สารทุติยภูมิของสเตรปโตมัยซิส TRA9851-2 จากตะกอนดินบริเวณป่าชายเลน

นางสาว สุชาดา สุนทรชัชเวช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

สาขาวิชาเภสัชเวท ภาควิชาเภสัชเวท

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2543

ISBN 974-346-489-1

SECONDARY METABOLITES OF *STREPTOMYCES* SP. TRA9851-2 FROM MANGROVE SEDIMENT

Miss Suchada Suntornchashwej

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy
Department of Pharmacognosy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2000

ISBN 974-346-489-1

Thesis Title	Secondary metabolites of *Streptomyces* sp. TRA9851-2 from mangrove sediment
By	Miss Suchada Suntornchashwej
Department	Pharmacognosy
Thesis Advisor	Mr. Khanit Suwanborirux, Ph.D.
Thesis Co-advisor	Mr. Prasat Kittakoop, Ph.D.

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

.....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Sunibhond Pummangura, Ph.D.)

Thesis committee

.....Chairman

(Associate Professor Kittisak Likhitwitayawuid, Ph.D.)

.....Thesis Advisor

(Mr. Khanit Suwanborirux, Ph.D.)

.....Thesis Co-advisor

(Mr. Prasat Kittakoop, Ph.D.)

.....Member

(Associate Professor Somboon Tanasupawat, Ph.D.)

.....Member

(Associate Professor Surattana Amnuoypol, M.Sc. in Pharm.)

สุชาดา สุนทรชัชเวช : สารทุติยภูมิของสเตรปโตมัยซิส TRA9851-2 จากตะกอนดินบริเวณป่าชายเลน (SECONDARY METABOLITES OF *STREPTOMYCES* SP. TRA9851-2 FROM MANGROVE SEDIMENT) อ. ที่ปรึกษา : อ. ดร. คณิต สุวรรณบริรักษ์, อ. ที่ปรึกษาร่วม : ดร. ประสาท กิตตะคุปต์; 138 pp. ISBN 974-346-489-1

ในการศึกษาเพื่อหาสารออกฤทธิ์ทางชีวภาพจากแบคทีเรียกลุ่มแอคติโนมัยซีท สามารถคัดเลือกเชื้อแอคติโนมัยซีทสายพันธุ์ TRA9851-2 จากตะกอนดิน ในป่าชายเลน จังหวัดตรัง และจากการศึกษาลักษณะทางสัณฐานวิทยา การเจริญ สรีรวิทยา ชีวเคมี และ องค์ประกอบของผนังเซลล์ สามารถพิสูจน์เอกลักษณ์ของสายพันธุ์ TRA9851-2 ได้เป็นแบคทีเรียในสกุล สเตรปโตมัยซิส สิ่งสกัดด้วยเอธิลอะซิเตทจากน้ำหมักของเชื้อสายพันธุ์นี้แสดงฤทธิ์ต้านเชื้อจุลชีพ *Bacillus subtilis* ATCC 6633 *Staphylococcus aureus* ATCC 25923 และ *Candida albicans* ATCC 10231 ได้อย่างมีนัยสำคัญ เมื่อทำการสกัดแยกสารให้บริสุทธิ์ด้วยวิธีทางโครมาโตกราฟี โดยทำการทดสอบฤทธิ์ต้านจุลชีพควบคู่ไปด้วย สามารถแยกสารได้ 5 ชนิด ได้แก่ สารกลุ่ม benzo[*c,d*]pyrene คือ resistomycin และอนุพันธ์อีกหนึ่งชนิด ซึ่งมีโครงสร้างที่เป็นไปได้ 2 ชนิด คือ 9-methyl-4,6,8,10,11,11-hexahydroxy-2H-benzo[*c,d*]pyrene-2,7(1H)-dione หรือ 9-methyl-4,6,8,10-tetrahydroxy-11-nor-2H-benzo[*c,d*]pyrene-2,7(1H)-dione สารในกลุ่ม diketopiperazine 1 ชนิด คือ *cyclo*-(propyl-leucyl) และ สารในกลุ่ม phenolic acid 2 ชนิด คือ syringic acid และ *p*-coumaric acid การพิสูจน์โครงสร้างทางเคมีของสารเหล่านี้ ใช้วิธีทางสเปคโตรสโคปี โดยเฉพาะ MS, UV, IR, และ NMR spectroscopy สาร resistomycin แสดงฤทธิ์ต้านเชื้อแบคทีเรีย *B. subtilis* ATCC 6633 และแสดงความเป็นพิษต่อ herpes-simplex viruses type I และ type II นอกจากนี้ resistomycin ยังแสดงฤทธิ์ทาง ชีวภาพที่น่าสนใจ คือ ฤทธิ์ต้านเชื้อมาลาเรีย *Plasmodium falciparum* (K_1, multidrug resistant strain) ซึ่งแสดงฤทธิ์แรงกว่า chloroquine และฤทธิ์ความเป็นพิษต่อ oral human epidermoid และ breast cancer cell lines

ภาควิชา เภสัชเวท ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชเวท ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4176586233 : MAJOR PHARMACOGNOSY
KEY WORD : MANGROVE/ *STREPTOMYCES* SP./ BENZO[*C,D*]PYRENE/ DIKETOPIPERAZINE/ PHENOLIC ACID/ ANTIBACTERIAL ACTIVITY/ CYTOTOXIC ACTIVITY/ ANTIMALARIAL ACTIVITY/ ANTI-HERPES SIMPLEX VIRUS ACTIVITY
SUCHADA SUNTORNCHASHWEJ: SECONDARY METABOLITES OF *STREPTOMYCES* SP. TRA9851-2 FROM MANGROVE SEDIMENT. THESIS ADVISOR: MR. KHANIT SUWANBORIRUX, PH.D., THESIS CO-ADVISOR: MR. PRASAT KITTAKOOP, PH.D. 138 pp. ISBN 974-346-489-1

In the course of our investigation for bioactive metabolites of the actinomycetes, we isolated an actinomycete strain TRA9851-2 from a sediment sample collected in a mangrove area of Trang Province. Based on morphological, cultural, physiological, biochemical, and cell wall component studies, the strain TRA9851-2 was identified as the genus *Streptomyces*. The ethyl acetate extract of fermentation broth of the strain significantly inhibited the growth of *Bacillus subtilis* ATCC 6633, *Staphyloccus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. Upon chromatographic separation guided by antimicrobial activity of the ethyl acetate extract yielded two benzo[*c,d*]pyrenes, resistomycin and a derivative with two possible structures, 9-methyl-4,6,8,10,11,11-hexahydroxy-2H-benzo[*c,d*]pyrene-2,7(1H)-dione or 9-methyl-4,6,8,10-tetrahydroxy-11-nor-2H-benzo[*c,d*]pyrene-2,7(1H)-dione; a diketopiperazine, *cyclo*-(propyl-leucyl); and two phenolic acids, syringic acid and *p*-coumaric acid. The structure elucidation of the isolated substances was achieved by analyses of spectroscopic data including MS, UV, IR, and NMR spectroscopy. Resistomycin exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633 and toxicity against herpes-simplex viruses type I and type II. Resistomycin possessed potent antimalarial activity against *Plasmodium falciparum* (K_1, multidrug resiststant strain), and also showed cytotoxicity against oral human epidermoid and breast cancer cell lines.

Department	Pharmacognosy	Student's signature.....
Field of study	Pharmacognosy	Advisor's signature.....
Academic year	2000	Co-advisor's signature.....

ACKNOWLEDGEMENTS

The author wishes to express her grateful appreciation to those who assisted her in the research as the followings.

To Dr. Khanit Suwanborirux, her major advisor, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for his guidance, suggestion, encouragement, and kindness throughout the research studies.

To Dr. Prasat Kittakoop, her co-advisor, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) for his helpful suggestion, and kindness throughout my research studies.

To Associate Professor Dr. Somboon Tanasupawat and Associate Professor Dr. Vimolmas Lipipun of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for microbiological assay and innumerable assistance, and for anti-herpes simplex virus assay, respectively.

To Dr. Hisayoshi Kobayashi of the University of Tokyo for his assistance in the FAB MS experiment.

To Bioassay Research Facility of the National Center for Genetic Engineering and Biotechnology (BIOTEC) for biological activities tests of antimalarial and cytotoxic assay.

To the thesis committee for their constructive suggestions and critical review of this thesis.

To Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA) for granting partial financial support to conduct this investigation.

Finally, the author wishes to express her infinite gratitude to her family, teacher and friends for their love, understand and encouragement.

CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF SCHEMES

Scheme **Page**

LIST OF ABBREVIATIONS

$[\alpha]^{20}_{D}$	=	specific rotation at 20° and sodium D line (589 nm)
acetone-d_6	=	deuterated acetone
ATCC	=	American Type Culture Collection, Maryland, USA
BC	=	Breast cancer cell line
br s	=	broad singlet
°C	=	degree Celsius
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
$CDCl_3$	=	deuterated chloroform
CD_3OD	=	deuterated methanol
$CHCl_3$	=	chloroform
cm	=	centimeter
conc.	=	concentrated
δ	=	chemical shift
DEPT	=	distortionless enhancement by polarization transfer
d	=	doublet
dd	=	doublet of doublets
ddd	=	doublet of doublets of doublets
DMSO-d_6	=	deuterated dimethylsulphoxide
EC_{50}	=	the concentration required for 50% of the activity
ED_{50}	=	50% effective dose
EtOAc	=	ethyl acetate
ϵ	=	molar absorptivity
ESI-TOF MS	=	Electronspray Ionisation Time of Flight Mass

μg	=	microgram
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence
^{1}H NMR	=	proton nuclear magnetic resonance
Hz	=	hertz
IR	=	infrared
J	=	coupling constant
KB	=	human epidermoid carcinoma cells of the nasopharynx
μl	=	microliter
L	=	liter
λ_{max}	=	wavelength at maximum absorption
$M^{+\bullet}$	=	molecular ion
$[M+1]^{+}$	=	protonated molecular ion
m	=	multiplet
μm	=	micrometer
MeOH	=	methanol
μg	=	microgram
MHz	=	mega Hertz
ml	=	milliliter
mm	=	millimeter
ν_{max}	=	wave number at maximum absorption
nm	=	nanometer
NMR	=	nuclear magnetic resonance
NOE	=	nuclear Overhauser effect

ppt	=	part per thousand
s	=	singlet
SDA	=	Sabouraud dextrose agar
sp.	=	species
t	=	triplet
TLC	=	thin layer chromatography
TSA	=	Trypticase soy agar
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

Actinomycetes are a proven source of structurally diverse secondary metabolites possessing broad ranges of biological activities such as antibiotics, erythromycin and tetracycline; anticancers, mitomycin C and daunomycin; immunomodulators, bestatin and erbstatin (Goodfellow, Williams, and Mordarski, 1988). The most useful in the chemical discoveries and biological activities reported from the actinomycetes are terrestrial actinomycetes. Although it is tempting to draw an analogy between actinomycetes in various terrestrial soils, they are generally not considered to be closely related microenvironments. Secondary metabolites of the terrestrial actinomycetes show wide varieties of chemical structures such as polycyclic quinones, quanolirones I and II (Qian-Cutrone *et al*., 1998); polyhydroxylated compounds, aflastatin A (Sakuda *et al*., 1996); peptides, polyoxypeptins A and B (Umezawa, 1999); macrolides, tetrin C (Ryu *et al*., 1999); terpenes, compound 11904w (Gansser, Pollak, and Berger, 1995). Since the frequency of novel bioactive compounds discovered from terrestrial actinomycete decreases with time, academic and industrial programs are increasingly screening actinomycetes from diverse environments for their ability to generate new metabolites such as estuarine environment.

Like the soil, in terms of sedimentation, estuaries are many complex environments (Burton, and Liss, 1976; Dawes, 1998). One principle reason for this is that the sediments themselves can originate from a number of areas. These include both upstream and marine sources. This characteristic makes estuarine sediments more nutrient-rich than terrestrial or marine sediments. Recent investigations have demonstrated that estuarine actinomycetes provide a diversity of biologically active metabolites. Although known compounds are frequently encountered, novel compounds are being reported at a high frequency. Examples include lagunapyrones A-C, the cytotoxic acetogenins of the new skeletal class from an unidentified actinomycete culture CNB-984 isolated from estuarine sediment collected in the Agua Hedionda Lagoon in Carlsbad, California (Lindel, Jensen, and Fenical, 1996);

wailupemycins A-C and 3-epi-5-deoxyenterocin, the α-pyrone containing metabolites from *Streptomyces* sp. BD-26T isolated from shallow water estuarine sediment in Wailupe beach park, Hawii (Sitachitta, Gadepalli, and Davidson, 1996); Halawanones A-D, the quinone-containing metabolites from *Streptomyces* sp. BD-18T isolated from a sediment sample collected in the estuarine environment at the mouth of Halawa stream, Oahu, Hawaii (Ford, Gadepalli and Davidson, 1998); the pentacyclic polyether arenaric acid from estuarine actinomycete culture CNH-248 isolated from sediment sample collected in an estuary near Doheny Beach, San Diego (Cheng, Jensen, and Fenical, 1999a); luisols A and B, the aromatic tetraols metabolites from *Streptomyces* sp. CNH-370 isolated from sandy sediment collected near San Diago in the San Luis Estuary (Cheng, Jensen, and Fenical, 1999b).

In the course of our screening for bioactive metabolites from actinomycetes, we planned to study the secondary metabolites of actinomycete strains isolated from mangrove forests in the estuarine environments because the sediments contain high organic matters. We found an interesting strain TRA9851-2 which had been isolated from a soil sample collected at the bottom of a canal in a mangrove forest (dominate in coastal zone), Trang Province, Thailand. It was determined to belong to the genus *Streptomyces* based on morphological and chemotaxonomic characteristics. Preliminary bioactivity screening on the ethyl acetate extract of the fermentation broth from this actinomycete strain showed antimicrobial activity in a disc diffusion assay against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 with 20, 10 and 23 mm zone of inhibition at 1 mg/6 mm disc, respectively.

The main objectives of this investigation are as follows:

1. Identification and characterization of the strain TRA 9851-2 isolated from mangrove sediment.
2. Purification and bioactivity evaluation of secondary metabolites from the strain TRA9851-2.
3. Structure elucidation of isolated secondary metabolites.

CHAPTER II

REVIEW OF LITERATURE

Microorganisms have had a profound effect on medicinal science since the discovery of not only the cause of infection but also the production of secondary metabolites, which can cure the infection diseases. Since the discovery of penicillin in 1929, between 30,000 and 50,000 natural products have been discovered from microorganisms (Fenical, and Jensen, 1993). Of these substances, more than 10,000 are biologically active and more than 8,000 are antibiotics (Betina, 1983). Despite the many discoveries, most antibiotics of microbial origin come from terrestrial bacteria belonging to one taxonomic group, the order Actinomycetales.

Organisms belonging to the group of actinomycetes are Actinobacteria, Actinoplanetes, Maduromycetes, Micropolysporas, Multilocular, Nocardioform, Streptomycetes, and Thermomonosporas. In many members of actinomycetes, the species in the genus *Streptomyces*, family Streptomycetaceae, have proven to be the major source for antibiotics and related bioactive metabolites (Nolan, and Cross, 1988; Goodfellow, and William, 1983). Its secondary metabolites show wide varieties in the chemical structures and are still regarded as a major potential source of new bioactive substance such as new macrodiolide antibiotics, 11-O-monomethylelaiophylin and 11-11′-O-dimethylelaiophylin (Ritzau *et al*., 1998); a novel antioxidant, diphenazithionin (Yoshiko *et al*, 1996); a new inhibitor of human cytomegalovirus protease, bripiodionen (Shu *et al*., 1996); a new antitumor antibiotic, UCE6 (Fujii *et al*., 1997); potent apoptosis-inducing peptides, polyoxypeptins A and B (Umezawa *et al*., 1999).

Although these bacteria continue to be studied extensively, it is clear that the rate of discovering novel metabolites from terrestrial actinomycetes is decreasing and that new sources of bioactive natural products must be explored including the actinomycetes from saline environments, including marine and estuary.

1. Characteristic of mangrove forests

Mangrove forest formations usually exist and thrive in estuarine environments with a range of salinity between 10-30 ppt (Goodfellow, and William, 1983), along the sea coast and estuary mouth between land and sea. Mangroves are typically highly productive regions because nutrients carried by rivers are deposited at the river mouths or deltas. Mangroves represent a rich and diverse living resource and are valuable to both the economy and protection of coastal environmental factors such as climate, tides, waves and current, salinity, dissolved oxygen, soils and nutrients.

Global geographical distribution of mangroves is mainly restricted to the tropics, but they are also found in some parts of the subtropics, particularly in Japan and New Zealand (Aksornkoae, 1993). The total mangrove area of the world has been assessed to be approximately 18.14 million hectares. There are 168,683 hectares of mangroves in Thailand, approximately 89% (150,272 hectares) is found on the south of Thailand especially on the West Coast of the peninsula (ประเสริฐ โพธิปักษ์, 2540).

In Thailand, mangroves occur on the sheltered muddy shores and low-lying bogs of river and stream estuaries at levels between low and high tides, along the banks of the Gulf of Thailand and on the west and east coasts of the peninsula. The best developed natural mangrove forests remain only along the west coast of the peninsula, especially in the provinces of Ranong (ระนอง), Phang-Nga (พังงา) and Trang (ตรัง). The mangroves along the coasts of the Gulf of Thailand are mainly classified as young growth because most of these mangrove forests have suffered heavy felling for many years, especially along the upper part of the Gulf of Thailand, in the provinces of Petchaburi (เพชรบุรี), Samut Sakorn (สมุทรสาคร) and Samut Songkram (สมุทรสงคราม) (Aksornkoae, 1993).

Mangrove sediments are formed by the accumulation of sediment derived from coastal or river banks erosion, or eroded soils from higher areas transported down along rivers

and canals, which make mangrove sediments contain a high percentage of high organic matter. Sediments that have accumulated in mangroves have different characteristics, depending on their origin. Sediment from rivers and canals is fine muddy soil, while coastal sediment is mainly sand. The degradation of organic matter deposited through time is also part of mangrove sediments.

Nutrients in mangroves are divided into two groups: inorganic nutrients and organic detritus (Aksornkoae, 1993). *Inorganic nutrients* are nitrogen, phosphorus, patassium, calcium, magnesium and sodium. These nutrients usually exist in adequate amounts, the only exception being nitrogen and phosphorus, the quantities of which are often limited. *Organic detritus* are organic nutrients derived from biogenic materials through several stages in the microbial degradation process.

Mangrove is the region where water and land meet. The species of mangrove microorganisms are only transitional members of terrestrial and true marine microorganisms. Since mangroves are areas of highly variable environmental parameters in terms of temperature, salinity, pH, organic loading and other factors, microorganisms in mangroves must be eurytolerant to many environmental factors. Mangrove microorganisms are bacteria and cyanobacteria, yeast, fungi, and microalgae (Suwanarit, 1998).

There are very few studies of the microbiological aspects of the mangroves. Accordingly, mangrove microorganisms are the prolific resources to discover the interesting strains, which may produce new biomedically important compounds.

2. Characteristics of the genus *Streptomyces*

The *Streptomyces* are gram-positive bacteria in the family Streptomycetaceae, order Actinomycetales (Table 1). Outgrowth from spores or fragments of mycelium, (colony forming unit, CFUs) develop into hyphae (branching filaments) that penetrate the agar (substrate mycelium) and hyphae that branch repeatedly and become cemented together on the surface of the agar to form a tough, leathery colony. In strains of *Streptomyces*, the colony becomes covered with aerial mycelium (free, erect hyphae surrounded by a

hydrophobic sheath that grow into the air away from the colony). These hyphae are initially white but assume a range of colors when spore formation begins. Colonies then appear powdery or velvety and can then be readily distinguished from the more typical bacteria colonies. *Streptomyces* strains produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia. Colored diffusible pigments may also be formed. Many strains produce one or more antibiotics. The cell wall peptidoglycan contains major amounts of L-diaminopimelic acid (L-DAP). *Streptomyces* strains use a wide range of organic compounds as sole sources of carbon for energy and growth (Goodfellow, 1989; Cross, 1994).

Cultural characteristics of the genus *Streptomyces* on various culture media are such characters as the colors of the soluble pigment, the color of the vegetative growth, the aerial mycelium and spores, and the micromorphology of the sporulation structures. Mycelia pigments and pigments that are produced in the substrate mycelium and diffuse out into the medium have been used as criteria for descriptions the species of *Streptomyces*.

Physiological and biochemical properties such as hydrolysis reaction (starch, gelatin or milk), nitrate reduction and melanin formation and utilization of carbon sources have been used extensively to characterize *Streptomyces* strains and species.

Organisms of the genus *Streptomyces* occur infrequently in soils but in relatively high numbers in aquatic habitats such as lake mud, river sediment and marine sediment. The *Streptomyces* have also been isolated from estuarine environment such as sandy sediment, intertidal sediment and sediment sample collected in the estuarine environment at the mouth of stream (Goodfellow, and William, 1983; Jensen, Dwight, and Fenical, 1991).

Table 1 Classification and morphological characteristics of the genus in Streptomycetaceae[a] (Cross, 1994).

Genus	Morphological characteristics
Intrasporangium	No aerial mycelium, substrate mycelium forms terminal and subterminal vesicles.
Kineosporia	No aerial mycelium, radiating vegetative hyphae is formed which spore is motile with polar tufts of flagella.
Sporichthya	No substrate mycelium is formed, aerial chains of motile, flagellated conidia are held to the surface of the substratum by holdfasts.
Streptomyces[b]	Aerial mycelium with chain (usually long) of non-motile conidia.
Streptoverticillium	Same as *Streptomyces*, but the aerial mycelium bears verticils consisting of at least three side branches, which may be chains of conidia or hold sporulating terminal umbels.

[a]Streptomycetaceae, All are aerobic sporoactinomycetes with cell wall type I (containing of L-diaminopimelic acid, L-DAP).

[b]*Streptomyces*, This genus includes the most common soil forms and most of the important producers of antibiotics.

3. Secondary metabolites from *Streptomyces* strains

3.1 Compounds of *Streptomyces* strains found in saline environments

Studies of secondary metabolites of actinomycetes have been mainly focused on terrestrial strains, with a small number of investigations on actinomycetes from other sources, especially those of marine or estuarine origin. Sediments from estuaries and bays are obviously organic rich and different chemical characteristics from terrestrials. Sediments from these habitats have provided the most fertile resource for the isolation of chemically prolific bacteria including bacteria in the genus *Streptomyces*. A review on the chemical discoveries and biological activities of *Sreptomyces* strains found in saline environments are shown in Table 2.

3.2 Chemistry of 2H-benzo[*c*,*d*]pyrene

Benzopyrenes are polycyclic aromatic hydrocarbons, which can be divided in to three classes, benzo[*a*]pyrene, benzo[*e*]pyrene, and benzo[*c*,*d*]pyrene. Derivatives of benzo[*a*] pyrene and benzo[*e*]pyrene are found mainly in coal tar and cigarette smoke, and in atmosphere as products of incomplete combustion (Richard, and Lewis, 1997). Some derivatives of benzo [*c,d*]pyrene are found as naturally occurring compounds especially in the rare group of 2H-benzo[*c,d*]pyrene. Up to the present, only two derivatives of 2H-benzo[*c,d*]pyrene have been found in nature, resistomycin **[50]** and resistoflavine **[51]**. The compounds resistomycin and resistoflavine, are polyhydroxyquinoid pentacyclic structure only found as secondary metabolites of *Streptomyces* spp. The chemical discovery and biological activity reported from 2H-benzo[*c,d*]pyrenes are as follows:

Resistomycin, $C_{22}H_{16}O_6$, was isolated from *Streptomyces resistomycificus* (Brockmann and Schmidt-Kastner, 1951); from *Streptomyces* JA 3733 (Eskardt, Bradler, and Fritzsche, 1970); from *S. erythrogriseus* var. *itamiceticus* as itamycin (Francisco *et al*., 1971); from *Streptomyces* strain as antibiotic A3733A (Eckardt *et al*., 1972); from *S. griseoflavus* (Bradler and Eckardt, 1972); from new species of actinomycete *A. atrovirens* as geliomycin (by Chugasova, and Preobrazhenskaya , 1975); from *S. griseoflavus* B 71 (Holfle and Wolf,

1983); from *S. heliomycini* as heliomycin (Vinogradava, Kirillaova, and Sokolova, 1991); and from culture of actinomycete strain DSM 7357 (Roggo *et al*., 1994).

The structure of resistomycin was established as 3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6 (1H)-dione (Rosenbrock, 1967; Hoefle, and Wolf, 1983). Biological activities of resistomycin were HIV-I protease inhibitory activity (Roggo *et al*., 1994) and antimicrobial activity against gram-positive bacteria and protozoa (Eckardt *et al*, 1972) such as *Bacillus subtilis*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* at a dilution of 1 : 20,000,000 and 1 : 1,000,000, respectively.

Several bromo derivatives of resistomycin were synthesized for elucidation its chemical structure such as 4,8,11-tribromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo [*c,d*]pyrene-2,6(1H)-dione **[52]**; 4,11-dibromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione **[53]**; 4,8-dibromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione **[54]**; and 4-bromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione **[55]** (Brockmann, and Meyer *et al*., 1969).

Resistoflavine, $C_{22}H_{16}O_7$, was isolated from *Streptomyces* sp. JA 3733 (Eckardt, Bradler, Fritzsche *et al*., 1970); from a *Streptomyces* strain (Eckardt, Bradler, Tresselt *et al*., 1972); and from *S. griseoflavus* (Hoefle, and Wolf, 1983).

The structure of resistoflavine was established as 3,5,7,11b-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione (Eckardt, Fritzsche, and Tresselt, 1970). Resistoflavine showed antibacterial activity against gram-positive bacteria, mycobacteria, viruses and protozoa (Eckardt, Bradler, Tresselt *et al*., 1972).

3.3 Chemistry of diketopepirazines (DKPs)

Diketopiperazines (DKPs), the closed-ring compounds produced by the condensation of two amino acids, the carboxyl group of each combining with the amino group of the other. DKPs are widely distributed in nature as secondary metabolites especially terrestrial yeast, lichen, fungi and also observed in marine bacteria and marine actinomycetes (Adamczeski, Reed, and Crews, 1995). DKPs are also found in culture broth of *Streptomyces* strains and some of them showed biological activity as shown in Table 3.

Table 2 Compounds produced by *Streptomyces* strains in saline environments.

No.	Compounds	Sources	Activities	References
1 2	Urauchimycin A **[1]** Urauchimycin B **[2]**	*Streptomyces* sp. Ni-80, unidentified sponge	antimycin antibiotic	Imamura *et al*., 1993
3	Altemicidin **[3]**	*S. sioyaensis* SA-1758, sea mud at Gamo, Miyagi Prefecture, Japan	antitumor against LI210, carcinoma, acaricidal activity and inhibit growth of gram-positive and gram-negative bacteria, yeasts and molds	Takahashi, 1990
4	Aburatubolactam A **[4]**	*Streptomyces* sp. SCRC-A20, derived from marine mollusk	potently inhibit superoxide anion generation	Bae *et al*., 1996
5 6 7	Salinamide A **[5]** Salinamide B **[6]** Salinamide D **[7]**	*Streptomyces* sp. CNB-091, surface of jellyfish *Cassiopeia xamachana* from the Frorida Keys	potent topical antiinflammatory activity, moderate antibiotic activity against gram-positive bacteria	Trischman *et al*., (1994)
8	Desotamide **[8]**	same extract that yielded **[5]**	inactive in bacterial RNA polymerase inhibitor assay while salinamide A active	Miao *et al*., 1997
9 10 11	Cyclomarin A **[9]** Cyclomarin B **[10]** Cyclomarin C **[11]**	*Streptomyces* sp. CNB-982, sediment samples collected in Mission Bay, CA.	**[9]** potent antiinflammatory agent	Renner *et al*., 1999
12	Anthranilamide **[12]**	*Streptomyces* sp. B7747, sediment from the Gulf of Mexico	antimicroalgal and phytotoxic agent	Biabani *et al*., 1998

Table 2 (continued)

No.	Compounds	Sources	Activities	References
13 14	Istamycin A **[13]** Istamycin B **[14]**	*S. tenjimariensis* SS-939, shallow-water mud sample from Sagami Bay, Japan	strong *in vitro* antibacterial activity against gram-negative and gram-positive bacteria	Okami *et al*., 1979
15	Halichomycin **[15]**	*S. hygroscopicus*, isolated from gastrointestinal tract of marine fish: *Helichoeres bleekeri*.	potent cytotoxic activity (ED_{50} 0.13 μg/ml) in the P-388 lymphocytic leukemia	Takahashi, 1994
16 17	Marinone **[16]** Debromomarinone **[17]**	*Streptomyces* sp. isolate CNB-632, sediment sample at the Torrey Pines Estuary, La Jolla, CA	antibacterial activity mainly gram-positive bacteria	Pathirana, Jensen *et al*., 1992
18 19 20 21	γ-indomycinone **[18]** Rubiflavinone C-1 **[19]** Rubiflavinone C-2 **[20]** β-indomycinone **[21]**	*Streptomyces* sp. PCI/B2, deep-sea sediment in University of Hawaii Corelab.	mixture of **[19]** and **[20]** cytotoxic to the human colon cancer cell line HCT-116	Schumacher *et al*., 1995
22 23 24 25	Halawanone A **[22]** Halawanone B **[23]** Halawanone C **[24]** Halawanone D **[25]**	*Streptomyces* sp. BD-18T, sediment sample collected in the estuarine environment at the mouth of Halawa stream, Oahu	ND	Ford, Gadepalli, and Davidson, 1998

Table 2 (continued)

No.	Compounds	Sources	Activities	References
26	δ-indomycinone **[26]**	*Streptomyces* sp. B8251, sediment sample collected at lagoon on the Gulf of Mexico	pluramycin-class antibiotic	Baibani, Laatsch *et al*., 1997
27 28 29 30 31 32	Wailupemycin A **[27]** Wailupemycin B **[28]** Wailupemycin C **[29]** Enterocin **[30]** 5-deoxyenterocin **[31]** 3-epi-5-deoxyenterocin **[32]**	*Streptomyces* sp. BD-26T, shallow water sediment at Wailupe beach park, Oahu Hawaii	**[30]** and **[31]** bacteriostatic against gram positive and gram-negative bacteria	Sitachitta, Gadepalli, and Davidson, 1996
33-36 37 38	Phenazine L-quinovose esters **[33]** – **[36]** 6-acetylphenazine-1-carboxylic acid **[37]** Saphenic acid **[38]**	*Streptomyces* sp. CNB-253, shallow sediment in Bodega Bay, CA	inactive in cytotoxicity test against murine and human cancer cell lines, broad spectrum activity against gram-negative and gram-positive bacteria while **[34]** more active overall, **[33]** shows potent activity against *Heamophilus influenzae* (MIC 1 μg/ml)	Pathirana *et al.*, 1992
39	Bioxalomycin α_2 **[39]**	*Streptomyces viridodiastaticus* subsp. *litoralis* LL-31F508, intertidal soil sample collected in Key West, Fla.	potent activity against gram-positive bacteria	Singh *et al*., 1994

Table 2 (continued)

No.	Compounds	Sources	Activities	References
40	Dihydrophencomycin methyl ester **[40]**	*Streptomyces* sp. B8251, marine sediment sample from the Gulf of Mexico	weakly antimicrobial activity	Puseker *et al*., 1997
41 42	Luisol A **[41]** Luisol B **[42]**	*Streptomyces* sp. CNH-370, sandy sediment collected near San Diago in the San Luis Estuary	ND	Cheng, Jensen, and Fenical , 1999
43	Actinoflavoside **[43]**	*Streptomyces* sp. CNB-689, intertidal sediment collected near Christchurch, New Zealand	weak antibacterial activity against gram-positive bacteria	Jiang, Jensen, and Fenical, 1997
44	Arenaric acid **[44]**	*Streptomyces* sp. CNH-248, sediment sample collected from the north of San Diego in an estuary near Doheny Beach	ND	Cheng, Jensen, and Fenical, 1999
45 46	Octalactin A **[45]** Octalactin B **[46]**	*Streptomyces* sp.PG-19, derived from the surface of the Sea of Cortez gorgonian octacoral *Pacifigorgia* sp.	**[45]** cytotoxic against B-16-F10 and HCT-116 with IC_{50} 7.2 x 10^{-3} μg/ml, and 0.5 μg/ml, respectively	Tapiolas, Roman, and Fenical, 1991

Table 2 (continued)

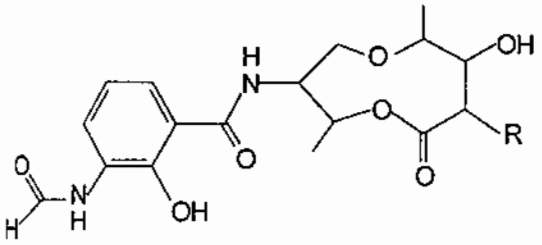
No.	Compounds	Sources	Activities	References
47 48 49	Aplasmomycin A **[47]** Aplasmomycin B **[48]** Aplasmomycin C **[49]**	*S. griseus* SS-20, shallow mud sample from Sagami Bay, Japan	antibiotic activity against gram-positive bacteria, antimalarial agent against *Plasmodium berghei*–infected mice	Okami *et al*., 1976

ND, No data

Table 3 Sources and biological activity of the *Streptomyces* derived diketopiperazines.

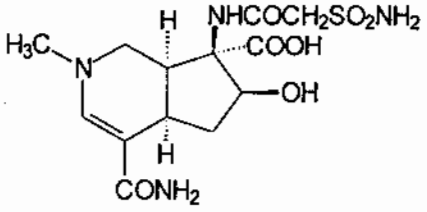
No.	Compounds	Sources	Activities	References
1	*cyclo*-(L-Pro-L-Leu) **[56]**	*Streptomyces griseus*	antimicrobial activity against *Staphylococcus aureus*	Johnson, Jackson, and Eble, 1951
2	*cyclo*-(L-Pro-L-Val) **[57]**	*Streptomyces* sp. No. K-73	ND	Ogura, Furuhata, and Furuhata, 1975
3	1-N-methylalbonoursin **[58]**	*Streptomyces albus*	ND	Robins and Sefton, 1984
4	3-benzylidene-6-isobutylidene-2,5-dioxopiperazine **[59]**	*Streptomyces noursei*	ND	Brown, Kelly, and Wiberley, 1965
5	3,6-dibenzylidene-2,5-dioxopiperazine **[60]**			
6	3-benzyl-6-benzylidine-2,5-dioxopiperazine **[61]**			
7	3,6-dibenzyl-2,5-dioxopiperazine **[62]**			
8	tryptophan-dehydrobutyrine diketopiperazine **[63]**	*Streptomyces spectabilis*	reverse transcriptase inhibitor	Kakinuma and Rinehart, 1974
9	diketopiperazine of N-methyltyrosine **[64]**	soil bacterium, *Streptomyces griseus* SC 488	calpain inhibitor	Alvarez *et al*., 1994
10	XR330 **[65]**	*Streptomyces* sp.	inhibitors of plasminogen activators	Bryans *et al*., 1996
11	XR334 **[66]**			

ND, No data

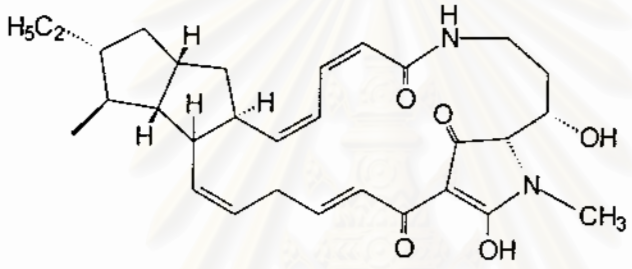


R

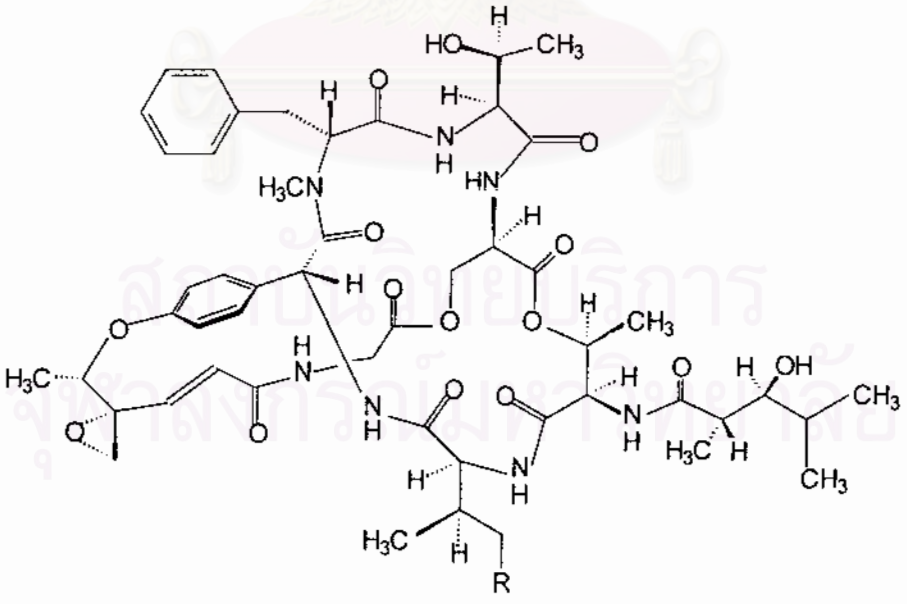
[1] Urauchimycin A $CH_2CH(CH_3)C_2H_5$
[2] Urauchimycin B $C_2H_4CH(CH_3)_2$



[3] Altemicidin

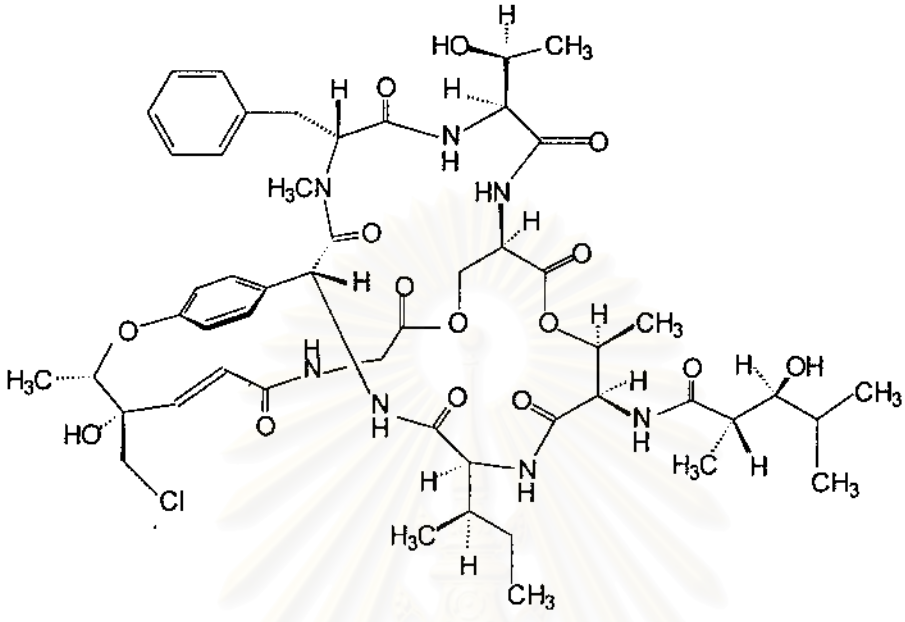


[4] Aburatubolactam A

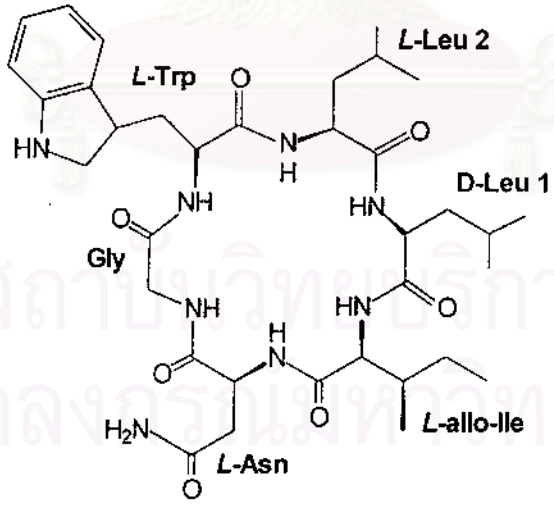


R

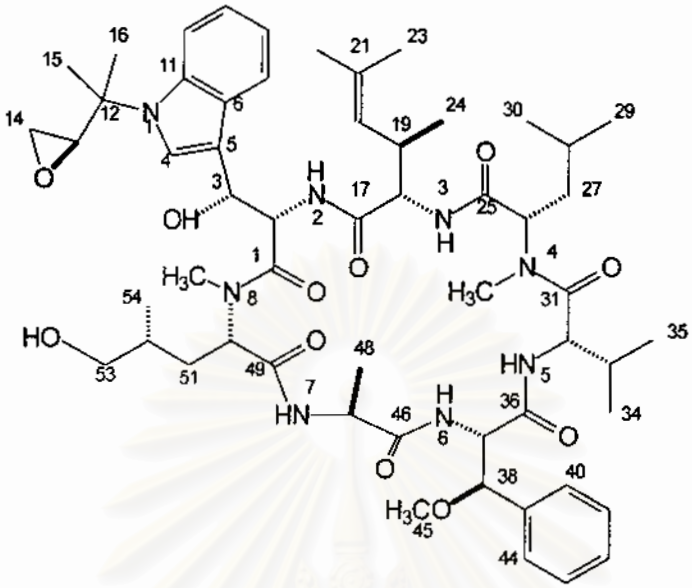
[5] Salinamide A CH_3
[7] Salinamide D H



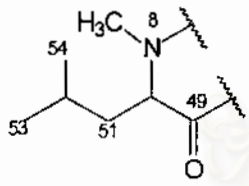
[6] Salinamide B



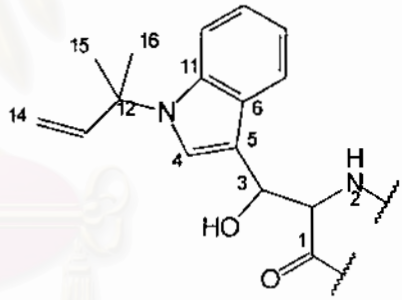
[8] Desotamide



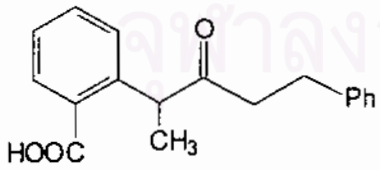
[9] Cyclomarin A



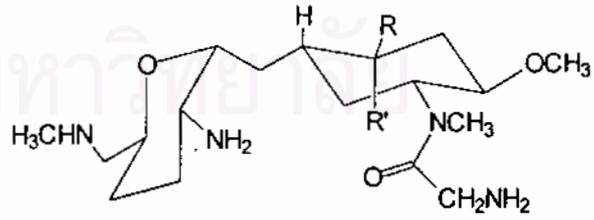
[10] Cyclomarin B



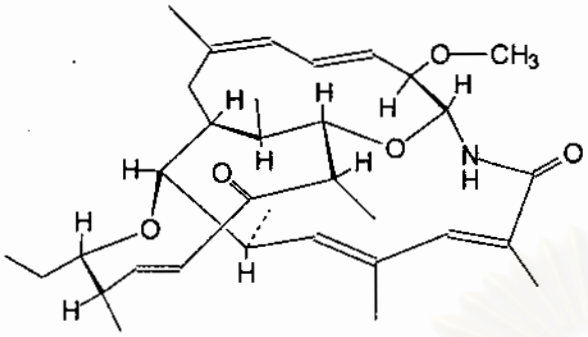
[11] Cyclomarin C



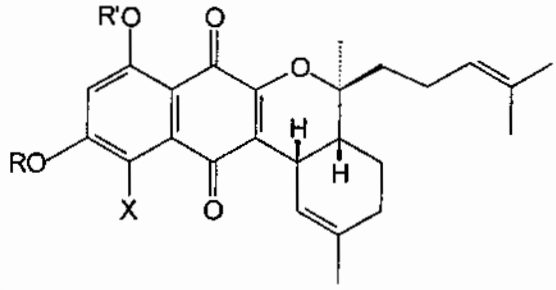
[12] Anthranilamide



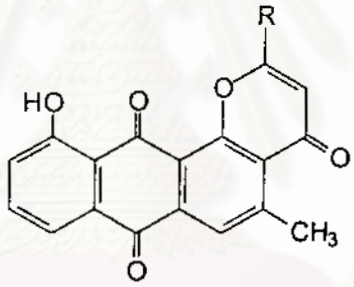
		R	R'
[13]	Istamycin A	H	NH_2
[14]	Istamycin B	NH_2	H



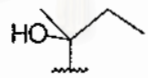
[15] Halichomycin



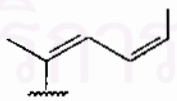
		R	R'	X
[16]	Marinone	H	H	Br
[17]	Debromomarinone	H	H	H



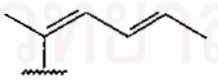
[18] *γ*-indomycinone



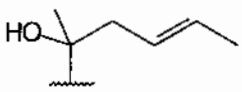
[19] Rubiflavinone C-1

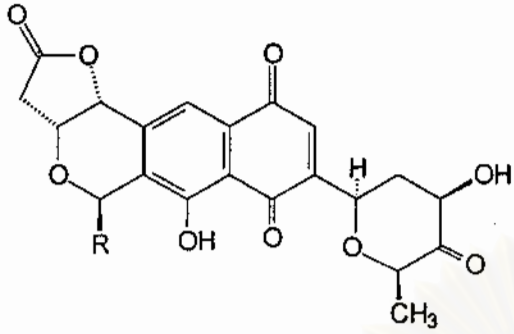


[20] Rubiflavinone C-2

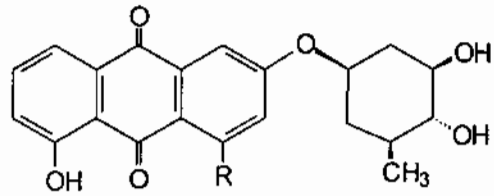


[21] *β*-indomycinone

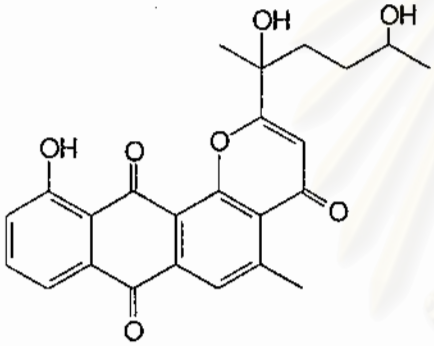




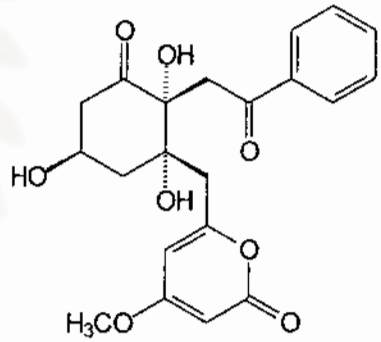
		R
[22]	Halawanone A	CH_2CH_3
[23]	Halawanone B	CH_3



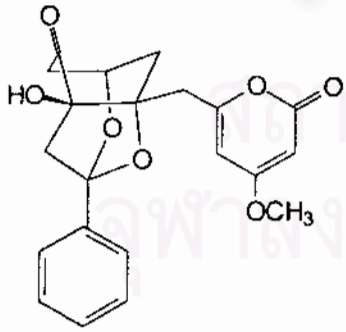
		R
[24]	Halawanone C	CH_3
[25]	Halawanone D	CH_2CH_3



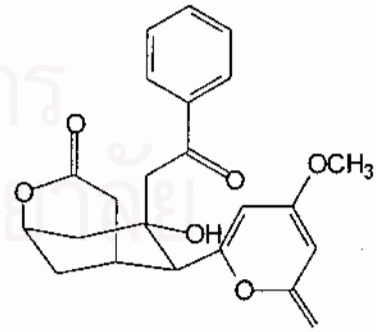
[6] δ-indomycinone



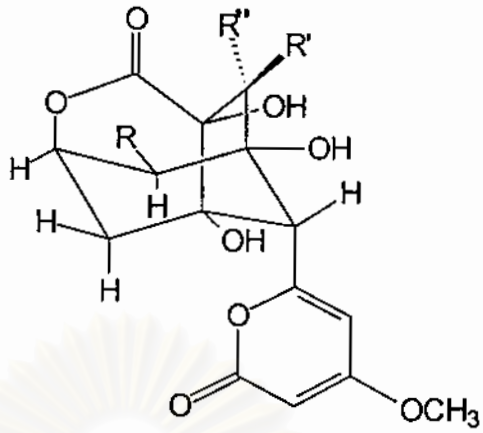
[27] Wailupemycin A



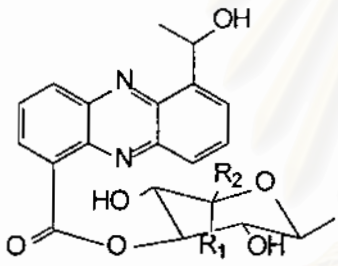
[28] Wailupemycin B



[29] Wailupemycin D

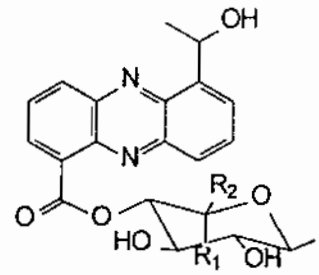


		R	R'	R"
[30]	Enterocin	OH	COPh	H
[31]	5-deoxyenterocin	H	COPh	H



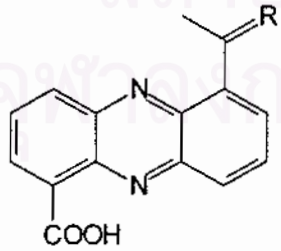
Phenazine L-quinovose esters

	R_1	R_2
[33]	OH	H
[36]	H	OH

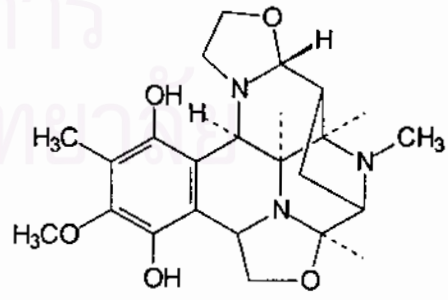


Phenazine L-quinovose esters

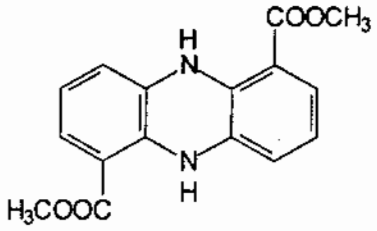
	R_1	R_2
[34]	OH	H
[35]	H	OH



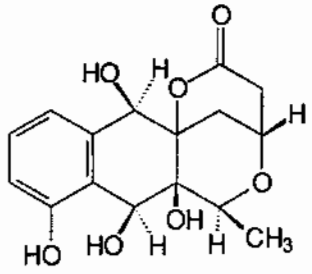
		R
[37]	6-acetylphenazine-1-carboxylic acid	H, OH
[38]	Saphenic acid	=O



