สารออกฤทธิ์ทางชีวภาพที่สร้างโดยราเอนโดไฟต์ที่คัดแยกจากผักบุ้งทะเล *Ipomoea pes-caprae* Linn.

นายวชิร ใจภักดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

BIOACTIVE COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGI ISOLATED FROM BEACH MORNING GLORY *Ipomoea pes-caprae* Linn.

Mr. Vachira Chaipackdee

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2011

Thesis Title	BIOACTIVE COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGI ISOLATED FROM BEACH MORNING GLORY *Ipomoea pes-caprae* Linn.
By	Mr. Vachira Chaipackdee
Field of Study	Biotechnology
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วชิร ใจภักดี : สารออกฤทธิ์ทางชีวภาพที่สร้างโดยราเอนโดไฟต์ที่คัดแยกจากผักบุ้งทะเล *Ipomoea pes-caprae* Linn. (BIOACTIVE COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGI ISOLATED FROM BEACH MORNING GLORY *Ipomoea pes-caprae* Linn) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. สุรชัย พรภคกุล, 100 หน้า.

ราเอนโดไฟ ต์จำนวน 150 ไอโซเลตแยกได้จาก ใบ ก้าน และเมล็ด ของผักบุ้งทะเล จาก ชายหาด ในประเทศไทย จากลักษณะทางสัณฐานวิทยาสามารถจำแนกราดังกล่าวได้เป็น สกุล *Alternaria*, *Aspergillus*, *Collectotrichum*, *Curvularia*, *Eurotium*, *Fusarium*, *Geotrichum*, *Penicillium*, *Phomopsis*, *Stemphylium*, *Trichoderma*, *Xylaria* และ *Mycelia sterilia* นำราเอนโดไฟต์ที่คัดแยกได้ทุก ไอโซเลตมาทดสอบคุณสมบัติการยับยั้งการเจริญของจุลินทรีย์ 5 ชนิด ได้แก่ *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* และ *Candida albicans* พบว่าราเอนโดไฟต์ไอโซเลต Cm1 และ Ch109 มีฤทธิ์ยับยั้งการเจริญของจุลินทรีย์ทดสอบสูง จึงนำราทั้ง 2 ไอโซเลตนี้มาทำการศึกษาต่อไป จากตรวจสอบด้วยกล้องจุลทรรศน์และการวิเคราะห์ลำดับเบสบริเวณ ITS ระบุว่า Cm1 คือสปีชีส์ *Stemphylium solani* และ Ch109 คือ สปีชีส์ *Eurotium amstelodami* จากการ คัดแยกสารออกฤทธิ์ทางชีวภาพโดยใช้เอทธิลอะซิเตทสกัดเส้นใยของรา Cm1 แล้วนำมาทดสอบฤทธิ์พบว่า สาร สกัด ME-10 มีฤทธิ์ยับยั้งการเจ ริญของจุลินทรีย์ทดสอบ ได้ดีที่สุดโดยให้ความกว้างของบริเวณยับยั้งใ นช่วง 13-25 มิลลิเมตร เมื่อนำมาทดสอบฤทธิ์ต้านอนุมูลอิสระพบว่าค่า IC_{50} ของสาร ME-10 เป็น 44 ไมโครกรัมต่อมิลลิลิตร และมีค่า LC_{50} ต่อไรทะเลเป็น 414 ไมโครกรัมต่อมิลลิลิตร ส่วนความเป็นพิษต่อเซลล์มะเร็ง 5 ชนิดได้แก่ BT474, CHAGO, HEP-G2, KATO-3 และ SW620 มีค่า IC_{50} เป็น 14.05, 10.40, 8.41, 8.91 และ 10.23 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สำหรับราไอโซเลต Ch109ได้ทำการสกัดสารออกฤทธิ์ทางชีวภาพจากเส้นใยด้วยเอทธิอะซิเตท เมื่อนำมาแยกให้บริสุทธิ์ พบว่าได้สารประกอบ 4 ชนิด ประกอบด้วย tetrahydroauroglaucin, flavoglaucin, physcion และ erythroglaucin การทดสอบ คุณสมบัติการยับยั้งการเจริญของจุลินทรีย์และฤทธิ์ต้านอนุมูลอิสระของสารประกอบทั้ง 4 ชนิดพบว่า IC_{50} ของสารประกอบ 1 (tetrahydroauroglaucin) สารประกอบ 2 (flavoglaucin) สารประกอบ 3 (physcion) และ สารประกอบ 4 (erythroglaucin) มีค่าเป็น 18, 35.5, 42 และ 32 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ เมื่อเทียบกับวิตามินอีซึ่งมีค่า IC_{50} เป็น 19 ไมโครกรัมต่อมิลลิลิตร คุณสมบัติการยับยั้งการเจริญของจุลินทรีย์ทดสอบ สารประกอบ 1 และ 2 มีค่า MIC ต่อ *B. subtilis* เท่ากันคือ 250 ไมโครกรัมต่อมิลลิลิตร และค่า MIC ต่อ *S. aureus* เท่ากันคือ 250 ไมโครกรัมต่อมิลลิลิตร สารประกอบ 3 และ 4 มีค่า MIC ต่อ *B. subtilis* เท่ากันคือ 50 ไมโครกรัมต่อมิลลิลิตร และค่า MIC ต่อ *S. aureus* เท่ากันคือ 80 ไมโครกรัมต่อมิลลิลิตร

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

4973888023 : MAJOR BIOTECHNOLOGY

KEYWORDS : endophytic fungi / *Ipomoea pes-caprae* Linn / bioactive compounds / biological assays

VACHIRA CHAIPACKDEE : BIOACTIVE COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGI ISOLATED FROM BEACH MORNING GLORY *Ipomoea pes-caprae* Linn.

ADVISOR: ASSOC. PROF. SURACHAI PORNPAKAKUL, Ph.D., 100 pp.

150 endophytic fungi were isolated from leaves, stems, flowers and seeds of *Ipomoea pes-caprae* Linn. collected from beaches of Thailand. The genera of isolated endophytic fungi comprised of *Alternaria*, *Aspergillus*, *Collectotrichum*, *Curvularia*, *Eurotium*, *Fusarium*, *Geotrichum*, *Penicillium*, *Phomopsis*, *Stemphylium*, *Trichoderma*, *Xylaria*. and *Mycelia sterilia*. All of selected endophytic fungi were tested antimicrobial activity against 5 microorganisms; *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Endophytic fungi isolate Cm1 and Ch109 were exhibited high antimicrobial activity against test microorganisms. Thus both fungi were selected for further studies. Based on light microscope identification and DNA sequencing of the ITS region, the fungus isolate Cm1 was identified as *Stemphylium solani* and Ch109 was identified as *Eurotium amstelodami.* Isolation of bioactive compounds from mycelial EtOAc extracted of Cm1 found that combined fraction ME-10 exhibited the best result of antimicrobial activity with 13-25 mm of clear zone width. The antioxidant test of ME-10 showed IC_{50} 44 µg/ml and LC_{50} of brine shrimp lethality test was 414 µg/ml. The cytotoxicity test of ME-10 with 5 cell lines; BT474, CHAGO, HEP-G2, KATO-3 and SW620, IC_{50} were 14.05, 10.40, 8.41, 8.91 and 10.23 µg/ml, respectively. Isolation of bioactive compounds from mycelial EtOAc extract of Ch109 by column chromatography gave 4 compounds including tetrahydroauroglaucin, flavoglaucin, physcion and erythroglaucin. **A**ntimicrobial and antioxidant assay of four compounds were tested. IC_{50} of compound 1 (tetrahydroauroglaucin), compound 2 (flavoglaucin), compound 3 (physcion) and compound 4 (erythroglaucin) were exhibited antioxidant with IC_{50} of 18, 35.5, 42 and 32 µg/ml, respectively while vitamin E was exhibited antioxidant with IC_{50} of 19 µg/ml. Antimicrobial activity of compound 1-2 against *B. subtilis* with MIC of 250 µg/ml and *S. aureus* with MIC of 250 µg/ml. Compound 3 and 4 showed antimicrobial activity against *B. subtilis* with the same MIC of 50 µg/ml and against *S. aureus* with MIC of 80 µg/ml

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and sincere gratitude to Associate Professor Surachai Pornpakakul, my thesis adisor, who gave me the comments and knowledge.

I would like to thank the Office of National Research Council of Thailand and Chulalongkorn University Graduate School for giving thesis scholarship.

Finally I am extremely grateful to my father, my mother and everyone who are my loves.

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LIST OF ABBREVIATIONS

ATCC	American Type
bp	base pair
°C	degree Celsius
Cm	centimeter
^{13}C NMR	carbon-13 nuclear magnetic resonance
COSY	Correlated Spectroscopy
GPS	Global Positioning System
HMBC	Heteronuclear Multiple Bond Correlation
^{1}H NMR	proton nuclear magnetic resonance
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
m	meter
min	minute
ml	milliliter
mm	millimeter
mg	milligram
µg	microgram
µm	micrometer
nm	nanometer
No.	number
ppm	part per million
sp.	Species
TLC	thin layer chromatography
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet
δ	Chemical shift

CHAPTER I

INTRODUCTION

Endophytic fungi are found in several plant species, it is estimated that there are more than 1 million species of endophytic fungi distributed around the world (Petrini, 1991). Natural products derived from endophytic fungi were recognized as an important bioactive compounds (Strobel et al., 2004). These potential compounds are very useful in medicine, agriculture, food industry and environment (Gunatilaka, 2006). Bioactive compounds of endophytic fungi comprise of antimicrobial, antitumor/cancer, antioxidant, antimalarial, pesticide, cytotoxic, immunosuppressant and other biological control agent (Keller et al., 2005; Pimentel et al., 2011). These compounds are classified as alkaloids, terpenoids, steroids, quinones, phenols and lactones (Zhang et al., 2006).

After discovery of gold bioactive compound named taxol from *Taxomyces andreanae* in 1993 (Stierle et al., 1993), many researchers have aimed to study endophytic fungi in various plants for extraction of the fungal bioactive compounds which can manipulate easier than in plants. Since some endophytic fungi can produce the same bioactive compounds as their host plants, it is beneficial to study the relationships between fungi and plants including to develop efficiency of bioactive compounds (Firakova et al., 2007).

As we know that there are biodiversity of endophytic fungi in the world, various host plants were chosen as the source of endophytic fungi isolation. In this research, *Ipomoea pes-caprae* Linn. (beach morning glory) was used as the source of endophytic fungi isolation and some bioactive compounds were investigated.

Objectives

1. To isolate and identify endophytic fungi from beach morning glory.

2. To investigate bioactive compounds produced by endophytic fungi which are isolated from beach morning glory.

3. To evaluate the biological activities of the isolated compounds

CHAPTER II

LITERATURE REVIEW

2.1 Introduction to endophytic fungi

2.1.1 Definition

Endophytic fungi are microbes living in plants and causing no apparent injury to the host. They are found in intercellular and/or intracellular of host plants (Strobel, 2003). These fungi are found in both bryophytes and tracheophytes and associated with roots, stem, leaves, flowers and seeds (Rodriguez et al., 2005). The relationship between endophytic fungi and host plant is symbiosis but sometimes endophytic fungi can be a pathogens and saprophytes (Fisher and Petrini, 1992). Endophytic fungi generate several kinds of secondary metabolites, usually involving in production of plant growth hormones (eg. auxin, abscisin, ethylene, gibberllin and kinetin) and defense mechanism against pathogenic organisms or other enemies of host plants (Dreyfuss and Chapela, 1994). The biological activities of these compounds are used in many therapeutic drugs such as antimicrobial, anticancer, antioxidant, antimalarial, and nematicide (Keller *et al*., 2005). The schematic of endophytic fungi in plant tissue is shown in Figure 2.1.

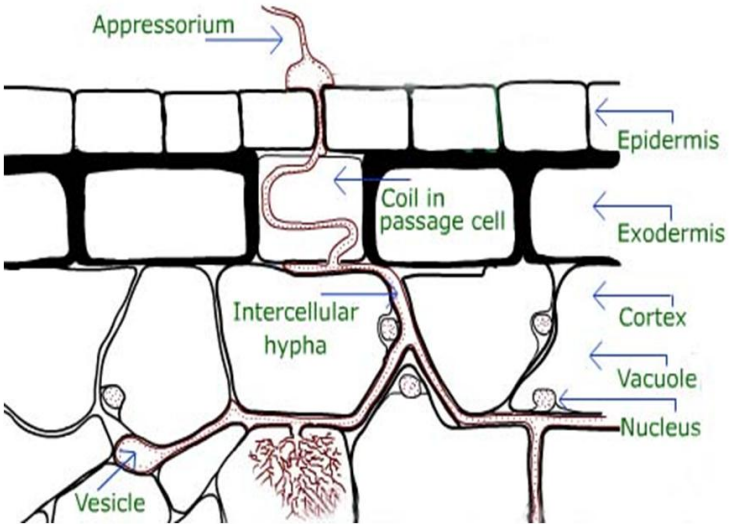


Figure 2.1 The schematic of endophytic fungi in plant tissue

2.1.2 Bioactive source

For decades, scientists have discovered that endophytic fungi play significant role as a sources of potential bioactive compounds (Martin and Gutierrez, 1992). The examples of endophyte species that produced bioactive compounds are showed in Table 2.1.

The researches concerning novel bioactive compounds such as antimicrobial, antimalarial, anticancer, antioxidant, etc., are studied in many ways to solve the problems of drug resistant organisms and various cancers (Strobel, 2003). The medicine derived from bioactive compounds of microorganisms is an interesting choice because they are easy to manipulate. Many researches of fungal metabolites have been investigated such as cytotoxicity, immunosuppressant, enzyme inhibitor, etc. Between 1993 and 2001, 1500 natural products were found and more than half of these had antibacterial, antifungal or anticancer activity (Keller *et al*., 2005).

Hawksworth (2001) estimated that the species of fungi are 1.5 million on the earth and only about 0.1 million species have been described. Strobel and Daisy (2003) reported that nearly 300,000 plant species are being on earth and each plant is the host of endophytic fungi. Schulz *et al* discovered from their research that bioactive compounds derived from endophytic fungi, 51% were new structures while bioactive compounds derived from other soil microbes, 38% were new structures.

Zhao et al. (2011) reviewed about the research progress on biological activities and action mechanisms of bioactive compounds such as paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin, and diosgenin were found from various endophytic fungi.

Daisy and Strobel (2002) revealed that *Muscodor vitigenus* isolated from soapberry could produce naphthalene effectively repelled wheat fly. The *Phomopsis phaseoli* isolated from leaf of the tropical tree produced 3-hydroxypropionic acid was a nematicide metabolite (Schwarz et al., 2004). Tansuwan et al. (2007) reported that there were 2 novel benzoquinone metabolites of *Xylaria* sp. isolated from *Sandoricum koetjape* exhibited antimalrial activity against *Plasmodium falciparum*.

Strobel and Daisy (2003) described an antidiabetic agent produced from endophytic fungus (*Pseudomassaria* sp.) a nonpeptidal metabolite acts as an insulin mimetic but is not destroyed in the digestive tract. Oral administration of this chemical to mouse models of diabetes showed significant lowering of blood glucose levels.

Table 2.1 The species of endophytes and their bioactive compounds.

Bioactive compounds	Endophyte species
Antimicrobials	
- Griseofulvin	- *Xylaria* sp.
- Jesterone	- *Pestalotiopsis jester*
- Decumbin	- *Penicillium decumbens*
- Cytoskyrines	- *Cuvularia lunata*
- Cytosporone	- *Cytospora* sp.
- Munumbicins	- *Streptomyces* spp.
Anticancers	
- Camptothecin	- *Neurospora* sp.
- Penicidone	- *Penicillium* sp.
- Taxol	- *Taxomyces andreanae*
Antioxidants	
- Isopestacin	- *Pestalotiopsis microspora*
- Graphis-lactone A	- *Cephalosporium* sp.
Immunosuppressants	
- Subglutiol A	- *Fusarium subglutinans*
- Mevinic acid	- *Phomopsis* sp.
Biological control agents	
- lotitrem B	- *Neotyphodium lolii*
- Munumbicin D	- *Streptomyces* sp.

Source: Martin and Gutierrez (1992); Joseph and Priya (2011); Zhao et al. (2011)

2.2 Ecology of endophytic fungi

Symbiotic relationships between endophytic fungi and plants are common in nature, beginning from highly parasitic to closely mutualistic. Grasses and woody plants are commonly and ecologically important components of many ecosystems worldwide often infected by endophytic fungi within the stems, leaves, flowers and seeds of annual and perennial plants (Owen and Hundley, 2004).

2.3 Groups of endophytic fungi

2.3.1 Grass endophytic fungi (Clavicipitales) (Bacon and White, 2000)

Members of the Ascomycota which are several fungi that associated with grasses (annual plant). The association includes pathogenic epibiotic (outside living tissue) fungus (e.g. *Claviceps, Myriogenospora*), a party endobiotic fungus with surface fruiting structures (some *Balansia* spp), and an entirely endobiotic fungus with unknown sexual stage. The grass endophytic fungi life cycle is shown in Figure 2.2

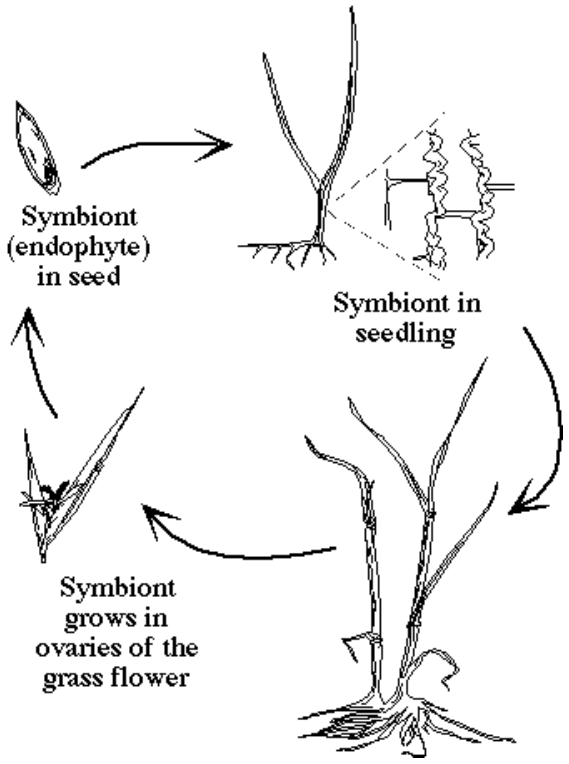


Figure 2.2 Grass endophytic fungi life cycle

2.3.1 Non - Grass endophytic fungi (Non - Clavicipitales) (Taylor, 1996)

Members of several fungi divisions found in perennial plants. Fungi live in leaves, stems, roots, flowers and seeds of host plants. There are both asexual (anamorph) and sexual stage (teleomorph). The non – grass endophytic fungi holomorph life cycle is shown in Figure 2.3.

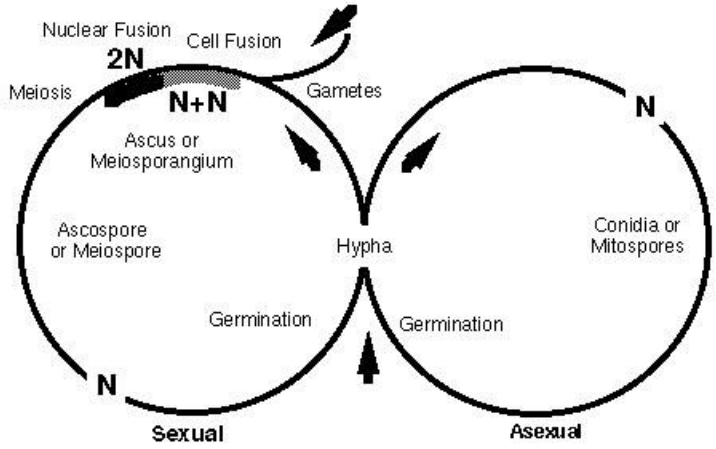


Figure 2.3 Non - Grass endophytic fungi holomorph life cycle

2.4 Bioactive compounds of endophytic fungi

Endophytic fungi have been recognized as a source of novel secondary metabolites, some of which had beneficial biological activities (Bills and Polishook, 1991; Strobel and Daisy, 2003). There were many reports about biological compounds produced by endophytic fungi in culture that were active against many organisms.

Pimentel et al. (2011) classified bioactive compounds produced by endophytic fungi into major 5 categories; antimicrobial, antitumor/anticancer, antioxidant, immunosuppressant and biological control agent. The researches of bioactive compounds from endophytic fungi are as follows.

2.4.1 Antimicrobial

Antimicrobial are defined as low molecular weight organic natural products made by microorganisms that are active at low concentration against other microorganisms (Strobel and Daisy, 2003).

Fan et al. (2010) observed two new metabolites 7-epiaustdiol and 8-O-methyl-epiaustdiol of *Talaromyces* sp. that were isolated from stem bark of *Kandelia candel* (L.) Druce, Rhizophoraceae. These compounds showed antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Sarcina ventriculi, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger,* and *Fusarium oxysporum.*

Wu et al. (2011) studied the high broad- spectrum antimicrobial activity of endophytic fungus, *Aspergillus aculeatus* YM311498 (CCTCC NO: M2010062), from *Azadirachta indica* that can inhibit *Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Hormodendrum compactum, Microsporum gypseum, Trichophyton gypseum, Aspergillus niger, Botrytis cinerea, Cunninghamella* sp.*, Fusarium avenaceum, Trichoderma* sp.*, Penicillium islandicum* and *Helminthosporium maydis*.

Park and his colleagues (2005) proved that *Xylaria* sp. isolated from Korean fir could produce griseofulvin which has strong antifungal activity against several plant pathogenic fungi (Structure of griseofulvin is shown in Figure 2.4).

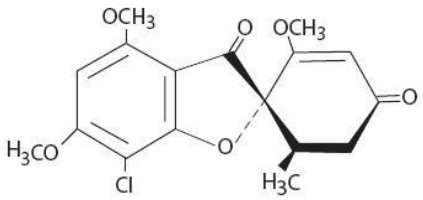


Figure 2.4 Griseofulvin

Raviraja et al. (2006) found that endophytic fungi, *Alternaria* sp., *Nigrospora oryzae* and *Papulospora* sp., from medicinal plant showed inhibitory activity against both Gram-positive and Gram-negative bacteria. Huang et al. (2008) isolated cytosporone from the mangrove endophytic fungi; *Phomopsis* sp. could inhibit growth of *Fusarium* sp. Lubertozzi and Keasling (2009) described the antibacterial activity of penicillium produced by endophytic fungi *Penicillium chrysogenum* isolated from marine red algae (Structure of penicillin is shown in Figure 2.5). Jin et al. (2011) studied 28 isolates of *Aquilaria sinensis* found that 13 isolates (46.4%) showed antibacterial activity against at least one of the test strains by agar well diffusion method. Chen et al. (2011) isolated endophytic fungi from *Agrimonia pilosa* Ledeb found

antibacterial activity of *Penicillium* sp., *Acremonium* sp., *Fusarium* sp., *Rhizopus* sp., *Arachniotus* sp., *Mucor* sp. and *Sclerrotinia* sp. against the growth of *Staphylococcus aureus*.

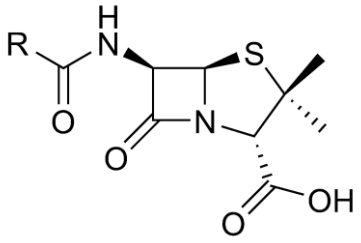


Figure 2.5 Penicillin

2.4.2 Anticancer and Antitumor

Paclitaxel (Taxol), a well- known and highly functionalized tetracyclic diterpenoid bioactive compound (Figure 2.6). In 1971, a paclitaxol producing endophytic fungus *Taxomyces andreanae* was found originally from the bark of *Taxus brevifolia* (Wani et.al., 1971). It has been proved with an efficient action against prostate, ovarian, breast and lung cancers. In 1993, paclitaxel production was successful discovered from the Pacific yew (*Taxus brevifolia*) is the world's first billion dollar anticancer drug (Stierle et al., 1993).

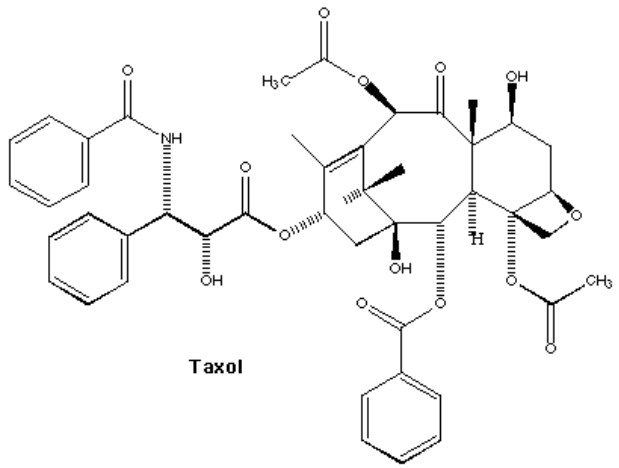


Figure 2.6 Paclitaxel (Taxol)

Stierle and Stierle (2000) showed that *Pestalotiopsis* spp., endophytic fungi isolated from pacific yew tree could produce paclitaxel, an efficient chemotherapeutic drug called taxol which are used to treat breast and ovarian cancers. Liangsakul (2003) reported that there were 8 bioactive compounds of endophytic fungi from *Croton oblongifolius* exhibited cytotoxic activity against several cancer cell lines. Kussari et al. (2009) reported that endophytic fungi isolated from *Camptotheca acuminate* (cancer tree) could produce camptothecin, a potent anticancer agent. Jin et al. (2011) found that 23 endophytic fungi isolated from *Aquilaria sinensis* has antitumor activity against at least one of five test cell lines by MTT assay. Zhang et al. (2010) extracted anthracenedione derivatives (Figure 2.7) from mangrove endophytic fungi *Halorosellinia* sp. and *Guignardia* sp. These compounds showed strong anticancer activity against KB and KBv200 cell lines with the IC_{50} values of 3.17 and 3.21 μM, respectively.

