สารออกฤทธิ์ทางชีวภาพจากรา *Trichoderma virens* และ *Alternaria porri*

นายจักรพันธุ์ วังสรรค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS FROM *Trichoderma virens* AND *Alternaria porri*

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จักรพันธุ์ รังสรรค์: สารออกฤทธิ์ทางชีวภาพจากรา *Trichoderma virens* และ *Alternaria porri* (BIOACTIVE COMPOUNDS FROM *Trichoderma virens* AND *Alternaria porri*) อ.ที่ปรึกษา: รศ. ดร. สันติ ทิพยางค์, อ.ที่ปรึกษาร่วม: ผศ. ดร. ปรีชา ภูวไพรศิริศาล, 72 หน้า

ราโรคพืชและราดินเป็นแหล่งของสารออกฤทธิ์ทางชีวภาพที่หลากหลาย *Trichoderma virens* และ *Alternaria porri* จึงถูกเลือกในการนำมาศึกษาต่อไป การแยกสิ่งสกัดไดคลอโรมีเทนจากอาหารเหลวเลี้ยงเชื้อ *T. virens* นำไปสู่การแยกสาร furanosteroid ใหม่คือ 9-*epi*-viridiol (4) พร้อมกับสารที่มีรายงานมาแล้ว 3 สารคือ viridin (1), 4-methylmevalonic acid (2) และ viridiol (3) สำหรับการแยกสารจากสิ่งสกัดเอทิลอะซีเตตของเชื้อ *A. porri* แยกสารใหม่ได้ 3 สาร คือ alterporriol F (9), zinnimide (10), deprenylzinnimide (16) พร้อมกับสารที่มีรายงานมาแล้ว 10 สารคือ zinnimidine (5), alterporriol D or E (6), alterporriol A (7), alterporriol C (8), macrosporin (11), 5-(3',3'-dimethylallyloxy)-3-methoxy-6-methylphthalide (12), porriolide (13), alternariol monomethyl ether (14), alternariol (15) และ demethylmacrosporin (17) การพิสูจน์ทราบโครงสร้างของสารทั้งหมดใช้วิธีทางสเปกโทรสโกปีและเปรียบเทียบข้อมูลกับสารที่เคยมีรายงานไว้แล้ว จากนั้นนำสารที่แยกได้ทั้งหมดไปทดสอบความเป็นพิษต่อเซลล์มะเร็งชนิด HeLa และ KB และฤทธิ์ในการยับยั้งการทำงานของ acetylcholinesterase พบว่าสาร 14 มีความเป็นพิษต่อเซลล์มะเร็งชนิด HeLa ได้สูงที่สุดที่ IC_{50} เท่ากับ 4.3 µg/mL ในขณะที่สาร 15 มีความเป็นพิษต่อเซลล์มะเร็งชนิด KB ได้สูงที่สุดที่ IC_{50} เท่ากับ 4.5 µg/mL นอกจากนี้สาร 3, 5, 10 และ 12 แสดงฤทธิ์การยับยั้งการทำงานของ acetylcholinesterase อย่างมีนัยสำคัญด้วยค่า MIC เท่ากับ 0.125, 0.250, 0.500 และ 1.000 mg/mL ตามลำดับ

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....จักรพันธุ์ รังสรรค์.....
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JAKAPHAN RANGSAN: BIOACTIVE COMPOUNDS FROM *Trichoderma virens* AND *Alternaria porri* THESIS ADVISOR: ASSOC. PROF. SANTI TIP-PYANG, Ph.D., THESIS CO-ADVISOR : ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 72 pp.

Plant pathogenic and soil fungi have been the source of a wide range of bioactive compounds. *Trichoderma virens* and *Alternaria porri* were selected for further investigation. The chromatographic separation of dichloromethane extract of liquid culture from *T. virens* led to the isolation of a novel furanosteroid, 9-*epi*-viridiol (**4**), along with three known compounds, viridin (**1**), 4-methylmevalonic acid (**2**) and viridiol (**3**). On the other hand, the isolation of ethyl acetate extract from *A. porri* afforded three new compounds, alterporriol F (**9**), zinnimide (**10**), deprenylzinnimide (**16**), together with ten known compounds, zinnimidine (**5**), alterporriol D or E (**6**), alterporriol A (**7**), alterporriol C (**8**), macrosporin (**11**), 5-(3',3'-dimethylallyloxy)-3-methoxy-6-methylphthalide (**12**), porriolide (**13**), alternariol monomethyl ether (**14**), alternariol (**15**) and demethylmacrosporin (**17**). The structures of all isolated compounds were elucidated by spectroscopic methods as well as comparison with the previous literature data. All compounds were evaluated for cytotoxicity on HeLa and KB cell lines and inhibitory activity of acetylcholinesterase. Compound **14** showed highest cytotoxicity against HeLa cell line with IC_{50} at 4.3 μg/mL, while compound **15** exhibited highest cytotoxicity with IC_{50} at 4.5 μg/mL for KB cell line. In addition, Compound **3**, **5**, **10** and **12** displayed significant inhibitory activity of acetylcholinesterase with MIC values of 0.125, 0.250, 0.500 and 1.000 mg/mL, respectively.

Field of Study.....Biotechnology.....Student's Signature Jakaphan Rangsan

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

Co-advisor's Signature P. Phuwapraisirisan

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List of Abbreviations

^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
° C	degree of Celsius
acetone-d_6	deuterated acetone
brs	broad singlet (NMR)
BSA	bovine serum albumin
c	concentration
calcd	calculated
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)
DMSO- d_6	deuterated dimethyl sulfoxide
e.g.	for example
ESIMS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
et al.	Et Alii/Alia (Latin: And Others)
g	gram (s)
h	hour
H_2O	water
HMBC	heteronuclear multiple bond correlation experiment
HPLC	high performance liquid chromatography
HRESIMS	high resolution electrospray ionization mass spectrometry
HSQC	heteronuclear single quantum correlation
Hz	hertz
IC_{50}	concentration that is required for 50% inhibition in vitro
J	coupling constant

L	liter (s)
m	multiplet (NMR)
M	molar
MeOH	methanol
mg	milligram (s)
MHz	megahertz
MIC	minimum inhibitory concentration
min	minute
mL	milliliter (s)
mult	multiplicity
NMR	nuclear magnetic resonance
TMS	tetramethylsilane
U	unit
UV	ultraviolet
δ	chemical shift
δ_C	chemical shift of carbon
δ_H	chemical shift of proton
ε	molar extinction coefficient
λ_{max}	maximum wavelength
µg	microgram (s)
µL	microliter (s)
µm	micrometer (s)
2D NMR	two dimentional nuclear magnetic resonance
$[\alpha]_D^{28}$	specific optical rotation at 28^{o}C

CHAPTER I

GENERAL INTRODUCTION

Natural products has served as an important source of drugs since ancient time and about half of the useful drugs today are derived from natural sources. Chemodiversity in nature, e.g. in plant, microorganism and marine organism, still offers a valuable source for novel lead discovery. Microbial secondary metabolites represent a large source of compounds endowed with ingenious structures and potent biological activities. Many of the products currently used for human or animal therapy, in animal husbandry and in agriculture are produced by microbial fermentation, or are derived from chemical modification of a microbial product. These products have been obtained after a few decades of intensive screening involving probably millions of microorganisms. These past successes make discovering new bioactive metabolites from microbial sources harder than ever, since thousands of compounds are described in the literature. Different strains generally produce different compounds. However, not all microorganisms are equally capable of producing secondary metabolites. In fact, this capability is at the moment restricted to a few groups of bacterial or eukaryotic microbes. In particular, the ability to produce a large number of chemically different secondary metabolites is associated mostly with the filamentous actinomycetes, the myxobacteria, the pseudomonads and the cyanobacteria within the prokaryotic world. We believe that novel antibiotics and other bioactive secondary metabolites can still be discovered from microbial sources.

Microorganism or microbe is an organism that is microscopic (too small to be visible to the naked eye). Microorganism can be bacteria, fungi, archaea or protist living almost everywhere in the world. A fungus is a eukaryotic organism which has a distinct nucleus and cytoplasmic organelles surround by membranes. The fungi live as heterotrops, utilizing organic compounds from other organisms. It is apparent that many organic compounds serve as an energy source or structural component of the fungi. These included many of the molecules such as carbohydrate, amino acid, protein and lipid that are primary metabolites. Primary metabolite characteristically predominates as the organisms actively growing, whereas secondary metabolite is

more likely to occur after most growth has occurred. The secondary metabolites play no obvious role in the economy to the cell nor are they involved in structural component of the cell such as the fungi also produce volatiles that give the fungi their distinctive odor, pigment that give the fungi their distinctive color and antibiotic and toxin that can be dangerous to the other organism. Structurally, the secondary metabolite can be unsaturated or saturated aliphatic compounds or aromatic compounds. They range from simple, small molecules to elaborated polycyclic compounds. Some secondary metabolites from fungi have powerful in many fields, such as antiviral agents, antifungals, antibacterials and immunosuppressants (Turner, 1971). Therefore, secondary metabolites that used in any field can still be discovered from fungi.

Fungi are an important component of the ecosystem. The majority of fungal species is saprophytic that feed on nonliving organic matter and decomposed the dead animal or plant by digestive enzyme to the environment. Parasite fungi invade a living plant or animal (host), feeding and multiplying within it at the host's expense while not contributing to the welfare of host. Several fungi obtain their liverhood by capturing animals (amoebae, rotifer, other protozoa and nematode) in a specialized trap and those feeding on them after their death found. In addition, many fungi can cause destruction of living cells, tissues and organs of birds, mammals, human and domesticated animals such as *Coccidioides imnitis* can cause of coccidioidomycosis in dog, cattle and sheep as well as in human. Moreover, some fungal as mutualistic symbiont such as lichen that association between a fungal partner and simple photosynthetic autotrop that resulting the development of a morphologically distinct thallus and mycorrhiza. From the previous data, fungus and animal or plant live together with relationship in any interaction. One of interaction was plant pathogenic fungi can benefit plant health by providing nutrient or fungi can cause harm to plants by parasitizing them, causing plant to become diseased (Moore-Landecker, 1996).

Plant pathogenic fungi have many species that depend on an alternation of host and environment. One of them is *Alternaria porri* that cause of purple blotch in onion. In chemical constituent research of *Alternaria porri*, many secondary metabolites were discovered. However, these compounds were only tested with the inhibition of seedling growth. The biological activity of isolated compounds was not investigated in the other field. This research interested in the isolation of secondary metabolites and the biological activities of isolated compounds. Cytotoxicity and

acetylcholinesterase inhibition were selected for new biological activities testing of all isolated compounds from *A. porri* in this experiment.

Another fungus that used in this experiment is *Trichoderma* species that act as a biological control agent to protect plants from pathogenic fungi. *Trichoderma harzianum* was the most investigated for biocontrol whereas the other species of *Trichoderma* was not well-known in worldwide. From literature review, a few secondary metabolites from *Trichoderma virens* have been reported and mostly showed the antifungal activity and plant growth regulator. In order to discover the bioactive compounds from secondary metabolites from other species of *Trichoderma*, *T. virens* was selected for isolation of bioactive compounds and interesting biological activities testing, such as cytotoxicity and acetylcholinesterase inhibition.

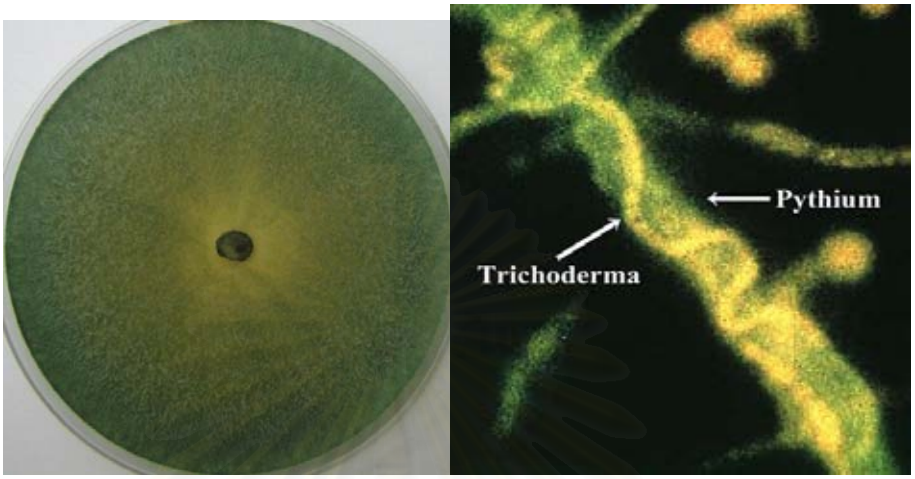
CHAPTER II

BIOACTIVE COMPOUNDS FROM *Trichoderma virens*

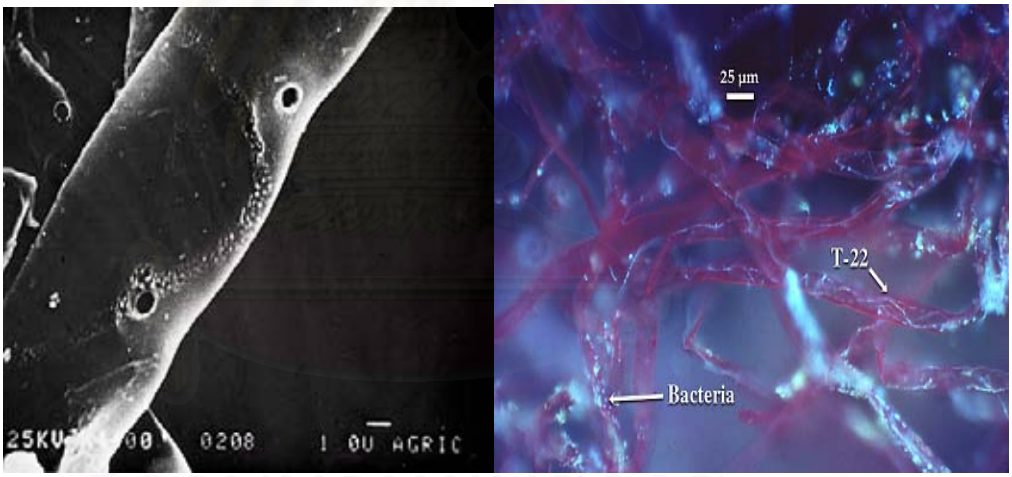
2.1 Introduction

Fungal species belonging to the genus *Trichoderma* are worldwide in occurrence and easily isolated from soil, decaying wood and other forms of plant organic matter. They are, for the most part, classified as imperfect fungi, in that they have no known sexual stage. The productions of numerous spores (conidia) are varying shades of green characterize fungi in this genus. *Trichoderma* species are generally considered to be nonpathogenic to humans. They have rapid growth rate in culture. The potential of *Trichoderma* species as biocontrol agents of plant diseases was first recognized in the early 1930s, and in subsequent years, control of many diseases has been discovered. In the past research indicated that the mechanisms are many and varied, even within the genus *Trichoderma*.

Mechanism involved in biocontrol with *Trichoderma* species had many pathways. Weindling described in detail the mycoparasitism of *Rhizoctonia solani* hyphae by the hyphae of the biocontrol agent, including coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm (Figure 2.1). This phenomenon occurred regardless of the supply of external nutrients to the host or mycoparasite (Weindling, 1932). Two years later, Weindling reported that a strain of *T. lignorum* produced gliotoxin demonstrated that it was toxic to both *R. solani* and *Sclerotinia americana* (Weindling, 1934). Subsequently, Stipanovic and Howell isolated a new antibiotic, gliovirin, from *T. virens* that was strongly inhibitory to *Pythium ultimum* and a *Phytophthora* species. One mechanism that has gained adherents in recent years is that of competition through rhizosphere competence. The difficulty in viewing competition through rhizosphere competence as a major mechanism in biological control is that strains of *T. koningii* that are excellent root colonizers exhibit little or no biocontrol activity against *R. solani* on cotton seedlings. One advanced idea is enzymes such as chitinases and/or glucanases produced by the



A) B)



C) D)

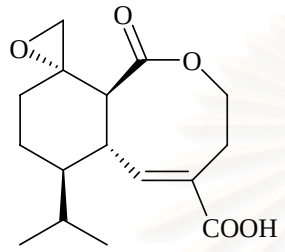
Figure 2.1 A) *T. virens* growth on semi-solid culture, B) Mycoparasitism by a *Trichoderma* strain on the *Pythium*, C) Scanning electron micrograph of the surface of a hyphae of the *R. solani* after mycoparasitic *Trichoderma* hyphae were removed, D) Colonization of root hairs of corn by the rhizosphere competent of *T. harzianum* T22.

biocontrol agent are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitins, and glucans that are responsible for the rigidity of fungal cell walls. For another interesting concept related to enzyme biosynthesis as a mechanism in the biocontrol process was found that *T. harzianum* produced proteases which inactivate the hydrolytic enzymes produced by *B. cinerea* on bean leaves. The protease enzymes break down hydrolytic enzymes into peptide chains and/or their constituent amino acids and thereby destroy their capacity to act on plant cells. On the other hand, another mechanism of *Trichoderma* species is that of induction of resistance in the host plant by treatment with the biocontrol agent plants. They also demonstrated that hyphae of the biocontrol fungus penetrated to the epidermis and upper cortex of the cucumber root. The plant response was occurring so, the plant pathogenic fungus was not infecting to host plant. *Trichoderma* species exhibit other characteristics during interactions with host plants that may contribute to disease resistance or tolerance. These characteristics manifest themselves by increasing in plant root and shoot growth, resistance to biotic and abiotic stresses, and changes in the nutritional status of the plant (Howell, 2003).

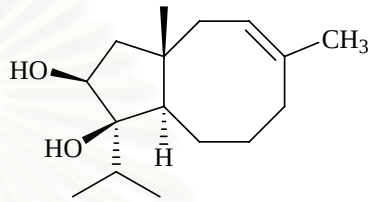
The fungus *Trichoderma virens* is a ubiquitous soil filamentous hyphomycete (a subclass of fungi) that has been applied as a biological control agent to protect plants from fungal pathogens. The other name of *Trichoderma virens* are *Hypocrea virens* and *Gliocladium virens* (Weinding and Emerson, 1936). *T. virens* are frequently isolated from forest, agricultural soils, wood and other fungi. Colony are radius on potato dextrose agar (PDA) 30 ^{o}C after 72 h ; conidiation effuse covering the entire surface of the plate, conidia (numerous spore) typically forming moderately well within 72 h at 25-30 ^{o}C on PDA in darkness, and sparsely at 35 ^{o}C; diffusing yellow pigment sometimes found in the original inoculum. Conidiophores (specialized fungal hypha that produces numerous spores) arise in clusters from aerial mycelium and branch toward the tip with a sterile stipe. This species is readily recognizable by the conidia that are borne in drops of clear, watery liquid and by the abundant chlamydospores, seen even in young cultures. Conidia on cornmeal dextrose agar are green, broadly ellipsoidal to obovoid and smooth.

Trichoderma virens produced several bioactive substances; most of them were sesquiterpenes. Heptelidic acid or koningic acid (**2.1**) was an antibiotic sesquiterpene lactone isolated from *T. virens* (Stipanovic *et al*., 1983). Its inhibitory effect against

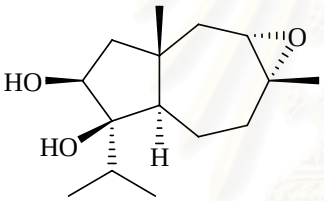
the enzyme glyceraldehydes-3-phosphate dehydrogenase (GAPDH) involved in antibiotic activity (Sakai *et al*., 1990). A carotene sesquiterpene, CAF-603 (**2.2**) showed antifungal activity against *Candida albicans* (Watanabe *et al*., 1990). The other carotane sesquiterpenes named trichocaranes A-D (**2.3**-**2.6**) inhibited the growth of etiolated wheat coleoptiles (Macias *et al*., 2000). In addition, the sesquiterpene gliovirin (**2.7**) was also isolated as a new potent dioxopiperazine antibiotic against *P. ultimum* (Stipanovic *et al*., 1982).



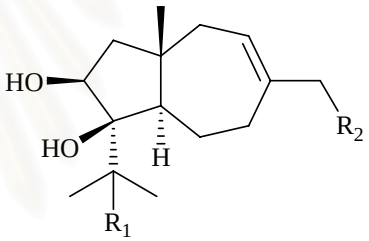
Heptelidic acid (**2.1**)



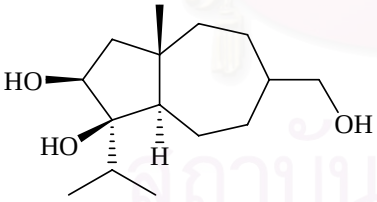
CAF-603 (**2.2**)



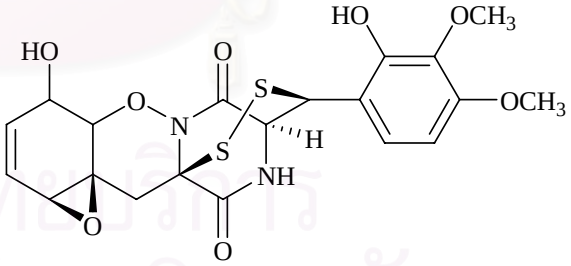
Trichocaranes A (**2.3**)



Trichocaranes B (**2.4**): R_1 = H, R_2 = OH
Trichocaranes D (**2.6**): R_1 = R_2 = OH

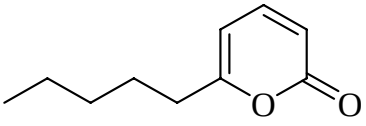


Trichocaranes C (**2.5**)

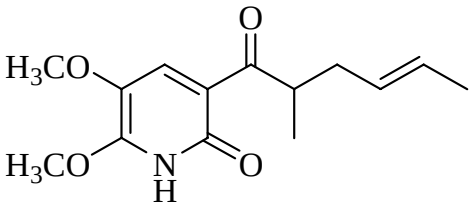


Gliovirin (**2.7**)

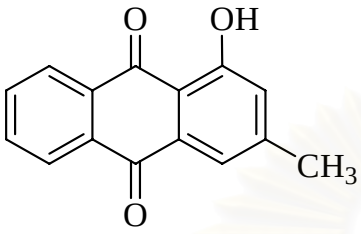
Trichoderma harzianum was the most investigated among the *Trichoderma* species for biocontrol and arguably the most effective. Many bioactive compounds included 6PP (**2.8**) (Ghisalberti and Sivasithamparam, 1991), pyridone (**2.9**) (Dickinson *et al*., 1989). The remaining groups were anthraquinone **2.10** and butenolide **2.11** (Almassi *et al*., 1991).



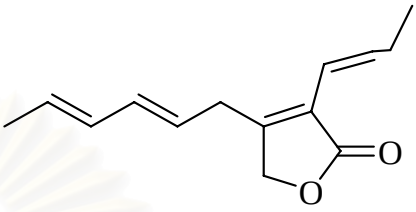
6PP (**2.8**)



Pyridone (**2.9**)

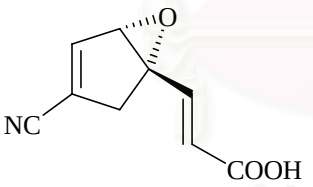


Anthraquinone **2.10**

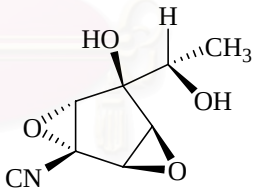


Butenolide **2.11**

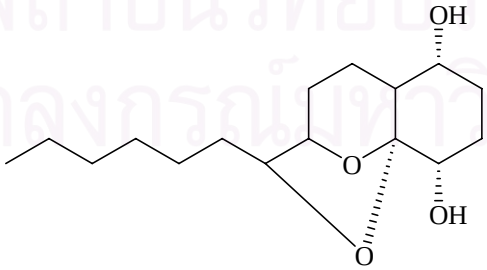
Tamura *et al.* reported the isolation of dermadin (**2.12**) and trichoviridin (**2.13**), metabolites containing an isocyanide group from *T. koningii* (Tamura *et al*., 1975). Dermadin showed antibiotic activity against gram-positive and negative bacteria and a wide variety of fungi while trichoviridin exhibited activity against *E. coli* (Pyke and Dietz, 1966). Moreover, some strains of *T. koningii* also produced 6PP and a ketal **2.14** (Culter *et al*., 1989).



