สารออกฤทธิ์ทางชีวภาพจากรา *Bipolaris oryzae*

นางสาวกนกวรรณ สว่าง

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BIOACTIVE COMPOUNDS FROM FUNGUS *Bipolaris oryzae*

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Thesis Title	BIOACTIVE COMPOUNDS FROM FUNGUS *Bipolaris oryzae*
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กนกวรรณ สว่าง : สารออกฤทธิ์ทางชีวภาพจากรา *Biopolaris oryzae* (BIOACTIVE COMPOUNDS FROM FUNGUS *Bipolaris oryzae*) อ.ที่ปรึกษา: รศ. ดร. สันติ ทิพยางค์, อ.ที่ปรึกษาร่วม: ผศ. ดร. ปรีชา ภูวไพรศิริศาล, 49 หน้า. ISBN 974-14-2539-2.

ในการศึกษาหาสารออกฤทธิ์ทางชีวภาพเบื้องต้นจากเชื้อราสาเหตุโรคพืช 11 สายพันธุ์ สามารถคัดเลือกเชื้อรา *Bipolaris oryzae* สำหรับใช้ในการทดลองครั้งนี้ ผลการแยกสารทางโครมาโทกราฟีของสิ่งสกัดเอทิลอะซีเตท ได้สารชนิดใหม่ 1 ชนิด และสารที่เคยมีรายงานมาก่อน 6 ชนิด แบ่งออกเป็น 2 กลุ่มคือ กลุ่มของ ophiobolins 4 ชนิด ได้แก่ 3-anhydroophiobolin A (1), ophiobolin I (2), ophiobolin A (3) และ 6-*epi*-ophiobolin A (4) และสารในกลุ่ม cochlioquinones 3 ชนิด ได้แก่ isocochlioquinone A (5), isocochlioquinone C (6) และ anhydrocochlioquinone A (7) โดยสาร 7 ยังไม่เคยมีการรายงานมาก่อน การพิสูจน์หาสูตรโครงสร้างของสารทั้งหมดโดยวิธีทางสเปกโทรสโกปีและเปรียบเทียบกับข้อมูลที่เคยมีการรายงานไว้แล้ว นอกจากนี้ยังได้ทดสอบความเป็นพิษต่อเซลล์มะเร็งและฤทธิ์การยับยั้งการทำงานของเอนไซม์ acetylcholinesterase สาร 2 มีฤทธิ์ในการยับยั้งเซลล์มะเร็งทั้ง HeLa และ KB ดีที่สุด โดยมีค่า IC_{50}<0.1 และ 0.89 μg/mL ตามลำดับ ในส่วนของฤทธิ์การยับยั้งการทำงานของเอนไซม์ acetylcholinesterase สาร 1, 2, 3, 4, 5 และ 7 แสดงฤทธิ์การยับยั้งที่ระดับความเข้มข้น 1 mg/mL

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....กนกวรรณ สว่าง.....

ปีการศึกษา.....2549..... ลายมือชื่ออาจารย์ที่ปรึกษา.....สันติ ทิพยางค์.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....ปรีชา ภูวไพรศิริศาล.....

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KANOKWAN SAWANG : BIOACTIVE COMPOUNDS FROM FUNGUS *Bipolaris oryzae*. THESIS ADVISOR: ASSOC. PROF. SANTI TIP-PYANG, Ph.D., THESIS COADVISOR : ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 49 pp. ISBN 974-14-2539-2.

In the course of preliminary screening test for bioactive compounds from 11 plant pathogenic fungi, *Bipolaris oryzae* was selected for further investigation. The chromatographic separation of the ethyl acetate crude extracts results in the isolation of one new and six known compounds. They can be divided into two groups, four ophiobolins; 3-anhydroophiobolin A (**1**), ophiobolin I (**2**), ophiobolin A (**3**) and 6-*epi*-ophiobolin A (**4**), and three cochlioquinones; isocochlioquinone A (**5**), isocochlioquinone C (**6**) and anhydrocochlioquinone A (**7**). Compound **7** has not been previously reported in the literature. The structures of all isolated compounds were elucidated on the basis of spectroscopic methods, as well as comparison with previously reported. In addition, the cytotoxic activity against cell lines and acetylcholinesterase inhibition were investigated Compound **2** showed highest cytotoxic activity against HeLa and KB cell lines at IC_{50}<0.1 and 0.89 μg/mL, respectively. For the acetylcholinesterase inhibition assay, compound **1**, **2**, **3**, **4**, **5**, and **7** showed the potential inhibition at concentration of 1 mg/mL.

Field of Study.....Biotechnology.....Student's Signature.....Kanokwan Sawang.....

Academic Year.....2006.....Advisor's Signature.....Santi Tip-pyang.....

Co-advisor's Signature.....P. Phuwapraisirisan.....

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CONTENTS

Pages

Content (Continued)

Pages

List of Figures

Figures **Pages**

List of Schemes

Schemes **Pages**

List of Tables

Tables **Pages**

List of Abbreviations

^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
° C	degree of Celsius
cat. no.	catalogue number
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane, methylene chloride
COSY	correlated spectroscopy
d	doublet (NMR)
dd	doublet of doublet (NMR)
EtOAc	ethyl acetate
g	gram (s)
H_2O	water
HMBC	heteronuclear multiple bond correlation experiment
HPLC	high performance liquid chromatography
HRESIMS	high resolution electrospray ionization mass spectrometry
HSQC	heteronuclear multiple-quantum coherence experiment
Hz	Hertz
IC_{50}	inhibitory concentration 50%
J	coupling constant
NMR	nuclear magnetic resonance
L	liter (s)
m	multiplet (NMR)
MeOH	methanol
mg	milligram (s)
MIC	minimum inhibitory concentration
mL	milliliter (s)
mm	millimeter (s)
m/z	mass to charge ratio
M^+	molecular ion
ng	nanogram (s)
NOESY	the nuclear overhauser enhancement spectroscopy
ppm	part per million

List of Abbreviations (Continued)

q	quartet (NMR)
qnt	quintet (NMR)
s	singlet (NMR)
t	triplet (NMR)
TLC	thin layer chromatography
δ	chemical shift
δ_C	chemical shift of carbon
δ_H	chemical shift of proton
µg	microgram (s)
µL	microliter (s)
λ_{max}	maximum wavelength

CHAPTER I

INTRODUCTION

The microorganism is the source of bioactive compounds that are used in medicine such as penicillin useful for treatment following a potent antibiotic against gram-positive bacteria, which was produced by *Penicillium notatum*. A further milestone in the history of fungal products for medicinal use was the discovery of the immunosuppressant cyclosporine which is produced, e.g., by *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Dreyfuss *et al*., 1976). It was first discovered as an antifungal metabolite and later found to be immunosuppressive which made cyclosporine useful for the treatment following organ transplantation (Goodman *et al*., 1985). Streptomycin, tetracycline and erythromycin are from the streptomyces bacteria group. Besides, ivermectin from bacteria is used as anti-parasite.

So far more than 5,000 taxonomic species have been studied with respect to their chemistry (Hawksworth, 1991). In 1995, Hawksworth *et al*. estimated the probable number of existing fungi to be 1.5 million species with only 70,000 species being described. Obviously, the majority of fungi inhabiting the world has not been described yet. This associates fungi has the potential to represent a tremendous source of natural products within various chemical structures and activities. There is significant worldwide interest in the potential of fungi, particularly the ability to produce new bioactive compounds.

Nevertheless, the natural function of secondary metabolites is often unknown. It is assumed that they play an important role in chemical defence and communication (Krohn, 1996). Many of them have been suggested acting as pheromones, antifeedants or repellents, and as regulators in the development of organism (Sterner, 1995). Gloer (1995) suggested that the biosynthesis of secondary metabolites did not occur randomly but was correlated with ecological factors.

Fungal pathogens of plants often produce disease symptoms by elaborating one or more phytotoxins. These phytotoxins are part of secondary metabolite compounds. We became interested in fungal pathogens of graminous plants and elected to study *Bipolaris oryzae* because it caused leaf spots on rice (*Oryzae sativa*), an important cereal crop in all tropical and semitropical areas of the world. *Biopolaris*

is a fungal genus well known for the production of bioactive compounds, and most of the plant pathogenic species belonging to this genus, as well as their secondary metabolites, have been widely studied as agents of very severe diseases of gramineous plants. Some secondary metabolites were also isolated from plant pathogenic fungi and proposed as potential natural bioactive compounds. Considering the potential of the genus in producing bioactive metabolites and interest in finding new natural bioactive compounds produced by plant pathogenic fungi. In this study, the production, isolation, chemical and biological characterization of metabolites produced by *B. oryzae* were investigated.

1.1 *Bipolaris oryzae*

Bipolaris oryzae is one of plant pathogenic fungi (formerly known as *Helminthosporium oryzae, Drechslera oryzae and Cochliobolus miyabeanus*). It can damage all plant parts and decrease yield of rice which is an economic plant of Thailand. The morphology and life cycle of the fungus are complex. Conidia of *Bipolaris oryzae* is very small, club shaped to cylindrical, generally curved, light brown to golden brown, with 6 to 14 transverse cell walls.

Bipolaris oryzae is seedborne. It can also survive on infected rice straw and stubble. It spreads from plant to plant in the field by airborne spores. *Bipolaris oryzae* can infect some species of susceptible 23 grass genera. Disease development depends on high relative humidities (86-100%) and temperatures between 21 to 26 °C. Leaves must be continuously wet around 8 to 24 hours for infection to occur.

Symptoms of brown spot primarily appear as small circular to oval spots on the first seedling leaves. The environmental conditions, age of the spots, and the degree of susceptibility of the rice variety are shown in the growing season and may vary in size, shape and color. Large spots have a light, reddish-brown or gray center being surrounded by a dark to reddish-brown margin while small spots are dark brown to reddish brown. A bright yellow halo surrounding the lesion may appear on older spots. Spots on hulls and the leaf sheath are similar to those on the leaves.

Early brown spot lesions are difficult to distinguish from blast disease lesions, but mature blast lesions are usually diamond shaped or spindle. Virulently infected leaves may die before maturity and these plants will produce chalky kernels or lightweight. Infection occurring directly on the kernels will significantly reduce grain yield and quality. Infected glumes and panicle branches have a black discoloration (Figure 1.1).

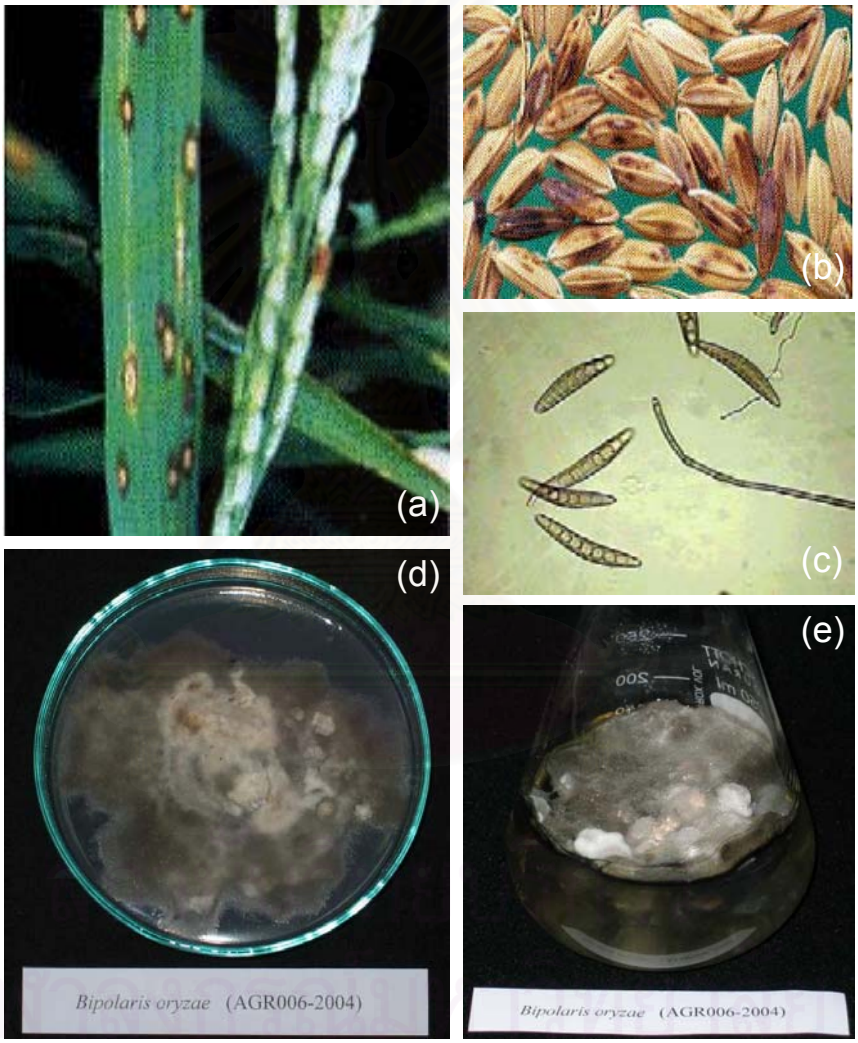
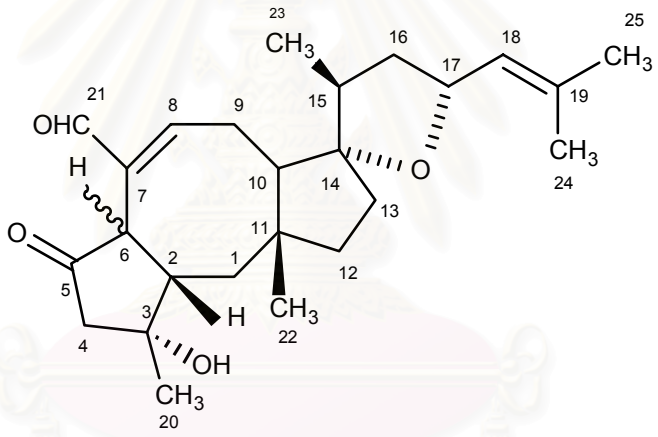


Figure 1.1 Brown spot symptoms on rice leaf (a) and grains (b), Conidia of rice brown spot disease pathogen (c), *B. oryzae* growth on semi-solid culture (d), *B. oryzae* growth on liquid broth culture (e).

1.2 Chemical constituent and biological investigations of *Bipolaris spp.*

1.2.1 Ophiobolins

Ophiobolins are sesterterpenoids having unique tricyclic 5-8-5 ring system (Evidente *et al*., 2006). They are produced mainly by *Bipolaris spp*. and other plant pathogenic fungi such as *Aspergilus ustus* and *Cephalosporium caerulens*. Ophiobolins have a broad spectrum of biological activity. They were identified as causative phytotoxins of brown spot lesions that attacked rice, maize and sorghum. Phytotoxic effect of ophiobolin is associated with inhibiting function of plasma membrane, β-1, 3 glucan synthelase, etc. However, Au *et al*. (2000) demonstrated an interaction between ophiobolin A and maize calmodulin and proposed that calmodulin was the target for the toxin.



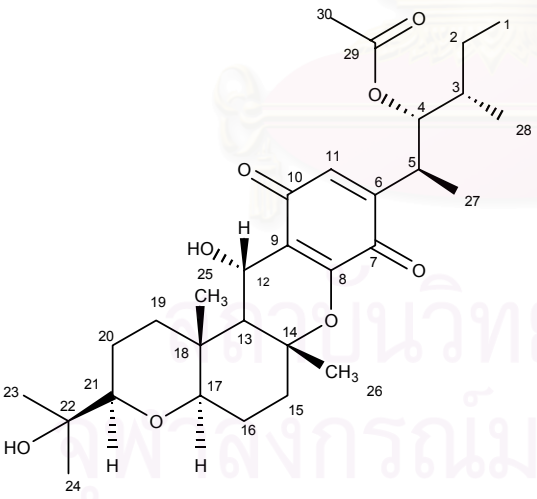
Ophiobolin A

Ophiobolins are also toxic to animals and microbes. Ophiobolin A showed cytotoxicity against cancer cell-lines A-549, HT-29, Mel-20 and cell-line P-388 (Shen *et al*., 1999) while ophiobolin K also showed cytotoxic activity against various tumor cell lines (NCI-H460, HOP18 (human lung carcinoma), MDA-MB-231, T-47D (human breast carcinoma), ACHN (human renal carcinoma), HCT116 (human colon carcinoma), P388 (mouse leukemia cells) and P388/ADR (adriamycin resistant cells) (Wei *et al*., 2004). Ophiobolins C, K and M possessed potent nematocidal activity (Au *et al*., 2000). Ophiobolins A and I were also reported as antibacterial and antifungal compounds (Li *et al*, 1995).

