สารเมแทบอไลต์ต้านมะเร็งจาก *Xylaria* sp. PB-30

นางสาวพรธนา ชนะปราชญ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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METABOLITES AGAINST CANCER CELL LINES FROM *Xylaria* sp. PB-30

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[METABOLITES AGAINST CANCER CELL LINES FROM *Xylaria* sp. PB-30]

อาจารย์ที่ปรึกษา : รศ. ดร.สุรชัย พรภคกุล, 154 หน้า

งานวิจัยนี้มุ่งศึกษาการสร้างสารเมแทบอไลต์ที่มีฤทธิ์ต้านเซลล์มะเร็งของราเอนโดไฟต์ *Xylaria* sp. PB-30 ในอาหารมาตราฐานทั้ง 5 ชนิด ได้แก่ Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Corn Meal Broth (CMB), Yeast Extract Sucrose Broth (YEB) และ Sabouraud's Dextrose Broth (SDB) โดยนำส่วนของเส้นใยและน้ำเลี้ยงจากอาหารแต่ละชนิดนำมาสกัดด้วย เฮกเซน เอทิลแอซีเทตและเมทานอล ตามลำดับ นำส่วนสกัดที่ได้ทั้งหมดไปวิเคราะห์องค์ประกอบของกลุ่มสารเมแทบอไลต์ด้วยวิธีทางสเปคโตรสโคปี (^{1}H-NMR) แล้วเลือกกลุ่มสารที่น่าสนใจจากส่วนสกัดเอทิลแอซีเทตจากน้ำเลี้ยงในอาหาร SDB ที่ 27 วัน, อาหาร MCzB ที่ 35 วัน อาหาร MEB ที่ 35 วัน และส่วนสกัดเอทิลแอซีเทตจากส่วนเส้นใยที่ได้จากการเลี้ยงในอาหาร MEB ที่ 35 วัน นำมาทดสอบความเป็นพิษต่อเซลล์มะเร็ง พบว่า ส่วนสกัดจากเอทิลแอซีเทตจากส่วนน้ำเลี้ยงในอาหาร MEB ที่ 35 วัน ให้ผลยับยั้งเซลล์มะเร็งทั้งหมดได้ดีที่สุด จึงนำเชื้อราเอนโดไฟต์ *Xylaria* sp. PB-30 ซึ่งเลี้ยงในอาหาร MEB 30 ลิตร เป็นเวลา 35 วัน มาแยกสารเมแทบอไลต์ของราเอนโดไฟต์ PB-30 ได้สารประกอบ 5 ชนิด ได้แก่ (4S,5S,6S)-5,6-epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one **1**, สารประกอบ **2**, cytochalasin D **3**, 4-hydroxymellein **4** และ 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **5**. ทดสอบความเป็นพิษของสารประกอบ **1-5** ต่อเซลล์มะเร็งของมนุษย์ 5 ชนิด ได้แก่ BT474 (เต้านม) CHAGO (ปอด) HEP-G2 (ตับ) KATO-3 (กระเพาะอาหาร) และ SW620 (ลำไส้ใหญ่) พบว่า สารประกอบ **4** ไม่มีฤทธิ์ในการยับยั้งเซลล์มะเร็งทุกชนิด สารประกอบ 1 และ 2 มีฤทธิ์ยับยั้งเซลล์มะเร็งทุกชนิด สารประกอบ **1** จะยับยั้งเซลล์มะเร็งลำไส้ใหญ่ที่ค่า IC_{50} ต่ำสุดเท่ากับ 5.31 µg/ml และยับยั้งเซลล์มะเร็งกระเพาะอาหารที่ค่า IC_{50} เท่ากับ 5.61 µg/ml สารประกอบ **2** จะยับยั้งเซลล์มะเร็งกระเพาะอาหารและเซลล์มะเร็งลำไส้ใหญ่ที่ค่า IC_{50} ใกล้เคียงกันเท่ากับ 5.41 และ 5.49 µg/ml ตามลำดับ สารประกอบ **3** จะยับยั้งเซลล์มะเร็งตับและเซลล์มะเร็งลำไส้ใหญ่ที่ค่า IC_{50} ใกล้เคียงกันเท่ากับ 6.29 และ 6.81 µg/ml ตามลำดับ สารประกอบ **5** จะยับยั้งเซลล์มะเร็งตับเพียงเซลล์เดียวที่ค่า IC_{50} เท่ากับ 3.39µg/ml และไม่มีฤทธิ์ในการยับยั้งเซลล์มะเร็งอื่น

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต พรธนา ชนะปราชญ์

ปีการศึกษา .....2551..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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The objective of this research is to investigate production of anticancer metabolites from the endophtytic *Xylaria* sp. PB-30 isolated from the healthy and mature leaves of *Sandoricum koetjape*. After culture grown on five basal media including Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Corn Meal Broth (CMB), Yeast Extract Sucrose Broth (YEB) and Sabouraud's Dextrose Broth (SDB). It was found that anticancer metabolites of culture on MEB were produced more than on MCzB, SDB, YEB, CMB and most of them contained in EtOAc extract of broth and mycelia. From ^{1}H-NMR analysis hexane extract of mycelia and broth cultured on mainly contained triglycerides while methanol extracts mainly contained glucose. Metabolites of this fungus exhibiting anticancer activity were produced during stationary phase of growth which was within 35 days of cultivation. Thus EtOAc extract of broth from culture on SDB 27 day, MCzB 35 day, MEB 35 day and EtOAc extracts of mycelia on MEB 35 day were tested for in vitro cytotoxicity activity against cancer cell lines. The EtOAc extracts of broth from culture on MEB showed the highest cytotoxicity activity against all cancer cell lines. The metabolites produced by the endophytic fungus *xylaria* sp. PB-30 was isolated to afford 5 compounds including (4S,5S,6S)- 5,6-epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one **1**, compound **2**, cytochalasin D **3**, 4-hydroxymellein **4** and 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **5**. Cytotoxic activities of compound **1-5** were examined against 5 cell lines including BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), SW620 (colon). Compound **4** was inactive against all cancer cell lines. Compound **1** exhibited cytotoxic activity against all cancer cell lines and was selective against HEP-G2, KATO-III and SW620 with the IC_{50} values 6.25, 5.61 and 5.31µg/ml, respectively. Compound **2** exhibited cytotoxicity activity against all cancer cell lines and was selective against CHAGO, HEP-G2, KATO-III and SW620 with the IC_{50} values 6.09, 5.79, 5.41 and 5.49 µg/ml, respectively. Compound **3** exhibited cytotoxicity activity against HEP-G2 and SW 620 with the IC_{50} values of 6.29 and 6.81µg/ml respectively, and inactive against other cell lines. Compound **5** exhibited only cytotoxicity activity against HEP-G2 with the IC_{50} value of 3.39 µg/ml

Field of study : .....Biotechnology..... Student's signature.....Porntana Chanaprat.....

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LIST OF ABBREVIATIONS

$[\alpha]_D$	optical rotation
ATCC	American Type Culture Collection
bp	base pair
^{o}C	degree Celsius
cm^{-1}	reciprocated centimeter (unit of wave number)
^{13}C NMR	carbon-13 nuclear magnetic resonance
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	doublet doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
EI	Electron impact
g	gravity (NMR)
h	hour
HMBC	Heteronuclear Multiple Bond Cerrelation
^{1}H NMR	proton nuclear magnetic resonance
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
IR	infared
J	coupling constant
m	multiplet (NMR)
m	medium (IR)
M^{+}	molecular ion
MHz	megahertz
mg	milligram
min	minute
mL	milliliter (s)
MS	mass spectroscopy
m/z	mass to change ratio

nm	namometer
No.	number
NOESY	Nuclear Overhauser Enhancement Spectroscopy
ppm	part per million
q	quartet
s	singlet (NMR)
sp.	species
t	tripet (NMR)
TLC	thin layer chromatography
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet
μg	microgram
μL	microliter
δ	Chemical shift
λ_{max}	the wavelength at maximum absorption (UV)
ν_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

Cancer or malignant disease is one of the major causes of death in humans (WHO, 2002). Malignant neoplasm is the third (12.4%) leading cause of death worldwide, the first (30%) being cardiovascular disease, and the second (18.8%) being infectious diseases, which include HIV/AIDS (Mathers *et al*., 2001). Between 2000 and 2020, the total number of cases of cancer is predicted to increase by 73% in the developing world and by 29% in the developed world (Parkin, 2001). Around 60% of the new drugs registered during the period 1981-2002 by the FDA as anticancer, antiplasmodial and antihypertensive agents are based on natural products. Plants have been utilized as medicines for thousands of years and have a long history of use in the treatment of cancer (Hartwell, 1982). However, many of the claims for the efficacy of such treatments should be viewed with some skepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine (Cragg *et al.* 1994).

The treatment of cancer may benefit from the introduction of novel therapies derived from natural products. Natural products have served to provide a basis for many of the pharmaceutical agents in current use in cancer therapy (Pietras and Weinberg, 2005). The use of chemotherapeutic drugs in cancer involves the risk of life threatening host toxicity. In recently years, scientists have discovered symbiotic microorganisms in the intercellular spaces of stems, petioles, roots and leaves of plants were called endophytes. Endophytes living in the healthy tissues of plants are relatively unstudied and potential sources of novel natural products for exploitation in medicine agriculture and industry (Strobel and Daisy 2003).Accordingly, endophytes are an important source of novel secondary metabolites for pharmaceutical potential as exemplified by taxol (Stierle et al., 1993). Paclitaxel (taxol), a natural bioactive diterpene derivative and a widely used anticancer drug, was first identified from the Pacific yew *Taxus brevifolia*. (Yuan et al., 2006)

In this study we investigated influence of culture basal media on anticancer metabolites from the endophytic fungus *Xylaria sp.* PB-30 and screening for anticancer activity against cancer cell lines for finding novel anticancer drug that warrant further studies.

Objectives

1. To investigate the influence of culture media on anticancer metabolites
2. To isolate and identify anticancer metabolites of *Xylaria* sp. PB-30

CHAPTER II

LITERATURE REVIEW

2.1 Definition of Fungal endophytes

Fungal endophytes live internally, either intercellularly or intracellularly, and asymptomatically (i.e. without causing overt signs of tissue damage) within plant tissues. Endophytes usually occur in above-ground plant tissues, but also occasionally in roots, and are distinguished from mycorrhizae by lacking external hyphae or mantels. The meaning of the term endophyte has undergone various transformations in the last decade and there is still considerable disagreement as what constitutes an endophyte. We refer here broadly to fungi that live for all, or at least a significant part, of their life cycle internally and asymptomically in plant parts. We thus include a wide range of fungi, from fungal plant pathogens and saprophytes that have extended latency periods before disease or external signs of infection appear to specialized fungi in grasses that are considered obligate mutualists. Our rationale, as well as that of others, is that the distinction between classical plant fungal pathogens and host plant are often variable among and within populations and communities. We contend that the blurring of boundaries between fungal pathogen and mutualists is supported by evolutionary and ecological theory and by accumulating empirical studies.

Although mycologists have long known that plants harbor fungal endophytes and that endophyte in certain grass species in the subfamily Pooideae are associated with toxicity to grazing liverstock, the causal link between fungal endophytes and toxicity to herbivores was not firmly established until the 1970s. Since then, ecological evolution ary, mycological and agronomic studies involving fungal endophytes in pooid grasses have proliferated. It is now well-established that clavicipitaceous fungal endophytes in cultivated turf and pasture, as well as some naturally occurring grasses, can have wide-ranging and often dramatic biological effects on growth and reproduction of host grasses, pathogens and herbivores of grasses and natural enemies of herbivores.

Fungal endopytes mutuality relationship benefits, their through provision of energy, nutrients and shelter and manifest itself as improved growth and survival of individual host plants. In some cases an endophyte may survive as a latent pathogen, causing infections for a long period and symptoms only when physiological or ecological conditions favours virulence. Recent reports indicate that fungal endophytes are responsible for the adaptation of plants to abiotic stresses such as light, droght and biotic stresess, such as herbivory, insect attack or tissue invading pathogens through the production of secondary metabolites (Stierle et al, 1993).

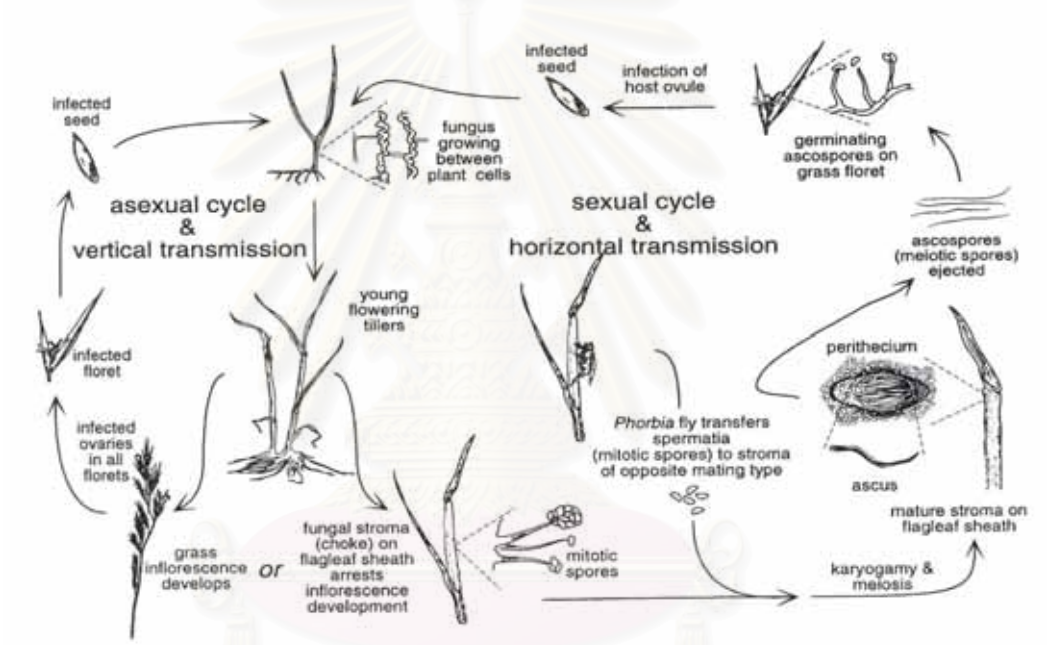


Figure 2.1 An example of balanced symbiosis involving coordinated life cycles of *Epichloe festucae* (MP-II) and one of its host grasses, *Fertuca rubra*.

In the past few decades, plant scientists have begun to realize that plants may serve as a reservoir of untold numbers of organisms known as endophytes (Bacon and White, 2000). By definition, these microorganisms (mostly fungi and bacteria) live in the intercellular spaces of plant tissues. Some of these endophytes may be producing bioactive substances that may be involved in host- endophytes relationship. As a direct result of the role that these Secondary metabolites may play in nature, they may ultimately be shown to have applicability in medicine. A worldwide scientific effort to

isolate endophytes and study their natural products is now under way. While there are myriads of epiphytic microorganisms associated with plants the endophytic ones now seem to be attracting more attention. This may be the case, since closer biological association may have developed between these organisms in their respective hosts than the epiphytes or soil-relate organisms. Hence, the result of this may be the production of a greater number and diversity of classes of biologically derived molecules, possessing a range of biological activities. In fact, a recent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown (Schutz, 2002). This compares with only 38% of novel substances from soil microflora.

One of the least studied biochemical-chemical systems in nature is the relationship between microorganisms and their plant hosts. For instance, it appears that all higher plants are hosts to one or more endophytic microbes. These microbes include the fungi, bacteria and actinomycetes, which primarily reside in the tissues beneath the epidermal cell layers and the host tissues, are transiently symptomless. It is well understood that endophytic infections are at least inconspicuous and as a result, the host tissues is internal to the surface of the plant (Stone and Bacon, 2000). The extract physical relationship of the endophyte to the plant has, in most cases remained obcure, because it is extremely difficult to find, by electron microscopy, an endophyte within plant tissues. Conceivably, the microbes live within the intercellular spaces of the tissues and it also seems likely that the penetration of living cells may occur, but it is not easy to observe under natural conditions.

These endophytic relationships may have begun to evolve from the time that higher plants first appeared on the earth hundreds of millions of year ago. Evidence of plant-associated microbes has been discovered in the fossilized tissues of stems and leaves (Bacon and White, 2000). As a result of these long-held associations, it is possible to imagine that some of these endophytic microbes may have devised genetic systems allowing for the transfer of information between themselves and the higher plants and vice versa (Stierle *et al,* 1993). Obviously, this would permit a more rapid and reliable mechanism for the endopyte to deal with everchanging environmental conditions and perhaps allow for more compatibility with the plant host. For these

reasons, it may have been the case that plant-associated microorganisms evolved biochemical pathways resulting in the production of plant growth hormones. Each of the five classes of these substances (auxin, abcisins, ethylene, gibberellins, and kinetins) is, in fact, known from a list of a range of representative plant-associated fungi and bacteria (Goodman *et al.,* 1986). In addition, independent evolution of the endophytic microbes may have allowed them to better adapt to a plant host and perhaps develop to point where they could contribute to the relationship by carrying out such functions as protection from pathogens, insects, and grazing animals. Thus, eventually various types of relationships may have formed, leading to symbiosis and ultimately to host specificity (Fisher and Petrini, 1992).

A relatively recent text presents an outstanding review of the biology of endophytes along with some aspect of their isolation, description, taxonomy, and uses in agriculture and forestry. It also describes the threats that some endophyte-plant associations have animal production (Bacon and White, 2000). On the other hand, in the same text, there is a comprehensive review on the uses and importance of the mycorrhizal fungi show the highest degree of plant compatibility, they exhibit relatively low host specificity, and useful products from them have not been isolated. It is also obvious that the earlier and numerous works of Petrini, Fisher and Carroll have led the way in showing how widespread, diverse and interesting these plant-associated microorganisms are in nature (Schutz, 2002).

2.2 Secondary metabolites from fungal endophyte

Endophytes colonizing inside plant tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain functional metabolites.

2.2.1 Growth promotion of the host plant

Endophyte-infected plants often grow faster than non-infected ones. This effect is at least in part due to the endophytes production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting sub-stances, and/or partly owing to the fact that endophytes could have enhanced the host uptake of

nutritional elements such as nitrogen and phosphorus. A culture broth of *Colletotrichum gloeosporioides,* an endophyte fungus of *Artemisia annua L*., has also been found to be able to promote the growth of the host callus (Tan and Zou, 2001).

2.2.2 Improvement of the host ecological adaptability

Certain endophytes improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens and/or herbivores including some insects feeding on the host plant. Endophyte-infected grasses usually possess an increased tolerance to drought and aluminium toxicity. Furthermore, some endophytes are able to provide the host plant with protection against some nematodes, mammal and insect herbivores as well as nematodes, bacterial and fungal pathogens. Some endophytes are capable of enhancing the hosts allelopathic effects on other species co-growing nearby, usually being competitor(s) for the nutrition and the space. This could be the reason why some plants with special endophytes are usually competitive enough to become dominant species in successional fields (Tan and Zou, 2001).

2.3 Secondary metabolites from Endophytes

In the world people having health problems caused by various cancers, drug-resistant bacteria, parasitic protozoans, and fungi is a cause for alarm. An intensive search for newer and more effective agents to deal with these disease problems is now under way.

Natural Products form plants, microbial and marine sources are important for discovery of new and potential drug molecules. Number of natural products with diverse chemical structures; have been isolated as biologically active compounds with great therapeutic potential, providing the molecular basis for most of drugs currently in clinical use, especially for cancer and infectious diseases. The search for novel secondary metabolites should be focused on endophytic microorganisms isolated from plants.

In the last few years cosiderable amount of knowledge has accumulated on the biology of endophytic microorganisms. The mutual relationship between endophytic microbes and their host plants, taxanomy and ecology of endophytes are being studied.

Recent reviews by Strobel (2003), Petrini (1991), Petrini *et al*. (1992) deal with biology of endophytes. Tan and Zou (2001) have summarized functional metabolites produced by endophytes, covering the years 1987–2000. The aim of our review is to briefly characterize endophytic microorganisms, summarize newly discovered endophytes and their structuraly different bioactive secondary metabolites and their host plants since the year 2001.

Recent studies have reported hundreds of natural products including substance of alkaloids, terpenoids, flavonoids, steroids, etc. from endophytes. Up to now, most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents and other bioactive compounds by their different functional roles.

Some examples of natural products obtained from endophyte microbes and their potential for exploitation in the pharmaceutical and agrochemical industries.

2.3.1 Endophytes producing antibiotics

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

2.3.1.1 Antibacterial and Antifungal compounds

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia.On petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. A unique peptide antimycotic, termed crytocandin, was isolated and characterized from *C. quercina* (Strobel *et al.,* 1999). This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid: 3-hydroxy-4-hydroxymethylpropine.

The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins (Walsh, 1992). As is generally true, not one but several bioactive and related compounds are produced by an endophytic microbe. Thus, other antifungal agents related to cryptocandin are also produced by *C.* cf. *quercina.* Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea.* Cryptocandin and its related compounds are currently being considered for use against a number of fungal-causing diseases of the skin and nails.

Pestalotiopsis microspora is a common rainforest endophyte. It turns out that enormous biochemical diversity does exist in this endophytic fungus, and many secondary metabolites are produced by various strains of this widely dispersed organism (Li *et al.,* 1996, Strobel *et al.,* 1996, Strobel, 2002a, Strobel, 2002b). One such secondary metabolite is ambuic acid, an antifungal agent, which has been recently described from several isolates of *P. microspora* found as representative isolates in many of the world's rainforests (Li *et al.,* 2001).This compound as well as another endophyte product, therein, have been used as models to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in disk diffusion assays (at a concentration of 4μg/disk) against *Bacillus subtilis, Salmonella gallinarum,* and *Staphylococcus aureus.* It also displays a moderate activity against the yeast *Candida tropicalis* (Horn *et al.,* 2001).

Colletotrichum sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed antimicrobial activity as well. *A. annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. The *Colletotrichum* sp. found in *A. annua* produced a new indole derivative 6-isoprenylindole-3-acetic acid, not only metabolite with activity against human pathogenic fungi and bacteria but also metabolite that was fungistatic to plant pathogenic fungi (Lu *et al.,* 2000).

2.3.1.2 Antiviral compounds

Another fascinating use of products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B have been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures were elucidated as *p*-tridepsides isomers by MS and NMR methods (Guo *et al.,* 2000). It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The fact, however, that some compounds have been found already is promising. The main limitation to compound discovery to date is probably related to the absence of common antiviral screening systems in most compound discovery programs.

2.3.1.3 Volatile antibiotics compounds

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) (Woropong *et al.,* 2001). This xylariaceaous (non-spore producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds. The majority of these compounds have been identified by GC/MS, synthesized or acquired, and then ultimately formulated into an artificial mixture. This mixture not only mimicked the antibiotic effects of the volatile compounds produced by the fungus but also was used to confirm the identity of the majority of the volatiles emitted by this organism (Strobel *et al.,* 2001). Each of the five classes of volatile compounds produced by the fungus had some microbial effects against the test fungi and bacteria, but none was lethal.However, collectively they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active. The composition of the medium on which *M. albus* grows dramatically influences the kind of volatile compounds that are produced (Ezra and Strobel, 2003). The ecological implications and potential practical benefits of the “mycofumigation” effects of *M. albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon be illegal in the United States. The potential use of mycofumigation to treat soil, seeds, and plants may soon be a reality. The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits, and other plant parts in storage and while

being transported. Using *M. albus* as a screening tool, it has now been possible to isolate other endophytic fungi producing volatile antibiotics. The newly described *M. roseus* was twice obtained from tree species growing in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus* (Woropong *et al.,* 2002). In addition, for the first time, a nonmuscodor species (*Gliocladium* sp.) was discovered as a volatile antibiotic producer. The volatile components of this organism are totally different than those of either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8]-annulene, formerly used as a rocket fuel and discovered for the first time as a natural product. However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or comprehensive as that of the *Muscodor* spp. (Stinson, Ezra, and Strobel, 2003).

2.3.2 Endophytes producing Antioxidants Activities

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage system of Papua New Guinea (Strobel *et al.,* 2002; Harper *et al.,* 2003a). Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity based on its structural similarity to the flavonoids. Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution (Strobel *et al.,* 2002). Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity (Harper *et al.,* 2003). The proposed antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C-H bond and, to a lesser extent, through O-H abstraction. The antioxidant activity of pestacin is at least 1 order of magnitude more potent than that of trolox, a vitamin E derivative (Harper *et al.,* 2003).

2.3.3 Endophytes producing Insecticidal Activities

Bioinsecticides are only a small part of the insecticide field, but their market is increasing (Demain, 2000). Several endophytes are known to have anti-insect

properties. Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). The two new compounds, 5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran, both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm (Findlay *et al.,* 1997).

2.3.4 Endophytes producing Antidiabetic Activities

A nonpeptidal fungal metabolite (L-783,281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo (Zhang *et al.,* 1999). This compound acts as an insulin mimetic but, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783,281 in two mouse models of diabetes resulted in significant lowering in blood glucose levels. These results may lead to new therapies for diabetes (Bensky and Gamble, 1999).

2.3.5 Endophytes producing Immunosuppressive Activities

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinols A and B (Lee et al., 1995a). Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) and thymocyte proliferation (TP) assays with an IC_{50} of 0.1 μM. In the same assay systems, the famed immunosuppressant drug cyclosporine A, also a fungal metabolite, was roughly as potent in the MLR assay and 104 more potent in the TP assay. Still, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants (Lee *et al.,* 1995a).

2.4 Regulation of secondary metabolism in fungi

Secondary metabolites (idiolites) are special metabolites usually possessing bizarre chemical structures and although not essential for the producing organism's

growth in pure culture, they have survival functions in nature. Secondary metabolites are produced only by some species of a genus. They possess unusual chemical linkages, such as lectam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings. Idiolites are produced typically as members of a particular chemical family because of the low specificity of some enzymes involved in secondary metabolism. They include mycotoxins, antibiotics, pigments, and pheromones. An important characteristic of secondary metabolism is that it is usually suppressed by high specific growth rates of the producing cultures. In addition to growth rate control, individual biosynthetic pathways are often affected by regulatory mechanisms such as induction, nutrient repression, synthetase decay, and end-product regulation. (Demain, 1986)

2.4.1 Carbon source regulation

Glucose, usually an excellent carbon source for growth but interferes with the biosynthesis of many secondary metabolites. Polysaccharides, oligosaccharides, and oils are often better carbon source for production than glucose. In a medium containing a rapidly used carbon source plus a more slowly utilized carbon source, the former usually is used first, idiolite production does not occur in this phase. After the favored carbon source is depleted, the second carbon source is used for idiolite biosynthesis.

2.4.2 Nitrogen source repression

A regulatory mechanism the controls the use of nitrogen sources is know in yeasts and molds. Ammonia (or some other readily used nitrogen source) represses enzymes involved in the use of alternate nitrogen source such as nitrite reductase, nitrate reductase, glutamate dehydrogenase, arginase, extracellular protease and acetamidase.

2.4.3 Phosphate regulation

Phosphate is the crucial growth-limiting nutrient in many secondary metabolite fermentations. Phosphate in the range of 0.3-300 mM generally supports extensive cell

growth, but concentrations of 10 mM and above suppress the biosynthesis of many secondary metabolites.

2.4.4 Enzyme induction

Many groups have reported on the stimulation of ergot alkaloid synthesis in *Claviceps* by the precursor tryptophan. Induction of a key enzyme by tryptophan was postulated by Floss and Mothes. This concept was supported by data showing stimulation

2.4.5 Growth rate

The mechanisms by which idiolite production is frequnently delayed until the end of the trophophase involves repression of the enzymes of secondary metabolism during growth (as described above) by sources of carbon, nitrogen and phosphorus. However, it also appears to involve growth rate.

2.5 Definition of the Xylariaceae

2.5.1 Biologycal diversity

The Xylariaceae (Xylariales, Ascomycotina) is a large family comprising of around 40 genera and although it has representatives in most countries of the world the Xylariaceae exhibits its greatest diversity in the tropics. As a result of comparatively recent in depth taxonomic studies, including biochemical, cultural and chemical approaches, there is now a reasonable understanding of species boundaries and intergeneric relationships within the. In early taxonomic studies in the Xylariaceae investigators were often confused by the wide variation in morphological form exhibited by many of the taxa. An extreme case being that of the dimorphic species *Camillea leprierii* Mont. In its upright or 'camilleoid' form it was placed in Camillea Fr. Whilst in its expanded or 'hypoxyloid' form it was referred to Hypoxylon Bull as H. melanspis Mont. The considerable variation in gross stromal morphology in certain species of Xylaria has in the past resulted in the same species being described as separate taxa. Furthermore, a lack of modern collections of tropical species has been an additional problem when attempting to understand diversity in certain genera. In developing a modern systematic

arrangement within the family teleomorphic features such as stromal form and colour, type of ostiole, structure of the ascus apical apparatus, shape and dimensions of ascospores, presence or absence and position of the germ slit, presence or absence of spore wall ornamentation have all proved to be useful taxonomic characters. Examination of the anamorphs, when these are produced, has also provided a valuable source of taxonomic characters which in the past were badly neglected. In the absence of a realistic inventory of the Xylariaceous genera it is still not possible to accurately assess the number of existing species or to predict with confidence how many might be expected. However on theasis of the recent revisions of Camillea Daldinia and Hypoxylon and a series of accounts of Xylaria from different regions it is possible to provide some indication of likely numbers for these Xylariaceaous genera. The figures for the number of known species are based on those provided in the current Dictionary of the Fungi subject to modifications for recently revised genera. The predicted number of species takes into account recent descriptions of new species and expectations for further new species predicted on the basis of percentage new species described following recent explorations in the tropics.

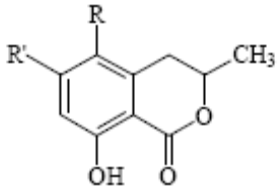
2.5.2 Chemical diversity

Unlike most ascomycete genera the Xylariaceae has received considerable attention over the past 25 years regarding production of secondary metabolites (Whalley and Edwards, 1995). In the earliest investigations of the family *Daldinia concentrica* (Bolt.:Fr.) Ces.& De Not. was found to contain 4,9-dihydroxyperylene quinone in the ascocarps (Allport and Bu'lock, 1958) whilst 1,8-dimethyoxynaphthalene and its corresponding ether were produced in culture (Allport and Bu'lock, 1960). During the same period Chen isolated rosellinic acid and diketopiperazine from cultures of Rosellinia necatrix Prill. And subsequently it was shown to produce cytochalasin E (Aldridge et al, 1972). Engleromycin, an epoxide of cytochalasin D was later isolated from the xylariaceous taxon, Engleromyces goetzii P. Henn. (Pedersen *et al.,* 1980). More recently *Hypoxylon fragiforme* (Pers.:Fr.) Kickx was found to owe its orange to brickred stromal colour to mitorubrin and its derivatives (Steglich et al, 1980) whilst *Xylaria polymorpha* Pers. produces a hydroxyphthalide derivative, xylaral, which

develops a violet purple colour with aqueous ammonia (Gunawan *et al,* 1990). Extensive studies in our laboratories have resulted in the characterization of many secondary metabolites from a range of representatives of the family and have demonstrated a remarkable diversity of chemical compound produced. A considerable number of these metabolites have proved to be new (Whalley and Edwards, 1995). The major metabolites produced by the representatives investigated can be grouped as dihydroisocoumarins and derivatives (Anderson et al, 1983), succinic acid and derivatives (Anderson et al, 1983) (Adeboya et al, 1996), butyrolactones (Edwards and whalley, 1979), cytochalasins (Edwards et al, 1989), sesquiterpene alcohols (punctaporonins) (Edwards *et al,* 1988) (Edwards et al, 1989), griseofulvin and griseofulvin derivatives, naphthalene derivatives (Whalley and Edwards, 1995) and long chain fatty acids (Edwards et al, 1995) (Adeboya *et al,* 1995).

Selected secondary metabolites from the Xylariaceae are as follows:

(1) Mellein. (2) 5-methyl-mellein. (3) 5-formyl-mellein. (4) 5-carboxy-mellein. (5) 5-methoxycarbonyl-mellein. (6) 5-hydroxymethyl-mellein. (7) 6-methoxy-5-methyl-mellein. (8) Iso-ochracein. (9) 4-hydroxyiso-ochracein. (10) Chromanone. (11) Ramulosin. (12) 2-butyl-3-methylsuccinic acid. (13) 2-hexylidene-3-methylsuccinic acid. (14) 4-(4`-methoxyphenoxy)-buta-1,2-diene. (15) Pyrenophorin. (16) Hymatoxin A. and its derivatives (17) B, (18) C, (19) D,(20) E. (21) 3,4,5,-trihydroxynaphthalenone. (22) 3,4,8,-trihydroxynaphthalenone. (23) Cytochalasin C. (24) Cytochalasin D. (25) Cytochalasin N. (26) Cytochalasin O. (27) Cytochalasin P. (28) Cytochalasin Q. (29) Cytochalasin R. (30) Punctaporonin B. (31) Hypoxylone. (32) Hypoxyxylerone. (33) Xylaral. (34) Cubensic acid. (35) Engleromycin. (36) Cytochalasin E. (37) Rosellichalasin. (38) 4,9-dihydroxyperylene quinone. (39) binaphthyl (40 and 41) Naphthalene derivatives.



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(2) R=CH 3, R'=H

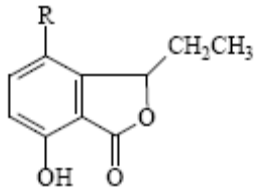
(3) R=CHO, R'=H

(4) R=CO 2H, R'=H

(5) R=CO 2CH3, R'=H

(6) R=CH 2OH, R'=H

(7) R=CH 3, R'=OCH 3



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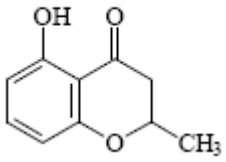
(9) R=OH



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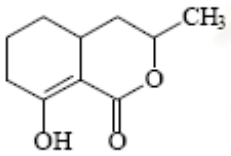

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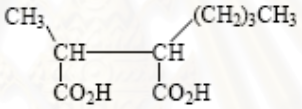

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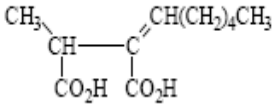

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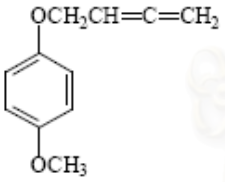

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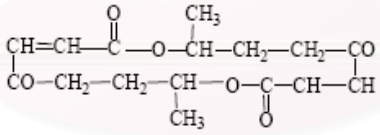

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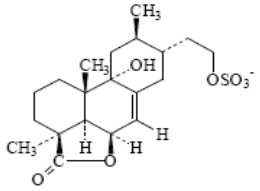

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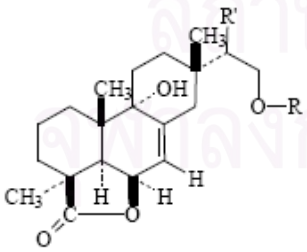

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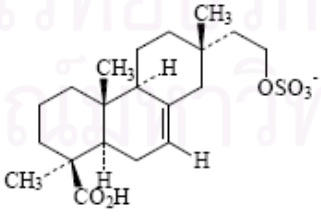

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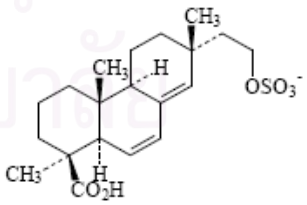

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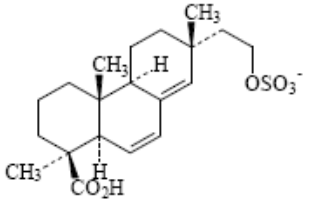

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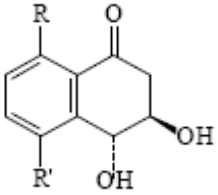

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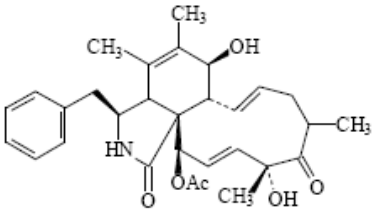
(22) R=OH, R'=H



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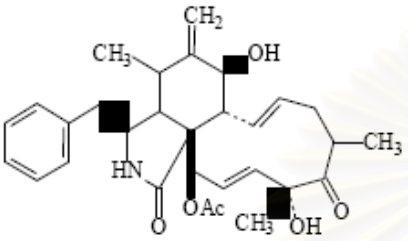

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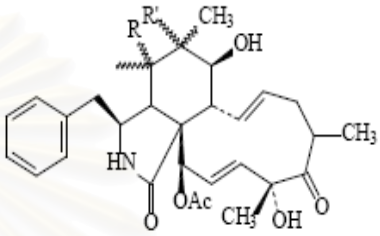

(25) R, R'= 

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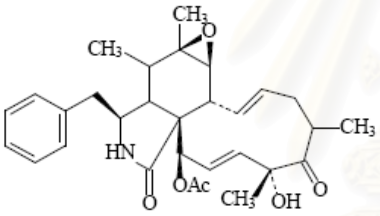

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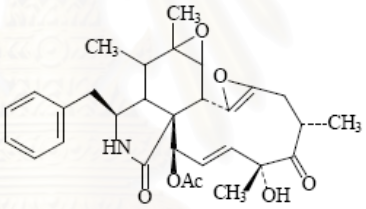

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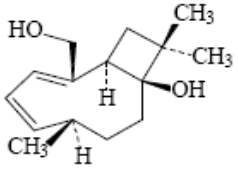

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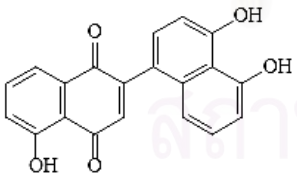

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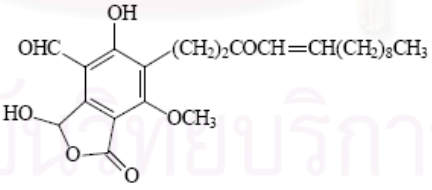

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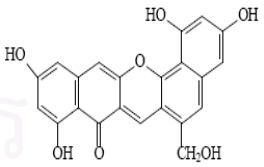

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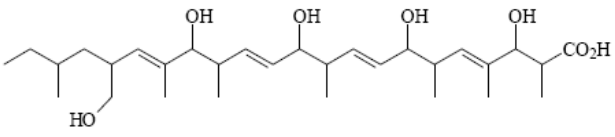

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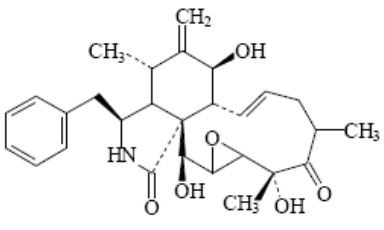

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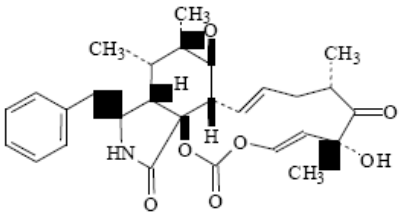

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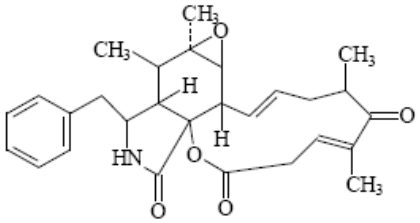

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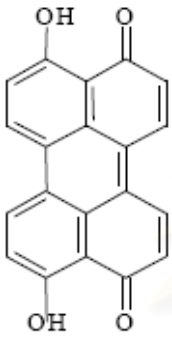

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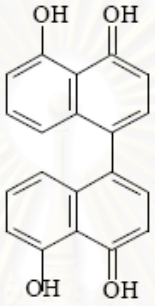

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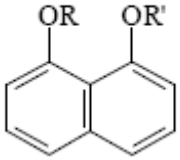


(40) R=H, R'=CH_3

(41) R=R'= CH_3

2.6 Definition of cancer

Cancer is a group of disease in which the cells in part of person's body grow uncontrollably. Cancer cells become aggressive, invasive and sometimes metastasis only malignant tumors are capable of invading other tissues or metastasizing (Chung *et al.,* 2006). Matastasis requires that cells pick up the ability to chew through the basal lamina (by activating extracellular proteinases called metalloproteases), enter the bloodstream, exit the bloodstream and survive in a new environment, or the lungs (Figure 1). They must be able to tolerate a very different environment in order to survive (Kleinsmith, 2005). Although there are many different types of cancer, they all develop as a result of uncontrolled growth of abnormal cells (Figure 2). The process involved both pro-oncogenes and tumor suppressor gene. Proto-oncogenes are involved in signal transduction by coding for a chemical "messenger" produced when a cell undergoes protein synthesis. These messengers send signals based on the amount of them present to the cell or other cells, telling them to undergo mitosis in order to divide and reproduce. When mutated they become oncogenes and over express the signals to

divide and thus cells have a higher chance to divide excessively (Cooper, 2000). While normal cells grow, divide and die in an orderly way. The rates of new cell growth and old cell death are kept in balance. In a healthy person, the cells divide at a rate to repair injuries and to replace depleted or dying cell, while the damaged cell self-destruct in process known as apoptosis. This normal balance is disrupted during the development of cancer. The growth rate of a tumor depends on the rate of cell division. When cell divides rapidly, Cancer may affect people at all ages, even fetuses, but risk for the tumor will grow quickly. The more common varieties tend to increase with age

Cancer is usually classified according to the tissue origin (location) or the normal cell type they most resemble (histology). A definitive diagnosis usually requires the histologic examination of a tissue biopsy specimen by a pathologist, although the initial indication of malignancy can be symptoms or radiographic imaging abnormalities. Cells in different parts of the body may look and work differently but most reproduce themselves in the same way (Skenhan *et al.,* 2007).

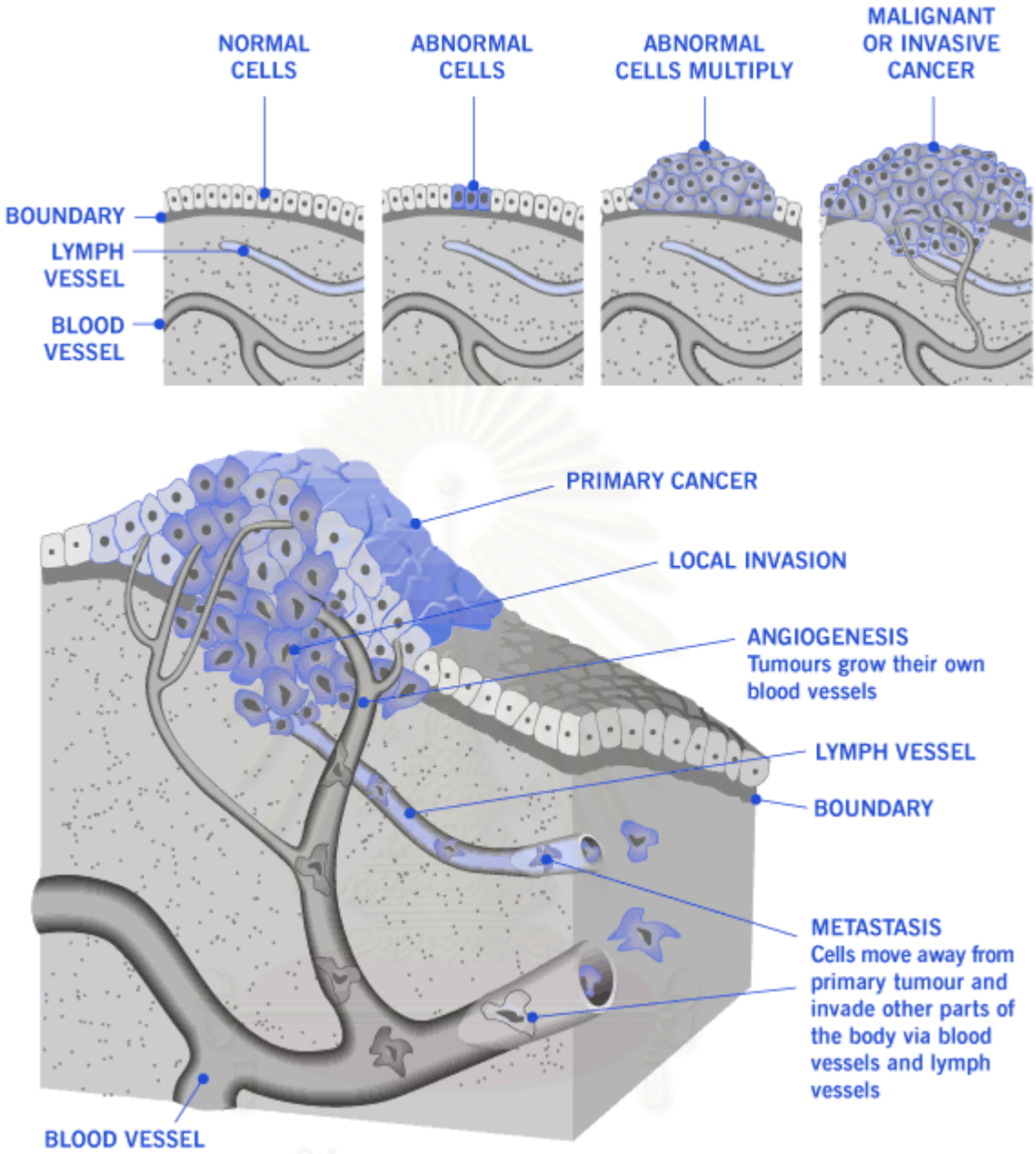


Figure 2 Metastasis developments (Kleinsmith, 2005).

(http://www.cancervic.org.au/aboutcancer/what_is_cancer.htm)

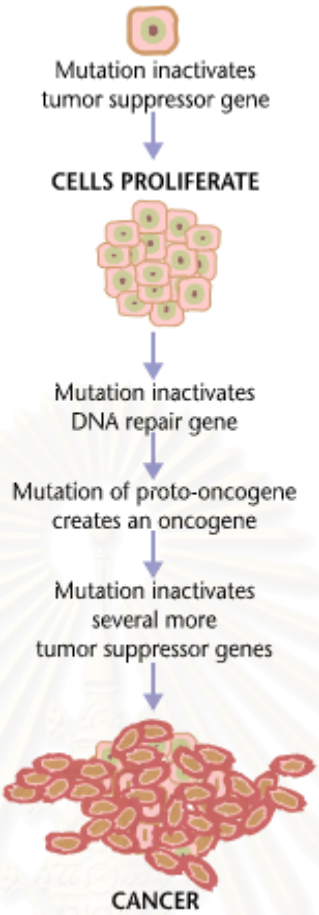


Figure 3 Series of mutation in cancer development, which each mutation alters behavior of the cell (Cooper, 2000). (http://en.wikipedia.org/wiki/Carcinogenesis).

Tumors can be either benign or malignant. Cancer is the name given to a malignant tumor. The tumor is benign or malignant by examining a small sample of cells under a microscope (Pisani *et al.,* 2006). In a benign tumor, cells do not spread to other parts of the body and so are not cancerous. However, if they continue to grow at the original site, they may cause a problem by pressing on the surrounding organs. A malignant tumor consists of cancer cells that have the ability to spread beyond the original area. If the tumor is left untreated, it may spread into and destroy surrounding tissues. Sometimes cells break away from the original (primary) cancer (Sharifah *et al.,* 2007). They may spread to other organs in the body through the bloodstream or lymphatic system. The lymphatic system is part of the immune system the body's natural defense against infection and disease. It is a complex system made up of organs, such as bone marrow, the thymus, the spleen, and lymph nodes. The lymph nodes

throughout the body are connected by a network of tiny lymphatic ducts. When the cancer cells reach a new area they may go on dividing and form a new tumor. This is known as a secondary cancer or metastasis. It is important to realize that cancer is not a single disease with a single type of treatment. There are more than 200 different kinds of cancer, each with its own name and treatment. Most cancers can be treated and some cured, depending on the specific type, location, and stage. Once diagnosed, cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. The treatments are becoming more specific for different varieties of cancer. There has been significant progress in the development of targeted therapy drugs that act specifically on detectable molecular abnormalities in certain tumors, and which minimize damage to normal cells (WHO, 2007).

2.6.1 Types of cancer

Cancer cells within a tumor are the descendents of a single cell, even after it has metastasized. Hence a cancer can be classified into 5 types by the type of cell in which it originated and by the location of the cell (Kleinsmith, 2005), (Ramonede, 2007).

2.6.1.1 Carcinomas The majority of cancers, about 85%, are carcinomas.They start in the epithelium, which is the covering of organs and of the body. Carcinomas are named after the type of epithelial cell that they started in and the part of the body that is affected. There are four different types of epithelial cells. Cancer that starts in squamous cells is called a squamous cell carcinoma. Cancer that starts in glandular cells is called an adenocarcinoma. Cancers that start in transitional cells are transitional cell carcinomas, and those that start in basal cells are basal cell carcinomas.

2.6.1.2 Sarcomas are malignant tumors derived from connective tissue, mesenchymal cells. They are a group of cancers that form in the supporting tissues of the body such as the bone, cartilage, fat, connective tissue and muscle.

2.6.1.3 Lymphomas is a general for malignancies derived from hematopoetic (blood-forming) cells, develop in the lymph node and the tissues of the immune system.

2.6.1.4 Teratoma is a type of tumor that derives from pluripotent germ cells. The word comes from a Greek term meaning roughly “monster tumor”. Teratomas (more correctly

teratomata) usually start from cells in the testes in men or the ovaries in women and in the sacrum in children. Teratomata involve cells from all three embryonic cell layers: ectoderm mesoderm and endoderm. They can be benign or malignant.

