องค์ประกอบทางเคมีของไลเคน *Usnea baileyi* (Stirt.) Zahlbr. จากเวียดนามและฤทธิ์ทางชีวภาพ

นายคิว หวัน เหวียน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHEMICAL CONSTITUENTS OF LICHEN *Usnea baileyi* (Stirt.) Zahlbr. FROM VIETNAM AND BIOLOGICAL ACTIVITIES

Mr. Kieu Van Nguyen

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
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By	Mr. Kieu Van Nguyen
Field of Study	Chemistry
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Vudhichai Parasuk, Ph.D.)

.....Thesis Advisor
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

.....Examiner
(Associate Professor Preecha Phuwapraisirisan, Ph.D.)

.....External Examiner
(Assistant Professor Ek Sangvichien, Ph.D.)

คิว หวัน เหวียน : องค์ประกอบทางเคมีของไลเคน *Usnea baileyi* (Stirt.) Zahlbr. จากเวียดนามและฤทธิ์ทางชีวภาพ (CHEMICAL CONSTITUENTS OF LICHEN *Usnea baileyi* (Stirt.) Zahlbr. FROM VIETNAM AND BIOLOGICAL ACTIVITIES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. วรินทร ชวศิริ, 101 หน้า.

เป็นที่ทราบกันเป็นอย่างดีว่า ไลเคนซึ่งเป็นสิ่งมีชีวิตที่เกิดจากการอยู่ร่วมกันของราและสิ่งมีชีวิตหุ้นส่วนที่สังเคราะห์แสงได้ ใช้เป็นสมุนไพรพื้นบ้าน ในการศึกษานี้ได้เก็บไลเคน *Usnea baileyi* (Stirt.) Zahlbr. จากภูเขา Tam Bo, Di Linh, Lam Dong ประเทศเวียดนาม ได้แยกและพิสูจน์เอกลักษณ์โดยอาศัยหลักฐานทางสเปกโทรสโกปีและเปรียบเทียบกับข้อมูลที่รายงานในเอกสารอ้างอิง 24 ชนิด ได้แก่ stictic acid (1), constictic acid (2), babartic acid (3), diffactaic acid (4), cryptostictic acid (5), hypoconstictic acid (6), menegazziaic acid (7), virensic acid (8), methylstictic acid (9), methyl 4-*O*-methyl haematomate (10), 8´-*O*-methylconstictic acid (11), 8´-*O*-methylmenegazziaic acid (12), 9´-*O*-methylprotocetraric acid (13), protocetraric acid (14), atranorin (15), methyl *b*-orsinol carboxylate (16), atranol (17), usnic acid (18), (20*R*,24*R*)-ocotillone (19), (20*S*,24*R*)-ocotillone (20), betulonic acid (21), dasypogalactone (22), 4-*O*-demethylbabartic acid (23) และ 7-hydroxy-5-methoxy-6-methylphthalide (24) ได้ศึกษาฤทธิ์ต้านแบคทีเรียของสารที่แยกได้ 19 ชนิด (1, 2, 4-9, 13-22, 24) ต่อ *Propionibacterium acnes* KCCM41747, *Staphylococcus aureus* ATCC25923, *Streptococcus sobrinus* KCCM11898, *Streptococcus mutans* ATCC25175 และ *Salmonella typhi* ATCC442 พบว่าที่ความเข้มข้น 1 mM ของ usnic acid (18) มีฤทธิ์ต้านแบคทีเรียทั้ง 5 ชนิด โดยแสดงบริเวณยับยั้งในช่วง 21-28 มิลลิเมตร นอกจากนี้ได้ศึกษาฤทธิ์ต้านอนุมูลอิสระของสาร 13 ชนิด (1, 4, 5, 7-11, 14, 17, 19, 20, 22, 24) ด้วยวิธี DPPH radical scavenging พบว่าสารทั้งหมดมีฤทธิ์ต้านอนุมูลอิสระระหว่าง 20-86 % จากสารที่นำมาทดสอบทั้งหมด พบว่า virensic acid (8) แสดงฤทธิ์ต้านอนุมูลอิสระสูงสุด โดยมี IC_{50} 0.41 mM.

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KIEU VAN NGUYEN: CHEMICAL CONSTITUENTS OF LICHEN *Usnea baileyi* (Stirt.) Zahlbr. FROM VIETNAM AND BIOLOGICAL ACTIVITIES. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 101 pp.

Lichens, symbiotic association of fungal and photosynthetic partners, have been well known used as folk and traditional medicine. In this study, lichen *Usnea baileyi* (Stirt.) Zahlbr., collected from Tam Bo mountain, Di Linh, Lam Dong, Vietnam was investigated. Twenty four constituents were isolated and identified based on spectroscopic evidence and compared the data with those reported in literatures as stictic acid (1), constictic acid (2), babartic acid (3), diffactaic acid (4), cryptostictic acid (5), hypoconstictic acid (6), menegazziaic acid (7), virensic acid (8), methylstictic acid (9), methyl 4-*O*-methyl haematomate (10), 8′-*O*-methylconstictic acid (11), 8′-*O*-methylmenegazziaic acid (12), 9′-*O*-methylprotocetraric acid (13), protocetraric acid (14), atranorin (15), methyl *b*-orsinol carboxylate (16), atranol (17), usnic acid (18), (20*R*,24*R*)-ocotillone (19), (20*S*,24*R*)-ocotillone (20), betulonic acid (21), dasypogalactone (22), 4-*O*-demethylbabartic acid (23), and 7-hydroxy-5-methoxy-6-methylphthalide (24). The antibacterial activities of nineteen compounds (1, 2, 4-9, 13-22, 24) were investigated against *Propionibacterium acnes* KCCM41747, *Staphylococcus aureus* ATCC25923, *Streptococcus sobrinus* KCCM11898, *Streptococcus mutans* ATCC25175 and *Salmonella typhi* ATCC442. Usnic acid (18) of 1 mM exhibited the highest activity against all bacteria with inhibition zone average in a range of 21-28 mm. Moreover, the antioxidant activity of thirteen compounds (1, 4, 5, 7-11, 14, 17, 19, 20, 22, 24) were investigated using DPPH radical scavenging assay. The scavenging effects of all compounds were in the range of 20-86 %. Among tested compounds, virensic acid (8) exhibited the highest DPPH radical scavenging activity with IC_{50} 0.41 mM.

Department: Chemistry Student's Signature .....
Field of Study: Chemistry Advisor's Signature .....
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LIST OF ABBREVIATION

1D	One dimensional
2D	Two dimensional
Ac	Acetone
AcOH	Acetic acid
br	Broad
calcd	Calculated
$CDCL_3$	deuterated chloroform
CC	Column chromatography
COSY	Homonuclear shift correlation spectroscopy
d	Doublet
Dc	dichloroform
dd	Doublet of doublets
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
EtOAc	Ethyl acetate
hexane	*n*-Hexane
HR-ESI-MS	High resolution electrospray ionization mass spectroscopy
m	Multiplet
MeOH	Methanol

NMR	Nuclear magnetic resonance
ppm	Parts per million (chemical shift value)
pTLC	Preparative thin-layer chromatography
q	Quartet
quint	Quintet
s	Singlet
sext	Sextet
t	Triplet
TLC	Thin-layer chromatography
UV	Ultraviolet

CHAPTER 1
INTRODUCTION

1.1 The lichen and usage of lichens

Lichens are symbiotic association of fungal partner (mycobiont) and photosynthetic partner (photobiont) such as greenalgae or cyanobateria. Lichens comprise over 25,000 species with around 98% Ascomycota fungal partners, and occur in a wide range of habitats like on or within rock, on soil, trees, shrubs, trucks, animal carapaces and on bricks, leather, wood [1]. Lichens are divided into three main types of thalli: crustose, foliose and fructicose (**Figure 1.1**) [2].

Xanthoria sp. (Crustose lichen) | *Xanthoparmelia* sp. (Foliose lichen) | *Usnea* sp. (Fructicose lichen)

Figure 1. 1 Types of lichen

1.2 Biological significance of lichen substances

Some of the biological meaning of the lichen metabolites were summarized by Huneck and Yoshimura [3] as follows:

- Lichens are slow-growing organisms, so the lichen metabolites are antibiotic and active protective substances to protect against lower and higher plant by themselves.
- The algae will be protected against too intensive irradiation by absorbing UV light of aromatic lichen substances.
- Symbiotic equilibrium promotion, which affects the cell wall permeability of photobionts.

- Lichen metabolites such as aliphatic and aromatic acids are strong chelating agents, which is very helpful for supplying the lichen with minerals from the substrate.
- Antifeedant activities which protect the lichen from insects and animals.
- Hydrophobic properties prevent the saturation of the medulla with water and to allow continuous gas exchange.

1.3 Usage of lichens

In the lichen division, lichens are comprised of at least 8 orders, 45 famillies, and 6,000 species [4].

Lichens have been used as folk and traditional medicine like traditional Indian medicine or traditional Chinese medicine. *Evernia furfuracea* (L.) Mann, family Pameliaceae was used as drug [4]. In Arabian medicine, *Alectoria usneoides* was used to treat enlarged spleen (splenomegaly) [4]. *Letharia vulpine* (L.) was used in stomach diseases in Northern California [4]. In India, *Parmelia chinense* was used as liniment for headache, and *P. sancti-angeli* was used to treat tinea. In Nepal, *P. nepalese* (Taylyor) Hale ex Sipman was used in the treatment of toothache and sore throat [4]. *Usnea*, belonging to Pameliaceae, is a fructicose lichen. *Usnea* generally grows by hanging from tree branches, resembling grey and greenish hair [4]. *Usnea* sp. was used in Homeopathic system of medicine and traditional medicine in Pacific island, New Zealand and traditional Chinese medicine. Around 500 A.D., *U. diffracta* Vain was used as medicine in China. *U. barbata* has been prescribed to use for uterine ailment by Hippocrates [4].

Lichens are used as basic material for perfume industry [3]. Up to 9,000 tons of two lichens: *Evernia prunastri* (L.) Ach. and *Pseudevernia furfuracea* (L.) Zopf. have been processed in Grasse, France. A typical ‘’mossy’’ flavor from the ethanol extract of both lichens is used not only like a component in certain perfumes, but also like a fixative which keeps the flavor for a long time [3].

Moreover, lichens were use as basic material for dyes. In 1966, dyes from *Roccella* species and other lichens were published by Kok [3]. Today, litmus is a complex mixture of pigment prepared mainly from *Roccella* species [3].

1.4 Biological activities of lichen substances

The biological activities of lichen substances have been shown extensively including antibiotic, antimycobacterial, antifungal, antiviral, antipyretic, anti-inflamatory, analgesic, antiproliferative, antitumour and cytotoxic effects. The biological activities in some recent studies are summarized in **Table 1.1**.

Table 1. 1 Biological activities of some lichen substances [5-9]

Antiviral activities	
Compounds	**Viruses and viral enzymes**
Depsidone: virensic acid and its derivatives	Human immunodeficiency virus.
Butyrolactone acid: protolichesterinic acid	HIV reverse transcriptase
(+)-Usnic acid and four orcinol depsides	Epstein-Barr virus (EBV)
Emodin, 7-chloroemodin, 7-chloro-1-*O*-methylemodin, 5,7-dichloroemodin, hypericin	HIV, cytomegalovirus and other viruses
Antibiotic and antifungal activities	
Compounds	**Organisms**
Usnic acid and its derivatives	Gram +ve bacteria, *Bacteroides* spp., *Clostridium perfringens, Bacillus subtilis, Staphylococcus aureus, Staphylococcus* spp., *Enterococcus* spp., *Mycobacterium aurum*

Methyl orsellinate, ethyl orsellinate, methyl β-orsellinate, methyl haematommate	*Epidermophyton floccosum, Microsporum canis, M. gypseum, Trichophyton rubrum, T. mentagrophytes, Verticillium achliae, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans*
Protolichesterinic acid	*Helicobacter pylori*
Pulvinic acid and its derivatives	*Drechslera rostrata, Alternaria alternate, Aerobic and anaerobic bacteria*
Antitumour and antimutagenic activities	
Compounds	**Activities/cell types**
(-)-Usnic acid	Antitumoral effect against Lewis Lung carcinoma, P388 leukaemia, mitotic inhibition, apoptotic induction, antiproliferative effect against human HaCaT keratinocytes
Scabrosin ester and its derivatives, euplectin	Cytotoxic effect against murine P815 mastocytoma and other cell lines
Hydrocarpone, salazinic acid, stictic acid	Apoptotic effect against primary culture of rat hepatocytes
Psoromic acid, chrysophanol, emodin and its derivatives	Antiproliferative effect against leukemia cells
Salazinic acid and stictic acid	Apoptotic effect against primary culture of rat hepatocytes

Enzyme inhibitory activities	
Compounds	**Enzymes**
Atranorin	Trypsin, Pankreaselastase, Phosphorylase
Chrysophanol	Glutathione reductase
Confluentic acid, 2β-*O*-methylperlatolic acid	Monoaminoxidase B
4-*O*-Methylcryptochlorophaeic acid	Prostataglandinsynthetase
(+)-Protolichesterinic acid	5-Lipoxygenase (HIV reverse transcriptase)
Vulpinic acid	Phosphorylase
Norsolorinic acid	Monoamino oxidase
Physodic acid	Arginine decarboxylase
Usnic acid	Ornithine decarboxylase

1.5 Lichen secondary metabolites

Lichen metabolites were divided into three main types based on their biosynthetic pathways as polyketide, shikimic acid, and mevalonic acid pathways (**Figure 1.2**) [10].