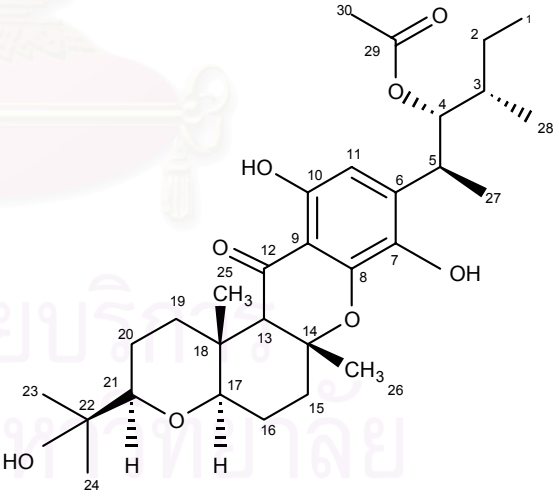
1.2.2 Cochlioquinones and Isocochlioquinones

Cochlioquinones and isocochlioquinones are terpenoid-derived quinones and hydroquinones isolated from *Bipolaris spp*. and related fungi. So far eleven compounds have been reported. Cochlioquinones A and B inhibited the growth of the malaria–causing protozoan *Plasmodium falciparum* while cochlioquinone B also reduced activity of enzyme tyrosine kinase $p56^{lek}$ (Osterhage *et al*., 2002).

Cochlioquinone A, cochlioquinone B and isocochlioquinone A were reported by Miyagawa *et al*. (1994). They inhibited the root growth of such plant seedlings as finger millet and rice. Especially, cochlioquinone A which exhibited the most potent activity against both plants subsequently cochlioquinone derivatives were studied by Lim *et al*. (1998), they inhibited the root growth and electron transfer in the mitochondrial respiratory system of gramineous plants. Jung *et al*. (2003) reported a newly isolated cochlioquinone A1 (CoA1) from the culture extract of *Bipolaris zeicola* as a potent anti-angiogenic agent of bovine aortic endothelial cells afterwards 17-methoxycochlioquinone A, cochlioquinone A, isocochlioquinone A, 11-O-methyl-epi-cochlioquinone A and *epi*-cochlioquinone A were discovered by Yoganathan *et al*. (2004) that are competed effectively with MIP-1α for binding to human CCR5.



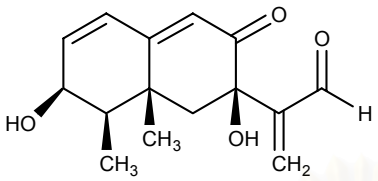
Cochlioquinone A



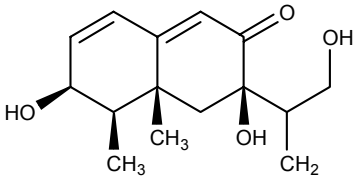
Isocochlioquinone A

1.2.3 Miscellaneous

In 1985, two sesquiterpenes named bipolaroxin and dihydrobipolaroxin were isolated from fungus *Bipolaris cynodontis* as phytotoxins against Bermuda grass (Sugawara *et al*.).

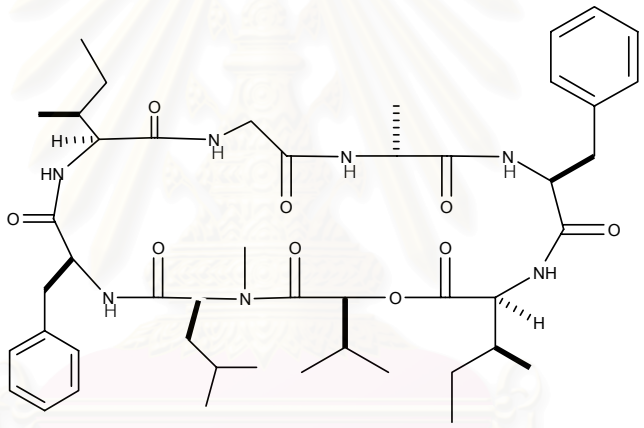


Bipolaroxin



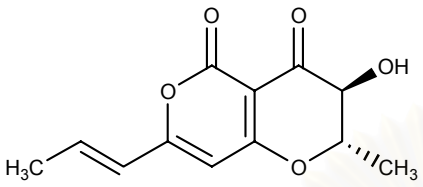
Dihydrobipolaroxin

BZR-Cotoxin IV (Ueda *et al*., 1995) was isolated from *Bipolaris zeicola* race 3, which is the pathogen of leaf spot disease in corn. It exhibited potent phytotoxicity and susceptibility-inducing activity in rice.

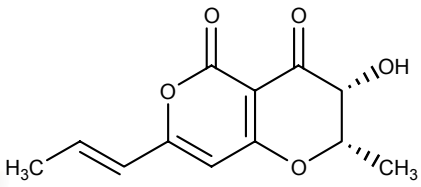


BZR-Cotoxin IV

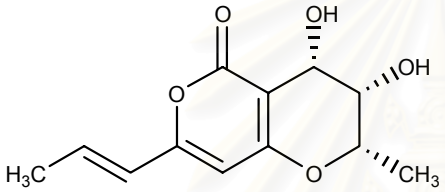
Radicinin and its diastereomers were isolated and characterized by Nakajima *et al*. (1997) from the phytopathogenic fungus *Bipolaris coicis H*-13-3. They were phytotoxic against Job's tears (*Coix lachryma-jobi*); a host of this fungus, but a diastereomer of radicinol and its epoxide were not phytotoxic.



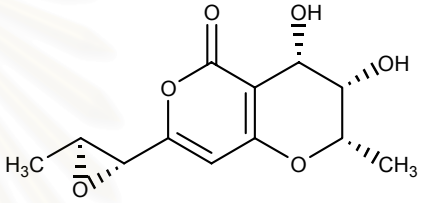
Radicinin



Compound 2

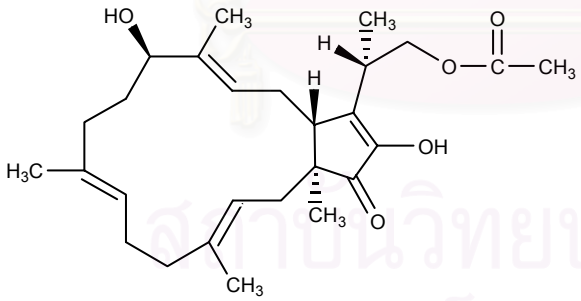


Compound 3a

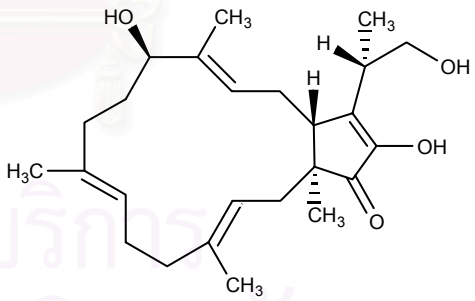


Compound 5

In 2002, 11-epiterpestacin was isolated from fungus *Bipolaris sorokiniana* by Nihashi *et al*. It is an epimer of terpestacin that has been isolated from *Arthrinium* sp.



11-Epiterpestacin



Terpestacin

As a result of preliminary screening, *B. oryzae* extract may contain compounds that could be developed for medicinal and agricultural uses. An attempt to identify bioactive metabolites present in *B. oryzae* is the principal goal of this research.

CHAPTER II

PRELIMINARY SCREENING FOR BIOACTIVE COMPOUNDS FROM PLANT PATHOGENIC FUNGI

Antibacterial and antifungal activity test have been widely used to accurately measure activity and are routinely used in susceptibility testing. These methods are simple and rapid for screening plant pathogenic fungi which have potent activity. Therefore, Antibacterial and antifungal activity test were chosen in the course of preliminary screening for bioactive compounds.

2.1 Preliminary screening results

The preliminary screening results of ethyl acetate extracts from culture broth of 11 plant pathogenic fungi (*Pestalotiopsis sp., Alternaria porri, Bipolaris oryzae, Phomopsis asparagi, Fusarium oxysporum, Corynespora cassiicola, Phytophthora palmivora, Colletotrichum gloeosporioides, Stemphylium vasicarium, Alternaria brassicola* and *Exserohilum turcicum*) are presented in Table 2.1.

Table 2.1 Antibacterial activity of ethyl acetate extracts from culture broth of plant pathogenic fungi.

Plant pathogenic fungi	Antibacterial activity (*)				
	Stephylococcus aureus	*Bacillus cereus*	*E. coli*	*Salmonella spp.*	*Listeria monocytogenes*
Pestalotiopsis sp.	0	8	0	0	0
A. porri	24	17	0	0	0
B. oryzae	12	15	0	0	8
P. asparagi	8	8	0	0	0
F. oxysporum	12	10	0	0	12
C. cassiicola	0	0	0	0	0
P. palmivora	0	0	0	0	0
C. gloeosporoides	0	0	0	0	0
S. vasicarium	11	14	0	0	0
A. brassicola	0	0	0	0	0
E. turcicum	0	0	0	0	0
Tetracycline (**) (30 µg)	27	20	18	20	32

* Inhibitory zones were recorded in mm.

** Positive antibacterial compound

Result from Table 2.1, *A. porri* and *B. oryzae* showed high activity against bacterial consequently both crude extracts were taken for preliminary antifungal screening test against pathogenic fungi *S. vesicarium, E. turcicum* and *A. brassicicola*. The antifungal results are summarized in Table 2.2.

Table 2.2 Antifungal activity of ethyl acetate extracts from culture broth of plant pathogenic fungi.

Fungal	Crude extract from *A. porri*	Crude extract of *B. oryzae*	Distilled water	Captan**
S. vesicarium	1.18*	1.33	< 0	0.65
E. turcicum	1.25	1.22	< 0	0.80
Pestalotiopsis sp.	0.48	0.30	< 0	0.25
A. brassicicola	1.28	1.32	< 0	0.77

* Inhibitory zones were recorded in mm.

** Positive antifungal compound

2.2 Experiment Section

2.2.1 Fungal material and culture condition

The plant pathogenic fungi were provided by Department of Plant Pathology, Faculty of Agriculture, Kasetsart University (*Pestalotiopsis sp., Phomopsis asparagi, Colletotrichum gloeosporioides, Phytophthora palmivora* and *Lasiodiplodia theobromae*) and Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand (*Bipolaris oryzae, Alternaria porri, Fusarium oxysporum, Corynespora cassiicola, Stemphylium vasicarium, Alternaria brassicola, Exserohilum turcicum*). Plant pathogenic fungi strain were maintained on malt extract agar (MEA) containing malt extract 20 g, agar 15 g, peptone 1 g, dextrose 20 g and distilled water 1 L. Each strain was cultured only one liter. After 3 days, Erlenmeyer flask (250 mL) containing malt extract broth (MEB) were inoculated with fungal mycelium (1 cm^2) taken from an agar plate containing MEA medium. The flasks had been shaken for 2 days. Then, they cultured under static condition at 25 ºC for 21 days.

2.2.2 Sample preparation for preliminary screening

The culture of MEB medium is filtered, and then the filtrate was extracted with ethyl acetate. The EtOAc layer was evaporated under reduced pressure to give an EtOAc extract.

2.2.3 Antibacterial activity test

Each crude extract from culture broth of plant pathogenic fungi was tested for antibacterial activity using paper disc method. This assay was performed at Food Research and Product development, Kasetsart University, Bangkok, Thailand. The results were shown in Table 2.1.

2.2.4 Antifungal activity test by using paper disc method

Paper disc method was used for antifungal assay. Potato dextrose agar (PDA) was prepared on Petri dish which containing with each fungi *A. brassicicola, E. turcicum, Pestalotiopsis sp.* and *Stemphylium vasicarium* on the center of Petri dish. Paper disc was dropped by crude extracts of *B. oryzae* and *A. porri* about 20 μl (concentration 5 mg/mL). Distilled water was used as a control paper disc and fungicide were used as a comparison paper disc. Paper dish was dried by hot air oven at 30 ºC for 5 minutes. Then, each paper disc was placed on PDA and incubated for 1 week. The antifungal activity was measured from the edge of mycelium to the edge of the inhibitory zones. The experiment was repeated twice and the results were averaged in mm. The results were shown in Table 2.2.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *Bipolaris oryzae*

The results from primary screening of ethyl acetate crude extract from culture broth of plant pathogenic fungi, *B. oryzae* showed high antibacterial and antifungal activities. It appears that, TLC pattern of fungi *B. oryzae* also shown the good candidate which met the selection criteria. Therefore, *B. oryzae* was selected for further investigation. The aim of this study was to investigate whether plant pathogenic fungi and *B. oryzae* could be an interesting and, perhaps, unique source of new secondary metabolites. The cultivation media was tested in order to find the most suitable one for the production of natural products. The culture media variation on malt extract broth (Smith and Onions, 1983), yeast extract-glucose-oatmeal broth (Yoganathan *et al*., 2004), rice polish broth (Tuite, 1969), potato glucose broth (Tuite, 1969) and potato dextrose broth (Smith and Onions, 1983) and produced yields 145.2, 119.5, 39.5, 34.5 and 31.0 mg/L, respectively.

Therefore, the media were based on malt extract for mass production of ethyl acetate crude extract from *B. oryzae* culture broth. The malt extract medium, artificial medium was added in order to adapt culture conditions to the natural environment of fungi. TLC comparison of culture extracts of media revealed that the *B. oryzae* has produced the highest activity of metabolites on malt extract medium, as evidenced by antibacterial and antifungal activity of substances. Following this investigation, it was decided to cultivate and then analysed extracts of fungi only grown on malt extract broth. After cultured fungus *B. oryzae* in malt extract agar 69 liters, 21 days (The maximum production of compounds occurred after 14 days of incubation, some compound was not detected until day 10). If cultures were incubated over 3 weeks, the extracts were no longer completely separable under HPLC analysis, (Sugawara *et al*., 1987), and 25 °C they gave ethyl acetate crude extract 10 g.

Ethyl acetate crude extracts (10 g) were preliminary separated by quick column chromatography to yield 6 fractions (F1-F6). All fractions were tested for antifungal activity using TLC bioautography assay (developed in 30% EtOAc-CH_2Cl_2 solvent system). After being developed on the TLC plate, fractions F1 and F2 did not show any antifungal activity and moved to solvent front. Fraction F3 and F4 showed inhibition zone which have a consistency of bioactive compounds against the fungal while fraction F5 and F6 are inactive compounds. (Figure 3.1).

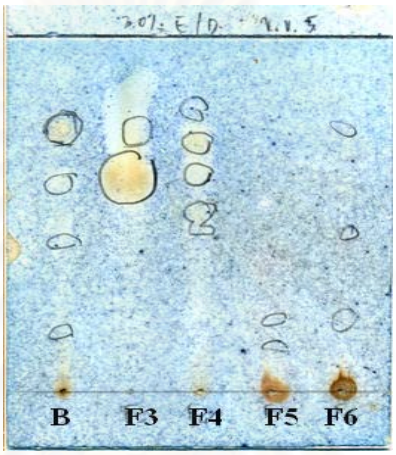


Figure 3.1 The result of TLC-bioautography screening of fraction F3 and F4. This figure showed the appearance of chromatogram after treatment with an inoculum of *Alternaria alternata*, indicating the location of fungal inhibition zone tended to be active compound.

