-tetraene			No data	
166.	3β-hydroxy-5α,8α-epidioxy-ergosta-6,22-diene			No data	
167.	3β-hydroxy-5α,8α-epidioxy-ergosta-6,9(11),22-triene			No data	

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
168.	3-oxo-ergosta-4-ene	*Collectotrichum* sp.	*Artemisia annua*	Antifungal	Lu et al., 2000
169.	3β-5β-dihydroxy-6β-acetoxy-			Antifungal	
170.	ergosta-7,22-diene				
171.	3β-5α-dihydroxy-6β-			Antifungal	
	phenylacetyloxy-ergosta-7,22-diene				
172.	6-isoprenylindole-3-carboxylic acid				
173.	Guanacasterpene	Unidentified st. CR 115.	*Daphnopsis americana*	Antibacterial	Brady et al., 2000 Brady et al., 2001
174.	Preussomarin G	Mycelia sterilia	*Atropa belladonma*	Antimicrobial	Krohn et al., 2001.
175.	Preussomarin H				
176.	Preussomarin I				
177.	Preussomarin J				
178.	Preussomarin K				
179.	Preussomarin L				

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
180.	Microcarpalide	Endophyte strain 112/13	*Ficus microcarpa*	Antimicrofilament	Ratnayake et al., 2001
181.	Nomofungin	Unidentified	*Ficus microcarpa*	Antimicrofilament	Ratnayake et al., 2001
182. 183.	Phomopxanthone A Phomopxanthone B	*Phomopsis* sp.	*Tectona grandis*	Vitro antimalarial, antitubercular and cytotoxicity	Isaka et al., 2001
184. 185. 186.	Dicerandrol A Dicerandrol B Dicerandrol C	*Phomopsis longicolla*	*Dicerandra frutescens*	Antibiotic and cytotoxic	Wagenaar et al., 2001
187.	Ambuic acid	*Pestalotiopsis* spp. and *Monochaetia* sp.	*Taxus* sp.	Antifungal	Li et al., 2001
188. 189.	Jesterone Hydroxyjesterone	*Pestalotiopsis jesteri*	*Fragraea bodenii*	Antimycotic	Li et al., 2001
190. 191.	Meroterpene A Meroterpene B	*Penicillium* sp.	*Melia azedarach*	Antibacterial	Geris dos Santos et al., 2002

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
192.	5-(E)-but-2-enylidene-3-propyl-5H-furan-2-one	*Galiella rufa* *Sarcosoma latahensis* *Urnula helvelloides* *Sarcosoma coryneoidea*	*Cistus salvifolius*	No data	Kopcke et al., 2002
193.	5-(E)-but-2-enylidene-3(E)-propyl-5H-furan-2-one				
194.	5-(E)-buta-1,3dienyl-3(E)propeyl-5H-furan-2-one				
195.	5-(E)-but-3-enyl-3(E)-propeyldihydrofuran-2-one				
196.	(-)-Pregaliellalactone				
197.	(+)-Deoxygalliellactone				
198.	(-)-Galliellactone				
199.	6-Pentyl-4-methoxy-6-pyran-2-one				
200.	6-(1-hydroxypentyl)-4-methoxy-6-pyran-2-one				
201.	Napthalene	*Muscodor vitigenus*	*Paullinia paulliniodes*	Insect repellent	Daisy et al., 2002

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
202.	Isopestacin	*Pestalotiopsis microspora*	*Terminalia morobensis*	Antimycotic	Strobel et al., 2002
203.	Brefeldin A	*Paecilomyces* sp. and *Aspergillus clavatus*	*Taxus mairei* and *Torreya grandis*	Cytitoxicity	Wang et al., 2002
204.	[8]annulene	*Gliocladium* sp.	*Eucryphia cordifolia*	antimycotic	Stinson et al., 2002
205.	Lepidimoide	*Collectotrichum* sp.	*Abelmoschus esculentum*	No data	Tanaka et al., 2002
206.	Pestacin	*Pestalotiopsis microspora*	Rainforest plant	Antifungal and antioxidant	Harper et al., 2003
207. 208.	1893A 1893B	Endophytic fungus (No.1893)	*Kandelia candel*	No data	Chen et al., 2003

Table 1 (continued)

No.	Compound	Endophytic fungi	Host plants	Activities	References
209.	7-Butyl-6,8-dihydroxy-3(R)-pent-11-enyisochroman-1-one	*Geotrichum* sp.	*Crassocephalum crepidioides*	Antimalarial, antituberculous, and antifungal	Kongsaeree et al., 2003
210.	7-But-15-enyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one				
211.	7-Butyl-6,8-dihydroxy-3(R)-pentylisochroman-1-one				

