สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ *Phomopsis* sp.จากผักหวานเมา *Urobotrya siamensis*
และไอโซเลต LRUB 20 จากกะตังใบ *Leea rubra*

นายพรเทพ ชมชื่น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI *Phomopsis* sp. FROM

Urobotrya siamensis AND ISOLATE LRUB 20 FROM *Leea rubra*

Mr. Porntep Chomcheon

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งานวิจัยนี้ทำการแยกสารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ไอโซเลต LRUB 20 ที่แยกได้จากกิ่งกะตังใบ และไอโซเลต USIA 5 ที่แยกได้จากใบผักหวานเมา โดยนำสารสกัดหยาบจากราเอนโดไฟต์ไอโซเลต LRUB 20 มาทำการแยกสารบริสุทธิ์โดยเทคนิคโครมาโทกราฟีได้สาร 3 ชนิด คือ asterric acid, 2-hydroxymethyl-3-methyl-cyclopent-2-enone และ 2-hydroxymethyl-3-methyl-cyclopentanone ในขณะที่สารสกัดหยาบจากราเอนโดไฟต์ไอโซเลต USIA 5 แยกสารบริสุทธิ์ได้ 1 ชนิด คือ 3-nitropropionic acid การพิสูจน์โครงสร้างทางเคมีของสารเหล่านี้ใช้วิธีการวิเคราะห์ข้อมูล UV, IR, MS, และ NMR ร่วมกับการเปรียบเทียบข้อมูลที่มีรายงานมาแล้ว เมื่อนำสารบริสุทธิ์ที่แยกได้ไปทดสอบฤทธิ์ทางชีวภาพ พบว่า สาร asterric acid, 2-hydroxymethyl-3-methyl-cyclopent-2-enone และ 3-nitropropionic acid แสดงฤทธิ์ต้านเชื้อ *Mycobacterium tuberculosis* H37Rv ด้วยค่า MIC เท่ากับ 200, 200 และ 0.39 μg/ml ตามลำดับ การศึกษาทางสัณฐานวิทยาและการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ ITS1-5.8S-ITS2 ของ rDNA สามารถจำแนกประเภทราเอนโดไฟต์ไอโซเลต USIA 5 คือ *Phomopsis* sp. ในวงศ์ Diaporthaceae ขณะที่การศึกษาทางสัณฐานวิทยาพบว่าราเอนโดไฟต์ไอโซเลต LRUB 20 ไม่สร้างสปอร์ จึงทำการจำแนกประเภทโดยการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ ITS1-5.8S-ITS2 ของ rDNA สามารถจำแนกประเภทราเอนโดไฟต์ไอโซเลต LRUB 20 ไว้ในวงศ์ Magnaporthaceae

	ลายมือชื่อนิสิต...
สาขาวิชา.......เทคโนโลยีชีวภาพ............	ลายมือชื่ออาจารย์ที่ปรึกษา..................................
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KEYWORD: ENDOPHYTIC FUNGI / BIOACTIVE COMPOUND / *Phomopsis* / MAGNAPORTHACEAE / ITS / ANTI-*Mycobacterium* ACTIVITY

PORNTEP CHOMCHEON: BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI *Phomopsis* sp. FROM *Urobotrya siamensis* AND ISOLATE LRUB 20 FROM *Leea rubra* THESIS ADVISOR: ASSOCIATE PROFESSOR NATTAYA NGAMROJANAVANICH, Ph.D., THESIS CO-ADVISOR: ASSOCIATE PROFESSOR NONGLUKSNA SRIUBOLMAS, Ph.D., PRASAT KITTAKOOP, Ph.D., 197 pp. ISBN 974-53-1551-6

The purpose of this research was to isolate bioactive compounds from endophytic fungi isolate LRUB 20 from *Leea rubra* Blume Ex Spreng. and isolate USIA 5 from *Urobotrya siamensis* Hiepko. Crude extract of endophytic fungus isolate LRUB 20 was purified by chromatographic techniques to afford three compounds, which were identified as asterric acid, 2-hydroxymethyl-3-methyl-cyclopent-2-enone, and 2-hydroxymethyl-3-methyl-cyclopentanone. The crude extract of endophytic fungus isolate USIA 5 provided 3-nitropropionic acid. The chemical structures of the isolated compounds were elucidated through extensive analyses of UV, IR, MS, and NMR and by comparison with literature. Asterric acid, 2-hydroxymethyl-3-methyl-cyclopent-2-enone, and 3-nitropropionic acid were found to exhibit activity against *Mycobacterium tuberculosis* H37Rv with the MIC values of 200, 200, and 0.39 μg/ml, respectively. Based on morphology and nucleotide sequences of ITS1-5.8S-ITS2 regions of rDNA, endophytic fungus isolate USIA 5 was identified as *Phomopsis* sp. in the family Diaporthaceae. While based on morphology, the fungus isolate LRUB 20 limited in spore formation. Nucleotide sequences of ITS1-5.8S-ITS2 regions of rDNA were applied to classify endophytic fungus isolate LRUB 20, which was found to be in the family Magnaporthaceae.

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LIST OF ABBREVIATIONS

acetone-*d*6	=	deuterated acetone
bp	=	Base pairs
^{0}C	=	degree Celsius
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
$CDCl_3$	=	deuterated chloroform
$CHCl_3$	=	chloroform
CH_2Cl_2	=	methylene chloride
CMA	=	Corn Meal Agar
δ	=	chemical shift
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublets (for NMR spectral data)
DNA	=	Deoxyribonucleic acid
DEPT	=	distortionless enhancement by polarization transfer
ε	=	molar absorptivity
e.g.	=	for example
et al.	=	and other
EtOAc	=	ethyl acetate
ESI-TOF MS	=	Electronspray Ionization Time of Flight Mass
g	=	gram
μg	=	microgram
h	=	hour
^{1}H-^{1}H COSY	=	Homonuclear (proton-proton) correlation spectroscopy
^{1}H NMR	=	proton nuclear magnetic resonance
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence

Hz	=	Hertz
IC_{50}	=	inhibitory concentration required for 50% inhibition of growth
IR	=	infrared
ITS	=	internally transcribed spacers
J	=	coupling constant
L	=	liter
μl	=	microliter
λ_{max}	=	wavelength at maximum absorption
M	=	Molar
$[M+Na]^+$	=	pseudomolecular ion
m	=	multiplet (for NMR spectral data)
MCzB	=	Malt Czapek Broth
MEA	=	Malt Extract Agar
MeOH	=	methanol
MES	=	Malt Extract Sucrose medium
mg	=	milligram
MIC	=	minimum inhibitory concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
MHz	=	megahertz
MS	=	mass spectroscopy
m/z	=	mass to charge ratio
ν_{max}	=	wave number at maximum absorption
nm	=	nanometer
NMR	=	nuclear magnetic resonance

NTP	=	Nucleotide triphosphate
PCR	=	polymerase chain reaction
PDA	=	Potato Dextrose Agar
PDB	=	Potato Dextrose Broth
ppm	=	part per million
q	=	quartet (for NMR spectral data)
rDNA	=	Ribosomal deoxyribonucleic acid
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid
s	=	singlet (for NMR spectral data)
SDA	=	Sabouraud's Dextrose Agar
SDB	=	Sabouraud's Dextrose Broth
sp.	=	species
t	=	triplet (for NMR spectral data)
TAE	=	Tris-HCl, acetate and EDTA
TE	=	Tris-HCl and EDTA
T_m	=	Melting temperature
TLC	=	thin layer chromatography
U	=	Unit
UV	=	ultraviolet
V	=	Volt
v	=	Volume
w	=	Weight
YCzB	=	Yeast Czapek Broth
YEA	=	Yeast Extract Agar
YES	=	Yeast Extract Sucrose medium

CHAPTER I

INTRODUCTION

An increase in the number of people in the world having health problems caused by various cancers, drug-resistant bacteria, parasitic protozoans, and fungi is a cause for alarm. Increased efforts are therefore needed to develop and search for new drugs from natural products. Microbes, especially fungi have been known to be a major source of bioactive compounds. Examples are *Metarhizium anisopliae* (microbial insecticide), *Penicillium chrysogenum* (penicillin), *Cephalosporium acremonium* (cephalosporin), *Penicillium griseofulvum* (griseofulvin), *Monascus ruber* and *Aspergillus terreus* (lovastatin) (Moore-Landecker, 1998). The estimated numbers of fungi on our planet are 1 million species and approximately 100,000 species have been described, as shown in Table 1 (Rossman, 1994). Fungi are important components of biological communities such as soil, marine, fresh water, litter, dung, and decaying remain of plants and animal (Charlie and Watkinson, 2001). Their influence is most prevalent in plant communities, where they are as biotrophic or necrotrophic parasites or pathogens, saprophytes, or facultative to obligate mutualists (Isaac, 1992). Among the least-known groups of plant-associated fungi are the fungal endophytes, the ubiquitous diverse Ascomycetes that grow asymptomatically within aerial plant tissues such as leaves and stems (Wilson, 1995). Hawksworth (1993) predicted that the vast majority of undescribed fungal diversity lies within tropical plant-associated fungi, yet the diversity and ecological roles of endophytes in tropical angiosperms are almost entirely unexplored. Thus, living plants are interesting source for screening of new microorganisms that may produce novel bioactive compounds.

Endophytic fungi are fungi which spend the whole or part of their life cycles colonizing inter-and/or intra-cellularly inside the healthy tissues of the host plant, as shown in Figure.1, typically causing no apparent symptoms of disease (Chanway, 1996). Some of these fungal endophytes may produce bioactive substances that may involve in a host-endophyte relationship. As a direct result of the role that these secondary metabolites may play in nature, they may ultimately have application in medicine. A worldwide scientific effort to isolate fungal endophytes and study their natural products is now under way. While there are myriads of epiphytic microorganisms associated with plants, the fungal endophytes now seem to attract more attention. This may be the case, since closer biological associations may have developed between these organisms in their respective hosts than the fungal epiphytes (fungi living on the outside of the plant) or soil-related organisms. Hence, the result of this may be the production of a greater number and diversity of classes of biologically derived molecules, possessing a range of biological activities. In fact, a recent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown. This compares with only 38% of novel substances from soil microflora (Strobel, 2003).

In Thailand, there are a few reports of endophytic fungi. For examples, endophytic fungi were isolated from indigenous dicotyledonous plants at Doi Suthep-Pui area from the northern Thailand (Lumyong *et al*., 1997). Studies by Wiyakrutta *et al*., (2004) have reported that endophytic fungi were isolated from 81 Thai medicinal plant species collected from forests in four geographical regions of Thailand, and crude extracts of these fungi were evaluated for biological activities.

The present research aims to study bioactive metabolites produced by endophytic fungi of Thai medicinal plants. During the course of study, the endophytic fungi isolate LRUB 20 (isolated from *Leea rubra* Blume ex Spreng) and isolate USIA 5 (isolated from *Urobotrya siamensis* Hiepko) exhibited interested ^{1}H NMR pattern. Chemical structures of the bioactive compounds were elucidated by spectroscopic methods and the isolated fungi were classified based on morphology and nucleotide sequence of ITS1-5.8S-ITS2 regions of rRNA gene.

The objectives of this study are as follows:

1. Isolation and characterization of bioactive compounds of the endophytic fungi isolate LRUB 20 from *L. rubra* and isolate USIA 5 from *U. siamensis*.

2. Classification of the endophytic fungi isolate *Lrub* 20 and isolate *Usia* 5.

3. Evaluation of biological activities of the isolated compounds.

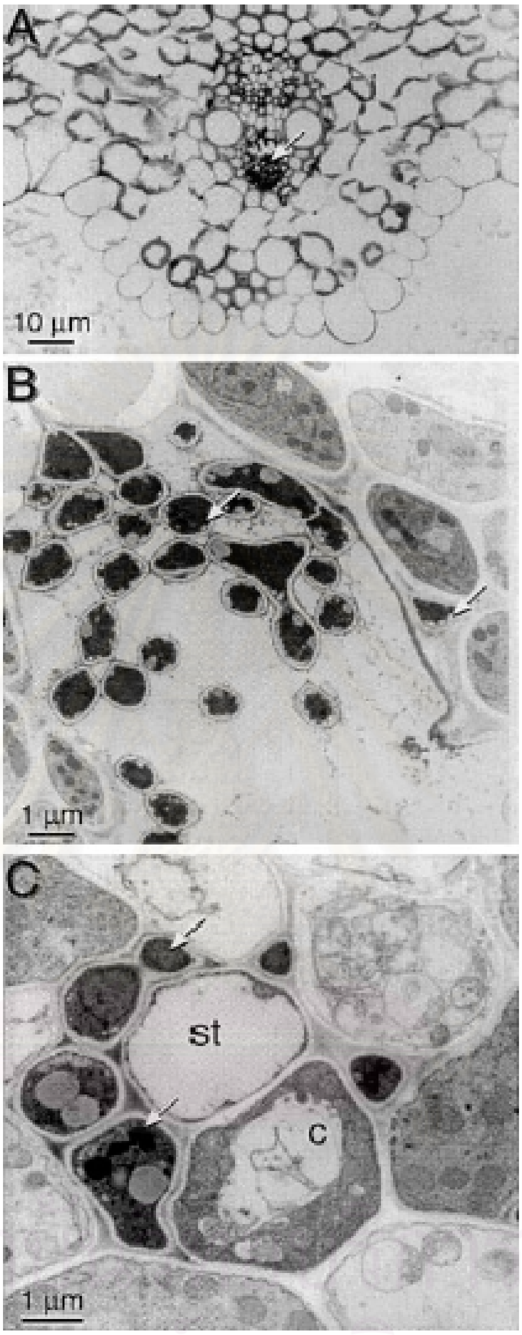
Table 1 Number of fungal species have been expected in this world (Rossman, 1994).

Group of Fungi		Number of species
Well-known	**Aphyllophorales** (saprophytes/facultative parasites)	20,000
	Macrolichens (symbiotic)	20,000
Moderately Well-known	**Agaricales** (mushrooms including secotioid and hypogeous relatives, saprophytic/ectomycorrhizal)	80,000
	Dematiaceous and aquatic hyphomycetes (primarily saprophytic, some plant pathogenic)	80,000
	Uredinales (rusts) (obligate parasites of vascular plants)	50,000
	Hypocreales and Xylariales (saprophytes on soil, rotting litter, and other fungi, some plant pathogens)	50,000
	Ustilaginales (smuts) (obligate parasites of vascular plants)	15,000
	Gasteromycetes (saprophytes on soil and rotting wood)	10,000
	Erysiphales (obligate parasites on vascular plants)	10,000
	Jelly fungi (saprophytes on rotting wood, possibly as parasites of invertebrates or other fungi)	5,000
	Ascomycetes-Pezizales (mostly saprophytic,some plant pathogenic and mycorrhizal)	3,000
	Myxomycetes (true slime molds) (saprophytic)	1,500

Table 1 Continued

Group of Fungi		Number of species
Moderately Well-known	Endomycetales (true yeasts)	1,000
Poorly Well-known	Non-dematiaceous hyphomycetes (excluding groups mentioned above)	200,000
	Coelomycetes (saprophytic on all substrates, some plantpathogens)	200,000
	Perithecial Ascomycetes and Loculoascomycetes (excluding Erysiphales, Hypocreales and Xylariales)	100,000
	Ascomycetes-Helotiales (saprophytic on all substrates, some plantpathogens)	70,000
	Insect-specificfungi (Entomophthorales, Laboulbenio-mycetes,Trichomycetes)	50,000
	Crustose lichenized ascomycetes (symbiotic)	20,000
	Mucorales (saprophytic)	20,000
	Oomycetes (some obligate parasites of vascular plants, nonspecizalized plant pathogens, saprophytes)	20,000
	Chytridiomycetes (some with specialized habitats)	2,000
	Endogonales and Glomales (vescicular mycorrhizal fungi)	1,000
Total		1,028,500

[Table adapted from: Rossman 1994 **Biodiversity and terrestrial ecosystems** Sinica Monograph Series No.14]



[Micrographs: Christensen *et al*. 1997 **Mycol. Res.** 100: 497]

Figure 1 Growth an *E. festucae* variant in the vascular tissue of meadow fescue.

(A) Cross section of a leaf sheath with hyphae (arrow) throughout the vascular bundle.

(B) Close-up of hyphae (arrow) in the air space.

(C) Hyphae (arrow) surrounding a phloem sieve tube element (st) and companion cell (c).

As shown in (B) and (C), plant cells adjacent to hyphae appear undamaged and exhibit no apparent response to the fungus (Christopher, 2001).

CHAPTER II

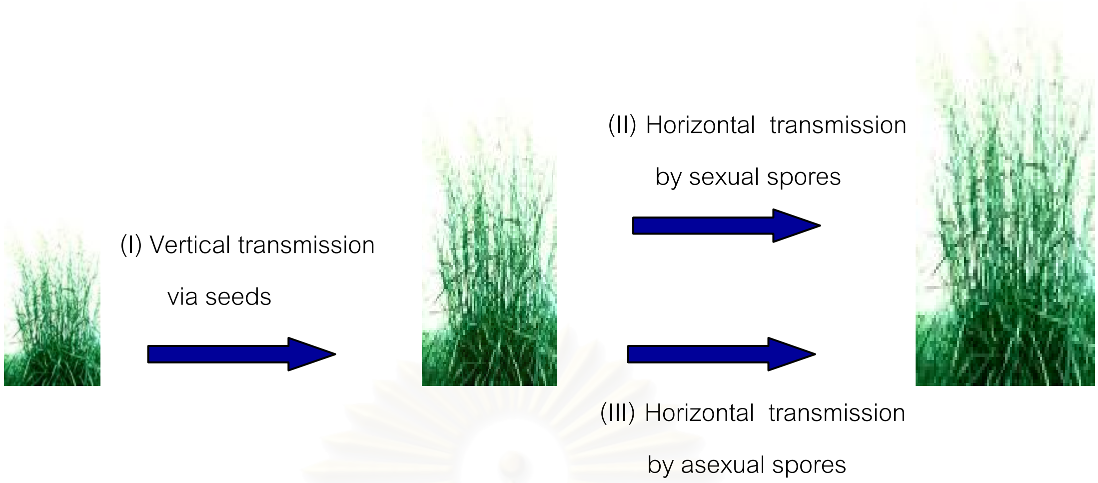
REVIEW OF LITERATURE

2.1 Association of the endophytic fungi and plants

As a matter of fact, fungal endophytes are important components of microbial biodiversity (Smith *et al.,* 1989), that occur in every host species sampled to date, including > 200 terrestrial and aquatic species representing > 20 families of such diverse taxa as marine macroalgae, mosses, fern, "gymnosperm", monocots, and herbaceous and woody dicots (Lodge *et al*, 1996). Commonly, several to hundreds of fungal endophyte species can be isolated from a single plant, among them, at least one species showing host specificity. The environment condition under which the host is growing also affect the fungal population, and the fungal endophytes profile may be more diversified in tropical areas. Most endophytic fungi belong to the Ascomycetes and Fungi imperfecti (Petrini, 1991). Fungal endophytes are different from pathogenic fungi on the basis of asymptomatic growth under most conditions, and from mycorrhiza-forming fungi on the basis of taxonomy and tissue-specificity. Endophytic fungi colonize living plant tissues by penetration of fungus hyphae between plants cells or may also grow intracellularly and must obtain nutrient materials through this intimate contact with the host (Isaac, 1992). Figure 2 shows evolution of endophyte-plant symbiosis (Saikkonen *et al*., 2004).

The relationship between the endophytic fungi and its host plant may range from mutualistic symbiosis, or commensalisms to borderline parasitism (Strobel and Long, 1998). Certain fungal endophytes improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens and/or herbivores including some insects feeding on the host plant. Endophyte-infected grasses usually possess an increased tolerance to drought and aluminium toxicity. Furthermore, some endophytes are able to provide the host plant with protection against some nematodes, mammal and insect herbivores as well as bacterial and fungal pathogens. (Tan and Zou, 2001).

(A) Life cycles of systemic grass endophytes



(B) Benefits to the partners

Benefits	
Plant	Fungus
Increased : - Growth - Reproduction - Resistance	- Refuge - Nutrition - Transmission

[Figure adapted from: Saikkonen *et al*. 2004 **Trends in Plant Science** 9: 276]

Figure 2 (A) Life cycles of systemic grass endophytes

(I) Hyphae grow internally and intercellularly throughout the above-ground tissues of the host plant and into the developing inflorescence and seeds and, thus, are transmitted the systemic fungi from plant to offspring via host seeds (Vertical transmission), e.g. *Neotyphodium* endophytes.

(II) *Epichloë* endophytes can also be transmitted sexually (spores) when the fungus forms external stromata with conidia around a developing inflorescence, causing abortion. Contagious spread should not be ruled out even in *Neotyphodium* endophytes because they produce asexual conidia on growth media and on living plants, and recent evidence indicates horizontal transmission in natural grass populations (III).

(B) Benefits to the partner

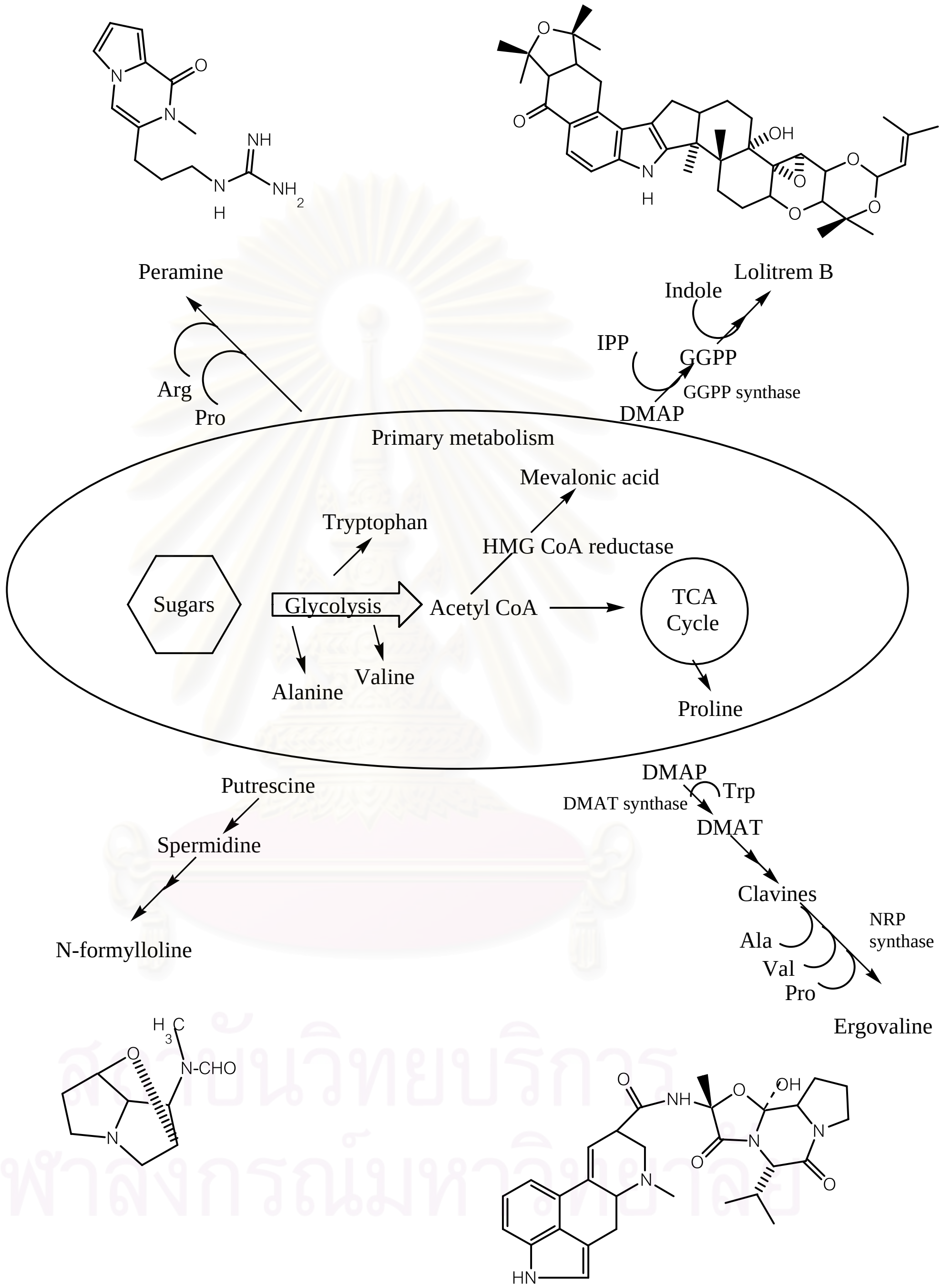
Grass endophytes are generally considered to be mutualists because the fungus subsists entirely on the resources of the host. The fitness of an endophytic symbiont that has lost or limited opportunities for contagious spread by spores depends largely on the fitness of the host plant. The host receives benefits through increased resistance to herbivores, pathogens and drought and flooding stress, and enhanced competitive abilities.

2.2 Study of bioactive compounds from the endophytic fungi

In the 1970's, endophytic fungi were initially considered only for identification and classification, not causing benefits nor showing detriment to plants. Until in the past two decades, the interest for endophytic fungi was as potential sources of novel bioactive compounds that exhibited interesting bioactivities such as anticancer, antifungal, insecticidal, antimicrobial, antimalarial, immunosuppressive, and antiviral activities (Azevedo *et al*, 2000).

For examples, Strobel *et al.* 1993 isolated paclitaxel (Taxol®, anticancer drug) from the endophytic fungus *Taxomyces andreanae* from Pacific yew *Taxus brevifolia.* Furthermore, taxol is also found in endophytic fungi, *Pestalotiopsis guepinii* from *Wollemia nobilis* (Strobel *et al,* 1997), *Periconia* sp. from *Torreya grandifolia* (Li *et al,* 1998b), *Pestalotiopsis microspora* from *Taxus wallachina* (Metz *et al.,* 2000, Li *et al.,* 1998a), *Tubercularia* sp. from *Taxus mairei* (Wang *et al.,* 2000), *Aspergillus niger* from *Taxus chinensis* (Wang *et al.,* 2001), and *Stegolerium kukenani* from *Stegolepis guianensis* (Strobel *et al.* 2001). The fungus *Pastalotiopsis jesteri* from *Fragraea bodenii* is found to produce jesterone and hydroxy-jesterone, which exhibite selective antimycotic activity against the oomycetous fungi. Isopestacin, an isobenzofuranone, possessing antifungal and antioxidant activities, is secondary metabolites of *Pestalotiopsis microspora* (Strobel *et al., 2002*). Peramine and *N*-formylloline, The bioactive compounds with insecticidal activities, whereas lolitrem B and ergovaline are mammalian toxins, are secondary metabolites of *Epjchlo*ë sp. from grass (Scott, 2001). Proposed pathways for biosynthesis of these metabolites are shown in Figure 3. A new antimicrobial metabolite, named colletotric acid, is isolated from *Colletotrichum gloeosporioides,* an endophytic fungus colonized inside the stem of *Artemisia mongolica* (Zou *et al*., 2000). Phomoxanthones A and B, two novel xanthone dimers with antimalarial activities are isolated from the endophytic fungus *Phomopsis* sp BCC 1323 that isolated from *Tectona glandis* leaf (Isaka, 2001). Subglutinols A and B, two immunosuppressive compounds, are isolated from *Fusarium subglutinols*, an endophytic fungus of *Tripterygium wilfordii* (Lee *et al*., 1995). Two novel *p*-tridepside antiviral compounds, cytonic acid A and B, are isolated from the endophytic fungus

Cytonaema sp. obtained from *Quercus* sp. (Guo *et al.* 2000a). The biological activities, sources and chemical compounds of secondary metabolites from fungal endophytes are summarized in Table A (in Appendix A).



[Figure adapted from: Scott 2001 **Microbiology** 4: 395]

Figure 3 Proposed pathways of secondary metabolites produced by *Epichloë* endophytes isolated from grass.

The primary metabolites is shown within the elipse. Proposed pathways for secondary metabolite systhesis are shown outside the elipse.

CHAPTER III

MATERIALS AND METHODS

3.1 Selection of endophytic fungal isolates

A total of forty five unidentified endophytic fungal isolates were studies. They were divided into two groups, the first seventeen isolates and the second twenty eight isolates. Seventeen isolates, as shown in Table 2, were selected based on their bioactivities in previous studies by Meevootisom *et al*., 2002 (in www.sc.mahidol.ac.th/scmi/epf/Home.htm.). Twenty eight isolates, as shown in Table 3, were new isolates that have not yet been tested for bioactivities.

Table 2 Endophytic fungal isolates selected based on their bioactivities (Meevootisom *et al.* 2002).

No.	Fungal code	Scientific name of plant host	Culture medium	Biological activities of fungal culture extract*
1	ACHI 4	*Anthocephalus chinensis* Rich. ex Walp.	MCz YES	Anti-C. Anti-F., C.
2	ALAK 6	*Artocarpus lakoocha* Roxb.	MCz YES	Not determine Anti-B., F., C.
3	COBL 1	*Croton oblongifolius* Roxb.	MCz YES	Anti-B., F., C. Anti-B., F.
4	DOLI 5	*Dalbergia oliveri* Gamble.	MCz YES	Anti-V., C. Anti-F., V., C.
5	FHIS 2	*Ficus hispida* Linn.	MCz YES	Anti-B., F., M., V., C. Anti-B., F., M., V.
6	GSPE 11	*Gardenia* sp.	MCz YES	Anti-B., F., M., C. Anti-C.

Table 2 Continue

No.	Fungal code	Scientific name of plant host	Culture medium	Biological activities of fungal culture extract*
7	HARO 1	*Homalomena aromatica* Schott.	MCz YES	Anti-B., F., C. Anti-B., F., V., C.
8	MFER 5	*Mesua ferrea* Linn.	MCz YES	Anti-B., F. Anti-B., F., C.
9	MSMI 11	*Myxopyrum smilacifolium* Bl.	MCz YES	Not determine Anti-C.
10	PSCA 1	*Paramignya scandens* Craib.	MCz YES	Anti-B., F., C. Anti-B., F., V., C.
11	SILL 10	*Streblus ilicifolius* Corner.	MCz YES	Anti-B., F. Anti-B., F.
12	SPIN 10	*Spondias pinnata* Kurz.	MCz YES	Anti-B., F., C. Anti-V., C
13	SSIA 2	*Shorea siamensis* Miq.	MCz YES	Anti-F., C. Anti-B., C
14	STUB 3	*Stemona tuberosa* Lour.	MCz YES	Anti-B., F., M., V., C. Anti-B., F., M., V., C.
15	TCAM 1	*Tetrastigma campylocarpum* Planch.	MCz YES	Anti-F., C. Anti-B., F., C.
16	TLAU 7	*Thunbergia laurifolia* Linn.	MCz YES	Anti-F., C. Anti-F., C.
17	USIA 5	*Urobotrya siamensis* Hiepko.	MCz YES	Anti-B., F. Anti-B., F.

*Anti-B: Antibacterial Anti-C: Anticancer Anti-F: Antifungal
Anti-M: Antimalarial Anti-V; Antiviral

Table 3 Selected new endophytic fungal isolates that have not been evaluated for bioactivities.

No.	Fungal code	Scientific name of plant host	Family	Culture medium*
1	AGSP 3	*Agapetes* sp.	Ericaceae	MCz, MID
2	CTOM 1	*Catunaregam tomentosa* (Bl. Ex DC.) Tirreng.	Rubiaceae	MID
3	CTOM8	*Catunaregam tomentosa* (Bl. Ex DC.) Tirreng.	Rubiaceae	MID
4	CTOM 11	*Catunaregam tomentosa* (Bl. Ex DC.) Tirreng.	Rubiaceae	MCz, MID
5	CTOM 12	*Catunaregam tomentosa* (Bl. Ex DC.) Tirreng.	Rubiaceae	MCz, MID
6	CTOM 21A	*Catunaregam tomentosa* (Bl. Ex DC.) Tirreng.	Rubiaceae	MCz, MID
7	GELL 3	*Gmelina elliptica* Sm.	Labiatae	MCz, MID
8	GELL 8	*Gmelina elliptica* Sm.	Labiatae	MCz,MID
9	GELL 12	*Gmelina elliptica* Sm.	Labiatae	MCz, MID
10	GELL 14	*Gmelina elliptica* Sm.	Labiatae	MCz
11	GLSP 11	*Grewia* sp.	Tiliaceae	SDB
12	GLSP 12	*Grewia* sp.	Tiliaceae	MCz, MID
13	GLSP 19	*Grewia* sp.	Tiliaceae	YCz, MCz, MID
14	GLSP 23	*Grewia* sp.	Tiliaceae	MCz
15	GLSP 30	*Grewia* sp.	Tiliaceae	YCz
16	LRUB 1	*Leea rubra* Blume ex Spreng.	Leeaceae	YES
17	LRUB 20	*Leea rubra* Blume ex Spreng.	Leeaceae	MCz
18	RLYI 1	*Rhododendron lyi* Levl.	Ericaceae	PDB, MCz, MID
19	RLYI 6	*Rhododendron lyi* Levl.	Ericaceae	YC_Z
20	RLYI 7	*Rhododendron lyi* Levl.	Ericaceae	YC_Z
21	SMON 6	*Sterculia monosperma* Vent.	Sterculiaceae	YES

Table 3 Continue

No.	Fungal code	Scientific name	Family	Culture medium*
22	SMON 7	*Sterculia monosperma* Vent.	Sterculiaceae	MC_Z
23	SMON 10	*Sterculia monosperma* Vent.	Sterculiaceae	YES
24	SMON 14	*Sterculia monosperma* Vent.	Sterculiaceae	MEB
25	TASP 5	*Tadehagi* sp.	Leguminosae	YC_Z, MC_Z
26	TASP 13	*Tadehagi* sp.	Leguminosae	SDB
27	TASP 15	*Tadehagi* sp.	Leguminosae	YC_Z, MC_Z, MID
28	TORI 2	*Trema orientalis* (L.) Blume.	Ulmaceae	MES

*MCz: Malt Czapek broth MEB: Malt Extract Broth

MES: Malt Extract Sucrose broth MID medium (Pinkerton and Strobel, 1976)

PDB: Potato Dextrose Broth SDB: Sabouraud's Dextrose Broth

YCz: Yeast Czapek broth YES: Yeast Extract Sucrose broth

3.2 Culture media and chemicals

3.2.1 Culture media

Culture media used for cultivation of endophytic fungi were Corn meal agar (CMA) (Difco), Malt extract agar (MEA) (Merck), Potato dextrose agar (PDA) (Merck), Sabouraud's dextrose agar (SDA) (Merck), malt extract powder (Merck), yeast extract powder (Merck), soytone (Merck) and agar base (agar-agar ultrapure granulated, Merck). Other mycological media were Tap water agar (TWA), Yeast extract sucrose medium (agar and broth) (YES), Malt Czapek medium (agar and broth) (MCz), Malt Extract Broth (MEB), Malt Extract Sucrose broth (MES), Potato Dextrose Broth (PDB), Sabouraud's Dextrose Broth (SDB), Yeast Czapek broth (YCz), and MID medium, the formula are shown in Appendix B.

3.2.2 Chemicals

Chemicals used in this study are as the following: boric acid (Merck, GR), ammonium tartrate (Merck, GR), sodium nitrate ($NaNO_3$) (BHD, AR), sodium chloride (NaCl) (Merck, GR), sodium hydrogen carbonate ($NaHCO_3$) (Merck, GR), sodium acetate (NaOAc) (Sigma, AR), disodium hydrogen phosphate (Na_2HPO_4) anhydrous (Merck, GR), potassium dihydrogen phosphate (KH_2PO_4) anhydrous (Merck, GR), magnesium chloride ($MgCl_2$) (Merck, GR), calcium dinitrate [$Ca(No_3)_2$] (Merck, GR), potassium nitrate (KNO_3) (Merck, GR), ferric chloride ($FeCl_3$) (Merck, GR), manganese sulphate ($MnSO_4$) (Merck, GR), potassium iodide (KI) (Merck, GR), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) (Merck, GR), potassium chloride (KCl) (RiedeldeHaen, AR), dipotassium hydrogen phosphate (K_2HPO_4) (Merck, GR), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (Merck, GR), copper sulphate pentahydrate ($CuSO_4.5H_2O$) (Merck, GR), ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Merck, GR), absolute ethanol (Merck, AR), 95 % ethanol (industrial grade), liquid paraffin (specific gravity of 0.83-0.89, medicinal grade), dichloromethane (CH_2Cl_2) (Labscan, AR), ethyl acetate (EtOAc) (Labscan, AR), phenol (C_6H_5OH) (Amersham, AR), Tris-HCl (Sigma), EDTA (Sigma, AR), methylene blue (Sigma), glycerol (Merck, GR), bromophenol blue (Sigma), chloroform-D, 99.9 atom %D (Labscan), acetone-d6, 99.9 atom %D (Labscan), and Sephadex LH-20 (Amersham).

Molecular biology grade reagent used were deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dUTP) (FINNZYMES), *Taq* DNA polymerase (FINNZYMES), *Pst*I (FINNZYMES), and LE agarose (Seakerm®, FMC).

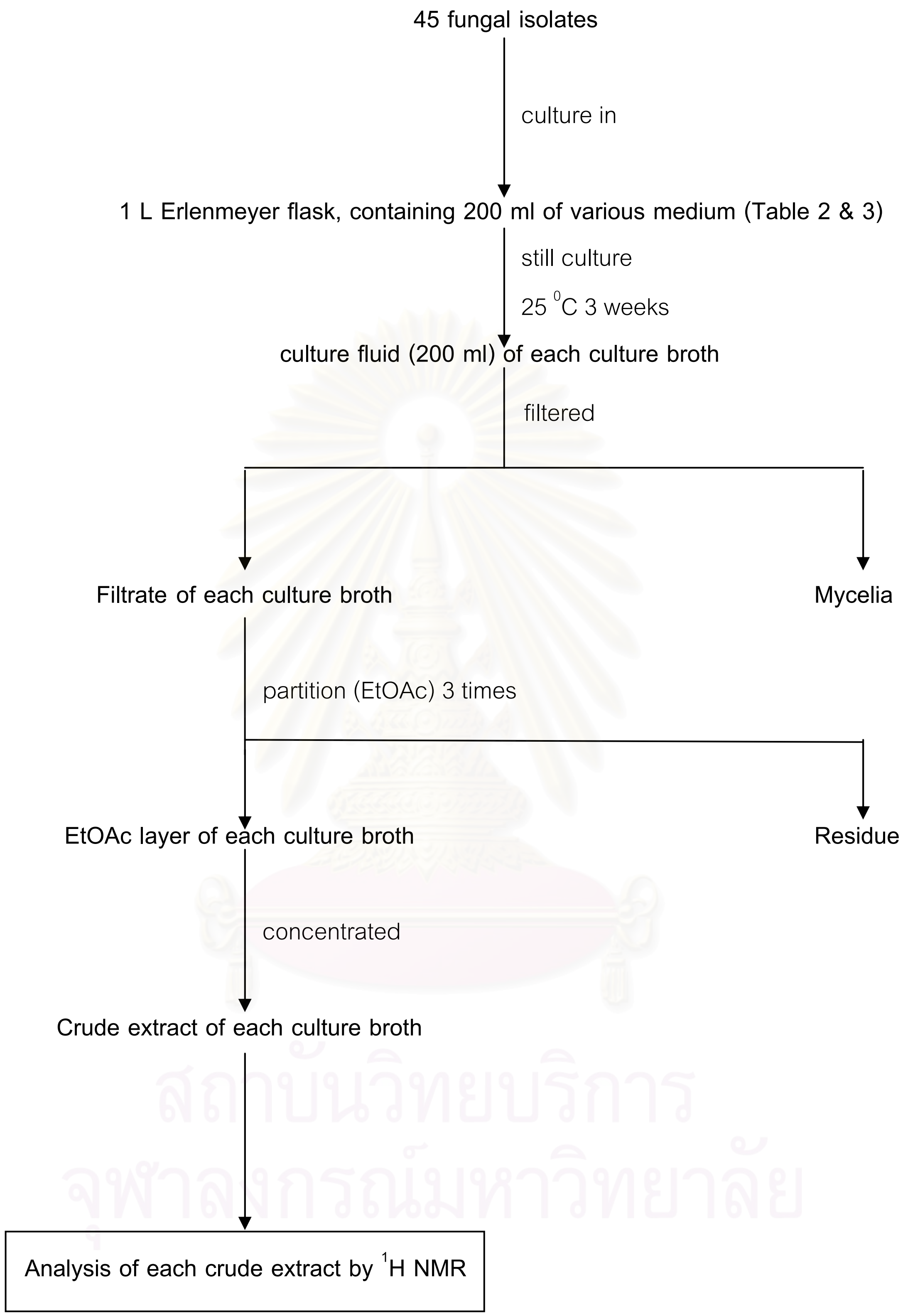
3.3 Screening of selected endophytic fungal isolates for expected novel compounds

A total of 45 fungal isolates were grown in 1-L Erlenmeyer flasks, containing 200 ml of various media, as shown in Tables 2 and 3. After 3 weeks of still culture at 25 ^{o}C, the culture fluid was passed through four layers of cheesecloth to remove mycelium. After ethyl acetate extraction, the culture extract of each fungal isolate was examined by analysis of its ^{1}H NMR spectrum data, together with the biological activities. Scheme 1 summarizes the whole process to get the crude extract.

Endophytic fungi isolate LRUB 20 from *Leea rubra* Blume ex Spreng. (Figure 4) and isolate USIA 5 from *Urobotrya siamensis* Hiepko. (Figure 5), were selected for further study due to their interesting ^{1}H NMR pattern (Appendix C). Further more, crude extract of isolate USIA 5 was found to exhibit activities against bacteria, e.g. *Staphylococcus aureus*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* with the MIC value of 100 μg/ml. The extract of USIA 5 also exhibited antifungal activity toward *Candida albicans* and *Trichophyton mentagrophytes*, and results are summarized in Table 2.

Figure 4 *Leea rubra* Blume ex Spreng. (Leeaceae) - กะตังใบ

Figure 5 *Urobotrya siamensis* Hiepko. (Opiliaceae) - ผักหวานเมา



Scheme 1 Experimental steps used to get crude extracts from fungal cultures.

Both isolates, LRUB 20 and USIA 5, were grown on four different medium, including malt Czapek (MCz) broth, potato dextrose broth (PDB), coconut broth and MID medium (Pinkerton and Strobel, 1976), as summarized in Table 4.

Table 4 Yields of crude extract (mg/100 ml) of fungi isolate LRUB 20 and isolate USIA 5 cultured on four different media

Fungal isolate	Types of medium			
	MCz broth	PDB	Coconut broth	MID medium
LRUB 20	32	16	13	25
USIA 5	17	9	5	47

The fungi isolate LRUB 20 and isolate USIA 5 grown on malt Czapek (MCz) broth and MID medium provided high yield of crude extract, and also their extracts showed interesting ^{1}H NMR spectra, therefore, these fermentation conditions were selected for further study.

3.4 Cultivation, extraction and deposition of fungi

3.4.1 Cultivation of fungi

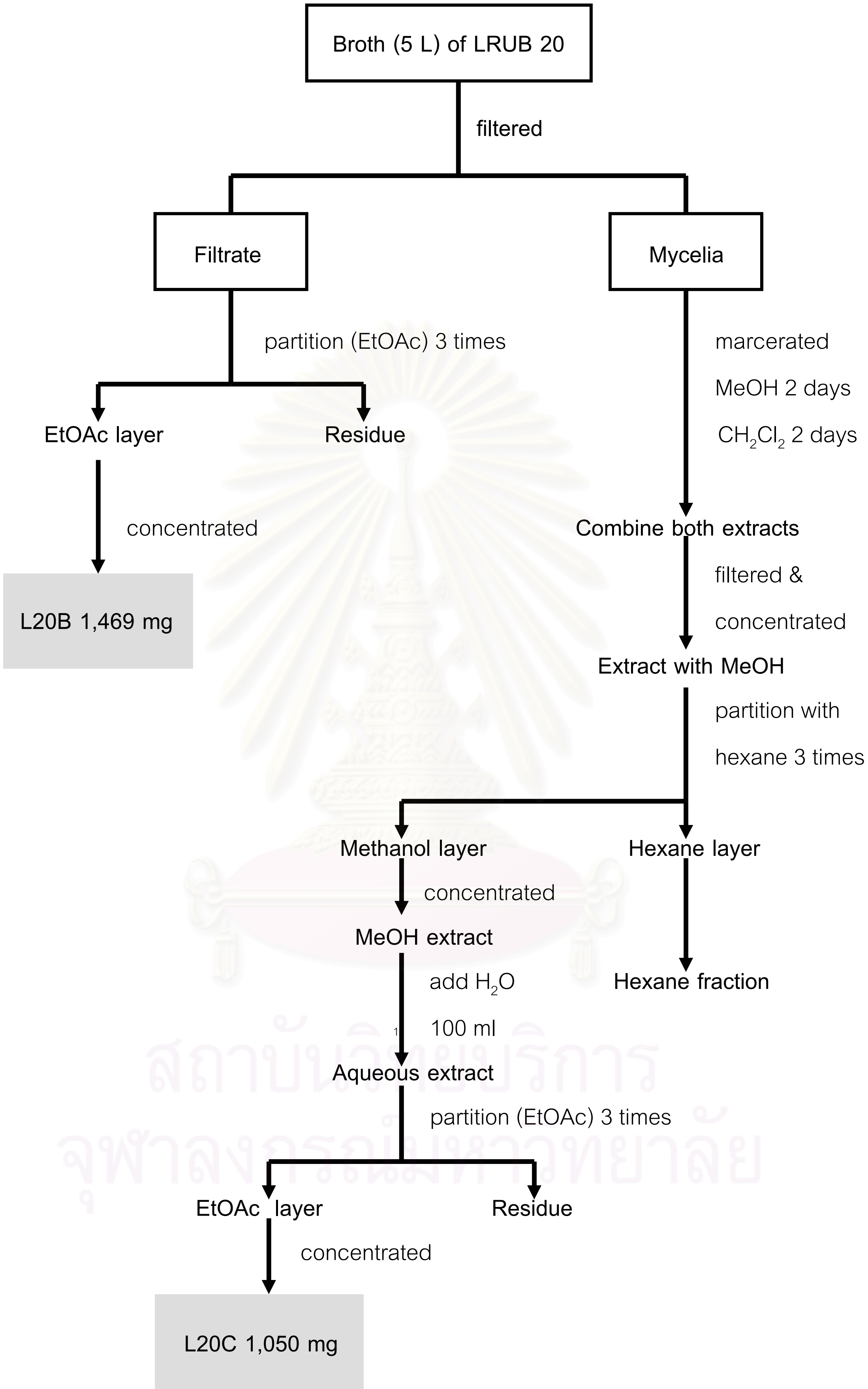
The fungi of interest were grown for three weeks at 25^{o}C in still conditions. They were cultivated in 1-L Erlenmeyer flasks containing 200 ml of MCz broth for isolate LRUB 20 and MID medium for isolate USIA 5. Several flasks of culture were prepared to obtain 5 L of MCz broth and 1.6 L of MID medium.

3.4.2 Extraction of fungi

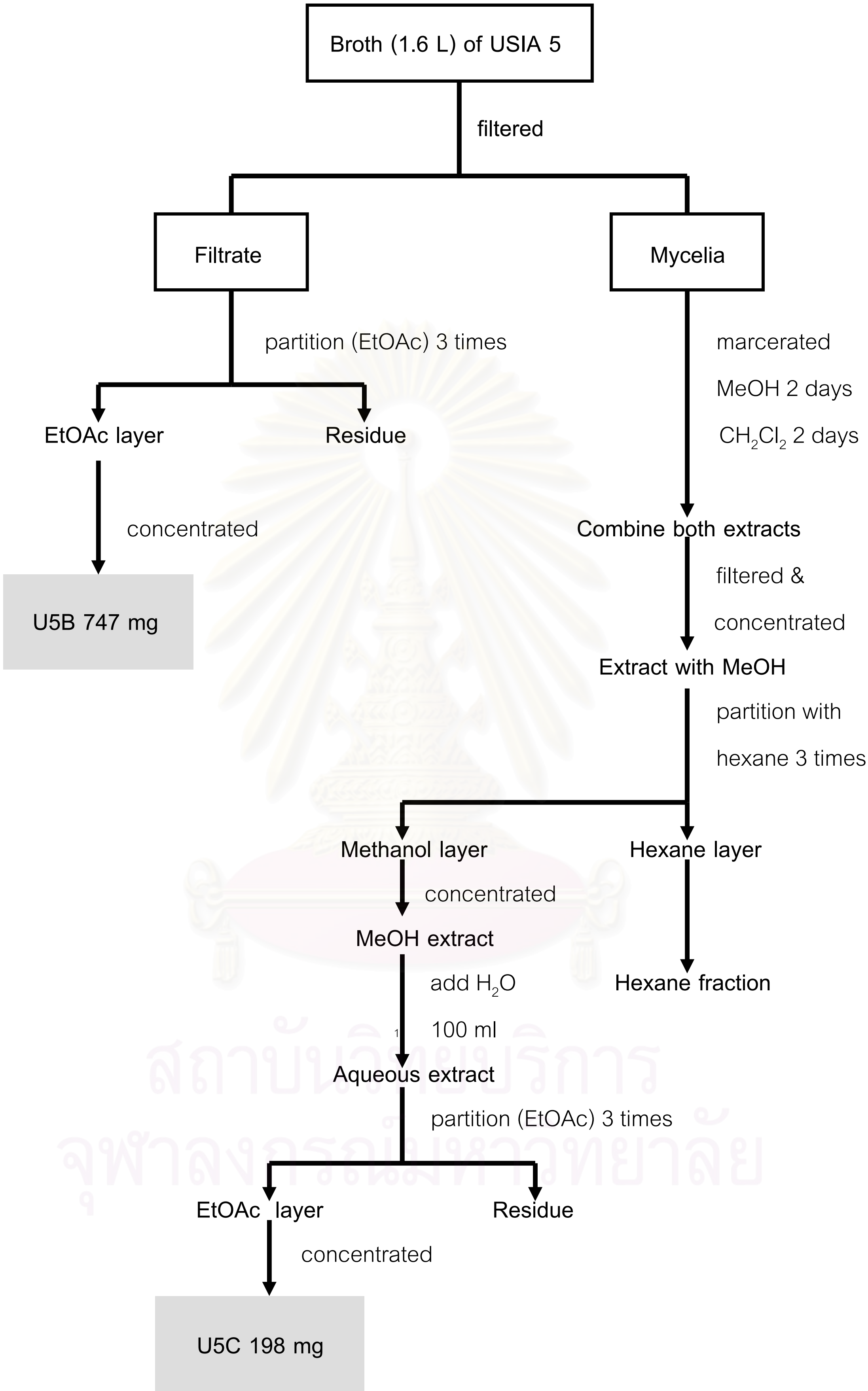
The culture broth was passed through four layers of cheese cloth and exhaustively pressed. The filtrate was extracted with an equal volume of ethyl acetate (EtOAc) 3 times. The solvent layers were then removed by evaporation at 40^{o}C to yield a residue. The residue was dissolved in methanol or methylene chloride (CH_2Cl_2), and transferred to a vial. The crude extracts of isolate LRUB 20 and isolate USIA 5 were obtained as brown viscous liquid (1,469 mg) and dark brown wax (747 mg), respectively. For the mycelium, they were extracted with MeOH (2 days) and CH_2CL_2 (2 days). The crude extracts from mycelium of isolate LRUB 20 and isolate USIA 5 were partitioned with EtOAc to yield extracts of 1050 mg and 198 mg (Figure C2 and C4 in Appendix C), respectively. The extractions of the culture broth and mycelium of the isolates LRUB 20 and USIA 5 are shown in Scheme 2 and Scheme 3, respectively.

3.4.3 Deposition of fungi

Endophytic fungi isolate LRUB 20 and isolate USIA 5 were deposited at the Bioactive Metabolite Unit (B600), Department of Microbiology, Faculty of Science, Mahidol University. For short-term storage (< 1 year), the fungi were placed in distilled H_2O, and for longer term storage they were kept frozen at -70^{o}C in 15% glycerol.



Scheme 2 Extraction of culture broth and mycelia of the fungus isolate LRUB 20



Scheme 3 Extraction of culture broth and mycelia of the fungus isolate USIA 5

3.5 Chromatographic techniques

3.5.1 Analytical thin-layer chromatography

Technique : one dimension ascending

Adsorbent : silica gel F_{254} coated on aluminium sheet (E. Merck)

Layer thickness : 250 μm

Distance : 5 cm

Temperature : laboratory temperature 25 ^{o}C

Detection : 1. Visual detection under daylight

2. Visual detection under ultraviolet light at wavelengths of 254 and 356 nm

3.5.2 Column chromatography

3.5.2.1 Gel filtration chromatography

Gel filter : Sephadex LH-20 (Amersham)

Packing method : Sephadex gel was suspended in the eluent and left overnight prior to use. It was then poured into the column and allowed to settle.

Sample loading : The sample was dissolved in a small amount of eluent then applied gently on the top of the column.

Detection : Fractions were examined by ^{1}H NMR (400 MH_{Z}) spectroscopy.

3.5.2.2 High performance liquid chromatography (HPLC)

Adsorbent : Reversed-phase column (LichroCARTRP C_{18})

Sample loading : The sample was dissolved in a small amount of eluent (MeOH and H_2O) then injected into the loop of the column.

Flow rate : 4.0 or 8.0 ml/min

Detection : UV-photodiode array detector

3.6 Isolation of bioactive compounds from endophytic fungi isolate LRUB 20 and isolate USIA 5.

3.6.1 Isolation of secondary metabolites from endophytic fungus isolate LRUB 20

Crude extract (1,469 mg) of the isolate LRUB 20 designated as L20B was purified by gel filtration chromatography using Sephadex LH-20 (column 3.0 x 60 cm), eluted with MeOH. Ten fractions (40 ml) were obtained and assigned as L20B1, L20B2, L20B3, L20B4, L20B5, L20B6, L20B7, L20B8, L20B9, and L20B10, as shown in Table 5

Table 5 Fractions obtained from Sephadex LH-20 column of crude extract L20B

Fraction code	Weight (mg)
L20B1	5.1
L20B2	73.9
L20B3	227.9
L20B4	294.9
L20B5	356.5
L20B6	135.8
L20B7	198.5
L20B8	28.8
L20B9	10.3
L20B10	16.4

Analysis of ^{1}H NMR spectral data as well as by X-ray crystallography revealed that fraction L20B7 was a pure compound and identified as asterric acid. Isolation of L20B7 is shown in Scheme 5. In addition, fraction L20B5 (356.5 mg) possessed high yield and exhibited interesting ^{1}H NMR pattern. It was then subjected to Sephadex LH-20 (2.5 x 52 cm) column using MeOH as mobile phase. Nine fractions (25 ml) were collected and assigned as L20B51, L20B52, L20B53, L20B54, L20B55, L20B56, L20B57, L20B58 and L20B59, as shown in Table 6.

Table 6 Fractions obtained from Sephadex LH-20 column of fraction L20B5

Fraction code	Weight (mg)
L20B51	17.3
L20B52	21.2
L20B53	85.2
L20B54	69.9
L20B55	54.9
L20B56	38.4
L20B57	17.6
L20B58	13.2
L20B59	12.4

Fractions L20B53 (85.2 mg) and L20B54 (69.9 mg) showed similar patterns of ^{1}H NMR spectral data. Both L20B53 and L20B54 were combined, and further purified by Sephadex LH-20 (1.5 x 43 cm) column using MeOH as mobile phase to obtain eight fractions (20 ml), as shown in Table 7.

Table 7 Fractions obtained from Sephadex LH-20 column of fractions L20B53 and L20B54

Fraction code	Weight (mg)
L20B5(34)1	2.6
L20B5(34)2	1.5
L20B5(34)3	2.4
L20B5(34)4	10.8
L20B5(34)5	82.9
L20B5(34)6	39.1
L20B5(34)7	12.8
L20B5(34)8	6.1

Fraction L20B5(34)5 (82.9 mg) was light brown viscous liquid and identified as 2-hydroxymethyl-3-methyl-cyclopentanone. Isolation of L20B5(34)5 is displayed in Scheme 4. In addition, fraction L20B5(34)5 was selected for further study, as displayed in Scheme 8.

Fraction L20B4 (294.9 mg) exhibited interesting ^{1}H NMR pattern in Table 5. It was then subjected to Sephadex LH-20 (2.5 x 52 cm) column using MeOH as mobile phase. Nine fractions (20 ml) were collected and assigned as L20B41, L20B42, L20B43, L20B44, L20B45, L20B46, L20B47, L20B48, and L20B49, as shown in Table 8.

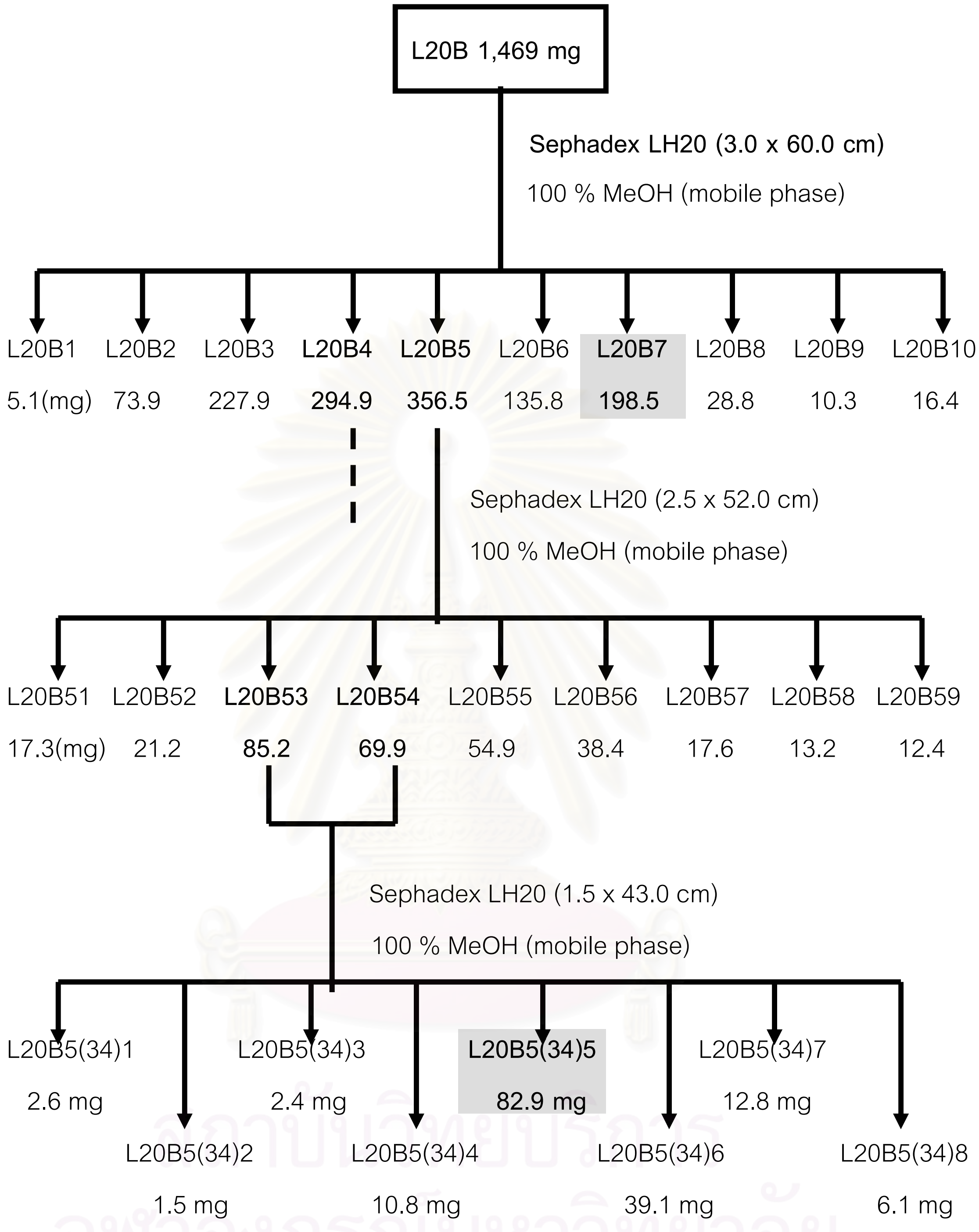
Table 8 Fractions obtained from Sephadex LH-20 column of fraction L20B4

Fraction code	Weight (mg)
L20B41	12.3
L20B42	19.2
L20B43	24.8
L20B44	38.5
L20B45	54.9
L20B46	114.4
L20B47	17.6
L20B48	13.2
L20B49	8.9

L20B46 fraction (114.4 mg) possessed high yield and showed interesting ^{1}H NMR pattern, and it was separated on Sephadex LH-20 (1.5 x 43 cm) using MeOH as mobile phase. Eight fractions were collected and assigned as L20B461, L20B462, L20B463, L20B464, L20B465, L20B466, L20B467, and L20B468, as shown in Table 9. Fraction L20B465 (65.7 mg) possessed high yield and exhibited interesting ^{1}H NMR pattern, which showed the presence of a mixture 2-hydroxymethyl-2-methyl-cyclopentanone and its derivative. However, this mixture could not separated by silica gel, Sephadex LH-20, and HPLC techniques. This fraction was derivatized with 2,4-dinitrophenylhydrazine, and their hydrazone mixture was further separated (Scheme 5).

Table 9 Fractions obtained from Sephadex LH-20 column of fraction L20B46

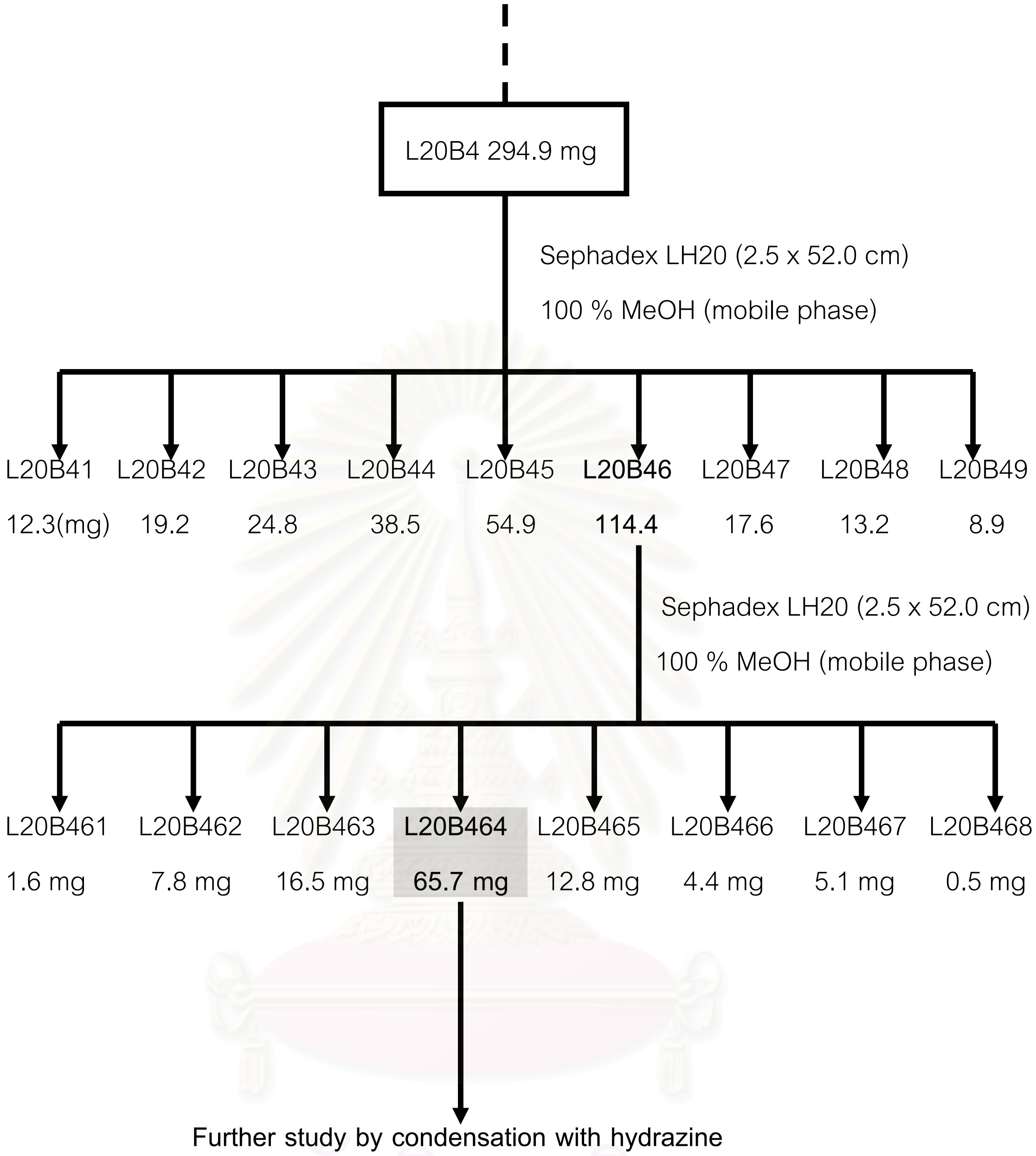
Fraction code	Weight (mg)
L20B461	1.6
L20B462	7.8
L20B463	16.5
L20B464	65.7
L20B465	12.8
L20B466	4.4
L20B467	5.1
L20B468	0.5



Scheme 4 Isolation of compounds L20B7 and L20B5(34)5

L20B7: Further elucidation by spectroscopic method

L20B5(34)5: Further elucidation by spectroscopic method and study by condensation with hydrazine



Scheme 5 Isolation of compounds L20B464

3.6.2 Condensation of compounds L20B5(34)5 and L20B464 with hydrazine

Fraction L20B5(34)5 (30 mg) was treated with 2,4-dinitrophenylhydrazine to give a hydrazone derivative (L20B5(34)5R) 44.5 mg. It was then subjected to Sephadex LH-20 (1.2 x 52 cm) column using MeOH as mobile phase. Five fractions (10 ml) were obtained and assigned as L20B5(34)5R1, L20B5(34)5R2, L20B5(34)5R3, L20B5(34)5R4 and L20B5(34)5R5, as shown in Table 10 and Scheme 6.