3.1 Extraction and purification

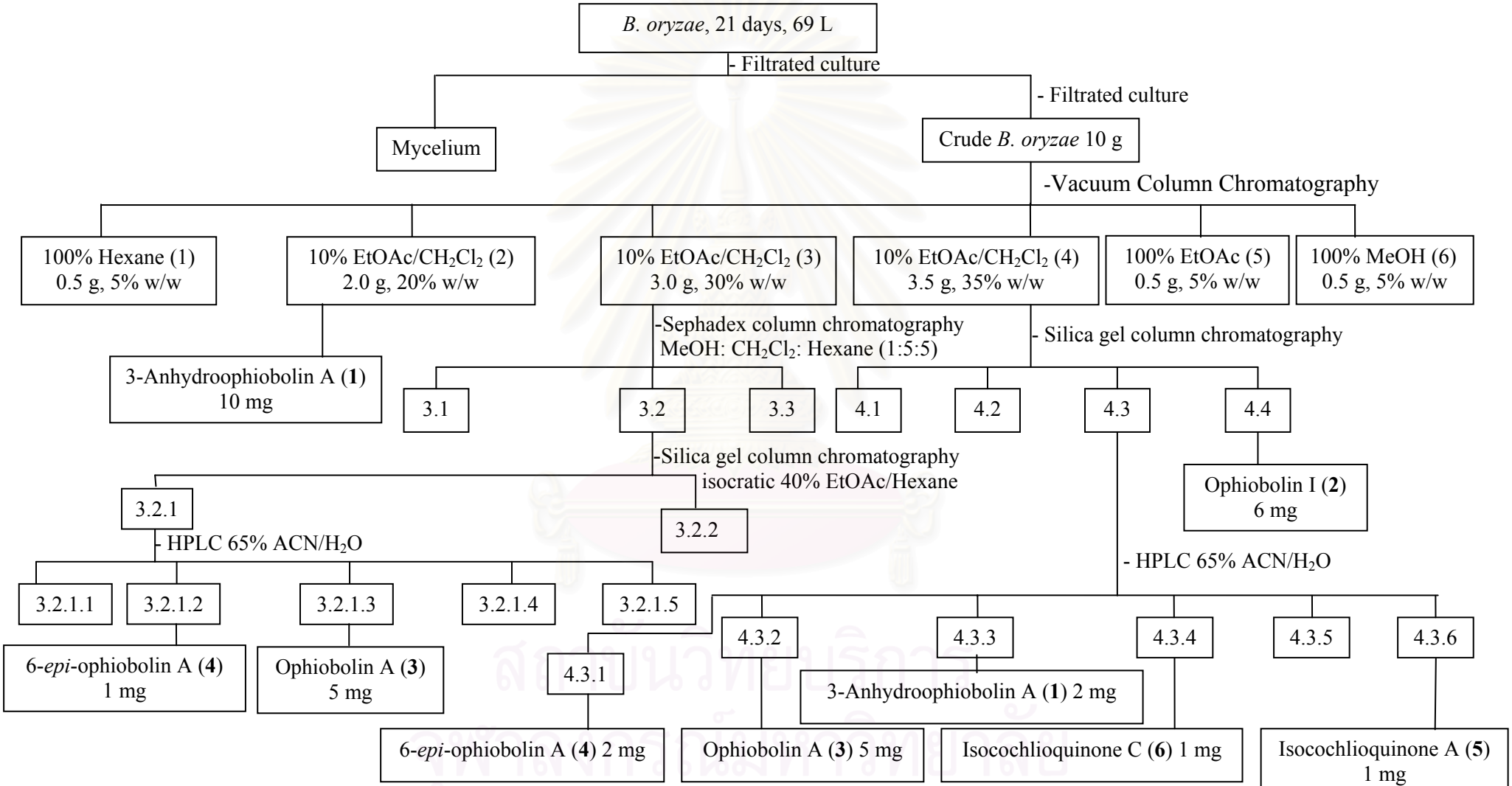
The crude EtOAc extract (10.0 g) was purified employing a combination of chromatographic techniques. First, it was passed over normal phase Si gel (vacuum liquid chromatography, VLC) using gradient elution from *n*-hexane to 10% EtOAc-CH_2Cl_2 to EtOAc to yield six fractions.

Fraction 2 yielded 3-anhydroophiobolin A (**1**, 10 mg) (Pena-Rodriguez and Chilton, 1989; Li *et al*., 1995).

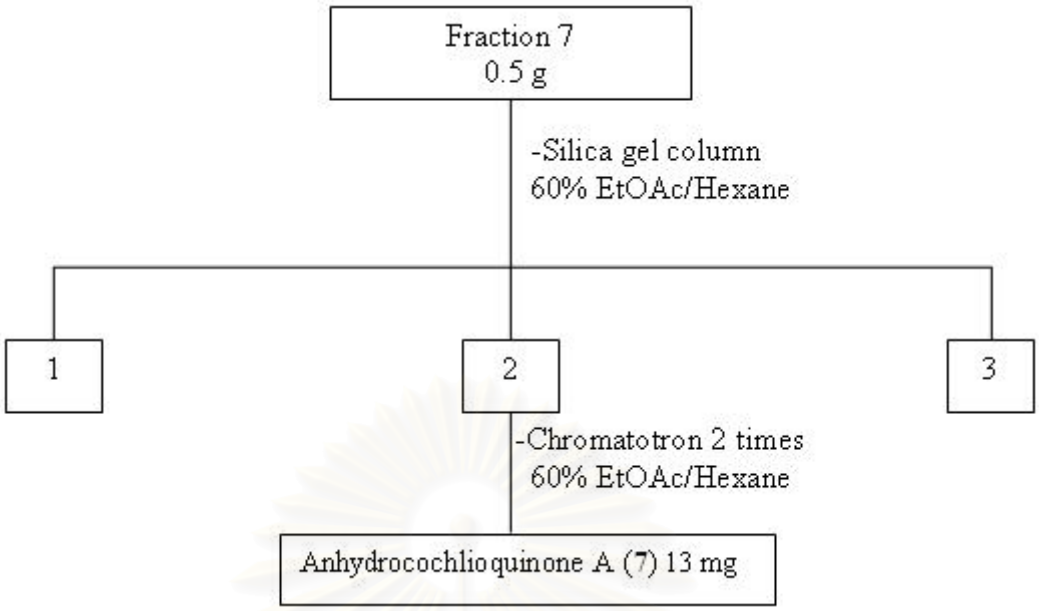
Fraction 3 was subsequently purified on Sephadex LH-20 [*n*-hexane-CH_2Cl_2-MeOH (5:5:1)], silica gel column chromatography 40% EtOAc-hexane and HPLC [Cosmosil 5C18-ARII, 10×250 mm, 65% ACN-H_2O] to obtain 6-*epi*-ophiobolin A (**4**, 1 mg) (Sugawara *et al*., 1987; Li *et al*., 1995) and ophiobolin A (**3**, 5 mg) (Li *et al*., 1995; Leung *et al*., 1984).

Fraction 4 was chromatographed on a silica gel column chromatography eluted with *n*-hexane-EtOAc to yield four fractions (4.1-4.4). Fraction 4.3 eluted with 60% EtOAc-hexane was further purified by HPLC [Cosmosil 5C18-ARII, 10×250 mm, 65% ACN-H_2O] to yield 6-*epi*-ophiobolin A (**4**, 1 mg), ophiobolin A (**3**, 5 mg), isocochlioquinone C (**6**, 1 mg) (Lim *et al*., 1994) and isocochlioquinone A (**5**, 1 mg) (Miyagawa *et al*., 1994), respectively. Fraction 4.4 eluted with 100% EtOAc afforded ophiobolin I (**2**, 6 mg) (Yun *et al*., 1988; Li *et a*l., 1995).

The remaining residue from all fractions of crude EtOAc extract were recombinded to get fraction 7 (0.5 g). Fraction 7 eluted with 60% EtOAc-hexane and the further purified by silica gel column chromatography. The final fraction was purified by chromatotron for twice to obtain anhydrocochlioquinone A (**7**, 13 mg). The extraction procedure was summarized in scheme 3.1 and 3.2.



Scheme 3.1 The isolation procedure of EtOAc crude extract.



Scheme 3.2 The isolation procedure of fraction 7

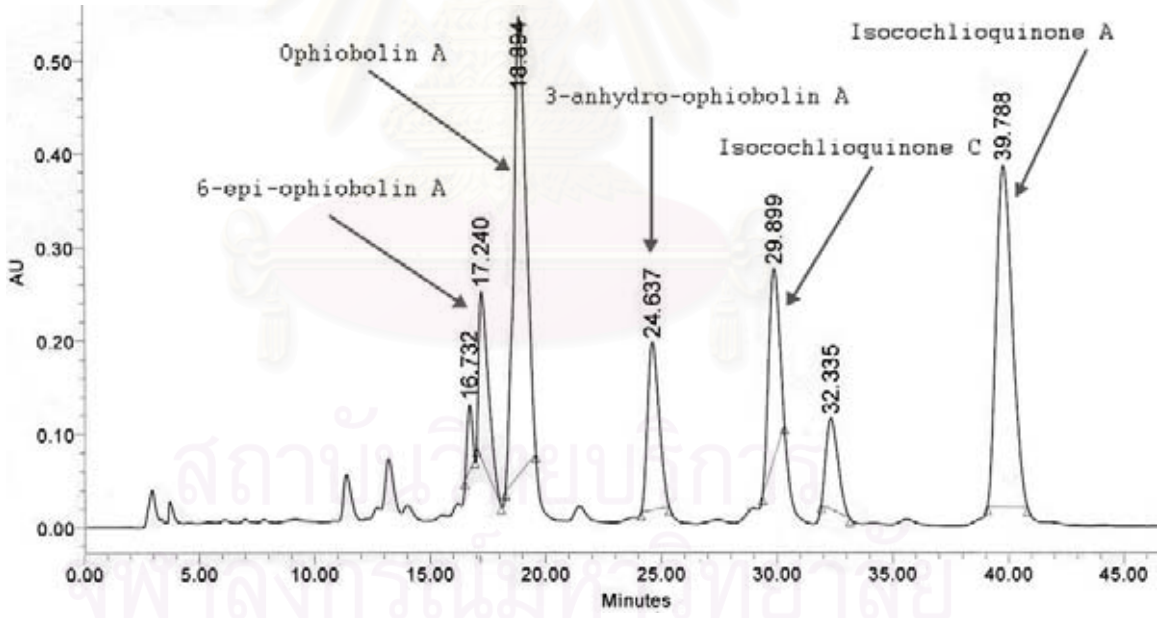
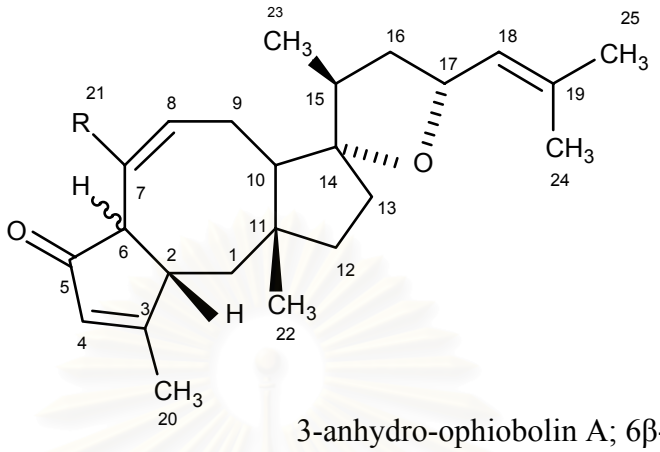


Figure 3.2 Chromatogram of 5 compounds separated by HPLC

The structural identification of known compounds (**1-6**) confirmed by comparison of their NMR data with those previously reported.



3-anhydro-ophiobolin A; 6β-H, R = CHO

Ophiobolin I; 6α-H, R = CH_2OH

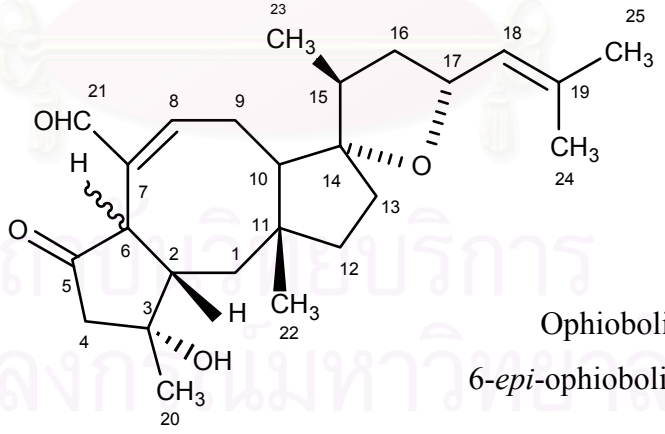
Figure 3.3 The structure of 3-anhydro-ophiobolin A (**1**) and ophiobolin I (**2**)

Table 3.1 The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of compound (**1**) and 3-anhydro-ophiobolin A in $CDCl_3$ (Pena-Rodriguez and Chilton, 1989; Li *et al*., 1995).

Position	Chemical shift (ppm)			
	^{13}C		^{1}H (mult, *J* in Hz)	
	compound (**1**)	3-anhydro-ophiobolin A	compound (**1**)	3-anhydro-ophiobolin A
1	46.8	36.4	-	-
2	49.3	48.2	-	-
3	177.3	176.1	-	-
4	130.4	131.4	6.05	6.11 m
5	207.1	207.2	-	-
6	49.2	47.9	3.45 d 4.0	3.85 d 6.9
7	141.0	139.4	-	-
8	155.3	159.7	6.85 m	7.06 dd 8.5, 8.5
9	30.7	30.7	-	-
10	53.9	53.8	-	-
11	42.5	44.3	-	-
12	41.8	39.4	-	-
13	42.2	42.8	-	-
14	96.1	94.7	-	-
15	35.5	36.7	-	-
16	35.5	30.7	-	-
17	72.0	71.4	4.58 m	4.43 ddd 6.7, 6.7, 6.7
18	126.8	126.7	5.13 m	5.13 m
19	135.3	134.5	-	-
20	17.2	25.7	2.07 s	2.12 s
21-CHO	192.9	193.8	9.33 s	-
22	22.4	18.2	-	-
23	16.3	17.4	1.06 d 6.8	1.01 d 7.0
24	18.2	18.2	1.68 d 0.8	1.63 d 1.2
25	25.9	25.7	1.72 s	1.69 s

Table 3.2 The ^{1}H NMR (400 MHz) data of compound (**2**) and ophiobolin I in $CDCl_3$ (Yun *et al*., 1988; Li *et a*l., 1995).

Position	Chemical shift (ppm)	
	compound (**2**)	ophiobolin I
2	2.71 d 12.4	2.71 m
4	5.89 s	5.94 brs
6	3.60 s	3.64 d 3.0
8	5.72 d 5.2	5.76 m
17	4.54 q	4.56 ddd 6.7, 6.7, 6.7
18	5.08 d 8.8	5.12 dq 8.7, 1.2
20	2.02 s	2.06 s
21-CH_2OH	3.87 d 12.0	3.88 d 1.9
	4.11 d 12.0	4.15 d 11.9
22	0.93 s	0.97 s
23	0.97 d 7.2	1.3 d 7.0
24	1.60 s	1.64 d 1.2
25	1.64 s	1.68 d 1.2



Ophiobolin A; 6β-H

6-*epi*-ophiobolin A; 6α-H

Figure 3.4 The structure of ophiobolin A (**3**) and 6-*epi*-ophiobolin A (**4**)

Table 3.3 The ^{1}H NMR (400 MHz) data of compound (**3**) and ophiobolin A in $CDCl_3$ (Li *et al*., 1995; Leung *et al*., 1984).