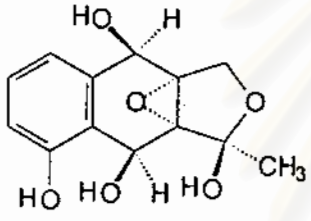
[39] Bioxalomycin α_2



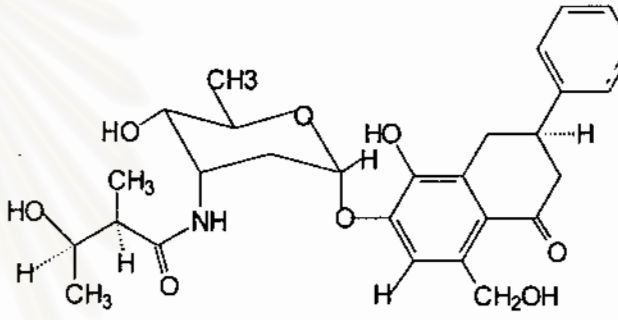
[40] Dihydrophencomycin methyl ester



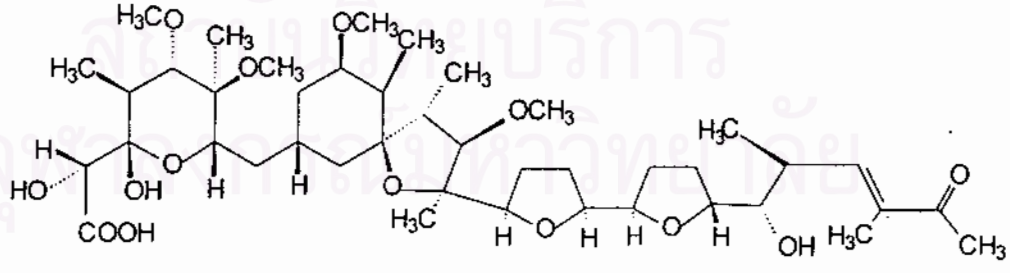
[41] Luisol A



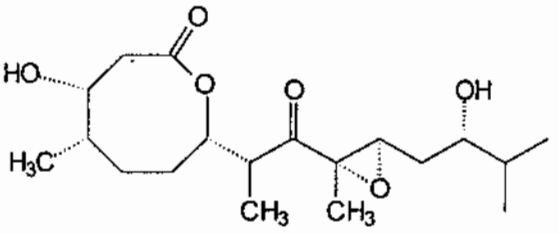
[42] Luisol B



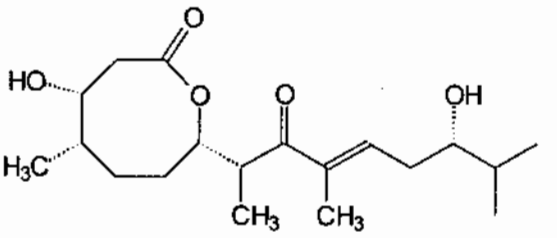
[43] Actinoflavoside



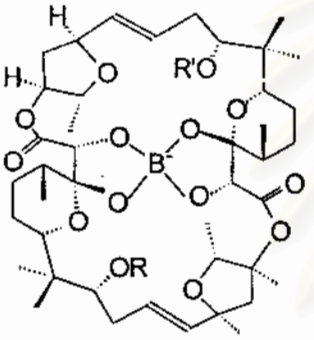
[44] Arenaric acid



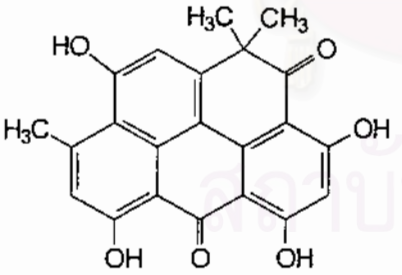
[45] Octalactin A



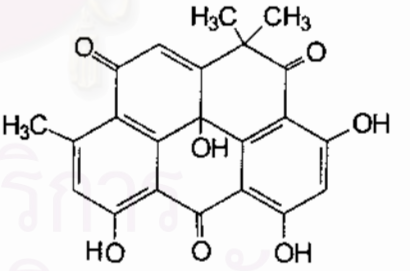
[46] Octalactin B



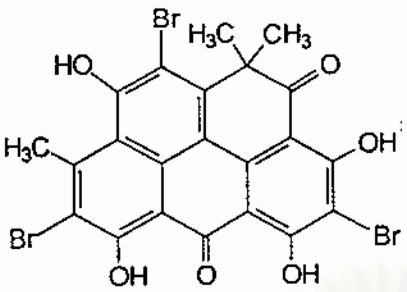
		R	R'
[47]	Aplasmomycin A	H	H
[48]	Aplasmomycin B	H	Ac
[49]	Aplasmomycin C	Ac	Ac



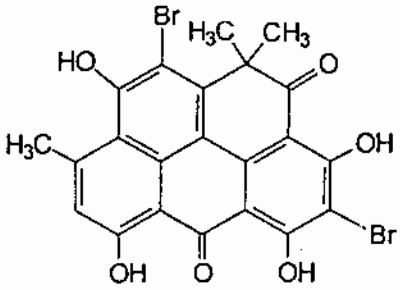
[50] Resistomycin



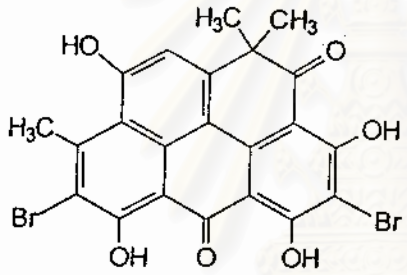
[51] Resistoflavine



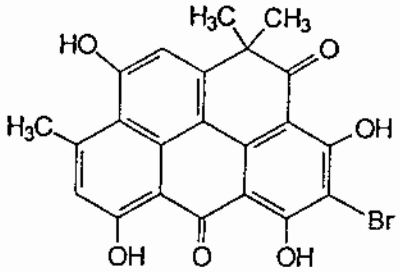
[52] 4,8,11-tribromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione



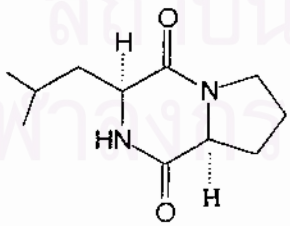
[53] 4,11-dibromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione



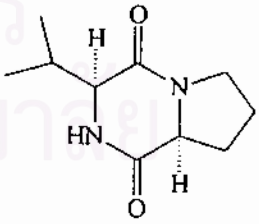
[54] 4,8-dibromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene-2,6(1H)-dione



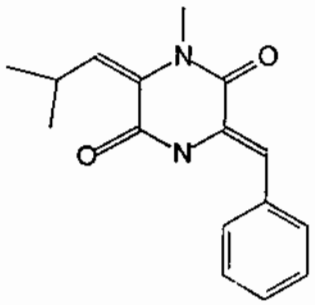
[55] 4-bromo-3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*c,d*]pyrene 2,6(1H)-dione



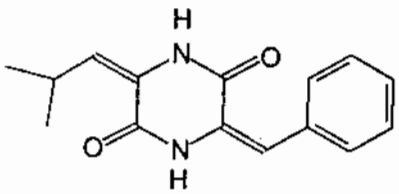
[56] *cyclo*-(L-Pro-L-leu)



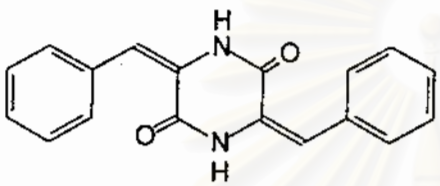
[57] *cyclo*-(L-Pro-L-Val)



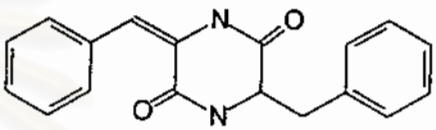
[58] 1-N-methylalbonoursin



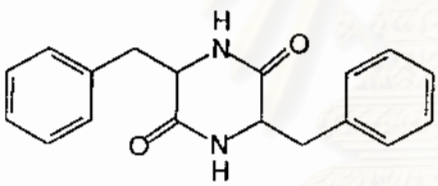
[59] 3-benzylidene-6-isobutylidene 2, 5-dioxopiperazine



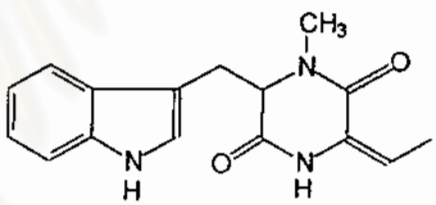
[60] 3, 6-dibenzylidene-2, 5-dioxopiperazine



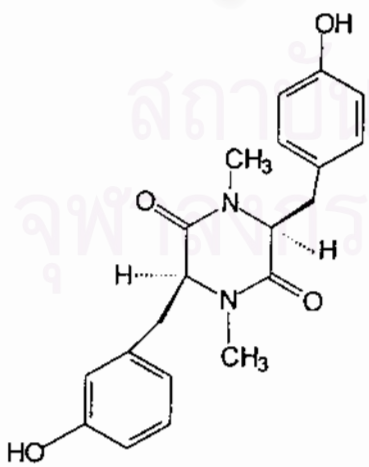
[61] 3-benzyl-6-benzylidene-2, 5-dioxopiperazine



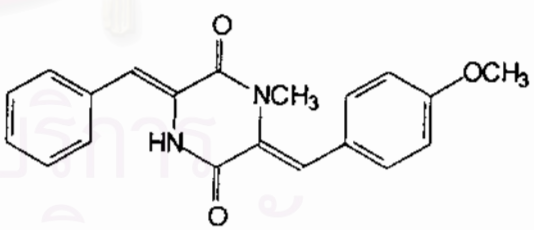
[62] 3, 6-dibenzyl- 2, 5- dioxopiperazine



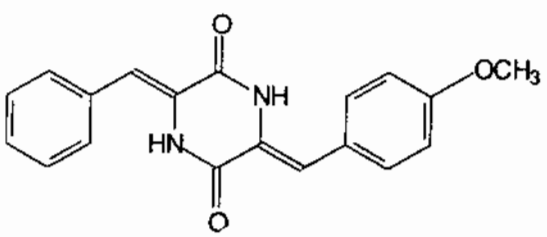
[63] Tryptophan-dehydrobutyrine diketopiperazine



[64] Diketopiperazine of N-methyltyrosine



[65] XR 330



[66] XR334

CHCPTER III

EXPERIMENTAL

1. Sample collection and isolation of actinomycete

A sediment sample of actinomycete (TRA9851-2) was collected from the bottom of a canal (3 meters in depth) in a mangrove forest on the West Coast of Trang Province, Thailand, in October 1998. Spread plate technique (Brock *et al*., 1993) was used to isolate the strain. The sediment sample (0.5 g) was suspended in 5.0 ml of sterile seawater. The suspension was diluted to 1:10 in sterile sea water and then heated on a water bath at 60°C for 5 minutes, and 0.1 ml of the dilution was spreaded on plates of sodium casienate agar (SCA) and potato carrot agar (PCA) added with antibiotics. The plates were incubated at room temperature for 7-14 days until white powdery colonies appeared. The colony was picked up and streaked on yeast extract-malt extract agar (YMA) to obtain several single colonies. Each single colony was transferred into YMA slants and incubated at room temperature for 7-14 days. The mature slants were kept at 15°C temperature.

The isolated actinomycete strain TRA9851-2 produced abundant white aerial mycelium, which afterwards transformed into dark-gray powdery spores. The stock culture of a strain TRA9851-2 was kept on YMA slants at 15°C temperature at the Departments of Pharmacognosy and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. Identification and characterization of actinomycete

The characterization of the actinomycete strain TRA9851-2 was carried out by the methods of Burgey's Manual of Systematic Bacteriology (Cross, 1994) and the methods of the International *Streptomyces* Project (ISP) (Shirling, and Gottlieb, 1966).

2.1 Morphological and cultural characteristics

2.1.1 Determination of morphological characteristics

Morphological characteristics, gram staining, spore bearing hyphae, spore chain, number of spores, spore morphology and spore surface were studied by using simple inclined coverslip technique (William, and Cross, 1971). The bacterium was cultured on YMA medium for 14 days until mature spores bearing hyphae were shown. For the scanning electron microscopic (SEM) examination, the selected strain was grown on YMA plate by crosshatch streak technique (Shirling, and Gottlieb, 1966) and incubated at room temperature for 10-14 days. The spores and mycelia were observed with a scanning electron microscope (SEM) using the method of De man, De man and Gupta (1986).

2.1.2 Cultural characteristics

Cultural characteristics were studied on the colors of mature aerial mycelium, substrate mycelium, spore and diffusible soluble pigment using crosshatch streak (Shirling, Gottlieb, 1966). The strain was cultured on five different agar media, inorganic salt-starch agar, glycerin asparagine agar, tyrosine agar, yeast extract-malt extract agar, and oatmeal agar. They were incubated at room temperature for 7-14 days, and examined for the color of the substrate mycelium by observing the reverse (under) side of mass growth on various media, color of spore and cultural characteristics.

2.2 Physiological and biochemical characteristics

2.2.1 Melanin formation

The strain TRA9851-2 was cultured on tyrosine agar slants and incubated at room temperature. Melanoid pigments of inoculated tubes and uninoculated controls were compared. Cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color shall be recorded as positive (+). Absence of brown to black

colors, or total absence of diffusible pigment, shall be recorded as negative (-) for melanoid pigment production.

2.2.2 Carbon utilization

Basal agar medium (carbon utilization medium) was prepared and a carbon source was added to give concentration of approximately 1%. The carbons required for the test were no carbon source (negative control), D-glucose (positive control), L-arabinose, sucrose, D-xylose, L-inosital, D-mannitol, D-fructose, rhamnose, and raffinose. Plates were observed at 10-16 days. Growth on a given carbon source was compared with the two controls, growth on basal medium alone and growth on basal medium plus glucose.

2.2.3 NaCl tolerance

Growth of the strain TRA9851-2 was observed on the cell grew on YMA plates containing 0%, 5%, 7%, 8%, 9%, 10%, 11% and 12% of NaCl and incubated at room temperature for 7-14 days.

2.2.4 pH tolerance test

Growth of the strain TRA9851-2 was observed on the cell grew on YMA plates adjusted to pH 3, pH 4, pH 5, pH 6, pH 7, pH 8, pH 9, pH 10, pH 11, pH 12, pH 13 and pH 14 and incubated at room temperature for 7-14 days.

2.2.5 Nitrate reduction

The strain TRA9851-2 was inoculated into peptone nitrate broth and incubated at room temperature for 4-6 days. On the forth day, 1 ml of the culture broth was transferred into a test tube, and three drops of the sulfanilic acid reagent and two drops of dimethyl-α-napthylamine solution were sequentially added. If nitrites were present, the mixture would become pink to red.

2.2.6 Starch hydrolysis

The strain TRA9851-2 were streaked on the surface of inorganic salt-starch agar plate and incubated at room temperature for 10 days. After incubation, Gram's iodine solution was poured on the surface of the agar plate. If starch hydrolysis was present, a dark blue color did not appear.

2.2.7 Gelatin liquefaction

The strain TRA9851-2 was inoculated into a test tube of nutrient gelatin broth and incubated at room temperature for 21 days. The appearance of inoculated tube was compared with uninoculated after incubating both tubes at 20°C for 30 minute; if the gelatin was hydrolyzed, it was liquid, not solid.

2.2.8 Milk coagulation and milk peptonization

The strain TRA9851-2 was inoculated in a tube of skim milk broth and incubated at room temperature for 21 days. If milk was peptonized, it would be then converted to clear solution. If milk was coagulated, it would be precipitated.

2.2.9 Cellulose decomposition

The strain TRA9851-2 was inoculated in a tube of cellulose decomposition medium and incubated at room temperature for 31 days. If the filter paper (Whatman NO.1) was digested, therefore the strain was considered to produce cellulase.

2.3 Cell wall analyses

The chemical analyses of cell wall diaminopimelic acid (DAP) isomers were carried out by the method of Komagata and Suzuki (1987). Dried cell of the strain TRA9851-2 was hydrolyzed by whole-cell analyses. DAP isomers were separated by thin layer

chromatography on cellulose plate (Merck no. 5577). The standard of DL-DAP (0.01 M) and the hydrolysate of two known strains that contained meso- and L-DAP were applied for reference purposes. TLC was developed with the solvent system methanol-water-6 N hydrochloric acid-pyridine (80:26:4:10, v/v). The spots were visualized by spraying with 0.2% ninhydrin solution in water-saturated n-butanol followed by heating at 100°C for 5 minutes. DAP isomers appear as dark-green spots with R_f 0.29 (L-isomer) and 0.24 (meso- and DL-isomer). Spots will gradually disappear in a few minutes.

3. Fermentation method

A loopful of the TRA9851-2 cultivated on YMA slants (7 days) was inoculated into a 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose beef extract, GBP). The flask was shaken on a rotary shaker and incubated at room temperature for 3 days. One milliliter of seed culture was inoculated into 250 ml of the production medium (glycerol peptone medium, GPM) in a 500-ml Erlenmeyer flask incubating on a rotary shaker (200 rpm) at room temperature for 5 days.

4. Chromatographic techniques

4.1 Analytical thin-layer Chromatography

Technique	: one dimension ascending
Adsorbent	: Silica gel F_{254}
Layer thickness	: 250 μm
Distance	: 5 cm
Temperature	: room temperature (25-30°C)
Detection	1. Visual detection under daylight
	2. Visual detection under ultraviolet light at wavelengths of 254 and 365 nm
	3. Visual detection in iodine vapor
	4. Visual detection under daylight after spraying with anisaldehyde reagent and heated until colors developed.

4.2 Column chromatography

4.2.1 Gel filtration chromatography

Gel filter : Sephadex LH-20 (Pharmacia Biotech AB)

Packing method : Sephadex gel was suspended in an eluant and left standing to swell for 24 hours prior to use. It was then poured into a 2.5 cm diameter column and allowed to settle tightly.

Sample loading : The sample was dissolved in a small volume of an eluant and loaded on top of a column.

Detection : Fractions were examined by TLC technique in the same manner as described in Section 4.1.

4.2.2 Flash column chromatography

Adsorbent : Silica gel 60 (No.7734), particle size 0.063-0.200 nm (70-230 mesh ASTM) (E. Merck)

Packing method : The adsorbent was suspended in an eluant. The adsorbent slurry was poured into a column, and then allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of an eluting solvent and loaded on top of a column.

Detection : Fractions were examined by TLC technique in the same manner as described in Section 4.1.

5. Crystallization technique

Compound TM-H01 were crystallized by dissolving in ethyl acetate until saturation and left standing at room temperature until yellow- needle crystal was formed.

Compound TM-2F21 was crystallized from a mixture of hexane and chloroform. It was dissolved in hexane until saturation, then chloroform was added. The solution was left standing at room temperature until colorless crystal was formed.

Compound TM-2F07 was crystallized from mixture of chloroform and acetone. It was dissolved in acetone until saturation, then chloroform was added. The solution was left standing at room temperature until dark-brown crystal was performed.

6. Spectroscopy

6.1 Ultraviolet (UV) Absorption Spectra

UV spectra were obtained from a Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

6.2 Infrared (IR) Absorption Spectra

IR spectra (KBr disc and NaCl cell) were obtained from a Perkin Elmer FT-IR 1760X spectrometer (the Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University).

6.3 Mass Spectra (MS)

FAB-MS spectra were obtained from JEOL JMS-HX 110 double focussing mass spectrometer of EBE arrangement with JMA-DA 7000 data system, 10 kV acceleration voltage, and fast-atom xenon gas accelerated at voltage of 3 kV. Glycerol and m-nitrobenzyl alcohol (mNBA) were used as the matrix, and sodium chloride was used as alkali metal cation source (the Institute of Molecular and Cellular Biosciences, the University of Tokyo). Electronspray Ionisation-Time of Flight mass spectra (ESI-TOF MS) were recorded on a Micromass LCT mass spectrometer. MeCN : H_2O (50 : 50) containing 0.02% of formic acid was used as a solvent (the National Center for Genetic Engineering and Biotechnology, BIOTEC). The Electron impact mass spectrum (EIMS) was measured on a Fission Micromass VG Platform II mass spectrometer. Methanol was used as a solvent (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

6.4 Proton and Carbon Nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) Spectra

^{1}H and ^{13}C NMR, DEPT 135, HMQC, and HMBC spectra were obtained from a Bruker AVANCE DPX-300 FT-NMR spectrometer operating at 300 MHz for proton and 75 MHz for carbon. Proton detected heteronuclear correlations were measured using HMQC (optimized for $^nJ_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ =3, 4, and 8 Hz) pulse sequences (Faculty of Pharmaceutical Sciences, Chulalongkorn University). Selective NOE difference spectra were recorded with a JEOL JMN-A 500 NMR spectrometer (the Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University).

Deuterated solvents; dimethylsulfoxide-d_6 (CD_3SOCD_3); chloroform-d ($CDCl_3$); methanol-d_4 (CD_3OD); and acetone-d_6 (CD_3COCD_3) were used in NMR experiments. Reference signals were the signals of residual undeuterated solvents at δ 2.49 (^{1}H) and 39.7 (^{13}C) for CD_3SOCD_3, 7.24 (^{1}H) and 77.0 (^{13}C) for $CDCl_3$, 3.30 (^{1}H) and 49.0 (^{13}C) for CD_3OD, and 2.04 (^{1}H) and 29.8 & 206.5 (^{13}C) for CD_3COCD_3.

6.5 Optical rotation

Optical rotation was measured on a Perkin-Elmer 341 polarimeter using a sodium lamp operating at 589 nm (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

7. Solvents

Throughout this work, all organic solvents were of commercial grade, which were redistilled prior to use.

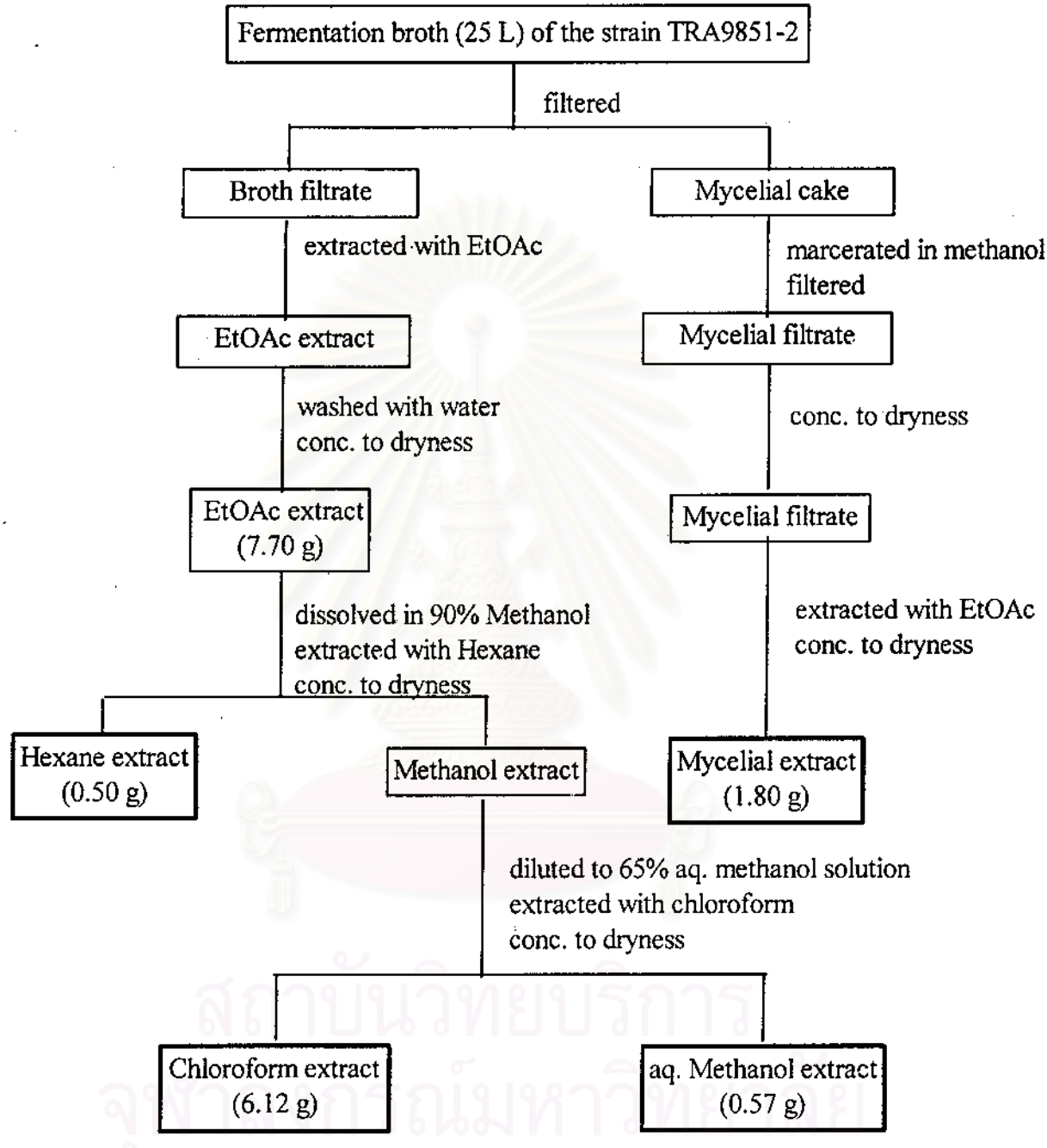
8. Extraction

The fermentation broth (25 liters) was filtered through a Buchner funnel packed with kieselguhr (diatomaceous earth or diatomite or bacillarieae earth). The mycelial cake was washed with a minimum amount of water that was subsequently combined with the original broth filtrate. The broth filtrate was extracted with ethyl acetate (4 x 25 liters), and the aqueous layer was discarded. The combined ethyl acetate extracts were washed with a minimum amount of water and concentrated under reduced pressure at temperature not exceeding 50°C to give an ethyl acetate extract (7.70 g), which was dissolved in 90% methanol solution (400 ml) and re-extracted with hexane (3 x 400 ml). The remaining methanol extract was diluted with water to give 65% aqueous methanol solution and then extracted with chloroform (3 × 200 ml). Concentration of the three fractions yielded 0.50 g of a hexane extract (yellow oil), 6.12 g of the chloroform extract (dark-brown oil), and 0.57 g of an aqueous methanol extract (dark-brown solid) as shown in Scheme 1. The mycelial cake was marcerated in methanol (500 ml) for 3 days and then filtered. The filtrate was evaporated to remove methanol. The residue (100 ml) was extracted with ethyl acetate (3 x 100 ml). The combined ethyl acetate extracts were concentrated to give a dried mycelial extract (yellow oil, 1.80 g) as shown in Scheme 1. All extracts were examined for antimicrobial activity by the method as described in this Chapter, Section 10.

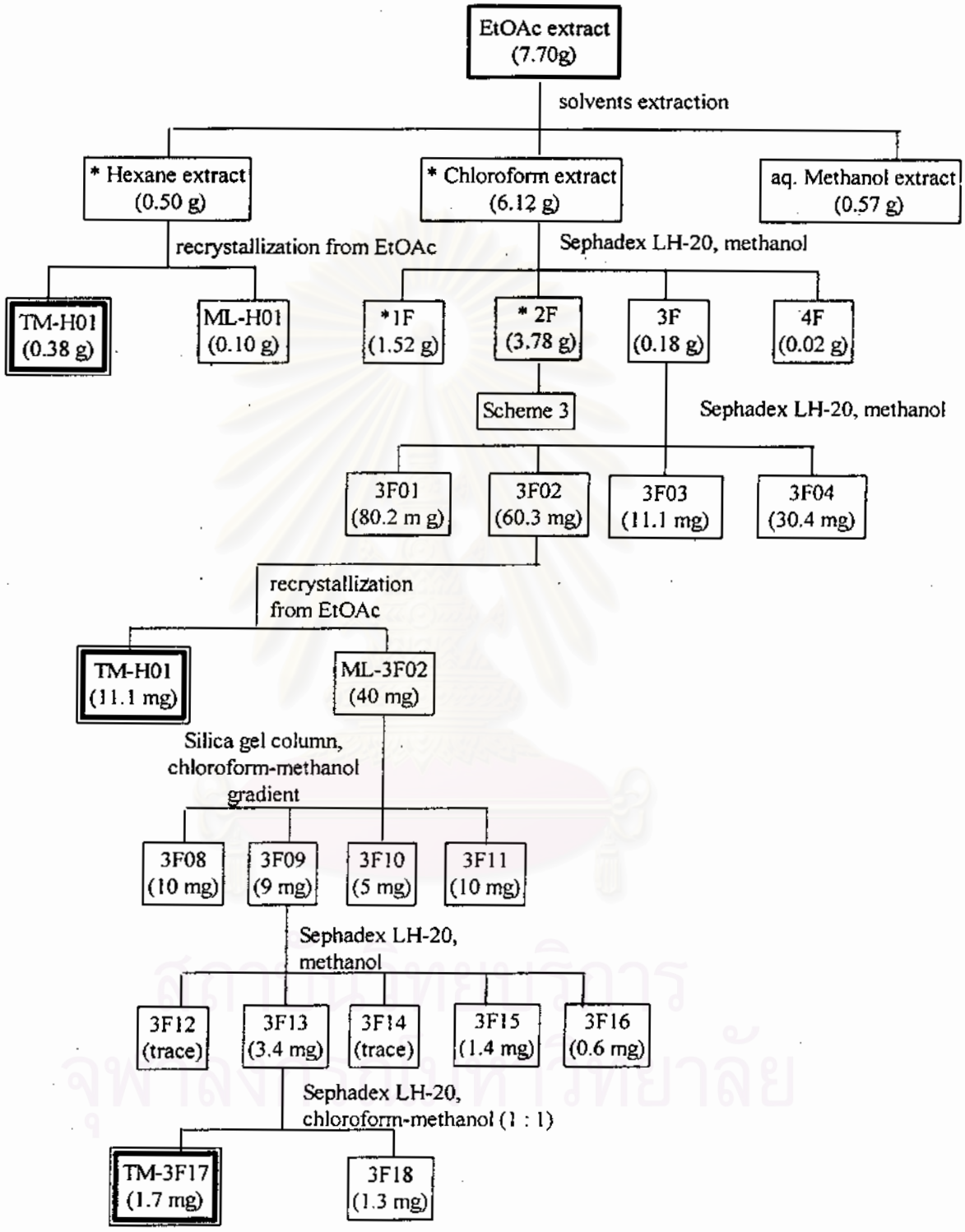
9. Isolation and purification of the extracts

Both crude hexane and chloroform extract from fermentation broth of the *Streptomyces* sp. TRA9851-2 showed antimicrobial activity in a disc diffusion assay at the concentration of 1 mg/6 mm disc. The crude hexane extract (0.50 g) exhibited antibacterial activity against *Bacillus subtilis* with 20 mm zone of inhibition, while the crude chloroform extract (6.12 g) was active against *Staphylococcus aureus* and *Candida albicans* with 10 mm and 20 mm zone of inhibition, respectively. The biologically active compounds of crude hexane and chloroform extracts were isolated by chromatographic technique as shown in Scheme II.

During evaporation of the hexane extract, there was a powder precipitated from the solution. This powder was recrystallized from ethyl acetate, yielding a pure compound TM-H01



Scheme 1 Extraction of the fermentation broth of the strain TRA9851-2.



(*) = positive antimicrobial activity

Scheme 2 Isolation of the ethyl acetate extract from the fermentation broth of the strain TRA9851-2.

(380 mg, 4.9% based on the ethyl acetate extract). Compound TM-H01 gave a yellow spot on TLC (Si gel, 10% methanol in chloroform, R_f = 0.6) and later identified as resistomycin (section 3.1, Chapter IV).

The concentrated brown oil of the chloroform extract (6.12 g) was diluted with a small amount of methanol and then fractionated on Sephadex LH-20 column (column 2 x 90 cm). The column was eluted with methanol, and fractions (25 ml each) were collected. The combined fractions were guided by TLC (Si gel) using 10% methanol in chloroform as a developing solvent. Fractions with similar chromatographic pattern were combined to give four fractions (1F-4F) as shown in Table 4. The fractions were evaporated to dryness and examined for antibacterial activity.

Table 4 Combination of fractions obtained from column chromatography of the chloroform extract from fermentation broth of the strain TRA9851-2.

Fraction code	Fractions	Volume of solvent (ml)	Total weight (g)
1F	1-11	275	1.52
2F	12-15	100	3.78
3F	16-28	325	0.18
4F	29-68	1000	0.02

Since the TLC chromatogram (Si gel, 10% methanol in chloroform) of fraction 3F (0.18g) showed spots of resistomycin (TM-H01, R_f = 0.6) and TM-3F17 at R_f between 0.2-0.4, fraction 3F was further purified by gel filtration chromatography. It was dissolved in a small amount of methanol and subjected to a Sephadex LH-20 column (column 2 x 90 cm) using methanol as an eluting solvent. Fractions (25 ml each) were collected. The combined fractions were guided by TLC (Si gel) using 10% methanol in chloroform as a developing solvent. Fractions with similar chromatographic pattern were combined to give four fractions (3F01-3F04) as shown in Table 5. The fractions were evaporated to dryness.

Table 5 Combination of fractions obtained from column chromatography of fraction 3F.

Fraction code	Fractions	Volume of solvent (ml)	Total weight (mg)
3F01	1-11	275	80.2
3F02	12-15	100	60.3
3F03	16-19	100	11.1
3F04	20-54	875	30.4

The 3F02 was recrystallized from ethyl acetate to yield yellow powders of TM-H01 (11.1 mg). The mother liquor containing TM-3F17 was evaporated to give ML-3F02 (40 mg). This residue was further purified on a silica gel column (column 2 x 20 cm, 30 g of Si gel) using a chloroform-methanol gradient as an eluting solvent. Fractions (25 ml each) were collected. Fraction combination was guided by TLC (Si gel) pattern with using 10% methanol in chloroform as a developing solvent. Fractions with the same TLC pattern were pooled and dried to give four fractions (3F08-3F11) as shown in Table 6.

Table 6 Combination of fractions obtained from column chromatography of fraction ML-3F02.

Fraction code	Fractions	Percentage of methanol in chloroform	Volume of solvent (ml)	Total weight (mg)
3F08	1-4	10	100	11
3F09	5-8	10	100	9
3F10	9-10	20	50	5
3F11	11-20	30	250	10
	21-30	50	250	
	31-40	100	250	

Fraction 3F09 (9 mg) containing compound TM-3F17 was separated on a Sephadex LH-20 column (column 1.5 x 50 cm) using methanol as an eluting solvent. Fractions (5 ml each) were collected, and fractions having similar TLC pattern were combined, yielding four fractions (3F12, 3F14, 3F15, and 3F16) as shown in Table 7.

Table 7 Combination of fractions obtained from column chromatography of fraction 3F09.

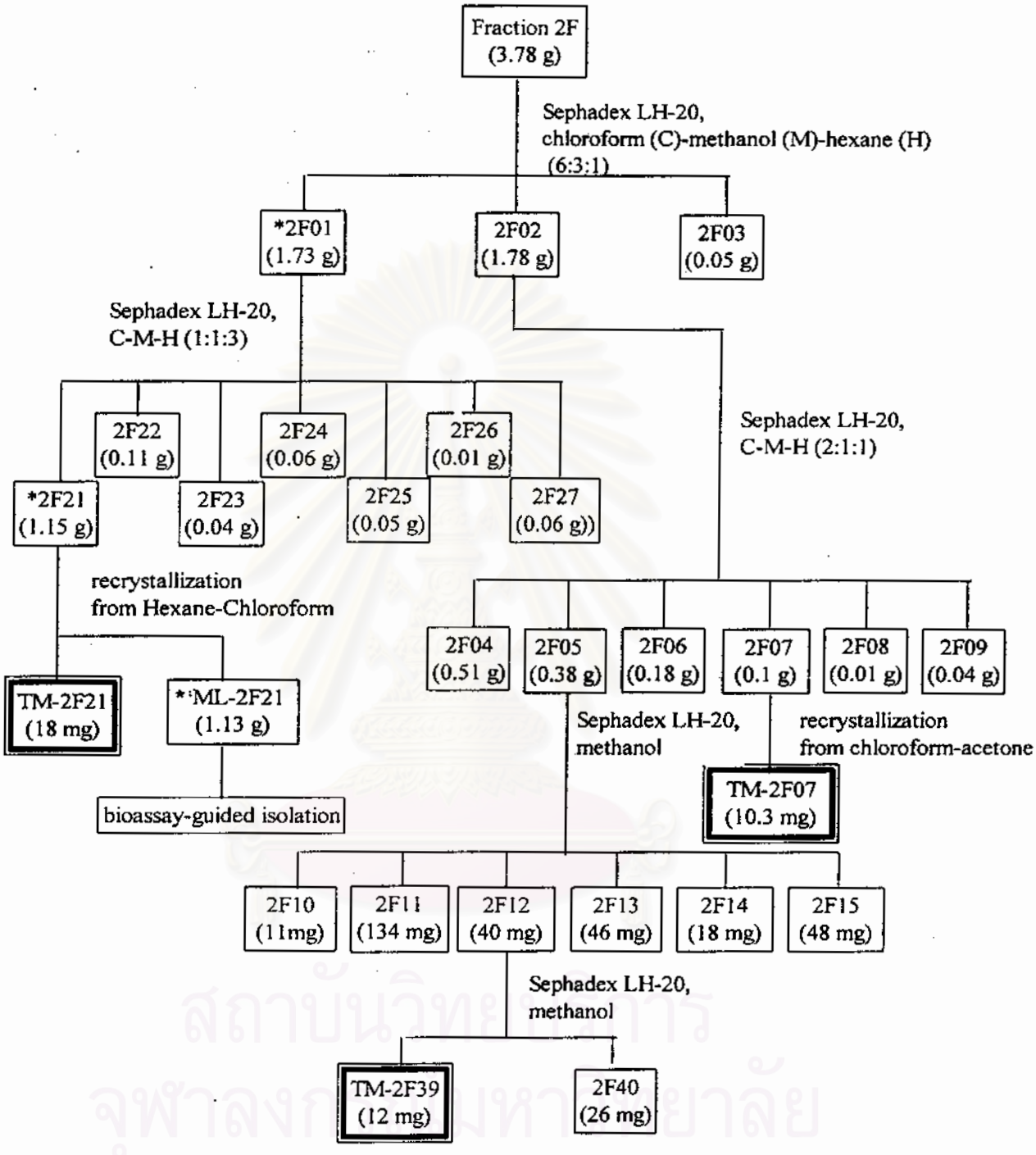
Fraction code	Fractions	Volume of solvent (ml)	Total weight (mg)
3F12	1-9	45	trace
3F13	10-16	35	3.4
3F14	17-23	35	trace
3F15	24-31	40	1.4
3F16	32-51	100	0.6

Fraction 3F13 (3.4 mg) was further purified by a Sephadex LH-20 column (column 1.5 x 50 cm) using a mixture of chloroform-methanol (1:1) as an eluting solvent. The fractions were collected according to a color of each band to give two fractions (3F17 and 3F018) as shown in Table 8. The first eluate, 3F17, gave a yellow spot on TLC (Si gel, 10% methanol in chloroform, R_f 0.24). This TLC chromatogram showed only one spot under detection as describe in Section 4.1. It was evaporated to dryness and assigned as TM-3F17 (1.7 mg, 0.02 % based on the ethyl acetate extract) and later identified as a benzo[*c,d*]pyrene derivative (Section 3.2 Chapter IV).

Table 8 Combination of fractions obtained from column chromatography of fraction 3F13.

Fraction code	Fractions	Volume of solvent (ml)	Total weight (g)
3F17	yellow	100	1.7
3F18	pale red	200	1.3

The active fraction, 2F (3.78 g) was investigated for its active pure compound against *Candida albicans* (23 mm inhibition zone at 1 mg/6 mm disc). This fraction was purified with the bioassay-guided isolation; one of these fractions exhibited antifungal activity, however the isolated yield was too low to carry out further study. A diketopiperazine, TM-2F21, was also isolated from this isolation as shown in Scheme 3.



(*) = positive antimicrobial activity

Scheme 3 Isolation of fraction 2F from the chloroform extract of the fermentation broth of the strain TRA9851-2.

Fraction 2F was applied to a Sephadex LH-20 column (column 2 x 90 cm) using chloroform-methanol-hexane (6:3:1) as an eluting solvent. Fractions (25 ml each) were collected. The eluates were pooled by TLC pattern (Si gel, 10% methanol in chloroform) and evaporated to give three fractions (2F01-2F03) as shown in Table 9.

Table 9 Combination of fractions obtained from column chromatography of fraction 2F.

Fraction code	Fractions	Volume of solvent (ml)	Total weight (g)
2F01	1-10	250	1.73
2F02	11-13	75	1.78
2F03	14-20	175	0.05

The fraction 2F01 (1.73 g) was further purified on Sephadex LH-20 column (column 2 x 80 cm) using chloroform-methanol-hexane (1:1:3) as an eluting solvent. Fractions (25 ml each) were collected. The eluates were pooled by TLC pattern (Si gel, 10% methanol in chloroform) and dried to give seven fractions (2F21-2F27) as shown in Table 10.

Table 10 Combination of fractions obtained from column chromatography of fraction 2F01.

Fraction code	Fractions	Volume of solvent (ml)	Total weight (g)
2F21	1-24	600	1.15
2F22	25-32	200	0.11
2F23	33-37	125	0.04
2F24	38-48	275	0.06
2F25	49-81	825	0.05
2F26	82-91	250	0.01
2F27	92-111	500	0.06

Fraction 2F21 (1.15 g) gave a colorless amorphous powder after removal of the solvent. It was washed with butanol and crystallized from a mixture solvent of hexane-chloroform. The TLC chromatogram of purified crystals showed only one spot under detection as described in Section 4.1 (R_f 0.66, 10% methanol in chloroform). It was assigned as compound TM-2F21 (18 mg, 0.23 % based on the ethyl acetate extract) and later identified as *cyclo*-(propyl-leucyl) (Section 3.3, Chapter IV). Although, the remaining mother liquor (ML-2F21) was active against *Candida albicans* ATCC10231, the active pure compound could not isolate.

Since the TLC chromatogram (Si gel, 10% methanol in chloroform) of fraction 2F02 (1.78 g) showed an interesting orange spot at R_f = 0.24 (TM-2F39) and the main spot which showed yellow color after spraying with anisaldehyde reagent at R_f = 0.1 (TM-2F07), this fraction was further separated by the method as shown in Scheme 3.

Fraction 2F02 (1.78 g) was divided into three portions (600 mg each), A, B and C. Each portion was separated on Sephadex LH-20 column (column 2 x 90 cm) using chloroform-methanol-hexane (2:1:1) as an eluting solvent. Fractions (25 ml each) were collected, and examined by TLC (Si gel) using 10% methanol in chloroform as a developing solvent. Fractions with the same TLC pattern were pooled and dried to give six fractions (2F04-2F09) as shown in Table 11.

Table 11 Combination of fractions obtained from column chromatography of fraction 2F02.

Fraction code	Fractions			Volume of solvent (ml)	Total weight (g)
	A	B	C		
2F04	1-10	1-10	1-8	700	0.51
2F05	11-16	11-15	9-14	425	0.38
2F06	17-21	16-19	15-19	325	0.18
2F07	22-25	20-22	20-23	275	0.10
2F08	26-27	23-24	24-25	150	0.01
*2F09	28-67	25-74	26-45	2000	0.04

* 2F09 was eluted with methanol.

cm) using chloroform-methanol-hexane (1:1:3) as an eluting solvent. Fractions (20 ml each) were collected, and then examined by TLC (Si gel) using 10% methanol in chloroform as a developing solvent. Fractions with the same TLC pattern were pooled and dried to give six fractions (2F10-2F15) as shown in Table 12.

Table 12 Combination of fractions obtained from column chromatography of fraction 2F05.

Fractions code	Fractions	Volume of solvent (ml)	Total weight (mg)
2F10	1-17	340	11
2F11	18-25	160	134
2F12	26-29	40	40
2F13	30-34	100	46
2F14	35-39	100	18
2F15	40-64	500	48

Fraction 2F12 (40 mg) was further purified by a Sephadex LH-20 column chromatography (column 1 x 50 cm), using methanol as an eluting solvent to yield two fractions (2F39-2F40) as shown in Table 13. The TLC chromatogram of 2F39 showed only one orange spot under detection as described in Section 4.1. It was assigned as compound TM-2F39 (12 mg, 0.16% based on the ethyl acetate extract) and later identified as syringic acid (Section 3.4, Chapter IV).

Table 13 Combination of fractions obtained from column chromatography of fraction 2F12.

Fractions code	Fractions	Volume of solvent (ml)	Total weight (mg)
2F39	8-9	20	12
2F40	1-7, 10-19	170	26

Fraction 2F07 (97.9 mg) was crystallized from a mixture of chloroform and acetone to give a brown crystal. The TLC chromatogram (Si gel, 10% methanol in chloroform) of this crystal showed yellow color after spraying with anisaldehyde reagent. It was assigned as compound TM-2F07 (10.3 mg, 0.13% based on the ethyl acetate extract) and later identified as *p*-hydroxyl coumaric acid (Section3.5, Chapter IV).

The active fraction 1F (1.52 g) against *Staphylococcus aureus* (15 mm zone of inhibition at concentration 500 μg/6 mm disc) was kept in a desiccator for further investigation.

10. Biological activity

10.1 Antimicrobial activity

The antimicrobial activities of the isolated fractions and pure compounds were examined by an agar disc diffusion method (Lorian, 1980). Evaluation of the antimicrobial activity was performed against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. All tested bacteria were cultivated on tryptic soy agar, TSA (Difco®), whereas the yeast strain was cultivated on Sabouraud's dextrose agar, SDA (Difco®) at 37^0C temperature for 24 hours. The cultures were then washed from agar by normal saline solution (NSS). The cell suspensions were adjusted to 0.5 turbidity, standard of McFarland NO.1, provided approximately $1x10^8$ CFU (colony forming unit/ml). Each 20 ml of molten was separately poured into 9-cm diameter petri dishes and allowed to solidify to form base agar. A loopful of each tested microorganism was spread on the surface of each plate. Test samples were dissolved in suitable solvent and then were applied on sterile paper disc for disc diffusion assay or Silica gel TLC aluminium sheet for autobiography assay. These paper discs or TLC plates were left in each sterile petridish until the solvent were completely dry. Then disc or plate was placed on the surface of the agar plate already spreaded with tested microorganisms and were incubated at 37^0C, 24 hours for bacteria and 48 hours for yeast. The diameter and R_f value of inhibition zones were measured. Fractions showing antimicrobial activity were subsequently selected for further study.

10.2 Anti-herpes simplex virus

Anti-herpes simplex virus was performed by plaque reduction method as follows. Cell-free HSV (30 plaque forming unit, PFU/well) was mixed with the maintenance medium containing various concentrations of sample and incubated at 37^0C for 1 hour. After incubation the mixtures were inocculated into monolayer Vero cell culture on 96-well microtiter plates and incubated at 37^0C for 2 days in CO_2 incubator. The cells were stained with 1% crystal violet in 10% formalin for 1 hour. The plaques were counted under an inverted microscope. Acyclovir was used as a positive control.

10.3 Antimalarial activity

Antimalarial assay was examined according to the method of Trager and Jensen (Arai, 1975) using continuous cell cultures (*in vitro*) of asexual erytrocytic stages of *Plasmodium falciparum* (K1, multidrug resistant strain). Quantitative assessment of antimalarial activity *in vitro* was determined by means of the microculture radioisotope technique. Effective concentration (EC_{50}) represents the concentration, which reduced 50% of parasite growth as indicate by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An EC_{50} value of 0.16 μg/ml (3.1 μM) was observed for the standard sample, chloroquine in the same condition.

10.4 Cytotoxic activity

Cytotoxic activity against breast cancer cell line (BC), human epidermoid carcinoma cell line of nasopharynx (KB) and Vero cell line was performed by sulforhodamine B (SRB) method (Ahmad *et al*., 1999).

