สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์จากฟ้าทะลายโจร *Andrographis paniculata*

(Burm. F.) Wall. ex Nees สาบเสือ *Chromolaena odoratum* (L.)

***R. M. King & H. Rob.* และหนอนตายหยาก *Stemona* sp.**

นายเริงฤทธิ์ สัปปพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2552

BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI FROM *Andrographis paniculata* (Burm. F.) Wall. ex Nees., *Chromolaena odoratum* (L.) R. M. King & H. Rob. AND *Stemona* sp.

Mr. Ruengrit Sappapan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI FROM *Andrographis paniculata* (Burm. F.) Wall. ex Nees., *Chromolaena odoratum* (L.) R. M. King & H. Rob. AND *Stemona* sp.
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Field of study	Biotechnology
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เริงฤทธิ์ สัปปพันธ์ : สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์จากฟ้าทะลายโจร สาบเสือ และหนอนตายหยาก. (BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI FROM *Andrographis paniculata* (Burm. F.) Wall. ex Nees., *Chromolaena odoratum* (L.) R. M. King & H. Rob. AND *Stemona* sp.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. ขนิษฐา พุดหอม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นงลักษณ์ ศรีอุบลมาศ, 119 หน้า.

ราเอนโดไฟต์ *Exserohilum rostratum* จากหนอนตายหยาก ผลิตสารใหม่ 1 ชนิด คือ 11-hydroxymonocerin (**2**) และสารที่มีรายงานแล้ว 2 ชนิด คือ monocerin (**1**) และ 12-hydroxymonocerin (**3**) หลังจากการสังเคราะห์สารอนุพันธ์ของ สาร **1** และ **2** ทำให้ได้สารใหม่อีก 8 ชนิด คือ 8-acetylmonocerin (**4**), diacetylmonocerin (**5**), 8-benzoylmonocerin (**6**), 8-methylmonocerin (**7**), 5-bromomonocerin (**8**), 8-hydroxy-6,7-dimethoxy-3-(pentan-2′-yl-acetate)-isocoumarin (**9**), 11-Oxomonocerin (**10**) and 11-carbonylmonocerin (**11**) พบว่า สาร **1, 2, 4, 5, 6** และ **10** มีฤทธิ์จำเพาะในการยับยั้งเชื้อมาลาเรียสายพันธุ์ดื้อยา *Plasmodium falciparum* K1 strain, IC_{50} = 0.69, 7.42, 0.82, 9.1, 7.85 และ 13.4 μM ตามลำดับ

นอกจากนี้ราเอนโดไฟต์จากฟ้าทะลายโจรผลิตสารที่มีรายงานแล้ว 3 ชนิดคือ *N*-Phenetyl-acetamide (**12**) tryptophol (**13**) และ indole-3-acetamide (**14**) และราเอนโดไฟต์จากสาบเสือผลิตสารที่มีรายงานแล้ว 2 ชนิดคือ cytochalasin D (**15**) และ cytochalasin H (**16**)

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....เริงฤทธิ์ สัปปพันธ์.....

ปีการศึกษา.....2552.....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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

RUENGRIT SAPPAPAN: BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI FROM *Andrographis paniculata* (Burm. F.) Wall. ex Nees., *Chromolaena odoratum* (L.) R. M. King & H. Rob. AND *Stemona* sp. THESIS ADVISOR : ASST. PROF. KHANITHA PUDHOM, Ph.D., THESIS CO-ADVISOR ASSOC. PROF. NONGLUKSNA SRIUBOLMAS, Ph.D., 119 pp.

Exserohilum rostratum, an endophytic fungus, from *Stemona* sp. produced a novel compound, 11-hydroxymonocerin (**2**), and two known compounds -- monocerin (**1**) and 12-hydroxymonocerin (**3**). Compounds **1** and **2** were derivatized to obtain **8** new compounds: 8-acetylmonocerin (**4**), diacetylmonocerin (**5**), 8-benzoylmonocerin (**6**), 8-methylmonocerin (**7**), 5-bromomonocerin (**8**), 8-hydroxy-6,7-dimethoxy-3-(pentan-2′-yl-acetate)-isocoumarin (**9**), 11-Oxomonocerin (**10**) and 11-carbonylmonocerin (**11**). Compounds **1**, **2**, **4**, **5**, **6** and **10** have specific activity against *Plasmodium falciparum* K1 strain with IC_{50} = 0.69, 7.42, 0.82, 9.1, 7.85 and 13.4 μM, respectively.

Endophytic fungal strains from *Andrographis paniculata* produced three known compounds: *N*-Phenetyl-acetamide (**12**), and tryptophol (**13**) and indole-3-acetamide (**14**). Endophytic fungal strains from *Chromolaena odoratum,* produced two known compounds: cytochalasin D (**15**) and cytochalasin H (**16**).

Field of Study……Biotechnology……Student's signature…Ruengrit Sapppan…

Academic year:……2009……Advisor's signature…Khanitha Pudhom

Co-advisor's signature Nongluksna Sriubolmas

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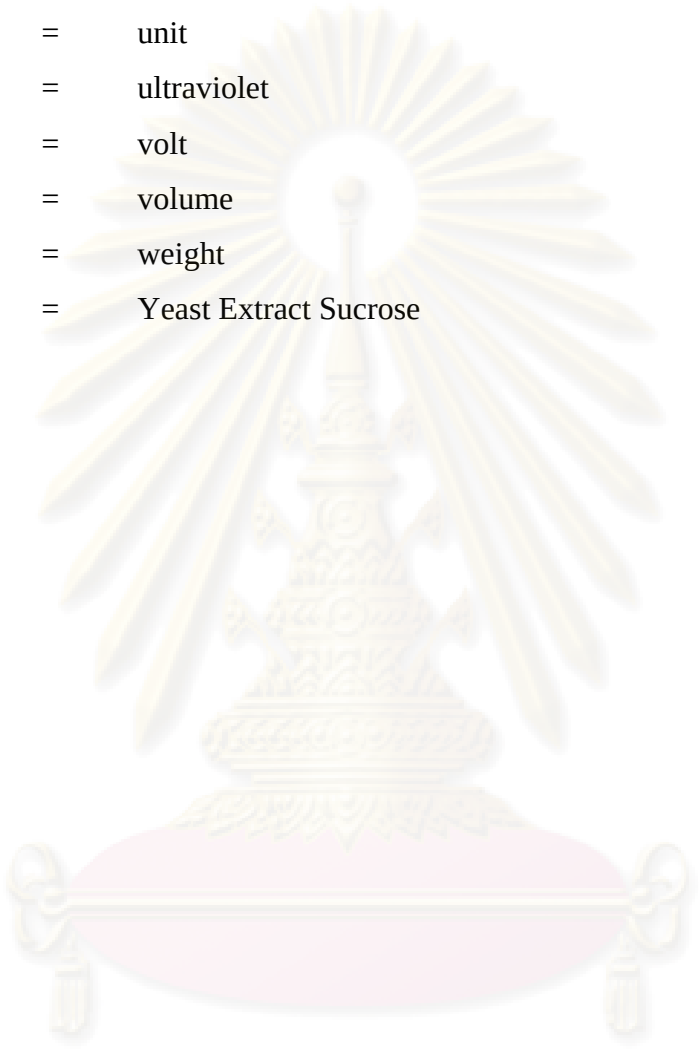
LIST OF ABBREVIATIONS

acetone-d_6	=	deuterated acetone
$^{\circ}$C	=	degree Celsius
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
$CDCl_3$	=	deuterated chloroform
$CHCl_3$	=	chloroform
CH_2Cl_2	=	methylene chloride
δ	=	chemical shift
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublets (for NMR spectral data)
DNA	=	Deoxyribonucleic acid
DEPT	=	distortionless enhancement by polarization transfer
ε	=	molar absorptivity
et al	=	and other
EtOAc	=	ethyl acetate
ESI-TOF MS	=	Electrospray Ionization Time of Flight Mass
g	=	gram
µg	=	microgram
h	=	hour
^{1}H-^{1}H COSY	=	Homonuclear (proton-proton) correlation spectroscopy
^{1}H NMR	=	proton nuclear magnetic resonance
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence
Hz	=	Hertz
IC_{50}	=	inhibitory concentration required for 50% inhibition of growth
IR	=	infrared
ITS	=	internally transcribed spacers
J	=	coupling constant
L	=	liter
µl	=	microliter

λ_{max}	=	wavelength at maximum absorption
M	=	Molar
$[M+Na]^+$	=	pseudomolecular ion
m	=	multiplet (for NMR spectral data)
MCzB	=	Malt Czapek Broth
MeOH	=	methanol
mg	=	milligram
MIC	=	minimum inhibitory concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
MHz	=	megahertz
MS	=	mass spectroscopy
m/z	=	mass to charge ratio
ν_{max}	=	wave number at maximum absorption
nm	=	nanometer
NMR	=	nuclear magnetic resonance
NTP	=	Nucleotide triphosphate
PCR	=	polymerase chain reaction
PDA	=	Potato Dextrose Agar
PDB	=	Potato Dextrose Broth
ppm	=	part per million
q	=	quartet (for NMR spectral data)
rDNA	=	Ribosomal deoxyribonucleic acid
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid
s	=	singlet (for NMR spectral data)
sp	=	species
t	=	triplet (for NMR spectral data)
TAE	=	*Tris*-HCl, acetate and EDTA

TE	=	*Tris*-HCl and EDTA
T_m	=	Melting temperature
TLC	=	thin layer chromatography
U	=	unit
UV	=	ultraviolet
V	=	volt
v	=	volume
w	=	weight
YES	=	Yeast Extract Sucrose



CHAPTER I

INTRODUCTION

Natural products have been the single most productive source of leads for the development of drugs (Harvey, 2008). Of the 20 best-selling non-protein drugs in 1999, nine were either derived from or developed as the result of leads generated by natural products – simvastatin, lovastatin, enalapril, pravastatin, atorvastatin, augmentin®, ciprofloxacin, clarithromycin and cyclosporine with combined annual sales of US$16 billion (Harvey, 2000).

Most of the leads from natural products have come from either plant or microbial sources (Baker *et al*., 2007; Harvey, 2000). Further advances in the ability to culture microbes could provide many novel chemicals for use in drug discovery assays. In the microbial area, the main sources to date have been fungi and terrestrial actinomycetes (Lam, 2006; Fenical and Jensen, 2006; Bull and Stach, 2007).

Endophytic microorganisms are an important source for the discovery of novel bioactive secondary metabolites. Endophytes are a rich source of natural products displaying a broad spectrum of biological activities. The natural product reports from endophytes include immunosupressants, anticancer compounds, antioxidant agents and other biologically active substances. The number of secondary metabolites produced by fungal endophytes is larger than that by other endophytic microorganisms. This may be a consequence of the high frequency of isolation of fungal endophytes from plants. Natural products from fungal endophytes have a broad spectrum of biological activity (Zhang *et al.*, 2006).

Recent interest has focused on endophytic microbes for their pharmaceutical potential (Strobel *et al.,* 1993). Thus, endophytic habitat is a niche that has been continued exploration (Bacon *et al.,* 2000).

The objectives of this study are as follows:

1. To isolate endophytic fungi from *Andrographis paniculata* (Burm. F.) Wall. ex Nees., *Chromolaena odoratum* (L.) R. M. King & H. Rob. and *Stemona* sp.
2. To isolate and chemically characterize bioactive compounds from selected endophytic fungi
3. To classify the selected endophytic fungi based on morphological characteristics and rRNA gene sequences
4. To evaluate biological activities of the isolated compounds

CHAPTER II

LITERATURE REVIEWS

2.1 Cancer

Cancer is one of the most dreaded diseases of the 20th century and spreading further with increasing incidence in 21st century (Balachandran and Govindarajan, 2005).

Cancer is an uncontrolled growth and spread of abnormal cells. It is caused by diet, alcohol, physical activity, infections, hormonal factors, radiation, chemicals, inherited mutations, immune conditions, etc. (American Cancer Society, 2008; WHO technical report series; 916, 2003).

2.2 Malaria

Malaria has been recognized as the most important parasitic disease of humans for centuries. Half of the world's population is at risk of malaria and an estimated 250 million cases led to nearly 1 million deaths (World Malaria Report 2008). Owing to widespread drug resistance, the drug is becoming increasingly ineffective in many parts of the world. Artemisinin resistance is expected to emerge in the near future (Gale *et al.*, 2007).

Malaria transmits by an infected female Anopheles mosquito each time it takes blood meal. Plasmodium parasites transfer from the mosquito to the blood circulation of the victim.

2.3 Endophytic fungi

Horizontally transmitted fungal endophytes reside in the tissues of plants without inducing any visual symptoms of their presence (Sánchez-Márquez *et al.*, 2007; Oses *et al.*, 2008; Rungjindamai *et al.*, 2008; Tao *et al.*, 2008). These fungi appear to have a capacity to produce an array of secondary metabolites exhibiting a variety of biological activity.

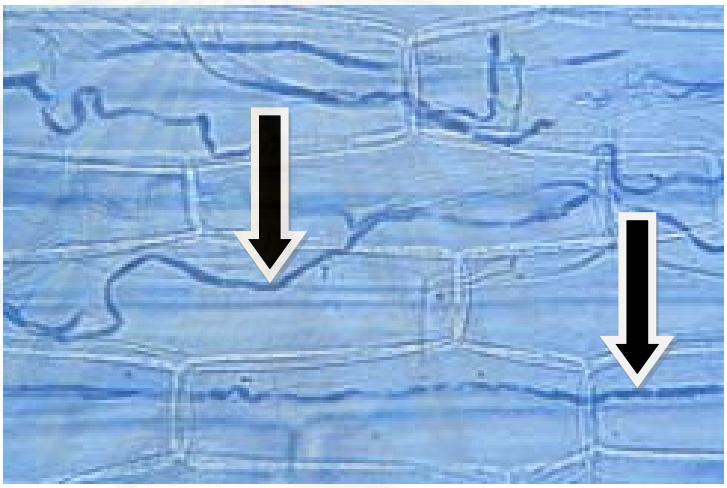


Figure 2.1 Endophytic fungal hyphae (arrows) in plant cells.
(Image from: http://www.peg.ethz.ch/research/Interactions/index_EN)

2.3.1 Biological activities of endophytic fungi

Paclitaxel (Taxol®), the world's first billion-dollar anti-cancer drug, was originally isolated from the bark of the Pacific yew, *Taxus brevifolia* (Wani *et al.*, 1971). Since Taxol® cannot meet the demand of the growing market; Taxol®-producing fungus through fungal fermentation can eliminate the problem (Yuan *et al.*, 2006). Endophytic fungi, associated with *Taxus brevifolia*, may also produce Taxol® (Stierle, Strobel, and Stierle, 1993).

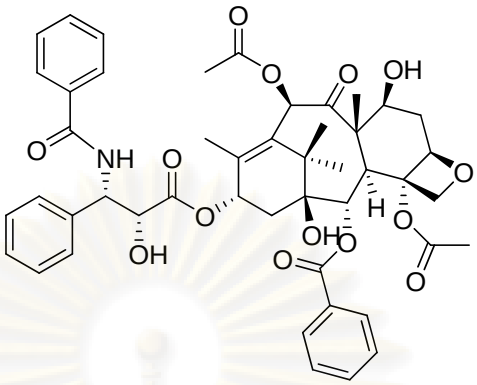
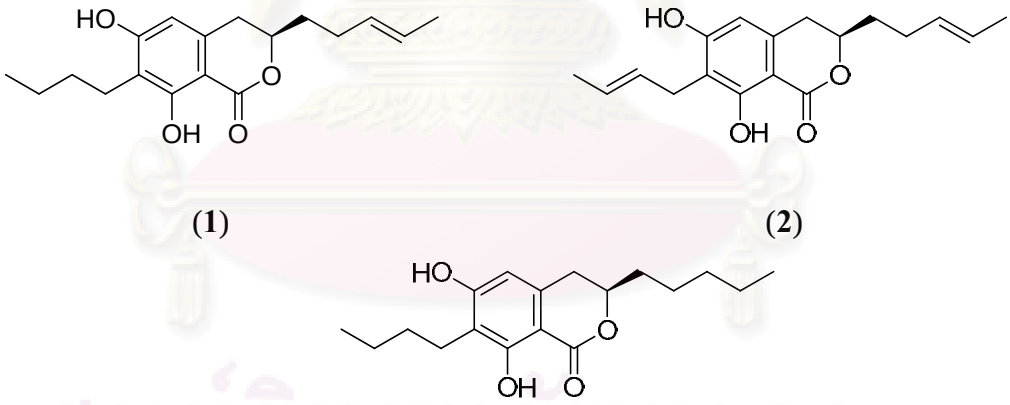


Figure 2.2 The chemical structure of Taxol®

Endophytic fungi are a new hope of anti-malarial drug and there are several compounds produced by them. Three novel dihydroisocoumarin derivatives **1**-**3** with anti-malarial, anti-tuberculous and anti-fungal activities were obtained from an endophytic fungus, *Geotrichum* sp., which was isolated from *Crassocephalum crepidioides* (Kongsaeree *et al.,* 2003).



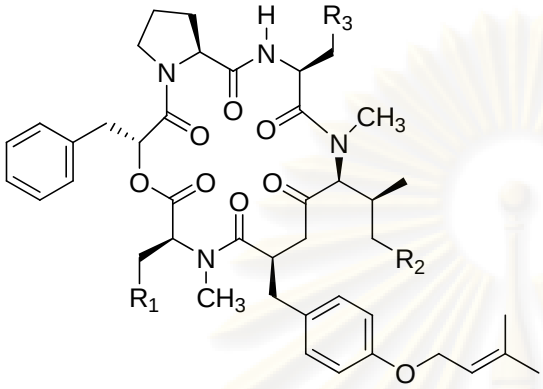
(**1**) (**2**)

(**3**)

Figure 2.3 The chemical structure of dihydroisocoumarin derivatives **1**-**3**

Four new cyclohexadepsipeptides, pullularins A–D (**4**-**7**), were obtained from the endophytic fungus *Pullularia* sp. BCC 8613, isolated from a leaf of *Culophyllum* sp. Pullularin A (**4**) exhibited activities against the malarial parasite *P. falciparum* K1

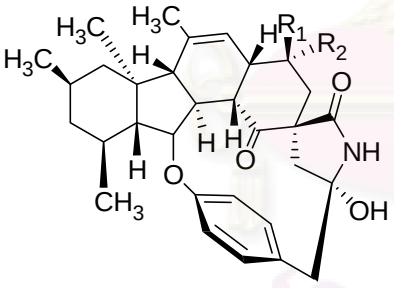
(IC_{50} 3.6 µg/mL) and herpes simplex virus type 1 (HSV-1; IC_{50} 3.3 µg/mL) (Isaka *et al.*, 2007).



(**4**) (R_1) = H, (R_2) = CH_3, (R_3) = OH
(**5**) (R_1) = CH_3, (R_2) = CH_3, (R_3) = OH
(**6**) (R_1) = H, (R_2) = H, (R_3) = OH
(**7**) (R_1) = H, (R_2) = CH_3, (R_3) = H

Figure 2.4 The chemical structure of pullularins A–D (**4-7**)

Pyrrospirones A and B (**8-9**) have been isolated from unpolished rice cultures of the endophytic fungus *Neonectria ramulariae* Wollenw KS-246. The compounds exhibited cytotoxicity and induced apoptosis in human promyelocytic leukemia HL-60 cells (Shiono *et al.*, 2008).

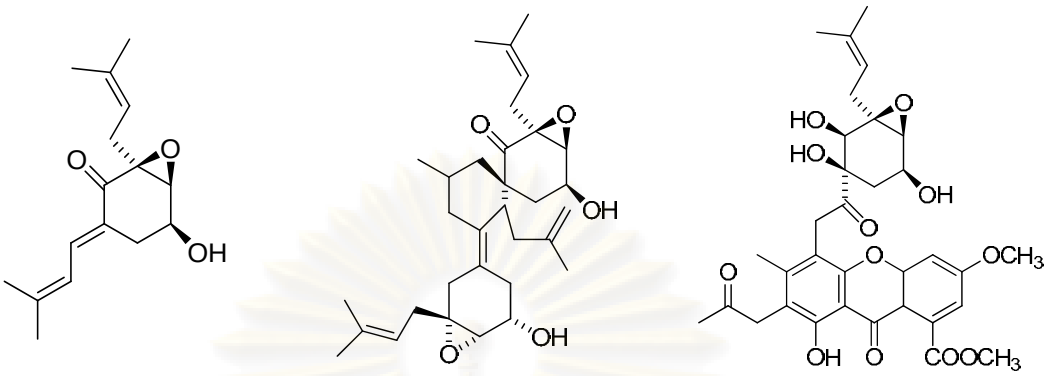


(**8**) (R_1) = H, (R_2) = OH

(**9**) (R_1) = OH, (R_2) = H

Figure 2.5 The chemical structure of pyrrospirones A and B (**8-9**)

Pestalofones A, B and E (**10-12**) have been isolated from cultures of the plant endophytic fungus *Pestalotiopsis fici*, and displayed inhibitory effects on HIV-1 replication in C8166 cells (Liu *et al.*, 2009).



(**10**) (**11**) (**12**)

Figure 2.6 The chemical structure of pestalofones A, B and E (**10-12**)

2.4 Botanical Aspects, Distribu tion and Ethnobotanical of *Andrographis paniculata, Chromolaena odorata* and *Stemona* spp.

2.4.1 *Andrographis paniculata*

Andrographis paniculata (Burm. F.) Wall. ex Nees (Acanthaceae), the Fha-Ta-Lai-Jone in Thai, is an important medicinal plant. (Chadha, 1985; http://en.wikipedia.org/wiki/Andrographis).

Figure 2.7 *Andrographis paniculata*

Taxonomy of *Andrographis paniculata*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Acanthaceae

Genus: *Andrographis*

Species: *Andrographis paniculata*

A. paniculata grows erect to a height of 100 cm with glabrous leaves 2.5 x 8 cm, lanceolate, pinnate and white flowers 1.9 x 0.3 cm with rose-purple spots on the petals.

A. paniculata is distributed in tropical Asian countries such as India, Sri Lanka, Malaysia, Indonesia and Thailand (http://en.wikipedia.org/wiki/Andrographis).

Since ancient times, *A. paniculata* is used in traditional Siddha and Ayurvedic systems of medicine and some other countries for multiple clinical applications. It has been reported to display anti-proliferative activity in human leukemia HL-60 cells (Chan *et al.,* 2008), immunomodulatory, anti-inflammatory (Carretta *et al.,* 2009), diabetic (Reyes *et al.,* 2006) and anti-malarial activities (Suwanakitti, 2006).

2.4.2 *Chromolaena odorata*

Chromolaena odorata (L.) R. M. King and H. Robinson, the Sab-Sua in Thai, is a perennial scandent or semi-woody shrub belonging to the Asteraceae family (Iwu *et al.,* 1993; Inya-Agha *et al.,* 1987).

Figure 2.8 *Chromolaena odorata*
(Image from: http://www.flickr.com/photos/91314344@N00/2112896548).

Taxonomy of *Chromolaena odorata*

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Asterales
Family: Asteraceae
Genus: *Chromolaena*
Species: *Chromolaena odoratum*

It may reach 1 m or more. Stems reach 2 cm in diameter. The opposite, three-nerved leaves are deltoid to ovate-lanciolate, usually with a dentate margin and a long pointed tip. The leaves are aromatic when crushed. The inflorescences are corymbs of cylindrical heads located on the terminals of lateral branches. There are 15 to 25 tubular florets per head, white, lavender, pink or blue in color. The seeds are a brownish gray to black achene that is 4 mm long with a pale brown pappus 5 or 6 mm long (Howard 1989; Liogier 1997).

This plant species is native to Central and South America. It is now distributed throughout Africa and tropical Asia (Muniappan and Marutani, 1991) and also widely distributed in many countries, including Thailand (Bunyapraphatsara and Chokechaijaroenporn, 2000).

In China it has been used as a herbal tea to treat chills and fever for more than 2,000 years (van Agtmael *et al.,* 1999; Klayman, 1985; Hien and White, 1993). It has been used in folk medicine for the treatment of cough, internal hemorrhage, malaria and diarrhea. It has activities such as anti-inflammatory, anti-hypertensive (Iwu *et al.,* 1993; Inya-Agha *et al.,* 1987), analgesic, anti-pyretic (Owoyele *et al.,* 2008), anti-oxidant activities (Phan *et al.,* 2001), hemostatic on wounds and skin anti-septic (Pisutthanan *et al.,* 2006).

A number of studies demonstrated that the extract of the leaves of *C. odorata* inhibited the growth of bacteria (*Pseudomonas aeroginosa*, *Escherichia coli*, *S. aureus* and *Neisseria gonorrhoea*) (Akah, 1990; Bamba *et al.,* 1993; Irobi, 1992; Le, 1995; Cáceres *et al.,* 1995). Enhancement of hemostasis and blood coagulation with use of *C. odorata* extract was also studied (Akah, 1990; Triaratana *et al.,* 1991). Compounds from *C. odorata* (L.), acacetin (5,7-dihydroxy-4'-methoxyflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone), displayed moderate cytotoxicity against human small cell lung cancer (NCI-H187) cells with the MIC value of 24.6 μM and 19.2 μM respectively (Suksamrarn *et al.,* 2004).

2.4.3 *Stemona* spp.

Stemona, known in the Thai vernacular as ‘‘Non-Tai-Yak’’, is the largest genus in the family Stemonaceae. This Family has represented a typical chemical character Stemona alkaloids that seem to be involved in apoptotic effects of chemo-resistant cancer cells (Tsi and Duyfjes 2000; Brem *et al.,* 2002; Kaltenegger *et al.,* 2003).

Genus *Stemona* has about 25 species occurring as subshrubs or twining herbs, mostly with perennial tuberous roots (Kimura and Kimura, 1975; Xu *et al.*, 1982; Terada *et al.*, 1982; Stöger 1999). Leaves whorled, opposite or alternate, petiolate or sessile; main veins 3 or more, transverse veinlets numerous. Inflorescences racemes or cymes. Flowers bisexual, actinomorphic. Seed appendages on or near funicle, arillate, testa leathery, embryo hard, albuminous (Zhanhe and Duyfjes, 2000).

Figure 2.9 *Stemona* sp.

Taxonomy of *Stemona* sp.

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Pandanales

Family: Stemonaceae

Genus: *Stemona*

Species: *Stemona* spp.

Scientific name: *Stemona* sp.

Their range of distribution is centered in Southeast Asia but extends also to tropical Australia and with one species even to southeast United States (Duyfjes, 1993; Tsi and Duyfjes, 2000).

The herb Radix Stemonae has been used as an anti-tussive and anthelmintic in traditional Chinese medicine for some 2,000 years (Committee for Pharmacopoeia of P. R. China, 2005).

The root extracts of *Stemona curtisii* effected on P-glycoprotein and MRP-1 function in multidrug-resistant cancer cells (Limtrakul *et al.,* 2007).

The roots of *Stemona tuberosa, Stemona japonica* and *Stemona sessilifolia* have long been recommended in Chinese, Japanese and Thai traditional medicine for the treatment of respiratory diseases. There have many activities such as anti-fungal, anti-cancer, against enteric helminthes and ectoparasites (Kimura and Kimura, 1975; Xu *et al.,* 1982; Terada *et al.,* 1982; Stöger, 1999; Pilli and de Oliveira, 2005). Compounds from roots of *S. tuberosa* inhibited the growth of various bacterial and fungal strains (Zhao *et al.,* 1995; Lin *et al.,* 2008). The root extracts of *S. tuberosa* and *S. collinsae* also affected on medullary and thyroid carcinoma cells (Rinner *et al.,* 2004).

It has been reported that 6-deoxyclitoriacetal from *Stemona collinsae* has potent cytotoxic activity against human breast carcinoma (BT 479), lung carcinoma (CHAGO), hepato-carcinoma (Hep-G2), gastric carcinoma (KATO3), colon carcinoma (SW620) (Roengsumran *et al.,* 2003) and lymphocytic leukaemia cell lines P388 (Fang *et al.,* 1998).

CHAPTER III

EXPERIMENTS

3.1 Plant sample collection

Healthy leaves and roots of a *Stemona* sp. were collected from Amphur Bangban, Ayutthaya Province; Nongkhai Province and Prajinburi Province; Thailand; in January 2007. Leaves of *Andrographis paniculata* were collected from Bangkok Province, Thailand, in February 2007. Leaves of *Chromolaena odoratum* were collected from Amphur Dankanun and Banprew, Phatthalung Province; Thailand; in January 2007 and Amphur Nongkae, Saraburi Province; Thailand; in December 2006.

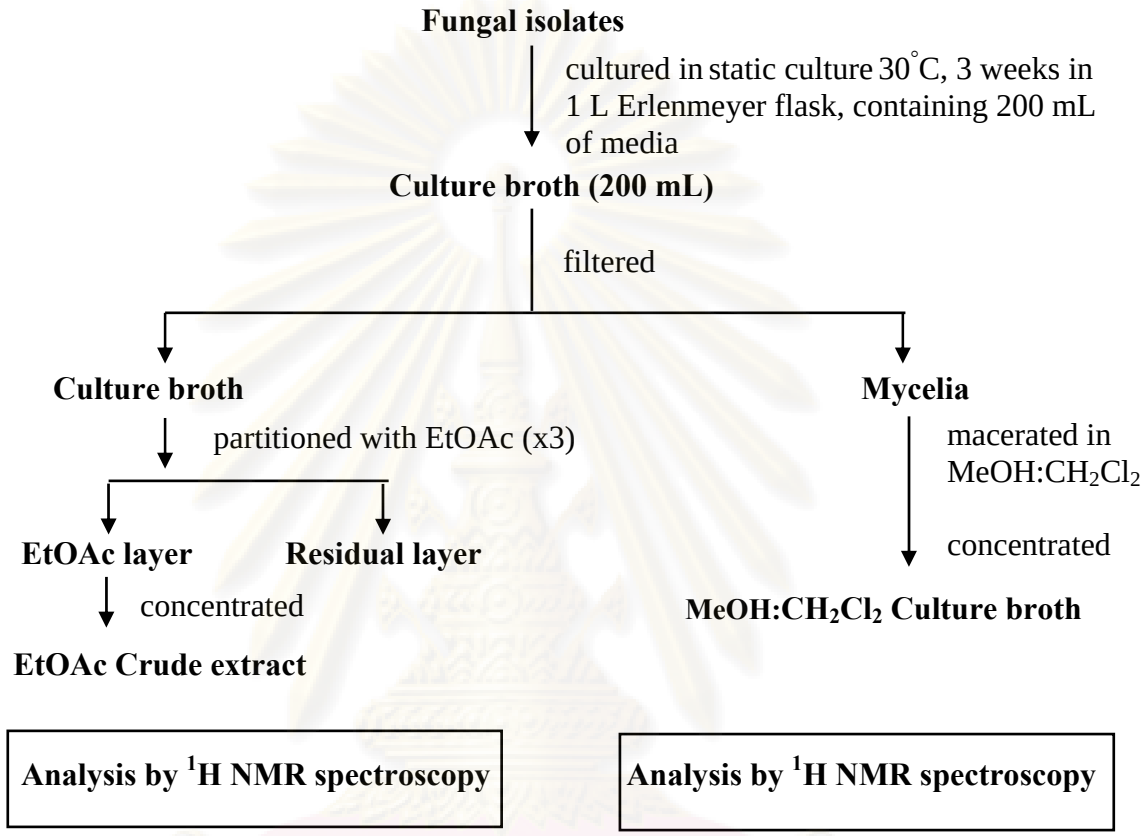
3.2 Fungal endophyte isolation

Plant samples were washed in tap water and air-dried. The cleaned leaf and root fragments were surface-sterilized as described by Schulz and co-workers (Schulz *et. al.*, 1995) with some modifications. Plant fragments were sequentially immersed in 70% EtOH for 1 min, NaOCl solution (6% available chloride) for 5 min, and sterile distilled water for 1 min (two times). Then, the surface-sterilized fragments were cut into small pieces (ca. 5 mm in length) using a sterile blade and placed on sterile water agar plates for further incubation at 30°C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile Pasteur pipet and transferred onto a potato dextrose agar (PDA) plate. After incubation at 30 °C for 7-14 days, culture purity was determined from colony morphology.

3.3 Screening of selected endophytic fung al isolates for the p roduction of bioactive compounds

Based on different morphology of fungi, 50 isolates were selected for cultivation on two types of media, malt Czapek broth and yeast extract sucrose broth. The 50 selected endophytic fungal isolates were grown in 1L Erlenmeyer flasks, containing 200 mL of appropriate culture medium. After 3 weeks of still culture at 30°C, the total of 200 culture broth was filtered through four layers of cheesecloth to remove mycelium. The culture broth was extracted with EtOAc, and the crude extract

was examined by analysis of its ^{1}H NMR spectrum. **Scheme 3.1** summarizes the whole extraction process.



Scheme 3.1 General procedure for extraction of fungal culture broth and mycelia.

3.4 Cultivation and extraction for large scale

The fungal isolate STEM3, APAN5, APAN15 and CODO58 grown on yeast extract sucrose broth (YES) and the isolate CODO23 cultured on malt extract broth (MCzB) provided crude extract in high yield and showed interesting ^{1}H NMR spectral profiles; therefore, these fermentation conditions were selected for further studies.

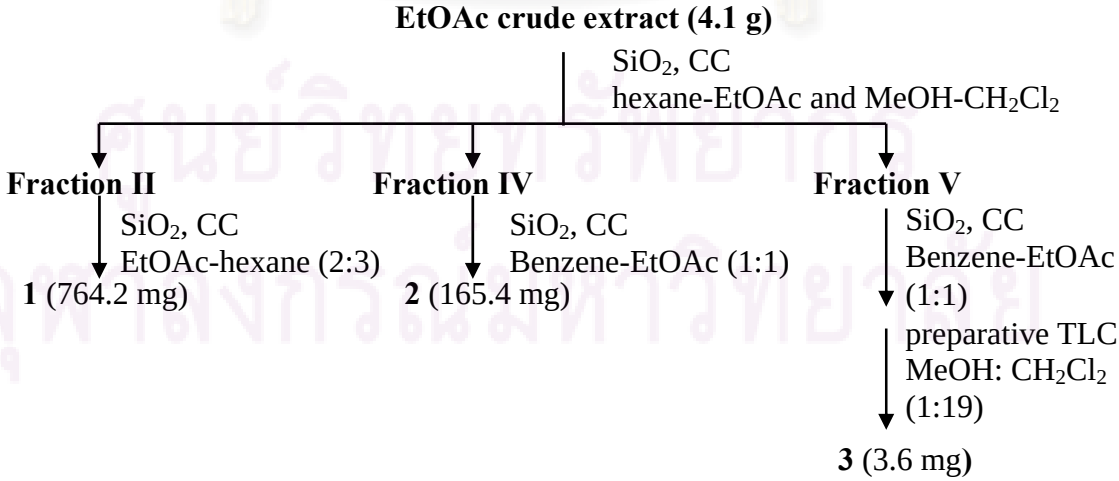
The fungi of interest were grown on PDA plate at 30°C for 7 days. Six pieces (6x6 mm^2) of the grown culture cut from the plate were inoculated into 1,000 mL Erlenmeyer flasks (x25) containing 200 mL of yeast extract sucrose broth (YES) or malt Czapek broth (MCzB) at 30°C for 3 weeks under static conditions.