2.6.1.5 Melanoma is a malignant tumor of melanocytes. Melanocytes predominantly occur in the skin but can be found elsewhere, especially the eye. The vast majority of melanomas originate in the skin.

2.6.2 Signs and symptoms of cancer

Cancer can cause a variety of signs and symptoms. The type of sign or symptom depends on the size of the cancer, the location of the cancer, how much it is affecting neighboring organs and other body parts, and whether it has spread (Buell and Dunn, 1999). Many of the signs and symptoms associated with cancer may also be caused by noncancerous condition. As cancer grows, it begins to affect nearby organs, blood vessels and nerves. When these areas are compromised, it may create a variety of signs and symptoms (Ramonede, 2007), (Buell and Dunn, 1999). Cancer may also cause generalized or nonspecific signs and symptoms. These may be caused by immune system reaction or by cancer cell releasing substances that alter the body's metabolism (Sinha et al., 2005).

General signs and symptoms of cancer include:

2.6.2.1 Local symptoms: unusual lumps or swelling, hemorrhage, pain and/or ulceration. Compression of surrounding tissues may cause symptoms such as jaundice.

2.6.2.2 Symptoms of metastasis: enlarged lymph nodes, cough and hemoptysis, hepatomegaly, bone pain, fracture of affected bones and neurological symptoms. Although advanced cancer may cause pain, it is often not the first symptom.

2.6.2.3 Systemic symptoms: weight loss, poor appetite, fatigue and cachexia, excessive sweating (night sweats), anemia and specific paraneoplastic phenomena, i.e. specific conditions that are due to an active cancer, such as thrombosis or hormonal changes.

2.6.3 Potential causes of cancer

2.6.3.1 Diet and lifestyle

Lifestyle is a factor that increases risk of developing cancer. For example, smoking is a major cause of lung cancer and is a factor in other cancers, such as bladder cancer and neck (Slattery *et al.,* 1999). Other factors that can influence our risk of developing cancers of the head and cancer include heavy alcohol consumption and exposure to sunlight. It is also thought that diet can influence the development of some cancers, although the evidence is less clear. Diets high in animal fats have been linked with breast cancer, bowel cancer and prostate cancer. A diet that is low in fresh fruit and vegetables may also increase the risk of developing some types of cancer. Obesity has been linked to some cancers, such as cancer of the breast or kidney (Larsson and Wolk, 2007).

2.6.3.2 Environmental and occupational

Contact with certain harmful substances in the environment or workplace can cause cancer. Substances that are known to cause cancer are called carcinogens. For example, 9 out of 10 people who developed mesothelioma have had contacted with asbestos. People who have worked in industries such as ship-building and construction may have come into contact with asbestos (Sinha *et al.,* 2005). Certain chemicals used in dye factories, rubber production, gas works and other chemical industries have all been linked to bladder cancer. Fortunately these chemicals have now been banned. Prolonged exposure to the sun causes skin cancer, including melanoma.

2.6.3.3 Viruses

Cancers are not infections however; there are a number of different viruses that are thought to be contributing factors in the development of cancer. For example, exposure to HPV (human papilloma virus) is known to increase the risk of cervical carcinoma. Epstein- Barr virus is linked to some of lymphoma. There is also a bacterial infection known types as *H. pylori* which is linked to a rare type of stomach cancer.

2.6.3.4 Genetics

Only 5-10% of cancers are thought to be caused by an inherited faulty gene (Chang et al., 2000). The genes which involve in carcinogenesis are as the followings:

2.6.3.4.1 Oncogenes

Oncogenes developed from the mutation of normal genes (proto-oncogenes). Proto-oncogenes normally produce proteins involved in growth control. Whole oncogenes also perform this function but in a distorted version or excessive amounts of the protein (Holland, 2003).

As a result, the presence of an oncogene causes a cell to continually grow, divide and develop cancer.

2.6.3.4.2 Tumor suppressor genes

Tumor suppressor genes are genes that instruct cell to manufacture proteins responsible for slowing cell growth and division (Chang *et al.,* 2000), (Holland, 2003). The absence of tumor suppressor genes has been linked to cancer development

The *p53* gene is a particular type of tumor suppressor gene that produces a protein that causes apoptosis, or cell suicide, which is a normal occurrence. When the DNA in a cell is damaged and can not enhance to be repaired, the p53 protein normally stops cell division by initiating apoptosis, thus prevent the damaged cell from growing uncontrollably (Weinberg, 1999).

Cancer may develop when a pair of tumor suppressor genes are lost from a cell or inactivated by mutation. Such an absence would cause cell to uncontrollably grow and divide. People who inherit an increased risk of developing cancer are frequently born with one defective copy of a tumor suppressor gene. Because genes come in pairs, an inherited defect in one copy will not lead to cancer because there is another normally functioning copy. Cancer may develop only when the second copy undergoes mutation, and the patient no longer has a normally functioning copy of the gene (Huber, 2001).

2.6.3.4.3 DNA repair genes

DNA repair genes are genes that instruct cell to produce proteins required to correct errors that may occur when cells duplicate their DNA prior to division. When these genes become mutated they fail to repair, allowing additional mutations to build

up. Usually multiple mutations must occur for cancer to develop. It may develop as the result of an accumulation of mutations involving oncogenes, tumor suppressor genes and DNA repair genes. Cancer can begin with a defect in one type of gene, such as a tumor suppressor gene, that allows excessive cell production. The cell often acquires additional mutations involving DNA repair genes, other tumor suppressor genes and several other growth related gene. Eventually the accounting mutations can produce a highly malignant (cancerous) tumor that is able to metastasize (Bertagnolli *et al.,* 2006).

2.6.3.5 Age

The main risk factor for cancer is age. Cancer is relatively rare in young people, but relatively common in the old. 65% of cancers happen to people over 65 (Bos, 1989).

2.7 Natural products in cancer therapy

The role of natural products as a source for remedies has been recognized since ancient times(Farnsworth et al., 1985), (Cragg et al., 1997). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural product still make an enormous contribution to drug discovery today. Table 1 shows some examples of agents derived from natural sources that are currently used in clinical practice (Balandrin *et al.,* 1993), (Farnsworth, 1990).

Nature is an attractive source of new therapeutic candidate compounds as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms. For many living organisms, this chemical diversity reflects the impact of evolution in the selection and conservation of self-defense mechanisms that represent the strategies employed to repel or destroy predators. The development of novel agents from natural sources presents obstacles that are not usually met when one deals with synthetic compounds. For instance, there may be difficulties in accessing the source of the samples, obtaining appropriate amounts of the sample, identification and isolation of the active compound in the sample, and problems in synthesizing the necessary amounts of the compound of interest. These problems became evident when the tubulin-interacting agent paclitaxel was introduced in clinical use. Initial antitumor activity was observed in various types of cancer, including ovarian

and breast adenocarcinoma. Paclitaxel was originally isolated from the bark of the yew tree *Taxus brevifolia* (Wani, 1971) a finite source of the compound. It took some years to develop a semi-synthetic analog (docetaxel) which is derived from a renewable source, the leaves of *Taxus baccata* (Cortes and Pazdur, 1995). Currently, total synthesis has been achieved for both agents and drug supply.

Table 2.1 Drugs developed from natural sources.

Drug	Medical use	Mechamism of action	Source
Aspirin	Analgesic, anti-inflammatory, antipyretic	Inhibition of COX	Plant
Atropine	Pupil dilator	Antagonist of Ach at muscarinic receptors at post-ganglionic parasympathetic neuroeffector sites	Plant
Caffeine	Stimulant	Adenosine receptor antagonist	Plant
Codeine	Analgesic, antitussive	Opioid receptor agonist	Plant
Digoxin	For atrial fibrillation and CHF	Inhibition of the Na^+/K^+ ATPase membrane pump	Plant
Eugenol	Toothache	Reduces excitability of sensory nerves (increased K^+ efflux and reduced Ca^{2+} influx)	Plant
Morphine	Analgesic	Opioid receptor agonist	Plant
Pilocarpine	Glaucoma	Muscarimic receptor agonist	Plant
Quinine	Malaria prophylaxis	Inhibition of protein syntesis in the malaria parasite	Plant
Taxol	Anticancer agent	Antimitotic agent (binds to and stabolizes microtubules)	Plant
Penicillin	antibiotic	Inhibition of synthesis of cell wall peptidoglycan	Microbe

Tetracyclin	antibiotic	Inhibition of protein syntesis to the ribosome 30s subunit	Microbe
Cyclosporin A	Immunosuppressant	Inhibition of clonal proliferation of T lymphocytes (via inhibition of lymphokine production)	Microbe
Aurantosides	Antifungal	Inhibition of tubulin polymerization	Marine organism
Spongistatin 1	Antifungal	Inhibition of tubulin polymerization	Marine organism
Manoalide	Analgesic, anti-inflammatory	Inhibittion of phospholipase A_2	Marine organism

2.7.1 Plants as a source of anticancer drugs

Vinblastine and vincristine were first introduced in the late 1960s and have contributed to long-term remissions and cures with childhood leukaemia, testicular teratoma, Hodgkin's disease and many other cancers. Several structural analogues are also in clinical use, and most notable of these are vinorelbine and vindesine5.Etoposide is inregular use for the effective treatment of testicular teratoma and small-cell lung cancer, whereas teniposide has efficacy against acute lymphocytic leukaemia and neuroblastoma in children, and against non-Hodgkin's lymphomas and brain tumours in adults. Much recent synthetic work has concentrated on the design of more water-soluble analogues6. But the undoubted star is Taxol, which shows efficacy against refractory breast and ovarian cancers. It is, at present, the bestselling anticancer drug; sales reached US $1.5 billion in 2000 and are still growing. But it took 20 years from its discovery in 1967 to the first real clinical responses observed with ovarian cancer in 1987, and even longer until its potential in refractory breast cancer was realized. Why did it take so long to reach the clinic? The early supply problems were enormous. About 4,000 Pacific yew trees (FIG. 2a) had to be sacrificed for their bark to provide 360 g of Taxol for the early clinical trials, and this rose to 38,000 trees for 25 kg of Taxol needed to treat 12,000 patients in the early 1990s. These difficulties were solved when it was discovered by Potier and co-workers that the foliage of the European yew,*Taxus*

baccata, contained greater amounts of a related chemical structure, 10deacetylbaccatin III, that could easily be converted into Taxol and into the more potent analogue taxotere8. In 1979, Susan Horwitz and colleagues showed that the mode of action of Taxol was different from that of any other anticancer agent in clinical use at the time.Unlike vinblastine and vincristine, which destabilize microtubules,Taxol stabilizes them during cell division9. This allowed structure–activity relationships to be established for hundreds of semi-synthetic analogues,with the result that several morepotent analogues are already in clinical trials10. The natural product, paclitaxel, has therefore provided not only an effective drug, but also the springboard for further developments. It has also been the object of commercial and political controversy, not least when Bristol-Myers Squibb was given permission to patent the name Taxol, depriving the scientific community of the name of a natural product. This led to some furious exchanges in the literature, perhaps most famously when a spokesman from Bristol-Myers Squibb responded to a critical editorial in *Nature* with an admonishment that the journal had stolen its trademark from Mother Nature.

Camptothecin, from the Chinese ornamental tree *Camptotheca accuminata*, had a similarly long gestation period. Its early promise in the 1970s was blighted by severe bladder toxicity, but chemical manipulation of its structure subsequently produced analogues, including topotecan (Hycamptin) and irinotecan (Camptosar), that have been approved for clinical use. These days camptothecin is valued as a biological tool to understand the functions of the enzyme topoisomerase I, for which it is a specific inhibitor. This enzyme is intimately involved in the unwinding of DNA before transcription and replication, and camptothecin and its structural analogues will not only help to unravel these complex processes but also act as lead structures for the design of other molecules that selectively inhibit topoisomerases.

(A) (B)

Figure 2.4 Two of the earliest plants to yield natural products with anticancer activities. **(A)** *Podophyllum peltatum* (the mayapple), which produces podophyllotoxin, **(B)** *Catharanthus roseus* (also known as *Vinca rosea* or the rosy periwinkle), which produces the *Vinca* alkaloids vinblastine and vincristine.

2.7.2 Anticancer drugs from microbes

Antitumor antibiotics are among the most important cancer chemotherapeutic agents, and include members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families (Cragg et al.,1997). Clinically useful agents from these families are the daunomycin-related agents (daunomycin itself, doxorubicin, idarubicin and epirubicin), the peptolides (exemplified by dactinomycin), the mitosanes (such as mitomycin C) and the glycosylated anthracenone mithramycin. The anthracyclines are among the most used antitumor antibiotics in the clinic and exert antitumor activity mainly by inhibiting topoisomerase II (Binaschi et al., 2000). Many pharmaceutical agents have been discovered by screening natural products from a wide range of micro-organisms. For example, cyclosporin A (CyA) and FK506 (Prograf, tacrolimus) were discovered at Sandoz and Fujisawa Pharmaceuticals, respectively, in screens for immunosupressive agents that would block a mixed lymphocyte response. These two immunosuppressant drugs have had a dramatic impact on clinical medicine and are

widely used to prevent and treat graft rejection and graft-versus-host disease following both solid-organ transplants and bone-marrow transplants. The immunosuppressant rapamycin (sirolimus) was originally discovered at Wyeth-Ayerst Pharmaceuticals in a screen for antifungal agents, and was later found to have potent immunosuppressive activity. In addition rapamycin, wortmannin and geldanamycin (also natural products) have been found to have antiproliferative actions and may therefore find clinical use as novel chemotherapeutic agents (Table 3),(Patrick, 1997). Rapamycin and its analogs are products of *Streptomyces hygroscopicus* and inhibit signaling pathways required for T-cell activation and proliferation. Rapamycin blocks progression of the cell cycle at middle-to-late G_1 phase in T cells and B cells, and osteosarcoma and rhabdomyosarcoma cell lines, among others (Alberts *et al.,* 1993). Geldanamycin is a benzoquinone ansamycin natural fermentation product that was originally thought to be a direct protein tyrosine kinase inhibitor. However, subsequent studies have revealed that geldanamycin binds to, and inhibits the 90 kDa heat-shock protein HSP 90 (Schulte and Necker, 1998). Wortmannin is a product of the fungus *Talaromyces wortmanni* and inhibits signal transduction pathways by forming a covalent complex with an active-site residue of phosphoinositide 3 kinase (PI3K), inhibiting PI3K activity (Cadenas, 1998).

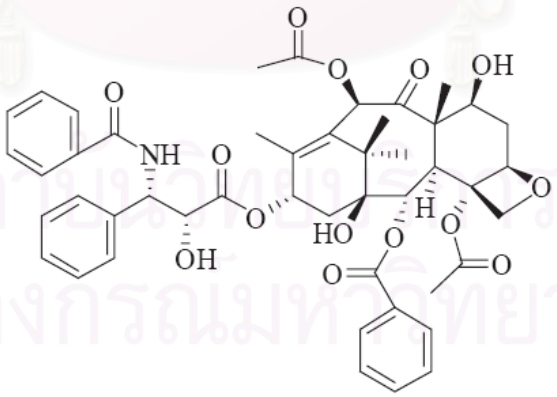
Table 2.2 Microbe-derived anticancer agents.

Compound	Cancer use	Status
Actinomycin	Sarcoma and germ-cell tumors	Phase III/IV
Bleomycin	Germ-cell, cervix, head and neck cancer	Phase III/IV
Daunomycin	Leukemia	Phase III/IV
Doxorubicin	Lymphoma, breast, ovary, lung and sarcomas	Phase III/IV
Epirubicin	Breast cancer	Phase III/IV
Idarubicin	Breast cancer and leukemia	Phase III/IV
Mitomycin C	Gastric, colorectal, and lung cancer	Phase III/IV
Streptozocin	Gastric and endocrine tumors	Phase III/IV
Wortmamin	Experimental	Preclimical
Rapamicin	Experimental	Preclimical
Geldanamycin	Experimental	Preclimical

2.8 Anticancer Agents from endophytic fungi

Paclitaxel (Taxol), a highly functionalized diterpenoid, originally isolated from the Pacific yew tree *Taxus brevifolia*. Its unique mode of action, of preventing the depolymerization of tubulin during the processes of cell division, made it a huge success clinically and commercially. Taxol (41) and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes. This compound is the world's first billion-dollar anticancer drug. The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well (Strobel *et al*., 2004). To overcome its unacceptably low yield, taxol was produced in *in vitro* culture by a new endophytic fungus, *Taxomyces andreanae*, which was isolated from a Pacific yew *T. brevifolia* in Montana, USA. Since then, a variety of endophytic fungi isolated from yew trees and other plants such as *T. brevifolia, T. wallachiana, T. yunnanensis, T. baccata, T. mairei, Taxodium distichum, Torreya grandifolia,* and *Wollemia nobilis* have been reported to be capable of producing taxol and/or taxane derivatives (Strobel *et al*., 2004).

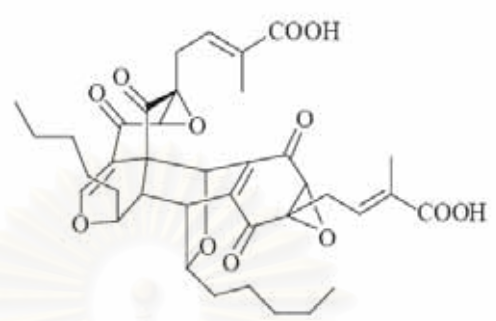
The greatest prospect of making microbial taxol a commercial reality may be the discovery of endophytes that make large quantities of one or more taxanes that could then be used as platforms for the organic synthesis of taxol or its anticancer relatives (Strobel *et al*., 2004).



Taxol (41)

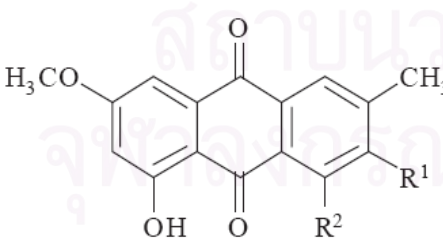
Torreyanic acid (42), a selectively cytotoxic quinone dimer, was isolated from the endophytic fungus *Pestalotiopsis microspora*. It (42) was tested in several cancer cell lines, and it demonstrated 5 to10 times more potency in those cell lines that are sensitive

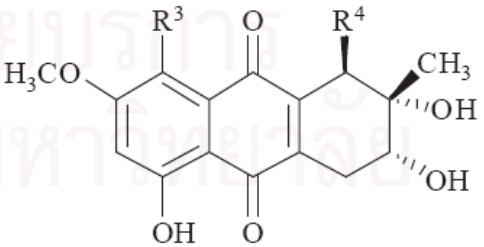
to protein kinase C (PKC) agonists and causes cell death by apoptosis (Lee *et al.,* 1996). Recently, a complete synthesis of torreyanic acid (42) has been successfully completed using the application of a biomimetic oxidation-dimerization cascade (Li *et al.,* 2003)



Torreyanic acid (42)

Six known metabolites,7-methoxy-2-methyl-3,4,5 trihydroxyanthraquinone (43), physcion (44), macrosporin (45), deoxybostrycin (46), altersolanol B (47) and dactylariol (48), together with a new hexahydroanthraquinone named pleospdione (49) were isolated from the culture of *Pleospora* sp. IFBE006, an endophytic fungus residing in the normal stem of *Imperata cylindrical* (Gramineae). Compounds 46-48 exhibited relatively high cytotoxic activities with IC50 values of 0.8 and 1.3 mg/mL, respectively, for compound 8, against human colon cancer (SW1116) and leukemia (K562) cell lines. However the cytotoxic activities of compounds 43, 44 and 49 were poor or moderate. These compare well to the IC50 value of 6.0 mg/mL for 5-fluorouracil used as a positive control (Ge *et al.,* 2005)





(43) $R^1 = R^2 = OH$

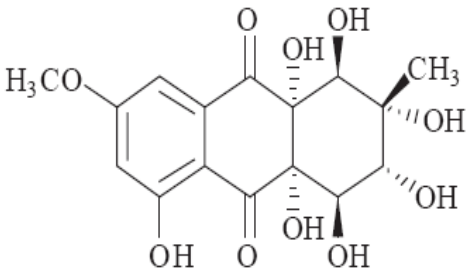
(44) $R^1 = H$, $R^2 = OH$

(45) $R^1 = OH$, $R^2 = H$

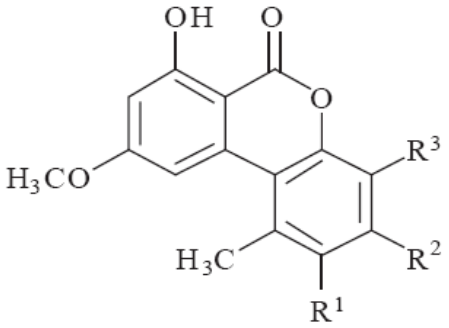
(46) $R^1 = OH$, $R^2 = H$

(47) $R^1 = R^2 = H$

(48) $R^1 = H$, $R^2 = OH$



(49)



(50) R^1= Cl, R^2 = OMe, R^4= H

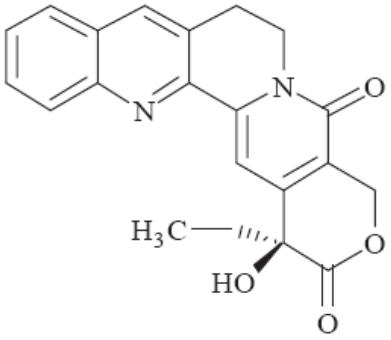
(51) R^1= H, R^2 = OMe, R^3= OMe

(52) R^1= H, R^2 = OMe, R^3= OH

(53) R^1= H, R^2 = OH, R^3= H

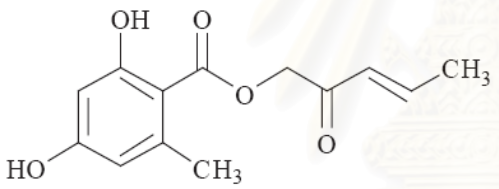
Two novel 6*H*-dibenzo[*b,d*]pyran-6-one derivatives, graphislactone G (50) and graphislactone H (51), together with graphislactone A (52) and alternariol monomethyl ether (53) were isolated from *Cephalosporium acremonium* IFB-E007, an endophytic fungus from roots of *Trachelospermum jasminoides* (LINDL). Anticancer tests showed that compounds 50-53 had pronounced activities against SW1116 cell with IC50 values of 21, 12, 8.5, and 14 mg/mL, respectively (Zhang *et al.,* 2005).

Camptothecin (54), a pentacyclic quinoline alkaloid, was isolated from endophytic fungus (RJMEF001) in the inner bark of the plant *Nothapodytes foetida* from the Western coast of India. The fungus, which belongs to the class Phycomycetes, produced the anticancer drug lead compound; camptothecin (54). It was also compared with an authentic example for its biological activity against a number of human cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer). Camptothecin and its derivatives showed strong antineoplastic activity. The drug is already used in China for the treatment of skin diseases. Hycamtin (topotecan) and Camptosar (irinotecan), semisynthetic derivatives of **54**, have been employed clinically for the treatment of ovarian and colon cancers. Compound 54 is also used as an insect chemosterilant, a plant regulator, and an inhibitor of the herpes virus. In addition, compound 54 prevents the replication of the influenza virus (Puri *et al.,* 2005)

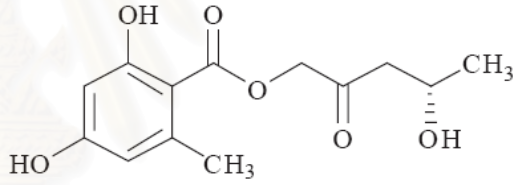


Camptothecin (54)

Globosumone A (55) and globosumone B (56), newly orsellinic acid esters from *Chaetomium globosum* endophytic in mormon tea *(Ephedra fasciculata)* exhibited moderate cytotoxicity against various cell lines, for example, non-small cell lung cancer (NCI-H460), breast cancer (MCF-7), CNS glioma (SF-268), pancreatic carcinoma (MIA Pa Ca-2), and normal human fibroblast cells (WI-38) (Bashyal *et al.,* 2005).



(55)



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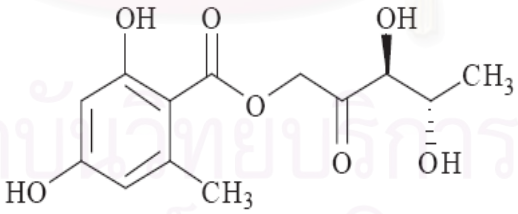
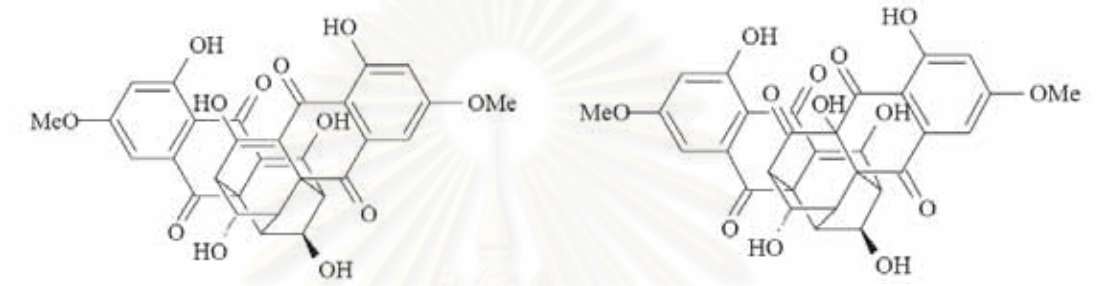


(57)

The biochemical induction assay (BIA) is a rapid (colorimetric) bacterial assay used to identify compounds that damage DNA or inhibit DNA synthesis and thereby identify potential natural product anticancer agents. The use of BIA, which measures the induction of the SOS response in bacteria, led to the isolation of the bisanthraquinones cytoskyrins A (58) and B (59), from *Cytospora* sp. CR200, a fungal strain endophytic in

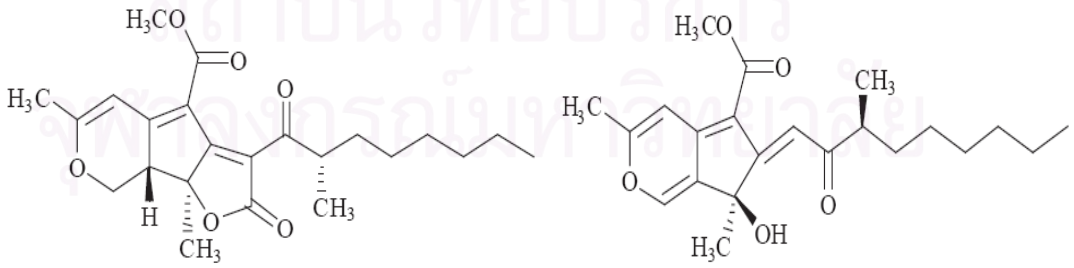
Conocarpus erecta. The *cis,cis* conformation for cytoskyrin A (18) was confirmed by the X-ray crystallography structure and therefore, by analogy, assumed to be true for cytoskyrin B (59). It was likely that the cytoskyrins arise from the dimerization of 1, 3, 6, 8-tetrahydroxyanthraquinone via oxidation and condensation. Cytoskyrin A (58) showed strong BIA activity down to 12.5 ng in the standard BIA assay while cytoskyrin B (59) showed no significant BIA response at any of the concentrations tested (<50 mg) (Brady *et al.,* 2000)



(58) (59)

Chemical investigation of *Aspergillus parasiticus,* an endophytic fungus in the inner bark of a coastal redwood tree, *Sequoia sempervirens*, also led to the isolation of sequoiatones A (60) and B (61). The absolute stereochemistry proof of sequoiatone A (60) was provided by X-ray crystallography (Stirerle *et al.,* 1999)

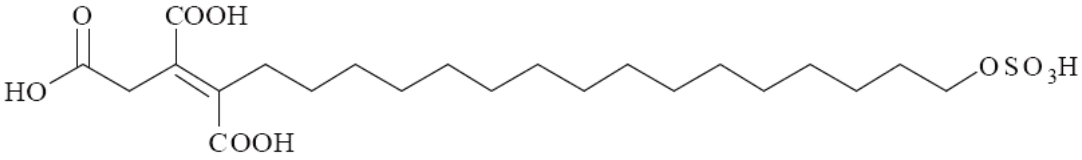
Sequoiatones were tested against a panel of 60 different human tumor cell lines. They showed moderate and somewhat selective inhibition of human tumor cells, with greatest efficacy against breast cancer cell lines. Most of the GI50 (concentrations required to inhibit growth by 50%) were between 4 and 10 mM with LC50 > 100 mM.



(60) (61)

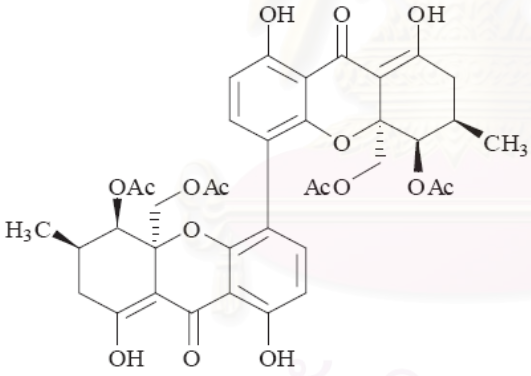
Oreganic acid (62), a tricarboxylated alkylsulfate and a specific inhibitor of farnesyl-protein transferase (FPTase) with an IC_{50} of 14 nM, was also found from the

extract of an endophytic fungus isolated from leaves of *Berberis oregano* (Jaysuriya *et al.,* 1996).



Oreganic acid (62)

Phomoxanthones A (63) and B (64), novel xanthone dimers, were isolated from the endophytic fungus *Phomopsi*s sp. BCC 1323. They exhibited significant activity against *Plasmodium falciparum* (K1, multi drug resistant strain) and against *Mycobacterium tuberculosis* (H37Ra strain), although weaker than standard drugs. However, these compounds are also cytotoxic to two cancer cell lines (KB, BC-1) and to Vero cells (Isaka *et al.,* 2001).



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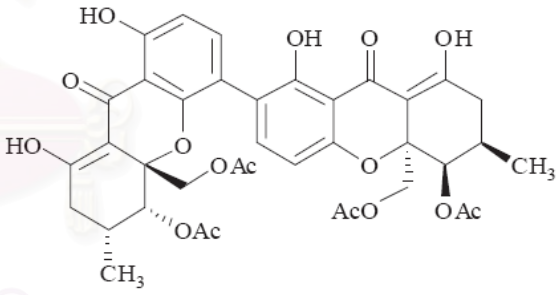
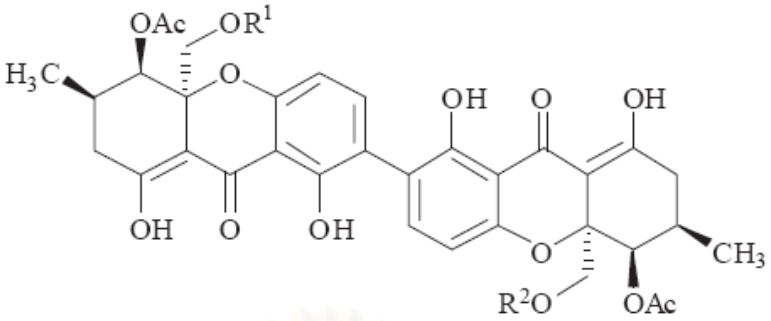


(64)

Dicerandrols A (65), B (66), and C (67), new antibiotic and cytotoxic dimers, were isolated from *Phomopsis longicolla*, an endophytic fungus of the endangered mint *Dicerandra frutescens* (Wagenaar and Clardy, 2001). These compounds (65-67) exhibited antibacterial activity against both *Staphylococcus aureus* and *Bacillus subtilis* but are inactive against the fungus *Geotrichum candidum* and the yeast *Saccharomyces cerevisiae* at 300 mg/disk. They also possess modest activity in two human cancer cell lines, A549 (human lung tumor cells) and HCT-116 (human colon tumor cells).

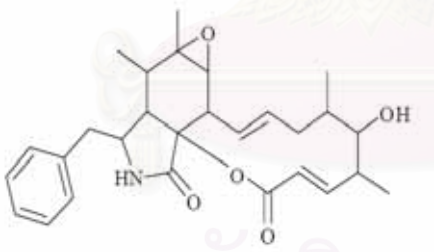


(65) $R^1 = R^2 = H$

(66) $R^1 = Ac$, $R^2 = H$

(67) $R^1 = R^2 = Ac$

A series of new cytotoxic cytochalasins (68-70) were isolated from a culture of the endophytic fungus *Rhinocladiella* sp. They exhibited a broad spectrum of antibiotic and antitumor activity, phytotoxic activity, and inhibitory activity of HIV-1 protease. Although they are widely used as biological probes,their therapeutic application has been limited by their toxicity (Wagenaar *et al.,* 2000).



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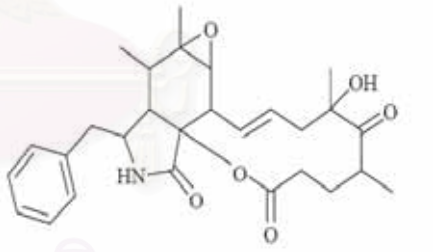

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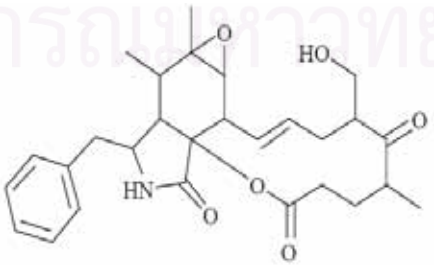


(70)

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments and equipments

3.1.1 Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H and ^{13}C NMR were performed on Varian Mercury +400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Deuterated solvents, chloroform-*d* ($CDCl_3$) and deuterium oxide (D_2O), were used for NMR experiments and chemical shifts (δ) were referenced the signals of residual solvents at 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C) for $CDCl_3$ and at 4.79 ppm for D_2O.

3.1.2 Ultraviolet-visible Spectrometer (UV-VIS)

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

3.1.3 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR analysis was obtained from Perkin Elmer system 200 FT-IR spectroscopy (Impact 410, Nicolet). The frequency range of 4000-400 cm^{-1} was observed using transmittance, Perkin Elmer, Nicolet (Impact 410) model. Silica was mixed with potassium bromide in agate mortar and pestle. The mixture were then transferred to a hydraulic pressing machine and pressed into a thin disc for FT-IR analysis.

3.1.4 Mass spectrometer (MS)

High resolution electro spray ionization was spectrometry (HRESIMS) were performed on a Micromass LCT (LC/MS) at National Science and Technology Development Agency Building (NSTDA) and Chemistry Department, Mahidol University.

3.1.5 Optical rotation

The optical rotations were measured on a Perkin-Elmer Model 341 Polarimeter, using a sodium lamp at wavelength 589 nm.

3.2 Chemicals reagents

3.2.1 Solvents

The solvents used for column chromatography were commercial grade and were distilled prior to use, such as hexane, ethyl acetate, dichloromethane and methanol.

The reagent grade solvents were used for re-crytallization.

The deuterated solvents for NMR experiments including $CDCl_3$ and D_2O were purchased from Merck.

3.2.2 Other chemicals

Silica gel 60 (0.040-0.063 mm) (Merck)

Silica gel 60 RP-18 (0.040-0.063 mm) (Merck)

TLC aluminium sheets, silica gel $^{60}F_{254}$ precoated 25 sheets, 20x20 cm^2, layer thickness 0.2 mm were used as adsorbent for TLC analysis. (Merck)

TLC spots were visualized under ultraviolet light at wavelengths 254 and 365 nm, in iodine vapour, and under daylight after spraying with vanillin reagent (Dissolve 0.5 g vanillin in 95 ml ethanol and add 4.5 ml concentrated sulfuric acid) and heating until the colors developed.

3.3 The endophytic fungus *Xylaria sp.* PB-30

The endophytic fungus *Xylaria sp.* PB-30 used in this study was isolated from the healthy and mature leaves of *Sandoricum koetjape* by Srinuan Tansuwan (Tansuwan *et al.,* 2007)

3.4 Culture media

As the basal media for isolation and cultivation of endophytic fungus, Potato Dextrose Agar (PDA) Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Sabouraud's Dextrose Agar (SDA), Yeast Extract Sucrose Agar (YES) and Corn Meal Agar (CMA) were used for study morphology characteristic and screening anticancer metabolites of *Xylaria* sp. PB-30 for then anticancer activity against cancer cell lines. Five basal media for cultivation for study anticancer metabolites of *Xylaria* sp. PB-30 included Malt extract broth (MEB), Malt Czapek broth (MCzB), Sabouraud's dextrose broth (SDB), Yeast Extract Sucrose broth (YES) and Corn meal broth (CMB).

3.5 Methods

3.5.1 Cultivation and Charateristic on five basal media

The endophytic fungus *Xylaria sp.* PB-30 was cultivated on five basal media including Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Sabouraud's Dextrose Agar (SDA), Yeast Extract Sucrose Agar (YES) and Corn Meal Agar (CMA) under static condition at room temperature. Morphology characteristic of *Xylaria* sp. PB-30 including colony characteristic, colony color and color pigment production on each media were observed and colony extent growing on each agar media were measured every week.

3.5.2 Growth profile of the endophytic fungus *Xylaria sp.* PB-30 on five basal media

The endophytic fungus *Xylaria sp.* PB-30 was cultivated in 250 mL flask containing 100 mL of five basal media including Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Sabouraud's Dextrose Broth (SDB), Yeast Extract Sucrose Broth (YES) and Corn Meal Broth (CMB) and cultured under static condition at room temperature. The culture broth was separated from mycelia by filtration through filter paper (Whatman No.1) using Buchner funnel and the mycelia were weighed. To obtain growth profile, the cell mass were measured every three days.

3.5.3 Cultivation and Screening for anticancer activity against cancer cell lines

The endophytic fungus *Xylaria sp.* PB-30 was cultivated into 250 mL each flask containing 100 mL of MEB, MCzB, SDB, YES and CMB and cultured under static condition at room temperature. After culture on each basal media enters a stationary phase the cultures was filtered through filter paper. The filtrate was partitioned with an equal volume of hexane (x3) and EtOAc (x3) and MeOH (x3), respectively. The extracts were concentrated by a rotary evaporation under reduced pressure at 30^{o}C to give hexane crude, EtOAc crude and MeOH crude. Fungal mycelia were ground using blender and then extracted with hexane, EtOAc (x5) and MeOH (x5) respectively.

All crude extract were analyzed by ^{1}H NMR spectroscopy and determined their cytotoxicity activity against cancer cell lines. ^{1}H NMR profiles of hexane crude extracts of each media revealed that oils and triglycerides. ^{1}H NMR profile of EtOAc crude extracts exhibited group of interesting compounds. Thus EtOAc crude broth extracts on SDB 27 day, MCzB 35 day, MEB 35 day and EtOAc extracts of mycelia on

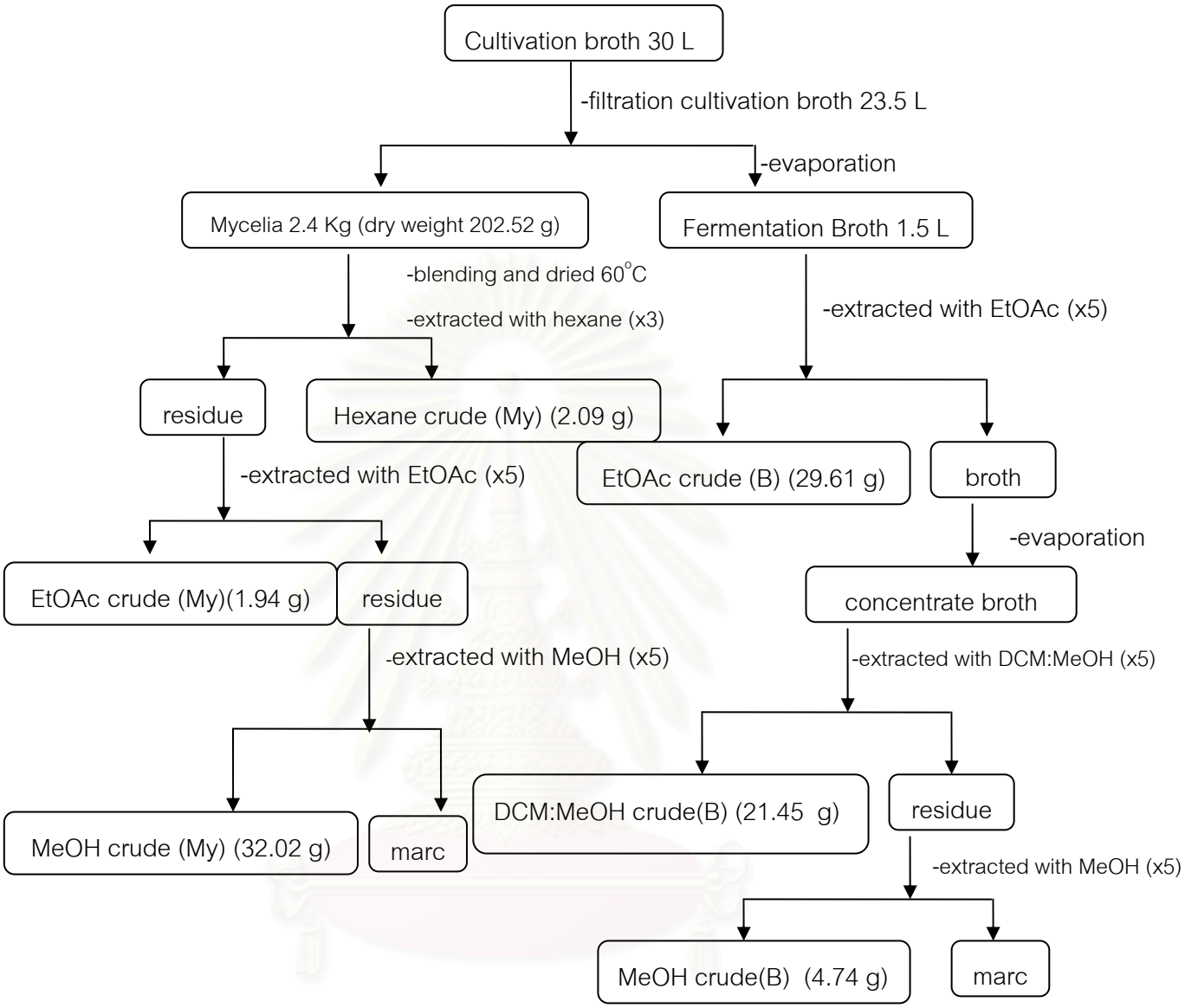
MEB 35 day were tested cytotoxic activity against 5 human tumor cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (Carmichael *et al*., 1987). Also their cytotoxicity activity were carried out at Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC) for in vitro anticancer activity against three cancer cell lines comprising of KB oral cavity cancer, MCF-7 Brest cancer and NCI-H187 small cell lung cancer using Resazurin Microplate assay (REMA).

3.5.4 Scale up cultivation for anticancer activity against cancer cell lines

The endophytic fungus *Xylaria sp.* PB-30 was cultivated in 250 ml flask (x300) each flask containing 100 ml of Malt Extract Broth (MEB) under static condition at room temperature for 35 days. Culture was filtered through filter paper (Whatman No.1). The culture broth (23 L) was evaporated by rotary evaporator *in vacuo* to remove partial water to give about 1.5 L of culture broth. Then was the broth of EtOAc (500 ml x5), CH_2Cl_2: MeOH (1:1)(500ml x5) and MeOH (500 mlx5) respectively. The extracts were concentrated by a rotary evaporation under reduced pressure at 30°C to give EtOAc crude as yellow viscous liquid (29.61 g), CH_2Cl_2: MeOH crude as brown viscous liquid (21.45 g) and MeOH crude as brown viscous liquid (4.74 g).

The fungal mycelia (202.52 g of dry weight) was ground using blender, dried in hot air oven at 60°C approximate 3-5 day and then extracted with hexane 500 ml (x3), EtOAc 500 ml (x3) and MeOH 500 ml (x3) respectively, The extracts were concentrated by a rotary evaporation under reduced pressure at 30°C to gave hexane crude as yellow viscous liquid (2.09 g), EtOAc crude as yellow brown viscous liquid (1.94 g) and MeOH crude as brown viscous liquid (32.02 g).

All crude extract were tested cytotoxic activity against 5 human tumor cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon) were performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (Carmichael *et al*., 1987).



Sheme3.1 Extraction diagram of culture broth and mycelia of *Xylaria sp.* PB-30

3.6 Cytotoxicity assay (Carmichael *et al*., 1987).

Cytotoxicity assay was carried out using a conventional method to estimate the number of viable cell growing in microtiter plate. The changing of colorimetric formation in wells was measured by automatic microplate reader as described by Mosmann. The living cells only could reduce the yellow tetrazoloium salt (MTT) [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] to insoluble blue formazan crystal by intracellular succinate dehydrogenase.

Cytotoxic activity against 5 human tumor cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon) was tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.

3.6.1 Cell culture treatment

BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon) were cultured in RPMI1640 containing 5 % fetal calf serum. 100 µl of the cell suspension approximate $5x10^3$cell/ml were seeded in 96-well tissue culture plates containing 100 µl culture media. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h.

3.6.2 Sample preparation

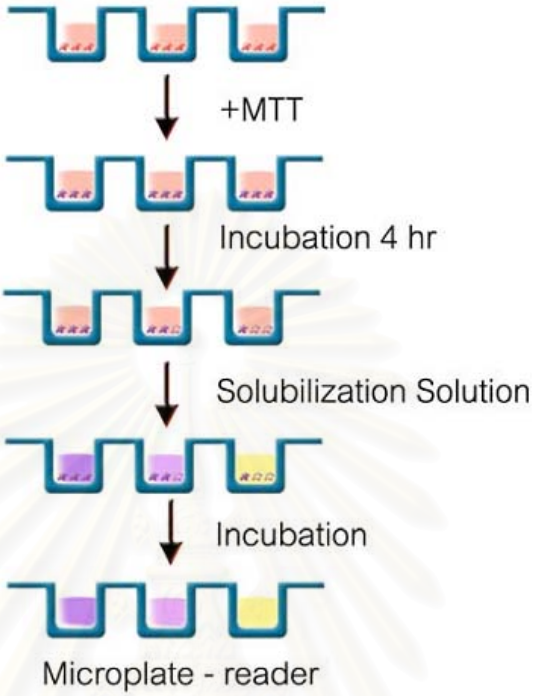
Each extracts was dissolved in DMSO to give final concentration of 10 µg/ml, and then added to the cell in each well. Final concentrate of compounds was in range of 10, 1, 0.1, 0.01 and 0.001µg/ml.

3.6.3 Cytotoxicity test

The sample solution (2 µl) was plated into each well of 96-well microtiterplate containing cell suspension ($5x10^3$cell/ml) and incubated at 37°C, 5% CO_2 for 72 h (3 replicate per concentration). After incubation, 100 µl of MTT was added to each well and incubated for another 4 h under darkness at 37°C in a humidified atmosphere of 5% CO_2. After definite time, the supernatant fluid of each well was discarded. 150 µl of DMSO and 25 µl of 0.1 M Glycine pH 10.5 were added to each well and mixed

thoroughly. The formation of formazan is measured by the optical density (OD) at wavelength 540 nm using microplate spectrophotometer.

Measurement of metabolic activity using the tetrazolium salts (MTT assay)



The percentage of cell viability was calculated according to the following formula.

$$\text{The percentage of cell viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

The IC_{50} value was obtained by plotting the percentage of cell viability versus the concentration.

3.7 Isolation of secondary metabolites from the EtOAc crude of broth extract

The EtOAc extract of broth (29.61g) was subjected to a column chromatography [Sephadex™ LH-20 (400g), column diameter 3.6 cm] using 5% dichloromethane in MeOH as eluent. 10 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vapor and vanillin/H_2SO_4 reagent to give 17 combined fractions as shown in table 3.1.

Table 3.1 The combined fractions obtained from the broth EtOAc crude extract.

Fraction code	Fraction No.	Appearance	Weight (mg)
EB-1	1-20	yellow viscous liquid	101.6
EB-2	21-22	yellow viscous liquid	63.3
EB-3	23-25	brown viscous liquid	281.8
EB-4	26	dark brown solid	91.8
EB-5	27	yellow solid	377.5
EB-6	28-34	yellow solid	1959.4
EB-7	35-38	brown viscous liquid	268.1
EB-8	43-45	yellow solid powder	530.3
EB-9	46-49	brown viscous liquid	1166.8
EB-10/1	50-59	soft yellow solid	2542.9
EB-10/2	50-59	yellow solid	5246.4
EB-11	60-66	yellow solid	6318.5
EB-12	67-72	shining black solid	3207.2
EB-13	73-79	black viscous liquid	702.7
EB-14	80-89	white solid and yellow viscous liquid	213.7
EB-15	90-92	brown viscous liquid	25.2
EB-16	93-99	dark brown viscous liquid	45.9
EB-17	100-126	brown viscous liquid	15.7

All of combined fractions were analyzed by ^{1}H NMR spectroscopy. Since ^{1}H NMR profile of fraction EB-5 and EB-6 were similar, both. Fractions were combined and crystallized from a 1:1 mixture of CH_2Cl_2 and Acetone to give a **compound 1** as colorless cystal (25 mg); m.p. 153-155 ^{o}C;

$[\alpha]_D^{20}$ -100^{o} (0.1, MeOH);

λ_{max} (MeOH) (log ε) 260 nm;

Fraction EB-10 was extracted by hexane and CH_2Cl_2 to give combined fraction EB-10/1 and EB-10/2, respectively which was analyzed by ^1H NMR spectroscopy. Result of ^1H NMR analysis and TLC analysis developed by 5% MeOH in CH_2Cl_2 revealed that this fraction was nearly of fraction EB-10/1 exhibited pure compound. Fraction EB-10/1 was crystallizaed from CH_2Cl_2 and acetone to obtain a **compound 2** as soft yellow solid (2.54 g);

m.p. 153-155 °C;

$[\alpha]_D^{20}$ -88° (0.1, MeOH);

λ_{max} (MeOH) (log ε) 260 nm;

3.8 Isolation of secondary metabolites from fraction EB-7, EB-8 and EB-9

Fraction EB-7, EB-8 and EB-9 (1.1g) were combined and then subjected to a column chromatography [silica gel 60 (40g), column diameter 2 cm] eluted with CH_2Cl_2, CH_2Cl_2in MeOH and MeOH, respectively. 20 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vapor and vanillin/H_2SO_4 reagent to give 29 combined fractions as shown in Table 3.2.