Position	Chemical shift (ppm)	
	compound (**3**)	ophiobolin A
1	1.29 s	1.28 dd 14.8, 12.5
2	2.32 m	2.30 m
4	2.52 d 19.2	2.44 d 19.3
	-	2.74 d 19.3
6	3.24 d 10.8	3.21 d 10.8
8	7.19 t 8.4	7.16 dd 8.5, 8.5
9	2.20 m	2.20 m
	2.41 m	2.40 m
12	1.36 m	1.36 m
15	1.76-1.81	1.60-1.80
	2.16 m	2.10 m
16	2.01 m	1.56 m
	-	2.00 ddd 13.8, 12.5, 8.7
17	4.38 m	4.37 ddd 8.7, 8., 6.0
18	5.13 d 8.4	5.10 d 8.7
20	1.32 s	1.31 s
21-CHO	9.23 s	9.18 s
22	0.81 s	0.78 s
23	1.09 d 7.2	1.04 d 7.0
24	1.65 s	1.65 d 1.2
25	1.7 d 1.2	1.69 d 1.2

Table 3.4 The ^{1}H NMR (400 MHz) data of compound (**4**) and 6-*epi*-ophiobolin A in $CDCl_3$ (Sugawara *et al*., 1987; Li *et al*., 1995)

Position	Chemical shift (ppm)	
	compound (**4**)	6-*epi*-ophiobolin A
2	2.10 m	-
4	2.39 s	2.38 m
	3.05 d 16.8	3.04 d 16.8
6	3.24 d 10.5	3.35 brd 10.5
8	6.84 d 6.4	6.87 m
9	2.75 d 19.6	2.77 m
10	2.61 dd 14.4, 4.0	2.60 dd 13.8, 4.2
17	4.61 dd 7.4, 15.6	4.60 ddd 9.0, 7.0, 7.0
18	5.14 d 8.4	5.13 dq 9.0, 1.5
20	1.40 s	1.41 s
21-CHO	9.20 s	-
22	0.84 s	0.84 s
23	1.04 d 6.8	1.03 d 6.9
24	1.66 s	1.66 d 1.2
	1.70 s	-
25	-	1.70 d 1.2

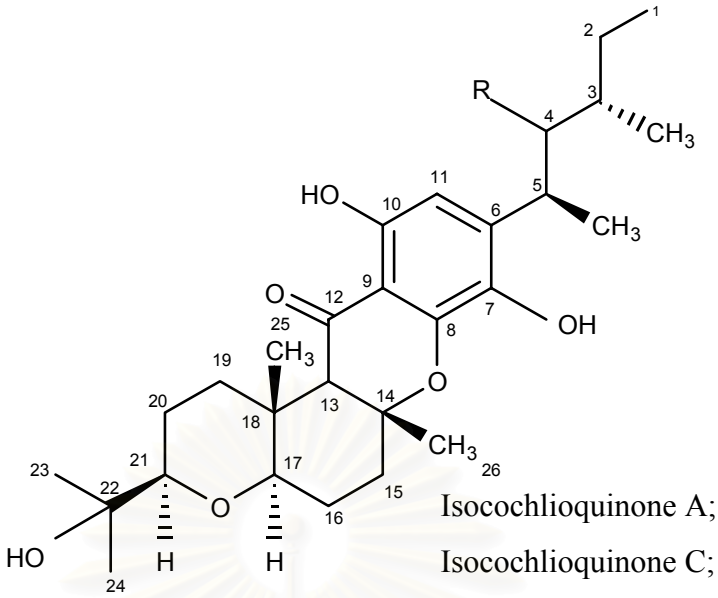


Figure 3.5 The structure of isocochlioquinone A (**5**) and isocochlioquinone C (**6**)

Table 3.5 The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of compound (**5**) and isocochlioquinone A in $CDCl_3$ (Miyagawa *et al*., 1994).

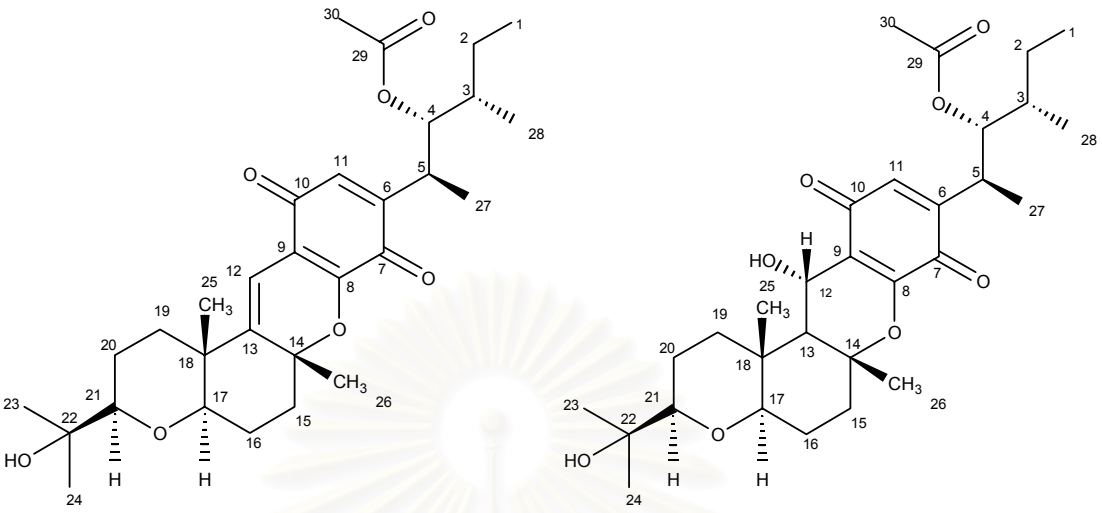
Position	Chemical shift (ppm)			
	^{13}C		^{1}H (mult, *J* in Hz)	
	compound (**5**)	isocochlioquinone A	compound (**5**)	isocochlioquinone A
1	17.2	17.5	0.92	0.89 t 7.0
2	26.4	26.5	1.25 s	1.25 t
3	36.3	36.2	1.64	1.63 t
4	79.1	79.0	5.17 dd 5.2, 6.8	5.18 dd 5.0, 7.5
5	35.4	35.5	3.47 qnt	3.46 qnt 7.0
6	140.0	140.2	-	-
7-OH	135.2	135.3	5.28 s	5.22
8	143.9	144.0	-	-
9	107.0	107.0	-	-
10-OH	153.2	153.2	10.76 s	10.76
11	108.2	108.2	6.40 s	6.40 s
12	194.4	198.5	-	-
13	60.4	60.5	2.76 s	2.77 s

14	83.5	83.3	-	-
15	37.6	37.6	2.06 *	2.06 t
16	25.0	25.0	-	1.60 t, 1.80 t
17	83.7	83.6	3.15 dd 11.6, 3.2	3.16 dd 12, 3.8
18	35.5	35.6	-	-
19	37.3	37.3	-	1.30 t, 2.75 t
20	21.3	21.3	1.58 s	1.60 t
21	85.3	85.5	3.26 dd 11.6, 2.4	3.27 dd 12.5, 2.5
22-OH	72.0	72.0	2.52 s	2.58 bs
23	23.7	23.8	-	1.19 s
24	26.0	26.0	-	1.19 s
25	12.3	12.5	1.17 s	1.13 s
26	22.0	22.0	1.50 s	1.46 s
27	17.3	17.5	1.20 d 8.0	1.20 d 7.0
28	13.4	13.5	0.90 d 6.8	0.88 d 6.8
29	170.6	170.5	-	-
30	20.9	20.8	1.97 s	1.93 s

***** overlapped

Isocochlioquinone C (6): yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85 (t, 1H), 0.94 (d, 1H, *J* =6.4 Hz), 1.29 (s, 1H), 1.19 (s, 1H), 1.25 (s, 1H), 1.34 (d, 1H, *J* =6.8 Hz), 1.49 (s, 1H), 2.09 (m, 2H), 2.57 (m, 1H), 2.72 (overlapped, 1H), 2.78 (s), 3.13 (dd, 1H, *J* =12.4, 3.6 Hz), 3.50 (dd, 1H, *J* =11.6, 0.8 Hz), 4.24 (q, 1H), 5.38 (s, 1H) and 6.26 (s, 1H), 10.80 (s, 1H) (Lim *et al*., 1994).

Structure elucidation of anhydrocochlioquinone A (7)



anhydrocochlioquinone A cochlioquinone A

Figure 3.6 The structure of anhydrocochlioquinone A (**7**, new compound) and cochlioquinone A

Compound (**7**) was obtained as a red solid and had the molecular formula $C_{30}H_{42}O_7$ (M^+ at *m/z* 514.2826) on the basis of HRESIMS. The UV (MeOH) showed the absorbance at λ_{max} 271, 483 nm.

The ^{1}H NMR spectrum ($CDCl_3$) of compound (**7**) showed three oxymethine groups (δ_H 4.95, dd, J = 5.2, 7.2 Hz, H-4; 3.14, m, H-17; 3.11, m, H-21), eight methyl groups (δ_H 0.82, overlapped, H-1; 1.14, s, H-23; 1.12, s, H-24; 1.05, s, H-25; 1.48, s, H-26; 1.08, d, J = 7.2 Hz, H-27; 0.80, overlapped, H-28; 1.91, s, H-30). From the ^{1}H NMR spectrum, cochiloquinone A (Miyagawa *et al*., 1994) contained 2 signals with chemical shifts at δ 4.93 (J = 10.0, 1.3 Hz) for H-12 and 3.78 (J = 1.3 Hz) for H-13 while compound (**7**) had only 1 proton signal observed at chemical shifts at δ 6.24 as a singlet of H-12 and had no proton signal at H-13. In this instance, the methine proton at C-12 of compound (**7**), suggesting the absence of hydroxyl group as is present in cochlioquinone A.

The ^{13}C NMR spectrum ($CDCl_3$) of compound (**7**) showed eight methylene carbon (δ_C 11.5, C-1; 26.1, C-23; 23.8, C-24; 20.2, C-25; 26.9, C-26; 17.5, C-27; 13.3, C-28; 20.8, C-30), and one acetate carbon (δ_C 170.6, C-29). The chemical shifts of 30 signals were similar to the signal from C-1 to C-11, C-14 to C-24 and C-26 to

C-30 of cochlioquinone A (Miyagawa *et al*., 1994), suggesting that compound (**7**) to be an analog or derivative of cochiloquinone A.

Structure of compound (**7**) was confirmed by HMBC cross peak of H-11 to C-5 (δ 34.80), C-7 (δ 181.10) and C-9 (δ 117.00), cross peak of H-5 to C-4 (δ 78.50), C-6 (δ 148.50), C-7 (δ 181.10) and C-11 (δ 132.40), cross peak of H-4 to C-2 (δ 26.30), C-3 (δ 36.20), C-5 (δ 34.80), C-6 (δ 148.50), C-27 (δ 17.50), C-28 (δ 13.30) and COO, and cross peak of H-12 to C-8 (δ 149.0), C-10 (δ 148.8), C-14 (δ 81.5), C-18 (δ 38.6). All the NMR data of (**7**) were shown in Table 3.1.

The relative configuration of compound (**7**) was deduced by NOESY data analysis; the correlations of H-12 and CH_3-25, and between CH_3-25 and CH_3-26 indicated that resided the same plane. From ^{13}C NMR and ^{1}H NMR spectrum of compound (**7**), representing the absence of OH and H at C-12 and C-13, respectively. The chemical shift of chochlioquinone A (Miyagawa *et al*., 1994) at C-25 showed value at δ 12.6 while compound (**7**) showed value at δ 20.2. The higher field chemical shift of the methyl group (C-25) cochlioquinone A might be shielded from the hydroxyl group. Based on 2-D NMR data (HMQC, HMBC, COSY and NOESY) and all above data, compound (**7**) was identified as anhydrocochlioquinone A. To our knowledge, compound (**7**) has not been previously reported in the literature (new compound).

Table 3.6 The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of anhydrocochlioquinone A (**7**) in $CDCl_3$

Position	Chemical shift (ppm)		
	Anhydrocochlioquinone A (**7**)		
	^{13}C	^{1}H (mult, *J* in Hz)	HMBC
1	11.5	0.82 *	C-2, 3
2	26.3	1.13 m	C-1, 3, 28
		1.27 m	C-1, 3, 28
3	36.2	1.54 m	C-2, 28
4	78.5	4.95 dd 5.2, 7.2	C-2, 3, 5, 6, 27, 28, COO
5	34.8	3.20 m	C-4, 6, 7, 11
6	148.5	-	
7	181.1	-	
8	149.0	-	
9	117.0	-	
10	184.8	-	
11	132.4	6.47 s	C-5, 7, 9
12	110.5	6.24 s	C-8, 10, 14, 18
13	147.5	-	
14	81.5	-	
15	37.6	1.95 m	C-14, 16
		2.22 m	C-13, 14, 17
16	24.5	1.63 m	
		1.75 m	
17	81.0	3.14 m	C-13, 19, 21, 25
18	38.6	-	
19	34.8	1.48 m	
		2.05 m	C-20, 21, 17
20	21.6	1.55 m	
		1.66 m	C-21, 22

21	84.5	3.11 m	C-17, 19, 22, 23, 24
22	71.8	-	
23	26.1	1.14 s	C-21, 22, 24
24	23.8	1.12 s	C-21, 22, 23,
25	20.2	1.05 s	C-13, 17, 18, 19
26	26.9	1.48 s	C-13, 14, 15
27	17.5	1.08 d 7.2	C-4, 5, 6
28	13.3	0.80 *	C-2, 3, 4
CO_2CH_3	170.6		
30	20.8	1.91 s	COO

* overlapped

Table 3.7 The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of Anhydrocochlioquinone A (**7**) compare with cochlioquinone A (Miyagawa *et al*., 1994) in $CDCl_3$.

Position	Chemical shift (ppm)			
	^{13}C		^{1}H (mult, *J* in Hz)	
	compound (**7**)	cochlioquinone A	compound (**7**)	cochlioquinone A
1	11.5	11.4	0.82 *	0.88 t 7.0
2	26.3	26.4	1.13 m	1.35
			1.27 m	
3	36.2	36.2	1.54 m	1.60
4	78.5	78.2	4.95 dd 5.2, 7.2	5.01 dd 5.0,7.0
5	34.8	34.6	3.20 m	3.22
6	148.5	148.3	-	
7	181.1	181.5	-	
8	149.0	151.5	-	
9	117.0	119.0	-	
10	184.8	188.5	-	
11	132.4	133.6	6.47 s	6.54 s
12	110.5	63.0	6.24 s	4.93 dd 10.0, 1.3

				3.78 d 1.3
13	147.5	51.8	-	1.72 d 10.0
14	81.5	83.0	-	
15	37.6	37.5	1.95 m	1.91 dt 13.3, 3.8
			2.22 m	2.09 dt 13.3, 3.3
16	24.5	25.2	1.63 m	1.56
			1.75 m	1.79
17	81.0	83.8	3.14 m	3.17 dd 12.5, 3.8
18	38.6	36.7	-	
19	34.8	38.5	1.48 m	1.40
			2.05 m	2.47 m
20	21.6	21.5	1.55 m	1.43
			1.66 m	1.65
21	84.5	85.1	3.11 m	3.25 dd 12.5, 2.5
22	71.8	71.7	-	2.55
23	26.1	24.0	1.14 s	1.17 s
24	23.8	25.9	1.12 s	1.18 s
25	20.2	12.6	1.05 s	1.01 s
26	26.9	21.0	1.48 s	1.32 s
27	17.5	17.2	1.08 d 7.2	1.14 d 6.8
28	13.3	13.2	0.80 *	0.87 d 7.0
CO_2CH_3	170.6	170.3		
30	20.8	20.7	1.91 s	1.98 s

