สารต้านมะเร็งจากราเอนโดไฟต์ที่คัดเลือกจากราชดัด *Brucea javanica* (L.) Merr.

นางสาวสุจิตรา หาญทนง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

ANTICANCERS FROM SELECTED ENDOPHYTIC FUNGI FROM RATCHADAT *Brucea javanica* (L.) Merr.

Miss Sujitra Hanthanong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2009

Thesis Title	ANTICANCERS FROM SELECTED ENDOPHYTIC FUNGI FROM RATCHADAT *Brucea javanica* (L.) Merr.
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Field of Study	Biotechnology
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สุจิตรา หาญทนง: สารต้านมะเร็งจากราแอนโดไฟต์ที่คัดเลือกจากราชดัด *Brucea javanica* (L.) Merr. (ANTICANCERS FROM SELECTED ENDOPHYTIC FUNGI FROM RATCHADAT *Brucea javanica* (L.) Merr.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.นาตยา งามโรจนวณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ขนิษฐา พุดหอม, 111 หน้า

จุดประสงค์ของงานวิจัยนี้คือเพื่อแยกสารที่มีความเป็นพิษต่อเซลล์มะเร็งจากราแอนโดไฟต์ในใบและเปลือกราชดัด โดยวิจัยนี้สามารถแยกราได้ทั้งหมด 34 ไอโซเลต หลังทำการเลี้ยงในอาหาร 5 ชนิด ได้แก่ potato dextrose broth (PDB), malt extract broth (MEB), malt czapek broth (MCzB), sabouraud's dextrose broth (SDB) และ glucose yeast extract broth (GYB) สารสกัดหยาบที่ได้นำมาทดสอบความเป็นพิษต่อเซลล์มะเร็งคน 5 ชนิด คือ มะเร็งตับ (Hep-G2), มะเร็งลำไส้ (SW-620), มะเร็งปอด (CHAGO), มะเร็งกระเพาะอาหาร (KATO-3) และ มะเร็งทรวงอก (BT-474) จากผลทดสอบความเป็นพิษต่อเซลล์และการวิเคราะห์ด้วยเทคนิค ^{1}H NMR ของสารสกัดเหล่านั้น ทำให้สามารถเลือกราที่จะนำมาเพาะเลี้ยงในขั้นต่อไปได้ 3 ชนิด ได้แก่ RB-20/2A, RB-20c และ RL-K3 ซึ่งเมื่อทำการแยกส่วนสกัดของราเหล่านี้ให้บริสุทธิ์ พบว่าได้สารที่มีการรายงานมาก่อน 8 ชนิดคือ macrosporin (**1**), alternariol monomethyl ether (**2**), alternariol (**3**), integric acid (**4**), cytochalasin C (**5**), cytochalasin Q (**6**), cytochalasin D (**7**) และ cyclo(D-tyrosyl-D-proline) (**8**)

จากนั้นนำสารที่แยกได้ทั้งหมดมาทำการทดสอบความเป็นพิษต่อเซลล์มะเร็ง พบว่าสาร (**1**) มีความเป็นพิษที่จำเพาะต่อเซลล์ BT-474 และ KATO-3 ในระดับสูง โดยมีค่า IC_{50} เท่ากับ 1.69 และ 8.69 μM ตามลำดับ, ส่วนสาร **2** แสดงความเป็นพิษต่อเซลล์ SW-620, KATO-3 และ BT-474 (ด้วยค่า IC_{50} เท่ากับ 3.01, 2.17 และ 12.19 μM ตามลำดับ) ในขณะที่สาร **3** มีความเป็นพิษที่จำเพาะต่อเซลล์ SW-620 และ CHAGO (IC_{50} เท่ากับ 9.33 และ 18.67 μM ตามลำดับ) และสาร **4** แสดงความเป็นพิษกับทุกเซลล์ที่ทดสอบ สำหรับสาร **7** และสาร **5** มีความเป็นพิษต่อเซลล์ Hep-G2, KATO-3 และ BT-474 ในระดับที่ใกล้เคียงกัน

สาขาวิชา .....เทคโนโลยีชีวภาพ.....ลายมือชื่อ.....

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

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

472533023 : MAJOR BIOTECHNOLOGY
KEY WORDS: ENDOPHYTIC FUNGI/ ANTICANCER/ CYTOTOXICITY

SUJITRA HANTHANONG: ANTICANCERS FROM SELECTED ENDOPHYTIC FUNGI FROM RATCHADAT *Brucea javanica* (L.) Merr. THESIS ADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., THESIS CO-ADVISOR: ASST. PROF. KHANITHA PUDHOM, Ph.D., 111 pp.

The purpose of this research was to isolate the secondary metabolites produced by the endophytic fungi from the leaves and barks of *Brucea javanica* (L.) Merr. and to study on their anticancer activity. Thirty-four fungal isolates were obtained from plant samples. After cultivation in five different media; potato dextrose broth (PDB), malt extract broth (MEB), malt czapek broth (MCzB), sabouraud's dextrose broth (SDB) and glucose yeast extract broth (GYB), crude extracts were investigated their cytotoxicity against five human cancer cell lines; hepato carcinoma (Hep-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), gastric carcinoma (KATO-3) and breast carcinoma (BT-474). Based on the results of cytotoxicity and ^{1}H NMR spectral data of crude extracts, RB-20/2A, RB-20c and RL-K3 grown in MEB, were selected to further cultivate for isolating cytotoxic compounds. The fractionation of their extracts led to the isolation of eight known compounds, which were identified as macrosporin (**1**), alternariol monomethyl ether (**2**), alternariol (**3**), integric acid (**4**), cytochalasins D (**5**), C (**6**) and Q (**7**), and cyclo(D-tyrosyl-D-proline) (**8**).

Pure isolated compounds were also evaluated for their cytotoxic effects. Compound **1** showed strongly and selectively cytotoxic activity against BT-474 and KATO-3 cell lines with IC_{50} values of 1.69 and 8.69 μM, respectively. Compound **2** displayed toxicity on SW-620, KATO-3 and BT-474 cell lines (IC_{50}: 3.01, 2.17 and 12.19 μM, respectively), while compound **3** showed selectively activity against SW-620 and CHAGO (IC_{50}: 9.33 and 18.67 μM, respectively). Compound **4** was active to all cell lines tested. Cytochalasins Q (**7**) and D (**5**) gave the similar results and they showed to be cytotoxic against only Hep-G2, KATO-3 and BT-474 cell lines.

Field of Study:.....Biotechnology.....Student's Signature.....Sujitra H.....
Academic Year:.....2009.....Advisor's Signature.....N. Ngamrojanavanich.....
Co-Advisor's Signature..Khanitha Pudhom

ACKNOWLEDGEMENTS

Concerning to the success of my thesis, I would like to express my deepest appreciation and sincere gratitude to my thesis advisor and co-advisor, Associate Professor Dr. Nattaya Ngamrojanavanich and Assistant Professor Dr. Khanitha Pudhom for excellent instruction, great valuable advice, kind guidance, financial aid and support throughout this dissertation. Without her ness and understanding, this thesis could not be accomplished. Sincere thanks are extended to the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University and the Department of Microbiology, Faculty of Science, Mahidol University for the support of chemicals and laboratory facilities.

In addition, appreciation is also extended the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for granting in partial financial support to conduct this research.

Special thanks are to Associate Professor Amorn Petsom for help of cytotoxicity test and chemical explanation.

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

Finally, for my family, I am deeply grateful to my parents for their care, love, understanding and great support throughout the entire study.

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LIST OF ABBREVIATIONS

ATCC	American type culture collection, Maryland, U.S.A
brd	Broad doublet
brs	Broad singlet
brt	Broad tripet
°C	Degree celsius
^{13}C NMR	Carbon-13 nuclear magnetic resonance
cm	Centimeter
COSY	Correlation spectroscopy
δ	Chemical shift
CH_2Cl_2	Dichloromethane
$CDCl_3$	Deutrated chloroform
d	Doublet (for NMR spectral data)
dd	Doublet of doublets (for NMR spectral data)
ddd	Doublet of doublets of doublets (for NMR spectral data)
CD_3OD	Deuterated methanol
DI water	Deionized water
DMSO	Dimethyl sulfoxide
DMSO-d_6	Dueterated dimethylsulfoxide
CH_2Cl_2	Dichloromethane
dt	Double of triplet
ε	Molar absorptivity
et al	and other
EI-MS	Electron impact mass spectrometry
ESI-MS	Electrospray ionization mass spectrometry
EtOAc	Ethyl acetate
g	Gram (s)
GYB	Glucose yeast extract broth
h	Hour (s)
HMBC	Heteronuclear multiple bond correlation

^{1}H NMR	Proton nuclear magnetic resonance
HSQC	Heteronuclear single quantum correlation
Hz	Hertz
IC_{50}	Median inhibitory concentration
IR	Infared
ITS	Internal transcribe spacers
J	Coupling constant
L	Liter
m	Multiplet (for NMR spectral data)
m	Medium (for IR spectral data)
MeOH	Methanol
min	Minute (s)
mm	Millimeter (s)
mL	Milliliter (s)
mg	Milligram
MHz	Megahertz
MS	Mass spectroscopy
m/z	Mass to charge
m.p.	Melting point
MCzB	Malt czapek broth
MW	Molecular weight
MEB	Malt extract broth
MeOH	Methanol
nm	Nanometer
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PCR	Polymerase chain reaction
ppm	Part per million
q	Quartet (for NMR spectral data)
RT	Room temperature

s	Singlet (for NMR spectral data)
s	Strong (for IR spectral data)
SD	Standard deviation
SDB	Sabouraud's dextrase broth
SEM	Scaning electron microscpoe
sept	Septet (for NMR spectral data)
sp.	Species
t	Triplet (for NMR spectral data)
td	Triplet of doublet
THF-d_8	Tertrahydrofuran-d_8
TLC	Thin layer chromatography
μL	Micro liter (s)
μg	Microgram
μm	Micromate
UV	Ultraviolet
w	Weak (for IR spectral data)
w/w	Weight by weight
λ_{max}	the wavelength at maximum absorption (UV)
ν_{max}	Wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

1.1 Cancer basics

1.1.1 What is cancer? (Theresa, Donald, and Marci, 2002)

The human body is made of millions of cells, each one containing 23 pairs of chromosomes. Chromosomes carry genetic information that cellular growth and functioning. In normal cell development, cells have the ability to reproduce themselves through a process celled mitosis. This process allows new cells to replace old cells, with a turnover rate of more than a billion cells a day. Cancer is a disease that originates from normal, healthy cells that lose their ability to control their growth and proceed to divide without control or order. When mutations in cellular division and chromosomal reproduction occur, the abnormal cell loses its ability to grow in an orderly fashion, and a mass or lump can result from the excessive cell division.

Cancer is not one disease, but a group of more than 200 different diseases resulting from uncontrolled abnormal cellular growth. During the earliest stages of abnormal cellular growth, cancer cells are confined to the particular layer of cells in which they first developed. This is known as carcinoma *in situ*, or noninvasive cancer. *In situ* cancers are generally curable if they are treated before the confined cancerous cells have had a chance to grow enough to became invasive. If the cancer cells continue grow, the tumor invades and destroys surrounding healthy tissue. When a cancer begins this spreading process, the growth proceeds to compress, invade, and damage normal tissue, often producing debilitating local and distant effects.

1.1.2 Staging of cancer (Dollinger, Rosenbaum, and Cable, 1997)

The seriousness or the severity of disease can range from minimal to life threatening, depending on the type of cancer, its location, the size of the initial tumor, its stage at the time of diagnosis, and whether the disease has spread (metastasized) to

other parts of the body. It is the staging of the disease that most directly indicates severity. There is a variety of staging systems that have been developed, but recent efforts to standardize the process have utilized the TNM system. In this system, T stands for the size of the tumor and the degree of local invasiveness, N for the degree of spread to the lymph nodes, and M for the presence of metastasis. From these categories, a composite of the TNM system is then converted to a stage, with the severity of disease increasing from stage I to stage IV (**Figure 1.1**). Treatment options and long-term prognoses are determined by the stage of the disease. Dollinger *et al*. (1997) provide an excellent reference to help understand cancer from a medical context.

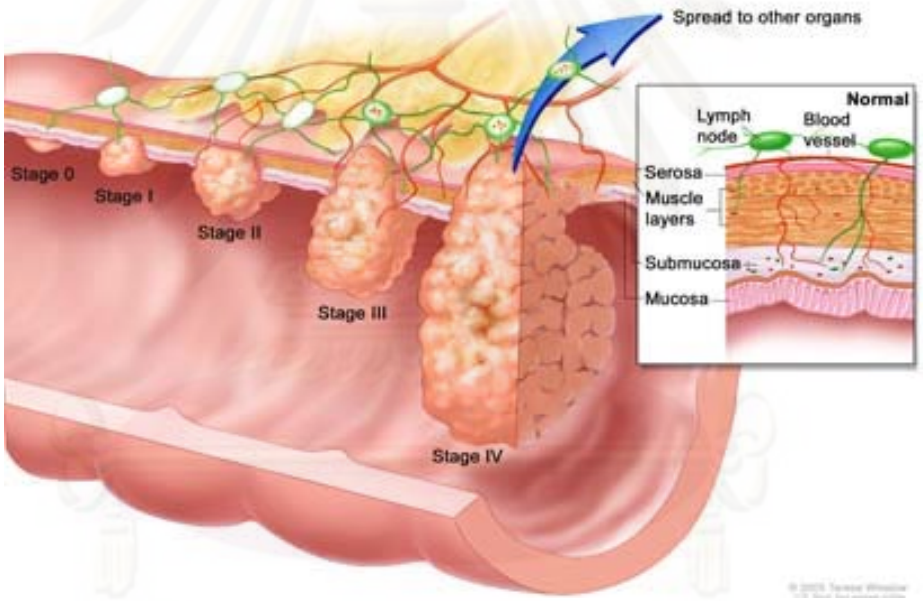


Figure 1.1 The cancer staging

1.1.3 Cancer symptoms and diagnosis

Every type of cancer is different, and has a unique set of symptoms associated with it. Some cancer symptoms are manifest outwardly, and are relatively easy to notice and identify (such as a lump in the breast for breast cancer, or blood in the stool corresponding to colorectal cancer). Other symptoms are observable, but harder to decipher. For instance, two of the major symptoms for lung cancer are a bronchitis-like deep cough and excessive shortness of breath. Few people would assume these

symptoms were serious and fewer would associate them with cancer. Still other forms of cancer produce no observable symptoms until they are at a very advanced (and therefore hard to treat) stage. Specific symptom detail for cancer subtypes is provided in our cancer subtype documents.

1.1.4 Treatment of cancer

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient (performance status). A number of experimental cancer treatments are also under development.

Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Radiation can also cause damage to normal tissue.

Because “cancer” refers to a class of diseases, it is unlikely that there will ever be a single “cure for cancer” any more than there will be a single treatment for all infectious diseases.

1.2 Fungi as a source of medicine

Many medicines in use today are derived from compounds originally produced by fungi. A number of modern drugs are the purified form of chemicals present in traditional medicines, but only a few macrofungi, such as *Cordyceps sinesis*, which grows on caterpillars of the moth *Hepilis fabricius*, have been used in traditional medicines. However, since the 1940s the pharmaceutical industry has relied upon microfungi as sources of new medicines. Of the current top-selling prescription medicines, pravastatin, simvastatin, and lovastatin (all used to lower plasma lipoprotein levels) are derived from a molecule, mevinolin, produced by *Aspergillus*

terreus. Three other top-selling medicines derived from fungi include the antibacterial antibiotics amoxicillin (a semisynthetic penicillin which is sold in combination with another compound) and ceftriaxone (a cephalosporin), and the immunosuppressive agent cyclosporin.

1.3 Endophytic fungi

Endophytes are microbes that inhabit such biotopes, namely, higher plants, which is why they are currently considered to be a wellspring of novel secondary metabolites offering the potential for medical, agricultural, and industrial exploitation. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it appears that these biotypical factors can be important in plant selection, since they may govern the novelty and biological activity of the pro duats associated with endophytic microbes (Strobel and Daisy, 2003).

1.4 Natural products from endophytic fungi

Endophytes have person to be a rich source of bioactive secondary metabolites. The following section shows some examples of natural products obtained from endophytic microbes and their potential in the pharmaceutical and agrochemical arenas.

1.4.1 Metabolites from endophytes as antibiotics

Antibiotics are defined as low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms (Demain, 1981). Often, endophytes are a source of these antibiotics. Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals (Strobel and Daisy, 2003).

An investigation of an unidentified endophytic from fungus Costa Rica for antibiotic activity yielded guanacastepene A (**Figure 1.2)** (Brady *et al*., 2000)**.** Displayed antibiotic activity against methicillin-sensitive and resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecalis.* Studies have suggested that the primary mode of bactericidal action is membrane damage. It was also found that guanacastepene A lysed human red blood cells and caused leakage of intracellular potassium (Singh *et al*., 2000).

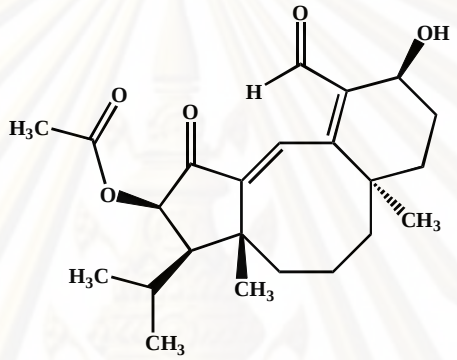


Figure 1.2 Structure of guanacastepene A

Cryptocin (**Figure 1.3**), a unique tetramic acid, was produced by *Cryptosporioosis quercina*, an endophytic fungus isolated from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia (Strobel *et al*., 1999). This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant-pathogenic fungi (Li *et al*., 2000).

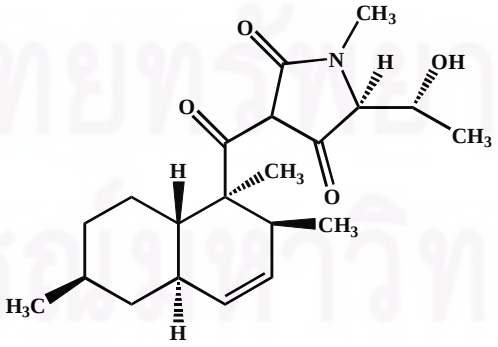
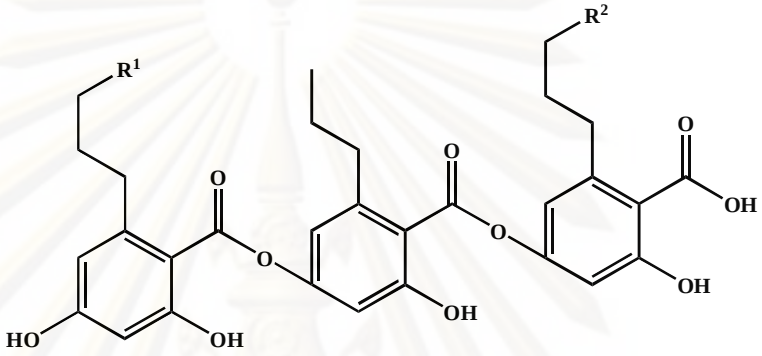


Figure 1.3 Structure of cryptocin

1.4.2 Metabolites from endophytes as antivirals

Another fascinating use of antibiotic products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus protease inhibitors, cytonic acids A and B (**Figure 1.4**), have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp. (Bingying *et* al., 2000; Strobel and Daisy, 2003; Silviya and Thomas, 2008).



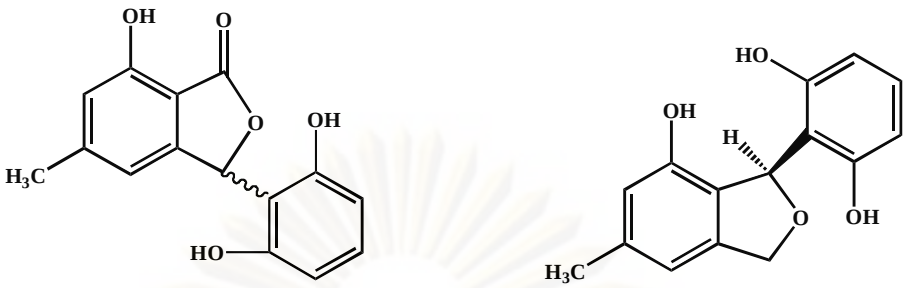
Cytonic acid A R^1 = Et, R^2 = H

Cytonic acid B R^1 = H, R^2 = Et

Figure 1.4 Structure of cytonic acids A and B

1.4.3 Metabolites from endophytes as antioxidants

Two compounds, pestacin and isopestacin (**Figure 1.5**), have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik river drainage of Papua New Guinea (Strobel *et al.*, 2002; Harper *et al*., 2003). Both pestacin and isopestacin displayed antimicrobial and antioxidant activity. Isopestacin was suspected of antioxidant activity based on its structural similarity to the flavonoids. White pestacin occurred naturally as a racemic mixture and also showed potent antioxidant activity (Harper *et al*., 2003).



(a) (b)

Figure 1.5 Structure of isopestacin (a) and pestacin (b)

1.4.4 Metabolites from endophytes as immunosuppressive agent

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such a rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus, *Fusarium subglutinans*, isolated from *Tripterygium wilfordii*, produced the immunosuppressive but noncytotoxic diterpene pyrone, subglutinol A (**Figure 1.6**) (Lee *et al.*, 1995).

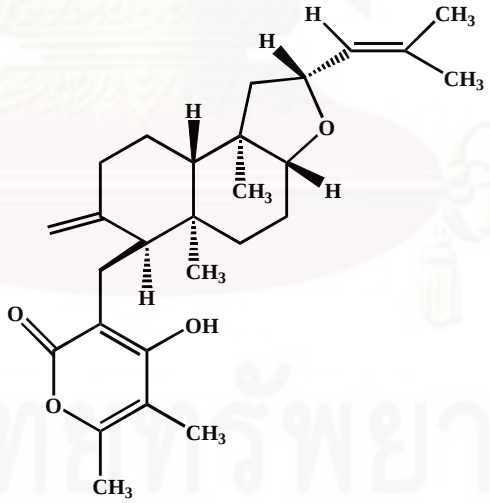


Figure 1.6 Structure of subglutinol A

1.4.5 Metabolites from endophytes as anticancer agents

Taxol is a highly functionalized, diterpene anticancer drug widely used in hospitals and clinics. The mode of action of paclitaxel is to preclude tubulin molecules from depolymerizing during the processes of cell division (Schiff and

Horowitz, 1980). It was originally isolated from the inner bark of Pacific yew, *Taxus brevifolia* (Wani *et al*., 1971).

In 1993, Strobel *et al.* firstly announced that taxol was produced by an endphytic fungus *Taxomyces andreanae* that was found growing on one particular specimen of yew tree (*T. brevifolia)* (Strobel *et al.*, 1996), thus the fungus can serve as a potential material for fungus engineering to improve the production of taxol. Since the first taxol-producing fungus was isolated, there have been a few reports on the isolation of taxol-producing endophytic fungi, were shown in **Table 1.1**.

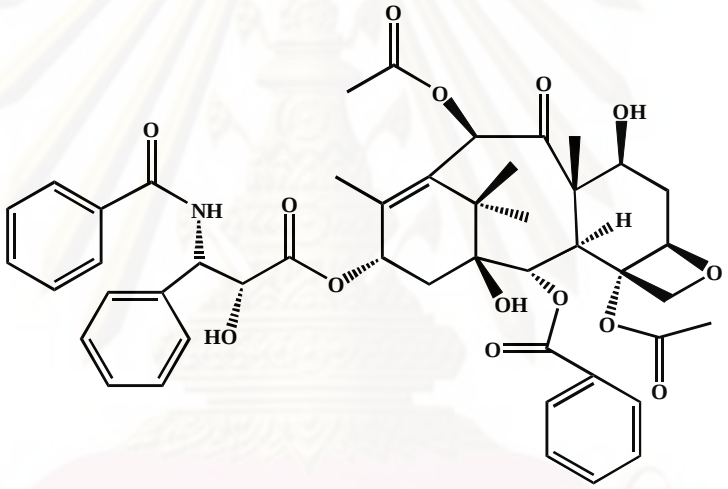


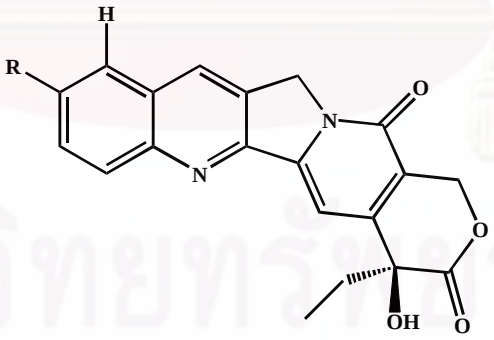
Figure 1.7 Structure of taxol (paclitaxel)

Table1.1 Endophytic fungi known to produce taxol

Fungus	Taxus source	Yield * mgL^{-1}
Monochaetia sp	*T. baccata*	102
Fusarium lateritium	*T. baccata*	130
Alternaria sp	*T. cuspidate*	157
Pestalotiopsis microspora	*T. cuspidate*	268
Pestalotiopsis microspora	*T. wallachiana*	500
Pithomyces sp	*T. sumatrana*	95
Pestalotia bicilia	*T. baccata*	1081

*Taxol measured quantitatively using monoclonal antibodies.

Camptothecin and 10-hydroxycamptothecin, have been isolated from an endophytic fungus *Entrophospora infrequens* that was isolated from an important Indian medicinal plant *Nathapodytes foetida*. Both of them showed strong antineoplastic activity (Lorence, Bolivar1, and Nessler, 2004; Satish *et* al., 2005; Li *et al*., 2010).

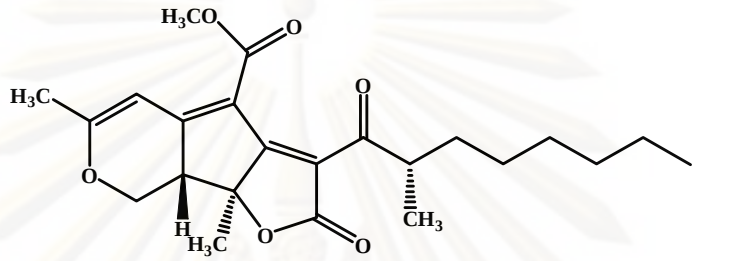


Camptothecin R = H

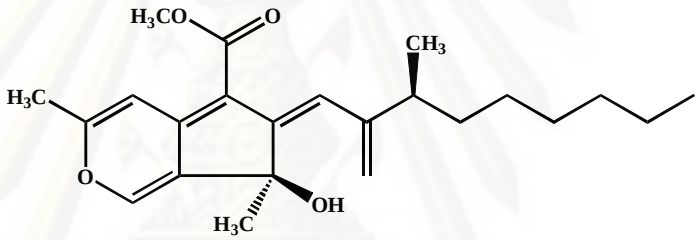
10-hydroxycamptothecin R = OH

Figure 1.8 Structure of camptothecin and 10-hydroxycamtothecin

Aspergillus parasiticus, an endophyte of the coastal redwood (*Sequoia sempervirens*), was found to produce sequoiatones A and B (**Figure 1.9**). These compounds showed moderate and selective inhibition of various human tumor cells, with the greatest activity against breast cancer cell lines (Stierle, A. A., Stierle, D. B., and Bugni, 1999).



(a)



(b)

Figure 1.9 Structure of sequoiatones A (a) and B (b)

1.5 Plant samples

1.5.1 Rationale for plant selection

It is important to understand the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms as well as ones making novel bioactive products. Thus, since the number of plant species in the world is so great, creative and imaginative, strategies must be used to quickly narrow the search for endophytes displaying bioactivity (Mittermeier *et al*., 1999).

A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is used. Several reasonable hypotheses govern this plant selection strategy and these are as follows:

1. Plants from unique environmental settings, especially those with an unusual biology, and possessing novel strategies for survival are seriously considered for study.
2. Plants that have an ethnobotanical history (use by indigenous peoples) that are related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, may have nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).
3. Plants that are endemic, having an unusual longevity, or that have occupied a certain ancient land mass, such as Gondwanaland, are also more likely to lodge endophytes with active natural products than other plants.
4. Plants growing in areas of great biodiversity also have the prospect of hosting endophytes with great biodiversity.

Just as plants from a distinct environmental setting are considered to be a promising source of novel endophytes and their compounds, so too are plants with an unconventional biology. For example, an aquatic plant, *Rhyncholacis penicillata*, was collected from a river system in Southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles (Stroble *et al.*, 1999). This created many portals through which common phytopathogenic oomycetes could enter the plant. Still, the plant population appeared to be healthy, possibly due to protection from an endophytic product. This was the environmental biological clue used to pick this plant for a comprehensive study of its endophytes.

Eventually, a potent antifungal strain of *Serratia marcescens*, was recovered from *R. penicillata* and was shown to produce oocydin A, a novel antioomycetous compound having the properties of a chlorinated macrocyclic lactone . It is conceivable that the production of oocydin A by *S. marcescens* is directly related to the endophyte's relationship with its higher plant host. Currently, oocydin A is being

considered for agriculture use to control the ever threatening presence of oomyceteous fungi such as pythium and phytophthora.

Plants with ethnobotanical history, as mentioned above, also are likely candidates for study since the medical uses to which the plant may have been selected relates more to its population of endophytes than to the plant biochemistry itself. For example, a sample of the snakevine, *Kennedia nigriscans*, from the Northern Territory of Australia was selected for study since its sap has traditionally been used as bush medicine for many years. In fact, this area was selected for plant sampling since it has been home to the world's long standing civilization-the Australian Aborigines. The snakevine is harvested, crushed and heated in an aqueous brew by local Aborigines in southwest Arnhemland to treat cuts, wounds and infections. As it turned out, the plant contained a novel endophyte, *Streptomyces* NRRL 30562, that produces wide spectrum novel peptide antibiotics called-munumbicins (Castillo *et al*., 2002). It is reasonable to assume that the healing processes, as discovered by indigenous peoples, might be facilitated by compounds produced by one or more specific plant-associated endophytes as well as the plant products themselves.

In addition, it is worthy to note that some plants generating bioactive natural products have associated endophytes that produce the same natural products. Such is the case with taxol, a highly functionalized diterpenoid and famed anticancer agent that is found in each of the world's yew tree species (*Taxus* spp.) (Suffness, 1995). In 1993, a novel taxol producing fungus, *Taxomyces andreanae*, from the yew, *Taxus brevifolia* was isolated and characterized (Strobel *et al*., 1993).

Thus, *Brucea javanica* (L.) Merr. (Simaroubaceae) was selected to use as a resource for isolating endophytic fungi. This plant, empirically, has been reported to be used as traditional medicine for the treatment of diseases like dysentery, malaria, leukemia and other cancers, including lung, cervical and skin cancer.

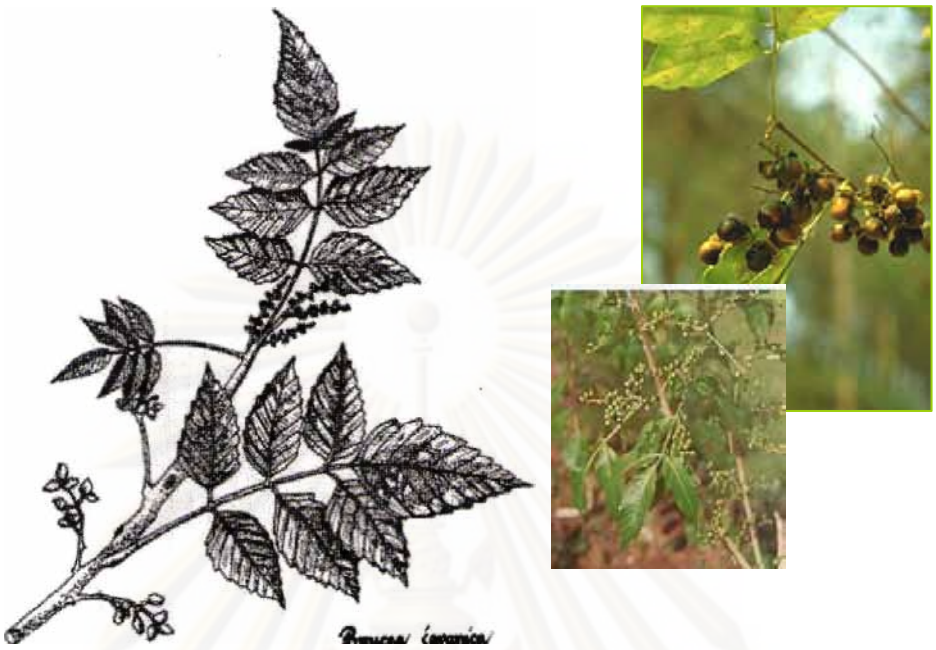


Figure 1.10 *Brucea javanica* (L.) Merr.

1.5.2 Botanical aspects of *Brucea javanica* (L.) Merr. (Roberts, 1994)

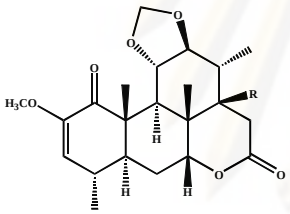
Brucea (Simaroubaceae) is widely distributed genus occurring in tropical Africa and tropical Asia. *Brucea* species are bitter monoecius or dioecius shrubs or small trees ranging from 0.3-10 m in height. Willis (1966) grouped *Brucea* into ten species; however, in the revision for the Flora Malesiana Nooteboom (1962) accepts only six species, consequently, some species' names which have appeared in the chemical literature are now accepted as synonyms of other species are.

B. javanica, known as Kho-sam, Ya-Tan-Tzu, and Macassar kernels. It is distributed from Sri-Lanka through southeast Asia to China and Taiwan, and throughout Malaysia to northern Australia. The synonyms of *B. javanica* are *B. sumatrana* Roxb., *B. sumatransis* Speng., *B. grscilis* DC., *B. glabruta* Decne., and *B. amarissima* Desv. and Merr.