APPENDIX B

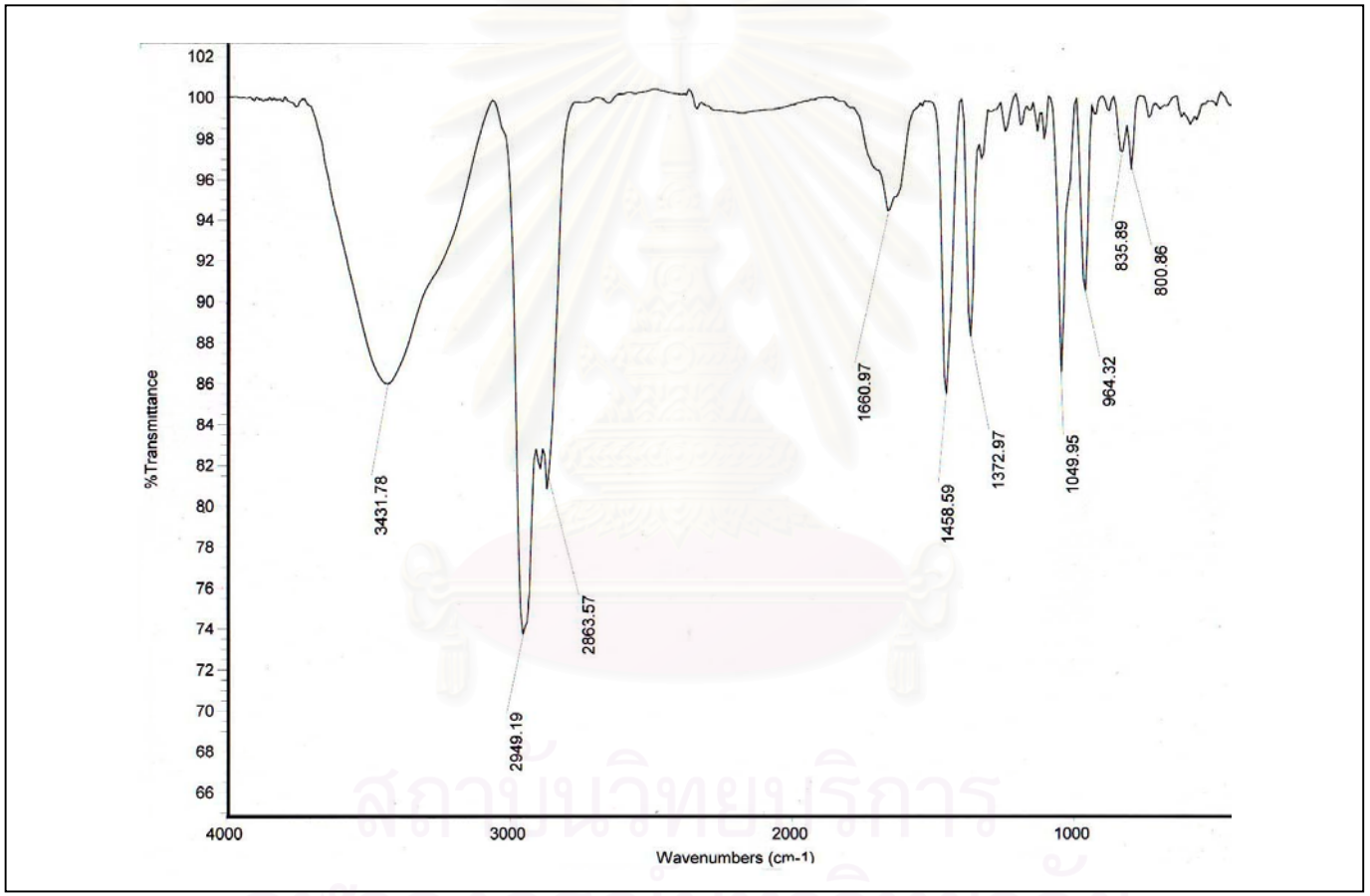

Figure B1 The IR spectrum of compound **1**

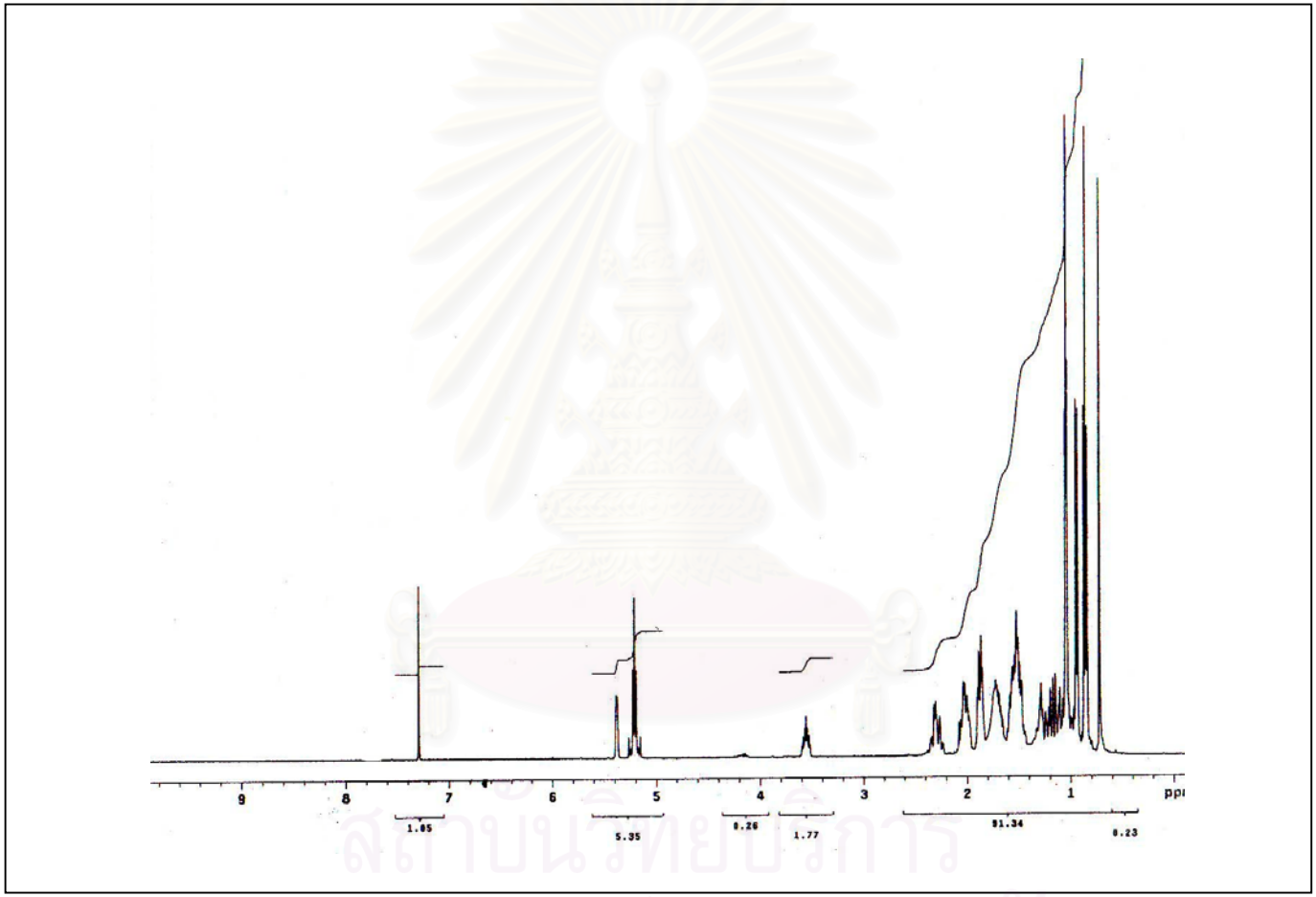

Figure B2 The ^{1}H-NMR spectrum of compound **1**

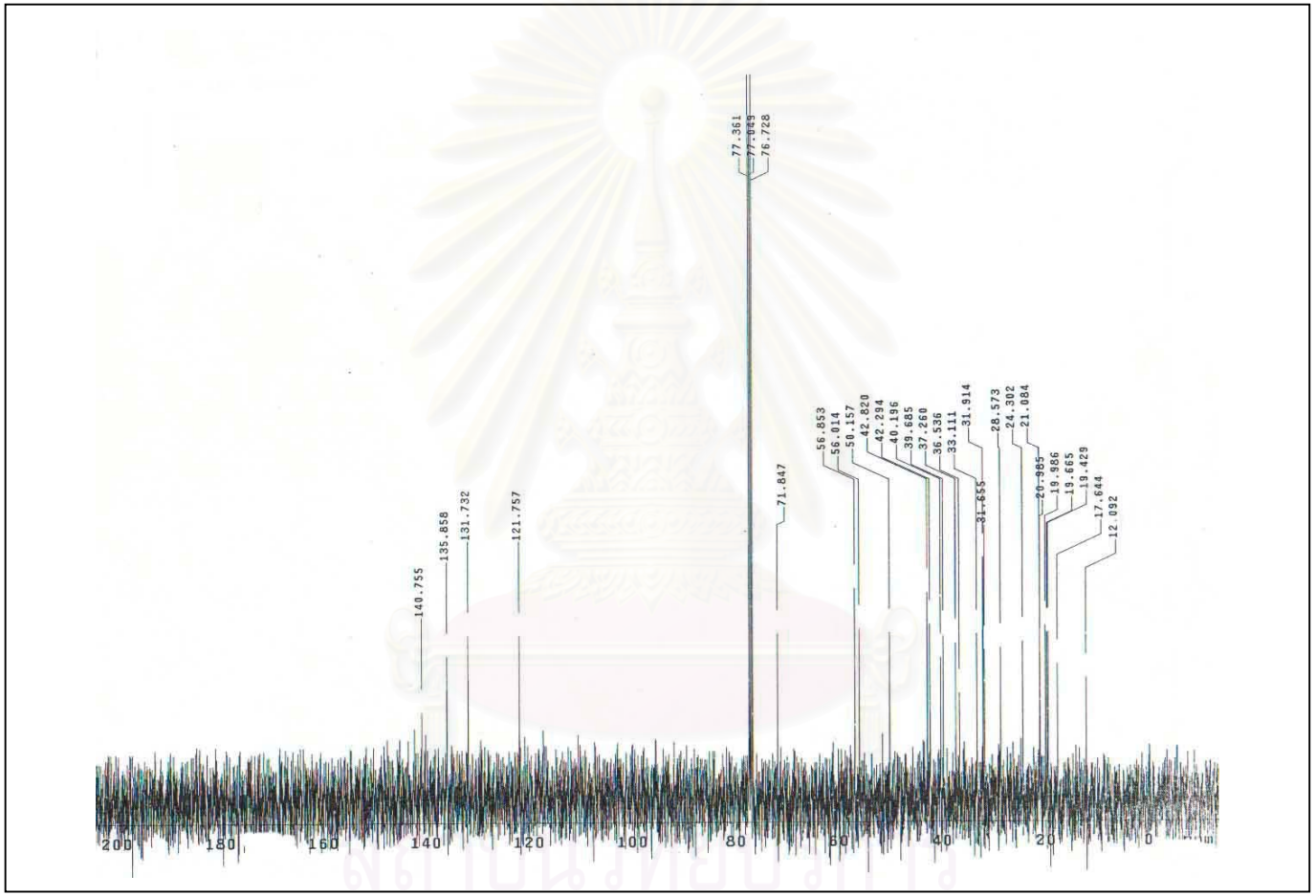

Figure B3 The ^{13}C-NMR spectrum of compound **1**

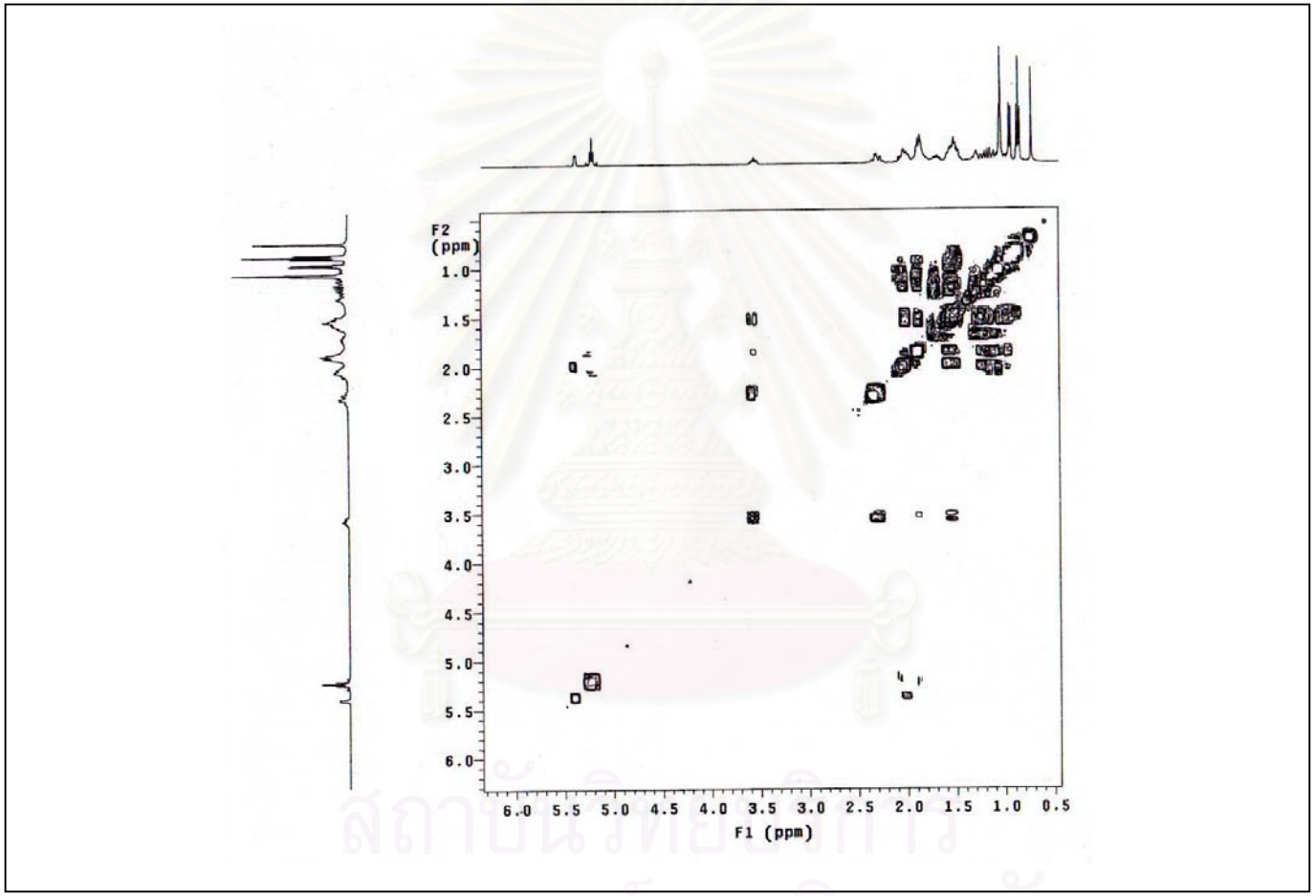

Figure B4 The gCOSY-NMR spectrum of compound **1**

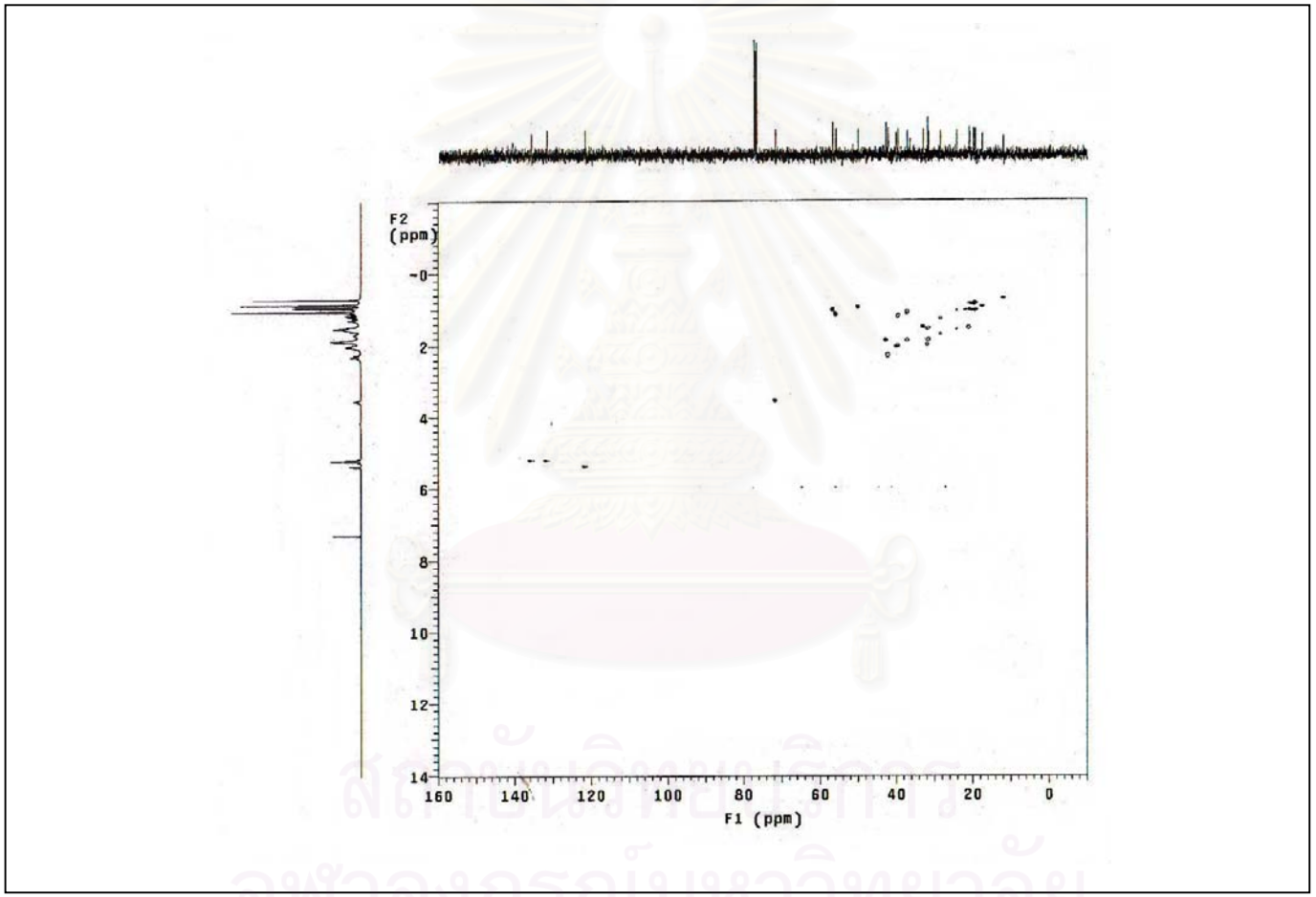

Figure B5 The gHSQC-NMR spectrum of compound **1**

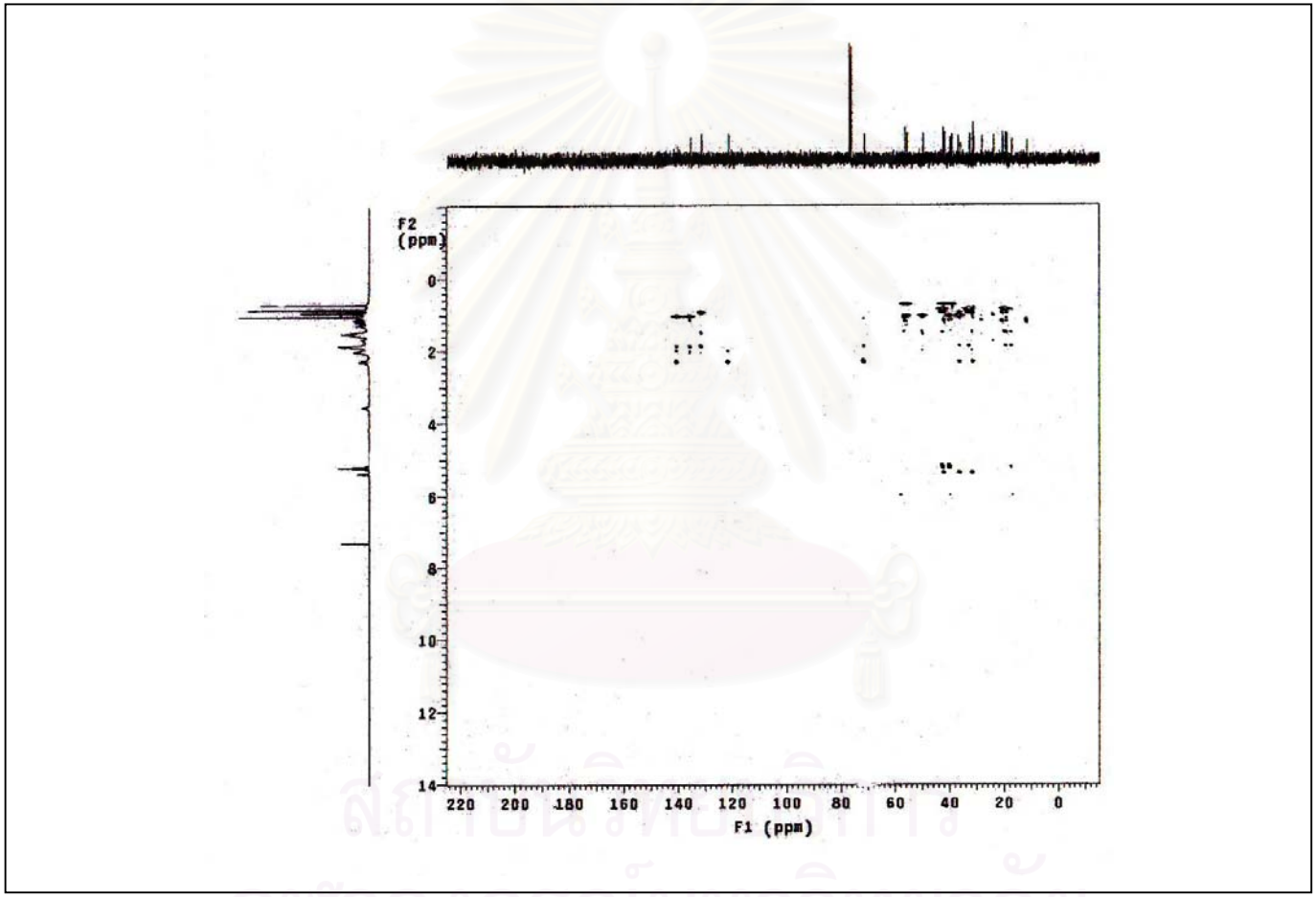

Figure B6 The gHMBC-NMR spectrum of compound **1**

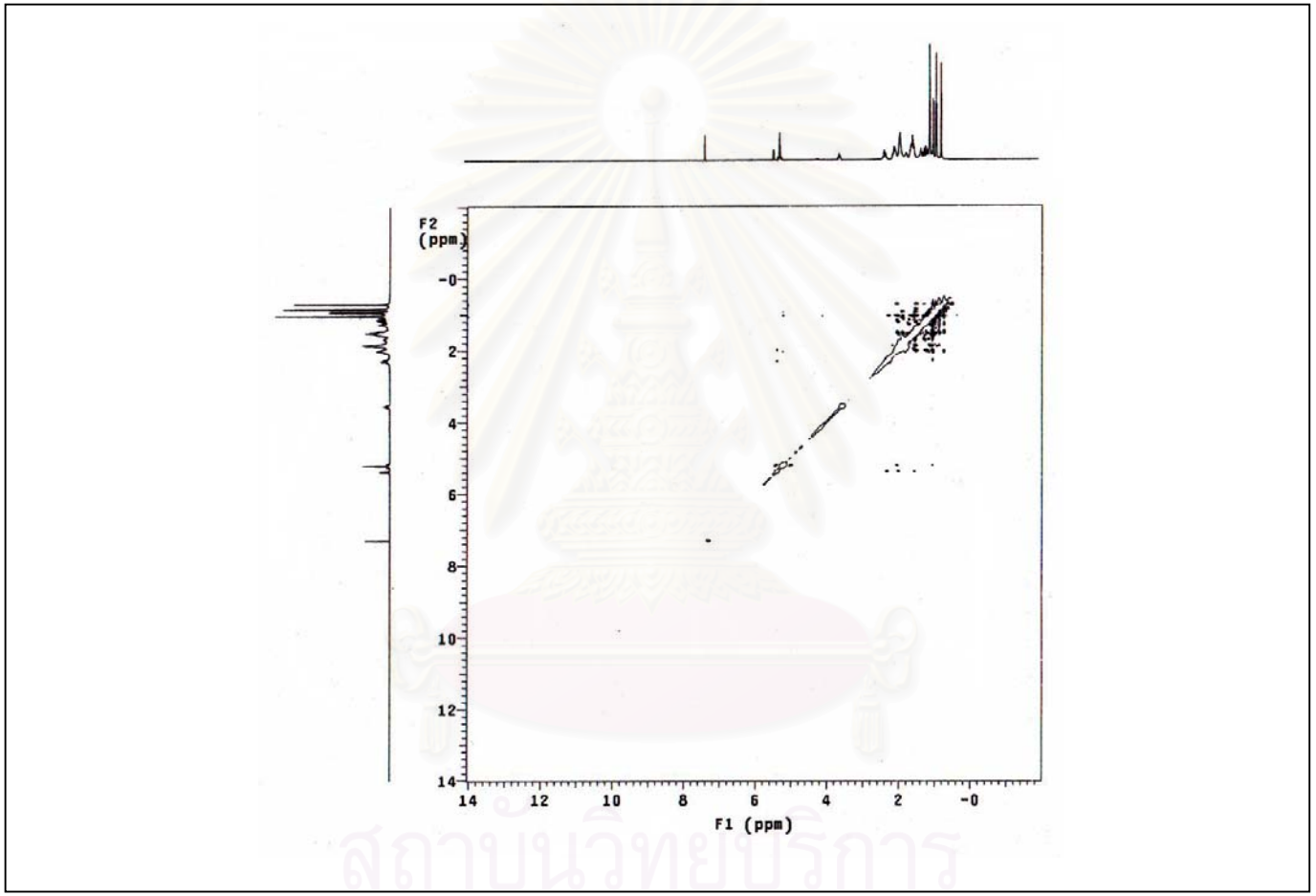

Figure B7 The NOESY-NMR spectrum of compound **1**

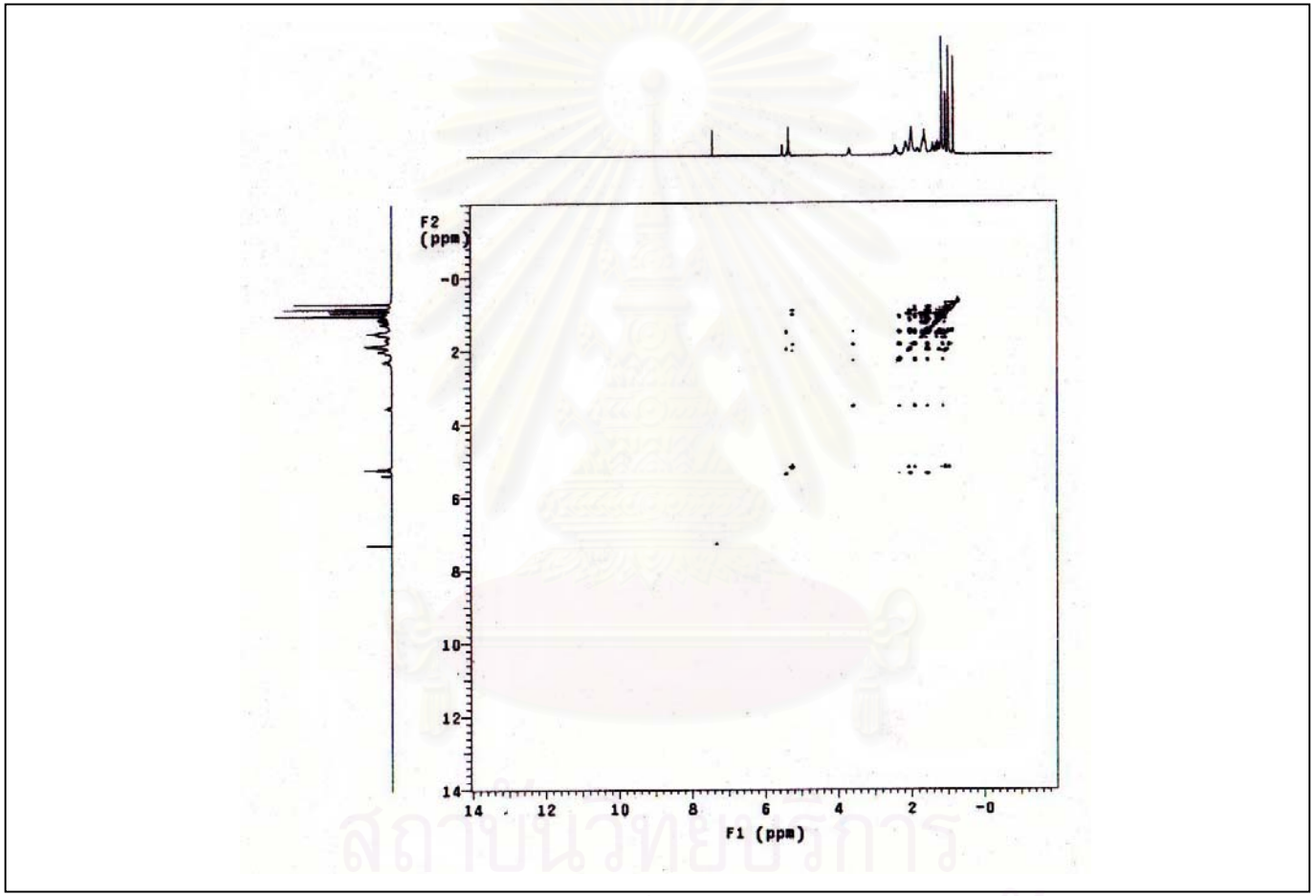

Figure B8 The TOCSY-NMR spectrum of compound 1

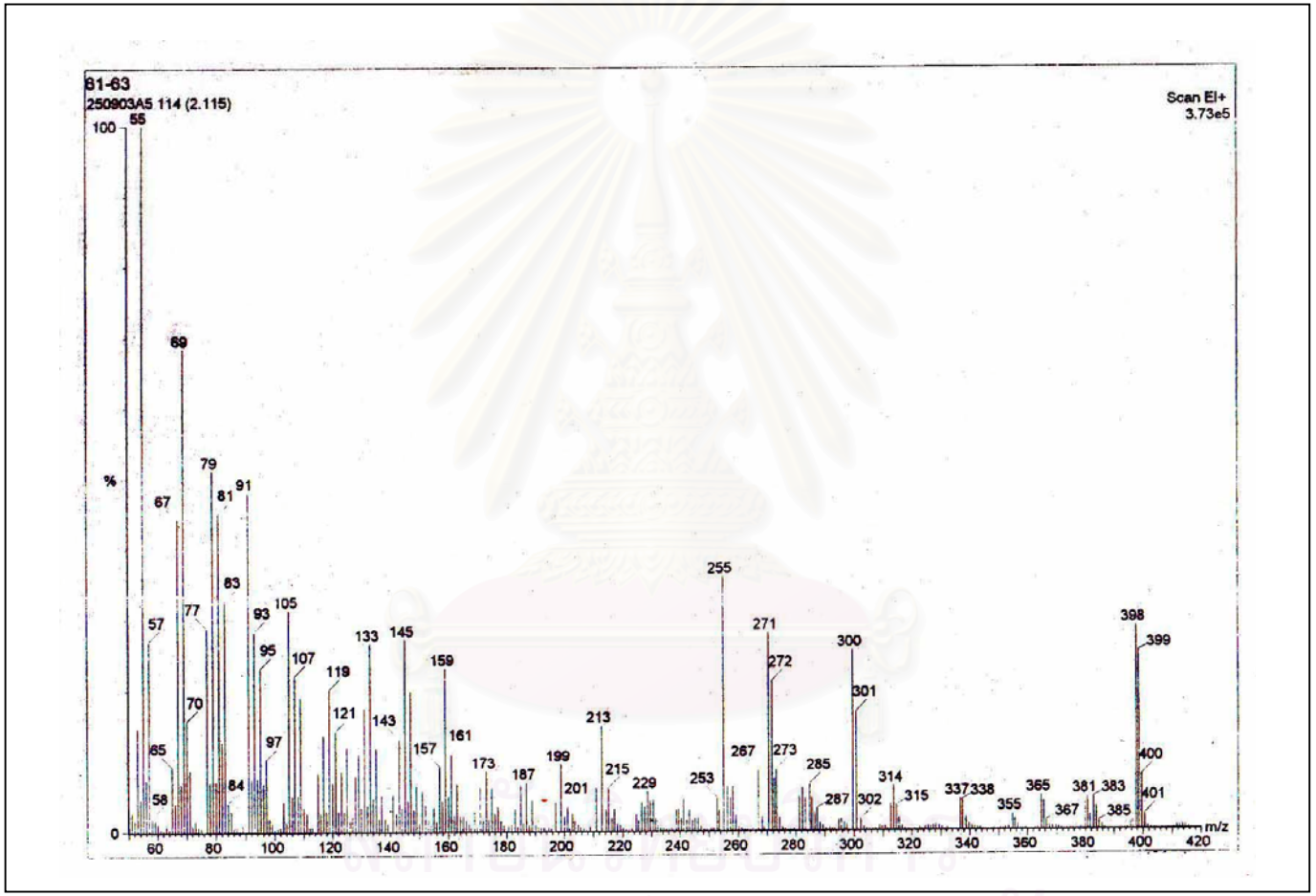

Figure B9 The EI-MS spectrum of compound 1

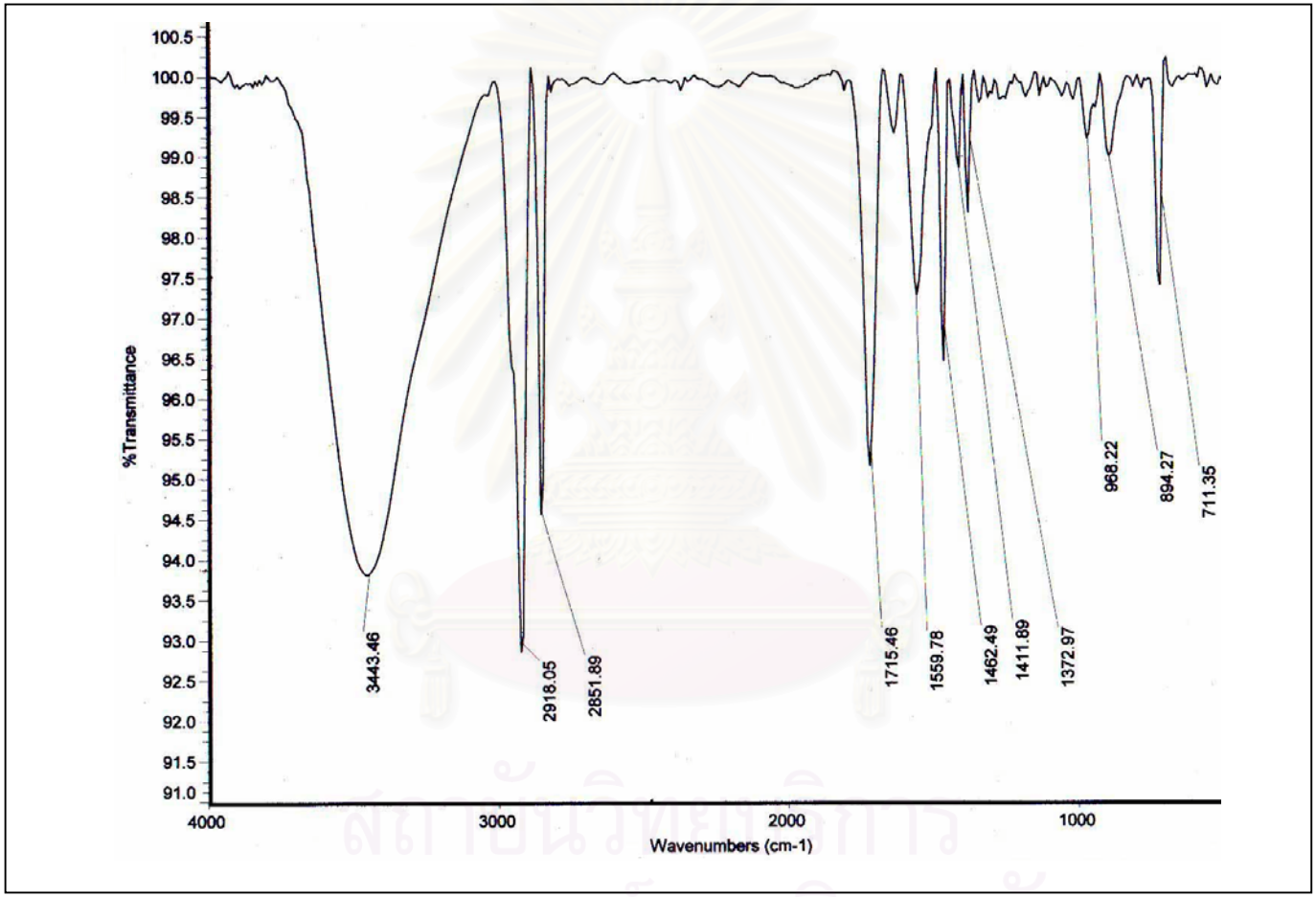

Figure B10 The IR spectrum of compound 2

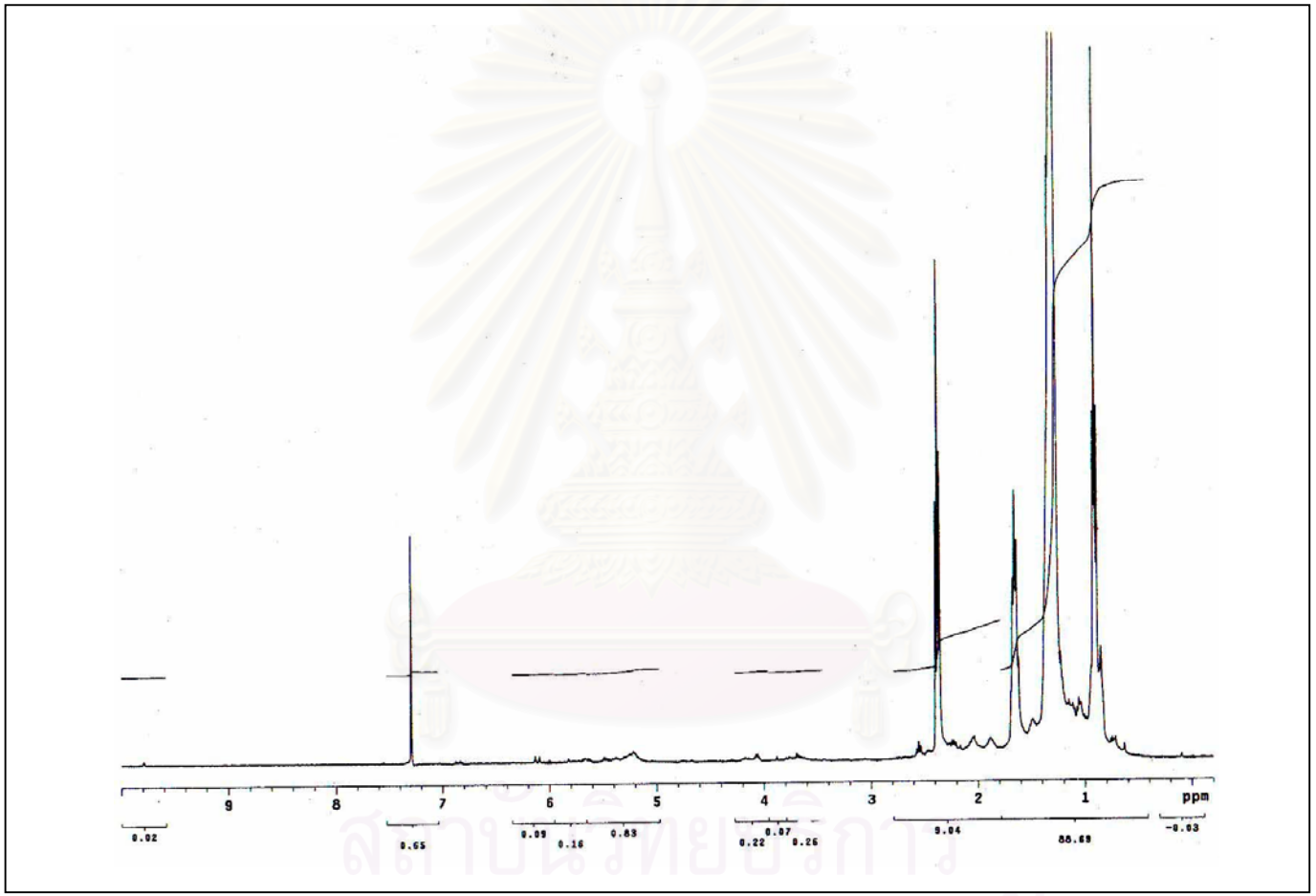

Figure B11 The ^{1}H-NMR spectrum of compound 2

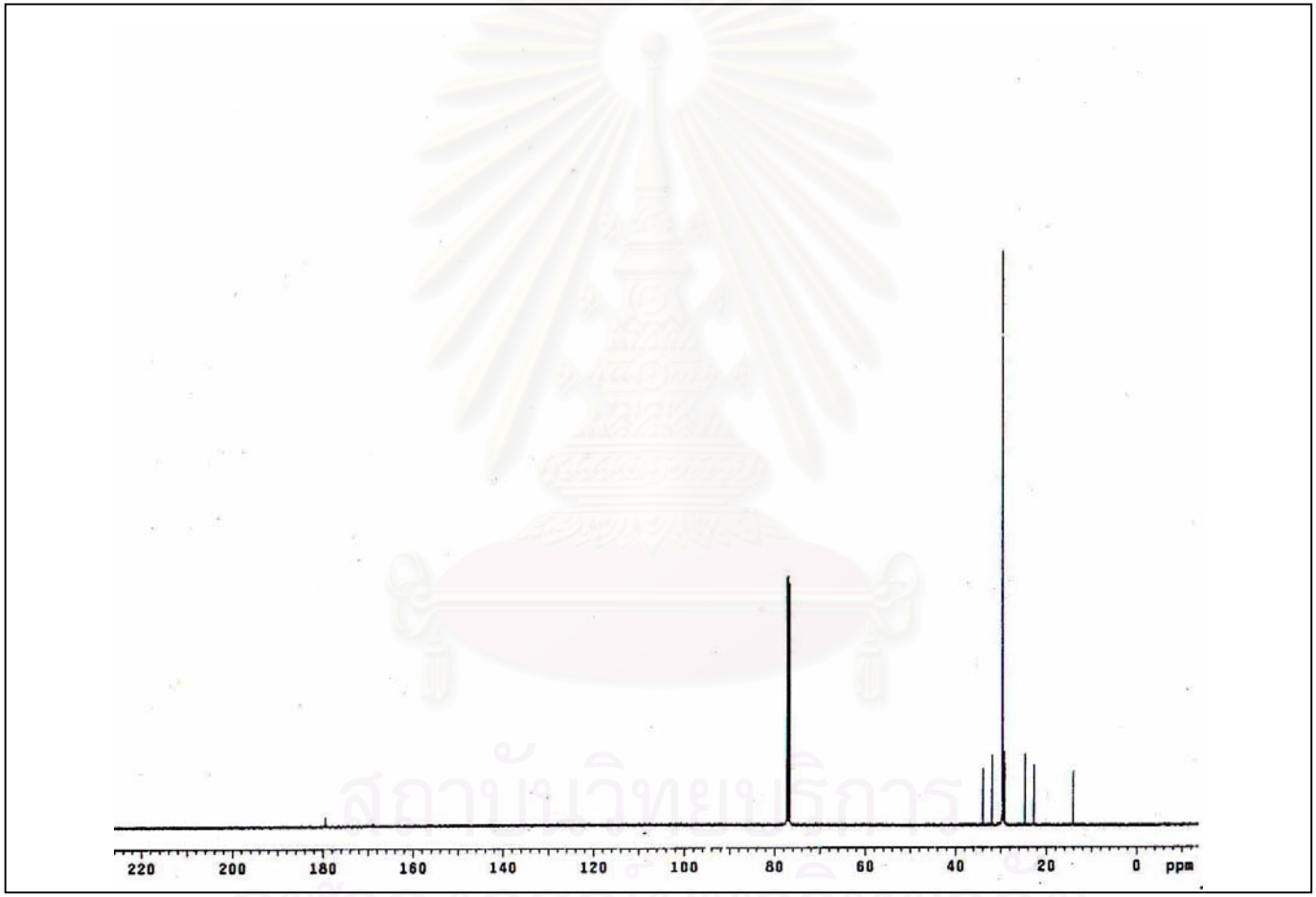