* overlapped

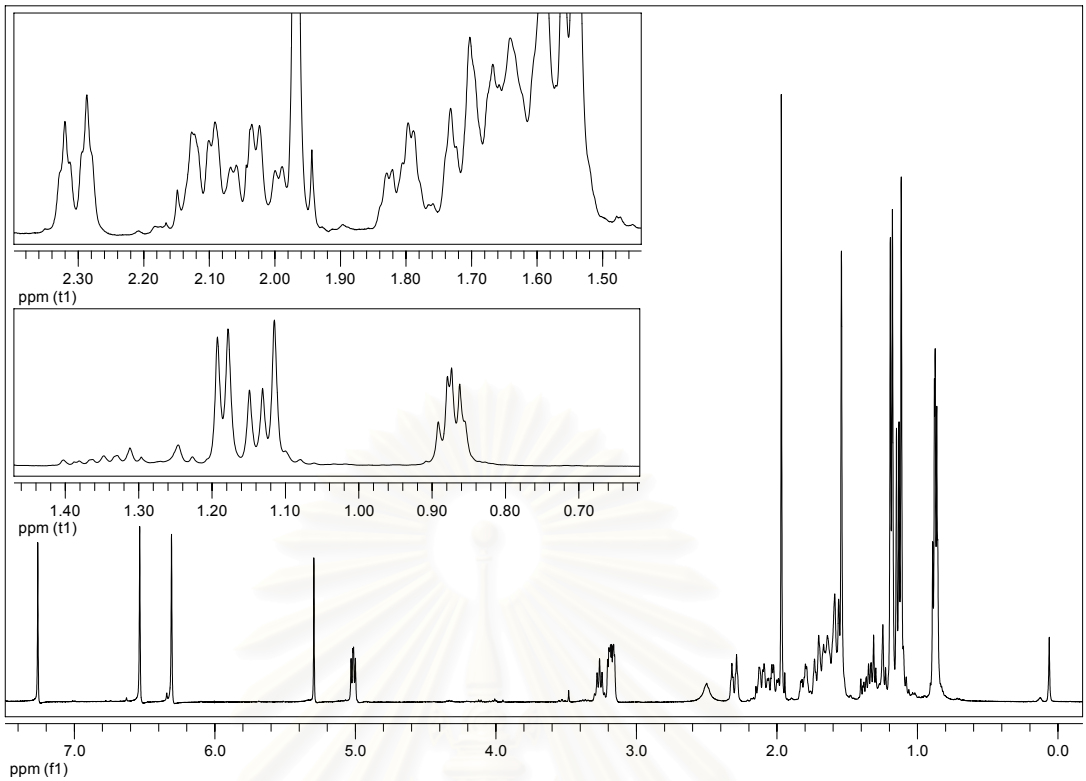


Figure 3.7 The ^{1}H NMR spectrum of compound (**7**) in $CDCl_3$

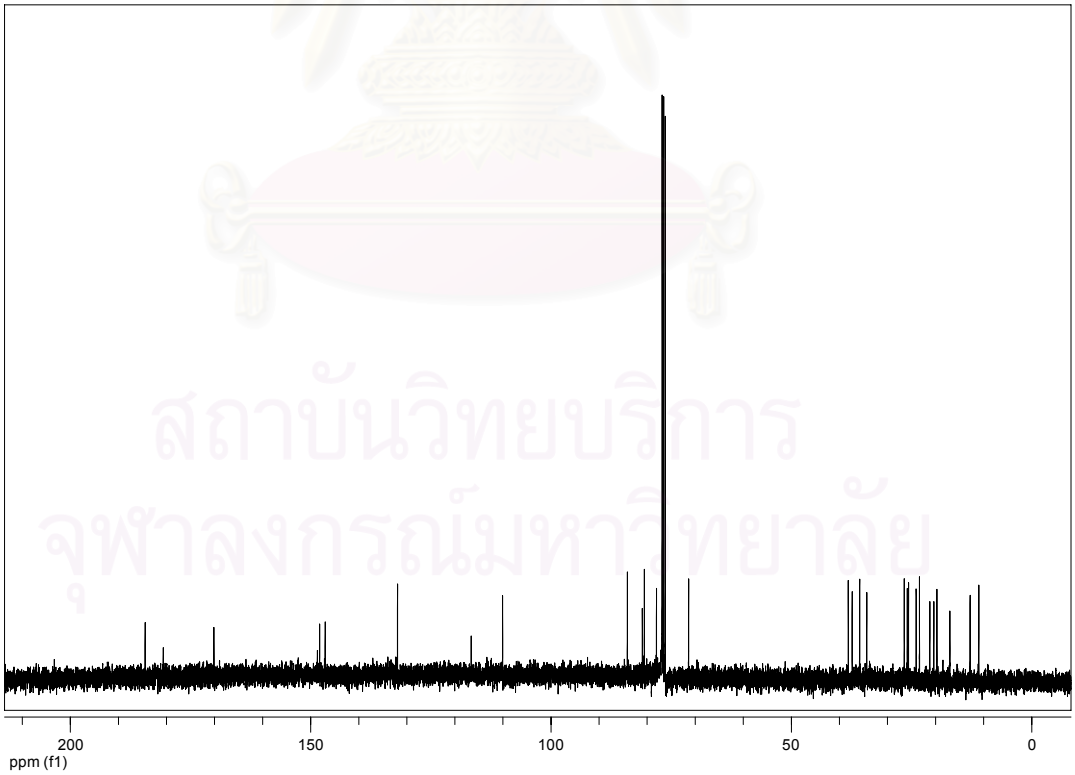


Figure 3.8 The ^{13}C NMR spectrum of compound (**7**) in $CDCl_3$

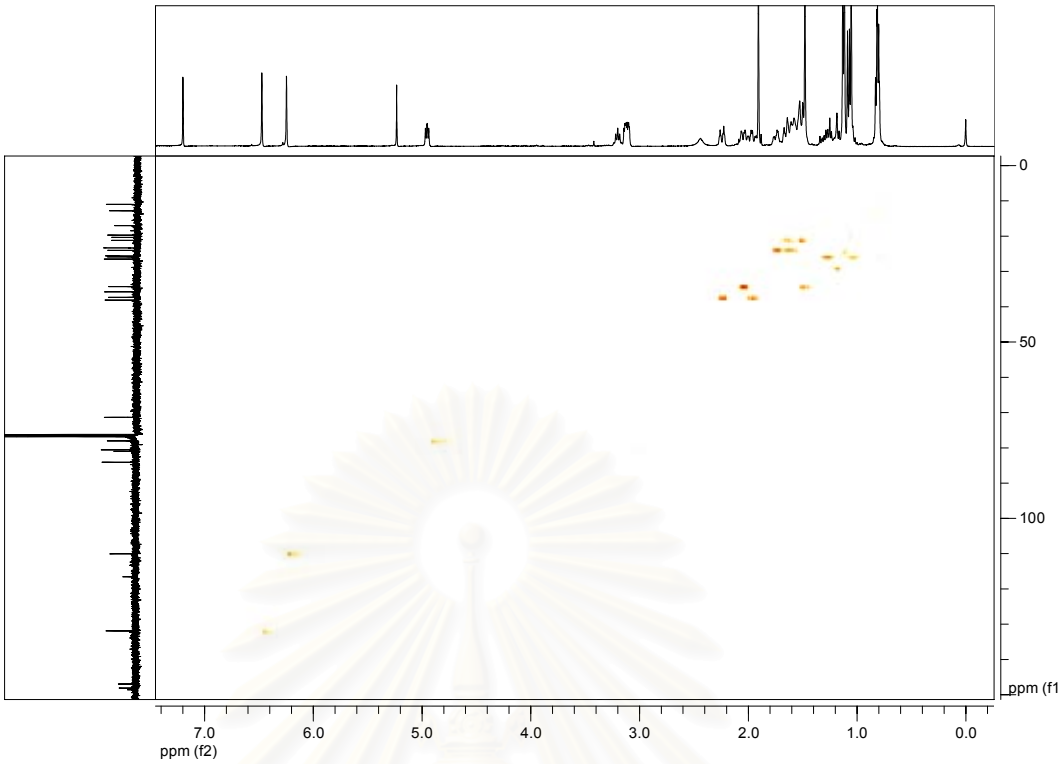


Figure 3.9 The HMQC ($CDCl_3$) spectrum of anhydrocochlioquinone A (**7**)

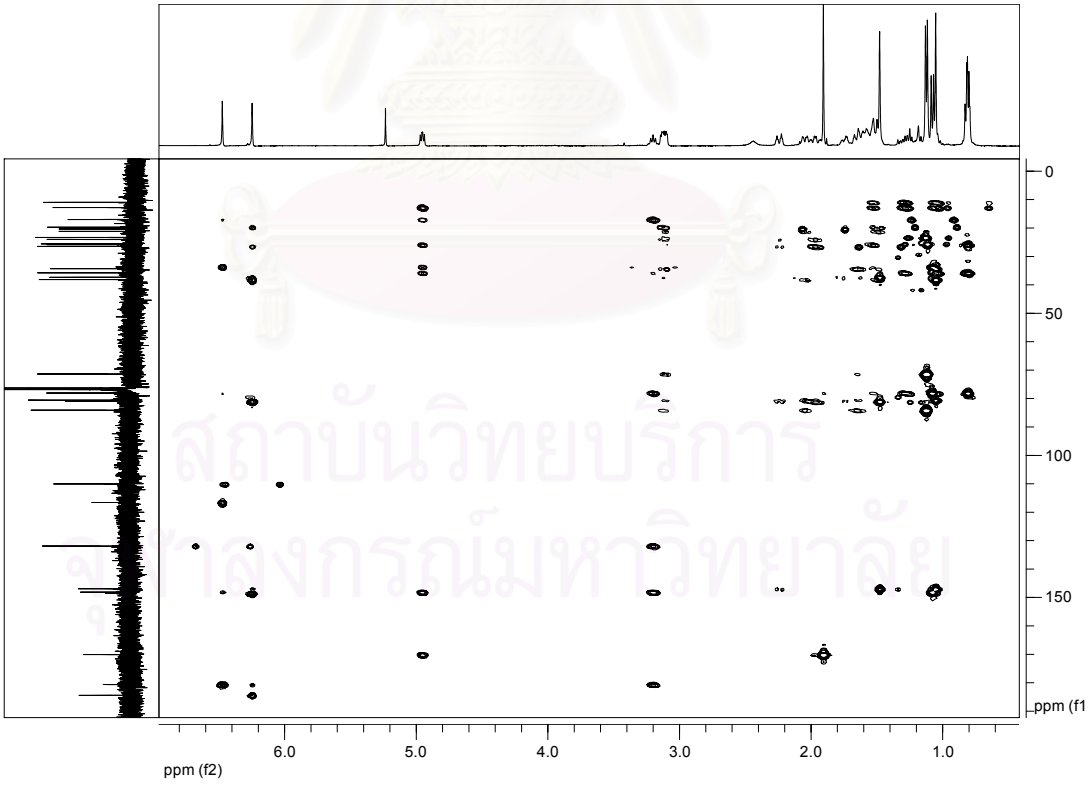


Figure 3.10 The HMBC ($CDCl_3$) spectrum of anhydrocochlioquinone A (**7**)

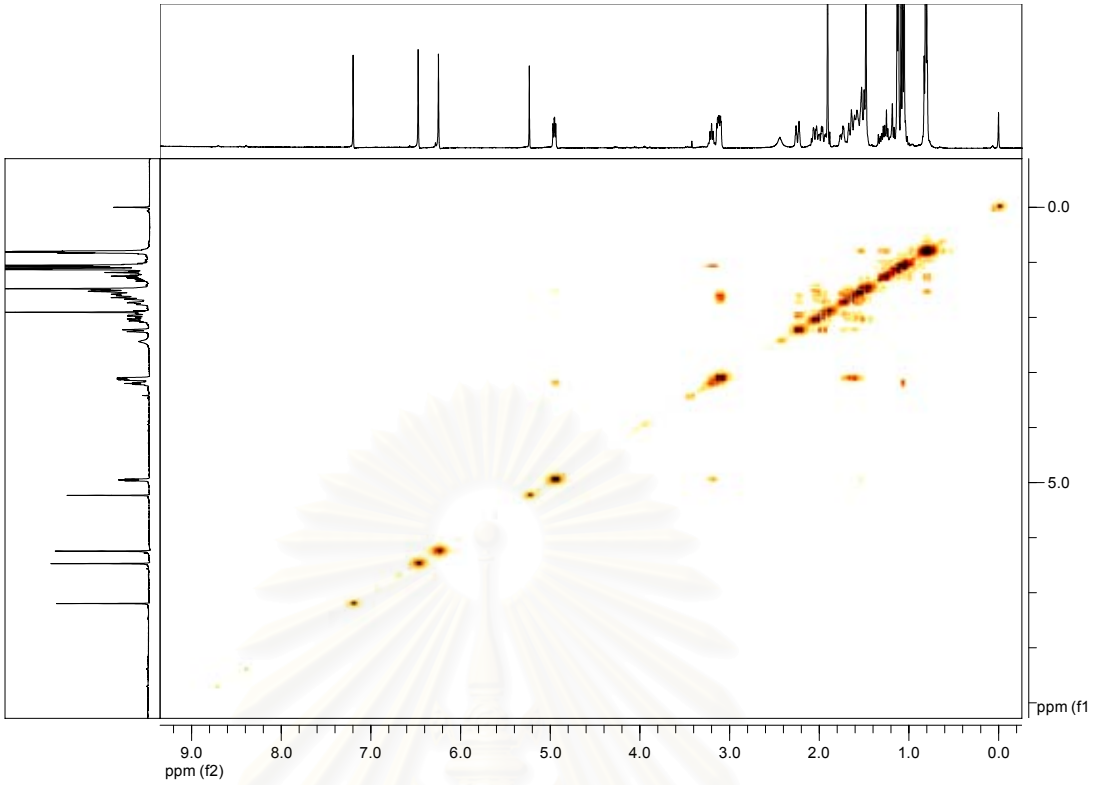


Figure 3.11 The COSY ($CDCl_3$) spectrum of anhydrocochlioquinone A (**7**)

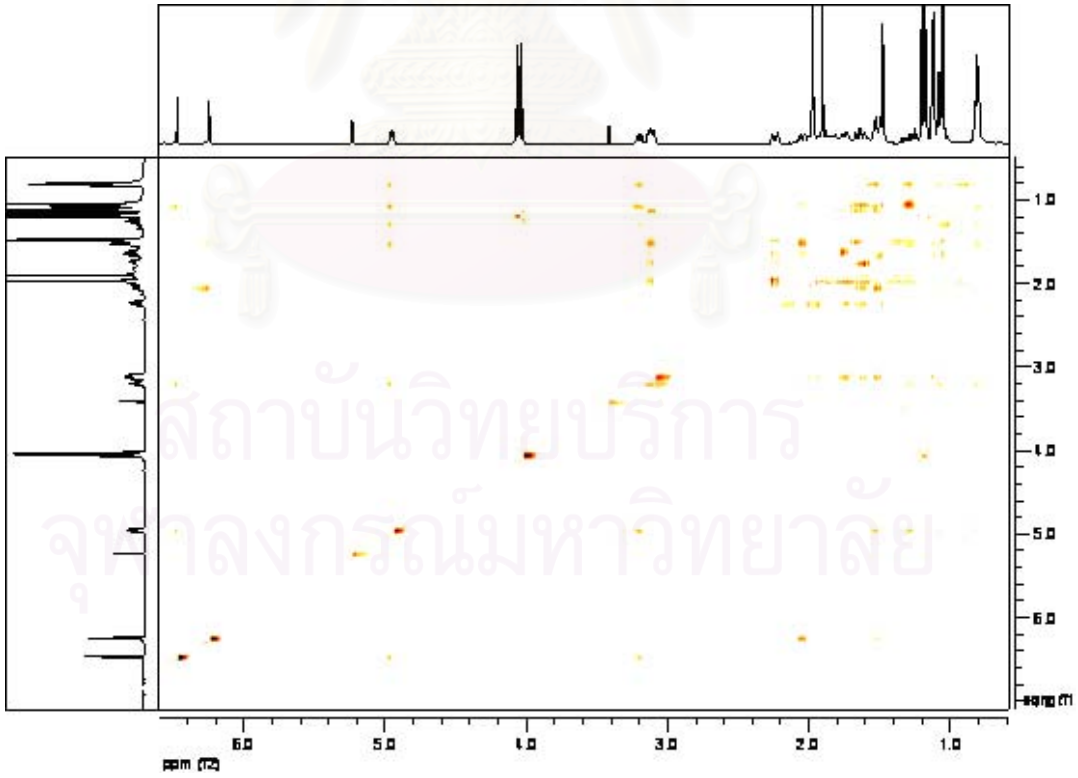


Figure 3.12 The NOESY ($CDCl_3$) spectrum of anhydrocochlioquinone A (**7**)