Dermadin (**2.12**)



Trichoviridin (**2.13**)

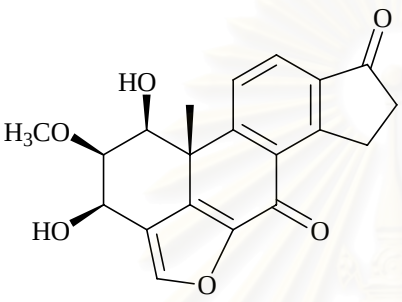


Ketal **2.14**

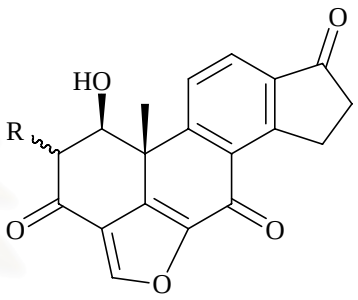
Dermadin, trichoviridin and isonitrin F were three isocyanide active compounds isolated from *T. hamatum* (Brewer *et al*., 1982). Isonitrin A was broadly

active against gram-positive and negative bacteria, yeast and filamentous fungi (Fugiwara *et al*., 1982; Okuda *et al*., 1982). In 2003, Wipf *et al*. reported the reassignment of TAEMC161, which is in fact identical to viridiol (**2.15**) (Wipf *et al*., 2003).

Viridin (**2.16**) and β-viridin (**2.17**) were also isolated from *Gliocladium flavofuscum* (Avent *et al*., 1993). Furthermore, viridiol, the antifungal metabolite of *Gliocladium deliquescens* was isolated by Hanson *et al* (Hanson *et al*., 1988).



Viridiol (**2.15**)



Viridin (**2.16**): R = α-OCH_3
β-viridin (**2.17**): R = β-OCH_3

The goal of this research will be as follows:

1. To extract and isolate the substances from liquid culture of *Trichoderma virens*
2. To elucidate the structures of the isolated compounds
3. To investigate the biological activity of isolated compounds

2.2 Extraction and isolation

The mycelial of *T. virens* was separated from culture by filtration through two layers of cheesecloth. The filtrate was extracted three times with equal volume of CH_2Cl_2, and the resulting organic layer was concentrated under vacuum to yield CH_2Cl_2 extract (5.0 g).

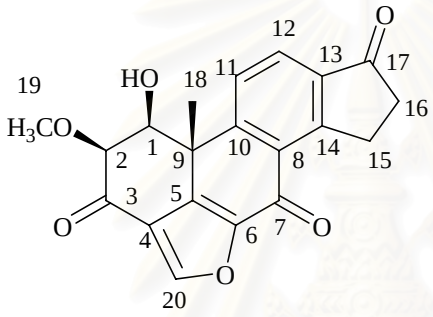
The CH_2Cl_2 extract mixed with silica gel (1:1) was loaded on the top of column and fractionated by vacuum column chromatography (VCC). The column was eluted by increasing polarity of solvent using gradient elution from *n*-hexane to MeOH. Fractions containing similar components were combined to yield 12 fractions.

The combined fractions 4-6 was chromatographed on silica gel column eluted with 100% CH_2Cl_2 to 10% EtOAc/CH_2Cl_2 to afford viridin (**1**, 5 mg) (Wipf *et al*., 2003) and 4-methylmevalonic acid (**2**, 5 mg).

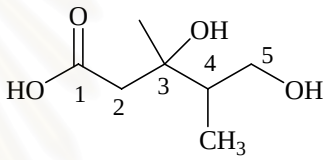
Fraction 9 was subjected to Chromatotron® eluted with EtOAc-*n*-hexane (0:1→1:1), giving viridiol (**3,** 15 mg) (Wipf *et al*., 2003).

Fraction 10 was subsequently purified on silica gel column chromatography eluted with EtOAc/CH_2Cl_2 (0:1→3:7) followed by Sephadex LH-20 [MeOH: CH_2Cl_2: *n*-hexane (0.5:1.5:3)] to obtain a novel furanosteroid named 9-*epi*-viridiol (**4**, 6 mg).

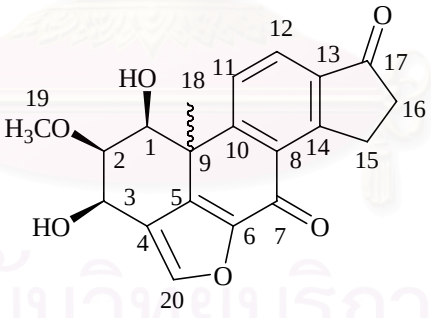
The isolation of CH_2Cl_2 extract of *T. virens* was summarized in Scheme 2.1



Viridin (**1**)

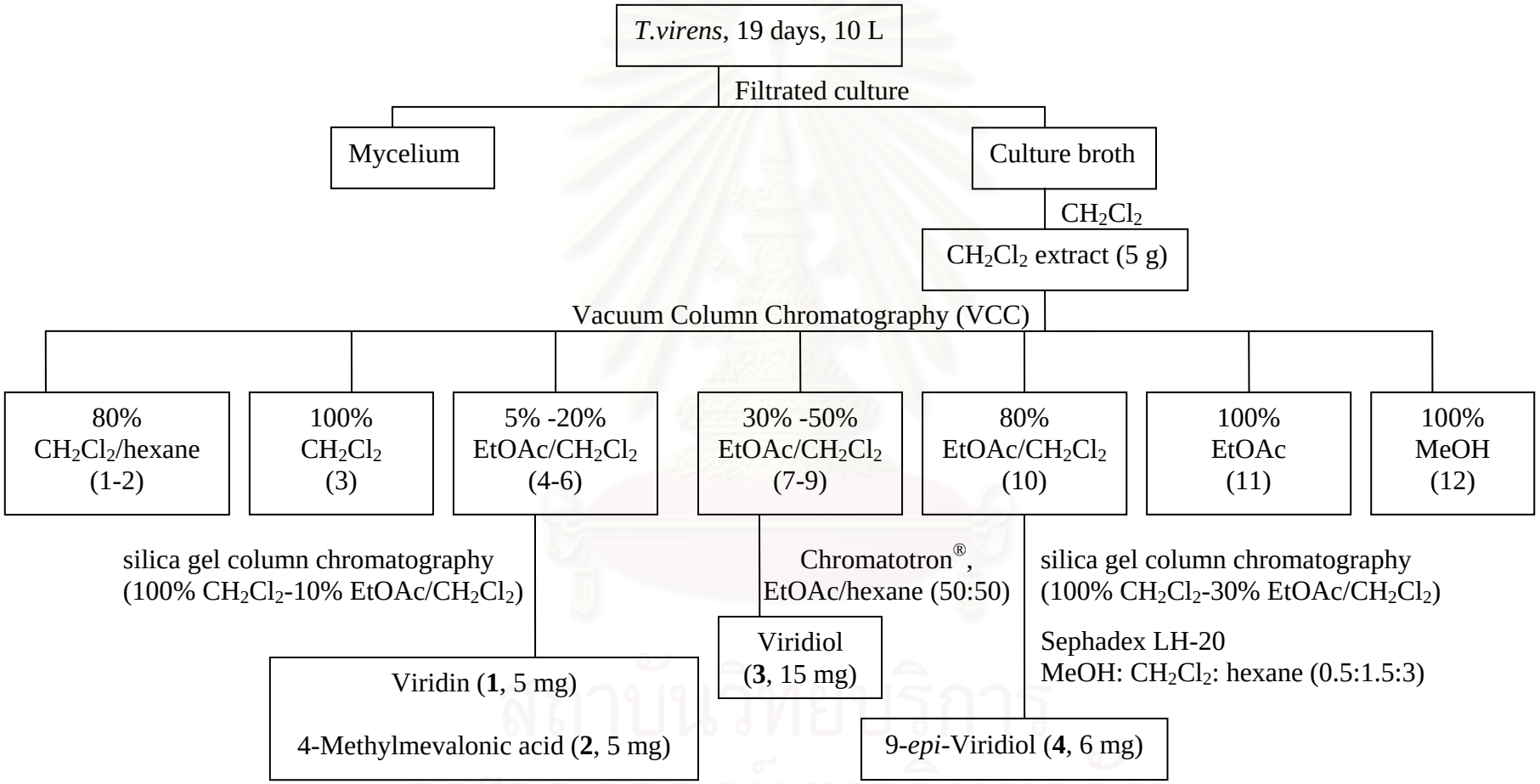


4-Methylmevalonic acid (**2**)



Viridiol (**3**): 18β-CH_3

9-*epi*-Viridiol (**4**): 18α-CH_3 (New)



Scheme 2.1 The isolation procedure of CH_2Cl_2 extract of *Trichoderma virens*

2.3 Structure elucidation of 9-*epi*-viridiol

9-*epi*-viridiol (**4**) was isolated as pale yellow amorphous. Its molecular formula was deduced as $C_{20}H_{18}O_6$ by high-resolution ESIMS; $[M + Na]^+$ 377.1032 (calcd 377.1001). The UV showed the maximum absorption band at 246 and 308 nm which suggested the presence of an aromatic carbonyl moiety. The ^1H NMR spectrum of compound **4** displayed signals of *ortho*-aromatic protons [7.90 (d, J = 8.2 Hz, 1H) and 7.58 (d, J = 8.2 Hz, 1H)], three contiguous oxygenated methines [5.08 (d, J = 6.0 Hz), 4.79 (d, J = 2.2 Hz) and 3.80 (dd, J = 2.2, 6.0 Hz)], one methoxy (3.65), one singlet methyl (1.65) and two sets of methylene protons [3.73 and 3.59 (m, each 1H); and 2.64 (m, 2H)]. The ^{13}C NMR showed 20 signals, one of which was readily accounted for ketone carbon (δ_C 206.7). On the basis of 2D NMR data, three partial structures (A-C) were constructed (Figure 2.2). Partial structure A consisted of three contiguous oxygenated methines (C-1 to C-3), in which C-1 and C-3 were flanked by two quaternary carbons at δ_C 43.7 and 122.2, respectively. The 19-OCH_3 was accommodated at C-2 (δ_C 83.7) while 18-CH_3 was located at C-9 (δ_C 43.7), as indicated by HMBC cross peaks. Partial structure B was established as a trisubstituted furan as evident by carbon signals typical to this moiety (Gavagnin *et al.*, 2003; McPhail *et al.*, 2001) and HMBC correlations between H-20 and C-4, C-5 and C-6. The HMBC correlations of H-3 to C-4, C-5 and C-20 and 18-CH_3 to C-1, C-5 and C-9 allowed the connection of partial structures A and B; C-3 was connected to C-4 whereas C-9 was assembled to C-5. Partial structure C included remaining signals ascribable to tetrasubstituted benzene. The HMBC correlations, particularly from H-12, H-15 and H-16 to C-14 and C-17, indicated the connectivity between benzene and cyclopentanone moieties. The partial structure C was assembled to partial structure A at C-9 as suggested by HMBC cross peak of H-11/C-9. The remaining unassigned signal at δ_C 173.1 should be placed at C-7, accounting for the aforementioned molecular formula.

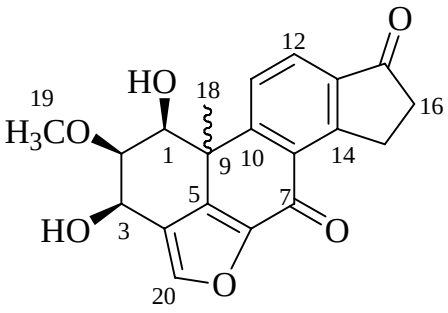
The NMR data of compound **4** turned out to be similar to those of viridiol (**3**), except for chemical shifts around chiral centers (C-1 to C-3 and C-9, Table 2.1). The small coupling constants of $J_{1,2}$ = 2.2 Hz and $J_{2,3}$ = 6.0 Hz were indicative of a *cis*-relation (Wipf *et al.*, 2003) between the hydroxy and methoxy groups. In NOESY spectrum, the cross peak between 18-CH_3 and H-1 supported their α-orientation and

confirmed that compound **4** was C-9 epimer of compound **3** (Figure 2.2). Therefore, the structure of compound **4** was described as 9-*epi*-viridiol (a novel furanosteroid).

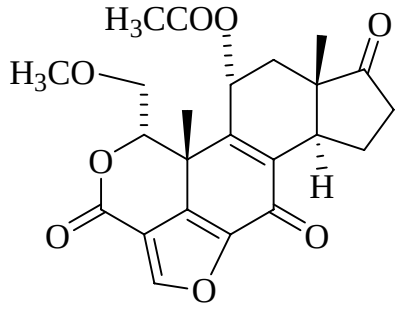
Table 2.1 NMR data of 9-*epi*-viridiol (**4**) in $CDCl_3$ (400 MHz for ^1H and 100 MHz for ^{13}C NMR)

Position	δ_C	δ_H (mult, *J* in Hz)[a]	HMBC
1	74.6	4.79 d (2.2) [4.26 d (5.8)]	C-2, 3, 5, 18
2	83.7	3.80 dd (2.2, 6.0) [3.58 dd (4.6, 5.8)]	C-1, 4, 9
3	60.2	5.08 d (6.0) [5.08 d (4.6)]	C-1, 4, 5, 20
4	122.2		
5	142.4		
6	146.1		
7	173.1		
8	137.2		
9	43.7		
10	154.9		
11	125.0	7.58 d (8.2)	C-8, 9, 13
12	127.1	7.90 d (8.2)	C-10, 14, 17
13	131.7		
14	159.1		
15	28.4	3.73 m, 3.59 m	C-8, 14, 16, 17
16	36.4	2.64 m (2H)	C-14, 15, 17
17	206.7		
18	31.2	1.65 s	C-1, 5, 9, 10
19	59.5	3.65 s	C-2
20	146.9	7.79 s	C-4, 5, 6

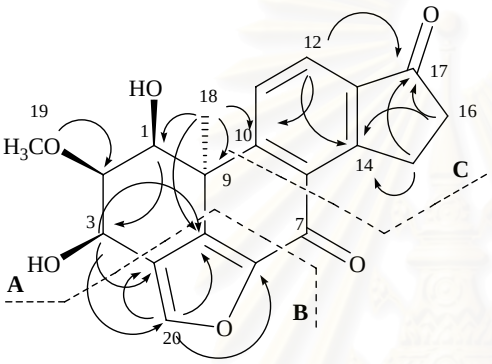
[a]Selected chemical shifts of viridiol (**3**) are shown in square brackets.



3: 18β-CH_3
4: 18α-CH_3



Wortmannin



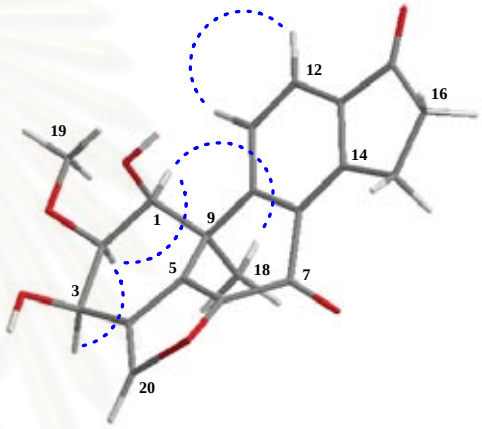


Figure 2.2 Partial structures and selected HMBC (solid arrows) and NOESY (dash curves) correlations of 9-*epi*-viridiol (**4**)

2.4 Biological activity of the isolated compounds

2.4.1 Cytotoxicity

Viridiol (**3**) and 9-*epi*-viridiol (**4**) showed moderate cytotoxicity against HeLa (human cervical carcinoma) (IC_{50} 7.8 and 19.0 μg/mL, respectively) and KB (human epidermoid carcinoma) cells (IC_{50} 17.0 and 50.0 μg/mL, respectively). Compound **4** is three-fold less active than compound **3**, suggesting that β-orientation of 18-CH_3 in compound **3** is possibly associated in exerting cytotoxicity against HeLa and KB cells. Compound **3** and **4** are structurally related to wortmannin, a potent inhibitor of PI-3 kinase, the enzyme that regulates cell growth and differentiation of cancer cells (Wipf and Halter, 2005). As a consequence of potent kinase inhibition, furanosteroids such as viridiol, 9-*epi*-viridiol and wortmannin would be useful tools in oncology.

Table 2.2 Cytotoxicity of isolated compounds against HeLa and KB cell lines

Compound	Cytotoxicity (IC_{50}, μg/mL)	
	HeLa	KB
Viridiol (**3**)	7.8	17.0
9-*epi*-Viridiol (**4**) (New)	19.0	50.0

2.4.2 Acetylcholinesterase inhibition assay

Acetylcholinesterase (AChE) inhibition was determined by many methods but TLC autographic assay was used in this experiment. The isolated compounds were tested using Ellman and Hostettmann methods in order to confirm their AChE inhibitory activity. Apparently, viridiol (**3**) showed inhibitory effect with MIC values of 0.125 mg/mL in both Ellman and Hostettmann methods (Table 2.3). However, viridin (**1**) and 9-*epi*-viridiol (**4**) demonstrated no inhibition against AChE at concentration of 1.000 mg/mL. Ellman and Hostettmann methods afforded the same results of AChE inhibition activity. Viridiol is distinct from the other isolated furanosteroids in having β-orientation of 18-CH_3 and OH group at C-3, suggesting that the orientation of these substituent groups possibly involved in binding to AChE function. Although other related steroids such as steroidal alkaloids (Kalauni *et al*., 2001) and alkylated steroids named haloxysterols A-D (Ahmed *et al*., 2006) have been reported as potent AChE inhibitors, this is first report of furanosteroids having AChE inhibition. According to these results, viridiol is a new candidate for further studies of AChE inhibitory activity.

Table 2.3 Minimal inhibitory concentrations (MICs) of isolated compounds of acetylcholinesterase inhibitor assay by Ellman method that compare with Hostettmann method

Compound	Ellman					Hostettmann				
	Concentration (mg/mL)					Concentration (mg/mL)				
	1.00	0.50	0.25	0.125	0.0625	1.00	0.50	0.25	0.125	0.0625
Viridin (**1**)	-	-	-	-	-	-	-	-	-	-
Viridiol (**3**)	++	++	++	++	-	++	++	++	++	-
9-*epi*-Viridiol (**4**) (New)	-	-	-	-	-	-	-	-	-	-
Eserine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

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

2.5 Experimental Section

2.5.1 General experimental procedures

The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Mercury+ 400 spectrometer in $CDCl_3$ with TMS as an internal standard. UV spectra were taken on a UV-160A spectrometer (SHIMADZU). ESIMS and HRESIMS were obtained by Micromass LCT mass spectrometer. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F254 plates (0.25 mm thick layer). Silica gel 60 Merck cat. No. 7731 and 7734 were used for vacuum column chromatography and open column chromatography, respectively. Chromatotron (model 7924 T, Harrison Research) on silica gel 60 F254 (Merck cat. No. 7749) plate of 1 mm thickness was carried out on centrifugal thin layer chromatography. Most solvents used in this research were commercial grade and were distilled prior to use.

2.5.2 Fungal material and culture condition

A strain of *Trichoderma virens* TV16 was obtained from Culture Collection at Department of Plant Pathology, Faculty of Agriculture, Kasetsart University Kamphaeng Saen Campus. The isolated culture was stored at 4 ºC in order to maintain biochemical metabolism. MEB medium (2% malt extract, 2% dextrose and 0.1% peptone) was used as seed and production media. The medium was sterilized in an

autoclave at 121 ºC for 20 min. The fungus was plated out on malt extract agar medium (MEB medium with 1.5% agar) at 25 ºC for 3 days. Fungus plug was cut into small pieces and transferred into 5 × 250 mL Erlenmeyer flask each containing 100 mL of seed medium, which was incubated at 25 ^{o}C for 2 days. Then, the broth culture was inoculated into the production medium (100 × 250 mL Erlenmeyer flask each containing 100 mL of medium). Cultivation was kept under static conditions at 25 ºC for 19 days.

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

Cytotoxicity of isolated compounds was evaluated using MTT assay at Natural Products Research Section, Research Division, National Cancer Institute, Thailand. The KB (human epidermoid carcinoma) and HeLa (human cervical carcinoma) cell lines were used in this experiment. Adriamycin was used as antibiotic antitumor agent. The results were shown in Table 2.2.

2.5.4 Acetylcholinesterase inhibitor assay

Acetylcholinesterase inhibitor assay was determined by TLC autographic assay which has 2 methods:

A. Hostettmann method (Marston *et al*., 2002)

Acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7) 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 ºC. Sample solution (10 μL) was spotted on TLC plate, which was then sprayed with enzyme solution and dried again. For incubation of the enzyme, the plate was laid flat on moist chamber at 37 ºC for 20 min. 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. Region of the TLC plate which contain AChE inhibitor show up as white spots against the purple background. The minimal inhibitory concentrations (MICs) of isolated compounds were measured by the minimal concentration that appears white spot on purple TLC plate.

B. Ellman method (Ellman *et al*., 1960; Rhee *et al*., 2001)

The TLC autographic assay for measuring AChE activity was modified from the method described by Ellman. AChE 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 ºC. Sample solution (10 μL) was spotted on TLC plate, which was then sprayed with mixture of 5,5′- dithiobis-(2-nitrobenzoic acid) (DNTB) and acetylthiocholine iodine (ATCI) (5 mM DNTB and 5 mM ATCI in buffer A). It was allowed to dry for 3-5 min and then 3 U/mL of enzyme solution was sprayed. A yellow background appeared, with white spots for inhibiting compounds becoming visible after 5 min. These were observed and recorded within 15 min because they disappeared in 20-30 min. The minimal inhibitory concentrations (MICs) of isolated compounds were measured by the minimal concentration that appears white spot on purple TLC plate.

Viridin (1): Yellow amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 8.58 (1H, d, *J* = 8.0 Hz, H-12), 8.24 (1H, s, H-20), 7.91 (1H, d, *J* = 8.0 Hz, H-11), 4.21 (1H, s, H-1), 3.82 (1H, m, H-2), 3.74 (3H, s, 19-OCH_3), 3.58 (1H, m, H-15b), 3.53 (1H, m, H-15a), 2.64 (2H, m, H-16), 1.67 (3H, s, 18-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 205.5 (C-17), 188.1 (C-3), 172.3 (C-7), 157.0 (C-14), 154.7 (C-10), 147.6 (C-20), 145.7 (C-6), 141.1 (C-5), 136.3 (C-8), 128.7 (C-13), 127.3 (C-12), 126.6 (C-11), 121.1 (C-4), 84.9 (C-2), 74.4 (C-1), 60.8 (C-19), 40.8 (C-9), 35.8 (C-16), 27.5 (C-15), 26.3 (C-18).

4-Methylmevalonic acid (2): Yellow amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 4.30 (2H, m, H-2), 2.68 (1H, d, *J* = 17.6 Hz, H-5b), 2.47 (1H, d, *J* = 17.6 Hz, H-5a), 1.94 (1H, m, H-4), 1.28 (3H, d, *J* = 6.8 Hz, 3-CH_3), 0.93 (3H, d, *J* = 6.8 Hz, 4-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 169.4 (C-1), 70.0 (C-5), 68.2 (C-3), 43.9 (C-2), 35.3 (C-4), 26.3 (3-CH_3), 8.1 (4-CH_3).

Viridiol (3): Yellow amorphous solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ_H 8.49 (1H, d, *J* = 8.0 Hz, H-12), 7.92 (1H, s, H-20), 7.81 (1H, d, *J* = 8.0 Hz, H-11), 5.04 (1H, d, *J* = 4.4 Hz, H-3), 4.38 (1H, s, H-1), 3.84 (1H, d, *J* = 4.4 Hz, H-2), 3.70 (3H, s, 19-OCH_3), 3.55 (2H, m, H-15), 2.63 (2H, m, H-16), 1.74 (3H, s, 18-CH_3); ^{13}C NMR (acetone-d_6, 100 MHz) δ_C 205.8 (C-17), 173.2 (C-7), 159.8 (C-10), 157.2 (C-14), 145.8 (C-6), 145.4 (C-20), 143.9 (C-5), 136.5 (C-8), 130.0 (C-13), 126.8 (C-12),

126.1 (C-11), 124.6 (C-4), 84.8 (C-2), 72.4 (C-1), 63.4 (C-3), 42.5 (C-9), 35.8 (C-16), 31.5 (C-18), 30.8 (C-19), 28.1 (C-15); Selected ^{1}H NMR data of compound **3** in $CDCl_3$ are in Table 2.1.