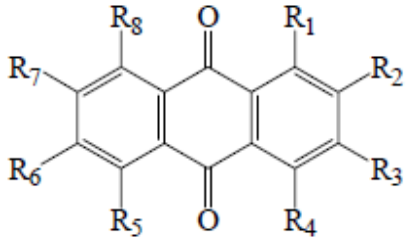


Figure 2.7 Anthracenedione; R = methyl, methoxy or hydroxyl

2.4.3 Antioxidant

Antidoxidant metabolites are often produced by endophytic fungi. Pestacin and isopestacin (Figure 2.8) were isolated from *Pestalotiopsis microspora* from plant *Termimalia morobensis*, native of the Papua New Guinea (Strobel et al., 2004). Chomcheon et al. (2009) extracted 3 compounds including corynesidones A and B and corynether A (Figure 2.9) from endophytic fungi *Corynespora cassiicola* these compounds exhibited antioxidant activity.

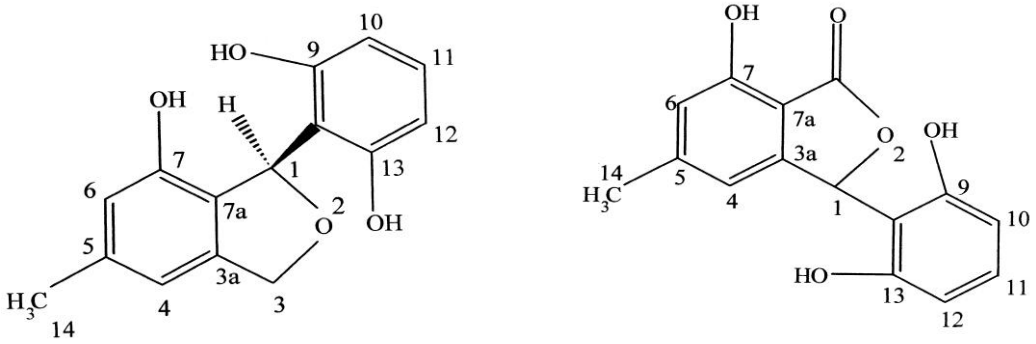


Figure 2.8 Pestacin (left) and isopestacin (right)

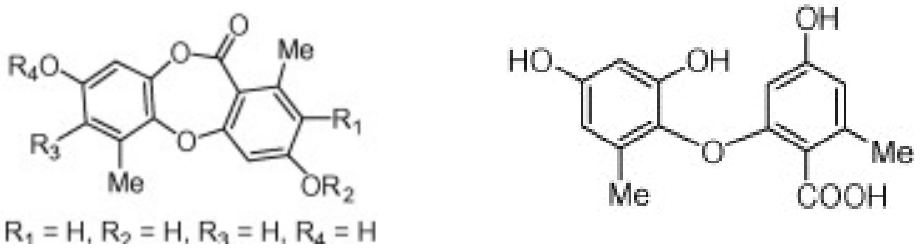


Figure 2.9 Corynesidones A (left) and corynether A (right)

2.4.4 Immunosuppressants

Immunosuppressive drugs are used to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such a rheumatoid arthritis and insulin-dependent diabetes (Lee et al., 1995). The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressant but noncytotoxic subglutinol A and B (Figure 2.10). Subglutinol A and B are efficient in the mixed lymphocyte reaction assay and thymocyte proliferation assay, with a 50% inhibitory concentration of 0.1 μM. (Lee et. al., 1995). Fujimoto et al. (1998) discovered new immunosupressants, kobiin and three kobifuranones which suppressed mouse spleen lymphocytes proliferation. The structures of kobiin and kobifuranone A, B and C are shown in figure 2.11.

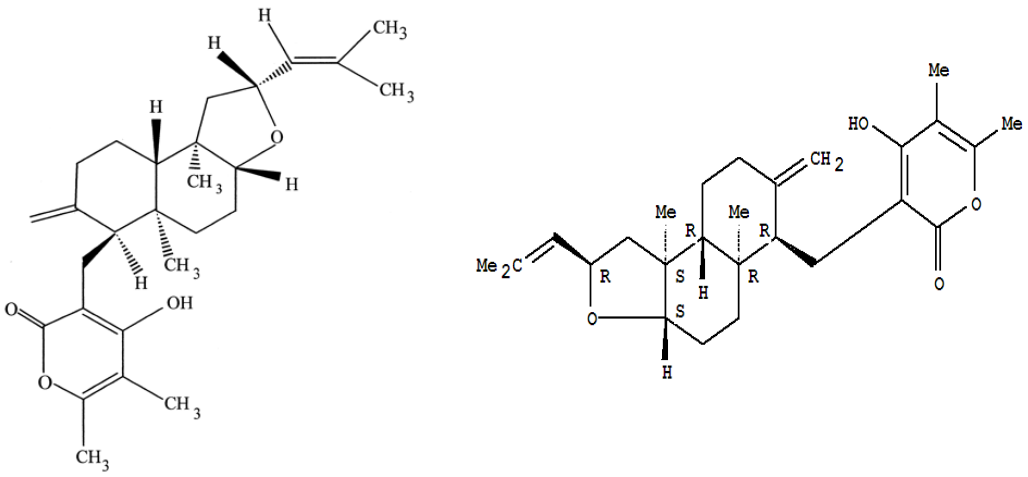


Figure 2.10 Subglutinol A (left) and B (right)

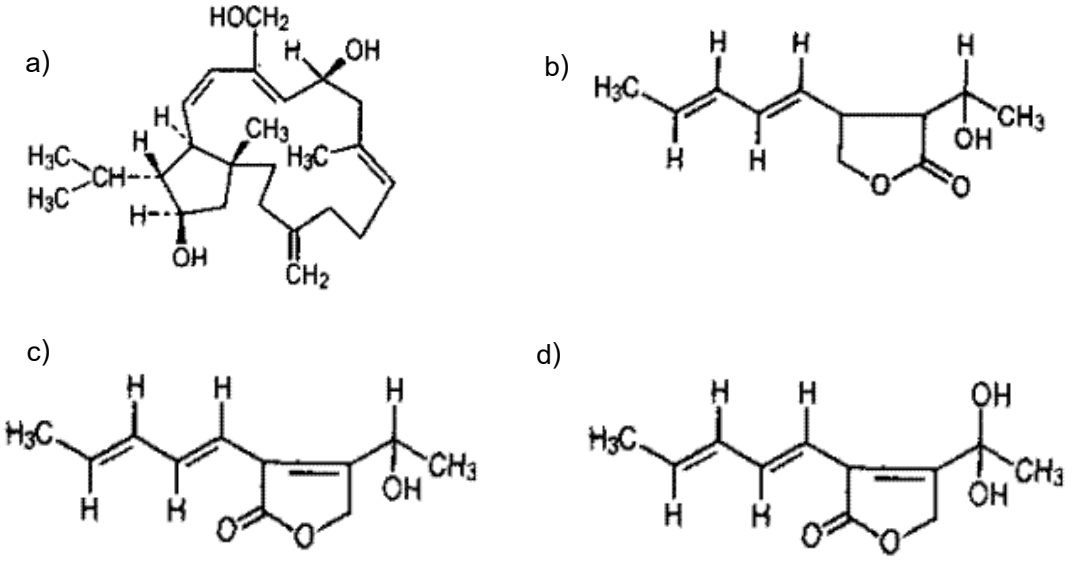


Figure 2.11 Kobiin (a), Kobifuranone A (b), Kobifuranone B (c) and Kobifuranone C (d)

2.4.5 Biological control agents

The symbiotic relationship between perennial ryegrass and its endophytic fungus, *Neotyphodium lolii*, is considerable agronomic significance in general livestock. *N. lolii* infects perennial ryegrass and secretes a neuromuscular mycotoxin, lolitrem B to protect plant from grazing animals (Ball et al., 1997) (Figure 2.12). Several endophytic fungi produce lytic enzymes which can hydrolyze polymer such as cellulose, hemicelluloses, chitin, protein and DNA for benefits of their lives directly and suppress plant pests and pathogens for indirectly benefits (Zhao et al., 2011).

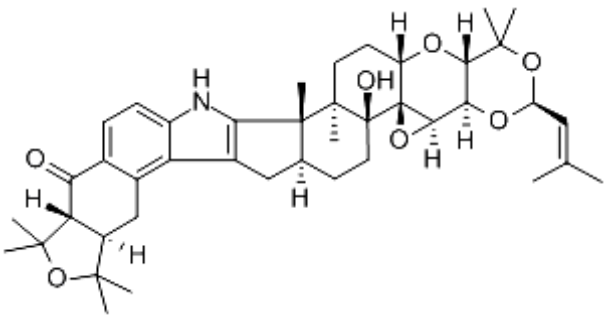


Figure 2.12 Lolitrem B

2.5 Plant sample (*Ipomoea pes-caprae* Linn.)

Plants Classification

Kingdom: Plantae

Class: Magnoliophyta

Class: Magnoliopsida

Order: Solanales

Family: Convolvulaceae

Genus: *Ipomoea*

Species**:** *I. pes-caprae*

Common name: beach morning glory or Goat's footprint

Description

A common creeping vine plant belonging to the genus *Ipomoea*. It distributes from tropical to subtropical regions, grows on the upper parts of beaches and may creep along the ground to 75 feet length. Their roots occasionally occur as branches from the nodes and develop a long thick, starchy root. The leaf shape reminds one of a goat's footprint and the 2 to 4 inch long leaves are thick, smooth and two-lobed. Beach morning glory is truly charming when in bloom. Funnel-shaped flowers that are 2 to 3 inches wide occur in the summer and fall. The flowers are pinkish lavender with purple red throats. They open in the early morning and close before noon each day that the plant is in bloom. Small, round seedpods that contain four velvety, dark-brown seeds appear on this plant after flowering. (Austin and Huáman, 1996). Beach morning glory is a medicinal plant used in many countries for the treatment of several ailments, involving inflammatory and analgesic processes. Some parts of the world use beach morning glory to treat fatigue, strain, arthritis and rheumatism (Tao et al., 2008). Bioactive compounds in beach morning glory exhibit antihistamine, anti-inflammatory and antiseptic activities. Its chemical compositions contain essential oils, eugenol, ergotamine, organic acids, resin glycosides, etc.. Beach morning glory has potential bioactivities such as antibacterial, antifungal and cytotoxicity (Yu et al., 2011).

Bioactive compounds from *Ipomoea pes-caprae* Linn.

Karthikeyan and Selvaraj (2009) isolated mycorrhizal endophytic fungi 6 genera; *Acaulospora, Entrophospora, Glomus, Gigaspora, Sclerocystis* and *Scutellospora* from beach morning glory roots. Beena et al. (2000) isolated 31 filamentous endophytic fungi species (19 Deuteromycetes, 6 Ascomycetes and 6 sterile fungi) from beach morning glory.

Barni et al. (2009) extracted flavonoid (hesperidin) from leaves and stems of beach morning glory that showed analgesic, anti-inflammatory and wound healing properties.

Yu et al. (2011) extracted pentasaccharide resin glycosides from the aerial parts of beach morning glory. Ten compounds were evaluated their potencies to modulate multidrug resistance in the human breast cancer cell line MCF-7/ADR. The combined use of these new compounds at a concentration of 5 μg/ml increased the cytotoxicity of doxorubicin by 1.5-3.7-fold.

Martinez et al. (2010) extracted pescapreins which are CHCl3 soluble resin glycosides from beach morning glory. Compounds were tested for antibacterial and resistance-modifying activity against strains of multidrug resistance *Staphylococcus aureus*. All of the pescapreins potentiated the action of norfloxacin against the NorA over-expressing strain by 4-fold.

Agoramoorthy et al. (2008) extracted polyphenols from beach morning glory which exhibited antioxidant activity. The DPPH radical scavenging activity was 83.7 μg/ml

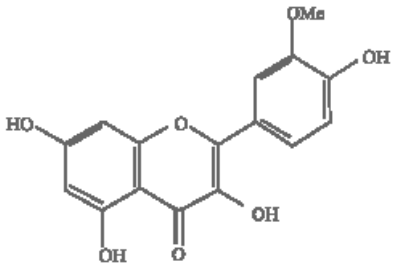
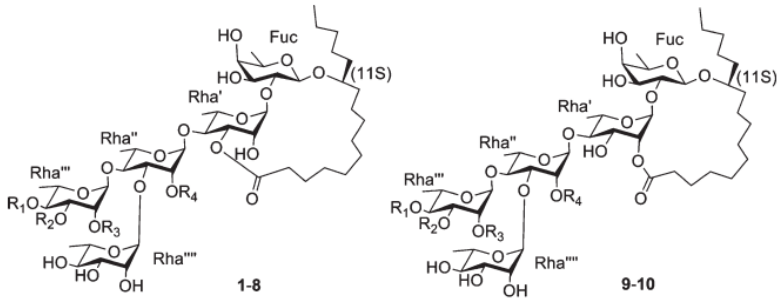
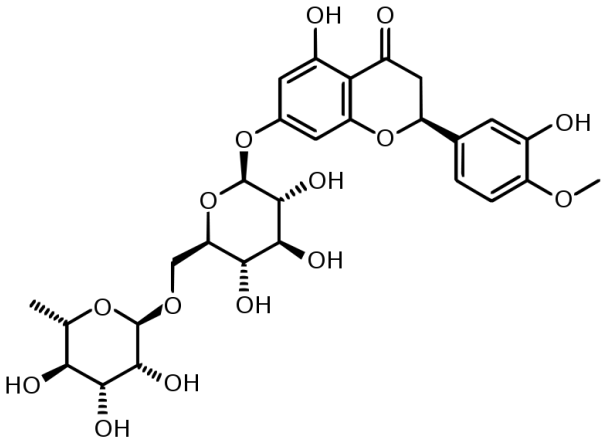
Liang et al. (2002) extracted phenylalanol derivatives from beach morning glory which used to prepare medical formulations for treating hepatitis B.

Wang et al. (2006) extracted 7 compounds from beach morning glory which are amarin, α-amyrin, β-sitosterol, stigmasterol, spinasterol, isoscopoletin and esculetin.

Marie et al. (2007) extracted essential oils from leaves of beach morning glory and found 11 major chemical components; 8-cedren-13-ol, nerolidol, guaiol, cadinol, limonene, caryophyllene, copaene, germacrene D, phytol, cadinene and humulene. The essential oils exhibited anti-hemorrhoidal activity.

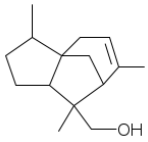
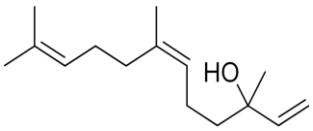
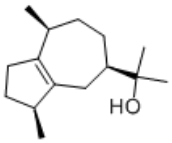
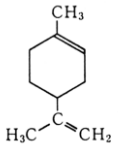
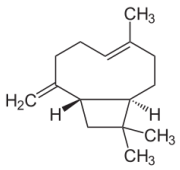
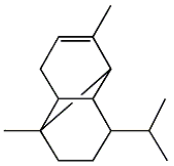
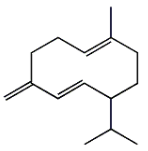
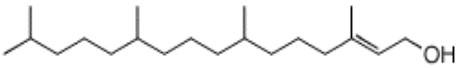
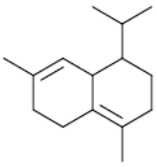
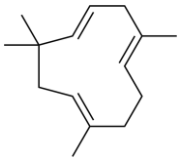
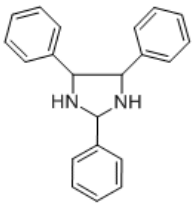
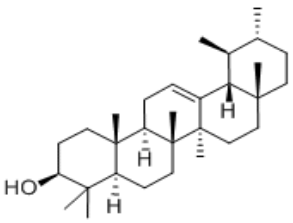
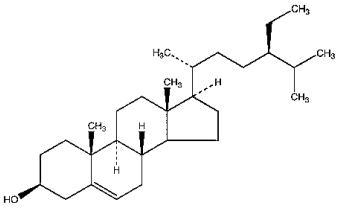
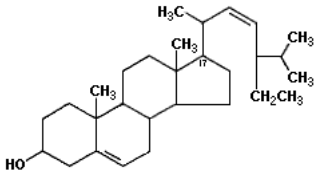
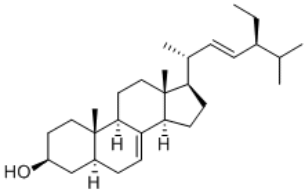
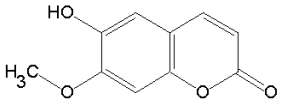
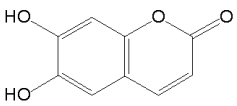
The structure of compounds extracted from beach morning glory are shown in Table 2.2

Table 2.2 Bioactive compounds extracted from *Ipomoea pes-caprae* Linn.

Compounds	References
 Polyphenol	Agoramoorthy et al. (2008)
	Yu et al. (2011)
 Hesperidin	Barni et al. (2009)

	R_1	R_2	R_3	R_4
1	2*S*-methylbutanoyl = Mba	*trans*-cinnamoyl = CA	H	*n*-dodecanoyl = Dodeca
2	Mba	H	CA	Dodeca
3	Mba	CA	H	*n*-decanoyl = Deca
4	Mba	H	CA	Deca
5	2-methylpropanoyl = Iba	CA	H	Dodeca
6	Iba	CA	H	Deca
7	Mba	CA	H	Mba
8	Mba	CA	H	*n*-octanoyl = Octa
9	Mba	CA	H	*n*-hexanoyl = Hexa
10	H	Mba	H	Deca

Table 2.2 (continued)

Compounds	References
 8-Cedren-13-ol  Nerolidol  Guaiol  Limonene  Caryophyllene  Copaene  Germacrene D  Phytol  Cadinene  Humulene	Marie et al. (2007)
 Amarin  α-Amyrin  β-Sitosterol  Stigmasterol  Spinasterol  Isoscopoletin  Esculetin	Wang et al. (2006)

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples preparation

Fresh leaves, stems, flowers and seeds of *Ipomoea pes caprae* Linn. were collected from 6 provinces located on the coast of gulf of Thailand; Prajuabkirikhunkirikhun, petchaburi, Songkla, Chonburi, Rayong and Chantaburi. Specimens were kept in bag and started the experiments within 24 hours after collection. The sites of samples collection are shown in Table 3.1.

Table 3.1 Global Positioning System (GPS) of collection

Province	District	GPS coordination	
Chantaburi	Laem Sing	12.480231° N	102.000625° E
Chonburi	Sattahip	12.660783° N	100.912439° E
Petchaburi	Cha Um	12.753523° N	99.969948° E
Prajuabkirikhun	Kui Buri	12.087772° N	99.955614° E
Rayong	Mueang	12.611228° N	101.384395° E
Songkla	Mueang	7.074201° N	100.693109° E

3.1.2 Fungal cultivation media

Potato dextrose agar (PDA), corn meal agar (CMA), malt extract agar (MEA), sabouraud's dextrose agar (SDA) and V8-juice agar were used for fungal morphology observation. PDA was also used for isolation of pure culture and malt extract broth (MEB) was used for production of fungal bioactive compounds. Yeast malt extract agar was used for growing yeast. Nutrient agar (NA) and Mueller Hinton

agar (MHA) were used for bacterial cultivation. PDA, CMA, MHA and malt extract powder were instant media from HIMEDIA®, the other media were belong to Difco. The formulae of culture media were shown in Appendix A.

3.1.3 Equipments

3.1.3.1 Column chromatography and TLC

- Silica gel 60 F_{254} and reverse phase silica gel (Merck)
- Sephadex LH20 (Merck)
- TLC aluminium sheets

3.1.3.2 UV-Vis spectrometer

UV-Vis spectra were measured in appropriate solvent and recorded on a varian cary 50 probe UV-VIS spectrophotometer.

3.1.3.3 Nuclear magnetic Resonance Spectrometer (NMR)

^{1}H and ^{13}C NMR data were performed on Varian Model Mercury +400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Deuterated solvent, chloroform-d ($CDCl_3$) was used for NMR experiments and chemical shifts (δ) were referenced the signals of residual solvent at 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C).

3.1.4 Solvents

Commercial grade of hexane, dichloromethane, ethyl acetate and methanol were distilled before use.

The deuterated solvent for NMR spectroscopy were purchased from Merck.

Clorox (6% NaOCl) was used for plant tissues surface sterilization.

3.2 Methods

3.2.1 Isolation of endophytic fungi

Plant tissues were washed in running tap water and air dried. Leaves, stems, flowers and seeds were cut into small pieces (Figure 3.1). The samples were

surface steriled using 1% Clorox for 2 minutes and 70% EtOH for 2 minutes. After rinsing with sterile distilled water, The surface sterile samples were placed on control PDA plates for 3 hours to examine the epiphyte contamination (Fungi grow on control plates are epiphyte.) and then transferred to another PDA plates and incubated at room temperature. The examination of the fungal growth was performed every day by using stereomicroscope. After the mycelia grew, picked up mycelial tips and transferred to another PDA plate for screening of pure cultures.

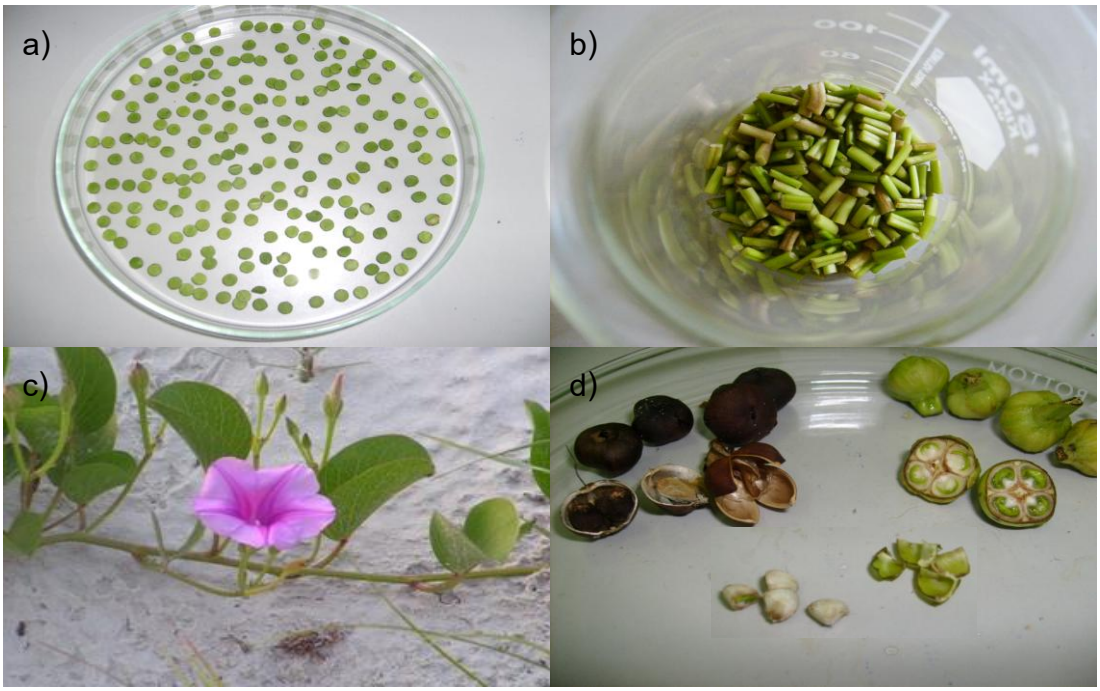


Figure 3.1 Plant tissues were prepared to make surface sterilization.

a) leaves b) stems c) flowers and d) seeds

3.2.2 Characterization of endophytic fungi

Endophytic fungi were identified and classified by observing macroscopic features (shape, size, color, and pigment) and microscopic feature (spores and mycelia) using both compound microscope and stereomicroscope.

3.2.3 Screening of endophytic fungi produced bioactive compounds

3.2.3.1 Fungal cultivation

Seven-day-old plate cultures of each endophytic fungi were cut into 7 mm diameter plugs with a flamed cork borer. Three plugs were inoculated into

250 ml flask containing 100 ml of MEB and cultured under static condition at room temperature for 4 weeks.

3.2.3.2 Solvent extraction of bioactive compounds

After 4 week cultivation, broth of each endophytic fungi isolate was filtered through Whatman No. 1 filter paper. The mycelia and the filtrates were separately extracted. The mycelia were extracted with EtOAc and MeOH while the filtrates were extracted with MeOH : CH_2Cl_2 (1:1). The solvents were evaporated under reduced pressure. All extracts were analyzed by TLC visualized using UV light, iodine vapor and vanillin/H_2SO_4 reagent. Mycelia and filtrates were kept under -20°C.

3.2.3.3 Investigation of antimicrobial activity of bioactive compound

The antimicrobial activity was examined by agar well diffusion method (National Committee for Clinical laboratory Standards; NCCLS, 2003 and 2004).

3.2.3.3.1 Sample preparation

1 mg of each culture broth extract and mycelium extract were dissolved in 1 ml of 10% DMSO in sterile distilled water.