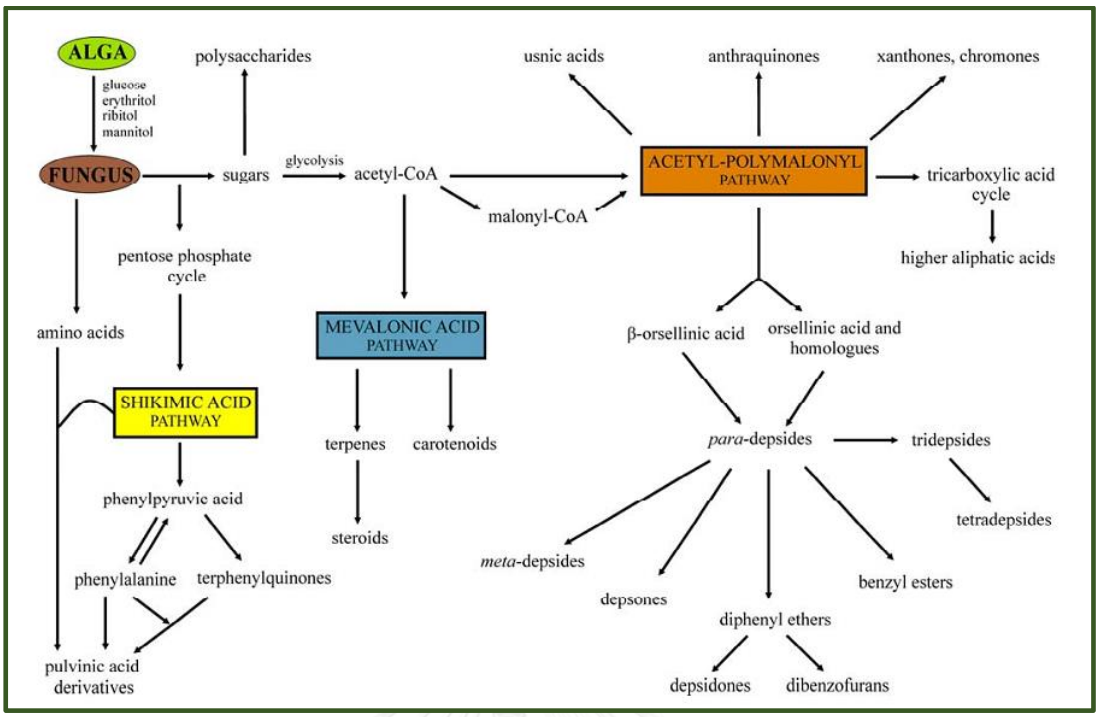


Figure 1. 2 Biosynthetic pathway of lichen substances [10]

1.5.1 Polyketide pathway

Aliphatic acids

In lichen, aliphatic acids are mostly the 5-membered lactones with alkyl chain such as lichesterinic acids, and some complex aliphatic acids such as roccelic acid (**Figure 1.3**) [11].

Monoaromatic compounds

Orsellinic acid, β-orsellinic acid and their derivatives are the most common monoaromatic compounds found in lichens (**Figure 1.3)** [11].

Diphenyl ethers

Diphenyl ethers are relatively rare in lichen. It is proposed to be the hydrolysis products of depsidones [12] as a result of their isolation or sometimes it can be called as "pseudodepsidones" because of their apparent biosynthetic relationship [10] (**Figure 1.3**) [11].

Dibenzofuran compounds

The third most abundant group in lichens after depsides and depsidones are dibenzofurans, which mostly are formed from orcinol-type monoaromatic units (**Figure 1.3**) [11].

Depsides

Depsides are consisted of two or more than two basic monocyclic aromatic moieties connecting by an ester bond. Depending on the correlate position between carboxyl group of first unit and hydroxyl group of the second unit, depsides can be divided into *para*- and *meta*-depsides. Because the monoaromatic units in lichen are common orsellinic acid, β-orsellinic acid and their derivatives, *o*-depsides are very rare, only isolecanoric acid has been known (**Figure 1.3**) [11].

Depsidones

Depsidones are formed by two monoaromatic units linked by an ester bond, the same as depsides with an additional ether bond, or diphenyl ethers to form a 7-membered ring which is between two aromatic rings (**Figure 1.3**) [11].

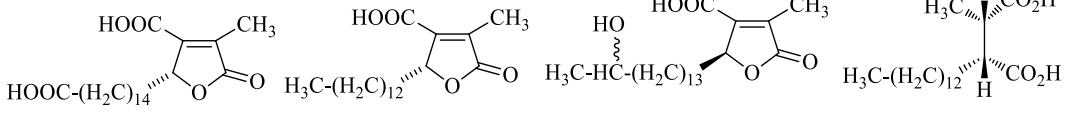
Depsones

Like diphenyl ethers, depsones compared to depsides and depsidones are very rare in lichen. In the A-ring, the aromaticity is lost while one bond between C-1 of A-ring and C-4′ of B-ring is formed (**Figure 1.3**) [11].

Quinones, chromones and xanthones

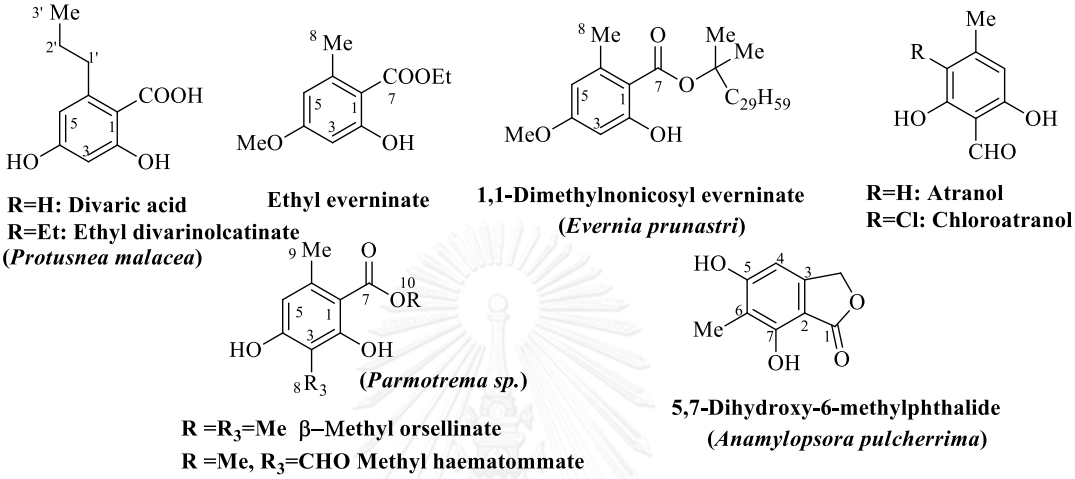
Sometime, binaphthoquinone, bixanthone, bianthraquinone can occur in lichen as quinone, and xanthone dimers, but it is very rare for bichromones (**Figure 1.3**) [11].

ALIPHATIC ACIDS



(+)-Praesorediosic acid **(+)-Protopraesorediosic acid** **(+)-Lichesterinic acid** **Roccellic acid**
(*Parmotrema praesorediosum*) **(*Roccella phycopsis*)**

MONOCYCLIC COMPOUNDS



R=H: Divaric acid
R=Et: Ethyl divarinolcatinate
(*Protusnea malacea*)

Ethyl everninate

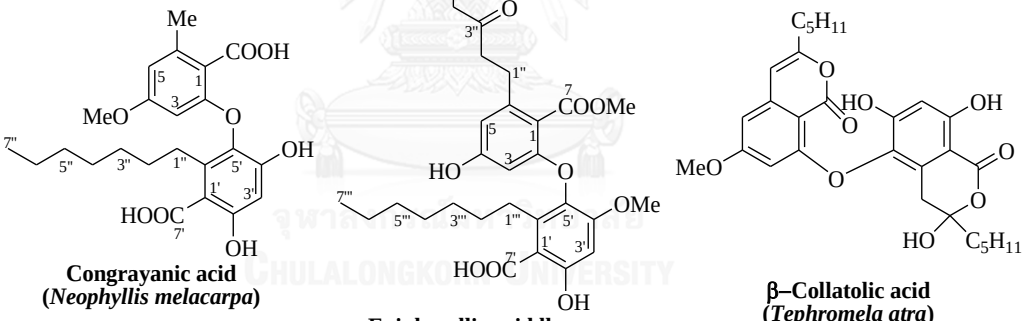
1,1-Dimethylnonicosyl everninate
(*Evernia prunastri*)

R=H: Atranol
R=Cl: Chloroatranol

R =R_3=Me β–Methyl orsellinate
R =Me, R_3=CHO Methyl haematommate
(*Parmotrema sp.*)

5,7-Dihydroxy-6-methylphthalide
(*Anamylopsora pulcherrima*)

DIPHENYL ETHERS



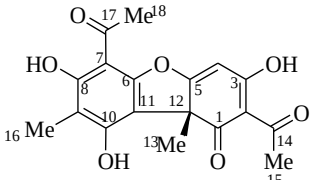
Congrayanic acid
(*Neophyllis melacarpa*)

Epiphorellic acid ll
(*Coelopogon epiphorella*)

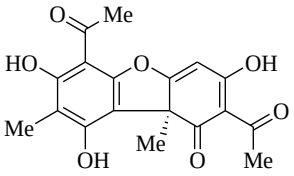
β–Collatolic acid
(*Tephromela atra*)

Figure 1. 3 Lichen secondary metabolites via polyketide pathway

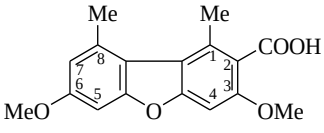
DIBENZOFURANS



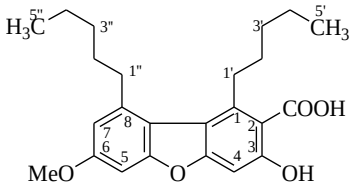
(+)-(12*R*)-Usnic acid
(*Evernia prunastri*)



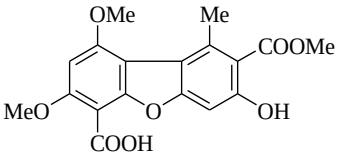
(-)-(12*S*)-Usnic acid
(*Cladonia stellaris*)



Ascomatic acid
(*Bunodophoron patagonicum*)

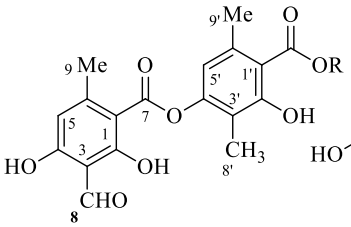


Condidymic acid
(*Clodonia rigida*)

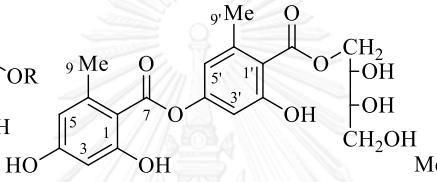


3-*O*-Demethylschizopeltic acid
(*Rocella hypomecha*)

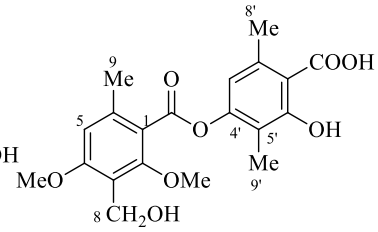
DEPSIDES



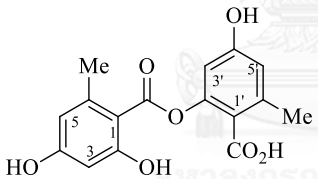
Atranorin (*Parmotrema sp.*)



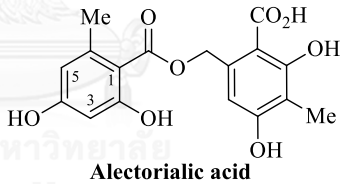
Erythrin (*Rocella sp.*)



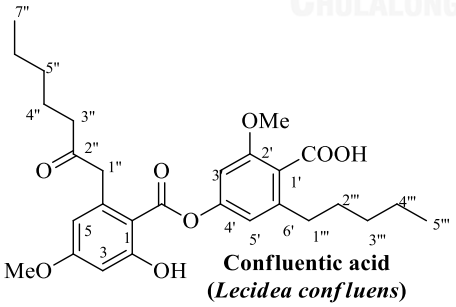
8-Hydroxydiffractaic acid
(*Usnea longissima*)



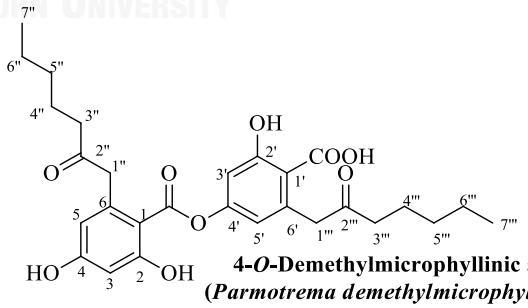
Isolecanoric acid



Alectorialic acid



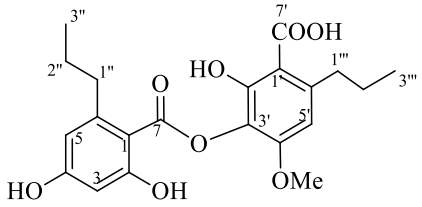
Confluentic acid
(*Lecidea confluens*)