Figure B12 The ^{13}C-NMR spectrum of compound 2

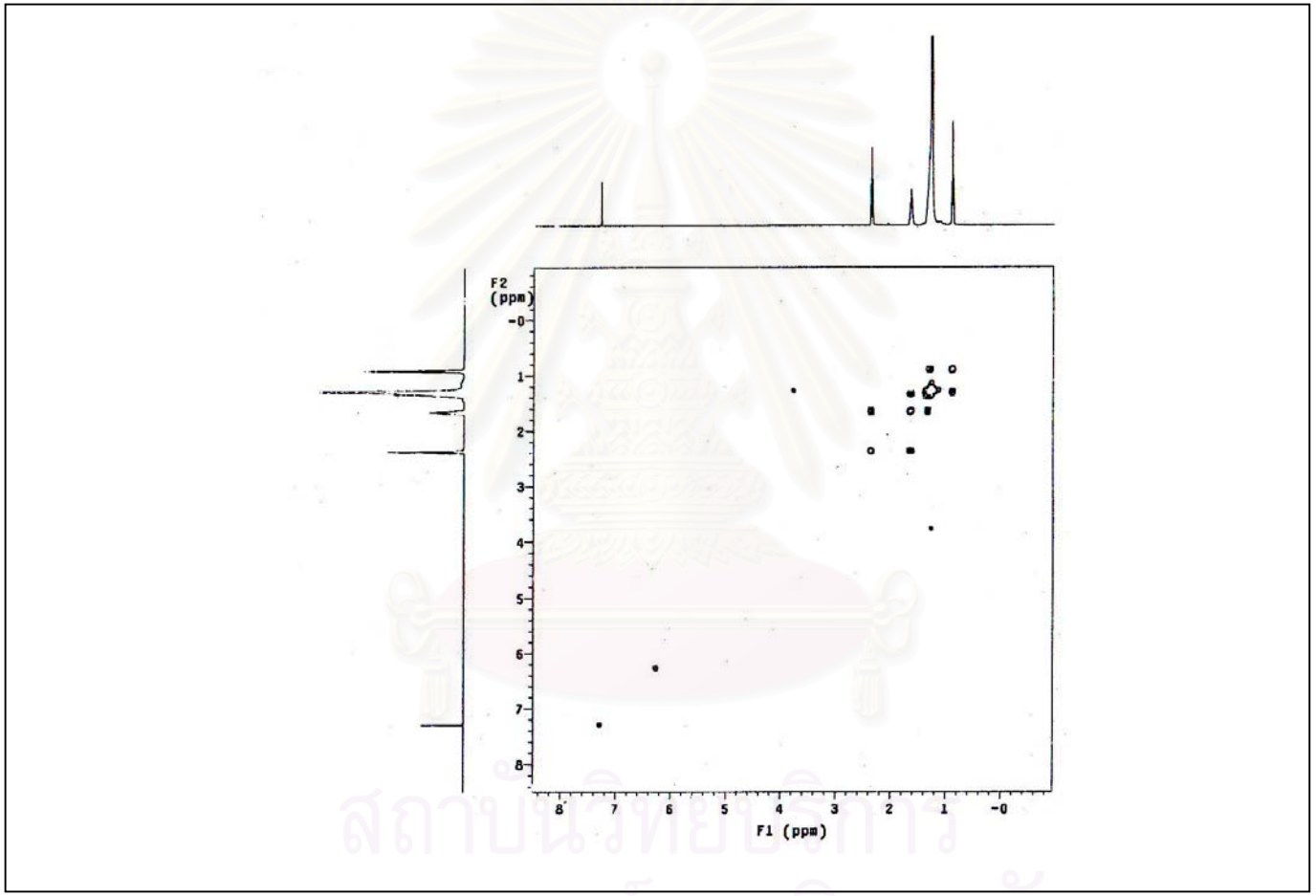

Figure B13 The gCOSY-NMR spectrum of compound **2**

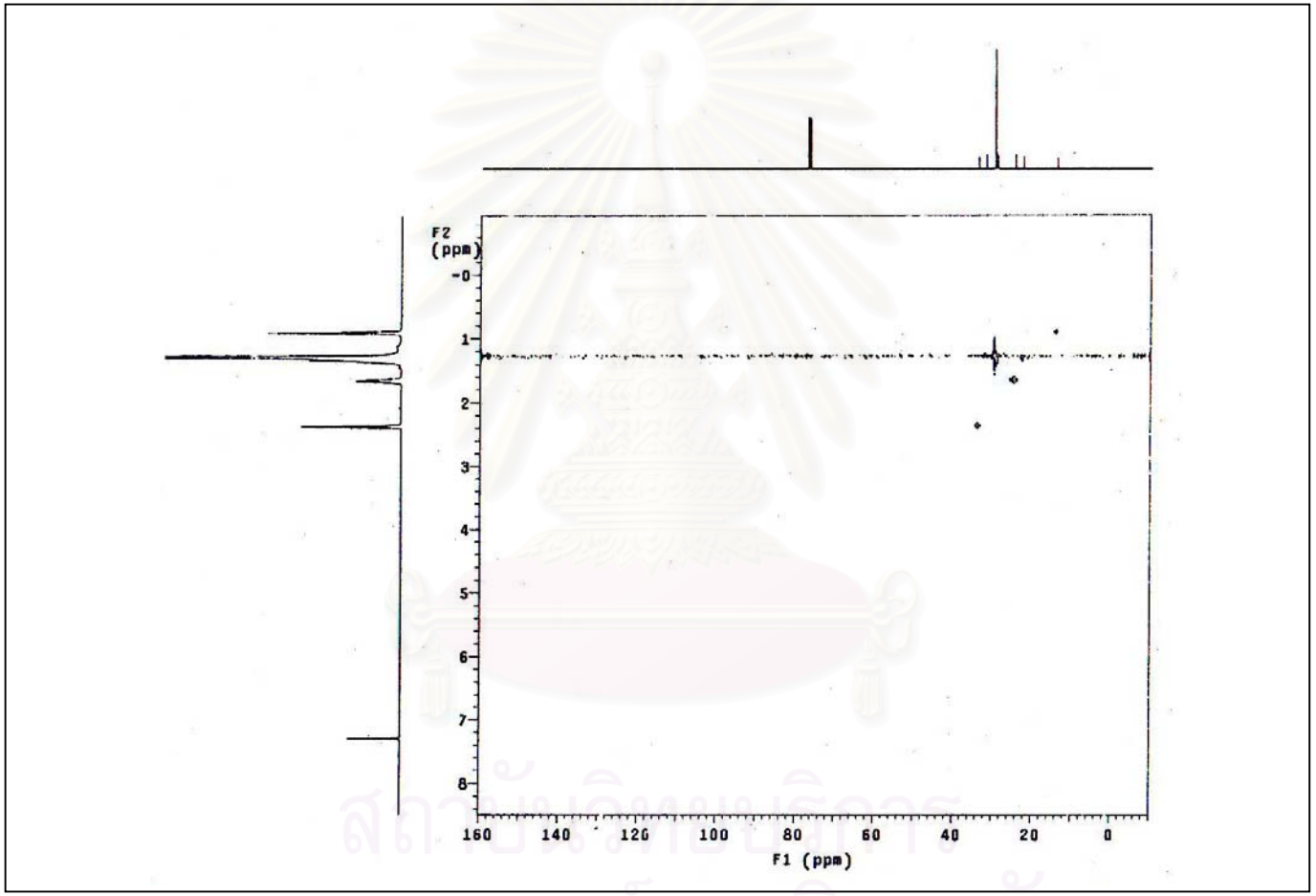

Figure B14 The gHSQC-NMR spectrum of compound **2**

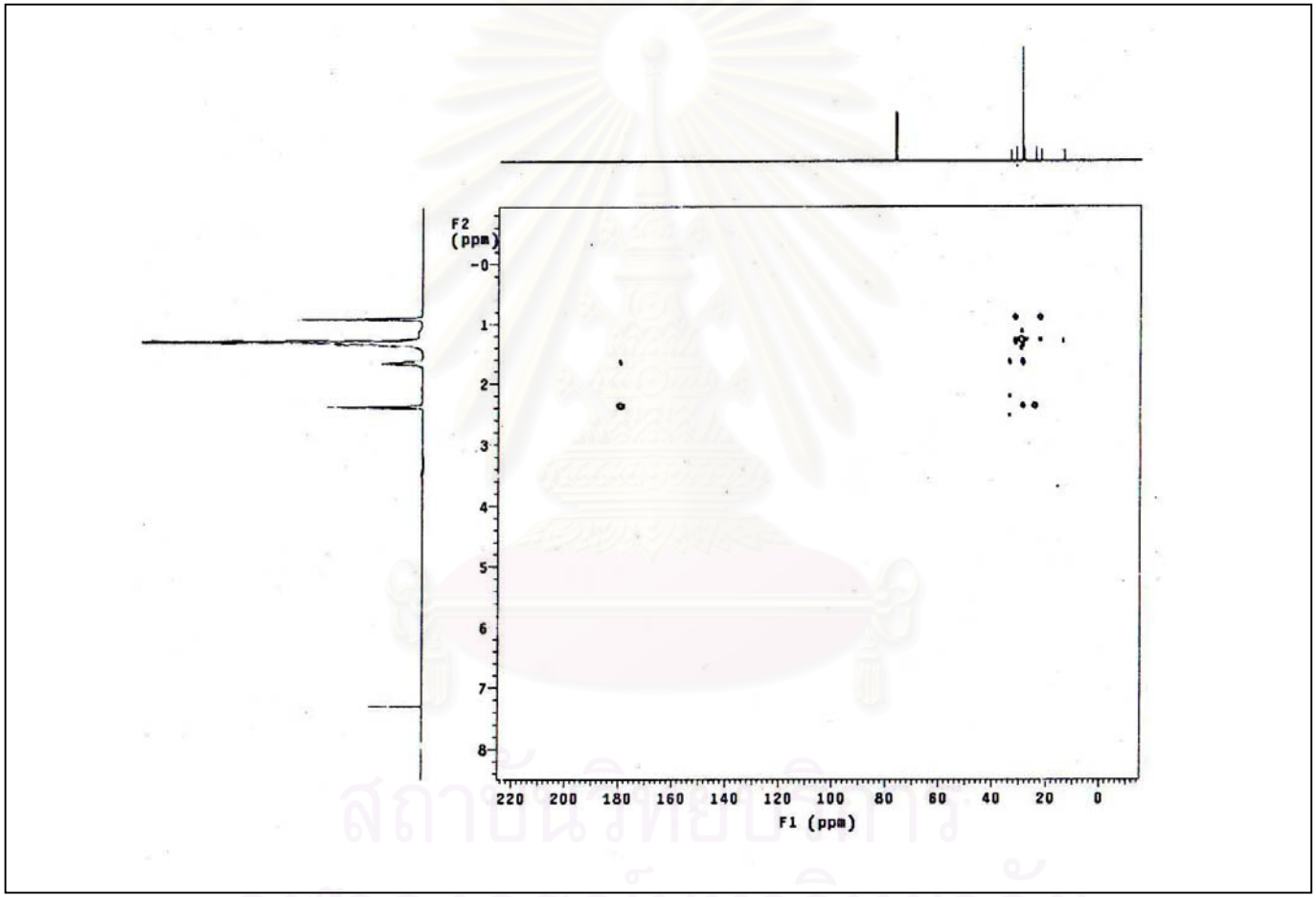

Figure B15 The gHMBC-NMR spectrum of compound 2

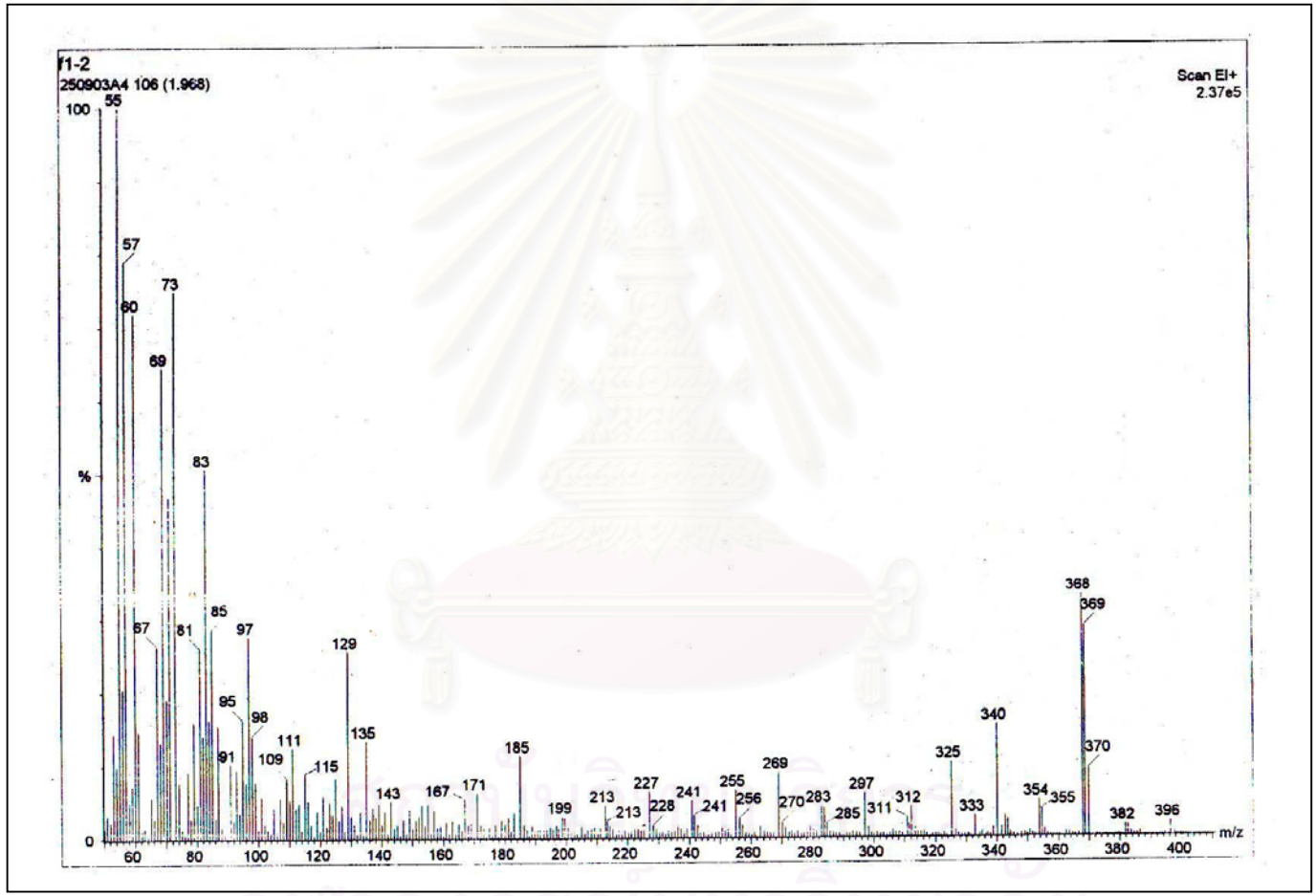

Figure B16 The EI-MS spectrum of compound **2**

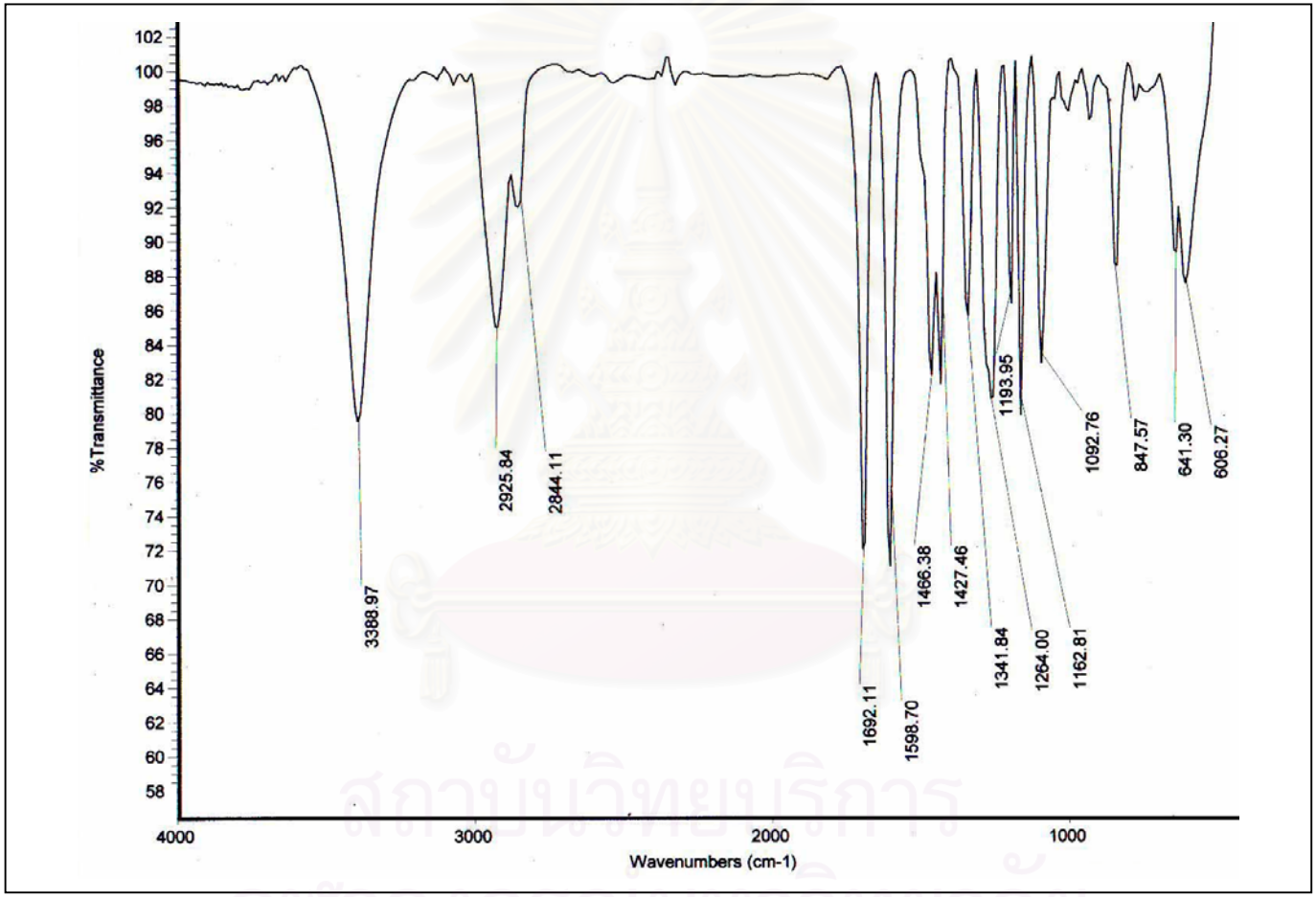

Figure B17 The IR spectrum of compound 3

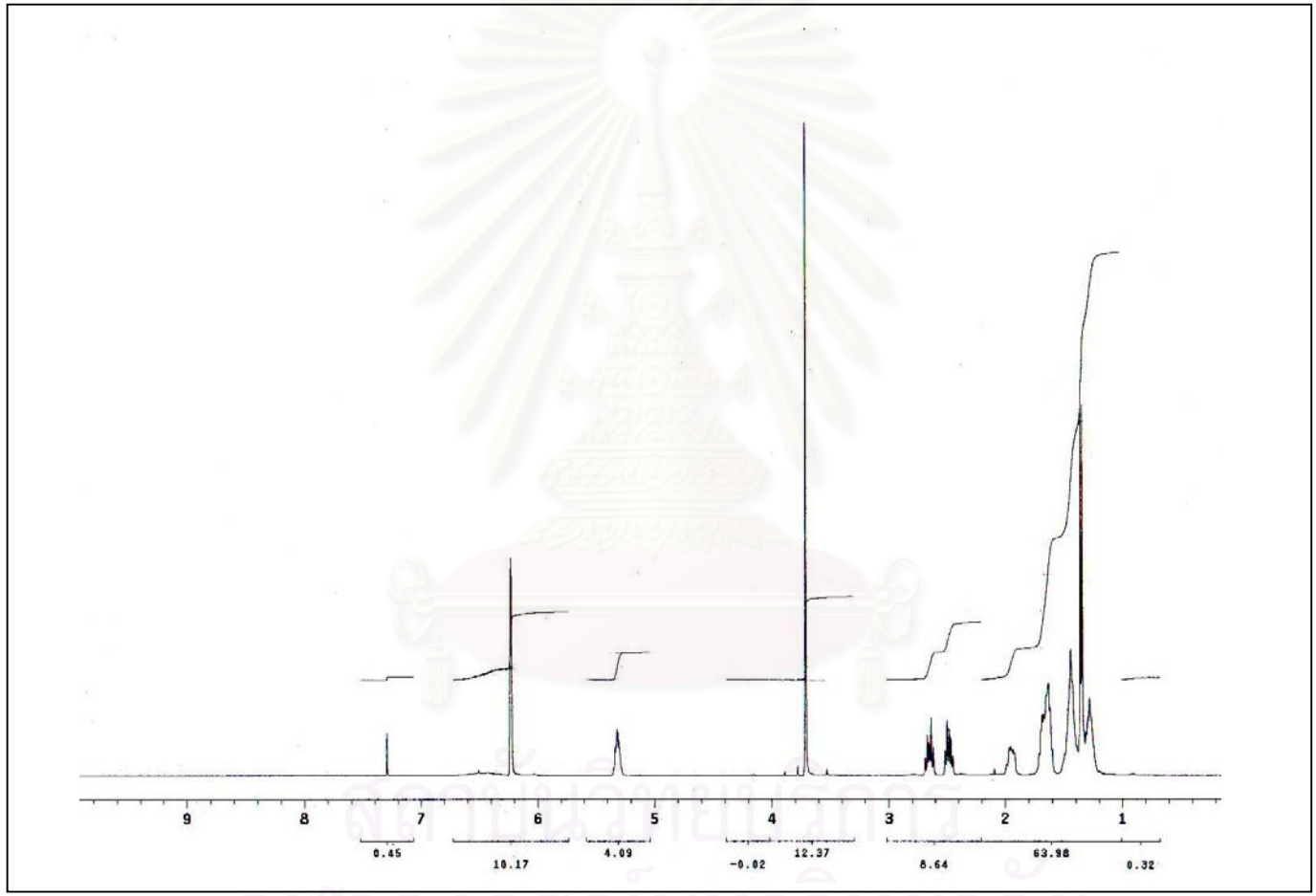

Figure B18 The ^{1}H-NMR spectrum of compound **3**

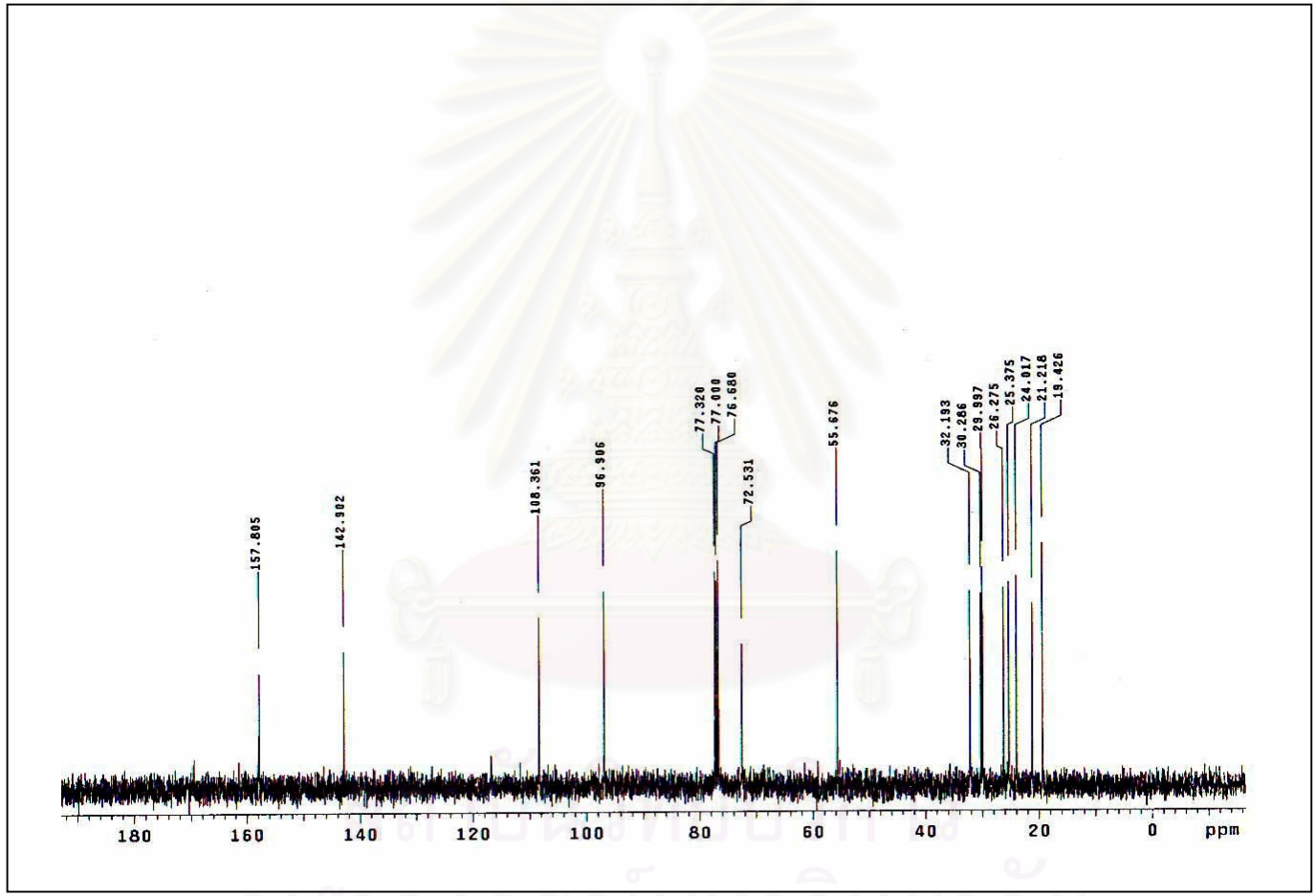

Figure B19 The ^{13}C-NMR spectrum of compound **3**

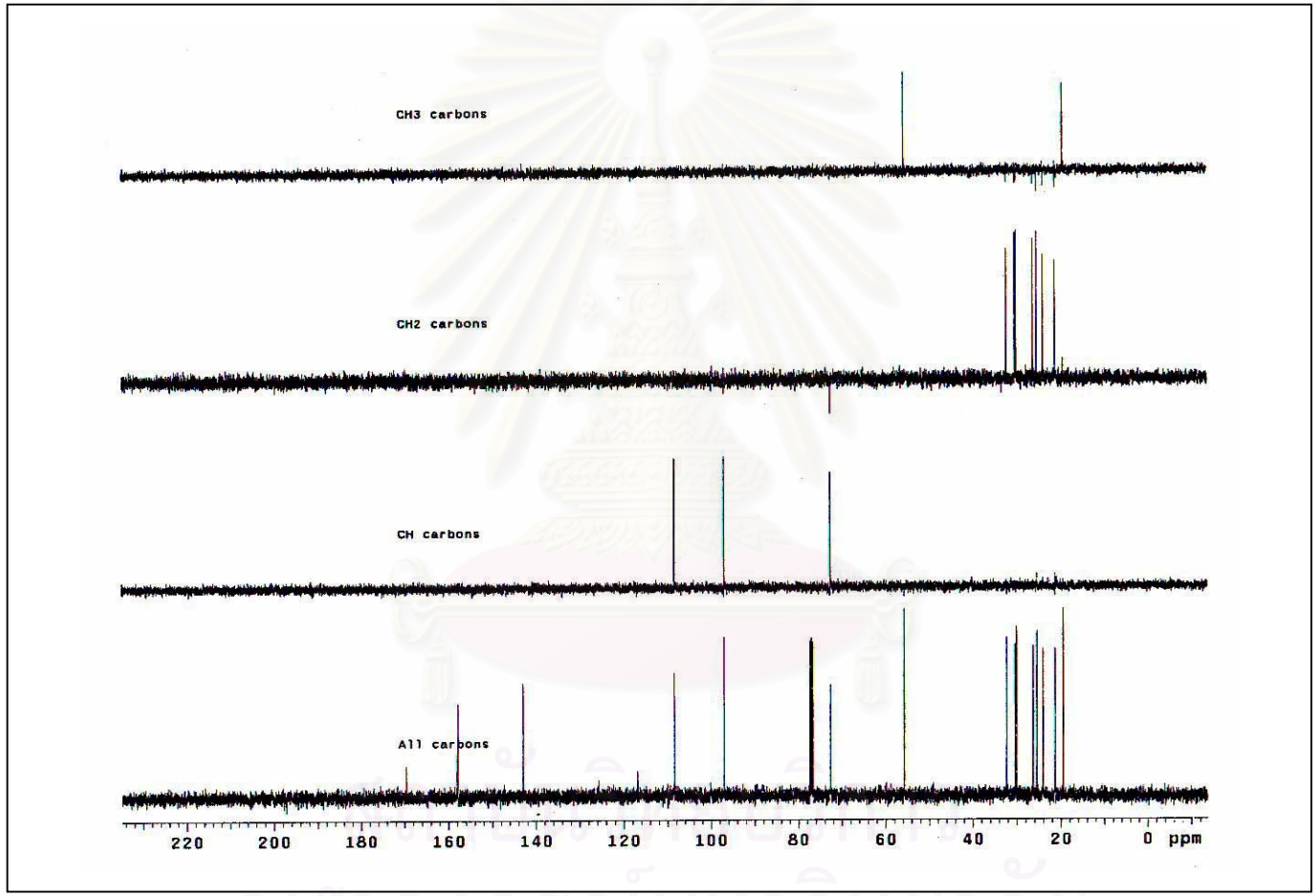

Figure B20 The DEPT, ^{13}C-NMR spectrum of compound 3

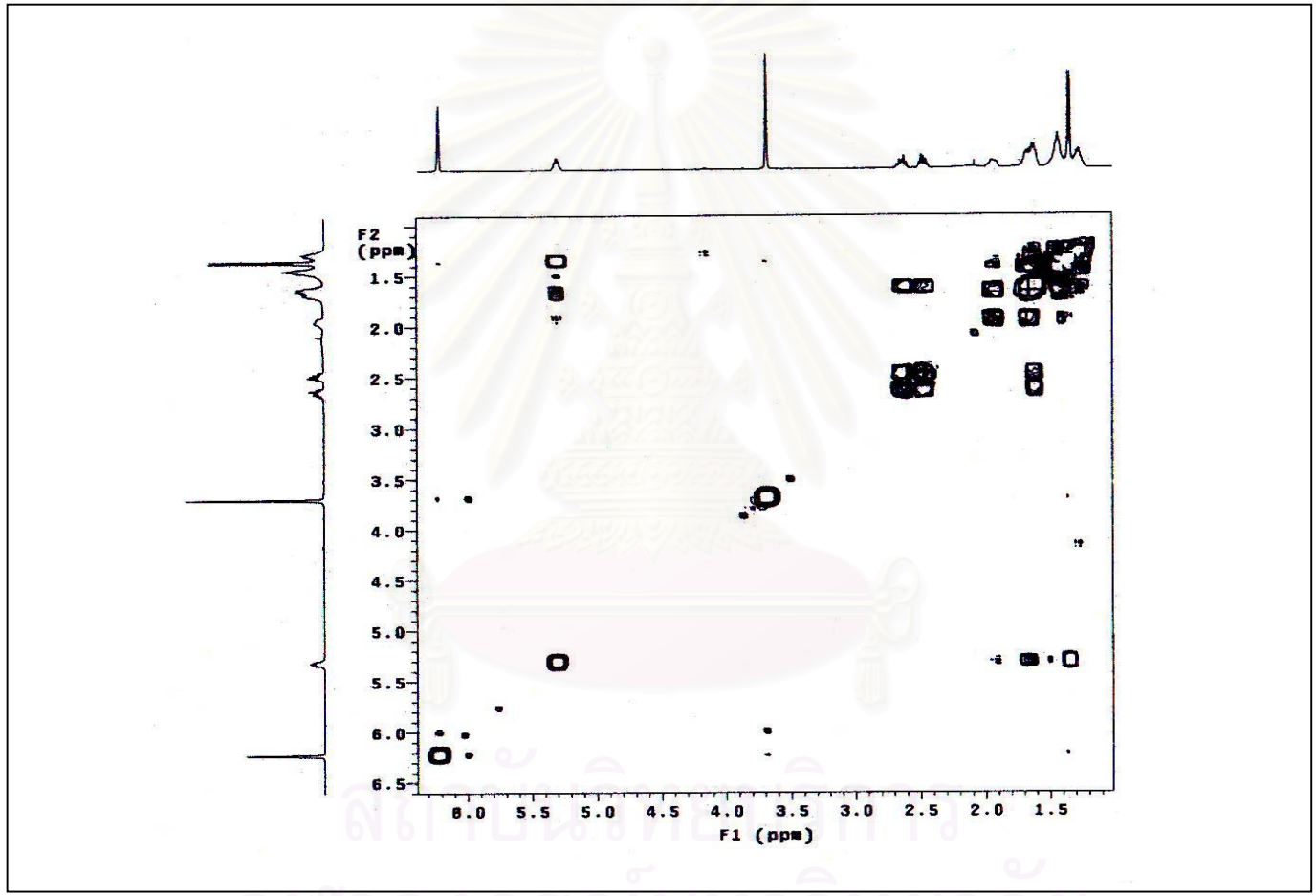

Figure B21 The gCOSY spectrum of compound **3**

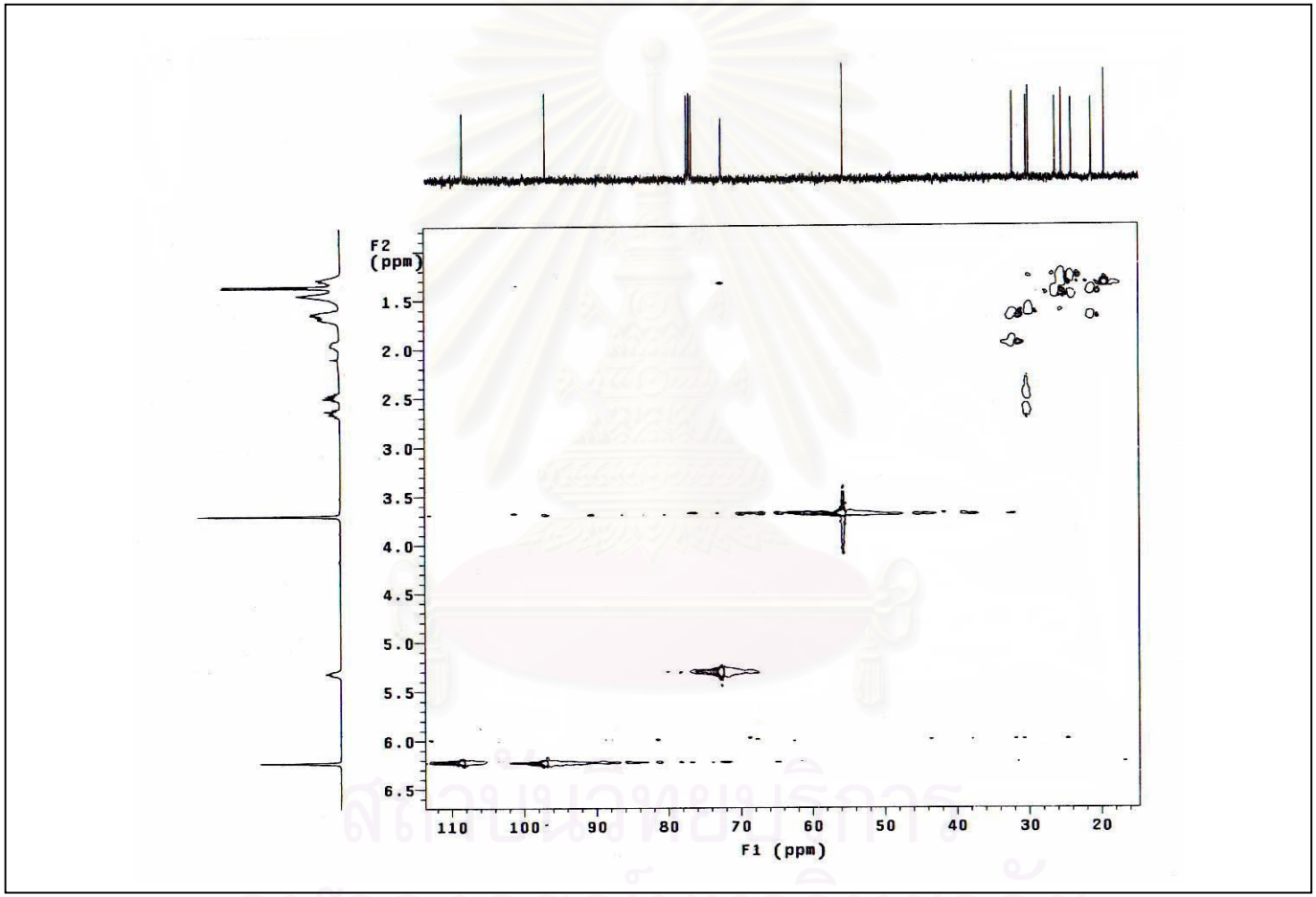

Figure B22 The gHSQC spectrum of compound 3

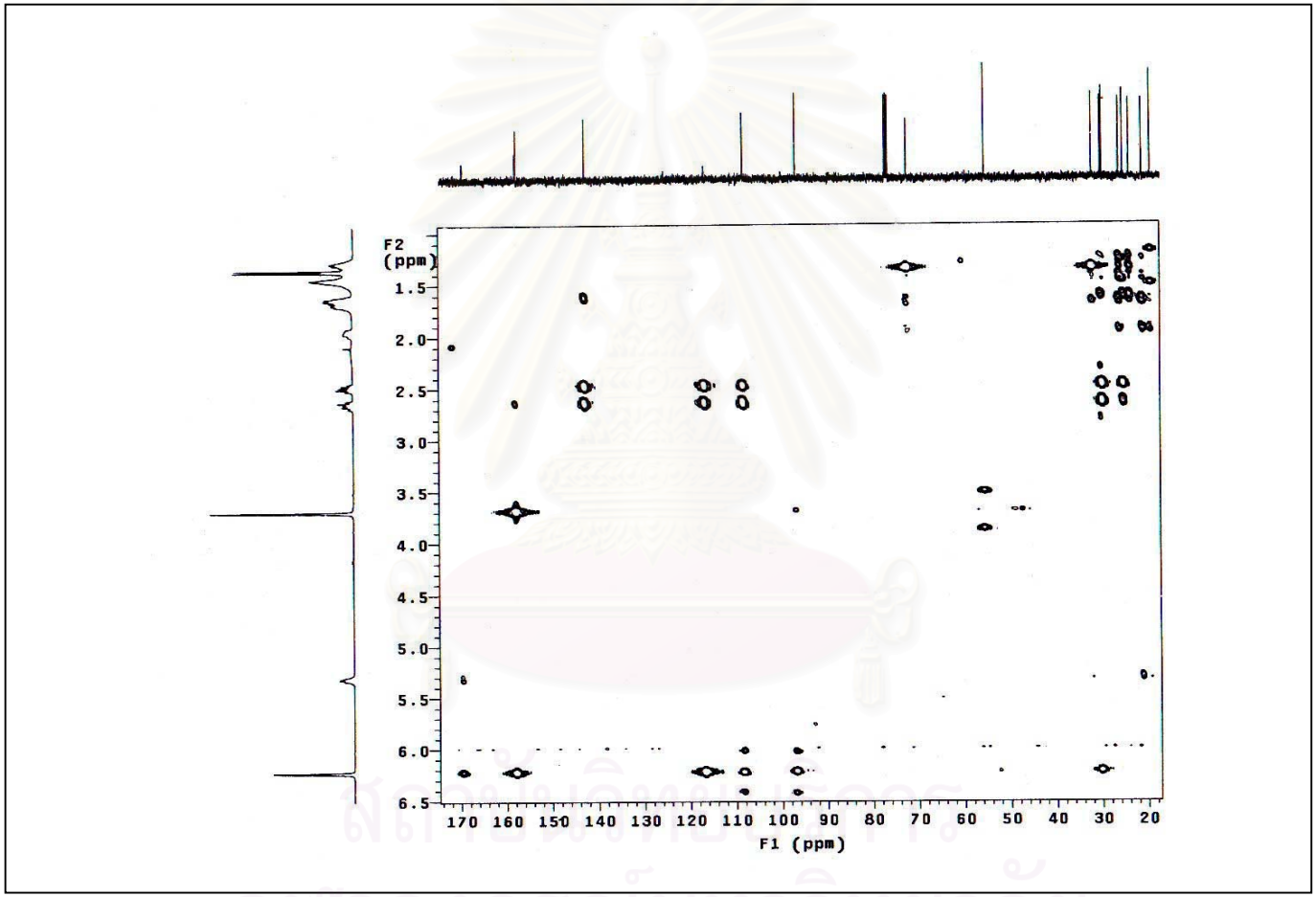

Figure B23 The gHMBC spectrum of compound **3**