The culture broth was passed through four layers of cheese cloth which were exhaustively pressed. The filtrate was extracted with an equal volume of EtOAc for 3 times. The organic layers were combined and evaporated at 40°C to yield a crude extract. Mycelia were sequentially macerated in MeOH and CH_2Cl_2, respectively, at room temperature, each of 2 days. Both MeOH and CH_2Cl_2 extracts were combined and evaporated under reduced pressure and partitioned twice with an equal volume of hexane. Then, the MeOH layer was added with 100 mL of H_2O and partitioned with EtOAc to obtain mycelium crude extract.

3.5 Isolation of bioactive compounds from selected endophytic fungi

3.5.1 Isolate from *Stemona* sp. (STEM3)

The 4.1 g of EtOAc crude broth extract from YES culture was subjected to SiO_2 column chromatography (CC) eluted with hexane-EtOAc and MeOH-CH_2Cl_2 mixtures of increasing polarity to afford eight fractions (I-VIII). Fraction II was rechromatographed over silica gel eluted with EtOAc-hexane (2:3) to afford monocerin (**1**, 764.2 mg). Fraction IV was further subjected to flash CC on silica gel benzene-EtOAc (1:1) to yield compound **2** (165.4 mg). Fraction V was rechromatographed by flash CC on SiO_2 eluted with benzene-EtOAc (3:2), followed by preparative TLC (MeOH: CH_2Cl_2, 1:19) to give compound **3** (3.6 mg). **Scheme 3.2** summarizes the whole extraction process.



Scheme 3.2 Isolation of a broth extract of STEM3 cultured on YES medium.

3.5.2 Isolate from *Andrographis paniculata* (APAN15)

The 1.01 g of EtOAc crude broth extract from YES culture was subjected to Sephadex LH-20 column eluted with MeOH to afford nine fractions. Fraction IX was rechromatographed over silica gel eluted with EtOAc-benzene (1:1) to afford 15 fractions (I- XV. Fraction XV was separated by preparative HPLC with MeOH to afford *N*-phenethyl acetamide (**12**, 11.4 mg). **Scheme 3.3** summarizes the whole extraction process.

EtOAc crude extract (1.01 g)

↓ Sephadex LH-20, MeOH

Fraction IX

↓ SiO_2, CC, Benzene-EtOAc

Fraction XV

↓ HPLC MeOH (2:3)

12 (11.4 mg)

Scheme 3.3 Isolation of a broth extract of APAN15 cultured on YES medium.

3.5.3 Isolate from *Andrographis paniculata* (APAN5)

The 4.53 g of EtOAc crude broth extract from YES culture was subjected to Sephadex LH-20 column eluted with MeOH to afford nine fractions. Fraction IX was rechromatographed over silica gel eluted with EtOAc-hexane (1:1) to afford tryptophol (**13**, 88.5 mg) and indole-3-acetamide (**14**, 120 mg). **Scheme 3.4** summarizes the whole extraction process.

EtOAc crude extract (4.53 g)

↓ Sephadex LH-20, MeOH

Fraction IX

SiO_2, CC, EtOAc-hexane (1:1)

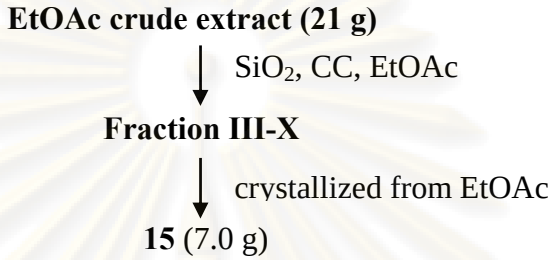
↓ ↓

13 (88.5 mg) **14** (120 mg)

Scheme 3.4 Isolation of a broth extract of APAN5 cultured on YES medium

3.5.4 Isolate from *Chromolaena odoratum* (CODO58)

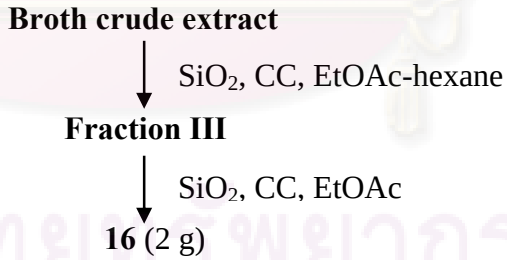
The 21 g of EtOAc crude broth extract from YES culture was subjected to SiO2 column chromatography (CC) eluted with EtOAc to afford ten fractions (I-X). Fraction III-V were crystallized from solvent to give cytochalasin D (**15**, 7.0 g)



Scheme 3.5 Isolation of a broth extract of CODO58 cultured on YES medium.

3.5.5 Isolate from *Chromolaena odoratum* (CODO23)

The 9.6 g of EtOAc crude broth extract from MCzB culture was subjected to SiO2 column chromatography (CC) eluted with EtOAc-hexane (1:1) to afford six fractions (I-VI). Fraction III was rechromatographed over silica gel eluted with EtOAc to afford cytochalasin H (**16**, 2.0 g)



Scheme 3.6 Isolation of a broth extract of CODO23 cultured on MCzB medium.

3.6 Chromatography

3.6.1 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) was carried out on a silica gel F254 coated on aluminium sheet (Merck). Detection was visualized under ultraviolet light at wavelengths of 254 and 356 nm.

3.6.2 Column chromatography

Column chromatography (CC) was performed using Sephadex LH-20 and Silica gel 60H (Merck code no. 7734 and no. 9385) as packing materials.

3.7 Structure elucidation

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

3.7.1 Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were calibrated using solvent signals (^{13}C: $CDCl_3$ 77.00 ppm, acetone-d_6 (CD_3COCD_3) 29.8 and 206.0 ppm, and DMSO-d_6 (CD_3SOCD_3) 39.5 ppm) or a signal of the portion of the partly or non deuterated solvent (^1H: $CHCl_3$ in $CDCl_3$ δ 7.26 ppm, acetone in acetone-d_6 δ 2.05 ppm, water (H_2O) in acetone-d_6 δ 2.80 ppm, DMSO in DMSO-d_6 δ 2.50 ppm, and water (H_2O) in DMSO-d_6 δ 3.31 ppm). ^1H NMR, ^{13}C NMR, DEPT, gCOSY, gHSQC, gHMBC, and NOESY pectra were recorded on a Varian Spectrometer operated at 400 MHz for ^1H nuclei and at 100 MHz for ^{13}C nuclei. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated chloroform at δ = 7.26 ppm.

3.7.2 Mass spectrometry (MS)

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

3.7.3 Ultraviolet-visible measurements (UV-vis)

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

3.7.4 Fourier transform infrared spectroscopy (FT-IR)

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

3.7.5 Optical rotation

Optical rotations were measured with using a sodium D line (589 nm) JASCO DPI-370 digital polarimeter equipped with a 1 mL cell (cell length 1.00 cm).

3.8 Chemicals

All solvents used in this research such as hexane, dichloromethane (CH_2Cl_2), acetone, ethyl acetate (EtOAc) and methanol (MeOH) were commercial grade and purified prior to use by distillation. The reagent grade solvents were used for recrystallization.

3.9 Culture media

Culture media used for cultivation of endophytic fungi were potato dextrose agar (PDA), agar, yeast extract sucrose broth (YES), malt Czapek broth (MCzB), Alphacel medium; the formula are shown in Appendix A.

3.10 Identification of absolute stereochemistry of 2 by Mosher's methods

Preparation of (*R*)-MTPA Ester (2a) and (*S*)-MTPA Ester (2b).

A reaction mixture of **2** (2 mg), (*S*)- or (*R*)-MTPACl (20 µL), and DMAP (catalytic amount) in pyridine (0.25 mL) was stirred at room temperature overnight. After removing the solvent under reduced pressure, the (*R*)- and (*S*)-MTPA esters (**2a** and **2b**) were purified by column chromatography on silica gel with EtOAc (1:4).

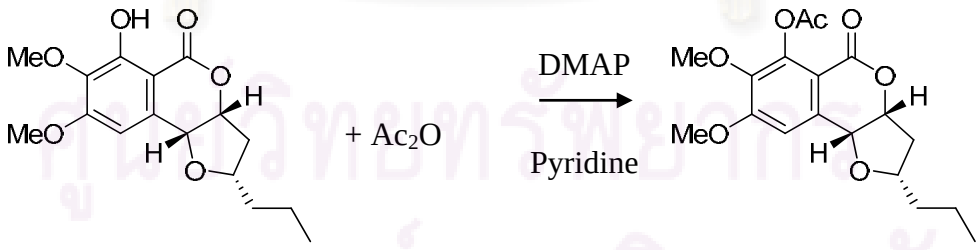
Compound **2a**: ^{1}H NMR of **2***R*-MTPA ester (400 MHz, $CDCl_3$) δ 6.57 (1H, s, H-5), 5.25 (1H, m, H-11), 5.03, (1H, m, H-3), 4.37 (1H, d, *J*= 3.6 Hz, H-4), 4.24 (1H, m, H-10), 3.98 (3H, s, 6-O*CH*$_3$), 3.91 (3H, s, 7- O*CH*$_3$), 2.56 (1H, m, H-9*β*), 2.15 (1H, dd, *J*=14.0, 6.2 Hz, H-9α), 2.10 (1H, m, H-12a), 1.74 (1H, m, H-12b), 1.17 (3H, t, *J* =7.2 Hz, H3-13).

Compound **2b**: ^{1}H NMR of **2***S*-MTPA ester (400 MHz, $CDCl_3$) δ 6.58 (1H, s, H-5), 5.23 (1H, m, H-11), 4.97, (1H, m, H-3), 4.50 (1H, d, *J* =3.2 Hz, H-4), 4.12 (1H, m, H-10), 3.96 (3H, s, 6- O*CH*$_3$), 3.94 (3H, s, 7-O*CH*$_3$), 2.29 (1H, m, H-9*β*), 2.12 (1H, m, H-12a), 2.04 (1H, dd, J=14.2, 5.8 Hz, H-9α), 1.92 (1H, m, H-12b), 1.26 (3H, t, *J* =7.0 Hz, H_3-13).

3.11 Derivertization of monocerin (1) and 11-hydroxymonocerin (2)

3.11.1 Acetylation of monocerin (1)

Acetic anhydride (0.3 mL) and DMAP (catalytic amount) were added to a solution of monocerin (**1**) (10 mg) in pyridine 1 mL, and the mixture was left stirring at room temperature for 5 h. After removing the solvent under reduced pressure, acetate **4** was purified by CC on silica gel with EtOAc-hexane (3:1); 95% yield (10.8 mg).



1 **4**

Scheme 3.7 Acetylation of monocerin (**1**)

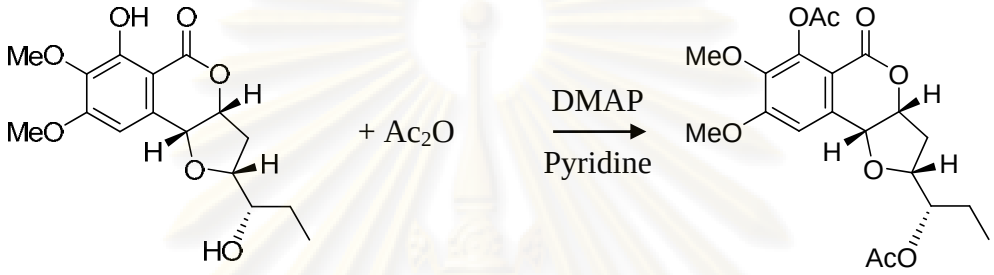
Compound 4

$C_{18}H_{22}O_7$; colorless oil; $[\alpha]^{20}_{D}$ -30.0 (*c* 0.1, in MeOH)

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.5/ Page 52**

3.11.2 Acetylation of 11-hydroxymonocerin (2)

In a similar fashion, compound **2** (10 mg), acetic anhydride (0.3 mL), and DMAP (catalytic amount) in pyridine (1 mL) were allowed to react at room temperature for 5 h, and the reaction mixture was processed as described above for **4** to afford diacetylmonocerin **5** (12.1 mg, 96% yield).



2 **5**

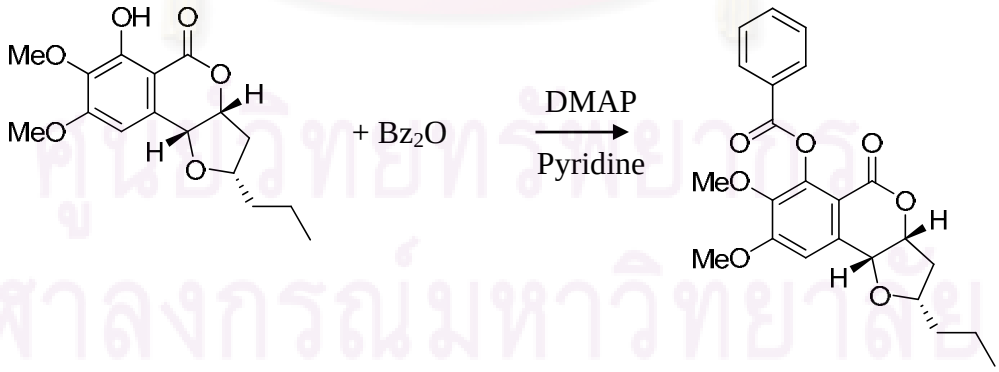
Scheme 3.8 Acetylation of 11-hydroxymonocerin (**2**)

Compound 5

$C_{20}H_{24}O_9$; colorless oil; $[\alpha]^{20}_D$ +24.0 (*c* 0.1, in MeOH)

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.6/ Page 53**

3.11.3 Benzylation of monocerin (1)



1 **6**

Scheme 3.9 Benzylation of monocerin (**1**)

A mixture of monocerin (32.0 mg, 0.1038 mmol) and pyridine (1.5 mL) was stirred under nitrogen atmosphere at room temperature. Then, benzoic anhydride (35.2 mg, 0.1557 mmol) and DMAP (catalytic amount) were added. The mixture was stirred overnight and worked up by adding $NaHCO_3$ (aq) and then it was extracted with EtOAc. Later, the organic phase was washed by $CuSO_4$ $4H_2O$ (aq) to remove excessive pyridine, followed by water and brine, dried with Na_2SO_4 and evaporated organic solvent to gain crude product. Hexane and a little of EtOAc were added to the crude product, causing white solid precipitated out. The precipitate was obtained by filtration and recrystallized from MeOH to get a white crystalline product (**6**) in 59.2% yield (25.3 mg, 0.0614 mmol). X-ray crystallographic analysis was also used to confirm the structure. The ORTEP diagram of this compound is shown in **Figure 3.1**. It might probably due to the low nucleophilicity of the hydroxyl group at the position 8 of monocerin. Because 8-hydroxy group in monocerin was phenolic and hydrogen bonded to the carbonyl group at the position 1.

Compound 6

$C_{23}H_{24}O_7$; colorless crystals; m.p. 162-164 $^{\circ}$C; $[\alpha]^{20}_D$ +10.2 (*c* 0.1, in MeOH)

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.7/ Page 55**

The crystal structure was solved by direct methods and refined by full-matrix least-squares methods. The non-hydrogen atoms were refined anisotropically. All hydrogen atoms were placed geometrically and allowed to ride on the parent atoms in the model.

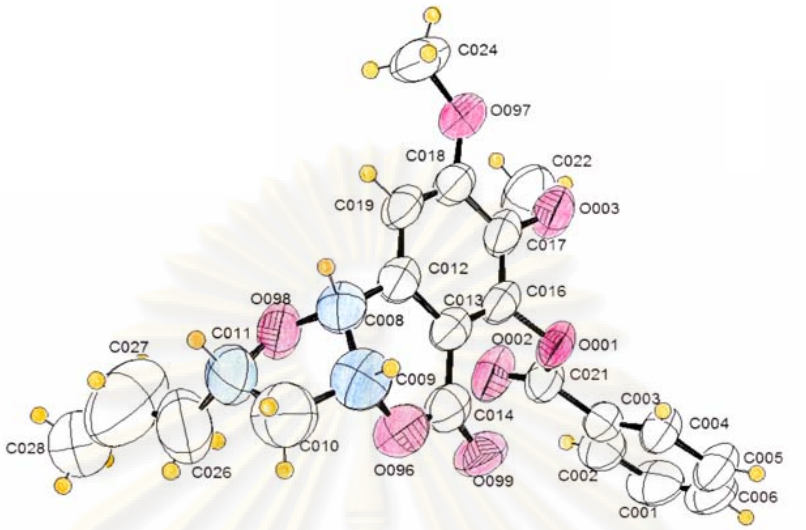
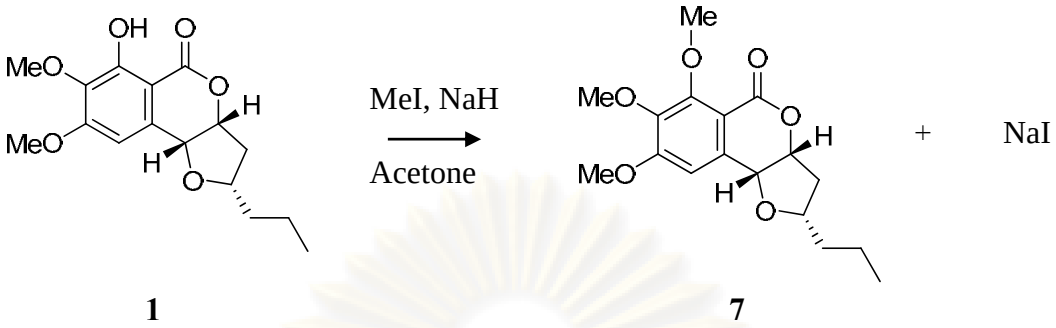


Figure 3.1 ORTEP drawing of 8-benzoylmonocerin (**6**) with atom labeling. Thermal ellipsoids are drawn at the 50% probability level.

3.11.4 Methylation of monocerin (1)

Sodium hydride (60%w/w 13.836 mg, 0.3500 mmol) was suspended in acetone (0.4 mL). Then, the solution of monocerin (71.1 mg, 0.2307 mmol) in acetone (0.4 mL) was added. Simultaneously with stirring, methyl iodide (0.0215 mL, 0.3459 mmol) was quickly added and the mixture was stirred under nitrogen atmosphere at room temperature until the reaction was completed (monitored by TLC).

To purify the product (**7**) from crude mixture, column chromatography technique was carried out by using 50%EtOAc-hexane as eluent. The yield of this reaction was 43.0%yield (32.0 mg, 0.0992 mmol).



1 **7**

Scheme 3.10 Methylation of monocerin (**1**)

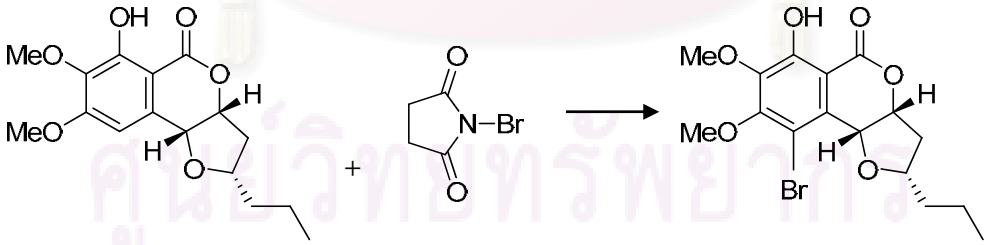
Compound 7

$C_{17}H_{22}O_6$; colorless oil; $[\alpha]^{20}_D$ -4.0 (*c* 0.1, in MeOH)

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.8/ Page 56**

3.11.5 Bromination of monocerin (1)

Monocerin (70.9 mg, 0.2300 mmol) was dissolved in dry CH_2Cl_2 (4.0 mL). NBS (53.7 mg, 0.3017 mmol) was then slowly added into the stirred mixture at room temperature and in the presence of light. After 6 hrs, the organic solvent was evaporated to dryness. The crude product was purified by column chromatography using 20% acetone-hexane as eluent to give brominated product (**8**) (37.4% yield, 33.3 mg, 0.0860 mmol).



1 **8**

Scheme 3.11 Bromination of monocerin (**1**)

Compound 8

$C_{16}H_{19}BrO_6$; yellow oil; $[\alpha]^{20}_D$ +42.0 (*c* 0.1, in MeOH)

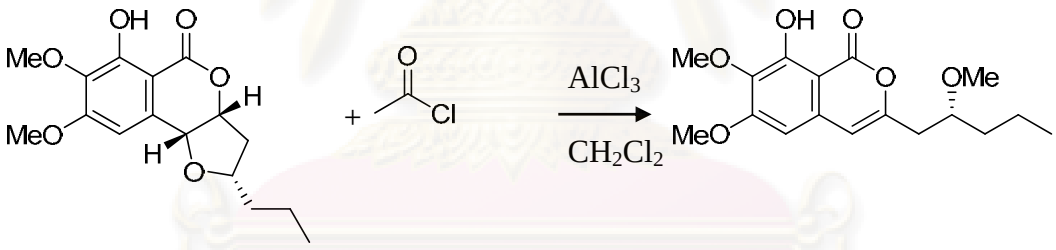
^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.9/ Page 57**

3.11.6 Friedel-Crafts acylation of monocerin (1)

Monocerin (90.1 mg, 0.2923 mmol) was dissolved in CH_2Cl_2 (1.0 mL), then acetyl chloride (0.1 mL, 1.414 mmol) and anh. $AlCl_3$ (excess amount) were added slowly. The mixture was stirred at room temperature for 6 hrs. The excessive $AlCl_3$ was removed by filtration, whereas the filtrate was collected and evaporated to dryness in order to obtain the crude product.

The major product (**9**) was separated by means of column chromatography using 20% EtOAc-hexane as eluent and followed by preparative-TLC using 20% EtOAc-benzene as eluent. There was 9.0% yield (8.4 mg, 0.0240 mmol) of this major product.

In addition, one minor product (**4**) could be obtained in 6.1%yield (6.2 mg, 0.0178 mmol).



1 **9**

Scheme 3.12 Friedel-Crafts acylation of monocerin (**1**)

Compound 9

$C_{18}H_{22}O_7$; colorless oil; $[\alpha]^{20}_D$ +17.0 (*c* 0.1, in MeOH)

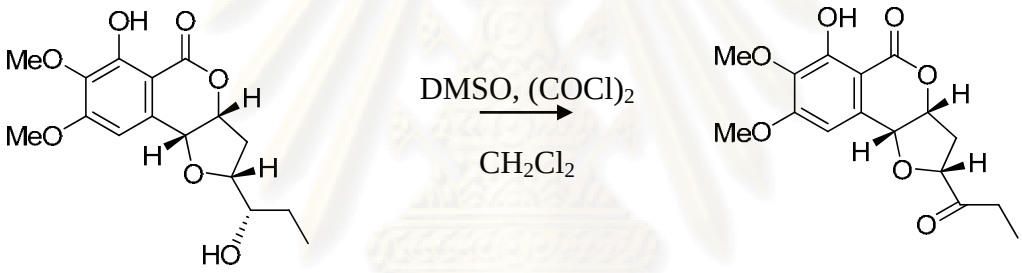
^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.10/ Page 59**

3.11.7 Oxidation of 11-hydroxymonocerin (2)

11-hydroxymonocerin (**2**) was converted to 11-oxomonocerin (**10**) by Swern oxidation.

A mixture of oxalyl chloride (0.05 mL, 0.5824 mmol) and CH_2Cl_2 (0.25 mL) was stirred under nitrogen atmosphere and cooled down to -78 ˚C. DMSO (0.1 mL, 1.408 mmol) was then slowly added. Afterwards, a solution of 11-hydroxymonocerin (50.2 mg, 0.1548 mmol) in CH_2Cl_2 (0.1 mL) was added dropwise and continued stirring for half an hour. Triethylamine (0.35 mL, 2.513 mmol) was added and the mixture was allowed to stir at room temperature for one more hour.

To work up, the reaction mixture was quenched with $NH_4Cl_{(aq)}$ and extracted with CH_2Cl_2, then washed with water and brine, and finally dried with Na_2SO_4. Ketone product was purified by column chromatography with 20% acetone-benzene as eluent. This reaction provided the product in 56.1%yield (28.0 mg, 0.0869 mmol).



2 **10**

Scheme 3.13 Oxidation of 11-hydroxymonocerin (**2**)

Compound 10

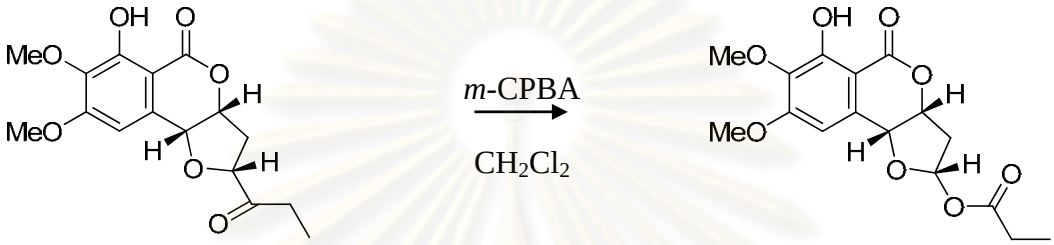
$C_{16}H_{18}O_7$; yellow viscous oil; $[\alpha]^{20}_D$ +62.0 (*c* 0.1, in MeOH)

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.11/ Page 60**

3.11.8 Baeyer-Villiger oxidation of 11-oxomonocerin (2)

The ketone product from the foregoing procedure (18.0 mg, 0.0558 mmol) was dissolved in CH_2Cl_2 (0.8 mL). *meta*-Chloroperoxybenzoic acid (40.2 mg, 0.2330 mmol) was then added and the mixture was stirred at room temperature until the reaction was completed (monitored by TLC).

The mixture was washed with $NaHCO_3$, H_2O and brine, dried with Na_2SO_4, and eventually purified by column chromatography using 20% acetone-benzene as eluent. The reaction provided the product (**11**) in 58.0%yield (10.9 mg, 0.0324 mmol).



10 **11**

Scheme 3.14 Baeyer-Villiger oxidation of 11-oxomonocerin (**2**)

Compound 11

$C_{16}H_{18}O_8$; whith solid; m.p. 165-167 °C; $[\alpha]^{20}_D$ -16.0 (*c* 0.1, in MeOH)

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.12/ Page 61**

3.12 Classification of the endophytic fungal isolate STEM3

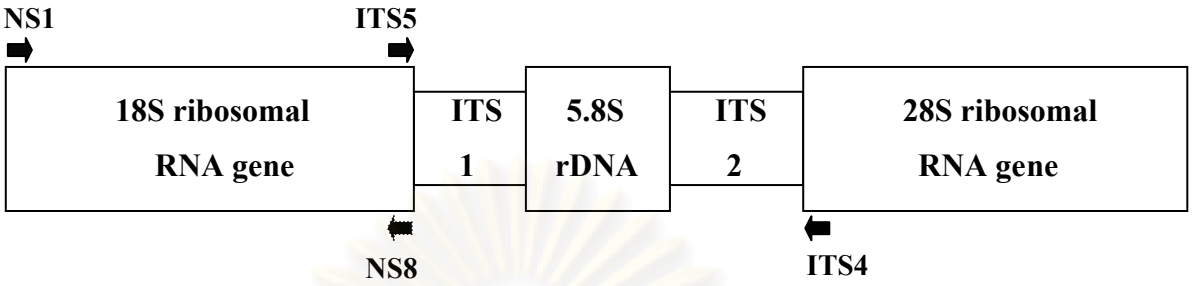
The fungal endophyte isolate STEM3 was identified on the basis of both morphology of the fungus grown on potato dextrose agar (PDA) and Alphacel medium (Sloan *et al*., 1961) at 25°C and analysis of the DNA sequences of the ITS region of the rRNA gene.

3.12.1 Conventional method

STEM3 isolate was grown on potato dextrose agar (PDA) for 14 days at room temperature and photographed. Microscorpic morphology of STEM3 grown on Alphacel medium was examined by light microscopy.

3.12.2 Molecular method

The STEM3 fungus was grown on potato dextrose broth. After cultivation for 7 days, 50 mg of fresh fungal mycelia were harvested, washed twice with normal saline solution, and homogenated in 250 µl of sterile water. The mycelial homogenate was applied evenly to an FTA card matrix, allowed to dry at room temperature, and a 2-mm disk containing the fungal mycelia was punched from the FTA card. Total cellular DNA of fungal mycelia on the disk was extracted and purified using the FTA® Plant Kit (Whatman®) according to the manufacturer's instruction. The disk was transferred to a PCR tube and the ITS1-5.8S-ITS2 ribosomal RNA gene region (**Figure 3.2**) of fungal genomic DNA was amplified using the ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al.,* 1990). PCR amplification was performed in a 50 µl reaction volume which contained Taq PCR Master Mix (USB Corp., USA) using an automated thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). The thermocycling program was as follows: 3 min at 95°C followed by 30 cycles of 50 s at 95°C, 40 s at 45°C and 50 s at 72°C, with a final extension period of 10 min at 72°C. The PCR products were purified and directly subjected to sequencing (Bioservice Unit, NSTDA, Bangkok, Thailand) in both directions primed with either of the two primers used to originally amplify the fragment. The DNA sequence of ITS1-5.8S-ITS2 rRNA gene obtained was used as query sequence to search for similar sequences in GenBank using BLASTIN 2.2.18 (Altschul *et al*., 1997). DNA sequence similarity was determined by the ClustalW 2 multiple sequence alignment program (Larkin *et al*., 2007).



[Diagram adapted from: White, *et al*. 1990 PCR protocols: 316]

Figure 3.2 Location on nuclear rDNAs of ITS5, ITS4, NS1, ands NS8 primers. The arrow heads represent the 3' end of each primer.

3.13 Evaluation of biological activities

The pure compounds were evaluated for their anti-bacterial, anti-cancer and anti-malarial activities.

3.13.1 Anti-bacterial activity

Both gram-positive and gram-negative bacteria were selected for 14 strains of bacteria for the *in vitro* antimicrobial activity. The test was performed by using microdilution assay as follows:

Table 3.1 Gram-positive and gram-negative bacteria tested

Gram-positive bacteria	Gram-negative bacteria
1. *Bacillus subtilis* ATCC 6633	1. *Escherichia coli* ATCC 35218
2. *Enterococcus faecalis* ATCC 29212	2. *Klebsiella pneumoniae* ATCC 27736
3. *Enterococcus faecalis* ATCC 51299 (vancomycin resistant)	3. *Klebsiella pneumonia* (ESBL producing) ATCC 700603
4. *Enterococcus faecium* UCLA 192	4. *Pseudomonas aeruginosa* ATCC 27853
5. *Salmonella typhimurium* ATCC 13311	5. *Proteus vulgaris* ATCC 13315
6. *Staphylococcus aureus* ATCC 25923	
7. MRSA ATCC 43300 (NCCLS) (Oxacillin resistant)	
8. *Staphylococcus epidermidis* ATCC 12228	
9. *Staphylococcus hominis* ATCC 27844	

3.13.1.1 Preparation of bacterial inocula

Bacteria were grown on Mueller Hinton agar (MHA) for 24 h at 37°C. Selected fresh single colonies were inoculated into 10 mL of Mueller Hinton broth (MHB) and incubated in shaking incubator for 2-3 h at 37°C. The turbidity of the bacterial suspension was adjusted with sterile normal saline solution to match the turbidity of 0.5 McFarland standard (OD 0.1 at 625 nm). Then the suspension was diluted 1:100 with Mueller Hinton broth (MHB) to contain $1x10^6$ CFU/mL.

3.13.1.2 Determination of minimum inhibitory concentration (MIC)

Solution of a test compound in DMSO (25.6 mg/mL) was diluted with MHB. The test compound was prepared at the concentration ranges of 0.5 to 256 µg/mL.

MIC is defined as the lowest concentration that inhibits growth of test microorganisms.

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

3.13.2 Anticancer activity

The MTT assay (Carmichael *et al*., 1987; Doyle and Griffiths, 1997; Mosmann, 1983; Tominaga et al., 1999) was applied for the evaluation of cytotoxicity against: BT 474 (Breast ductol carcinoma), Chago (Human undifferentiated lung carcinoma), Hep-G2 (Liver hepatoblastoma), KATO-3 (Human gastric carcinoma) and SW620 (Colon adenocarcinoma) cancer cell lines. Cells were plated in a 96-well microplate (Costar No. 3599, USA, 100 µL/well at a density of 5 $\times 10^3$–2×10^4 cells/well), and incubated for 24 h at 37°C under 5% CO_2 and 95% humidity. The tested compounds at various concentrations were added to the cell lines, which were incubated further for 48 h. Cell viability was determined by staining with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The MTT stock solution (5 mg/mL) was prepared in phosphate buffered saline (PBS), which was diluted (1:10) with a culture medium prior to use. After removing the culture medium, the diluted MTT solution (50 µL) was added to the adhesive cells, and plates were incubated at 37°C under 5% CO_2 and 95% humidity for 2–4 h. Subsequently, DMSO (100 µL) was added to dissolve the resulting formazan by sonication. The plates were

read on a microplate reader (Molecular Devices, CA, USA), using a test wavelength of 550 nm and a reference wavelength of 650 nm.

3.13.3 Antimalarial activity (Deharo, *et al.*, 2000).

The in vitro test was modified from the original technique as follows: The malaria-infected erythrocytes were plated at 1% parasitemia (3% hematocrit), in 96 well micro titer plates and exposed to different concentrations of the extracts and incubated for 24 h. After the initial incubation, ^{3}H-hypoxanthine (0.8 mCi) was added and incubation continued for a further 24 h. Each well was harvested onto a glass fibber filter, which was allowed to dry for 24 h at 37°C and the incorporated ^{3}H-hypoxanthine was then determined. Controls were performed to assess the effect of solvents on parasite growth. Growth curves were obtained and the concentration of drug required to inhibit growth by 50% (IC_{50}) was determined graphically by plotting concentration versus percentage inhibition. Tests were made in triplicate.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of fungal endophytes

A total of 92 pure isolates of endophytic fungi were isolated from *A. paniculata, C odoratum* and *Stemona* sp. Fifty eight, eighteen and sixteen isolates were from *C. odoratum, A. paniculata a*nd *Stemona* sp., respectively, as shown in **Table 4.1**.

