# สารออกฤทธิ์ต้านจุลินทรีย์จากราเอนโดไฟต์ที่แยกจากกรวยป่า *Casearia grewiaefolia* Vent. กอมขม *Picrasma javanica* Bl. เพกา *Oroxylum indicum* (L.) Vent. และทองหลางลาย *Erythrina variegata* Linn.

นางสาวพูนลาภ ป้อมเป็ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

# ANTIMICROBIAL COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Casearia grewiaefolia* Vent., *Picrasma javanica* Bl., *Oroxylum indicum* (L.) Vent. AND *Erythrina variegata* Linn.

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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|---|---|
| Thesis Title | ANTIMICROBIAL COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Casearia grewiaefolia* Vent., *Picrasma javanica* Bl., *Oroxylum indicum* (L.) Vent. AND *Erythrina variegata* Linn. |
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| Field of study | Biotechnology |
| Thesis Advisor | Associate Professor Nattaya Ngamrojanavanich, Ph.D. |
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พูนลาภ ป้อมเป็ง : สารออกฤทธิ์ต้านจุลินทรีย์จากราแอนโดไฟต์ที่แยกจากกรวยป่า *Casearia grewiaefolia* Vent กอมขม *Picrasma javanica* Bl เพกา *Oroxylum indicum* (L.) Vent และทองหลางลาย *Erythrina variegata* Linn (ANTIMICROBIAL COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Casearia grewiaefolia* Vent., *Picrasma javanica* Bl., *Oroxylum indicum* (L.) Vent. AND *Erythrina variegata* Linn.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร. นาตยา งามโรจนวณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รองศาสตราจารย์ ดร. นงลักษณ์ ศรีอุบลมาศ, 123 หน้า.

งานวิจัยนี้ทำการหาสารออกฤทธิ์ต้านเชื้อแบคทีเรียที่แยกจากราแอนโดไฟต์ที่แยกจากกรวยป่า กอมขม เพกา และทองหลางลาย โดยนำสารสกัดหยาบจากราแอนโดไฟต์มาทำการแยกสารบริสุทธิ์โดยใช้เทคนิคทางโครมาโทกราฟี ได้สารบริสุทธิ์ 9 ชนิด ดังนี้ alternariol monomethyl ether (**1**), 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**), altersolanol A (**3**), macrosporin (**4**), 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**), alternariol (**6**), altenusin (**7**), 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol (**8**) และ 5'-epialtenuene (**9**) การพิสูจน์โครงสร้างทางเคมีของสารเหล่านี้ใช้วิธีวิเคราะห์ข้อมูล NMR ร่วมกับการเปรียบเทียบข้อมูลที่มีการรายงานมาก่อน สาร (**3**), (**5**) และ(**7**) แสดงฤทธิ์ต้านเชื้อแบคทีเรียเมื่อทดสอบด้วยวิธี MIC จากการประเมินฤทธิ์ร่วมของสารบริสุทธิ์และยา โดยใช้วิธี checkerboard พบว่าเมื่อสาร (**5**) แสดงผลเสริมฤทธิ์ (synergistic) กับเตตร้าซัยคลินในการต้านเชื้อ *S. aureus* (FIC index 0.12) และสาร (**7**) แสดงฤทธิ์เสริมกับเซฟไตรอะโซนในการต้านเชื้อ *P. vulgaris* (FIC index 0.09) จากการจำแนกชนิดของราโดยใช้สัณฐานวิทยา และ ไรโบโซมอล RNA ยืนพบว่าราแอนโดไฟต์ 10B จัดอยู่ในสายพันธุ์ *Alternaria* sp.



สาขาวิชา.....เทคโนโลยีชีวภาพ....ลายมือชื่อนิสิต.....พูนลาภ ป้อมเป็ง.....

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

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

## 5072397423 : MAJOR BIOTECHNOLOGY

KEYWORDS : Fungal metabolites / Antibacterial activity/ Drug combination assay

PHUNLAP POMPENG : ANTIMICROBIAL COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Casearia grewiaefolia* Vent., *Picrasma javanica* Bl., *Oroxylum indicum* (L.) Vent. AND *Erythrina variegata* Linn (สารออกฤทธิ์ต้านจุลินทรีย์จากราเอนโดไฟต์ที่แยกจากกรวยป่า กอมขม เพกา และ ทองหลางลาย) THESIS ADVISOR : ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., THESIS CO-ADVISOR : ASSOC. PROF. NONGLUKSNA SRIUBOLMAS, Ph.D., 123 pp.

The purpose of this research was to isolate antimicrobial compounds from endophytic fungi isolated from *Casearia grewiaefolia* Vent., *Picrasma javanica* Bl., *Oroxylum indicum* (L.) Vent. and *Erythrina variegate* Linn. Crude extract of endophytic fungus isolate 10B was purified by chromatographic techniques to afford nine known compounds which were identified as alternariol monomethyl ether (**1**), 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**), altersolanol A (**3**), macrosporin (**4**), 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**), alternariol (**6**), altenusin (**7**), 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol (**8**) and 5'-epialtenuene (**9**). The chemical structures of the isolated compounds were elucidated through extensive analyses of NMR and by comparison with literatures. Compound (**3**), (**5**) and (**7**) exhibited antibacterial activity by MIC assay. By checkerboard technique, compound (**5**) and compound (7) showed synergistic activity with tetracycline against *S. aureus* (FIC index 0.12) and with ceftriaxone against *P. vulgaris* (FIC index 0.09), respectively. Identification of fungus 10B based on conventional method and molecular method suggested that 10B was *Alternaria* sp.

Field of Study.....Biotechnology..... Student's Signature.....Phunlap Pompeng.....

Academic Year.....2009.....Advisor's Signature.....N. Ngamrojanavanich.....

Co-Advisor's Signature.....Nongluksna Sriubolmas.....

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| acetone-$d_6$ | = | deuterated acetone |
| $^{\circ}$C | = | degree Celsius |
| $^{13}$C NMR | = | carbon-13 nuclear magnetic resonance |
| $CDCl_3$ | = | deuterated chloroform |
| $CHCl_3$ | = | chloroform |
| $CH_2Cl_2$ | = | methylene chloride |
| CzYA | = | czapak yeast autolysate agar |
| $\delta$ | = | chemical shift |
| *d* | = | doublet (for NMR spectral data) |
| *dd* | = | doublet of doublets (for NMR spectral data) |
| DMSO-$d_6$ | = | dimethyl-$d_6$-sulfoxide |
| DNA | = | Deoxyribonucleic acid |
| DEPT | = | distortionless enhancement by polarization transfer |
| ε | = | molar absorptivity |
| *et al* | = | and other |
| EtOAc | = | ethyl acetate |
| ESI-TOF MS | = | Electrospray Ionization Time of Flight Mass |
| FIC | = | Fraction Inhibitory Concentration |
| g | = | gram |
| µg | = | microgram |
| h | = | hour |
| $^1$H-$^1$H COSY | = | Homonuclear (proton-proton) correlation spectroscopy |
| $^1$H NMR | = | proton nuclear magnetic resonance |
| HMBC | = | $^1$H-detected heteronuclear multiple bond correlation |
| HSQC | = | $^1$H-detected heteronuclear single quantum coherence |
| Hz | = | Hertz |
| $IC_{50}$ | = | inhibitory concentration required for 50% inhibition of growth |
| IR | = | infrared |
| ITS | = | internally transcribed spacers |

| | | |
|---|---|---|
| *J* | = | coupling constant |
| L | = | liter |
| µl | = | microliter |
| $\lambda_{max}$ | = | wavelength at maximum absorption |
| M | = | Molar |
| $[M+Na]^+$ | = | pseudomolecular ion |
| *m* | = | multiplet (for NMR spectral data) |
| MCzA | = | Malt Czapak agar |
| MCzB | = | Malt Czapek broth |
| MEA | = | Malt extract agar |
| MEB | = | Malt extract broth |
| MeOH | = | methanol |
| mg | = | milligram |
| MHA | = | Mueller Hinton agar |
| MHB | = | Mueller Hinton broth |
| MIC | = | minimum inhibitory concentration |
| min | = | minute |
| ml | = | milliliter |
| mm | = | millimeter |
| mM | = | millimolar |
| MHz | = | megahertz |
| MS | = | mass spectroscopy |
| m/z | = | mass to charge ratio |
| $\nu_{max}$ | = | wave number at maximum absorption |
| nm | = | nanometer |
| NMR | = | nuclear magnetic resonance |
| NTP | = | Nucleotide triphosphate |
| PCR | = | polymerase chain reaction |
| PDA | = | Potato Dextrose Agar |
| PDB | = | Potato Dextrose Broth |
| ppm | = | part per million |

| | | |
|---|---|---|
| *q* | = | quartet (for NMR spectral data) |
| rDNA | = | Ribosomal deoxyribonucleic acid |
| rpm | = | Round per minute |
| rRNA | = | Ribosomal ribonucleic acid |
| *s* | = | singlet (for NMR spectral data) |
| SDA | = | Sabouraud's dextrose broth |
| sp | = | species |
| *t* | = | triplet (for NMR spectral data) |
| TAE | = | *Tris*-HCl, acetate and EDTA |
| TE | = | *Tris*-HCl and EDTA |
| TLC | = | thin layer chromatography |
| U | = | unit |
| V | = | volt |
| v | = | volume |
| w | = | weight |
| WA | = | water agar |
| YES | = | Yeast Extract Sucrose |

# CHAPTER I

# INTRODUCTION

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever-growing. In the areas of cancer and infectious disease, 60 % and 75 % of new drugs originated from natural sources between 1981 and 2002 (Newman *et al*., 2003). Between 2001 and 2005, 23 new drugs derived from natural products were introduced for the treatment of disorders such as bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic diseases such as tyrosinaemia and Gaucher disease (Kin, 2007). Increased efforts are therefore needed to develop and search for new drugs from natural products.

Most of the leads from natural products that are currently in development have come from either plant or microbial sources (Baker *et al*., 2007; Harvey, 2000). Since the discovery of the penicillin, soil microorganisms have been extensively prospected in the search of bioactive compounds. However, endophytes, marine microorganisms, microorganisms from extreme environments and microorganisms found in association with other microorganisms or insects have been less studied, and should be considered as under-explored sources of new useful natural products.

Endophytes are microorganisms which spend the whole or part of their life cycles colonizing inter-and/or intra-cellularly inside the healthy tissues of host plant and causing no apparent symptoms of disease (Chanway, 1996). Endophytes not only can mediate interactions between host plants and their competitors, herbivores and pathogens (Carroll, 1988; Clay, 1990; Clay and Holah, 1999), but also can control food-web structure by disrupting the transfer of energy from plants to upper trophic levels (Omacini *et al*., 2001). In addition, fungal endophytes have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Bills and Polishook, 1991; Strobel and Daisy, 2003).

A recent comprehensive study has indicated that 51 % of bioactive substances isolated from endophytic fungi were previously unknown (Schutz, 2001). Hence, the endophytic fungi are expected to be a potential source for new natural bioactive products.

**The objectives of this study are as follows:**

1. Isolation and screening for antimicrobial activity of the endophytic fungi from *Casearia grewiaefolia* Vent, *Picasma javanica* Bl. and *Oroxylum indicum* (L.) Vent.
2. Isolation and characterization of bioactive compounds of the endophytic fungus isolate 10B from *Erythrina variegata* Linn.
3. Classification of the endophytic fungi isolate 10B base on morphological characteristics and rRNA gene sequences.
4. Evaluation of biological activities of the isolated compounds.

# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Natural products and traditional medicine

Throughout the ages, humans have relied on nature for their basic needs including the production of foodstuffs, shelter, clothing, fertilizers, flavours, fragrances and medicines. Nature have been exploited for human use for thousands of the years and plants have been the chief source of compounds used for medicine. Even today, the largest users of traditional medicines are the Chinese with over 5000 plants, and plant products in their pharmacopoeia (Bensky and Gamble, 1993).

Natural products are naturally derived metabolites and/or by products from microorganisms, plants or animals (Baker, Mocek and Garr, 2000.). Aspirin is the world's best known and most universally used medicinal agent, which has its origin from salicin that found in *Salix* sp. and *Populus* sp.

About 3000 years ago, the Mayans used fungi grown on roasted green corn to treat intestinal ailments (Buss and Hayes, 2000). About 800 AD ago, the Benedictine monks began to apply *Papever somniferum* as an anesthetic and pain reliever (Grabley and Thiericke, 1999.).

In recent years an interest in obtaining biological active compounds from natural sources has been observed, notwithstanding the impressive progress of new competing methodologies, such as combinatorial chemistry and high throughput screening or genetic engineering. Contributing to this worldwide attention towards formulations based on natural products, which are less or non toxicity, complete biodegradability and availability from renewable sources.

## 2.2 Endophytic fungi

Endophytic fungi was discovered in 1904 by Darnel (Tan and Zou, 2001), since then, endophytes were studied in different ways which is usually depended on the perspective from which the endophytes were isolated and subsequently examined. Endophyte, which mean the microbes that colonize internal tissues of live plants (**Figure 2.1**) without causing any

immediate, overt negative effects (Bacon, White and Stone, 2000). Their biological diversity, especially in temperate and tropical rain forests is large. Each plant species may harbour a number of endophytes (Strobel, 2003).

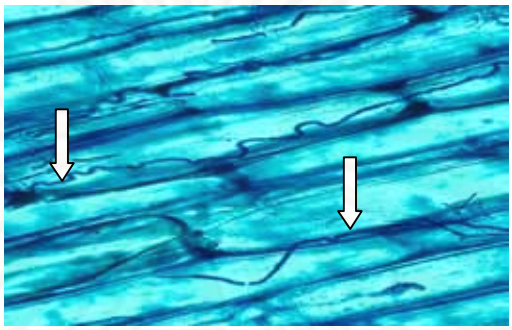


**Figure 2.1** Endophytic fungal hyphae (arrows) in plants cells.

(Image from: http://www.ars.usda.gov/is/graphics/photos/jul00/k8931-2.htm).

### 2.3 Novel secondary metabolites from endophytes

Paclitaxel (Taxol®) (**Figure 2.2**), one of the most important drugs available for the treatment of breast and ovarian cancers was found in the bark of yew tree species (*Taxus* spp.) and *Taxomyces andreanae*, an endophytic fungus associated with Pacific yew *Taxus brevifolia* (Stierle *et al*., 1993; Suffness and Wall, 1995). This incidence might be related to a genetic recombination of the endophyte with the host that occurs in evolutionary time (Stierle *et al*., 1993).

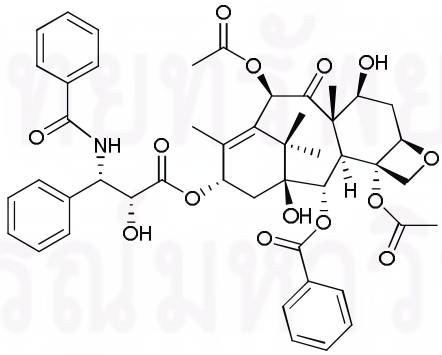



**Figure 2.2** The chemical structure of paclitaxel (Taxol®)

Natural products from endophytic fungi have been observed to inhibit or kill a wide variety of harmful microorganisms such as bacteria, fungi, viruses and protozoans that affect humans and animals. For example, cryptocandin A (**Figure 2.3**), a lipopeptide antifungal compound obtained from *Cryptosporiopsis* cf. *quercing*, the imperfect stage of *Pezicula cinnamomea*, which is an endophytic fungus isolated from *Triptergeun wilfordii* (Strobel *et al*., 1999).

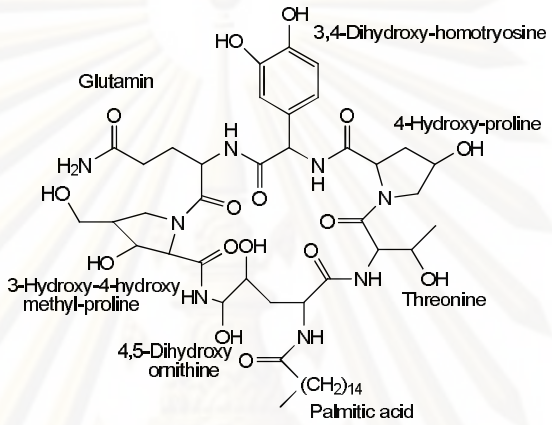



**Figure 2.3** The chemical structure of cryptocandin A

Phomopsichalasin (**Figure 2.4**), a metabolite from an endophytic *Phomopsis* sp., presents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This compound exhibits antibacterial activity in disk-diffusion assays (at a concentration of 4$\mu$g/disk) against *Bacillus subtilis, Salmonella gallinarum*, and *Staphylococcus aureus*, and also displays a moderate activity against *Candida tropicalis* (Horn *et al*., 1995).

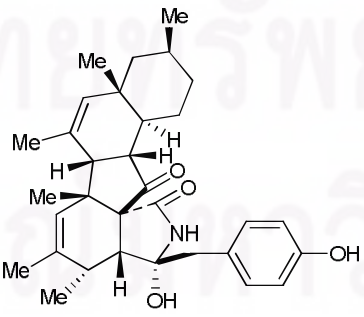
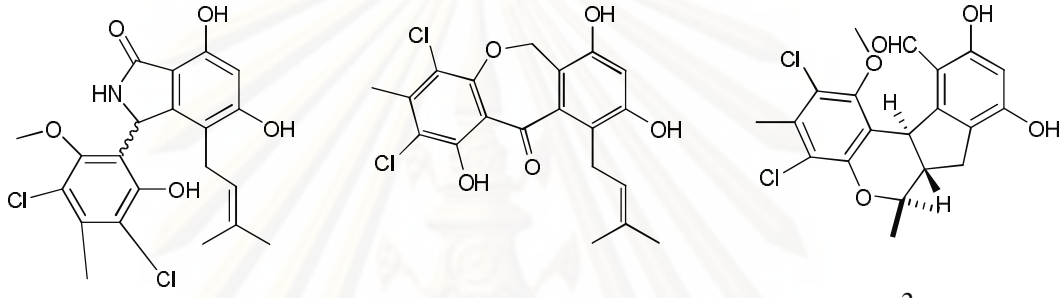


**Figure 2.4** The chemical structure of phomopsichalasin

Three new chlorinated benzophenone derivatives, pestalachlorides A-C (1-3) (**Figure 2.5**), were isolated from *Pestalotiopsis adusta*. Pestalachloride A, a mixture of two inseparable atropisomers (1a and 1b), showed potent antifungal activities against plant pathogenic fungus, *Fusarium culmorum* (CGMCC 3.4595) with an $IC_{50}$ value of 0.89 $\mu$M, while pestalachloride B exhibited remarkable activity against *Gibberella zeae* (CGMCC 3.2873) with an $IC_{50}$ value of 1.1 $\mu$M (Erwei *et al*., 2008).




1a and 1b (Atropisomers)

2

3

**Figure 2.5** The chemical structure of pestalachlorides A-C (1-3)

**2.4 Botanical Aspects, Distribution and Ethnobothanical of *Casearia grewiaefolia* Vent., *Erythrina variegata* Linn, *Oroxylum indicum* (L.) Vent., and *Picrasma javanica* Bl.**

2.4.1 *Casearia grewiaefolia* Vent.

*Casearia grewiaefolia* Vent. (**Figure 2.6**), in Thailand is commonly known as "Gruay pa". It is found in the Northeast of Thailand. This plant is belonging to family Flacourtiaceae. There are some reports showed that plants in family Flacourtiaceae have often been found to contain clerodane, kolovane diterpene, ester and phenolic glycosides; some of which have cytotoxic, insect antifeedant and LEA-1/ICAM binding inhibitory activities (Beutler *et al.,* 2000), while methanol crude extracts from some of Australian Flacourtiaceae species showed antioxidant, cytotoxic and antimicrobial activities (Mosaddik *et al*., 2004).

**Figure 2.6** *Casearia grewiaefolia* Vent.

Taxonomy of *Casearia grewiaefolia* Vent.

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Violales

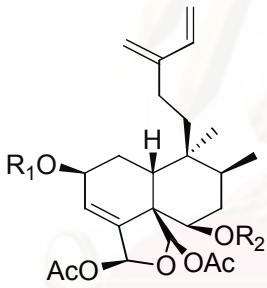
Family: Flacourtiaceae

Genus: *Casearia*

Species: *Casearia grewiaefolia* Vent

*Casearia grewiaefolia* Vent is a small tree about 7-10 m tall with smooth brown gray bark. Leaf simple, alternate, oblong 8-10 cm, long 3-6 cm midth, acute or obtuse, attenuate, crenate, abaxial pubescent. Influrescense cyme, axillary, petal 4-5 perianth, white, green or yellow, unegual. Elliptic fruit, diameter 2.5-3 cm long, three lobes, fusiform, ripe yellow, aril orange red. Its bark and flowers are used traditionally as a tonic and febrifuge (Smitinand, 2001).

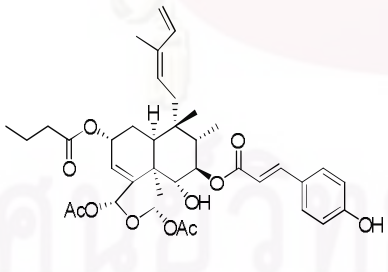
The extract of *C. grewiifolia* bark afforded four new clerodane diterpenes, caseargrewiins A-D (4-7), and two known clerodane diterpenes (8-9) (**Figure 2.7**). All compounds not only exhibited promising antimalarial (evaluated against parasite *Plasmodium falciparum*, K1) and antimycobacterial (assessed against *Mycobacterium tuberculosis*, H37Ra) activities but also showed cytotoxicity against three cancer cell line: human epidermoid carcinoma (KB), human breast cancer (BC1), and human small cell lung cancer (NCI-H187) (Kanokmedhakul *et al*., 2005).



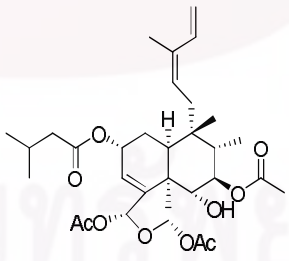

**4** $R_1 = (CH_3)_2CHCH_2CO$, $R_2 = CH_3$

**8** $R_1 = CH_3CH_2CH(CH_3)CO$, $R_2 = CH_3$

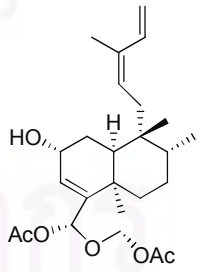
**9** $R_1 = CH_3CH_2CH(CH_3)CO$, $R_2 = OH$




**5**




**6**




**7**

**Figure 2.7** The chemical structure of caseargrewiins A-D (4-7), and clerodane diterpenes (8-9)

2.4.2 *Erythrina variegata* Linn

*Erythrina variegata* Linn, is called in Thai as "Tong Lang Lai", belongs to family Fabaceae which is distributed throughout the topics and subtropics. This plant is the most commonly cultivated member of this genus. Most plants in this family have been found to contain lectins (Tapash and Pranab, 1981; Lis, Joubert and Sharon, 1985; Fukuda, Hidaka and Yomo, 1990).

**Figure 2.8** *Erythrina variegata* Linn

(Image from: http://www.traditionaltree.org)

Taxonomy of *Erythrina variegata* Linn

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Erythrina* L.

Species: *Erythrina variegata* L.

*E. variegata* L., is 20 meter height with trunk 35 cm diameter, quick growing, trunk and branches armed with conical prickles till about 4 years old. Its leaves are mostly ovate (widest toward the base) and sometimes variegated with yellow. Its crimson to orange flowers, 5-8 cm long and its pod is 5-10 seeded. This plant is occasionally used as folk medicine in many countries, such as in Tongo, its bark is used in a formula with other herbs to treat stomachache. In Samoa, its leaves are used to treat eye ailments and bark for cure swellings. In India, china, and Southeast Asia, bark and leaves of this plant are used to treat wind-damp obstruction syndrome-manifested as rheumatic joint pain, spasm of limbs, and stimulate lactation and menstruation for women (Whistle and Craig, 2006).

The previous chemical studies on *E. variegate* presented many isolations of isoflavones, flavonones, and a cinnamyl phenol (Deshpande *et al*., 1977; Telikepalli *et al.*, 1990; Hegde *et al*., 1997), some of which have antimicrobial (Telikepalli *et al.*,1990) and anti-inflammatory activities (Hegde *et al*., 1997).

2.4.3 *Oroxylum indicum* (L.) Vent

*Oroxylum indicum* (L.) Vent. , is called as Phea ka or Maridmai in Thai, is a medicinally important, medium sized, deciduous tree belonging to family Bignoniaceae (Chumsri, 1999; Singhabutra, 1997).


**Figure 2.9** *Oroxylum indicum* (L.) Vent.

Taxonomy of *Oroxylum indicum* (L.) Vent

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Scrophulariales

Family: Bignoniaceae

Genus: *Oroxylum* Vent.

Specices: *Oroxylum indicum* (L.) Vent.

This plant can reach a height of 12 m with few branches and small open crown. The bark is soft, light, brownish-grey and corky outsides. Leaves are opposite, 3-pinnate about the middle and simply pinnate towards apex, very large up to 150 mm in length, rachis stout and cylindric; leaflets 2 to 4 paired, ovate or elliptic, acuminate, entire and glabrous with a rounded or cordate base, appearing to look like a pile of broken limb bones. The flowers are white or purplish and numerous in large and erect racemes, which are a night-bloomer and adapted to natural pollination by bats. They form enormous seed pods that hang down from bare branches. The seeds are round with papery wings (Oudhi, 2003).

The pods, seeds, stem and root bark of this plant contains many flavonoes and traces of alkaloids (Uddin *et al.,* 2003; Dalal and Rai, 2004). Leaves are emollient and contain anthraquinone and aloe-emodin (Nakahara *et al.,* 2002). The government of India categorized this plant as vulnerable medicinal plant (Ravi kumar and Ved, 2000). In India roots are used in Ayurvedic preparation called “Dasamoola” used as an astringent, anti-inflammatory, anti-helminthic, anti-bronchitic, anti-leucodermatic, anti-rheumatic, anti-anorexic and for treatment of leprosy and tuberculosis (Manonmani *et al*., 1995). The fruits are used to treat bronchitis, leucoderma and helminthosis (Dalal and Rai, 2004). Seed extract exhibits anti-microbial, analgesic, anti-tussive and anti-inflammatory properties (Rasadah *et al*., 1998). In Thailand, this plant is used to treat various kinds of diseases, such as stomach

disorders, diarrhea, and dysentery, as a diaphoretic and as a styptic and antipyretic for the treatment of rheumatic swellings (Perry and Metzger, 1980).

In 2008, different crude extracts (crude ethyl acetate, methanolic and water) of leaves of this plant were evaluated for antioxidant activity by using DPPH assay. The $IC_{50}$ values of each crude extract are shown as 49.0, 55.0, and 42.5 µg/mL, respectively, while compared to $IC_{50}$ values of standard (ascorbic acid, $IC_{50}$ 24.0 µg/mL) found that all crude extracts showed less activity than standard (Gupta *et al*., 2008).

2.4.4 *Picrasma javanica* Bl.

*Picrasma javanica* Bl., known in Thai as "Kom Khom", is in the family Simaroubaceae. Alkaloids and quassinoids are found in the plants in this family, some of which showed useful biological activity, such as antifungal activity toward mycotoxin producing fungi, antimalarial and antitumor activities

**Figure 2.10** *Picrasma javanica* Bl.

(Image from http://home.hiroshima-u.ac.jp/shoyaku/photo/Japan/Gunma/020815nurude.jpg)

Taxonomy of *Picrasma javanica* Bl.

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Rutales

Family: Simaroubaceae

Genus: *Picrasma*

Species: *Picrasma javanica* Bl.

*P. javanica* Bl. is a monoecious or dioecious tree that can reach up to 25 m tall, with flute liked bole, up to 35 cm in diameter. Leaves are arranged spirally and imparipinnate. The petiole is 2-6 cm long while stipules are early caducous and foliaceous. The leaflets are opposite or subopposite with a size of 4-20 cm x 1-10 cm, wedge-shapedbase, blunt-acuminate apex and entire. The flowers are white to yellow or green. Fruit is consisting of 1-4, hardly flesh drupelets. Drupe is ovoid to depressed-spherical with green to red or blue color, thin exocarp, fleshy, wrinkled when dry and hard endocarp (Lemmens and Bunyaprapratsara, 2003). In Indonesia, this plant is used to treat malaria in traditional medicine. There are some reports presented that crude extract from leave, bark and stem parts from P. javanica afford quassinoids and alkaloids (Ishii, Koike and Ohmoto, 1991; Koike *et al.*, 1991; Koike *et al.,* 1995)

In 1988, primary screening of chloroform extract from bark of this plant showed the highest yield of anti-malarial activity (*Plasmodium falciparum*; multi-drug resistant Thai isolates) (Pavanand *et al*., 1988). Later it was found that the hexane crude extract of stembark of this plant also exhibited in vitro anti-malarial activity with $IC_{50}$ values 3.3 µg/mL (Saiin *et al*., 2003).

# CHAPTER III

# EXPERIMENTS

## 3.1 Chemicals

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

## 3.2 Culture media

Culture media used for cultivation of endophytic fungi were Potato dextrose agar (PDA), agar, Czapek yeast autolysate agar (CzYA), malt Czapek agar (MCzA), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA) and yeast extract sucrose agar (YES). The formula are shown in **Appendix A**.

## 3.3 Plant sample collection

Healthy leaves and stem were collected from *Casearia grewiaefolia* Vent. (CGV), *Oroxylum indicum* (L.) Vent. (OIV) and *Picrasma javanica* Bl. (PJB) in the forest area of Lam Phun Province, Thailand, in July 2007. They were identified based on their morphological characteristics. The fresh-cut ends of plant samples were wrapped with Parafilm M (3M Co.Ltd.) before they were placed in zip-lock plastic-bags and stored less than 72 h prior to isolation of endophytic fungi.

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## 3.4 Fungal endophyte isolation

Plant samples were washed in tap water and air-dried. The cleaned leaf and root fragments were surface-sterilized as described by Schulz and co-workers (Schulz *et. al.,* 1995) with some modifications. Plant fragments were sequentially immersed in 70% EtOH for 1 min, NaOCl solution (6% available chloride) for 5 min, and sterile distilled water for 1 min (two times). Then, the surface-sterilized fragments were cut into small pieces (ca. 5 mm in length) using a sterile blade and placed on sterile water agar plates for further incubation at

30°C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile Pasteur pipet and transferred onto a potato dextrose agar (PDA) plate. After incubation at 30 °C for 7-14 days, culture purity was determined from colony morphology.

**3.5 Screening of antimicrobial activities**

Antimicrobial activities of endophytic fungi and crude extracts were determined by dual-culture agar diffusion assay (Sriubolmas *et al.,* 2001) and disk diffusion test as described in the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) M7-A8 method, respectively.

3.5.1 Preparation of test microorganisms and test plates

All endophytic fungal isolates were screen for antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231. Each bacterial strain was grown in tryptic soy broth at 37 °C for 2-3 h. *C. albicans* was grown on Sabouraud's dextrose agar at 30 °C for 24 h and suspended in 0.85% NaCl. The turbidity of microbial suspension was adjusted to match that of 0.5 McFarland standard. A sterile cotton swab was used to apply each bacterial and yeast inocula onto the entire surface of the Mueller Hinton agar plate and SDA plate, respectively.

3.5.2 Dual-culture agar diffusion assay

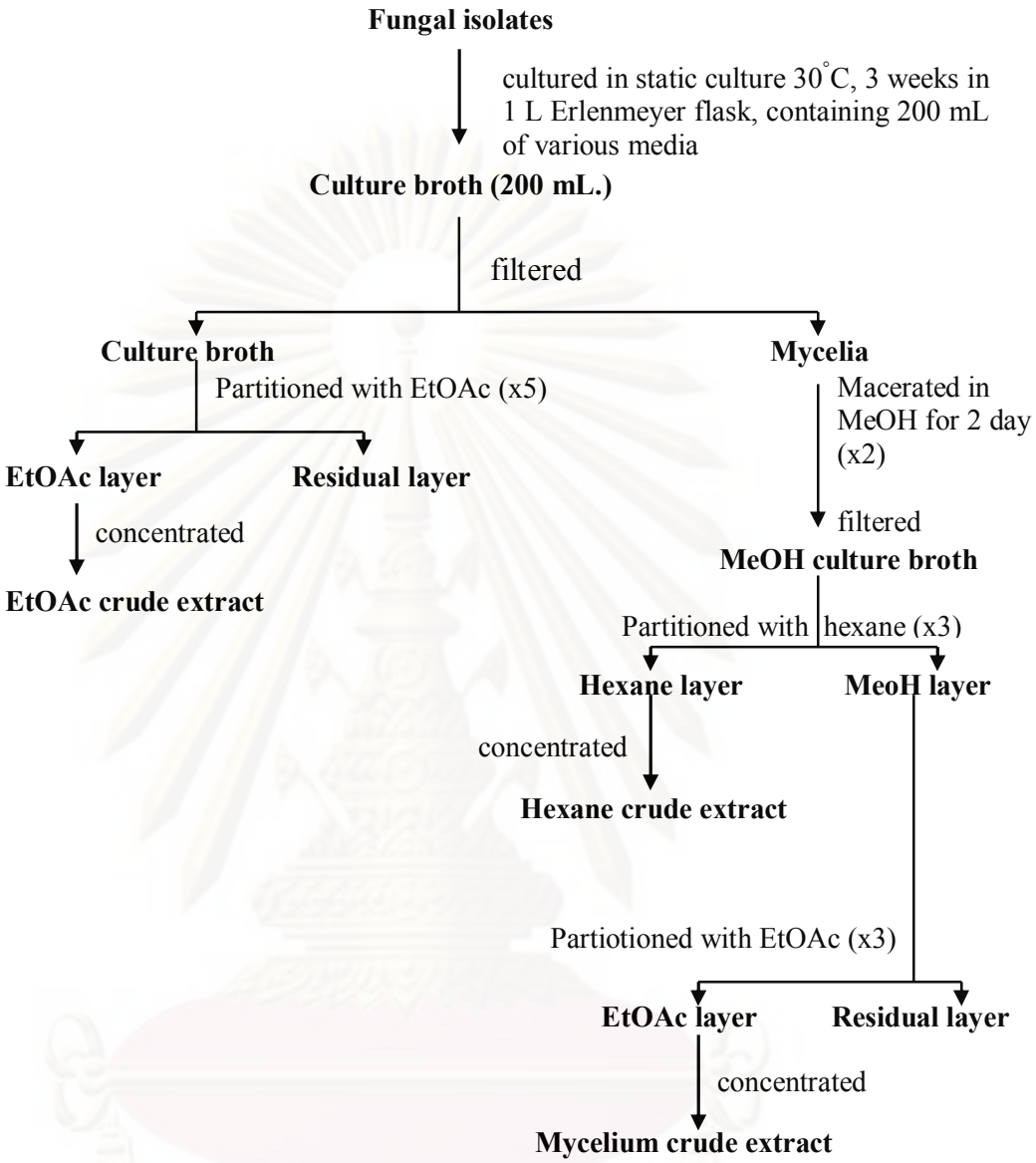
Endophytic fungal isolates were grown at room temperature for 14 days on six different mycological media: Czapek yeast autolysate agar (CzYA), malt Czapek agar (MCzA), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA) and yeast extract sucrose agar (YES). Then they were cut into small agar block with sterile cork borer (8-mm diameter). Endophytic fungal agar block were placed on inoculated test plate. After keeping at room temperature for 1 h, the test plates were incubated at 37 °C for 24 h. Inhibition zone around the agar block indicated antimicrobial activities of endophytic fungi.

3.5.3 Disk diffusion assay

Six sterile blank disks (6 mm diameter) were distributed evenly onto the inoculated agar surface. One mg of each crude extract in 10 µl DMSO was applied onto the blank disks. After keeping at room temperature for 1 h, the test plates were incubated at 37 $^{o}$C for 24 h. The inhibition zone around the paper disk indicated antimicrobial activities of crude extracts.

**3.6 Fermentation and extraction**

Endophytic fungal isolates that showed antimicrobial activities were selected for further study by cultivation in appropriate liquid medium under standstill condition at 30 °C for 21 days. The fungal cultures were filtered to remove mycelium. The filtered broths were then extracted with ethyl acetate 5 times and evaporated to dryness using rotary evaporator and weighed to constitute the crude broth extract. The mycelium was extracted twice by soaking in methanol (2 days/time). The pooled methanol crude extracts were extracted with hexane for 3 times, evaporated to dryness and weighed to constitute the crude hexane extract. The part that could not be extracted with hexane was extracted again with ethyl acetate 3 times, evaporated to dryness and weighed to constitute the crude mycelium-ethyl acetate extract. **Scheme 3.1** summarizes the whole extraction process.




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

### 3.7 Selection of endophytic fungal isolate

All endophytic fungi which were isolated from *Casearia grewiaefolia* Vent. (CGV), *Oroxylum indicum* (L.) Vent. (OIV) and *Picrasma javanica* Bl. (PJB), along with fifteen endophytic fungi as shown in **Table 3.1**. were selected based on its NMR spectrum and antimicrobial activities in previous studies by Phunmod and Phuthong, 2008. All selected fungi were cultured on appropriate medium for producing secondary metabolites, then

measured crude extract by $^{1}$H NMR. The crude extracts of the fungi that show characteristic interesting $^{1}$H NMR signals were selected for large scale of cultivation and extraction.

**Table 3.1** Endophytic fungal isolates selected based on their antimicrobial activities (Phunmod and Phuthong, 2008).

| No. | Fungal code | Scientific name of plant host | Culture medium | Inhibited microorganisms |
|---|---|---|---|---|
| 1 | 1AA | *Avicennia officinalis* | MEA | *C. albicans* |
| 2 | 6Eb | *Sonneratia caseolaris* | MEA<br>YES | *S. aureus*<br>*S. aureus* |
| 3 | 8C | *Xylocarpus obovatos* | YES | *E. coli, C. albicans* |
| 4 | 8D | *Xylocarpus obovatos* | YES | *E. coli* |
| 5 | 9E | *Heritiera Pryand* | YES | *S. aureus* |
| 6 | 10B | *Erythrina variegata* | MEA | *S. aureus* |
| 7 | 10F | *Erythrina variegata* | MEA | *S. aureus* |
| 8 | 11J | *Solanum indicum* Linn. | YES | *C. albicans* |
| 9 | 11DB | *Solanum indicum* Linn. | MEA | *S. aureus* |
| 10 | 14K | *Pluchea indica* Less. | MEA<br>YES | *S. aureus*<br>*S. aureus* |
| 11 | 15B | *Pithecolobium* Bulce | MEA | *C. albicans* |
| 12 | 15G | *Pithecolobium* Bulce | MEA | *C. albicans* |
| 13 | 19CB | *Nipa fruticans* Wurmb | MEA<br>YES | *S. aureus*<br>*S. aureus* |
| 14 | 21L | *Azima sarmentosa* Benth. | YES | *S. aureus* |
| 15 | 21B | *Azima sarmentosa* Benth. | PDA<br>MEA<br>YES | *E. faecalis*<br>*E. faecalis*<br>*E. faecalis, S. aureus, C. albicans* |

* MEA: Malt Extract Agar YES: Yeast Extract Sucrose agar
PDA: Potato Dextrose Agar

### 3.8 Cultivation and isolation of the pure compounds

The fungi of interest were grown on PDA plate at 30ºC for 7 days. Six pieces (6x6 $mm^2$) of the grown culture cut from the plate were inoculated into 1000 mL Erlanmeyer flask (x 25) containing 200 mL of appropriate medium at 30ºC for 21 days under static conditions.

The culture broth was passed through four layers of cheese cloth which were exhaustively pressed. The filtrate was subjected to MCI-gel CHP 20 eluted with $H_2O$-MeOH and acetone. Mycelia were sequentially macerated in MeOH at room temperature, 2days (x2). Combined MeOH extracts and evaporated under reduced pressure and partitioned three time with equal volume of hexane. Then, the MeOH layer was added with $H_2O$ and partitioned with EtOAc to obtain mycelium crude extract.

### 3.9 Chromatography

#### 3.9.1 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) was carried out on a silica gel F254 coated on aluminium sheet (Merck) and silica gel RP-18 F254 coated on mirror sheet (Merck). Detection was visualized under ultraviolet light at wavelengths of 254 and 356 nm.

#### 3.9.2 Column chromatography

Column chromatography (CC) was performed using Sephadex LH-20, Diaion HP-20 resin; MCI-gel CHP 20 (Mitsubishi chemical corporation), Wakogel-reverse phase silica gel-C18 (Wako pure chemical industry; size 63-212 $\mu$m) and Silica gel 60H (Merck code no. 7734 and no. 9385) as packing materials.

### 3.10 Isolation of bioactive compounds from selected endophytic fungi

Endophytic fungi isolate 10B from *Erythrina variegata* (Figure 3.1), was selected for further study due to their interesting $^1$H NMR (Appendix B) when grown on malt extract broth (MEB). Furthermore, crude extract of isolate 10B was found to exhibit activities against *Staphylococcus aureus* with the MIC value of 100 $\mu$g/mL.

**Figure 3.1** *Erythrina variegate* Linn
(Image from: http://www.forest.go.th/nursery/puttaprawat/newpage23.htm)

The 10 L of dark brown culture broth medium was subjected to Diaion HP-20 resin (MCI-gel CHP 20) column chromatography (CC) using $H_2O$, followed by a gradient elution of $H_2O$-MeOH as a solvent to afford eleven fractions (A-K).