4-*O*-Demethylmicrophyllinic acid
(*Parmotrema demethylmicrophyllicum*)

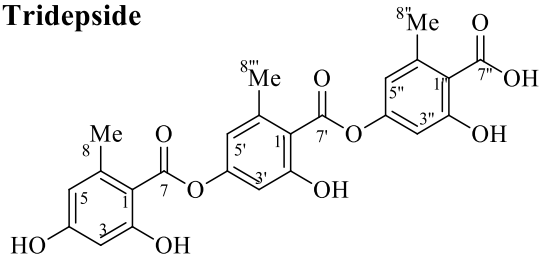
Figure 1.3 (continued)

***Meta*-depside**



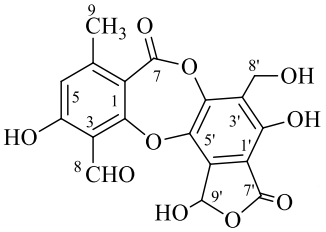
Sekikaic acid
(*Ramalina boulhautiana*)

Tridepside

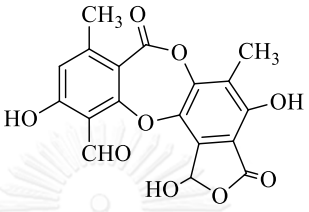


Gyrophoric acid
(*Lasallia pustulata*)

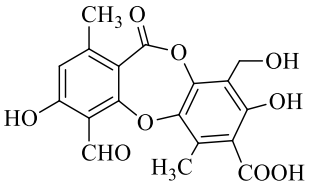
DEPSIDONES



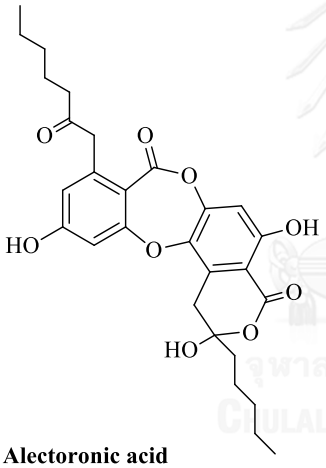
Salazinic acid



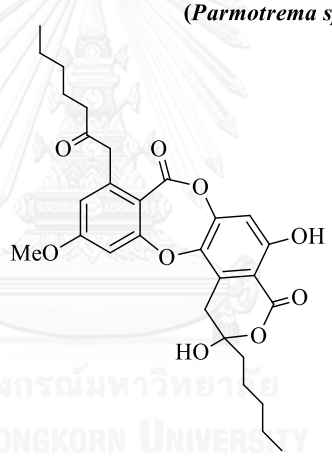
Norstictic acid
(*Parmotrema sp.*)



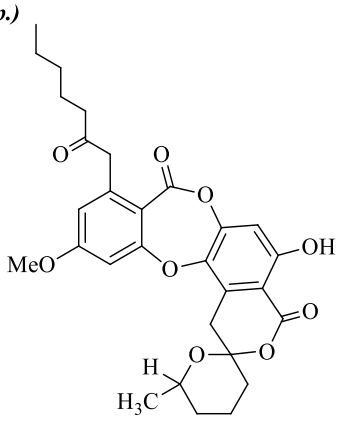
Protocetraric acid



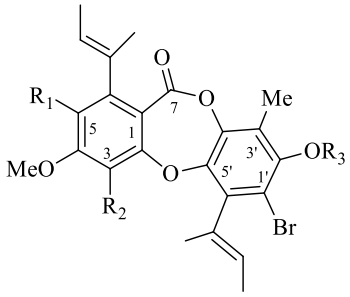
Alectoronic acid



α-Collatolic acid
(*Parmotrema sp.*)



Dehydrocollatolic acid



R_1=R_3=H, R_2=Me **Acarogobien A**

R_1=Br, R_2=CHO,
R_3= **Acarogobien B**

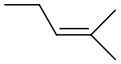
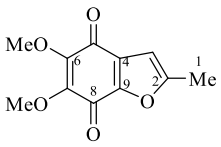


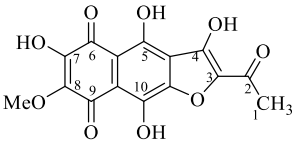
Figure 1.3 (continued)

QUINONES, CHROMONES, AND XANTHONES

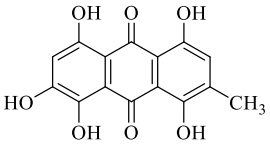
Quinones and *bis*-quinones



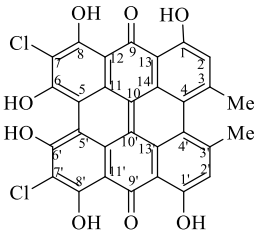
Benzoquinone
(***Graphis desquamescens***)



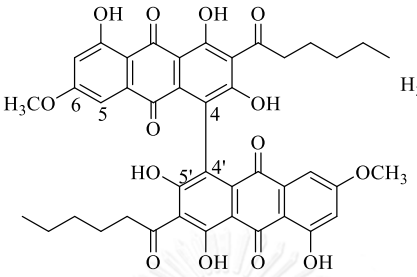
Chiodectonic acid
(***Cryptothecia robrocincta***)



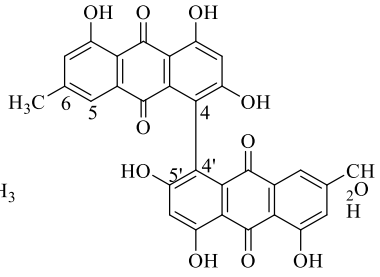
Asahinin
(***Asahinea chrysanta***)



7,7'-Dichlorohypericin
(***Nephroma laevigatum***)

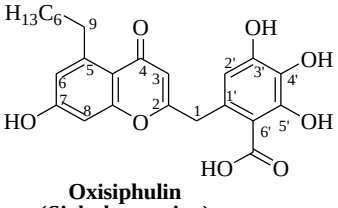


4,4'-Disolorinic acid
(***Solorina crocea***)

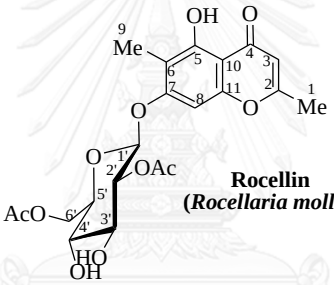


Oxyskyrin
(***Trypertheliopsis bonibensis***)

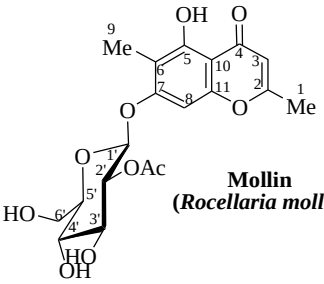
Chromones



Oxisiphulin
(***Siphula ceratites***)

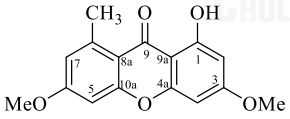


Rocellin
(***Rocellaria mollis***)

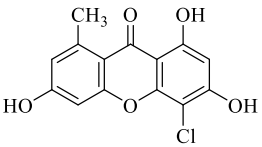


Mollin
(***Rocellaria mollis***)

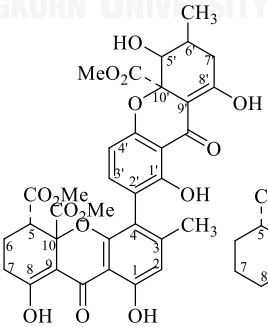
Xanthones and *bis*-xanthones



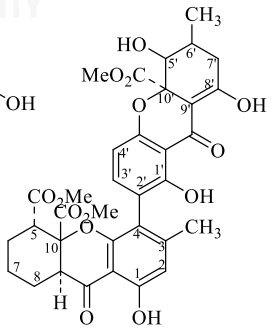
Lichexanthone (*Parmotrema sp.*)



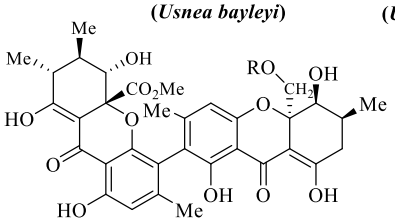
4-Chloronorlixanthone
(***Lecanora straminea***)



Eumitrin A1
(***Usnea bayleyi***)



Eumitrin B
(***Usnea bayleyi***)



R=H: Hirtusneanine
R=L-rhamnose: Hirtusneanoside
(***Usnea hirta***)

Figure 1.3 (continued)

1.5.2 Shikimic acid pathway

In this pathway, the common examples are terphenylquinones, phenylalanine, or pulvinic acid and derivatives which often contain two benzene rings with non- or mono-substituents (**Figure 1.4**) [11].

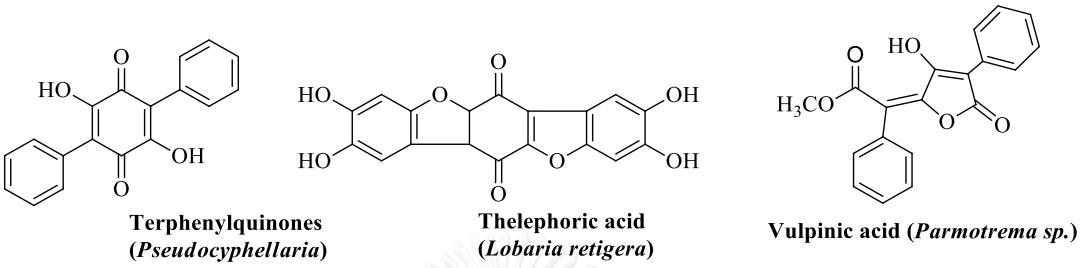


Figure 1. 4 Lichen secondary metabolites via shikimic acid pathways

1.5.3 Mevalonic acid pathway

In lichen, triterpenoids which hopane or fern-9(11)-ene skeletons were isolated (**Figure 1.5**) [11]. Other terpenoids, steroids, and carotenoids were also found.

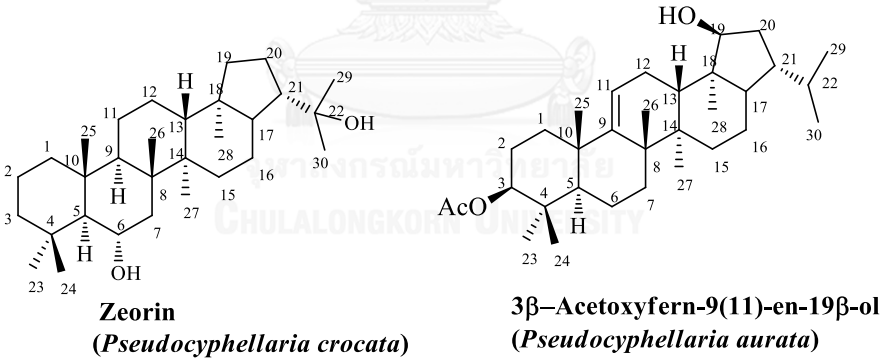


Figure 1. 5 Hopane or fern-9(11)-ene skeletons

1.6 Chemical constituents of lichens in *Usnea* genus

Usnea, appeared on host trees as a shrub-like, generally grows hanging from tree branches, resembling grey and greenish hair (**Figure 1.1**). In the middle of the thallus, an elastic chord or axis running through that can be indicated by carefully pulling a filament apart from either end [13]. It is being one of the largest genera in the

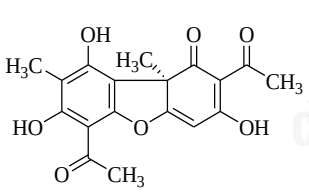
Parmeliaceae with more than 600 species [14]. Many secondary metabolites of *U*snea genus were reported.

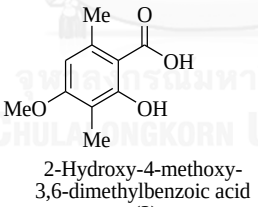
Seven compounds: (+)-usnic acid (**1**), 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**2**), ethyl 2,4-dihydroxy-3,6-dimethylbenzoate (**3**), ethyl 2-hydroxy-4-methoxy-3,6-dimethylbenzoate (**4**), evernic acid (**5**), barbatic acid (**6**) and diffractaic acid (**7**) were isolated from *U. emidotteries* [15] (**Figure 1.6**)

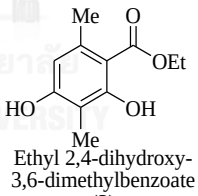
In addition, Devehat and Boustie [16] isolated two new β-orcinol depsidones, depsidone **1** (**8**) and cryptostictinolide (**9**), together with 13 known compounds: barbatic acid (**6**), atranorin (**10**), norstictic acid (**11**), stictic acid (**12**), fumarprotocetraric acid (**13**), constictic acid (**14**), cryptostictic acid (**15**), menegazziaic acid (**16**), peristictic acid (**17**), methyl β-orcinolcarboxylate (**18**), (+)-usnic acid (**1**) and ergosterol peroxide (**19**) from *U. articulata* collected in Indonesia (**Figure 1.6**).