CHAPTER IV

RESULTS AND DISCUSSION

1. Sample collection and isolation of actinomycete

The actinomycete strain TRA9851-2 was isolated from a sediment sample collected from a mangrove forest on the West Coast of Trang Province, Thailand, in October 1998. The strain was isolated by spread plate technique (Brock *et al*., 1993).

2. Identification and characterization of actinomycete

The characteristics described in Bergey's Manual of Systematic Bacteriology (Cross, 1994) and the method in International *Streptomyces* Project (ISP) (Shirling, and Gottlieb, 1996) were employed in the identification and characterization of the strain.

2.1 Morphological and cultural characteristics

2.1.1 Morphological characteristics

The morphological characteristics were observed after cultivation on yeast extract-malt extract agar (YMA) plate at room temperature for 14 days. The strain TRA9851-2 was a gram-positive bacterium. Vegetative mycelium of the strain TRA9851-2 growing abundantly on YMA medium was initially white and then changed to dark-gray during the incubation term as shown in Figure 1. Examination by light microscopy revealed that the aerial mycelium was gray, branched and did not show fragmentation into bacillary or coccoid forms. Scanning electron micrograph, as shown in Figure 2, indicated that the spore was cylindrical in shape, 0.2 ~ 0.6 × 0.3 ~ 1 μm in size with a smooth surface.

Figure 1 The colonial appearance of the strain TRA9851-2.

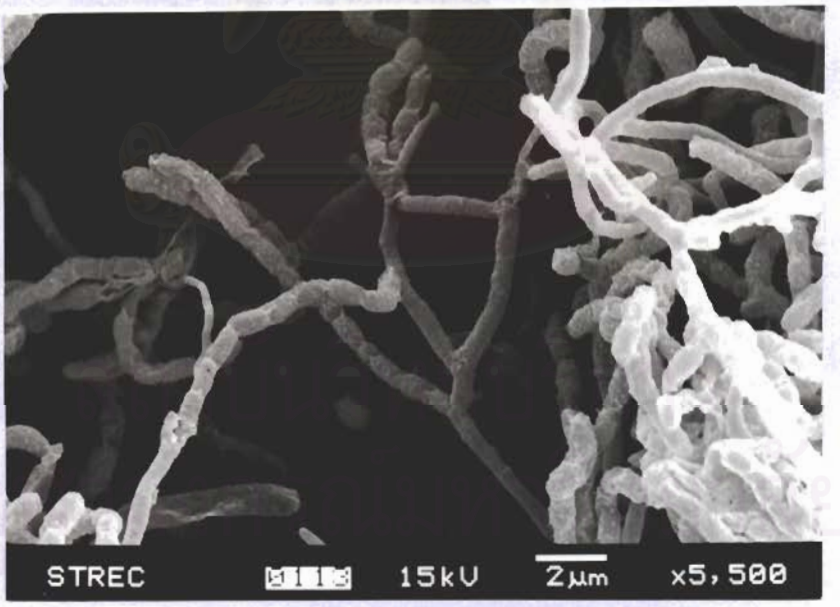


Figure 2 Scanning electron micrograph of spore-bearing substrate mycelium of the strain TRA9851-2.

2.1.2 Cultural characteristics

Cultural characteristics of the strain TRA9851-2 on various media at room temperature for 14 days are shown in Table 14. Melanoid pigment was observed in YMA medium.

Table 14 Cultural characteristics of the strain TRA9851-2[a].

Medium	Growth	Color tone of:	
		Substrate mycelium	Soluble pigment
Inorganic salts-starch agar	Good (sandy, dry)	Pale yellow	None
Glycerine-asparagine agar	Good (sandy, dry)	Pale yellow	None
Tyrosine agar	Good (wrinkled, dry)	Orange yellow	None
Yeast extract-malt extract agar	Good (sandy, dry)	Pale yellow	Reddish brown
Oatmeal agar	Good (sandy, dry)	Light brown	None

[a]Color names used in this table were based on the Jacal Color Card L2200 (Japan Color Research Institute).

2.2 Physiological and biochemical characteristics

Physiological and biochemical characteristics of the strain TRA9851-2 are shown in Table 15 while the utilization of carbon sources are shown in Table 16.

Table 15 Physiological and biochemical characteristics of the strain TRA 9851-2.

Characteristic	Result	Characteristic	Result
Optimum growth temperature	25-30°C	Melanin formation	Positive
Nitrate reduction	Negative	Tyrosinase reaction	Negative
Liquefaction of gelatin	Positive	Cellulolytic activity	Negative
Starch hydrolysis	Positive	NaCl tolerance	≥ 0, < 11 %
Coagulation of milk (37°C)	Positive	pH tolerance	6-11
Peptonization of milk (37°C)	Positive		

Table 16 Utilization of carbon sources of the strain TRA9851-2.

Carbon source	Result	Carbon source	Result
No carbon sources	Positive	L-inositol	Positive
D-glucose	Positive	D-mannitol	Positive
L-arabinose	Positive	D-fructose	Positive
sucrose	Positive	Rhamnose	Positive
D-xylose	Positive	Raffinose	Positive

2.3 Cell wall analyses

The chemical analyses of cell wall diaminopimelic acid (DAP) isomers revealed the presence of the L-isomer of diaminopimelic acid, indicating the cell wall type I.

The actinomycete strain TRA9851-2 was identified as *Streptomyces* sp. based on its morphological and cultural characteristics. Specifically, the organism produced abundant dark-gray aerial mycelia, which transformed into chains of smooth, cylindrical spores, as shown in scanning electron micrograph. The presence of L-DAP in cell wall peptidoglycan supported the identification of this strain in the genus *Streptomyces*. This *Streptomyces* strain also demonstrated the ability of starch and gelatin hydrolysis, milk coagulation, milk peptonization and melanin production.

The *Streptomyces* sp. TRA9851-2 has been preserved by freeze-drying and deposited at the Department of Pharmacognosy and the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

3. Structure elucidation of the isolated compounds

The fermentation broth of the *Streptomyces* sp. TRA9851-2 was extracted with ethyl acetate. The ethyl acetate extract was further separated to give hexane, chloroform and aqueous methanol extracts, respectively. The hexane extract (yellow gum) was further purified by recrystallization from ethyl acetate to give yellow crystals of TM-H01 (380 mg). The chloroform extract was separated using several chromatographic techniques (Section 9, Chapter III) to give five compounds including, TM-H01 (11.1 mg), TM-3F17 (1.7 mg), TM-2F21 (18 mg), TM-2F39 (12 mg), and TM-2F07 (10.3 mg). The percent yielded of these compounds were 4.9%, 0.02%, 0.23%, 0.16%, and 0.13% based on the ethyl acetate extract, respectively. These isolated compounds were characterized by analyses of their spectroscopic data including MS, UV, IR, and NMR spectral data.

3.1 Structure elucidation of compound TM-H01

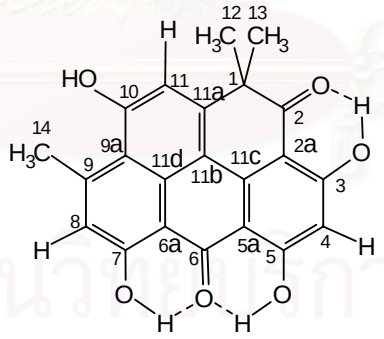
Compound TM-H01 was obtained as yellow crystals. The FAB-MS of TM-H01 (Figure 4) showed a protonated molecular ion $[M+H]^+$ peak at *m/z* 377 corresponding to the formula $C_{22}H_{16}O_6$, which indicated fifteen degrees of unsaturation. It was identified as resistomycin. The ^1H NMR spectrum of TM-H01 (Figure 7) showed eight singlet signals. Six equivalent protons of a geminal dimethyl group appeared as a singlet at δ 1.54, which was assigned to CH_3-12 and CH_3-13. The three-proton singlet at δ 2.87 was readily assigned to the methyl group (CH_3-14) connected to a double bond. The aromatic protons appearing as three singlets at δ 6.30, 6.95, and 7.20 were assigned to H-4, H-8, and H-11, respectively. The exchangeable proton signals at δ 13.95 (5-OH), 14.32 (3-OH), and 14.45 (7-OH) could be assigned to chelated phenolic hydroxyl protons. Although one phenolic hydroxyl proton (10-OH) was not observed, it was recognized based on its molecular weight.

The ^{13}C NMR spectral data (Figure 8) revealed twenty-two carbon signals confirming the molecular formula $C_{22}H_{16}O_6$. The DEPT-135 (Figure 9) and HMQC (Figure 10) techniques were employed for the classification of the type of carbons and their connectivity with nearby protons, respectively. Accordingly, the signals were classified into one methyl carbon at δ 26.1 (CH_3-14) and two equivalent carbons of the geminal dimethyl groups at δ 28.9 (CH_3-12 and CH_3-13); three aromatic methine carbons at δ 100.6 (C-4), 119.7 (C-8), and 109.9 (C-11); one sp^3 quaternary carbon at δ 46.5 (C-1) and thirteen aromatic quaternary carbons. The signals at δ 170.3 (C-3), 169.8 (C-5), 168.0 (C-7), and 162.6 (C-10) appeared in the more downfield region than other aromatic carbons, suggesting the connection to the hydroxyl groups. The downfield carbon signals at δ 204.9 (C-2) and 184.6 (C-6) were assigned to carbonyl carbons. This information suggested that the structure of TM-H01 should be a polyhydroxy quinoid polycyclic structure that was substituted with three methyl (CH_3-12, CH_3-13, and CH_3-14) and four hydroxyl groups (3-OH, 5-OH, 7-OH, and 10-OH). The presence of the polyhydroxyquinoid structure was supported by the UV and IR spectral data. The UV spectrum of compound TM-H01 (Figure 5) showed maximum absorption, λ_{max} (log ϵ), at 266 (4.78), 289 (4.73), 318 (4.55), 366 (4.43), and 454 (4.59), confirming the presence of conjugated aromatic rings. The absorption bands at 3248, 1639 and 1597 cm^{-1} in the IR spectrum of compound TM-H01 (Figure 6) indicated the presence of hydroxyl group and conjugated ketone carbonyl group, respectively.

The HMBC spectra [$^nJ_{HC}$ 8 Hz (Figures 11-14) and $^nJ_{HC}$ 3 Hz (Figures 15-19)] of compound TM-H01 providing the long-range heteronuclear correlations, are the most useful spectra for establishing the chemical structure of compound TM-H01. The correlations of both CH_3-12 and CH_3-13 (δ_H 1.54) to C-1 (δ 46.5), C-11a (δ 152.6), and C-2 (δ 204.9) clearly confirmed the nearby relations of the geminal dimethyl groups and the ketone carbonyl C-2. Furthermore, the correlations of the 3-OH (δ 14.32) to C-2 (δ 204.9), C-2a (δ 102.9), C-3 (δ 170.3), and C-4 (δ 100.6) supported the assignment of the hydroxyl group at C-3. In addition, the correlations of H-4 (δ 6.30) to C-2 (δ 204.9), C-2a (δ 102.9), C-3 (δ 170.3), C-5 (δ 169.8), C-5a (δ 106.0), and C-6 (δ 184.6); 5-OH (δ 13.95) to C-4 (δ 100.6), C-5 (δ 169.8), C-5a (δ 106.0), and C-6 (δ 184.6); 7-OH (δ 14.45) to C-6 (δ 184.6), C-6a (δ 106.7), C-7 (δ 168.0), C-8 (δ 119.7), and C-9 (δ 152.1); H-8 (δ 6.95) to C-6a (δ 106.7), C-7 (δ 168.0),

C-9a (δ 114.4), and C-14 (26.1); and CH_3-14 (δ_H 2.87) to C-8 (δ 119.7), C-9 (δ 152.1), C-9a (δ114.4) established the carbon connectivity of C-3/C-4/C-5/C-5a/C-6/C-6a/C-7/C-8/C-9 (CH_3-14)/C-9a and also defined the assignments of H-4, H-8; 5-OH, 7-OH, and CH_3-14. Finally, the correlations of H-11 (δ 7.20) to C-1 (δ 46.5) and C-10 (δ 162.6) revealed the position of 10-OH and the carbon connectivity of C-9a/C-10/C-11/C-11a/C-11b and C-11a/ C-1. The correlation of H-11 to C-11c was also observed. Selective NOE difference experiment (Figure 20) was used to confirm the close proximity of the CH_3-14 and H-8. Irradiation at CH_3-14 (δ_H 2.87) resulted in NOE enhancement at H-8 (5.8 %, δ_H 7.22), confirming that H-8 was nearby to CH_3-14.

Compound TM-H01 was therefore proposed as 1,1,9-trimethyl-3,5,7,10-tetrahydroxy-1,2-dihydro-2H-Benzo[*cd*]pyrene-2,6(1H)-dione **[50]**. Comparison of its ^1H and ^{13}C spectral data with the reported values (Holf, and Wolf, 1983) confirmed its chemical structure as resistomycin. Resistomycin had been previously isolated from several culture broths of *Streptomyces* strains (see Section 3.2, Chapter II). The ^1H and ^{13}C NMR data of resistomycin and compound TM-H01 with long-range correlations are summarized in Table 17.



[50]

Table 17 ^{1}H and ^{13}C NMR spectral data of TM-H01 and resistomycin, and the ^{1}H-^{13}C long-range correlations in the HMBC spectra.

Posi-tion	Resistomycin[a]		TM-H01[b]			
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	long-range correlated carbons	
					HMBC ($^{n}J_{HC}$ = 8 Hz)	HMBC ($^{n}J_{HC}$ = 3 Hz)
1	49.5	-	46.5	-	-	-
2	204.6	-	204.9	-	-	-
2a	102.5	-	102.9	-	-	-
3	170.2	-	170.3	-	-	-
4	100.1	6.29	100.6	6.30	C-2a, C-3, C-5, C-5a	C-2a, C-3, C-5, C-5a, C-2, C-6
5	169.7	-	169.8	-	-	-
5a	105.7	-	106.0	-	-	-
6	184.5	-	184.6	-	-	-
6a	106.4	-	106.7	-	-	-
7	167.9	-	168.0	-	-	-
8	119.2	6.97	119.7	6.95	C-6a, C-7, C-9a, C-14	C-6a, C-7
9	151.5	-	152.1	-	-	-
9a	114.1	-	114.4	-	-	-
10	162.3	-	162.6	-	-	-
11	109.5	7.27	109.9	7.20	C-1, C-9a, C-10, C-11a, C-11b	C-1, C-9a, C-10, C-11a, C-11b, C-11c
11a	152.2	-	152.6	-	-	-
11b	107.4	-	107.6	-	-	-
11c	139.5	-	139.8	-	-	-
11d	128.5	-	128.7	-	-	-

Table 17 (continued)

Posi-tion	Resistomycin		TM-H01			
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	long-range correlated carbons	
					HMBC ($^nJ_{HC}$ = 8 Hz)	HMBC ($^nJ_{HC}$ = 3 Hz)
12 13	28.4	1.61	28.9	1.54	C-1, C-2, C-11a	C-1, C-2, C-11a
14	25.4	2.96	26.1	2.87	C-8, C-9, C-9a	C-9a
3-OH	-	14.00	-	14.32	C-3, C-4, C-2a	C-3, C-2, C-2a,
5-OH	-	11.20	-	13.95	C-4, C-5, C-5a	C-4, C-5, C-5a, C-6
7-OH	-	14.23	-	14.45	C-6a, C-7, C-8	C-6a, C-7, C-6, C-9
10-OH	-	14.57	-	ND	-	-

[a]Resistomycin from Hoefle and Wolf, 1983

^{13}C NMR spectral data recorded in DMSO-d_6 + $CDCl_3$ (8:2)

^{1}H NMR spectral data of hydroxy protons recorded in $CDCl_3$ + DMSO-d_6 (9:1); aromatic protons recorded in $CDCl_3$ + DMSO-d_6 + CD_3OD (9:1:1).

[b]TM-H01 recorded in DMSO-d_6

ND, Not determined

3.2 Structure elucidation of compound TM-3F17

Compound TM-3F17 was obtained as a yellow powder. The ESI-TOF MS of TM-3F17 (Figure 22) showed a protonated molecular ion $[M+H]^+$ peak at *m/z* 381 corresponding to the tentative molecular formula $C_{20}H_{12}O_8$ (fifteen double-bond equivalents).

The ^{1}H NMR spectrum of compound TM-3F17 (Figure 25), recorded in DMSO-d_6, showed one methyl group attached to a double bond at δ 2.54 (CH_3-12); three singlets in the olefinic region at δ 6.57 (H-5), 7.04 (H-3), and 7.53 (H-1); and a singlet of a hydrogen-bonded phenolic hydroxyl proton at δ 13.19 (6-OH) (D_2O exchangeable). While the signals of H-3 and H-5 were singlets in this spectrum, they appeared as two doublets with J = 2.2 Hz at δ 7.08 and 6.53 in the ^{1}H NMR spectrum recorded in CD_3OD (Figure 27), indicating their *meta* coupling relation.

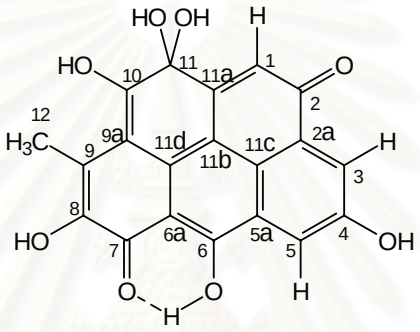
The ^{13}C NMR spectrum (Figure 26, recorded in DMSO-d_6; Figure 28, recorded in CD_3OD) of compound TM-3F17 showed only nineteen signals. The DEPT-135 (Figure 29) and HMQC (Figure 30) spectra of TM-3F17 were used to classify type of carbons and their connectivity with nearby protons. The ^{13}C NMR spectrum, recorded in CD_3OD, established that TM-3F17 possessed a methyl group connecting to a double bond at δ 19.7 (CH_3-12) and three sp^2 methine carbons at δ 113.0 (C-1), 108.2 (C-3), and 109.3(C-5). The downfield quaternary carbons at δ 166.1 (C-6), 165.3 (C-4), 159.0 (C-10), and 141.3 (C-8) were assignable to hydroxylated sp^2 carbons. The carbon signals at δ 139.2 (C-2a), 122.8 (C-5a), 138.0 (C-9), 124.7 (C-9a), 138.2 (C-11a), 123.6 (C-11b), 111.8 (C-11c), and 135.6 (C-11d) were defined as non-oxygenated sp^2 quaternary carbons. The most downfield carbon signals at δ 183.4 (C-2) and 189.4 (C-7) were carbonyl carbons. However, the upfield shifts of these carbonyl carbons implied that TM-3F17 contained two quinoid carbonyls. The presence of the polyhydroxyquinoid structure was suggested by the UV and IR spectral data. The UV spectrum of TM-3F17 (Figure 23) showing maximum absorption, λ_{max} (log ϵ), at 268 (3.36), 286 (3.41), 308 (3.25), and 435 (2.65), confirmed the presence of conjugated aromatic rings. The IR absorption bands at 3804-2260 and 1702-1620 cm^{-1} in the IR spectrum of TM-3F17

(Figure 24) indicated the existence of the hydroxyl group and conjugated ketone carbonyls, respectively.

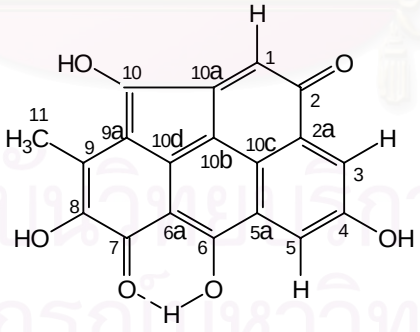
The ^{1}H and ^{13}C NMR assignments of TM-3F17 were achieved through analyses of the HMBC spectra optimized for $^{n}J_{HC}$ = 8 Hz and 4Hz (Figures 31-36 and Figures 37-40, respectively). From the HMBC spectral data recorded in CD_3OD (Figures 31-34), the signals of non-oxygenated quaternary carbons at δ 138.2 (C-11a), 123.6 (C-11b), 139.2 (C-2a) were distinguished by their long-range correlations to H-1 (δ 7.56); and at δ 111.8 (C-11c) showing correlations to H-3 (δ 7.08). The upfield *meta*-coupled aromatic protons at C-3 (δ_H 7.08, δ_C 108.2), and C-5 (δ_H 6.53, δ_C 109.3) were assigned to position *ortho* to the phenolic group C-4 (δ 165.3) which was confirmed by the HMBC correlations between H-3 and H-5 to C-4. Furthermore, the correlations between H-1 and H-3 to the quinoid carbonyl at 183.4 (C-2) were also observed. These spectral evidences suggested the linkages of C-11c/C-11b/C-11a/ C-1/C-2/C-2a/C-3/C-4/C-5. The proton signal of 6-OH appeared in the downfield region at δ 13.19 ppm, suggesting the hydrogen-bonded phenolic hydroxyl proton. Accordingly, the carbonyl carbon at δ 189.4 was assigned to C-7, which chelated the 6-OH. From the HMBC spectral data recorded in DMSO-d_6 ($^{n}J_{HC}$ = 8 Hz, Figures 35 and 36), the 6-OH showed long-range correlations to C-6 (δ 164.3), C-5 (δ 108.3), and C-6a (111.3), suggesting the 6-OH was in peri-position to H-5. The HMBC correlations between the methyl proton, CH_3-12 (s, δ_H 2.62), to quaternary carbons at δ 124.7, 138.0 and a hydroxylated carbon at δ 141.3 revealed the assignments of carbon signals to C-9a, C-9, and C-8, respectively. Furthermore, the HMBC spectrum (optimized for $^{n}J_{HC}$ 4 Hz, recorded in DMSO-d_6) revealed the correlation of H-1 to the hydroxylated sp^2 carbon at δ 158.2 (C-10). The carbon signals at δ 122.8 and δ 134.1, they were assigned to C-5a and C-11d, respectively. Based on the above information, the partial structure of TM-3F17 corresponding to $C_{19}H_{10}O_6$. The remaining fragment of CH_2O_2 should be assigned as a ketal group placing between C-10 and C-11a. Then the structure of TM-3F17 is proposed as 9-methyl-4,6,8,10,11,11-hexahydroxy-2H-benzo[*c*,*d*]pyrene-2,7(1H)-dione **[67]**. However, the carbon signal of C-11 was not observed in the ^{13}C NMR spectrum. Therefore, one possible structure of TM-3F17 is proposed as 9-methyl-4,6,8,10-tetrahydroxy-11-nor-2H-benzo[*c*,*d*]pyrene-2,7(1H)-dione **[68]**. Selective NOE difference experiment (Figure 41, in DMSO-d_6) was used to confirm the position of

CH_3-12; irradiation at CH_3-12 (δ 2.54) resulted in no enhancement at H-1 (δ 7.53), H-3 (δ 7.04), and H-5 (δ 6.57) which confirming that these aromatic protons were not nearby to 12-CH_3.

The assignments of protons and carbons of TM-3F17 for the structure **[67]** are summarized in Table 18.



[67]



[68]

Table 18 ^{1}H and ^{13}C NMR spectral data and ^{1}H-^{13}C long-range correlations in the HMBC spectra of TM-3F17.

Posi-tion	TM-3F17 (in DMSO-d_6)		TM-3F17 (in CD_3OD)			
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	long-range correlated carbons	
					HMBC ($^{n}J_{HC}$ = 8 Hz)	HMBC ($^{n}J_{HC}$ = 4 Hz)
1	112.5	7.53 (s)	113.0	7.56 (s)	C-2,C-10,C-11a, C-11b	C-2,C-2a,C-10, C-11b
2	182.0	-	183.4	-	-	-
2a	138.2	-	139.2	-	-	-
3	107.1	7.04 (s)	108.2	7.08, d (2.2)	C-2, C-4, C-5, C-11c	C-2, C-4, C-11c
4	163.9	-	165.3	-	-	-
5	108.3	6.57 (s)	109.3	6.53, d (2.2)	C-3, C-4, C-6, C-11c	C-3, C-4, C-6, C-11c
5a	121.2	-	122.8	-	-	-
6	164.3	-	166.1	-	-	-
6a	111.3	-	112.8	-	-	-
7	187.7	-	189.4	-	-	-
8	139.5	-	141.3	-	-	-
9	136.2	-	138.0	-	-	-
9a	124.5	-	124.7	-	-	-
10	158.2	-	159.0	-	-	-
11	ND	-	ND	-	-	-
11a	137.1	-	138.2	-	-	-
11b	122.4	-	123.6	-	-	-
11c	110.3	-	111.8	-	-	-
11d	134.1	-	135.6	-	-	-

Table 18 (continued)

Posi-tion	TM-3F17 (in DMSO-d_6)		TM-3F17 (in CD_3OD)			
	δ_C (ppm)	δ_H (ppm), mult. (J in Hz)	δ_C (ppm)	δ_H (ppm), mult. (J in Hz)	long-range correlated carbons	
					HMBC ($^nJ_{HC}$ = 8 Hz)	HMBC ($^nJ_{HC}$ = 4 Hz)
12	19.4	2.54	19.7	2.62	C-8, C-9, C-9a	C-8, C-9, C-9a
4-OH	-	ND	-	ND	-	-
6-OH	-	13.19	-	ND	C-5, C-6a, C-6[a]	-
8-OH	-	ND	-	ND	-	-
10-OH	-	ND	-	ND	-	-
11-OH 11-OH	-	ND	-	ND	-	-

[a]Correlation in HMBC spectrum recorded in DMSO-d_6.

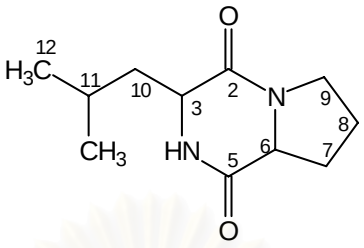
ND, Not determined

3.3 Structure elucidation of compound TM-2F21

Compound TM-2F21 was isolated as a colorless amorphous solid, and identified as *cyclo*-(propyl-leucyl) **[69]**, a natural diketopiperazine (DKP) derivative by comparison its spectral data with the reported data (Jaruchoktaweechai, 1999; Adamczeski, Reed, and Crews, 1995).

Compound TM-2F21 had the molecular formula $C_{11}H_{18}N_2O_2$ as deduced by ESI-TOF MS (Figure 43), showing an abundant pseudomolecular ion peak of $[M+H]^+$ at *m/z* 211, which indicated four degrees of unsaturation. The UV spectrum (Figure 44) exhibited λ_{max} (log ϵ) at 205 (3.08), 260 (2.67), 292 (2.66), and 320 (2.54) nm. The IR spectrum (Figure 45) showed absorption bands for amide NH at 3265, cycloalkane at 2955 and amide carbonyl at 1674 and 1641 cm^{-1}, supporting the presence of an amide functionality in the diketopiperazine TM-2F21.

The ^1H NMR spectrum (Figure 46) of compound TM-2F21 showed a signal of amide NH at δ 5.98 which was assigned to 4-NH. Analyses of the ^{13}C (Figure 47) and DEPT-135 (Figure 48) NMR spectra revealed that compound TM-2F21 contained two methyl carbons at δ 23.4 (CH_3-12) and 21.3 (11-Me); four methylene carbons at δ 28.2 (C-7a, C-7b), δ 22.9 (C-8a, C-8b), δ 45.6 (C-9a, C-9b), and δ 38.7 (C-10a, C-10b); three methine carbons at δ 53.4 (C-3), 59.0 (C-6) and 24.8 (C-11); and two amide carbonyl at δ 165.9 (C-2) and 170.0 (C-5), respectively. The presence of the diketopiperazine ring system in compounds TM-2F21 was evident from the characteristic ^{13}C NMR chemical shifts of two CONH groups (δ_C 165-170) of C-2 and C-5: and ^1H NMR chemical shifts of the two α-methine residues (δ_H 3.9-4.3) of C-3 and C-6. Diketopiperazine containing proline typically exhibited three broad 2H multiplets (δ_H 1.8-3.7), C-7a and 7b, C-8a and 8b, and C-9a and 9b. Comparison these spectral data with reported values conclusively confirmed that compound TM-2F21 is *cyclo*-(propyl-leucyl). Complete assignments of protons and carbons of compound TM-2F21 are in Table 19.



[69]

Table 19 Comparison of ^{1}H and ^{13}C NMR spectral data of TM-2F21 with the published values.

Position	TM-2F21[a]		*cyclo*-(L-propyl-D-luecyl)[b]		*cyclo*-(D-propyl-D-luecyl)[c]	
	δ_C (ppm)	δ_H (ppm),mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm),mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm),mult. (*J* in Hz)
2	165.9	-	166	-	169.6	-
3	53.4	3.99, dd (3.4, 9.6)	53.4	3.95, dd (3.3, 9.3)	56.4	3.92, ddd (4.5, 5.4, 9.9)
4	-	5.98, br s	-	6.18, br s	-	6.68, br s
5	170.0	-	170.0	-	166.4	-
6	59.0	4.09, t (8.2)	59.0	4.11, t (8.1)	58.1	4.07, dd (1.5, 6.9)
7a 7b	28.2	2.10, m 2.32, m	28.3	2.10, m 2.32, m	29.1	2.02, m 2.37, ddd (2.4, 6.4, 8.7)
8a 8b	22.9	1.89, 2H, m	22.8	1.89, 2H, m	23.1	1.96, m 1.88, m
9a 9b	45.6	3.54, 2H, m	45.5	3.52, 2H, m	45.7	3.52, dt (2.7,9.8) 3.62, dt (4.5,9.0)
10a 10b	38.7	1.50, m 1.99, m	38.6	1.50, m 1.99, m	42.6	1.63, ddd (1.8, 6.5, 11.1) 1.75, q (6.3)
11	24.8	1.79, m	24.7	1.79, m	24.5	1.60-1.66, m (under H-10)
11-Me[d]	21.3	0.92, 3H, d (6.5)	21.3	0.90, 3H, d (6.3)	21.4	0.97, d (6.3)
12[d]	23.4	0.97, 3H, d (6.5)	23.4	0.95, 3H, d (6.3)	22.3	0.94, d (6.3)

[a]TM-2F21 recorded in $CDCl_3$

[b]*Cyclo*-(L-propyl-D-leucyl) recorded in $CDCl_3$ (Jaruchoktaweechai, 1999).

[c]*Cyclo*-(D-propyl-D-leucyl) recorded in $CDCl_3$ (Adamczeski, Reed, and Crews, 1995).

[d]Assignments may be interchangeable.

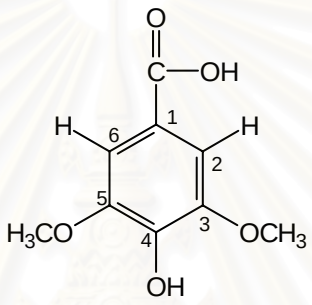
3.4 Structure elucidation of compound TM-2F39

Compound TM-2F39 was obtained as a pale yellow powder. The EIMS of compound TM-2F39 (Figure 50) showed a molecular ion peak of $M^{+\bullet}$ at *m/z* 198, suggesting a molecular formula of $C_9H_{10}O_5$ (five double equivalents). The UV spectrum (Figure 51) showed maximum absorptions, λ_{max} (log ϵ), at 257 (3.75) and 292 (3.77), and 320 (3.68) nm. The IR spectrum (Figure 52) exhibited absorption bands for a hydroxyl group at 3377, and a carbonyl group at 1702 cm^{-1}, indicating the existence of carboxylic functional group. From the UV and IR spectral data, the proposed structure for the compound TM-2F39 was a derivative of benzoic acid.

The ^1H NMR spectrum (Figure 53) of compound TM-2F39 showed signals for aromatic protons (both at δ 7.32, H-2 and H-6) and two methoxy protons (δ 3.87, 3-OCH_3 and 5-OCH_3). Although one signal of phenolic hydroxyl proton was not observed, the assignment was performed based on its molecular weight. Analyses of the ^{13}C (Figure 54) and DEPT-135 (Figure 55) NMR spectra revealed the presence of six signals for nine carbons which were classified into two methoxy carbons at δ 56.3 (3-OCH_3 and 5-OCH_3); six aromatic carbons of two methine carbon at δ 107.6 (C-2, C-6), four quaternary carbon at δ 141.0 (C-1), 121.1 (C-4) and 147.8 (C-3, C-5); and one carboxylic carbonyl carbon at δ 167.0 (1-COOH). From the HMQC spectrum (Figure 56) all protonated carbons could be assigned. The HMBC experiment (Figure 57) revealed correlations of H-2 and H-6 (δ_H 7.32) to C-1, C-2, C-6, C-3, C-5, and 1-COOH, while 3-OCH_3 and 5-OCH_3 (δ_H 3.9) having correlations to C-3 and C-5. The correlations of HMBC spectrum indicated that compound TM-2F39 was 4-hydroxy-3,5-dimethoxy benzoic acid (syringic acid) **[70]**. The ^1H and ^{13}C NMR data of compound TM-2F39 with long-range correlations are summarized in Table 20.

Syringic acid is a natural phenolic acid derivative of benzoic acid which are found mainly in plant species (Torssel, 1997). While syringic acid was reported as the constituent of cane molasses (combination of fermentation broth used in this study) (Takeshi, Tohru, and Yukiko, 1967), data in the literature data suggested that some microorganisms also produced syringic acid in its metabolic pathway. The bacterium, *Arthrobacter* species isolated from

soil converted gallic acid into 3,4-dimethoxy-5-hydroxybenzoic acid, 4,5-dihydroxy-3-methoxyybenzoic acid, 3,4-dimethoxy-5-hydroxybenzoic acid, and 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) (Catroux, and Fournier, 1969). The *Alternaria* sp. isolated from soil, when grown in malt extract medium (combination of fermentation broth used in this study), rapidly metabolized various cinnamic acid and benzoic acid to *p*-hydroxybenzoic acid and protocatechuic acid (Nambudiri, Rao, and Bhat, 1970). Finally, syringic acid is also found in metabolic pathway in fungi (Cain, Bilton, and Darrah, 1968).



[70]

Table 20 ^{1}H and ^{13}C NMR spectral data and ^{1}H-^{13}C long-range correlations in the HMBC spectrum of TM-2F39[a].

Position	δ_C (ppm)	δ_H (ppm),mult.	HMBC ($^{n}J_{HC}$ = 8 Hz)
1	141.0	-	-
2	107.6	7.32, s	C-1, C-2 & C-6, C-3, C-4 1-COOH
3	147.8	-	-
4	121.1	-	-
5	147.8	-	-
6	107.6	7.32, s	C-1, C-2 & C-6, C-4, C-5, 1-COOH
1-COOH	167.0	-	-
3-OCH_3	56.3	3.87, s	C-3
5-OCH_3	56.3	3.87, s	C-5

[a]TM-2F39 recorded in acetone-d_6

3.5 Structure elucidation of compound TM-2F07

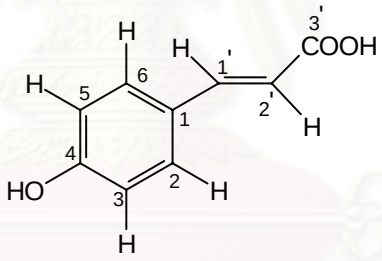
Compound TM-2F07 was obtained as a brown crystal, and its spot on TLC (Si gel) showed yellow color after spraying with anisaldehyde reagent. The ESI-TOF MS (Figure 59) of compound TM-2F07 showed a molecular ion peak of $[M+H]^+$ at *m/z* 164 corresponding to the molecular formula $C_9H_8O_3$. The UV spectrum (Figure 60) showed absorption at λ_{max} (log ϵ) at 223 (4.18), and 309 (4.32) nm, indicating an aromatic system. The IR spectrum (Figure 61) displayed absorption bands for a hydroxyl group at 3382, and a carboxylic group at 1674 cm^{-1}. Aliphatic alkene was also observed at 1629 cm^{-1} (C=C st), while strong absorption band at 805 cm^{-1} (=C-H bending) indicated compound TM-2F07 bearing the disubstituted *trans*-alkene.

The ^1H NMR spectrum (Figure 62) showed four signals including two signals for four aromatic protons (indicating the molecular was symmetrical), two signals for olefenic protons and one phenolic hydroxyl proton at δ 8.72 (4-OH). The aromatic protons at δ 6.83 (H-3, H-5) appeared as a doublet with *ortho*-coupling (J = 8.5 Hz) to the protons at δ 7.41 (H-2, H-6). The olefenic proton at δ 7.56 (H-1′) appeared as a doublet with *trans*-coupling (J = 15.9 Hz) to the proton at δ 6.24 (H-2′). Analyses of the spectral data suggested that compound TM-2F07 had two symmetrical fragments of a disubstituted aromatic ring with a *trans*-alkene residue.

The ^{13}C (Figure 63), DEPT-135 (Figure 64), HMQC (Figure 65) and HMBC (Figure 66) techniques were used to assign protons and carbons in compound TM-2F07 (Table 22). The HMBC revealed correlations of H-2 and H-6 (δ 7.41) to C-4 (δ 158.4), C-2 and C-6 (δ 128.9), and C-1′ (δ 143.9); H-3 and H-5 (δ 6.83) to C-1 (δ 125.1), C-3 & C-5 (δ 114.9), C-4 (δ 158.4), and C-1′ (δ 143.9); H-1′ (δ 7.56) to C-2 & C-6 (δ 128.9), C-2′ (δ 113.8), and C-3′ (δ 166.9); and H-2′ (δ 6.24) to C-1 (δ 125.1), C-1′ (δ 143.9), and C-3′ (δ 166.9). These HMBC correlations conclusively supported the connection of aromatic ring and *trans*-alkene residue. This proposed structure was additionally support by UV spectrum, which showed the molar absorbtivity (ϵ) more than 10,000 (logϵ > 4), indicating the conjugated aromatic system linked with the *trans*-alkene residue. The signal of C-1′ (δ 143.9)

was more downfield than C-2′ (δ 113.8), due to the *beta*-position to carboxylic group, C-3′ (δ 166.9). The signals at δ 6.83 (H-3 and H-5) were *ortho*-position to phenolic hydroxyl proton (4-OH), thus having upfield shift signals. Analyses of the spectral data indicated that compound TM-2F07 was 4-hydroxy cinnamic acid (*p*-coumaric acid) **[71]**. The ^{1}H and ^{13}C NMR data of compound TM-2F07 with long-range correlations are summarized in Table 21.

p-Coumaric acid, is a natural phenolic acid derivative of cinnamic acid, which is found mainly in plant species (Torssel, 1997). While *p*-coumaric acid was reported as a constituent of cane molasses (combination of fermentation broth used in this study) (Takeshi, Tohru, and Yukiko, 1967), data in the literature data suggested that some microorganisms also produced *p*-coumaric acid in its metabolic pathway such as an *Alternaria* species (Nambudiri, Rao, and Bhat, 1970).



[71]

Table 21 ^{1}H and ^{13}C NMR spectral data and ^{1}H-^{13}C long-range correlations in the HMBC spectrum of TM-2F07[a].

Position	δ_C (ppm)	δ_H (ppm),mult.	HMBC ($^{n}J_{HC}$ = 8 Hz)
1	125.1	-	-
2	128.9	7.41, d (8.5)	C-4, C-6, C-1′
3	114.9	6.83, d (8.5)	C-1, C-4, C-5, C-1′
4	158.4	-	-
5	114.9	6.83, d (8.5)	C-1, C-3, C-4, C-1′
6	128.9	7.41, d (8.5)	C-2, C-4, C-1′
1′	143.9	7.56, d (15.9)	C-2 & C-6, C-2′, C-3′
2′	113.8	6.24, d (15.9)	C-1, C-1′, C-3′
3′	166.9	-	-
4-OH	-	8.72, br s	-

[a]TM-2F07 recorded in acetone-d_6 + $CDCl_3$.

4. Biological activity

Results from the preliminary bioactivity screening of the ethyl acetate extract from the fermentation broth of *Streptomyces* sp. TRA9851-2 showed antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231 and potent cytotoxic activity against Vero cell line (LD_{50} < 0.1 μg/ml). The ethyl acetate extract was therefore further separated to yield hexane, chloroform, and aqueous methanol extracts, respectively. Each extract was subsequently investigated for the biologically active metabolites using bioassay-guided isolation. Finally, five chemical compounds were obtained including TM-H01 (resistomycin), TM-3F17 (a benzo[*c,d*]pyrene derivative), TM-2F21 [*cyclo*-(propyl-leucyl)], TM-2F07 (syringic acid), and TM-2F39 (*p*-coumaric acid). The autobiographic technique (on TLC, Si gel) was then employed for examining the inhibition zones of the fractions to determine the active spots, using 10% methanol in chloroform as a developing solvent. The TLC exhibited a clear zone only on *B. subtilis* ATCC 6633 agar plate at R_f 0.5-0.7, which was related toTM-H01 (resistomycin). The mycelial extract of the strain also showed the activity against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923, similarly to the ethyl acetate extract. Both extracts showed the main spot of resistomycin on TLC chromatogram (Si gel, 10% methanol in chloroform).

Based on the bioassay-guided isolation, its active pure compound was isolated from either hexane or chloroform extract. However, the active pure compound against *C. albicans* ATCC 10231 from fraction ML-2F21 could not be obtained because of limited amount of the sample.

The biological activities of crude extracts and pure compounds including antimicrobial, cytotoxicity, antimalarial, and anti herpes-simplex virus type I (anti-HSV I) and type II (anti-HSV II) activity are shown in Tables 22 and 23.

Table 22 Antimicrobial activity of crude extracts and pure compounds obtained from *Streptomyces* sp. TRA9851-2.

Fraction or compound	Concentration (μg/6 mm disc)	Inhibition zone (mm)			
		B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
EtOAc extract	1,000	20	10	-	23
Hexane extract	1,000	20	-	-	-
•TM-H01	100	15	+ -	-	-
	50	9	+ -	-	-
	10	+ -	+ -	-	-
$CHCl_3$ extract	1,000	-	10	-	20
1F	500	-	15	ND	+ -
2F	500	-	+ -	ND	23
•TM-2F21	100	ND	-	ND	-
•ML-2F21	500	ND	ND	ND	20
•TM-2F07	100	-	-	-	-
•TM-2F39	100	-	-	-	-
3F	500	-	+ -	ND	-
•TM-3F17	←		ND		→
4F	500	-	-	-	-
aq. MeOH extract	1,000	-	+-	-	-
Mycelial extract	1,000	28	10	-	

ND, Not determined

+ -, 6-8 mm inhibition zone

-, Inactive

Table 23 Cytotoxic, antimalarial, and anti-HSV I and II activities of the isolated compounds from *Streptomyces* sp. TRA 9851-2.

Compound	Cytotoxic activity ED_{50} (μg/ml)		Antimalarial activity EC_{50} (μg/ml)	Anti HSV activity	
	KB	BC		HSV I	HSV II
TM-H01	0.52	0.65	0.77	toxic at 20 μg/ml	toxic at 20 μg/ml
TM-2F21	-	-	-	ND	ND
TM-2F07	-	-	-	ND	ND
TM-2F39	-	-	-	ND	ND
TM-3F17	←		ND		→

ND, Not determined

-, Inactive

Resistomycin (TM-H01) has previously been reported as anti HIV-I protease inhibitor and antibacterial agent against gram-positive and mycobacterium, however, the present work reported, for the first time, the antimalarial and cytotoxic activities of resistomycin.

Malaria is one of the most devastating diseases causing enormous health problems in Thailand. The emergence of parasite resistant to almost all the available antimalarials has made it necessary for new antimalarials effective against the drug-resistant parasite to be developed (World Health Organization [WHO], Malaria unit, 1993). Secondary metabolites from *Streptomyces* strains have been known in rich sources of biologically active substances but so far the discovering of new antimalarials candidate from *Streptomyces* strains have not been of interest for scientists. In our screening, we found that resistomycin was active against *Plasmodium falciparum* (K_1, multidrug resistant strain). The compound was incorporated

is stronger than that of chloroquine (EC_{50} = 0.16 μg/ml, 3.1 μM), a well-known antimalarial agent. Although, resistomycin possessed potent antimalarial activity against *P. falciparum*, this activity was due to cytotoxicity.

Resistomycin was found to be toxic against HSV I and II Vero cell lines, and also exhibited strong cytotoxicity against KB and BC cancer cell lines with ED_{50} = 0.52 and 0.65 μg/ml, respectively. This cytotoxic activity is the first report from resistomycin. The other pure compounds including *cyclo*-(propyl-leucyl) (TM-2F21), syringic acid (TM-2F39), and *p*-coumaric acid (TM-2F07) did not shown any biological activities, while compound TM-3F17 (a benzo[*c,d*]pyrene derivative) was not determined for its biological activities due to the limited amount of sample. Previous reports of biological activities of a series of *cyclo*-(propyl-leucyl) diketopiperazine are in Table 24. Syringic acid and *p*-coumaric acid were reported its activities such as antibacterial (Fernandez, Garcia, and Saenz, 1996), antioxidant (Hosny, and Rosazza, 1999) and anti-inflammatory activity (Fernandez, Saenz, and Garcia, 1998). Syringic acid was also reported for radical-scavenging activity (Hirota *et al*., 2000). Coumaric acid was reported for antimicrobial activity (Hegazi, Hady, and Allah, 2000) and cytoprotective agent (Vieira *et al*, 2000). Recently, *p*-coumaric acid was characterized as the principal tyrosinase inhibitor from the fresh leaves of *Panax ginseng* (Araliaceae) (Lim, Shiguro, and Kubo, 1999).

Table 24 Sources and biological activities of *cyclo*-(propyl-leucyl) diketopiperazine.

Compound	Source	Activity	Reference
cyclo-(L-Pro-D-Leu)	cyanobacterium (symbiosis with sponge *Calyx* cf. *podatypa*)	ND	Adamczeski *et al*., 1995
	marine *Bacillus* sp. Sc026	anti-HSV I and II activities	Jaruchokta-weechai, 1999
cyclo-(L-Pro-L-Leu)	bacterium *Streptomyces griseus* and fungus *Aspergillus fumigatus* and algae *Scenedesmus* sp.	antimicrobial activity against *Staphyloccus aureus*	Johnson, Jackson, and Eble, 1951
	sponge, *Tendania ignis*	ND	Luedemann *et al*., 1961
	sponge-associated bacterium, *Pseudomanas aeruginosa*	inactive against cytotoxicity and antimicrobial assay	Jayatilake *et al*., 1996
cyclo-(D-Pro-D-Leu)	cyanobacterium (symbiosis with sponge *Calyx* cf. *podatypa*)	ND	Adamczeski *et al*., 1995

ND, No data

CHAPTER V

CONCLUSION

A sample of mangrove sediments from Trang Province was investigated for actinomycete strains with biologically active metabolites. We found an interesting strain TRA9851-2 that was identified as *Streptomyces* strain based on morphological, cultural, physiological, biochemical and cell wall component studies. The preliminary antimicrobial screening on the ethyl acetate extracts of cell free culture filtrate of the strain TRA9851-2 showed antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. A large-scale fermentation broth of the *Streptomyces* sp. TRA9851-2 was extracted with ethyl acetate and the ethyl acetate extract was partitioned to give hexane, chloroform, and aqueous methanol extracts. Directed by antibacterial activity, resistomycin (TM-H01) was obtained from the hexane extract, while a benzo[*c,d*]pyrene derivative (TM-3F17) was isolated from the chloroform extract. Further isolation of the chloroform extract yielded *cyclo*-(propyl-leucyl) (TM-2F21), syringic acid (TM-2F39), and *p*-coumaric acid (TM-2F07).

Resistomycin exhibited antimicrobial activity against the gram-positive bacterium, *Bacillus subtilis*, with 15 mm zone of inhibition at concentration 100 μg/disc, and showed toxicity against herpes-simplex viruses type I and type II at concentration of 20 μg/ml. Resistomycin also possessed potent cytotoxicity against oral human epidermoid and breast cancer cell lines with the ED_{50} = 0.52 and 0.65 μg/ml, respectively. The antimalarial activity of resistomycin against *Plasmodium falciparum* (K_1, multidrug resistant strain) at EC_{50} = 0.77 μg/ml (2.0 μM) was stronger than that of chloroquine (EC_{50} = 3.1 μM), a well-known antimalarial agent. However, the antimalarial activity of resistomycin might be due to its cytotoxicity. The present work is the first report for antimalarial and cytotoxic activities of resistomycin.

Compound TM-3F17 was a derivative of benzo[*c,d*]pyrene class, which its two possible chemical structures were closely related to resistomycin. The two possible structures, i.e. 9-methyl-4,6,8,10,11,11-hexahydroxy-2H-benzo[*c,d*]pyrene-2,7(1H)-dione and 9-methyl-4,6,8,10-tetrahydroxy-11-nor-2H-benzo[*c,d*]pyrene-2,7(1H)-dione were proposed. However, it could not be tested for any biological activities due to the limited amount of sample. The isolated *cyclo*-(propyl-leucyl), syringic acid, and *p*-coumaric acid were tested for antimicrobial, antimalarial, and cytotoxic activities, but none of them showed activities.

REFERENCES

ประเสริฐ โพธิปักษ์. 2540. ป่าชายเลน. กรุงเทพมหานคร: โครงการปลูกป่าเฉลิมพระเกียรติ. (อัดสำเนา)

Adamczeski, M., Reed, A. R., and Crews, P. 1995. New and known diketopiperazines from the Caribbean sponge, *Calyx* cf. *podatypa*. J. Nat. Prod. 58(2): 201-208.

Ahmad, S., Malik, A., Afza, N., and Yasmin, R. 1999. A new withanolide glycoside from *Physalis peruviana*. J. Nat. Prod. 62: 493-494.

Aksornkoae, S. 1993. Ecology and management of mangroves. Thailand: Dyna Print.

