Doctoral Dissertation

Screening of Novel Secondary Metabolites from Endophytic Fungi by Chemical Library Analysis

化合物データベースを利用した植物内生菌からの新規生理活性物質の探索

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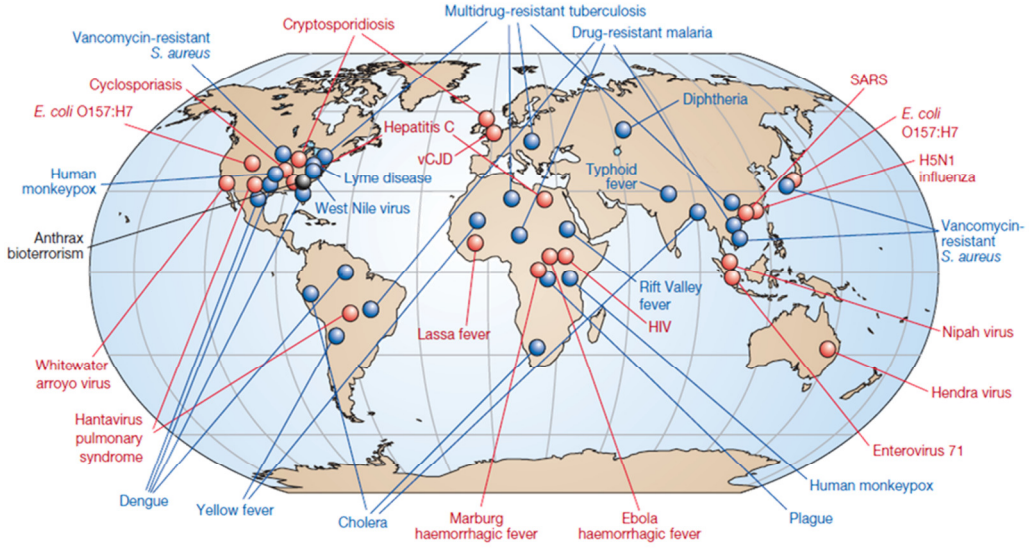
CHAPTER 1

General Introduction

1.1 Critical dearth of new substances for human application

There are currently more over 10 million compounds available for application to the myriad aspects of human life. However, there is an unlimited demand for new substances due to the daily emergence of new diseases or drug-resistant pathogens as well as the continued presence of untreatable diseases, which together cause 15 million (>25%) of the 57 million annual deaths worldwide. The emergence of new pathogens has been recorded from ancient times as an important thread of human history, and has been responsible, of course, for an incalculable number of illnesses and deaths. In recent decades, we have been faced with severe new diseases and re-emergent conditions, including tuberculosis, cancer, AIDS, SARS, avian influenza (H5N1 and H7N9), 2009 pandemic flu/swine flu (H1N1), and infections caused by multidrug-resistant microorganisms.[2,3] In addition, new therapeutic agents have been required for the cure of opportunistic infections such as *Aspergillus*, *Cryptococcus*, and *Candida*, which can exploit patients with a weakened immune system but are normally not a major problem in healthy humans. At the same time, new medicinal agents are needed to treat parasitic protozoan and nematode infections such as malaria, leishmaniasis, trypanomiasis, and filariasis. Similarly, in the field of agriculture, there has been a demand for chemical substances which can function as growth supplements, disease treatments, or pesticides. Natural compounds are regarded as a hopeful choice for

innovation in both medicine and agrochemistry, particularly because of their greater ecological safety and greater variety of chemical structures compared with synthetic chemical compounds.



Red: newly emerging diseases; blue: re-emerging/resurging diseases; black: a "deliberately emerging" disease.

Figure 1.1 Global diagram of emerging and re-emerging infectious diseases[1]

1.2 Natural products as a potential source for new compounds

Since the discovery of the first antibiotic in 1929, there has been an ever increasing demand for substances to cope with medicinal and agricultural problems. Research into the novel metabolites of natural products, and especially microorganisms, was begun to meet this demand, and continues to be a fascinating area of investigation. In the early "golden age" of this research, numerous novel metabolites were explored from new natural sources together with semi-synthetic antibiotics from the pharmaceutical industry. The number of new antibiotics increased almost exponentially. However, from the end of the golden age to the present time, the number of newly discovered compounds significantly decreased. On the other hand, the number of

resistant microbes has expanded, causing an imbalance in the ratio of new compounds to resistant pathogens. Based on this depressed status of compound discovery, we should now refocus on such research, improving the methodology in order to explore new natural resources and untap the chemical diversity of nature.

Table 1.1 Approximate number of known synthetic compounds, natural products, and microbial products

	Synthetic chemical compounds	**Natural products**	**Microbial products**
Numbers	8-10 millions	~500,000	~70,000
Drugs	2,000-2,500	1,200-1,300	450-500
Percentage	0.005	0.6	1.6

A report by Bérdy in 2012 described that over 10 million known chemical compounds had been reported, mainly from the field of synthetic chemistry (Table 1.1). Although the number of compounds from natural sources is only about one-tenth of the number from synthetic compounds, the compounds from nature, especially those from microorganisms, tend to be much more bioactive than synthetic compounds (Table 1.2). In addition, the natural products can be obtained with less stress to the environment, and are more structurally diverse. Hence, natural products are expected to serve as a powerful and promising resource of novel compounds.[4]

Table 1.2 Approximate numbers of known natural products (2010)[5]

Natural products	All known	All bioactive	Antibiotic[a]	Prospects
	Over 500,000	**80-100,000**	**30-40,000**	**+++**
Microbial metabolites:	60-80,000	32- 34,000	25-27,000	++
Actinobacteria	~20,000	~12,000	~10,000	++
Fungi[b]	~30,000	~15,000	~10,000	++
Unicellular bacteria[c]	~20,000	~7,000	~5,000	+
Higher organisms:				
Plant kingdom				
Lower plants (algae, etc.)	50-60,000	~6,000	~100-200	?
Higher plants	300-350,000	~25,000	~15,000	+
Animal kingdom				
Marine invertebrates	25-30,000	~10,000	~5,000	+ +
Terrestrial animals	4-5,000?	~200	~100	?
Synthetic compounds:				
Semisynthetic antibiotics	Over 200,000	?	Almost all	+ +
Mimics, analogues	Over 50,000	?	Few	+ ?
Total synthesis products	Millions?	?	~1,000	±

[a]Anti-infective, antitumor and antiviral compounds.

[b]Microscopic and basidimicetous fungi, myxomycetes, slime molds, yeasts.

[c]Eukaryotes including cyanobacteria and myxobacteria.

Since the discovery of penicillin, the first antibiotic, the majority of antibiotics have been derived from *Streptomyces*, a gram positive bacteria. In the early age of microbial metabolites development (1940-1974), 62% of all microbial metabolites came from actinobacteria, mainly *Streptomyces*, while other bacterial and fungal sources

accounted for smaller amounts of 15% and 23%, respectively. In the mid-age of microbial metabolite development (1975-2000), the ratio of the original sources was changed. The role of fungi as secondary metabolite producers has continued to expand, especially from the early 80s, and today the metabolites from fungal sources account for the same percentage (45%) of metabolites as do those from actinobacteria. From the mid-era to the present (2000-2010), the percentages of antibiotic sources have shifted further. The fungal origin has become a major source of natural compounds (61%), while actinobacteria account for less than one-third (28.5%) (Table 1.3).

Table 1.3 Approximate numbers of bioactive microbial metabolites from 1940 to 2010[5]

Species \ Periods	**1940-1974**	**%**	**1975-2000**	**%**	**2001-2010**	**%**	**Total**
Actinobacteria	3,400		7,200		3,100		13,700
Streptomyces sp.	2,900	62	5,100	42	2,400	28.5	10,400
Other actinobacteria	500		2,100		700		3,300
All microscopic bacteria	800	15	2300	13	1100	10	4200
Myxobacteriales	25		400		210		635
Cyanobacteria	10		30		1250		1290
All fungi	1300	23	7700	45	6600	61	156000
Microscopic fungi	950		5400		4900		11250
Basidiomycetes	300		1800		1500		3600
Other fungi	20		200		160		380
Total per year	5500	100	17000	100	10800	100	33500

1.3 Endophytic fungi

1.3.1 General information and definition

The word “endophyte” comes from the Greek “endon,” meaning inside or within, and “phyton,” meaning plant. Endophytic fungi are fungi that colonize inside the plant’s tissue without causing symptoms of disease to the hosts. They exist in most plant parts, such as the leaves, stems, and roots, and are ubiquitously isolated from almost every terrestrial and aquatic plant studied so far. Ascomycota in the anamorph stage are commonly found endophytes. Endophytes are closely related to pathogenic fungi and can sometimes be opportunistic pathogens.

Endophytic fungi can be divided into 2 major groups: clavicipitaceae and non-clavicipitaceae. The clavicipitaceae, which include *Epichloe* sp. and *Neotyphodium* sp., are generally distributed in grass hosts, and several species of clavicipitaceae are systemically transmitted to the next generation vertically by way of the seeds of the host grasses.[5] In contrast, non-clavicipitaceae are composed of a broad range of species in several families, all of which are members of Dikarya (Ascomycota or Bisidiomycota). Non-clavicipitaceae are not systemically distributed, but are present in almost all host plant species, including grasses.[6]

1.3.2 Plant-fungal interaction and relationship

Endophytes inhabit most of the more than 300,000 species around the world. Endophytes exist symbiotically within their host plants without harming them. The interaction between plants and endophytes and their taxonomic relationship will be described in this section.

1.3.2.1 Mechanisms in the establishment of endophytic symbiosis

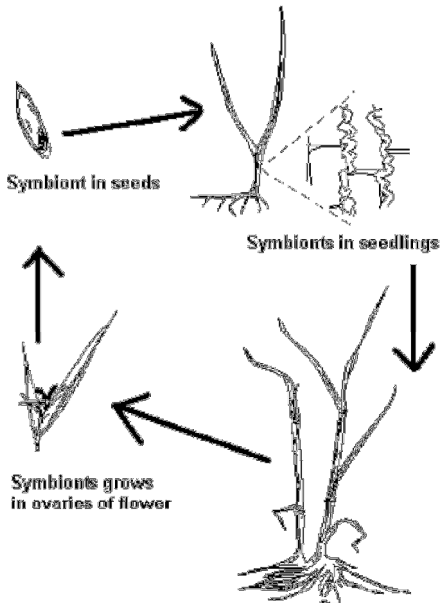


Figure 1.2 Endophyte asexual life cycle[1]

The initial steps of host establishment, including recognition, germination and penetration, are same as infection of fungal plant pathogens. During the pathway, the fungi have to overcome plant defense mechanisms. The fungal spore often recognizes the host via lectin-like molecules. After germination, the fungi penetrate into plant tissue by softening the cuticle and the wall of epidermal cells or breaching the cuticle by mechanical force. Since the penetrated fungi shift to a silent state, the defense mechanism of the host plant is not activated. In the review of Sieber, this phenomenon is explained by a Gene-for-Gene (GFG) model, in which an avirulence gene (avr) of endophyte encoded an elicitor is recognized by the product of the resistance gene (R) of hosts and then a hypersensitive reaction of host plants and subsequent quiescence occurs via a signal transduction pathway. In contrast, the pathogen does not contain the avr gene, and thus the R product is not produced and the disease symptoms do not develop.[6,7]

The interaction of endophytic fungi with host plants results in a compromise between mutualism and antagonism to create a harmonious symbiotic system. Plants can limit the growth of endophytes, and thus endophytes may use a wide range of mechanisms to survive. Endophytes not only decompose some plant metabolites with ectoenzymes in order to retrieve essential nutrients and energy to survive, but produce beneficial compounds and/or support or promote the growth of host plants to achieve a balanced living environment. Furthermore, the interaction requires that the morphological and physiological changes and the life cycle of endophytes be matched for symbiosis.[8] The symbiosis brings benefit in a variety of areas, such as increased resistance to herbivores, pathogen drought and flooding stress, enhanced competitive abilities, and enhanced growth.

1.3.2.2 Plant and fungi co-evolution

Endophytes and their host plants are thought to be co-evolved, judging by the fact that the closely related endophyte species are present in host species of the same plant family. Clear evidence of the co-evolution between plant and fungi has been observed in the relation between gymnosperms and angiosperms. Most of the endophyte species from angiosperms, broad leaf-plants, such as Aceraceae, Betulaceae, and Fagaceae, belong to the order Diaporthales, whereas endophyte species from gymnosperms, needle-like leaf-plants, such as Cupressaceae and Pinaceae, belong to the order Helotiales. In the evolution of plants, the divergence between angiosperms and gymnosperms appears to have occurred around 300 million years ago based on molecular data,[9] which corresponds to the divergence between Diaporthalean and Halotialean ascomycetes.[10,11] Thus, the dominant endophytes, Diaporthales in

angiosperms and Helotiales in gymnosperms, appear to have co-evolved with their hosts for 300 million years. The bitunicate endophytes also exist in both angiosperms and gymnosperms such as Dothideales, Pleosporales, Mycosphaerellales, and Xylariales. The fact that the bitunicate endophytes inhabit both plant groups might be attributable to the early divergence of the common ancestor of Helotiales and Diaporthales, meaning that this divergence should have occurred before the divergence of gymnosperms and angiosperms (Figure 1.3). The host-endophyte co-evolution is also suggested by the high relationship of dominant endophytes in *Abies*, *Tsuga* and *Pinus* species. *Phyllosticta* spp. are dominant only in *Abies* and *Tsuga*, which are more closely related to each other than *Pinus*, whereas *Cyclaneusma* spp. are only found in *Pinus.* On the other hand, some endophytes have been obtained from non-specific host plant such as Xylariales and Dothideales. They exist as endophytes in a wide range of plant species. The host non-specific colonization might result from some mutualistic benefits to a host, probably by enhancing fitness, or protection from biotic or abiotic stress. The host non-specific, apathogenic species *Collectotrichum* provides benefits for disease resistance, drought tolerance and growth enhancement.[12] The species *Curvularia* belongs to Dothideales and provides thermal protection for *Dichanthelium lanuginosum* against increases in soil temperature.

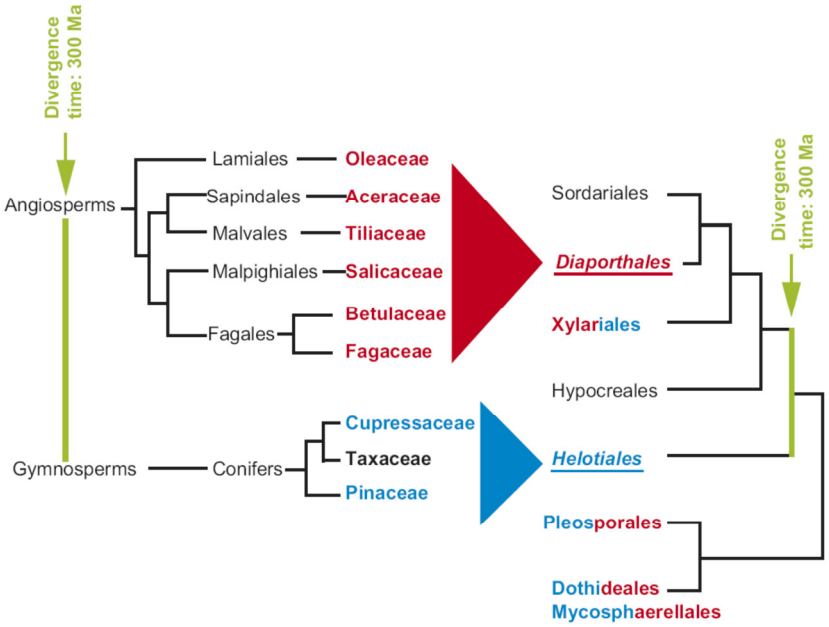


Figure 1.3 Comparison of the genealogical trees of the fungal orders and plant families

1.3.3 Rationale for endophytic fungal isolation

The rational selection of host plant is crucial to increase the chances of isolation of novel microorganisms which may produce new bioactive compounds. The plant sources should be selected based on the following criteria: 1) high biodiversity; 2) unique habitats; 3) use as traditional medicines; 4) high rate of infestation by pathogens. Plants growing in an area of great biodiversity are expected to house various endophytes. Hence, tropical rain forests are regarded as an endophyte storehouses with great biodiversity. The endophytes which inhabit unique plants might possess special capabilities for survival, and hence might produce unique secondary metabolites. Medicinal plants are plants that have been used for their medicinal properties in herbalism. Medicinal plants have been utilized for a wide range of properties, such as anti-inflammatory, anti-fungal, insect repellent, antiseptic, antibacterial, fever reduction, antihistamine and pain relief properties. Endophytic fungi from medicinal plants can be

regarded as a rich source of functional metabolites because the active components of such medical function. The last criterion is included because healthy plants that are surrounded by infectious pathogens are expected to house endophytes which might secrete antipathogenic compounds.[13] All these criteria would enhance the possibility of obtaining endophytes with the potential to produce novel metabolites.

1.4 Bioactive compounds from endophytic fungi

Although the complex relationship between endophytic fungi and plants has not been clarified, their unique symbiotic relationship gives endophytes the powerful ability to produce novel bioactive substances. Over the last 20 years, endophytic fungi have been viewed as an outstanding source of secondary metabolites from natural product. A variety of biological activities and chemical structures have been discovered.

1.4.1 Anticancer activity

The most famous and fascinating compound in the history of secondary metabolites from endophytic fungi is paclitaxel (taxol). This complex diterpenoid compound is an active anticancer drug found in the bark of a yew tree species. The mode of action of paclitaxel is unique in that it prevents the depolymerization of tubulin during the process of cell division. Not only paclitaxel production, but also the production of other potential anticancer compounds has been reported. Torreyanic acid, a specific cytotoxic quinone dimer, was identified from *Pestalotiopsis microspora* isolated from the endangered tree *Torreya taxifolia.* Torreyanic acid showed 5-10 times more potent cytotoxicity against several cancer cell lines sensitive to protein kinase C agonists, and caused cell death via apoptosis. An alkaloid, 22-oxa-12-cytochalasins,

which displayed antitumor activity, was isolated from *Rhinocladiella* sp., an endophyte on *Triptergium wilfordii*.[14,15]

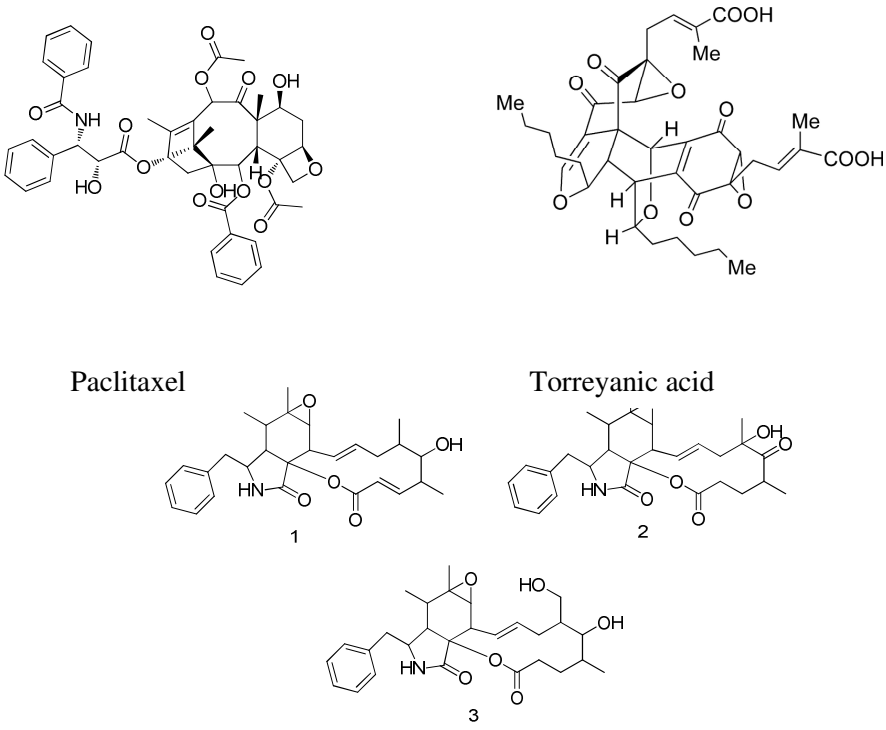
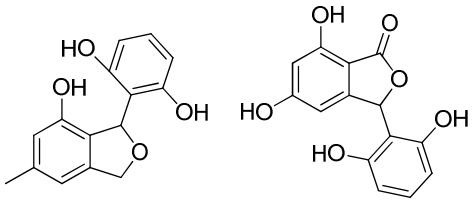


Figure 1.4 Anticancer compounds from endophytic fungi

1.4.2 Antioxidant activity

Many antioxidant compounds have been obtained from endophytic fungi. Pestacin and isopestacin were obtained from *Pestalotiopsis microspora*, endophytes isolated from the Papua New Guinea plant, *Terminalia merobensis*. Based on its structural similarity to the flavonoids, the antioxidant activity of isopestacin was attributed to the scavenging ability of both superoxide and hydroxyl free radicals. The antioxidant activity of pestacin has been evaluated to be at least ten times higher than that of trolox, a vitamin E derivative, via cleavage of an unusually reactive C-H bond and to a lesser extent, through O-H abstraction.[16,17]



pestacin isopestacin

Figure 1.5 Antioxidant compounds from endophytic fungi

1.4.3 Antimicrobial activity

So far, a large number of metabolites displaying antimicrobial activity have been isolated from endophytic fungi. The compounds are classified into several chemical structural groups such as alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids.[13] They include compounds showing antibacterial, antifungal and antiviral activities. Examples of antimicrobial agents with antifungal activity include cryptocandin, cryptocin, ecomycins, pseudomycins, pestaloside, and pestalopyrone; examples of antimicrobial agents with antibacterial activity include periconicins A and B, phomopsichalasin, and javanicin; and examples of antimicrobial agents with antiviral activity include cytonic acid A and B.[1,17,18]

Some of the antimicrobial agents from endophytic fungi are active not only against human pathogens but also against plant pathogens, leading to their application in agriculture fields.

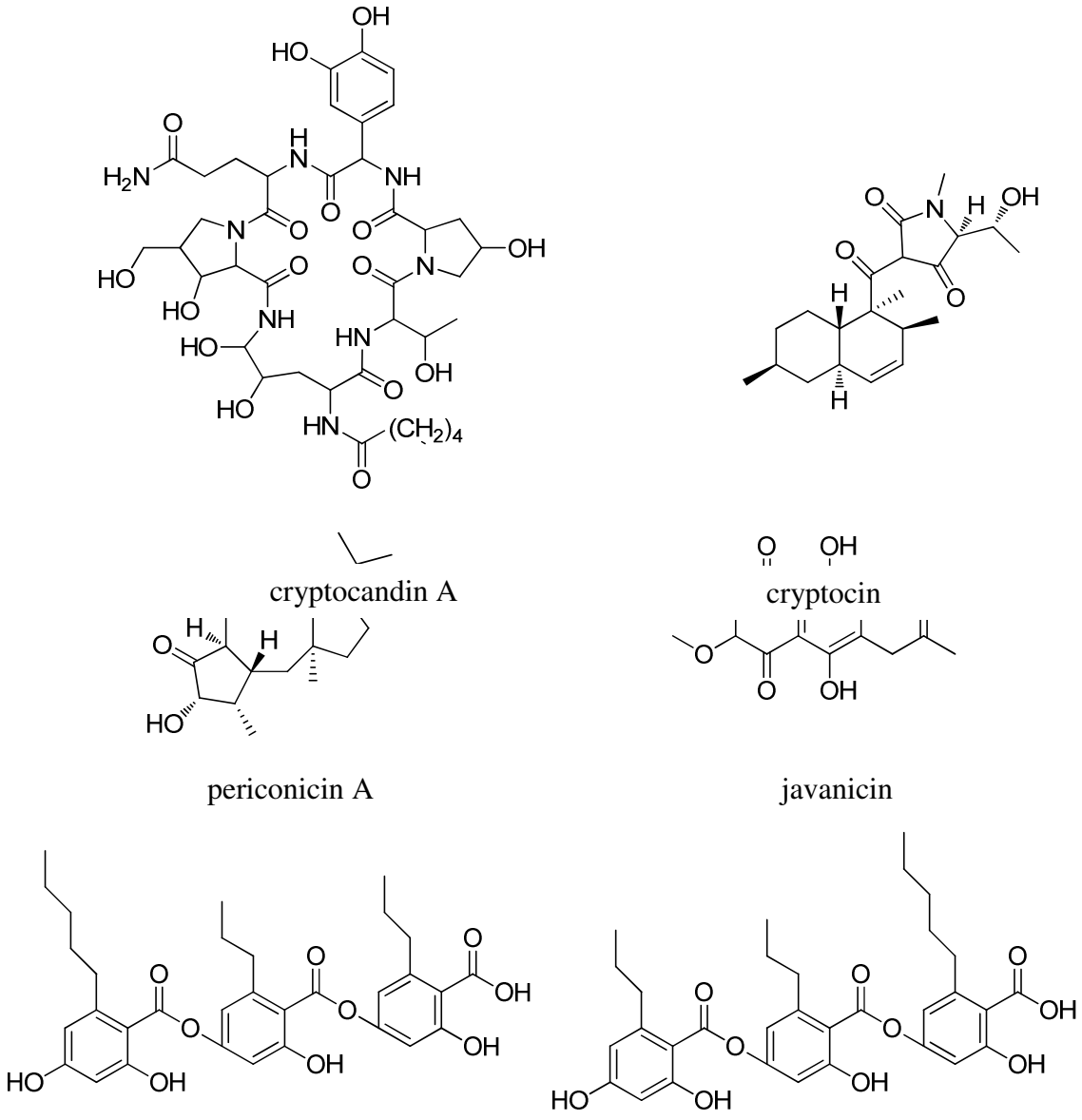


Figure 1.6 Antimicrobial compounds from endophytic fungi

1.4.4 Other activities

In addition to the activities mentioned above, several compounds from endophytic fungi display a variety of activities. Nodulisporic acids, which are novel indole diterpenes, exhibit potent insecticidal properties against a larvae of the blowfly.