**10B medium cultured (10L; MEB)**

↓ MCI-gel CHP 20
100%$H_2O$ to 100%MeOH

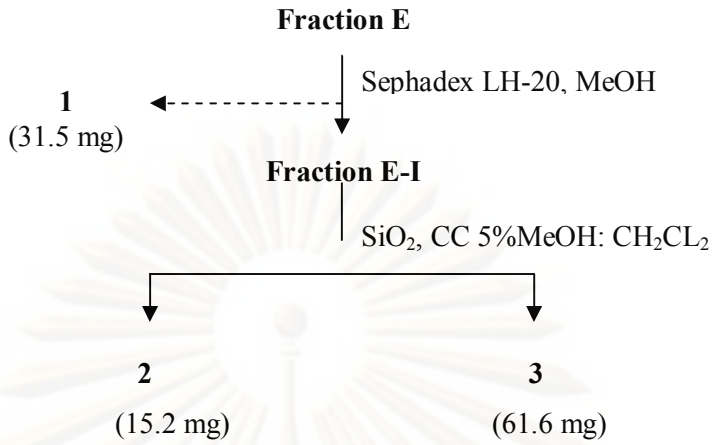
**Fraction A-K**

**Scheme 3.2** Isolation of 10B cultured on MEB medium

3.10.1 Fraction E

Fraction E crystalized to afford compound **1** (31.5 mg). The 468.1 mg. of fraction E was rechromatographed over sephadex LH-20 column eluted with MeOH to afford four fractions. Fraction E-I was further subjected to flash CC on silica gel (MeOH:$CH_2CL_2$, 5:95) to yield compound **2** (15.2 mg) and compound **3** (61.6 mg).

The isolation of fraction E is briefly summarized in **Scheme 3.3**.

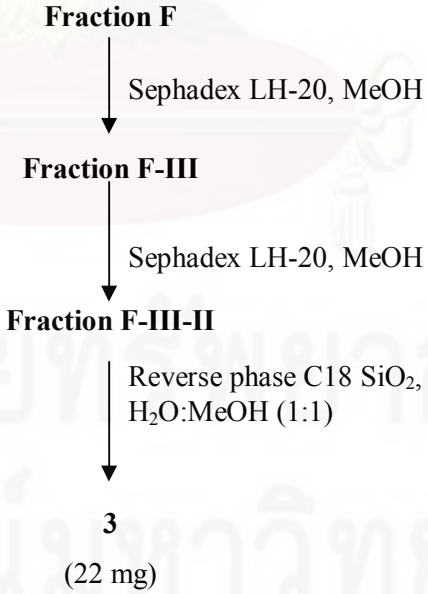



**Scheme 3.3** Isolation of fraction E

3.10.2 Fraction F

The 596.3 mg of fraction F was subjected to sephadex LH-20 column euluted with MeOH to afford three fractions. Fraction F-III was rechromatographed over sephadex LH-20 eluted with MeOH to afford two fractions. Fraction F-III-II was further subjected to flash reverse phase C-18 silica gel eluted with $H_2O$:MeOH (1:1) to afford compound **3** (22 mg)

The isolation of fraction F is briefly summarized in **Scheme 3.4**.

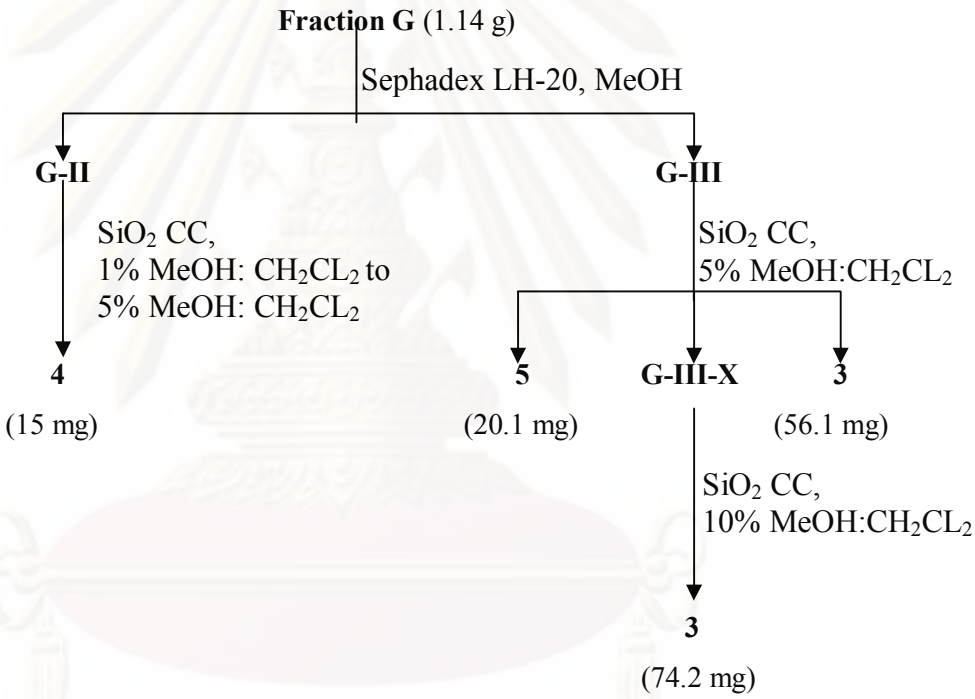



**Scheme 3.4** Isolation of fraction F

3.10.3 Fraction G

The 1.14 g of fraction G was subjected to sephadex LH-20 column chromatography eluted with MeOH to provide five fractions along with solid part. Fraction G-II was further subjected on $SiO_2$ CC eluted from 1% MeOH: $CH_2CL_2$ to 5% MeOH: $CH_2CL_2$ to afford compound **4** (15 mg). Fraction G-III was rechromatographed on $SiO_2$ CC eluted with 5% MeOH: $CH_2CL_2$ to provide compound **5** (20.1 mg) and more compound 3 (56.1 mg). Fraction G-III-X was further subjected on $SiO_2$ CC eluted with 10% MeOH: $CH_2CL_2$ to give more compound **3** (74.2 mg).

The isolation of fraction G is briefly summarized in **Scheme 3.5**.

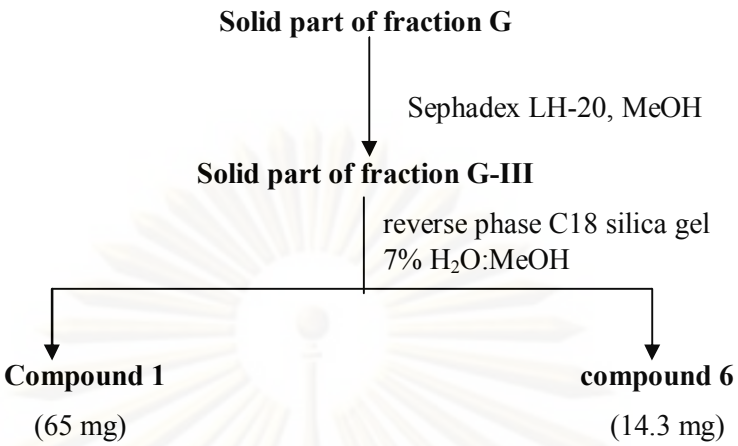



**Scheme 3.5** Isolation of fraction G

3.10.4 Fraction G-solid part

The 183.5 mg of solid from fraction G was subjected to sephadex LH-20 eluted with MeOH to afford three fraction. Fraction G_S-III was rechromatographed over reverse phase C18 silica gel eluted with $H_2O$:MeOH (7:93) to give **compound 1**(65 mg) and **compound 6**( 14.3 mg).

The isolation of fraction G-crystallized is briefly summarized in **Scheme 3.6**.




**Scheme 3.6** Isolation of crystallized part of fraction G

3.10.5 Fraction H-K

The 8.83 g of fraction H-K was subjected to $SiO_2$ column chromatography (CC) gradient elution from EtOAc:hexane (1:1) to 100% EtOAc to give five fractions.

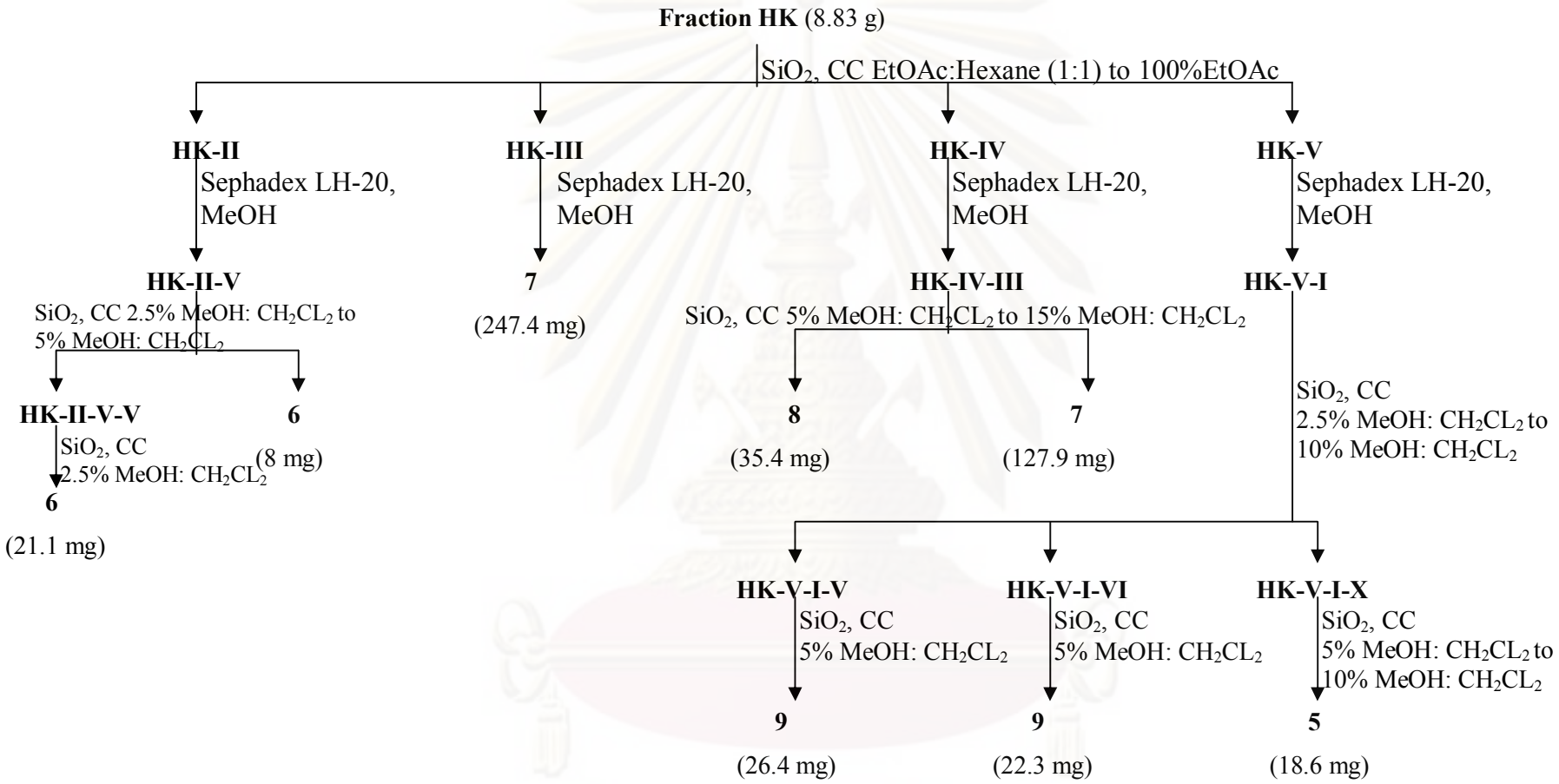
Fraction HK-II was further subjected to sephadex LH-20 eluted with MeOH to give three fractions. Fraction HK-II-V was rechromatographed by flash $SiO_2$ CC eluted from 2.5% MeOH: $CH_2CL_2$ to 5% MeOH: $CH_2CL_2$ to afford compound **6** (8 mg). Fraction HK-II-V-V was further subjected on $SiO_2$ CC eluted with 2.5% MeOH: $CH_2CL_2$ to afford more compound **6** (21.1 mg)

Fraction HK-III was further subjected to sephadex LH-20 eluted with MeOH to afford compound **7** (247.4 mg).

Fraction HK-IV was rechromatographed over sephadex LH-20 eluted with MeOH to give three fractions. Fraction HK-4-III was further subjected on $SiO_2$ CC eluted from 5% MeOH: $CH_2CL_2$ to 15% MeOH: $CH_2CL_2$ to afford three pure compounds; compound **7** (127.9 mg) and compound **8** (35.4 mg).

Fraction HK-V was subjected to sephadex LH-20 eluted with MeOH to afford two fractions. Fraction HK-V-I was further subjected on flash $SiO_2$ CC eluted from 2.5% MeOH: $CH_2CL_2$ to 10% MeOH: $CH_2CL_2$ to give ten fractions. Fraction HK-V-I-V was rechromatographed on $SiO_2$ CC eluted with 5% MeOH: $CH_2CL_2$ to afford compound **9** (26.4 mg). Fraction HK-V-I-VI was rechromatographed on $SiO_2$ CC eluted with 5% MeOH: $CH_2CL_2$ to afford more compound **9** (22.3 mg). Fraction HK-V-I-X was rechromatographed on $SiO_2$ CC eluted from 5% MeOH: $CH_2CL_2$ to 10% MeOH: $CH_2CL_2$ to afford more compound **5** (18.6 mg).

The isolation of fraction H-K is briefly summarized in **Scheme 3.7**




**Scheme 3.7** Isolation of fraction H-K



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### 3.11 Structure elucidation

Structures were elucidated by the interpretation of NMR spectra.

3.11.1 Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were calibrated using solvent signals ($^{13}C$: $CDCl_3$ 77.00 ppm, acetone-$d_6$ ($CD_3COCD_3$) 29.8 and 206.0 ppm, and DMSO-$d_6$ ($CD_3SOCD_3$) 39.5 ppm) or a signal of the portion of the partly or non deuterated solvent ($^1H$: $CHCl_3$ in $CDCl_3$ $\delta$ 7.26 ppm, acetone in acetone-$d_6$ $\delta$ 2.05 ppm, water ($H_2O$) in acetone-$d_6$ $\delta$ 2.80 ppm, DMSO in DMSO-$d_6$ $\delta$ 2.50 ppm, and water ($H_2O$) in DMSO-$d_6$ $\delta$ 3.31 ppm). $^1H$ NMR, $^{13}C$ NMR, DEPT, gCOSY, gHSQC, gHMBC, and NOESY pectra were recorded on a Varian Spectrometer operated at 400, 600 MHz for $^1H$ nuclei and at 100 MHz for $^{13}C$ nuclei. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated chloroform at $\delta$ = 7.26 ppm.

### 3.12 Structural elucidation of pure compounds

3.12.1 Compound **1**

(Alternariol momomethyl ehter)

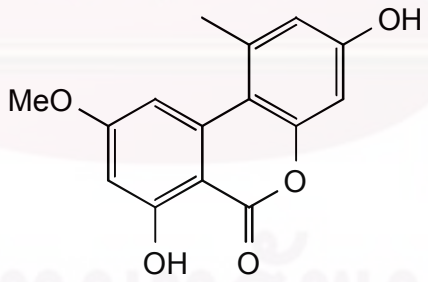



**Figure 3.2** The chemical structure of compound **1**

$C_{15}H_{12}O_5$

Cream powder

$^1H$ NMR and $^{13}C$ NMR data in DMSO-$d_6$ , shown in **Table 4.1 page 39**

3.12.2 Compound **2**

(5-carbomethoxymethyl-2-heptyl-7-hydroxychromone)

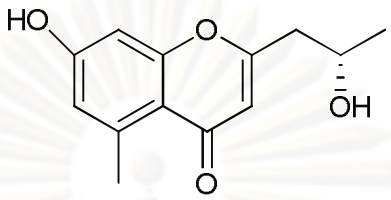



**Figure 3.3** The chemical structure of compound **2**

$C_{13}H_{14}O_4$

Colorless needles

$^1$H NMR and$^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.2 page 41**

3.12.3 Compound **3**

(Altersolanol A)

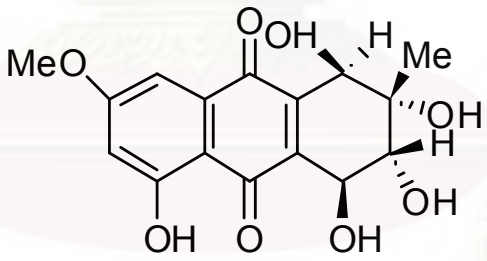



**Figure 3.4** The chemical structure of compound **3**

$C_{16}H_{16}O_8$

deep red cubic

$^1$H NMR and$^{13}$C NMR data in acetone-$d_6$, shown in **Table 4.3 page 44**

3.12.4 Compound **4**

(macrosporin)

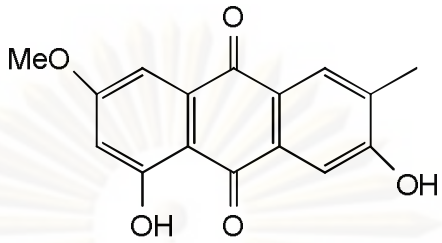



**Figure 3.5** The chemical structure of compound **4**

$C_{16}H_{12}O_5$

Yellow solid

$^1$H NMR and$^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.4 page 46**

3.12.5 Compound **5**

(1,2,4,5-tetrahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthracene-9,10-dione)

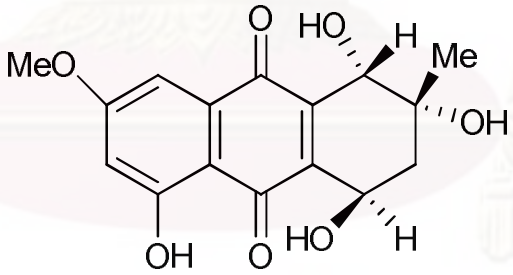



**Figure 3.6** The chemical structure of compound **5**

$C_{16}H_{16}O_7$

Yellow to orange solid

$^1$H NMR and$^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.5 page 48**

3.12.6 Compound **6**

(Alternariol)

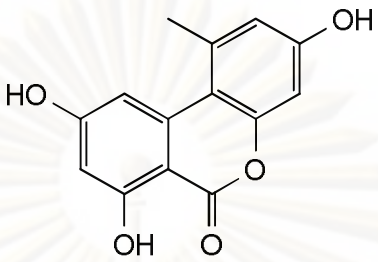



**Figure 3.7** The chemical structure of compound **6**

$C_{14}H_{10}O_5$

yellowish powder

$^1$H NMR and$^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.6 page 50**

3.12.7 Compound **7**

(Altenusin)

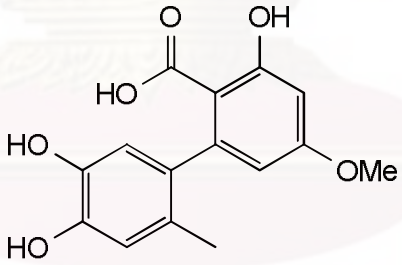



**Figure 3.8** The chemical structure of compound **7**

$C_{15}H_{14}O_6$

Brown solid

$^1$H NMR and$^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.7 page 52**

3.12.8 Compound **8**

(3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol)

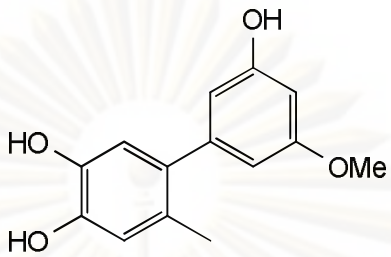



**Figure 3.9** The chemical structure of compound **8**

$C_{14}H_{14}O_4$ ;

Brown liquid

$^1$H NMR and $^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.8 page 54**

3.12.9 Compound **9**

(5'-epialtenuene)

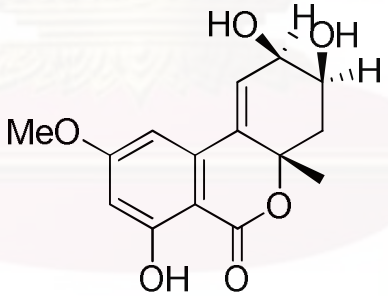



**Figure 3.10** The chemical structure of compound **9**

$C_{15}H_{16}O_6$

Colourless solid

$^1$H NMR and $^{13}$C NMR data in DMSO-$d_6$ , shown in **Table 4.9 page 56**

### 3.13 Evaluation of biological activities

The pure compounds were evaluated for their anti-bacterial activity.

3.13.1 Antibacterial activity

A total of 12 strains of gram-positive and gram-negative bacteria (**Table 3.2**) were selected for *in vitro* antimicrobial assay. The test was performed by using microdilution assays as follows:

**Table 3.2** Gram-positive and gram-negative bacteria tested

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

*3.13.1.1 Preparation of bacterial inocula*

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

*3.13.1.2 Determination of minimum inhibitory concentration (MIC)*

Solution of a test compound in DMSO (25.6 mg/mL) was diluted with MHB. The test compound was tested at the concentration ranges of 0.5 to 256 µg/mL. MIC is defined as the lowest concentration that inhibits growth of test microorganisms.

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

*3.13.1.3 Antimicrobial combinations assay*

The following antibiotic powders were used in this study: ampicillin, ceftriaxone disodium salt hemi(heptahydrate), gentamicin sulfate salt, tetracycline hydrochloride, and vancomycin hydrochloride (Sigma-Aldrich)

*3.13.1.3.1 Determination of checkerboard technique*

In this test (Satish and Robert, 2005), serial dilutions of antimicrobial agent and antimicrobial compound were mixed together in a microtiter plates that each row and column contained a fixed amount of one agent and increasing amounts of the second agent. The concentrations of antimicrobial ranged from approximately twice the MIC to seven serial twofold dilutions below this amount. Each plate also contained a row and column in which a serial dilution of each agent was present alone (**Figure 3.11**).

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 2/0 | 2/1/32 | etc | | | | | |
| | 1 | 1/0 | etc | | | | | | |
| | 1/2 | | | | | | | | |
| Test | 1/4 | | | | | | | | |
| Compound | 1/8 | | | | | | | | |
| ( A ) | 1/16 | | | | | | | | |
| | 1/32 | | | | | | | | |
| | 0 | | | | | | | | |
| | (xMIC) | 0 | 1/32 | 1/16 | 1/8 | 1/4 | 1/2 | 1 | 2 |

Drug (B)

**Figure 3.11** Checkerboard teachnique, serial dilution of two drug concentration proportional to MICs of the test compound and drug being tested.

A 25 µL volume of MHB containing the test compound at the same concentration were dispensed into each well of microtiter plates (96-flat-bottom wells) in the *x* axis from well number one to eight, while 25 µL volume of MHB containing drug were dispensed into each well in the *y* axis from well alphabet A to H. The final adjusted bacterial suspensions (1x $10^6$ CFU/mL) were inoculated into each well with volumes of 50 µL. The experiments were done in duplicate. After incubation at 37°C for 24 h, a 20 µL of *p*-iodonitrotetrazolium (INT) solution (1 mg/mL) was added into each well. The antibacterial assay plates were further incubated for 1 h. Growth in each well was indicated by a color change from colorless to violet. Compounds that inhibit microbial growth would prevent the development of a violet color. The well that shows no change in color indicates antimicrobial activity of the test compound.

Interpreting the checkerboard result by using the pattern they form on the isobologram (**Appendix C**) and the FIC index which can be viewed as a mathematical restatement of the isobologram (**Table 3.3**).

**Table 3.3** Calculation of the Fractional Inhibitory Concentration (FIC) index for combination of two antimicrobials

$$\frac{(A)}{(MIC_A)} + \frac{(B)}{(MIC_B)} = FIC_A + FIC_B = FIC$$

(A) is the concentration of test compound in a well that is the lowest inhibitory concentration in its row.

($MIC_A$) is the MIC of test compound alone.

$FIC_A$ is the fractional inhibitory concentration of test compound.

(B) is the concentration of drug in a well that is the lowest inhibitory concentration in its column. ($MIC_B$) and $FIC_B$ are defined in the same fashion for drug A

To interpret FIC index as follow

| | |
|---|---|
| Synergism | FIC index $\leq$ 0.5 |
| Indifference | 0.5 < FIC index $\leq$ 4.0 |
| Antagonism | FIC index > 4.0 |

**3.14 Classification of the endophytic fungal isolate 10B**

The fungal endophyte isolate 10B was identified on the basis of both morphology of the fungus grown on potato dextrose agar (PDA) and water agar with banana leaf pieces at 25°C and analysis of the DNA sequences of the ITS region of the rRNA gene.

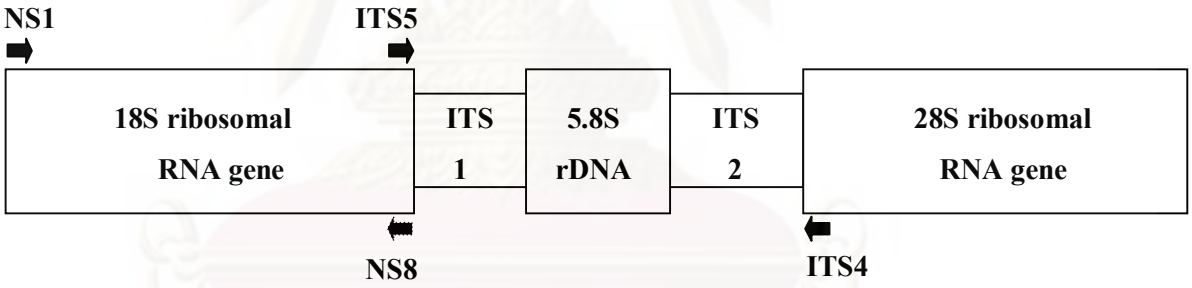
3.14.1 Conventional method

10B isolate was grown on potato dextrose agar (PDA) for 14 days at room temperature and photographed. Microscorpic morphology of 10B grown on water agar with banana leaf pieces was examined by light microscopy.

3.14.2 Molecular method

The 10B fungus was grown on potato dextrose broth. After cultivation for 7 days, 50 mg of fresh fungal mycelia were harvested, washed twice with normal saline solution, and homogenated in 250 µl of sterile water. The mycelial homogenate was applied evenly to an FTA card matrix, allowed to dry at room temperature, and a 2-mm disk containing the fungal mycelia was punched from the FTA card. Total cellular DNA of fungal mycelia on the disk

was extracted and purified using the FTA® Plant Kit (Whatman®) according to the manufacturer's instruction. The disk was transferred to a PCR tube and the ITS1-5.8S-ITS2 ribosomal RNA gene region (**Figure 3.12**) of fungal genomic DNA was amplified using the ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al.,* 1990). PCR amplification was performed in a 50 µl reaction volume which contained Taq PCR Master Mix (USB Corp., USA) using an automated thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). The thermocycling program was as follows: 3 min at 95°C followed by 30 cycles of 50 s at 95°C, 40 s at 45°C and 50 s at 72°C, with a final extension period of 10 min at 72°C. The PCR products were purified and directly subjected to sequencing (Bioservice Unit, NSTDA, Bangkok, Thailand) in both directions primed with either of the two primers used to originally amplify the fragment. The DNA sequence of ITS1-5.8S-ITS2 rRNA gene obtained was used as query sequence to search for similar sequences in GenBank using BLASTIN 2.2.18 (Altschul *et al*., 1997). DNA sequence similarity was determined by the ClustalW 2 multiple sequence alignment program (Larkin *et al*., 2007).




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

**Figure 3.12** Location on nuclear rDNAs of ITS5, ITS4, NS1, ands NS8 primers. The arrow heads represent the 3' end of each primer.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Isolation of fungal endophytes

A total of 40 pure isolates of endophytic fungi were isolated from *Casearia grewiaefolia* Vent., *Oroxylum indicum* (L.) Vent. and *Picrasma javanica* Bl. Fifteen, fifteen and ten isolates were from *Casearia grewiaefolia* Vent., *Oroxylum indicum* (L.) Vent, and *Picrasma javanica* Bl., respectively, as shown in **Table 4.1**.

**Table 4.1** Sources of endophytic fungi

| Plant | Part | Numbers of Isolate |
|---|---|---|
| *Casearia grewiaefolia* Vent. | Leaf | 5 |
| | Stem | 10 |
| *Oroxylum indicum* (L.) Vent | Leaf | 7 |
| | Stem | 8 |
| *Picrasma javanica* Bl. | Leaf | 3 |
| | Stem | 7 |

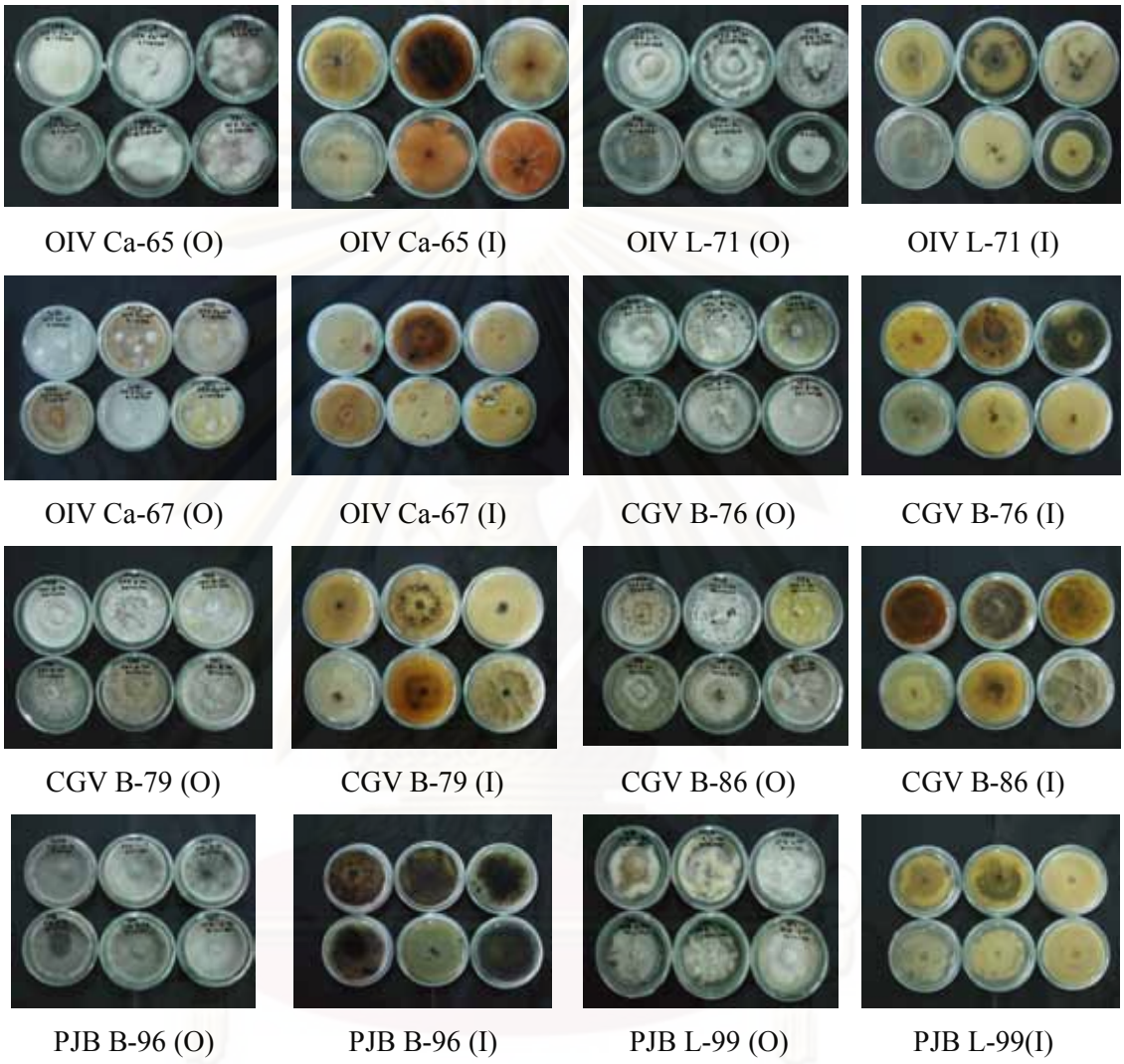



**Figure 4.1** Some endophytic fungi grown on six difference mycological media.
Code: CGV are endophytic fungi isolated from *Casearia grewiaefolia* Vent, OIV are endophytic fungi isolated from *Oroxylum indicum* (L.) Vent., PJB are endophytic fungi isolated from *Picrasma javanica* Bl.

**4.2 Isolation of pure compounds**

The crude extract of endophytic fungi isolate 10B obtained from *Erythrina variegata* Linn. was subjected to several steps of chromatographic fractionations to provide nine known compounds; alternariol monomethyl ether (**1**), 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**), altersolanol A (**3**), macrosporin (**4**), 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**), alternariol (**6**), altenusin (**7**), 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol (**8**) and 5'-epialtenuene (**9**).

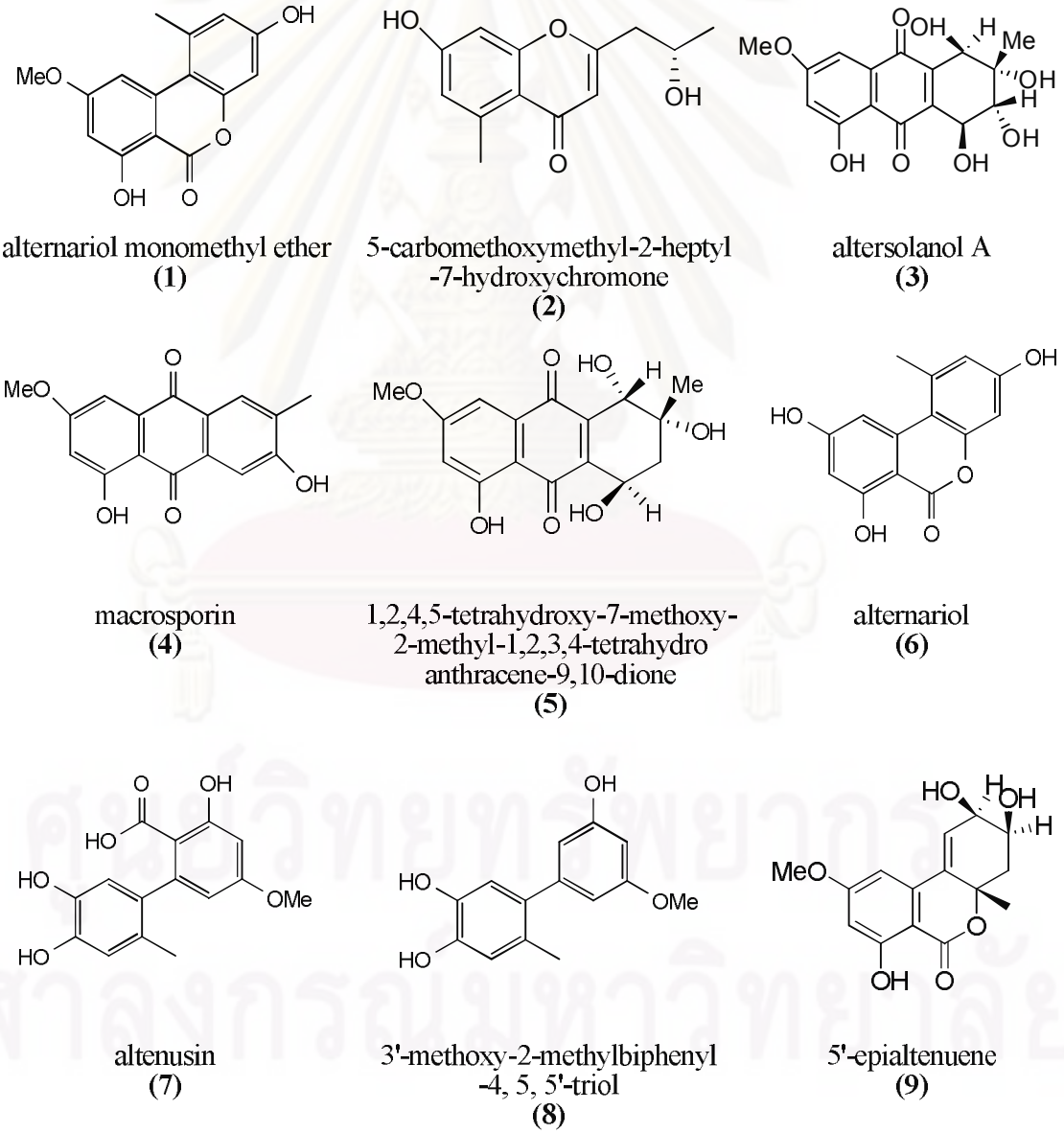



**Figure 4.2** Chemical structures of pure compounds from endophytic fungi isolate 10B

**4.3 Structure elucidation of pure compounds**

4.3.1 Compound **1**

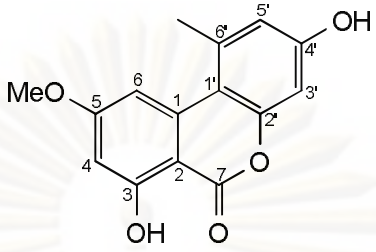



**Figure 4.3** The chemical structure of compound **1**

Compound **1** was isolated as a cream powder and its molecular formula was assigned to be $C_{15}H_{12}O_5$ by 1D NMR and HSQC data, which indicated 10 degrees of unsaturation.

The $^1$H NMR spectrum of **1** displayed signals of four aromatic protons ($\delta_H$ 6.60, 6.65, 6.73 and 7.22), a methoxy ($\delta_H$ 3.92) and a methyl ($\delta_H$ 2.74) group.

The $^{13}$C NMR revealed the presence of an ester carbonyl ($\delta_C$ 166.6), four oxygenated aromatic carbons ($\delta_C$ 153.1, 159.1, 164.6 and 165.1), eight aromatic carbons ($\delta_C$ 99.0, 99.6, 102.1, 103.9, 109.3, 118.1, 138.3 and 138.9) and a methoxy ($\delta_C$ 56.3).

These NMR data indicated that seven of the 10 elements of unsaturation come from six carbon-carbon aromatic double bonds and one carbonyl. Therefore, the molecule possesses a tricyclic skeleton. The structure of **1** was corroborated by the observed HMBC correlations as shown in **Figure 4.4**. Comparison of the $^1$H and $^{13}$C NMR data of **1** with those reported in the literatures (**Table 4.2**), confirmed that **1** is alternariol monomethyl ether (Wen, 2009).

Alternariol monomethyl ether is one of the well-known metabolites produced by several species of the genus *Alternaria*, such as *A. altarnata, A. tenuissima* and *A. solani* (Stinson *et al.,* 1980) and it has been reported as a chemotaxonomic marker for the classification of *Alternaria* species (Andersen *et al.,* 2008). In addition, it also showed to be mutagenic and cytotoxic to bacterial and mammalian cells (Patriarca *et al.,* 2007).

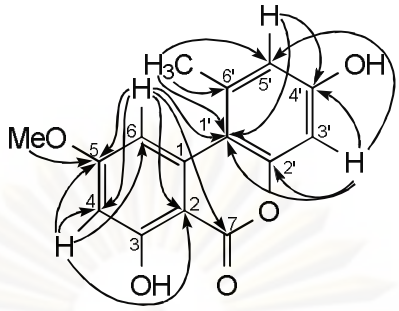



**Figure 4.4** HMBC correlations of compound **1**

**Table 4.2** The $^{1}H$ and $^{13}C$ spectral data of compound **1** and alternariol monomethylether

| | **Compound 1** (DMSO-$d_6$) | | **Alternariol monomethyl ether** (DMSO-$d_6$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) |
| 1 | 138.3, qC | - | 137.8, qC | - |
| 2 | 99.0, qC | - | 98.5, qC | - |
| 3 | 165.1, qC | - | 164.0, qC | - |
| 4 | 99.6, CH | 6.60, s | 99.3, CH | 6.6, d (2.3) |
| 5 | 164.6, qC | - | 164.7, qC | - |
| 6 | 103.9, CH | 7.22, s | 103.5, CH | 7.20, d (2.0) |
| 7 | 166.6, qC | - | 165.5, qC | - |
| 1’ | 109.3, qC | - | 108.8, qC | - |
| 2’ | 153.1, qC | - | 152.6, qC | - |
| 3’ | 102.1, CH | 6.65, s | 101.6, CH | 6.71, d (2.5) |
| 4’ | 159.1, qC | - | 158.5, qC | - |
| 5’ | 118.1, CH | 6.73, s | 117.7, CH | 6.64, d (2.5) |
| 6’ | 138.9, qC | - | 138.5, qC | - |
| 3-OH | - | 11.82 | - | 11.81, s |
| 5-OMe | 56.3, $CH_3$ | 3.92, s | 55.8, $CH_3$ | 3.90, s |
| 4’-OH | - | 10.36 | - | 10.36, s |
| 6’-Me | 25.5, $CH_3$ | 2.74, s | 25.0, $CH_3$ | 2.71, s |

4.3.2 Compound **2**

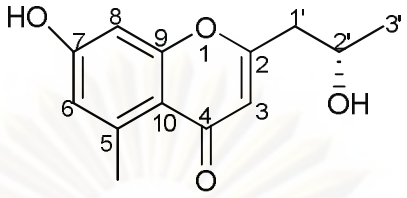



**Figure 4.5** The chemical structure of compound **2**

Compound **2** was obtained as colorless needles and its molecular formula was assigned to be $C_{13}H_{14}O_4$ by 1D NMR and HSQC data.

The $^1$H NMR spectrum of **2** exhibited signals due to a secondary methyl ($\delta_H$ 1.16), an allyl methylene ($\delta_H$ 2.58), a methyl carbinol ($\delta_H$ 4.03), an olefinic ($\delta_H$ 5.97), and two aromatic protons ($\delta_H$ 6.61 and 6.63).

The $^{13}$C NMR signals revealed the presence of secondary methyl group ($\delta_C$ 23.9), a hydroxyl bearing carbon ($\delta_C$ 64.5), a methylene ($\delta_C$ 43.3), a carbonyl ($\delta_C$ 178.7), three oxygenated aromatic carbons ($\delta_C$ 159.7, 161.3, and 165.3), and five aromatic carbons ($\delta_C$ 101.1, 112.0, 115.0, 116.9, and 141.9).