Table 4.1 Sources of endophytic fungi

Plant	Part	Place	Numbers of Isolate
C. odoratum	Leaf	Amphur Nongkae, Saraburi Province	16
C. odoratum	Leaf	Amphur Dankanun, Phatthalung Province	22
C. odoratum	Leaf	Amphur Banprew, Phatthalung Province	20
A. paniculata	Leaf	Bangkok Province	18
Stemona sp.	Leaf	Amphur Bangban, Ayutthaya Province	10
Stemona sp.	Root	Nongkhai Province	4
Stemona sp.	Root	Prachinburi Province	2

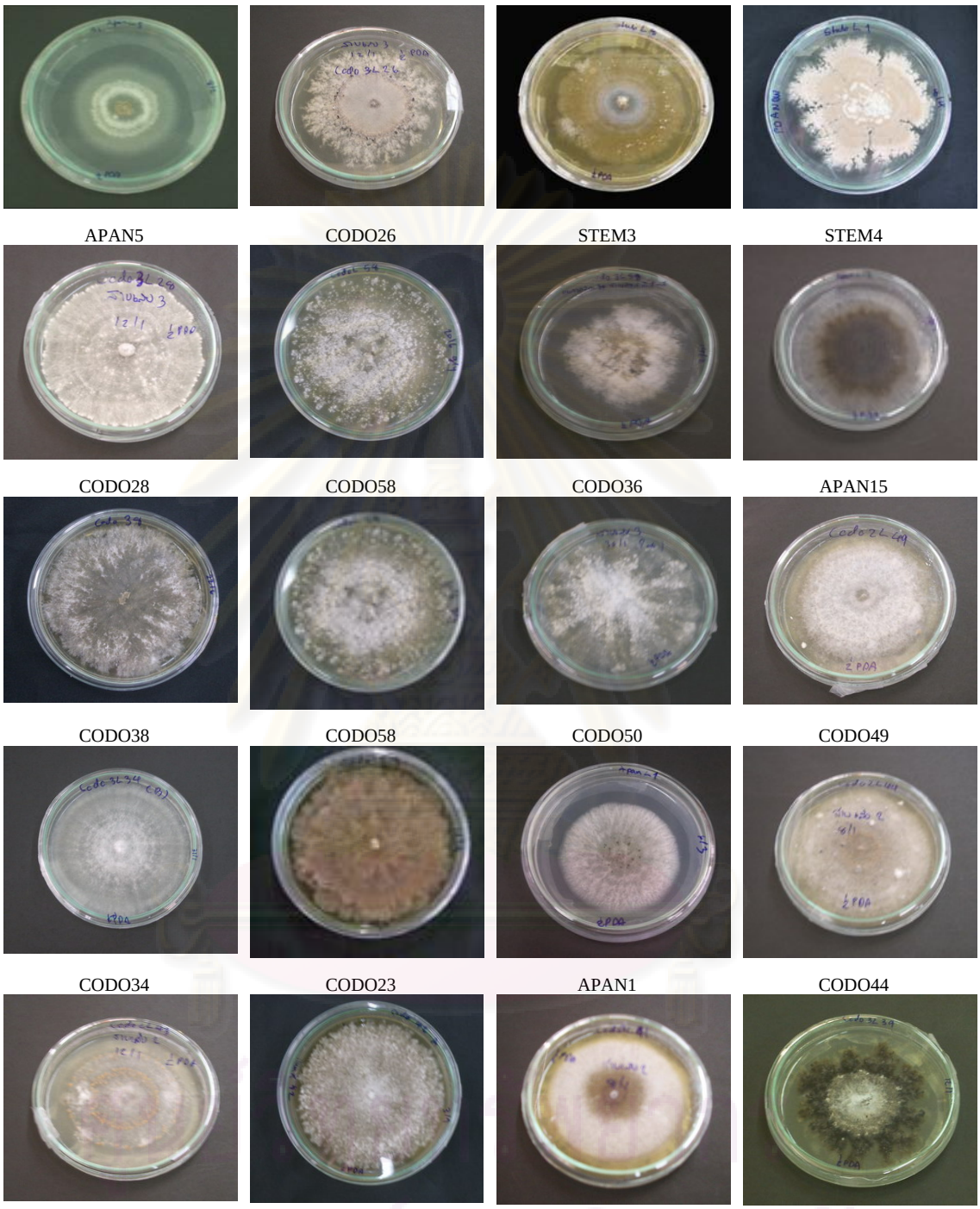


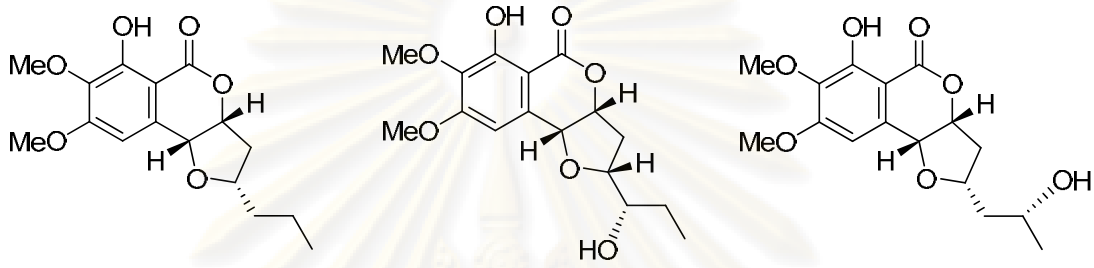
Figure 4.1 Some endophytic fungi with different morphology.

Code: CODO are endophytic fungi isolated from *C. odoratum*, STEM are endophytic fungi isolated from *Stemona* sp., APAN are endophytic fungi isolated from *A. paniculata.*

4.2 Isolation of pure compounds

4.2.1 Isolation of pure compounds from STEM3

A novel (11-hydroxymonocerin **2**) and two known (monocerin **1** and 12-hydroxymonocerin **3**) compounds from broth of STEM3, an endophytic fungus obtained from the leaves of *Stemona* sp. from Ayutthaya Province.

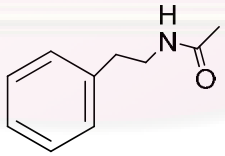


monocerin **1** 11-hydroxymonocerin **2** 12-hydroxymonocerin **3**

Figure 4.2 Chemical structure of pure compounds from STEM3

4.2.2 Isolation of pure compounds from APAN15

One known (*N*-Phenetyl-acetamide **12**) from broth of APAN15, an endophytic fungus obtained from the leaves of *A. paniculata* from Bangkok Province.



N-Phenetyl-acetamide **12**

Figure 4.3 Chemical structure of *N*-Phenetyl-acetamide (**12**)

4.2.3 Isolation of pure compounds from APAN5

Two known (tryptophol **13** and indole-3-acetamide **14**) compounds were isolated from the crude extract 4.53 g of 10 L of yeast extract sucrose broth of an endophytic fungus strain APAN5 obtained from the leaves of *A. paniculata*. The compound **12** was reported to have anti-oxidant activity, antimicrobial activities

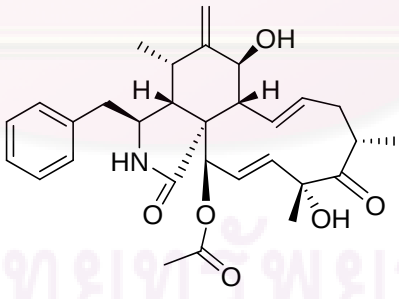
against *E. coli, Bacillus* spp., yeast, and to induce apoptosis in U937 cells (human leukemic monocyte lymphoma cell line) (Guzmán-López1 *et. al*., 2007; Inagaki *et. al*., 2007).

tryptophol **13** indole-3-acetamide **14**

Figure 4.4 Chemical structure of tryptophol (**13**) and indole-3-acetamide (**14**)

4.2.4 Isolation of pure compounds from CODO58

Cytochalasin D (**15**) was isolated from an endophytic fungus CODO58. Cytochalasin D has well documented for anti-cancer and anti-HIV activities (Xu, *et al*., 2001; Petrovas, *et al*., 2007).

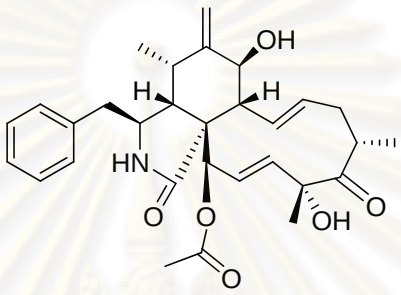


cytochalasin D **15**

Figure 4.5 Chemical structure of cytochalasin D (**15**)

4.2.5 Isolation of pure compounds from CODO23

Cytochalasin H (**16**) was isolated from an endophytic fungus strain CODO23 that was isolated from the leaves of *C. odoratum* from Banprew district, Phatthalung Province.

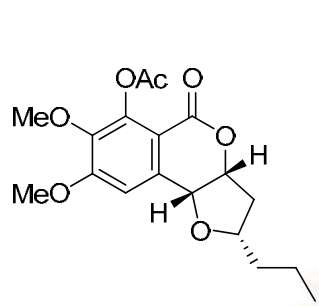


Cytochalasin H **16**

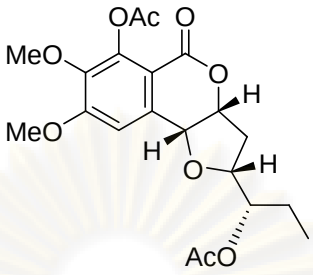
Figure 4.6 Chemical structure of cytochalasin H (**16**)

4.3 Derivatives of monocerins

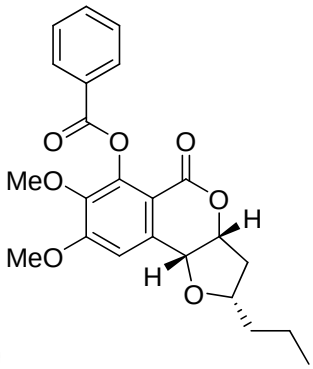
Additionally, eight new (**4**-**11**) monocerin derivatives were obtained from various reactions of monocerin **1** and 11-hydroxymonocerin **2**. Acetylated Product **4**, **5**; the esterification of monocerin with benzoic anhydride gave 8-benzoylmonocerin **6**, as white crystals. The methylation of monocerin gave 8-methylmonocerin **7** and the bromination of monocerin yielded 5-bromomonocerin **8**. Fridel-Craft acetylation of **1** gave opening products **9**. Swern oxidation of **1** afforded **10**, which was further subjected to Bayer-Villiger oxidation to yield **11**.



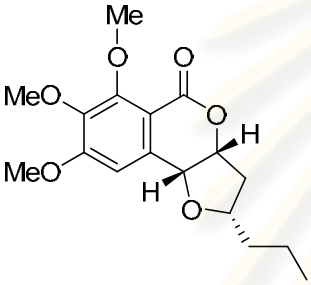
8-acetylmonocerin **4**



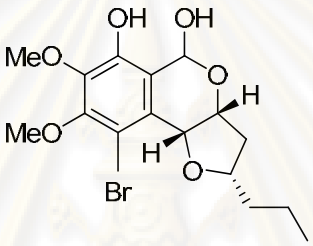
diacetylmonocerin **5**



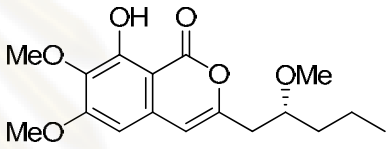
8-benzoylmonocerin **6**



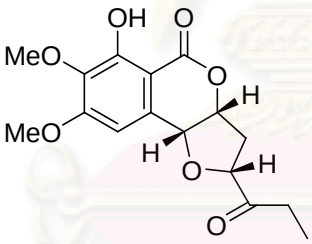
8-methylmonocerin **7**



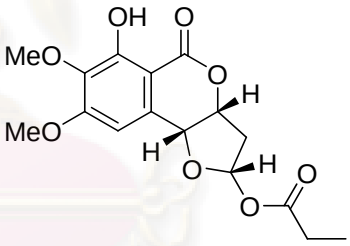
5-bromomonocerin **8**



oxidizing product **9**



11-oxomonocerin **10**



11-carbonylmonocerin **11**

Figure 4.7 Chemical structure of monocerin derivatives

Compound 1 (monocerin)

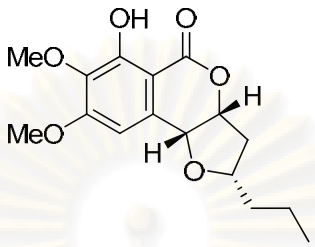
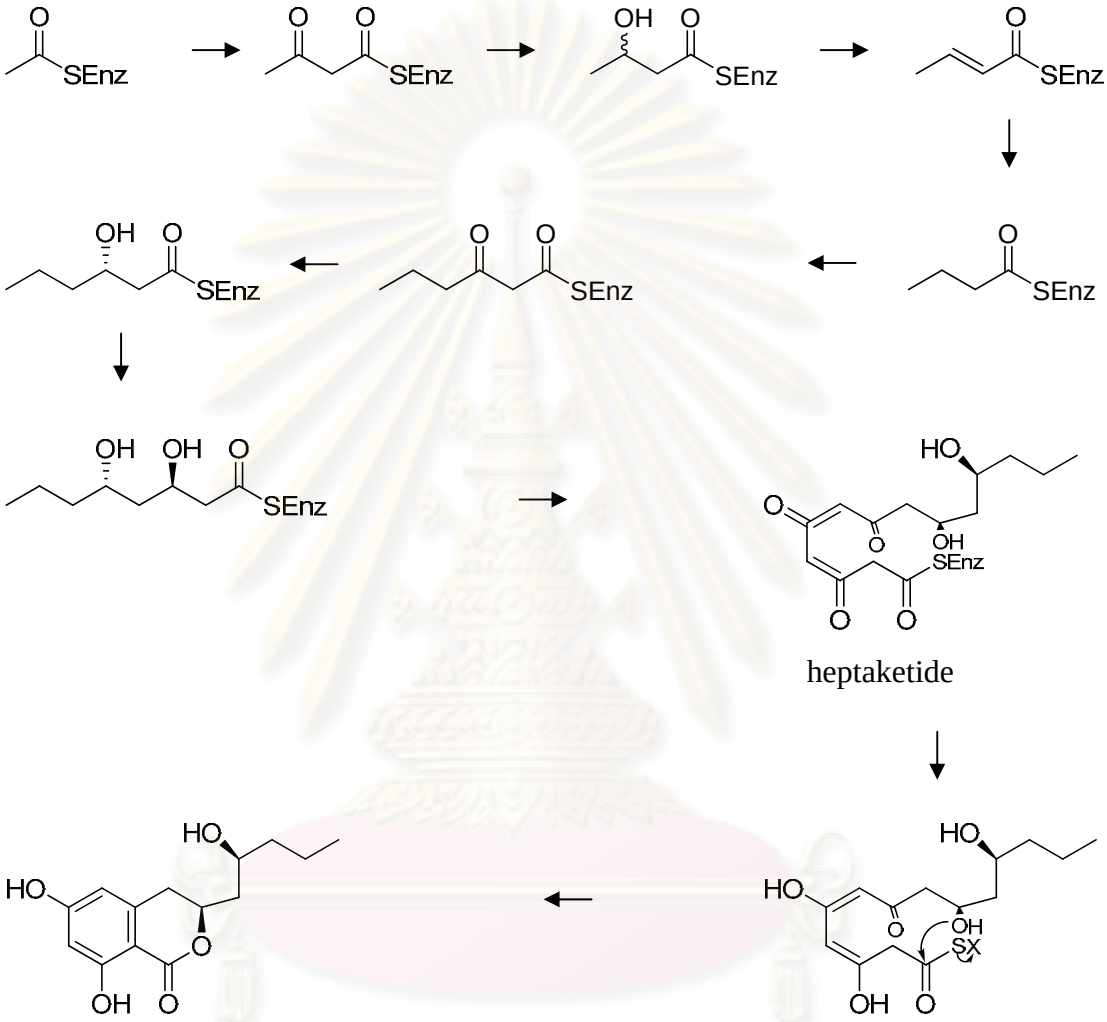


Figure 4.8 compound **1**

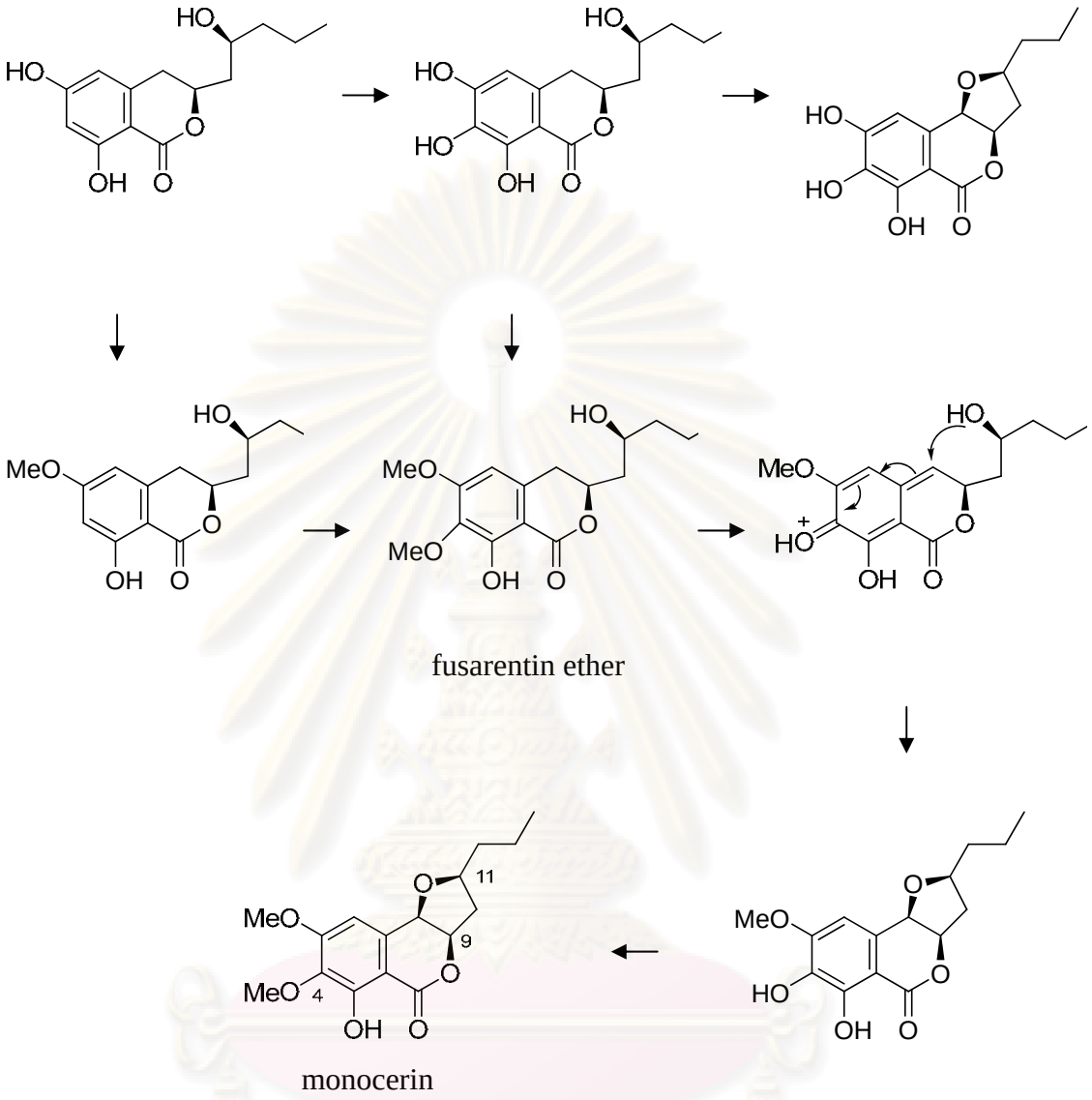
$C_{16}H_{20}O_6$; colorless oil; $[\alpha]^{20}_D$ +70.0 (*c* 0.1, in MeOH)
^1H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.2/ Page 46**

Monocerin a polyketide compound. Its biosynthesis was studied in *Dreschlera ravenelii* (Simpson, 1998) Incorporations of ^{13}C, ^2H and ^{18}O-labelled acetates and $^{18}O_2$ gas and analysis by ^{13}C and ^2H NMR showed inter alia that the oxygen atoms attached to C-9 and C-11 were acetate derived, so that successive ketoreductions occur with opposite stereochemistry; that both hydrogens at C-10 are acetate derived, consistent with ketoreduction occurring during chain assembly; and that the'extra' oxygen at C-4 is derived aerobically (Scott *et al*., 1984). These results are consistent with the polyketide assembly sequence shown in **Scheme 4.1** to give the heptaketide as the product of the assembly phase. This would cyclise and aromatise and finally lactonisation of the C-9 hydroxyl onto the thioester would give the dihydroisocoumarin as the first PKS-free intermediate. This would be further converted into the final metabolite as outlined in **Scheme 4.2**, in which the exact sequence of the necessary modification steps is unclear. Support for this sequence does come from the isolation of the known fusarentin ether as a co-metabolite of monocerin. In order to establish the exact sequence, an enantioselective synthesis of the fusarentins which will allow preparation of the necessary di- and trioxygenated dihydroisocoumarin intermediates doubly labelled with ^{13}C has been developed (McNicholas *et al*., 1996). A number of putative assembly intermediates have been

prepared in ^{2}H labelled forms for feeding studies to confirm the assembly sequence shown in **Scheme 4.1**.



Scheme 4.1 Polyketide assembly sequence



Scheme 4.2 Biosynthesis of monocerin

Compound 2 (11-hydroxymonocerin)

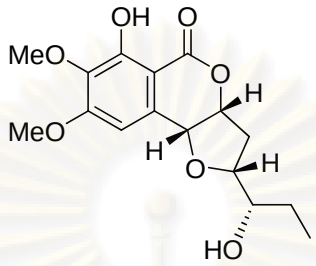


Figure 4.9 compound **2**

$C_{16}H_{20}O_7$; white solid; m.p. 118-121°C; $[\alpha]^{20}_D$ +50.0 (*c* 0.1, in MeOH); UV (EtOAc) λmax (log ε) 308 (3.78), 273 (4.23) nm; IR (KBr) ν_{max} 3431, 2930, 1665, 1455, 1276, 1117 cm^{-1}

^1H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.3/ Page 48**

Compound 3 (12-hydroxymonocerin)

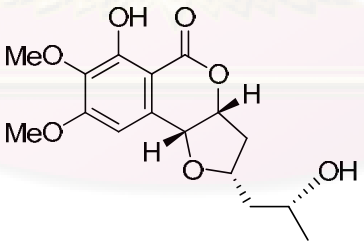


Figure 4.10 compound **3**

$C_{17}H_{20}O_7$; colorless oil; $[\alpha]^{20}_D$ +30.0 (*c* 0.1, in MeOH)

^1H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.4/ Page 50**

Compound 12

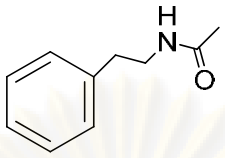


Figure 4.11 compound **12**

$C_{10}H_{13}NO$; white solid

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.13/ Page 62**

Compound 13

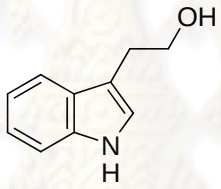


Figure 4.12 compound **13**

$C_{10}H_{11}NO$; white solid

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.14/ Page 63**

Compound 14

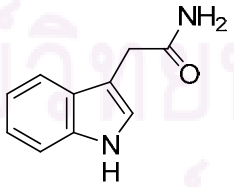


Figure 4.13

$C_{10}H_{10}N_2O$; white solid

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.15/ Page 64**

Compound 15 (cytochalasin D)

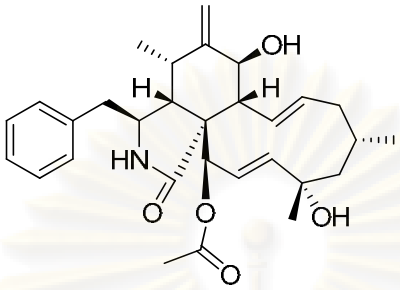


Figure 4.14 compound **15**

$C_{30}H_{37}NO_6$; white solid

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.16/ Page 65**

Disruption of actin filaments affects multiple cell functions including motility, signal transduction and cell division, ultimately culminating in cell death. Cytochalasin D disrupts actin filaments, mouse mesangial cells, to undergo apoptosis. Cytochalasin D induces the pro-apoptotic pathways, p38 and stress-activated protein kinase (SAPK)/jun amino-terminal kinase (JNK), in both cell types (Ailenberg and Silverman, 2003).

Compound 16 (cytochalasin H)

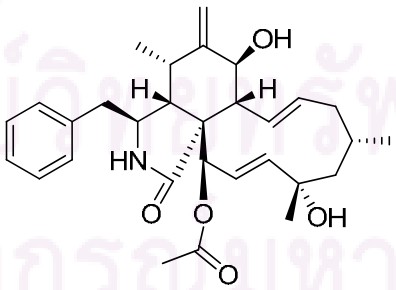


Figure 4.15 compound **16**

$C_{30}H_{37}NO_6$; white solid

^{1}H NMR and ^{13}C NMR data in $CDCl_3$: see **Table 4.17/ Page 67**

4.4 Structural elucidation of pure compounds

4.4.1 Compound **1**

Monocerin **1** was isolated as colorless oil and was optically active. The molecular formula was determined to be $C_{16}H_{20}H_6$ by analysis of its HRESIMS (*m/z* 331.1151 $[M + Na]^+$.

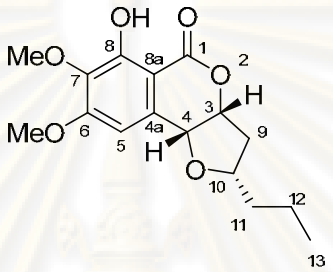


Figure 4.16 Compound **1**

Monocerin (**1**), the major metabolite, was characterized by analyses of its spectroscopic data and by comparison with data reported in the literature (Zhang *et. al*., 2008). The ^{1}H NMR and ^{13}C NMR spectral data for compound **1** are shown in **Table 4.2**.

Table 4.2 NMR spectral data for compound **1**

	Monocerin		**Compound 1** ($CDCl_3$)	
Position	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1	167.8	-	167.8	-
2	-	-	-	-
3	81.3	4.98 (brs)	81.3	5.03 (brs)
4	74.3	4.46 (brs)	74.7	4.52 (brs)
4a	131.3	-	131.2	-
5	104.9	6.53 (s)	104.9	6.57 (s)
6	158.6	-	158.7	-
7	137.2	-	137.2	-
8	156.0	11.21	156.2	11.25
8a	101.9	-	101.9	-
9	39.0	2.03 (ddd)	39.0	2.12 (dd)
		J=14.5, 6.0, 1.1		*J*=14.4, 5.2
		2.52 (m)		2.58 (m)
10	78.5	4.08 (m)	78.7	4.08 (m)
11	38.1	1.49 (m)	38.0	1.55 (m)
		1.60 (m)		1.66 (m)
12	19.0	1.33 (m)	19.1	1.38 (m)
13	13.9	0.82 (t) *J*=7.4	13.9	0.88 (t) *J*=7.0
6-OMe	56.2	3.86 (s)	56.2	3.92 (s)
7-OMe	60.6	3.79 (s)	60.7	3.86 (s)
8-OH	-	11.25 (s)	-	11.25 (s)

4.4.2 Compound **2**

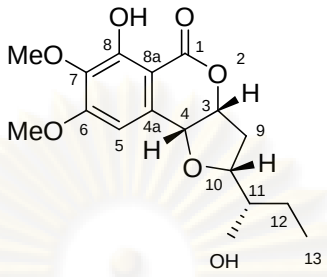


Figure 4.17 Compound **2**

11-hydroxymonocerin (**2**) was isolated as a white solid and was optically active. The molecular formula was determined to be $C_{16}H_{20}H_7$ by analysis of its HRESIMS (*m/z* 347.1128 $[M + Na]^+$, Δ -0.1 mmu), implying seven double-bond equivalents. The IR spectrum showed a strong broadened OH absorption band at 3431 cm^{-1} and characteristic absorption bands for ester carbonyl (1665 cm^{-1}) and aromatic ring (1521, 1455, and 1276 cm^{-1}) groups. The ^1H NMR spectrum showed a chelated OH signal (δ_H 11.24, OH-8) and signals for an aromatic proton (δ_H 6.61), four oxygen-bearing methine groups (δ_H 5.10, 4.63, 4.02, and 3.53), and two OCH_3 groups (δ_H 3.97 and 3.91). Analysis of ^{13}C NMR and HSQC experiments revealed the presence of a conjugated ester carbonyl (δ_C 167.6) strongly hydrogen-bonded with an OH, six aromatic carbons of which three were oxygenated (δ_C 158.7, 156.2, and 137.4), and one protonated (δ_C 104.6), four oxymethine (δ_C 81.8, 80.9, 75.0, and 74.6), two methylene (δ_C 35.9 and 25.9), and a methyl (δ_C 9.9) carbon. The ^1H and ^{13}C NMR spectra were very similar to those of monocerin (**1**), except for the marked differences in chemical shift values corresponding to position 11. In the ^1H NMR spectrum of **2**, the signal attributable to an oxygen-bearing methine at δ_H 3.53 replaced those corresponding to the methylene signal of **1** at δ_H 1.55 and 1.66. Treatment of **2** with acetic anhydride resulted in the formation of a diacetate **5**, and the ^1H NMR spectrum of **5** revealed two acetate methyl singlets at δ_H 2.09 and 2.40, respectively. The shift in the signal corresponding to H-11 (δ_H 4.92) indicated that one OH group was attached to C-11 in **2**. The proposed structure was confirmed by a proton spin system from H-4 to H^3-13, established by ^1H-^1H correlation observed in

the COSY spectrum and by HMBC correlations (**Figure 4.18**) of H-4 to δ_C 130.6 (C-4a), 101.9 (C-8a), 104.5 (CH-5), and 80.9 (CH-3) as well as H-3 to δC 38.9 (CH2-9). The full assignments and connectivity were determined by ^{1}H-^{1}H COSY correlations as indicated by bold lines and HMBC correlations shown by arrows (**Figure 4.18**). By analysis of NOESY data, the compound exhibited NOEs between H-3 and H-4 and between H-4 and H-10, indicating that these protons are all on the same face of the ring system. The ^{1}H NMR and ^{13}C NMR spectral data for compound **2** are shown in the **Table 4.3**.

Table 4.3 NMR spectral data for compound **2**

	Compound 2 ($CDCl_3$)			
Position	δ_C	δ_H (mult., *J* in Hz)	COSY	HMBC
1	167.6	-	-	-
2	-	-	-	-
3	80.9	5.10 (brs)	4, 9	4
4	74.6	4.63 (brs)	3	3, 5, 4a, 8a
4a	130.6	-	-	-
5	104.6	6.61 (s)	-	4a, 6, 7, 8a,
6	158.7	-	-	-
7	137.4	-	-	-
8	156.2	11.24 (s)	-	-
8a	101.9	-	-	-
9	35.9	2.27 (dd, 5.0, 14.4)	3, 10	3, 10
		2.57 (m)		
10	81.8	4.02 (m)	9, 11	-
11	75.0	3.53 (s)	10, 12	-
12	25.9	1.45 (m)	11, 13	11, 13
		1.53 (m)		
13	9.9	1.02 (t)	12	11, 12
6-OMe	56.3	3.97 (s)	-	6
7-OMe	60.3	3.91 (s)	-	7
8-OH	-	11.24 (s)	-	7, 8, 8a

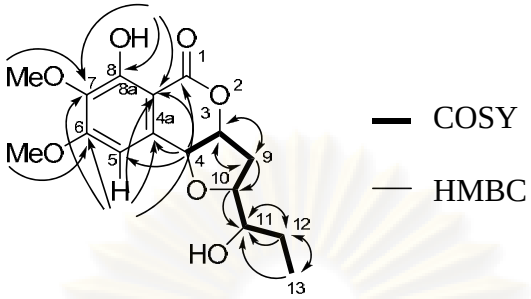


Figure 4.18 The COSY spectrum and by HMBC correlations of compound **2**

The absolute configuration of **2** was assigned by application of the modified Mosher method. Treatment of **2** with (*S*)- and (*R*)-MTPACl afforded the (*R*)-MTPA ester (**2a**) and (*S*)-MTPA ester (**2b**), respectively. The difference in chemical shift values ($\Delta\delta$) δS - δR) for the diastereomeric esters **2b** and **2a** was calculated in order to assign the absolute configuration at C-11. Calculation for all relevant signals suggested the *R* absolute configuration at C-11, as shown in **Figure 4.19**. Therefore, compound **2** was determined to be 11(*R*)-hydroxymonocerin.

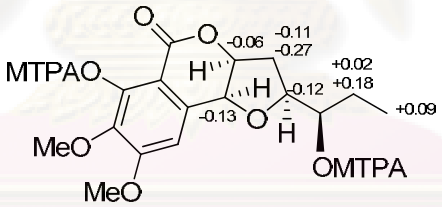


Figure 4.19 $\Delta\delta$ values (in ppm) δ_S - δ_R for (*S*)- and (*R*)-MTPA esters in **2a** and **2b**.

4.4.3 Compound **3**

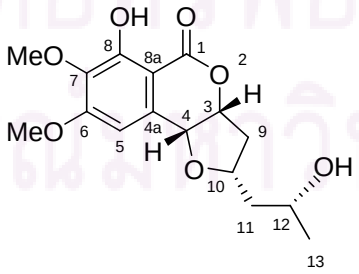


Figure 4.20 Compound **3**

12-hydroxymonocerin **3** had the same molecular formula as **2**, $C_{16}H_{20}H_7$. Comparison of the optical rotation and its NMR spectroscopic data with those in the literature indicated that compound **3** is 12(*R*)- hydroxymonocerin (Zhang *et. al*., 2008). The ^{1}H NMR and ^{13}C NMR spectral data for compound **3** are shown in the **Table 4.4**.

Table 4.4 NMR spectral data for compound **3**

Position	**Compound 3** ($CDCl_3$)	
	δ_C	δ_H (mult., *J* in Hz)
1	167.6	-
2	-	-
3	80.8	5.06 (dd) *J*= 6.0, 3.0
4	75.0	4.60 (d) *J*=3.0
4a	130.6	-
5	104.5	6.57 (s)
6	158.7	-
7	137.5	-
8	156.3	-
8a	102.0	-
9	39.6	2.22 (dd) *J*=14.5, 5.5
		2.67 (ddd) *J*=14.5, 8.5, 6.0
10	78.4	4.35 (m)
11	44.8	1.75 (dt) *J*= 14.0, 4.0
		1.84 (ddd) 14.0, 9.0, 6.0
12	67.2	3.98 (m)
13	23.6	1.81 (d) 6.0
6-OMe	56.3	3.95 (s)
7-OMe	60.7	3.91 (s)
8-OH	-	11.23 s

Structural elucidation of monocerin derivatives

4.4.4 Compound **4**

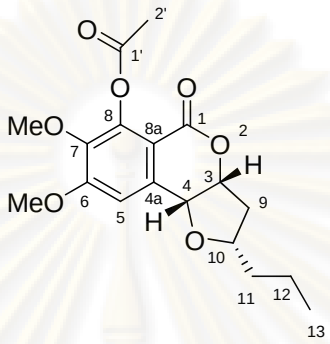


Figure 4.21 Compound **4**

8-acetylmonocerin **4** was obtained from acetylation reaction of monocerin (**1**) by using acetic anhydried in the present of DMAP as a catalyst. The ^{1}H NMR and ^{13}C NMR spectral data for compound **4** are shown in the **Table 4.5**.