Subglutinols A and B, noncytotoxic diterpene pyrones, are potent as immunosuppressants. L-783,281, a nonpeptidal fungal metabolite, is used as an antidiabetic agent as an insulin mimetic. Chlorinated, epimeric 1,3-oxazinane derivatives isolated from the endophytic fungal strain *Geotrichum* sp. AL4, showed clear bioactivities against the nematodes *Bursaphelenchus xylophilus* and *Panagrellus redivivus*.[3,1,19]

1.5 Endophytic fungi producing bioactive compounds from medicinal plants in Thailand (tropical endophytic fungi)

Based on studies on the diversity of tropical endophytic fungi in several tropical regions, it has been hypothesized that tropical endophytic fungi tend to be less host-specific than their temperate counterparts, because the highly diverse ecosystem could act against host specificity due to the close distribution of hosts. Many reports on the discovery of novel compounds have demonstrated that the tropical endophytic fungi have great potential as a source of novel chemical substances. Thailand is a tropical country with high biological diversity and abundant bioresources, suggesting that Thai endophytic fungi could be a rich source of bioactive compounds. Although since 2000-2013, the reports of new compound isolation of endophytic fungi isolated from Thai medicinal plants were obtained in few reports when compared with a total number of the medicinal plants recorded over 1,157 spp. in Thailand (PHARM database (MedPlant Online), The Medicinal Plant Information Center (MPIC), Faculty of Pharmacy, Mahidol University, http://www.medplant.mahidol.ac.th/index.asp (*In Thai*)). Several reports have exhibited the isolation of novel bioactive compounds from Thai medicinal

plants, such as xylariaquinone A, scoparasins A and B, 11-hydroxymonocerin, and phomoenamide.[20,21,22,23] (Figure 1.7)

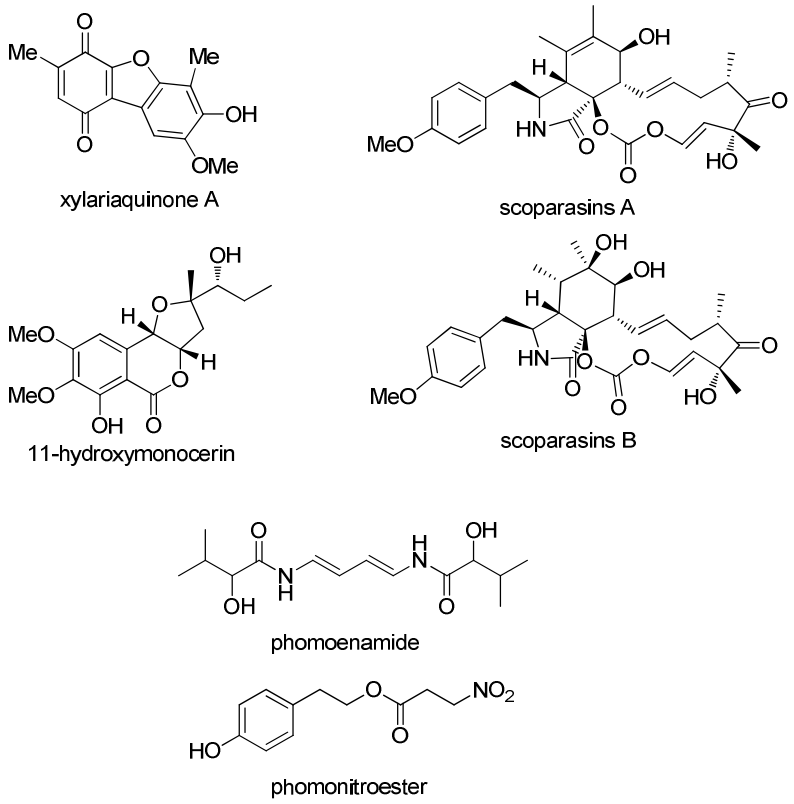


Figure 1.7 Compounds from Thai endophytic compounds

1.6 Overview and objective of this study

In this study, Thailand, a tropical country rich in biodiversity and biological resources, was selected as a source of medicinal plants to obtain endophytic fungi. The aim of this research was to obtain novel compounds and assess the potential of endophytic fungi inhabiting medicinal plants in Thailand. In Chapter 2, the isolation and the taxonomic study of endophytic fungi, and the screening criteria are described. Eighty-six endophytic fungi were isolated from the leaves of 30 *spp.* of medicinal plants in the botanical garden of Mahidol University in Bangkok, Thailand. Taxonomic

identification was used to identify the fungi in order to assess the biological characteristics and metabolic production of each isolate. The screening criteria were established in order to identify novel chemical substances. The results of the preliminary screening are also discussed in this chapter. In Chapter 3, the details of the isolation, characterization, and biological activity of four novel compounds, xylaropyrone, mycoleptione, and bipolamides A and B, are described. Xylaropyrone, a novel pyrone compound consisting of 2 unique branch chains, was obtained from *Xylaria feejeensis* MU18. Mycoleptione was isolated from *Mycoleptodiscus* sp. MU41. Mycoleptione is a chromone derivative compound with a modification of an ethyl side chain instead of a methyl group. Bipolamides A and B, two triene fatty acid amide chains, were characterized from a culture of *Bipolaris* sp. MU34. Finally, in Chapter 4, a summary and future perspective is given.

Chapter 2

Isolation and taxonomic identification of endophytic fungi from medicinal plants in Thailand, and screening of novel compounds from the isolates by chemical library analysis

2.1 General introduction

Fungal entophytes are microorganisms that live asymptomatically in plant tissue and can be found in almost all terrestrial and aquatic plants.[24,25] Judging from the fact that more than one endophyte often inhabits a single plant, new and interesting endophytic microorganisms are likely to be found from the nearly 300,000 plant species that inhabit the diverse environments and ecosystems of the earth. Moreover, a great number of secondary metabolites with diverse chemical structures and various biological activities[26,27,28] have been discovered from endophytes. Also, the ability of endophytes to precisely or partially imitate the production of compounds in plant metabolites has been an attractive feature for natural product researchers.[29-31]

Tropical plants are considered to be highly diverse not only because they grow under a great abundance of nutrients and proper abiotic environments, but because the complicated food chains and the complex overlapping of ecological niches in tropical areas require high competition and adaptation in order for organisms to survive. These factors make tropical plants a fascinating potential source of endophytic fungi. Hence plants in Thailand, particularly medicinal plants which have been used as traditional herbal medicines, are one of the hopeful areas for obtaining highly diverse endophytic

fungi, because several novel bioactive substances were recently discovered from fungal endophytes isolated from Thai medicinal plants, e.g., 3-nitropropionic acid, cis-2-hydroxym-ethyl-3-methylcyclopentanone, xylariaquinone A, scopararanes A and B, cyclopentenones, and 11-hydroxymonocerin.[31,32,21,22,23]

Historically, taxonomical identification of filamentous fungi has been a very difficult task. The conventional method for fungal identification is based on morphological differences, and thus specialized skills are needed to accurately classify fungi at the species level in the conventional manner. To overcome this limitation, molecular identification is currently used as a more efficient method of fungal classification.

Since the nucleotide sequence of rDNA changes very slowly, rDNA sequencing is useful as a molecular taxonomical tool for evolutionary comparison among relatively distant organisms. rDNA consists of highly conserved regions and variable regions such as internal transcribed spacer (ITS) regions and 18S DNA. Fungal rRNA operons contain two ITS regions (Figure 2.1). One is located between the 18S and 5.8S rRNA genes (ITS1) and the other exists between the 5.8S and 28S rRNA genes (ITS2). The sequence of the two ITS regions are accumulate mutations at a faster rate than the 5.8S, 18S, and 28S rRNA genes because the two ITS sequences are excised and are not required for any functional purpose after the transcription of rRNA operon. Hence the analysis of ITS regions has been useful for clarifying a number of problems associated with fungal classification, such as determining the phylogenetic relationship among isolates.

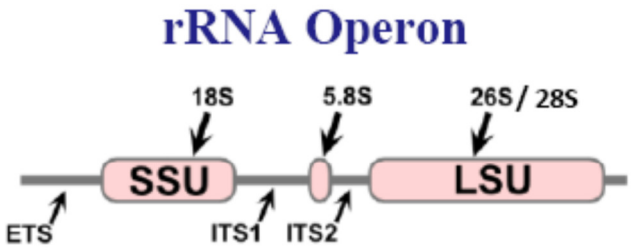


Figure 2.1 Use of the ITS regions for fungal systematics and classification.

In this study, 30 spp. of medicinal plants in Thailand were selected as the source of endophytic fungi. The isolates were taxonomically identified based on the 18S and ITS rDNA sequences and were used as fungal source for screening and isolation of novel bioactive compounds.

Although the screening strategy is the key to obtaining novel metabolites, the majority of current screenings are still conducted using conventional bioassay methods. The target compounds are relatively easily isolated and purified by tracing the target activity, while the bioassay-based screenings are likely to hit known compounds with higher activity. Moreover, bioassay-based screening theoretically may overlook novel compounds which do not display target-activity but have novel chemical structures. Hence, instead of bioassay-based screening, screening dependent on physiochemical properties might be used as an alternative method. Each compound has particular physiochemical properties. The molecular structure can be investigated by several methods based on quantum mechanics, such as infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, UV/Vis spectroscopy, and mass spectrometry (MS). Although those methods which measure the physiochemical properties can be used as tools for finding novel compounds efficiently among various known metabolites by comparing the properties of the molecule with a database, screening with the UV/Vis spectrum is regarded as an outstanding technique for the establishment of a database,

because the data are simply acquired by high performance liquid chromatography (HPLC) analysis using a photodiode array. In this study, the metabolites extracted from the culture of endophytes will be analyzed by HPLC by comparing each spectrum to an in-house UV-Vis library database in which the UV/Vis spectra of registered known metabolites. The similarity of the UV spectrum to a spectrum in the database will be used as the criterion to judge whether the compound should be purified as a candidate for a structurally novel compound. This process, known as chemical library analysis, was used for the screenings in this study.

In this chapter, I describe the isolation of endophytic fungi from the leaves of Thai medicinal plants, and their taxonomic identification via 18S and ITS rDNA sequence analysis. Lastly, the screening criteria are noted.

2.2 Materials and methods

2.2.1 Isolation of endophytic fungi from Thai medicinal plants

Healthy leaves of Thai medicinal plants (30 species) were collected at the botanical garden in Mahidol University, Bangkok, Thailand. The leaves were sterilized with 1% NaOCl solution followed by 70% ethanol, and were incubated on water agar plates containing streptomycin (20 µg/ml) and chloramphenicol (30 µg/ml) at 28^{o}C. Fungi emerging within a week were not regarded as endophytes and discarded. After 2 weeks, the developed mycelia on the plate were picked up and transferred to potato dextrose agar (PDA) plates as stock culture.

2.2.2 Taxonomic identification

2.2.2.1 Genomic DNA extraction and PCR amplification

The mycelia of 86 endophytic fungi were harvested from the agitated cultures in 5 ml YPD medium at 28°C for 5-7 days, and then the genomic DNAs were extracted using ISOPLANT II (Nippon Gene Co., Ltd., Tokyo, Japan). Their partial 18S rDNA and ITS sequences were amplified with the primer pairs NS1-1 (5'-TAGTCATWTGCTTGTCTYAAA-3') / SR6-1 (5'TTTTASTTCCTCTAAAYGACC-3'),[24] and ITS1 (TCCGTAGGTGAACCTGCGG) / ITS4 (TCCTCCGCTTATTGATATGC),[33] respectively. The PCR amplifications were performed using Ex *Taq* polymerase (Takara, Shiga, Japan) in Wizard® SV Gel and a PCR Clean-Up System (Promega, Madison, WI) under both partial 18S and ITS rDNA primer conditions. The detailed conditions were as follows: 5 min of initial denaturation at 94°C; followed by 35 amplification cycles of 30 sec at 94°C for denaturation, 30 sec at 50°C for annealing, and 1 min at 72°C for extension; and then a final extension for 7 min at 72°C.

2.2.2.2 Partial 18S and ITS rDNA sequencing and strain identification

The PCR products of the partial 18S and ITS rDNA sequences were sequenced using an ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) with a BigDye® Terminator Cycle Sequencing kit version 3.1. Sequences were analyzed with the Genetyx program version 7.0.5 (Genetyx Corp., Tokyo, Japan) as well as with the BLAST program through the database at the National Center for Biotechnology Information (NCBI) in order to characterize the isolates.

2.2.3 Secondary metabolic screening from endophytic fungi using chemical library analysis

2.2.3.1 Fermentation and extraction

All chemicals, media, and reagents were purchased from Wako (Osaka, Japan) unless stated otherwise. In order to screen novel compounds produced by endophytic fungi, liquid static cultivation was adopted. Media 2 and 6 were selected from among 11 media (Appendix 1) which yielded higher average secondary metabolite production and the highest levels of growth of endophytic fungi. The components of the media are shown in Table 2.1.

In the first screening, 86 endophytic fungi were cultured with optimized media, media 2 and 6. Pre-culture of static cultivation was conducted at 28^{o}C for 3 days with 3 ml of liquid media using test tubes of Ø 12.5 x 105 mm. Static cultivation was started by transferring 0.2 ml of pre-cultured broth to 10 ml of new media with the same compositions in test tubes of Ø 16.5 x 150 mm and cultivated at 28^{o}C for 21 days. After liquid static cultivation of all endophytic fungi, 10 ml of whole culture broth was transferred to a Falcon tube, mixed with 5 ml of *n*-butanol and shaken at room temperature for 1 hr. The *n*-butanol layer was separated by centrifugation and 1 ml of the extract was put into 1.5 ml microcentrifuge tubes and dried by vacuum centrifugation for 4-6 hr.

For the second screening, endophytic fungi were cultured in the same optimized medium. The pre-culture for static cultivation was conducted at 28^{o}C for 3 days with 5 ml of liquid media using test tubes of Ø 16.5 x 150 mm. Static cultivation was started by transferring 0.6 ml of pre-cultured broth to 30 ml of new media with the same

compositions in a 100 ml Erlenmeyer flask. The cultures were incubated for 21 day at 28°C. After 21 days, 30 ml of whole culture broth was transferred to a Falcon tube, mixed with an equal volume of ethyl acetate (EtOAc) and shaken at room temperature for 1 hr. The EtOAc layer was separated by centrifugation and 1 ml of the extract was put into 1.5 ml microcentrifuge tubes and dried by vacuum centrifugation for 1-2 hr.

Table 2.1 Optimized media compositions for liquid static cultivation

Media	Compositions
2	Soluble starch 5%, Pharmamedia (Southern Cotton Oil Co., Memphis, TN) 2%, oatmeal 0.5%, KH_2PO_4 0.35%, Na_2HPO_4 0.25%, $(NH_4)_2SO_4$ 0.6%
6	Glucose 1%, mannitol 10%, Pharmamedia 3.5%, KH_2PO_4 0.9%

2.2.3.2 Data-couple-HPLC analysis

After evaporation of the extraction solvent, residues of extract were re-suspended with dimethyl sulfoxide (DMSO) and filtrated through 0.45 µm filter membranes before analysis by HPLC-DAD. The extract metabolites were separated by reversed phase-HPLC and a photodiode array detector (DAD) detector (200-600 nm) was used to record their characteristic UV spectra. The conditions for the HPLC analysis were as follows: Varian Microsorb C_{18} column (4.6 x 100 mm) on an Agilent HP1100 system with a binary pump equipped with DAD for 200 to 600 nm. The mobile phase used the following stepwise gradient of acetonitrile (CH_3CN) and phosphate buffer at pH 3.5: 15% (CH_3CN) for 0-3 min, 15-40% CH_3CN for 6-12 min, 40-55% CH_3CN for 12-19 min, 55-85% CH_3CN for 19-22 min, 85% CH_3CN for 22-29 min and 85-15% CH_3CN for 29-32 min, with a flow-rate of 1.2 ml/min. The absorption spectra of characteristic

peaks were analyzed by comparison with those in the HPLC-UV/Vis database which containing over 1600 registered compounds. The peaks were compared with UV/Vis spectra and the retention times (optional) to characteristic peaks in an in-house library database.

2.2.3.3 LC/MS analysis

In the second screening, the LC/MS technique was used as an additional tool to predict the molecular weights of target peaks. The dried extracts were re-suspended with dimethyl sulfoxide (DMSO) and filtrated through 0.45 µm filter membranes before analysis by HPLC-DAD. The LC/MS was performed using a time of flight mass spectrometer (Bruker microTOF; Bruker Daltonics, Bremer, Germany) equipped with an electrospray ionization (ESI) source. The HPLC separation was carried out on an Agilent HPLC1200 system with a binary pump using a Cosmosil 5C18-AR-II column (Ø 2.0 x 150 mm; Nacalai Tesque Inc., Kyoto, Japan). The mobile phases were water with 0.1% formic acid and acetonitrile, respectively. The gradient elution at a flow rate of 0.2 ml/min was performed as follows: 15% CH_3CN for 0-5 min, 15-100% CH_3CN for 5-40 min, 100% CH_3CN for 40-50 min, 100-15% CH_3CN for 50-51 min, and 15% CH_3CN for 51-60 min. The full-scan mass spectra were obtained within a range of 50-2000 m/z. Data acquisition was conducted in positive and negative ion modes. Molecular ions from the LC/MS of target peaks were examined to estimate the molecular weight.

2.2.3.4 Screening tools

Five information sources were used as the tools for the first and second screenings: 1) taxonomic identification, 2) metabolic profiles from HPLC-DAD, 3) the in-house HPLC-UV/Vis database, 4) MS data, and 5) the dictionary of natural products on DVD version 20:1 (DNP) (Taylor & Francis Group, CRC Press, UK). In the first screening, three information sources were used. In the second screening, two additional information sources, MS data and the DNP, were combined with the three data sources from the first screening to improve the possibility of isolating new compounds. The details for each of the five screening tools are given below.

2.2.3.5 Criteria of the first screening

By using the three information sources, taxonomic information, metabolic profiles from HPLC data and HPLC-UV/Vis database analysis, five screening steps were established as follows: (1) excluding well-studied genera, (2) selecting highly produced metabolites, (3) excluding same compounds produced in the same genus (4) excluding compounds that matched with data in the UV-Vis database, and (5) excluding compounds displaying non-interesting UV spectra.

2.2.3.5.1 Excluding well-studied genera

By taxonomy-based information, four genera were judged to be well-studied: *Aspergillus* spp., *Furasium* spp., *Alternaria* spp. and *Colletotrichum* spp. Thus the isolates which belong to these genera were eliminated from selection.

2.2.3.5.2 Selecting high metabolic productions / Excluding duplicate compounds

Using metabolic profiles obtained from the reverse phased HPLC experiment, the high reproducible production profiles of compound peaks (area ≥100 mAU*s) were selected as candidate peaks. Furthermore, compounds that showed the same UV spectrum and retention time were eliminated from the screening flow.

2.2.3.5.3 Selecting compounds having unique UV spectra by comparing them with the in-house library / Excluding compounds displaying non-interesting UV spectra

Candidate compounds were finally selected from compounds for which the UV spectra did not match those registered in the in-house library in order to exclude known compounds (Figure 2.2). In addition, in this study, an attempt was made not to isolate compounds which showed UV spectra similar to those of complex aromatic polyketides (UV/Vis >400 nm), because such compounds could be easily detected and had already been isolated during the long history of screening discovery of filamentous fungi.

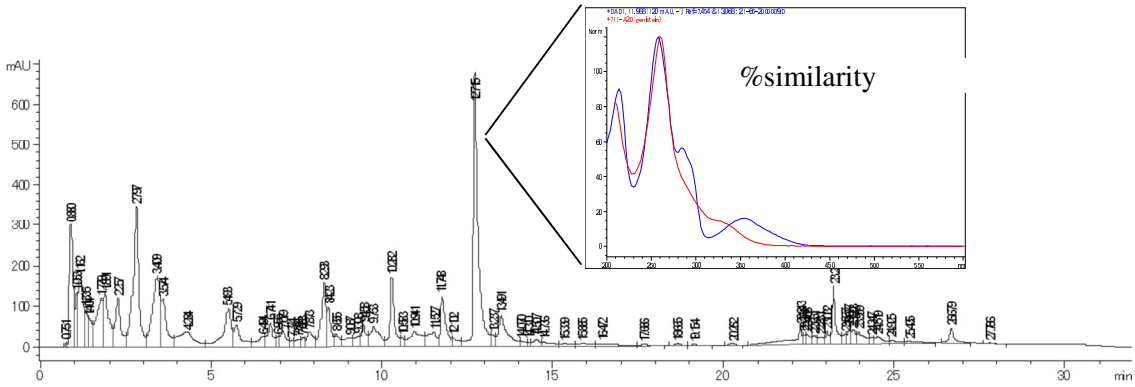


Figure 2.2 Data-couple-HPLC analysis

2.2.3.6 Criteria of the second screening

In the second screening, two additional tools, MS analysis and reference to the data from the DNP, were added to the screening flow. Using five different types of information, the following screening steps were established: (1) selecting metabolites with relatively high-level production, (2) excluding the duplicated compounds (3) excluding compounds that matched with data in the UV-Vis database, (4) excluding compounds displaying non-interesting UV spectra, (5) excluding compounds with small molecular weights, (6) excluding known compounds with DNP, and (7) selecting dominant compounds from a rare genera. The details of the second screening are described below.

2.2.3.6.1 Selecting highly produced metabolites / Excluding same compounds produced in same genus

The productivity of compounds was evaluated by the height of the peaks in the HPLC profile. The secondary metabolites for which the peaks were higher than those of medium components were regarded as highly produced, and were selected as candidates to be targeted. To avoid redundant isolation of the same compound, the peaks that appeared at the same retention time and showed the same UV-Vis spectra were assumed to be the same compound. The higher producer was selected as the representative source of the compound.

2.2.3.6.2 Excluding compounds matching with data in the UV-Vis database / Excluding compounds displaying non-interesting UV spectra

Just as in the first screening, the candidate compounds were selected when the UV/Vis spectra of the compounds failed to match those of the compounds registered in the in-house library. In addition, the compounds that displayed an absorption maxima at around 400-600 nm were excluded to avoid the isolation of known aromatic polyketide compounds, because many polyketide compounds from filamentous fungi possess absorption maxima in that range.

2.2.3.6.3 Excluding small compounds

The molecular weights of metabolites selected by the above criteria were checked by LC/MS analysis. In order to avoid the isolation of compounds with simple chemical structures, metabolites larger than 300 atomic mass units (amu) were targeted.

2.2.3.6.4 Excluding known compounds

The metabolites selected by the above-described criteria were further analyzed using the DNP to prevent the inclusion of known compounds. This database contained more than 250,000 reported compounds. The UV/Vis spectrum, molecular weight and genus of fungal producer were used as queries to check with the DNP. If the data of compounds were identical with compounds registered in the DNP, the compounds were not entered into the final screening.

2.2.3.6.5 Selecting compounds dominantly from rare genera

To increase the possibility of obtaining new compounds, the purification was conducted dominantly from the metabolites produced by a genus from which only small numbers of compounds have been reported in the DNP.

2.3 Results and discussion

2.3.1 Isolation of endophytic fungi from Thai medicinal plants

Healthy leaves of Thai medicinal plants (30 species) were collected at the botanical garden in Mahidol University, Bangkok, Thailand. Sterilized surface-leaves of 30 species of Thai medicinal plants were cultured on water-agar plates containing streptomycin (20 µg/ml) and chloramphenicol (30 µg/ml) as antibacterial agents. The cultures were incubated at 28^{o}C, and the mycelia of fungi emerged within a week assuming as non-endophytes were removed. The mycelia that emerged after two weeks were transferred to PDA plates and collected for endophytic culture stock. From 30 spp. of plants, we obtained 86 endophytic isolates (average 2-3 fungal isolates/plant) named MU01-MU86, which were deposited at ICBiotech, Osaka University, Osaka, Japan. The list of endophytic isolates and their plant hosts is shown in Table 2.3.

2.3.2 Taxonomic identification

Eighty-six endophytic fungi were identified by their partial 18S and ITS rDNA sequences. The sequences were analyzed with BLAST in order to identify their taxonomic information. From the partial sequence of 18S rDNA, the similarity of sequences showed a high conserved region between each isolates. It was difficult to

make the genus-level identification by using only a partial 18S rDNA sequence because the sequence was highly reserved. The ITS rDNA sequences contained partial 18S rDNA, ITS1 (internal transcribed spacer1), 5.8S rDNA, ITS2 and partial 28S rDNA sequences. The sequence lengths were approximately 500-600 bp. The conserved sequences from rDNA of the 18S, 5.8S and 28S regions could be observed while a variation in the ITS region was also clearly noted. Based on the ITS sequence, the BLAST results identified the genera of the endophytes, some isolates could be identified until species level depend on the taxonomic history of each fungi.

86 endophytic fungi were identified by ITS rDNA sequences and were grouped into 18 genera (Table 2.2 and 2.3). All isolates belonged to the phylum Ascomycota, which is the largest phylum of fungi, and exhibits a characteristic ascus formation Ascus is a microscopic sexual structure in which non-motile spores, called ascospores, are formed. All 86 isolates belonging to ascomycota could be divided into 3 classes: 61 isolates into sordariomycetes, 22 isolates into dothideomycetes, and 3 isolates into eurotiomycetes. The majority class of 86 isolates was sordariomycetes composed of 5 orders; glomerellales 36 isolates, xylariales 12 isolates, diaporthales 9 isolates, hypeocreales 2 isolates, and magnaporthales 2 isolates. Interestingly, the majority of isolates (36 out of 86) were from the genus *Colletotrichum* belonging to the order glomerallales. This genus was obtained from 20 spp. of total 30 spp. of plants, indicating that *Colletotrichum* is a dominant common endophyte in this fungal source as in the case of a previous report in which *Colletotrichum* was distributed in a tropical area as a common endophyte.[34] The second group belonging to dothideomycetes consisted of 2 orders: 7 isolates from pleosporales and 15 isolates from

botryosphaeriales. The smallest group was eurotiomycetes, in which there was only 1 order, i.e., eurotiales: 3 isolates were classified into the genus *Aspergillus*.