9-*epi*-Viridiol (4): Pale yellow amorphous powder; $[\alpha]_D^{28}$ -1.6° (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε) 246 (4.15), 308 (3.81); HRESIMS *m/z* $[M + Na]^+$ 377.1032 (calcd for $C_{20}H_{18}O_6Na$, 377.1001); ^{1}H and ^{13}C NMR (Table 2.1).

Supporting information

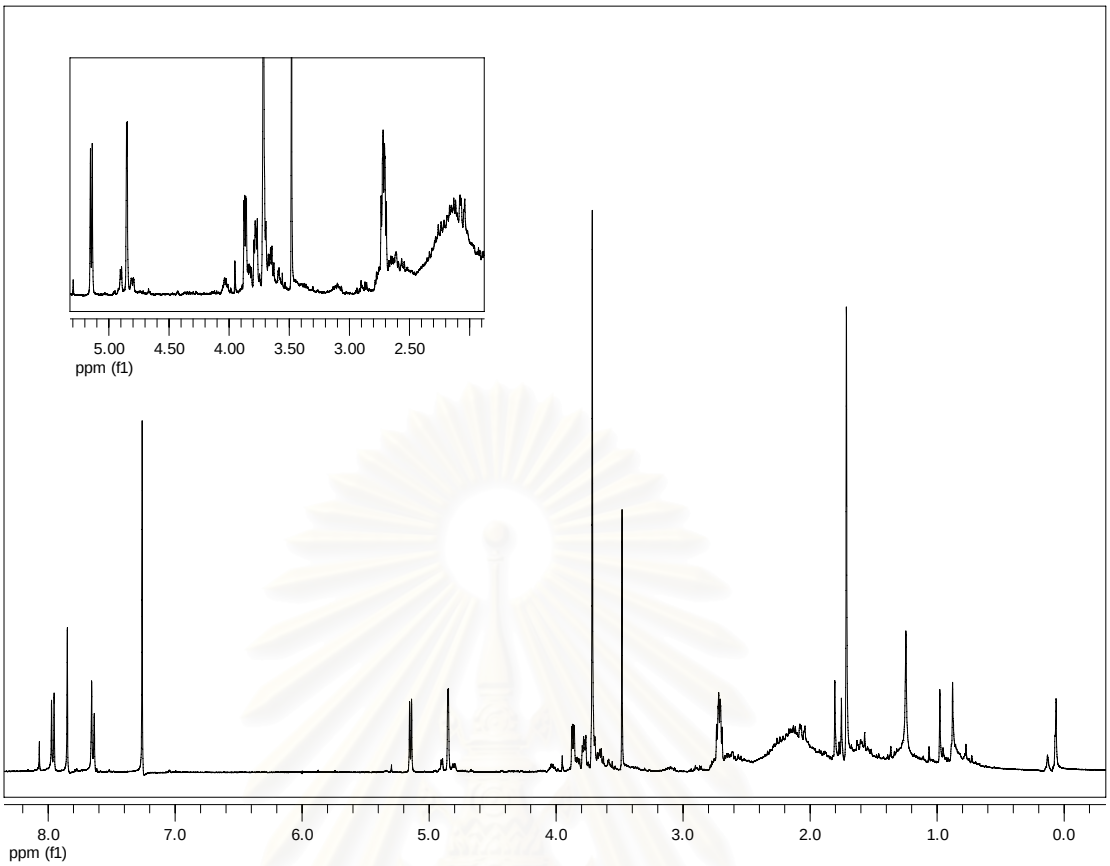


Figure S-2.1 The ^{1}H NMR spectrum of 9-*epi*-viridiol (**4**) in $CDCl_3$

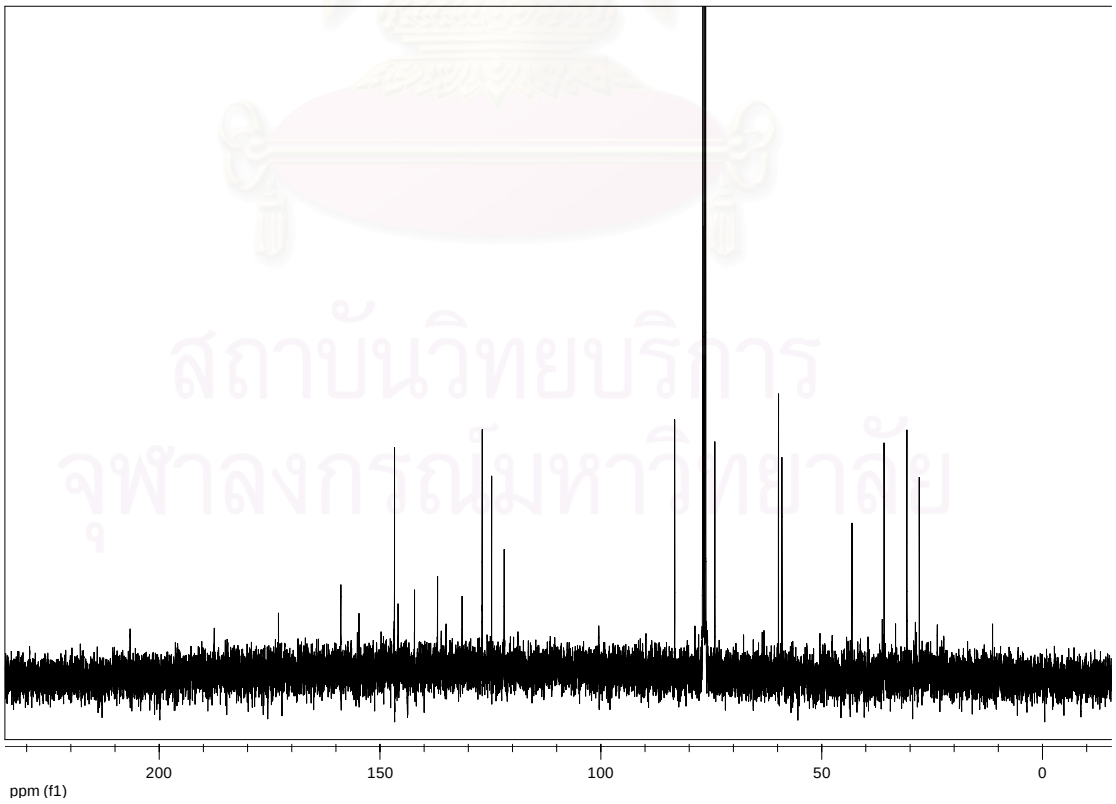


Figure S-2.2 The ^{13}C NMR spectrum of 9-*epi*-viridiol (**4**) in $CDCl_3$

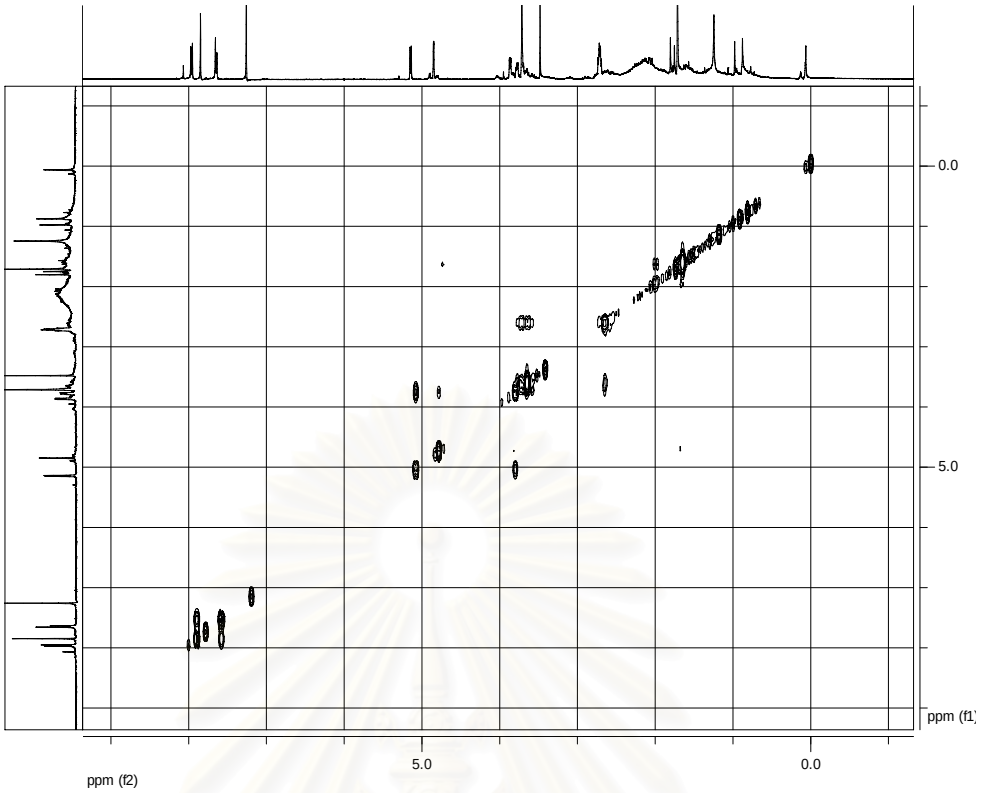


Figure S-2.3 The COSY spectrum of 9-*epi*-viridiol (**4**) in $CDCl_3$

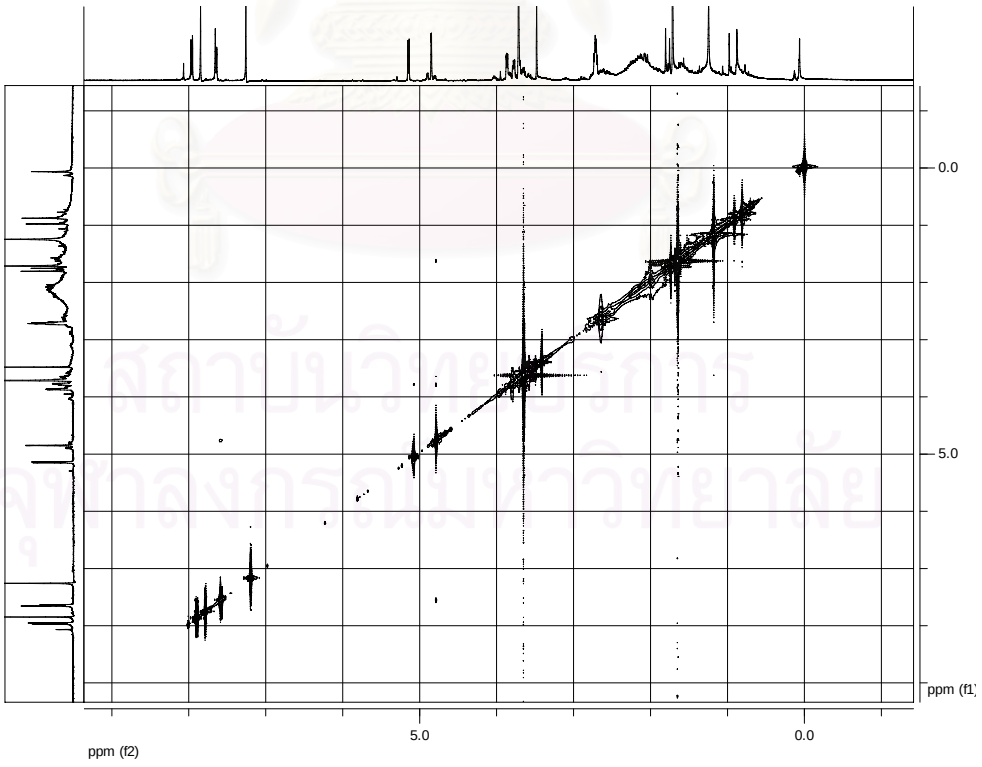


Figure S-2.4 The NOESY spectrum of 9-*epi*-viridiol (**4**) in $CDCl_3$

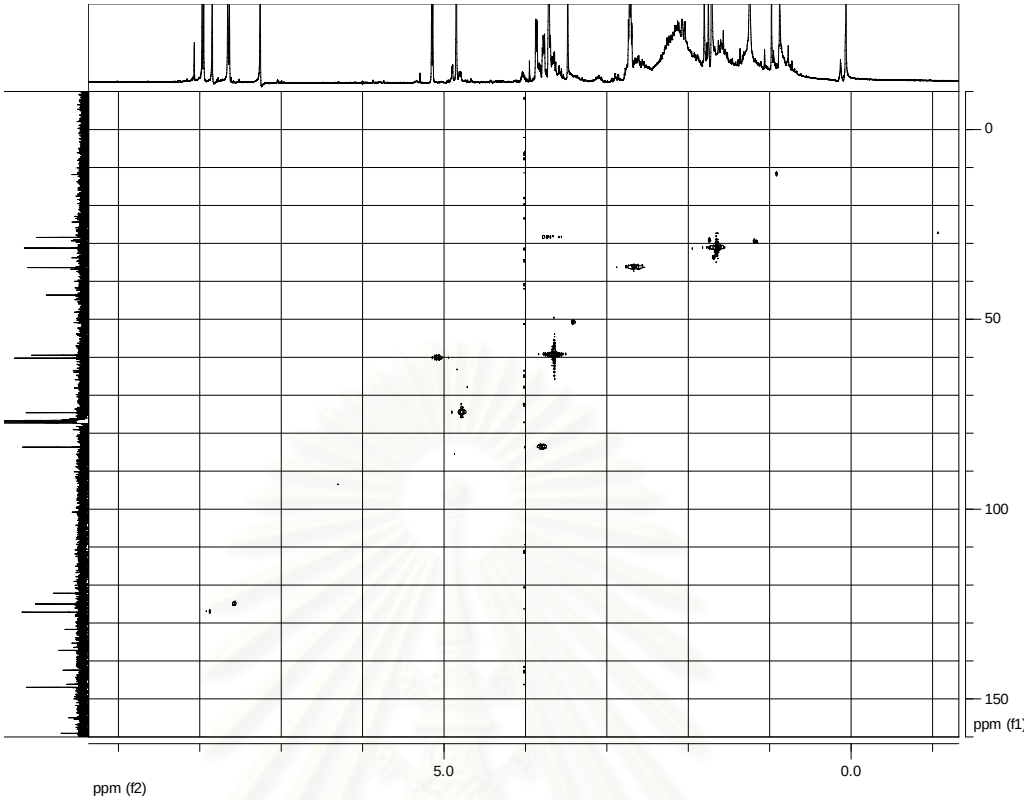


Figure S-2.5 The HSQC spectrum of 9-*epi*-viridiol (**4**) in $CDCl_3$

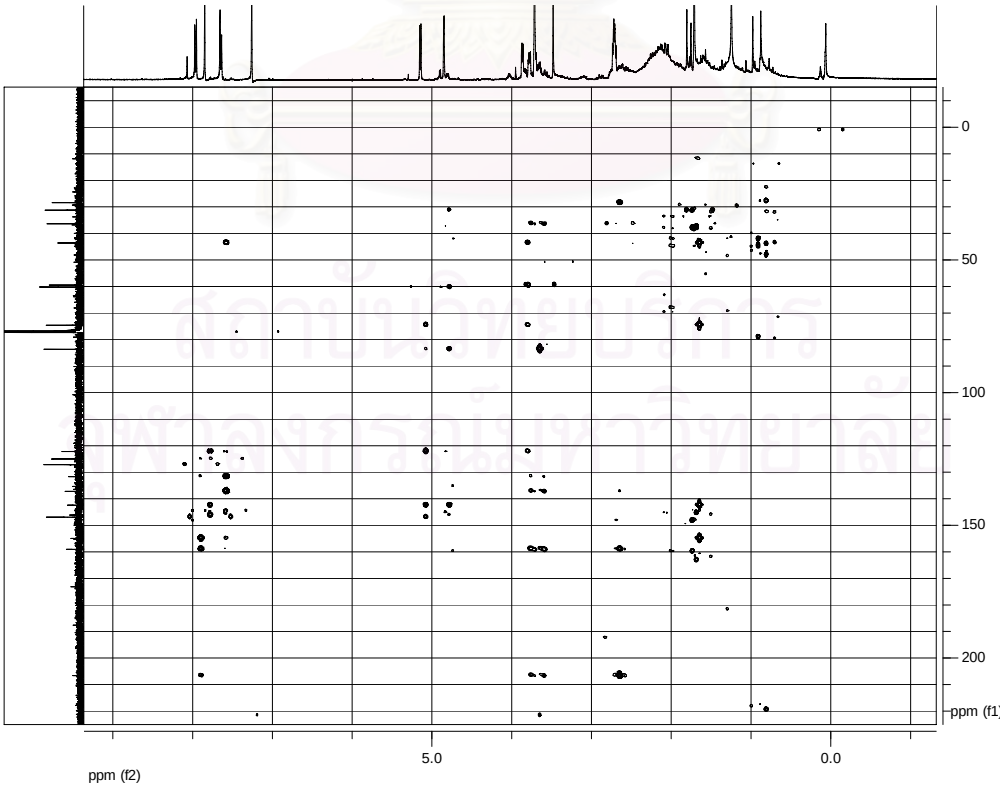


Figure S-2.6 The HMBC spectrum of 9-*epi*-viridiol (**4**) in $CDCl_3$

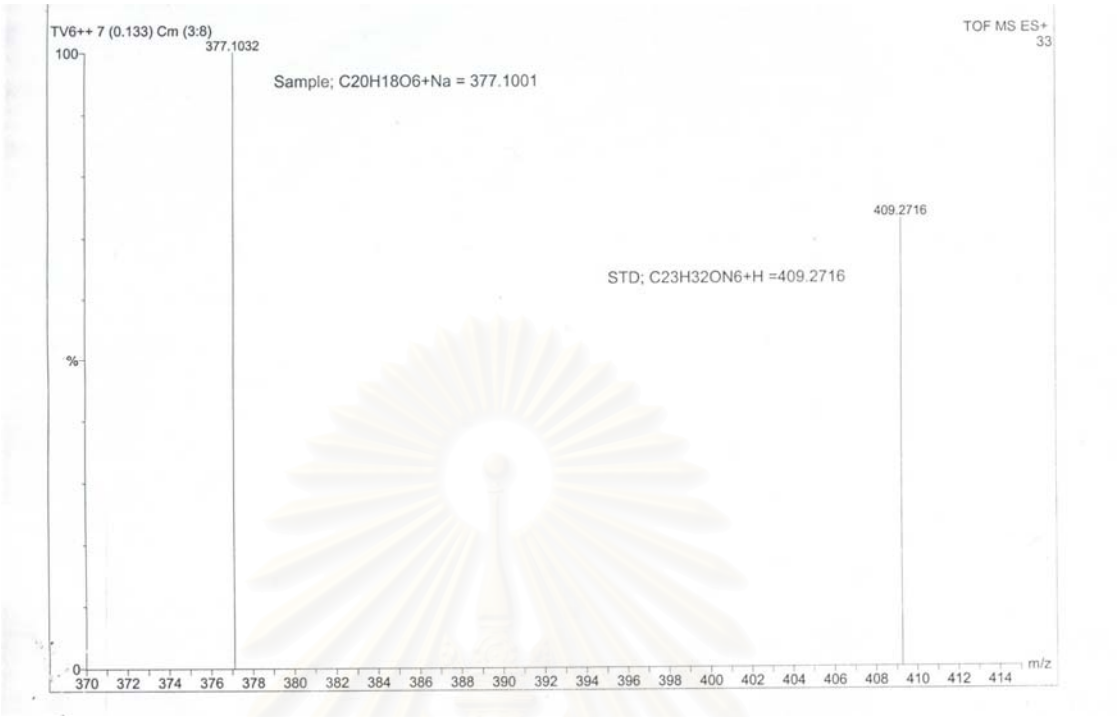


Figure S-2.7 The high resolution mass spectrum of 9-*epi*-viridiol (**4**)

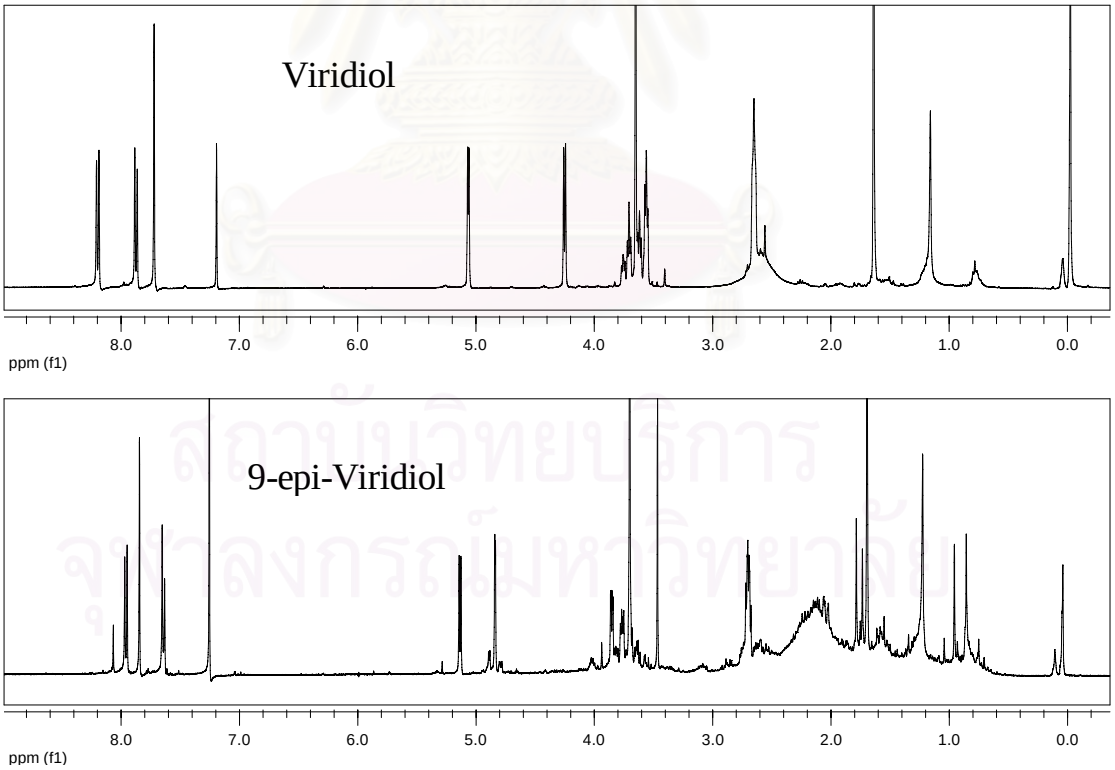


Figure S-2.8 The ^{1}H NMR spectrum of viridiol (**3**) compare with 9-*epi*-viridiol (**4**) in $CDCl_3$

CHAPTER III

BIOACTIVE COMPOUNDS FROM *Alternaria porri*

3.1 Introduction

The various species of *Alternaria* cause decay on most of fresh fruits and vegetables either before or after harvest. The symptoms appear as brown or black, flat or sunken spots with definite margins, or as diffuse, large, decayed areas that are shallow or extend deep into the flesh of fruits and vegetables. The fungus develops well as a wide range of temperatures, even in the refrigerator, although at a slower rate.

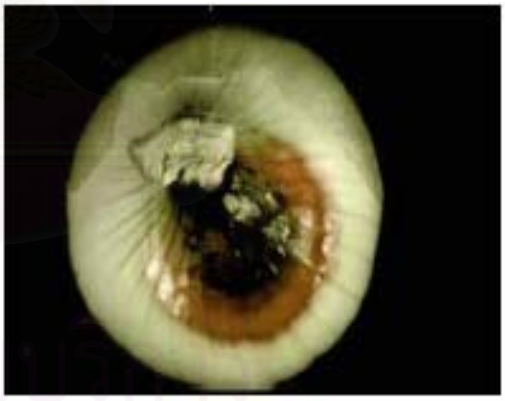
The other names of phytopathogenic fungus *Alternaria porri* are *Alternaria alii* Nolla or *Macrosporium porri*. *Alternaria porri* is causal fungus of purple blotch of common onion, Egyptian onion, Welsh or Spanish onion, garlic, leek, false shallot, and possibly other members of the onion family may become infected. It is a deuteromycetes fungus that morphology of conidiophores (specialized fungal hypha that produces numerous spores) are single or in groups, straight or flexuous. Sometimes conidiophores are geniculate, septate, pale to mid brown, up to 120 μm long, 5-10 μm thick, with 1 or several well-defind conidial scars. Conidia (numerous spores) are usually solitary, straight or curved, obclavate or with the body of the conidium ellipsoidal, tapering to the beak which is commonly about the same length as the body but may be shorter or longer, pale to mid-golden brown, smooth or minutely verrucose. Overall length of conidia usually are 100-300 μm, 15-20 μm thick in the broadest part, with 8-12 transverse and 20 to several longitudinal or oblique septa, beak flexuous, pale, 2-4 μm thick and tapering (Holliday, 1995).