1.5.3 Chemical constituents of *Brucea javanica* (L.) Merr.

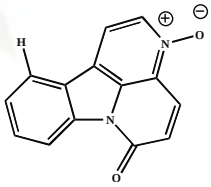
The seeds of *B. javanica* having been used for the treatment of dysentery, malaria and cancer, are also known as a rich source of quassinoids, some of which have been investigated for their biological properties including antitumor, antiamoebic, antitrypanosomal, antimalarial, anti-HIV antitubercular, antibabesial and cancer chemopreventive (Toshiro *et al.*, 1985; Toshiro *et al.*, 1986; Melanie *et al*., 1987; Wright *et* al., 1988; Narihiko *et al*., 1992; Ik *et* al., 2003; Froelich *et al*., 2007; Saw *et al*., 2008).

From the literature surverys, *B. javanica* has been widely studied. Many alkaloids, nigakilactones, flavonoids, triterpenoids and steroids have been isolated and their structures are showed in **Figure 1.11** (Ohnishi *et al*., 1995; Fukamiya *et* al., 2005; Jian *et al*., 2009; Li *et al*., 2009).

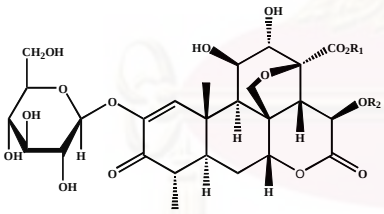


Picrasin E R = OH

Picrasin D R = H



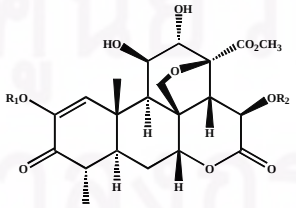
Canthin-6-one- *N*-oxide



Bruceoside D R_1 = H , R_2=

Bruceoside E R_1 = H , R_2 =

Bruceoside F R_1 = H , R_2 =



Bruceoside A R_1 = β-Glc , R_2 =

Yadanzioside A R_1 = β-Glc , R_2 =

Yadanzioside G R_1 = β-Glc , R_2 =

Yadanzioside K R_1 = β-Glc , R_2 =

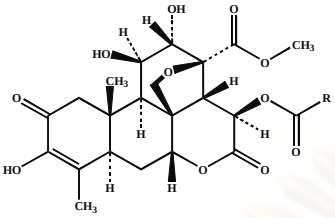
Bruceantinol R_1 = H , R_2 =

Bruceantarin R_1 = H , R_2 =

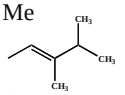
Bruceantin R_1 = H , R_2 =

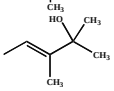
Yadanzioside O aglycone R_1 = H , R_2=

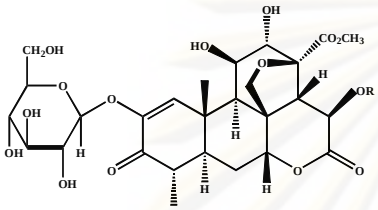
Figure 1.11 The chemical constituents of *B. javanica*.

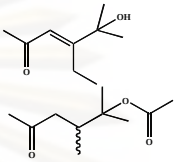


Brucein B R = Me

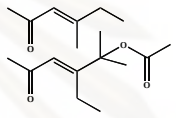
Bruceantin R =  , Brusatol R = 

Brucein A R =  , Brucein C R = 

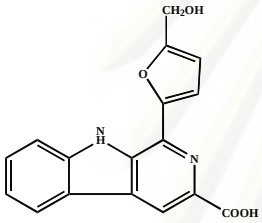


Javanicoside D R = 

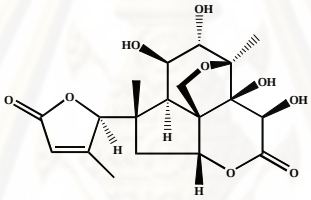
Javaniocoside E R =

Javaniocoside F R = 

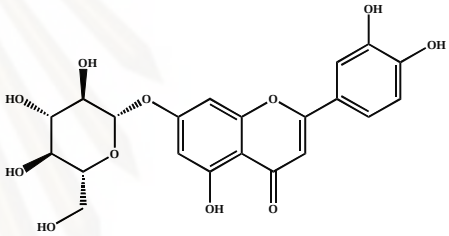
Yadanzioside O R =



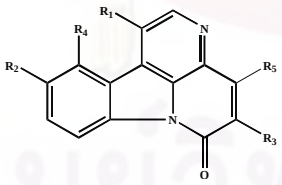
Flazine



Yadanziolode D



Luteolin-7-*O*-β-glucoside



Canthin-6-one

R_1 = H , R_2 = H , R_3 = H , R_4 = H , R_5 = H

5-Methoxycanthin-6-one

R_1 = H , R_2 = H , R_3 = OMe , R_4 = H , R_5 = H

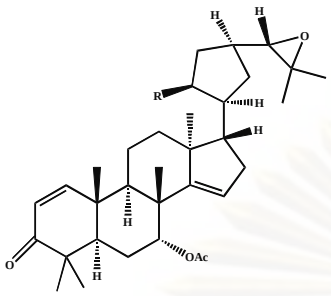
11-Methoxycanthin-6- one

R_1 = H , R_2 = H , R_3 = OMe , R_4 = OMe , R_5 = H

Picrasidine Q

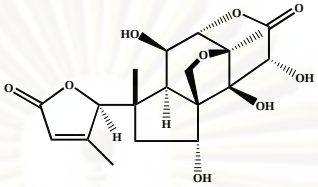
R_1 = H , R_2 = H , R_3 = OMe , R_4 = H , R_5 = OH

Figure1.11 The chemical constituents of *B. javanica*. (continue)

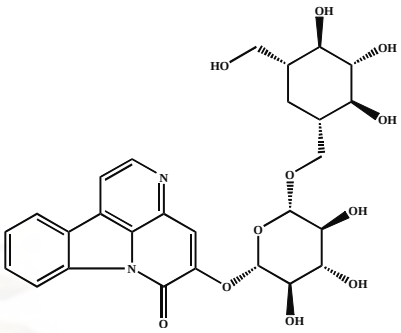


Bruceajavanin A R = OAc

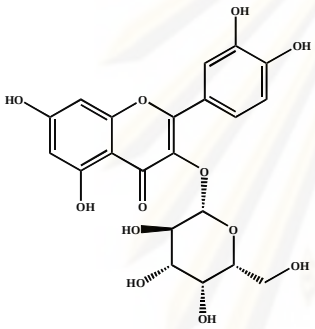
Bruceajavanin B R = OMe



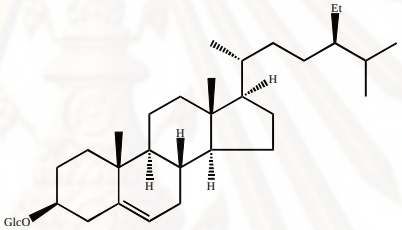
Javanicolide A



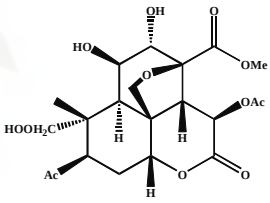
Bruceolline B



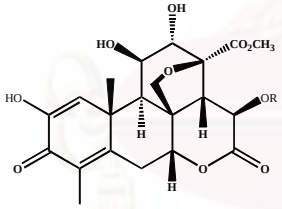
Quercetin- 3- *O*- D- galactoside



Daucosterol



Bruceaketolic acid



Dehydrobrusatol R =

Dehydrobruceantinol R =

Dedrobruceantin R =

Figure1.11 The chemical constituents of *B. javanica*. (continue)

1.6 Constituents of media

Various media have been used to grow fungi for studies of bioactive compound. Media selection is very important when fungi are grown because these organisms differ in their nutritional requirements. Differences in media composition can produce very diverse growth and behavioral responses. Media generally contain a source of carbon, nitrogen and vitamins.

A. Carbon is one of the elements that are necessary for growth which made up half of the dry weight of the fungal cells. It is required in greater quantities than any other essential elements by the fungus, and this nutrition is of important to the fungus (Moore-Landecker, 1996; Cooney, 1981). Low carbon concentration affects product formation, while increasing carbon concentration supports product synthesis (Moore-Landecker, 1972; 1996).

B. Nitrogen is required by all organisms in the synthesis of amino acids and, from these, proteins, which are required to build protoplasm. So, without protein, growth cannot occur. Fungi may use inorganic nitrogen in the from of nitrates, nitrites, ammonia, or organic nitrogen in the form of amino acids (Moore-Landeckar, 1996). Many fungi can utilize nitrate as a nitrogen source. However, basidiomycetes are not able to utilize nitrates for growth (Griffin, 1994). Nitrites are the least utilizable source of nitrogen and are normally toxic to most species of fungi, especially if they accumulate in the medium. Natrites exert their toxic effects by delaminating used in fungal media (Moore-Landecker, 1972). Numerous fungi use ammonium ion, or in the from organic nitrogen, such as an ammonium salt, which has the same oxidation level as the ammonium ion.

C. Vitamins are organic compounds that play a role as coenzymes or constituent of coenzymes, which catalyze specific reaction. These compounds are required in very small quantities, usually in the range 10^{-5}-10^{-6} M. Some fungi are able to synthesize vitamins, but some species acquire them from the environment or medium (Moore-Landecker, 1972; 1996; Zable and Morell, 1992; Griffin, 1994).

Dextrose (glucose) is the most widely utilizable carbon source, and hence is the most commonly used in growth media. Sucrose (table sugar) may be used in some media. Nitrogen sources include peptone, yeast extract, malt extract, amino acids, ammonium and nitrate compounds. Fungi have natural deficiencies for vitamins that are satisfied at μM to nM concentrations. Other organic nutrients such as glucose are often contaminated with vitamins sufficient to supply the growth requirements of fungi (Stamets and Chilton, 1983).

We will use ‘Semi-synthetic’ media, containing both natural ingredients and defined components include malt extract, potato extract, yeast extract and peptone.

1. Malt czapek broth (MCzB): This media are prepared with only inorganic sources of nitrogen and chemically defined sources of carbon. Malt extract which provides the carbon, protein, and nutrient sources required for growth of fungi.
2. Malt extract broth (MEB): It contains a high concentration of maltose and other saccharides as energy sources. Dextrose is included as a source of fermentable carbohydrate and peptone serves as a nitrogen sources.
3. Potato dextrose broth (PDB): The nutritionally rich base (potato broth) encourages a very rich fungal. Dextrose is the fermentable carbohydrate as carbon sources (MacFaddin, 1985).
4. Glucose yeast extract broth (GYB): Yeast extract is the water-soluble portion of hydrolyzed yeast and is a source of vitamins, particularly the B-group. Glucose is a carbon sources (Frank, and Bullerman, 1993).
5. Sabouraud’s dextrose broth (SDB): Dextrose is the carbohydrate energy sources and peptone mixture is the nitrogen, vitamins, minerals and amino acids sources. (Davidson, Dowding, and Buller, 1932).

From the five media test, the composition of MEB contain highest carbon sources from malt extracts and dextrose but lowest nitrogen sources when compared with MCzB that contained both carbon and nitrogen sources. Nitrogen sources of MEB were less than those of MCzB. While PDB and GYB, both media contain carbon sources, nitrogen sources and other mineral sources but nitrogen sources of GYB were less than PDB. Among of them, SDB contain highest carbon and nitrogen sources.

1.7 The objectives of this study

1. To isolate endophytic fungi from leaves and barks of *Brucea javanica* (L.) Merr.
2. To screen of endophytic fungi metabolites by anticancer activity.
3. To isolate and chemically characterize bioactive compounds from selected endophytic fungi.
4. To identify selected endophytic fungi based on morphology method.

CHAPTER II

EXPERIMENTS

2.1 Materials

2.1.1 Plant samples

Healthy and mature leaves and barks of *Brucea javanica* (L.) Merr., were carefully collected from Rayong province, Thailand. Fresh specimens were kept in a plastic bag and then immediately brought to the laboratory and processed within 24 h after collection.

2.1.2 Culture media for endophytic fungi cultivation

Water agar (WA) were culture medium for isolation of endophytic fungi. Potato dextrose agar (PDA) was used for observing morphology of isolated endophytic fungi. In addition, potato dextrose broth (PDB), malt extract broth (MEB), malt czapek broth (MCzB), glucose yeast extract broth (GYB) and sabouraud's dextrose broth (SDB) were used for growing isolated endophytic fungi.

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

2.1.3 Equipments

2.1.3.1 Ultraviolet-visible spectrophotometer (UV-VIS)

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

2.1.3.2 Fourier transform infrared spectrophotometer (FT-IR)

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

2.1.3.3 Mass spectrometer (MS)

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

2.1.3.4 Nuclear magnetic resonance spectrometer (NMR)

^{1}H NMR and ^{13}C NMR data were performed on Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Deuterated solvents, chloroform-*d* ($CDCl_3$), dimethysulfoxide-d_6 (DMSO-d_6) and acetone-d_6, were used for NMR experiments and chemical shifts (δ) were referenced by the signals of residual solvents at 7.26 (s) ppm (^{1}H NMR) and 77.00 (t) ppm (^{13}C NMR) for $CDCl_3$, at 2.50 (t) ppm (^{1}H NMR) and 39.5 (sept) ppm (^{13}C NMR) for DMSO-d_6 and at 2.09 ppm (^{1}H NMR) and 29.9, 206.7 ppm (^{13}C NMR) for acetone-d_6.

2.1.4 Chemicals used in the experiments

2.1.4.1 Solvent

All commercial grade solvents, used in this research such as hexane, chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), acetone, benzene, Ethanol (EtOH) and methanol (MeOH), distillated prior to use.

The deuterated solvents for NMR experiments including $CDCl_3$, DMSO-d_6 and acetone-d_6 were purchased from Merck.

2.1.4.2 Other chemicals

- Sephadex LH-20 and Merck silica gel 60 GF 254 NO. 9385 was used as adsorbents for column chromatography.

- Merck TLC aluminum and glass sheets, silica gel ^{60} F_{254} precoated 25 sheets, 20x20 mm^2, layer thickness 0.2 mm were used for TLC analysis.

- Clorox® (12% NaOCl) was used as a detergent for surface sterilization.

2.2 Methods

2.2.1 Isolation endophytic fungi

The plant samples were cleaned, then the endophytic fungi were isolated using the surface sterilization method which was modified from the modified method by Petrini (1991).

Plant leaves and barks were washed in running tap water and dried in laminar air flow. The samples were cut into 5x5 cm^2 and were surface steriled respectively in 70% EtOH for 1 min, followed with a solution of 12% Clorox® for 2-5 min and rinsing twice with sterile distilled water. The surface sterilized samples were dried on sterile filter papers and cut into 5x5 mm^2 before putting on WA. All Petri dishes were incubated at room temperature and examined the fungal growth every day under a stereomicroscope. The fungal hyphal tips were transferred to the PDA plate and cultured in same conditions. The purity of isolated endophytic fungi was determined by colony morphology.

2.2.2 Preservation of endophytic fungi

Fungal endophyte isolates were kept at 4 ^oC by storage as agar slants under liquid paraffin or as agar blocks in sterile water and 15% glycerol in water as described by Smith and Onions (1994). They were kept in duplicate.

2.2.2.1 Storage under liquid paraffin

Fungal endophyte isolates were grown on PDA slant at 25 ^oC for 10 days. The mature culture were then covered up 10 mm height with sterile liquid paraffin and sealed. The liquid paraffin was steriled and autoclaving twice at 121 ^oC for 15 min.

2.2.2.2 Storage in water and 15% glycerol in water

Endophytic fungi grown on agar medium were cut in cryotubes containing 1 mL of sterile water and 15% glycerol in water.

2.2.3 Metabolite production of the endophytic fungi

2.2.3.1 Fungal cultivation

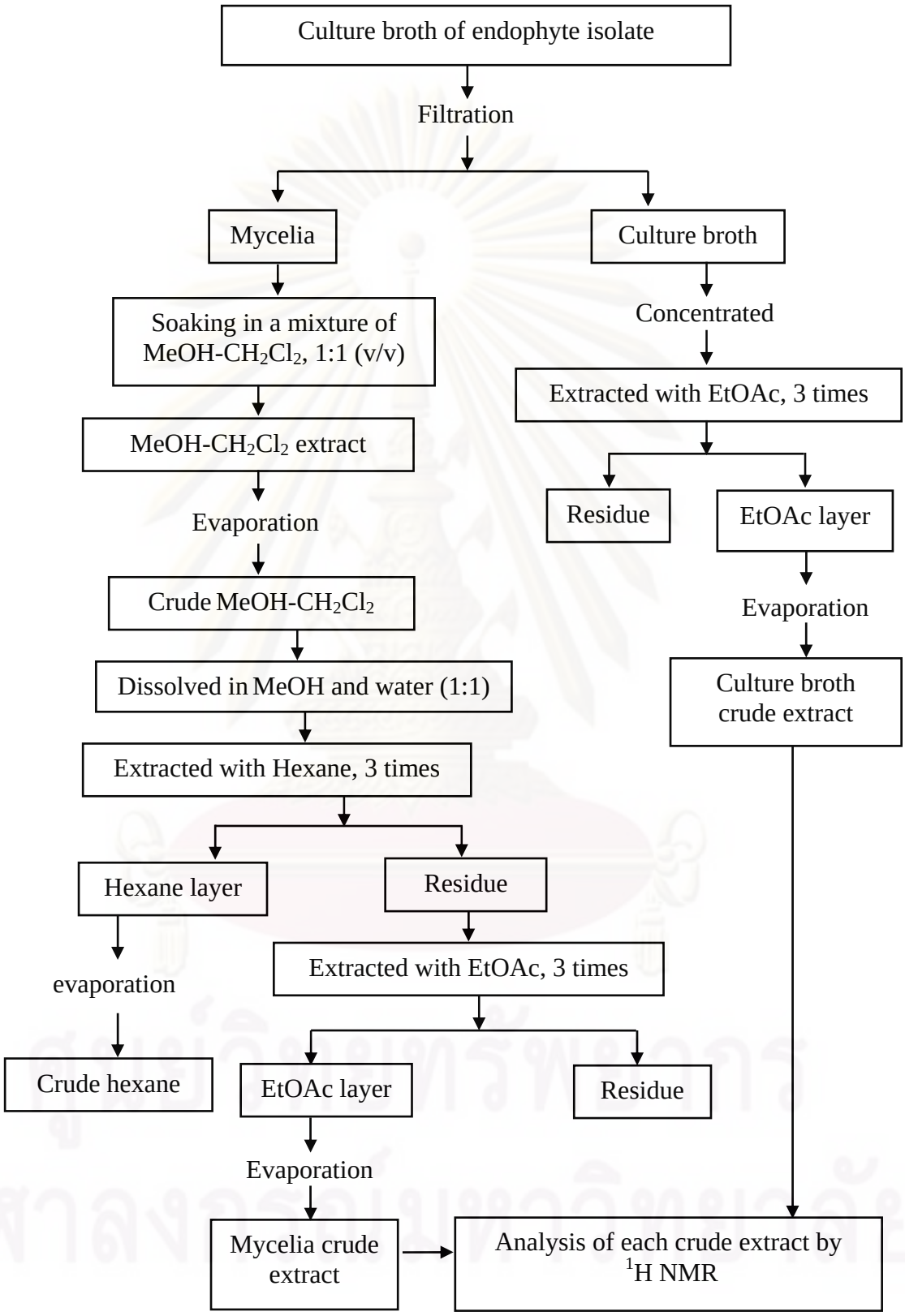
The inocula were prepared by introducing the 7 to 14-day-old plate cultures of each endophyte. The agar culture was cut into 8 mm diameter disks by a flamed cork borer. Five disks were inoculated into 250 mL flask containing 100 mL of culture media; PDB, MEB, MCzB, GYB, SDB, and cultured under static condition at room temperature for 3 weeks.

2.2.3.2 Fungal metabolite extraction and preparation of crude extract

Culture broth of each endophyte isolate was filtered through filter paper (Whatman No. 1). The filtrates were extracted with EtOAc for 3 times. The EtOAc layer was concentrated in vacuum to yield a crude extract.

Mycelia was extracted by soaking in a mixture of CH_2Cl_2 and MeOH 1:1 (v/v) for 2 days, 3 times. The solvent was evaporated under reduced pressure and dissolved in MeOH and water (1:1) v/v. Then, extracted with hexane for 3 times, hexane layer was combined and evaporated to yield a crude extract. For the residue was extracted with EtOAc for 3 times, the EtOAc layer was concentrated in vacuum to yield a crude extract.

All dried crude extracts of each fungal isolate were examined by ^1H NMR analysis for preliminarily screening of interesting compounds. The procedure of the extraction was summarized in **Scheme 2.1**



Scheme 2.1 Experimental steps used to get crude extract

2.2.3.3 TLC analysis of metabolites

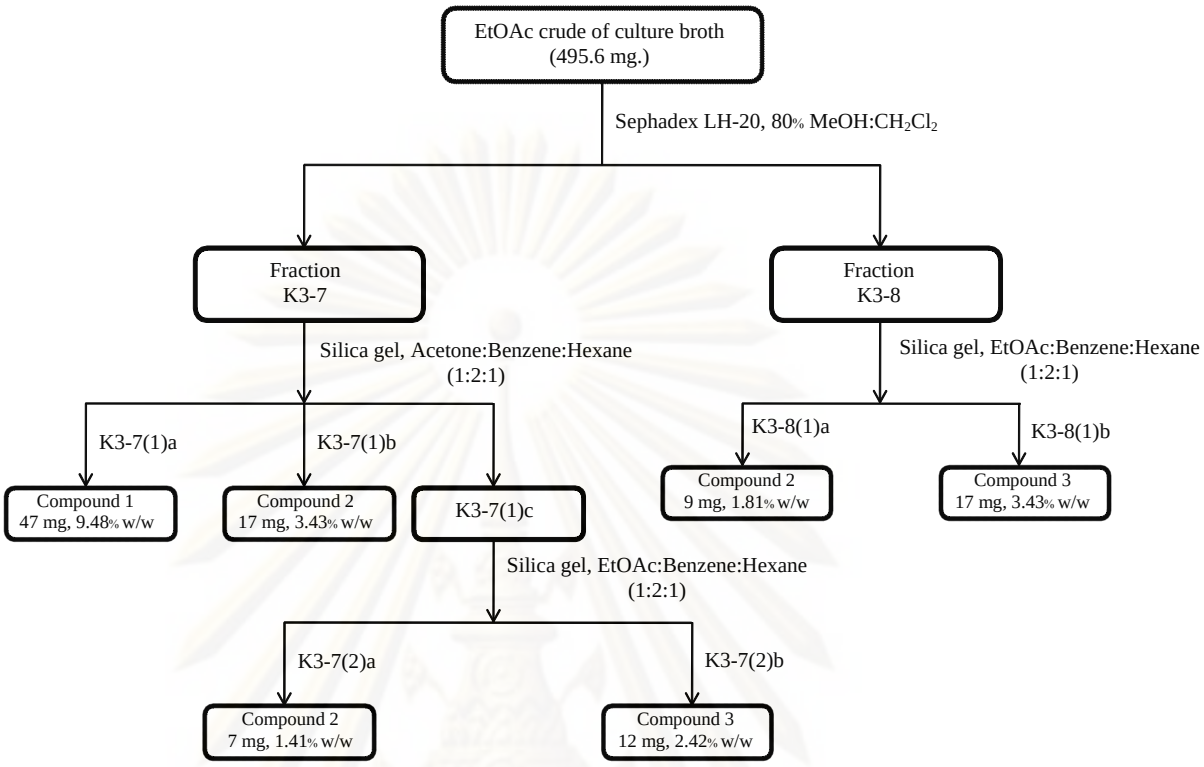
TLC analysis of the compound was performed on the silica-gel plates in order to optimize the best eluent system for the chromatographic separation. Various combinations of ratios of the following solvents, hexane, benzene, chloroform, dichloromethane, ethyl acetate, acetone and methanol were applied in mobile phases and visualized under UV light at 254 nm before dipping with phosphomolybdic acid solution in EtOH, followed by heating the plate at 150 ^{o}C.

2.2.4 Isolation and purification of selected fungus

2.2.4.1 Extraction procedure of endophytic fungus strain RL-K3

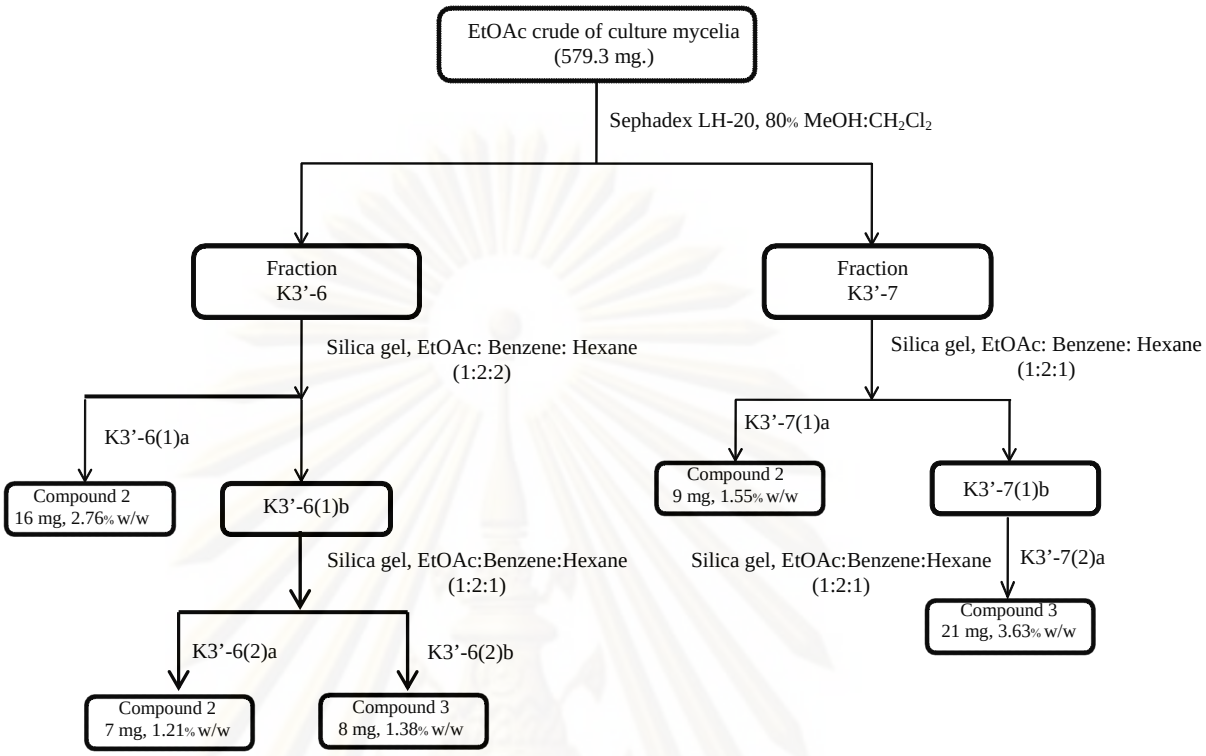
Endophytic fungus strain RL-K3 were cultured in malt extract broth (1.0 L) for 3 weeks at room temperature.

The EtOAc crude extract (495.6 mg) of culture broth of endophytic fungal isolate RL-K3 was fractionated by gel filtration chromatography using Sephadex LH-20, eluted with 80% MeOH:CH_2Cl_2 to give 12 fractions, K3-1 to K3-12. Fraction K3-7 was rechromatographed on SiO_2 flash column, eluted with acetone:benzene:hexane (1:2:1) to give macrosporin (**1**, 47 mg) and alternariol monomethyl ether (**2**, 17 mg), respectively. Subfraction K3-7(1)c which was further purified by another SiO_2 column, eluted with EtOAc:benzene:hexane (1:2:1) to give alternariol monomethyl ether (**2**, 7 mg) and alternariol (**3**, 12 mg). Fraction K3-8 was purified by SiO_2 flash column chromatography, eluted with EtOAc:benzene:hexane (1:2:1) to furnish alternariol monomethyl ether (**2**, 9 mg) and alternariol (**3**, 17 mg). The isolation and purification procedure were briefly summarized in **Scheme 2.2**.



Scheme 2.2 The isolation and procedure of culture broth of the fungus isolate RL- K3

The EtOAc crude extract (579.3 mg) of mycelia of endophytic fungal isolate RL-K3 was fractionated by gel filtration chromatography using Sephadex LH-20, eluted with 80% MeOH:CH_2Cl_2 to give 11 fractions, K3'-1 to K3'-11. Fraction K3'-6 was rechromatographed on SiO_2 flash column, eluted with EtOAc:benzene:hexane (1:2:2) to give alternariol monomethyl ether (**2**, 16 mg) and subfraction K3'-6(1)b was further purified by another SiO_2 column, eluted with EtOAc:benzene:hexane (1:2:1) to give alternariol monomethyl ether (**2**, 7 mg) and alternariol (**3**, 8 mg). Fraction code K3'-7 was purified by SiO_2 flash column, eluted with EtOAc:benzene:hexane (1:2:1) to give alternariol monomethyl ether (**2**, 9 mg) and subfraction K3'-7(1)b was further purified by another SiO_2 column, eluted with same condition to give alternariol (**3**, 21 mg). The isolation and purification procedure were briefly summarized in **Scheme 2.3**.

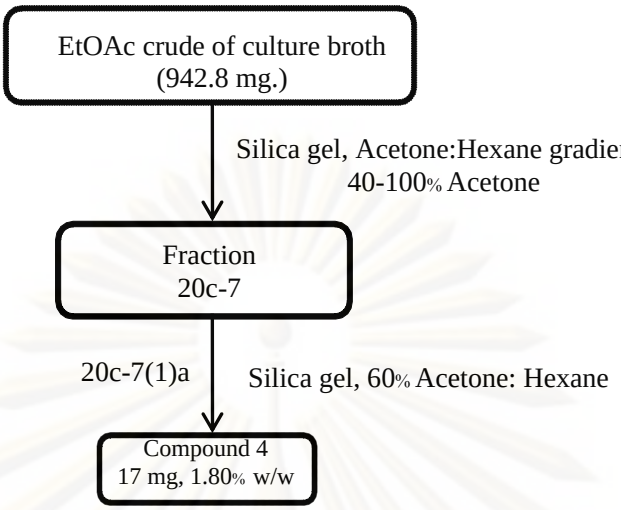


Scheme 2.3 The isolation and procedure of culture mycelia of the fungus isolate RL-K3

2.2.4.2 Extraction procedure of endophytic fungus strain RB-20c

Endophytic fungi strain RB-20c, cultured in malt extract broth (2.0 L) for 3 weeks at room temperature.

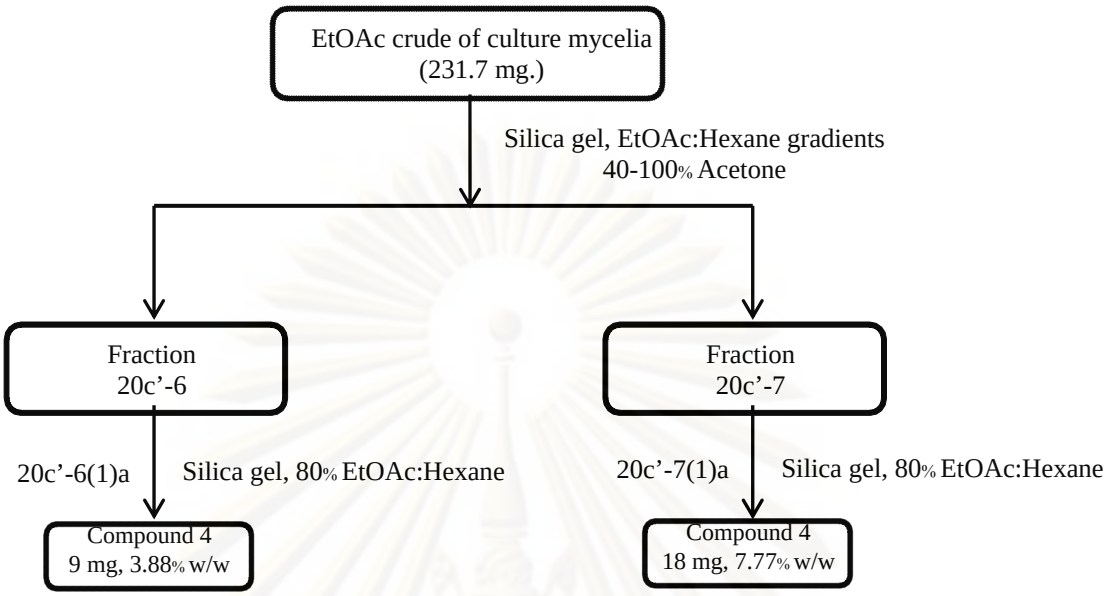
The EtOAc crude extract (942.8 mg) of culture broth of endophytic fungal isolate RB-20c was fractionated by column chromatography using Silica gel 60 GF 254. Elution system was acetone: hexane gradients (40-100% acetone to give 11 fractions, 20c-1 to 20c-11. Fraction 20c-7 was purified by SiO_2 flash column, eluted with 60% acetone:hexane to give integric acid (**4**, 17 mg). The isolation and purification procedure were briefly summarized in **Scheme 2.4**.



Scheme 2.4 The isolation and procedure of culture broth of the fungus isolate RB-20c

The EtOAc crude extract (942.8 mg) of culture broth of endophytic fungal isolate RB-20c was fractionated by column chromatography using Silica gel 60 GF 254. Elution systems were acetone:hexane gradients (40-100% acetone) to give 11 fractions, 20c-1 to 20c-11. Fraction 20c-7 was purified by SiO_2 flash column, eluted with 60% acetone:hexane to give integric acid (**4**, 17 mg). The isolation and purification procedure were briefly summarized in **Scheme 2.4**.

The EtOAc crude of culture mycelia (231.7 mg) was fractionated by column chromatography using Silica gel 60 GF 254. Elution systems were EtOAc:hexane gradients (50-100% EtOAc) to give 10 fractions, 20c'-1 to 20c'-10. Fraction 20c'-6 was recolumn by SiO_2 flash column, eluted with 80% EtOAc:hexane to give integric acid (**4**, 9 mg) and fraction code 20c'-7 was rechromatographed on SiO_2 flash column, eluted with same condition to give integric acid (**4**, 18 mg). The isolation and purification procedure were briefly summarized in **Scheme 2.5**.