3.2.3.3.2 Bacterial and yeast inoculums

The test bacteria were *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. *P. aeruginosa* was grown on MHA and the others were grown on NA, all bacteria are incubated at room temperature for 24 hours. Single colony was inoculated into 5 ml of each medium and incubated at 37°C for 2-3 hours. Turbidity of bacterial suspension was adjusted to 0.5 McFarland (OD_{625} = 0.1)

The test yeast *Candida albicans* ATCC 10231 was grown on YMA at room temperature for 24 hours. Single colony was inoculated into YMB 5 ml and incubated at 37°C for 2-3 hours. Turbidity of bacterial suspension was adjusted to 0.5 McFarland (OD_{625} = 0.1)

3.2.3.3.3 Inoculation of test microorganisms

The test plates were inoculated by dipping cotton swab into microbial suspension and streaking the cotton swab across the entire surface. This was repeated twice, turning the plate at right angle between each streaking. Let the surface dry before close plate.

3.2.3.3.4 Antimicrobial assay by agar well diffusion method

Agar media were made holes and removed cut plugs (7 mm diameter). Each crude of broth extract and mycelia extract [1mg/ml] 100 µl was pipette into the agar well. The test plates were incubated at room temperature over night.

3.2.3.3.5 Antibiotic drug stock preparation

Streptomycin was used as a positive control for antibacterial and ketoconazole was used as a positive control for yeast. 1 mg of each antibiotic drug standard was dissolved in 1 ml of 10% DMSO in sterile distilled water and kept in a refrigerator at 4°C before use.

3.2.4 Identification of the selected endophytic fungi

3.2.4.1 Morphological identification

Endophytic fungi were characterized by observing macroscopic features (shape, size, color, and pigment) and microscopic feature (spores and mycelia) using both compound microscope and stereomicroscope as described by Barnett and Hunter (1998).

3.2.4.2 Molecular identification

Endophytic fungi were cultured in 250 ml flask containing 100 ml of PDB at room temperature for a few weeks under static condition then filtered through Whatman No. 1 filter paper. The mycelia were kept at 4°C for genomic DNA extraction as describe by Zhou et al. (1999).

3.2.4.2.1 DNA extraction

The mycelia were homogenized with a pestle in a mortar in 1 ml of washing buffer solution (0.1 M Tris-HCL (pH 8.0), 2% 2-mercaptoethanol, 1% polyvinylpyrolidone and 0.05 M ascorbic acid). Then the sample was transferred to 1.5 ml micro tube. Sample mixture was centrifuged at 15,000 g for 3 minutes. Supernatant was removed and the pellet was washed 4-5 times using the washing buffer then centrifuged at 15,000 g for 3 minutes. DNA was extracted from pellet by adding 700 µl of cetyltrimethylammonium bromide (CTAB) lysis buffer and incubation in water bath at 65°C for 1 hour then extracted twice with a mixture of chloroform and isoamyl alcohol (24:1, v/v). DNA was precipitated in ice bath with isopropanol and centrifuged at 4°C, 8,000 rpm for 10 minutes. Supernatant was removed and 80% cool ethanol was added to wash the DNA. TE buffer was added to dissolve the fungal DNA in final step and kept at -20°C. The genomic DNA was checked by 1.2% agarose gel electrophoresis.

3.2.4.2.2 Amplification of Internal transcribed spacer region (ITS)

The ITS region was amplified by 2 primers; ITS1f (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). 100 ng genomic DNA and 1x PCR Master Mix (BioLab) were mixed in total volume 50 µl. The PCR amplification was process in a thermal cycler (LifePro) with 94°C for 5 minutes, followed by 38 cycles of 94°C for 1 minute, 51°C for 1 minute and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. PCR product was purified and subcloned with BAC cloning kit**.**

3.2.4.2.3 DNA Sequencing

The DNA sequences were analyzed by Prism sequencer using ITS4 as sequencing primer.

3.2.5 Cultivation and chemical investigation of selected endophytic fungi

Two endophytic fungi isolates; Cm1 and Ch109 were selected for cultivation and investigation of bioactive compounds. Cm1 was isolated from stem of beach morning glory collected from Petchaburi province. Ch109 was isolated from seed of beach morning glory collected from Petchaburi province.

3.2.5.1 Cultivation and chemical investigation of Cm1

Cm1 grown on PDA at room temperature for 7 days was cut with a flamed cork borer into 7 mm diameter 3 plugs and transferred to 100 ml of MEB per flask (x300). After cultivation under static condition at room temperature for 4 weeks, the culture was filtered through Whatman No. 1 filter paper. The filtrate (22 L) was extracted three times with an equal volume of MeOH : CH_2Cl_2 (1:1). After evaporation of the solvents by a rotary evaporation under reduced pressure at 42°C, a dark brown viscous residue (18.7 g) was obtained.

Fungal mycelia (823 g of dry weight) were ground using blender and then extracted with 1 L of hexane (x3), 1 L of EtOAc (x3) and 1 L of MeOH (x3) respectively. After extractions, the solvents were evaporated under reduced pressure at 40°C. The hexane extract gave a brown viscous oil (11 g), the EtOAc extract gave a dark brown residue (20 g) of and the MeOH extract gave a brown residue (26 g). All of the crudes were kept in a refrigerator at 4°C before use. Extraction procedure of mycelia and broth of Cm1 were shown in Figure 3.2.

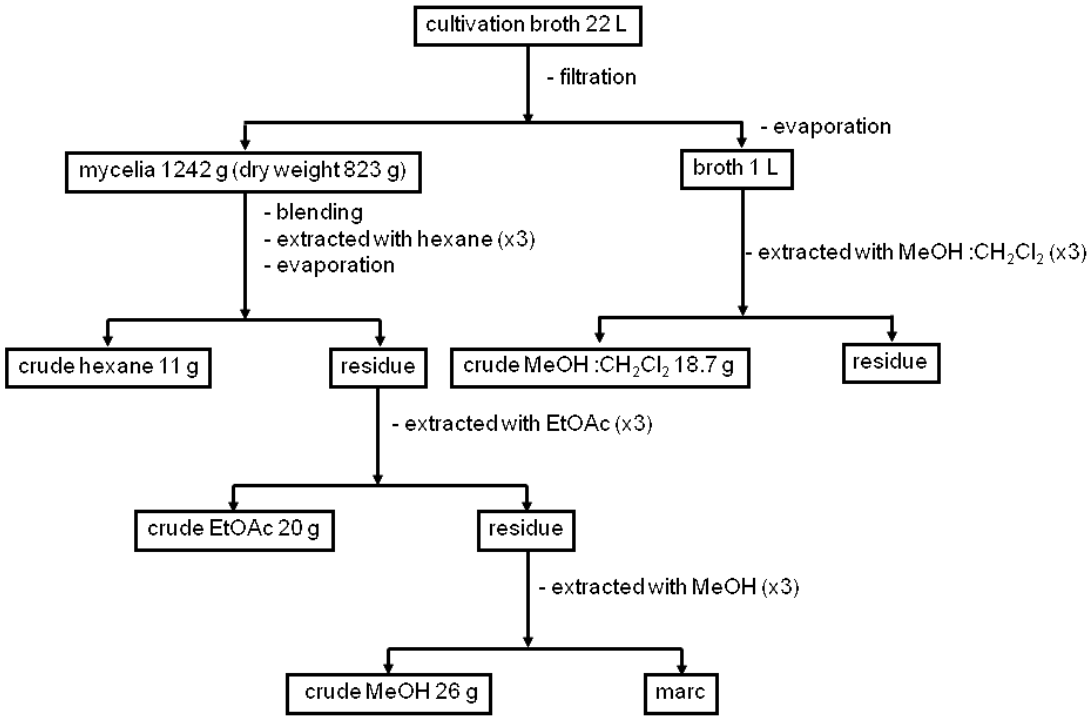


Figure 3.2 Solvent extractions of mycelia and broth of endophytic fungus Cm1

3.2.5.2 Cultivation and chemical investigation of Ch109

Ch109 grown on PDA at room temperature for 7 days was cut with a flamed cork borer into 7 mm diameter 3 plugs and transferred to 100 ml of MEB per flask (x300). After cultivation under static condition at room temperature for 4 weeks, total of cultured media (25 L of broth) were filtered through Whatman No. 1 filter paper. The filtrate was extracted with an equal volume of MeOH : CH_2Cl_2 (1:1) (trice) and then was concentrated by a rotary evaporation under reduced pressure at 42°C to give a dark brown viscous residue (12.3 g).

Fungal mycelia (760 g of dry weight) were ground using blender and then extracted with 1 L of hexane (x3), 1 L of EtOAc (x3) and 1 L of MeOH (x3) respectively. After extractions, the solvents were evaporated under reduced pressure at 40°C. The hexane fraction gave a brown viscous oil (19 g), the EtOAc fraction gave a dark brown residue (25 g) and the MeOH fraction gave a brown residue (10 g). All of the crudes were kept in a refrigerator at 4°C before use. The extraction procedure of mycelia and broth of Ch109 were shown in Figure 3.3.

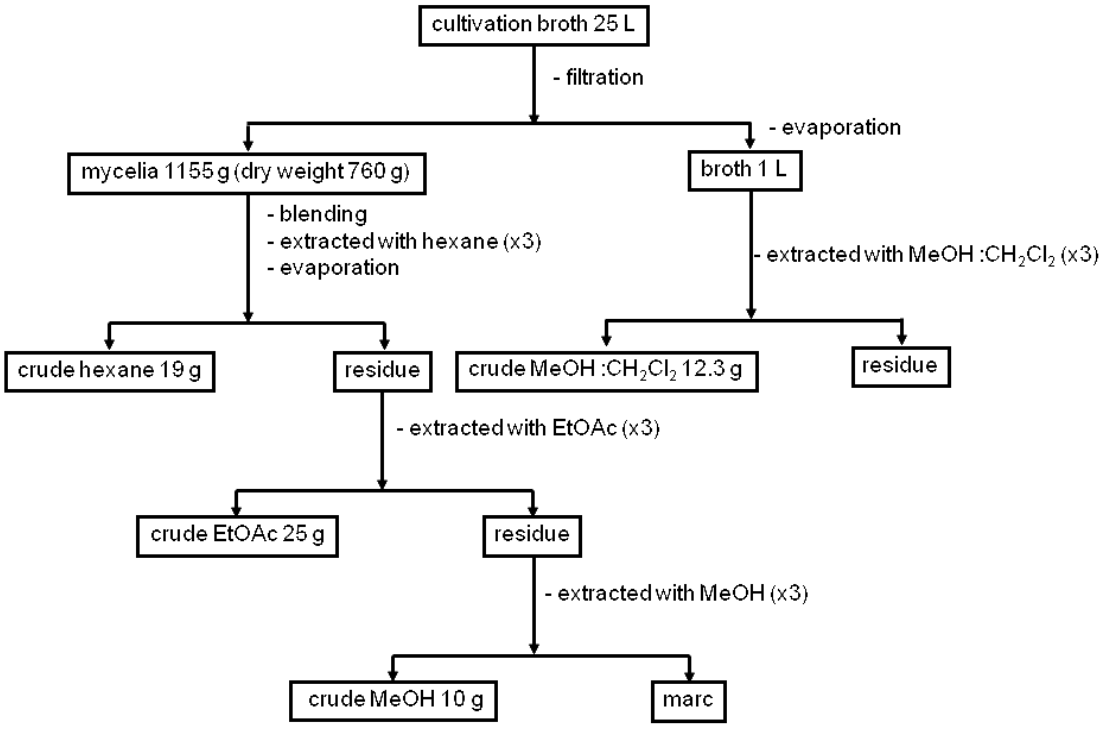


Figure 3.3 Solvent extractions of mycelia and broth of endophytic fungus Ch109

3.2.6 Chemical investigation of the mycelia EtOAc extracted crude of Cm1

The EtOAc crude of Cm1 was subjected to a sephadex LH20 column chromatography, eluted with MeOH and 50 ml of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV and vanillin/H_2SO_4 reagent. Combined fractions of Cm1 were shown in table 3.1.

Table 3.2 The combined fractions of Cm1 obtained from the mycelia EtOAc extracted crude

Combined fractions	Fraction No.	Appearance	Weight (mg)
ME-1	1-12	Yellow viscous liquid	82
ME-2	13-15	Yellow viscous liquid	41
ME-3	16-20	Yellow viscous liquid	75
ME-4	21-24	Dark-gray viscous liquid	29
ME-5	25-32	Dark-gray viscous liquid	181
ME-6	33-40	Gray viscous liquid	134
ME-7	41-55	Brown viscous liquid	315
ME-8	56-61	Brown viscous liquid	98
ME-9	62-72	Orange viscous liquid	511
ME-10	73-75	Orange viscous liquid	430
ME-11	76-86	Brown viscous liquid	356
ME-12	87-95	Red-orange viscous liquid	286
ME-13	96-102	Red-orange viscous liquid	114
ME-14	103-111	Red-orange viscous liquid	192
ME-15	112-120	Brown viscous liquid	34
ME-16	121-130	Brown viscous liquid	83
ME-17	131-140	Red-orange viscous liquid	220
ME-18	141-165	Red-orange viscous liquid	347
ME-19	166-184	Gray viscous liquid	198
ME-20	185-200	Gray viscous liquid	247

All of combined fractions of Cm1 were tested antimicrobial activity and combined fraction ME-10 (orange viscous liquid) was tested antioxidant activity, brine shrimp lethality and cytotoxicity.

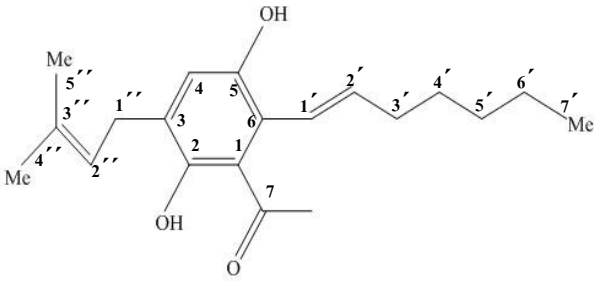
3.2.7 Chemical investigation of the mycelia EtOAc extracted crude of Ch109

The EtOAc crude of Ch109 was subjected to a silica gel 60 column chromatography, eluted with hexane, EtOAc and MeOH, 50 ml of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV and vanillin/H_2SO_4 reagent. Combined fractions of Cm1 were shown in table 3.2.

Table 3.3 The combined fractions of Ch109 obtained from the mycelia EtOAc extracted crude

Combined fractions	Fraction No.	Eluents	Appearance	Weight (mg)
ME-1	1	hexane (100)	Yellow viscous liquid	935
ME-2	2-6	hexane : EtOAc (90:10)	Dark-orange viscous liquid	4,120
ME-3	7-25	hexane : EtOAc (80:20)	Orange viscous liquid	286
ME-4	26-31	hexane : EtOAc (70:30)	Orange viscous liquid	55
ME-5	32-39	hexane : EtOAc (60:40)	Orange viscous liquid and orange solid	156
ME-6	40-54	hexane : EtOAc (50:50)	Red viscous liquid and orange solid	721
ME-7	55-60	hexane : EtOAc (40:60)	Red viscous liquid and orange solid	365
ME-8	61-72	hexane : EtOAc (30:70)	Dark red viscous liquid	1,110
ME-9	73-82	EtOAc (100)	Dark red viscous liquid	715
ME-10	83-89	EtOAc : MeOH (80:20)	Brown viscous liquid	395
ME-11	90-95	EtOAc : MeOH (60:40)	Brown viscous liquid	380
ME-12	96-104	EtOAc : MeOH (40:60)	Dark brown viscous liquid	928
ME-13	105-125	EtOAc : MeOH (20:80)	Black viscous liquid	2,980
ME-14	126-140	MeOH (100)	Black viscous liquid	5,110

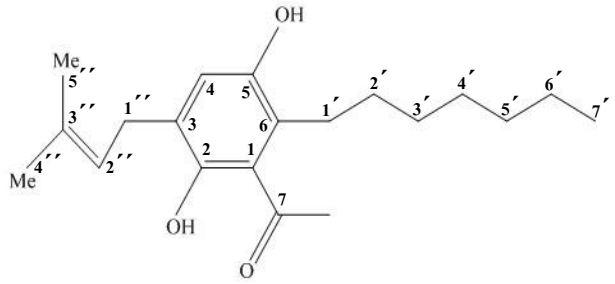
Combined fraction ME-1 eluted from pure hexane (yellow viscous liquid) was crystallized by hexane and CH_2Cl_2 to give compound **1** and compound **2**. NMR spectra were shown in appendix D. Chemical shifts of proton and carbon of compound **1** and compound **2** were as the following.



Compound 1

Compound **1** ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 11.73 (1H, s, OH-2), 10.09 (1H, s, CHO-1), 7.01 (1H, s, H-4), 6.48 (1H, br.d, 16.0 Hz, H-1′), 5.98 (1H, dt, 16.0, 6.8 Hz, H-2′), 5.29 (1H, br.t, 7.2 Hz, H-2′′), 5.05 (1H, br.s, OH-5), 3.31 (2H, d, 7.2 Hz, H-1′′), 2.32 (2H, br.q, 7.2 Hz, H-3′), 1.75 (3H, s, H-4′′), 1.69 (3H, s, H-5′′), 1.51 (2H, m, H-4′), 1.35 (2H, m, H-6′), 1.34 (2H, m, H-5′), 0.91 (3H, t, 6.8 Hz, H-7′) ppm.

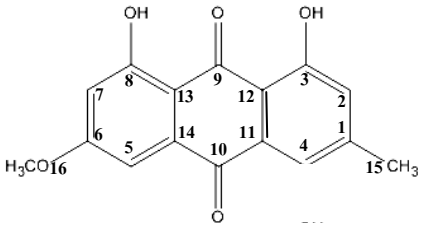
^{13}C NMR ($CDCl_3$, 400 MHz) δ: 196.30 (d, CHO), 155.05 (s, C-2), 144.74 (s, C-5), 142.69 (d, C-2′), 133.95 (s, C-3′′), 130.29 (s, C-3), 125.03 (d, C-4), 123.95 (s, C-6), 120.93 (d, C-2′′), 120.04 (d, C-1′), 117.07 (s, C-1), 33.45 (t, C-3′), 31.43 (t, C-5′), 28.70 (t, C-4′), 27.18 (t, C-1′′), 25.79 (q, C-4′′), 22.45 (t, C-6′), 17.76 (q, C-5′′), 14.01 (q, C-7′) ppm.



Compound 2

Compound **2** ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 11.93 (1H, s, OH-2), 10.25 (1H, s, CHO-1), 6.89 (1H, s, H-4), 5.28 (1H, m, H-2′′), 4.43 (1H, s, OH-5), 3.29 (2H, d, 7.2 Hz, H-1′′), 2.88 (2H, t, 7.6 Hz, H-1′), 1.76 (3H, s, H-5′′), 1.69 (3H, s, H-4′′), 1.57 (2H, q, 7.6 Hz, H-2′), 1.39 (2H, q, 7.2 Hz, H-3′), 1.32 (2H, m, H-4′), 1.27 (4H, m, H-5′ and H-6′), 0.87 (3H, t, 6.8 Hz, H-7′) ppm.

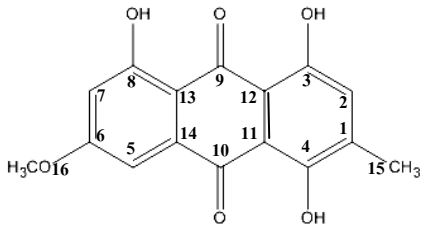
Combined fraction ME-4 eluted from hexane: EtOAc (70:30) (orange viscous liquid) was crystallized by hexane and CH_2Cl_2 to give compound **3** and compound **4**. NMR spectra were shown in appendix D. Chemical shifts of proton and carbon of compound **3** and compound **4** were as the following.



Compound 3

Compound **3** ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 12.32 (1H, s, OH-8), 12.12 (1H, s, OH-3), 7.63 (1H, s, H-4), 7.38 (1H, s, H-5), 7.09 (1H, s, H-2), 6.69 (1H, s, H-7), 3.94 (3H, s, OCH_3-6), 2.45 (3H, s, CH_3-1) ppm.

^{13}C NMR ($CDCl_3$, 400 MHz) δ: 190.85 (C-9), 166.60 (C-6), 165.23 (C-8), 162.54 (C-1), 148.44 (C-3), 124.50 (C-2), 121.28 (C-4), 108.21 (C-5), 106.80 (C-7), 56.06 (C-16), 22.12 (C-15) ppm.



Compound 4

Compound **4** ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 13.37 (1H, s, OH-4), 12.45 (1H, s, OH-8), 12.37 (1H, s, OH-3), 7.41 (1H, s, H-5), 7.13 (1H, s, H-2), 6.70 (1H, s, H-7), 3.94 (3H, s, OCH_3-6), 2.36 (3H, s, CH_3-1) ppm.

3.2.8 Bioassay of the isolated bioactive compounds

3.2.8.1 Antimicrobial activity

Antimicrobial activity was performed by agar well diffusion method (National Committee for Clinical laboratory Standards; NCCLS, 2003 and 2004). The test microorganisms, inoculum preparation and antibiotic drug preparation were as same as in the section 3.2.3.3.

3.2.8.2 Antioxidant activity

Antioxidant test was performed by DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay by the method of Osawa and Namiki (1981).

3.2.8.3 Brine shrimp lethality

Modified by the method of Fatope *et al.* (1993). Sea salt solution was made by dissolving 3 g in 100 ml distilled water. 50 mg of *Artemia salina* eggs was added in a hatching chamber. The hatching chamber was lighten for 48 hours for the eggs to hatch into shrimp larvae. 10 larvae were dropped into each well of 96 well plate. The crude extracts were dissolved with 5% DMSO in sea salt solution and varied concentrations at 10, 50, 100, 500 and 1,000 µg/ml in final volume 200 µl. Crude samples were added into well plate and allowed to expose the air 24 hours at room temperature after that calculated LC_{50} value of bioactive compounds.

3.2.8.4 Cytotoxic activity

Cytotoxicity test was carried out at The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. The test was against 5 human cancer cell lines which were breast (BT474), lung (CHAGO), hepatoma (HEP-G2), gastric (KATO-3) and colon (SW620). The method was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenultetrazolium bromide] assay (Carmichael *et al*., 1987).

3.2.8.5 Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration is the lowest concentration of an antimicrobial that will inhibit the growth of microorganisms after overnight incubation. MIC is used to confirm resistance of microorganisms to antimicrobial agents. The MIC was perform by method of Jorgensen (1999).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

Beach morning glory collected from 6 provinces; Chantaburi, Chonburi, Petchaburi, Prajuabkirikhun, Rayong and Songkla were used for endophytic fungi isolation by surface sterilization method (Smith et al, 1996). The result showed 150 endophytic fungi isolated from leaves, stems and seeds of plant samples as shown in Table 4.1.

Table 4.1 Number of endophytic fungi isolated from beach morning glory tissues.

Province	Leaves	Stems	Flowers	Seeds	Total
Chantaburi	6	13	-	8	27
Chonburi	11	14	-	17	42
Petchaburi	2	4	-	18	24
Prajuabkirikhun	10	2	-	2	19
Rayong	5	8	-	3	16
Songkla	2	14	-	6	22
Total	36	60	0	54	150

There were 60 endophytic fungi isolated from stems, 36 isolates derived from leaves and 54 isolates derived from seeds of beach morning glory. For the part of flowers there was no endophytic fungal isolated because of contamination. The germinations of fungal mycelia from beach morning glory tissues were shown in figure 4.1.

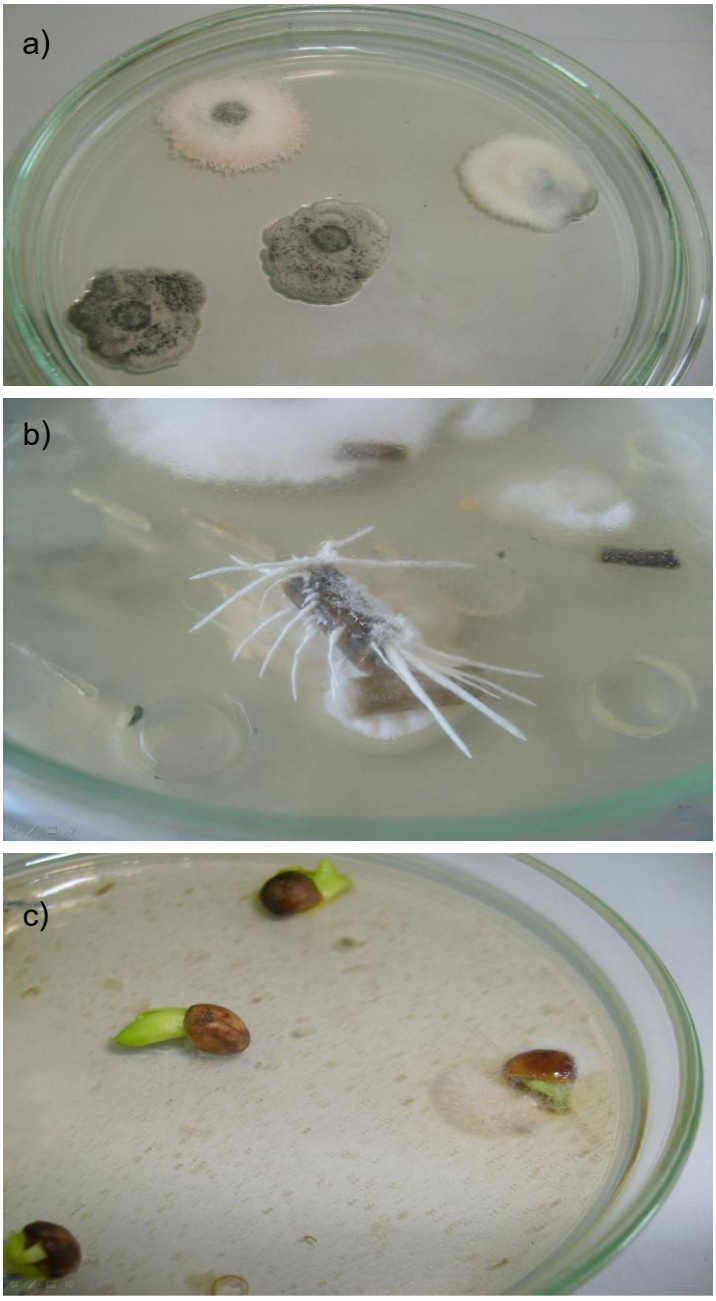


Figure 4.1 Germination of fungal mycelia from Beach morning glory tissues

a) leaves b) stems and c) seeds

4.2 Identification and classification of endophytic fungi

Endophytic fungi isolates were grown on PDA, for 14 days at room temperature. The fungi were classified by morphology (macroscopic and microscopic feature). There were 83 isolates of endophytic fungi be classified as mycelia sterilia (no spore formation). The genera of all isolates composed of *Alternaria* (3 isolates), *Aspergillus* (5 isolates), *Collectotrichum* (7 isolates), *Curvularia* (8 isolates), *Eurotium* (1 isolate), *Fusarium* (10 isolates), *Geotrichum* (2 isolates), *Penicillium* (4 isolates), *Phomopsis* (9 isolates), *Stemphylium* (1 isolate), *Trichoderma* (2 isolates), *Xylaria* (15 isolates) and *Mycelia sterilia* (83 isolates). The results of the identification of the endophytic fungi from 6 provinces were shown in Table 4.2 and figure 4.2.