Table 3.2 The combined fractions obtained fraction EB-7, EB-8 and EB-9.

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
A-1	3	CH_2Cl_2 (100)	yellow viscous liquid	1.0
A-2	4-6	CH_2Cl_2:MeOH (99:1)	yellow solid	3.6
A-3	7	CH_2Cl_2:MeOH (99:1)	yellow viscous liquid	5.0
A-4	8-9	CH_2Cl_2:MeOH (99:1)	yellow solid	4.5
A-5	10-11	CH_2Cl_2:MeOH (99:1)	yellow solid	3.9
A-6	12-14	CH_2Cl_2:MeOH (98:2)	white solid and yellow viscous liquid	40.3
A-7	15-20	CH_2Cl_2:MeOH (97:3)	yellow viscous liquid	16.3
A-8	21-22	CH_2Cl_2:MeOH (96:4)	yellow viscous liquid	32.4

A-9	23-29	CH_2Cl_2:MeOH (95:5)	yellow viscous liquid	26.7
A-10	30	CH_2Cl_2:MeOH (93:7)	yellow viscous liquid	8.5
A-11	31-34	CH_2Cl_2:MeOH (93:7)	yellow viscous liquid	31.3
A-12	35-41	CH_2Cl_2:MeOH (93:7)	yellow viscous liquid	29.7
A-13	42	CH_2Cl_2:MeOH (90:10)	white solid and brown viscous liquid	6.4
A-14	43-44	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	7.2
A-15	45	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	8.3
A-16	46	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	3.4
A-17	47-49	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	20.2
A-18	50-52	CH_2Cl_2:MeOH (89:11)	yellow viscous liquid	10.9
A-19	53-59	CH_2Cl_2:MeOH (89:11)	yellow viscous liquid	13.4
A-20	60-63	CH_2Cl_2:MeOH (87:13)	yellow viscous liquid	7.2
A-21	64-69	CH_2Cl_2:MeOH (85:15)	yellow viscous liquid	13.0
A-22	70-79	CH_2Cl_2:MeOH (84:16)	yellow viscous liquid	8.2
A-23	80-87	CH_2Cl_2:MeOH (83:17)	yellow viscous liquid	10.4
A-24	88-90	CH_2Cl_2:MeOH (83:17)	yellow viscous liquid	11.3
A-25	91-96	CH_2Cl_2:MeOH (83:17)	yellow viscous liquid	6.3
A-26	97-100	CH_2Cl_2:MeOH (82:18)	yellow solid	4.4
A-27	101-109	CH_2Cl_2:MeOH (82:18)	brown solid	8.5
A-28	110-117	CH_2Cl_2:MeOH (80:20)	brown solid	2.9
A-29	118-119	CH_2Cl_2:MeOH (75:25)	brown solid	39.4

A-6 (fraction 12-14) was washed with CH_2Cl_2 and **compound 3** which is white solid (18.3 mg); m.p. 267°C;

$[\alpha]_D^{20}$ -43° (c = 0.10,$CHCl_3$);

λ_{max} (MeOH) (log ε) 259 nm.

3.9 Isolation of secondary metabolites from fraction EB-12, EB-13 and EB-14

Fraction EB-12, EB-13 and EB-14 (3.1g) was subjected to a column chromatography [Sephadex™ LH-20 (80g), column diameter 1.8 cm] using 10% dichloromethane in MeOH as eluent. 10 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vapor and vanillin/H_2SO_4 reagent to give 17 combined fractions.

Table 3.3 The combined fractions obtained fraction EB-12, EB-13 and EB-14.

Fraction code	Fraction No.	Appearance	Weight (mg)
B-1	1-12	dark brown solid	36.8
B-2	13	yellow viscous liquid	32.1
B-3	14	yellow viscous liquid	65.7
B-4	15	yellow viscous liquid	104.4
B-5	16	white solid and brown viscous liquid	130.2
B-6	17-19	white solid and brown viscous liquid	897.4
B-7	20-21	yellow viscous liquid	658.3
B-8	22	yellow viscous liquid	292.9
B-9	23	yellow viscous liquid	116.3
B-10	24-26	yellow viscous liquid	193.4
B-11	27	brown viscous liquid	2.8
B-12	28	brown viscous liquid	16.7
B-13	29	brown viscous liquid	1.7
B-14	30-33	dark brown solid	36.3
B-15	34-36	dark brown solid	27.2
B-16	37-39	dark brown solid	1.6
B-17	40-52	dark brown solid	26.5

3.10 Isolation of secondary metabolites from fraction B-6, B-7 and B-8

Fractions B-6, B-7 and B-8 (1.8 g) were combined and then subjected to flash column chromatography [Silica gel 60 RP-18 (50g), column diameter 3 cm] using MeOH in DI water and 100% MeOH as eluent, respectively. 5 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine and vanillin/H_2SO_4 reagent to give 14 combined fractions.

Table 3.4 The combined fractions obtained fraction B-6, B-7 and B-8

Fraction code	Fraction No.	Eluted	Appearance	Weight (mg)
FC-1	1-7	90:10 MeOH: DI water	brown viscous liquid	140
FC-2	8-18	90:10 MeOH: DI water	brown viscous liquid	40.7
FC-3	19-29	80:20 MeOH: DI water	brown viscous liquid	110
FC-4	30-39	80:20 MeOH: DI water	yellow viscous liquid	61.4
FC-5	40-47	70:30 MeOH: DI water	yellow viscous liquid	2.9
FC-6	48-54	60:40 MeOH: DI water	yellow viscous liquid	51.5
FC-7	55-88	50:50 MeOH: DI water	dark brown solid	60.9
FC-8	89-93	40:60 MeOH: DI water	brown viscous liquid	12.4
FC-9	94-99	30:70 MeOH: DI water	brown viscous liquid	5.3
FC-10	100-110	30:70 MeOH: DI water	brown viscous liquid	10.5
FC-11	111-117	20:80 MeOH: DI water	dark brown solid	8.3
FC-12	117-124	10:90 MeOH: DI water	dark brown solid	8.1
FC-13	125-130	10:90 MeOH: DI water	dark brown solid	3.2
FC-14	131-139	100% MeOH	brown viscous liquid	20.9

3.11 Isolation of secondary metabolites investigation from fraction FC-6

Fraction FC-6 (51.5 mg) was subjected to flash column chromatography [Silica gel 60 RP-18 (10g), column diameter 1.5 cm] eluted with MeOH in DI water and 100% MeOH, respectively. 5 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vapor and vanillin/H_2SO_4 reagent to give 6 combined fractions.

Table 3.5 The combined fractions obtained fraction FC-6

fraction code	fraction no.	Eluted	Appearance	weight (mg)
FCC-1	1-17	90:10 MeOH: DI water	violet solid	4.0
FCC-2	18-29	90:10 MeOH: DI water	dark violet solid	1.8
FCC-3	30-35	70:30 MeOH: DI water	violet solid	15.3
FCC-4	36-40	70:30 MeOH: DI water	dark violet solid	2.7
FCC-5	41-45	50:50 MeOH: DI water	violet solid	5.9
FCC-6	46-52	100%MeOH	yellow viscous liquid	18.1

BB-4 (fraction no. 36-40) was washed with CH_2Cl_2 and **compound 4** as white powder (3.8 mg) Compound **4** is soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 149-151 $^{\circ}$C;

λ_{max} (MeOH) (log ε) 278 (4.12), 206 (3.87) nm.

3.12 Isolation of secondary metabolites from fraction FC-3

Fraction FC-3 (110 mg) was subjected to flash column chromatography [Silica gel 60 RP-18 (10g), column diameter 1.5 cm] using MeOH in DI water and 100% MeOH as eluent. 5 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vaporand vanillin/H_2SO_4 reagent to give 7 combined fractions.

Table 3.6 The combined fractions obtained fraction FC-3.

fraction code	fraction no.	Eluted	Appearance	weight (mg)
FCB-1	1-5	90:10 MeOH: DI water	brown viscous liquid	30
FCB-2	6-7	90:10 MeOH: DI water	violet solid	15.6
FCB-3	8-14	90:10 MeOH: DI water	violet solid	9.2
FCB-4	15-18	90:10 MeOH: DI water	violet solid	5.9
FCB-5	19-28	80:20 MeOH: DI water	violet solid	3.6
FCB-6	27-32	70:30 MeOH: DI water	violet solid	1.1
FCB-7	33-39	50:50 MeOH: DI water	brown solid	2.4

FCB-4 (fraction 15-18) was washed with CH_2Cl_2 and gave **compound 5** to obtain a yellow orange solid (4.8 mg). Compound **5** was soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 149-151 ^{o}C;

λ_{max} (MeOH) (logε) 278 (4.12), 206 (3.87) nm.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Morphology Characteristic of the endophytic fungus *Xylaria* sp. PB-30 on five basal media

The endophytic fungus *Xylaria sp.* PB-30 was cultivated on five basal media Malt Extract Agar (MEA), Malt Czapek Agar (MCzA), Sabouraud's Dextrose Agar (SDA), Yeast Extract Sucrose Agar (YES) and Corn Meal Agar (CMA). Morphological characteristic of *Xylaria* sp. PB-30 were observed colony characteristic, colony color, color pigment production on each media were observed and growth rate on each media every weeks was also measured.

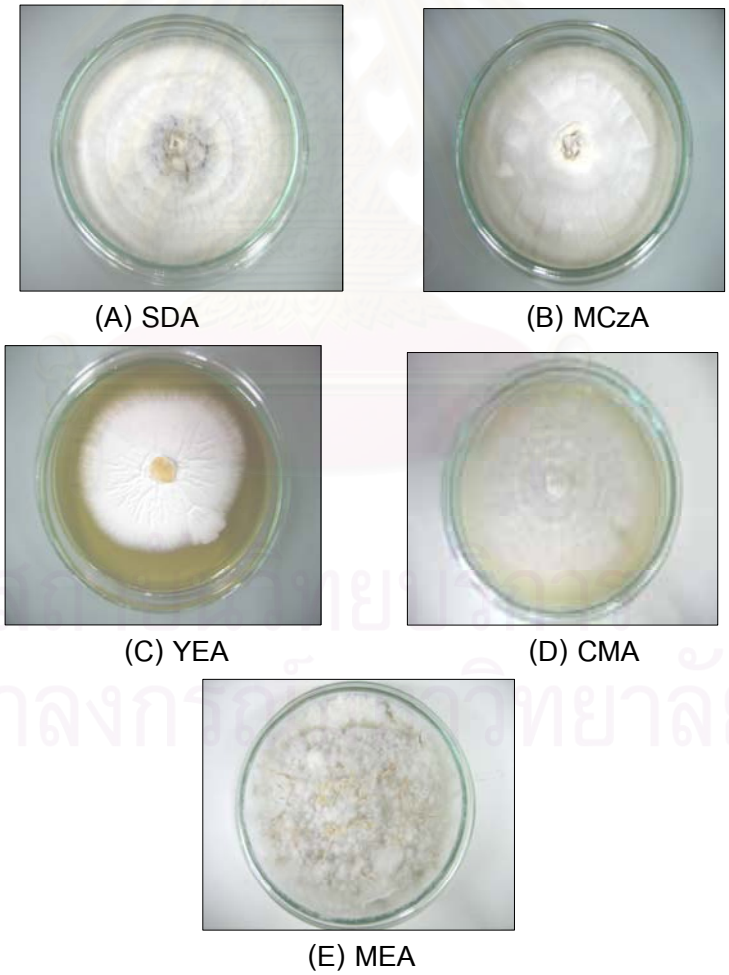


Figure 4.1 Colony characteristic of endophytic fungus *Xylaria sp.* PB-30 on five basal media after cultivation for 2 weeks at room temperature.

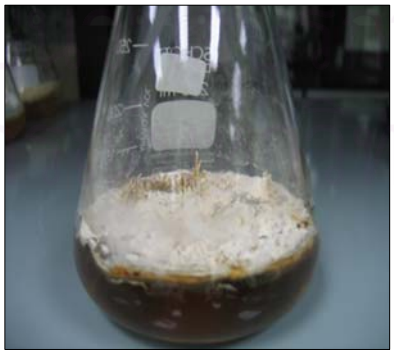
The endophytic fungus *Xylaria* sp. PB-30 was cultivated on five basal media Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Sabouraud's Dextrose Broth (SDB), Yeast Extract Sucrose Broth (YEB) and Corn Meal Broth (CMB) under static condition at room temperature.

(A) SDA

(B) MCzA

(C) YEA

(D) CMA



(E) MEA

Figure 4.2 Colony Characteristic of endophytic fungus *Xylaria sp.* PB-30 on five basal media after cultivation.

Table 4.1 Characteristics of colony *Xylaria* sp. PB-30 on five basal media.

kinds of media	*Xylaria* sp. PB-30 characteristic on basal media			
	Colony characteristic	Colony color	Color pigment production on basal media	Mycelium expansion (cm/week)
SDA	cottony	white	Black	2.6
MCzA	cottony	white	not produce	3.0
MEA	produce stroma	white	Black	2.6
YEA	cottony	white	not produce	1.6
CMA	cottony	white	not produce	2.1

Morphology characteristics of *Xylaria* sp. PB-30 on five basal media were showed in Table 4.2. Colony growth of *Xylaria* sp. PB-30 on MCzA reached margin of Petri plate in 2 week. Mycelia were initially white cottony on all media when growing to edge of Petri plate black pigments was produced on MEA and SDA. *Xylaria* sp. PB-30 on MEA produced mature stoma in 2-3 week after cultivation. Growing on YEA colony growth was very slow and did not reach the edge of plate within 3-4 weeks.

4.2 Growth profile of the endophytic fungus *Xylaria sp.* PB-30 on five of basal media

The endophytic fungus *Xylaria* sp. PB-30 was cultured in 250 mL flask containing 100 mL of five basal media Malt Extract Broth (MEB), Malt Czapek Broth (MCzB), Sabouraud's Dextrose Broth (SDB), Yeast Extract Sucrose Broth (YES) and Corn Meal Broth (CMB) under static condition at room temperature. The cultures were filtered though filter paper (Whatman No.1) and the cell mass of the culture on these media were measured every three day for analysis of growth profile. The results were presented in Figure 4.3.

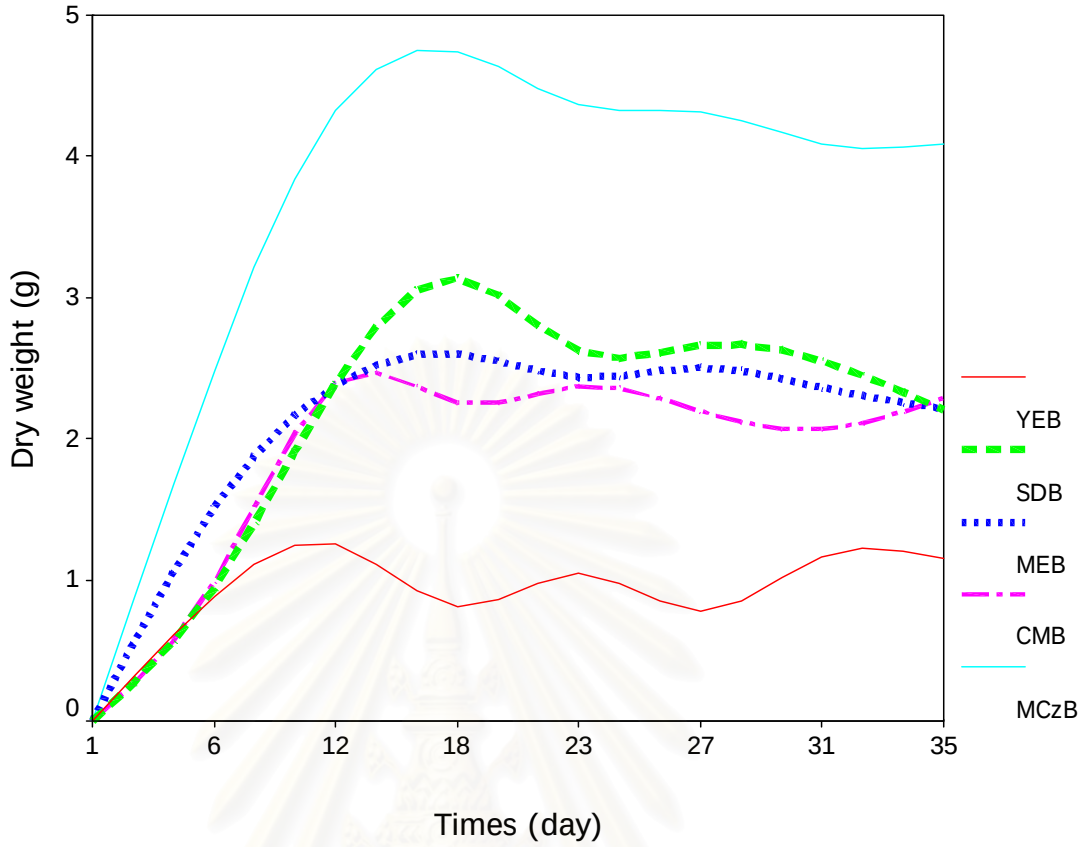


Fig. 4.3 Growth profile of endophytic fungus *Xylaria sp.* PB-30 on five basal media.

Growth rate of *Xylaria sp.* PB-30 on five media were different as presented in Fig.4.3. *Xylaria sp.* PB-30 on MCzB growth rate fast more than other media and was reached a stationary phase at 15 days after cultivated. *Xylaria* sp. PB-30 on SDB, MEB, CMB was reached a stationary phase at 18, 21, 15 days of cultivation, respectively.

4.3 Cytotoxicity activity of the EtOAc crude extracts

All crude extract of the culture on five basal media were analyzed by ^{1}H NMR spectroscopy. ^{1}H NMR profile of hexane crude extracts of the mycelia from each media revealed that these extracts mainly contained oils and triglycerides. ^{1}H NMR spectra of EtOAc crude extracts of the broth and mycelia revealed signals of compounds which may exhibit biologically activity (Figure 4.4). ^{1}H-NMR spectra of MeOH extracts of mycelia and broth these culture mainly contained glucose. The EtOAc crude broth extracts on SDB 27 day, MCzB 35 day, MEB 35 day and EtOAc crude mycelia extracts on MEB 35 day were tested cytotoxic activity against 5 human cancer cell lines comprising of BT474 (breast),

CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (Carmichael *et al.*, 1987)(Table 4.2). In vitro anticancer activity against three cancer cell lines comprising of KB oral cavity cancer, MCF-7 Brest cancer and NCI-H187 small cell lung cancer using Resazurin Microplate assay (REMA) (Table 4.3)

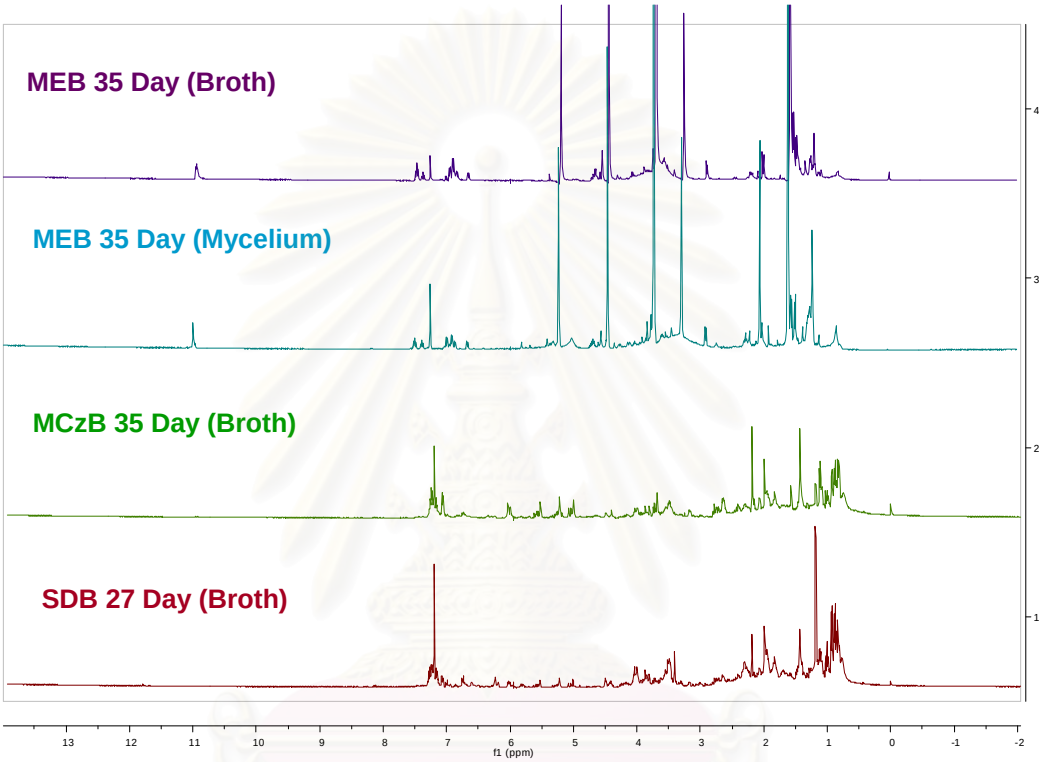


Figure 4.4 ^{1}H NMR spectra of the EtOAc crude extracts (broth and mycelium) on SDB cultured for 27 day, MCzB cultured for 35 day and MEB cultured for 35 day.

Table 4.2 Cytotoxicity activity of the EtOAc crude extracts against cancer cell lines by the MTT colorimetric method.

EtOAc curde extracts	Final concentration (µg/ml)	%Inhibition				
		BT474 (Breast)	CHAGO (lung)	HEP-G2 (hepatoma)	KATO-III (gastric)	SW 620 (colon)
SDB (B) 27day	10	Inactive	Inactive	54.48	63.57	52.58
MC_ZB (B) 35day	10	Inactive	Inactive	68.79	66.6	85.81
MEB (B) 35day	10	59.74	73.92	86.04	75.76	89.10
MEB (My) 35day	10	57.49	59.05	83.77	71.87	89.87

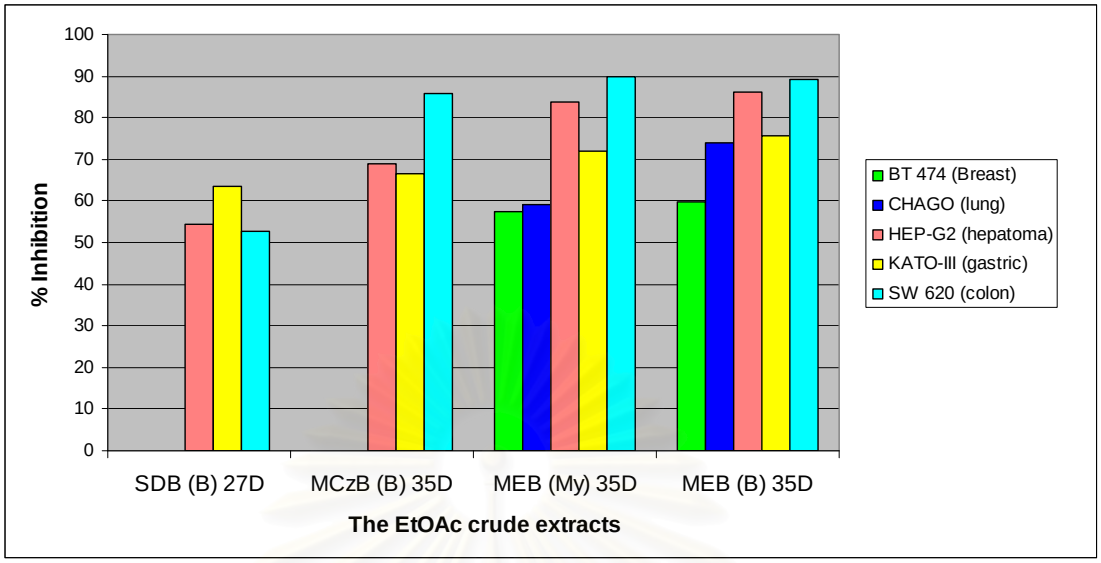


Figure 4.5 Comparison of %Inhibition of the EtOAc crude extracts on basal media against cancer cell lines.

The EtOAc extracts of broth on SDB (cultured for 27 day), MCzB (cultured for 35 day), MEB (cultured for 35 day) and EtOAc extracts of mycelia on MEB (cultured for 35 day) tested for in vitro cytotoxicity activity against 5 human cancer cell lines were compared as shown in (Figure 4.4). The EtOAc extracts of broth and mycelia on MEB media (cultured for 35 day) exhibited cytotoxicity activity against cancer cells higher than EtOAc crude broth extracts on SDB (cultured for 27 day) and MCzB (cultured for 35 day) media. And the EtOAc extracts of broth on MEB media (cultured for 35 day) exhibited highest cytotoxicity activity against cancer cells.

The EtOAc extracts of broth on MEB media (cultured for 35 day) inhibited human cancer cell lines higher than the EtOAc extracts of mycelia on MEB media and percent inhibition of BT-474 with 59.74%, CHAGO with 73.92%, HEP-G2 with 86.04%, KATO-III with 75.76% and SW 620 with 89.10%, respectively. Thus, Malt Extract Broth media was selected for investigation of anticancer metabolites of EtOAc extracts of broth on SDB (cultured for 27 day), MCzB (cultured for 35 day), MEB (cultured for 35 day) and EtOAc crude mycelia extracts on MEB (cultured for 35 day) for in vitro anticancer activity against KB oral cavity cancer, MCF-7 Brest cancer and NCI-H187 small cell lung cancer using Resazurin Microplate assay (REMA) (Table 4.3).

Table 4.3 Cytotoxicity activity of the EtOAc crude extracts against cancer cell lines by Resazurin Microplate assay (REMA)*

EtOAc curde extracts	Final concentration(µg/ml)	%Inhibition		
		KB (oral cavity)	NCI-187 (small cell lung)	MCF-7 (Brest)
SDB (B) 27 day	50	78	Inactive	Inactive
MC_ZB (B) 35 day	50	70	Inactive	Inactive
MEB (B) 35 day	50	94	80	83
MEB (My) 35 day	50	93	80	83

*Results were obtained from Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC)

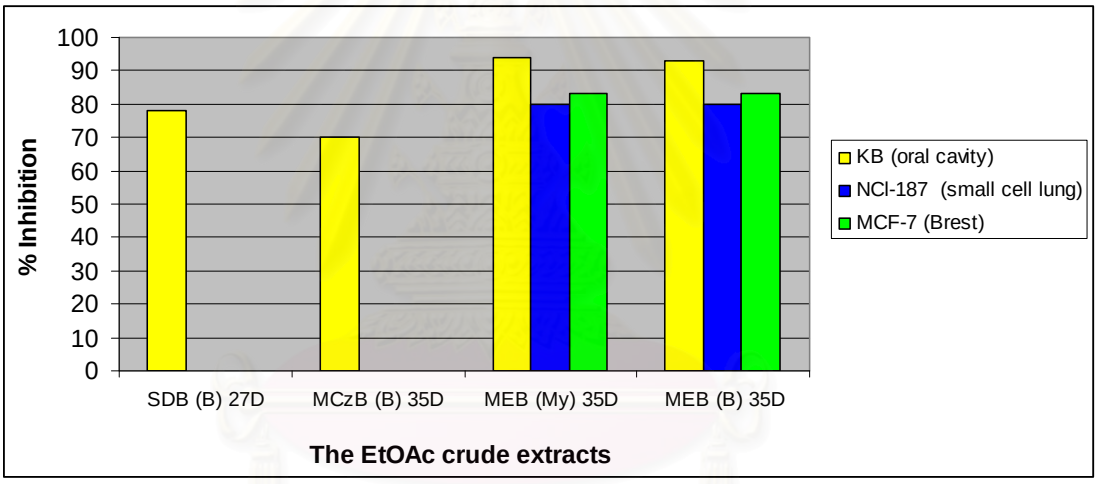


Figure 4.6 Comparison of %Inhibition of the EtOAc crude extracts on basal media against cancer cell lines.

The results (Figure 4.5) showed that the EtOAc extract of broth cultured on MEB media (cultured for 35 day) exhibited strongest anticancer activity against KB, MCF-7 and NCL-H187 with percent inhibition of 94, 83 and 80, respectively. The EtOAc extract of mycelia on MEB (cultured for 35 day) showed anticancer activity against KB, MCF-7 and NCL-H187 with percent inhibition of 93, 83 and 80, respectively. The EtOAc extract of broth on SDB (cultured for 27 day), MCzB (cultured for 35 day) exhibited only anticancer activity against KB with percent inhibition of 78 and 70, respectively.

Table 4.4 Cytotoxicity activity of the EtOAc crude extracts on MEB media (cultured for 24, 28, 35, 54 Day) against cancer cell lines by the MTT colorimetric method.

Compounds	Final concentration (µg/ml)	% Inhibition				
		BT 474 (Breast)	Chago (lung)	Hep-G2 (hepatoma)	KATO-III (gastric)	SW 620 (colon)
MEB (B) 24D	10	inactive	inactive	inactive	inactive	inactive
MEB (B) 28D	10	inactive	inactive	inactive	inactive	inactive
MEB (B) 35D	10	59.74	73.92	86.04	75.76	89.1
MEB (B) 54D	10	61.19	inactive	76.13	74.37	87.1

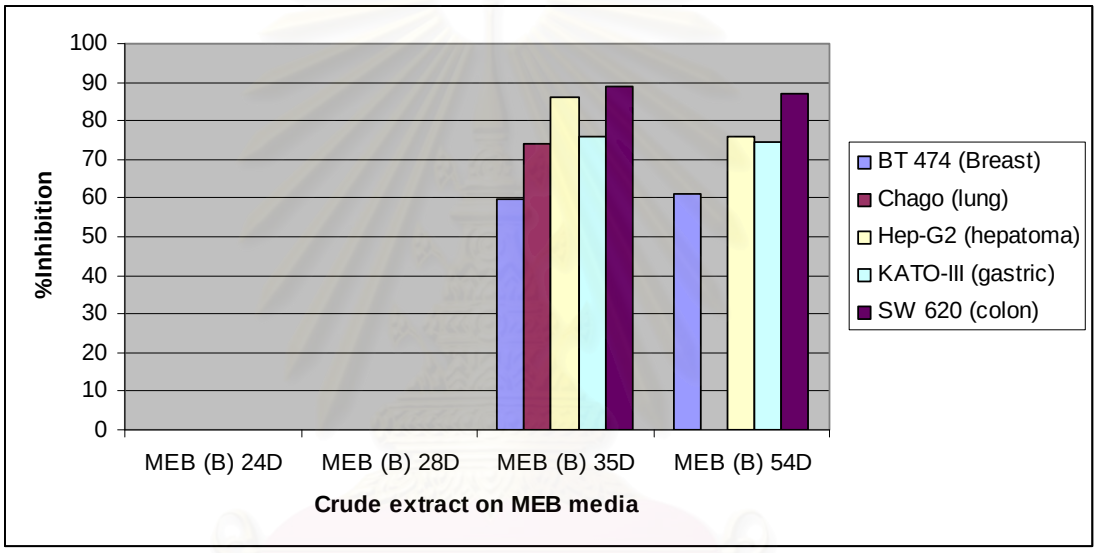


Figure 4.7 Comparison of %Inhibition of the EtOAc crude extracts on MEB media (cultured for 24, 28, 35, 54 Day) against cancer cell lines.

Future more the EtOAc extracts of broth cultured on MEB media for 24, 28, 35 and 54 day were examined for in vitro cytotoxicity activity against 5 human cancer cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon). The results (Figure 4.6) showed that the EtOAc extracts of broth on MEB media (cultured for 35 day) exhibited cytotoxicity activity against these cell lines which EtOAc extracts of broth on MEB media (cultured for 24 and 28 day) was inactive.

4.4 Scale up cultivation for anticancer activity against cancer cell lines

The endophytic fungus *Xylaria sp.* PB-30 was cultivated (30 L) into 250 ml flask each flask containing 100 ml on Malt Extract Broth (MEB) under static condition at room temperature for 35 days. Culture was filtered through filter paper (Whatman No.1). The culture broth (23 L) was concentrated by rotary evaporator *in vacuo* to give 1.5 L culture broth. And then extracted with EtOAc (x5), CH_2Cl_2: MeOH (1:1) (x5) and MeOH (x5) respectively. The extracts were concentrated by a rotary evaporation under reduced pressure at 30°C to give EtOAc crude as yellow viscous liquid (29.61 g), CH_2Cl_2: MeOH crude as brown viscous liquid (21.45 g) and MeOH crude as brown viscous liquid (4.74 g).

The fungal mycelia (202.52 g of dry weight) was ground using blender, dried in hot air oven at 60°C approximate 3-5 days and then extracted with hexane (500ml x3), EtOAc (500ml x3) and MeOH (500mlx3) respectively, The extracts were concentrated by a rotary evaporation under reduced pressure at 30°C to give hexane crude mycelia to obtain yellow viscous liquid (2.09 g), EtOAc crude as yellow brown viscous liquid (1.94 g) and MeOH crude as brown viscous liquid (32.02 g).

4.4.1 Isolation of secondary metabolites from the EtOAc extract of broth

The EtOAc extract of broth (29.61g) was subjected to a column chromatography [Sephadex™ LH-20 (400g), column diameter 3.6 cm] using 5% dichloromethane in MeOH as eluent. 10 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vapor and vanillin/H_2SO_4 reagent to give 14 combined fractions as shown in Table 4.5.

Table 4.5 The combined fractions obtained from the EtOAc extract of broth.

Fraction code	Fraction No.	Appearance	Weight (mg)
EB-1	1-20	yellow viscous liquid	101.6
EB-2	21-22	yellow viscous liquid	63.3
EB-3	23-25	brown viscous liquid	281.8
EB-4	26	dark brown solid	91.8

EB-5	27	yellow solid	377.5
EB-6	28-34	yellow solid	1959.4
EB-7	35-38	brown viscous liquid	268.1
EB-8	43-45	yellow solid powder	530.3
EB-9	46-49	brown viscous liquid	1166.8
EB-10/1	50-59	soft yellow solid	2542.9
EB-10/2	50-59	yellow solid	5246.4
EB-11	60-66	yellow solid	6318.5
EB-12	67-72	shining black solid	3207.2
EB-13	73-79	black viscous liquid	702.7
EB-14	80-89	white solid and yellow viscous liquid	213.7
EB-15	90-92	brown viscous liquid	25.2
EB-16	93-99	dark brown viscous liquid	45.9
EB-17	100-126	brown viscous liquid	15.7

All of combined fraction from EtOAc crude extract was analyzed by ^{1}H NMR spectroscopy. Since results of ^{1}H NMR data of fraction EB-5 and EB-6 were both fractions similar were combined and purified by crystallization with 1:1 mixture of CH_2Cl_2: Acetone to obtain a **compound 1** as colorless crystal (25.8 mg). This compound was characterized by NMR, MS, FT-IR, UV, optical rotation and melting point.

4.4.1.1 The chemical structure of compound 1

Compound **1** is soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 153-155^{o}C;

$[\alpha]_D^{20}$ -100 ^{o} (c = 0.1, MeOH);

λ_{max} (MeOH) (log ε) 260 nm;

HRESIMS (Figure B 1-7) *m/z* 363.0579 $[2M+Na]^+$ *calc.* for $(C_8H_{10}O_4)_2$ Na 363.1055

The FT-IR spectrum (NaCl) (Figure B1 -1) ν_{max} (cm^{-1}): 3445, 3070, 3033, 2987, 2940, 2917, 2857, 1642, 1605, 1456, 1386, 1300, 1253, 1213, 1070, 1014, 931, and 864;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure B1 -2) 1.64 (3H, s), 2.58 (1H, d, *J*=6.0Hz), 3.32 (1H, d, *J*=2.0 Hz), 4.48 (1H, d, *J*=6.3 Hz) and 5.25 (1H, d, *J*= 2.0 Hz) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure B 1-3) 18.9 (C-7), 56.6 (C-8), 59.4 (C-5), 60.5 (C-6), 69.1 (C-4), 98.2 (C-2), 171.3 (C-3) and 193.4 (C-1) ppm;

The FT-IR spectrum (NaCl) (Figure B1-1) of compound **1** showed the absorption peak of OH stretching vibration at 3445 cm^{-1}, of CH stretching vibration at 3070, 2987 and 2854 cm^{-1}, of C=O stretching vibration at 1642 cm^{-1}, of C=C stretching vibration of aromatic ring at 1605 cm^{-1} and of C-O stretching vibration at 1213, 1070 and 1014 cm^{-1}

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) compound **1** (Figure B1-2) showed two oxygenated methine protons [δ_H 3.32 (1H, d, *J*=2.0 Hz) and 4.48 (1H, dd, *J*=6.0Hz)], one methine protone [δ_H 5.25 (1H, d, *J*= 1.6 Hz)] attached to a double bond, a methyl attached to a quaternary sp^2 carbon [δ_H 1.64 (3H, s)], and a doublet signal at δ_H 2.58 (1H, d, *J*=6.0Hz).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) of compound 1 (Figure B1-3) showed the presence of one α,β-unsaturated ketone (δ_C 193.4) two quarternary sp^2 carbons (δ_C 171.3 and 98.2), two methlys (δ_C 18.9 and 56.6), two oxygenated methines (δ_C 60.5 and 59.4) and a quarternary carbon (δ_C 59.4) bearing an oxygen atom.

In order to determine the connection of the partial structures and assign the NMR signals, the 2D NMR experiment HMBC and HSQC were preformed (Table 4.6) Long-range correlation in HMBC between H_3-7 and C-4, C-5, C-6 and between the methoxy protons (H_3-8) and C-3 showed the methyl group at C-5 and the OMe group at C-3. Furthermore, HMBC correlations between H-6 and C-1, C-2 and C-7, OH-4 and C-3 and C-4, and H-2 and C-1, C-4 and C-6 established the positions of the epoxide (C-5 and C-6) and hydroxyl (C-4) groups. The chemical structure of compound **1** showed in (figure 4.8). This structure of compound **1** showed spectral data identical to that (4S,5S,6S)-5,6-epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one which was

isolated from unpolished rice fermented with Xylariaceous endophytic fungus (strain YUA-026) and its minimum inhibitory concentrations of (4S,5S,6S)-5,6,epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one exhibited against *Staphylococcus aureus* and *Pseudomonas aeruginosa* at 100 µg/ml (Yosshito *et al.,* 2005). Comparison $[\alpha]_D^{20}$ of compound **1** and $[\alpha]_D^{20}$ of (4S,5S,6S)-5,6-epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one indicated that both compounds have the same stereochemistry (see Figure 4.7)

Table 4.6 ^{1}H and ^{13}C-NMR Data and HMBC Correlations for compound **1**

Position	δ_C	δ_H	HMBC
1	193.4 (s)	-	-
2	98.2 (d)	5.25 (1H, d, 2.0)	C-1, C-3, C-4, C-6
3	171.3 (s)	-	-
4	69.1 (d)	4.48 (1H, d, 6.3)	C-3, C-7
5	59.4 (s)	-	-
6	60.5 (d)	3.32 (1H, d, 2.0)	C-1, C-2, C-5, C-7
7	18.9 (q)	1.64 (3H, s)	C-4, C-5, C-6
8	56.6 (q)	-	C-3
4-OH	-	2.58 (1H, d)	C-3, C-4

Table 4.7 ^{1}H and ^{13}C NMR data for compound **1** and (4S, 5S, 6S)-5,6,epoxy-4-hydroxy-3- methoxy-5-methyl-cyclohex-2-en-1-one.

Position	Compound 1		(4S,5S,6S)-5,6-epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one	
	δ_C	δ_H	δ_C (100 Hz)	δ_H (400 Hz)
1	193.4 (s)	-	194.4 (s)	-
2	98.2 (d)	5.25 (1H, d, 2.0)	98.6 (d)	5.11 (1H, d, 2.0)
3	171.3 (s)	-	174.6 (s)	-
4	69.1 (d)	4.48 (1H, d, 6.3)	69.6 (d)	4.53 (1H, d, 6.3)
5	59.4 (s)	-	60.8 (s)	-
6	60.5 (d)	3.32 (1H, d, 2.0)	61.2 (d)	3.11 (1H, d, 2.0)

7	18.9 (q)	1.64 (3H, s)	19.7 (q)	1.51 (3H, s)
8	56.6 (q)	-	57.2 (q)	3.68 (3H, s)
4-OH	-	2.58 (1H, d, 6.3)	-	4.85 (1H, d, 6.3)

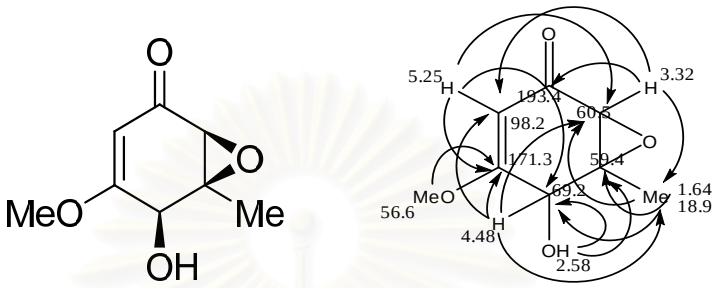


Figure 4.8 The chemical structure of compound **1** and gHMBC correlation.

Fraction EB-10 was washed with hexane and CH_2Cl_2 to give fraction EB-10/1 and EB-10/2, respectively. Result of ^{1}H NMR analysis of fraction EB-10/1 exhibited pure compound and a single spot on TLC which developed by 5% MeOH in CH_2Cl_2 showed that compound **1** was almost pure. Crystallization of fraction EB-10/2 gave compound **2** as soft yellow solid (2.54 g). Compound **2** was characterized by NMR, MS, FT-IR, UV, optical rotation and melting point:

4.4.1.2 The chemical structure of compound 2

Compound **2** is soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 153-155^{o}C;

$[\alpha]_D^{20}$ -88 ^{o} (c = 0.1, MeOH);

λ_{max} (MeOH) (log ε) 260 nm;

HRESIMS (Figure B 2-7) *m/z* 363.0579 $[2M+Na]^+$ *calc.* for $(C_8H_{10}O_4)_2$ Na 363.1055;

The FT-IR spectrum (NaCl) (Figure B2 -1) ν_{max} (cm^{-1}): 3402, 3030, 2993, 2940, 2884, 1652, 1608, 1459, 1369, 1266, 1233, 1174, 1004 and 835;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure B1 -2) 1.64 (3H, s), 3.32 (1H, d, *J*=2.0 Hz), 4.48 (1H, d, *J*=6.3 Hz) and 5.25 (1H, d, *J*= 2.0 Hz) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure B 1-3) 18.9 (C-7), 56.6 (C-8), 59.4 (C-5), 60.5 (C-6), 69.1 (C-4), 98.1 (C-2), 171.5 (C-3) and 193.5 (C-1) ppm;

The FT-IR spectrum (NaCl) of compound **2** (Figure B2-1) showed the absorption peak of OH stretching vibration at 3402 cm^{-1}, of CH stretching vibration at 3030, 2993 and 2884 cm^{-1}, of C=O stretching vibration at 1652 cm^{-1}, of C=C stretching vibration of aromatic ring at 1608 cm^{-1} and of C-O stretching vibration at 1266, 1170, 1074 and 1004 cm^{-1}

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure B2-2) compound **2** showed two oxygenated methine protons[δ_H 3.32 (1H, d, *J*=2.0 Hz) and 4.48 (1H, d, *J*=6.3 Hz)], one methine protone [δ_H 5.25 (1H, d, *J*= 2.0 Hz)] attached to a double bond, a methyl attached to a quaternary carbon [δ_H 1.64 (3H, s)].

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure B2-3) of compound **2** showed the presence of one α, β-unsaturated ketone (δ_C 193.5, 171.5 and 98.1), two methlys (δ_C 18.9 and 56.6), two oxygenated methines (δ_C 60.5 and 61.2) and a quarternary carbon (δ_C 59.4) bearing an oxygen atom.

In order to determine the connection of the partial structures and assign the NMR signals, the 2D NMR spectra (HMBC) (Table 4.7) long-range correlation between H_3-7 and C-4, C-5, C-6 and between the methoxy protons (H_3-8) and C-3 showed the methyl group at C-5 and the OMe group at C-3. Furthermore, HMBC correlations between H-6 and C-1, C-2 and C-7, OH-4 and C-3 and C-4, and H-2 and C-1, C-4 and C-6 established the positions of the epoxide (C-5 and C-6) and hydroxyl (C-4) groups. The chemical structure of compound **2** showed in (figure 4.7). This structure of compound *2* showed spectral data similar of compound **1**. Due to no hydroxyl signal at at δ_H 2.58 (1H, d, *J*=6.0Hz) in compound **2** and different $[\alpha]_D^{20}$ -88 ^{o} (c = 0.1, MeOH), Stereochemistry of compound **2** may be different from compound **1**. The stereochemistry of compound **2** was carried out using Mosher's modification precursor.
It found that compound **2** was unstable in basic and acid condition or under Mosher's modification process. Thus the stereochemistry of compound **2** was could not be indentified.

Table 4.8 ^{1}H and ^{13}C-NMR Data and HMBC Correlations for compound **2**

Position	δ_C	δ_H	HMBC
1	193.5 (s)	-	-
2	98.1 (d)	5.24 (1H, d, 2.0)	C-1, C-3, C-4, C-6
3	171.5 (s)	-	-
4	69.0 (d)	4.47 (1H, d, 6.3)	C-3, C-7
5	59.4 (s)	-	-
6	60.5 (d)	3.31 (1H, d, 2.0)	C-1, C-2, C-5, C-7
7	18.9 (q)	1.63 (3H, s)	C-4, C-5, C-6
8	56.6 (q)	3.75 (3H, s)	C-3
4-OH	-	-	C-3, C-4

Table 4.9 ^{1}H and ^{13}C NMR data for compound **2** and (4S, 5S, 6S)-5,6-epoxy-4-hydroxy-3- methoxy-5-methyl-cyclohex-2-en-1-one.

Position	Compound 2		(4S,5S,6S)-5,6-epoxy-4-hydroxy-3-methoxy-5-methayl-cyclohex-2-en-1-one	
	δ_C	δ_H	δ_C (100 Hz)	δ_H (400 Hz)
1	193.5 (s)	-	194.4 (s)	-
2	98.1 (d)	5.24 (1H, d, 2.0)	98.6 (d)	5.11 (1H, d, 2.0)
3	171.5 (s)	-	174.6 (s)	-
4	69.0 (d)	4.47 (1H, d, 6.3)	69.6 (d)	4.53 (1H, d, 6.3)
5	59.4 (s)	-	60.8 (s)	-
6	60.5 (d)	3.31 (1H, d, 2.0)	61.2 (d)	3.11 (1H, d, 2.0)
7	18.9 (q)	1.63 (3H, s)	19.7 (q)	1.51 (3H, s)
8	56.6 (q)	3.75 (3H, s)	57.2 (q)	3.68 (3H, s)
4-OH	-	-	-	4.85 (1H, d, 6.3)

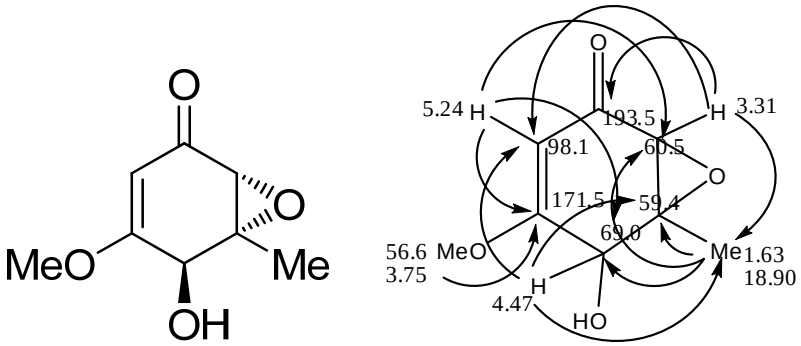


Figure 4.9 The chemical structure of compound **2** and gHMBC correlation.

4.4.2 Isolation of secondary metabolites of fraction EB-7, EB-8 and EB-9

Fraction EB-7, EB-8 and EB-9 (1.1g) were combined and then subjected to a column chromatography [silica gel 60 (40g), column diameter 2 cm] using CH_2Cl_2, CH_2Cl_2 in MeOH and MeOH eulent. 20 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine and vanillin/H_2SO_4 reagent to give 29 combined fractions.

Table 4.10 The combined fractions obtained fraction EB-7, EB-8 and EB-9.