Alvarez, M. E., *et al*. 1994. Isolation and structure elucidation of two new calpain inhibitors from *Streptomyces griseus*. J. Antibiot. 47(11): 1195-1201.

Arai, T. 1975. Culture media for actinomycetes. Japan: The Society for Actinomycetes. (Mimeographed)

Bae, M. A. *et al*. 1996. Heterocyclic comm. 2:315. cited in John Faulkner. Marine natural products. Nat. Prod. Rep. 15: 113-157. 1998.

Betina, V. 1983. The chemistry and biology of antibiotics. Amsterdam: Elsevier. cited in Fenical Willium and Paul R. Jensen. Marine microorganisms: A new Biomedical resource. In D. H. Attaway, and O. R. Zaborsky (ed), Pharmaceutical and bioactive natural products, pp. 419-457. New York: Plenum Press. 1993.

Biabani, M. A. F., Baake, M., Lovisetto, B., Laatsch, H., Helmke, E., and Weyland, H. 1998. J. Antibiot. 51: 333 cited in John Faulkner. Marine natural products. Nat. Prod. Rep. 17: 7-55. 2000.

Biabani, M. A. F., Laatsch, H., Helmke, E., and Weyland, H. 1997. J. Antibiot. 50: 874. cited in John Faulkner. Marine natural products. Nat. Prod. Rep. 16: 155-198. 1999.

Bradler, G., and Eckardt, K. 1972. Characterization of a *Streptomyces* strain producing resistomycin. Isolation and properties of the new antibiotic. Allg. Mikrobiol. 12(7): 535-545. (cited in CA 78: 1206)

Brock, T. D., Madigan, M. T., Martinko, J. M., and Parker, J. 1993. Biology of microorganisms (7^{th} ed.). New Jersey: Prentice Hall.

Brockmann, H., Meyer, Ernst., Schrempp, K., Reiners, F., and Reschke, T. 1969. Resistomycin. IV. Constitution of resistomycin. Chem. Ber. 102(4): 1224-1246. (cited in CA 71: 3176p)

Brockmann, H., and Schmidt-Kastner, G. 1951. Naturwissenschaften. 20: 479. cited in William Hosenbrook. The structure of resistomycin. J. Org. Chem. 32(9): 2024-2025. 1967.

Brown, R., Kelley, C., and Wiberley, S. E. 1965. The production of 3-benzylidene-6-isobutylidene-2, 5-dioxopiperazine, 3, 6-dibenzlidene-2, 5-dioxopiperazine. 3-benzl-6-benzylidene-2, 5-dioxopiperazine, and 3, 6-dibenzyl-2, 5-dioxopiperazine by a variant of *Streptomyces noursei*. Experientia. 30: 1965.

Bryans, J., *et al*. 1996. Inhibition of plasminogen activator inhibitor-1 activity by two diketopiperazines, XR330 and XR334 produced by *Streptomyces* sp. J. Antibiot. 49(10): 1014-1021.

Burton, J. D., and Liss, P. S. 1976. Estuarine chemistry. London: Academic Press.

Cain, R. B., Bilton, R. F., and Darrah, J. A. 1968. Metabolism of aromatic acids by microorganisms. Metabolic pathways in the fungi. Biochem. J. 108(5): 797-828. (cited in CA 69: 65308a)

Catroux, G., and Fournier, J. C. 1969. Methylation of the phenolic hydroxyl groups of gallic acid by two bacterial strains isolated from soil. C. R . Acad. Sci. 269(24): 2460-2463. (cited in CA 72: 52053a)

Cheng, X. C., Jensen, P. R., and Fenical, W. 1999. Arenaric acid, a new pentacyclic polyether produced by a marine bacterium (Actinomycetales). J. Nat. Prod. 62: 605-607.

Cheng, X. C., Jensen, P. R., and Fenical, W. 1999. Luisols A and B, new aromatic tetraols produced by an estuarine marine bacterium of the genus *Streptomyces* (Actinomycetales). J. Nat. Prod. 62: 608-610.

Chugasova, V. A., Preobrazhenskaya, T. P., and Blinov, N. 1975. Biologically active pigments of *Actinomyces atrovirens*. Antibiotiki (Moscow). 20(5): 415-423. (cited in CA 83-75159)

Cross, T. 1994. Growth and examination of actinomycetes-some guidelines. in J. B. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. William (eds.). Bergey's manual of determinative bacteriology. (9th ed.), pp. 605-623. Baltimore: The William and Wilkins.

De man, J. M., De man, L., and Gupta, S. 1986. Texture and microstructure of soybean curd (tofu) as affected by different coagulants. Food Micro. Struct. 5: 83-89.

Eckardt, K., Bradler, G., and Fritzsche, H. 1970. Resistoflavine, a new antibiotic from an actinomycete. Z. Chem. 10(6): 221.

Eckardt, K., Bradler, G., and Tresselt, D. 1972. Resistoflavine, a new antibiotic of the resistomycin family. in Heigzlar M. (ed.), Int. Congr. Chemother. Meeting Date 7 (Vol. 1). Issue Pt. 2: 1205-1207. (cited in CA 79: 28661)

Eckardt, K., Fritzsche, H., and Tresselt, D. 1970. Structure of the antibiotics, resistoflavine. Tetrahedron. 26: 5875-5883.

Fenical, W., and Jensen, P. R. 1993. Marine microorganisms: A new Biomedical resource. In D. H. Attaway, and O. R. Zaborsky (ed), Pharmaceutical and bioactive natural products, pp. 419-457. New York: Plenum Press.

Fernandez, M. A., Garcia, M. D., and Saenz, M. T. 1996. Antibacterial activity of the phenolic acids fractions of *Scrophularia frutescens* and *Scrophularia sambucifolia*. J. Ethnopharmacol. 53(1): 11-14.

Fernandez, M. A., Saenz, M. T., and Garcia, M. D. 1998. Anti-inflammatory acitivity in rats and mice of phenolic acids isolated from *Scrophularia frutescens*. J. Pharmcy and Pharmacology. 50(10): 1183-1186.

Ford, P. W., Gadepalli, M., and Davidson, B. S. 1998. Halawanones A-D, new polycyclic quinones from a marine-derived Streptomycete. J. Nat. Prod. 61: 1232-1236.

Francisco de Mello, J., Delle monache, F., Marini-Betollo, G. B., Lyra, F. A., Fernandes de Albuquerque, M. M., and Goncalves, L. 1971. Istamycin, a new antibiotic

produced by *Streptomyces erythrogriseus*. II. Identification with resistomycin. Rev. Inst. Antibiotic. 11(2): 3-6. (cited in CA79: 122869)

Fujii, N., Tanaka, F., Yamashita, Y., Ashizawa, T., Chiba, S., and Nakano, H. 1997. UCE6, a new antitumor antibiotics with topoisomerase I-mediated DNA cleavage activity produced by actinomycetes: Producing organisms, fermentation, isolation and biological activity. J. Antibiot. 50(6): 490-495.

Gansser, D., Pollak, F. C., and Berger, R. G. 1995. 111904w A sesquiterpene alcohol from *Streptomyces citreus* CBS 109.60. J. Nat. Prod. 58(11): 1790-1793.

Goldberg, D. M., Hoffman, B., Yang, J., and Soleas, G. J. 1999. Phenolic constituents, furans, and total antioxidant status of distilled spirits. J. Agri. Food. Chem. 47 (10): 3978-3985.

Goodfellow, M. 1989. The actinomycetes I : Supragenic classification of actinomycetes. In S. T. Williams, M. E. Sharpe, and J. G. Holt (eds), Bergey's manual of systematic bacteriology (vol. 4), pp. 1333-1339. Baltimore: the Williams and Wilkins.

Goodfellow, M., and William, S. T. 1983. Ecology of actinomycetes. Ann. Rev. Microbiol. 37: 189-216.

Goodfellow, M., Williams, S. T., and Mordarski, M. 1988. Actinomycetes in biotechnology. Great Britain: T. J. (Padstow) Press.

Hegazi, A. G., Hady, E. I., and Allah, F. A. M. 2000. Chemical composition and antimicrobial activity of European propolis. J. Biosciences. 55(1-2): 70-75.

Hirota, A., Taki, S., Kawaii, S., Yano, M., and Abe, N. 2000. 1,1-diphenyl-2-picrylhydrazyl radical-scavenging compounds from soybean miso and antiproliferative activity of isoflavone from soybean miso toward the cancer cell lines. Bioscience biotechnology and biochemistry. 64(5): 1038-1040.

Hofle, G., and Wolf, H. 1983. Isolation, carbon-13 NMR spectra, and biosynthesis of resistomycin and resistoflavine from *Streptomyces griseoflavus* B 71 (Actinomycetales). Liebigs Ann. Chem. 5: 835-843.

Hosny, M., and Rosazza, J. P. N. 1999. Novel isoflavone, cinnamic acid, and triterpenoid glycosides in soybean molasses. J. Nat. Prod. 62: 853-858.

Imamura, N., Nishijima, M., Adachi, K., and Sano, H. 1993. J. Antibiot. 46: 241. cited in John Faulkner. Marine natural products. Nat. Prod. Rep. 16: 155-198. 1999.

Jaruchoktaweechai, J. 1999. Bioactive constituents from marine *Bacillus* strains. Ph. D. Thesis, Department of Pharmaceutical Chemistry and Natural Products, Faculty of Pharmaceutical Sciences, Chulongkorn University.

Jayatilake, G. S., Thornton, M. P., Leonard, A. C., Grimwade, J. E., and Baker, B. J. 1996. Metabolites from an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa.* J. Nat. Prod. 59: 293-296.

Jensen, P. R., Dwight, R., and Fenical, W. 1991. Distribution of actinomycetes in near-shore tropical marine sediments. Appl. Microbiol. 57(4): 1102-1108.

Jiang, Z., Jensen, P. R., and Fenical, W. 1997. Actinoflavoside, a novel flavonoid-like glycoside produced by a marine bacterium of the genus *Streptomyces*. Tetrahedron Lett. 38(29): 5065-5068.

Johnson, J. L., Jackson, W. G., and Eble, T. E. 1951. Isolation of L-leucyl-L-proline anhydride from microbiological fermentation. J. Am. Chem. Soc. 73: 2947-2949.

Kakinuma, K., and Rinenart, K. L. 1974. Tryptophan-dehydrobutyrine diketopiperazine, a metabolite of *Streptomyces spectabilis*. J. Antibiot. 27(10): 733-737.

Komagata, K., and Suzuki, K. 1987. Lipid and cell-wall analysis in bacterial systematics. In Methods in microbiology (vol. 19). London: Academic Press.

Lim, J. Y., Ishiguro, K., and Kubo, I. 1999. Tyrosinase inhibitory *p*-coumaric acid from ginseng leaves. Phytother. Res. 13(5): 371-375.

Lindel, T., Jensen, P. R., and Fenical, W. 1996. Lagunapyrones A-C: Cytotoxic acetogenins of a new skeletal class from a marine sediment bacterium. Tetrahedron Lett. 37(9): 1327-1330.

Lorian, V. 1980. Antibiotics in laboratory medicine. Baltimore: William & Wilkins Company.

Luedemann, G., *et al*. 1961. Microbiological transformation of steroids. IX. The transformation of Ruichstein's compound S by *Scennedesmus* sp. diketopiperazine metabolites from *Scenedesmus* sp. J. Am. Chem. Soc. 26: 1428-1430.

Miao, S., Anstee, M. R., LaMarco, K., Matthew, J., Huang, L. H. T., and Brasscur, M. M. 1997. Inhibition of bacterial RNA polymerase. Peptide metabolites from the cultures of *Streptomyces* sp. J. Nat. Prod. 60: 858-861.

Nambudiri, A. M. D., Rao, P. V. S., and Bhat, J. V. 1970. Metabolism of aromatic compounds by an *Alternaria* species. Phytochemistry. 9(4): 987-993. (cited in CA 73: 32599a)

Nolan, R. D., and Cross, T. 1988. Isolation and screening of actinomycetes. In M. Goodfellow, S. T. Williums, and M. Mordaski (ed.), Actinomycetes in biotechnology, pp. 1-32. Great Britain: T. J. Press (Padstow).

Ogura, H., Furuhata, K., and Furuhara, K. 1975. Studies on lactams, VI. Stereochemistry of L-propyl-L-valine anhydride. Chem. Pharm. Bull. 23(11): 2474-2477.

Okami, Y., Hotta, K., Yoshida, M., Ikeda, D., Kondo, S., and Umezawa, S. 1979. J. Antibiot. 32: 964. cited in William Fenical. Chemical studies of marine bacteria: Developing a new resource. Chem. Rev. 93: 1673-1683. 1993.

Okami, Y., Okazaki. T., Kitaehara, T., Umezawa, H. 1976. J. Antibiot. 29: 1019. cited in William Fenical. Chemical studies of marine bacteria: Developing a new resource. Chem. Rev. 93: 1673-1683. 1993.

Pathirana, C., Jensen, P. R., Dwight, R., and Fenical, W. 1992. Marinone and debromomarinone: Antibiotic sesquiterpenoid naphthoquinones of a new structure class from a marine bacterium. Tetrahedron Lett. 33 (50): 7663-7666.

Pathirana, C., Jensen, P. R., Dwight, R., and Fenical, W. 1992. Rare phenazine L-quinovose esters from a marine actinomycete. J. Org. Chem. 57: 740-742.

Puseker, K., Laatsch, H., Helmke, E., and Weyland, H. 1997. J. Antibiot. 50: 479. cited in John Faulkner. Marine natural products. Nat. Prod. Rep. 16: 155-198. 1999.

Qian-Cutrone, J., Kolb, J. M., McBrien, K., Huang, S., Gustavson, D., Lowe, S. E., and Manly, S. P. 1998. Quanolirones I and II, two new human cytomegalovirus

protease inhibitors produced by *Streptomyces* sp. WC76535. J. Nat. Prod. 61: 1379-1982.

Renner, M. K., *et al*. 1999. Cyclomarins A-C, new antiinflammatory cyclic peptides produced by a marine bacterium (*Streptomyces* sp.). J. Am. Chem. Soc. 121: 11273-11276.

Richard, J., and Lewis, S. 1997. Condensed chemical dictionary (13 th). USA: John Wiley & Sons.

Ritzau, M., Heinze, S., Friedrich, F., Martin, H., and Grafe, U. 1998. New macrodiolides antibiotics, 11-o-monomethyl- and 11-11′-o-dimethylelaiophylins, from *Streptomyces* sp. HKI-0113 and HKI-0114. J. Nat. Prod. 61: 1337-1339.

Robin, D. J., and Sefton, M. A. 1984. 1-N-methyl-(6E)-(2-methylpropylidene)-(3Z)-3-(phenylmethylene)-2, 5-piperazinedione, a metabolite from *Streptomyces albus*. Phytochemistry. 23(1): 200-201.

Roggo, *et al*., 1994. 3-Alkanoyl-5-hydroxymethyl tetronic acid homologs and resistomycin: New inhibitors of HIV-1 protease. I. Fermentation, isolation and biological activity. J. Antibot. 47(2): 136-142. (cited in CA 120: 265434)

Rosenbrook, W. 1967. The structure of resistomycin. J. Org. Chem. Sep: 32(9): 2924-2925.

Ryu, G., Choi, W., Hwang, S., Yeo, W., Lee, C., and Kim, S. 1999. Tetrin C, a new glycosylated polyene macrolide antibiotic produced by *Streptomyces* sp. GK9244. J. Nat. Prod. 62: 917-919.

Sakuda, S., Ono, M., Furihata, K., Nakayama, J., Suzuki, A., and Isogai, A. 1996. Aflastatin A, a novel inhibitor of aflatoxin production of *Aspergillus parasiticus*, from *Streptomyces*. J. Am. Chem. Soc. 118: 7855-7856.

Schumacher, R. W., Davidson, B. S., Montenegro, D. A., and Bernan, V. S. 1995. γ-Indomycinone, a new pluramycin metabolite from a deep-sea derived actinomycete. J. Nat. Prod. 58(4): 613-617.

Shirling, B. E., and Gottlieb, D. 1966. Method for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313-340.

Shu, Y., *et al*. 1997. Bripiodionen, a new inhibitor of human cytomegalovirus protease from *Streptomyces* sp. WC76599. J. Nat. Prod. 60: 529-532.

Singh, M. P., Petersen, P. J., Jacobus, N. V., Maiese, W. M., Greenstein, M., and Steinberg, D. A. 1994. Mechanistic studies and biological activity of bioxalomycin α_2, a novel antibiotic produced by *Streptomyces viridodiastaticus* subsp. Litoralis LL-31F508. Antimicrob. Agents Chemother. 38(8): 1808-1812.

Sitachitta, N., Gadepalli, M., and Davidson, B. S. 1996. New α-pyrone containing metabolites from a marine-derived actinomycete. Tetrahedron Lett. 52(24): 8073-8080.

Suwanarit, P. 1998. Biodiversity of microorganisms in mangrove forest (Machinereadable data file). Poonpilai Suwanarit: Department of Microbiology, Faculty of Sciences, Kasetsart University, Bangkok (Producer and Distributor).

Takahashi, A., Kurasawa, S., Ikeda, D., Okami, Y., and Takeuchi, T. 1990. Altemicidin, a new acaricidal and antitumor substance I. Taxonomic, fermentation, isolation and physico-chemical and biological properties. Antibiot. XLII (11): 1556-1561.

Takahashi, C., *et al*. 1994. Halichomycin, a new class of potent cytotoxic macrolide produced by an actinomycete from a marine fish. Tetrahedron Lett. 35(28): 5013-5014.

Takeshi, H., Tohru, Y., and Yukiko, S. 1967. Constituents of cane molassses. II. Separation and identification of phenolic compounds. Agr. Biol. Chem. (Tokyo) 31(3): 324-329. (cited in CA 67: 12778f)

Tapiolas, D. M., Roman, M., and Fenical, W. 1991. Octalactins A and B: Cytotoxic eight-membered ring lactones from marine bacterium, *Streptomyces* sp. J. Am. Chem. Soc. 113: 4682-4683.

Torssell, K. B. G. 1997. The shikimic acid pathway. In Natural product chemistry: A mechanistic, biosynthetic and ecological approach. p. 147. Kristianstads Boktryckeri AB: Sweden.

Trischman, J. A., Tapiolas, D. M., Jensen, P. R., Dwight, R., and Fenical, W. 1994. Salinamide A and B: Anti-inflammatory depsipeptides from a marine Streptomycete. J. Am. Chem. Soc. 116: 757-758.

Umezawa, K., Nakazawa, K., Ikeda, Y., Naganawa, H., and Kondo, S. 1999. Polyoxypeptins A and B produced by *Streptomyces*: Apoptosis-inducing cyclic depsipeptides containing the novel amino acid (2R, 3R)-3-hydroxy-3-methylproline. J. Org. Chem. 64: 3034-3038.

Vieira, *et al*. 1998. Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. British J. Pharm. 123(3): 565-573.

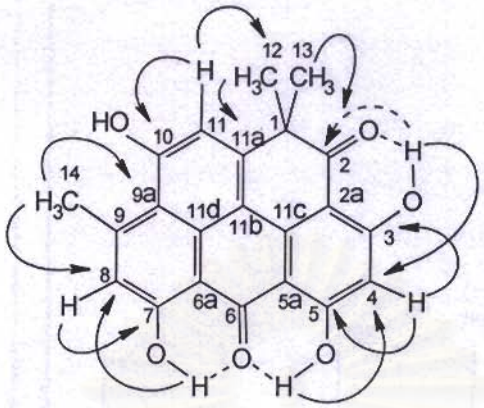
Vinogradova, K. A., Kirillova, N. P., and Sokolova, Z. G. 1991. Structure of heliomycin produced by *Streptomyces heliomycin* and antibiotic 11-98 produced by *Streptomyces olivocinereus*. Antibiot. Khimioter. 36(3): 28-29. (cited in CA 114: 225333.

Willium, S. T., and Cross, T. 1971. Actinomycetes: Slide and coverslip methods. In Methods in microbiology (vol. 4). London: Academic Press.

World Health Organization [WHO], malaria unit. 1993. Global malaria control. Bull. WHO. 71: 281-284. cited in Sumalee Kamchonwongpaisan, and Yongyyuth Yuthavong. Antimalarial screening. Workshop on Investigation of Bioactive Substances in Plants and Microorganisms, pp. 15-16. Bangkok: National Science and Technology Development Agency [NSTDA]. 1998.

Yoshiko, H., *et al*. 1996. The structure of diphenazithionin, novel antioxidant from *Streptomyces griseus* ISP 5236. Tetrahedron Lett. 37(51): 9227-9228.

APPENDIX A



⟶ $^{n}J_{HC}$ = 8 Hz; ----▸ $^{n}J_{HC}$ = 3 Hz

Figure 3 The long-range heteronuclear correlations observed in the HMBC spectra of resistomycin (TM-H01).

Spectral data of compound TM-H01

FAB-MS : *m/z* (% relative intensity); Figure 4

377 ([M+H]$^{+}$, 24), 307 (22), 289 (18), 246 (27), 185 (18), 154 (100), 137 (98)

UV : λ_{max} nm (log ϵ), in methanol; Figure 5

454 (4.59), 366 (4.43), 318 (4.55), 289 (4.73), 266 (4.78)

IR : ν_{max} cm^{-1}, KBr disc; Figure 6

3248 (br), 1629, 1597, 1570, 1253, 848, 571

^{1}H NMR : δ_{ppm}, 300 MHz, in DMSO-d_6; see Figure 7 and Table 17

^{13}C NMR : δ_{ppm}, 75 MHz, in DMSO-d_6; see Figure 8 and Table 17

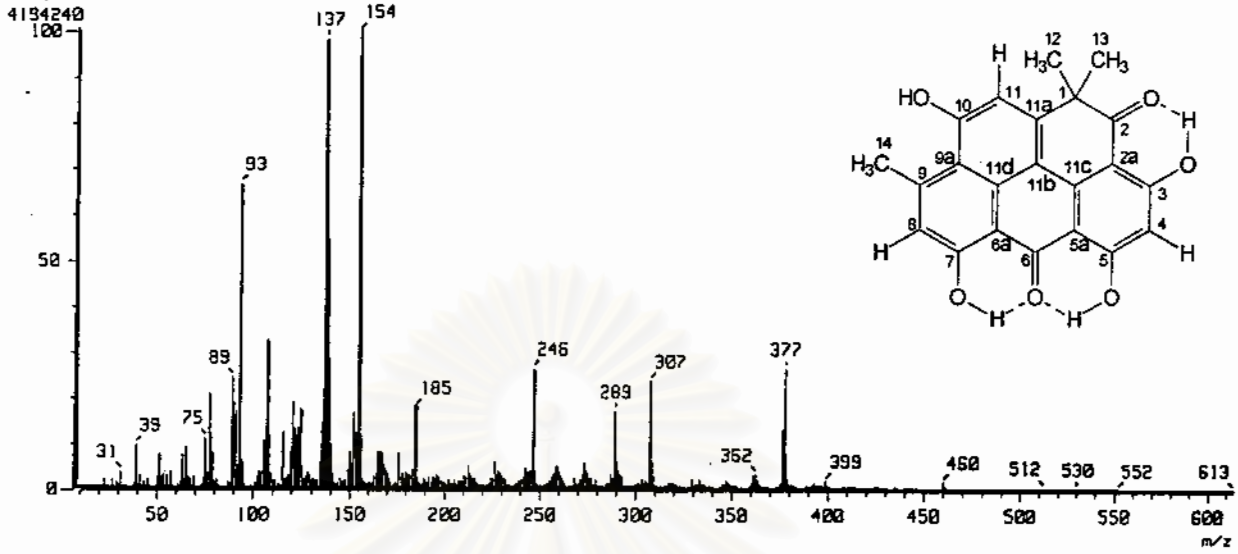


Figure 4 The FAB-MS spectrum of compound TM-H01.

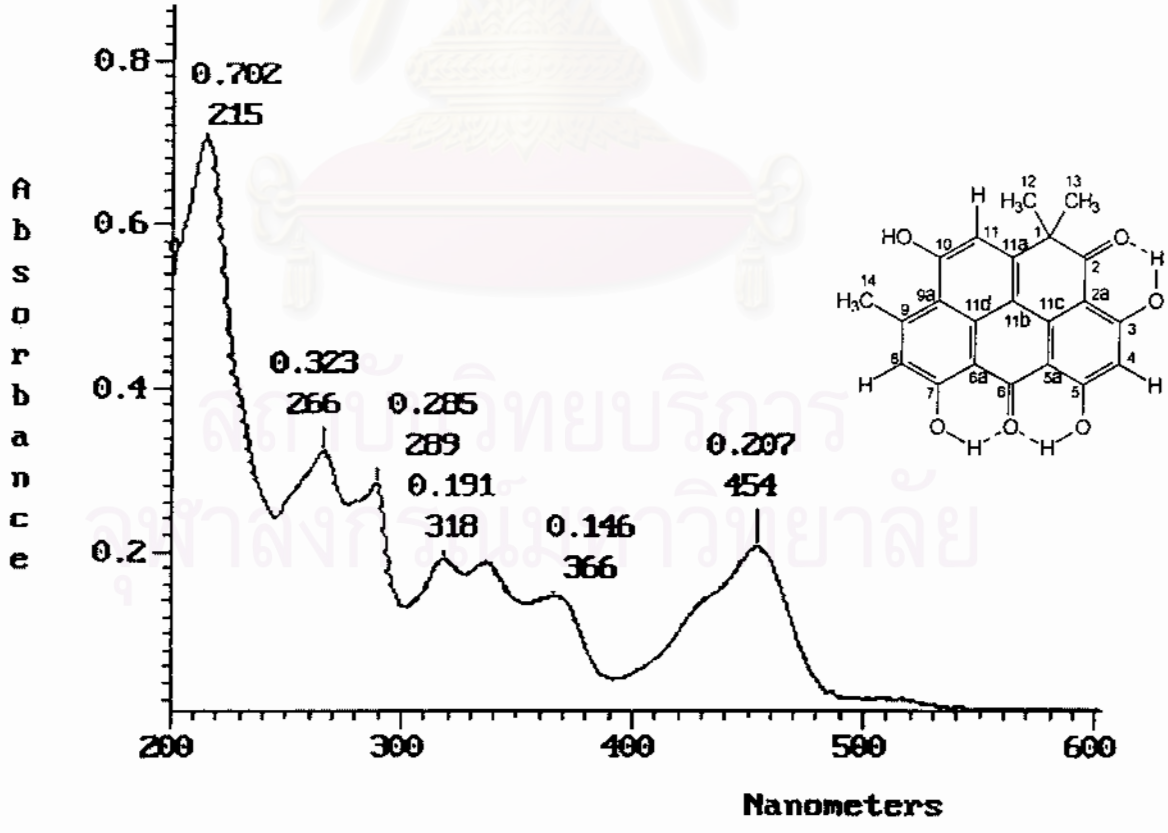


Figure 5 The UV spectrum of compound TM-H01 (in MeOH).

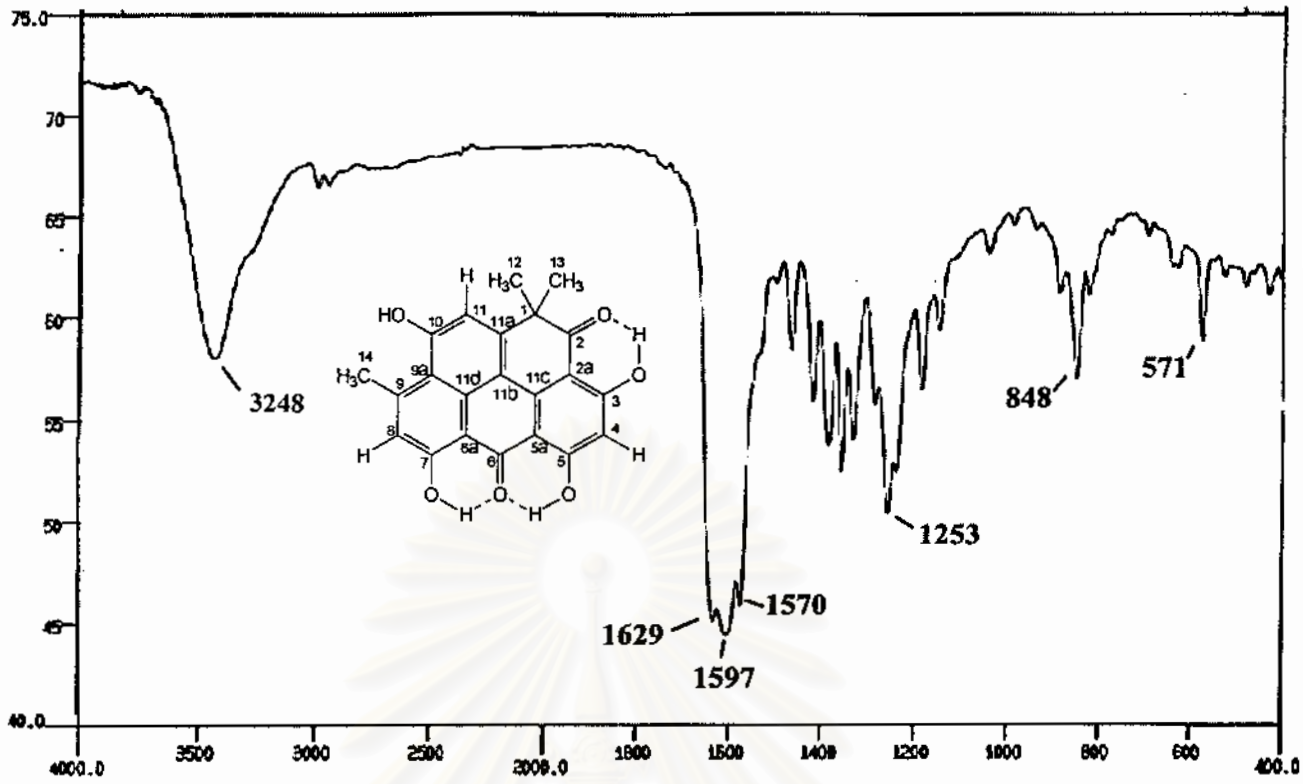


Figure 6 The IR spectrum of compound TM-H01 (KBr disc).

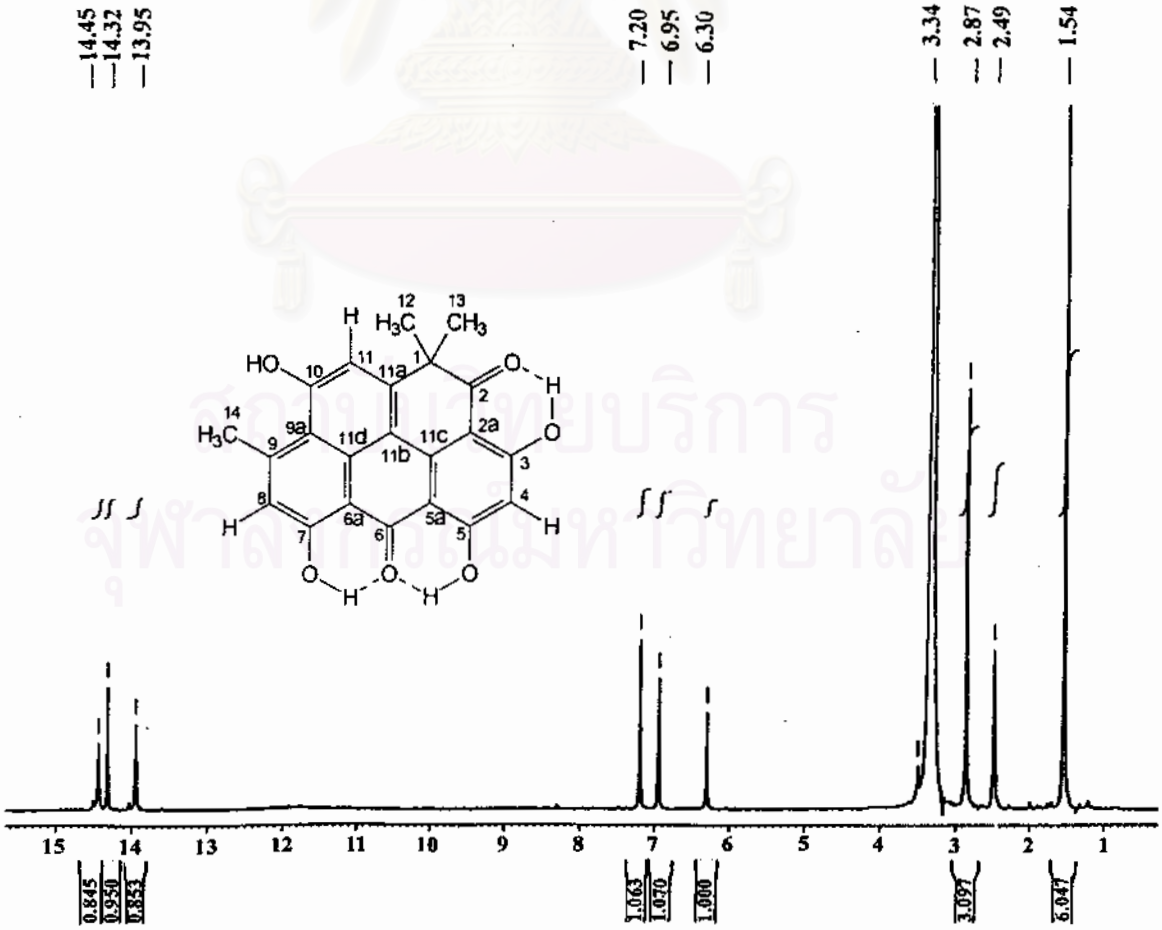


Figure 7 The 300 MHz ^{1}H NMR spectrum of compound TM-H01 (in DMSO-d_6).

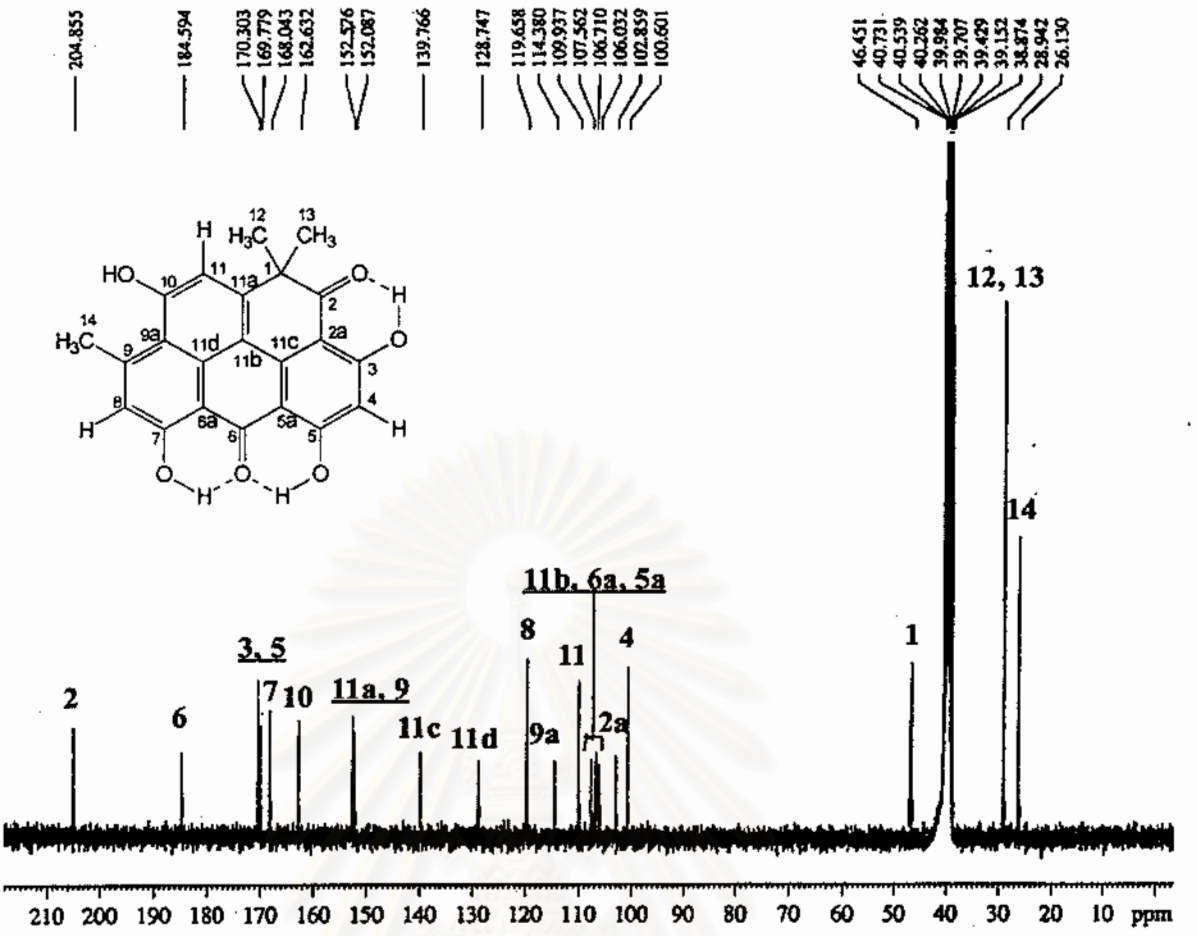


Figure 8 The 75 MHz ^{13}C NMR spectrum of compound TM-H01 (in DMSO-d_6).

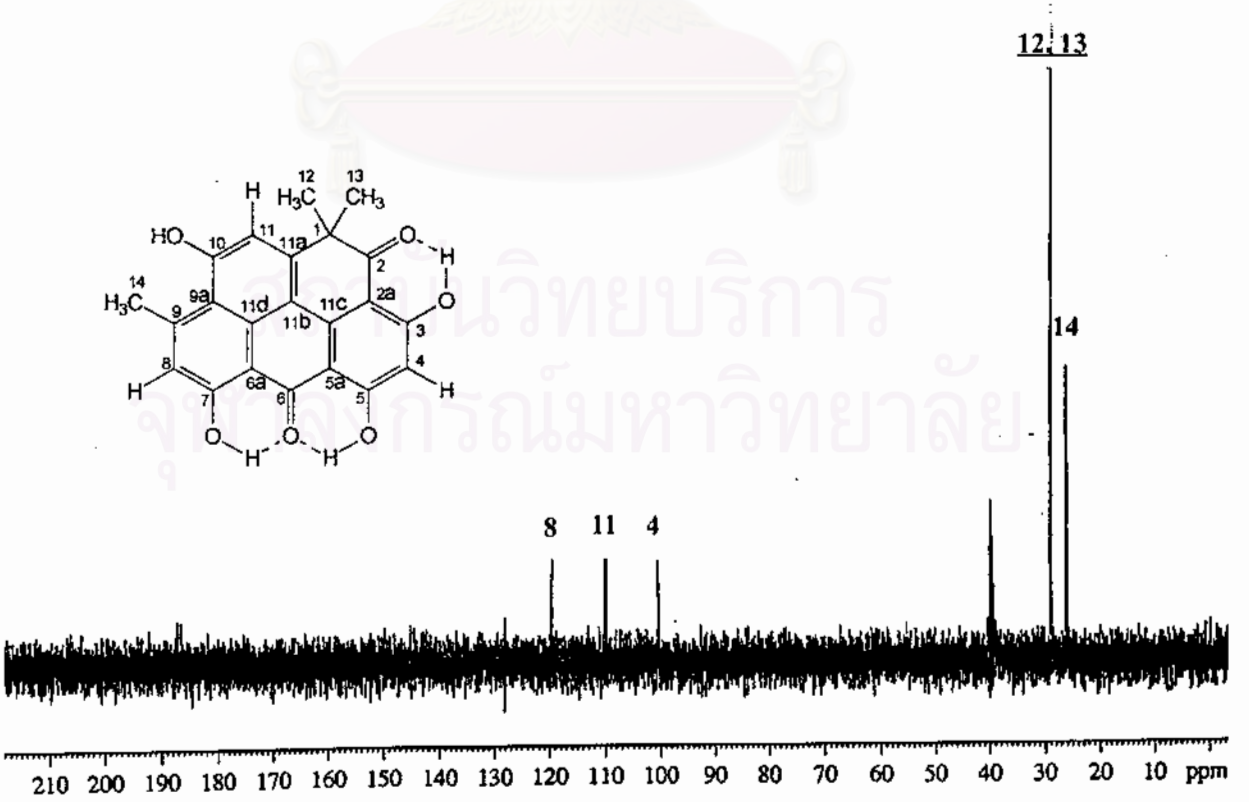


Figure 9 The 75 MHz DEPT-135 spectrum of compound TM-H01 (in DMSO-d_6).

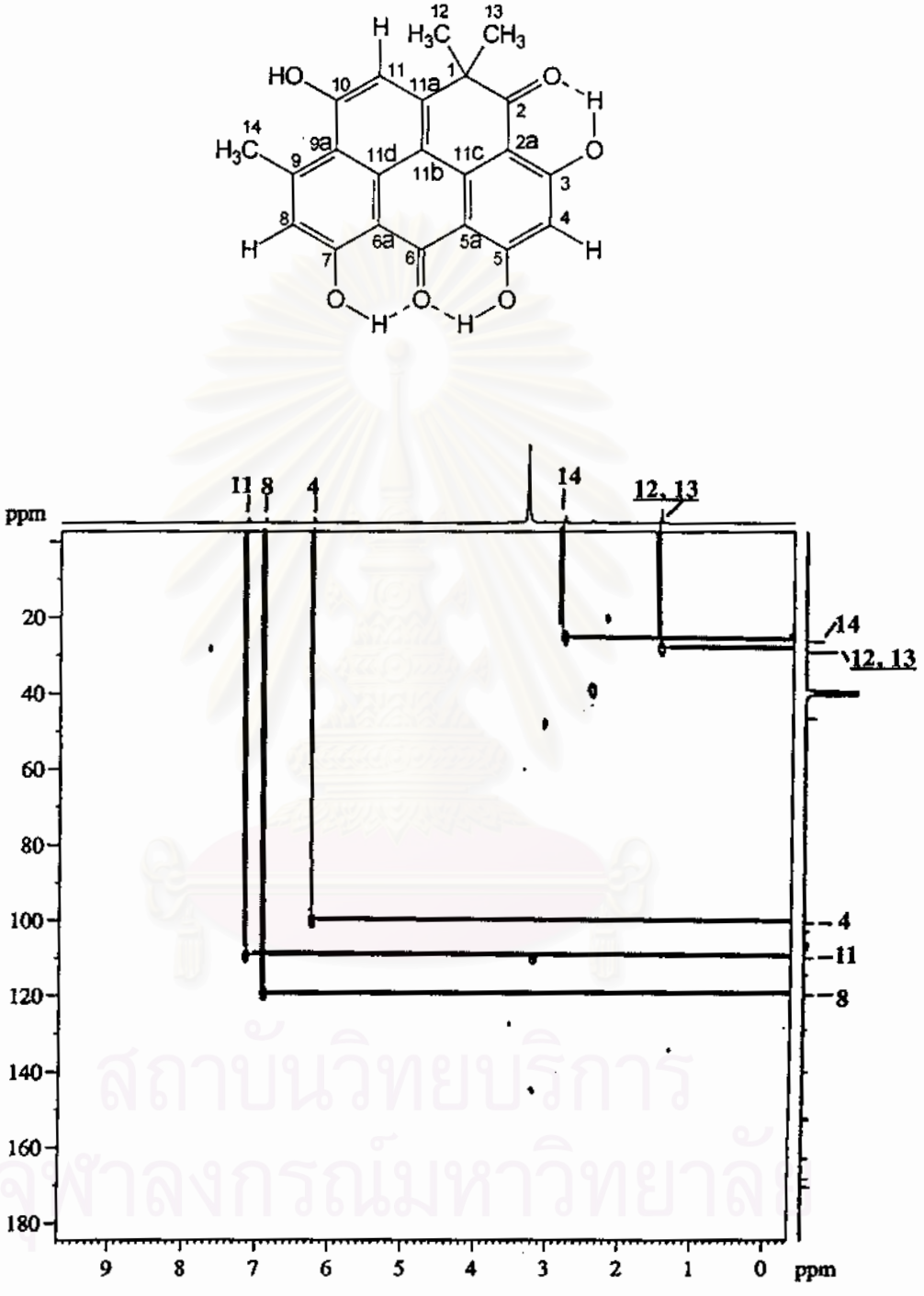


Figure 10 The 300 MHz HMQC spectrum of compound TM-H01 (in DMSO-d_6).

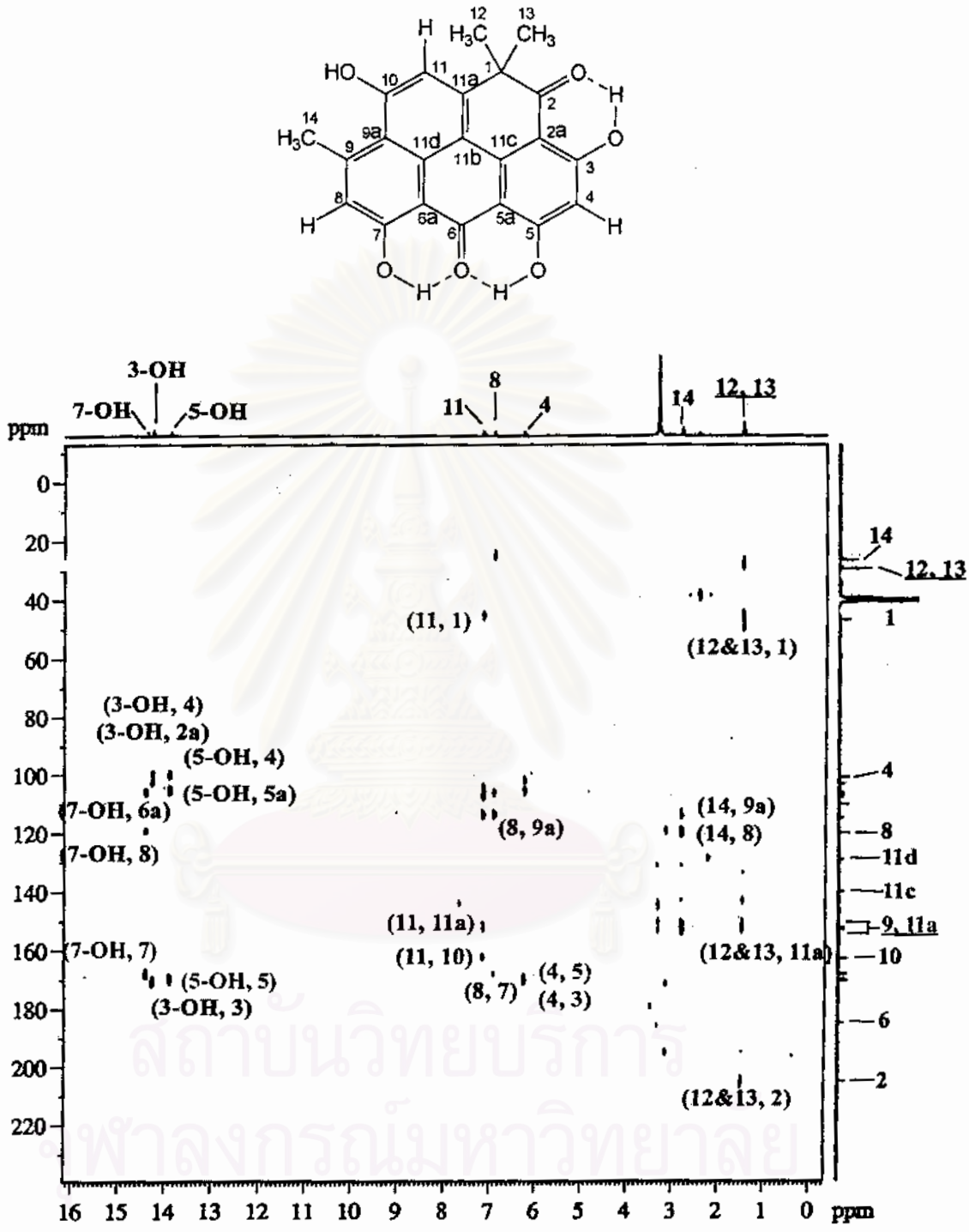


Figure 11 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-H01 (in DMSO-d_6).