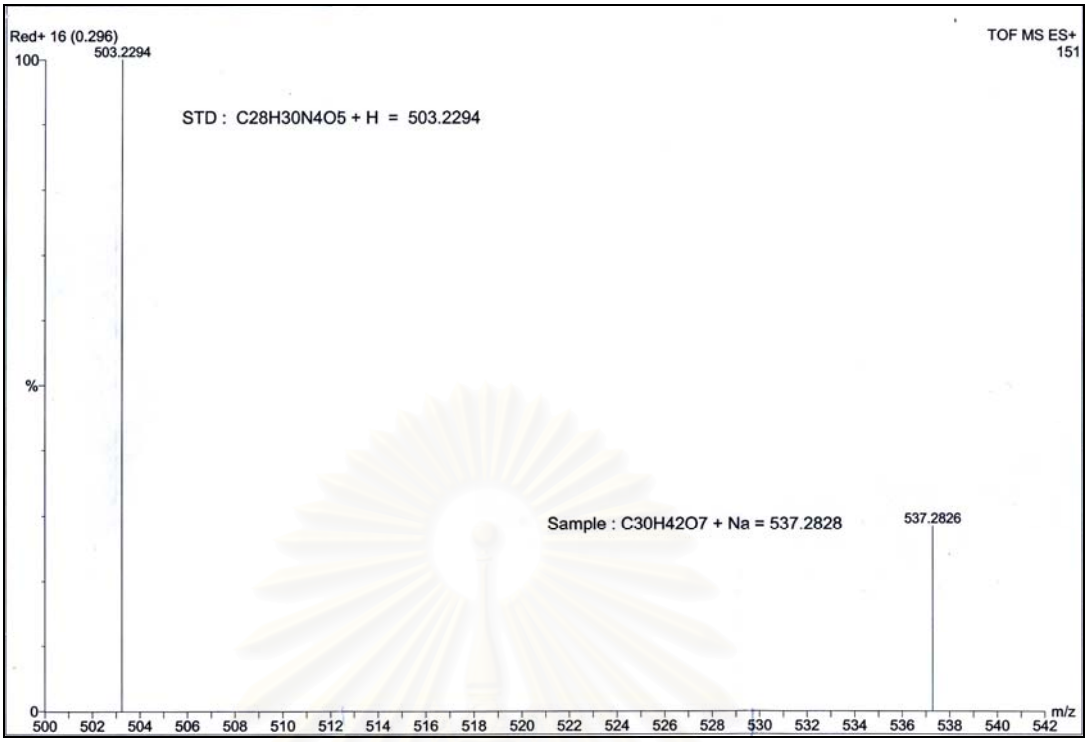


Figure 3.13 The high resolution mass spectrum of compound (**7**)

The fractionation and purification of EtOAc crude extracts of *B. oryzae* by using chromatographic techniques could be elucidated 7 compounds and divided into two groups. They were four ophiobolins, 3-anhydroophiobolin A (**1**), ophiobolin I (**2**), ophiobolin A (**3**) and 6-ophiobolin A (**4**), and three cochlioquinones, isocochlioquinone A (**5**), isocochlioquinone C (**6**) and anhydrocochlioquinone A (**7**). Considering the small amount of isolated compounds available and literature review of these two types of isolated compounds showed the cytotoxicity activity against various cell lines, it was considered judicious to determine the cytotoxicity activity against HeLa and KB cell lines and acetylcholinesterase inhibitor assay.

The cytotoxic activity against HeLa and KB cell lines of all compounds were determined using MTT assay. Compound **2** showed highest cytotoxic activity against HeLa and KB cell lines with IC_{50} values of 0.1 and 0.89 μg/mL, respectively (Table 3.7). In addition, all compounds were also evaluated for acetylcholinesterase inhibitory effect. Compounds **1**, **2**, **3**, **4**, **5**, and **7** showed inhibitory affect against acetylcholinesterase with MIC values of 1.0 mg/mL (Table 3.8).

Table 3.8 Cytotoxic activity of **1**-**7** against HeLa and KB cell lines

Sample	HeLa IC_{50} (μg/mL)	KB IC_{50} (μg/mL)
3-anhydroophiobolin A (**1**)	3.8	4.6
ophiobolin I (**2**)	0.1	0.89
ophiobolin A (**3**)	25.0	31.0
6-*epi*-ophiobolin A (**4**)	5.7	8.9
isocochlioquinone A (**5**)	26.0	95.0
isocochlioquinone C (**6**)	6.5	18.0
anhydrocochlioquinone A (**7**)	5.9	> 100

Note: Standard agent (Adriamycin IC_{50} = 0.018 μg/mL)

Table 3.9 Minimal inhibitory concentrations (MICs) of Compounds **1**, **2**, **3**, **4**, **5** and **7** from acetylcholinesterase inhibitor assay.

Compounds	Concentration (mg/mL)			
	1.000	0.500	0.250	0.125
3-anhydroophiobolin A (**1**)	+++	-	-	-
ophiobolin I (**2**)	+	-	-	-
ophiobolin A (**3**)	+	-	-	-
6-*epi*-ophiobolin A (**4**)	+	-	-	-
isocochlioquinone A (**5**)	+++	++	-	-
anhydrocochlioquinone A (**7**)	++	+	-	-

Note: Standard acetylcholinesterase inhibitor=Phytostigmine (Eserine); Inhibition zone: ++++ = strong inhibition, +++ = moderate inhibition, ++ = weak inhibition, + = very weak inhibition and - = no inhibition.

3.2 Experiment section

3.2.1 TLC bioautography

The procedure of Homans and Fuchs (1970) was adapted. Briefly, fractions (5 mg/mL, 10 μl) were spotted on TLC plate which was developed in 30% EtOAc-CH_2CL_2 . The presence of components was marked under UV light. After completing

solvent removal, spraying an inoculum of *Alternaria brassicicola* on TLC plate and incubated in a moist atmosphere at room temperature for 2 days. The bioautographic procedure described above was used to locate the active component in mixtures. The appearance of a growth inhibition zone around the spot indicated the fungitoxic activity.

3.2.2 Mass production of *Bipolaris oryzae* for extraction and purification

Fungus *B. oryzae* was cultured in malt extract broth (MEB) at 25 ° C for 21 days. The cultured broth (69 L) was filtrated, and the filtration was extracted with equal amount of EtOAc. The organic layer was evaporated in vacuum to afford EtOAc crude extract (145.20 mg/L).

3.2.3 Equipments

NMR spectra were recorded with a Varian model Mercury+ 400 which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. HPLC was performed on Water® 600 controllers equipped with a Water® 2996 photodiode array detector (USA). Cosmosil 5C18-ARII (10 × 250 mm) reverse phase column (Alltech Associates, IL, USA) was used for separation (65% ACN-H_2O, UV λ 274 nm). Most solvents used in this research were commercial grade and were distilled prior to use. Absorbents such as silica gel 60 Merck cat. No. 7731, 7734, and 7749 were used for quick column chromatography, open column chromatography, chromatotron, respectively. Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F254 plates (0.25 mm thick layer). Chromatotron (mode 7924 T, Harrison Research) on silica gel plate of 1 mm thickness was used on centrifugal thin layer chromatography.

3.2.4 The cytotoxic activity against HeLa and KB cell lines by MTT assay

All compounds (1 mg) were examined cytotoxic activity against HeLa and KB cell lines by MTT assay. This assay was performed at Natural Products Research Section, Research Division, National Cancer Institute, Thailand. The results were shown in Table 3.2 (chapter III).

3.2.5 Acetylcholinesterase inhibitor assay (Marston *et al*., 2002)

Acetylcholinesterase was dissolved in 150 mL of 0.005 M Tris-HCl acid buffer at pH 7.8; BSA (150 mg) was added to the solution in order to stabilize the enzyme during the bioassay. The stock solution was kept at 4 °C. Direct deposition of sample on TLC plate. The plate was then sprayed with enzyme stock solution and dried again. For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing a little water; water did not directly into contact with the plate. The cover was placed on the tank for being kept humid and incubation was performed at 37 °C for 20 min. The enzyme had satisfactory stability under these conditions. Solutions of 1-naphthyl acetate (250 mg) in ethanol (100 mL) and of Fast Blue B salt (400 mg) in distilled water (160 mL) were prepared immediately before use (in order to prevent decomposition) for detection of the enzyme. After incubation of the TLC plate, 10 mL of the naphthyl acetate solution and 40 ml of the Fast Blue B salt solution were mixed and sprayed onto the plate to occur a purple coloration after 1-2 min and white spots were generally easier to observe for the inhibiting compounds. The minimum concentration which could be recognized by eye was considered as the detection limit and measured from obvious white spots.

3-anhydro-ophiobolin A (1): yellow amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.06 (d, 3H, *J* = 6.8 Hz, Me-23), 1.72 (s, 3H, Me-25), 1.68 (d, 3H, *J* = 0.8 Hz, Me-24), 2.07 (s, 3H, Me-20), 3.45 (d, *J* = 4.0 Hz, H-6), 4.58 (m, 1H, H-17), 5.13 (m, 1H, H-18), 6.05 (s, 1H, H-4), 6.85 (m, 1H, H-8) and 9.33 (s, 1H, 21-CHO); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 46.8 (C-1), 49.3 (C-2), 177.3 (C-3), 130.4 (C-4), 207.1 (C-5), 49.2 (C-6), 141.0 (C-7), 155.3 (C-8), 30.7 (C-9), 53.9 (C-10), 42.5 (C-11), 41.8 (C-12), 42.2 (C-13), 96.1 (C-14), 35.5 (C-15), 35.5 (C-16), 72.0 (C-17), 126.8 (C-18), 135.3 (C-19), 17.2 (C-20), 192.9 (21-CHO), 22.4 (C-22), 16.3 (C-23), 18.2 (C-24) and 25.9 (C-25).

Ophiobolin I (2): amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93 (s, 3H, Me-22), 0.97 (d, 3H, *J* = 7.2 Hz, Me-23), 1.60 (s, 3H, Me-24), 1.64 (s, 3H, Me-25), 2.02 (s, 3H, Me-20), 2.71 (d, 1H, *J* = 12.4 Hz, H-2), 3.60 (s, 1H, H-6), 3.87 (d, 1H, *J* = 12.0 Hz, 21-CH_2OH), 4.11 (d, 1H, *J* = 12.0 Hz, 21-CH_2OH), 4.54 (q, 1H, H-17), 5.08 (d, 1H, *J* = 8.8 Hz, H-18), 5.72 (d, 1H, *J* = 5.2 Hz, H-8) and 5.89 (s, 1H, H-4).

Ophiobolin A (3): white crystals; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.81 (s, Me-22), 1.09 (d, *J* = 7.2 Hz, Me-23), 1.29 (s, H-1), 1.33 (s, Me-20), 1.35 (m, 1H, H-12), 1.65 (d, Me-24), 1.70 (d, 3H, *J* = 1.2 Hz, Me-25), 1.76-1.81 (5H, H-15), 2.16 (m, 1H, H-15), 2.01 (m, 1H, H-16), 2.20 (m, 1H, H-9), 2.41 (m, 1H, H-9), 2.52 (d, 1H, *J* = 19.2 Hz, H-4), 3.24 (d, 1H, *J* = 10.8 Hz, H-6), 4.38 (m, 1H, H-17), 5.13 (d, 1H, *J* = 8.4 Hz, H-18), 7.19 (t, 1H, *J* = 8.4 Hz, H-8) and 9.23 (s, 1H, H-21).

6-*epi*-ophiobolin A (4): amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84 (s, Me-22), 1.04 (d, *J* = 6.8 Hz, Me-23), 1.40 (s, Me-20), 1.66 (s, Me-24), 1.70 (s, Me-25), 2.11 (m, H-2), 2.39 (s, 1H, H-4), 2.61 (dd, 1H, *J* = 14.4, 4 Hz, H-10), 2.75 (d, 1H, *J* = 19.6 Hz, H-9), 3.06 (d, 1H, *J* = 16.1 Hz, H-4), 3.36 (d, 1H, *J* = 10.5 Hz, H-6), 4.61 (dd, *J* = 7.4, 15.6 Hz, H-17), 5.14 (d, 1H, *J* = 8.4 Hz, H-18), 6.86 (d, 1H, *J* = 6.4 Hz, H-8) and 9.20 (s, 1H, 21-COH).

Isocochlioquinone A (5): yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (d, *J* = 6.8 Hz, Me-28), 0.92 (H-1), 1.17 (s, Me-25), 1.20 (d, 3H, *J* = 8.0 Hz, Me-27), 1.25 (s, H-2), 1.50 (s, Me-26), 1.58 (s, 1H, H-20), 1.64 (H-3), 1.97 (s, 1H, H-30), 2.06 (overlapped, 2H, H-15), 2.52 (s, 1H, 22-OH), 2.76 (s, 1H, H-13), 3.15 (dd, 1H, *J* = 11.6, 3.2 Hz, H-17), 3.26 (dd, 1H, *J* = 11.6, 2.4 Hz, H-21), 3.47 (qnt, H-5), 5.17 (dd, *J* = 5.2, 6.8 Hz, H-4), 5.28 (s, 1H, 7-OH), 6.40 (s, 1H, H-11) and 10.76 (s, 1H, 10-OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 17.2 (C-1), 26.4 (C-2), 36.3 (C-3), 79.1 (C-4), 35.4 (C-5), 140.0 (C-6), 135.2 (7-OH), 143.9 (C-8), 107.0 (C-9), 153.2 (10-OH), 108.2 (C-11), 198.4 (C-12), 60.4 (C-13), 83.5 (C-14), 37.6 (C-15), 25.0 (C-16), 83.7 (C-17), 35.5 (C-18), 37.3 (C-19), 21.3 (C-20), 85.3 (C-21), 72.0 (22-OH), 23.7 (C-23), 26.0 (C-24), 12.3 (Me-25), 22.0 (Me-26), 13.5 (Me-28), 170.6 (C-29) and 20.9 (C-30).

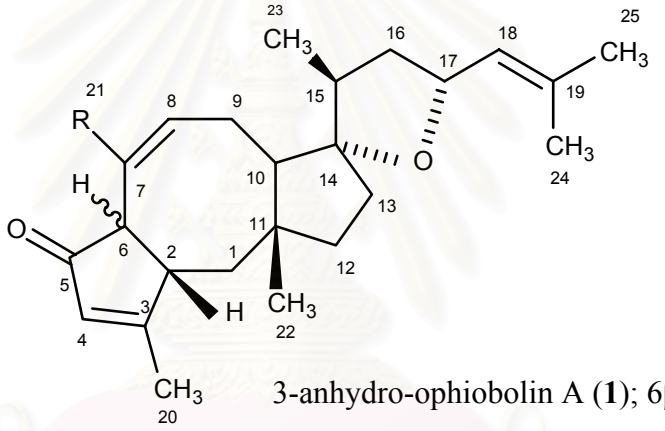
Isocochlioquinone C (6): yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85 (t, 1H), 0.94 (d, 1H, *J* =6.4 Hz), 1.29 (s, 1H), 1.19 (s, 1H), 1.25 (s, 1H), 1.34 (d, 1H, *J* =6.8 Hz), 1.49 (s, 1H), 2.09 (m, 2H), 2.57 (m, 1H), 2.72 (overlapped, 1H), 2.78 (s), 3.13 (dd, 1H, *J* =12.4, 3.6 Hz), 3.50 (dd, 1H, *J* =11.6, 0.8 Hz), 4.24 (q, 1H), 5.38 (s, 1H) and 6.26 (s, 1H), 10.80 (s, 1H).

Anhydrocochlioquinone A (7): red solid; ^{1}H and ^{13}C NMR see Table 3.6; UV (MeOH) λ_{max} 271, 483 nm; $[\alpha]^{28}_{D}$ -18.50° (c 0.085, MeOH); HRESIMS *m/z* 514.2826 (calcd for $C_{30}H_{42}O_7$, 514.2826).