Table 10 Fractions obtained from Sephadex LH-20 column of fraction L20B5(34)5R

Fraction code	Weight (mg)
L20B5(34)5R1	5.1
L20B5(34)5R2	16.2
L20B5(34)5R3	16.5
L20B5(34)5R4	3.8
L20B5(34)5R5	1.6

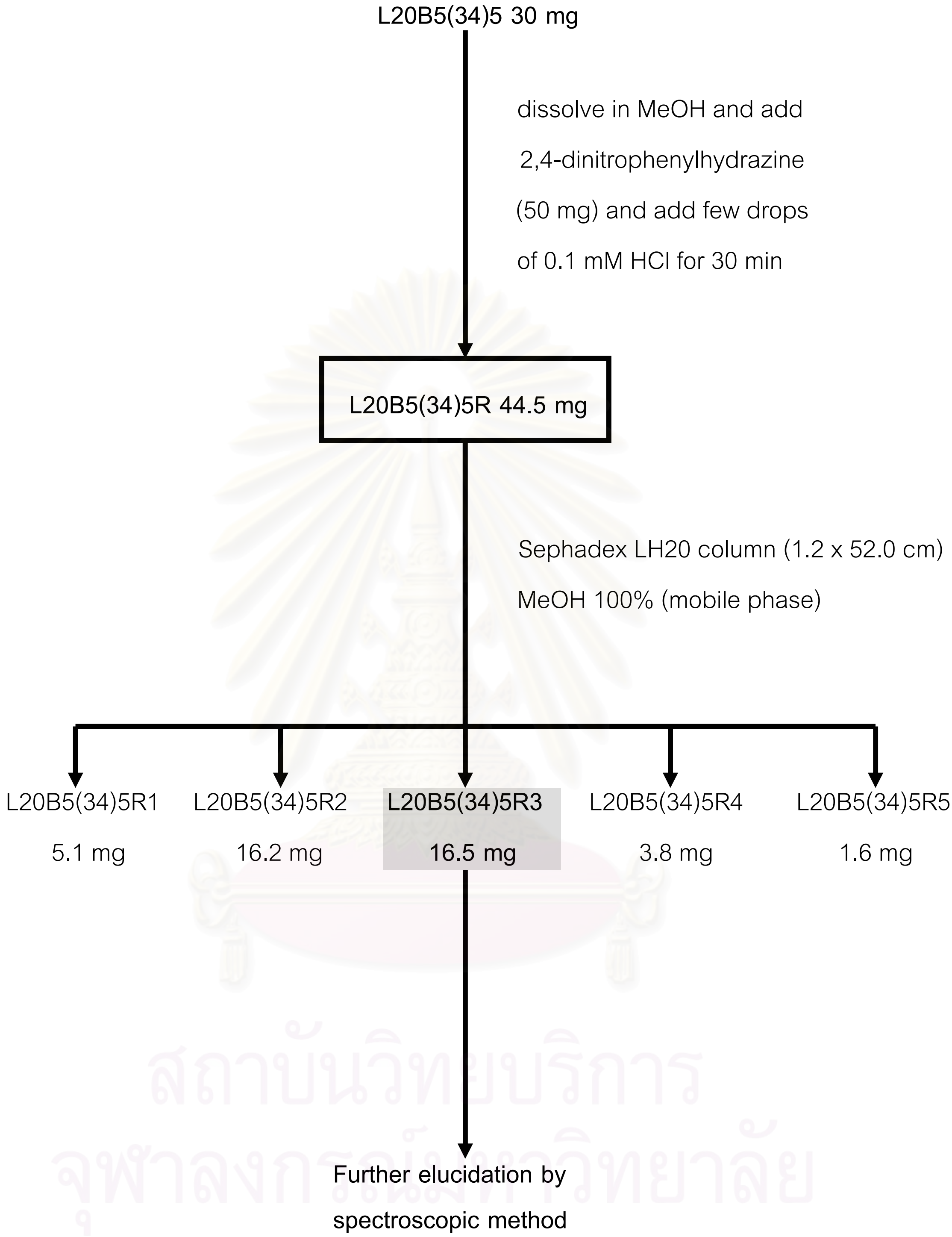
Fraction L20B5(34)5R3 (16.5 mg) was a pure compound and identified as {2-methyl-5-[(4-methyl-2-nitro-phenyl)-hydrazono]-cyclopent-1-enyl}-methanol.

Fraction L20B464 (30 mg) was reacted with 2,4-dinitrophenylhydrazine to give a hydrazone derivative (L20B464R) 43.3 mg. It was then subjected to Sephadex LH-20 (1.2 x 52 cm) column using MeOH as mobile phase. Five fractions (10 ml) were obtained and assigned as L20B464R1, L20B464R2, L20B464R3, L20B464R4, and L20B464R5, as shown in Table 11 and Scheme 7.

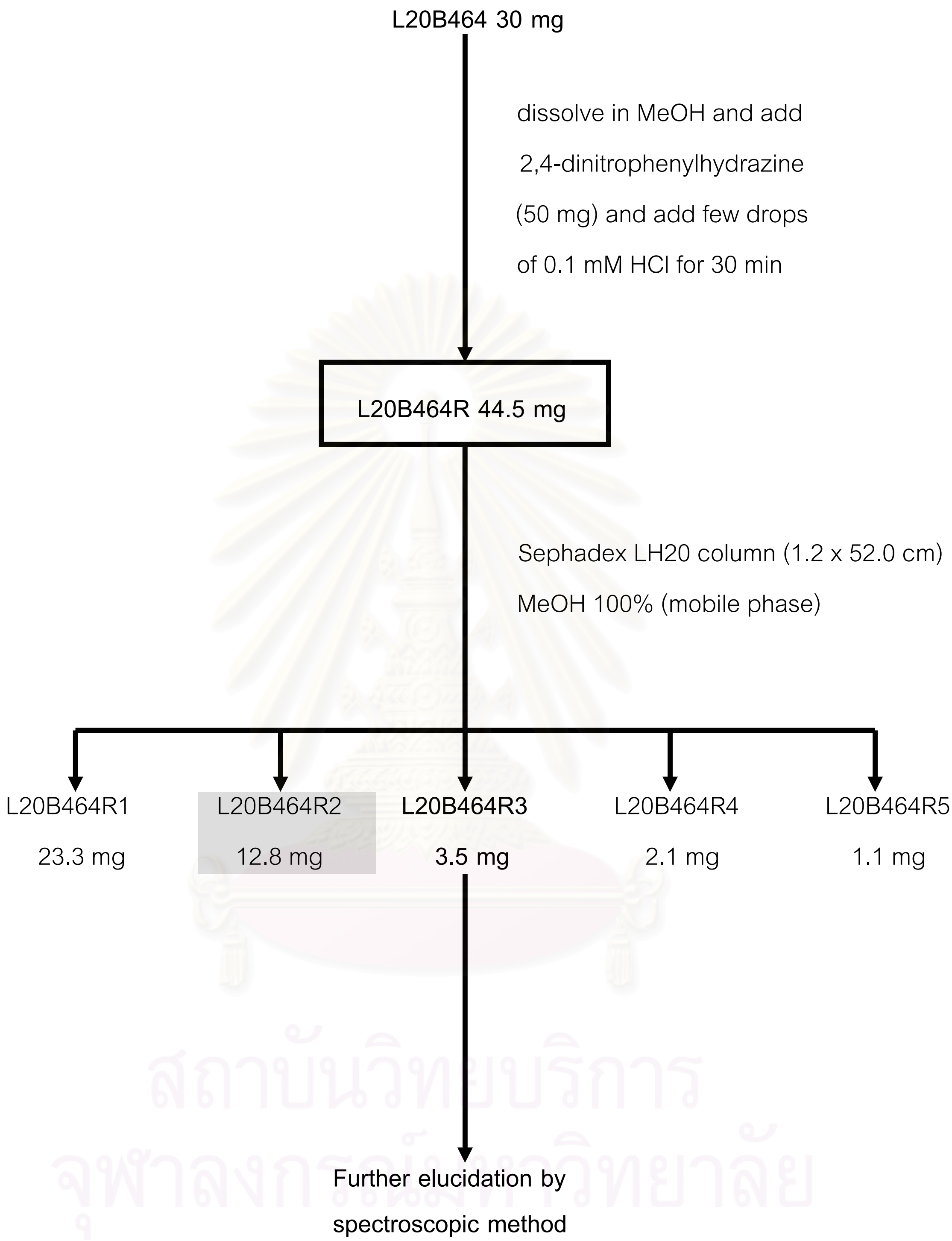
Table 11 Fractions obtained from Sephadex LH-20 column of fraction L20B464R

Fraction code	Weight (mg)
L20B464R1	23.3
L20B464R2	12.8
L20B464R3	3.5
L20B464R4	2.1
L20B464R5	1.1

Fraction L20B464R2 (12.8 mg) was a pure compound, and identified as {2-[(2,4-dinitro-phenyl)-hydra-zono]-5-methyl-cyclopentyl}-methanol.



Scheme 6 Isolation of compound L20B5(34)5R3



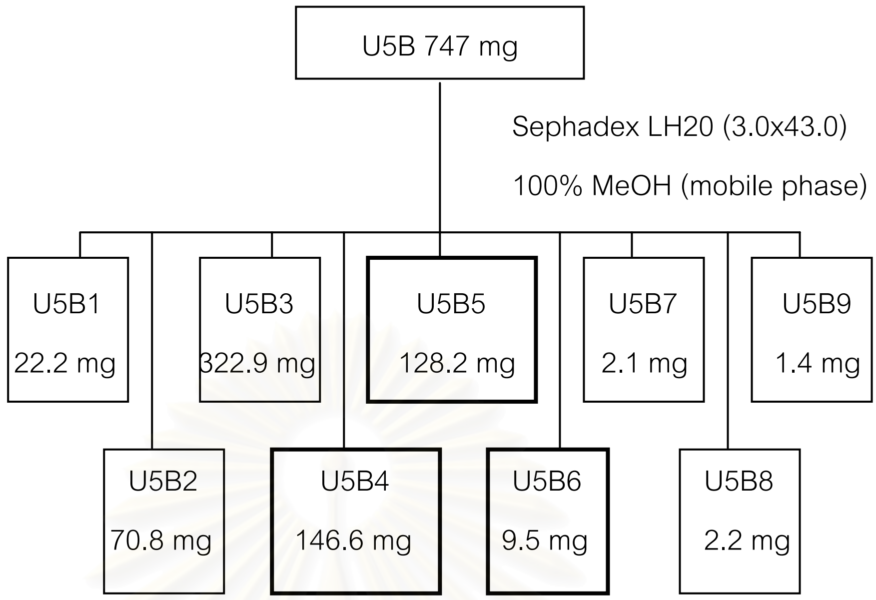
Scheme 7 Isolation of compound L20B464R2

3.6.3 Isolation of bioactive compounds from endophytic fungus isolate USIA 5

Crude extract (U5B) (747 mg) of the isolate USIA 5 *was* purified by gel filtration chromatography using Sephadex LH-20 (column 3.0 x 43 cm), eluted with MeOH. Nine fractions (30 ml) were obtained and assigned as U5B1, U5B2, U5B3, U5B4, U5B5, U5B6, U5B7, U5B8 and U5B9, as shown in Scheme 8 and Table 12. Fractions U5B4 (146.6 mg), U5B5 (128.2 mg) and U5B6 (9.5 mg) were pure compound and identified as 3-nitropropionic acid.

Table 12 Fractions obtained from Sephadex LH-20 column of crude extract U5B

Fraction code	Weight (mg)
U5B1	22.2
U5B2	70.8
U5B3	322.9
U5B4	146.6
U5B5	128.2
U5B6	9.5
U5B7	2.1
U5B8	2.2
U5B9	1.4



Scheme 8 Isolation of compounds U5B4, U5B5 and U5B6

3.7 Spectroscopy

3.7.1 Ultraviolet (UV) spectroscopy

UV (in MeOH) spectra were obtained from a CARY 1 E UV-vis spectrophotometer, at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand.

3.7.2 Infrared (IR) spectroscopy

IR spectra of pure compounds (film technique) were obtained from a Bruker Vector 22 FT-IR spectrophotometer, at the Bioresources Research Unit (BRU), the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand.

3.7.3 Mass spectroscopy (MS)

Electrospray ionization time of flight mass spectra (ESI-TOF-MS) were obtained on a Micromass LTC mass spectrometer, at the Bioresources Research Unit (BRU), the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand.

3.7.4 Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectroscopy

^{1}H (500 MH_Z) and ^{13}C NMR (125 MH_Z), DEPT 135, COSY, HMQC, HMBC and NOESY spectra were obtained from a Bruker ADVANCE DRX-500 FT-NMR spectrometer, at the Bioresources Research Unit (BRU), the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand.

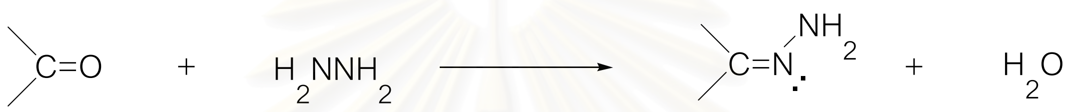
Deuterated solvents; chloroform-*d* ($CDCl_3$), methanol-*d*4 (CD_3OD) and acetone-*d*6 were used in NMR experiments. Reference signals were the signals of residual undeuterated solvents at δ7.24 ppm (^{1}H) and 77.0 ppm *t* (^{13}C) for $CDCl_3$; 3.35 ppm (^{1}H) and 49.0 ppm *spet* (^{13}C) for CD_3OD; and 2.05 ppm (^{1}H) and 29.8 ppm *sept* (^{13}C) and 206.0 ppm *s* (^{13}C) for acetone-*d*6.

3.8 Derivatization of the isolated compounds

3.8.1 Condensations with hydrazine

Compounds L20B5(34)5 and L20B464 possess ketone functionality. Ketones normally condense with other ammonia derivatives, such as substituted hydrazines, to give imine derivatives. The equilibrium constants for these reactions are usually more favorable than those for reaction with simple amines. Ketone reacts with hydrazine derivatives react to fom hydrazones (Solomon and Fryhle, 2004).

Example

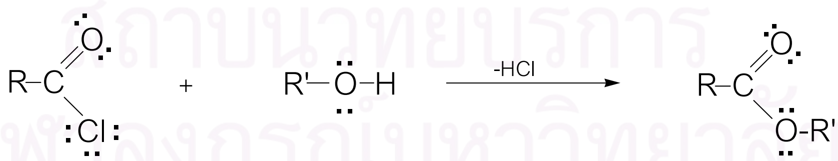


Ketone Hydrazine Hydrazone

3.8.2 Condensations of acids with alcohols: The Fischer esterification

Compound U5B4-6 possesses a secondary alcohol moiety. Carboxylic acids are directly converted to esters by the Fischer esterification, an acid-catalyzed nucleophilic acyl substitution by alcohol. The net reaction is replacement of the acid OH group by the OR group of the alcohol. Acid chlorides of carboxylic acids also condense with alcohols.

Example



Acid chloride Alcohol Ester

3.9 Physical properties of bioactive compounds

3.9.1 Fraction L20B7 of fungus isolate LRUB 20

UV : λ_{max} nm (ε) in methanol; Figure C6 in Appendix C
213 (57052), 248 (14210), 314 (8421)

IR : ν_{max} cm^{-1}; Figure C7 in Appendix C
1053, 1358, 1603, 1689, 3005, 3419

ESI-TOF MS : *m/z*; Figure C5 in Appendix C
m/z 371.0734 (found)
371.0743 (calculated for $C_{17}H_8O_{16}Na^+$)

^1H NMR : δH (ppm), 500 MHz, in acetone-*d*6
see Figure C8 in Appendix C

^{13}C NMR : δC (ppm), 125 MHz, in acetone-*d*6
see Figure C9 in Appendix C

3.9.2 Fraction L20B5(34)5 of fungus isolate LRUB 20

UV : λ_{max} nm (ε) in methanol; Figure C20 in Appendix C
207 (5000)

IR : ν_{max} cm^{-1}; Figure C21 in Appendix C
1066, 1254, 1644, 1689, 2879, 2925, 3423

ESI-TOF MS : *m/z*; Figure C19 in Appendix C
m/z 149.0586 (found)
149.0578 (calculated for $C_7H_{10}O_2Na^+$)

^1H NMR : δH (ppm), 500 MHz, in $CDCl_3$
see Figure C22 in Appendix C

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$
see Figure C23 in Appendix C

3.9.3 Fraction L20B5(34)5R3 of fungus isolate LRUB 20

UV : λ_{max} nm (ε) in methanol; Figure C29 in Appendix C
215 (28125), 255 (28579), 285 (16207), 384 (44886)

ESI-TOF MS : *m/z*; Figure C30 in Appendix C

m/z 307.1050 (found)

307.1042 (calculated for $C_{13}H_{14}O_5N_4Na^+$)

^{1}H NMR : δH (ppm), 500 MHz, in $CDCl_3$

see Figure C31 in Appendix C

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$

see Figure C32 in Appendix C

3.9.4 Fraction L20B464R2 of fungus isolate LRUB 20

UV : λ_{max} nm (ε) in methanol; Figure C40 in Appendix C

227 (50308), 251 (39435), 366 (72974)

IR : ν_{max} cm^{-1}; Figure C41 in Appendix C

919, 1066, 1269, 1335, 1504, 2931, 3443

ESI-TOF MS : *m/z*; Figure C39 in Appendix C

m/z 309.1190 (found)

309.1199 (calculated for $C_{13}H_{16}O_5N_4Na^+$)

^{1}H NMR : δH (ppm), 500 MHz, in $CDCl_3$

see Figure C42 in Appendix C

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$

see Figure C43 in Appendix C

3.9.5 Fraction U5B5 of fungus isolate USIA 5

UV : λ_{max} nm (ε) in methanol; Figure C51 in Appendix, C

205 (9967)

IR : ν_{max} cm^{-1}; Figure C53 in Appendix C

1242, 1555, 1724, 3021

ESI-TOF MS : *m/z*; Figure C50 in Appendix C

m/z 142.0108 (found)

142.0116 (calculated for $C_3H_5O_4NNa^+$)

^{1}H NMR : δH (ppm), 500 MHz, in $CDCl_3$

see Figure C54 in Appendix C

^{13}C NMR : δC (ppm), 125 MHz, in $CDCl_3$

see Figure C55 in Appendix C

3.10 Determination of biological activities

Determination of biological activities (Table 13) were performed by the Bioassay Research Facility (BRF), the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand. Brief methods of each assay were shown below.

Table 13 Biological activities tested in this study.

Biological activities	
Anticancer	BC cell line (IC_{50}, µg/ml)
	KB cell line (IC_{50}, µg/ml)
	NCI-H187:Small cell lung cancer (IC_{50}, µg/ml)
Antiviral	Anti HSV-1 (IC_{50}, µg/ml)
Antifungal	Anti *Candida albicans* (IC_{50}, µg/ml)
Antibacterial	Anti *Mycobacterium tuberculosis* (MIC, µg/ml)
Antimalarial	Anti *Plasmodium falciparum* (IC_{50}, µg/ml)
Cytotoxicity	Vero cell line (IC_{50}, µg/ml)

3.10.1 Cytotoxicity and Anticancer assays

The cytotoxic assay employed the colorimetric method reported by Skehan *et al*. (1990). Activities against KB cell line (human epidermoid carcinoma of cavity, ATTC CCL-17) and BC cell line (breast cancer cell line) were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan *et al*. (1990). Elliptine was used as positive control. DMSO (10%) was used as negative control. Briefly, cells at a logarithmic growth phase were harvested and diluted to 10^5 cells/ml with fresh medium and gently mixed. Testing compound was dissolved in DMSO (concentration at 20 mg/ml), and this solution was then diluted with distilled water to obtain a stock solution at 0.4 mg/ml (with 10% DMSO). The stock solution (10 µl) and cell suspension (190 µl) were transferred into microtiter plates (concentration at 20 µg/ml with 0.05% DMSO). If the compound is active at 20 µg/ml, a series of solutions were prepared by two-fold dilution of the stock solution (diluted with 10% DMSO solution), and exposed to cells as mentioned above, in order to obtain IC_{50} value. Plates were incubated at 37^0C under 5% CO_2 atmosphere for 72 h. After incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at 4^0C for 30 min, washed with water, and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B (SRB) dissolved in 1% acetic for 30 min. After staining period, SRB was removed with 1% acetic acid. Plates were air-dried before bound dye was solubilized with 10mM Tris base for 5 min on shaker. Optical density was read in a microtiter plate reader at wavelength 510 nm. Ellipticine, the reference substance, exhibited activity toward BC and KB cell lines, both with the IC_{50} of 0.3 µg/ml.

3.10.2 Antimalarial assay

The parasite *Plasmodium falciparum* (K1, multidrug resistant strain) was cultured continuously according to the method of Trager and Jensen (1976). Quantitative assessment of antimalarial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method described by Desjardins *et al*. (1979). Briefly, a mixture of 200 µl of 1.5% of erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 µl of the medium containing a test sample dissolved in DMSO (0.1% final concentration) for 24 h employing the incubation conditions described above. Subsequently, 25 µl of [^{3}H]hypoxanthine (Amersham, USA) in culture medium (10 µCi) was added to each well and plates were incubated for an additional 24 h. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the TopCount microplate scintillation counter (Packard, USA). An IC_{50} value of 1.2±0.02ng/ml (n=3) was observed for the standard compound, dihydroartemisinin.

3.10.3 Antifungal assay

The antifungal activity was assessed employing a colorimetric method (Scudiero *et al*., 1988; Plumb *et al*., 1989). *Candida albicans* (ATCC 90028) was grown on a potato dextrose agar (PDA) plate at 30^{0}C for 3 days. Three to five single colonies were then suspended in RPMI640 and cultured in a shaking flask until cell density reaches 2×10^{6} CFU/ml. One hundred µl of the culture was added to each well of 96-well plate containing 100 µl of test sample and incubated at 37^{0}C for 4 h. Fifty µl of 0.5 mg/ml MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; thiazolyl blue) in RPMI 1640 was added to each well and incubated at 37^{0}C for an additional 4 h. After incubation period, the microplates were spinned down at 200xg for 5 min. MTT was then removed from the wells and the formazan crystals were dissolved in 200 µl of 100% DMSO and 25 µl of Sorensen' glycine buffer. Subsequently absorbance at 570 nm was determined using the multilabel counter Victor^{3}V. Amphotericin B and 10% DMSO were used as a positive and a negative control, respectively. In our system, the IC_{50} value of the standard drug, amphotericin B, was 0.04±0.01 µg/ml (n=3).

3.10.4 Anti-Mycobacterium assay

Activity against *Mycobacterium tuberculosis* H37Rv was assessed using the Microplate Alamer Blue Assay (MABA) (Collins and Franzblau, 1997). *M. tuberculosis* H37Rv was growth in 100 ml of 7H9GC containing 0.005% Tween 80. Culture was incubated in 500 ml plastic flask on a rotary shaker at 200 rpm and 37^{0}C until they reached an optical density of 0.4-0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate buffered saline and passed through an 8-µm-pore-size filter to eliminate clumps. The filtrates were aliquot, stored at -80^{0}C. Antimicrobial susceptibility testing was performed in 96-well microplates. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial screened-sample dilutions were prepared in either DMSO or distilled deionized water. The dissolved-screened samples were then diluted by Middlebrook 7H9 media containing 0.2 % v/v glycerol and 1.0 g/l casitone (7H9GC), and subsequent two-fold dilutions were performed in 0.1 ml of 7H9GC in the microplates. Frozen inocula were diluted 1:100 in

7H9GC. Addition of 0.1 ml to the well resulted in final bacterial titers of about $5x10^4$ CFU/ml. Wells containing sample only were used to determine whether the tested samples themselves could reduce the dye or not. Additional control wells consisting of bacteria (B) or medium (M) were included. Plates were incubated at 37^0C. Starting at day 6 of incubation, 20 µl of Alamar Blue solution and 12.5 µl of 20% Tween 80 were added to B and M wells, and plates were re-incubated at 37^0C. Wells were observed at 24 h for a colour change from blue to pink. If the B wells became pink by 24 h, Alamar Blue solution was add to all testing plates. However, if a colour (blue) of M and B wells did not change, both wells were tested daily until a colour of B wells change from blue to pink. After the change of B well colour, Alamar Blue solution was subsequently added to all remaining wells. Plates were then incubated at 37^0C for 24 h, and the results were recorded with a fluorescence multi-well reader (CytoFluor, Series 4000) at the excitation and emission wavelengths of 530 and 590 nm, respectively. The standard drugs, isoniazid and kanamycin sulfate, showed respective MIC values of 0.040-0.090 and 2.0-5.0 µg/ml.

3.10.5 Antiviral assay

The colorimetric method previously described by Skehan and Coworkers (1990) was employed for antiviral assay. Herpes simplex virus type 1 (HSV-1) was maintained in the Vero cell line (kidney fibroblast of an African green monkey), which was cultured in the Eagle's minimum essential medium (MEM) with the addition of heat-inactivated fetal bovine serum (FBS) (10%) and antibiotics. The test samples were put into wells of a microtiter plate at the final concentrations ranging from 20 to 50 µg/ml. The viral HSV-1 (30 PFU) was added into 96-well plate, followed by plating of Vero cells ($1x10^5$ cells/ml); the final volume was 200 µl. After incubation at 37^0C for 72 h, under 5% of CO_2 atmosphere, cells were fixed and stained, and optical density was measured at 510 nm. Under the screening conditions, the reference compound, Acyclovir, typically exhibited the antiviral HSV-1 with the IC_{50} of 2-5 µg/ml.

3.11 Classification of the endophytic fungi isolate LRUB 20 and isolate USIA 5

3.11.1 Conventional method

3.11.1.1 Macroscopic morphology

Both LRUB 20 and USIA 5 isolates were grown on five different media, including corn meal agar (CMA), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA), and yeast extract sucrose agar (YEA). After cultivation for 14 days at room temperature they were photographed. Colony morphology of specimens such as shape, size, color, margin, pigment, and others were examined.

3.11.1.2 Microscopic morphology

Both LRUB 20 and USIA 5 isolates were grown on water agar and small pieces of sterilized banana leaves at room temperature for 2 months. Fungal spores and fruiting bodies appearing on the banana leaf fragments were examined by light microscopy.

3.11.2 Molecular method

3.11.2.1 DNA extraction

Both LRUB 20 and USIA 5 isolates were grown on potato dextrose broth at 25^{0}C for 7 days. The mycelium were harvested by centrifugation and washed 3 times with sterile distilled water. The pellet were lyophilized and then ground into fine powder using a mortar and pestle. The ground powder would be further subjected to DNA extraction.

The ground mycelium was filled up to one third of a 1.5 ml microfuge tube and subjected to DNA extraction according to Lee and Taylor (1990). A 400-µl volume of lysis buffer (Appendix B) was added and the mixture was mixed with vortex until being homogeneous. The tube was then incubated at 65 ^{0}C for 1 h. A 400-µl volume of chloroform: phenol (Appendix B) was added to the mixture and the tube was inverted several times. The mixture was centrifuged at 10,000 rpm (Sigma 202MC) for

15 min at room temperature. The aqueous (top) phase containing the DNA was transferred to a new tube. Then, 10 µl of 3M sodium acetate was added to the aqueous phase followed by 0.54 volume of cold isopropanol. The tube was inverted gently and DNA precipitate was spun down at room temperature as previously for 2 min. The pellet was washed once with cold 70% ethanol before leaving dry. The DNA pellet was resuspended in100 µl TE (10mM Tris HCl pH 8.0, 0.1 mM EDTA) buffer.

3.11.2.2 Polymerase chain reaction (PCR) amplification

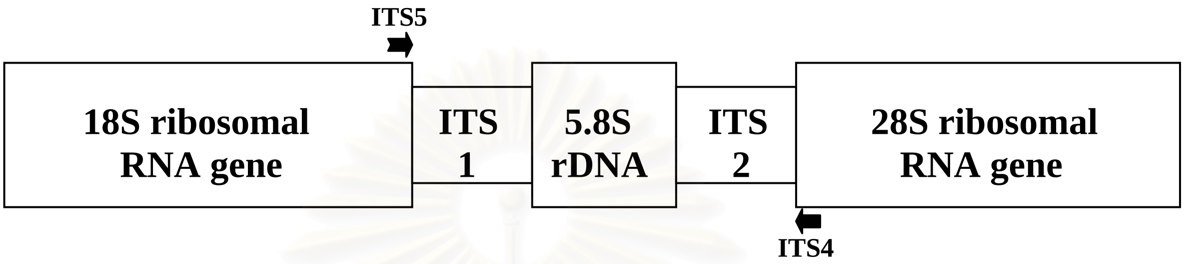
ITS1-5.8-ITS2 regions of ribosomal DNA (rDNA) (Figure 6) were amplified by PCR using the forward primer ITS5 and the reverse primer ITS4 according to White *et al*, (1990). The primer sequences are shown in Table 14. Oligonucleotide primers were synthesized using ABI PRISM™, DNA/RNA synthesizer model 392, Perkin Elmer, by the Bioservice Unit (BSU) at the National Center for Genetic Engineering and Biotechnology (BIOTEC). The reaction mixture was prepared on ice. The amplification reaction was performed in the total volume of 50 µl: 2 ng/µl of template DNA , 0.5 mM of each primer, 0.2 mM of individual dNTP, 3 mM of $MgCl_2$, 50 mM KCl, 10 mM of Tris-HCl at pH 8.8 and 1.0 U of *Taq* DNA polymerase (Appendix B). For each test, a primer negative control was included without template DNA. Ice-cold PCR reaction tubes were transferred to an Eppendrof Mastercycler Gradient PCR machine.

The thermal cycling program was as follow: 3 min initial denaturation at 95 ^{0}C, followed by 30 cycles of 50s denaturation at 95 ^{0}C, 40s primer annealing at 48 ^{0}C, 40s extension at 72 ^{0}C, and a final 10 min extension at 72 ^{0}C.

Four microlitres of PCR products from each PCR reaction were examined by electrophoresis at 100V (4 V cm^{-1}) for 2 h in a 2% (w/v) agarose gel in Tris-acetate-EDTA (TEA) buffer (Appendix B) and visualized with UV light after staining with ethidium bromide (0.5 µg/ml).

3.11.2.3 DNA sequencing

PCR products were purified using minicolumns (Wizard® PCR Preps DNA Purification System, Promega) according to the manufacture's protocol (Guo *et al*., 2003). Primers ITS5 and ITS4 were used in the sequencing reactions. Both DNA strands were sequenced. Purified PCR products were sequenced using dye terminator cycle sequencing and reactions were resolved on the ABI Prism 3100 Genetic Analyzer (AME Bioscience). This was done at the Bioservice Unit (BSU), the National Center for Genetic Engineering and Biotechnology (BIOTEC).



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

Figure 6 Location on nuclear rDNAs of primers ITS5 and ITS4. The arrow heads represent the 3' end of each primer.

Table 14 Primers for amplification of ribosomal RNA genes of fungi isolate LRUB 20 and isolate USIA 5

rRNA	GenePrimer[a]	Product Size (bp)[b]	*Tm* (°C)
Nuclear, ITS			
ITS5	GGAAGTAAAAGTCGTAACAAGG	620	65
ITS4	TCCTCCGCTTATTGATATGC		58

[a] Primer ITS5 is forward primer; ITS 4 is reward primer.

[b] Product sizes are approximated based on the rRNA genes of *Saccharomyces cerevisiae*; the side of the region amplified is the product size minus the primers.

[c] Tm's were calculated by the method of Meinkoth and Wahl (1988).

3.11.3 Phylogenetic Analysis

ITS1-5.8S-ITS2 DNA sequence was used as query sequence to search for similar sequence from GenBank using BLASTN 2.2.10 (Altschul *et al*., 1997). The similar reference sequences with query sequences were obtained and used for subsequent phylogenetic analyses. DNA sequence alignment and identity were performed and determined, respectively, using ClustalW (1.82) multiple sequence alignment program (Thompson *et al*. 1994). The alignment results were adjusted manually where necessary to maximize alignment using BioEdit. The alignment data were subsequently used for maximum-parsimony analysis in which searches for most parsimonious trees were conducted with the heuristic search algorithms with tree-bisection-reconnection (TBR) branch swapping in PAUP® (v 4.0b10) (Swofford, 2003). For each search, 10 replicates of random stepwise sequence addition were performed and 100 trees were saved per replicate. Gaps were treated as missing data. Character states were treated as unordered. Statistical support for the internal branches was estimated by bootstrap analysis with 1000 replications.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Structure elucidation of the isolated compounds from endophytic fungi isolate LRUB 20 and isolate USIA 5

The ethyl acetate extract (L20B 1,469 mg) of MCz fermentation broth (5L) of the endophytic fungus isolate LRUB 20 gave three secondary metabolites, which were identified as asterric acid (L20B7, 198.5 mg, 13.51% of EtOAc extract), 2-hydroxymethyl-3-methyl-cyclopent-2-enone (L20B5(34)5, 82.9 mg, 5.64% of EtOAc extract), and 2-hydroxymethyl-3-methyl-cyclopentanone. While a secondary metabolite, 3-nitropropionic acid (U5B4-6, 284.3 mg, 38% of ethyl acetate extract), was obtained from EtOAc extract (U5B 747 mg) of MID fermentation broth (1.6L) of the endophytic fungus isolate USIA 5.

4.1.1 Structure elucidation of asterric acid (L20B7)

The compound L20B7 was obtained as white solid. The ESI-TOF MS of the compound L20B7 (Figure C5 in Appendix C) displayed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 371.0734 (calculated for $C_{17}H_{16}O_8Na^+$ at *m/z* 371.0743). The UV spectrum in MeOH (Figure C6 in Appendix C) of the compound L20B7 showed λ_{max} (ε) at 213 (57052), 248 (14210), and 314 (8421) nm. The IR absorption spectrum (Figure C7 in Appendix C) exhibited characteristic bands at 1053 cm^{-1} (C-O stretching), 1358 cm^{-1} (C-C stretching), 1603 cm^{-1} (C=C stretching), 1689 cm^{-1} (C=O stretching), 3005 cm^{-1} (C-H stretching), and 3419 (O-H stretching).

The 500 MHz ^{1}H-NMR spectrum of the compound L20B7 in acetone-d_6 (Figure C8-C11 in Appendix C) (δ, ppm) showed signal attributable to: 2.15 (3H, *s*, $ArCH_3$), 3.74 (3H, *s*, OMe), 3.81 (3H, *s*, OMe), 5.91 (1H, *s*, ArH), 6.47 (1H, *s*, ArH), 6.91 (1H, *d*, ArH), and 7.06 (1H, *d*, ArH).

The 125 MHz ^{13}C-NMR spectrum of compound L20B7 in acetone-d_6 (Figure C12 in Appendix C) gave seventeen carbon signals. The carbon signals were classified by DEPT 135 spectrum (Figure C13 in Appendix C) and HMQC spectrum (Figure C14 in Appendix C) as three methyl carbon signals at δ 21.11 ppm (C-16), 51.85 (C-9), and 55.77 ppm (C-7); four methine carbon signals at δ 104.50 (C-13), 105.18 (C-5), 108.35 (C-3), and 111.67 ppm (C-15); and ten quaternary carbonyl carbon signals at δ 164.81 (C-8), 170.78 ppm (C-17), 99.86 (C-11), 124.86 (C-2), 133.84 (C-1), 146.84 (C-14), 153.93 (C-6), 156.03 (C-12), 158.62 (C-4), and 163.33 (C-10).

The ^{1}H-^{1}H COSY spectra of the compound L20B7 in acetone-d_6 (Figure C20 in appendix C) established the correlation from H-16 to H-13 and H-15, and H-3 to H-5, as shown in Figure 7.

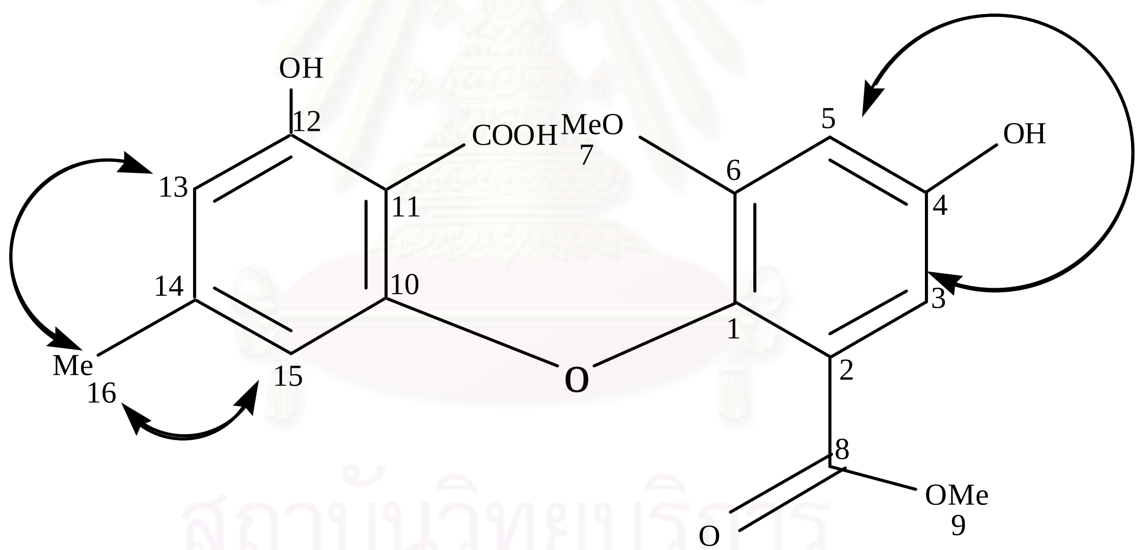


Figure 7 The correlations ^{1}H-^{1}H COSY spectrum (arrow) of compound L20B7

The complete ^{13}C assignments of the compound L20B7 were obtained from the HMBC spectra ($^{n}J_{HC}$ = 8 Hz) (Figure 15-19 in Appendix C) showing the following long-range correlations; H-3 (δ 7.06) to C-5 (δ 105.18), C-1 (δ 133.84), C-4 (δ 158.62), and C-8 (δ

164.81); H-5 (δ 6.91) to C-3 (δ 108.35), C-1 (δ 133.84), and C-4 (δ 158.62); H-7 (δ 3.81) to C-6 (δ 153.93); H-9 (δ 3.71) to C-8 (δ 164.81); H-13 (δ 5.91) to C-11 (δ 99.86), C-15 (δ 111.67), and C-16 (δ 21.11); H-15 (δ 6.47) to C-10 (δ 163.33), C-11 (δ 99.86), C-13 (δ 104.5), and C-16 (δ 21.11); and H-16 (δ 2.10) to C-13 (δ 104.5), and C-15 (δ 111.67), and C-14 (δ 146.84).

The ^{1}H-^{13}C long-range correlations of compound L20B7 in acetone-d_6 are summarized in Figure 8 and Table 15.

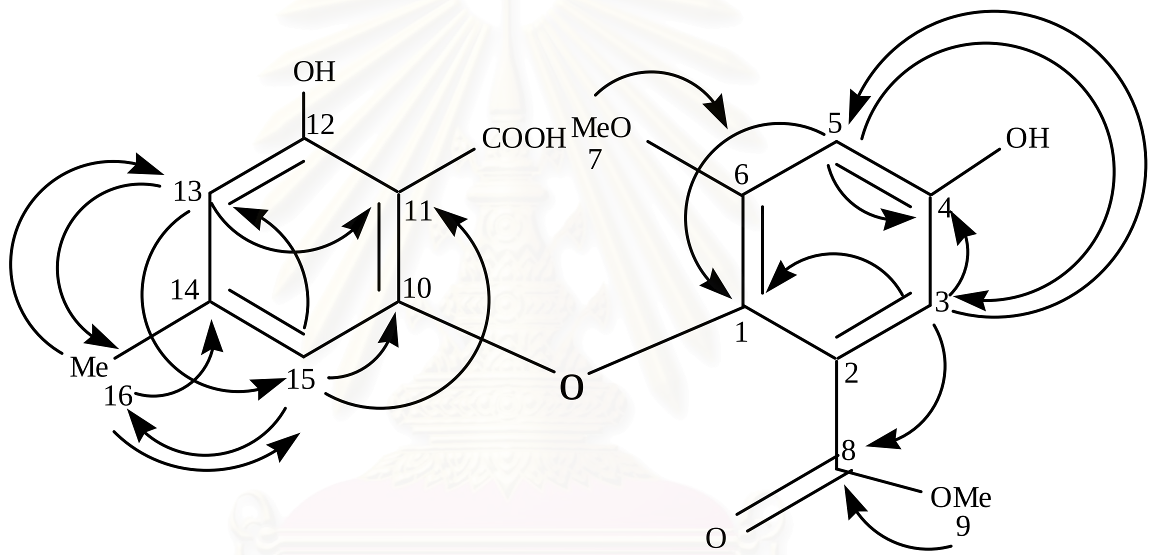


Figure 8 Long-range correlations from HMBC ($^{n}J_{HC}$ = 8 Hz) spectral data of the compound L20B7 in acetone-d_6.

Chemical structure of compound L20B7 could not be assembled by analysis of NMR data, therefore a single crystal of L20B7 was prepared and subjected to X-ray crystallographic analysis. Additional structural information needed to complete NMR shift assignments (i.e. heteroatom, positions, and connections), and the structure of L20B7 was finally solved by X-ray crystallographic analysis, its ORTREP plot is as shown in Figure 9. The X-ray analysis revealed an ether bond between aromatic rings, and compound L20B7 was identified as asterric acid, which was previously reported as fungal metabolite (from *Scytalidium* sp. and *Aspergillus* sp.).

Table 15 The ^{1}H, ^{13}C-NMR and HMBC spectral data of compound L20B7 in acetone-d_6

Position of carbon	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlations in HMBC $^{n}J_{HC}$ = 8 Hz
1	-	133.84	-
2	-	124.86	-
3	7.06, *d*, (2.8)	108.35	C-1, C-4, C-5, C-8
4	-	158.60	-
5	6.91, *d*, (2.8)	105.18	C-1, C-3, C-4
6	-	153.93	-
7	3.81, *s*	55.77	C-6
8	-	164.81	-
9	3.74, *s*	51.85	C-8
10	-	163.33	-
11	-	99.86	-
12	-	156.03	-
13	5.91, *s*	104.50	C-11, C-15, C-16
14	-	146.84	-
15	6.47, *s*	111.67	C-10, C-11, C-13, C-16
16	2.10, *s*	21.11	C-13, C-14, C-15
17	-	170.78	-

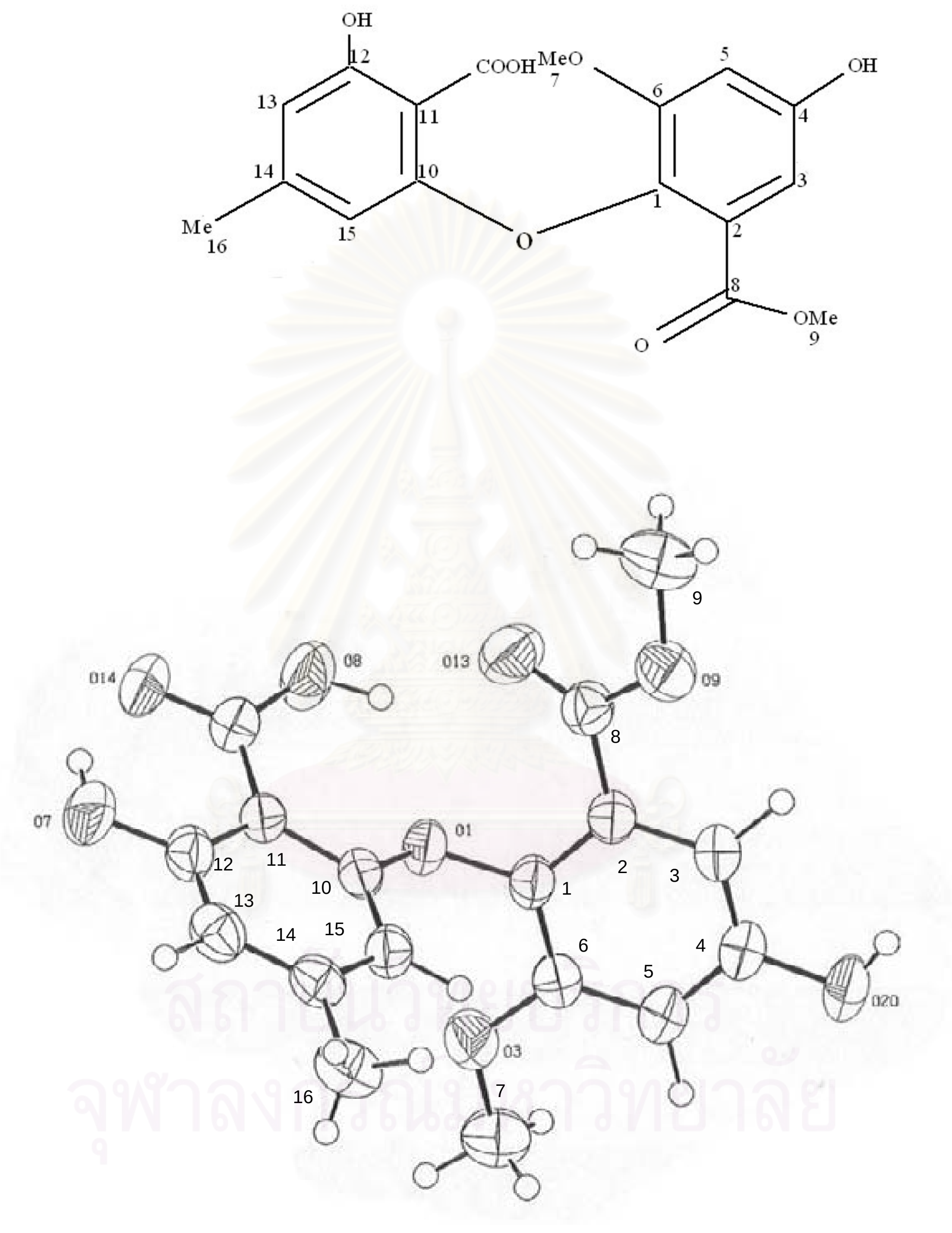


Figure 9 ORTREP plot of asterric acid

Stermitz *et al.* (1973) have reported that the fungus *Scytalidium* sp. grown on Bacto malt extract medium could produce asterric acid. In 2002, Jaih *et al*. isolated and characterized asteric acid, a secondary metabolite from the fermentation of *Aspergillus* sp. Comparison with the compound C20B7 are shown in Table 16.

Table 16 The ^{1}H-NMR spectral data of L20B7 and asterric acid in acetone-d_6

Position of carbon	δH (ppm), *mult* of compound (L20B7)	δH (ppm), *mult* of asterric acid (Stermitz *et al*. 1973)	δH (ppm), *mult* of asterric acid (Jaih *et al*. 2001)
1	-	-	-
2	-	-	-
3	7.06, *d*	7.10, *d*	7.04, *d*
4	-	-	-
5	6.91, *d*	6.95, *d*	6.92, *d*
6	-	-	-
7	3.81, *s*	3.85, *s*	3.81, *s*
8	-	-	-
9	3.74, *s*	3.78, *s*	3.73, *s*
10	-	-	-
11	-	-	-
12	-	-	-
13	5.91, *s*	5.95, *s*	5.91, *s*
14	-	-	-
15	6.47, *s*	6.51, *s*	6.47, *s*
16	2.10, *s*	2.19, *s*	2.16, *s*
17	-	-	-

4.1.2 Structure elucidation of 2-hydroxymethyl-3-methyl-cyclopent-2-enone [L20B5(34)5]

Compound L20B5(34)5 was obtained as light brown viscous liquid, and its ESI-TOF MS of the compound L20B5(34)5 (Figure C21 in Appendix C) displayed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 149.0586 (calculated for $C_7H_{10}O_2H^+$ at *m/z* 149.0578). The UV spectrum in MeOH (Figure C22 in Appendix C) of the compound L20B5(34)5 showed λ_{max} (ε) at 207 (5000). The IR absorption spectrum (Figure C23 in Appendix C) exhibited characteristic bands at 1066 cm^{-1} (C-O stretching), 1254 cm^{-1} (C-C stretching), 1644 cm^{-1} (C=C stretching), 1689 cm^{-1} (C=O stretching), 2879, and 2925 cm^{-1} (C-H stretching), and 3423 (O-H stretching).

The 500 MHz ^1H-NMR spectrum of compound L20B5(34)5 in $CDCl_3$ (Figure C24 and C25 in Appendix C) showed: one methyl proton signal at δ 2.12 ppm and three methylene proton signals at δ 2.39, 2.55, and 4.31 ppm.

The 125 MHz ^{13}C-NMR spectrum of compound L20B5(34)5 in $CDCl_3$ (Figure C26 in Appendix C) gave seven carbon signals, which carbon signals were classified by DEPT 135 (Figure C27 in Appendix C) and HMQC spectral data (Figure C28 in Appendix C) as one methyl carbon signal at δ 19.17 ppm (C-7); three methylene carbon signals at δ 32.05 (C-4), 34.42 (C-5), and 54.92 ppm (C-6); three quaternary carbon signals at δ 138.62 (C-2), and 173.68 (C-3), and 210.60 ppm (C-1).

The ^1H-^1H COSY spectra of compound L20B5(34)5 in $CDCl_3$ (Figure C30 in Appendix C) established the correlation between H-4 and H-5, as shown in Figure 10.

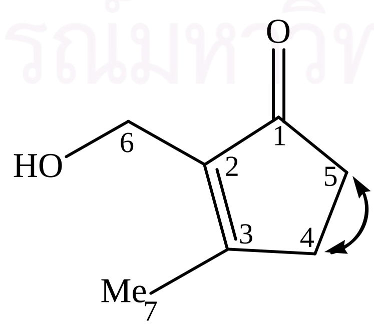


Figure 10 The correlation of ^1H-^1H COSY spectrum (arrow) of compound L20B5(34)5

The complete ^{13}C assignments of compound L20B5(34)5 were established from the HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (Figure 29 in Appendix C) showing the following long-range correlations; H-4 (δ 2.55) to C-5 (δ 34.42), C-2 (δ 138.62), and C-3 (δ 173.68); H-5 (δ 2.39) to C-4 (δ 32.05), C-1 (δ 210.60), and C-3 (δ 173.68); H-6 (δ 4.31) to C-1 (δ 210.60), C-2 (δ 138.62), and C-3 (δ 173.68); and H-7 (δ 2.12) to C-4 (δ 32.05), C-2 (δ 138.62), and C-3 (δ 173.68).

The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of compound L20B5(34)5 in $CDCl_3$ are shown in Figure 11 and summarized in Table 17.

Based upon these spectral data, L20B5(34)5 was identified as 2-hydroxymethyl-3-methyl-cyclopent-2-enone that is previously found to be chemically synthesized from 2-bromo-3-methyl-2-cyclopenten-1-one ethylene ketal (Cho *et al*., 2004). This is the first report of 2-hydroxymethyl-3-methyl-cyclopent-2-enone as a fungal metabolite.

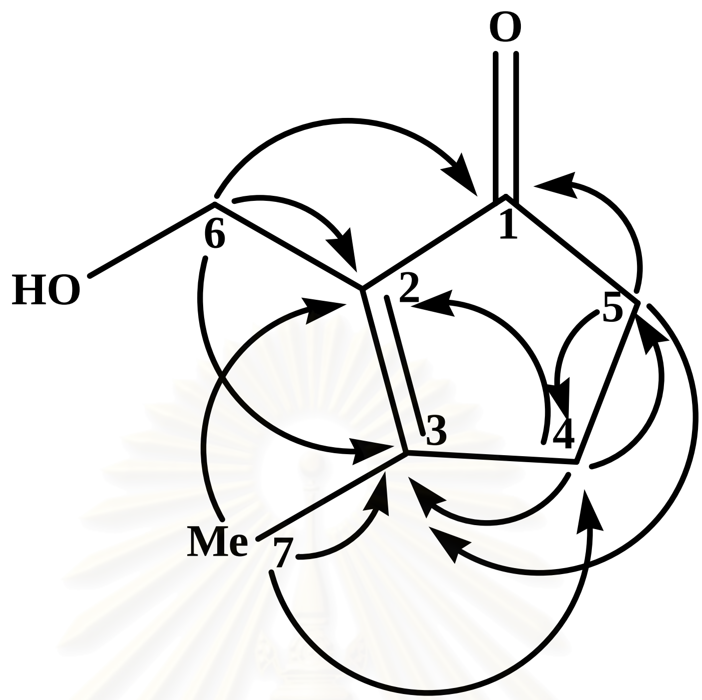


Figure 11 Long-range correlations from HMBC ($^{n}J_{HC}$ = 8 Hz) spectral data of compound L20B5(34)5

Table 17 The ^{1}H, ^{13}C-NMR and HMBC spectral data ($CDCl_3$) of compound L20B5(34)5

Position of carbon	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlation in HMBC $^{n}J_{HC}$ = 8 Hz
1	-	210.60	-
2	-	138.62	-
3	-	173.68	-
4	2.55, *m*, (4.6)	32.05	C-2, C-3, C-5
5	2.39, *m*, (4.6)	34.42	C-1, C-3, C-4
6	4.31, *s*	54.92	C-1, C-2, C-3
7	2.12, *s*	19.17	C-2, C-3, C-4

4.1.3 Structure elucidation of {2-methyl-5-[(4-methyl-2-nitro-phenyl)-hydrazono]-cyclopent-1-enyl}-methanol [L20B5(34)5R3]

The compound L20B5(34)5R3, red powder solid, was obtained after treating L20B5(34)5)R3 with 2,4-dinitrophenylhydrazine to give its corresponding hydrazone derivative. . The ESI-TOF MS of compound L20B5(34)5R3 (Figure C31 in Appendix C) displayed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 307.1050 (calculated for $C_{13}H_{14}O_5N_4Na^+$ at *m/z* 307.1042). The UV spectrum in MeOH (Figure C32 in Appendix C) of compound L20B5(34)5R3 showed λ_{max} (ε) at 215 (28125), 255 (28579), 285 (16207), and 385 (44886) nm

The 500 MHz ^{1}H-NMR spectrum of compound L20B5(34)5R3 in $CDCl_3$ (Figure C33-36 in Appendix C) exhibited: one methyl proton signal at δ 2. ppm; three methylene proton signals at δ 2.74, 2.74, and 4.51 ppm; and three aromatic proton signals at δ 7.85, 8.33, 9.15, and an exchangeable proton at δ 10.93 ppm.

The 125 MHz ^{13}C-NMR spectrum of compound L20B5(34)5R3 in $CDCl_3$.(Figure C37 in Appendix C) showed thirteen carbon signals, which were classified by DEPT 135 spectrum (Figure C38 in Appendix C) and HMQC spectrum (Figure C39 in Appendix C) as one methyl carbon signal at δ 18.48 ppm (C-7); three methylene carbon signals at δ 25.55 (C-4), 34.86 (C-5), and 56.08 ppm (C-6); six quaternary carbon signals at δ 135.28 (C-2), 160.43 (C-3), 129.06 (C-9), 137.64 (C-11), 144.78, and 169.82; and three methine carbon signals at δ 115.91 (C-13), 123.52 (C-10), and 130.09 (C-12).

The ^{1}H-^{1}H COSY spectrum of compound L20B5(34)R3 in $CDCl_3$. (Figure C41 and C42 in Appendix C) showed correlation between H-4 and H-5; and H-12 and H-13, as shown in Figure 12.

Analysis of HMBC spectrum (Figure C40 in Appendix C) assisted in assignments of compound L20B5(34)5R3 from which the following correlations were observed: H-4 (δ 2.74) to C-5 (δ 34.86), C-2 (δ 135.28), and C-3 (δ 160.43); H-5 (δ 2.74) to C-4 (δ 25.55), C-1 (δ 169.82), and C-3 (δ 160.43); H-6 (δ 4.51) to C-1 (δ 169.82), C-2 (δ 135.28), and C-3 (δ 160.43); and H-7 (δ 2.08) to C-4 (δ 25.55), C-2 (δ 135.28), and C-3 (δ 160.43); NH (δ 10.93) to C-13 (δ 115.91), C-1 (δ 169.82), and C-8 (δ 144.78); H-10 (δ 9.15) to C-12 (δ 130.09), C-8 (δ 144.78), and C-11 (δ 137.64); H-12 (δ 8.33) to C-10 (δ 123.52), C-8 (δ 144.78), and C-11 (δ 137.64); and H-13 (δ 7.85) to C-12 (δ 130.09), and C-11 (δ 137.64).

The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of compound L20B5(34)5R3 in $CDCl_3$ are summarized in Figure 13 and Table 18.

Table 18 The ^{1}H, ^{13}C-NMR and HMBC spectral data ($CDCl_3$) of compound L20B5(34)5R3

Position of carbon	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	HMBC correlations
1	-	169.82	-
2	-	135.28	-
3	-	160.43	-
4	2.74, *m*, (4.2)	25.55	C-2, C-3, C-5
5	2.74, *m*, (4.2)	34.86	C-1, C-3, C-4
6	4.51, *s*	56.08	C-1, C-2, C-3
7	2.08, *s*	18.48	C-2, C-3, C-4
8	-	144.78	-
9	-	129.06	-
10	9.15, *d*, (2.6)	123.52	C-8, C-11, C-12
11	-	137.64	-
12	8.33, *dd*	130.09	C-8, C-10, C-11
13	7.85, *d*, (9.6)	115.91	C-11, C-12
NH	10.93, *s*	-	C-1, C-13

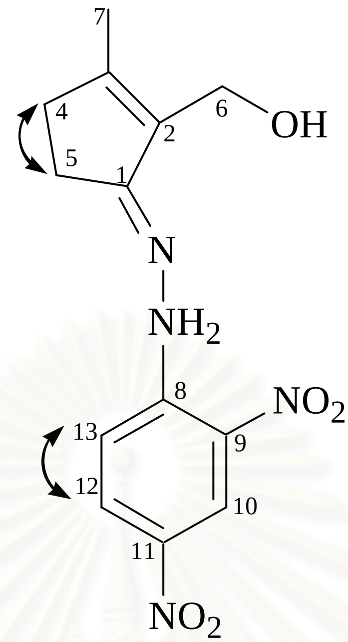


Figure 12 The correlation from ^{1}H-^{1}H COSY spectrum (arrow) of compound L20B5(34)5R3

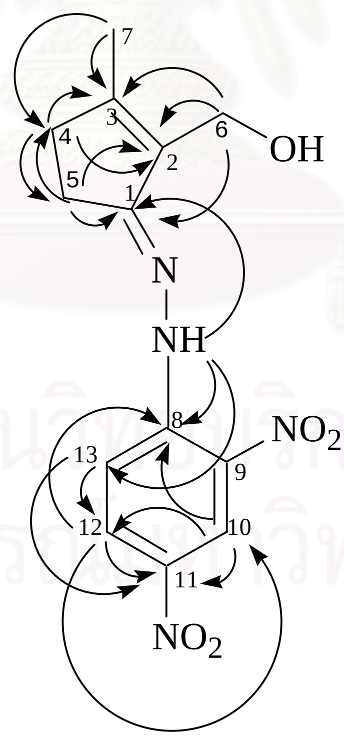


Figure 13 Long-range correlations from HMBC ($^{n}J_{HC}$ = 8 Hz) spectral data of compound L20B5(34)5R3

On the basis of these spectral data, compound L20B5(34)5R3 was identified as {2-methyl-5-[(4-methyl-2-nitro-phenyl)-hydrazono]-cyclopent-1-enyl}-methanol. Hydrazone L20B 5(34)5R3 was prepared because we need to transform L20B5(34)5R3, which is liquid, to be solid. This hydrazone derivative is expected to be crystallized to obtain single crystals for X-ray crystallographic analysis. However, a good single crystal could not be obtained for X-ray crystallographic analysis.

4.1.4 Structure elucidation of {2-[(2,4-dinitro-phenyl)-hydrazono]-5-methyl-cyclopentyl}-methanol (L20B464R2)

L20B464R2 (red solid) was a hydrazone derivative, which was obtained from reaction of fraction L20B464 with 2,4-dinitrophenylhydrazine. The ESI-TOF MS of compound L20B464R2 (Figure C43 in Appendix C) displayed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 309.1190 (calculated for $C_{13}H_{16}O_5N_4Na^+$ at *m/z* 309.1199). The UV spectrum in MeOH (Figure C44 in Appendix C) of compound L20B464R2 showed λ_{max} (ε) at 227 (50308), 251 (39435), and 366 (72974) nm. The IR spectrum (Figure C45 in Appendix C) exhibited characteristic bands at 919 cm^{-1} (C-N stretching), 1066 cm^{-1} (C-O stretching), 1269 cm^{-1} (C-C stretching), 1335 cm^{-1} (C=N stretching), 1504 cm^{-1} (C=C stretching), 2931 cm^{-1} (C-H stretching), and 3443 (O-H stretching). The optical rotation of compound L20B464R2 displayed the value of -79.6460 in MeOH at wavelength 589 nm.

The 500 MHz ^1H-NMR spectrum ($CDCl_3$) of compound L20B464R2 (Figure C46-49 in Appendix C) demonstrated methyl proton signal at δ 1.20. ppm; three methylene proton signals at δ 1.55 and 2.23, 2.46, and 2.71 ppm; three aromatic proton signals at δ 7.81, 8.33, and 9.15; two methine proton signals at δ 1.96, and 2.46 ppm, and exchangeable proton at δ 10.90 ppm.

The 125 MHz ^{13}C-NMR spectrum of compound L20B464R2 in $CDCl_3$ (Figure C50 in Appendix C) gave thirteen carbon signals. The carbon signals were classified by DEPT 135 spectrum (Figure C51 in Appendix C) and HMQC spectrum (Figure C52 in Appendix C) as one methyl carbon signal at δ 18.46 ppm (C-7); three methylene carbon signals at δ 31.56

(C-4), 28.12 (C-5), and 62.23 ppm (C-6); four quaternary carbon signals at δ 129.40 (C-9), 138.04 (C-11), 144.84 (C-8), and 169.80 (C-1); and five methine carbon signals at δ 54.16 (C-2), 35.66 (C-3),115.98 (C-13), 123.62 (C-10), and 130.10 (C-12).

The ^{1}H-^{1}H COSY spectrum of compound L20B464R2 in $CDCl_3$ (Figure C55 and C56 in Appendix C) established the connectivity from H-2 to H-5, and also showed the correlations between H-2 and H-6, H-3 and H-7, and H-12 and H13, as shown in Figure 14.

HMBC correlations (Figure C53 and C54 in Appendix C) well assembled the structure of compound L20B464R2 showing the following long-range correlations; H-2 (δ 2.46) to C-6 (δ 62.23), and C-1 (δ 169.80); H-3 (δ 1.96) to C-7 (δ 18.46); H-4 δ (1.55 and 2.23) to C-3 (δ 35.66), C-7 (δ 18.46), and C-1 (δ 169.80); H-5 δ (2.46 and 2.71), to C-4 (δ 31.56), C-1 (δ 169.80), C-2 (δ 54.16), C-3 (δ 35.66), and C-4 (δ 31.56); H-6 (δ 4.00) to C-1 (δ 169.80), C-2 (δ 54.16), and C-3 (δ 35.66); H-7 (δ 1.20) to C-2 (δ 54.16), C-3 (δ 35.66), and C-4 (δ 31.56); NH (δ 10.90) to C-13 (δ 115.98), C-1 (δ 169.80), and C-8 (δ 144.84); H-10 (δ 9.15) to C-12 (δ 130.10), C-8 (δ 144.84), and C-11 (δ 138.04); H-12 (δ 8.33) to C-10 (δ 123.62), C-8 (δ 144.84), and C-11 (δ 138.04); and H-13 (δ 7.81) to C-12 (δ 130.10) and C-11 (δ 138.04).

The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of compound L20B464R2 are summarized in Figure 15 and Table 19.