Table 2.2 Results of strain identification

No.	Genus	No. of isolates
1	*Lasiodiplodia*	1
2	*Leptosphaerulina*	1
3	*Nemania*	1
4	*Diaporthe*	2
5	*Fusarium*	2
6	*Guignardia*	2
7	*Hypoxylon*	2
8	*Mycoleptodiscus*	2
9	*Alternaria*	3
10	*Aspergillus*	3
11	*Cochliobolus*	3
12	*Diatractium*	3
13	*Daldinia*	4
14	*Phomopsis*	4
15	*Xylaria*	5
16	*Bipolaris*	6
17	*Phoma*	6
18	*Colletotrichum*	36

Table 2.3 Classification of 86 endophytic fungi and their host plants

No	Class	Order	Family	Genus	Isolate No.	Host plant
1	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	MU22	*Quassia amara L.*
2					MU85	*Ficus religiosa* L.
3					MU86	*Ficus religiosa* L.
4	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	*Guignardia*	MU35	*Gynura hispida* Thwaites
5					MU48	*Dillenia indica* L.
6				*Lasiodiplodia*	MU82	*Pithecellobium dulce* (Roxb.)
7			Pleosporaceae	*Alternaria*	MU20	*Quassia amara L.*
8					MU32	*Solanum sanitwongsei* Craib
9					MU33	*Solanum sanitwongsei* Craib
10				*Bipolaris*	MU34	*Gynura hispida* Thwaites
11					MU42	*Tinospora crispa (L.)* Miers ex Hook.f.f.& Thoms
12					MU43	*Tinospora crispa (L.)* Miers ex Hook.f.f.& Thoms
13					MU55	*Croton stellatopilosus* Ohba
14					MU58	*Senna alata* Roxb.
15					MU83	*Ficus religiosa* L.
16				*Cochliobolus*	MU21	*Quassia amara L.*
17					MU26	*Solanum trilobatum L.*
18					MU65	*Piper nigrum* Linn
19		Pleosporales	Didymellaceae	*Leptosphaerulina*	MU09	*Piper retrofractum* Vahl.
20				*Phoma*	MU19	*Eryngium foetidum* Linn.
21					MU61	*Ixeris debilis* A. Grey
22					MU62	*Ixeris debilis* A. Grey
23					MU63	*Ixeris debilis* A. Grey
24					MU78	*Pithecellobium dulce* (Roxb.)
25					MU79	*Pithecellobium dulce* (Roxb.)
26	Sordariomycetes	Diaporthales	Diaporthaceae	*Diaporth*	MU45	*Plantago major* Linn.
27					MU46	*Plantago major* Linn.
28			Valsaceae	*Phomopsis*	MU29	*Lonicer japonica* Thunb
29					MU30	*Lonicer japonica* Thunb
30					MU31	*Lonicer japonica* Thunb
31					MU57	*Senna alata* Roxb.

Table 2.3 Classification of 86 endophytic fungi and their host plants (continued)

No	Class	Order	Family	Genus	Isolate No.	Host plant
32	Sordariomycetes	Diaporthales	Valsaceae -	*Diatractium*	MU08	*Laurentia longiflora* Peterm.
33					MU36	*Spilanthes acmella* Murr.
34					MU47	*Plantago major* Linn.
35		Glomerellales	Glomerella	*Colletotrichum*	MU01	*Blumea napifolia* DC
36					MU02	*Blumea napifolia* DC
37					MU05	*Laurentia longiflora* Peterm.
38					MU06	*Laurentia longiflora* Peterm.
39					MU07	*Laurentia longiflora* Peterm.
40					MU10	*Piper retrofractum* Vahl.
41					MU12	*Acanthus ilicifolius*
42					MU13	*Acanthus ilicifolius*
43					MU14	*Acanthus ilicifolius*
44					MU15	*Clerodeendrum serratum var. serratum Schan*
45					MU16	*Clerodeendrum serratum var. serratum Schan*
46					MU17	*Eryngium foetidum* Linn.
47					MU23	*Quassia amara L.*
48					MU25	*Solanum trilobatum L.*
49					MU27	*Myristica fragrans* Houtt
50					MU28	*Maytenus marcanii*
51					MU44	*Plantago major* Linn.
52					MU49	*Dillenia indica* L.
53					MU51	*Pandanus amaryllifolious* Roxb.
54					MU54	*Croton stellatopilosus* Ohba
55					MU56	*Croton stellatopilosus* Ohba
56					MU59	*Senna alata* Roxb.
57					MU60	*Ixeris debilis* A. Grey
58					MU66	*Artocarpus heterophyllus* Lam.
59					MU67	*Artocarpus heterophyllus* Lam.
60					MU68	*Artocarpus heterophyllus* Lam.
61					MU70	*Artocarpus heterophyllus* Lam.
62					MU71	*Artocarpus heterophyllus* Lam.

Table 2.3 Classification of 86 endophytic fungi and their host plants (continued)

No	Class	Order	Family	Genus	Isolate No.	Host plant
63	Sordariomycetes	Glomerellales	Glomerella	*Colletotrichum*	MU72	*Artocarpus heterophyllus* Lam.
64					MU73	*Murranya paniculata* (L.) Jack
65					MU74	*Murranya paniculata* (L.) Jack
66					MU75	*Murranya paniculata* (L.) Jack
67					MU76	*Pithecellobium dulce* (Roxb.)
68					MU77	*Pithecellobium dulce* (Roxb.)
69					MU80	*Pithecellobium dulce* (Roxb.)
70					MU81	*Pithecellobium dulce* (Roxb.)
71		Hypocreales	Nectriaceae	*Fusarium*	MU24	*Solanum trilobatum L.*
72					MU64	*Piper nigrum* Linn
73		Magnaporthales	Magnaporthaceae	*Mycoleptodiscus*	MU03	*Blumea napifolia* DC
74					MU41	*Tinospora crispa (L.)* Miers ex Hook.f.f.& Thoms
75		Xylariales	Xylariaceae	*Daldinia*	MU04	*Laurentia longiflora* Peterm.
76					MU39	*Broussonetia papyrifera* (L.) Vent
77					MU40	*Broussonetia papyrifera* (L.) Vent
78					MU84	*Ficus religiosa* L.
79				*Hypoxylon*	MU52	*Pandanus amaryllifolious* Roxb.
80					MU53	*Pandanus amaryllifolious* Roxb.
81				*Nemania*	MU38	*Broussonetia papyrifera* (L.) Vent
82				*Xylaria*	MU11	*Acanthus ilicifolius*
83					MU18	*Eryngium foetidum* Linn.
84					MU37	*Spilanthes acmella* Murr.
85					MU50	*Pandanus amaryllifolious* Roxb.
86					MU69	*Artocarpus heterophyllus* Lam.

2.3.3 Preliminary test by data-couple-HPLC analysis

The 86 endophytic fungi obtained were cultured in media 2 and 6 statically at 28°C for 21 days. All the cultures were extracted with *n*-butanol and the extracts were analyzed with HPLC-DAD. Each metabolic peak observed in HPLC was evaluated for novelty by comparing the UV/Vis spectrum with those registered in our in-house database. After the analysis, 65 metabolites from 36 isolates were expected to have new chemical structures (Appendix 2). These 36 isolates were further selected for first and second screening.

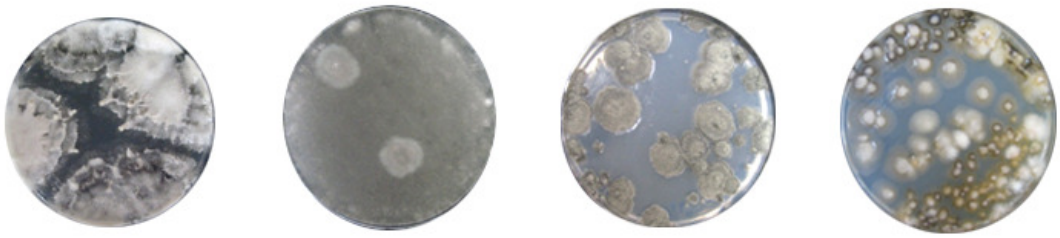
The second screening was used to obtain new chemical structures in a more effective way and to provide easy scale-up for large-scale purification. 100 ml Erlenmeyer flasks were selected instead of test tubes in order to avoid non-reproducible results for compounds due to the use of different culture conditions such as aeration. The extraction solvent was changed to EtOAc (77.1°C) instead of *n*-butanol (118°C) due to the lower boiling point.

2.3.4 Chemical library analysis of candidate isolates from the first screening

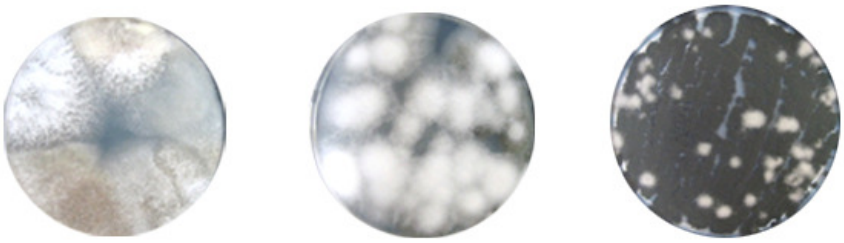
Using three sources of information, i.e., taxonomic information, metabolic profiles from HPLC data and data-couple-HPLC-DAD analysis, the following screening steps were established: (1) excluding well-studied genera, (2) selecting highly produced metabolites, (3) excluding same compounds produced in the same genera (4) excluding compounds with matching data in the UV/Vis database, and (5) excluding compounds displaying non-interesting UV spectra (Figure 2.5).

Based on the first analysis of data-couple-HPLC, 36 isolates had a total of 65 metabolites that were expected to have novel chemical structures (Appendix 2). The

metabolites were then screened using the five-step selection strategy described above, using taxonomic information, metabolic profiles from HPLC data and HPLC-UV/Vis database analysis. According to a report on secondary metabolite isolation, four genera were judged to be well-studied, *Aspergillus*, *Furasium, Alternari*a and *Collectotrichum*.[29] Based on the taxonomic classification, isolates which belong to well-studied genera (*Aspergillus* spp., *Furasium* spp., *Alternaria* spp. and *Collectotrichum* spp.) were excluded from candidate producers in order to decrease the possibility of isolating known compounds. After excluding well-studied genera, 25 isolates (50 candidate compounds) were forwarded to the next selection stage. In the second selection, metabolites showing higher reproducible productivity (area ≥100 mAU*s) were selected. Then the same compounds produced in same genus were excluded. Finally, 20 isolates (45 candidate compounds) remained for evaluation by the last criterion. In data coupled-HPLC analysis, each candidate peak was analyzed by comparing its UV/Vis spectrum and retention time with those of known compounds in an in-house database library. As a result of the final selection, 7 candidate isolates producing 15 putative new compounds were selected for further isolation and characterization of the compounds in chapter 3. The candidate producers were the *Xylaria* sp. MU18, *Cochliobolus* sp. MU21, *Guignardia* sp. MU35, *Daldinia* sp. MU41, *Phomopsis* sp. MU46, *Phoma* sp. MU79, and *Bipolaris* sp.MU83 (Figure 2.3, Figure 2.5 and Table 2.4).

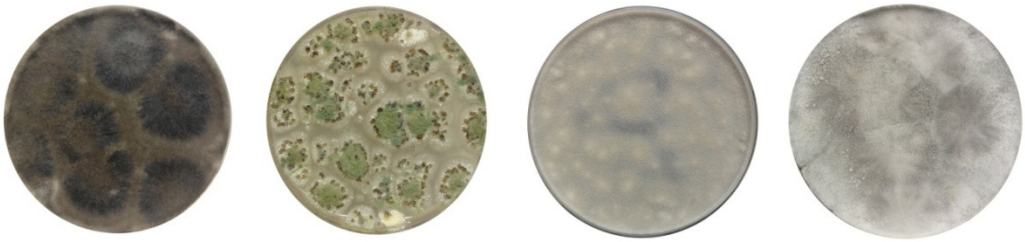


Xylaria sp. MU18 *Cochliobolus* sp. MU21 *Guignardia* sp. MU35 *Daldinia* sp. MU41

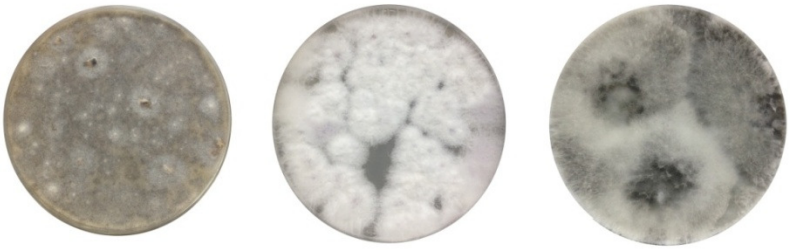


Phomopsis sp. MU46 *Phoma* sp. MU79 *Bipolaris* sp. MU83

Figure 2.3 Seven candidate producers from the first screening



Alternaria sp. MU20 *Aspergillus* sp. MU22 *Bipolaris* sp. MU34 *Daldinia*sp. MU39



Phoma sp. MU62 *Fusarium* sp. MU64 *Lasiodiplodia* sp.MU82

Figure 2.4 Seven candidate producers from the second screening

Table 2.4 Fifteen candidate compounds of seven endophytic isolates from the first screening

No.	Genus	Isolates	Optimized media	Candidate compounds	
				RT (min)	UV/Vis absorption (λ_{max} nm)
1.	Xylaria	MU18	2	2.797	230
				3.409	250, 290
				8.293	205, 242.5
				10.282	207.5, 250
				11.748	227.5, 262.5
				12.175	215.3, 252
2.	*Cochliobolus*	MU21	6	7.223	265, 305
3.	*Guignardia*	MU35	2	13.190	225, 300
				17.871	210, 248, 345
4.	*Daldinia*	MU41	6	2.282	200, 380
				11.287	206.7
				12.485	205,250
5.	*Phomopsis*	MU46	6	22.526	265, 340
6.	*Phoma*	MU79	6	10.072	260
7.	*Bipolaris*	MU83	2	3.118	270, 280

2.3.5 Chemical library analysis of candidate isolates from the second screening

In the second screening, two additional tools, MS analysis and analysis with reported metabolites from the dictionary of natural products (DNP), were added to the screening flow. Using this combination of five information sources, seven screening

steps were established as follows: (1) selecting compounds exhibiting relatively high production of metabolites, (2) excluding duplicated compounds, (3) excluding compounds that matched the UV/Vis database, (4) excluding compounds displaying non-interesting UV spectra, (5) excluding compounds with small molecular weight, (6) excluding known compounds with DNP, (7) selecting compounds dominantly from rare genera.

The 36 isolates (65 compounds with potentially novel chemical structures) selected in the first screening were cultured in 100 ml Erlenmeyer flasks for easy scale-up. The cultures were extracted with EtOAc and the extracts were evaporated *in vacuo*. The dried residues were dissolved in MeOH and analyzed with HPLC-DAD and LC/MS analysis. Among the detected metabolites, only highly produced peaks were selected. Then duplicated compounds were investigated, and the species with the highest yields were selected to obtain compounds for purification. The remaining 43 peaks from 24 isolates were analyzed with the UV/Vis database as described previously (2.3.4). In this study the compounds that displayed absorption maxima at around 400-600 nm were excluded to avoid isolation of known aromatic polyketide compounds and to increase the possibility of obtaining new compounds. Consequently 26 metabolites from 15 isolates met the fourth criterion. LC/MS were used to eliminate small compounds (less than 300 amu). The remaining candidate compounds were checked against the DNP to avoid the isolation of known compounds. If all features of a compound, such as the UV spectrum, the molecular weight and the biological source, were related or identical to those of a compound registered in the DNP, that compound was excluded from further screening. Finally, from all steps of screening, 11 putative compounds from 7 isolates (*Alternaria* sp. MU20, *Aspergillus* sp. MU22, *Bipolaris* sp. MU34, *Daldinia* sp. MU39,

Phoma sp. MU62, *Fusarium* sp. MU64, and *Lasiodiplodia* sp. MU82) were selected for further purification (Figure 2.4, Figure 2.5 and Table 2.5).

Table 2.5 Seven candidate compounds of seven strains as fungal producers

No.	**Genus**	**Isolates**	**Optimized media**	**Candidate compounds**		
				RT (min)	**UV-Vis absorption λ_{max} (nm)**	**Predicted MW (amu)**
1	*Alternaria*	MU20	6	11.6	210, 260, 290, 370	332
				14.1	210, 275, 380	330
2	*Aspergillus*	MU22	2	17.7	220, 280	336
				20.9	230, 280	380
				22.5	230, 280	419
				24.3	230, 280	501
3	*Bipolaris*	MU34	2	14.8	310	323
				15.4	310	309
4	*Daldinia*	MU39	6	16.1	219, 330	432
5	*Phoma*	MU62	6	15.58	250,330	
6	*Fusarium*	MU64	6	20.1	310	462
7	*Lasiodiplodia*	MU82	6	13.9	226,288,300	336
				14.7	226,288,300	334
				17.6	224,256,300	334

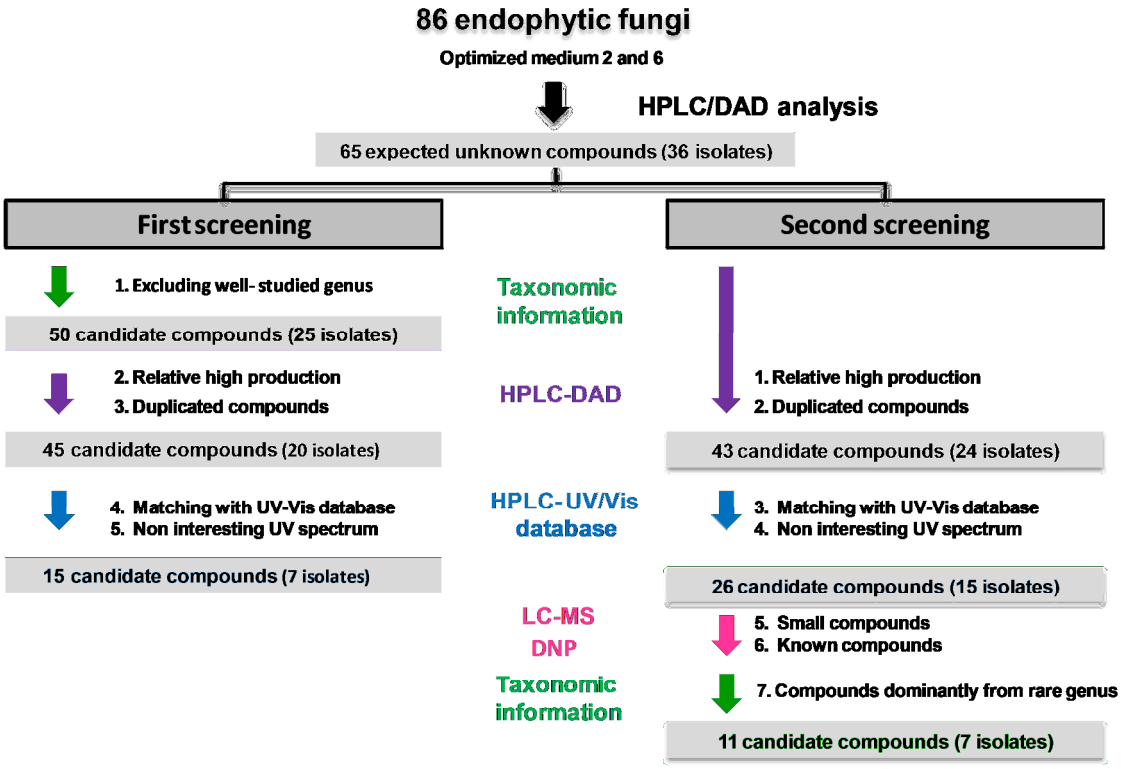


Figure 2.5 Flowchart of the strategies for the first and second screenings

2.4 Summary

In this chapter, 86 endophytic fungi from 30 spp. of Thai medicinal plants were identified by partial 18S and ITS rDNA sequences and were grouped into 18 genera. In this chapter, two screening strategies were established. The first screening was composed of 5 steps and used 3 information sources: taxonomic information, metabolic profiles from HPLC data and HPLC-UV/Vis database analysis. This screening yielded 15 compounds with putatively novel chemical structures from 7 candidate producers, *Xylaria* sp. MU18, *Cochliobolus* sp. MU21, *Guignardia* sp. MU35, *Daldinia* sp. MU41, *Phomopsis* sp. MU46, *Phoma* sp. MU79 and *Bipolaris* sp. MU83. In the second screening, 2 additional tools, LC/MS analysis and comparison with the DNP data, were added to the strategy of the first screening in order to increase its efficiency. As a result

of the second screening, 11 putative compounds from 7 isolates (*Alternaria* sp. MU20, *Aspergillus* sp. MU22, *Bipolaris* sp. MU34, *Daldinia* sp. MU39, *Phoma* sp. MU62, *Fusarium* sp. MU64, and *Lasiodiplodia* sp. MU82) were selected. The isolation and purification of candidate compounds will be described in chapter 3.

CHAPTER 3

Novel chemical structures and bioactive metabolites from the candidate endophytic producers

3.1. Introduction

In this chapter, the candidate endophytic fungi selected by the screening process in chapter 2 as the producer of novel compounds were cultured in optimized medium in order to obtain target putative novel compounds. Herein, the isolation, structure elucidation and biological activity assay of four newly identified compounds together with seven known compounds from endophytic fungi are described.

3.2. Isolation, structure elucidation, and biological activity of xylaropyrone, a novel compound from *Xylaria feejeensis* MU18

Endophytic fungi from the genus *Xylaria* have been identified. in many herbs and woody plants and shown to be efficient producers of compounds with a range of chemical structures that have potential as novel metabolites.[35–40] As a result of the screening described in chapter 2, the putative novel compounds were screened from the endophytic fungus *Xylaria feejeensis* MU18 isolated from *Eryngium foetidum* Linn. From the crude extract, one novel compound was isolated and its chemical structure was determined. The structure of the compound was elucidated on the basis of the spectroscopic data of NMR and MS. This compound, named xylaropyrone (**1**), possesses a novel chemical structure comprising a γ-pyrone with a hydroxymethyl

group and a methylpentyl group at C-2 and C-5, respectively. This is the first report of a natural or even a synthetic compound possessing a γ-pyrone moiety having these two side chains. In this section, the detailed characteristics of xylaropyrone (**1**) and its production, isolation and purification are described.

3.2.1. Compound production, isolation and purification

All chemicals, media, and reagents were purchased from Wako unless stated otherwise. For seed culture preparation, the mycelia of *X. feejeensis* MU18 grown on a PDA slant were inoculated into 5 ml of medium 2 in test tubes (Ø 12.5 mm × 10.5 cm), and incubated for 3 days at 28°C on a reciprocal shaker at 120 rpm. The seed culture (2 ml) was inoculated into 100 ml of medium 2 in 500-ml baffled flasks and cultivated for 21 days at 28°C under a static condition. After cultivation, the culture broth (100 ml x 10 flasks) was mixed with an equal amount of EtOAc and left to stir for 1 hr at room temperature. Mycelia were removed by filtration with Miracloth© (Calbiochem, La Jolla, CA), and the EtOAc layer was recovered from a separation funnel, dried over anhydrous Na_2SO_4 and evaporated to afford a crude extract (brown gum, 650 mg). The crude extract (200 mg, 2 time repeat) was first separated on a C_{18} cartridge (Sep-Pak© Vac 35cc; Waters, Milford, MA) by stepwise elution with increasing MeOH concentrations (MeOH:H_2O=2:8, 4:6, 6:4, 8:2 and 1:0 v/v). The 60% MeOH fractions containing compound **1** were combined and evaporated (50 mg from 2 repeats). Compound **1** was further purified by using a preparative reversed-phase C_{18} HPLC column (PU-1570; Jasco, Tokyo, Japan; equipped with a UVIDEC-100-V detector) and a CAPCELL PAK C_{18} column (UG80S5; Shiseido, Tokyo, Japan) with a shallow

MeOH gradient in 0.1% TFA (a 60% to 75% MeOH gradient over a period of 20 min) to yield 10 mg of pure compound.

3.2.2. Structure elucidation

Xylaropyrone (**1**) was obtained as a yellow oil. The molecular formula was determined to be $C_{12}H_{18}O_3$ based on HRFABMS (obs. *m/z* 211.1335 $[M+H]^+$, calcd. 211.1334 for $C_{12}H_{19}O_3$), ^1H and ^{13}C NMR spectra data (Table 3.1). The IR spectrum showed a broadened OH absorption band at 3390 cm^{-1}. ^1H NMR data of **1** showed two methyl signals at δ 0.82 (t, *J*= 6.9 Hz, 3H) and 0.85 (d, *J*= 6.9 Hz, 3H), eight methylene protons at δ 1.14 (ddq, *J*= 7.6, 15.0, 11.0 Hz, 1H), 1.31 (m, 1H), 1.41 (m, 1H), 1.61 (dddd, J= 6.7, 7.6, 13.4, 16.6 Hz, 1H), 2.48 (m, 2H), and 4.42 (s, 2H), one methine proton at δ 1.33 (m, 1H) and two aromatic protons at δ 7.74 (s,1H) and 6.20 (s,1H). The^{13}C NMR spectrum of **1** indicated one carbonyl carbon at δ 180.4, two quaternary carbons at δ 126.8 and 171.4, two methyl carbons at δ 11.2 and 18.3, four sp^3 methylene carbons at δ 29.1, 31.3, 33.4, and 58.3, one sp^3 methine carbon at δ 33.8 and two sp^2 methine carbons at δ 113.4 and 152.4.