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
A-1	3	CH_2Cl_2 (100)	yellow viscous liquid	1.0
A-2	4-6	CH_2Cl_2:MeOH (99:1)	yellow solid	3.6
A-3	7	CH_2Cl_2:MeOH (99:1)	yellow viscous liquid	5.0
A-4	8-9	CH_2Cl_2:MeOH (99:1)	yellow solid	4.5
A-5	10-11	CH_2Cl_2:MeOH (99:1)	yellow solid	3.9
A-6	12-14	CH_2Cl_2:MeOH (98:2)	white solid and yellow viscous liquid	40.3
A-7	15-20	CH_2Cl_2:MeOH (97:3)	yellow viscous liquid	16.3
A-8	21-22	CH_2Cl_2:MeOH (96:4)	yellow viscous liquid	32.4
A-9	23-29	CH_2Cl_2:MeOH (95:5)	yellow viscous liquid	26.7
A-10	30	CH_2Cl_2:MeOH (93:7)	yellow viscous liquid	8.5
A-11	31-34	CH_2Cl_2:MeOH (93:7)	yellow viscous liquid	31.3

A-12	35-41	CH_2Cl_2:MeOH (93:7)	yellow viscous liquid	29.7
A-13	42	CH_2Cl_2:MeOH (90:10)	white solid and brown viscous liquid	6.4
A-14	43-44	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	7.2
A-15	45	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	8.3
A-16	46	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	3.4
A-17	47-49	CH_2Cl_2:MeOH (90:10)	yellow viscous liquid	20.2
A-18	50-52	CH_2Cl_2:MeOH (89:11)	yellow viscous liquid	10.9
A-19	53-59	CH_2Cl_2:MeOH (89:11)	yellow viscous liquid	13.4
A-20	60-63	CH_2Cl_2:MeOH (87:13)	yellow viscous liquid	7.2
A-21	64-69	CH_2Cl_2:MeOH (85:15)	yellow viscous liquid	13.0
A-22	70-79	CH_2Cl_2:MeOH (84:16)	yellow viscous liquid	8.2
A-23	80-87	CH_2Cl_2:MeOH (83:17)	yellow viscous liquid	10.4
A-24	88-90	CH_2Cl_2:MeOH (83:17)	yellow viscous liquid	11.3
A-25	91-96	CH_2Cl_2:MeOH (83:17)	yellow viscous liquid	6.3
A-26	97-100	CH_2Cl_2:MeOH (82:18)	yellow solid	4.4
A-27	101-109	CH_2Cl_2:MeOH (82:18)	brown solid	8.5
A-28	110-117	CH_2Cl_2:MeOH (80:20)	brown solid	2.9
A-29	118-119	CH_2Cl_2:MeOH (75:25)	brown solid	39.4

A-6 (fraction 12-14) was washed with CH_2Cl_2 and gave **compound 3** which is white solid. This compound was characterized by NMR, MS, FT-IR, UV, optical rotation and melting point.

4.4.2.1 The chemical structure of compound 3

Compound **3** is soluble in CH_2Cl_2, MeOH and slightly soluble in hexane, m.p. 267°C;

$[\alpha]_D^{20}$ -43° (c = 0.10, $CHCl_3$);

λ_{max} (MeOH) (log ε) 259 nm

HRESIMS (Figure B 3-7) *m/z* 530.2518 $[M+Na]^+$ *calc.* for $C_{30}H_{37}NO_6$ 507.262088;

The FT-IR spectrum (NaCl) (Figure B3 -1) ν_{max} (cm^{-1}): 3418, 2967, 2917, 2847, 1735, 1698, 1685, 1642, 1456, 1373, 1270, 1236, 1114, 1054, 1011, 957, and 904;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure B3 -2) 0.944 (1H,d, H-16), 1.191 (1H,d, H-16), 1.503 (3H,s), 2.061 (2H,dd, H-16 -CH_3), 2.140 (1H, dd, *J* = 3.6 and 4.8 Hz), 2.25 (3H,s), 2.50 (2H, q, *J* = 11.2 Hz), 2.66 (2H, dd, *J* = 9.6 and 13.6 Hz), 2.72 (1H, d, *J* = 6 Hz), 2.81 (2H, d, *J* = 4.8 and 13.6 Hz), 3.22 (1H, dt, *J* = 3.6 and 9.2 Hz), 3.80 (1H, d, *J* = 10.8 Hz), 5.084 (2H,s), 5.13 (1H, dd, *J* = 2.4 and 16 Hz), 5.29 (2H,s), 5.33 (1H, ddd, *J* = 5.2 and 10.8 Hz), 5.62 (1H, t, *J* = 2.4 Hz), 5.68 (1H, dd, *J* = 10 and 15.6 Hz), 7.12 (1H, dd, *J* = 7.2 Hz), 7.23 (1H, t, *J* = 7.2 Hz) and 7.31 (1H, t, *J* = 7.2 Hz) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure B 3-3) 13.66(C-11), 19.36 (C-22), 20.86(C-CH_3(Ac)), 24.16(C-23), 32.65(C-5), 37.70(C-15), 42.31(C-16), 45.30(C-10), 46.96(C-8), 49.99(C-4), 53.25(C-9), 53.54(C-3), 69.08(C-7), 77.09(C-21), 77.69(C-18), 114.52(C-12), 127.08(C-19), 127.57(C-4'), 128.93(C-3' and 5'), 129.06(C-2' and 6'), 130.57(C-13), 132.26(C-20), 134.13(C-14), 137.22(C-1'), 147.45(C-6), 169.71(C=O(Ac)), 173.64(C-1) and 210.26(C-17) ppm;

The FT-IR spectrum of **3** (Figure B 3-1, Table 4.10) showed the absorption peak of OH stretching vibration at 3418 cm^{-1}, of C=O stretching vibration at 1735 and 1698 cm^{-1}, of N-H stretching vibration at 1685 cm^{-1}, of C-H bending vibration at 1456 cm^{-1}, of C-O stretching vibration at 1054 and 1011 cm^{-1}

Table 4.11 The IR absorption assignment of compound **3**

Wave numbers (cm^{-1})	Tentative assignment
3418	O-H stretching vibration
1735	C=O stretching vibration
1698	C=O stretching vibration
1270 and 1236	C-N
1054 and 1011	C-O stretching vibration

The ^{1}H-NMR data of compound **3** (Figure B 3-2, Table 4.11) indicated that it possesses three aromatic proton at δ_H 7.12, 7.31 and 7.23 ppm; eleven methine proton at δ_H 6.10, 5.68, 5.62, 5.33, 5.13, 3.22, 2.84, 2.74, 2.72 and 2.14 ppm; six methylene protons at δ_H 5.29, 5.08, 2.81, 2.66, 2.50 and 2.01 ppm; four methyl protons at δ_H3.802, 2.25, 1.50, and 0.94 ppm.

The ^{13}C-NMR and HSQC spectra of compound **3** (Figure B 3-3, Table 4.11) showed 30 signals consisting of five aromatic methine carbon at δ_C 129.06 (x2), 128.93 (x2), and 127.08 ppm; a quarternary aromatic carbons at δ_C 137.22 ppm; four methine sp^2-carbons at δ_C 134.13, 132.26, 130.57, and 127.57 ppm; an methylene sp^2-carbon at δ_C 114.5 ppm; an qurternary sp^2-carbon at δ_C 147.4 ppm; two oxygenated methine sp^3-carbons at δ_C 77.69 and 69.80 ppm; an oxygenated quarternary sp^3-carbons at δ_C , 77.09 ppm; a methine sp^3-carbon attached to NH at δ_C 53.54 ppm; methine sp^3-carbons at δ_C 49.99, 46.96, 42.31 and 32.65 ppm; two methylene carbons at δ_C 45.30 and 37.70 ppm; four methyl carbons at δ_C24.16, 20.86, 19.39 and 13.66 ppm; one quarternary sp^3-carbon at δ_C 53.25 ppm; and three carbonyl groups at δ_C210.26, 173.64 and 169.71 ppm.

2D-NMR data analysis with gHSQC, gHMBC and g COSY to perform ^{1}H-NMR and ^{13}C-NMR shown in Table 4.12, 4.13, and 4.14

Table 4.12 ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **3**

^{13}C-NMR (ppm)	^{1}H-NMR and coupling constant (Hz)
210.26	-
173.64	-
169.71	-
147.45	-
137.22	-
134.13	5.33 (1H, ddd, *J* = 5.2 and 10.8 Hz)
132.26	6.10 (1H, dd, *J* = 2.8 and 15.6 Hz)
130.57	5.68 (1H, dd, *J* = 10 and 15.6 Hz)
129.06	7.12 (1H, dd, *J* = 7.2 Hz)

128.93	7.31 (1H, t, *J* = 7.2 Hz)
127.57	5.13 (1H, dd, *J* = 2.4 and 16 Hz)
127.08	7.23 (1H, t, *J* = 7.2 Hz)
114.52	5.29 (2H,s), 5.084 (2H,s)
77.69	-
77.09	5.62 (1H, t, *J* = 2.4 Hz)
69.08	3.80 (1H, d, *J* = 10.8 Hz)
53.54	3.22 (1H, dt, *J* = 3.6 and 9.2 Hz)
53.25	-
49.99	2.14 (1H, dd, *J* = 3.6 and 4.8 Hz)
46.96	2.84 (1H, t, *J* = 10 Hz)
45.3	2.66 (2H, dd, *J* = 9.6 and 13.6 Hz)
	2.81 (2H, d, *J* = 4.8 and 13.6 Hz)
42.31	2.74 (1H, dd)
37.7	2.50 (2H, q, *J* = 11.2 Hz)
	2.01 (2H, dd, *J* = 4.8 and 12.8 Hz)
32.65	2.72 (1H, d, *J* = 6 Hz)
24.16	1.5 (3H, s)
20.86	2.25 (3H, s)
19.39	1.19 (1H, d, *J* = 6.8 Hz)
13.66	0.94 (1H, d, *J* = 6.8 Hz)

Table4.13 Comparison of ^{13}C-NMR and ^{1}H-NMR chemical shifts of compound **3** and Cytochalasin D

Position		Compound 3		Cytochalasin D	
		δ_C (100 Hz)	δ_H (400 Hz)	δ_C (100 Hz)	δ_H (400 Hz)
1	C	173.64	-	-	-
2	N	-	5.587	8.92	174.90
3	C	53.54	3.226	3.54	

4	C	49.99	2.140	2.43	54.00
5	C	32.65	2.723	2.70	50.00
6	C	147.45	-	-	151.40
7	C	69.80	3.802	4.36	71.20
8	C	46.96	2.846	3.34	48.70
9	C	53.25	-	-	54.37
10	C	45.30	2.665	2.92	45.49
		-	2.814	-	-
11	C	13.66	0.944	0.97	13.65
12	C	114.52	5.291	5.42	112.20
		-	5.084	5.07	-
13	C	130.57	5.682	6.23	132.10
14	C	134.13	5.330	5.64	132.70
15	C	37.70	2.505	2.70	38.58
		-	2.016	1.95	-
16	C	42.31	2.740	2.00	42.45
17	C	210.26	-	5.55	210.70
18	C	77.69	-	-	78.32
19	C	127.57	5.133	-	127.70
20	C	132.26	6.101	6.79	133.70
21	C	77.09	5.625	5.98	77.92
22	C	19.39	1.191	1.09	19.44
23	C	24.16	1.503	1.54	24.64
1'	C	137.22	-	-	138.30
2', 6'	C	129.06	7.126	7.24	129.90
3', 5'	C	128.93	7.312	7.24	128.70
4'	C	127.08	7.238	7.24	126.80
C=O(Ac)	C	169.71	-	-	170.30
CH_3(Ac)	C	20.86	2.259	2.35	20.61

Table 4.14 The correlation of gHMBC and NOESY of compound **3**

Position		δ_C	δ_H	gHBC (H to C)	gCOSY
1	C	173.64	-	-	-
2	N		5.587	C-3, C-4, C-12	-
3	C	53.54	3.226	C-1, C-4, C-5, C-10	H-4, H-10
4	C	49.99	2.140	C-1, C-2, C-5, C-6, C-9, CH_3(Ac)	H-3, H-5, H-16
5	C	32.65	2.723	C-4, C-7, C-11	H-4, H-15
6	C	147.45	-	-	-
7	C	69.80	3.802	C-5, C-6, C-12, C-13	H-8, H-10
8	C	46.96	2.846	C-1, C-7, C-13, C-14, C-16, C-21	H-7, H-10
9	C	53.25	-	-	-
10	C	45.30	2.665	C-3, C-4, C-7, C-9, C-1'	H-3, H-7, H-8
			2.814	C-3, C-4, C-15, C-1'	H-10, H-3, H-10
11	C	13.66	0.944	C-4, C-5, C-6	H-16
12	C	114.52	5.291	C-4, C-5, C-6, C-7, C-12	H-13, H-19
			5.084	C-4, C-5, C-6, C-7, C-8, C-12, C-15	
13	C	130.57	5.682	C-4, C-5,C-7, C-8, C-9	H-12, H-14
14	C	134.13	5.330	C-6, C-7, C-8, C-15	H-13
15	C	37.70	2.505	C-13, C-14,C-16, C-17	H-5, H-16
16	C	42.31	2.016	C-13, C-14,C-16, C-17, C-16 (CH_3)	H-4, H-11, H-15, H-16 (CH_3)
			2.740	C-3, C-6,C-12, C-14, C-15, C-16(CH_3)	
17	C	210.26	-	-	-
18	C	77.69	-	-	-
19	C	127.57	5.133	C-18, C-20, C-21, C-18(CH_3)	H-12, H-20, H-21

20	C	132.26	6.101	C-18, C-19, C-21	H-19, H-21
21	C	77.09	5.625	C-8, C-9, C-16, C-20, C=O(CH_3)	H-19, H-20
22	C	19.39	1.191	C-15, C-16, C-17	H-16
23	C	24.16	1.503	C-17, C18, C19, C-20, C-21, C-2', C-3', C-4'	-
1'	C	137.22	-	-	-
2', 6'	C	129.06	7.126	C-10, C-4'	H-3'
3', 5'	C	128.93	7.312	C-1', C-2', C-3'	H-2', H-4'
4'	C	127.08	7.238	C-2', C-3'	H-3'
C=O(Ac)	C	169.71	-	-	-
CH_3(Ac)	C	20.86	2.259	C-21, C=O(CH_3)	-

From spectroscopic data analysis, compound **3** was indicated as cytochalasin D which was discovered first in 1966 as a known class of mold metabolites (Aldridge *et al.*, 1967). There are now more than 20 known cytochalasins, isolated from a variety of fungal species, including *Helminthosporium* sp., *Phoma* sp., *Xylaria* sp., *Hypoxylon* sp., *Chalara* sp., and *Rhinocladiella* sp. (Buckingham, 1994; Dagne *et al.*,1994; Wipapan *et al.*, 2007). Bioactivities of cytochalasin D including cytotoxicity activity against standard Vero cell with IC_{50} values 0.19 μM and a weak antimycobacterial activity against *Mycobacterium tuberculosis* with MIC values of 394.3 μM (Wipapan *et al.*, 2007) have been reported.

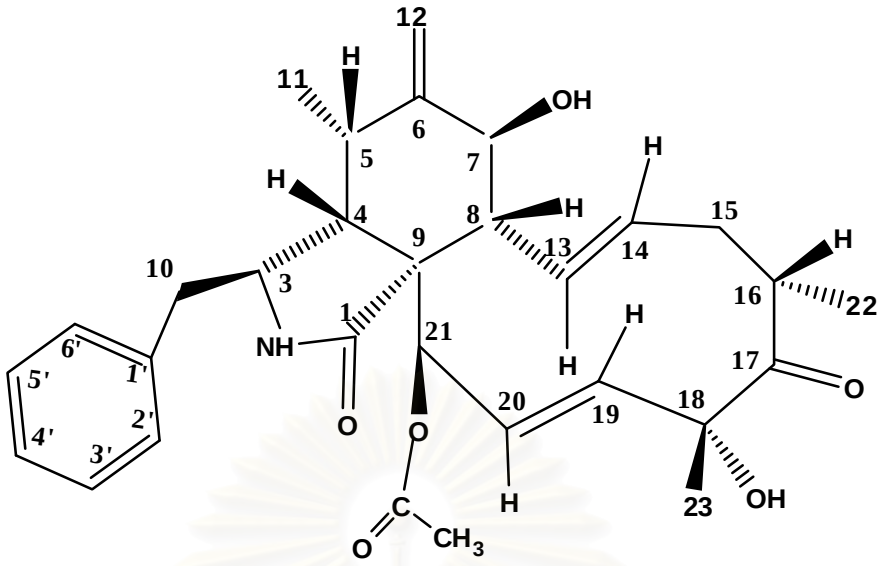


Figure 4.10 The chemical structure of compound **3**

4.4.3 Isolation of secondary metabolites of fraction FC-6

Fraction FC-6 (51.5 g) was subjected to flash column chromatography [Silica gel 60 RP-18 (10g), column diameter 1.5 cm] eluted with MeOH in DI water and 100% MeOH. 5 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine vapor and vanillin/H_2SO_4 reagent to give 6 combined fractions.

Table 4.15 The combined fractions obtained fraction FC-6

fraction code	fraction no.	Eluted	Appearance	weight (mg)
FCC-1	1-17	90:10 MeOH: DI water	violet solid	4.0
FCC-2	18-29	90:10 MeOH: DI water	dark violet solid	1.8
FCC-3	30-35	70:30 MeOH: DI water	violet solid	15.3
FCC-4	36-40	70:30 MeOH: DI water	dark violet solid	2.7
FCC-5	41-45	50:50 MeOH: DI water	violet solid	5.9
FCC-6	46-52	100%MeOH	yellow viscous liquid	18.1

BB-4 (fraction 36-40) was extracted with solvent CH_2Cl_2. The CH_2Cl_2 extraction gave compound **4** to obtain a white powder (3.8 mg). This compound was characterized by NMR, MS, FT-IR, UV, optical rotation and melting point.

4.4.3.1 The chemical structure of compound 4

Compound **4** is soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane,

m.p. 95-97^{o}C;

$[\alpha]_D^{20}$+47 (c = 0.075, $CHCl_3$);

λ_{max} (MeOH) (log ε) 219 (4.86) nm;

HRESIMS (Figure D 4-9) *m/z* 195.0657 $[M+H]^+$ *calc.* for $C_{10}H_{11}O_4$ 195.1953.

The FT-IR spectrum (NaCl) (Figure B 4-1) ν_{max} (cm^{-1}): 3395, 3069, 2995, 2917, 2821, -3200, 1673, 1582, 1478, 1413, 1295, 1195, 1091, 1021, 960, 917, 821, 747, 673, 591, 482;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure B 4-2) 1.59 (3H, s, Me-3), 3.49 (1H, s, OH-4), 4.59 (1H, s, H-4), 4.65 (1H, d, *J* = 8.58 Hz, H-3), 6.93 (1H, d, *J* = 7.0 Hz, H-5), 7.04 (1H, d, *J* = 8.6 Hz, H-7), 7.52 (1H, dd, *J* = 7.8 Hz, H-6) and 11.03 (1H, s, OH-8) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure B 4-3) 16.02 (OMe), 67.23 (C-4), 78.27 (C-3), 106.58 (C-8a), 118.34 (C-7), 118.50 (C-5), 136.81 (C-6), 140.57 (C-4a), 162.07 (C-8) and 169.25 (C-1) ppm;

R_f 0.54 developed on silica gel TLC using 2% MeOH in CH_2Cl_2 as the mobile phase and the spot was visualized with UV lamp (365 nm).

The IR spectrum of compound **4** were indicate the absorption band at 3700-3100 cm^{-1} was broad and showed the O-H stretching vibration, the signals absorption band at 3069 cm^{-1} showed the =C-H stretching vibration, the absorption band at 2995 cm^{-1} showed the -C-H stretching vibration (for asymmetric stretching of methyl group), the absorption broad band at ~2900 cm^{-1} showed the –C-H stretching vibration (for symmetric stretching of methyl group), the strong absorption band at 1673

$cm^{-1}$of the C=O stretching vibration of carbonyl group, the absorption bands at 1478, 1195 cm^{-1} and of C-O stretching vibration at 1091, 1021 cm^{-1}

The ^{1}H NMR spectrum of compound **4** indicated that it one methyl group at δ_H 1.59 ppm, two protons attached with oxygenated carbons group at δ_H 3.49 ppm, five methines at δ_H 4.59, 4.65, 6.93, 7.04 and 7.52 ppm and one hydroxy proton at δ_H 11.03 ppm.

The ^{13}C NMR spectrum of compound **4** displayed 10 carbon resonances. The signals at δ_C 169.25 and 162.07 ppm could be ascribed to a carbonyl group of ketone and an aromatic carbon bound to hydroxy group, respectively. The resonances at δ_C 140.57 and 106.58 ppm were ascribed to aromatic carbons. Three signals of quaternary carbon at δ_C 136.81, 118.50, and 118.34 ppm. One signal of methine carbon appeared at δ_C 78.27 ppm, a methylene carbon of hydroxy group at δ_C 67.23 ppm and a methyl carbon at δ_C 16.02 ppm.

Table 4.16 ^{1}H and ^{13}C NMR data for compound **4** and 4-hydroxymellein.

Position	Compound 4		4-hydroxymellein (Cloe et al., 1971)	4-hydroxymellein (Devys et al., 1980)
	δ_C	δ_H	δ_H (mult., *J* in Hz)[a]	δ_H (mult., J in Hz)[b]
1	169.25	-	-	-
2	-	-	-	-
3	78.27	4.65 (1H, d, *J* = 8.53 Hz)	4.4 (1H, s)	4.56 (1H, dd, *J* = 6.0, $J_{3,4}$ = 2.0)
3-Me	16.02	1.59 (3H, d, *J* = 7.0)	1.22 (3H, d, *J* = 3.0)	1.55 (3H, d, *J* = 6.0)
4	67.23	4.59 (1H, s)	4.45 (1H, d, *J* = 2.0)	4.50 (1H, d, *J* = 2.0)
4-OH	-	3.49 (1H, s)	2.61(1H, d, *J* =3.0)	3.26 (1H, s, large)
4a	140.57	-	-	-
5	118.50	6.93 (1H, d, *J* = 7.0)	6.85 (1H, d, *J* = 4.0)	6.95 (1H, d, *J* = 4)
6	136.81	7.52 (1H, d, *J* = 7.8)	7.42 (1H, t, *J* = 4.0)	7.52 (1H, dd, *J* = 4.0)

		7.04 (1H, d,		
7	118.34	*J* = 8.6)	6.82 (1H, d, *J* = 4.0)	6.88 (1H, d, *J* = 3.5)
8	162.07	-	-	-
8-OH	-	11.03 (1H, s)	11.03 (1H, s)	11.00 (1H, s)
8a	106.58	-	-	-

[a,b] Not showed the NMR frequency.

From spectroscopic data analysis of compound **4** was indicated to **4,8-dihydroxy-3-methyl-3,4-dihydroisochroman-1-one** or **4-hydroxymellein**, an isocoumarin metabolite. This metabolite was isolated from microorganism such as *Aspergillus oniki* (Sasaki *et al.,* 1970), *Aspergillus ochraceus* (Cole *et al*., 1971; Moore *et al*., 1972), *Cercospora taiwanensis* (Camarda *et al*., 1976), *Cercospora* sp. (Assante *et al*., 1977), *Lasiodiplodia theobromae* (Devys *et al*., 1980), *Microsphaeropsis* sp. (Höller *et al*., 1999) and endophytic fungi including *Xylaria longiana* (Rehm.) (Edwards *et al*., 1999) and *Diplodia corticola* (Evidente *et al*., 2006). More recently, this metabolite had found in plant, the stem bark of *Uvaria hamiltonii* (Asha, *et al*., 2004).

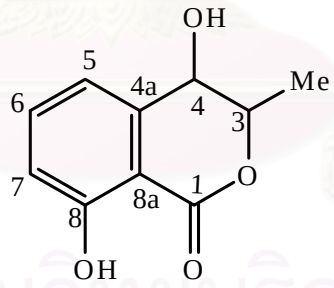


Figure 4.11 The chemical structure of compound **4**

4.4.4 Isolation of secondary metabolites of fraction FC-3.

Fraction FC-3 (110 mg) was subjected to flash column chromatography [Silica gel 60 RP-18 (10g), column diameter 1.5 cm] eluted with MeOH in DI water and 100% MeOH. 5 mL of each fraction was collected. The similar fractions were combined on the basis of TLC profile and monitored by UV, iodine and vanillin/H_2SO_4 reagent to give 7 combined fractions.

Table 4.17 The combined fractions obtained fraction FC-3

fraction code	fraction no.	Eluted	Appearance	weight (mg)
FCB-1	1-5	90:10 MeOH: DI water	brown viscous liquid	30
FCB-2	6-7	90:10 MeOH: DI water	violet solid	15.6
FCB-3	8-14	90:10 MeOH: DI water	violet solid	9.2
FCB-4	15-18	90:10 MeOH: DI water	violet solid	5.9
FCB-5	19-28	80:20 MeOH: DI water	violet solid	3.6
FCB-6	27-32	70:30 MeOH: DI water	violet solid	1.1
FCB-7	33-39	50:50 MeOH: DI water	brown solid	2.4

FCB-4 (fraction 15-18) was extracted with solvent CH_2Cl_2. The CH_2Cl_2 extraction gave **compound 5** to obtain a yellow orange solid (4.8 mg). This compound was characterized by NMR, MS, FT-IR, UV, optical rotation and melting point.

4.4.4.1 The chemical structure of compound 5

Compound **5** was soluble in EtOAc, CH_2Cl_2, MeOH and slightly soluble in hexane;

m.p. 149-151 $^{\circ}$C;

λ_{max} (MeOH) (log ε) 278 (4.12), 206 (3.87) nm;

ESI-TOF *m/z* 191.0301 $[M+Na]^+$ *calc.* for $C_8H_9O_4$ 169.0549;

The FT-IR spectrum (NaCl) (Figure B5 -1) ν_{max} (cm^{-1}): 3445, 3070, 3033, 2987, 2940, 2917, 2857, 1642, 1605, 1456, 1386, 1300, 1253, 1213, 1070, 1014, 931, and 864;

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure B5 -2) 1.94 (3H, s, Me-3), 3.86 (3H, s, OMe), 5.84 (1H, s, H-6) and 7.27 (1H, s, OH) ppm;

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure B5 -3) 7.8 (Me-3), 56.8 (OMe), 102.1 (C-6), 114.8 (C-3), 151.6 (C-2), 161.2 (C-5), 182.0 (C-4) and 184.5 (C-1) ppm;

R_f value 0.63 on TLC plate using 10% MeOH in CH_2Cl_2 as the mobile phase.

The FT-IR spectrum (Figure B5-1) showed IR absorption bands at 3445 cm^{-1} were showed the O-H stretching vibration, the signals absorption band at 3070 cm^{-1} showed the C-H stretching vibration, the absorption band at 2940 cm^{-1} showed the C-H stretching vibration (for asymmetric stretching of methyl group), the absorption broad band at 2854 cm^{-1} showed the C-H stretching vibration (for symmetric stretching of methyl group), the strong absorption bands at 1642 and 1605 $cm^{-1}$of the C=O stretching vibration of carbonyl group, the absorption bands at 1456, 1386, 1300 $cm^{-1}$of the C=C stretching vibration and the absorption bands at 1213, 1070 $cm^{-1}$of C-O stretching vibration.

The ^{1}H NMR spectrum of compound **5** indicated that it one methyl group at δ 1.94 ppm, one methoxy group at δ 3.86 ppm, one olefinic proton at δ 5.84 ppm and one hydroxy group at δ 7.27 ppm.

The ^{13}C NMR spectrum of compound **5** displayed 8 carbon resonances. Two signals at δ 184.5 and 182.0 ppm could be ascribed to carbonyl group of ketone. The signals at δ 161.2 and 114.8 ppm were aromatic carbon attached with methoxy group and methyl group, respectively. A signal at δ 102.1 ppm was a methine carbon. The resonances at δ 56.8 and 7.8 ppm were methoxy carbon and methyl carbon, respectively.

The structure of compound **5** was identified as **2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione** Compound **5** is a known compound, which has been synthesized by reacting 2,5-dihydroxy-3-methylbenzoquinone with boiling MeOH (Kiuchi, Takashima and Tsuda, 1998). The first report of **2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione** found as a natural product. This compound isolated from an endophytic fungus, *Xylaria* sp. (Tansuwan, 2007).

Table 4.18 ^{1}H and ^{13}C NMR data for compound **5**

Position	δ_H (mult., J in Hz)	δ_C	gHMBC
1		182.6	
2		151.6	
2-OH	7.26 (1H, s)		C-1, C-2

3		114.8	
3-Me	1.94 (3H, s)	7.8	C-2, C-3, C-4
4		182.0	
5		161.2	
5-OMe	3.86 (3H, s)	56.8	C-5
6	5.84 (1H, s)	102.1	C-2, C-4, C-5

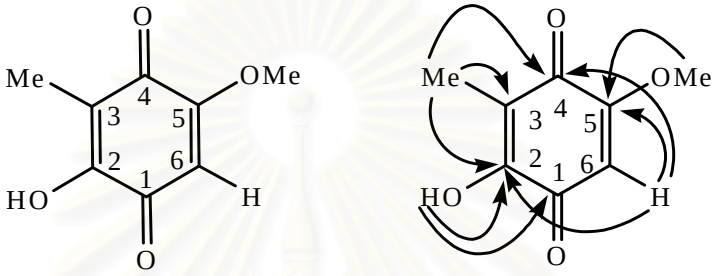


Figure 4.12 The chemical structure of compound **5** and gHMBC correlation.

4.5 Cytotoxicity test

Compounds **1-5** were tested for cytotoxic activity against five human cancer cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (Carmichael *et al*., 1987)

The results were shown in Table 4.19 for cytotoxic activity against five human cancer cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon). Their IC_{50} against different cell lines were calculated as the dose at which 50% cell death occurred relative to the untreated cells (Table 4.19).

Table 4.19 Cytotoxic activities against cell line of pure compounds.

Compounds	IC_{50} (μg/ml)				
	BT474 (breast)	CHAGO (lung)	HEP-G2 (hepatoma)	KATO-III (gastric)	SW620 (colon)
1	10.51	11.11	6.25	5.61	5.31
2	22.43	6.09	5.79	5.41	5.49
3	inactive	inactive	6.29	inactive	6.81
4	inactive	inactive	inactive	inactive	inactive
5	inactive	inactive	3.39	inactive	inactive

Cytotoxic activities of compound **1-5** were examined against 5 cell lines including BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), SW620 (colon). Compound **4** was inactive against all cancer cell lines. Compound **1** exhibited cytotoxic activity against all cancer cell lines and was selective against HEP-G2, KATO-III and SW620 with the IC_{50} values 6.25, 5.61 and 5.31μg/ml, respectively. Compound **2** exhibited cytotoxicity activity against all cancer cell lines and was selective against CHAGO, HEP-G2, KATO-III and SW620 with the IC_{50} values 6.09, 5.79, 5.41 and 5.49 μg/ml, respectively. Compound **3** exhibited cytotoxicity activity against HEP-G2 and SW 620 with the IC_{50} values of 6.29 and 6.81μg/ml respectively, and inactive against other cell lines. Compound **5** exhibited only cytotoxicity activity against HEP-G2 with the IC_{50} value of 3.39 μg/ml.

CHAPTER V

CONCLUSION

In the present study, production of anticancer metabolites from the endophtytic fungus *Xylaria sp.* PB-30 was investigated. This fungus was cultured in five media (MEB, MCzB, SDB, YEB and CMB). Anticancer metabolites of cultured in MEB were produced more than in MCzB, SDB, YEB, CMB and most of them contained in EtOAc extract of broth and mycelia. From ^{1}H-NMR analysis of mycelia and broth cultured on mainly contained triglycerides which methanol extracts mainly contained glucose. Metabolites of this fungus exhibited anticancer activity were produced during stationary phase of growth which was within 35 days of cultivation. Thus EtOAc crude broth extracts on SDB 27 day, MCzB 35 day, MEB 35 day and EtOAc crude mycelia extracts on MEB 35 day were tested for in vitro cytotoxicity activity against cancer cell lines. The EtOAc extracts of broth from culture on MEB showed the highest cytotoxicity activity against all cancer cell lines. The metabolites produced by the endophytic fungus *xylaria* sp. PB-30 was isolated to afford 5 compounds including (4S,5S,6S)- 5,6-epoxy-4-hydroxy-3-methoxy-5-methyl-cyclohex-2-en-1-one **1**, compound **2**, cytochalasin D **3**, 4-hydroxymellein **4** and 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione **5**. Cytotoxic activities of compound **1-5** were examined against 5 cell lines including BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), SW620 (colon). Compound **4** was inactive against all cancer cell lines. Compound **1** exhibited cytotoxic activity against all cancer cell lines and was selective against HEP-G2, KATO-III and SW620 with the IC_{50} values 6.25, 5.61 and 5.31μg/ml, respectively. Compound **2** exhibited cytotoxicity activity against all cancer cell lines and were selective against CHAGO, HEP-G2, KATO-III and SW620 with the IC_{50} values 6.09, 5.79, 5.41 and 5.49 μg/ml, respectively. Compound **3** exhibited cytotoxicity activity against HEP-G2 and SW 620 with the IC_{50} values of 6.29 and 6.81μg/ml respectively, and inactive against other cell lines. Compound **5** exhibited only cytotoxicity activity against HEP-G2 with the IC_{50} value of 3.39 μg/ml.

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Appendix A

Media

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

1. Corn Meal Agar (CMA)

Suspened 17.0 g in 1 L distilled H_2O

2. Malt Extract Agar (MEA)

Malt extracts	20	g
Peptone	1	g
Glucose	20	g
Agar	15	g

3. Potato Dextrose Agar (PDA)

Potato (pelled and diced)	200	g
Glucose	20	g
Agar	15	g

Boil the potatoes for one hour in a litre of water. Filter, add the glucose and agar make up the filtrate to 1 L.

4. Sabouraud's Dextrose Agar (SDA)

Peptone	10	g
Glucose	40	g
Agar	15	g

5. Yeast Surcose Extract Agar (YES)

Yeast extracts	20	g
Surcose	15	g
Agar	15	g

6. Malt Czapek Agar (MCzB)

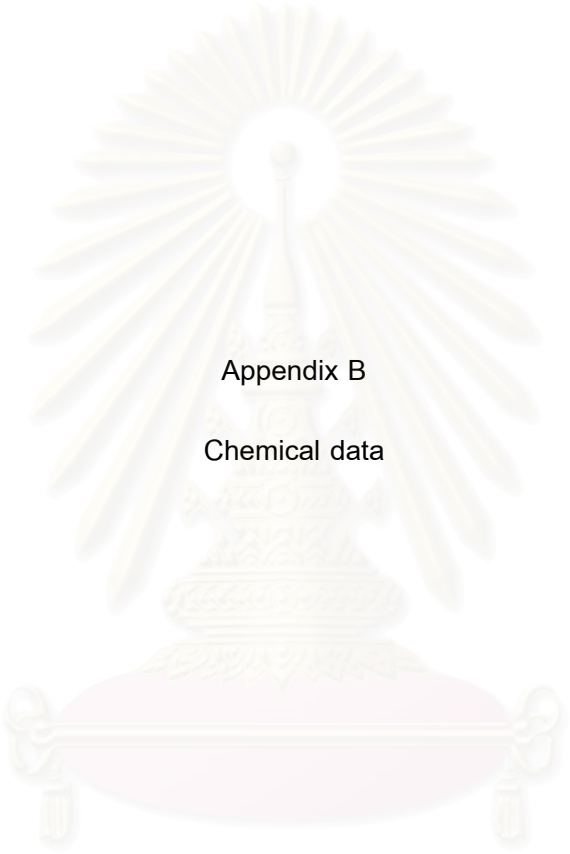
Solution A

$NaNO_3$	40	g
KCl	10	g
$MgSO_4 \bullet 7H_2O$	10	g
$Fe\ SO_4 \bullet 7H_2O$	0.2	g
Distilled H_2O	1	L

Solution B

K_2HPO_4	20	g
Distilled H_2O	1	L

Stock Czapek solution A	50	ml
Stock Czapek solution B	50	ml
Sucrose	30	g
Malt Extract	40	g
Oxoid Agar N^{o} 3	15	g
Distilled H_2O	1	L