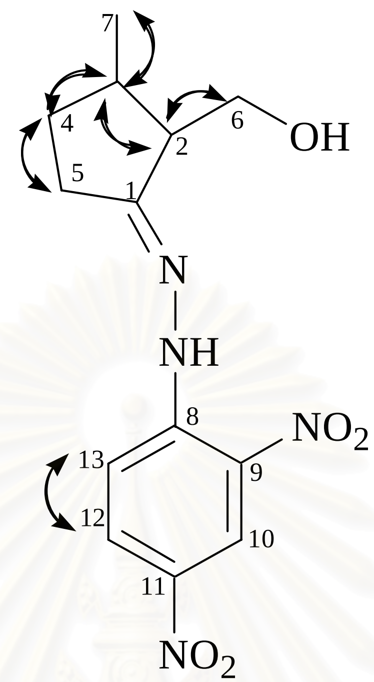


Figure 14 The correlations from ^{1}H-^{1}H COSY spectrum (arrow) of compound L20B464R2

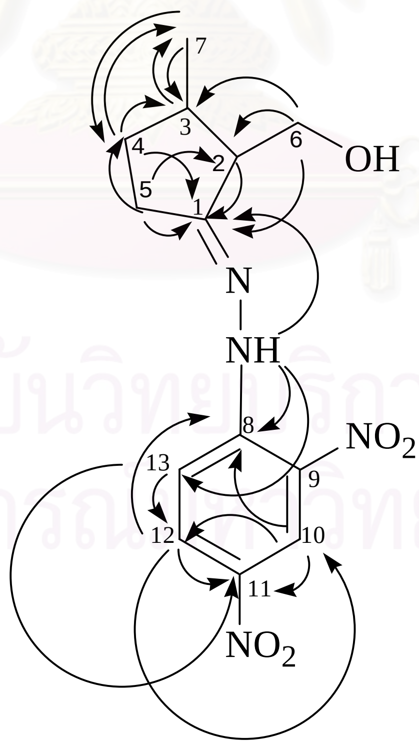


Figure 15 Long-range correlations from HMBC ($^{n}J_{HC}$ = 8 Hz) spectral data of compound L20B464R2

Table 19 The [1]H, [13]C-NMR and HMBC spectral data ($CDCl_3$) of compound L20B464R2

Position of carbon	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlation in HMBC $^{n}J_{HC}$ = 8 Hz
1	-	169.80	-
2	2.46, *m*	54.16	C-1, C-2
3	1.96, *m*	35.66	C-7
4	1.55, *m* 2.23, *m*	31.56	C-3, C-7 C-1, C-7
5	2.46, *m* 2.71, *m*	28.12	C-1, C-4 C-1, C-2, C-3, C-4
6	4.00, *dd*, (6.0)	62.23	C-1, C-2, C-3
7	1.20, *d*, (6.5)	18.46	C-2, C-3, C-4
8	-	144.84	-
9	-	129.40	-
10	9.15, *d*, (2.6)	123.62	C-8, C-11, C-12
11	-	138.04	-
12	8.33, *dd*	130.10	C-8, C-10, C-11
13	7.81, *d*, (9.6)	115.98	C-11, C-12
N-H	10.90, *s*	144.84	C-1, C-8, C-13

Based on these spectral data, hydrazone L20B464R2 was identified as {2-[2,4-dinitrophenyl)-hydrazono]-5-methyl-cyclopentyl}-methanol. Therefore a fungal metabolite was 2-hydroxymethyl-3-methyl-cyclopentanone, and its structure is shown below (Figure 16).

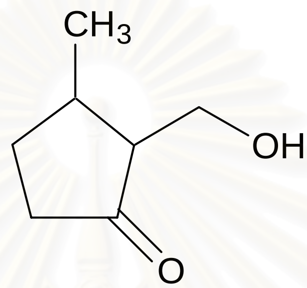


Figure 16 Structure of {2-[2,4-dinitrophenyl)-hydrazono]-5-methyl-cyclopentyl}-methanol, a secondary metabolite from the fermentation of fungal isolate *Lrub* 20.

4.1.5 Structure elucidation of 3-nitropropionic acid (U5B4-6)

Compound U5B4-6 was obtained as white solid. The ESI-TOF MS of compound U5B4-6 (Figure C57 in Appendix C) displayed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 142.0108 (calculated for $C_3H_5NO_2Na^+$ at *m/z* 142.0116). The UV spectrum in MeOH (Figure C58 in Appendix C) of the compound U5B4-6 showed λ_{max} (ε) at 205 (9967). The IR spectrum (Figure C59 in Appendix C) exhibited characteristic bands at 3021 cm^{-1} (O-H stretching); 1724 cm^{-1} (C=O stretching); 1266 cm^{-1} (C-C stretching); and 1555 cm^{-1} (C-N stretching).

Compound U5B4-6 was identified as 3-nitropropionic acid (3-NPA, in Figure 30) by NMR spectroscopy (500 MHz for ^1H and 125 MHz for ^{13}C NMR). Only two signals appearing as triplets (both with the coupling constant *J* = 6.02) with the same intensity could be observed at 4.67 and 3.07 ppm in the ^1H NMR spectrum ($CDCl_3$)(Figure 60-62) ^{13}C NMR spectrum contained three signals at 174.18 (C-1), 30.72 (C-2), and 69.33 (C-3) ppm (Figure C63). ^{1}H-^{1}H COSY spectrum of U5B4-6 (Figure C67 in Appendix C) showed that the two triplets coupled with each other, and the HMBC spectrum (Figure C66) demonstrated that the signals at 4.67 and 3.07 ppm were attached to the carbons at δ 69.33 (C-2) and 30.72 (C-3) ppm, respectively. The carbon at 174.18 (C-1) ppm was not protonated. In HMBC experiments, both proton signals gave long-range correlations to the carbon at δ 174.18 ppm, as shown in Table 20.

The downfield shift of methylene protons (at δ 4.67) suggested the attachment between this group and heteroatom (e.g. NO_2 and OH functionality). However compound U5B4-6 could not react with acid chloride (4-bromobenzenesulfonylchloride), suggesting that the attach is not OH group. The ESI-TOF MS data revealed the presence of NO_2 group in compound U5B4-6. Therefore, compound U5B4-6 was identified as 3-nitropropionic acid. Comparison of NMR spectra of U5B4-6 with those of authentic sample (sigma) readily confirmed (Figure C68 in Appendix C) that U5B4-6 is 3-nitropropionic acid, as shown in Figure 17.

Table 20 The ^{1}H, ^{13}C-NMR and HMBC spectral data ($CDCl_3$) of the compound U5B4-6

Position of carbon	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	HMBC correlation
1	-	174.18	-
2	3.07, *t*, (6.02)	30.72	C-1, C-2
3	4.67, *t*, (6.02)	69.33	C-1, C-3

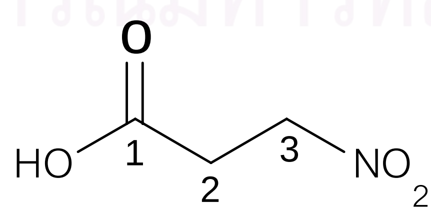


Figure 17 The structure of 3-nitropropionic acid (U5B4-6)

4.2 Biological activities of the isolated compounds

The isolated substances, asterric acid (L20B7), 2-hydroxymethyl-3-methyl-cyclopent-2-enone [L20B5(34)5], and 3-nitropropionic acid (U5B4-6) were tested for biological activities, while 2-hydroxymethyl-3-methyl-cyclopentanone was not biologically evaluated. The biological activities are summarized in Table 21.

Table 21 Summary of biological activities of the compounds from endophytic fungi isolate LRUB 20 and isolate USIA 5

Biological activity	Compounds		
	L20B7	L20B5(34)5	U5B4-6
Anticancer (IC_{50}, µg/ml)			
- BC cell line	IA	IA	IA
- KB cell line	IA	IA	IA
- NCI-H187:Small cell lung cancer	IA	IA	IA
Antiviral (IC_{50}, µg/ml)			
- HSV-1	IA	IA	IA
Antifungal (IC_{50}, µg/ml)			
- *Candida albicans*	IA	IA	IA
Antimycobacterial (MIC)			
- *Mycobacterium tuberculosis*	200	200	0.39
Antimalarial (IC_{50}, µg/ml)			
- *Plasmodium falciparum*	IA	IA	IA
Cytotoxicity (IC_{50}, µg/ml)			
- Vero cell line	> 50	> 50	> 50

* IA: Inactive at 20 µg/ml

Asterric acid (L20B7) was found to exhibit activity against *Mycobacterium tuberculosis* (MIC value 200 µg/ml), but inactive toward other activities tested (Table 21). Recently, asterric acid was isolated from culture filtrates of *Aspergillus* sp. and was the first non-peptide endothelin (ET) binding inhibitor discovered. It specifically inhibited (IC_{50} 10^{-5} M) binding of ET-1 to the ETA receptor of A 10 cells. It is a secondary metabolite of unidentified fungal strain B90911 and exhibits potent and long-lasting vasoconstrictive activity (Ohashi *et al*., 1992), and its derivatives inhibit vascular endothelial growth factor (VEGF)-induced tube formation of HUVECs (Lee *et al*., 2002). A number of derivatives of asterric acid have been claimed to be useful in the treatment of myocardial infarction and renal insufficiency (Ishimaru *et al*., 1992). The chlorinated derivatives of asterric acid have phosphodiesterase inhibitory activity (Katano *et al*., 1985) and inhibit the formation of melanins in cultured human melanocytes (Yada *et al*., 1994).

2-Hydroxymethyl-3-methyl-cyclopent-2-enone [L20B5(34)5] was found to exhibit activity against *Mycobacterium tuberculosis* (MIC value 200 µg/ml), but inactive against other cells tested (Table 21).

3-Nitropropionic acid (U5B4-6) was found to inhibit the growth of *Mycobacterium tuberculosis* (MIC value 0.39 µg/ml), but had no antimalarial, antiviral, anticancer, and cytotoxic activities (Table 21). In addition, this compound was produced by several endophytic fungi in this study, which were examined by ^{1}H NMR spectra of crude extracts. 3-Nitropropionic acid producing strains are listed in Table 22.

Table 22 List of endophytic fungal isolates capable of producing 3-nitropropionic acid.

Fungal isolate	Culture medium	Scientific - name	Family	Plant source
1) GRSP 11	SDB	*Grewia* sp. (no Thai name)	Tiliaceae	Pisanulok
2) GRSP 12	MID	*Grewia* sp. (no Thai name)	Tiliaceae	Pisanulok
3) GRSP 19	MID	*Grewia* sp. (no Thai name)	Tiliaceae	Pisanulok
4) MFER 5 (Phomopsis sp.)	MID	*Mesua ferrea* Linn. (บุนนาค)	Guttiferae	Chiangmai
5) RLYI 1	MID	*Rhododendron lyi* Levl. (กุหลาบขาว)	Ericaceae	Pisanulok
6) TASP 15	MID	*Tadehagi* sp. (ไชหิน)	Leguminosae	Pisanulok
7) GELL 14	MCz	*Gmelina elliptica* Sm. (ทองแมว)	Labiatae	Pisanulok
8) USIA 5 (Phomopsis sp.)	MID	*Urobotrya siamensis* Hiepko. (ผักหวานเมา)	Opiliaceae	Nakornratchasima

3-Nitropropionic acid is a toxic metabolite produced by plants of the family *Fabeaceae*, in which it occurs both in the free from and as a component of the glycoside hiptagin (Carter and McChesney, 1949) and by fungi of the *Penicillium* and *Aspergillus* genera (Turner, 1979). The compound has been shown to be a suicide inhibitor of mammalian succinate dehydrogenases, being converted into 3-nitroacrylate which subsequently inactivates the enzyme by alkylation of an essential cysteine sulfydryl (Coles. *et al*., 1982). Furthermore, several species of fungi from the genera *Aspergilus*, *Penicillium*, and *Neurospora* are capable of catalyzing the oxidation of aliphatic nitro compounds by O_2

(Doxtader and Alexander, 1966). Specifically, *Aspergilus flavus* and *Penicillium atrovenetum*, which synthesize the toxic antibiotic 3-nitropropionate, catalyze the oxidation of this nitroalkane by O_2 (Birkinshaw and Dryland, 1964).

Based on the biological activities summarized in Table 21, it is to be noted that Asterric acid (L20B7), 2-hydroxymethy-3-methy-cyclopent-2-enone (L20B5(34)5), and 3-nitropropionic acid (U5B4-6) were isolated from culture broth, while bioactive metabolites had not isolated from mycelial extracts in this study. Thus, the bioactive metabolites were mostly produced and secreted into the extracellular fluid. Perhaps this may explain the biological role of endophytic fungi in their host plants. They may survive in the plants as symbionts and provide protective substances that can accumulate in plant tissues to inhibit or kill invading pathogens.

4.3 Classification of the endophytic fungi isolate LRUB 20 and isolate USIA 5

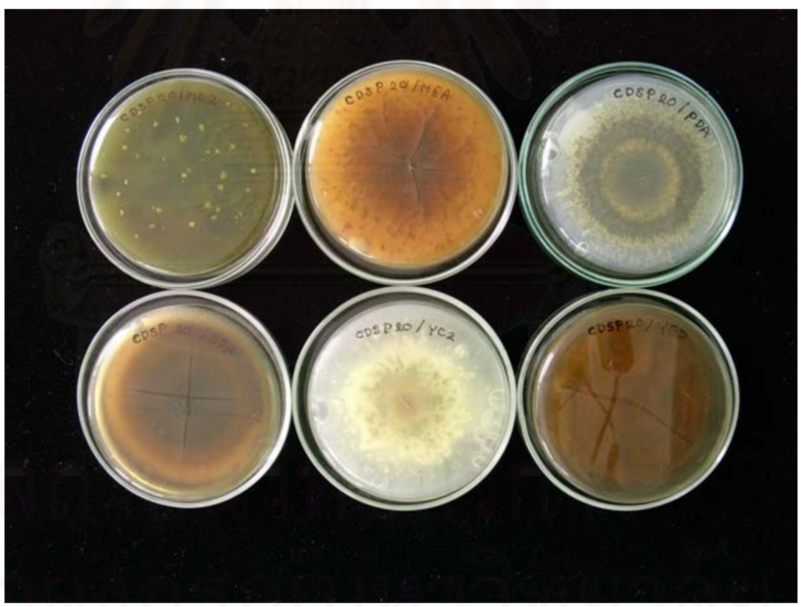
Endophytic fungus isolate LRUB 20 was isolated from *Leea rubra* Blume Ex Spreng., while the isolate USIA 5 was obtained from *Urobotrya siamensis* Hiepko. Conventional and molecular methods were applied to classify the isolate LRUB 20 and isolate USIA 5.

4.3.1 Conventional method

The endophytic fungus isolate LRUB 20 did not produce conidia or spore on common mycological media, including corn meal agar (CMA), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA), yeast Czapek agar (YCz) and yeast extract sucrose agar (YES), after cultivation for 14 days at room temperature, as shown Figure 17. The fungus isolate LRUB 20 did not sporulate when grown for 2 months on water agar and small pieces of banana leaves, a nutritionally weak medium. This condition is suggested for promoting sporulation (Smith and Onions, 1994). Therefore, LRUB 20 was classified as mycelia sterilia, and nucleotide sequences of rRNA genes provided an attractive approach in its taxonomy.

The endophytic fungus isolate USIA 5 did not produce conidia or spore on common mycological media, including corn meal agar (CMA), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA), and Yeast extract sucrose agar (YES) (Figure 18). On banana leaf agar, it developed black pycnidia (Figure 19) with two morphological distinct conidia, α-conidia (hyaline fusiform with biguttulate) and β-conidia (hyaline fusiform), as shown in Figure 20. It was found that isolate USIA 5 produced α-conidia in common than β-conidia that were infrequently found. Based on its microscopic morphology, isolate USIA 5 could be classified in genus *Phomopsis*. General morphology of *Phomopsis* sp. is the production of two basic types α-or/and β-conidia such as *Phomopsis abdita*, α conidia; *P. archeri*, α and β conidia; *P. lantanae*, α conidia; *P. diachenii*, α and β conidia; and *P. obscurans*, α conidia (Sution, 1980).

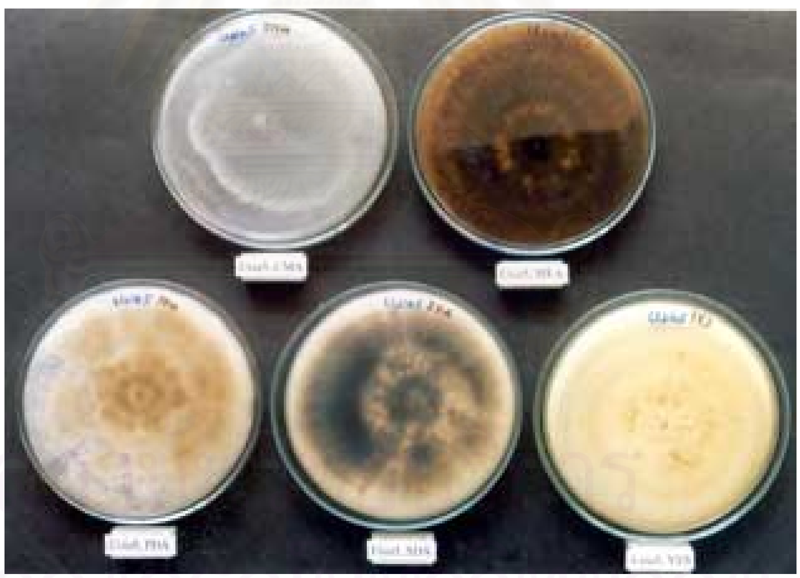
Obverse



Reverse

Figure 18 Colony morphology of endophytic fungus isolate LRUB 20 on six different media Culture: top left, Corn meal ager (CMA); top middle, Malt extract agar (MEA); top right, Potato dextrose agar (PDA); bottom left, Sabouraud’s dextrose agar (SDA); bottom middle, Yeast Czapek agar (YCzA) and bottom right, Yeast extract agar (YEA).

Obverse



Reverse

Figure 19 Colony morphology of endophytic fungus isolate USIA 5 on five different media culture: top left, Corn meal ager (CMA); top right, Malt extract agar (MEA); bottom left, Potato dextrose agar (PDA); bottom middle, Sabouraud's dextrose agar (SDA); and bottom right, Yeast extract agar (YEA).

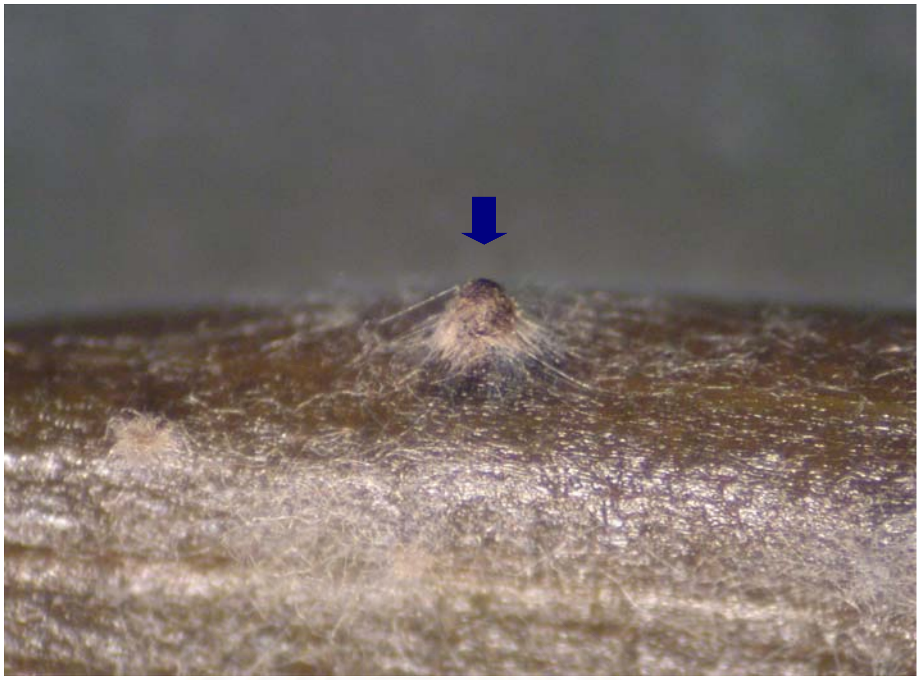


Figure 20 Conidioma (arrow) of endophytic fungus isolate USIA 5 on banana leaf.

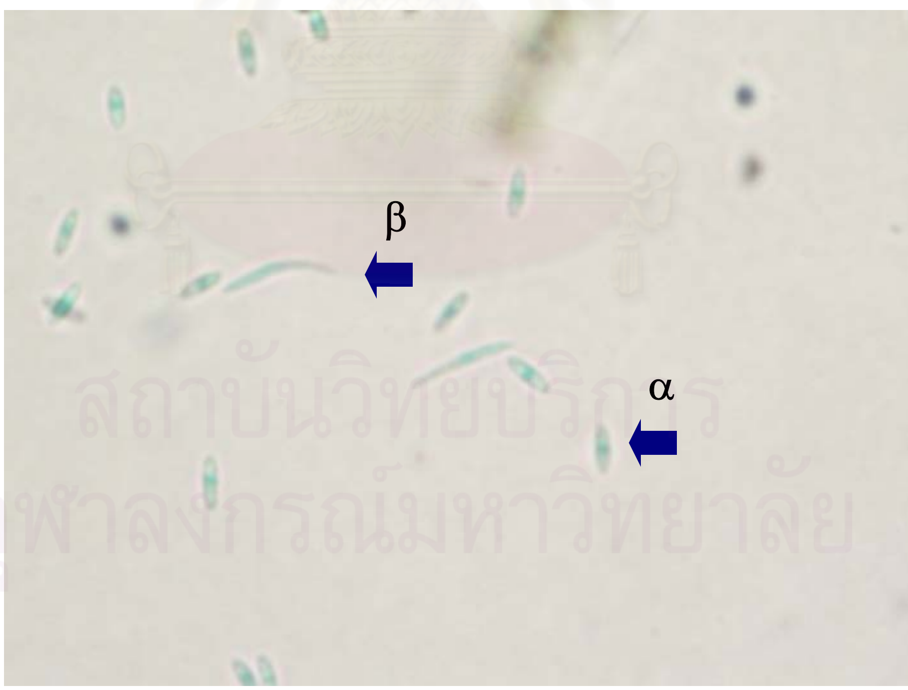


Figure 21 α and β conidia (arrow) of endophytic fungus isolate USIA 5.

4.3.2 Molecular method

Further efforts to taxonomically classify the endophytic fungal isolates LRUB 20 and USIA 5 were carried out with molecular method by determining the nucleotide sequence of ITS1-5.8S-ITS2 region of rRNA gene. Nucleotide sequence of 5.8S region is highly conserved, and it is used for the phylogenetic analysis at higher taxonomic levels (Phylum and Class). Whereas the highly variable internal transcribed spacers (ITS1 and ITS2) were used for phylogenetic analysis at lower taxonomic levels (order to species) (Mitchell *et al.,* 1995).

4.3.2.1 The PCR product of ITS1-5.8S-ITS2 region of rRNA gene

PCR conditions were optimized to amplify rRNA gene of the isolates LRUB 20 and USIA 5. The oligonucleotide primers ITS5 and ITS4 (White *et al*., 1990) were used to amplify a DNA fragment at 3' end of 18S, ITS1-5.8S-ITS2, and 5' end of 28S rDNA. Figure 11 shows the PCR product for 30-amplication cycles by 2 % agarose gel electrophoresis. The optimization condition was previously described in the material and method section. The sizes of PCR products were compared with λ*Pst*1 the molecular marker. The PCR products amplified from chromosomal DNA of isolate LRUB 20 and isolate USIA 5 were found as single band with size between 600 to 700 bp, as shown in Figure 21, lanes 1 and 5, respectively.

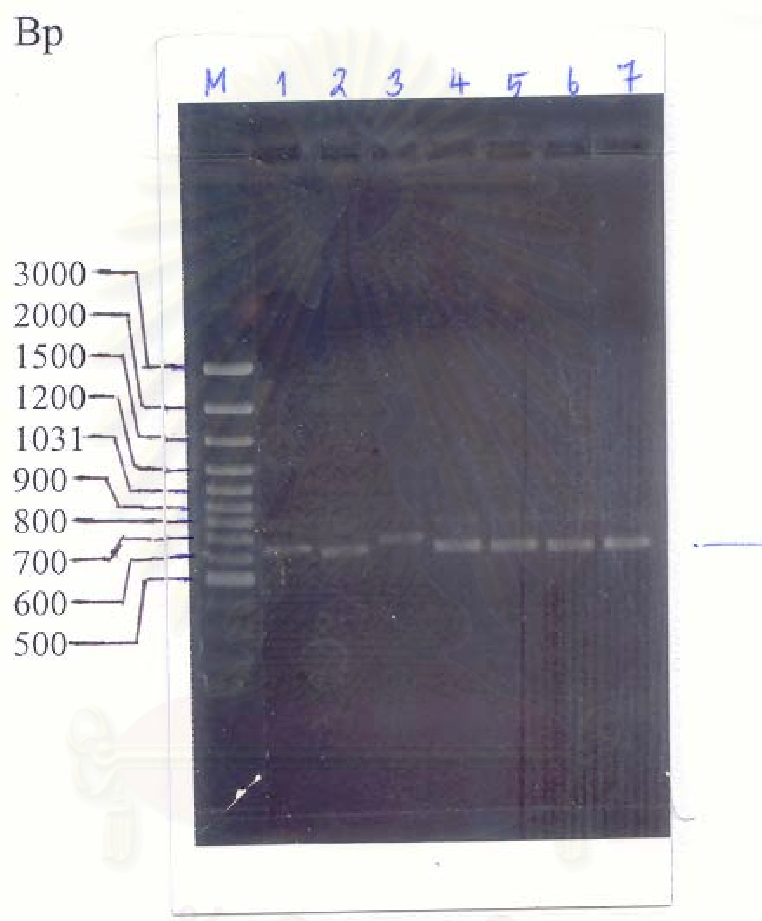


Figure 22 Agarose gel electrophoresis analysis of the PCR product from amplification of ITS1, 5.8S, and ITS2 regions of rDNA. Lanes M, 1, and 5 were the standard marker (λ*Pst*1), the PCR product of LRUB 20, and the PCR product of USIA 5, respectively.

4.3.2.2 Nucleotide sequence of partial 18S and 28S sequences and complete ITS-5.8S-ITS2 sequences of isolate USIA 5 and phylogenetic analysis

Sequencing of the PCR product amplified from chromosomal DNA of isolate USIA 5 resulted in a 554 bp fragment. This comprised partial of the 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial of the 28S sequence, as shown in Figure 12.

```
                                18S ←——→ ITS1
1    GTTGGTGAAC CAGCGGAGGG ATCATTGCTG GAACGCGCCC CAGGCGCACC    50
51   CAGAAACCCT TTGTGAACTT ATACCTTACT GTTGCCTCGG CGCAGGCTGG    100
101  TCCTCCGGGG CCCCTCACCC GCCACGGGTG TTGAGACAGC CCGCCGGCGG    150
151  CCAACCTAAC TCTTGTTTTT ACACTGAAAC TCTGAGAATA AACATAAATG    200
 ITS1 ←——→ 5.8S
201  AATCAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC    250
251  GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG    300
301  AATCTTTGAA CGCACATTGC GCCCTCTGGT ATTCCGGAGG GCATGCCTGT    350
          5.8S ←——→ ITS2
351  TCGAGCGTCA TTTCAACCCT CAAGCCTGGC TTGGTGATGG GGCACTGCTT    400
401  TTACACAAAA GCAGGCCCTG AAATTCAGTG GCGAGCTCGC CAGGACCCCG    450
451  AGCGCAGTAG TTAAACCCTC GCTTTGGAAG GCCCTGGCGG TGCCCTGCCG    500
                ITS2 ←——→ 28S
501  TTAAACCCCC AACCTTTGAA AATTGACCTC GGATCAGGTA GGAATACCCG   550
     CTGA
```

Figure 23 Nucleotide sequences of the partial 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial 28S sequence of the isolate USIA 5

The complete ITS1-5.8S-ITS2 sequences of isolate the USIA 5 was used as the query sequence to search for similar sequences from GenBank. It was found that *Phomopsis* and its teleomorph, Diaporthe, are the closest matches. A total of 23 known species (Table 23) with relative high % identity (88-97%) were selected for phylogenetic analysis.

Table 23 Twenty three known species (taxa) with relatively high sequence similarity to isolate USIA 5 that were selected for phylogenetic analysis.

Known species	Taxa (GenBank)
1	*Phomopsis amygdali*
2	*Phomopsis quercina*
3	*Phomopsis magnoliae*
4	*Phomopsis vaccinii*
5	*Phomopsis juniperivora*
6	*Diaporthe vaccinii*
7	*Phomopsis asparagi*
8	*Diaporthe caulivola*
9	*Phomopsis bougainvilleicola*
10	*Phomopsis liquidambari*
11	*Phomopsis phyllanthicola*
12	*Phomopsis averrhoae*
13	*Diaporthe phaseolorum*
14	*Diaporthe meridionalis*
15	*Diaporthe angelicae*
16	*Diaporthe arctii*
17	*Phomopsis chimonanthi*
18	*Phomopsis micheliae*
19	*Diaporthe helianthi*
20	*Phomopsis columnaris*
21	*Phomopsis glabrae*
22	*Phomopsis vexans*
23	*Phomopsis sclerotioides*

Figure 23 shows % identity between complete ITS1-5.8S-ITS2 region of USIA 5 and the reference taxa. It was found that the isolate USIA 5 had relatively higher sequence similarity to *Phomopsis amygdali*, *P. quercina*, and *P. magnoliae* with 97% identity than with any other sequences. The isolate USIA 5 also had relatively high nucleotide similarity with 96% identity to that of *P. vaccinii* and *P. juniperivora*. The isolate USIA 5 also had relatively high sequence similarities with seven *Diaporthe* species (90-95 % identity), These results confirmed that USIA 5 is *Phomopsis* sp.

Alignment of ITS1-5.8S-ITS2 sequences of USIA 5 and 24 reference taxa including outgroup by ClustalW multiple alignment program and by manually resulted in a data matrix of 527 base sites, as shown in Appendix (Figure D1). The phylogenetic relationship inferred from these data is shown in Figure 24. This inferred phylogenetic trees was 50% majority rule consensus trees with 61 steps tree length, with consistency index (CI), retention index (RI) and rescaled consistency index (RC) of 0.5062, 0.7539, and 0.4662, respectively. Evolution of isolate USIA 5 was found to be most closely related to *P*. *amygdaii*, *P*. *asparagi*, *P*. *quercina*, *P*. *magnoliae*, *P*. *vaccinii*, *P*. *juniperivora*, and *D*. *vaccinii* with 95% bootstrap support, as shown in Figure 24.

	USIA 5	Phomopsis amygdali	P. quercina	P. magnoliae	P. vaccinii	P. juniperivora	Diaporthe vaccinii	P. asparagi	D. caulivola	P. bougainvilleicola	P. liquidambari	P. phyllanthicola	P. averrhoae	D. phaseolorum	D. meridionalis	D. angelicae	D. arctii	P. chimonanthi	P. micheliae	D. helianthi	P. columnaris	P. glabrae	P. vexans
Phomopsis amygdali	97																						
P. quercina	97	99																					
P. magnoliae	97	99	99																				
P. vaccinii	96	98	98	98																			
P. juniperivora	96	99	99	98	97																		
Diaporthe vaccinii	95	96	96	95	97	95																	
P. asparagi	94	95	95	94	95	94	94																
D. caulivola	93	93	93	93	94	92	92	93															
P. bougainvilleicola	93	94	94	93	93	93	92	91	91														
P. liquidambari	93	92	92	92	93	92	92	93	95	93													
P. phyllanthicola	93	93	93	93	93	93	91	92	95	94	97												
P. averrhoae	93	93	93	93	93	92	91	92	92	97	95	95											
D. phaseolorum	92	92	92	92	93	91	92	93	94	92	94	93	94										
D. meridionalis	91	92	92	92	93	92	91	92	95	92	95	94	92	93									
D. angelicae	90	90	90	90	90	89	89	90	93	89	93	91	90	90	93								
D. arctii	90	91	91	91	91	90	90	90	94	89	93	92	90	90	93	97							
P. chimonanthi	90	90	90	90	91	90	89	89	93	89	92	91	89	90	91	94	95						
P. micheliae	90	90	90	90	91	90	89	89	93	89	92	91	89	90	91	94	95	90					
D. helianthi	90	91	91	91	91	90	89	90	93	89	93	92	90	90	93	96	97	96	96				
P. columnaris	89	89	89	89	90	89	88	89	92	89	92	91	90	90	92	95	95	94	94	96			
P. glabrae	89	90	90	90	90	89	88	89	92	88	92	92	89	89	92	95	95	95	95	96	96		
P. vexans	88	89	88	88	89	89	88	89	92	89	92	91	89	89	92	95	95	94	94	95	93	94	
P. sclerotioides	88	88	88	88	89	89	88	88	91	89	92	90	89	89	92	94	94	92	92	94	98	95	92

Figure 24 The alignment scores (% identity) of complete ITS1-5.8S-ITS2 sequences of the isolate USIA 5 and 23 reference taxa from GenBank.

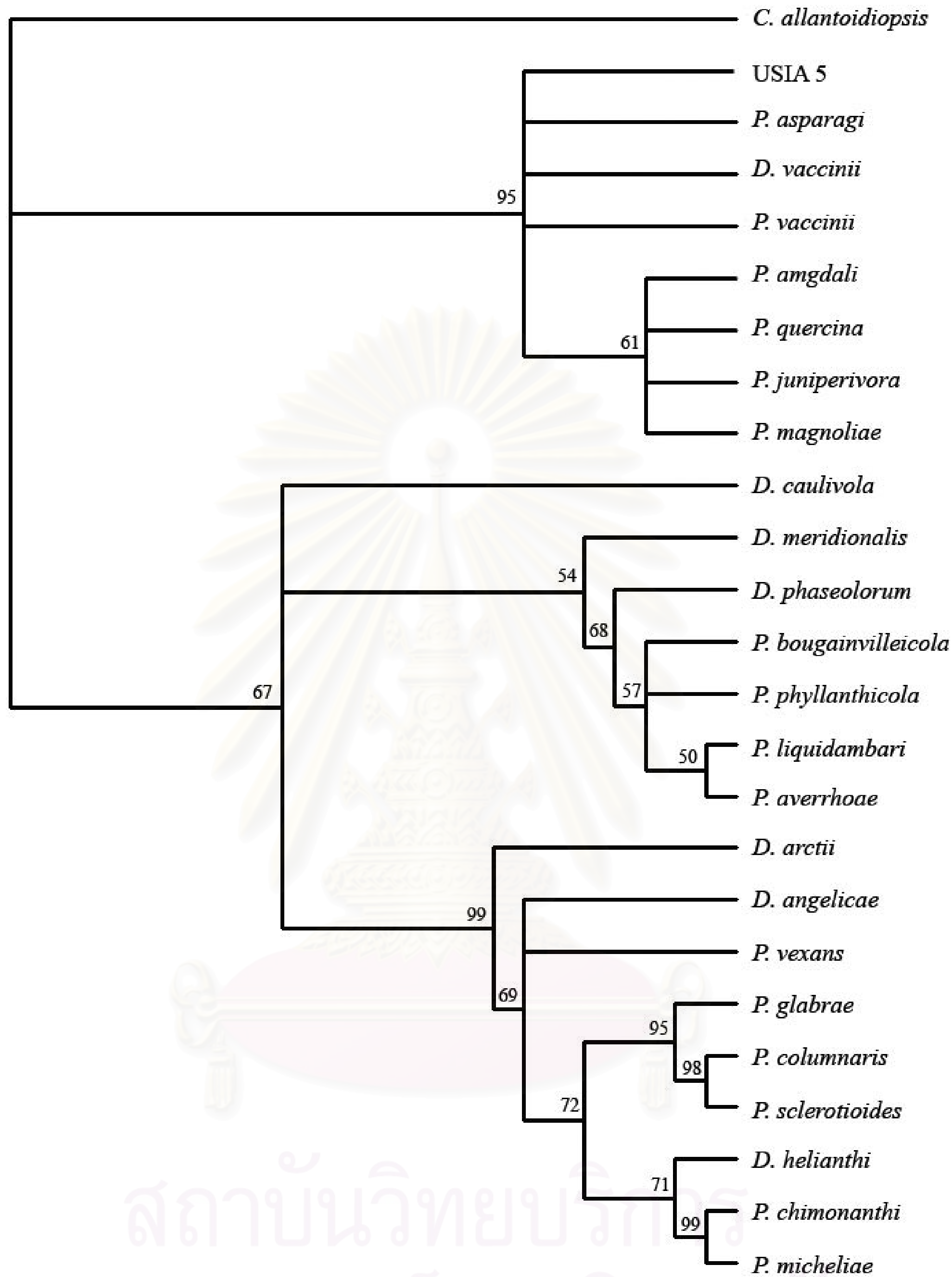


Figure 25 Maximum-parsimony tree (50% majority-rule consensus tree) generated from the ITS1-5.8S-ITS2 sequences of 25 taxa (CI=0.5062, RI=0.7539, RC=0.4662, tree length=61 steps) showing the evolutionary relationship of USIA 5 with reference taxa. The numbers at internal node indicate the percentages of trees from 1,000 bootstrap replications. *Cytospora allantoidiopsis* was used as an outgroup.

This study shows that the fungal isolate USIA 5 could be species of *Phomopsis* and its telemorph, *Diaporthe*, whose several members were known to be plant pathogens. *D. meridionalis* was known to cause stem canker in soybean and *D. helianthi* causes brown stem canker in sunflower (Gulya and Masirevic, 1993). *P. amygdali* causes sunken canker in peach (Jones and Sutton, 2004; Mostert and Crous, 2004), and *P. vaccinii* causes twig blight in cranberry (Mcmanus, 2004). Despite of these evidences of plant pathogenic nature of *Phomopsis* sp. and *Diaporthe* sp., the USIA 5 is considered to be an endophytic fungus because it is also capable of living as a symptomless endophyte for prolonged periods within its host plant, *Urobotrya siamensis* Hiepko., and it did not sporulate when grown on common mycological media (normal condition) such as CMA, MEA, PDA, SDA, and YES except sporulating only on plant material.

It should be noted that some fungi are considered to be both endophytes and plant pathogens. For example, *M. betulinum* and other *Melanconium* spp. are known as endophytes and as causal agents of diebacks and cankers of various broad-leaved trees, including *Betula* spp. (Sieber *et al*., 1991; Belisario, 1999; and Elamo *et al*., 1999).

4.3.2.3 Nucleotide sequence of partial 18S and 28S sequences and complete ITS1-5.8S-ITS2 sequences of isolate LRUB 20 and phylogenetic analysis

Sequencing of the PCR product amplified from chromosomal DNA of isolate LRUB 20 resulted in a 572 bp fragment. This comprised partial of the 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial of the 28S sequence, as shown in Figure 25.

```
                    18S <---|---> ITS1
10    TGAACCTGCG GAAGGATCAT TACAAGTTGA AACGGTTGCC CTCGCGGTGA    50
51    CCGGTTCTTC AAACCTCTGC GTACCAAACC TTTCAGTTGC CTCCGGCGGC    100
101   CCTGGGCCGG CGCGGCGCGC GACCTCCCCC TCGCGGGCGG GGCCGCTCCT    150
151   CGCGGCGGAC CACCCGCCGG GCGGTCATAA ACAAAACCTT TTCGTCGAGA    200
                                      ITS1 <---|---> 5.8S
201   TGGCATCGTC TAATTTCTTC ATATCAAAAT ATGAAATACA ACTTTCAACA    250
251   ATGGATCTCT TGGCTCCGGC ATCGATGAAG AACGCAGCGA AATGCGATAA    300
301   CTAGTGTGAA TTGCAGATTT CAGTGAATCA TCGAGTCTTT GAACGCACAT    350
                                             5.8S <---|---> ITS2
351    TGCGCCTCTT GGTATTCCTC GAGGCATGCC TGTTCGAGCG TCGTTACGCC   400
401   CCTCAAGCGC GAGCTTGGTG TTGGGGATCG CCCCTGAGAT ACGGCGGCGG    450
451   CCCTTAAATG CATCGGCGGT GCTGGTGTCA GCCCGGAGCG CAGCAGACAT    500
501   GCGGCTTCCA GGCGACCACG CGCCCGCCGG ACAACGACCC GACCTTCAAA    550
ITS2 <---|---> 28S
550    CGTCGACCTC GGATCAGGTA GG    572
```

Figure 26 Nucleotide sequences of the partial 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial 28S sequence of the isolate LRUB 20

The ITS1-5.8S-ITS2 sequence was used as the query sequence to search for similar sequences from GenBank using BLASTN 2.2.10 program (Altschul *et al*., 1997). It was noticed that all 100 blast hit sequences show no similar sequence to ITS1 region of isolate LRUB 20 and some hit sequences show similarity in some region of ITS2 sequence. A total of 40 known species from 100 blast hits were selected. *Mycoleptodiscus terrestris* was found to be the species that show the highest sequence similarity (72% identity). The % identity of ITS1-5.8S-ITS2 sequence of LRUB 20 and the other sequences was found to be 55-64%.

Alignment of ITS1-5.8S-ITS2 sequences of LRUB 20, 40 reference taxa and 2 outgroup taxa by ClustalW multiple alignment program and by manually resulted in a data matrix of 677 base sites, as shown in Appendix D (Figure D2). The phylogenetic relationship inferred from these data using maximum parsimony algorithm is shown in Figure 26. This inferred phylogenetic tree was 50% majority-rule consensus trees with 1,808 steps tree length, with consistency index (CI), retention index (RI) and rescaled consistency index (RC) of 0.5492, 0.6337, and 0.3480, respectively. It revealed that isolate LRUB 20 had evolution related to *Mycoleptodiscus terrestris* in Family Magnaporthaceae, with 95% bootstrap support, as shown in Figure 26. According to the low similarity between ITS1-5.8S-ITS2 sequences of LRUB 20 and the known blast hit species, 5.8S sequence of isolate LRUB 20 was used as the query sequence. A total of 20 known species from 100 blast hits were selected as representative (Table 24). Multiple sequence alignment by ClustalW program showed that LRUB 20 had relative highest identity (98%) to *M. terrestris*, as shown in Figure 27. Alignment of 5.8S sequences of LRUB 20, 20 reference taxa and 2 outgroup taxa by ClustalW multiple alignment program and by manually resulted in a data matrix of 165 base sites, as shown in Appendix D (Figure D3). The phylogenetic relationship inferred from these data using maximum parsimony algorithm is shown in Figure 28. This inferred phylogenetic trees was 50% majority-rule consensus trees with 62 steps tree length, with consistency index (CI), retention index (RI) and rescaled consis tency index (RC) of 0.7419, 0.7895, and 0.5857, respectively. Maximum parsimony tree based on 5.8S sequences also showed evolutionary relationship of LRUB 20 to *M. terrestris* with 98% bootstrap support, as shown in Figure 28. In addition, It was found that LRUB 20 and *M. terrestris* clade was a sister clade to *Aspergillus* clade.

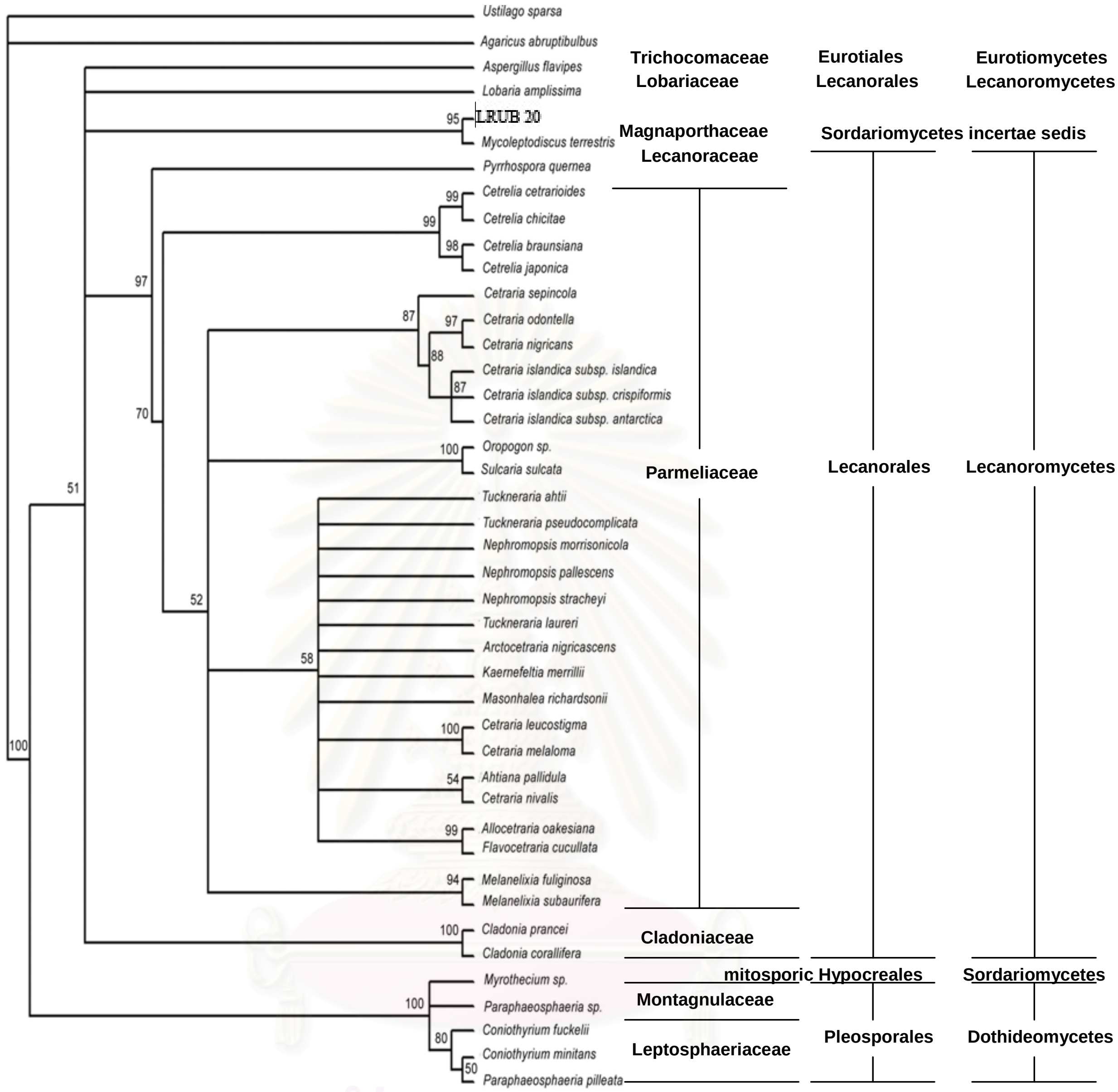


Figure 27 Maximum-parsimony tree (50% majority-rule consensus tree) generated from the ITS1-5.8S-ITS2 sequences of 43 taxa (CI=0.5062, RI=0.7539, RC=0.4662, tree length=1,808 steps) showing the evolutionary relationship of LRUB 20 with reference taxa. The numbers at internal node indicate the percentages of trees from 1,000 bootstrap replications. *Ustilago sparsa* and *Agaricus abruptibulbus* were used as outgroups.

Table 24 Twenty known species (taxa) selected as representatives from 100 blast hits that obtained from GenBank when 5.8S sequence of LRUB 20 was used as the query sequence.

Known species	Taxa (GenBank)
1	*Mycoleptodiscus terrestris*
2	*Myrothecium* sp. Z16
3	*Coniothyrium sporulosum*
4	*Montagnula opulenta*
5	*Paracoconiothyrium cyclothyrioides*
6	*Paraphaeosphaeria* sp.
7	*Paraphaeosphaeria pilleata*
8	*Conithyrium fuckelii*
9	*Conithyrium minitans*
10	*Massarina bipolaris*
11	*Massarina lacustris*
12	*Paraphaeosphaeria michotii*
13	*Lophiostoma arundinis*
14	*Aspergillus flavipes*
15	*Aspergillus niger*
16	*Aspergillus ellipticus*
17	*Fennellia nivea* strain SRRC 333
18	*Tuber rufum* morphotype 5
19	*Aporospora terricola*
20	*Humicola fuscoatra*

LRUB 20

98	*Mycoleptodiscus terrestris*																			
94	92	*Myrothecium* sp. Z16																		
94	92	100	*Coniothyrium sporulosum*																	
94	92	100	100	*Montagnula opulenta*																
94	92	100	100	100	*Paracoconiothyrium cyclothyrioides*															
94	92	100	100	100	100	*Paraphaeosphaeria* sp.														
94	92	100	100	100	100	100	*Paraphaeosphaeria pilleata*													
94	92	100	100	100	100	100	100	*Conithyrium fuckelii*												
94	92	100	100	100	100	100	100	100	*Conithyrium minitans*											
93	92	97	97	97	97	97	97	97	97	*Massarina bipolaris*										
93	91	99	99	99	99	99	99	99	99	96	*Massarina lacustris*									
93	91	98	98	98	98	98	98	98	98	96	98	*Paraphaeosphaeria michotii*								
92	91	96	96	96	96	96	96	96	96	99	96	96	*Lophiostoma arundinis*							
93	91	93	93	93	93	93	93	93	93	91	94	93	92	*Aspergillus flavipes*						
92	91	93	93	93	93	93	93	93	93	91	93	93	92	99	*Aspergillus niger*					
92	91	93	93	93	93	93	93	93	93	91	93	93	92	99	100	*Aspergillus ellipticus*				
92	91	93	93	93	93	93	93	93	93	91	93	93	92	99	100	100	*Fennellia nivea* strain SRRC 333			
91	89	96	96	96	96	96	96	96	96	94	95	95	94	92	91	91	91	*Tuber rufum* morphotype 5		
94	92	100	100	100	100	100	100	100	100	97	99	98	96	93	93	93	93	96	*Aporospora terricola*	
93	91	98	98	98	98	98	98	98	98	96	98	98	96	93	93	93	93	95	98	*Humicola fuscoatra*

Figure 28 The alignment scores (% identity) of complete 5.8S sequence of the isolate LRUB 20 and 20 reference taxa from GenBank

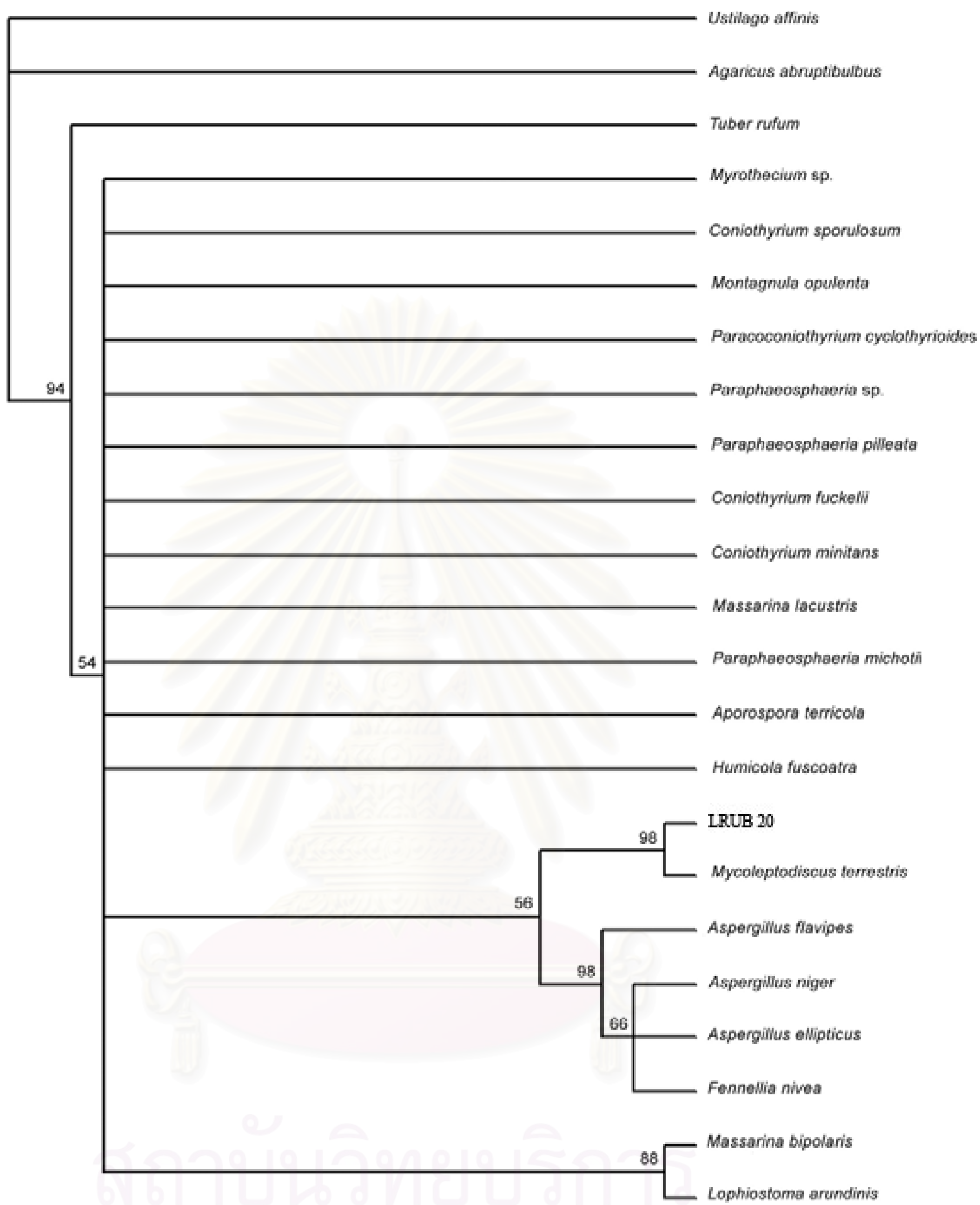


Figure 29 Maximum-parsimony tree (50% majority-rule consensus tree) generated from the 5.8S sequences of 23 taxa (CI=0.7419, RI=0.7895, RC=0.5857, tree length=62 steps) showing the evolutionary relationship of LRUB 20 with reference taxa. The numbers at internal node indicate the percentages of trees from 1,000 bootstrap replications. *Ustilago sparsa* and *Agaricus abruptibulbus* were used as outgroups.

In order to confirm evolutionary relationship of LRUB 20 and *M. terrestris*, other six representative species of Magnaporthaceae were further selected for phylogenetic analysis together with *Aspergillus* species, as shown in Table 25. Alignment of 5.8S sequences of LRUB 20 and these reference taxa including outgroup taxa by ClustalW multiple alignment program and by manually resulted in a data matrix of 158 base sites, as shown in Appendix D (Figure D4). The phylogenetic relationship inferred from these data is shown in Figure 30. This inferred phylogenetic trees was 50% majority-rule consensus tree with 54 steps tree length, with consistency index (CI), retention index (RI) and rescaled consistency index (RC) of 0.7407, 0.7846, and 0.5812, respectively. Phylogenetic anlysis based on 5.8 sequence of LRUB 20, selected representative species from Magnaporthaceae and Trichocomaceae also showed that LRUB 20 and *M. terrestris* were in the same clade with 99% bootstrap support that was sister clade to *Aspergillus* species, as shown in Figure 30.

Molecular method is a possible tool to classify the endophytic fungal isolate LRUB 20 because it is sterile. There are several studies to identify endophytic fungi using molecular techniques (e.g. Arnold *et al*., 2000; Okane, 2001; and Baayen *et al*., 2002). However, there are limitations in the identification of mycelia sterilia by means of DNA sequence analyses (Guo *et al*. 2000b, 2001). All phylogenetic analyses and sequence similarity attempted suggested that LRUB 20 should be novel species in family Magnaporthaceae, class Sordariomycetes, and subphylum Pezizomycotina, phylum Ascomycota. The endophytic fungus isolate LRUB 20 in this study that was given taxonomic placement at family level (could not be classified to lower taxonomic level) could be further resolved once more references are available in the databases. Nevertheless, molecular identification based on nucleotide sequences is a powerful tool that could potentially become a routine approach in future studies of fungal diversity, especially for sterile mycelia.

Table 25 Representative species of families Magnaporthaceae and Trichocomaceae obtained from GenBank sequences used for phylogenetic analysis.

Known species	Taxa (GenBank)
1	*Mycoleptodiscus terrestris*
2	*Aspergillus flavipes*
3	*Aspergillus niger*
4	*Aspergillus ellipticus*
5	*Fennellia nivea*
6	*Buergenerula spartinea*
7	*Gaeumannomyces amomi*
8	*Magnaporthe grisea*
9	*Pyricularia angulata*
10	*Harpophora maydis*
11	*Phialophora bofulispora*

Lrub 20											
98	*Mycoleptodiscus terrestris*										
93	**91**	*Aspergillus flavipes*									
92	**91**	**99**	*Aspergillus niger*								
92	**91**	**99**	**100**	*Aspergillus ellipticus*							
92	**91**	**99**	**100**	**100**	*Fennellia nivea*						
88	**87**	**94**	**94**	**94**	**94**	*Buergenerula spartinea*					
87	**87**	**94**	**94**	**94**	**94**	**99**	*Gaeumannomyces amomi*				
88	**87**	**94**	**94**	**94**	**94**	**100**	**99**	*Magnaporthe grisea*			
88	**87**	**94**	**94**	**94**	**94**	**100**	**99**	**100**	*Pyricularia angulata*		
88	**88**	**93**	**93**	**93**	**93**	**96**	**97**	**96**	**96**	*Harpophora maydis*	
89	**89**	**93**	**93**	**93**	**93**	**96**	**96**	**96**	**96**	**94**	*Phialophora bofulispora*

Figure 30 The alignment scores (% identity) of complete 5.8S sequence of the isolate LRUB 20 and 11 reference taxa from GenBank

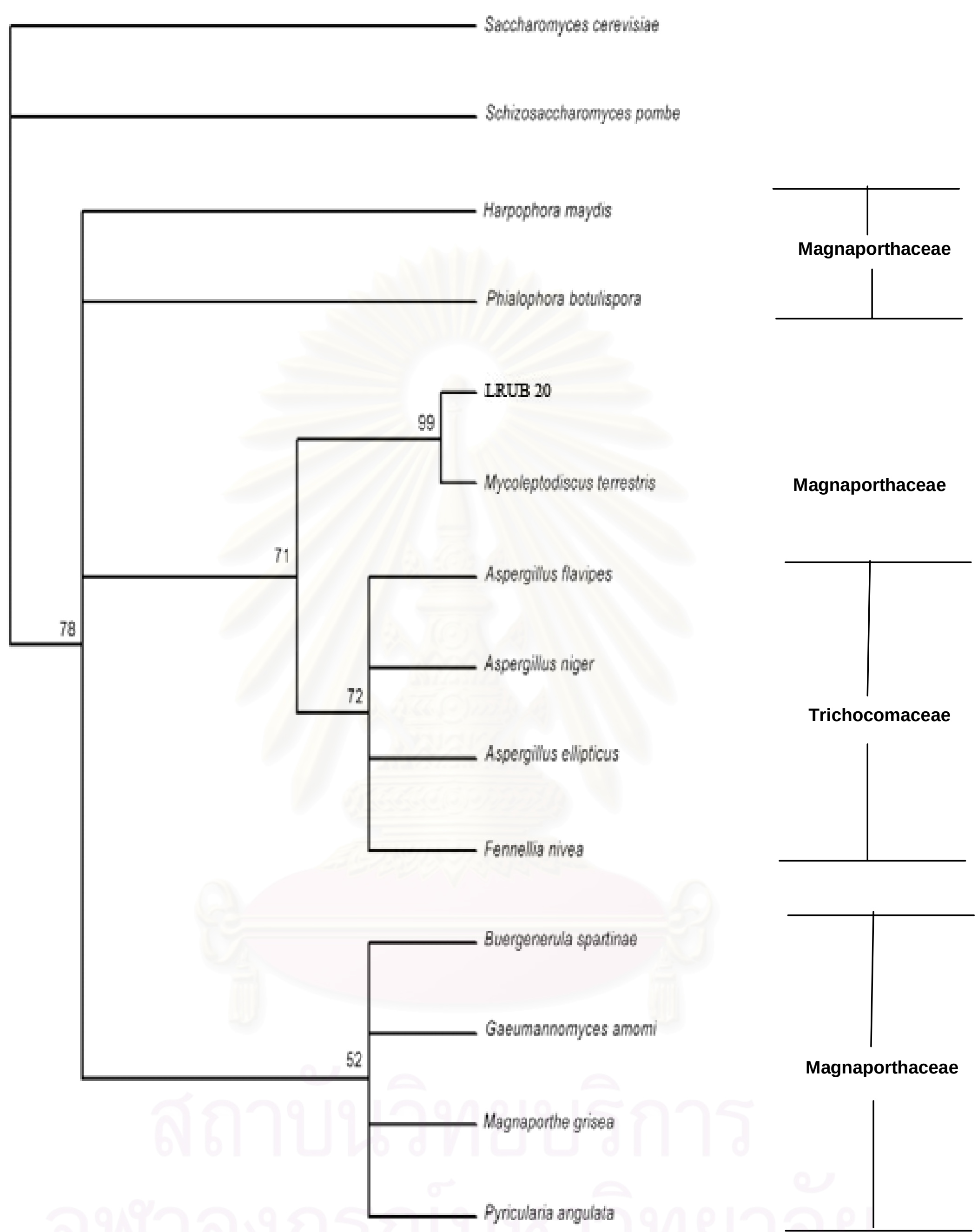


Figure 31 Maximum-parsimony tree (50% majority-rule consensus tree) generated from the 5.8S sequences of 14 taxa (CI=0.7407, RI=0.7846, RC=0.5812, tree length=54 steps) showing the evolutionary relationship of LRUB 20 with reference taxa. The numbers at internal node indicate the percentages of trees from 1,000 bootstrap replications. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used as outgroups.

CHAPTER V

CONCLUSION

The endophytic fungus isolate LRUB 20 was isolated from the surface-sterilized stem of *Leea rubra* Blume Ex Spreng. (Leeaceae). In the present investigation, three compounds were isolated from MCz culture of the endophytic fungus isolate LRUB 20. The isolated compounds include asterric acid, 2-hydroxymethyl-3-methyl-cyclopent-2-enone, and 2-hydroxymethyl-3-methyl-cyclopentanone. Asterric acid and 2-hydroxymethyl-3-methyl-cyclopent-2-enone were found to exhibit activity against *Mycobacterium tuberculosis* H37Rv with the MIC value of 200 µg/ml. Based on conventional method, the fungal isolate LRUB 20 limited in spore formation. Nucleotide sequencing of ITS1-5.8S-ITS2 sequences of rDNA was applied to classify the endophytic fungal isolate LRUB 20. It was found to be in the family Magnaporthaceae. However, the fungal isolate LRUB 20 could not be identified at the taxonomic level of genus and species due to the highly variable internal transcribed spacers (ITS1 and ITS2) of rDNA sequence that did not match with any known fungi in the GenBank database.

The endophytic fungus isolate USIA 5 was isolated from the surface-sterilized leaf of *Urobotrya siamensis* Hiepko. (Opiliaceae). In the present investigation, 3-nitropropionic acid was isolated from MID culture of the endophytic fungus isolate USIA 5. 3-Nitropropionic acid exhibited activity against *Mycobacterium tuberculosis* H37Rv with the MIC value of 0.39 µg/ml. The endophytic fungus isolate USIA 5 produced black pycnidia with α-conidia and β-conidia (rarely) on banana leaf. Based on the microscopic morphology and the nucleotide sequencing of ITS1-5.8S-ITS2 sequences of rDNA, endophytic fungus isolate USIA 5 was identified as *Phomopsis* sp. in the family Diaporthaceae.

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APPENDICES

APPENDIX A

Table A The chemical compounds, sources, biological activities of bioactive compounds of endophytic fungi.