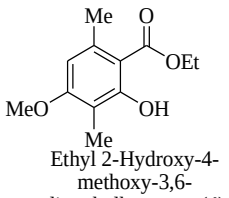
Paranagama and Gunatilaka (2007) [17] isolated herbarin (**20**) and a heptaketide, 1-hydroxydehydroherbarin (**21**) from lichen *U. cavernosa* (**Figure 1.6**).

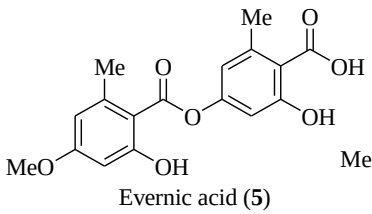
From lichen *U. alata* growing on trees in La Carbonera, state of Mérida, Venezuela, Keeton and Keogh (1973) [18], norstictic acid (**11**), stictic acid (**12**) and caperatic acid (**22**) were isolated (**Figure 1.6**).

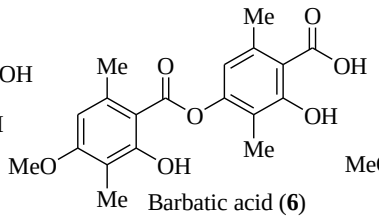

(+)-Usnic acid (**1**)

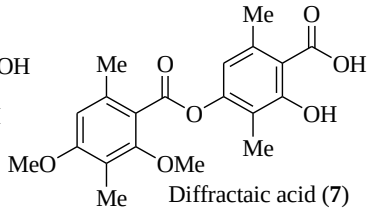

2-Hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**2**)


Ethyl 2,4-dihydroxy-3,6-dimethylbenzoate (**3**)


Ethyl 2-Hydroxy-4-methoxy-3,6-dimethylbenzoate (**4**)


Evernic acid (**5**)


Barbatic acid (**6**)


Diffractaic acid (**7**)

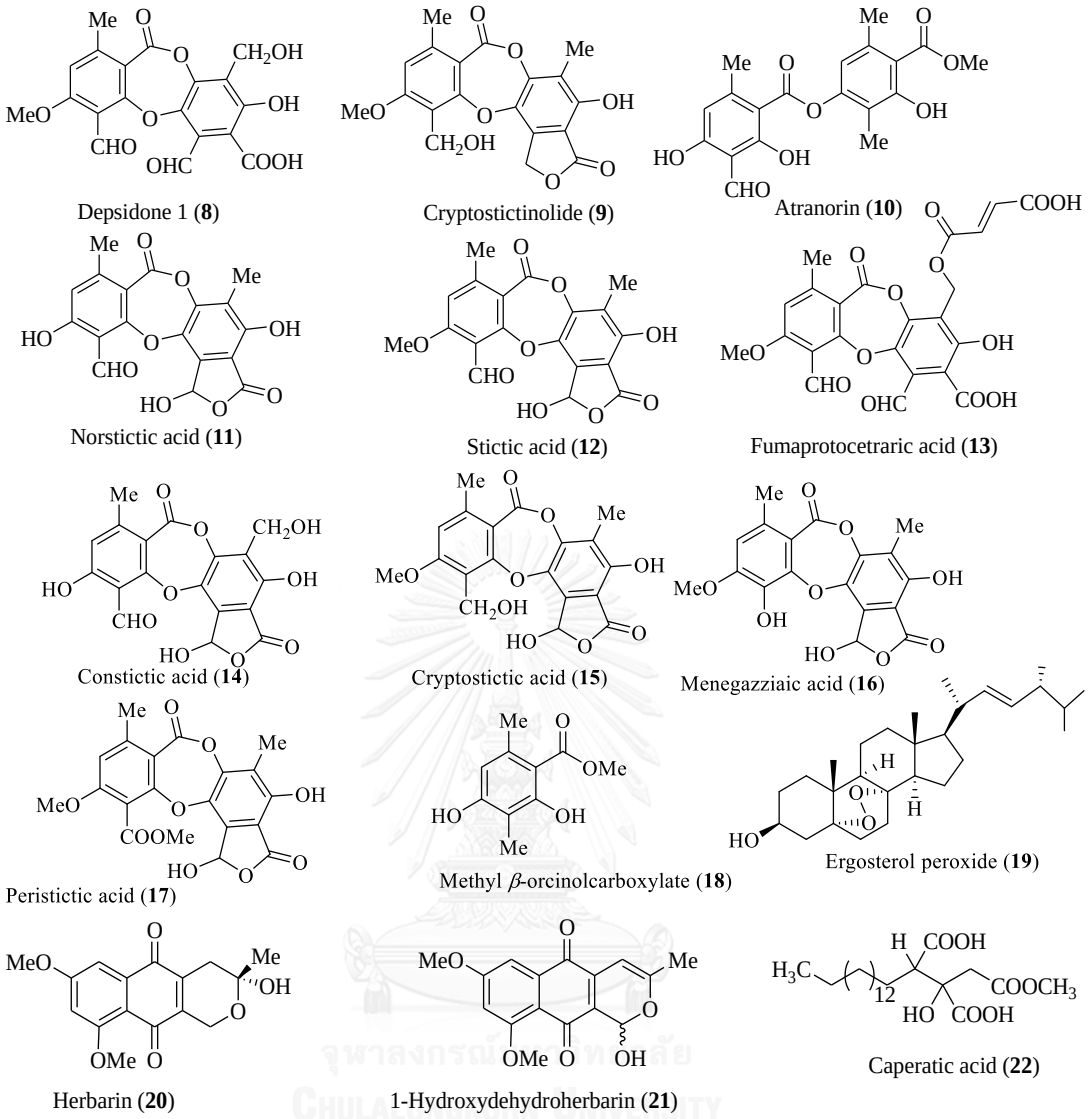
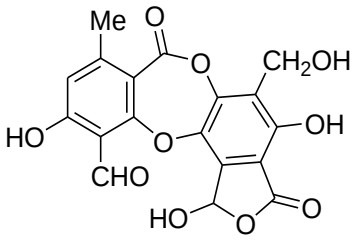


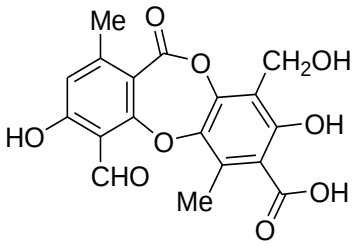
Figure 1. 6 Chemical constituents (**1-22**) from *Usnea* genus

Several species of *Usnea* genus were investigated; nonetheless, there are a few paper reported for *U. baileyi*.

In 2010, Din and Elix [19] reported the presence of (+)-usnic acid (**1**), salazinic acid (**23**), norstictic acid (**11**), atranorin (**10**) and protocetraric acid (**24**) (**Figure 1.6** and **1.7**) from lichen *U. baileyi* collected in Bukit Larut, Taiping, Malaysia.



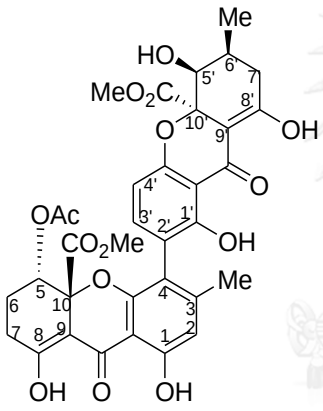
Salazinic acid (**23**)



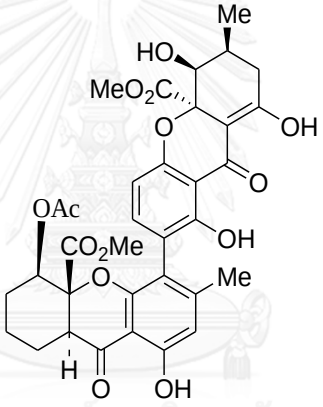
Protocetraric acid (**24**)

Figure 1. 7 Chemical constituents (**23-24**) from *U. baileyi*

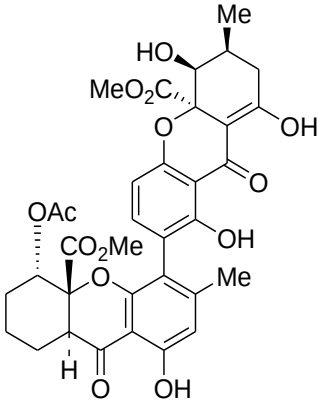
In 1973, Yang and Shibata isolated eumitrin A_1 (**25**), eumitrin A_2 (**26**) and eumitrin B (**27**) from yellow pigment of lichen *U. baileyi* (Stirt.) Zahlbr collected at Yuriagehama (**Figure 1.8**) [20].



Eumitrin A_1 (**25**)



Eumitrin A_2 (**26**)



Eumitrin B (**27**)

Figure 1. 8 Chemical constituents (**25-27**) from *U. baileyi*

1.7 Research scope and objectives

In Vietnam, the tropical monsoon climate is very suitable for lichen developing [21]. Vietnam has a number of diverse tropical lichen, but only a few species have been studied [21]. The chemical constituents of Vietnamese lichen are worth for further investigation in order to isolate novel compounds and/or biologically active compounds according to the diversity of Vietnamese lichen. Thus, the major purpose of this thesis is to investigate the chemical constituents of Vietnamese lichen, *Usnea baileyi* (Stirt.) Zahlbr. collected in highland.

CHAPTER 2
EXPERIMENTAL

2.1 Instruments and materials

2.1.1 Instruments and chemicals

The solvent was evaporated from the extracts using rotatory evaporator Buchi-111. ^{1}H, ^{13}C and 2D NMR spectra were acquired on Bruker Avance (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) spectrometers. All instruments are in Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Thin layer chromatography (TLC) was performed on silica gel Merck Kieselgel 60 PF_{254}. Silica gel (No. 7729, 7734, and 9385, Merck) was used for quick and open column, respectively with solvent system including hexane, dichloromethane, ethyl acetate, acetone, methanol, and acetic acid. Visualizing reagents for TLC were 10% solution of H_2SO_4 and vanillin/H_2SO_4.

2.1.2 Lichen material *Usnea baileyi*

In June 2015, lichen *Usnea baileyi* (Stirt.) Zahlbr. (**Figure 2.1**) was collected from the barks of trees in Tam Bo mountain, Di Linh, Lam Dong, Vietnam where is 1000 m alt. The scientific name of this lichen was identified by Ms. Natwida Dangphui and Assistant Professor Dr. Ek Sangvichien, Lichen Research Unit, Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand.

Figure 2. 1 The lichen *Usnea baileyi*

2.2 Extraction

The air-dried lichen powder (800 g) was extracted with acetone at room temperature by maceration to get acetone extract (80 g) after evaporating acetone under reduced pressure. The acetone extract (80 g) was washed many times with acetone to obtain two parts: precipitate (23.7 g) and the acetone solution which was further evaporated to afford the acetone fraction (56.2 g).

The acetone fraction (56.2 g) was applied to silica gel quick column eluting with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), acetone and methanol (MeOH) to obtain four fractions: **DC** (31.2 g), **EA** (9.6 g), **AC** (6.5 g) and **ME** (4.6 g).

Fraction **EA** (9.6 g) was washed by hot acetone to get two sub-fractions: **EP** (1.0 g) and **EL** (7.8 g). **EP** (1.0 g) was applied on normal phase silica gel column with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06) (solvent system S1) yielding two compounds: **1** (506.2 mg) and **2** (307.0 mg). **EL** (7.8 g) was separated into three subfractions: **EL1** (100.0 mg), **EL2** (1.2 g), and **EL3** (6.5 g) using solvent system S1 for chromatographic column with normal silica gel (CC/S1). From **EL2** (1.2 g), two compounds: **3** (14.1 mg) and **4** (18.4 mg) were isolated. Four compounds: **5** (12.2 mg), **6** (6.8 mg), **7** (9.5 mg), and **8** (3.0 mg) were obtained from **EL3** (6.5 g) using CC/S1.