Three partial structures of **1**, namely, a hydroxyl methyl group, a methyl pentyl group and a 2,5-disubstituted γ-pyrone, were deduced by comprehensive interpretation of its ^1H,^{13}C NMR, COSY, HSQC and HMBC spectra and other spectroscopic data (Figure 3.1). The observed carbon signals at δ 113.4, 126.8, 152.3, 171.4 and 180.4 in the ^{13}C NMR spectrum suggested the presence of a γ-pyrone moiety.[41] This was further supported by the maximum UV absorption at 252 nm and strong absorption band at 1660 cm^{-1} in the IR spectrum.[41]

Table 3.1 NMR spectroscopic data of xylaropyrone (**1**) in $CDCl_3$[81]

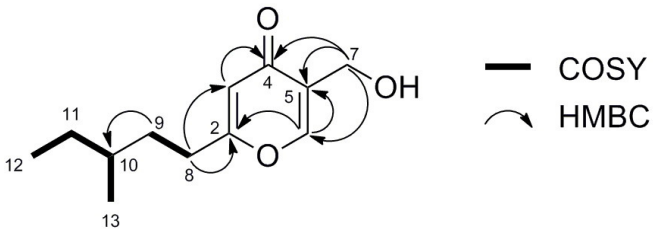
Position	δ_C	δ_H	H-H COSY	HMBC
2	171.4	-		H-3, H-6, H-8
3	113.4 CH	6.20 (s)		H-8
4	180.4	-		H-3, H-6, H-7
5	126.8	-		H-3, H-6, H-7
6	152.4 (CH)	7.74 (s)	H-7	H-7
7	58.3 (CH_2)	4.42 (s)	H-6	H-6
8	31.3 (CH_2)	2.48 (m)	H-9	H-3, H-9
9	33.4 (CH_2)	1.41 (m)	H-8, H-9	H-8, H-10, H-12, H-13
		1.61 (dddd, J= 6.7,7.6, 13.4, 16.6 Hz)	H-8, H-9	
10	33.8 (CH)	1.33 (m)	H-11, H-13	H-9, H-11, H-12, H-13
11	29.1 (CH_2)	1.14 (ddq, *J*= 7.6, 15.0, 11.0 Hz)	H-10, H-12, H-13	H-9, H-10, H-12, H-13
		1.31 (m)	H-10, H-12, H-13	
12	11.2 (CH_3)	0.82 (t, *J*= 6.9 Hz)	H-11	H-11
13	18.8 (CH_3)	0.85 (d, *J*= 6.9 Hz)	H-10, H-11	H-9, H-10, H-11

^1H, ^{13}C NMR and 2D NMR spectra were obtained on JOEL JNM-ECS400 NMR spectrometer, in $CDCl_3$ at room temperature, and the solvent peak was used as an internal standard (δ_H 7.26 and δ_C 77.0 in $CDCl_3$).

The key long range connections of H-8 (δ2.48) with C-2 (δ171.4) and C-3 (δ113.4) and of H-7 (δ4.42) with C-4 (δ180.4), C-5 (δ126.8), and C-6 (δ152.4) indicated that the hydroxyl, methyl and methyl pentyl groups were connected to C-5 and C-2 of the γ-pyrone nucleus, respectively. Regarding the absolute configuration at C-10, it was deduced to be *R*, from the comparison of optical rotations on compounds having a similar aliphatic chain with a 3-methyl or 3-hydroxymethyl group: the rotations of the *R*-configuration were all negative [(*R*)-2-(3-(hydroxymethyl)pentyl)-4H-*pyran*-4-one

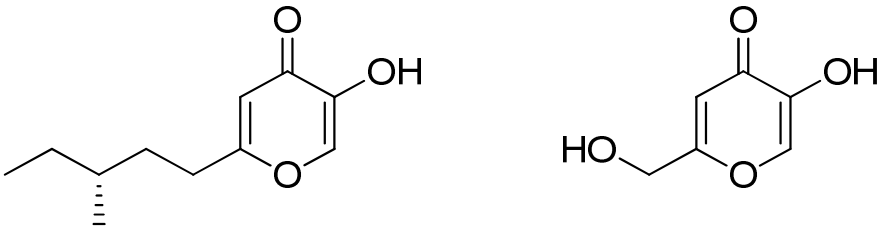
([α]$^{20}_{D}$ -0.567), (*R*)-(3-methylpentyl)benzene ([α]$^{25}_{D}$ -5.52), (*R*)-4-methylhexan-1-ol ([α]$^{20}_{D}$ -8.1)],[42–44] while those of the *S*-configuration were all positive.[45–47] Thus the structure of **1** was elucidated as *(R)*-5-(hydroxymethyl)-2-(3-methylpentyl)-4*H*-pyran-4-one (Figure 3.2).

To the best of our knowledge, xylaropyrone, which consists of a γ-pyrone moiety, a hydroxyl methyl group and a methyl pentyl group, is a novel compound that has not previously been identified in natural resources or derived from chemical synthesis.



(**1**)

Figure 3.1 Partial COSY and HMBC correlation of xylaropyrone (**1**)[81]



(**1**) (**2**)

Figure 3.2 Structure of xylaropyrone (**1**) and kojic acid (**2**)[81]

3.2.3. Physico-chemical and biological properties

Xylaropyrone (**1**): a yellow oil; [α]$^{26}_{D}$ -4.1 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (logε) 215 (3.48), 252 (3.85); HRFABMS *m/z* $[M + H]^+$ 211.1335 (calcd. for $C_{12}H_{19}O_3$, 211.1334). IRν_{max} (film) 3161-3502 (OH), 2958, 2523, 2858, 1654 (C=O), 1596, 1419,

1338, 1184, 1128, 1029 cm^{-1}. See Table 3.1 for details of the ^{1}H ($CDCl_3$, 400 MHz), ^{13}C ($CDCl_3$, 100 MHz), H-H COSY and HMBC.

3.2.4. Antimicrobial activity

Because no antimicrobial data is available on a γ-pyrone compound possessing two side chains, and especially for evaluating the effects of the two side chains on the biological activities of γ-pyrone, the antimicrobial activities against typical prokaryotes and eukaryotes were measured using kojic acid, which has a similar pyrone structure, as a reference. Xylaropyrone showed moderate activity against *S. cerevisiae* (MIC=32 μg/ml), while kojic acid did not show any inhibition even at a concentration of 128 μg/ml. Neither xylaropyrone nor kojic acid showed any inhibitory activity against *E. coli, P. aeruginosa, S. aureus* or *E. faecalis* when used at a concentration of 512 μg/ml, or against *A. niger, R. oryzae* or *G. candidum* when administered at 128 μg/ml (Table 3.2).

Table 3.2 Antimicrobial activities of xylaropyrone (**1**) and kojic acid (**2**)

Indicator strains	**Minimum inhibitory concentration (MIC) μg/ml***		
	xylaropyrone (**1**)	kojic acid (**2**)	chloramphenicol
Escherichia coli ATCC 25922	>512	>512	2
Pseudomonas aeruginosa ATCC 27853	>512	>512	>128
Stapphylococcus aureus ATCC 29923	>512	>512	8
Enterococcus faecalis ATCC 29212	>512	>512	4
Indicator strains	**Minimum inhibitory concentration (MIC) μg/ml****		
	xylaropyrone (**1**)	kojic acid (**2**)	amphotericin B
Saccharomyces cerevisiae ATCC 25922	32	>64	0.5
Candida albicans OUT 6266	>64	>64	1
Aspergillus niger ATCC 6275	>64	>64	1
Rhisopus oryzae ATCC 10404	>64	>64	1
Geotrichum candidum IFO 4598	>64	>64	1

*The reading time was 18-24 hr.
**The reading time was 24-48 hr.

3.2.5. General materials and methods

3.2.5.1. General experimental procedures

The UV spectrum was recorded on a Hitachi U-3200 spectrophotometer. NMR spectra were recorded on a JEOL JNM-ECS400 (JEOL, Tokyo, Japan) at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent signal (δH 7.26 and δC 77.0 in $CDCl_3$). HRFABMS was recorded on a JEOL JMS-700 spectrometer. Optical rotation was measured on a JASCO P-1020 polarimeter (Jasco). IR spectra were recorded on a FTIR-8400S (Shimadzu, Kyoto, Japan).

3.2.5.2. Fungal material

The endophytic fungus MU18 was isolated from leaves of *Eryngium foetidum* Linn., which were obtained from Mahidol University, Bangkok, Thailand. The fungus was identified as *Xylaria feejeensis* based on the DNA sequences of the ITS rRNA region (DDBJ accession number AB569622). A GenBank search for similar ITS sequences confirmed that the fungus was *Xylaria feejeensis*, with 99% sequence identity.[48] The fungus was deposited as *X. Feejeensis* MU18 at the culture collection of the International Center for Biotechnology (ICBiotech; Osaka University, Osaka, Japan).

3.2.5.3 Antimicrobial activity

The minimum inhibitory concentration (MIC) of xylaropyrone (**1**) was determined by a two-fold broth micro-dilution method in three individual experiments according to the procedures of the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial, anti-yeast and antifungal activity.[49] Kojic acid was used as a reference

for the γ-pyrone compound. The MIC is defined as the lowest concentration of the compound at which there is no visible growth of the indicator strains: *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29923, *Enterococcus faecalis* ATCC 29212, *Saccharomyces cerevisiae* ATCC 6275, *Candida albicans* OUT 6266, *Aspergillus niger* ATCC 6275, *Rhizopus oryzae* ATCC 10404, and *Geotrichum candidum* IFO 4598.

3.3. Isolation, structure elucidation, and biological activity of mycoleptione, a novel compound from *Mycoleptodiscus* sp. MU41

As a result of the screening in chapter 2, an endophytic fungus, *Mycoleptodiscus* sp. MU41 isolated from *Tinospora crispa*. *T crispa* has been used as a traditional medicine in rural regions in Thailand in order to treat fever, cholera, snake bites, rheumatism and fever due to malaria. Furthermore, this plant is currently being investigated for its antioxidant, antidiabetic, antimalarial and cosmetic effects.[50]

The genus *Mycoleptodiscus*, which is classified as a plant-associated fungus, has also attracted great attention because of its several interesting abilities. For example, several reports have proposed that *M. terrestris* can be used as a biological control agent for management of the world's aquatic weeds, including hydrilla and Eurasian watermilfoil,[51–53] and that *M. indicus* produces the compound eugenitin, which can activate glucoamylase from *Aspergillus niveus*.[54] Based on these facts, *Mycoleptodiscus* spp. might be regarded as an interesting unexplored target for screening novel secondary metabolites.

The preliminary screening for novel compounds with HPLC-DAD analysis detected two unknown metabolites in *n*-BuOH extract from the static culture of *Mycoleptodiscus*

sp. MU41 which have maximum UV absorptions at 210, 242, 250, and 288 nm and at 204, 256, and 380 nm, respectively. Since the comparison of these spectra with the data registered in our in-house database suggested the discovery of new compounds, and since only limited metabolites have been reported from *Mycoleptodiscus* spp., we determined to isolate and identify the compounds from *Mycoleptodiscus* sp. MU41. Herein, we describe the isolation, structure elucidation and biological activity of one derivative chromone, mycoleptione (**3**), together with a known compound, 7-epiaustdiol (**4**).

3.3.1 Compound production, isolation and purification

All chemicals, media, and reagents were purchased from Wako unless stated otherwise. The mycelia of *Mycoleptodiscus* sp. MU41 were inoculated into 100 ml of medium 6 in 500-ml baffled flasks (20 flasks), and cultivated for 21 days at 28°C under a static condition to obtain **3** and under an agitated condition on a reciprocal shaker at 120 rpm to yield **4**. After static cultivation, the 2L culture was extracted with an equal amount of EtOAc, which was then evaporated to afford a crude extract (brown gum, 4 g). The crude extract was separated on a C_{18} chromatograph column by stepwise elution with increasing MeOH concentrations (MeOH:H_2O = 0:10, 2:8, 4:6, 5:5, 6:4, 8:2, 9:1 and 10:0 v/v). The 50% MeOH fractions containing compound **3** were evaporated (67.7 mg). Compound **4** was further purified by preparative reversed-phase HPLC with a shallow MeOH gradient in 0.1% TFA (a 55% to 65% MeOH gradient over a period of 20 min), yielding pure **3** (6.1 mg). To obtain **4** from the agitated culture, the whole culture (200 ml) was directly filtrated by filter paper (90 mm; Advantec, Tokyo, Japan). The culture filtrate (125 ml) was subjected to HP-20

(Mitsubishi Chemical Co., Tokyo, Japan) column chromatography and was fractionated by stepwise elution with increasing MeOH concentrations (MeOH:H_2O=2:8, 4:6, 6:4, 8:2 and 10:0 v/v). The 60% and 80% MeOH fractions containing **4** were combined and evaporated. The residue (61.8 mg) was further purified by two rounds of preparative reversed-phase HPLC; the first round was conducted with a MeOH gradient in 0.1% TFA (a 30% to 100% MeOH gradient over a period of 35 min) (12.6 mg); the second round was performed with a shallow MeOH gradient in 0.1% TFA (a 35% to 45% MeOH gradient over a period of 20 min), yielding pure **4** (8.1 mg).

3.3.2 Structure elucidation

Compound (**3**) was obtained as a yellow powder. The molecular formula was determined to be $C_{14}H_{16}O_4$ based on HREIMS (obs. *m/z* 248.1046 $[M]^+$, calcd. 248.1043 for $C_{14}H_{16}O_4$), and ^1H and ^{13}C NMR spectra data (Table 1). The IR spectrum showed a broadened OH absorption at 3398 cm^{-1}. The ^1H NMR spectrum of **3** indicated two methyl signals at δ 1.01 (t, *J*= 7.4 Hz, 3H) and 2.71 (s, 3H), two methylene protons at δ 1.57 (m, 2H), 2.60 (dd, *J*= 8.7, 14.5 Hz, 1H) and 2.76 (dd, *J*= 4.0, 14.5 Hz, 1H), one methine proton at δ 3.91 (m, 1H) and three aromatic protons at δ 6.06 (s, 1H), 6.62 (d, *J*= 2.2 Hz) and 6.65 (d, *J*= 2.2 Hz, 1H). The ^{13}C NMR and DEPT data of **3** revealed the presence of 14 carbon signals, including one carbonyl carbon at δ 182.0, five quaternary carbons at δ 115.9, 143.6, 161.5, 163.0, and 167.4, two methyl carbons at δ 10.3 and 23.1, two sp^3 methylene carbons at δ 31.2 and 42.4, one sp^3 methine carbon at δ 71.5 and three sp^2 methine carbons at δ 101.7, 112.5 and 118.0.

Table 3.3 NMR spectroscopic data of mycoleptione (**3**) in CD_3OD[82]

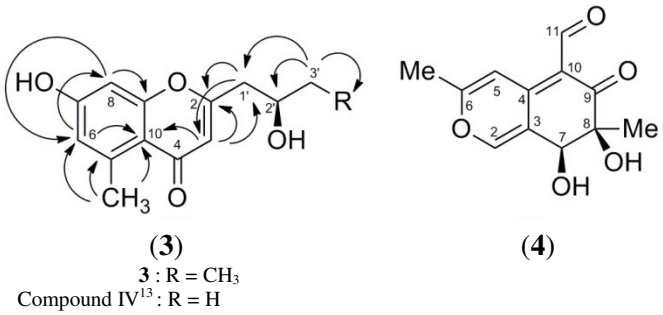
Position	δ_C	δ_H	H-H COSY	HMBC
2	167.4	-		
3	112.5 (CH)	6.06 (s)		C-2, C-10, C-1’
4	182.0	-		
5	143.6	-		
6	118.0 (CH)	6.62 (dd, *J*= 2.2 Hz)	H-(5-CH_3)	C-8, C-10, 5-CH_3
7	163.0	-		
8	101.7 (CH)	6.65 (d, *J*= 2.2 Hz)		C6, C9
9	161.5	-		
10	115.9	-		
5-CH_3	23.1 (CH_3)	2.71 (s)	H-6	C-5, C-6, C-10
1’	42.4 (CH_2)	2.60 (dd, *J*= 8.7, 14.5 Hz)	H-2’	C-2, C-3, C-2’, C-3’
		2.76 (dd, *J*= 4.0, 14.5 Hz)	H-2’	C-2, C-3, C-2’
2’	71.5 (CH)	3.91 (m)	H-1’, H-3’	
3’	31.2 (CH_2)	1.57 (m)	H-2’, H-4’	C-2’, C-1’, C-4’
4’	10.3 (CH_3)	1.01 (t, *J*= 7.4 Hz)	H-3’	C-3’, C-2’

^1H, ^{13}C NMR and 2D NMR spectra were obtained on JOEL JNM-ECS400 NMR spectrometer, in CD_3OD at room temperature, and the solvent peak was used as an internal standard (δ_H 3.31 and δ_C 49.0 in CD_3OD).

In addition to the characteristic UV and IR spectra, such as the maximum UV absorptions at 242, 250 and 288 nm, and a strong absorption band at 1645 cm^{-1} in the IR spectrum,[55] the observed carbon signals at δ 101.7, 112.5, 115.9, 118.0, 143.6, 161.5, 163.0, 167.4 and 182.0 in the ^{13}C NMR spectrum suggested the presence of a 5,7-disubstituted chromone moiety.[56] The complete structure of **3** was deduced by

comprehensive interpretation of its ^{1}H, ^{13}C NMR, HH-COSY, HSQC and HMBC spectra and other spectroscopic data.

The physicochemical data of **3** resembled those of the first chromone compound (compound IV; Figure 3.3) reported by Kashiwada, except for the signals (C-3' δ 42.4 and C-4'δ 10.3) originating from the ethyl group connected to the C-2', while a methyl group was attached in compound IV.[55] The substitution of the methyl group in compound IV for an ethyl group at the 2-hydroxybutyl chain was further confirmed by HH-COSY correlation of H-3' (δ 1.57) with H-2' (δ 3.91), and also by long range correlation of H-3' (δ 1.57) with C-2' (δ 71.5). Judging from these data, the structure of compound **3** was assigned as a chromone bearing a 2-hydroxybutyl chain.



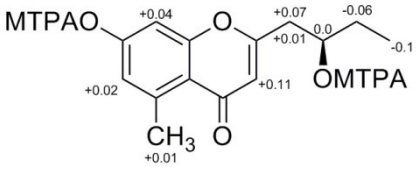
(**3**) (**4**)

3 : R = CH_3

Compound IV[13] : R = H

Figure 3.3 Structure of mycoleptione (**3**), compound IV and 7-epiausdiol (**4**)[82]

The absolute configuration at C-2' was deduced by the Mosher ester method.[57] Negative Δ δ S-R values were observed for H-3' (-0.06) and H-4' (-0.1), while positive Δ δ S-R values were observed for H-1' (+0.01, +0.07), H-3 (+0.11), H-6 (+0.02), H-8 (+0.04) and H-5-CH_3 (+0.01), indicating a 2'R absolute configuration for **3** (Figure 3.4). Consequently, the structure of **3** was determined to be (*R*)-7-hydroxy-2-(2-hydroxybutyl)-5-methyl-4*H*-chromen-4-one and compound **3** was designated to be mycoleptione (Figure 3.4).



(**3**)

Figure 3.4 Δ δ S-R values of MTPA esters of mycoleptione (**3**)[82]

3.3.3 Physico-chemical and biological properties

Compound (**3**): a yellow powder; $[\alpha]^{26}_{D}$ -26 (*c* 0.10, MeOH); m.p. 158-162 ^{o}C; UV (MeOH) λ_{max} (logε) 210 (4.34), 242 (4.21), 250 (4.23), 288 (4.08) nm; HREIMS *m/z* $[M]^{+}$ 248.1046 (calcd. for $C_{14}H_{16}O_4$, 248.1043). IRν_{max} (film) 3151-3398 (OH), 2972, 2931, 2881, 1645 (C=O), 1577, 1396, 1340, 1276, 1159 cm^{-1}. See Table 3.3 for the ^{1}H (CD_3OD, 400 MHz), ^{13}C (CD_3OD, 100 MHz), HH COSY and HMBC.

Compound (**4**): a yellow powder; $[\alpha]^{26}_{D}$ +99.5 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (logε) 204 (4.08), 256 (4.17), 380 (4.37) nm; HRFABMS m/z $[M+H]^{+}$ 237.0767 (calcd. for $C_{12}H_{13}O_5$, 237.0771). See Appendix 7 for the ^{1}H (CD_3CO_2D, 400 MHz), ^{13}C (CD_3CO_2D, 100 MHz).

3.3.4 Antimicrobial activity

The antimicrobial activity of mycoleptione was evaluated by the procedure of the Clinical and Laboratory Standards Institute (CLSI) using *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29923, *Enterococcus faecalis* ATCC 29212, *Saccharomyces cerevisiae* ATCC 6275, *Candida albicans* OUT 6266, *Aspergillus niger* ATCC 6275, *Rhizopus oryzae* ATCC 10404, and *Geotrichum candidum* IFO 4598 as indicator strains. However, no activities against

these strains were detected (MIC >512 μg/ml for antibacterial and >128 μg/ml for anti-yeast and antifungal assays). Subsequently, the anti-oomycetes activities against *Aphanomyces cochlioides* AC-5 and *Phytophthora sojae* P6497 were measured by the agar plug method of Strobel with some modifications.[58] Mycoleptione showed moderate activity against *A. cochlioides*, with an MIC value of 6.25 μg/ml, but had no effect against *P. sojae* even at 100 μg/ml.

In addition to the determination of **3**, compound **4** was identified as 7-epiaustdiol based on its spectroscopic data (Figure 3.3 and Appendix 7).[59,60] The anti-oomycetes assay revealed that **4** displayed strong activity against both *A. cochlioides* and *P. sojae*, with MIC values of <0.39 and 1.56 mg/ml, respectively. This is the first study to report that 7-epiaustdiol (**4**) is produced in this genus and exhibits the anti-oomycete activities.

Table 3.4 Antimicrobial activity of mycoleptione (**3**) and 7-epiaustdiol (**4**)

Indicator strains	**Minimum inhibitory concentration (MIC) μg/ml***		
	mycoleptione (**3**)	7-epiaustdiol (**4**)	chloramphenicol
Escherichia coli ATCC 25922	>128	n/a	2
Pseudomonas aeruginosa ATCC 27853	>128	n/a	>128
Stapphylococcus aureus ATCC 29923	>128	n/a	4
Enterococcus faecalis ATCC 29212	>128	n/a	2
Indicator strains	**Minimum inhibitory concentration (MIC) μg/ml****		
	mycoleptione (**3**)	7-epiaustdiol (**4**)	amphotericin B
Saccharomyces cerevisiae ATCC 25922	>64	n/a	>64
Candida albicans OUT 6266	>64	n/a	0.5
Aspergillus niger ATCC 6275	>64	n/a	0.5
Rhisopus oryzae ATCC 10404	>64	n/a	0.5
Geotrichum candidum IFO 4598	>64	n/a	2
Indicator strains	**Minimum inhibitory concentration (MIC) μg/ml****		
	mycoleptione (**3**)	7-epiaustdiol (**4**)	hygromycin B
Aphanomyces cochlioides AC-5	6.25	<0.39	<0.39
Phytophthorasojae P6497	>100	1.56	12.5

*The reading time was 18-24 hr.
**The reading time was 24-48 hr.

3.3.5 General materials and methods

3.3.5.1 General experimental procedures

The UV spectrum was recorded on a Hitachi U-3200 spectrophotometer. NMR spectra were recorded on a JEOL JNM-ECS400 (JEOL) for ^{1}H (400 MHz) and ^{13}C (100 MHz). The ^{1}H and ^{13}C chemical shifts were referenced to the solvent signals (δ H 3.31 and δ C 49.0 in CD_3OD for compound **3** and δ H 2.04 and δ C 20.0 in CD_3CO_2D for compound **4**). HREIMS was recorded on a JEOL JMS-700 spectrometer. Optical rotation was measured on a JASCO P-1020 polarimeter (Jasco). IR spectra were recorded on a FTIR-8400S (Shimadzu). Melting points were determined on a Yanaco MP-S3 micro-melting point apparatus. HPLC was performed on a PU-1570 with a UVIDEC-100-V detector (Jasco) using a CAPCELL PAK C_{18} column (Ø 10 x 250 mm, UG80S5; Shiseido) for preparative purification, and on a 1200 Series instrument with a binary pump equipped with a photodiode array detector (Agilent 1200 Series; Agilent**,** *Böblingen*, Germany) using a Microsorb-MV100-3 C_{18} column (Ø 4.6 x 100 mm; Varian, Palo Alto, CA) for Mosher ester analysis.

3.3.5.2 Fungal material

The endophytic fungus MU41 was isolated from healthy leaves of *T. crispa* collected at the botanical garden in Mahidol University, Bangkok, Thailand. The leaves were sterilized with 1% NaOCl solution followed by 70% ethanol, and were incubated on water agar plates containing streptomycin (20 µg/ml) and chloramphenicol (30 µg/ml) at 28°C. After 2 weeks, the developed mycelia on the plate were picked up and transferred to PDA plates as stock culture. The fungus was identified as a

Mycoleptodiscus sp. based on the homology of the ITS rRNA region (DDBJ accession number AB694751), with the highest identity being that of 98% with *Mycoleptodiscus indicus* in GenBank. The fungus was deposited as *Mycoleptodiscus* sp. MU41 at the culture collection of the International Center for Biotechnology (ICBiotech; Osaka University, Osaka, Japan).