Table 4.5 NMR spectral data for compound **4**

Compound 4 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	159.5	-
2	-	-
3	79.4	4.96 (brs)
4	74.0	4.54 (d)
4a	132.2	-
5	109.5	6.89 (s)
6	157.5	-
7	142.6	-
8	145.5	-
8a	109.8	-
9	38.5	2.52 (m)
		2.10 (dd, 14.4, 5.6)
10	78.4	4.14 (m)
11	37.6	1.53 (m)
		1.65 (m)
12	18.6	1.34 (m)
13	13.5	0.88 (t, 7.2)
6-OMe	55.8	3.94 (m)
7-OMe	60.7	3.82 (s)
1´	168.8	2.39 (s)
2´	20.5	-

4.4.5 Compound **5**

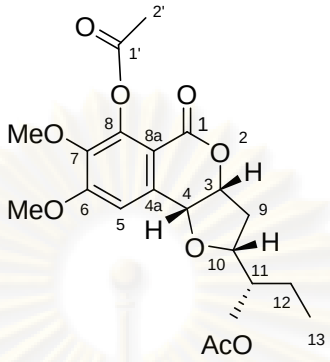


Figure 4.22 Compound **5**

Diacetylmonocerin **5** was synthesized by adding acetic anhydride and DMAP to a solution of 11-hydroxymonocerin (**2**) in pyridine. The ^{1}H NMR and ^{13}C NMR spectral data for compound **5** are shown in the **Table 4.6**.

Table 4.6 NMR spectral data for compound **5**

Compound 5 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	160.0	-
2	-	-
3	78.9	4.98 (brs)
4	74.4	4.59 (d 3.6)
4a	132.2	-
5	110.0	6.90 (s)
6	158.0	-
7	143.2	-
8	146.0	-
8a	110.4	-
9	35.6	2.21 (dd) 14.4, 6.0
		2.48 (m)
10	78.7	4.26 (m)
11	74.7	4.92 (m)
12	23.7	1.62 (m)
13	9.9	0.87 (t) *J*=7.6
6-OMe	56.3	3.97 (s)
7-OMe	61.2	3.84 (s)
1´	169.5	-
2´	21.0	2.09 (s)
1´´	171.5	-
2´´	21.0	2.40 (s)

4.4.6 Compound **6**

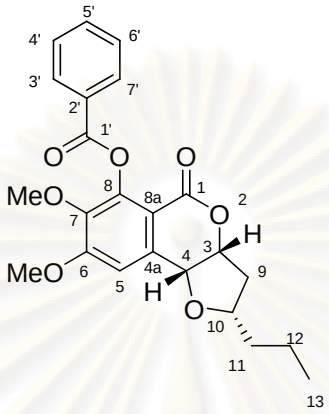


Figure 4.23 Compound **6**

8-benzoylmonocerin **6** was synthesized by adding benzoic anhydride and DMAP to a solution of monocerin (**1**) in pyridine. The ^{1}H NMR and ^{13}C NMR spectral data for compound **6** are shown in the **Table 4.7**.

Table 4.7 NMR spectral data for compound **6**

Compound 6 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	ND	-
2	-	-
3	79.8	4.99 (brs)
4	74.6	4.58 (d, 2.7)
4a	129.4	4.58 (d, 2.7)
5	110.7	6.95 (s)
6	158.0	-
7	146.2	-
8	ND	-
8a	110.0	-
9	39.0	2.10 (dd, 14.2, 5.2), 2.52 (m)
10	79.0	-
11	38.1	1.55 (m), 1.67 (m)
12	19.1	1.37 (m)
13	14.0	0.89 (t, 7.2)
1´	ND	-
2´	ND	-
3´, 7´	130.5	8.24 (d, 7.4)
4´, 6´	128.5	7.50 (d, 7.8)
5´	133.5	7.62 (t, 7.4)
6-OMe	56.3	3.98 (s)
7-OMe	61.3	3.85 (s)

4.4.7 Compound **7**

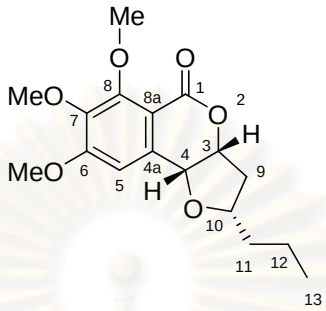


Figure 4.24 Compound **7**

8-methylmonocerin **7** was methylated by using methyl iodide in the present of sodium hydride as base. The ^{1}H NMR and ^{13}C NMR spectral data for compound **6** are shown in the **Table 4.8**.

Table 4.8 NMR spectral data for compound **7**

Compound 7 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	160.0	-
2	-	-
3	79.5	4.94 (brs)
4	75.0	4.51 (brs)
4a	132.5	-
5	111.1	6.78 (s)
6	157.9	-
7	144.2	-
8	108.1	-
8a	156.4	
9	39.0	2.16 (dd, 14.5, 5.3)
		2.51 (m)
10	78.9	4.14 (m)
11	38.0	159
		1.71 (m)
12	19.1	1.38 (m)
13	13.9	0.90 (t, 7.3)
6-OMe	56.2	3.94 (s)
7-OMe	61.1	3.88 (s)
8-OH	61.8	3.96 (s)

4.4.8 Compound **8**

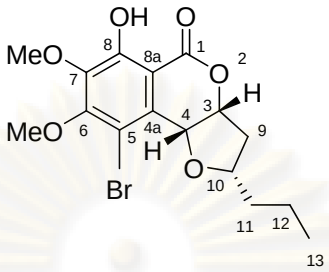


Figure 4.25 Compound **8**

5-bromomonocerin **8** was synthesized by bromination of monocerin (**1**) with NBS. The ^{1}H NMR and ^{13}C NMR spectral data for compound **5** are shown in the **Table 4.9**.

Table 4.9 NMR spectral data for compound **8**

Compound 8 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	167.8	-
2	-	-
3	81.3	5.03 (brs)
4	73.6	4.84 (d)
4a	129.7	
5	109.5	-
6	167.0	-
7	142.5	-
8	156.3	11.59 (s)
8a	105.0	-
9	38.5	2.17 (dd)
		2.59 (m)
10	78.6	4.17 (m)
11	37.9	1.70 (m)
		1.56 (m)
12	19.0	1.37 (m)
13	14.0	0.91 (t) *J*= 7.3
6-OMe	61.1	4.03 (s)
7-OMe	61.4	3.93 (s)
8-OH	-	11.59 (s)

4.4.9 Compound **9**

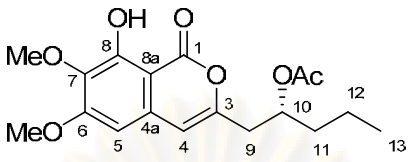


Figure 4.26 Compound **9**

8-hydroxy-6,7-dimethoxy-3-(pentan-2´-yl-acetate)-isocoumarin (**9**) was obtained from Friedel-Crafts acylation of monocerin (**1**) with acetyl cholide and $AlCl_3$ in excess amount. The structure of the ring-opening derivative **9** was characterized on the basis of spectroscopic data. The ^{1}H NMR and ^{13}C NMR spectral data are shown in the **Table 4.10**, and its COSY and HMBC correlations are shown in the **Figure 4.27**

Figure 4.27 The COSY (left) spectrum and by HMBC (right) correlations of compound **9**.

Table 4.10 NMR spectral data for compound **9**

Position	**Compound 9** ($CDCl_3$)	
	δ_C	δ_H (mult., *J* in Hz)
1	166.0	-
2	-	-
3	153.0	-
4	105.7	6.23 (s)
4a	135.4	
5	98.4	6.36 (s)
6	160.0	-
7	133.9	-
8	154.7	-
8a	111.1	
9	38.1	2.73 (m)
10	71.2	5.19 (m)
11	36.1	1.64 (m)
12	18.5	1.38 (m)
13	13.8	0.93 t *J*= 7.0
6-OMe	56.2	3.94 (s)
7-OMe	60.8	3.90 (s)
8-OH	-	11.03 (s)
10-OMe	170.6	-
	21.1	-

4.4.10 Compound **10**

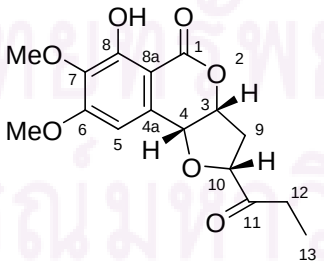


Figure 4.28 Compound **10**

11-Oxomonocerin **10** was synthesized by Swern oxidation of 11-hydroxymonocerin (**2**). The ^{1}H NMR and ^{13}C NMR spectral data for compound **5** are shown in the **Table 4.11**.

Table 4.11 NMR spectral data for compound **10**

Compound 10 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	167.7	-
2	-	-
3	83.0	4.75 (brs)
4	75.9	5.06 (brs)
4a	130.5	-
5	104.8	6.61 (s)
6	159.0	-
7	137.8	-
8	156.6	-
8a	101.9	-
9	38.0	2.73 (m)
10	80.2	4.54 dd 10.1, 4.3
11	211.6	-
12	31.3	2.50 (m)
13	7.2	0.99 (t) 7, 2
6-OMe	56.6	3.97 (s)
7-OMe	61.1	3.93 (s)
8-OH	-	11.3 (s)

4.4.11 Compound **11**

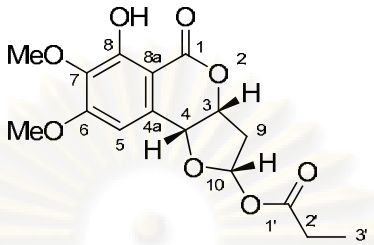


Figure 4.29 Compound **11**

11-carbonylmonocerin **11** was synthesized by Baeyer-Villiger oxidation of 11-oxomonocerin (**10**). The ^{1}H NMR and ^{13}C NMR spectral data for compound **5** are shown in the **Table 4.12**.

Table 4.12 NMR spectral data for compound **11**

Compound 11 ($CDCl_3$)		
Position	δ_C	δ_H (mult., *J* in Hz)
1	167.0	-
2	-	-
3	78.3	5.22 (m)
4	75.7	4.98 d *J*=4.0
4a	130.4	
5	104.6	6.59 (s)
6	158.8	-
7	137.4	-
8	156.2	-
8a	101.2	-
9	40.5	2.61 (m)
		2.73 (dd) *J*= 14.9
10	97.0	6.42 (d) *J*=5.3
1´	173.6	-
2´	27.6	2.23 (m)
3´	8.5	1.03 (t) *J*= 7.5
6-OMe	55.7	3.94 (s)
7-OMe	60.8	3.90 (s)
8-OH	-	-

4.4.12 Compound **12**

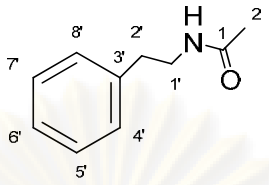


Figure 4.30 Compound **12**

N-Phenetyl-acetamide **12** was obtained from an endophytic fungus strain APAN15. Its structure was determined by analysis of the ^{1}H NMR and ^{13}C NMR spectral data (**Table 4.13**).

Table 4.13 NMR spectral data of compound **12**

Position	Compound 12	
	δ_C	δ_H (multiplicity *J* in Hz)
1	170.2	-
2	23.3	1.93 (s)
1´	40.7	3.50 (dd) *J*=13.02, 6.80
2´	35.6	2.81 (t), *J*=6.96
3´	138.8	-
4´, 8´	128.6	7.23-7.25 (m)
5´, 7´	128.7	7.29-7.33 (m)
6´	126.5	7.18-7.17 (m)

4.4.13 Compound **13**

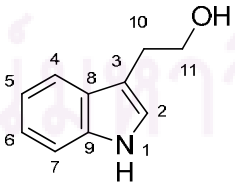


Figure 4.31 Compound **13**

Tryptophol **13** was obtained from an endophytic fungus strain APAN15. Its structure was determined by analysis of the ^{1}H NMR and ^{13}C NMR spectral data (**Table 4.14**).

Table 4.14 NMR spectral data of compound **13**

Position	Compound 13	
	δ_C	δ_H (multiplicity *J* in Hz)
1	-	-
2	122.8	7.44 (brs)
3	111.9	-
4	118.8	7.66 (d) *J*=7.86
5	119.3	7.24 (m)
6	122.0	7.19 (m)
7	111.4	7.34 (d) *J*=8.10
8	127.4	-
9	136.5	-
10	28.7	3.03 (t) *J*=6.46
11	62.6	3.90 (t) *J*=6.46

4.4.14 Compound **14**

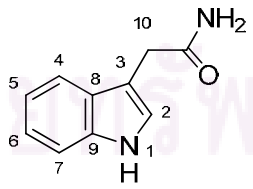


Figure 4.32 Compound **14**

Indole-3-acetamide **14** was obtained from an endophytic fungus strain APAN5. Its structure was determined by analysis of the ^{1}H NMR and ^{13}C NMR spectral data (**Table 4.15**).

Table 4.15 NMR spectral data of compound **14**

Position	Compound 14	
	δ_C	δ_H (multiplicity *J* in Hz)
1	-	-
2	124.4	7.21 (d) *J*=2.25
3	108.1	-
4	118.8	7.48 (d) *J*=7.90
5	119.0	7.06 (m)
6	121.4	6.96 (m)
7	111.9	7.32 (brs)
8	127.6	-
9	136.5	-
10	31.4	3.62 (s)
11	173.7	-

4.4.15 Compound **15**

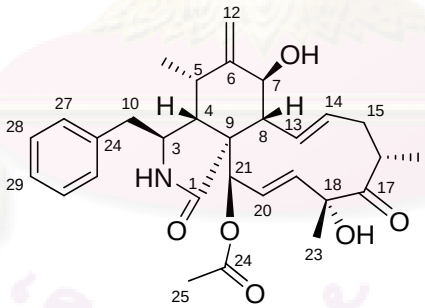


Figure 4.33 Compound **15**

Compound **15**, a white powder, was obtained from an endophytic fungus isolate CODO58. Its molecular formula was established to be $C_{30}H_{37}NO_6$ by analysis of its HRESIMS (*m/z* 507.261 $[M + Na]^+$. Comparison of spectroscopic data of **15** with those published in the literature (Jikai *et. al.* 2002; Kakeya *et. al.* 1997) (**Table 4.16)** indicated that compound **15** was cytochalasin D.

Table 4.16 NMR spectral data of compound **15**

Position	Cytochalasin D		Compound 15	
	δ_C	δ_H (multiplicity *J* in Hz)	δ_C	δ_H (multiplicity *J* in Hz)
1	173.7	-	173.6	-
2	-	-	-	-
3	53.5	3.20 (m)	53.5	3.23 (m)
4	49.9	2.15 (m)	50.0	2.15 (m)
5	32.6	2.73(m)	32.7	2.74 (m)
6	147.6	-	147.5	-
7	69.8	3.80 d, *J* = 10.5	69.8	3.81 d, *J* =10.28
8	46.9	2.82 (m)	47.0	2.83 (m)
9	53.3	-	53.2	-
10	45.3	2.59, 2.82 (m)	45.3	2.53, 2.83 (m)
11	13.6	0.94 d, *J*=6.8	13.7	0.95 d, *J* = 6.5
12	114.4	5.29 (s) 5.08 (s)	114.5	5.30 (s) 5.09 (s)
13	127.6	5.62 dd *J*=15.7, 9.8	127.6	5.64 brs
14	130.6	5.13 dd *J*= 15.7, 2.3	130.6	5.12 brs
15	37.7	2.02 dd *J*= 5.1, 13.0	37.7	2.02 dd, *J* = 4.3, 12.9
16	42.3	2.73 (m)	42.3	2.74 (m)
17	210.2	-	210.2	-
18	77.6	-	77.7	-
19	132.3	5.32 (m)	132.3	5.33 (m)
20	134.1	6.11 dd, *J*=15.7, 2.7	134.1	6.11 d, *J* = 15.6
21	76.7	4.64 (s)	76.8	4.65 (s)
22	19.4	1.19 d, *J*= 6.8	19.4	2.00
23	24.2	1.51 (s)	24.1	1.51
24	169.6	-	169.7	-
25	20.8	2.26 (s)	20.8	2.27 (s)
26	137.2	-	137.2	-
27,31	129.1	7.13 (m)	129.1	7.13 (m)
28, 30	128.9	7.32 (m)	128.9	7.34 (m)
29	127.0	7.26 (m)	127.0	7.25 (m)

4.4.16 Compound **16**

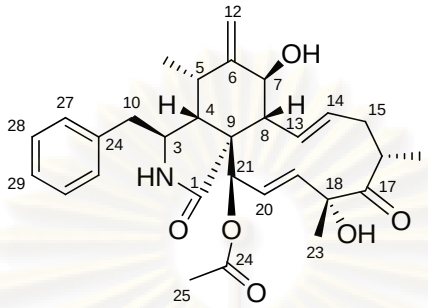


Figure 4.34 Compound **16**

Compound **16**, a white powder, was obtained from an endophytic fungus isolate CODO23. Its molecular formula was established to be $C_{30}H_{37}NO_6$ by the NMR spectroscopic data. Comparison of spectroscopic data of **16** with the published in the literature (Ondeyka *et. al.* 1992; Kakeya *et. al.* 1997) (**Table 4.17)** indicated that compound **16** was cytochalasin H.

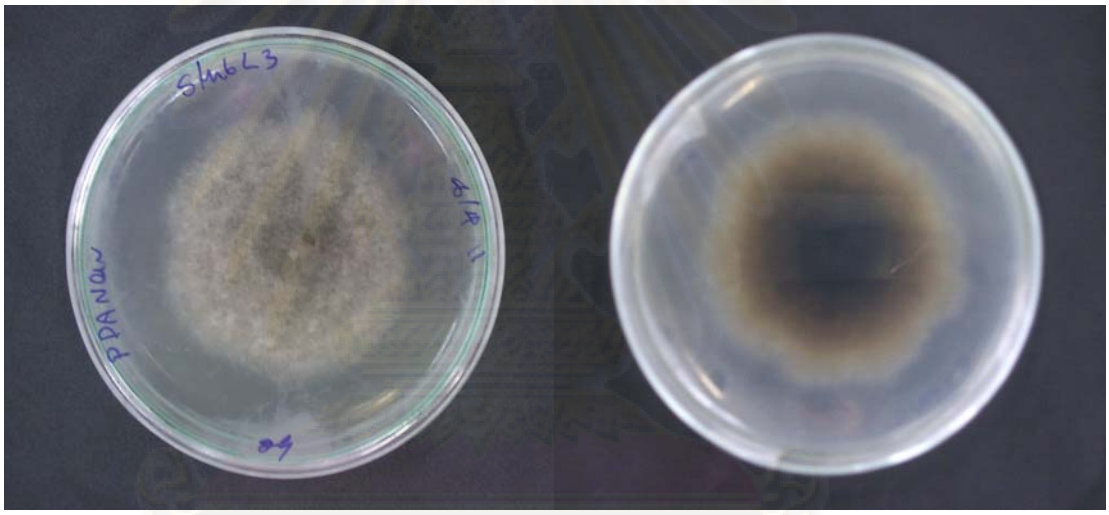
Table 4.17 NMR spectral data of compound **16**

Position	Cytochalasin H		compound 16	
	δ_C	δ_H (multiplicity *J* in Hz)	δ_C	δ_H (multiplicity *J* in Hz)
1	174.3	-	174.3	-
2	-	5.6	-	5.54 (m)
3	53.8	3.23 m	53.8	3.25 (dd) *J*=4.08, 8.68
4	50.3	2.12 t	50.3	2.12 (m)
5	32.8	2.77 m	32.8	2.76 (m)
6	148.0	-	147.9	-
7	69.7	3.82 d 10.8	69.7	3.82 (d) *J*=10.75
8	47.2	2.93 t	47.2	2.93 (t) *J*=10.22
9	51.8	-	51.8	-
	45.6	2.62 dd 9.5, 13.3	45.5	2.65 (dd) *J*=9.61, 13.19
10		2.85 dd 4.4, 13.5		2.84 (dd) *J*=4.46, 13.42
11	14.1	1.00 d 6.7	14.0	0.97 (d) *J*=6.61
	114.1	5.15 s	114.1	5.10 (brs)
12		5.35 s		5.33 (brs)
13	125.9	5.75 ddd 1.2, 9.2, 15.6	125.9	5.72 (m)
	139.1		138.7	5.39 (m)
14		5.40 ddd 5.4, 10.5, 15.6		
	42.8	1.77 m	42.7	2.03 (m)
15		2.00 m		1.77 (m)
16	31.1	1.8 m	31.1	1.79 (m)
	53.8	1.56 dd 2.0, 14.0	53.8	1.56 (d) *J*=13.56
17		1.86 dd 2.4, 14.2		1.86 (d) *J*=14
18	74.3	-	74.3	
19	127.0	5.56 dd 2.1, 13.2	127.0	5.54 (m)
20	138.6	5.86 dd 3.3, 13.6	138.7	5.84 (d) *J*=18.66
21	77.5	5.55 dd 2.4, 2.4	77.5	5.54 (m)
22	26.5	1.05 d 6.3	26.4	1.03 (d) *J*=5.73
23	28.4	1.32 s	28.4	1.33 (s)
24	170.1	-	170.1	-
25	20.9	2.23	20.9	2.23 (s)
26	137.4	-	137.3	-
27, 31	128.9	7.14 d	128.9	7.13 (d) *J*=7.27
28, 30	128.9	7.30 dd	129.0	7.30 (m)
29	127.0	7.25 t	127.0	7.36 (m)

4.5 Classification of the endophytic fungal isolate STEM3

4.5.1 Conventional method

The fungal endophyte isolate STEM3 grew on PDA as brown filamentous colonies, as shown **Figure 4.35**. On Alphacel medium, STEM3 produced dark septate hyphae and straight to slightly curved brown conidia with strongly protruding, truncate hilum, as shown in **Figure 4.36**. Conidia contained 5-12 distosepta and had prominent dark basal and distal septa. Conidia were measured 27-87 μm x 10-17 μm. These are the characteristics of *Exserohilum rostratum* (Sivanesan, 1987).



Obverse Reverse

Figure 4.35 Colony morphology of endophytic fungus isolate STEM3 on PDA.

Figure 4.36 Conidia of endophytic fungus isolate STEM3.

4.5.2 Molecular method

Sequencing of the PCR product amplified from chromosomal DNA of isolate STEM3 resulted in a 564 bp fragment. This comprised partial of the 18S sequence, complete ITS1-5.8S-ITS2 sequence, and partial of the 28S sequence, as shown in **Figure 4.37**. A GenBank search for DNA sequence similarity revealed that ITS1-5.8S-ITS2 of the STEM3 was 99% homology to those of three strains of *E. rostratum* (AF163066, AJ853741 and EF222027) and 98% homology to that of a teleomorphic fungus *Setosphaeria rostrata* ATCC 32197 (AF071342) which is known to produce *E. rostratum* anamorph (Leonard, 1976). The ITS1-5.8S-ITS2 DNA sequence of STEM3 was also found to be 99% homology to that of *E. mcginnisii* (AF081453). However, conidia morphology of STEM3 was different from that of *E. mcginnisii* (Padhye *et al.,* 1986). Based on microscopic morphological characteristics and DNA sequence of the ribosomal RNA gene region, this endophytic fungus is classified as *Exserohilum rostratum* STEM3. The ITS1-5.8S-ITS2 DNA sequence of the STEM3 fungus has been submitted to GenBank with the accession number of EU571210.

```
....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50
               18S                        ITS1
CCGTAGGTGA ACCTGCGGAG GGATCATTAC ACAACAAAAA TATGAGGGTG
                          ITS1
TGGTTTGCTG GCAACAGCGT CCGCCCCAAG TATTTTTCAC CCATGTCTTT
                          ITS1
TGCGCACTTT TTGTTTCCTG GGCGAGTTCG CTCGCCACCA GGACCCAACC
                          ITS1
ATAAACCTTT TTTTATGCAG TTGCAATCAG CGTCAGTATA ATAATTCAAT
                               5.8S
TTATTAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA
                          5.8S
CGCAGCGAAA TGCGATACGT AGTGTGAATT GCAGAATTCA GTGAATCATC
                          5.8S
GAATCTTTGA ACGCACATTG CGCCCTTTGG TATTCCAAAG GGCATGCCTG
      5.8S                      ITS2
TTCGAGCGTC ATTTGTACCC TCAAGCTTTG CTTGGTGTTG GGCGTCTTTT
                          ITS2
TGTCTCTCCC CTTGTTGGGG GAGACTCGCC TTAAAACGAT TGGCAGCCGA
                          ITS2
CCTACTGGTT TTCGGAGCGC AGCACAAATT TGCGCCTTCC AATCCACGGG
                          ITS2                    28S
GCGGCATCCA GCAAGCCTTT GCTTTCTATA GCAAATCCAC ATTTTGACCT
       28S
CGGATCAGGT AGGG
```

Figure 4.37 Nucleotide sequences of 18S (partial), ITS1-5.8S-ITS2 (complete) and 28S (partial) ribosomal RNA genes of endophytic fungus isolate STEM3.

Taxonomy of *Exserohilum rostratum* (Leonard and Suggs, 1974)

Kingdom: Fungi

Phylum: Ascomycota

Class: Euascomycetes

Order: Pleosporales

Family: Pleosporaceae

Genus: *Exserohilum*

4.6 Biological activities of isolated compounds

4.6.1 Antibacterial activities

A total of 200 crude extracts were determined for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against five bacterial strains: *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The result is shown in **Table 4.18**

The crude extracts that have activity against *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were determined for minimum inhibitory concentration (MIC) at the final concentration at 256 µg/mL toward 14 bacterial strains: *B. subtilis*, *E. faecalis*, *E. faecalis* (vancomycin resistant), *E. faecium*, *S. typhimurium*, *S. aureus*, MRSA (NCCLS) (Oxacillin resistant), *S. epidermidis*, *S. hominis*, *E. coli*, *K. pneumoniae*, *K. pneumonia* (ESBL producing), *P. aeruginosa* and *P. vulgaris.* The result is shown in **Table 4.19**

Table 4.18 MIC and MBC of crude extracts

Crude extracts	*B. subtilis*		*S. aureus*		*E. faecalis*	
	MIC (µg/mL)	**MBC (µg/mL)**	**MIC (µg/mL)**	**MBC (µg/mL)**	**MIC (µg/mL)**	**MBC (µg/mL)**
S1MM	16	64	256	>256	128	>256
S1YM	32	64	256	>256	128	>256
S2MB	256	256	I	I	I	I
S2YB	256	256	I	I	I	I
S2MM	64	64	256	>256	I	I
3CB	128	>256	256	>256	I	I
S4MM	128	128	I	I	I	I
4CM	32	>256	I	I	I	I
4CB	128	>256	I	I	I	I
4MB	256	>256	I	I	I	I
8MM	128	128	I	I	I	I
8YB	64	64	I	I	I	I
S11YM	256	256	I	I	I	I
S11MM	128	128	I	I	I	I
16MM	128	128	I	I	I	I
S16YB	I	I	I	I	128	>256
A17MB	256	>256	I	I	I	I
31MM	64	256	128	256	128	256
47MM	256	256	I	I	I	I
58MB	256	>256	I	I	I	I

I = inactive

Table 4.19 MIC of crude extracts

Crude extracts	*E. faecalis* (vancomycin resistant)	*E. faecium*	*S. epidermidis*	*S. hominis*
	MIC (µg/mL)	**MIC (µg/mL)**	**MIC (µg/mL)**	**MIC (µg/mL)**
S1MM	256	128	I	I
S1YM	256	128	I	I
3CB	I	I	128	256

Sixteen pure compounds (compounds **1-16**) were determined for minimum inhibitory concentration (MIC) at the final concentration at 256 µg/mL against 14 bacterial strains: *B. subtilis, E. faecalis, E. faecalis* (vancomycin resistant), *E. faecium, S. typhimurium, S. aureus,* MRSA (NCCLS) (Oxacillin resistant), *S. epidermidis, S. hominis, E. coli, K. pneumoniae, K. pneumonia* (ESBL producing), *P. aeruginosa* and *P. vulgaris*. All of them were not active against the bacteria tested.

4.6.2 Cytotoxic activity

A total of 29 interesting crude extracts determined by ^{1}H NMR were applied for the evaluation of cytotoxicity against: BT 474, Chago, Hep-G2, KATO-3 and SW620 cancer cell lines. The result is shown in **Table 4.20**

Table 4.20 Cytotoxicity screening of crude extracts

Active	**Inactive**
S1YB	S1MM, A2YB, 3CB, S3YB, A3YB, A3YM, 4YB, 4CM(A), A5YB, A7MB, A8MM, 9MM, S10YB, S12YB, A12MM, A12YB, S13YB, A15MB, A15YB, A18MB, A18YB, 23MB, 23YB, 23MM, 26MB, 47MM, 47YB, 58YB

Sixteen pure compounds (compound **1-16**) were applied for the evaluation of cytotoxicity against: BT 474, Chago, Hep-G2, KATO-3 and SW620 cancer cell lines. Compounds **15** and **16** inhibited Hep-G2 (hepatoma) cancer cell line as reported in **Table 4.21**. Compound **15** inhibited KATO-3 cancer cell line as reported in **Table 4.22**

Table 4.21 Cytotoxic activity against Hep-G2 cell line of compounds **15**, and **16**.

Compound	IC_{50} (µg/mL)	IG_{50} (µg/mL)	TGI (µg/mL)	LD_{50} (µg/mL)
15	4.674	0.625	3.14	8.751
16	6.80	0.765	5.52	>10

Table 4.22 Cytotoxic activity against Kato-3 cell line of compound **15**.

Compound	IC_{50} (µg/mL)	IG_{50} (µg/mL)	TGI (µg/mL)	LD_{50} (µg/mL)
15	3.799	0.625	0.451	>10

4.6.3 Antimalarial activity test

Ten pure compounds were found to exhibit anti-malarial activity, as shown in **Table 4.23**.

Table 4.23 Antimalarial activity (*P. falciparum*) of compounds **1**, **2, 4**, **5, 6, 7, 8, 9, 10** and **11**.

Compound	Antimalarial activity (IC_{50}, µM)
1	0.69
2	7.42
4	0.82
5	9.10
6	7.95
7	I
8	I
9	I
10	13.4
11	I
Dihydroartemisinnine	0.004

I = inactive

CHAPTER V

CONCLUSION

The objectives of this research are to investigate bioactive compounds from endophytic fungi isolated from *A. paniculata, C. odoratum* and *Stemona* sp. for anti-bacterial activities by broth microdilution assay against *B. subtilis, E. faecalis, E. faecalis* (vancomycin resistant), *E. faecium, S. typhimurium, S. aureus*, MRSA (NCCLS) (Oxacillin resistant), *S. epidermidis, S. hominis, E. coli, K. pneumoniae, K. pneumoniae* (ESBL producing), *P. aeruginosa* and *P. vulgaris*; anti-malarial activity against *P. falciparum* K1 strain and cytotoxicity against cancer cell lines: HEP G-2 (hepatoma), SW 20 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) and CH-liver (normal cell lines); to characterize the bioactive compounds; and to identify of the fungi.

In the present investigation, a total of 92 pure isolates of endophytic fungi were isolated from *A. paniculata, C odoratum* and *Stemona* sp. Fifty eight isolates were obtained from *C. odoratum*, 18 isolates were isolated from *A. paniculata,* and 16 isolates were obtained from *Stemona* sp. Based on ^{1}H NMR, 50 isolates were selected for cultivation on 2 types of media: malt Czapek broth and yeast extract sucrose broth. Two hundreds crude extracts have been investigated for anti-bacterial activities. Certain crude extracts could inhibit bacteria with minimum inhibition concentration (MIC) of 16-256 µg/ml, minimum bactericidal concentration (MBC) of 64->256 µg/ml. The crude extracts with characteristic interesting ^{1}H NMR signals were selected for large scale cultivation.

Monocerin (**1**), 11-OH monocerin (**2**), the synthesized derivatives of monocerins (**4, 5, 6,** and **10**) have specificity in anti-malarial activity without any activity against bacteria and cytotoxicity. By SAR, replacing OAc with OH at the C-11, the anti-malarial activity decreases 10 times. Replacing OH with small ester at the C-8, the anti-malarial activity slightly decreases. Replacing OH with big ester at the

C-8, the anti-malarial activity is none. Replacing H with Br at the C-5, the anti-malarial activity is none. Opening ring has no anti-malarial activity.

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APPENDICES

APPENDICES

APPENDIX A

1. Media

1.1 Yeast extract sucrose broth (YES)

Yeast extract	20 mL
Sucrose	150 g
Distilled water up to	1 L

1.2 Malt Czapek Broth (MCz)

Czapek stock solution A	50 mL
Czapek stock solution B	50 mL
Sucrose	30 g
Malt Extract	40 g
Distilled water up to	1 L

Czapek stock solution A

$NaNO_3$	4.0 g
KCl	1.0 g
$MgSO_4.7H_2O$	1.0 g
$FeSO_4.7H_2O$	0.02 g
Dissolved in distilled water up to	100 mL

Czapek stock solution B

K_2HPO_4	2.0 g
Dissolved in distilled water up to	100 mL

A solution

$ZnSO_4.7H_2O$	1.0 g
Dissolved in distilled water up to	100 mL

B solution

$CuSO_4.5H_2O$	1.0 g
Dissolved in distilled water up to	100 mL

1.3 Potato Dextrose Agar (PDA)

Potato	200 g
Dextrose	20 g
Agar	15g
Distilled water up to	1 L

1.4 Water Agar

Agar	15 g
Distilled water up to	1 L

1.5 Alphacel medium (Sloan et al. 1961)

Alpha-cellulose (non nutritive)	20 g
$MgSO_4$.7H2O	1 g
KH_2PO_4	1.5 g
$NaNO_3$	1 g
Coconut milk	50 mL
Agar	15 g
Distilled water up to	1 L
Tomato paste	10 g
Oatmeal	10 g

2. Reagent and buffer for DNA amplification by PCR

2.1 Lysis buffer

Tris-HCl (pH 7.2)	50 mM
EDTA	50 mM
SDS	3%
2-mercaptoethanol	1%

2.2 Chlorofrom : TE-saturated phenol 1:1,v/v

2.3 TE for resuspending pellet

Tris-HCl	10 mM
EDTA	0.1 mM

2.4 Gel loading buffer

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

2.5 5-X Tris-Borate-EDTA (TBE)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20 mL

The working solution was 1X TBE, diluted with 4 volume of distilled water.

2.6 10X Buffer

Tris HCl pH 9.0	100 mL
KCL	500 mM
Triton X-100	1%

2.7 2mM dNTP (dATP, dCTP, dGTP, dTTP mix)

dATP	100 mM
dCTP	100 mM
dGTP	100 mM
dTTP	100 mM

Mixed equal volume of each dNTP to get 25 mM dNTP, then dilute to 2 mM dNTP with sterile double distilled water.