Purple blotch is an important disease and worldwide, especially in warm and humid environments. Early symptoms appear as small, whitish sunken lesions. Almost immediately, the spots turn brown, enlarge, and become zoned, somewhat sunken, and more or less purplish (Figure 3.1). The lesions occur on the leaves, flower

A)

B)

C)



D)

Figure 3.1 A) High-power microscope of conidiophores of *Alternaria porri*, B) Close-up of purple blotch lesions on onion leaves, C) Purple blotch and necrosis lesion on onion leaves, D) *Alternaria* bulb rot on onion by infected of *Alternaria porri*

stalks, and floral parts of seed onions. The lesion borders are reddish and surrounded by a yellow “halo”. Affected leaves and stems may turn yellow, die back, collapse and die within several weeks after the first lesions appear. In moist weather, diseased tissues are covered with a dense, dark purplish black mold composed of large numbers of microscopic, dark multicelled spores. The conidia are carried to other onion leaves by air currents, splashing rains, tools, and so on. When the spores land on susceptible onion tissue they germinate in a film of water, and the germ tubes penetrate to the stomates or penetrate directly through the epidermis. Early symptoms can appear 1 to 4 days after penetration has occurred. A new generation of conidia may be produced every 5 days in warm, moist weather. Infection, reproduction, and spread of the disease may follow in rapid succession as long as favorable conditions persist. Free moisture, in the form of rain, persistent fog, or dew, is required for infection and spore production. Mycelial growth of the *Alternaria* fungus occurs over a temperature range of 6 to 34 ºC (optimum 25 to 27 ºC) at a relative humidity of 90 percent. Almost no infection occurs below 24 ºC. Onion bulbs become infected at harvest or later in storage through the neck or through wounds in the fleshy bulb scales. The rot is first semiwatery and a deep yellow but gradually turns a wine-red, finally becoming dark brown to black. Often only one or two outer scales are affected. Diseased bulb tissue gradually dries out and becomes “papery.” Sweet Spanish onions are very susceptible, while varieties with waxy foliage have some resistance (University of Illinois, Department of Crop Sciences, 1990).

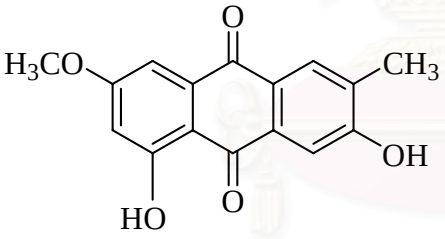
Studies on the involvement of toxin in plant pathogenic microorganism date from the second half of the 19th century. As a result, many new phytotoxin, pesticide, fungicide, antibiotic, plant growth regulator and mycotoxin have been reported. Most of plant pathogenic fungi produce toxins in culture and in their hosts. Frequently, these compounds play a role in the pathogenesis and reproduce some, or even all, of the symptoms of the diseases. The chemical nature of these toxins ranges from low molecular weight compounds includes all classes of natural products such as terpenes, chromamones, butanolides, pyrones, macrolides, aromatic derivatives, amino acids etc. to high molecular weight compounds such as proteins, glycoproteins and polysaccharides (Tringali, 2001). Phytotoxins can spread in plants by diffusion from the site of fungal infection to adjacent tissue or through transport via the plant’s xylem. Two classes of phytotoxins can be distinguished: The majority belongs to the

non-selective or non-host-specific toxin. These are poisonous to all plants, not only the plant serving as host for the toxin-producing pathogen. The so-called host-selective or host-specific toxin, belong to the second and smaller class of phytotoxins. They are active only on host plants carrying genetically determined sensitivity for the particular toxin.

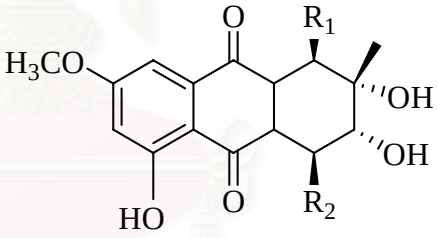
Alternaria sp. can produce non-host-specific toxin and host-specific toxin. Only non-host-specific toxin was isolated from culture of *Alternaria porri* because host-specific toxin represents the signal recognized by the sensor of the host plant (host recognition). The bioactive compounds or phytotoxins of *A. porri* can be separated into 2 groups.

A. Anthraquinones and bianthraquinone derivatives

Suemitsu *et al.* reported the isolation of three anthraquinones; macrosporin (**3.1**), 3-methylxanthopurpurin-6-methylether and 1, 2, 8-trihydroxy-6-methoxy-3-methylanthraquinone. In addition, altersolanols A-C (**3.2-3.4**) and dactylariol were also obtained from *A. porri* (Suemitsu *et al*., 1990). Altersolanols A-C showed antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtillis*, *Micrococcus luteus* and *Pseudomonas aeruginosa* (Yagi *et al*., 1993).

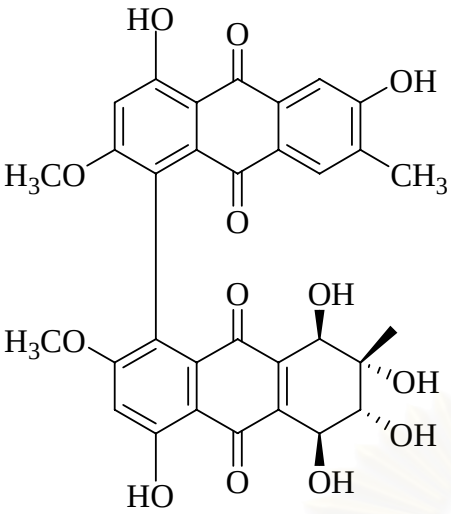


Macrosporin (**3.1**)

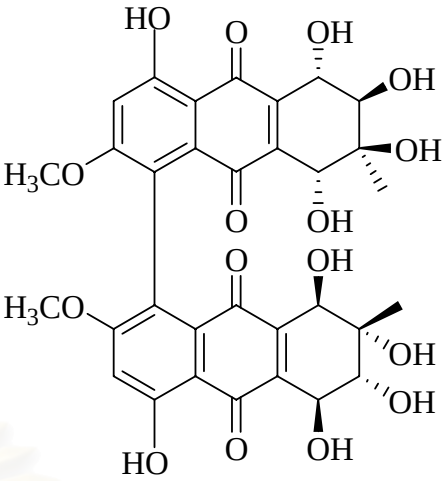


Altersolanol A (**3.2** ;R_1 = OH, R_2 = OH)
Altersolanol B (**3.3** ;R_1 = H, R_2 = H)
Altersolanol C (**3.4** ;R_1 = OH, R_2 = H)

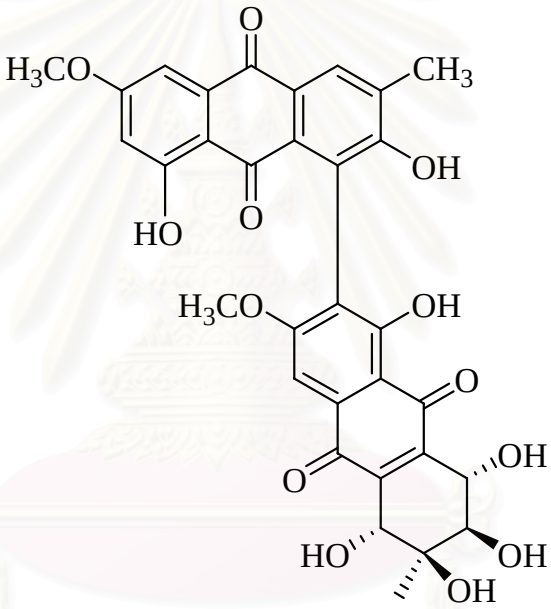
The fungal pigments, bianthraquinones were also reported. Alterporriols A-C (**3.5-3.7**) consisted of two anthraquinones, macrosporin and altersolanol A, connected through C-C bond. The restrict rotation around the C-C bond generated two separable atropisomers such as alterporriols A (**3.5**) and B (**3.6**) as well as alterporriols D (**3.8**) and E (**3.9**) (Suemitsu *et al*., 1987; 1988; 1989).



Alterporriols A (**3.5**) and B (**3.6**)



Alterporriols D (**3.8**) and E (**3.9**)

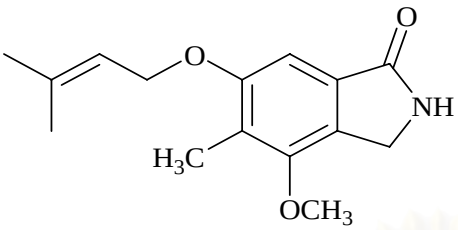


Alterporriol C (**3.7**)

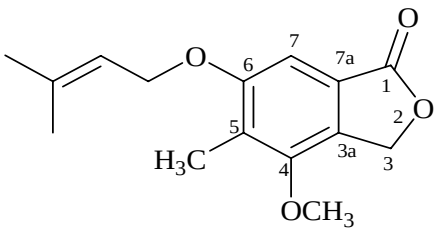
B. Phthalides and phthalates

The other types of phytotoxin, zinnimidine (**3.10**), 6-(3′,3′-dimethylallyloxy)-4-methoxy-5-methylphthalide (**3.11**) and 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (**3.12**) were also investigated from *A. porri*. They displayed no inhibitory activity toward lettuce and stone-leek seedlings (Suemitsu *et al*., 1995). Porritoxin sulfonic acid (**3.13**) and 2-(2′′-hydroxyethyl)-4-methoxy-5-methyl-6-(3′-methyl-2′-butenyloxy)-2,3-dihydro-1*H*-isoindol-1-one (**3.14**) isolated from liquid culture of *A. porri* showed potent activity in seedling-growth assay against stone leek and lettuce (Horiuchi *et al*., 2003). Tentoxin and zinniol (**3.15**) were also isolated in

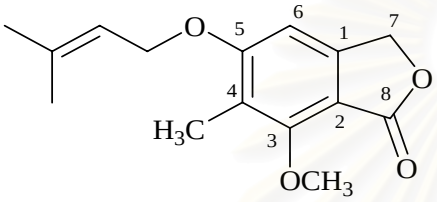
A. porri, in which zinniol was known additionally in other *Alternaria* species. The porritoxin and porritoxinol showed the inhibition of seedling growth in lettuce at concentration of 10.0 and 12.5 ppm, respectively (Suemitsu *et al*., 1992; 1994).



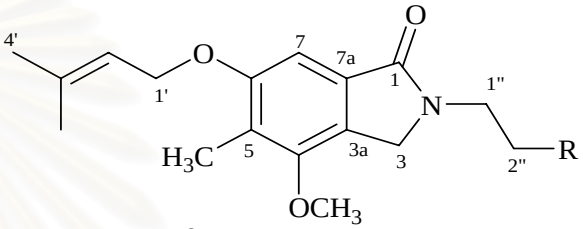
Zinnimidine (**3.10**)



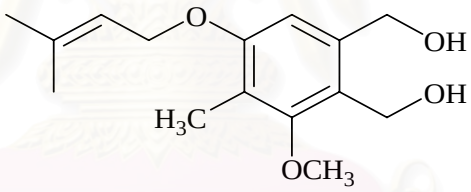
6-(3' ,3' -Dimethylallyloxy)-4-methoxy-5-methylphthalide (**3.11**)



5-(3' ,3' -Dimethylallyloxy)-3-methoxy-4-methylphthalide (**3.12**)



Porritoxin sulfonic acid **(3.13** ;R = SO_3H)
2-(2''-hydroxyethyl)-4-methoxy-5-methyl-6-(3'-methyl-2'-butenyloxy)-2,3-dihydro-1*H*-isoindol-1-one (**3.14** ;R = OH)



Zinniol (**3.15**)

From the previous data, isolated compounds from *A. porri* show potent biological activity. In order to discover bioactive compound for used in any field, the aims of this research are:

1. To extract and isolate the substances from liquid culture of *Alternaria porri*
2. To elucidate the structures of the isolated compounds
3. To investigate the biological activity of isolated compounds

3.2 Extraction and isolation

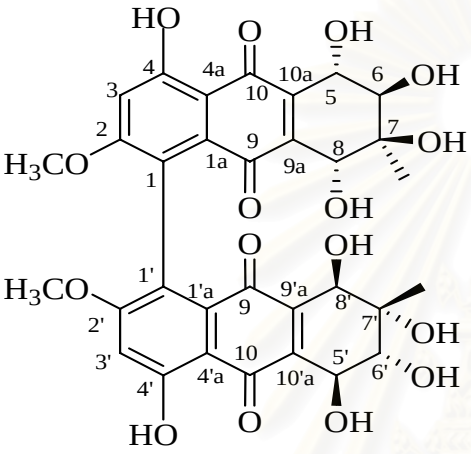
After harvesting, the culture broth (10 L) was filtered, and the culture filtrate was extracted three times with equal volume of CH_2Cl_2. The CH_2Cl_2 layer was evaporated under reduced pressure, yielding 5.0 g of CH_2Cl_2 extract. The aqueous layer was partitioned three times with equal volume of EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to yield 5.0 g of EtOAc extract.

The CH_2Cl_2 extract was fractionated by VCC eluted with gradient of CH_2Cl_2 and EtOAc. Zinnimidine (**5**, 185 mg) (Suemitsu *et al*., 1995) was obtained from the fraction eluted with 30% EtOAc/CH_2Cl_2 of VCC. The EtOAc extract was fractionated by VCC using gradient elution from *n*-hexane to MeOH to afford 7 fractions (Scheme 3.1). Fraction 6 was subsequently purified on silica gel column chromatography (CC SiO_2) eluted with gradient from CH_2Cl_2 to MeOH and then followed by HPLC [Nova-Pak RP-C_{18} (8 × 100 mm), 40% MeOH/H_2O at flow rate 6 mL/min, λ 254 nm] to afford alterporriol D or E (**6**, 20 mg) (Suemitsu *et al*., 1989; Okamura *et al*., 1996). Fraction 5 was subjected to silica gel column chromatography (CC SiO_2), eluted with gradient from CH_2Cl_2 to MeOH, and then was further rechromatographed on Sephadex LH-20 [MeOH: CH_2Cl_2: hexane (0.5:2:2)] to obtain alterporriol A (**7**, 270 mg) (Suemitsu *et al*., 1987) and alterporriol C (**8**, 40 mg) (Suemitsu *et al*., 1988). Fraction 4 was purified on Sephadex LH-20 using elution of MeOH: CH_2Cl_2: hexane (1:2:2), giving 2 fractions (4.1 and 4.2). The former fraction was seperated on Chromatotron® eluted with 80% EtOAc/hexane to obtain a novel compound named alterporriol F (**9**, 7 mg).

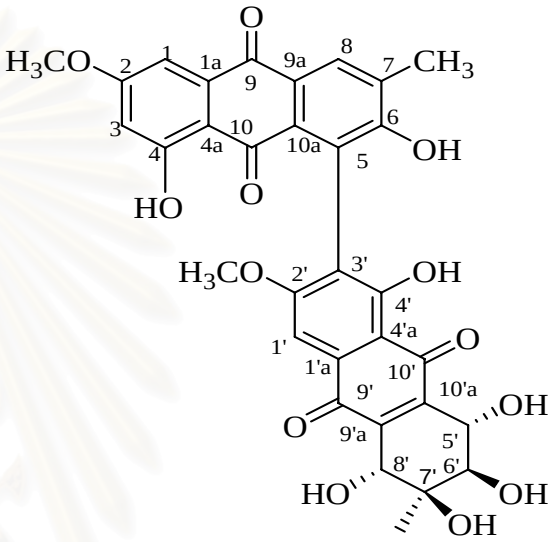
Fraction 3 was fractionated by silica gel column chromatography (CC SiO_2) eluted with gradient of CH_2Cl_2-MeOH, resulting in 6 fractions (3.1-3.6). Fraction 3.1 was purified on silica gel column chromatography (CC SiO_2) using a gradient of CH_2Cl_2 to MeOH and then on Chromatotron® eluted with 10% EtOAc/hexane, yielding a new compound named zinnimide (**10**, 7 mg) and macrosporin (**11**, 230 mg) (Suemitsu *et al*., 1987). 5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**, 2 mg) (Suemitsu *et al*., 1995) was obtained from Sephadex LH-20 [MeOH: CH_2Cl_2: hexane (1:5:5)] of fraction 3.3. Fraction 3.4 was further purified on Chromatotron® using elution of 30% EtOAc/hexane to afford fractions 3.4.1 and 3.4.2. Fraction 3.4.1 was seperated on Sephadex LH-20 using elution of MeOH: CH_2Cl_2: hexane (1:5:5) to

afford porriolide (**13**, 10 mg) (Suemitsu *et al*., 1993) and alternariol monomethyl ether (**14**, 15 mg) (Koch *et al*., 2005). Fraction 3.5 was purified on Sephadex LH-20 [MeOH: CH_2Cl_2: hexane (1:5:5)], yielding novel compound, deprenylzinnimide (**16**, 7 mg), along with alternariol (**15**, 5 mg) (Koch *et al*., 2005) and demethylmacrosporin (**17**, 5 mg) (Hosoe *et al*., 1990).

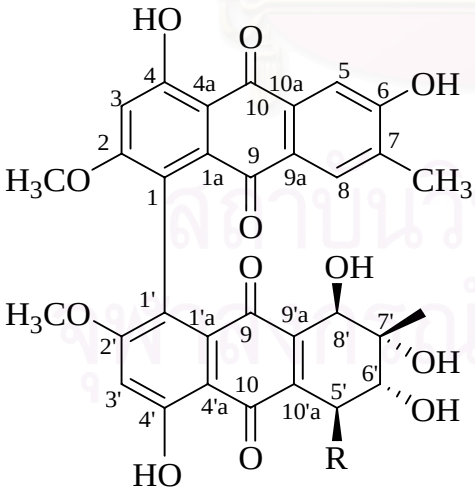
The isolation procedure of *A. porri* was summarized in Scheme 3.1.



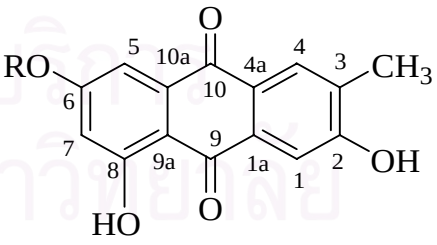
Alterporriol D or E (**6**)



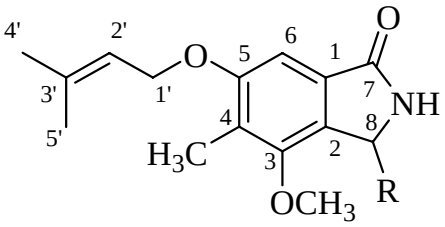
Alterporriol C (**8**)



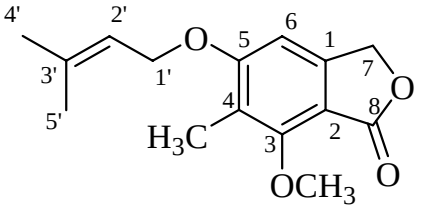
Alterporriol A (**7**): R = OH
Alterporriol F (**9**): R = H



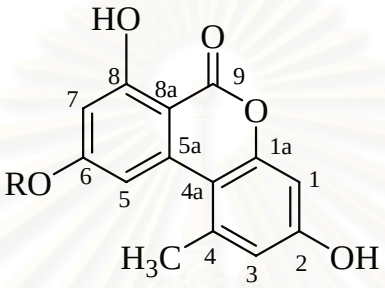
Macrosporin (**11**): R = CH_3
Demethylmacrosporin (**17**): R = H



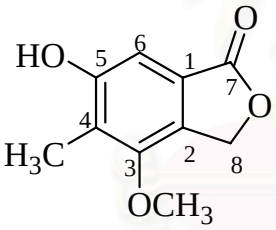
Zinnimidine (**5**): R = H
Zinnimide (**10**): R = O═



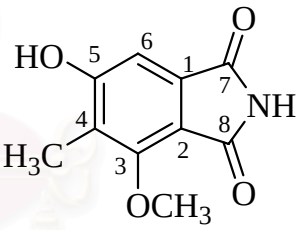
5-(3', 3' -Dimethylallyloxy)-
3-methoxy-4-methylphthalide (**12**)



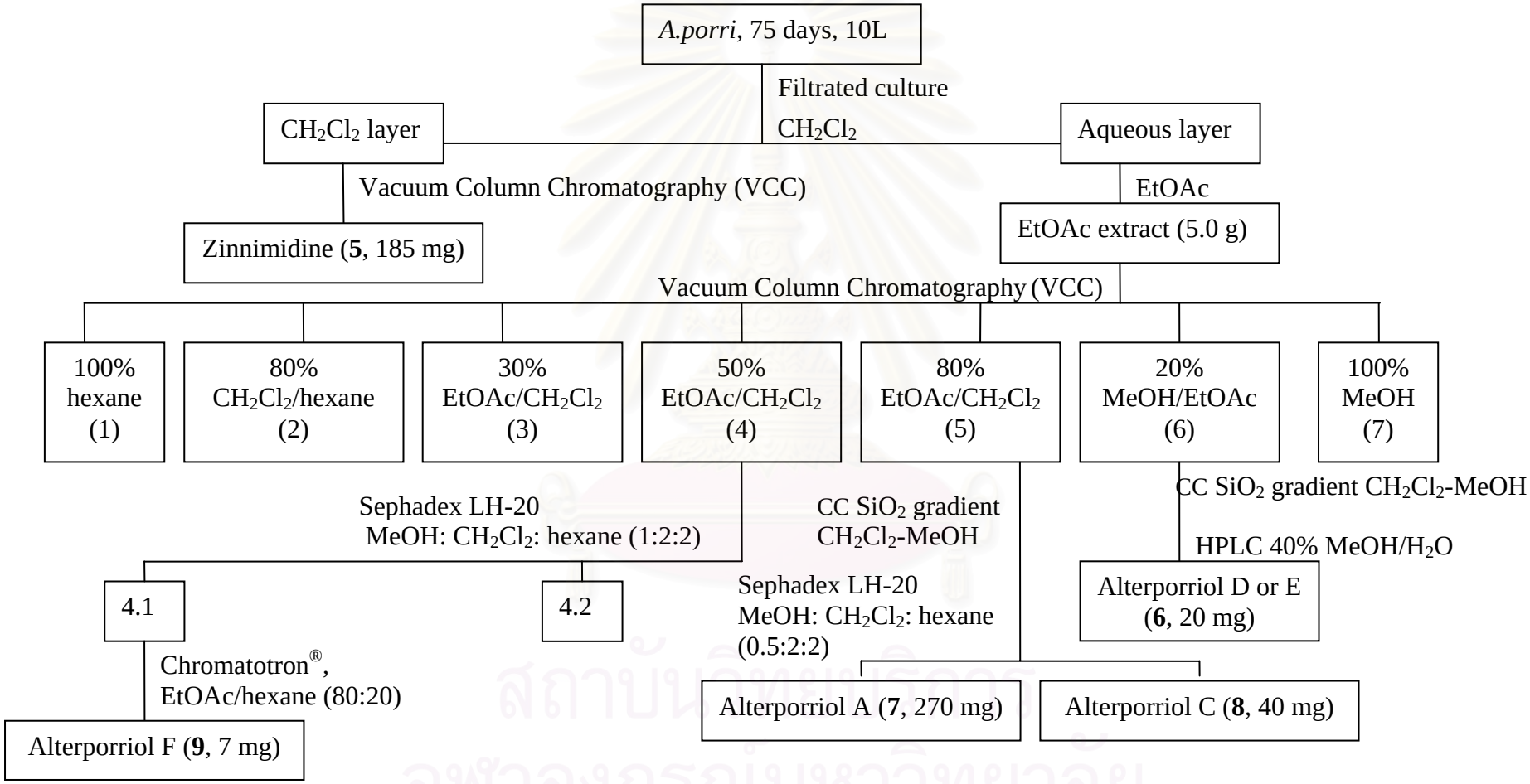
Alternariol monomethyl ether (**14**): R = CH_3
Alternariol (**15**): R = H