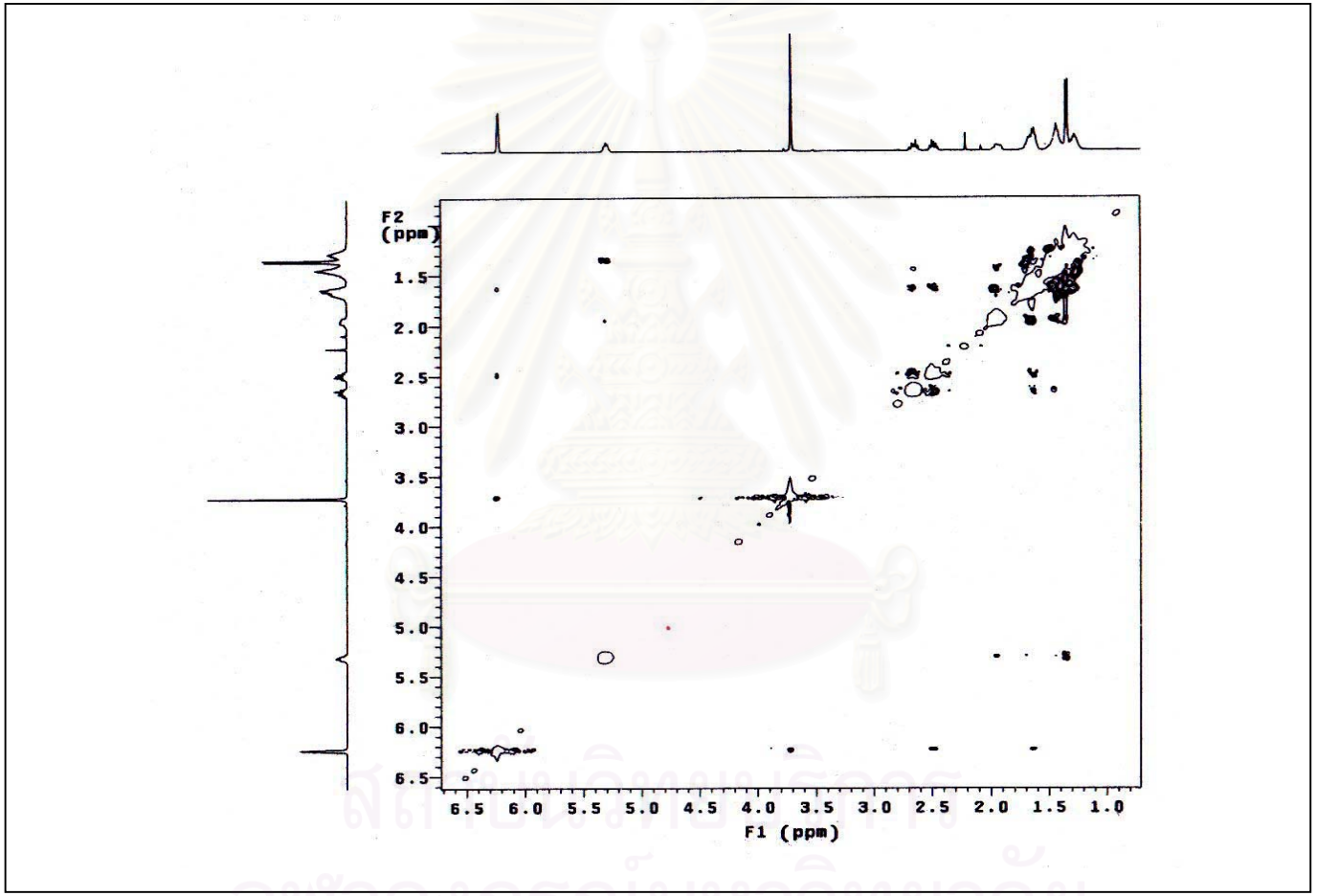

Figure B24 The NOESY spectrum of compound 3

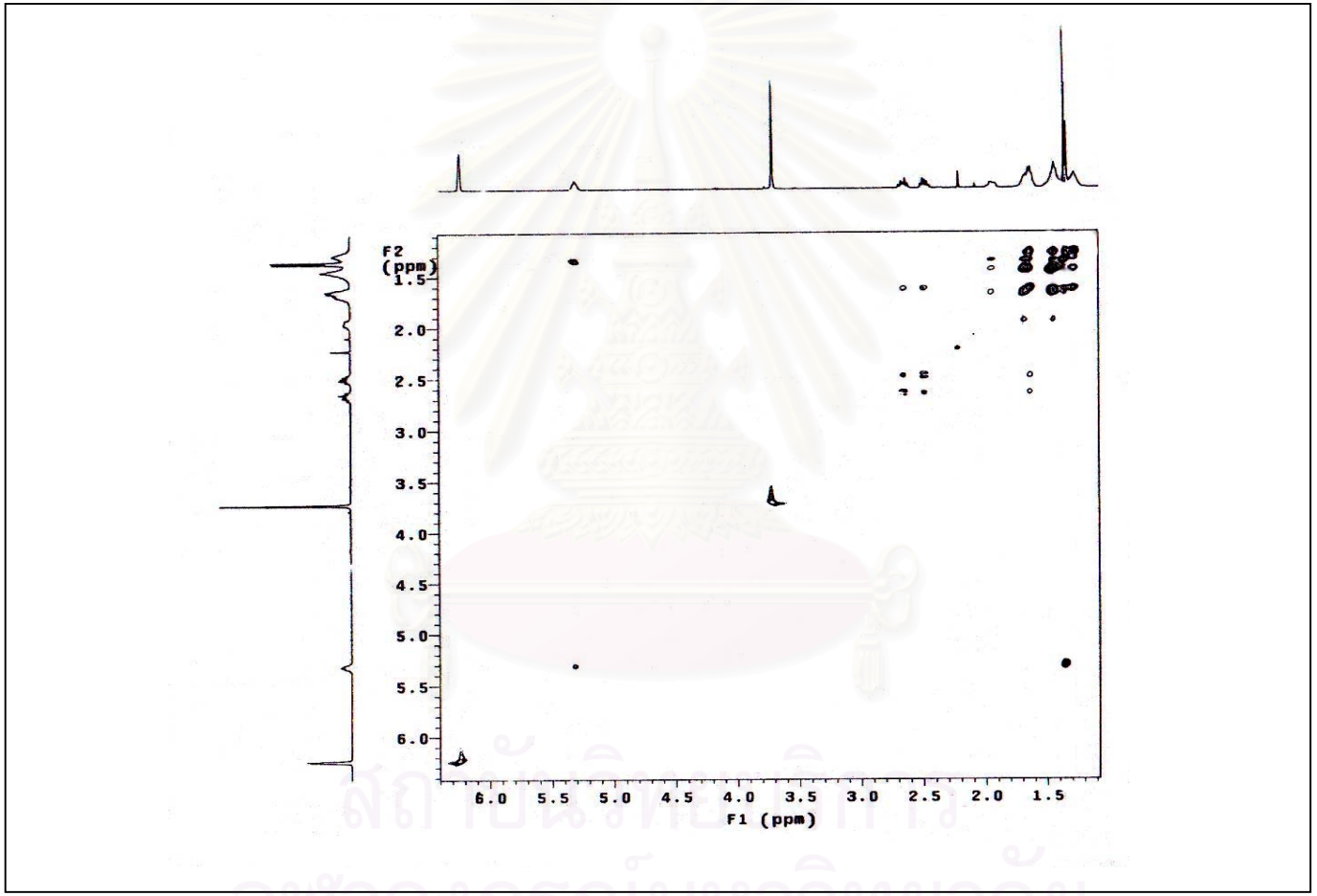

Figure B25 The TOCSY spectrum of compound 3

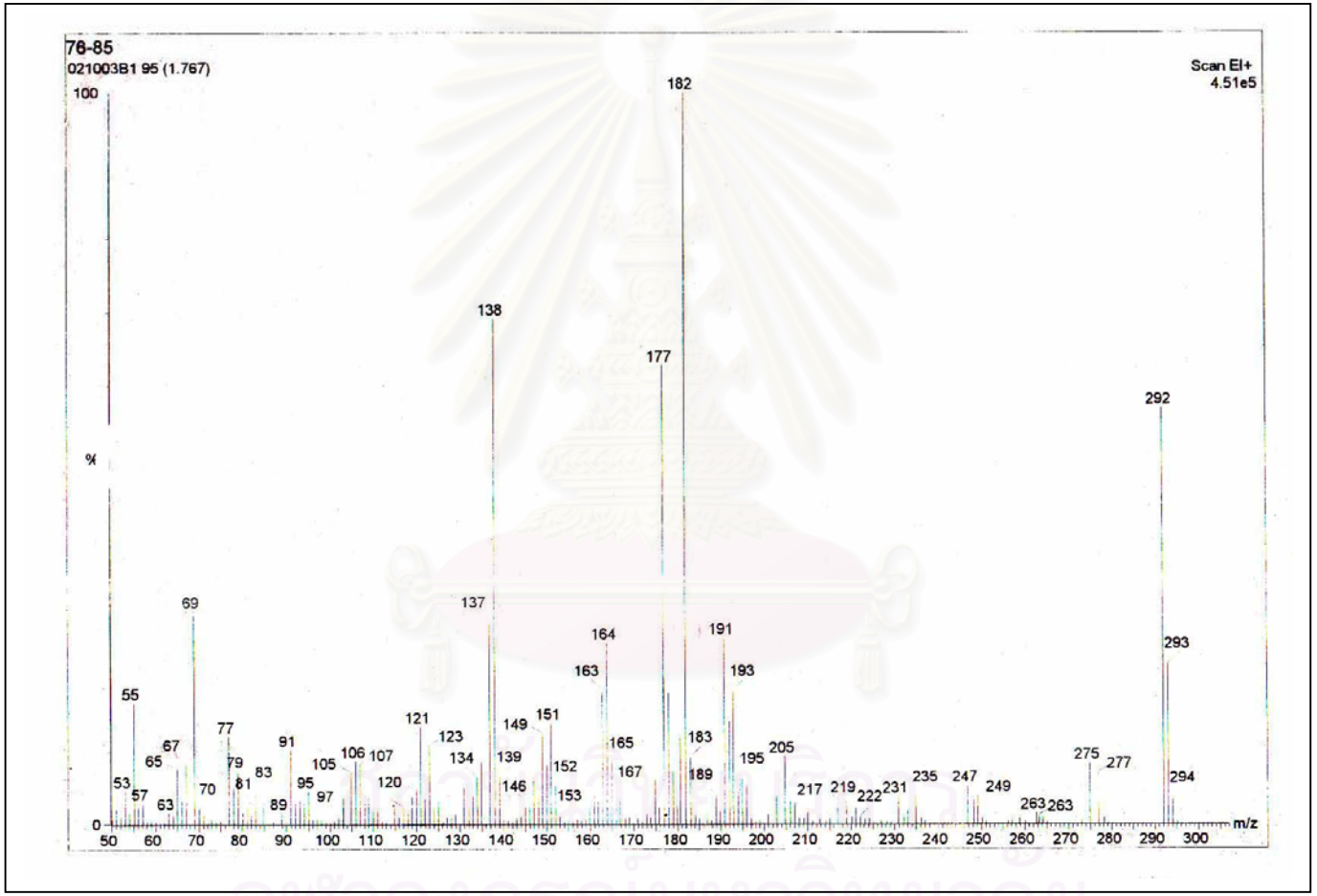

Figure B26 The MS spectrum of compound **3**

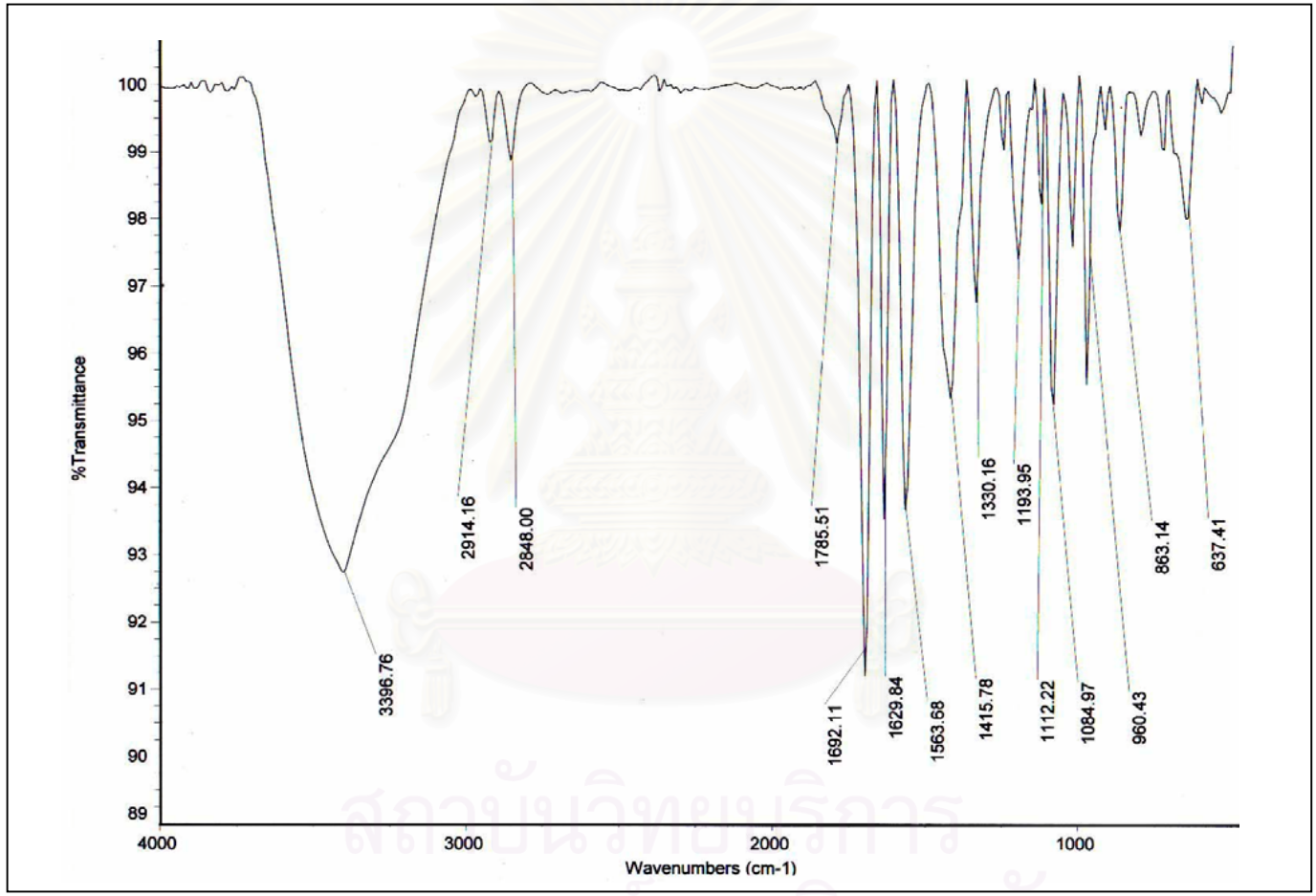

Figure B27 The IR spectrum of compound **4**

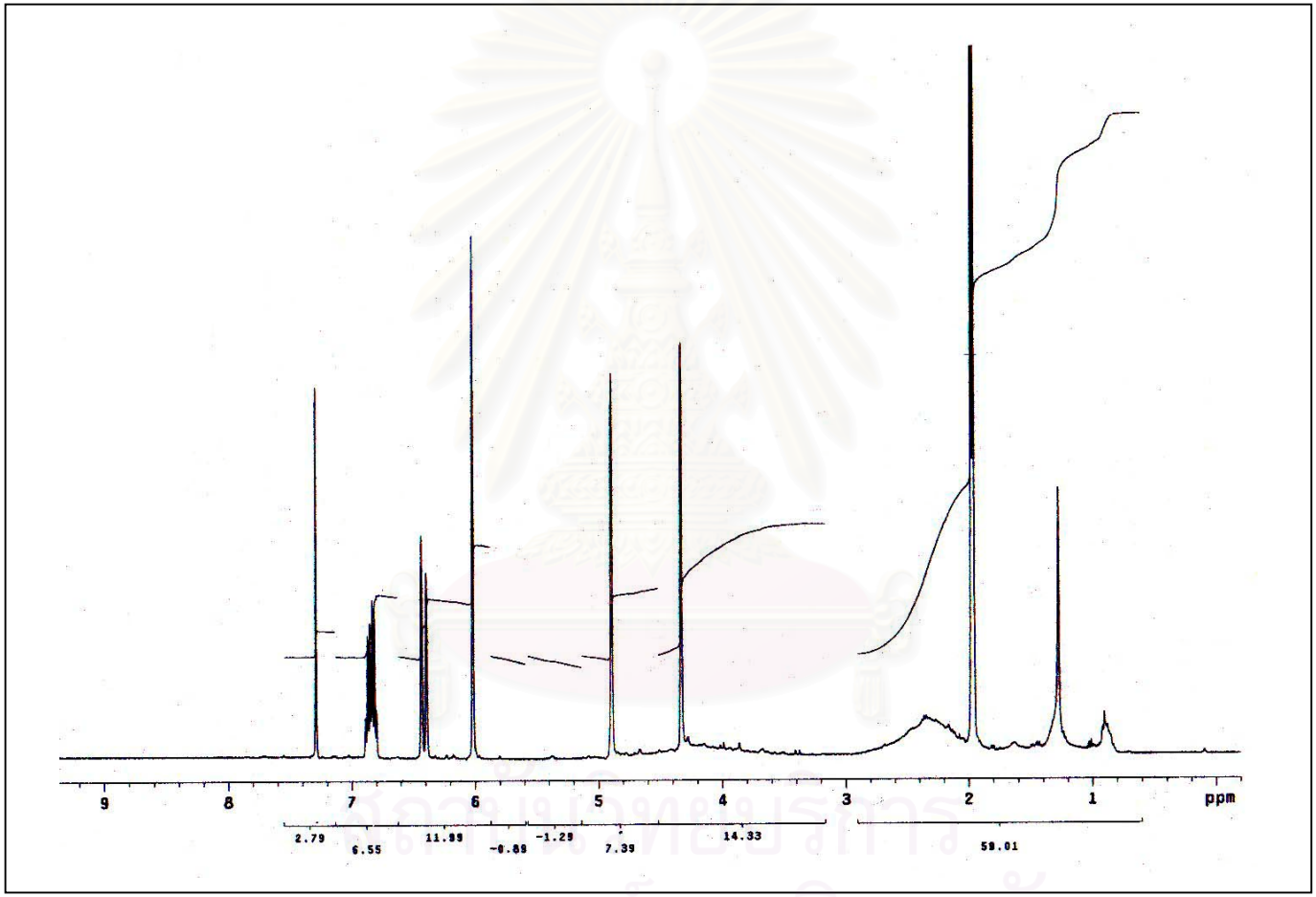

Figure B28 The ^{1}H-NMR spectrum of compound **4**

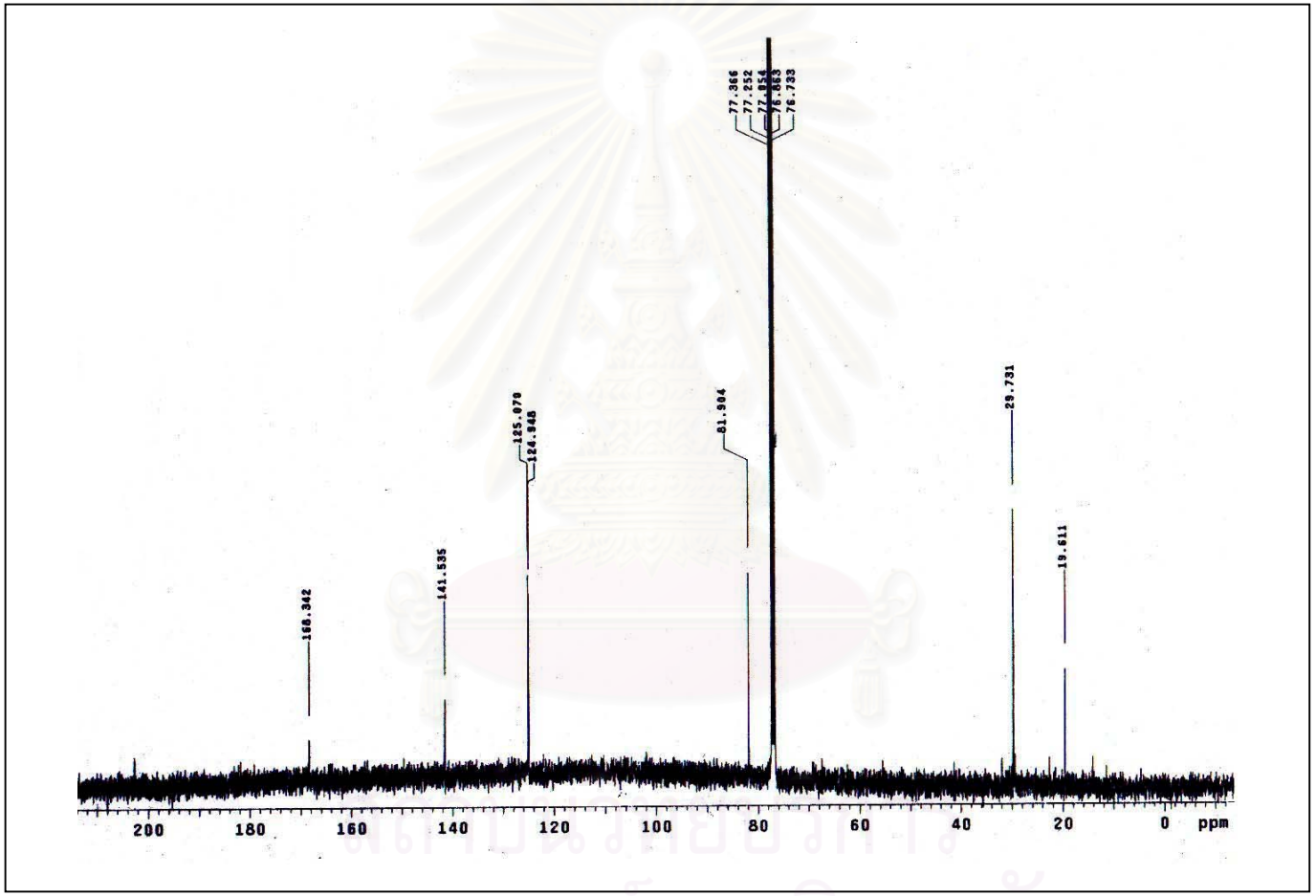

Figure B29 The ^{13}C-NMR spectrum of compound 4

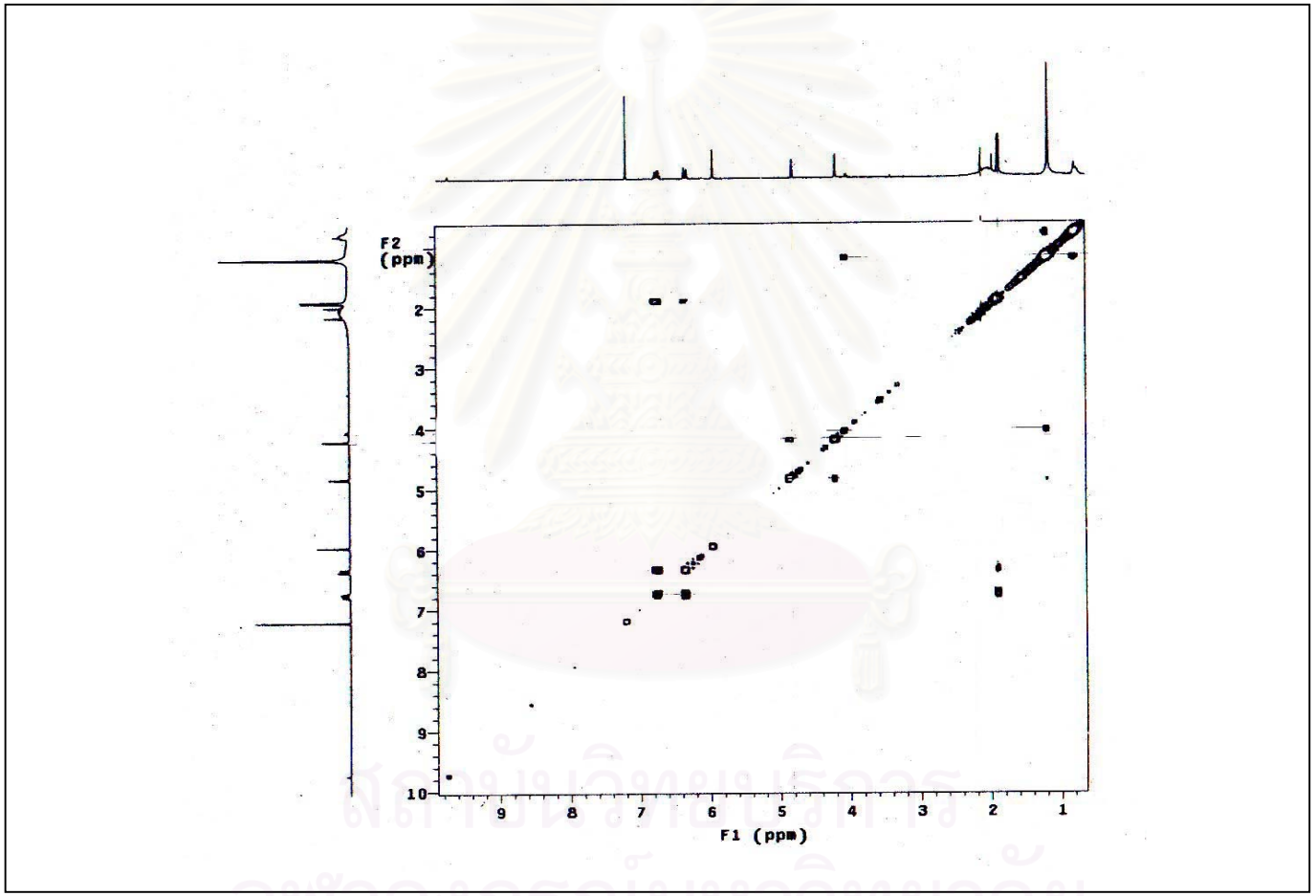

Figure B30 The gCOSY spectrum of compound 4

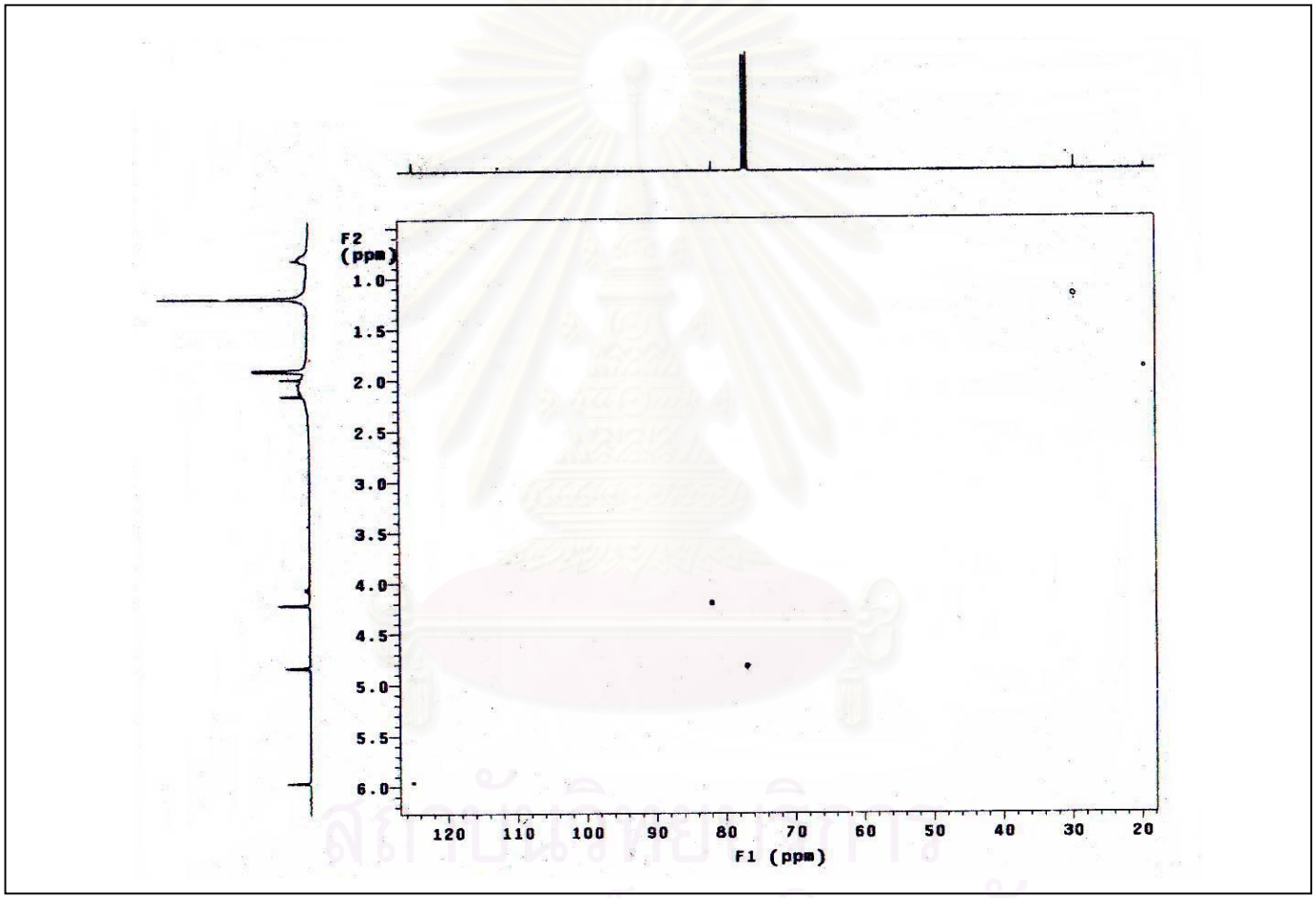

Figure B31 The gHSQC spectrum of compound 4

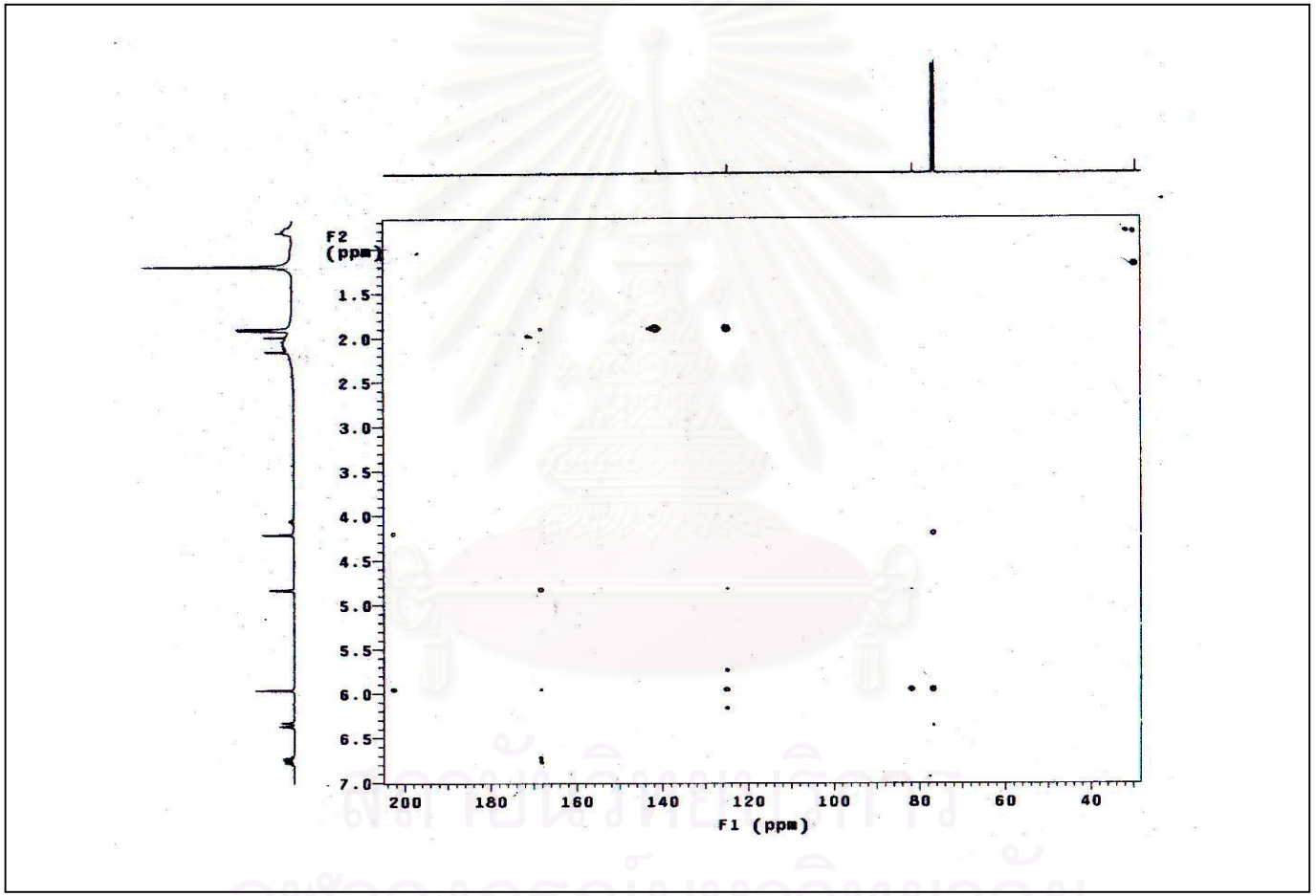

Figure B32 The gHMBC spectrum of compound **4**

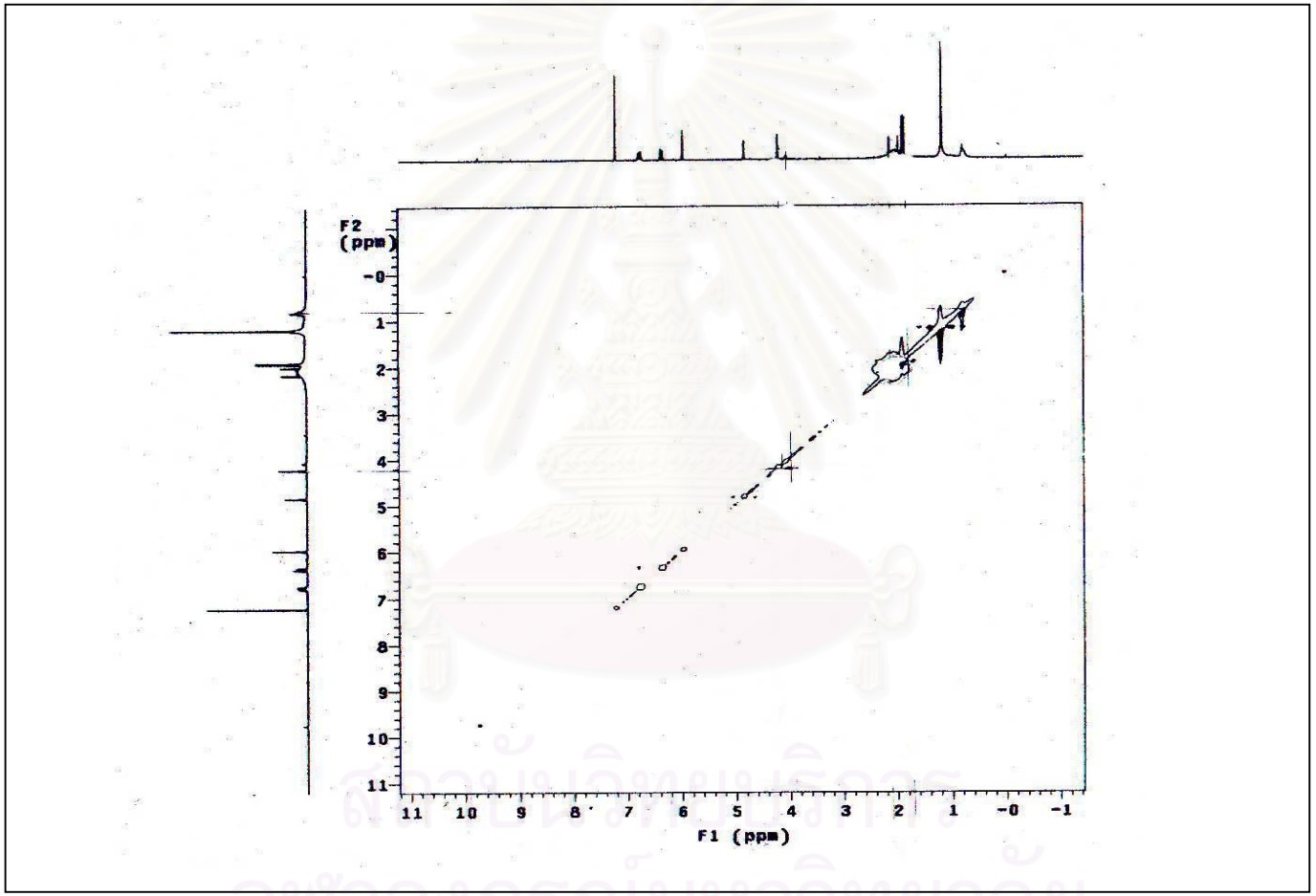

Figure B33 The NOESY spectrum of compound **4**

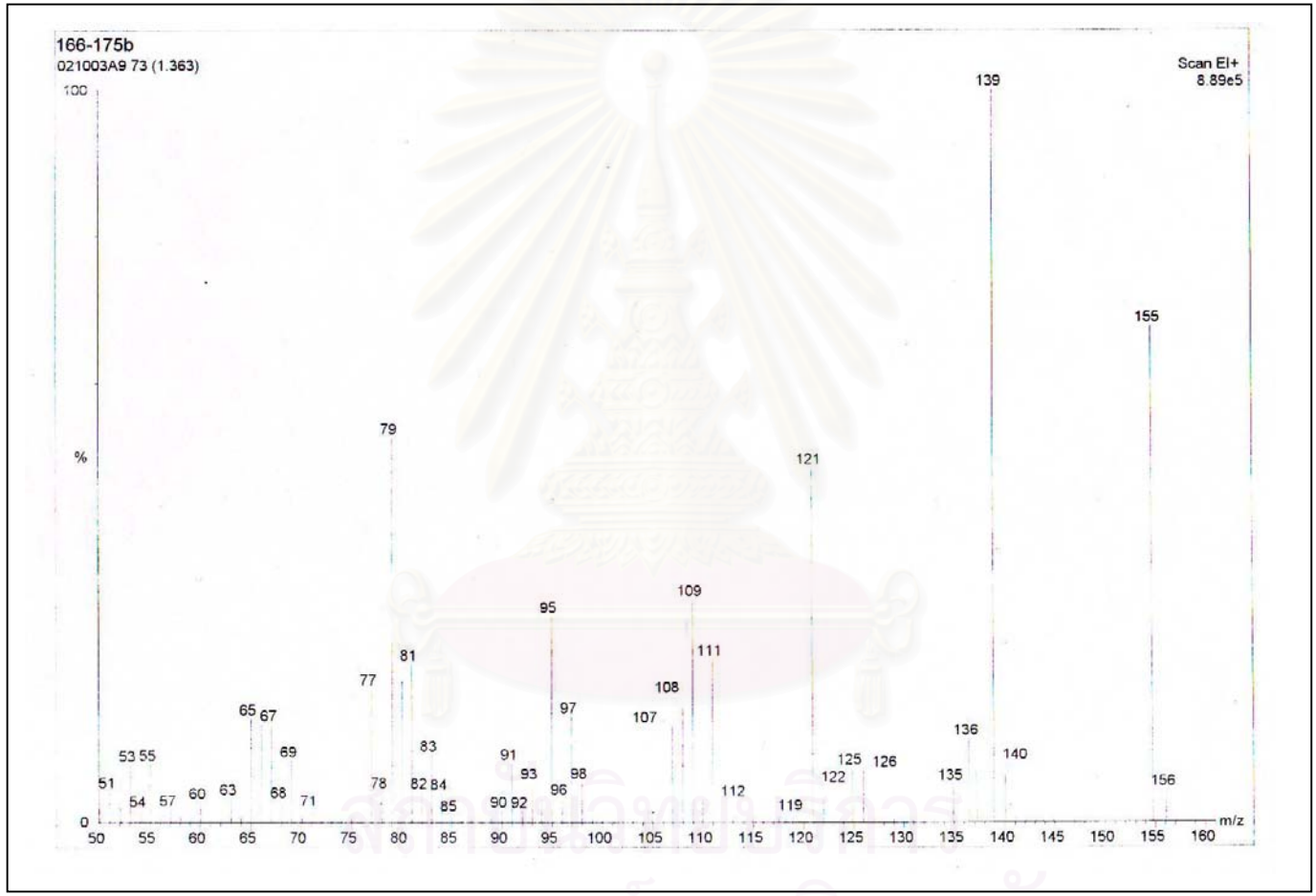

Figure B34 The MS spectrum of compound **4**

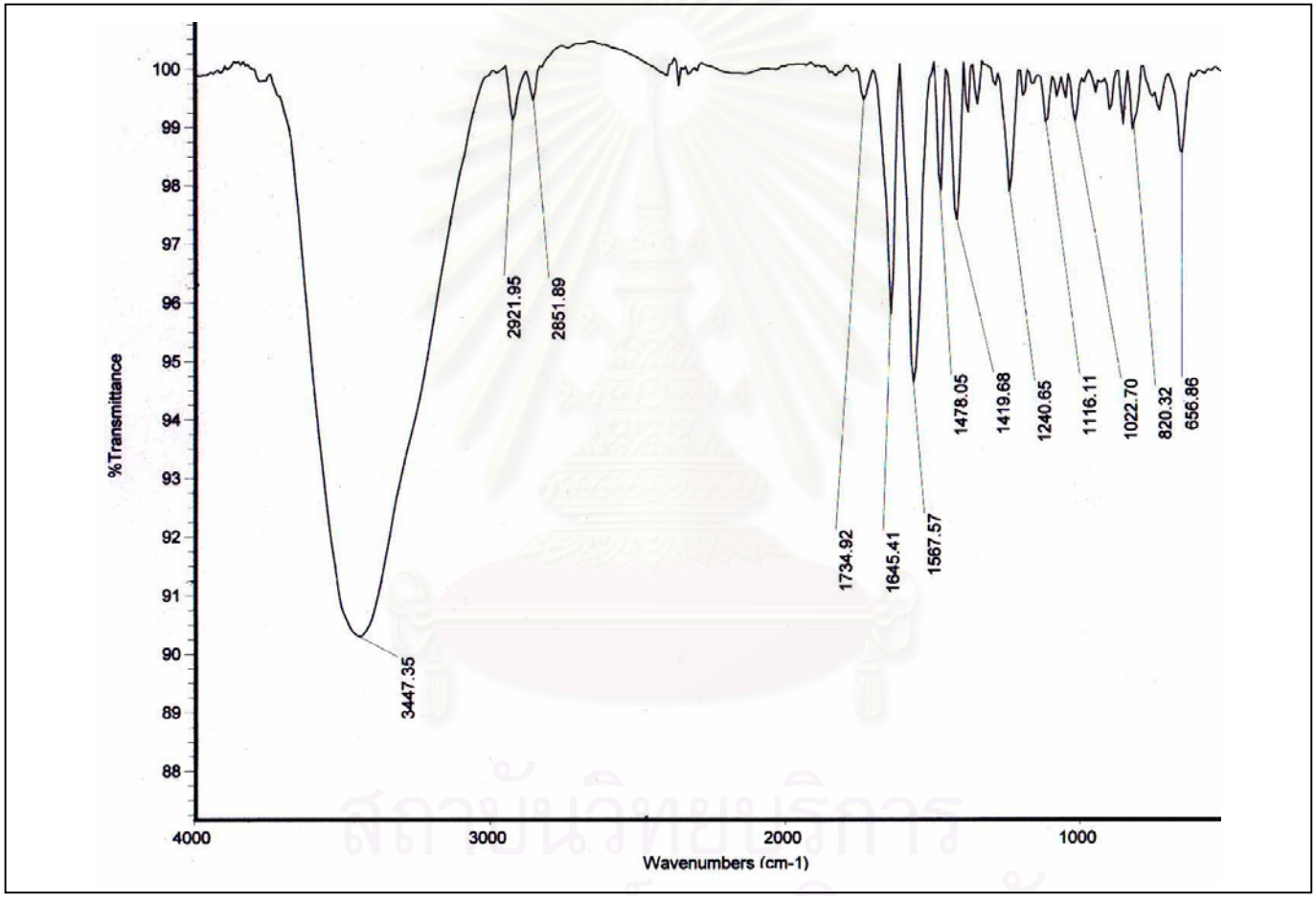

Figure B35 The IR spectrum of compound 5