APPENDIX B

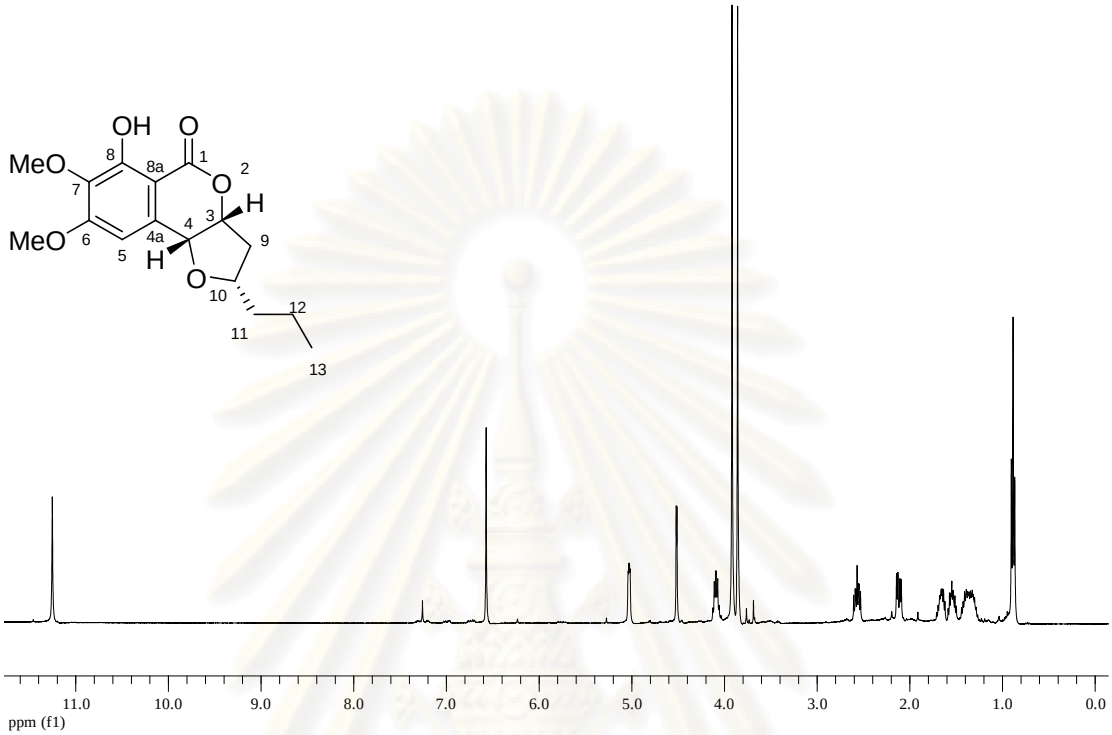

Figure B1 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of monocerin (**1**)

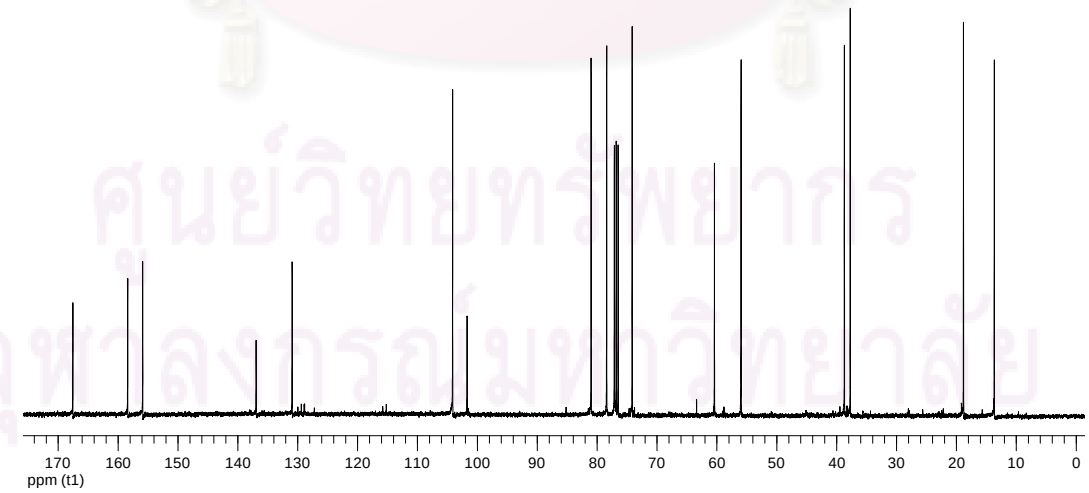

Figure B2 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of monocerin (**1**)

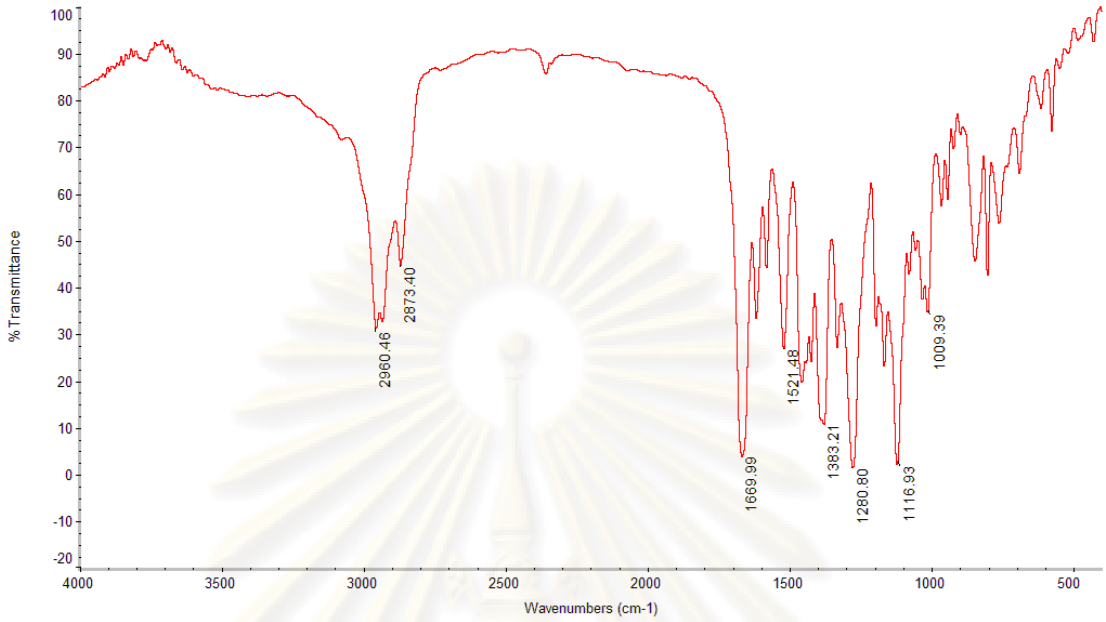

Figure B3 IR spectrum of monocerin (**1**)

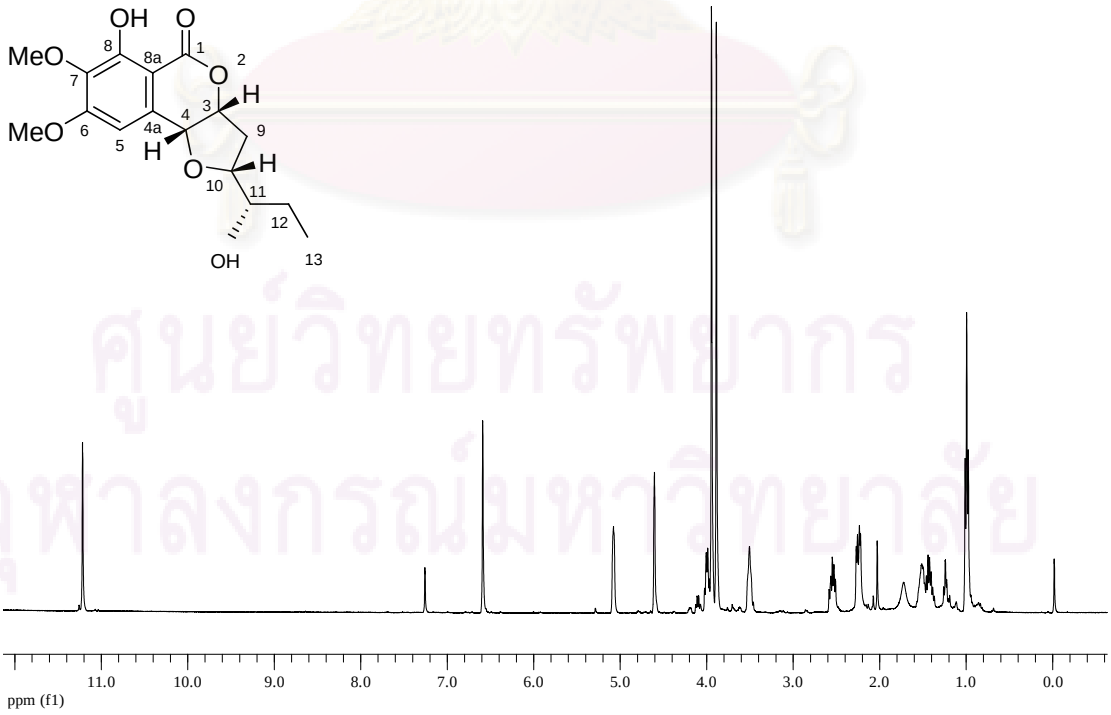

Figure B4 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 11-hydroxymonocerin (**2**)

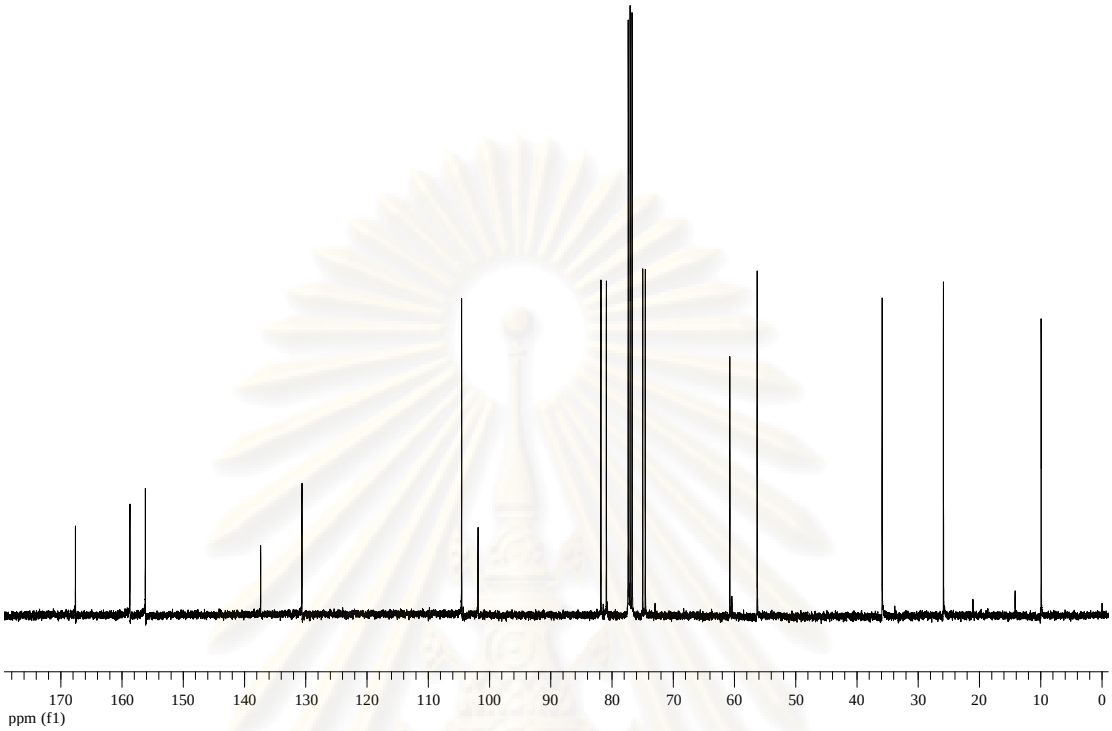

Figure B5 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 11-hydroxymonocerin (**2**)

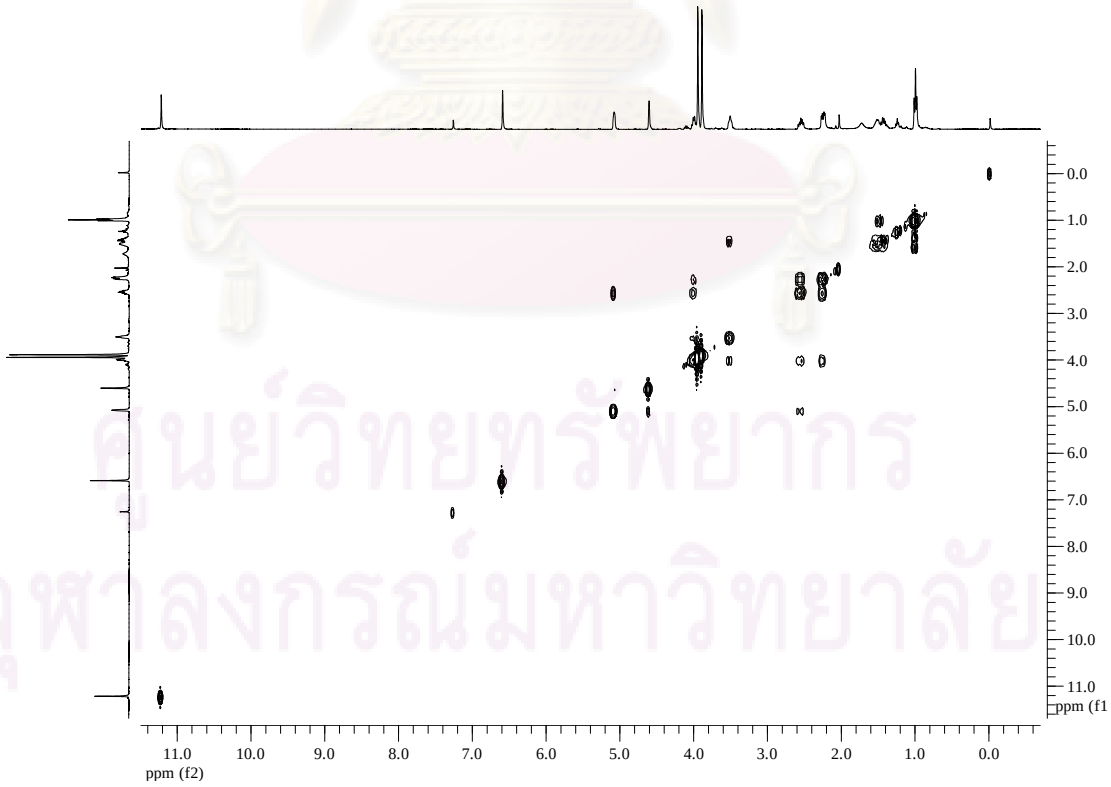

Figure B6 COSY of 11-hydroxymonocerin (**2**)

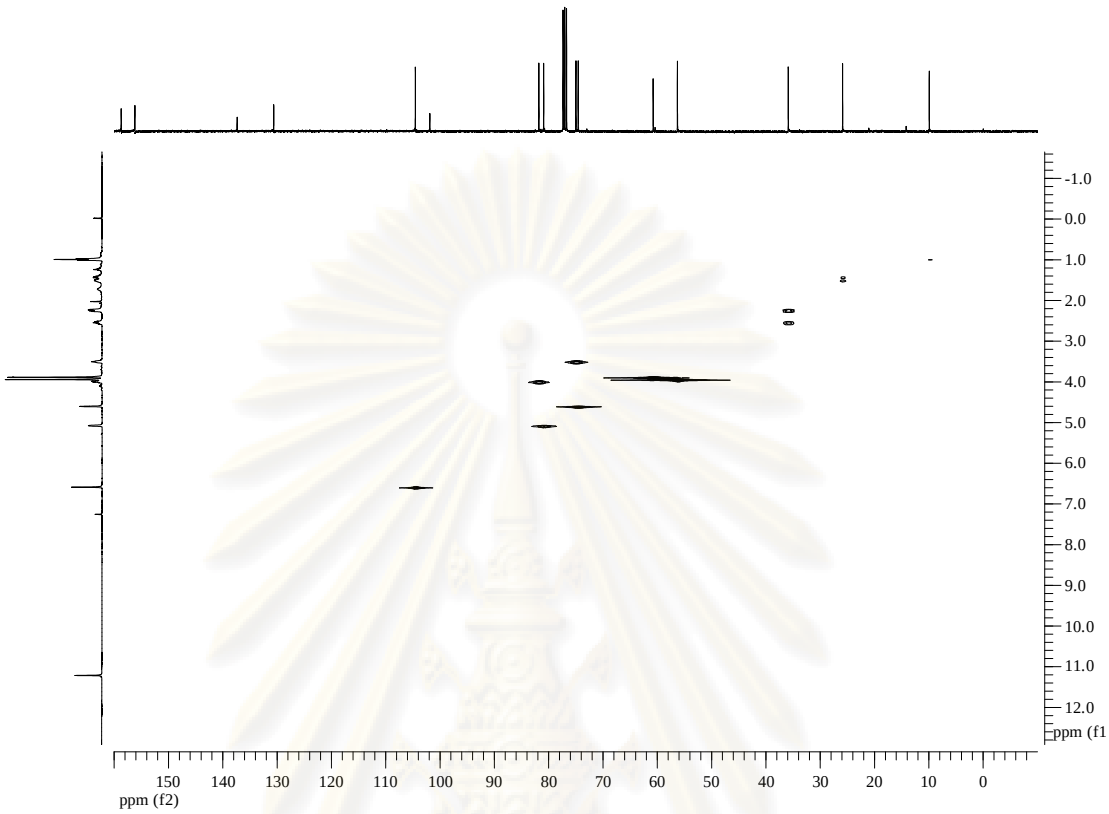

Figure B7 HSQC of 11-hydroxymonocerin (**2**)

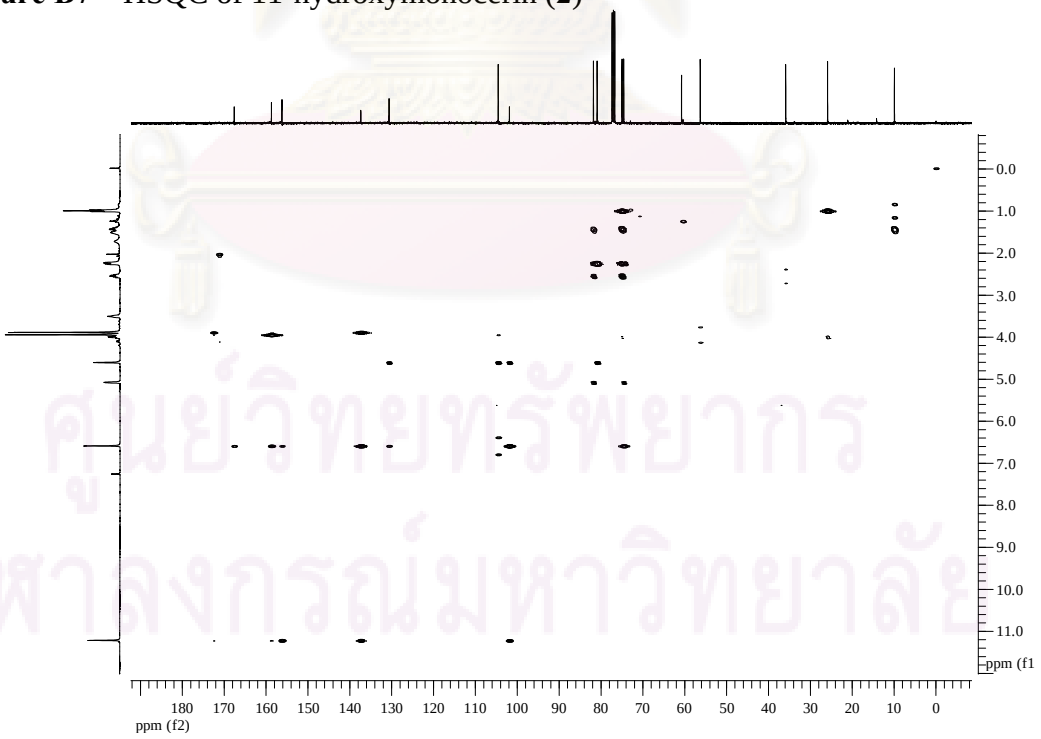

Figure B8 HMBC of 11-hydroxymonocerin (**2**)

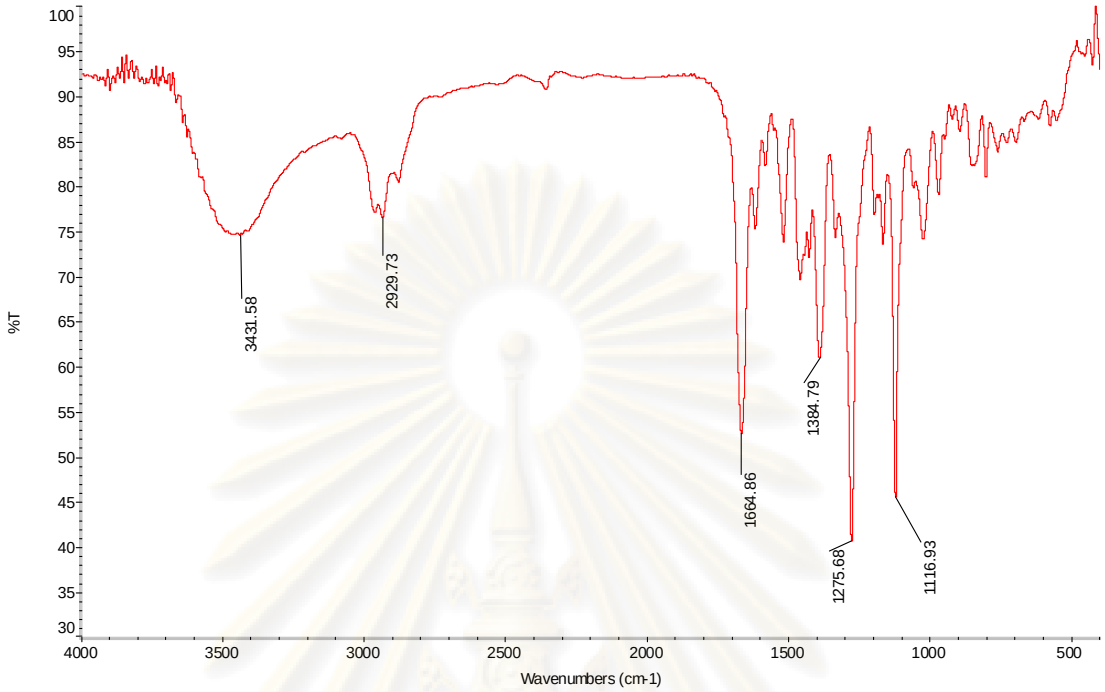

Figure B9 IR spectrum of 11-hydroxymonocerin (**2**)

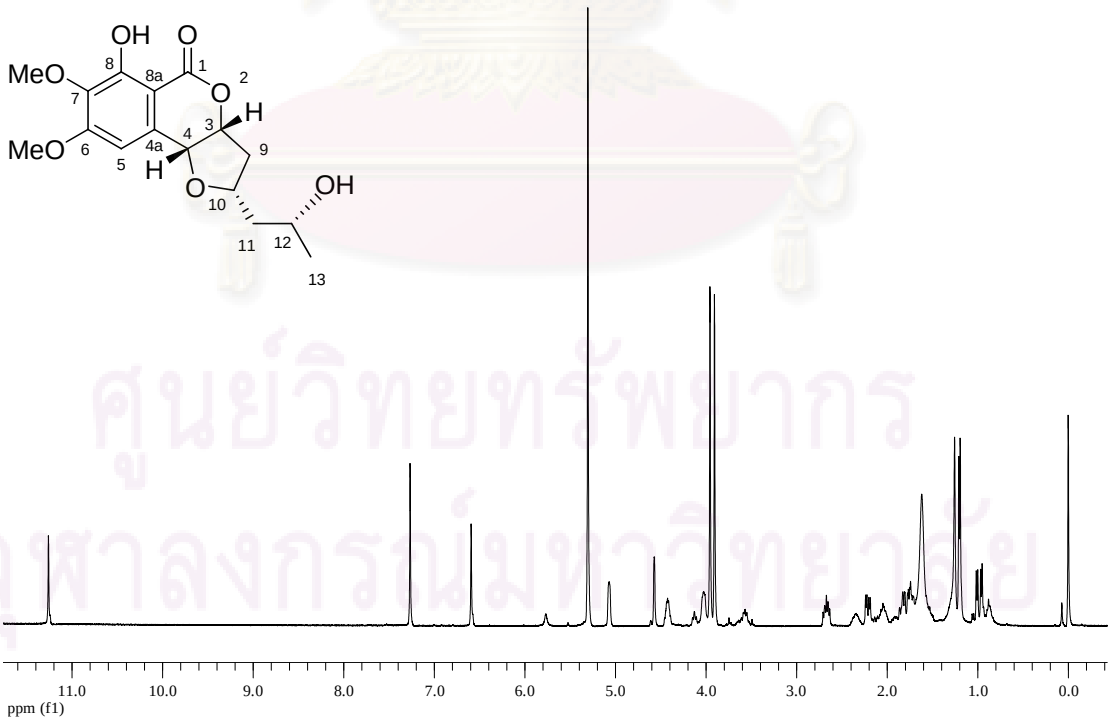

Figure B10 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 12-hydroxymonocerin (**3**)

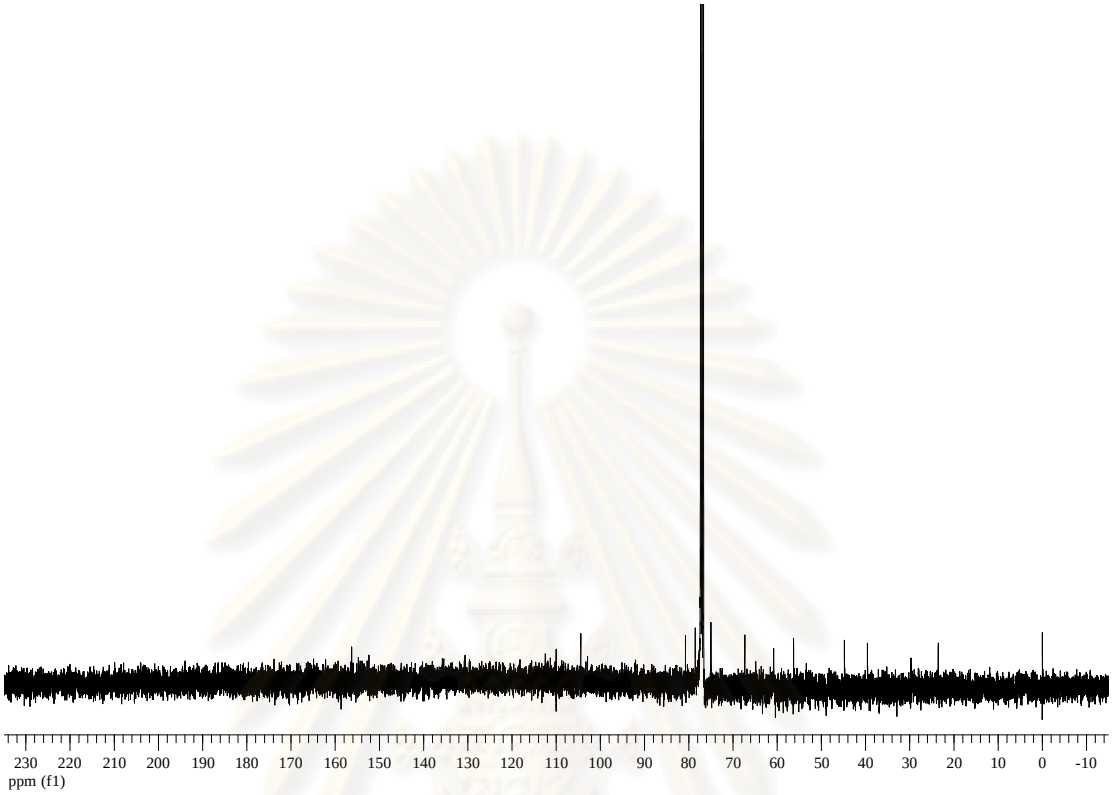

Figure B11 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 12-hydroxymonocerin (**3**)

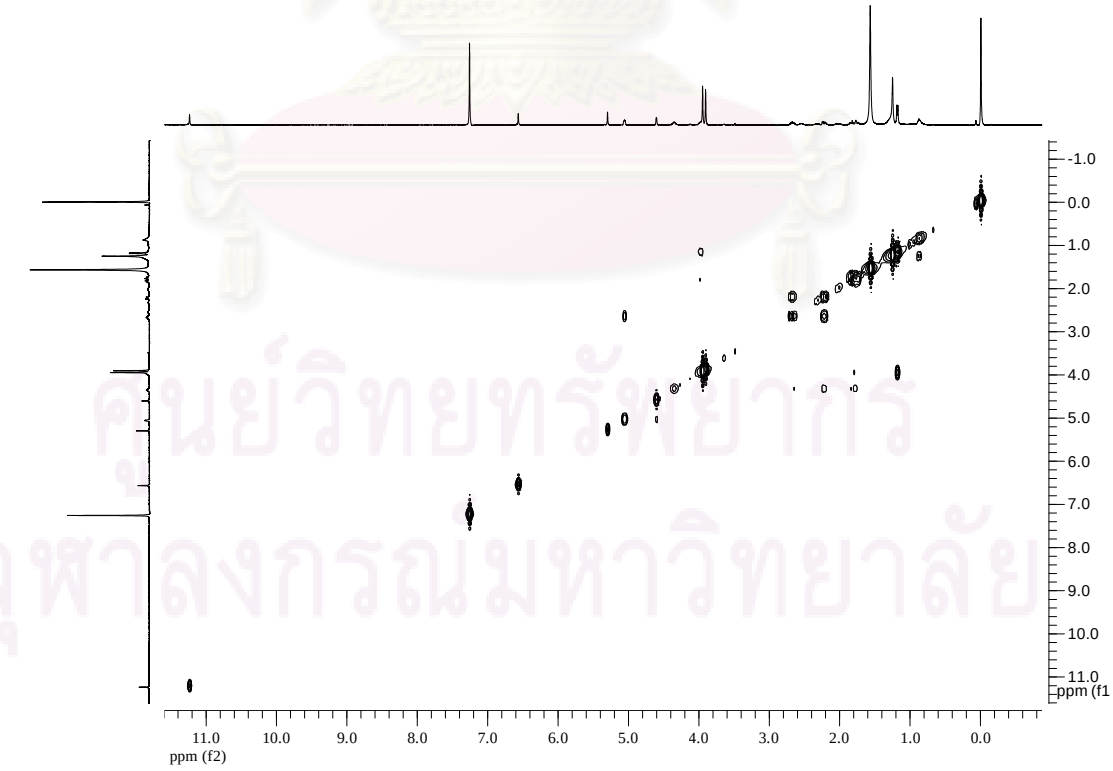

Figure B12 COSY of 12-hydroxymonocerin (**3**)

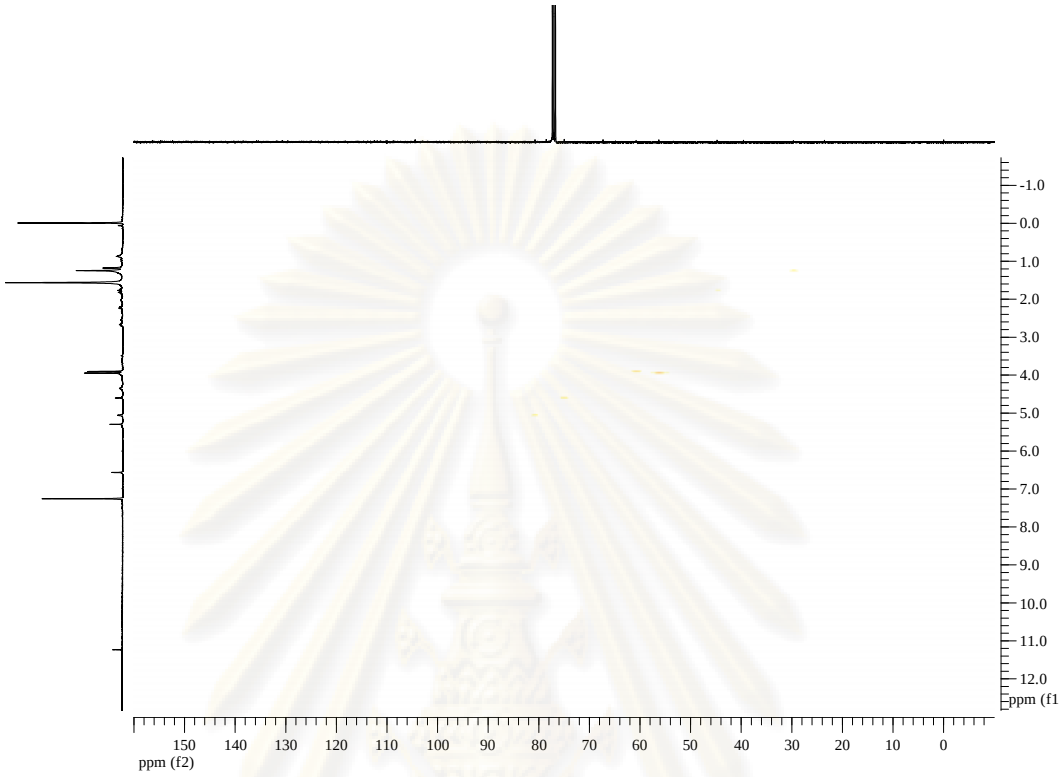

Figure B13 HSQC of 12-hydroxymonocerin (**3**)

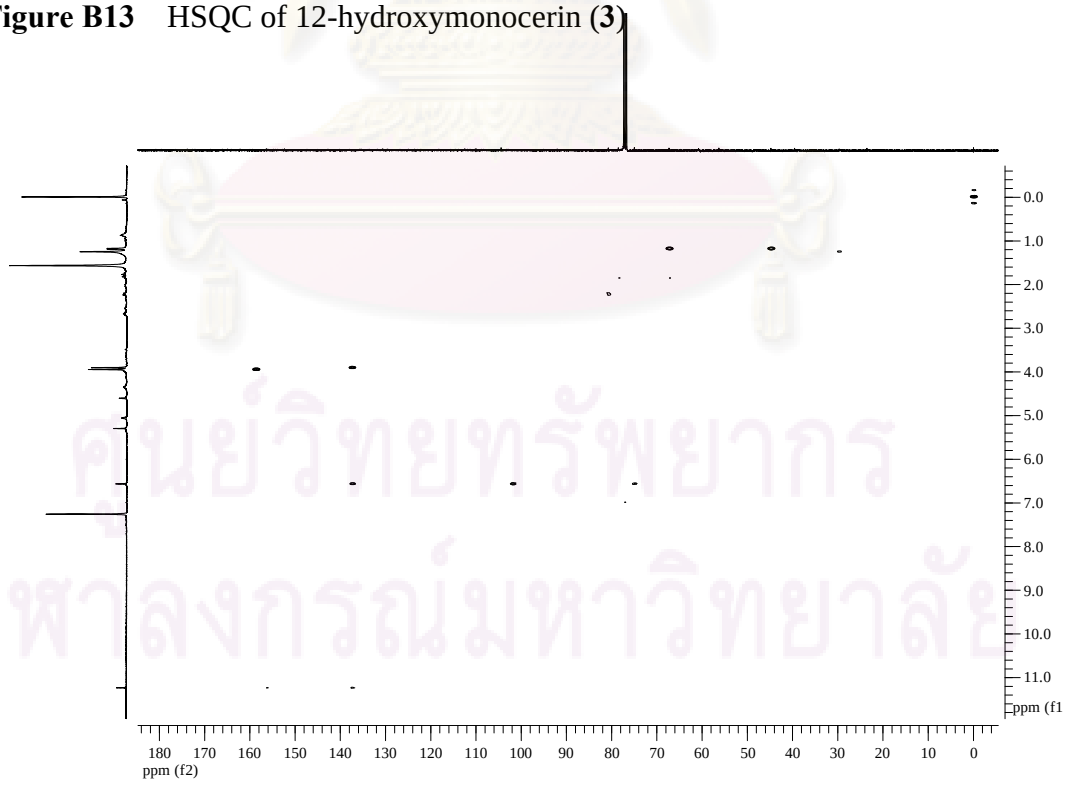

Figure B14 HMBC of 12-hydroxymonocerin (**3**)