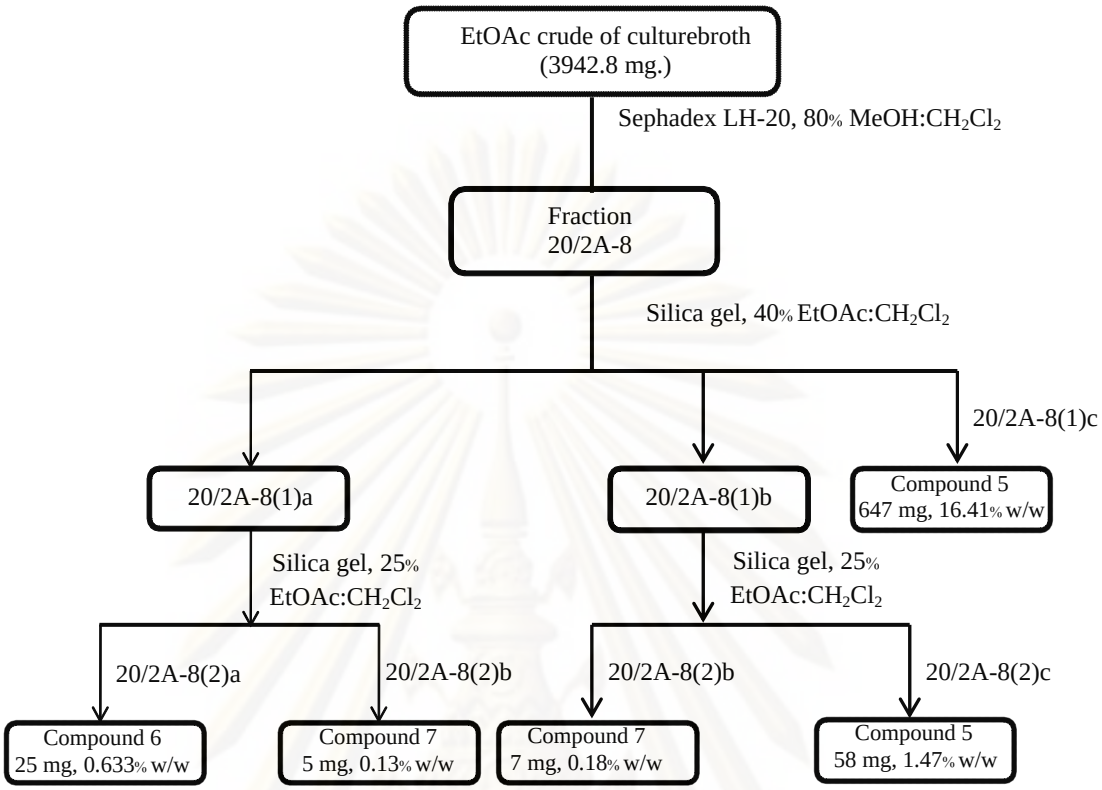


Scheme 2.5 The isolation and procedure of culture mycelia of the fungus isolate RB-20c

2.2.4.3 Extraction procedure of endophytic fungus strain RB-20/2A

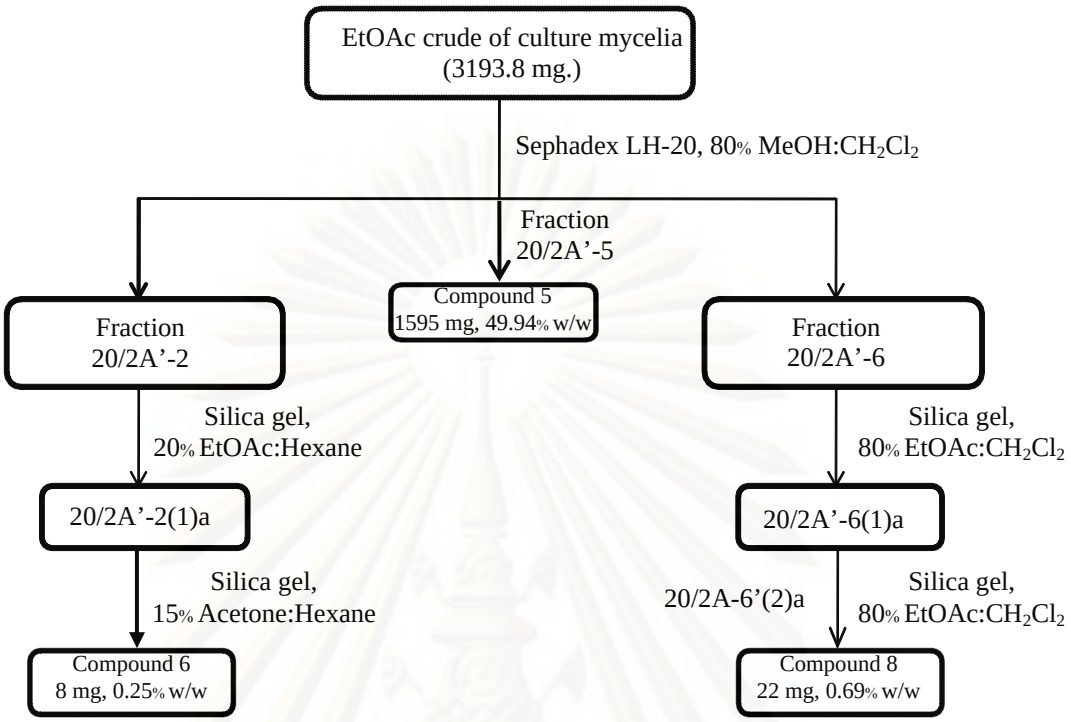
Endophytic fungi strain RB-20/2A, cultured in malt extract broth (10.0 L) for 3 weeks at room temperature.

The EtOAc crude extract (3942.8 mg) of culture broth of endophytic fungal isolate RB-20/2A was fractionated by gel filtration chromatography using Sephadex LH-20. 80% MeOH:CH_2Cl_2 to give 13 fractions, 20/2A-1 to 20/2A-13. Fraction 20/2A-8 was rechromatographed on SiO_2 flash column, eluted with 40% EtOAc:CH_2Cl_2 to give cytochalasin D (**5**, 647 mg) and subfractions 20/2A-8(1)a which was further purified by another SiO_2 column, eluted with 25% EtOAc:CH_2Cl_2 to give cytochalasin C (**6**, 25 mg) and cytochalasin Q (**7**, 5 mg), respectively. Then, fraction 20/2A-8(1)b was purified by SiO_2 column, eluted with same condition to give cytochalasin Q (**7**, 7 mg) and cytochalasin D (**5**, 58 mg), respectively. The isolation and purification procedure were briefly summarized in **Scheme 2.6**.



Scheme 2.6 The isolation and procedure of culture broth of the fungus isolate RB-20/2A

The EtOAc crude extract (3193.8 mg) of culture mycelia of endophytic fungal isolate RB-20/2A was fractionated by gel filtration chromatography using Sephadex LH-20, eluted with 80% MeOH:CH_2Cl_2 to give 9 fractions, 20/2A'-1 to 20/2A,-9 and fraction 20/2A'-5 was cytochalasin D (**5**, 1595 mg). Fraction 20/2A'-2 was rechromatographed on SiO_2 flash column, eluted with 20% EtOAc:hexane and subfraction 20/2A'-2(1)a which was further purified by another SiO_2 column, eluted with 15% acetone:hexane to give cytochalasin C (**6**, 8 mg). Also fraction 20/2A'-6 was rechromatographed on SiO_2 flash column chromatography, eluted with 80% EtOAc:CH_2Cl_2 and subfraction 20/2A'-6(1)a which was further purified by another SiO_2 column, eluted with same condition to give dipeptide 1 (**8**, 22 mg). The isolation and purification procedure were briefly summarized in **Scheme 2.7**.



Scheme 2.7 The isolation and procedure of culture mycelia of the fungus isolate RB-20/2A

2.3 Experimental section

2.3.1 Cytotoxicity assay

Cytotoxicity test was carried out at the institute of Biotechnology and Genetic Engineering, Chulalongkorn University. Bioassay of cytotoxic activity was performed in vitro by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltrazolium bromide) calorimetric method (Mossmann, 1983; Carmichael et al., 1987; Doyle and Griffiths, 1997; Tominaga et al., 1999) against hepato carcinoma (Hep-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), gastric carcinoma (KATO-3) and breast carcinoma (BT-474). In principle, the viable cell number/well was directly proportional to the production of formazan, followed by solubilization, and could be measured spectrophotometrically.

The human cancer cell line was harvested from exponential-phase maintenance cultures (T-25 cm2 flask), counted by trypan blue exclusion, seed cells in a 96-well culture plates at a density of 1x105 cells/well in 200 μL of culture medium without compounds to be tested. Cells were cultured in a 5% CO2 incubator at 37 oC, 100% relative humidity for 24 h. Culture medium containing the sample was dispensed into the appropriate wells (control cells group, N = 3; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 3) and medium/DMSO blank (N = 3) “background” determination. Culture plates were then incubated for 3 days prior to the addition of tetrazolium reagent. MTT stock solution in a concentration of 5 mg/ml in PBS was sterilized by filtering through 0.45 μL filter units. MTT working solution was prepared just prior to culture application by dilution of MTT stock 1:5 (V/V) in prewarmed standard culture medium. The freshly prepared MTT reagent in a volume of 10 μL was added into each well and mixed gently for 1 minute on an orbital shaker. The cells were further incubated for 4 h at 37 ^{o}C in a 5% CO2 incubator. After incubation, the formazan produced in the cells will capture as dark crystals in the bottom of the wells. All of the culture medium supernatants was removed from wells and 150 μL of DMSO was added to dissolve the resulting formazan. Samples in the culture plate were mixed for 5 minutes on an orbital shaker. Subsequently, 25 μL of

0.1 M glycine pH 10.5 was added and the culture plate was shaken for 5 minutes. Following formazan solubilization, the absorbance was measured using a microculture plate reader at 510 nm (single wavelength, calibration factor = 1.00).

The concentration of each extract reduced cell survival by 50% was determined from cell survival curves.

Percentage of cell survival is expressed as:

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

The extracts displaying cytotoxicity were further purified by column chromatography. Thereafter, all pure compounds were retested their toxicity against cancer cells using the same procedure as mentioned in the screening stage, by five concentrations of 10.0, 5.0, 2.5, 1.25, 0.625 mg/mL of each compound in three replicates. The half maximal inhibitory concentrations (IC_{50}) were calculated from cell survival curves.

2.4 Identification and classification of the endophytic fungi isolated

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

2.4.1 Morphological identification

2.4.1.1 Macroscopic characteristic

Endophytic fungal were cultured on PDA. Each isolates were observed colony characteristics, for example, shape, size, color, margin, pigment, and others were studied.

2.4.1.2 Microscopic characteristic

Preparation of the specimens for light microscope

All fungal isolates were grown on water agar and small pieces of sterilized banana at room temperature for 2 months. Fungal spores and fruiting bodies appearing on the banana leaves were examined by light microscopy

The specimens for light microscopy were mounted in lactophenol cotton blue or lactophenol aniline blue for observation the characteristic spore arrangements and other characteristics necessary for a definitive identification on an Olympus CH2 research microscope. Examine the preparation microscopically, first under the low-power (10x) objective and the under high-power (40x), or under oil immersion (100x) if suspicious fungal structures were seen.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation of endophytic fungi from *Brucea javanica* (L.) Merr. (Simaroubaceae)

Based on different morphology of fungi, a total of 34 pure isolates of endophytic fungi were obtained from leaves and barks of *B. javanica* (L.) Merr. (Simaroubaceae) collected from Rayong province, by using a modified Wilson and Carroll's procedure (Wilson *et al.,* 1994). Isolated fungi are given as code "RL" for the fungi obtained from leaves and "RB" for those from bark of *B. javanica*, as shown in **Table 3.1**. It was noticed that leaf sections gave a slightly higher amounts of isolated fungi than the bark sections; however, both sections gave the different fungi. Thus, it means that the parts of plant samples should also affect the kind of endophytic fungi isolated.

Table 3.1 The strain code of endophytic fungi from *B. javanica*

Part of plant sample	Strain code	Total
Bark	RB-7A, RB-9A, RB-14A, RB-2B, RB-7B, RB-14B, RB-15B, RB-20B, RB-5C, RB-7C, RB-20C, RB-2/2A, RB-15/2A, RB-20/2A, RB-6/2B, RB-3/Q2	16
Leaf	RL-K1, RL-K2, RL-K3, RL-K4, RL-K5, RL-K6, RL-K7, RL-K8, RL-K9, RL-K10, RL-K11, RL-K12, RL-K13, RL-2N, RL-5N, RL-24/1, RL-24/2, RL-15/2A	18
	Total	34

After isolation of endophytic fungi, all pure strains were placed on potato dextrose agar (PDA) until grown fully on petri dish, to observe their morphological characteristics including colony, color, produced pigment and sporulation. The characteristics of each strain are shown in **Table 3.2**, **Figure 3.1** and **Figure 3.2**.

Table 3.2 Characteristics of each endophytic fungi on potato dextrose agar (PDA) medium.

Strain code	Characteristics		
	Colony	Color	Produced pigment
RB-7A	cottony	white	not produce
RB-9A	flat	yellow	not produce
RB-14A	cottony	gray and black	black
RB-2B	flat	white and yellow	yellow
RB-7B	cottony	gray and black	black
RB-14B	cottony	gray and black	not produce
RB-15B	cottony	white	green
RB-20B	cottony	white and gray	not produce
RB-5C	cottony	white and gray	not produce
RB-7C	cottony	white and brown	not produce
RB-20C	like as flower	white and brown	not produce
RB-2/2A	cottony	white	not produce
RB-15/2A	like as flower	white and black	not produce
RB-20/2A	cottony	white	not produce
RB-6/2B	cottony	white and yellow	not produce
RB-3/Q2	like as flower	white and black	not produce

Table 3.2 Characteristics of each endophytic fungi on potato dextrose agar (PDA) medium. (continued)

Strain code	Characteristics		
	Colony	**Color**	**Produced pigment**
RL-K1	flat	white and yellow	not produce
RL-K2	cottony	white and gray	not produce
RL-K3	cottony	white and yellow	yellow
RL-K4	cottony	white and brown	not produce
RL-K5	cottony	brown	not produce
RL-K6	cottony	gray and black	not produce
RL-K7	cottony	white and gray	not produce
RL-K8	cottony	brown	not produce
RL-K9	cottony	white and brown	yellow
RL-K10	cottony	white and gray	yellow
RL-K11	cottony	brown	not produce
RL-K12	cottony	gray	not produce
RL-K13	cottony	black	not produce
RL-2N	flat	white and yellow	not produce
RL-5N	cottony	brown and black	brown
RL-24/1	flat	white	not produce
RL-24/2	flat	brown	not produce
RL-15/2A	like as flower	white and black	not produce

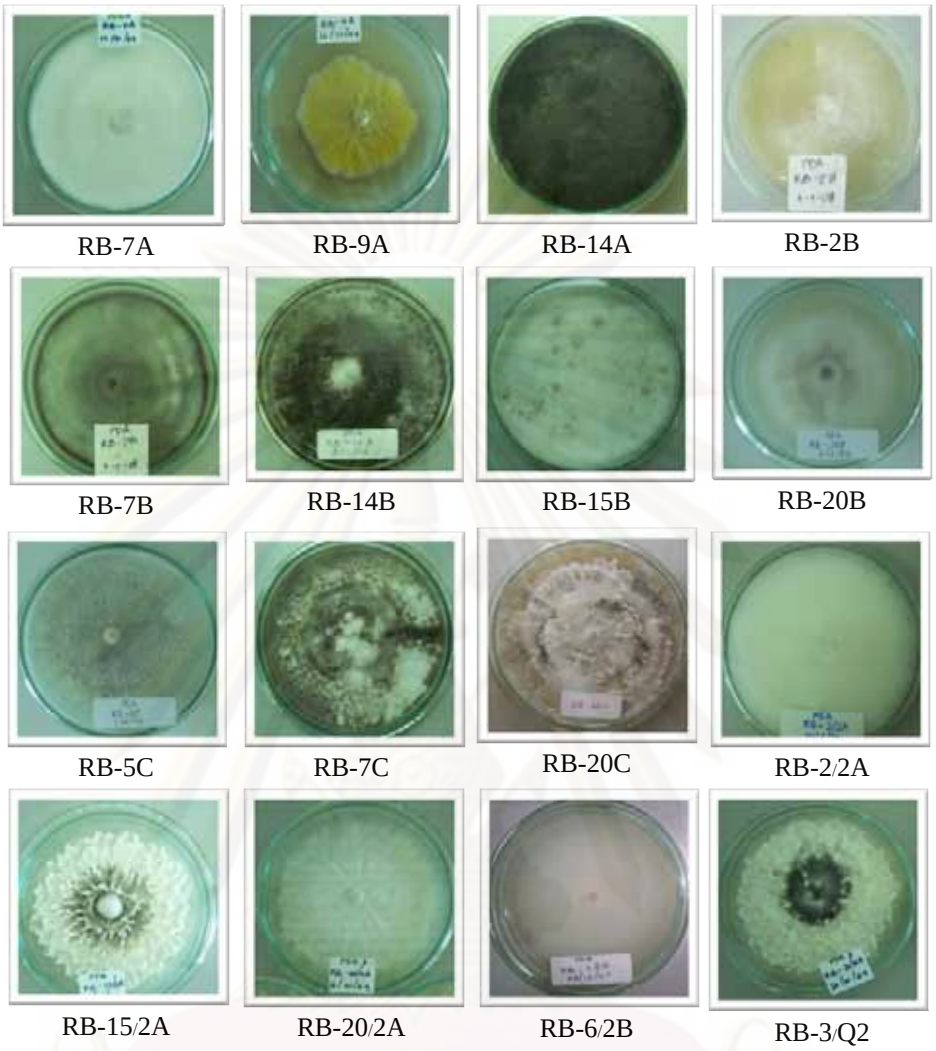


Figure 3.1 Colony characteristics of endophytic fungi isolated from bark sections of *B. javanica*, after cultivation on potato dextrose agar (PDA) for 1-3 weeks at room temperature.

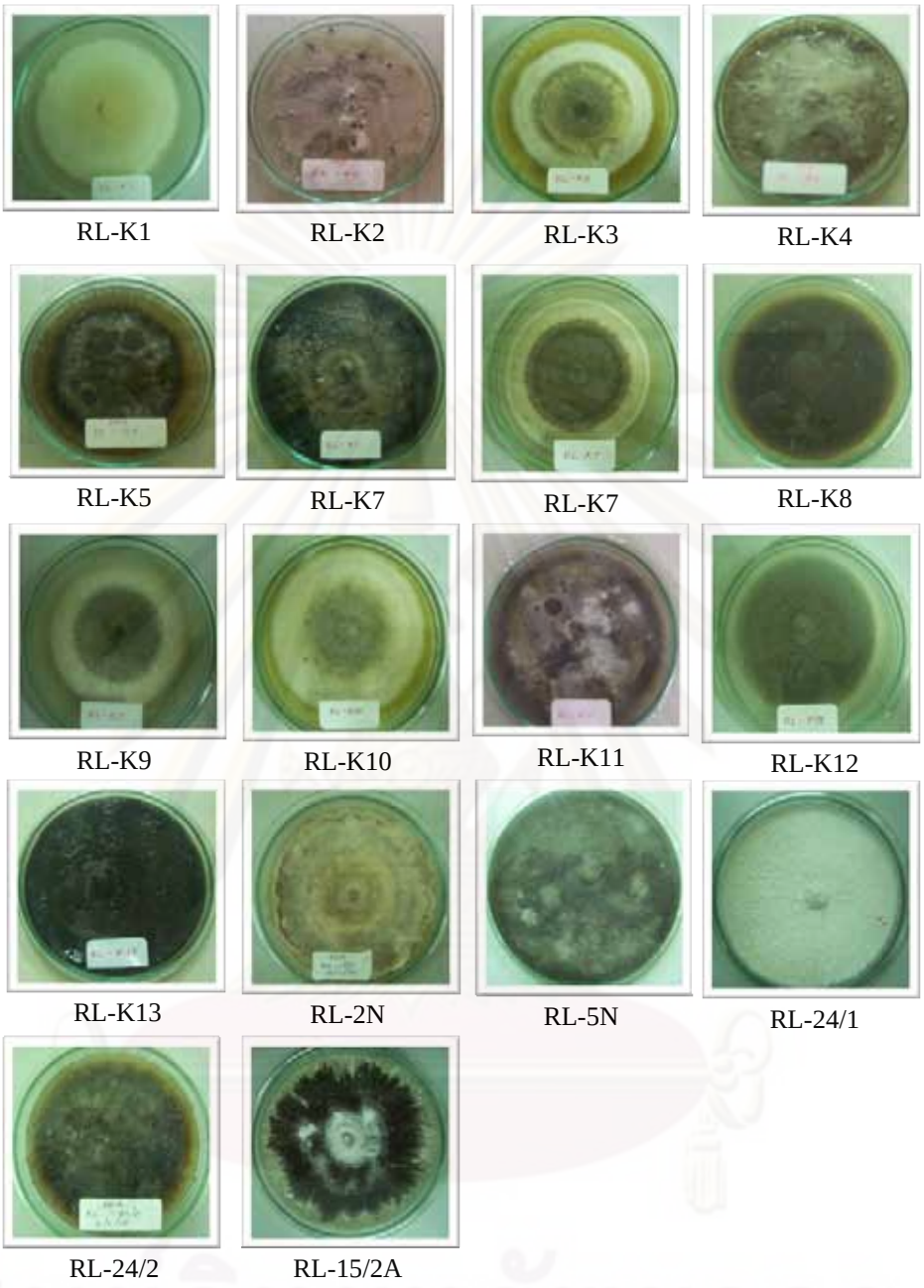


Figure 3.2 Colony characteristics of endophytic fungi isolated from leaf sections of *B. javanica*, after cultivation on potato dextrose agar (PDA) for 1-3 weeks at room temperature.

3.2 Screening for interesting endophytic fungi

3.2.1 Cultivation and extraction

Since it has been reported that culture medium affected the secondary metabolite production of the fungi. All of isolated strains were thus cultivated in five different media; potato dextrose broth (PDB), malt extract broth (MEB), malt czapek broth (MCzB), glucose yeast extract broth (GYB) and sabouraud's dextrose broth (SDB), for 21 days under static condition. Then, the culture broths were extracted according to **Scheme 2.1** to yield the EtOAc crude extracts from broth (B) and mycelium (M) parts.

3.2.2 Evaluation for cytotoxic activity of crude extracts

The dried EtOAc extracts were subjected to cytotoxic activity assay to evaluate their cytotoxic properties by using MTT colorimetric method. The cancer cell lines used in this study included hepato carcinoma (Hep-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), gastric carcinoma (KATO-3) and breast carcinoma (BT-474) cancer cells. The concentration of extract was 1 mg/mL in DMSO. The results were reported as %survival of cancer cells. The "active" extracts were considered to be those hich gave less than 50% survival (Xiaoqing and Jianbo, 2006) and it means that the extract showed cytotoxicity against cell lines tested. The extracts displaying the cytotoxic activity are shown in **Table 3.3**.

Table 3.3 Cytotoxic activities of EtOAc crude extracts on five human cancer cell lines at a dose of 1 mg/mL.

Strain code	**Culture medium/ type of crude**	% **Survival**				
		Hep-G2	SW-620	CHAGO	KATO-3	BT-474
RB-6/2B	PDB/B[a]	48.52	I	I	I	I
RB-6/2B	GYB/B	9.89	I	I	I	I
RB-2/2A	PDB/B	46.10	I	I	I	I
RB-2/2A	MEB/M[b]	45.84	I	I	23.74	3.58
RB-20/2A	PDB/M	46.01	I	I	22.98	33.04
RB-20/2A	MEB/M	45.12	I	I	22.88	32.98
RB-20c	MEB/M	9.76	9.12	9.26	8.71	8.22
RB-20c	MCzB/M	10.68	ND	ND	ND	ND
RL-K1	GYB/B	21.11	I	I	I	I
RL-K1	GYB/M	14.92	15.69	I	I	I
RL-K3	MEB/B	17.62	11.47	13.78	11.53	22.36
RL-K3	GYB/B	I	37.23	42.38	I	I
RL-K7	MEB/B	35.71	14.39	23.73	I	19.88
RL-K7	GYB/B	15.24	9.42	14.19	37.45	24.16
RL-K8	GYB/M	I	8.12	I	I	I
RL-K10	GYB/B	I	46.75	I	I	I
RL-K10	GYB/M	15.71	13.20	26.53	I	22.36
RL-K12	MEB/B	42.22	15.58	17.41	I	I
RL-K12	MEB/M	42.13	17.96	34.30	37.35	I
RL-K13	GYB/B	47.30	I	I	I	I

I = Inactive (The extracts with %survival values < 50% were considered “active”)

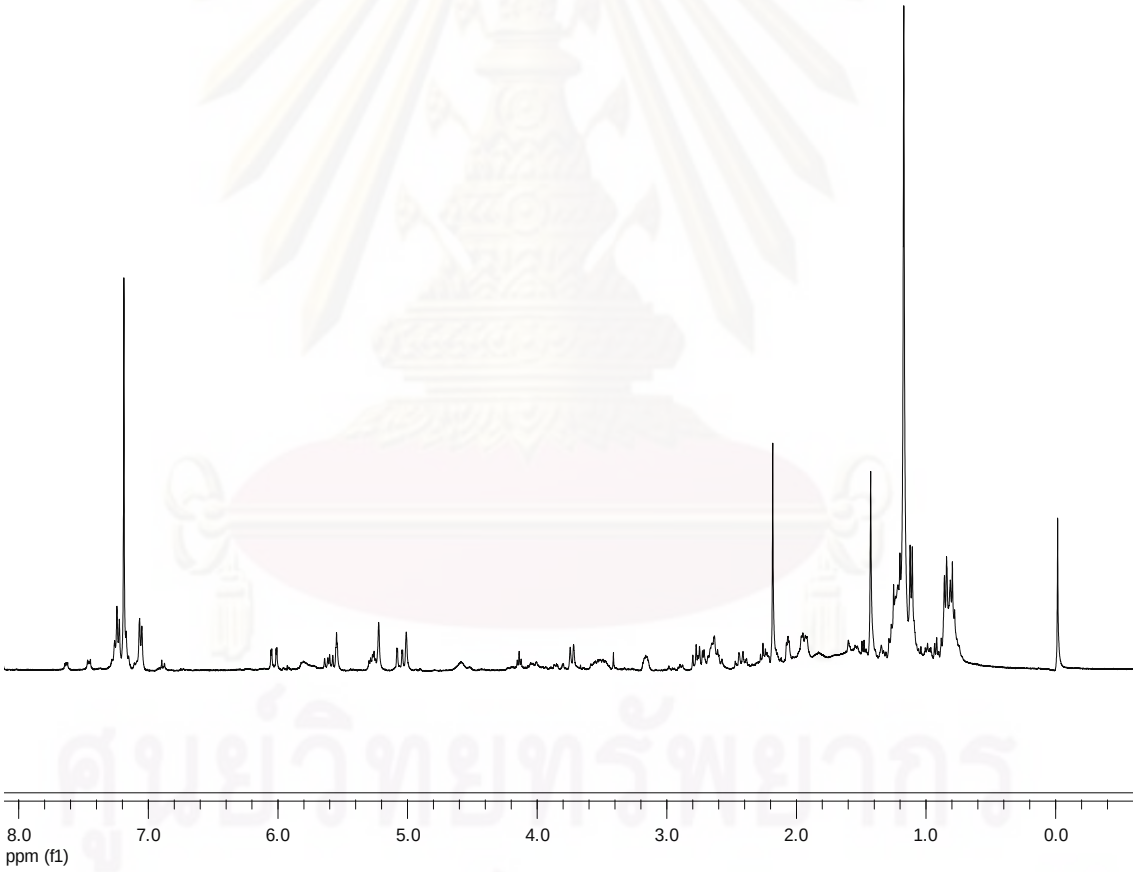
ND = not determined

[a] = extract from broth

[b] = extract from mycelium

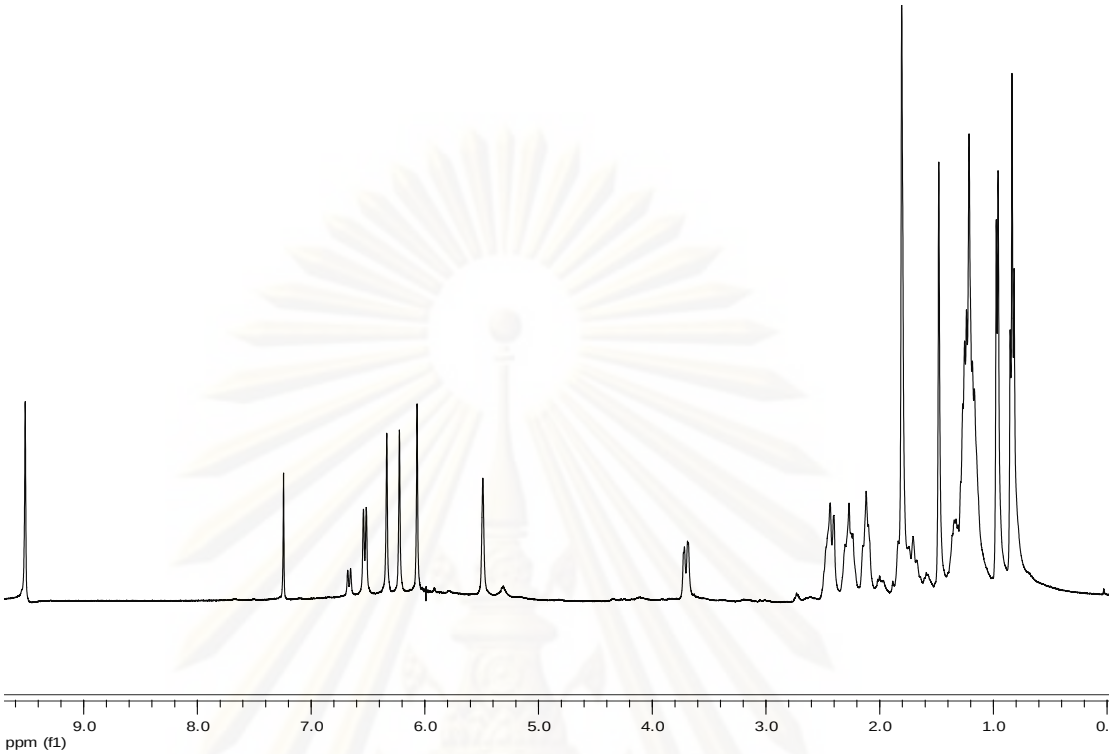
3.2.3 Selection of interesting secondary metabolite producing endophytic fungi from ^{1}H NMR spectroscopic analysis

A total of 20 crude extracts displaying cytotoxicity (**Table 3.3**) were further subjected to ^{1}H NMR experiment to examine their chemical constituents. Based on ^{1}H NMR spectral data, RB-20/2A, RB-20c and RL-K3 which were grown in malt extract broth (MEB), were selected to cultivate in large scale (10 L) for isolating cytotoxic compounds in the further step, due to the signals of various functionality inclulding aromatic (6-8 ppm), olefinic (5-6 ppm) and oxygenated (3-4 ppm) protons as shown in **Figure 3.3.**

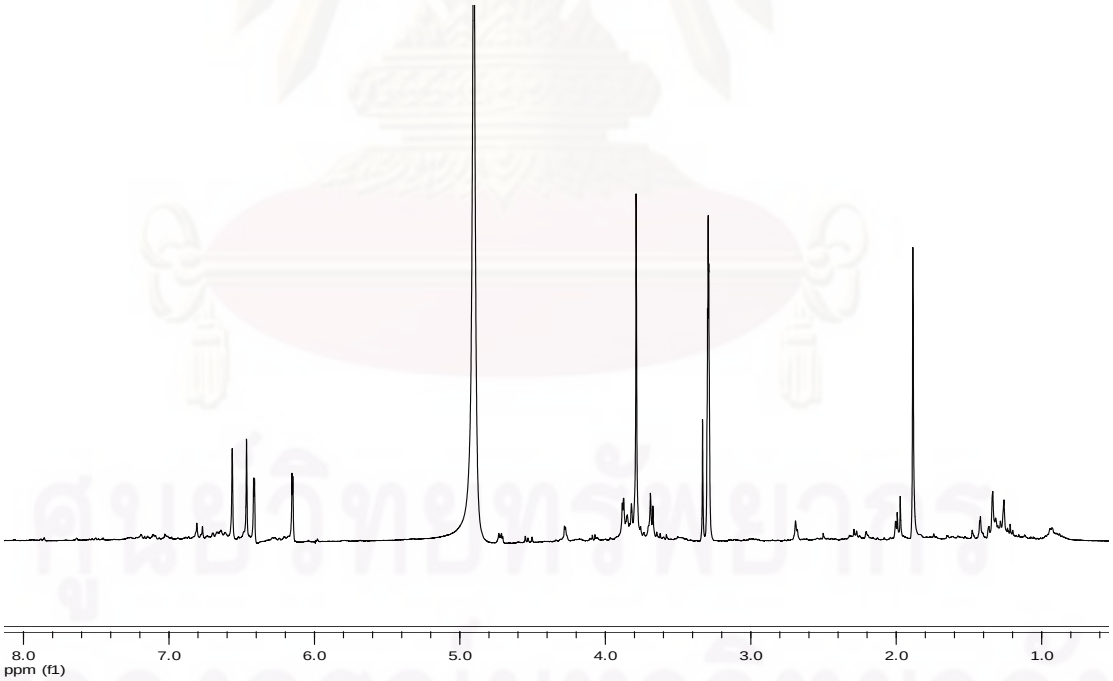


(a)

Figure 3.3 ^{1}H NMR spectrum of RB-20/2A (a), RB-20c (b), and RL-K3 (c)



(b)



(c)

Figure 3.3 ^{1}H NMR spectrum of RB-20/2A (a), RB-20c (b), and RL-K3 (c) (continued)

3.4 Extraction and isolation of secondary metabolites

After the large scale (10 L) cultivation of selected fungi in suitable medium, the culture broths were extracted according to the procedure in **Scheme 2.1.**

3.5 Structural elucidation of pure compounds

3.5.1 Compounds isolated from RL-K3 strain

3.5.1.1 Compound 1

Compound **1** was obtained as a yellow solid and its molecular formula was assigned to be $C_{16}H_{12}O_5$ by 1D NMR and HSQC data, which indicated 11 degrees of unsaturation. The ^{1}H NMR spectrum showed typical signals of two phenolic protons (δ_H 10.26 and 12.87), four aromatic protons (δ_H 6.57, 7.24, 7.57 and 7.91), a methoxy (δ_H 3.86) and a methyl (δ_H 2.27) groups. The ^{13}C NMR spectrum displayed 16 non-equivalent signals including two carbonyls (δ_C 181.5 and 187.1), three oxygenated aromatic carbons (δ_C 161.6, 164.9 and 166.0), nine aromatic carbons (δ_C 105.7, 107.2, 110.8, 111.6, 125.5, 130.5, 132.3, 133.4 and 135.4) and a methoxy (δ_C 55.9) group. These NMR data indicated that eight of the 11 units of unsaturation come from six carbon-carbon aromatic double bonds and two carbonyls. Therefore, the remaining three degrees required **1** to comprise a tricyclic core. The structure of **1** was mainly deduced from the analysis of observed HMBC correlations as shown in **Figure 3.4**. The singlet signal appearing at δ_H 12.87 was attributable to the chelated phenolic OH at C-5. These data suggested compound **1** is anthraquinone type compound as shown in **Figure 3.5.** Comparison of the ^{1}H and ^{13}C NMR data of **1** with those of published in the literature (**Table 3.4**), (Suemitsu *et al*., 1989a; Suemitsu *et al*., 1989b) confirmed that **1** is macrosporin. Macrosporin has been reported as a typical metabolite produced by several strains of *Alternaria* sp. such as *Alternaria porri, A. cucumerina, A. bataticola* and *A. solani* as well as *Stemphylium globuliferum* (Birgitte *et al.,* 2008). There were some reports that it showed cytotoxicity against L5178Y mouse lymphoma cells, but no antibacterial activity toward *Staphylococcus epidermidis, S. aureus, Enterococus faecalis, Escherichia coli* and *Pseudomonas aeruginosa* (MIC > 50 μg/mL) (Amal *et al.,* 2008a).