These NMR data suggested that **2** should be a chromone derivative. The COSY spectrum clarified connections from $H_2$-1' to $H_3$-3' of 2-hydroxypropyl unit (**Figure 4.6**). This part was located at C-2 by HMBC correlations from $H_2$-1' to C-2 and C-3. The full assignment for structure of **2** was mainly determined by the observed HMBC correlations as shown in **Figure 4.6**. Comparison of the $^1$H and $^{13}$C NMR data of **2** with those of published in the literature (**Table 4.3**) (Kashiwada, Nanoka and Nishioka, 1984), confirmed that **2** is 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone.

5-carbomethoxymethyl-2-heptyl-7-hydroxychromone, is a previously known metabolite afford from medicinal rhubarb *Rhei Rhizoma* (*Rheum officinale*).

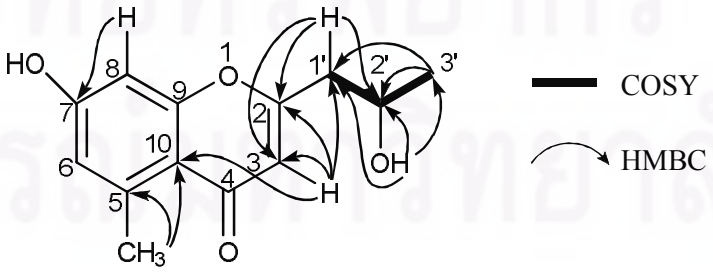



**Figure 4.6** $^1$H-$^1$H COSY and HMBC correlations of compound **2**

**Table 4.3** The $^{1}H$ and $^{13}C$ spectral data of compound **2** and 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone

| | **Compound 2** (DMSO-$d_6$) | | **5-carbomethoxymethyl-2-heptyl-7-hydroxychromone** (DMSO-$d_6$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) |
| 2 | 161.3, qC | - | 164.9, qC | - |
| 3 | 112.0, CH | 5.97, s | 116.4, CH | 5.97, s |
| 4 | 178.7, qC | - | 178.9, qC | - |
| 5 | 141.9, qC | - | 141.5, qC | - |
| 6 | 116.9, qC | 6.61, s | 111.4, qC | 6.61, s |
| 7 | 165.3, qC | - | 160.6, qC | - |
| 8 | 101.1, CH | 6.63, s | 100.4, CH | 6.61, s |
| 9 | 159.7, qC | - | 159.1, qC | - |
| 10 | 115.0, qC | - | 114.4, qC | - |
| 1’ | 43.3, CH | 2.58,dd (6.5, 10.0) | 42.7, CH | 2.58, d (7.0) |
| 2’ | 64.5, CH | 4.03, m | 64.0, CH | 4.02, m |
| 3’ | 23.9, $CH_3$ | 1.16, d (7.5) | 23.2, $CH_3$ | 1.15, d (7.0) |
| 5-Me | 22.9, $CH_3$ | 2.66, s | 22.3, $CH_3$ | 2.66, s |
| 7-OH | - | 10.54, br s | - | 10.51, s |
| 2’-OH | - | 4.85, d (6.5) | - | - |

4.3.3 Compound **3**

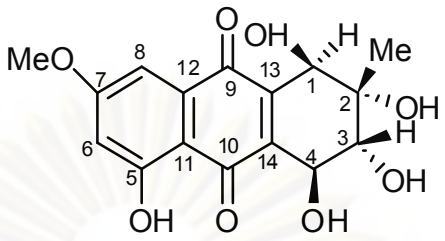



**Figure 4.7** The chemical structure of compound **3**

Compound **3** was a deep red solid and from analysis of 1D NMR and HSQC data could assign its molecular formula as $C_{16}H_{16}O_8$.

The $^1$H NMR spectrum of **3** showed the presence of three hydroxyl ($\delta_H$ 4.10, 4.78, and 12.10), two aromatic ($\delta_H$ 6.63 and 7.00), three oxymethine ($\delta_H$ 3.80, 4.46 and 4.64), a methyl ($\delta_H$ 0.71) and a methoxy ($\delta_H$ 3.87) protons.

The $^{13}$C NMR revealed the presence of two carbonyls ($\delta_C$ 183.7 and 189.9), two oxygenated aromatic carbons (164.5 and 166.4), four oxygenated methines ($\delta_C$ 69.1, 70.0, 73.2 and 73.9) and six aromatic carbons ($\delta_C$ 105.5, 107.4, 109.7, 133.7, 142.6 and 143.5).

The presence of naphthaquinone moiety was suggested by a pair of carbonyls coupled with eight aromatic carbons. Thus, the remaining four oxygenated carbons could be assembled to naphthaquinone portion to afford the gross structure of anthraquinone as shown in **Figure 4.7**. The hydroxyl doublet protons at $\delta_H$ 4.78 and 4.10 were assigned as 1-OH and 2-OH, by their HMBC correlations to C-1 and C-2, respectively. The remaining hydroxyl proton at $\delta_H$ 12.10 was attributable to chelated phenolic OH at C-5.

The structure of **3** was mainly deduced from the analysis of observed HMBC correlations as shown in **Figure 4.8**. By comparing NMR data of **3** with those of in literature (**Table 4.4**) indicated that **3** is altersolanol A (Okamura *et al.*, 1996).

The structure and relative stereochemistry of **3** was confirmed by the X-ray study as shown in **Figure4.9**.

Altersolanol A was reported as antibiotics and phytotoxic activity against some Gram-positive and Gram-negative bacteria with growth inhibitory activities on lettuce and stone-leek seedling, along with cytotoxicity activity on L5178Y (mouse lymphoma cells) (Suemitsu *et al.*, 1984; Aly *et al*., 2008).

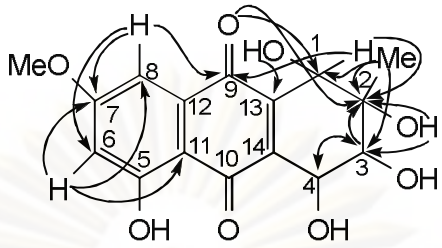



**Figure 4.8** HMBC correlations of compound **3**

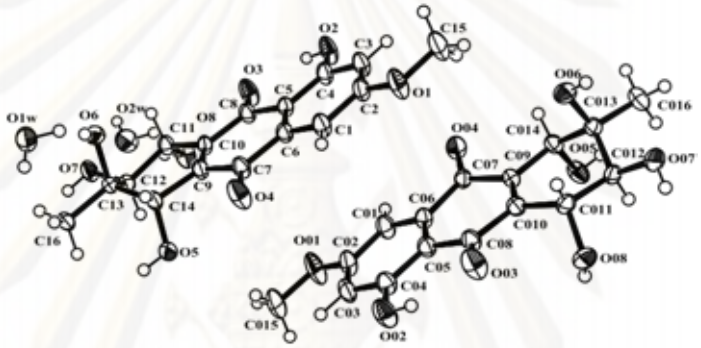


**Figure 4.9** The relative stereochemistry of compound **3**

**Table 4.4** The $^{1}H$ and $^{13}C$ spectral data compare between compound **3** and altersolanol A

| | **Compound 3** (acetone-$d_6$) | | **Altersolanol A** (DMSO-$d_6$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult., $J$ in Hz) | $\delta_C$ | $\delta_H$ (mult., $J$ in Hz) |
| 1 | 69.1, CH | 4.46, d (6.0 ) | 68.3, CH | 4.32, d (5.9) |
| 2 | 73.2, qC | - | 72.7, qC | - |
| 3 | 73.9, CH | 3.80, m | 73.6, CH | 3.64, m |
| 4 | 70.0, CH | 4.64, m | 68.3, CH | 4.48, m |
| 5 | 164.5, qC | - | 165.1, qC | - |
| 6 | 105.5, CH | 6.63, d (2.4) | 105.6, CH | 6.83, d (2.4) |
| 7 | 166.4, qC | - | 162.9, qC | - |
| 8 | 107.4, CH | 7.00, d (2.4) | 106.4, CH | 7.02, d (2.4) |
| 9 | 183.7, qC | - | 183.3, qC | - |
| 10 | 189.9, qC | - | 188.1, qC | - |
| 11 | 109.7, qC | - | 109.2, qC | - |
| 12 | 133.7, qC | - | 132.9, qC | - |
| 13 | 142.6, qC | - | 141.8, qC | - |
| 14 | 143.5, qC | - | 144.2, qC | - |
| MeO | 55.9, $CH_3$ | 3.87, s | 56.1, $CH_3$ | 3.91, s |
| 1-OH | - | 4.78, d (5.6) | - | 5.70, d (5.9) |
| 2-OH | - | 4.10, d (5.6) | - | 4.48, s |
| 2-Me | 21.8, $CH_3$ | 0.71, s | 22.2, $CH_3$ | 1.24, s |
| 3-OH | - | - | - | 4.90, d (6.8) |
| 4-OH | - | - | - | 5.05, d (5.9) |
| 5-OH | - | 12.10, s | - | 12.15, s |

4.3.4 Compound **4**

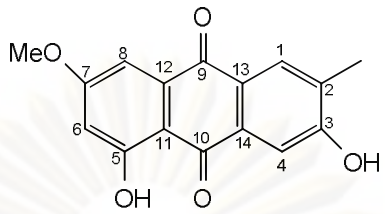



**Figure 4.10** The chemical structure of compound **4**

Compound **4** was obtained as yellow solid (15 mg). By using 1D NMR and HSQC data could assign molecular formula as $C_{16}H_{12}O_5$.

The NMR data of **4** were very similar to those of **3,** except for the presence of two additional aromatic protons ($\delta_H$ 7.49 and 7.90) and the absence of three oxymethine signals at C-1, C-3, and C-4 in **4**. Further, the structure of **4** was confirmed by HMBC correlations as shown in **Figure 4.11**. By comparing the $^{1}H$ and $^{13}C$ NMR data of **4** with those of published in the literatures (**Table 4.5**), indicated that **4** is macrosporin (Suemitsu *et al*., 1989).

Macrosporin has been reported as a typical metabolite produced by several strains of *Alternaria* sp. such as *Alternaria porri*, *A. cucumerina, A. bataticola* and *A. solani* as well as *Stemphylium globuliferum* (Anderson, *et al.,* 2008). There were some reports that it showed cytotoxicity against L5178Y mouse lymphoma cells, but no antibacterial activity toward *Staphylococcus epidermidis, S. aureus, Enterococus faecalis, Escherichia coli* and *Pseudomonas aeruginosa* (MIC > 50 $\mu$g/mL) (Aly *et al.,* 2008).

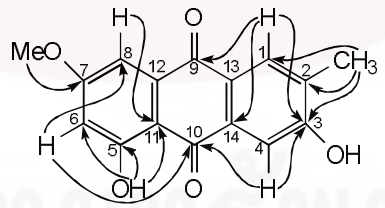



**Figure 4.11** HMBC correlations of compound **4**

**Table 4.5** The $^{1}H$ and $^{13}C$ spectral data compare between compound **4** and macrosporin

| | **Compound 4** (DMSO-$d_6$) | | **macrosporin** ($CDCl_3$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult., *J* in Hz) | $\delta_C$ | $\delta_H$ (mult., *J* in Hz) |
| 1 | 130.5, CH | 7.90, s | 130.8, CH | 7.95, s |
| 2 | 132.7, qC | - | 133.0, qC | - |
| 3 | 163.8, qC | - | 162.5, qC | - |
| 4 | 111.9, CH | 7.49, s | 111.8, CH | 7.65, s |
| 5 | 165.0, qC | - | 165.8, qC | - |
| 6 | 106.0, CH | 6.82, d (3.6) | 106.0, CH | 6.80, d (2.5) |
| 7 | 166.4, qC | - | 167.0, qC | - |
| 8 | 107.4, CH | 7.16, d (3.6) | 107.8, CH | 7.19, d (2.5) |
| 9 | 187.4, qC | - | 187.6, qC | - |
| 10 | 180.5, qC | - | 181.2, qC | - |
| 11 | 110.8, qC | - | 111.1, qC | - |
| 12 | 135.8, qC | - | 136.2, qC | - |
| 13 | 124.2, qC | - | 126.0, qC | - |
| 14 | 133.6, qC | - | 134.1, qC | - |
| 2-Me | 16.7, $CH_3$ | 2.26, s | 16.4, $CH_3$ | 2.34, s |
| 3-OH | - | - | - | - |
| 5-OH | - | - | - | - |
| 7-OMe | 56.7, $CH_3$ | 3.93, s | 56.7, $CH_3$ | 4.00, s |

4.3.5 Compound 5

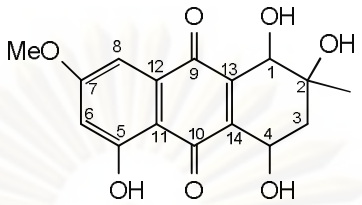



**Figure 4.12** The chemical structure of compound **5**

Compound **5** was obtained as yellow solid and its molecular formulation was $C_{16}H_{16}O_7$, which was assigned from the data of 1D NMR and HSQC.

The $^{1}H$ and $^{13}C$ NMR data of **5** were virtually identical to those of **3** with the difference only being the appearance of a methylene signals at $\delta_H$ 2.34 (dd, J= 14.4, 18.0) and 2.78 (dd, J = 9.0, 19.81) instead of an oxymethine at C-3 in **3**. The structure of **5** was confirmed by using HMBC correlations as shown in **Figure 4.13,** and by comparing the $^{1}H$ and $^{13}C$ NMR data of **5** with those of published in the literatures (**Table 4.6**) (Burns, Gill and Giménez, 1991), indicated that **5** was 1,2,4,5-tetrahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthracene-9,10-dione (Burns, Gill and Giménez, 1989).

In the 1D NOE experiment (Appendix **B** : **Figure B22** ), when the methyl group at $\delta_H$ 1.27 was irradiated, NOE intensities were observed for H-1 ($\delta_H$ 4.32) and OH-4 ($\delta_H$ 3.75) . Therefore, Me-2, H-1 and OH-4 were all β-oriented, and signals at $\delta_H$ 3.75, 4.4 and 5.51 were assigned to H-4α, OH-2α and OH-1α. From these data, structure and relative stereochemistry of **5** possibly were considered as shown in **Figure 4.14**.

1,2,4,5 – tetrahydroxy – 7 – methoxy – 2 – methyl -1,2,3,4-tetrahydroanthracene-9, 10-dione, the first trihydroxylated tetrahydroanthraquinone, was discovered in Basidiomycetes by Burns, *et al*. in 1989. This was the first naturally occurring tricyclic analogues of anthracyclinones of the rhodomycinone – citromycinone group (Burns *et al.,* 1989).

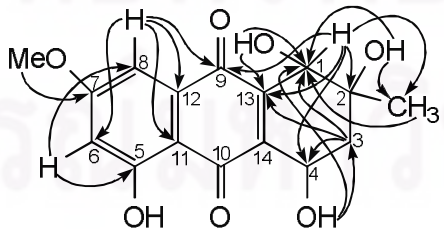



**Figure 4.13** HMBC correlations of compound **5**

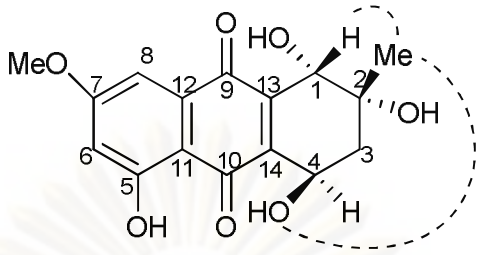



**Figure 4.14** The chemical structure of compound **5** from 1D NOE experiment

**Table 4.6** The $^{1}H$ and $^{13}C$ spectral data compare between compound **5** and 1,2,4,5-tetrahydroxy -7-methoxy-2-methyl-1,2,3,4-tetrahydroanthracene-9,10-dione

| | **Compound 5** (DMSO-$d_6$) | | **1,2,4,5-tetrahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthracene-9,10-dione** ($CDCl_3$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult., *J* in Hz) | $\delta_C$ | $\delta_H$ (mult., *J* in Hz) |
| 1 | 69.71, CH | 4.32, d (9.6) | 61.3, CH | 4.57, d (2.2 ) |
| 2 | 72.4, qC | - | 71.0, qC | - |
| 3 | 29.5, $CH_2$ | 2.34 dd (12.5, 24.0) | 38.2, CH | 2.39 dd (14.6, 2.9) |
| | | 2.78, dd (16.5, 24.0) | | 1.81, ddd (14.6, 4.4, 2.2) |
| 4 | 67.2, CH | 3.75, m | 71.1, CH | 4.9, ddd (8.8, 4.4, 2.9) |
| 5 | 163.8, qC | - | 164.6, qC | - |
| 6 | 106.1, CH | 6.80, d (3.0) | 106.6, CH | 6.67, d (2.2) |
| 7 | 166.1, qC | - | 166.2, qC | - |
| 8 | 107.4, CH | 7.02, d (3.0) | 108.5, CH | 7.18, d (2.2) |
| 9 | 183.4, qC | - | 187.1, qC | - |
| 10 | 188.6, qC | - | 187.4, qC | - |
| 11 | 109.6, qC | - | 109.5, qC | - |
| 12 | 133.9, qC | - | 133.3, qC | - |
| 13 | 144.3, qC | - | 144.2, qC | - |
| 14 | 143.5, qC | - | 140.3, qC | - |
| MeO | 56.8, $CH_3$ | 3.9, s | 56.1, $CH_3$ | 3.92, s |
| 1-OH | - | 5.51, d (10.2) | - | 4.47, d (2.2) |
| 2-OH | - | 4.4, s | - | 3.28, d (2.2) |
| 2-Me | 22.3, $CH_3$ | 1.27, s | 29.7, $CH_3$ | 1.42, s |
| 4-OH | - | 4.7, d (12.0) | - | 4.42, d (8.8) |
| 5-OH | - | - | - | 12.25, s |

4.3.6 Compound **6**

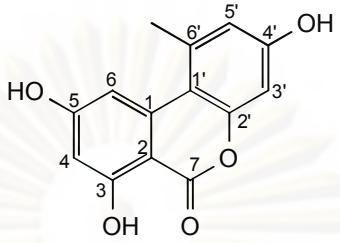



**Figure 4.15** The chemical structure of compound **6**

Compound **6** was obtained as yellowish powder and by analysis of 1D NMR and HSQC data, its molecular formula was assigned as $C_{14}H_{10}O_5$.

The NMR data of **6** were virtually identical to those of **1**, except for the absence of methoxy signals at C-5 in **6**. The structure of **6** was further confirmed by HMBC correlations as shown in **Figure 4.16**, and by comparing both $^{1}H$ and $^{13}C$ NMR data of **6** with the data from literatures as shown in **Table 4.7,** indicated that **6** is alternariol (Tan *et al*., 2008; Freeman, 1965).

Alternariol was reported an acute cytotoxic effects on NIH/3T3 cells, inhibited cell proliferation, exhibited antifungal and phytotoxic activity (Wollenhaupt *et al*., 2008; Magnani, Souza and Filho, 2007; Tiemann *et al*., 2009; Zhao, Ma and Yang, 2009).

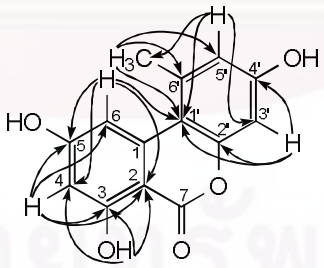



**Figure 4.16** HMBC correlations of compound **6**

**Table 4.7** The $^{1}H$ and $^{13}C$ spectral data of compound **6** and alternariol

| | **Compound 6** (DMSO-$d_6$) | | **Alternariol** (DMSO-$d_6$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) |
| 1 | 138.6, qC | - | 138.6, qC | - |
| 2 | 97.8, qC | - | 97.7, qC | - |
| 3 | 164.5, qC | - | 164.9, qC | - |
| 4 | 100.76, CH | 6.36, s | 100.6, CH | 6.36, d (1.5) |
| 5 | 165.1, qC | - | 165.3, qC | - |
| 6 | 101.5, CH | 7.24, s | 101.3, CH | 7.23, d (1.5) |
| 7 | 165.9, qC | - | 165.5, qC | - |
| 1’ | 109.4, qC | - | 109.5, qC | - |
| 2’ | 153.1, qC | - | 153.1, qC | - |
| 3’ | 104.2, CH | 6.62, s | 104.2, CH | 6.62, d (2.5) |
| 4’ | 158.9, qC | - | 158.3, qC | - |
| 5’ | 117.4, CH | 6.70, s | 117.0, CH | 6.70, d (2.4) |
| 6’ | 138.8, qC | - | 138.4, qC | - |
| 4’-OH | - | - | - | 10.91, s |
| 6’-Me | 25.1, $CH_3$ | 2.69, s | 24.4, $CH_3$ | 2.69, s |
| 5-OH | - | - | - | 10.34, s |
| 3-OH | - | 11.76, s | - | 11.76, s |

4.3.7 Compound **7**

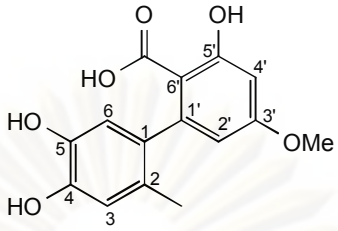



**Figure 4.17** The chemical structure of compound **7**

Compound **7** was obtained as a brown solid. The molecular formula of **7** was assigned as $C_{14}H_{14}O_4$ by analysis of 1D NMR and HSQC data.

The $^1$H NMR spectrum of **7** showed the presence of two hydroxyl groups ($\delta_H$ 8.64 and 8.60), four aromatic protons ($\delta_H$ 6.42, 6.12, 6.53 and 6.41), one methoxy group ($\delta_H$ 3.76) and a methyl group ($\delta_H$ 1.85).

The $^{13}$C NMR spectrum of **7** revealed the presence of a carboxylic carbon at $\delta_C$ 172.0, a methoxy at $\delta_C$ 55.8, four oxygenated aromatic carbons ($\delta_C$ 142.6, 144.4, 162.0 and 162.4), and eight additional aromatic carbons ($\delta_C$ 100.1, 109.3, 109.4, 116.4, 117.1, 125.4, 132.9 and 145.4).

The structure of **7** was further confirmed by HMBC correlations as shown in **Figure 4.18**, along with comparing $^1$H and $^{13}$C NMR data of **7** with data from literatures as shown in **Table 4.8**, indicated that **7** is altenusin (Nakanishi *et al*., 1995).

Altenusin was reported as antifungal penicillide, non-competitive, specific neutral sphingomyelinase (N-SMase) and strong pp60c-Src inhibitor, along with inhibited cFMS receptor tyrosine kinase (CSF-1/m-CSF receptor tyrosine kinase) which was implicated in cancer and bone diseases, and exhibited anti-HIV-1 integrase activity (Rosett *et al*., 1957; Thomas *et al*., 1961; Coombe *et al*., 1970; Nishimura *et al*., 1983; Ayer *et al*., 1990; Singh *et al*., 2003; Cota *et al*., 2008).

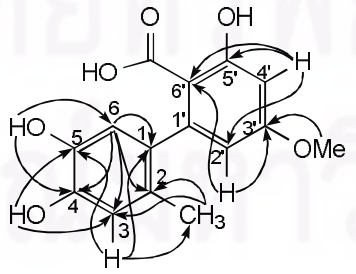



**Figure 4.18** HMBC correlations of compound **7**

**Table 4.8** The $^{1}H$ and $^{13}C$ spectral data of compound **7** and altenusin

| | **Compound 7** (DMSO-$d_6$) | | **Altenusin** (DMSO-$d_6$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) |
| 1 | 132.9, qC | - | 132.4, qC | - |
| 2 | 125.4, qC | - | 124.9, qC | - |
| 3 | 117.1, CH | 6.53 s | 116.6, CH | 6.54 s |
| 4 | 144.4, qC | - | 143.9, qC | - |
| 5 | 142.6, qC | - | 142.1, qC | - |
| 6 | 116.4, CH | 6.41 s | 115.6, CH | 6.42 s |
| 1’ | 145.4, qC | - | 145.0, qC | - |
| 2’ | 109.3, CH | 6.10, d (2.4) | 108.9, CH | 6.1, d (2.7) |
| 3’ | 162.0, qC | - | 162.0, qC | - |
| 4’ | 100.1, CH | 6.42, d (2.4) | 99.6, CH | 6.43, d (2.7) |
| 5’ | 162.4, qC | - | 161.6, qC | - |
| 6’ | 109.4, qC | - | 108.8, qC | - |
| 2-Me | 19.4, $CH_3$ | 1.85 s | 18.8, $CH_3$ | 1.86 s |
| 4-OH | - | 8.64 br s | - | 8.61 br s |
| 5-OH | - | 8.60 br s | - | 8.57 br s |
| 3’-OMe | 55.8, $CH_3$ | 3.76 s | 55.3, $CH_3$ | 3.76 s |
| 6’-COOH | 172.0, qC | - | 171.6, qC | - |

4.3.8 Compound **8**

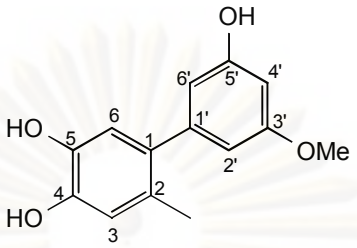



**Figure 4.19** The chemical structure of compound **8**

Compound **8** was as a brown liquid and by using 1D NMR and HSQC data analysis, its molecular formula was assigned as $C_{14}H_{14}O_4$.

The NMR data of **8** were virtually identical to those of **7** except for the absence of a carboxy group at C-6' (appeared in $^{13}C$ NMR of **7** at $\delta_C$ 172.0 on the aromatic carbon at $\delta_C$ 109.4), which was replaced by aromatic carbon at $\delta_C$ 106.2 with proton signal at $\delta_H$ 6.20 in **8**.

The structure of **8** was further confirmed by HMBC correlations as shown in **Figure 4.20**, and indicated that it was 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol, which was firstly reported in 1974 (Kameda, Aoki and Namiki, 1974).

The 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol, was reported as a synthesis compound which was produced by treating dehydroaltenusin with zinc-powder in acetic acid. This is the first report of 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol as fungal metabolites.

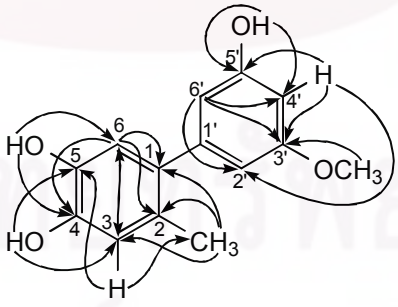



**Figure 4.20** HMBC correlations of compound **8**

**Table 4.9** The $^{1}H$ and $^{13}C$ spectral data of compound **8**

| | **Compound 8** (DMSO-$d_6$) | |
|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult, *J* in Hz) |
| 1 | 132.7, qC | - |
| 2 | 125.3, qC | - |
| 3 | 118.0, CH | 6.61, s |
| 4 | 144.0, qC | - |
| 5 | 143.3, qC | - |
| 6 | 117.1, CH | 6.55, s |
| 1’ | 144.8, qC | - |
| 2’ | 109.3, CH | 6.22, s |
| 3’ | 160.4, qC | - |
| 4’ | 99.7, CH | 6.25, s |
| 5’ | 158.4, qC | - |
| 6’ | 106.2, CH | 6.20, s |
| 2-Me | 19.3, $CH_3$ | 2.05, s |
| 4-OH | - | 8.80, br s |
| 5-OH | - | 8.75, br s |
| 3’-OMe | 55.4, $CH_3$ | 3.70, s |
| 5’-OH | - | 9.43, s |

4.3.9 Compound **9**

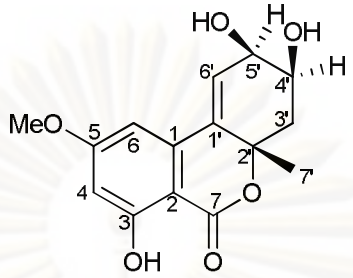



**Figure 4.21** The chemical structure of compound **9**

Compound **9** was obtained as a colorless solid and its molecular formula was assigned as $C_{15}H_{16}O_6$ by analysis of 1D NMR and HSQC data.

The NMR data of **9** was similar to those of **1** except for the appearance of an olefinic proton ($\delta_H$ 6.30), a methylene ($\delta_H$ 1.95, 2.27 ; $\delta_C$ 39.0), two oxymethines ($\delta_H$ 3.70, 3.95; $\delta_C$ 69.3, 70.0) and an additional hydroxyl group ($\delta_H$ 5.30), and the absence of two aromatic protons in **1**. These data strongly suggested that the molecule of **9** differed from that of **1** only at the C ring part.

The C ring was corroborated by $^1$H-$^1$H COSY correlations from $H_2$-3' to H-6', which was connected to B ring at C-1' and C-2 due to the HMBC cross peaks of H-6'/C-1', H-6'/C-2' and $H_2$-3'/C-2' (**Figure 4.22**).

The relative stereochemistry of **9** was determined by 1D NOE experiment (Appendix **B** : **Figure B39** ). When the H-5' at $\delta_H$ 3.95 was irradiated, NOE intensities were observed for two proton at H-4' and H-6'($\delta_H$ 3.70 and 6.30). From these data, it was indicated that H-4' and H-5' were all the same α-face, so Me-7', OH-4' and OH-5' were all the same β-face.

By comparing its data with literatures (**Table 4.10**), and from 1D NOE experiment, compound **9** turned out to be 5'-epialtenuene (**Figure 4.22**) (Bradburn *et al*., 1994). 5'Epialtenuene was first discovered from the extract of *Alternaria alternate* cultured on rice (Bradburn *et al*., 1994). This compound is an isomer of altenuene.

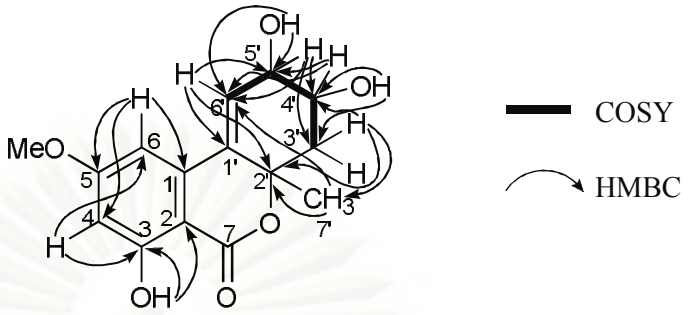



**Figure 4.22** $^{1}$H-$^{1}$H COSY and HMBC correlations of compound **9**

**Table 4.10** The $^{1}$H and $^{13}$C spectral data of compound **9** and 5'-epialtenuene

| | **Compound 9** (DMSO-$d_6$) | | **Altenuene** ($CDCl_3$-DMSO-$d_6$) | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ (mult., *J* in Hz) | $\delta_C$ | $\delta_H$ (mult., *J* in Hz) |
| 1 | 132.3, qC | - | 133.9, qC | - |
| 2 | 101.4, qC | - | 100.9, qC | - |
| 3 | 163.5, qC | - | 166.0, qC | - |
| 4 | 100.5, CH | 6.5, d (2.4) | 100.9, CH | 6.43 d (2.2) |
| 5 | 166.3, qC | - | 163.9, qC | - |
| 6 | 102.8, CH | 6.74, d (2.4) | 102.6, CH | 6.56 d (2.2) |
| 7 | 168.7, qC | - | 168.6, qC | - |
| 1' | 139.7, qC | - | 138.6, qC | - |
| 2' | 81.7, qC | - | 81.1, qC | - |
| 3' | 39.0, $CH_2$ | 2.27 dd (3.2,14.0) | 39.8, $CH_2$ | 2.47, dd (5.4, 13.9) |
| | | 1.95, dd (7.6, 14.0) | | 2.22, d (1.4, 13.9) |
| 4' | 69.3, CH | 3.70, m | 66.9, CH | 4.20, br m (5.4) |
| 5' | 70.0, CH | 3.95, m | 67.3, CH | 4.40, m |
| 6' | 131.5, CH | 6.30, d (3.2) | 128.2, CH | 6.14, d (3.0) |
| 7' | 27.9, $CH_3$ | 1.47, s | 27.8, $CH_3$ | 1.67, s |
| 3-OH | - | 11.30, s | - | - |
| 5-OMe | 56.4, $CH_3$ | 3.86, s | 55.7, $CH_3$ | 3.86, s |
| 4'-OH | - | 5.14 d (3.6) | - | 3.85, d (3.2) |
| 5'-OH | - | 5.30 d (6.0) | - | 4.05, d (6) |

**4.4 Classification of the endophytic fungal isolate 10B**

4.4.1 Conventional method

Endophytic fungus 10B grew rapidly on PDA as greyish cottony colony, as shown in **Figure 4.23**. On water agar with banana leaf pieces it produced acropetal chains of brown multicelled conidia with 3-7 transverse and 1 or 2 longitudinal septa., as shown in **Figure 4.24**. Conidia were obclavate with a slightly swallen tip. These structures corresponded with the *Alternaria* morphology (Ellis, 1971).

4.4.2 Molecular method

Sequencing of the PCR product amplified from chromosomal DNA of isolate 10B resulted in a 509 bp fragment. This comprised partial of the 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial of the 28S sequence of rRNA gene, as shown in **Figure 4.25**. The complete ITS1-5.8S-ITS2 sequences of the 10B fungus was used as the query sequence to search for similar sequences from GenBank. It was found that DNA sequence of the ITS1-5.8S-ITS2 rRNA gene of 10B was perfectly matched (100% homology) with that of *Alternaria* sp. and *Alternaria tenuissima*. This suggests that 10B fungus is *Alternaria* sp.

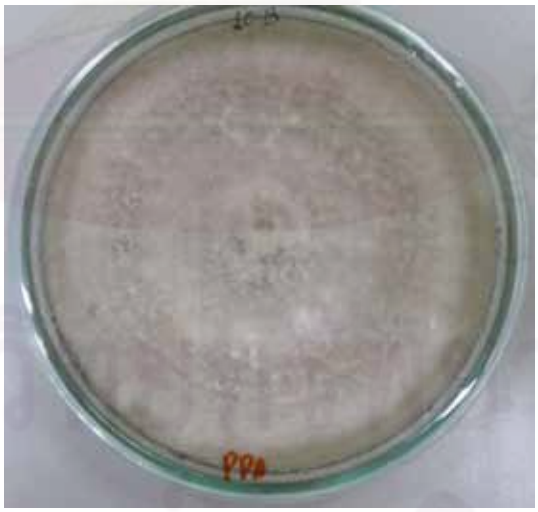


**Figure 4.23** Colony morphology of endophytic fungus 10B grown on PDA for 14 days

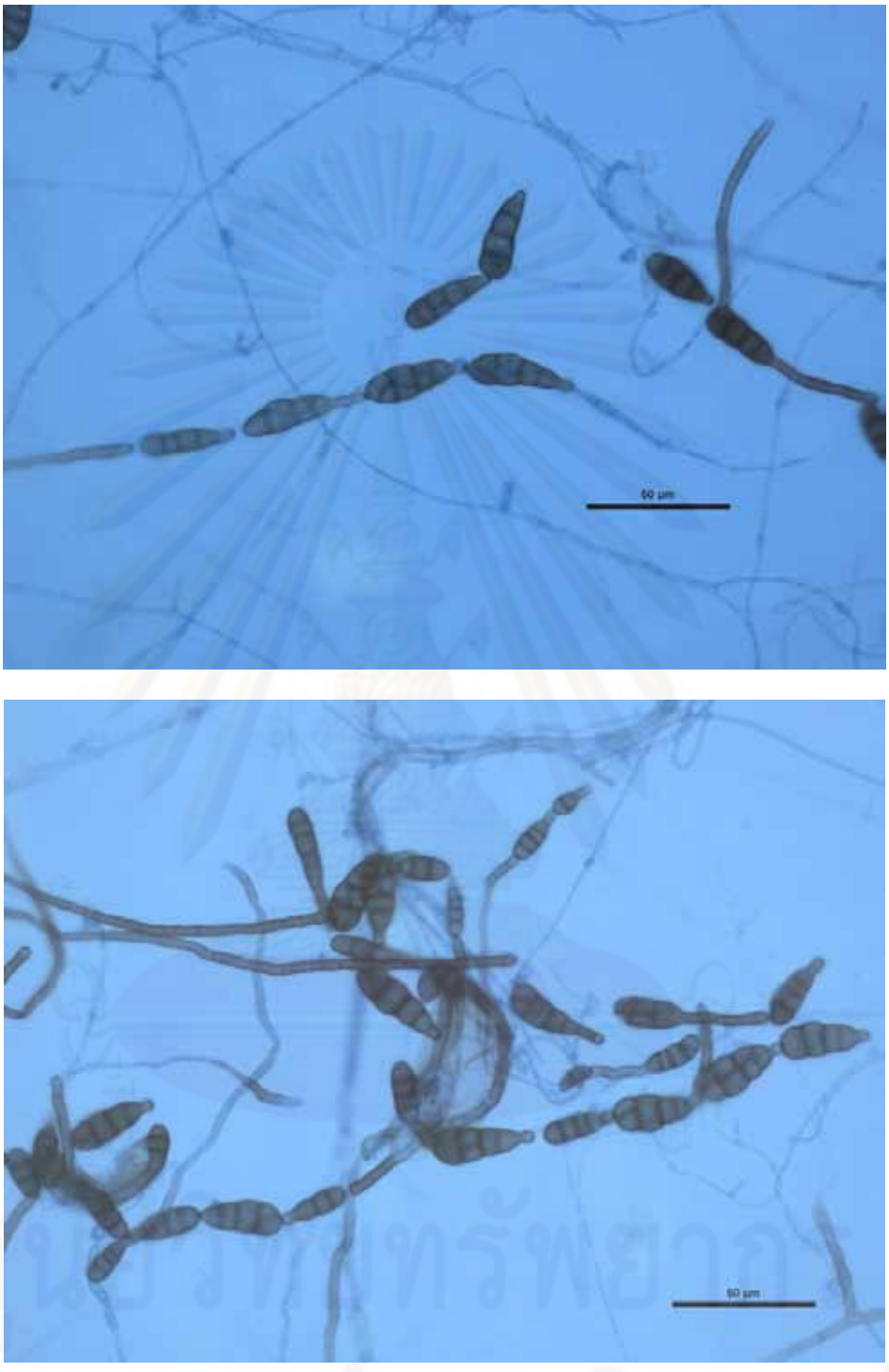


**Figure 4.24** Microscopic morphology of endophytic fungus 10B showing dematiaceous fungus with acropetal chains of brown multicelled conidia with 3-7 transverse and 1 or 2 longitudinal septa.

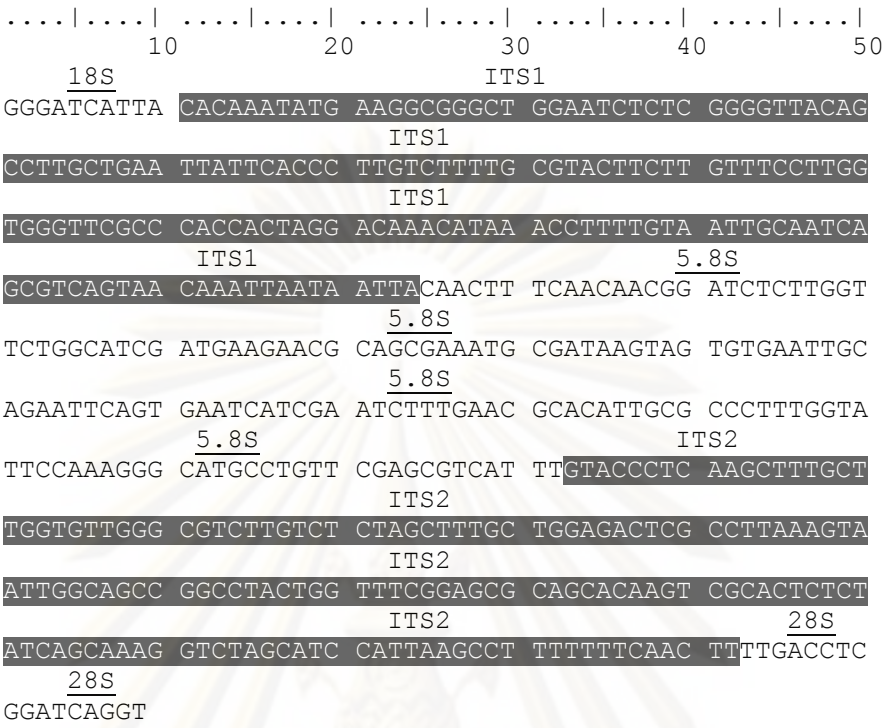



**Figure 4.25** Nucleotide sequences of the partial 18S rRNA gene, complete ITS1-5.8S-ITS2, and partial 28S rRNA gene of the endophytic fungus 10B

## 4.5 Biological activities of isolated compounds

### 4.5.1 Antibacterial activities

#### *4.5.1.1 Screening of antimicrobial activities*

From the primary screening by using dual-culture agar diffusion assay, it was found that 15 isolates exhibited antimicrobial activities, as shown in **Appendix D**. Most of them (11 isolates) showed only anti-*S. aureus*, and isolate PJB B96 could exhibited anti-*E. coli* and isolate PJB B97 presented anti-*E. feacalis*, while 2 isolates of endophytic fungal could exhibited more than one test microorganism. Isolate PJB B92 could inhibited both *S. aureus* and *E. feacalis*, and isolate OIV V65 showed anti-*S.aureus* and anti-*C. albicans*.

Determination of crude extracts obtained from active isolates cultivated in liquid media by using disk diffusion assay revealed that most of active isolates, except CGV L87 and CGV L89, had stable anti-*S. aureus* phenotype. Anti-*E. faecalis* and anti-*E. coli* activities were not detected in crude extracts of PJB B92 and PJB B97, and PJB B96, respectively.