Appendix B

Chemical data

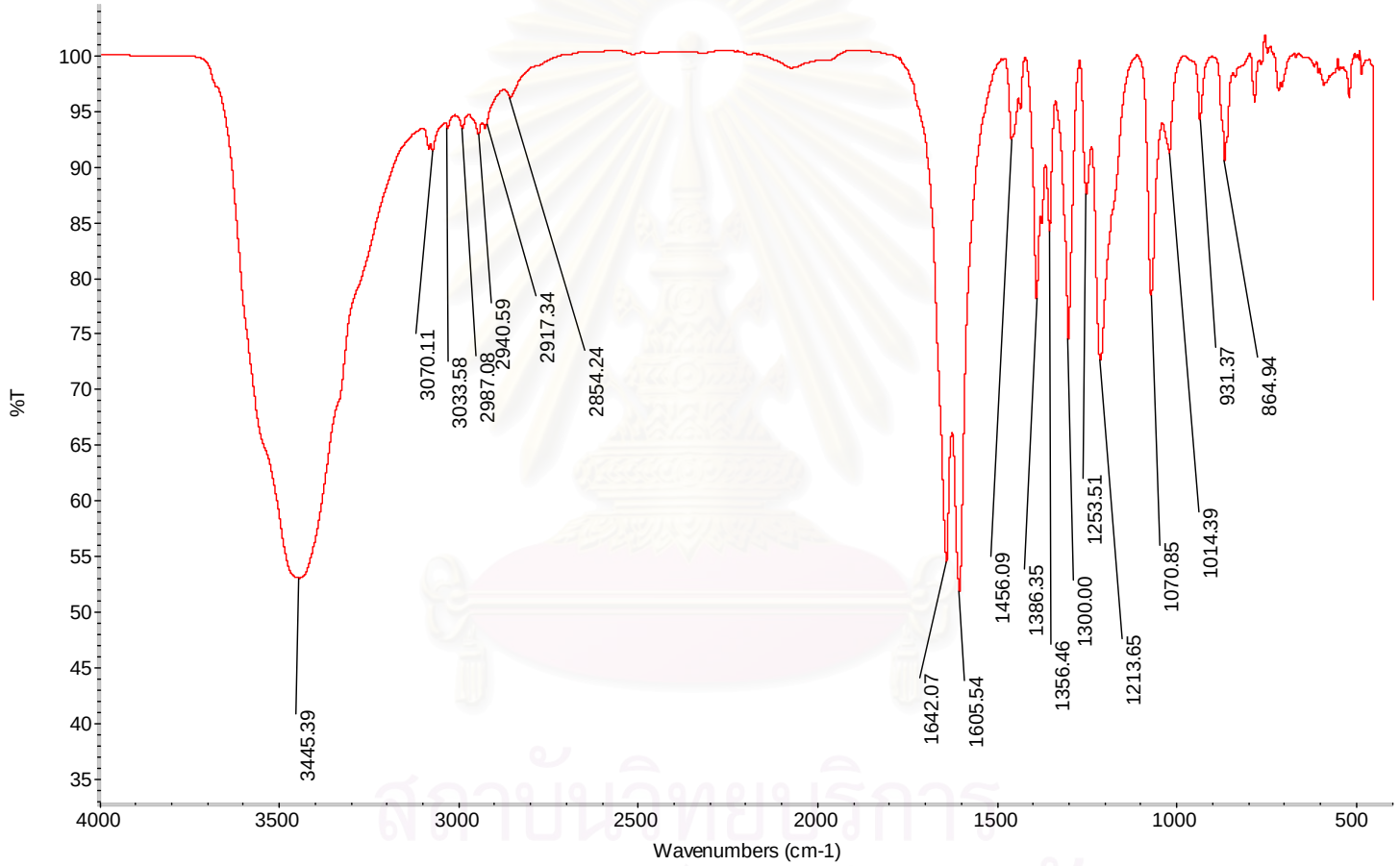


Figure B1-1 The IR spectrum of compound 1

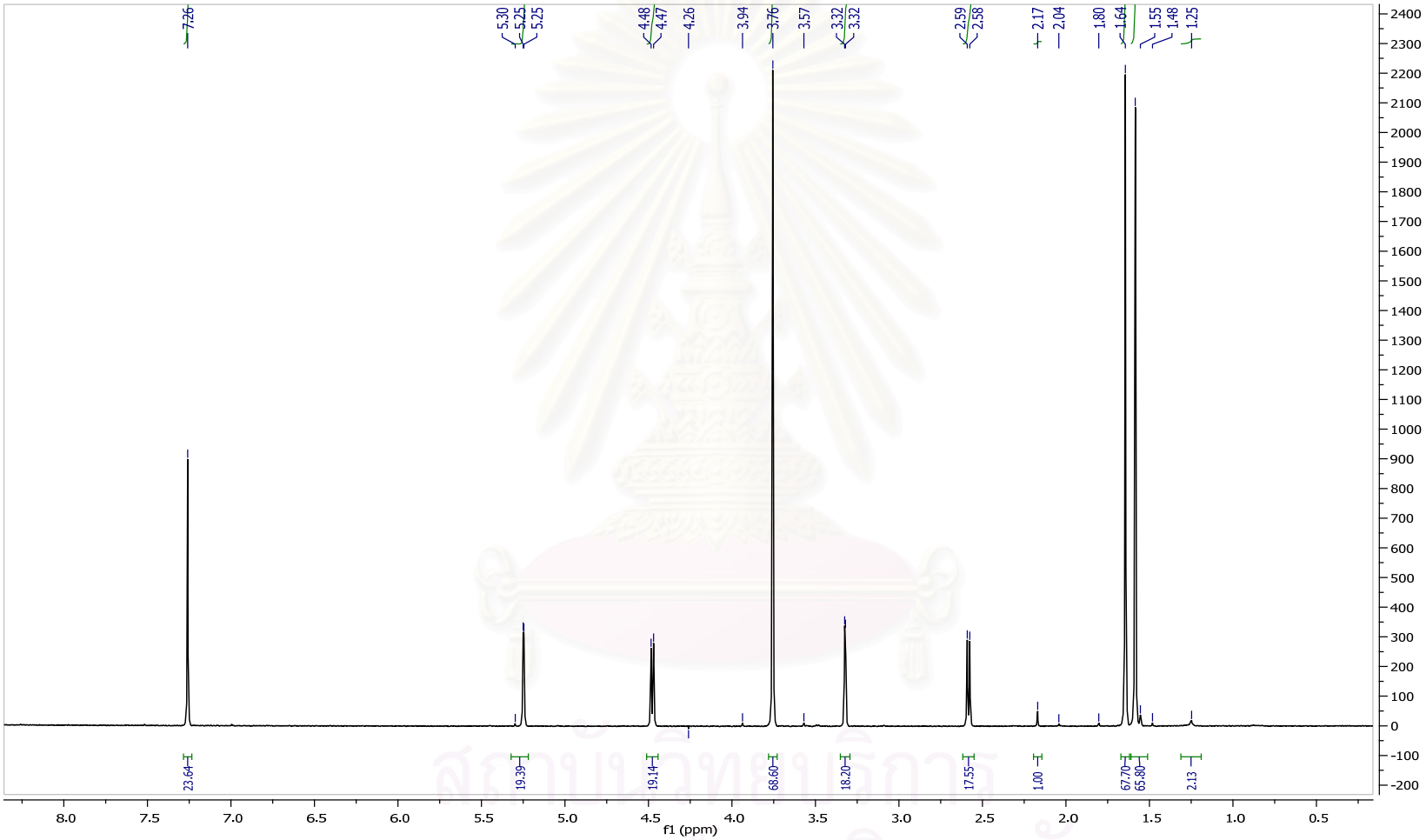


Figure B1-2 ^{1}H NMR spectrum of compound 1

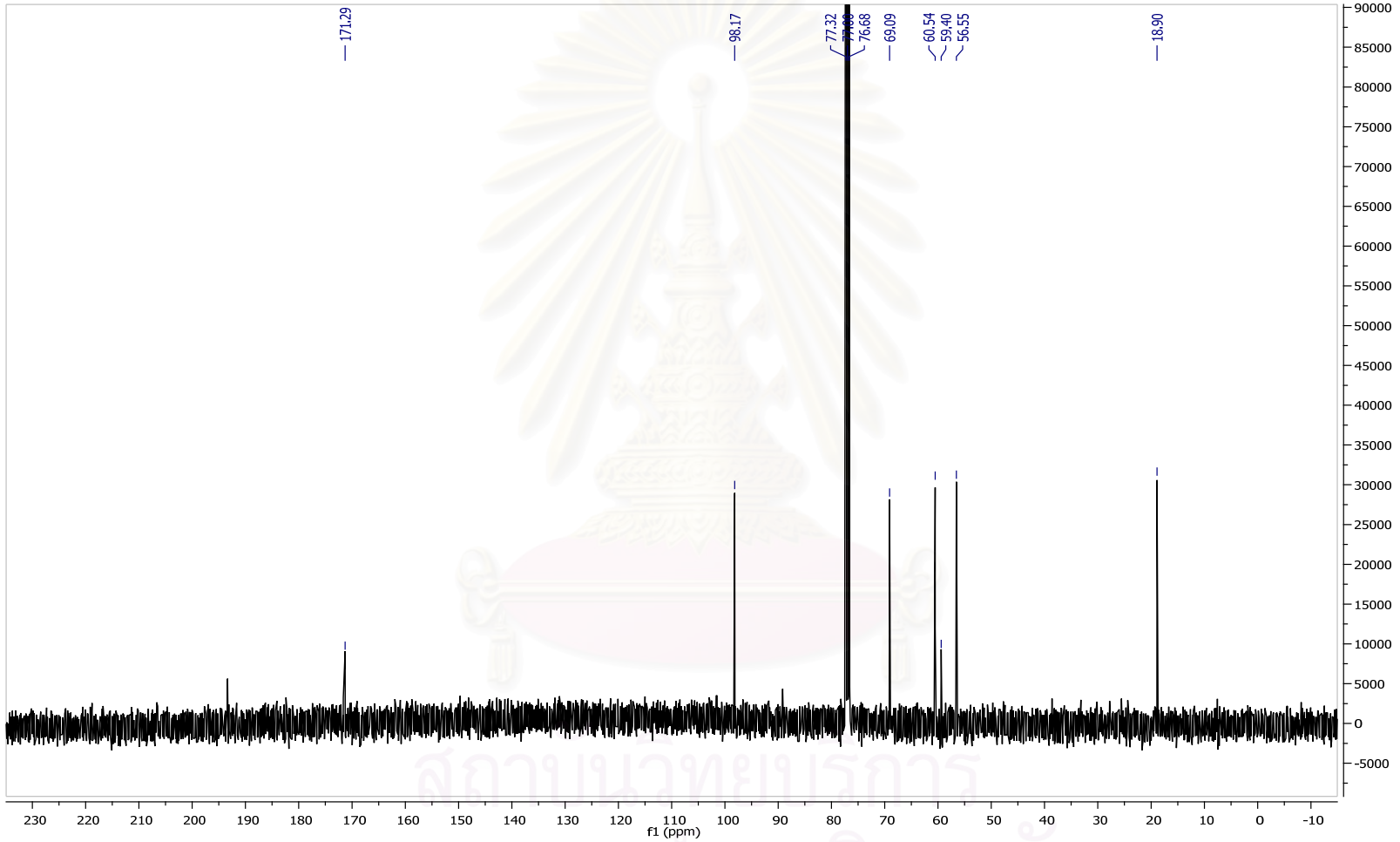


Figure B1-3 ^{13}C NMR spectrum of compound 1

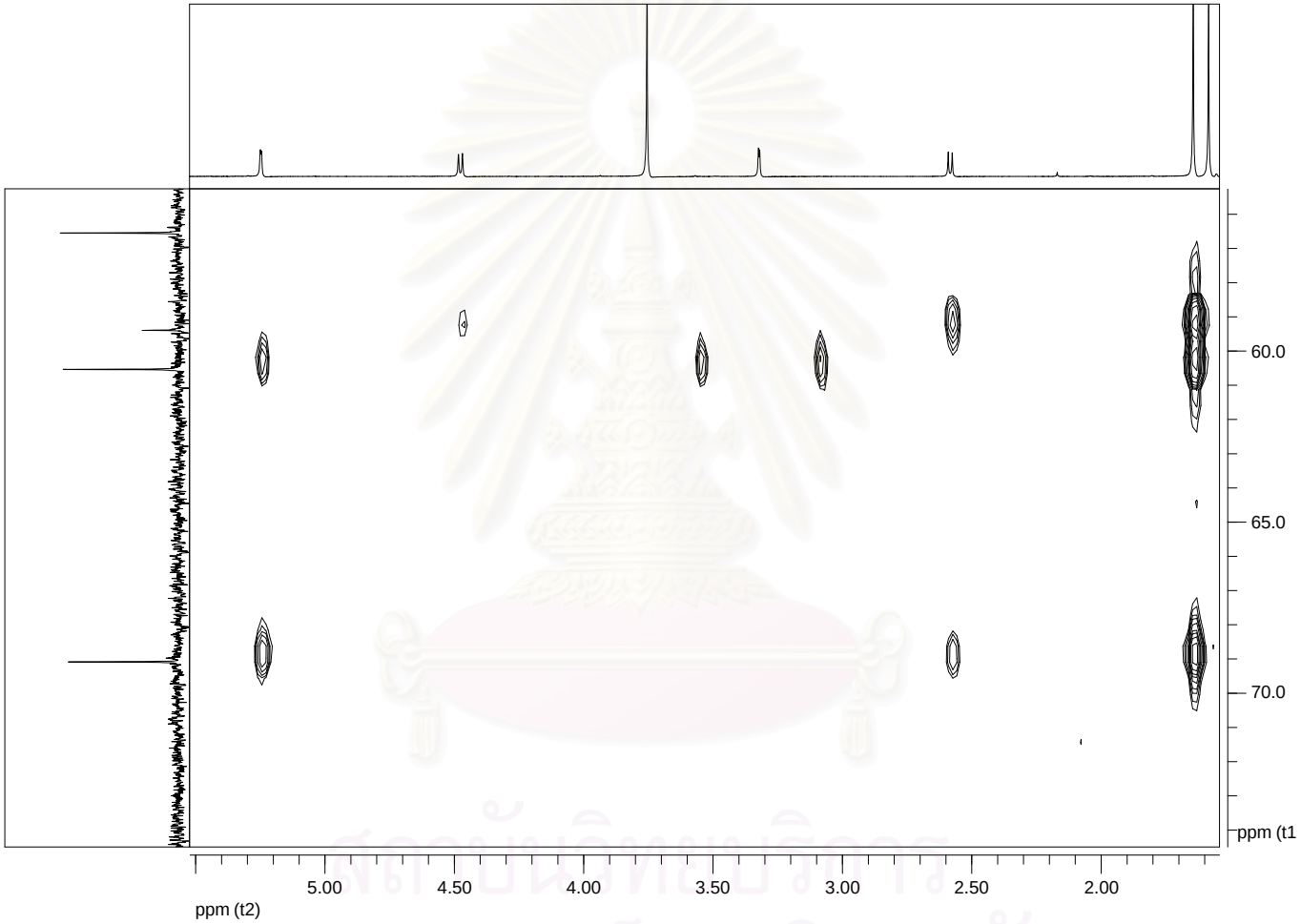


Figure B1-4 HMBC spectrum of compound 1

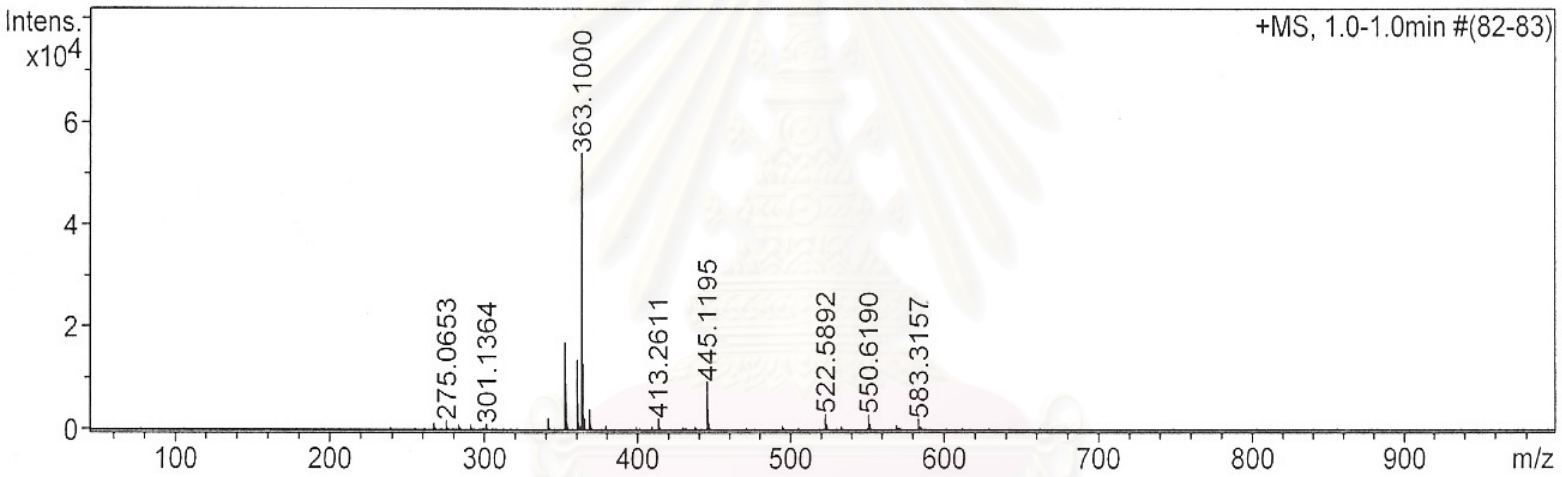


Figure B1-5 The profile mass spectrum of compound 1

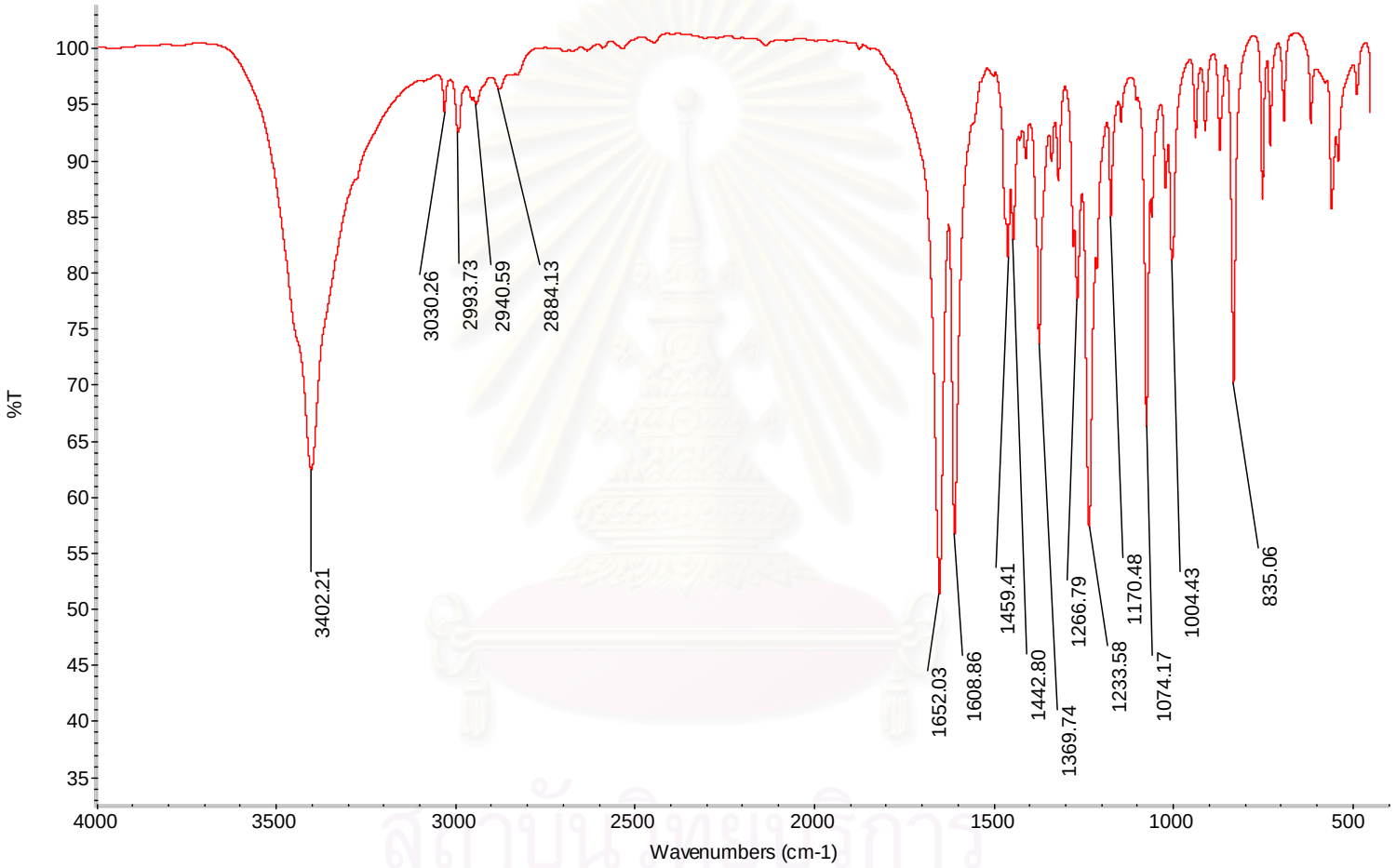


Figure B2-1 The IR spectrum of compound 2

101

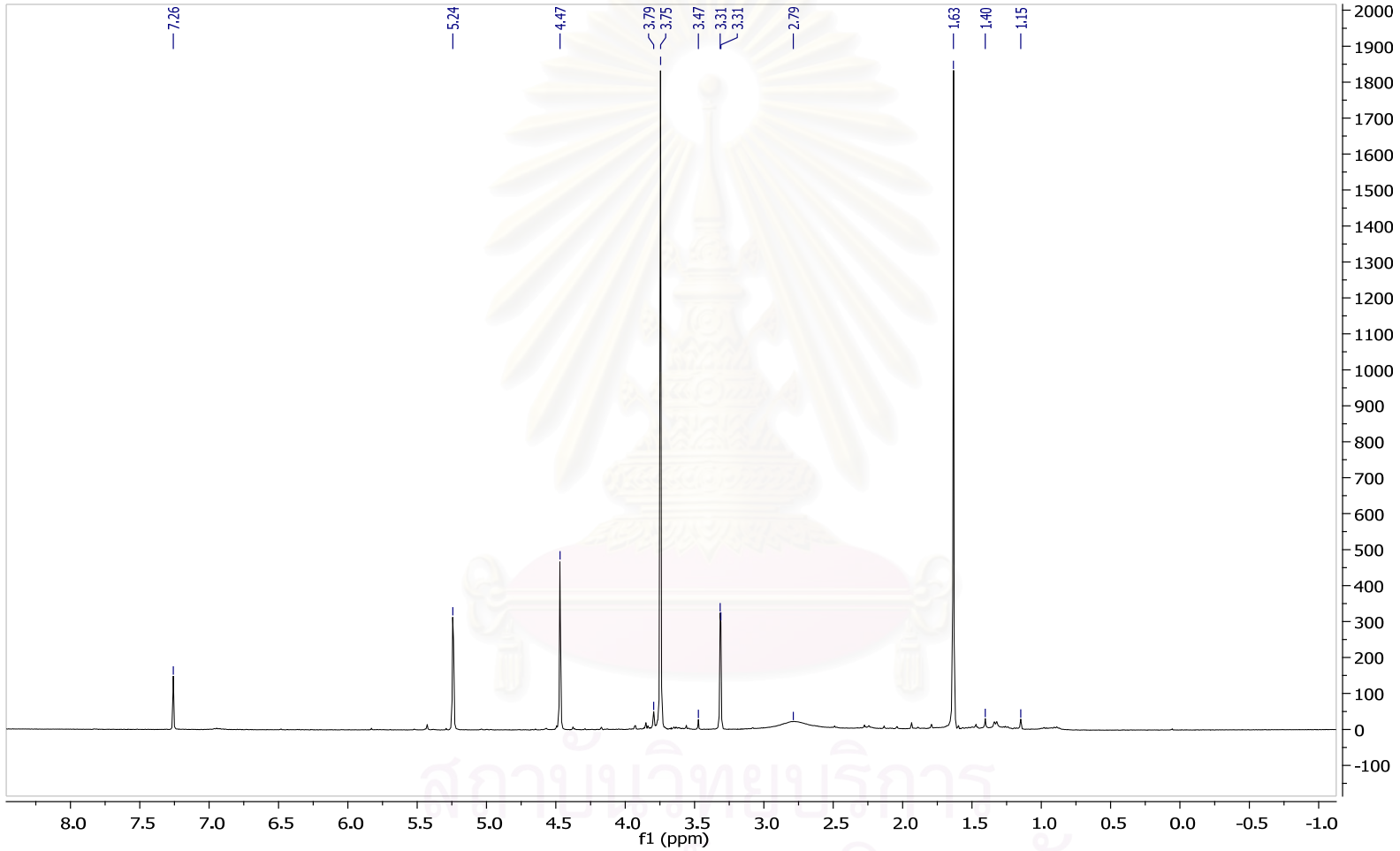


Figure B2-2 ^{1}H NMR spectrum of compound 2

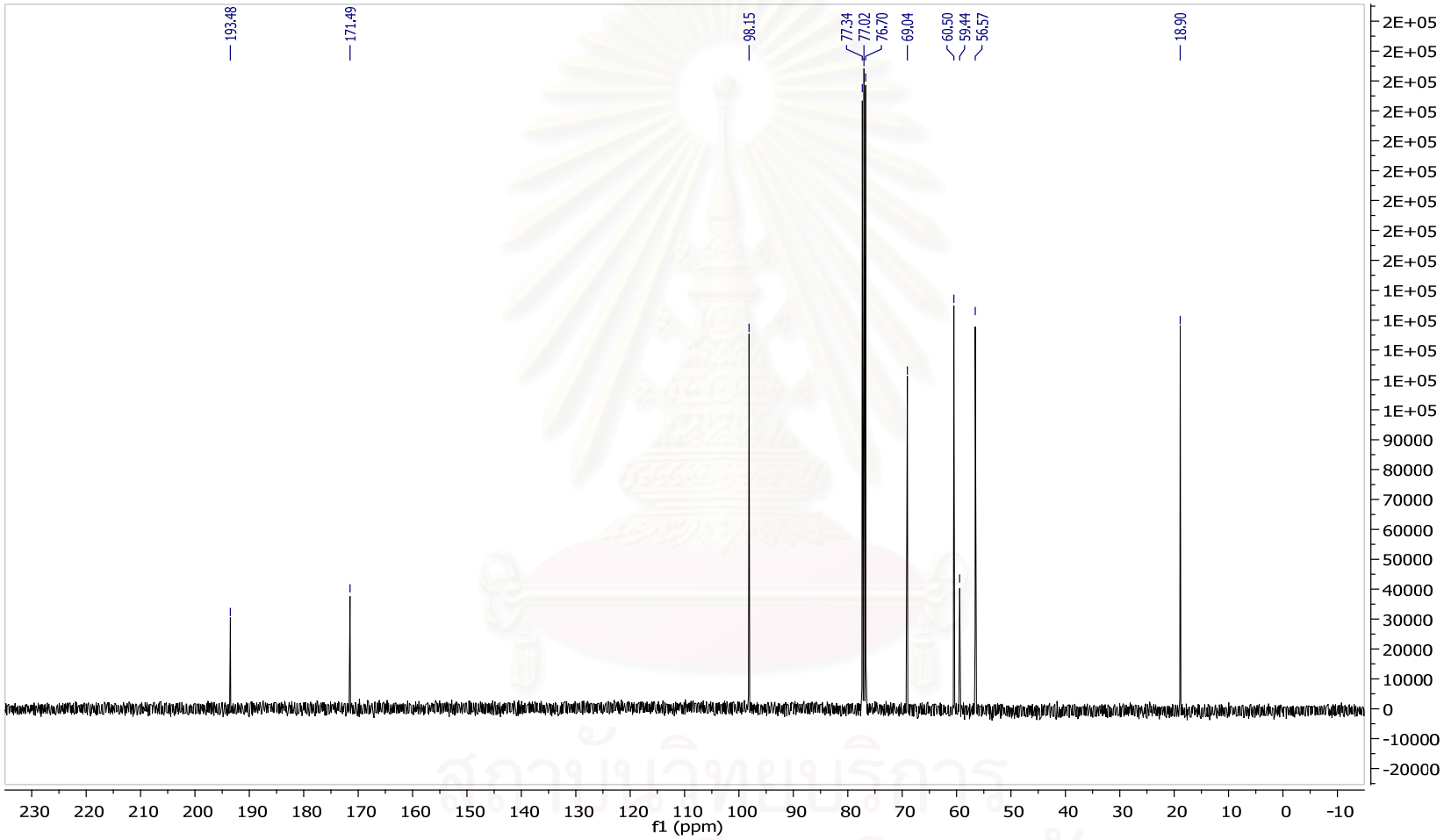


Figure B2-3 ^{13}C NMR spectrum of compound 2

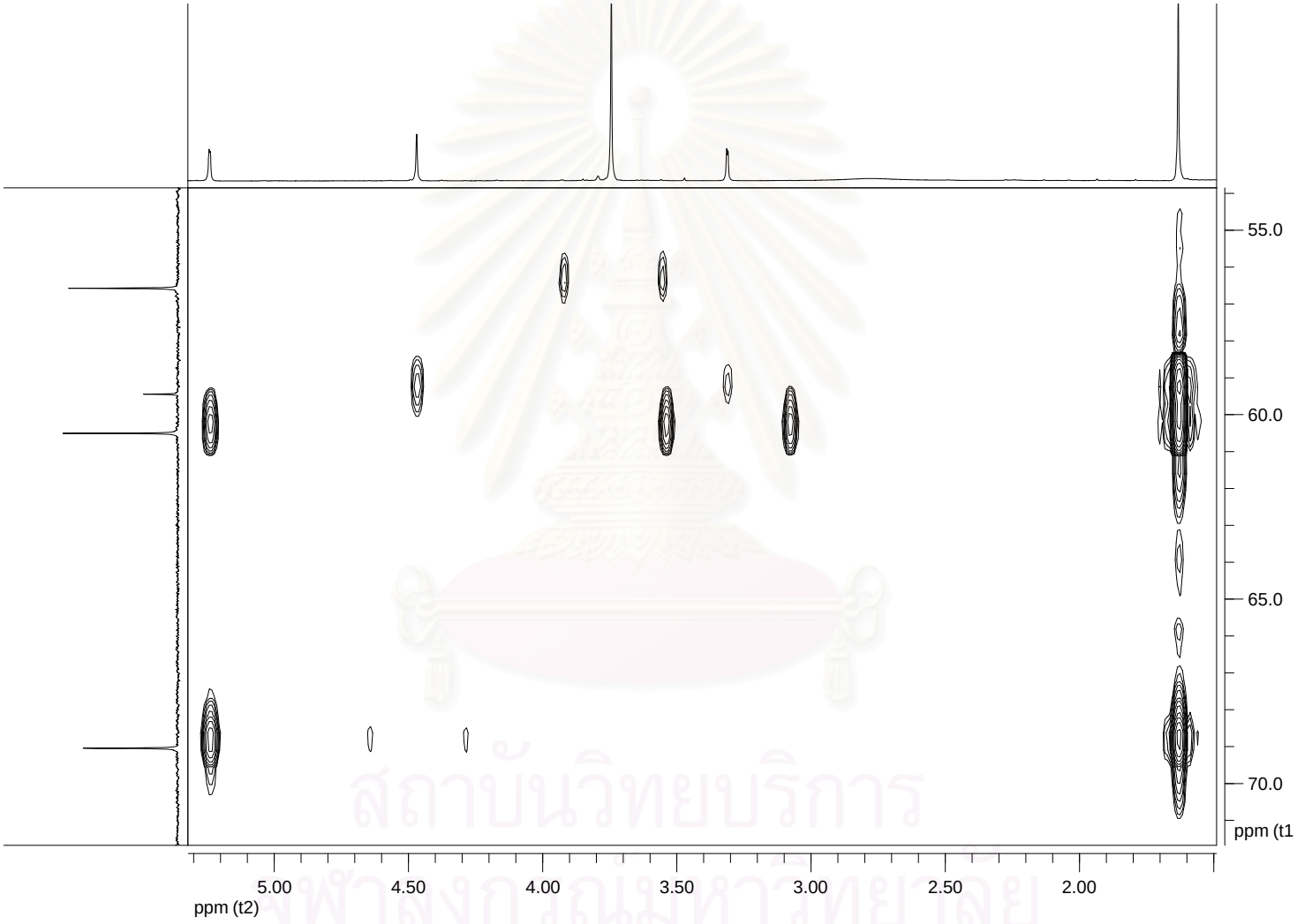


Figure B2-4 HMBC spectrum of compound 2

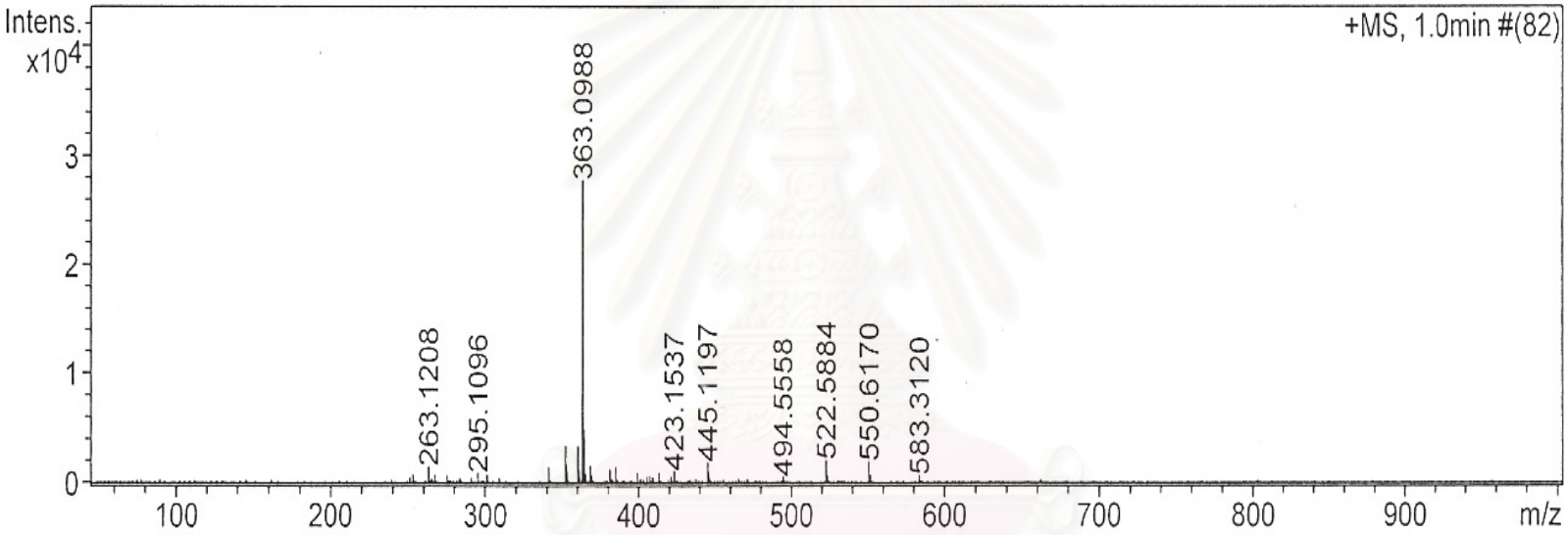


Figure B2-5 The profile mass spectrum of compound 2

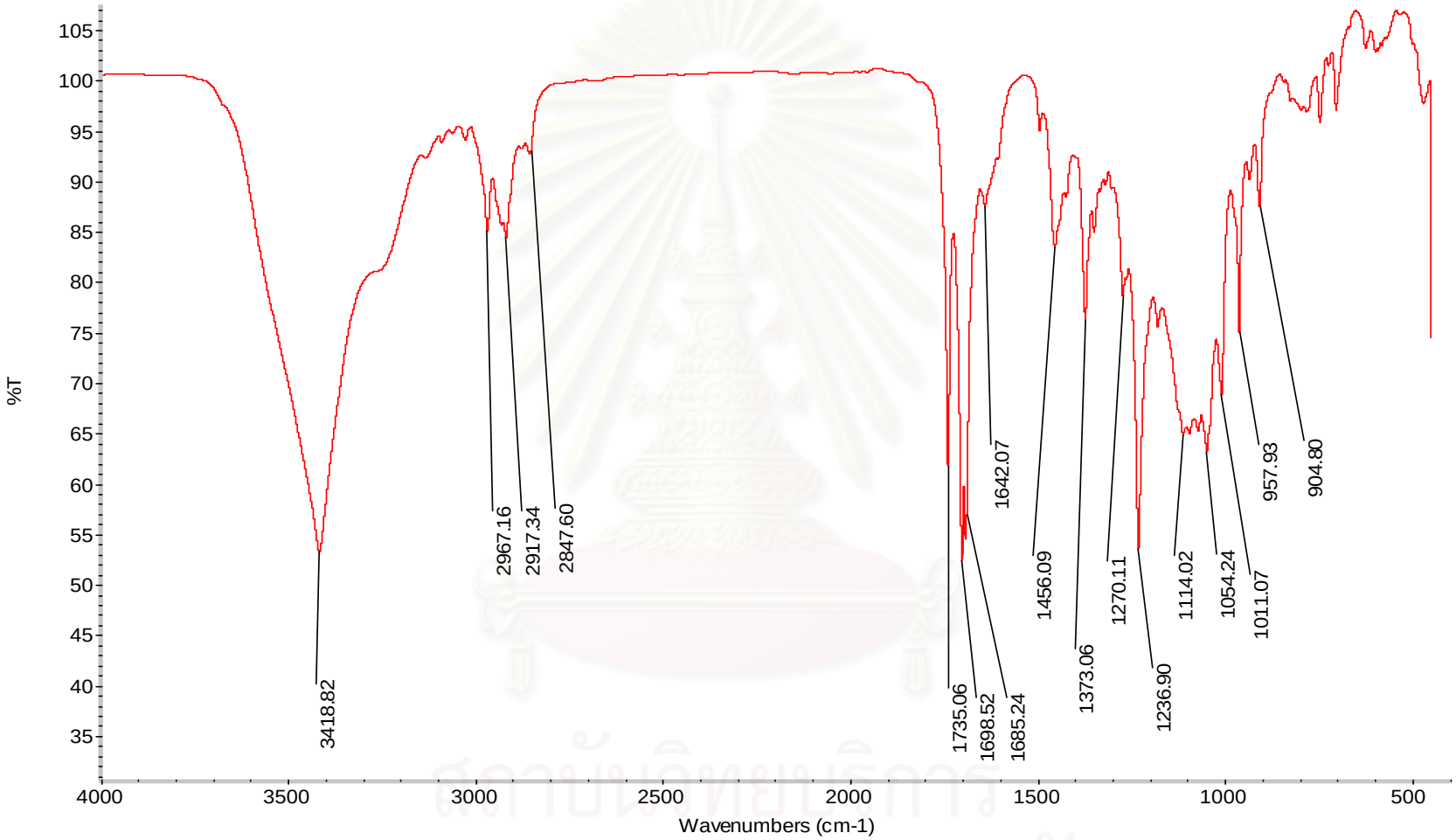


Figure B3-1 The IR spectrum of compound 3

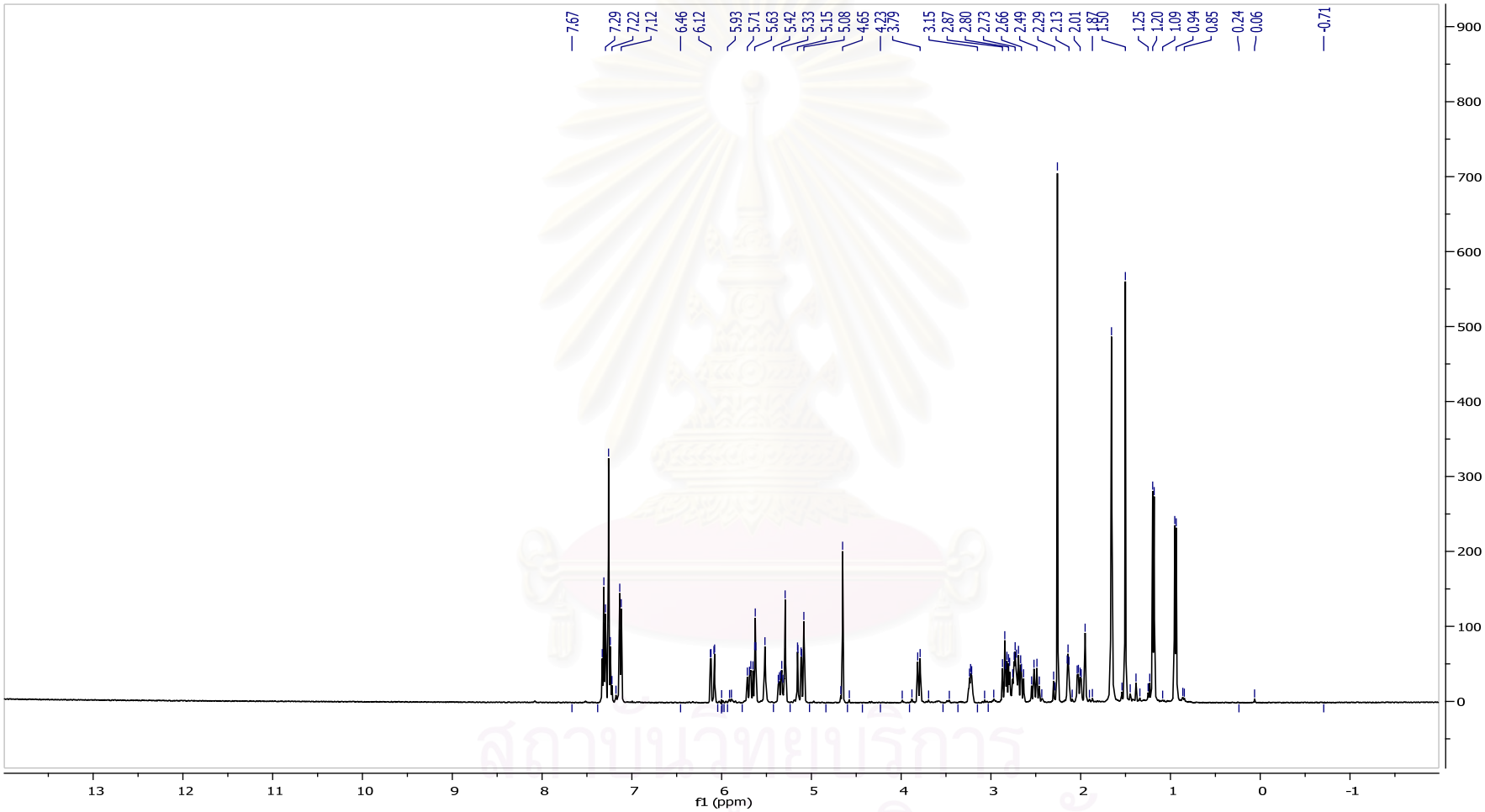


Figure B3-2 ^{1}H NMR spectrum of compound 3

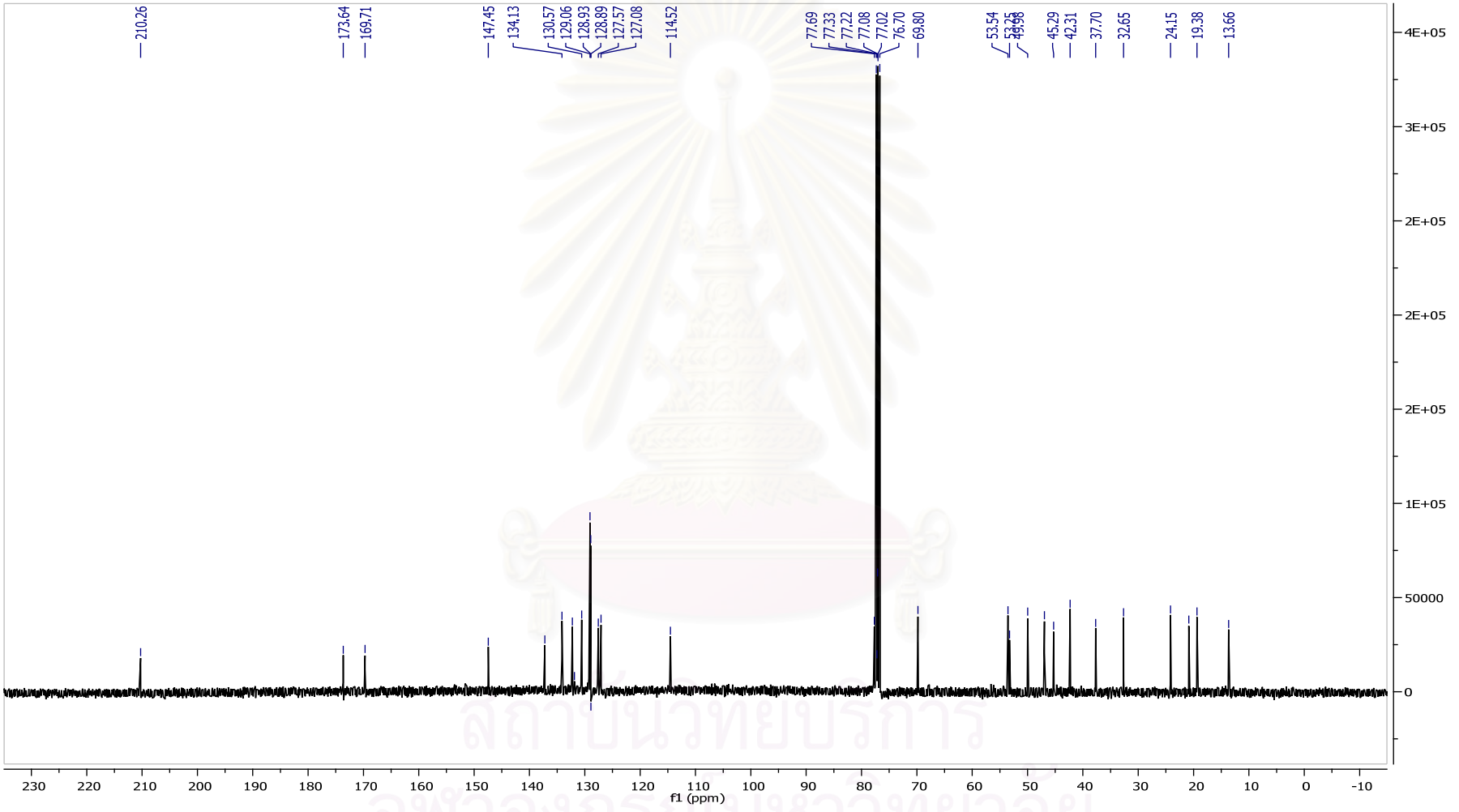


Figure B3-3 ^{13}C NMR spectrum of compound 3

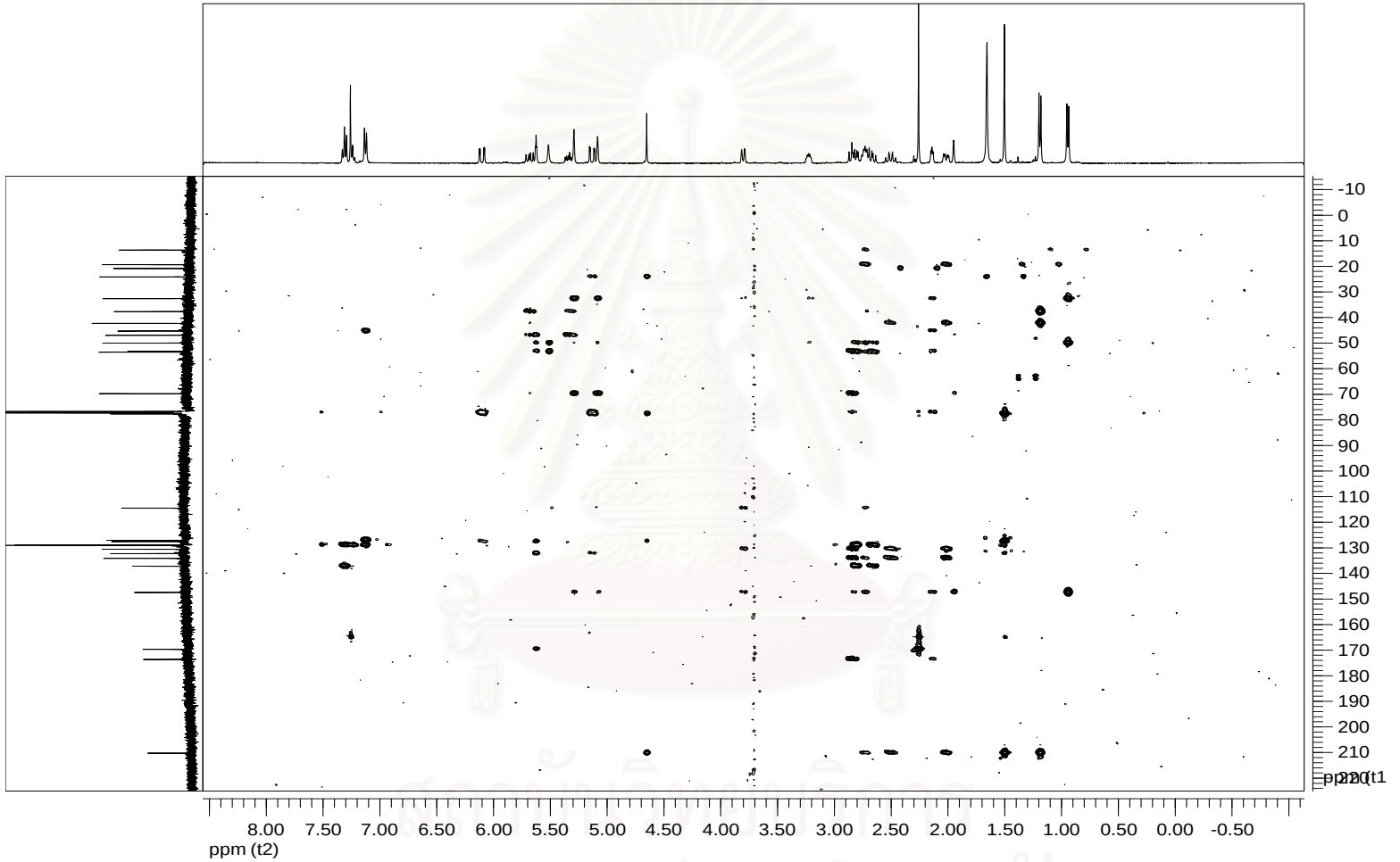


Figure B3-4 HMBC spectrum of compound 3

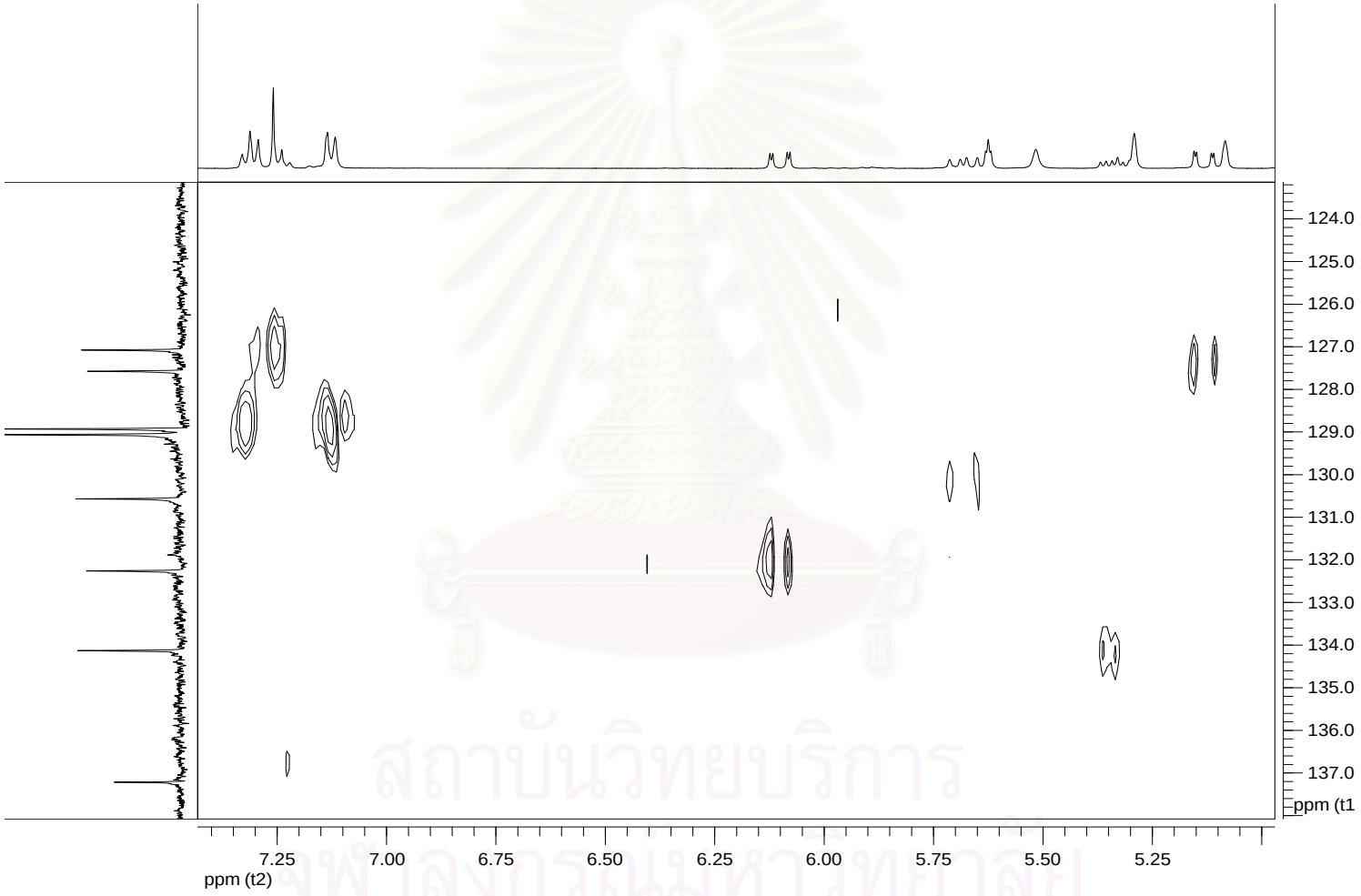


Figure D3-5 HSQC spectrum of compound 3

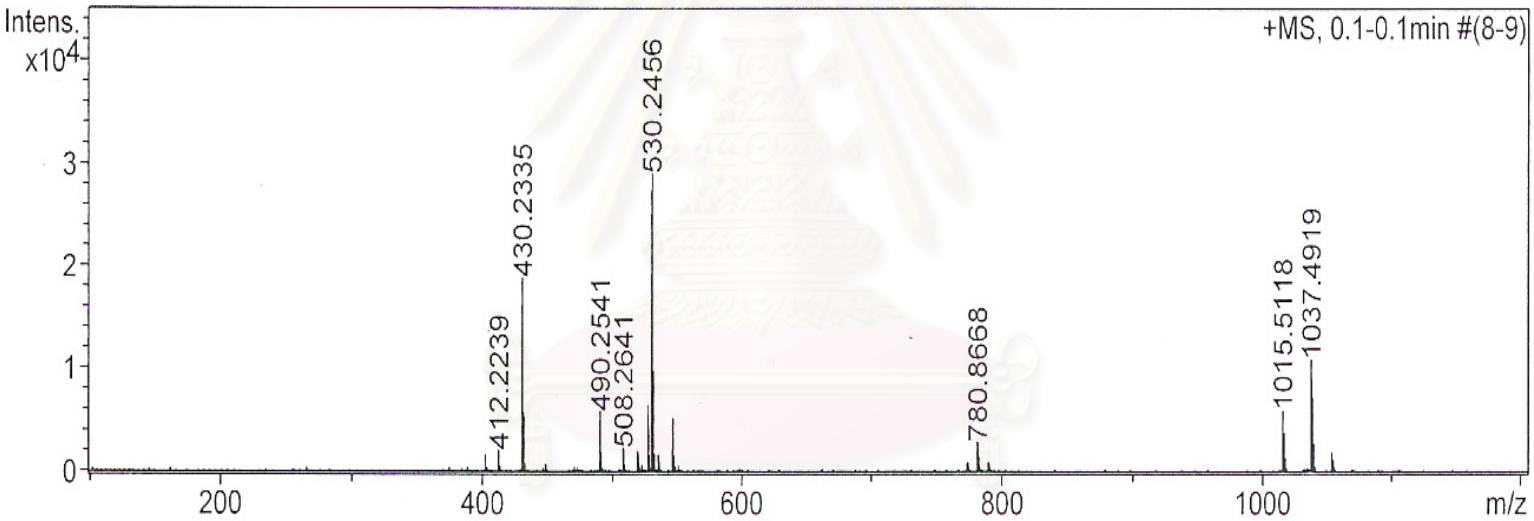