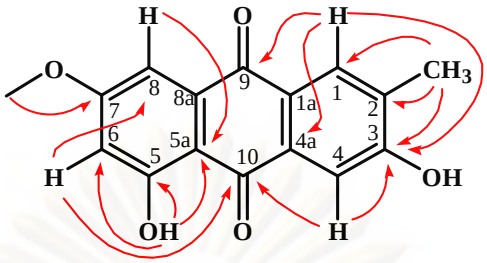


Figure 3.4 HMBC correlations for compound **1**.

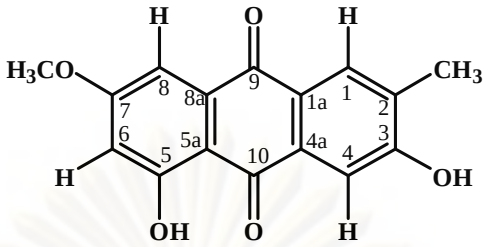


Figure 3.5 Structure of macrosporin (**1**)

Table 3.4 The ^{1}H and ^{13}C NMR spectral data of macrosporin (THF- d_8) and compound **1** in $CDCl_3$+DMSO-d_6.

position	**1**		**macrosporin**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	7.91 s	130.5, CH	7.95 s	130.8, CH
1a	-	125.5, qC	-	126.0, qC
2	-	132.3, qC	-	133.0, qC
3	-	161.6, qC	-	162.5, qC
4	7.57 s	111.6, CH	7.65 s	111.8, CH
4a	-	133.4, qC	-	134.1, qC
5	-	164.9, qC	-	165.8, qC
5a	-	110.8, qC	-	111.1, qC
6	6.57 d, (2.4)	105.7, CH	6.80 d, (2.5)	106.0, CH
7	-	166.0, qC	-	167.0, qC
8	7.24 d, (2.4)	107.2, CH	7.19 d, (2.5)	107.8, CH
8a	-	135.4, qC	-	136.0, qC
9	-	187.1, qC	-	187.6, qC
10	-	181.5, qC	-	181.2, qC
2-Me	2.27 s	16.4, CH_3	2.34 s	16.4, CH_3
7-OMe	3.86 s	55.9, CH_3	4.00 s	56.7, CH_3
3-OH	10.26 br s	-	-	-
5-OH	12.87 s	-	-	-

3.5.1.2 Compound 2

Compound **2** was obtained as yellowish powder and its molecular formula was assigned to be $C_{15}H_{12}O_5$ by 1D NMR and HSQC data, which indicated 10 degrees of unsaturation. The ^{1}H NMR spectrum of **2** indicated two phenolic protons (δ_H 9.22 and 11.84), four aromatic protons (δ_H 6.43, 6.57, 6.67 and 7.15), a methoxy (δ_H 3.84) and a methyl (δ_H 2.67) group. The ^{13}C NMR revealed the presence of an ester carbonyl (δ_C 165.2), four oxygenated aromatic carbons (δ_C 153.2, 158.5, 165.0 and 166.3), eight aromatic carbons (δ_C 99.0, 99.1, 101.8, 103.6, 110.0, 117.5, 138.2 and 138.8) and a methoxy (δ_C 55.3). These NMR data indicated that seven of the 10 elements of unsaturation come from six carbon-carbon aromatic double bonds and one carbonyl. Therefore, the molecule possesses a tricyclic skeleton. The structure of **2** was corroborated by the observed HMBC correlations as shown in **Figure 3.6**. Comparison of the ^{1}H and ^{13}C NMR data of **2** with those reported in the literature (**Table 3.5**), confirmed that **2** is alternariol monomethyl ether (Stinson *et* al., 1985; Wen, 2009). Alternariol monomethyl ether is one of the well-known metabolites produced by several species of the genus *Alternaria*, such as *A. altarnata*, *A. tenuissima* and *A. solani* (Stinson *et al.*, 1980) and it had been reported as chemotaxonomic markers for the classification of *Alternaria* species (Andersen, Dongo, and Pryor, 2008). In addition, it also showed to be mutagenic and cytotoxic to bacterial and mammalian cells (Patriarca *et al.*, 2007).

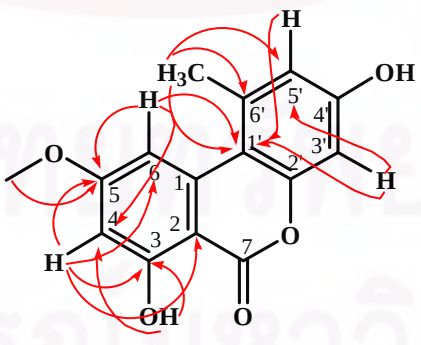


Figure 3.6 HMBC correlations for compound **2**.

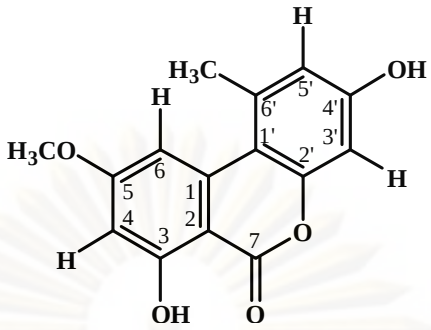


Figure 3.7 Structure of alternariol monomethyl ether (**2**)

Table 3.5 The ^{1}H and ^{13}C NMR spectral data of alternariol monomethyl ether (DMSO- d_6) and compound **2** in DMSO-d_6.

position	**2**		**alternariol monomethyl ether**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	-	138.8, qC	-	137.8, qC
2	-	99.1, qC	-	98.5, qC
3	-	165.0, qC	-	164.0, qC
4	6.43 d, (2.0)	99.0, CH	6.60 d, (2.3)	99.3, CH
5	-	166.0, qC	-	164.7, qC
6	7.15 d, (2.0)	103.6, CH	7.20 d, (2.3)	103.5, CH
7	-	165.2, qC	-	165.5, qC
1’	-	110.0, qC	-	108.8, qC
2’	-	153.2, qC	-	152.6, qC
3’	6.57 d, (2.0)	101.8, CH	6.71 d, (2.5)	101.6, CH
4’	-	158.5, qC	-	158.5, qC
5’	6.67 d, (2.4)	117.5, CH	6.64 d, (2.5)	117.7, CH
6’	-	138.2, qC	-	138.5, qC
4’-OH	9.22 s	-	10.36 s	-
6’-Me	2.67 s	24.7, CH_3	2.71 s	25.0, CH_3
5-OMe	3.84 s	55.3, CH_3	3.90 s	55.8, CH_3
3-OH	11.84 s	-	11.81 s	-

3.5.1.3 Compound 3

Compound **3** was isolated as yellowish powder and its molecular formula was assigned as $C_{14}H_{10}O_5$ on the basis of analysis of 1D NMR and HSQC data. The NMR data of **3** were virtually identical to those of **2**, except for the appearance of an additional phenolic proton at δ_H 10.36 and the absence of a methoxy signal in **2**. This indicated the methoxy group had been replaced by the hydroxyl group at C-5 (**Figure 3.7**). Further, the structure of **3** was confirmed by HMBC correlations as shown in **Figure 3.8**. By comparing the ^{1}H and ^{13}C NMR data of **3** with those of published in the literatures (**Table 3.6**), compound **3** is alternariol (Wen, 2009). Alternariol is also a major metabolite of *Alternaria* sp. (Andersen *et al.*, 2008). There were some reports that it showed inhibition of cell proliferation and clastogenicity of alternariol in Ishikawa and V79 cells *in vitro* (Lehmann, Wagner, and Metzler, 2006).

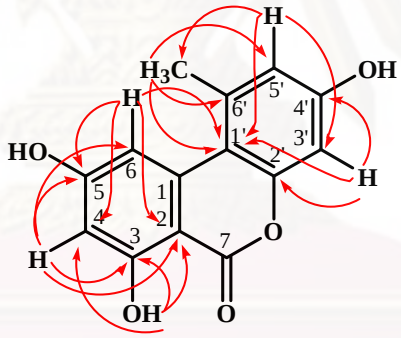


Figure 3.8 HMBC correlations for compound **3**.

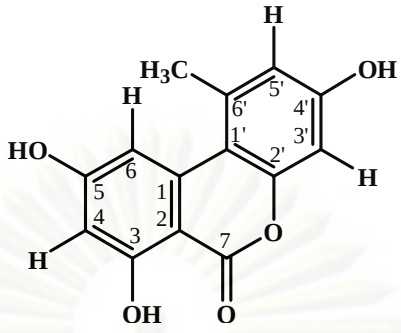


Figure 3.9 Structure of alternariol (**3**)

Table 3.6 The ^{1}H and ^{13}C NMR spectral data of alternariol (DMSO- d_6) and compound **3** in acetone-d_6

position	**3**		**alternariol**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	-	138.8, qC	-	138.6, qC
2	-	97.8, qC	-	97.7, qC
3	-	164.5, qC	-	164.9, qC
4	6.33 s	101.3, CH	6.36 d (1.5)	100.6, CH
5	-	165.1, qC	-	165.3, qC
6	7.21 s	104.7, CH	7.23 d (1.5)	101.3, CH
7	-	165.8, qC	-	165.5, qC
1’	-	109.4, qC	-	109.5, qC
2’	-	153.0, qC	-	153.1, qC
3’	6.60 s	102.0, CH	6.62 d (2.5)	104.2, CH
4’	-	158.8, qC	-	158.3, qC
5’	6.68 s	117.9, CH	6.70 d (2.4)	117.0, CH
6’	-	138.6, qC	-	138.4, qC
4’-OH	10.93 br s	-	10.91 s	-
6’-Me	2.67 s	25.6, CH_3	2.69 s	24.4, CH_3
5-OH	10.36 s	-	10.34 s	-
3-OH	11.73 s	-	11.76 s	-

3.5.2 Compounds isolated from RB-20c strain

3.5.2.1 Compound 4

Compound **4** was obtained as an off-white solid and its molecular formula was established to be $C_{25}H_{34}O_6$ by 1D NMR and HSQC data. The molecular formula suggested 9 degrees of unsaturation in the molecular structure of **4**. The ^{1}H NMR spectrum of **4** displayed distinct signals for methyl groups (δ_H 0.86, 0.99, 1.51 and 1.83), olefinic methane doublet of a quartet (δ_H 6.54), vinyl group singlets (δ_H 6.35 and 6.09) and an aldehyde group singlet (δ_H 9.53). The spin systems in ^{1}H NMR spectrum were assigned by 2D ^{1}H-^{1}H COSY correlation, which indicated only three contiguous fragments as shown in **Figure 3.10**. The ^{13}C NMR spectrum of **4** showed carbon signals for four methyls (δ_C 12.6, 14.1, 19.5 and 19.9), two olefinic methines (δ_C 129.6 and 149.7), an olefinic methylene (δ_C 136.6), six methylenes (δ_C 20.1, 22.7, 29.6, 29.7, 36.5 and 43.2), three aliphatic methines (δ_C 33.3, 43.0 and 53.2), an oxygenated methine (δ_C 72.6), three olefinic quaternaries (δ_C 125.8, 147.6 and 158.9), an aliphatic quaternary (δ_C 38.2), an aldehyde (δ_C 193.2), two carboxy type carbonyls (δ_C 166.8 and 177.6) and a deshielded carbonyl (δ_C 196.9). An HMBC experiment was used for establishing the connectivity between the COSY derived fragments with the remainder of the molecule (**Figure 3.10**). Comparison of the ^{1}H and ^{13}C NMR data of **4** with those of published in the literatures (**Table 3.7**) revealed the gross structure of **4** was assigned to integric acid. In addition, the NOESY data of **4** clarified the same relative stereochemistry as reported (Sheo *et al*., 1999) which is shown in **Figure 3.11**. Integric acid is a compound produced by the fungus *Xylaria* sp., such as *X. persicaria*. It was identified as an inhibitor of HIV-1 integrasa, the enzyme responsible for provirus entry into the host cell nucleus and integration in to the genome (Singh *et al*., 2000).

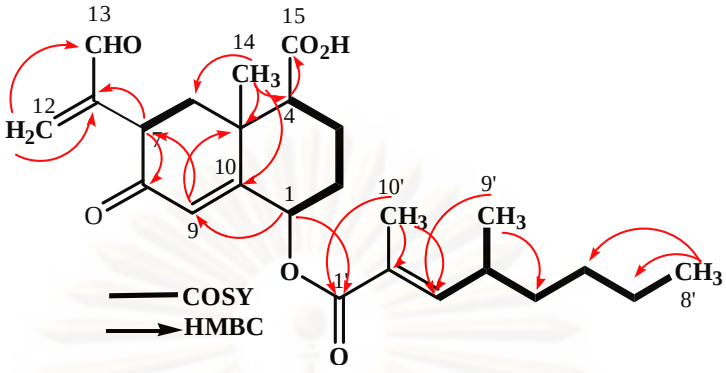


Figure 3.10 ^{1}H-^{1}H COSY and selected HMBC correlations of compound **4**.

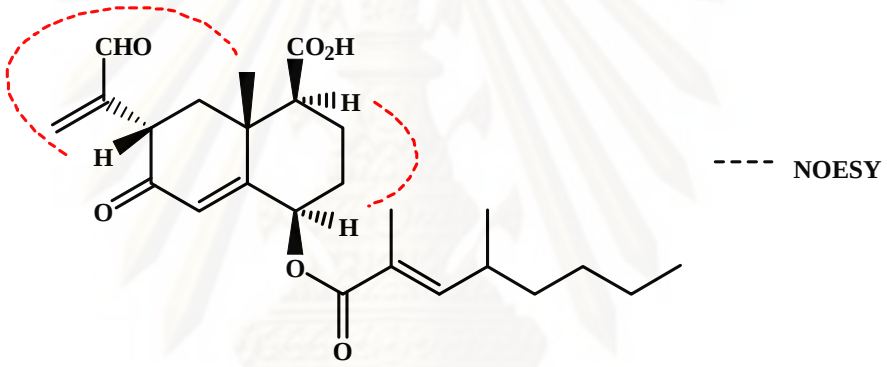


Figure 3.11 Selected NOESY correlations of compound **4** in $CDCl_3$.

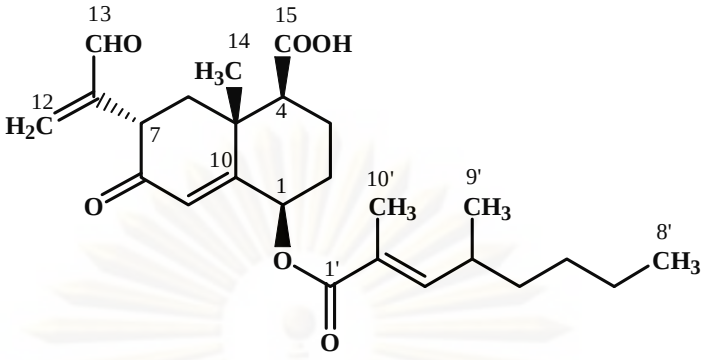


Figure 3.12 Structure of integric acid (**4**)

Table 3.7 The ^{1}H and ^{13}C NMR spectral data of integric acid ($CDCl_3$) and compound **4** in $CDCI_3$.

position	**4**		**integric acic**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	5.51 br s	72.6, CH	5.25 t, (2.8)	72.7, CH
2	2.16 m	29.7, CH_2	2.15 m	29.9, CH_2
	1.73 m	-	1.70 m	-
3	1.85 m	20.1, CH_2	1.90 m	20.1, CH_2
	2.30 m	-	2.30 m	-
4	2.45 dd, (12.8, 2.4)	53.2, CH	2.46 dd, (13.2, 3.2)	53.3, CH
5	-	38.2, qC	-	38.3, qC
6	2.13 dd, (13.2, 4.4)	43.2, CH_2	2.12 dd, (13.2, 4.4)	43.2, CH_2
	2.29 m	-	2.26 t, (13.6)	-
7	3.73 dd, (14.8, 4.4)	43.0, CH	3.73 dd, (14.8, 4.4)	43.1, CH
8	-	196.9, qC	-	196.9, qC
9	6.09 s	129.6, CH	6.10 s	129.6, CH
10	-	158.9, qC	-	158.9, qC
11	-	147.6, qC	-	147.7, qC
12	6.35 s	136.6, CH_2	6.35 s	136.5, CH_2
	6.24 s	-	6.25 s	-
13	-	193.2, qC	-	193.1, qC
14	1.51 s	19.5, CH_3	1.50 s	19.5, CH_3
15	-	177.6, qC	-	177.9, qC
1’	-	166.8, qC	-	166.8, qC
2’	-	125.8, qC	-	125.8, qC
3’	6.54 d, (10.0)	149.7, CH	6.54 dq, (10.0, 1.2)	149.7, CH
4’	2.50 m	33.3, CH	2.50 m	33.3, CH
5’	1.37 m	36.5, CH_2	1.30 m	36.5, CH_2
	1.29 m	-	1.40 m	-
6’	1.28 m	22.7, CH_2	1.30 m	22.8, CH_2
7’	1.19 m	29.6, CH_2	1.20 m	29.7, CH_2
8’	0.86 t, (7.2)	14.1, CH_3	0.87 t, (7.2)	14.1, CH_3
9’	0.99 d, (6.4)	19.9, CH_3	1.00 d, (6.6)	19.9, CH_3
10’	1.83 s	12.6, CH_3	1.80 d, (1.6)	12.7, CH_3

3.5.3 Compounds isolated from RB-20/2A strain

3.5.3.1 Compound 5

Compound **5** was isolated as white powder, while inspection of the 1D NMR and HSQC data suggested a molecular formula of $C_{30}H_{37}NO_6$. The ^{1}H NMR spectrum exhibited the presence of an amide group (δ_H 5.46), monosubstituted benzene ring (δ_H 7.06, 7.19 and 7.24), two oxygenated protons (δ_H 3.75 and 5.56), two olefinic protons belonging to an enone system (δ_H 5.08 and 6.05), two exomethylene protons (δ_H 5.02 and 5.23), two further olefinic protons (δ_H 5.62 and 5.28), two secondary methyls (δ_H 0.88 and 1.11) and a tertiary methyl group (δ_H 1.44) attached to an oxygenated carbon. The ^{13}C NMR and HSQC spectrum indicated the presence of 28 carbon signals including three carbonyls (δ_C 169.7, 173.7 and 210.3), six aromatic carbons (δ_C 127.1, 128.9, 129.1 and 137.2), six olefinic carbons (δ_C 114.5, 127.6, 130.6, 132.3, 134.1 and 147.5), two oxygenated carbons (δ_C 69.8 and 77.7), one quaternary carbon (δ_C 53.5), four methyls (δ_C 13.7, 19.4, 20.8 and 24.2), two methylenes (δ_C 37.7 and 45.3) and five methines (δ_C 32.7, 42.3, 47.0, 50.0 and 53.3) and an oxygenated methine (δ_C 77.1). The structure of **5** was corroborated by the observed ^{1}H-^{1}H COSY and HMBC correlations as shown in **Figure 3.13**. Comparison of the ^{1}H and ^{13}C NMR data of **2** with those reported in the literature (**Table 3.8**) comfirmed that **5** is cytochalasin D (Hong *et al.*, 2001; Liu *et al*., 2002). The relative configuration of **5** was assigned on the basis of 1D NOE data. The irradiation of H-23 methyl protons suggested that CH_3-23, H-16, H-5 and OH-7 were on the same face **Figure 3.14**. This information helped to exactly confirm **5** is cytochalasin D.

Cytochalasin D is one of the well-known metabolites produced by several species of the genus *Xylaria* sp., such as *X. hypoxylon, X. obovata, X. cubensis* as well as *Engleromyces goetzii* and *Hypocrella bambusae* (Hong *et al.*, 2001; Liu *et al*., 2002). There were some reports that it showed a marked inhibitory effect on murphy-sturm lymphosarcoma, and a slight affect on walker carcinosarcoma 256 in rats (Katagiri and Matsuura, 1971).

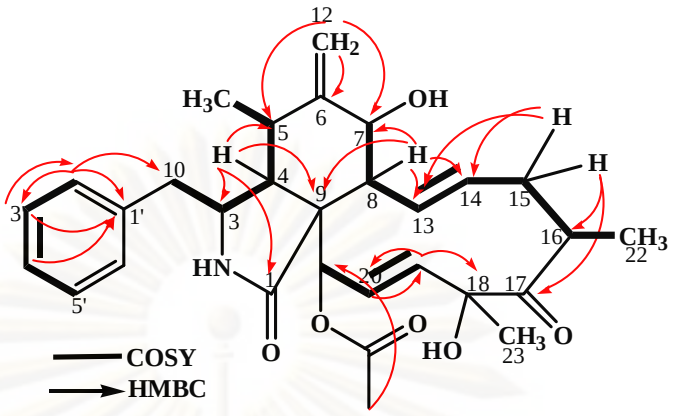


Figure 3.13 ^{1}H-^{1}H COSY and HMBC correlations for compound **5**.

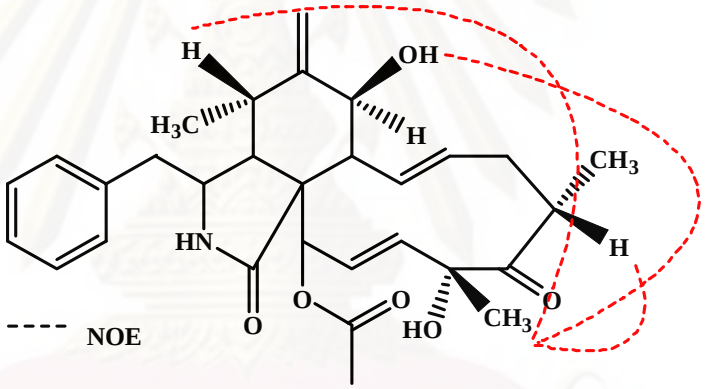


Figure 3.14 Selected NOE correlations of compound **5** in $CDCl_3$

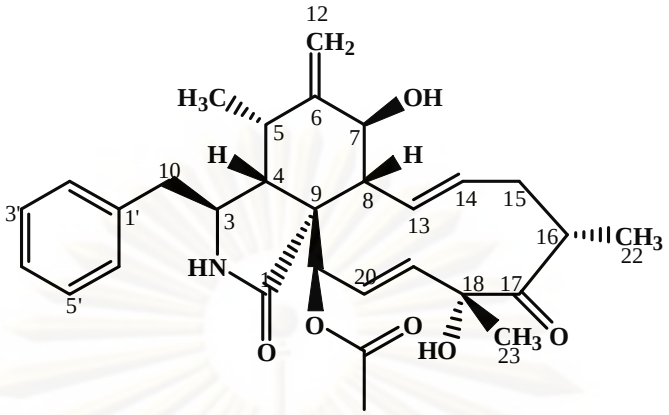


Figure 3.15 Structure of cytochalasin D (**5**)

Table 3.8 The ^{1}H and ^{13}C NMR spectral data of cytochalasin D ($CDCl_3$) and compound **5** in $CDCl_3$.

position	**5**		**cytochalasin D**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	-	173.7, qC	-	173.7, qC
2	5.46 s	-	5.53 br s	-
3	3.16 m	53.3, CH	3.22 m	53.5, CH
4	2.08 m	50.0, CH	2.84 m	46.9, CH
5	2.64 m	32.7, CH	2.14 m	49.9, CH
6	-	147.5, qC	-	147.6, qC
7	3.75 d, (10.8)	69.8, CH	3.80 d, (10.5)	69.8, CH
8	2.78 m	47.0, CH	2.83 m	32.64, CH
9	-	53.5, qC	-	53.2, qC
10	2.06 t, (4.4)	45.3, CH_2	2.65 dd, (13.5, 9.5)	45.3, CH_2
	2.75 m	-	2.83 m	-
11	0.88 d, (6.4)	13.7, CH_3	0.97 d, (7.0)	13.6, CH_3
12	5.02 s	114.5, CH_2	5.09 s	114.5, CH_2
	5.23 s	-	5.29 s	-
13	5.62 m	130.6, CH	5.35 m	134.1, CH
14	5.28 m	134.1, CH	5.65 dd, (15.5, 10.0)	130.6, CH
15	1.91 dd, (12.4, 4.4)	37.7, CH_2	2.02 dd, (13.0, 5.0)	37.7, CH_2
	2.44 m	-	2.51 dd, (13.5, 11.0)	-
16	2.68 m	42.3, CH	2.73 m	42.3, CH
17	-	210.3, qC	-	210.2, qC
18	-	77.7, qC	-	77.7, qC
19	5.08 m	127.6, CH	5.15 dd, (16.0, 2.5)	127.1, CH
20	6.05 dd, (15.2, 1.2)	132.3, CH	6.11 dd, (15.7, 2.5)	132.3, CH
21	5.56 m	77.1, CH	5.63 m	77.3, CH
22	1.11 d, (6.4)	19.4, CH_3	1.20 d, (7.0)	19.4, CH_3
23	1.44 s	24.2, CH_3	1.51 s	24.2, CH_3
1’	-	137.2, qC	-	137.2, qC
2’, 6’	7.06 m	129.1, CH	7.13 m	129.1, CH
3’, 5’	7.19 m	127.1, CH	7.26 m	128.9, CH
4’	7.24 m	128.9, CH	7.32 m	127.6, CH
7-OH	4.58 s	-	-	-
18-OH	-	-	-	-
O<u>C</u>OCH$_3$	-	169.7, qC	-	169.6, qC
OCO<u>C</u>H$_3$	2.19 s	20.8, CH_3	2.26 s	20.8, CH_3

3.5.3.2 Compound 6

Compound **6** was obtained as a white powder and its molecular formula $C_{30}H_{37}NO_6$ was established as the same that of **5** by 1D NMR and HSQC data. The NMR data of **6** similar to those of **5,** except for the presence of an additional methyl group (δ_H 1.39 ; δ_C 17.2) in place of the absence of the exomethylene in **5** (δ_H 5.02 and 5.23). This was confirmed by HMBC correlations from CH_3-12 to C-5, C-6 and C-7 (**Figure 3.15**). Therefore, the whole structure of **6** was established by ^{1}H-^{1}H COSY and HMBC as shown in **Figure 3.16**. On the basis of these findings, and comparing its NMR data with those published in the literature strongly suggested that **6** is cytochalasin C (Raymond and Derek, 1989). Such compound was found to be originated from xylariaceous such as *Xylaria mellisii*, *Hypoxylon terricola*, *Halorosellinia oceanica*, *Engleromyces goetzei* (Raymond and Derek, 1989; Jiang 2000; Zha-Jin *et al*., 2003; Pattama *et al*., 2005). Cytochalasin C acts as a potent inhibitor of actin filament and contractile microfilaments (Walling *et al*., 1988).

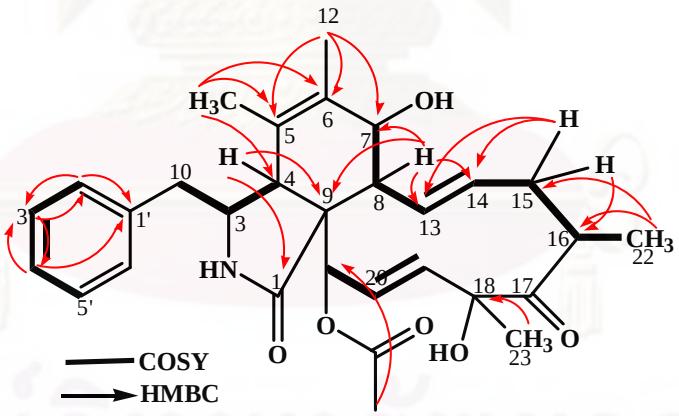


Figure 3.16 ^{1}H-^{1}H COSY and HMBC correlations for compound **6**.

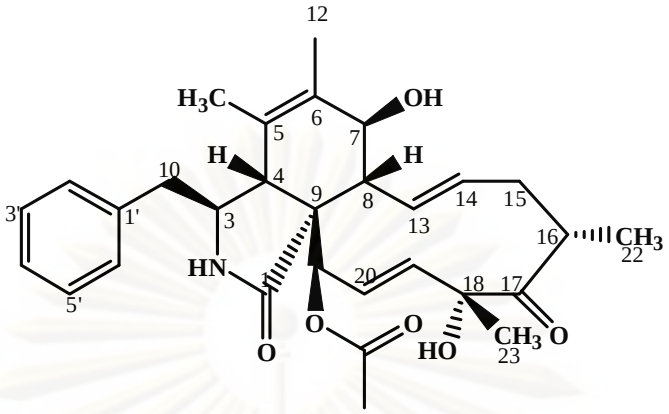


Figure 3.17 Structure of cytochalasin C (**6**)

Table 3.9 The ^{1}H and ^{13}C NMR spectral data of cytochalasin C ($CDCl_3$) and compound **6** in $CDCl_3$.

position	**6**		**cytochalasin C**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	-	174.3, qC	-	175.7, qC
2	-	-	9.41 s	-
3	3.27 t, (7.6)	60.1, CH	3.69 ddt, (7.5, 7.5, 1.5)	61.1, CH
4	2.44 d, (12.0)	50.1, CH	2.88 d, (12.5)	50.7, CH
5	-	126.3, qC	-	126.7, qC
6	-	131.6, qC	-	134.1, qC
7	3.73 d, (9.6)	67.9, CH	4.40-4.51 m	69.1, CH
8	2.37 m	49.6, CH	3.09 dd, (10.0, 10.0)	50.7, CH
9	-	52.4, qC	-	53.8, qC
10	2.90 m	44.6, CH_2	3.16 dd, (13.0, 7.5)	45.4, CH_2
	-	-	3.22 dd, (13.0, 7.5)	-
11	1.39 s	17.2, CH_3	1.95 s	17.3, CH_3
12	1.62 s	13.9, CH_3	1.41 s	14.9, CH_3
13	5.80 t, (10.8)	131.2, CH	6.59 ddd, (15.4, 10.0, 1.0)	132.9, CH
14	5.28 m	134.1, CH	5.54 ddd, (15.4, 10.0, 5.5)	133.5, CH
15	1.92 m	37.6, CH_2	1.93-1.96 m	38.7, CH_2
	-	-	2.60-2.75 m	-
16	2.67 m	42.3, CH	2.60-2.75 m	42.5, CH
17	-	210.0, qC	-	210.9, qC
18	-	77.8, qC	-	78.6, qC
19	5.10 dd, (15.6, 2.0)	128.1, CH	5.66 dd, (15.5, 2.5)	127.0, CH
20	5.96 dd, (15.6, 2.4)	131.9, CH	6.84 dd, (15.5, 2.5)	132.8, CH
21	5.80 t, (10.8)	75.0, CH	6.41 dd, (2.5, 2.5)	76.4, CH
22	1.15 d, (6.8)	19.4, CH_3	1.05 d, (6.3)	19.5, CH_3
23	1.44 s	24.2, CH_3	1.58 s	24.8, CH_3
1’	-	137.5, qC	-	138.9, qC
2’, 6’	7.24 m	128.9, CH	7.22-7.34 m	130.0, CH
3’, 5’	7.11 m	129.0, CH	7.22-7.34 m	129.1, CH
4’	7.19 m	127.0, CH	7.22-7.34 m	128.6, CH
7-OH	-	-	5.79 d, (5.5)	-
18-OH	-	-	6.02 s	-
$O\underline{C}OCH_3$	-	169.9, qC	-	170.9, qC
$OCO\underline{C}H_3$	2.20 s	20.9, CH_3	2.38 s	20.7, CH_3

3.5.3.3 Compound 7

Compound **7** was also obtained as an amorphous solid and had the molecular formula $C_{30}H_{37}NO_6$ equivalent to that of **5** and **6**, which was determined by 1D NMR and HSQC data. Their formula indicated that they had the same 13 degrees of unsaturation, but compound **7** had one carbon-carbon double less than compound **5** and **6**. Therefore, this required **7** to have one more ring in the molecule relative to **5** and **6**. From its ^{1}H and ^{13}C NMR data, it was clear that compound **7** is a very close derivative of **6** with the difference only being the appearance of an additional oxygenated quaternary carbon (δ_C 57.1, C-6) and a methine group (δ_H 1.62 m, δ_C 36.8, C-5), and the absence of carbon-carbon double in **6**. This strongly suggested that there is an epoxide ring located at C-6 and C-7 in the molecule of **7**. In addition, the structure of **7** was completely corroborated by the analysis of ^{1}H-^{1}H COSY and HMBC data as shown in **Figure 3.18**. Comparison of its NMR data with those published in the literatures (**Table 3.10**) revealed that **7** is cytochalasin Q (Richard, Milbra, and Bruce, 2003).