*4.5.1.2 Determination of minimum inhibitory concentration*

A total nine pure compounds (compounds **1-9**) afford from endophytic fungi isolate 10B, were determined for minimum inhibitory concentration (MIC) at the final concentration at 256 µg/mL against 12 bacterial strains: *E. faecalis, E. faecalis* (vancomycin resistant), *E. faecium, S. typhimurium, S. aureus, S. epidermidis, S. hominis, E. coli, K. pneumoniae, K. pneumonia* (ESBL producing), *P. aeruginosa* and *P. vulgaris*. The MICs of active compounds are summarized in **Table 4.11.** All active compounds exhibited against Gram-positive bacteria, while only two active compounds (compound **3** and **7** ) could inhibited Gram-negative bacteria, and none of them showed antibacterial activity against *K. pneumoniae, K. pneumonia* (ESBL producing), and *P. aeruginosa*, respectively.

**Table 4.11** MIC of active compounds

| Compound | EF | VEF | EFa | EC | SA | SE | SH | ST | PV |
|---|---|---|---|---|---|---|---|---|---|
| | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) | MIC (µg/mL) |
| **3** | 128 | 64 | 32 | 128 | I | 32 | 16 | 256 | 32 |
| **5** | 128 | 128 | 128 | I | 64 | 256 | 32 | I | I |
| **6** | I | I | I | I | I | 256 | 256 | I | I |
| **7** | I | I | I | I | I | 128 | I | I | 256 |
| **8** | I | I | I | I | 256 | 256 | 256 | I | I |
| **9** | I | I | I | I | I | 256 | 256 | I | I |

* EF : *E. faecalis*, VEF : *E. faecalis* (vancomycin resistant), EFa : *E. faecium*, EC : *E. coli*, SA : *S. aureus*, SE : *S. epidermidis*, SH : *S. hominis*, ST : *S. typhimurium*, and PV : *P. vulgaris*

** I = inactive at MIC 256 µg/mL

*4.5.1.3 Determinations of antimicrobial combinations assay using checkerboard technique*

The present study was carried out to screen the combination effect of pure compound with antibiotic against clinical isolated bacteria; enterococcus, staphylococcus, and *P. vulgaris*. This combination effect might be used advantageously to improve the efficiency to treat infectious disease. The isobolograms obtained from plotting of checkerboard MIC determinations are summarized in **Appendix E**.

The FICs index of compound **3, 5**, and **7** plus five selected antibiotics against clinical isolates are shown in **Table 4.12.** Compound **7** when combined with ceftriaxone against *P. vulgaris* had significantly reduced their MIC values and exhibited FICs index of 0.09, these result showed synergy effect. Compound **5** when combined with tetracycline against *S. aureus* had significantly reduced their MIC values and exhibited FICs index of 0.12, these result showed synergy effect, while the other combination showed no significant when in combination with FICs index between 0.5 to 4, which mean indifference effect (**Table 4.12**).

**Table 4.12** Summary of the FICs for checkerboard assays of compound **3**, **5**, and **7** against the test organisms

| Test organism | Agent for combination (MIC of individual compound, µg/mL ) | | MIC in combination (µg/mL) | Approximate FICs | Type of interaction |
|---|---|---|---|---|---|
| *E. faecalis* | Compound **3** (128) | Ceftriaxone (512) | 32 : 1024 | 2.25 | Indifference |
| | | Gentamicin (8) | 32 : 16 | 2.25 | Indifference |
| | | Tetracycline (8) | 16 : 8 | 0.62 | Indifference |
| *E. faecalis* (vancomycin resistant) | Compound **3** (64) | Ceftriaxone (1024) | 32 : 2048 | 2.5 | Indifference |
| | | Vancomycin (8) | 64 : 2 | 0.75 | Indifference |
| *E. faecium* | Compound **3** (32) | Ampicillin (64) | 8 : 32 | 0.75 | Indifference |
| | | Vancomycin(512) | 16 : 512 | 1.5 | Indifference |
| *S. epidermidis* | Compound **3** (32) | Ceftriaxone (1) | 8 : 0.5 | 0.75 | Indifference |
| | | Tetracycline (128) | 2 : 64 | 0.56 | Indifference |
| | Compound **7** (128) | Ceftriaxone (1) | 64 : 1 | 1.5 | Indifference |
| | | Tetracycline (128) | 64 : 64 | 1 | Indifference |
| *S. hominis* | Compound **3**(16) | Tetracycline (32) | 8 : 8 | 0.75 | Indifference |
| | Compound **5**(32) | Tetracycline (32) | 8 : 8 | 0.5 | Indifference |
| *S. aureus* | Compound **5** (64) | Ampicillin(0.125) | 16 : 0.06 | 0.75 | Indifference |
| | | Gentamicin(2) | 16 : 1 | 0.75 | Indifference |
| | | Tetracycline(0.125) | 4 : 0.005 | 0.12 | Synergism |
| *P. vulgaris* | Compound **7** (256) | Ampicillin (32) | 128 : 16 | 1 | Indifference |
| | | Ceftriaxone (2) | 16 : 0.06 | 0.09 | Synergism |
| | | Gentamicin (0.5) | 32 : 0.25 | 0.62 | Indifference |
| | | Tetracycline (0.25) | 128 : 0.12 | 1.0 | Indifference |
| | | Vancomycin (1024) | 32 : 512 | 0.62 | Indifference |

* MIC presented as Geomean of 2 observations

# CHAPTER V

## CONCLUSION

The objectives of this research are to (i) investigate antibacterial activities from endophytic fungi isolated from *Casearia grewiaefolia* Vent., *Picrasma javanica* Bl., *Oroxylum indicum* (L.) Vent.by dual-cuture agar diffusion assay and disk diffusion assay against *E. faecalis, S. aureus*, *E. coli, P. aeruginosa* and *C. albicans*, (ii) investigate pure compounds from endophytic fungi isolated 10B from *Erythrina variegate* Linn by broth microdilution assay and checkerboard assay against *E. faecalis, E. faecalis* (vancomycin resistant), *E. faecium, S. typhimurium, S. aureus*, *S. epidermidis, S. hominis, E. coli, K. pneumoniae*, *K. pneumoniae* (ESBL producing), *P. aeruginosa* and *P. vulgaris*, (iii) characterize the bioactive compounds; and (iv) identify the fungus.

In the present investigation, 15 pure isolates of endophytic fungi were isolated from *Casearia grewiaefolia* Vent., 15 isolates from *Oroxylum indicum* (L.) Vent. and 10 isolates from *Picrasma javanica* Bl. Each endophytic fungus was grown on different mycological media and tested for antimicrobial activities, fifteen isolates were found to have antimicrobial activities. Most of them presented anti *S. aureus* with inhibition zone between 10-25 mm. $^{1}$H NMR spectrum of the crude extracts from active isolates exhibited no interesting spectrum. The crude extract of endophytic fungus 10B isolated from *Erythrina variegata* Linn showed antibacterial activity and interesting $^{1}$H NMR spectrum. Large scale cultivation crude extract from 10B was isolated to afford pure compounds.

A total nine known pure compound ; alternariol monomethylether (**1**), 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**), altersolanol A (**3**), macrosporin (**4**), 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**), alternariol (**6**), altenusin (**7**), 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol (**8**), and 5'-epialtenuene (**9**) were collected. Most of them inhibited at least one microorganisms; both compound **3** and **5** showed broad spectrum activity against Gram-positive and Gram-negative bacteria at MIC between 16 -256 µg/mL; compound **6**, **7**, **8** and **9** showed weak antibacterial activity at MIC of 128- 256 µg/mL. Compound **2** showed no antibacterial and anti-cancer activities.

Compound **3**, **5** and **7** were further test for combination assay, compound **5** combined with tetracycline and compound **7** combined with ceftriaxone showed synergistic interaction

against *S. aureus* and against *P. vulgaris* with FIC index of 0.12 and 0.09, respectively. Time kill assay will be further conducted to determine bactericidal activity of synergistic action.

Identification of fungus 10B by conventional and molecular methods indicated that this fungus was *Alternaria* sp.

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# APPENDICES

# APPENDIX A

## 1. Media

### 1.1 Yeast extract sucrose Agar (YEA)

| | |
|---|---|
| Yeast extract | 20 mL |
| Sucrose | 150 g |
| Agar | 15 g |
| Distilled water up to | 1 L |

### 1.2 Malt extract Agar (MEA)

| | |
|---|---|
| Malt extract | 20 g |
| Peptone | 1 g |
| Agar | 15 g |
| Distilled water up to | 1 L |

### 1.3 Malt Czapek Agar (MCzA)

| | |
|---|---|
| Czapek stock solution A | 50 mL |
| Czapek stock solution B | 50 mL |
| Zinc solution | 1 mL |
| Copper solution | 1 mL |
| Sucrose | 30 g |
| Malt Extract | 40 g |
| Agar | 15 g |

| | |
|---|---|
| Distilled water up to | 1 L |

**1.4 Czapek Yeast Autolysate (CzYA)**

| | |
|---|---|
| Czapek stock solution A | 50 mL |
| Czapek stock solution B | 50 mL |
| Zinc solution | 1 mL |
| Copper solution | 1 mL |
| Yeast Extract | 20 g |
| Agar | 15 g |
| Distilled water up to | 1 L |

**Czapek stock solution A**

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

**Czapek stock solution B**

| | |
|---|---|
| $K_2HPO_4$ | 2.0 g |
| Dissolved in distilled water up to | 100 mL |

**Zinc solution**

| | |
|---|---|
| $ZnSO_4.7H_2O$ | 1.0 g |

| | |
|---|---|
| Dissolved in distilled water up to | 100 mL |

**Copper solution**

| | |
|---|---|
| $CuSO_4.5H_2O$ | 0.5 g |
| Dissolved in distilled water up to | 100 mL |

**1.5 Potato Dextrose Agar (PDA)**

| | |
|---|---|
| Potato dextrose broth | 240 g |
| Agar | 15g |
| Distilled water up to | 1 L |

**1.6 Sabouraud's Dextrose Agar (SDA)**

| | |
|---|---|
| Sabouraud's dextrose broth | 30 g |
| Agar | 15 g |
| Distilled water up to | 1 L |

**1.7 Mueller Hinton Agar (MHA)**

| | |
|---|---|
| Mueller hinton broth | 21 g |
| Agar | 15 g |
| Distilled water up to | 1 L |

**1.8 Water Agar**

| | |
|---|---|
| Agar | 15 g |

| | |
|---|---|
| Distilled water up to | 1 L |

**2. Reagent and buffer for DNA amplification by PCR**

**2.1 TE for resuspending pellet**

| | |
|---|---|
| Tris-HCl | 10 mM |
| EDTA | 0.1 mM |

**2.2 Gel loading buffer**

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

**2.3 5-X Tris-Borate-EDTA (TBE)**

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

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

**2.4 2mM dNTP (dATP, dCTP, dGTP, dTTP mix)**

| | |
|---|---|
| dATP | 100 mM |
| dCTP | 100 mM |
| dGTP | 100 mM |
| dTTP | 100 mM |