Table 4.2 Genera of isolates endophytic fungi from tissues of *Ipomoea pes-caprae* Linn.

Province Genera	Chantaburi	Chonburi	Petchaburi	Prajuabkirikhun	Rayong	Songkla	Total
Alternaria	-	1	-	2	-	-	3
Aspergillus	2	-	-	-	-	3	5
Collectotrichum	1	1	1	1	1	2	7
Curvularia	2	1	2	2	1	-	8
Eurotium	-	-	1	-	-	-	1
Fusarium	2	2	2	1	2	1	10
Geotrichum	-	-	1	1	-	-	2
Penicillium	1	1	-	-	1	1	4
Phomopsis	1	1	2	1	1	3	9
Stemphylium	-	-	1	-	-	-	1
Trichoderma	-	2	-	-	-	-	2
Xylaria	3	2	3	2	2	3	15
Mycelia sterilia	15	31	11	9	8	9	83
Total	27	42	24	19	16	22	150

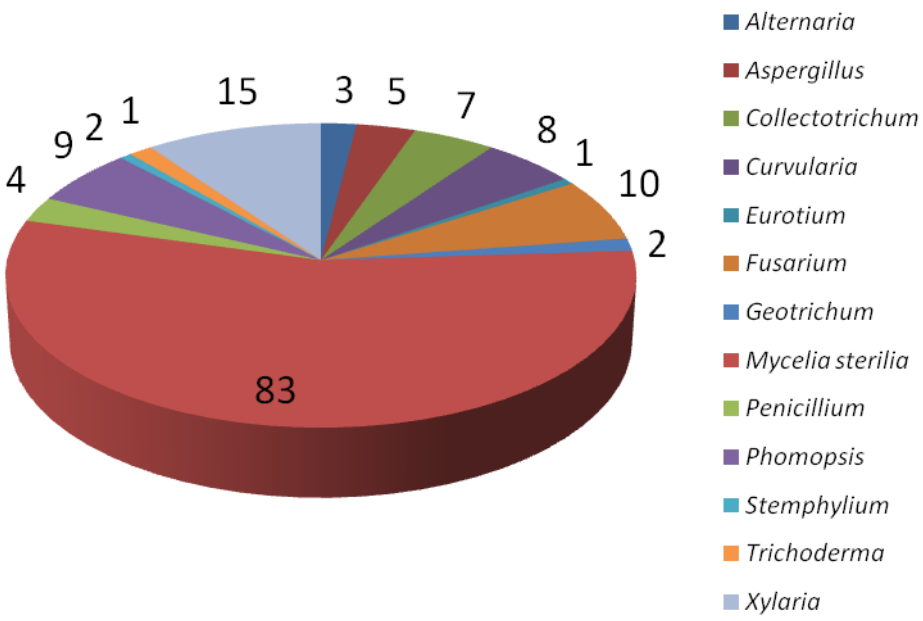


Figure 4.2 Genera and amount of all endophytic fungi isolates

4.3 Screening of bioactive compounds produced by endophytic fungi

Bioactive compounds produced by 150 endophytic fungi isolates were examined the antimicrobial activity against 5 microorganisms (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231) using an agar diffusion method (NCCLS, 2003 and 2004). The endophytic fungi isolates showed antimicrobial activity with diameter of clear zones were over 10 mm against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231 were 74.7%, 68.0%, 34.0%, 41.3% and 23.3%, respectively. There were 14.0% showed antimicrobial activity against all test microorganisms and 22.7% showed no activity. The result of antimicrobial activity of all endophytic fungi isolates was shown in figure 4.3

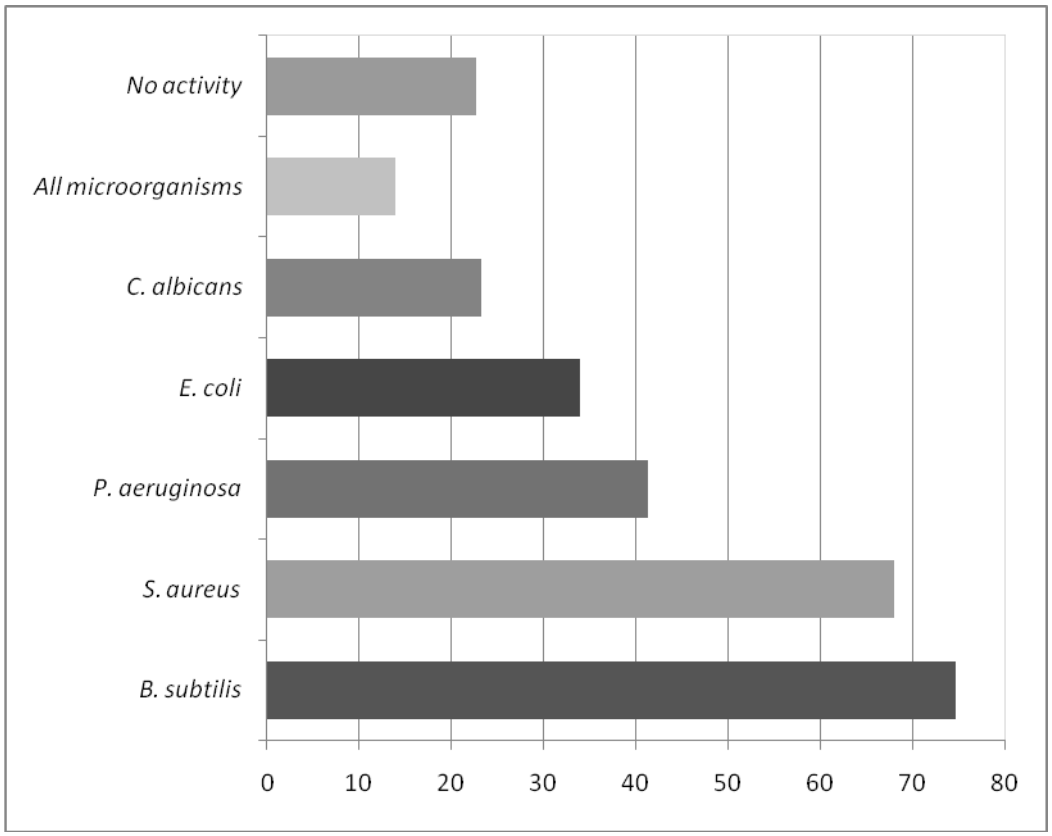


Figure 4.3 Percentage of 150 endophytic fungi isolates that showed antimicrobial activity

There were large number of endophytic fungi isolated from 6 provinces showed antimicrobial activity against gram positive bacteria (*B. subtilis* and *S. aureus*) more than against gram negative bacteria *(E. coli* and *P. aeruginosa*) including yeast (*C. albicans*). The result was shown in Table 4.3.

Table 4.3 Number of endophytic fungi produced antimicrobial metabolites against 5 microorganism: classified by provinces.

Province	Number of endophytic fungi which against test microorganisms				
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
Chantaburi	12	15	3	8	1
Chonburi	25	20	10	4	7
Petchaburi	17	14	2	6	5
Prajuabkirikhun	9	6	8	11	-
Rayong	4	7	4	-	3
Songkla	13	15	2	6	2
Total	80	77	29	35	18

Many genera of isolated endophytic fungi exhibited antimicrobial activity against test microorganisms more than 2 species. *Aspergillus*, *Xylaria* and *Mycelia sterilia* could inhibit growth of all test microorganisms. *Collectotrichum*, *Penicillium* and *Stemphylium* could inhibit growth of 4 test microorganisms. *Eurotium*, *Fusarium* and *Phomopsis* could inhibit growth of 3 test microorganisms. The results were shown in Table 4.4.

Table 4.4 Number of endophytic fungi produced antimicrobial metabolites against 5 microorganism: classified by genera of fungi

Genera	Number of endophytic fungi which against test microorganisms				
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
Altermaria	1	1	-	-	-
Aspergillus	4	4	2	3	1
Collectotrichum	3	3	1	2	-
Curvularia	2	-	-	-	-
Eurotium	1	1	-	-	1
Fusarium	2	7	-	2	-
Geotrichum	-	2	-	-	-
Penicillium	3	2	3	1	-
Phomopsis	5	6	-	5	-
Stemphylium	1	1	1	1	-
Trichoderma	-	-	-	-	-
Xylaria	9	7	8	3	4
Mycelia sterilia	49	43	14	18	12
Total	80	77	29	35	18

4.4 Identification of selected endophytic fungal

From the data of antimicrobial activity using agar well diffusion assay of concentration of 1 mg/ml, there were 2 endophytic fungi isolates; Cm1 and Ch109 (Cm1 was isolated from stem of beach morning glory collected from Petchaburi province, Ch109 was isolated from seed of beach morning glory collected from Petchaburi province), exhibited wide clear zone against test microorganisms. The inhibition zone width of Cm1 against *B. subtilis, S. aureus, E. coli* and *P. aerugenosa* were 20, 20, 12 and 11 mm, respectively. The inhibition zone

width of Ch109 against *B. subtilis, S. aureus* and *C.albican* were 16, 16 and 13 mm, respectively. Cm1 and Ch109 were selected for further studies.

Selected endophytic fungi isolates, Cm1 and Ch109, were grown on 7 media; CMA, MEA, PDA, SDA, V8A, YMA and MEB for morphology examination. The data were described in Table 4.5.

Table 4.5 Morphology of Cm1 and Ch109 cultured on different media

Media	Cm1		Ch109	
	mycelium color	pigment color	mycelium color	pigment color
CMA	white	no	white	no
MEA	white	no	black and white	brown
PDA	grey	no	brown	brown
SDA	white	no	brown	brown
V8A	grey	no	white	no
YMA	white	no	white	no
MEB	brown	no	brown	no

Morphology of Cm1 and Ch109 cultured on different media were shown in figure 4.4 and 4.5.

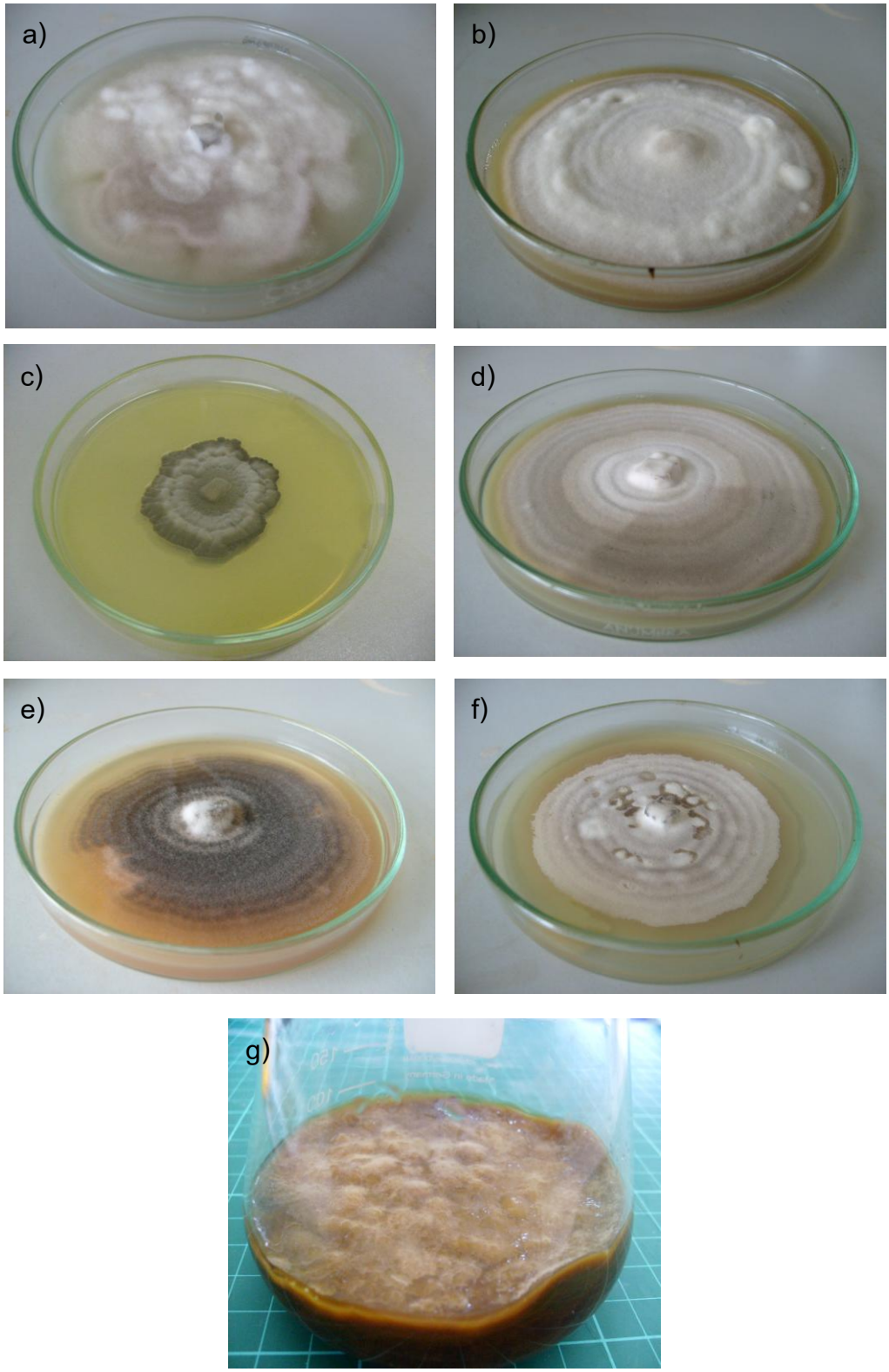


Figure 4.4 Colony of endophytic fungi isolate Cm1 was grown on CMA (a), MEA (b), PDA (c), SDA (d), V8A (e), YMA (f) and MEB (g) for 4 weeks at room temperature.

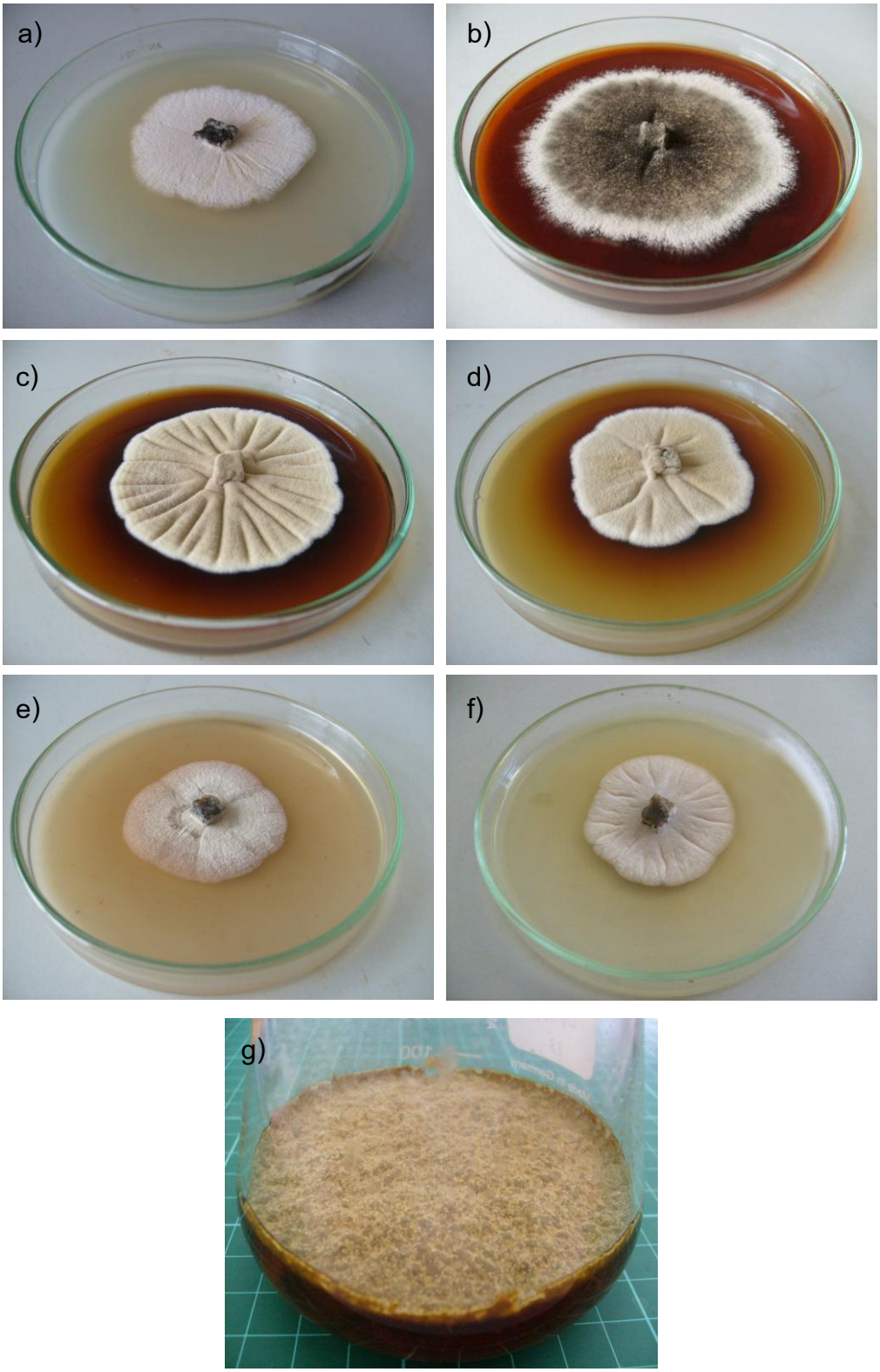


Figure 4.5 Colony of endophytic fungi isolate Ch109 was grown on CMA (a), MEA (b), PDA (c), SDA (d), V8A (e), YMA (f) and MEB (g) for 4 weeks at room temperature.

4.4.1 Identification of the endophytic fungus Cm1

Endophytic fungi Cm1 produced spore (Fig 4.6) which was characterized as *Stemphylium* genera (Barnett and Hunter, 1998). Cm1 was identified based on fungal morphology and analysis of the DNA sequence of the ITS region (Bruns et al, 1992). Total DNA was extracted from fungal mycelium grown in MEB followed in 3.2.4.1. Primer ITS4 was used to amplify the ITS1-5.8S-ITS2 region from total DNA extracted. The thermal cycle program was as follows: 94°C for 5 min, followed by 38 cycle of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min. The amplified DNA was purified and directly subjected to sequencing by Faculty of Science, Mahidol University using primer ITS4. BLASTN 2.2.25+ was used to search for similar sequences in the database (GenBank/ EMBL/ DDBT and PDB).

The ITS fragment length of fungal isolate Cm1 was 486 bp fragment as shown in Figure 4.7. A blast search was performed to find a similar sequence of ITS region of fungal isolate Cm1 in the DNA database. The ITS region of isolate Cm1 was similar to 100% identity of *Stemphylium solani*. Alignment data of ITS region of isolates Cm1 was showed in Appendix C.

Figure 4.6 Spores of endophytic fungus isolate Cm1 (compound microscope 400x).

ATTGTGCTGC GCTCCGAAAC CAGTAGGTCG GCTGCCAATG ATTTTAAGGC GAGTCTCGTG
AGAGACAAGA CGCCCAACAC CAAGCAAAGC TTGAGGGTAC AAATGACGCT CGAACAGGCA
TGCCCTTTGG AATACCAAAG GGCGCAATGT GCGTTCAAAG ATTCGATGAT TCACTGAATT
CTGCAATTCA CACTACGTAT CGCATTTCGC TGCGTTCTTC ATCGATGCCA GAACCAAGAG
ATCCGTTGTT GAAAGTTGTA ATAATTACAT TGTTTACTGA CGCTGATTGC AATTACAAAA
AGGTTTATGG TTTGGTCCTG GTGGCGGGCG AACCCGCCCA GGAAACAAGA AGTGCGCAAA
AGACATGGGT GAATAATTCA GACAAGCTGG AGCCCTCACC GAAGTAAGGT CCCAGCCCGC
TTTCATATTG TGTAATGATC CCTCCGCAGG TTCACCTACG GAGACCTTGT TACGACTTTT
ACTTCC

Figure 4.7 Nucleotide sequences of complete ITS region of the isolate Cm1.

4.4.2 Identification of the endophytic fungus Ch109

Endophytic fungi Ch109 produced spore (Fig 4.8) which was characterize as *Stemphylium* genera (Barnett and Hunter, 1998). Ch109 was identified based on fungal morphology and analysis of the DNA sequence of the ITS region (Bruns et al., 1992). Total DNA was extracted from fungal mycelium grown in MEB followed in 3.2.4.1. Primer ITS4 was used to amplify the ITS1-5.8S-ITS2 region from total DNA extracted. The thermal cycle program was as follows: 94°C for 5 min, followed by 38 cycle of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min. The amplified DNA was purified and directly subjected to sequencing by Faculty of Science, Mahidol University using primer ITS4. BLASTN 2.2.25+ was used to search for similar sequences in the database (GenBank/ EMBL/ DDBT and PDB).

The ITS fragment length of fungal isolate Ch109 was 500 bp fragment as shown in Figure 4.9. A blast search was performed to find a similar sequence of ITS region of fungal isolate Ch109 in the DNA database. The ITS region of isolate Ch109 was similar to 100% identity of *Stemphylium solani*. Alignment data of ITS region of isolates Ch109 was showed in Appendix C.

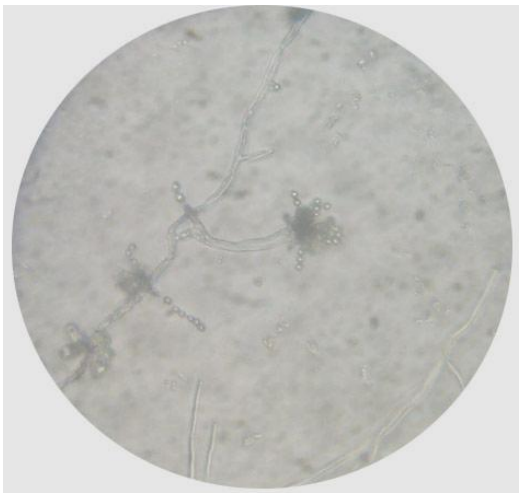


Figure 4.8 Spores of endophytic fungus isolate Ch109 (compound microscope 400x).

TGCGAGGCTA GCTGCCAGCT GGACCTACGG GAGCGGGTGA CAAAGCCCCA TACGCTCGAG

GACCAGACAT GGTGCCGCCA CTGCCTTTTG GGCCCGTCCC CGTTGCCAGC GACGGAAGCC

CAACACACAA GCCGTGCTTG AGGGCAGCAA TGACGCTCGG ACAGGCATGC CCCCCGGAATT

ACCAGGGGGC GCAATGTGCG TTCAAAGACT CGATGATTCA CTGAATTCTG CAATTCACAT

TAATTATCGC ATTTCGCTGC GTTCTTCATC GATGCCGGAA CCAAGAGATC CGTTGTTGAA

AGTTTTAACG ATTGTTTAAC TAAAAACTCA GATGCAAAC TTCAGACAGC GTTCAAATGT

TAGTCTCCGG CGGGCCGTGG CCACGCCGAA GCAACAGGGT ACAGATAGAC ACGGATGGGA

GGTTGGACCC AGAGGGCCCG CACTCGGTAA TGATCCTTCC GCAGGTTCAC CTACGGAAAC

CTTGTTACGA CTTTTACTTC

Figure 4.9 Nucleotide sequences of complete ITS region of the isolate Ch109.

4.5 Growth curve and antimicrobial activity test of mycelial crude extract

4.5.1 Growth profile and biological activity test of Cm1 culture mycelium

The endophytic fungi Cm1 was inoculated into 250 ml flask containing 100 ml of MEB and culture under static condition at room temperature for over 1 month. After one week

of culture, dry weight of mycelia were measured every 3 days. The mycelia were extracted with EtOAc and tested the antimicrobial activity against 5 microorganisms every 3 days. The results were shown in figure 4.10.