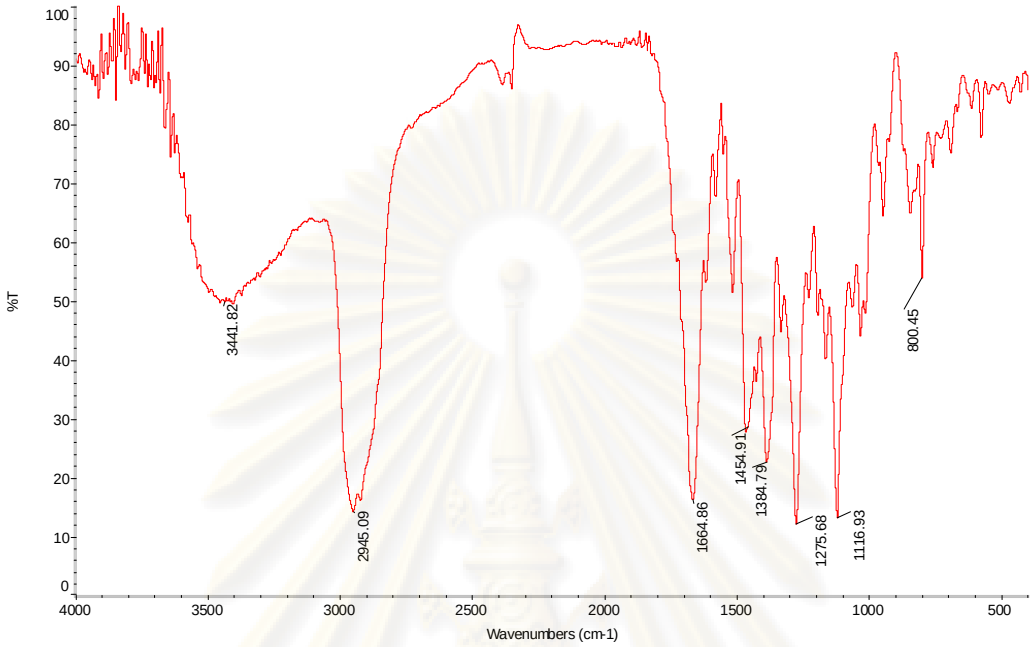

Figure B15 IR spectrum of 12-hydroxymonocerin (**3**)

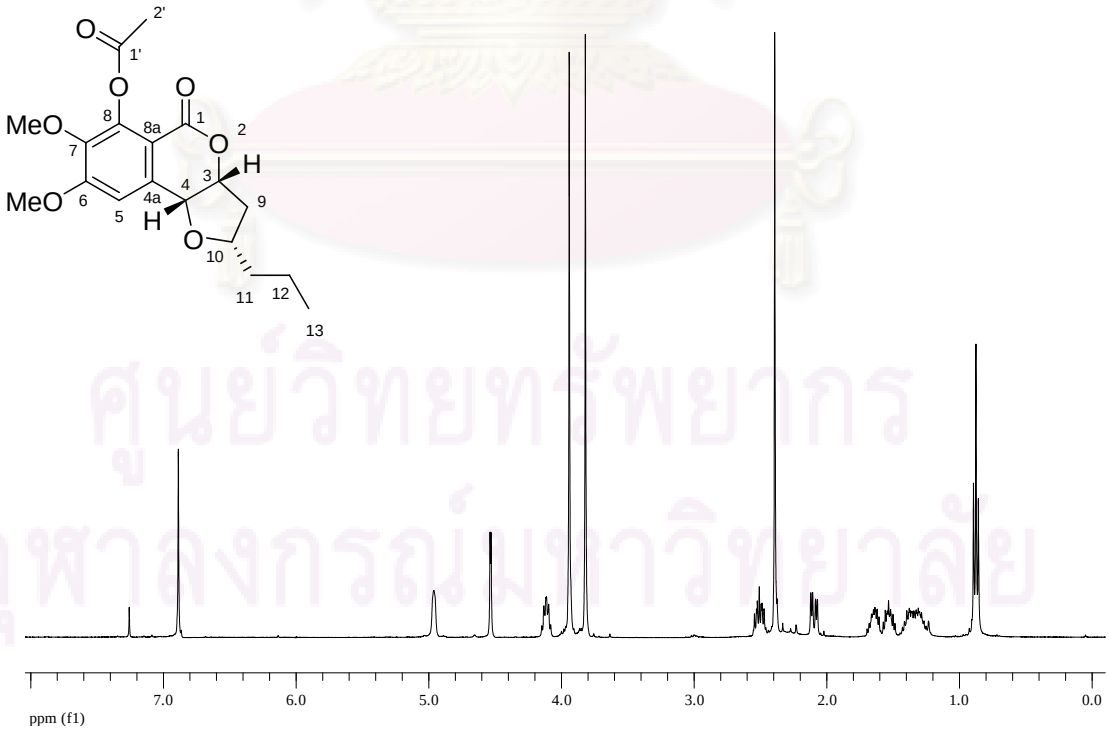

Figure B16 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 8-acetylmonocerin (**4**)

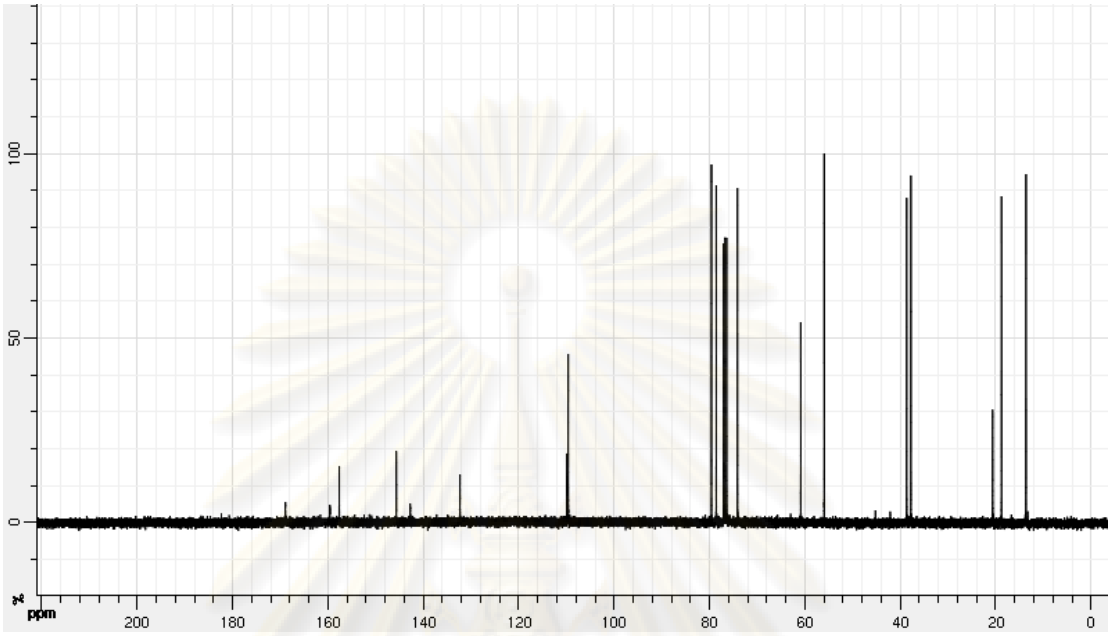

Figure B17 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 8-acetylmonocerin (**4**)

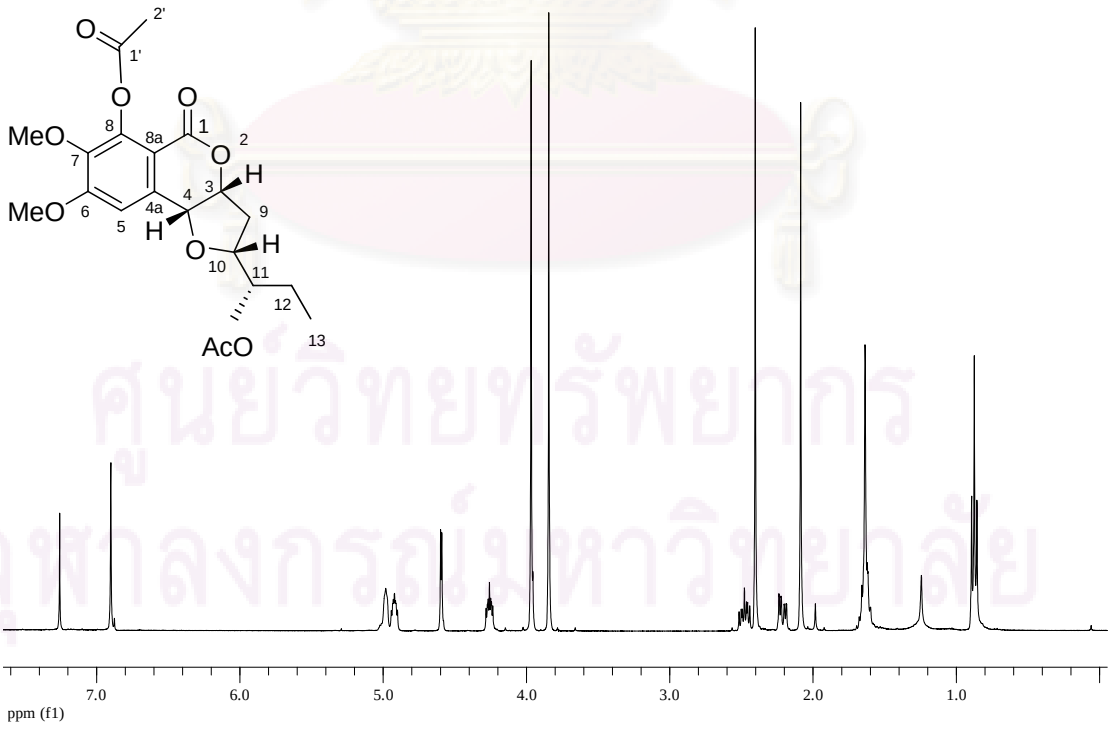

Figure B18 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound **5**

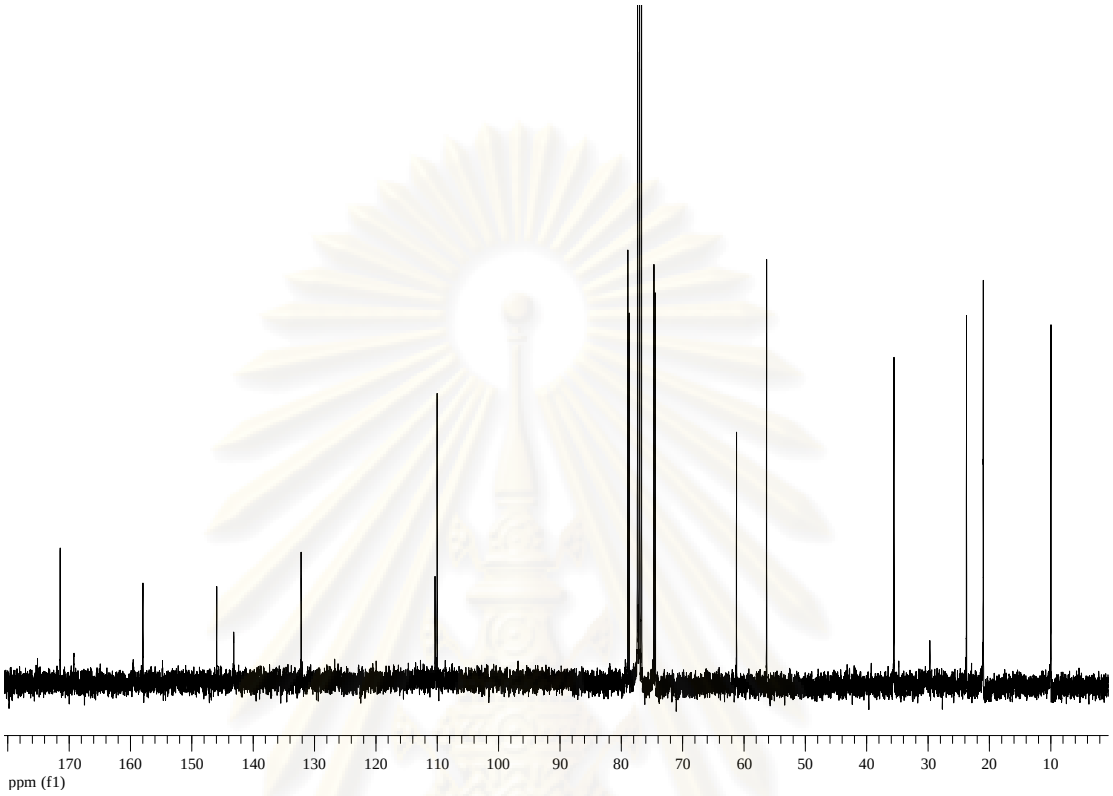

Figure B19 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of compound **5**

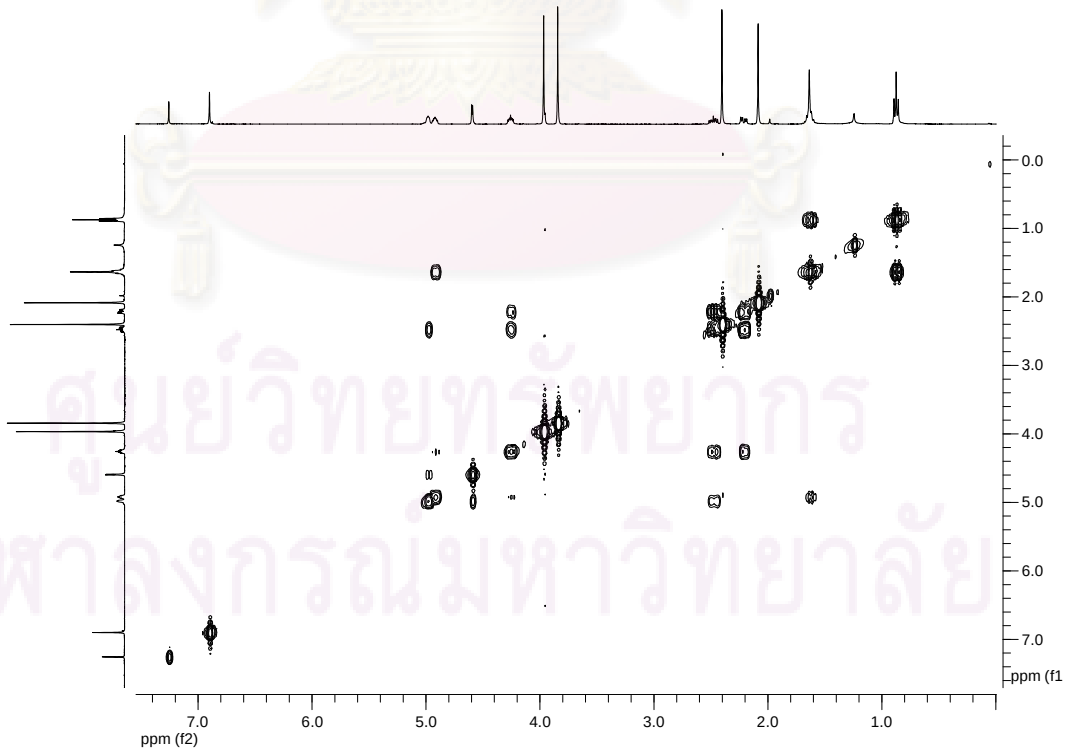

Figure B20 COSY of compound **5**

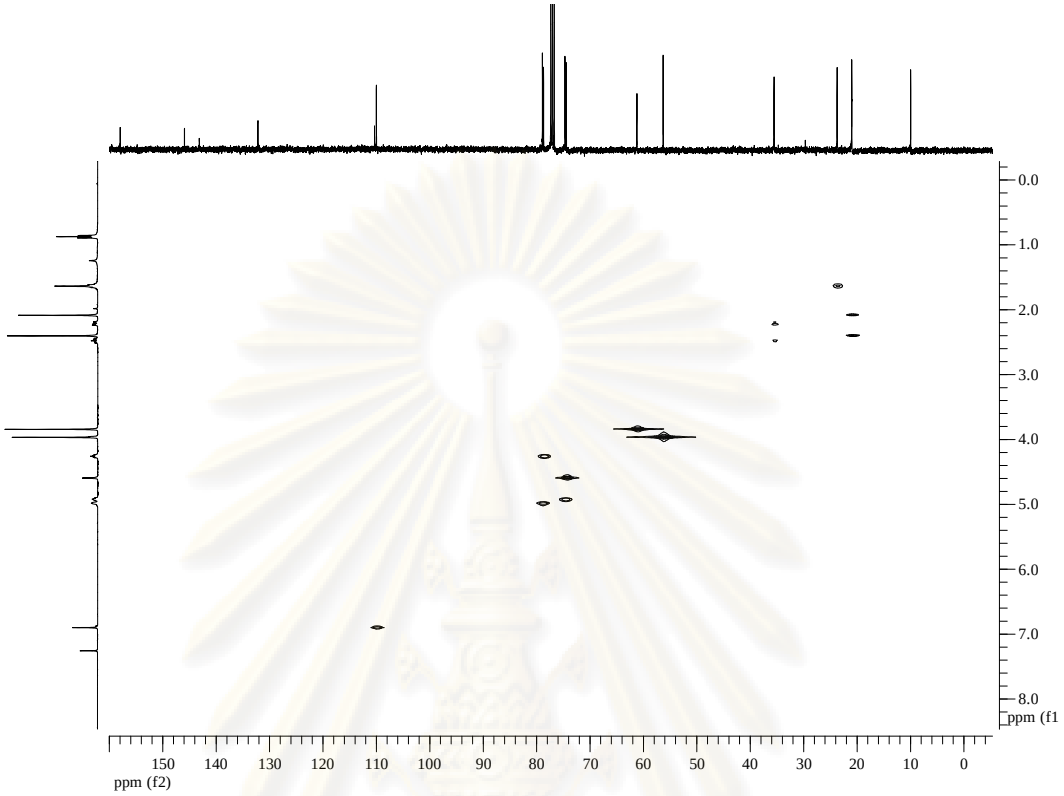

Figure B21 HSQC of compound **5**

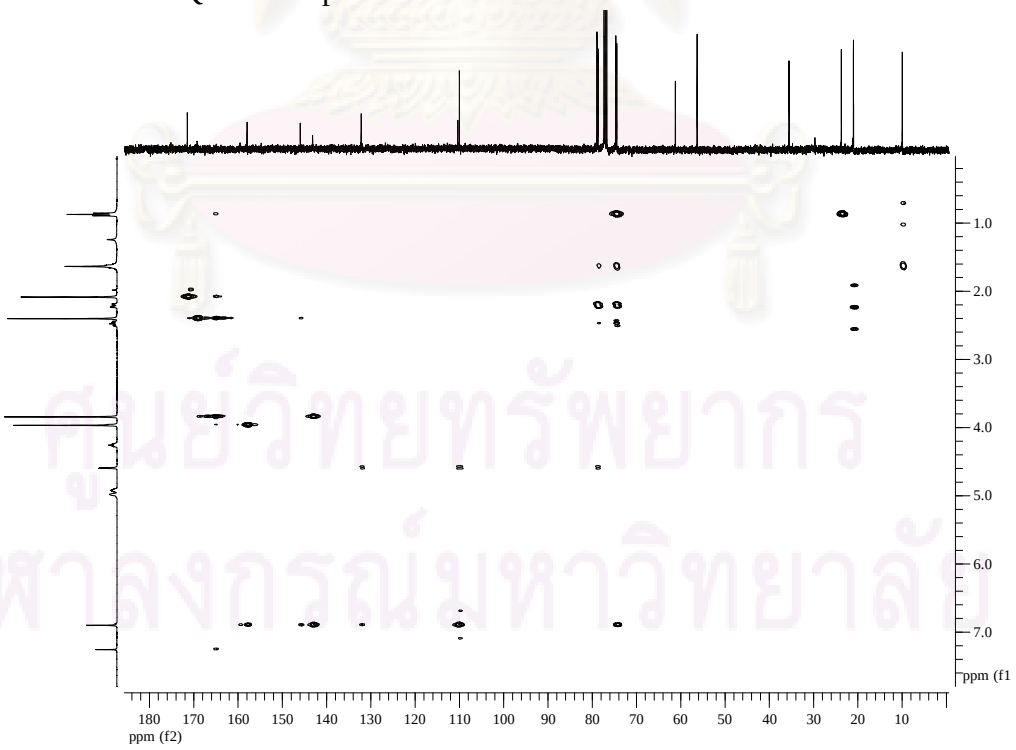

Figure B22 HMBC of compound **5**

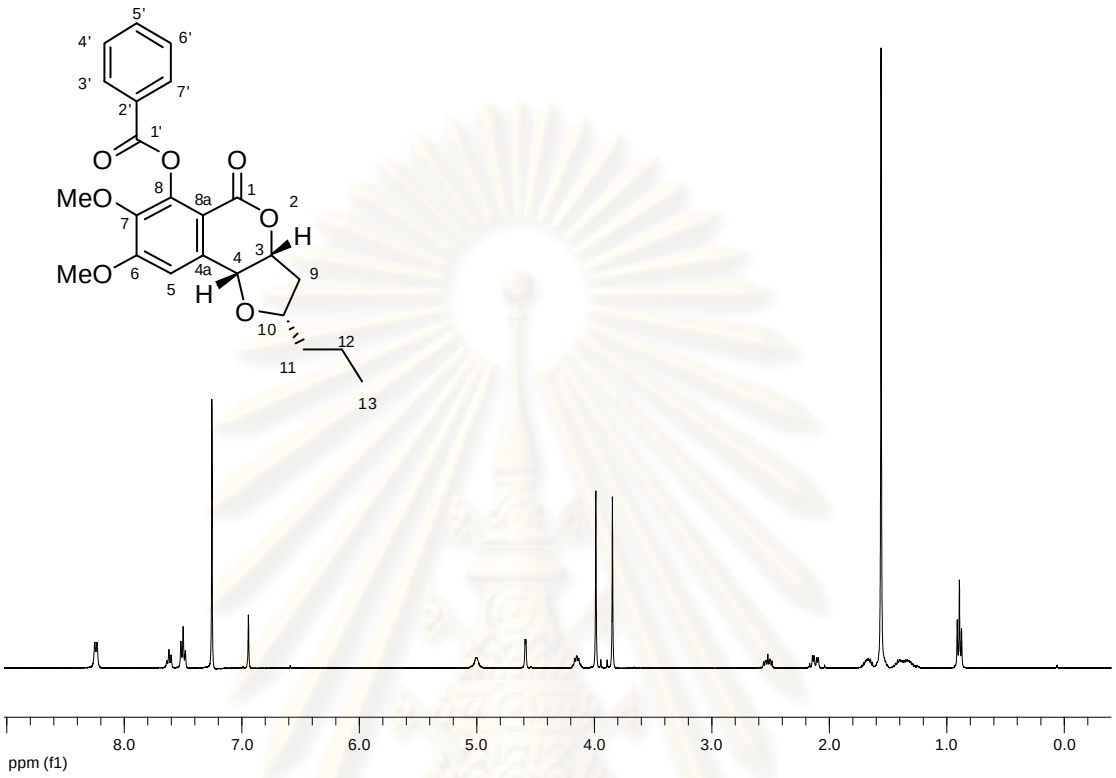

Figure B23 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 8-benzoylmonocerin (**6**)

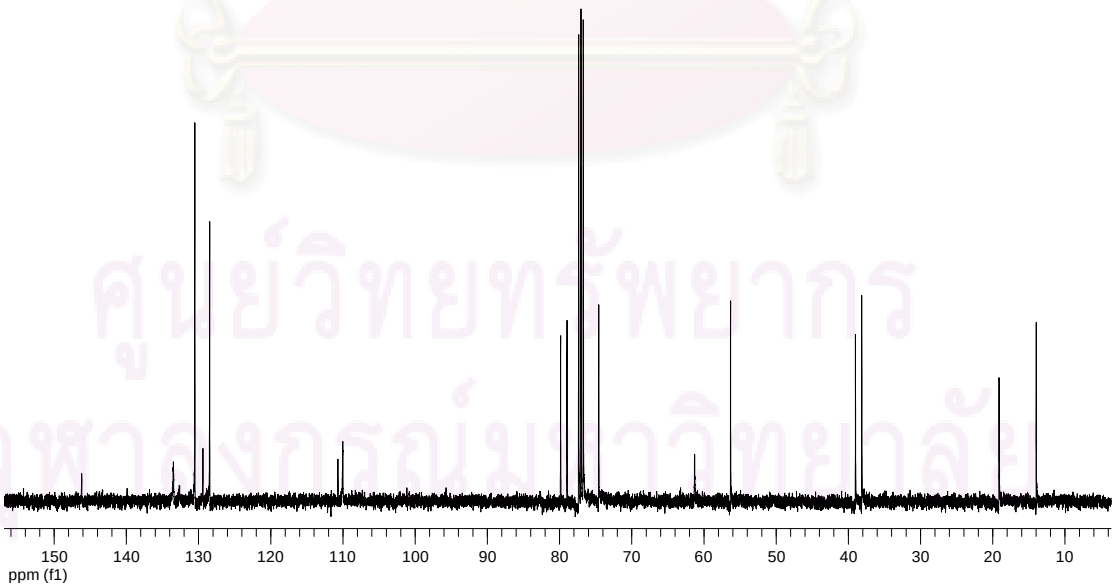

Figure B24 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 8-benzoylmonocerin (**6**)

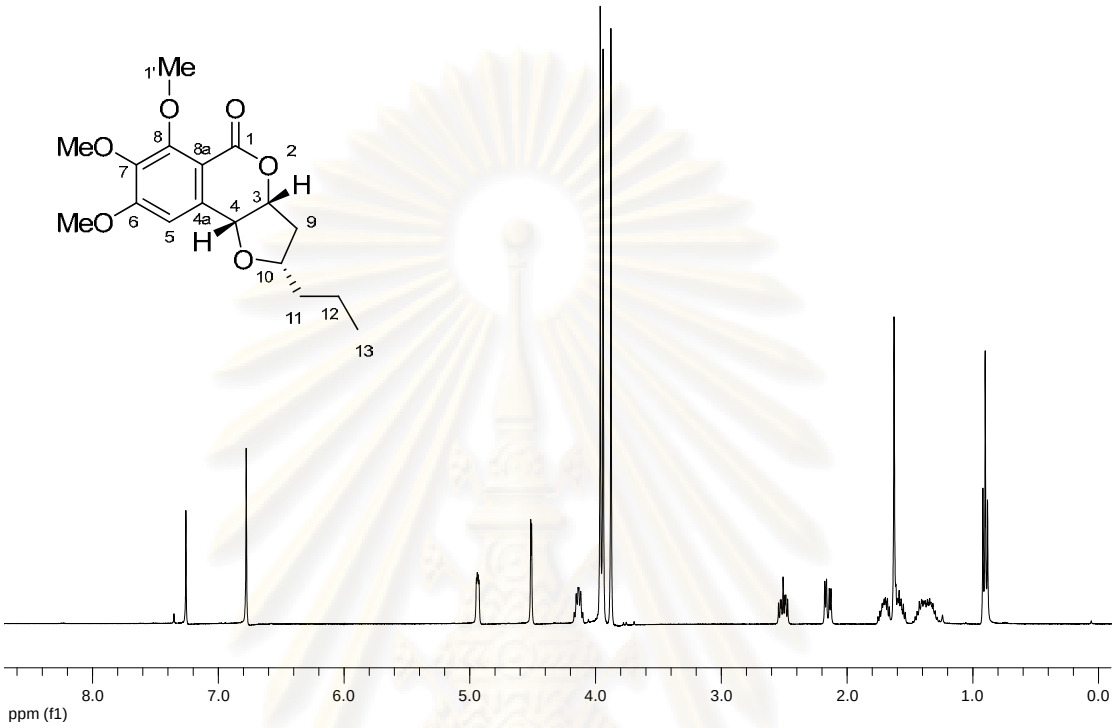

Figure B25 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 8-methylmonocerin (**7**)

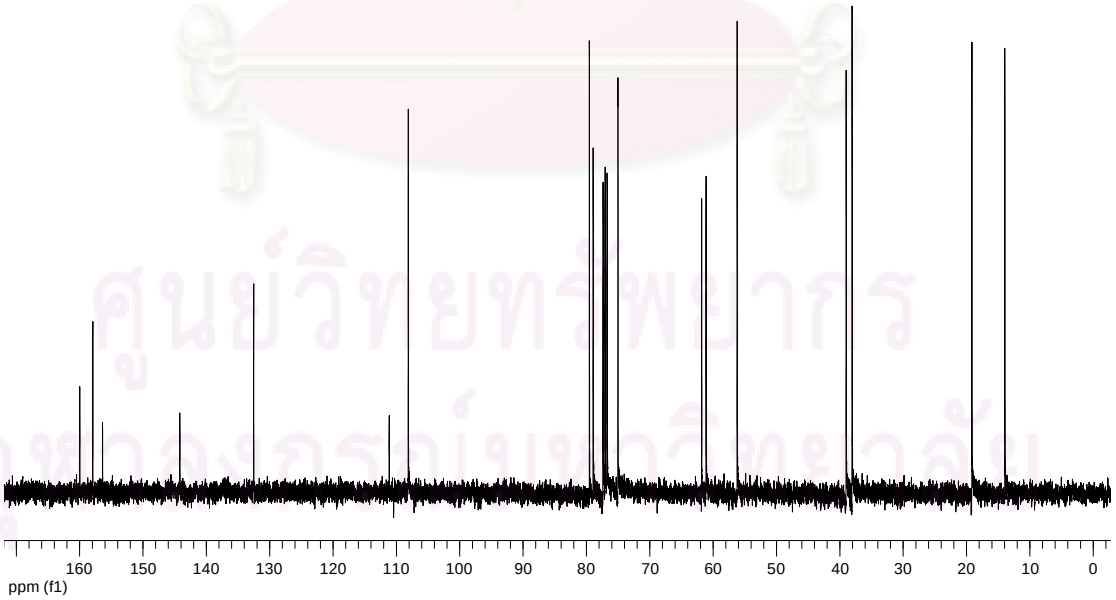

Figure B26 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 8-methylmonocerin (**7**)

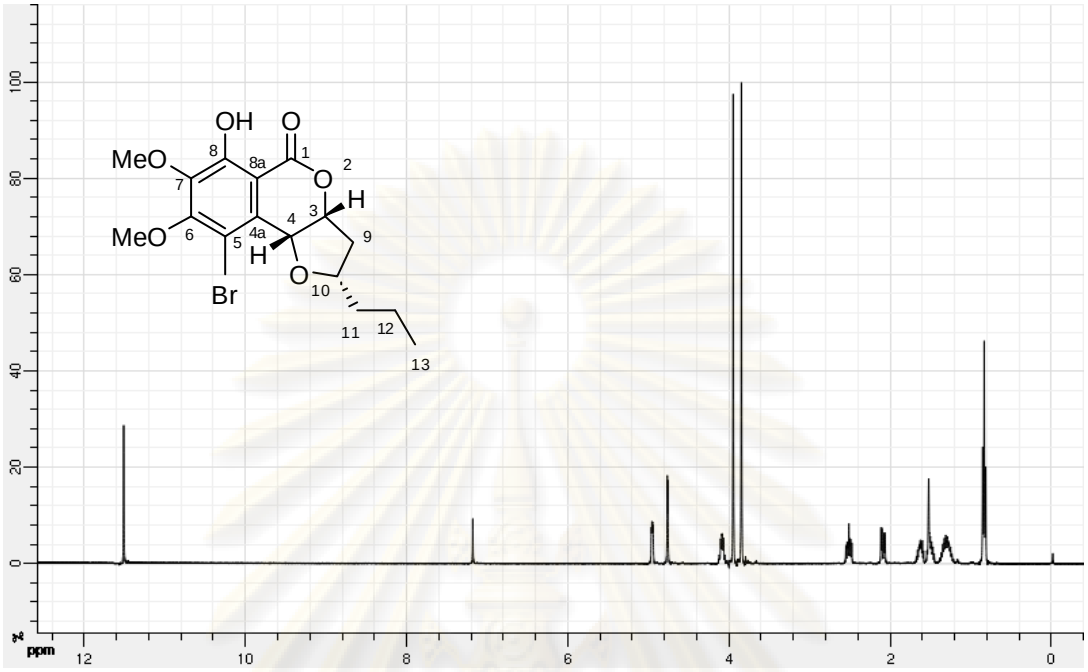

Figure B27 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5-bromomonocerin (**8**)

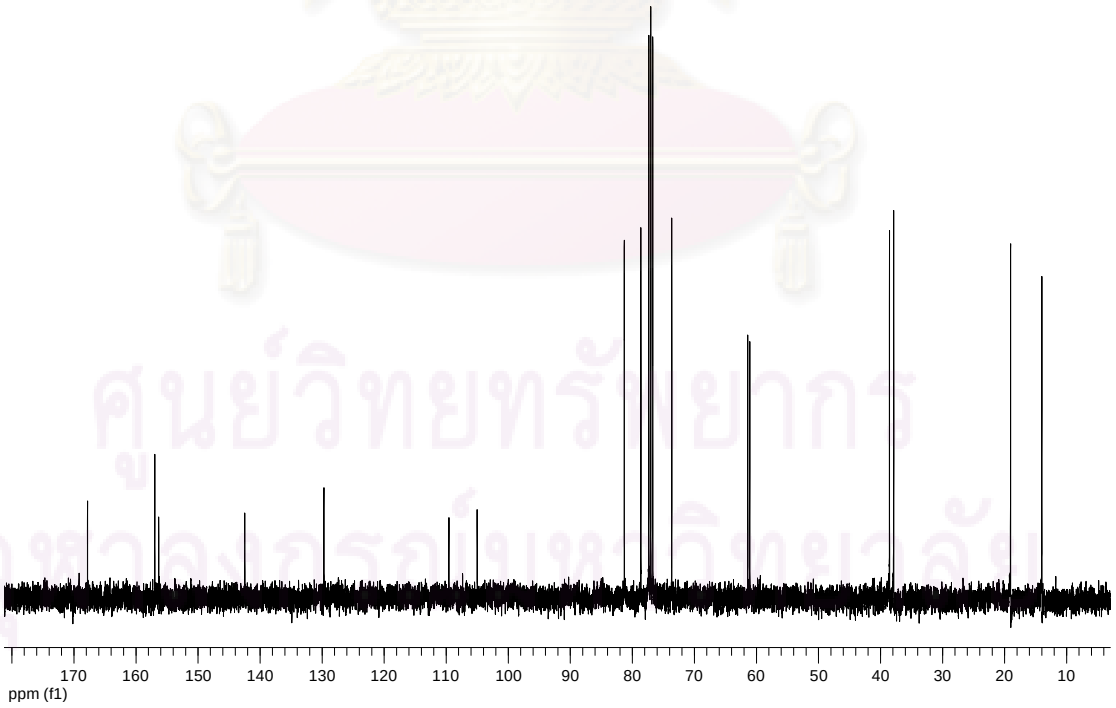

Figure B28 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 5-bromomonocerin (**8**)