3.3.5.3 Preparation of the (*R*)- and (*S*)-MTPA ester derivatives of mycoleptione (3)

Compound **3** (1 mg) and dry pyridine (1.6 µl) in anhydrous CH_2Cl_2 (100 µl) at room temperature, *S*-(-)-MTPA-Cl (2.3 µl) was added. The reaction progress was monitored by thin-layer chromatography (TLC) on silica gel (1:1; Hex: EtOAc). After complete consumption of compound **3** (~2 hr), the reaction mixture was quenched by the addition of water (100 µl) and ether (300 µl). The aqueous layer was extracted with two additional portions of ether (300 µl), and the combined organic layers were dried *in vacuo.* The crude product mixture was purified by HPLC chromatography with a CH_3CN gradient in 0.1% formic acid (a 15% to 85% CH_3CN gradient over a period of 35 min) to give the *S*- MTPA ester of **3**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.91 (t, *J*= 7.4 Hz, H-4'), δ 1.75 (m, H-3'), δ 2.84 (s, H-5-CH_3), δ 2.87 (d, *J*= 6.9 Hz , H-1'), δ 5.45 (m, H-2'), δ 6.06 (s, H-3), δ 6.89 (d, *J*= 2.2 Hz, H-6), δ 7.07 (d, *J*= 2.2 Hz, H-8). The *R*-MTPA ester of **3** was prepared in the same way from compound **3**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.01 (t, *J*= 7.4 Hz, H-4'), δ 1.81 (m, H-3'), δ 2.83 (s, H-5-CH_3), δ 2.80 (m, H-1'), δ 2.86 (m, H-1'), δ 5.45 (m, H-2'), δ 5.95 (s, H-3), δ 6.87 (d, *J*= 2.2 Hz, H-6), δ 7.03 (d, *J*= 2.2 Hz, H-8).

3.3.5.4 Antimicrobial activity

The MIC of mycoleptione (**3**) was determined by a two-fold broth micro-dilution method in three individual experiments according to the procedures of the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial, anti-yeast and antifungal activity. The MIC is defined as the lowest concentration of the compound at which there is no visible growth of the indicator strains: *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29923, *Enterococcus faecalis* ATCC 29212, *Saccharomyces cerevisiae* ATCC 6275, *Candida albicans* OUT 6266, *Aspergillus niger* ATCC 6275, *Rhizopus oryzae* ATCC 10404, and *Geotrichum candidum* IFO 4598.

3.3.5.5 Antioomycetes assay

The MIC values of **3** and **4** against two plant pathogenic oomycetes, *A. cochlioides* AC-5 and *P. sojae* P6497, were determined with a 96-well microplate (Zellkultur Test Plate 96F; Zellkultur, Basel, Switzerland) using the method modified from Strobel *et al.* [58] The compound was dissolved in methanol by serial two-fold dilution and a 3 µl aliquot was dispensed into a microplate containing 300 µl of potato dextrose broth (PDB) and V8 for *A. cochlioides* and *P. sojae*, respectively, for a final concentration ranging from 0.39 - 100 µg/ml. An agar plug (5 mm diameter) from the 7-day-old culture of the oomycetes was placed into each well. The anti-oomycetes activity was evaluated after incubation for 5-6 days by comparing with a negative control containing culture broth and microorganisms without the compounds. Hygromycin B dissolved in MeOH was used as the positive control, and **3** µl of MeOH was used as the negative

control. The lowest concentration of the compound under which the microorganism could not grow was considered the MIC.

3.4 Isolation, structure elucidation, and biological activity of bipolamides A and B, novel compounds from *Bipolaris* sp. MU34

As the result of the screening in Chapter 2, *Bipolaris* sp. MU34 from *Gynura hispida* was revealed to produce unknown metabolites which had the maximum UV/Vis spectrum at 300 nm. *Bipolaris* sp. is known to be a plant-associated fungus, although reports of bioactive compounds from *Bipolaris* sp. have been limited.[61-63] *Bipolaris* sp. has attracted attention and has been regarded as a promising untapped source of novel secondary metabolites because its products exhibit intriguing activities, such as antagonism of the human chemokine receptor CCR5 in HIV-1,[64] and antitumor activities.[65] Therefore, I isolated unknown metabolites from *Bipolaris* sp. MU34, identified their chemical structures, and determined their biological activities. In the section below I describe the isolation, structure elucidation and biological activity of two triene amides, bipolamides A (**5**) and B (**6**).

3.4.1 Compound production, isolation and purification

Bipolaris sp. MU34 was cultivated under static conditions in liquid medium 2 for 21 days at 28°C. The whole culture was extracted with EtOAc. Compounds **5** and **6** were purified by a series of steps with C_{18} column chromatography and preparative reversed-phase HPLCs.

All chemicals, media, and reagents were purchased from Wako unless stated otherwise. For the preparation of seed culture, the mycelia of *Bipolaris* sp. MU34 grown

on a PDA slant was inoculated into 5 ml of medium 2 in test tubes (Ø 12.5 mm X 10.5 cm), and incubated for 3 days at 28°C on a reciprocal shaker at 120 rpm. Two ml of the seed culture was inoculated into 100 ml of medium 2 in 500-ml baffled flasks, and cultivated for 21 days at 28°C under static conditions to obtain **5** and **6**. After static cultivation, the whole cultures (100 ml x 10 flasks) were mixed with an equal amount of EtOAc and stirred for 2 hr at room temperature. Mycelia were removed by filtration with a Miracloth© (Calbiochem, La Jolla, CA), and the EtOAc layer was recovered with a separation funnel, dried over anhydrous Na_2SO_4 and evaporated to afford a crude extract (brown gum, 2 g).

The crude extract (2 g) was separated by C_{18} column chromatography using stepwise elution with increasing MeOH concentrations (MeOH:H_2O= 1:9, 2: 8,3: 7,4: 6,5:5, 6:4, 7:3, 8:2, 9:1 and 10:0 v/v). The mixture of compounds **5** and **6** was obtained by evaporation of the fractions eluted with 90% MeOH (140 mg). Compounds **5** and **6** were further purified by preparative reversed-phase HPLC with a shallow MeCN gradient in 0.1% TFA (a 40% to 70% MeCN gradient over a period of 30 min with a flow rate of 5 ml/min), yielding pure **5** (17.3 mg), and a mixture containing **6** (19.0 mg). Compound **6** was further purified with the second round HPLC (32% MeCN isocratic elution over a period of 55 min with a flow rate of 5 ml/min), yielding pure **6** (8.1 mg).

3.4.2 Structure elucidation

Compound **5** was obtained as a pale yellow powder. The molecular formula was determined to be $C_{18}H_{29}NO_4$ based on high-resolution fast atom bombardment mass spectrometry (HRFABMS) (obs. *m/z* 324.2166 $[M+H]^+$, calcd. 324.2140 for $C_{18}H_{30}NO_4$). The IR spectrum showed absorptions at 3340, 1715, 1646 and 1603

cm^{-1}, which are characteristic of hydroxyl groups, carbonyl groups, amide carbonyl groups and double bonds, respectively. Because of the instability of **5** during NMR measurements, **5** was acetylated, yielding compound **7** as monoacetate. The structure of **5** was elucidated based on the monoacetate form.

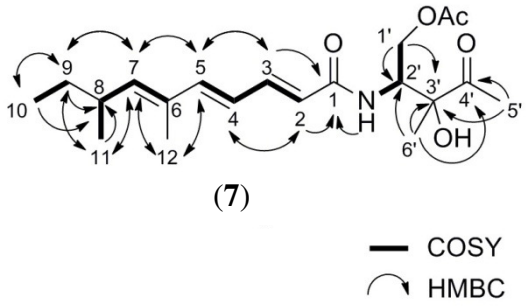
The ^{1}H NMR spectrum of **7** indicated six methyl signals at δ 0.83 (t, *J*= 7.5 Hz), 0.96 (d, *J*= 6.6 Hz), 1.41 (s, 3H), 1.77 (s, 3H), 2.03 (s, 3H) and 2.28 (s, 3H), two methylene protons at δ 1.29 (m, 1H), 1.38 (m, 1H) and 4.26 (dd, *J*= 8.4, 11.4 Hz, 1H), 4.43 (dd, *J*= 4.2, 11.4 Hz, 1H), two methine protons at δ 2.40 (m, 1H) and 4.77 (ddd, *J*= 4.2, 8.4, 9.6 Hz,), five olefinic protons at δ 5.45 (d, *J*= 10.2 Hz, 1H), 5.74 (d, *J*= 15.0 Hz, 1H), 6.17 (dd, *J*= 11.4, 15.6 Hz, 1H), 6.53 (d, *J*= 15.6 Hz, 1H) and 7.22 (dd, *J*= 11.4, 15.0 Hz, 1H), an NH proton at 5.70 (d, *J*= 9.6 Hz, 1H) and an OH proton at 4.31 (s, br). The ^{13}C NMR and DEPT data of **3** revealed the presence of 20 carbon signals, including three carbonyl carbons at δ 210.4, 171.1, and 166.2, two quaternary carbons at δ 79.4 and 132.4, six methyl carbons at δ 11.9, 12.5, 20.3, 20.8, 22.6 and 23.5, two sp^3methylene carbons at δ 30.1 and 62.7, two sp^3methine carbons at δ 34.7 and 52.5, and five sp^2 methine carbons at δ 121.0, 123.4, 142.9, 144.6 and 145.9 (Table 3.5).

Table 3.5 NMR spectroscopic data of monoacetate bipolamide A (**7**) and bipolamide B (**6**) in $CDCl_3$[83]

Position	monoacetate bipolamide A (7)				bipolamide B (6)			
	δ_C	δ_H	HH COSY	HMBC	δ_C	δ_H	HH COSY	HMBC
1	166.2 (C)	-	-	-	168.3 (C)	-	-	-
2	121.0 (CH)	5.74 (d, *J*= 15.0 Hz)	H-3	C-1, C-4	120.8 (CH)	5.89 (d, *J*= 15.0 Hz)	H-3	C-1, C-4
3	142.9 (CH)	7.22 (dd, *J*= 11.4, 15.0 Hz)	H-2, H-4	C-1, C-5	143.0 (CH)	7.29 (dd, *J*= 11.1, 15.0 Hz)	H-2, H-4	-
4	123.4 (CH)	6.17 (dd, *J*= 11.4, 15.6 Hz)	H-3, H-5	C-2	123.4 (CH)	6.21 (dd, *J*= 11.1, 15.0 Hz)	H-3, H-5	-
5	145.9 (CH)	6.53 (d, *J*= 15.6 Hz)	H-4	C-3, C-7, C-12	145.9 (CH)	6.55 (d, *J*= 15.0 Hz)	H-4	C-3, C-7, C-12
6	132.4 (C)	-	-	-	132.4 (C)	-	-	-
7	144.6 (CH)	5.45 (d, *J*= 10.2 Hz)	H-8	C-5, C-9, C-11, C-12	144.6 (CH)	5.46 (d, *J*= 9.7 Hz)	H-8	C-12
8	34.7 (CH)	2.40 (m)	H-7, H-11	-	34.7 (CH_2)	2.40 (m)	H-7, H-9, H-11	-
9	30.1 (CH_2)	1.29, 1.38 (m)	H-10	C-7, C-8, C-11	30.1 (CH)	1.27, 1.37 (m)	H-8, H-10	C-7, C-8, C-10, C-11
10	11.9 (CH_3)	0.82 (t, *J*= 7.5 Hz)	H-9	C-8, C-9	11.9 (CH_3)	0.82 (t, *J*= 7.6 Hz)	H-9	C-8, C-9
11	20.3 (CH_3)	0.96 (d, *J*= 6.6 Hz)	H-8	C-7, C-8, C-9	20.4 (CH_3)	0.96 (d, *J*= 6.5 Hz)	H-8	C-7, C-8, C-9
12	12.5 (CH_3)	1.77 (s)	-	C-5, C-7	12.5 (CH_3)	1.76 (s)	-	C-5, C-6, C-7
NH	-	5.70 (d, *J*= 9.6 Hz)	H-2’	C-1	-	-	-	-
NH_2	-	-	-	-	-	5.60 (s, br)	H-2’	-
1’	62.7 (CH_2)	4.26, 4.43 (dd, *J*=8.4, 11.4; dd, *J*= 4.2, 11.4 Hz)	H-2’	C-2’, C-3’	-	-	H-2’, H-4’	-
2’	52.5 (CH)	4.77 (ddd, *J*= 4.2, 8.4, 9.6 Hz)	H-3’, H-13	-	-	-	H-3’, H-13	-
3’	79.4 (C)	-	-	-	-	-	-	-
4’	210.4 (C)	-	-	-	-	-	-	-
5’	23.5 (CH_3)	2.28 (s)	-	C-3’, C-4’	-	-	-	C-3’, C-4’
6’	22.6 (CH_3)	1.41 (s)	-	C-2’, C-3’, C-4’	-	-	-	C-2’, C-3’, C-4’
3’-OH	-	4.31 (s, br)	-	-	-	-	-	-
4’-OCH_3	-	-	-	-	-	-	-	C-4’
1’-O*CO*CH_3	171.1 (C)	-	-	-	-	-	-	-
1’-OCO*CH_3*	20.8 (CH_3)	2.03 (s)	-	-	-	-	-	-

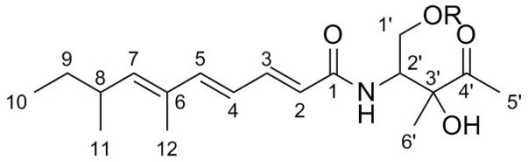
^1H, ^{13}C NMR and 2D NMR spectra were obtained on Varian UNITY INOVA 600 spectrometer, in $CDCl_3$ at room temperature, and the solvent peak was used as an internal standard (δ_H 7.26 and δ_C 77.0 in $CDCl_3$).

Comprehensive interpretation of its UV, IR, ^{1}H-NMR, ^{13}C-NMR, HH-COSY, HSQC and HMBC spectra indicated that **7** contains a decatriene fatty acid amide moiety. The partial structure was identical to the reported triene fatty acid moiety of dictyopanine C[66], judging from the matched ^{1}H-NMR and ^{13}C-NMR spectra, except for the signal from a secondary amide. The presence of NH was confirmed by the data of MS and HMBC correlation between the proton on the nitrogen (δ 5.70) and C1 (δ 166.2). The remaining part of **7** was deduced to contain one carbonyl group, one acetoxy group, one hydroxyl group, two methyls, one methylene, one methine and one quaternary carbon. The fatty acid amide part was linked to the methine C2' by HH-COSY correlation of NH (δ 5.70) to H-2' (δ 4.77). The remaining units and functional groups were connected on the basis of HH-COSY and HMBC correlations. HH-COSY and HMBC demonstrated the connection between C1' and C2', HMBC of H6' revealed the correlation with methine C2', quaternary C3' and carbonyl C4', and HMBC of H5' displayed the correlation with carbonyl C4' and quaternary C3'. All these findings indicated that the partial structure consisted of a short branched five carbon unit. Consequently, the complete structure of **7** was elucidated, as depicted in Figure 3.5. According to the structure of compound **7**, which was estimated as the monoacetylated form of compound **5**, the complete structure of compound **5** was also identified (Figure 3.6).

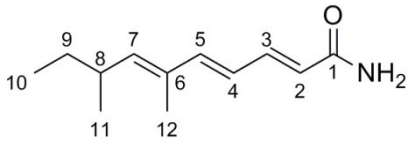


(**7**)

Figure 3.5 HH COSY and HMBC correlation of monoacetae bipolamide A (**7**)[83]



(**5**): R= H
(**7**): R= Ac



(**6**)

Figure 3.6 Bipolamide A (**5**), monoacetate bipolamide A (**7**) and bipolamide B (**6**)[83]

The stereochemistry of the triene in the fatty acid amide moiety was deduced from the vicinal coupling constants between olefinic protons, and the detection of peaks by nuclear Overhauser effect spectroscopy (NOESY). The constants of $J_{2,3}$ and $J_{4,5}$ were 15.0 and 15.6 Hz, respectively. Peaks of NOE were detected for H4/H6-CH_3, H3/H5, and H5/H7, while H4 and H6-CH_3 did not show any effects on the H3, H5 and H7 groups. All these data supported the notion that the geometry of all three olefins was in an (*E*)-configuration (Figure 3.7).

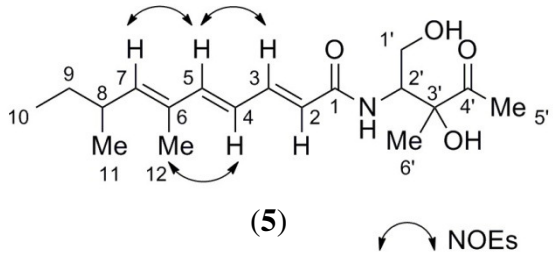


Figure 3.7 Partial NOEs correlation of bipolamide A (**5**)[83]

Compound **6** was obtained as a colorless powder. The molecular formula was determined to be $C_{12}H_{19}NO$ based on HREIMS (obs. *m/z* 193.1470 $[M]^+$, calcd. 193.1490 for $C_{12}H_{19}NO$). The UV and IR spectra suggested that it was a conjugated primary amide. The ^1H and ^{13}C NMR spectra of **6** closely resembled those of the fatty acid amide moiety of compound **7** (Table 3.5). The structure was finally elucidated as a linear triene primary amide, as supported by the findings of HH-COSY, HSQC and HMBC correlation. Based on the fact that **6** had the same vicinal coupling constants of olefinic protons as compound **5**, and probably also the same biosynthetic pathway, **6** was deduced to possess the same (*E*)-configurations.

To the best of our knowledge, bipolamide A (**5**), which consists of a triene fatty acid amide and a unique short five-carbon unit, exhibited a rare chemical structure due to the five carbon unit that has not previously been reported, together with bipolamide B (**6**), which consisted of a triene fatty acid secondary amide. These two metabolites are novel compounds that have not previously been identified in natural resources or derived from chemical synthesis. Thus, the biosynthesis pathway of bipolamide A (**5**), and particularly the shot carbon chain, would be of interest for future study.

3.4.3 Physico-chemical properties

Compound (**5**) was obtained as a pale yellow powder: $[\alpha]^{28}_{D}$ -28 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (logε) 301 (4.69) nm; HRFABMS *m/z* 324.2166 $[M+H]^+$ (calcd. for $C_{18}H_{30}NO_4$, 324.2140). IRν_{max} (film) 3340 (OH), 2961, 2926, 1715 (C=O), 1646 (C=O), 1603, 1539, 1455, 1362, 1258, 1145, 1120, 997 cm^{-1}. Appendices 11 and 12 show the ^1H and ^{13}C data.

Compound (**6**) was obtained as a colorless powder: $[\alpha]^{28}_{D}$ -27 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (logε) 300 (4.41) nm; HREIMS *m/z*193.1470$[M]^+$(calcd. for $C_{12}H_{19}NO$, 193.1490). IRν_{max} (film) 3332 (NH), 3166 (NH), 2959, 2924, 1670 (C=O), 1595, 1455, 1398, 1290, 991 cm^{-1}. See Table 3.5 for the ^1H, ^{13}C, H-H COSY and HMBC data.

Compound (**7**) was obtained as a white powder: $[\alpha]^{27}_{D}$ -46 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (logε) 302 (4.28) nm; HRCIMS *m/z* 366.2274 $[M+H]^+$ (calcd. for $C_{20}H_{32}NO_5$, 366.2256). IRν_{max} (film) 3290 (OH), 2961, 2925, 1742 (C=O), 1716 (C=O), 16516 (C=O), 1606, 1540, 1520, 1507, 1456, 1362, 1228, 1151, 1041, 997 cm^{-1}. See Table 3.5 for the ^1H, ^{13}C, H-H COSY and HMBC data.

3.4.4 Antimicrobial activity

The antimicrobial activities of compounds **5** and **6** were evaluated by the procedure of the Clinical and Laboratory Standards Institute (CLSI). No activities were detected against bacterial strains (MIC >512 μg/ml) for either compound. However, compound **6** showed moderate antifungal activities against *Cladosporium cladosporioides* FERMS-9, *Cladosporium cucumerinum* NBRC 6370, *Saccharomyces cerevisiae* ATCC 6275, *Aspergillus niger* ATCC 6275 and *Rhizopus oryzae* ATCC 10404, with MIC

values of 16, 32, 32, 64, and 64 µg/ml, respectively, while no activities were observed in compound **5** (MIC> 128 µg/ml) (Table 3.6).

Table 3.6 Antibacterial, anti-yeast, and antifungal activities of bipolamides A (**5**) and B (**6**)

Indicator strains	**Minimum inhibitory concentration (MIC) µg/ml***		
	5	**6**	**Chloramphenicol**
Escherichia coli ATCC 25922	>512	>512	4
Pseudomonas aeruginosa ATCC 27853	>512	>512	>256
Stapphylococcus aureus ATCC 29923	>512	>512	8
Enterococcus faecalis ATCC 29212	>512	>512	4
Escherichia coli ATCC 25922	>512	>512	4
Indicator strains	**Minimum inhibitory concentration (MIC) µg/ml***		
	5	**6**	**Amphotericin B**
Candida albicans OUT 6266	>128	128	2
Aspergillus niger ATCC 6275	>128	64	2
Rhisopus oryzae ATCC 10404	>128	64	2
Geotrichum candidum IFO 4598	>128	>128	2
Cladosporium cladosporioides FERMS-9	>128	16	4
Alternaria mali NBRC 8984	>128	>128	4
Cladosporium cucumerinum NBRC 6370	>128	32	2
Furasium oxysporum NBRC 31224	>128	128	8

*The reading time was 18-24 hr.
**The reading time was 24-48 hr.

3.4.5 General materials and methods

3.4.5.1 General experimental procedures

UV spectra were recorded on a Hitachi U-3200 spectrophotometer. NMR spectra were recorded on a Varian UNITY INOVA 600 for ^{1}H (600 MHz) and ^{13}C (150 MHz). The ^{1}H and ^{13}C chemical shifts were referenced to the solvent signals (δ H 7.26 and δ C 77.0 in $CDCl_3$). HREIMS, HRFABMS and HRCIMS were recorded on a JEOL JMS-700 spectrometer. Optical rotation was measured on a JASCO P-1020 polarimeter

(Jasco). IR spectra were recorded on a FTIR-4100 (Jasco). HPLC was performed on a PU-1570 with a UVIDEC-100-V detector (Jasco) using a CAPCELL PAK C_{18} column (Ø 10 x 250 mm, UG80S5; Shiseido) for preparative purification, and on a 1200 Series instrument with a binary pump equipped with a photodiode array detector (Agilent 1200 Series; Agilent) using a Cadenza CD-C18 (Ø 4.6 x 75 mm; Imtakt, Kyoto, Japan) for acetate bipolamide A (**7**) purification.

3.4.5.2 Fungal material

The endophytic fungus MU34 was isolated from leaves of *Gynura hispida* Thwaites obtained at the botanical garden in Mahidol University in Bangkok, Thailand. The leaves were sterilized with 1% NaOCl solution followed by washing with 70% ethanol, and were incubated on water agar plates containing streptomycin (20 µg/ml) and chloramphenicol (30 µg/ml) at 28°C. After 2 weeks, the developed mycelia on the plate were picked and transferred to a PDA plate as stock culture. The fungus was identified as a *Bipolaris* sp. based on the homology of the ITS rRNA region (DDBJ accession number AB813872), which showed the highest identity (98%) to a *Bipolaris* sp. in GenBank. The fungus was deposited as *Bipolaris* sp. MU34 at the culture collection of the International Center for Biotechnology (ICBiotech; Osaka University, Osaka, Japan).

3.4.5.3 Acetylation of compound 5

Six milligrams of compound (**5**) in dry pyridine (0.2 ml) was mixed with 0.2 ml of acetic anhydride, and left for 2 hr with stirring at room temperature. The reaction mixture was evaporated to dryness under reduced pressure to afford white powder. The

residue was purified by reversed-phase HPLC using the following conditions: MeCN gradient in 0.1% TFA (a 15% to 85% MeCN gradient over a period of 32 min with a flow rate of 1.2 ml/min), yielding pure monoacetate compound (**7**) (3.5 mg).

3.4.5.4 Antimicrobial activity

The MIC values of bipolamides (**5** and **6**) were determined by the two-fold broth micro-dilution method according to the procedure of the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial, anti-yeast and antifungal activities. The MIC is defined as the lowest concentration of the compound at which there is no visible growth of the indicator strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29923, *Enterococcus faecalis* ATCC 29212, *Saccharomyces cerevisiae* ATCC 9804, *Candida albicans* OUT 6266, *Aspergillus niger* ATCC 6275, *Rhizopus oryzae* ATCC 10404, *Geotrichum candidum* IFO 4598, *Cladosporium cladosporioides* FERMS-9, *Alternaria mali* NBRC 8984, *Cladosporium cucumerinum* NBRC 6370 and *Fusarium oxysporum* subsp. *Cucumerinum* NBRC 31224.

3.5 Seven known bioactive compounds from five candidate producers, *Cochliobolus* sp. MU21, *Guignardia* sp. MU35, *Phomopsis* sp. MU46, *Phoma* sp. MU79, and *Lasiodiplodia* sp. MU82

During my isolation of novel compounds, seven known bioactive compounds were isolated from five candidate producers, *Cochliobolus* sp. MU21, *Guignardia* sp. MU35, *Phomopsis* sp. MU46, *Phoma* sp. MU79 and *Lasiodiplodia* sp. MU82. The purification procedures were established for each candidate compound. The structures of all the

isolated compounds were elucidated on the basis of the spectroscopic data of NMR and MS. The details of the isolation and structure elucidation, bioactivity and compound distribution are described in this section.

3.5.1 General experimental procedures

The purified compounds were analyzed based on their NMR spectra on a JEOL ECP400SL spectrometer for compounds **8**-**12** and on a Varian UNITY INOVA 600 for compounds **13** and **14**. The chemical shift of ^{1}H and ^{13}C NMR were referenced to solvent signals. HREIMS and HRFEBMS were recorded on a JEOL JMS-700 spectrometer. Consequently, the purified compounds were analyzed and their chemical structures elucidated by using spectroscopic data. HPLC was performed on a PU-1570 with a UVIDEC-100-V detector (Jasco) using a CAPCELL PAK C_{18} column (Ø 10 x 250 mm, UG80S5; Shiseido) for preparative purification, and on a 1200 Series instrument with a binary pump equipped with a photodiode array detector (Agilent 1200 Series; Agilent) using a Cadenza CD-C18 (Ø4.6 x 75 mm; Imtakt) for checking purity.