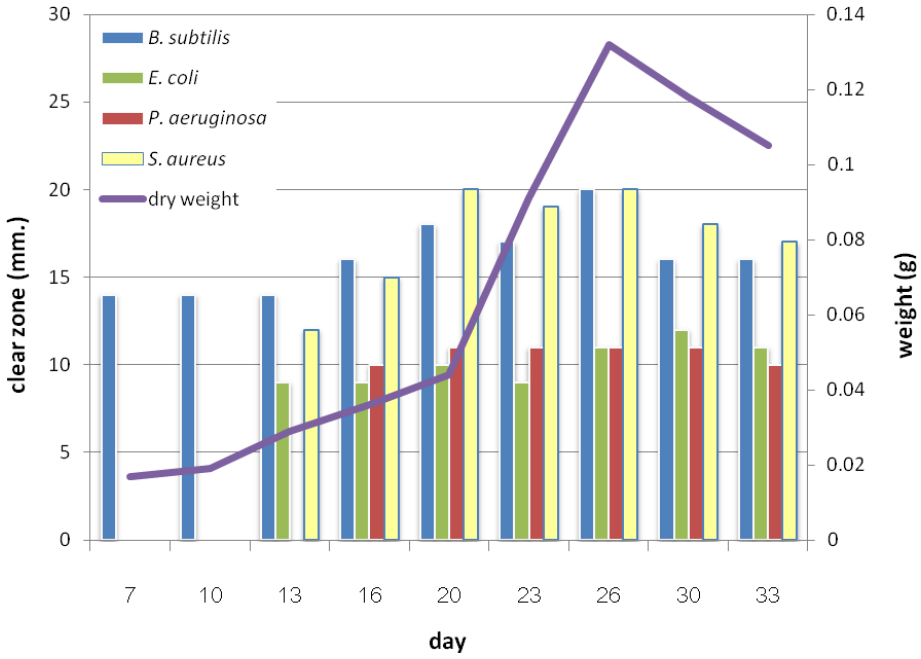


Figure 4.10 Dry weight and clear zone of Cm1 mycelial crude extract [1 mg/ml] against test microorganisms.

From period examination dry weight was maximum (0.135 g/flask) at the day 26 and the antimicrobial activity against test microorganisms were maximum at the day between 20-30. Cm1 mycelial crude extract could inhibit gram positive bacteria (*B. subtilis* and *S. aureus*) better than gram negative bacteria (*E. coli* and *P. aerogenosa*). NMR profiles of Cm1 mycelial crude extract were examined every week for 5 weeks; see in appendix D.

4.5.2 Growth profile and biological activity test of Ch109 culture mycelium

The endophytic fungi Ch109 was inoculated into 250 ml flask containing 100 ml of MEB and cultured under static condition at room temperature for over 1 month. After one week of culture, dry weight of mycelia were measured every 3 days and extracted with EtOAc, each EtOAc extract was tested the antimicrobial activity against 5 microorganisms. The results were shown in figure 4.11.

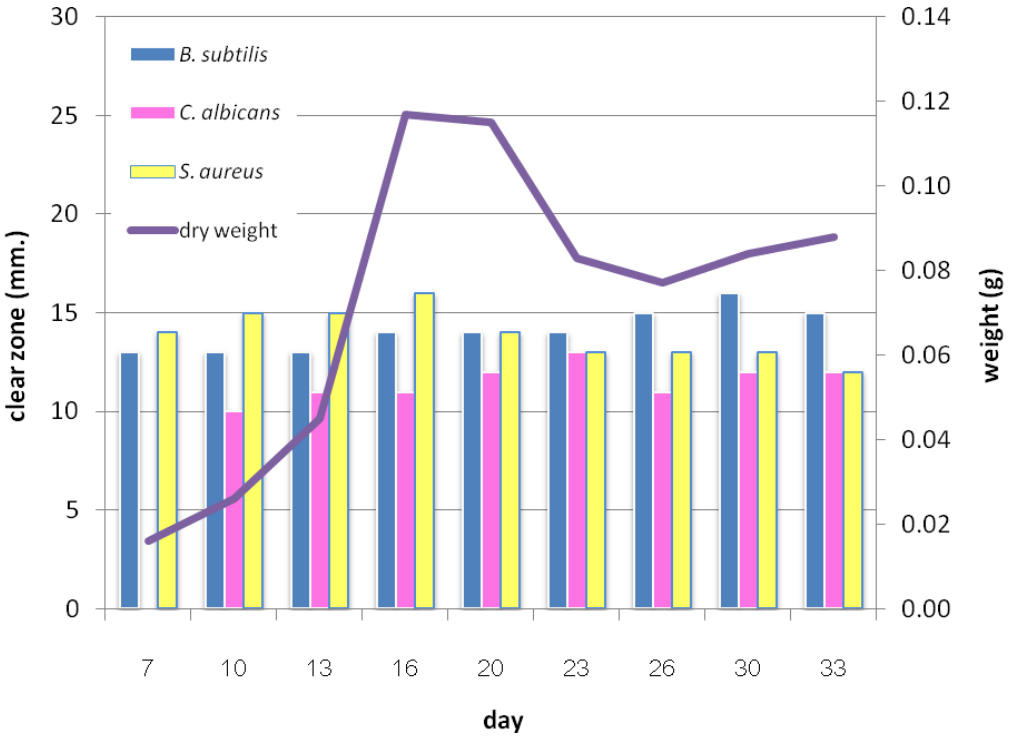


Figure 4.11 Dry weight and clear zone of Ch109 mycelial crude extract [1 mg/ml] against test microorganisms.

From period examination dry weight was maximum (0.11 g/flask) at the day between 16-20 and the antimicrobial activity against *B. subtilis, S. aureus* and *C. albicans* were maximum at the day 30, 16 and 23, respectively. Ch109 mycelial crude extract could inhibit only gram positive bacteria (*B. subtilis* and *S. aureus*) and yeast (*C. albicans*). NMR profiles of Ch109 mycelial crude extract were examined every week for 5 week; see in appendix D.

4.6 Chemical investigation of bioactive compounds produced by Cm1 and biological assays

4.6.1 Cultivation and chemical investigation of the metabolite produced by the endophytic fungus Cm1

The endophytic fungus Cm1 was cultivated in malt extract broth under static condition at room temperature for 4 weeks (the appropriate time for bioactive compound production by NMR profile; appendix D), and then the whole culture (22 L of broth) was filtered through whatman no. 1 filter paper. After the filtrate was concentrated under reduced pressure to 1 L the concentrated filtrate was extracted with an equal volume of MeOH : CH_2Cl_2 followed by removal of the solvent to give a yield (18.7 g) of dark brown viscous liquid.

Fungal mycelia (823 g) were extracted with hexane, EtOAc and MeOH to give yields of brown viscous liquid (11 g), dark brown residue (20 g) and brown residue (26 g) respectively.

The EtOAc crude obtained from mycelia was isolated by sephadex column chromatography eluted with 100% MeOH. The similar fractions were combined on the basis of TLC profile to give 20 combined fractions as shown in Table 4.6.

Table 4.6 The combined fractions of Cm1 obtained from the mycelia EtOAc extracted crude

Combined fractions	Fraction No.	Appearance	Weight (mg)
ME-1	1-12	Yellow viscous liquid	82
ME-2	13-15	Yellow viscous liquid	41
ME-3	16-20	Yellow viscous liquid	75
ME-4	21-24	Dark-gray viscous liquid	29
ME-5	25-32	Dark-gray viscous liquid	181
ME-6	33-40	Gray viscous liquid	134
ME-7	41-55	Brown viscous liquid	315
ME-8	56-61	Brown viscous liquid	98
ME-9	62-72	Orange viscous liquid	511
ME-10	73-75	Orange viscous liquid	430
ME-11	76-86	Brown viscous liquid	356
ME-12	87-95	Red-orange viscous liquid	286
ME-13	96-102	Red-orange viscous liquid	114
ME-14	103-111	Red-orange viscous liquid	192
ME-15	112-120	Brown viscous liquid	34
ME-16	121-130	Brown viscous liquid	83
ME-17	131-140	Red-orange viscous liquid	220
ME-18	141-165	Red-orange viscous liquid	347
ME-19	166-184	Gray viscous liquid	198
ME-20	185-200	Gray viscous liquid	247

4.6.2 Biological assays of Cm1 combined fractions

EtOAc extracted crude of Cm1 combined fractions 1-20 were test antimicrobial activity against 5 microorganisms. The results showed that clear zone of all combined fractions were 10-25 mm (Table 4.7) which ME-10 exhibited the largest inhibition zone against test microorganisms, combined fraction ME-10 was test antioxidant activity, brine shrimp lethality and cytotoxicity.

Table 4.7 Antimicrobial activity test of Cm1

Combined fractions	Clear zone (mm) against tested microorganisms				
	Basillus	*Staphylococcus*	*Escherichia*	*Pseudomonas*	*Candida*
ME-1	-	-	-	-	-
ME-2	-	-	-	-	-
ME-3	-	-	-	-	-
ME-4	-	-	-	-	-
ME-5	-	-	-	-	-
ME-6	-	-	-	-	-
ME-7	-	-	-	-	-
ME-8	-	-	-	-	-
ME-9	20	21	10	12	-
ME-10	25	22	16	13	-
ME-11	22	23	15	12	-
ME-12	18	13	13	10	-
ME-13	19	12	12	-	-
ME-14	18	-	-	-	-
ME-15	16	-	-	-	-
ME-16	14	-	-	-	-
ME-17	-	-	-	-	-
ME-18	-	-	-	-	-
ME-19	-	-	-	-	-
ME-20	-	-	-	-	-
Streptomycin	32	31	29	30	-

Combined fraction ME-10 was tested DPPH assay and the IC_{50} was 44 µg/ml compared with vitamin E (control) which was 17 µg/ml.

Combined fraction ME-10 was tested brine shrimp lethality and the LC_{50} was 414 µg/ml.

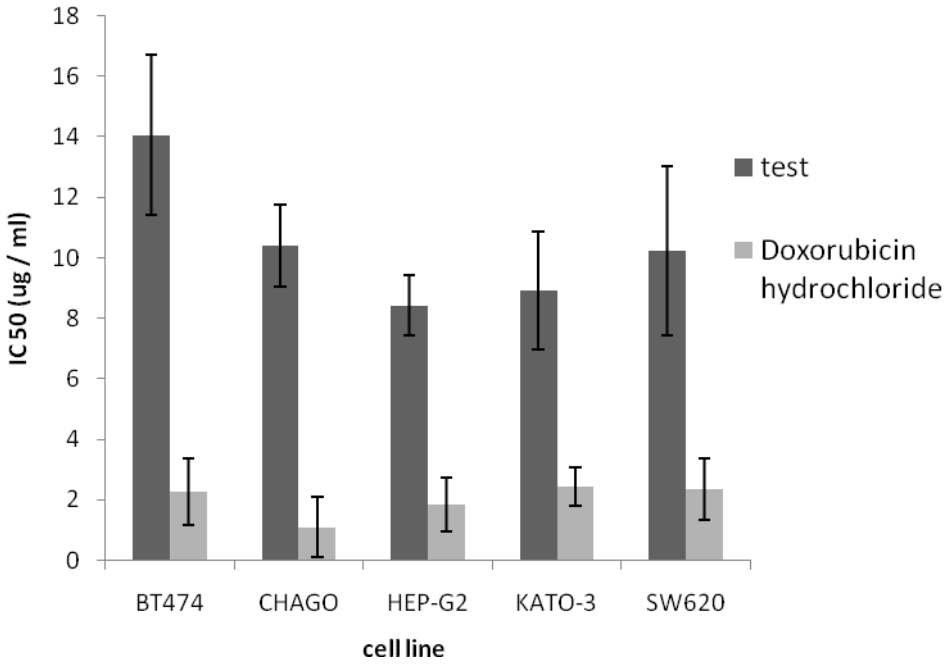


Figure 4.12 Cytotoxicity test of combined fraction ME-10

Combined fraction ME-10 was tested MTT assay with 5 cell lines (BT474, CHAGO, HEP-G2, KATO-3 and SW620). The IC_{50} were 14.05, 10.40, 8.41, 8.91 and 10.23 µg/ml, respectively while IC_{50} of doxorubicin hydrochloride (control) were 2.28, 1.09, 1.84, 2.43 and 2.36 µg/ml, respectively.

4.7 Chemical investigation of bioactive compounds produced by Ch109

The endophytic fungus Ch109 was cultivated in malt extract broth under static condition at room temperature for 4 weeks and then the whole culture (25 L of broth) was filtered through whatman no. 1 filter paper. After the filtrate was concentrated under reduced pressure to 1 L the concentrated filtrate was extracted with an equal volume of MeOH : CH_2Cl_2 followed by removal of the solvent to give a dark brown viscous residue (12.3 g).

Fungal mycelia (760 g) were extracted with hexane, EtOAc and MeOH. Evaporation of the solvents gave hexane crude as a brown viscous liquid (19 g), EtOAc crude as a dark brown residue (25 g) and methanol crude as a brown residue (10 g) respectively.

The EtOAc crude obtained from mycelia was isolated by silica gel column chromatography eluted with hexane, EtOAc and MeOH. The similar fractions were combined on the basis of TLC profile to give 14 combined fractions as shown in Table 4.8.

Table 4.8 The combined fractions of Ch109 obtained from the mycelia EtOAc extracted crude

Combined fractions	Fraction No.	Eluents	Appearance	Weight (mg)
ME-1	1	hexane (100)	Yellow viscous liquid	935
ME-2	2-6	hexane : EtOAc (90:10)	Dark-orange viscous liquid	4,120
ME-3	7-25	hexane : EtOAc (80:20)	Orange viscous liquid	286
ME-4	26-31	hexane : EtOAc (70:30)	Orange viscous liquid	55
ME-5	32-39	hexane : EtOAc (60:40)	Orange viscous liquid and orange solid	156
ME-6	40-54	hexane : EtOAc (50:50)	Red viscous liquid and orange solid	721
ME-7	55-60	hexane : EtOAc (40:60)	Red viscous liquid and orange solid	365
ME-8	61-72	hexane : EtOAc (30:70)	Dark red viscous liquid	1,110
ME-9	73-82	EtOAc (100)	Dark red viscous liquid	715
ME-10	83-89	EtOAc : MeOH (80:20)	Brown viscous liquid	395
ME-11	90-95	EtOAc : MeOH (60:40)	Brown viscous liquid	380
ME-12	96-104	EtOAc : MeOH (40:60)	Dark brown viscous liquid	928
ME-13	105-125	EtOAc : MeOH (20:80)	Black viscous liquid	2,980
ME-14	126-140	MeOH (100)	Black viscous liquid	5,110

Combined fraction ME-1 eluted from pure hexane (yellow viscous liquid) was crystallized by hexane and CH_2Cl_2 to give compound **1** and compound **2**. The NMR spectra were shown in appendix D. Compound **1** was characterized by 2D NMR including COSY, TOCSY, HSQC and HMBC. The structure of compound **1** was identified as tetrahydroauroglaucin as described by Smetanina et al. (2007) (figure 4.13). Comparison of ^{1}H and ^{13}C NMR data of compound **1** with tetrahydroauroglaucin were shown in Table 4.9.

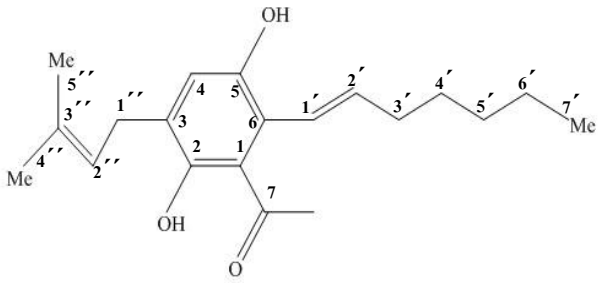


Figure 4.13 Compound **1** purified from Ch109 mycelia

Table 4.9 Comparison of proton and carbon chemical shifts between compound **1** and tetrahydroauroglaucin (Smetanina et al., 2007)

C atom		compound **1**		tetrahydcroauroglaucin	
		δ_C	δ_H	δ_C	δ_H
1	C	117.07		117.2	
2	C	155.05		155.1	
3	C	130.29		130.4	
4	CH	125.03	7.01 (s)	125.1	7.02 (s)
5	C	144.74		144.8	
6	C	123.95		124.0	
7	CH	196.30	10.09 (s)	196.2	10.10 (s)
1′	CH	120.04	6.48 (br.d, 16.2)	120.1	6.47 (br.d, 16.2)
2′	CH	142.69	5.98 (dt, 16.1, 6.9)	142.7	5.98 (dt, 16.1, 6.9)
3′	CH_2	33.45	2.32 (br.q, 7.1)	33.4	2.30 (br.q, 7.1)
4′	CH_2	28.70	1.51 (m)	28.7	1.52 (m)
5′	CH_2	31.43	1.34 (m)	31.4	1.37 (m)
6′	CH_2	22.45	1.35 (m)	22.4	1.37 (m)
7′	CH_3	14.01	0.91 (t, 6.8)	14.0	0.92 (t, 6.8)
1′′	CH_2	27.18	3.31 (d, 7.4)	27.2	3.31 (d, 7.4)
2′′	CH	120.93	5.29 (br.t, 7.4)	121.0	5.30 (br.t, 7.4)
3′′	C	133.95		133.9	
4′′	CH_3	25.79	1.75 (s)	25.8	1.76 (s)
5′′	CH_3	17.76	1.69 (s)	17.76	1.70 (s)

Tetrahydroauroglaucin was reported to the sources of isolation and bioactivity, the data were shown in table 4.10.

Table 4.10 Source of isolation and bioactivity of tetrahydroglaucin

Microorganisms	Bioactivity	Reference
Eurotium herbariorum	antioxidant with EC_{50} = 47.3 ± 3.8 µM	Miyake et al., 2009
E. repens	cytotoxic to sea urchin egg at 0.5 µg/ml	Smetanina et al., 2007
E. repens	binding affinity for human opioid receptor	Gao et al., 2011
E. amstelodami, E. rubrum and *E. herb*	anticancer and weakly cytotoxic to Hela cell	Slack et al., 2009
E. rubrum	anticancer against P-388, K-562, HL-60 and A-549 with IC_{50} > 10 µg/ml	Li et Al., 2008
E. repens	antioxidant with EC_{50} = 41 ± 1.4 µg/ml	Miyake et al., 2010
E. herb	antitumor with IC_{50} = 291 µg/ml	Miyake et al., 2010

^{1}H and ^{13}C NMR of compound **2** were similar to compound **1** except the side chain at C-6 of compound **2** was saturated alkyl group. In comparison ^{1}H NMR data of compound **2** with flavoglaucin (Table 4.11) as described by Miyake et al. (2009) (figure 4.14).

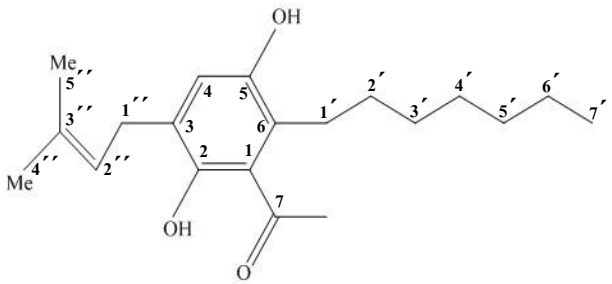


Figure 4.14 Compound **2** purified from Ch109 mycelia

Table 4.11 Comparison of proton chemical shifts between compound **2** and flavoglaucin (Miyake et al., 2009)

C atom		compound 2	flavoglaucin	
		δ_H	δ_C	δ_H
1	C		117.3	
2	C		155.8	
3	C		128.6	
4	CH	6.89 (s)	125.7	6.89 (s)
5	C		145.0	
6	C		128.6	
7	CH	10.25 (s)	195.5	10.25 (s)
1′	CH_2	2.88 (t, 7.6)	23.9	2.88 (t, 7.6)
2′	CH_2	1.57 (q, 7.6)	32.0	1.58 (q, 7.6)
3′	CH_2	1.39 (q, 7.2)	29.6	1.40 (q, 7.6)
4′	CH_2	1.32 (m)	29.1	1.3 (m)
5′	CH_2	1.76 (s)	31.8	1.75 (s)
6′	CH_2	1.27 (m)	22.6	1.28 (m)
7′	CH_3	0.87 (t, 6.8)	14.1	0.88 (t, 7.0)
1′′	CH_2	3.29 (d, 7.2)	27.0	3.29 (d, 7.3)
2′′	CH	5.28 (m)	121.2	5.28 (m)
3′′	C		133.8	
4′′	CH_3	1.69 (s)	17.8	1.70 (s)
5′′	CH_3	1.76 (s)	25.8	1.75 (s)

Flavoglaucin was reported to the sources of isolation and bioactivity, the data were shown in table 4.12.

Table 4.12 Source of isolation and bioactivity of flavoglaucin

Microorganisms	Bioactivity	Reference
E. herbariorum	antioxidant with EC_{50} = 93 ± 1.2 µM	Miyake et al., 2009
E. repens	binding affinity for human opioid receptor	Gao et al., 2011
E. amstelodami, E. rubrum and *E. herb*	anticancer and weakly cytotoxic to Hela cell	Slack et al., 2009
E. rubrum	anticancer P-388, K-562, HL-60, A-549 IC_{50} > 10 µg/ml	Li et Al., 2008
E. repens	antioxidant with EC_{50} = 29.2 ± 1.3 µg/ml	Miyake et al., 2010
E. herb	antitumor with IC_{50} = 299 µg/ml	Miyake et al., 2010

Combined fraction ME-4 eluted from hexane: EtOAc (70:30) (orange viscous liquid) was crystallized by hexane and CH_2Cl_2 to give compound 3 and compound 4. The NMR spectra were shown in appendix D. Compound **3** was characterized by 2D NMR including COSY, HSQC and HMBC. The structure of compound **3** was identified as physcion as described by Smetanina et al. (2007) (figure 4.15). Comparison of ^{1}H and ^{13}C NMR data of compound **3** with physcion were shown in Table 4.13.

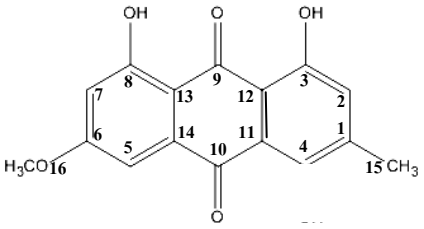


Figure 4.15 Compound **3** purified from Ch109 mycelia

Table 4.13 Comparison of proton and carbon chemical shifts between compound **3** and physcion (Smetanina et al., 2007)

C atom		compound **4**		physcion	
		δ_C	δ_H	δ_C	δ_H
1	C	162.54		162.5	
2	CH	124.50	7.09 (br.s)	124.5	7.08 (br.s)
3	C	148.44		148.4	
4	CH	121.28	7.63 (br.s)	121.3	7.62 (br.s)
5	CH	108.21	7.38 (d, 2.5)	108.2	7.36 (d, 2.5)
6	C	166.60		166.5	
7	CH	106.80	6.69 (d, 2.5)	106.8	6.69 (d, 2.5)
8	C	165.23		165.2	
9	C	190.85		190.8	
10	C			182.0	
11	C			133.2	
12	C			113.7	
13	C			110.3	
14	C			135.2	
15	CH_3	22.12	2.45 (s)	22.1	2.45 (s)
16	OCH_3	56.06	3.94 (s)	56.1	3.94 (s)

Physcion was reported to the sources of isolation and bioactivity, the data were shown in table 4.14.

Table 4.14 Source of isolation and bioactivity of physcion

Organisms	Bioactivity	Reference
Aspergillus glaucus	antibacterial against *B. brevis* with MIC > 20 µg/ml	Anke et al., 1980
E. repens	cytotoxic to sea urchin egg 25 µg/ml	Smetanina et al., 2007
A. glaucus	toxicity to mouse and chicken embryo	Bachmann et al., 1979
unknown	antifungal against *B. dinerea* and *P. grisea* with EC_{50} = 3.74 and 1.14 mg/L	Yang et al., 2010
E. repens	anticancer against HeLa cells with IC_{50} = 0.1 µg/ml	Podojil et al., 1978
L. coromandelica	toxicity to brine shrimp with LC_{50} = 53.59 ± 1.33 µg/ml	Rahman et al., 2008

^{1}H and ^{13}C NMR of compound **4** were similar to compound **3** except the side chain at C-4 of compound **4** was hydroxyl group. In comparison ^{1}H NMR data of compound **4** with erythroglaucin (Table 4.15) as described by Keller and Steglich (1987) (figure 4.16).

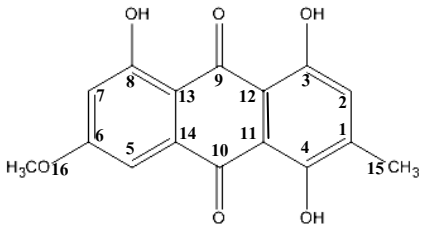


Figure 4.16 Compound **4** purified from Ch109 mycelia

Table 4.15 Comparison of proton chemical shifts between compound **4** and erythroglaucin (Keller and Steglich, 1987)

C atom		δ_H (compound **3**)	δ_H (erythroglaucin)
1	C		
2	CH	7.13 (s)	7.14 (br.s)
3	C		
4	CH	7.41 (s)	7.42 (d, 2.6)
5	CH		
6	C		
7	CH	6.69 (s)	6.71 (d, 2.6)
8	C		
9	C		
10	C		
11	C		
12	C		
13	C		
14	C		
15	CH_3	2.45 (s)	2.36 (d, 0.6)
16	OCH_3	3.94 (s)	3.95 (s)

Erythroglaucin was reported to the sources of isolation and bioactivity, the data were shown in table 4.16.