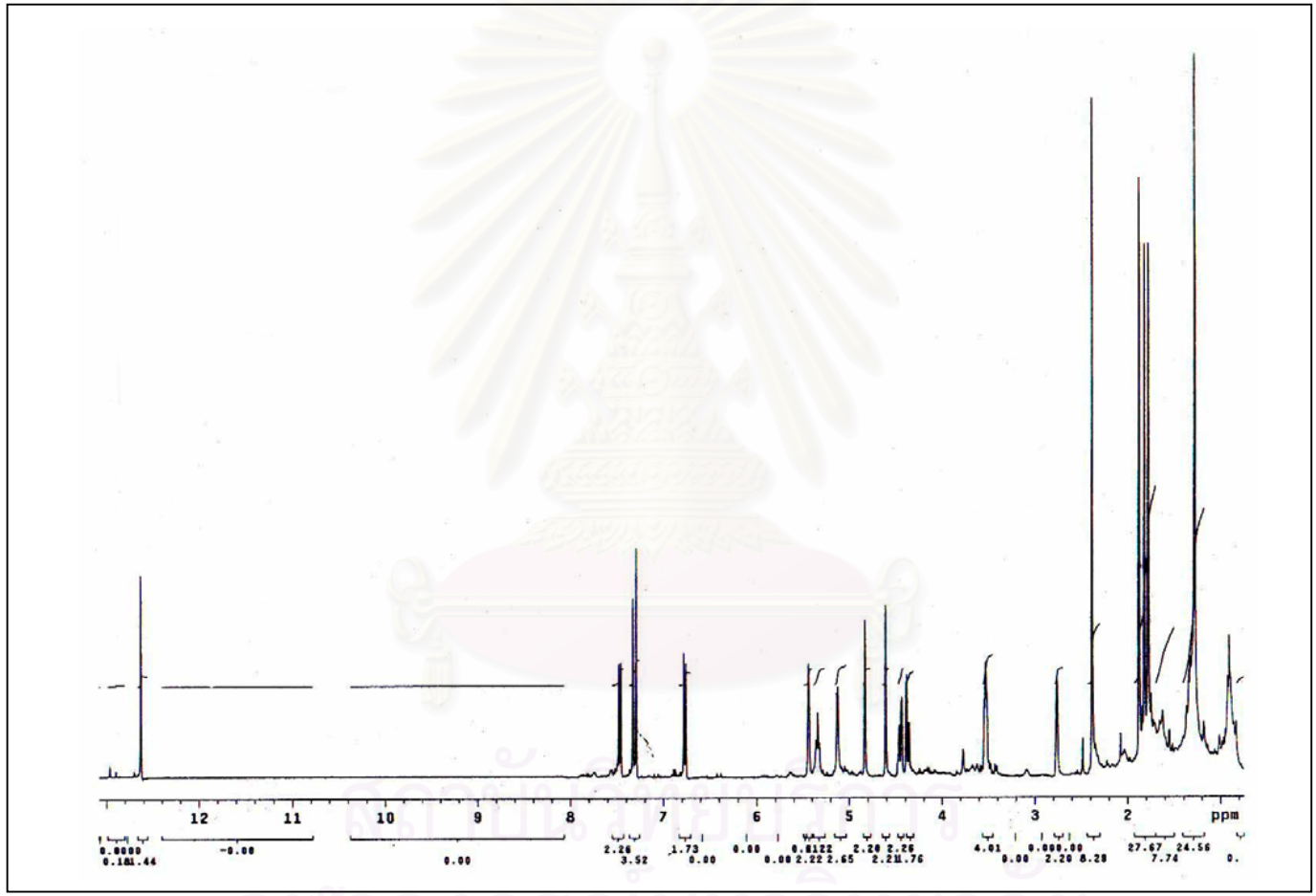

Figure B36 The ^{1}H-NMR spectrum of compound **5**

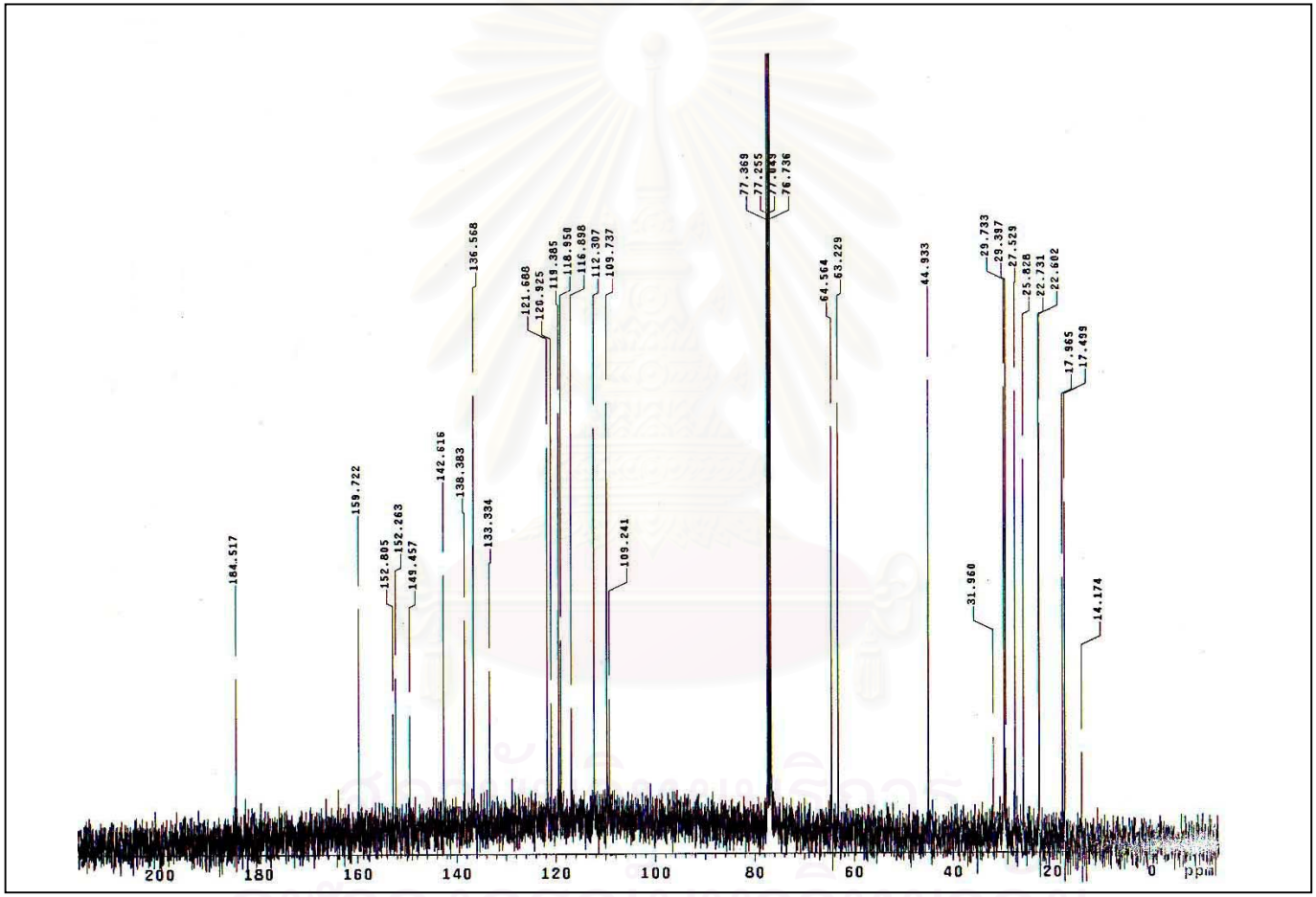

Figure B37 The ^{13}C-NMR spectrum of compound 5

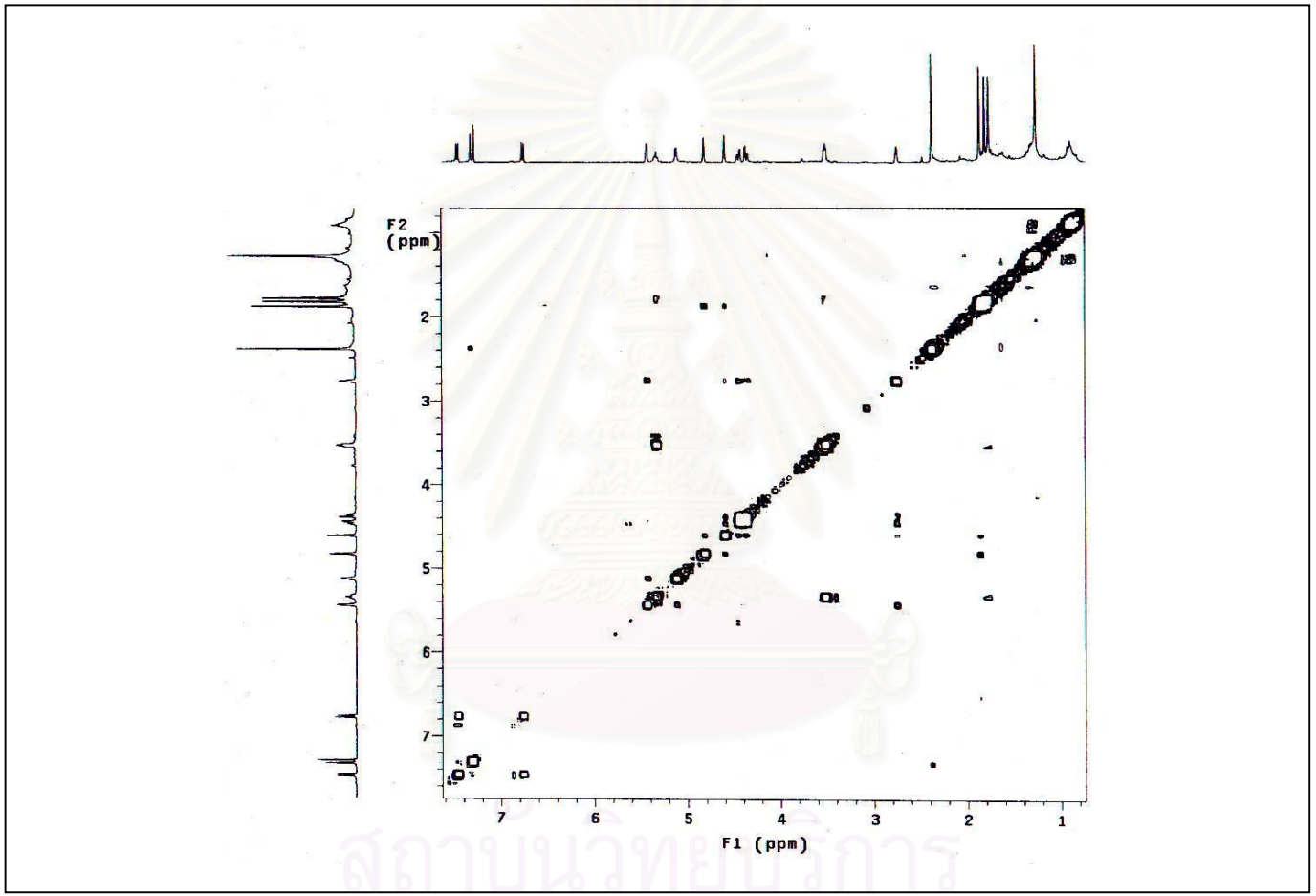

Figure B38 The gCOSY spectrum of compound 5

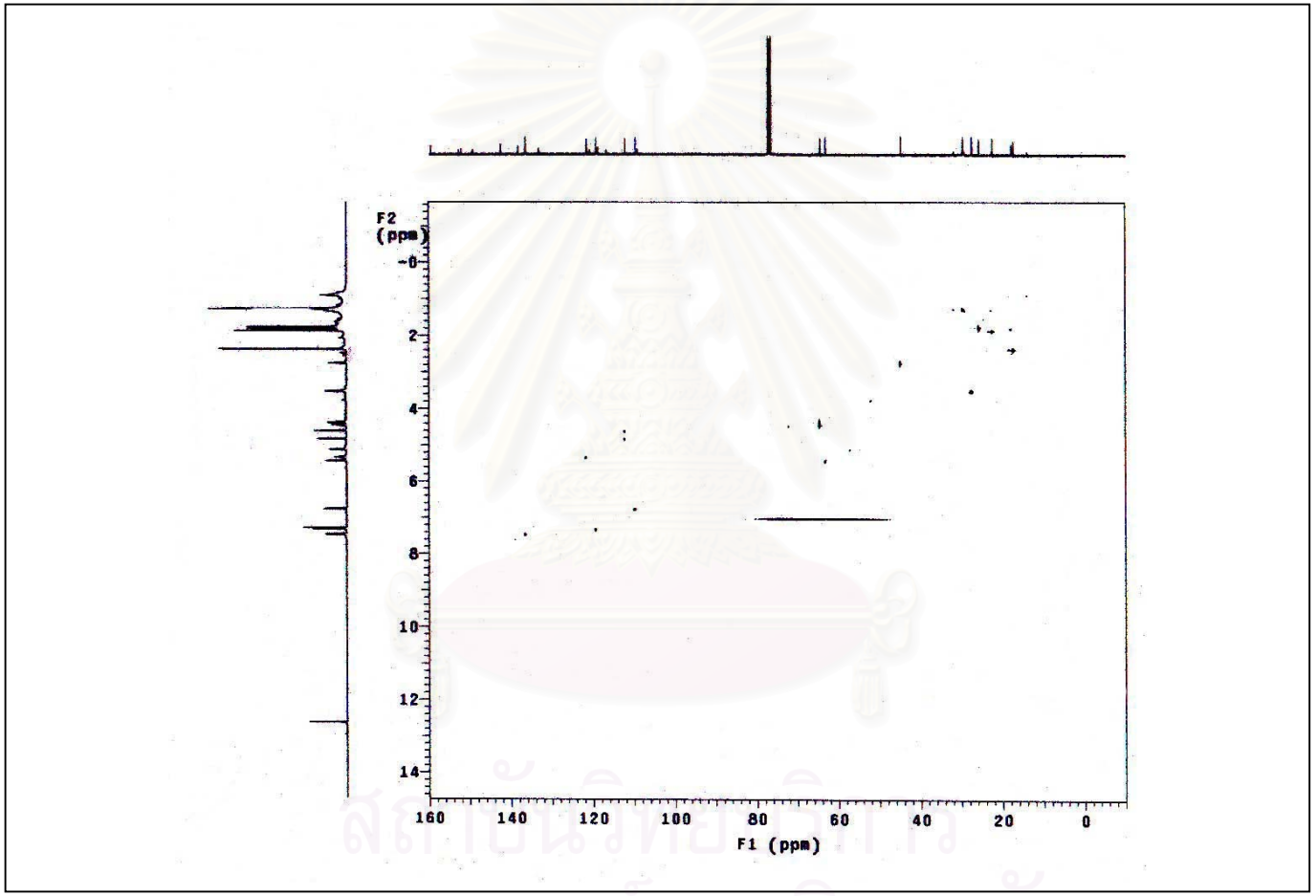

Figure B39 The gHSQC spectrum of compound 5

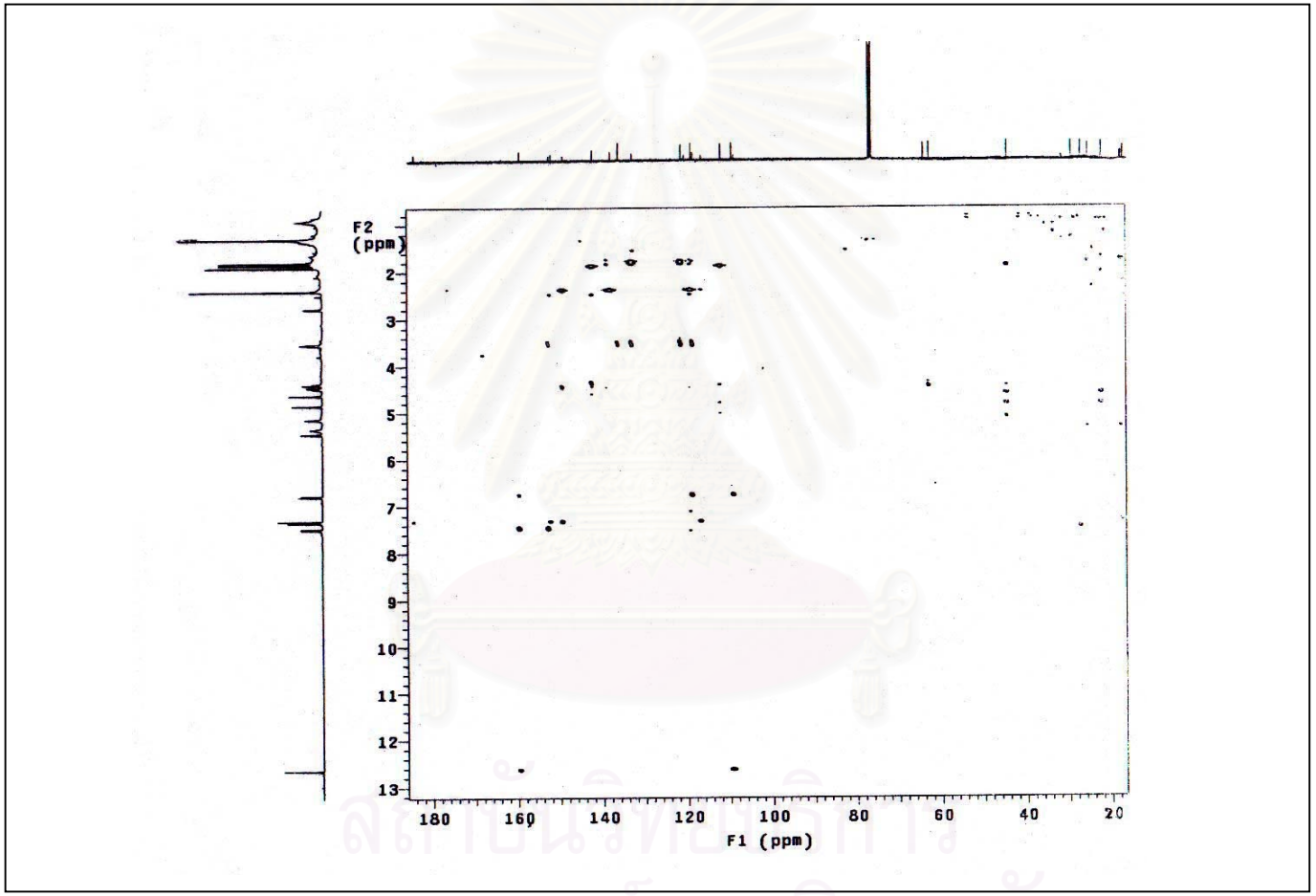

Figure B40 The gHMBC spectrum of compound 5

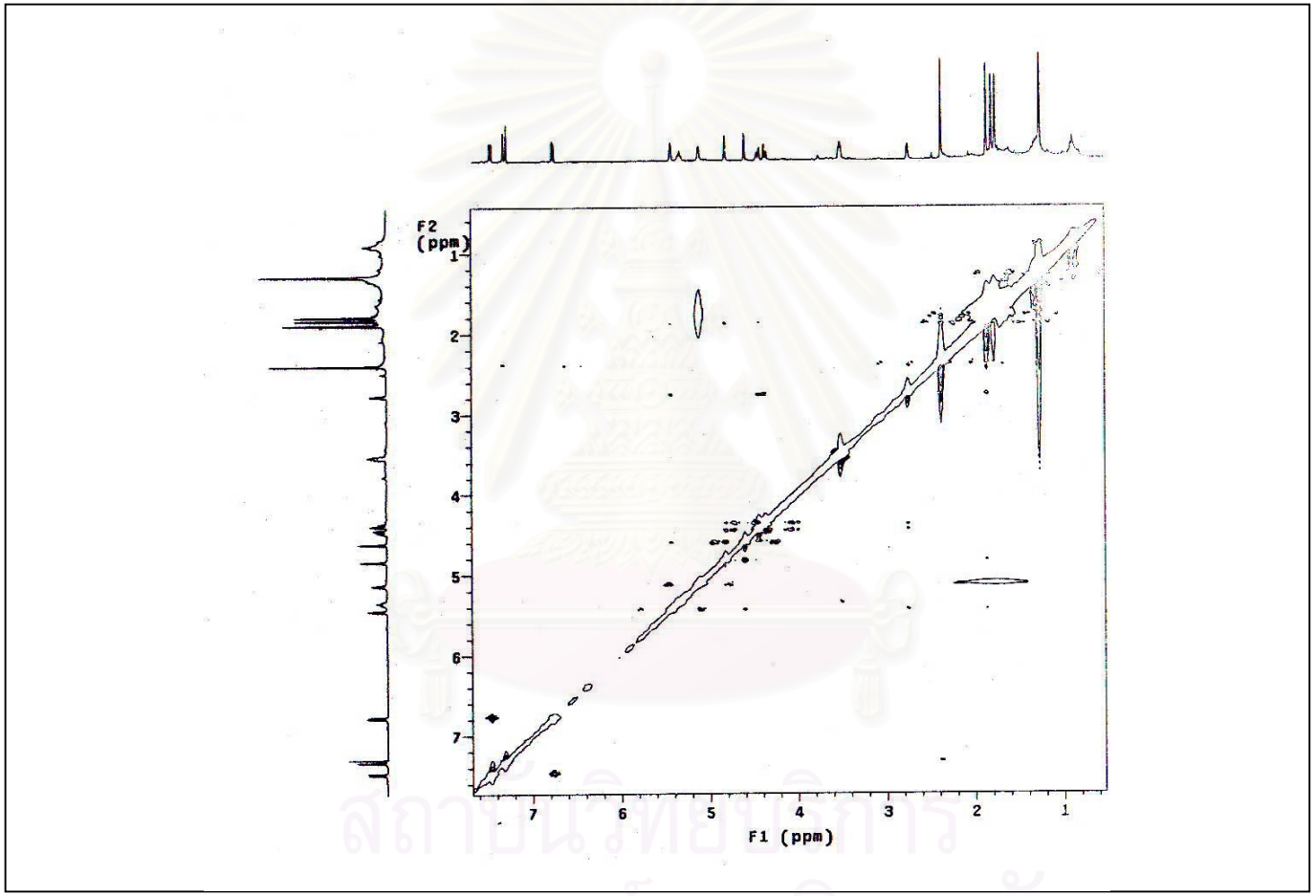

Figure B41 The NOESY spectrum of compound **5**

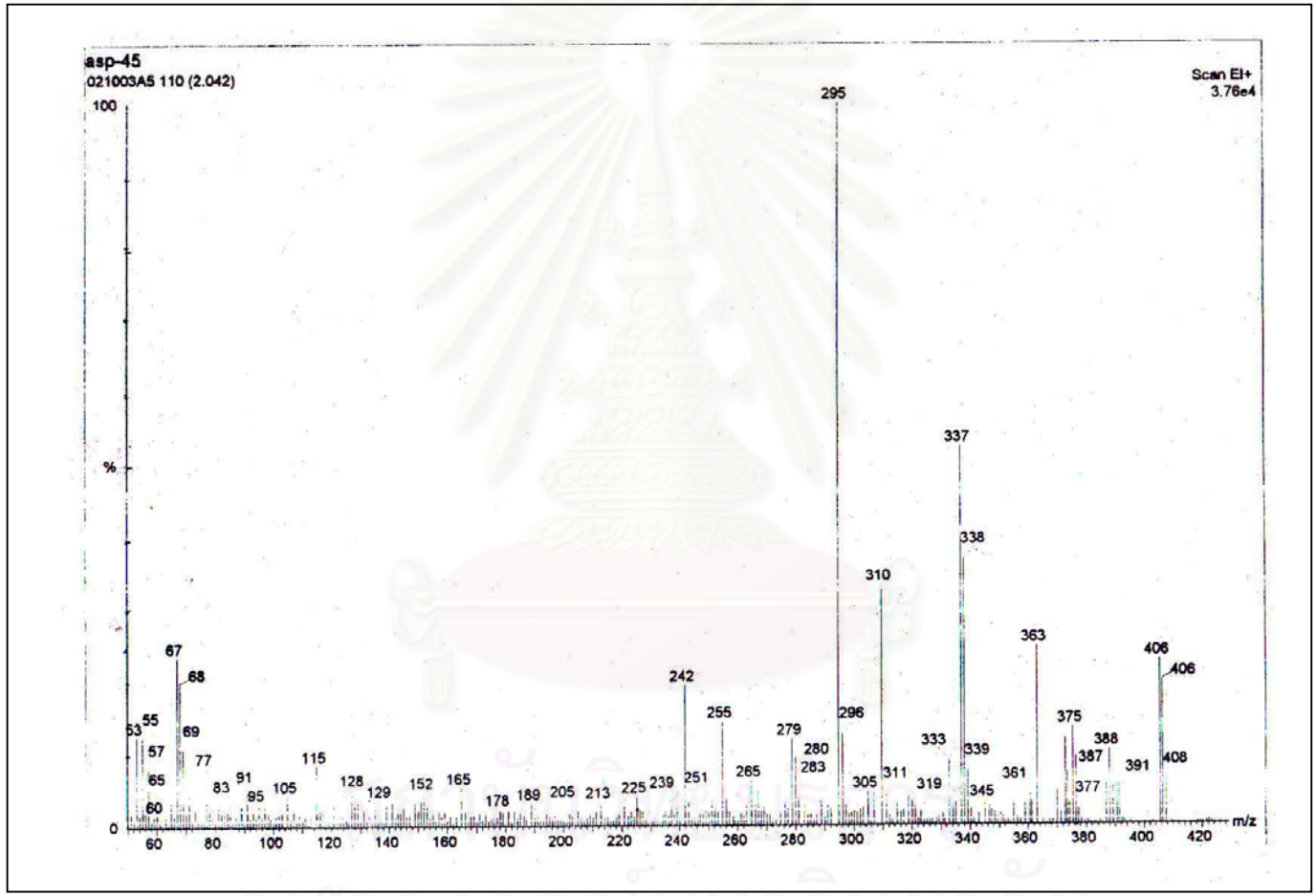

Figure B42 The MS spectrum of compound **5**

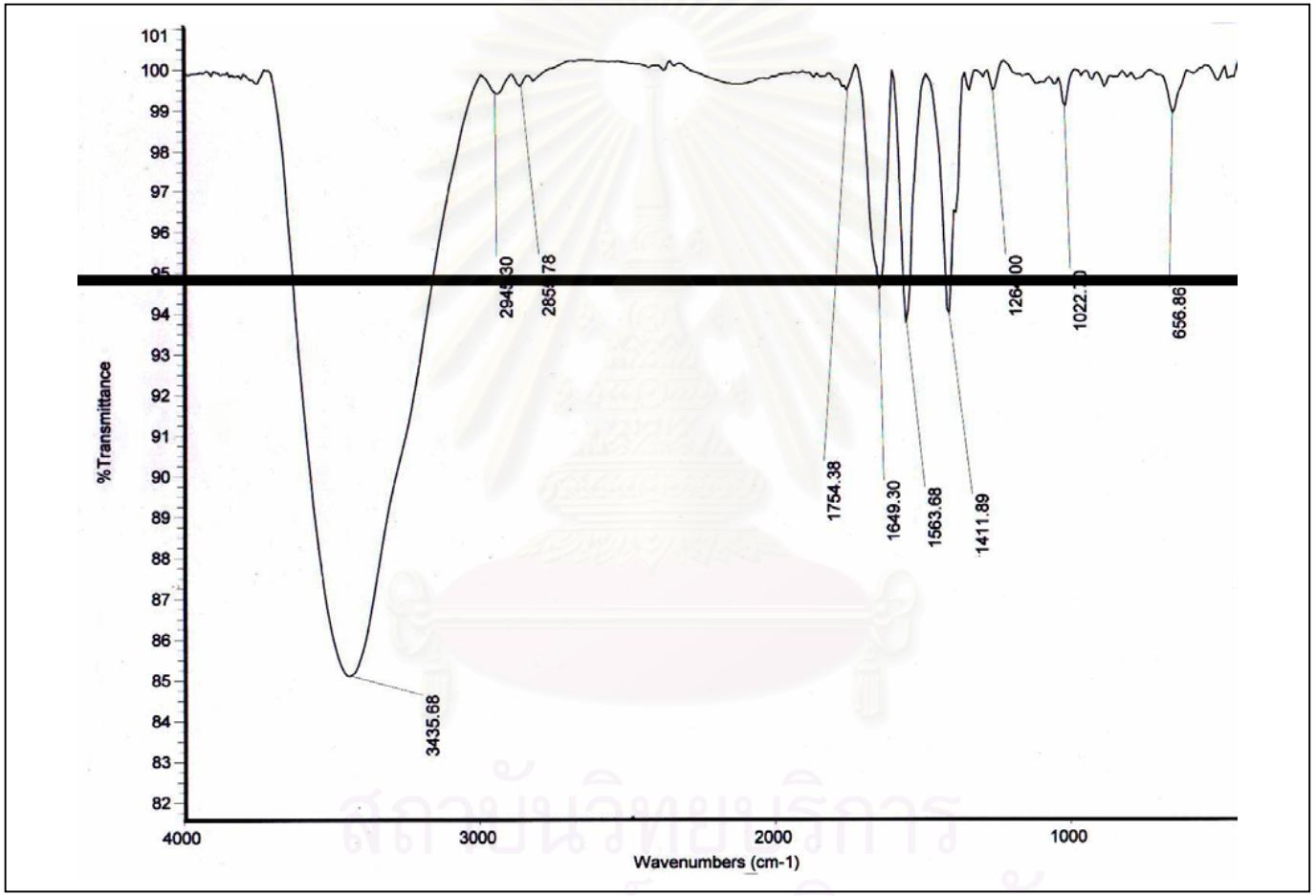

Figure B43 The IR spectrum of compound 6

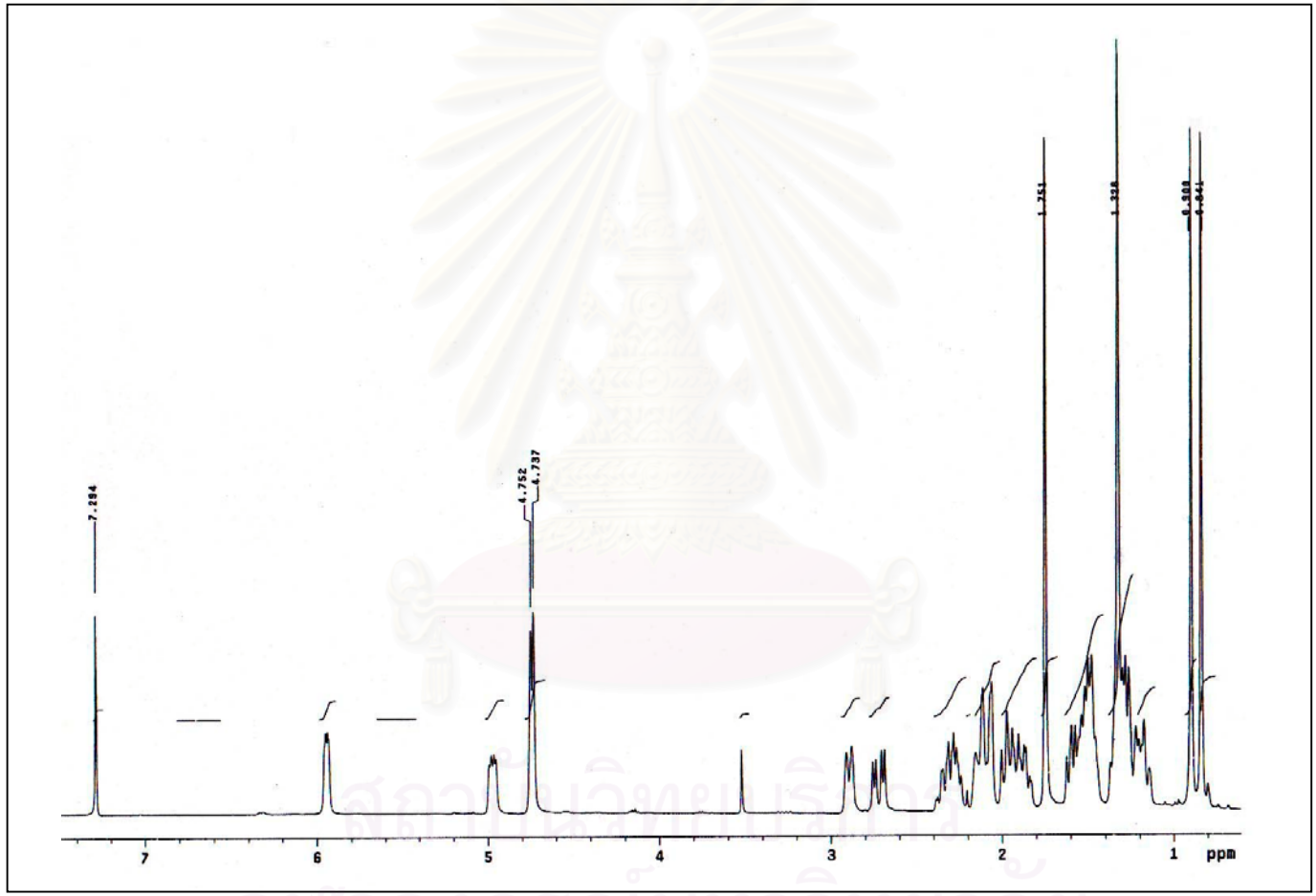

Figure B44 The ^{1}H-NMR spectrum of compound **6**

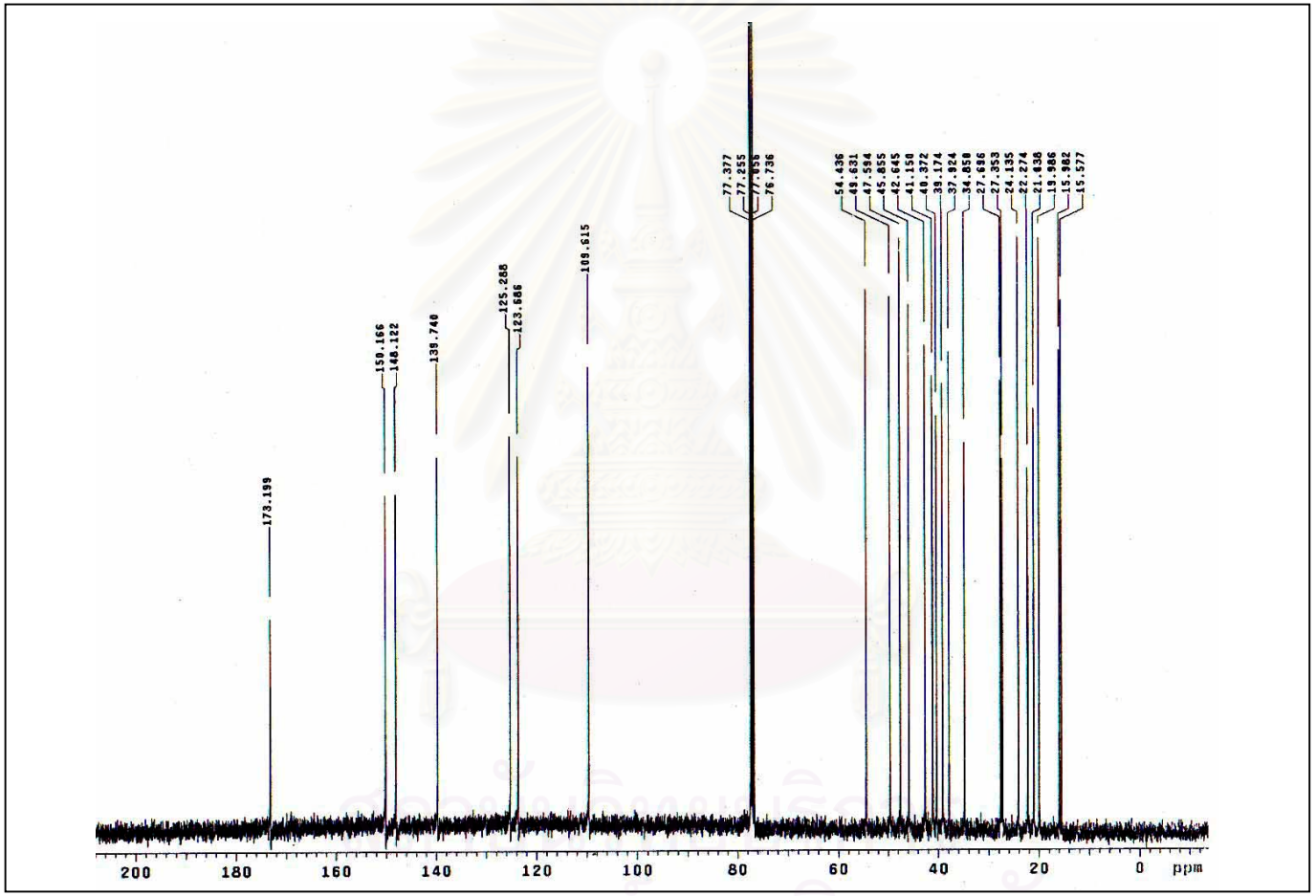

Figure B45 The ^{13}C-NMR spectrum of compound 6

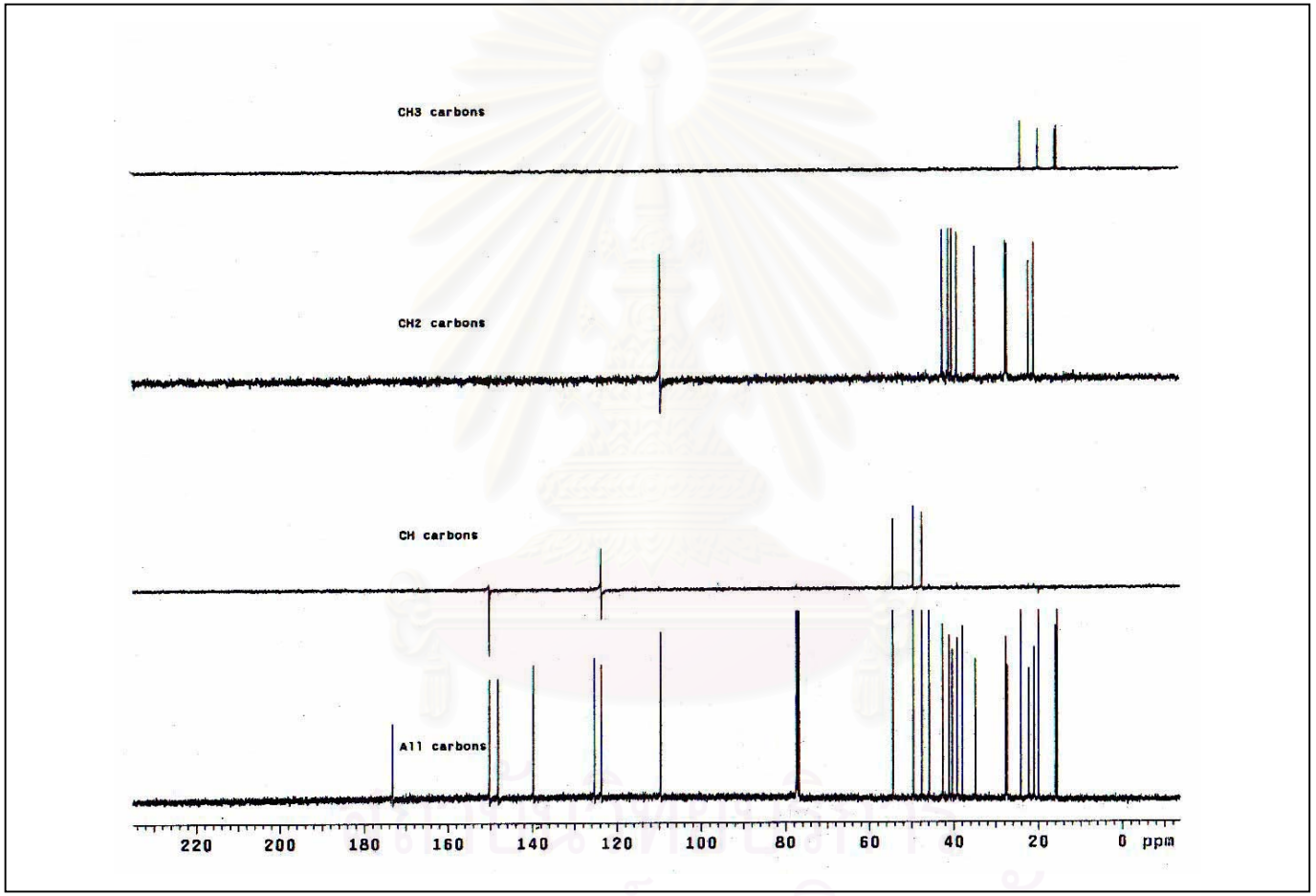

Figure B46 The DEPT, ^{13}C-NMR spectrum of compound 6

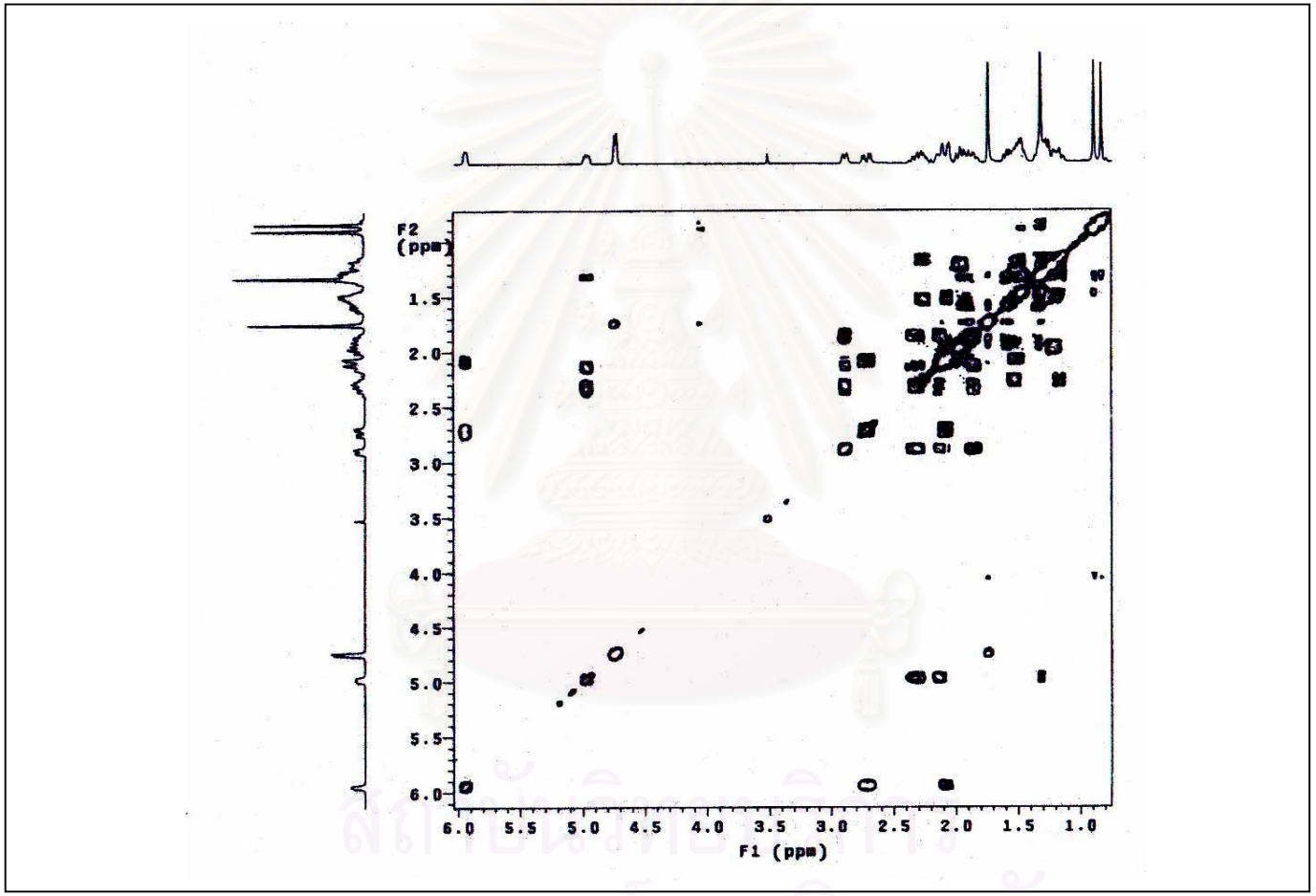

Figure B47 The gCOSY spectrum of compound **6**