Figure B3-6 The profile mass spectrum of compound 3

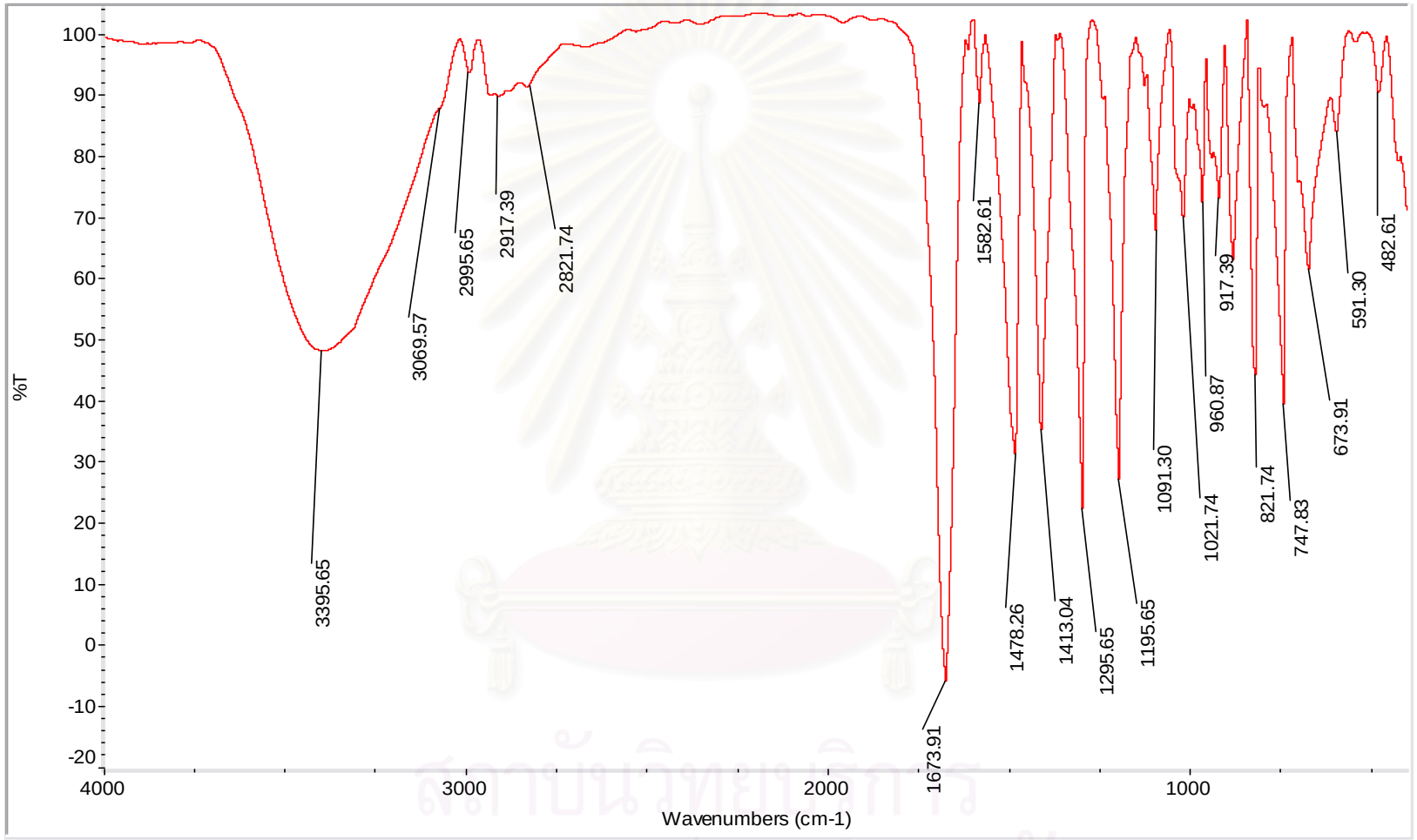


Figure B4-1 The IR spectrum of compound 5

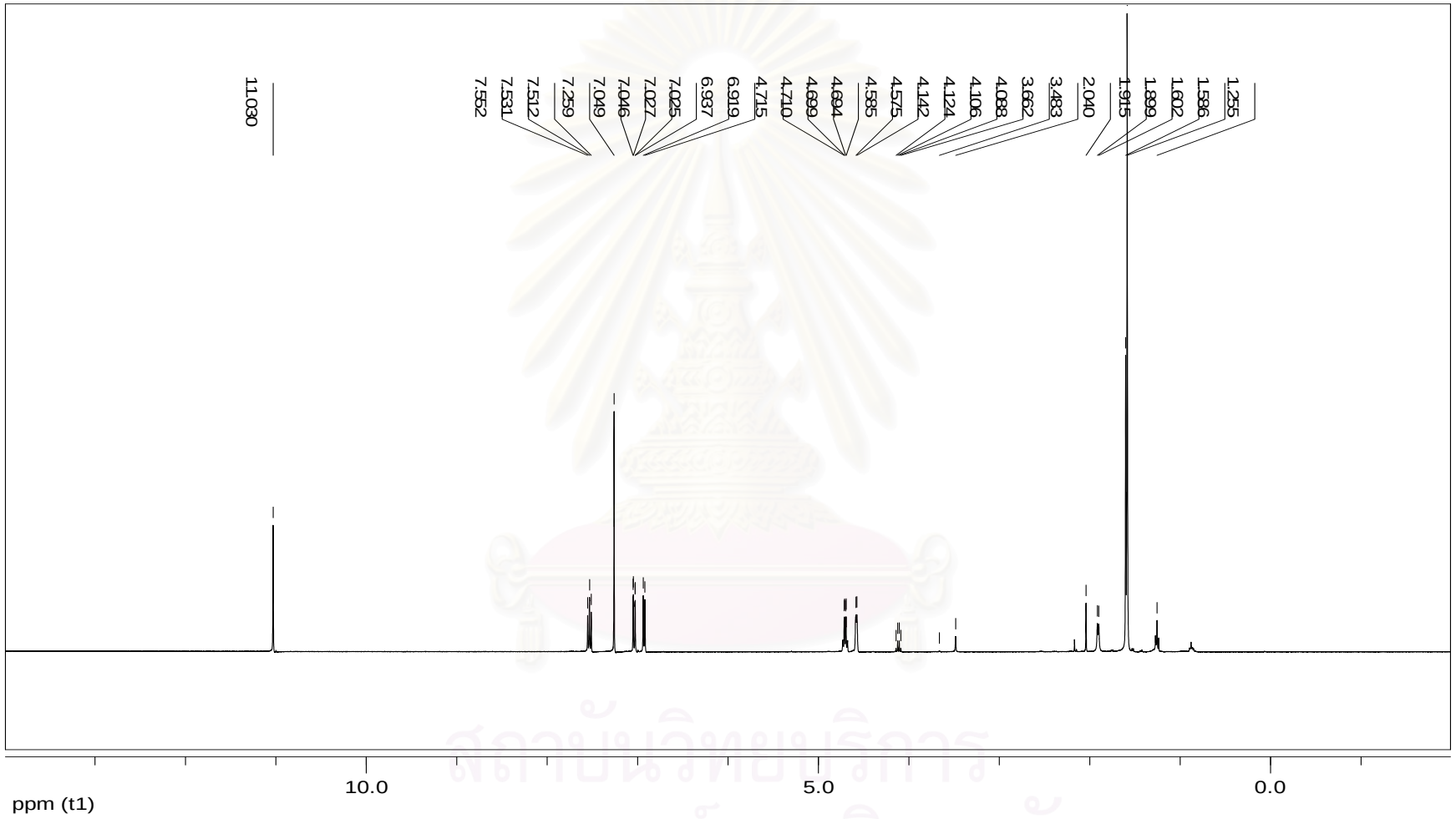


Figure B4-2 ^{1}H NMR spectrum of compound 4

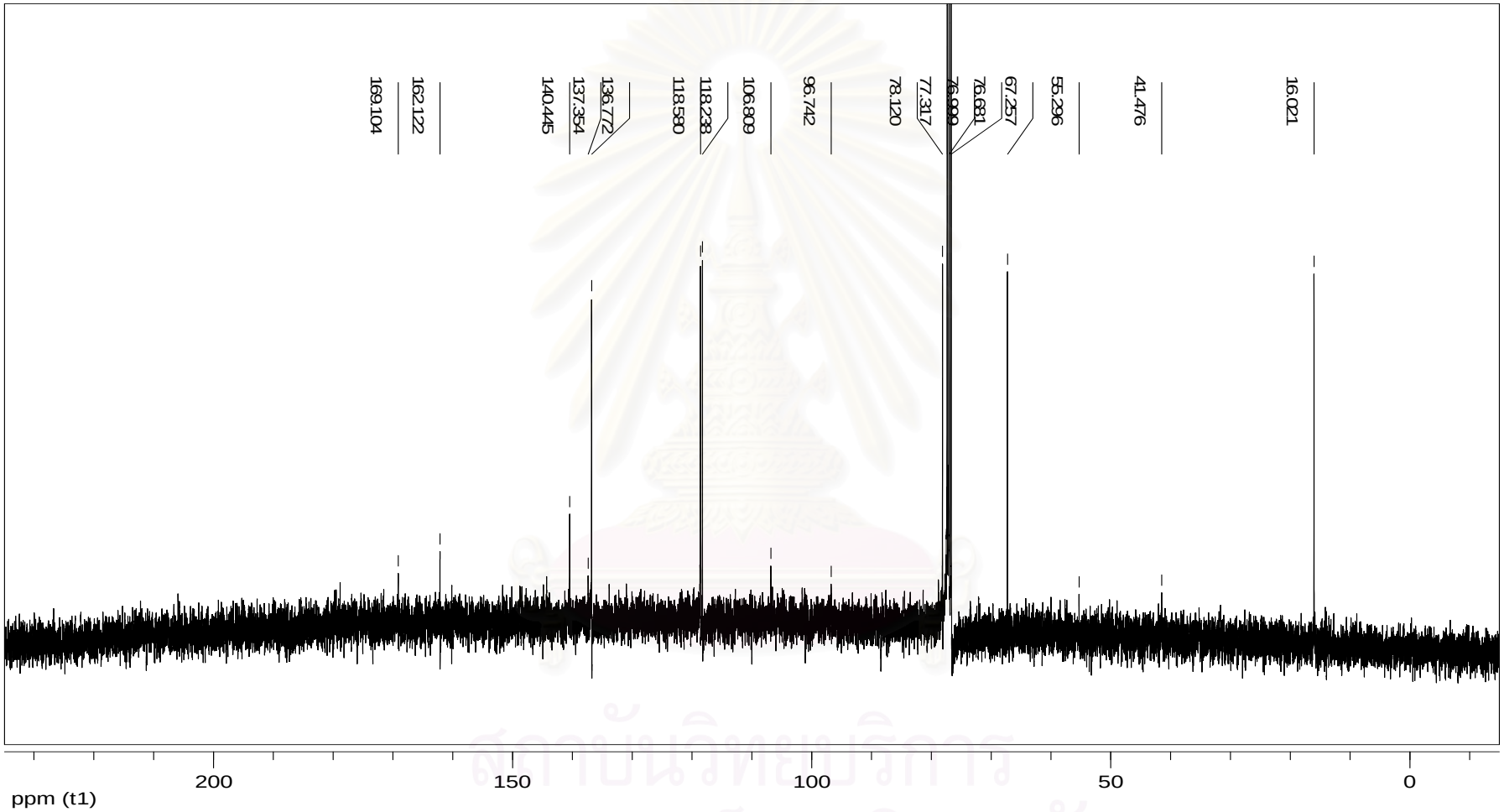


Figure B4-3 ^{13}C NMR spectrum of compound 4

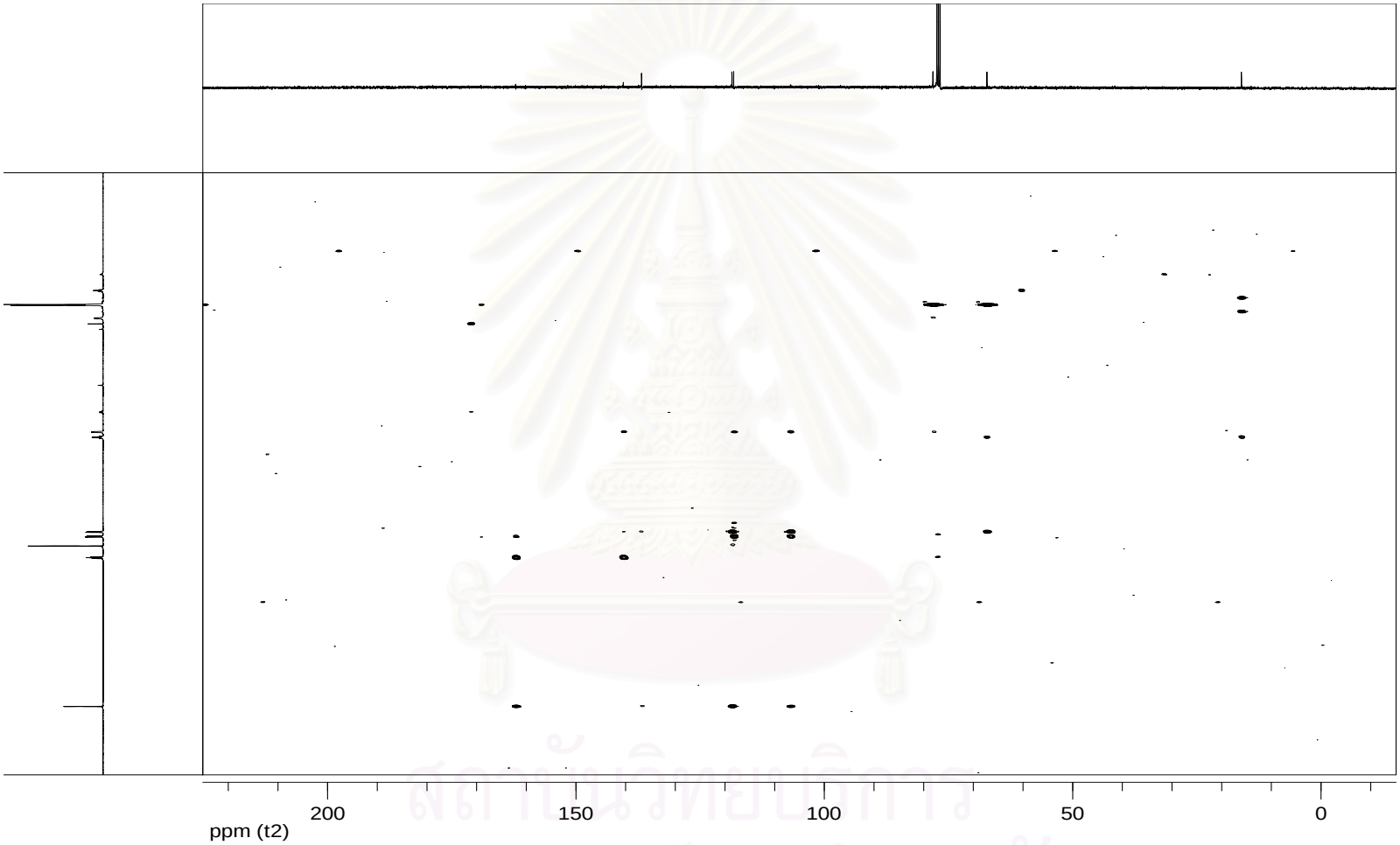


Figure B4-4 HMBC spectrum of compound 4

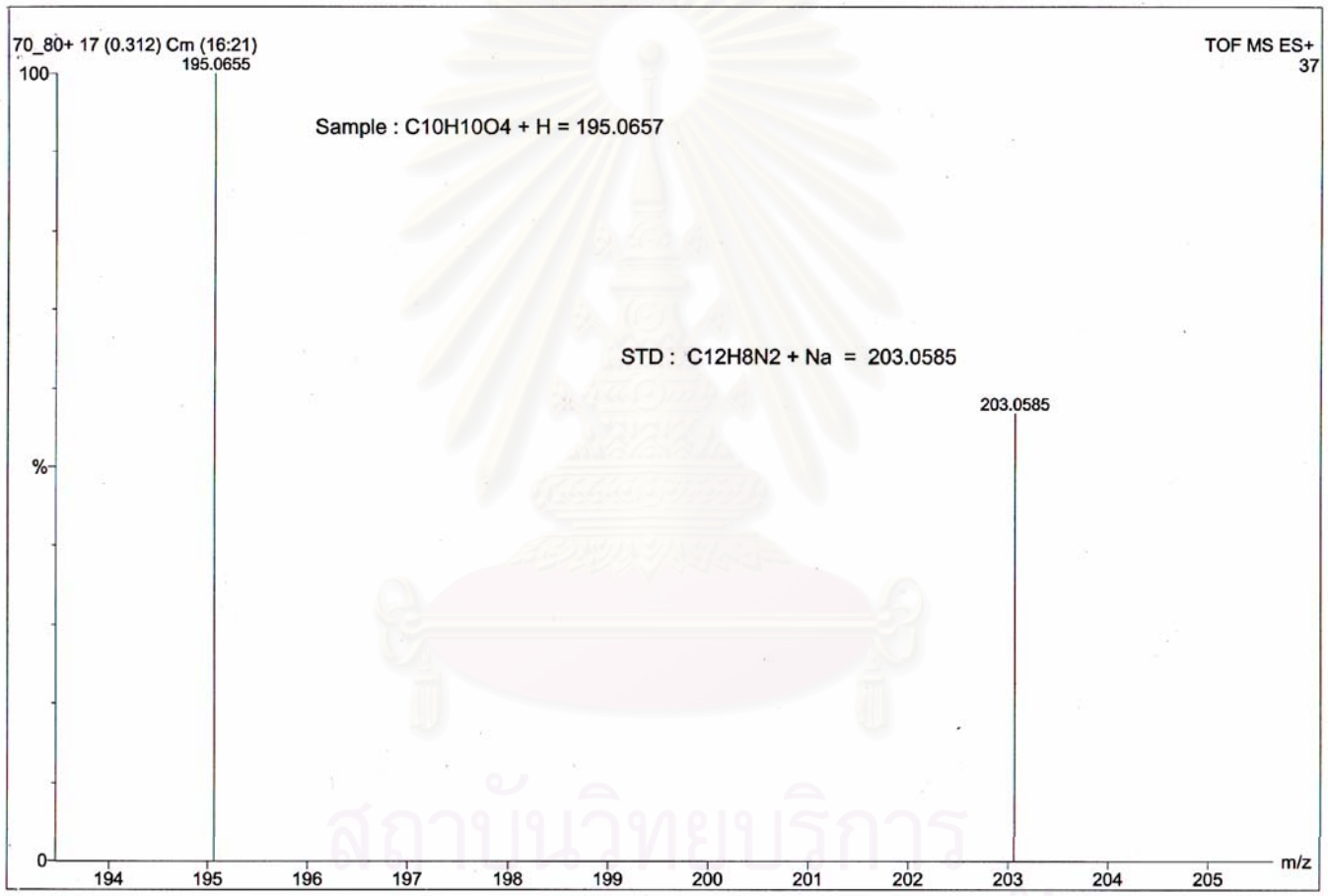


Figure B4-5 The profile mass spectrum of compound 4

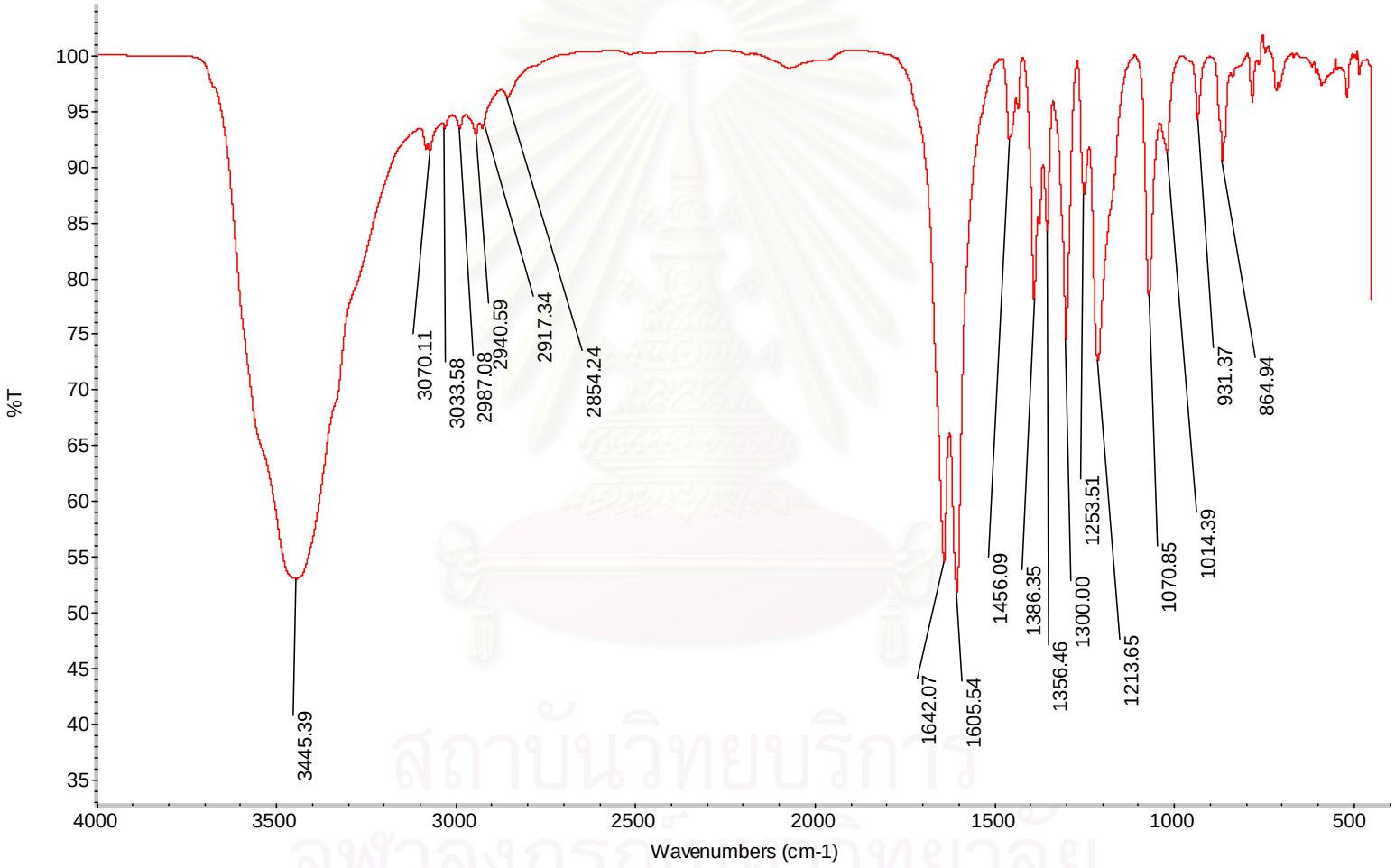


Figure B5-1 The IR spectrum of compound 5

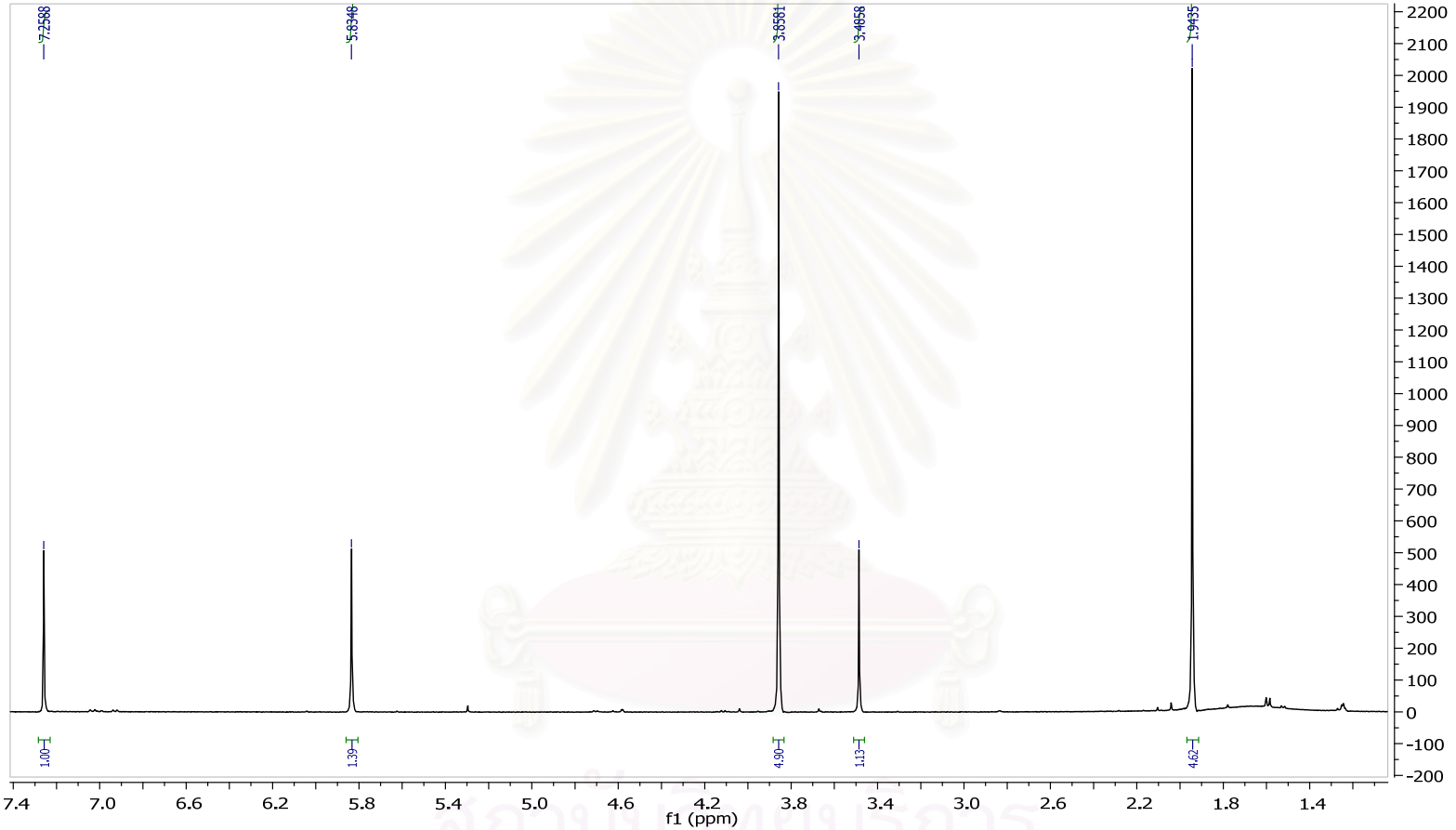


Figure B5-2 ^{1}H NMR spectrum of compound 5

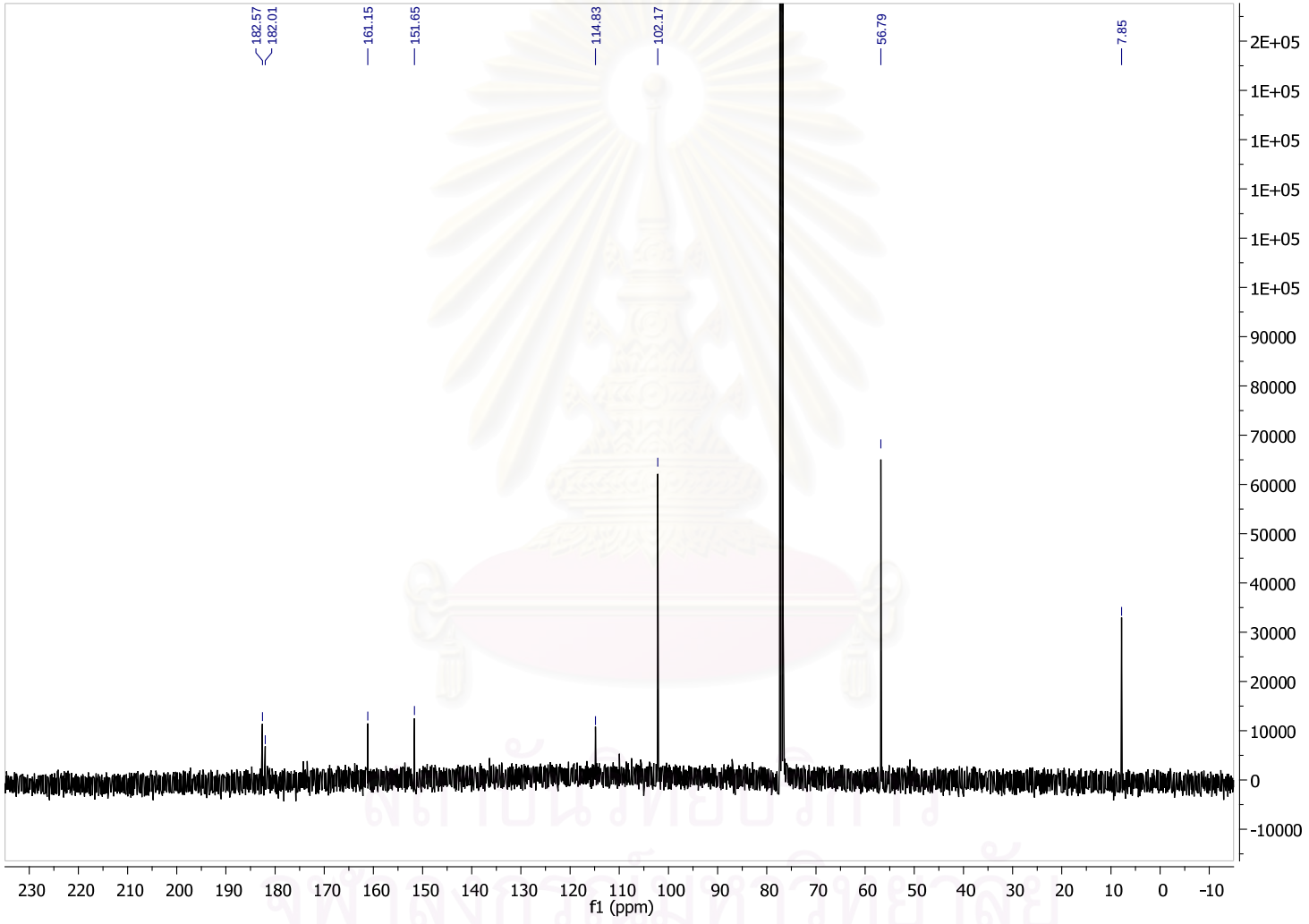


Figure B5-3 ^{13}C NMR spectrum of compound 5

119

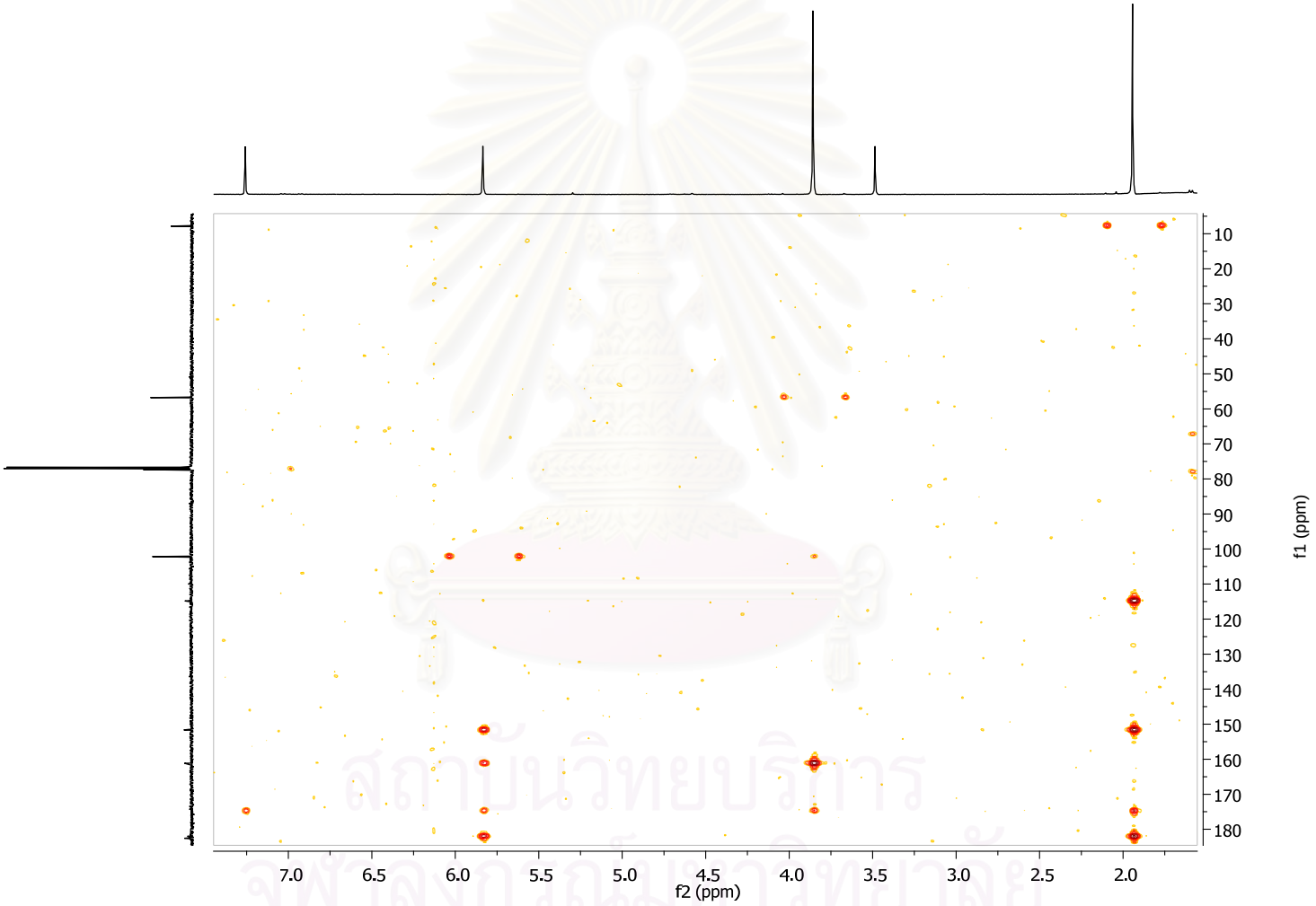


Figure B5-4 HMBC spectrum of compound 5

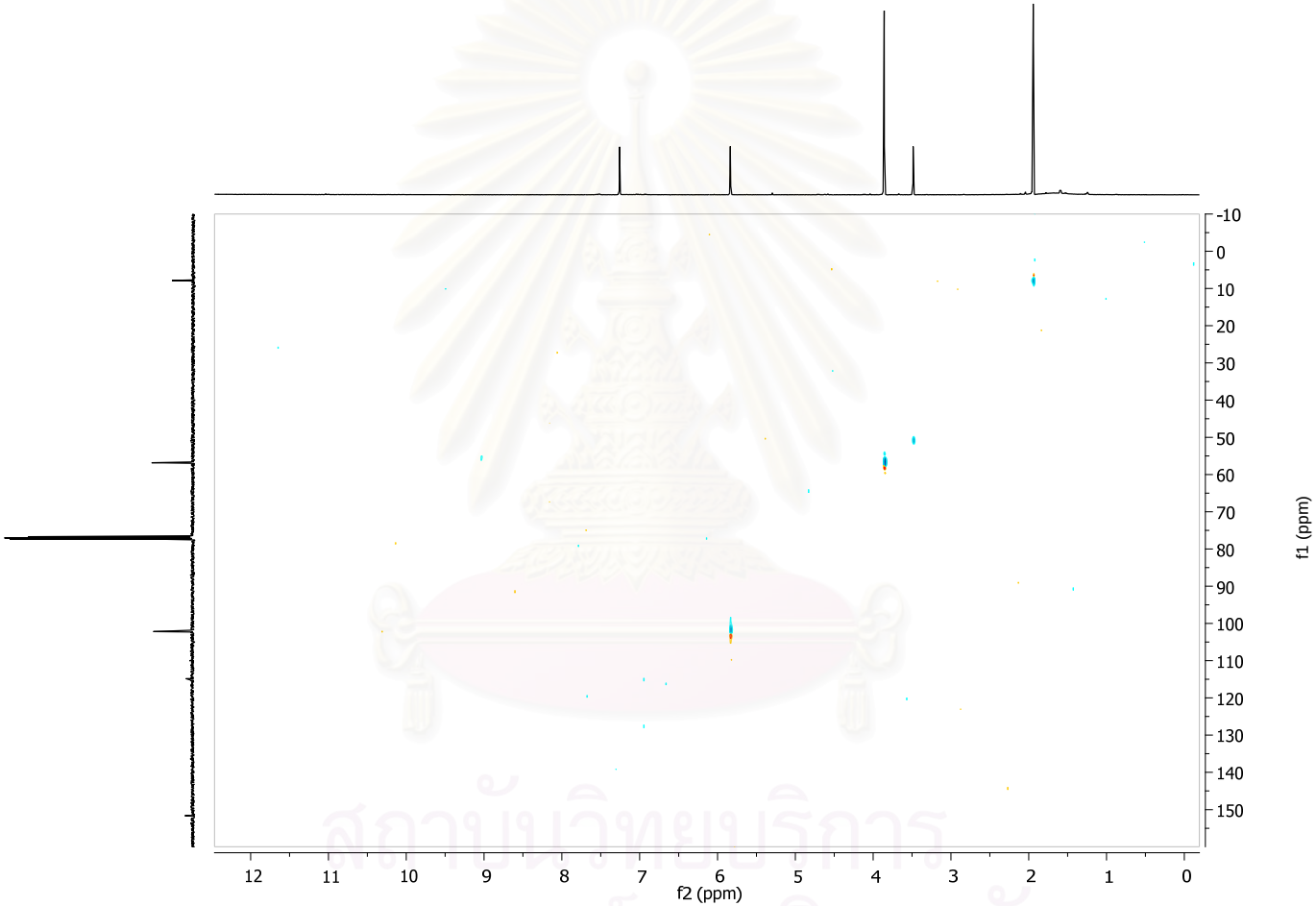


Figure B5-5 HSQC spectrum of compound 5

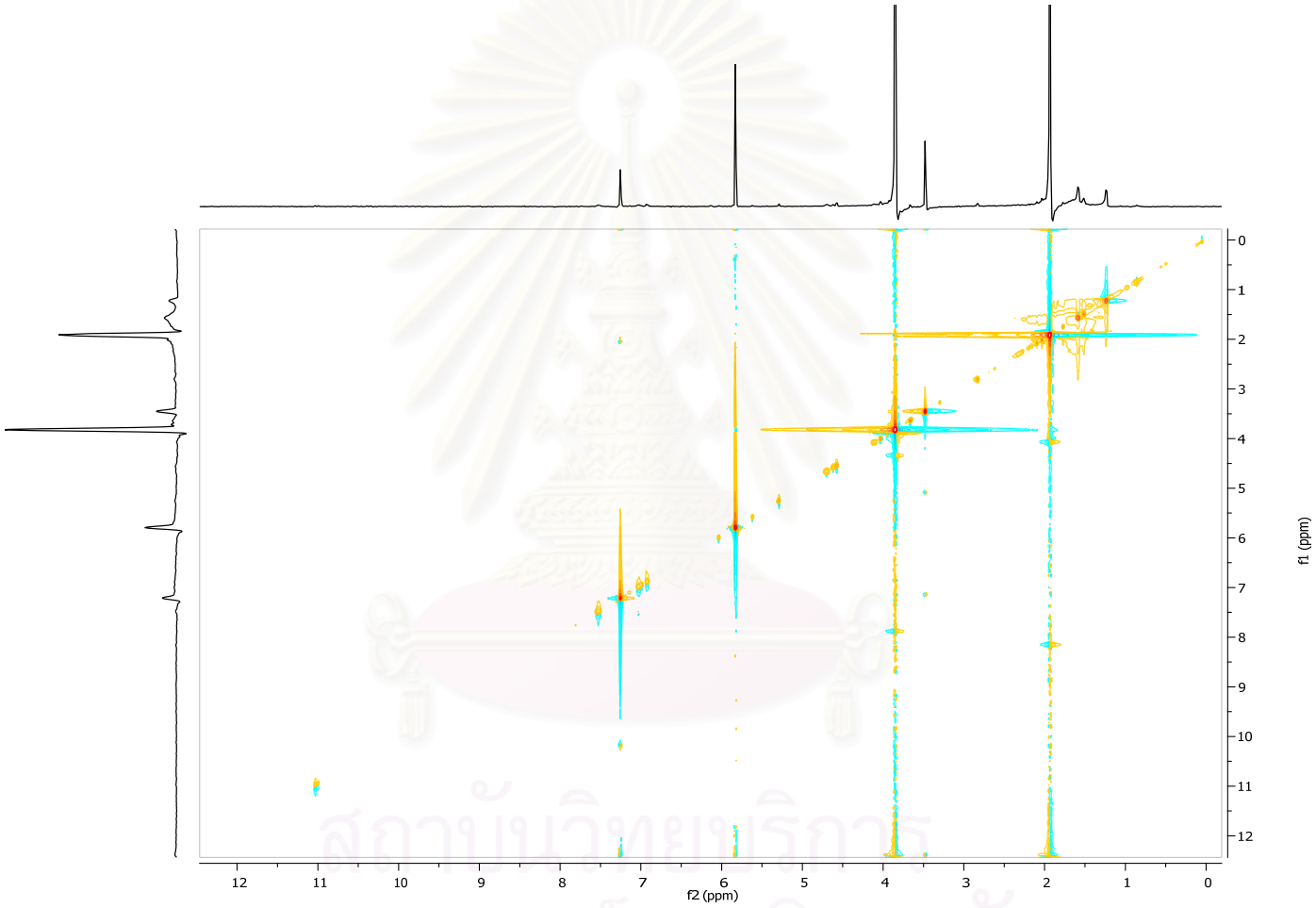


Figure B5-6 COSY spectrum of compound 5

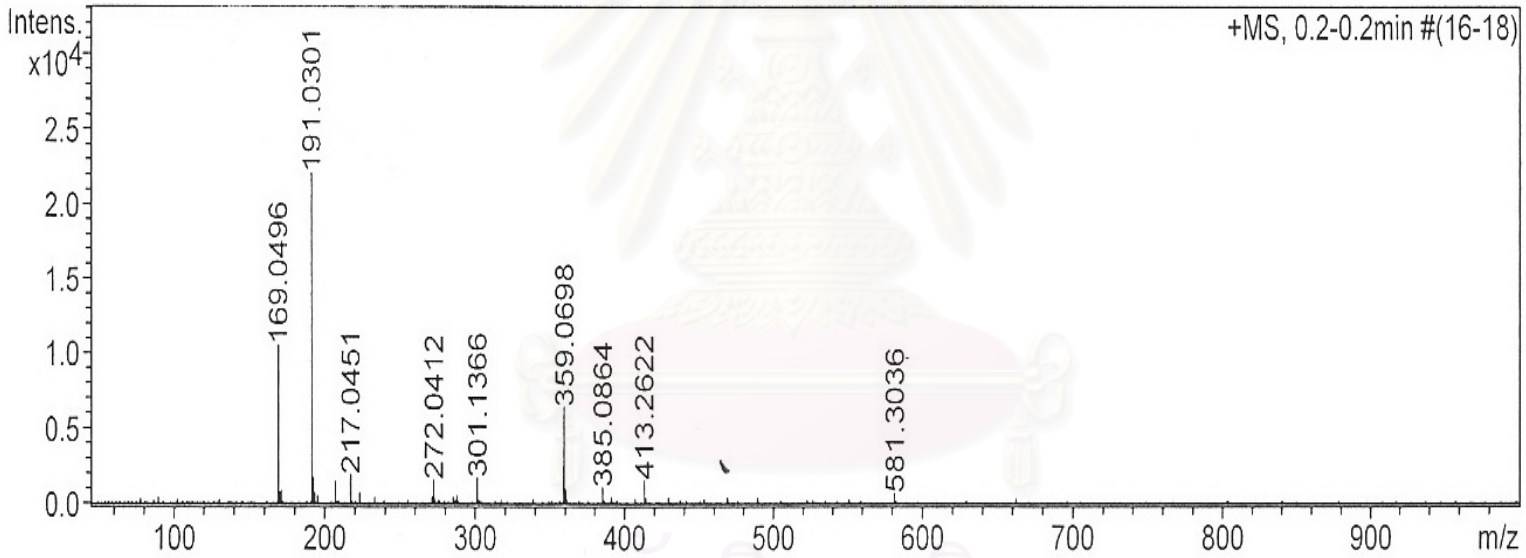


Figure B5-7 The profile mass spectrum of compound 5

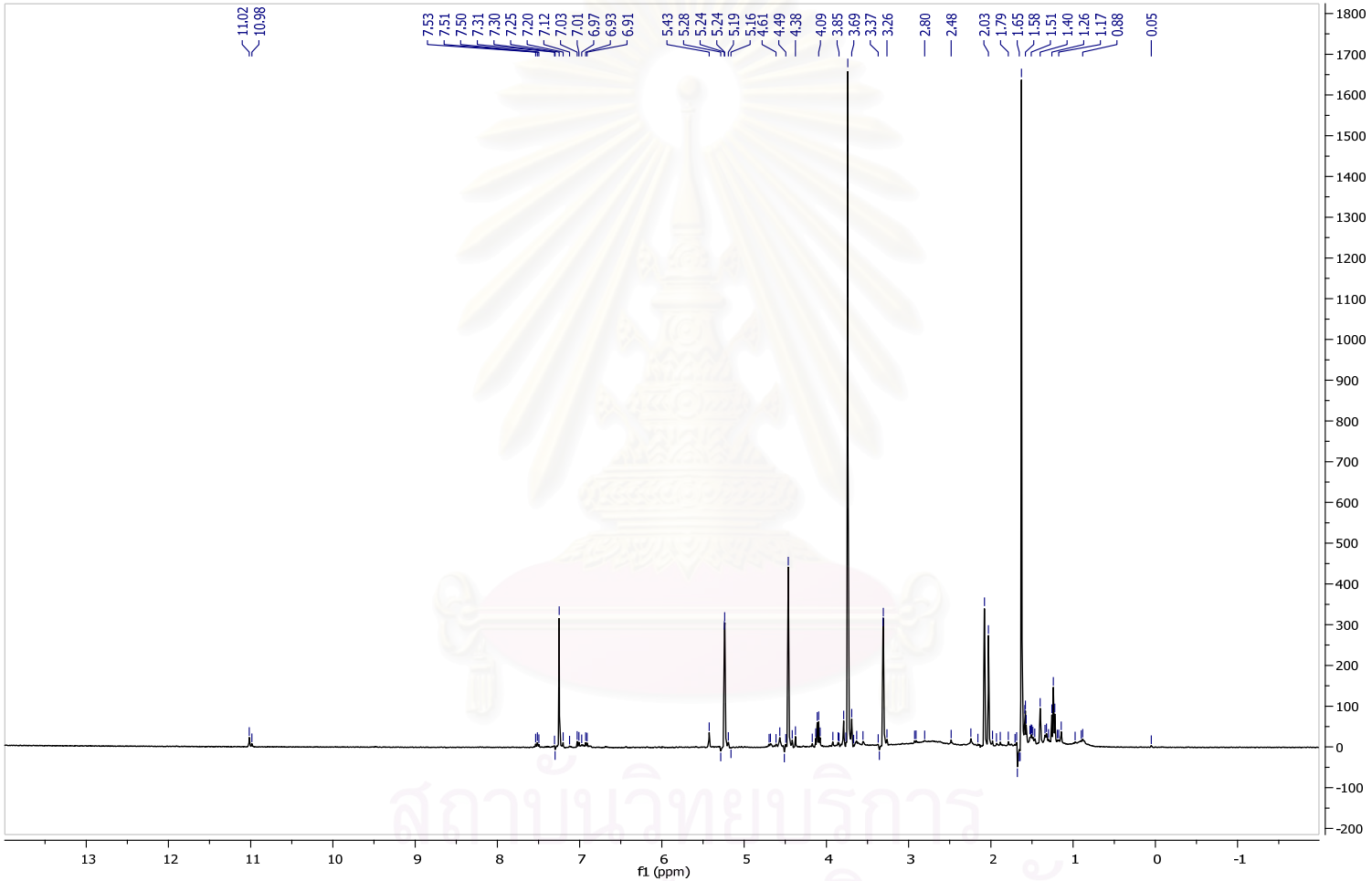


Figure B6-1 The EtOAc crude broth extract (up scale)

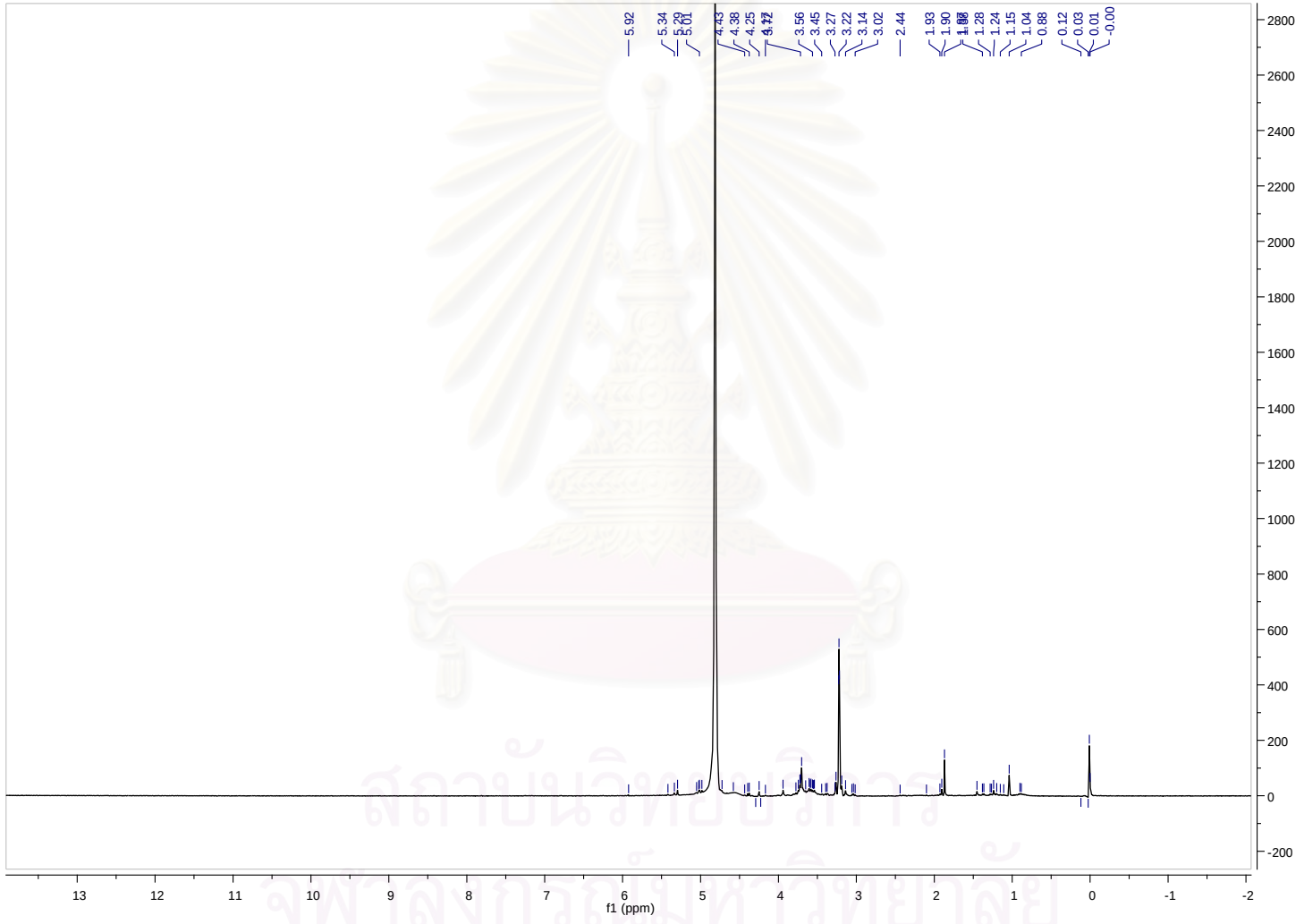


Figure B6-2 The DCM:MeOH crude broth extract (scale up)

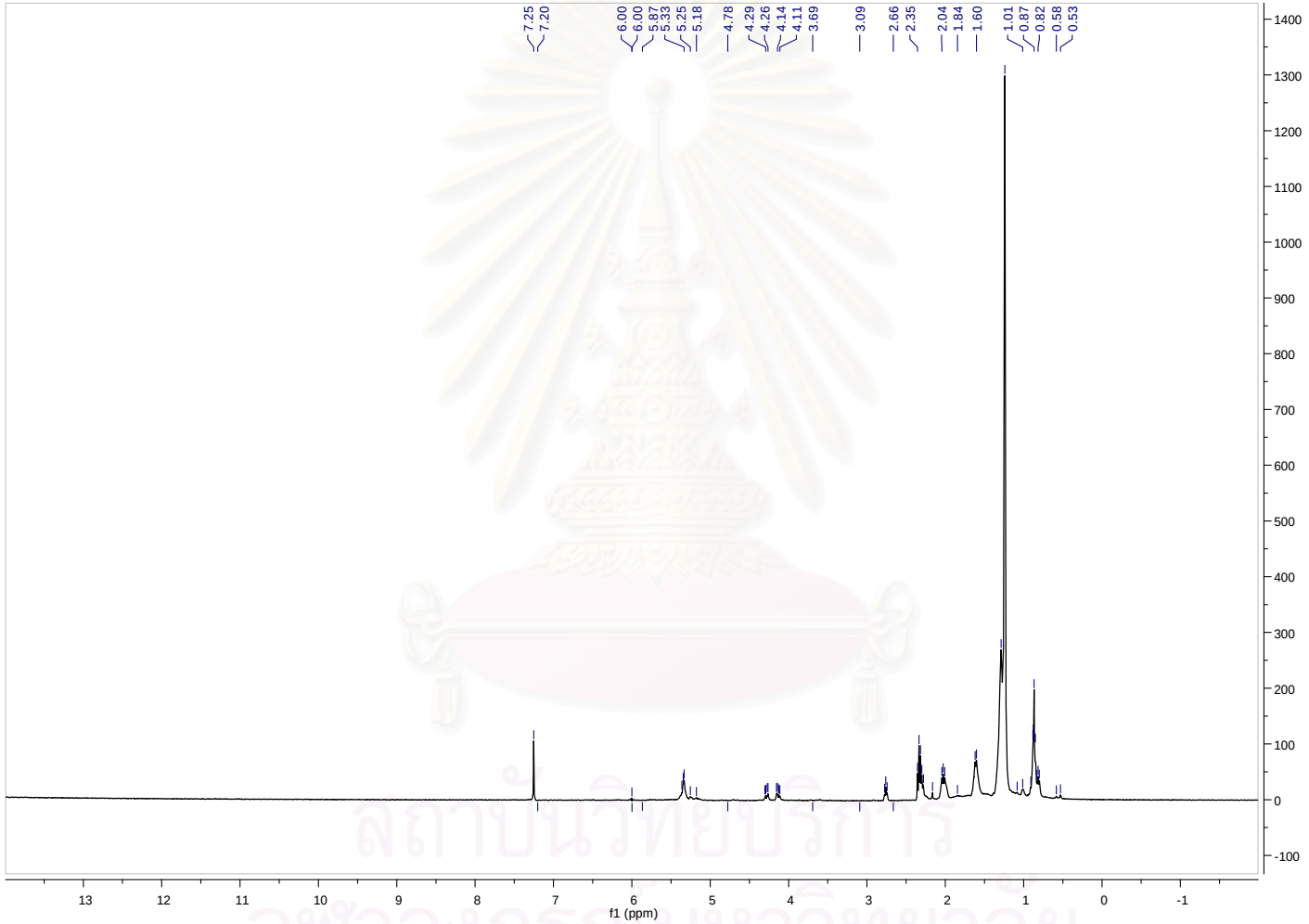


Figure B6-3 The Hexane crude mycelium extract (scale up)