Table 4.16 Source of isolation and bioactivity of erythroglaucin

Microorganisms	**Bioactivity**	**Reference**
Aspergillus glaucus	antibacterial B. brevis > 20 μg/ml (MIC)	Anke et al., 1980
A. glaucus	toxicity to mouse and chicken embryo	Bachmann et al., 1979
C. globosum	antioxidant that exhibited moderate cytotoxicity towards several cell lines	Wang et al., 2006

4.8 Bioactivity assay of pure compounds

Four compounds isolated from Ch109 were tested antioxidant activity and MIC. The results were shown in Table 4.17 and 4.18.

Table 4.17 Antioxidant activity of compound **1-4**

Compound	**IC_{50} (µg/ml)**	**Vitamin E equivalent (fold)**
1	18	0.95
2	35.5	1.87
3	42	2.21
4	32	1.68
Vitamin E (control)	19	1

Table 4.18 MIC of compound **1-4**

Test microorganisms	**MIC (µg/ml)**			
	Compound 1	**Compound 2**	**Compound 3**	**Compound 4**
B. subtilis	250	250	50	50
S. aureus	250	250	80	80

CHAPTER V

CONCLUSION

150 endophytic fungi were isolated from leaves, stalks, flower and seeds of *Ipomoea pes-caprae* Linn. collected in 6 provinces of Thailand; Prajuabkirikhun, petchaburi, Songkla, Chonburi, Rayong and Chantaburi. There were *Alternaria* sp. (3 isolates), *Aspergillus* sp. (5 isolates), *Collectotrichum* sp. (7 isolates), *Curvularia* sp. (8 isolates), *Eurotium amstelodami* (1 isolate), *Fusarium* sp. (10 isolates), *Geotrichum* sp. (2 isolates), *Penicillium* sp. (4 isolates), *Phomopsis* sp. (9 isolates), *Stemphylium solani* (1 isolate), *Trichoderma* sp. (2 isolates), *Xylaria* sp. (15 isolates) and *Mycelia sterilia* (83 isolates).

All of selected endophytic fungi were tested antimicrobial activity against 5 microorganisms; *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231.

Endophytic fungus isolate Cm1 was exhibited high antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* with inhibition zone of 20, 20, 12, and 11 mm respectively. Endophytic fungus isolate Ch109 was exhibited high antimicrobial activity against *B. subtilis*, *S. aureus* and *C. albicans* with inhibition zone of 16, 16 and 13 mm respectively. Thus both fungi were selected for further studies. Based on light microscope identification and DNA sequencing of the ITS region, the fungus isolate Cm1 was identified as *Stemphylium solani* and Ch109 was identified as *Eurotium amstelodami.*

Isolation of bioactive compounds from mycelial EtOAc extracted of Cm1 found that combined fraction ME-10 exhibited the best result of antimicrobial activity with 13-25 mm of clear zone width. The antioxidant test of ME-10 showed IC_{50} 1.74 µg/ml and LC_{50} of brine shrimp lethality test was 414 µg/ml. The cytotoxicity test of ME-10 with 5 cell lines; BT474, CHAGO, HEP-G2, KATO-3 and SW620, IC_{50} were 14.05, 10.40, 8.41, 8.91 and 10.23 µg/ml, respectively. However, isolation bioactive compound from combined fraction ME-10 by column chromatography was unsuccessful.

Isolation of bioactive compounds from mycelial EtOAc extract of Ch109 by column chromatography gave 4 compounds including tetrahydroauroglaucin, flavoglaucin, physcion and erythroglaucin.

Antimicrobial and antioxidant DPPH assay of four compounds were tested. IC_{50} of compound 1 (tetrahydroauroglaucin), compound 2 (flavoglaucin), compound 3 (physcion) and

compound 4 (erythroglaucin) were exhibited antioxidant with IC_{50} of 18, 35.5, 42 and 32 µg/ml, respectively while vitamin E was exhibited antioxidant with IC_{50} of 19 µg/ml. Antimicrobial activity of compound 1-2 against *B. subtilis* with MIC of 250 µg/ml and *S. aureus* with MIC of 250 µg/ml. Compound 3 and 4 showed antimicrobial activity against *B. subtilis* with the same MIC of 50 µg/ml and against *S. aureus* with MIC of 80 µg/ml

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APPENDICES

APPENDIX A

Media

All of media were add distilled H_2O to a final volume of 1 L and sterile in the autoclave at 121 °C for 15 min. The pH was adjusted with NaOH or HCl before addition if agar and sterilization. The broth media didn't add the agar in the formula.

1. Corn meal agar (CMA)

Suspense 17.0 g in 1 L distilled H_2O

2. Malt extract agar (MEA)

Malt extracts	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	15.0 g

3. Mueller Hinton agar (MHA)

Suspense 21.0 g in 1 L distilled H_2O

4. Nutrient agar (NA)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g

5. Potato dextrose agar (PDA)

Suspense 39.0 g in 1 L distilled H_2O

6. Sabouraud's dextrose agar (SDA)

Peptone	10.0 g
Glucose	40.0 g
Agar	15.0 g

7. V8-juice agar (V8A)

V8 Vegetable juice	200.0 g
Calcium carbonate	4.0 g

Agar	15.0 g

8. Yeast-malt extract agar (YMA)

Malt extracts	3.0 g
Peptone	5.0 g
Yeast extracts	3.0 g
Glucose	10.0 g
Agar	15.0 g

APPENDIX B

Chemicals for molecular assay

1. Washing buffer

PVP (polyvinyl pyrrolidone)	1 %
Ascorbic acid	0.05 M
Tris-HCl (pH 8.0)	0.1 M
2-mercaptoethanol	2 %

2. 2x CTAB lysis buffer

CTAB	2 %
Tris-HCl (pH 8.0)	0.1 M
EDTA (pH 8.0)	20.0 mM
NaCl	1.4 M
2-mercaptoethanol	0.5 %

3. Chloroform : isoamyl alcohol (24:1 v/v)

Chloroform	192.0 ml
Isoamyl alcohol	8.0 ml
Final volume	200.0 ml

4. 20 % Polyethylene glycol (PEG)

Polyethylene glyvol	20 %
NACl	2.5 M
Autoclave 121 °C, 20 min	

5. TE buffer

Tris-HCl (pH 8.0)	10.0 mM
EDTA	1.0 mM

6. TBE buffer

Tris-base	54.0	g
EDTA	4.65	g
Boric acid	27.5	g

Add deionized H2O to a final volume of 500 ml

7. 6x loading dye

Bromophenol blue	25.0	mg
Glycerol	4.0	ml

APPENDIX C

Alignment data

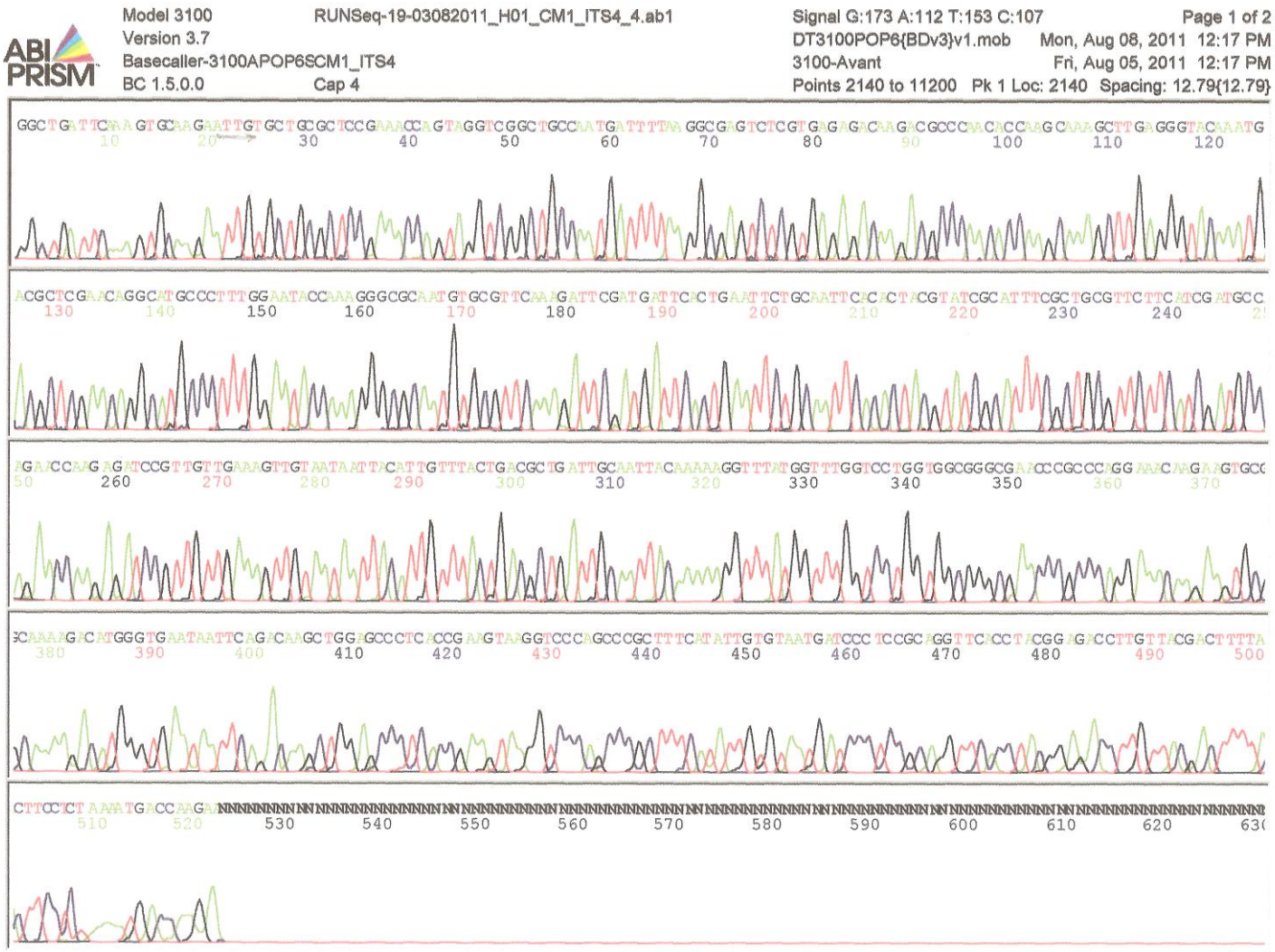

Figure C1 Nucleotide sequence of ITS region of isolate Cm1

CM1

Program BLASTN 2.2.25+

Description

All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)

Query Length 500

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF203451.1	Stemphylium solani strain SS1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	898	898	97%	0.0	100%
GQ999516.1	Uncultured fungus clone LX042234-122-013-F08 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	893	893	96%	0.0	100%
GQ999477.1	Uncultured fungus clone L042883-122-063-B12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	887	887	96%	0.0	99%
HQ649918.1	Pleospora sp. s008 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	883	883	95%	0.0	100%
GQ120980.1	Fungal endophyte isolate 281B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	881	881	100%	0.0	98%
GQ120976.1	Pleospora sp. 286A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	881	881	100%	0.0	98%
HQ533146.1	Stemphylium sp. CNU094002 18S ribosomal RNA gene, partial	880	880	98%	0.0	98%

Figure C2 Alignment data of ITS region of Cm1

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
	sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence					
AF203450.1	Stemphylium solani strain SS31 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	872	872	97%	0.0	98%
AF203449.1	Stemphylium solani strain SS28 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	872	872	97%	0.0	98%
AF203448.1	Stemphylium solani strain SS21 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	872	872	97%	0.0	98%
HQ650091.1	Uncultured Stemphylium clone CNU094013 internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	867	867	96%	0.0	98%
HQ650090.1	Uncultured Stemphylium clone CNU094007 internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	867	867	96%	0.0	98%
HQ650089.1	Uncultured Stemphylium clone CNU094005 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	867	867	96%	0.0	98%
HQ650085.1	Uncultured Stemphylium clone CNU094008 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	867	867	96%	0.0	98%
HQ650084.1	Uncultured Stemphylium clone CNU094004 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	867	867	96%	0.0	98%

Figure C2 (Continued)

gb|AF203451.1| Stemphylium solani strain SS1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Length=605

Score = 898 bits (486), Expect = 0.0

Identities = 486/486 (100%), Gaps = 0/486 (0%)

Strand=Plus/Minus

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Query  1    ATTGTGCTGCGCTCCGAAACCAGTAGGTCGGCTGCCAATGATTTTAAGGCGAGTCTCGTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  ATTGTGCTGCGCTCCGAAACCAGTAGGTCGGCTGCCAATGATTTTAAGGCGAGTCTCGTG  428

Query  61   AGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  AGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCA  368

Query  121  TGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATT  308

Query  181  CTGCAATTCACACTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAG  240
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Sbjct  7    ACTTCC  2
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Figure C2 (Continued)

Figure C3 Alignment data of ITS region of isolate Ch109

Ch109

Program BLASTN 2.2.25+

Description

All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)

Query Length 500

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ723257.1	Eurotium amstelodami isolate JH03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
GU721877.1	Uncultured fungus clone f3Fc82 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
GU721577.1	Uncultured fungus clone f2Fc36 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
GQ120984.1	Fungal endophyte isolate 253 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
AM901691.1	Eurotium sp. BF78 ITS region containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene	924	924	100%	0.0	100%
EF151446.1	Eurotium amstelodami isolate F21 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
DQ658184.1	Fungal sp. DQY-9 18S ribosomal RNA gene, partial sequence;	924	924	100%	0.0	100%

Figure C4 Alignment data of ITS region of Ch109

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
	internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence					
AF455536.1	Eurotium amstelodami isolate wb145 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
AF455520.1	Eurotium amstelodami isolate wb212 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
AF455470.1	Eurotium amstelodami isolate wb338 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
AF455464.1	Eurotium amstelodami isolate wb345 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	924	924	100%	0.0	100%
GQ999257.1	Uncultured fungus clone LX042399-122-056-E06 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	920	920	99%	0.0	100%
GQ999254.1	Uncultured fungus clone LX042234-122-013-H12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	920	920	99%	0.0	100%

Figure C4 (Continued)

gb|HQ728257.1| Eurotium amstelodami isolate JH03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Length=594

Score = 924 bits (500), Expect = 0.0

Identities = 500/500 (100%), Gaps = 0/500 (0%)

Strand=Plus/Minus

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Query  121  CAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAAT  180
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Query  181  ACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACAT  240
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Query  301  AGTTTTAACGATTGTTTAACTAAAAACTCAGACTGCAAACTTCAGACAGCGTTCAAATGT  360
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Query  361  TAGTCTCCGGCGGGCCGTGGCCACGCCGAAGCAACAGGGTACAGATAGACACGGATGGGA  420
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Sbjct  97   GGTTGGACCCAGAGGGCCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAAC  38

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Sbjct  37   CTTGTTACGACTTTTACTTC  18
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Figure C4 (Continued)

APPENDIX D

NMR data

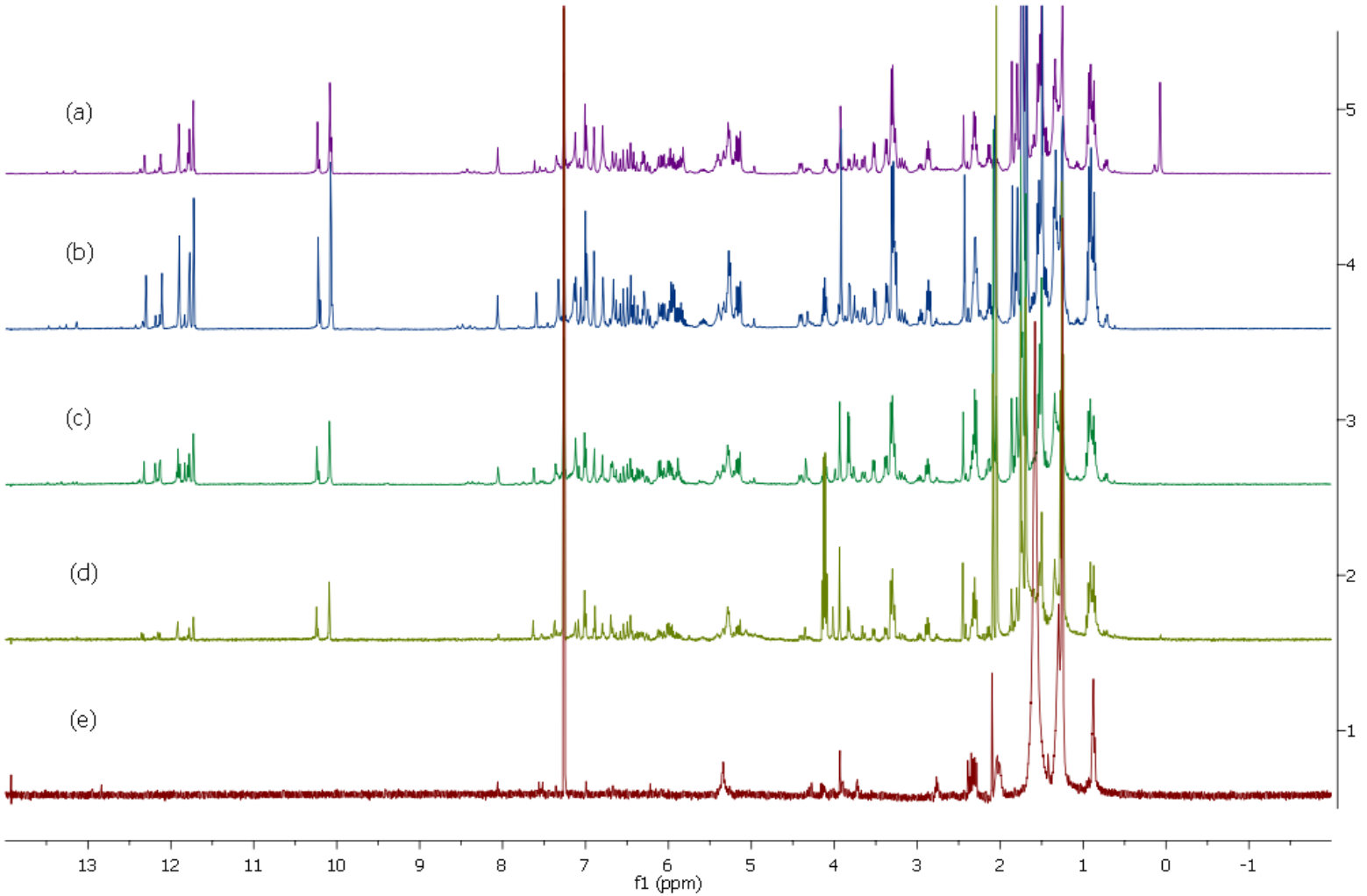

Figure D1 Proton NMR of Cm1 crude extract for 5 weeks. (a) week 1 (b) week 2 (c) week 3 (d) week 4 and (e) week 5

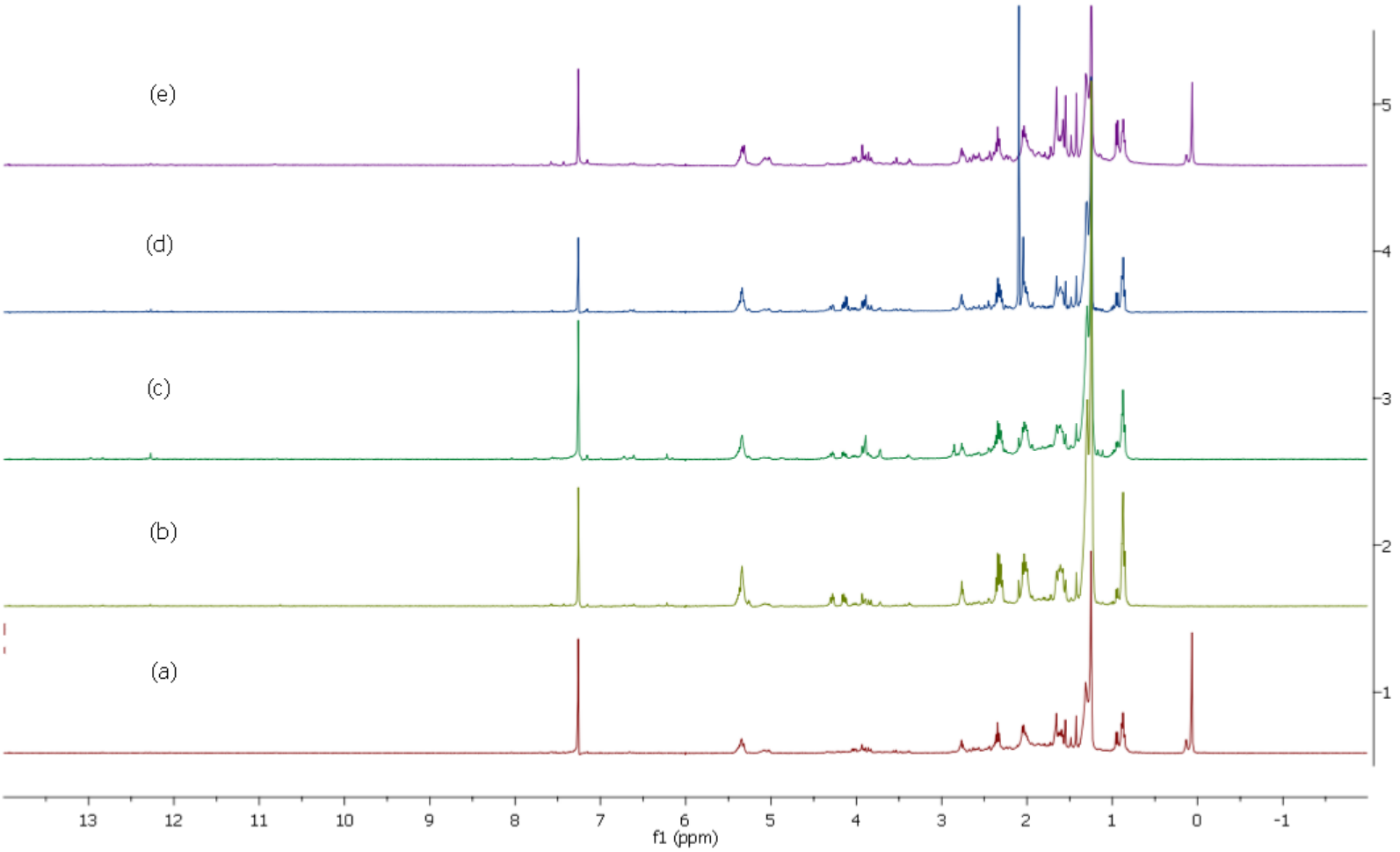

Figure D2 Proton NMR of Ch109 crude extract for 5 weeks. (a) week 1 (b) week 2 (c) week 3 (d) week 4 (e) week 5