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
1	Taxol	*Taxomyces andreanae*	*Taxus brevifolia*	Anticancer	Strobel *et al*., 2003, Stierle and Strobel,1995, Stierle *et al*., 1993, Strobel and Stierle, 1993
		Stegolerium kukenani	*Stegolepis guianensis*	Anticancer	Strobel *et al*., 2001
		Aspergillus niger	*Taxus chinensis*	Anticancer	Wang *et al*., 2001
		Tubercularia sp.	*Taxus mairei*	Anticancer	Strobel *et al*., 2003, Wang *et al*., 2000
		Pestalotiopsis microspora	*Taxus wallachina*	Anticancer	Strobel *et al*., 2003, Metz *et al*., 2000, Li *et al*., 1998, Strobel *et al*., 1996
			Taxodium distichum	Anticancer	Li *et al*.,*1996*
		Periconia sp.	*Torreya grandifolia*	Anticancer	Li *et al*., 1998
		Pestalotiopsis guepinii	*Wollemia nobilis*	Anticancer	Strobel *et al*., 1997

Table A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
2	1,3,5,7 cyclooctatetraene or [8]annulene	*Gliocladium* sp.	*Eucryphia cordifolia*	Antimicrobial	Stinson *et al*., 2003
3 4	Lactone 1893 A Lactone 1893 B	Endophytic fungus No. 1893	*Kandelia candel*	Cytotoxic	Chen *et al*., 2003
5	Pestacin	*Pestalotiopsis microspora*	Rainforest	Antioxidant and antimycotic	Harper *et al*., 2003
6 7 8	7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one 7-Butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one 7-Butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one	*Geotrichum* sp.	*Crassocephalum crepidioides*	Antimalarial, antituberculous and antifungal	Kongsaeree *et al*., 2003

Table A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
9	Brefeldin A	*Paecilomyces* sp. and *Aspergillus clavatus*	*Taxus mairei and Torreya grandis*	Cytotoxic	Wang *et al*., 2002
10	Isopestacin	*Pestalotiopsis microspora*	*Terminalia morobensis*	Antifungal and antioxidant	Strobel *et al*., 2002
11 12 13	Preaustinoid A Preaustinoid B Alkaloid verruculogen	*Penicillium* sp.	*Melia azedarach*	Bacteriostatic	Santos and Rodrigues-Fo, 2002
14	Ambuic acid	*Pestalotiopsis* spp., *Monochaetia* sp.	Rainforests	Antifungal	Li *et al*., 2001
15 16	Jesterone hydrosy-jesterone	*Pestalotiopsis jesteri*	*Fragraea bodenii*	Antioomycete	Li *et al*., 2001
17 18 19	Preussomerin G Preussomerin H Preussomerin I	Mycelia sterile	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn *et al*., 2001

Table A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
20 21 22	Preussomerin J Preussomerin K Preussomerin L	Mycelia sterile	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn *et al*., 2001
23 24 25	Dicerandrol A Dicerandrol B Dicerandrol C	*Phomopsis longicolla*	*Dicerandra frutescens*	Antibiotic and cytotoxic	Wagenaar and Clardy, 2001
26	Microcarpalide	Unidentified endophytic fungus	*Ficus microcarpa*	Microfilament disrupting agent	Ratnayake *et al*., 2001
27	Nomofungin	Unidentified endophytic fungus	*Ficus microcarpa* L.	Microfilament disruptin agent and cytotoxic	Ratnayake *et al*., 2001
28	Isoprenylindole-3-carboxylic acid	*Collectotrichum* sp.	*Artemisia annua*	Antibacterial and antifungal	Lu *et al*., 2000

Table A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
29	3beta,5alpha-Dihydroxy-6beta-acetoxy-ergosta-7,22-diene	*Collectotrichum* sp.	*Artemisia annua*	Antibacterial and antifungal	Lu *et al*., 2000
30	3beta,5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7,22-diene				
31	Indole-3-acetic acid (IAA)	*Epichloe/Neotyphodium* spp.	Grasses	Antifungal	Yue *et al*., 2000
32	Indole-3-ethanol (IEtOH)				
33	Methylindole-3-carboxylate				
34	Indole-3-carboxaldehyde				
35	Diacetamide				
36	Cyclonerodiol				
37	Colletotric acid	*Colletotrichum gloeosporioides*	*Artemisia mongolica*	Antimicrobial	Zou *et al*., 2000

Table A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
38	CR377, pentaketide	*Fusarium* sp.	*Selaginella pallescens*	Antifungal	Brady and Clardy, 2000
39	Cytochalasin 1	*Rhinocladiella* sp.	*Tripterygium wilfordii*	Cytotoxic	Wagenaar *et al*., 2000
40	Cytochalasin 2				
41	Cytochalasin 3				
42	Cytochalasin E				
43	Cryptocandin	*Cryptosporiopsis* cf. *quercina*	*Tripterigeum wilfordii*	Antimycotic	Strobel *et al*., 1999
44	Geniculol	*Geniculosporium* sp.	*Teucrium scorodania*	Antialgal	Konig *et al*., 1999
45	Cytochalasin F				
46	Sequoiatone A	*Aspergillus parasiticus*	*Sequoia sempervirens*	Antitumor	Stierle *et al*., 1999
47	Sequoiatone B				
48	Terpendole M	*Neotyphodium lolii*	*Lolium perenne*	neurotoxins	Gatenby *et al*., 1999
49	Tricin (1)	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998
50	7-O-(B-D-glucopyranosyl) tricin				
51	Isoorientin (3)				

Table A (continued)

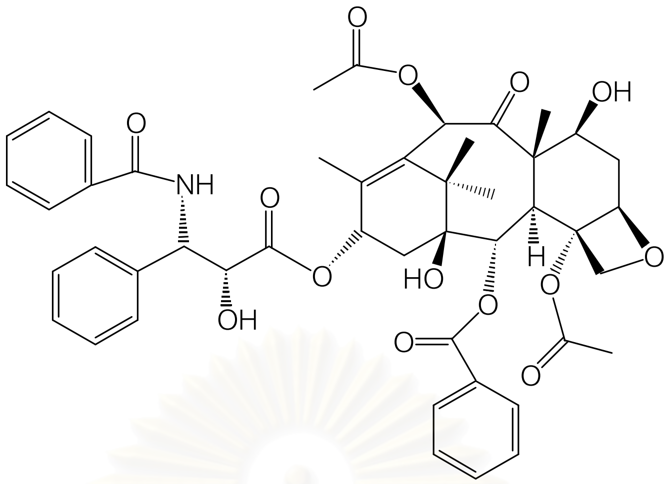
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
52	7-o-[α-L-Rhamnopyranosyl(1-6)-β-D-glucopy-ranosyl]tricin	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju *et al*., 1998
53	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxic	Berny *et al*., 1997
54	Leucinostatin A	*Acremoium* sp.	*Taxus baccata*	Antifungal and anticacer	Strobel *et al*., 1997
55 56 57 58	Oreganic acid (1) Trimethyester (2) Desulfated analog (3) Desulfated analog (4)	Endophytic fungus (MF 6046)	*Berberis oregana*	Anticancer	Jayasuriya *et al*., 1996
59 60	Pestalotiopsin A Pestalotiopsin B	*Pestalotiopsis* sp.	*Taxus brevifolia*	-	Pulici *et al*., 1996
61 62	(R)-mellein (-)-mycorrhizin A	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995

Table A (continued)

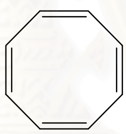
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
63	2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde	*Pezicula* sp.	Deciduous and coniferous trees	Fungicidal, herbicidal, algicidal and antibacterial	Schulz *et al*., 1995
64	(+)-cryptosporiopsin				
65	4-epi-ethiosolide				
66	Altersolanol A	*Phoma* sp.	*Taxus wallachiana*	Antibacterial	Yang *et al*., 1994
67	2-hydroxy-6-methylbenzoic acid				
68	Preussomerin D	*Hormonema dematioides*	Conifer wood	Antifungal	Polishook *et al*., 1993
69	Lolitrem C	*Acremonium lolii*	*Lolium perenne*	Neurotoxic and insect antifeedant	Rowan *et al*., 1993
70	Peramine R=H				
71	Diacetylperamine R=Ac				
72	Paxilline				
73	Loline alkaloid				
74	Ergovaline				

Table A (continued)

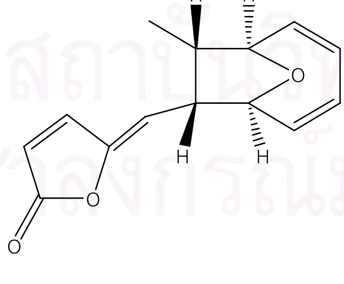
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
75	Lysergic acid	*Acremonium coenophialun*	*Festuca arundinacea*	Toxin	Garner *et al*., 1993
76	Isolysergic acid				
77	Pospalic acid				
78	Lysergol				
79	Lysergic acid amide				
80	Lysergic acid diethyl-amide				
81	Lycergic acid-2-propanolamide or (Ergonovine)				



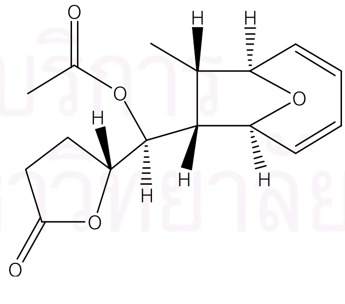
[1] Taxol



[2] 1,3,5,7 cyclooctatetraene or (8)-annulene

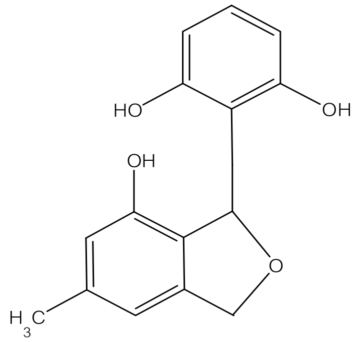


[3] Lactone 1893 A

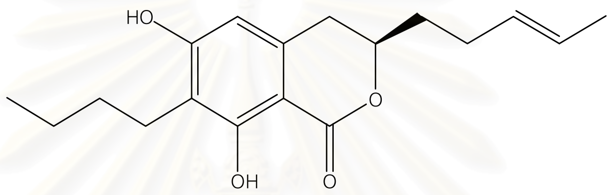


[4] Lactone 1893 B

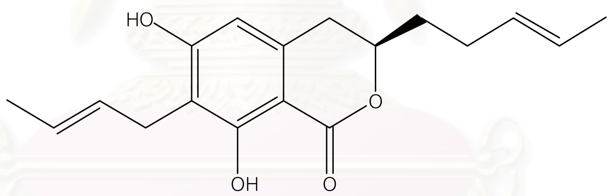
Figure A Structure of bioactive compounds of endophytic fungi of listed in Table A.



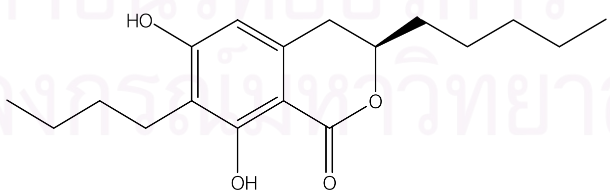
[5] Pestacin



[6] 7-Butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



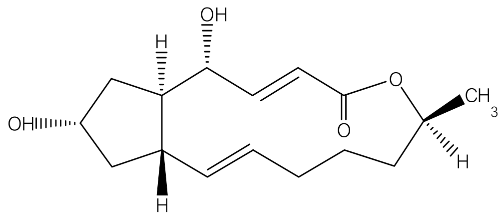
[7] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



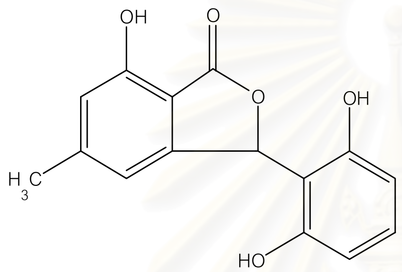
[8] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

Dihydroisocumarins [6-8]

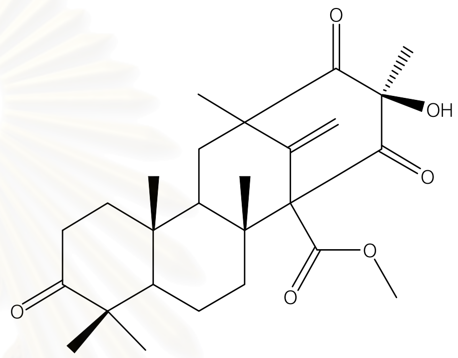
Figure A (continued)



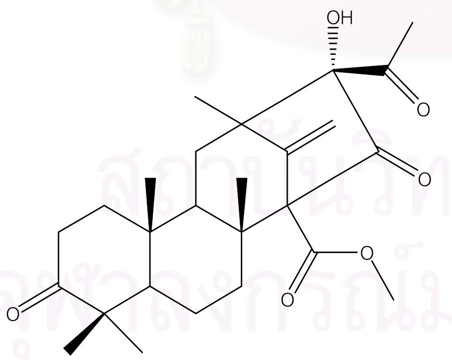
[9] Brefeldin A



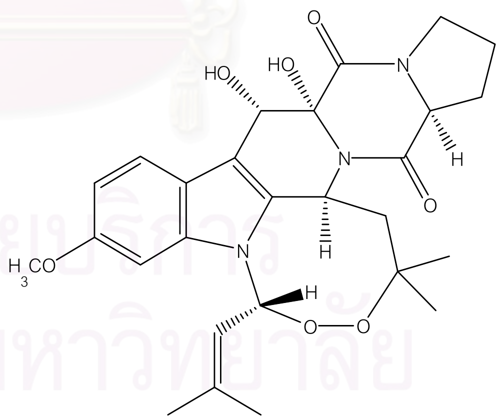
[10] Isopestacin



[11] Preaustinoid A

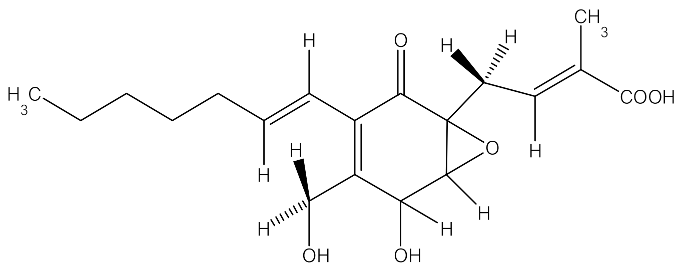


[12] Preaustinoid B

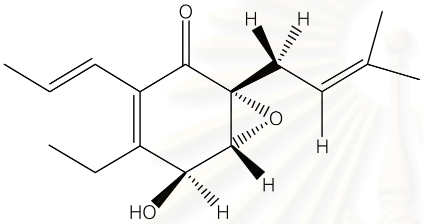


[13] Alkaloid verruculogen

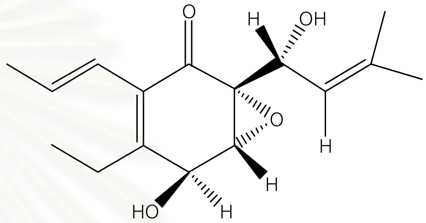
Figure A (continued)



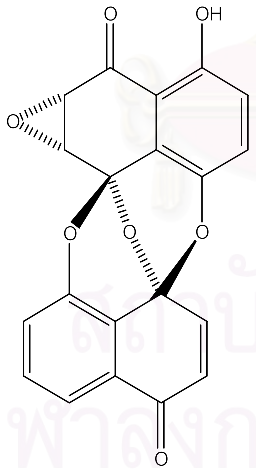
[14] Ambuic acid



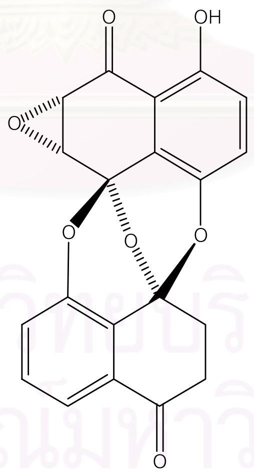
[15] Jesterone



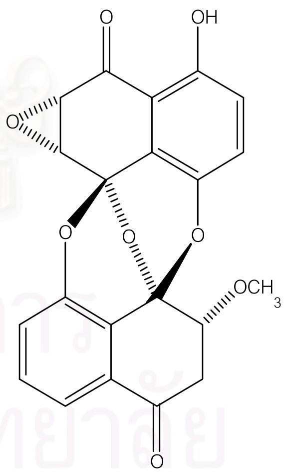
[16] Hydroxy-jesterone



[17] Preussomerin G

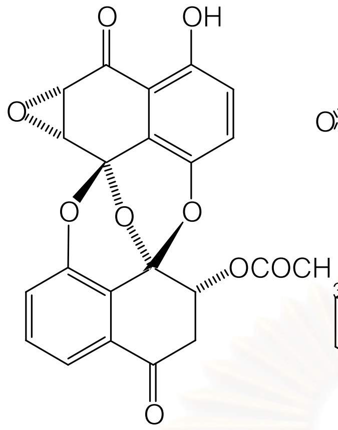


[18] Preussomerin H

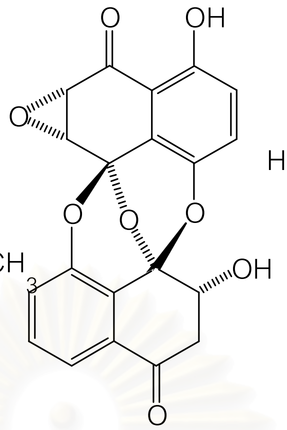


[19] Preussomerin I

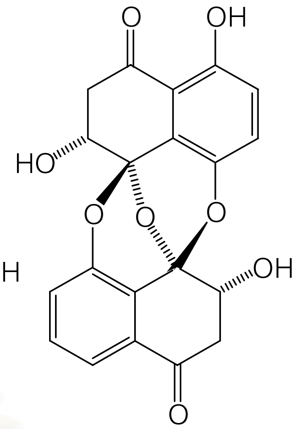
Figure A (continued)



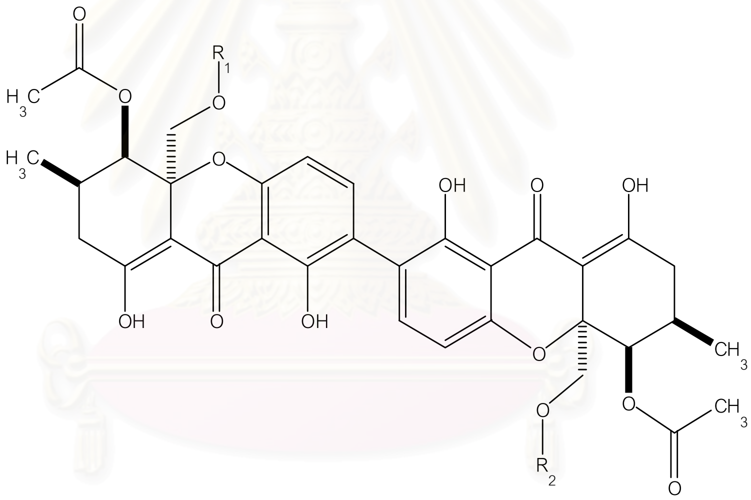
[20] Preussomerin J



[21] Preussomerin K



[22] Preussomerin L

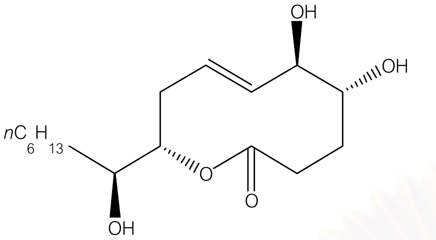


[23] Dicerandrol A, R1=R2=H

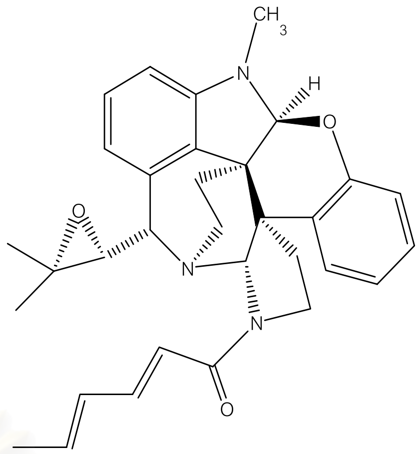
[24] Dicerandrol B, R1=Ac, R2=H

[25] Dicerandrol C, R1=R2=Ac

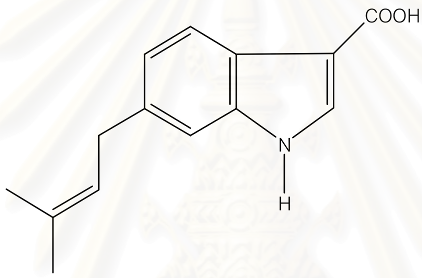
Figure A (continued)



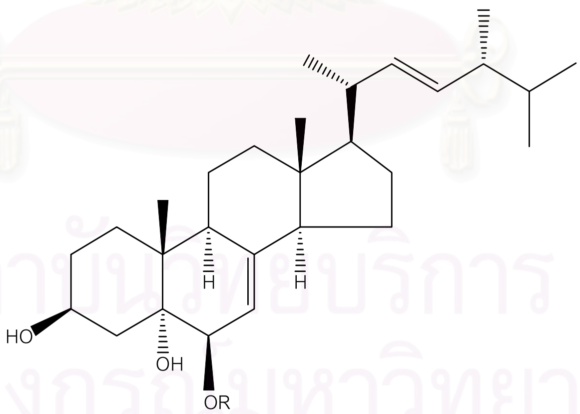
[26] Microcarpalide



[27] Nomofungin



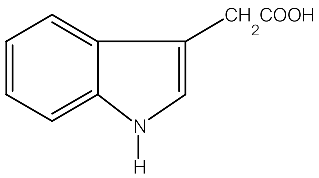
[28] Isoprenylindole-3-carboxylic acid



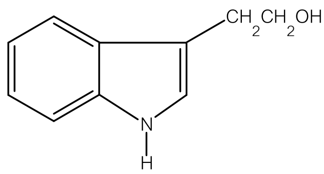
[29] 3beta,5alpha-Dihydroxy-6beta-acetoxy-ergosta-7,22-diene, R=$COCH_3$

[30] 3beta,5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7,22-diene, R=$COCH_2C_6H_5$

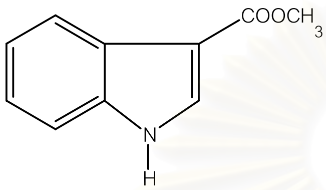
Figure A (continued)



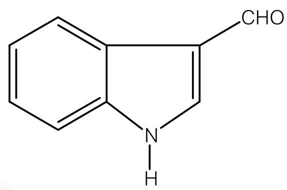
[31] Indole-3-acetic acid (IAA)



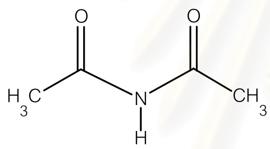
[32] Indole-3-ethanol (IEtOH)



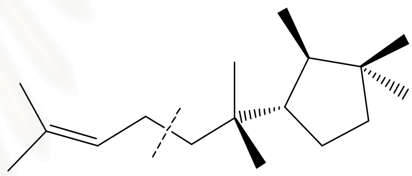
[33] Methylindole-3-carboxylate



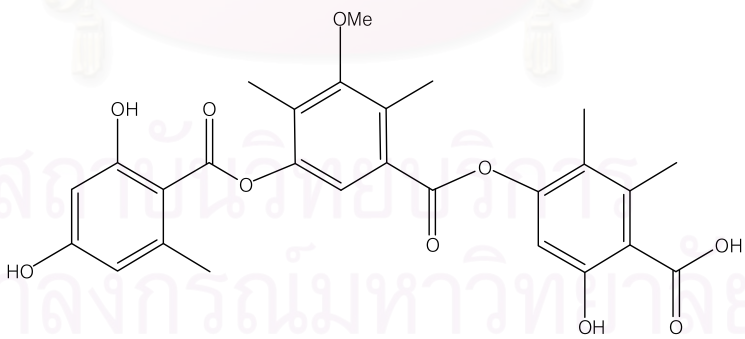
[34] Indole-3-carboxaldehyde



[35] Diacetamide

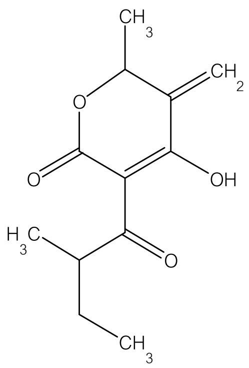


[36] Cyclonerodiol

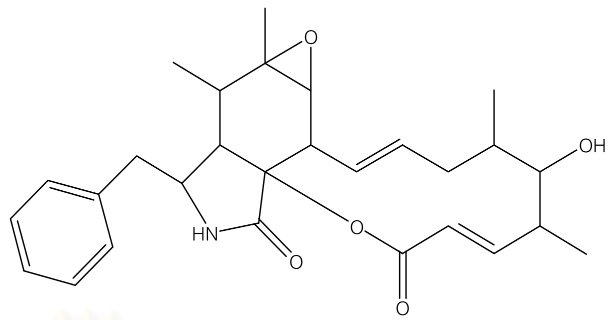


[37] Colletotric acid

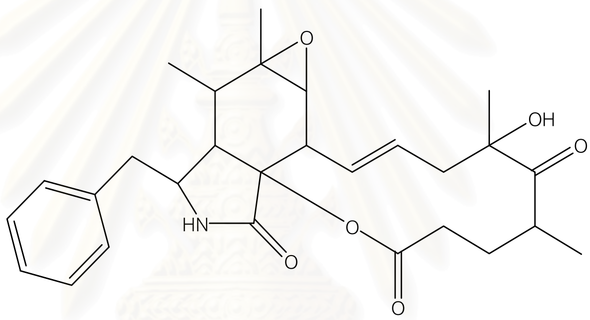
Figure A (continued)



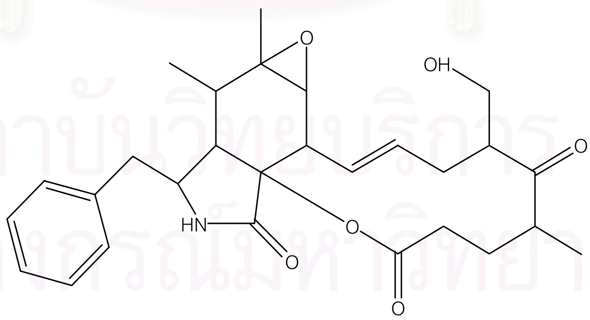
[38] CR377, pentaketide



[39] Cytochalasin 1

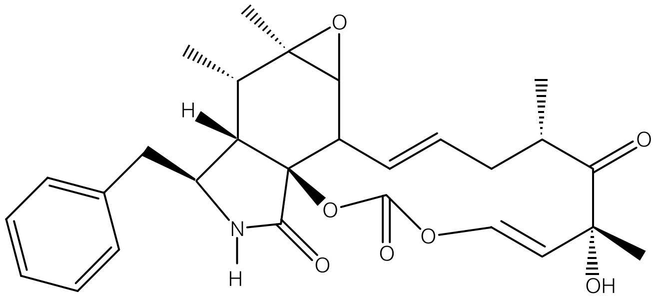


[40] Cytochalasin 2

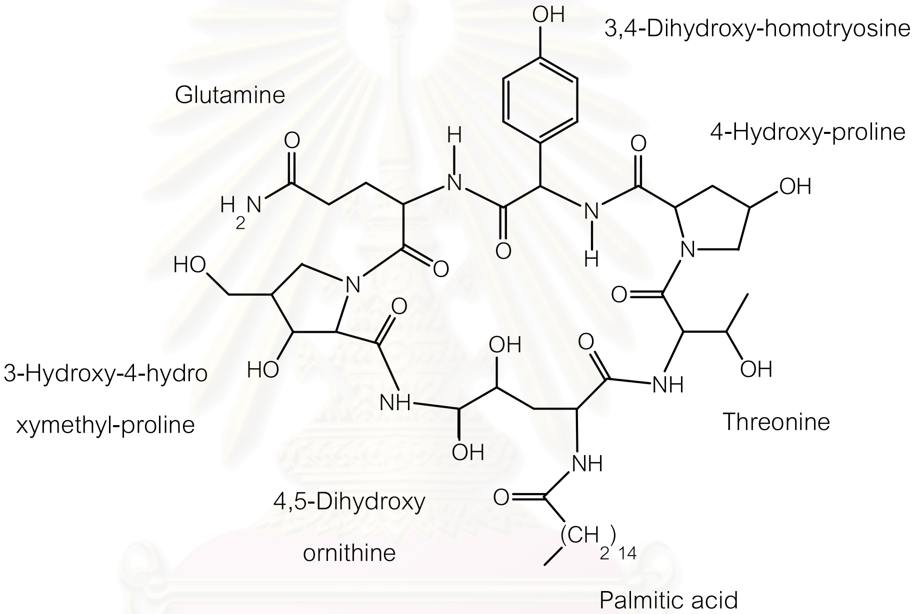


[41] Cytochalasin 3

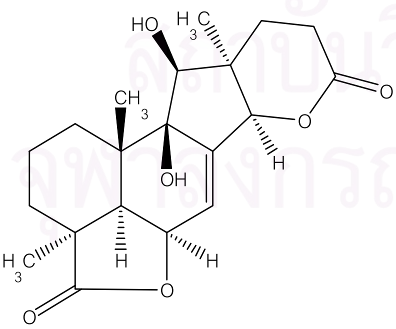
Figure A (continued)

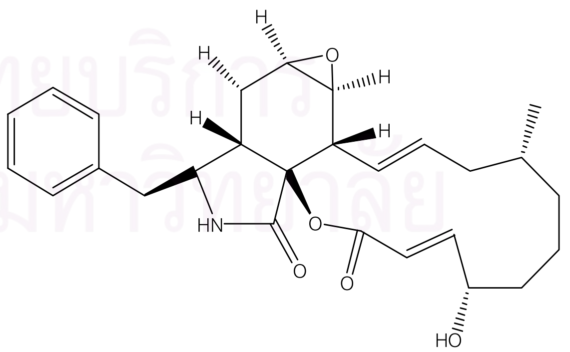


[42] Cytochanlasin E



[43] Cryptocandin

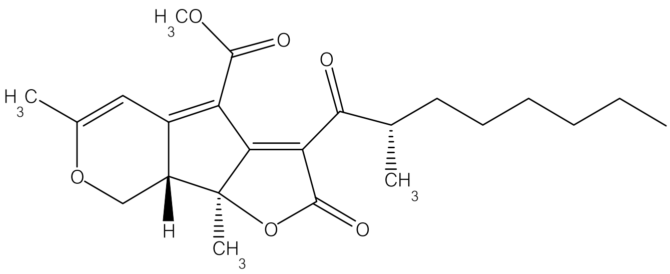




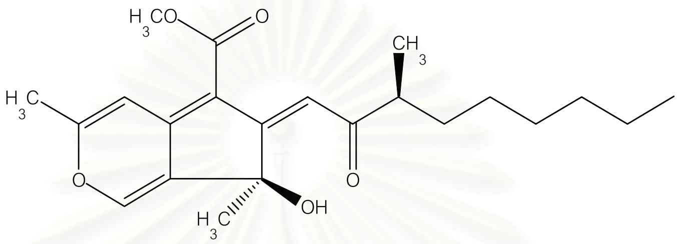
[44] Geniculol

[45] Cytochalasin F

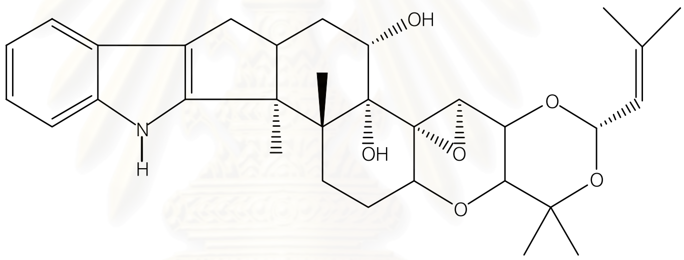
Figure A (continued)



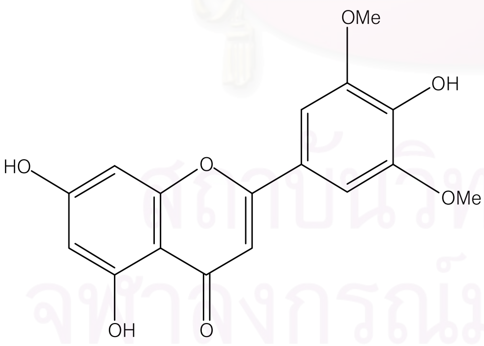
[46] Sequoiatone A



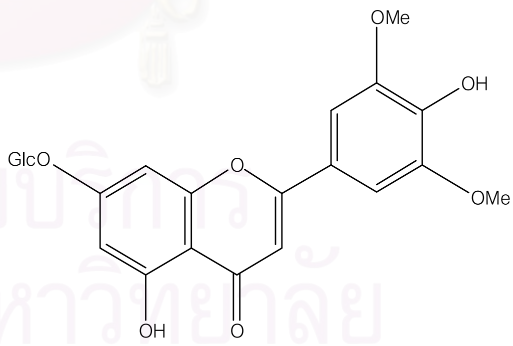
[47] Sequoiatone B



[48] Terpendole M

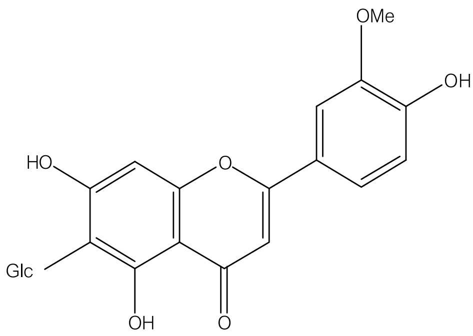


[49] Tricin

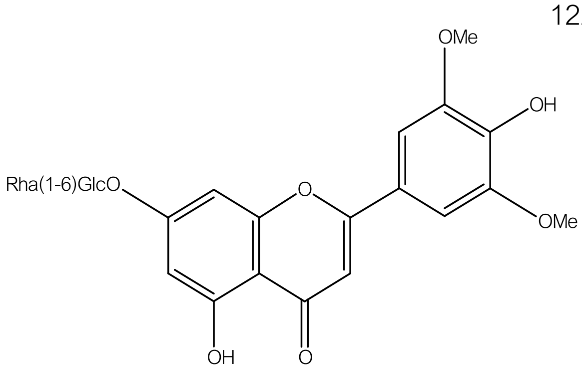


[50] 7-O-(B-D-glucopyranosyl)tricin

Figure A (continued)

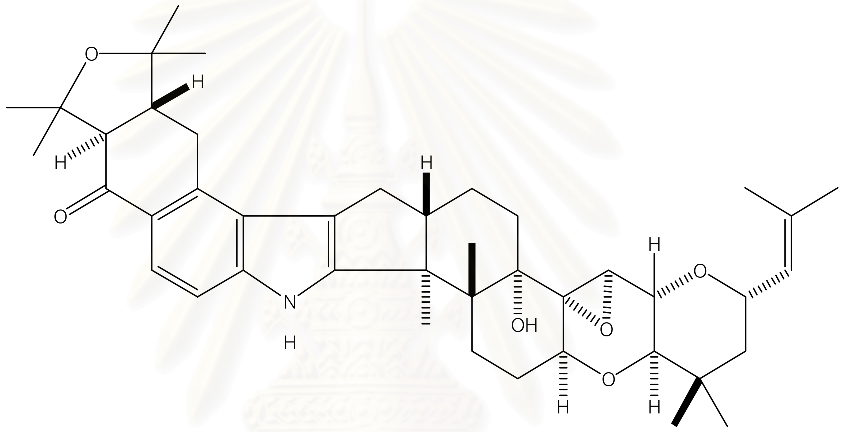


[51] Isoorientin

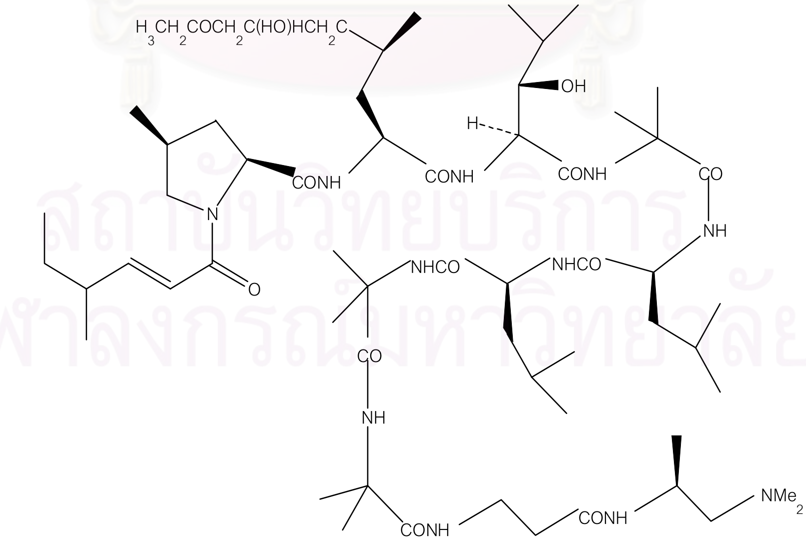


[52] 7-**o**-[α-L-Rhamnopyranosyl(1-6)-

β-D-glucopy-ranosyl]tricin

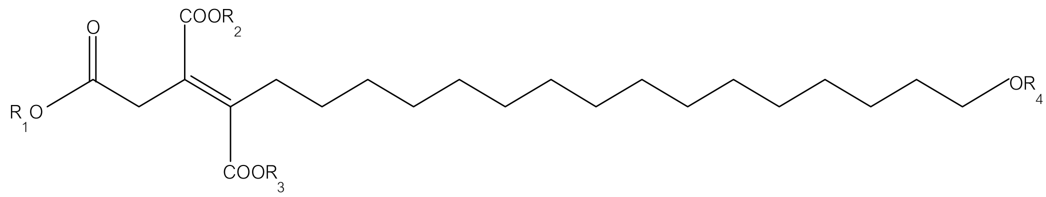


[53] Lolitrem B



[54] Leucinostatin A

Figure A (continued)

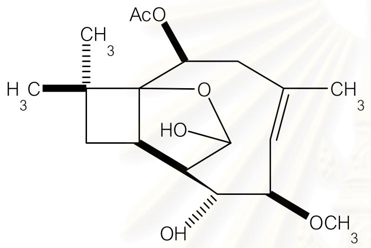


[55] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H

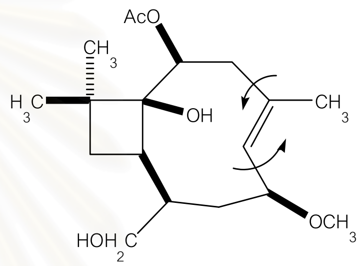
[56] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H

[57] 3: Desulfated analog, R1=R2=R3=CH3, R4=H

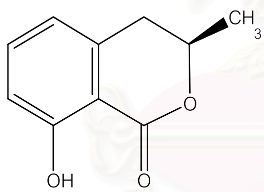
[58] 4: Desulfated analog, R1=R2=R3=R4=H



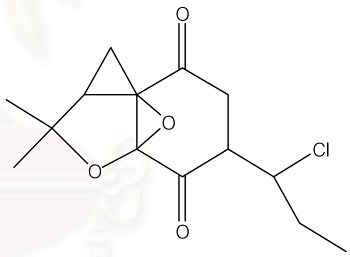
[59] Pestalotiopsin A



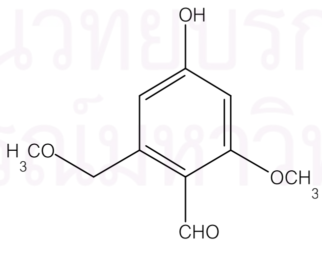
[60] Pestalotiopsin B



[61] (R)-mellein

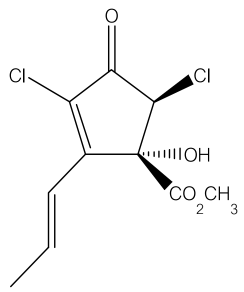


[62] (-)-mycorrhizin A

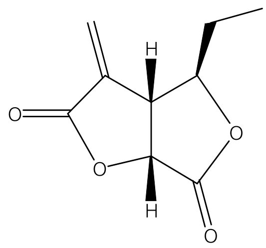


[63] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

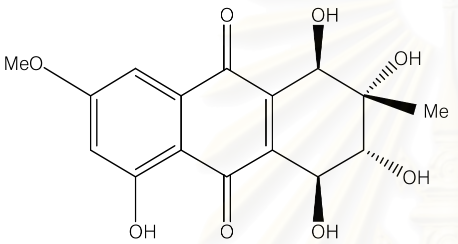
Figure A (continued)



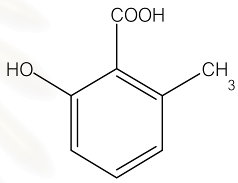
[64] (+)-cryptosporiopsin



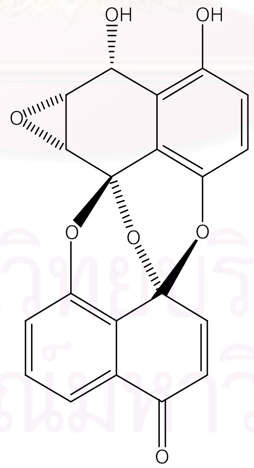
[65] 4-epi-ethiosolide



[66] Altersolanol A

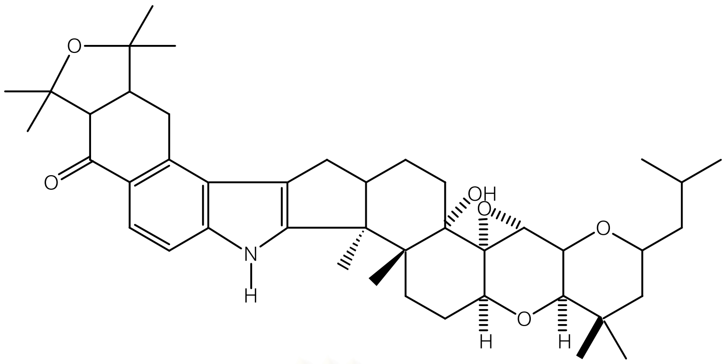


[67] 2-hydroxy-6-methyl benzoic acid

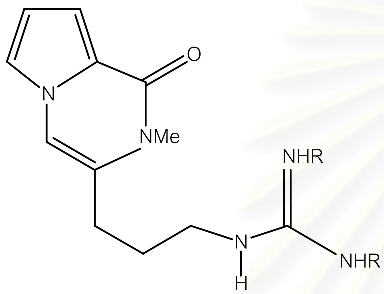


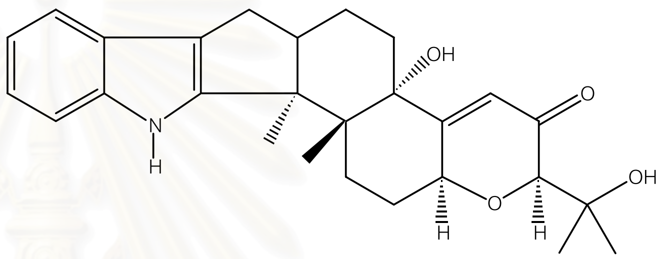
[68] Preussomerin D

Figure A (continued)



[69] Lolitrem C

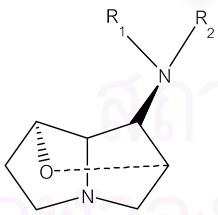


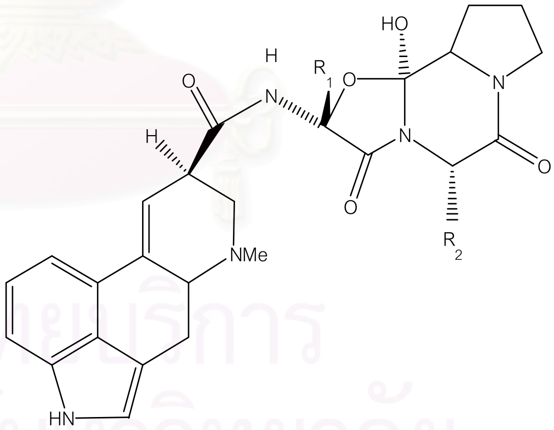


[70] Peramine R=H

[72] Paxilline

[71] Diacetylperamine R=Ac





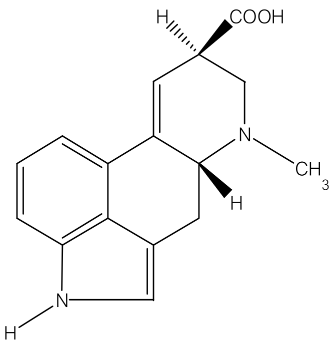
[73] Loline alkaloid, R_1=H, Me,

R_2= H, HCO, Ac

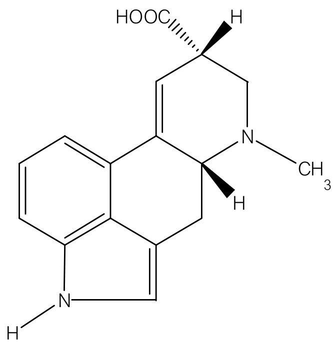
[74] Ergopeptine alkaloids

Ergovaline R_1=Me, R_2=i-Pr

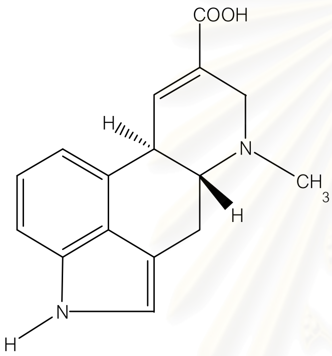
Figure A (continued)



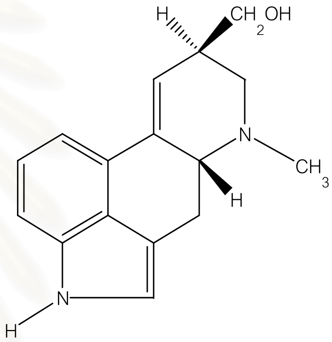
[75] Lysergic acid



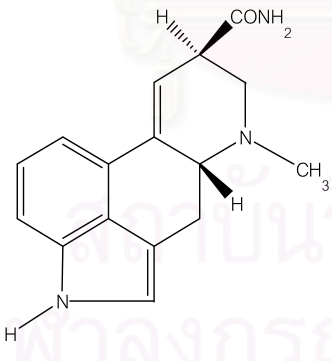
[76] Isolysergic acid



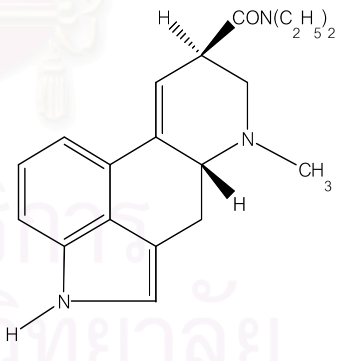
[77] Paspalic acid



[78] Lysergol

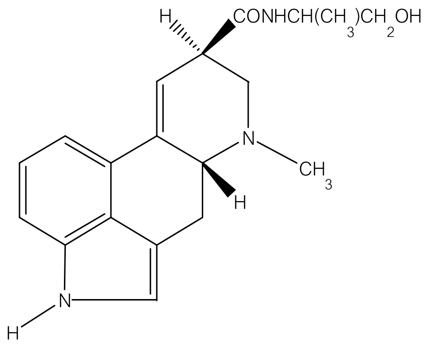


[79] Lysergic acid amide



[80] Lysergic acid diethyl amide

Figure A (continued)



[81] Lysergic acid-2-propanolamide (Ergonovine)

Figure A (continued)

APPENDIX B

1. Media

1.1 Yeast Extract Sucrose Agar (YEA)

Yeast extract	20 g
Sucrose	150 g
Distilled water up to	1 L

Composition of Yeast Extract Sucrose (YES) is similar to YEA but not supplemented with agar.

1.2 Malt Czapek Broth (MCz)

Czapek stock solution A	50 ml
Czapek stock solution B	50 ml
Sucrose	30 g
Malt Extract	40 g
Distilled water up to	1 L

Czapek stock solution A

$NaNO_3$	4.0 g
KCL	1.0 g
$MgSO_4.7H_2O$	1.0 g
$FeSO_4.7H_2O$	0.02 g
Dissolved in distilled water up to	100 ml

Keep in a refrigerator.

Czapek stock solution B

K_2HPO_4	2.0 g
A solution	1.0 g
B solution	1.0 g

Dissolved in distilled water up to	100 ml
Keep in a refrigerator	

A solution

$ZnSO_4.7H_2O$	1.0 g
Dissolved in distilled water up to	100 ml

B solution

$CuSO_4.5H_2O$	1.0 g
Dissolved in distilled water up to	100 ml

1.3 Sabouraud's Dextrose Agar (SDA)

Dextrose	40 g
Neopeptone	10 g
Distilled water up to	1 L

Composition of Sabouraud's Dextrose Broth (SDB) is similar to SDA but not supplemented with agar.

1.4 Potato Dextrose Agar (PDA)

Potato	200 g
Dextrose	20 g
Distilled water up to	1 L

Composition of Potato Dextrose Agar (PDB) is similar to PDA but not supplemented with agar.

1.5 Yeast Czapek Broth (Ycz)

Czapek solution agar	49.0 g
Yeast extract	4.9 g
Distilled water up to	1 L

1.6 Malt Extract Sucrose Broth (MES)

Yeast extract	20 g
Sucrose	200 g
Distilled water up to	1 L

1.7 Malt Extract Agar (MEA)

Malt extract	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Distilled water up to	1 L

Composition of Malt Extract Agar (MEB) is similar to MEA but not supplemented with agar.

1.8 MID Medium (Pinkerton and Strobel, 1976)

$Ca(NO_3)_2$	1.2 mM
KNO_3	0.79 mM
KCl	0.87 mM
$MgSO_4$	3.0 mM
$NaH_2PO_4.H_2O$	0.007mM
$FeCl_3$	0.0074 mM
$MnSO_4$	0.03 mM
$ZnSO_4.H_2O$	0.0087 mM
H_3BO_3	0.0022 mM
KI	0.0045 mM
Sucrose	87.6 mM
Ammonium Tartrate	27.1 mM
Yeast Extract	0.5 g
Soytone	1.0 g
Distilled water up to	1 L

pH = 5.5 with 1 N HCl

1.9 Water Agar

Agar	15 g
Distilled water up to	1 L

1.10 Corn Meal Agar

Corn meal	30 g
Agar	15 g
Distilled water up to	1 L

2. Reagent and buffer for DNA amplification by PCR.

2.1 Lysis buffer

Tris-HCl (pH 7.2)	50 mM
EDTA	50 mM
SDS	3%
2-mercaptoethanol	1%

2.2 Chlorofrom : TE-saturated phenol 1:1,v/v

2.3 TE for resuspending pellet

Tris-HCl	10 mM
EDTA	0.1 mM

2.4 Gel loading buffer

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

Store temperature at 4^0C

2.5 5-X Tris-Borate-EDTA (TBE)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20 ml

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

6.6 10X Buffer

Tris HCl pH 9.0	100 ml
KCL	500 mM
Triton X-100	1%

6.7 2mM dNTP (dATP, dCTP, dGTP, dTTP mix)

dATP	100 mM
dCTP	100 mM
dGTP	100 mM
dTTP	100 mM

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

APPENDIX C

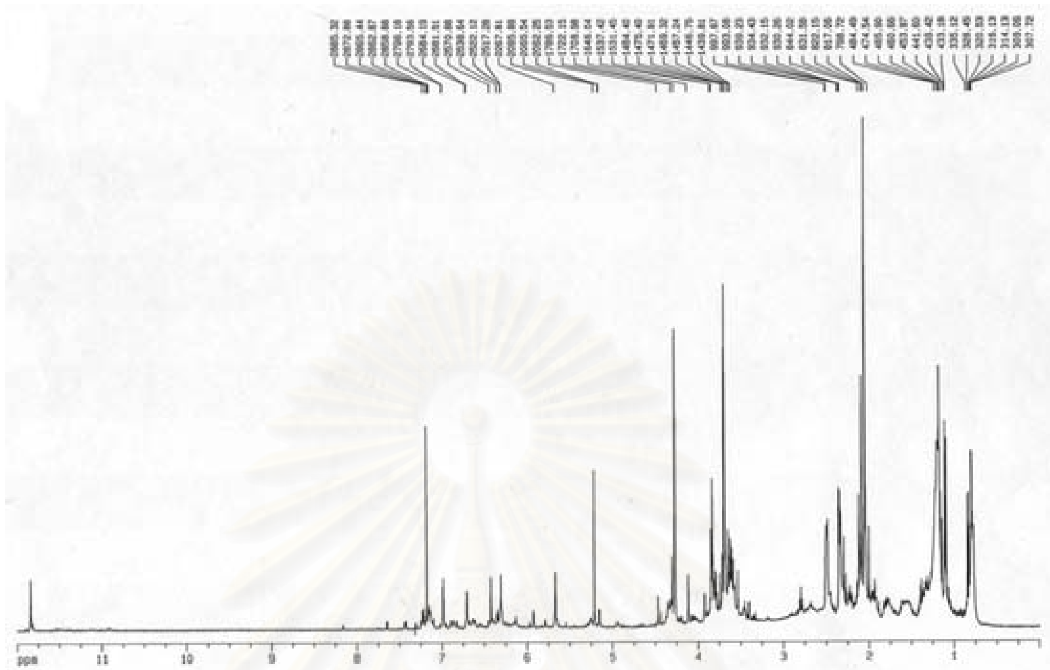


Figure C1 The 400 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of crude extract L20B of endophytic fungus isolate LRUB 20

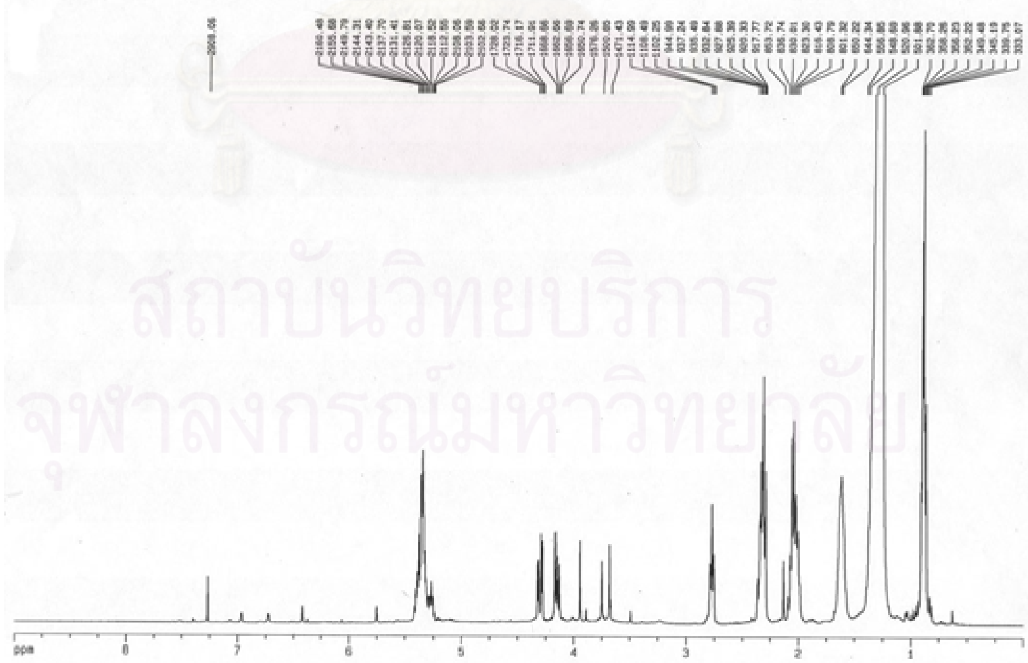


Figure C2 The 400 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of mycelia extract L20C of endophytic fungus isolate LRUB 20

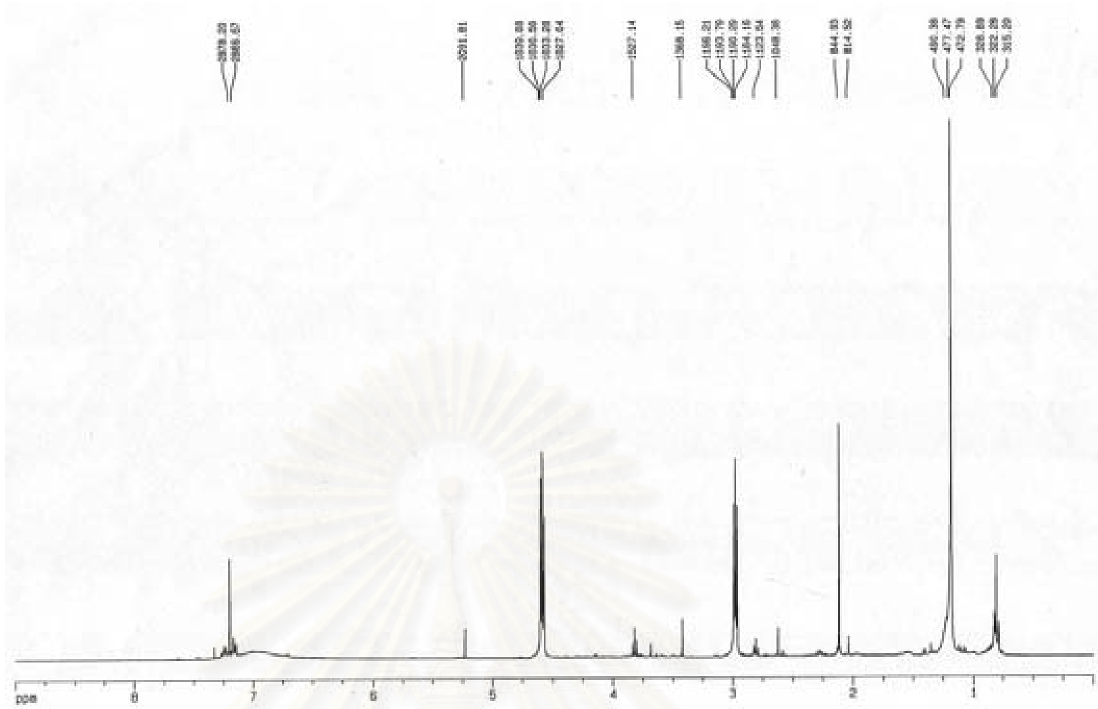


Figure C3 The 400 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of crude extract U5B of endophytic fungus isolate USIA 5

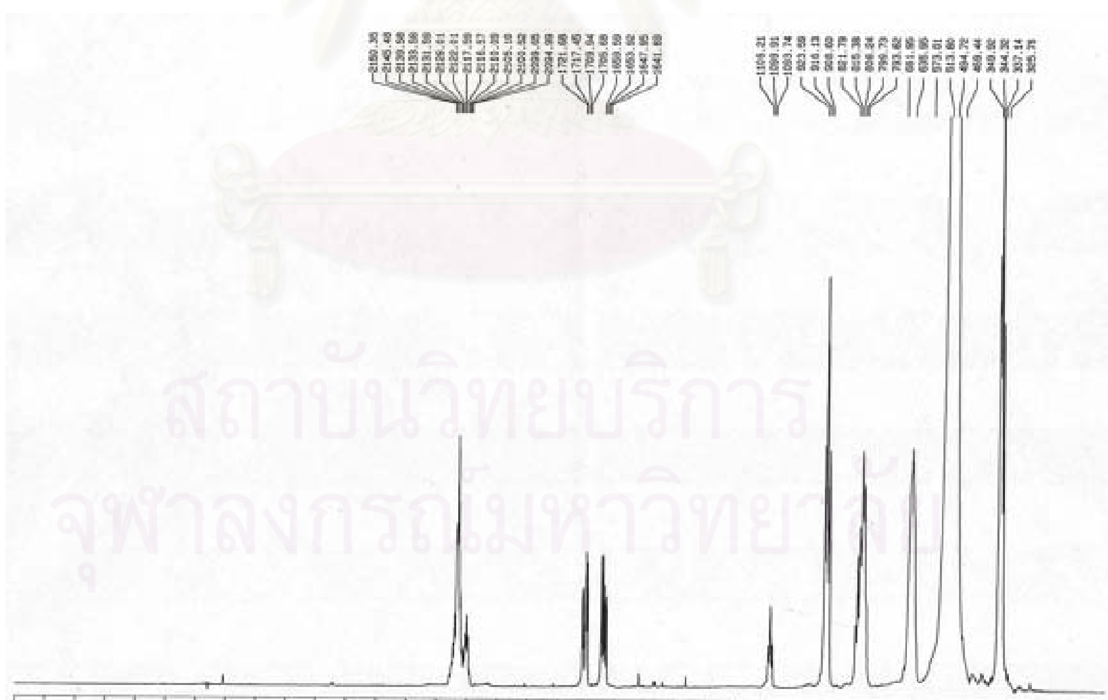


Figure C4 The 400 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of mycelia extract U5C of endophytic fungus isolate USIA 5

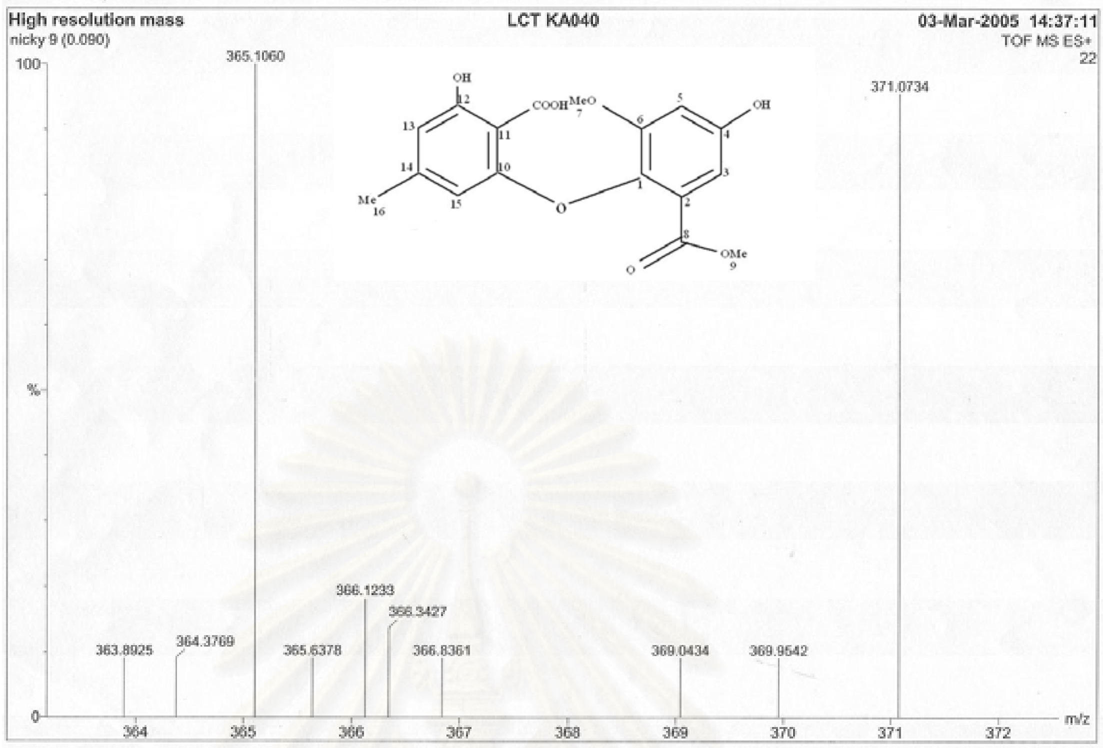


Figure C5 The ESI-TOF spectrum of compound L20B7

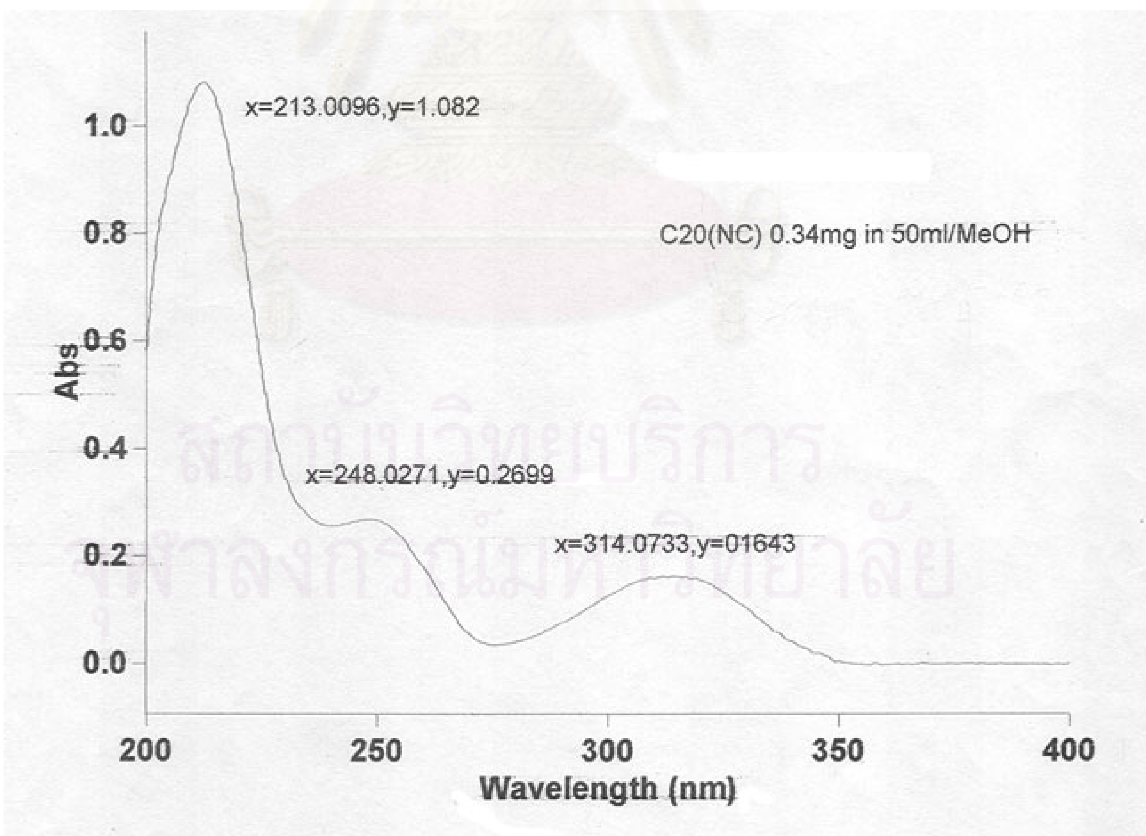


Figure C6 The UV spectrum of compound L20B7 in methanol

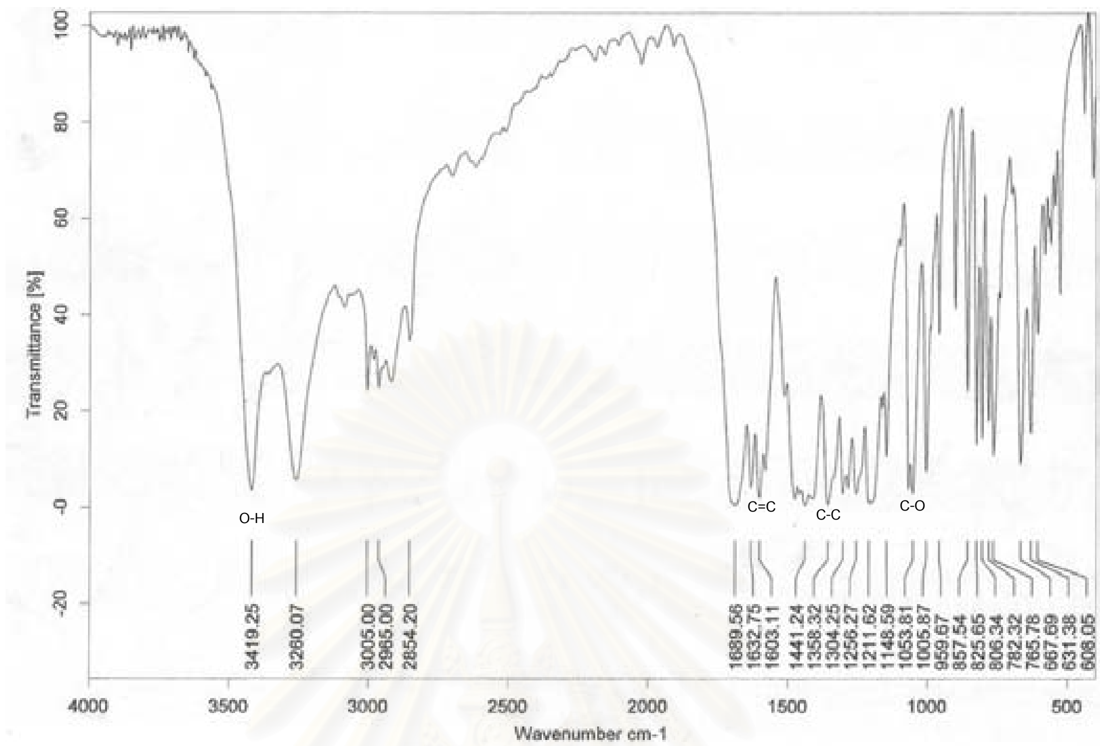


Figure C7 The IR spectrum of compound L20B7

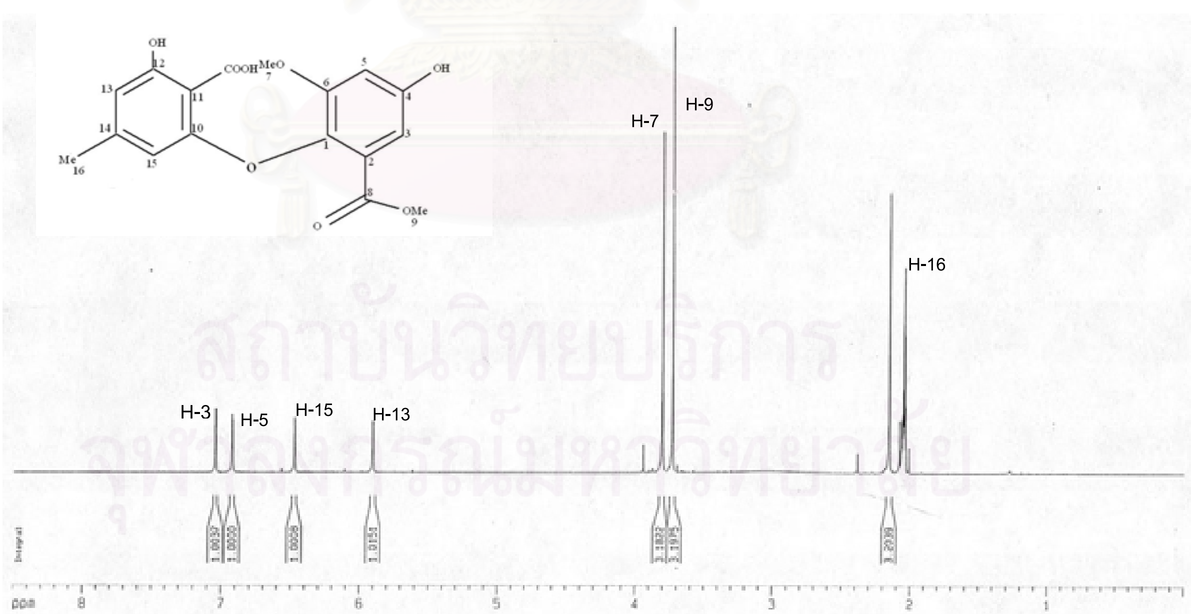


Figure C8 The 500 MHz ^{1}H-NMR (in acetone–*d*6) spectrum of compound L20B7

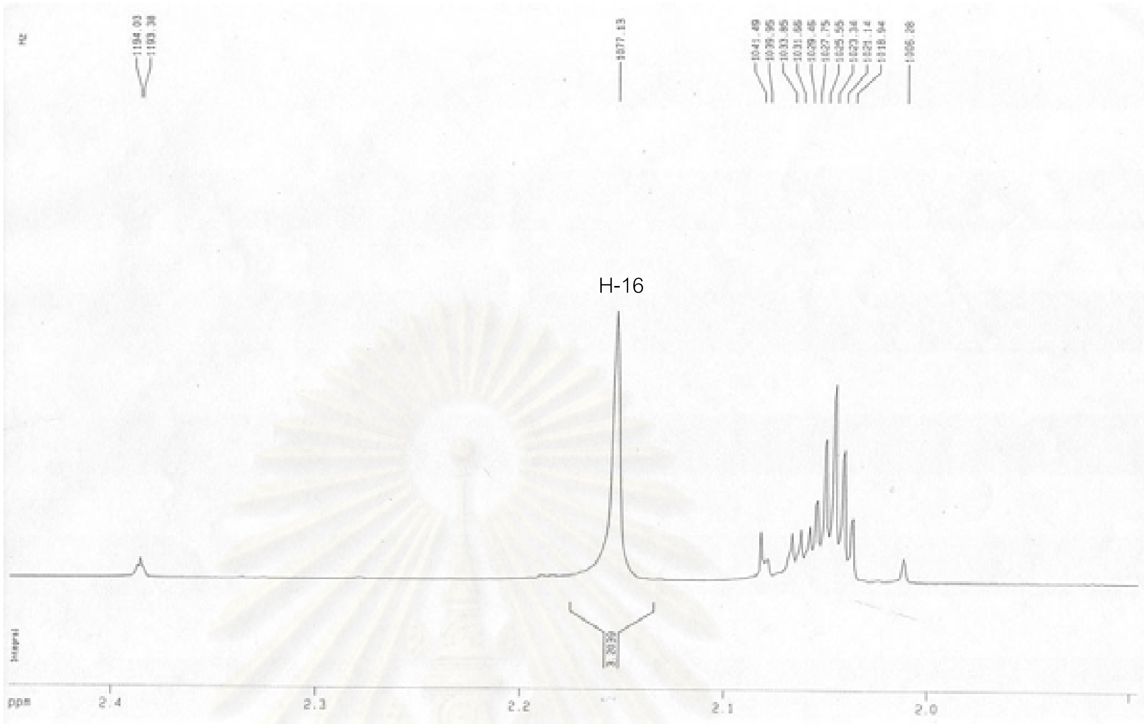


Figure C9 Expansion 500 MHz ^{1}H-NMR (in acetone – *d*6) spectrum of compound L20B7 (δ = 0-2.4 ppm)