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

# APPENDIX B

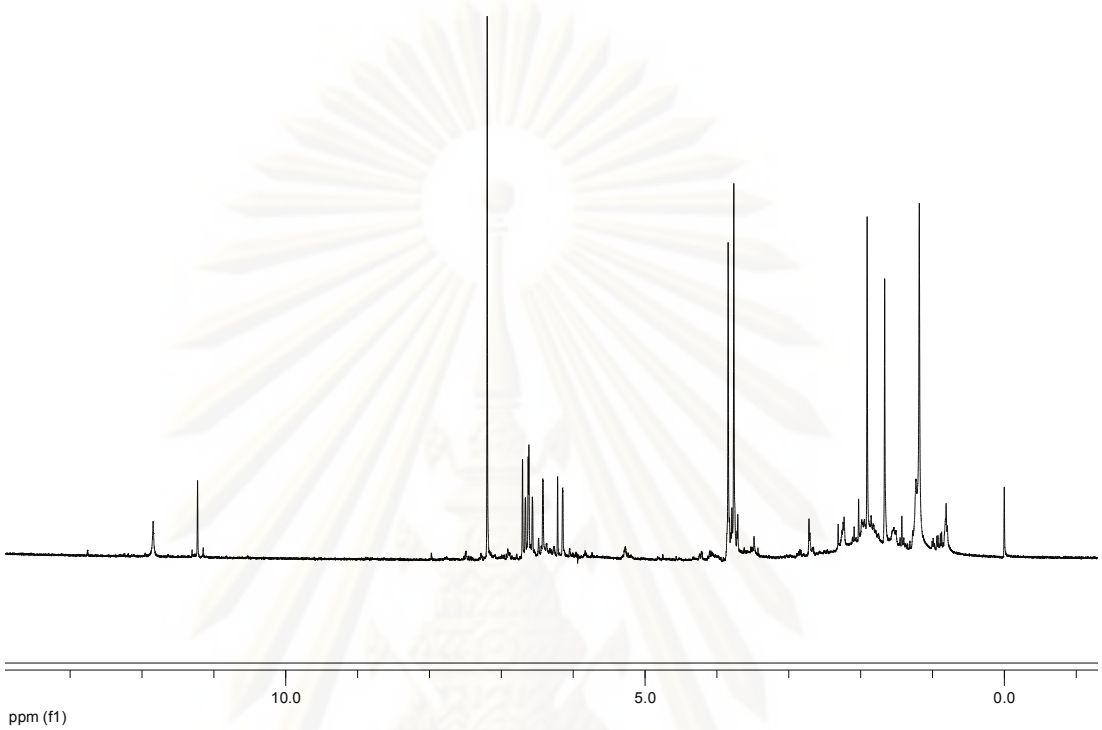



**Figure B1** 400 MHz $^{1}$H NMR ($CDCl_3$) spectrum of 10B crude EtOAc extract

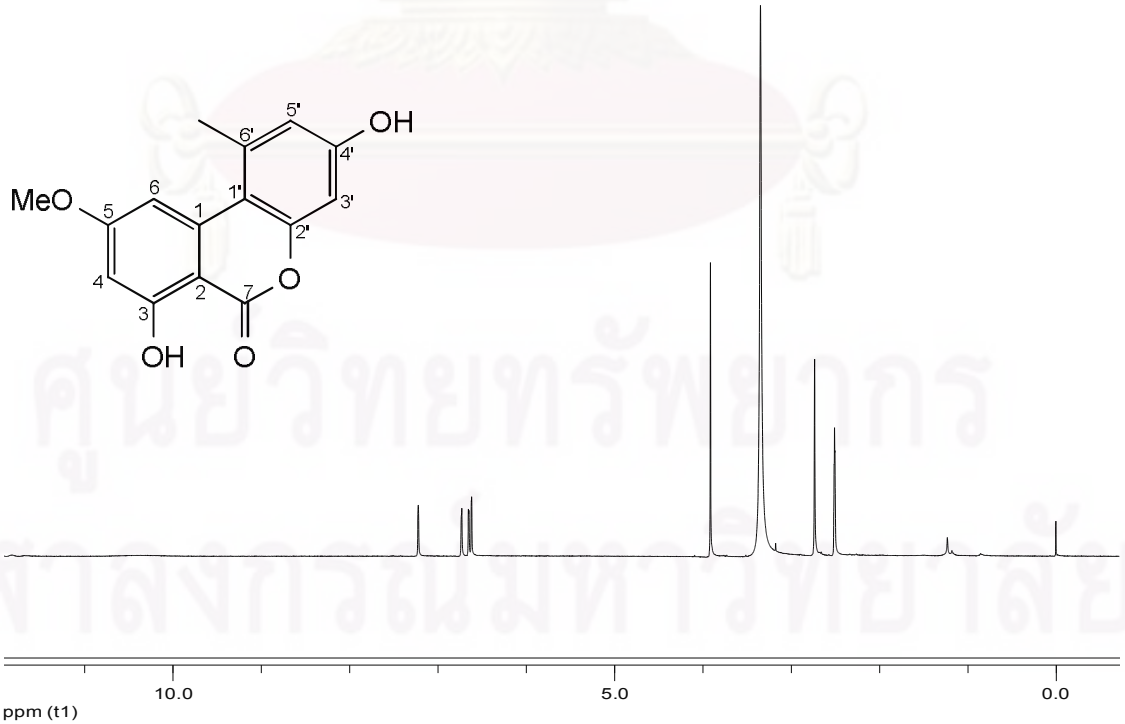



**Figure B2** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of alternariol monomethyl ether(**1**)

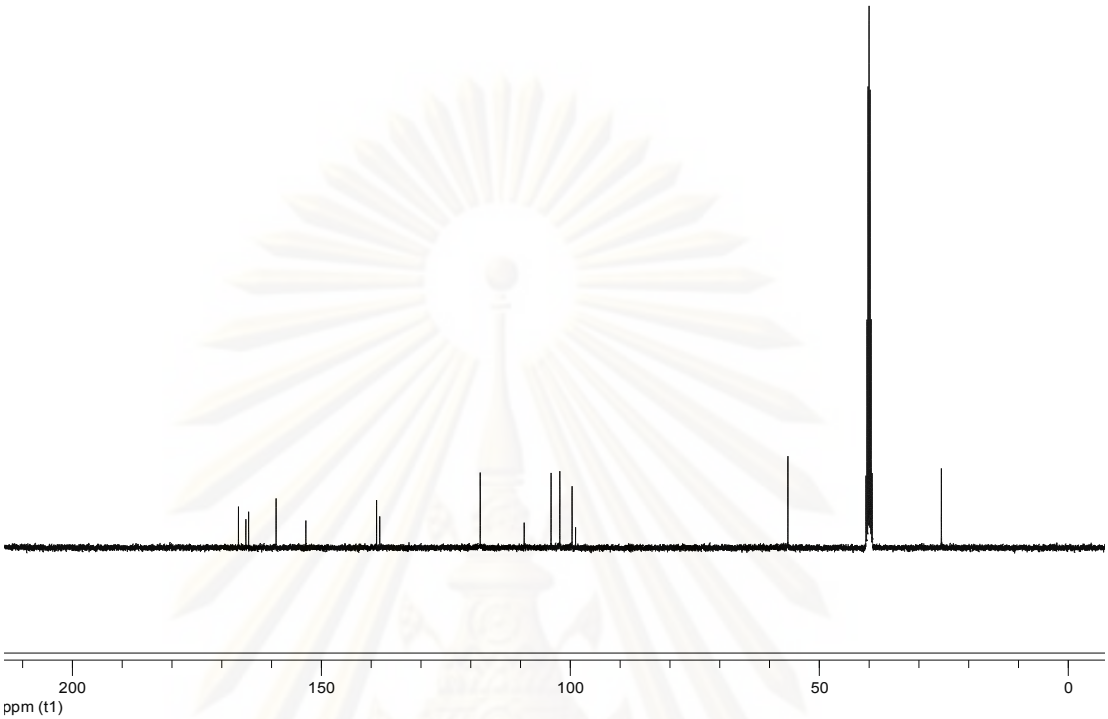



**Figure B3** 600 MHz $^{13}$C NMR (DMSO-$d_6$) spectrum of alternariol monomethyl ether (**1**)

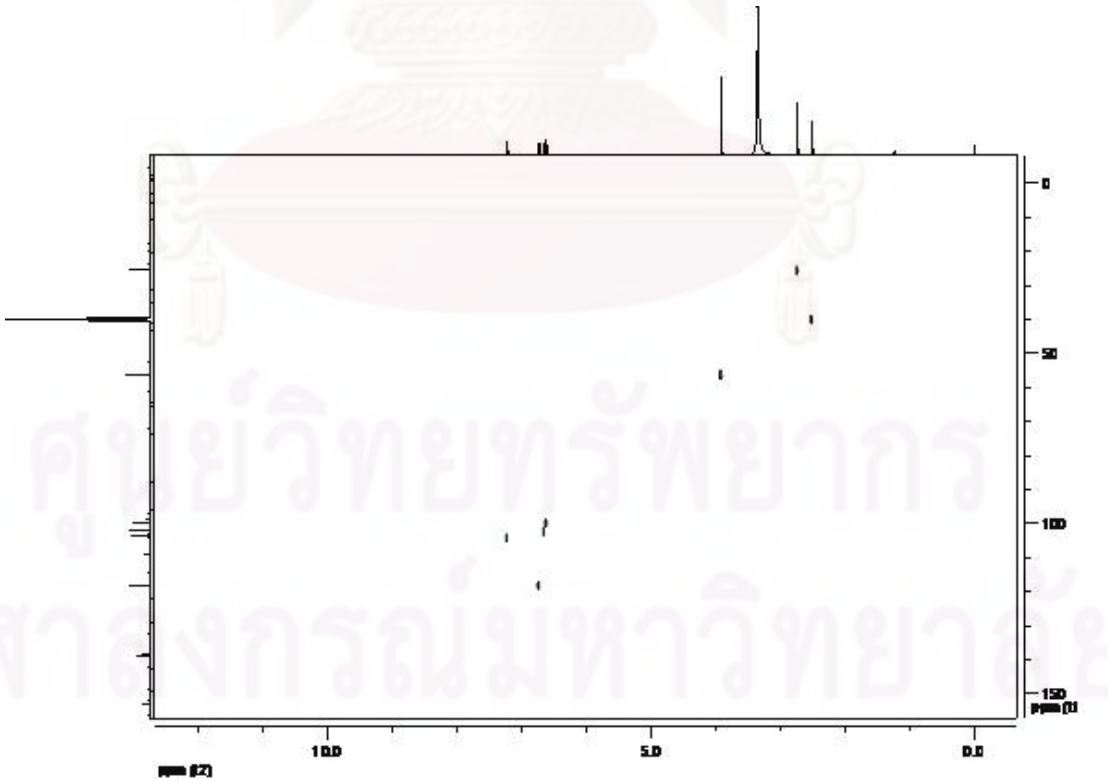



**Figure B4** HSQC of alternariol monomethyl ether (**1**)

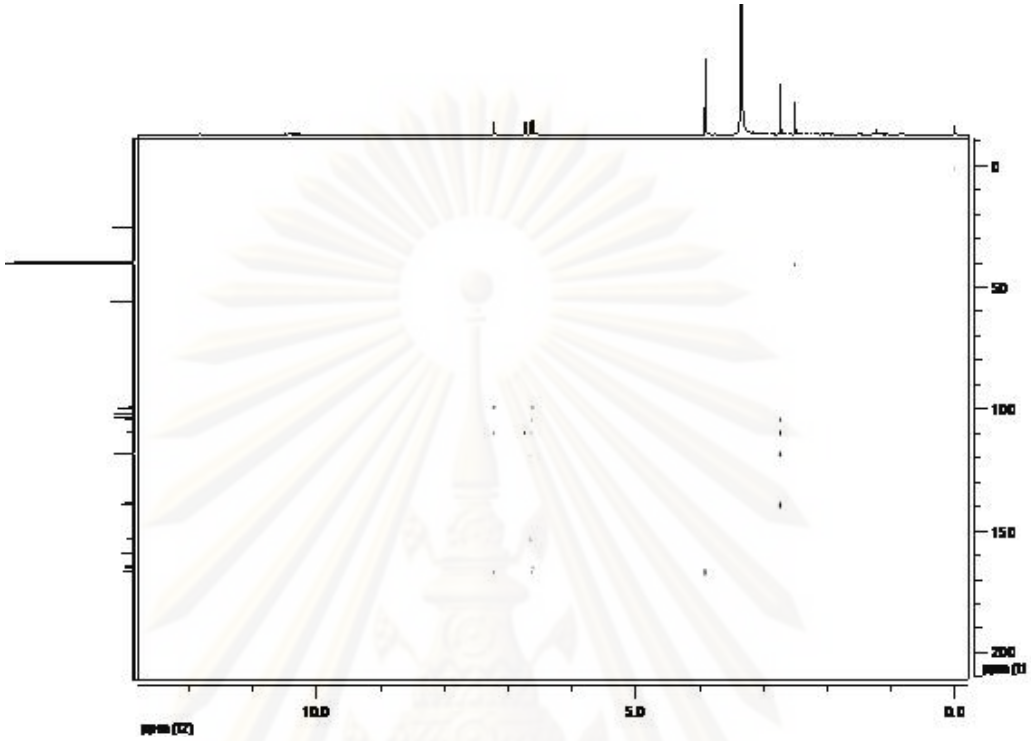



**Figure B5** HMBC of alternariol monomethyl ether (**1**)

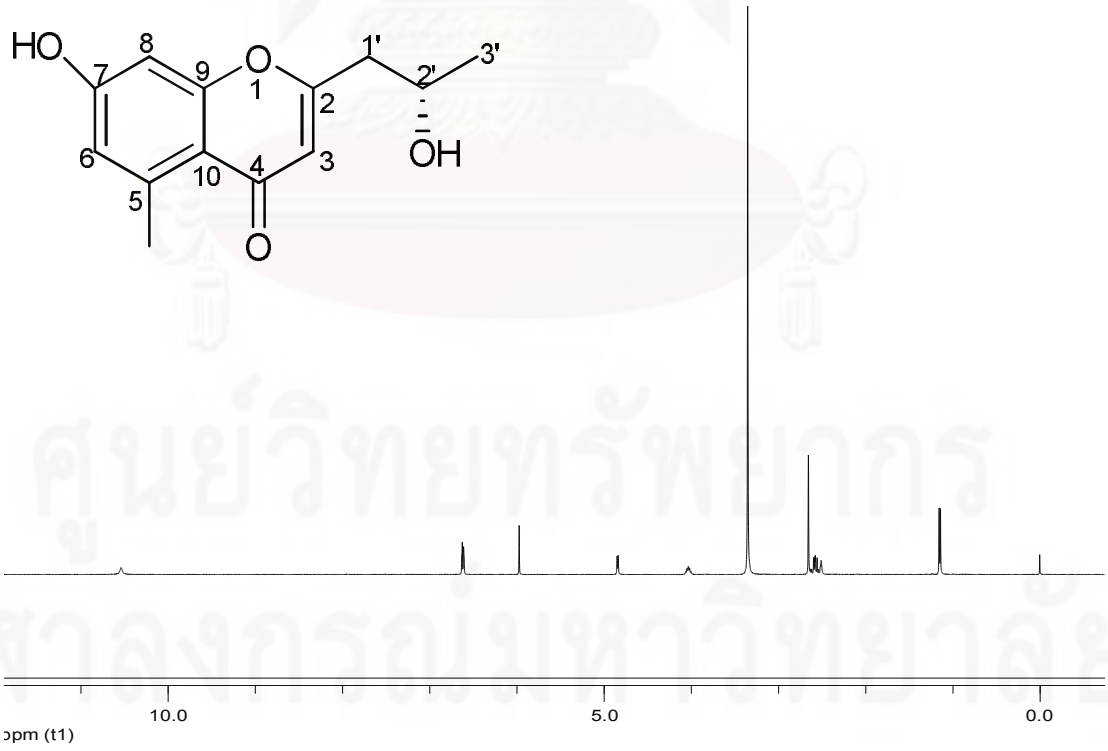



**Figure B6** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**)

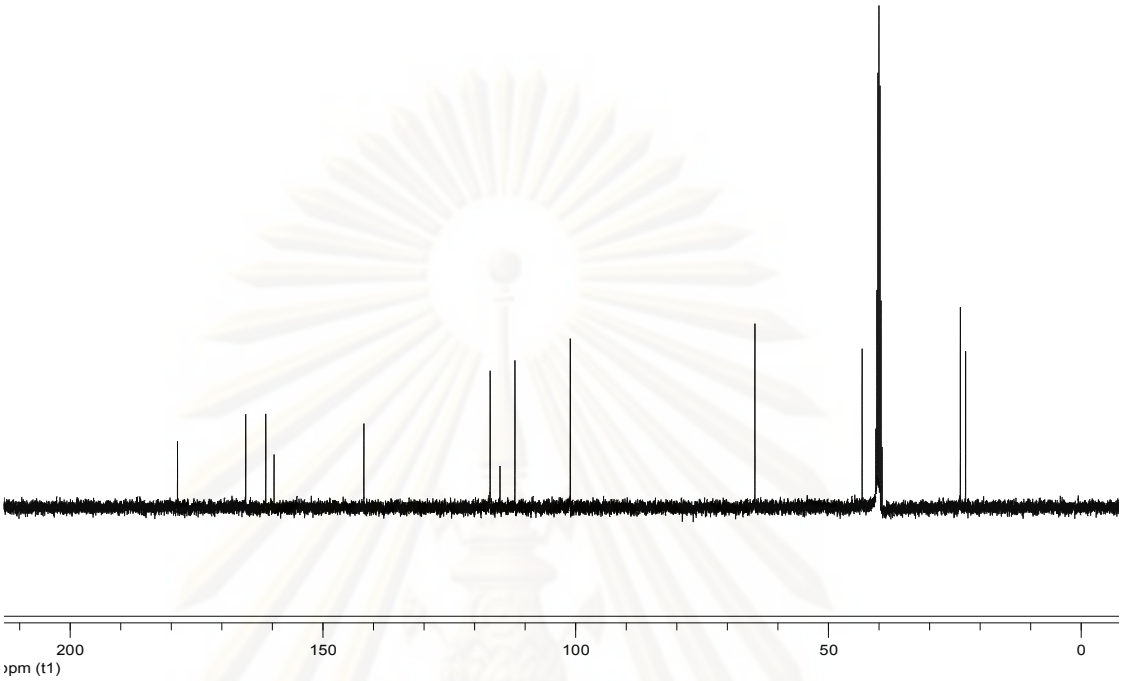



**Figure B7** 600 MHz $^{13}$C NMR (DMSO-$d_6$) spectrum of 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**)

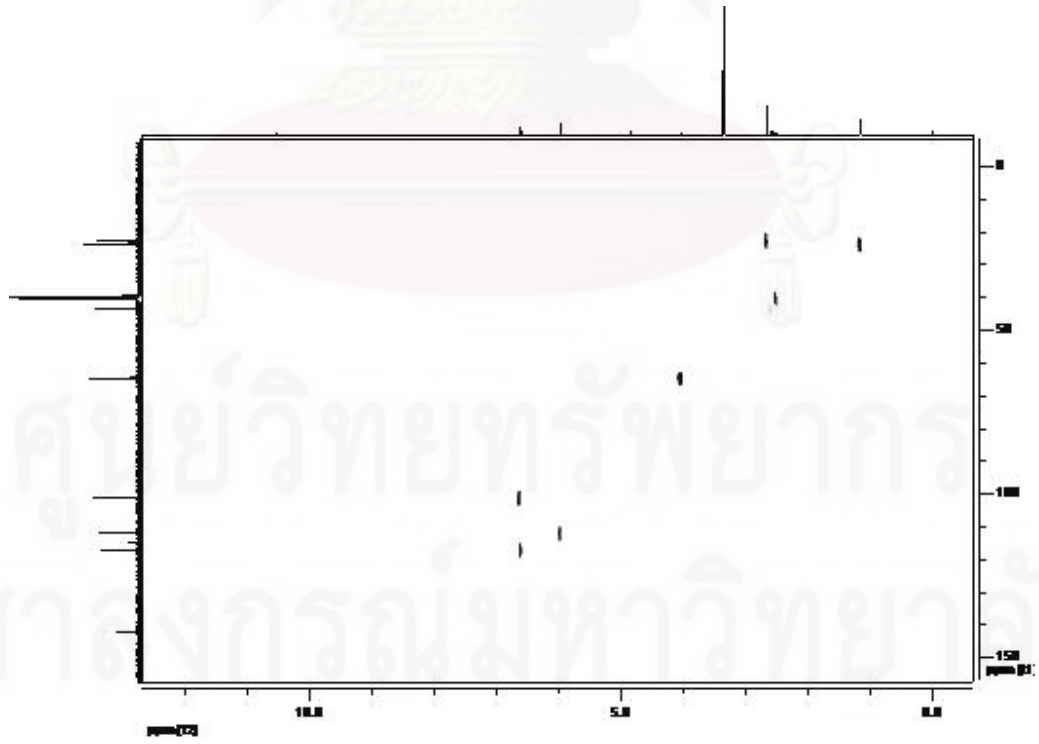


**Figure B8** HSQC of 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**)

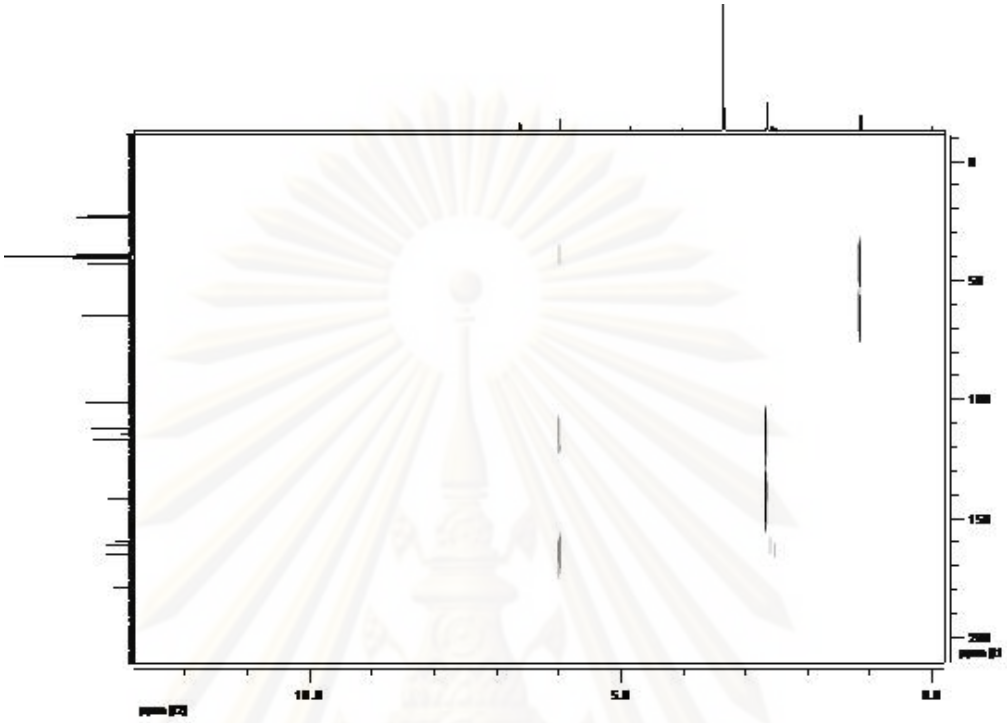


**Figure B9** HMBC of 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**2**)

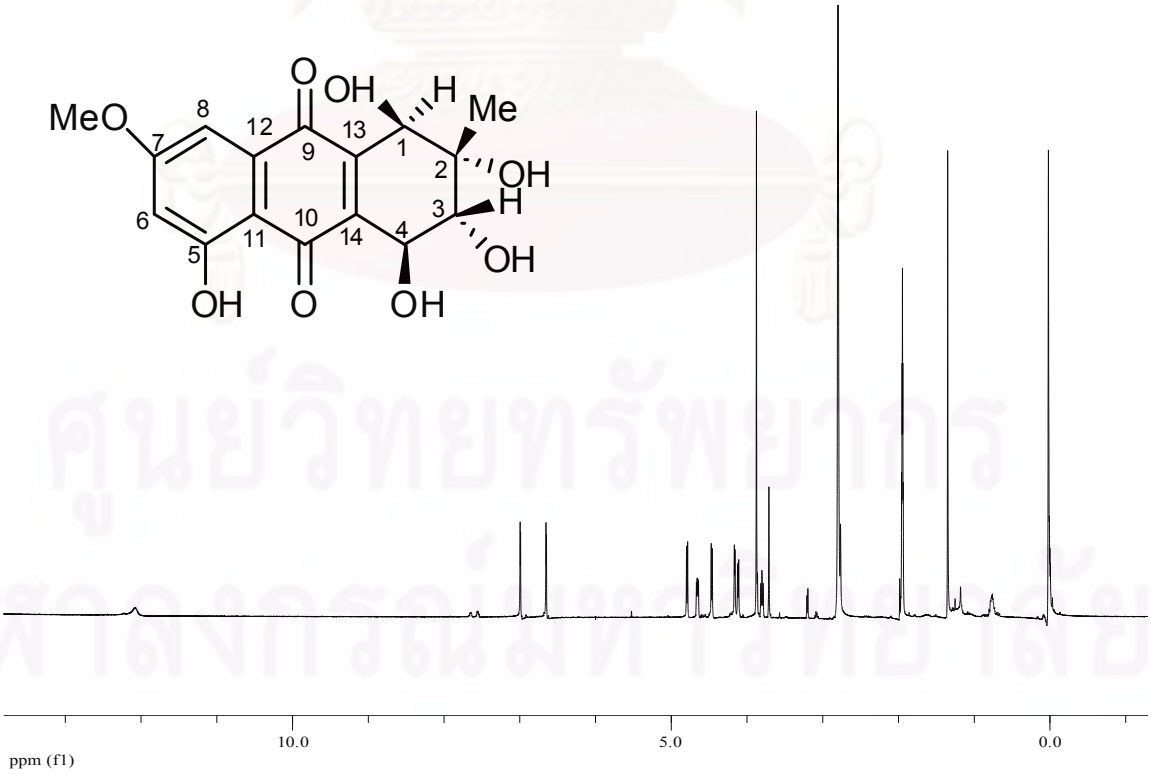



**Figure B10** 400 MHz $^{1}$H NMR (acetone-$d_6$) spectrum of altersolanol A (**3**)

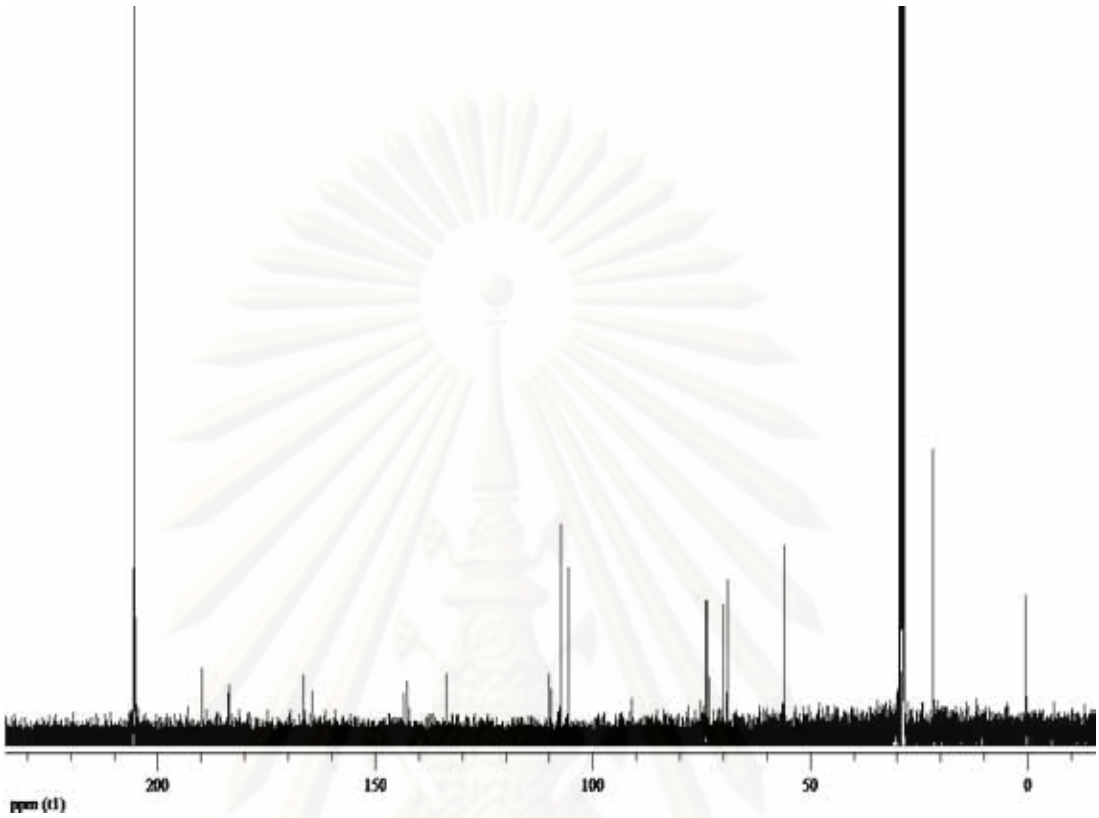



**Figure B11** 400 MHz $^{13}C$ NMR (acetone-$d_6$) spectrum of altersolanol A (**3**)

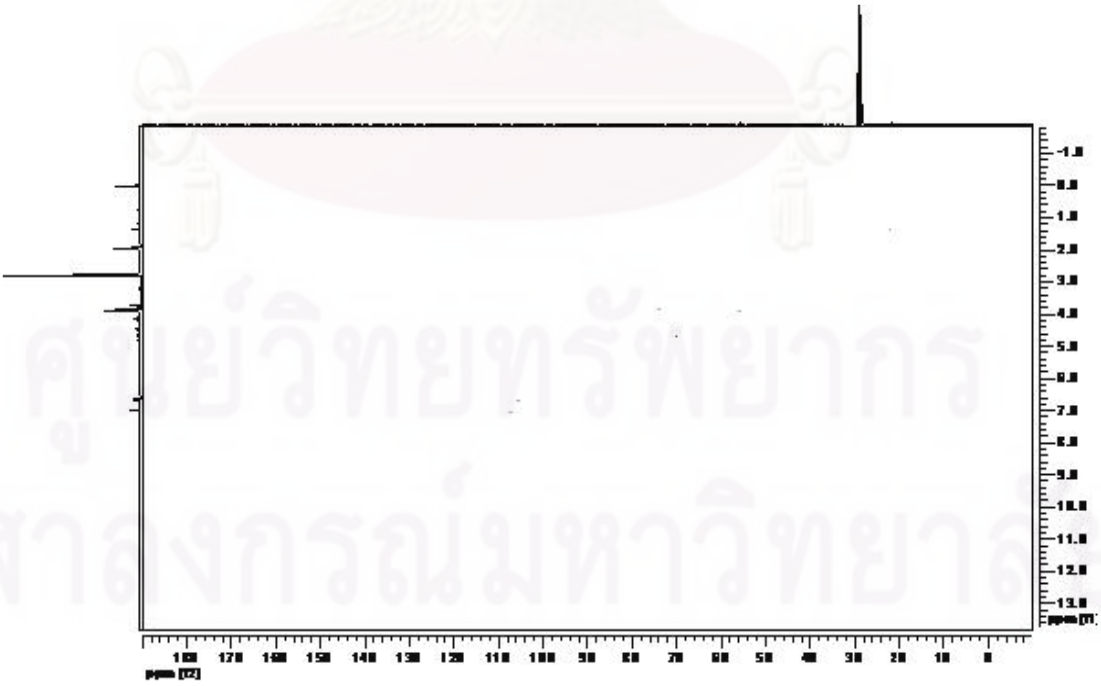


**Figure B12** HSQC of altersolanol A (**3**)

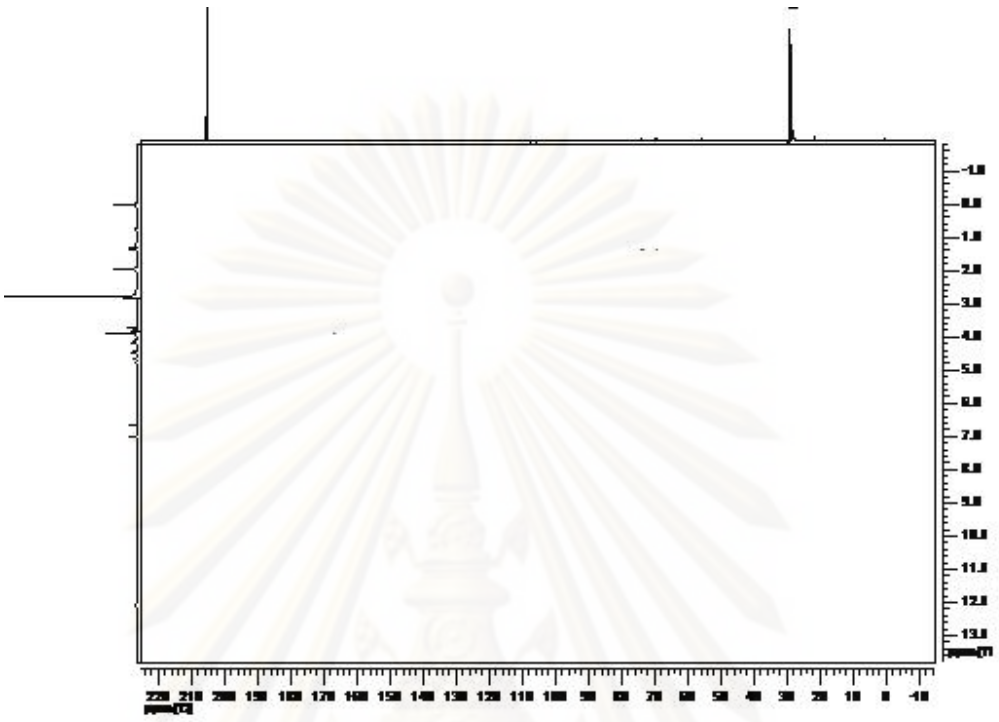


**Figure B13** HMBC of altersolanol A (**3**)

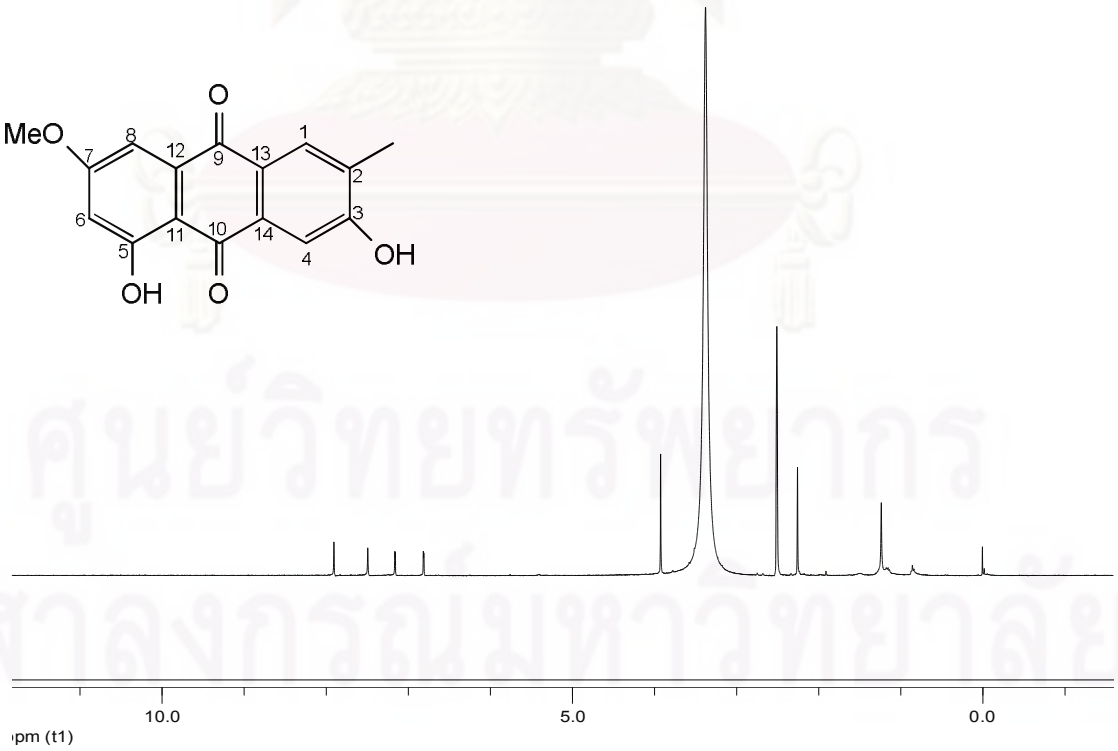



**Figure B14** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of macrosporin (**4**)

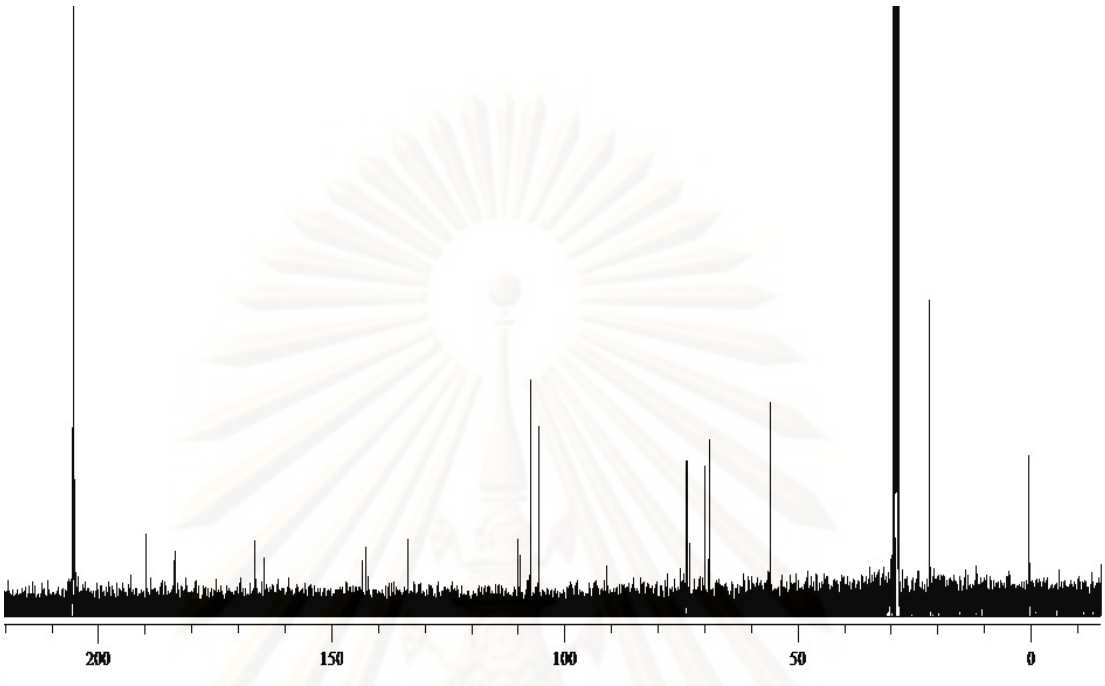



**Figure B15** 600 MHz $^{13}C$ NMR (DMSO-$d_6$) spectrum of macrosporin (**4**)

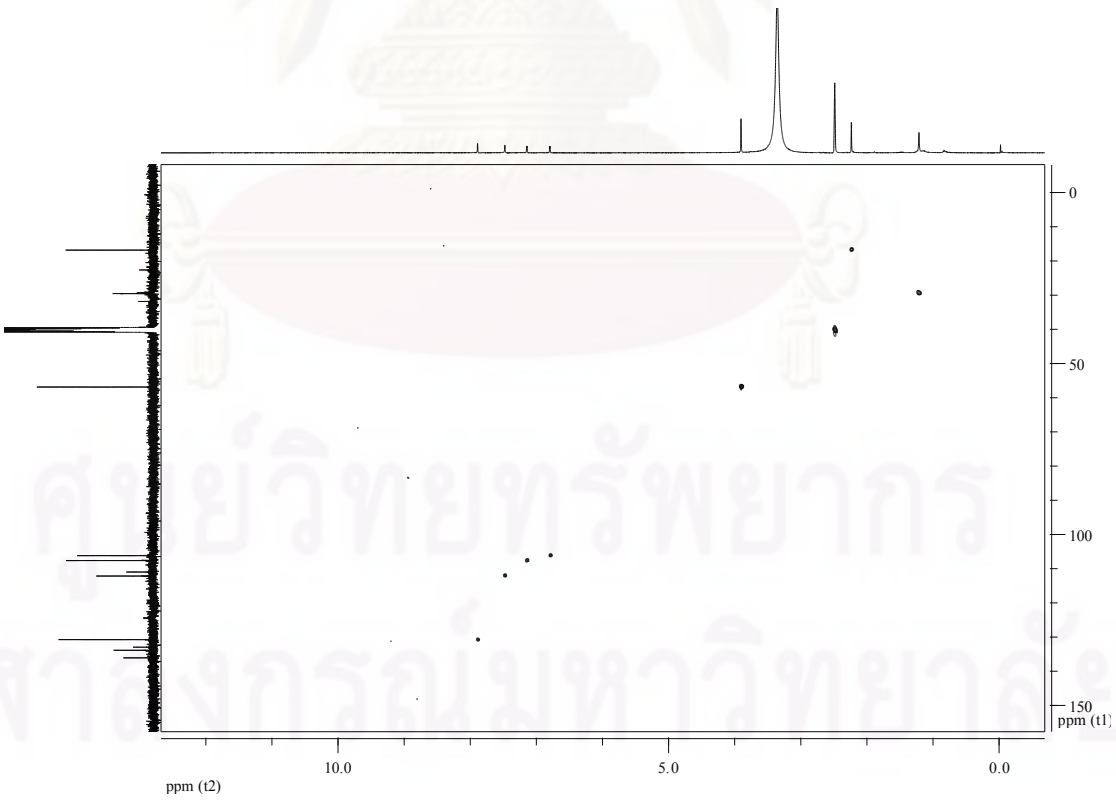



**Figure B16** HSQC of macrosporin (**4**)

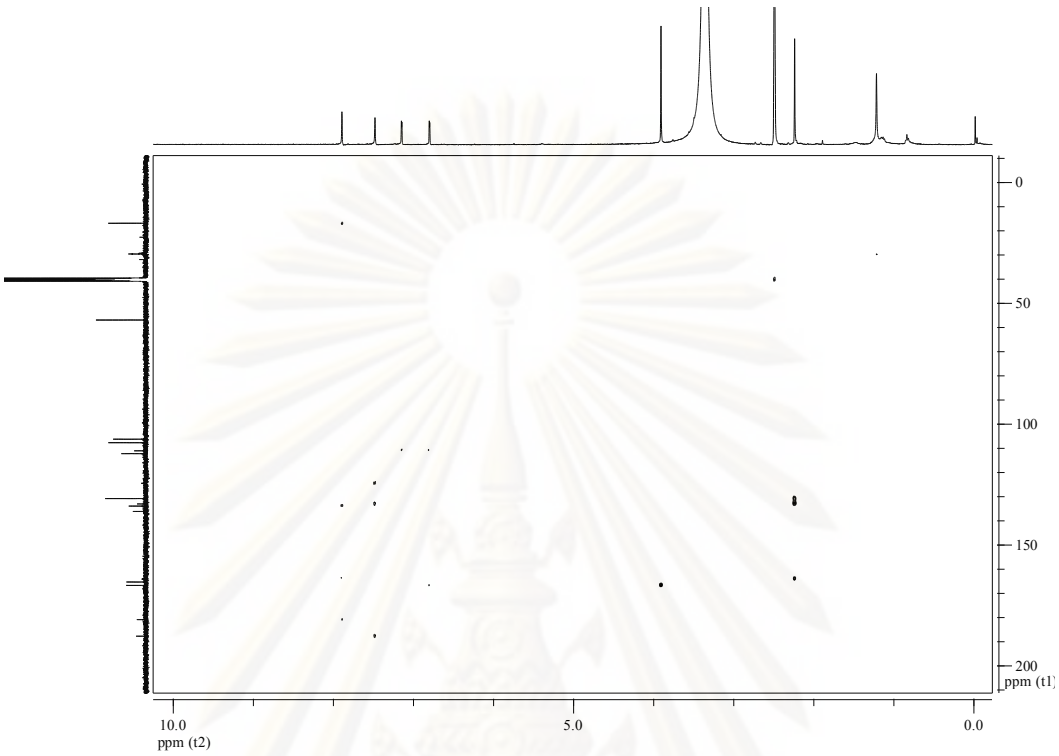



**Figure B17** HMBC of macrosporin (**4**)

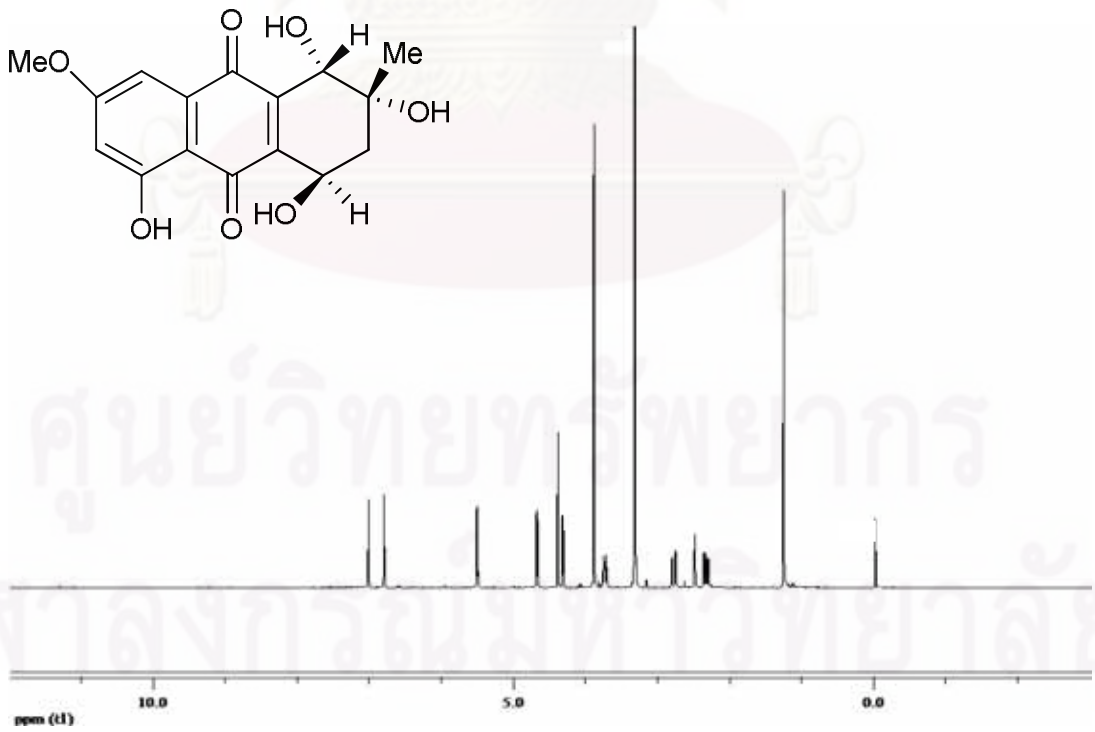



**Figure B18** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**)

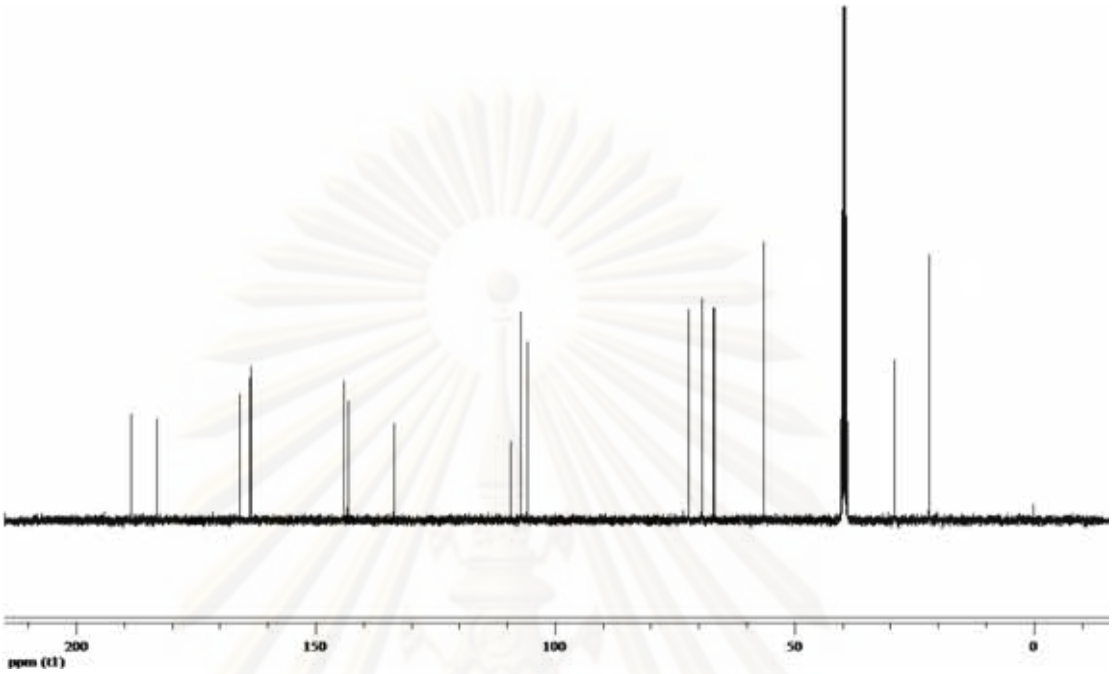



**Figure B19** 600 MHz $^{13}C$ NMR (DMSO-$d_6$) spectrum of 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**)

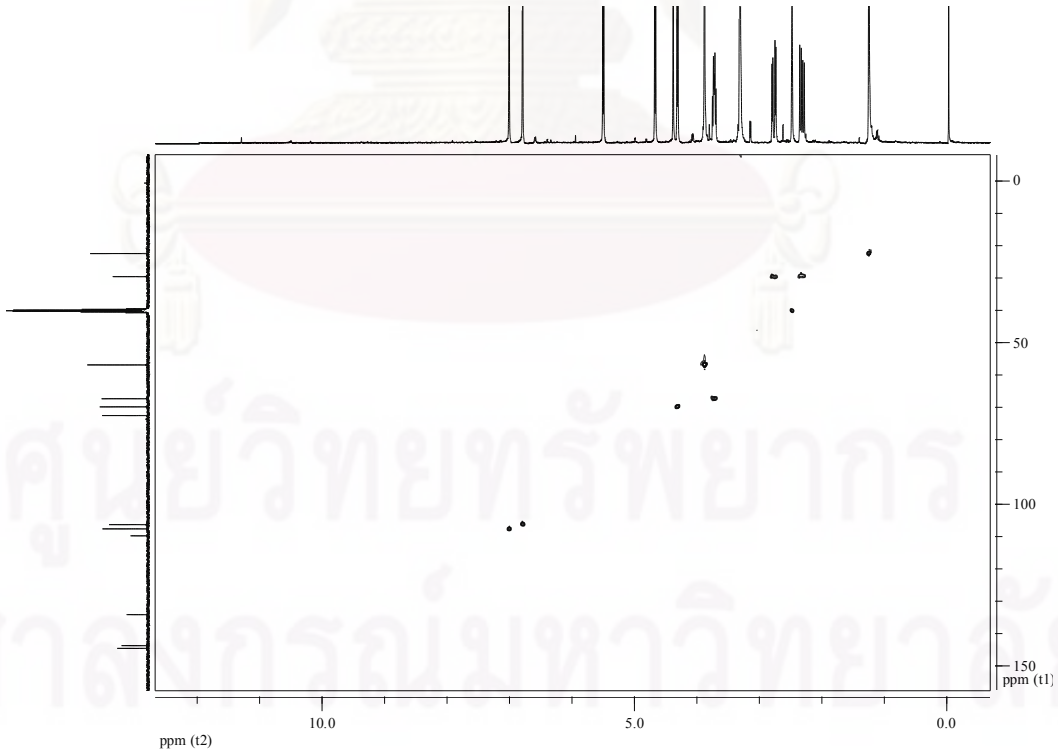



**Figure B20** HSQC of 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**)

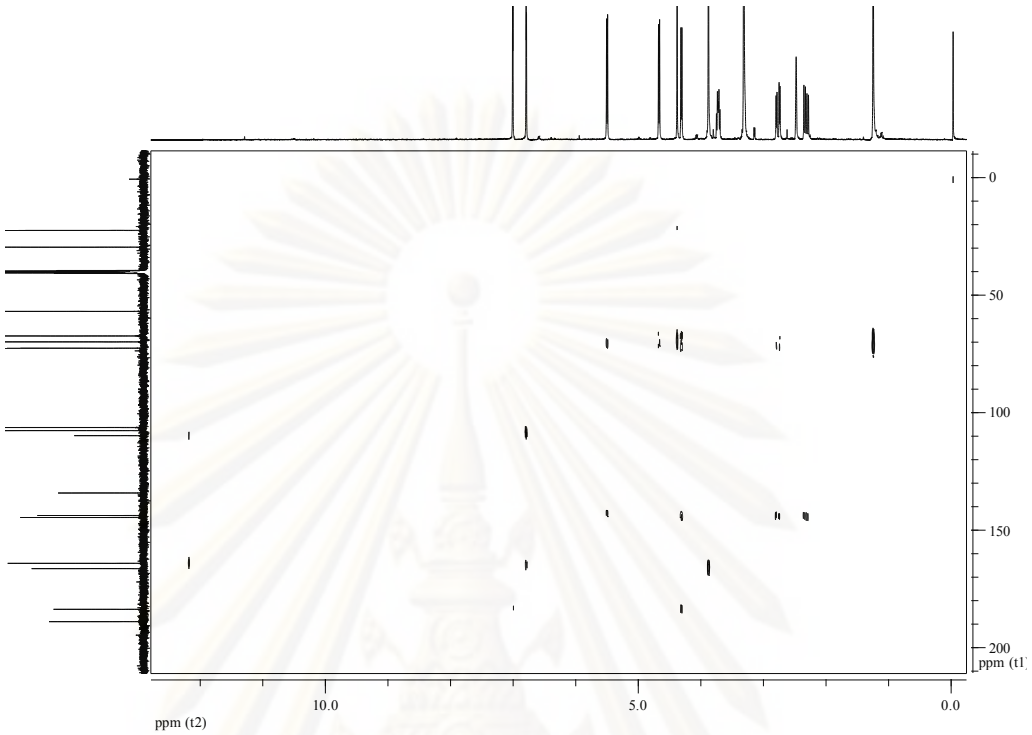



**Figure B21** HMBC of 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**)

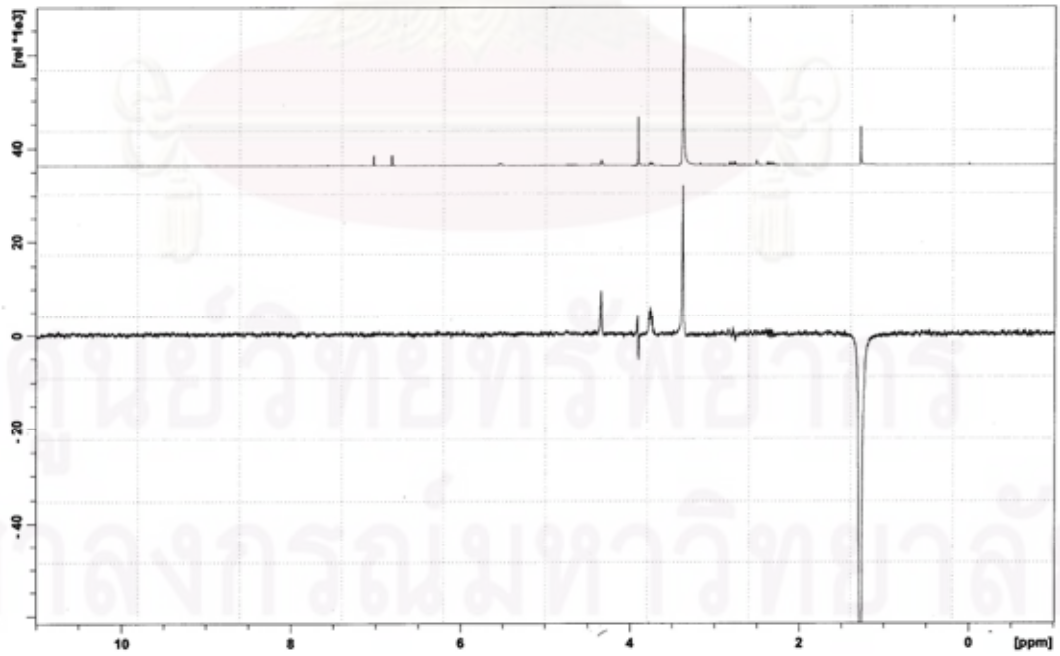



**Figure B22** 1D NOE experiment of 1, 2, 4, 5-tetrahydroxy-7-methoxy-2-methyl-1, 2, 3, 4-tetrahydroanthracene-9, 10-dione (**5**)