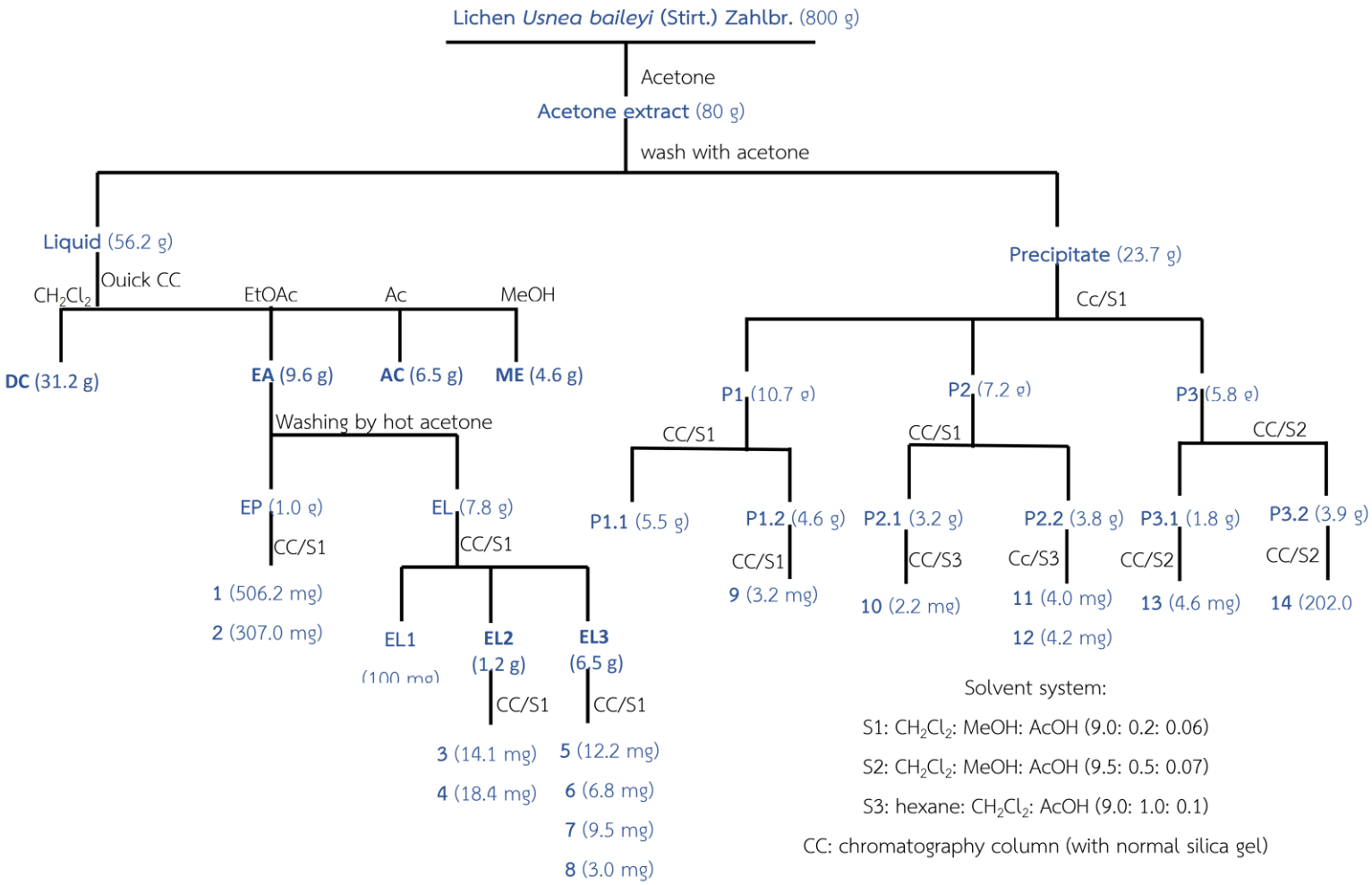
Three fractions: **P1** (10.7 g), **P2** (7.2 g), and **P3** (5.8 g) were obtained from the separation of precipitate fraction (23.7 g) using CC/S1. Each fraction was further separated into two subfractions: **P1.1** (5.5 g) and **P1.2** (4.6 g) from **P1** with CC/S1; **P2.1** (3.2 g) and **P2.2** (3.8 g) from **P2** with CC/S1, and **P3.1** (1.8 g) and **P3.2** (3.9 g) from **P3** with CC/CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07) (solvent system S2).

Compound **9** (3.2 mg) was obtained from the separation of **P1.2** (4.6 g) using CC/S1. Using CC/hexane: CH_2Cl_2: AcOH (9.0: 1.0: 0.1) (solvent system S3) to purify **P2.1** furnished compound **10** (2.2 mg). The purification of **P2.2** using silica gel column and solvent system: S1 led to the isolation of two compounds: **11** (4.0 mg) and **12** (4.2 mg) while compounds **13** (4.6 mg) and **14** (2.2 g) were achieved from the separation of **P3.1** and **P3.2** using silica gel column and solvent system S2. The summary of the separation of **EA** and precipitate fractions can be depicted as shown in **Scheme 2.1**.

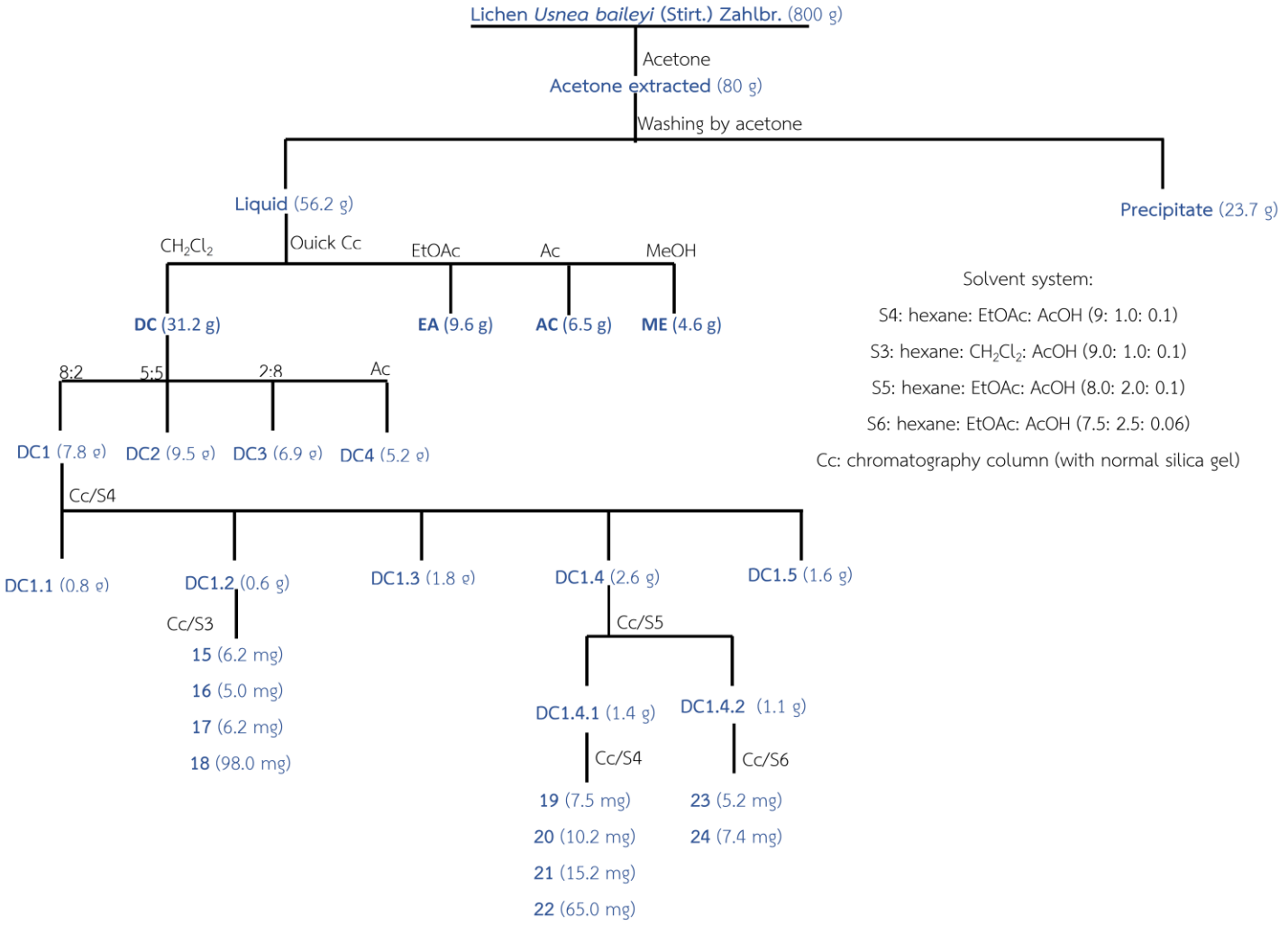
The dichloromethane fraction (**DC**, 31.2 g) was separated by silica gel column with hexane: EtOAc (8:2, 5:5, 2:8), then the column was cleaned by acetone to obtain four fractions: **DC1** (7.8 g), **DC2** (9.5 g), **DC3** (6.9 g), and **DC4** (5.2 g). Using CC/hexane: EtOAc: AcOH (9.0: 1.0: 0.1) (solvent system S4), **DC1** (7.8 g) was separated into five subfractions: **DC1.1** (0.8 g), **DC1.2** (0.6 g), **DC1.3** (1.8 g), **DC1.4** (2.6 g), and **DC1.5** (1.6 g).

Four compounds: **15** (6.2 mg), **16** (5.0 mg), **17** (6.5 mg), and **18** (3.4 mg) were obtained from the separation of **DC 1.2** (0.6 g) using CC/S3.

DC1.4 (2.6 g) was separated into two subfractions: **DC1.4.1** (1.4 g) and **DC1.4.2** (1.1 g) with CC/hexane: EtOAc: AcOH (8.0: 2.0: 0.1) (solvent system S5). The purification of **DC1.4.1** (1.4 g) using silica gel column and solvent system S4 led to the isolation of four compounds: **19** (7.5 mg), **20** (10.2 mg), **21** (15.2 mg) and **22** (65.0 mg) while two compounds: **23** (5.2 mg), and **24** (7.4 mg) were obtained from the separation of **DC1.4.2** (1.1 g) using silica gel column and hexane: EtOAc: AcOH (7.5: 2.5: 0.06) (solvent system S6). The summary of the separation of **EA** and precipitate fractions can be depicted as shown in **Scheme 2.2**.



Scheme 2. 1 Procedure for the separation of EA and precipitate fraction of *U. baileyi*



Scheme 2. 2 Procedure for the separation of DC fraction of *U. baileyi*

2.3 Biological activities

2.3.1 Antibacterial activity

The antibacterial testing was performed by diffusion agar method. Five bacterial pathogens including *Propionibacterium acnes* KCCM41747 (*P. acnes*), *Staphylococcus aureus* ATCC25923 (*S. aureus*), *Streptococcus sobrinus* KCCM11898 (*S. sobrinus*), *Streptococcus mutans* ATCC25175 (*S. mutans*), and *Salmonella typhi* ATCC5442 (*S. typhi*) were tested. The evaluation was based on the antibiotic sensitivity of bacteria. When the compounds were put into an agar plate in the presence of bacteria, the zone of inhibition was formed if the bacteria were killed or not grown enough. A larger zone will be formed by the stronger antibiotics. In details, the nutrient broth with the inoculated test organisms were incubated for 24 h at room temperature. Then, 0.6 mL of these broth was added to 60 mL of molten agar and poured into a sterile Petri dish. After that, the bacteria was spread to the petri dish nutrient agar. 1 mM of sample was introduced into the well and incubated for 24 h at room temperature. The diameter of the zone of inhibition was recorded to evaluate the antibacterial activity of compounds.

2.3.2 Antioxidant activity

The antioxidant activity of lichen compounds were measured by DPPH radical scavenging assay. The method used was slightly modified from that described in reference [22]. Samples were diluted in DMSO, excepted ascorbic acid (diluted in MeOH) with different concentrations: 1000, 500, 250, 136 and 62.5 µg/mL. 50 µL of samples were places into 96 well plate and followed by addition of 100 µL of DPPH in MeOH solution with concentration 0.05 mg/mL. The mixtures were incubated for 30 minutes at room temperature in dark room. Positive controls were generated by using ascorbic acid. The absorbance was measured by micro plate reader Biotek at wavelength 517 nm. The DPPH free radical concentration was calculated by using the equation showed below:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

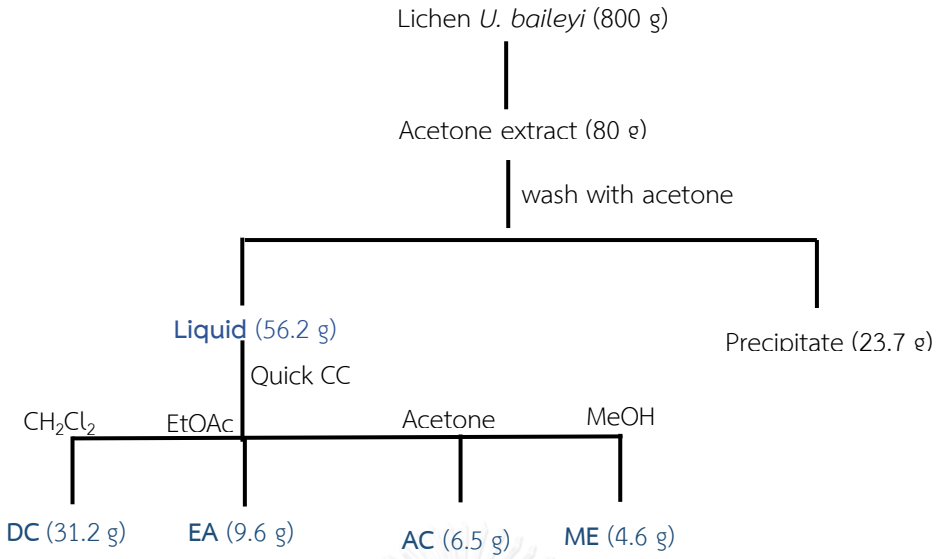
Where A_0 is the absorbance of the negative control, A_1 is the absorbance of the standard or the mixture between samples and DPPH. The inhibition concentration 50% (IC_{50}), which was the concentration of samples reducing the DPPH radical about 50%, was used to compare the radical scavenging activity of tested samples.