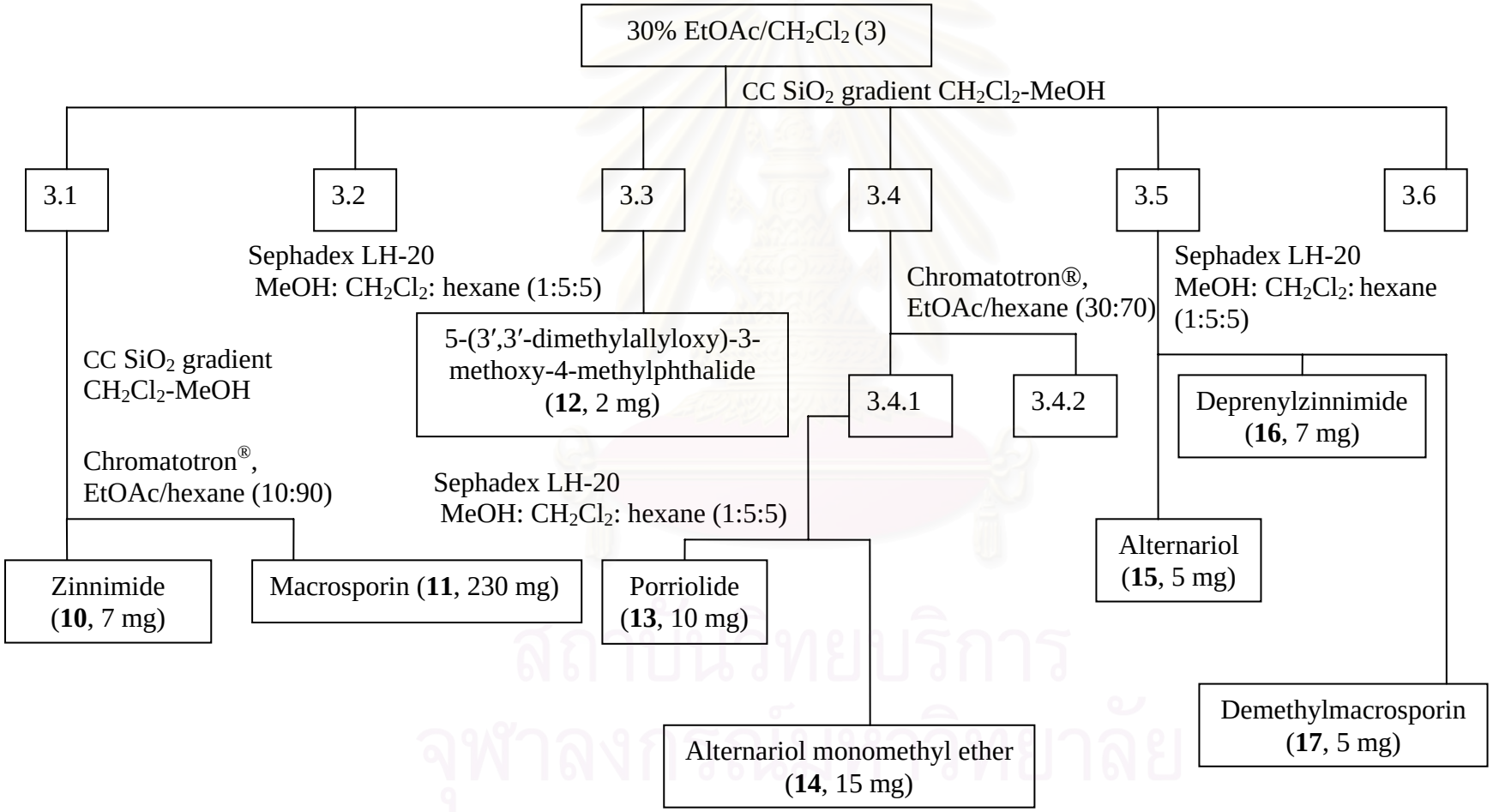
Porriolide (**13**)



Deprenylzinnimide (**16**)



Scheme 3.1 The isolation procedure of the compounds from *Alternaria porri*



Scheme 3.1 The isolation procedure of the compounds from *Alternaria porri* (continued)

3.3 Structure elucidation of new compounds

3.3.1 Alterporriol F

Alterporriol F (**9**) was obtained as a deep red amorphous powder. The ^{1}H NMR spectrum displayed signals apparently arising from two closely related isomers in a 1:1 ratio. The isomer having more downfield signals was arbitrarily assigned as compound **9a** while the other was **9b**. The molecular formula of compound **9** was deduced as $C_{32}H_{26}O_{12}$ by HRESIMS; $[M+Na]^{+}$ 625.1324 (calcd 625.1322). As for compound **9a**, the presence of two hydrogen-bonded hydroxyl protons [δ_H 13.65 (4-OH) and 13.13 (4′-OH)], two sets of aromatic protons [δ_H 7.67 (H-8), 7.53 (H-5) and 6.92 (2H, H-3 and H-3′)] and molecular formula, suggested that it had two anthraquinone moieties (Okamura *et al*., 1993; Yagi *et al*., 1993). Comparing the ^{1}H NMR spectral data of compound **9a** with isolated compounds from the *Alternaria* species, it was similar to those of macrosporin and altersolanol C; both are metabolic pigments of *Alternaria porri*. Compound **9a** was presumed to be a modified bianthraquinone consisting of macrosporin and altersolanol C. The disappearance of COSY correlation between H-5 and H-8, and HMBC correlations of H-3, H-5 and H-8 to their adjacent carbons (Table 3.1) exhibited that one of anthraquinone moiety in alterporriol F was macrosporin. In addition, the COSY correlation between methylene (H-5′) and oxygenated methine protons (H-6′), together with HMBC correlations of 7′-CH_3/C-6′, C-7′, C-8′ and the presence of methylene protons [δ_H 2.78 (dd, J = 5.6, 19.6 Hz) and 2.30 (dd, J = 9.6, 19.6 Hz)], indicated that the remaining moiety in alterporriol F was altersolanol C (Okamura *et al*., 1993) in compound **9**. The above data also confirmed that alterporriol F consisted of macrosporin (**I**) and altersolanol C (**II**).

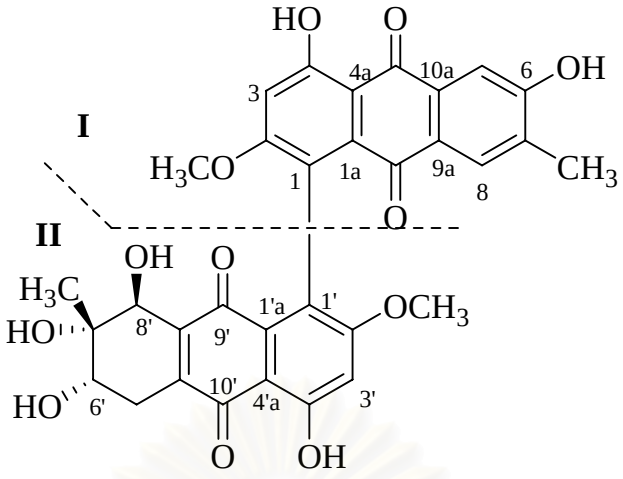
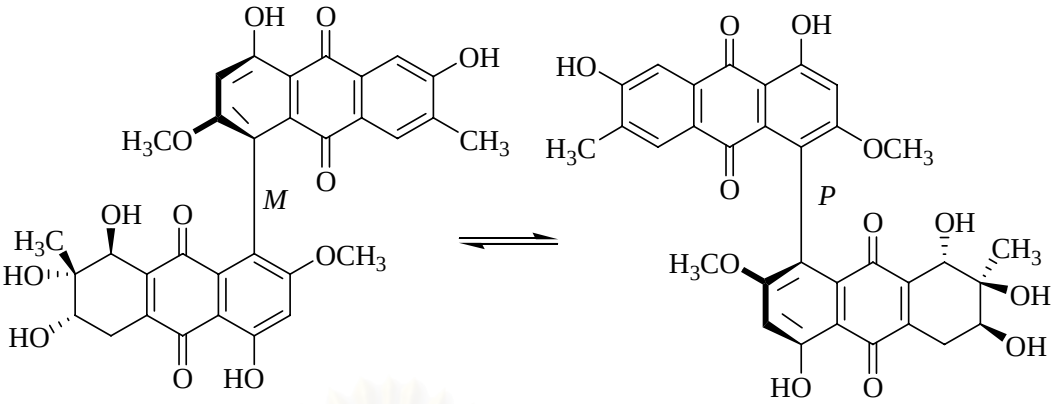


Figure 3.2 Partial structures (**I** and **II**) of alterporriol F (**9**)

The connectivity between partial structures **I** and **II** was inferred from HMBC correlations of H-3 and H-3′ to their adjacent carbons (Table 3.1) although intercorrelations among these moieties were not observed in 2D NMR spectra. The HMBC cross peaks of H-3 to quaternary C-1 and H-3′ to quaternary C-1′ and the lack of aromatic proton on C-1 and C-1′ suggested that **I** was connected at C-1 with **II** at C-1′ though C-C linkage, therefore completing overall structure of alterporriol F. Alterporriol F exhibited most diagnostic resonances similar to those of alterporriol A (Suemitsu *et al*., 1987), except for the presence of methylene protons [δ_H 2.78 (dd, J = 5.6, 19.6 Hz) and 2.30 (dd, J = 9.6, 19.6 Hz)] (Figure 3.3). Apparently, the presence of methoxy groups in *ortho* position prevented free rotation of C-C single bond (Suemitsu *et al*., 1989), causing the existence of two atropisomers (**9a** and **9b**) in alterporriol F. According to the Helical nomenclature (Eliel and Wilen, 1994), the axial configuration of compound **9a** and **9b** was assigned as *M* and *P*, respectively.



9a **9b**

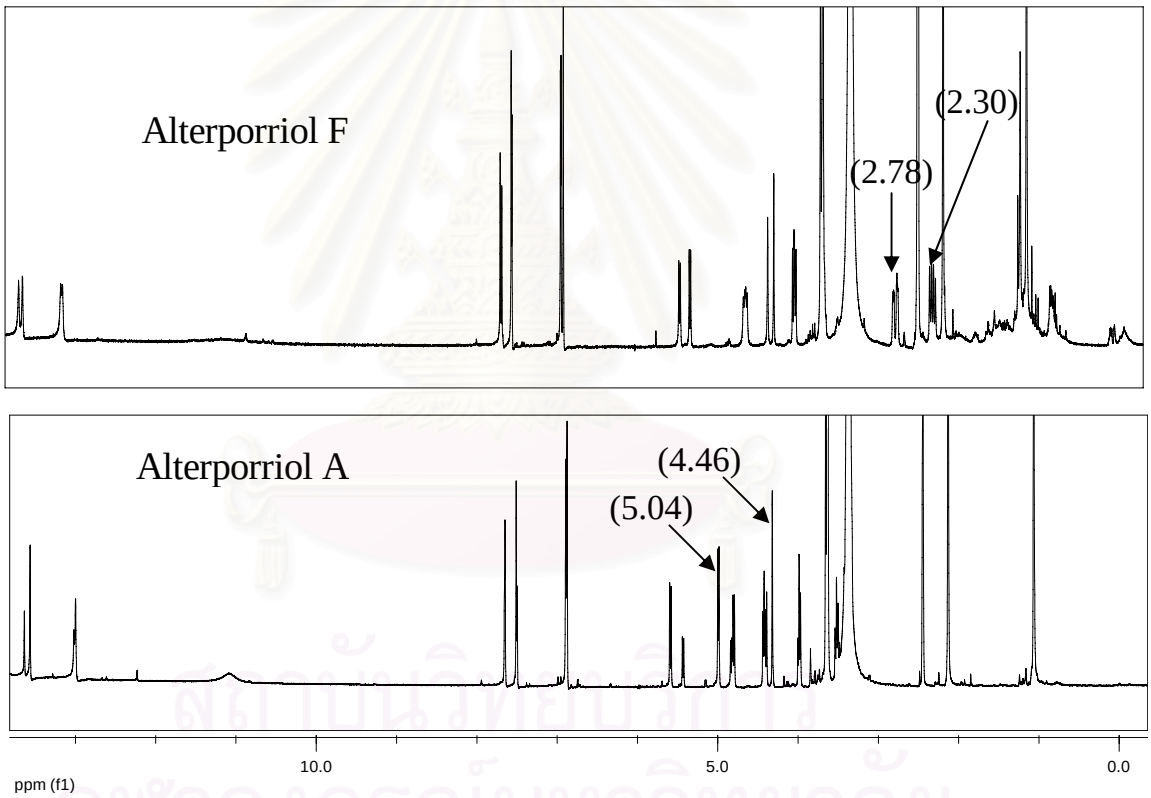


Figure 3.3 The ^{1}H NMR spectrum of alterporriol F (**9**) compare with alterporriol A (**7**) in DMSO-d_6

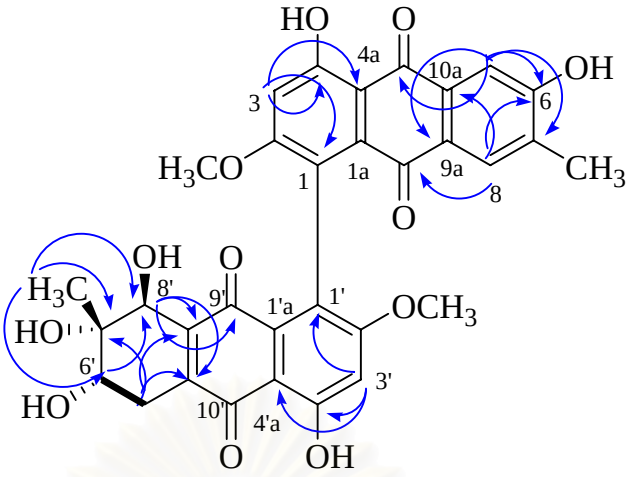


Figure 3.4 Selected HMBC (arrow curve) and COSY (solid line) correlations of **9**

Table 3.1 The ^{1}H and ^{13}C NMR[a] data of alterporriol F (**9a** and **9b**) in DMSO-d_6

Position of carbon	δ_C		δ_H (mult, *J* in Hz)		HMBC
	9a	**9b**	**9a**	**9b**	
1	108.9	108.9			
1a	124.3	124.3			
2	165.9	165.9			
3	104.2	104.2	6.92 m	6.89 m	C-1, C-4, C-4a
4	164.2	164.2			
4a	110.8	110.8			
5	111.0	111.0	7.53 s	7.52 s	C-6,C-7, C-9a, C-10
6	161.7	161.7			
7	131.4	130.3			
8	133.1	133.1	7.67 s	7.65 s	C-6, C-9, C-10a
9	181.3	181.3			
9a	125.3	125.3			
10	186.9	186.9			
10a	126.4	126.4			
2-OCH_3	57.2	57.2	3.69 m	3.69 m	C-2
4-OH			13.65 s	13.60 s	C-3, C-4, C-4a
6-OH					
7-CH_3	16.5	16.5	2.17 s	2.17 s	C-6, C-7, C-8
1´	110.0	110.0			
1´a	109.0	109.0			
2´	163.9	163.9			
3´	103.8	104.2	6.92 m	6.89 m	C-1´, C-4´, C-4´a
4´	165.2	165.2			
4´a	110.8	110.8			
5´	29.0	29.0	2.78 dd (5.6, 19.6) 2.30 dd (9.6, 19.6)	2.73 dd (5.6, 19.6) 2.30 dd (9.6, 19.6)	C-6´, C-7´, C-9´a, C-10´a
6´	67.2	67.2	3.66 m (overlap)	3.66 m (overlap)	C-5´, C-8´
7´	72.3	72.3			
8´	69.7	69.3	4.03 d (7.6)	4.01 d (7.6)	C-9´, C-9´a, C-10´a
9´	184.1	184.1			
9´a	143.2	143.2			
10´	189.4	189.4			
10´a	144.1	144.1			
2´-OCH_3	57.1	57.1	3.69 m	3.69 m	C-2´
4′-OH			13.13 s	13.11 s	C-3´, C-4´, C-4´a
6´-OH			4.64 d (6.8)	4.61 d (6.8)	C-6´, C-7´
7´-OH			4.35 s	4.27 s	C-6´, C-8´
7´-CH_3	22.0	22.6	1.13 s	1.13 s	C-6´, C-7´, C-8´
8´-OH			5.44 d (7.6)	5.31 d (7.6)	C-8´, C-9´a

[a]Measured at 400 MHz (^{1}H) and 100 MHz (^{13}C).

3.3.2 Zinnimide

Zinnimide (**10**) was obtained as a yellow amorphous solid. Its molecular formula was deduced as $C_{15}H_{17}NO_4$ by HRESIMS; $[M + Na]^+$ 298.1065 (calcd 298.1055), indicating eight degrees of unsaturation.

The ^1H NMR spectrum of compound **10** showed signals ascribable to NH amide proton [9.64 (brs, 1H)], pentasubstituted aromatic proton [7.01 (s, 1H)], one methoxy (3.94), singlet methyl (2.04) and oxygenated prenyl group [5.30 (1H), 4.66 (2H) and 1.67 (6H)]. The HMBC correlation between H-1′ and C-5 allowed the connection of oxygenated prenyl group at C-5 of benzene ring. The remaining methoxy and methyl groups were placed at C-3 and C-4, respectively, as evident from HMBC cross peaks of OCH_3/C-3 and CH_3/C-3, C-4 and C-5. The HMBC correlation especially from H-6 to carbonyl carbon C-7 (δ_C 168.2) indicated the connectivity between benzene ring and amide. By default, the remaining element CO (δ_C 167.0) must be amide carbonyl that connected benzene ring at C-2 and formed cyclic diamide to account for the molecular formula. Therefore the structure of zinnimide was described as compound **10**.

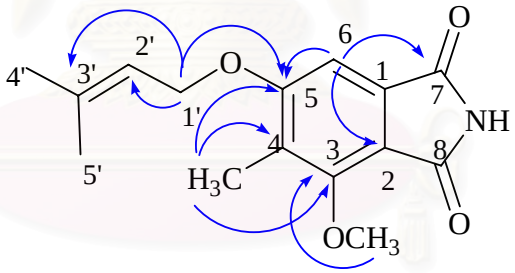


Figure 3.5 Selected HMBC correlation of zinnimide (**10**)

Table 3.2 The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of zinnimide (**10**) in acetone-d_6

Position of carbon	δ_C	δ_H (mult, *J* in Hz)	HMBC
1	134.0		
2	114.5		
3	156.8		
4	125.2		
5	162.8		
6	101.4	7.01 s	C-1, C-2, C-4, C-5, C-7
7	168.2		
8	167.0		
3-OCH_3	61.4	3.94 s	C-3
4-CH_3	8.5	2.04 s	C-3, C-4, C-5
1′	65.9	4.66 d (6.4)	C-5, C-2', C-3'
2′	119.2	5.40 m	C-4', C-5'
3′	138.3		
4′	24.9	1.67 s	C-2', C-3', C-4', C-5'
5′	17.3	1.67 s	C-2', C-3', C-4', C-5'
NH		9.64 brs	

3.3.3 Deprenylzinnimide

Deprenylzinnimide (**16**) was isolated as a yellow amorphous solid. Its molecular formula was deduced as $C_{10}H_9NO_4$ by HRESIMS; $[M + Na]^+$ 230.0439 (calcd 230.0424).

The ^1H NMR spectrum of compound **16** displayed signals of hydroxyl proton [9.85 (s, 1H)], NH amide proton [9.65 (brs, 1H)], pentasubstituted aromatic proton [6.91 (s, 1H)], one methoxy (3.95) and singlet methyl (2.03). The HMBC cross peaks of OCH_3/C-3 and CH_3/C-3, C-4 and C-5 indicated that the methoxy and methyl groups were accommodated at C-3 and C-4, respectively. The hydroxyl proton was placed at C-5, as evident from HMBC cross peaks of 5-OH/C-4, C-5 and C-6. The HMBC correlations particularly between H-6 and carbonyl carbon C-7 (δ_C 168.0) indicated that amide was flanked by quaternary carbon C-1 (δ_C 134.0) of the benzene ring. By default, the remaining element CO must be amide carbonyl that must be connected with benzene ring at C-2, forming cyclic diamide to account for the molecular formula. Therefore the structure of deprenylzinnimide was described as compound **16**.

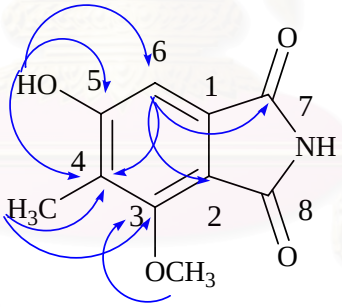


Figure 3.6 Selected HMBC correlations of deprenylzinnimide (**16**)

Table 3.3 The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of deprenylzinnimide (**16**) in acetone-d_6

Position of carbon	δ_C	δ_H (mult, *J* in Hz)	HMBC
1	134.0		
2	113.2		
3	157.0		
4	123.4		
5	161.7		
6	105.0	6.91 s	C-2, C-4, C-7
7	168.0		
8[a]			
3-OCH_3	61.5	3.95 s	C-3
4-CH_3	8.5	2.03 s	C-2, C-3, C-4, C-5
5-OH		9.85 s	C-4, C-5, C-6
NH		9.65 brs	

[a]carbon chemical shift was not detected.

3.4 Biological activity of isolated compounds

3.4.1 Cytotoxicity

From comparison of cytotoxicity results between HeLa and KB cell lines in Table 3.4, all isolated compounds showed only slightly different IC_{50} values. Therefore, cytotoxicity of all isolated compounds against HeLa and KB cell lines were generally nonspecific. For structure activity relationship study, all isolated compounds were categorized into 4 groups: phthalides and phthalimides, anthraquinones, bianthraquinones and dibenzo-α-pyrones. The first group, phthalides and phthalimides, they were zinnimidine (**5**), zinnimide (**10**), 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**), porriolide (**13**) and deprenylzinnimide (**16**). All of five compounds in this type displayed no cytotoxicity against HeLa and KB cell lines, compared with antibiotic antitumor agent (adriamycin showed IC_{50} at 0.018 μg/mL). The second group, anthraquinones, there were macrosporin (**11**) and demethylmacrosporin (**17**). Only compound **17** showed moderate cytotoxicity in both cell lines, while compound **11** exhibited no cytotoxicity. These results suggesting that the hydroxyl group on oxygenated C-6 of compound **17** possibly associated in exerting cytotoxicity against HeLa and KB cells. The third group, bianthraquinones, there were alterporriols D or E (**6**), A (**7**), C (**8**) and F (**9**). Interestingly, novel compound **9** (IC_{50} = 6.5, 7.0 μg/mL), which was differ

structurally from compound **7** in the absent of only hydroxyl group at C-5′, was more potent cytotoxic activity than compound **7** (IC_{50} = 18.0, 19.0 μg/mL). Therefore, it was suggested that the disappearance of hydroxyl group at C-5′ seem to be an important in cytotoxic activity of bianthraquinones.

Table 3.4 Cytotoxicity of isolated compounds against HeLa and KB cells

Compound	Cytotoxicity (IC_{50}, μg/mL)	
	HeLa	KB
Zinnimidine (**5**)	45.0	46.0
Alterporriol D or E (**6**)	50.0	47.0
Alterporriol A (**7**)	18.0	19.0
Alterporriol C (**8**)	5.9	17.0
Alterporriol F (**9**)	6.5	7.0
Zinnimide (**10**)	13.0	27.0
Macrosporin (**11**)	>100.0	>100.0
5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**)	36.0	14.0
Porriolide (**13**)	>100.0	59.0
Alternariol monomethyl ether (**14**)	4.3	5.5
Alternariol (**15**)	6.6	4.5
Deprenylzinnimide (**16**)	>100.0	90.0
Demethylmacrosporin (**17**)	7.3	8.6
Positive control (Adriamycin)	0.018	0.018

Furthermore, alterporriol D or E (**6**), which consists of two units of tetrahydroanthraquinones exhibited lowest activity in this type, while compounds **7**-**9** were presence of only one tetrahydroanthraquinone moiety fused with one anthraquinone unit. This suggests that the unit of tetrahydroanthraquinone had affect in cytotoxicity. Finally, the remaining group was dibenzo-α-pyrones: alternariol monomethylether (**14**) and alternariol (**15**) which were found to be the most potent in cytotoxic activity. For this type, substitution on oxygenated C-6 had little an influence on the cytotoxicity as seen from the structure of compounds **14** and **15**.