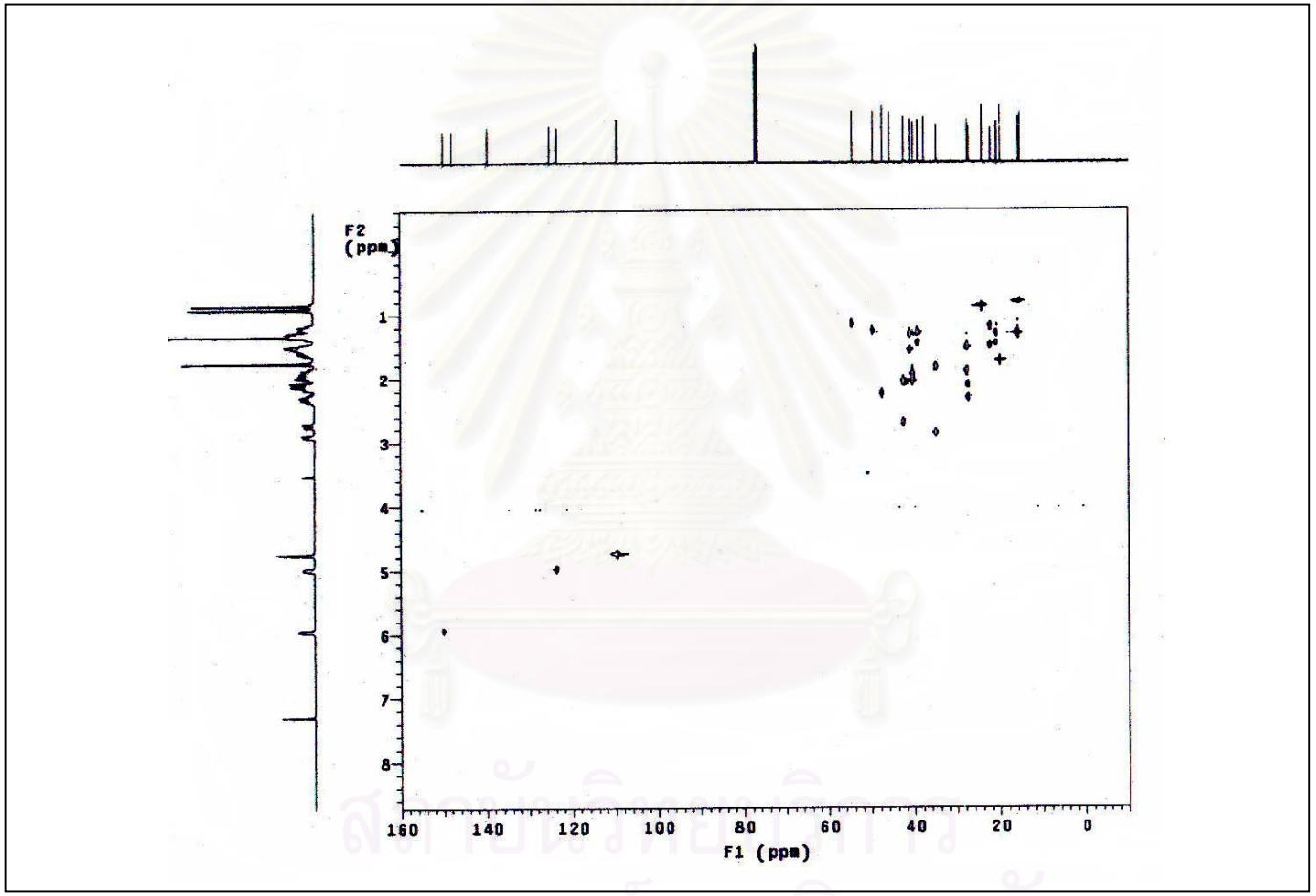

Figure B48 The gHSQC spectrum of compound 6

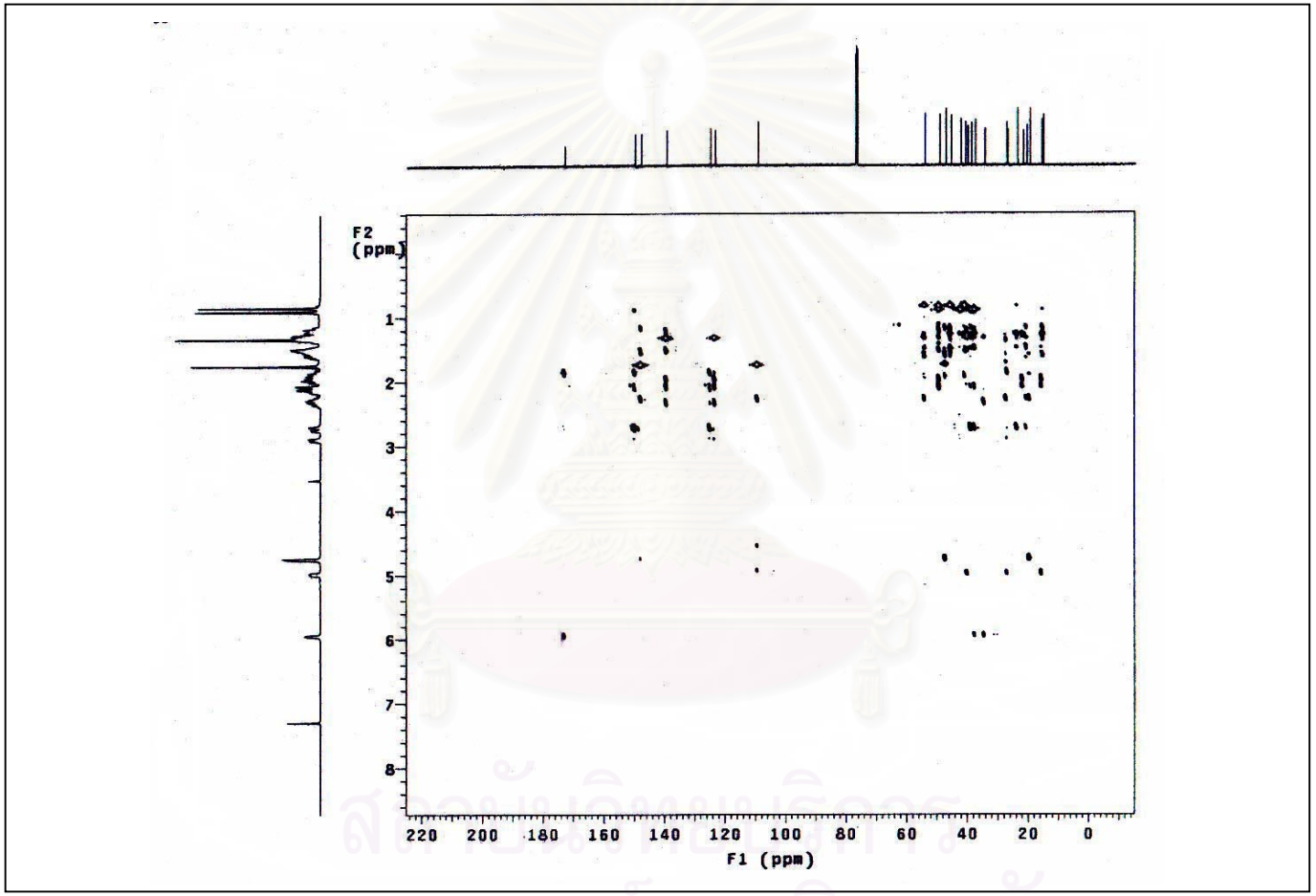

Figure B49 The gHMBC spectrum of compound 6

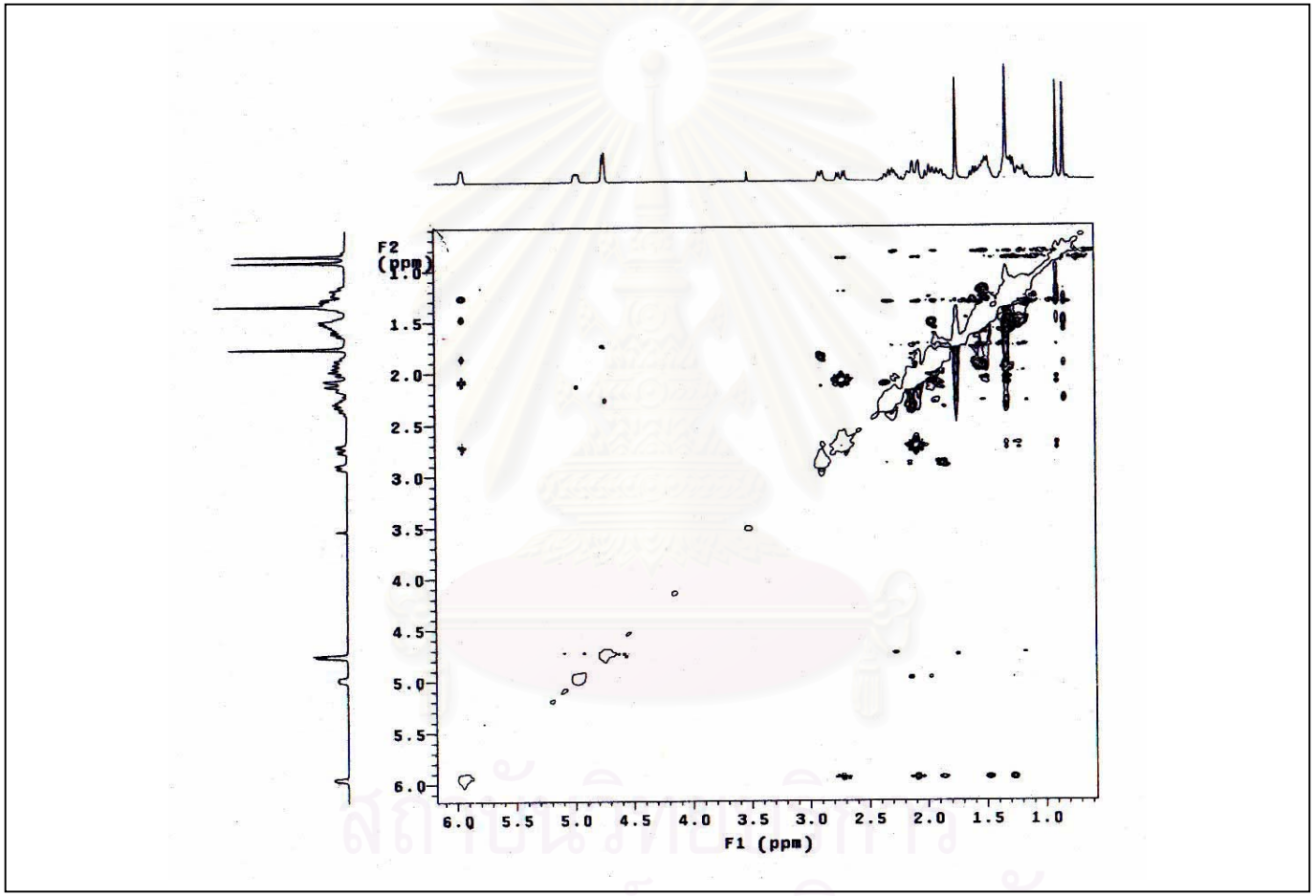

Figure B50 The NOESY spectrum of compound 6

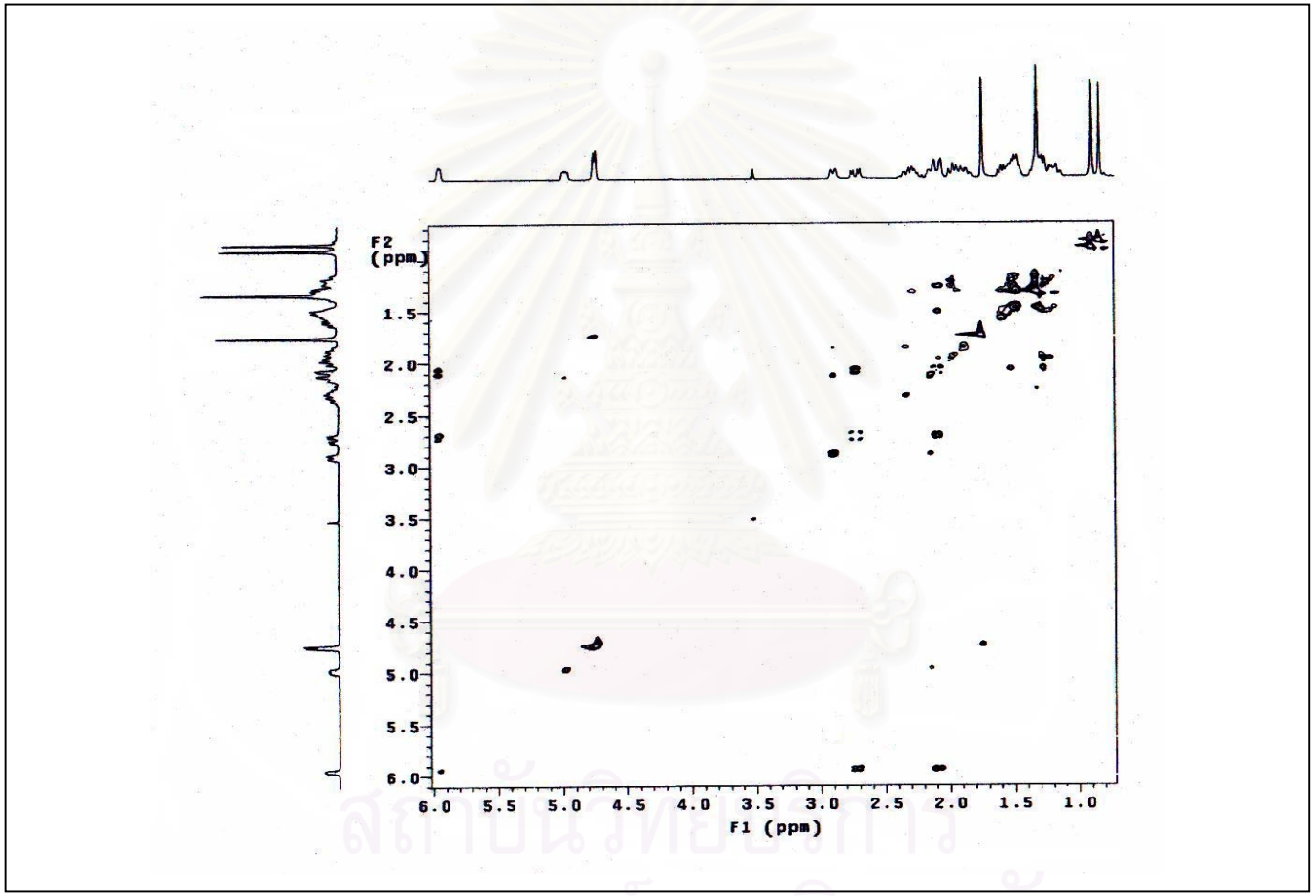

Figure B51 The TOCSY spectrum of compound 6

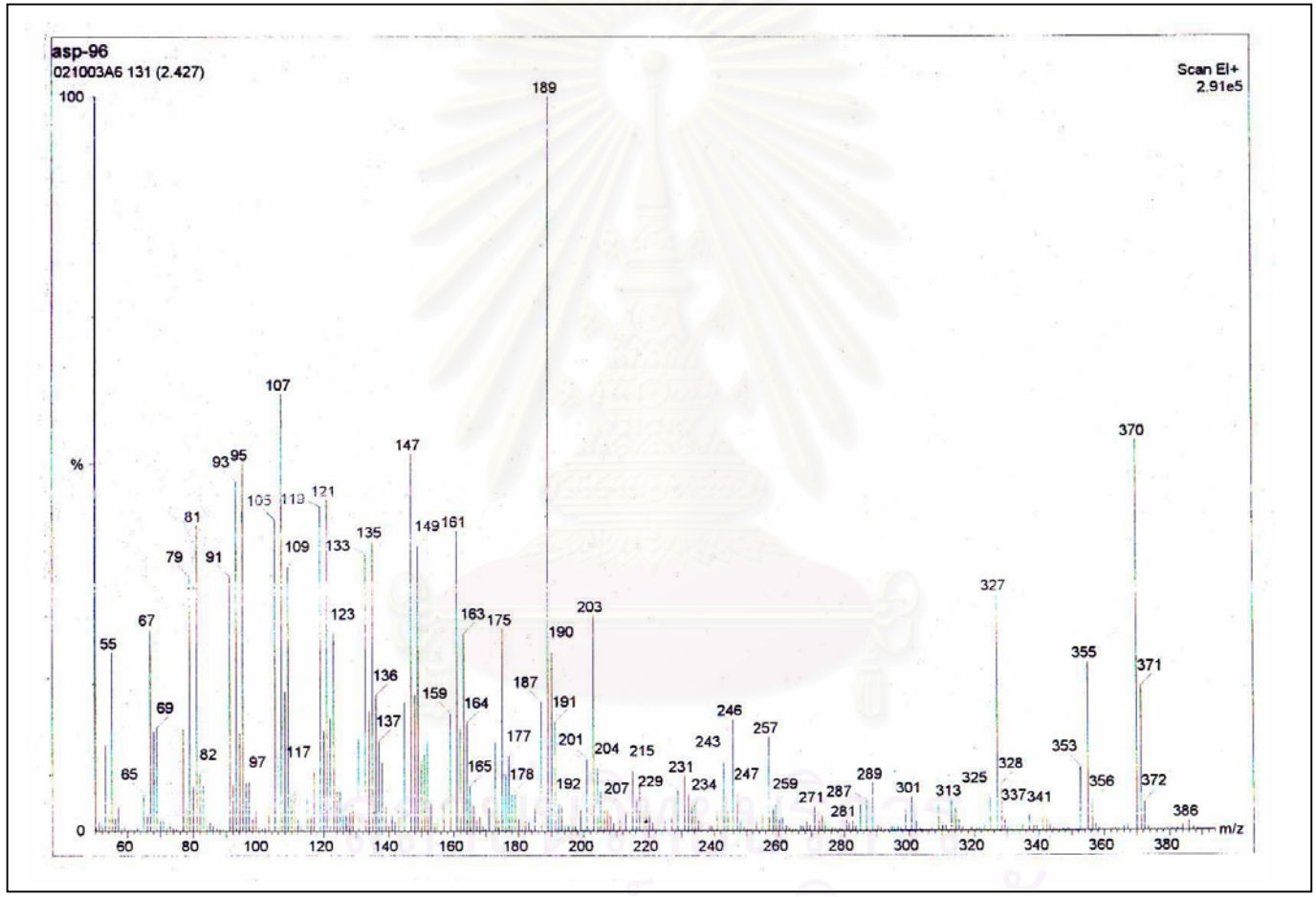

Figure B52 The MS spectrum of compound 6

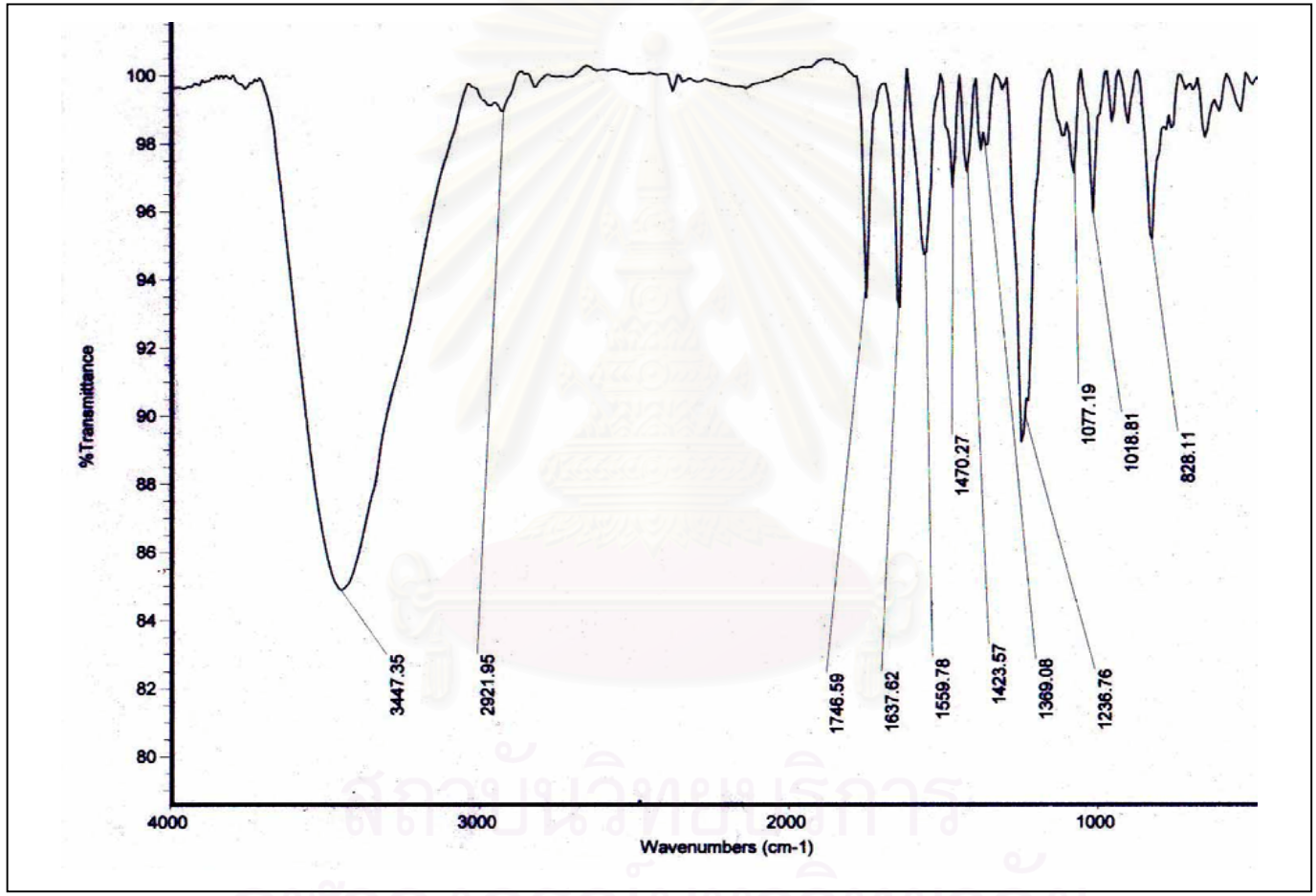

Figure B53 The IR spectrum of compound 7

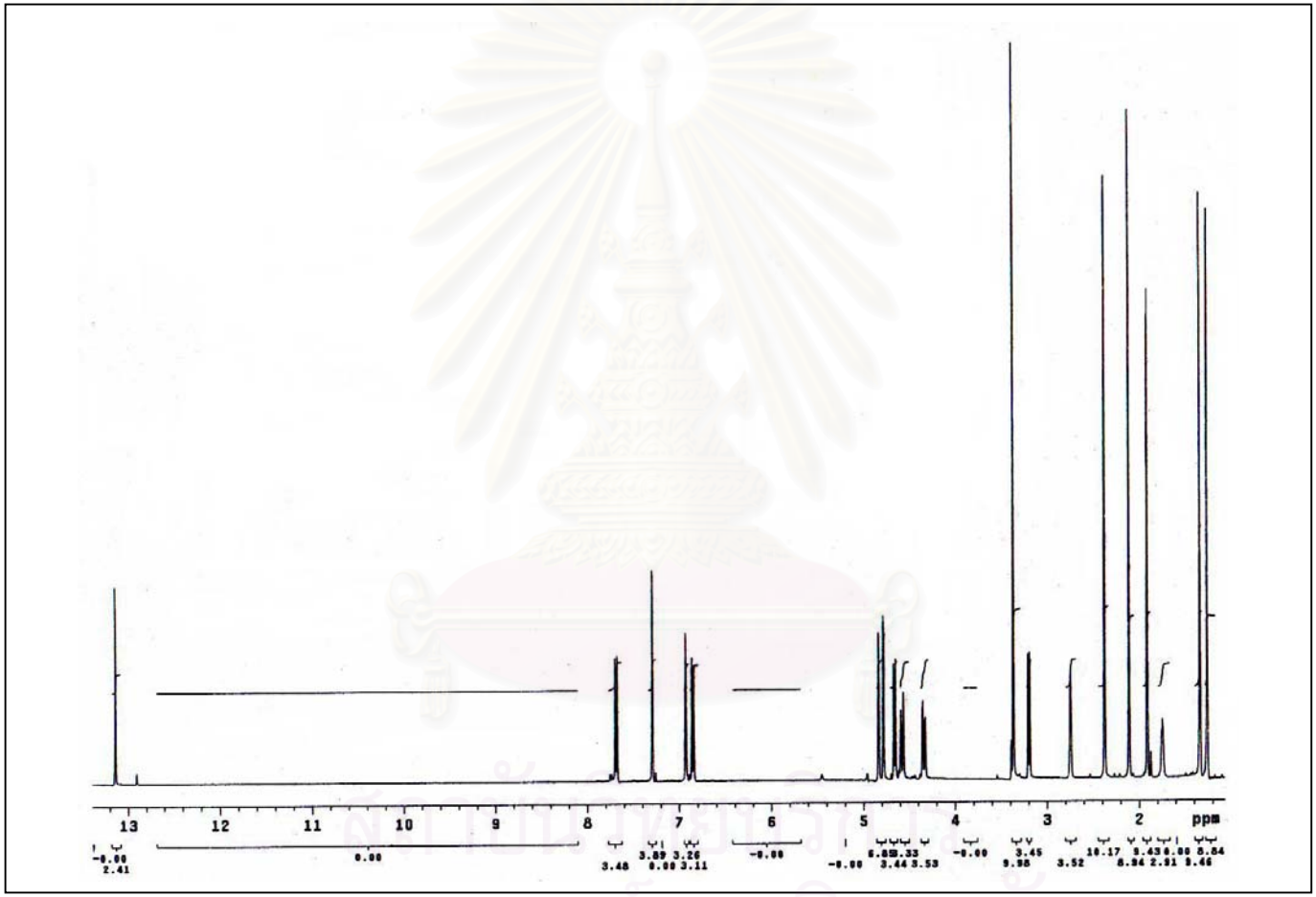

Figure B54 The ^{1}H-NMR spectrum of compound **7**

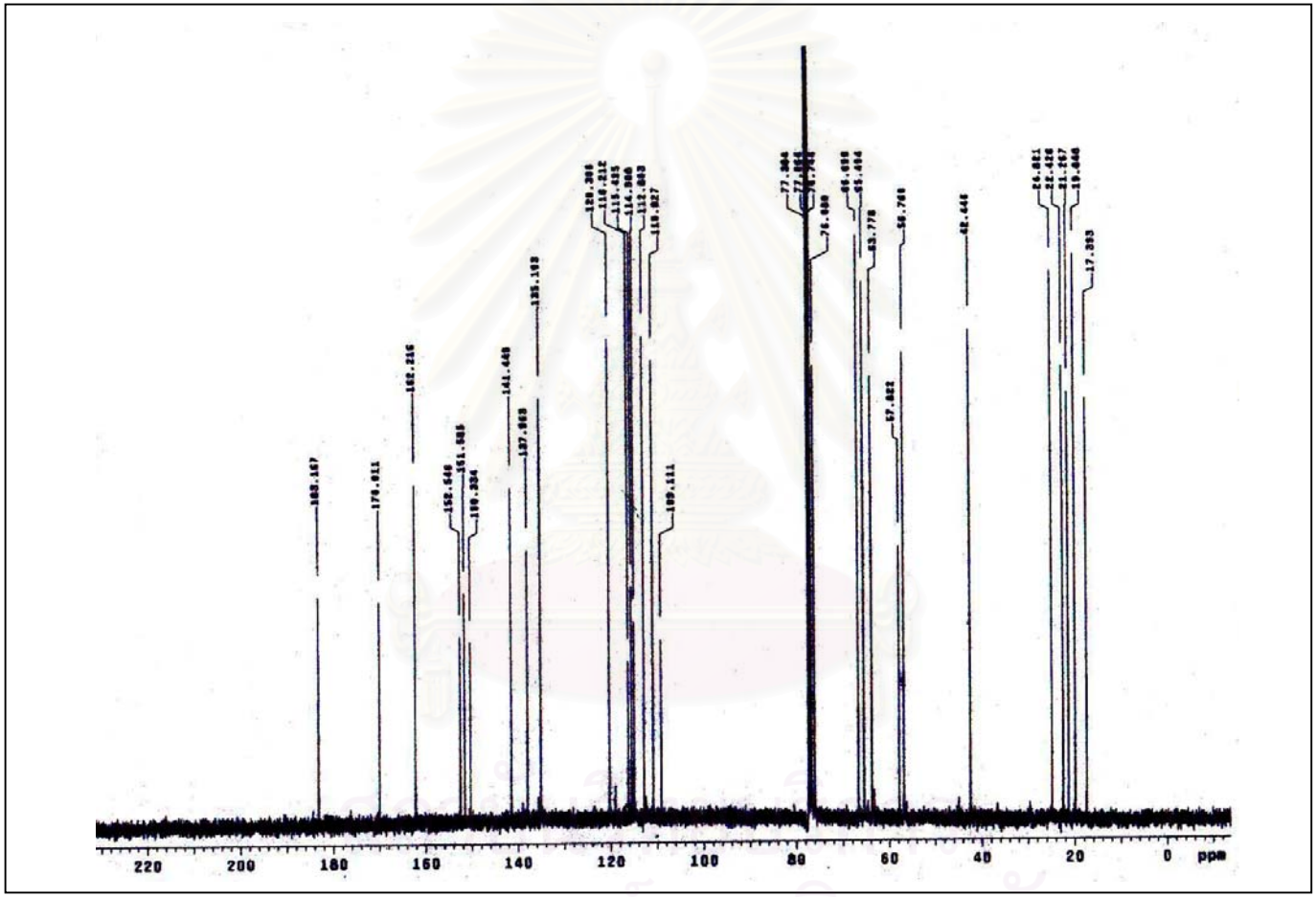

Figure B55 The ^{13}C-NMR spectrum of compound **7**

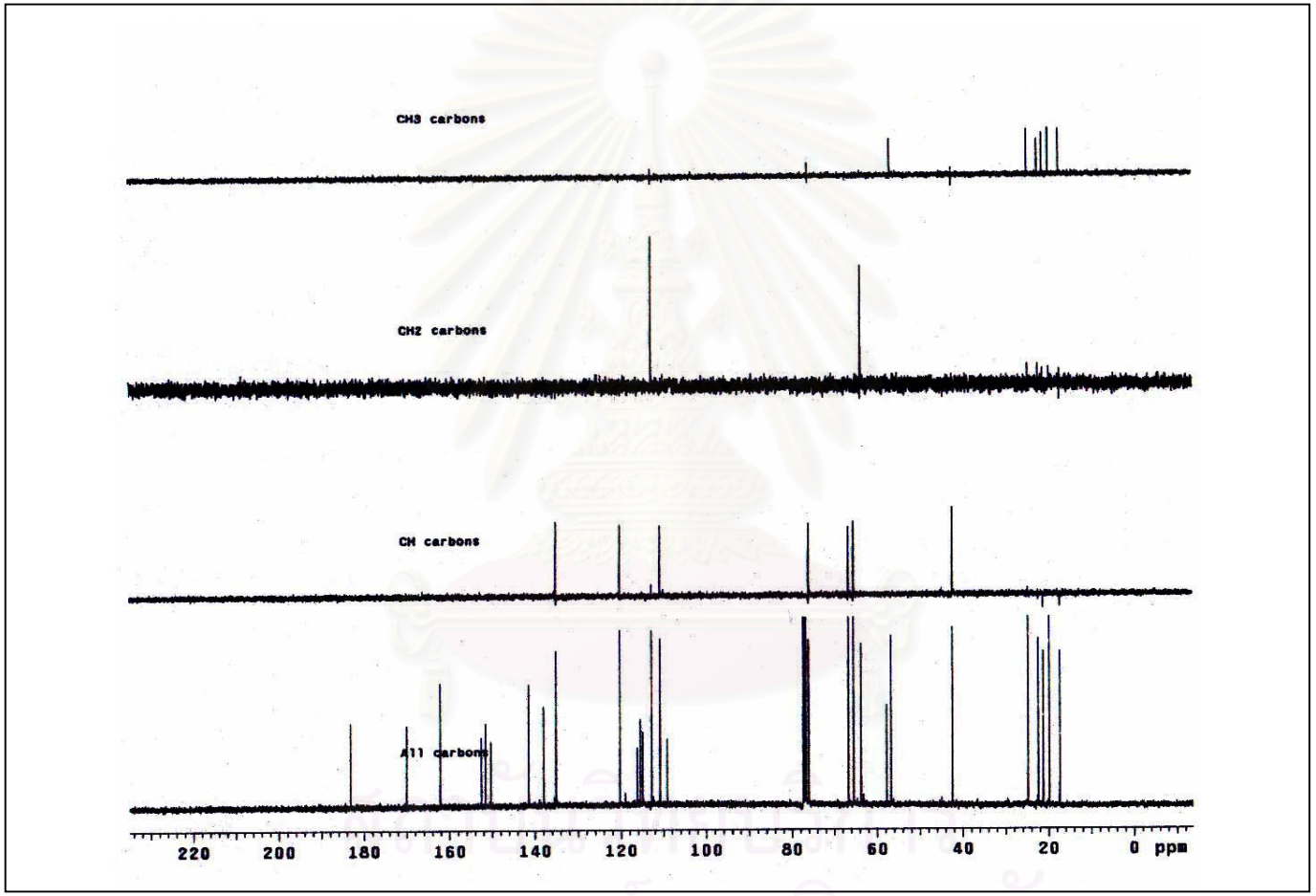

Figure B56 The DEPT, ^{13}C-NMR spectrum of compound 7

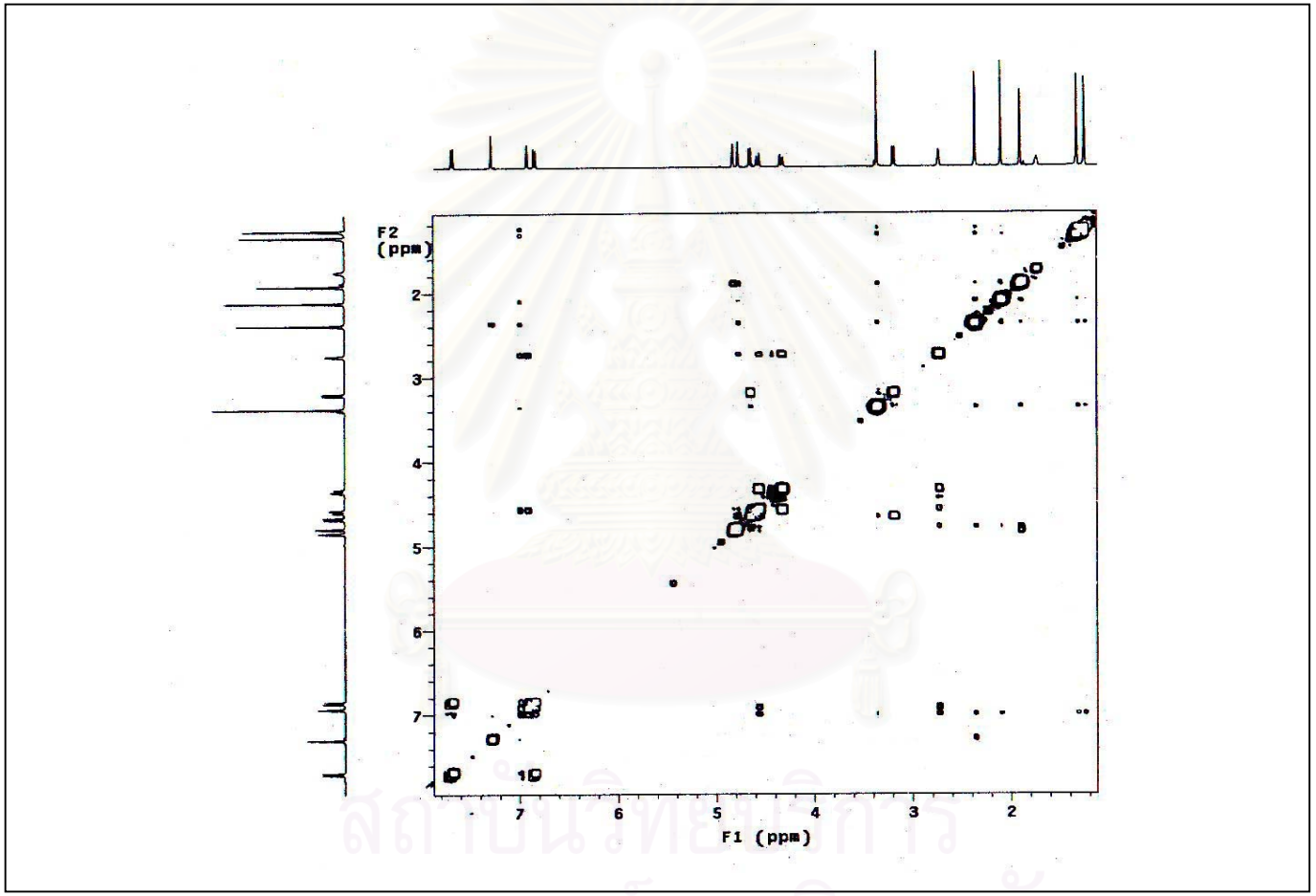

Figure B57 The gCOSY spectrum of compound **7**

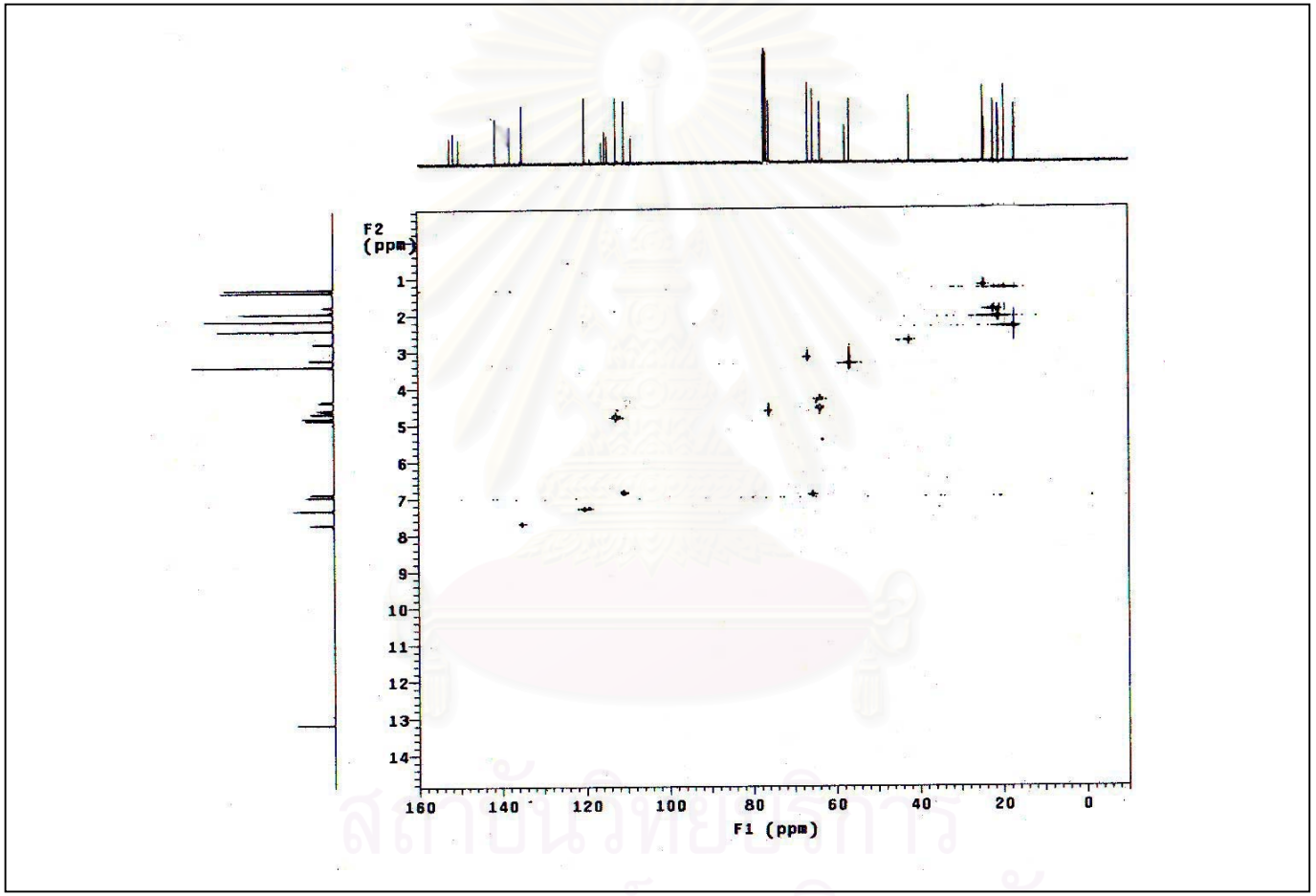

Figure B58 The gHSQC spectrum of compound 7

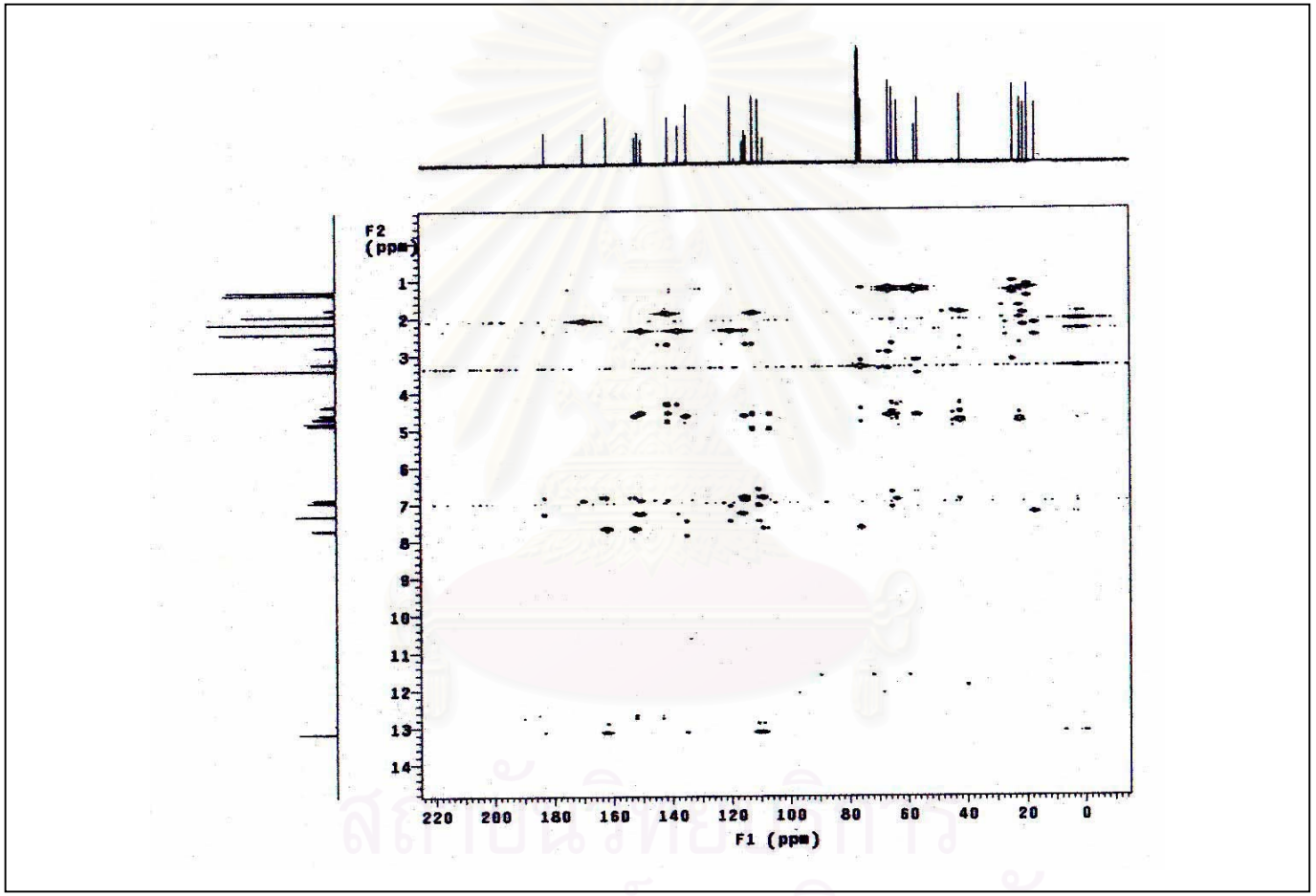

Figure B59 The gHMBC spectrum of compound 7