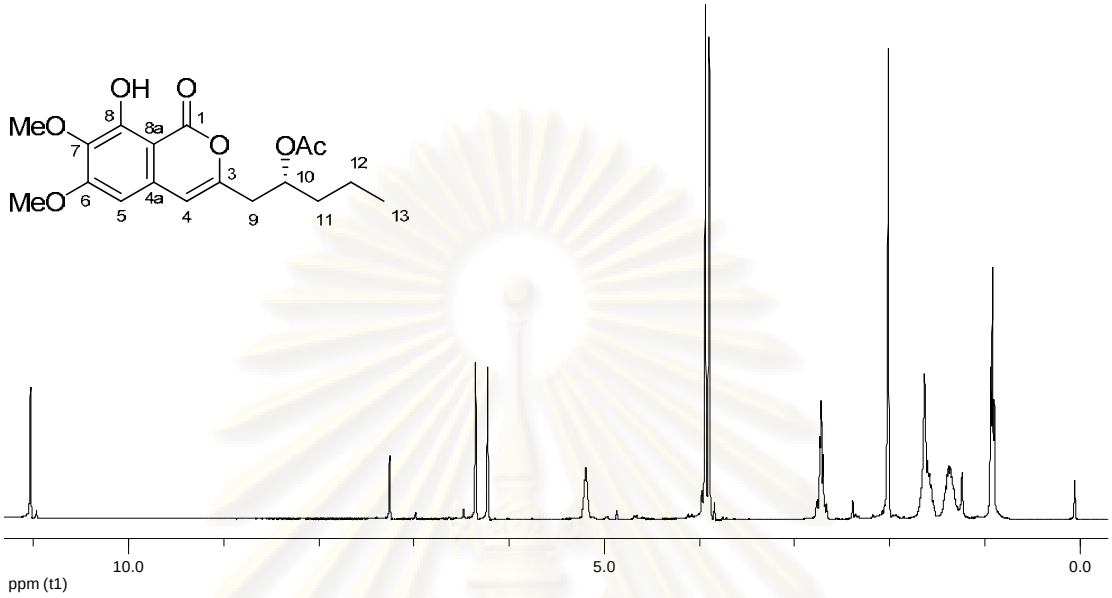

Figure B29 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound **9**

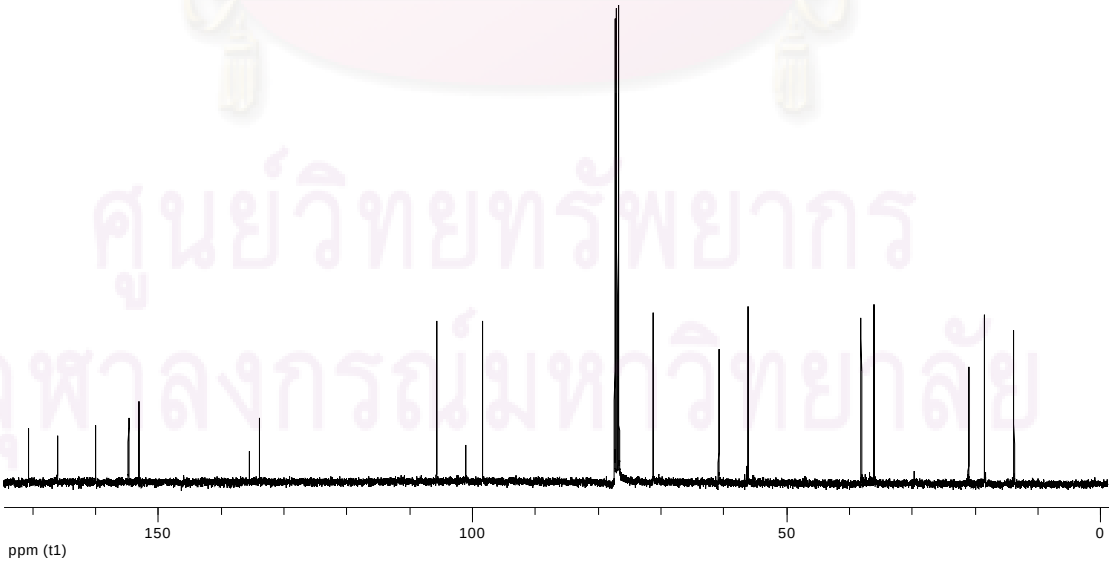

Figure B30 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of compound **9**

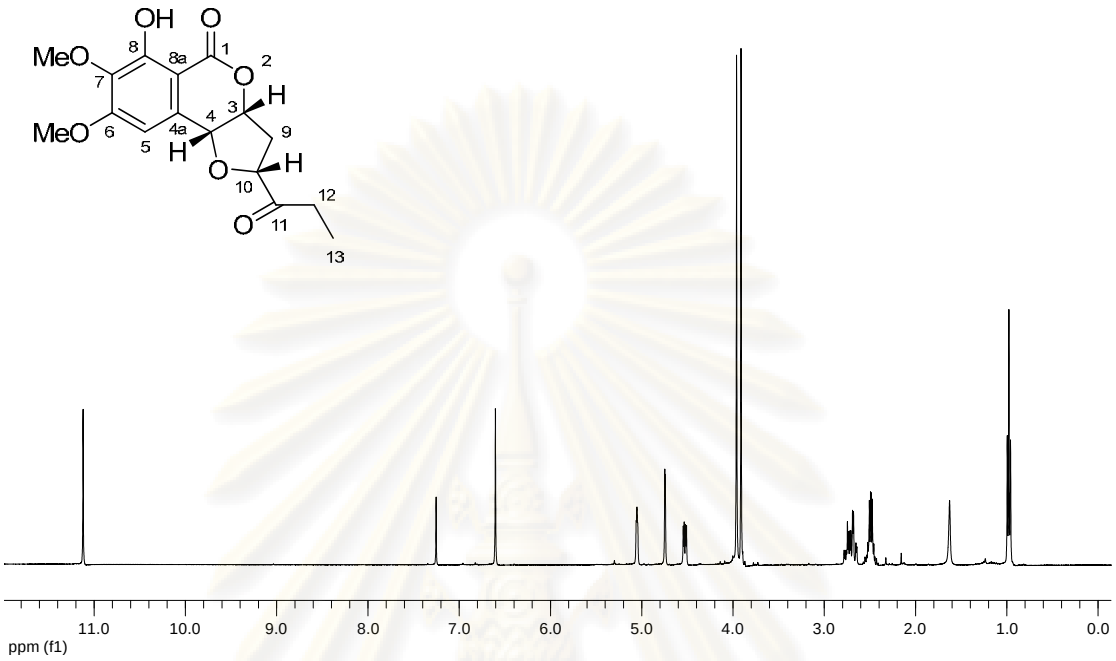

Figure B31 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of 11-oxomonocerin (**10**)

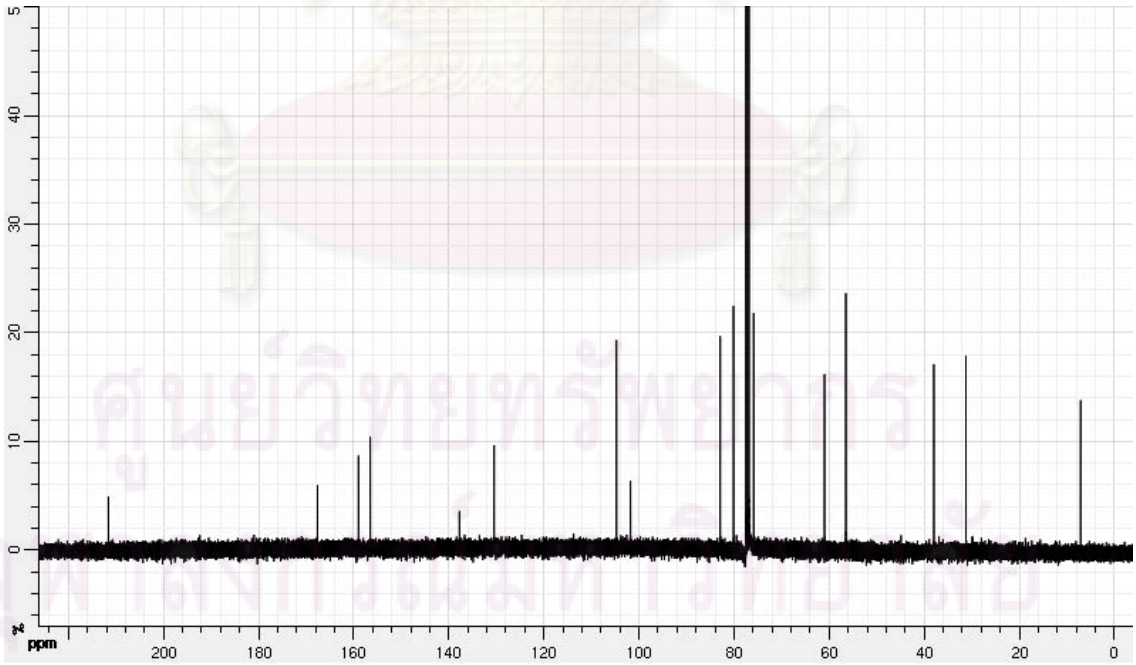

Figure B32 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 11-oxomonocerin (**10**)

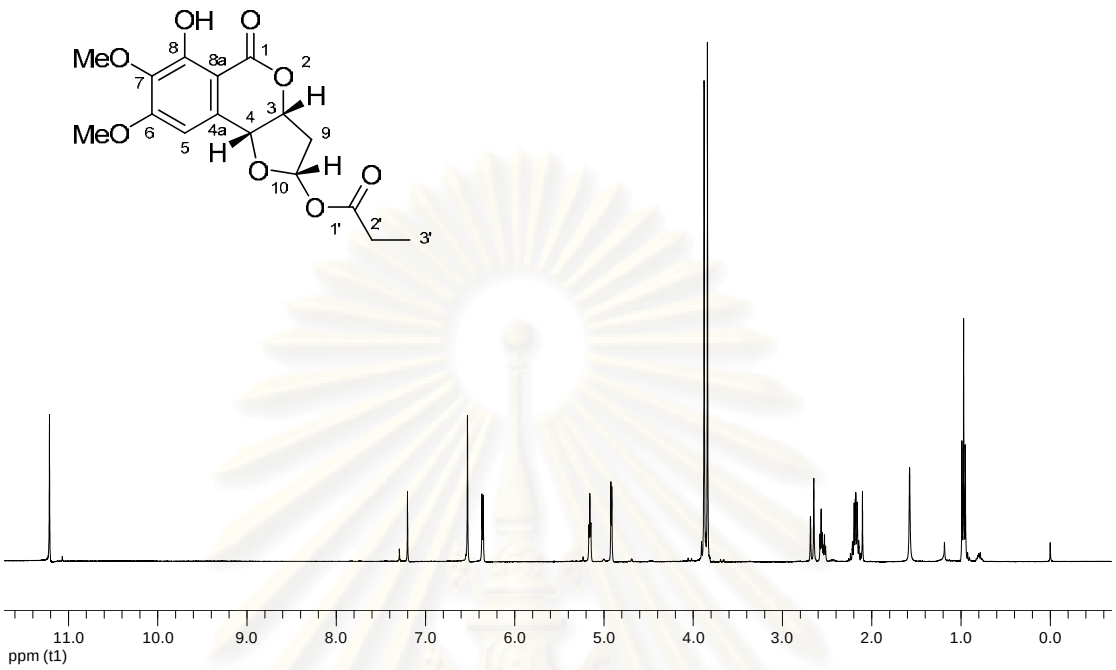

Figure B33 400 MHz ^{1H} NMR ($CDCl_3$) spectrum of 11-oxomonocerin (**11**)

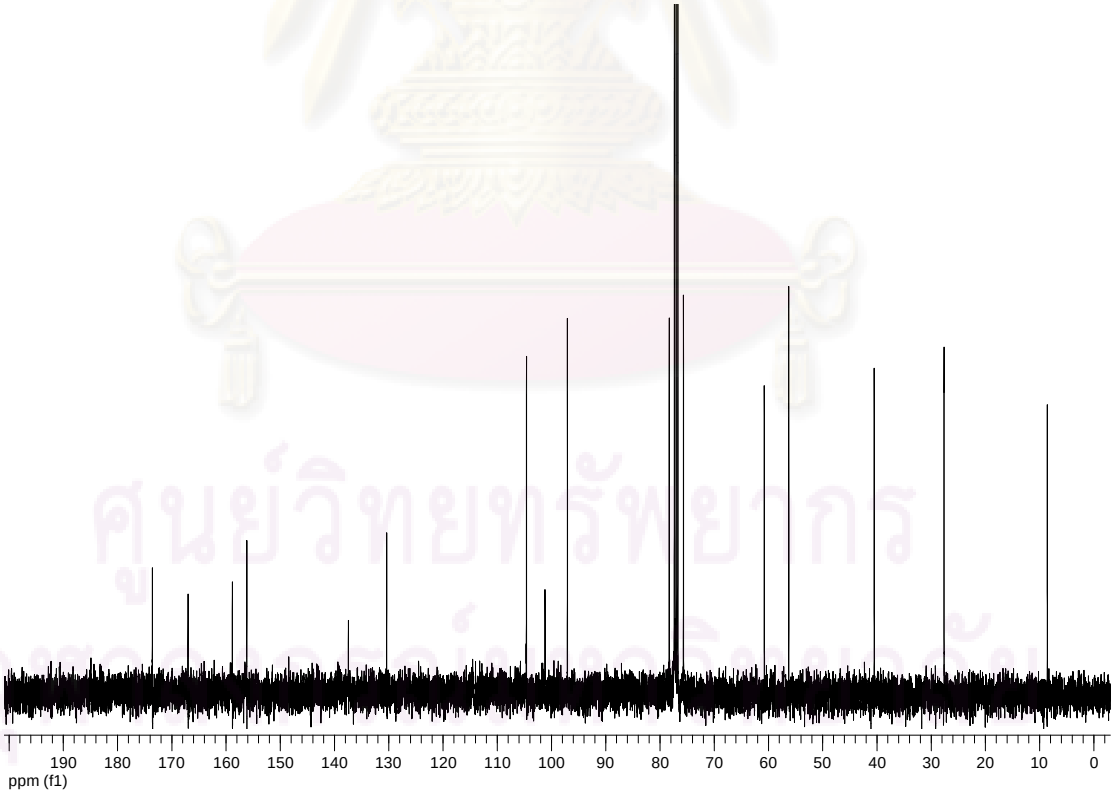

Figure B34 400 MHz ^{13}C NMR ($CDCl_3$) spectrum of 11-oxomonocerin (**11**)

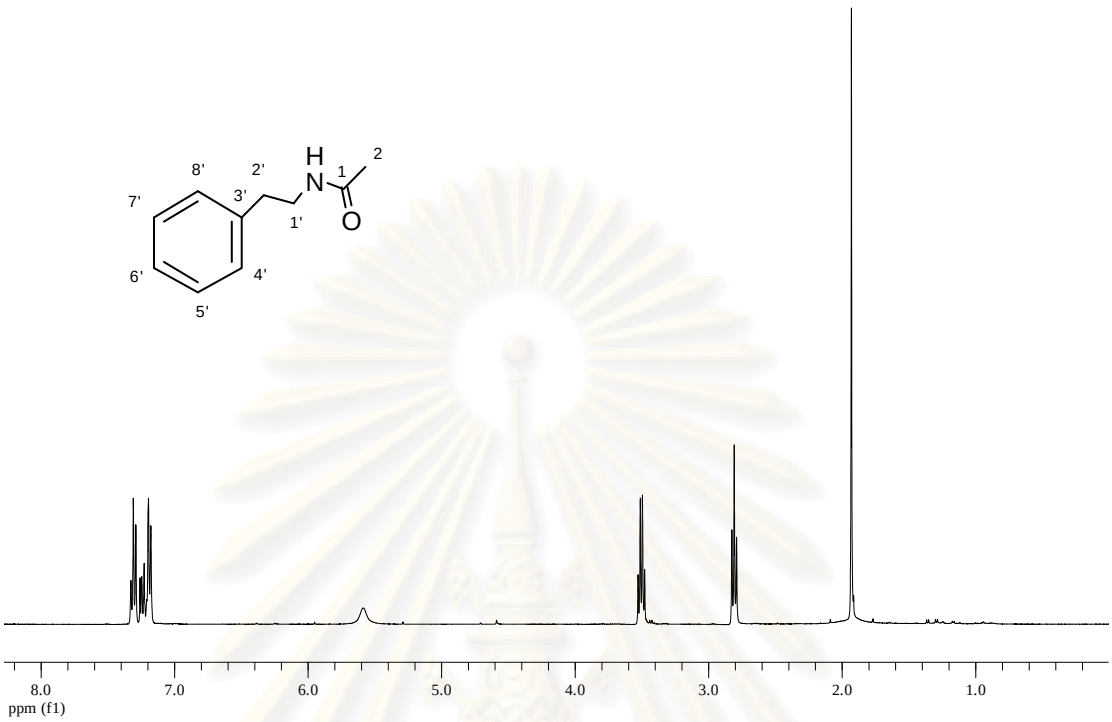

Figure B35 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of *N*-phenetyl-acetamide (**12**

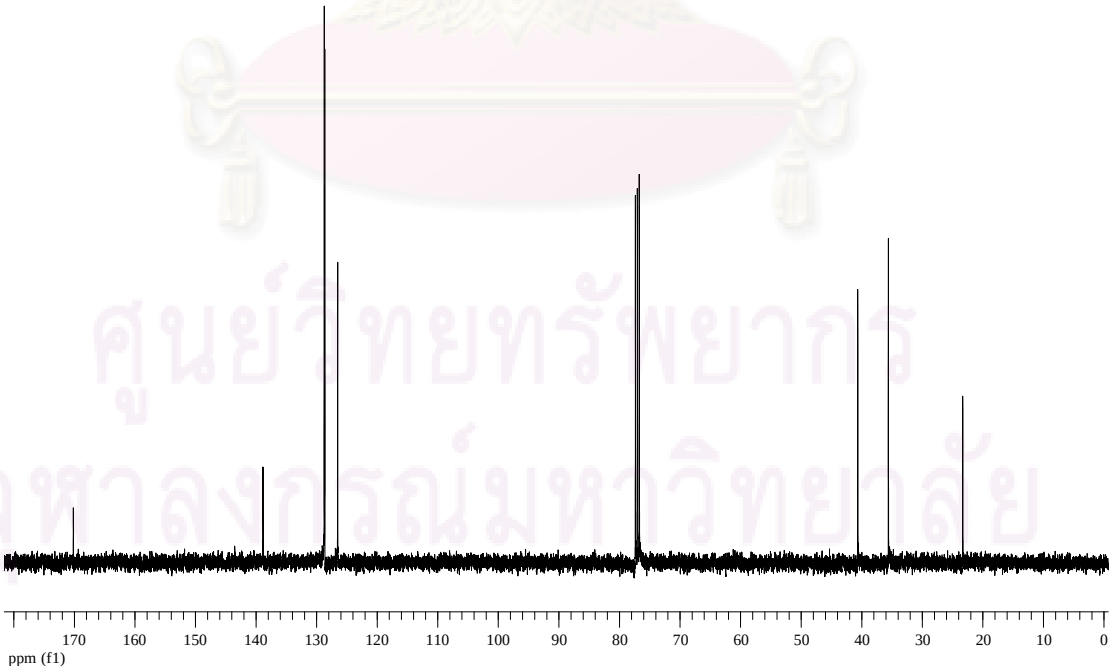

Figure B36 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of *N*-phenetyl-acetamide (**12**)

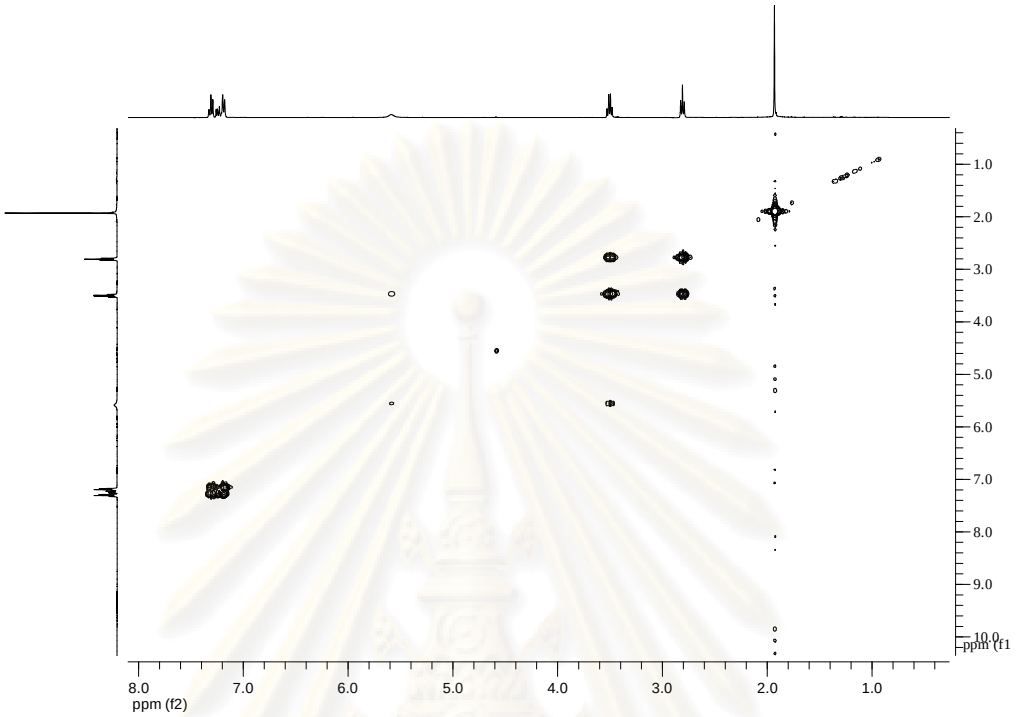

Figure B37 COSY of *N*-phenetyl-acetamide (**12**)

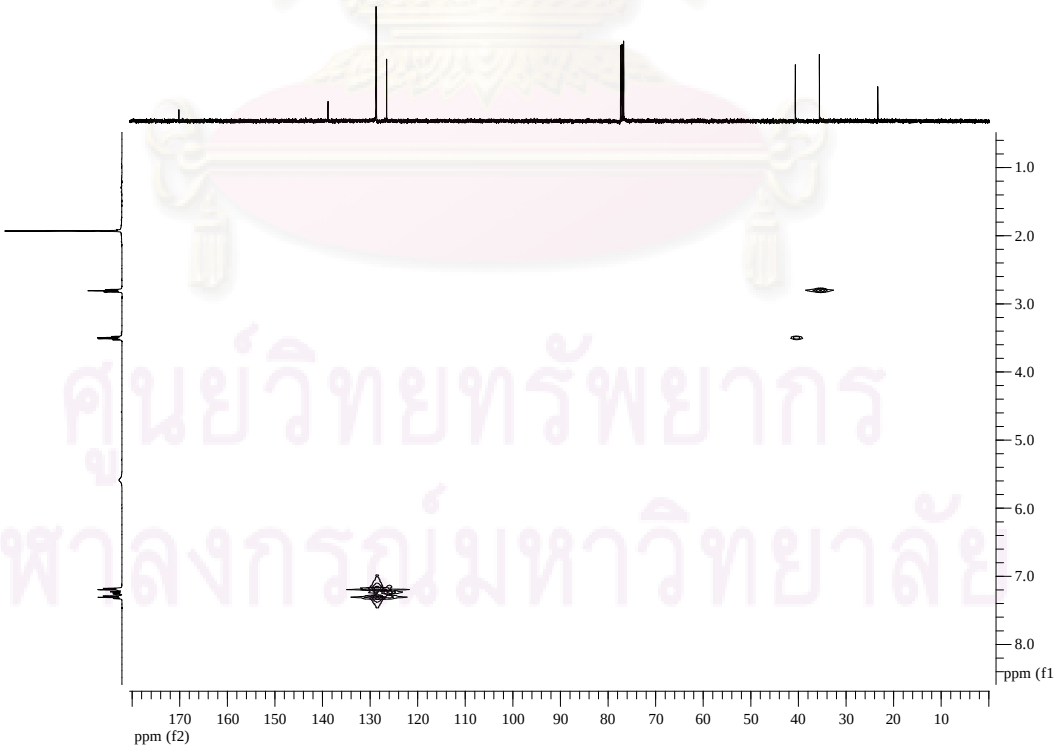

Figure B38 HSQC of *N*-phenetyl-acetamide (**12**)

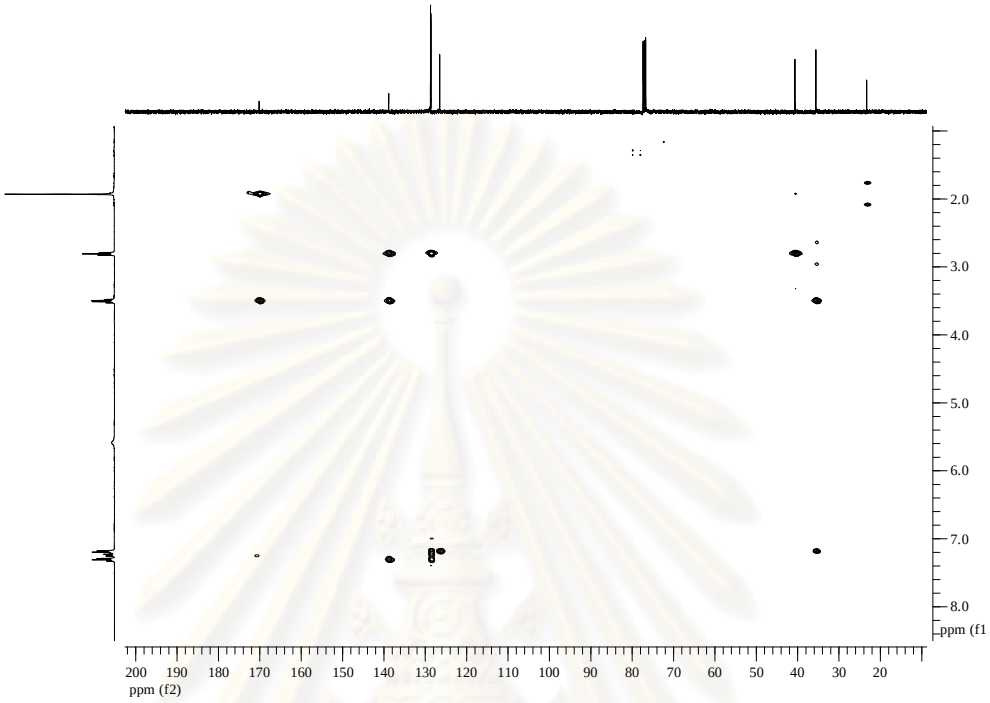

Figure B39 HMBC of *N*-phenetyl-acetamide (**12**)

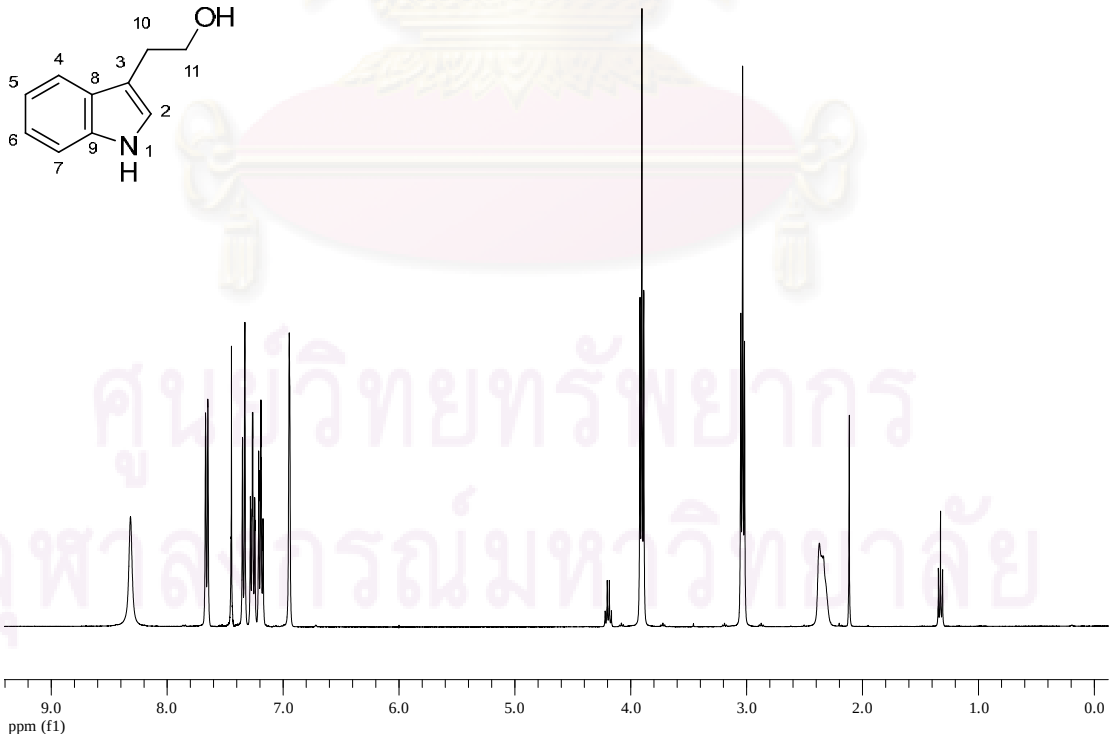

Figure B40 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of tryptophol (**13**)

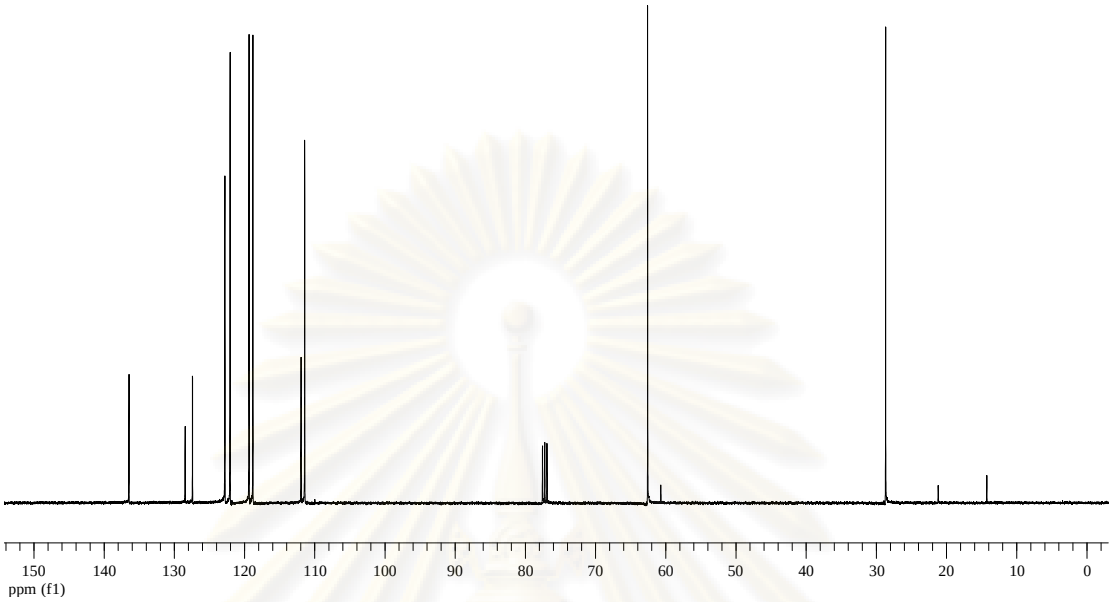

Figure B41 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of tryptophol (**13**)

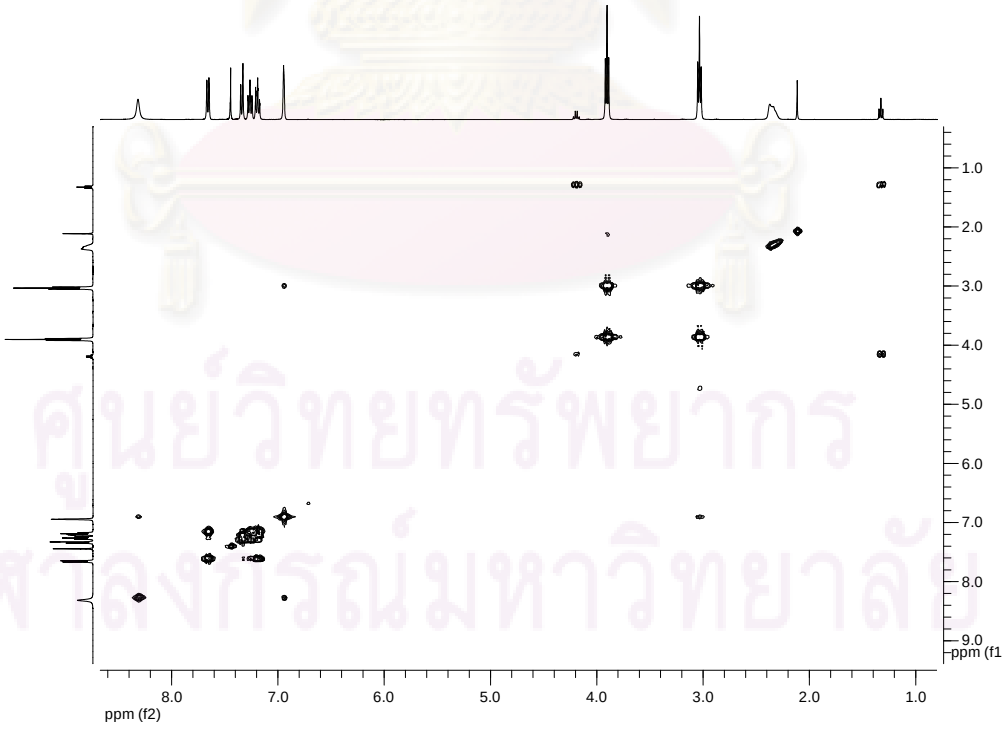

Figure B42 COSY of tryptophol (**13**)