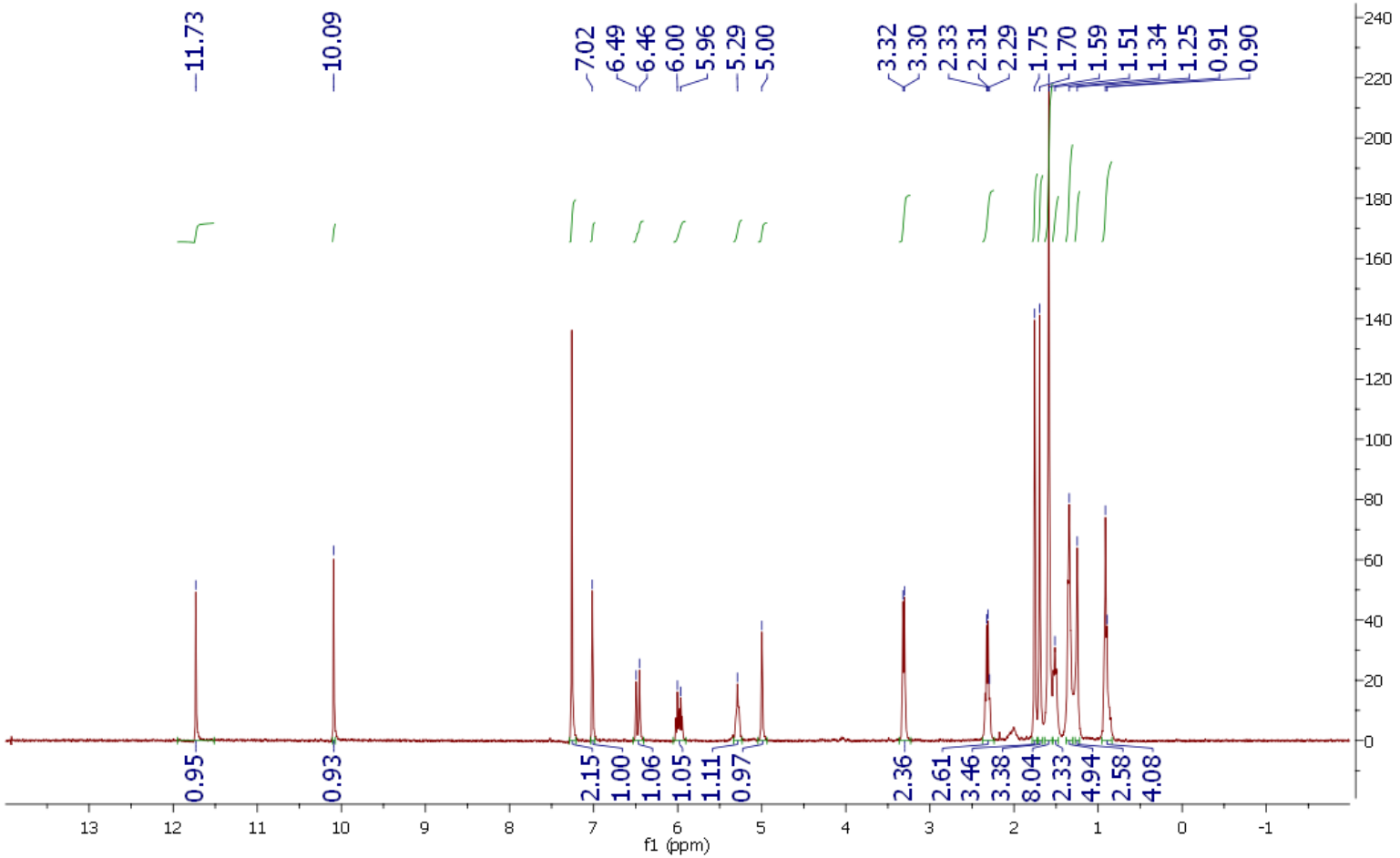

Figure D3 Proton NMR of Compound 1

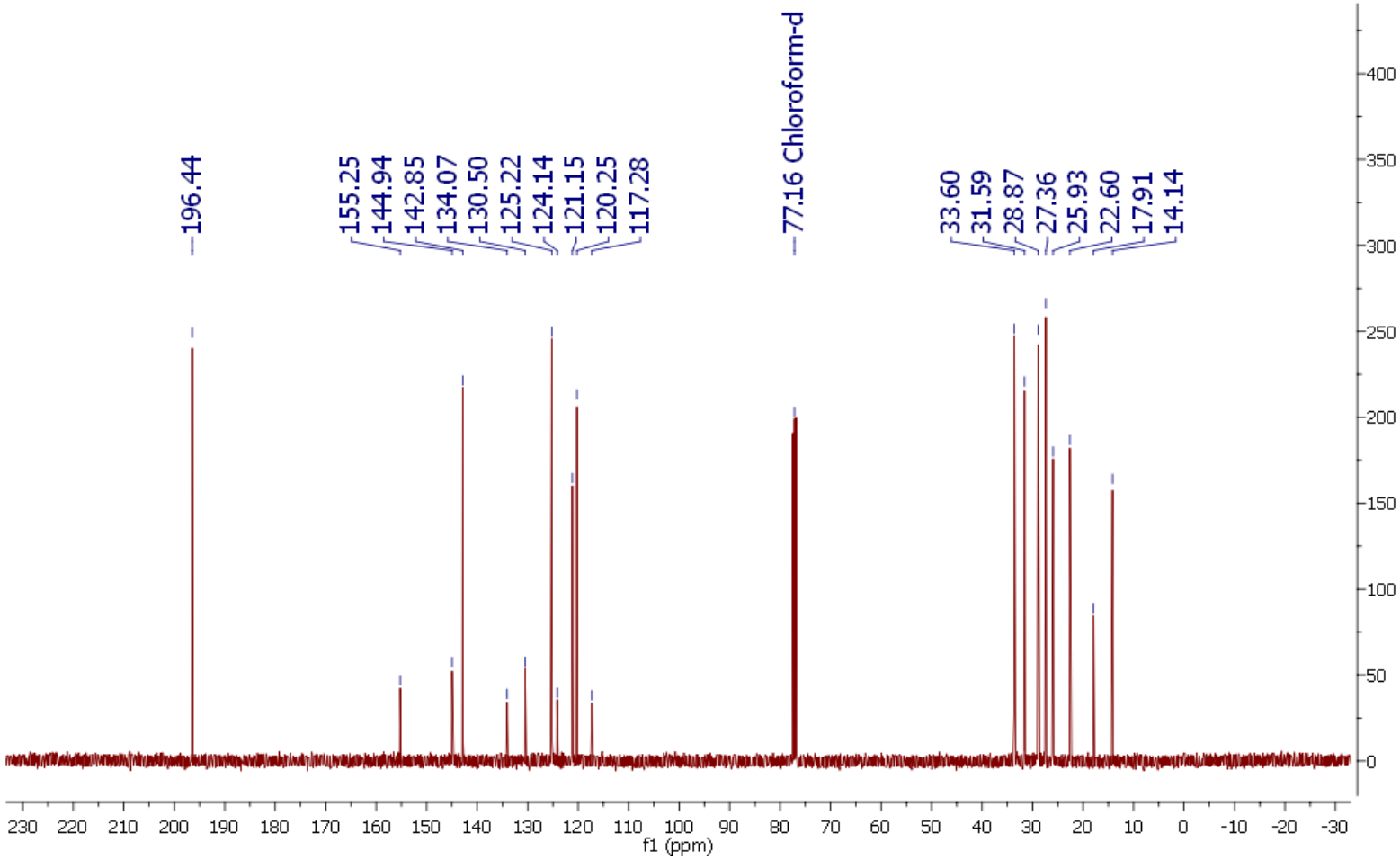

Figure D4 Carbon NMR of Compound 1

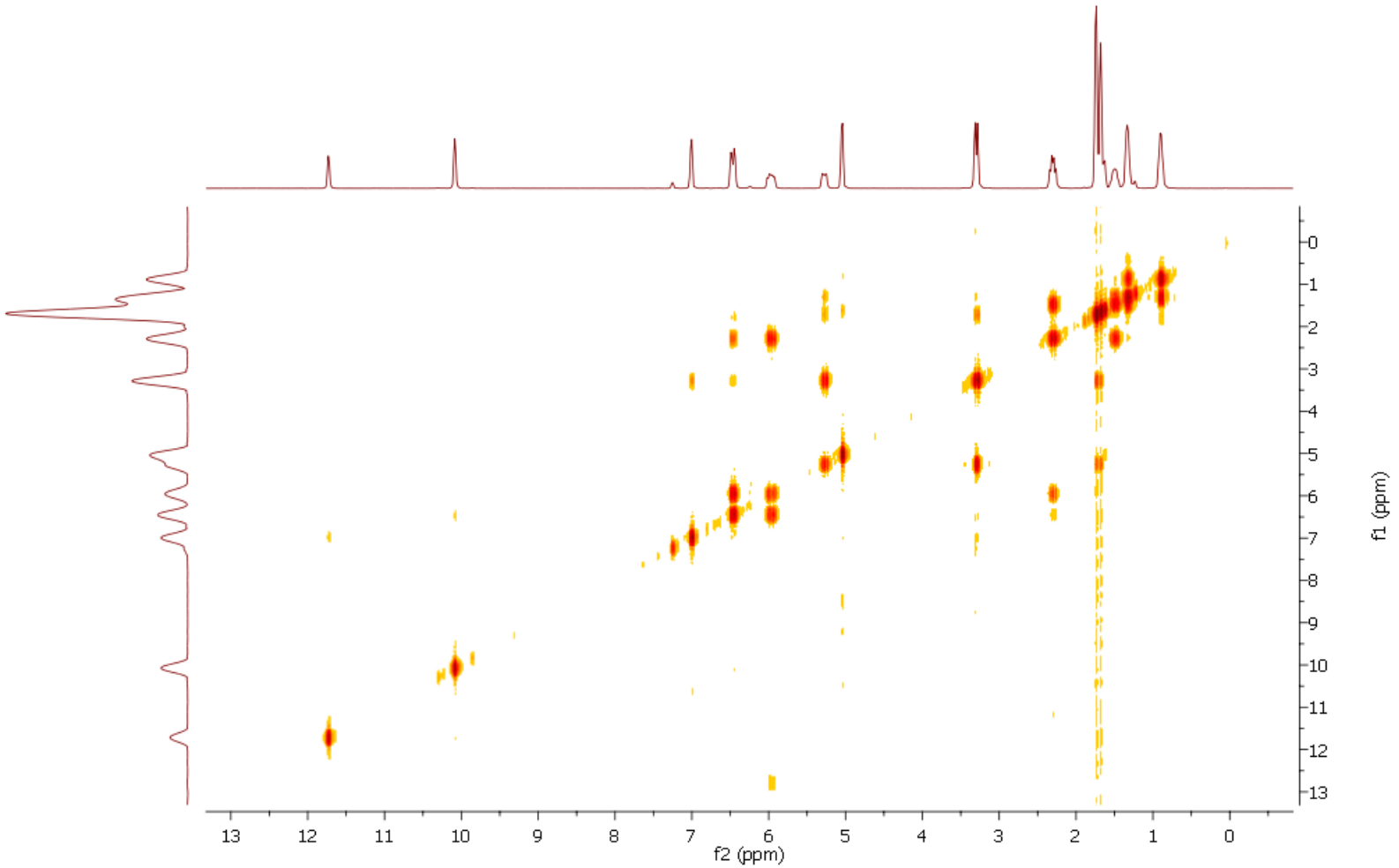

Figure D5 2D NMR COSY of Compound 1

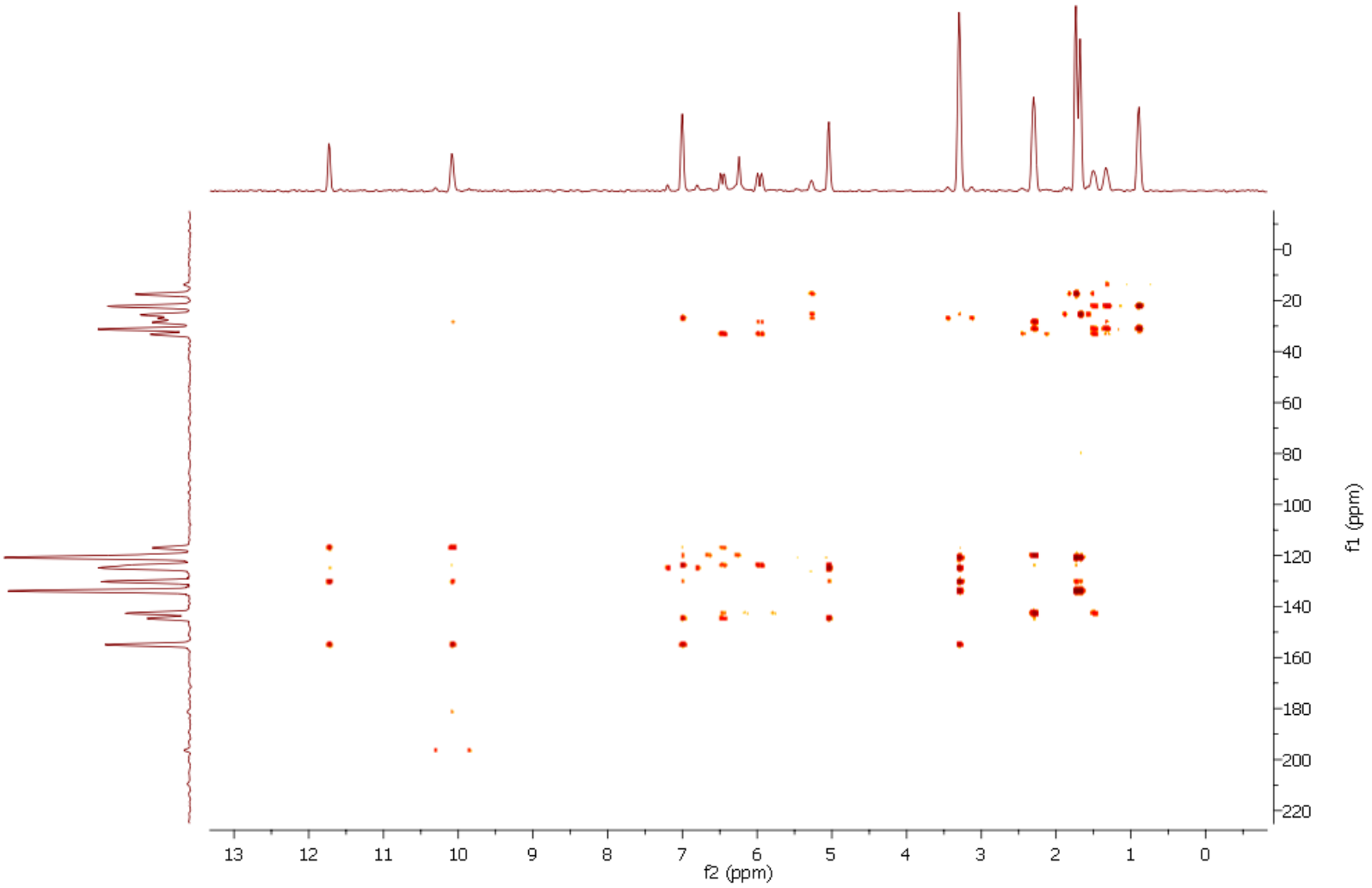

Figure D6 2D NMR HMBC of Compound 1

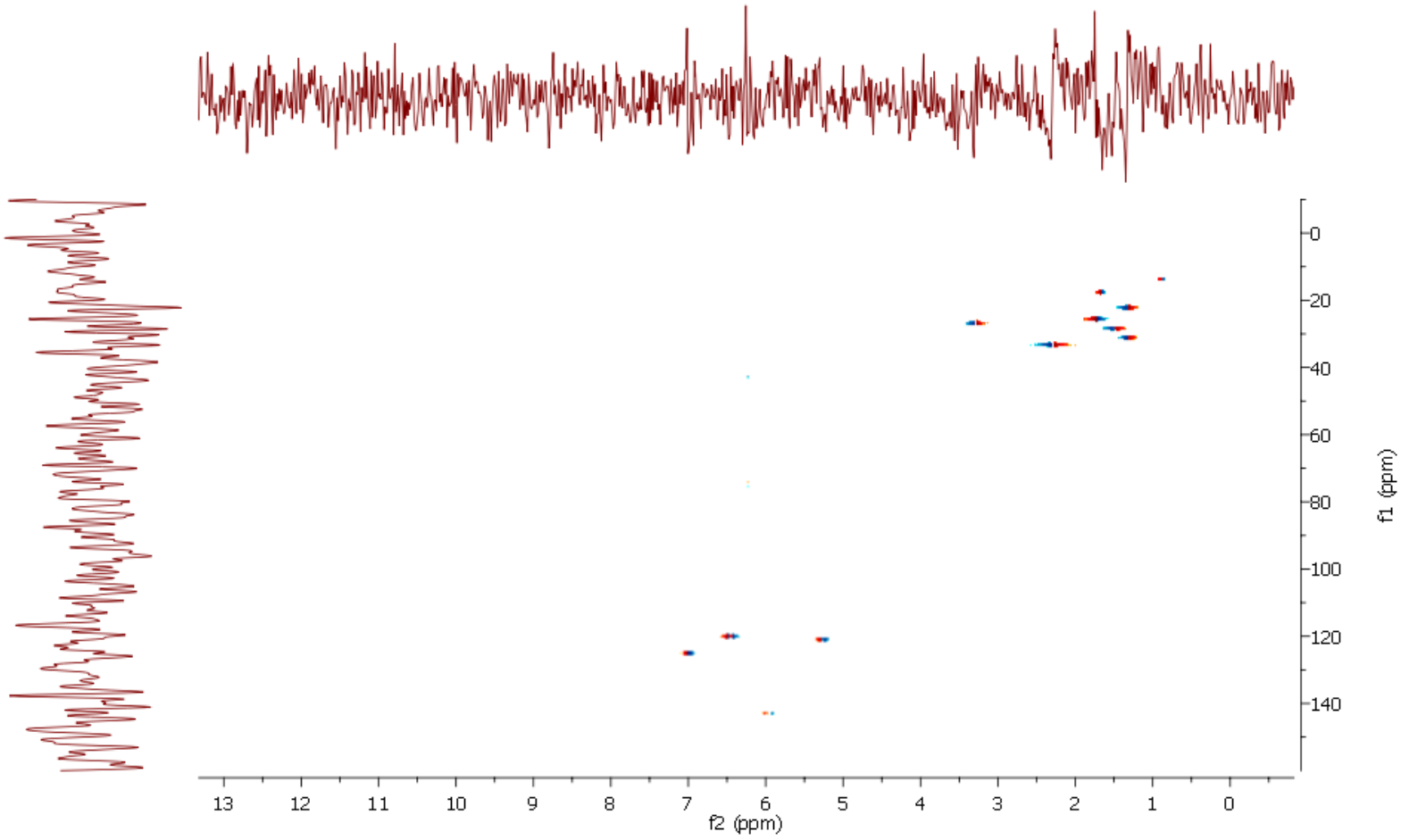

Figure D7 2D NMR HSQC of Compound 1

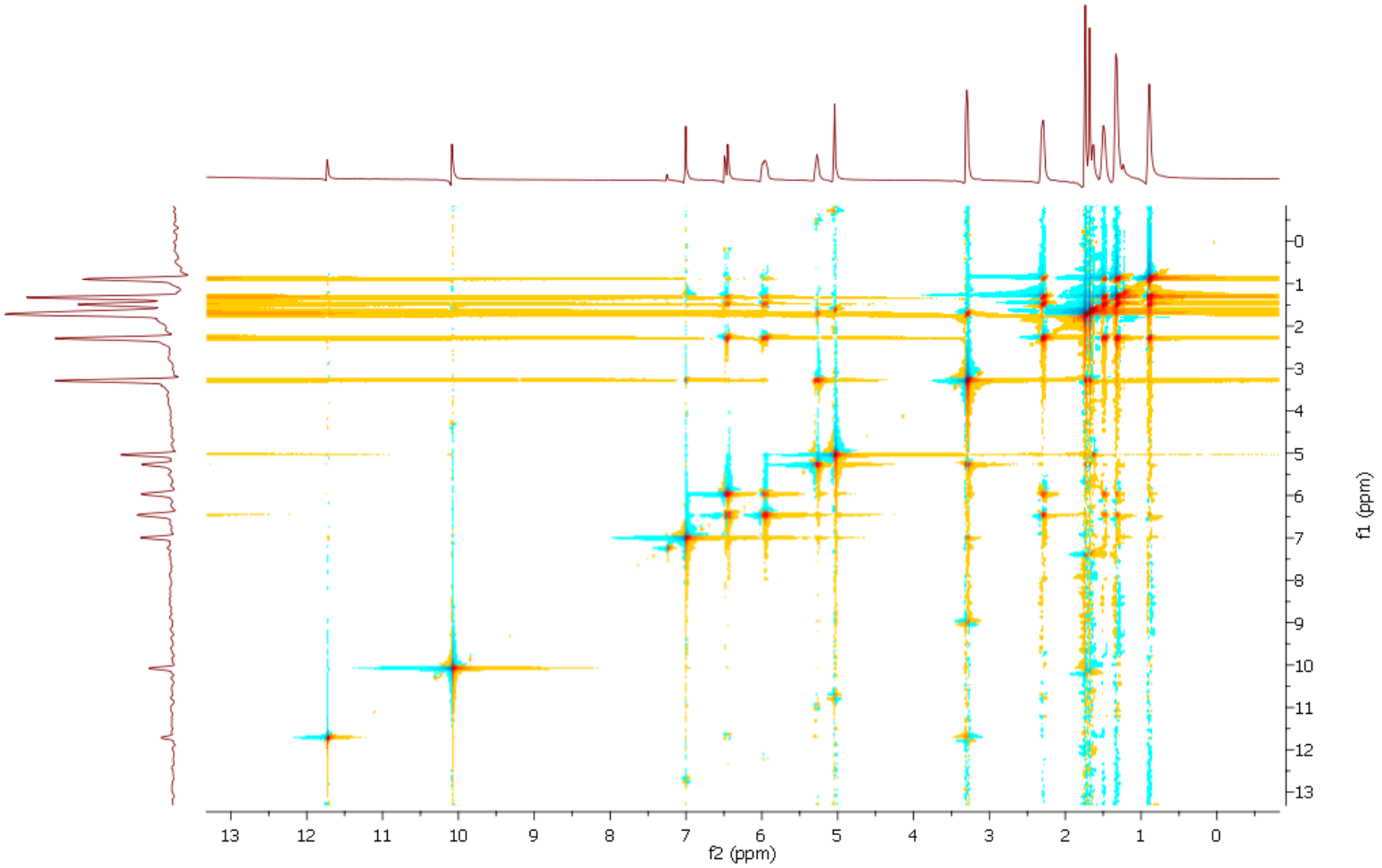

Figure D8 2D NMR TOCSY of Compound 1

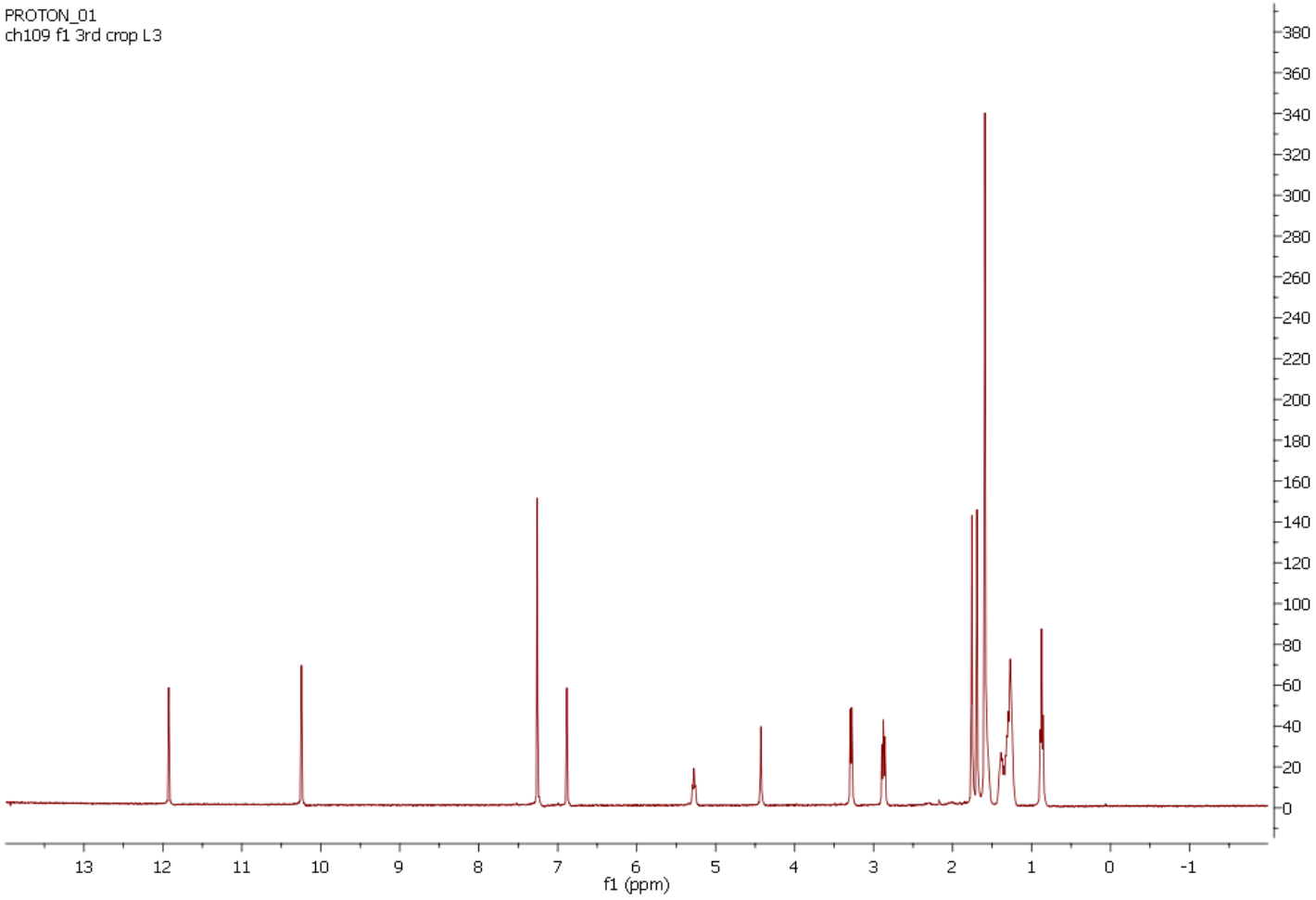

Figure D9 Proton NMR of Compound 2

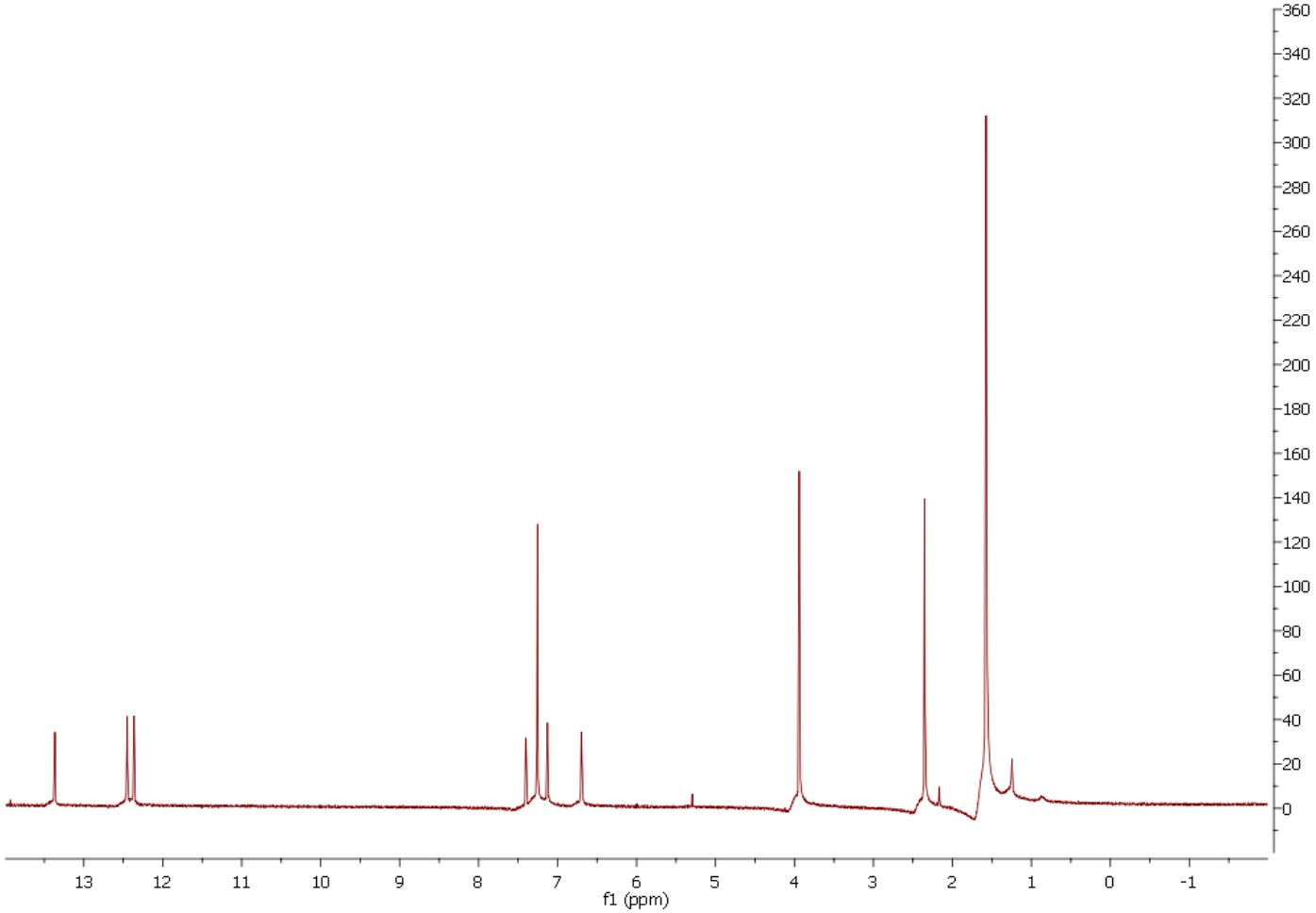

Figure D10 Proton NMR of Compound 3

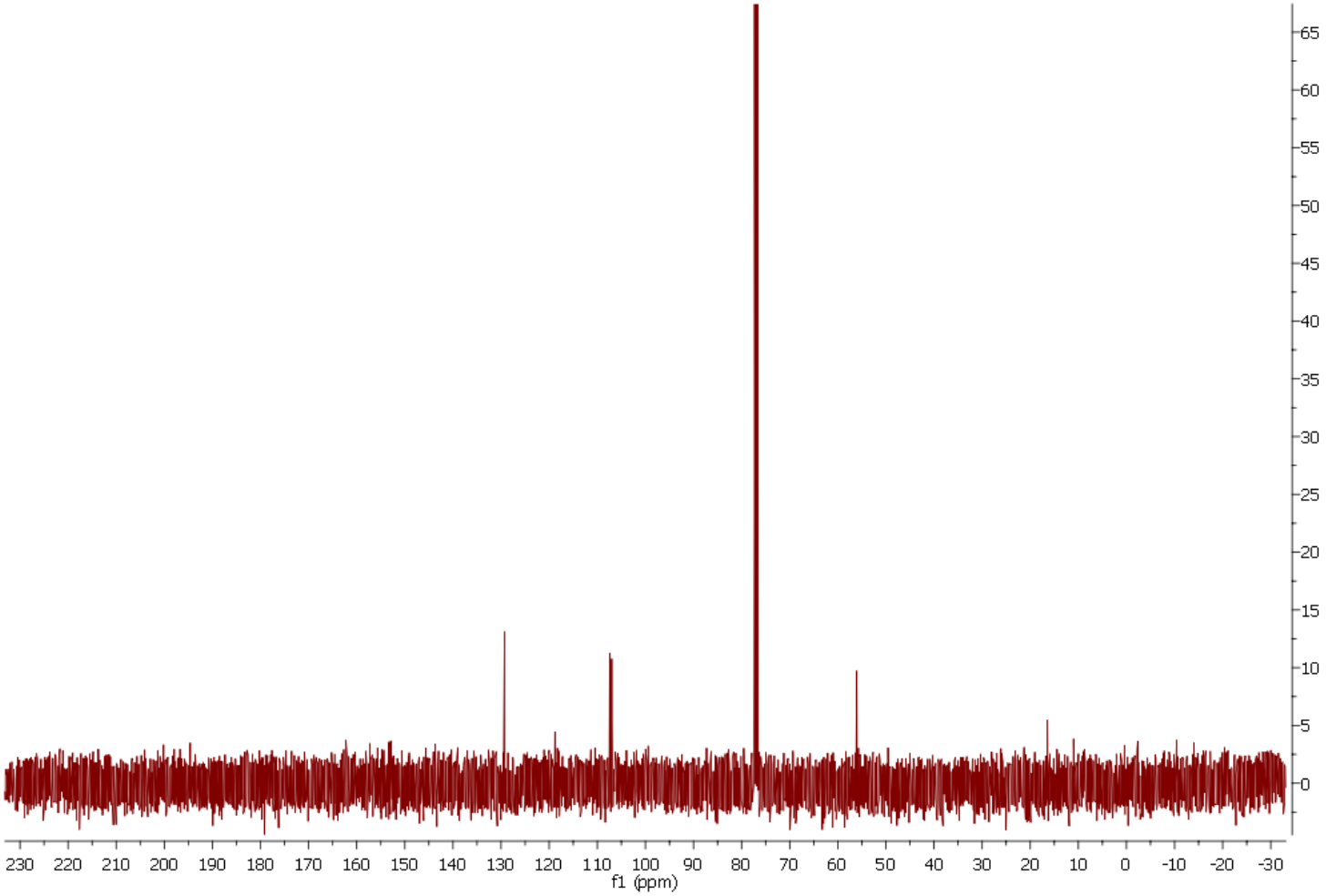

Figure D11 Carbon NMR of Compound 3

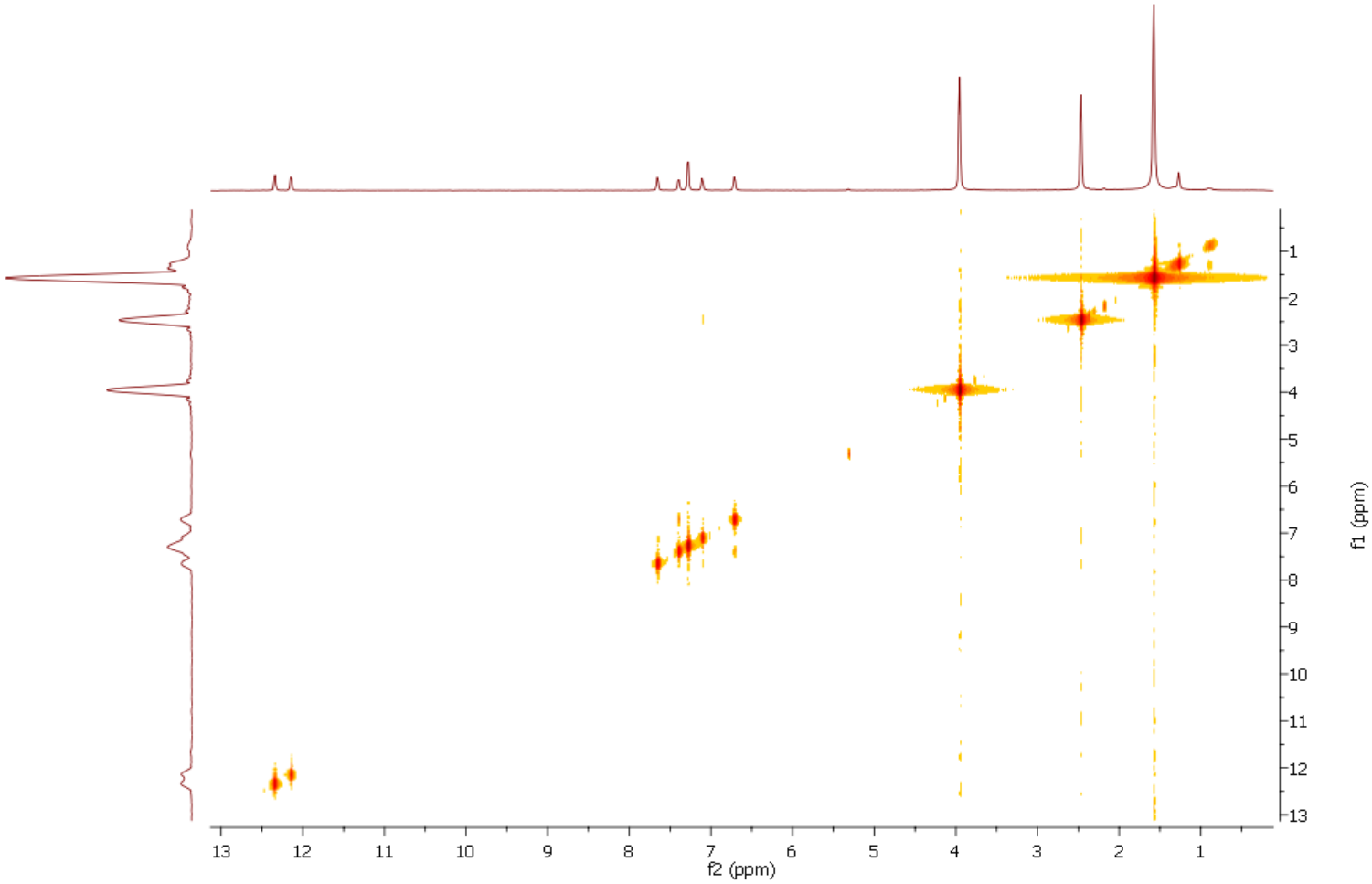