CHAPTER IV

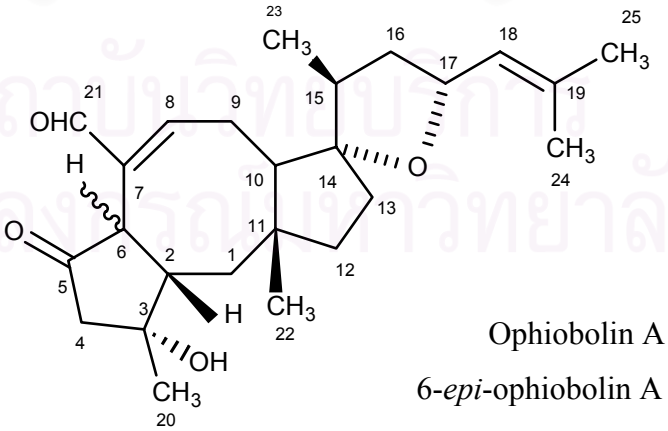
CONCLUSION

In conclusion, the isolation of the ethyl acetate crude extract yielded seven compounds, which could be divided into two groups; ophiobolins and cochlioquinones. They are 3-anhydroophiobolin A (**1**), ophiobolin I (**2**), ophiobolin A (**3**), 6-*epi*-ophiobolin A (**4**), isocochlioquinone A (**5**), isocochlioquinone C (**6**) and a new compound named anhydrocochlioquinone A (**7**). The chemical structures of all isolated compounds were characterized by means of NMR, MS experiments and comparison with previous reports.



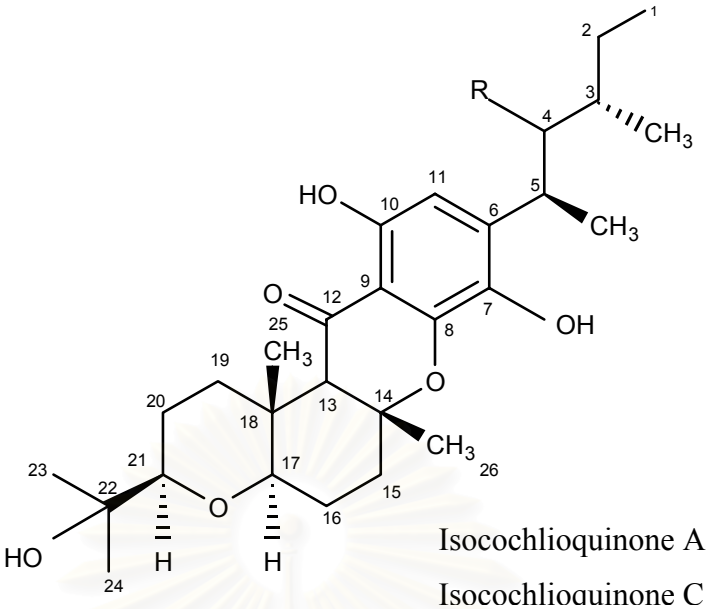
3-anhydro-ophiobolin A (**1**); 6β-H, R = CHO

Ophiobolin I (**2**); 6α-H, R = CH_2OH



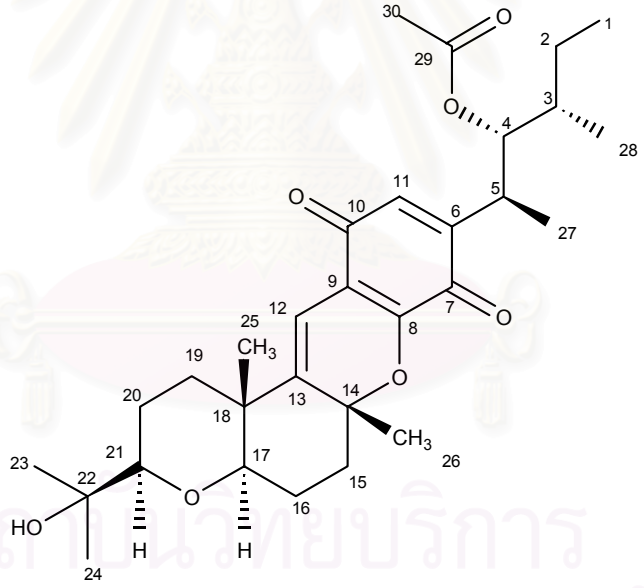
Ophiobolin A (**3**); 6β-H

6-*epi*-ophiobolin A (**4**); 6α-H



Isocochlioquinone A (**5**); R = α-OAc

Isocochlioquinone C (**6**): R = =O



Anhydrocochlioquinone A (**7**, new compound)

In this research it could be separated bioactive compounds into two groups. First group is ophiobolins and the second group is cochlioquinones. The cytotoxic activity against HeLa cell lines by MTT assay revealed that ophiobolin I (**2**) showed highest cytotoxic activity against HeLa and KB cell lines with IC_{50} <0.1 and 0.89 μg/mL, respectively as shown in table 3.2. These results suggest possible involvement of hydroxyl methyl group in exerting cytotoxic activity. 3-anhydroophiobolin A (**1**) also showed cytotoxic activity against HeLa cell lines higher than KB cells lines with IC_{50} = 3.8 and 4.6 μg/mL, respectively. Besides, 6-*epi*-ophiobolin A (**4**), isocochlioquinone C (**6**) and anhydrocochlioquinone A (**7**) showed mild cytotoxic activity against HeLa cell lines at IC_{50} 5.7, 6.5 and 5.9 μg/mL, respectively when compared with reference, Adriamycin (show high activity at IC_{50} = 0.018 μg/mL). Previous studies on biological activities of the ophiobolins showed a variety of activity and also posses antimicrobial activity (Au *et al*., 2000). The antifungal and antimicrobial activities of the naturally occurring ophiobolins and the microbial metabolite were evaluated. For example, ophiobolin I act strong activity against *Trichopyton mentagrophytes*, being moderate activity against *Cryptococcus neoformans* and *Microbacterium intracellular* in an agar-well diffusion assay (Li *et al*., 1995). In 1999, Shen *et al*. had reported ophiobolin A showed activity against cancer cell-lines A-549, HT-29, Mel-20 and P-388 at IC_{50} 50 *n*g/mL, and ophiobolin K showed cytotoxic activity against various tumor cell lines (NCI-H460, HOP18 (human lung carcinoma), MDA-MB-231, T-47D (human breast carcinoma), ACHN (human renal carcinoma), HCT116 (human colon carcinoma), P388 (mouse leukemia cells) and P388/ADR (adriamycin resistant cells) (Wei *et al*., 2004).

According to an acetylcholinesterase inhibitor assay, 3-anhydroophiobolin A (**1**), ophiobolin I (**2**), ophiobolin A (**3**), 6-*epi*-ophiobolin A (**4**), isocochlioquinone A (**5**), and a new compound named anhydrocochlioquinone A (**7**) are powerful inhibited at MIC value of 1 mg/mL. In addition, isocochlioquinone A (**5**) and anhydrocochlioquinone A (**7**) also showed inhibitory affect against acetylcholinesterase with MIC values of 0.5 mg/mL when compared with reference, Eserine. Therefore, this is the first report in acetylcholinesterase inhibition of ophiobolins and cochlioquinones. Acetylcholine is a neuronal transmitter. It is metabolized by the enzyme acetylcholinesterase (AChE). The three drugs donepezil, rivastigmine and galantamine inhibit AChE, and they raise the concentration of acetylcholine at sites of neurotransmission. Alzheimer's disease is the most common

cause of senile dementia in later life. It is estimated that up to 4 million people are affected this disease in the USA. Inhibitors of acetylcholinesterase are currently form the basis of the newest drugs available for the management of this disease (Marston *et al.*, 2002). As for the chemical investigation from *Bipolaris oryzae*, anhydrocochlioquinone A (**7**) was reported as a new compound in this research. The chemical structure of anhydrocochlioqinone A (**7**) has similar to cochlioquinone A (Miyagawa *et al*., 1994) and clearly different from cochlioquinone A by absence H_2O (M^+-H_2O, OH at C-12, H- at C-13). Therefore, anhydrocochlioqinone A (**7**) presented the same skeleton as cochiloquinone A.

In the future work may be synthesized for increasing quantity of 3-anhydrocphiobolin A (**1**), ophiobolin I (**2**), isocochlioquinone A (**5**) and anhydrocochlioquinone A (**7**) and testing with appropriate activities for developing a new drug candidate based on their activities.