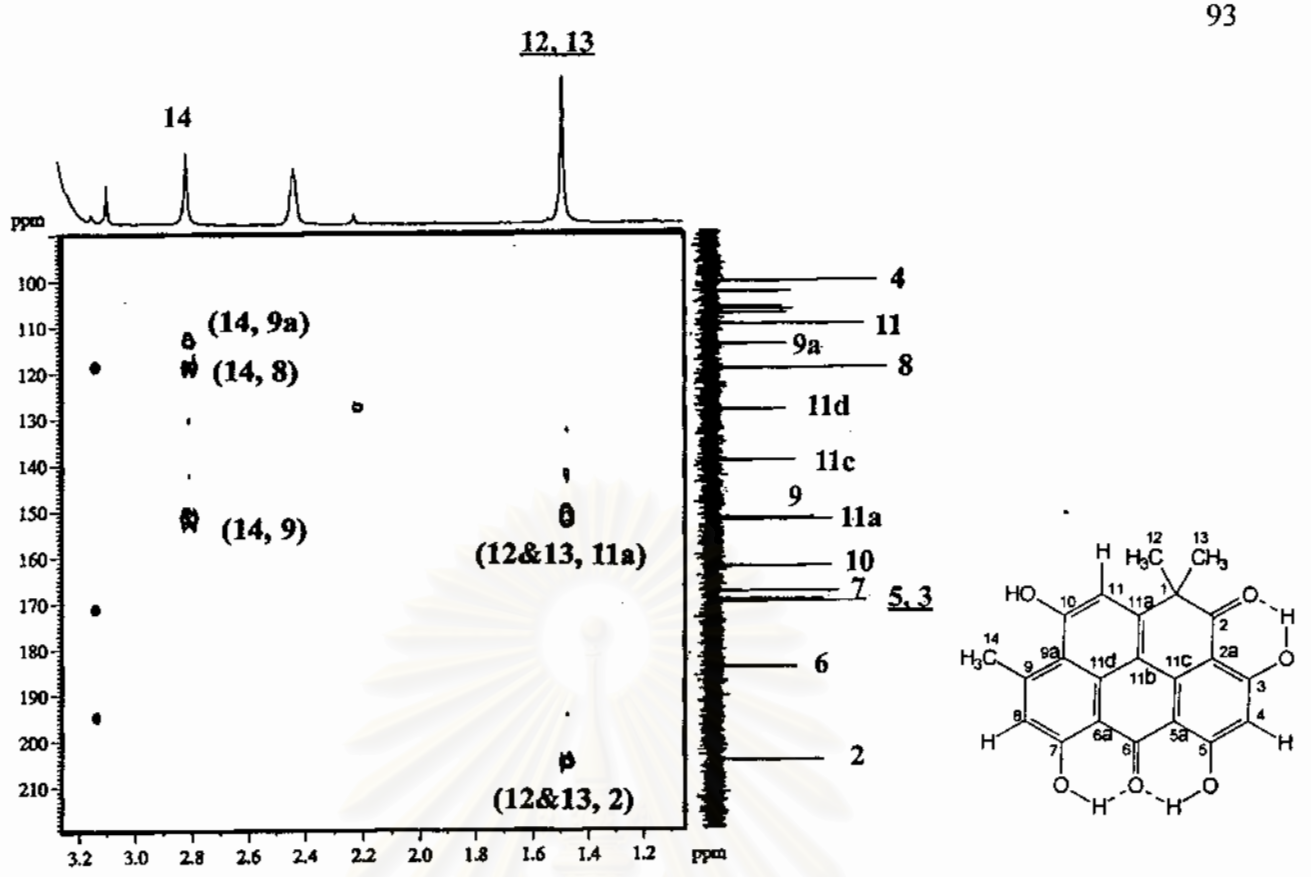


Figure 12 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 1.1-3.3 ppm; δ_C 90-220 ppm].

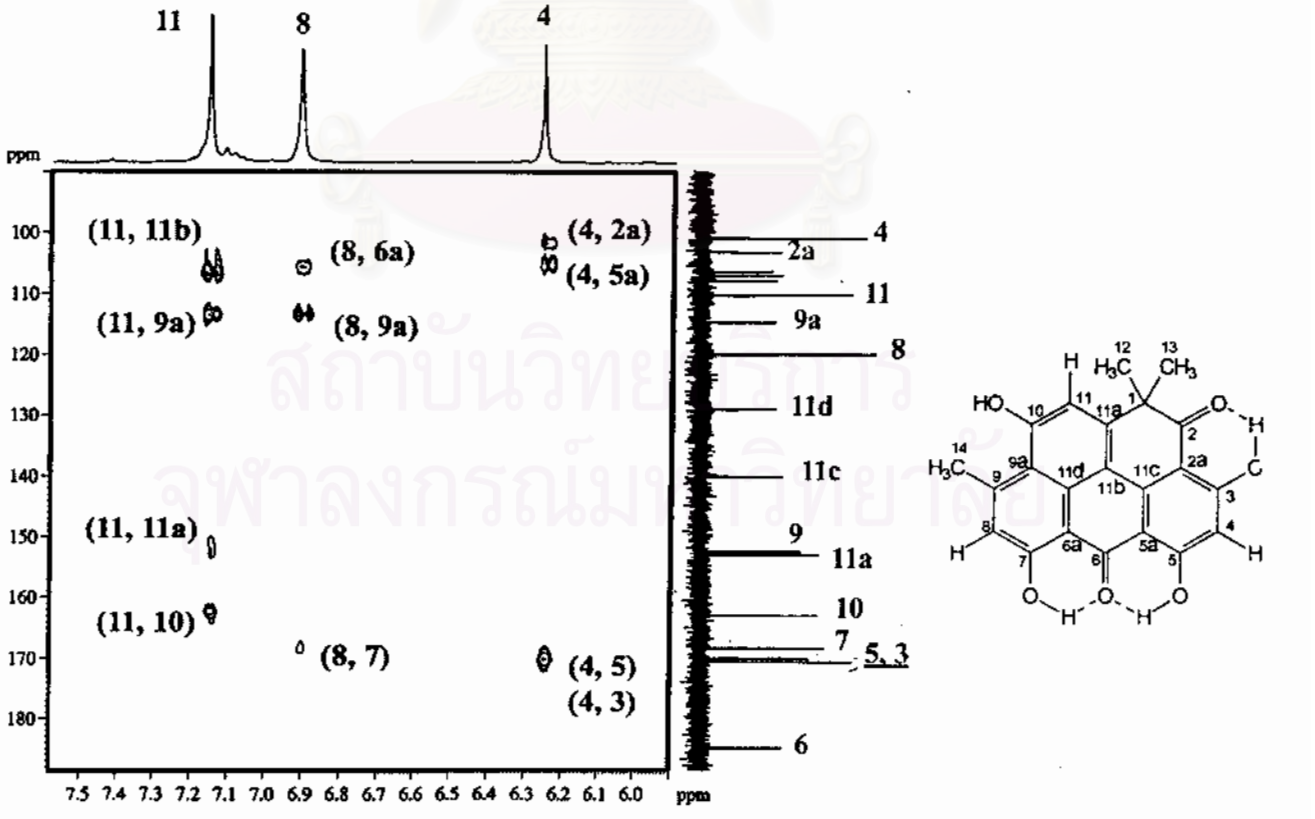


Figure 13 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 5.9-7.6 ppm; δ_C 90-190 ppm].

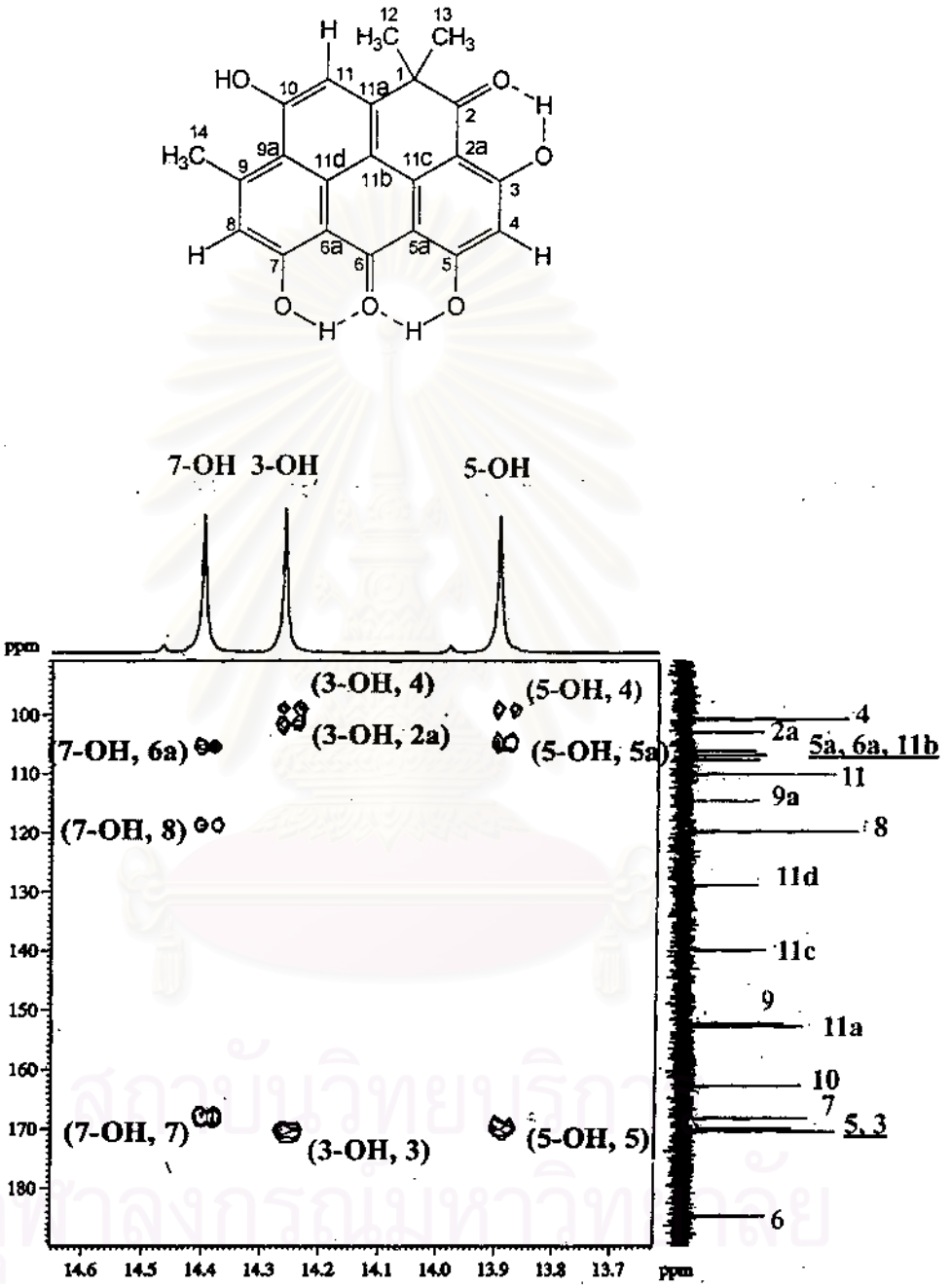


Figure 14 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 13.6-14.7 ppm; δ_C 90-190 ppm].

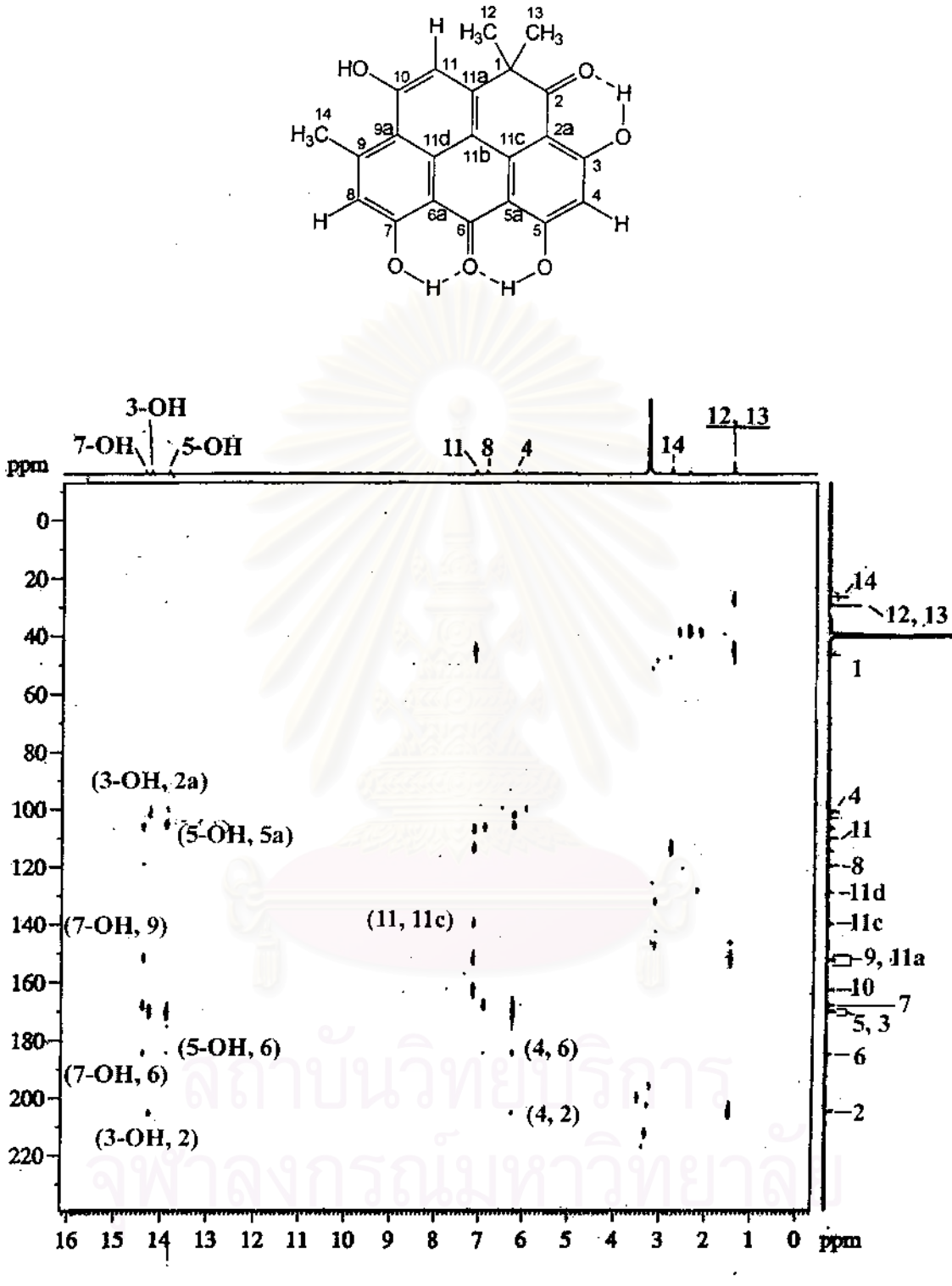


Figure 15 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 3 Hz) of compound TM-H01 (in DMSO-d_6).

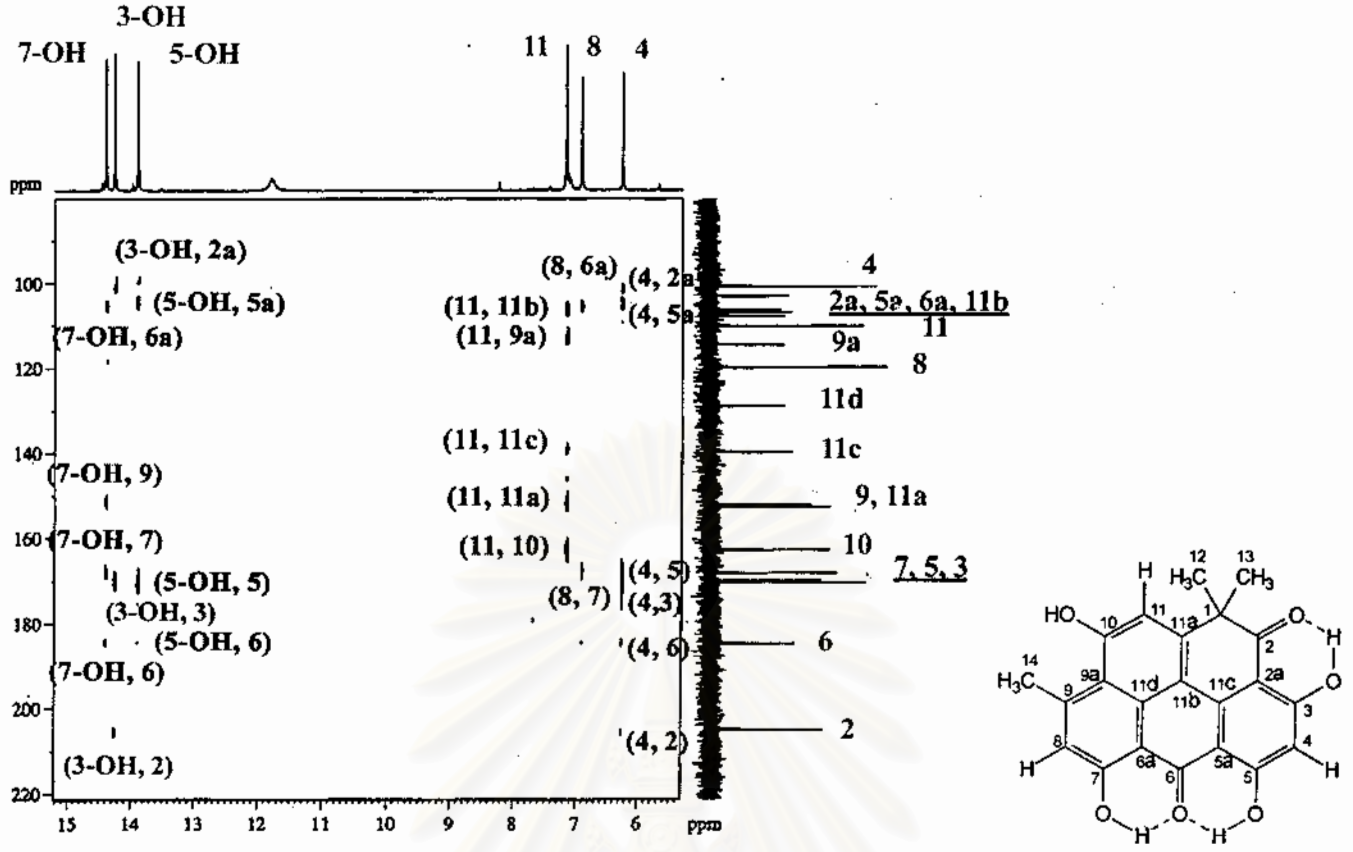


Figure 16 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 3 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 5-15 ppm; δ_C 90-220 ppm].

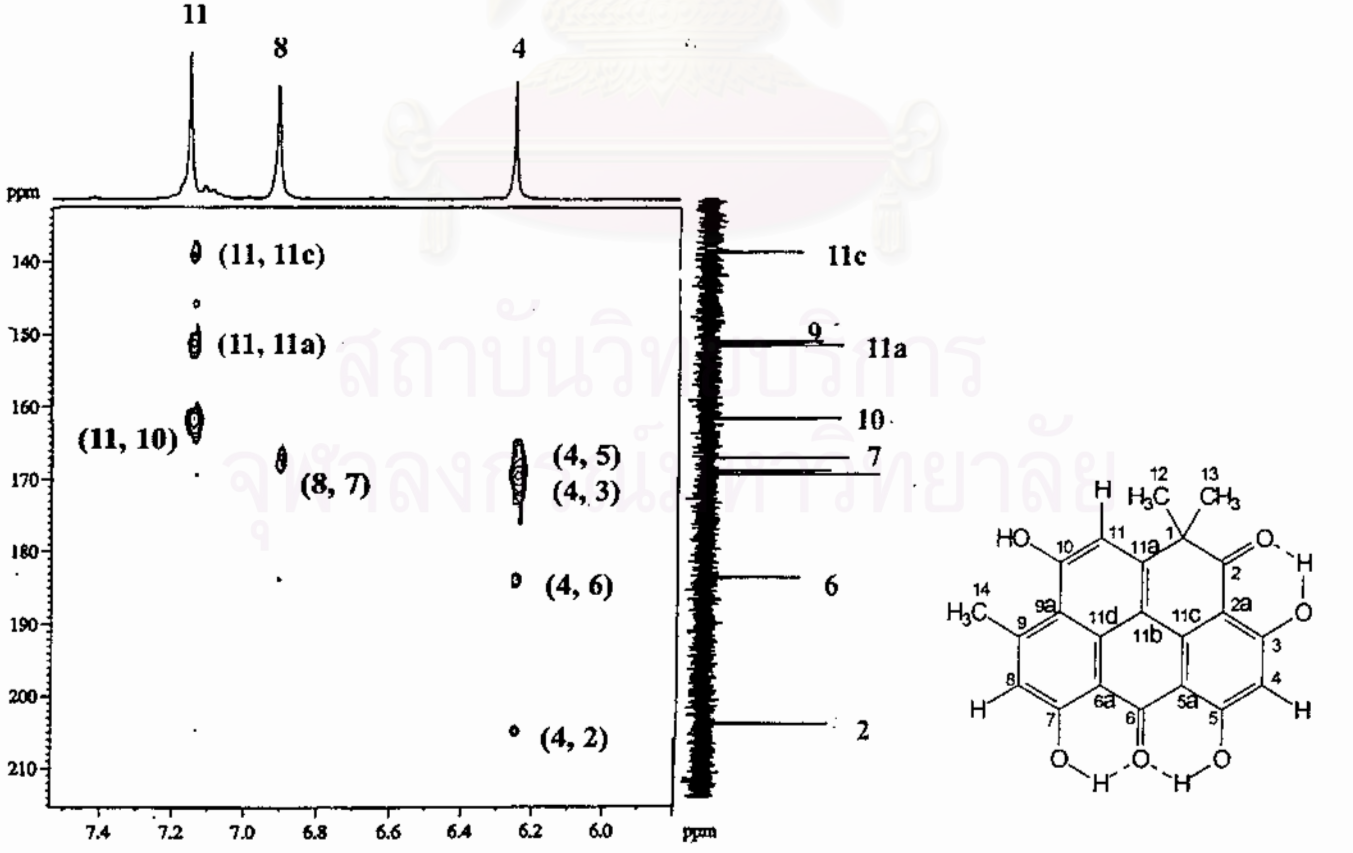


Figure 17 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 3 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 5.9-7.5 ppm; δ_C 130-220 ppm].

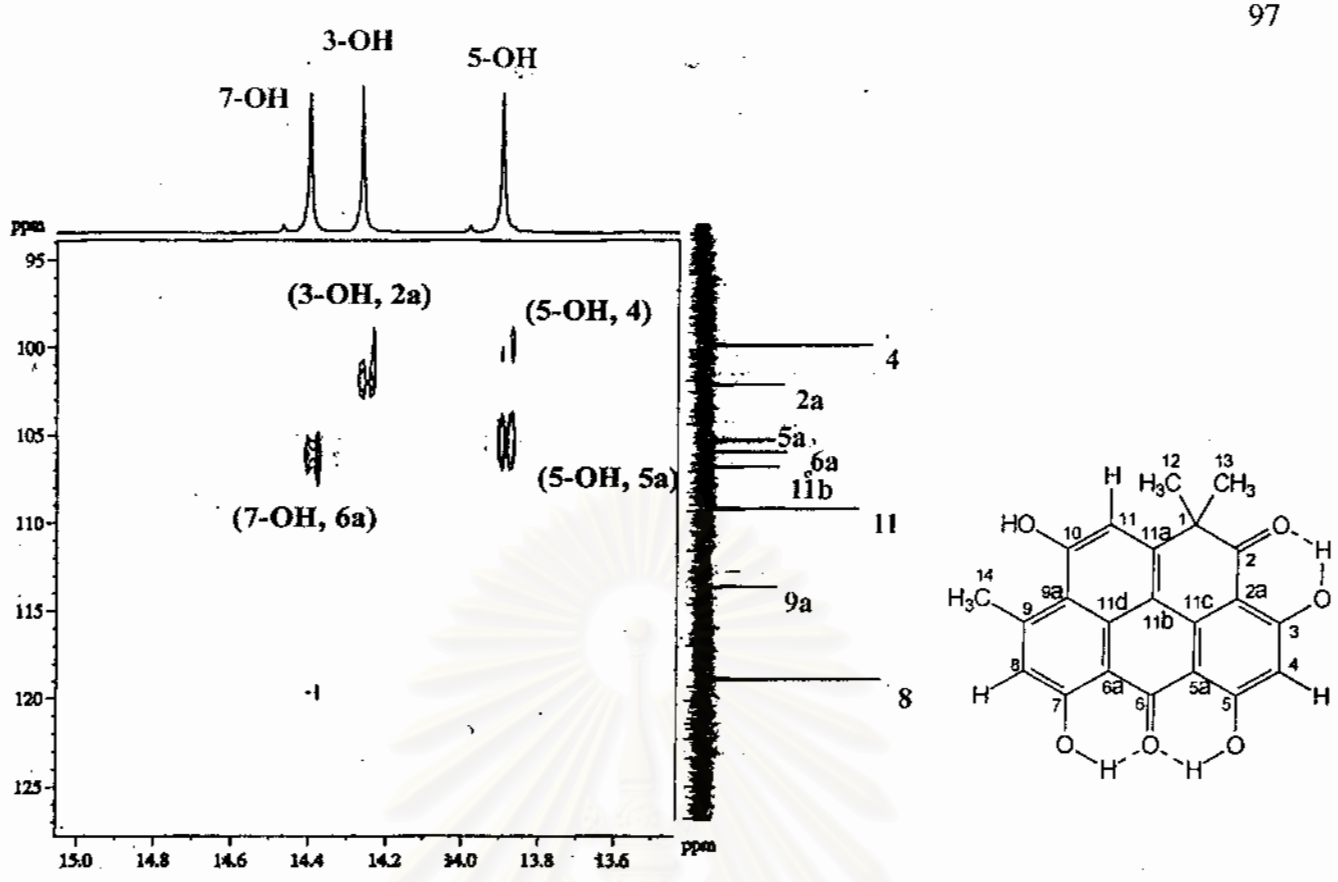


Figure 18 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 3 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 13.5-15 ppm; δ_C 95-125 ppm].

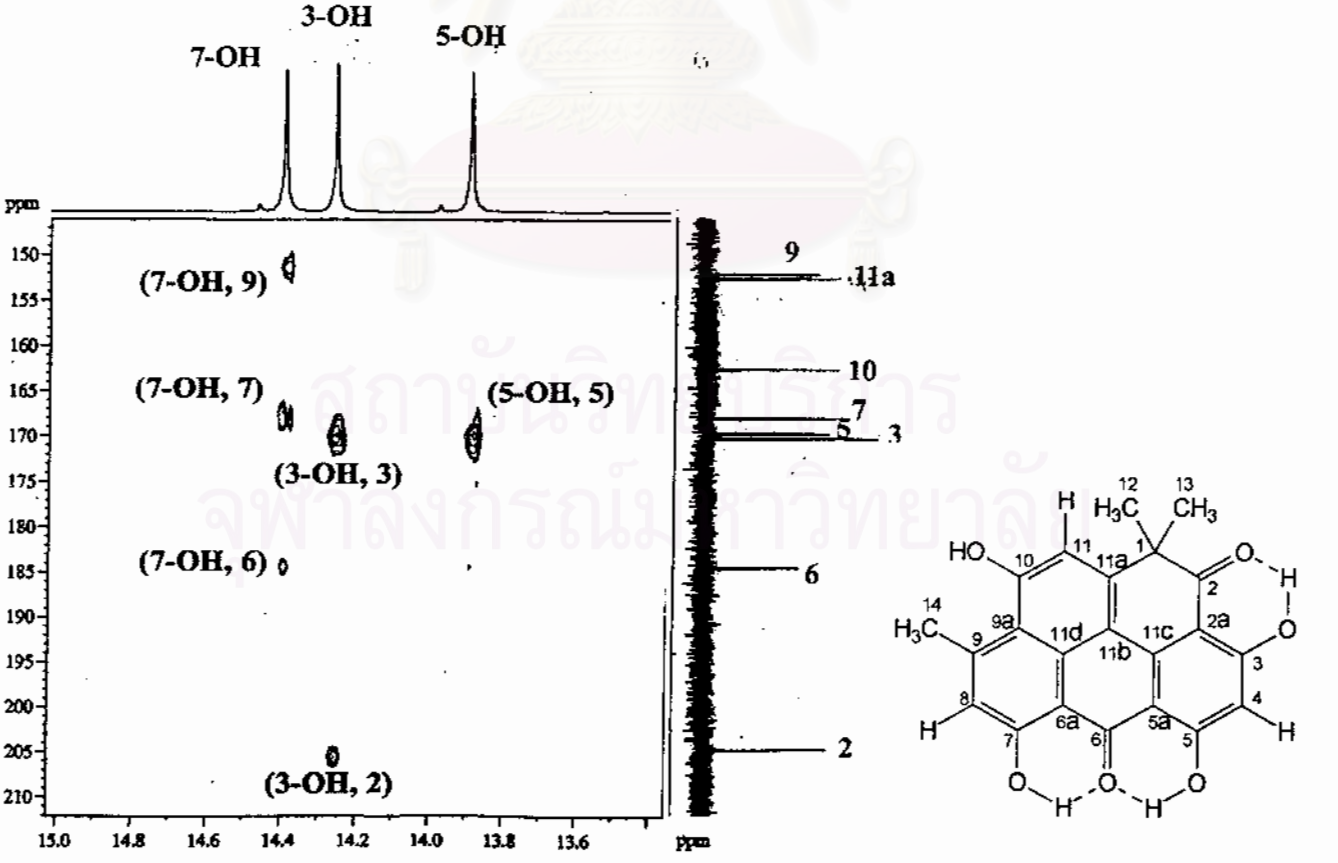
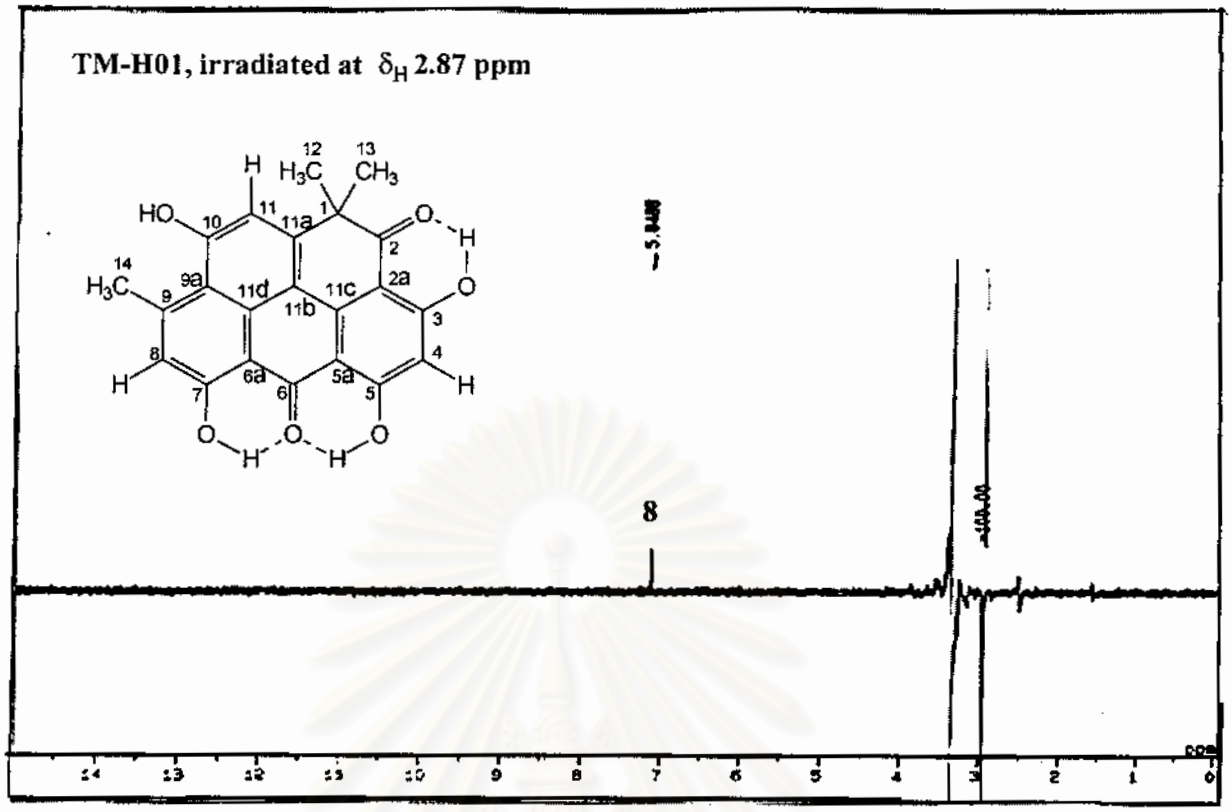
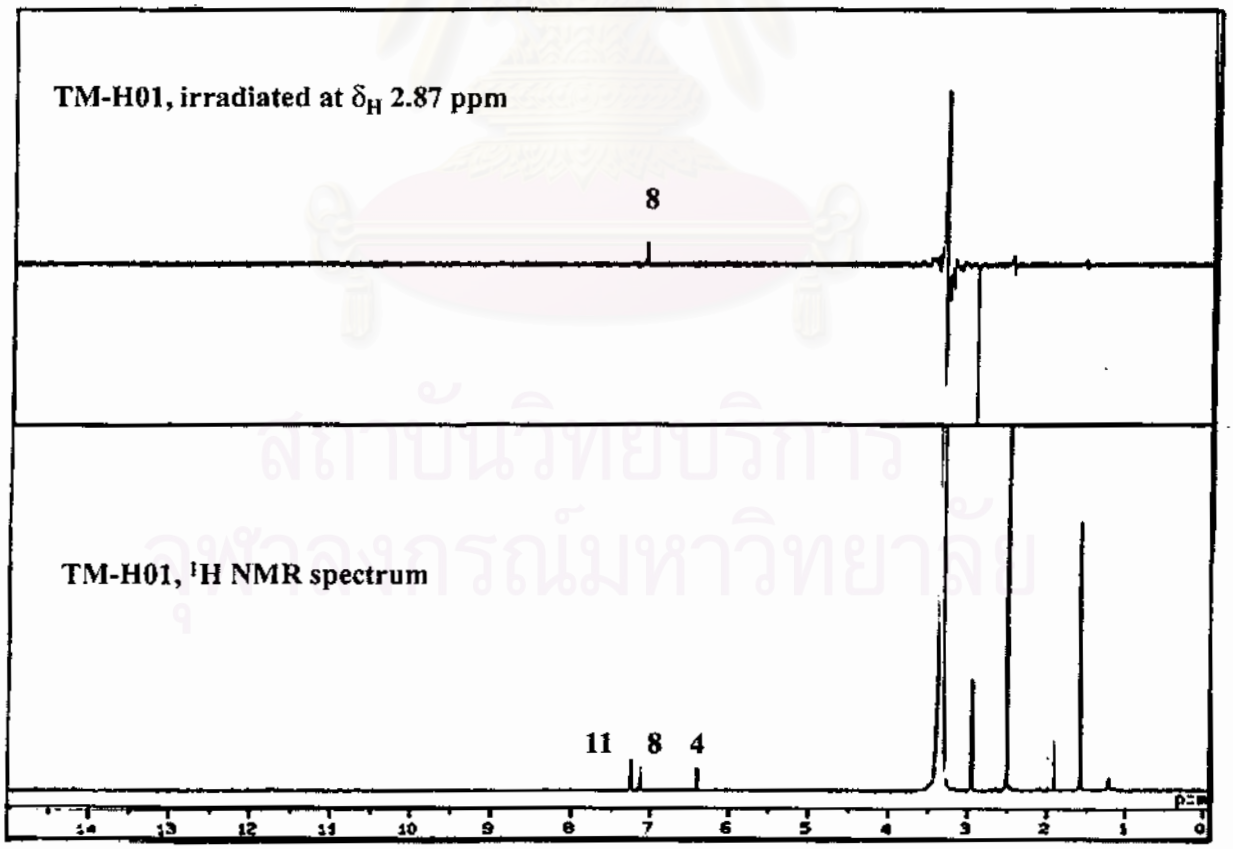


Figure 19 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 3 Hz) of compound TM-H01 (in DMSO-d_6) [expanded: δ_H 13.5-15 ppm; δ_C 145-210 ppm].



(A)



(B)

Figure 20 The one-dimensional difference NOE spectrum of compound TM-H01 (in DMSO-d_6).

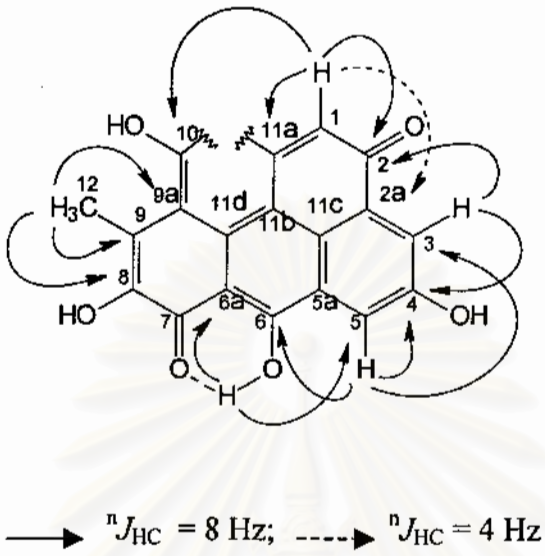


Figure 21 The long-range heteronuclear correlations observed in the HMBC spectra of compound TM-3F17.

Spectral data of compound TM-3F17

ESI-TOF MS	:	*m/z* (% relative intensity); Figure 22 381 ($[M+H]^+$, 100), 359 (15), 353 (43)
UV	:	λ_{max} nm (log ε), in methanol; Figure 23 435 (2.65), 308 (3.25), 286 (3.41), 268 (3.36)
IR	:	ν_{max} cm^{-1}, NaCl cell; Figure 24 3804-2260, 1702, 1671, 1620, 1574, 1355, 1290, 1235, 1025, 1000, 761, 621
^1H NMR	:	δ_{ppm}, 300 MHz, in DMSO-d_6 and CD_3OD; see Figure 25 and 27, and Table 18
^{13}C NMR	:	δ_{ppm}, 75 MHz, in DMSO-d_6 and CD_3OD; see Figure 26 and 28, and Table 18

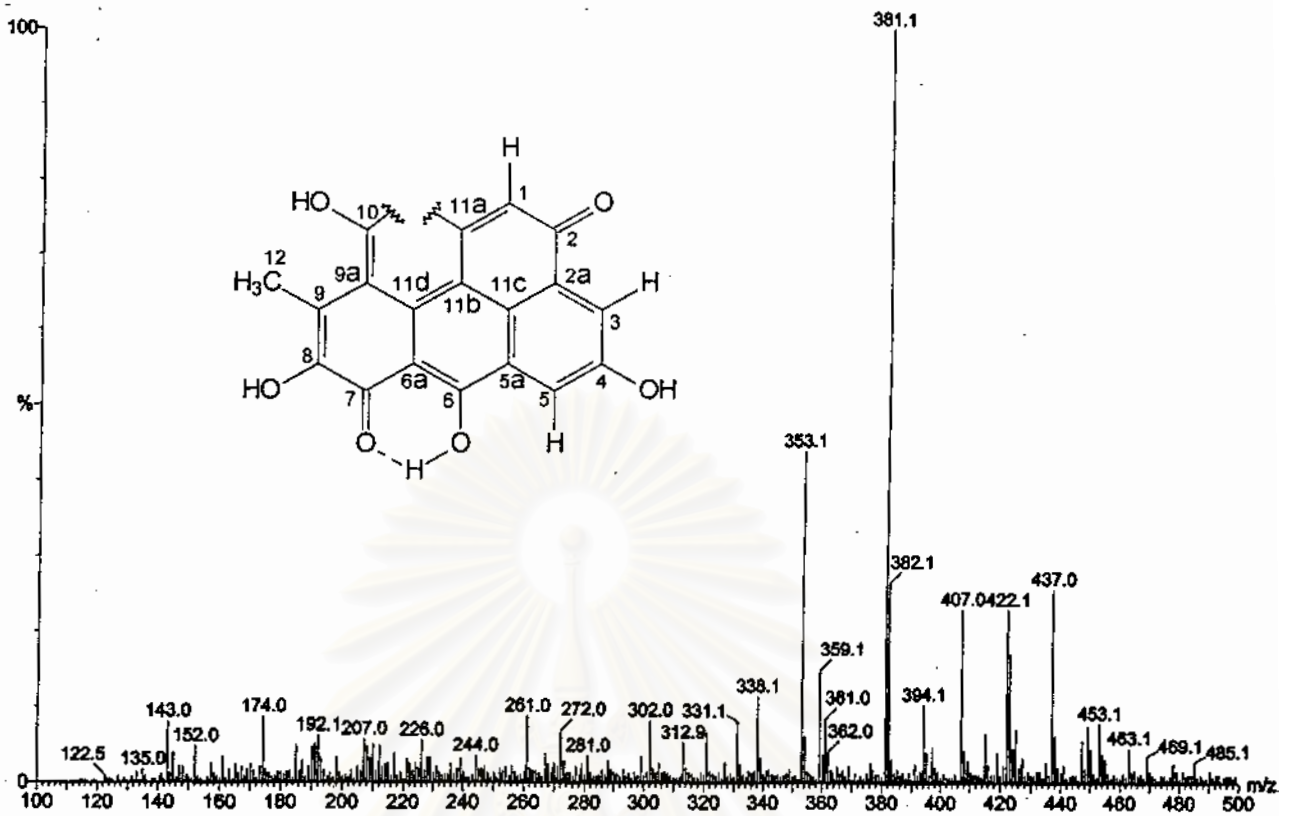


Figure 22 The ESI-TOF MS spectrum of compound TM-3F17.

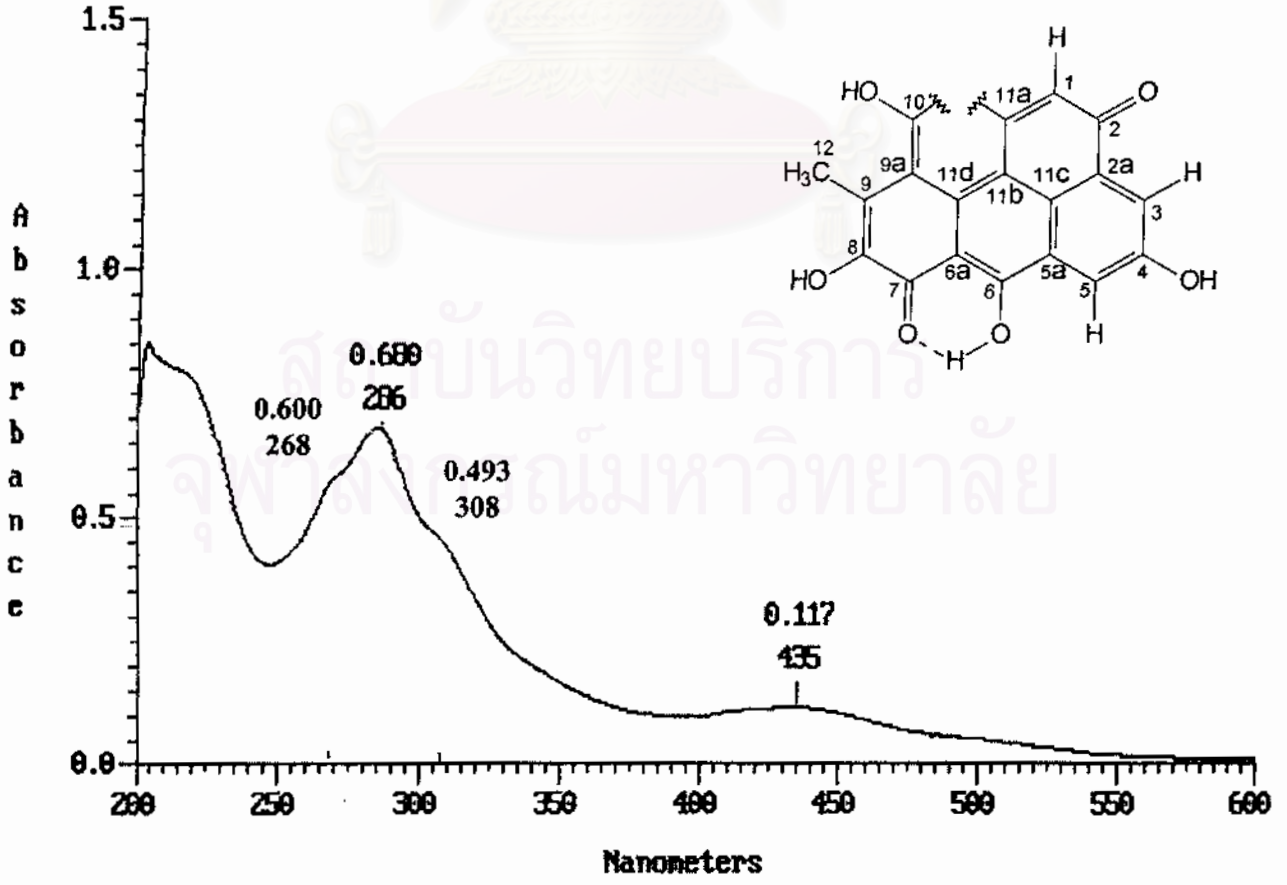


Figure 23 The UV spectrum of compound TM-3F17 (in MeOH).

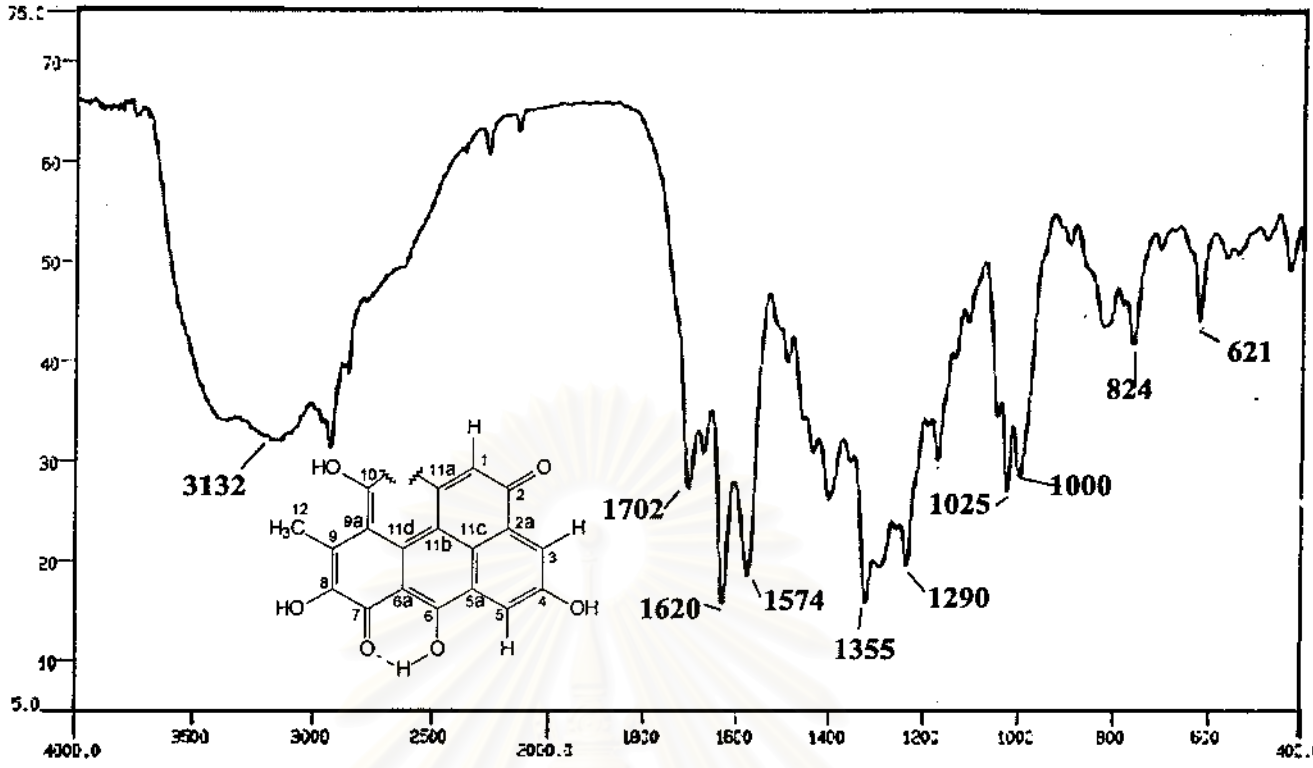


Figure 24 The IR spectrum of compound TM-3F17 (NaCl cell).

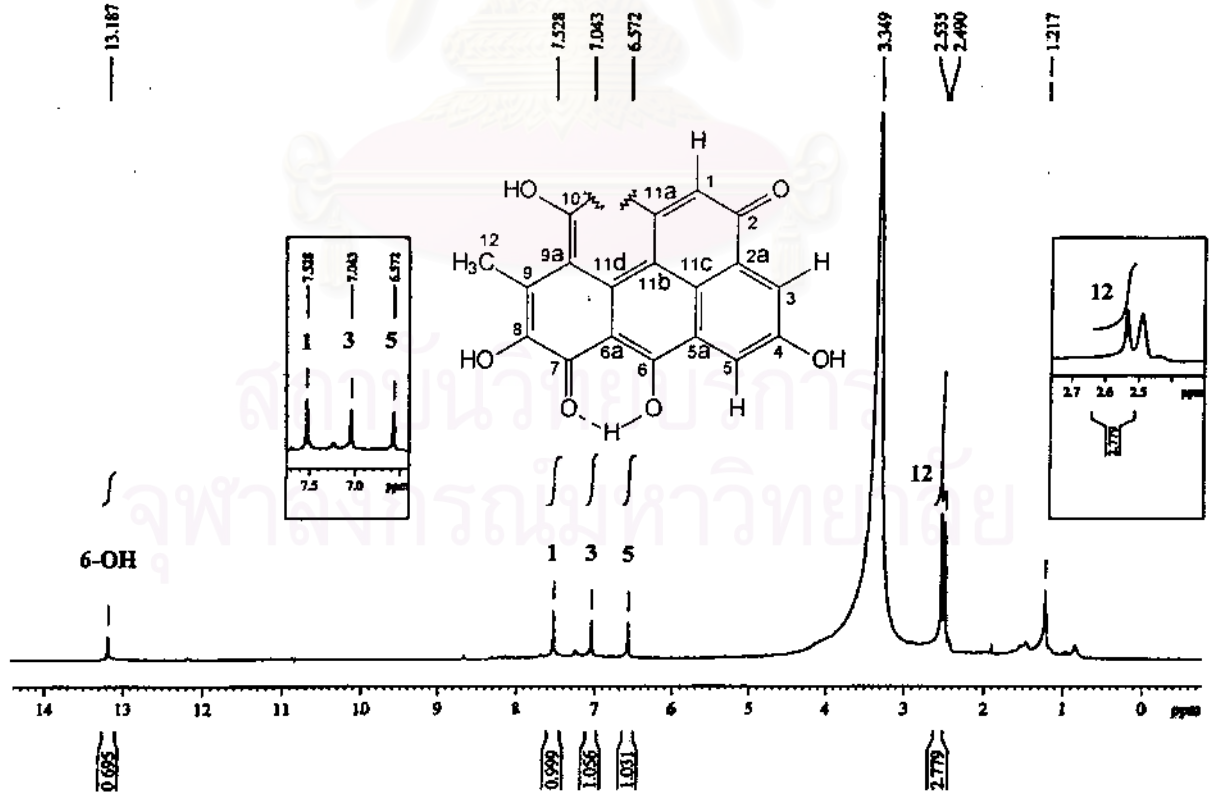


Figure 25 The 300 MHz ^{1}H NMR spectrum of compound TM-3F17 (in DMSO-d_6).