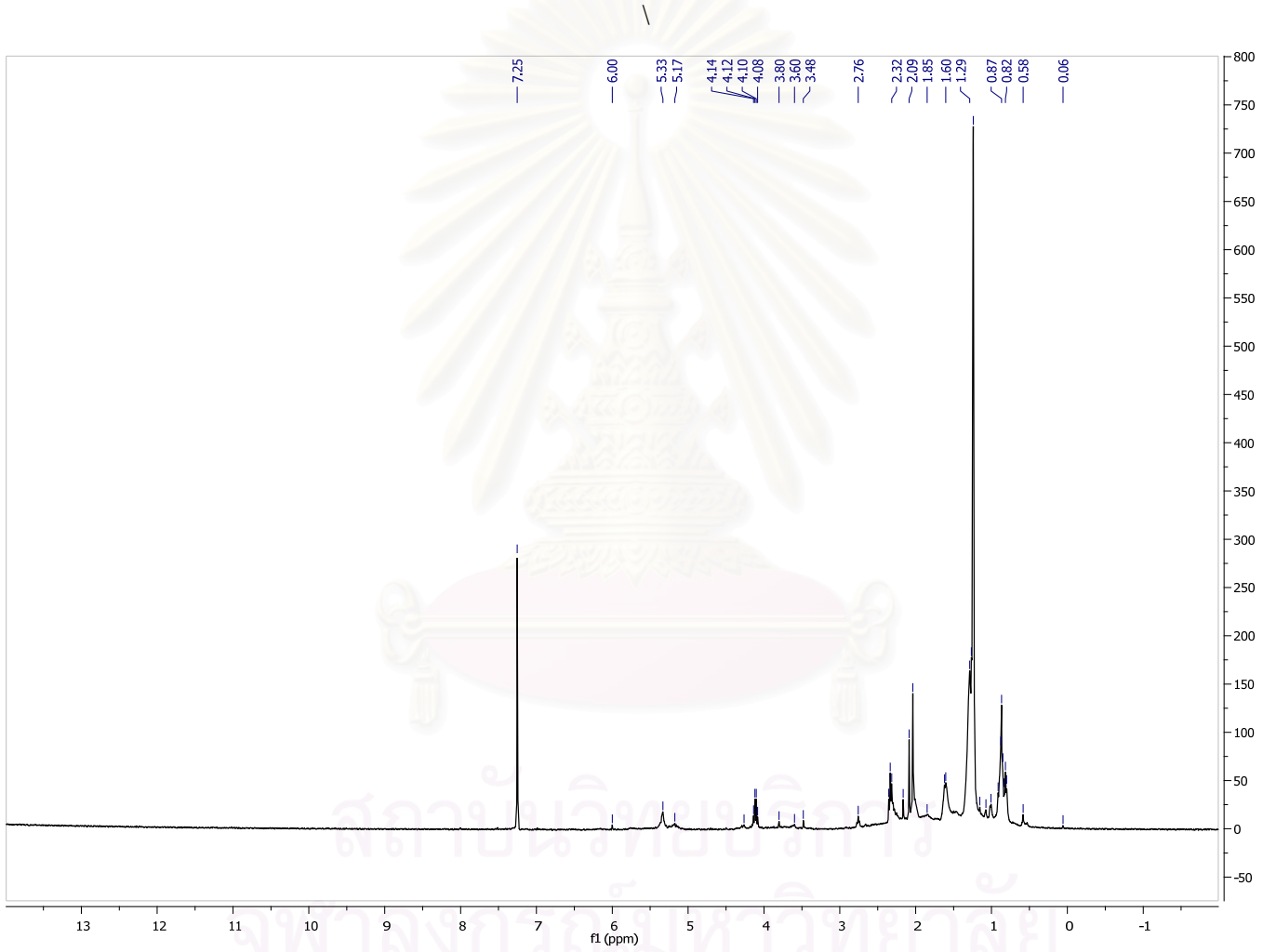


Figure B6-4 The EtOAc crude mycelium extract (scale up)

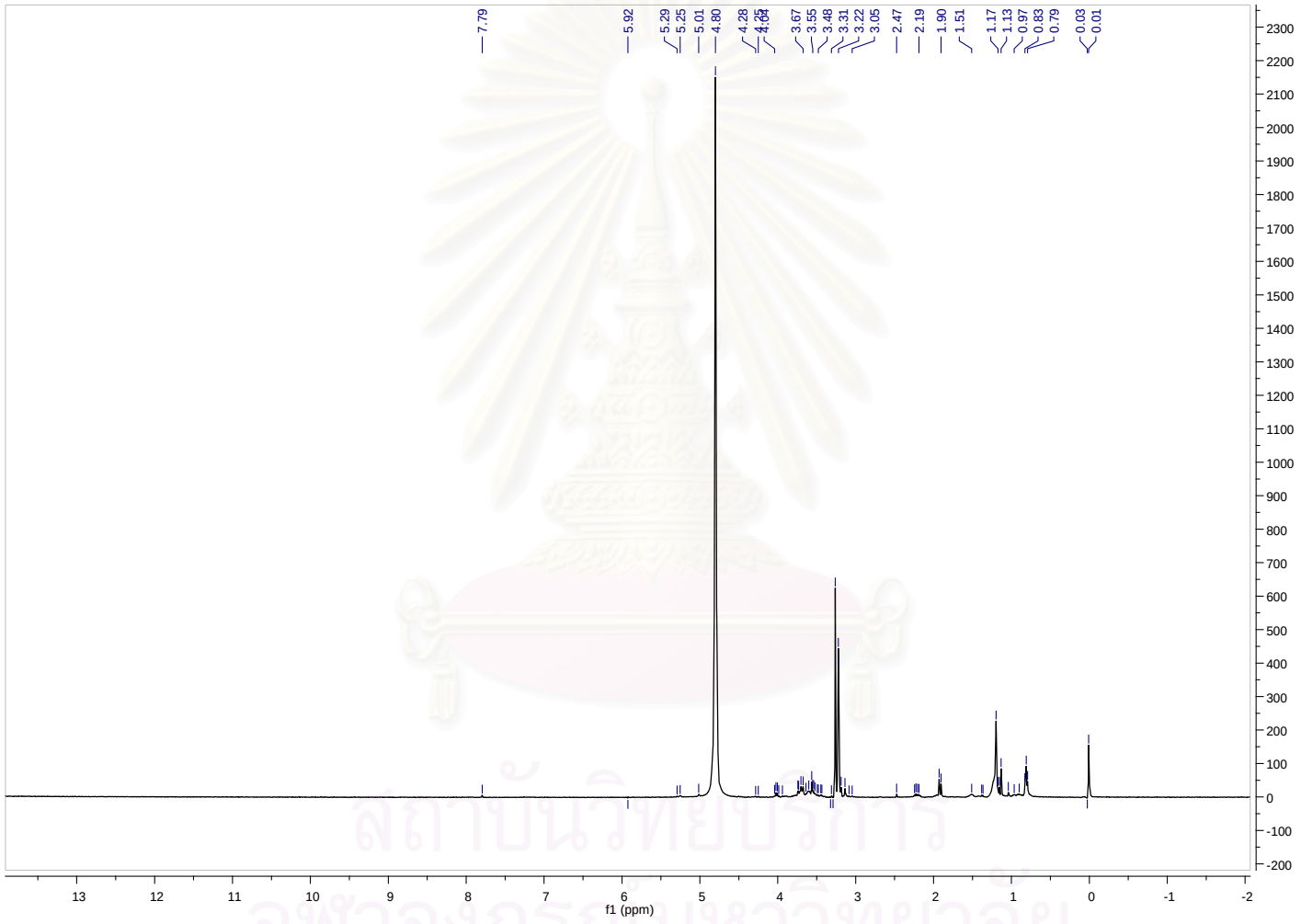


Figure B6-5 The MeOH crude mycelium extract (scale up)

Appendix C

Cytotoxicity data

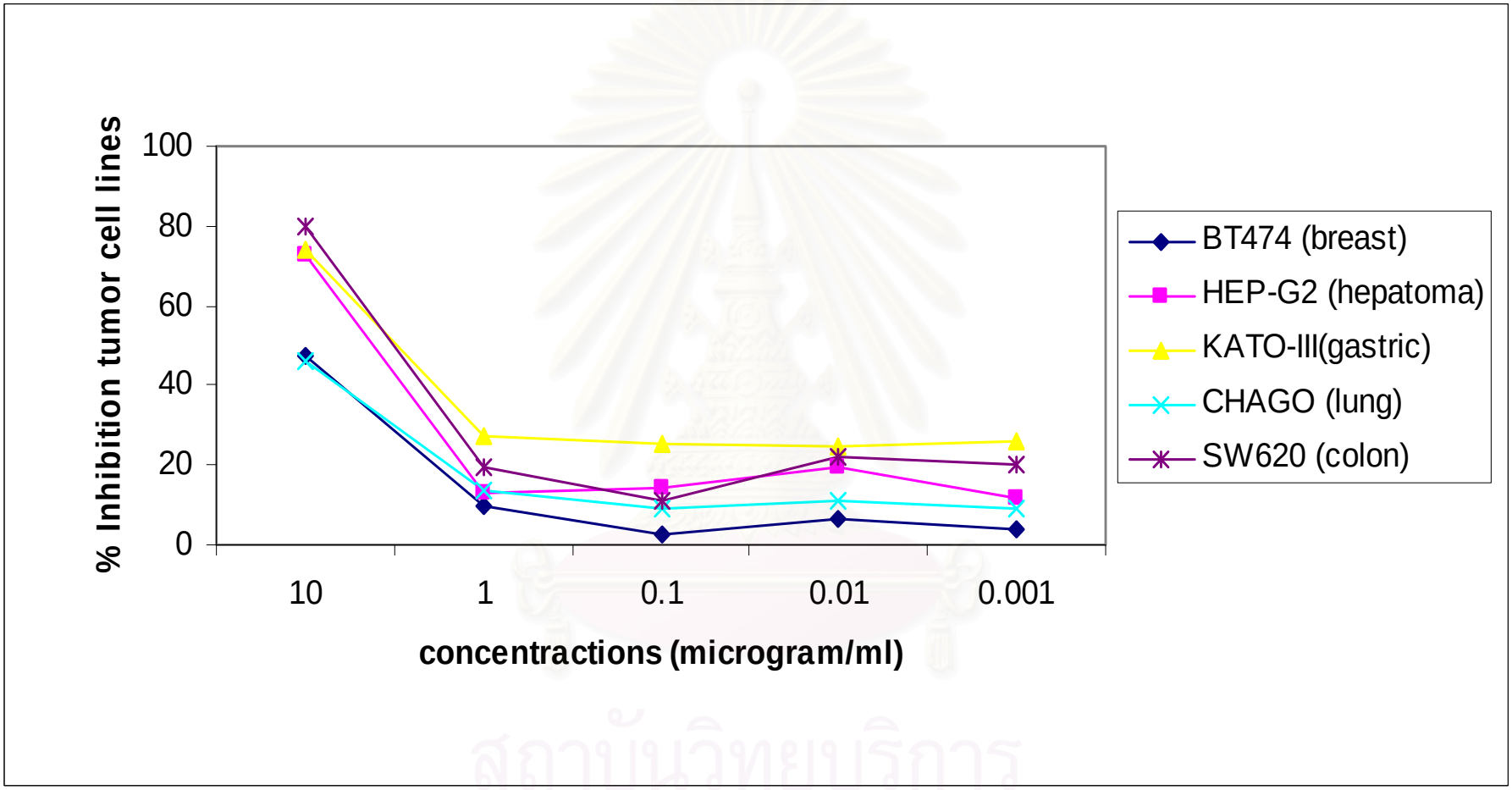


Figure C-1 Cytotoxicity test of compound 1 against human tumor cell lines.

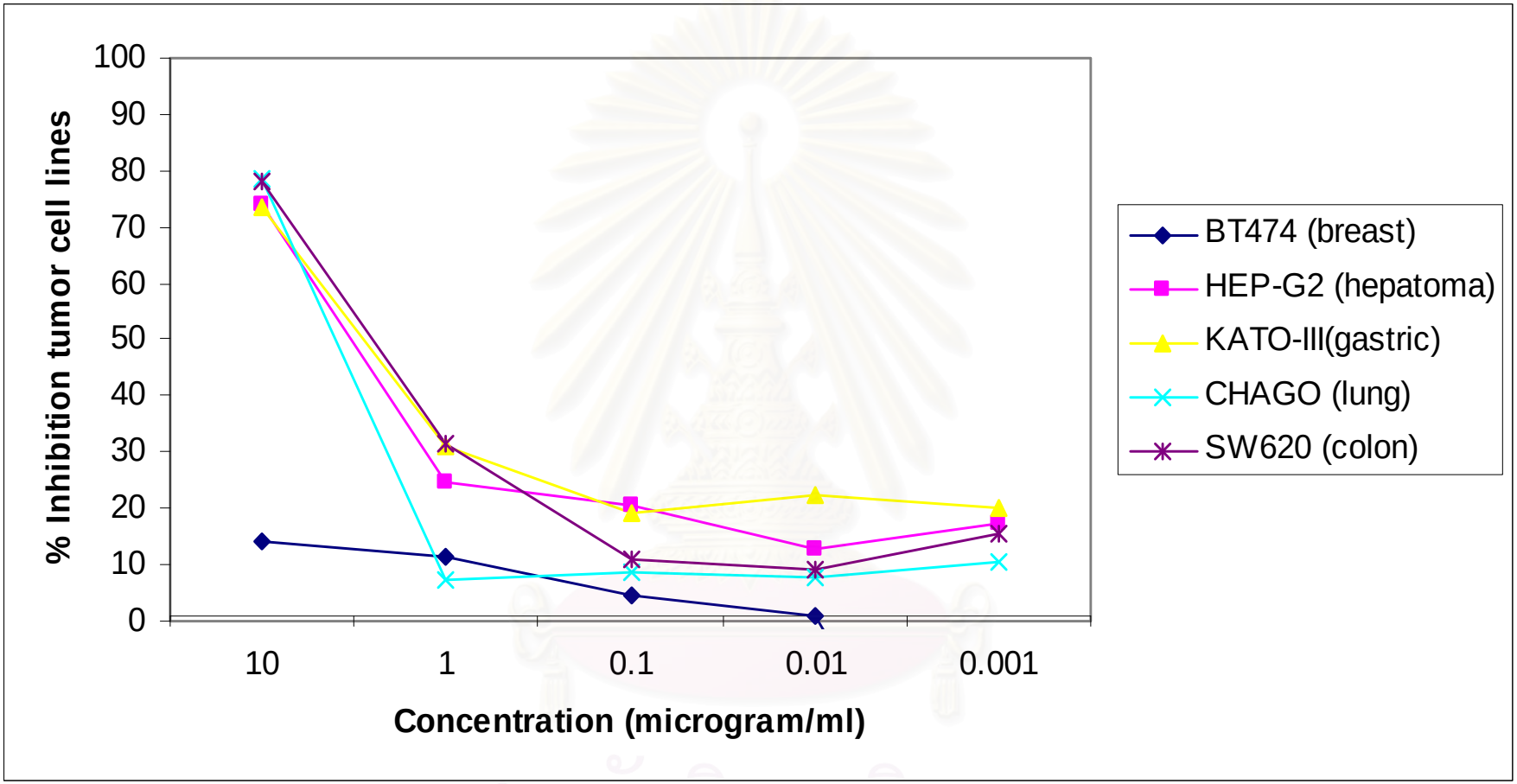


Figure C-2 Cytotoxicity test of compound 2 against human tumor cell lines.

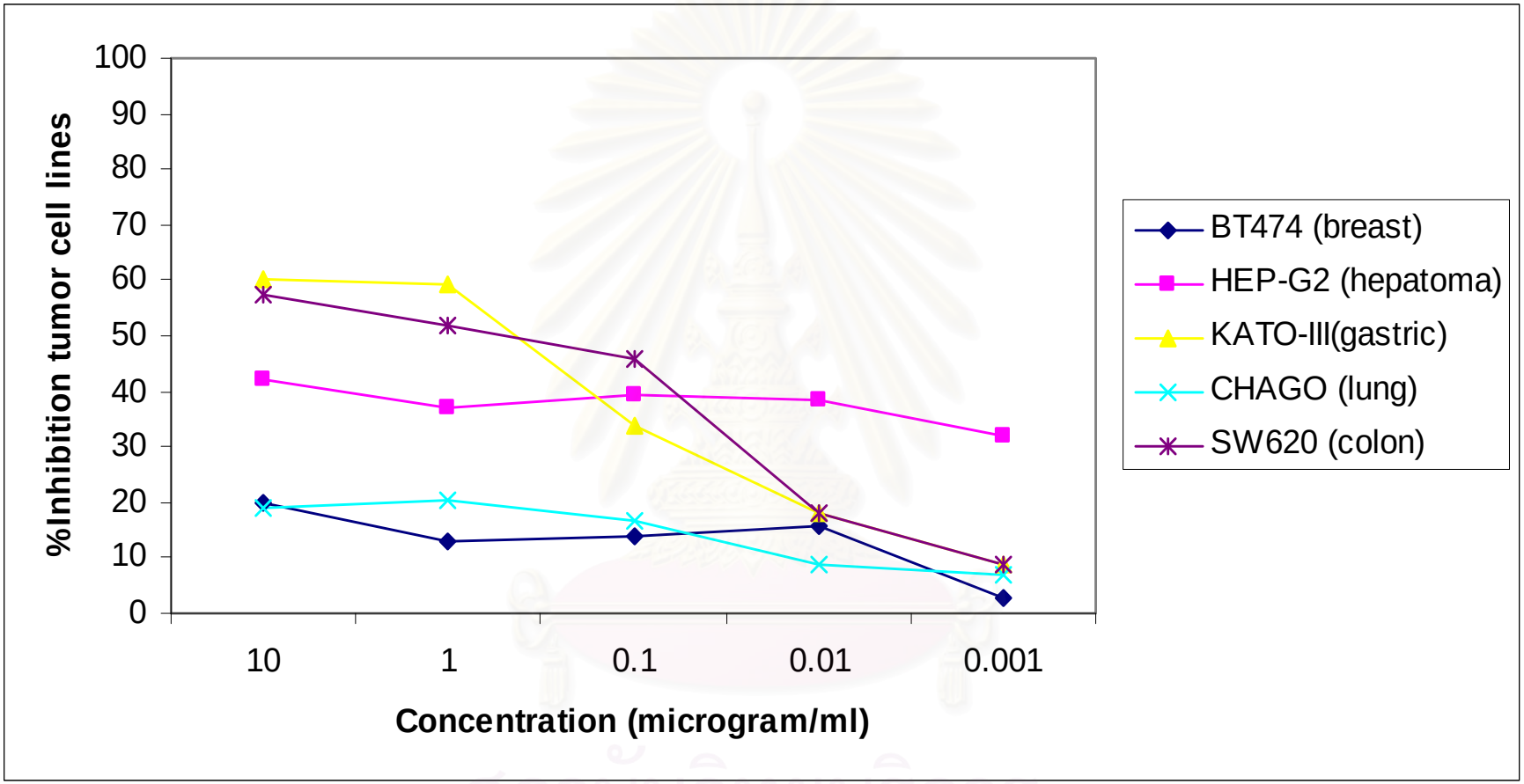


Figure C-3 Cytotoxicity test of compound 3 against human tumor cell lines.

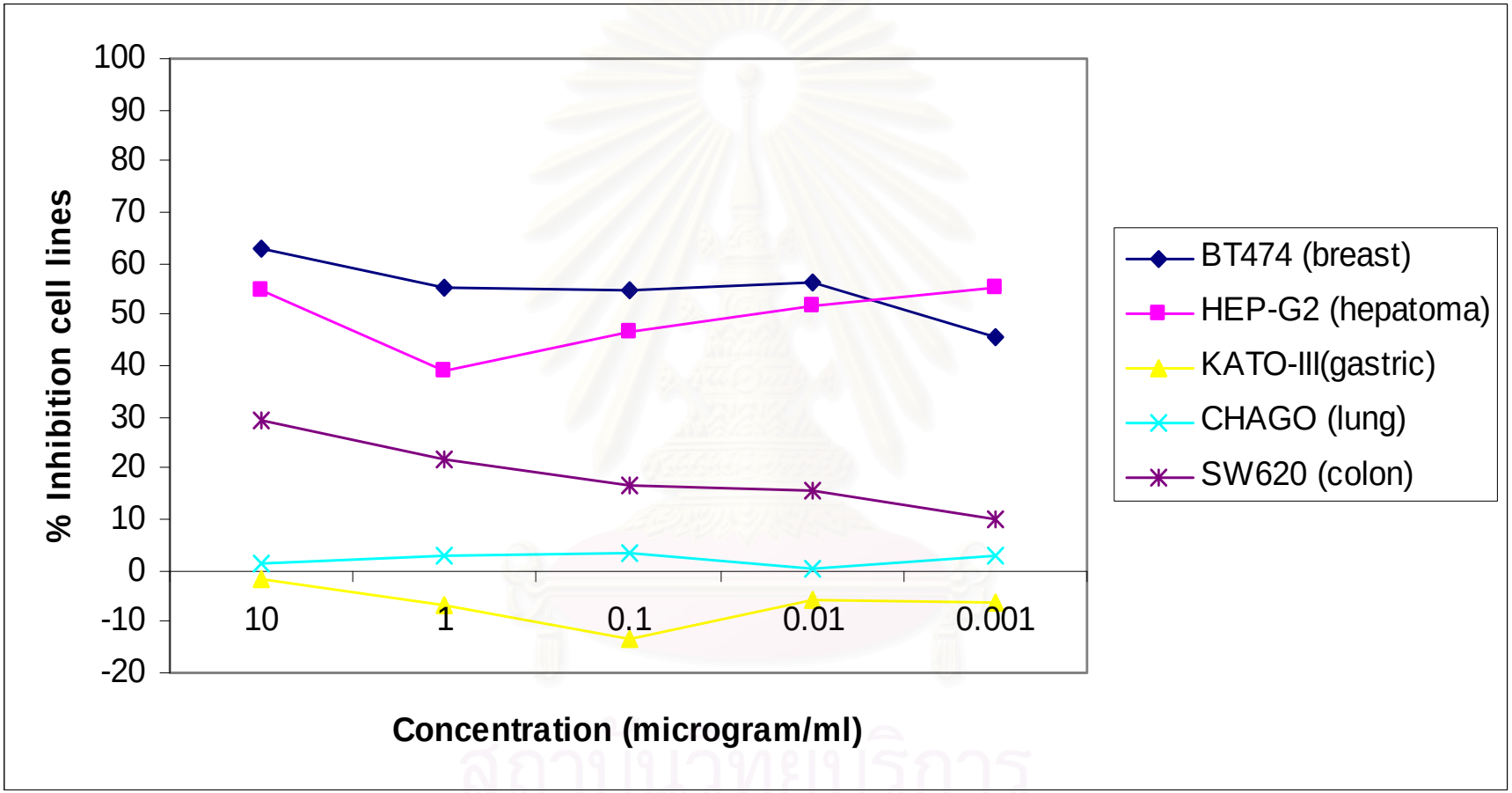
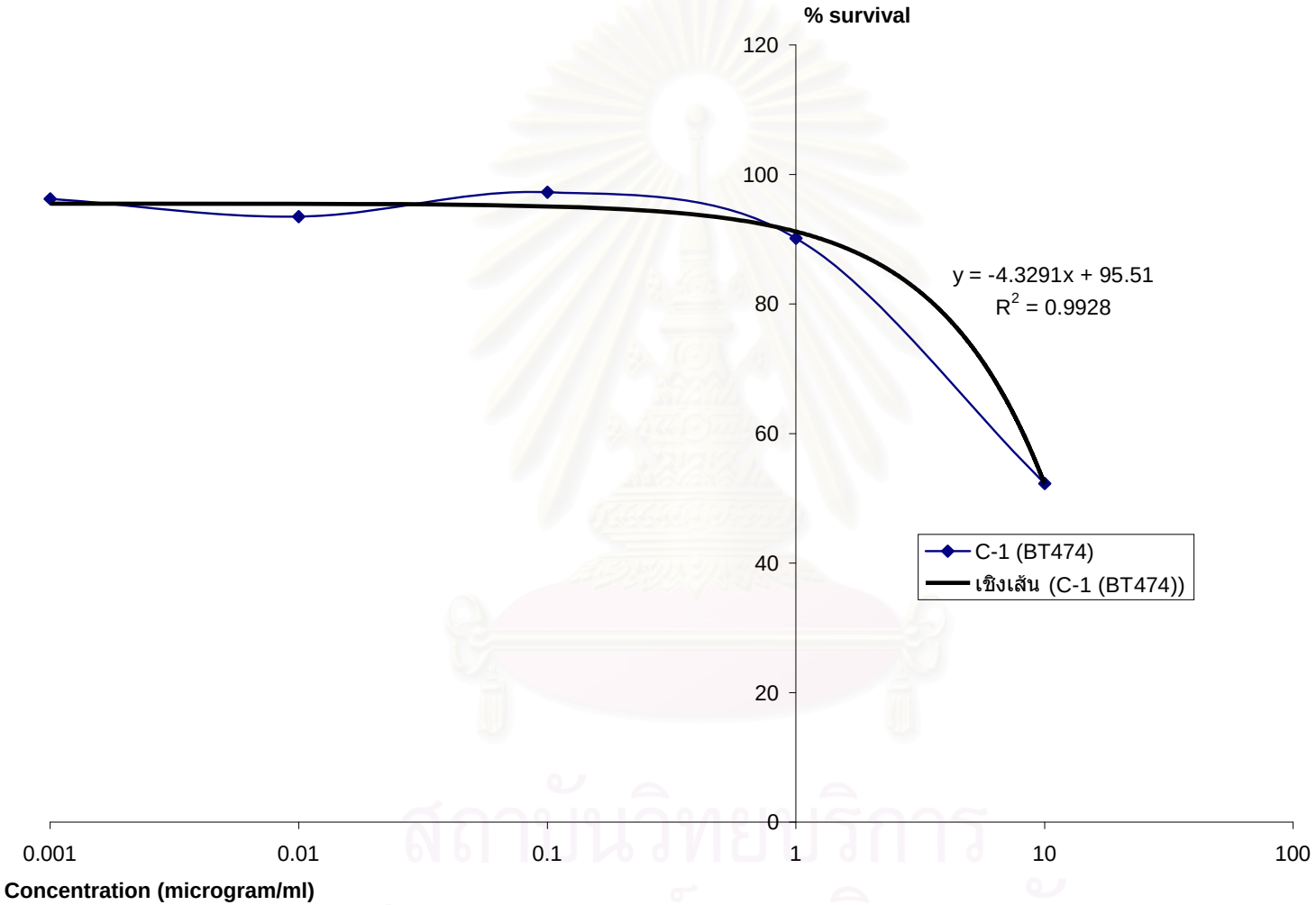
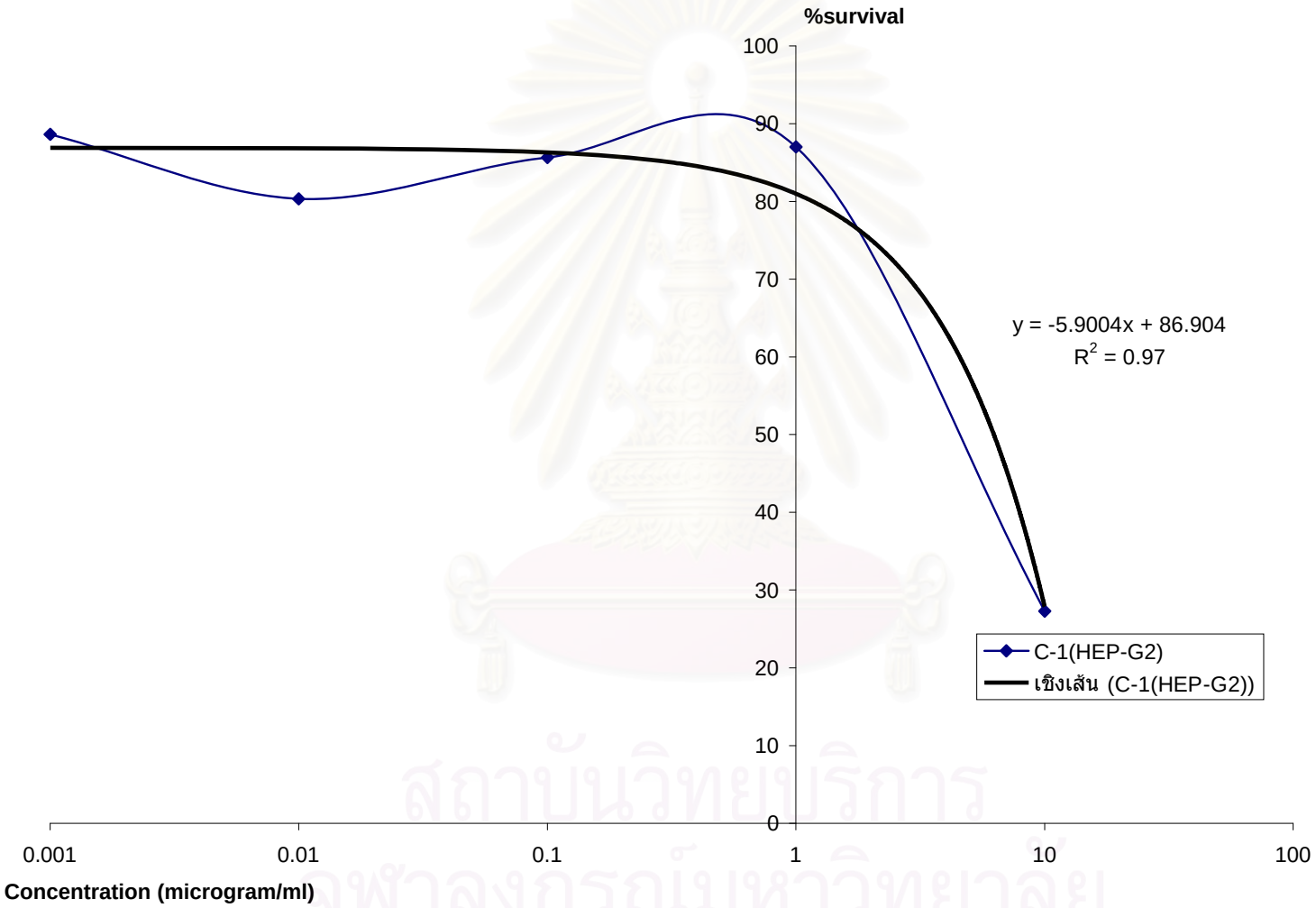
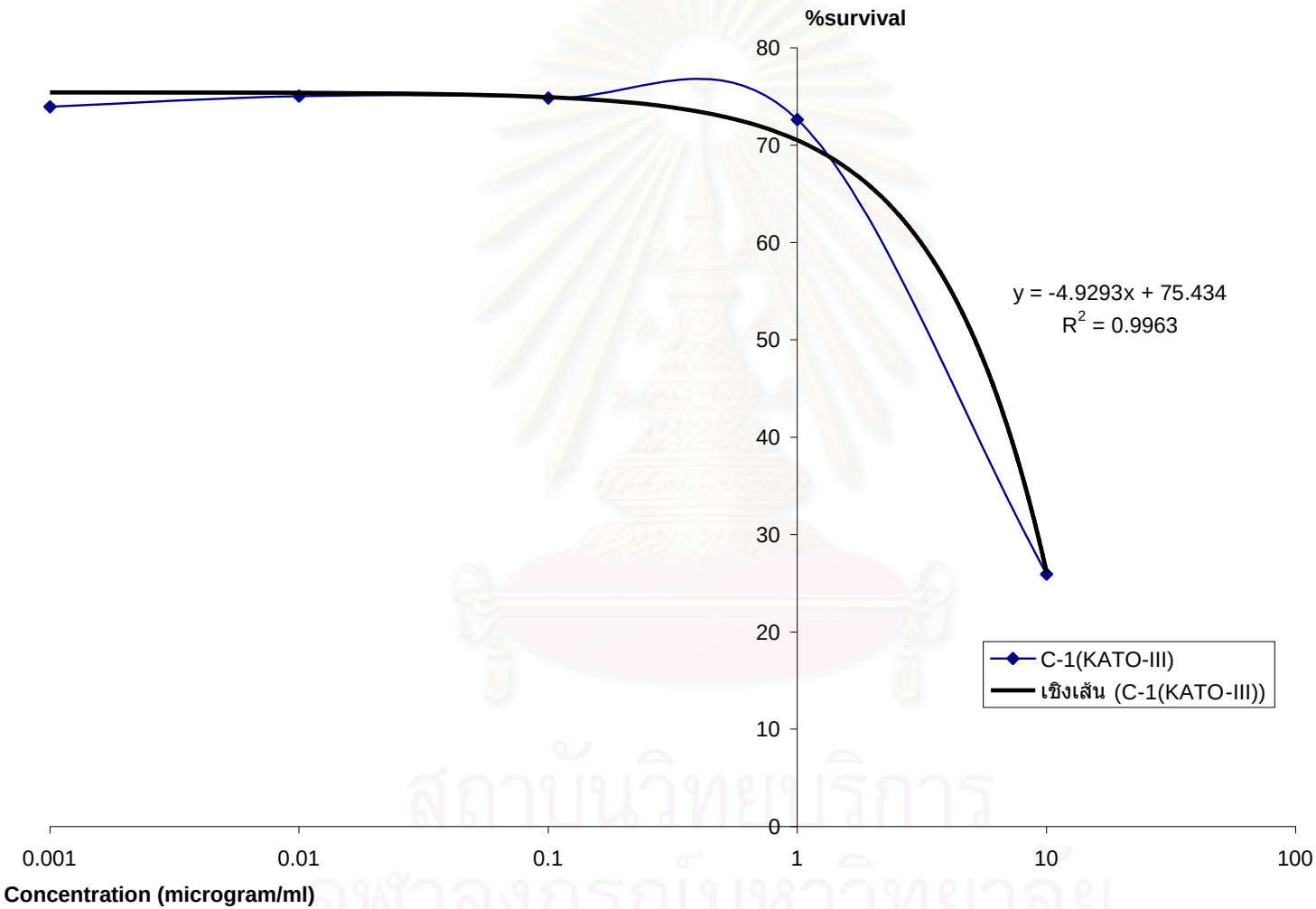
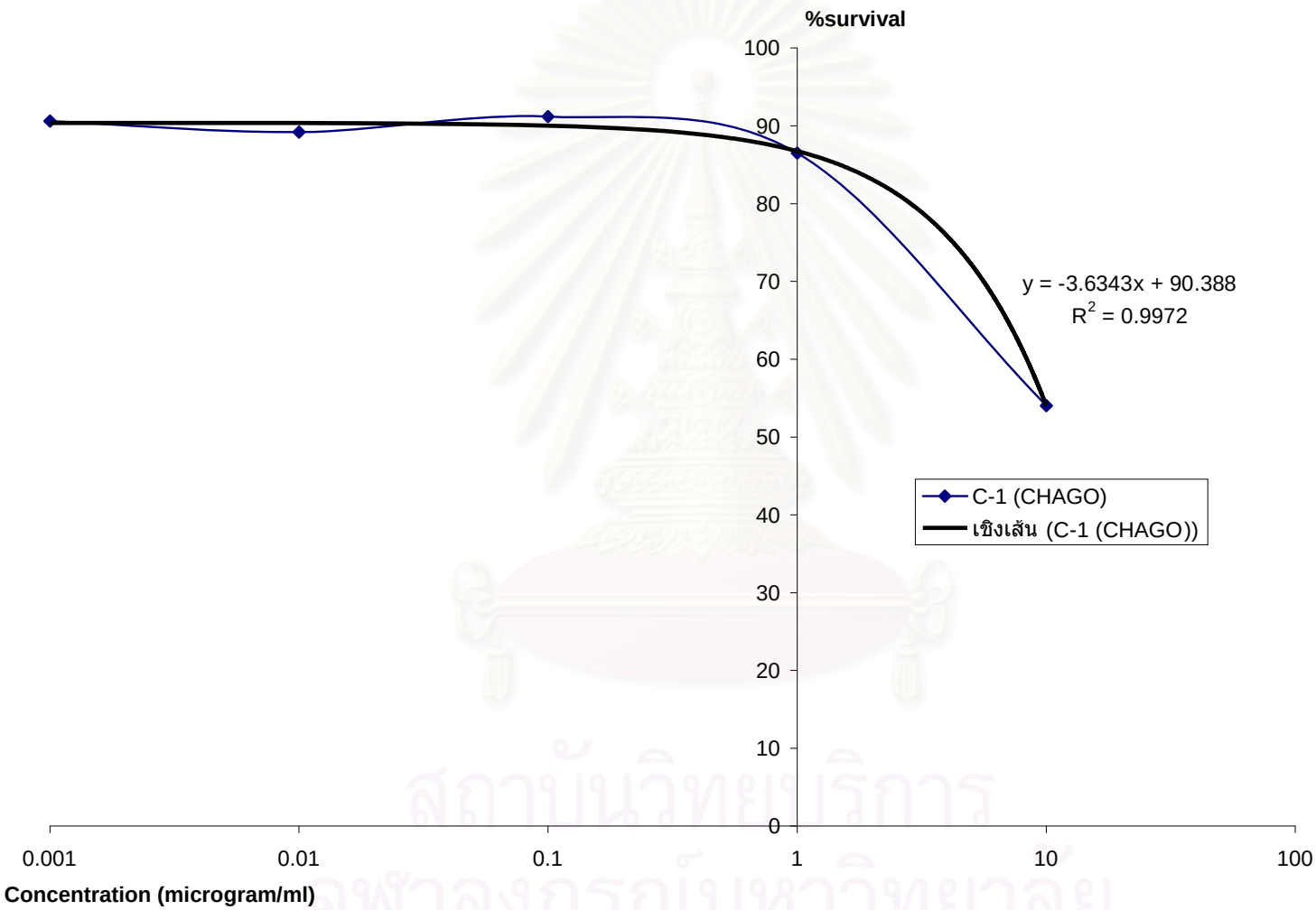


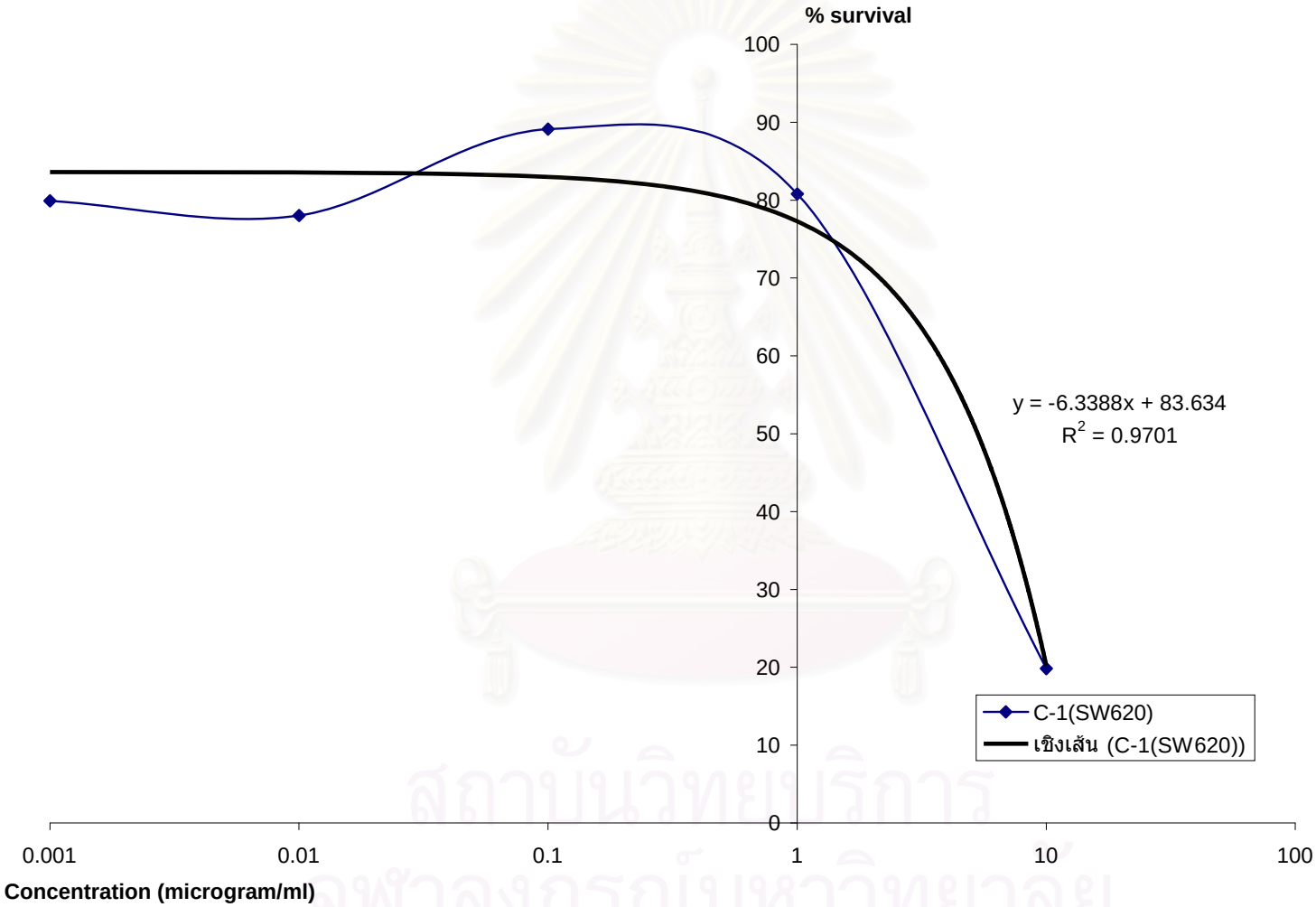
Figure C-5 Cytotoxicity test of compound 5 against human tumor cell lines.