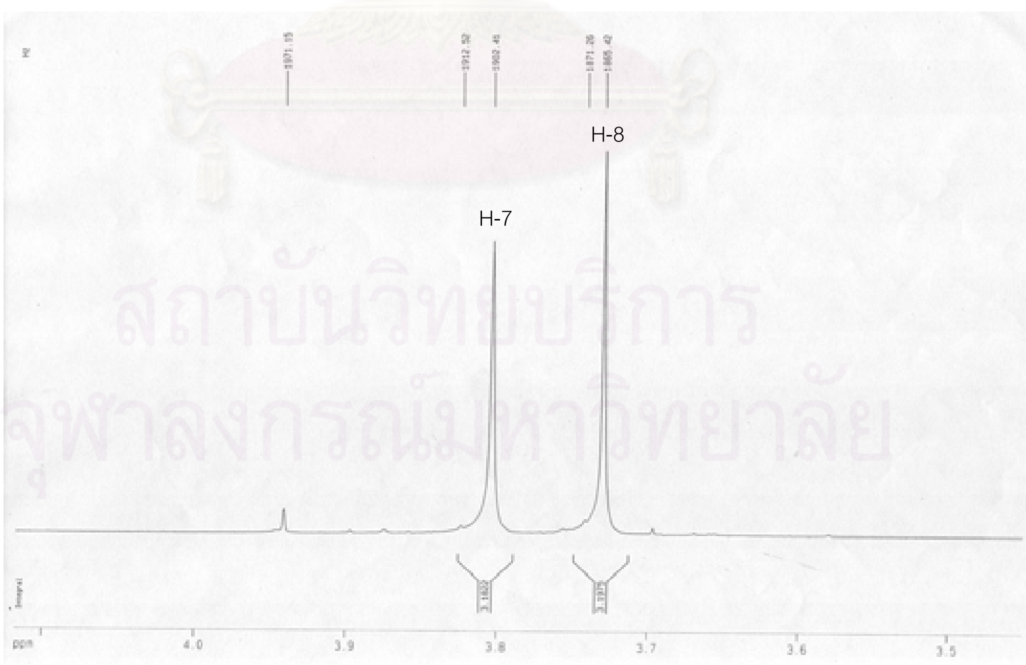


Figure C10 Expansion 500 MHz ^{1}H-NMR (in acetone – *d*6) spectrum of compound L20B7 (δ = 3.5-4.0 ppm)

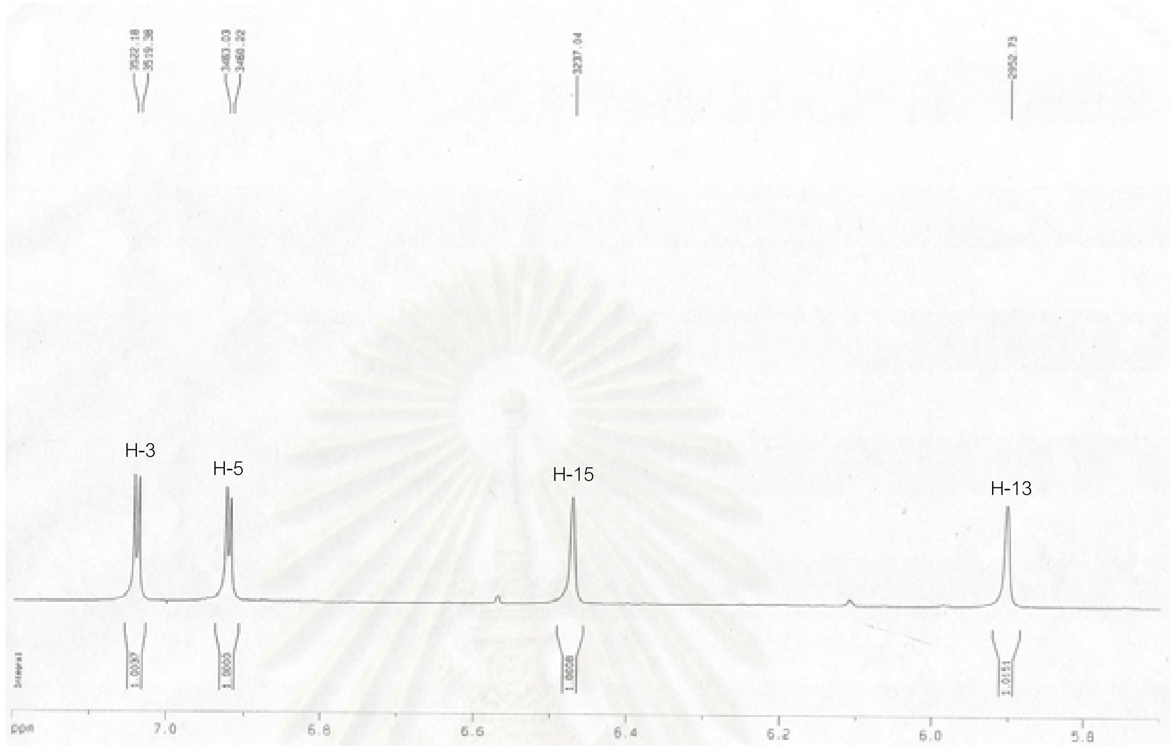


Figure C11 Expansion 500 MHz [1]H-NMR (in acetone – *d*6) spectrum of compound L20B7 (δ = 5.7-7.2 ppm)

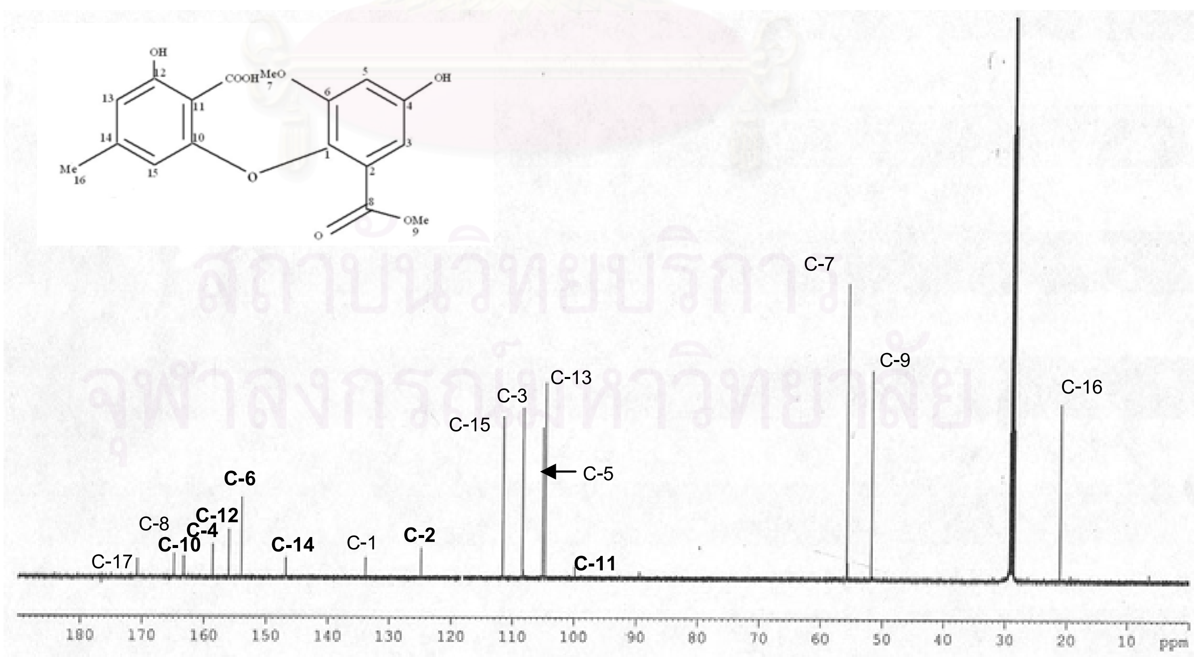


Figure C12 The 125 MHz [13]C-NMR spectrum of compound L20B7

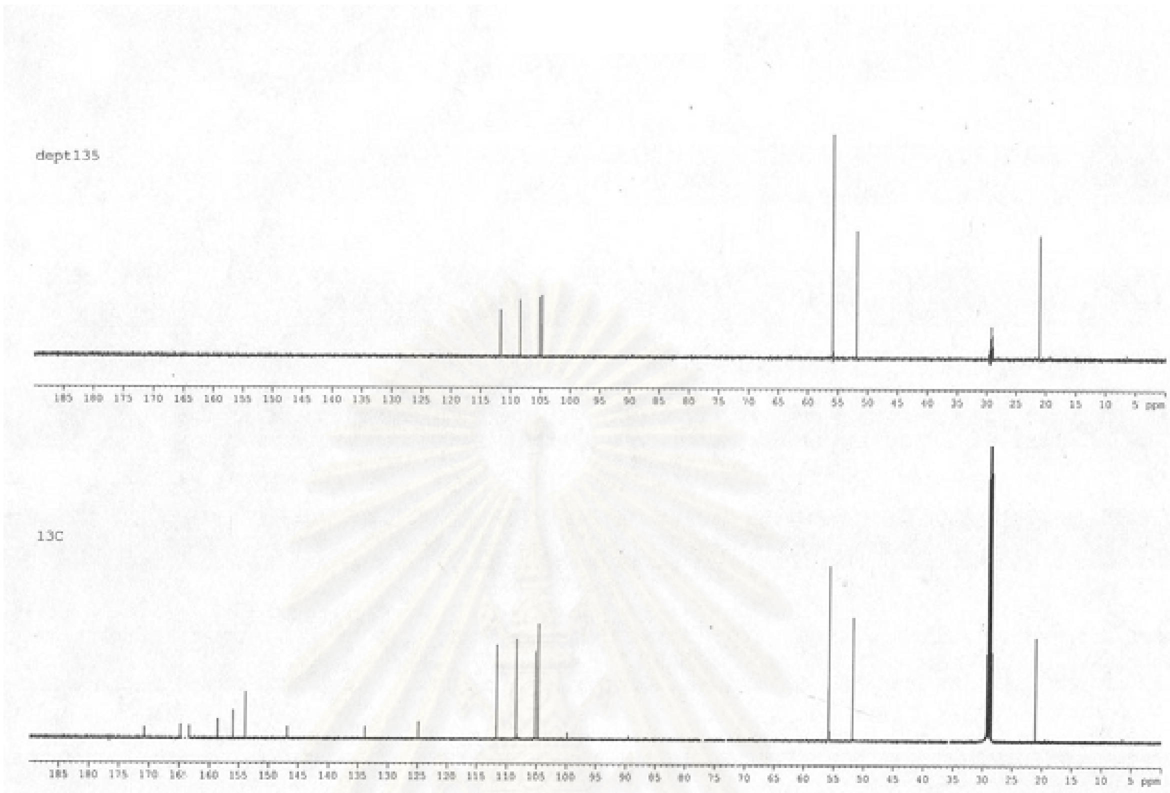


Figure C13 The DEPT 135 spectrum of compound L20B7

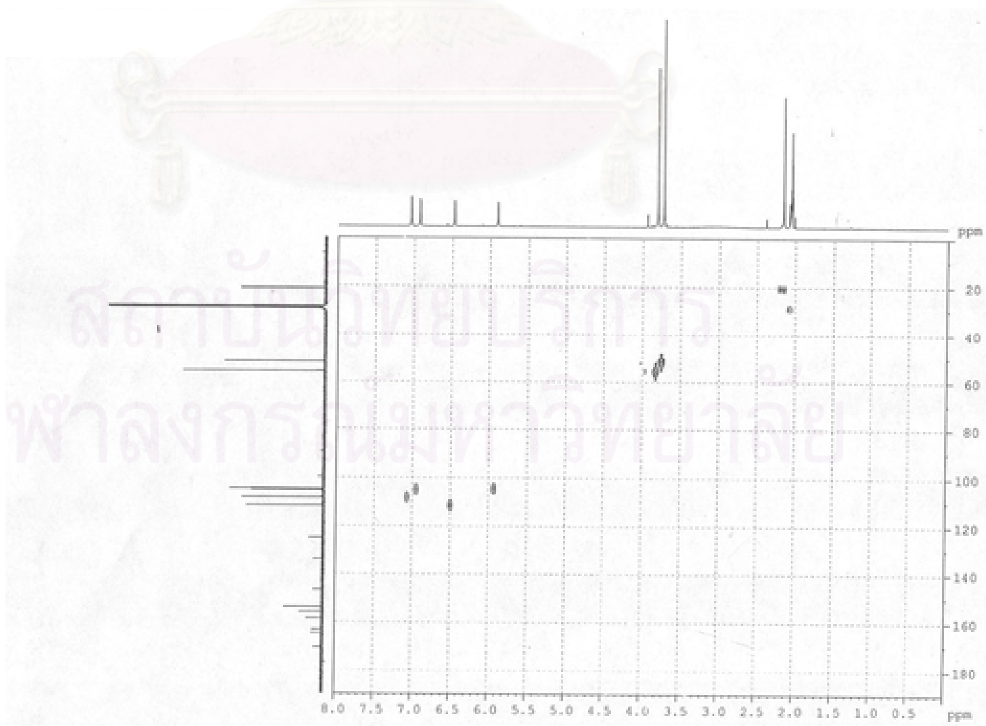


Figure C14 The HMQC spectrum of compound L20B7

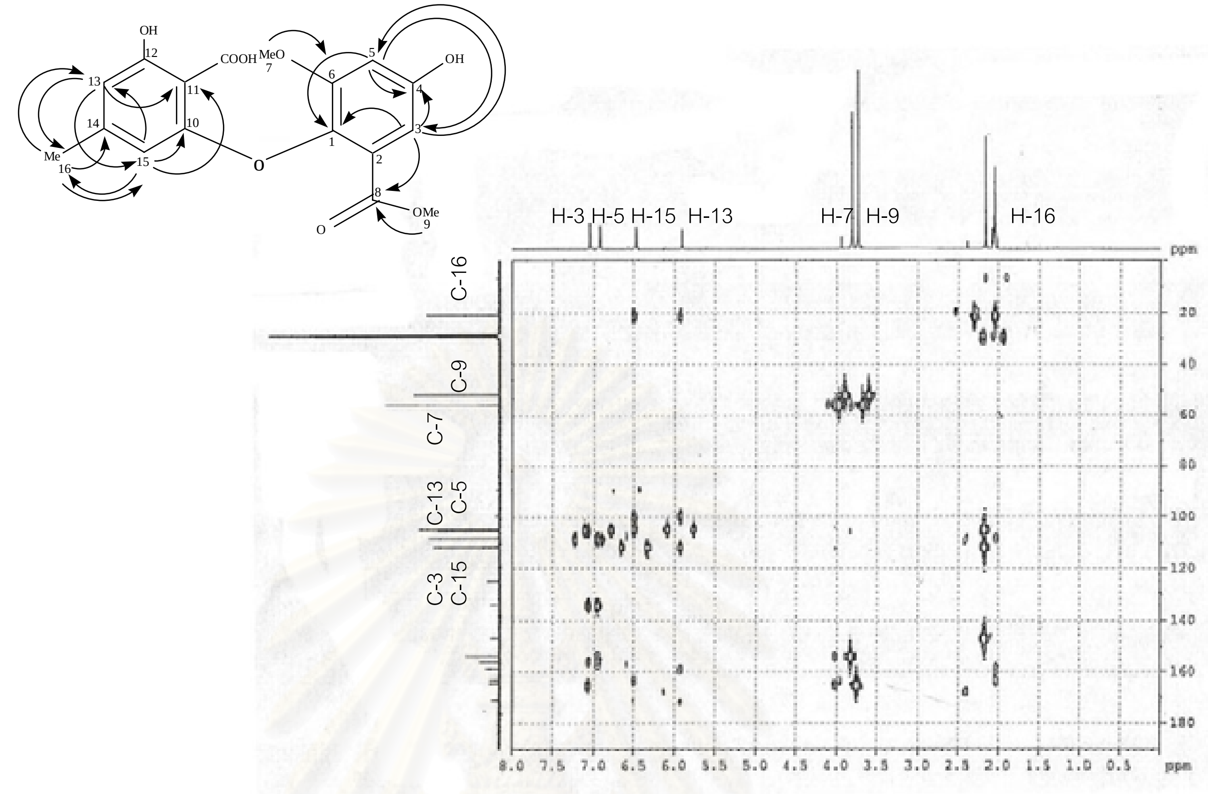


Figure C15 The HMBC spectrum of compound L20B7

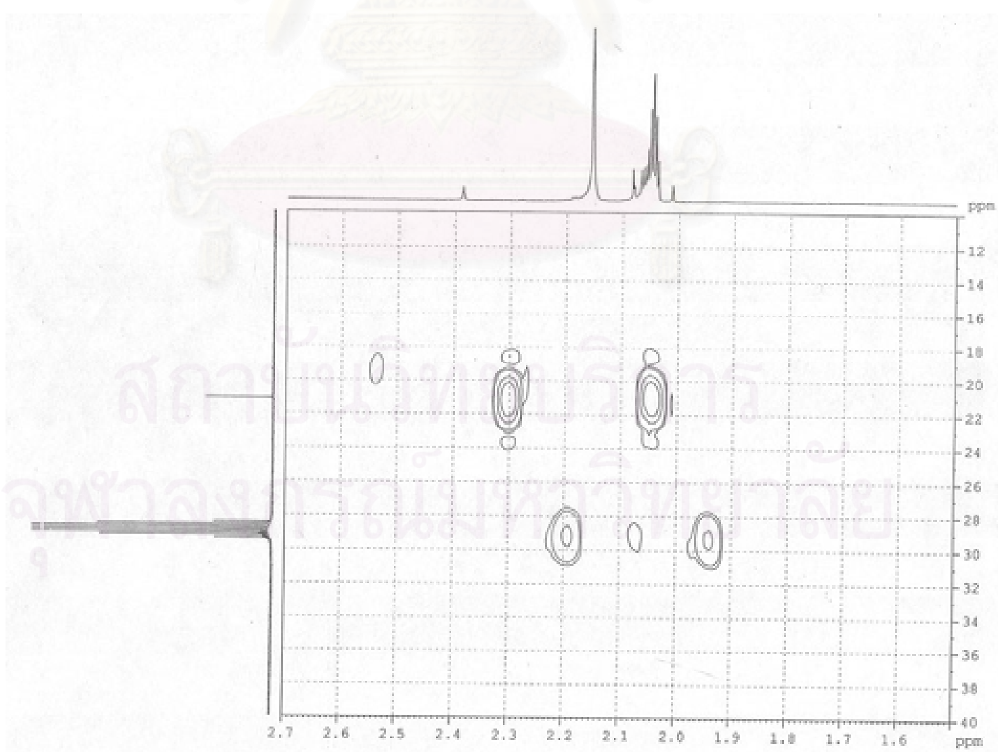


Figure C16 The HMBC spectrum of compound L20B7 (partial expanded: δH 0-2.7 ppm, δC 0-40 ppm)

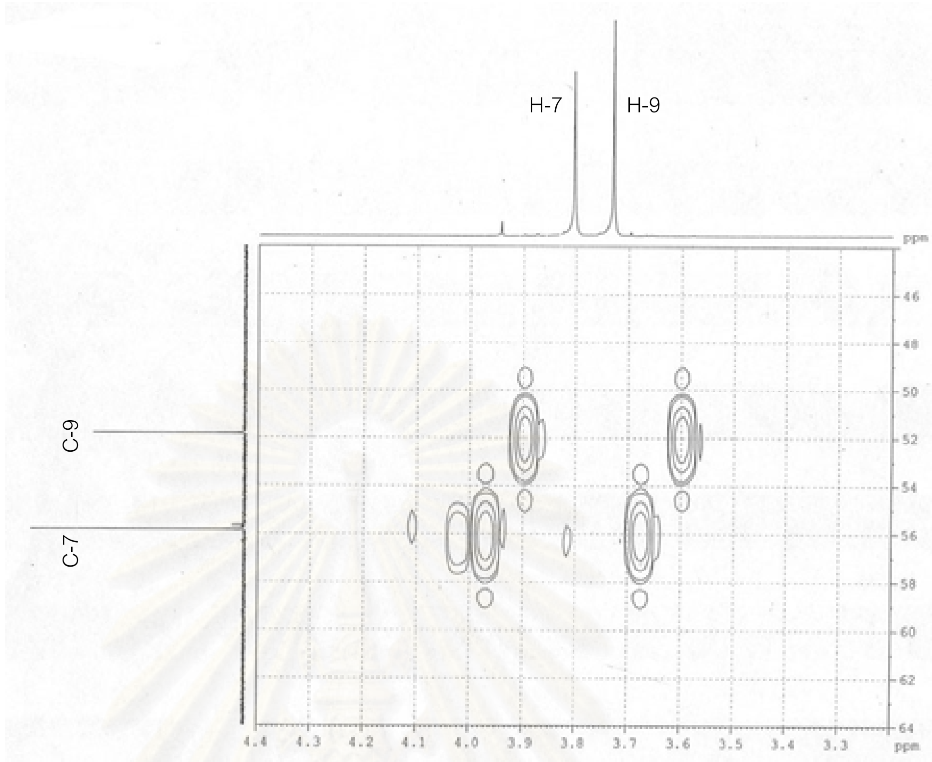


Figure C17 The HMBC spectrum of compound L20B7 (partial expanded: δH 3.2-4.4 ppm, δC 45-64 ppm)

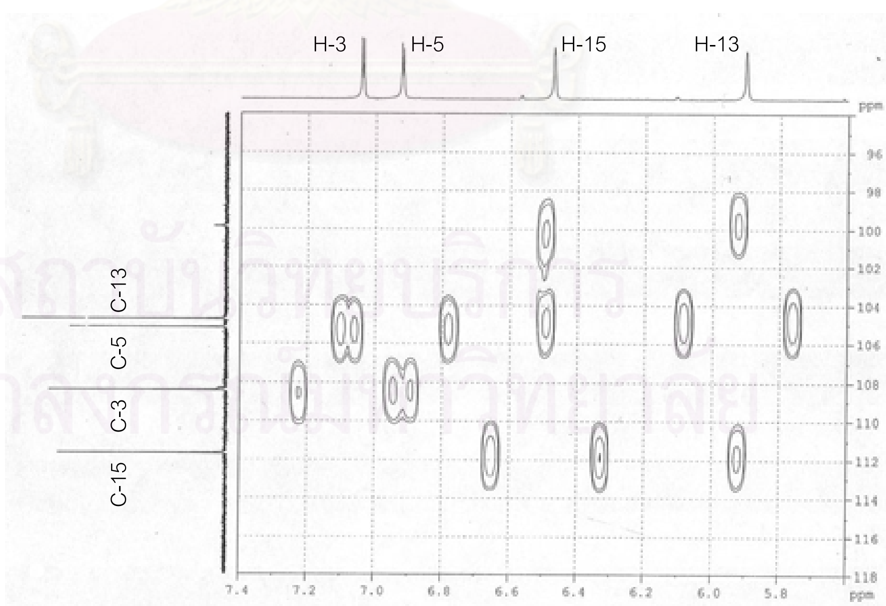


Figure C18 The HMBC spectrum of compound L20B7 (partial expanded: δH 5.6-7.4 ppm, δC 94-118 ppm)

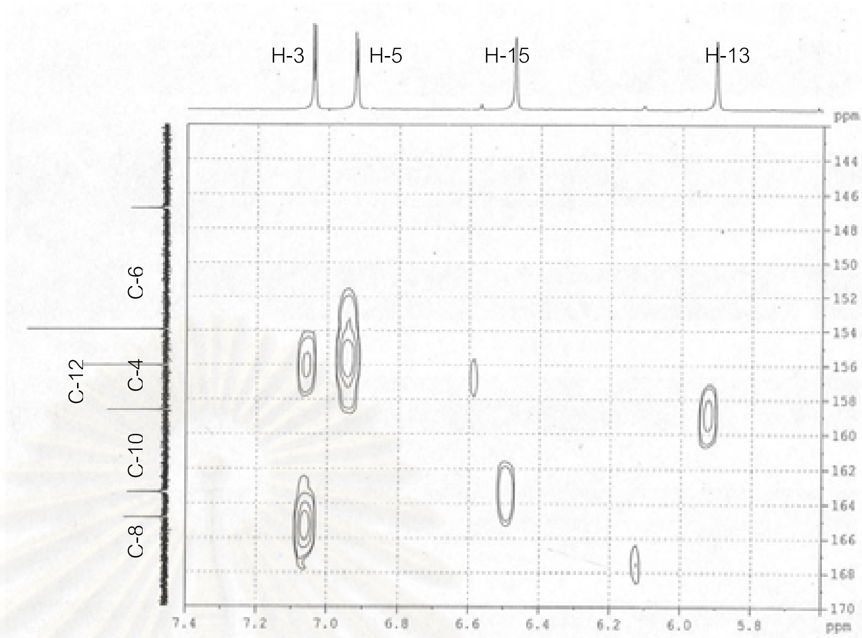


Figure C19 The HMBC spectrum of compound L20B7 (partial expanded: δH 5.6-7.4 ppm, δC 142-170 ppm)

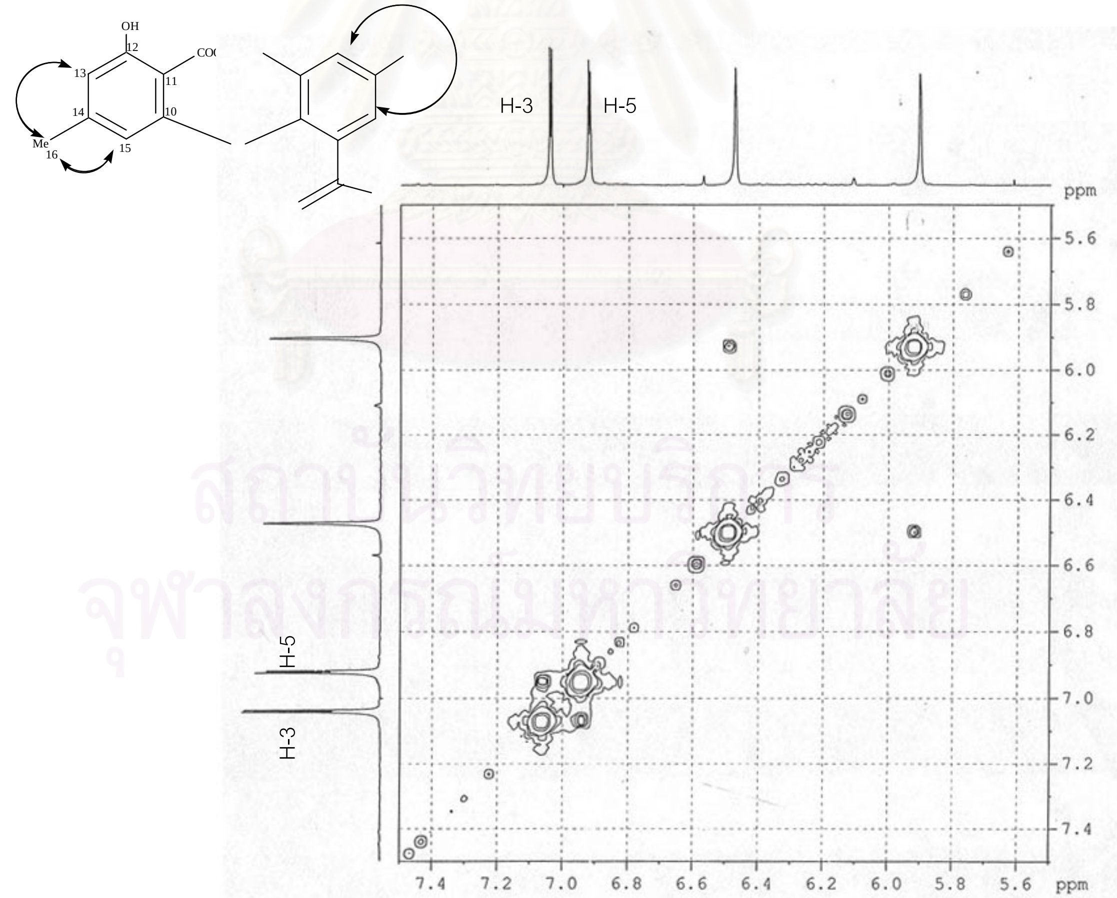


Figure C20 Expansion ^{1}H-^{1}H COSY spectrum of compound L20B7

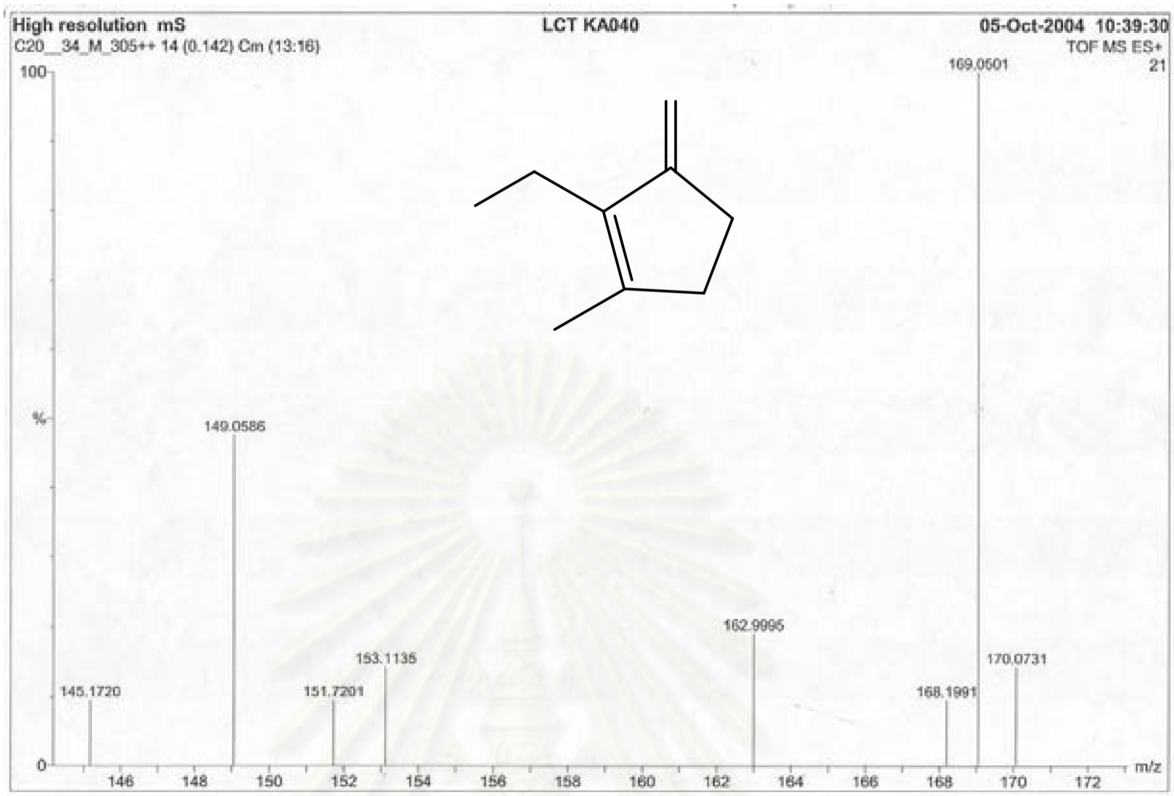


Figure C21 The ESI-TOF spectrum of compound L20B5(34)5

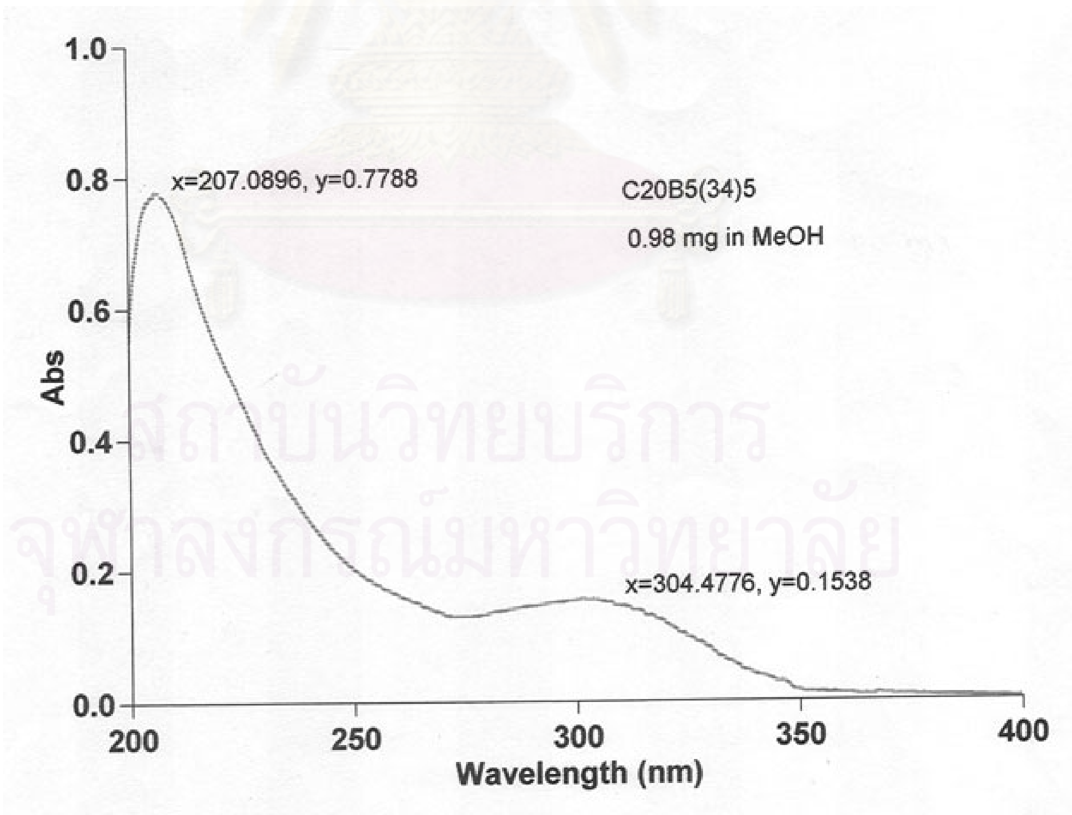


Figure C22 The UV spectrum of compound L20B5(34)5 in methanol

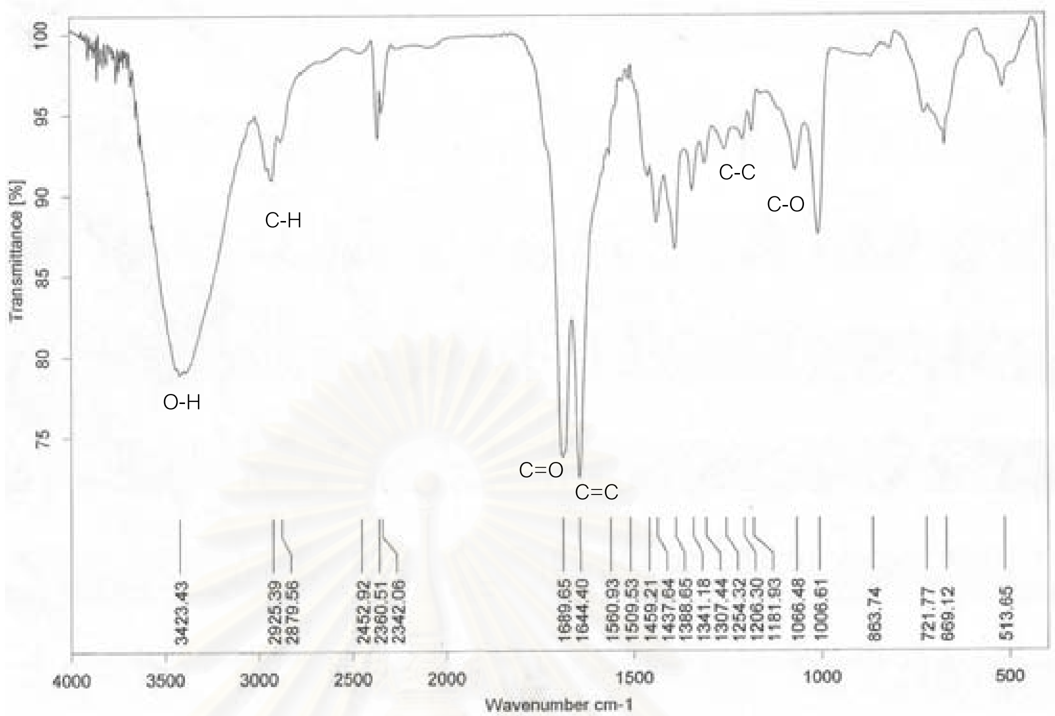


Figure C23 The IR spectrum of compound L20B5(34)5

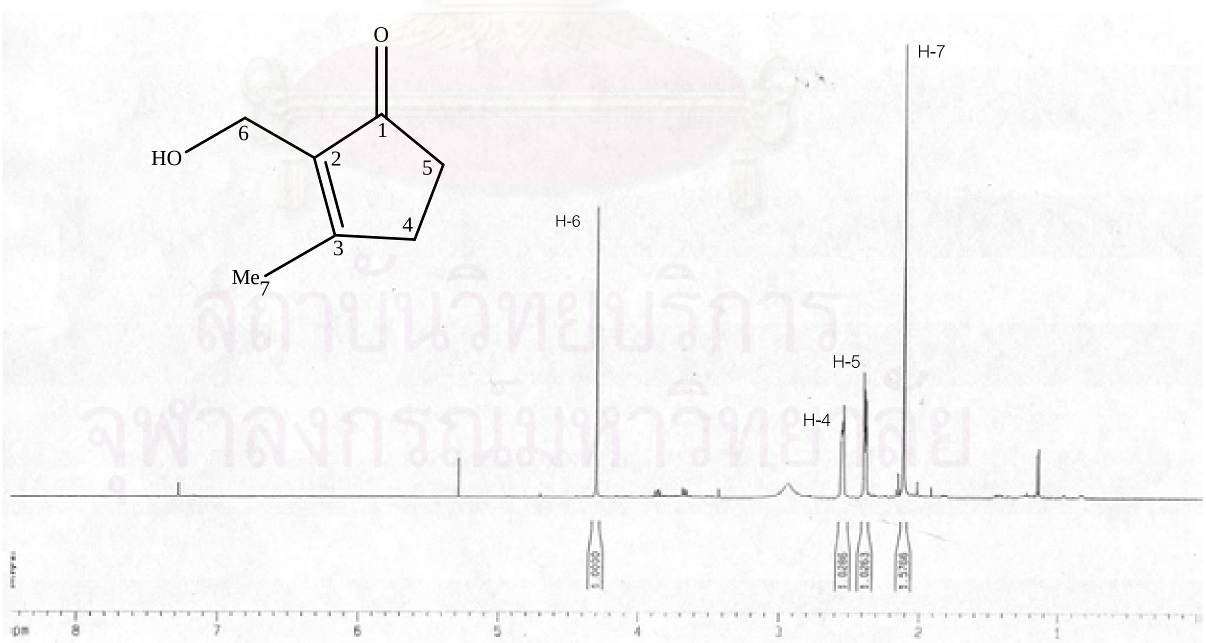


Figure C24 The 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B5(34)5

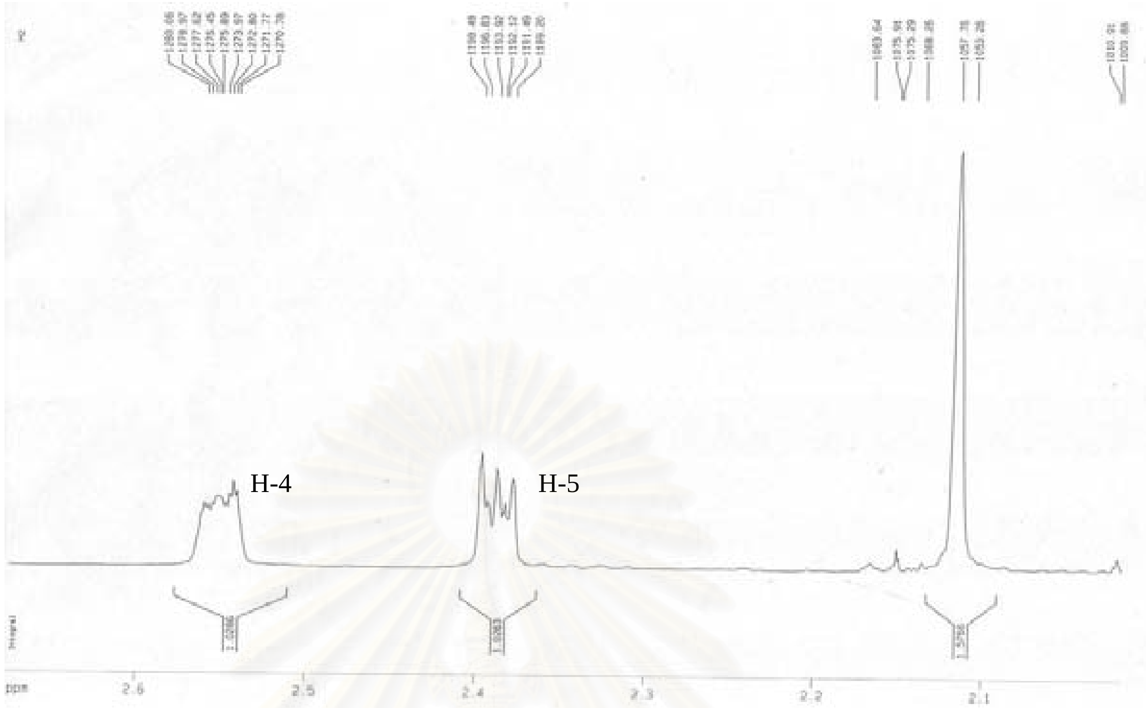


Figure C25 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B5(34)5 (δH = 2.0-2.7 ppm)

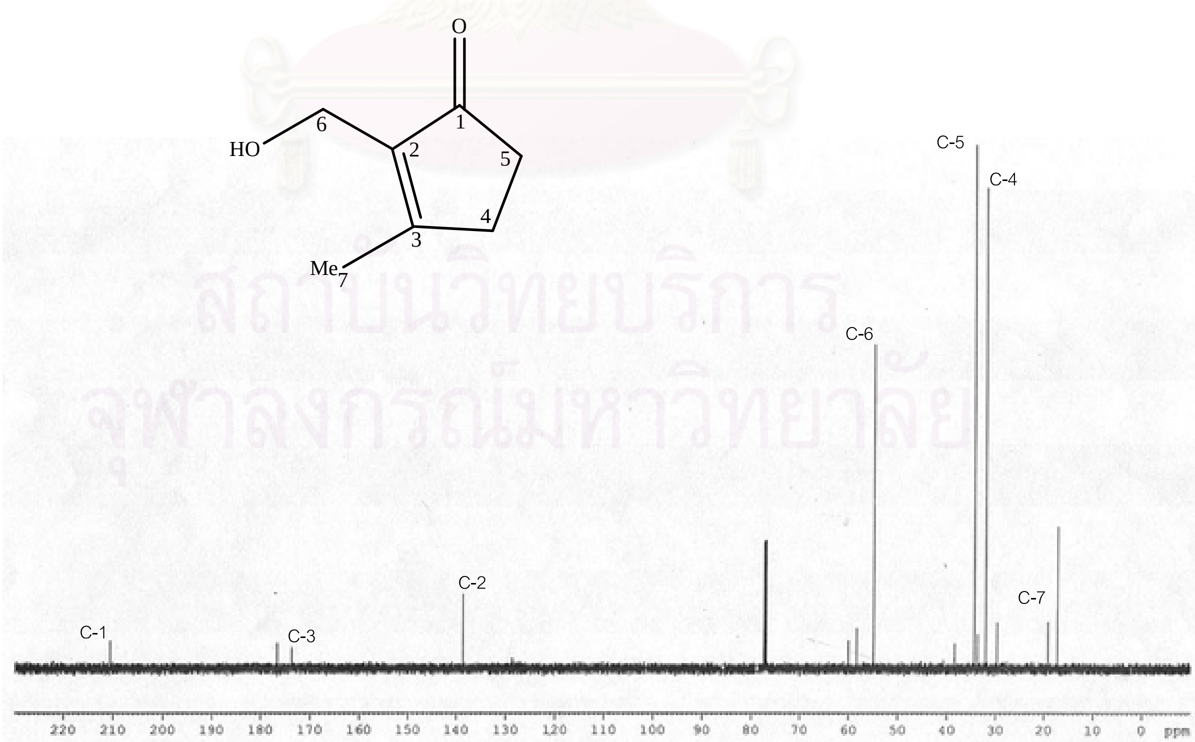


Figure C26 The 125 MHz ^{13}C-NMR spectrum of compound L20B5(34)5

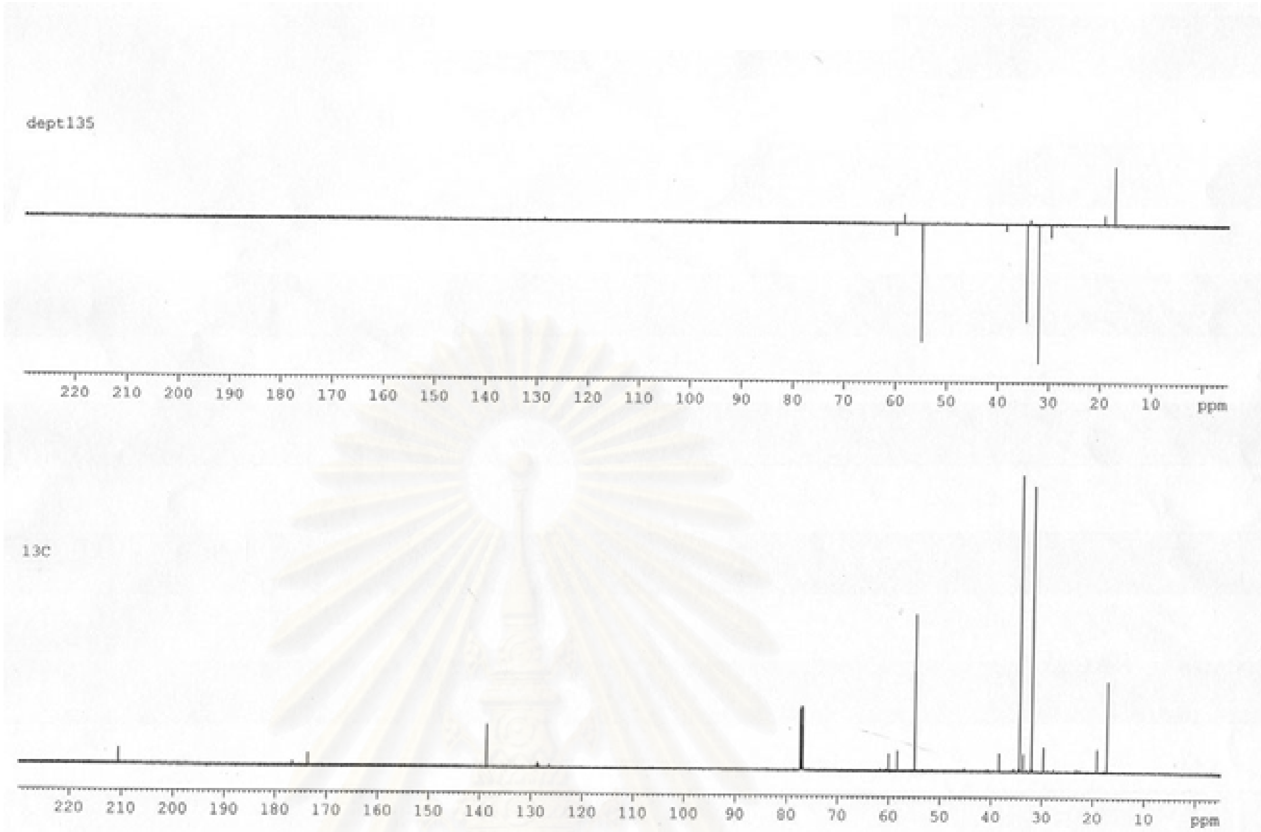


Figure C27 The DEPT 135 spectrum of compound L20B5(34)5

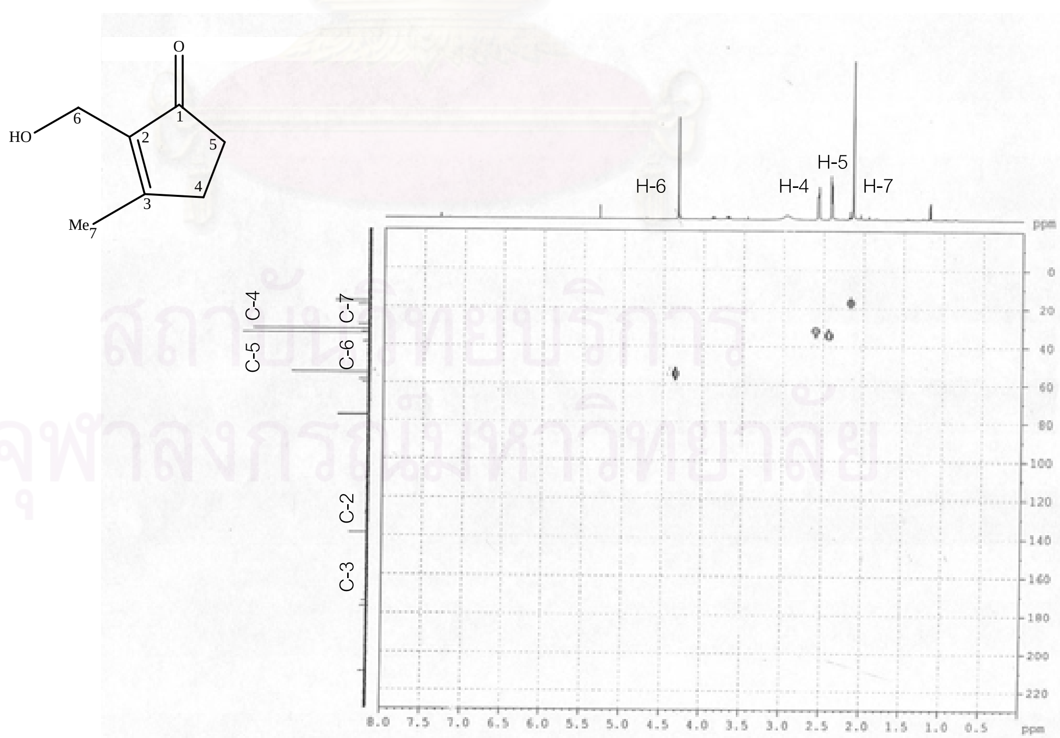


Figure C28 The HMQC spectrum of compound L20B5(34)5

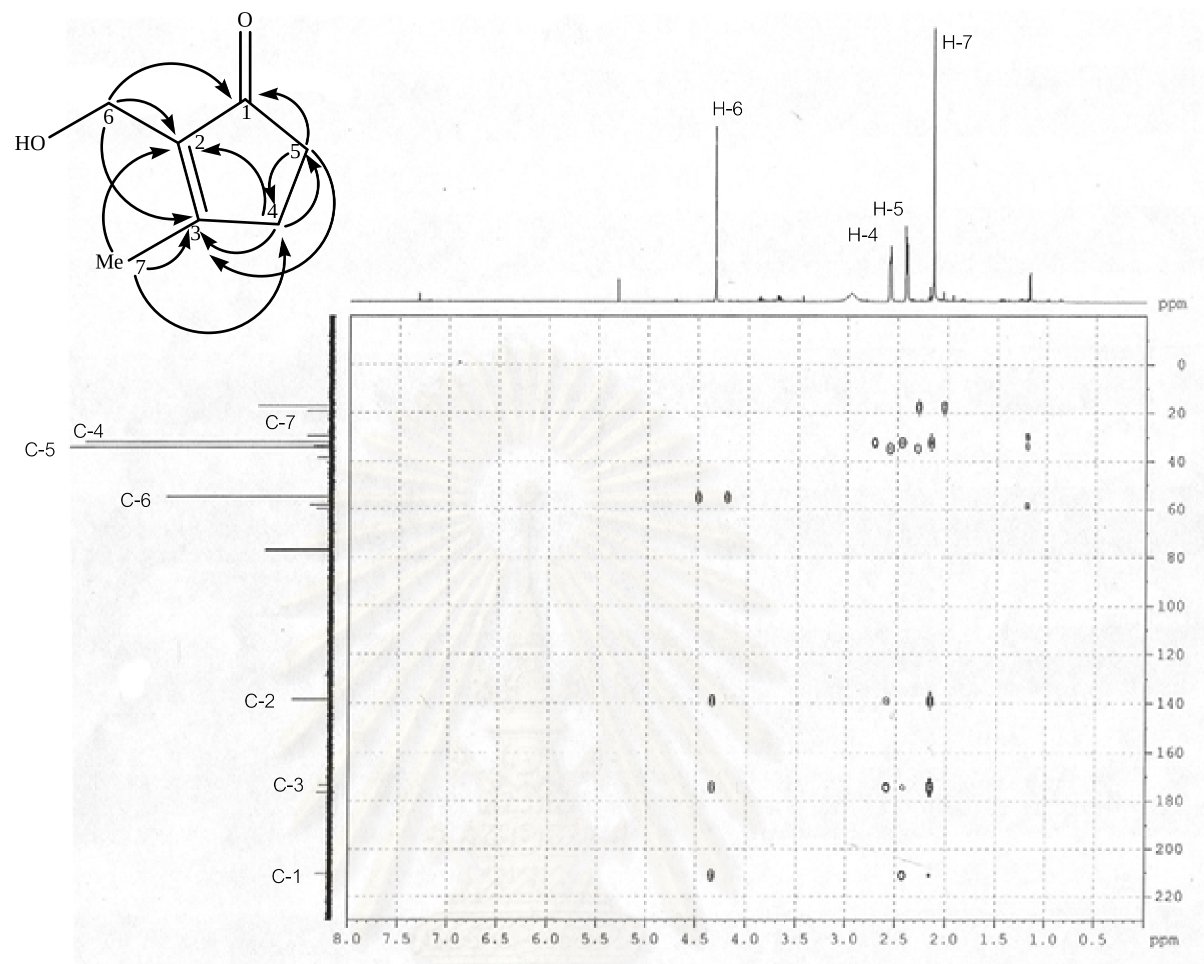


Figure C29 The HMBC spectrum of compound L20B5(34)5

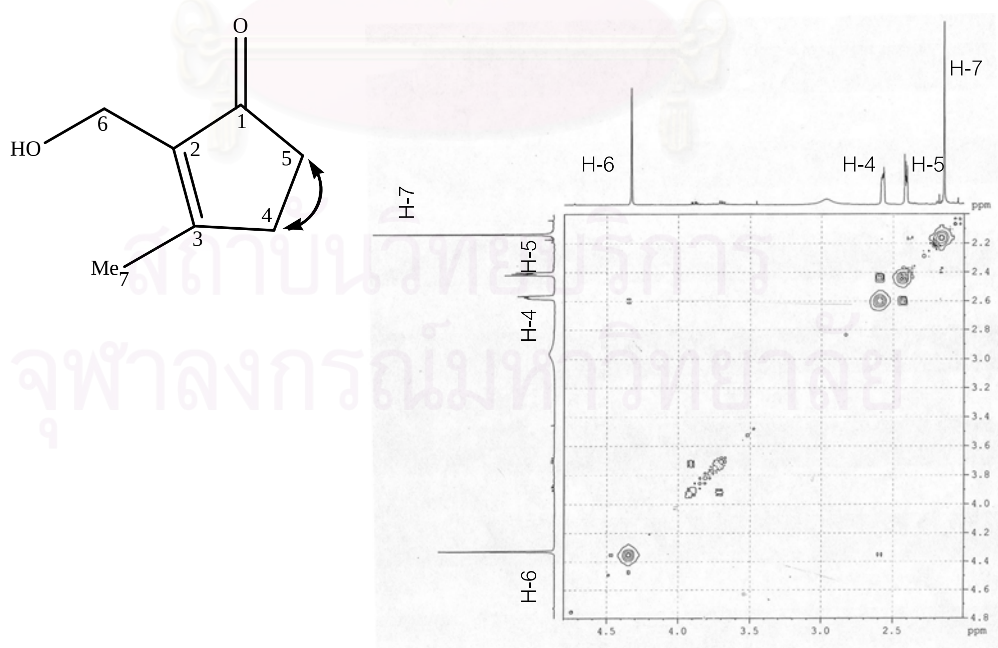


Figure C30 Expansion ^{1}H-^{1}H COSY spectrum of compound L20B5(34)5

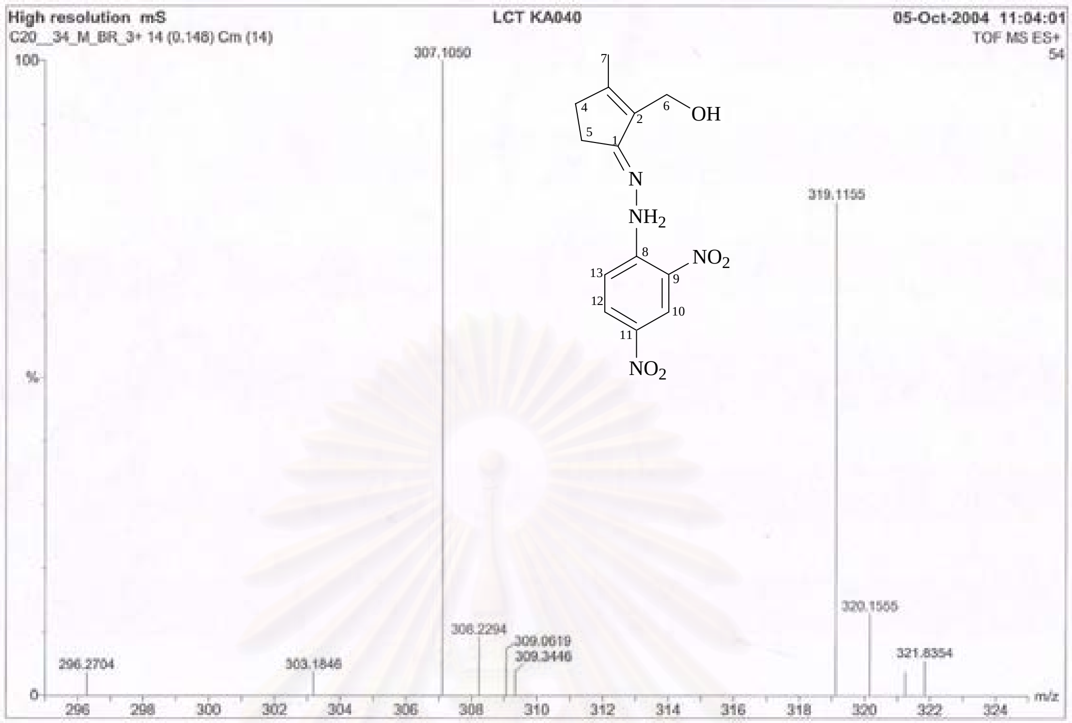


Figure C31 The ESI-TOF spectrum of compound L20B5(34)5R3

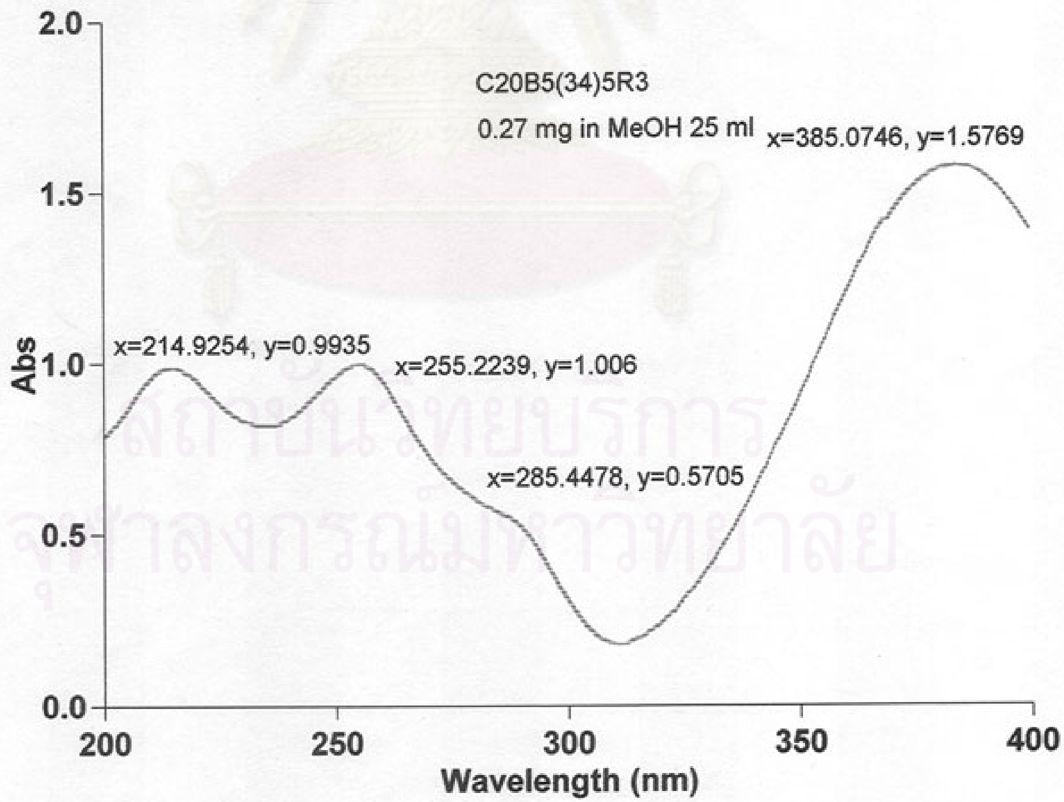


Figure C32 The UV spectrum of compound L20B5(34)5R3 in methanol

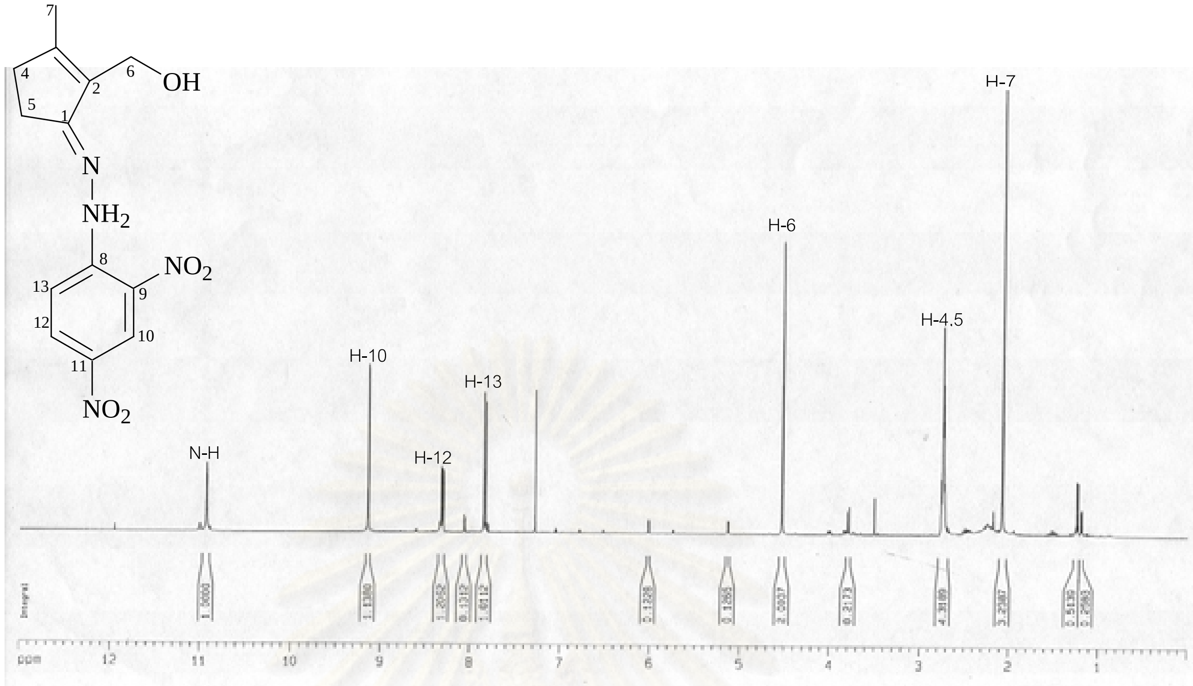


Figure C33 The 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B5(34)5R3

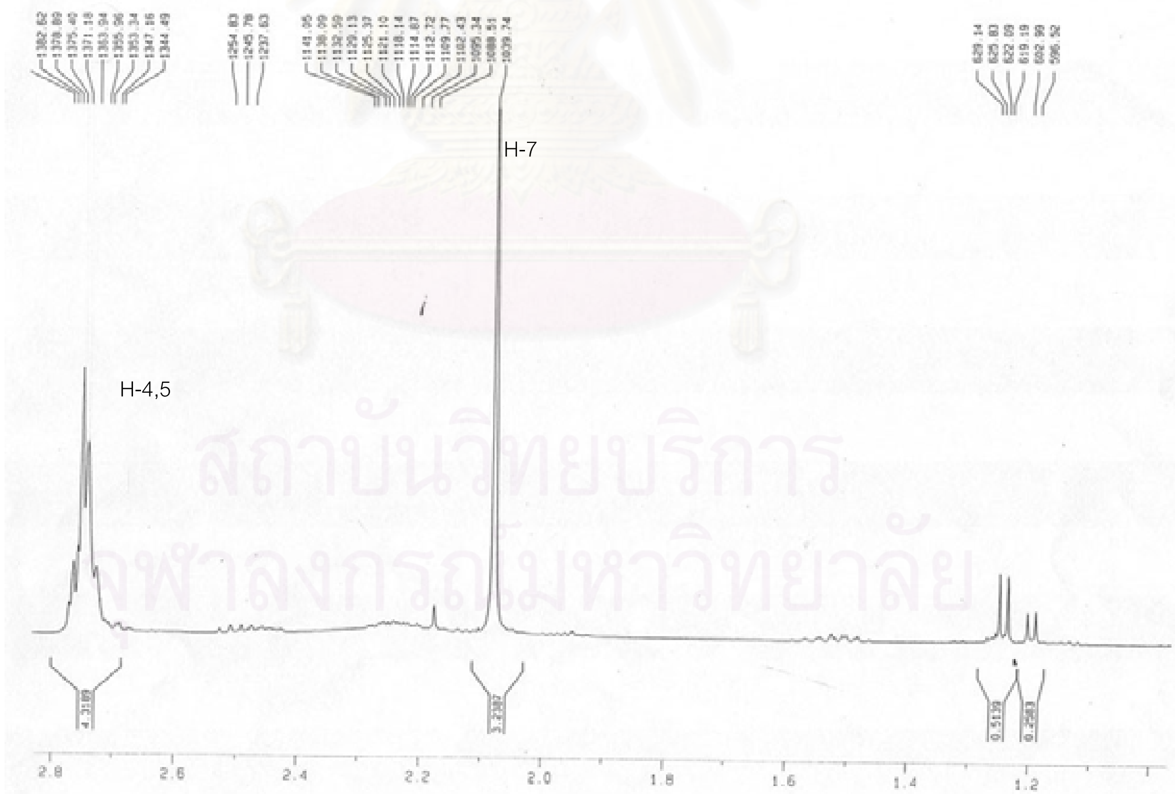


Figure C34 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B5(34)5R3 (δH = 1.0-2.8 ppm)