CHAPTER 3
RESULTS AND DISCUSSION

Lichens have been long-known used as folk and traditional medicine [4]. Since the tropical monsoon climate in Vietnam is very suitable for lichen developing [21], a large biodiversity of Vietnamese lichen is recognized. Lichen *Usnea baileyi* was collected from Tam Bo mountain, Di Linh, Lam Dong, Vietnam to investigate with the main aim to isolate and elucidate the structures of their chemical constituents. In addition, the biological activities such as antibacterial and antioxidant of isolated compounds will be evaluated.

3.1 Extraction and fractionation of lichen *U. baileyi*

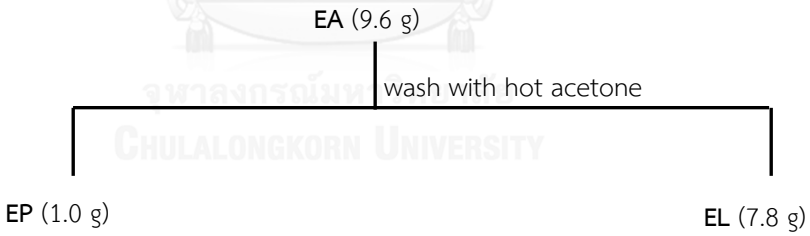
800 g of air-dried lichen powder was extracted with acetone at room temperature by maceration to obtain 80 g of acetone extract after evaporating the solvent under reduced pressure. The acetone extract was further washed with acetone many times to yield the liquid (56.2 g, 70.25 %) and precipitate fractions (23.7 g, 29.62 %). The liquid fraction was subjected to silica gel quick column and eluted with CH_2Cl_2, EtOAc, acetone and MeOH to gain four fractions: CH_2Cl_2 (31.2 g, 55.52 %), EtOAc (9.6 g, 17.08 %), acetone (6.5 g, 11.57 %) and MeOH (4.6 g, 8.19 %) fractions. The details of the extraction and fractionation are summarized in **Scheme 3.1**.



Scheme 3. 1 The extraction and fractionation of lichen *U. baileyi*

3.2 Separation of ethyl acetate (EA) fraction

After extraction the **EA** fraction (9.6 g) with hot acetone, two fractions: **EP** (1.0 g, 10.42 %) and **EL** (7.8 g, 81.25 %) were obtained as detailed in **Scheme 3.2**.



Scheme 3. 2 Fractionation and isolation of **EA** fraction

3.2.1 Isolation and structural elucidation of the constituents from EP fraction.

Fraction **EP** was separated by silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06). Three sub-fractions were obtained as shown in **Table 3.1**.

Table 3. 1 The separation of **EP**

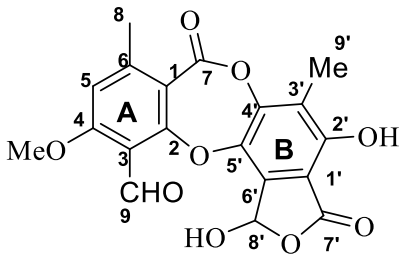
Solvent system	fraction	weight (mg)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**EP1**	506.2	White solid (compound **1**)
	EP2	37.3	White solid (mixture of **1** and **2**)
	EP3	307.0	White solid (compound **2**)

3.2.1.1 Structural elucidation of compound 1

Compound **1** was isolated as white powder (506 mg, 50.62 %) from **EP**. (**Table 3.1**). The TLC showed only single spot with R_f 0.51 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The ^{1}H NMR spectrum (**Figure A.1**) showed seven signals including a formyl proton at δ_H 10.45 (3-CHO), a singlet signal belonging to a hydroxy group at δ_H 8.19 (8′-OH), and a methoxy signal at δ_H 3.89 (3H, 4-OMe). Two methyl signals with 3H integration were detected at δ_H 2.48 (C-8) and 2.17 (C-9′).

The ^{13}C NMR spectrum (**Figure A.2**) of **1** revealed nineteen carbons, including one aldehyde carbon at δ_C 186.6 (C9), two carboxyl carbons at δ_C 163.0 (C-7′) and 160.7 (C-7), twelve aromatic carbons in the range of δ_C 108-167 implying two six-membered aromatic rings, one oxygenated carbon at δ_C 56.8 belonging to a methoxy group, one hemiacetal carbon at δ_C 95.1 (C8′), and two methyl carbons at δ_C 21.5 (C8), and 9.6 (C9′).

The ^{1}H and ^{13}C NMR spectra of this compound were compared with those of stictic acid [11] and assured that the structure of **1** was stictic acid, a depsidone. The tentative assignment of ^{1}H and ^{13}C NMR chemical shifts are tabulated in **Tables 3.2** and **3.3**.



Compound **1**: stictic acid

Table 3. 2 The tentative assignment of ^{1}H NMR chemical shifts of **1**, **2**, **5**, **6**, **7**, and **8**

Position	**1**	**2**	**5**	**6**	**7**	**8**
5	7.10 s	7.08 s	6.95 s	6.87 s	6.82 s	6.73 s
8	2.48 s	2.46 s	2.45 s	2.39 s	2.30 s	2.33 s
9	10.45 s	10.42 s	4.80 d, 11.2	2.23 s	9.10 s	10.49 s
			4.62 d, 11.2			
4-OMe	3.89 s	3.88 s	3.87 s	3.85 s	3.81 s	
8′	6.60 s	6.62 s	-	6.67 s	6.70 s	2.31 s
9′	2.17 s	4.60 s	2.19 s	4.60 s	2.11 s	2.05 s
8′-OH	8.19 s	8.25 s	8.22 s	8.29 s	8.25 s	
4-OH						11.85

Table 3. 3 The tentative assignment of ^{13}C NMR chemical shifts of **1**, **2**, **5**, **6**, **7**, and **8**

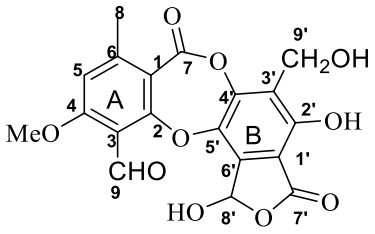
Position	**1**	**2**	**5**	**6**	**7**	**8**
1	113.1	114.4	111.6	110.8	113.3	117.0
2	166.3	166.2	166.5	166.1	166.5	164.0
3	114.5	114.5	118.5	114.5	138.2	112.3
4	162.4	162.4	161.7	161.1	152.4	155.3
5	112.8	112.8	112.7	112.5	111.7	115.1
6	150.8	150.7	148.2	148.3	152.4	152.0
7	160.7	160.6	158.9	158.7	161.4	161.2
8	21.5	21.4	20.8	20.7	19.8	21.4
9	186.6	186.6	51.3	8.6		191.7
4-OMe	56.8	56.8	56.2	56.2	56.2	
1′	109.2	113.2	109.0	109.1	109.0	111.8
2′	152.0	152.7	151.5	152.3	151.4	163.8
3′	120.8	123.2	120.3	122.1	120.4	115.7
4′	148.0	148.2	144.2	141.8	148.3	144.7
5′	137.5	138.1	137.9	137.8	132.4	141.8
6′	135.9	137.4	135.8	136.9	134.9	127.6
7′	163.0	162.4	161.4	161.1	161.4	170.8
8′	95.1	95.3	95.4	95.3	95.4	14.3
9′	9.6	52.6	9.5	52.7	9.5	9.3

3.2.1.2 Structural elucidation of compound 2

Compound **2** (307 mg, 30.70 %) was obtained as white amorphous from fraction **EP** with R_f 0.26 [CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07)]. The ^{1}H NMR spectrum (**Figure A.3**) displayed one formyl group at δ_H 10.42 (3-CHO), one singlet signal belonging to a hydroxy group at δ_H 8.25 (8′-OH). It also showed another singlet signal of oxygenated methylene group at δ_H 4.60 (2H, 8′-CH_2O-), one methoxy signal at δ_H 3.88 (3H, 4-OMe), and one signal of methyl group at δ_H 2.46 (8-Me).

The ^{13}C NMR spectrum (**Figure A.4**) of **2** showed total nineteen carbons. By comparison of this NMR spectrum with that of **1**, the chemical shift pattern was very similar. This implied that the structure of ring A should contain the substituents as one aldehyde carbon at δ_C 186.6 (C-9), one carboxyl carbon at 160.6 (C7), one methoxy group at δ_C 56.8 (4-OMe) and one methyl group at δ_C 21.4 (C8). In the ring B, the appearance of signal at δ_C 52.6 along with the disappearance of signal at δ_C 9.6 was strongly suggested that the substituents in ring B were the same as those of **1**, but the methyl group at C-9 was replaced by an oxygenated methylene group (-CH_2OH).

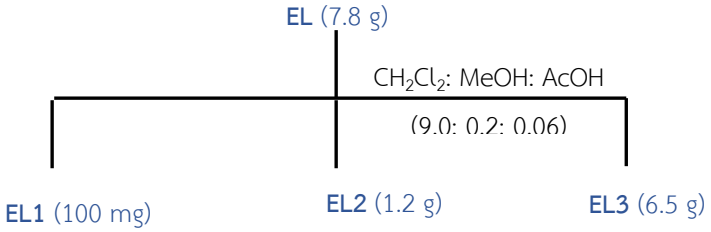
After comparing the ^{13}C NMR spectrum of **2** with that of constictic acid [23], **2** was elucidated as constictic acid. The tentative ^{1}H and ^{13}C NMR chemical shift assignment is presented in **Tables 3.2** and **3.3**.



Compound **2**: constictic acid.

3.2.2 Isolation and structural elucidation of compounds from EL fraction.

EL (7.8 g) was applied to silica gel column and eluted with solvent system S1 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)] to separate **EL** into three fractions: **EL1** (0.1 g, 1.28 %), **EL2** (1.2 g, 15.4 %), and **EL3** (6.5 g, 83.3 %) (**Scheme 3.3**, **Table 3.4**).



Scheme 3. 3 Fractionation and isolation of **EL**

Table 3. 4 The separation of **EL**

solvent system	fraction	weight (g)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**EL1**	0.1	White solid
	EL2	1.2	White solid
	EL3	6.5	White solid

Separation of fraction EL2

Fraction **EL2** 1.2 g was re-separated using silica gel column with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06). The results were presented in **Table 3.5.**

Table 3. 5 The separation of **EL2**

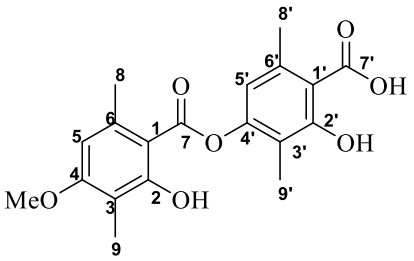
Solvent system	subfractions	weight (mg)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**EL2.1**	960	White solid
	EL2.2	14.1	White needle (compound **3**)
	EL2.3	18.4	White needle (compound **4**)

3.2.2.1 Structural elucidation of compound 3

Compound **3** was isolated from **EL2** as colorless needle (14.1 mg, 0.18 %). With vanillin stain, dark-pink color on TLC was detected which was a characteristic color of depside. This compound showed a single spot at R_f 0.28 [CH_2Cl_2: MeOH (9:1)]. The ^{1}H NMR spectrum (**Figure A.5**) exhibited total seven signals including one chelated hydroxy signal at δ_H 10.74 (1H, 2′-OH), one methoxy signal at δ_H 3.85 (3H, 4-OMe), and four methyl signals at δ_H 2.56 (3H, H-8), 2.48 (3H, H-8′), 2.00 (3H, H-9), and 1.98 (3H, H-9′).

In the ^{13}C NMR spectrum (**Figure A.6**), two carboxyl signals at δ_C 172.9, 168.6, twelve aromatic carbons in the range of δ_C 105-162, one methoxy signal at δ_C 55.7, and four methyl signals at δ_C 23.0, 22.7, 9.1, and 8.0 were detected.

The comparison of the NMR spectrum of this compound with literature [24] confirmed that **3** should be barbatic acid.



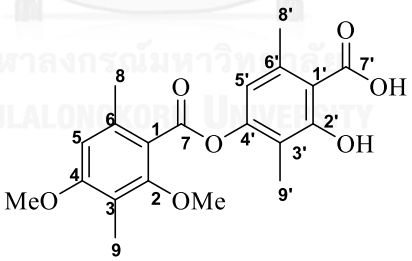
Compound **3**: barbatic acid

3.3.2.2 Structural elucidation of compound 4

Compound **4** (18.4 mg, 0.23 %) was obtained from **EL2** as colorless needle with dark-pink color on TLC of depside characteristic with R_f 0.3 [CH_2Cl_2: MeOH (9:1)]. In the ^{1}H NMR spectrum (**Figure A.7**), the signals of two methoxy groups at δ_H 3.60 (3H, 2-OMe), 3.68 (3H, 4-OMe) and four methyl groups at δ_H 2.24 (3H, H-8), 2.34 (3H, H-8′), 1.90 (3H, H-9), and 1.92 (3H, H-9′) were visualized. Twenty carbon signals were detected in the ^{13}C NMR spectrum (**Figure A.8**), including two carboxyl signals at δ_C 173.1, 165.5, twelve aromatic carbons at δ_C 108-162, two methoxy signals at δ_C 61.7 and 55.8, and four methyl signals at δ_C 22.8, 19.5, 8.9, and 8.7.