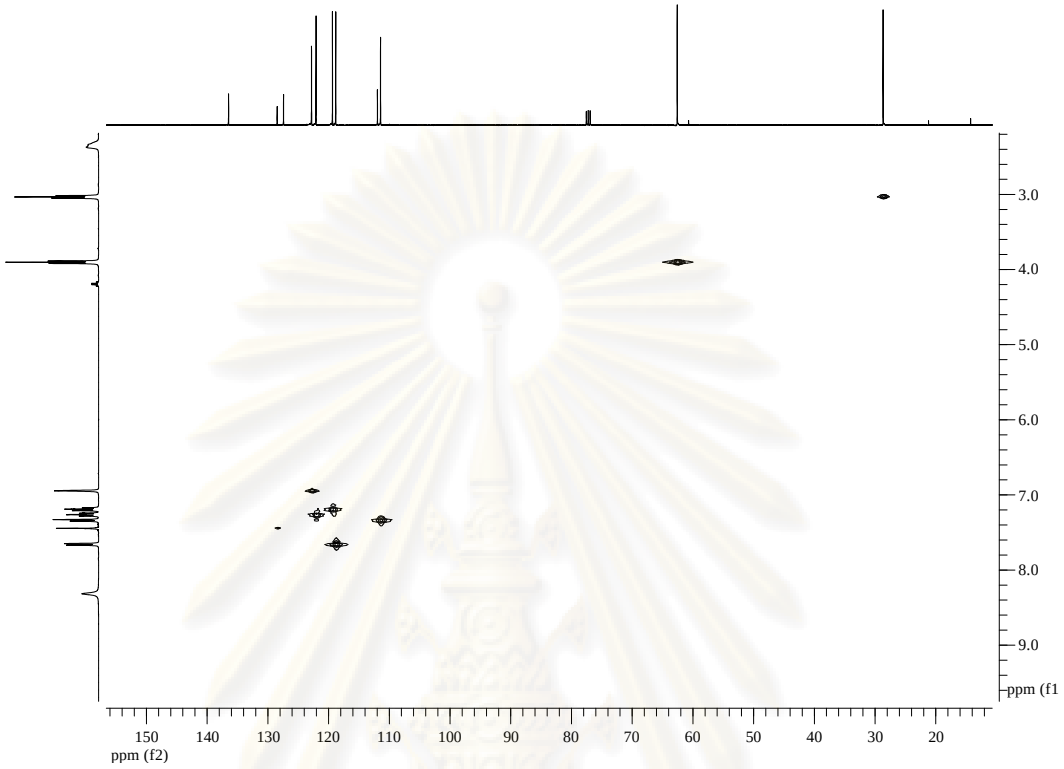

Figure B43 HSQC of tryptophol (**13**)

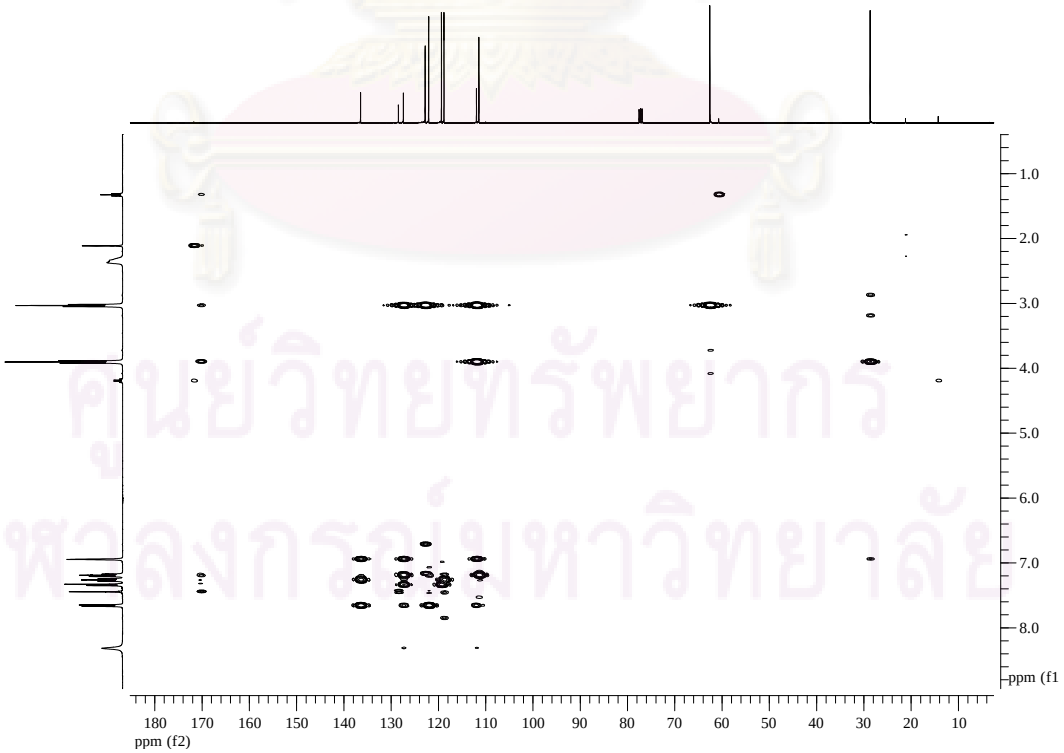

Figure B44 HMBC of tryptophol (**13**)

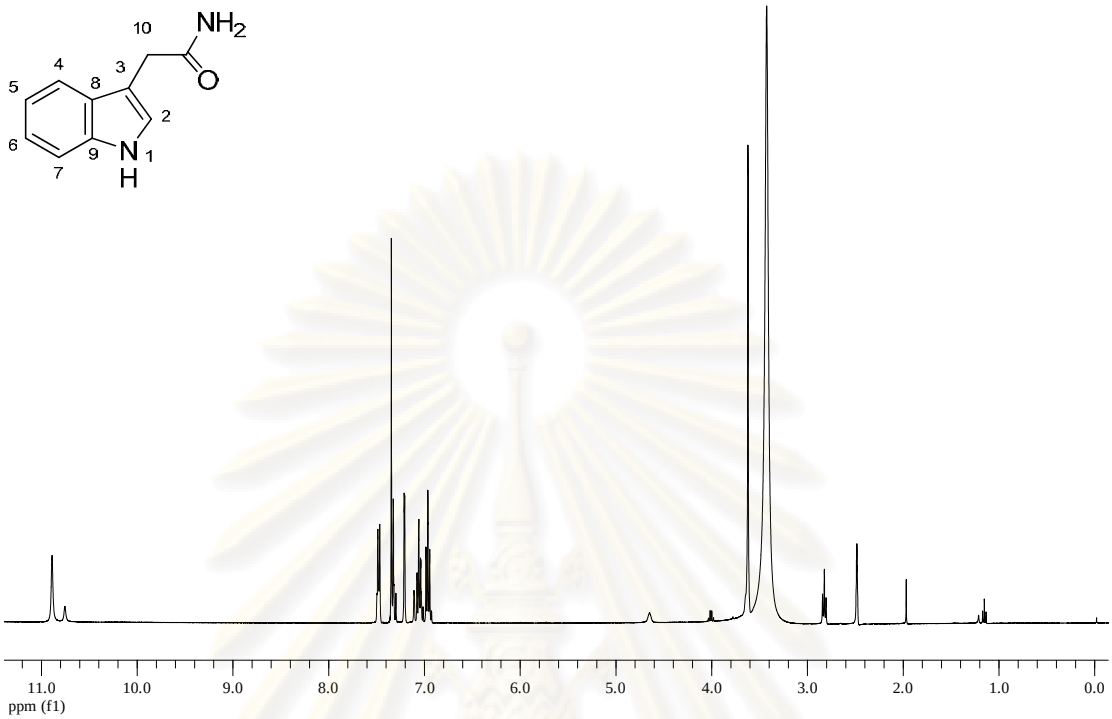

Figure B45 400 MHz ^{1}H NMR (DMSO) spectrum of indole-3-acetamide (**14**)

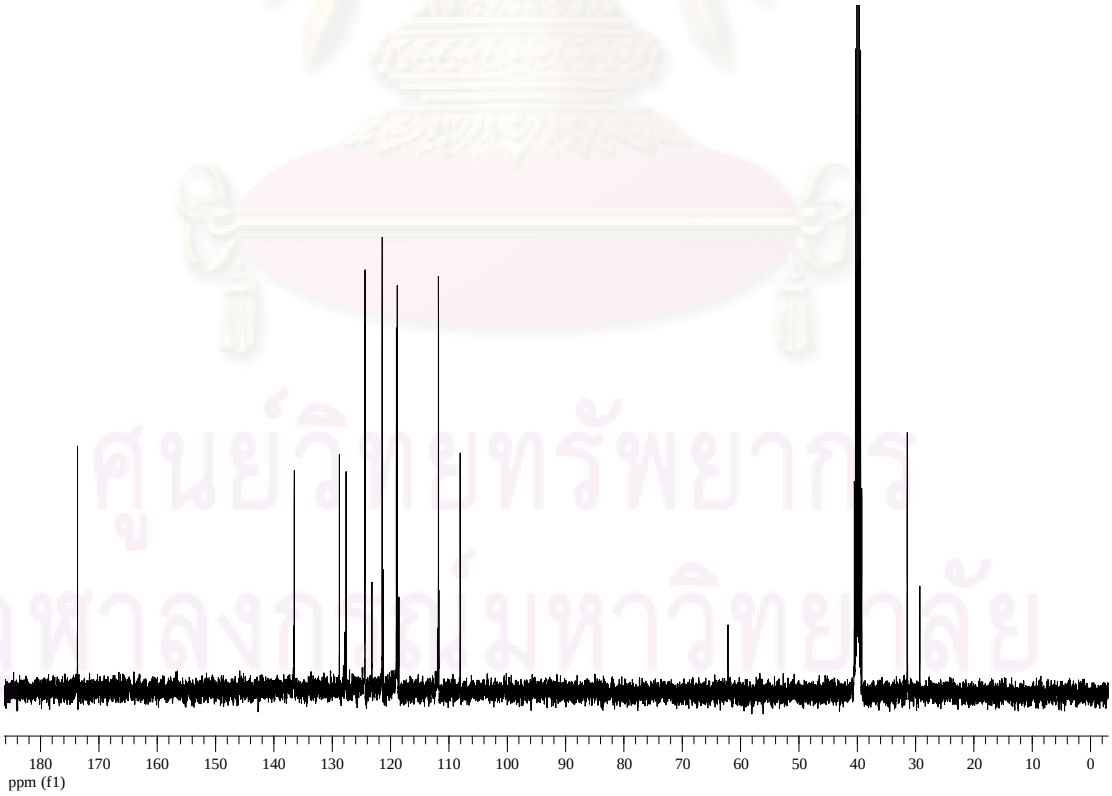

Figure B46 400 MHz ^{1}H NMR (DMSO) spectrum of indole-3-acetamide (**14**)

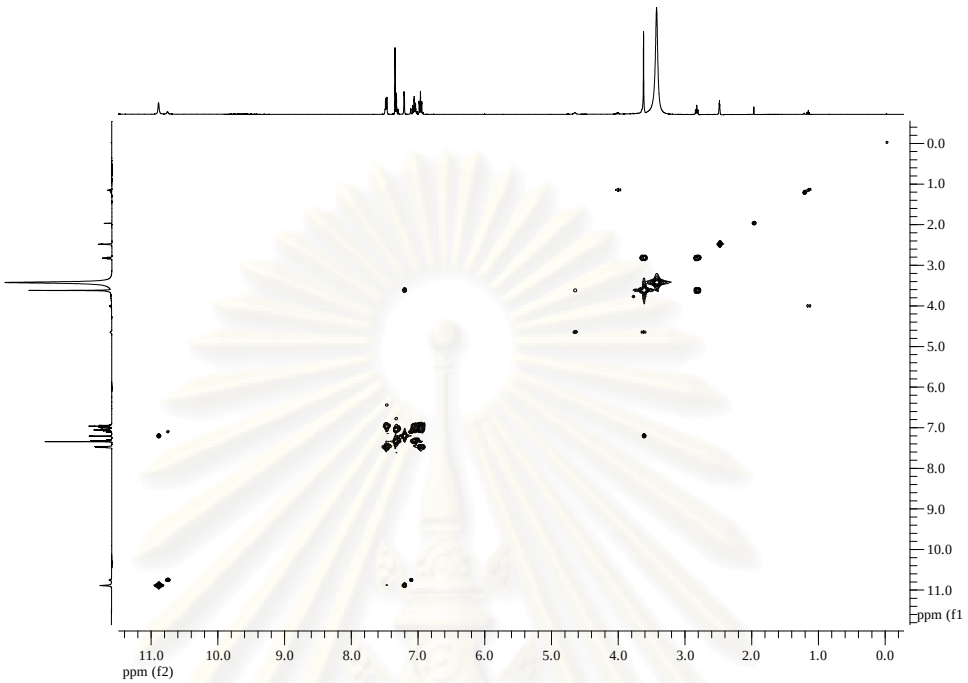

Figure B47 COSY of indole-3-acetamide (**14**)

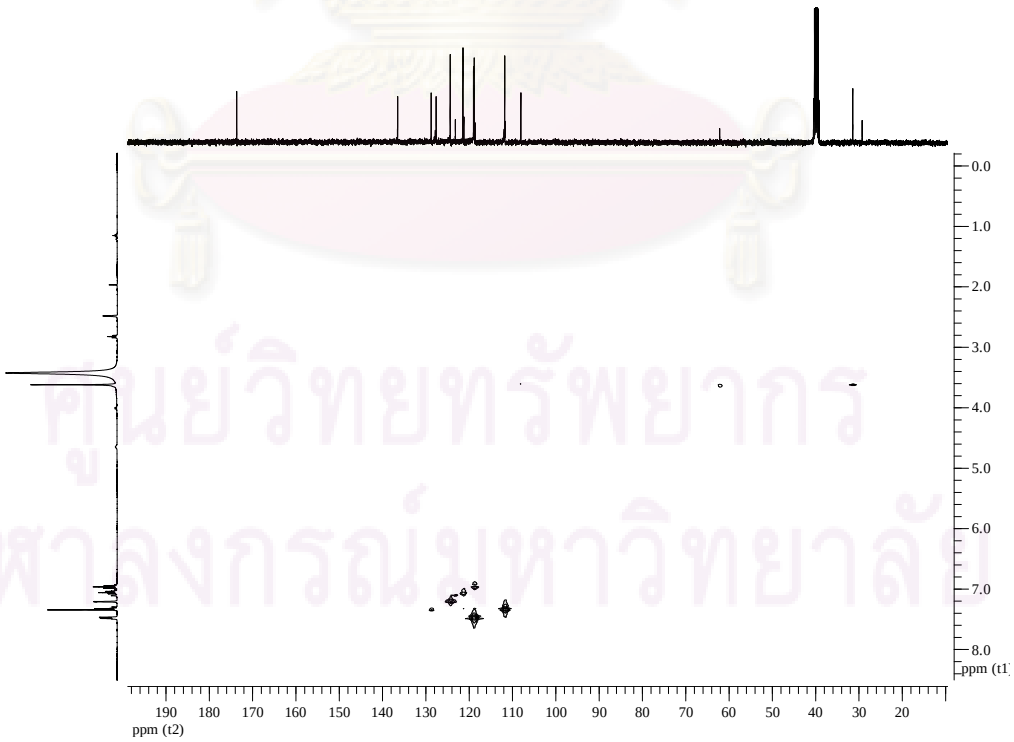

Figure B48 HSQC of indole-3-acetamide (**14**)

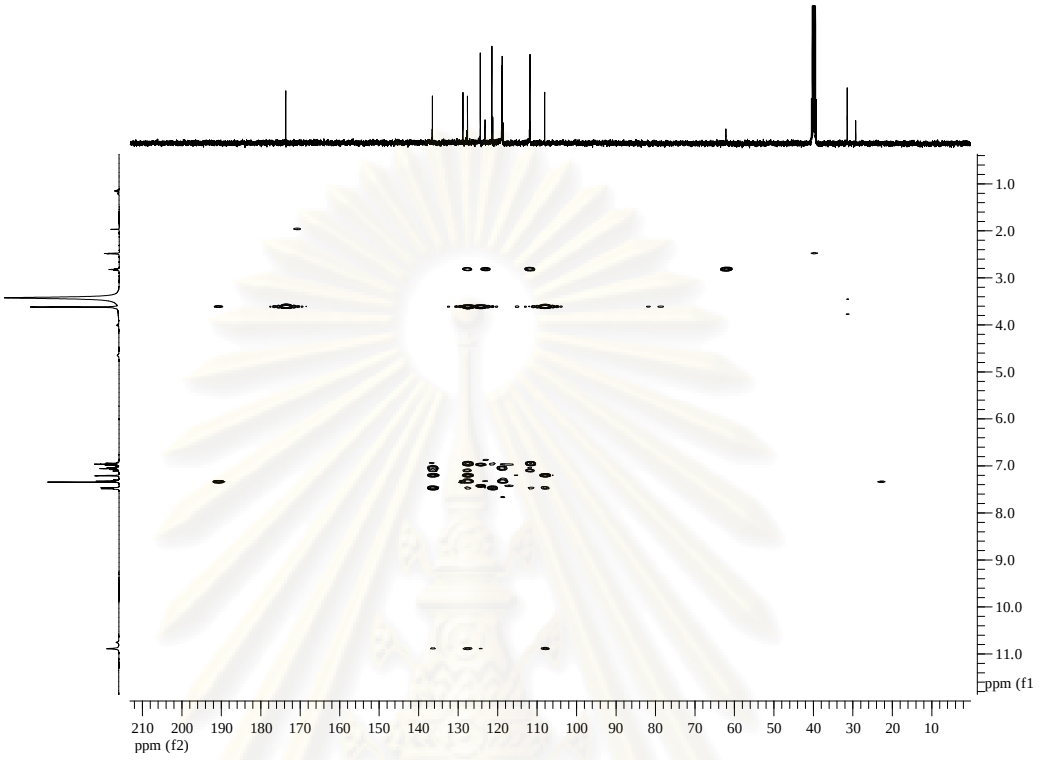

Figure B49 HMBC of indole-3-acetamide (**14**)

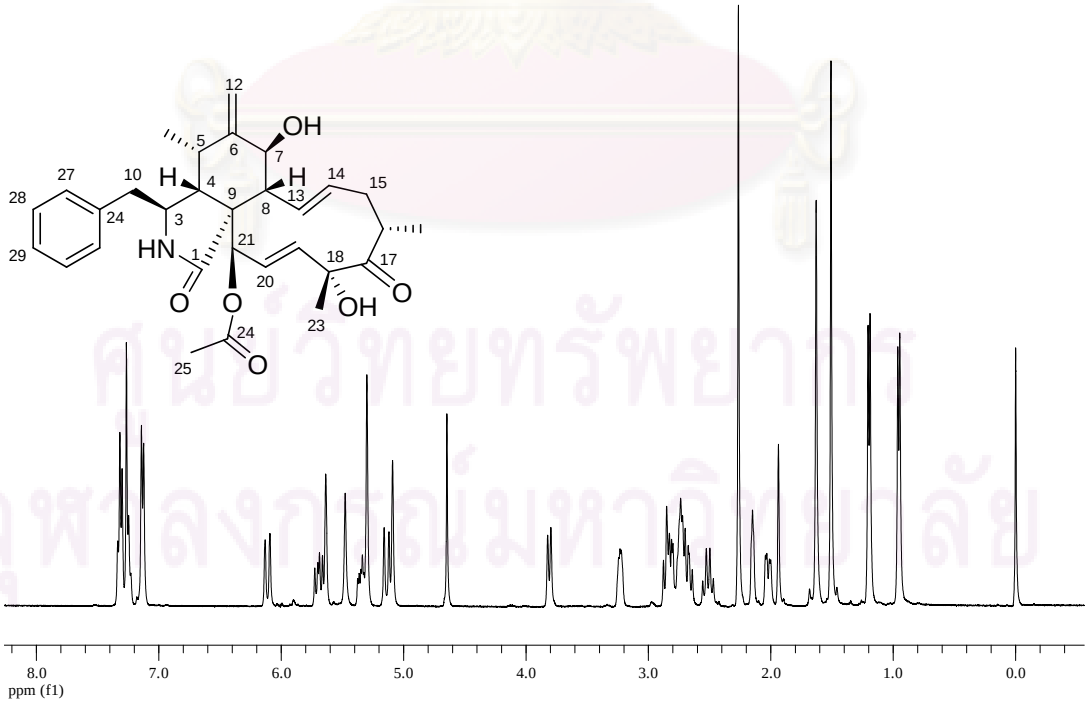

Figure B50 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of cytochalasin D (**15**)

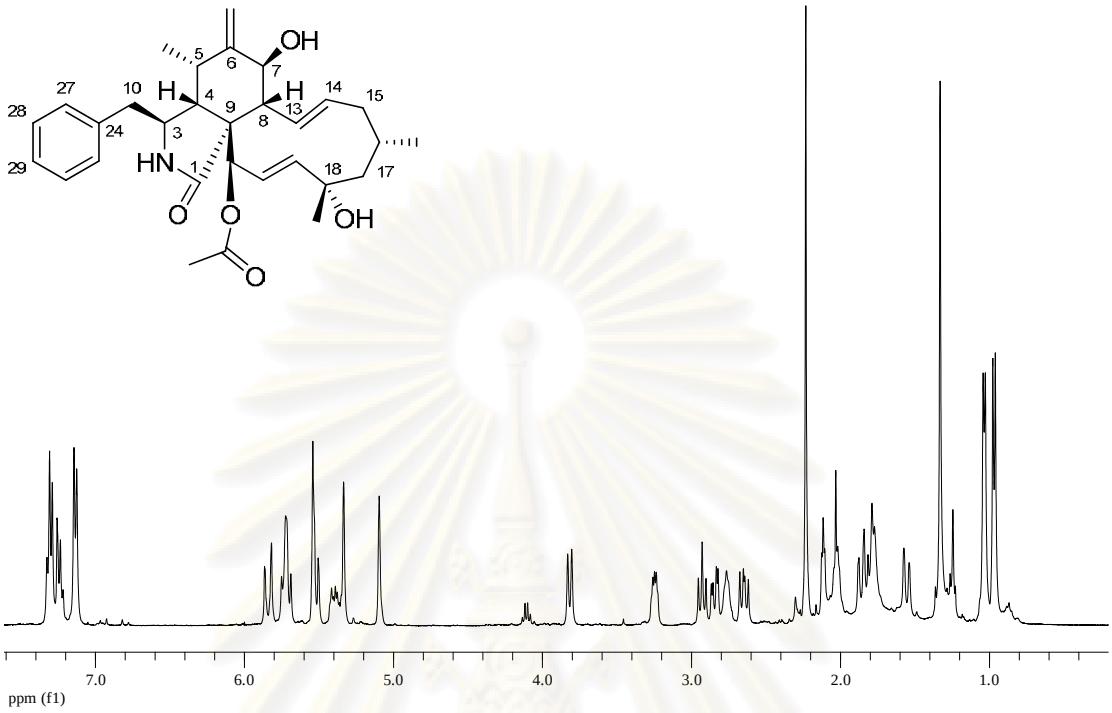

Figure B51 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of cytochalasin H (**16**)

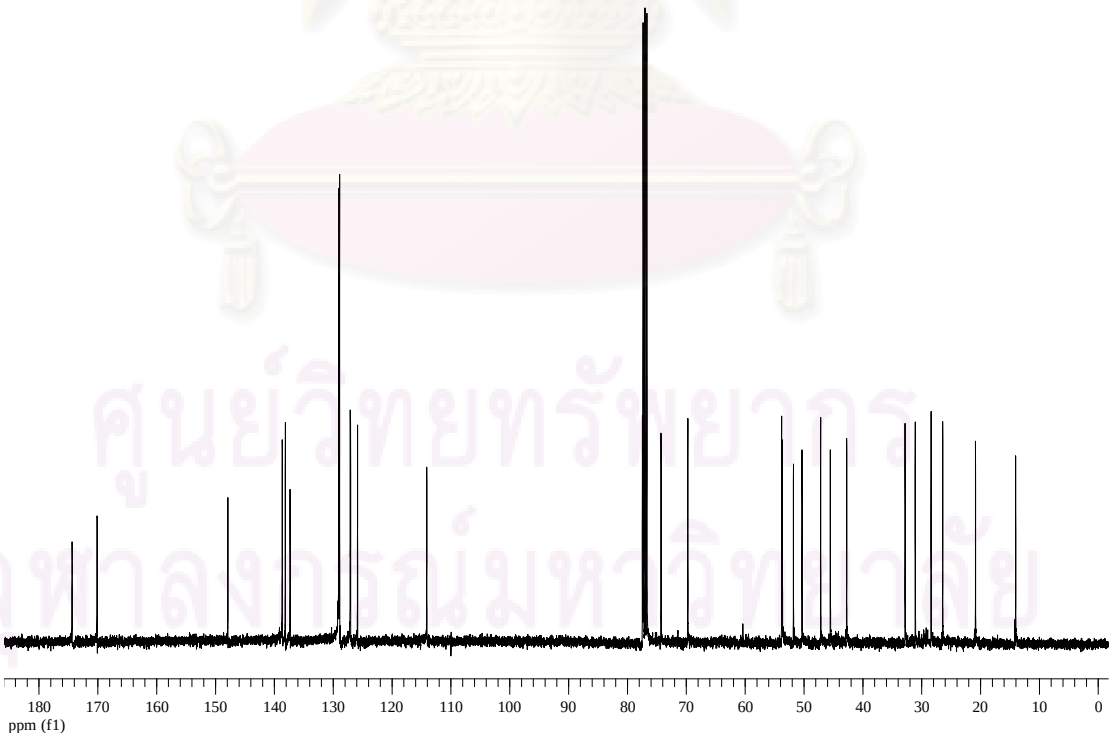

Figure B52 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of cytochalasin H (**16**)

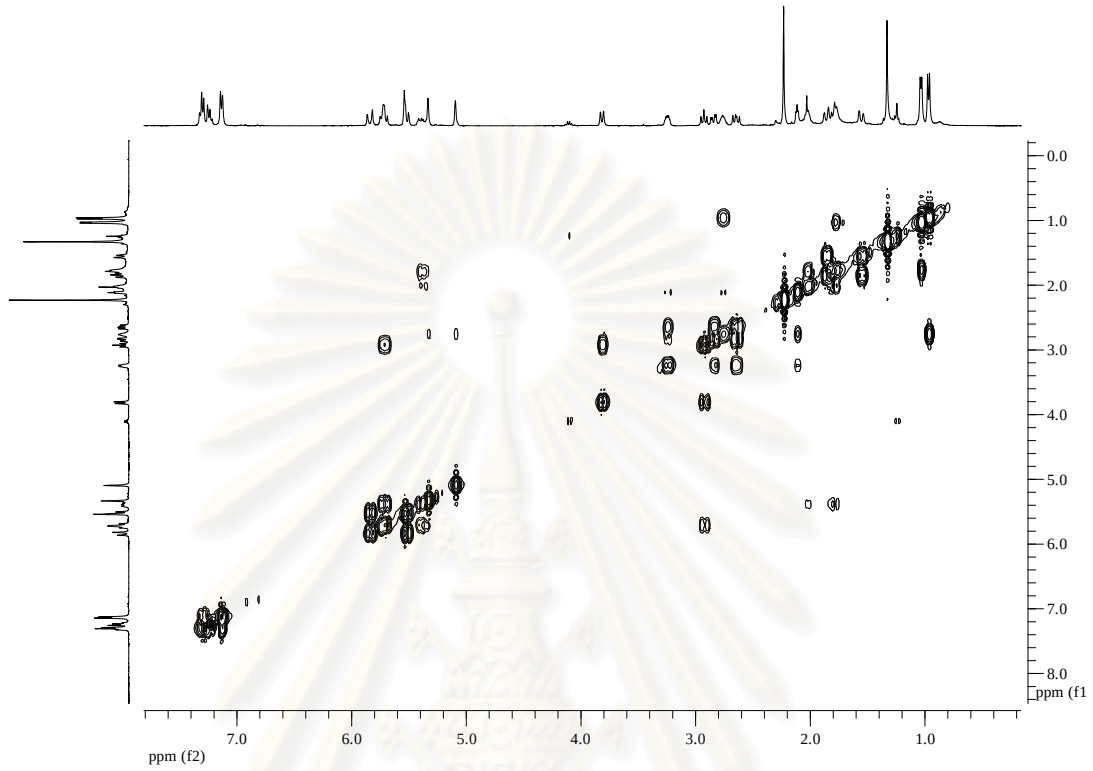

Figure B53 COSY of cytochalasin H (**16**)

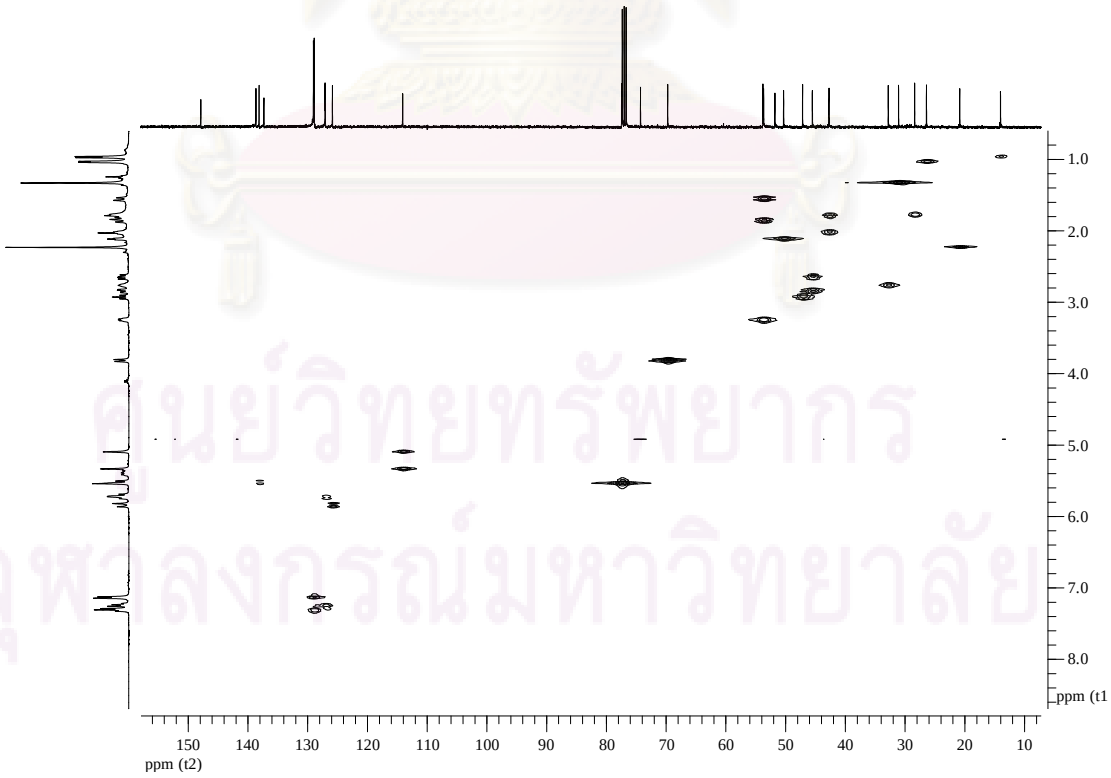

Figure B54 HSQC of cytochalasin H (**16**)

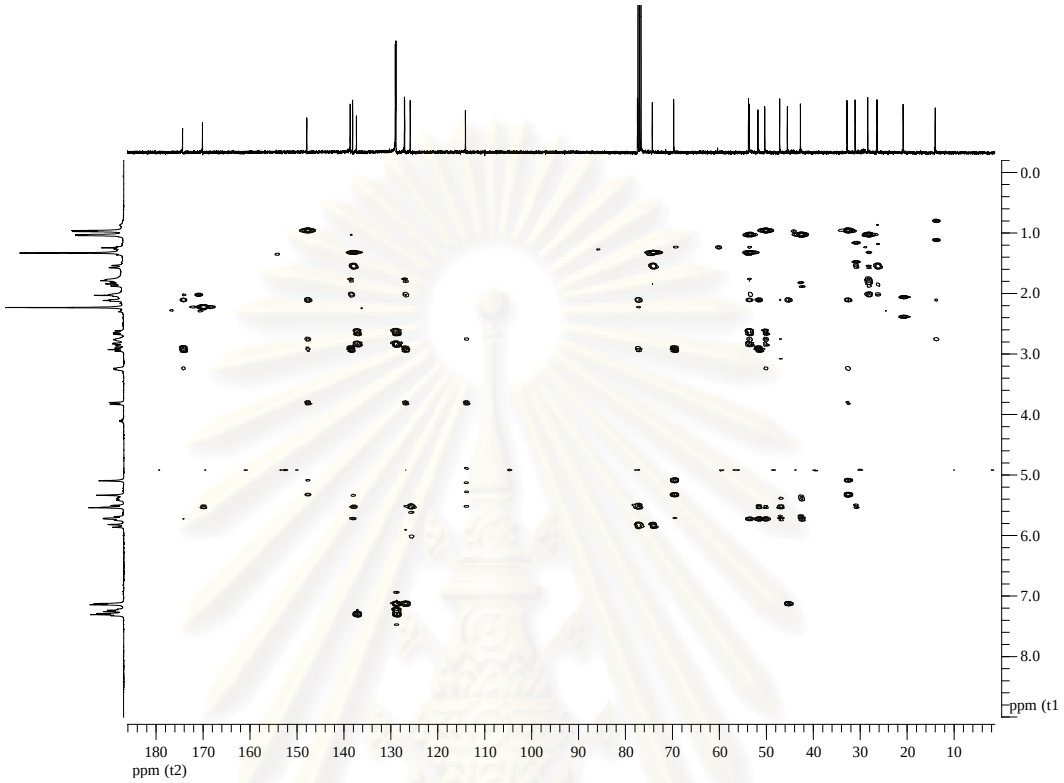

Figure B55 HMBC of cytochalasin H (**16**)

BIOGRAPHY

Mr. Ruengrit Sappapan was born on July 16, 1977 in Pattani Province, Thailand.

His education:

- 2003: The Master of Science, Department of Zoology (Microbiology), Faculty of Science, Kasetsart University. Thesis Subject: Bactericidal Efficiency of the Thai Freshwater Crocodile (*Crocodylus siamensis*) Serum.

-1999: The Bachelor of Education in Biology, Faculty of Education, Kasetsart University.

His research articles:

- 2009. Cytotoxic 3,4-seco-Cycloartane Triterpenes from *Gardenia sootepensis*. *Journal of Natural Products*. 72(6): 1161–1164.

- 2008. 11-Hydroxymonocerin from the Plant Endophytic Fungus *Exserohilum rostratum*. *Journal of Natural Products*. 71(9): 1657-1659.

- 2003. Bactericidal Efficiency of the Thai Freshwater Crocodile Serum. *Journal of Medicinal Technology & Physical Therapy*. 15 supp 1: S120.

His awards:

- Good Oral Presentation Award (Health Science) on The 10th National Graduate Research Conference, Sukhothai Thammathirat Open University

- Outstanding Student Award (5As), Kasetsart University

- Pan-Prapai Inkasuwan's Award (First class of Human Physiology)

- First class of Microbilogy, Kasetsart University

His interests:

- Cancer and infectious diseases

- Drug discovery and screening

- Microbial metabolites