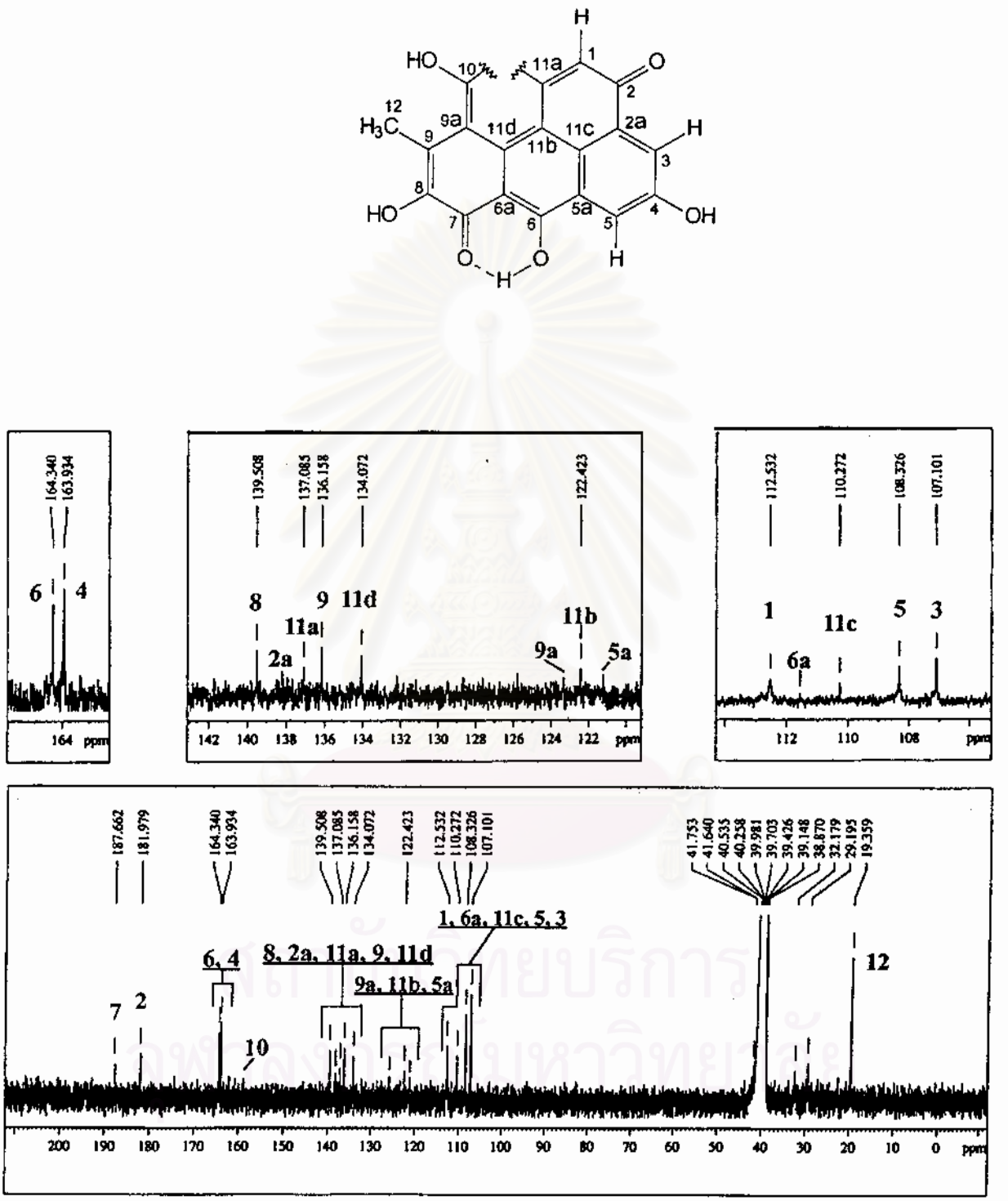
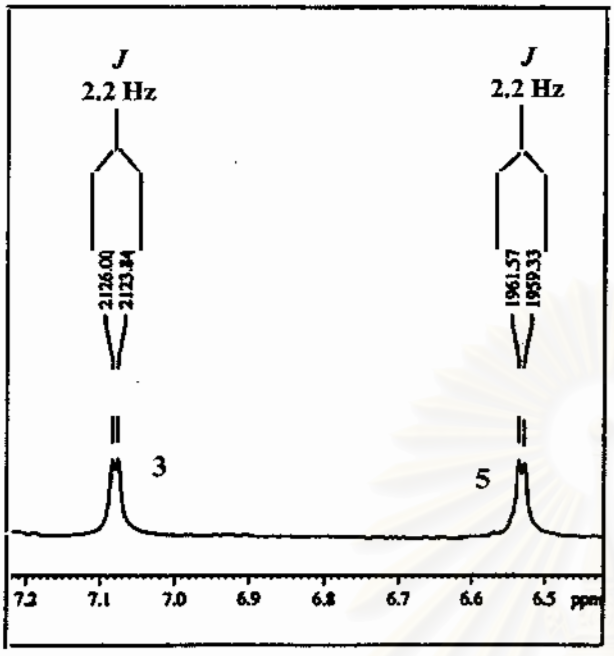
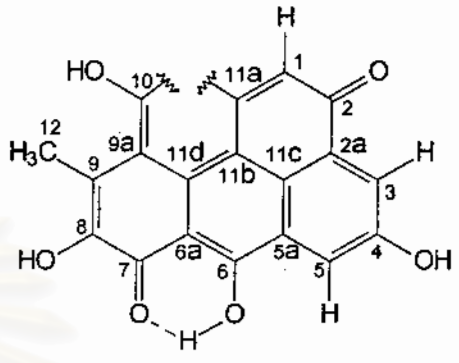


Figure 26 The 75 MHz ^{13}C NMR spectrum of compound TM-3F17 (in DMSO-d_6).





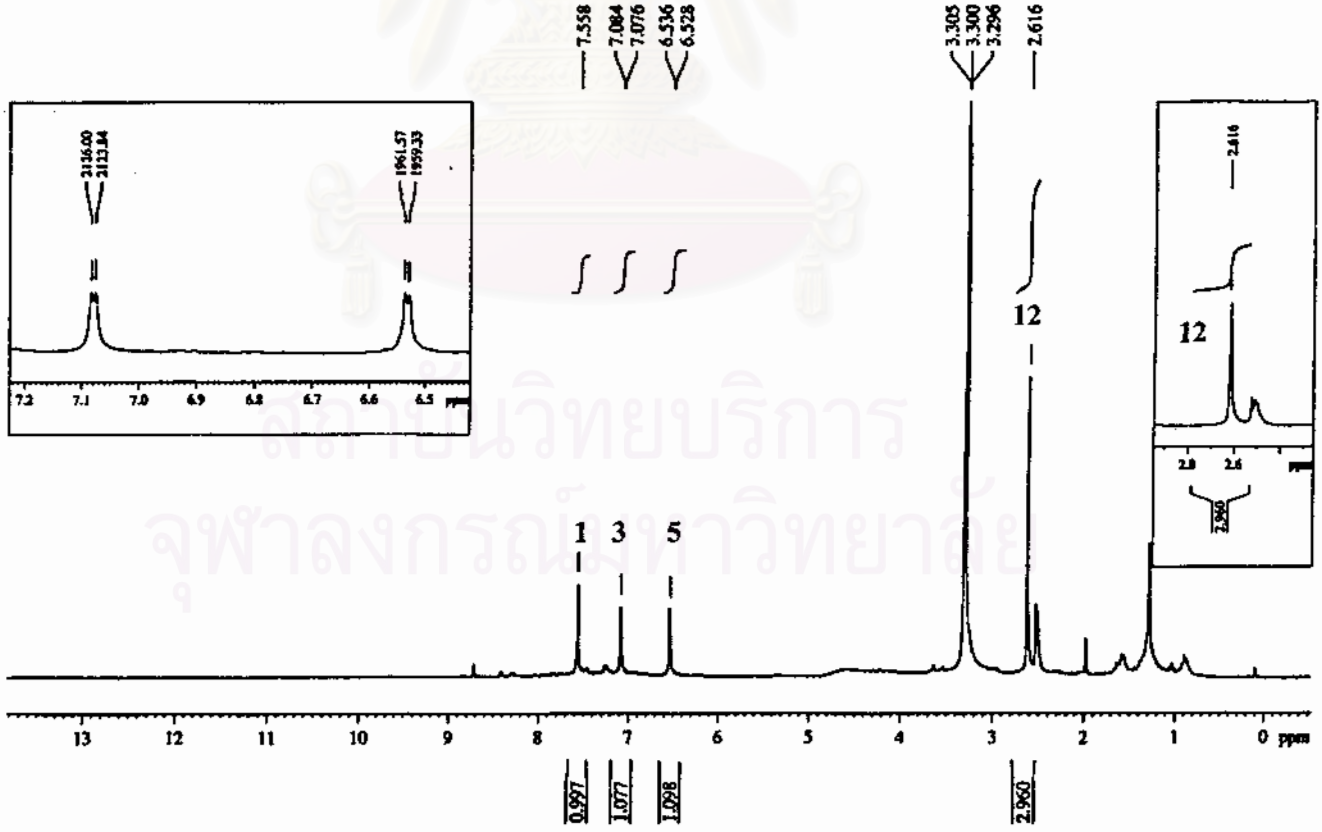


Figure 27 The 300 MHz ^{1}H NMR spectrum of compound TM-3F17 (in CD_3OD).

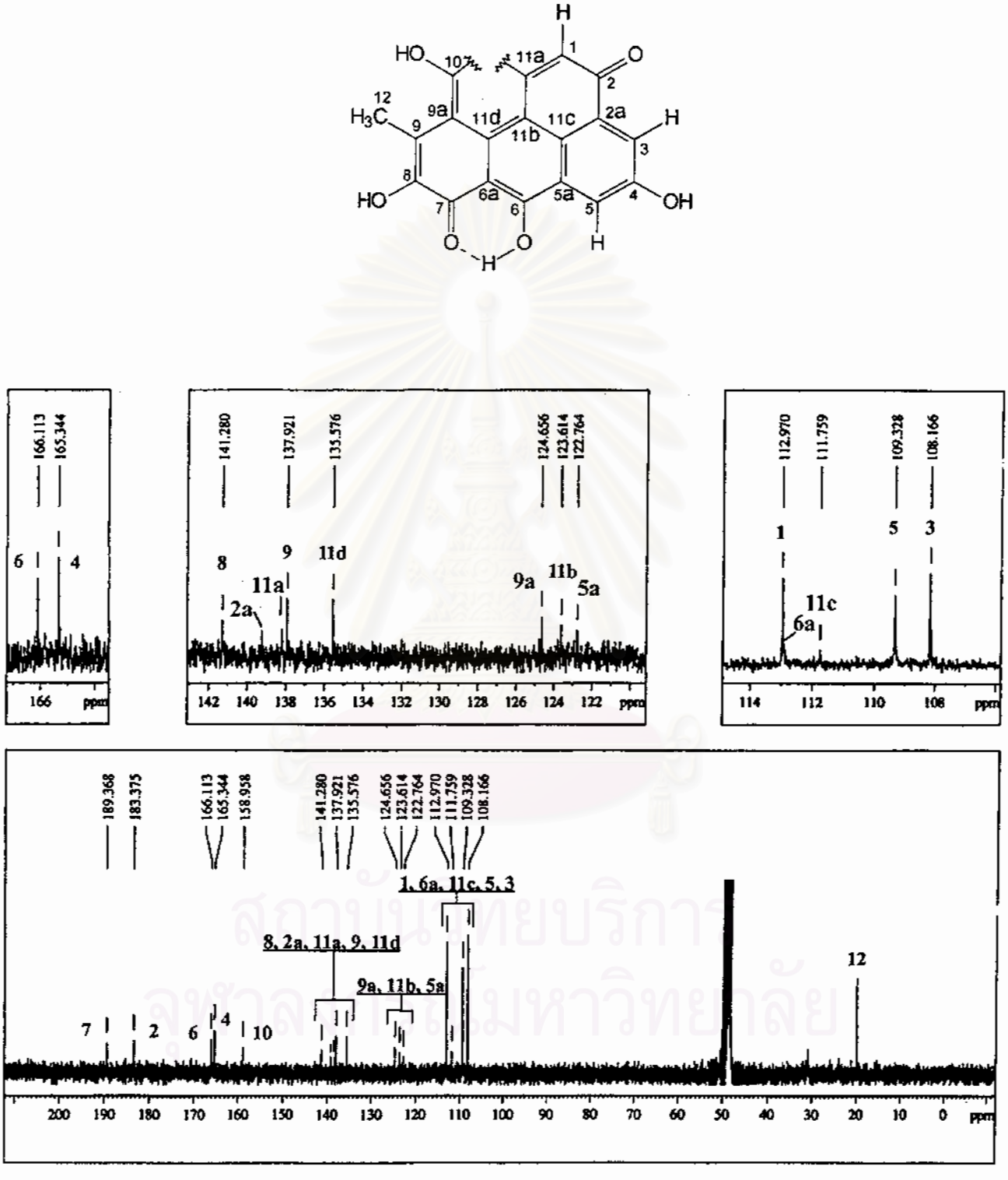


Figure 28 The 75 MHz ^{13}C NMR spectrum of compound TM-3F17 (in CD_3OD).

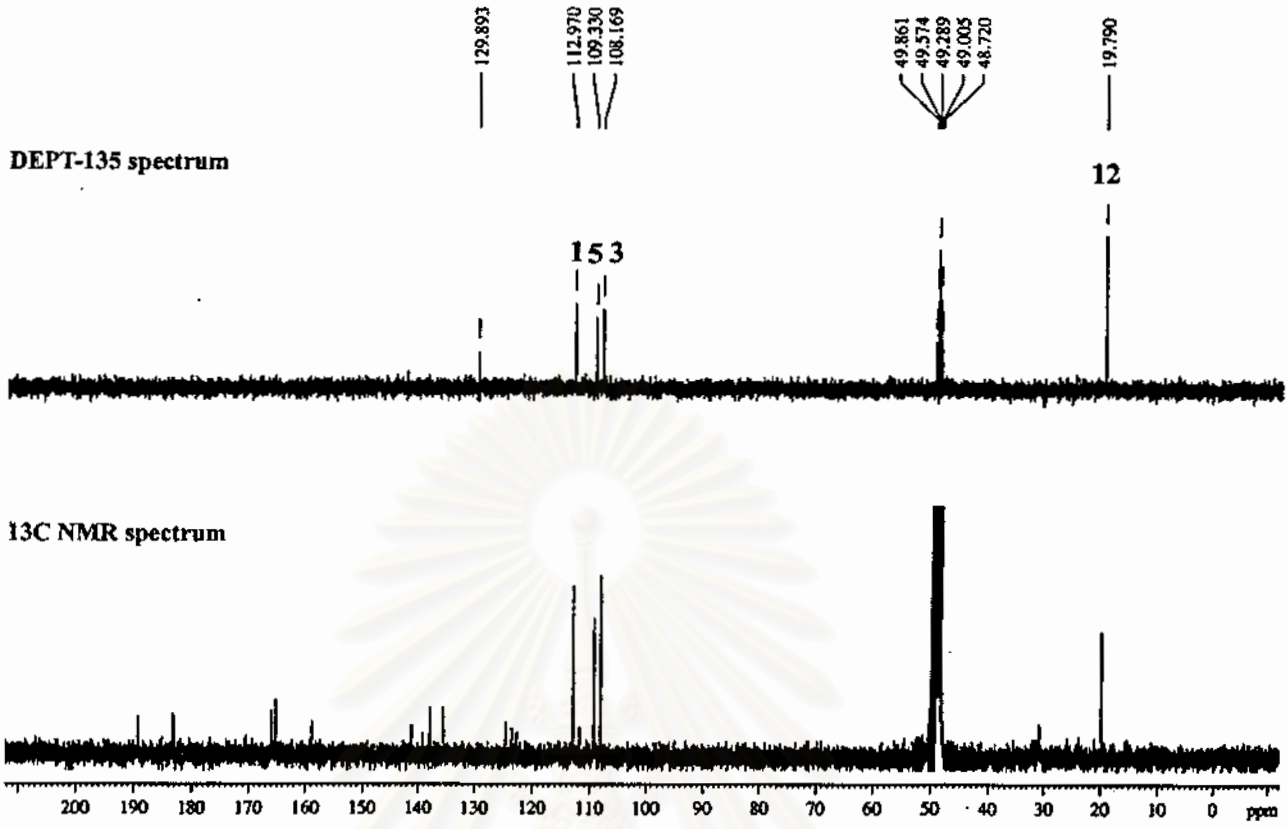


Figure 29 The 75 MHz ^{13}C NMR and DEPT-135 spectra of compound TM-3F17.

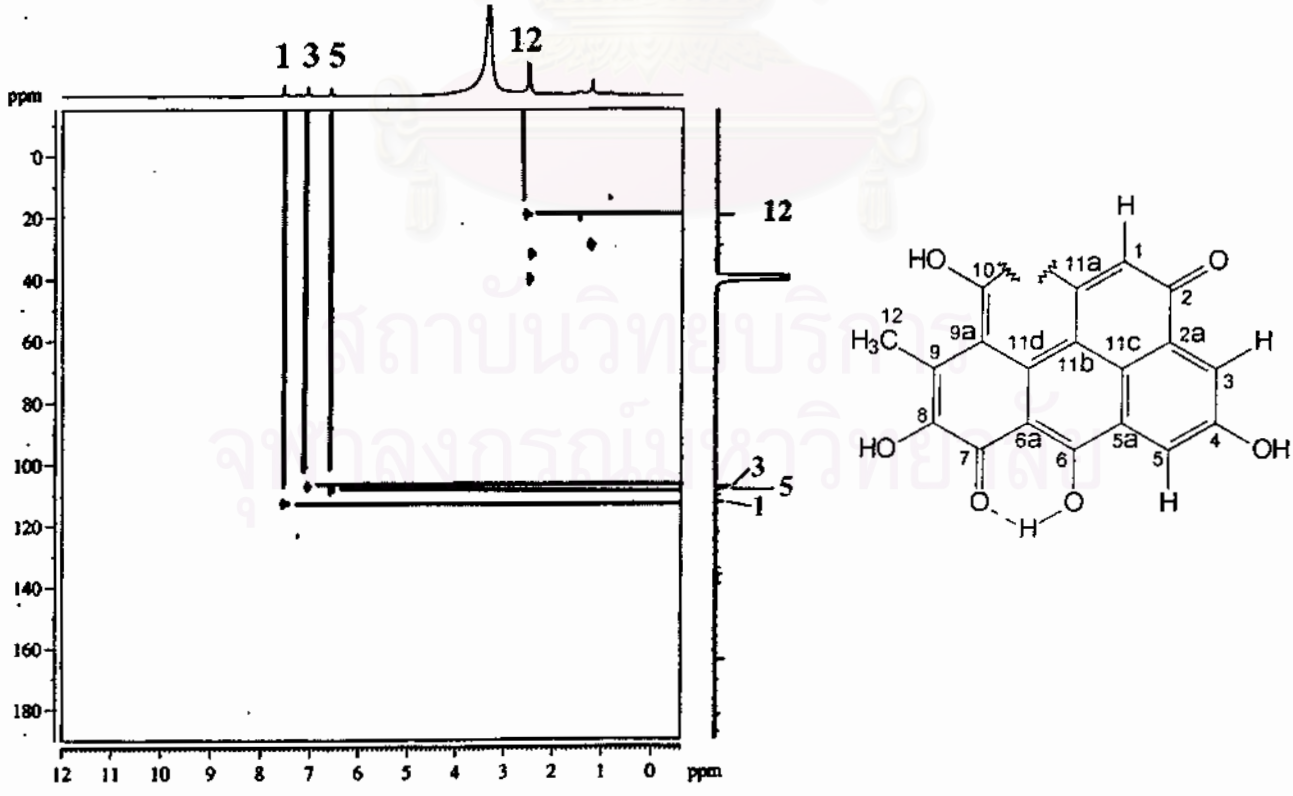


Figure 30 The 300 MHz HMQC spectrum of compound TM-3F17 (in DMSO-d_6).

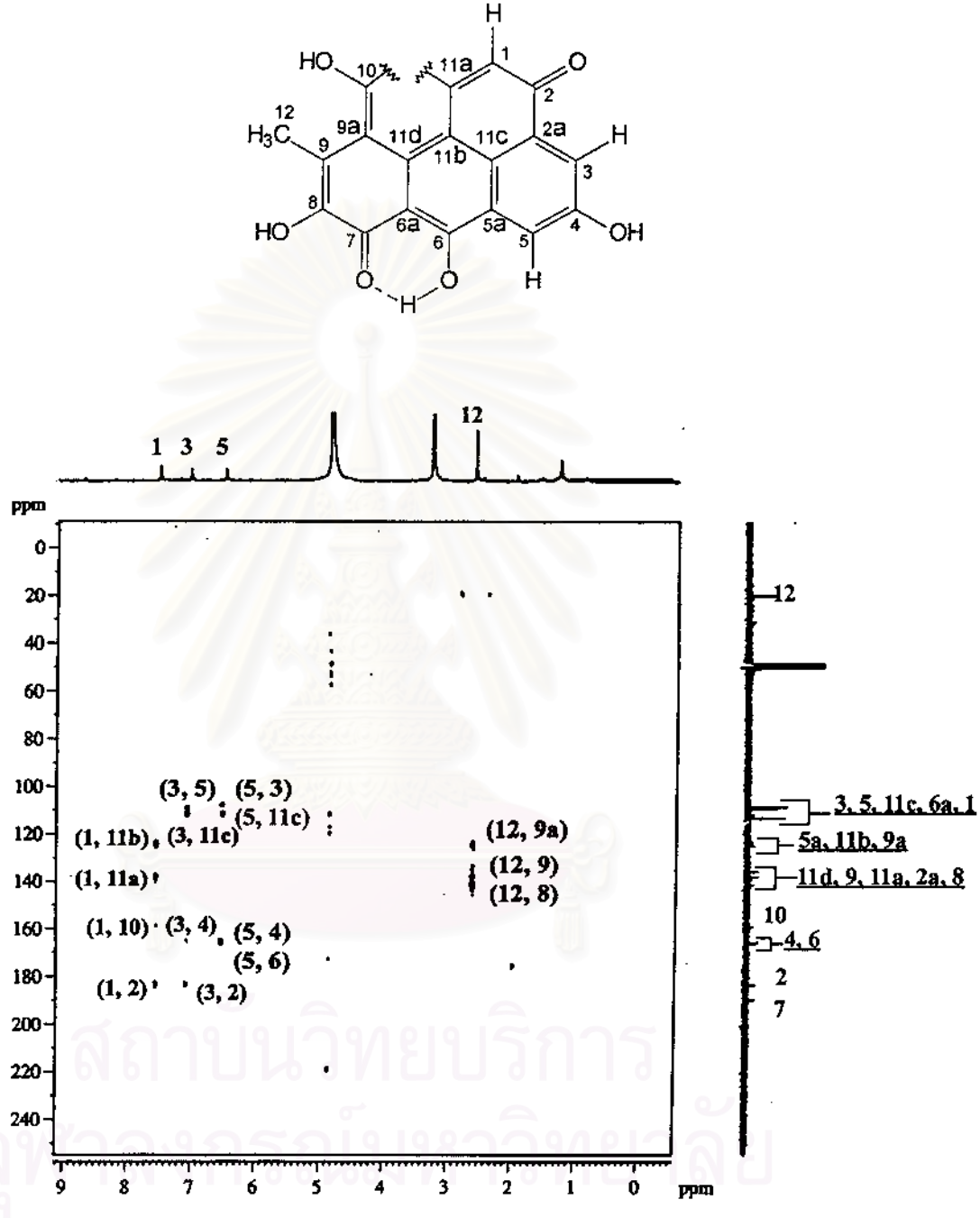


Figure 31 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-3F17 (in CD_3OD).

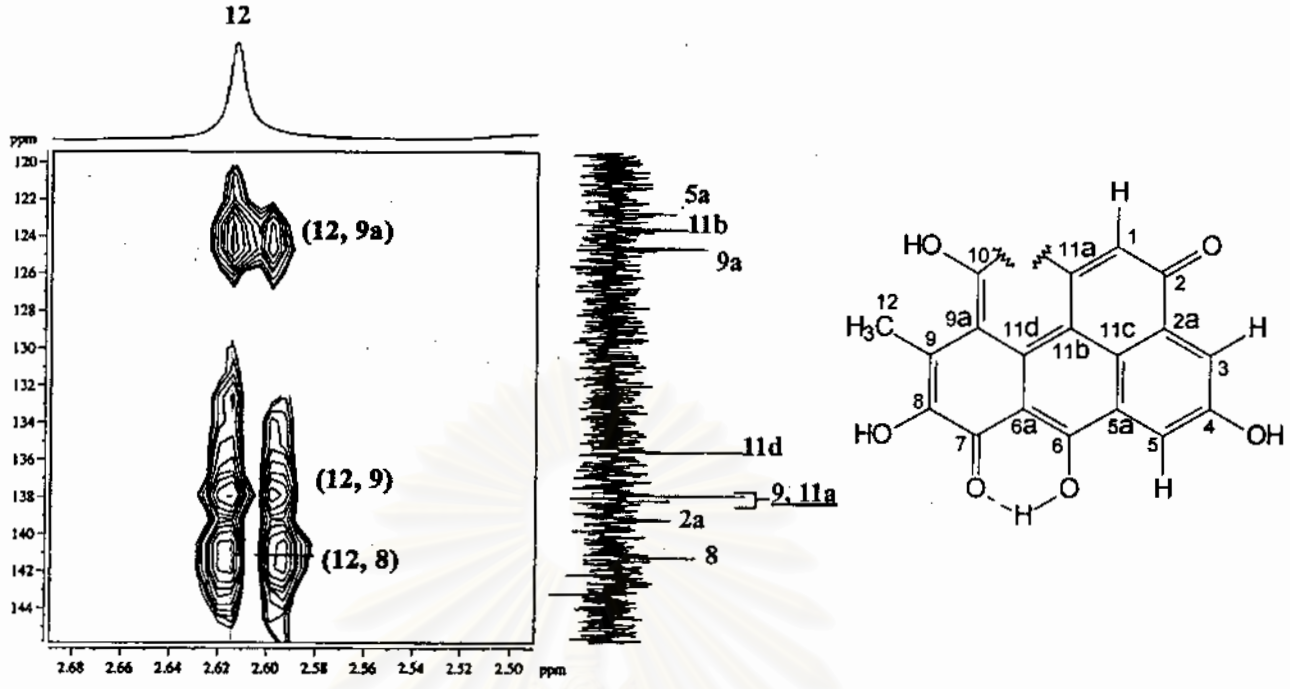


Figure 32 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-3F17 (in CD_3OD) [expanded: δ_H 2.5-2.7 ppm; δ_C 120-145 ppm].

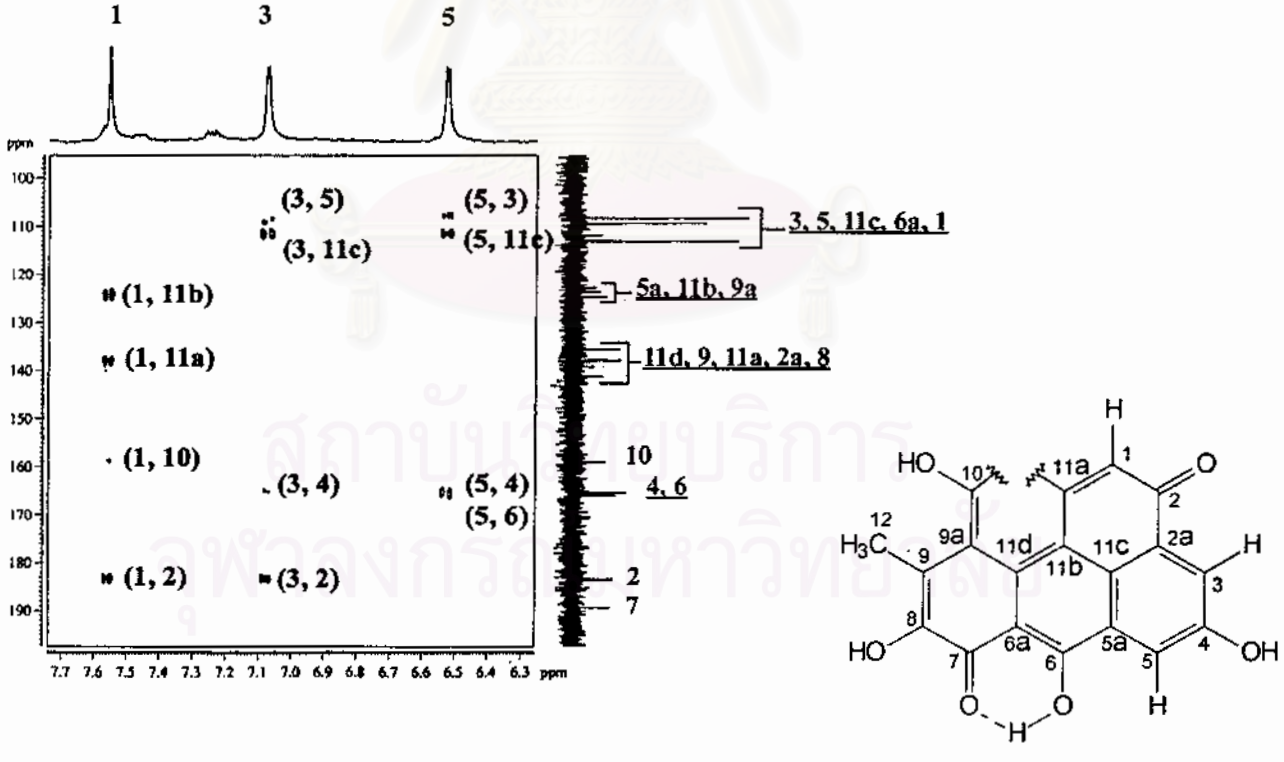
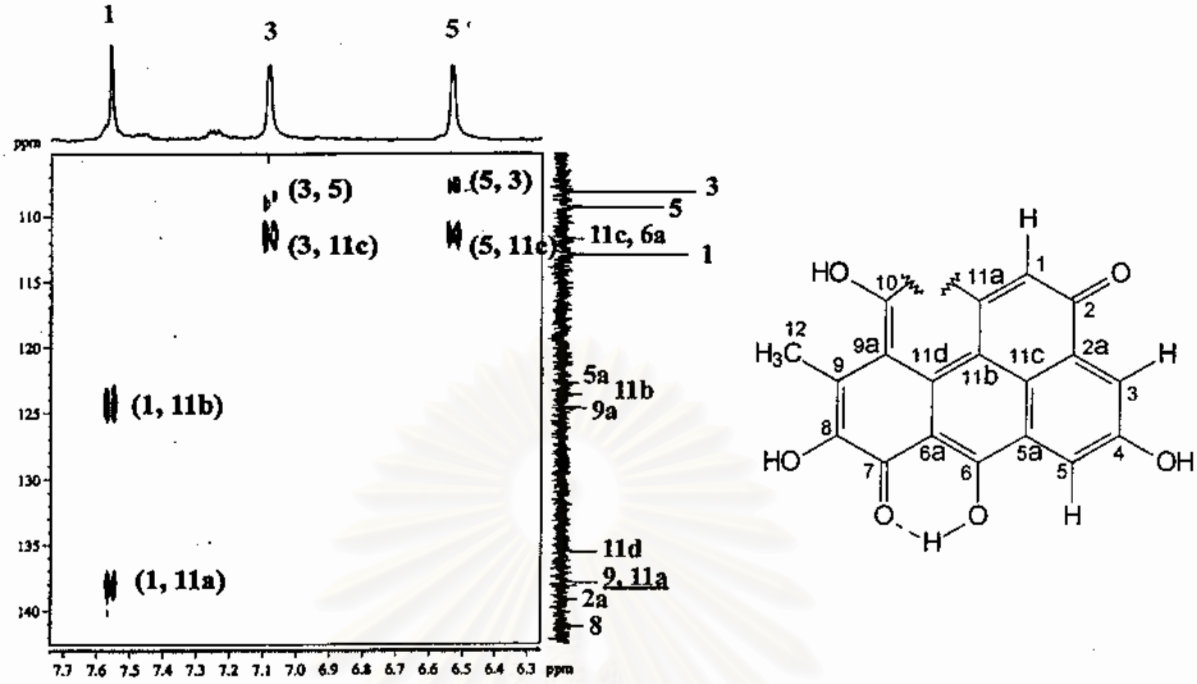
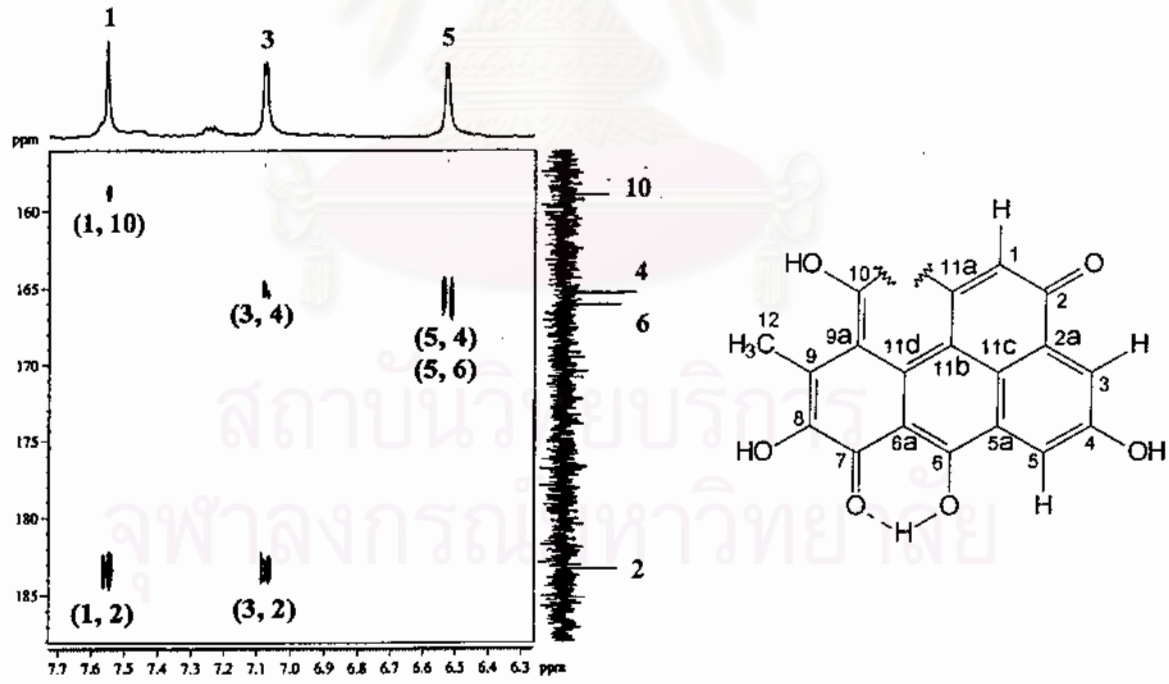


Figure 33 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-3F17 (in CD_3OD) [expanded: δ_H 6.3-7.7 ppm; δ_C 100-190 ppm].



(A) Expanded: δ_H 6.3-7.7 ppm; δ_C 105-145 ppm.



(B) Expanded: δ_H 6.3-7.7; $\delta_{C\ 155\text{-}190\ ppm}$

Figure 34 The 300 MHz HMBC spectrum ($^nJ_{HC}$ = 8 Hz) of compound TM-3F17 (in CD_3OD) [expanded: δ_H 6.3-7.7 ppm; δ_C 105-190 ppm].

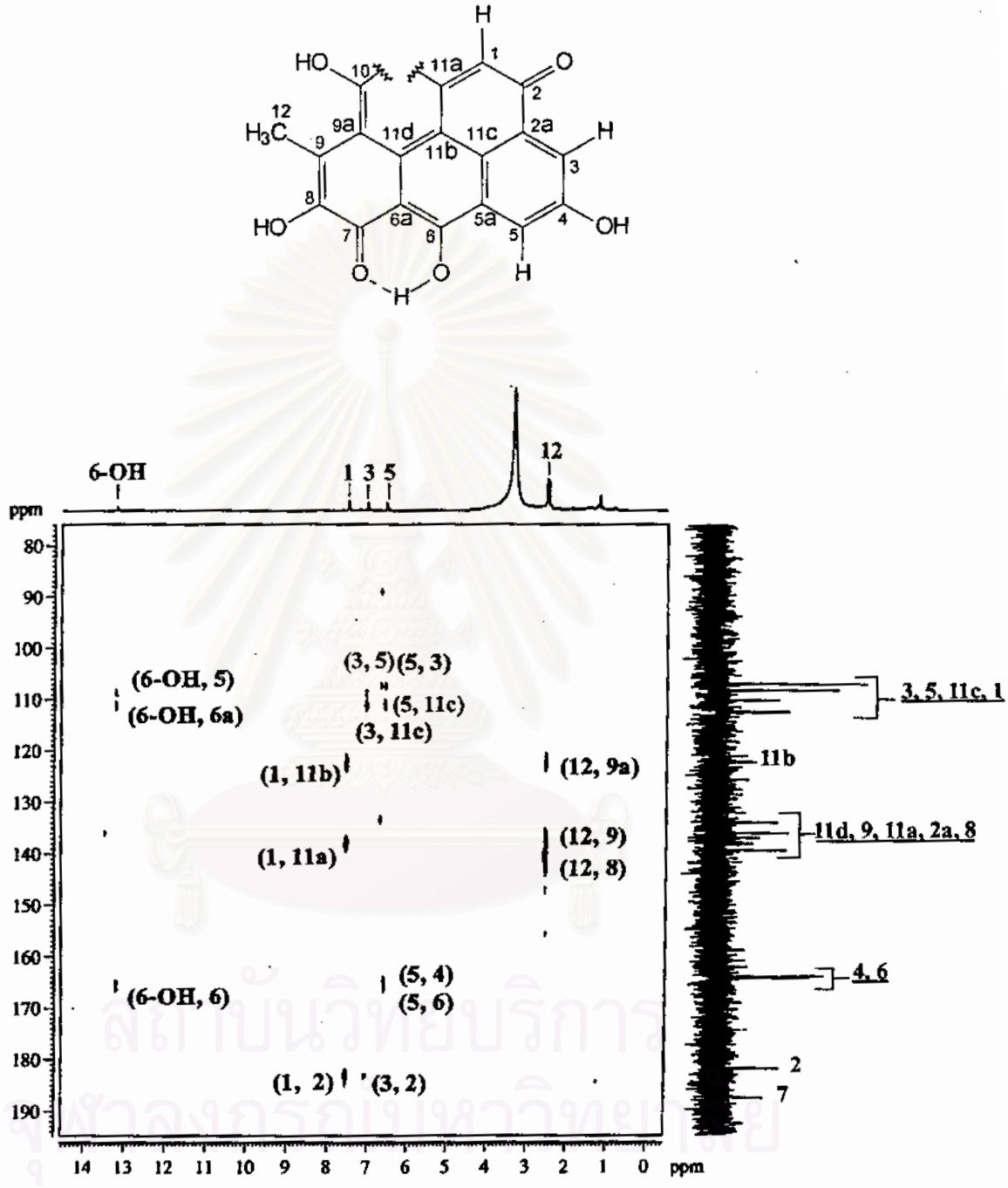
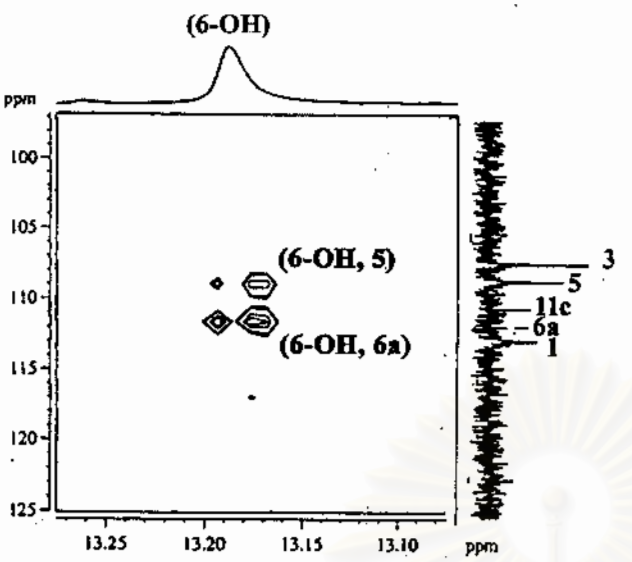
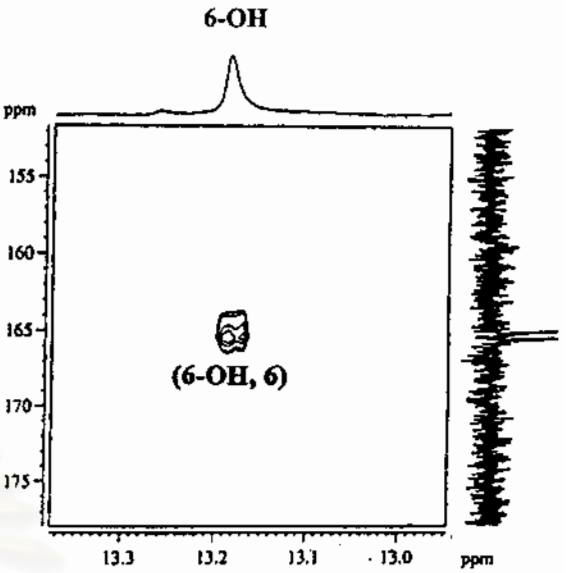


Figure 35 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-3F17 (in DMSO-d_6).



(A) Expanded: δ_H 13.1-13.3 ppm; δ_C 95-125 ppm



(B) Expanded: δ_H 13.1-13.3 ppm; δ_C 150-180 ppm

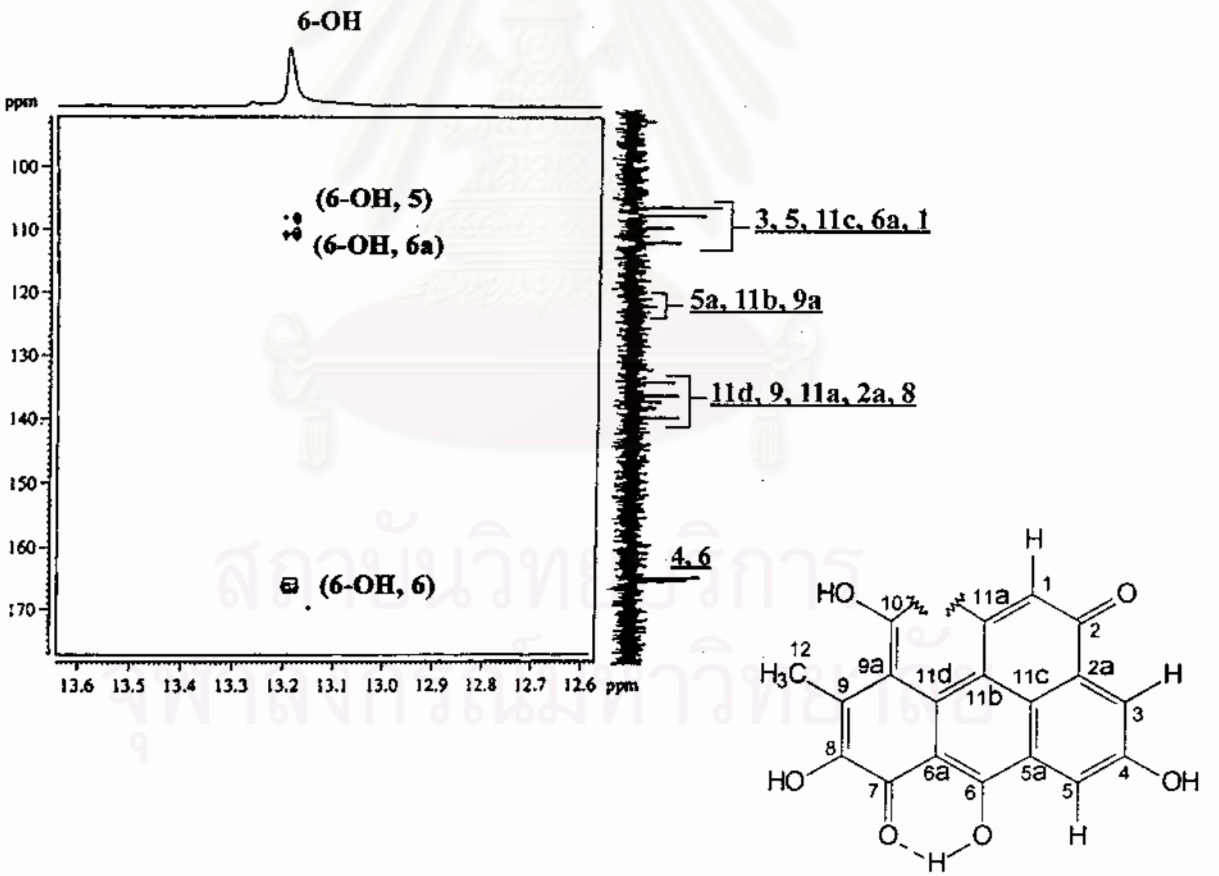


Figure 36 The 300 MHz HMBC spectrum ($^nJ_{HC}$ = 8 Hz) of compound TM-3F17 (in DMSO-d_6) [expanded: δ_H 12.6-13.6 ppm; δ_C 90-180 ppm].

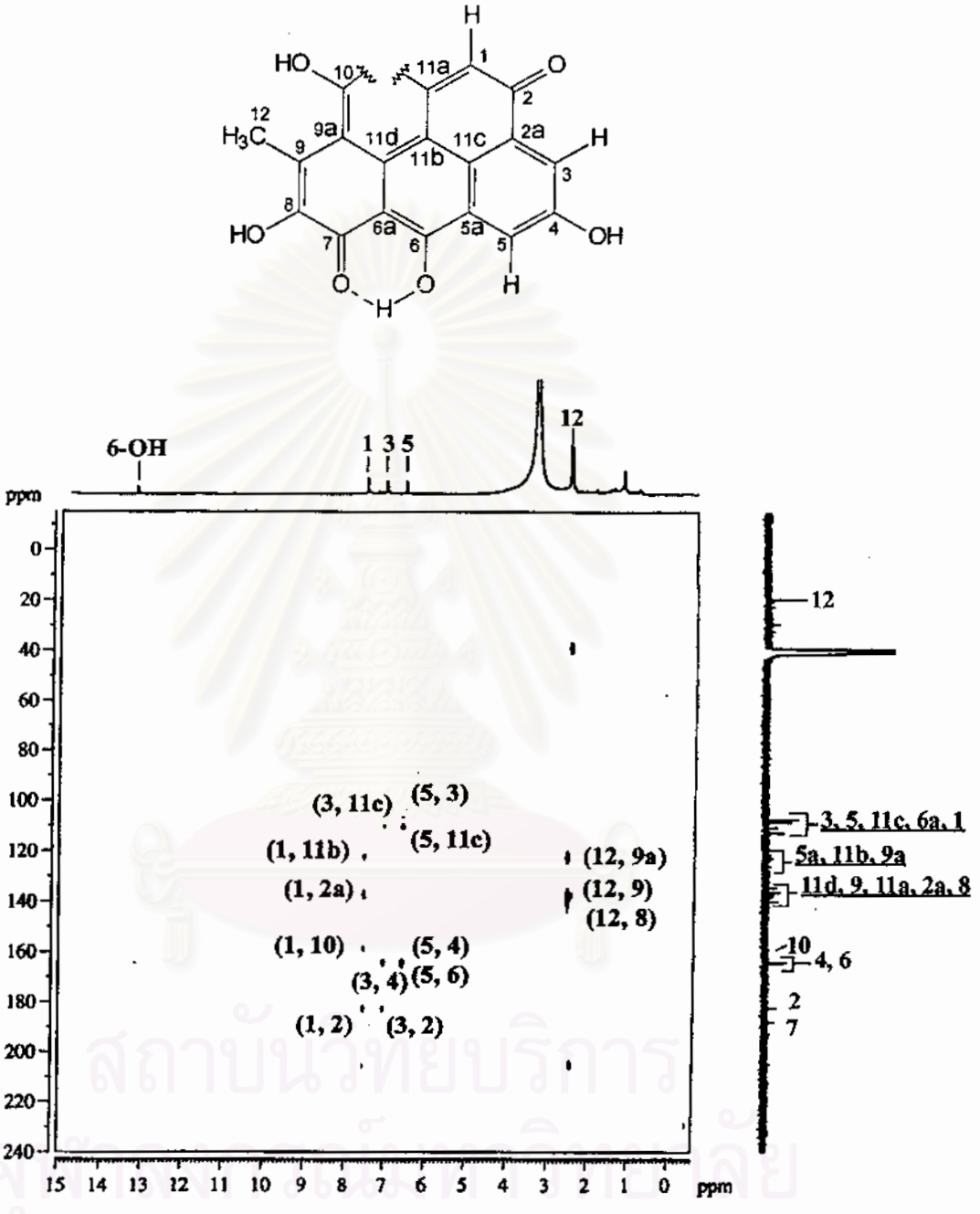


Figure 37 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) of compound TM-3F17 (in DMSO-d_6).