Cytochalasin Q is one of the well-known metabolites produced by several species of the genus xylariaceous (Raymond and Derek, 1989). There were some reports that it showed cytotoxicity against KB and BC-1 cell lines, and moderate antimalarial activity. While compound was inactive on antimycobacteria (Maneekarn *et al*., 2001).

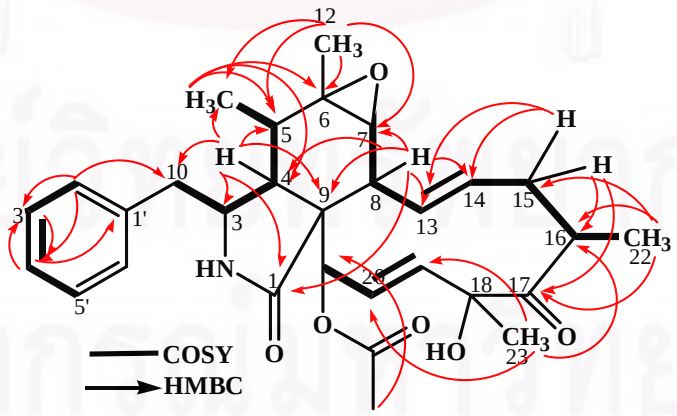


Figure 3.18 ^{1}H-^{1}H COSY and selected HMBC correlations of compound **7**.

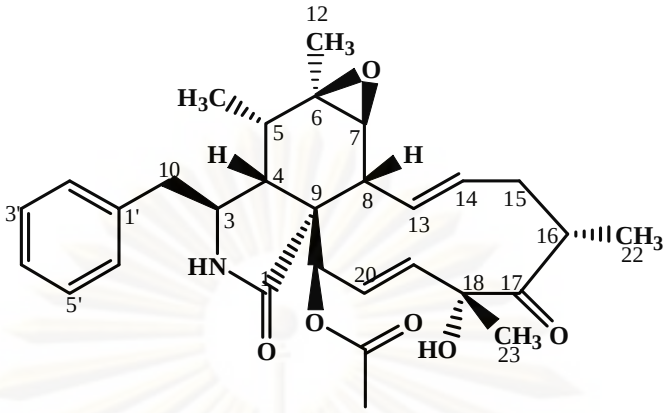


Figure 3.19 Structure of cytochalasin Q (**7**)

Table 3.10 The ^{1}H and ^{13}C NMR spectral data of cytochalasin Q (C_5D_5N) and compound **7** in $CDCl_3$.

position	**7**		**cytochalasin Q**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	-	174.6, qC	-	175.5, qC
2	-	-	9.50 s	-
3	3.50 t, (6.8)	54.1, CH	3.92 dd, (6.6, 7.9)	54.5, CH
4	2.03 dd, (5.6, 2.4)	50.5, CH	2.45 dd, (5.7, 1.8)	46.0, CH
5	1.62 m	36.8, CH	1.90-2.05 dq, (7.3, 5.7)	50.5, CH
6	-	57.1, qC	-	56.1, qC
7	2.64 d, (6.0)	62.5, CH	3.06 d, (5.8)	63.2, CH
8	2.45 m	45.1, CH	2.97 dd, (9.9, 5.7)	37.4, CH
9	-	55.1, qC	-	*
10	2.71 m	45.8, CH_2	2.84 dd, (13.2, 7.9)	46.2, CH_2
	2.75 m	-	3.09 dd, (13.2, 6.6)	-
11	0.77 d, (7.6)	12.5, CH_3	0.74 d, (7.3)	12.9, CH_3
12	1.10 s	19.6, CH_3	1.21 s	19.6, CH_3
13	5.78 m	130.8, CH	6.46 dd, (15.6, 10.1)	133.0, CH
14	5.18 m	131.7, CH	5.46 ddd, (15.6, 10.4, 5.2)	131.9, CH
15	1.92 dd, (12.8, 4.8)	37.9, CH_2	1.90-2.05 m	38.3, CH_2
	2.40 m	-	2.64-2.77 m	-
16	2.66 m	42.2, CH	2.64-2.77 m	42.5, CH
17	-	210.5, qC	-	210.8, qC
18	-	77.8, qC	-	78.2, qC
19	5.03 dd, (16.0, 2.4)	127.6, CH	5.62 dd, (15.8, 2.4)	127.1, CH
20	6.00 dd, (16.0, 2.8)	131.8, CH	6.85 dd, (15.8, 2.4)	132.1, CH
21	5.71 s	75.8, CH	6.24 dd, (2.2, 2.4)	76.6, CH
22	1.10 d, (6.8)	19.3, CH_3	1.07 d, (6.2)	19.4, CH_3
23	1.42 s	24.2, CH_3	1.57 s	24.7, CH_3
1’	-	136.9, qC	-	138.3, qC
2’, 6’	7.09 d, (7.2)	129.2, CH	7.20-7.40 m	130.1, CH
3’, 5’	7.26 m	128.9, CH	7.20-7.40 m	129.0, CH
4’	7.19 m	127.1, CH	7.20-7.40 m	128.6, CH
7-OH	-	-	-	-
18-OH	-	-	-	-
$OC\underline{O}CH_3$	-	169.8, qC	-	170.7, qC
$OCOC\underline{H}_3$	2.19 s	20.8, CH_3	2.38 s	20.6, CH_3

* not reported in the literature.

3.5.3.4 Compound 8

Compound **8** was isolated as white powder and its molecular formula was assigned as $C_{14}H_{16}N_2O_3$ on the basis of analysis of 1D NMR and HSQC data. The ^{1}H NMR spectrum of **8** exhibited signals for four aromatic protons as two doublets (δ_H 6.72 and 6.99), this indicated the presence of a *para*-substituted benzene ring., two methine protons (δ_H 4.02 and 4.15) and a phenolic proton (δ_H 6.45). The ^{13}C NMR spectrum contain signals from two quaternary aromatic carbon atoms one of which (δ_C 155.5) was bound to the hydroxyl group, two methines (δ_C 56.2 and 59.1), four methylenes (δ_C 22.5, 28.3, 35.9 and 45.4) and the peptide nature of the compound is indicated by the presence of signals from two carboxamide carbons (δ_C 169.7 and 165.1). On the basis of NMR data, it was proved that **8** is a cyclo(D-tyrosyl-D-proline). The structure of **8** was mainly deduced from the analysis of observed HMBC correlations as shown in **Figure 3.20**. Comparison of the ^{1}H and ^{13}C NMR data of **8** with those reported in the literature (**Table 3.11**), confirmed that **8** is cyclo(D-tyrosyl-D-proline) (Sobolevskaya *et al*., 2008). This compound was described as the metabolite of the marine bacterium *Pseudomonas aerugonosa* (Jayatilake *et al*., 1996).

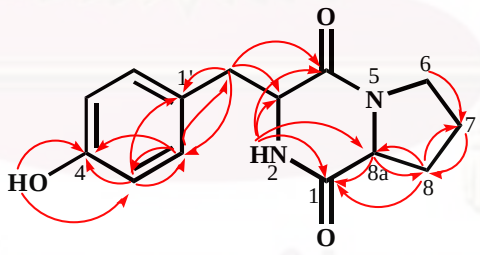


Figure 3.20 HMBC correlations for compound **8**.

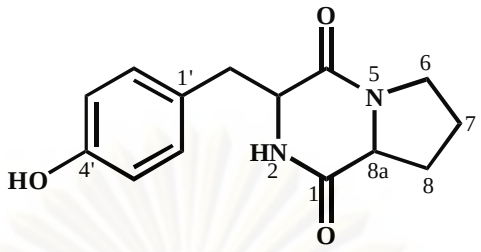


Figure 3.21 Structure of cyclo(D-tyrosyl-D-proline) (**8**)

Table 3.11 The ^{1}H and ^{13}C NMR spectral data of cyclo(D-tyrosyl-D-proline) (CD_3OD) and compound **8** in $CDCl_3$.

position	**8**		**cyclo(D-tyrosyl-D-proline)**	
	δ_H mult, (*J* in Hz)	δ_C	δ_H mult, (*J* in Hz)	δ_C
1	-	169.7, qC	-	172.0, qC
2	-	-	-	-
3	4.15 td	56.2, CH	4.13 td	59.8, CH
4	-	165.1, qC	-	168.2, qC
5	-	-	-	-
6	3.53 m	45.4, CH_2	3.28 m	46.7, CH_2
	-	-	3.32 m	-
7	1.86 m	22.5, CH_2	1.64 m	23.0, CH_2
	1.98 m	-		-
8	1.93 m	28.3, CH_2	1.90 m	30.4, CH_2
	2.28 m	-	2.05 m	-
8a	4.02 dd, (10.4, 2.8)	59.1, CH	3.53 dd, (-)	60.6, CH
1’	-	127.0, qC	-	127.7, qC
2’	6.99 d, (8.8)	130.3, CH	6.97 d, (8.5)	132.9, CH
3’	6.72 d, (8.4)	116.1, CH	6.71 d, (8.5)	117.0, CH
4’	-	155.5, qC	-	158.8, qC
5’	6.72 d, (8.4)	116.1, CH	6.71 d, (8.5)	117.0, CH
6’	6.99 d, (8.8)	130.3, CH	6.97 d, (8.5)	132.9, CH
3-CH_2	2.68 m	35.9, CH_2	3.28 m	40.8, CH_2
	3.43 m	-	3.32 m	-
4-OH	6.45 s	-	-	-

3.6 Cytotoxic activities of pure compound

Pure isolated compounds were evaluated for their cytotoxic effects against five cancer cell lines: hepato carcinoma (Hep-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), gastric carcinoma (KATO-3) and breast carcinoma (BT-474) by MTT colorimetric method. Results are presented in **Table 3.12**. A caused no cytotoxic effect even at 20 μM (Ichiji *et al*. 2000). Macrosporin (**1**) showed selectively cytotoxic activity against BT-474 and KATO-3 cell lines with IC_{50} values of 1.69 and 8.69 μM, respectively. Which strong activity on KATO-3 when compared with doxorubicin. While alternariol monomethyl ether (**2**) displayed the most potent activity on SW-620 and KATO-3 cell lines (IC_{50}: 3.01 and 2.17 μM, respectively) and weak activity on BT-474 with IC_{50} 12.19. Alternariol (**3**) showed selectively activity against SW-620 and CHAGO (IC_{50}: 9.33 and 18.67 μM, respectively). Also integric acid (**4**) was moderately active to all cell lines tested. Cytochalasin Q (**7**) and D (**5**) gave the similar results, they showed to be cytotoxic against only Hep-G2, KATO-3 and BT-474 cell lines. However, cytochalasin D was stronger than cytochalasin Q with all tested cell lines especially against Hep-G2, cytochalasin Q gave very weak cytotoxicity. While cytochalasin D strong activity on KATO-3.

Table 3.12 Cytotoxic activity of pure compounds on Hep-G2, SW-620, CHAGO, KATO-3 and BT-474 cell lines.

Cell lines / **Pure compounds**	**IC_{50} (μM)**				
	Hep-G2	SW-620	CHAGO	KATO-3	BT-474
Macrosporin (**1**)	>20	>20	>20	8.69	1.69
Alternariol monomethyl ether (**2**)	>20	3.01	>20	2.17	12.19
Alternariol (**3**)	>20	9.33	18.67	>20	>20
Integric acid (**4**)	5.64	6.64	5.74	4.72	4.83
Cytochalasin D (**5**)	9.19	>20	>20	1.24	4.89
Cytochalasin C (**6**)	ND	ND	ND	ND	ND
Cytochalasin Q (**7**)	17.18	>20	>20	7.49	9.10
Cyclo(D-tyrosyl-D-proline) (**8**)	ND	ND	ND	ND	ND
Doxorubicin (positive)	0.91	0.81	0.14	0.98	0.91

ND = not determined

Although all isolated compounds are the known compounds and the cytotoxicity of some compounds, for example, macrosporin, alternariol monomethyl ether, alternariol, cytochalasin D, have already been reported, their toxicities evaluated in the present study was performed with the different cell lines reported.

- Macrosporin was reported not cytotoxic against HeLa, KB (Preecha *et al*., 2009), L5178Y (Abdessamad *et al*., 2009; Amal *et al*., 2010), SW1116 and K562 (Ge *et al*., 2005). Thus, this research is the first report that macrosporin shows cytotoxic against BT-474 and KATO-3 cell lines.

- Alternariol monomethyl ether were shown to be substantially cytotoxic against SW1116 cells with IC_{50} values of 8.5 μg/mL. (Hua, Yong, and Ren, 2006). Which SW1116 cells is colon carcinoma same SW-620 cells in this research, both of them give the similar results. And all alternariol derivatives exhibited activity toward the L5178Y cells. The most active compound detected was alternariol, with an EC_{50} value of 1.7 μg/mL (Amal *et al*., 2008b).

- Cytochalasin D exhibited potent cytotoxicity against Vero, HeLa, NIH 3T3 and CHO cell lines with an IC_{50} value of 0.190, 0.204, 1.011 and 1.084 μM, respectively. Furthermore, cytochalasin D displayed higher toxicity than the standard drug, ellipticine. (Wipapan *et al*., 2007). Thus, can summarize that cytochalasin D showed strongly cytotoxic activity but not selectively same this research.

3.7 Identification and classification of the endophytic fungi isolated

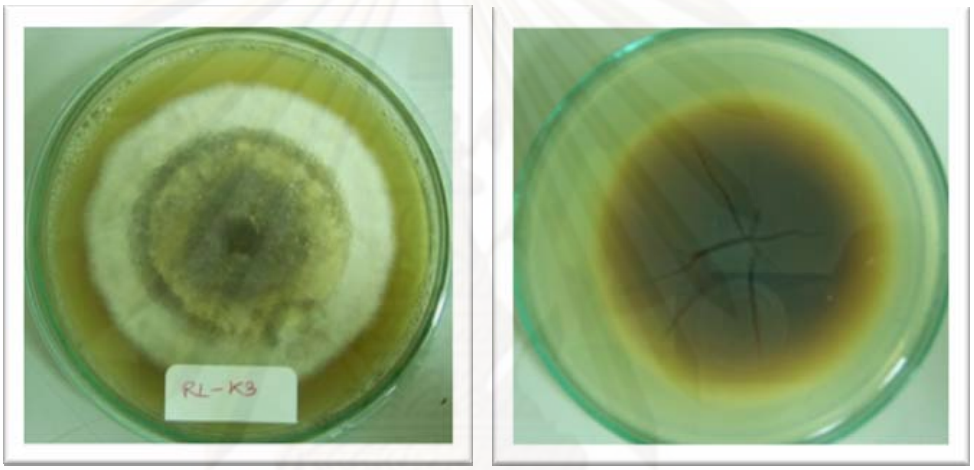
3.7.1 Identification and classification of RL-K3

3.7.1.1 Macroscopic characteristic of RL-K3

The endophytic fungus isolate RL-K3 that grown on potato dextrose agar (PDA) showed flat, downy to woolly and eventually covered to grayish. The grayish colony was short, aerial hyphae in time. The surface was grayish white at the beginning which later darkens and becomes greenish black. The reverse side was typically brown to black due to pigment production (yellow), as shown in **Figure 3.22**.

3.7.1.2 Microscopic characteristic of RL-K3

The endophytic fungus isolate RL-K3 that grown on water agar with small pieces of banana leafs, a condition suggested for promotion fruiting body production (Smith and Onions, 1990), it produced acropetal chains of yellowish brown to dark brown conidia with 3-7 transverse and had a few longitudinal septa in addition to cross septa., as shown in **Figure 3.23**. Conidia were narrow claviform to moniliform with a slightly swallen tip. These structures corresponded with the typical *Alternaria* morphology (Ellis, 1971).



Obverse Reverse

Figure 3.22 Colony morphology of endophytic fungus isolate RL-K3 (7 days) on potato dextrose agar (PDA)

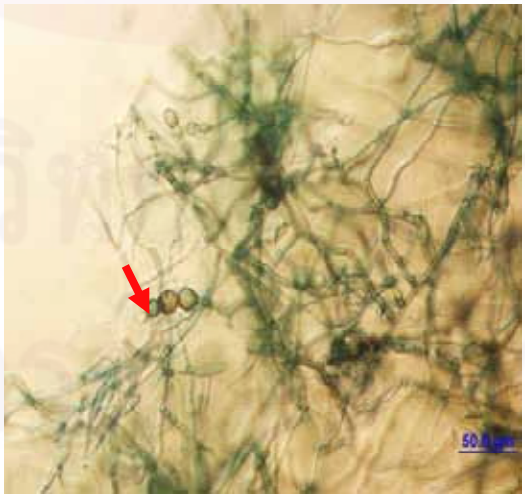


Figure 3.23 Conidia dark (arrow) of endophytic fungus isolate RL-K3 (Bar size = 50 µm)

3.7.1.3 The genus *Alternaria*: Biological diversity

Many species of the genus *Alternaria* are widely distributed in the soil as normal components of its microflora, and they also occur ubiquitously in the air, as a most important part of the ‘‘air spora’’ worldwide.

The taxonomy of *Alternaria* has been extensively discussed. The genus comprises more than 100 species, and there is much nomenclature confusion. Conidial size and shape have been the major criteria used for the identification of Alternaria species, but the importance of the three-dimensional sporulation pattern has also been emphasized. In addition to the traditional morphological and physiological characteristics, it was suggested that the profile of mycotoxins and other metabolites produced by *Alternaria* would offer advantages in the identification of *Alternaria* species and species-groups.

3.7.1.4 Chemistry of the *Alternaria*

Different species of *Alternaria* produce different compounds and most of the known compounds or secondary metabolites have been reported from fungi ideated as *A. alternata* (King and Schade, 1984). *A. alternata* (syn. *A. tenuis*) has been reported to produce altenuene, alternariol, alternariol monomethyl ether, altertoxin I, tentoxin and tenuazonic acid (Bottalico and Logrieco, 1998), while *A. gaisen* (syn. *A. kikuchiana*, Simmons, 1993) coude produce 3,6,8-trihydroxy-3-methyl-3,4-dihydroisocoumarin, alternariol, alternariol monomethyl ether, tentoxin and tenuazonic acid (Kameda *et al*., 1973, Montemurro and Visconti, 1992, Bottalico and Logrieco, 1998). *A. longipes* has only been reported to produce alternariol monomethyl ether, tenuazonic acid and tentoxin.

3.7.2 Identification and classification of RB-20c and RB-20/2A

3.7.2.1 Macroscopic characteristic of RB-20c and RB-20/2A

The endophytic fungus isolate RB-B20c that grown on potato dextrose agar (PDA). Colony growth slowed, at first white, then appearing in areas which have become underliain with black mycelial aggregations, with surface lannose to felty and edge was curly as shown in **Figure 3.24**. While, isolate RB-20/2A showed flat and white colony **(Figure 3.25**).

Figure 3.24 Colony morphology of endophytic fungus isolate RB-20c (14 days) on potato dextrose agar (PDA)

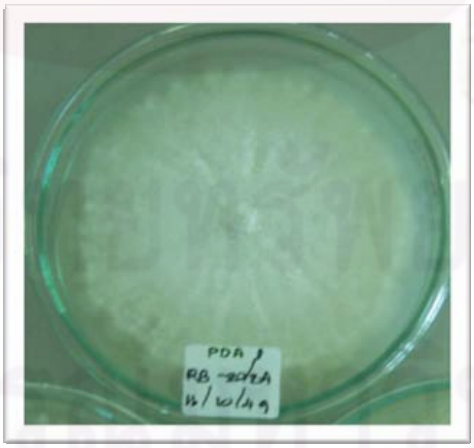


Figure 3.25 Colony morphology of endophytic fungus isolate RB-20/2A (10 days) on potato dextrose agar (PDA)

3.7.2.2 Microscopic characteristic of RB- 20c and RB-20/2A

The endophytic fungus isolate RB-20c that grown in potato dextrose broth (PDB) it produced stomata. Neither upright stromata nor conidia produced, its character resembles the coral as shown in **Figure 3.26**. And isolate RB-20/2A was also but the stromata was character resembles to antler (**Figure 3.27**). The stromata is particularities of *Xylaria* sp.

Figure 3.26 Stromata (circle) of endophytic fungus isolate RB-20c in potato dextrose broth (PDB)

Figure 3.27 Stromata (circle) of endophytic fungus isolate RB-20/2A in potato dextrose broth (PDB)

3.7.2.3 The Xylariaceae: Biological diversity

The Xylariaceae (Xylariales, Ascomycotina) is a large family comprising of around 40 genera and although it has representatives in most countries of the world the Xylariaceae exhibits its greatest diversity in the tropics. As a result of comparatively recent in depth taxonomic studies, including biochemical, cultural and chemical approaches, there is now a reasonable understanding of species boundaries and intergeneric relationships within the family.

For survival and propagation, many xylariaceous endophytes are thought to colonize the living plants opportunistically, decomposing the cellulose and lignin for fuel after the plant dies. There is, however, evidence that some Xylariaceae may inhabit plants solely as endophytes of living plants and decrease in number as the plant begins to decay. There has been no reported benefit to the host in relationship with *Xylaria* endophytes.

3.7.2.4 Chemistry of the Xylariaceae

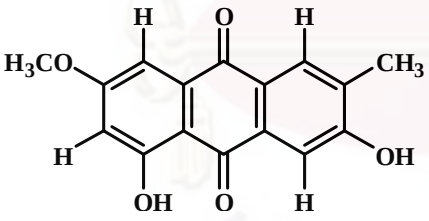
The chemistry of the xylariaceae has been studied in much detail relative to other fungal families. Roughly 30 years ago, 4,9-dihydroxypeylene,1,8-dimethoxynaphthalene, rosellnic acid, and diketeopiperazine were discovered in species. *Rosellinia necatrix* was later found to produce cytochalasin E. To date, the major metabolites discovered (which have been found in roughly a dozen genera) can be grouped as dihydroisocoumarins and derivatives, succinic acid and derivatives, butyrolactones, cytochalasins, sesquiterpene alcohols (punctaporonins), griseofulvin and griseofulvin and griseofulvin derivatives, naphthalene derivatives, and long chain fatty acids. The study of metabolites has proved to be invaluable in sorting out the taxonomy of this family.

CHAPTER IV

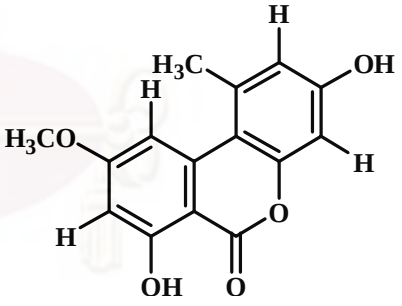
CONCLUSION

The objective of this thesis was the investigation of secondary metabolites produced by endophytic fungi isolated from *Brucea javanica* (L.) Merr. and the study on their anticancer activities against five human cancer cell lines inclulding hepato carcinoma (Hep-G2), colon carcinoma (SW-620), lung carcinoma (CHAGO), gastric carcinoma (KATO-3) and breast carcinoma (BT-474) cell lines.

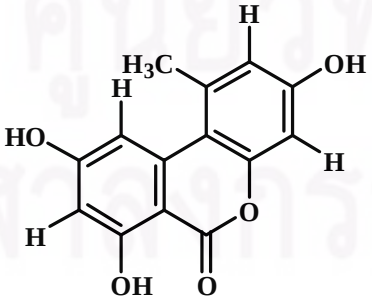
In the present investigation, a total of 34 pure isolates of endophytic fungi were obtained from leaves and barks of *B. javanica.* Based on the results of cytotoxic activities and ^{1}H NMR spectral data, RB-20/2A, RB-20c and RL-K3 which were grown in malt extract broth (MEB), were selected to cultivate in large scale (10 L) for isolating cytotoxic compounds. The fractionation of their EtOAc crude extracts led to the isolation of eight known compounds and the structures of them are represented as follows.



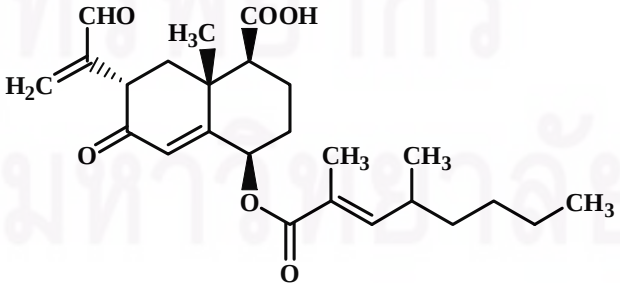
Macrosporin (**1**)



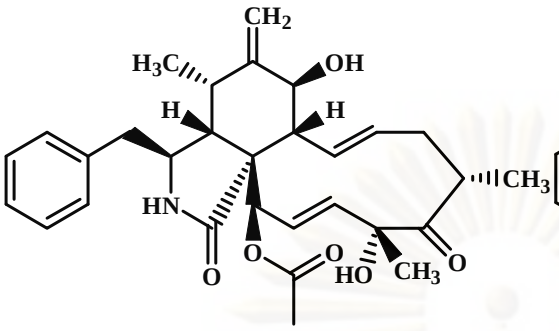
Alternariol monomethyl ether (**2**)



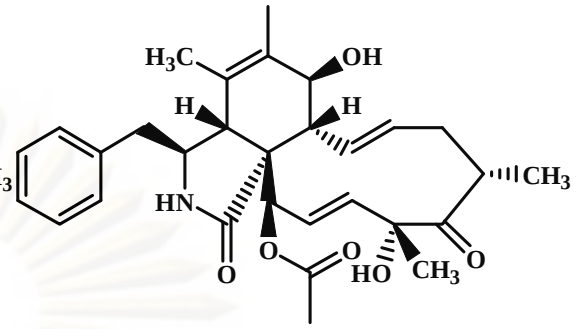
Alternariol (**3**)



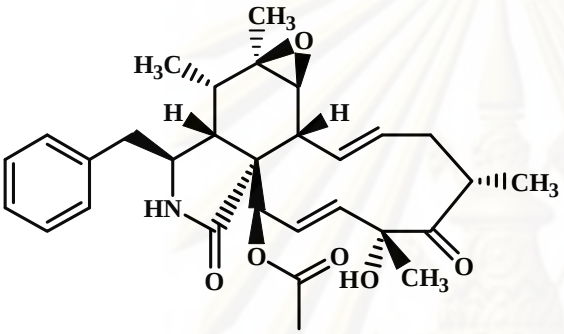
Integric acid (**4**)



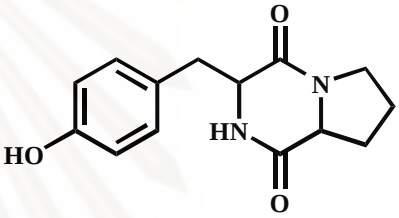
Cytochalasin D (**5**)



Cytochalasin C **(6)**



Cytochalasin Q **(7)**



Cyclo(D-tyrosyl-D-proline) (**8**)

Pure isolated compounds were evaluated for their IC_{50} values for each cell lines. Macrosporin (**1**) showed strongly and selectively cytotoxic activity against BT-474 and KATO-3 cell lines with IC_{50} values of 1.69 and 8.69 μM, respectively. Alternariol monomethyl ether (**2**) displayed toxicity on SW-620, KATO-3 and BT-474 cell lines (IC_{50}: 3.01, 2.17 and 12.19 μM, respectively), while alternariol (**3**) showed selectively activity against SW-620 and CHAGO (IC_{50}: 9.33 and 18.67 μM, respectively). Integric acid (**4**) was active to all cell lines tested. Cytochalasins Q (**7**) and D (**5**) gave the similar results and they showed to be cytotoxic against only Hep-G2, KATO-3 and BT-474 cell lines.

The selected fungi were identified by macroscopic and microscopic characteristics, endophytic fungal isolate RL-K3 was identified as *Alternaria* sp., whereas isolates RB-20/2A and RB-20c were identified as *Xylaria* sp.

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APPENDICES

APPENDIX A
MEDIA

The media were sterilized by autoclaving at 121 ^{o}C, 15 lb/ in^{2} for 15 minutes

1. Malt czapek broth (MCzB)

Czapek stock solution A	50.0	mL
Czapek stock solution B	50.0	mL
Malt extracts	40.0	g
Sucrose	30.0	g
Distilled water up to	1000	mL

Czapex stock solution A

$NaNO_3$	4.0	g
KCl	1.0	g
$MgSO_4.7H_2O$	10.0	g
$FeSO_4.7H_2O$	0.02	g
Distilled water up to	1000	mL

Keep in a refrigerator

Czapex stock solution B

K_2HPO_4	20.0	g
Distilled water up to	1000	mL

Keep in a refrigerator

2. Malt extract broth (MEB)

Malt extracts	20.0	g
Dextrose	20.0	g
Peptone	1.0	g
Distilled water up to	1000	mL

3. Sabouraud's dextrose broth (SDB)

Dextrose	40.0	g
Peptone	10.0	g
Distilled water up to	1000	mL

4. Glucose yeast extract broth (GYB)

Yeast extracts	3.0	g
Glucose (dextrose)	20.0	g
Distilled water up to	1000	mL

5. Potato dextrose broth (PDB)

Potatoes extracts	24.0	g
Dextrose	20.0	g
Distilled water up to	1000	mL

6. Water agar (WA)

Agar	13.0	g
Distilled water up to	1000	mL

7. Potato dextrose agar (PDA)

Potatoes, peeled and diced	200.0	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water up to	1000	mL

Boil 200 g of peels, diced potatoes for 1 hr in 1000 mL of distilled water. Filter, and adjust the filtrate to 1000 mL. Add the dextrose and agar and dissolve by steaming and sterilize by autoclaving at 121 ^{o}C for 15 min.