Compound **14** and **15** also showed wide range of antibacterial activity against Gram positive and negative bacteria (Freeman, 1965). Moreover, compound **15** also displayed inhibition toward human endometrial adenocarcinoma cell line and Chinese hamster cell (Lehmann *et al*., 2006). On the other hand, compound **14** administered subchronically was slightly toxic to rats and foetotoxic to golden hamster (Pollock *et al*., 1982). By the way, compound **14** and **15** were the main mycotoxins formed in foods such as tomatoes, apples, olives, etc that infected by *Alteraria alternata.* These data revealed that these two compounds were well-known compounds worldwide. In addition, compound (**15**) was commercially available substance from Sigma (CAS No. 641-38-3).

3.4.2 Acetylcholinesterase inhibitor assay

The isolated compounds were evaluated for their acetylcholinesterase (AChE) inhibitory effect on TLC autographic assay using Ellman and Hostettmann methods. This is the first report on AChE inhibition of compounds isolated from *A. porri*. From the result of AChE inhibition, clear zones of acetylcholinesterase inhibitors observed in Hostettmann method could be easier detected than Ellman method. However, only clear zones detected by both methods were reported. The effect of isolated compounds on the activity of AChE using Ellman and Hostettmann methods was summarized in Table 3.5 and 3.6.

Table 3.5 Minimal inhibitory concentrations (MICs) of isolated compounds of acetylcholinesterase inhibitor assay by Ellman method

Compound	Concentration (mg/mL)				
	1.000	0.500	0.250	0.125	0.0625
Zinnimidine (**5**)	+	+	+	-	-
Alterporriol D or E (**6**)	-	-	-	-	-
Alterporriol A (**7**)	-	-	-	-	-
Alterporriol C (**8**)	-	-	-	-	-
Alterporriol F (**9**)	-	-	-	-	-
Zinnimide (**10**)	+	+	-	-	-
Macrosporin (**11**)	-	-	-	-	-
5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**)	+	-	-	-	-
Porriolide (**13**)	-	-	-	-	-
Alternariol monomethyl ether (**14**)	-	-	-	-	-
Alternariol (**15**)	-	-	-	-	-
Deprenylzinnimide (**16**)	-	-	-	-	-
Demethylmacrosporin (**17**)	-	-	-	-	-
Eserine	+	+	+	+	+

Note: Standard acetylcholinesterase inhibitor = Phytostigmine (Eserine);
Inhibition zone: + = inhibition and - = no inhibition.

Table 3.6 Minimal inhibitory concentrations (MICs) of isolated compounds of acetylcholinesterase inhibitor assay by Hostettmann method

Compound	Concentration (mg/mL)				
	1.000	0.500	0.250	0.1250	0.0625
Zinnimidine (**5**)	+	+	+	+	-
Alterporriol D or E (**6**)	-	-	-	-	-
Alterporriol A (**7**)	-	-	-	-	-
Alterporriol C (**8**)	-	-	-	-	-
Alterporriol F (**9**)	-	-	-	-	-
Zinnimide (**10**)	+	+	-	-	-
Macrosporin (**11**)	+	-	-	-	-
5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**)	+	-	-	-	-
Porriolide (**13**)	+	-	-	-	-
Alternariol monomethyl ether (**14**)	+	-	-	-	-
Alternariol (**15**)	-	-	-	-	-
Deprenylzinnimide (**16**)	+	-	-	-	-
Demethylmacrosporin (**17**)	-	-	-	-	-
Eserine	+	+	+	+	+

Note: Standard acetylcholinesterase inhibitor = Phytostigmine (Eserine);
Inhibition zone: + = inhibition and - = no inhibition.

Of compounds examined, zinnimidine (**5**), zinnimide (**10**) and 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**) significantly inhibited AChE activity with MIC values of 0.250, 0.500 and 1.000 mg/mL, respectively, while the others were not active. In inhibition of AChE activity, the potential of compound **5** and **10** might be due to the presence of phthalimide ring. From literature review (Alonso *et al*., 2005; Sussman *et al*., 1993), it has been reported that phthalimide moiety acts as efficient ligand for the peripheral site of AChE because of the carbonyl groups of phthalimide ring form a direct hydrogen-bond contact with residue of peripheral site of AChE. There was no evidence about mechanism of AChE activity of compound **12**. From the results of AChE activity, compounds **5**, **10**, **12** are worth for further study on inhibition of AChE activity for Alzheimer's disease treatment.

3.5 Experiment Section

3.5.1 General experimental procedures

Most instruments used in this experiment were the same as in Chapter II. HPLC was carried out on a Waters 600E Delivery system pump, equipped with a Waters 2487 dual wavelength absorbance detector, and a Nova-Pak RP-C_{18} (8 × 100 mm) column.

3.5.2 Fungal material and culture condition

A strain of *Alternaria porri* was obtained from Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand. The isolated culture was stored at 4 ^{o}C in order to maintain biochemical metabolism. Potato dextrose broth (PDB), which was prepared by dissolving dextrose (20 g) in 1 L of potato extract (200 g) solution, was used as seed and production medium. The medium was sterilized in an autoclave at 121 ºC for 20 min. The fungus was plated out on potato dextrose agar medium (PDB with 1.5% agar) at 25 ºC for 3 days. Fungus plug was cut into small pieces and transferred into 5 × 250 mL Erlenmeyer flask each containing 100 mL of seed medium which, was incubated at 25 ºC for 3 days. Then, the broth culture was inoculated into the production medium (100 × 250 mL Erlenmeyer flask each containing 100 mL of medium). Cultivation was kept under static conditions at 25 ºC for 75 days.

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

Cytotoxicity of isolated compounds was evaluated using MTT assay at Natural Products Research Section, Research Division, National Cancer Institute, Thailand. The KB (human epidermoid carcinoma) and HeLa (human cervical carcinoma) cell lines were used in this experiment. Adriamycin was used as antibiotic antitumor agent. The results were shown in Table 3.4.

3.5.4 Acetylcholinesterase inhibitor assay

Acetylcholinesterase inhibition assay was the same as that described in Chapter II.

Zinnimidine (5): Pale yellow crystal; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.54 (1H, brs, NH), 7.04 (1H, s, H-6), 5.45 (1H, m, H-2′), 4.50 (2H, d, *J* = 6.4 Hz, H-1′), 4.42 (2H, s, H-8), 3.80 (3H, s, 3-OCH_3), 2.14 (3H, s, 4-CH_3), 1.72 (3H, s, H-4′), 1.65 (3H, s, H-5′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 172.2 (C-7), 158.6 (C-3), 153.7 (C-5), 137.8 (C-3′), 131.1 (C-2), 126.2 (C-1), 123.7 (C-4), 119.6 (C-2′), 101.1 (C-6), 65.7 (C-1′), 59.8 (3-OCH_3), 43.9 (C-8), 25.8 (C-4′), 18.3 (C-5′), 9.6 (4-CH_3).

Alterporriol D or E (6): Deep red solid; ^{1}H NMR (DMSO-*d*$_6$, 400 MHz) δ_H 6.98 (2H, s, H-3 and H-3′), 5.73 (2H, d, *J* = 7.2 Hz, 6-OH and 6′-OH), 5.10 (2H, d, *J* = 5.6 Hz, 5-OH and 5′-OH), 4.91 (2H, d, *J* = 5.2 Hz, 8-OH and 8′-OH), 4.53 (2H, d, *J* = 6.0 Hz, H-5 and H-5′), 4.47 (2H, s, 7-OH and 7′-OH), 4.15 (2H, d, *J* = 6.8 Hz, H-8 and H-8′), 3.74 (6H, s, 2-OCH_3 and 2′-OCH_3), 3.65 (2H, d, *J* = 5.6 Hz, H-6 and H-6′), 1.21 (6H, s, 7-CH_3 and 7′-CH_3).

Alterporriol A (7): Deep red solid; ^{1}H NMR (DMSO-*d*$_6$, 400 MHz) δ_H 13.74 (1H, s, 4-OH), 13.10 (1H, s, 4′-OH), 7.70 (1H, s, H-8), 7.55 (1H, s, H-5), 6.80 (1H, s, H-3), 6.78 (1H, m, H-3′), 4.63 (1H, dd, *J* = 12.4, 6.5 Hz, 5′-OH), 4.26 (1H, m, H-8′), 3.69 (6H, m, 2-OCH_3 and 2′-OCH_3), 3.68 (1H, m, H-6′), 2.23 (3H, s, 7-CH_3), 1.27 (3H, s, 7′-CH_3); ^{13}C NMR (DMSO-*d*$_6$, 100 MHz) δ_C 190.7 (C-10′), 187.9 (C-10), 185.7 (C-9′), 182.0 (C-9), 166.7 (C-2), 165.4 (C-4), 165.3 (C-4′), 162.7 (C-6), 145.3 (C-10′a), 144.1 (C-9′a), 133.5 (C-1a), 133.4 (C-1′a), 132.7 (C-10a), 131.4 (C-7), 131.1 (C-8), 127.4 (C-9a), 111.6 (C-4′a), 111.5 (C-5), 111.1 (C-1), 109.3 (C-1′), 105.1 (C-4a), 104.5 (C-3), 104.3 (C-3′), 76.6 (C-7′), 75.2 (C-6′), 70.7 (C-5′), 70.1 (C-8′), 57.1 (2′-OCH_3), 57.0 (2-OCH_3), 22.4 (7′-CH_3), 16.5 (7-CH_3).

Alterporriol C (8): Deep red solid; ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 13.06 (1H, s, 4′-OH), 12.50 (1H, s, 4-OH), 8.01 (1H, s, H-8), 7.17 (1H, d, *J* = 2.4 Hz, H-1), 6.93 (1H, s, H-1′), 6.72 (1H, d, *J* = 2.0 Hz, H-3), 5.70 (1H, d, *J* = 7.2 Hz, 8′-OH), 5.08 (1H, d, *J* = 5.6 Hz, 5′-OH), 4.87 (1H, d, *J* = 6.8 Hz, 6′-OH), 4.45 (1H, m, H-5′), 4.35 (1H, s, 7′-OH), 4.05 (1H, d, *J* = 7.2 Hz, H-8′), 3.88 (3H, s, 2-OCH_3), 3.68 (3H, s, 2′-OCH_3), 3.50 (1H, m, H-6′), 2.32 (3H, s, 7-CH_3), 1.10 (3H, s, 7′-CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ_C 189.5 (C-10′), 187.9 (C-10), 184.7 (C-9′), 181.3 (C-9), 166.0 (C-2), 164.9 (C-4), 164.4 (C-4′), 159.0 (C-6), 143.9 (C-9′a), 143.0 (C-10′a), 131.9 (C-8), 131.3 (C-7), 123.3 (C-4′a), 110.7 (C-4a), 107.0 (C-1), 106.5 (C-3), 104.5 (C-1′), 74.1 (C-6′), 73.7 (C-8′), 68.8 (C-5′), 68.6 (C-7′), 57.2 (2′-OCH_3), 56.7 (2-OCH_3), 22.7 (7′-CH_3), 17.6 (7-CH_3).

Alterporriol F (9): Deep red solid; $[\alpha]_D^{28}$ -25° (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 439 (3.30), 280 (4.18), 226 (4.28); HRESIMS *m/z* $[M + Na]^+$ 625.1324 (calcd for $C_{32}H_{26}O_{12}Na$, 625.1322); ^{1}H NMR and ^{13}C NMR see Table 3.1.

Zinnimide (10): Yellow amorphous solid; $[\alpha]_D^{28}$ -6° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 320 (3.54), 284 (3.47), 242 (4.32); HRESIMS *m/z* $[M + Na]^+$ 298.1065 (calcd for $C_{15}H_{17}NO_4Na$, 298.1055); ^{1}H NMR and ^{13}C NMR see Table 3.2.

Macrosporin (11): Yellow amorphous solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ_H 12.75 (1H, brs, 8-OH), 7.86 (1H, s, H-4), 7.53 (1H, s, H-1), 7.12 (1H, s, H-5), 6.62 (1H, s, H-7), 3.86 (3H, s, 6-OCH_3) , 2.24 (3H, s, 3-CH_3).

5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (12) : Yellow amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 6.55 (1H, s, H-6), 5.42 (1H, m, H-2′), 5.10 (2H, s, H-7), 4.52 (2H, d, *J* = 6.4 Hz, H-1′), 3.96 (3H, s, 3-OCH_3), 2.08 (3H, s, 4-CH_3), 1.74 (3H, s, H-4′), 1.68 (3H, s, H-5′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 169.0 (C-8), 163.8 (C-5), 157.9 (C-3), 148.0 (C-1), 138.8 (C-3′), 120.8 (C-4), 118.9 (C-2′), 109.6 (C-2), 99.1 (C-6), 68.8 (C-7), 66.0 (C-1′), 62.1 (3-OCH_3), 25.8 (C-4′), 18.3 (C-5′), 8.8 (4-CH_3).

Porriolide (13): Yellow amorphous solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ_H 9.05 (1H, s, 5-OH), 6.99 (1H, s, H-6), 5.50 (2H, s, H-8), 3.97 (3H, s, 3-OCH_3), 2.17 (3H, s, 4-CH_3).

Alternariol monomethyl ether (14): Yellow amorphous solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ_H 11.84 (1H, s, 8-OH), 9.21 (1H, s, 2-OH), 7.15 (1H, s, H-5), 6.67 (1H, s, H-3), 6.57 (1H, s, H-1), 6.43 (1H, s, H-7), 3.84 (3H, s, 6-OCH_3) 2.67 (3H, s, 4-CH_3).

Alternariol (15): Yellow amorphous solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ_H 11.80 (1H, s, 8-OH), 10.02 (1H, s, 6-OH), 9.50 (1H, s, 2-OH), 7.22 (1H, s, H-5), 6.66 (1H, s, H-3), 6.57 (1H, s, H-1), 6.20 (1H, s, H-7), 2.67 (3H, s, 4-CH_3); ^{13}C NMR (acetone-d_6, 100 MHz) δ_C 165.5 (C-8), 158.6 (C-2), 153.5 (C-5a), 138.5 (C-4), 117.6 (C-3), 109.6 (C-1a), 104.5 (C-5), 101.8 (C-1), 101.0 (C-7), 98.5 (C-8a), 22.0 (4-CH_3).

Deprenylzinnimide (16): Yellow amorphous solid; UV (MeOH) λ_{max} (log ε) 322 (3.14), 286 (3.08), 245 (3.86); HRESIMS *m/z* $[M + Na]^+$ 230.0439 (calcd for $C_{15}H_{17}NO_4Na$, 230.0424); ^{1}H NMR and ^{13}C NMR see Table 3.3.

Demethylmacrosporin (17): Yellow amorphous solid; ^{1}H NMR (acetone-d_6, 400 MHz) δ_H 12.71 (1H, brs, 8-OH), 10.00 (1H, brs, 6-OH), 9.80 (1H, brs, 2-OH), 7.85 (1H, s, H-4), 7.53 (1H, s, H-1), 7.10 (1H, s, H-5), 6.49 (1H, s, H-7), 2.24 (3H, s, 3-CH_3); ^{13}C NMR (acetone-d_6, 100 MHz) δ_C 186.7 (C-9), 181.7 (C-10), 165.5 (C-6), 165.0 (C-8), 161.5 (C-2), 133.5 (C-1a), 132.2 (C-3), 130.2 (C-4), 126.0 (C-4a), 111.1 (C-1), 110.0 (C-8a), 107.9 (C-5), 107.0 (C-7), 15.4 (3-CH_3).