The ^{13}C NMR spectrum was compared with that of compound **3**, and was found that **4** contained all signals as those found in **3**. Moreover, **4** had one more methoxy signal at δ_C 61.7. In compound **3**, only one methoxy group was appeared at δ_C 55.8 for 4-OMe, this manifestly suggested that the methoxy signal at δ_C 61.7 of compound 4 can be as either 2-OMe or 2′-OMe.

By comparison with literature [25], **4** was proposed to have the methoxy group substituted at δ_C 61.7 in 2-OMe as diffactaic acid, a major substance in *Usnea* genus.



Compound **4**: diffractaic acid

Table 3. 6 The tentative ^{1}H and ^{13}C NMR chemical shift assignment of **3**, **4**, **15**, and **23**

Position	**3** (DMSO-d_6)		**4** (DMSO-d_6)		**15** ($CDCl_3$)		**23** ($CDCl_3$)	
	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C
1		1118		116.4		108.7		108.6
2		161.1		161.3		169.2		161.9
3		107.1		111.5		110.4		115.7
4		159.5		156.4		167.7		160.7
5	6.65 s	106.4	6.45 s	108.5	6.51, s	116.2	6.63, s	110.9
6		138.9		134.8		152.2		139.0
7		168.6		165.5		169.8		169.2
8	2.56 s	22.7	2.23 s	19.5	2.69, s	25.7	2.44, s	23.5
9	2.00 s	8.0	1.90 s	8.7	10.36, s	194.0	1.94, s	8.0
2-OMe			3.68 s	61.8				
4-OMe	3.85 s	55.8	3.60 s	55.8				
2-OH	10.74 s				12.54, s		11.13, s	
4-OH					12.50, s			
1′		115.6		119.3		103.0		111.6
2′		161.5		159.5		163.0		161.3
3′		110.1		116.0		116.9		115.9
4′		151.6		152.2		152.6		151.7
5′	6.60 s	115.7	6.62 s	115.7	6.40, s	113.0	6.36, s	110.9
6′		139.0		139.0		140.0		139.0
7′		173.0		173.1		172.3		173.1
8′	2.48 s	23.0	2.34 s	22.8	2.54 s	24.1	2.44 s	22.7
9′	1.98 s	9.1	1.98 s	8.9	2.10 s	9.5	1.94 s	9.1
2′-OH					11.94 s		10.33 s	
COOMe					3.97 s	52.3		

3.2.3 Fractionation and elucidation of compounds from EL3 fraction

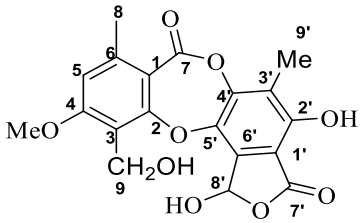
EL3 (6.5 g) was subjected to silica gel column and eluted the column with [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The results of separation are shown in **Table 3.7.**

Table 3. 7 Isolation compounds from **EL3**

solvent system	subfractions	weight (mg)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**EL3.1**	5.8 (g)	White solid
	EL3.2	12.2	White solid (compound **5**)
	EL3.3	6.8	White solid (compound **6**)
	EL3.4	12.5	White solid (Mixture **6** + **7**)
	EL3.5	9.5	White solid (compound **7**)
	EL3.6	3.0	White solid (compound **8**)

3.2.3.1 Structural elucidation of compound 5

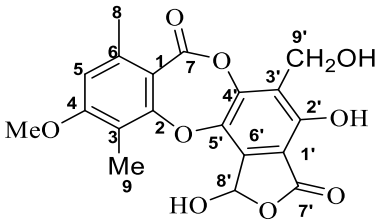
Compound **5** (12.2 mg, 0.16 %) was acquired as white amorphous from fraction **EL2** (1.2 g). This compound showed a spot on TLC with R_f 0.33 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The ^{1}H NMR spectrum (**Figure A.9**) exhibited signals for seven protons belonging to one hydroxy signal at δ_H 8.22 (8′-OH), two oxygenated signals of one methylene group at δ_H 4.80 (1H, *d*, 11.2, H-9a), 4.62 (1H, *d*, 11.2, H-9b), one methoxy singlet signal at δ_H 3.87 (3H, 4-OMe), two singlet signals of two methyl groups at δ_H 2.45 (H-8) and 2.19 (H-9′). The ^{13}C NMR spectrum (**Figure A.10**) displayed nineteen carbon signals the same as that of compound **1**, but without signal in the range of δ_C 180-198 implying that the formyl group was absent in compound **5**. Addition to the chemical shift in the range of δ_C 50-60 of compound **5**, two oxygenated carbon signals were observed including one methoxy group at δ_C 56.2 (4-OMe) and one methylene group δ_C 51.3 (C-9). On the basis of above information as well as comparison of the ^{1}H NMR spectrum of **5** with that of cryptostictic acid [23], compound **5** was assigned as cryptostictic acid.



Compound **5**: cryptostictic acid

3.2.3.2 Structural elucidation of compound 6

Compound **6** (6.8 mg, 0.09 %) was obtained as white amorphous from fraction **EL2**. TLC showed a single spot at R_f 0.16 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The ^{1}H NMR spectrum (**Figure A.11**) revealed one hydroxy group at δ_H 8.29 (8′-OH), one methylene group at δ_H 4.60 (2H, H-8′), one methoxy group at δ_H 3.84 (3H, 4-OMe), two signals of methyl groups at δ_H 2.39 (H-8) and δ_H 2.23 (H-9). Moreover, the ^{13}C NMR spectrum (**Figure A.12**) also indicated nineteen carbon signals including two carbonyl carbons (δ_C 161.1, C-7′ and 158.7, C-7), twelve aromatic carbons (δ_C 109-167), one hemiacetal carbon (δ_C 95.3, C-8′), two oxygenated carbons (δ_C 56.1, 4-OMe and δ_C 52.6, C-9′), and two methyl groups (δ_C 20.7, C-8 and δ_C 8.6, C-9). On the basis of the above data, it was strongly suggested that **6** contain the same skeleton as **2**. Furthermore, the comparison of the NMR data of **6** with those of **2** confirmed that they were close except for the appearance of methyl signal at δ_C 8.3 in **6** instead of formyl group at δ_C 186.6 in **2**. Thus, **6** was considered as hypoconstictic acid [26].



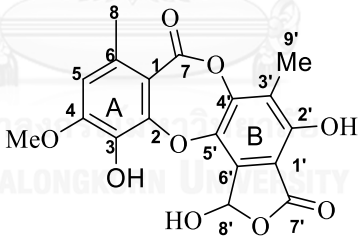
Compound **6**: hypoconstictic acid

3.2.3.3 Structural elucidation of compound 7

Compound **7** (9.5 mg, 0.12 %) was yielded as white powder from fraction **EL2** (1.2 g) with R_f 0.25 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The ^{1}H NMR spectrum (**Figure**

A.13) not only showed one singlet signal at δ_H 8.25 (8′-OH) which belonged to hydroxy group, one methoxy group at δ_H 3.81 (3H, 4-OMe), and two methyl groups at δ_H 2.30 (H-8) and δ_H 2.11 (H-9′), but also detected one hydroxy phenol at δ_H 9.1 (3-OH).

As depsidones, the ^{13}C NMR spectrum (**Figure A.14**) of **7** confirmed two carbonyl carbons (δ_C 161.4, C7′ and 161.4, C-7), twelve aromatic carbons in the range of δ_C 109-167, one hemiacetal carbon (δ_C 95.3, C-8′), one oxygenated carbon (δ_C 56.2, 4-OMe), and two methyl groups (δ_C 19.8, C-8 and δ_C 9.5, C-9′). In ring B, the positions of the substituents in **7** were not different compared with those of **1** (**Tables 3.2** and **3.3**). Morever, the aldehyde signal in **1** at δ_C 186.6 was disappeared and no carbon signal appeared in **7**. Furthermore, the downfield methyl at δ_C 19.8 belonging to C8 [21.5 (C-8) in **1**] was appeared as the effect from electron donating group at *para*-position [δ_H 9.1 (3-OH)]. Based on this information as well as the comparison the ^{13}C NMR spectrum of **7** with that reported in literature [27], **7** was designated as menegazziaic acid.



Compound **7**: menegazziaic acid.

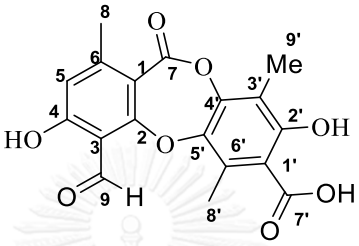
3.2.3.4 Structural elucidation of compound 8

Compound **8** (3.0 mg, 0.04 %) was isolated as white amorphous from fraction **EL2** with R_f 0.43 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. In the ^{1}H NMR spectrum (**Figure A.15**), the signals of one chelated hydroxy group at δ_H 11.85 (4-OH), one formyl group at δ_H 10.49 (3-CHO), methyl groups at δ_H 2.33 (H-8), δ_H 2.31 (H-8′), and δ_H 2.04 (H-9′) were observed.

In the ^{13}C NMR spectrum (**Figure A.16**), eighteen carbon signals belonging to one aldehyde group (δ_C 191.7, C-9), two carbonyl carbons (δ_C 170.8, C-7′ and 161.2, C-7),

twelve aromatic carbons in the range of δ_C 110-165, and three methyl groups at δ_C 21.4 (C-8), 14.3 (C-8′), and 9.3 (C-9′) were detected. The disapperance of hemiacetal carbon at δ_C 95 (in compound **1**) indicated that the five-member ring lactone was not formed in compound **8**.

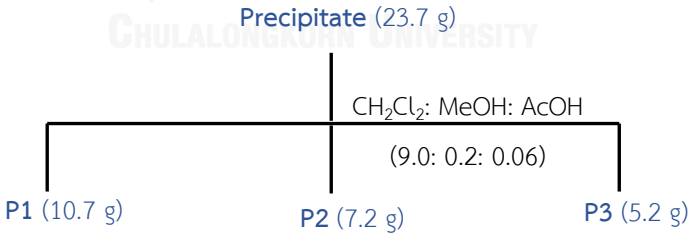
Comparing the ^{13}C NMR spectrum of **8** with that of virensic acid [28], all evidence indicated that **8** was virensic acid.



Compound **8**: virensic acid

3.3 Isolation and structure elucidation of chemical constituents in precipitate fraction

Three fractions: **P1** (10.7 g, 45.15 %), **P2** (7.2 g, 30.38 %), and **P3** (5.2 g, 21.94 %) were obtained when the precipitate fraction (23.7 g) was separated by silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06). The details for the separation of this fraction are presented in **Table 3.8** and Scheme **3.4**.



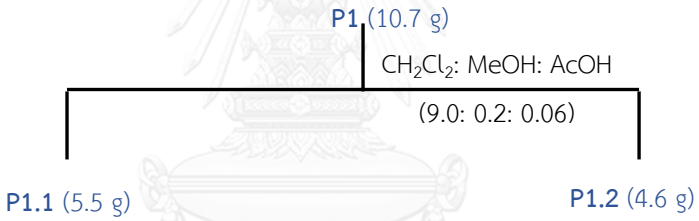
Scheme 3. 4 Fractionation and isolation of precipitate fraction

Table 3. 8 The separation of precipitate fraction

solvent system	fractions	weight (g)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**P1**	10.7	White solid
	P2	7.2	Brown solid
	P3	5.2	Brown solid

3.3.1 Structural elucidation of compound 9

P1 was applied to silica gel column and eluted by CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06) to separate into two sub-fractions: **P1.1** (5.5 g, 51.4 %) and **P1.2** (4.6 g, 43.0 %). The results are shown in **Scheme 3.5** and **Table 3.9**.



Scheme 3. 5 Fractionation and isolation of **P1**

Table 3. 9 The separation of **P1**

Solvent system	fractions	weight (g)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**P1.1**	5.5	White solid
	P1.2	4.6	White solid

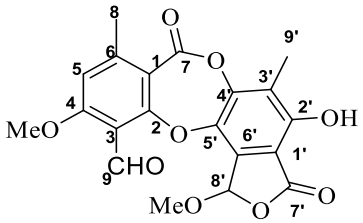
Fraction **P1.2** was reseparated by silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06) to yield compound **9** as presented in **Table 3.10**.

Table 3. 10 The isolation of compound **9** from **P1.2**

Solvent system	subfractions	weight (mg)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**P1.2.1**	2.5 g	White solid
	P1.2.2	3.2	White powder (Compound **9**)
	P1.2.3	1.2 g	White solid

Compound **9** (3.2 mg, 0.07 %) as white powder from **P1.2** displayed a single spot on TLC with R_f 0.47 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The ^{1}H NMR spectrum (**Figure A.17**) exhibited one formyl group at δ_H10.41 (3-CHO), two methoxy groups at δ_H 3.92 (3H, 4-OMe), and δ_H 3.44 (3H, 8′-OMe). The signals belonging to two methyl groups at δ_H 2.49 (H 8) and δ_H 2.19 (H 9′) were also observed.