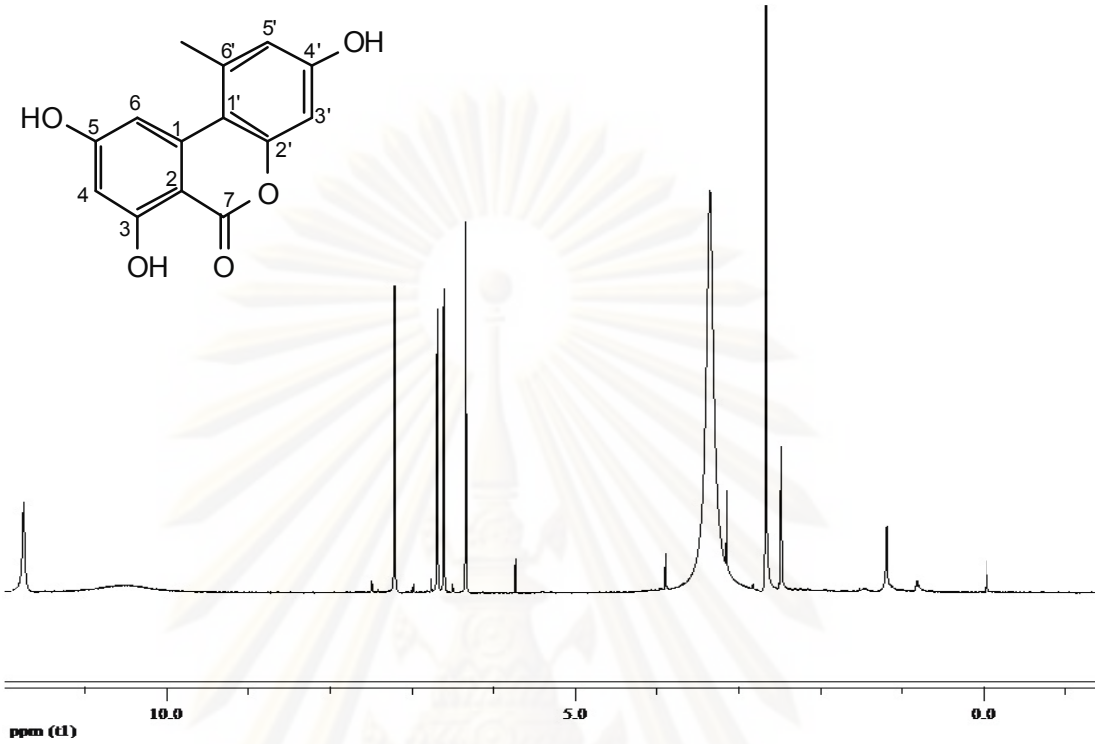



**Figure B23** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of alternariol (**6**)

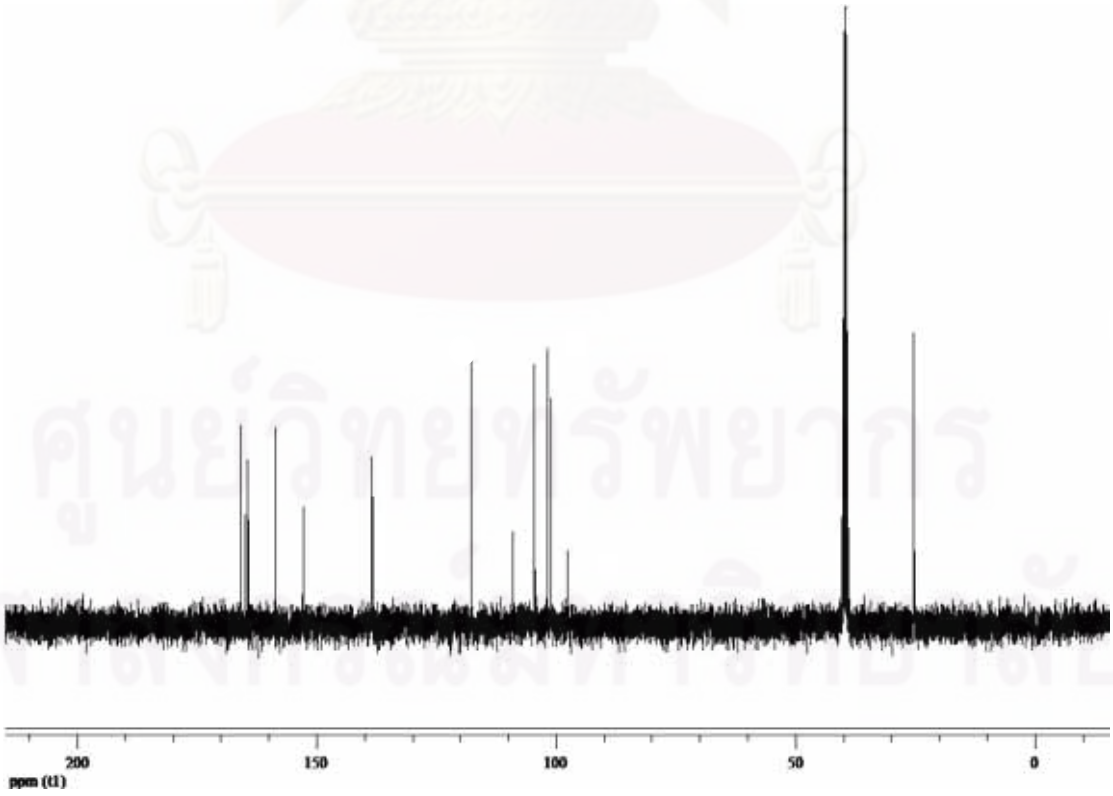



**Figure B24** 600 MHz $^{13}$C NMR (DMSO-$d_6$) spectrum of alternariol (**6**)

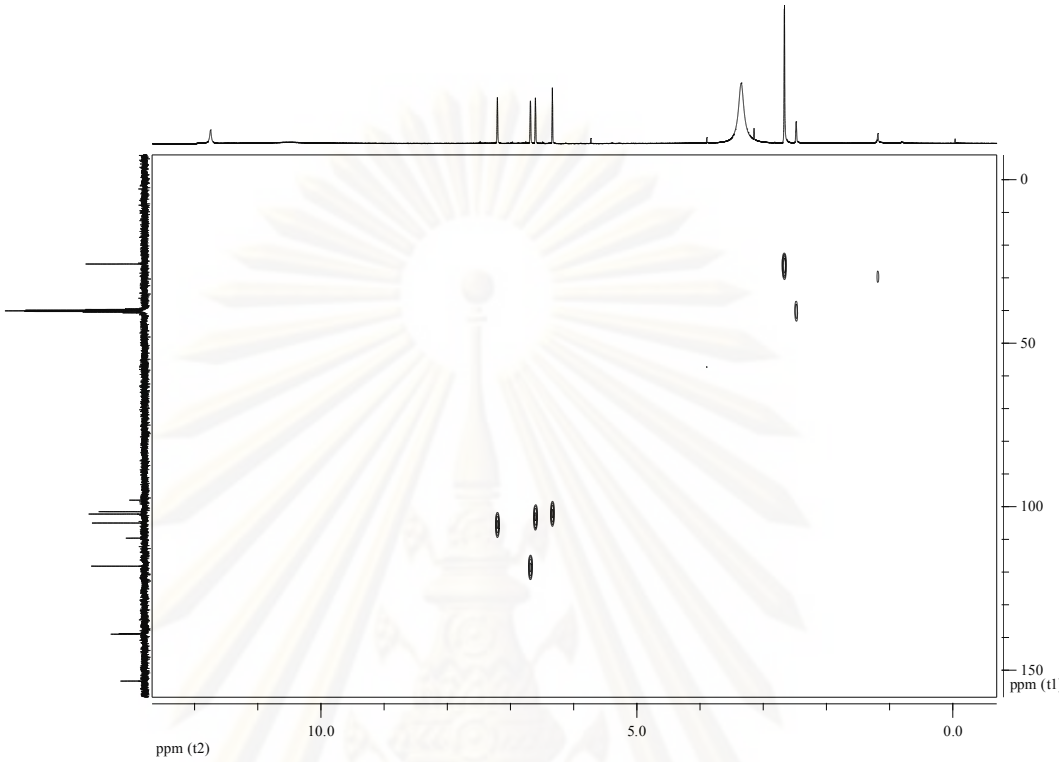



**Figure B25** HSQC of alternariol (**6**)

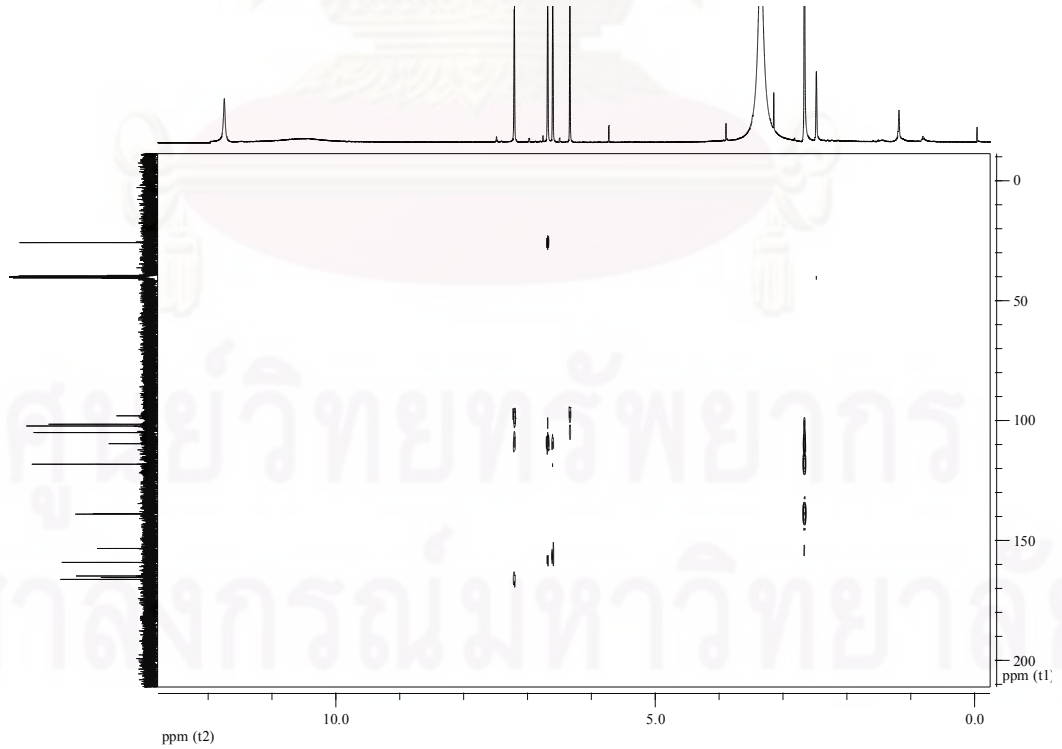



**Figure B26** HMBC of alternariol (**6**)

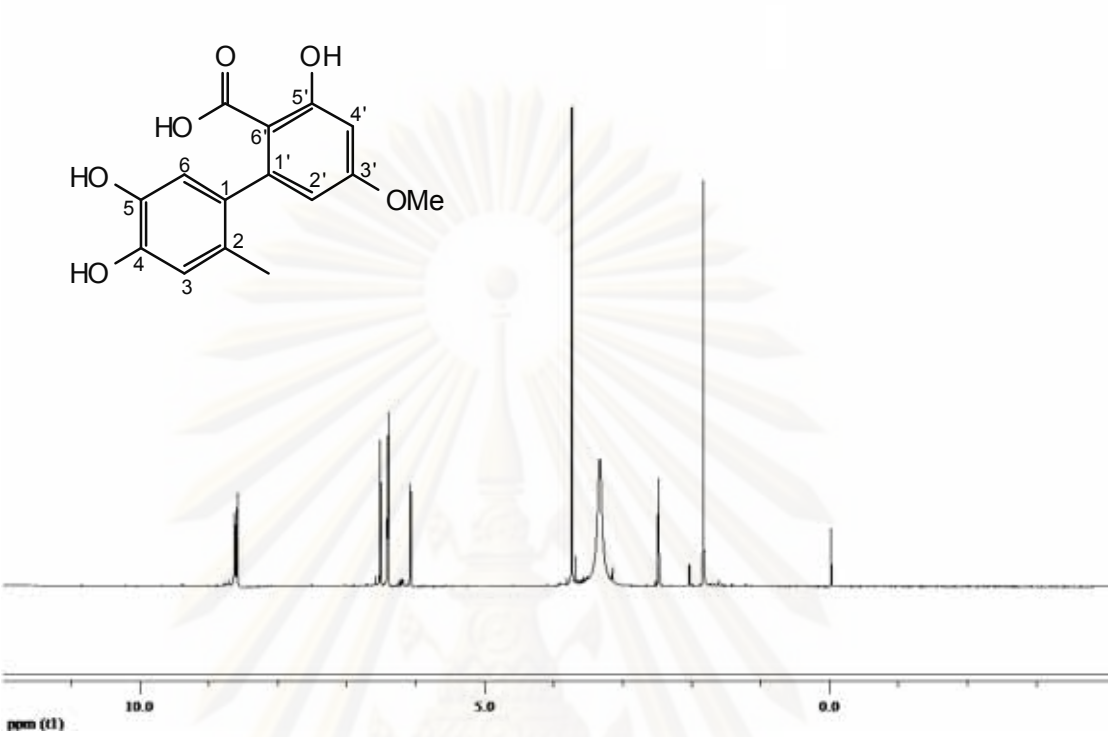



**Figure B27** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of altenusin (**7**)

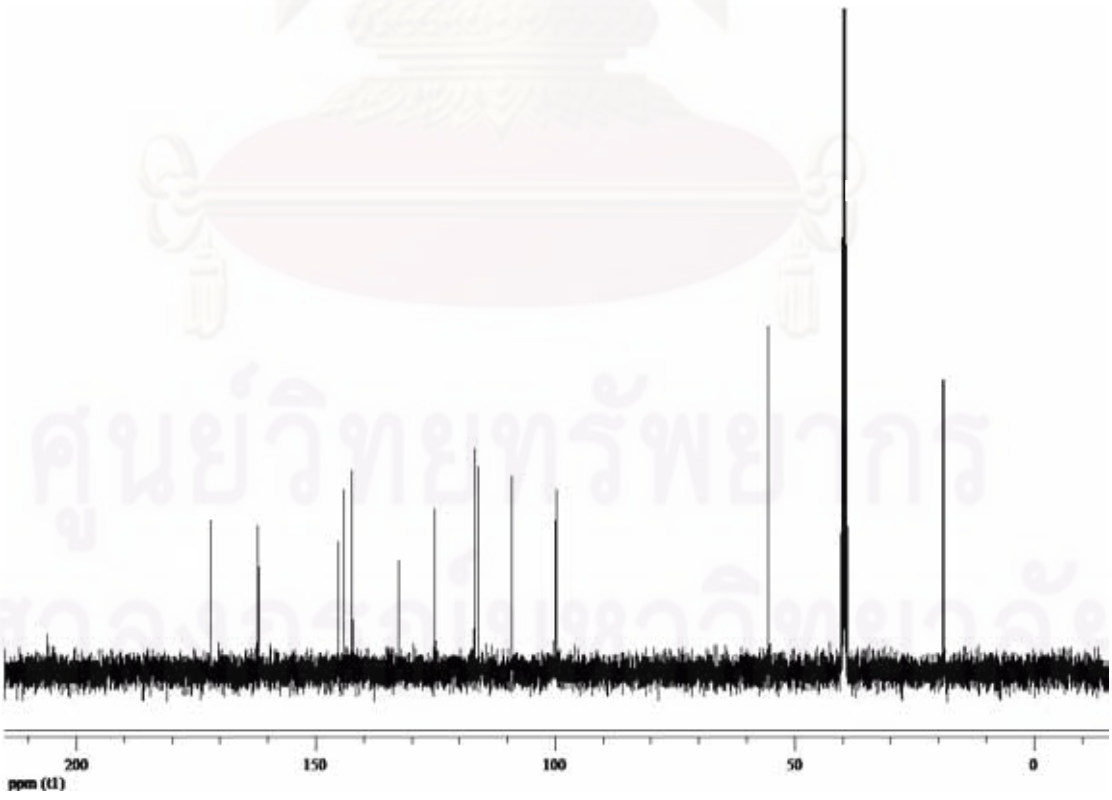



**Figure B28** 600 MHz $^{13}$C NMR (DMSO-$d_6$) spectrum of altenusin (**7**)

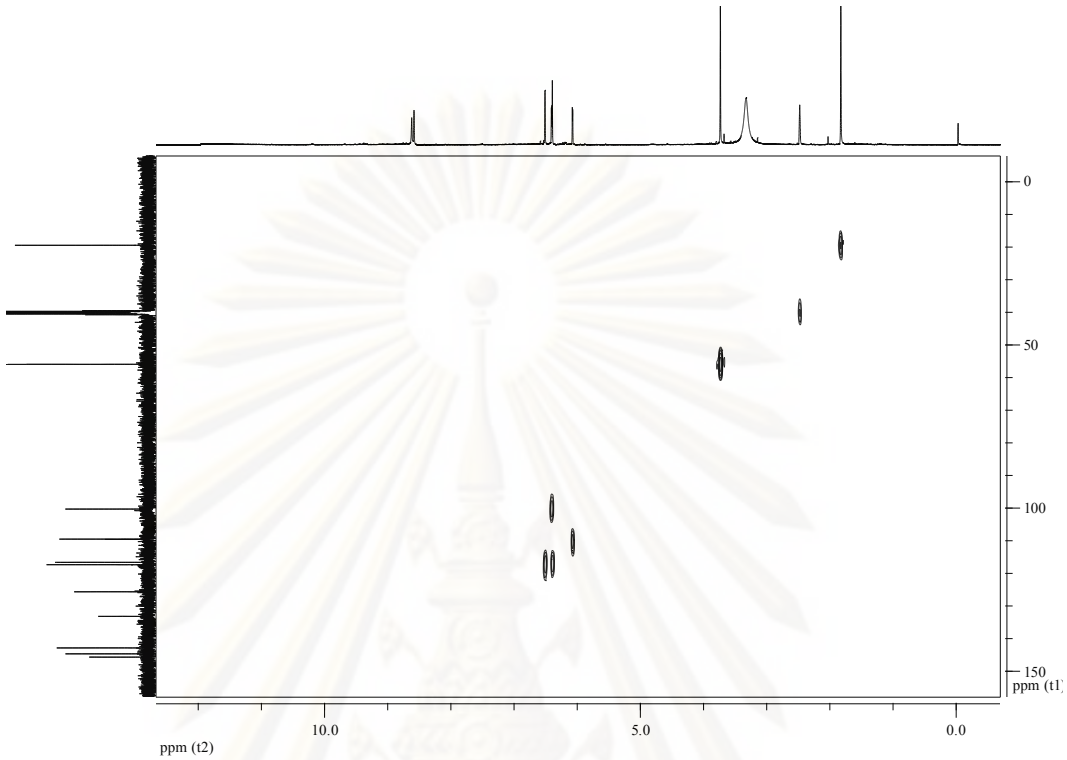



**Figure B29** HSQC of altenusin (**7**)

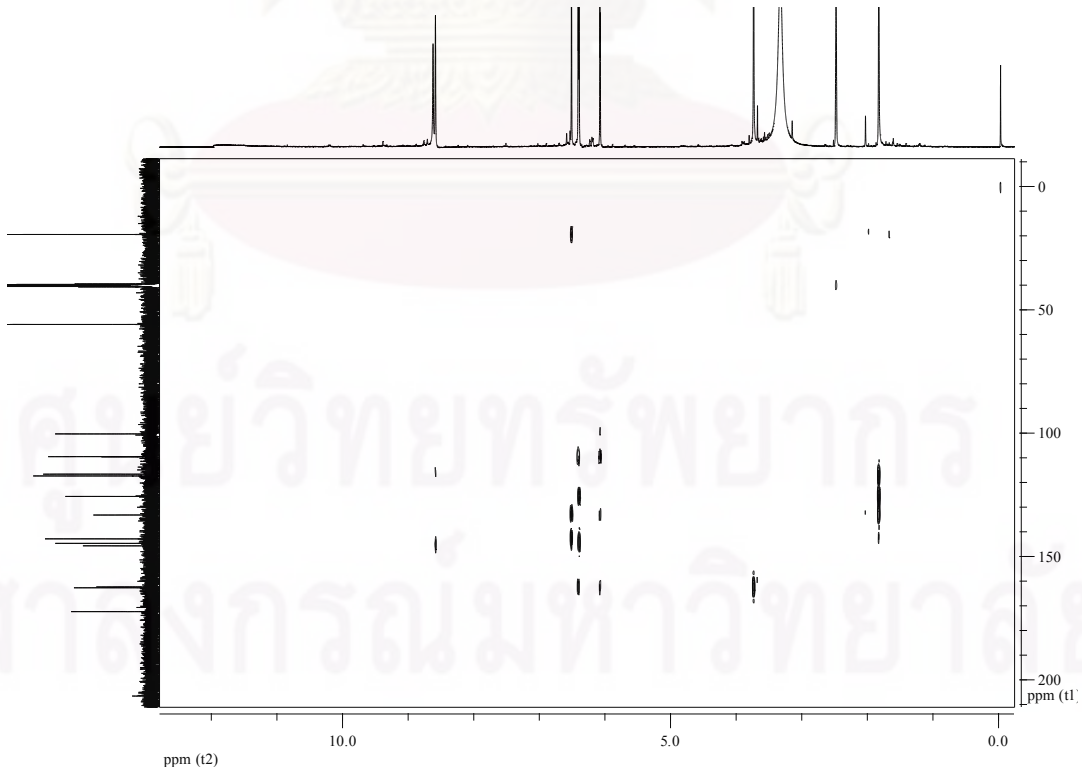



**Figure B30** HMBC of altenusin (**7**)

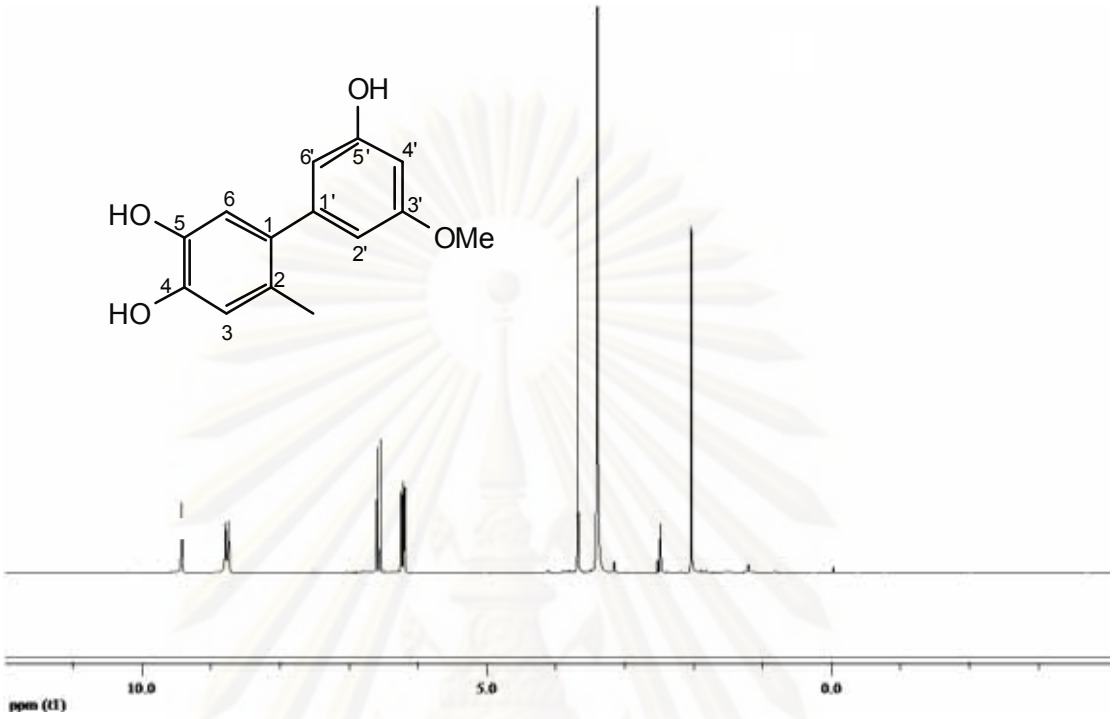



**Figure B31** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol (**8**)

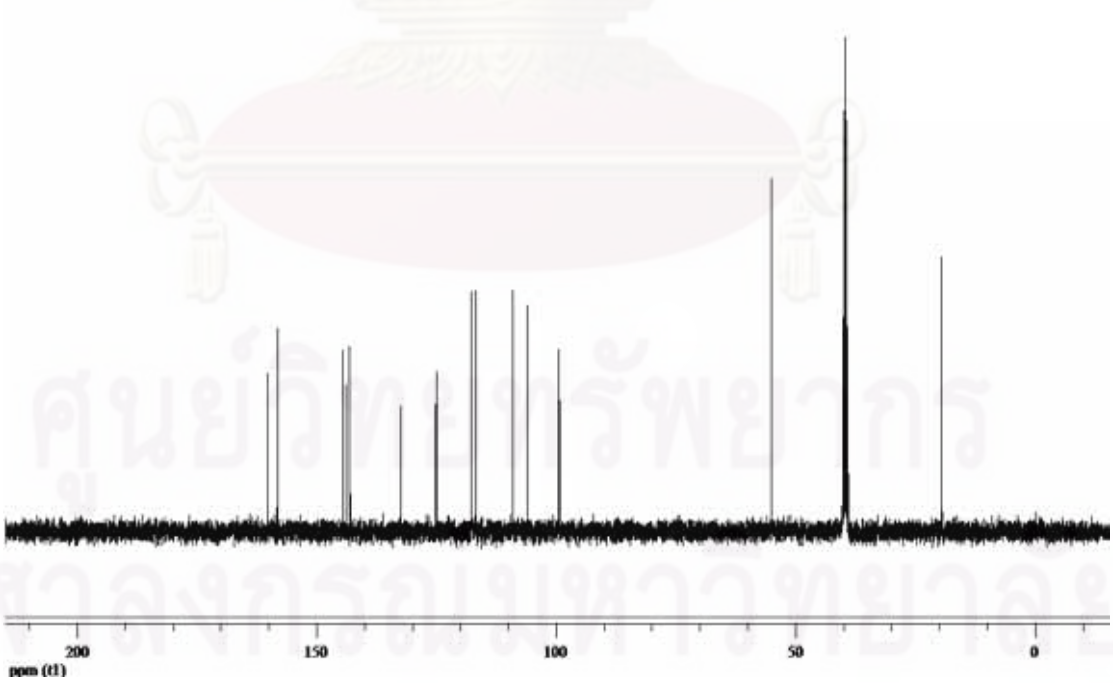



**Figure B32** 600 MHz $^{13}$C NMR (DMSO-$d_6$) spectrum of 3'-methoxy-2-methylbiphenyl-4, 5, 5'-triol (**8**)

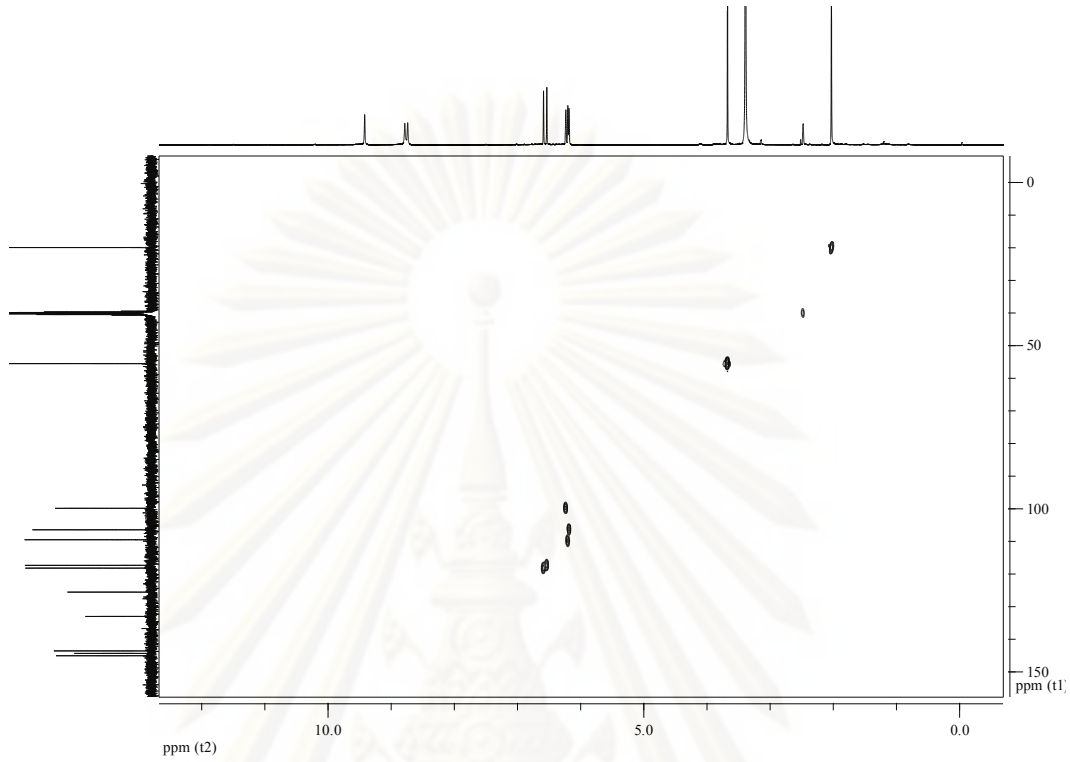



**Figure B33** HSQC of 3’-methoxy-2-methylbiphenyl-4, 5, 5’-triol (**8**)

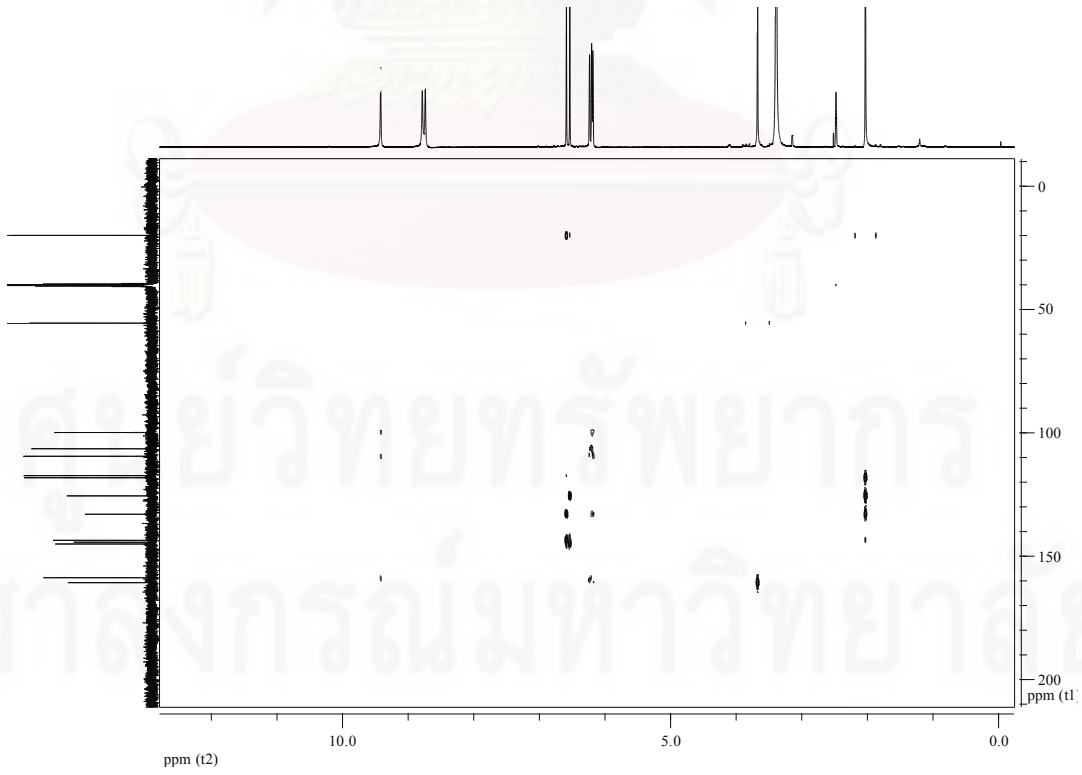



**Figure B34** HMBC of 3’-methoxy-2-methylbiphenyl-4, 5, 5’-triol (**8**)

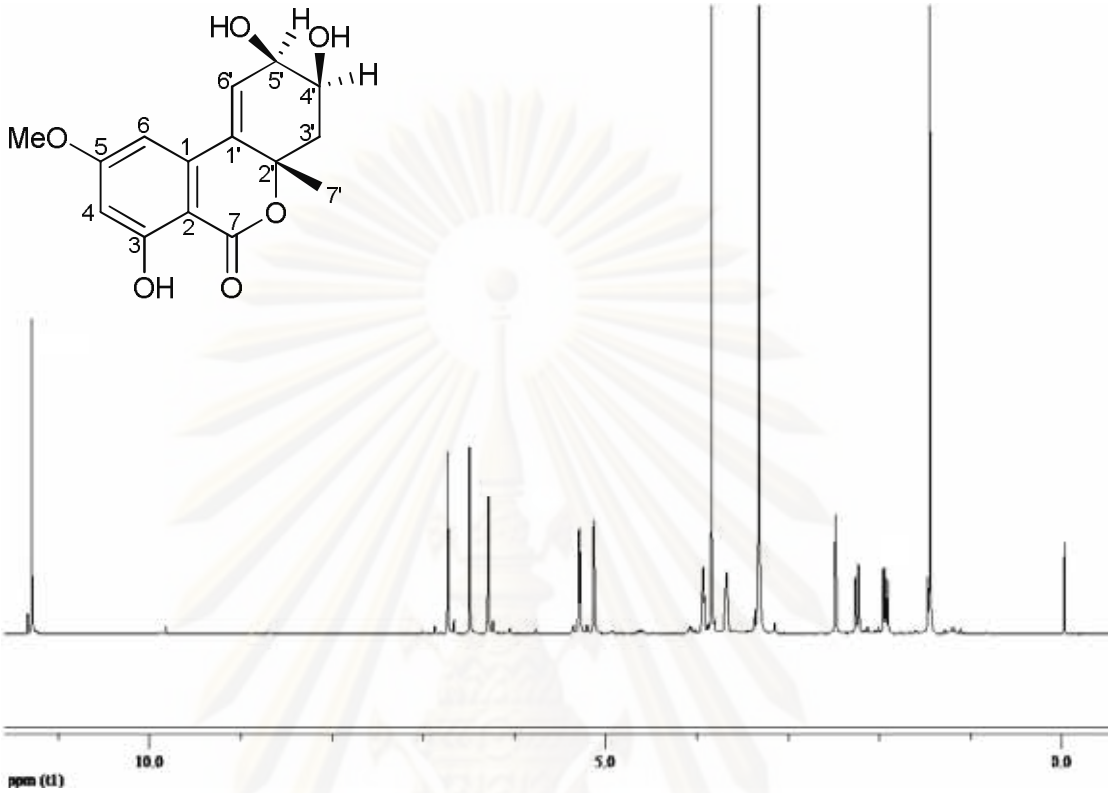



**Figure B35** 600 MHz $^{1}$H NMR (DMSO-$d_6$) spectrum of 5'-epialtenuene (**9**)

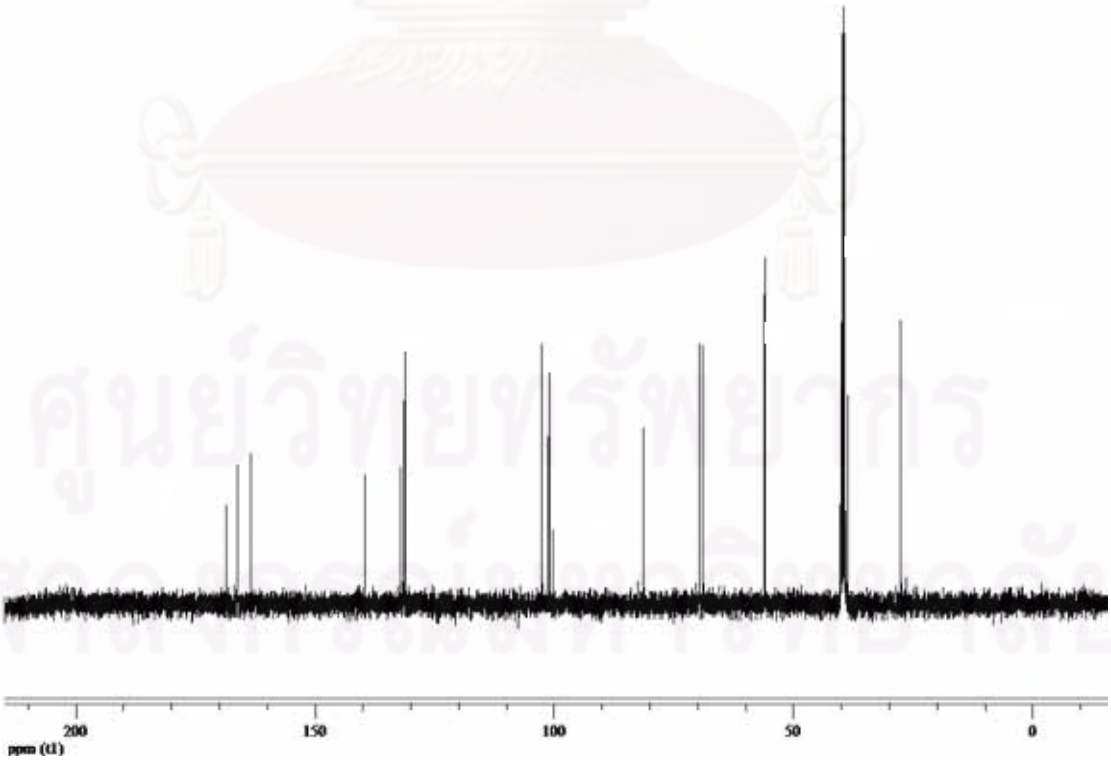



**Figure B36** 600 MHz $^{13}$C NMR (DMSO-$d_6$) spectrum of 5'-epialtenuene (**9**)

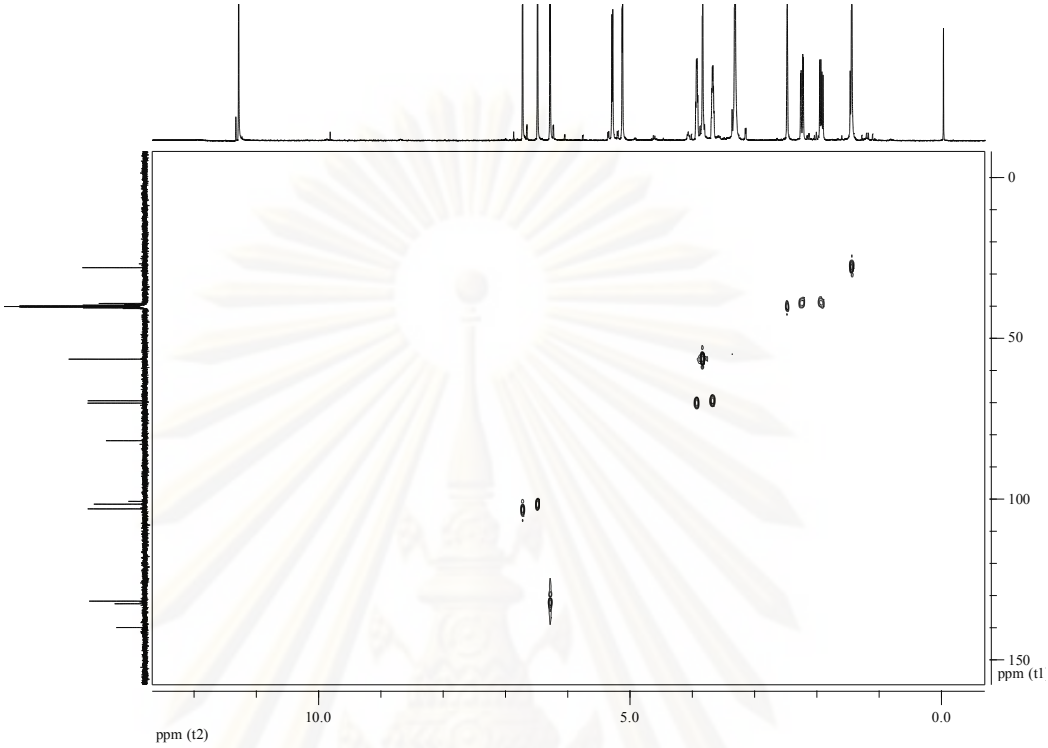



**Figure B37** HSQC of 5'-epialtenuene (**9**)

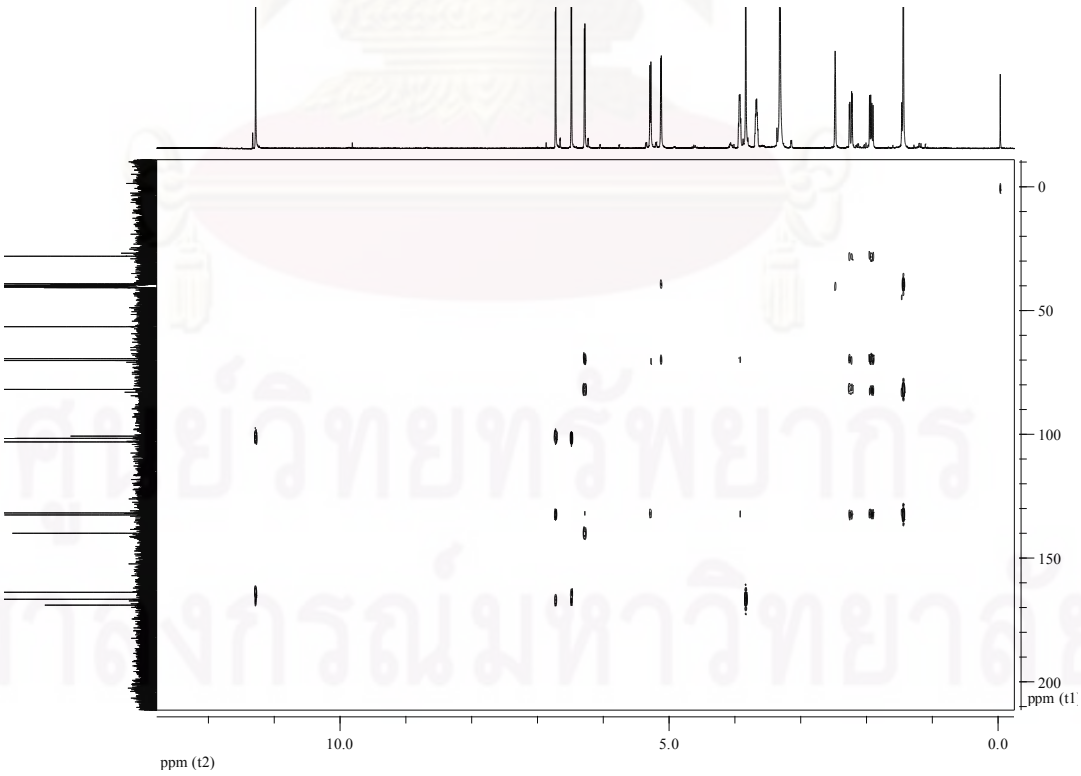



**Figure B38** HMBC of 5'-epialtenuene (**9**)

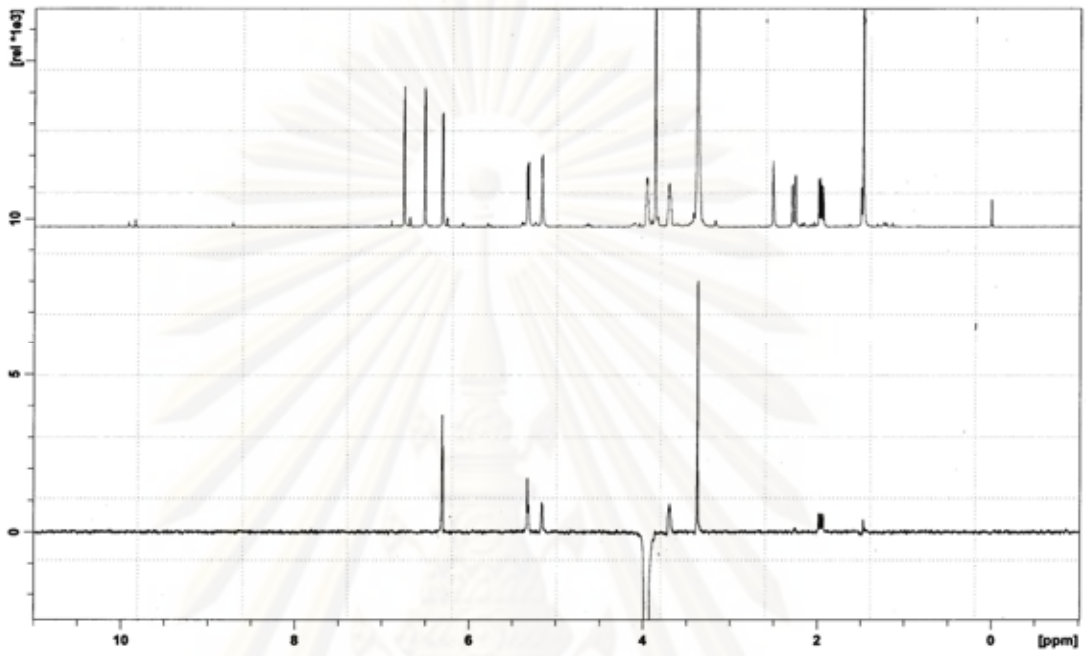
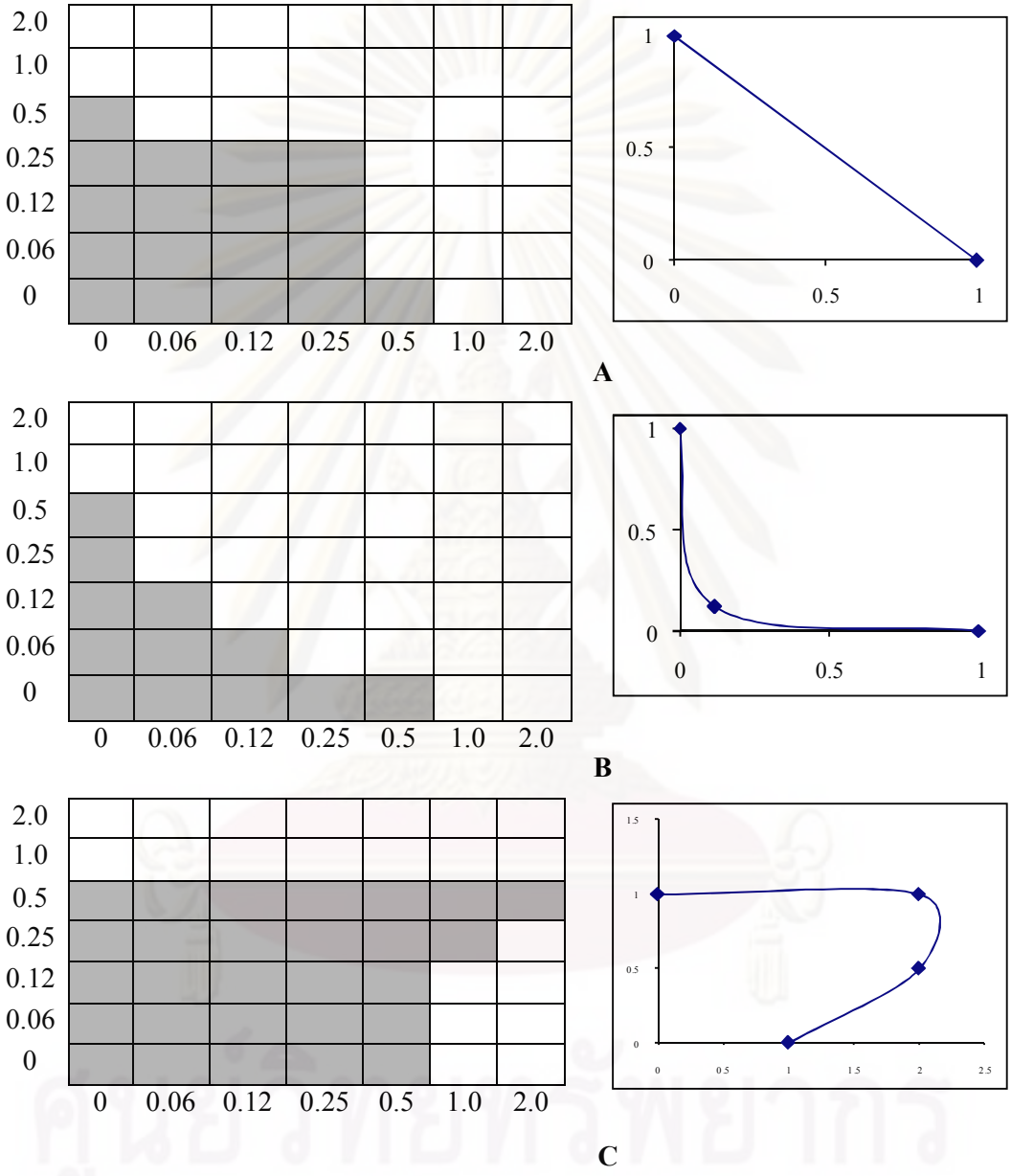



**Figure B39** 1D NOE experiment of 5’-epialtenuene (**9**)

# APPENDIX C

Checkerboard interpreted isobolograms




Assessment of antimicrobial combinations with the checkerboard method. **A, B** and **C** showed results of testing combinations of two drugs (diluted in geometric twofold increments along the *x* and *y* axes). Shading, visible growth. Concentrations are expressed as multiples of the MIC. Graph on the left side are isobologram. A showed indifference effect; B showed synergism; C showed antagonism. (Lorian, 2005, **Figure 9.2**, p. 369.)

## APPENDIX D

**Table D1** Active endophytic fungus isolates and culture media promoting antimicrobial metabolite(s) production

| Endophytic fungus isolates | Inhibited microorganisms | Culture media promoting antimicrobial metabolite(s) production (potency*) |
|---|---|---|
| OIV B63 | *S. aureus* | MEA (++), PDA (++) |
| OIV B64 | *S. aureus* | MEA (++), PDA (++) |
| OIV V65 | *S. aureus* | MEA (+++), YES (++) |
| | *C. albicans* | MEA (++) |
| CGV B76 | *S. aureus* | MEA (++), PDA (++) |
| CGV B80 | *S. aureus* | MCzA (++), PDA (++) |
| CGV B81 | *S. aureus* | MEA (++), PDA (++), YES (++) |
| CGV B83 | *S. aureus* | MEA (++), PDA (++) |
| CGV B84 | *S. aureus* | MEA (++) |
| CGV B85 | *S. aureus* | MEA (++) |
| CGV L87 | *S. aureus* | MCzA (++), MEA (++), YES (++) |
| CGV L89 | *S. aureus* | MEA (++) |
| CGV L90 | *S. aureus* | CzYA (++), SDA (++) |
| PJB B92 | *S. aureus* | MCzA (++), MEA (++), PDA (++) |
| | *E. faecalis* | MEA (++), PDA (++) |
| PJB B96 | *E. coli* | MEA (++) |
| PJB B97 | *E. faecalis* | MEA (++), YES (+++) |

* ++: the inhibition zone diameter is 10 mm to 20 mm

+++: the inhibition zone diameter is larger than 20 mm

**Table D2** Antimicrobial activity of endophytic fungi from crude extract

| **Endophytic fungus isolates** | **Inhibited microorganisms** | **Crude extract promoting antimicrobial metabolite(s) production (potency*)** |
|---|---|---|
| OIV B63 | *S. aureus* | E (++) |
| OIV B64 | *S. aureus* | B (+) |
| OIV V65 | *S. aureus* | B (++) |
| | *C. albicans* | B (++), E (++) |
| CGV B76 | *S. aureus* | B (++), E (++) |
| CGV B80 | *S. aureus* | B (+) |
| CGV B81 | *S. aureus* | B (+) |
| CGV B83 | *S. aureus* | B (+) |
| CGV B84 | *S. aureus* | B (++) |
| CGV B85 | *S. aureus* | E (++) |
| CGV L90 | *S. aureus* | B (++), E (+) |
| PJB B92 | *S. aureus* | B (++) |
| | *E. faecalis* | B (+) |
| PJB B96 | *S. aureus* | B (++), E (+) |
| | *C. albicans* | B (++), E (+++) |
| PJB B97 | *S. aureus* | B (++) |

B : broth crude extract, H : fungal mycelium crude extract via hexane and E : fungal mycelium crude extract via ethyl acetate

+ : the inhibition zone is less than 10 mm

++ : the inhibition zone is from 10 mm to 20 mm

+++ : the inhibition zone is above 20 mm

## APPENDIX E

**Table E1** Minimum inhibitory concentration (µg/mL) of the following antibiotics against clinical isolated bacterial of enterococcus, staphylococcus and *P. vulgaris*.

| antibiotic | EF | VEF | EFa | SA | SE | SH | PV |
|---|---|---|---|---|---|---|---|
| **Ampicillin** | 0.5< | 1 | 64 | 0.125 | 16 | 0.5< | 32 |
| **Ceftriaxone** | 512 | 1024 | I | 0.5< | 1 | 0.5< | 2 |
| **Gentamicin** | 8 | I | I | 2 | 0.5< | 0.5< | 0.5 |
| **Tetracycline** | 8 | 0.5< | 0.5< | 0.125 | 128 | 32 | 0.25 |
| **Vancomycin** | 0.5< | 8 | 512 | 0.5< | 0.5< | 0.5< | 1024 |

* EF : *E. faecalis*, VEF : *E. faecalis* (vancomycin resistant), EFa : *E. faecium*, SA : *S. aureus*, SE : *S. epidermidis*, SH : *S. hominis*, and PV : *P. vulgaris*

** I : Inactive at 1024 µg/mL

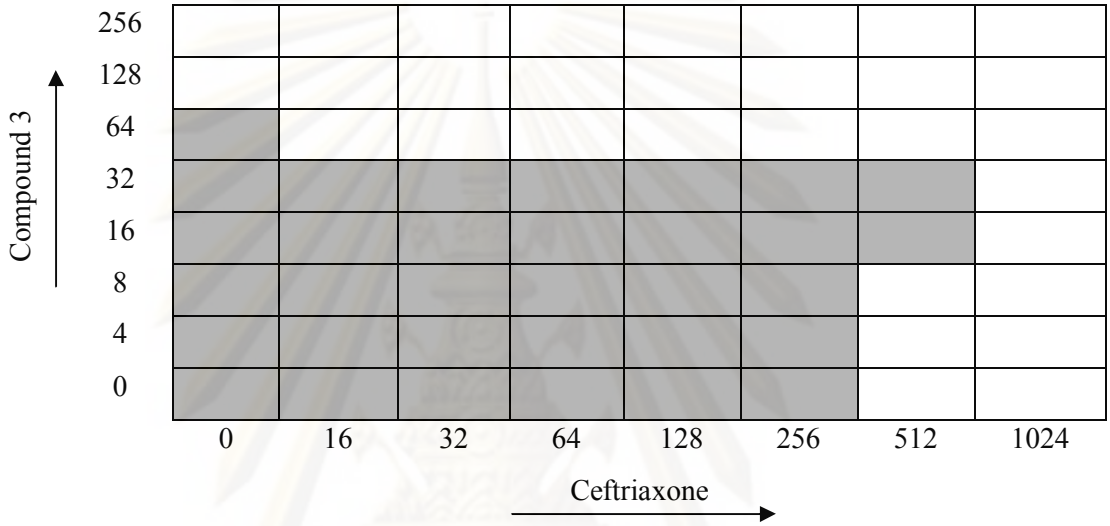
*** MIC presented as Geomean of 3 observations

**FIGURE E1** The result of compound 3 combined with ceftriaxone against *E. faecalis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 128 µg/mL, MIC of ceftriaxone = 512 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|
| FIC of ceftriaxone | 0 | 0 | 2 | 2 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 2.25 | 2.12 | 1.06 | 1.03 | 1 | 2 |