Figure D12 2D NMR COSY of Compound 3

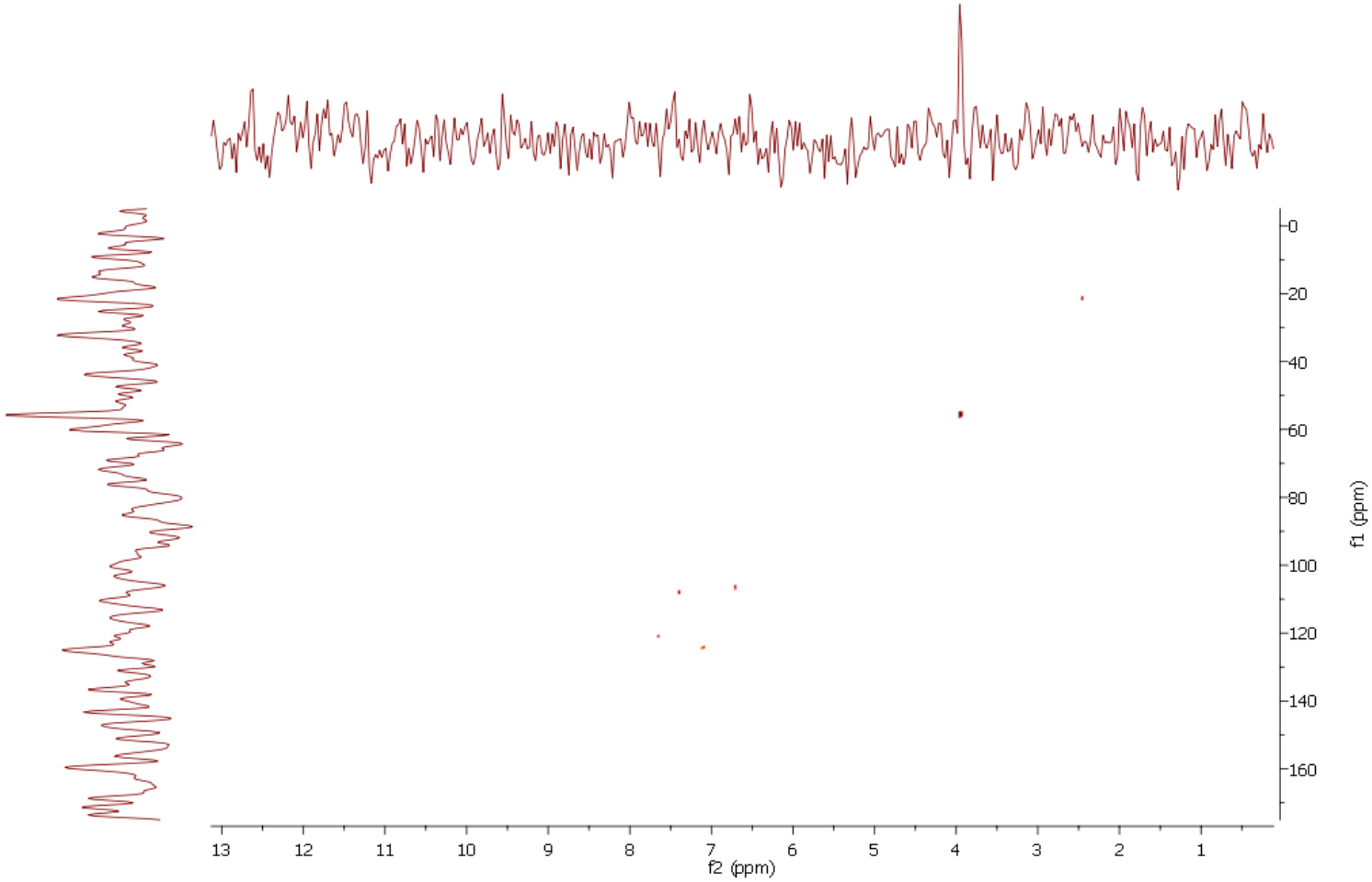

Figure D13 2D NMR HSQC of Compound 3

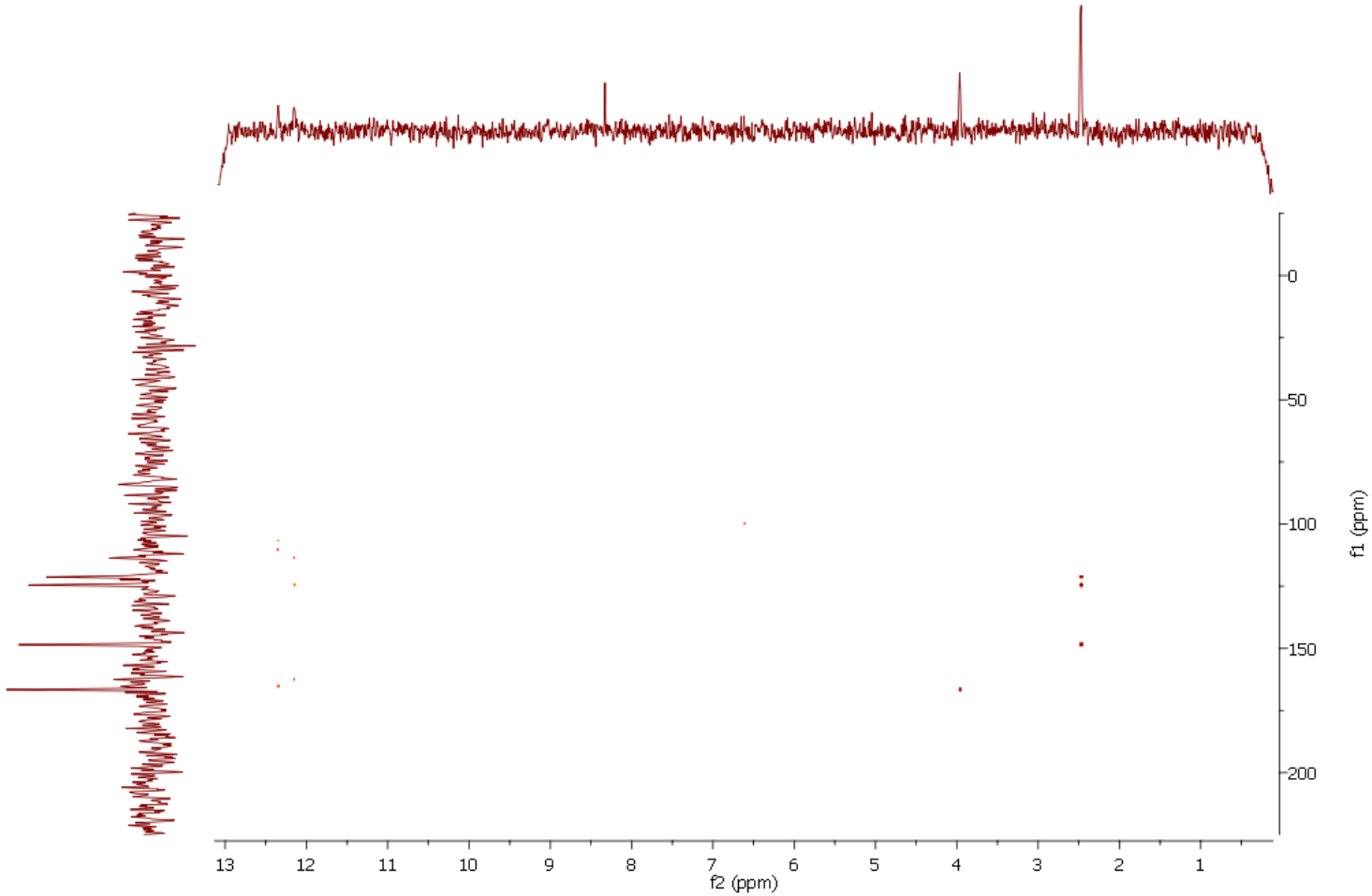

Figure D14 2D NMR HMBC of Compound 3

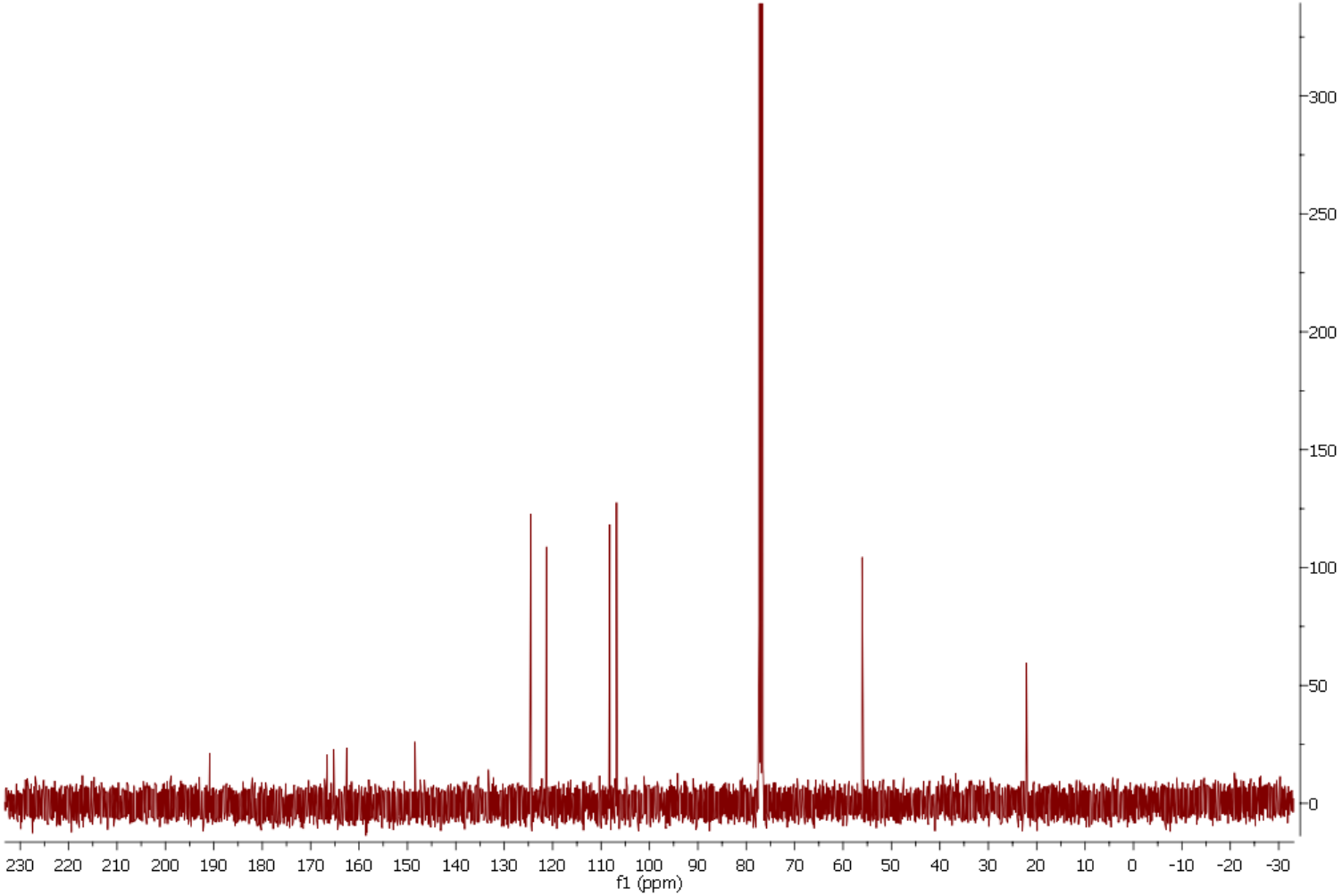

Figure D15 Carbon NMR of Compound 4

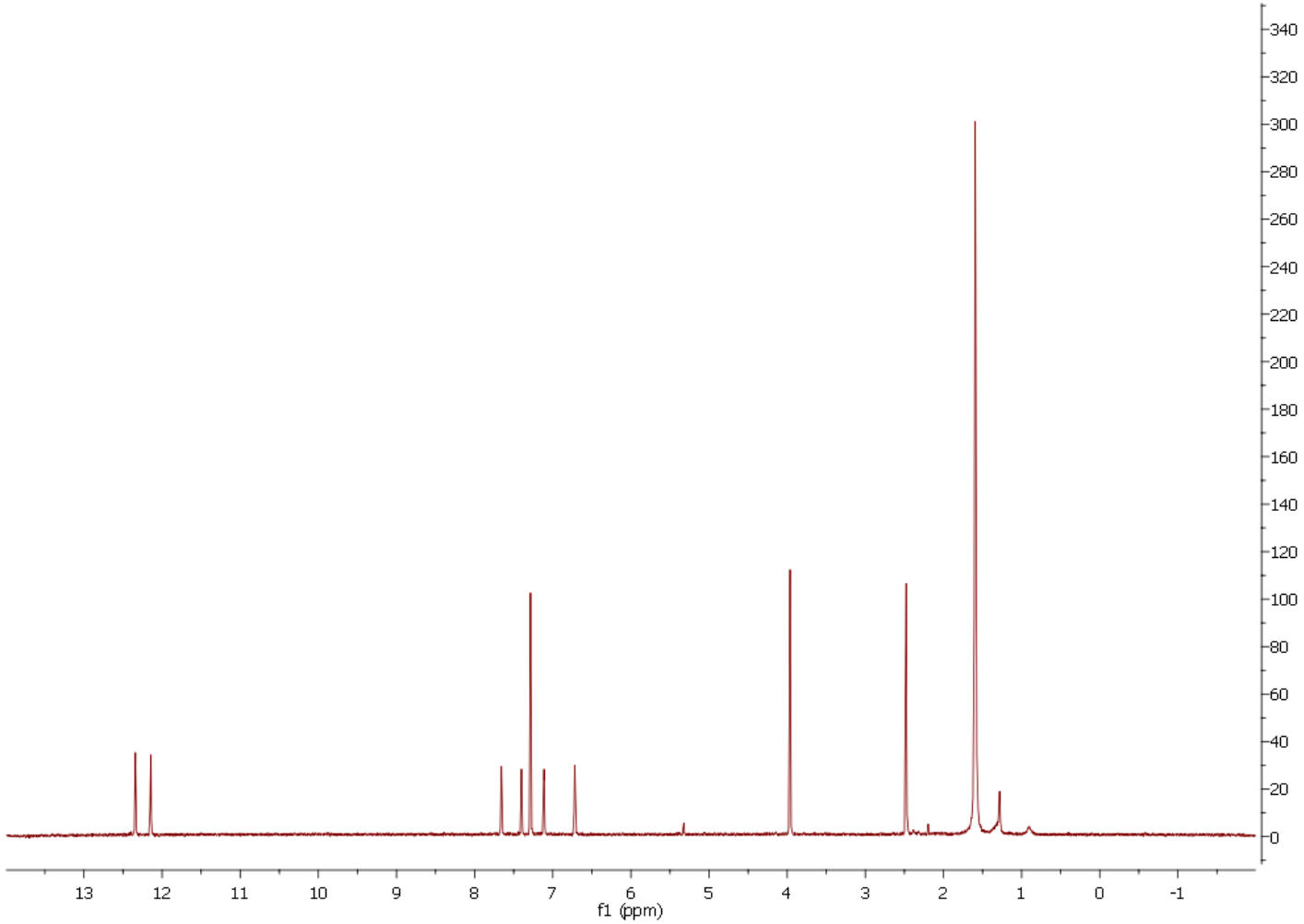

Figure D16 Proton NMR of Compound 4

BIOGRAPHY

Mr. Vachira Chaipackdee was born on January 23, 1975 in Bangkok, Thailand. He was graduated with a Bachelor Degree (Biology) from the Faculty of Science, Kasetsart University in 1997. In 2001, he was graduated with a Master Degree (Genetics) from the Graduate School, Kasetsart University. He has been studying for a degree of Doctoral Philosophy of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2006.