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APPENDICES

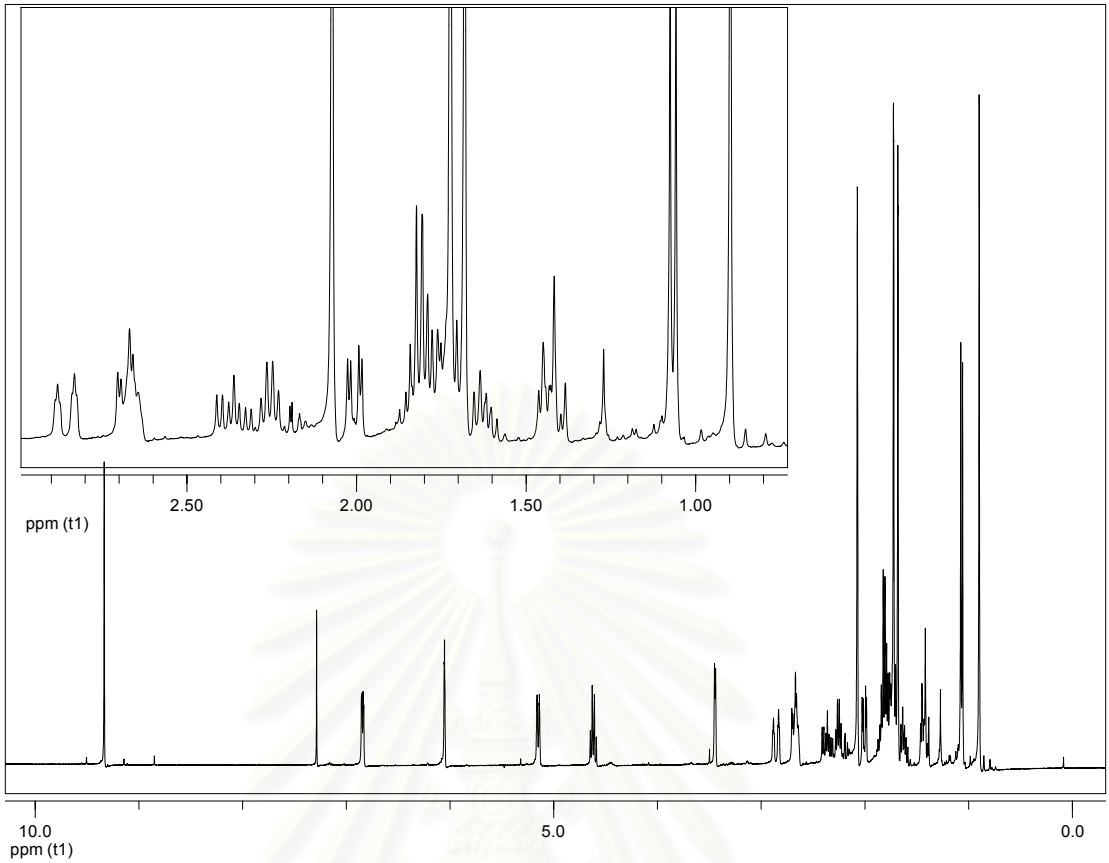

Figure A The ^{1}H NMR spectrum of compound (**1**) in $CDCl_3$

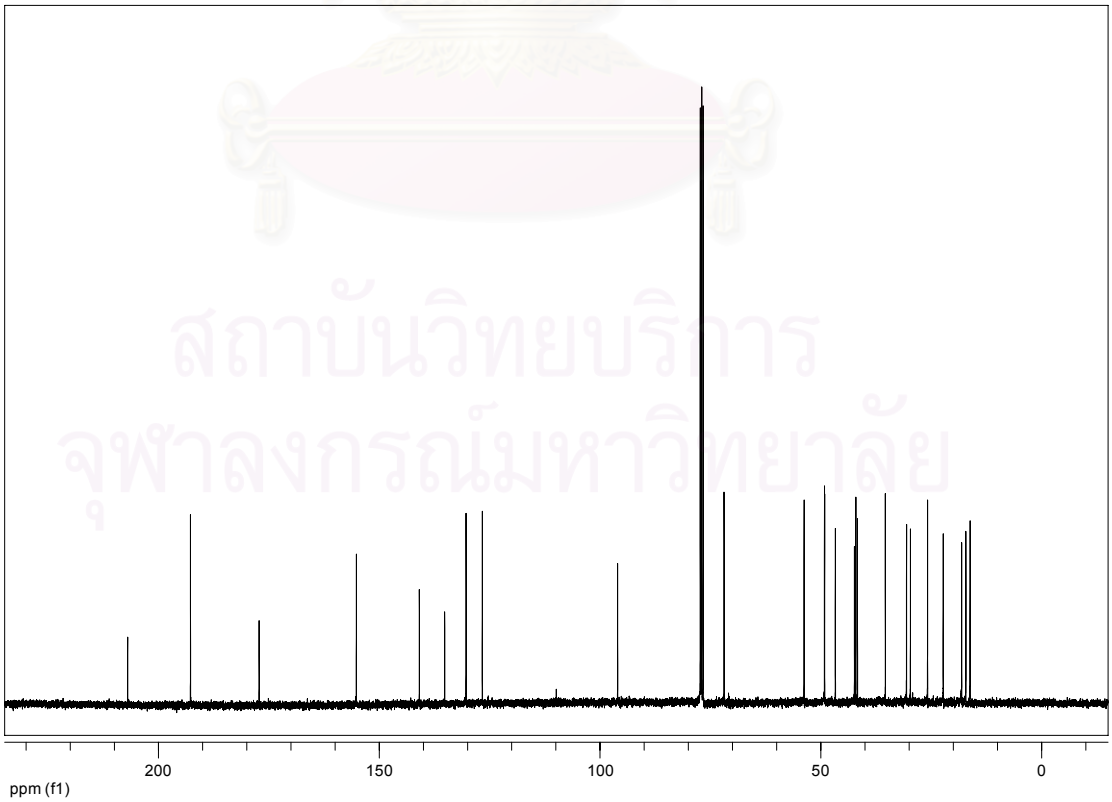

Figure B The ^{13}C NMR spectrum of compound (**1**) in $CDCl_3$

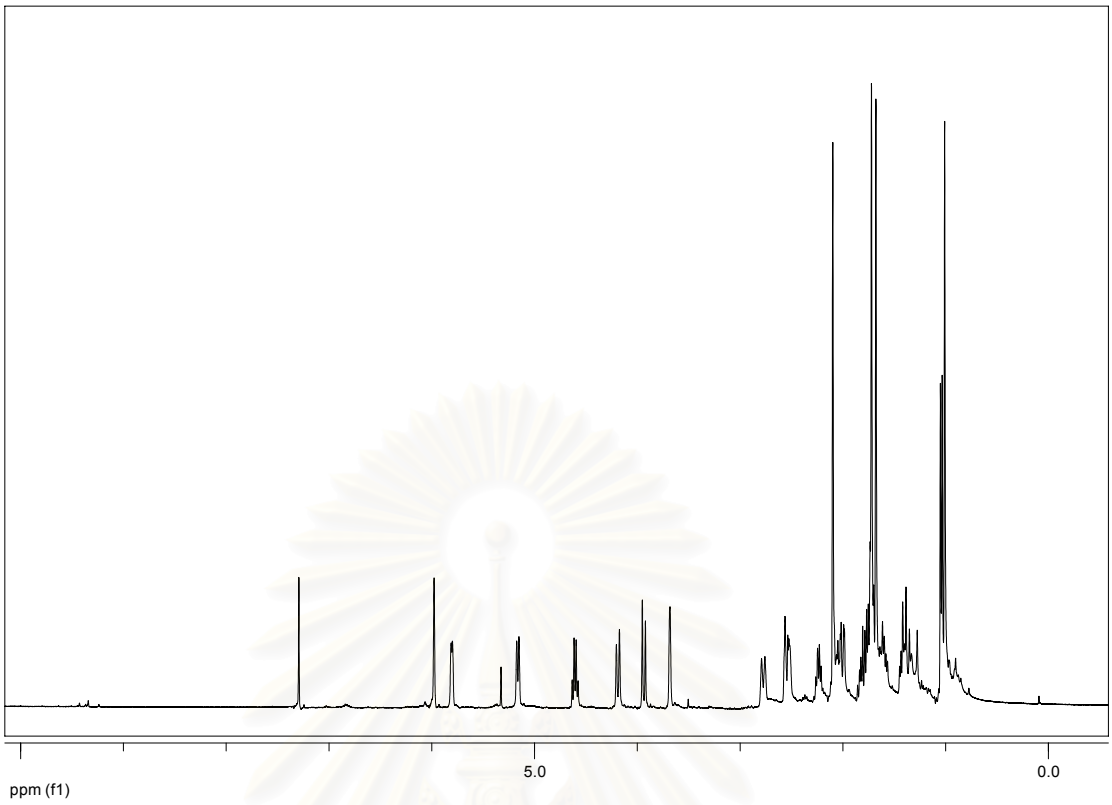

Figure C The ^{1}H NMR spectrum of compound (**2**) in $CDCl_3$

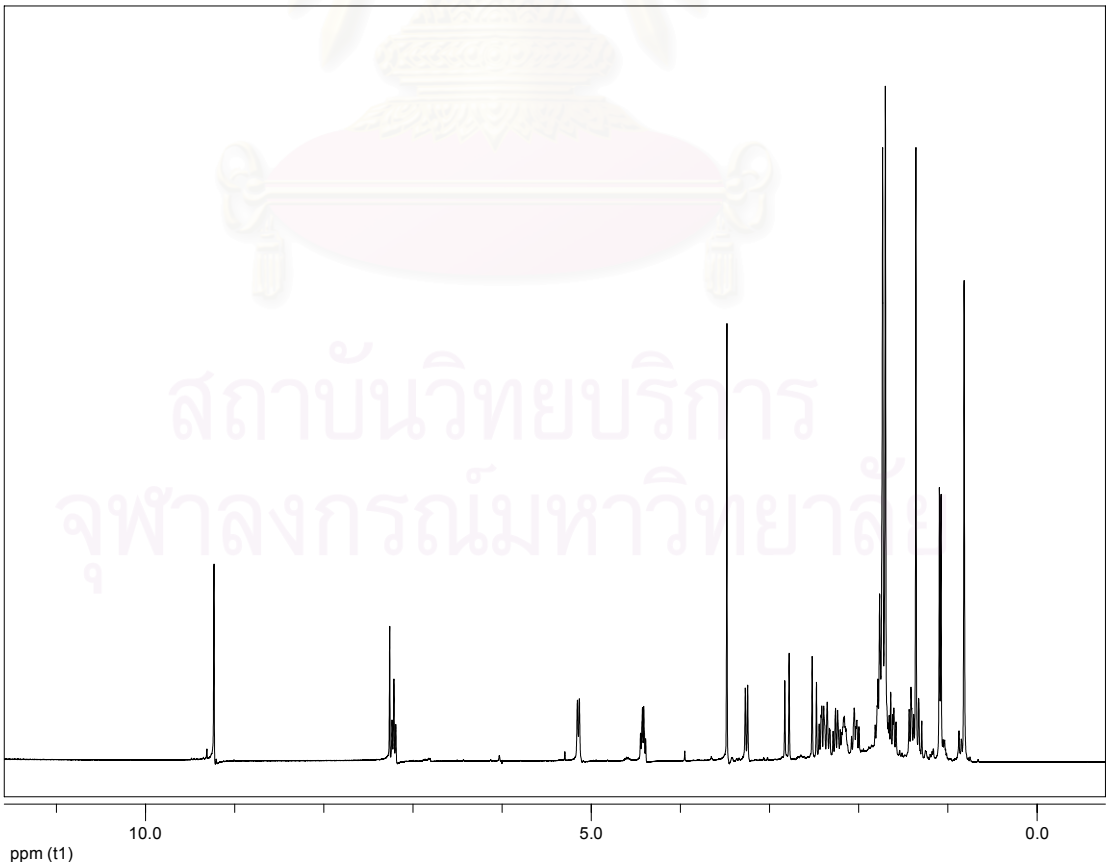

Figure D The ^{1}H NMR spectrum of compound (**3**) in $CDCl_3$

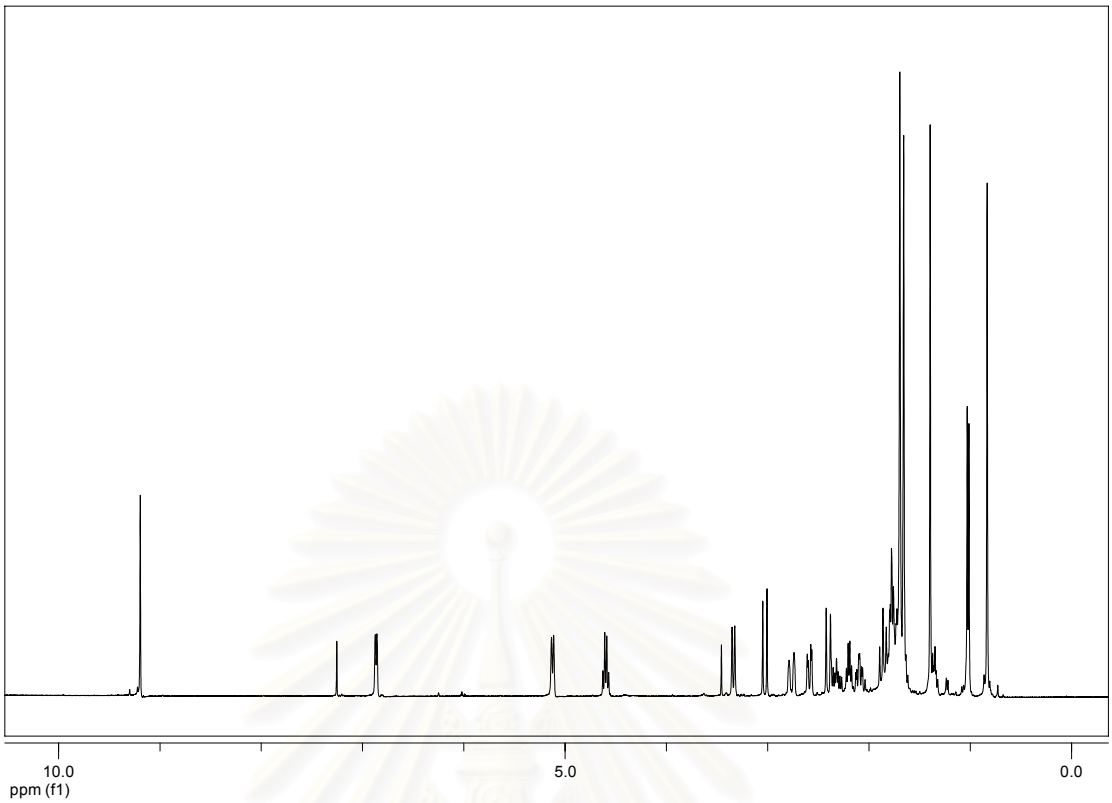

Figure E The ^{1}H NMR spectrum of compound (**4**) in $CDCl_3$

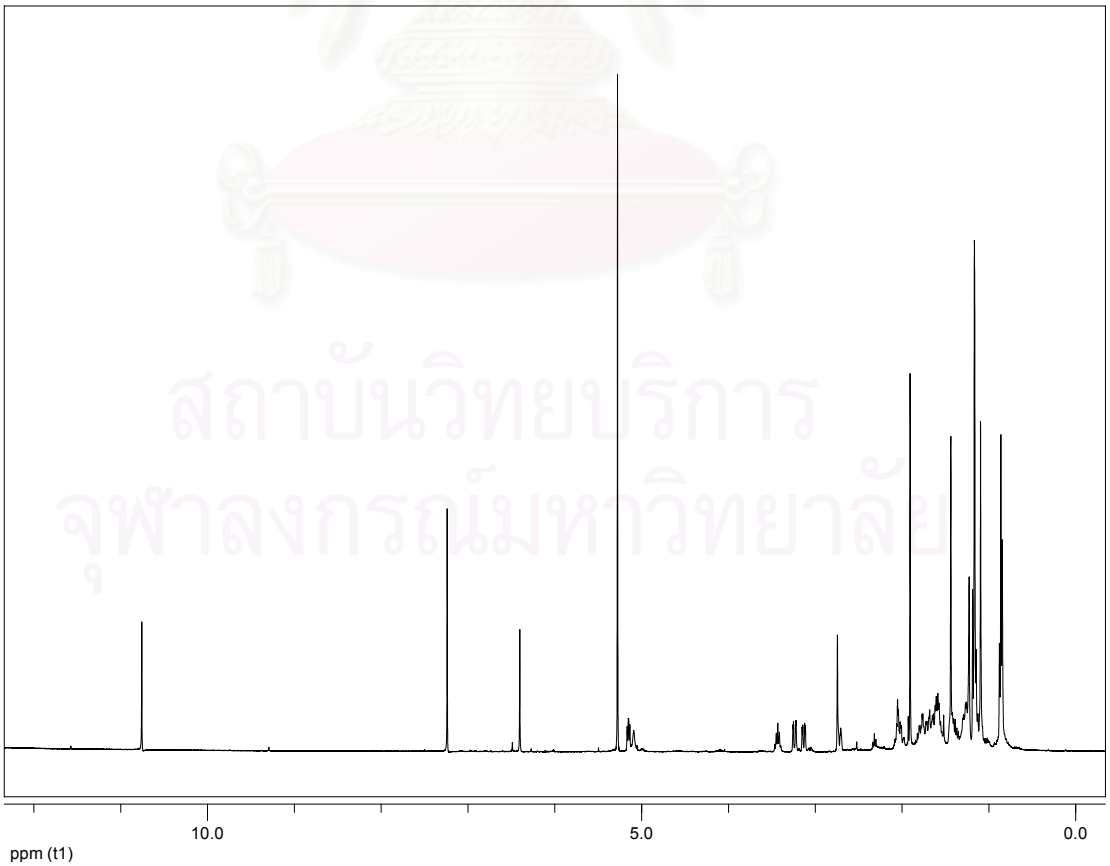

Figure F The ^{1}H NMR spectrum of compound (**5**) in $CDCl_3$

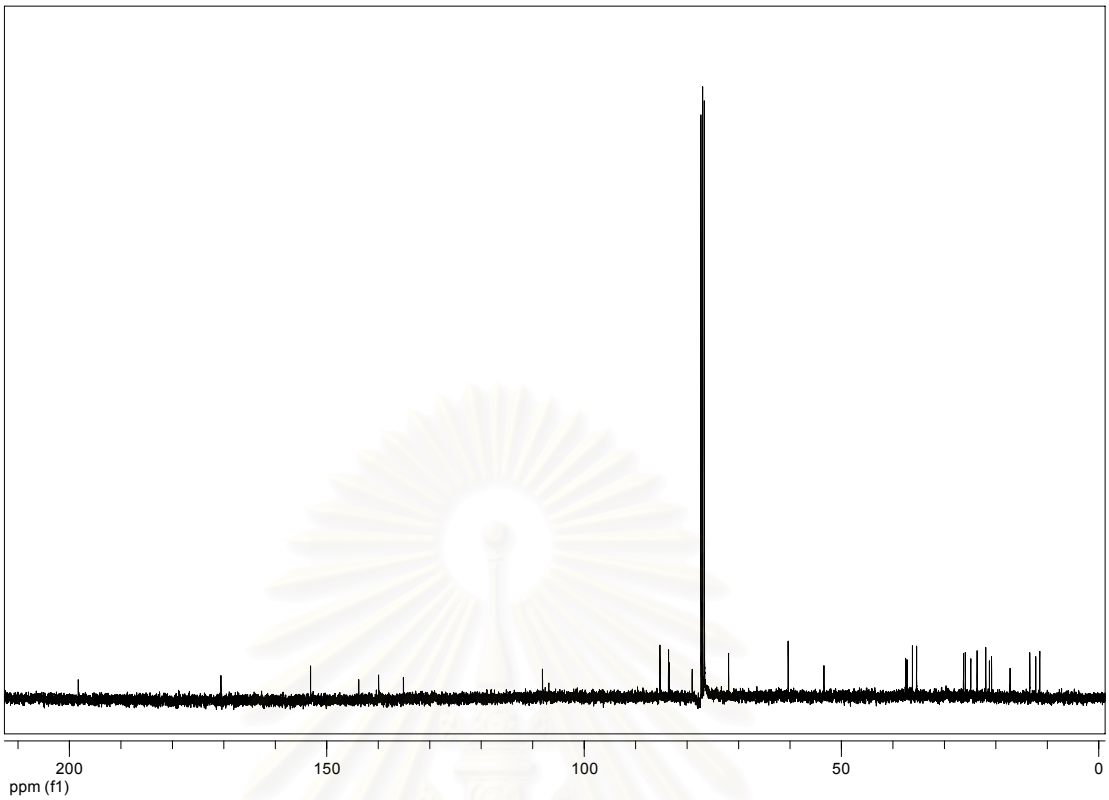

Figure G The ^{13}C NMR spectrum of compound (**5**) in $CDCl_3$

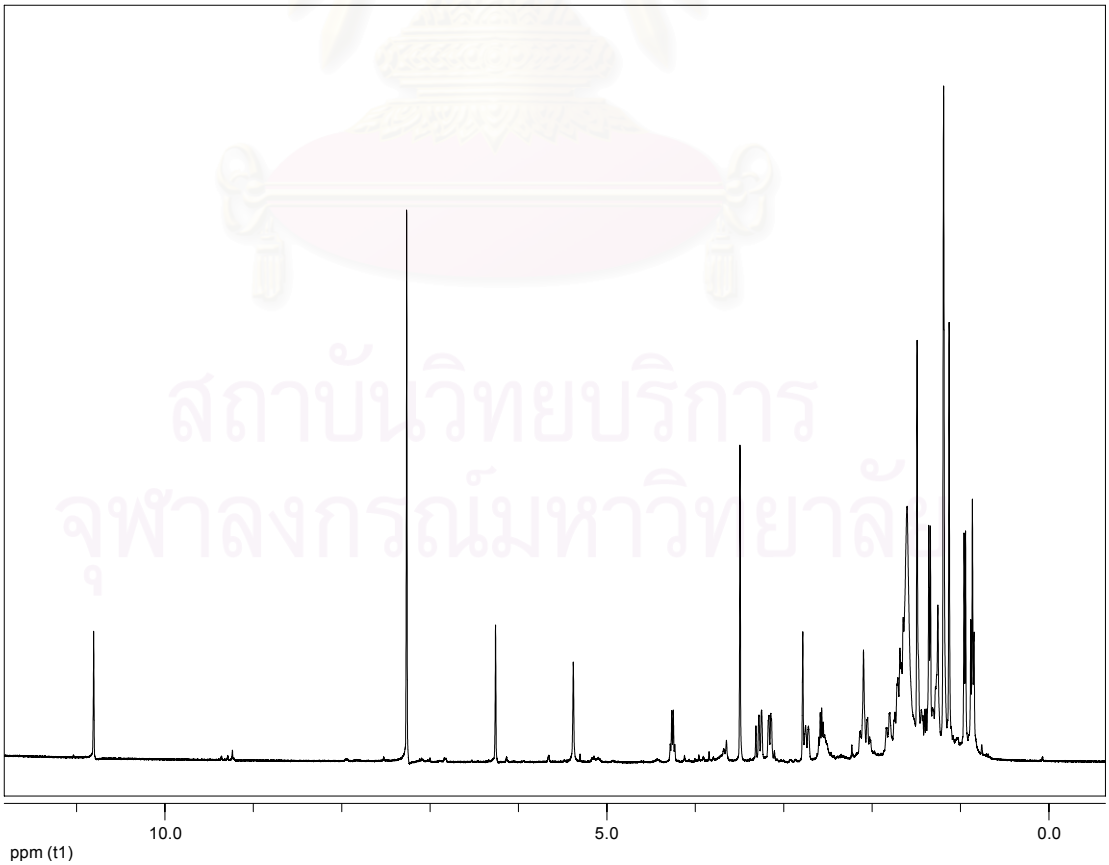

Figure H The ^{1}H NMR spectrum of compound (**6**) in $CDCl_3$

VITA

Miss Kanokwan Sawang was born on August 15, 1977 in Nakhonphanom Province, Thailand. She graduated with Bachelor Degree of Science in Plant Pathology from Faculty of Agriculture, Kasetsart University, in 2001. During she was studying in Master Degree in Biotechnology program, she received a research fund for her thesis from Graduate School, Chulalongkorn University.