APPENDIX B

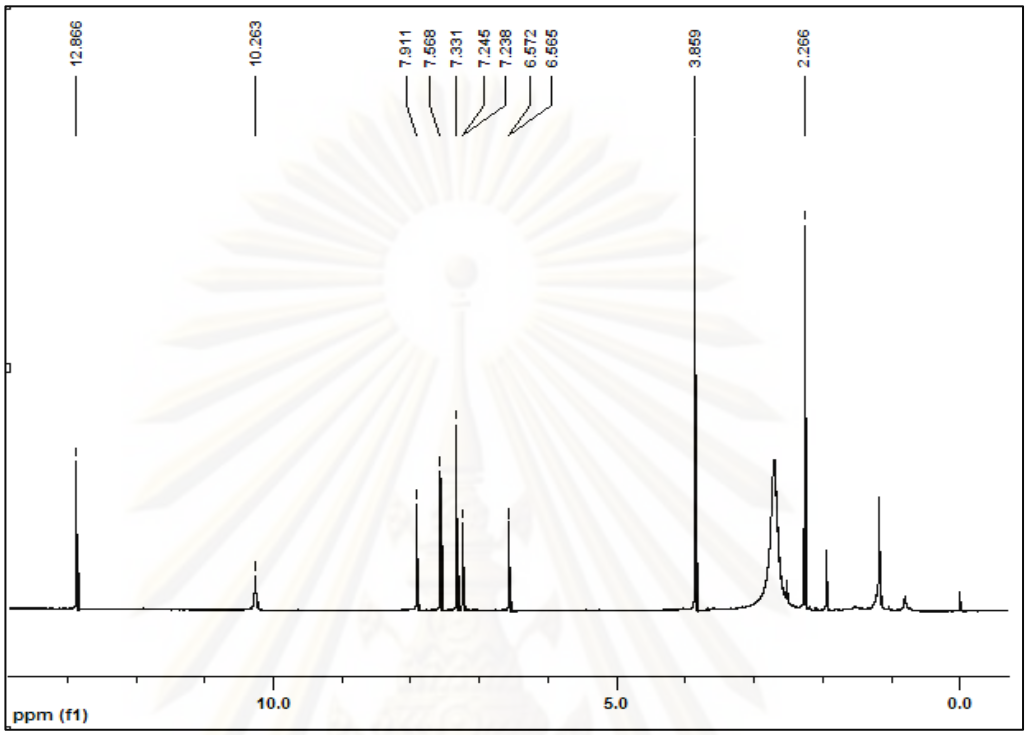

Figure B1 The ^{1}H NMR spectrum of macrosporin (**1**) in $CDCl_3$+DMSO-d_6

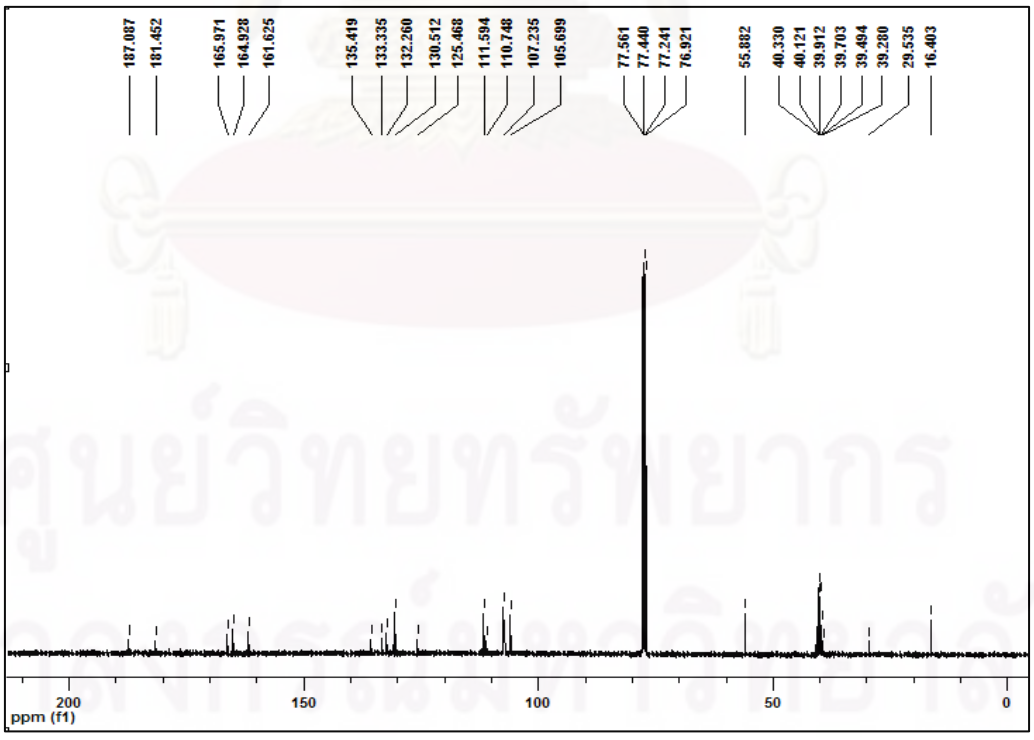

Figure B2 The ^{13}C NMR spectrum of macrosporin (**1**) in $CDCl_3$+DMSO-d_6

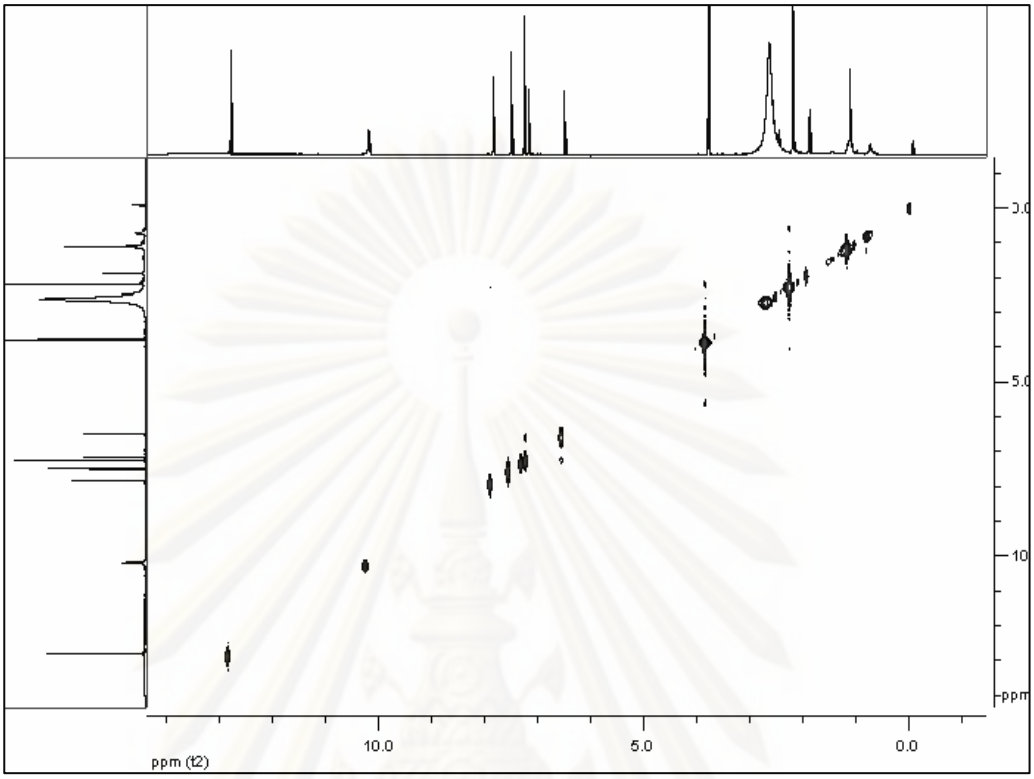

Figure B3 The ^{1}H-^{1}H COSY spectrum of macrosporin (**1**) in $CDCl_3$+DMSO-d_6

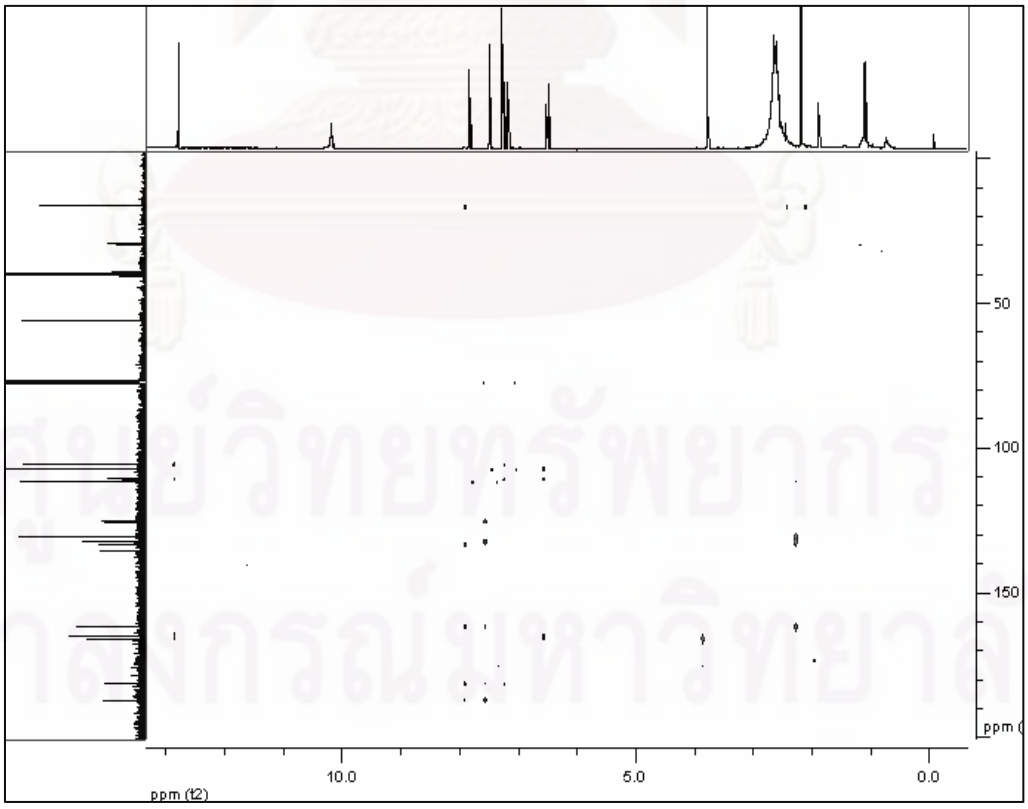

Figure B4 The HMBC spectrum of macrosporin (**1**) in $CDCl_3$+DMSO-d_6

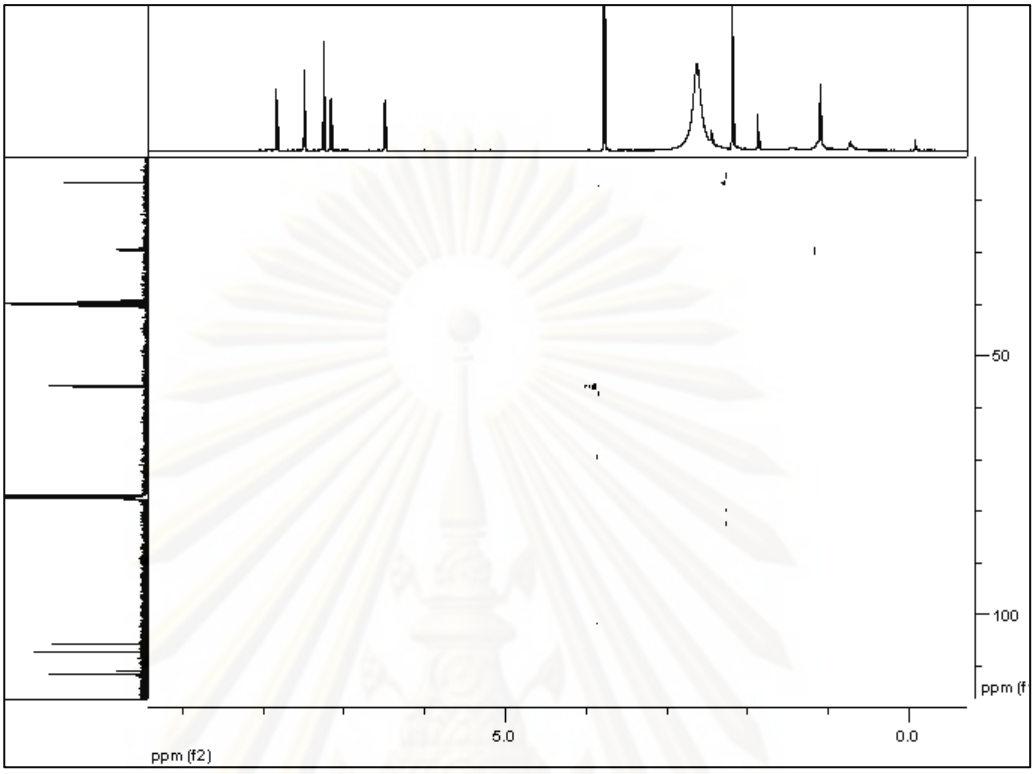

Figure B5 The HSQC spectrum of macrosporin (**1**) in $CDCl_3$+DMSO-d_6

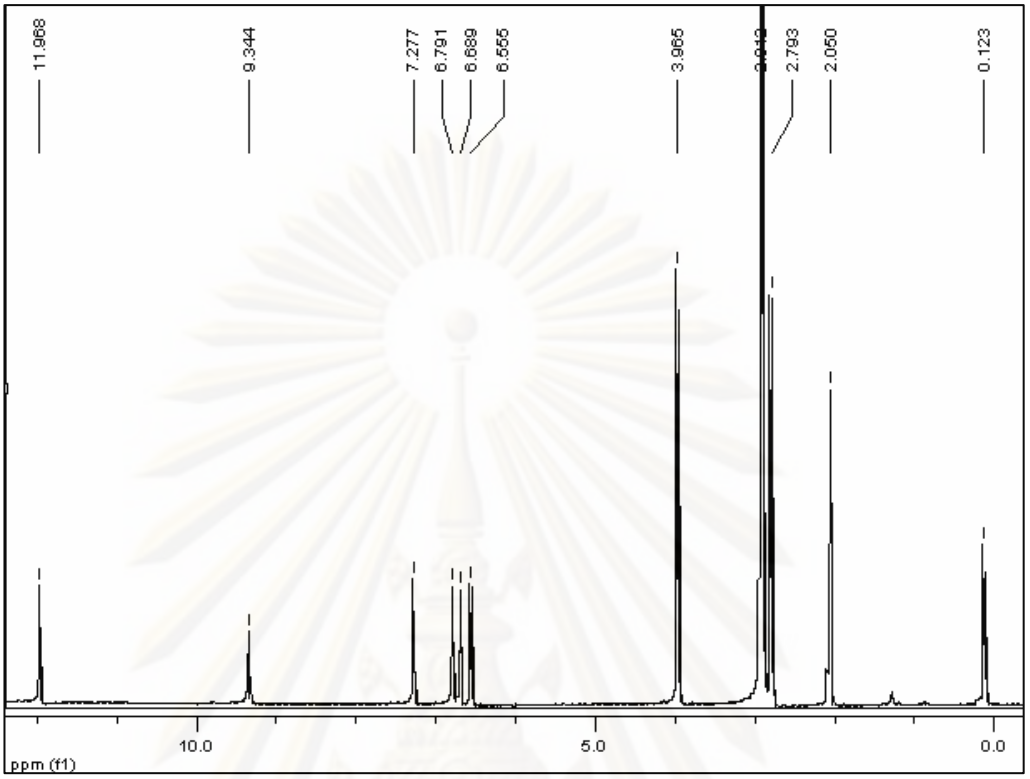

Figure B6 The ^{1}H NMR spectrum of alternariol monomethyl ether (**2**) in acetone-d_6

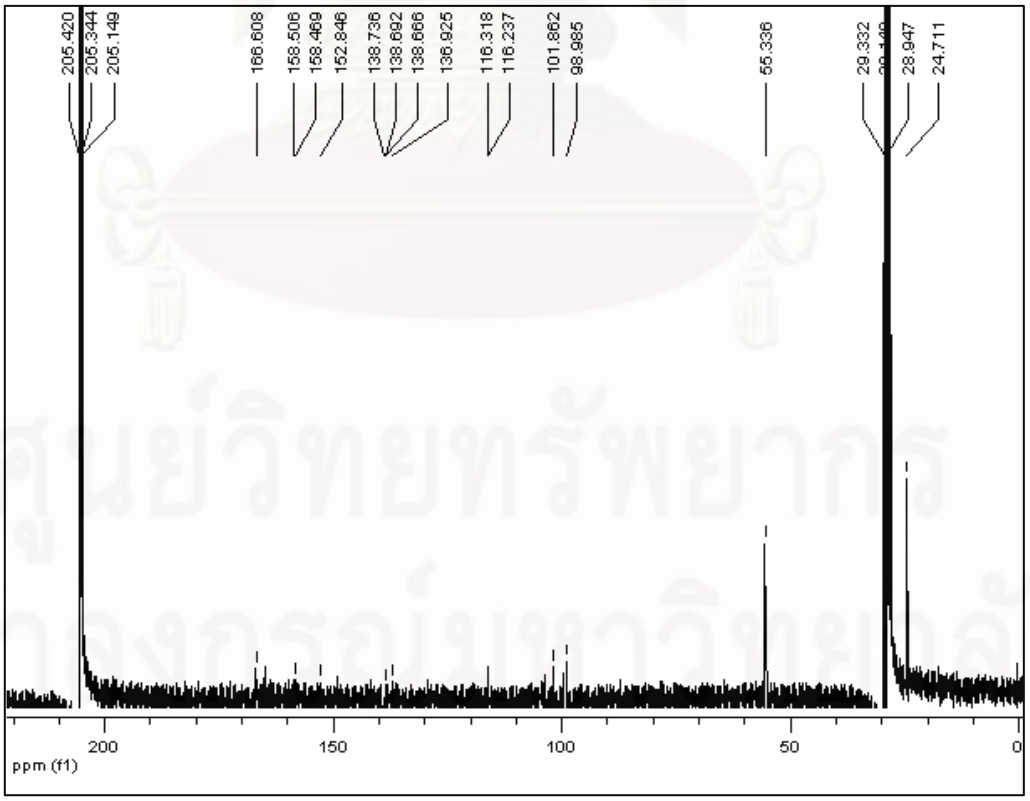

Figure B7 The ^{13}C NMR spectrum of alternariol monomethyl ether (**2**) in acetone-d_6

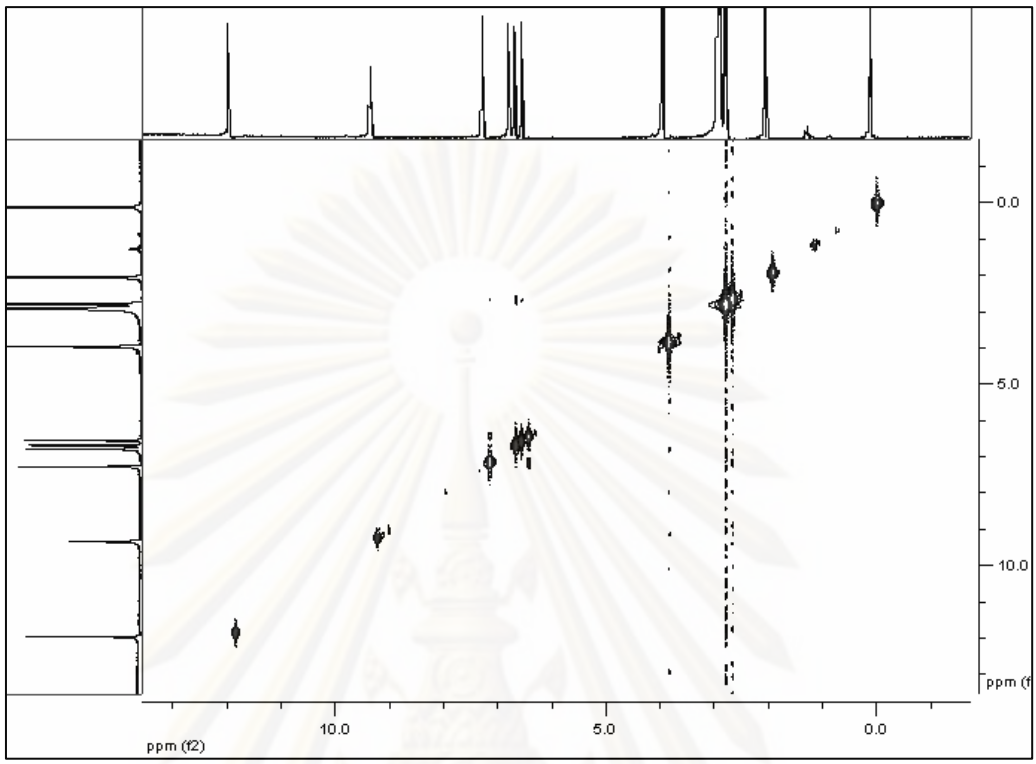

Figure B8 The ^{1}H-^{1}H COSY spectrum of alternariol monomethyl ether (**2**) in acetone-d_6

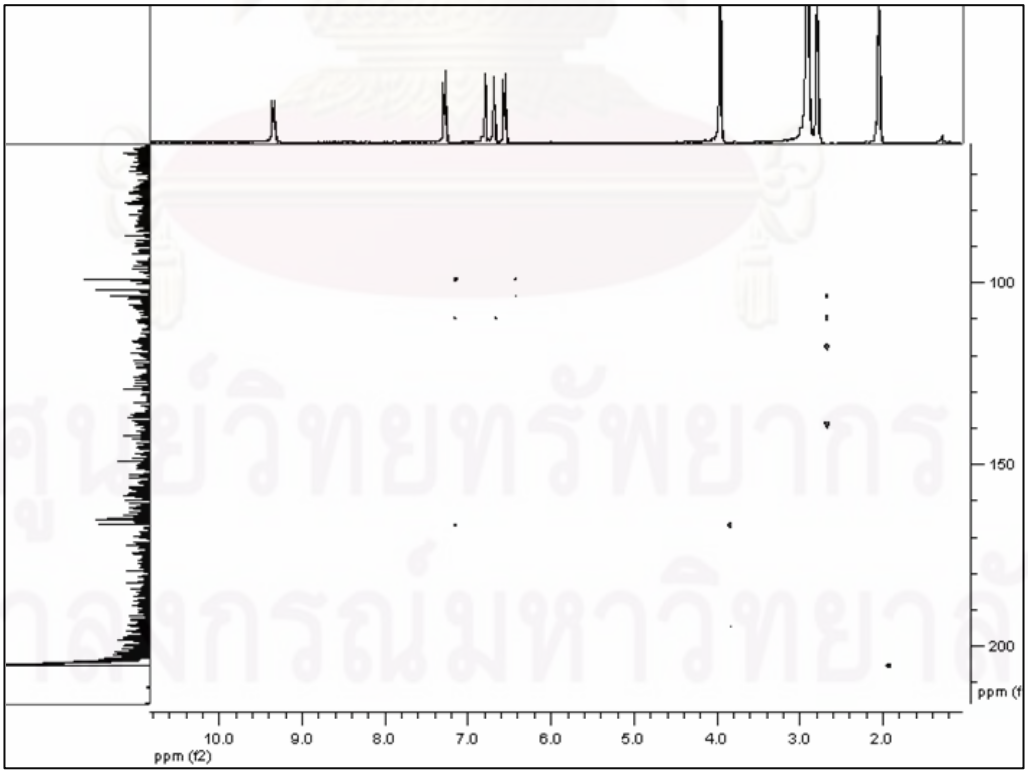

Figure B9 The HMBC spectrum of alternariol monomethyl ether (**2**) in acetone-d_6

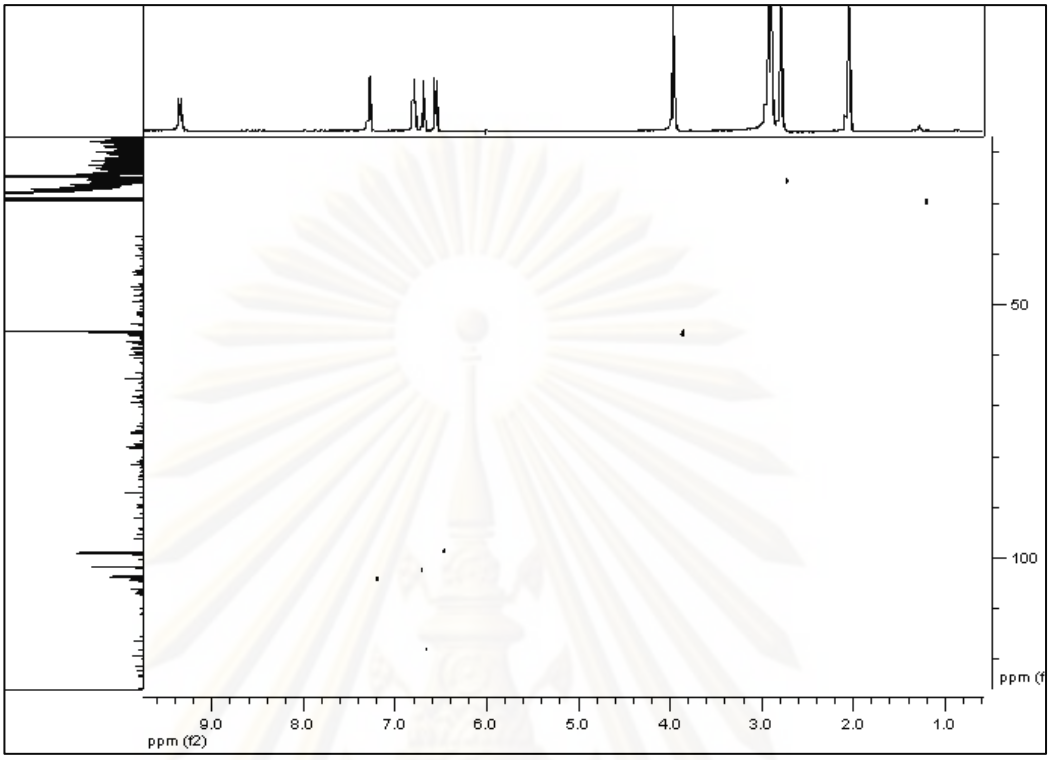

Figure B10 The HSQC spectrum of alternariol monomethyl ether (**2**) in acetone-d_6