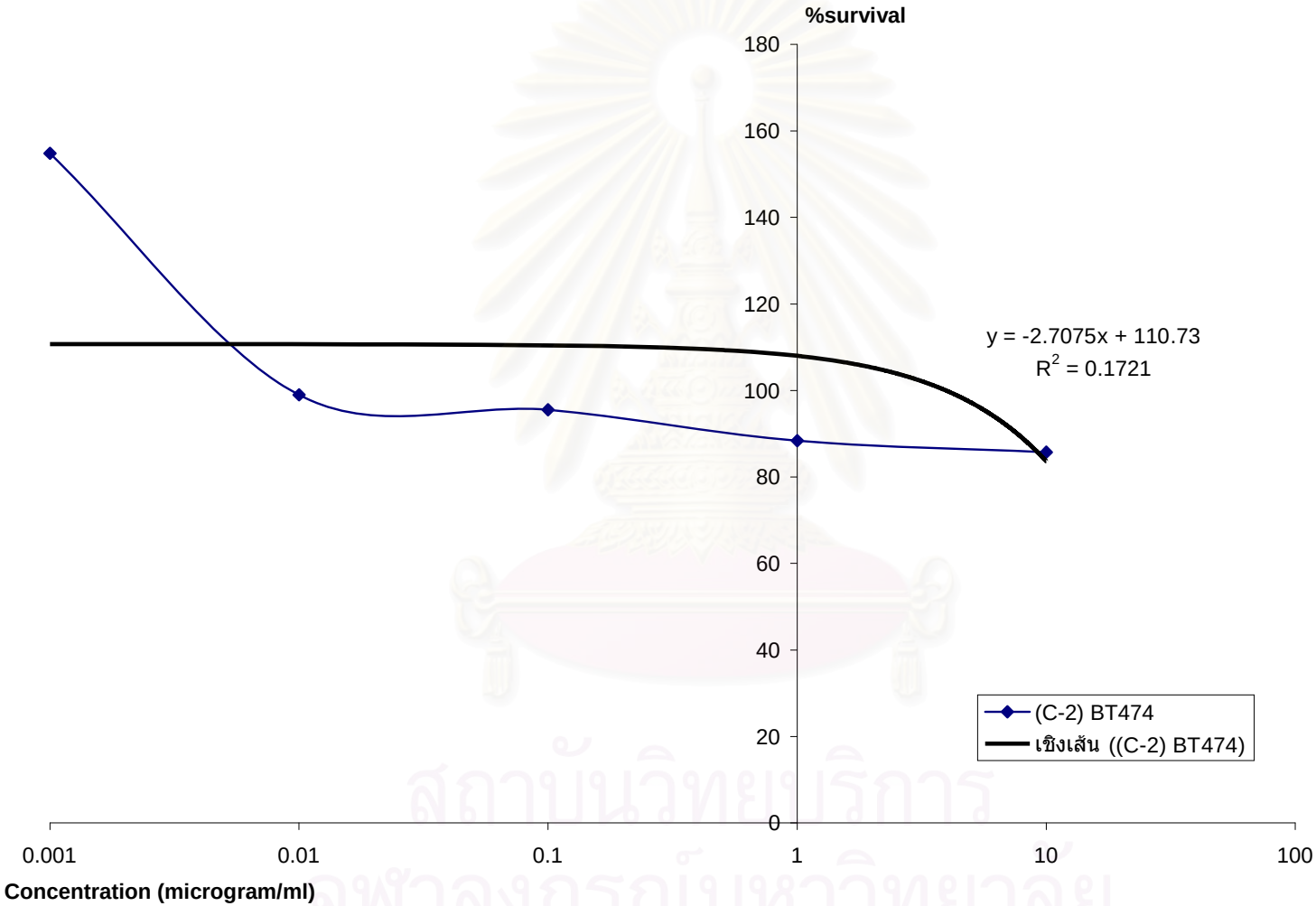


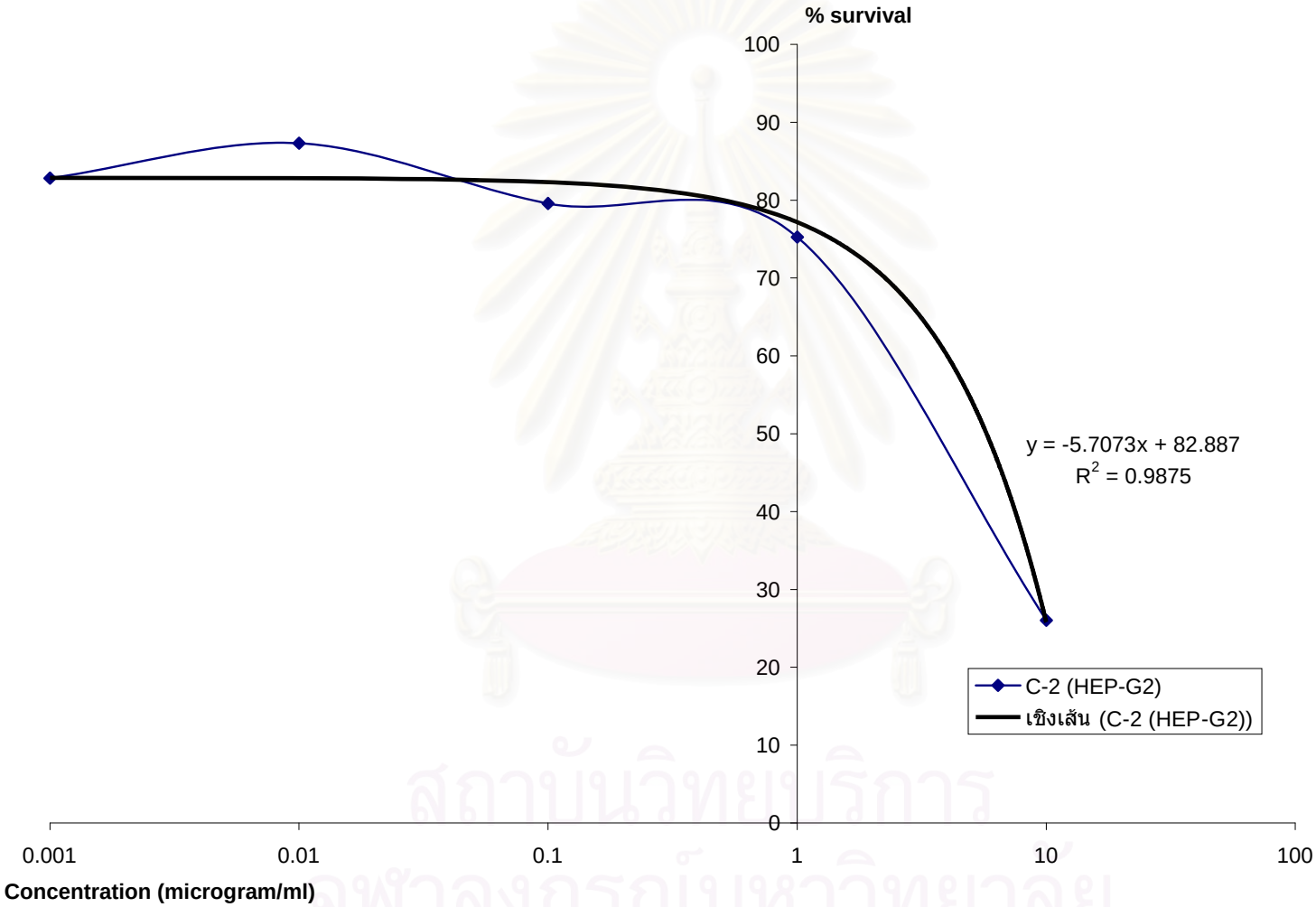


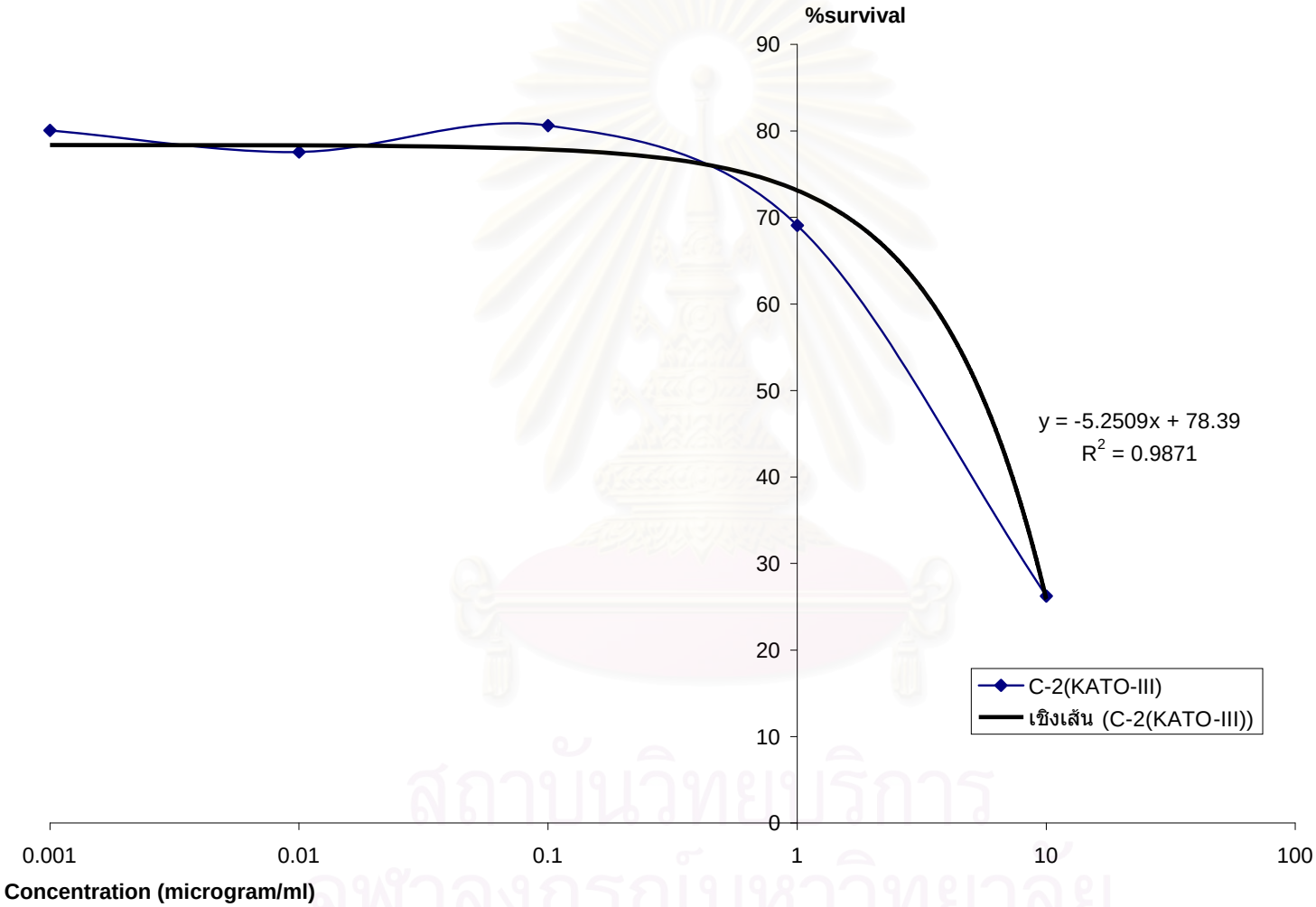


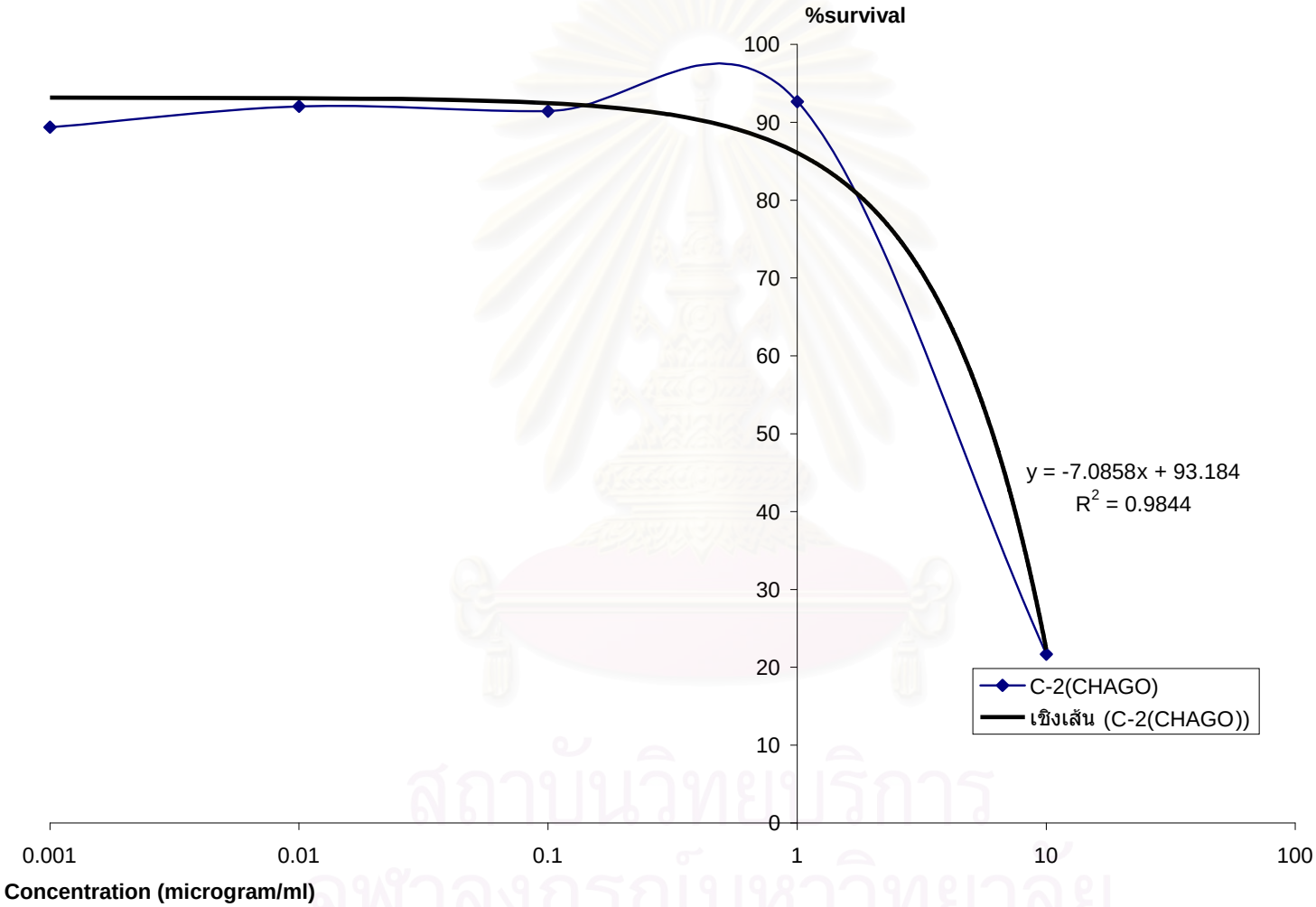


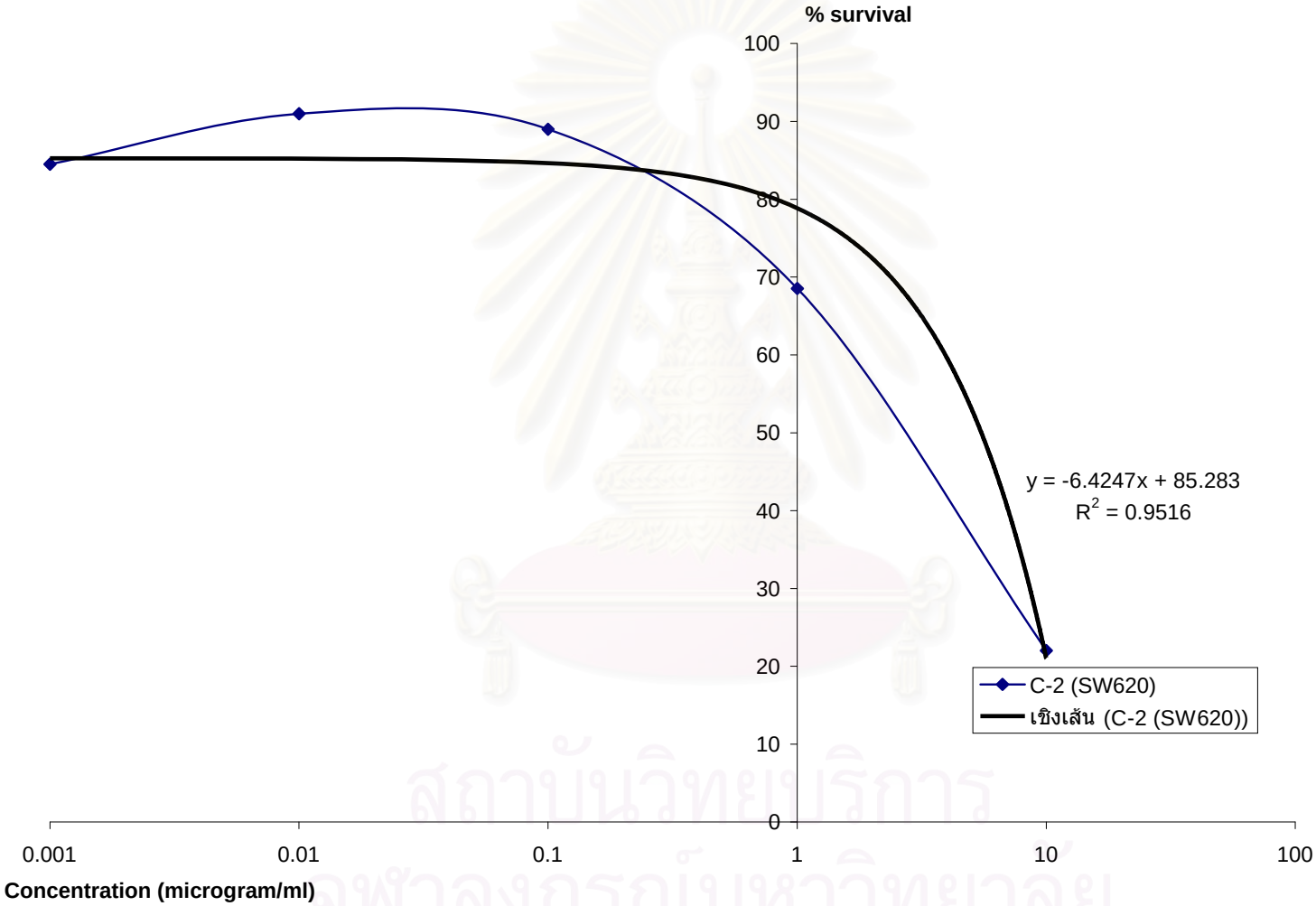


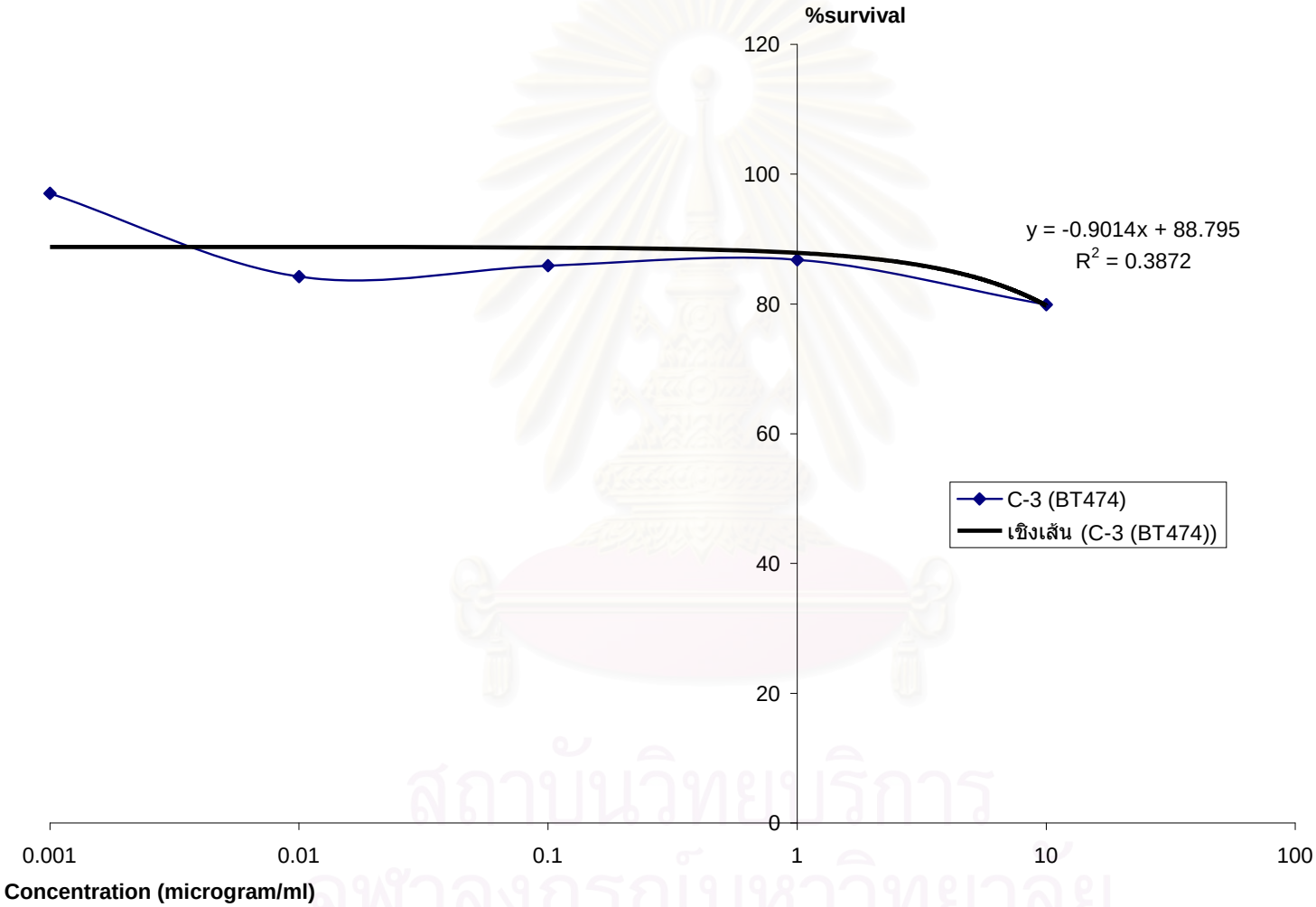


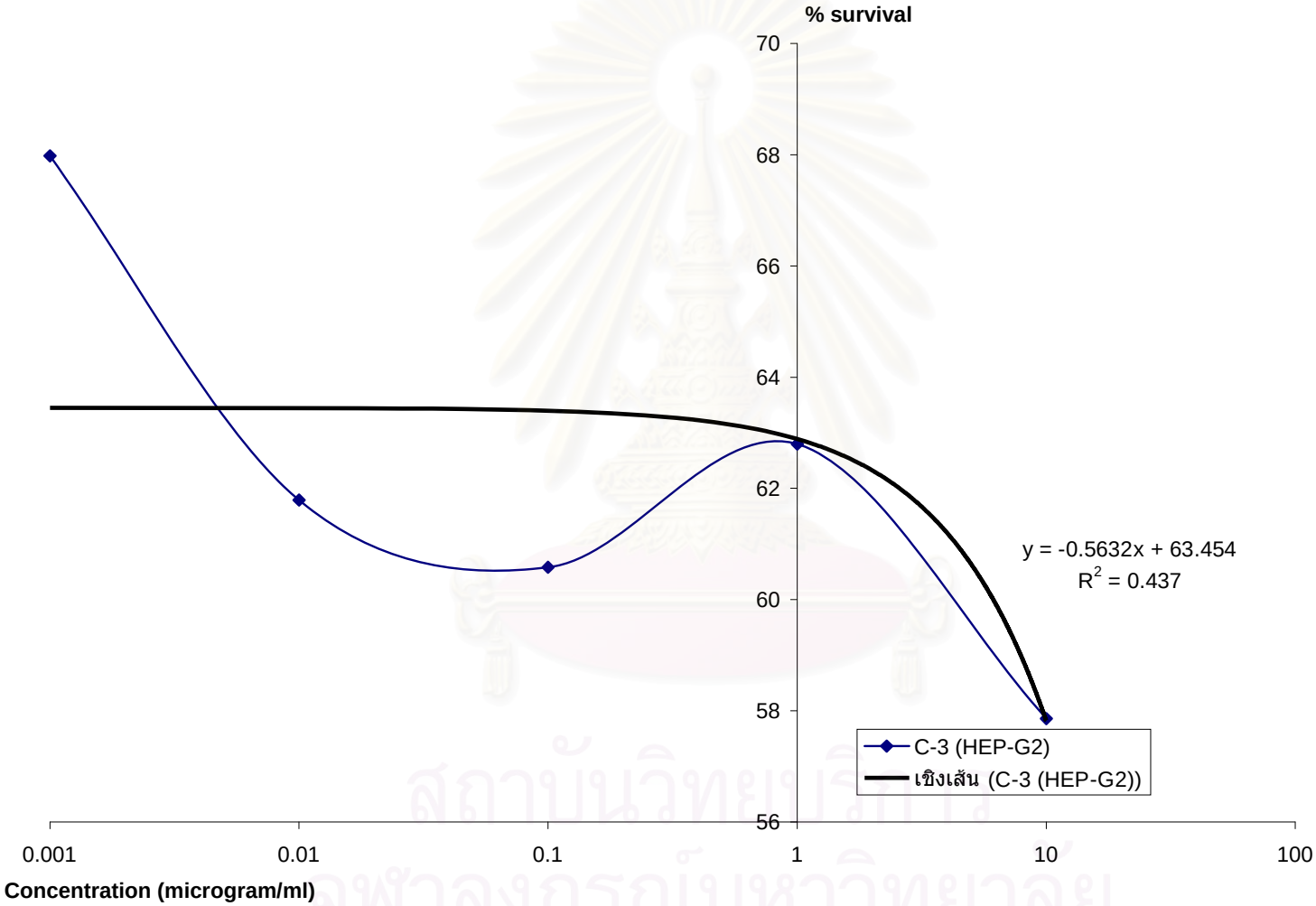


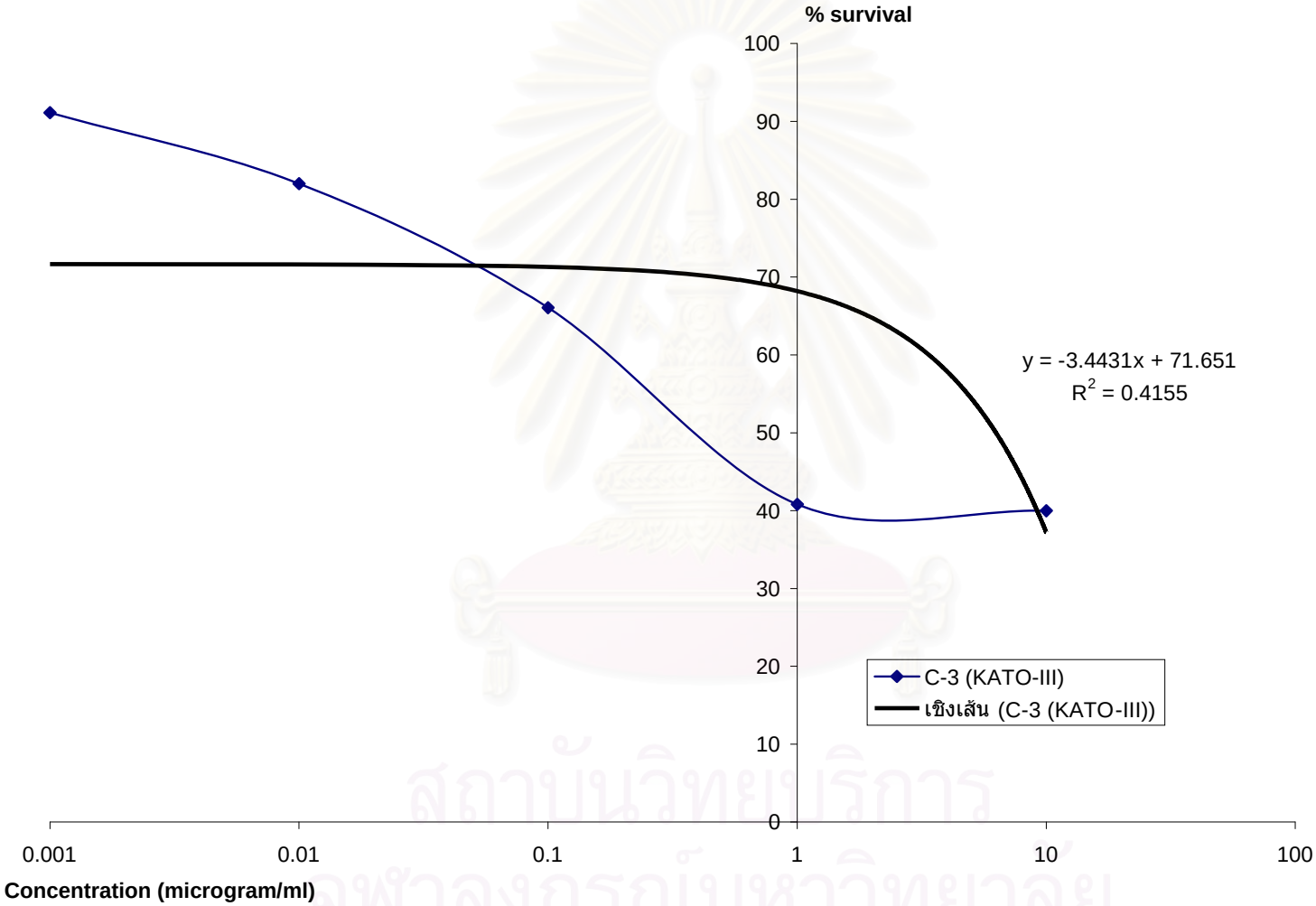


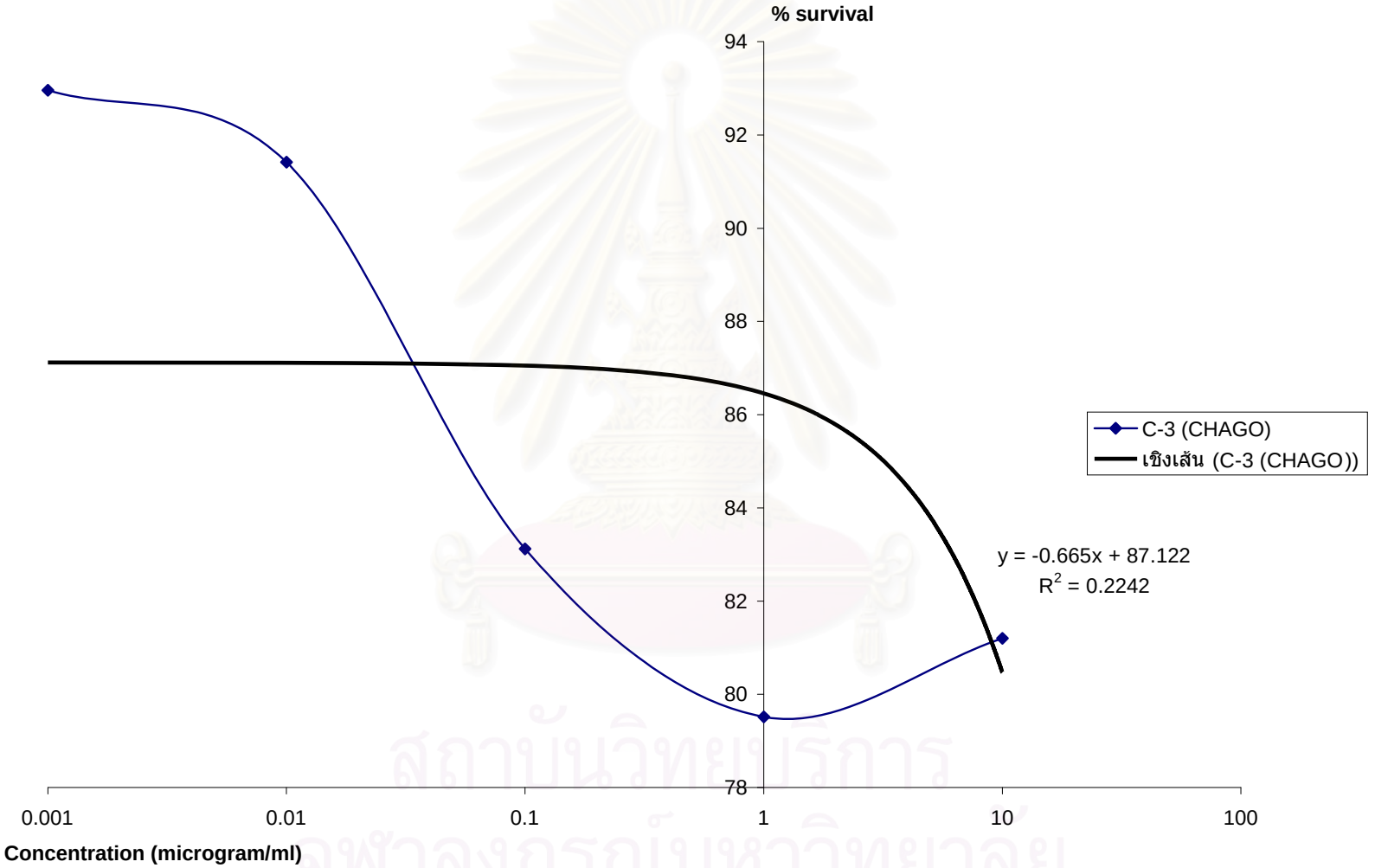


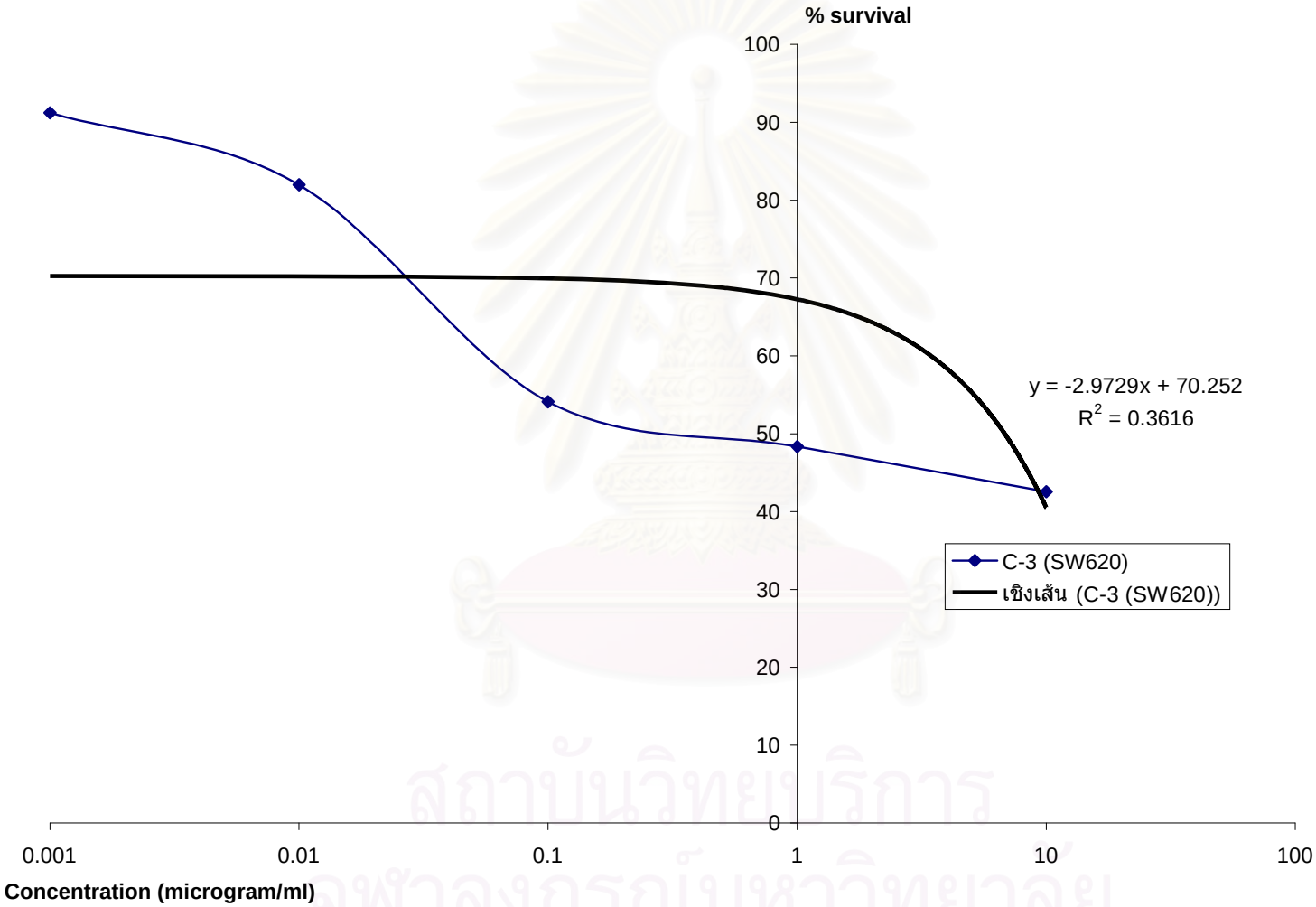


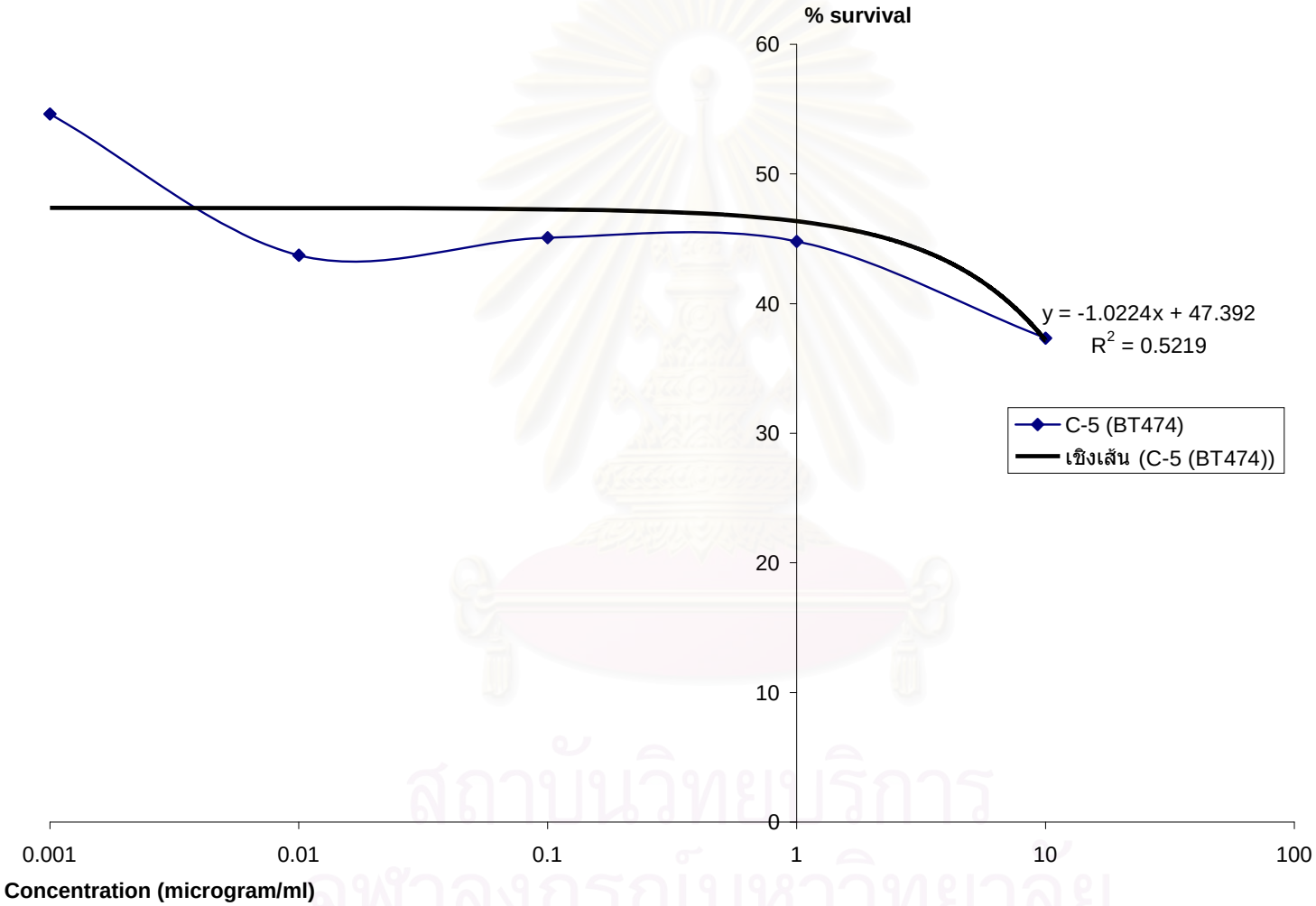


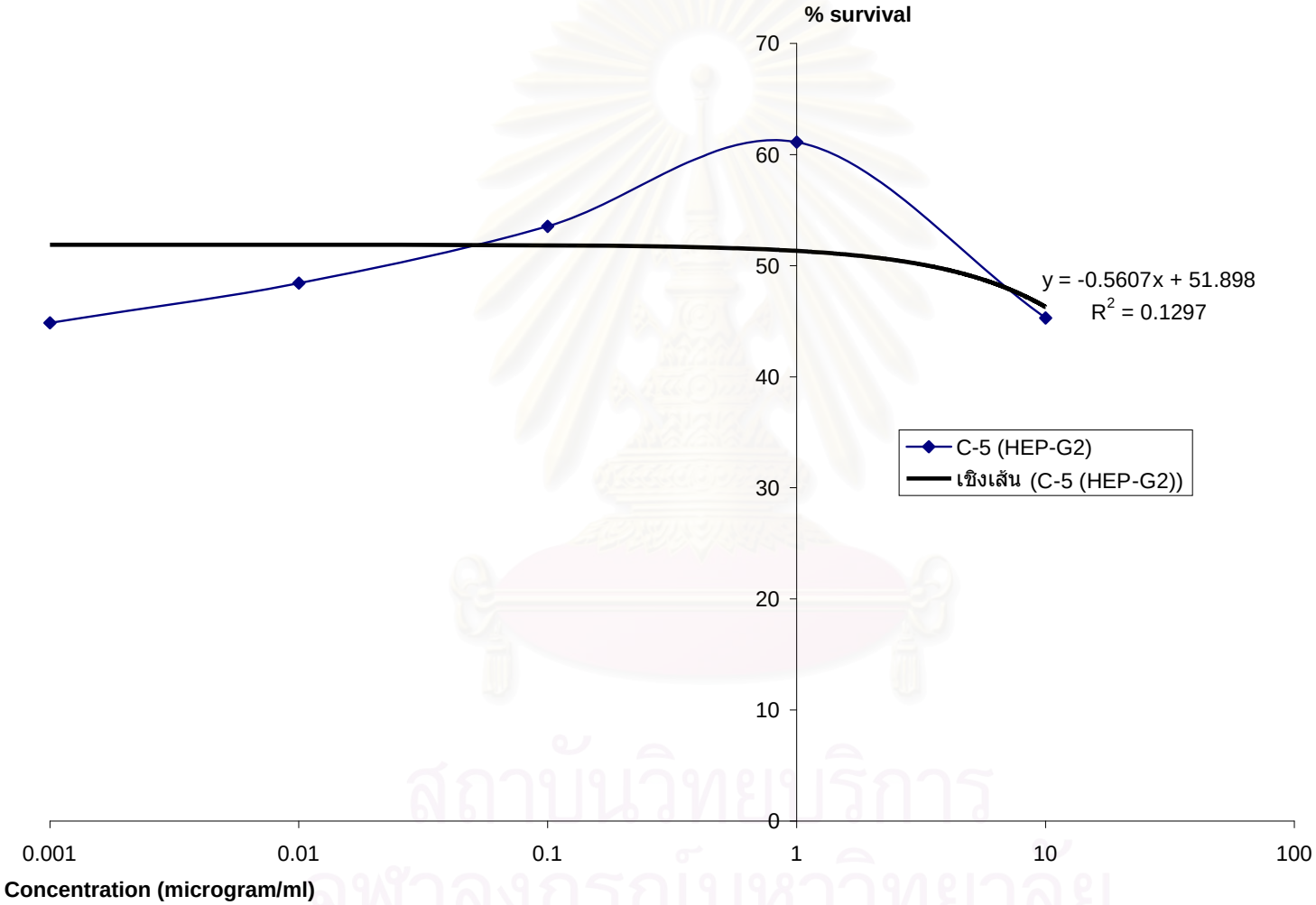


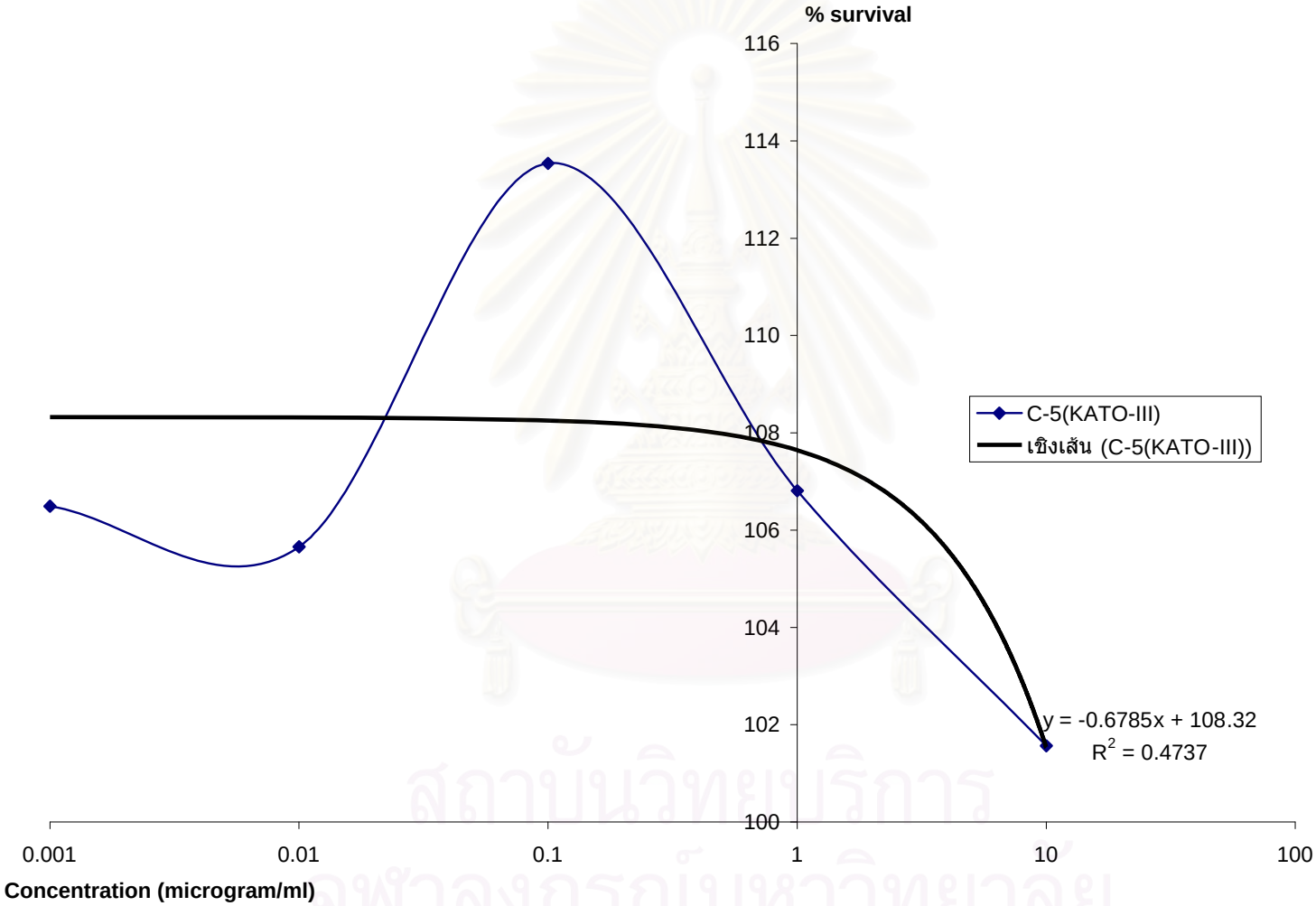


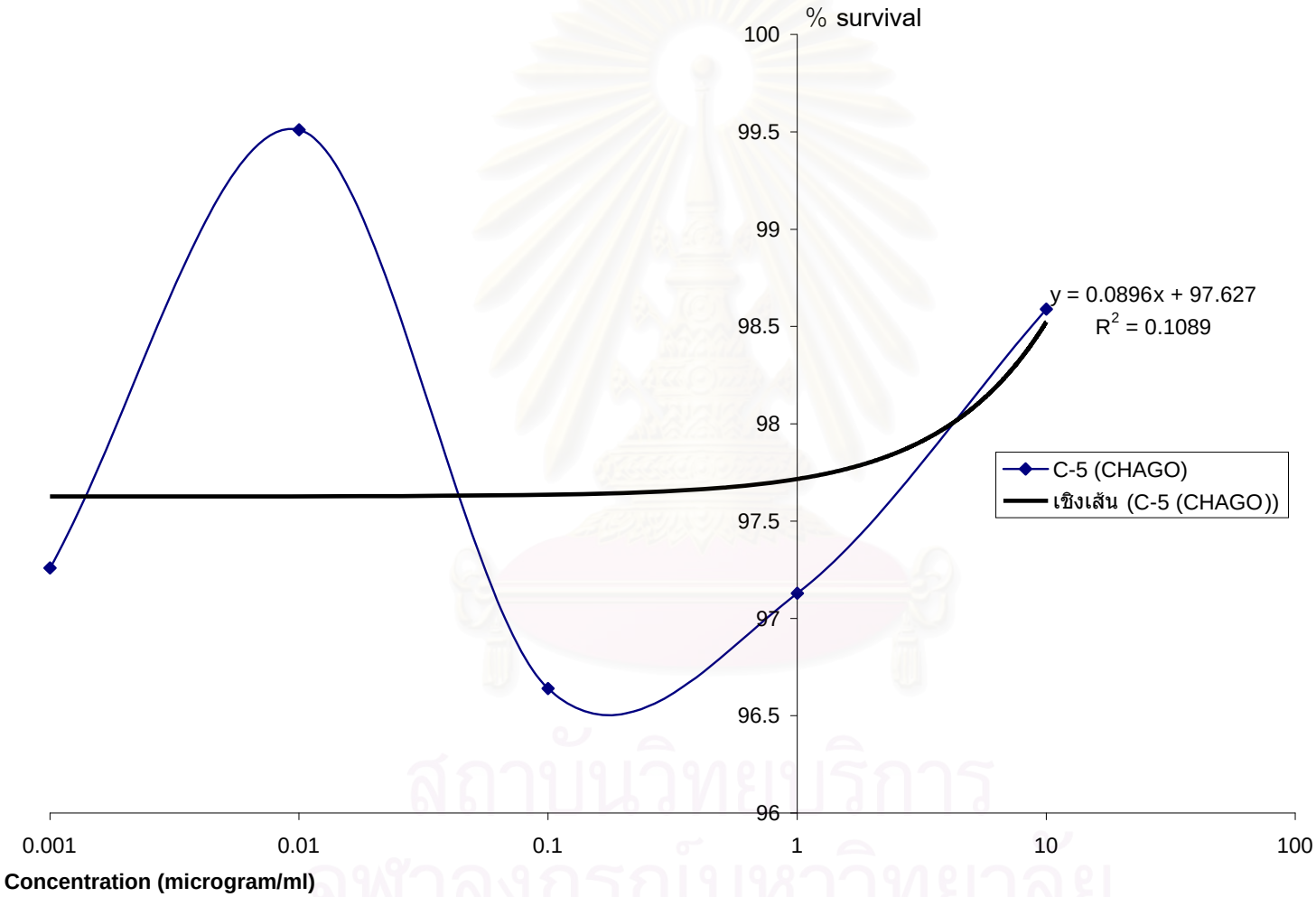


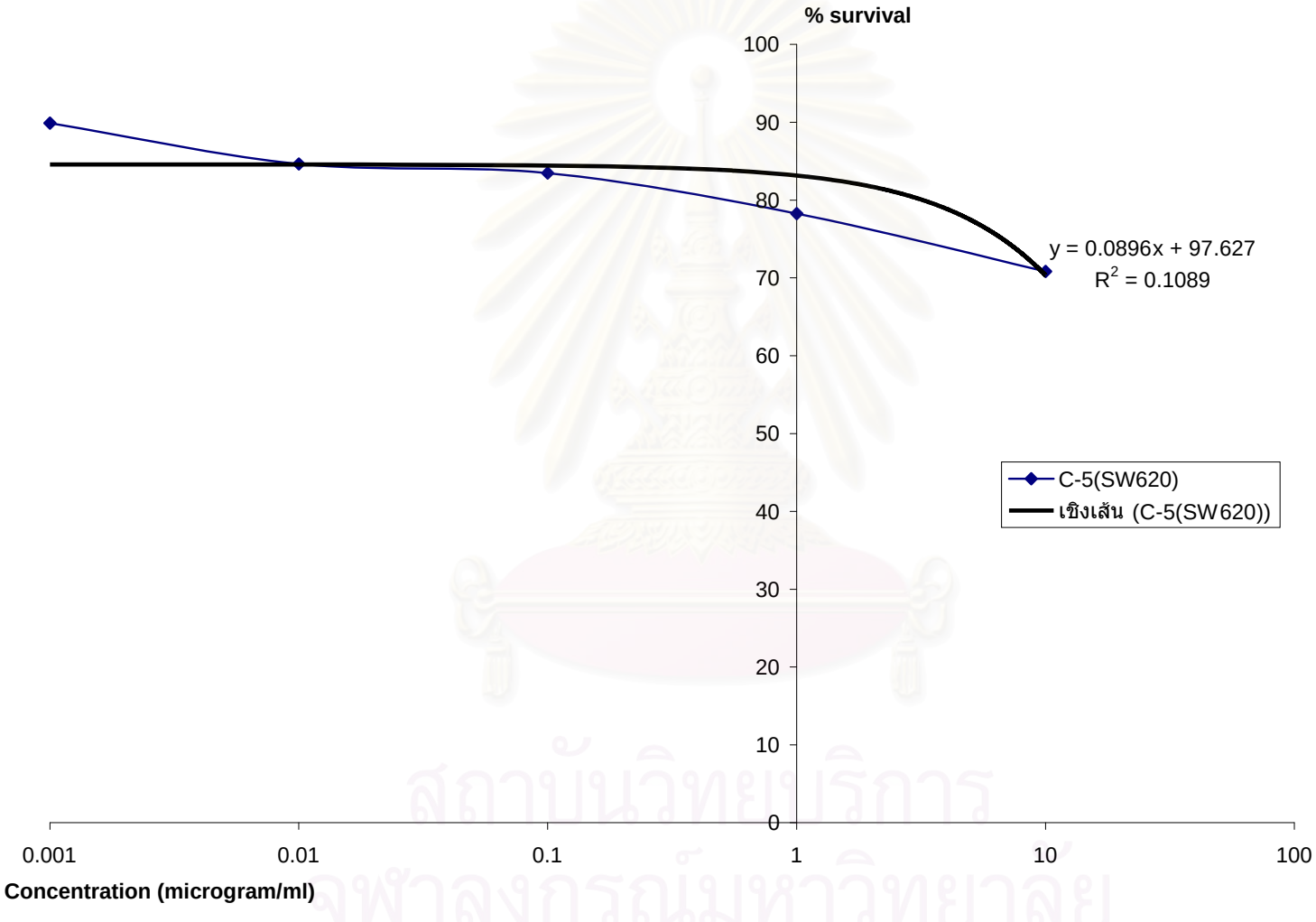












BIOGRAPHY

Miss. Porntana Chanapart was born on September 28, 1982 in Trang, Thailand. She was graduated with a Bachelor's Degree in Industrial Microbiology from the Faculty of science, King Mongkut's Institute of Technology Ladkrabang (KMITL) in 2004.In 2006 she was graduated with a Master Degree in Biotechnology from the Faculty of Science, Chulalongkorn University.