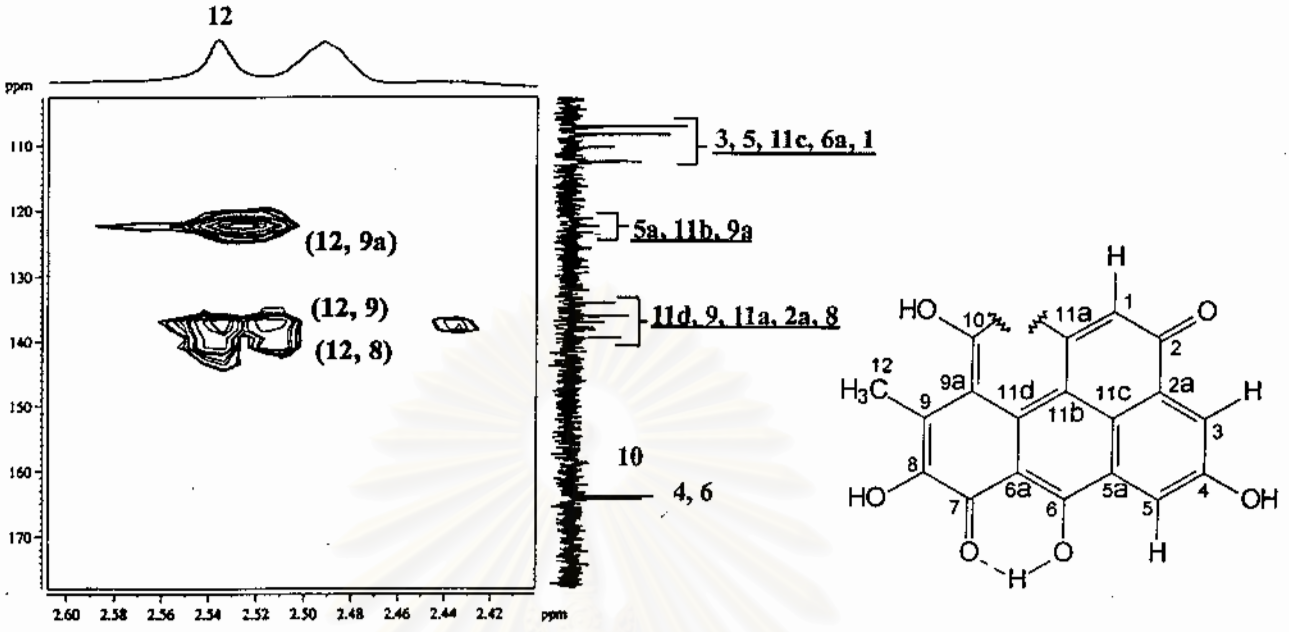


Figure 38 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) of compound TM-3F17 (in DMSO-d_6) [expanded: δ_H 2.4-2.6 ppm; δ_C 100-180 ppm].

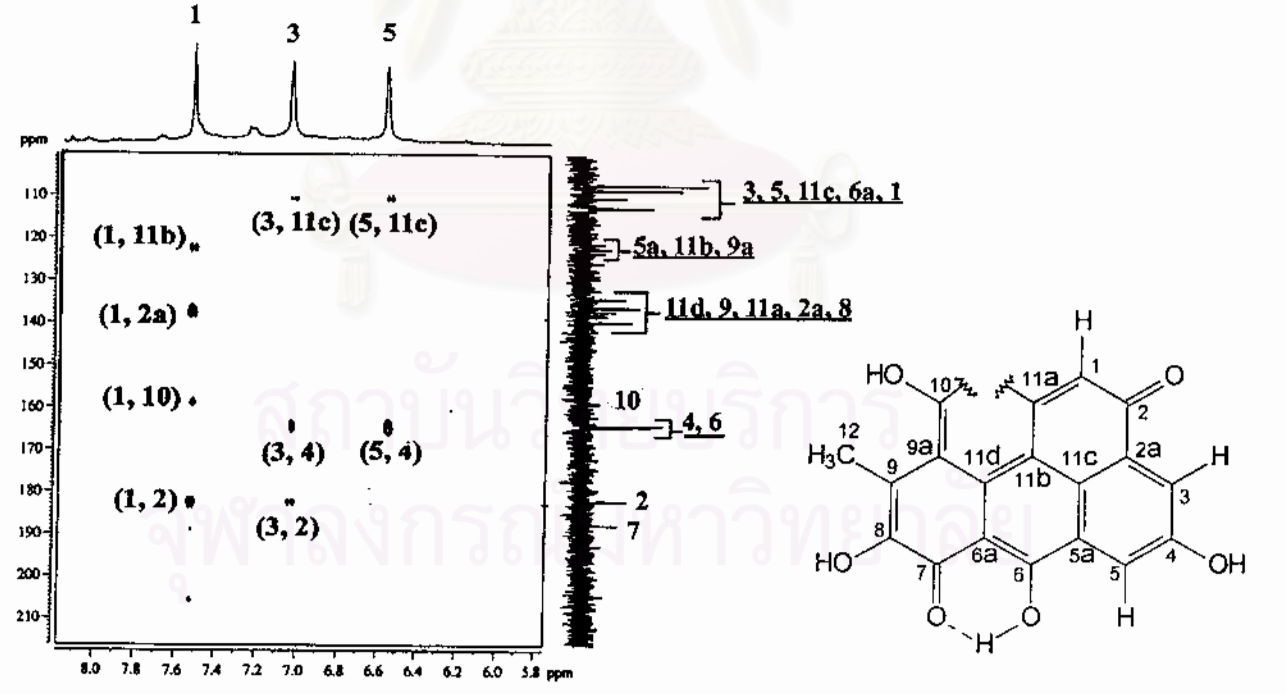
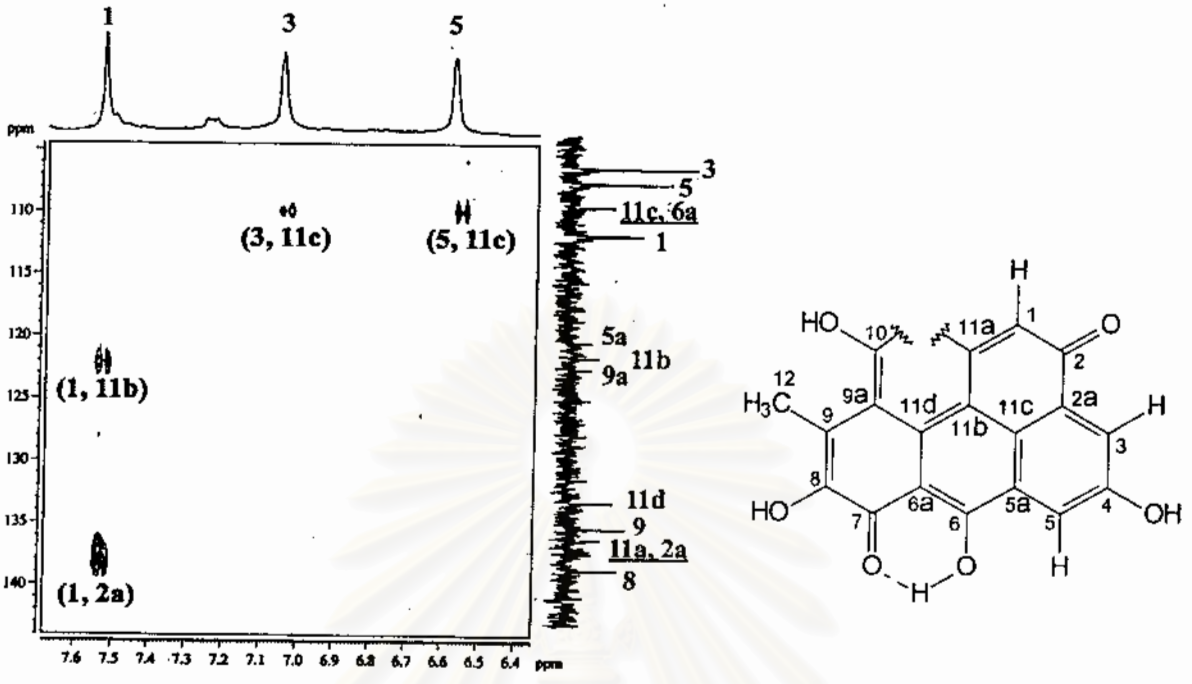
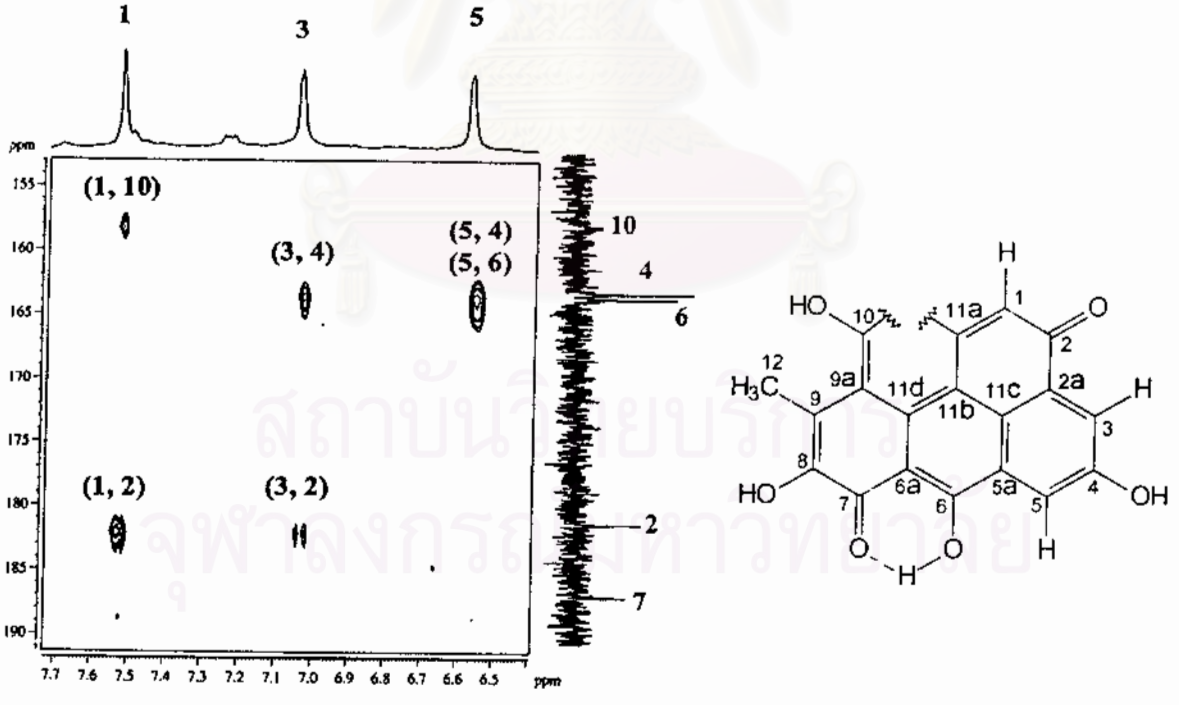


Figure 39 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) of compound TM-3F17 (in DMSO-d_6) [expanded: δ_H 5.8-8.0 ppm; δ_C 100-210 ppm].

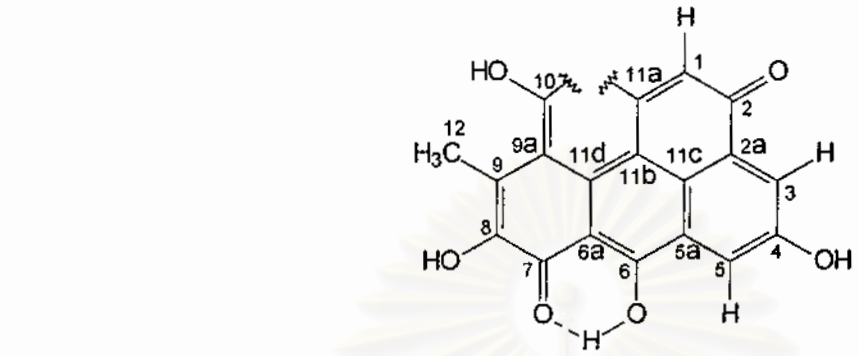


(A) Expanded: δ_H 6.4-7.7 ppm; δ_C 105-145 ppm.



(B) Expanded: δ_H 6.4-7.7 ppm; δ_C 150-190 ppm.

Figure 40 The 300 MHz HMBC spectrum ($^nJ_{HC}$ = 4 Hz) of compound TM-3F17 (in DMSO-d_6) [expanded: δ_H 6.4-7.7 ppm; δ_C 100-190 ppm].



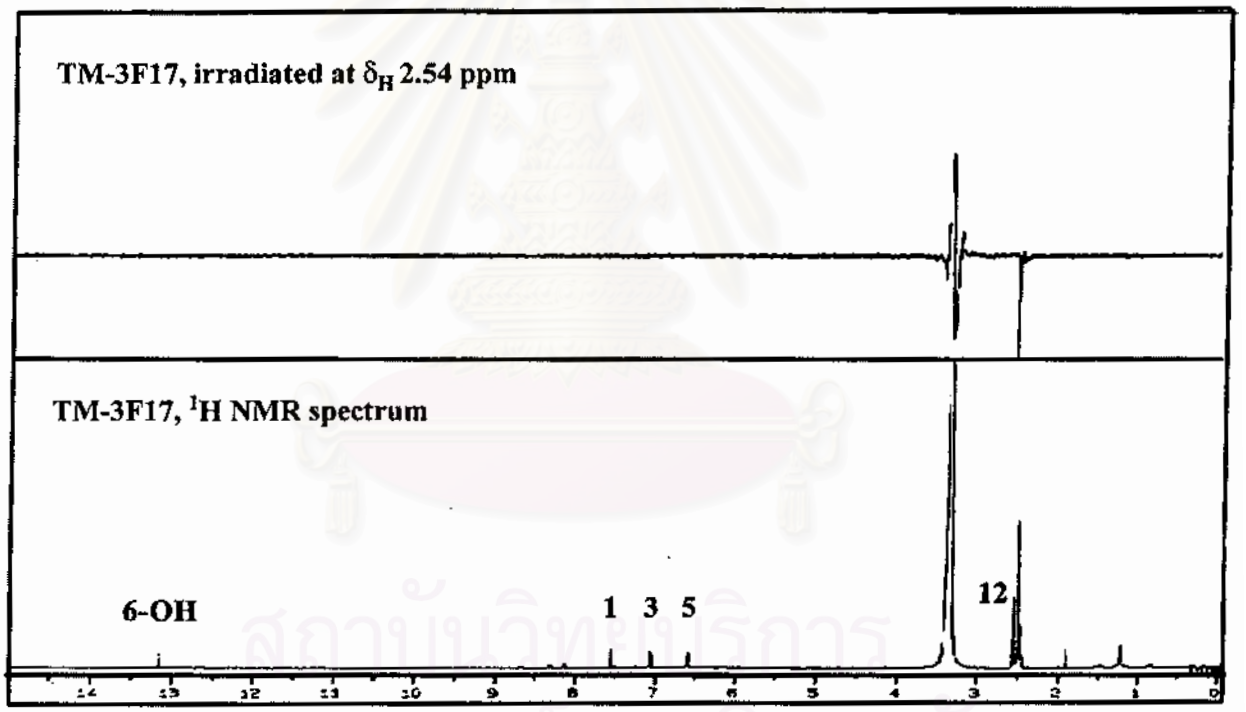


Figure 41 The one-dimensional difference NOE spectrum of compound TM-3F17 (in DMSO-d_6).

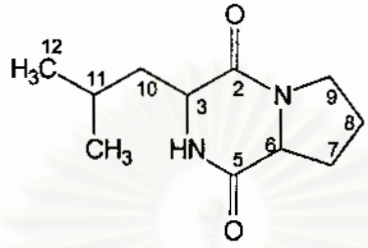


Figure 42 The structure of *cyclo*-(propyl-leucyl) (TM-2F21).

Spectral data of compound TM-2F21

$[\alpha]^{20}_D$	:	+ 6.4° (c 0.14 g/ 100 ml, in methanol)
ESI-TOF MS	:	*m/z* (% relative intensity); Figure 43
		211 ($[M+H]^+$, 100)
UV	:	λ_{max} nm (log ϵ), in methanol; Figure 44
		320 (2.54), 292 (2.66), 260 (2.67), 205 (3.08)
IR	:	ν_{max} cm^{-1}, KBr disc; Figure 45
		3265, 2955, 2877, 1674, 1641, 1434, 1306, 710, 640
^1H NMR	:	δ_{ppm}, 300 MHz, in $CHCl_3$; see Figure 46 and Table 19
^{13}C NMR	:	δ_{ppm}, 75 MHz, in $CHCl_3$; see Figure 47 and Table 19

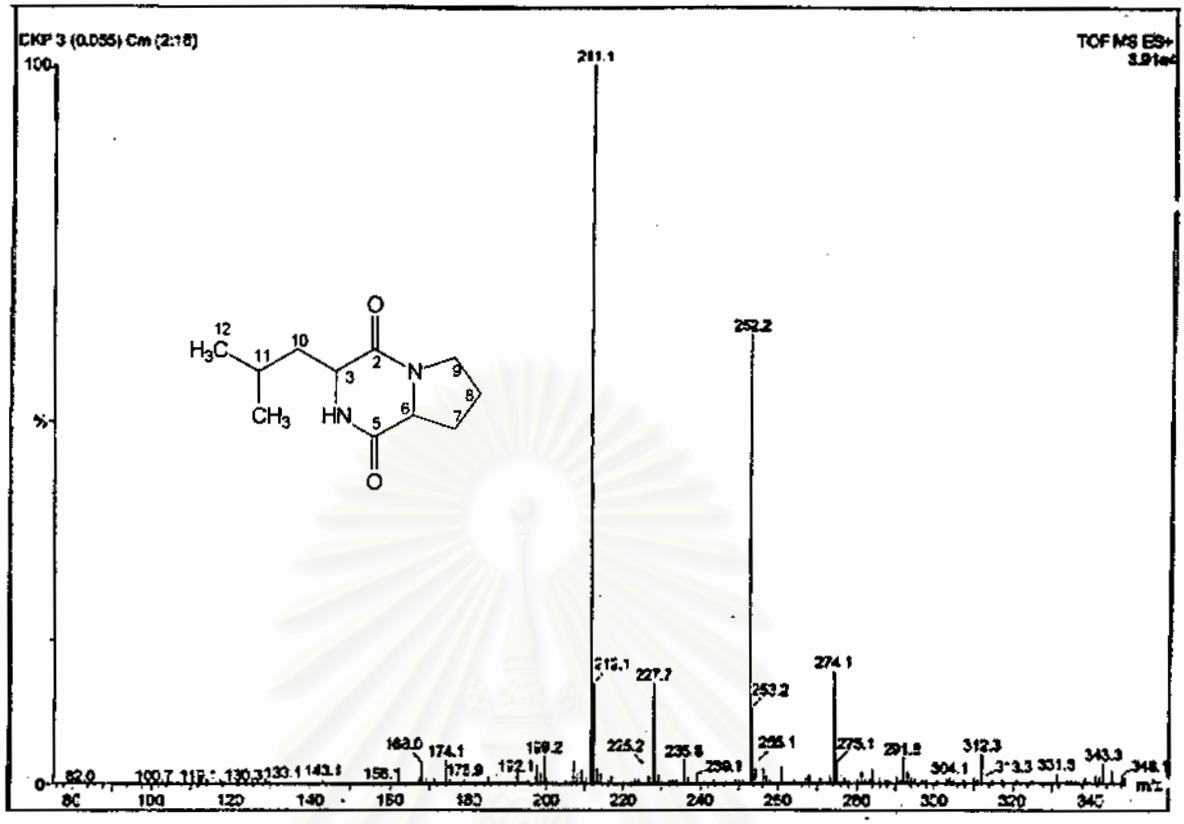


Figure 43 The ESI-TOF MS spectrum of compound TM-2F21.

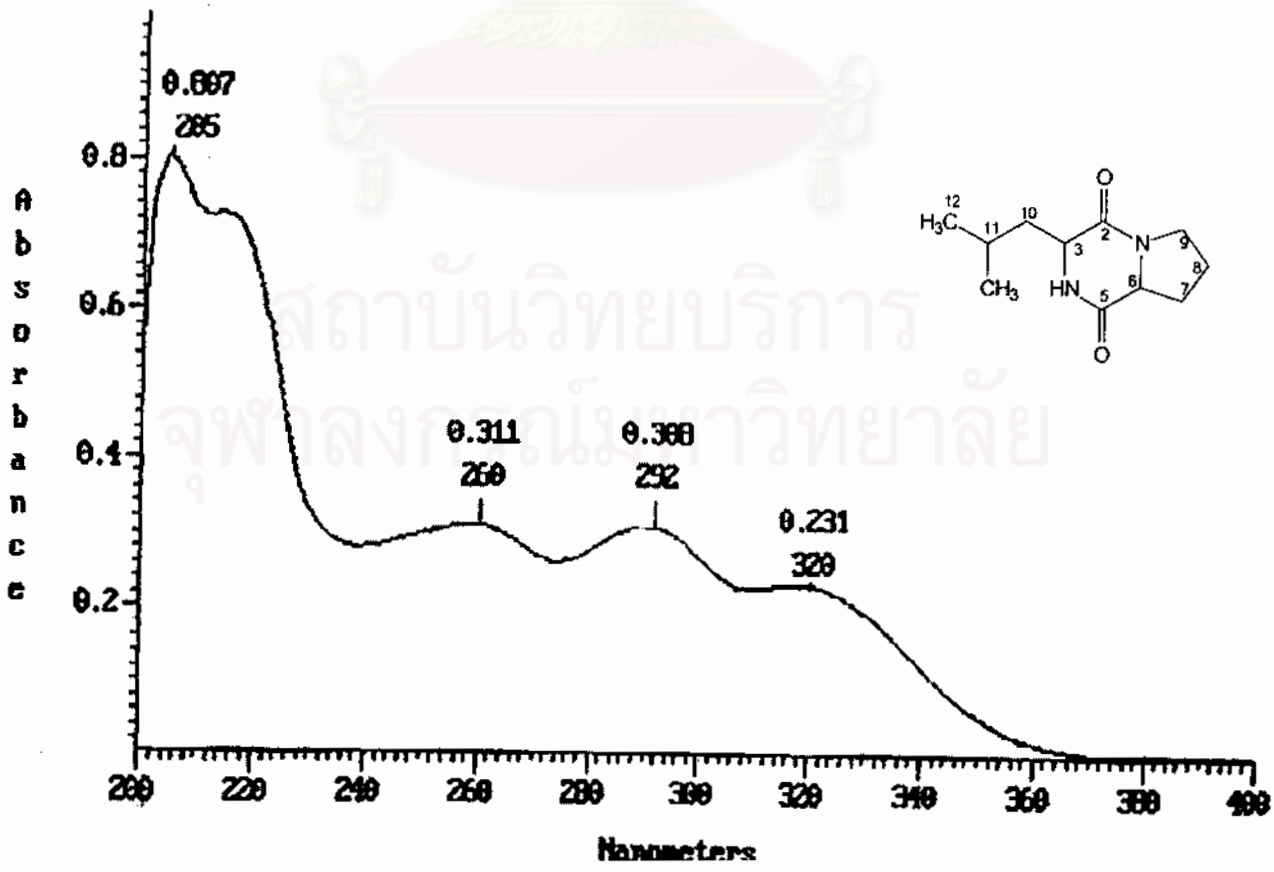


Figure 44 The UV spectrum of compound TM-2F21 (in MeOH).

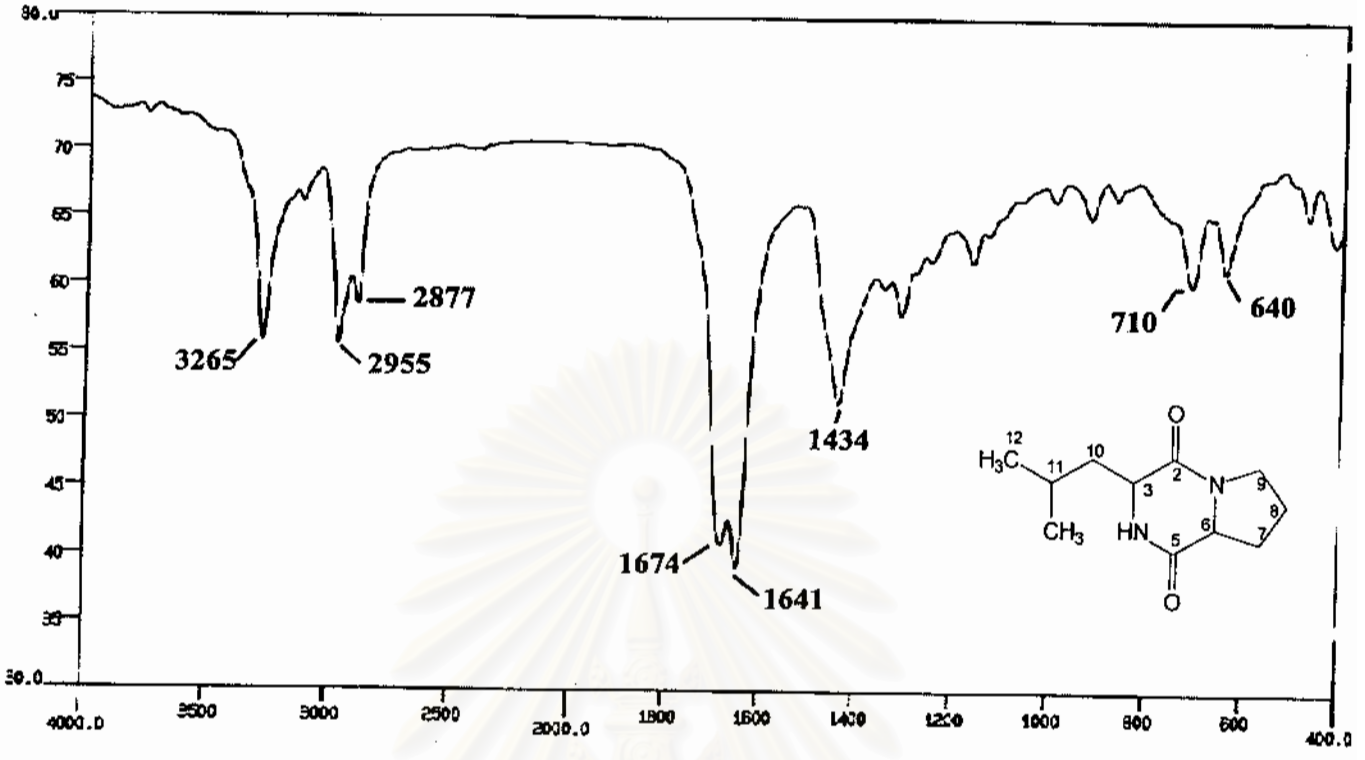


Figure 45 The IR spectrum of compound TM-2F21(KBr disc).

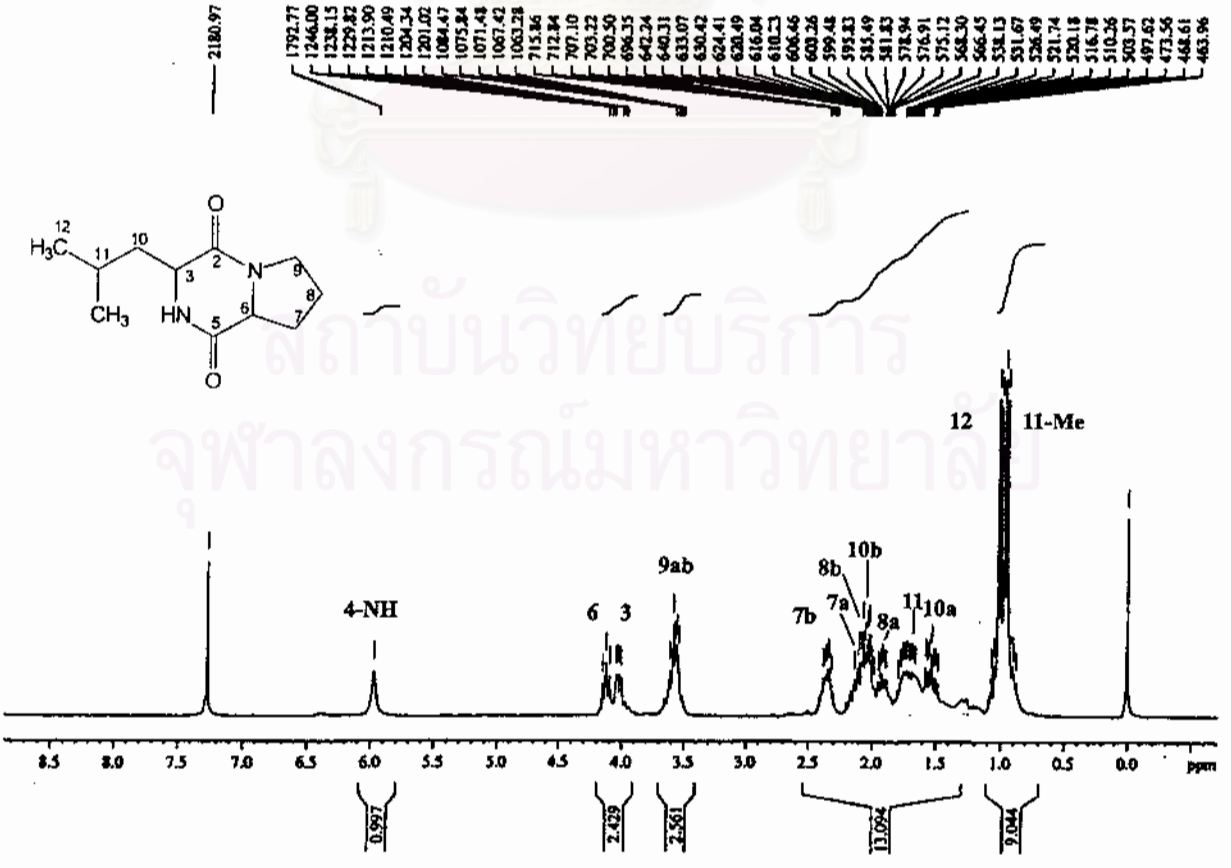


Figure 46 The 300 MHz ^{1}H NMR spectrum of compound TM-2F21 (in $CDCl_3$).

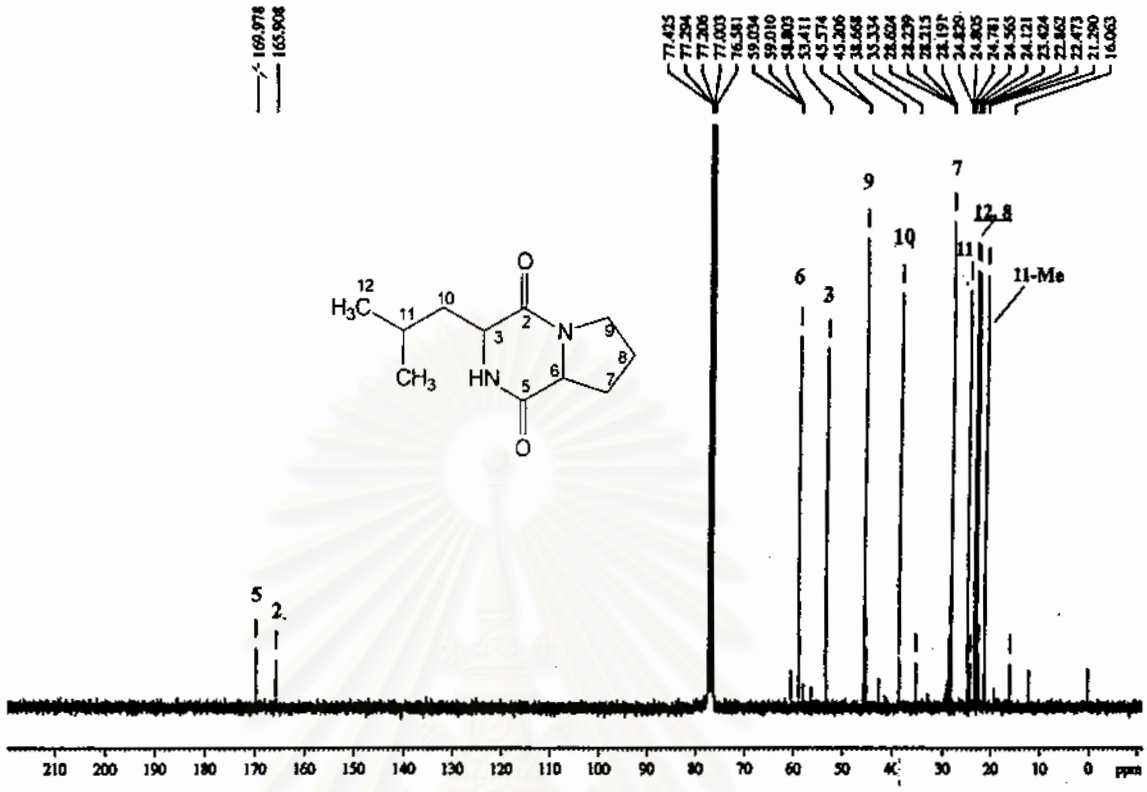


Figure 47 The 75 MHz ^{13}C NMR spectrum of compound TM-2F21 (in $CDCl_3$).

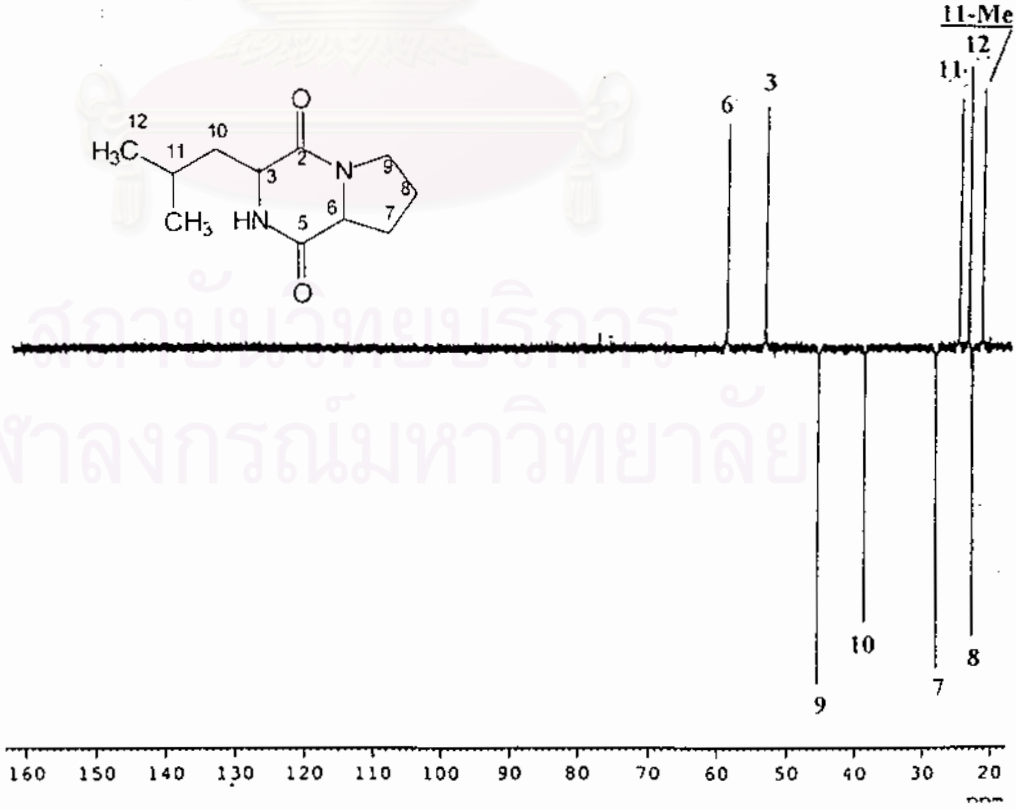


Figure 48 The 75 MHz DEPT-135 spectrum of compound TM-2F21 (in $CDCl_3$).

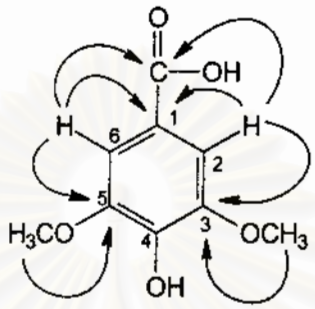


Figure 49 The long-range heteronuclear correlations observed in the HMBC spectrum of syringic acid (TM-2F39).

Spectral data of compound TM-2F39

EIMS	:	*m/z* (% relative intensity); Figure 50
		198 ($[M]^{+\bullet}$, 11), 127 (19), 109 (12), 93 (13), 84 (19), 81 (26),
		79 (37), 69 (43), 67 (66), 65 (59), 55 (98), 53 (100)
UV	:	λ_{max} nm (log ε), in methanol; Figure 51
		320 (3.68), 292 (3.77), 257 (3.75)
IR	:	ν_{max} cm^{-1}, KBr disc; Figure 52
		3377 (br), 1702, 1620, 1523, 1462, 1420, 1378, 1324, 1211,
		1180, 772, 691
^{1}H NMR	:	δ_{ppm}, 300 MHz, in acetone-d_6; see Figure 53 and Table 20
^{13}C NMR	:	δ_{ppm}, 75 MHz, in acetone-d_6; see Figure 54 and Table 20

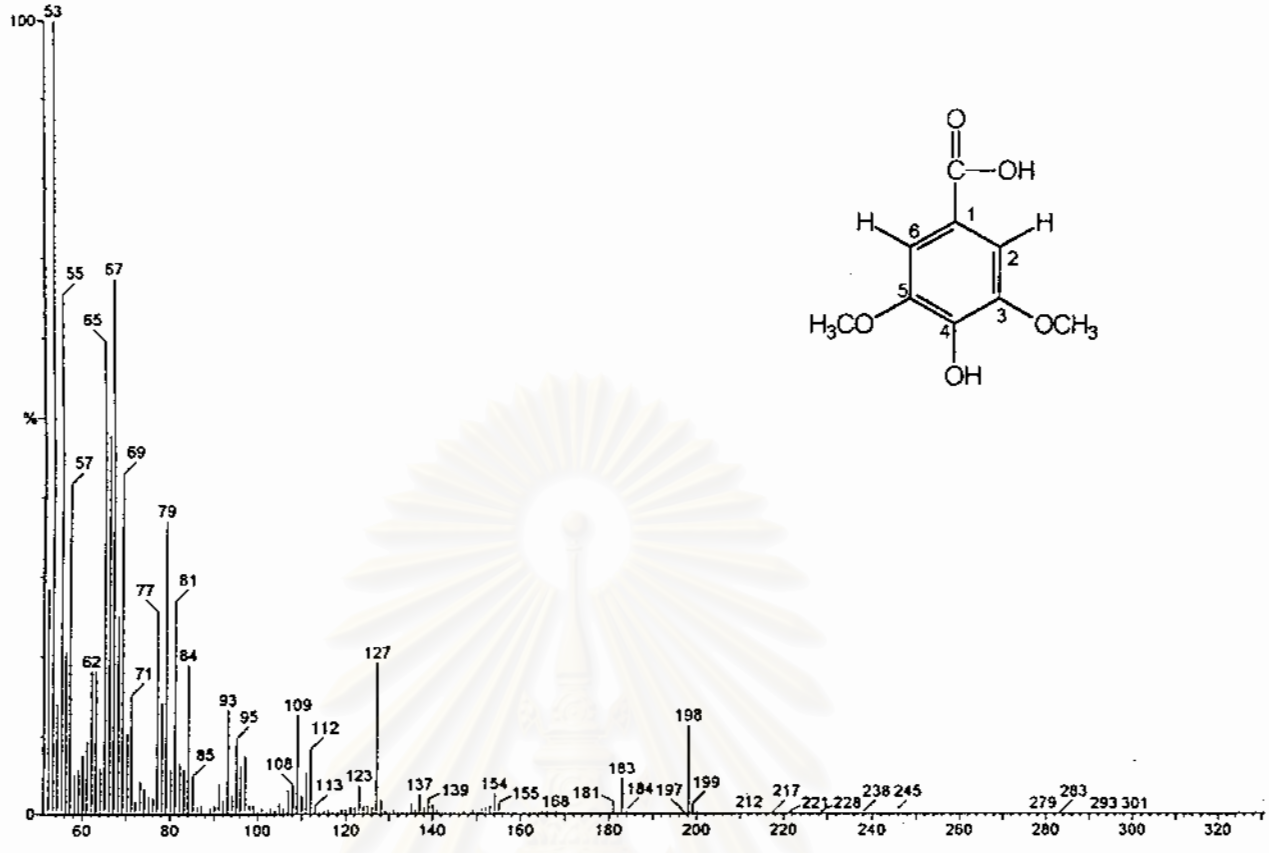


Figure 50 The EIMS spectrum of compound TM-2F39.

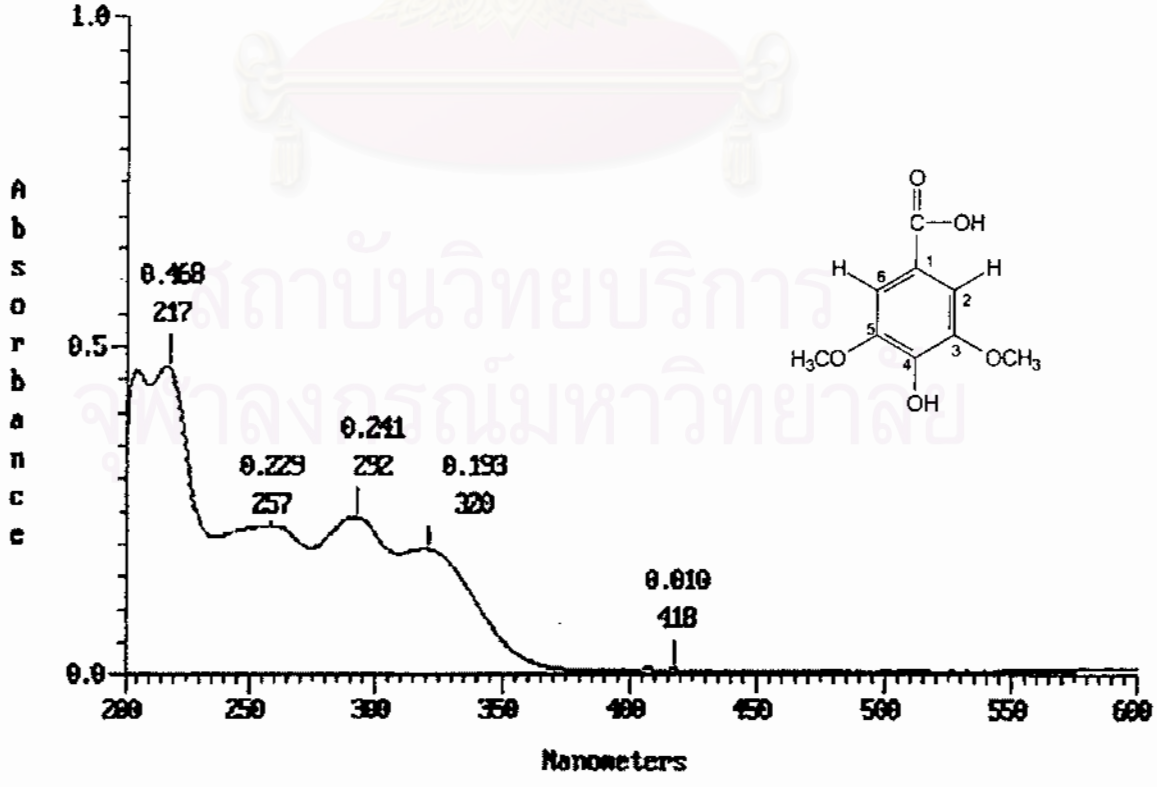


Figure 51 The UV spectrum of compound TM-2F39 (in MeOH).

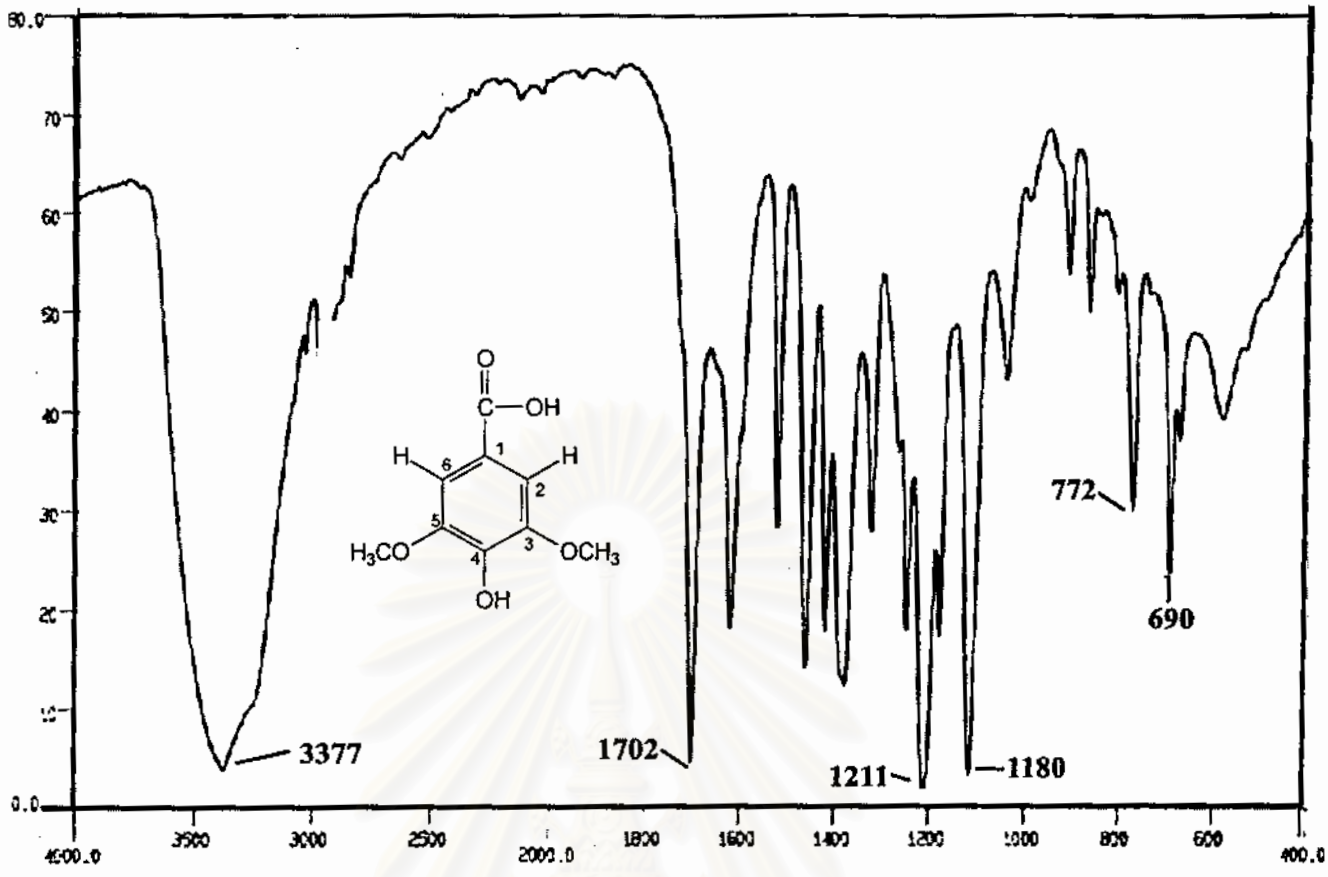


Figure 52 The IR spectrum of compound TM-2F39 (KBr disc).