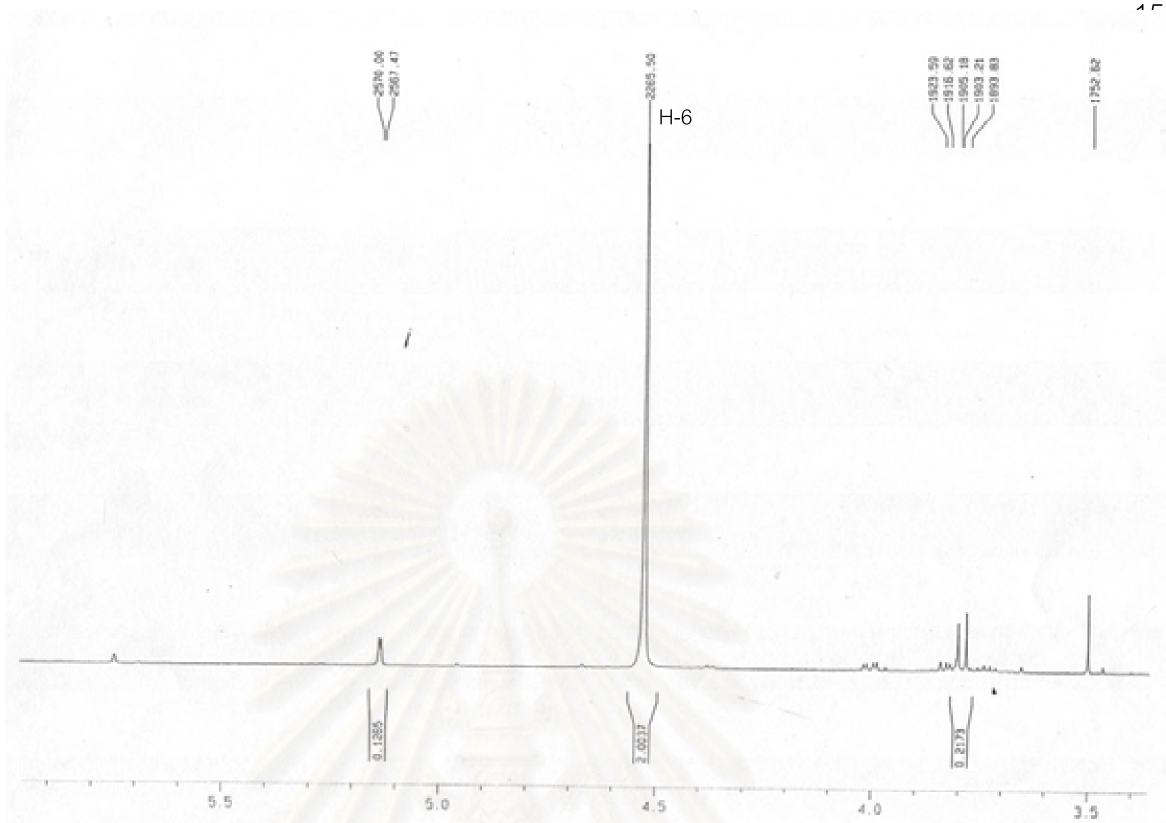


Figure C35 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B5(34)5R3 (δH = 3.4-6.0 ppm)

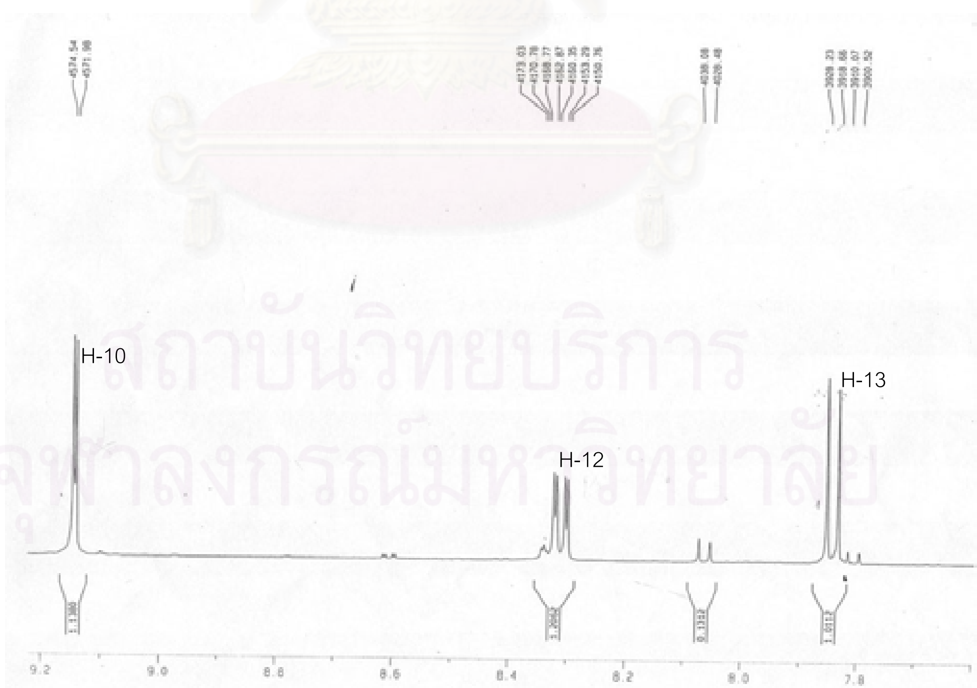


Figure C36 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B5(34)5R3 (δH = 7.6-9.2 ppm)

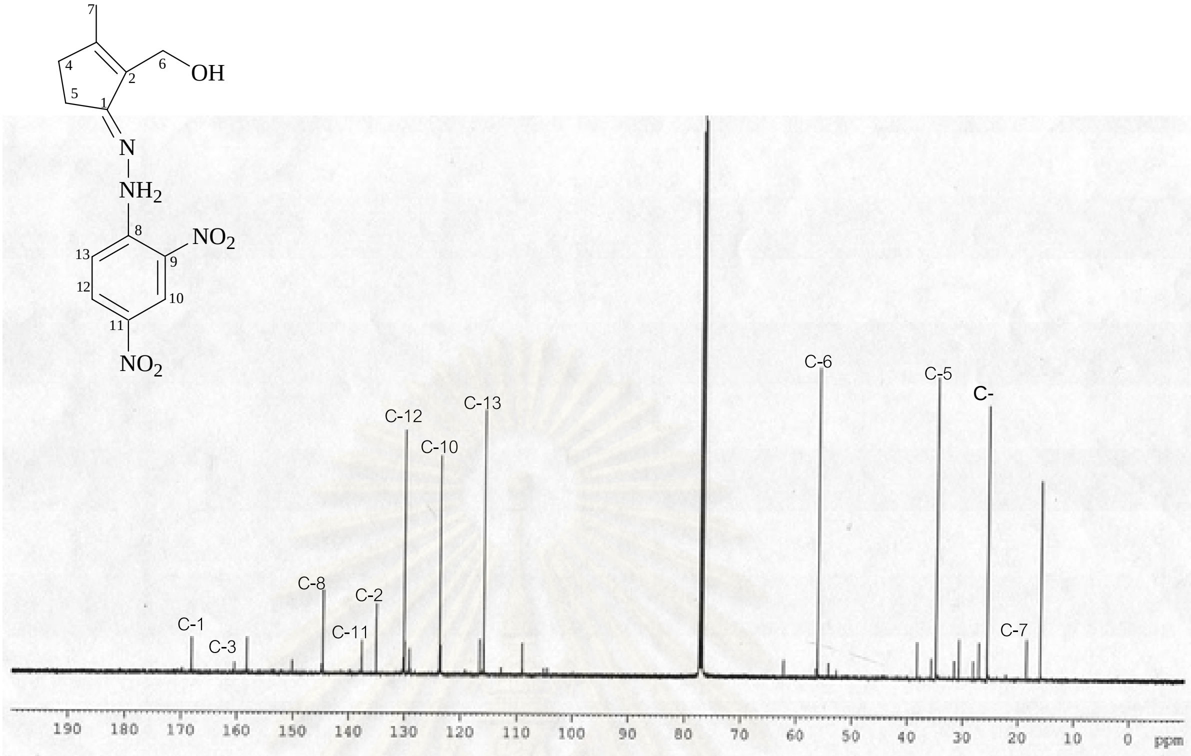


Figure C37 The 125 MHz ^{13}C-NMR spectrum of compound L20B5(34)5R3

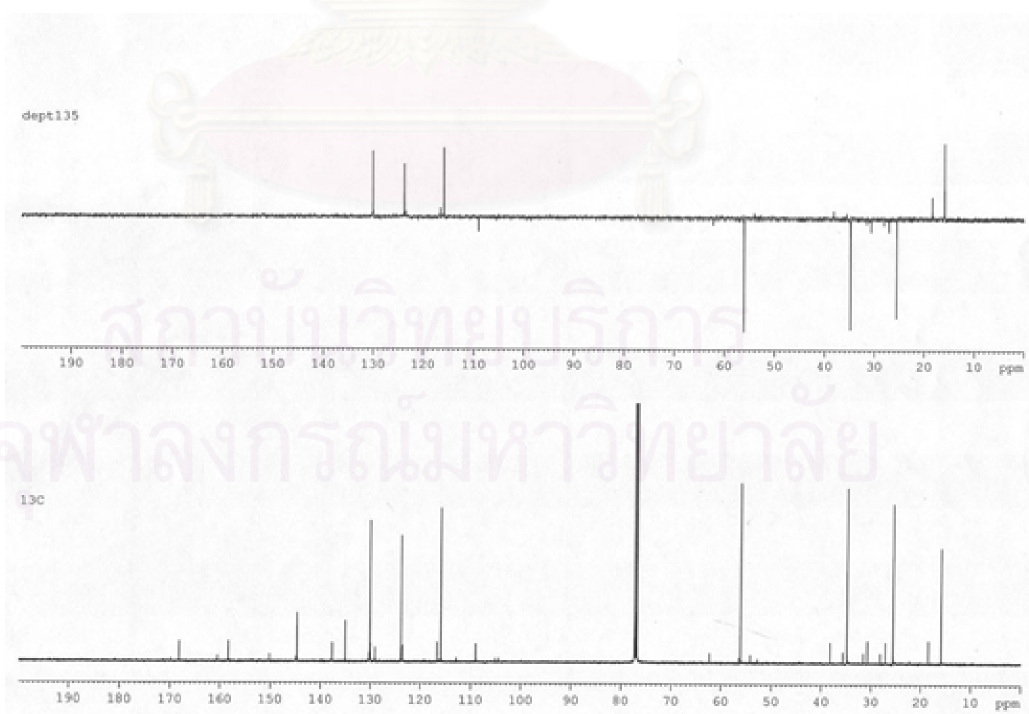


Figure C38 The DEPT 135 spectrum of compound L20B5(34)5R3

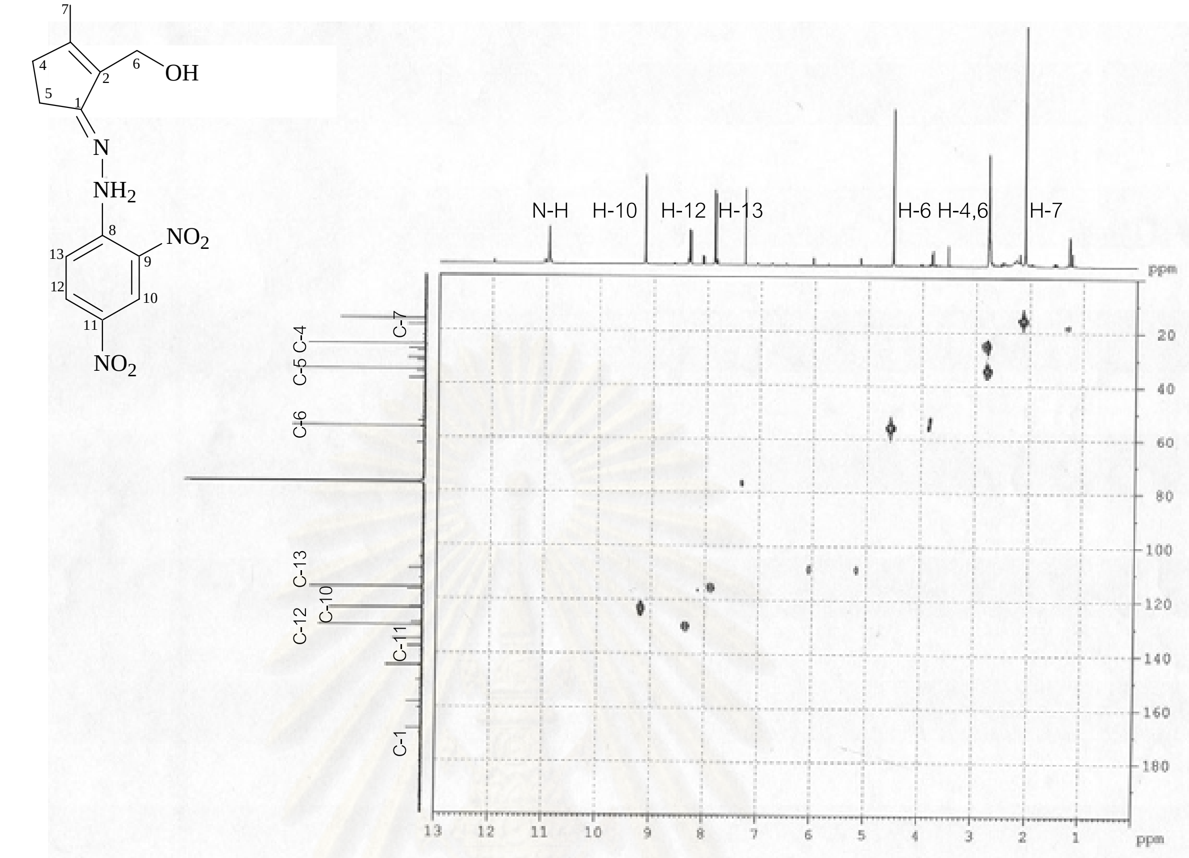


Figure C39   The HMQC spectrum of compound L20B5(34)5R3

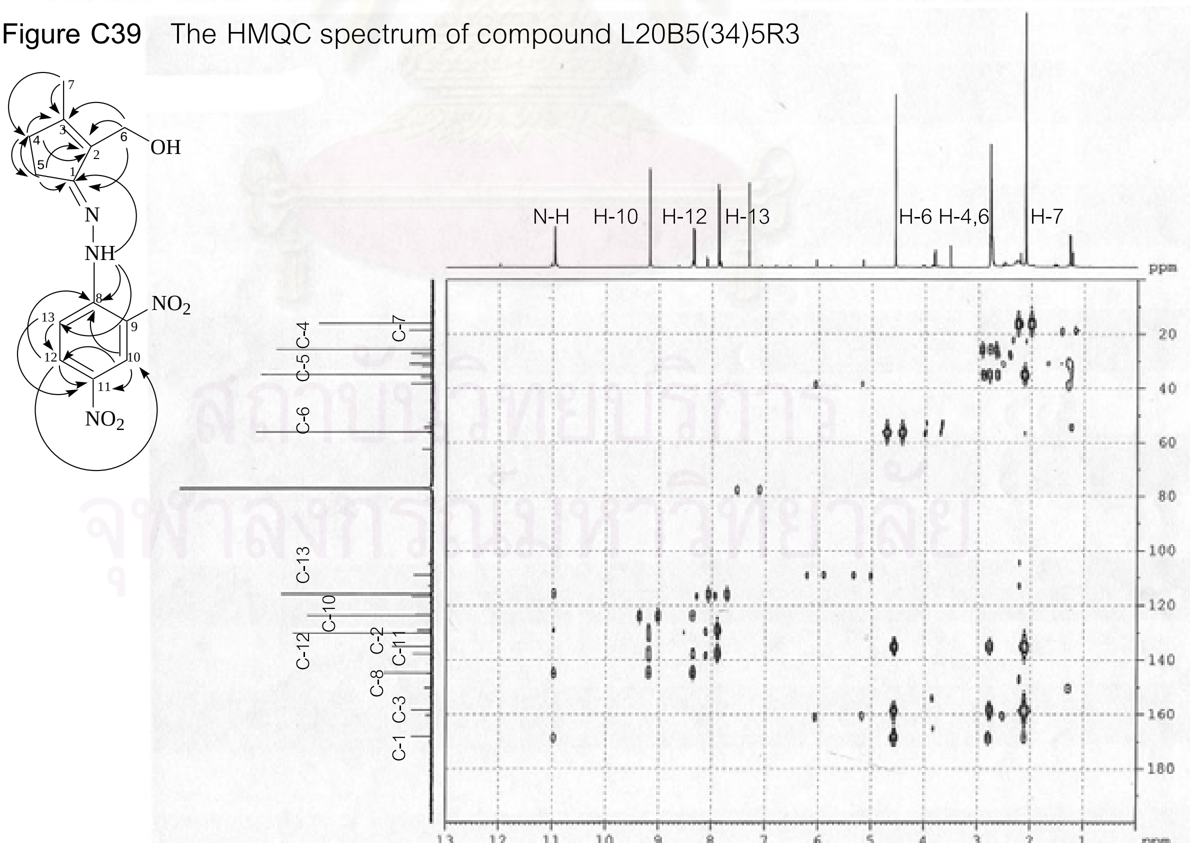


Figure C40   The HMBC spectrum of compound L20B5(34)5R3

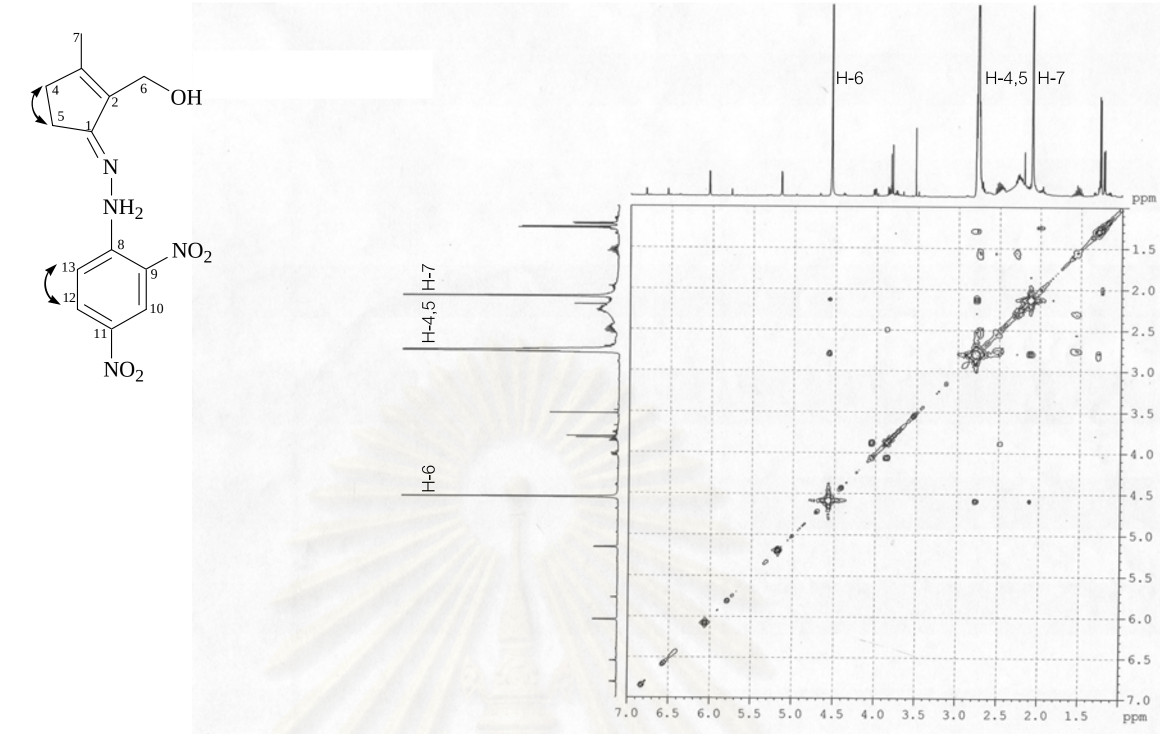


Figure C41 Expansion [1]H-[1]H COSY spectrum of compound L20B5(34)5R3 (δH = 0-7.0 ppm)

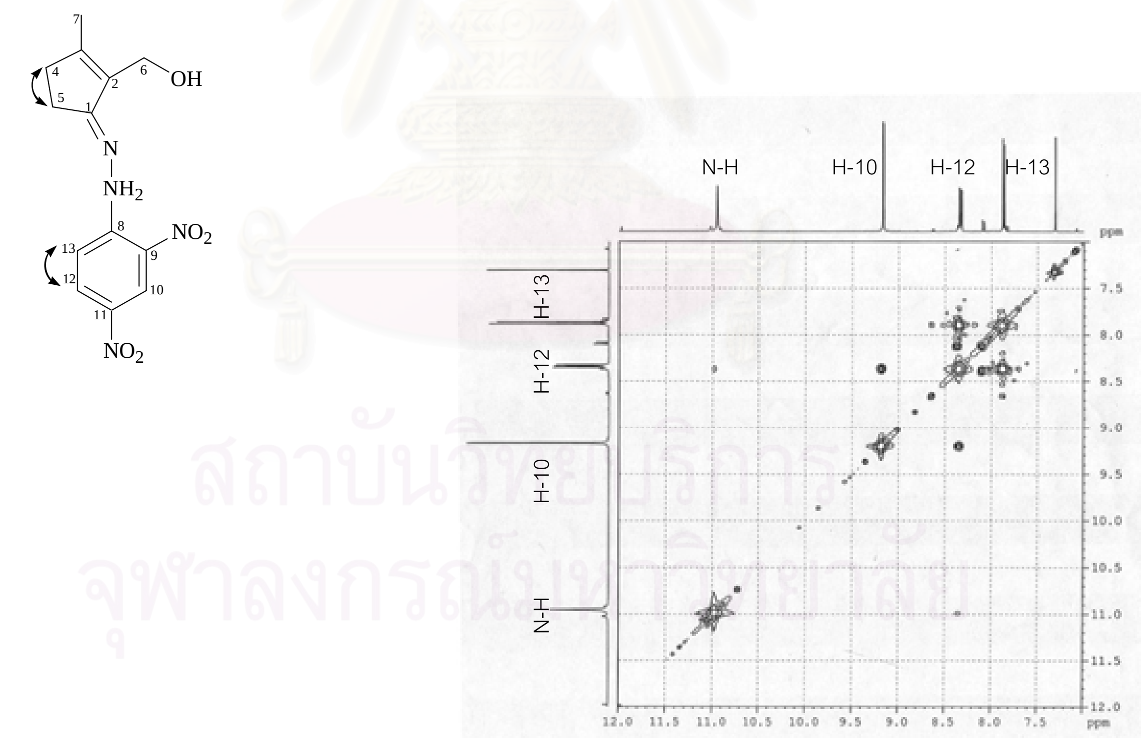


Figure C42 Expansion [1]H-[1]H COSY spectrum of compound L20B5(34)5R3 (δH = 7.0-12.0 ppm)

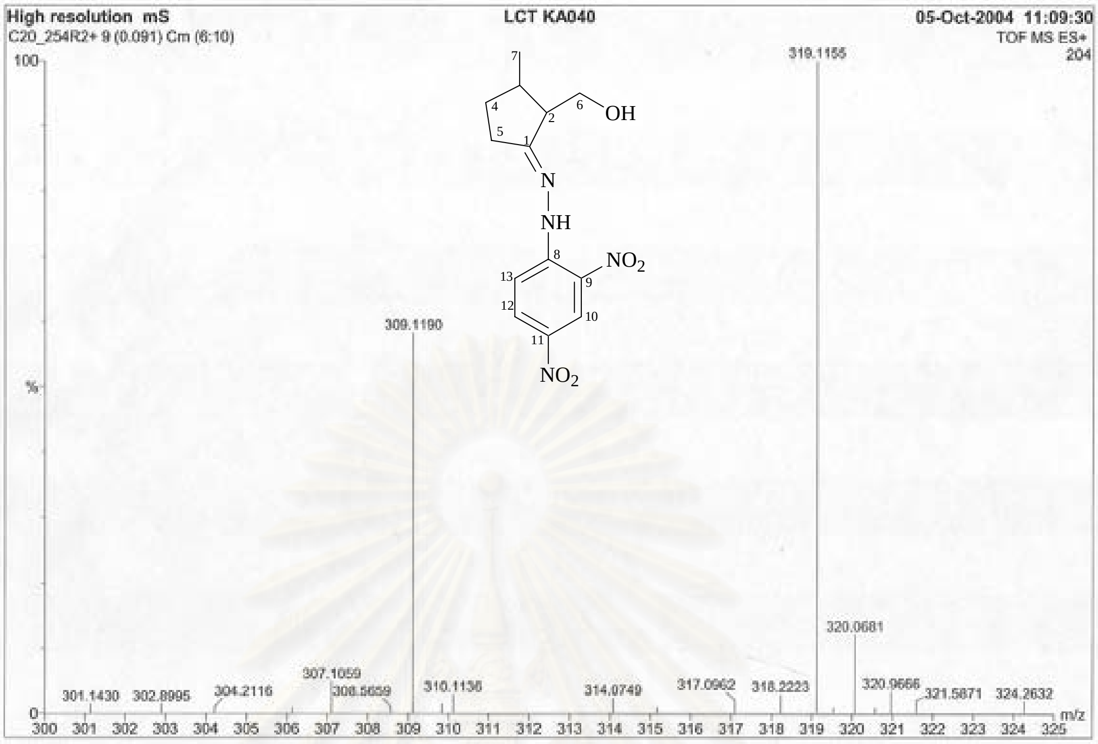


Figure C43 The ESI-TOF spectrum of compound L20B464R2

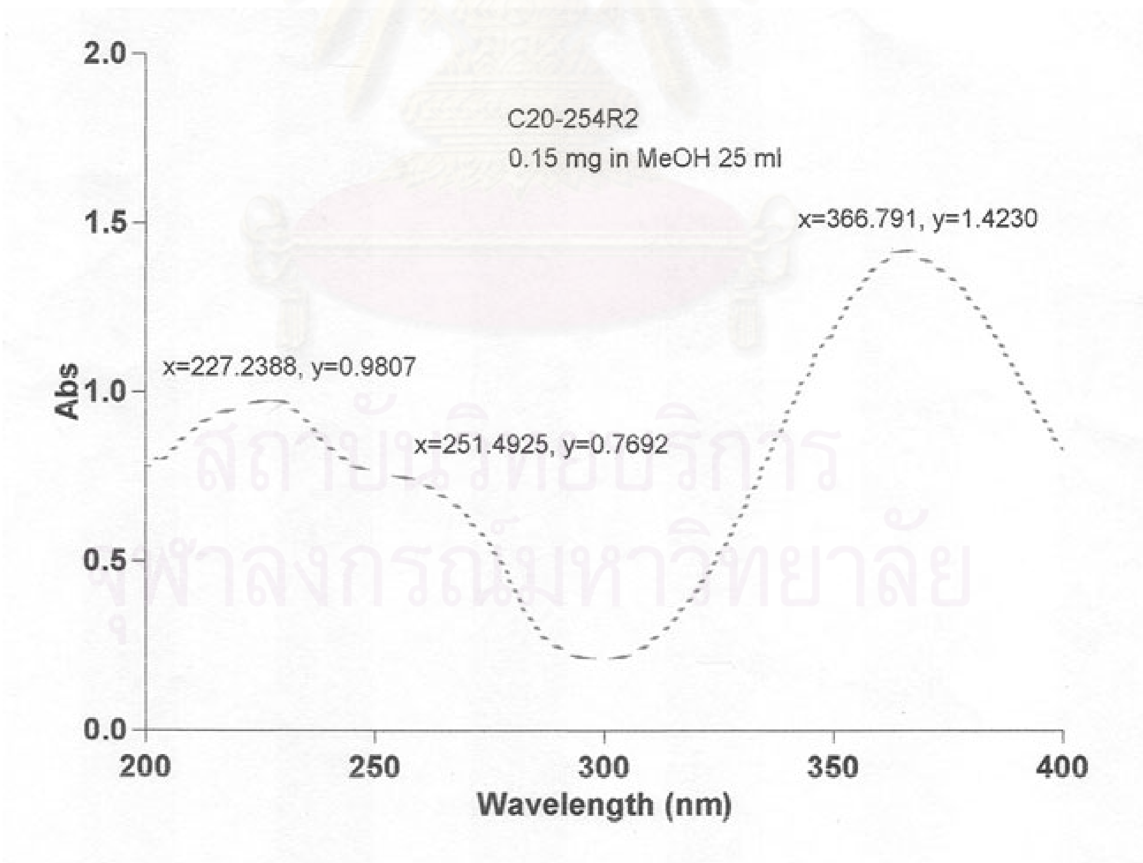


Figure C44 The UV spectrum of compound L20B464R2 in methanol

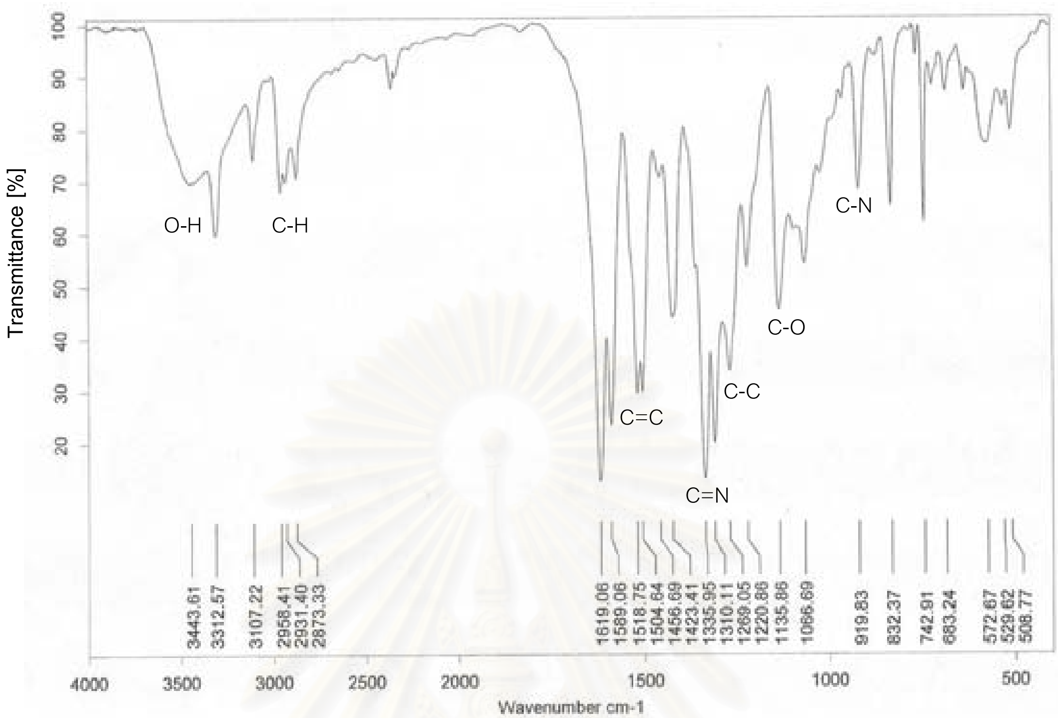


Figure C45 The IR spectrum of compound L20B464R2

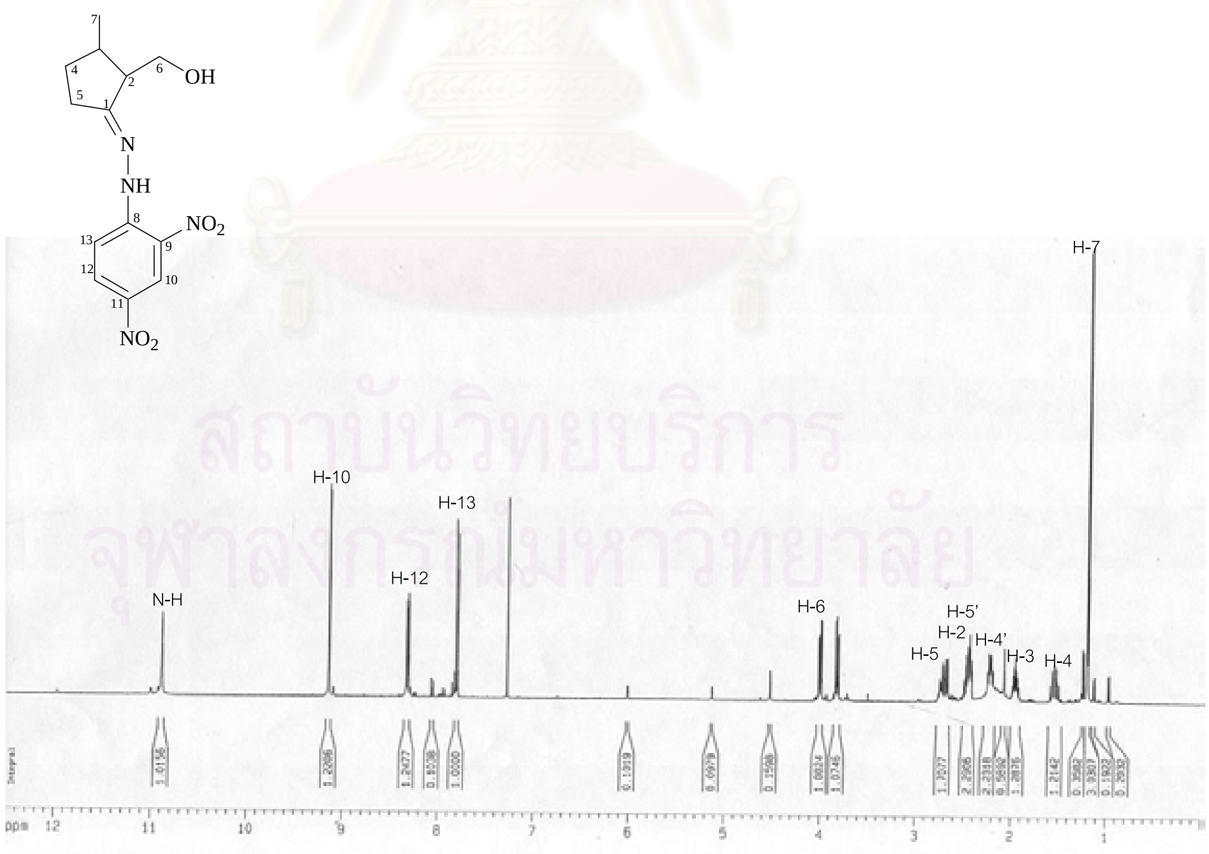


Figure C46 The 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B464R2

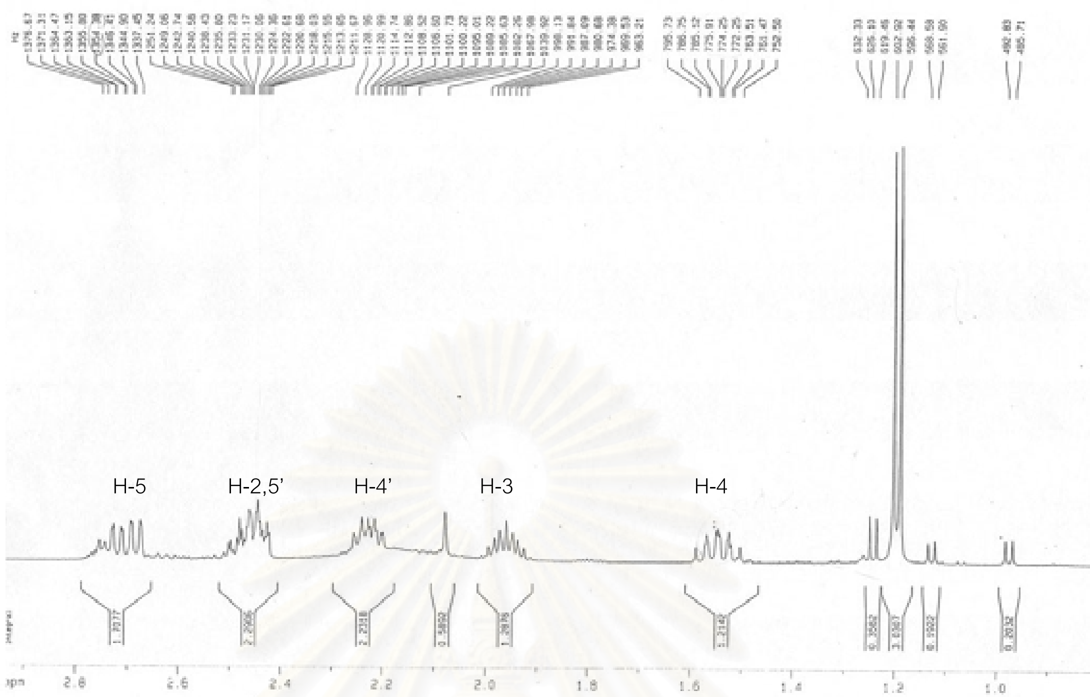


Figure C47 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B464R2 (δH = 0-3.0 ppm)

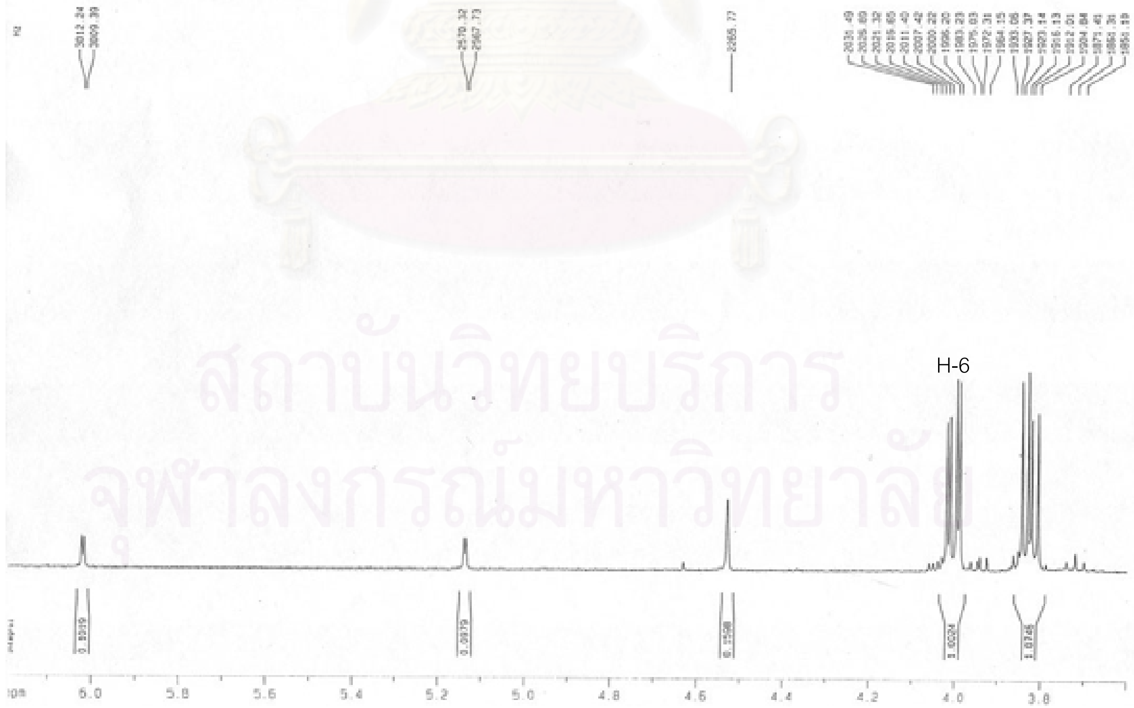


Figure C48 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B464R2 (δH = 3.6-6.2 ppm)

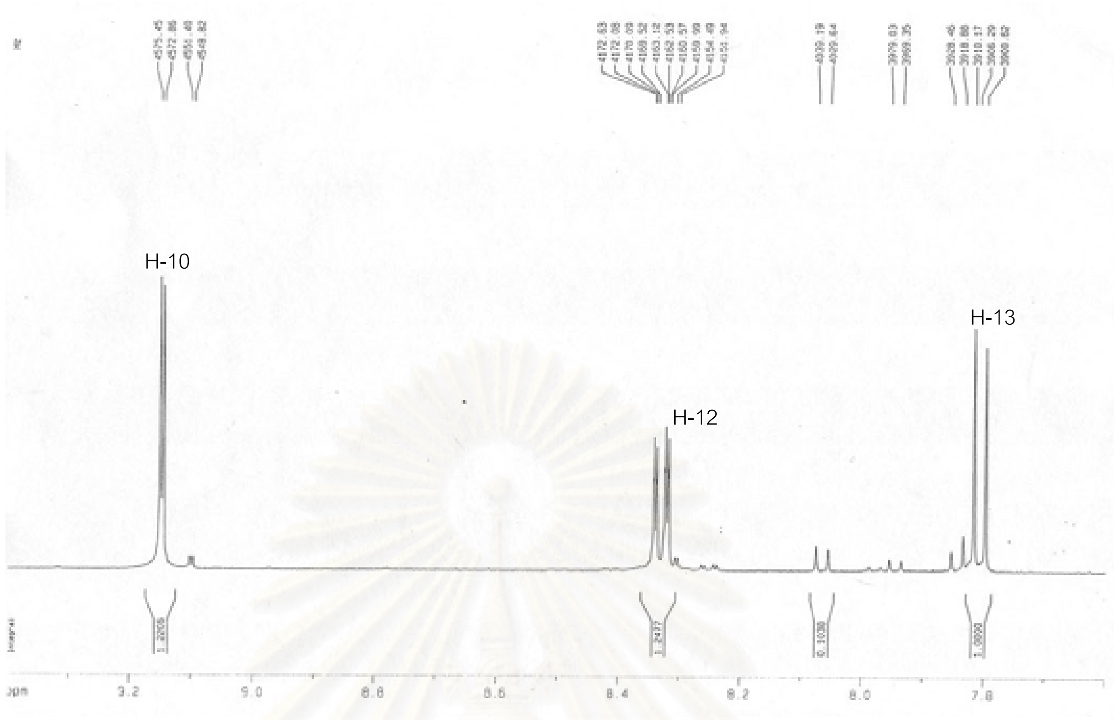


Figure C49 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound L20B464R2 (δH = 7.6-9.4 ppm)

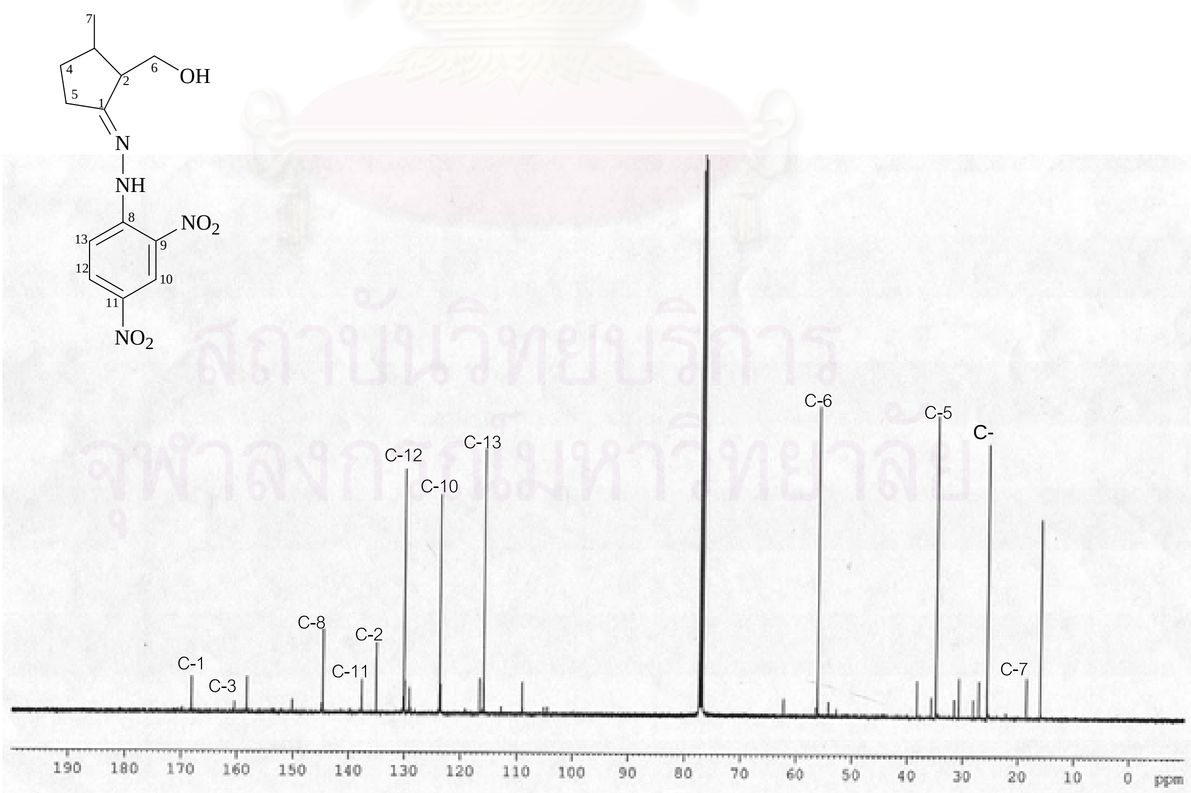


Figure C50 The 125 MHz ^{13}C-NMR spectrum of compound L20B464R2

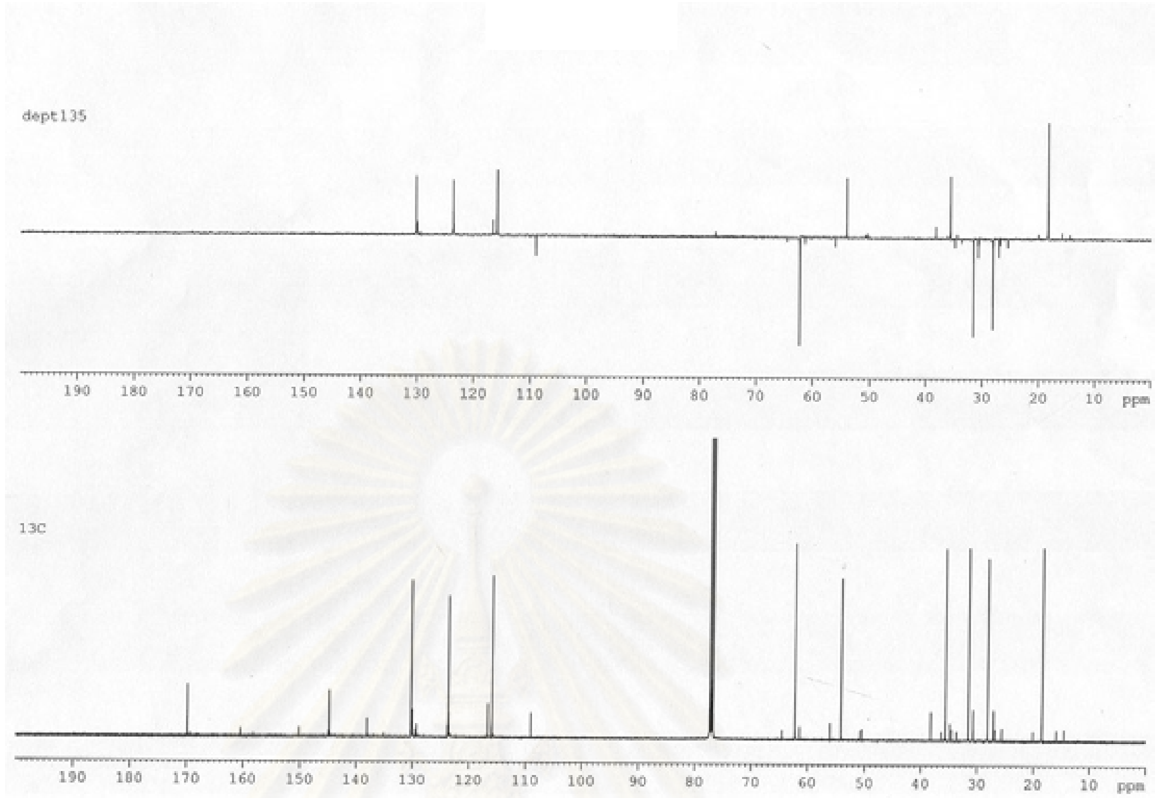


Figure C51 The DEPT 135 spectrum of compound L20B464R2

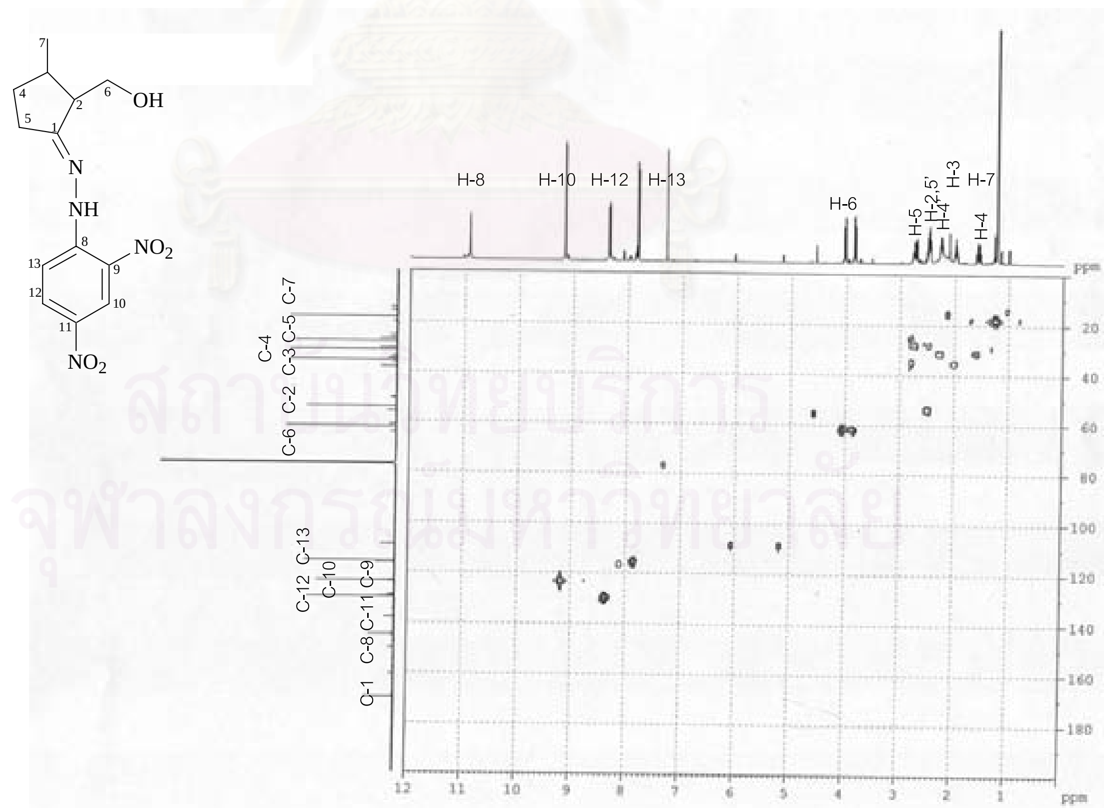


Figure C52 The HMQC spectrum of compound L20B464R2

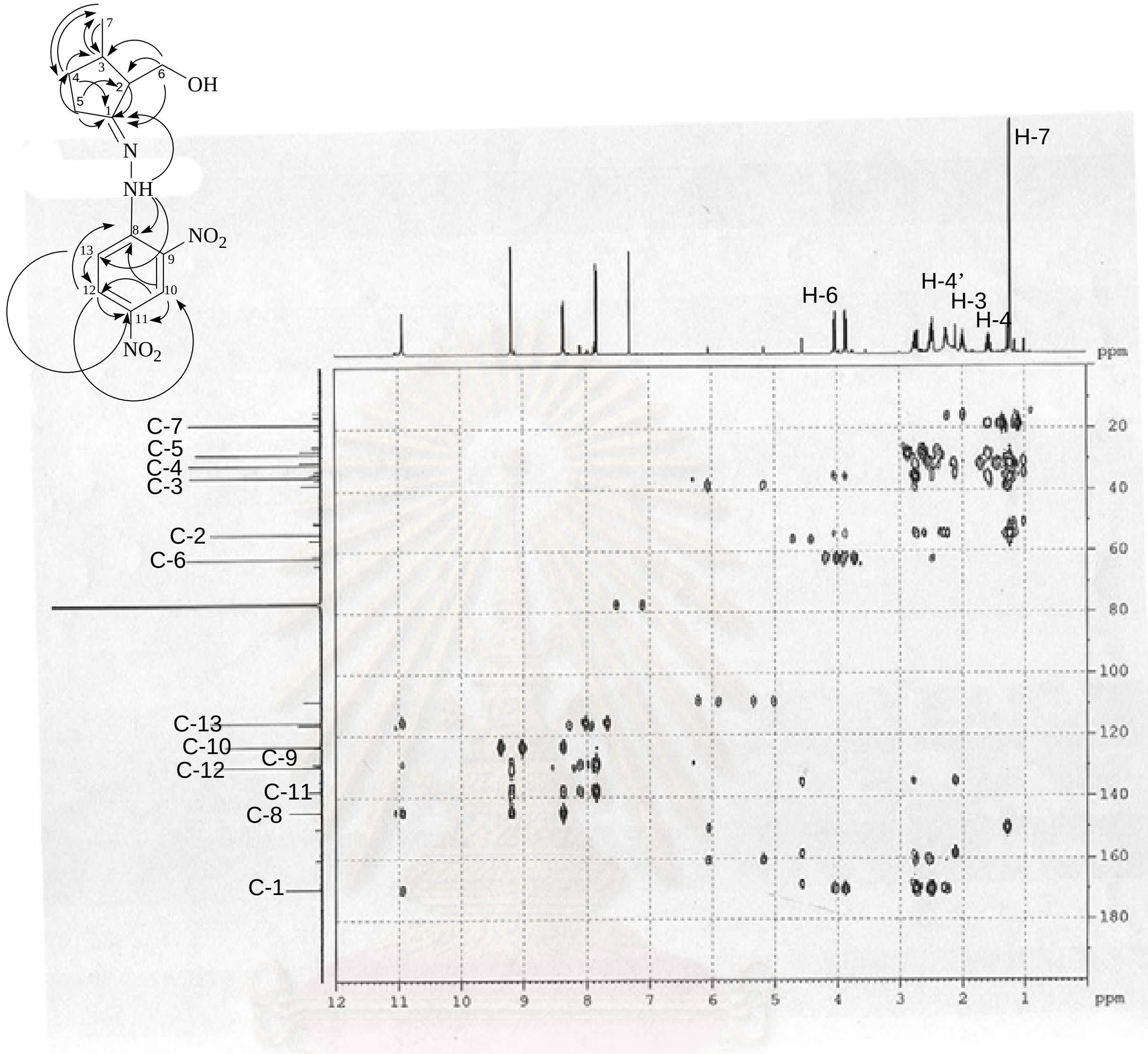


Figure C53 The HMBC spectrum of compound L20B464R2

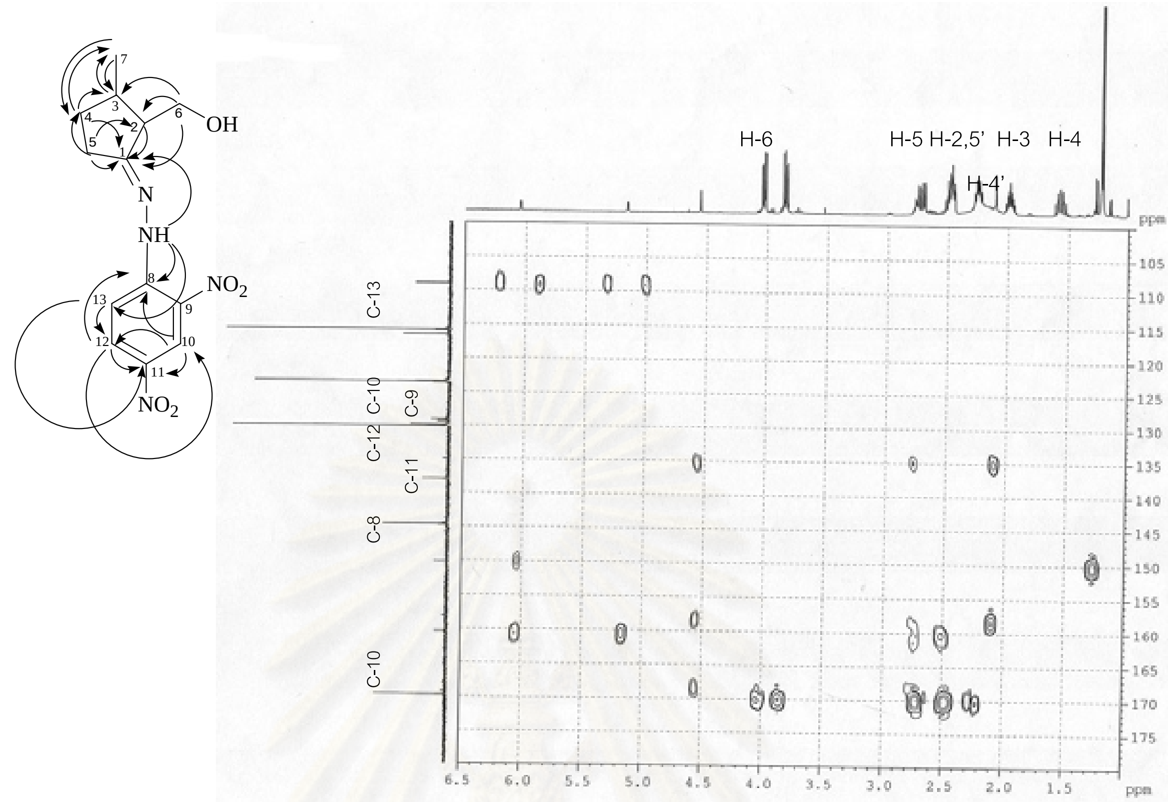


Figure C54 Expansion HMBC spectrum of compound L20B464R2 (δH=0-6.5 ppm, δC=100-180 ppm)