3.5.2 Strains, media cultivation conditions and extraction

Each isolate was grown under static liquid cultivation in a suitable optimized media 2 and 6. First, the pre-culture for a static cultivation was incubated at 28^{o}C for 3 days with 5 ml of liquid media using test tubes. Static cultivation was started by transferring 2% of pre-culture broth to 100 ml of new media with the same compositions in a 500 ml flask containing 100 ml of medium for 21 days. After the

cultivation, the whole culture broth was extracted with an equal volume of ethyl acetate (EtOAc). The organic layer was dried over sodium sulfate and evaporated to dryness.

3.5.3 Purification procedure and structure elucidation compound of seven candidate compounds from five producers

From five candidate producers, *Cochliobolus* sp. MU21, *Guignardia* sp. MU35, *Phomopsis* sp. MU46, *Phoma* sp. MU79, and *Lasiodiplodia* sp. MU82, isolated from two screenings, seven bioactive compounds were isolated (Table 3.7). All seven of these compounds have diverse structure groups, e.g. xanthone, alkaloid and pyrone, and also have various kinds of specific biological activities. The chemical structures of the purified compounds were characterized by means of spectroscopic data.

3.5.3.1 Isolation of the antibiotic LL-Z1640-2 (8) from *Cochliobolus* sp. MU21

Compound (**8**) was produced by *Cochliobolus* sp. MU21 isolated from *Quassia amara L.* The whole culture was extracted by 1:1 EtOAc. The purification procedure consisted of two separation steps. First, in the preliminary separation, the ethyl acetate extract was fractionated by reversed-phase C_{18} column chromatography using a Sep-Pak© Vac 35cc C_{18} cartridge (Waters) with a step gradient of CH_3CN-H_2O, and then the eluted compound fraction (40% CH_3CN) was purified by preparative reversed-phase C_{18} HPLC using a CAPCELL PAK C_{18} UG80S5 column (Ø10 x 250 mm; Shiseido) with isocratic concentration (40% CH_3CN). ^{1}H-NMR suggested that compound **8** was the antibiotic LL-Z1640-2, which was first isolated in 1978 from the fungus *Lederle* culture Z1640 and exhibit as protein kinase inhibitor.[67]

3.5.3.2 Isolation of pestalamide A (9) and kotanin (10) from *Guignardia* sp. MU35

Another candidate producer, *Guignardia* sp. MU35, isolated from *Gynura hispida* Thwaites, produced 2 candidate compounds, compounds **9** and **10**. The whole culture was extracted using an equal volume of EtOAc. First, the extract was separated by reversed-phase C_{18} column chromatography using a Sep-Pak© Vac 35cc C_{18} cartridge (Waters) with a step gradient of CH_3CN-H_2O. Compound **9** was eluted with 25% CH_3CN and compound **10** with 40% CH_3CN. Secondly, the purification was performed using a reversed-phase C_{18} HPLC column (Ø 10 x 250 mm) with gradient conditions as follows: 40-55% MeOH- H_2O+0.05%TFA 20 min for compound **9** and 65-85% MeOH-H_2O+0.05%TFA 20 min for compound **10**. Compounds **9** and **10**, which were obtained as a pale brown powder and colorless cubes, were analyzed with NMR analysis and suggested to be pestalamide A (**9**) and kotanin (**10**), respectively. Pestalamide A (**9**) was discovered in 2008 in the plant pathogenic fungus, *Pestalotiopsis theae.*[68] This is the first report of the isolation of pestalamide A (**9**) from an endophytic fungus. Kotanin (**10**) was first isolated from *Aspergillus clavatus* and exhibits both antibacterial activity and anti-mycotoxin activity.[69]

3.5.3.3 Isolation of dicerandrol A (11) from *Phomopsis* sp. MU46

Compound **11** was produced by *Phomopsis* sp. MU46 isolated from *Plantago major* Linn. The whole culture was extracted with an equal volume of EtOAc. The purification procedure began with three separation steps. First, for the preliminary separation, the ethyl acetate extract was fractionated by reversed-phase C_{18} column chromatography using a Sep-Pak© Vac 35cc C_{18} cartridge (Waters) with a step gradient

of CH_3CN-H_2O. And then the compound eluted with 55% CH_3CN was further purified by 2 steps of preparative reversed-phase C_{18} HPLC (Ø 10 x 250 mm). For the first step, 60% CH_3CN was used and then the eluate was intensively purified under gradient conditions (52-58% of CH_3CN). NMR analysis indicated that compound **11**, which was obtained as an amorphous yellow powder, was dicerandrol A. Dicerandrol A was reported in 2001 and shown to have both antibacterial activity and cytotoxicity.[70]

3.5.3.4 Isolation of pseurotin A (12) from *Phoma* sp. MU79

One of the candidate producers, *Phoma* sp. MU79, was isolated from *Pithecellobium dulce* (Roxb.) and produced compound **12** as a candidate novel compound. The extraction of the whole culture was performed using an equal volume of EtOAc. First, the extract was separated by reversed-phase C_{18} column chromatography using a Sep-Pak© Vac 35cc C_{18} cartridge (Waters) with a step gradient of CH_3CN-H_2O. Compound **12** was eluted with 40% CH_3CN. Secondly, the purification was performed using a reversed-phase C_{18} HPLC column (10 mm x 250 mm) under isocratic conditions with 40% CH_3CN- H_2O. Compound **12** was obtained as a white solid. From NMR structural analysis, it was concluded that compound **12** was pseurotin A. Pseurotin A was first isolated in 1976 from *Pseudeurotium ovalis* STOLK (ascomycetes).[71] It showed neuritogenic activity in PC12.[72]

3.5.3.5 Isolation of the palmarumycins BG2 (13) and JC2 (14) from *Lasiodiplodia* sp. MU82

Lasiodiplodia sp. MU82 was cultivated under a static condition in medium 2 for 21 days and the culture filtrate was extracted by EtOAc, followed by the first round of a

Sep-Pak© Vac 35cc C_{18} cartridge (Waters) with a step gradient of CH_3CN-H_2O (300 mg X 4 times). The candidate compounds were eluted with 60% MeOH for compound **13** and 80% MeOH for compound **14** and were further purified by preparative reversed-phase C_{18} HPLC with an MeOH gradient in 0.1% TFA (a 50-65% gradient for the 60% fraction and a 55-85% of CH_3CN for the 80% of CH_3CN, both over a period of 35 min), yielding compounds **13** and **14**. From 1D and 2D NMR analysis, compound **13** was suggested to be palmarumycin BG2. The structure of compound **14** was elucidated from ^{1}H, ^{13}C and COSY. The chemical shifts of ^{1}H and ^{13}C were the same as those of compound **13** except for the presence of carbonyl carbon (δ 199.1) and the low magnetic field of 1H (δ 2.8, δ 3.17). All information indicated that compound **14** was the palmarumycin JC2. Palmarumycins have unique structures and interesting biological properties and are a relatively new and rare class of natural products found mainly in filamentous fungi. This class of compound displays a wide range of biological activities, including antibacterial, antifungal, algicidal, nematicidal and anti-leishmanial activity. The palmarumycin BG2 (**13**)[73] was discovered in 2011, whereas the palmarumycin JC2 (**14**)[74] is an antibacterial agent isolated from *Jatropha curcas* reported in 2004. To the best of our knowledge, palmarumycins BG2 and JC2 were first discovered in plants, and this is the first report of the isolation of either compound from an endophytic fungus. These data support the ability of endophytes to produce metabolites similar or identical to those made by plants.

3.6 Summary

In this chapter, the isolation of novel compounds and known bioactive compounds from a group of screened candidate producers was discussed in detail. From the first

and second screening, four novel compounds were discovered. In the first screening, one unique compound having a γ-pyrone structure, 5-(hydroxymethyl) -2-(3-methylpentyl)-4H-pyran-4-one (**1**), was obtained from the *Xylaria feejeensis* MU18 isolated from Mexican coriander, *Eryngium foetidum* Linn. This is the first report of a γ-pyrone moiety of a natural compound having a hydroxyl methyl and methyl pentyl attached in these positions. The results of MIC showed weak activity against *S. serevisiae* at 32 μg/ml. And from *Mycoleptodiscus* sp. MU 41, one new chromone derivative, mycoleptione (**3**), was discovered together with one known compound, 7-epiaustdiol (**4**). The antimicrobial assay revealed that mycoleptione (**3**) showed moderate growth-inhibitory activity against an oomycete strain, *Aphanomyces cochlioides*, with an MIC value of 6.25 μg/ml. As a result of the second screening for new metabolites, two new triene fatty acid amides, the bipolamides A (**5**) and B (**6**), were discovered from *Bipolaris* sp. MU34. Bipolamide A possessed the rare structure of a five carbon unit connected to a decatriene fatty acid amide. The antimicrobial assay revealed that bipolamide B (**6**) displayed moderate antifungal activity against *Cladosporium cladosporioides* FERMS-9, *Cladosporium cucumerinum* NBRC 6370, *Saccharomyces cerevisiae* ATCC 9804, *Aspergillus niger* ATCC 6275 and *Rhisopus oryzae* ATCC 10404 with MIC values of 16, 32, 32, 64, 64 μg/ml, respectively. Moreover, six bioactive known compounds (pestalamide A, kotanin, dicerandrol, antibiotic LL-Z1640-2, pseurotin A, and palmarumycins BG2 and JC2) were isolated from five endophytic fungi in the first screening and two compounds, palmarumycin BG2 and JC2, were isolated from the second screening. Those compounds show a diversity of chemical structures and have various biological activities, especially in the case of the palmarumycis BG2 and JC2, which were isolated in filamentous fungi for

the first time herein. All the isolates showed highly diverse chemical structures and various biological activities, implying that endophytic fungi have great potential for the production of unknown compounds with chemically unique structures and novel bioactivities.

Table3.7 Summary of general properties and bioactivity of the 8 bioactive compounds

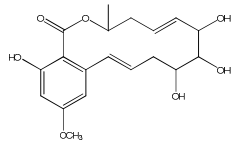
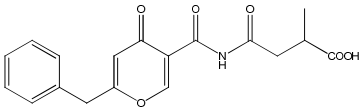
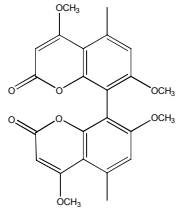
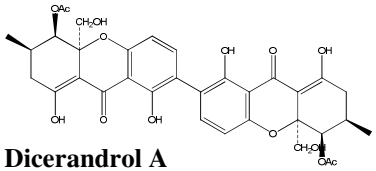
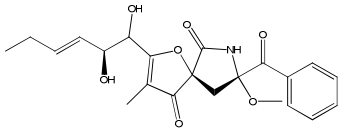
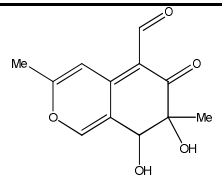
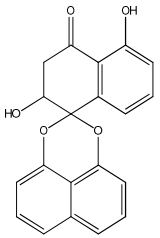
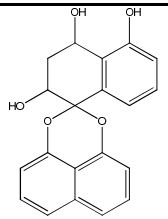
Endophytic fungi	Compound	Molecular formula & M.W.	Appearance	Compound	Discovered report	Bioactivity
Cochliobolus sp. MU21	**8**	$C_{19}H_{22}O_7$ 362.37	crystal	 **Antibiotic LL-Z1640-2**	1977 *Lederle* culture Z1640	-Protein kinase inhibitor -Mycotoxicity
Guignardia sp. MU35	**9 (first reported in endophytic fungus)**	$C_{18}H_{17}NO_6$ 366.09	pale brown powder	 **Pestalamide A**	2008 *Pestalotiopsis theae* (plant pathogenic fungus)	-Inhibitory effects on HIV-1replication in C8166 cells
Guignardia sp. MU35	**10**	$C_{24}H_{22}O_8$ 438.13	cubes	 **Kotanin**	1971 *Aspergillus clavatus*	-Antibacterial activity -Mycotoxicity
Phomopsis sp. MU46	**11**	$C_{34}H_{34}O_{14}$ 666.63	yellow powder	 **Dicerandrol A**	2001 *Phomopsis longicolla*	-Antibiotic activity -Cytotoxicity
Phoma sp. MU79	**12**	$C_{22}H_{25}NO_8$ 431.40	white solid	 **Pseurotin A**	1976 *Pseudeurotium ovalis* STOLK (ascomycetes)	-Neuritogenic activity in PC12phaechromocytoma cells -Chitinase inhibition

Table3.7 Summary of general properties and bioactivity of the 5 candidate compounds (continued)

Endophytic fungi	Compound	Molecular formula & M.W.	Appearance	Compound	Discovered report	Bioactivity
Mycoleptodiscus sp. MU41	**4**	$C_{12}H_{13}O_5$ 237.07	yellow powder	 **7-epiaustdiol**	2010 *Talaromyces sp. ZH-154*	-Wide range of antimicrobial activity
Lasiodiplodia sp. MU82	**13 (first reported in fungus)**	$C_{20}H_{14}O_5$ 362.32	white solid	 **Palmarumycin JC2**	2004 *Jatropha curcas* **(plant)**	-Wide range of antimicrobial activity -Antitumor activity
	14 (first reported in fungus)	$C_{20}H_{16}O_5$ 336.34	white solid	 **Palmarumycin BG2**	2011 *Bruguiera gymnorrhiza* **(plant)**	-Wide range of antimicrobial activity -Antitumor activity

CHAPTER 4

General conclusions

The extensive demand of new substances for various human applications has led to continuous screening for novel chemical structures. Natural products produced by microorganisms have served as potential resources to fulfill this important demand, because they are highly effective, possess low toxicity, and have a minor environmental impact. Nevertheless, for further efficient screening of bioactive compounds in the future, the rational selection of high-quality screening sources is essential. In this study, endophytic fungi, which are expected to be a potential source of new compounds, were isolated from Thai medicinal plants serving as natural producers.

In chapter 2, among over 1,157 spp. of the medicinal plants in Thailand [recorded on PHARM database (MedPlant Online), The Medicinal Plant Information Center (MPIC), Faculty of Pharmacy, Mahidol University, http://www.medplant.mahidol.ac.th/index.asp (*In Thai*)], 30 spp. of medicinal plants having various reports on the bioactivity and long history as herbal remedies were selected as the source of endophyte isolation. Majority of the selected 30 spp. have never been examined for their residing endophytes except for *B. papyrifera* and *T. crispa*. According to the fact that the same species of plant, if differs on location and season of collection, could result in different endophyte species,[70,71,72] in this study, the leave of *B. papyrifera* and *T. crispa* were also included as the sample for endophyte isolation.[73] Isolated endophytic fungi were classified by a molecular taxonomic

technique by using partial 18S and ITS rDNA sequences. All the 86 isolates were taxonomically identified and classified into 18 genera (~average 4 strains per 1 genus). Eighty-six of the isolates were ascomycetes, the major group of endophytic fungi studied to date. In this study, dominant genus (*Collectotrichum* spp.) was found to reside in almost all the collected plants (20 spp. of all 30 spp. host plants). Combined with previous reports on Thai endophytic fungi, this study indicated that the dominant endophytes among Thai medicinal plants can be concluded to be *Colletotrichum*, *Fusarium*, *Promopsis* and *Xylariacous*. In addition, rare endophytic fungi, such as *Mycoleptodiscus*, *Lasiodiplodia*, *Diaporthe* and *Leptosphaerulina*, were also isolated, implying that Thai medicinal plants can be a good source for isolating rare endophytic fungi.

From literatures covering the year 2000-2013, reports on endophytic fungi of Thailand were found only in a few articles (less than 30 articles), and majority of the studies focused on biodiversity and antimicrobial assessment.[73,74,75] Although more than 1,000 isolates were examined for their ecological distribution and antimicrobial activities, unfortunately, a few articles led to achieve new metabolites.[32,71] Following the taxonomic identification, profiles of secondary metabolites were examined on all the isolates. The isolates were cultivated in optimum media 2 and 6 under static conditions to promote the production of secondary metabolites. These optimum conditions could promote the fast and high production of metabolites, resulting in the increased possibility of forcing endophytes to produce new and different secondary metabolites from previous reports.

Chemical library analysis using UV/Vis database-coupled HPLC was adopted to analyze the novelty of all metabolites. These preliminary data were utilized to establish

criteria for selecting candidate isolates that produced putative novel compounds. The chemical library analysis based on UV/Vis spectrum comparison with those of over 1,600 registered metabolites, most from actinomycetes and filamentous fungi. From database-coupled HPLC analysis, the metabolic profiles were matched with the in-house database, by which more than 10 known or similar compounds were detected per each isolate (data not shown). Using the in-house UV/Vis database, the possibility to isolate known compound(s) was highly reduced. The screening of novel chemical structures based on the combination of chemical library analysis (database-coupled HPLC, LC/MS and DNP) with metabolic information based on taxonomic data resulted in quicker selection of targets and increased the possibility to obtain novel chemical structures. From the screening process, over 10 known or similar compounds per each isolate were excluded. For example, the peak at 15.9 min from *Alternaria* sp. MU32 was excluded due to its high similarity in UV-Vis spectrum to that of alternariol and the same molecular weight in LC/MS, together with the report of the compound produced by endophytic fungi in DNP. At the same time, database-coupled HPLC analysis usually revealed more than 20 unmatched metabolites per isolate. The analysis shown in this study indicates that endophytic fungi from Thai medicinal plants should be regarded as good source of unexplored valuable metabolites for further development.

In chapter 3, the candidate endophytes selected in chapter 2 were further examined, putative novel compounds were isolated, and their chemical structures and biological activities were determined. In the first screening, we obtained two new compounds, xylaropyrone and mycoleptione, together with six known compounds, pestalamide A, kotanin, dicerandrol, antibiotic LL-Z1640-2, pseurotin A and 7-epiaustdiol, which gave ~33% possibility of obtaining a compound with a novel structure. The possibility for

obtaining compounds with a novel structure increased to 50% in the second screening, in which both the LC/MS analysis and the DNP database was used, resulting in 2 novel compounds, bipolamides A and B, together with 2 known compounds, the palmarumycins BG2 and JC2.

Xylaropyrone isolated from *Xylaria feejeensis* MU18 was a novel pyrone compound consisting of 2 unique branch chains: a hydroxymethyl group and a methylpentyl group. Mycoleptione, isolated from *Mycoleptodiscus* sp. MU41, was a chromone derivative compound with a modification of a hydroxyl butyl side chain. Lastly, bipolamides A and B, two decatriene fatty acid amide chains, were characterized from the culture of *Bipolaris* sp. MU34. Bipolamide A displayed a rare structure of five carbon units connecting with the fatty acid amide chain. The fact that the four identified compounds exhibited weak to moderate antimicrobial activities demonstrated that the developed strategy for screening in this study is useful to discover structurally novel compounds without easily detectable activity.

Screening compounds based on bioassay may be inefficient and time-consuming way to achieve compounds of novel structure, in addition to the necessity to maintain laborious bioassay systems. The chemical library analysis is one tool to improve for faster and more efficient isolation of novel structures. The system is simple and fast to establish and accumulate the metabolic data, and can be easily applicable to several research purposes. In the future, combination of chemical library analysis with bioassay-based targeting will improve further the screening efficiency for novel bioactive compounds.

Under this study, new compounds having novel structures have been obtained by fermentation of filamentous fungi: xylaropyrone containing the unique branch-chain structure, mycoleptione (a chromone derivative bearing a 2-hydroxybutyl chain), and bipolamide A composed of an acyloin moiety and a triene fatty acid secondary amide. These novel compounds should have a lot of potentials for use. As described in this thesis, four novel compounds displayed moderate antimicrobial activities. However, chemical modification study would explore the potential of these novel compounds for finding any useful biological activity. These are exemplified by the case of pritinamycin IA and pristinamycin IIA. Their water solubility was improved by hemisynthetic and mutasynthetic approach to develop human-disease applicable derivatives, resulting in quinupristin (type B) and dalfopristin (type A) with the approval of the Food and Drug Administration (FDA, USA) in 1999.[84,85] The four new compounds are now easily available as starting materials by fermentation of the fungi in this study, if necessary by increasing the fermentation size to obtain large quantities, chemical modifications can be performed, with necessary quantities provided, to test in many different bioassay systems.

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List of publications

1. Ratklao Siriwach, Hiroshi Kinoshita, Shigeru Kitani,Yasuhiro Igarashi, Kanokthip Pansuksan,Watanalai Panbangred, Takuya Nihira . Xylaropyrone, a new γ-pyrone from the endophytic fungus *Xylaria feejeensis* MU18. *J Antibiot*. 64, 217–219 (2011).

2. Ratklao Siriwach, Hiroshi Kinoshita, Shigeru Kitani,Yasuhiro Igarashi, Kanokthip Pansuksan,Watanalai Panbangred, Takuya Nihira. Mycoleptione, a new chromone derivative isolated from the endophytic fungus *Mycoleptodiscus* sp. MU41. *J Antibiot*. 65, 627-629 (2012).

3. Ratklao Siriwach, Hiroshi Kinoshita, Shigeru Kitani,Yasuhiro Igarashi, Kanokthip Pansuksan,Watanalai Panbangred, Takuya Nihira. Bipolamides A and B, triene amides isolated from the endophytic fungus *Bipolaris* sp. MU34. *J Antibiot*. (Submitted).

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Appendices

Appendix 1 Optimization of the medium for static cultivation

In order to efficiently obtain anti-fungal compounds produced by endophytic fungi for further analysis, a liquid static cultivation was adopted. Twenty-one random isolates that were considered representative of endophytic fungi were cultured in 11 different types of media as shown in Table 1. Pre-culture of static cultivation was conducted at 28^{o}C for 3 days with 3 ml of liquid media using test tubes of Φ12.5 mm × 10.5 cm. Static cultivation was started by transferring 0.2 ml of pre-cultured broth to 10 ml of new media with the same compositions in test tubes of size Φ16.5 mm × 13 cm. One milliliter of culture broth was collected each week for three weeks, and the supernatants of the culture broths were prepared by removing the mycelium by centrifugation at 14,000 rpm for 5 min. After liquid static cultivation of all endophytic fungi, the culture broths were extracted with *n*-butanol for further analysis by the following method. Ten milliliters of whole culture broth was transferred to a Falcon tube, then supplemented with 5 ml of *n*-butanol and vortexed at room temperature for 1-2 min. The *n*-butanol layer was separated by centrifugation and 1 ml of the extract was put into a sample tube. After the evaporation of butanol, the residues were further analyzed by high performance liquid chromatography (HPLC).

In the liquid static cultivation, the endophytic fungi showed individual differences in growth in each type of media. Judging from the rapid growth rate and high production of secondary metabolites, Media number 2 and 6 were selected for static cultivation in further experiments.

Appendix 1. Media compositions for liquid static cultivation (continued)

Media	Compositions
A-3M	Glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia 1.5%, yeast extract 0.3%, HP20 0.1%, pH 7
A-11M	Soluble starch 2.5%, glucose 0.2%, yeast extract 0.5%, polypeptone 0.5%, NZ-amine 0.5%, $CaCO_3$ 0.3%, HP20 0.1%, pH 7
A-16M	Glucose 2%, Pharmamedia 1%, $CaCO_3$ 0.5%, HP20 0.1%, pH 7
1	Glycerol 3%, oatmeal 2%, yeast extract 1%, KH_2PO_4 1%, Na_2HPO_4 1%, $MgCl_2.6H_2O$ 0.5%
2	Soluble starch 5%, Pharmamedia 2%, oatmeal 0.5%, KH_2PO_4 0.35%, Na_2HPO_4 0.25%, $(NH_4)_2SO_4$ 0.6%
3	Corn starch 2%, glucose 1%, Pharmamedia 1%, soybean meal 0.5%, yeast extract 0.5%
4	Glycerol 2%, sucrose 1%, ammonium acetate 0.5%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.05%, KCl 0.05%, yeast extract 0.2%
5	Sucrose 8.0%, yeast extract 4.0%, $CaCO_3$ 0.5%
6	Glucose 1%, mannitol 10%, Pharmamedia 3.5%, KH_2PO_4 0.9%
PDB	Potato dextrose broth powder 2.4%
SMY	Maltose 4%, yeast extract 1%, peptone 1%

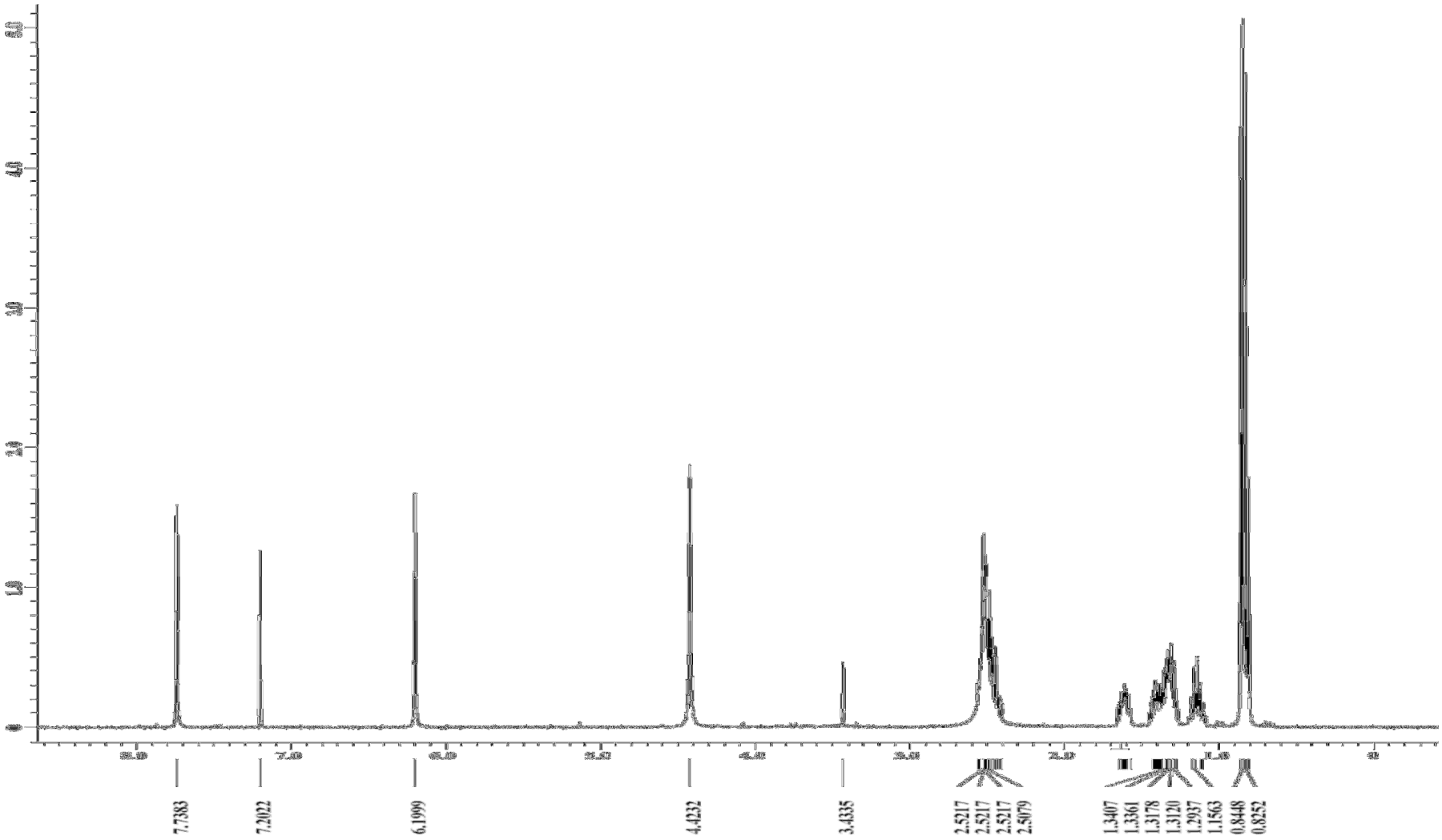
Appendix 2. Analysis of the secondary metabolite profiles of endophytic fungi isolated from Thai medicinal plants cultured in media number 2 and 6 by HPLC.