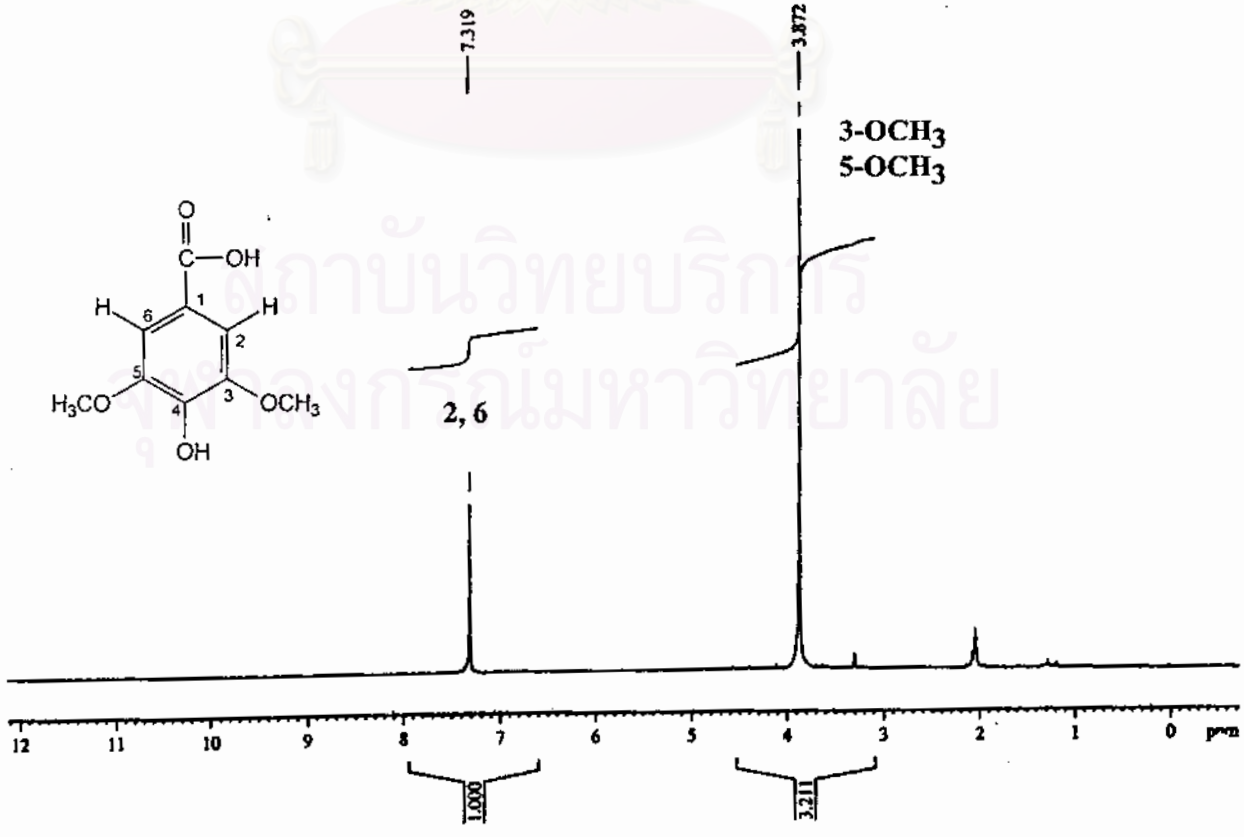


Figure 53 The 300 MHz ^{1}H NMR spectrum of compound TM-2F39 (in acetone-d_6).

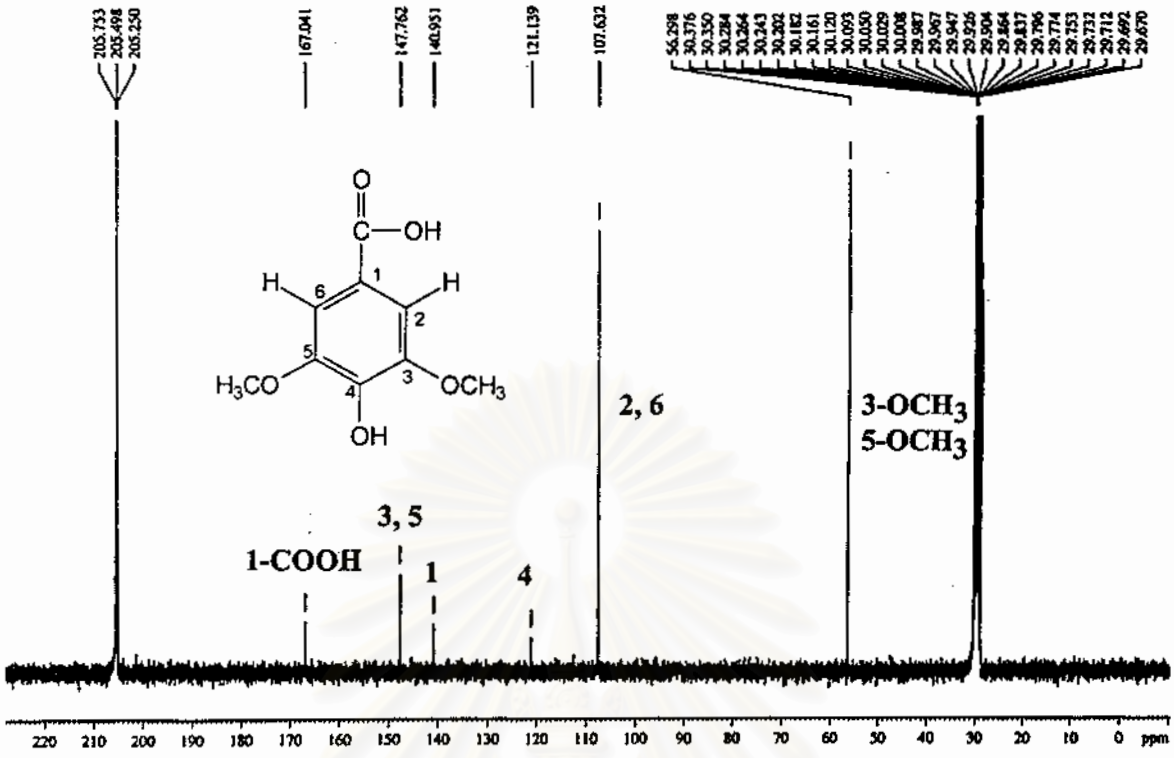


Figure 54 The 75 MHz ^{13}C NMR spectrum of compound TM-2F39 (in acetone-d_6).

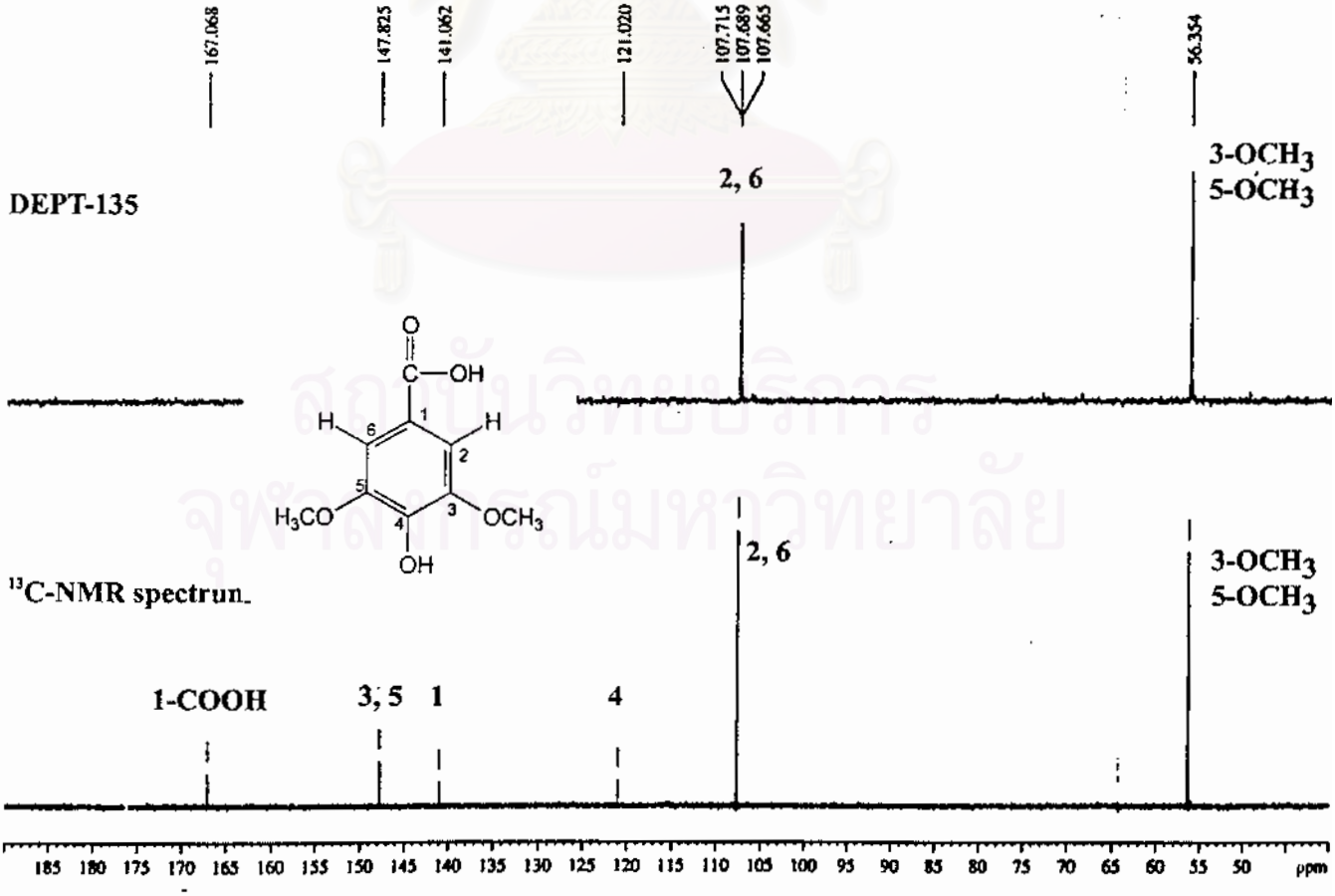


Figure 55 The 75 MHz ^{13}C and DEPT-135 spectra of compound TM-2F39 (in acetone-d_6).

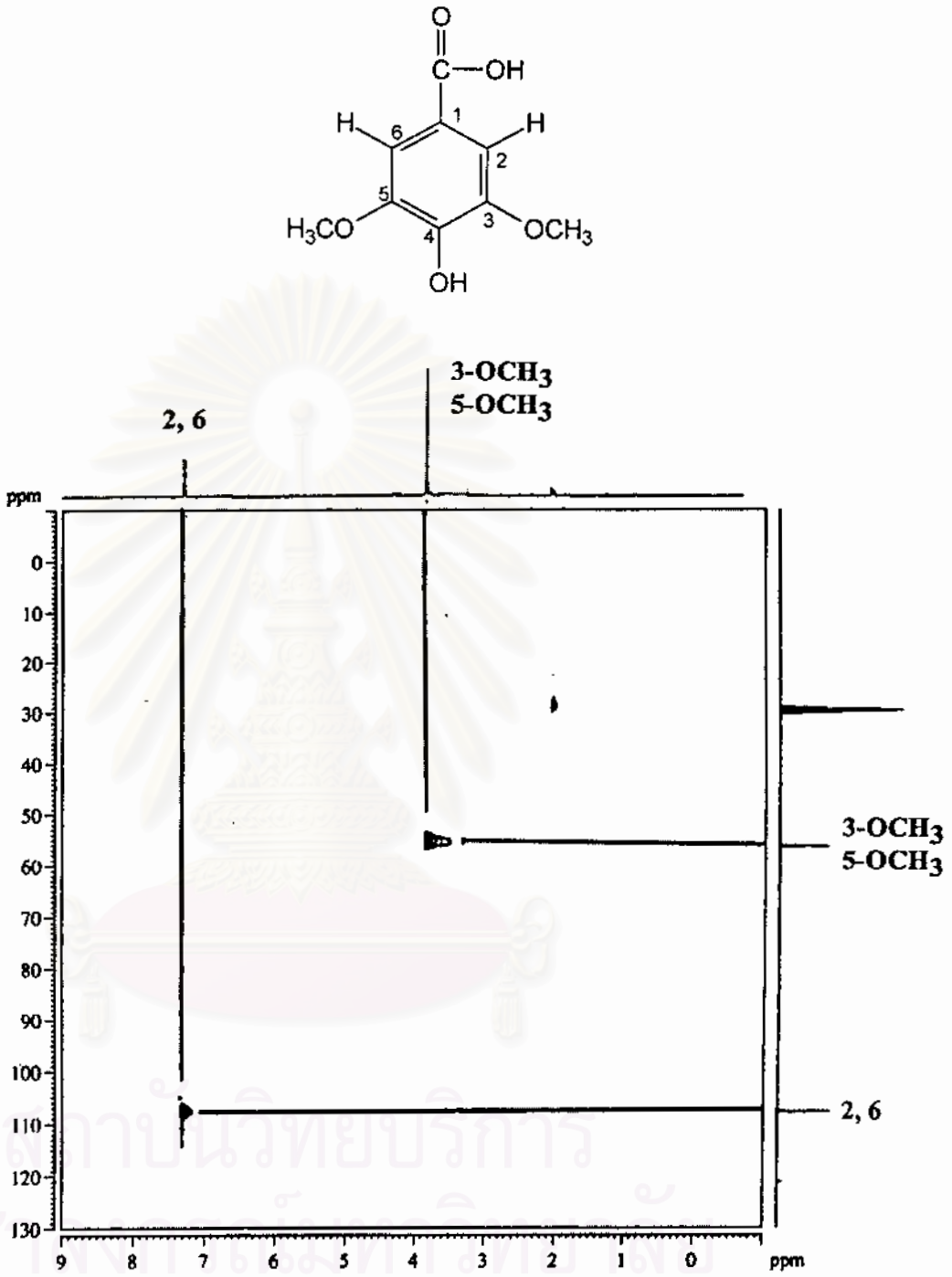


Figure 56 The 300 MHz HMQC spectrum of compound TM-2F39 (in acetone-d_6).

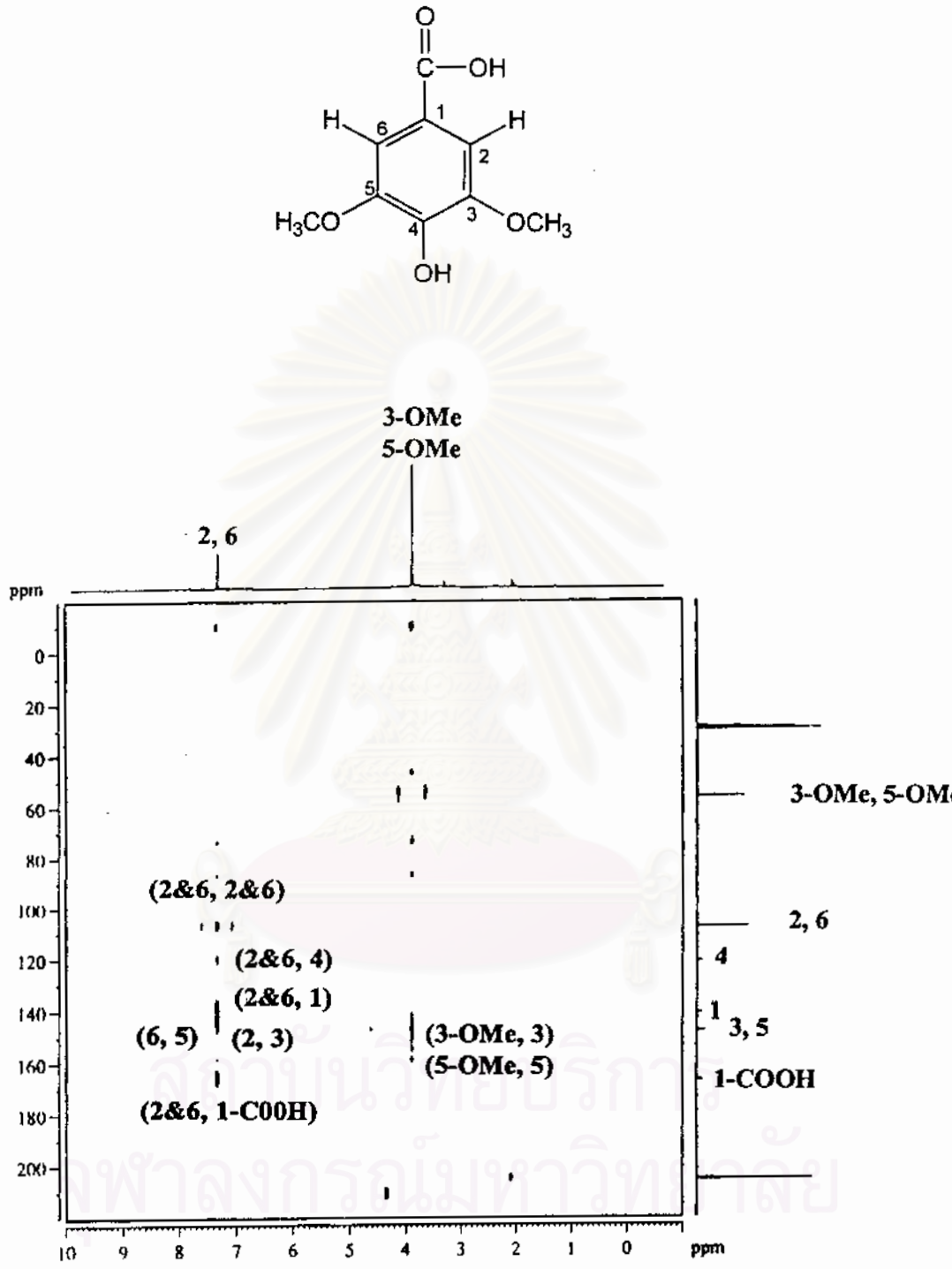


Figure 57 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-2F39 (in acetone-d_6).

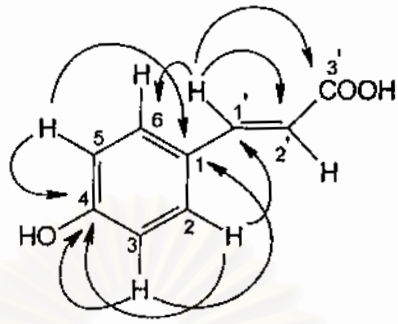


Figure 58 The long-range heteronuclear correlations observed in the HMBC spectrum of *p*-coumaric acid (TM-2F07).

Spectral data of compound TM-2F07

ESI-TOF MS	:	*m/z* (% relative intensity); Figure 59
		165 ($[M+H]^+$, 42), 147 (28)
UV	:	λ_{max} nm (log ϵ), in methanol; Figure 60
		309 (4.32), 223 (4.18)
IR	:	ν_{max} cm^{-1}, KBr disc; Figure 61
		3382 (br), 1674, 1629, 1602, 1513, 1450, 1380, 1246, 1216, 1173, 980, 834
^1H NMR	:	δ_{ppm}, 300 MHz, in acetone-d_6 + $CDCl_3$; see Figure 62 and Table 21
^{13}C NMR	:	δ_{ppm}, 75 MHz, in acetone-d_6 + $CDCl_3$; see Figure 63 and Table 21

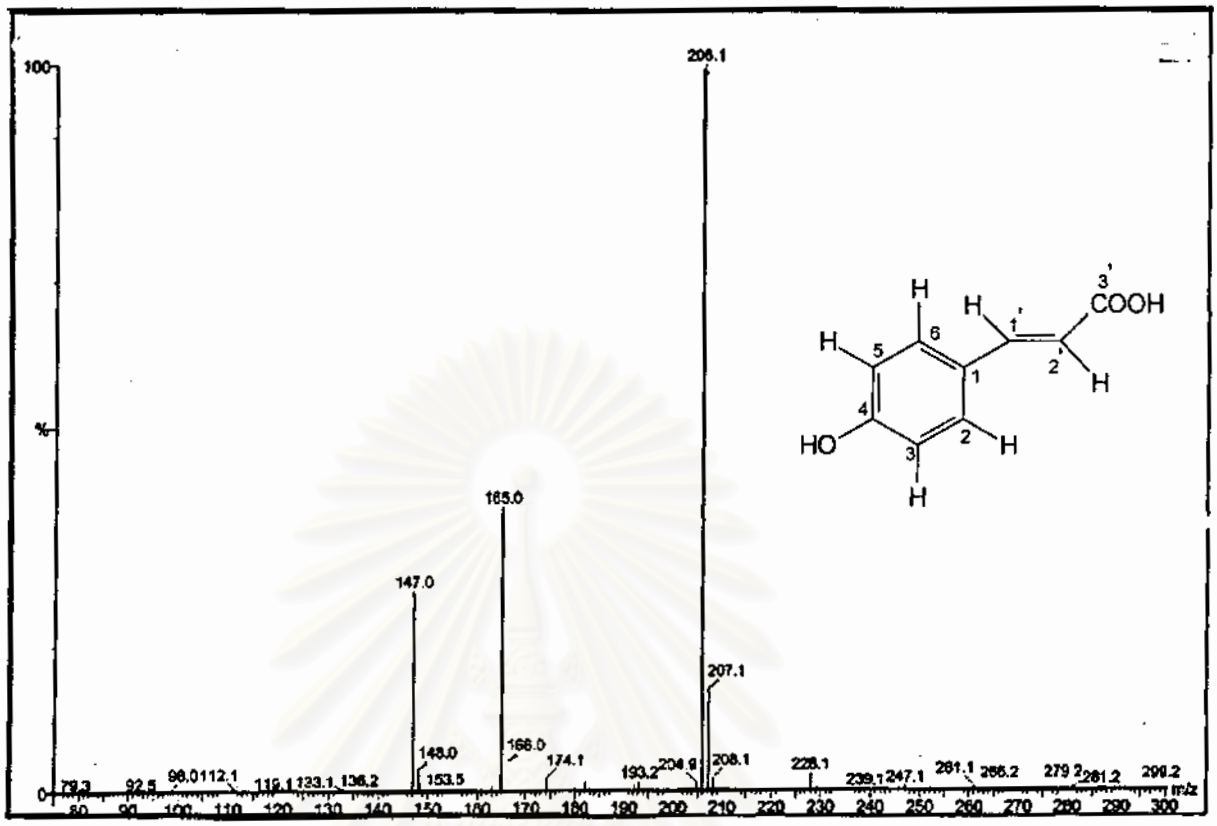


Figure 59 The ESI-TOF MS spectrum of compound TM-2F07.

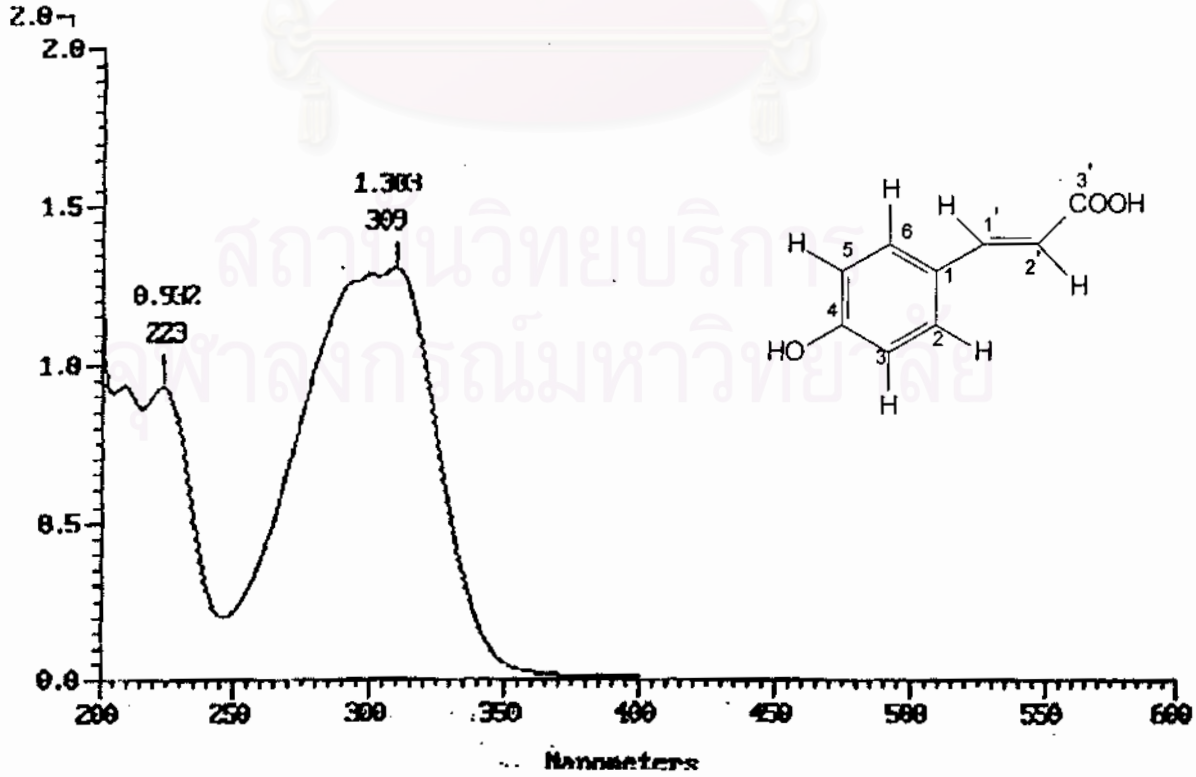


Figure 60 The UV spectrum of compound TM-2F07 (in MeOH).

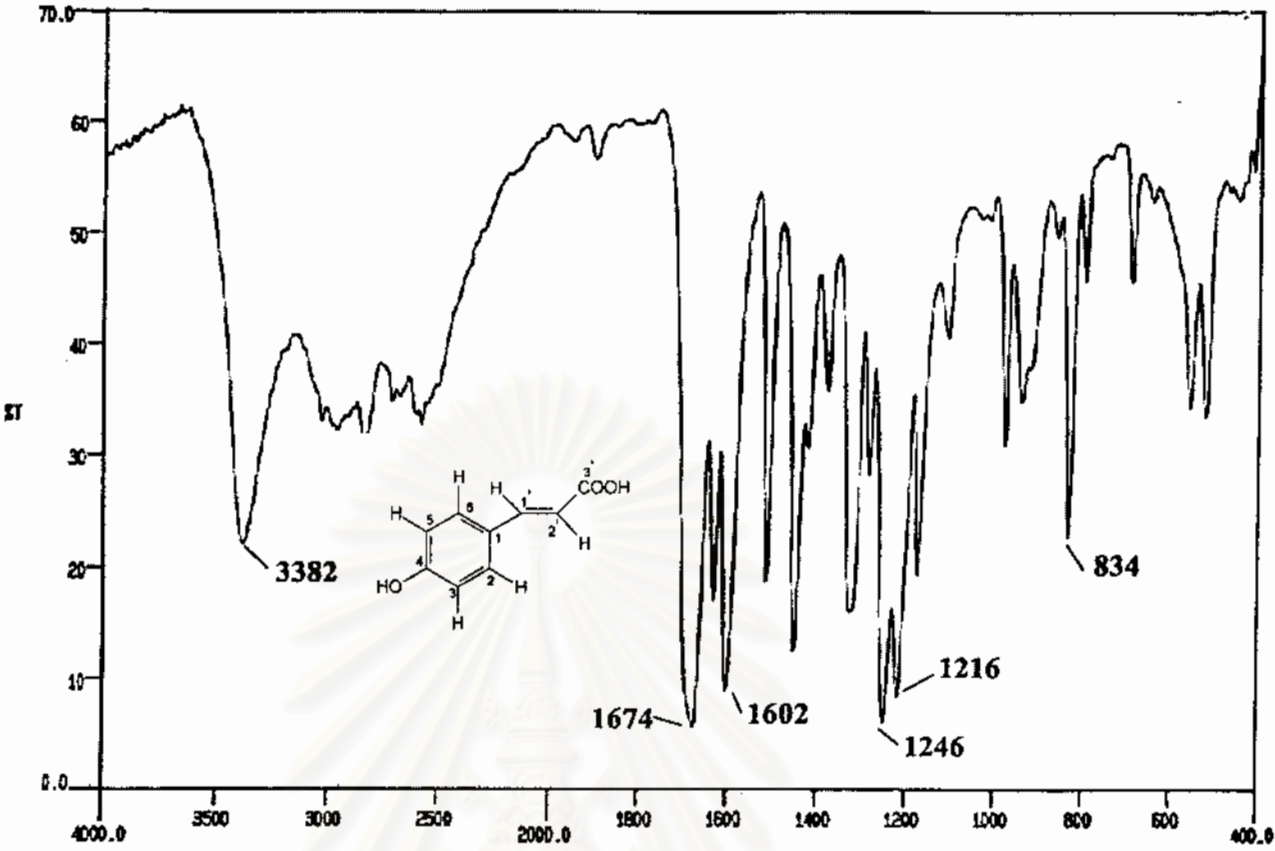


Figure 61 The IR spectrum of compound TM-2F07 (KBr disc).

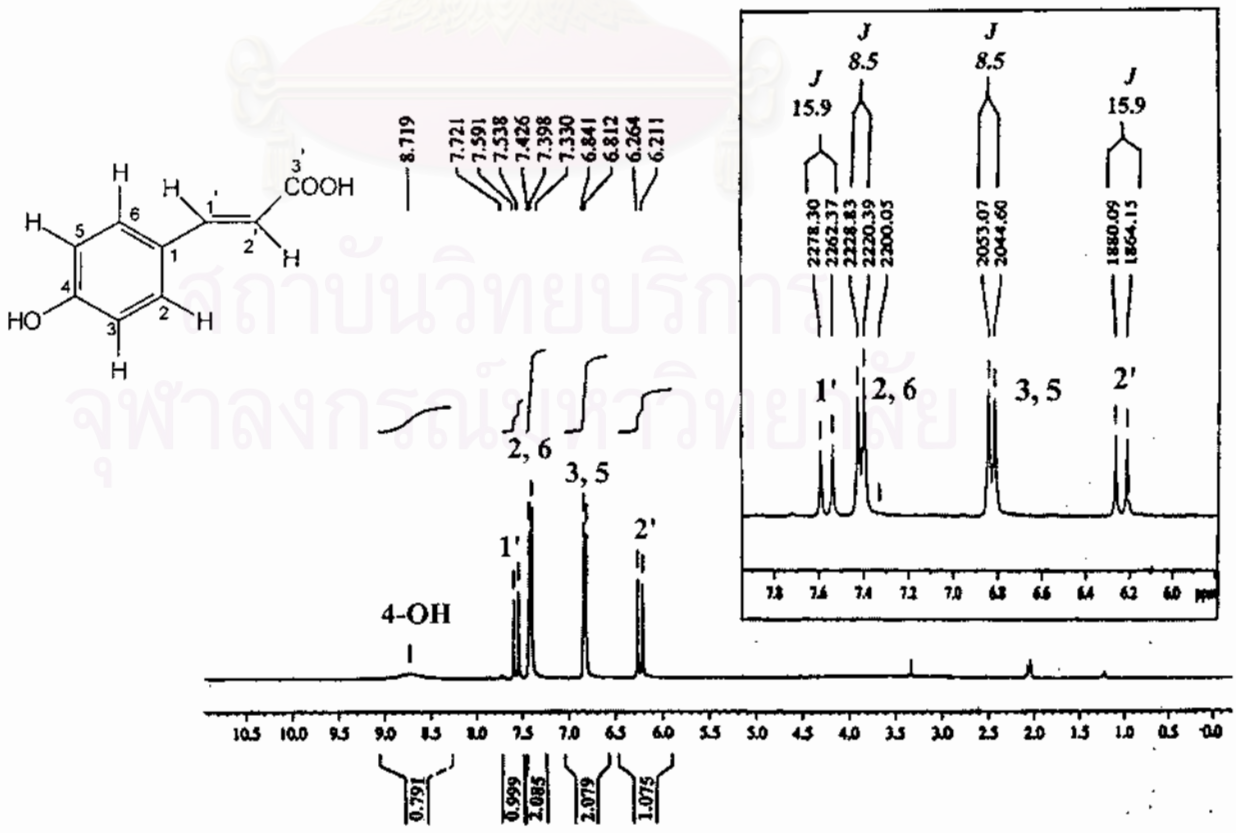


Figure 62 The 300 MHz ^{1}H NMR spectrum of compound TM-2F07 (in acetone-d_6 +$CDCl_3$).

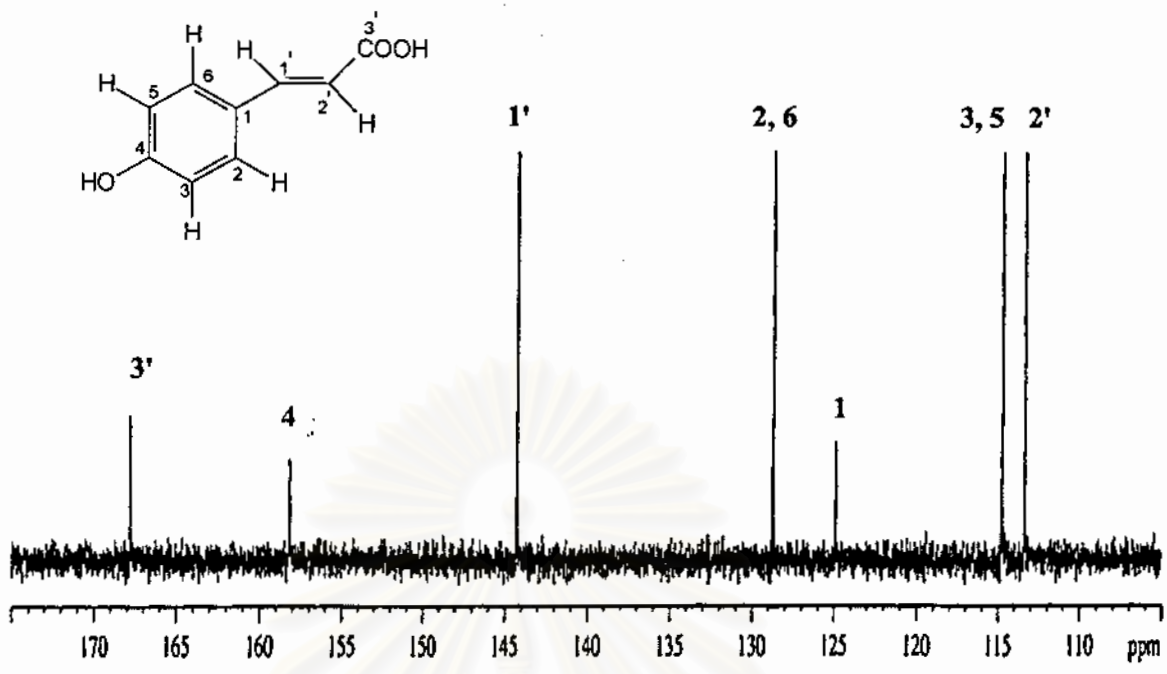


Figure 63 The 75 MHz ^{13}C NMR spectrum of compound TM-2F07 (in acetone-d_6 + $CDCl_3$)

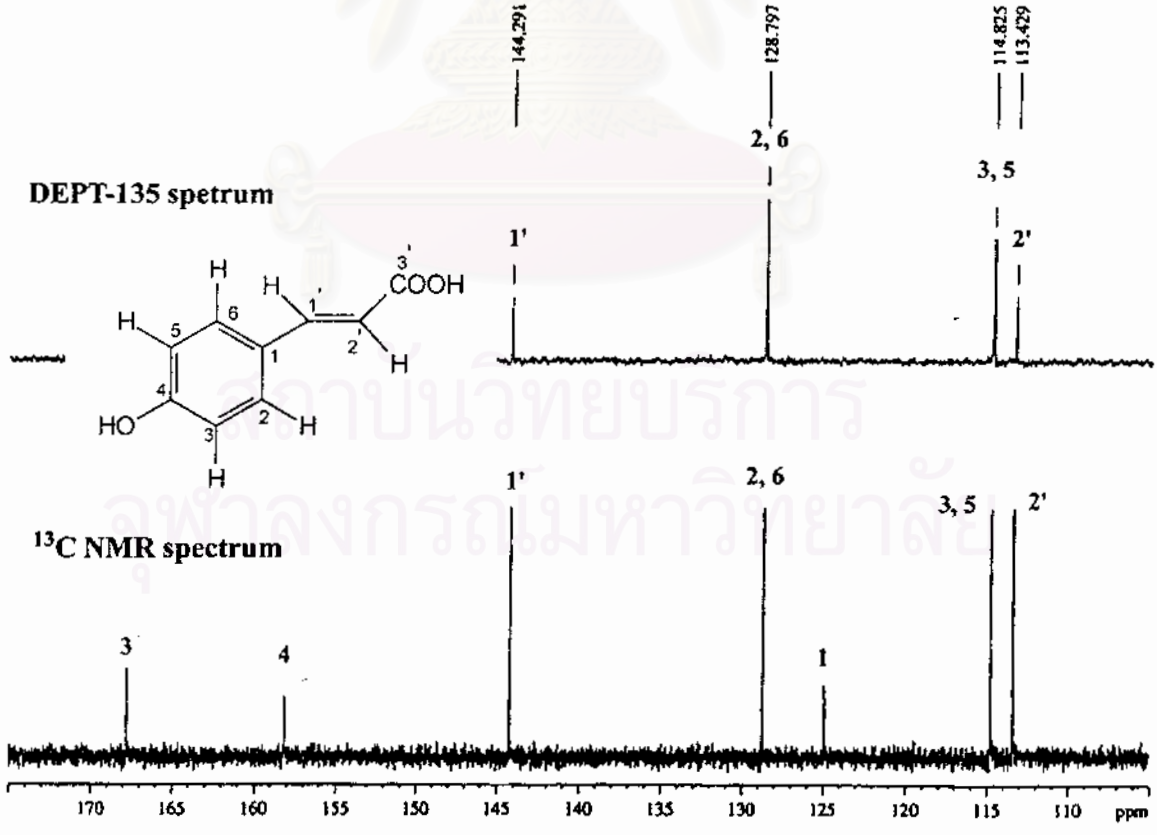


Figure 64 The 75 MHz ^{13}C and DEPT-135 spectra of compound TM-2F07 (in acetone-d_6 + $CDCl_3$

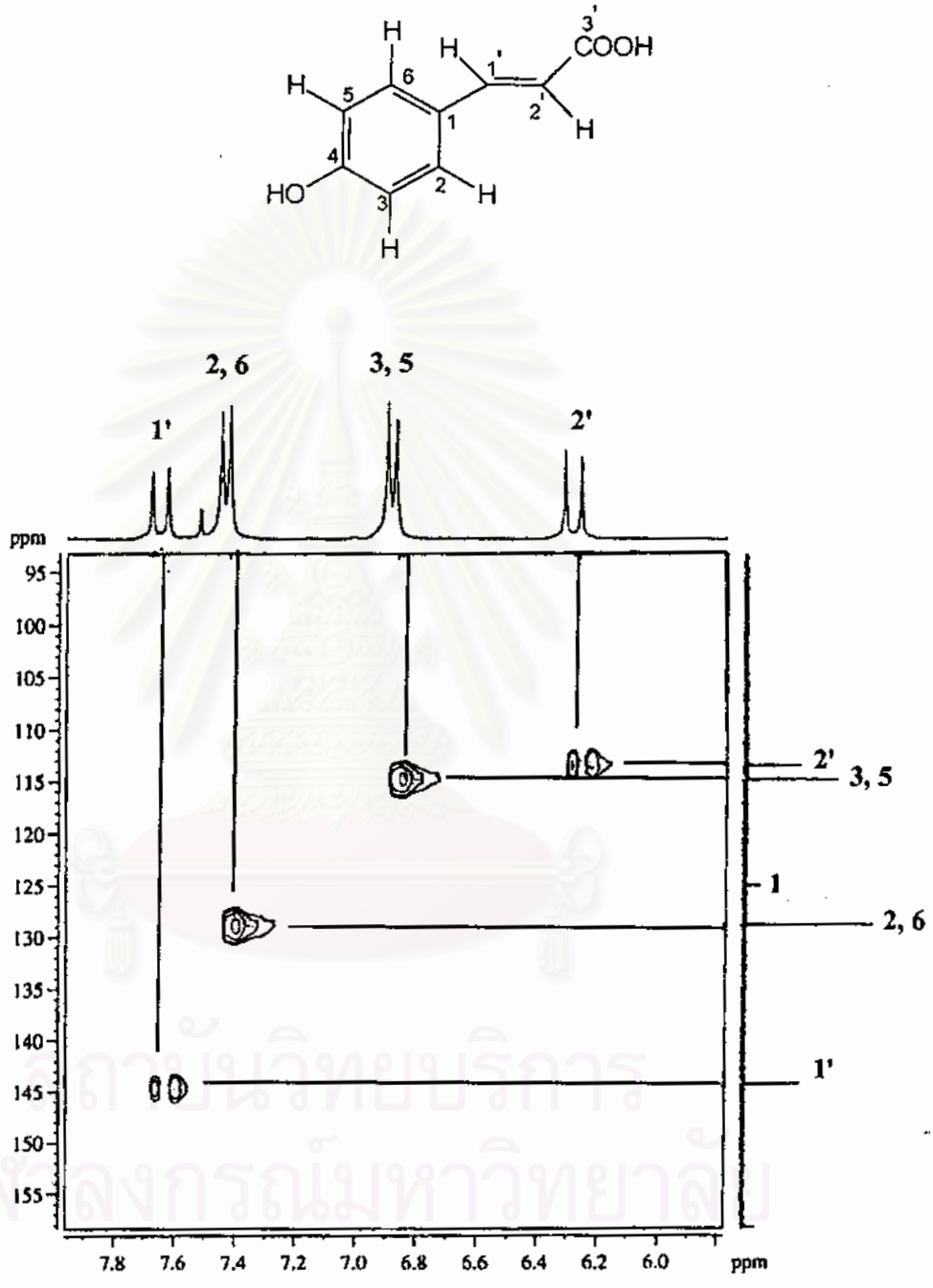


Figure 65 The 300 MHz HMQC spectrum of compound TM-2F07 (in acetone-d_6 + $CDCl_3$).

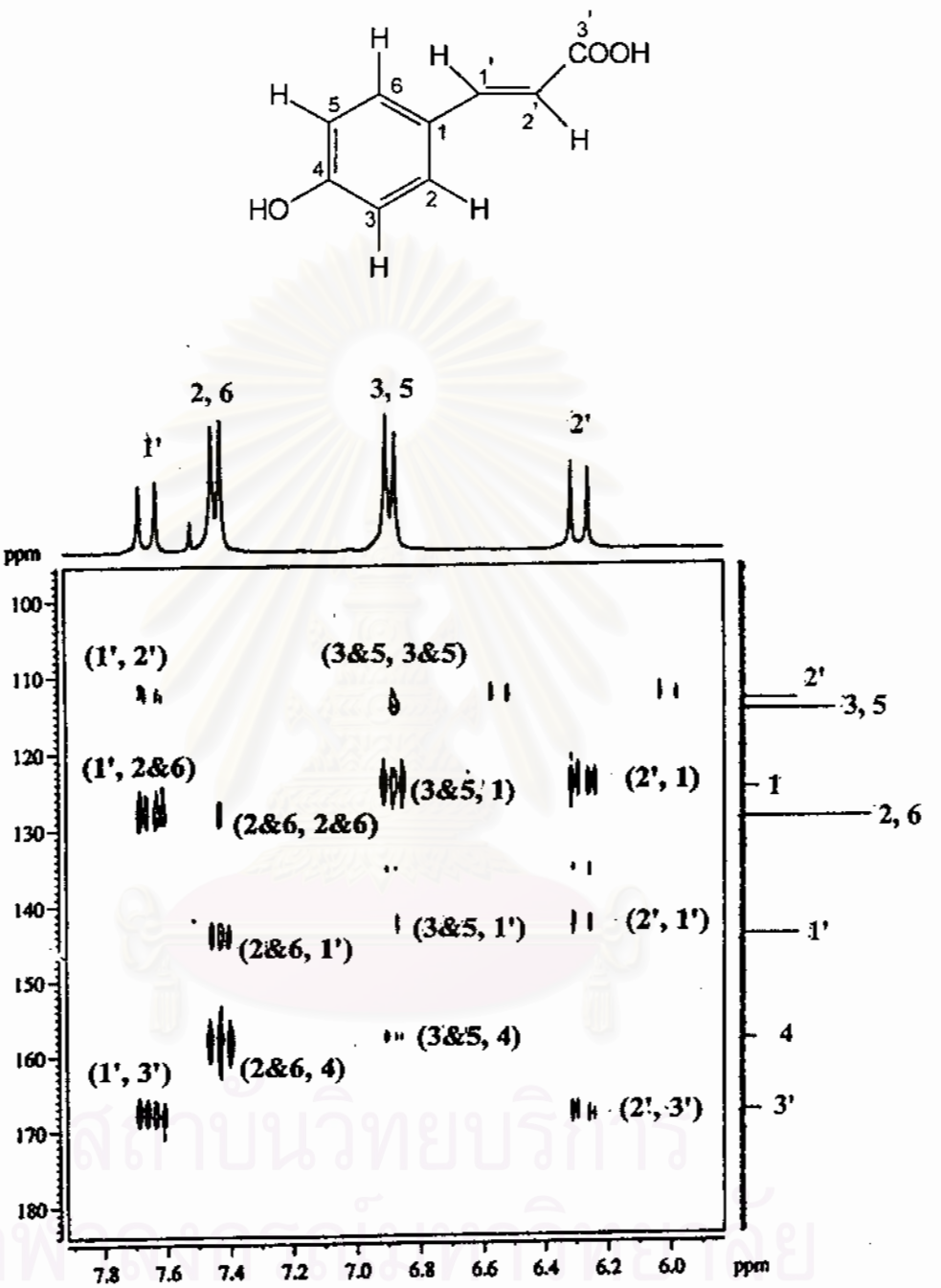


Figure 66 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of compound TM-2F07 (in acetone-d_6 + $CDCl_3$

APPENDIX B

MEDIA

All media were dispensed and sterilized in autoclave for 15 minutes at 15 pound pressure (121 °C) except for media for carbon utilization test which was sterilized at 110 pound for 10 minutes.

1. Carbon utilization medium (ISP-9)

carbon source

No carbon source (negative control)

D - glucose (positive control)

L- arabinose

Sucrose	D – fructose
D –xylose	Rhamnose
Inosital	Raffinose
D – manitol	Cellulose

Pridham and Gottlieb trace salt

$CuSO_4.5H_2O$	0.64	g
$FeSO_4.7H_2O$	0.11	g
$MnCl_2.4H_2O$	0.79	g
$ZnSO_4.7H_2O$	0.15	g
Distilled water	1,000	ml

Basal mineral salt agar

$(NH_4)_2SO_4$	2.64	g
K_2HPO_4(anhydrous)	2.38	g
$K_2HPO_4.3H_2O$	5.65	g
$MgSO_4.7H_2O$	1.00	g
Pridham and Gottlieb trace salt	1.0	ml
Distilled water	1,000	ml
Agar	15-20	g

2. Cellulose decomposition medium

Czapek's solution, free from sucrose.

3. Glucose beef extract peptone medium (GBP)

Glucose	15	g
Beef extract	3	g
Peptone	6	g
Yeast extract	3	g
Seawater 35 ppt	1,000	ml
$MgSO_4.7H_2O$	2.5	g
pH 7.0-7.4		

4. Glycerol asparagine agar (ISP-5)

L- asparagine (anhydrous)	1.0	g
K_2HPO_4	1.0	g
Glycerol	10.0	g
Distilled water	1,000	ml
Trace salt solution	1	ml
Agar	15-20	g
pH 7.0 – 7.4		

5. Glycerol peptone media (GPM)

Glycerol	20.0	g
Molasses	10.0	g
Beef extract	5.0	g
Peptone	5.0	g
$CaCO_3$	4.0	g
Seawater 35 ppt	1,000	ml

6. Inorganic salt-starch agar

Difco soluble starch	10.0	g
K_2PO_4(anydrous)	1.0	g
$MgSO_4.7H_2O$	1.0	g
NaCl	1.0	g
$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
Distilled water	1,000	ml
Trace salt solution	1	ml
Agar	15-20	g
pH 7.0 – 7.4		

7. Nutrient gelatin broth

Peptone	10.0	g
Meat extract	5.0	g
NaCl	5.0	g
Gelatin	150.0	g
Distilled water	1,000	ml
pH 7.0-7.2		

8. Oatmeal agar

Oatmeal agar	18.0	g
Distilled water	1,000	ml
pH 7.2		

9. Peptone nitrate broth

Peptone	10.0	g
KNO_3	1.0	g
NaCl	5.0	g
Distilled water	1,000	ml
pH 7.0		

10. Potato carrot agar (PCA)

Potato	30.0	g
Carrot	25.0	g
Tap water	1000	ml
Agar	15-20	g
pH 7.0		

11. Sabouraud's dextrose agar, SDA (Difco® Sabouraud dextrose agar)

Neopeptone	10.0	g
Dextrose	40.0	g
Agar	15	g
Purified water	1,000	ml
pH 5.60 $\pm$ 0.2		

12. Skim milk

Bacto skim milk. dehydrated (Difco®)	100	g
Distilled water	1,000	ml

13. Sodium casienate agar (SCA)

Sodium casienate	20.0	g
Glucose	10.0	g
K_2PO_4(anhydrous)	0.2	g
$MgSO_4.7H_2O$	0.2	g
$FeSO_4.7H_2O$	trace	
Distilled water	1,000	ml
Agar	15-20	g
pH 7.0		

14. Tryptic soy agar, TSA (Difco® Tryptic agar)

Tryptone peptone	15.0	g
(pancreatic digest of casien)		
Soytone peptone	5.0	g
(papaic digest of soybean meal)		
Sodium chloride	5.0	g
Agar	15	g
Purified water	1,000	ml
pH 7.3$\pm$0.2		

15. Tyrosinase reaction agar

Peptone	5.0	g
Meat extract	3.0	g
L – tyrosine	5.0	g
Distilled water	1,000	ml
Agar	15-20	g

16. Tyrosine agar

Glycerol	15.0	g
L – tyrosine	0.5	g
L – asparagine	1.0	g
K_2PO_4(anhydrous)	0.5	g
$MgSO_4.7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4.7H_2O$	0.01	g
Distilled water	1,000	ml
Trace salt solution	1 ml	
Agar	15-20	g
pH 7.2 - 7.4		

17. Yeast extract - malt extract agar (YMA)

Glucose	10.0	g
Peptone	5.0	g
Yeast extract	3.0	g
Malt extract	3.0	g
Distilled water	1,000	ml
Agar	15-20	g

VITA

Miss Suchada Suntornchashwej was born on April 13, 1972 in Bangkok, Thailand. She received her Bachelor's Degree of Science in Pharmacy in 1995 from the Faculty of Pharmacy, Mahidol University, Thailand. After graduation, she had worked in the drug store as a community pharmacist for 2 years. During her study in Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, she has been a recipient of the scholarship from Thailand Graduate Institute of Science and Technology (TGIST).