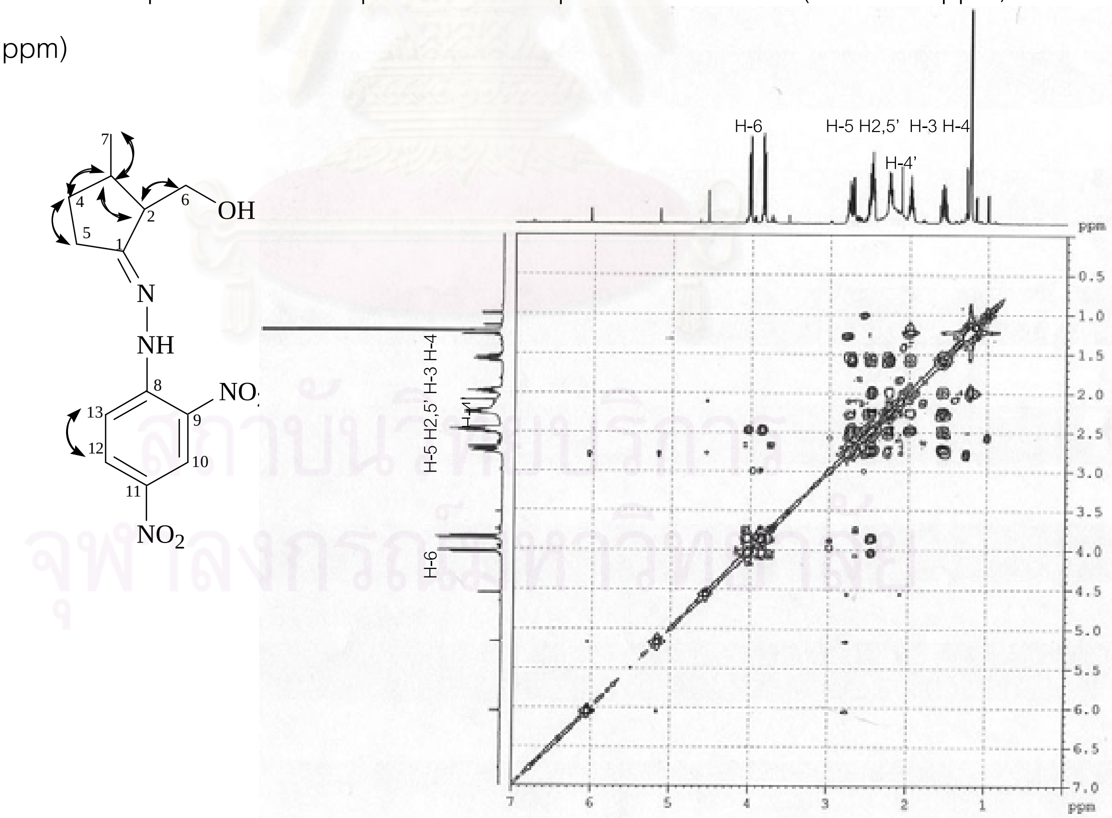


Figure C55 Expansion ^{1}H-^{1}H COSY spectrum of compound L20B464R2 (δH=0-7.0 ppm)

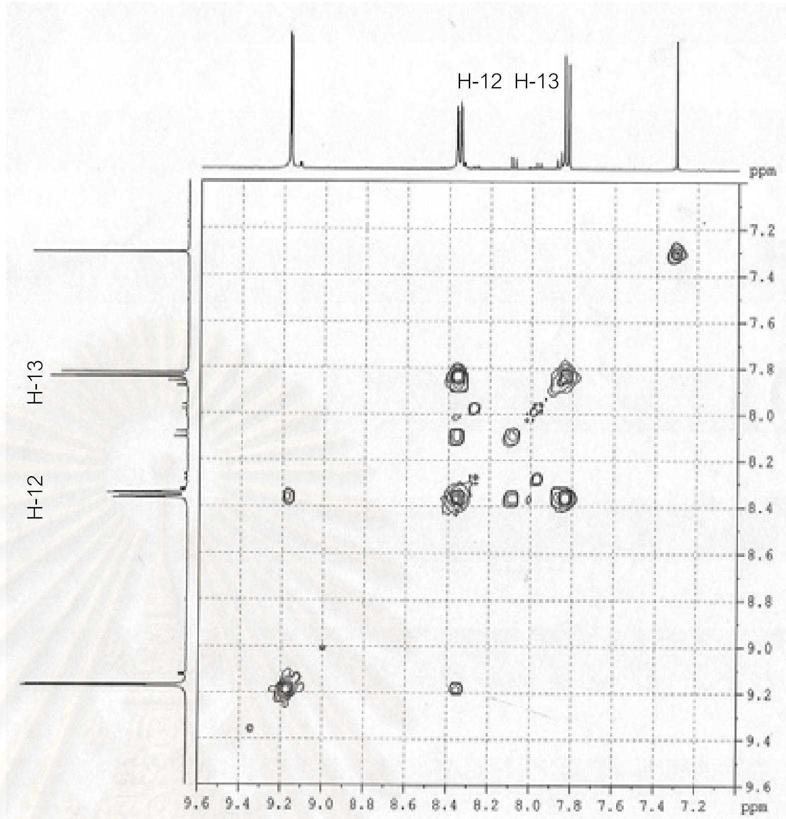


Figure C56 Expansion [1]H-[1]H COSY spectrum of compound L20B464R2 (δH=7.0-9.6 ppm)

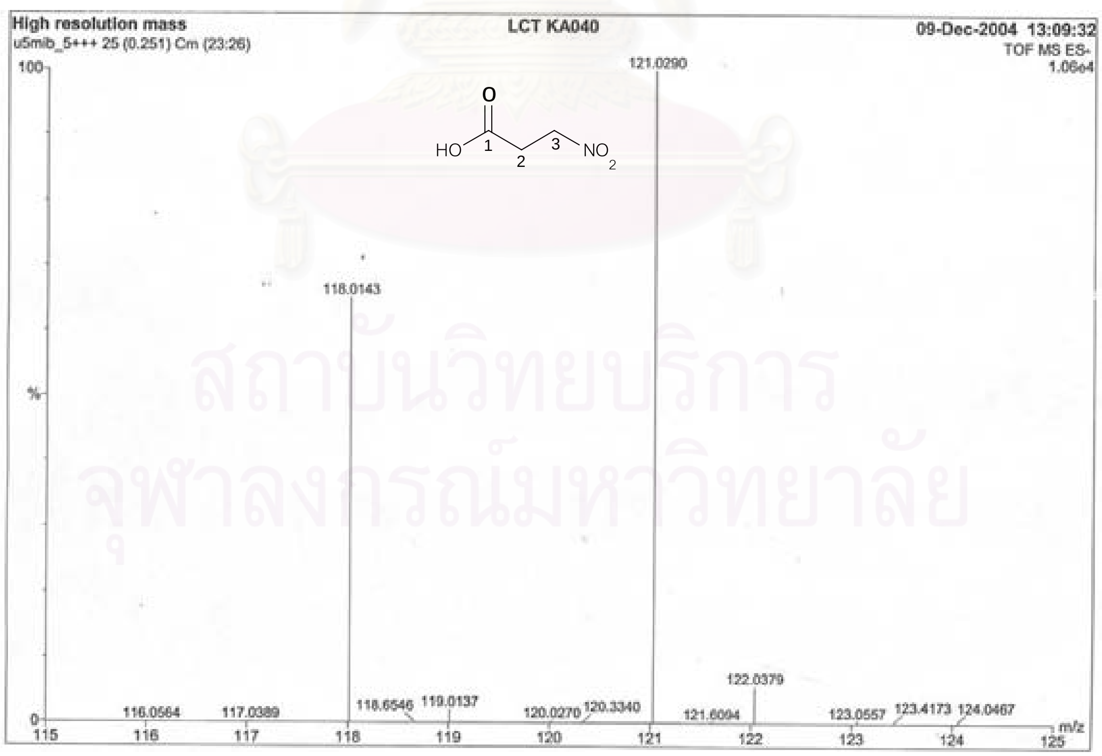


Figure C57 The ESI-TOF spectrum of compound U5B4-6

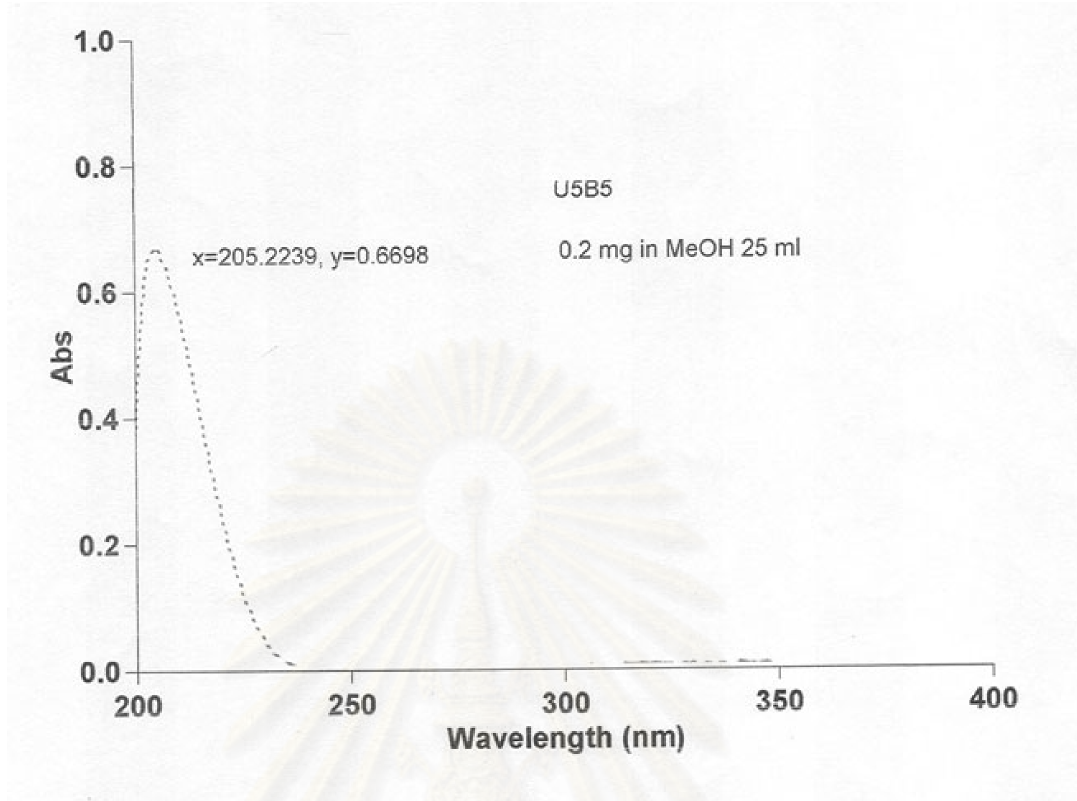


Figure C58 The UV spectrum of compound U5B4-6 in methanol

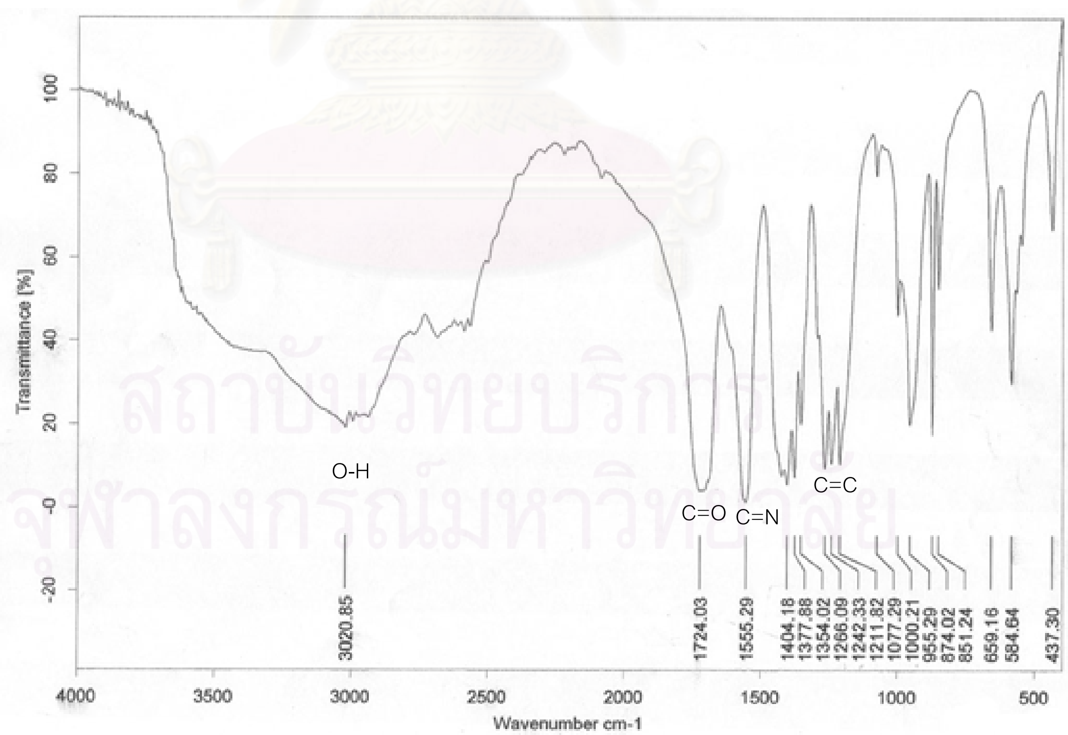


Figure C59 The IR spectrum of compound U5B4-6

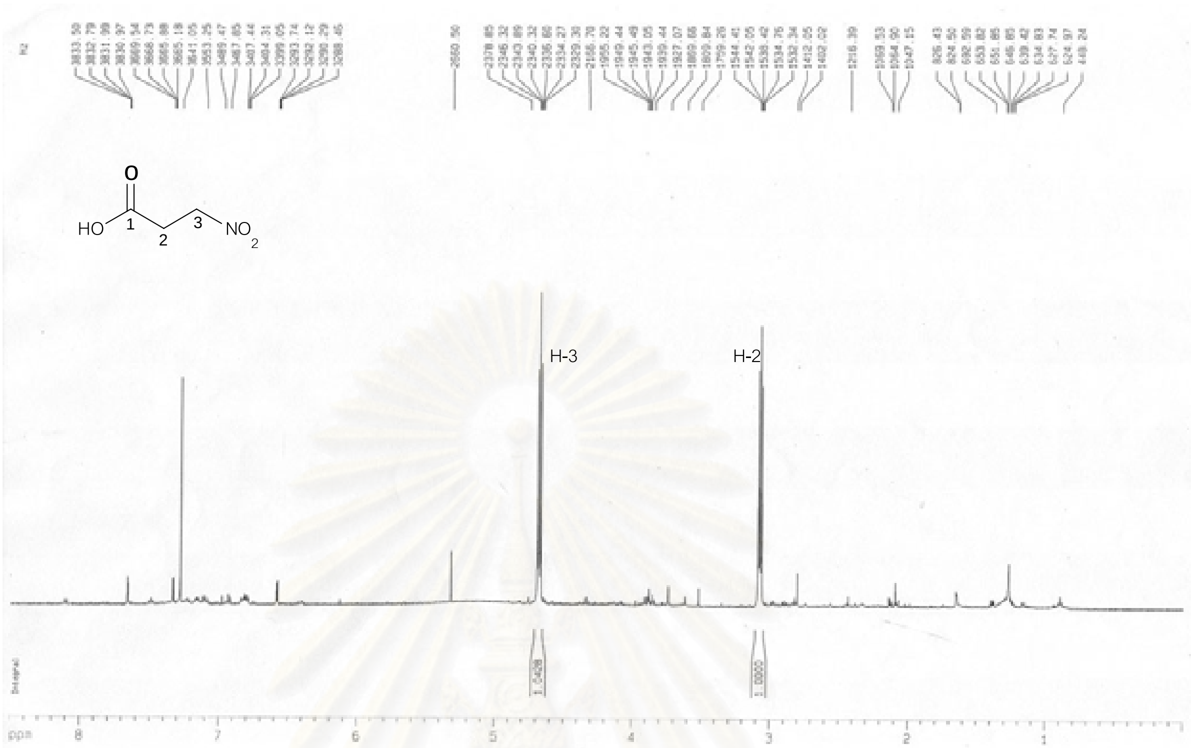


Figure C60 The 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound U5B4-6

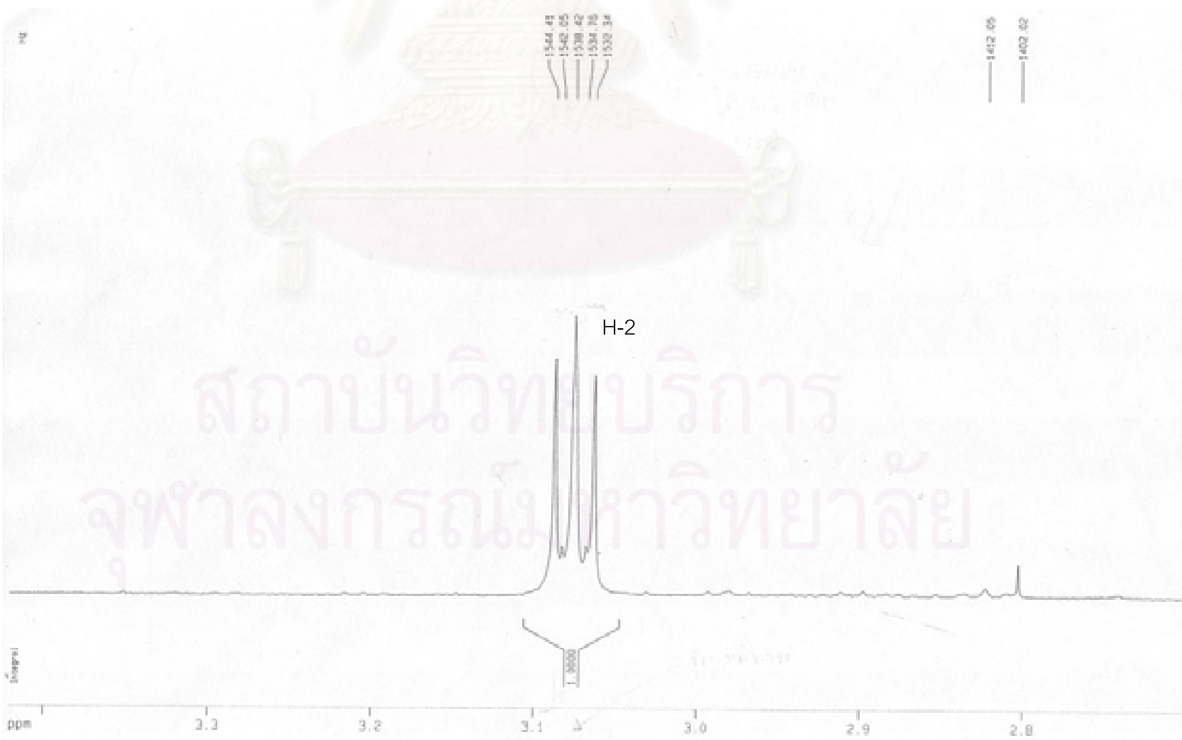


Figure C61 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound U5B4-6 (δH = 2.7-3.4 ppm)

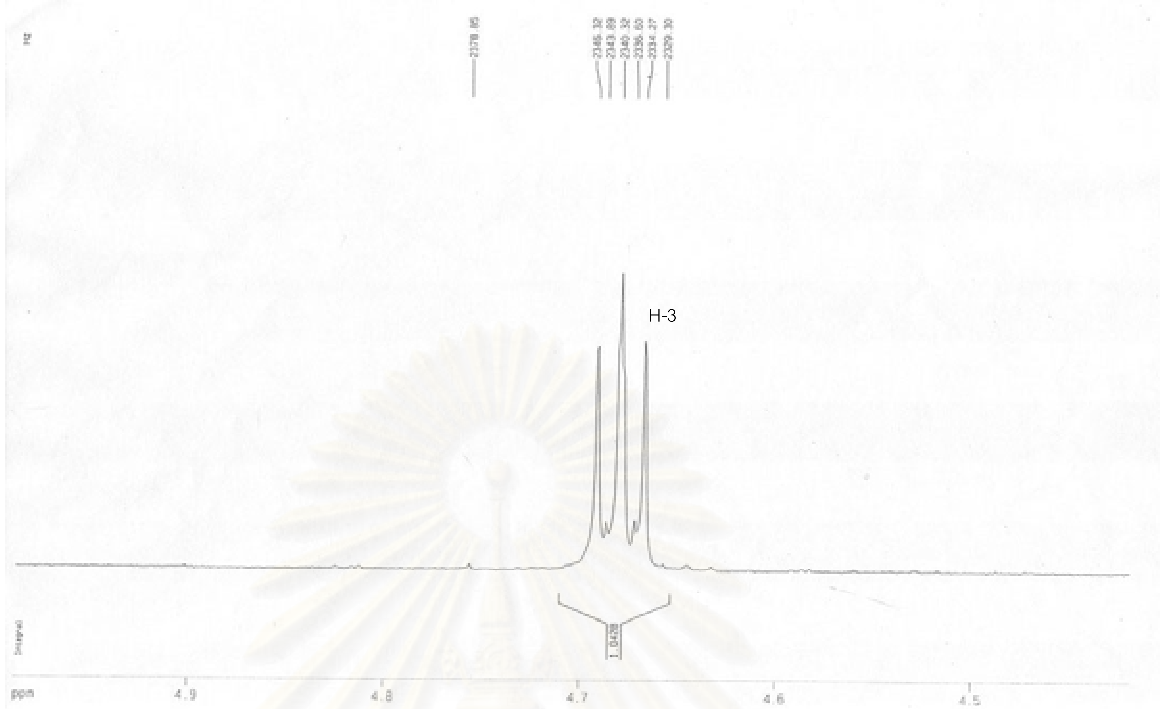


Figure C62 Expansion 500 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of compound U5B4-6 (δH = 4.4-5.0 ppm)

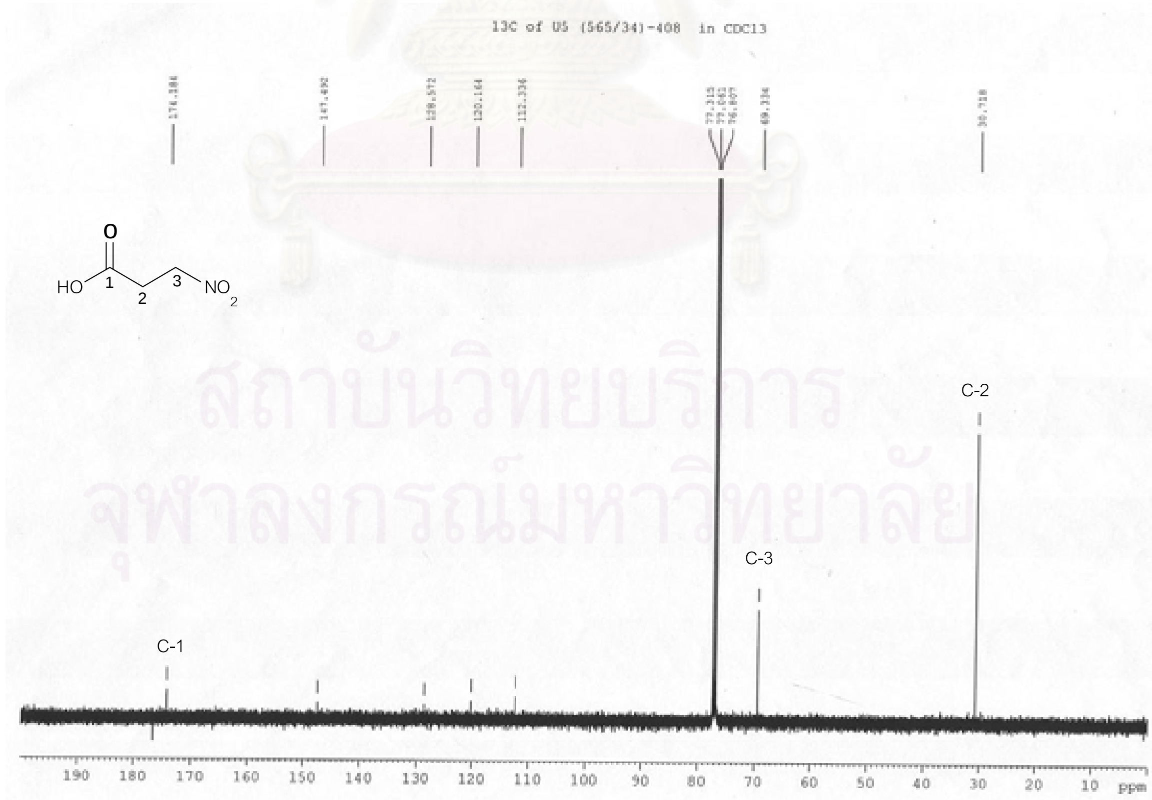


Figure C63 The 125 MHz ^{13}C-NMR spectrum of compound U5B4-6

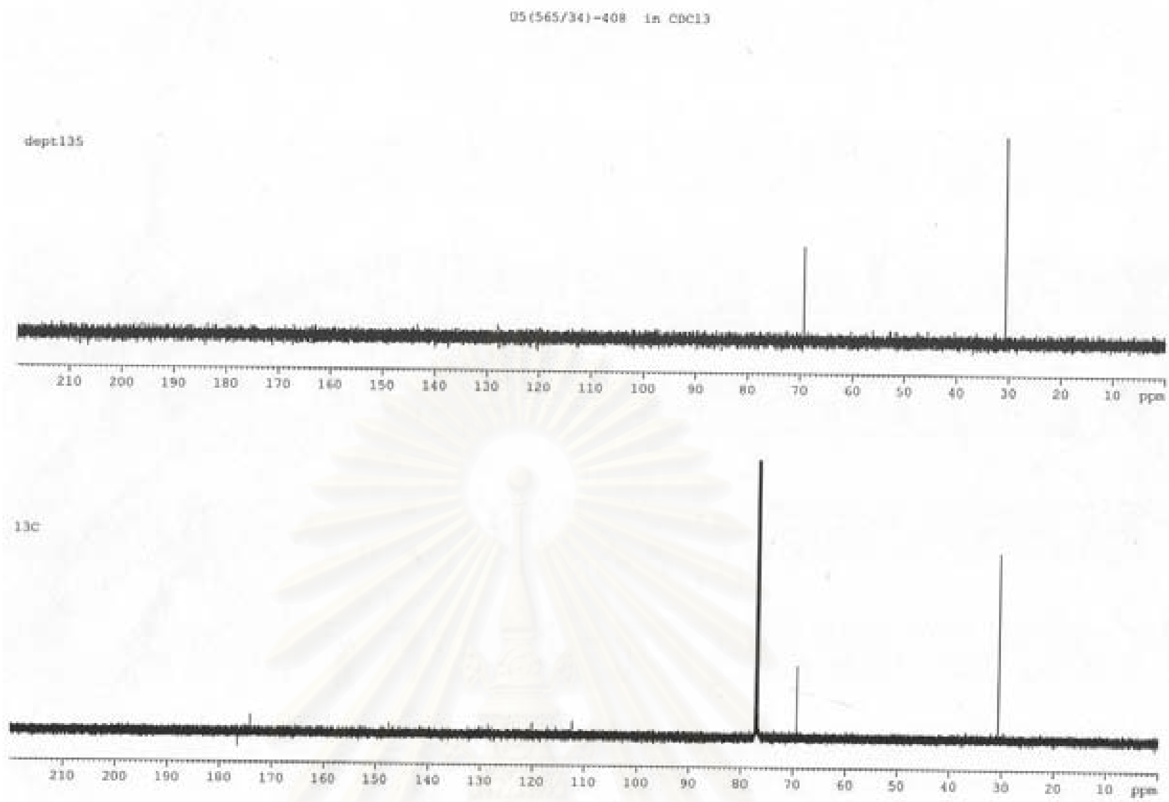


Figure C64 The DEPT 135 spectrum of compound U5B4-6

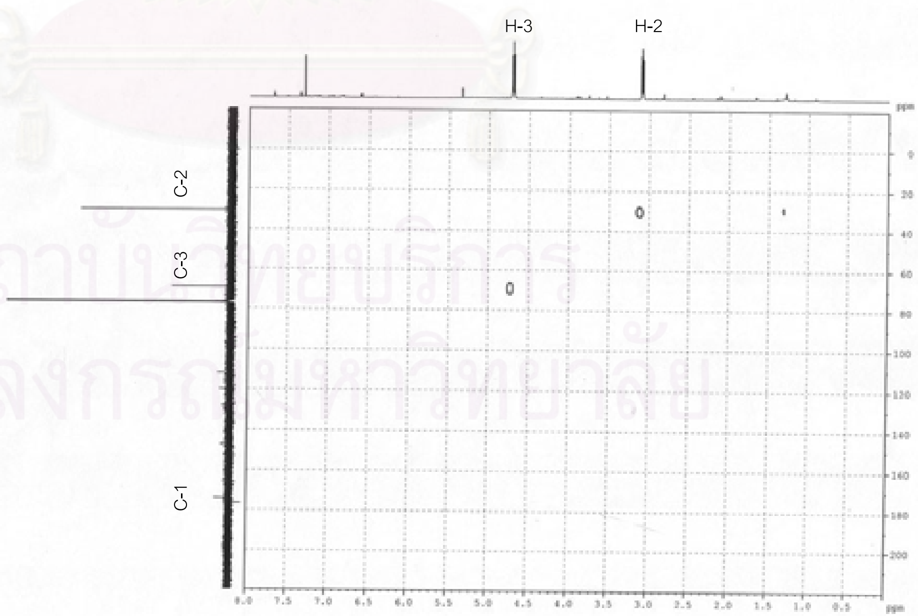


Figure C65 The HMQC spectrum of compound U5B4-6

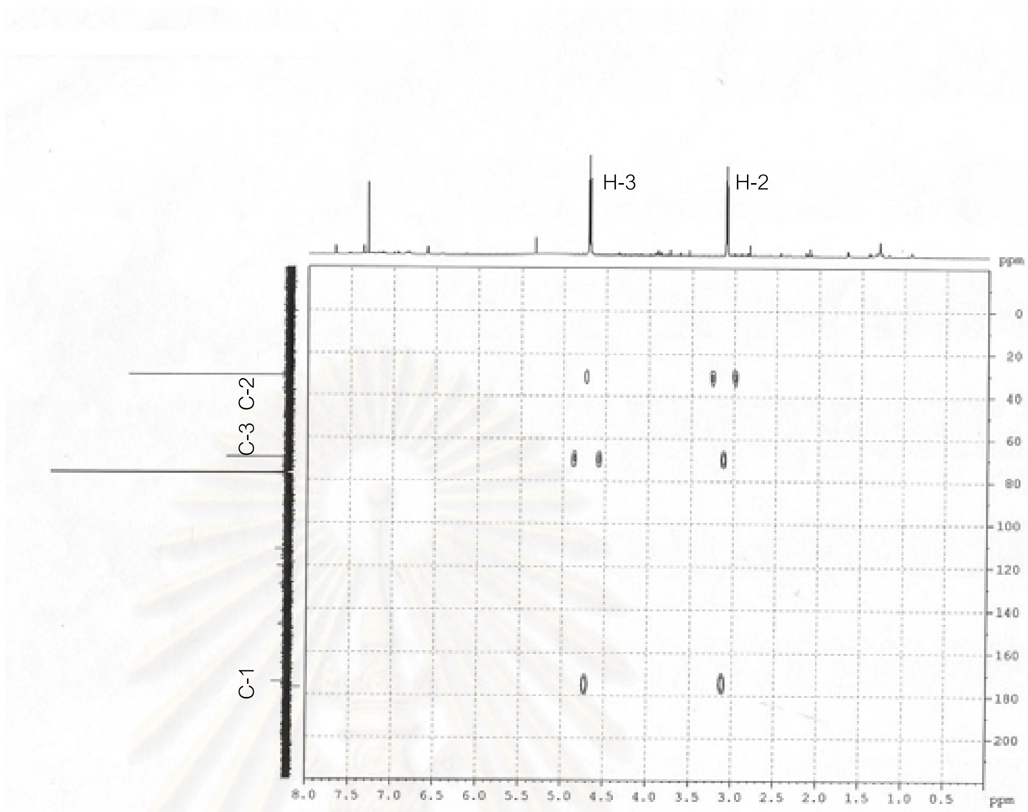


Figure C66 The HMBC spectrum of compound U5B4-6

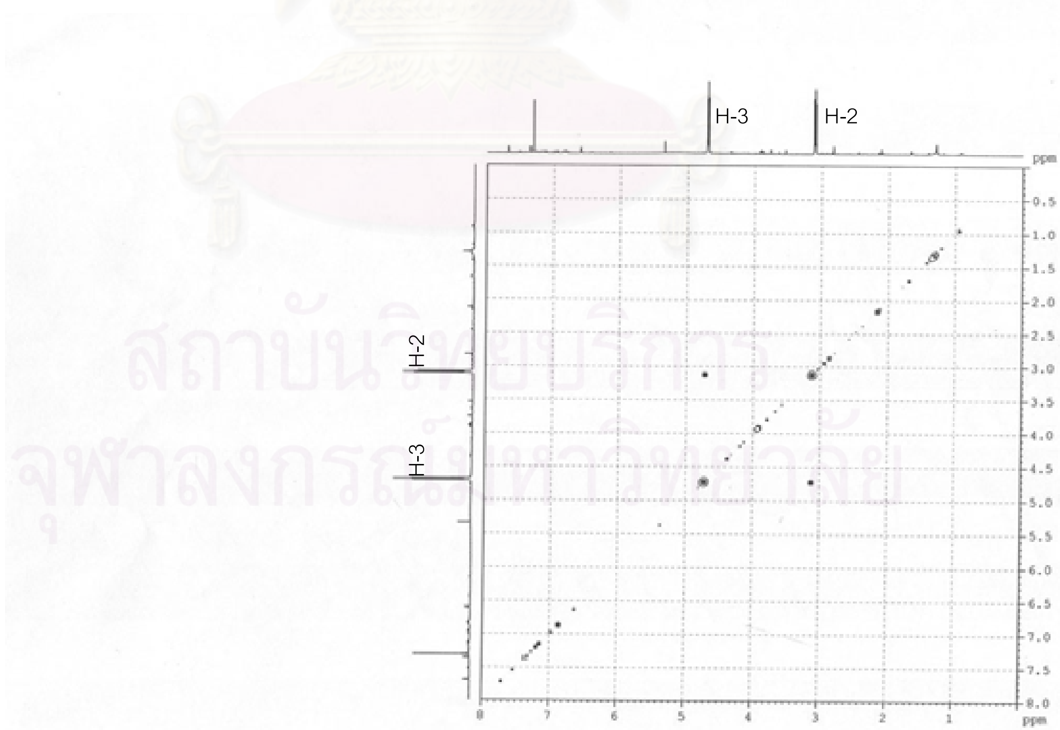


Figure C67 The ^{1}H-^{1}H COSY spectrum of compound U5B4-6

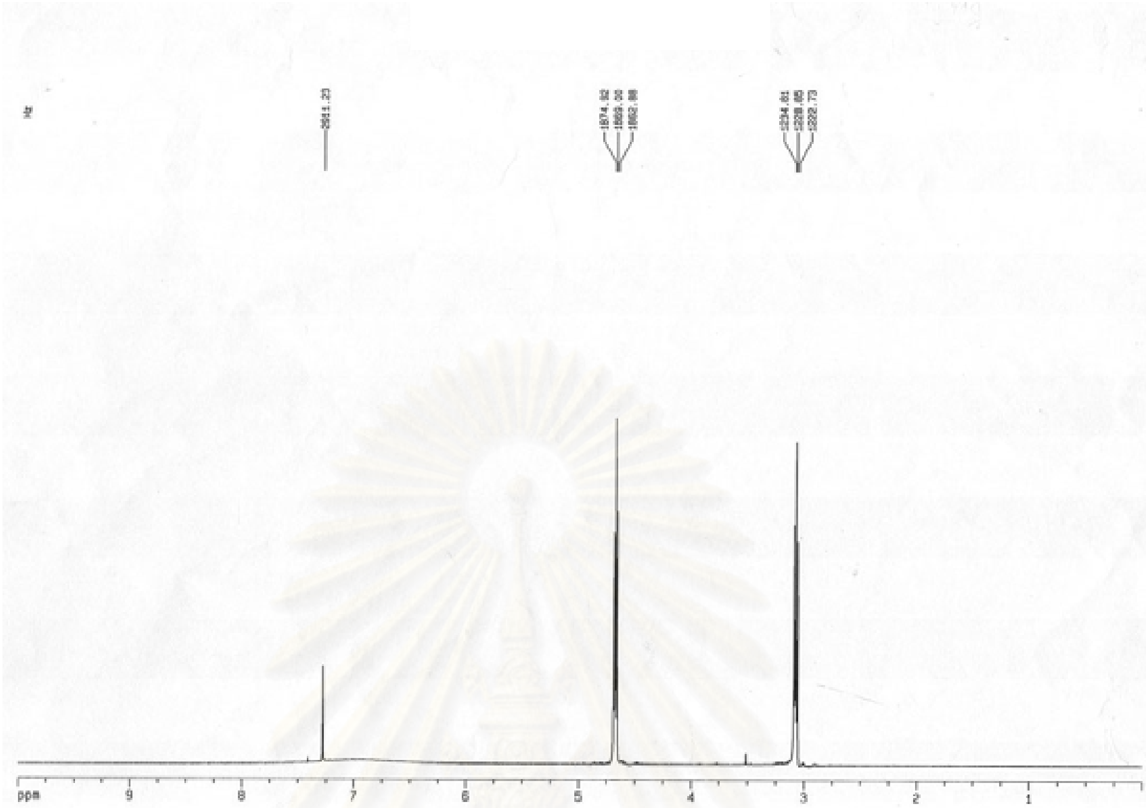


Figure C68 The 400 MHz ^{1}H-NMR (in $CDCl_3$) spectrum of 3-nitropropionic acid from Sigma

APPENDIX D

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            10         20         30         40         50
1C. allantoidiopsis TTGGAACGCG CTC----CGC ACCTCCAGAC AACCC-TTTG TGAACTTATA
USIA 5              CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. amygdali         CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCCATTTG TGAACTTATA
P. quercina         CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. magnoliae        CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. vaccinii         CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. junipervora      TTGGAACGCG CCCCAGGGGC ACC--CA-A- AACCC-TTTG TGAACTGATA
D. vaccinii         CTGGAA-GCC CCCCAGAAGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. asparagi         CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
D. caulivola        CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. bougainvilleicolaCTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. liquidambari     CTGGAACGCG CCCTAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. phyllanthicola   CTGGAACGCG CCCTAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. averrhoae        CTGGAACGCG CCCTAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
D. phaseolorum      CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
D. meridionalis     CTGGAACGCG CCCCAGGCGC ACC--CAGA- AACCC-TTTG TGAACTCATA
D. angelicae        CTGGAACGCG CC-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
D. arctii           CTGGAACGCG CC-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. chimonanthi      CTGGAACGCG CT-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. micheliae        CTGGAACGCG CT-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
D. helianthi        CTGGAACGCG CT-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. columnaris       CTGGAACGCG CT-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. glabrae          CTGGAACGCG CT-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. vexans           CTGGAACGCG CC-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
P. sclerotioides    CTGGAACGCG CT-TCGGCGC ACC--CAGA- AACCC-TTTG TGAACTTATA
                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            60         70         80         90        100
1C. allantoidiopsis CCTATACTGT TGCCTCGGCG TC-GGCTGGC CCCCCTCGG- -GGGGTCCC-
USIA 5              CCT-TACTGT TGCCTCGGCG -CAGGCTGGT CC---TCC-- -GGGGCCCC-
P. amygdaliae       CCT-TACTGT TGCCTCGGCG -CTAGCTGGT CC--TTC--- --GGGCCCC-
P. quercina         CCT-TACTGT TGCCTCGGCG -CTAGCTGGT CC--TTC--- -GGGGCCCC-
P. magnoliae        CCT-TACTGT TGCCTCGGCG -CTAGCTGGT CC--TTC--- -GGGGCCCC-
P. vaccinii         CCT-TACTGT TGCCTCGGCG -CTAGCTGG- -CCCCTC--- -GGGGCCCC-
P. juniperivora     CCT-TACTGT TGCCTCGGCG -CTAGCTGGT CC--TTC--- -GGGGCCCC-
D. vaccinii         CCT-TATCGT TGCCTCGGCG -CTAGCTGG- -CCCCTC--- -GGGGCCCC-
P. asparagi         CCT-TACCGT TGCCTCGGCG -CTAGCTGGT CC---TCC-- -GGGGCCCCC
D. caulivola        CCT-TACTGT TGCCTCGGCG -CAGGCCGGC CCCCTT-GG- --GGGCCCCC
P. bougainvilleicolaCCTTT--TGT TGCCTCGGCG -CATGCTGGT C--TCT-AGT AGG--CCCC-
P. liquidambari     CCT-TACTGT TGCCTCGGCG -CATGCTGG- CCCCCTC--- -GGGGCCCCC
P. phyllanthicola   CCT-TACTGT TGCCTCGGCG -CATGCTGGC CCCCTT---- -GGGGTCCCC
P. averrhoae        CCT-TACTGT TGCCTCGGCG -CATGCTGGT C--TCT-AGT AGG--CCCC-
D. phaseolorum      CCT-TATTGT TGCCTCGGCG T-ACGCTGG- -CCCCT-AG- --GGGTCCC-
D. meridionalis     CCT-TACTGT TGCCTCGGCG -CAGGCCGGC CCCCCC-AG- -GGGGCCCC-
D. angelicae        CCTATACTGT TGCCTCGGCG -CAGGCCGGC CTTTCTCGGT AAAGGCCCCC
D. arctii           CCCAAACTGT TGCCTCGGCG -CAGGCCGGC CCCTCTCGTT AGGGGCCCCC
P. chimonanthi      CCT--ATTGT TGCCTCGGCG TCAGGCCGGC CTC-TTCACT -GAGGCCCCC
P. micheliae        CCT--ATTGT TGCCTCGGCG TCAGGCCGGC CTC-TTCACT -GAGGCCCCC
D. helianthi        CCC--ACTGT TGCCTCGGCG -CAGGCCGGC CTC-TTCACT -GAGGCCCCC
P. columnaris       CCT-TACTGT TGCCTCGGCG -CAGGCCGGC ----CTCACT -GAGGCCCC-
P. glabrae          CCTATACTGT TGCCTCGGCG -CTGGCCGGC CTC-CTCACC -GAGGCCCCC
P. vexans           CCT--ATTGT TGCCTCGGCG -CAGGCCGGC CTCTCCTGGC AGAGGCCCCC
P. sclerotioides    CCT-TACTGT TGCCTCGGCG -CAGGCCGGC ----CTCACC -GAGGCCCC-
```

Figure D1 Alignment data of complete ITS1-5.8S-ITS2 sequences of isolate USIA 5 and 23 refernce taxa from GenBank (1*C* =*Cytospora*)

```
                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
1C. allantoidiopsis TCACCATCT- --CGGT---- ------GAGG AGCAGGCCCG CCGGCGGCCA
USIA 5              TCACCCGCCA C-GGGTGTT- GAGACAG--- ------CCCG CCGGCGGCCA
P. amygdali         TCACCCTC-- --GGGTGTT- GAGACAG--- ------CCCG CCGGCGGCCA
P. quercina         TCACCCTC-- --GGGTGTT- GAGATAG--- ------CCCG CCGGCGGCCA
P. magnoliae        TCACCCTC-- --GGGTGTT- ---------G AGACAGCCCG CCGGCGGCCA
P. vaccinii         TCACCCTC-- --GGGTGTT- GAGACGG--- ------CCCG CCGGCGGCCA
P. junipervora      TCACCCTC-- --GGGTGTT- GAGACAG--- ------CCCG CCGGCGGCCA
D. vaccinii         TCACCCTCG- ---GGT-T-- GAGACGG--- ------CCCG CCGGCGGCCA
P. asparagi         TCACC-TCGC CAGGGTGTC- --------GG AGAGAGCACG CCGGCGGCCA
D. caulivola        ---------- --------CG GAGAC-GGGG AGCAG-CCCG CCGGCGGCCA
P. bougainvilleicolaTCACCC---- --CGGTG-AG GAGACGG--- ------CACG CCGGCGGCCA
P. liquidambari     T--------- ---------G GAGACAG-GG AGCAGGCACG CCGGCGGCCA
P. phyllanthicola   T--------- ---------G GAGACAG-GG AGCAGGCACG CCGGCGGCCA
P. averrhoae        TCA--CTC-- --CGGTG-AG GAGA------ ---AGGCACG CCGGCGGCCA
D. phaseolorum      TCA--CTC-- --CGGT---- ------GAGG AGCAGGCGCG CCGGCGGCCA
D. meridionalis     TC-------- ---------G GAAAC-GAGG AGCAGGCCCG CCGGCGGCCA
D. angelicae        T--------- ---------G GAGACAG-GG AGCAG-CCCG CCGGCGGCCA
D. arctii           T--------- ---------G GAGACAG-GG AGCAG-CCCG CCGGCGGCCG
P. chimonanthi      -C-------- ---------G GAGAC-GGGG AGCAG-CCCG CCGGCGGCCA
P. micheliae        -C-------- ---------G GAGAC-GGGG AGCAG-CCCG CCGGCGGCCA
D. helianthi        T--------- ---------G GAAACAG-GG AGCAG-CCCG CCGGTGGCCA
P. columnaris       TC-------- ---------G GAAAC-GAGG AGCAG-CCCG CCGGCGGCCA
P. glabrae          T--------- ---------G GAGACAG-GG AGCAG-CCCG CCGGCGGCCA
P. mvexans          T--------- ---------G GAGACAG-GG AGCAGCTCCG CCGGCGGCCA
P. sclerotioides    TC-------- ---------G GAAAC-GAGG AGCAG-CCCG CCGGCGGCCG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            160        170        180        190        200
1C. allantoidiopsis AGTTAACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CAAAAATGAA
USIA 5              ACCTAACTCT TGTTTTTACA CTGAAACTCT GAGAAT-AAA CATAAATGAA
P. amygdale         ACCCAACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CATAAATGAA
P. quercina         ACCCAACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CATAAATGAA
P. magnoliae        ACCCAACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CATAAATGAA
P. vaccinii         ACCCAACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CATAAATGAA
P. junipervora      ACCCAACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CATAAATGAA
D. vaccinii         ACC-AACTCT TGTTTTTACA CTGAAACTCT GAGAATAAAA CATAAATGAA
P. asparagi         GCCTAACTCT TGTTTTTACA CTGAAACTCT GAGGATAAAA CATAAATGAA
D. caulivola        AGCTAACTCT TGTTTTTACA CTGAAACTCT GAGAAATAAA CATAAATGAA
P. bougainvilleicolaAGTTAACTCT TGTTTTTACA CTGAAACTCT GAGAAAAAA- CACAAATGAA
P. liquidambari     AGTTAACTCT TGTTTTTACA CTGAAACTCT GAGAAAAAAA CACAAATGAA
P. phyllanthicola   AGTTAACTCT TGTTTTTACA CTGAAACTCT GAGAAAAAA- CACAAATGAA
P. averrhoae        AGTTAACTCT TGTTTTTACA CTGAAACTCT GAGAAAAAA- CACAAATGAA
D. phaseolorum      AGTTAACTCT TGTTTTTACA CTGAAACTCT GAGAAAAAAA CA-AAATGAA
D. meridionalis     AGCCAACTCT TGTTTTTACA CCGAAACTCT GAGCAAAAAA CACAAATGAA
D. angelicae        GCCAAACTCT -GTTTCTATA GTGGATCTCT GAGTAAAAAA CATAAATGAA
D. arctii           ACCAAACTCT -GTTTCTATA GTGAATCTCT GAGTAAAAAA CATAAATGAA
P. chimonanthi      ACTAAACTCT TGTTTCTATA GTGAATCTCT GAGTAAAAAA CATAAATGAA
P  micheliae        ACTAAACTCT TGTTTCTATA GTGAATCTCT GAGTAAAAAA CATAAATGAA
D. helianthi        ACTAAACTCT -GTTTCTATA GTGAATCTCT GAGTAAAAAA CATAAATGAA
P. columnaris       ACCAGACTCT TGTTTCT-TA GTGGATCTCT GAGTAAAAAA CATAAATGAA
P. glabrae          AACAAACTCT TGTTTCT-TA GTGAATCTCT GAGTAAAAAA CATAAATGAA
P. vexans           GCTAAACTCT TGTTTCTACA GTGAATCTCT GAGTAAAAA- CATAAATGAA
P. sclerotioides    ACCAAACTCT TGTTTCT-CA GTGGATCTCT GAGTAAAAAA -AAAAATGAA
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Figure D1 (Continued)

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             210        220        230        240        250
1C. allantoidiopsis TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
USIA 5              TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. amygdali         TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. quercina         TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. magnoliae        TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. vaccinii         TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. junipervova      TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
D. vaccinii         TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. asparagi         TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
D. caulivola        TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. bougainvilleicolaTCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. liquidambari     TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. phyllanthicola   TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. averrhoae        TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
D. phaseolorum      TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGG TGAAGAACGC
D. meridionalis     TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
D. angelicae        TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
D. arctii           TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. chimonanthi      TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. micheliae        TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
D. helianthi        TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. columnaria       TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. glabrae          TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. vexans           TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
P. sclerotioides    TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           260        270        280        290        300
1C. allantoidiopsis AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
USIA 5              AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. amygdali         AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. quercina         AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. magnoliae        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. vaccinii         AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. junipervora      AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. vaccinii         AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. asparagi         AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. caulivola        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. bougainvilleicolaAGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. liquidambari     AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. phyllanthicola   AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. averrhoae        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. phaseolorum      AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. meridionalis     AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. angelicae        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. arctii           AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. chimonanthi      AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. micheliae        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
D. helianthi        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. columnaris       AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. glabrae          AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. vexans           AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. sclerotiodes     AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
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Figure D1 (Continued)

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          310        320        330        340        350
1C. allantoidiopsis TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGAAGGGC ATGCCTGTTC
USIA 5              TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. amygdali         TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. quercina         TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. magnoliae        TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. vaccinii         TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. junipervora      TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
D. vaccinii         TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. asparagi         TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
D. caulivola        TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. bougainvilleicolaTCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. liquidambari     TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. phyllanthicola   TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. averrhoae        TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
D. phaseolorum      TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
D. meridionalis     TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
D. angelicae        TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
D. arctii           TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. chimonanthi      TCTTTGAACG CACATTGCGC CCCCTGGTAT TCCGGGGGGC ATGCCTGTTC
P. micheliae        TCTTTGAACG CACATTGCGC CCCCTGGTAT TCCGGGGGGC ATGCCTGTTC
D. helianthi        TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. columnaris       TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. glabrae          TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. vexans           TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC
P. sclerotioides    TCTTTGAACG CACATTGCGC CCTCTGGTAT TCCGGAGGGC ATGCCTGTTC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          360        370        380        390        400
1C. allantoidiopsis GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACTTGCCTT
USIA 5              GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCTTT
P. amygdali         GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GC-TT
P. quercina         GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GC-TT
P. magnoliae        GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCTTC
P. vaccinii         GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCTT
P. junipervora      GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GC-TT
D. vaccinii         GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCTT
P. asparagi         GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCT-
D. caulivola        GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGTTGGGG CACT-GCCT-
P. bougainvilleicolaGAGCGTCATT TCAACCCTCA AGCACTGCTT GGTGTTGGGG CACT-GC-TT
P. liquidambari     GAGCGTCATT TCAACCCTCA AGCATTGCTT GGTGTTGGGG CACT-GCCT-
P. phyllanthicola   GAGCGTCATT TCAACCCTCA AGCATTGCTT GGTGTTGGGG CACT-GC-TT
P. averrhoae        GAGCGTCATT TCAACCCTCA AGCATTGCTT GGTGTTGGGG CACT-GC-TT
D. phaseolorum      GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGTTGGGG CACT-GCCT-
D. meridionalis     GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGTTGGGG CACT-GCCT-
D. angelicae        GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCT-
D. arctii           GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCT-
P. chimonanthi      GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GC-TT
P. micheliae        GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GC-TT
D. helianthi        GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCT-
P. columnaris       GAGCGTCATT TCAACCCTCA AGCACTGCTT GGTGTTGGGG CACC-GCCT-
P. glabrae          GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGTTGGGG CACC-GCCTT
P. vexans           GAGCGTCATT TCAACCCTCA AGCCTGGCTT GGTGATGGGG CACT-GCCT-
P. sclerotioides    GAGCGTCATT TCAACCCTCA AGCACTGCTT GGTGTTGGGG CACC-GCCT-
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Figure D1 (Continued)

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             410        420        430        440        450
1C. allantoidiopsis CGGTAA-GAA ---GGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
USIA 5              T---ACACAA A--AGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. amygdali         T--TACCCAA --GAGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. quercina         T--TACCCAA --GAGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. magnoliae        T--TACCCAA -GAAGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. vaccinii         ---TACCCAA A-G-GCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. junipervora      T--TACCCAA --GAGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
D. vaccinii         ---TACAGAA A-GGGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. asparagi         -G-TA---AA A-GGGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
D. caulivola        -G-TA---AA A-GGGCAGGC CCTGAAATTC ATTGGCGAGC TCGCCAGGAC
P. bougainvilleicola--TAACG-- --GAGCAGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. liquidambari     -G-TA---AA A-GGGCAGGC CCTGAAATCT AGTGGCGAGC TCGCTAGGAC
P. phyllanthicola   T--TAACCAA ----GCAGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. averrhoae        T--TAACGAA ----GCAGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
D. phaseolorum      -GTTA---AA --GGGCAGGC CCTCAAATAT AGTGGCGAGC TCGCCAGGAC
D. meridionalis     -G-TA---AA A-GGGCAGGC CCTGAAATCT AGTGGCGGGC TCGCCAGGAC
D. angelicae        -G-T---GAA A-GGGCAGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
D. arctii           -GTT----AA A-GGGCAGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. chimonanthi      CG------AA AGGAGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
P. micheliae        CG------AA AGGAGCAGGC CCTGAAATTC AGTGGCGAGC TCGCCAGGAC
D. helianthi        -G-TA---AA A-GGGCAGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. columnaris       -G-TA---AA A-GGGCGGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. glabrae          TG----CAAA A-GGGCGGGC CCTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. vexans           -G-T---GAA A-GGGCAGGC CTTGAAATCT AGTGGCGAGC TCGCCAGGAC
P. sclerotioides    -G-TA---AA A-GGGCGGGC CCTGAAATCT AGTGGCGAGC TCGCCGGGAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             460        470        480        490        500
1C. allantoidiopsis CCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGACTGTACT GGTGCGG-GC
USIA 5              CCCGAGCGCA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
P. amygdale         CCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGAAGGCCCT GG--CGGTGC
P. quercina         CCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGAAGGCCCT GG--CGGTGC
P. magnoliae        CCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGAAGGCCCT GG--CGGTGC
P. vaccinii         CCCGAGCGCA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
P. junipervora      CCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGAAGGCCCT GG--CGGTGC
D. vaccinii         CCCGAGCGCA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GGCGCGGTG-
P. asparagi         CCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGAAGGCCCT GG--CGGTGC
D. caulivola        CCCGAGCGTA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
P. bougainvilleicolaCCCGAGCGCA GTAG-TTAAA CCCTCGCTCT GGAAGGCCCT GG--CGGTGC
P. liquidambari     CCCGAGCGTA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
P. phyllanthicola   CCCGAGCGCA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
P. averrhoae        CCCGAGCGTA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
D. phaseolorum      CCCGAGCGTA GTAG-TTAAA CCCTCGCTTT GGAAGGCCCT GG--CGGTGC
D. meridionalis     CCCGAGCGCA GTAG-TTAAA CCCTCGCTC- GGGAGGCCCT GG--CGGTGC
D. angelicae        CCCGAGCGTA GTAG-TTACA -TCTCGCTCT GGGAGGCCCT GG--CGGTGC
D. arctii           CCCGAGCGTA GTAG-TTACA -TCTCGCTCT GGAAGGCCCT GG--CGGTGC
P. chimonanthi      CCCGAGCGTA GTAG-TTATA -TCTCGCTTT GGAAGGCCCT GG--CGGTGC
P. micheliae        CCCGAGCGTA GTAG-TTATA -TCTCGCTTT GGAAGGCCCT GG--CGGTGC
D. helianthi        CCCGAGCGTA GTAG-TTATA -TCTCGCTCT GGAAGGCCCT GG--CGGTGC
P. columnaris       CCCGAGCGTA GTAA-TTATA -TTTCGTTCT GGAAGGCCCT GG--CGGTGC
P. glabrae          CCCGAGCGTA GTAG-TTATA -TCTCGTTCT GGAAGGCCCT GG--CGGTGC
P. vexans           CCCGAGCGTA GTAG-TATTA -TCTCGCCCT GGAAGGCCCT GG--CGGTGC
P. sclerotioides    CCCGAGCGTA GTAAATTATA -TTTCGTTCT GGAAGGCCCC GG--CGGTGC
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Figure D1 (Continued)

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                    ....|....| ....|....| ....|....|
                             510        520       530
1C. allantoidiopsis CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
USIA 5              CCTGCCGTTA AACCCCC-AA CCTTTGAAAA
P. amygdale         CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. quercina         CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. magnoliae        CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. vaccinii         CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. junipervora      CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
D. vaccinii         -CTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. asparagi         CCTGCCGTTA AACCCCC-AA CTTTTGAAAA
D. caulivola        CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. bougainvilleicolaCCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. liquidambari     CCTGCCGTTA AACCCCC-AA CTTTTGAAAA
P. phyllanthicola   CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. averrhoae        CCTGCCGTTA AACCCCC-AA CTTTTGAAAA
D. phaseolorum      CCTGCCGTTA AACCCCC-AA CTTTTGAAAA
D. meridionalis     CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
D. angelicae        CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
D. arctii           CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. chimonanthi      CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. micheliae        CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
D. helianthi        CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. columnaris       CCTGCCGTTA AACCCCC-AA CTTCTGAAAA
P. glabrae          CCTGCCGTTA AACCCCC-AA CTTCTGAAAT
P. vexans           CCTGCCGTTA AACCCCCCAA CTCCTGAAAA
P. sclerotioides    CCTGCCGTTA AACCCCC-AA CTCCTGAAAA
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Figure D1 (Continued)

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                    .....|....|.....|....|.....|....|.....|....|.....|....|
                              10        20        30        40        50
Ustillago sparsa     C-GA----TG AAAC-CC-TT TTTTCTTGAG GTGTGGCT-- CGCACCT-GT
Agaricus abrupti     TTGAATTATG TTTCTAAATG GGTTGTAGCT GGCT--CTTT AGAGCAT-GT
LRUB 20              TTGAAACGGT TGCCCTCGCG GTG---ACCG GTT---CTTC --------AA
4C. fuckeli          TCCATC--TC AACC-AGGTG CGGT--CGCG G-----CCCT CGG-----GG
Myrothecium sp.      TCTAT---TC CATG-AGGTG CGGT--CGCG G-----CCCT CGG----CGG
Paraphaeosphaeria sp.CCAAT---TC AAC---GGTG TGGT--CGCG G-----CCTC CGG-----GG
4C. minitans         TCCATCC-TT AAC--AGGTG CGGT--CGCG G-----CCCC TGG-----GG
P. pilleata          TCCATCT-TT AACC-AGGTG CGGT--CGCG G-----CCTC CGG-----GT
2M. terrestris       --GAAAAGGG TGCC-TCGCG GCCC--CGAT T--------- -----CTCAA
Aspergillus flavipes CCGAGTGAGG GTCC-TCGTG GCCC--AAC- ---------- ----------
1C. cetrarioides     CCGAGAGCGG GGCT-TCATG CTCC--CGGA GG----CTTC -GG-CCTCTA
1C. chicitae         CCGAGAGCGG GGCT-TCATG CCCC--CGGA GG----CTCC -GG-CCTCTA
1C. braunsiana       CCGAGAGCGG GGCT-CTATG CTCC--CGGA GG----CTTC -GG-CCTCTA
1C. japonica         CCGAGAGCGG GGCT-CTATG CTCC--CGGA GG----CTTC -GG-CCTCTA
1P. quernea          CTGAGAGAGG GGCT-TCGCG CCCC--CGGG GG----CTCC -GG-CCTCCA
3C. prancei          CGGCGGGTGT TTGT-CCAAG CCCT--AGCG GG----CTT- -GGACAGCGA
3C. corallifera      CGGCGGGTGT TTGT-CCAAG CCCT--AGTG GG----CTT- -GGACAGCGA
Lobaria amplissima   TCGAGAACGA GGCG-CCCCG CCTC--CGGG GGGG--CTCC -GGCCCCCCC
Cetraria odontella   CTGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----TCTC -GG-CCTCTA
Cetraria nigricans   CTGAGAGAGG GGCT-TCGCG CTCC--TGGG GG----TCTC -GG-CCCCTA
Oropogon sp.         CCGAGAGAGG GGCT-CCGCG CCCC--CGGG GG----CTTC -GG-CCCTCG
Sulcaria sulcata     CCGAGAGAGG GGCT-CCGCG CCCC--CGGG GG----CTTC -GG-CCCTCG
Cetraria leucostigma CCGAGAGAGG GGCT-TCGCG CCCC--CGGA GG----CTCC -GG-CCTCCA
Cetraria melalom     CCGAGAGAGG GGCT-TCGCG CCCC--CGGA GG----CTCC -GG-CCTCCA
Tuckneraria ahtii    CCGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----CTAC -GA-CCCTCA
T. pseudocomplicata  ATGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----CTTC -GG-CCCTCA
N. morrisonicola     ATGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----CTTC -GGGCCCTCA
N. pallescens        CTGAGAGAGG GGCT-CCGCG CTCC--CGGG GG----CTCC -GG-CCCCCA
N. stracheyi         CTGAGAG--G GGCT-TCGCG CTCC--CGGG GG----CTCC -GG-CCCCCC
Tuckneraria laureri  ATGAGAGAGG GCCT-CCGCG CTCC--CGGG GG----CTTC -GG-CCCCTA
Ahtiana pallidula    CTGAGAGAGG GGCC-TCGTG CTCC--CGGG GG----CTCC CG--CCTCCA
A. nigricascens      TTGAGAGAGG GGCT-TCGTG CTCC--CGGG GG----TTTC -GG-CCTCCA
Cetraria nivalis     CTGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----CTTC -GG-CCTCCA
K. merrillii         TCGAGAGAGG GGCT-TCGTG CTCC--CGGG GG----TTTC -GG-CCTCCA
1A. oakesiana        CTGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----TTTC -GG-CCTCTA
F. cucullata         CTGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----TTTC -GG-CCTCTA
M. richardsonii      CTGAGA--GG GGCT-TCGCG CTCC--CCGG GG----CTTC -GG-CCCCTA
2C. islandica        CTGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----TCTC -GG-CCCCTA
2C. crispiformis     CTGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----TCTC -GG-CCCCTA
2C. antarctica       CTGAGAGAGG GGCT-TCGCG CTCC--TGGG GG----TCTC -GG-CCCCTA
Cetraria sepinco     CTGAGA--GG GGCT-TCGCG CTCC---GGG GG----TCTC -GG-CCCCCA
1M. fuliginosa       CCGAGAGAGG GGCT-TCGCG CTCC--CGGG GG----TTTC -GG-CCCCCG
1M. subauri          CCGAGAGAGG GGCT-TCGGG CTCC--GGGG GG----TTTC -GG-CCCCCG
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 Alignment data of complete ITS1-5.8S-ITS2 sequences of isolate LRUB 20 and 42 refernce taxa from GenBank.

```
                         ....|....|.....|....|.....|....| ....|....|.....|....|
                                   60         70         80         90        100
Ustillago sparsa         CTAACTAAAC TTGAGCTA-- CCTTTTTTCA ACACG--GTT G-CATCGGTT
Agaricus abrupti         GCACGCCTGT TTGGACTT-- CATTTTCAT- CCACC--TGT G-CACCTATT
LRUB 20                  AC--CTCT-- ---------G CGTACCAA-A CCTTTCAGTT G------CCT
4C. fuckeli              G----TTCTC TCCC-GGGTG -GTAGGGGTA ---------- -ACAC--CCT
Myrothecium sp.          GAGCA----- ACAGCTGCCG TCGGGCGGTA GAGGTA---- -ACACT--TT
Paraphaeosphaeria sp.CTCC CCCC-GGGCG -GTAGAGGTA ---------- -ACACT--CT
4C. minitans             G-----CTCC -CCC-AGGTG -GTA-AGGT- GAAATA---- --CCCT--AT
P. pilleata              GAAAG----- -CCC-GGGTG -GTTT----- ---------- ----------
2M. terrestris           AC-------C AC---TGTT- --TACCAA-A CGTTTC-GTT G-------CC
Aspergillus flavipes     ----CTCC-C ACCC--GT-G ACTACTGT-A CCACT--GTT G-------CT
1C. cetrarioides         AC---TCTTC ACCCC-ATTG CCTATCT-TA CCTTT--GTT G-------CT
1C. chicitae             AC---TCTTC ACCCC-ATTG CCTATCT-TA CCTTT--GTT G-------CT
1C. braunsiana           AC---TCTTC ACCCA-AT-G CCTACCT-TA CCTTT--GTT G-------CT
1C. japonica             AC---TCTTC ACCCG-AT-G CCTACCT-TA CCTTT--GTT G-------CT
1P. quernea              AC---TCTTC ACCC--TTTG ACTACC--TA -CTTT--GTT G-------CT
3C. prancei              TC-------- ------GTCG TGTATCTCGA CCCCAT-GTT TACCATACCT
3C. corallifera          TC-------- ------GTCG TGTATCTCGA CCCCAT-GTT TACCATACCT
Lobaria amplissima       AC--CTCTTC ACCC--GATG GGTACC--CA GCAGC--GTT T-------CT
Cetraria odontella       AC---TCTTC ACCC--TTTG CGTACC--AA CCTTT--GTT G-------CT
Cetraria nigricans       AC---TCTTC ACCC--TTTG CGTACC--AA CCTTT--GTT G-------CT
Oropogon sp.             AC---TCTTC CCCC--TCTG CGTACCC-TA CCTTT--GTT G-------CT
Sulcaria sulcata         AC---TCTTC CCCC--TCTG CGTACCC-TA CCTTT--GTT G-------CT
Cetraria leucostigma     AC---TCTTC ACCC--GTTG CCTACC--TA CCTTT--GTT G-------CT
Cetraria melalom         AC---TCTTG ACCC--GTTG CCTACC--TA CCTTT--GTT G-------CT
Tuckneraria ahtii        AC---TCTTC ACCC--ACTG TCTACC--TA CCTTT--GTT G-------CT
T. pseudocomplicata      AC---TCTTC ACCC--GTGG ACTATC--TA CCTTT--GTT G-------CT
N. morrisonicola         AT---TCTTC ACCC--ATTG TCTACC--TA CCTTT--GTT G-------CT
N. pallescens            AC---TCTTC ACCC--GTTG TCTACC--TA CCTTC--GTT G-------CT
N. stracheyi             AC---TCTTC ACCC--GTTG TCTACC--TA CCTTT--GTT G-------CT
Tuckneraria laureri      AC---TCTTC ACCC--TTTG TCTACC--TA CCTTT--GTT G-------CT
Ahtiana pallidula        AC---TCTTC ACCC--ATTG TCTACC--TA CCTAT--GTT G-------CT
A. nigricascens          AC---TCTTC ACCC--ATTG TCTACC--TA CCTTT--GTT G-------CT
Cetraria nivalis         AC---TCTTC ACCC--ATTG TCTACC--TA CCTAT--GTT G-------CT
K. merrillii             AC---TCTTC ACCC--ATTG TCTACC--TA CCTAT--GTT G-------CT
1A. oakesiana            GC---TCTTC GCCC--ATTG TCTACA--TA CCTTT--GTT G-------CT
F. cucullata             GC---TCTTC ACCC--ATTG TCTACA--TA CCTTT--GTT G-------CT
M. richardsonii          AC---TCTTC ACCC--ATTG TCTACA--TA CCTTT--GTT G-------CT
2C. islandica            AC---TCTTC ACCC--TTTG TGTACC--AA CCTCT--GTT G-------CT
2C. crispiformis         AC---TCTTC ACCC--TTTG TGTACC--AA CCCTT--GTT G-------CT
2C. antarctica           AC---TCTTC ATCC--TTTG TGTACC--AA CCTTT--GTT G-------CT
Cetraria sepinco         AC---TCTTC ACCC--ATTG ACTACC--TA CCTTT--GTT G-------CT
1M. fuliginosa           AC---TCTTC ACCC--GTTG CATACCA-TA CCTTT--GTT G-------CT
1M. subauri              AC---TCTTC ACCC--GTTG CATATCG-TA CCTTT--GTT G-------CT
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