Isolates	Media	RT (min)	Area (mAU*s)	Peaks analysis λ_{max} (nm)	Remark with database HPLC
MU02	2	14.807	346.14	240	unknown
		23.228	471.72	240, 282.5	unknown
MU04	2	11.564	3601.59	206.7,228.3,251.7,295	unknown
		14.917	1541.41	205, 230, 255, 300, 375	unknown
MU05	2	11.422	309.67	305	unknown
		13.294	75.68	225,330	unknown
MU06	6	7.653	269.01	206.7	unknown
		22.796	370.47	236.7, 258.3	unknown
MU12	6	7.678	291.49	253.3	unknown
MU18	2	13.491	1318.35	215, 265	unknown
MU20	6	6.663	6688.85	206.7, 216.7, 223.3, 231.7, 250, 243.3	unknown
		11.441	662.66	220, 256.7, 288.3, 336.7	unknown
MU21	6	7.223	829.38	238.3, 273.3, 313.3	unknown
MU22	2	12.206	1019.12	205, 225, 270, 290	unknown
MU32	2	11.188	729.52	203.3, 253.3,285, 338.3	unknown
		11.538	308.86	213.3, 260, 340	unknown
		18.364	297.43	255, 288.3, 298.3, 338.3	unknown
	6	7.414	494.76	213.3, 260, 285, 355	unknown
		7.642	754.28	255, 288.3, 298.3, 340	unknown
		11.672	4614.79	203.3, 253.3, 286.7, 296.7, 335	unknown
MU34	2	19.737	297.36	265, 305	unknown
MU36	6	9.326	371.46	268.3, 321.7	unknown
MU39	2	7.802	720.11	225, 300	unknown
	6	15.114	115.67	210, 247.5, 345	unknown
MU40	2	8.744	1114.75	237.5, 285, 337.5	unknown
	6	5.516	995.32	206.7, 256.7, 350	unknown
		6.750	492.84	215, 265, 338.3	unknown
		8.730	370.44	238.3	unknown
		12.032	191.03	235, 245	unknown
MU41	6	11.287	3101.67	206.7	unknown
MU42	2	12.092	473.01	245, 312.5	unknown
MU43	2	8.627	474.51	210, 231.7, 260	unknown
MU45	2	7.340	1879.75	218.5, 265.1, 288.5, 298.5, 396.8	unknown
		10.156	4399.75	240, 263.4, 286.3, 298.5,396.8	unknown
MU46	6	11.165	593.58	223.3, 246.7, 316.7	unknown
MU46	6	14.086	678.72	250, 347.5	unknown
		22.526	270.33	265, 340	unknown
MU47	2	23.220	352.69	232.5, 305	unknown
MU49	2	8.307	593.73	217.5, 280, 335	unknown
	6	8.226	338.41	212.5, 277.5, 335	unknown
MU51	2	9.090	216.22	206.8, 230, 283.4	unknown
MU53	2	11.163	1002.63	265, 275	unknown

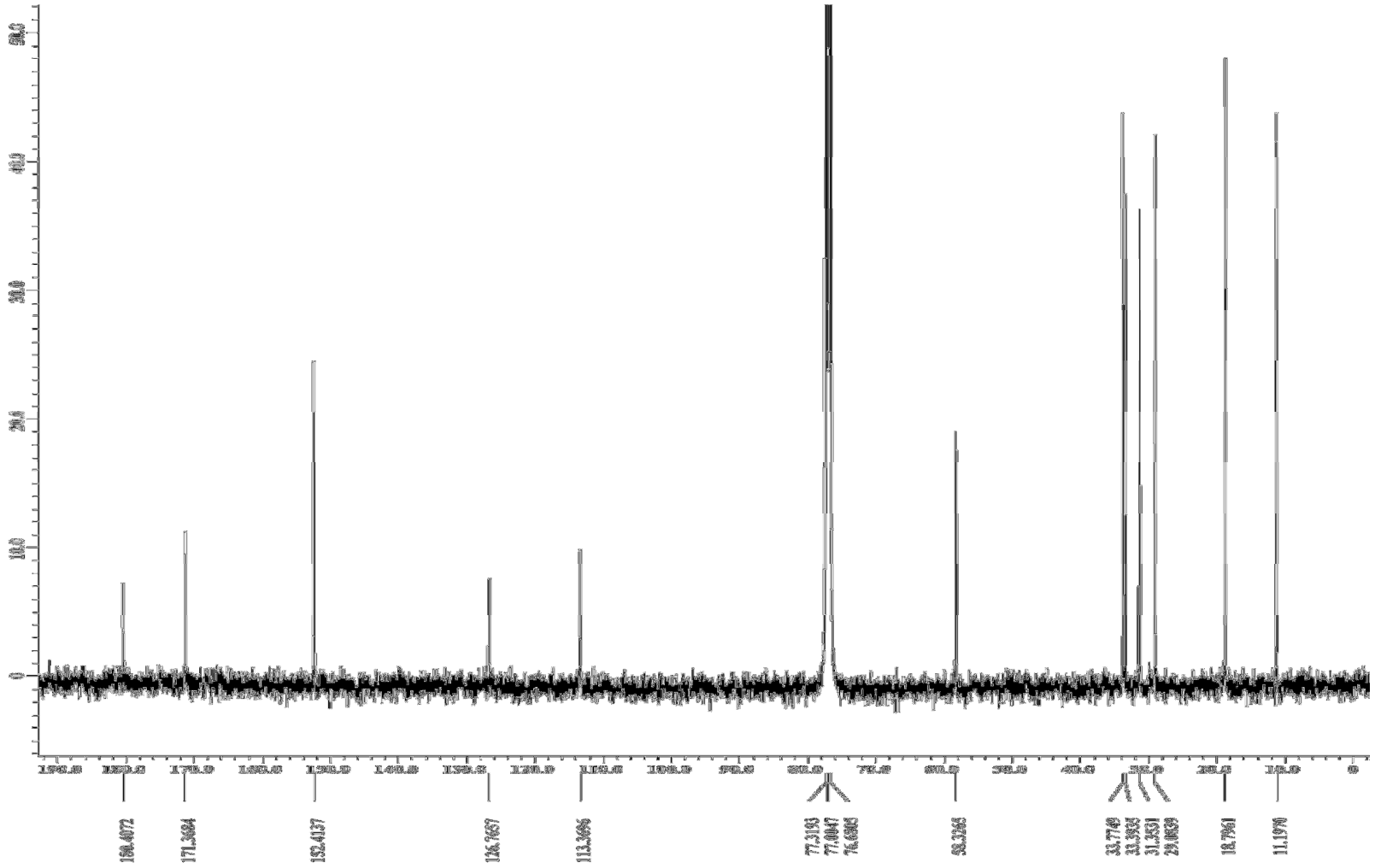
Isolates	Media	RT (min)	Area (mAU*s)	Peaks analysis λ_{max} (nm)	Remark with database HPLC
MU56	2	24.261	150.76	277.5	unknown
MU62	2	7.713	165.18	201.7, 285.5	unknown
	6	10.298	694.17	216.7, 295	unknown
MU64	2	10.786	604.07	225, 262.5, 370, 485	unknown
		12.092	1060.73	205, 247.5, 317.5, 487.5	unknown
		23.600	767.24	217.5, 277.5	unknown
MU67	6	23.949	1056.53	213.3, 275	unknown
MU69	6	13.500	1594.7	225, 265	unknown
MU70	2	28.200	155.21	272.5	unknown
MU73	2	8.206	216.39	260, 356.7	unknown
MU75	2	13.412	402.47	215	unknown
	6	13.382	3845.3	205, 315	unknown
MU77	6	23.954	1222.09	245	unknown
MU79	6	10.072	14607.90	260	unknown
MU80	2	24.581	45.42	240, 285	unknown
MU82	2	9.559	40.96	228.3, 235, 300, 313.3, 326.7	unknown
MU83	2	26.385	409.81	270, 280	unknown
MU84	2	9.009	2367.55	272.5	unknown
		10.256	823.94	254.5, 442.5	unknown
		10.717	1006.59	212.5, 310	unknown
	6	8.917	17299.10	205, 270	unknown
		9.487	7963.22	230, 275, 320, 330, 405	mithramycin-like (RT= 7.709)
		10.711	6025.27	207.5, 310	unknown
		14.912	12548.20	235, 282.5, 322.5, 332.5, 405	mithramycin-like (RT= 7.709)
		16.594	8254.51	225, 280, 405	HF326_C (RT=6.358)
		18.368	164375.00	230, 285, 320, 330, 400	mithramycin-like (RT= 7.709)
		21.055	14196.10	232.5, 275	unknown[1/]
		21.338	16923.10	230, 280, 405	unknown[1/]
		22.207	11905.90	230, 280, 405	unknown[1/]
		23.087	4240.39	230, 280, 406	unknown[1/]
		24.240	3712.95	235	unknown

[1/] Similar peak spectrum with HF326_C at RT=6.358 □max= 225, 280, 405 nm

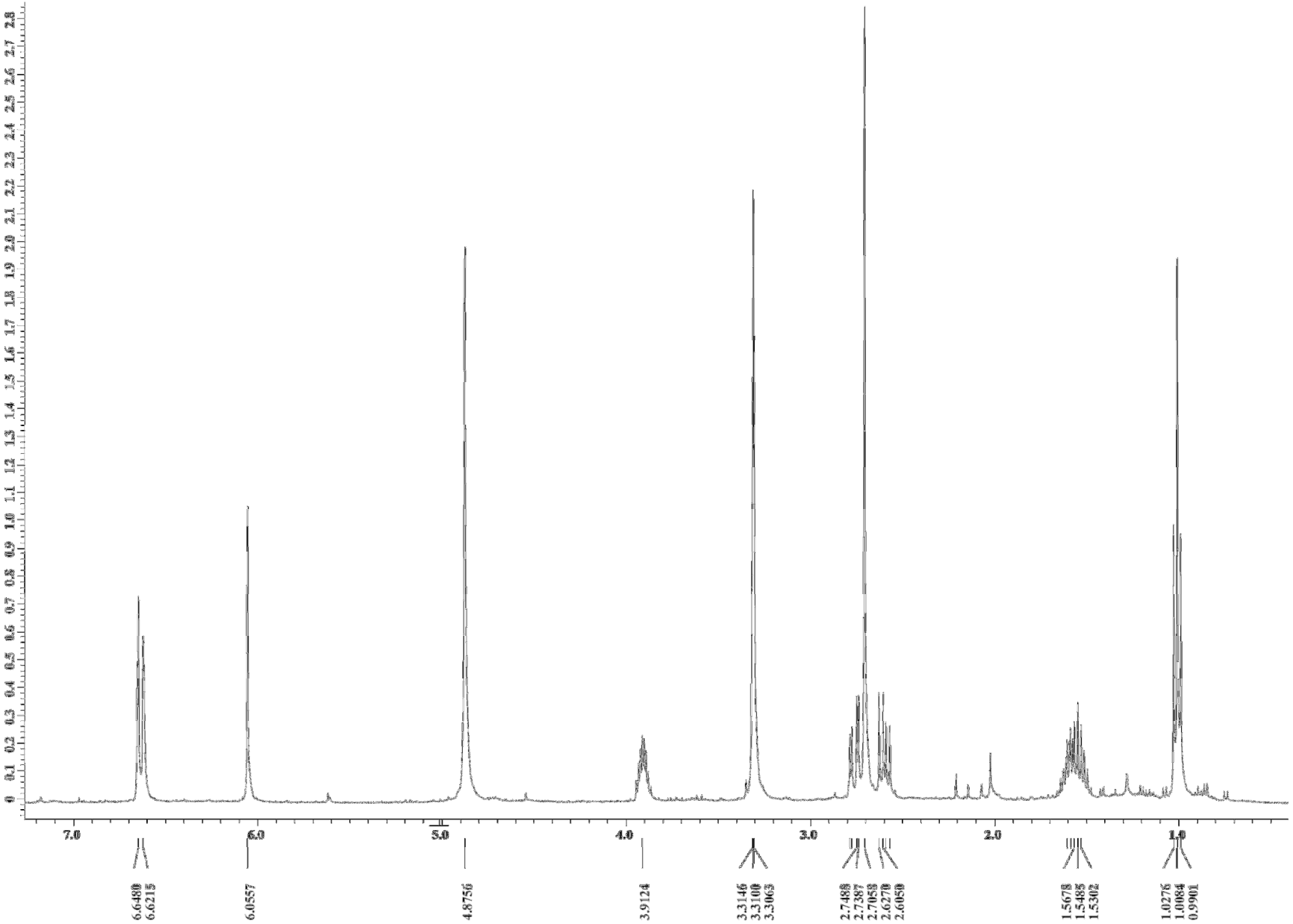
Appendix 3. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of xylaropyrone (1)



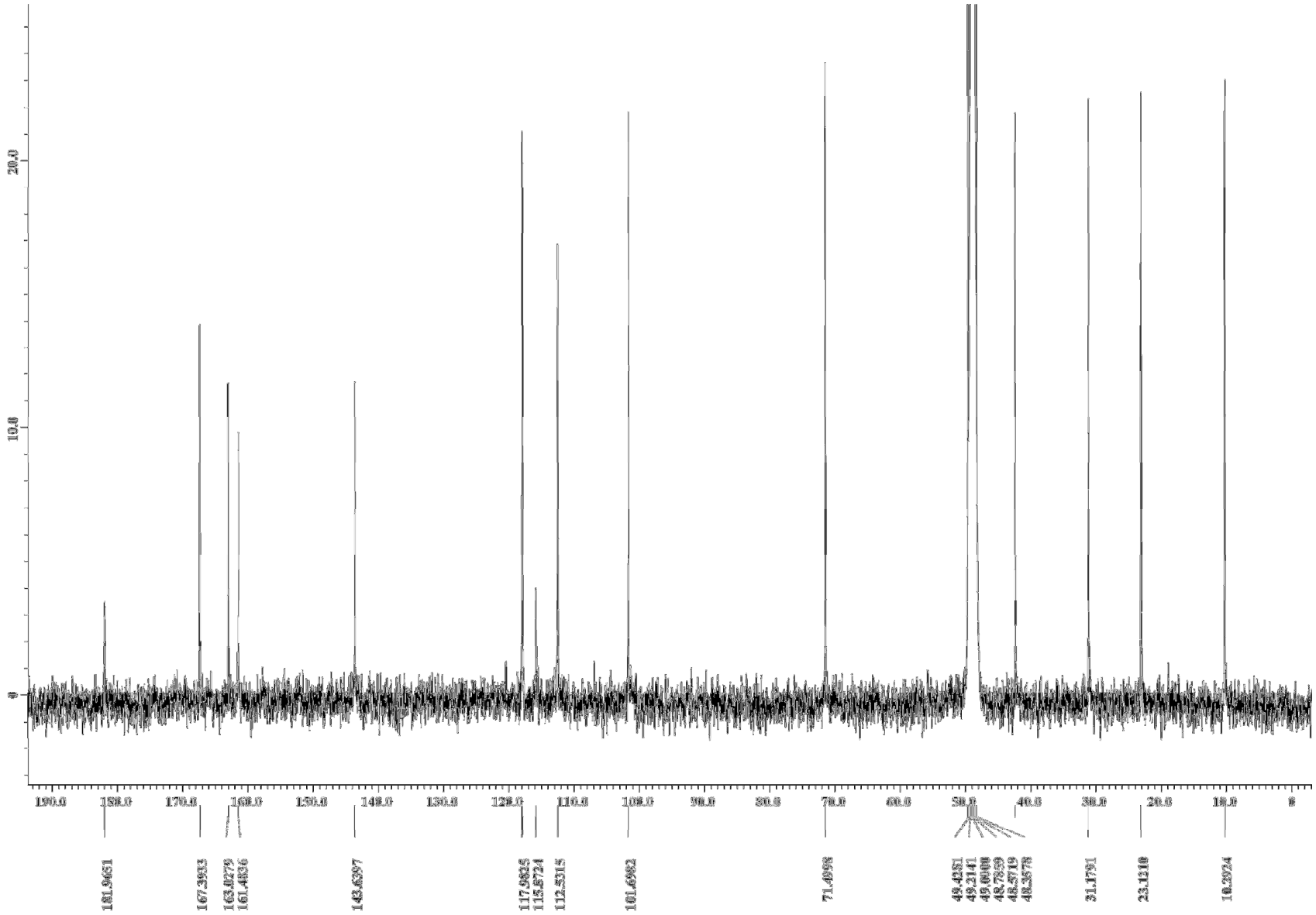
Appendix 4. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of xylaropyrone (1)



Appendix 5. ^{1}H NMR spectrum (400 MHz, CD_3OD) of mycoleptione (1)



Appendix 6. ^{13}C NMR spectrum (100 MHz, CD_3OD) of mycoleptione (1)

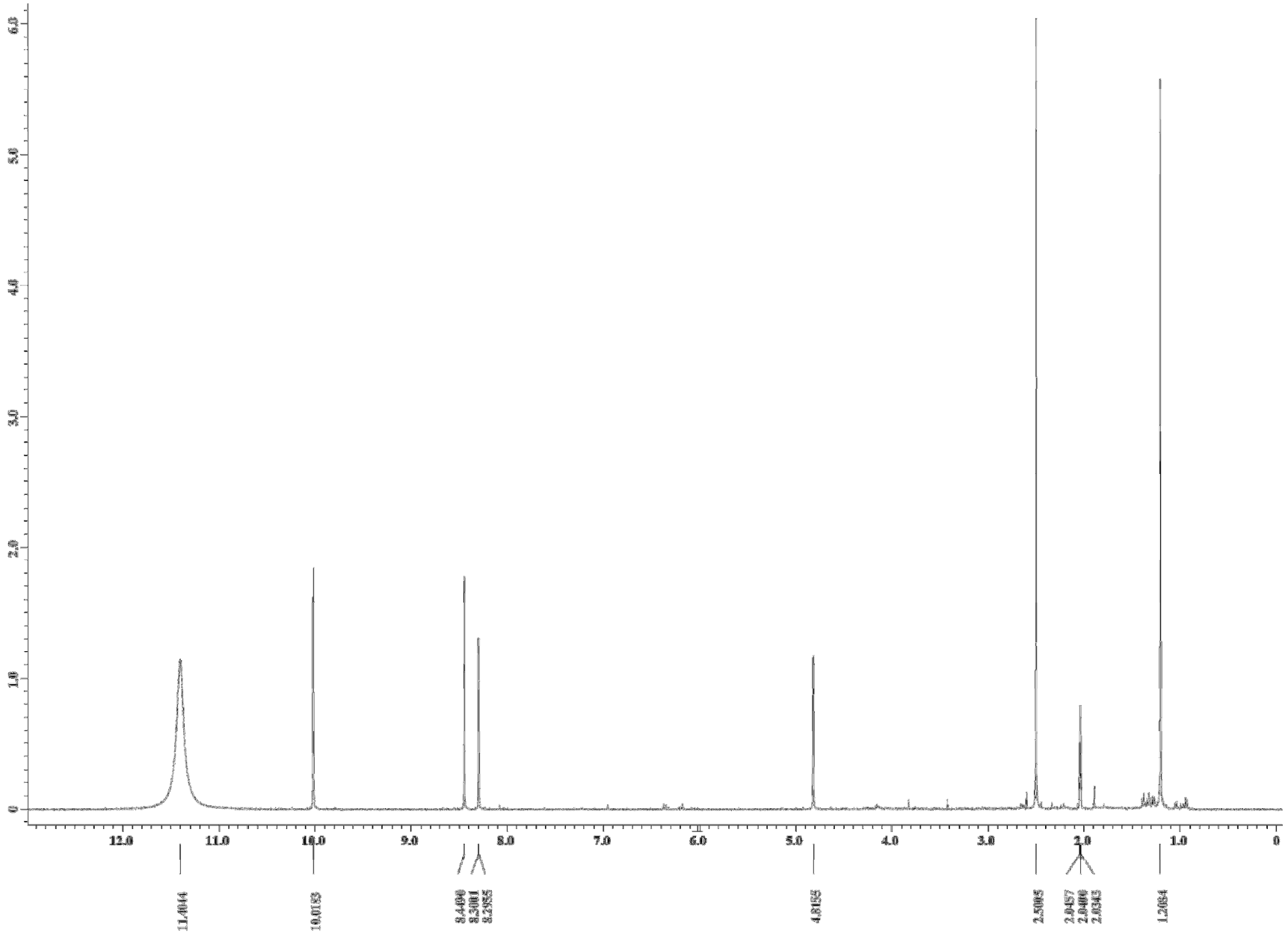


Appendix 7 Table of NMR spectroscopic data of 7-epiaustdiol (2) in CD_3CO_2D

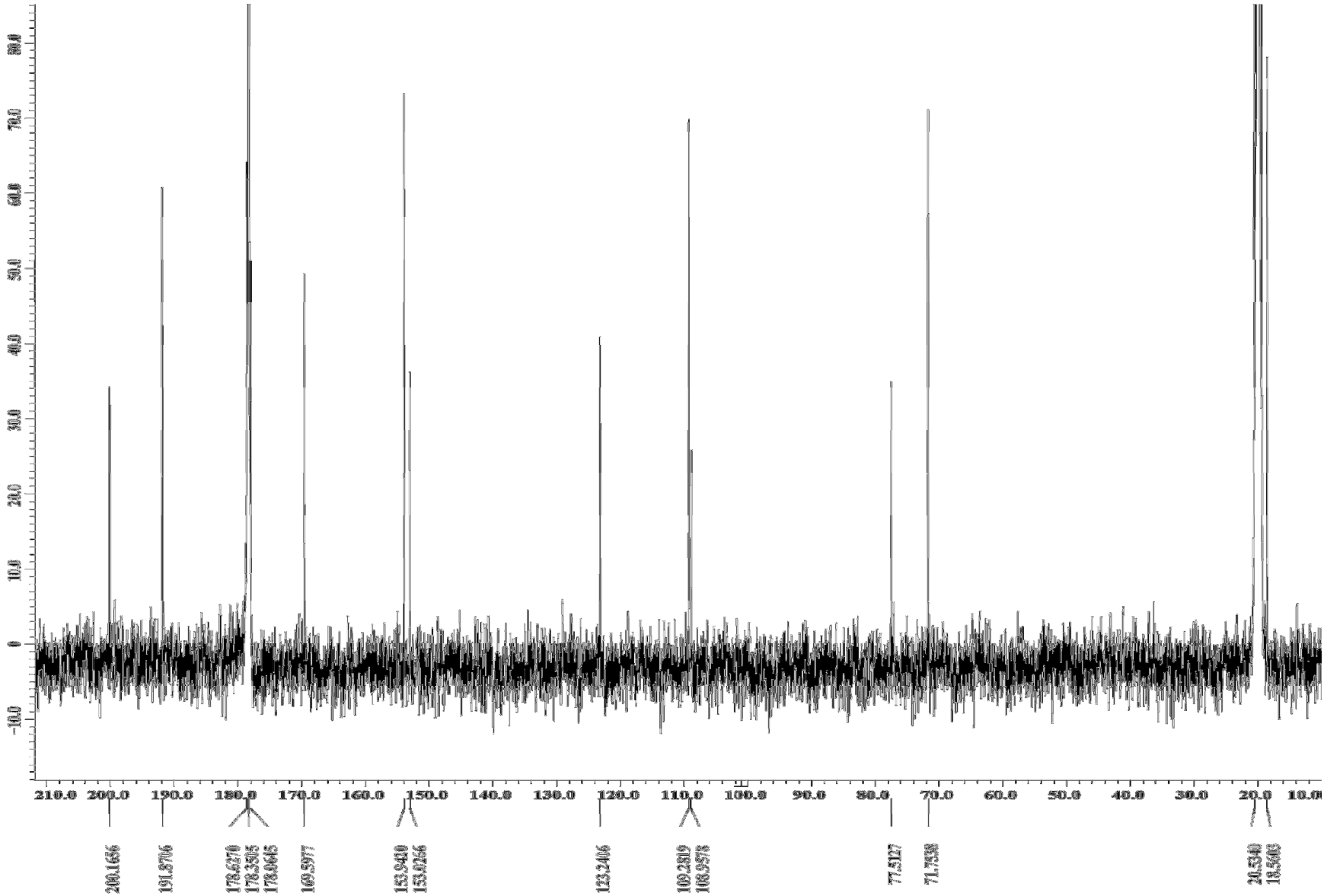
Position	δ_C	δ_H
2	153.9 (CH)	8.30 (d, *J*=1.6 Hz)
3	123.2	-
4	153.0	-
5	109.3 (CH)	8.45 (s)
6	169.6	-
7	71.8 (CH)	4.82 (d, *J*=1.6 Hz)
8	77.5	-
9	200.2	-
10	108.9	-
11	191.9 (CH)	10.02 (s)
6-CH_3	20.5 (CH_3)	2.50 (s)
8-CH_3	18.6 (CH_3)	1.21 (s)

^1H, ^{13}C NMR and 2D NMR spectra were obtained on JOEL JNM-ECS400 NMR spectrometers, in CD_3O_2D at room temperature, and the solvent peak was used as an internal standard (δ_H 2.04 and δ_C 20.0 in CD_3OD).