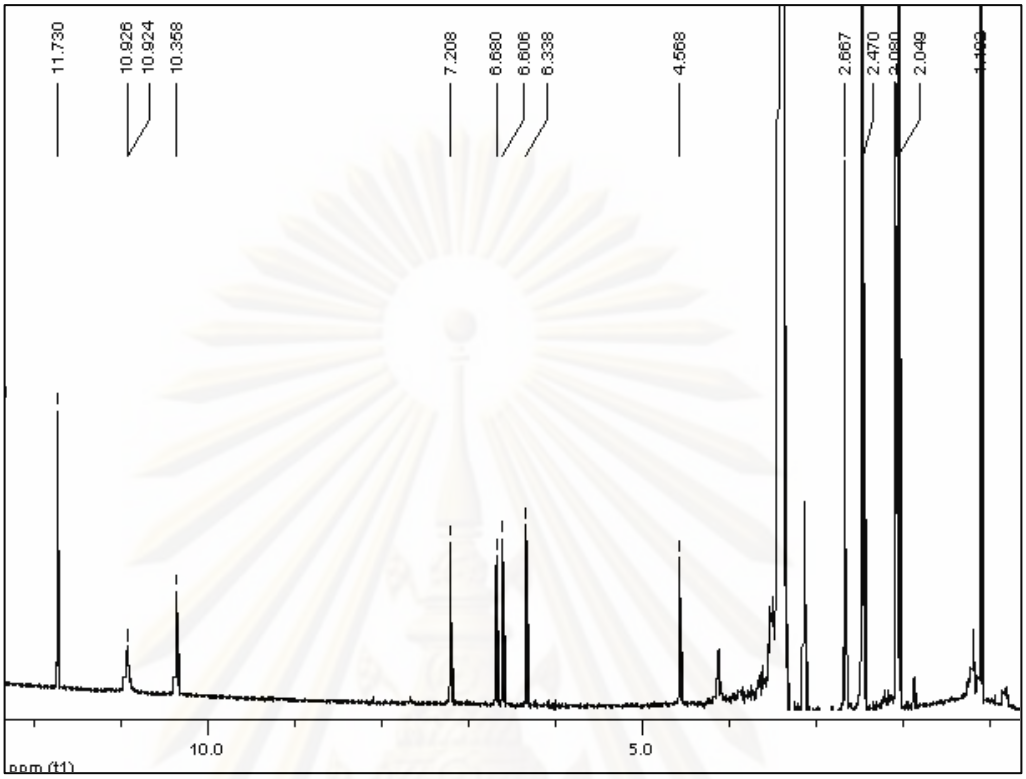

Figure B11 The ^{1}H NMR spectrum of alternariol (**3**) in DMSO-d_6

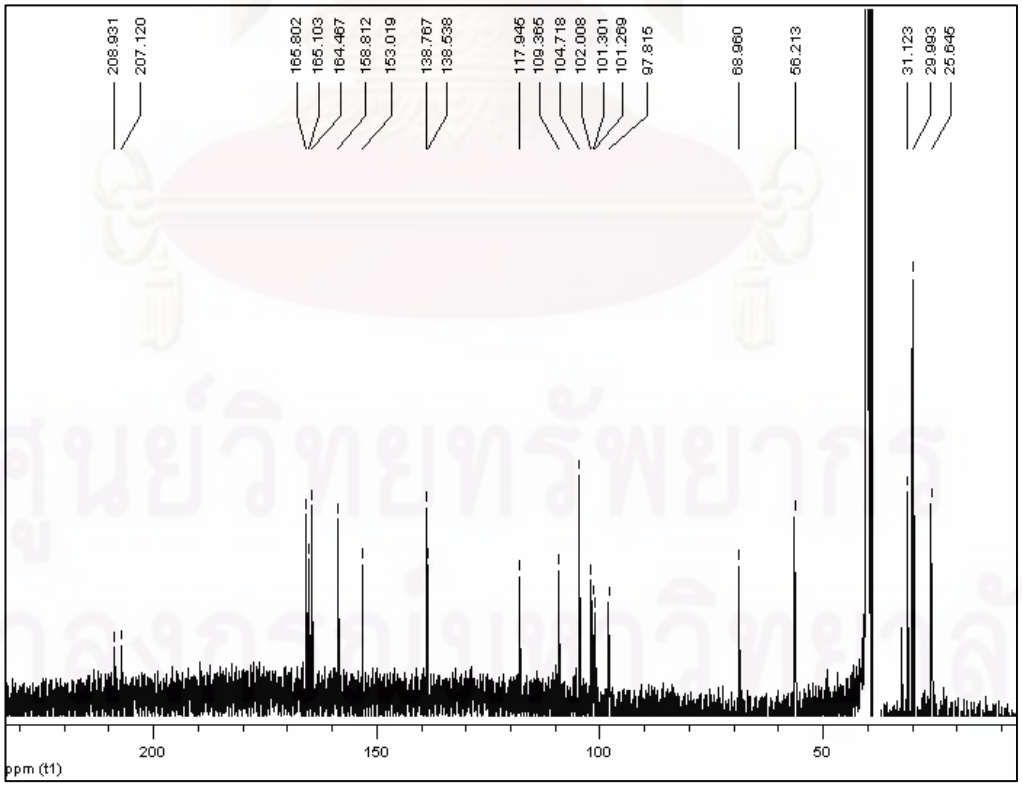

Figure B12 The ^{13}C NMR spectrum of alternariol (**3**) in DMSO-d_6

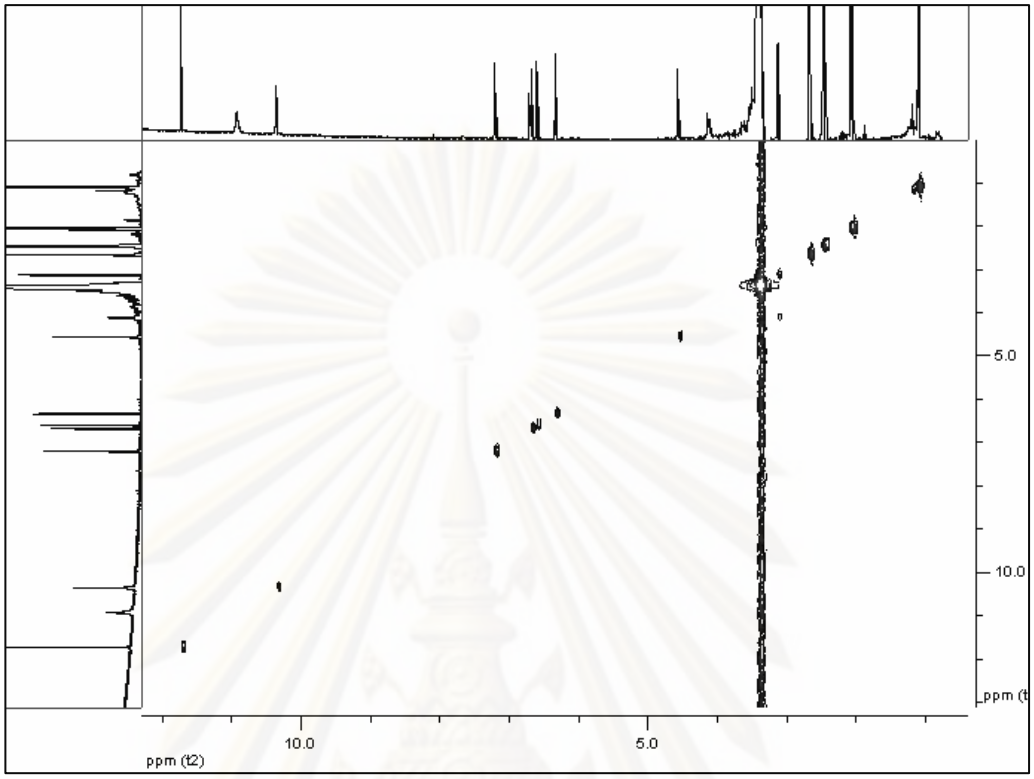

Figure B13 The ^{1}H-^{1}H COSY spectrum of alternariol (**3**) in DMSO-d_6

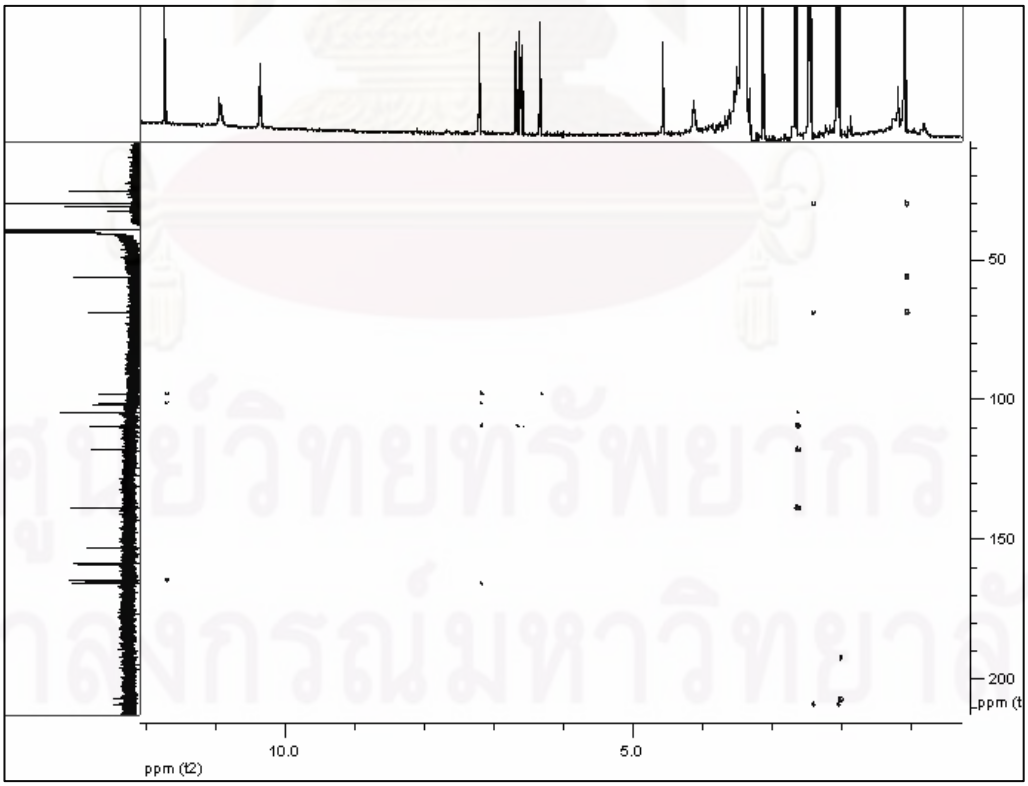

Figure B14 The HMBC spectrum of alternariol (**3**) in DMSO-d_6

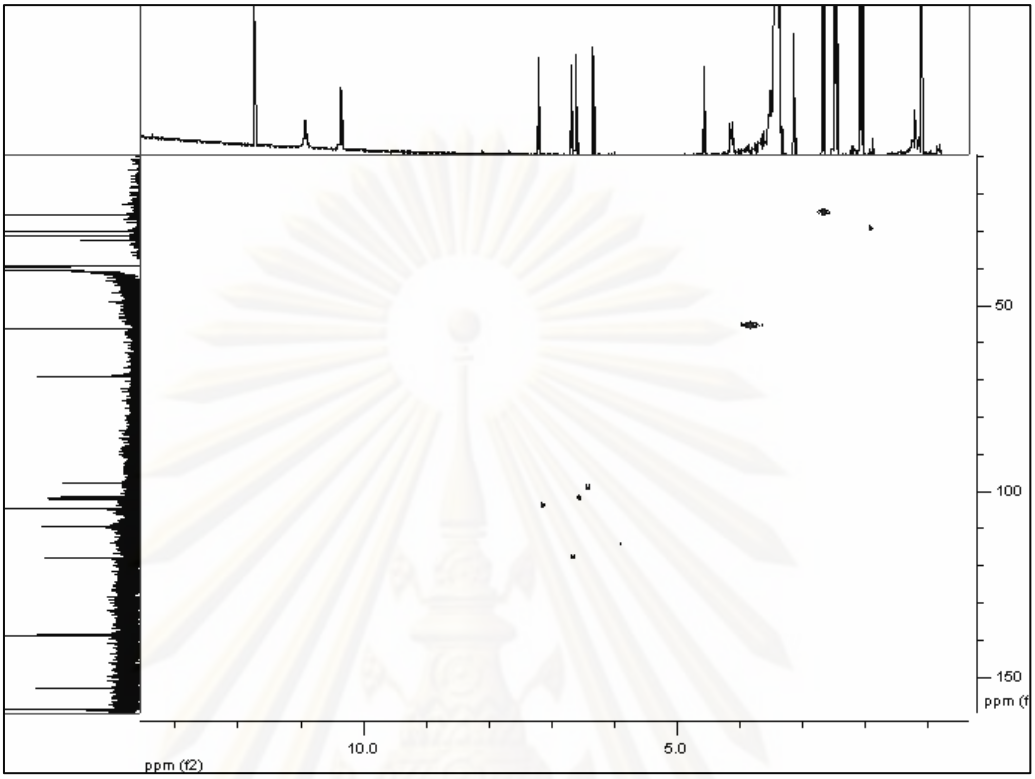

Figure B15 The HSQC spectrum of alternariol (**3**) in DMSO-d_6

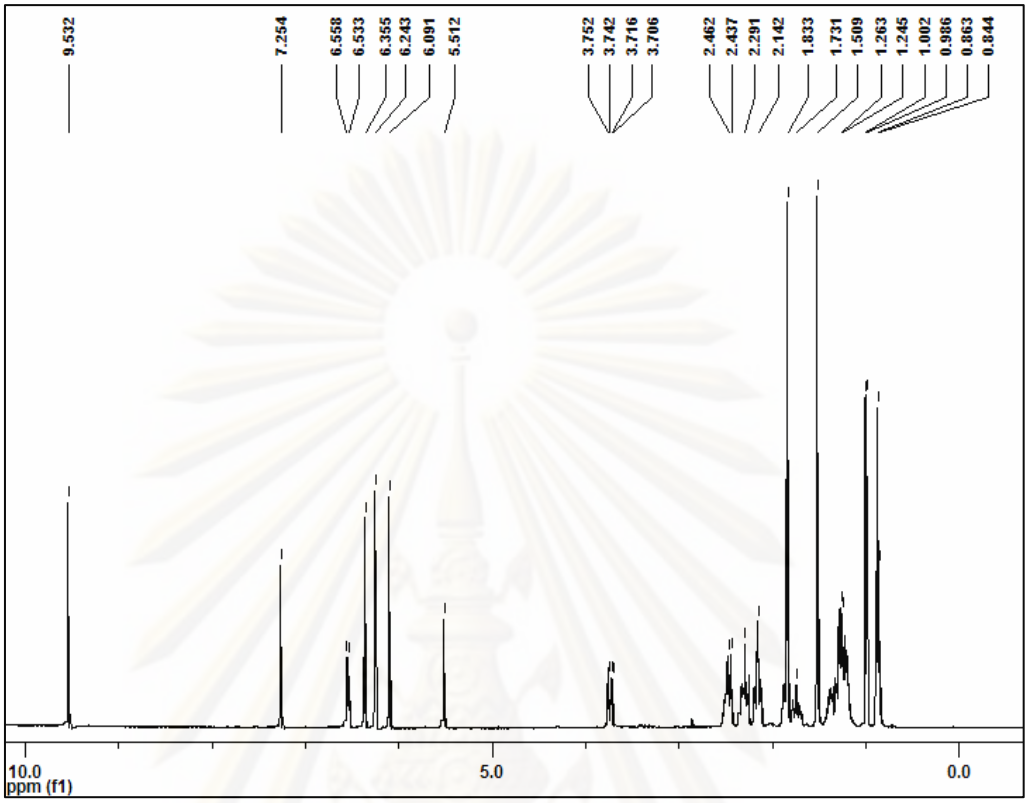

Figure B16 The ^{1}H NMR spectrum of integric acid (**4**) in CH_2Cl_2

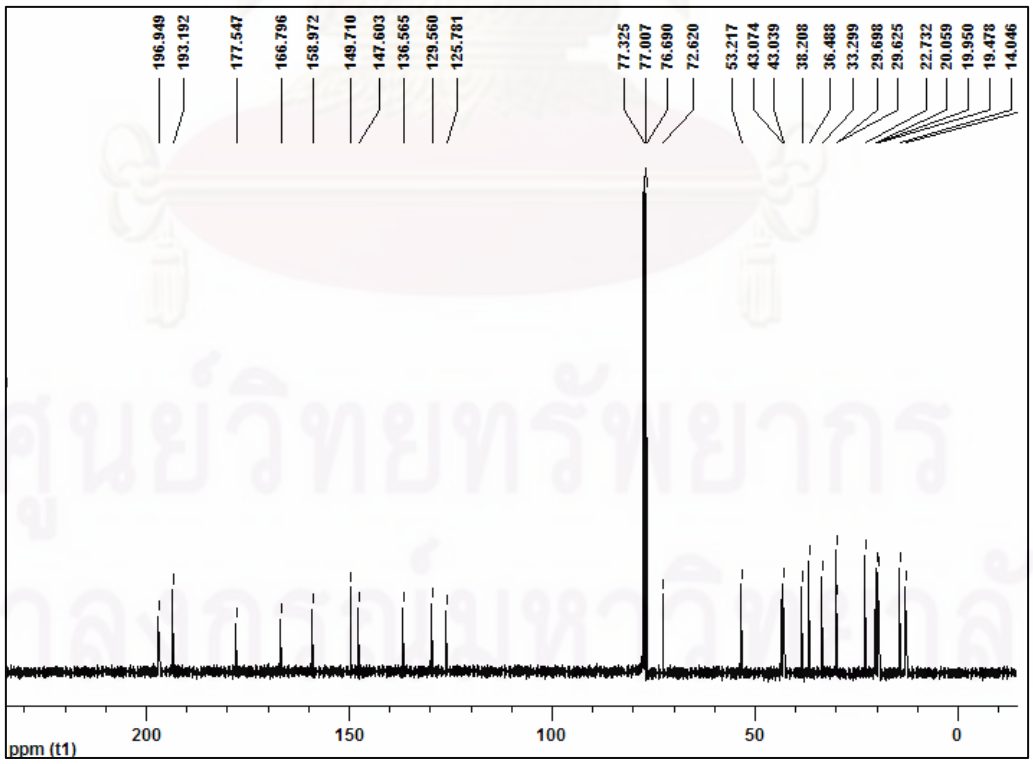

Figure B17 The ^{13}C NMR spectrum of integric acid (**4**) in CH_2Cl_2

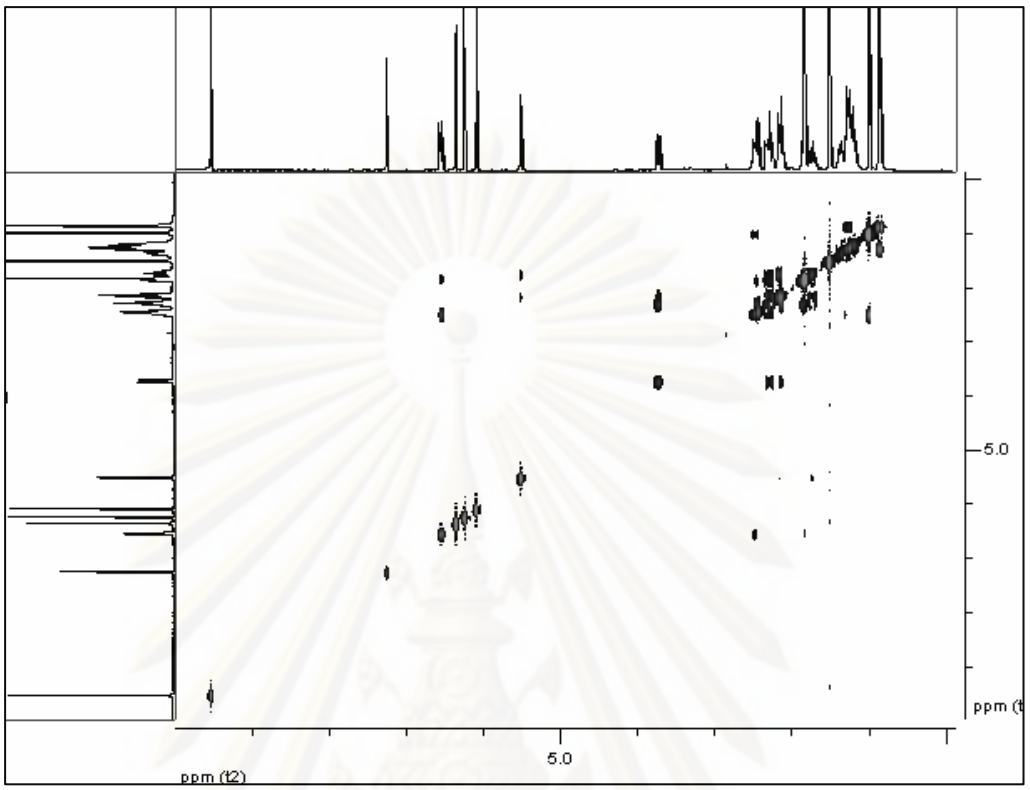

Figure B18 The ^{1}H-^{1}H COSY spectrum of integric acid (**4**) in CH_2Cl_2

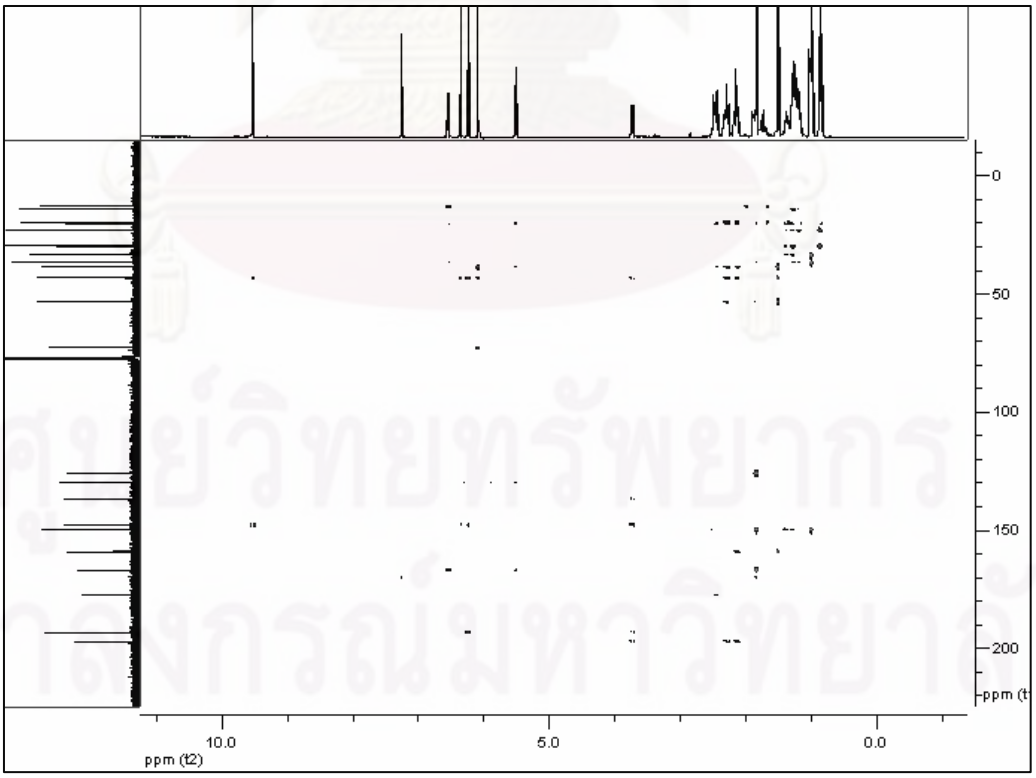

Figure B19 The HMBC spectrum of integric acid (**4**) in CH_2Cl_2

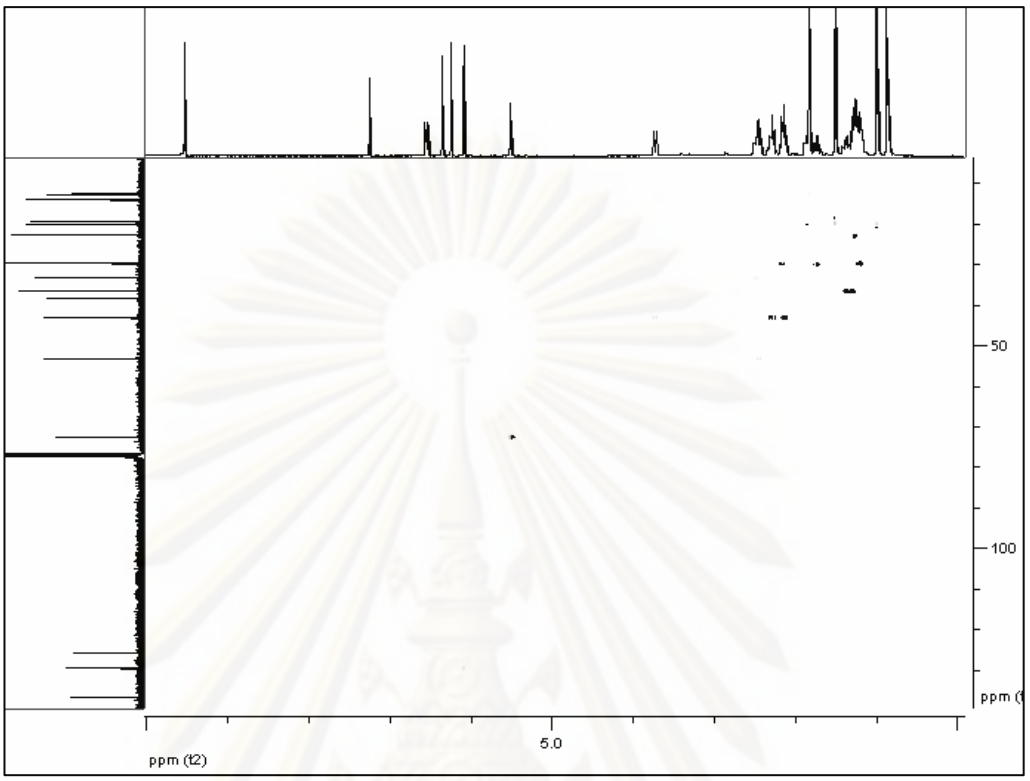

Figure B20 The HSQC spectrum of integric acid (**4**) in CH_2Cl_2

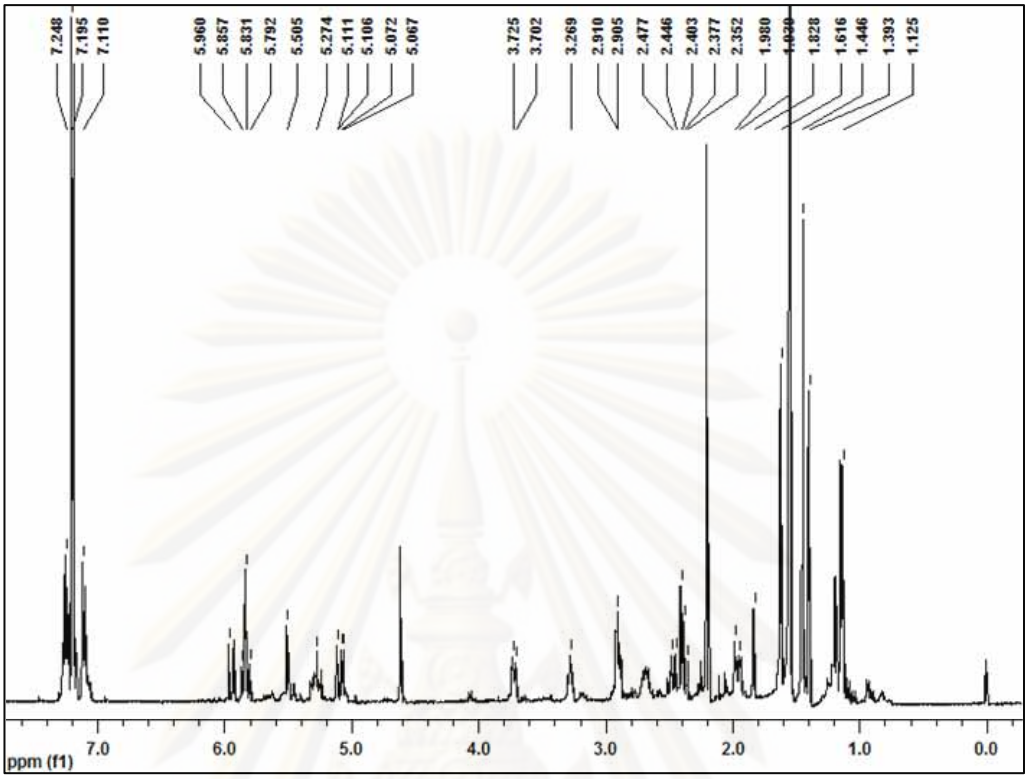

Figure B21 The ^{1}H NMR spectrum of cytochalasin C (**5**) in CH_2Cl_2

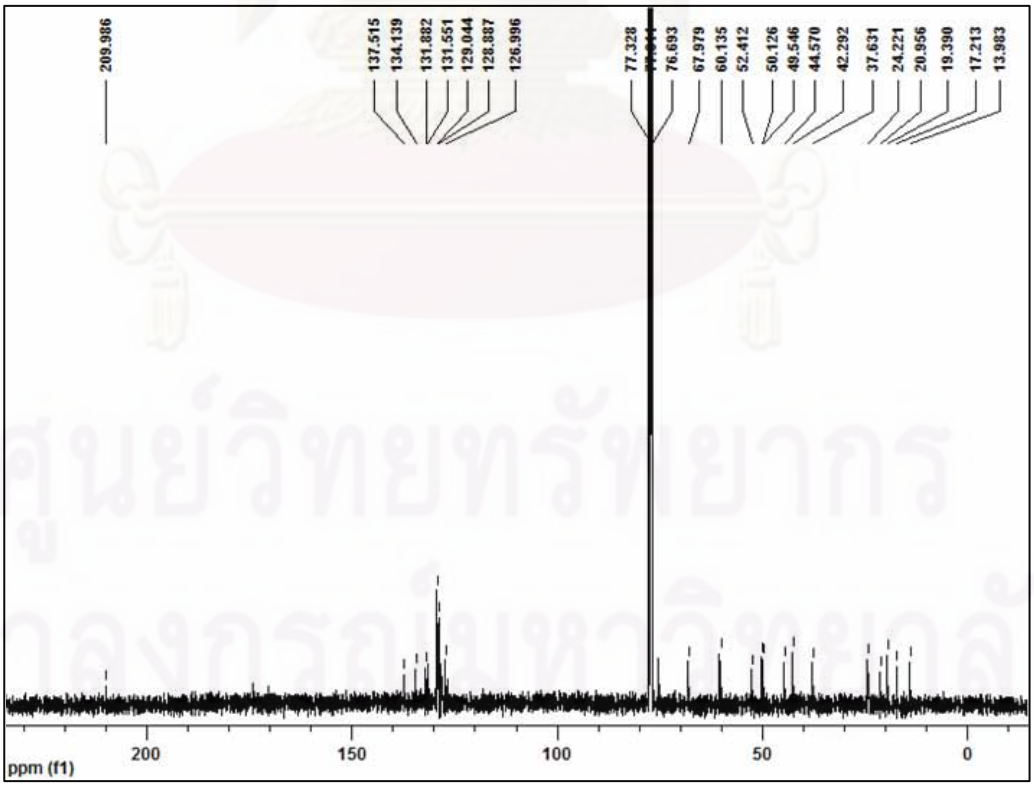

Figure B22 The ^{13}C NMR spectrum of cytochalasin C (**5**) in CH_2Cl_2

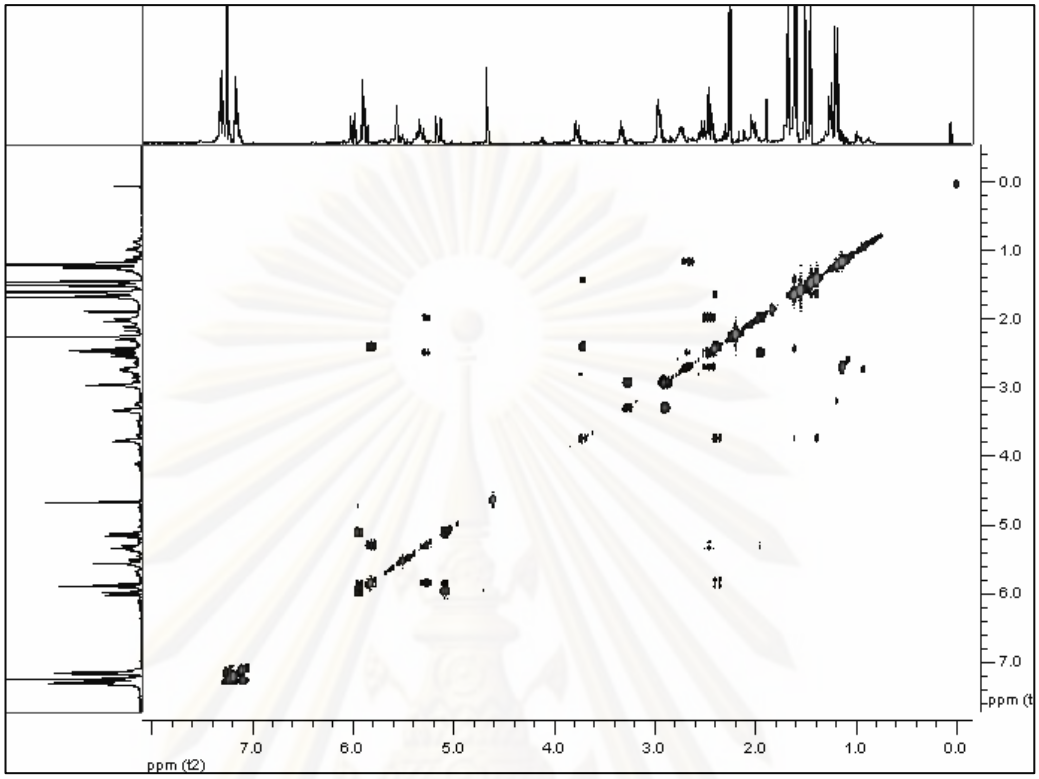

Figure B23 The ^{1}H-^{1}H COSY spectrum of cytochalasin C (**5**) in CH_2Cl_2

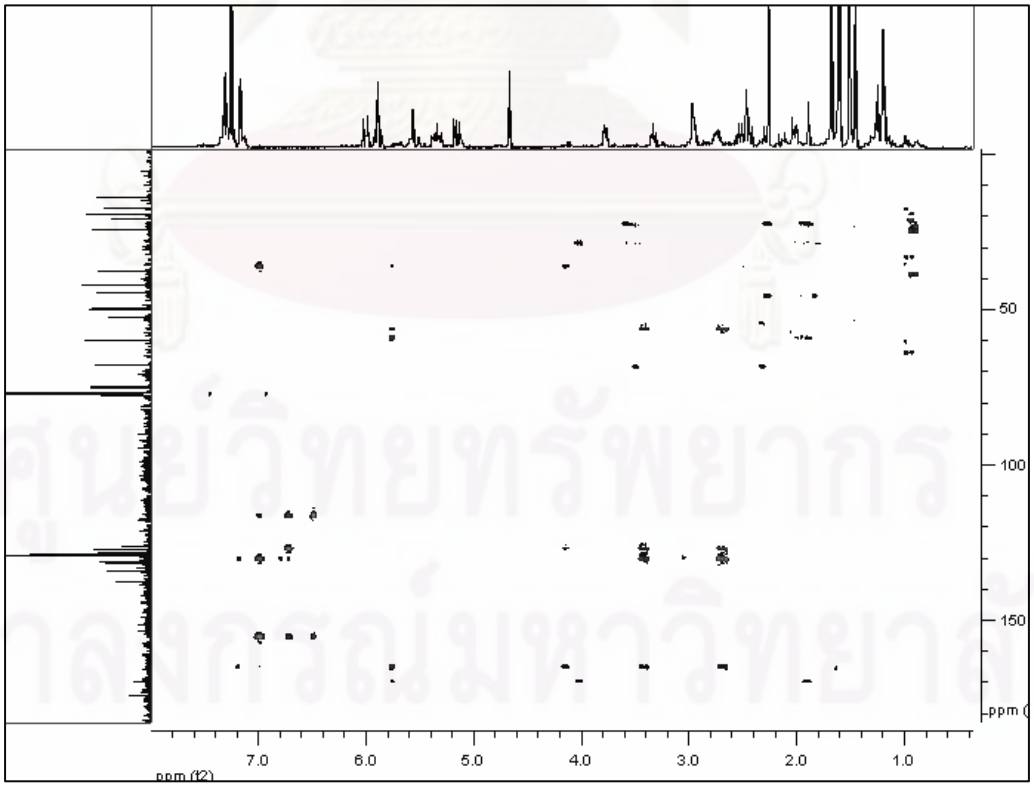

Figure B24 The HMBC spectrum of cytochalasin C (**5**) in CH_2Cl_2

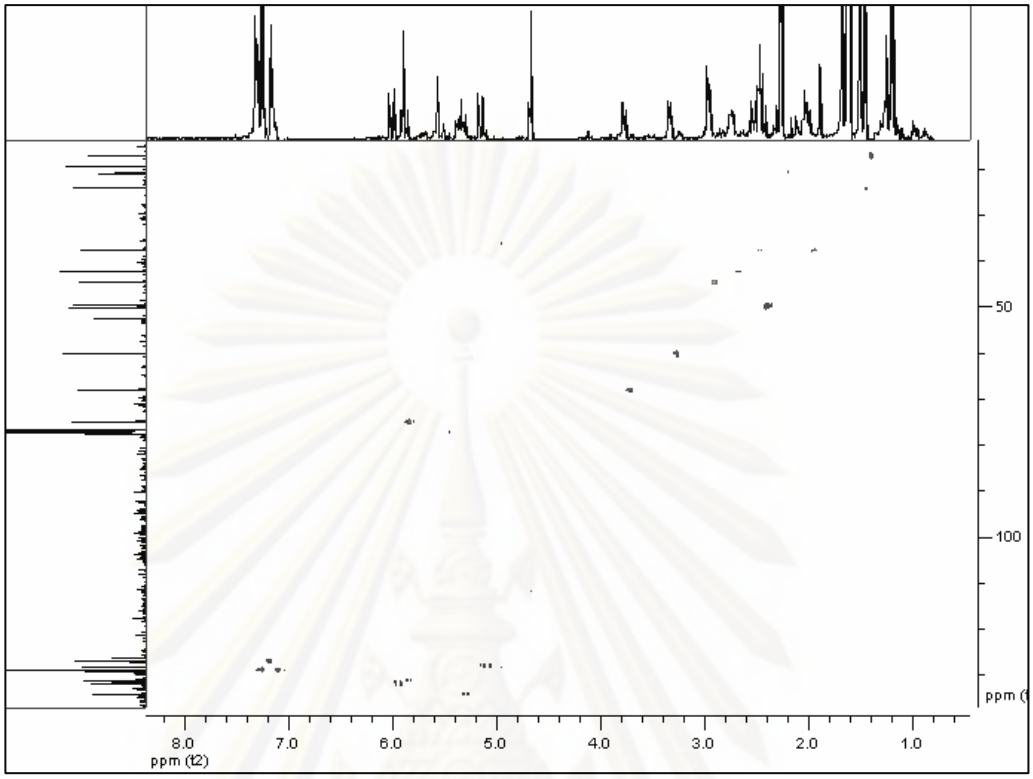

Figure B25 The HSQC spectrum of cytochalasin C (**5**) in CH_2Cl_2

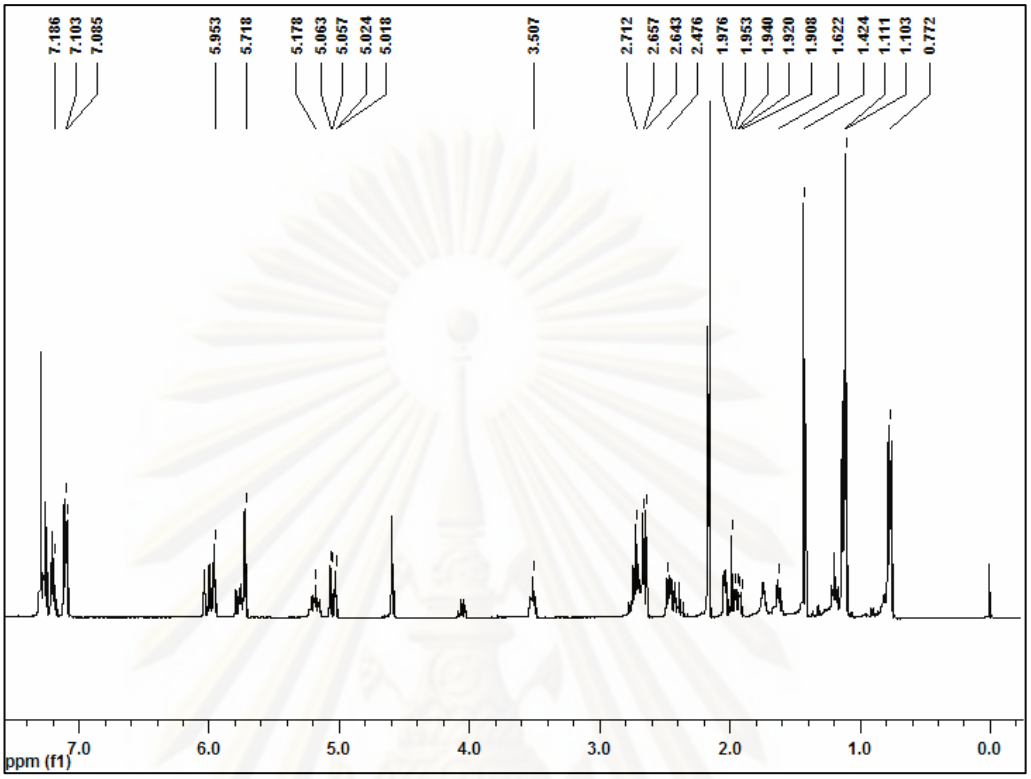

Figure B26 The ^{1}H NMR spectrum of cytochalasin Q (**6**) in CH_2Cl_2

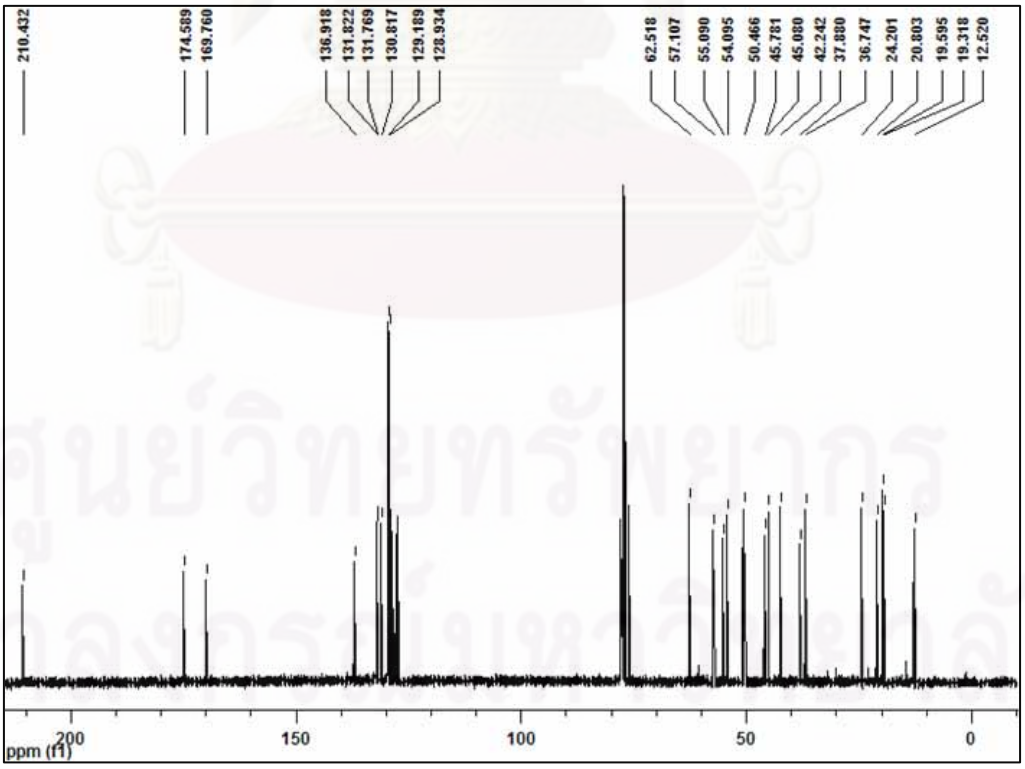

Figure B27 The ^{13}C NMR spectrum of cytochalasin Q (**6**) in CH_2Cl_2