Supporting information

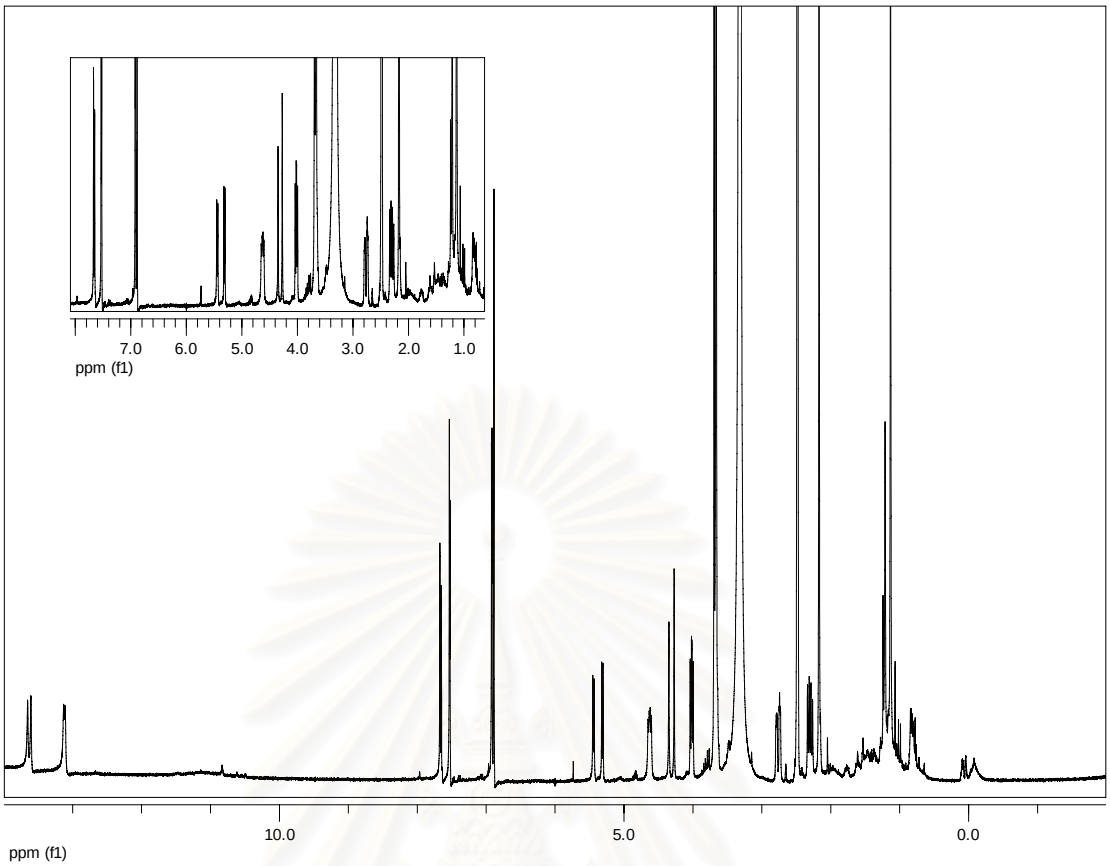


Figure S-3.1 The ^{1}H NMR spectrum of alterporriol F (**9**) in DMSO-d_6

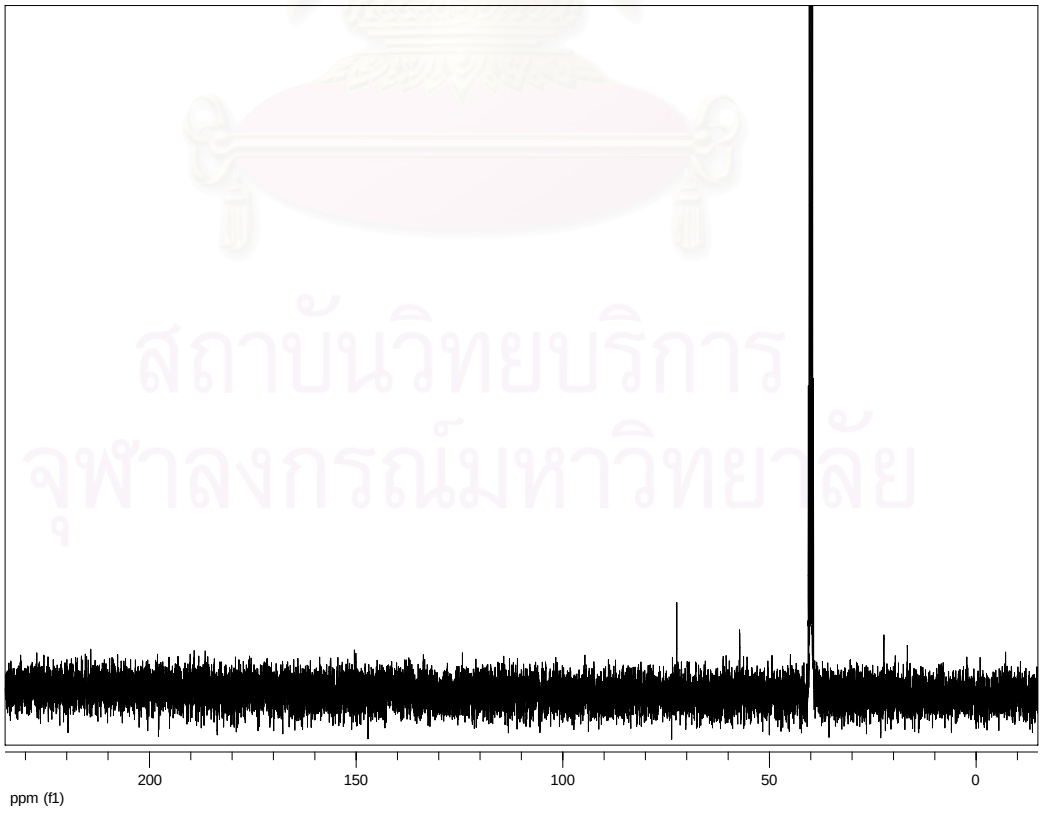


Figure S-3.2 The ^{13}C NMR spectrum of alterporriol F (**9**) in DMSO-d_6

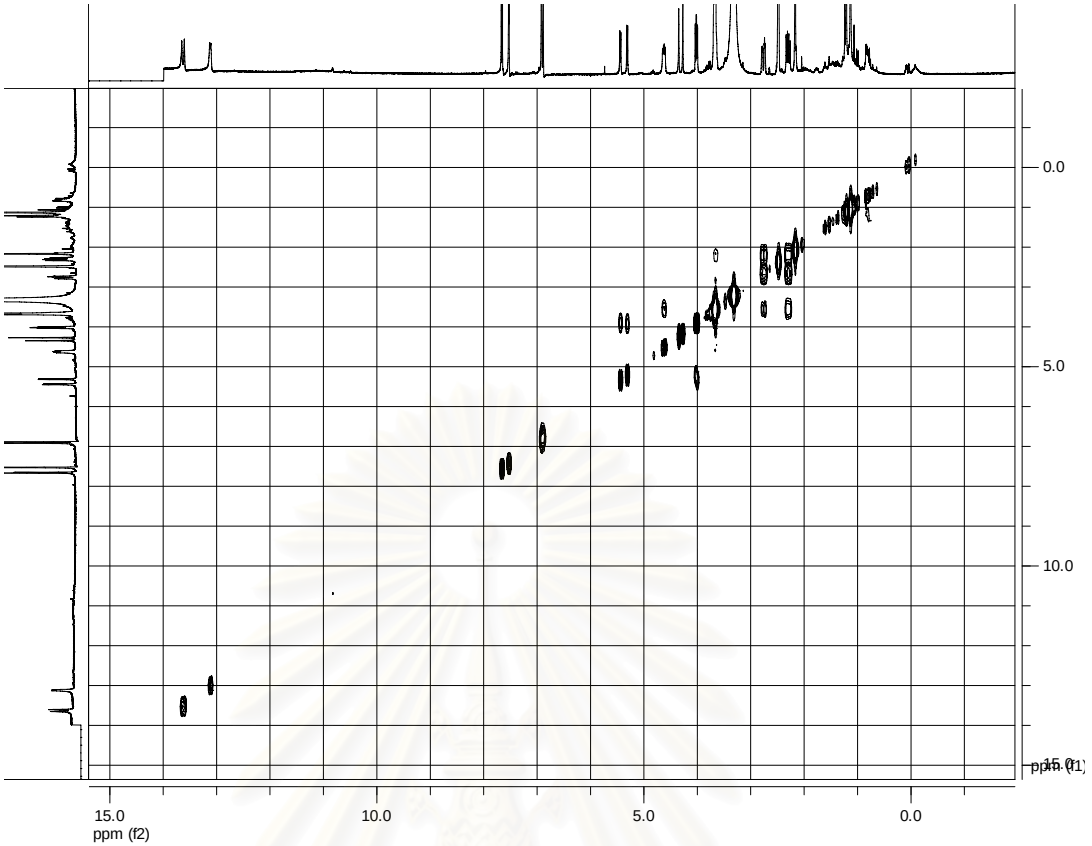


Figure S-3.3 The COSY spectrum of alterporriol F (**9**) in DMSO-d_6

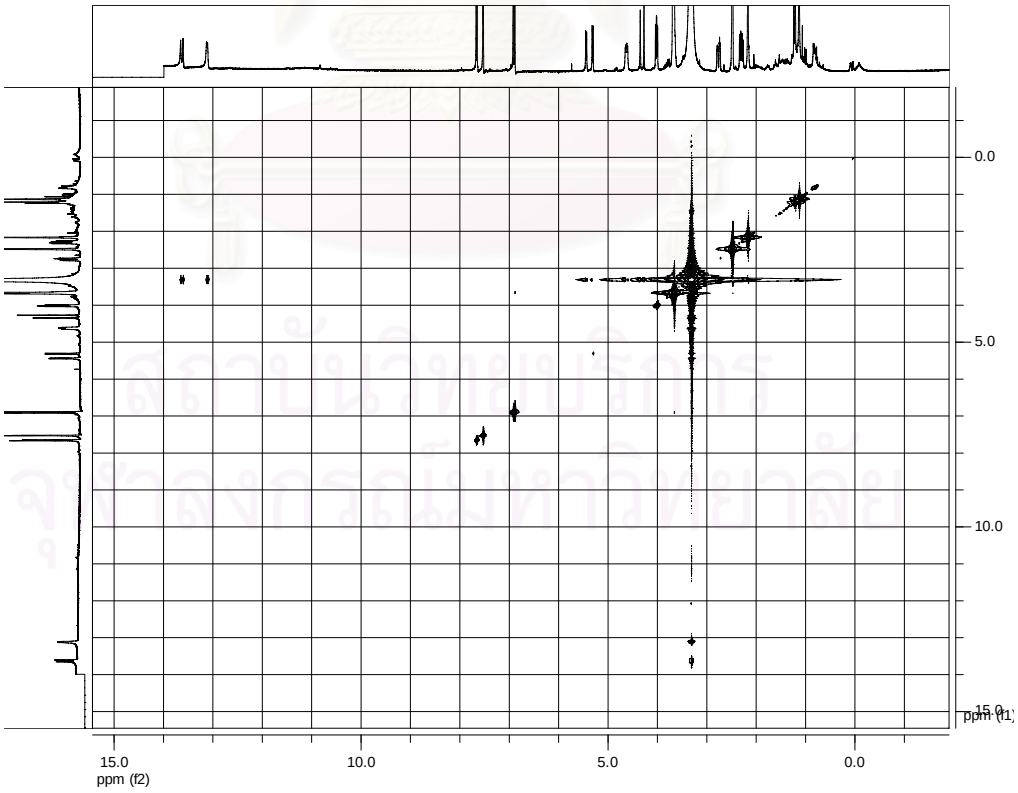


Figure S-3.4 The NOESY spectrum of alterporriol F (**9**) in DMSO-d_6

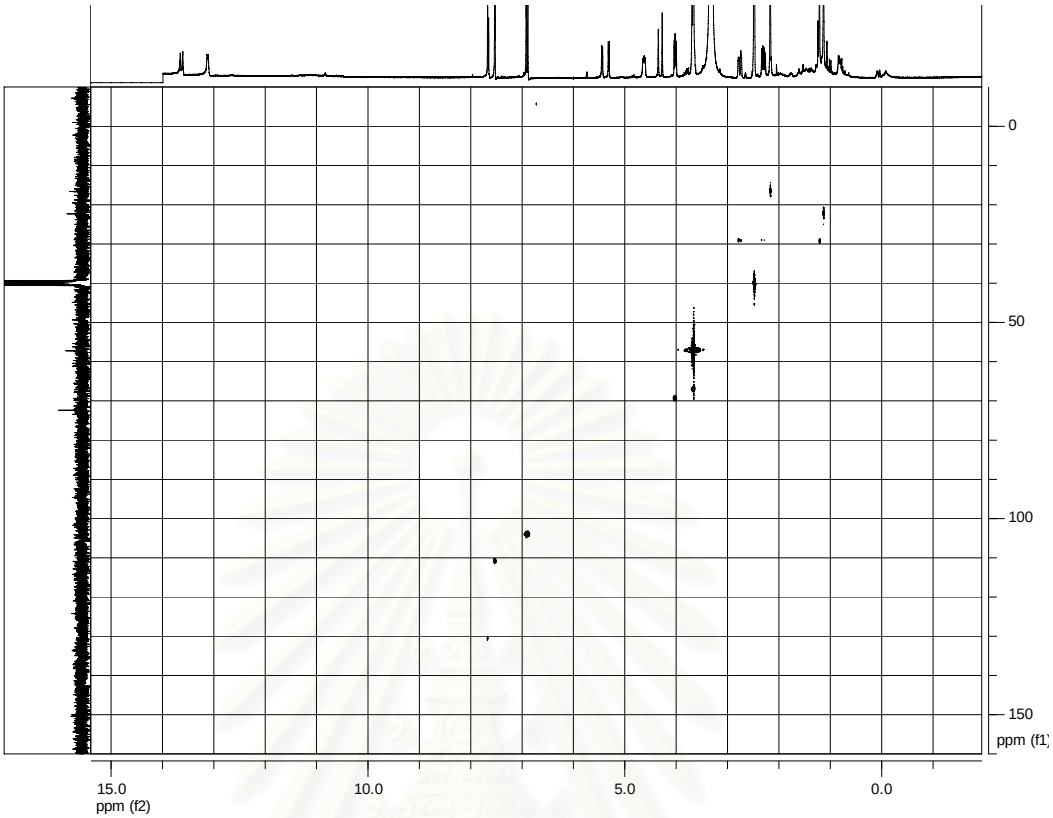


Figure S-3.5 The HSQC spectrum of alterporriol F (**9**) in DMSO-d_6

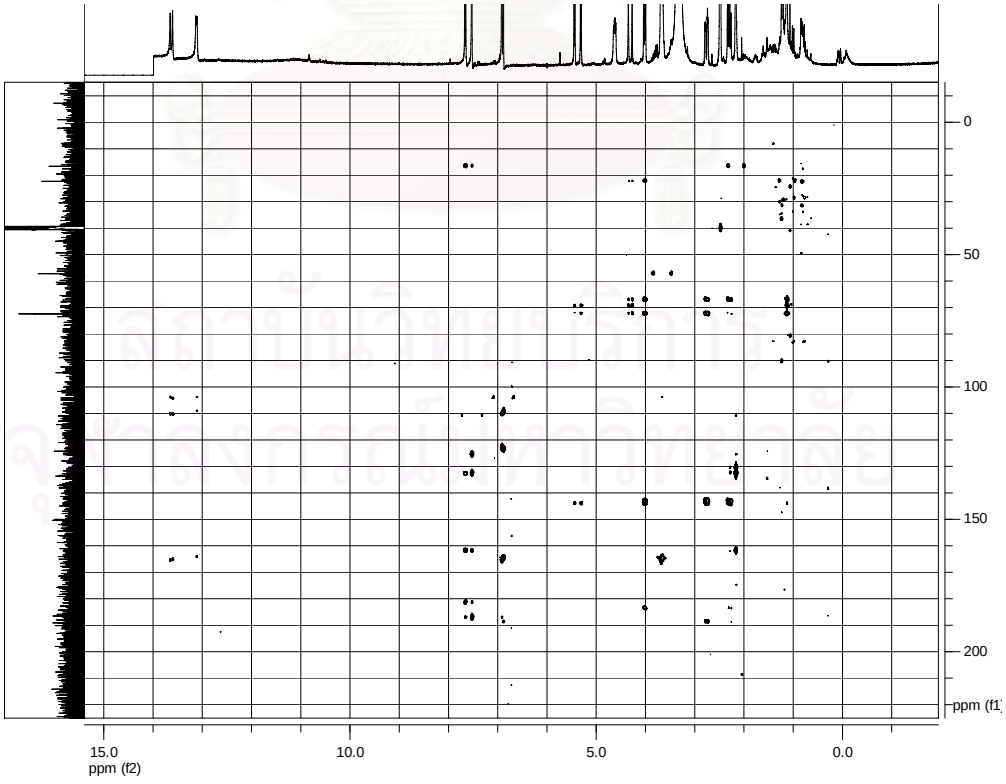


Figure S-3.6 The HMBC spectrum of alterporriol F (**9**) in DMSO-d_6

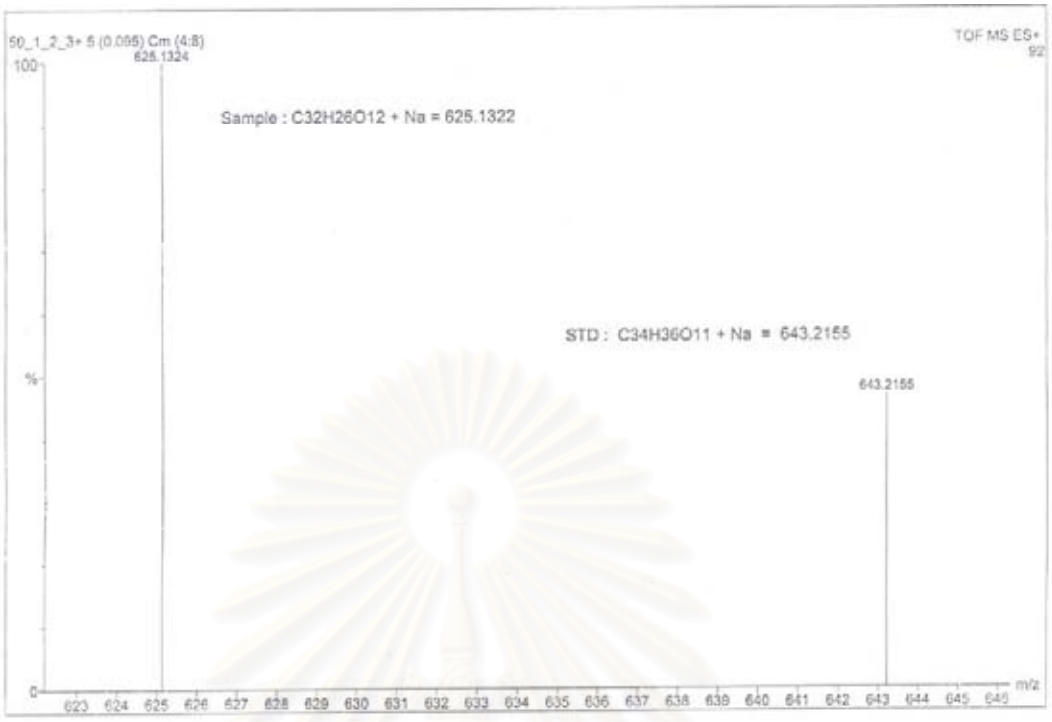


Figure S-3.7 The high resolution mass spectrum of alterporriol F (**9**)

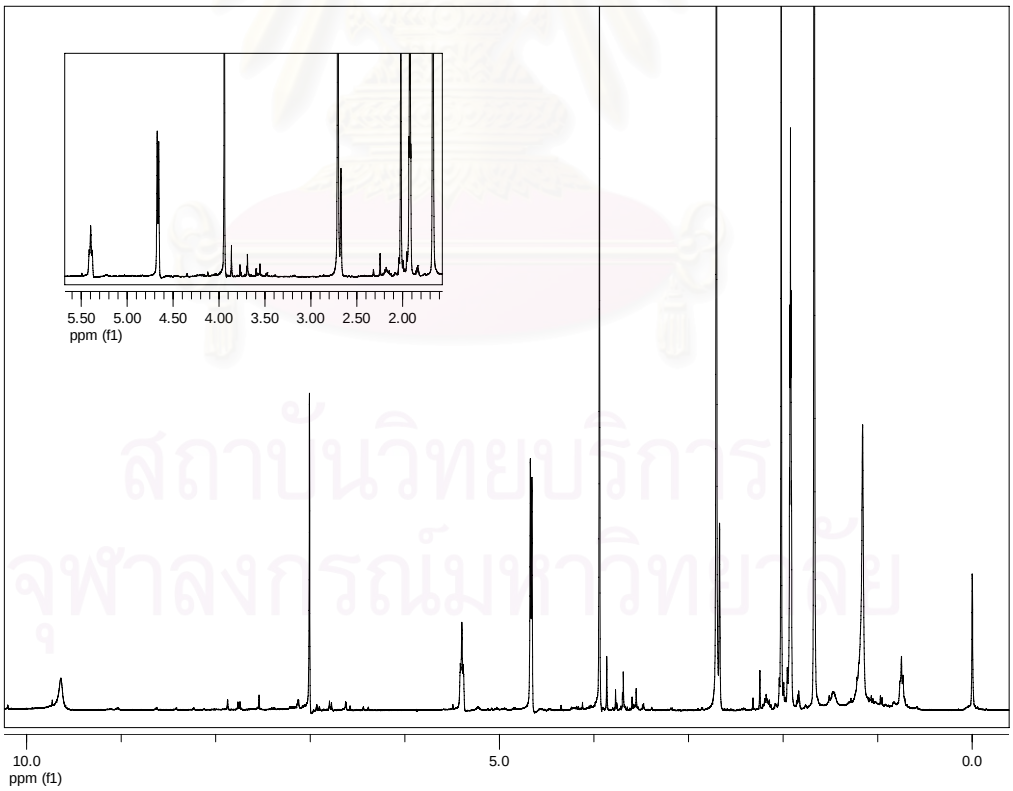


Figure S-3.8 The ^{1}H NMR spectrum of zinnimide (**10**) in acetone-d_6

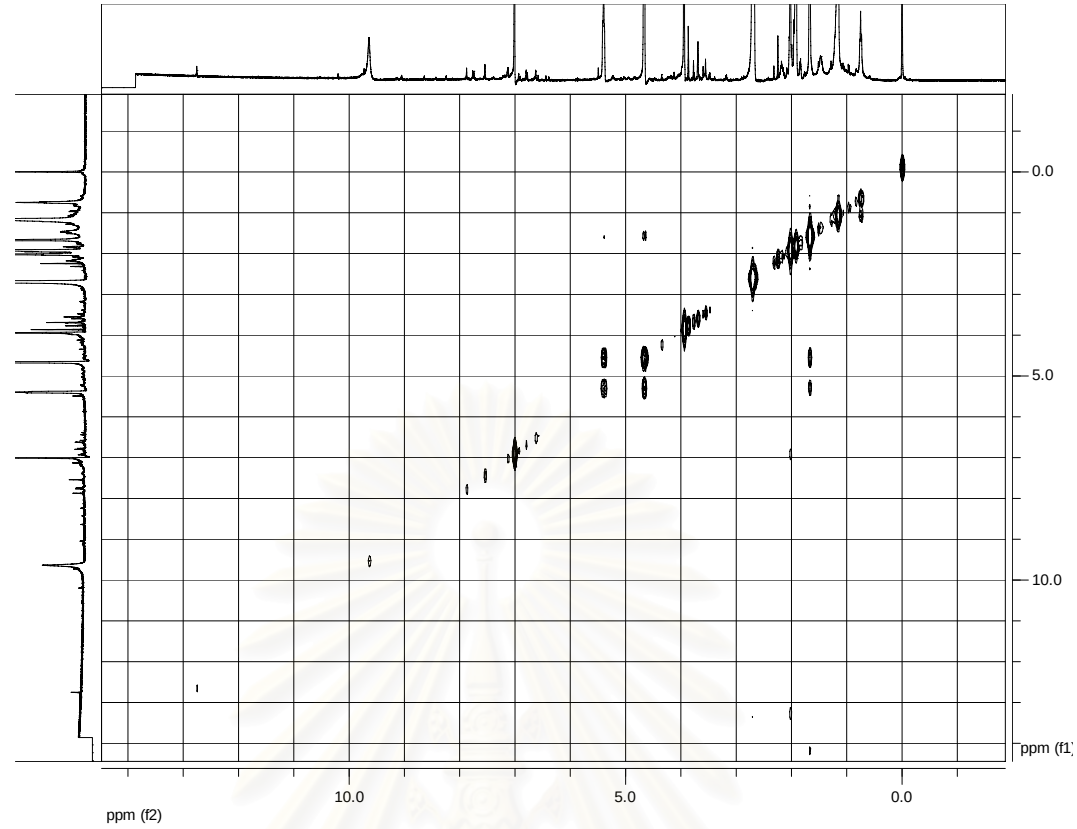


Figure S-3.9 The COSY spectrum of zinnimide (**10**) in acetone-d_6

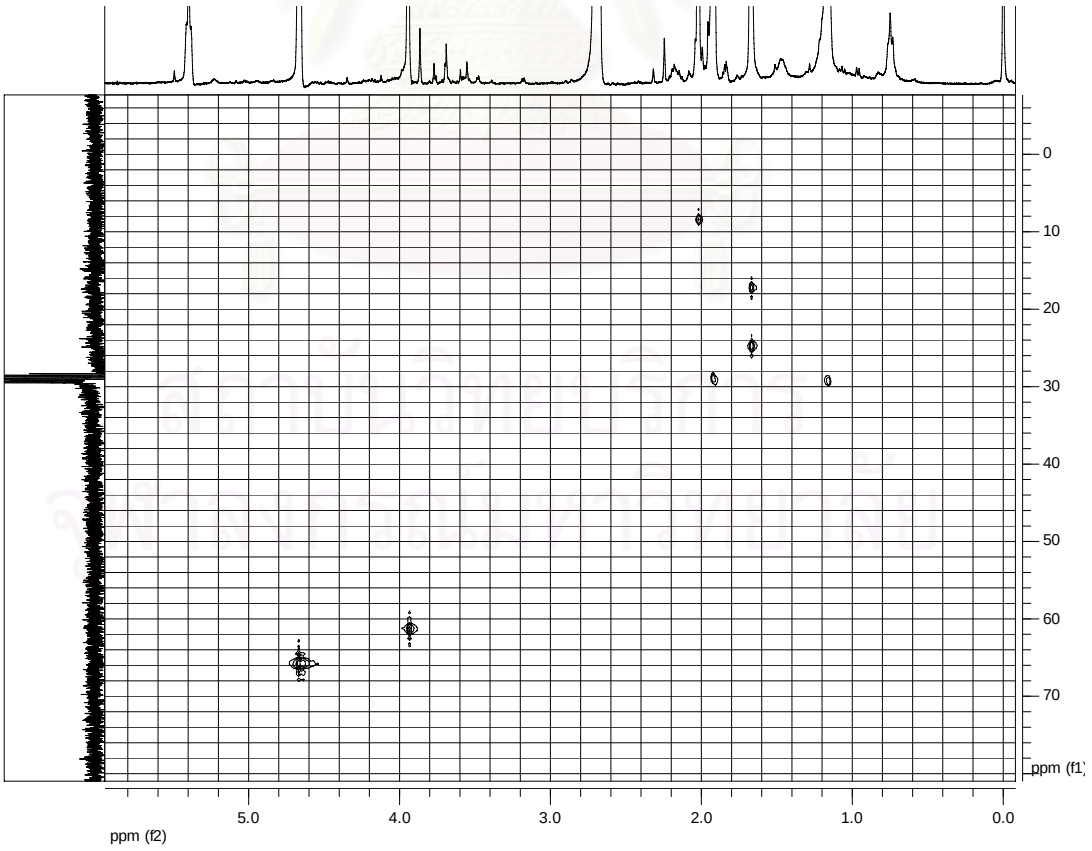


Figure S-3.10 The HSQC spectrum of zinnimide (**10**) in acetone-d_6

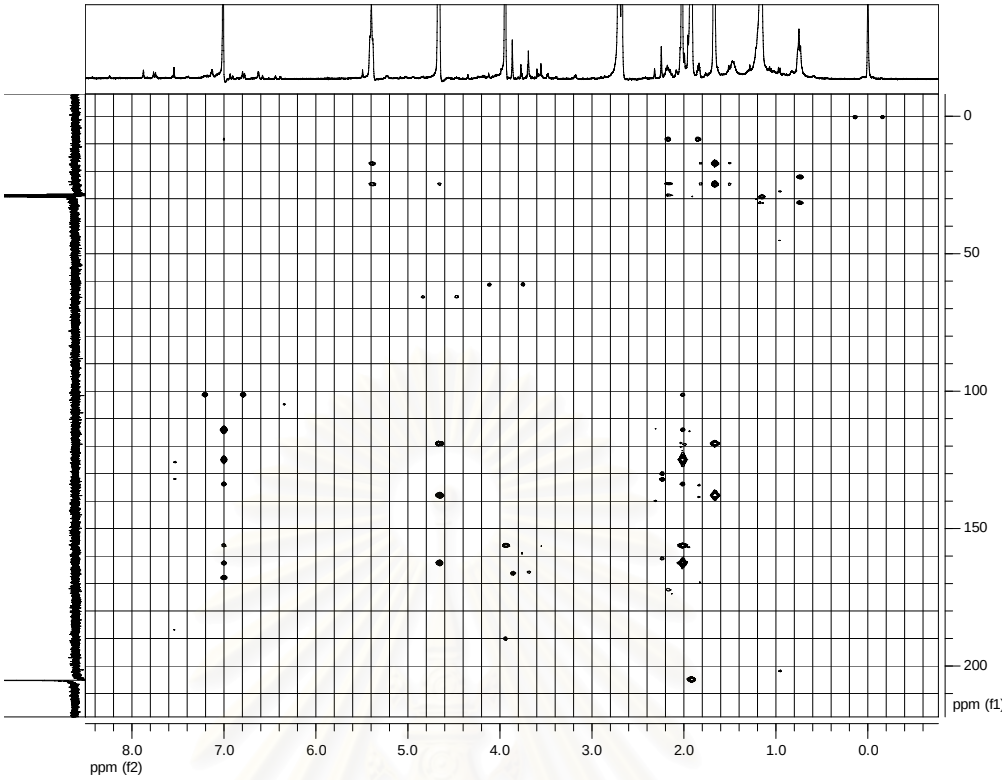


Figure S-3.11 The HMBC spectrum of zinnimide (**10**) in acetone-d_6

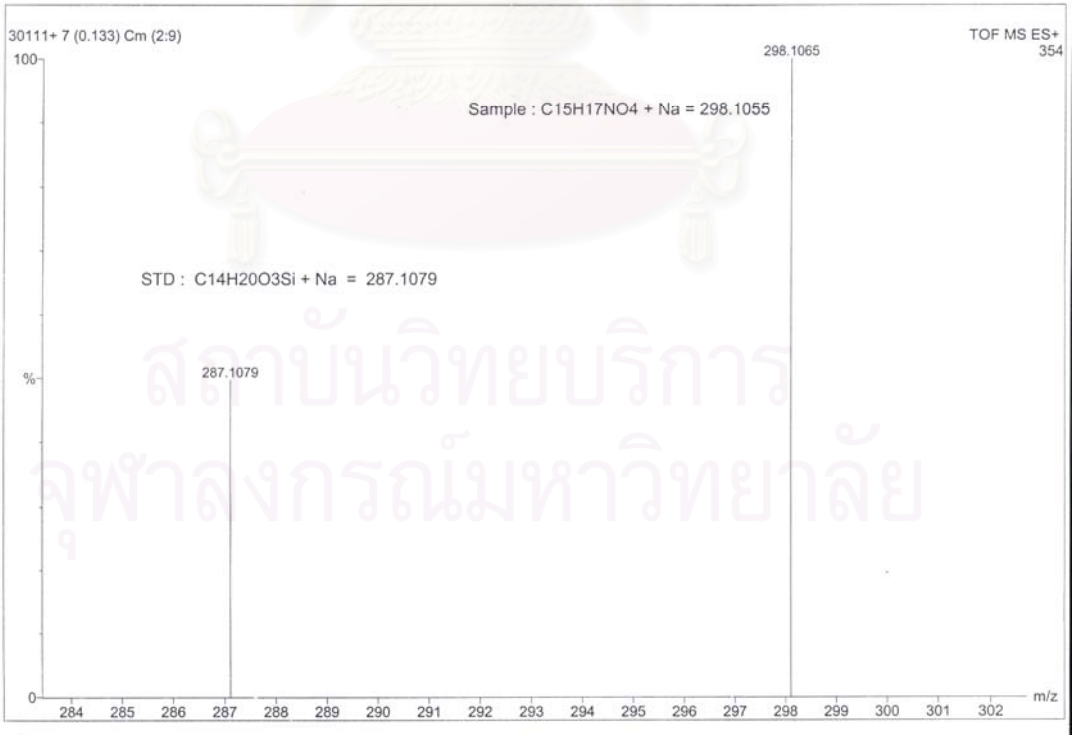


Figure S-3.12 The high resolution mass spectrum of zinnimide (**10**)