Appendix 8 ^{1}H NMR spectrum (400 MHz, CD_3COOD) of 7-epiaustdiol (2)

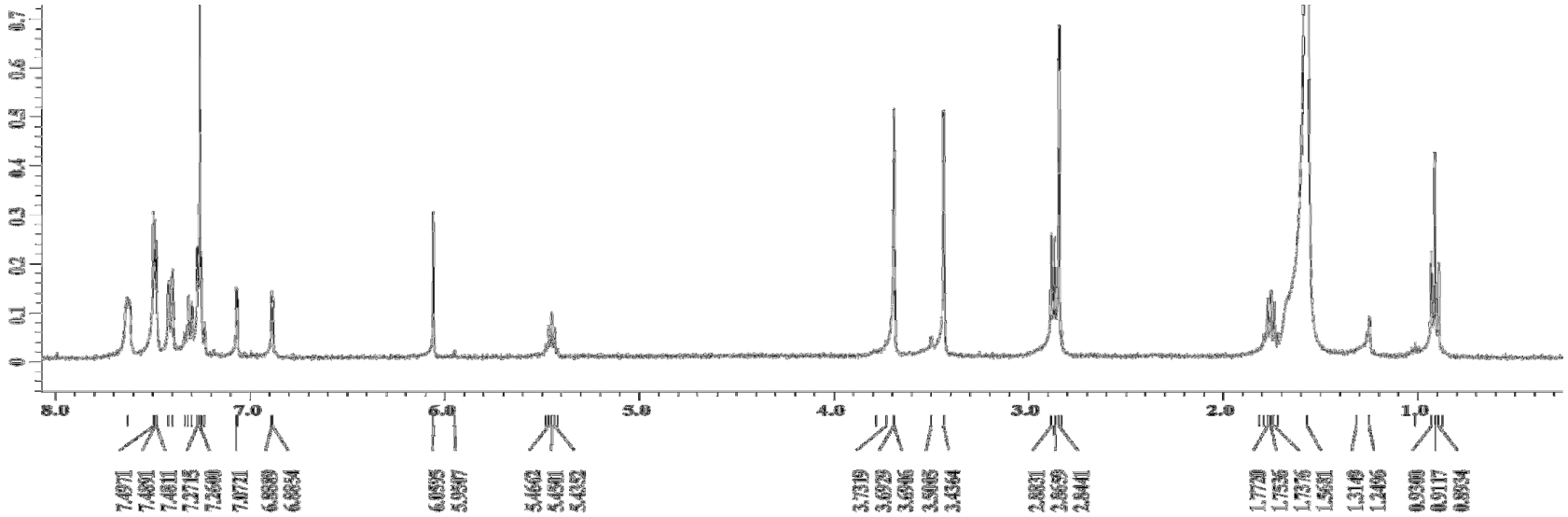


Appendix 9. ^{13}C NMR spectrum (100 MHz, CD_3COOD) of 7-epiaustdiol (2)

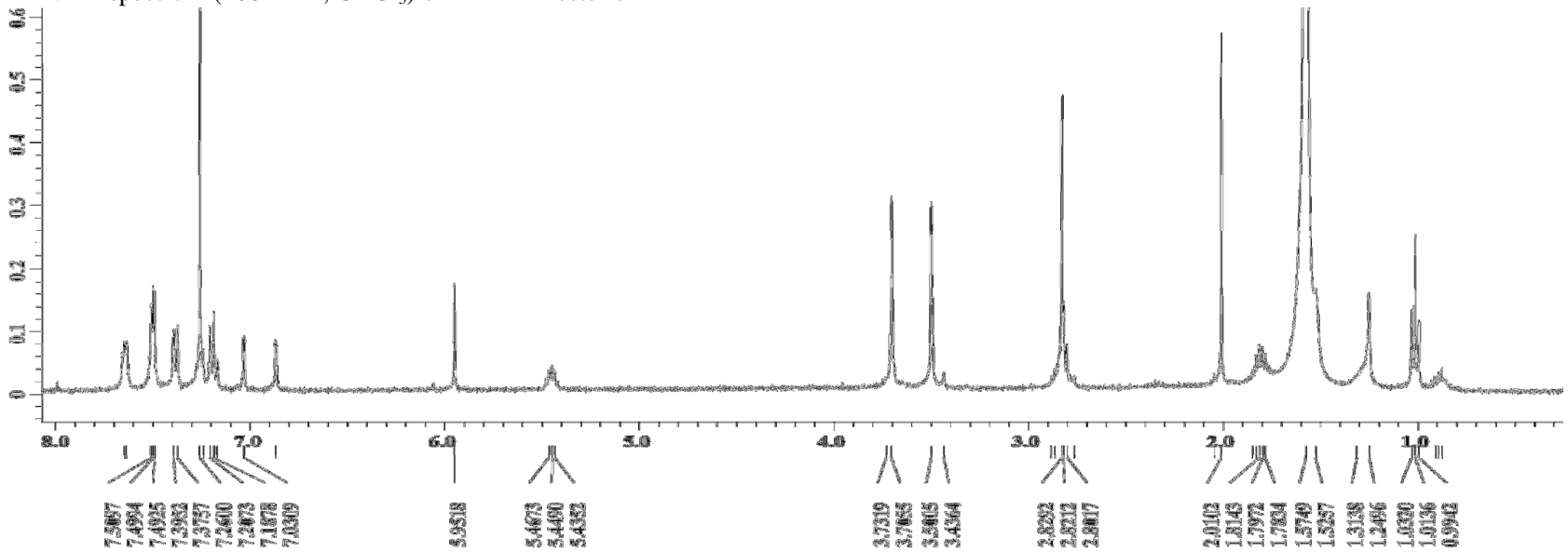


Appendix 10. Preparation of the (*R*)- and (*S*)-MTPA ester derivatives of mycoleptione (1)

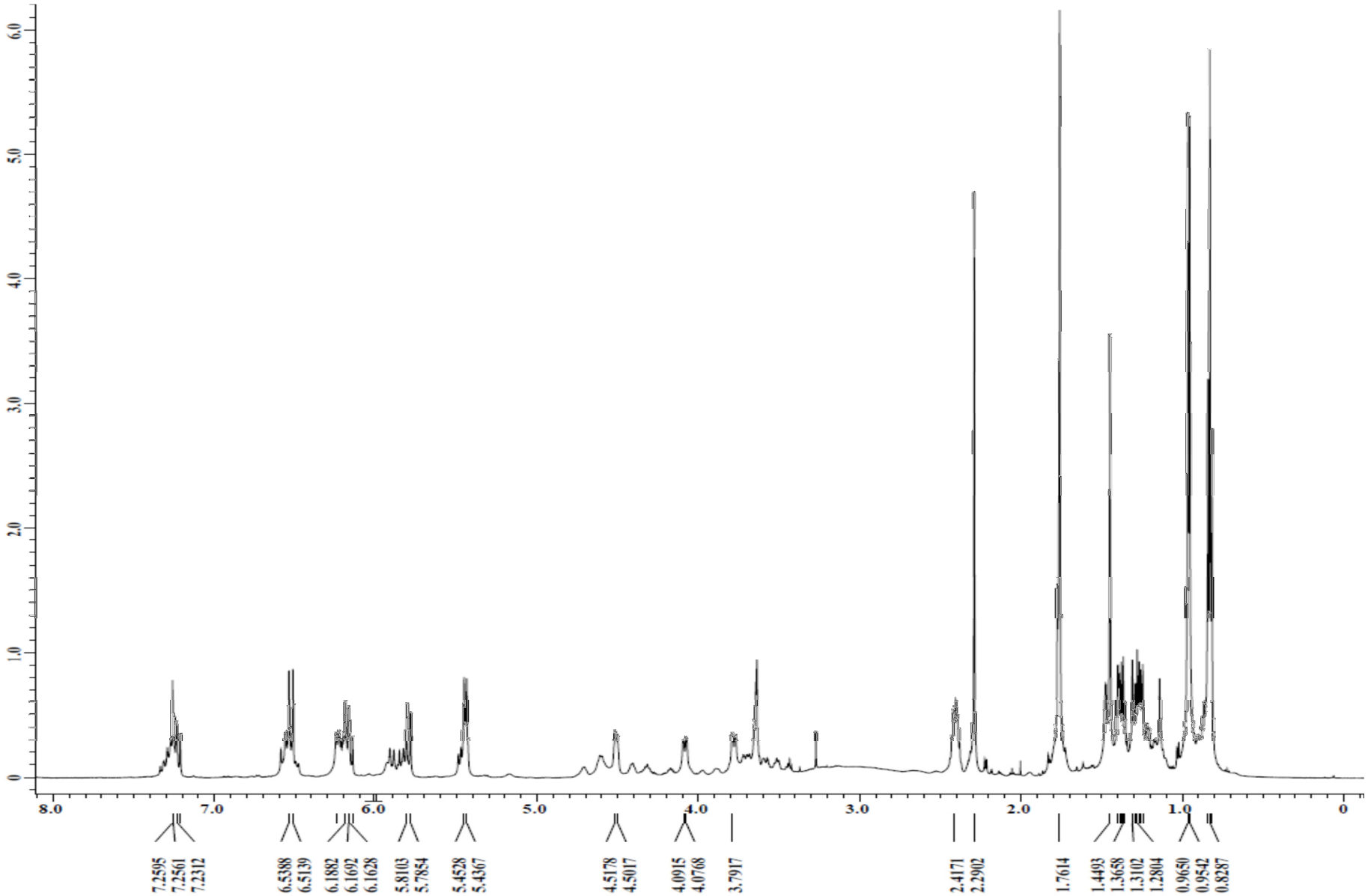
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of *S*-MTPA ester of 1



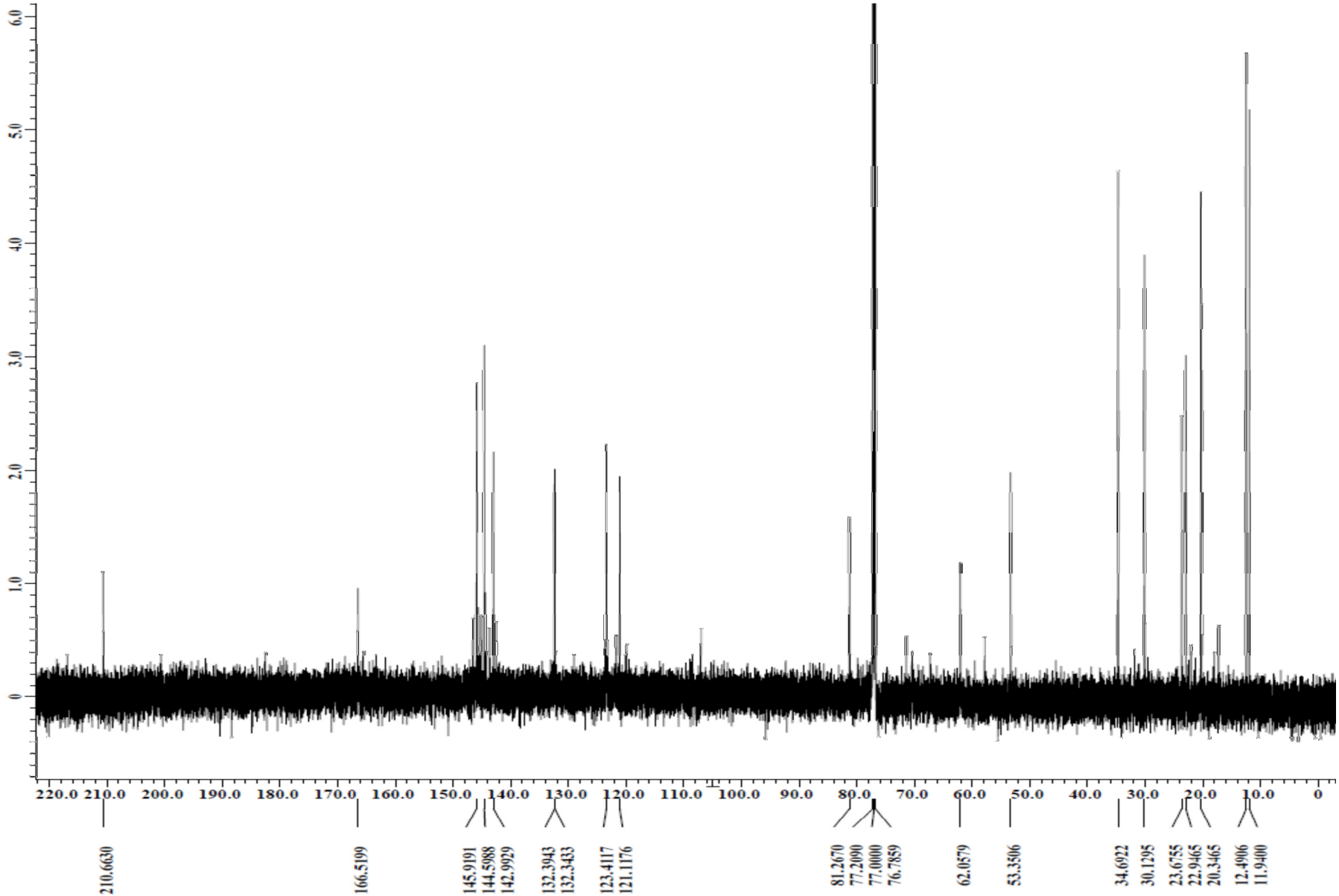
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of *R*-MTPA ester of 1



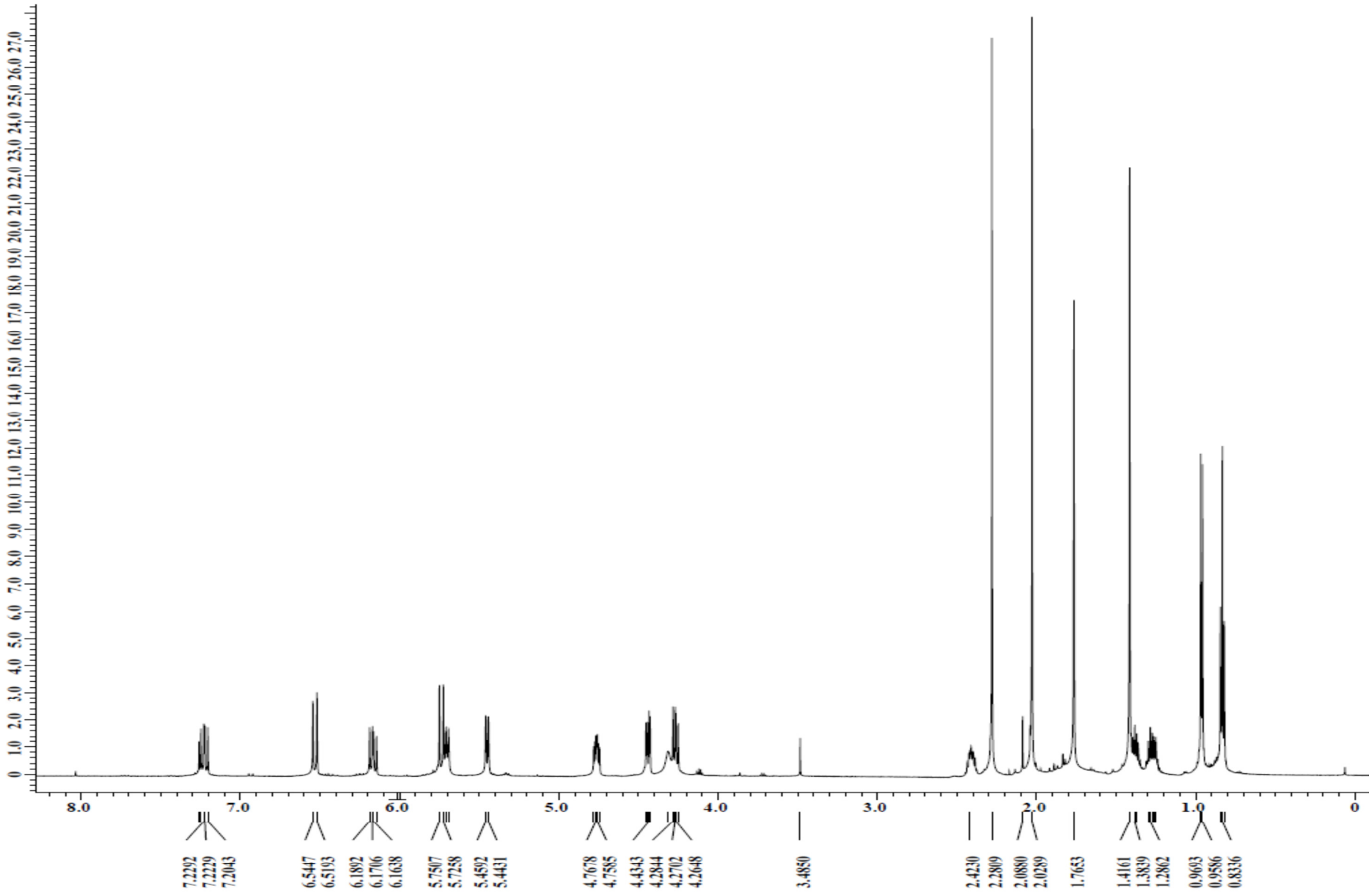
Appendix 11. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of bipolamide A (1)



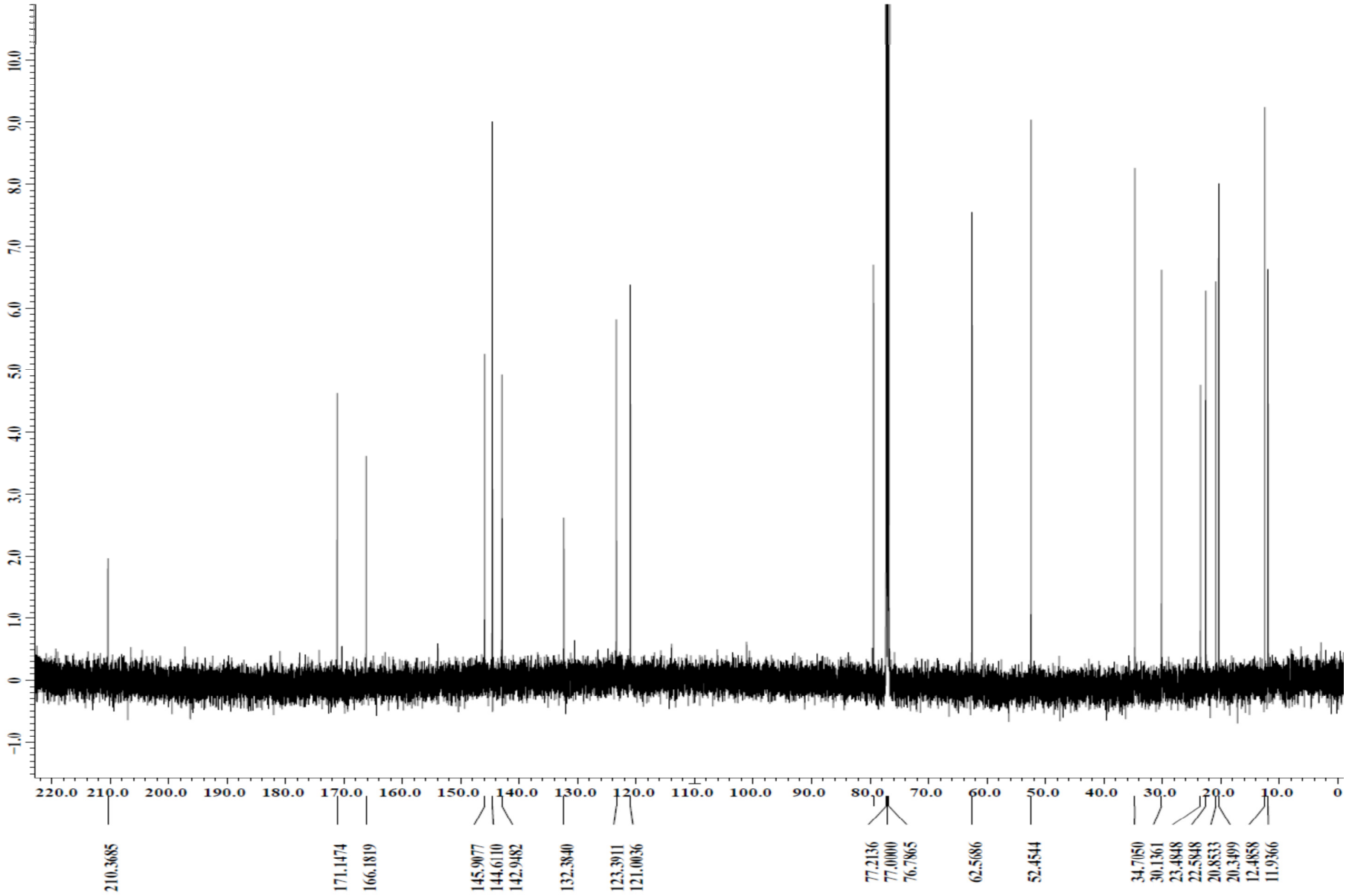
Appendix 12. ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of bipolamide A (1)



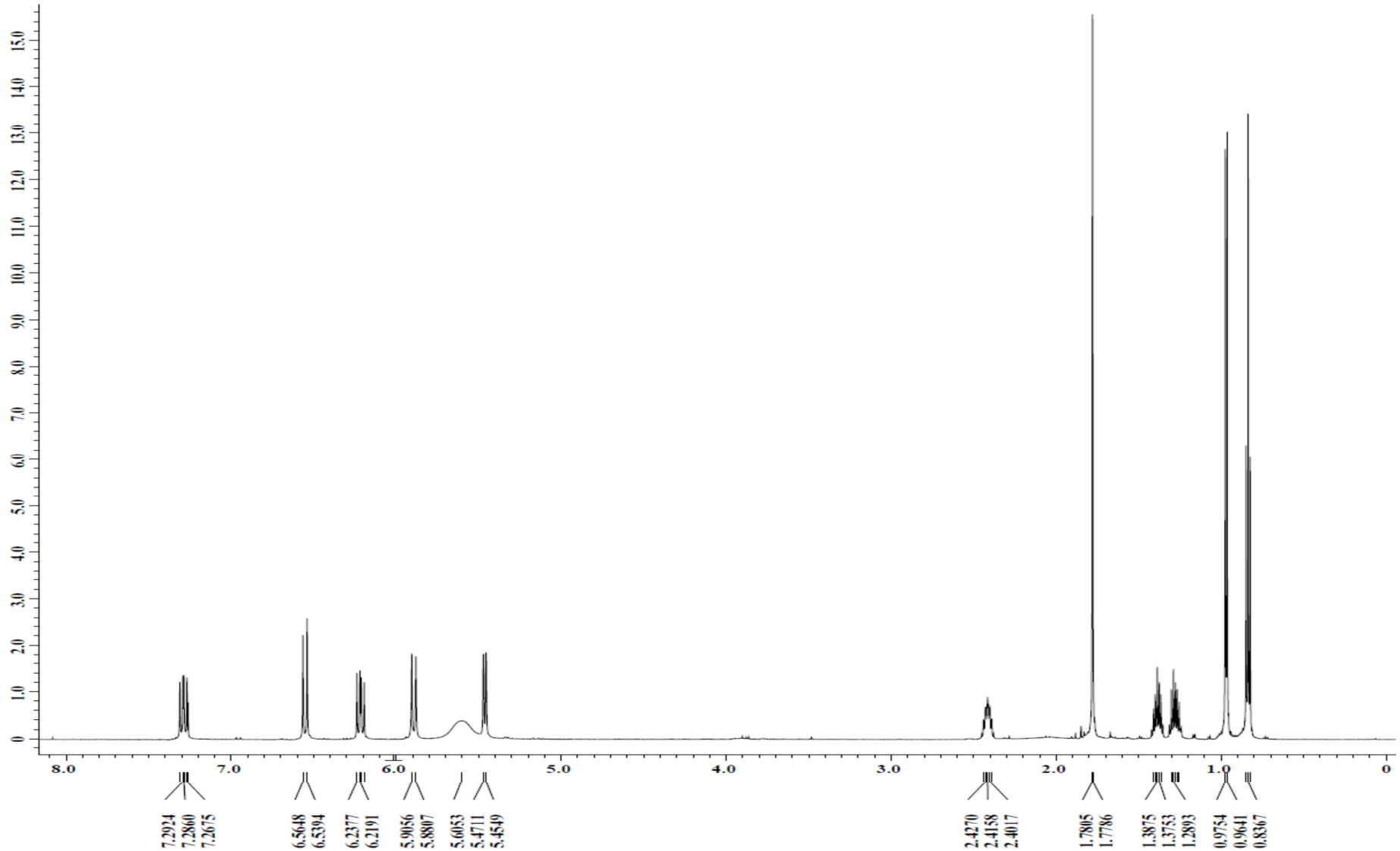
Appendix 13. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of acetate bipolamide A (3)



Appendix 14. ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of acetate bipolamide A (3)



Appendix 15. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of bipolamide B (2)



Appendix 16. ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of bipolamide B (2)