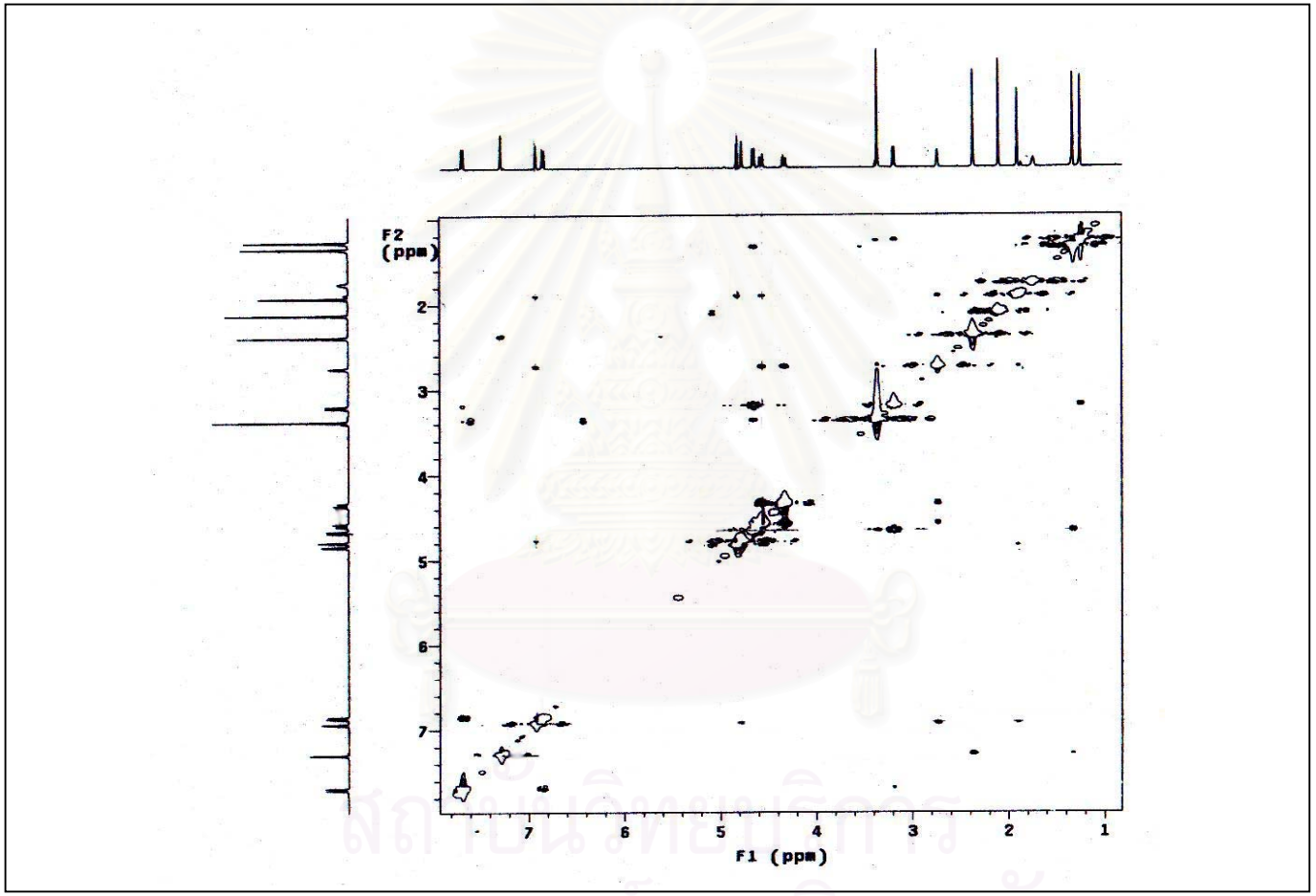

Figure B60 The NOESY spectrum of compound 7

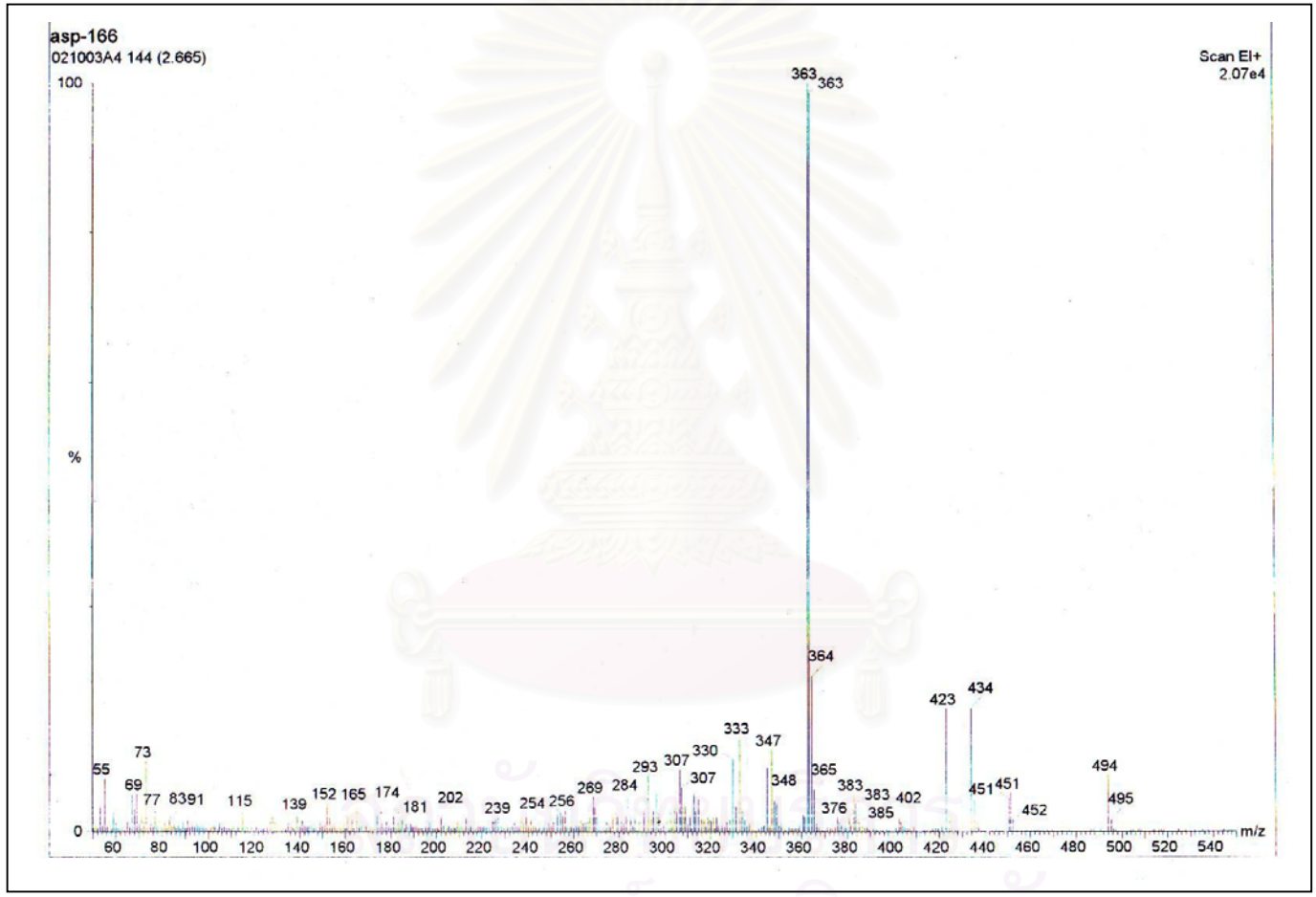

Figure B61 The MS spectrum of compound **7**

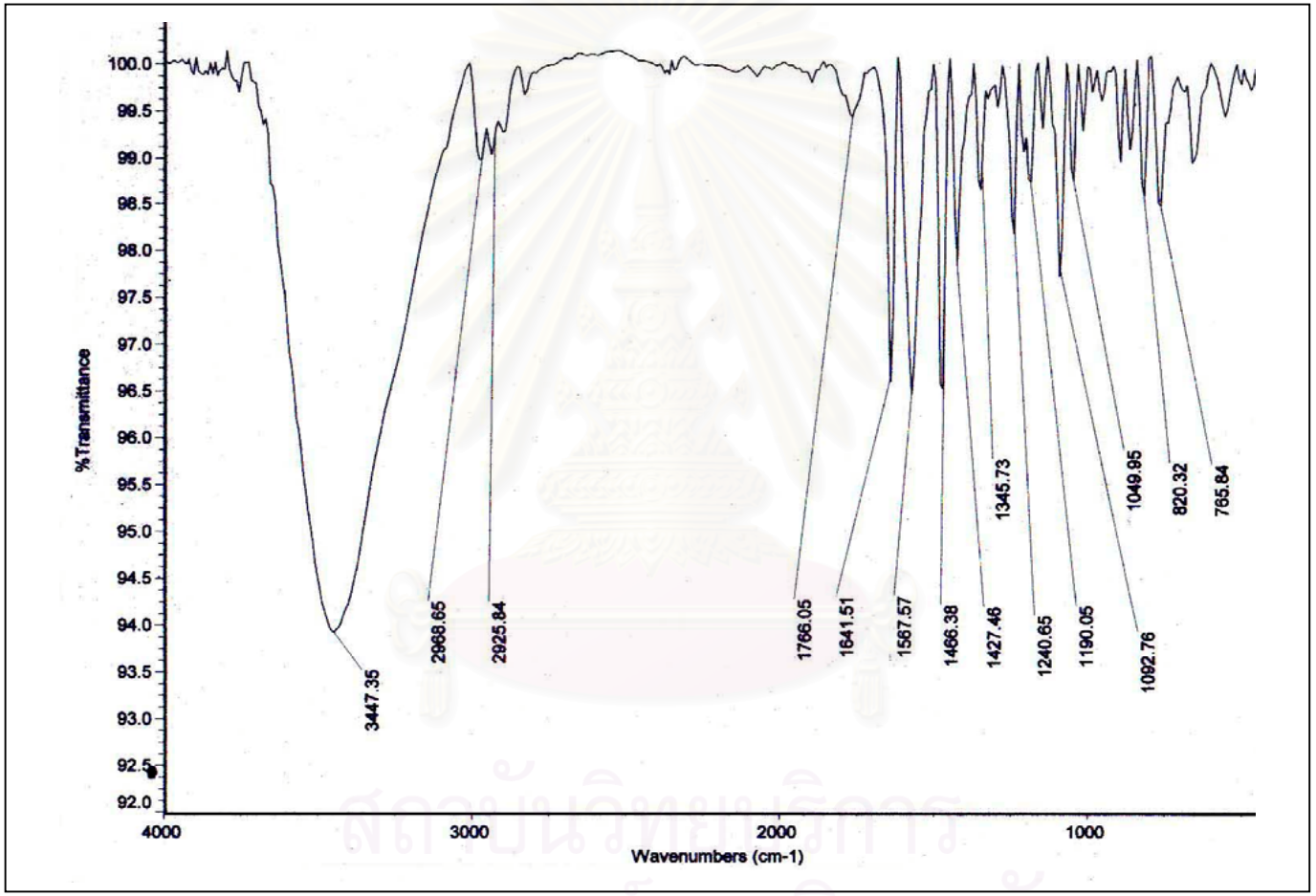

Figure B62 The IR spectrum of compound 8

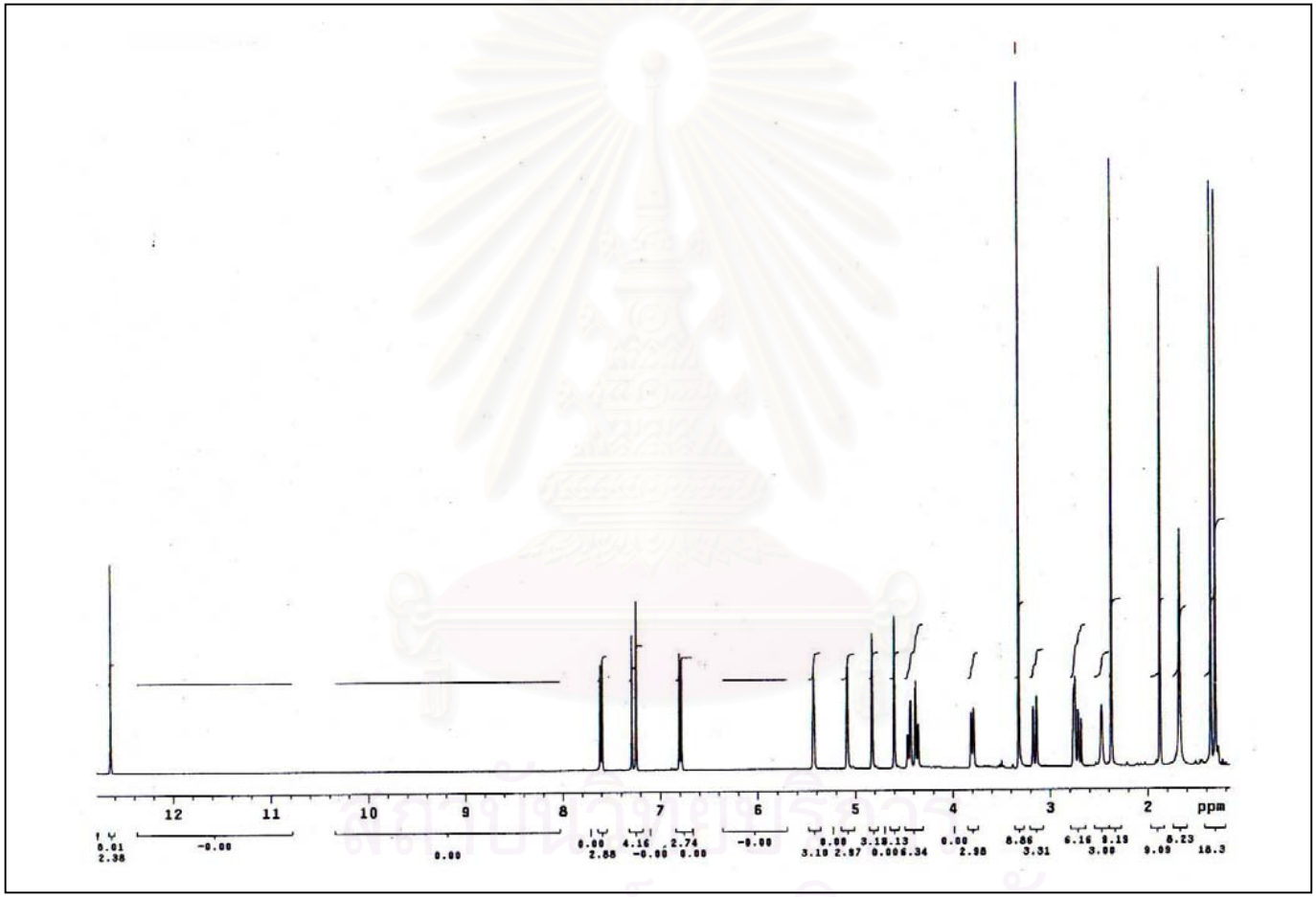

Figure B63 The ^{1}H-NMR spectrum of compound **8**

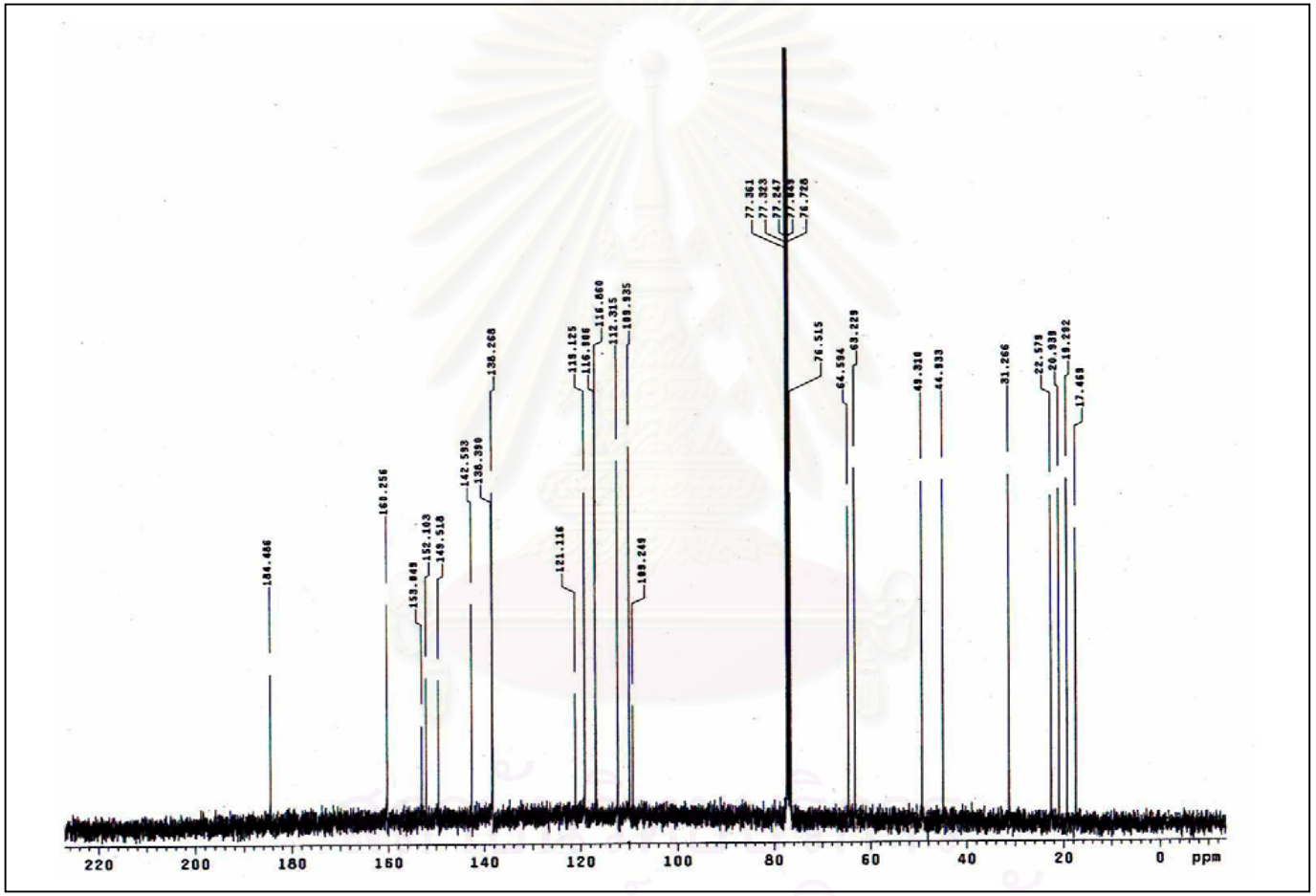

Figure B64 The ^{13}C-NMR spectrum of compound 8

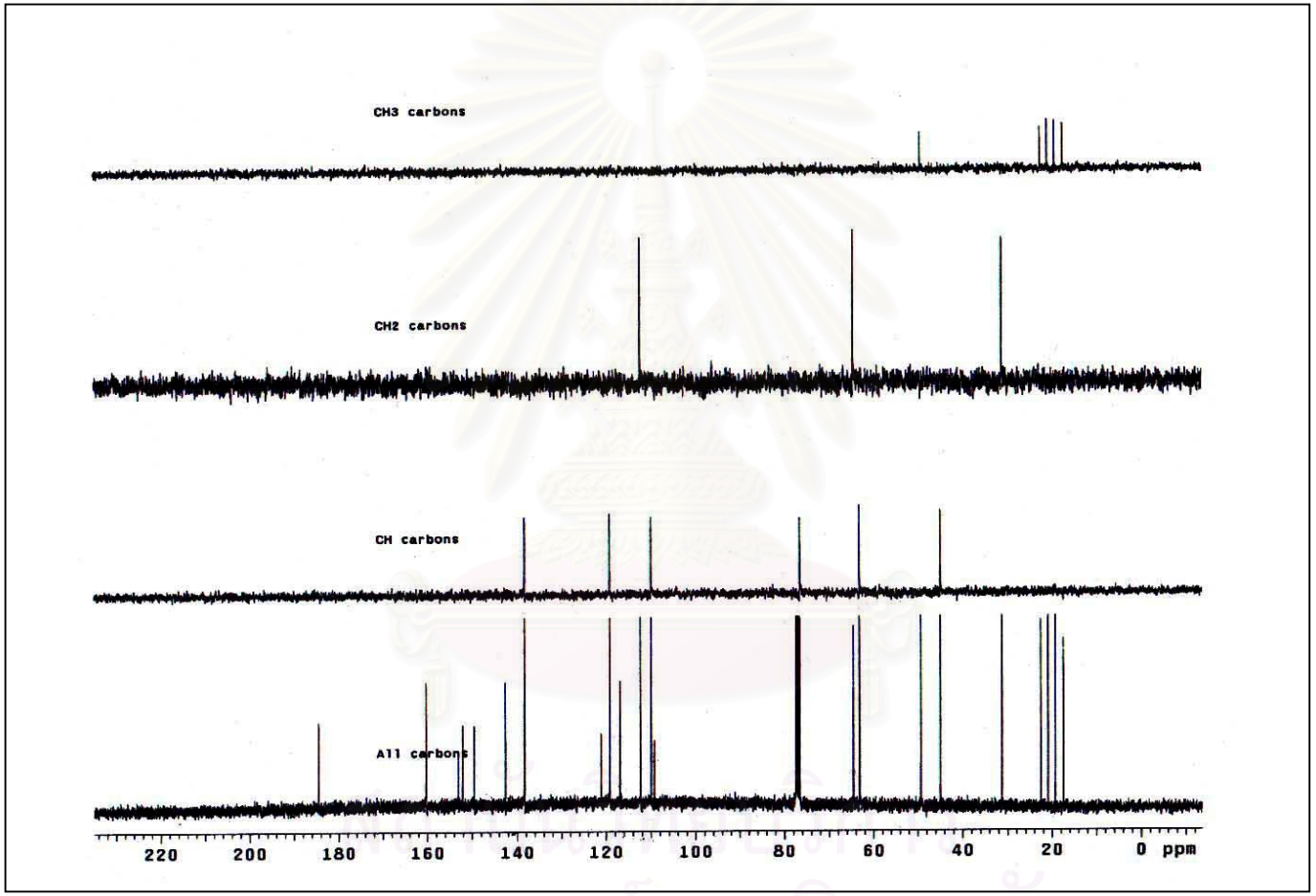

Figure B65 The DEPT, ^{13}C-NMR spectrum of compound 8

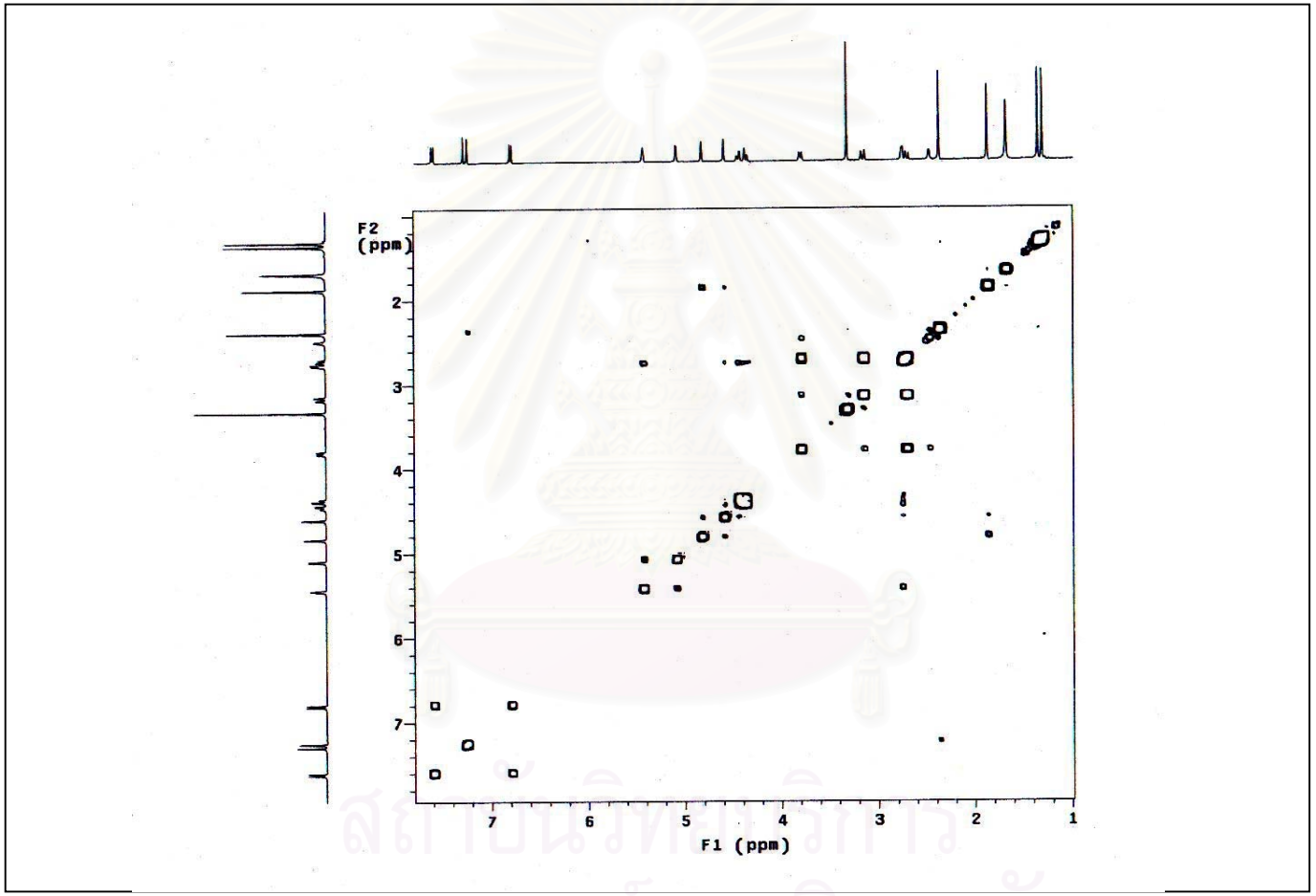

Figure B66 The gCOSY spectrum of compound 8

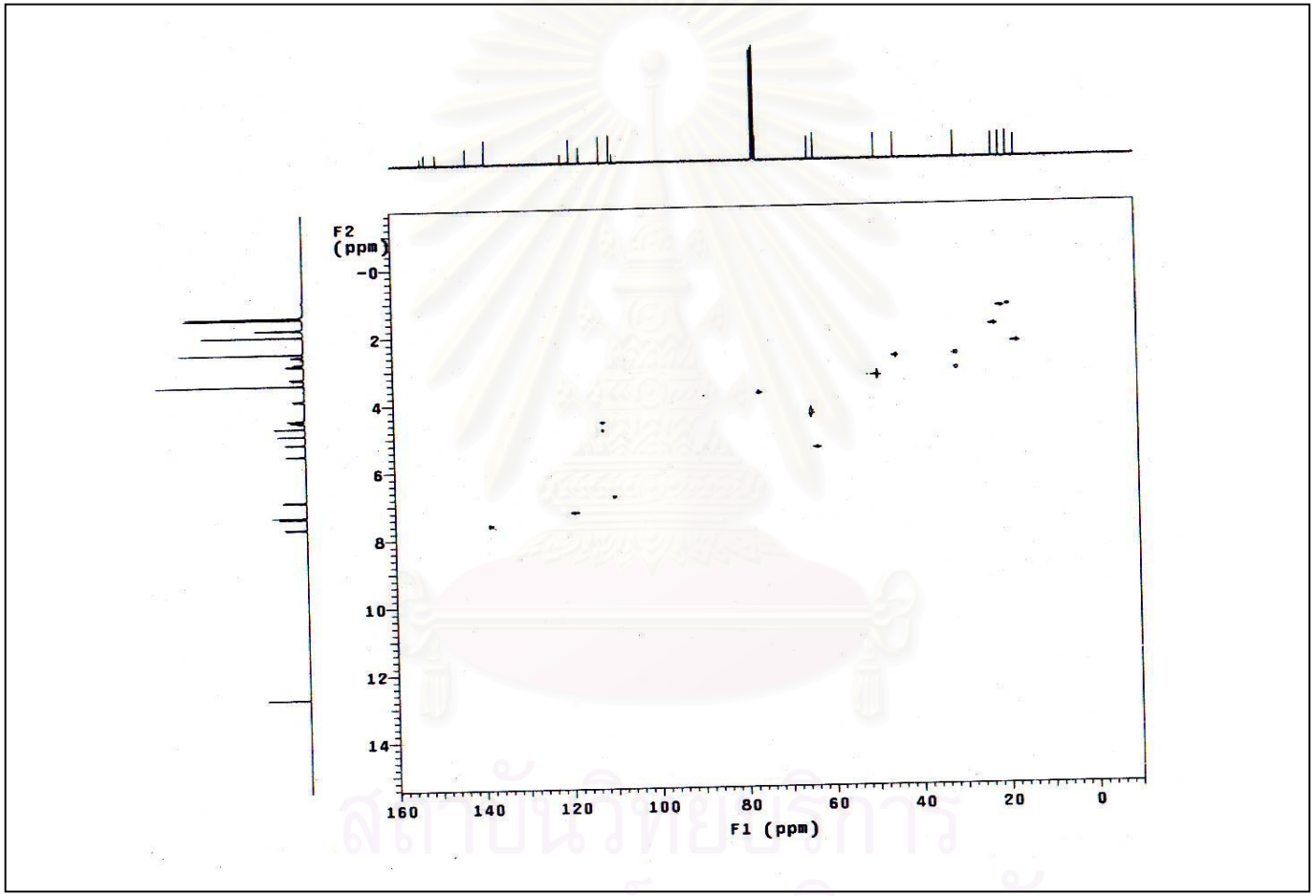

Figure B67 The gHSQC spectrum of compound 8

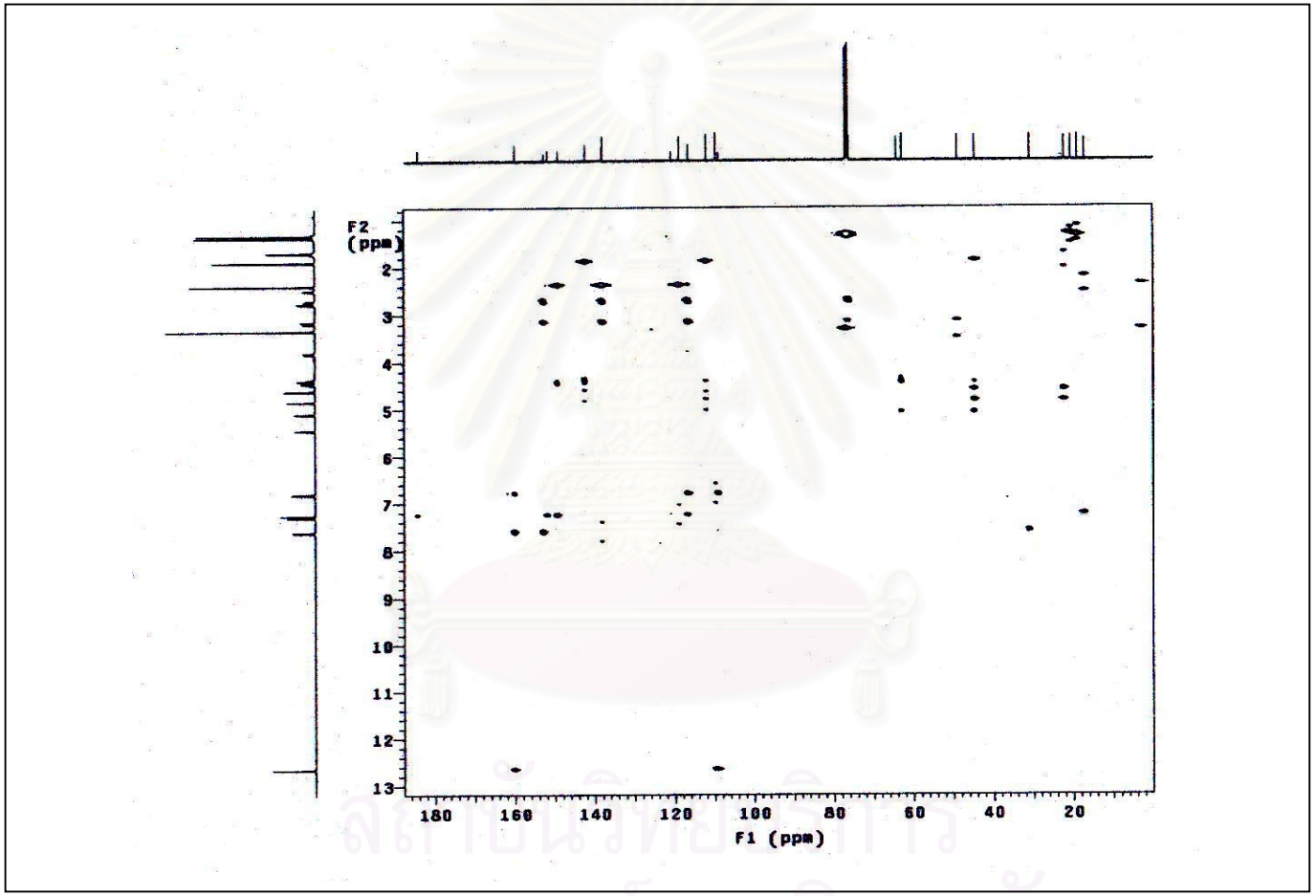

Figure B68 The gHMBC spectrum of compound 8

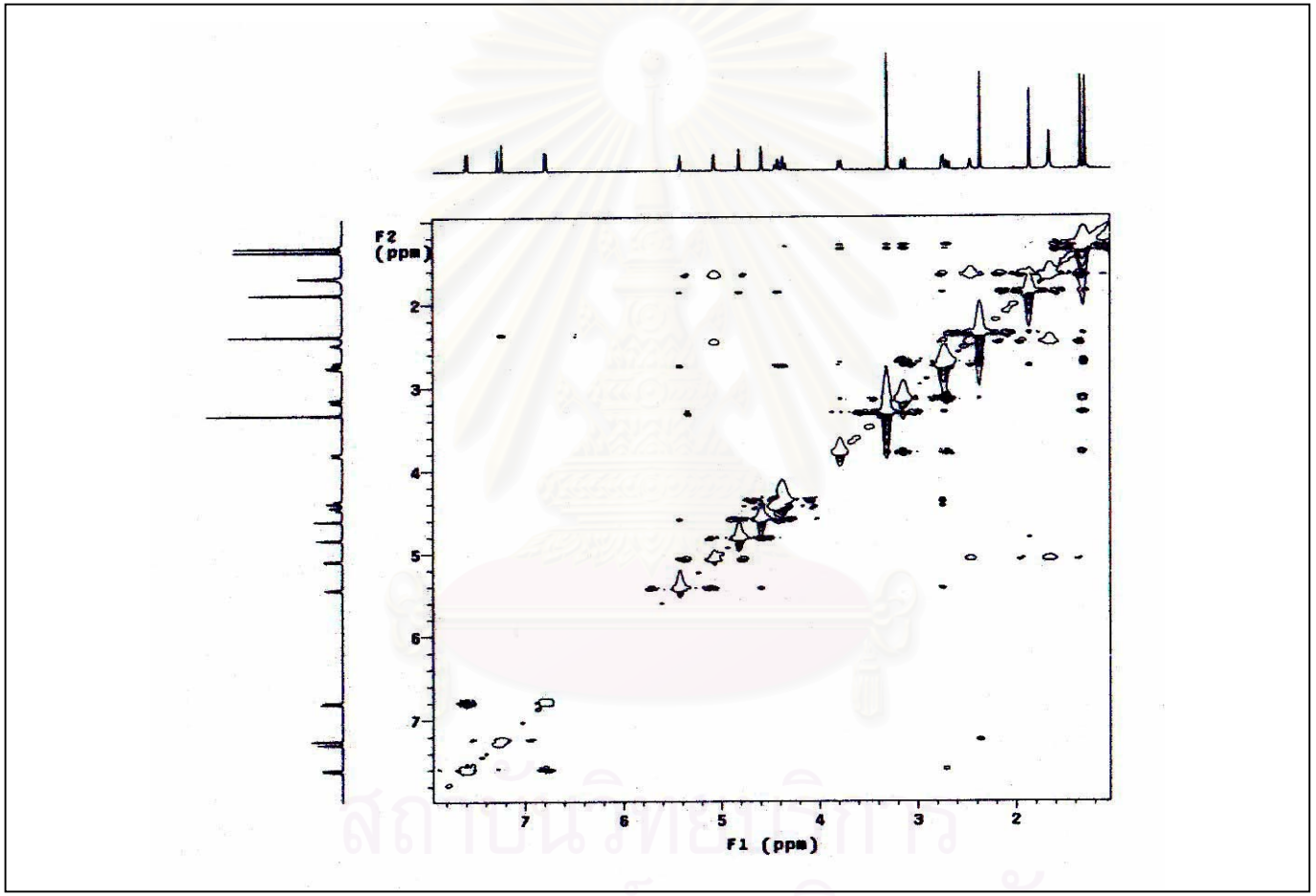

Figure B69 The NOESY spectrum of compound 8

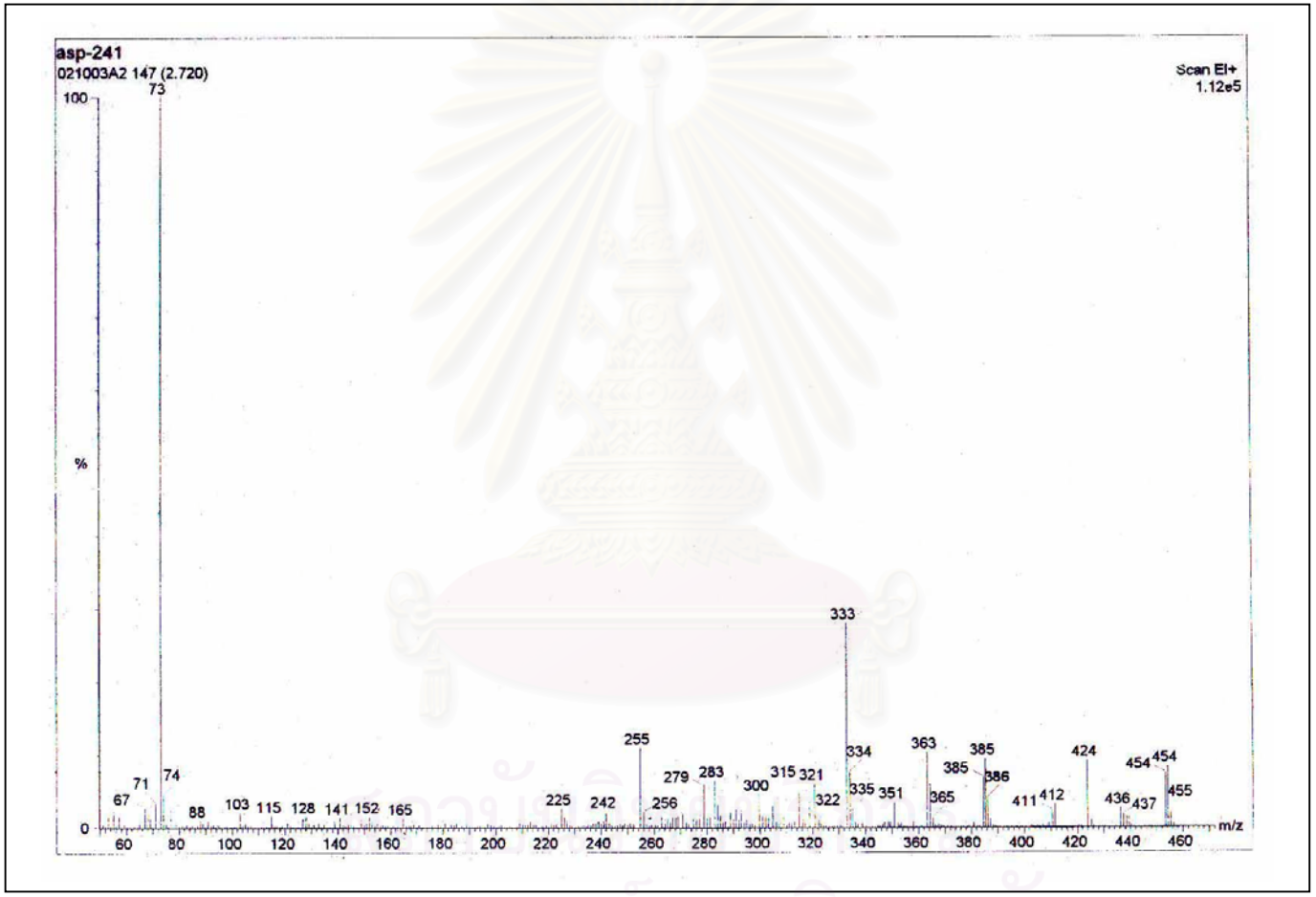

Figure B70 The MS spectrum of compound 8

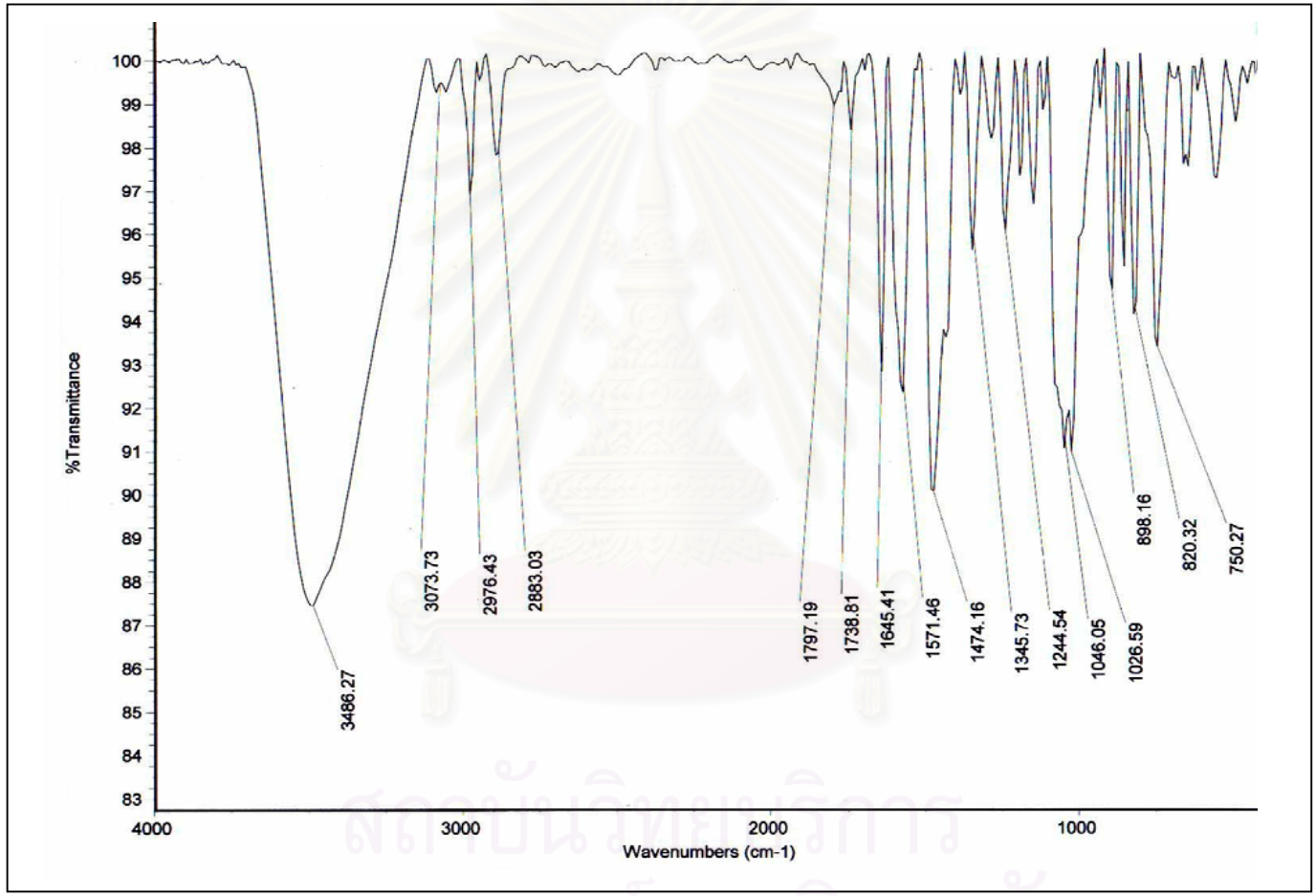

Figure B71 The IR spectrum of compound **9**