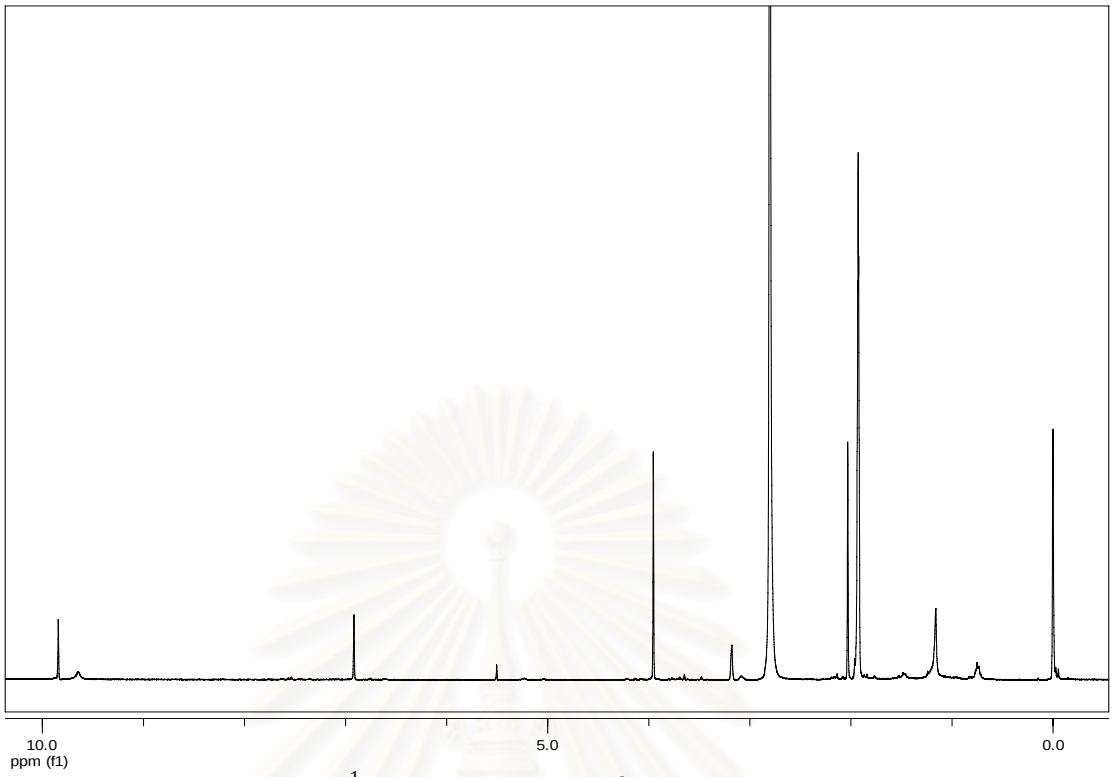


Figure S-3.13 The ^{1}H NMR spectrum of deprenylzinnimide (**16**) in acetone-d_6

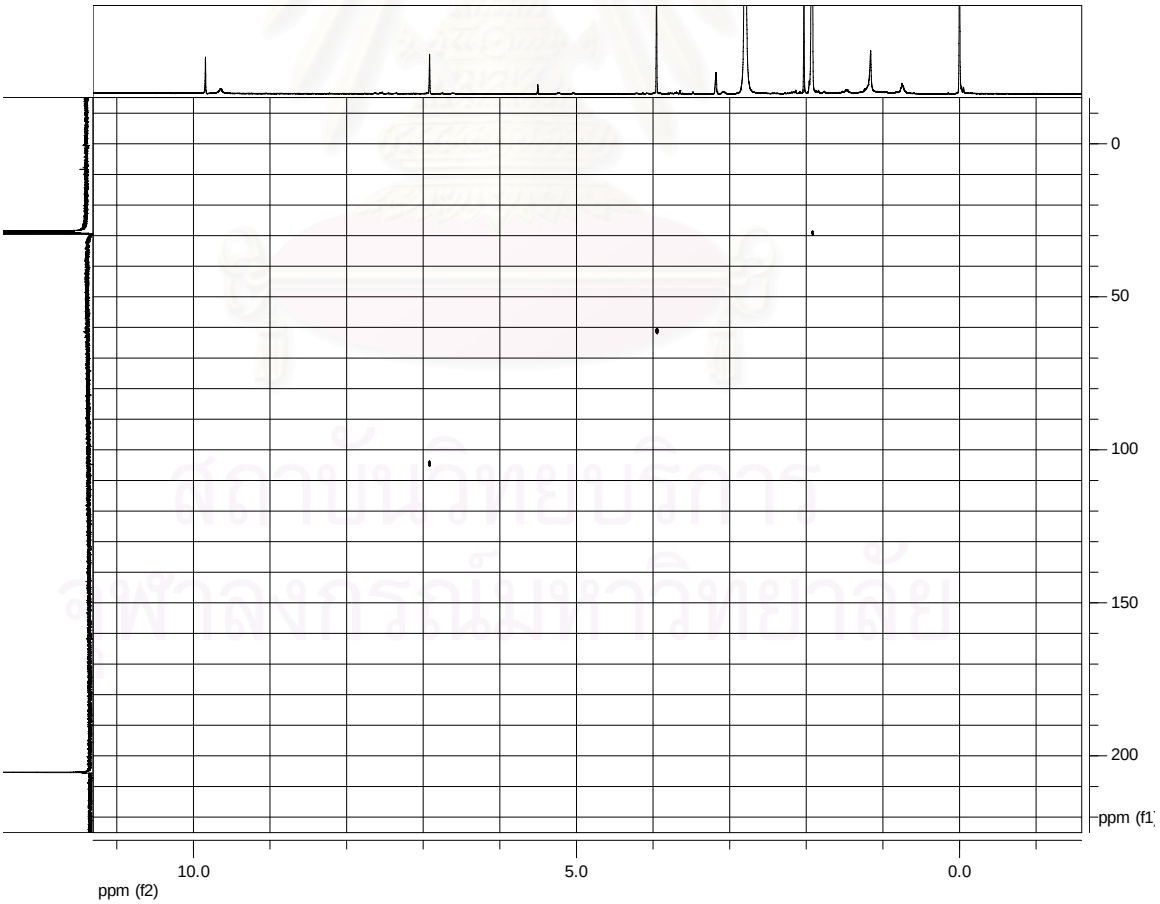


Figure S-3.14 The HSQC spectrum of deprenylzinnimide (**16**) in acetone-d_6

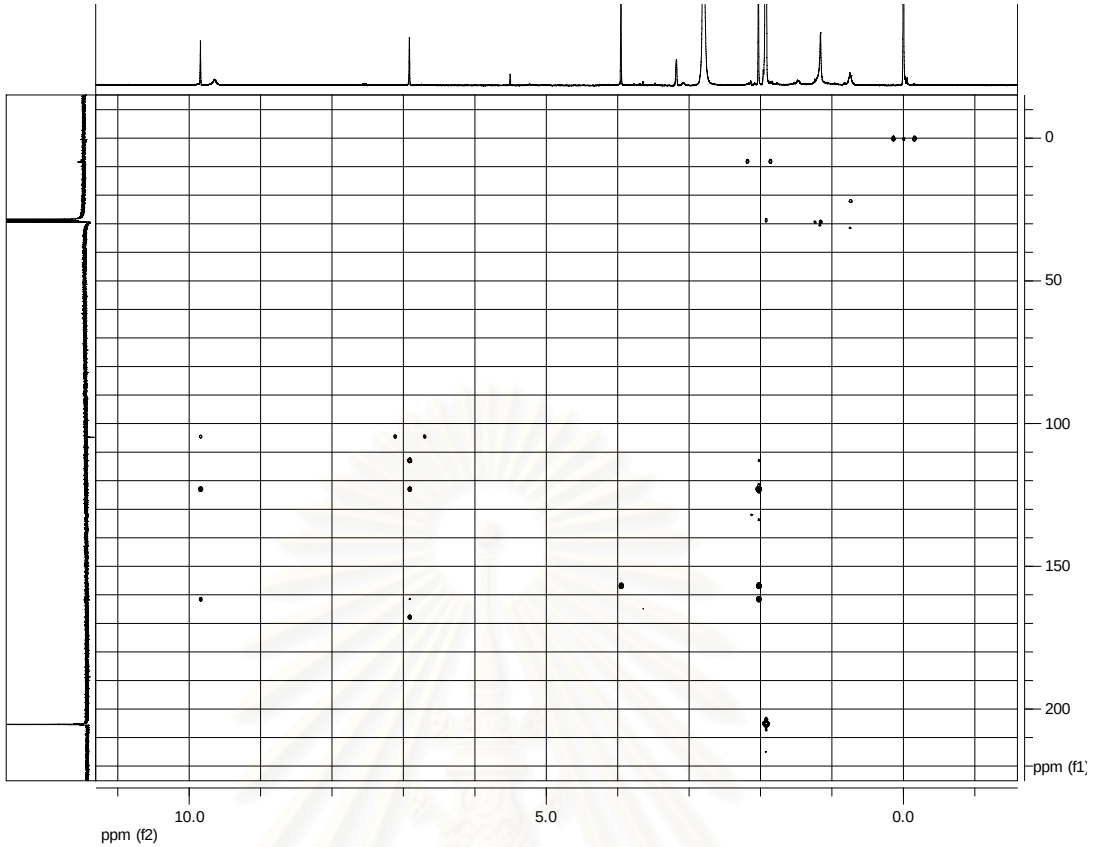


Figure S-3.15 The HMBC spectrum of deprenylzinnimide (**16**) in acetone-d_6

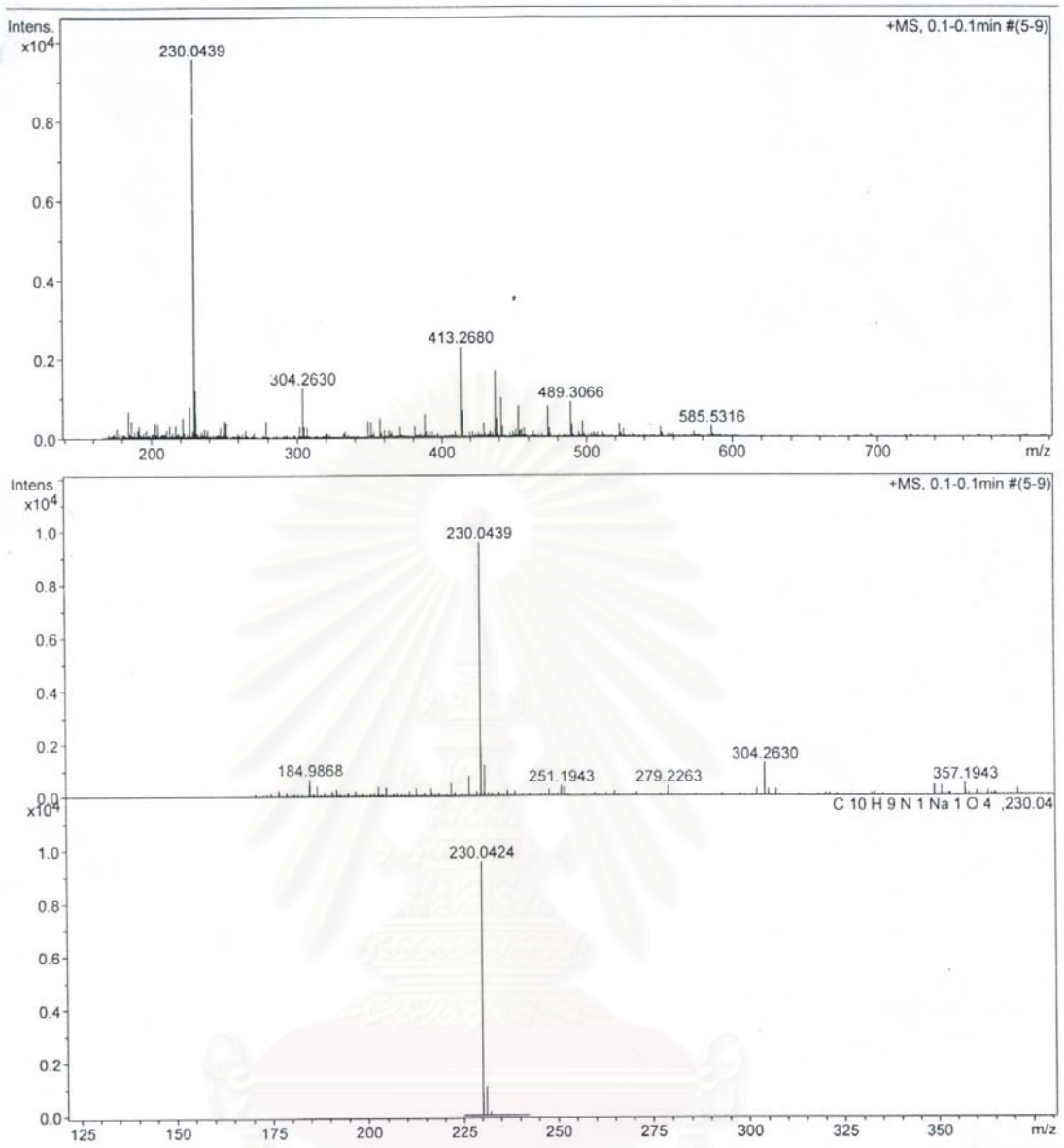
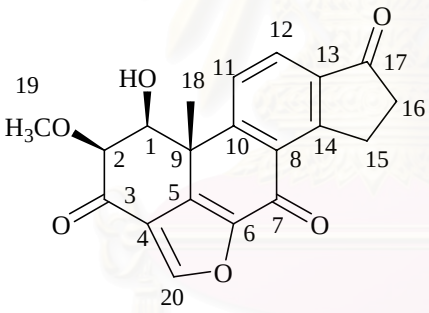


Figure S-3.16 The high resolution mass spectrum of deprenylzinnimide (**16**)

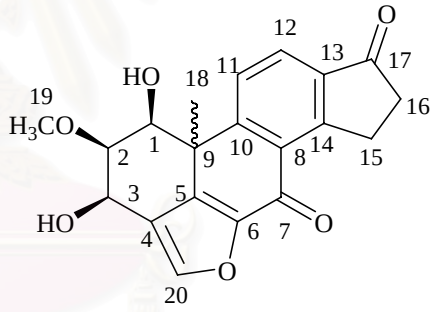
CHAPTER IV

CONCLUSION

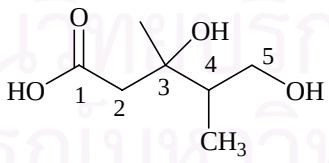
This research investigated bioactive compounds isolated from liquid culture of *Trichoderma virens* and *Alternaria porri*. A novel furanosteroid named 9-*epi*-viridiol (**4**), along with viridin (**1**), viridiol (**3**) and 4-methylmevalonic acid (**2**), were isolated from CH_2Cl_2 extract of *Trichoderma virens*. For *A. porri*, the isolation of ethyl acetate extract affords three novel compounds named alterporriol F (**9**), zinnimide (**10**) and deprenylzinnimide (**16**) together with 10 known compounds (Compounds **5-8**, **11-15**, **17**). The chemical structures of all isolated compounds were determined by spectroscopic data (^{1}H NMR, ^{13}C NMR, 2D NMR and MS) as well as compared with previous reports. The structures of all isolated compounds are summarized as followed.



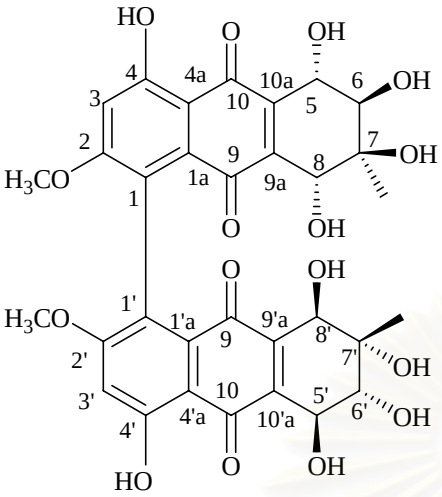
Viridin (**1**)



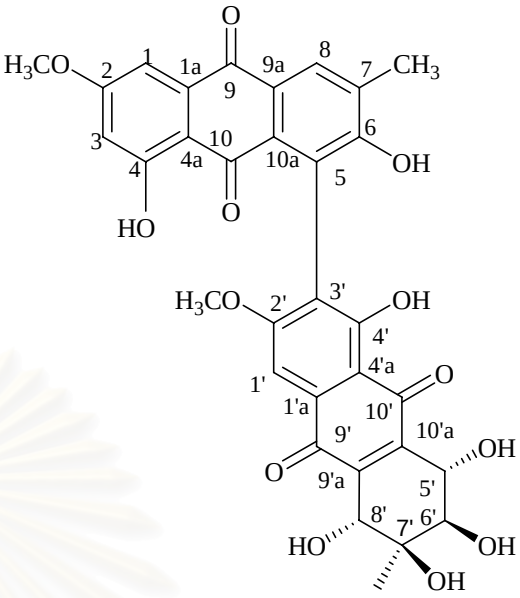
Viridiol (**3**): 18β-CH_3
9-*epi*-Viridiol (**4**): 18α-CH_3



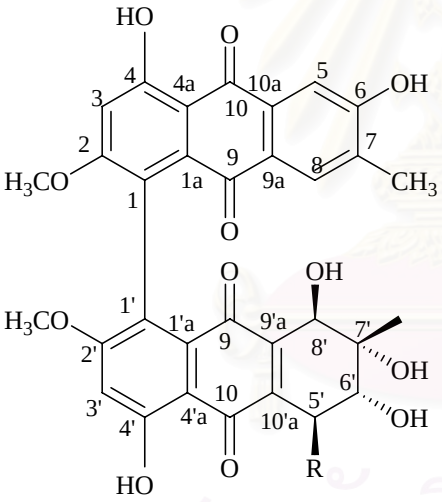
4-Methylmevalonic acid (**2**)



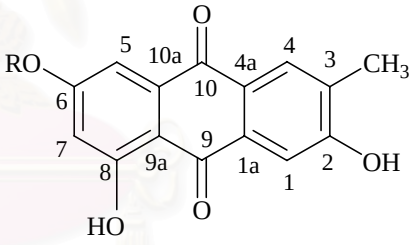
Alterporriol D or E (**6**)



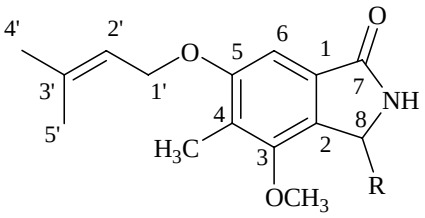
Alterporriol C (**8**)



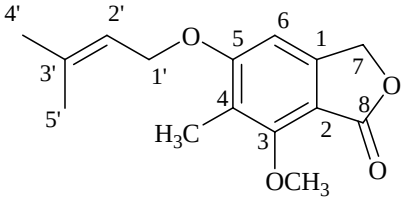
Alterporriol A (**7**): R = OH
Alterporriol F (**9**): R = H



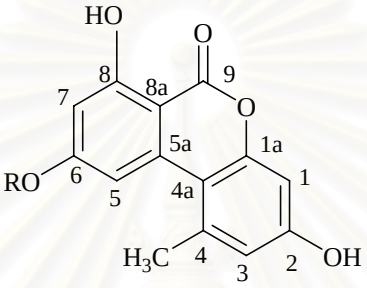
Macrosporin (**11**): R = CH_3
Demethylmacrosporin (**17**): R = H



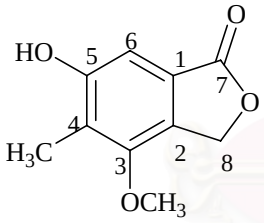
Zinnimidine (**5**): R = H
Zinnimide (**10**): R = O



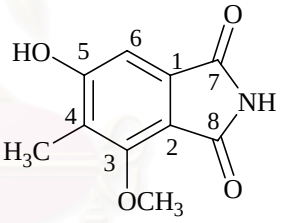
5-(3',3' -Dimethylallyloxy)-
3-methoxy-4-methylphthalide (**12**)



Alternariol monomethyl ether (**14**): R = CH_3
Alternariol (**15**): R = H



Porriolide (**13**)



Deprenylzinnimide(**16**)

The cytotoxic activity against HeLa and KB cell lines of isolated compounds was evaluated using MTT assay. Viridiol (**3**) and 9-*epi*-viridiol (**4**) which isolated from *T. virens* showed moderate cytotoxicity, although compound **4** is three-fold less active than compound **3**. For *A. porri*, alterporriols C (**8**), F (**9**), alternariol monomethyl ether (**14**), alternariol (**15**) and demethylmacrosporin (**17**) exhibited slightly strong cytotoxicity against HeLa and KB cell lines, particularly compound **14** showed highest IC_{50} at 4.3 μg/mL for HeLa cell line, while compound **15** showed highest IC_{50} at 4.5 μg/mL for KB cell line.

From the results of acetylcholinesterase (AChE) inhibitory assay by TLC autographic method, viridiol (**3**) that isolated from *T. virens* showed the strongest

activity with MIC values at 0.125 mg/mL. On the other hand, zinnimidine (**5**), zinnimide (**10**) and 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**) that isolated from *A. porri* exhibited activity with MIC values of 0.250, 0.500 and 1.000 mg/mL, respectively.

In conclusion, four novel compounds named 9-*epi*-viridiol (**3**), alterporriol F (**9**) zinnimide (**10**) and deprenylzinnimide (**16**) were isolated from *T. virens* and *A. porri*. Biological activities of all isolated compounds were evaluated by cytotoxicity and acetylcholinesterase inhibition assay. According to cytotoxic activity against HeLa and KB cell lines, alternariol monomethylether (**14**), alternariol (**15**), alterporriols C (**8**), F (**9**) and demethylmacrosporin (**17**) showed slightly strong cytotoxicity which could be further study on cytotoxicity for the other cell lines to discover appropriate target cell lines. In addition, this is first report that all isolated compounds from *T. virens* and *A. porri* were evaluated for cytotoxicity and AChE inhibitory activity. Interestingly, viridiol (**3**), zinnimidine (**5**), zinnimide (**10**) and 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (**12**), which exhibited AChE inhibitor activity can be tested with other models for acetylcholinesterase inhibitory assay. Therefore, these compounds could be synthesized to increase amounts and varities of compounds for AChE inhibitory testing. Furthermore, the all isolated compounds could be tested with suitable activities in order to discover new bioactive compounds.

Alzheimer’s disease is the most common neuro-degenerative disorder of this century and the most prevalent cause of dermentia with aging. An important approach to treat Alzheimer’s disease is directed to the inhibition of AChE. Some AChE inhibitors like phytostigmine or tacrine are known to have limitations such as short half-life or side-effects like hepatotoxicity. Galanthamine, a long-acting, selective, reversible, and competitive AChE inhibitor, is considered to be more effective in the treatment of Alzheimer’s disease and to have fewer limitations. Inhibitors of acetylcholinesterase are currently from the basis of the newest drugs available for the management of Alzheimer’s disease. The search for new AChE inhibitors is still of great interest and natural products such as plant and microorganism are an important potential source of such compounds.

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