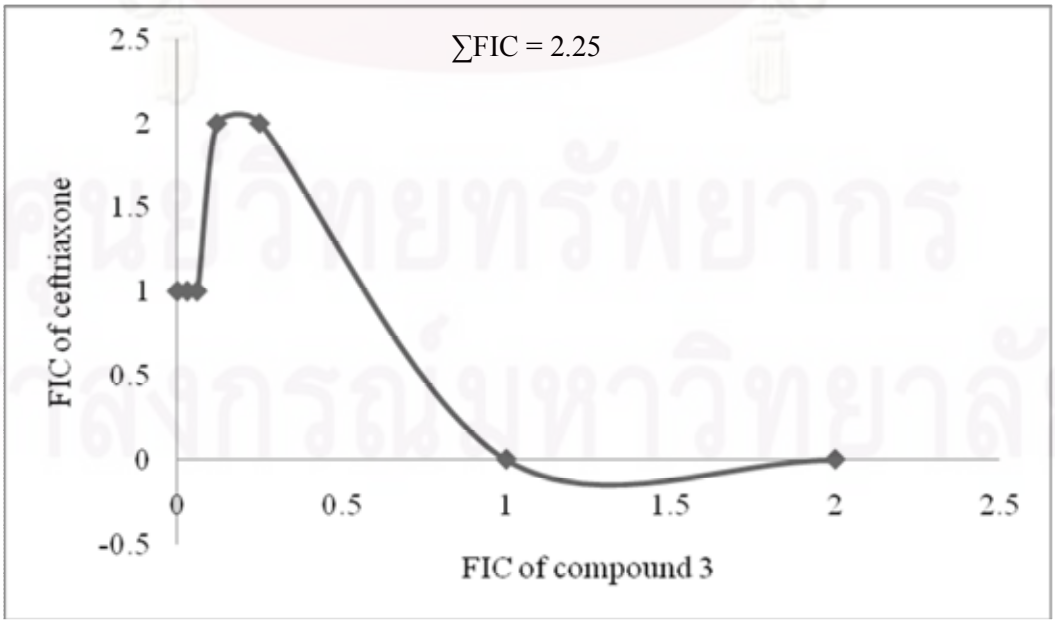
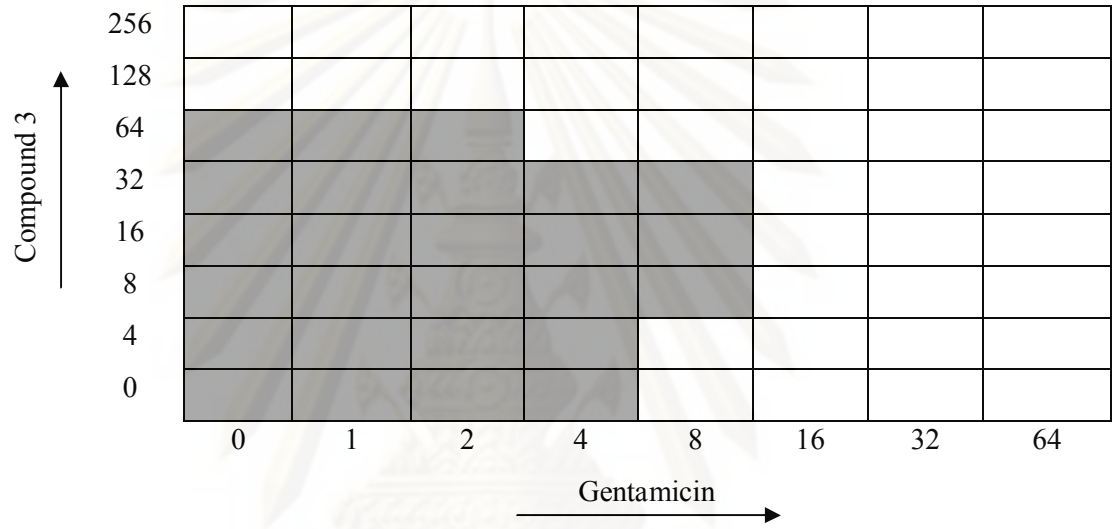


**FIGURE E2** The result of compound 3 combined with gentamicin against *E. faecalis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 128 µg/mL, MIC of gentamicin = 8 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| FIC of Gentamicin | 0 | 0 | 2 | 2 | 2 | 1 | 1 | 2 | 4 | 8 |
| ∑FIC | 2 | 1 | 2.25 | 2.12 | 2.06 | 1.03 | 1 | 2 | 4 | 8 |

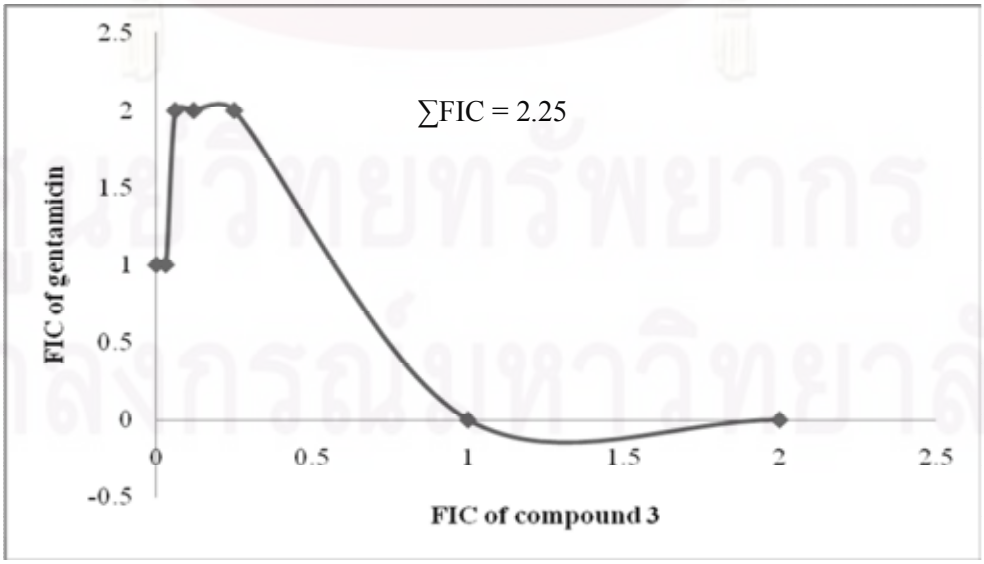
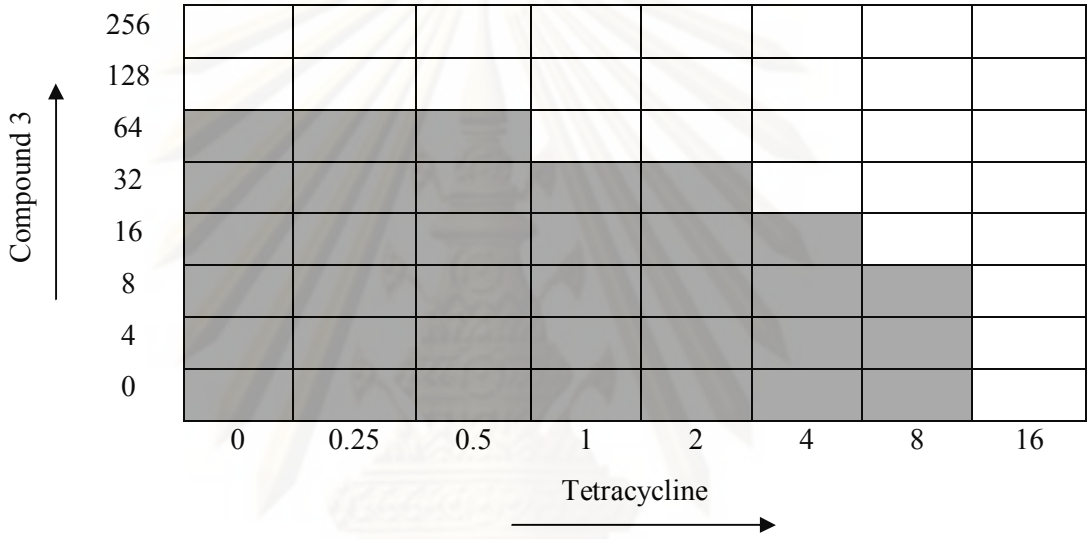


**FIGURE E3** The result of compound 3 combined with tetracycline against *E. faecalis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 128 µg/mL, MIC of tetracycline = 8 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.12 | 0.06 | 0.03 | 0 |
|---|---|---|---|---|---|---|
| FIC of tetracycline | 0 | 0 | 0.5 | 1 | 1 | 1 |
| ∑FIC | 2 | 1 | 0.62 | 1.06 | 1.03 | 1 |

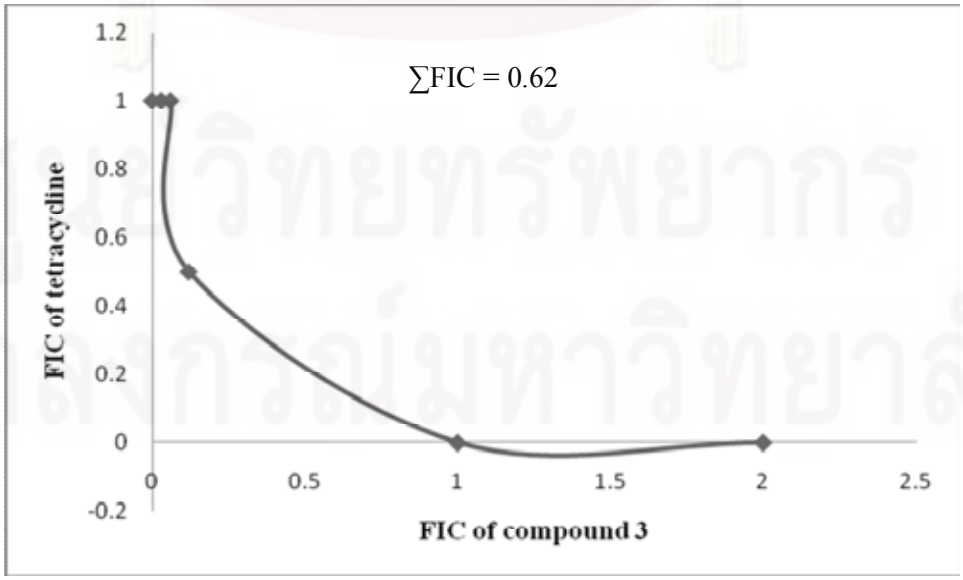
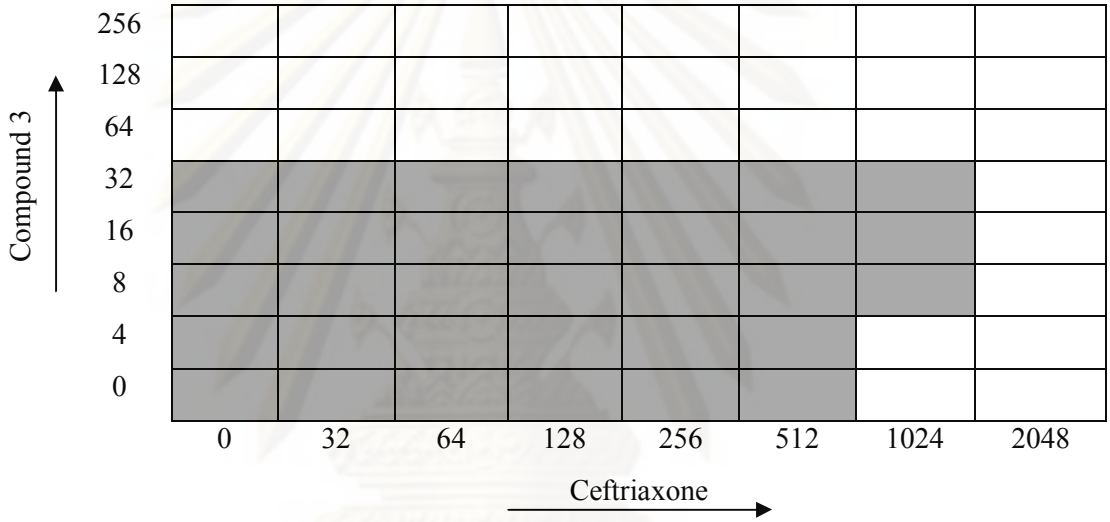


**FIGURE E4** The result of compound 3 combined with ceftriaxone against *E. faecalis* (vancomycin resistant), calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 64 µg/mL, MIC of ceftriaxone = 1024 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|
| FIC of ceftriaxone | 0 | 0 | 2 | 2 | 2 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 2.5 | 2.25 | 2.12 | 1.06 | 1 | 2 |

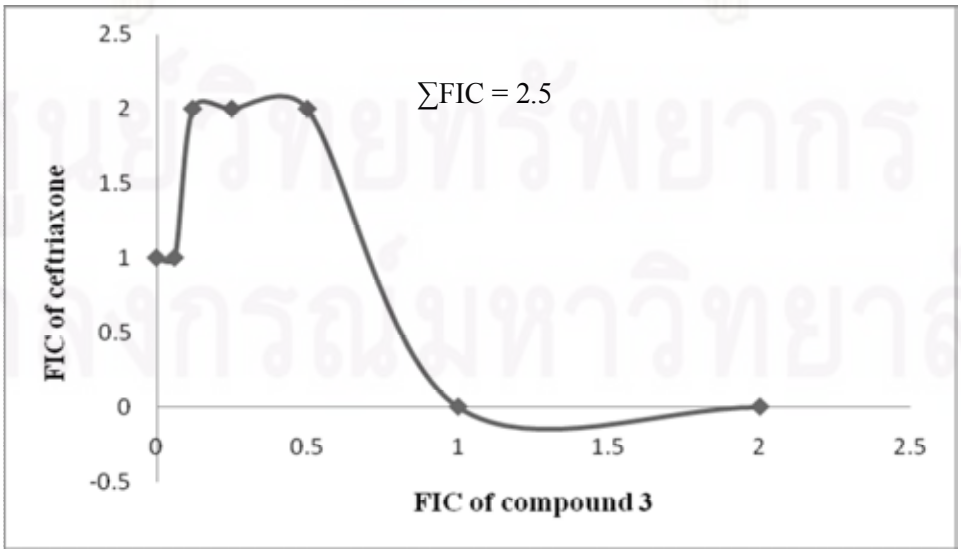
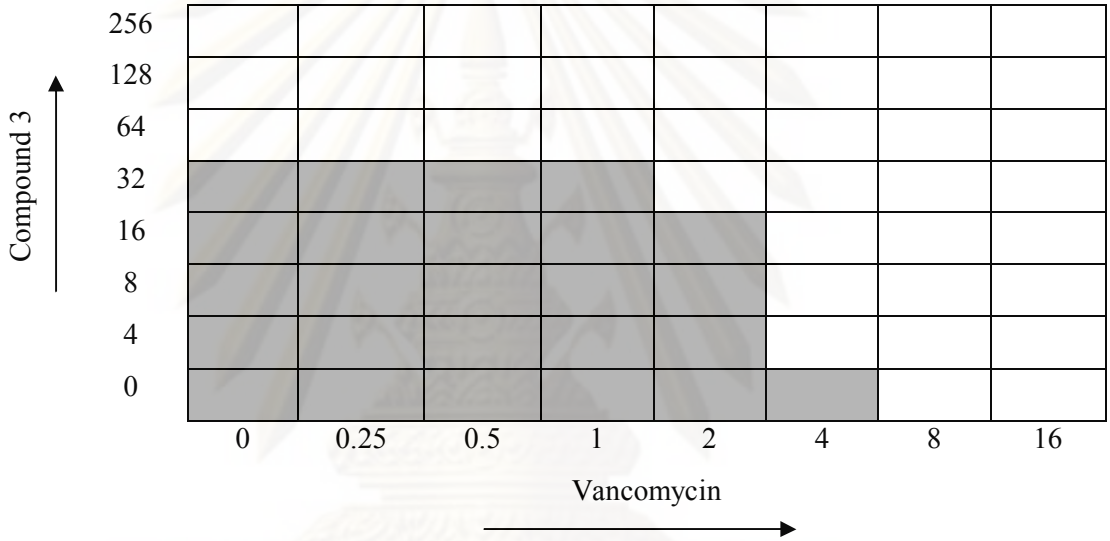


**FIGURE E5** The result of compound 3 combined with vancomicin against *E. faecalis* (vancomycin resistant), calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 64 µg/mL, MIC of vancomicin = 8 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 4 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of vancomycin | 0 | 0 | 0 | 0.25 | 0.5 | 0.5 | 0.5 | 1 | 2 |
| ∑FIC | 4 | 2 | 1 | 0.75 | 0.75 | 0.62 | 0.56 | 1 | 2 |

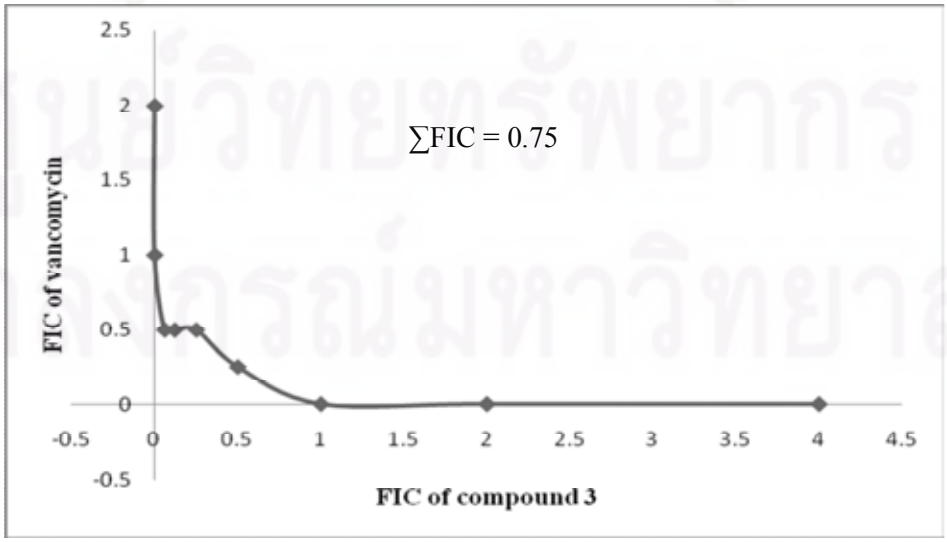
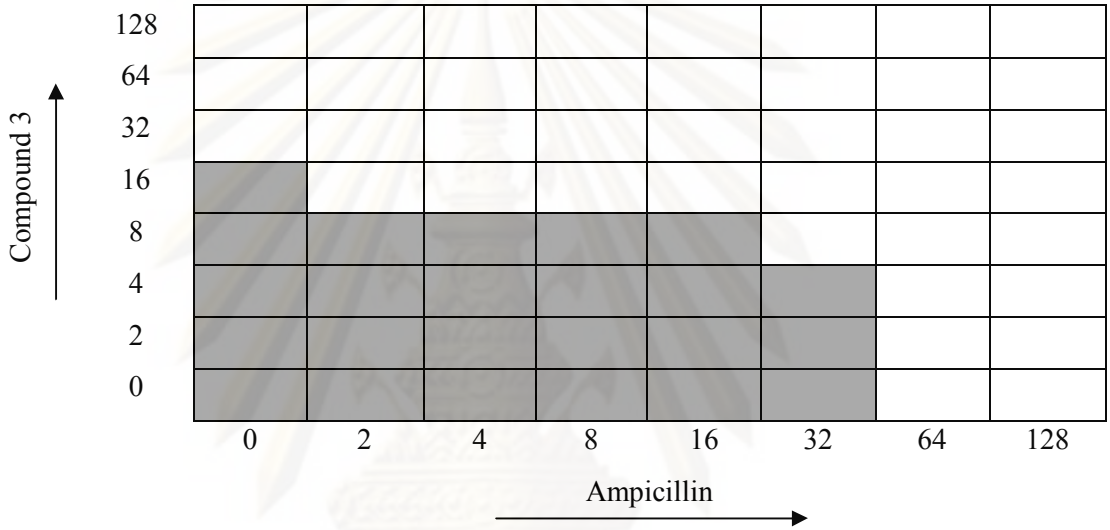


**FIGURE E6** The result of compound 3 combined with ampicillin against *E. feacium*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 32 µg/mL, MIC of ampicillin = 64 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 4 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0 |
|---|---|---|---|---|---|---|---|
| FIC of ampicillin | 0 | 0 | 0 | 0.5 | 1 | 1 | 1 |
| ∑FIC | 4 | 2 | 1 | 0.75 | 1.12 | 1.06 | 1 |

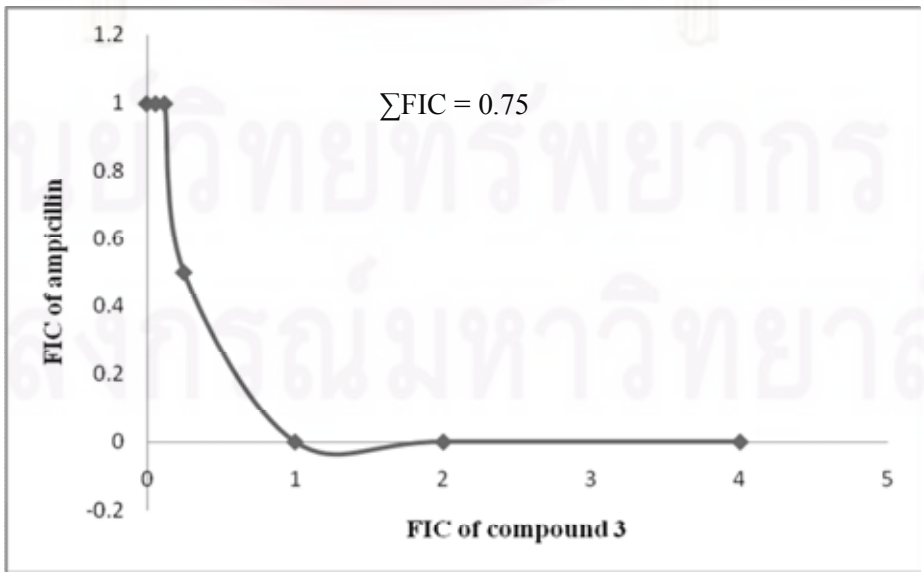
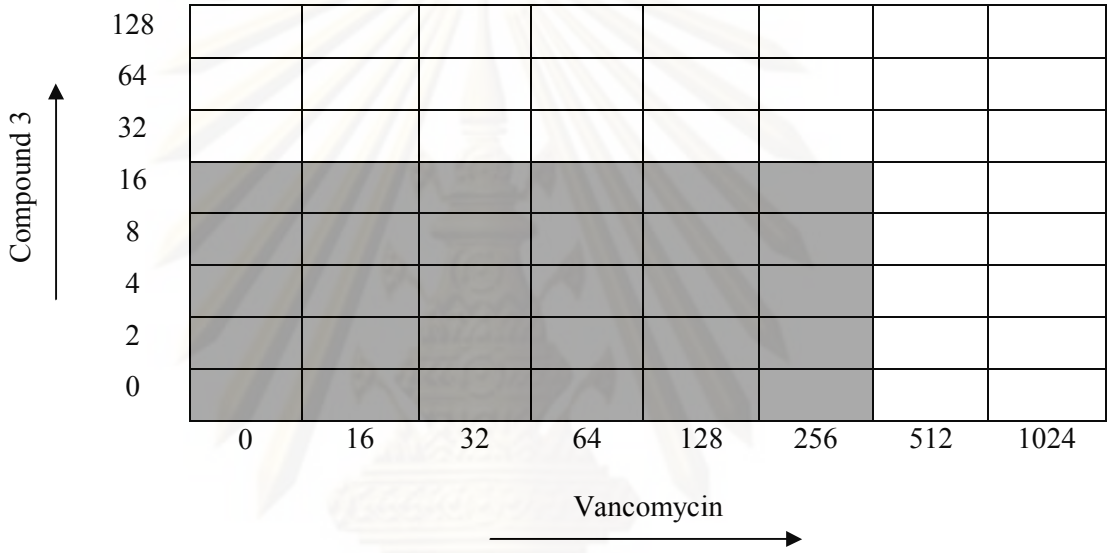


**FIGURE E7** The result of compound 3 combined with vancomycin against *E. feacium*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 32 µg/mL, MIC of vancomycin = 512 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.5 | 0.12 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|
| FIC of vancomycin | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 1.5 | 1.12 | 1.06 | 1.03 | 1 | 2 |

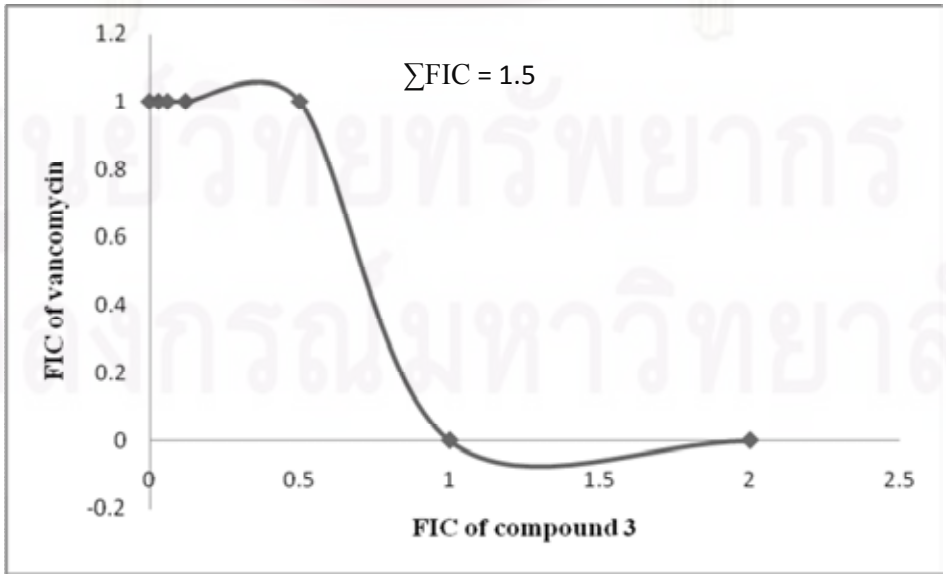
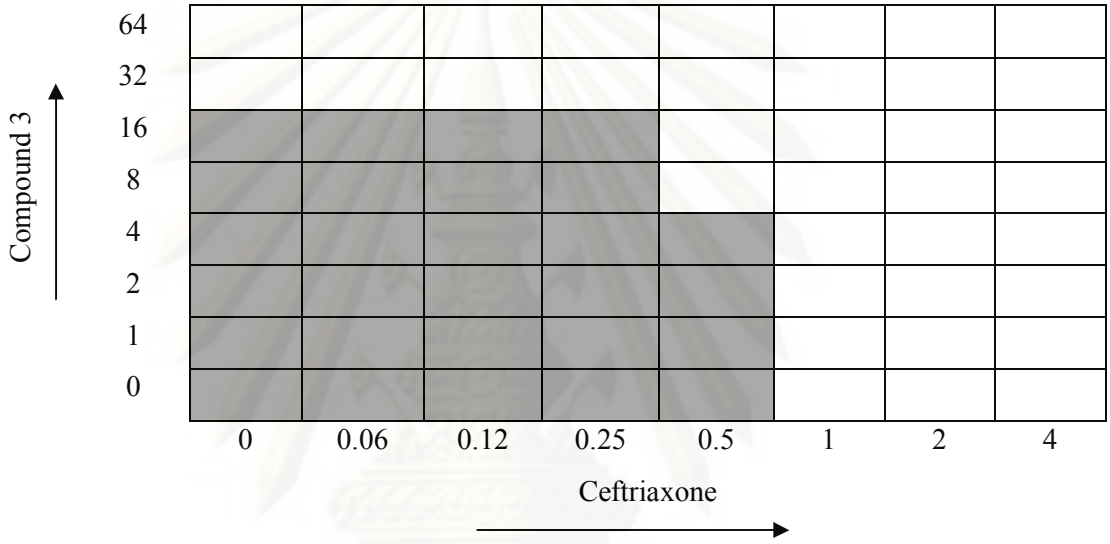


**FIGURE E8** The result of compound 3 combined with ceftriaxone against *S. epidermidis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 32 µg/mL, MIC of ceftriaxone = 1 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of ceftriaxone | 0 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.75 | 1.12 | 1.06 | 1.03 | 1 | 2 | 4 |

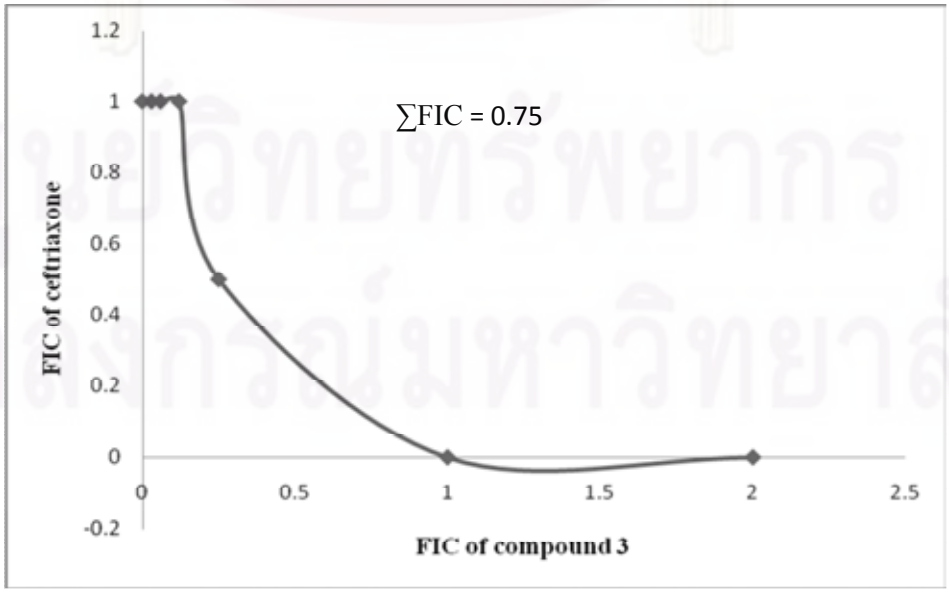
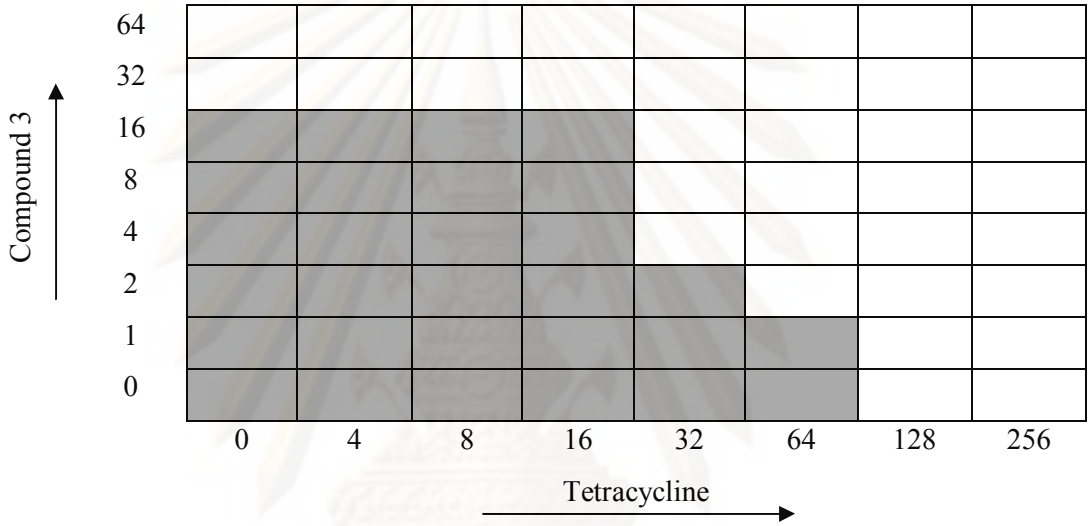


**FIGURE E9** The result of compound 3 combined with tetracycline against *S. epidermidis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 32 µg/mL, MIC of tetracycline = 128 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|
| FIC of tetracycline | 0 | 0 | 0.5 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 0.56 | 1.03 | 1 | 2 |

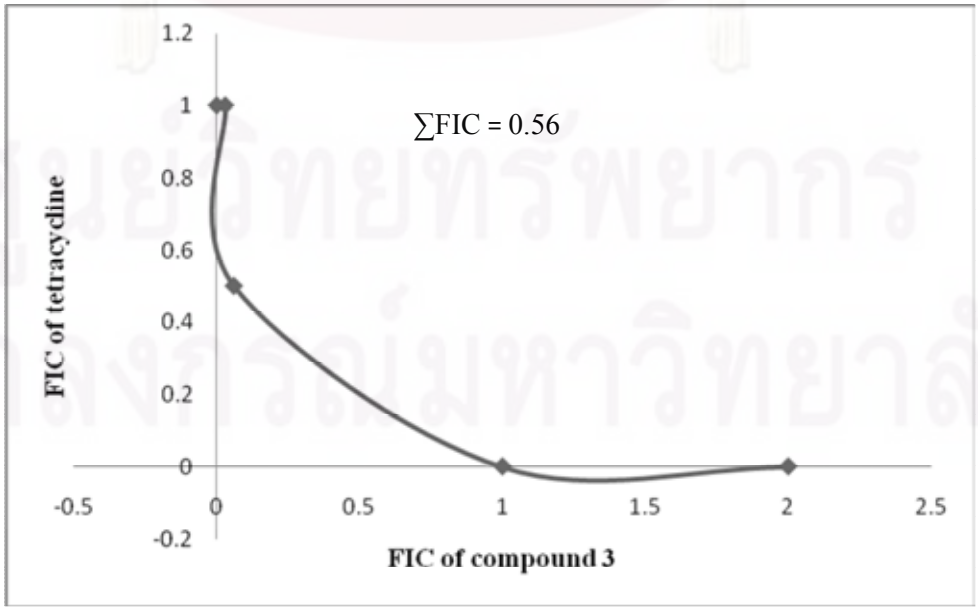
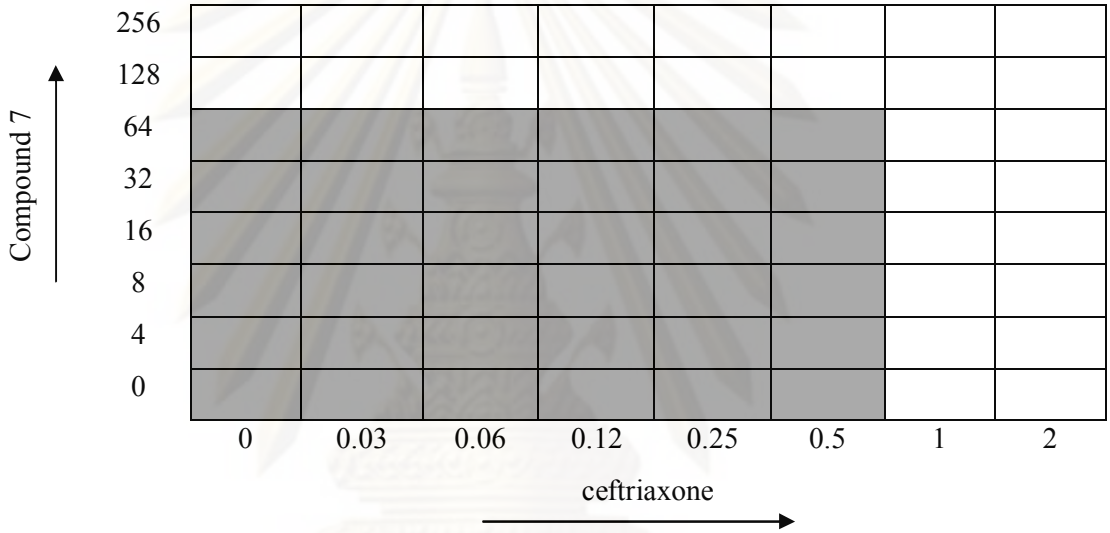


**FIGURE E10** The result of compound **7** combined with ceftriaxone against *S. epidermidis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 7 = 128 µg/mL, MIC of ceftriaxone = 1 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 7 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|
| FIC of ceftriaxone | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 1.5 | 1.25 | 1.12 | 1.06 | 1 | 2 |

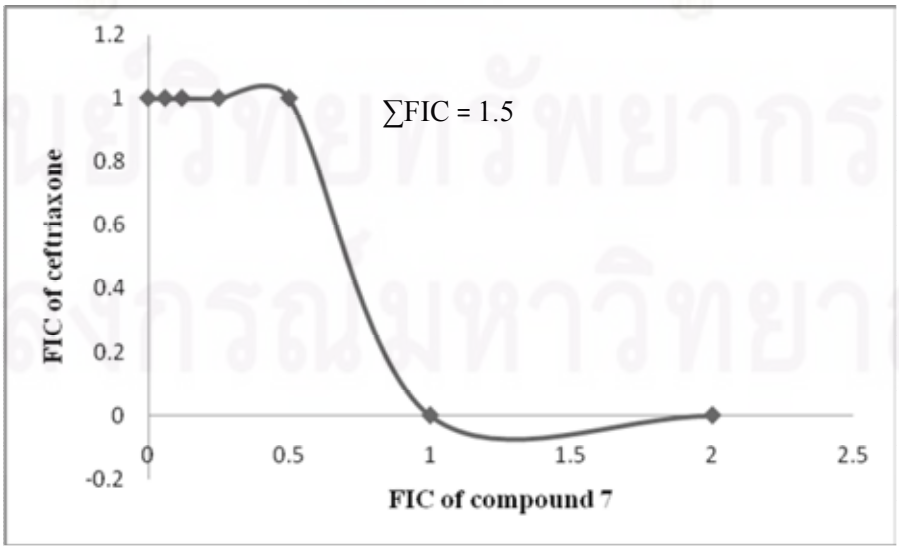
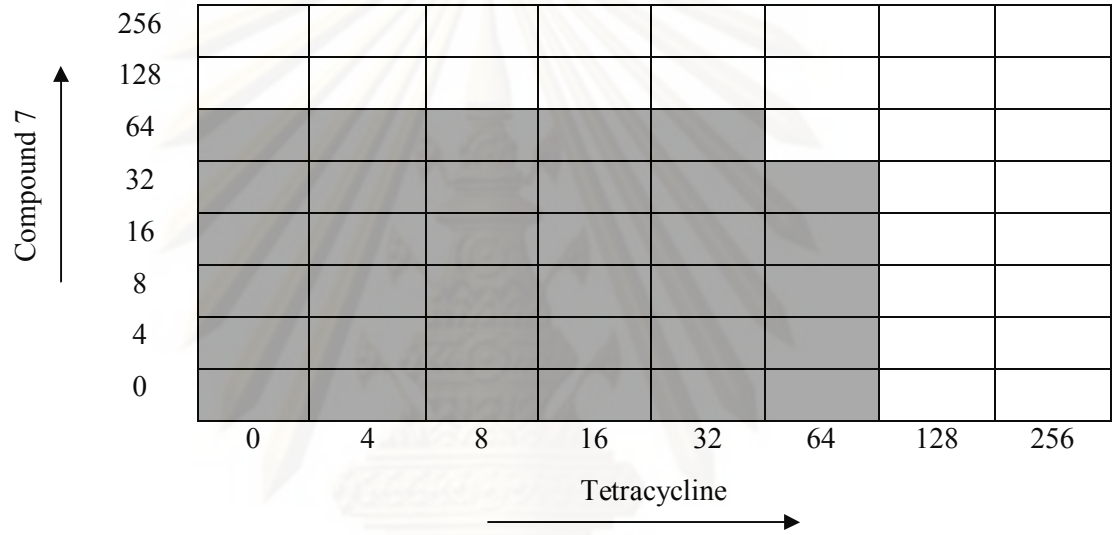


**FIGURE E11** The result of compound 7 combined with tetracycline against *S. epidermidis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 7 = 128 µg/mL, MIC of tetracycline = 128 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 7 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of tetracycline | 0 | 0 | 0.5 | 1 | 1 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 1 | 1.25 | 1.12 | 1.06 | 1.03 | 1 | 2 |

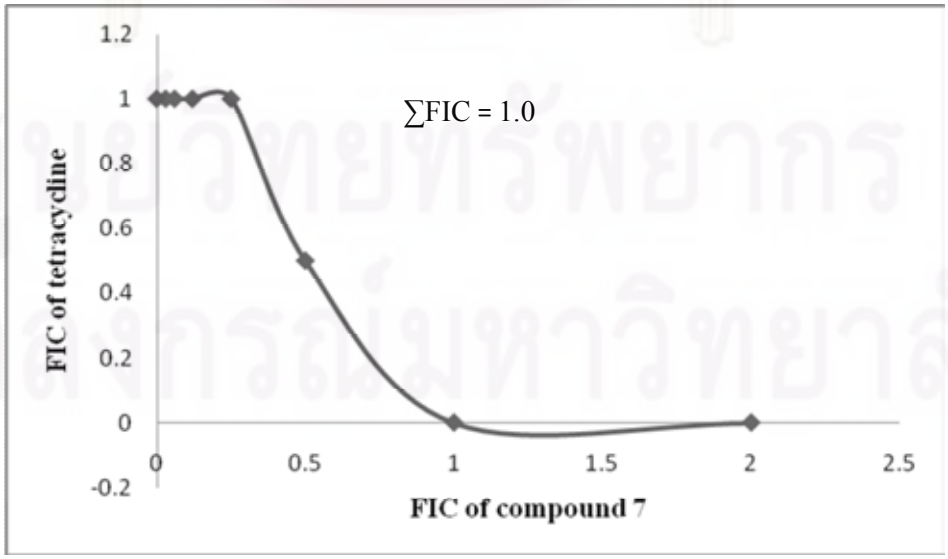
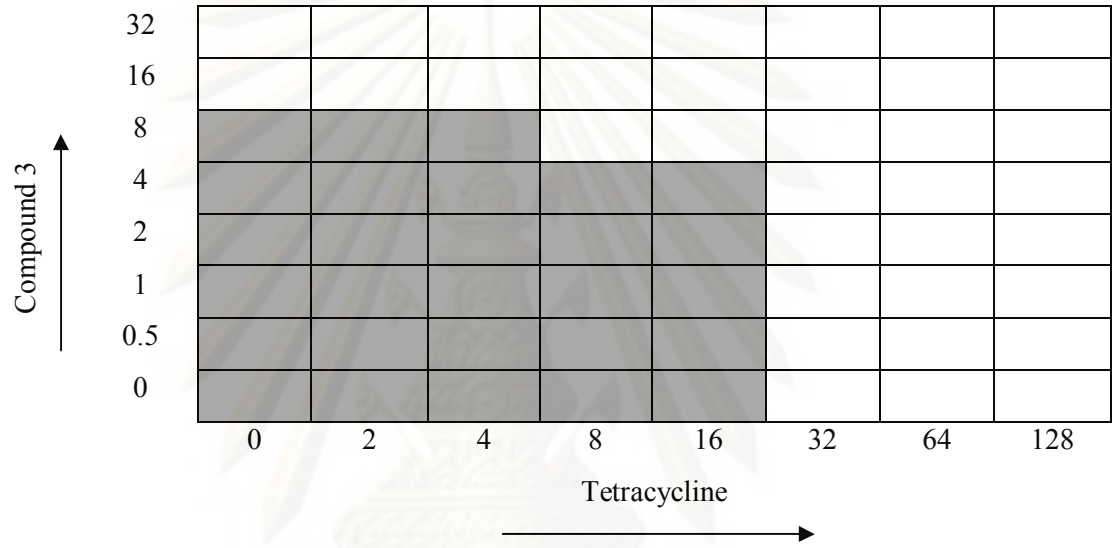