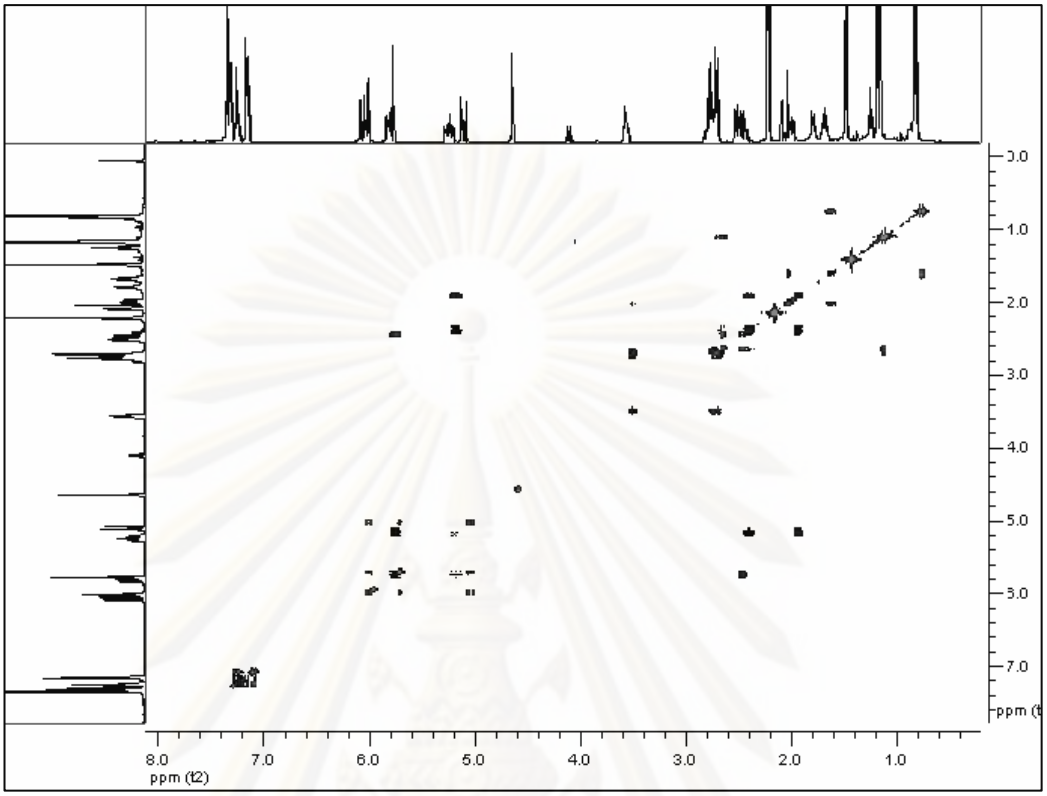

Figure B28 The ^{1}H-^{1}H COSY spectrum of cytochalasin Q (**6**) in CH_2Cl_2

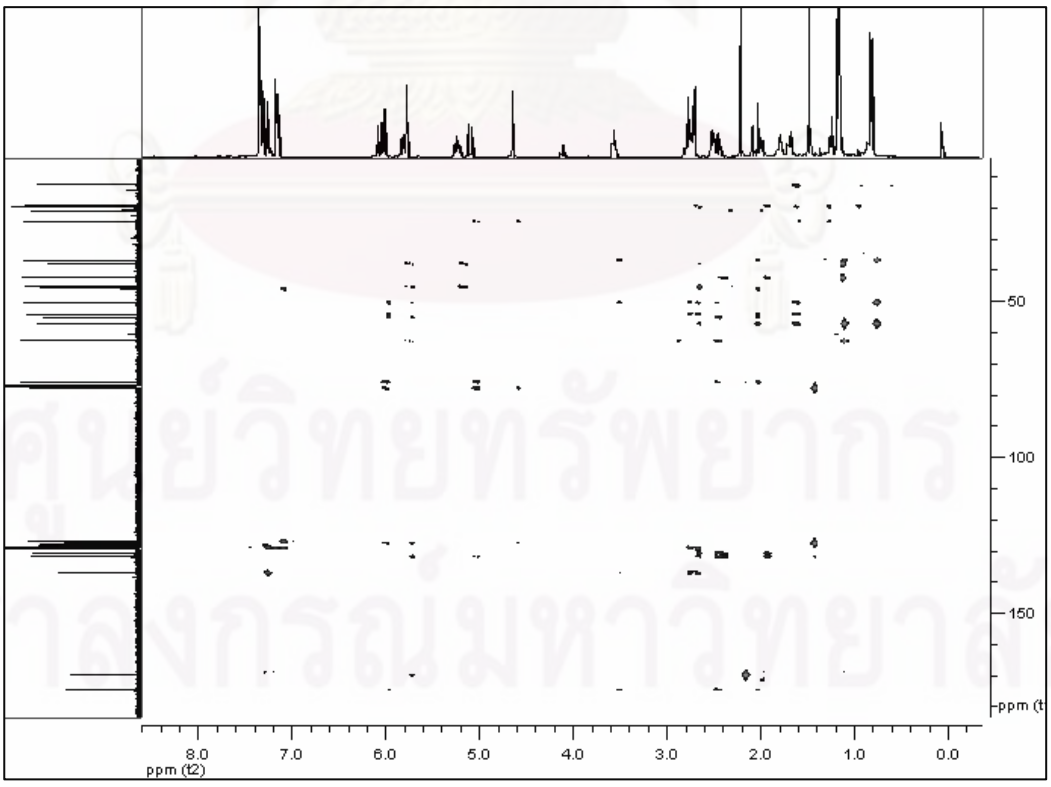

Figure B29 The HMBC spectrum of cytochalasin Q (**6**) in CH_2Cl_2

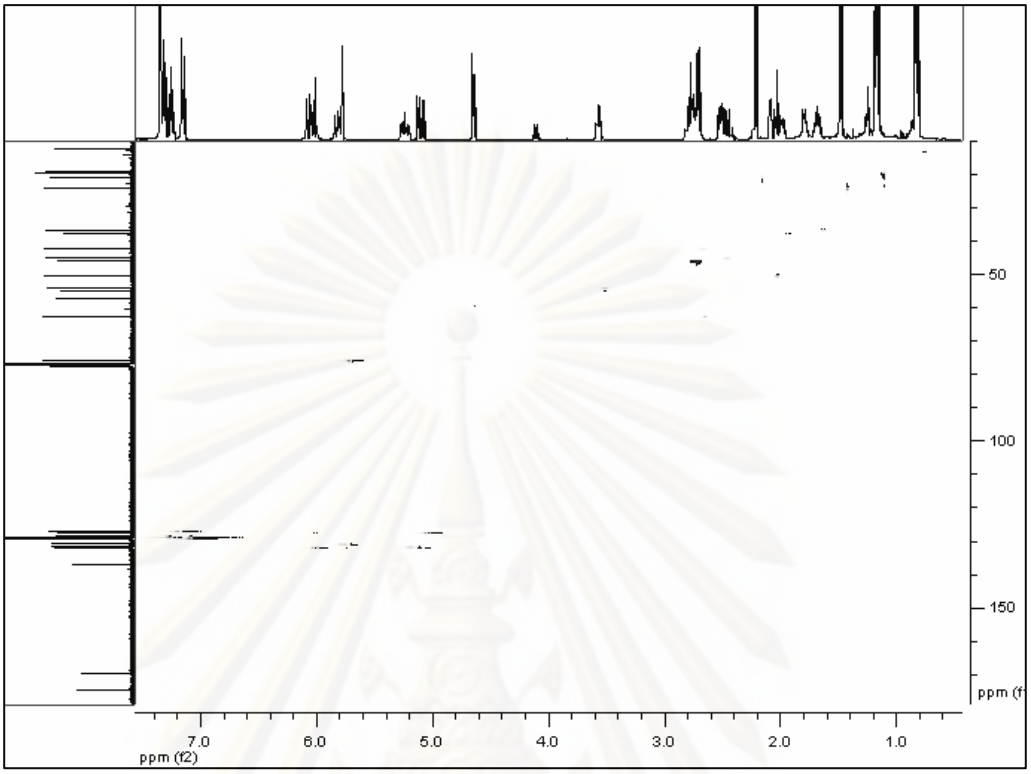

Figure B30 The HSQC spectrum of cytochalasin Q (**6**) in CH_2Cl_2

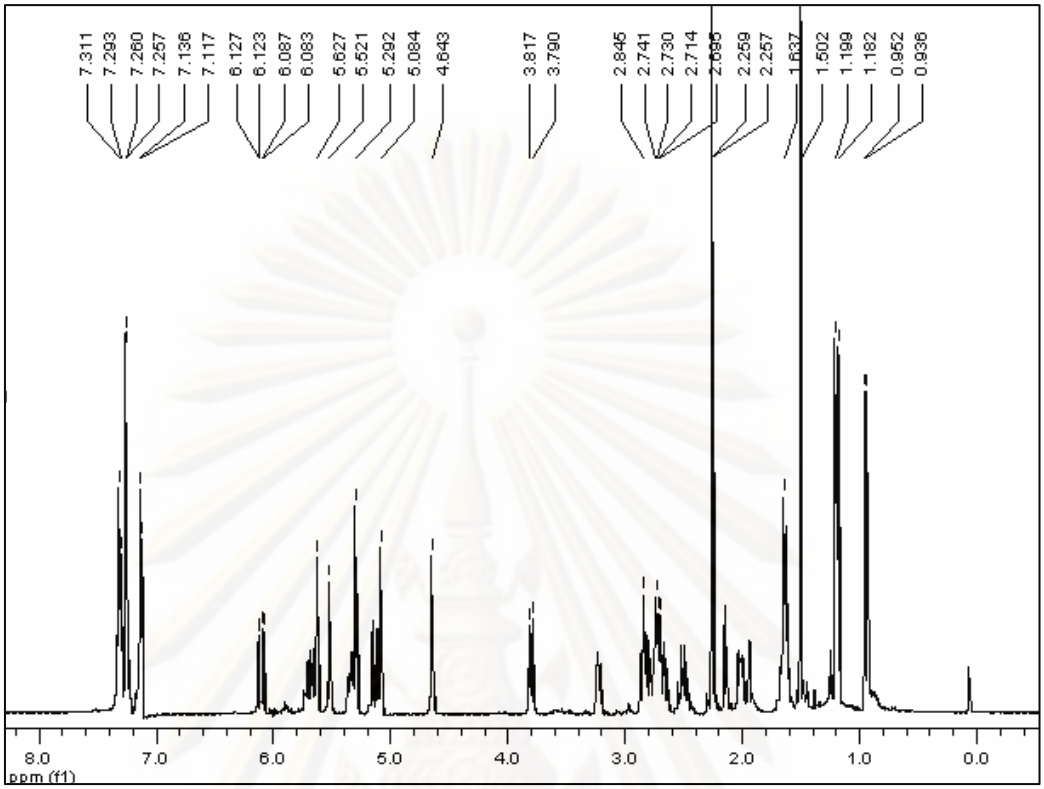

Figure B31 The ^{1}H NMR spectrum of cytochalasin D (**7**) in CH_2Cl_2

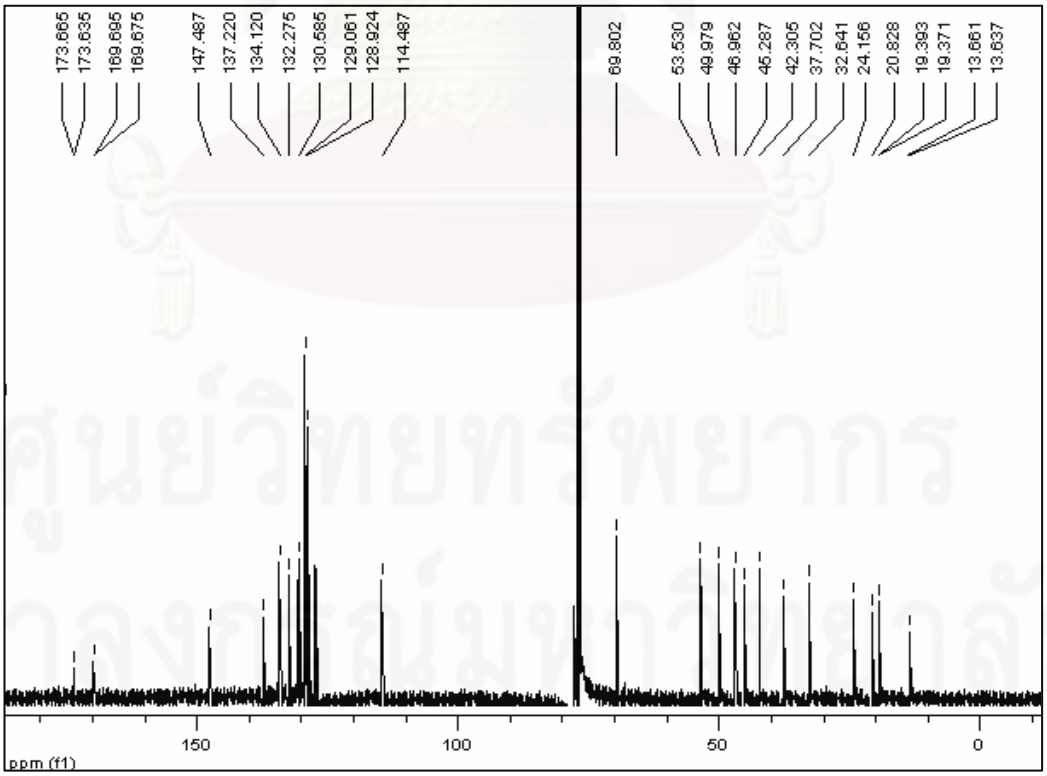

Figure B32 The ^{13}C NMR spectrum of cytochalasin D (**7**) in CH_2Cl_2

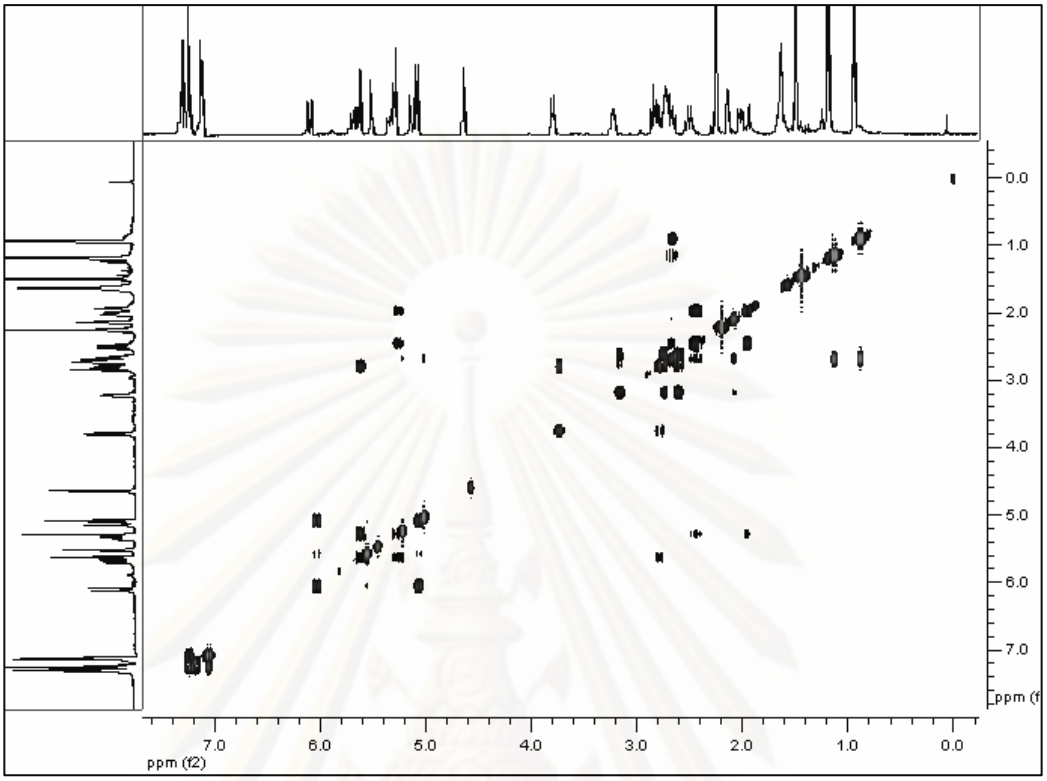

Figure B33 The ^{1}H-^{1}H COSY spectrum of cytochalasin D (**7**) in CH_2Cl_2

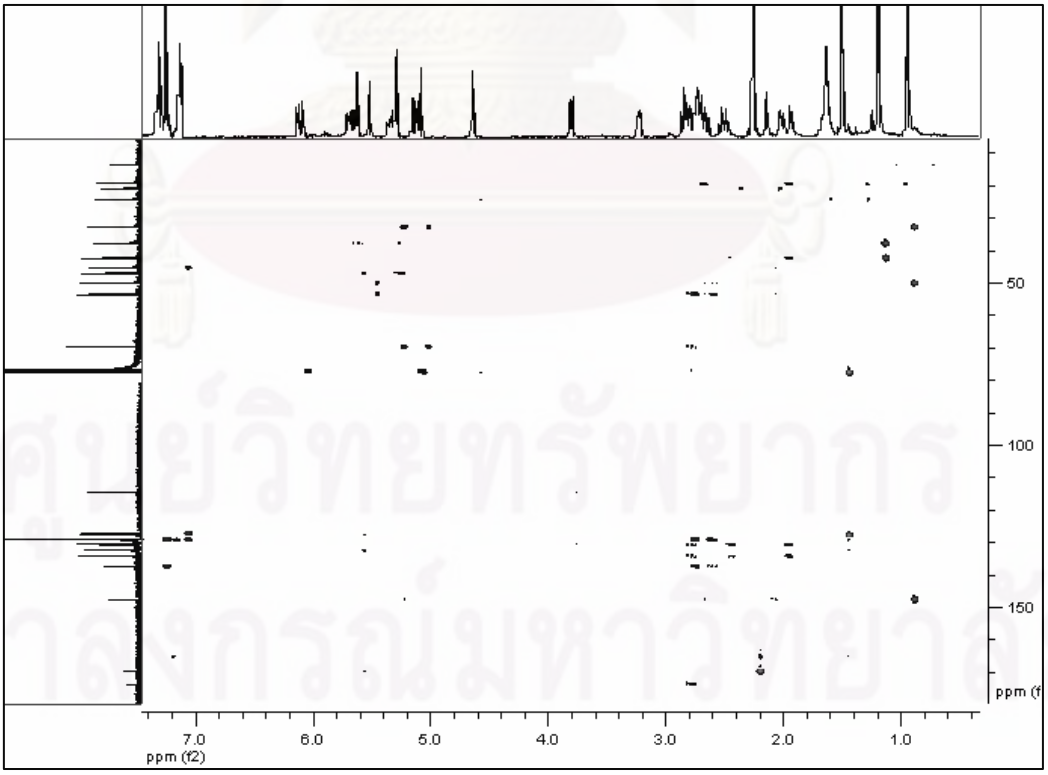

Figure B34 The HMBC spectrum of cytochalasin D (**7**) in CH_2Cl_2

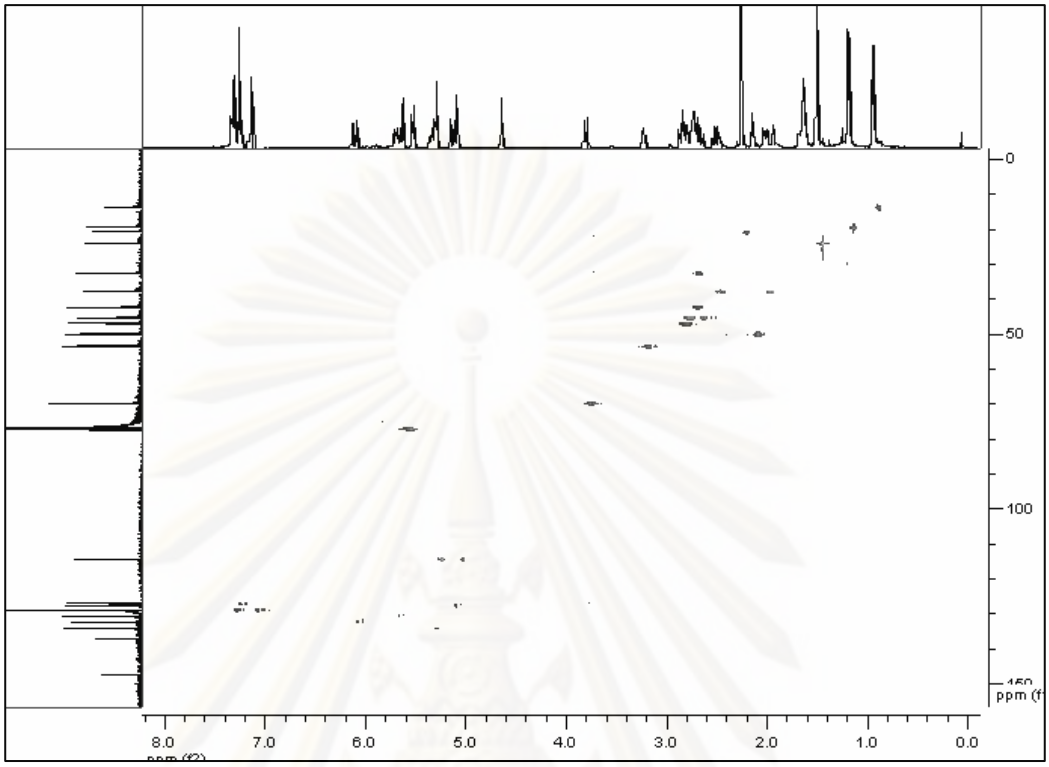

Figure B37 The HSQC spectrum of cytochalasin D (**7**) in CH_2Cl_2

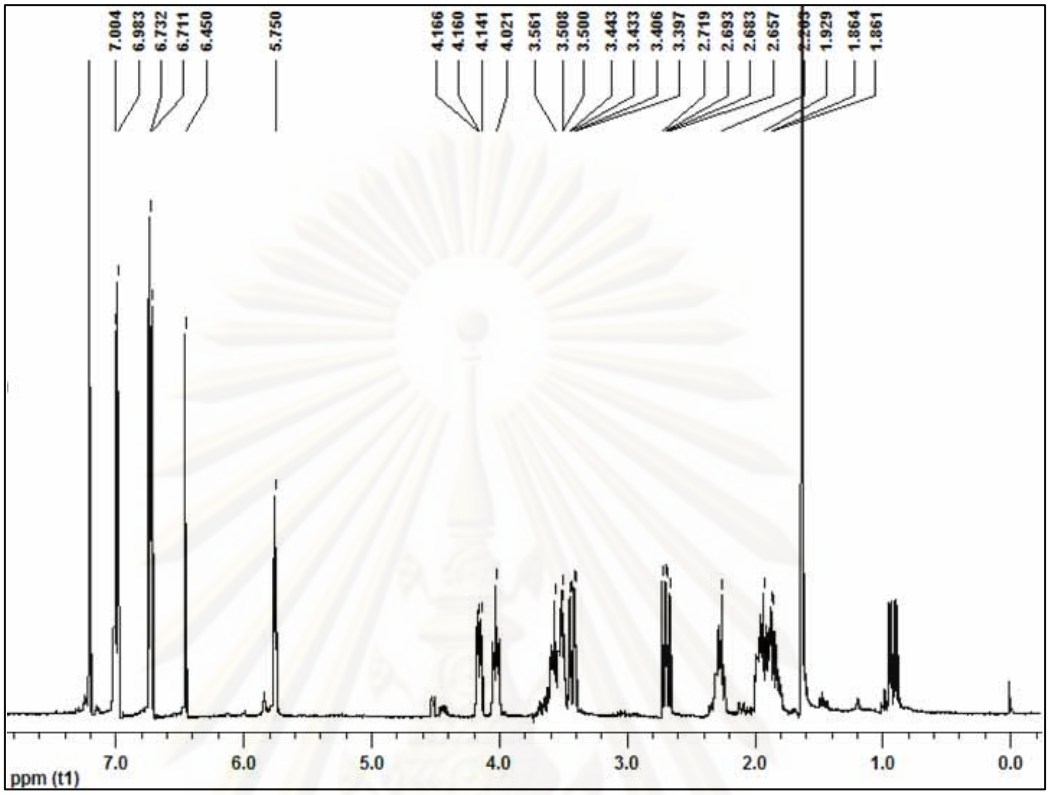

Figure B38 The ^{1}H NMR spectrum of cyclo(D-tyrosyl-D-proline) (**8**) in CH_2Cl_2

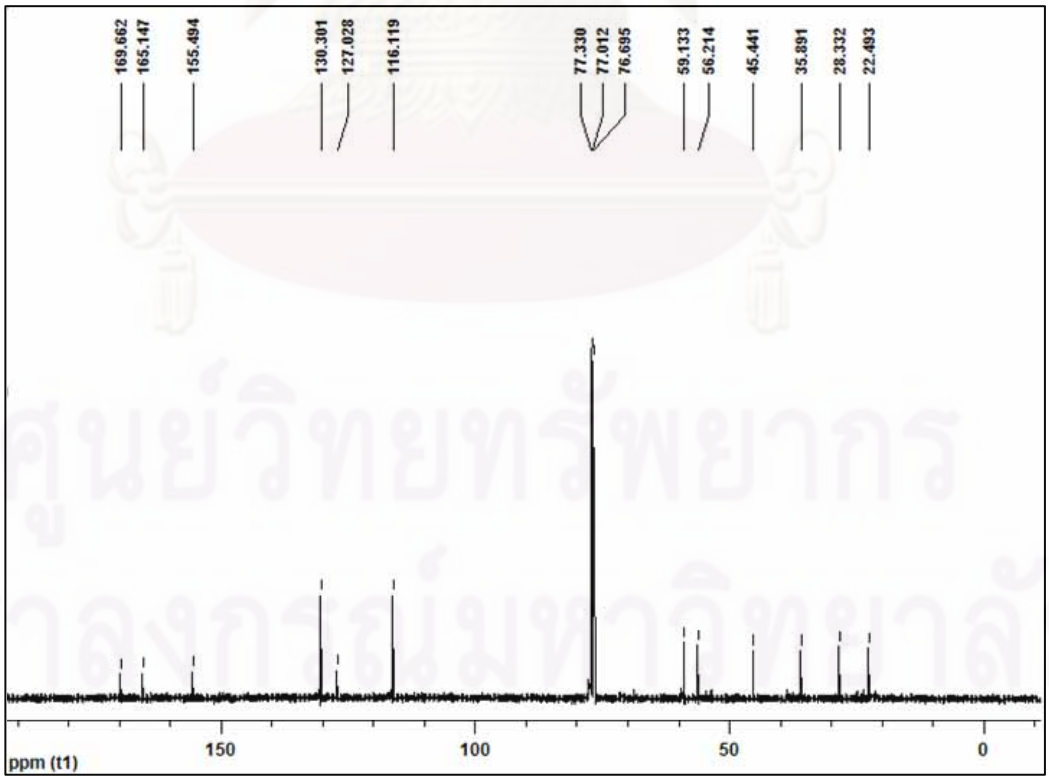

Figure B39 The ^{13}C NMR spectrum of cyclo(D-tyrosyl-D-proline) (**8**) in CH_2Cl_2

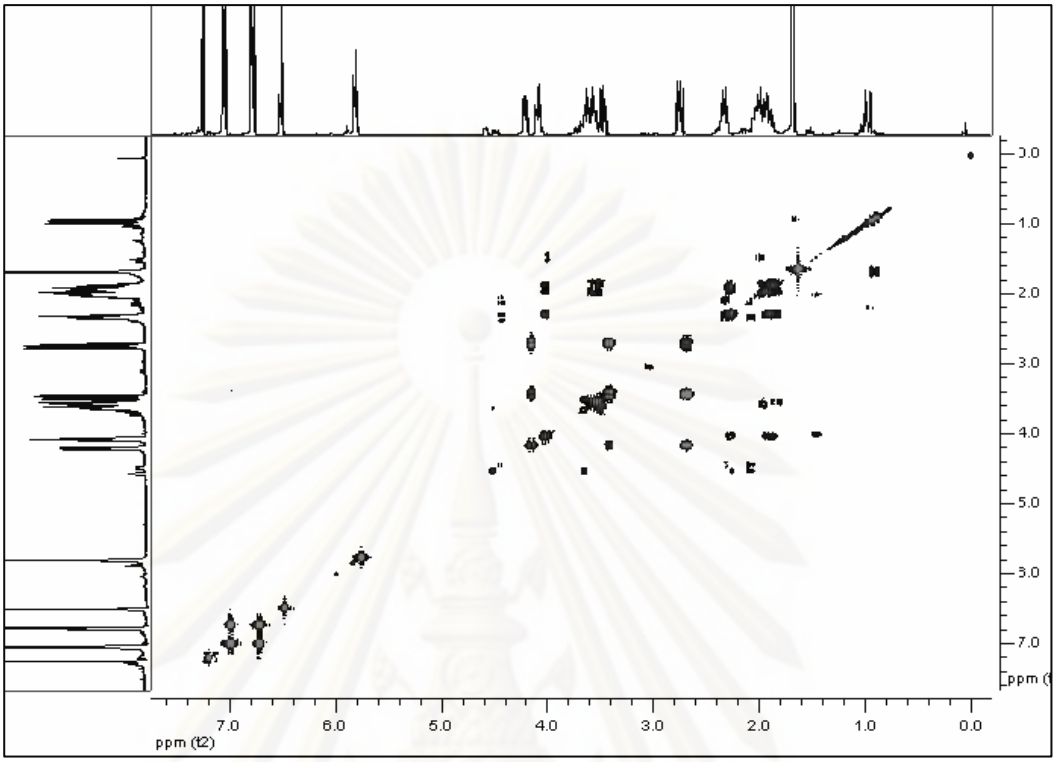

Figure B40 The ^{1}H-^{1}H COSY spectrum of cyclo(D-tyrosyl-D-proline) (**8**) in CH_2Cl_2

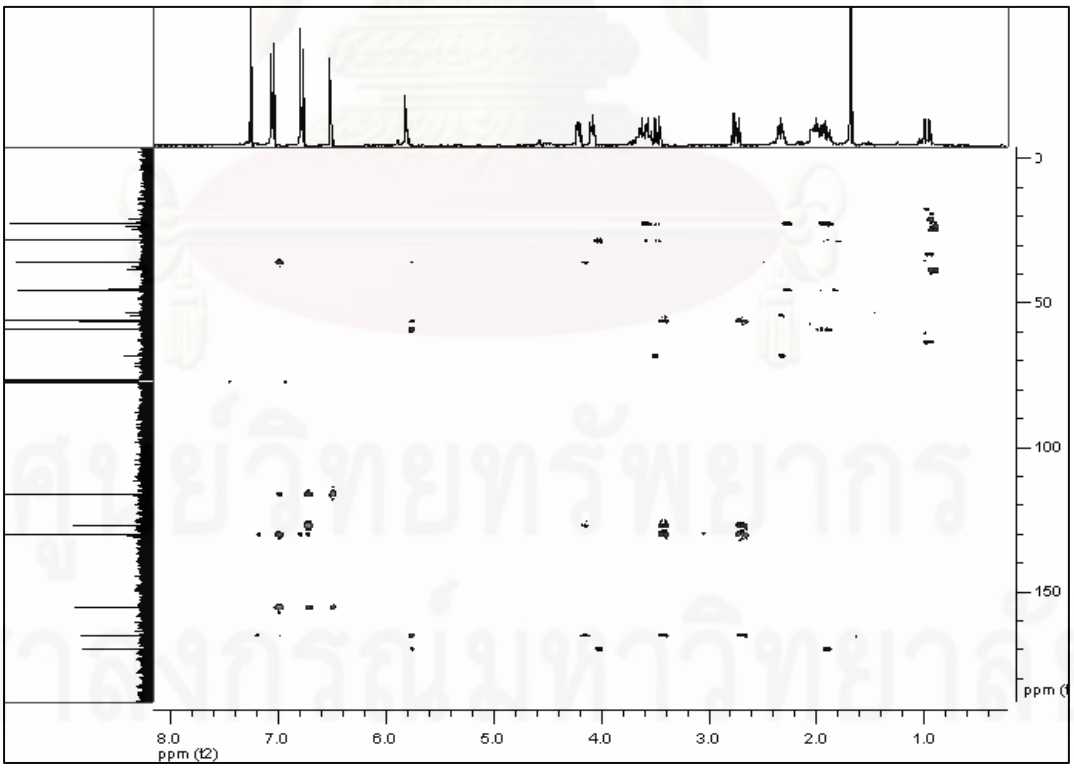

Figure B41 The HMBC spectrum of cyclo(D-tyrosyl-D-proline) (**8**) in CH_2Cl_2

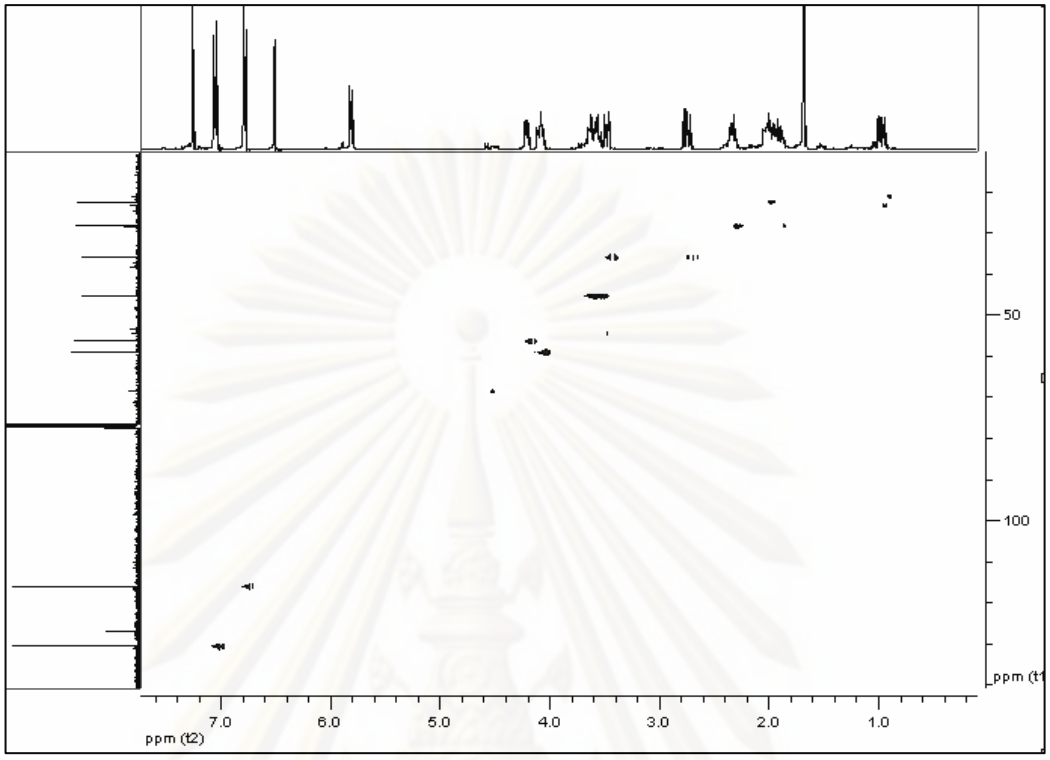

Figure B42 The HMQC spectrum of cyclo(D-tyrosyl-D-proline) (**8**) in CH_2Cl_2

VITA

Miss Sujitra Hanthanong was born on September 12th, 1981. She held Bachelor Degree of Science in Biotechnology from the School of Agriculture Technology, Walailak University in 2004. While she studying in Master Degree program, she received a financial support from Graduate School, Chulalongkorn University.

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