```
                         ....|....| ....|....| ....|....| ....|....| ....|....|
                                110        120        130        140        150
Ustillago sparsa         GGCCTGTCAA ACAGTGCG-G CGGTCGC-GA AATTGATTTT TC-GCAGCTG
Agaricus abrupti         GTA--GTCTT TGGTTGGGTT AGGAGGAAGT GGTCATCCTG TCAGCATTTG
LRUB 20                  CCGGCGGCCC T---GGGCC- GGCGC----- GGC---GCGC G-ACCTCCC-
4C. fuckeli              CACGCGCCGC ----ATTCC- TGCATCCTTT TTTTACGAGC --ACCTTTCG
Myrothecium sp.          CACGCGCCGC ----ATGTC- TGAATCCTTT TTTTACGAGC --ACCTTTCG
Paraphaeosphaeria sp.TACGCGCCAC ----ATGTC- TGAATCCTTT TTTTACGAGC --ACCTTTCG
4C. minitans             --CGCGCCGC ----ATACC- TGCATCCTTT TTTTACGAGC --ACCTTTCG
P. pilleata              --CGCGCCGC ----ATTCC- TGCACCCTTT TTATACGAGC --ACCTTTCG
2M. terrestris           TCGGCGGGCC -----GGCCA -------TTT GGCT-CGACC --AGCGGCCC
Aspergillus flavipes     TCGGCGGGCC CGCCA-GCC- TAGCT----- GGC--CG-CC G-GGGGGC--
1C. cetrarioides         TTGGCGGGCC -TCGAGGTTC ----CCTC-- GCG-CCGACC C-TCGGGTCG
1C. chicitae             TTGGCGGGCC -TCGAGGTCC ----CCTC-- GCG-CCGACC C-TCGGGTCG
1C. braunsiana           TTGGCGGGCC -TCGGGGTCT ----CCTC-- GCG-CTGACC T-TCCGGTCG
1C. japonica             TTGGCGAGCC -TCGGGGTCT ----CCCC-- GCG-TTGGCC T-TTGGGTCG
1P. quernea              TTGGCGGGAC -TTGGGGCAA ---GCCTC-- ACA-CCGGCT TCTCCGGCCG
3C. prancei              TTTGTTGCTT TGGCGGGCCT TGAGTA---- GGCTATACGG CTCATGCCAG
3C. corallifera          TTTGTTGCTT TGGCGGGCCT TGAGTA---- GGCTATACGG CTCATGCCAG
Lobaria amplissima       TTGGCGG--C TCGCACGCC- ---G-CCC-- GAAGACCCCC CCCCAAACTC
Cetraria odontella       TTGGCGGGCC --CGAGGACC T--CTC---- GCG-CCG--C GTACAAACCG
Cetraria nigricans       TTGGCGGGCC --CGAGGACC T--CTC---- GCG-CCG--C GTACAAACCG
Oropogon sp.             TTGGCGGG-T CCCGGGGCTT G--CTCCC-- GCA-CCGGCC GCGCC---CG
Sulcaria sulcata         TTGGCGGG-T CCCGGGGCTT G--CTCCC-- GCA-CCGGCC GCGCC---CG
Cetraria leucostigma     TTGGCGGG-T CTCGGGTACC ---ATCCC-- GTG-CCGACC G-ACCGGTCG
Cetraria melalom         TTGGCGGG-T CTCGGGTACC ---ATCCC-- GTG-CCGACC G-ACCGGTCG
Tuckneraria ahtii        TTGGCGGGCC -TCGGGTACC ---CTCCC-- GTG-CCGACT T-ACCGGTCG
T. pseudocomplicata      TTGGCGGGCC -TCGGGCACC ---ATCCC-- GTG-CCGACC G-ACCGGTCG
N. morrisonicola         TTGGCGGGCC -TCGGGCATC ---TTCCC-- GTG-CCGGCC G-ACCGGTCG
N. pallescens            TTGGCGGGCC -TCGGGTACC ---ATCCT-- GTG-CCGGCC C-AGCGGTCG
N. stracheyi             TTGGCGGGCC -TCGGGTACC ---ATCCC-- GTG-CCGGCT G-ATCGGTCG
Tuckneraria laureri      TTGACGGG-T CTCGGACATC ---GTTCC-- GTG-CCGACC C-ACCGGTCG
Ahtiana pallidula        TTGGCGGGCC -TCGGGTACC ---ATCCC-- GTG-TCGGCC T-ACCGGCCG
Cetraria nivalis         TTGGCGGGCC -TCGGGTACC ---ATCCC-- GTG-TCGGCC T-ACCGGTCG
K. merrillii             TTGACGGGTC -TCGGGTACC ---ATCCC-- GTG-TCGGCT T-ACCGGTCG
1A. oakesiana            TTGGCGGGCC -TCGGGCACC ---GTCCC-- GTG-TCGACT G-ACTGGTCG
F. cucullata             TTGGCGGGCC -TCGGGCACC ---GTCCC-- GTG-TCGACT G-ACTGGTCG
M. richardsonii          TTGGCGGG-T CTCGGG-GTT ---ATCCC-- GCG-TCGGCT T-TCGGGTCG
2C. islandica            TTGGCGGG-T C-CGAGGACC ----TCTC-- GCG-CCG-CC C-ACAGGCCG
2C. crispiformis         TTGGCGGG-T C-CGAGGACC ----TCTC-- GCG-CCG-CC C-ACAGGCCG
2C. antarctica           TTGGCGGG-T C-CGAGGACC ----TCTC-- GCG-CCG-CC C-CCAGGCCG
Cetraria sepinco         TTGGCGGGCC C-CGAGGACC ----TCTC-- GCG-CCG-CG T-ACAGGCCG
1M. fuliginosa           TTGGCGGGCC C-CGGG--TC ---GCCCC-- GCG-CCGGCC T-CTGGGCCG
1M. subauri              TTGGCGGACC C-CGGG--TC ---GCCCC-- GCG-CTGGTT T-TCGGGCCG
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

```
                         ....|....| ....|....| ....|....| ....|....| ....|....|
                                  160        170        180        190        200
Ustillago sparsa         CCCAACTCGG CGACGGACC- GACACTTTTT ACCAAACACT TTT-GATGAT
Agaricus abrupti         CTGGATGTGA GGACTTGCAT TGTGAAAACT GTGC-TGTCT TTATG-TGAT
LRUB 20                  ---CCT-CGC GGG-CGGGGC CGCTCCTC-- --GCG-GCG- GACCACCCGC
4C. fuckeli              TTCT--CC T--TCGGCGG GGC----AAC CTGCC-GCT- ----------
Myrothecium sp.          TTCT--CC T--TCGGCGG GGC----AAC CTGCC-GTT- ----------
Paraphaeosphaeria sp.--TTCT--CC T--TCGGTGG GGC----AAC CTGCC-GTT- ----------
4C. minitans             --TTCT--CC T--TCGGCGG GGC----AAC CTGCC-GCT- ----------
P. pilleata              --TTCT--CC T--TCGGCGG GGC----AAC CTGCC-GCT- ----------
2M. terrestris           CCCCCTCCGC CCCTCGGGGC GAGGA--AGG GAGCA-GCCC GCCCA-----
Aspergillus flavipes     --TTCT--GC CCC-CGGGCC CGCGC----- ---CC-GCC- ----------
1C. cetrarioides         GCGAGCGCCC --GCCAGAGG TCC--ATTAA AT--TCTATT T-ATC-----
1C. chicitae             GCGAGCGTCC --GCCAGAGG TCC--ATTAA AT--TCTACT TT--------
1C. braunsiana           GCGAGTGTCC --GTCAGAGG TCC--ATTAA AT--TCTATT T-ATC-----
1C. japonica             GCGAGTGTCC --GTCAGAGG TCC--ATTAA AT--TCTATT T-ATC-----
1P. quernea              GTGAGCGTCC --GTCAGAGG CCCCCTTTAA A----CTCTT T-ATC-----
3C. prancei              CCCCCAGCGT T-TTCTTGCT GG-----AGG GGGCTCGCGC CCGCC-----
3C. corallifera          CCCCCAGCGT T-TTCTTGCT GG-----AGG GGGCTCGCGC CCGCC-----
Lobaria amplissima       CAGTGATCCC T-GTC-GTC- GGAGCC---- ATA-TCGAAT ACGCA-----
Cetraria odontella       GCGAGCGCCC --GCCAGAGG CCC--ATTAA AA--TCTGCT T-ATT-----
Cetraria nigricans       GCGAGCGCCC --GCCAGAGG CCC--ATTAA AA--TCTGCT T-ATT-----
Oropogon sp.             GTGAGCGCCC --GCCAGAGG CCT--ATTGC AT--TCCGAT TTATC-----
Cetraria leucostigma     GCGAGCGCCC --GTCAGAGG CCC--ATCAA AT--TCT-CT T---C-----
Cetraria melalom         GCGAGCGCCC --GTCAGAGG CCC--ATCAA AT--TCT-AT T---C-----
Tuckneraria ahtii        GCGAGCGCCC --GTCAGAGG CCC---TCAA AT--TCTATT TCATC-----
T. pseudocomplicata      GCGAGCGCCC --GTCAGAGG CCC---TCAA AT--TCTATT TTATC-----
N. morrisonicola         GCGAGCGCCC --GTCGAAGG CTC--TTTAA AT--TCGATT T-ATC-----
N. pallescens            GCGAGCGCCC --GTCGGAGT CCC--ATGAA AT--TCTCCT CTATC-----
N. stracheyi             GCGAGCGCCC --GTCAGAGG CCC--TTTAA AT--TCTACT CTATC-----
Tuckneraria laureri      GCGAGCGCCC --GTCAGAGG CCC--TTTAA AT--CCTATT T-ATC-----
Ahtiana pallidula        GCGAGCGCCC --GTCAGAGG CCA--ATCAA AT--TCTATT T-ATT-----
Cetraria nivalis         GCGAGCGCCC --GTCAGAGG CCA--ATCAA AT--TCTATT T-ATC-----
K. merrillii             GCGAGCGCCC --GTCGGAGG CCA--ATCAA AT--CCTATT T-ATT-----
1A. oakesiana            GCGAGCGCCC --GTCAGAGG CCC--ATTAA AT--CCTGTT TTATC-----
F. cucullata             GCGAGCGCCC --GTCAGAGG CCA--ATCAA AT--TCTATT T-ATC-----
M. richardsonii          GCGAGCGCCC --GTCAGAGG CCA--ATCAA AT--TCTATT T-ATC-----
2C. islandica            GCGAGCGCCC --GTCAGAGG CCA--TTTAA AC--TCTGTT T-ATC-----
2C. crispiformis         GCGAGCGCCC --GCCAGAGG CCC--ATTAA AA--TCTGCT T-ATT-----
2C. antarctica           GCGAGCGCCC --GCCAGAGG CCC--ATTAA AA--TCTGCT T-ATT-----
Cetraria sepinco         GCGAGCGCCC --GCCAGAGG CCC--ATTAA AA--TCTGCT T-ATT-----
1M. fuliginosa           GCGAGCGCCC --GCCAGAGG CCC--ATTCA AT--TCTGTT T-ATC-----
1M. subauri              GCGAGTGTCC --GTCAGAGG CCC--ATTAC AT--TCTGTT T-ATT-----
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

```
                       ....|....| ....|....| ....|....| ....|....| ....|....|
                               210        220        230        240        250
Ustillago sparsa       CTAGGATT-- TGAATGAGAA AAGTTCATTT TTACAAATGA AATCGACTGG
Agaricus abrupti       CATGAAATCA CTTTCT-CAC CAGAGTCTAT GTCTTTCATT ATACTCTGTC
LRUB 20                CGGGCGGTCA TAAACAAAAC C-TTTTCGT- -CGAG-ATGG CATCGTCTA-
4C. fuckeli            -GGAACTT-- --AACAAAAC C-TTTTTT-- -------GCA TCTAGCATT-
Myrothecium sp.        -GGAACCT-- --ATCAAAAC C-TTTTTTTT -------GCA TCTAGCATT-
Paraphaeosphaeria sp.-GGAACTT-- --ATCAAAAC C-TTTTTTT- -------GCA TCTAGCATT-
4C. minitans           -GGAACCT-- -GAT--AAAC C-TTTTTT-- -------GCA TCTAGTATT-
P. pilleata            -GGAACTT-- --AACAAAAC C-TTTTTTT- -------GCA TCTAGCATT-
2M. terrestris         -GGACGCT-- --ACAAAAAC CATTCCGTT- -CGAAGAACG TCTGATTTT-
Aspergillus flavipes -GGAGACC-- --CCAACACG AACACTGTT- TCT--GAAAG CCTG-TATGA
1C. cetrarioides       ---------- ---------- ---------- --------AG TG--------
1C. chicitae           ---------- ---------- ---------- --------AG TG--------
1C. braunsiana         ---------- ---------- ---------- --------CA TG--------
1C. japonica           ---------- ---------- ---------- --------CG TG--------
1P. quernea            ---------- ---------- ---------- ------ACAA TG--------
3C. prancei            -GGAGGTT-- CAACCACATC C-TGTTTAT- TAG--TGAAG TC-CGAGTAA
3C. corallifera        -GGAGGTT-- CAACCACATC C-TGTTTAT- TAG--TGAAG TC-CGAGTAA
Lobaria amplissima     ---------- ---------- ---------- ---------- ----------
Cetraria odontella     ---------- ---------- ---------- --------AG TG--------
Cetraria nigricans     ---------- ---------- ---------- --------AG TG--------
Oropogon sp.           ---------- ---------- ---------- --------CG TG--------
Sulcaria sulcata       ---------- ---------- ---------- --------CG TG--------
Cetraria leucostigma ---------- ---------- ---------- --------AG TG--------
Cetraria melalom       ---------- ---------- ---------- --------AG TG--------
Tuckneraria ahtii      ---------- ---------- ---------- --------AG TG--------
T. pseudocomplicata    ---------- ---------- ---------- --------GG TG--------
N. morrisonicola       ---------- ---------- ---------- --------AG TG--------
N. pallescens          ---------- ---------- ---------- --------AG TG--------
N. stracheyi           ---------- ---------- ---------- --------AG TG--------
Tuckneraria laureri    ---------- ---------- ---------- --------AG TG--------
Ahtiana pallidula      ---------- ---------- ---------- --------AG TG--------
A. nigricascens        ---------- ---------- ---------- --------AG TG--------
Cetraria nivalis       ---------- ---------- ---------- --------AG TG--------
K. merrillii           ---------- ---------- ---------- --------AG TG--------
1A. oakesiana          ---------- ---------- ---------- --------AG TG--------
F. cucullata           ---------- ---------- ---------- --------AG TG--------
M. richardsonii        ---------- ---------- ---------- --------AG TG--------
2C. islandica          ---------- ---------- ---------- --------AG TG--------
2C. crispiformis       ---------- ---------- ---------- --------AG TG--------
2C. antarctica         ---------- ---------- ---------- --------AG TG--------
Cetraria sepinco       ---------- ---------- ---------- --------AG TG--------
1M. fuliginosa         ---------- ---------- ---------- --------AG TG--------
1M. subauri            ---------- ---------- ---------- --------AG AG--------
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

```
                         ....|....| ....|....| ....|....| ....|....| ....|....|
                                260        270        280        290        300
Ustillago sparsa         TAATGCGGTC GTCTAATTTT TAAA------ ---------- ----------
Agaricus abrupti         GAATGTCATT GAATGTCTTT ACATGGGCTT GTATGCCTAT GAAAATTGTA
LRUB 20                  -ATTTCTTC- ------ATAT CAAA------ ---------- ---ATATGAA
4C. fuckeli              -ACCTGTTC- ----TGATA- CAAA------ ---------- ---CAATC-G
Myrothecium sp.          -ACCTGTTC- ----TGATA- CAAA------ ---------- ---CAATC-G
Paraphaeosphaeria sp.-ACCTGTTC- ----AGATA- CAAA------ ---------- ---CAATC-G
4C. minitans             -ACCTGTTC- ----TGATA- CAAA------ ---------- ---CAATC-G
P. pilleata              -ACCTGTTC- ----TGATA- CAAA------ ---------- ---CAATC-G
2M. terrestris           -ACC--TTCG CGAATGCGA- TAAA------ ---------- ----------
Aspergillus flavipes ATCCGATTC- ----TTTG-- ---------- ---------- ---TAATCAG
1C. cetrarioides         ---GTGTCCG AG------TC AAAA------ ---------- -CACAAATAG
1C. chicitae             ---GTGTCCG AG------TC AAAA------ ---------- -CACAAATAG
1C. braunsiana           ---GTGTCTG AG------TC GAAA------ ---------- -CGCAAATAG
1C. japonica             ---GTGTCCG AG------TC CAAA------ ---------- -TACAAATAG
1P. quernea              ---TTGTCCG AG------TT ACACG----- ---------- -CAAACA-GT
3C. prancei              ---------- ---------- -AAA------ ---------- -TTAAAT-AA
3C. corallifera          ---------- ---------- -AAA------ ---------- -TTAAAT-AA
Lobaria amplissima       ---------- ---------- ---------- ---------- ----------
Cetraria odontella       ---ATGTCCG AG-----TGA AAAA------ ---------- -CACAATAAA
Cetraria nigricans       ---ATGTCCG AG-----TGA AAAA------ ---------- -CACAATAAA
Oropogon sp.             ---CCGTCCG AG-----TAC CAAA------ ---------- -CACAATA-G
Sulcaria sulcata         ---CCGTCCG AG-----TAC CAAA------ ---------- -CACAATA-G
Cetraria leucostigma ---ATGTCGG AG------CA AAAC------ ---------- -CT-AATAAT
Cetraria melalom         ---ATGTCGG AG------CA AAAC------ ---------- -CT-AATAAT
Tuckneraria ahtii        ---ATGTCCG AG------CG AAAA------ ---------- -CAATAATCT
T. pseudocomplicata      ---ATGTCCG AG------CG AAAA------ ---------- -CACAATAAT
N. morrisonicola         ---ATGTCCG AG------CA AAAA------ ---------- -CACAATAAT
N. pallescens            ---ACGTCCG AG------CG AAAA------ ---------- -CACAATAAT
N. stracheyi             ---ATGTCCG AG------CG AACAA----- ---------- -CCCAATAAT
Tuckneraria laureri      ---ACGTCCG AG------CG AAAA------ ---------- -CACAATAAT
Ahtiana pallidula        ---ATGTCCG AG------CT AAAA------ ---------- -CACAATAAT
A. nigricascens          ---ATGTCCG AG------CC AAAA------ ---------- -CA---TAAT
Cetraria nivalis         ---ATGTCCG AG------TA AAAA------ ---------- -CACAATAGT
K. merrillii             ---ATGTCCG AG------CA AAAA------ ---------- -CACAATAAT
1A. oakesiana            ---ATGTCCG AG------CA AAAA------ ---------- -CACAATAAT
F. cucullata             ---ATGTCCG AG------CA AAAA------ ---------- -CGCAATAAT
M. richardsonii          ---AAGTCCG AG------CA AAAGA----- ---------- -CACAATAAT
2C. islandica            ---ATGTCCG AG------CG AAAAA----- ---------- -CACAATAAA
2C. crispiformis         ---ATGTCCG AG------CG AAAAA----- ---------- -CACAATAAA
2C. antarctica           ---ATGTCCG AG------CG AAAAA----- ---------- -CACAATAAA
Cetraria sepinco         ---ATGTCCG AG------TG AAAA------ ---------- -CACAATCAA
1M. fuliginosa           ---ACGTCCG AG------TA CAAAC----- ---------- -CACAATAGT
1M. subauri              -TGACGTCCG AG------TA TAAAC----- ---------- -CACAATAAT
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

```
                     ....|....| ....|....| ....|....| ....|....| ....|....|
                              310        320        330        340       350
Ustillago sparsa     -AACAACTTT TGGCAACGGA TCTCTTGGTT CTCCCATCGA TGAAGAACGC
Agaricus abrupti     ATACAACTTT CAGCAACGGA TCTCTTGGCT CTCGCATCGA TGAAGAACGC
LRUB 20              ATACAACTTT CAACAATGGA TCTCTTGGCT CCGGCATCGA TGAAGAACGC
4C. fuckeli          TTACAACTTT CAACAATGGA TCTCTTGGCT CTGGCATCGA TGAAGAACGC
Myrothecium sp.      TTACAACTTT CAACAATGGA TCTCTTGGCT CTGGCATCGA TGAAGAACGC
Paraphaeosphaeria sp.TTACAACTTT CAACAATGGA TCTCTTGGCT CTGGCATCGA TGAAGAACGC
4C. minitans         TTACAACTTT CAACAATGGA TCTCTTGGCT CTGGCATCGA TGAAGAACGC
P. pilleata          TTACAACTTT CAACAATGGA TCTCTTGGCT CTGGCATCGA TGAAGAACGC
2M. terrestris       -TACAACTTT CAACAATGGA TCTCTTGGCT CCAGCATCGA TGAAGAACGC
Aspergillus flavipes TTAAAACTTT CAACAATGGA TCTCTTGGTT CCGGCATCGA TGAAGAACGC
1C. cetrarioides     TAAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1C. chicitae         TCAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1C. braunsiana       TAAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1C. japonica         TCAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1P. quernea          TAAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
3C. prancei          TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
3C. corallifera      TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
Lobaria amplissima   -CAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC
Cetraria odontella   T-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Cetraria nigricans   T-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Oropogon sp.         TAAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Sulcaria sulcata     TAAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Cetraria leucostigma C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Cetraria melalom     C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Tuckneraria ahtii    CAAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
T. pseudocomplicata  CTAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
N. morrisonicola     C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
N. pallescens        C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
N. stracheyi         C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Tuckneraria laureri  C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Ahtiana pallidula    C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
A. nigricascens      C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Cetraria nivalis     C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
K. merrillii         C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1A. oakesiana        C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
F. cucullata         C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
M. richardsonii      C-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
2C. islandica        T-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
2C. crispiformis     T-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
2C. antarctica       T-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
Cetraria sepinco     TCAAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1M. fuliginosa       A-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
1M. subauri          A-AAAACTTT CAACAACGGA TCTCTTGGTT CCAGCATCGA TGAAGAACGC
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                        ....|....| ....|....| ....|....| ....|....| ....|....|
                                 360        370        380        390        400
Ustillago sparsa        AGCGAATTGC GATAAGTAAT GTGAATTGCA GAA----GTG AATCATCGAA
Agaricus abrupti        AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
LRUB 20                 AGCGAAATGC GATAACTAGT GTGAATTGCA GATTTCAGTG AATCATCGAG
4C. fuckeli             AGCGAAATGC GATAAGTAGT GTGAATTGCA GAATTCAGTG AATCATCGAA
Myrothecium sp.         AGCGAAATGC GATAAGTAGT GTGAATTGCA GAATTCAGTG AATCATCGAA
Paraphaeosphaeria sp.AGCGAAATGC GATAAGTAGT GTGAATTGCA GAATTCAGTG AATCATCGAA
4C. minitans            AGCGAAATGC GATAAGTAGT GTGAATTGCA GAATTCAGTG AATCATCGAA
P. pilleata             AGCGAAATGC GATAAGTAGT GTGAATTGCA GAATTCAGTG AATCATCGAA
2M. terrestris          AGCGAAATGC GATAACTAGT GTGAATTGCA GATTTCAGTG AATCATCGAG
Aspergillus flavipes    AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
1C. cetrarioides        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
1C. chicitae            AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
1C. braunsiana          AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
1C. japonica            AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
1P. quernea             AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
3C. prancei             AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
3C. corallifera         AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
Lobaria amplissima      AGCGAAATGC GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA
Cetraria odontella      AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Cetraria nigricans      AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Oropogon sp.            AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Sulcaria sulcata        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Cetraria leucostigma    AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Cetraria melalom        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Tuckneraria ahtii       AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
T. pseudocomplicata     AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
N. morrisonicola        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
N. pallescens           AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
N. stracheyi            AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Tuckneraria laureri     AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Ahtiana pallidula       AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
A. nigricascens         AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Cetraria nivalis        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
K. merrillii            AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
1A. oakesiana           AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
F. cucullata            AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
M. richardsonii         AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
2C. islandica           AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
2C. crispiformis        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
2C. antarctica          AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
Cetraria sepinco        AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
1M. fuliginosa          AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
1M. subauri             AGCGAAATGC GATAACTAAT GTGAATTGCA GAATTCAGTG AATCATCGAG
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                         ....|....| ....|....| ....|....| ....|....| ....|....|
                                   410        420        430        440        450
Ustillago sparsa         TCTTTGAACG CACCTTGCGC TCCCGGCAGA TCTAATCTGG GGAGCATGCC
Agaricus abrupti         TCTTTGAACG CATCTTGCGC TCCTTGG--- --TATTCCGA GGAGCATGCC
LRUB 20                  TCTTTGAACG CACATTGCGC CTCTTGGTAT TCCTCGAGGC ATGCCTGTTC
4C. fuckeli              TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCATGGGGC ATGCCTGTTC
Myrothecium sp.          TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCATGGGGC ATGCCTGTTC
Paraphaeosphaeria sp.TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCATGGGGC ATGCCTGTTC
4C. minitans             TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCATGGGGC ATGCCTGTTC
P. pilleata              TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCATGGGGC ATGCCTGTTC
2M. terrestris           TCTTTGAACG CACATTGCGC CTCTTGGTAT TCCTCGAGGC ATGCCTAT-C
Aspergillus flavipes TCTTTGAACG CACATTGCGC CCCCTGGTAT TCCGGGGGGC ATGCCTGTCC
1C. cetrarioides         TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGGGGGGC ATGCCTGTTC
1C. chicitae             TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGGGGGGC ATGCCTGTTC
1C. braunsiana           TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGAGGGGC ATGCCTGTTC
1C. japonica             TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGAGGGGC ATGCCTGTTC
1P. quernea              TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGTGGGGC ATGCCTGTTC
3C. prancei              TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGGGGGGC ATGCCTGTTC
3C. corallifera          TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGGGGGGC ATGCCTGTTC
Lobaria amplissima       TCTTTGAACG CACATTGCGC CCCTTGGTAT TCCGAGGGGC ATGCCTGTCC
Cetraria odontella       TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Cetraria nigricans       TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Oropogon sp.             TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Sulcaria sulcata         TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Cetraria leucostigma TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Cetraria melalom         TTTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Tuckneraria ahtii        TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
T. pseudocomplicata      TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
N. morrisonicola         TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
N. pallescens            TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
N. stracheyi             TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Tuckneraria laureri      TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Ahtiana pallidula        TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGAGGGGC ATGCCTGTTC
A. nigricascens          TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Cetraria nivalis         TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
K. merrillii             TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
1A. oakesiana            TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
F. cucullata             TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
M. richardsonii          TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
2C. islandica            TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
2C. crispiformis         TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
2C. crispiformis         TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
Cetraria sepinco         TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
1M. fuliginosa           TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
1M. subauri              TCTTTGAACG CACATTGCGC CCCTCGGTAT TCCGGGGGGC ATGCCTGTTC
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                         ....|....| ....|....| ....|....| ....|....| ....|....|
                                   460        470        480        490        500
Ustillago sparsa         TGTTTGAGGG CCGCGAATTG TTTCGAAC-- -GACAACTTT TTTC----AC
Agaricus abrupti         TGTTTGAGTG TCATTAAAT- TCTCAACTCT CTTATACTGT GTT-----GT
LRUB 20                  GAGCGTCGT- TACGCCCCTC AAGCGCGA-- -GCT--TGGT GTTGG--GGA
4C. fuckeli              GAGCGTCATC TACA-CCCTC AAGCTCT--- -GCT--TGGT GTTGGG-CGT
Myrothecium sp.          GAGCGTCATC TACA-CCCTC AAGCTCT--- -GCT--TGGT GTTGGG-CGT
Paraphaeosphaeria sp.GAGCGTCATC TACA-CCCTC AAGCTCT--- -GCT--TGGT GTTGGG-CGT
4C. minitans             GAGCGTCATC TACA-CCCTC AAGCTCT--- -GCT--TGGT GTTGGG-CGT
P. pilleata              GAGCGTCATC TACA-CCCTC AAGCTCT--- -GCT--TGGT GTTGGG-CGT
2M. terrestris           GAGCGTCGT- TTCGACCATC AAGCGCA--- -ACT--TGGT GTTGG-GGAC
Aspergillus flavipes GAGCGTCAT- TACTGCCCTC AAGCCCG--- -GCT--TG-T ATTGGGTCCT
1C. cetrarioides         GAGCGTCAT- TACACCCCTC AAGCGTC--- -GCT--TGGT ATTGGG-TTT
1C. chicitae             GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-TTT
1C. braunsiana           GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-TCT
1C. japonica             GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-TCT
1P. quernea              GAGCGTCAT- TACACCCCTC AAGCGCG--- -GCT--TGGT GTTGGGCTCT
3C. prancei              GAGCGTCAT- TACACCCCTC AAGCGCA--- -GCT--TGGT ATTGGA-CGT
3C. corallifera          GAGCGTCAT- TACACCCCTC AAGCGCA--- -GCT--TGGT ATTGGA-CGT
Lobaria amplissima       GAGCGTCAT- TACACCCGTC AAGCGCGT-- -GCT--TGGT GTTGGG-CCG
Cetraria odontella       GAGCGTCAT- TATACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-TCT
Cetraria nigricans       GAGCGTCAT- TATACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-TCT
Oropogon sp.             GAGCGTCAT- TACACCCCTC AAGCGCG--- -GCT--TGGT ATTGGGTCCT
Sulcaria sulcata         GAGCGTCAT- TACACCCCTC AAGCGCG--- -GCT--TGGT ATTGGGTCCT
Cetraria leucostigma GAGCGTCAT- TACACCCCTC AAGCGCA--- -GCT--TGGT ATTGGG-CCT
Cetraria melalom         GAGCGTCAT- TACACCCCTC AAGCGCA--- -GCT--TGGT ATTGGG-CCT
Tuckneraria ahtii        GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
T. pseudocomplicata      GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
N. morrisonicola         GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CGT
N. pallescens            GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
N. stracheyi             GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CTT
Tuckneraria laureri      GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-TCT
Ahtiana pallidula        GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
A. nigricascens          GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CTT
Cetraria nivalis         GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT CTTGGG-CCT
K. merrillii             GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
1A. oakesiana            GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CTT
F. cucullata             GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CTT
M. richardsonii          GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CTT
2C. islandica            GAGCGTCAT- TATACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
2C. crispiformis         GAGCGTCAT- TATACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
2C. crispiformis         GAGCGTCAT- TATACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CGT
Cetraria sepinco         GAGCGTCAT- TACACCCCTC AAGCGTA--- -GCT--TGGT ATTGGG-CCT
1M. fuliginosa           GAGCGTCAT- TACACCCCTC AAGCGCA--- -GCT--TGGT ATTGGGCCAT
1M. subauri              GAGCGTCAT- TACACCCCTC AAGCTCA--- -GCT--TGGT ATTGGGTCCT
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                     ....|....| ....|....| ....|....| ....|....| ....|....|
                             510        520        530        540        550
Ustillago sparsa     AAA-GAGTTG GCGGATCGGT ATTGAGAGT- ---------T TTTTTGC-CA
Agaricus abrupti     AAAGGAGAGC TTGGAT-TGT GGAGGCTTGC TGGCCACTTG TTTGGGGTCA
LRUB 20              TCGCCCC--- TGAGA--TAC GGCG-GCGGC CCTT-AAAT- GCATCGG---
4C. fuckeli          CTGTCCCGCC TT-----CGC GCGCGGACTC GCCC-CAAAT TCATTGGCAG
Myrothecium sp.      CTGTCCCGCC TC-----TGC GCGCGGACTC GCCC-CAAAT TCATTGGCAG
Paraphaeosphaeria sp.CTGTCCCGCC TC-----TGC GCGTGGACTC GCCC-CAAAT TCATTGGCAG
4C. minitans         CTGTCCCGCC TT-----TGC GCGCGGACTC GCCC-CAAAC TCATTGGCAG
P. pilleata          CTGTCCCGCC TC-----TGC GCGCGGACTC GCCC-CAAAT TCATTGGCAG
2M. terrestris       CCGCCCC--- TGAAATACGC GA--GGCGGC CCTT-GAA-T CCATCGGC--
Aspergillus flavipes CGTCCCCCC- -----GGGG- -ACGGGCCC- GAAA-GGCA- GCGGCGGCAC
1C. cetrarioides     C-GTCCCT-- ---------- --GAGGCGT- GCCC-GAAAG TTAGTGG---
1C. chicitae         C-GTCCCT-- ---------- --GAGGCGT- GCCC-GAAAG TTAGTGG---
1C. braunsiana       C-GTCCCT-- ---------- --GAGGCGT- GCCC-GAAAG TCAGTGG---
1C. japonica         C-GTCCCT-- ---------- --GAGGCGT- GCCC-GAAAG TCAGTGG---
1P. quernea          C-GCCCCCG- ---------- --TAGGCGG- GCCC-GAAAG TCAGTGG---
3C. prancei          TCGCGGGCCC TCTT-TTGGG GGCCTGCGT- GCCC-GAAAA ACAGTGG---
3C. corallifera      TCGCGGGCCC TCTT-TTGGG GGCCTGCGT- GCCC-GAAAA ACAGTGG---
Lobaria amplissima   GCGTCCCCCC ---------- --GGGACGG- GTCC-GAATG GCAGTGG---
Cetraria odontella   C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAGTGG---
Cetraria nigricans   C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAGTGG---
Oropogon sp.         C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
Sulcaria sulcata     C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
Cetraria leucostigma C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
Cetraria melalom     C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
Tuckneraria ahtii    C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
T. pseudocomplicata  C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
N. morrisonicola     C-GCCCCA-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGC---
N. pallescens        C-GCTCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
N. stracheyi         C-GCCCCC-- ---------- --GCGGCGT- GTCC-GAAAA ACAGTGG---
Tuckneraria laureri  C-GCCCCC-- ---------- --GCGGCGT- ACCC-GAAAA GCAGTGG---
Ahtiana pallidula    C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA TCAGCGG---
A. nigricascens      C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
Cetraria nivalis     C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
K. merrillii         C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA TCAGTGG---
1A. oakesiana        C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
F. cucullata         C-GCCCCC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
M. richardsonii      C-GTCCTC-- ---------- --GCGGCGT- GCCC-GAAAA GCAGTGG---
2C. islandica        C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAATGG---
2C. crispiformis     C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAGTGG---
2C. crispiformis     C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAGTGG---
Cetraria sepinco     C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAGTGG---
1M. fuliginosa       C-GCCCCC-- ---------- --GTGGCGT- GCCC-GAAAA GCAGTGG---
1M. subauri          C-GCCTCCC- ---------- --GGGGCGT- GCCC-GAAAA TTAGTGG---
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                     ....|....| ....|....| ....|....| ....|....| ....|....|
                             560        570        580        590        600
Ustillago sparsa     -TTCA-CCGT GGC------- TCTCTCGAAA T-GCATTAGC GCATCCATTT
Agaricus abrupti     GCT---CCTC TGA-AATGCA TTA-GCGGAA CCGTCTGCGA TCTGCCACAA
LRUB 20              CG-GTGCT-- GGTGTCAGCC CG---GAGCG CAGCAGACA- -TG----CGG
4C. fuckeli          CG-GT-CCTT GCC-T---CC TCTC---GCG CAGCACAA-- TTG----CGT
Myrothecium sp.      CG-GT-CCTT GCC-T---CC TCTC---GCG CAGCACA--- TTG-----CG
Paraphaeosphaeria sp.CG-GT-CTTT GCC-T---CC TCTC---GCG CAGCACA--- TTG-----CG
4C. minitans         CG-GT-TTTT GCC-T---CC TCTC---GCG CAGCACAA-- TTG----CGT
P. pilleata          CG-GT-CTTT GCC-T---CC TCTC---GCG CAGCACAA-- TTG----CGT
2M. terrestris       -G-GTGCC-- GGTGT-AGCC TG---GAGCG CAGCAGCAA- -TG----CAG
Aspergillus flavipes CGCGT-CC-- GGT-----CC TC---GAGCG TA-TGGGGCT TTGTCACCCG
1C. cetrarioides     CG-GT-CC-- GGCG-TGAC- TTT--AAGCG TAGTAAAA-T TTATC--CCG
1C. chicitae         CG-GT-CC-- GGCG-TGAC- TTT--AAGCG TAGTAAAA-T TTATC--CCG
1C. braunsiana       CG-GT-CC-- GGCG-TGAC- TTT--AAGCG TAGTAAAA-T TTATC--CCG
1C. japonica         CG-GT-CC-- GGCG-TGAC- TTT--AAGCG TAGTAAAA-T TTATC--CCG
1P. quernea          CG-GT-CC-- GGCG-TGAC- -TTC-GAGCG TAGTAAAT-T TTATC--CCG
3C. prancei          CG-GT-CC-- -CCGGGGA-- TTTC-GCGCG TAGTAAATC- TTCTC--CCG
3C. corallifera      CG-GT-CC-- -CCGGGGA-- TTTC-GCGCG TAGTAAATC- TTCTC--CCG
Lobaria amplissima   CG-GT-CC-- GGCG-TGAC- -TTC-GAGCG CAGTAGAACC TTGTT--TCG
Cetraria odontella   CG-GT-CC-- GG-G-CGAC- TTT--AAGCG TAGTAAAA-- TCATC--CCG
Cetraria nigricans   CG-GT-CC-- GGGG-CGAC- TTT--AAGCG TAGTAAAA-- TTATC--CCG
Oropogon sp.         CG-GT-CC-- GGTG-CGGC- TTT--AAGCG TAGTAATTTT TCATC--CCG
Sulcaria sulcata     CG-GT-CC-- GGTG-CGGC- TTT--AAGCG TAGTAATTTT TCATC--CCG
Cetraria leucostigma CG-GT-CC-- GGTG-TGAC- TTT--AAGCG TAGTAAAACT TCATC--CCG
Cetraria melalom     CG-GT-CC-- GGTG-TGAC- TTT--AAGCG TAGTAAAACT TCATC--CCG
Tuckneraria laureri  CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAACT TCGTC--CCG
T. pseudocomplicata  CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAACT TCATC--CCG
N. morrisonicola     CG-GC-CC-- GGTG-CGGC- TTT--AAGCG TAGTAAAACT TCATC--CCG
N. pallescens        CG-GT-CC-- GGCG-TGAC- TTT--AAGCG TAGTAAAACC TCATC--CCG
N. stracheyi         CG-GT-CC-- GGTG-CGAC- TTC--AAGCG TAGTAAAACT TCCTC--CCG
Tuckneraria laureri  CG-GT-CC-- GGCG-CGAC- TTT--AAGCG TAATAAAACT CCATC--CCG
Ahtiana pallidula    CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAT-T TCATC--CCG
A. nigricascens      CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAT-T TCATC--CCG
Cetraria nivalis     CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAT-T TCATC--CCG
K. merrillii         CG-GT-CC-- GGTG-CTAC- TTT--AAGCG TAGTAAAT-T TCATC--CCG
1A. oakesiana        CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAT-T TCATC--CCG
F. cucullata         CG-GT-CC-- GGTG-CGAC- TTT--AAGCG TAGTAAAT-T TTATC--CCG
M. richardsonii      CG-GT-CC-- GGGG-CGAC- TTT--AAGCG TAGTAAAT-T TCATC--CCG
2C. islandica        AG-GT-CC-- GGGG-TGAC- TTT--AAGCG TAGTAAAA-- TTATC--CCG
2C. crispiformis     CG-GT-CC-- GGGG-TGAC- TTT--AAGCG TAGTAAAA-- TTATC--CCG
2C. crispiformis     CG-GT-CC-- GGGG-CGAC- TTT--AAGCG TAGTAAAA-- TTATC--CCG
Cetraria sepinco     CG-GT-CC-- GTGG-TGGC- -TTC-AAGCG TAGTAAAA-- TCATC--CCG
1M. fuliginosa       CG-GT-CC-- GGAG-CGGC- TTT--AAGCG TAGTAATA-T TTATC--CCG
1M. subauri          CG-GT-CC-- GGAG-CGAC- TTT--AAGCG TAGTAAAA-T TTATC--CCG
```

Codes of genus are shown in Figure D2

A=Arctocetraria   *1A=Allocetraria*   *1C=Cetrelia*   *2C=Cetraria islandica* subsp.

3C=Cladonia   *4C=Coniothyrium*   *F=Flavocetraria*   *K=kaernefeltia*

M=Masonhalea   *1M=Melanelixnia*   *2M=Mycoleptodiscus*   *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

```
                     ....|....| ....|....| ....|....| ....|....| ....|....|
                              610        620        630        640        650
Ustillago sparsa     GATAGGCAAG ACGGACGAA- -----AGCTC ATCTTTTCGC TCTCTCTTCC
Agaricus abrupti     GTGTG---AT AAATTATCTA CAC-TGGCGA GGG-GATTGC TCTCTGTGAT
LRUB 20              CTTCC----- AGGCGACCA- --CGCG-CCC GCCGG----A CAACG--ACC
4C. fuckeli          CTGCG----G GGGGGCGT-- -----GGCCC G-CGTCCA-C GAAGC-----
Myrothecium sp.      CTTCTC---G AGGGGCGC-- -----GGCCC G-CGTCCA-C GAAGC-----
Paraphaeosphaeria sp.CTTCA----G AGGGGTGT-- -----GGGCC G-CGTCCA-C GAAGC-----
4C. minitans         CTGCG----A GGGGGCGT-- -----GGCCC G-CGTCCA-C GAAGC-----
P. pilleata          CTGCG----A GGGGGCGT-- -----GGCCC G-CATCCA-C GAAGC-----
2M. terrestris       CTTCTT---- -GGGGCA--- -----G-CCC G-AAGCCA-G CCGGACAAT-
Aspergillus flavipes CTCTGT---- AGGCCC---- -----GGCCG G-CG-CCA-- ---GCCCA--
1C. cetrarioides     CCTTTA--A- GTTCGCGCC- ---GTGGCCC G----CCA-- GACA------
1C. chicitae         CCTTTA--A- GTTCGCGCC- ---GTGGCCC G----CCA-- AACAA-----
1C. braunsiana       CCGTTA--A- GTTCGCGCC- ---GTGGCCC G----CCA-- GACAA-----
1C. japonica         CCTTTA--A- GTTCGCGCC- ---GTGGCCC G----CCA-- GACAA-----
1P. quernea          CTTTGG--AG TTTCGCGTC- ---GCGGCTG G----CCA-- GGATGCC---
3C. prancei          CGTTGG---- ---------- ---------- ---------- ----------
3C. corallifera      CGTTGG---- ---------- ---------- ---------- ----------
Lobaria amplissima   CTCGGG--AG GCACGC-CC- ---GGGTCCG G----CCAGT CAACCGTGAA
Cetraria odontella   CTTTGA--AA GTTCGCTTC- ---GTGGCCG G----CCA-- GACA---ACC
Cetraria nigricans   CTTTGA--AA GTTCGCTTC- ---GTGGCCG G----CCA-- GACA---ACC
Oropogon sp.         CTTTGA--AG GCCCGCCCC- ---GAGGCTG G----CCA-- GACA---ACC
Sulcaria sulcata     CTTTGA--AG GCCCGCCCC- ---GAGGCTG G----CCA-- GACA---ACC
Cetraria leucostigma CTTTGA--AA GCTCGCCCC- ---GCGACCG G----CCA-- GACA---ACC
Cetraria melalom     CTTTGA--AA GCTCGCCCC- ---GCGACCG G----CCA-- GACA---ACC
Tuckneraria laureri  CTTTGA--AA GCTCGCCCC- ---GCGACCG G----CCA-- GACA---ACC
T. pseudocomplicata  CTTTGA--AA GTCCGCCCC- ---GCGACCG G----CCA-- GACA---ACC
N. morrisonicola     CTTTGA--AA GCCCGCCCC- ---GCGGCCG G----CCA-- GACA---ACC
N. pallescens        CTTTGA--AA GTCTGCCCC- ---GCGACCG G----CCA-- GACA---ACC
N. stracheyi         CTCTGG--AA GTTCGCCCC- ---GCGATCG G----CCG-- GACA---ACC
Tuckneraria laureri  CTTTGA--AA GTTCGCCTC- ---GCGACCG G----CCA-- GACA---ACC
Ahtiana pallidula    CTTTGA--AA GTTCGCCTC- ---GTGGCCG G----CCA-- GACA---GCC
A. nigricascens      CTTTGA--AA GTTCGCCTC- ---GTGGCCG G----CCA-- GACA---ACC
Cetraria nivalis     CTTTGA--AA GTTCGCCCC- ---GTGGCCG G----CCA-- GACA---ACC
K. merrillii         CTTTGA--AA GTTCGCCCC- ---GTGGCTG G----CCA-- GACA---ACC
1A. oakesiana        CTTTGA--AA GTTCGCCCC- ---GTGGCTG G----CCA-- GACA---ACC
F. cucullata         CTTTGA--AA GTTCGCCCC- ---GTGGCTG G----CCA-- GACA---ACC
M. richardsonii      CTTTGA--AA GTTCGCCCC- ---GCGGCTG G----CCA-- GATA---ACC
2C. islandica        CTTTGA--AA GTTCGCCTC- ---GTGGCCT G----CCA-- GACA---ACC
2C. crispiformis     CTTTGA--AA GTTCGCCTC- ---GTGGCCT G----CCA-- GACA---ACC
2C. crispiformis     CTTTGA--AA GTTCGCCTC- ---GTGGCCT G----CCA-- GACA---ATC
Cetraria sepinco     CTTTGA--AA GCTCGTCTC- ---GTGGCCG G----CCA-- GACA---ACC
1M. fuliginosa       CTTTGA--AA GTCCGCCCC- ---GTGGCCT G----CCA-- GGTA---ACC
1M. subauri          CTTTGA--AA GTTCGCTCC- ---GCGGCTG G----CCA-- AGTA---ACC
```

Codes of genus are shown in Figure D2

A=Arctocetraria   *1A=Allocetraria*   *1C=Cetrelia*   *2C=Cetraria islandica* subsp.

3C=Cladonia   *4C=Coniothyrium*   *F=Flavocetraria*   *K=kaernefeltia*

M=Masonhalea   *1M=Melanelixnia*   *2M=Mycoleptodiscus*   *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                     ....|....| ....|....| ....|..
                              660        670
Ustillago sparsa     CTGCCGGGTT TTGATAATAT CAGGACT
Agaricus abrupti     GTTCAGCTTC TAATC-GTCT ACGGACA
LRUB 20              CG---A-CCT TCA------- --AA-CG
4C. fuckeli          ----AA-CAT T-A-CCG--T CT-----
Myrothecium sp.      ----AA-CAT T-A-CCG--T CT-----
Paraphaeosphaeria sp.----AA-CAT T-A-TCG--T CT-----
4C. minitans         ----AA-CAT T-A-CCG--T CT-----
P. pilleata          ----AA-CAT T-A-CCG--T CT-----
2M. terrestris       CG--AAACCT TCATTTTTTT CTCA-CG
Aspergillus flavipes CG-CAGATCA TCCTTTTTTT C--A-GG
1C. cetrarioides     -----ATAA- TTTTTATTTT C-CATAA
1C. chicitae         ------TAA- TTTTTATTTT C-CATAA
1C. braunsiana       ------CATT -CGTTTTCCT C-AATAA
1C. japonica         ------TATT -CGTTTTCCT C-AATAA
1P. quernea          -G--AAAGGC TTC-----AT CTCA-CA
3C. prancei          ----AAAG-- ---------- --AACCG
3C. corallifera      ----AAAG-- ---------- --AACCG
Lobaria amplissima   CCC---CAT- --CA-----T CT-GT--
Cetraria odontella   CCG------- TACAT---TT CAAATCA
Cetraria nigricans   CCG------- TACAT---TT CAAATCA
Oropogon sp.         CCA------- -A-AT--TTT CCACGA-
Sulcaria sulcata     CCA------- -A-AT--TTT CCA-CGA
Cetraria leucostigma CCA------- -ACAC---TT CAA-TCA
Cetraria melalom     CCA------- -ACAC---TT CAA-TCA
Tuckneraria laureri  CCA------- -ACAC---TT CAA-TCA
T. pseudocomplicata  CCA------- -CCAC---TT CAA-TTA
N. morrisonicola     CCA------- -ACAC--TTT CA--TCA
N. pallescens        CCA------- -ACAT--TTT -AA-CCA
N. stracheyi         CCA------- -ACGCC---T CGA-CAA
Tuckneraria laureri  C-----TCA- -ACATC---T CAA-TA-
Ahtiana pallidula    C--------- --CATT-TTT CAA-TAA
A. nigricascens      C--------- --CATTACTT CTA-TAA
Cetraria nivalis     CCA------- -TCATT---T CAA-TAA
K. merrillii         C--------- --CATTATTT CAA-TAA
1A. oakesiana        C--------- --CA-TACTT CAA-TAA
F. cucullata         C--------- --CA-TACTT CAA-TAA
M. richardsonii      C--------- -ACCA-ATTT CAA-TAA
2C. islandica        C--------- -CGTACATTT CAAATCA
2C. crispiformis     C--------- -CGTACATTT CAAATCA
2C. crispiformis     C--------- -CGTACATTT CAAATCA
Cetraria sepinco     C--------- -CATATCTTC CATATCA
1M. fuliginosa       C--------- -CGATGACTT CAA-TAA
1M. subauri          C--------- -CGATTACTT CAA-TAA
```

Codes of genus are shown in Figure D2

A=Arctocetraria *1A=Allocetraria* *1C=Cetrelia* *2C=Cetraria islandica* subsp.

3C=Cladonia *4C=Coniothyrium* *F=Flavocetraria* *K=kaernefeltia*

M=Masonhalea *1M=Melanelixnia* *2M=Mycoleptodiscus* *N=Nephromopsis*

1P=Pyrrhospora

Figure D2 (Continued)

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                      ....|....|....|....|....|....|....|....|....|....|
                                10        20        30        40        50
Ustillago affinis     TAACTTTGGG CAACGGATCT CTTGGTTCTC CCATCGATGA AGAACGCAGC
A. abruptibulbus      CAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
LRUB 20               CAACTTTCAA CAATGGATCT CTTGGCTCCG GCATCGATGA AGAACGCAGC
M. terrestris         CAACTTTCAA CAATGGATCT CTTGGCTCCA GCATCGATGA AGAACGCAGC
Myrothecium sp.       CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
C. sporulosum         CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
Montagnula opulenta   CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
1P. cyclothyrioides   CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
Paraphaeosphaeria sp.CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
P. pilleata           CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
C. fuckelii           CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
C. minitans           CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
Massarina bipolaris   CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
Massarina lacustris   CAACTTTCAA CAATGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC
P. michotii           CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
Lophiostoma arundinisAAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
1A. flavipes          AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
1A. niger             AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
1A. ellipticus        AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
Fennellia nivea       AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
Tuber rufum           AAACTTTCAA CAACGGATCT CTTGGCTCTC GTATCGATGA AGAACGCAGC
Aporospora terricola  CAACTTTCAA CAATGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
Humicola fuscoatra    CAACTTTCAA CAATGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC

                      ....|....|....|....| ....|....| ....|....| ....|....|
                               60        70        80        90       100
Ustillago affinis     GAATTGCGAT AAGTAATGTG AATTGCAGA- ---AGTGAAT CATCGAATCT
A. abruptibulbus      GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
LRUB 20               GAAATGCGAT AACTAGTGTG AATTGCAGAT TTCAGTGAAT CATCGAGTCT
M. terrestris         GAAATGCGAT AACTAGTGTG AATTGCAGAT TTCAGTGAAT CATCGAGTCT
Myrothecium sp.       GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
C. sporulosum         GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Montagnula opulenta   GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
1P. cyclothyrioides   GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Paraphaeosphaeria sp.GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
P. pilleata           GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
C. fuckelii           GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
C. minitans           GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Massarina bipolaris   GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Massarina lacustris   GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
P. michotii           GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Lophiostoma arundinisGAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
1A. flavipes          GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
1A. niger             GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
1A. ellipticus        GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
Fennellia nivea       GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
Tuber rufum           GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Aporospora terricola  GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Humicola fuscoatra    GAAATGCGAT AAGTAGTGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
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Figure D3 Alignment data of complete 5.8S sequences of isolate LRUB 20 and 22 refernce taxa from GenBank (*A=Agaricus*, 1*A=Aspergillus*, *C=Coniothyrium*, *M=Mycoleptodiscus* *1P=Paracoconiothyrium*).

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                     ....|....|....|....|....|....|....|....|....|....|
                             110       120       130       140       150
Ustillago affinis    TTGAACGCAC CTTGCGCTCC C-GGCAGATC TAATCTGGGG AGCATGCCTG
A. abruptibulbus     TTGAACGCAT CTTGCGCTCC TTGG------ TATTCCGAGG AGCATGCCTG
LRUB 20              TTGAACGCAC ATTGCGCCTC TTGG------ TATTCCTCGA GGCATGCCTG
M. terrestris        TTGAACGCAC ATTGCGCCTC TTGG------ TATTCCTCGA GGCATGCCTA
Myrothecium sp.      TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
C. sporulosum        TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
Montagnula opulenta  TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
1P. cyclothyrioides  TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
Paraphaeosphaeria sp.TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
P. pilleata          TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
C. fuckelii          TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
C. minitans          TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
Massarina bipolaris  TTGAACGCAC ATTGCGCCCT TTGG------ TATTCCTTAG GGCATGCCTG
Massarina lacustris  TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
P. michotii          TTGAACGCAC ATTGCGCCCC TCGG------ TATTCCGTGG GGCATGCCTG
Lophiostoma arundinisTTGAACGCAC ATTGCGCCCT TTGG------ TATTCCTTAG GGCATGCCTG
1A. flavipes         TTGAACGCAC ATTGCGCCCC CTGG------ TATTCCGGGG GGCATGCCTG
1A. niger            TTGAACGCAC ATTGCGCCCC CTGG------ TATTCCGGGG GGCATGCCTG
1A. ellipticus       TTGAACGCAC ATTGCGCCCC CTGG------ TATTCCGGGG GGCATGCCTG
Fennellia nivea      TTGAACGCAC ATTGCGCCCC CTGG------ TATTCCGGGG GGCATGCCTG
Tuber rufum          TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCTTGG GGCATGCCTG
Aporospora terricola TTGAACGCAC ATTGCGCCCC TTGG------ TATTCCATGG GGCATGCCTG
Humicola fuscoatra   TTGAACGCAC ATTGCGCCCC TCGG------ TATTCCTTGG GGCATGCCTG

                     .....|....| ....|
                              160
Ustillago affinis    TTTGAGGGCC GCGAA
A. abruptibulbus     TTTGAGTGTC AT-TA
LRUB 20              TTCGAGCGTC GT-TA
M. terrestris        T-CGAGCGTC GT-TT
Myrothecium sp.      TTCGAGCGTC ATCTA
C. sporulosum        TTCGAGCGTC ATCTA
Montagnula opulenta  TTCGAGCGTC ATCTA
1P. cyclothyrioides  TTCGAGCGTC ATCTA
Paraphaeosphaeria sp.TTCGAGCGTC ATCTA
P. pilleata          TTCGAGCGTC ATCTA
C. fuckelii          TTCGAGCGTC ATCTA
C. minitans          TTCGAGCGTC ATCTA
Massarina bipolaris  TTCGAGCGTC AT-TT
Massarina lacustris  TTCGAGCGTC ATCTA
P. michotii          TTCGAGCGTC ATCTA
Lophiostoma arundinisTTCGAGCGTC AT-TT
1A. flavipes         TCCGAGCGTC AT-TA
1A. niger            TCCGAGCGTC AT-TG
1A. ellipticus       TCCGAGCGTC AT-TG
Fennellia nivea      TCCGAGCGTC AT-TG
Tuber rufum          TTCGAGCGTC A-CTA
Aporospora terricola TTCGAGCGTC ATCTA
Humicola fuscoatra   TTCGAGCGTC ATCTA
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Figure D3 (Continued)

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                       ....|....|.....|....|.....|....|.....|....|....|....|
                                 10          20          30          40         50
S. cerevisiae          AAACTTTCAA CAACGGATCT CTTGGTTCTC GCATCGATGA AGAACGCAGC
1S. pombe              AAACTTTCAG CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC
LRUB 20                CAACTTTCAA CAATGGATCT CTTGGCTCCG GCATCGATGA AGAACGCAGC
B. spartinae           AAACTTTCAA CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC
Gaeumannomyces amomi   AAACTTTCAA CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC
Magnaporthe grisea     AAACTTTCAA CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC
Harpophora maydis      AAACTTTCAA CAACGGATCT CTTGGCTCTG GCATCGATGA AGAACGCAGC
1M. terrestris         CAACTTTCAA CAATGGATCT CTTGGCTCCA GCATCGATGA AGAACGCAGC
1P. botulispora        AAACTTTCAA CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC
Pyricularia angulata   AAACTTTCAA CAACGGATCT CTTGGTTCTG GCATCGATGA AGAACGCAGC
Aspergillus flavipes   AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
A. niger               AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
A. ellipticus          AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
Fennellia nivea        AAACTTTCAA CAATGGATCT CTTGGTTCCG GCATCGATGA AGAACGCAGC
                       .....|....| ....|....| ....|....| ....|....|....|....|
                                 60          70          80          90        100
S. cerevisiae          GAAATGCGAT ACGTAATGTG AATTGCAGAA TTCCGTGAAT CATCGAATCT
1S. pombe              GAAATGCGAT ACGTAATGTG AATTGCAGAA TTCCGTGAAT CATCGAATCT
LRUB 20                GAAATGCGAT AACTAGTGTG AATTGCAGAT TTCAGTGAAT CATCGAGTCT
B. spartinae           GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Gaeumannomyces amomi   GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Magnaporthe grisea     GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Harpophora maydis      GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGCGAAT CATCGAATCT
1M. terrestris         GAAATGCGAT AACTAGTGTG AATTGCAGAT TTCAGTGAAT CATCGAGTCT
1P. botulispora        GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Pyricularia angulata   GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
Aspergillus flavipes   GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
A. niger               GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
A. ellipticus          GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
Fennellia nivea        GAAATGCGAT AACTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAGTCT
                       .....|....| ....|....| ....|....| ....|....|....|....|
                                 110         120         130         140       150
S. cerevisiae          TTGAACGCAC ATTGCCCCCT T-GGTATTCC AGGGGGCATG CCTGTTTGAG
1S. pombe              TTGAACGCAC ATTGCGCCTT TGGGTTCTAC CAAAGGCATG CCTGTTTGAG
LRUB 20                TTGAACGCAC ATTGCGCCTC TTGGTATTCC TCGAGGCATG CCTGTTCGAG
B. spartinae           TTGAACGCAC ATTGCGCCCG CCGGTATTCC GGCGGGCATG CCTGTTCGAG
Gaeumannomyces amomi   TTGAACGCAC ATTGCGCCCG CCGGTATTCC GGCGGGCATG CCTGTCCGAG
Magnaporthe grisea     TTGAACGCAC ATTGCGCCCG CCGGTATTCC GGCGGGCATG CCTGTTCGAG
Harpophora maydis      TTGAACGCAC ATTGCGCCCG CTGGTATTCC AGCGGGCATG CCTGTCCGAG
1M. terrestris         TTGAACGCAC ATTGCGCCTC TTGGTATTCC TCGAGGCATG CCTAT-CGAG
1P. botulispora        TTGAACGCAC ATTGCGCCCT GTGGTATTCC GCAGGGCATG CCTGTTCGAG
Pyricularia angulata   TTGAACGCAC ATTGCGCCCG CCGGTATTCC GGCGGGCATG CCTGTTCGAG
Aspergillus flavipes   TTGAACGCAC ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTCCGAG
A. niger               TTGAACGCAC ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTCCGAG
A. ellipticus          TTGAACGCAC ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTCCGAG
Fennellia nivea        TTGAACGCAC ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTCCGAG
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Figure D4 Alignment data of complete 5.8S sequences of isolate LRUB 20 and 13 refernce taxa from GenBank (*B=Buergenerula*, *1M=Mycoleptodissus*, *1P=Phialophora* S=*Saccharomyces*, *1S=Schizosaccharomyces*)

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                    ....|.....|
                             158
S. cerevisiae        CGTCATTT
1S. pombe            TGTCATTA
Lrub 20              CGTCGTTA
B. spartinae         CGTCATTT
Gaeumannomyces amomi CGTCATTT
Magnaporthe grisea   CGTCATTT
Harpophora maydis    CGTCATTT
1M. terrestris       CGTCGTTT
1P. botulispora      CGTCATTT
Pyricularia angulata CGTCATTT
Aspergillus flavipes CGTCATTA
A. niger             CGTCATTG
A. ellipticus        CGTCATTG
Fennellia nivea      CGTCATTG
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Figure D4 (Continued)

BIOGRAPHY

Mr. Porntep Chomcheon was born on December 4, 1977 in Uttaradit province, Thailand. He graduated with a Bachelor Degree of Science in Biotechnology from the Faculty of Science and Technology, Thammasat University, Thailand in 2000. He has been studying for a Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand since 2002.