**FIGURE E12** The result of compound 3 combined with tetracycline against *S. hominis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 3 = 16 µg/mL, MIC of tetracycline = 32 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 3 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| FIC of tetracycline | 0 | 0 | 0.25 | 1 | 1 | 1 | 1 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.75 | 1.25 | 1.12 | 1.06 | 1.03 | 1 | 2 | 4 |

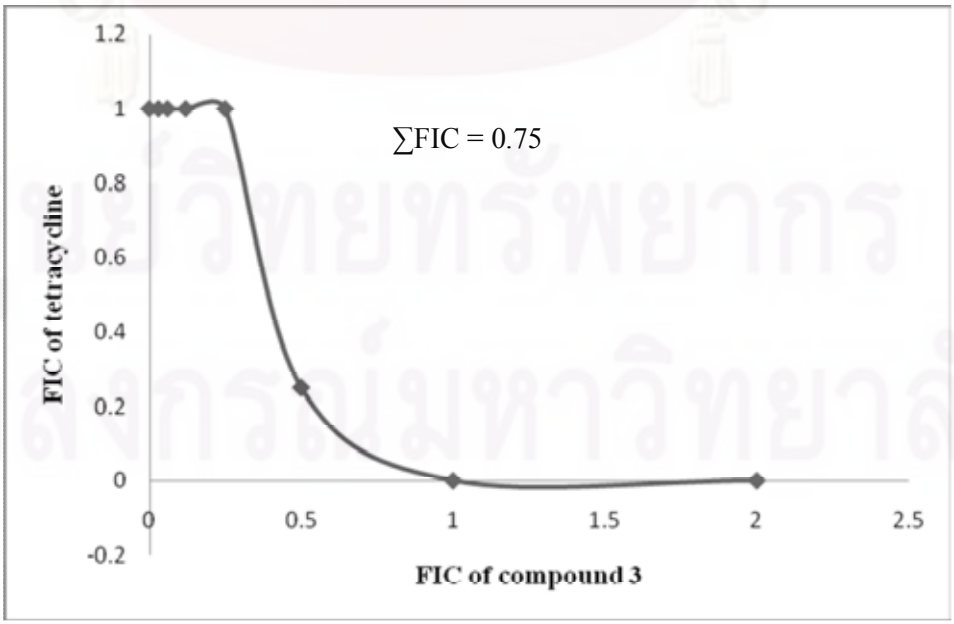
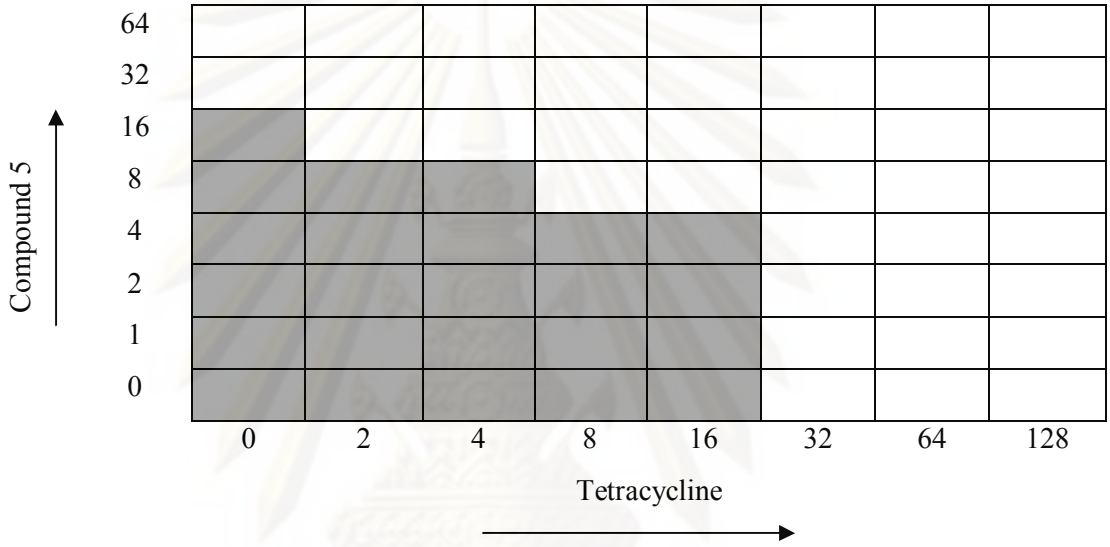


**FIGURE E13** The result of compound 5 combined with tetracycline against *S. hominis*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 5 = 32 µg/mL, MIC of tetracycline = 32 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 5 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of tetracycline | 0 | 0 | 0.25 | 1 | 1 | 1 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.5 | 1.12 | 1.06 | 1.03 | 1 | 2 | 4 |

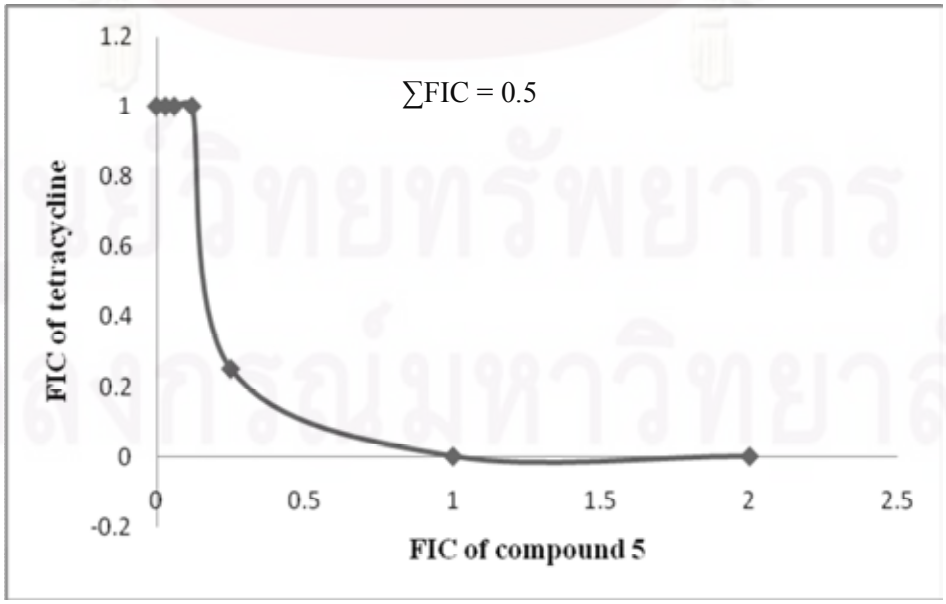
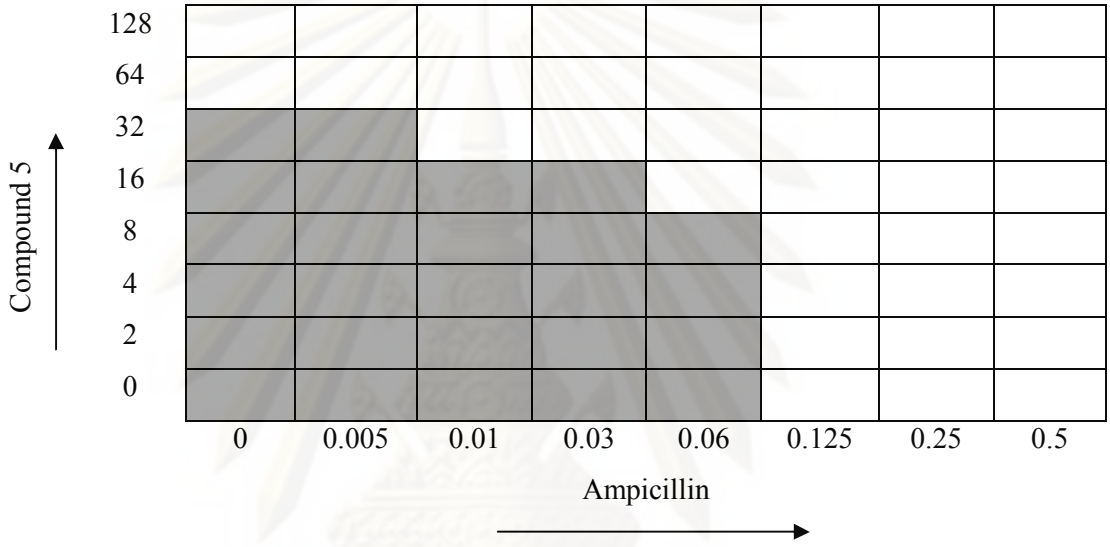


**FIGURE E14** The result of compound 5 combined with ampicillin against *S. aureus*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 5 = 64 µg/mL, MIC of anpicillin = 0.125 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 5 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of ampicillin | 0 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.75 | 1.12 | 1.06 | 1.03 | 1 | 2 | 4 |

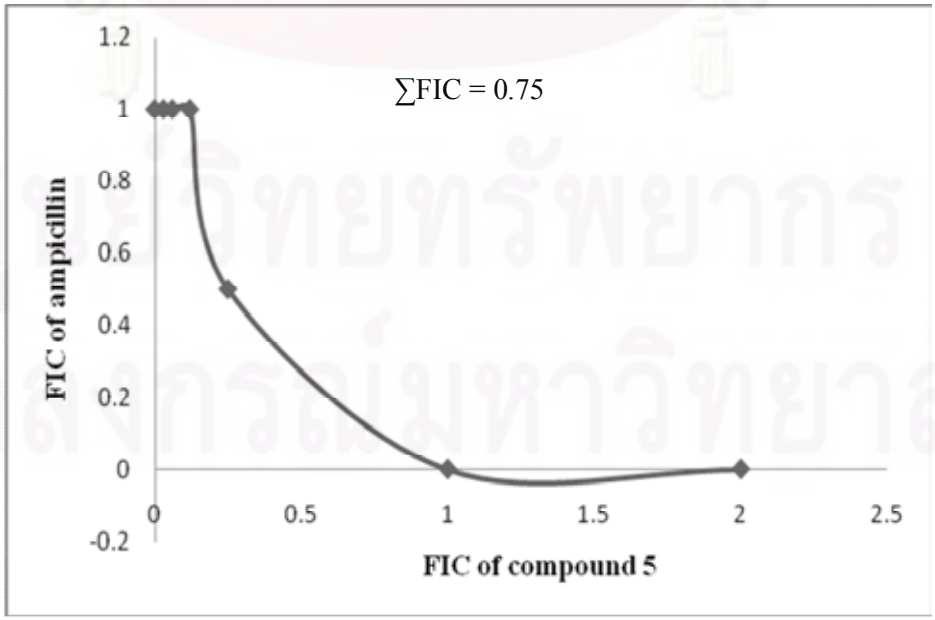
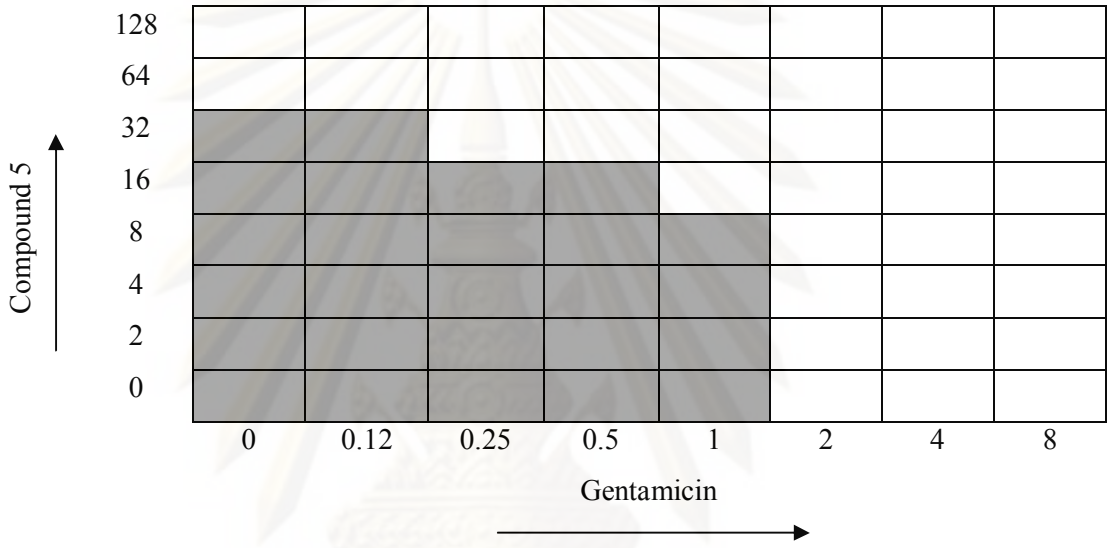


**FIGURE E15** The result of compound 5 combined with gentamicin against *S. aureus*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 5 = 64 µg/mL, MIC of gentamicin = 2 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 5 | 2 | 1 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of gentamicin | 0 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.75 | 1.12 | 1.06 | 1.03 | 1 | 2 | 4 |

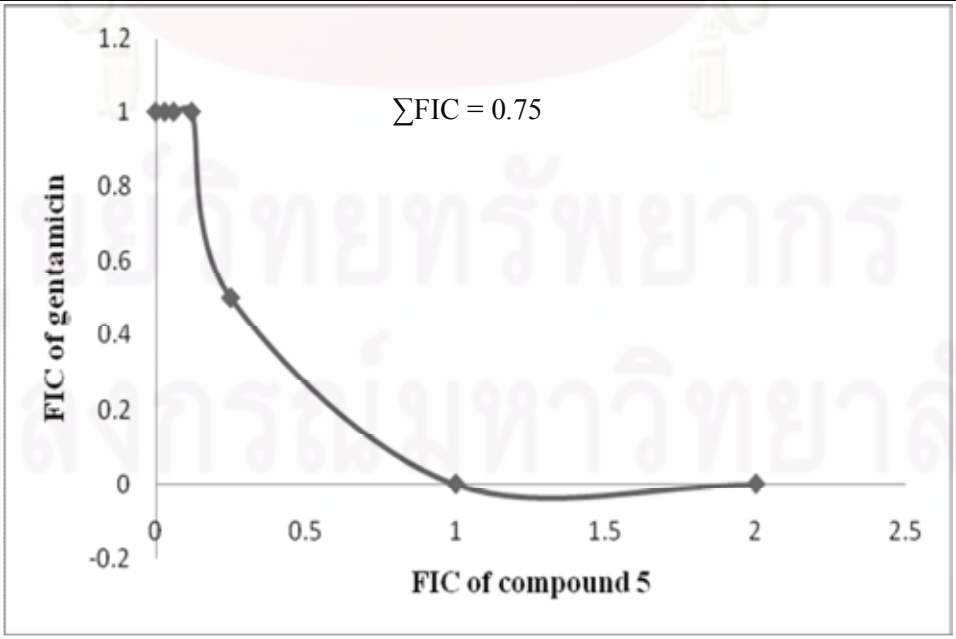
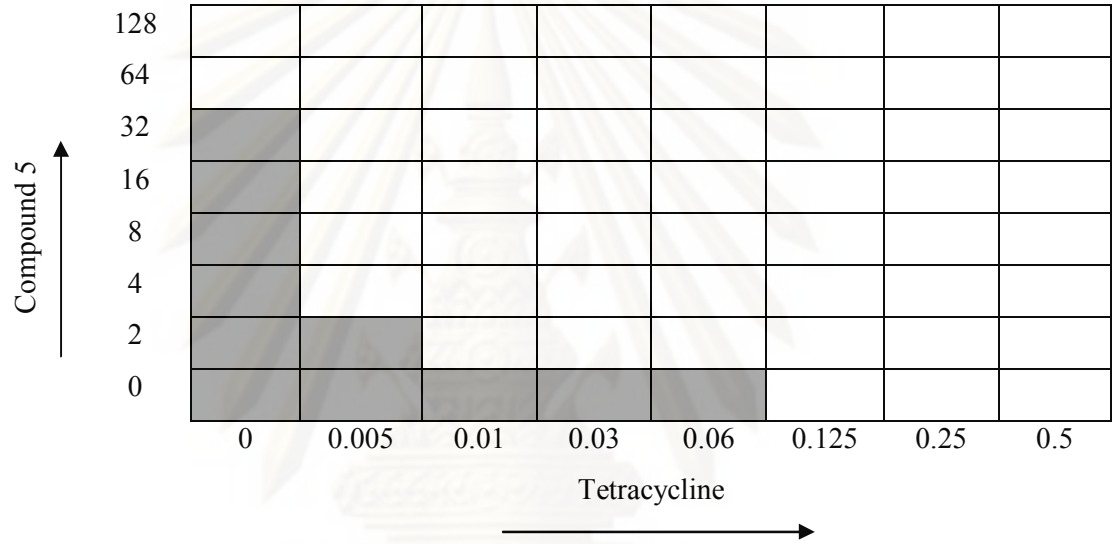


**FIGURE E16** The result of compound 5 combined with tetracycline against *S. aureus*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 5 = 64 µg/mL, MIC of tetracycline = 0.125 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FIC of compound 5 | 2 | 1 | 0.06 | 0.03 | 0 | 0 | 0 |
| FIC of tetracycline | 0 | 0 | 0.06 | 0.12 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.12 | 0.15 | 1 | 2 | 4 |

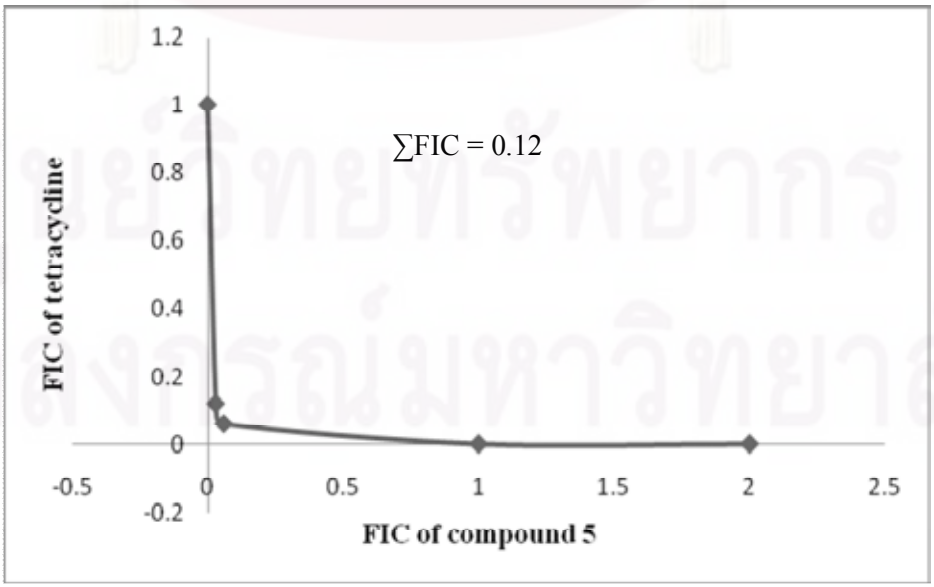
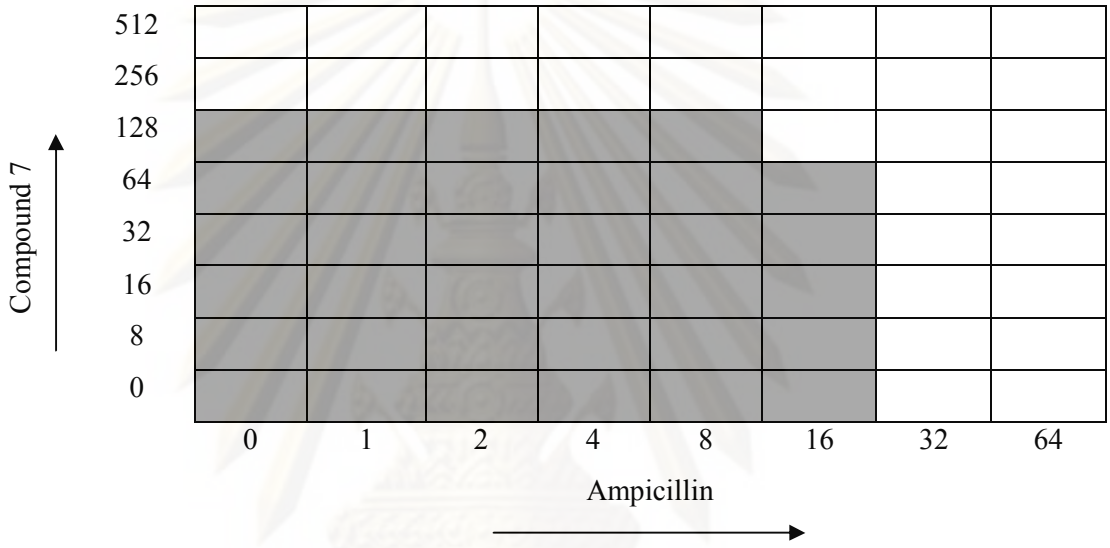


**FIGURE E17** The result of compound 7 combined with ampicillin against *P. vulgaris*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 7 = 256 µg/mL, MIC of ampicillin = 32 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 7 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of ampicillin | 0 | 0 | 0.5 | 1 | 1 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 1 | 1.25 | 1.12 | 1.06 | 1.03 | 1 | 2 |

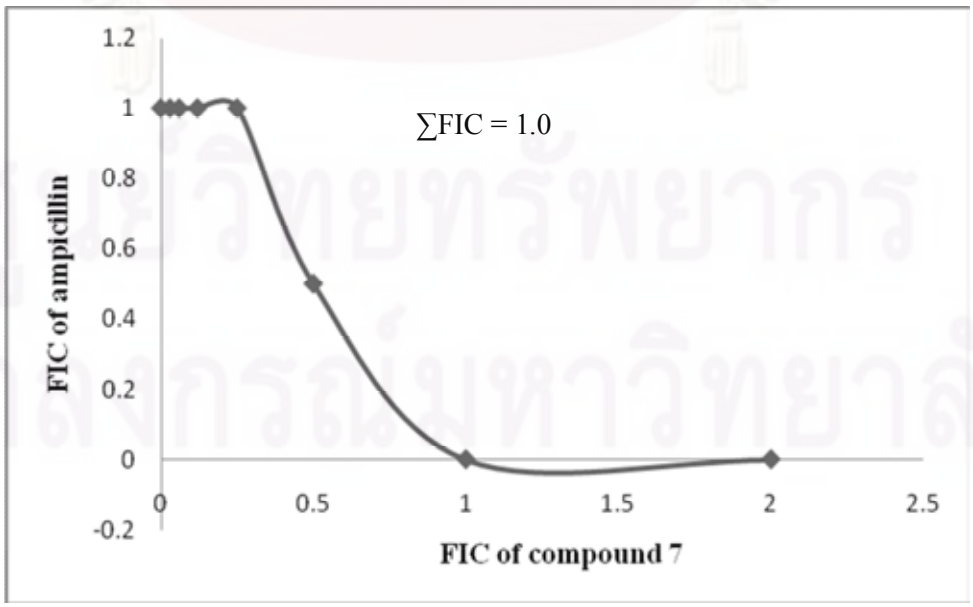
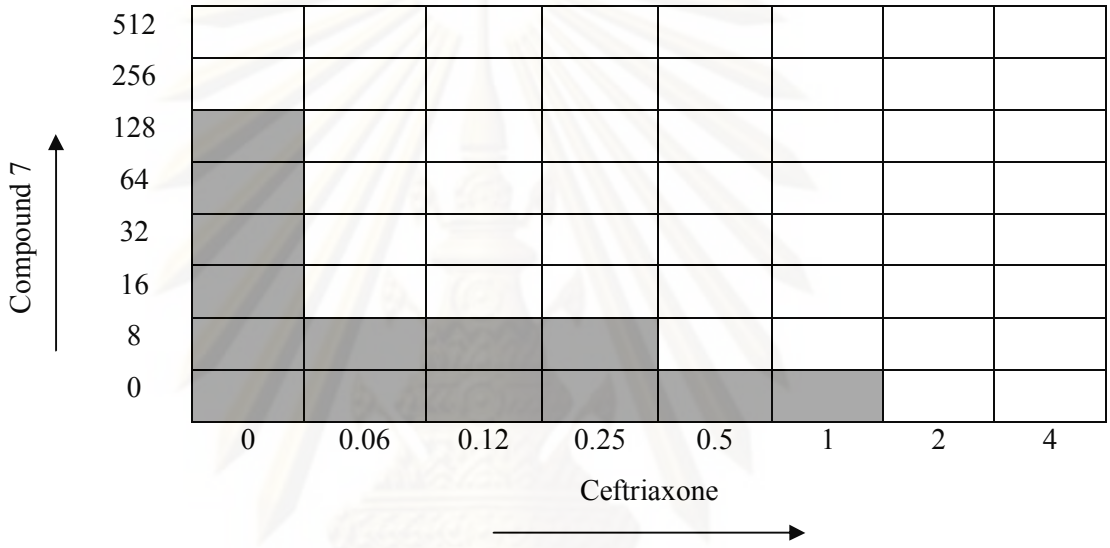


**FIGURE E18** The result of compound 7 combined with ceftriaxone against *P. vulgaris*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 7 = 256 µg/mL, MIC of ceftriaxone = 2 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 7 | 2 | 1 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|
| FIC of ceftriaxone | 0 | 0 | 0.03 | 0.25 | 1 | 2 |
| ∑FIC | 2 | 1 | 0.09 | 0.28 | 1 | 2 |

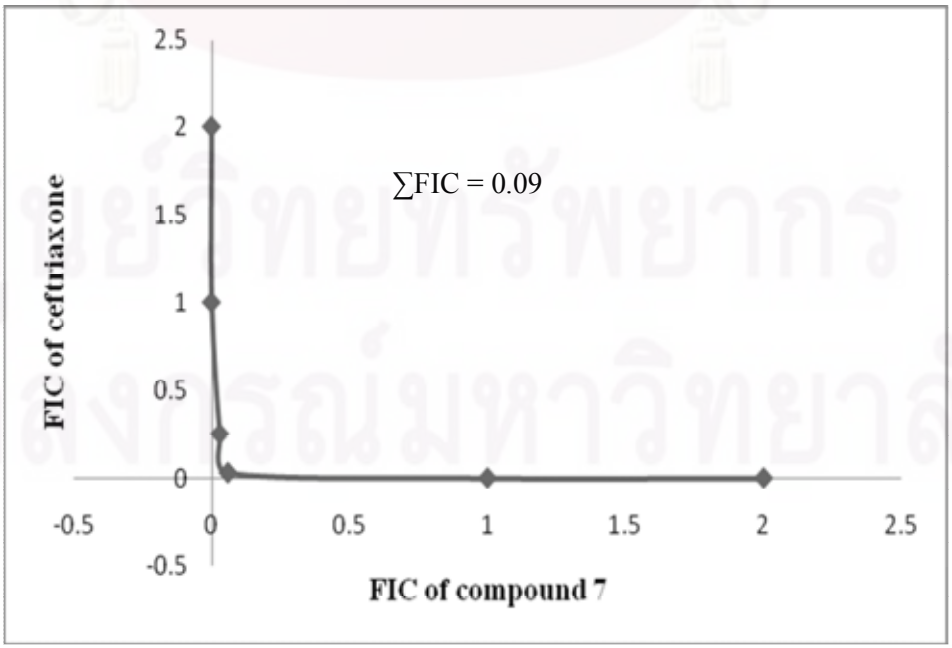
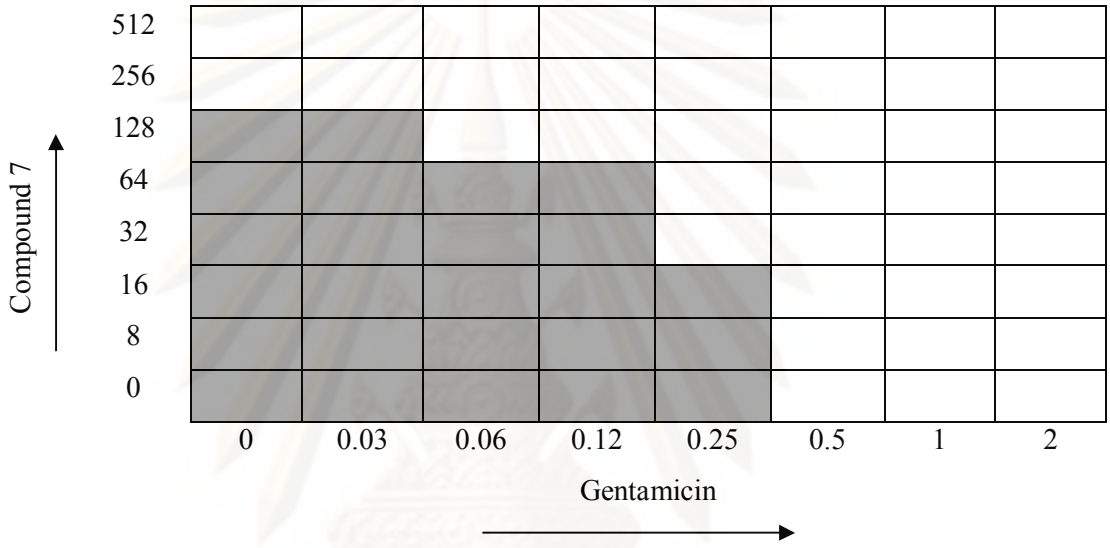


**FIGURE E19** The result of compound 7 combined with gentamicin against *P. vulgaris*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 7 = 256 µg/mL, MIC of gentamicin = 0.5 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 7 | 2 | 1 | 0.12 | 0.06 | 0.03 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|
| FIC of gentamicin | 0 | 0 | 0.5 | 1 | 1 | 1 | 2 | 4 |
| ∑FIC | 2 | 1 | 0.62 | 1.06 | 1.03 | 1 | 2 | 4 |

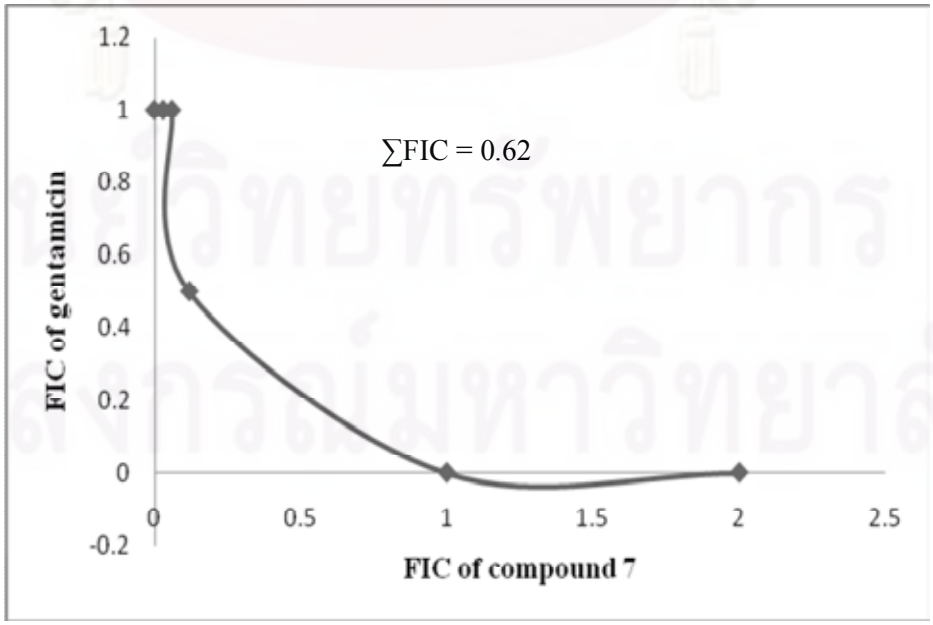
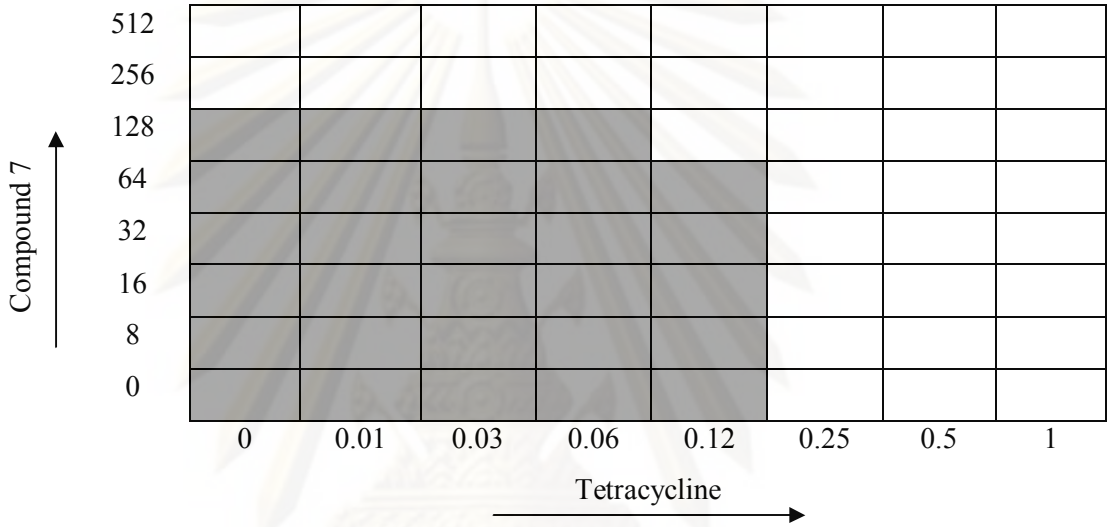


**FIGURE E20** The result of compound 7 combined with tetracycline against *P. vulgaris*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

(MIC of compound 7 = 256 µg/mL, MIC of tetracycline = 0.25 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| FIC of compound 7 | 2 | 1 | 0.5 | 0.25 | 0.12 | 0.06 | 0.03 | 0 | 0 |
|---|---|---|---|---|---|---|---|---|---|
| FIC of Tetracycline | 0 | 0 | 0.5 | 1 | 1 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 1 | 1.25 | 1.12 | 1.06 | 1.03 | 1 | 2 |

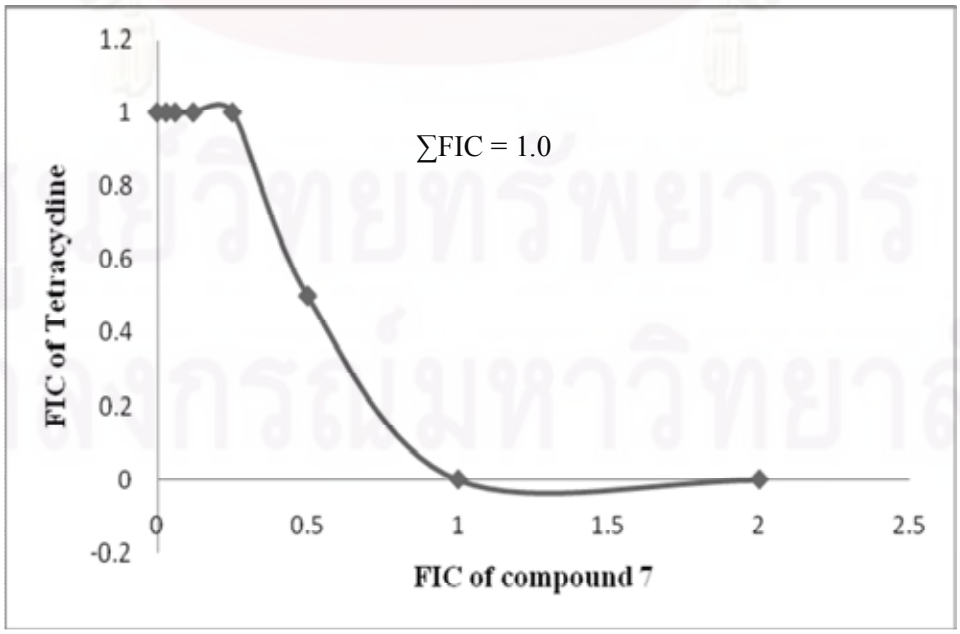
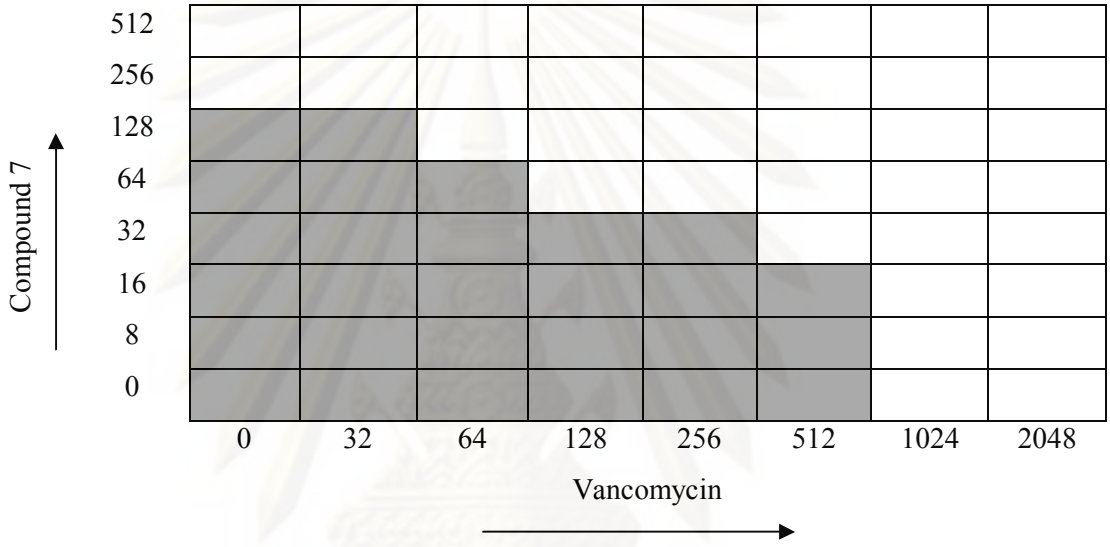


**FIGURE E21** The result of compound 7 combined with vancomycin against *P. vulgaris*, calculation of ∑FIC, and isobologram constructed from checkerboard MIC data

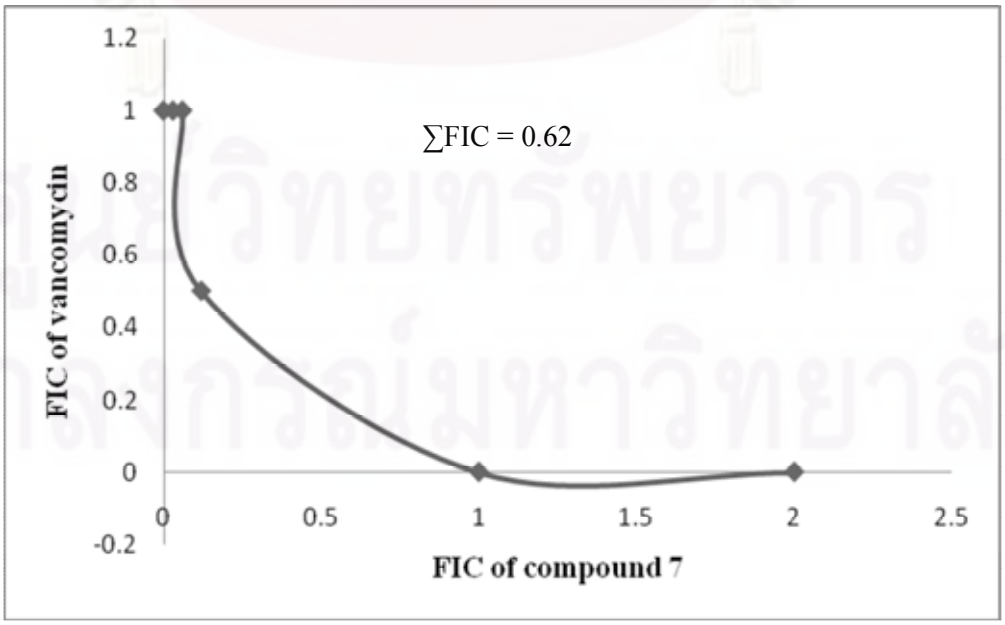
(MIC of compound 7 = 256 µg/mL, MIC of vancomycin = 1024 µg/mL)

* shading area showed growth of organism

** no shading area showed no growth of organism




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FIC of compound 7 | 2 | 1 | 0.12 | 0.06 | 0.03 | 0 | 0 |
| FIC of vancomycin | 0 | 0 | 0.5 | 1 | 1 | 1 | 2 |
| ∑FIC | 2 | 1 | 0.62 | 1.06 | 1.03 | 1 | 2 |



# BIOGRAPHY

MissPhunlap Pompeng was born on December 2nd, 1983 in Chiang Mai Province, Thailand. She received her Bachelor's degree of Science Program in Physical Therapy, Faculty of Allied Health Sciences, Chulalongkorn University in 2006. In year 2007, she was admitted into a Master Degree program in biotechnology, Faculty of Science, Chulalongkorn University and completed the program in 2009.