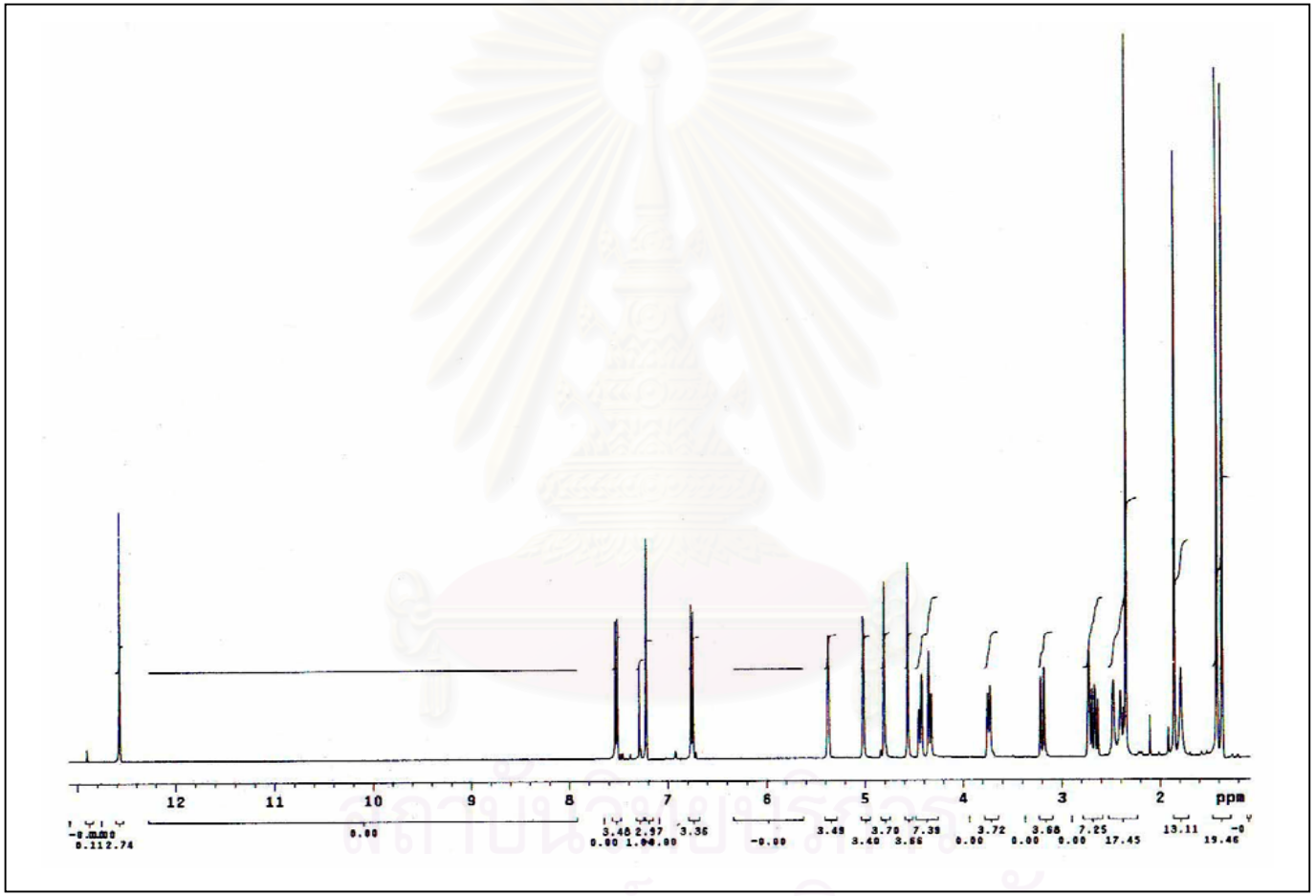

Figure B72 The ^{1}H-NMR spectrum of compound 9

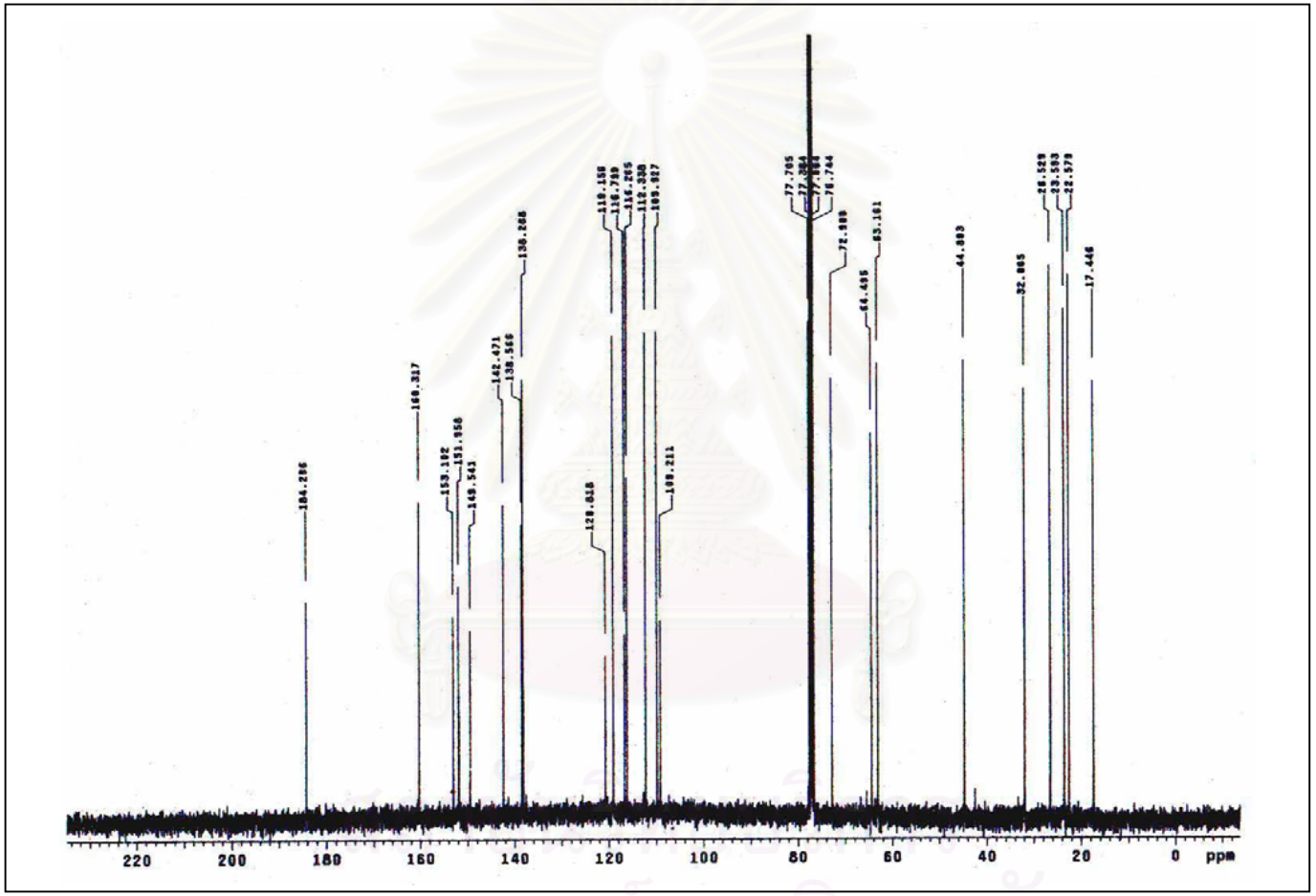

Figure B73 The ^{13}C-NMR spectrum of compound 9

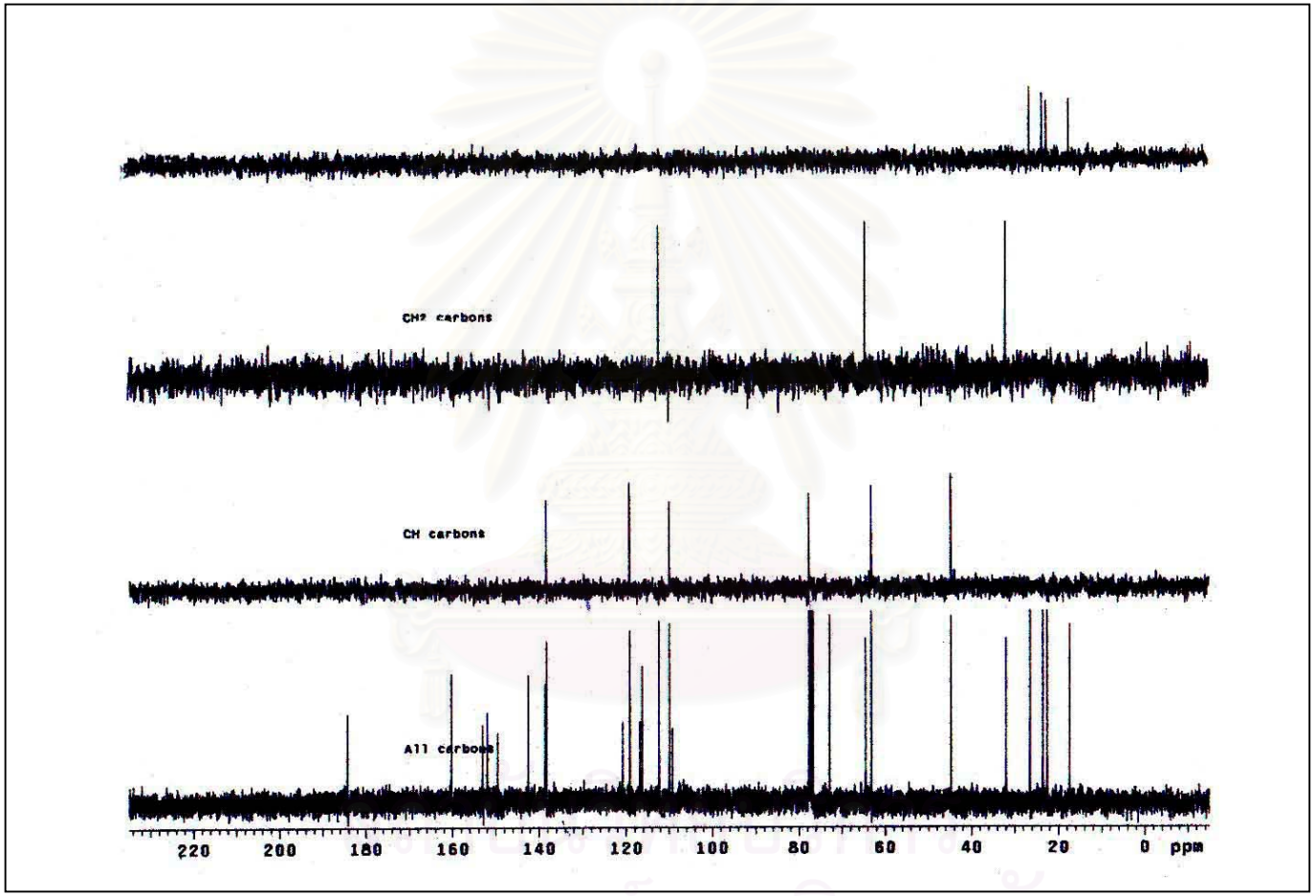

Figure B74 The DEPT, ^{13}C-NMR spectrum of compound 9

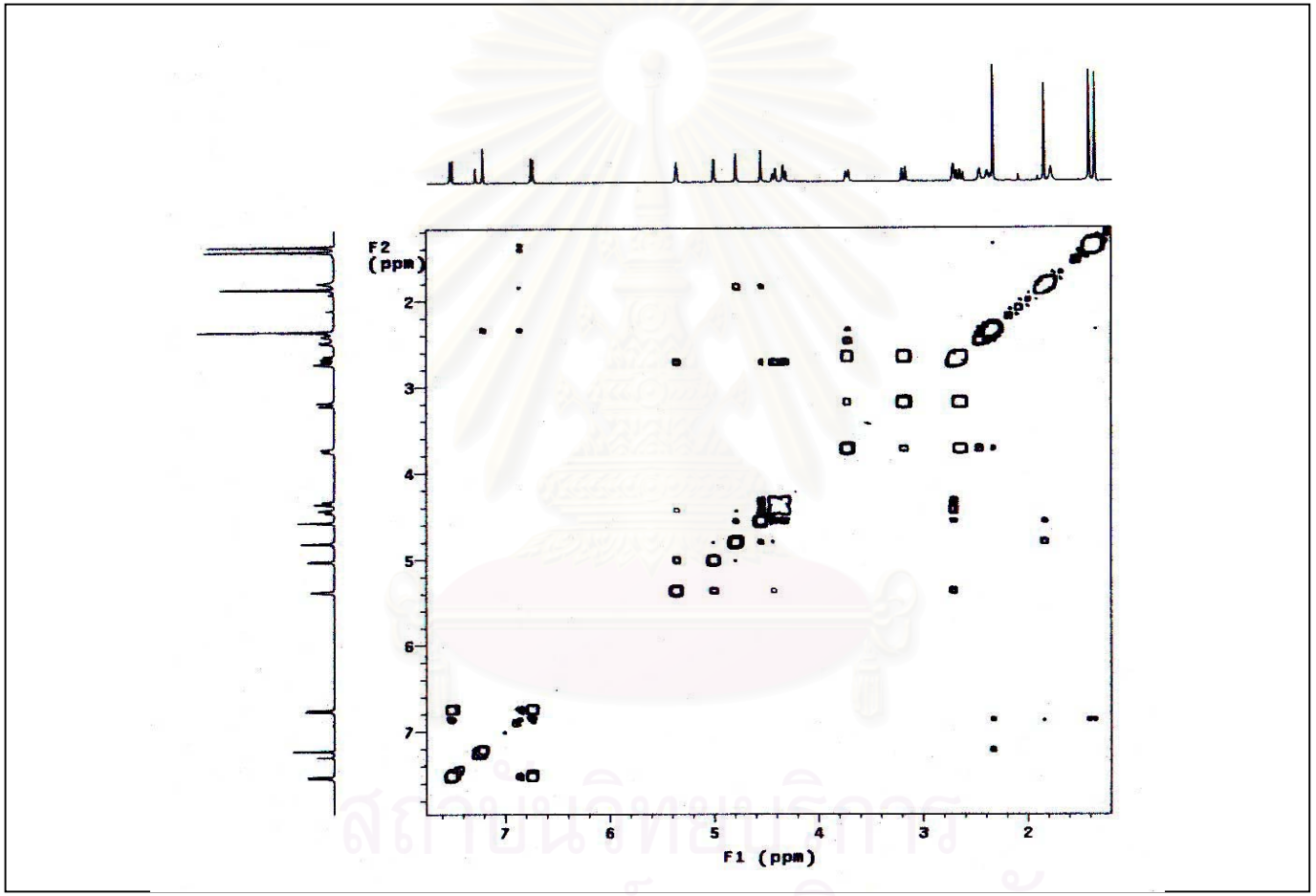

Figure B75 The gCOSY spectrum of compound 9

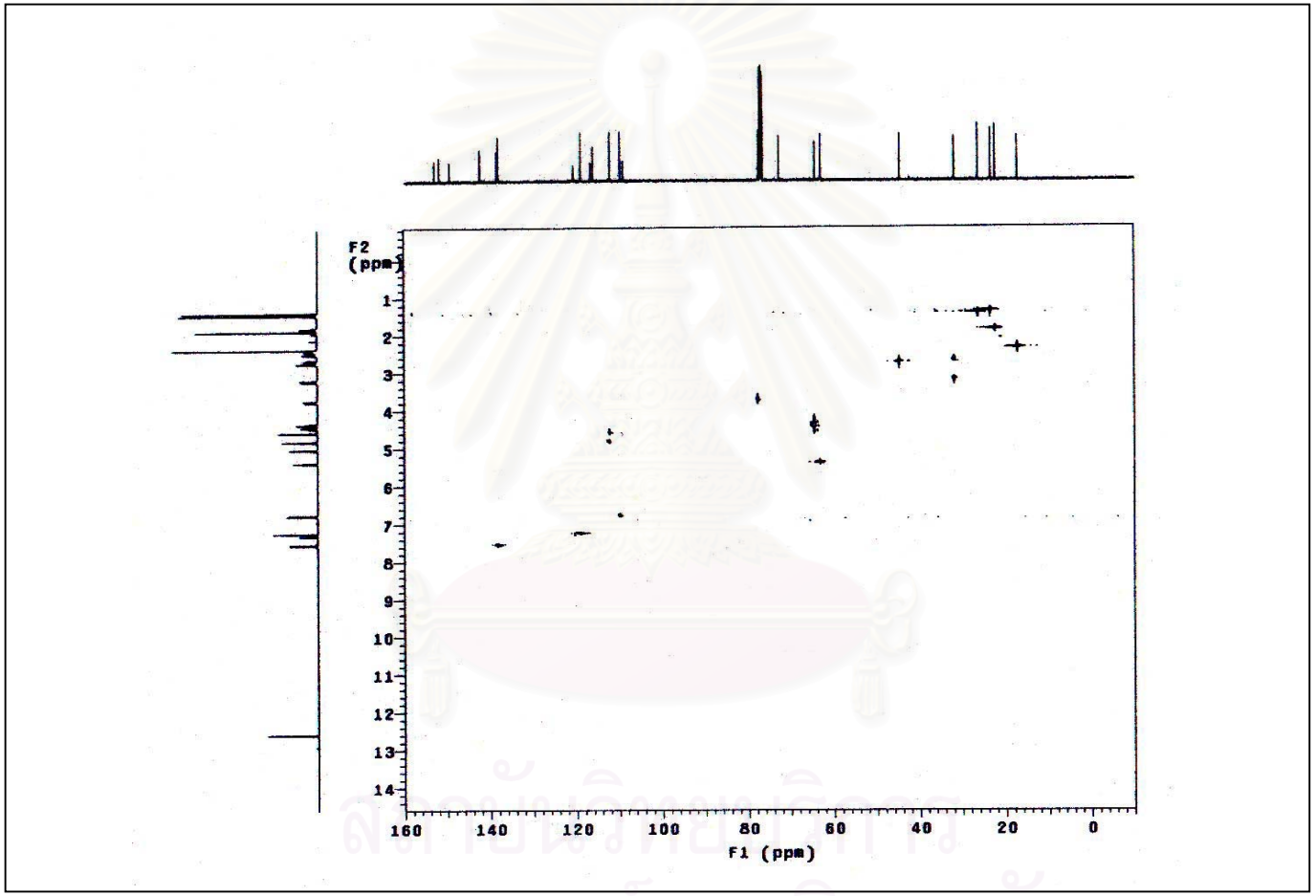

Figure B76 The gHSQC spectrum of compound 9

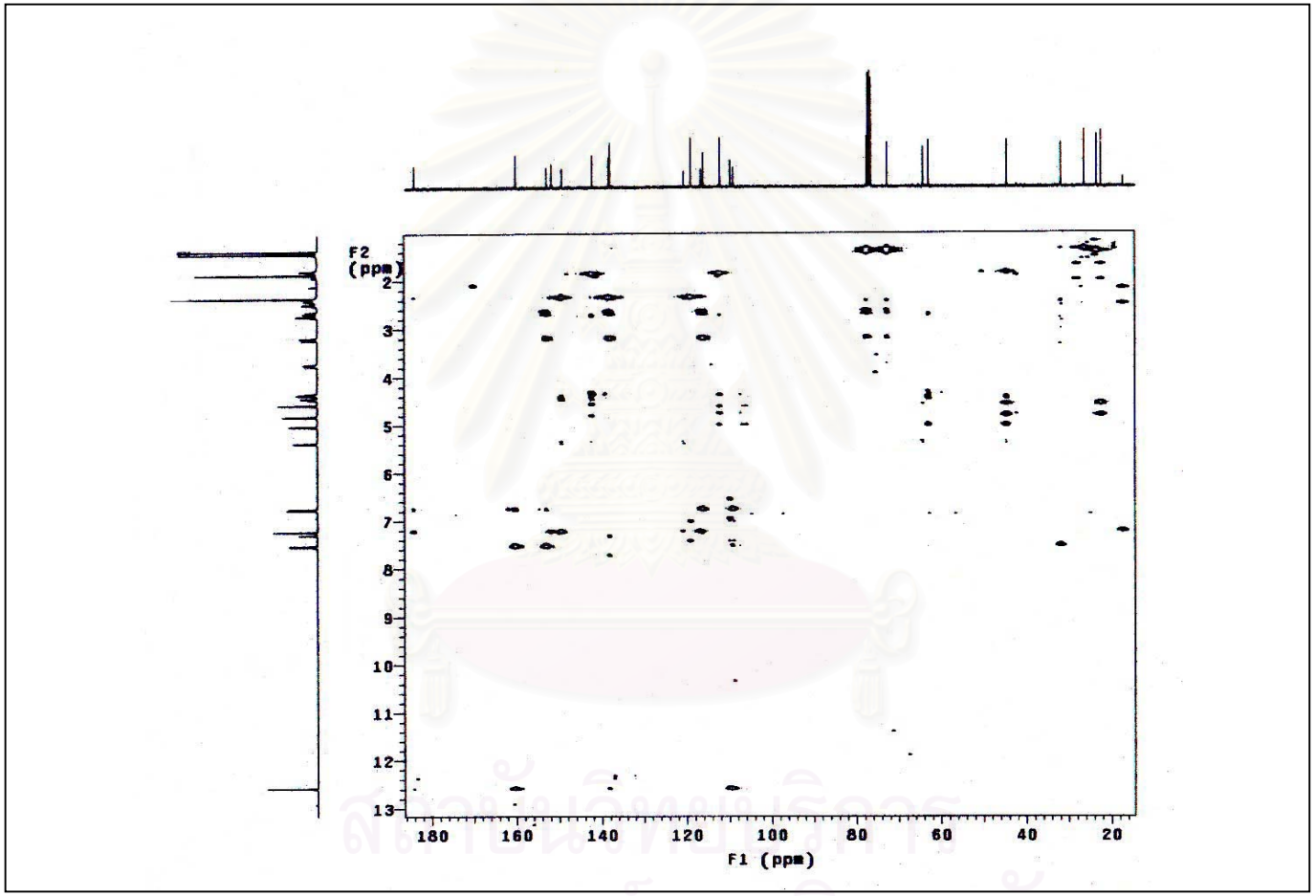

Figure B77 The gHMBC spectrum of compound 9

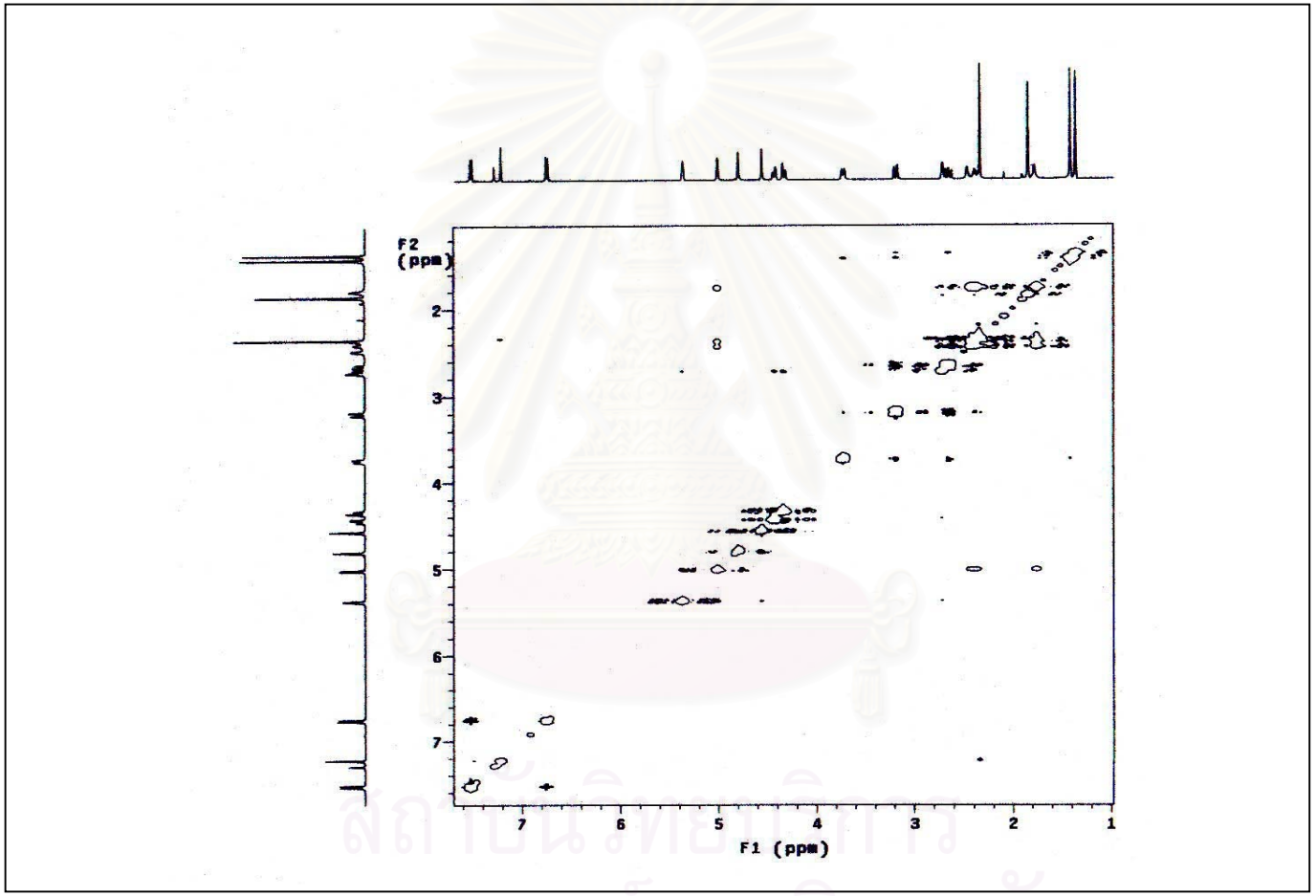

Figure B78 The NOESY spectrum of compound 9

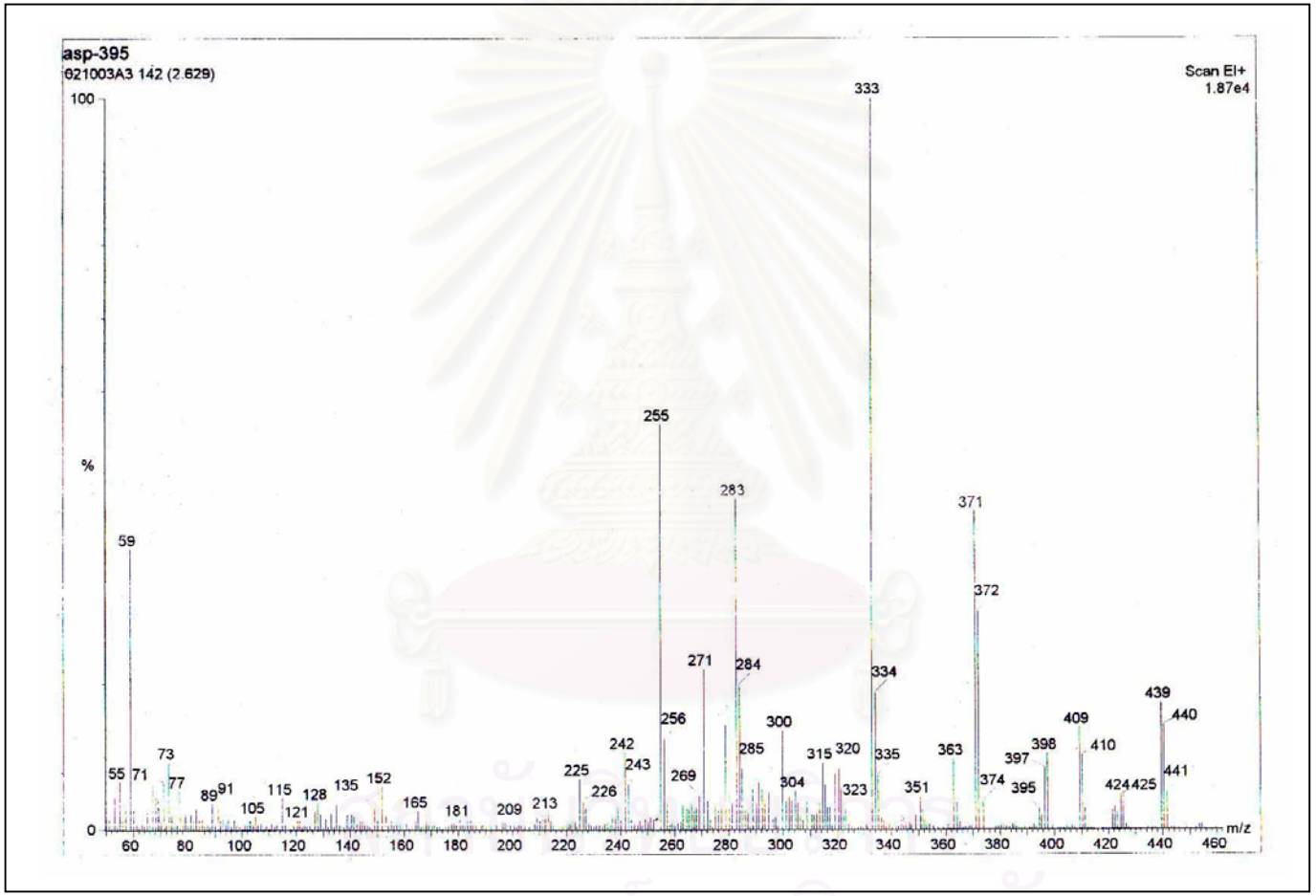

Figure B79 The MS spectrum of compound 9

BIOGRAPHY

Mr.Jatupol Liangsakul was born on October 22, 1978 in Takuapa, Phang-nga Province, Thailand. He graduated with Bachelor Degree of Science in Science and Technology Faculty (Chemistry and Biology) from Prince of Songkhla University in 1999, and he has been studying for a Master Degree of Science in Biotechnology since 2003.