Twenty carbon signals were displayed in the ^{13}C NMR spectrum (Figure 3.18). When the NMR data of **9** were compared with those of **1**, it was found that the ^{13}C NMR spectrum of **9** was very close to that of **1** including one aldehyde carbon at δ_C 186.7 (C9), two carboxyl carbons at δ_C 162.5 (C7′) and 160.5 (C7), twelve aromatic carbons in the range of 108-166, one methoxy carbon at δ_C 56.3 (4-OMe), one acetal carbon at δ_C 99.9 (C-8′), and two methyl carbons at δ_C 21.5 (C-8), and 9.7 (C-9′). However, one more methoxy group at δ_C 56.8 (8′-OMe) was detected in compound **9** which was in consistent with the signal at δ_H 3.44 in the ^{1}H NMR spectrum. Moreover, in the range of δ_H 8.0-9.0, the hydroxy signal (8′-OH in **1**) was not appeared along with the appearance of proton hemiacetal at δ_H 6.42. This suggested that the substituent on C-8′ (δ_C 99.9) be a methoxy group. All evidence from NMR data and comparison with those of literature [29] indicated that **9** was methylstictic acid. The tentative assignment of ^{1}H and ^{13}C NMR chemical shifts are tabulated in **Table 3.11**.



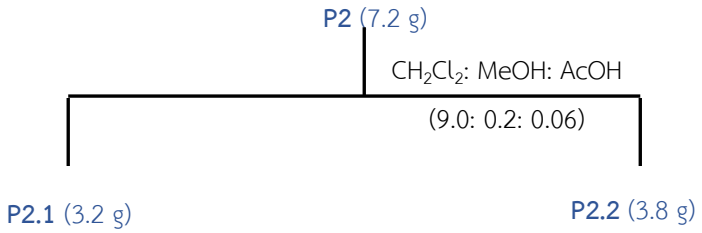
Compound **9**: methylstictic acid

Table 3. 11 The ^{1}H and ^{13}C NMR of compounds **9**, **11**, **12**, **13**, and **14** (DMSO-d_6)

	9		**11**		**12**		**13**		**14**	
	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C
1		112.9		113.1		113.6		116.9		117.1
2		165.9		165.6		166.8		164.4		164.8
3		114.3		114.4		135.0		112.2		112.6
4		152.4		162.5		152.5		155.1		155.8
5	7.10 s	113.2	7.11 s	113.4	6.85 s	111.7	6.77 s	115.1	6.82 s	115.6
6		151.0		151.1		152.5		151.7		152.2
7		160.6		160.6		161.6		161.3		161.5
8	2.49 s	21.5	2.49 s	21.6	2.33 s	19.9	2.45 s	21.3	2.42 s	21.5
9	10.41 s	186.7	10.41 s	186.9	9.38 s		10.54 s	191.7	10.58 s	191.9
4-OMe	3.92 s	56.3	3.92 s	56.5	3.84 s	56.3				
1′		108.8		109.4		109.0		111.8		111.9
2′		151.0		153.2		151.4		163.8		163.9
3′		121.7		124.0		120.4		115.4		116.4
4′		148.3		148.6		148.3		145.1		144.5
5′		132.6		135.5		132.6		141.6		140.7
6′		133.1		137.6		135.1		131.5		127.5
7′		162.7		162.9		161.3		170.4		170.3
8′	6.47 s	99.9	6.49	99.9	6.47 s	100.7	2.34 s	14.4	2.41 s	14.4
9′	2.19 s	9.7	4.64 s	53.0	2.15 s	9.7	4.43 s	62.4	4.58 s	52.9
8′-OMe	3.44 s	56.8	3.45 s	57.0	3.56 s	56.7				
9′-OMe							3.17 s	57.3		
4-OH							11.95 s			

3.3.2 Structural elucidation of isolated compounds from P2

P2.1 (3.2 g, 44.44 %) and P2.2 (3.8 g, 52.78 %) were obtained from the separation of **P2** by silica gel column with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06). The fractionation of **P2** is displayed in **Scheme 3.6** and **Table 3.12**.



Scheme 3. 6 Fractionation of **P2**

Table 3. 12 The separation of **P2**

solvent system	Fraction	weight (g)	remarks
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**P2.1**	3.2	White solid
	P2.2	3.8	White solid

3.3.2.1 Structural elucidation of compound 10

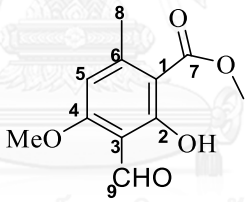
After separation of **P2.1** (3.2 g) by silica gel column eluting with hexane: CH_2Cl_2: AcOH (9.0: 1.0: 0.1), compound **10** was isolated. The results of the isolation of compound **10** are displayed in **Table 3.13**.

Table 3. 13 The separation of **P2.1**

solvent system	fraction	weight (mg)	remarks
hexane: CH_2Cl_2: AcOH (9.0: 1.0: 0.1)	**P1.2.1**	220.2	White solid (Compound **10**)
	P1.2.2	2.8 g	White solid
	P1.2.3	5.2	Colorless syrup

Compound **10**, white powder (2.2 mg, 0.07 %) from **P2.1** showed only one red spot on TLC with R_f 0.21 in hexane: CH_2Cl_2: AcOH (8.0: 2.0: 0.1). The ^{1}H NMR spectrum (**Figure A.19**) displayed the signals of one chelated hydroxy group at δ_H 12.54 (1H, 2-OH), one formyl group at δ_H 10.25 (1H, 3-CHO), two methoxy groups at δ_H 3.92 (3H, 4-OMe), and δ_H 3.91 (3H, -COOMe), and one methyl group at δ_H 2.38 (3H, H-8).

The ^{13}C NMR spectrum (**Figure A.20**) confirmed that total eleven carbon signals including one aldehyde carbon at δ_C 193.4 (C-9), one carbonyl carbon δ_C 167.4 (C-7′), six aromatic carbons (δ_C 103-163), two methoxy groups at δ_C 56.1 (4-OMe), and δ_C 52.4 (-CO-OMe), and one methyl group at δ_C 21.8 (C-8). All obtained information was well consistent with the proton signals detected in the ^{1}H NMR. According to six aromatic carbons (δ_C 103-163), **10** was in good agreement with a mono-aromatic ring. Compound **10** was elucidated as methyl 4-*O*-methylhaematomate after comparing the NMR data with those reported for methyl haematomate [30]. The tentative assignment of ^{1}H and ^{13}C NMR of **10** was detailed in **Table 3.14**.



Compound **10**: methyl 4-O-methylhaematomate

Table 3. 14 The spectroscopic data of compounds **10**, **16**, **17** ($CDCl_3$)

Position	**10**		**16**		**17**	
	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C
1		108.9		108.7		118.9
2		162.1		163.3		150.9
3		149.1		105.4	6.21, s	108.6
4		163.1		158.2		136.6
5	6.24 s	103.5	6.20, s	110.7	6.21, s	108.6
6		128.9		140.3		150.9
S7		167.4		172.8		193.4
8	2.38 s	21.8	2.45, s	24.2	2.27, s	22.6
9	10.25 s	193.4	2.10, s	7.8	10.28, s	
2-OH	12.54 s		12.03, s			
4-O-Me	3.92 s	56.0				
-COOMe	3.91 s	52.3	3.92, s	51.9		

Isolation and structural elucidation of compounds 11, 12

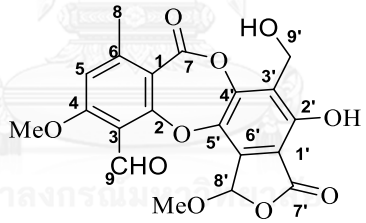
Compounds **11** and **12** were isolated from **P2.2** (3.8 g) which was applied to silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06). The results of the separation are presented in **Table 3.15**.

Table 3. 15 The separation of **P2.2**

solvent system	**fraction**	**weight (mg)**	**remarks**
CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)	**P1.2.1**	4.0	Compound **11**
	P1.2.2	4.2	Compound **12**
	P1.2.3	3.2 (g)	Brown solid

3.3.2.2 Structural elucidation of compound 11

Compound **11** (4.0 mg, 0.11 %) was obtained as white powder from **P2.2** with R_f 0.29 [hexane: CH_2Cl_2: AcOH (5.0: 5.0: 0.1)]. The ^{1}H NMR spectrum revealed one formyl group at δ_H 10.41 (3-CHO), one methylene group at δ_H 4.64 (2H, H-9′), one methoxy group at δ_H 3.92 (3H, 4-OMe), one signal of methyl groups at δ_H 2.49 (H-8). The main observed signals were close to those of **2**. In addition, this spectrum exhibited one more methoxy proton at δ_H 3.45 (3H, 8′-OMe). A hydroxy group at δ_H 8.25 (8′-OH from **2**) was disappeared when it was compared with that of **2**. Furthermore, the NMR spectra (**Figures A.21 and A.22**) of **11** were compared with those of **2**. The ^{13}C NMR spectrum of **11** contained nineteen signals as found in **2**. In addition, the ^{13}C NMR of **11** revealed an extra methoxy signal at δ_C 57.0 (8′-OMe). When the ^{1}H and ^{13}C NMR spectra of **11** were compared with those of 8′-*O*-methylconstictic acid [31], it was ascertained that **11** was elucidated as 8′-*O*-methylconstictic acid.



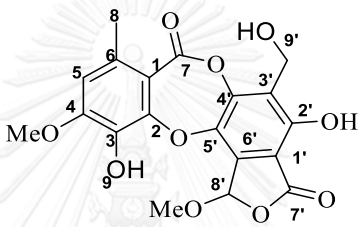
Compound **11**: 8′-*O*-methylconstictic acid

3.3.2.3 Structural elucidation of compound 12

Compound **12** was isolated as white powder (0.11 %yield) from **P2.2** with R_f 0.26 [hexane: CH_2Cl_2: AcOH (5.0: 5.0: 0.1)]. The ^{1}H NMR spectrum (**Figure A.23**) showed one singlet signal belonging to hydroxy group at δ_H 9.38 (3-OH), two methoxy groups at δ_H 3.84 (3H, 4-OMe) and δ_H 3.56 (3H, 9′-OMe), and two methyl groups at δ_H 2.33 (H-8) and δ_H 2.15 (H-9′). In addition, the ^{13}C NMR spectrum (**Figure A.24**) also revealed characteristic resonances of a depsidone skeleton including two conjugated carboxyl carbons (δ_C 161.3, C-7′ and 161.3, C-7), twelve aromatic carbons, two oxygenated carbons (δ_C 56.2, 4-OMe) and δ_C 56.2 (8′-OMe), two methyl groups (δ_C 19.8, C-8 and

δ_C 9.5, C-9′) along with the disappearance of one hemiacetal carbon (δ_C 95.3, C-8′ in 7). Comparison of the spectroscopic data of **12** with those of **7**, the position of substituents on rings A and B were determined the same as compound **7**. In five-membered ring lactone, however, the substituent at C-8′ (δ_C 95.3) must be a methoxy group displaying at δ_C 56.2 (8′-OMe) because of the disappearance of the signal in the range of δ_H 8-9 [as in compound 7: 8′-OH (δ_H 8.30)].

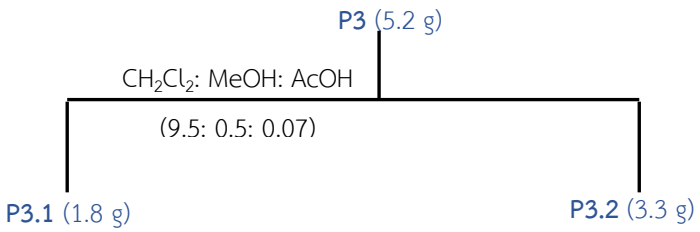
Based on the above information along with the comparison of the ^{13}C NMR spectrum of **12** with literature [29], **12** was completely identified as 8′-*O*-methylmenegazziaic acid.



Compound **12**: 8′-*O*-methylmenegazziaic acid

3.3.3 Structural elucidation of isolated compounds from P3

Fraction **P3** collected from the precipitate fraction (**Scheme 3.4**) was re-separated by silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07). The fractionation of **P3** was detailed in **Table 3.16** and **Scheme 3.7**.



Scheme 3. 7 Separation of **P3**

Table 3. 16 The separation of **P3**

solvent system	fractions	weight (g)	remarks
CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07)	**P3.1**	1.8	White solid
	P3.2	3.3	Brown solid

3.3.3.1 Isolation and structural elucidation of compound 13

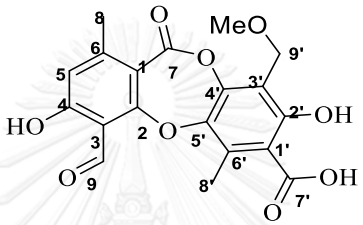
Fraction **P3.1** was further separated by silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07) to furnish compound **13**. The results of isolation are displayed in **Table 3.17**.

Table 3. 17 The separation of **P3.15**

solvent system	fractions	weight (mg)	remarks
CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07)	**P3.1.1**	230.0	White power
	P3.1.2	4.6	White powder (Compound 13)
	P3.1.3	1.4 (g)	Brown solid

Compound **13** was isolated as white powder (4.6 mg, 0.26 %). Its TLC displayed a single spot at R_f 0.36 [CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07)]. The ^{1}H NMR spectrum (**Figure A.25**) showed one singlet signal belonging to hydroxy group at δ_H 11.95 (4-OH) chelated with one formyl group at δ_H 10.54 (3-CHO). It also showed one oxygenated methylene group at δ_H 4.43 (2H, H-9′), one methoxy group at δ_H 3.17 (3H, 9′-O-Me), and two signals of methyl groups at δ_H 2.45 (H-9) and 2.34 (H-8′). The ^{13}C NMR spectrum (**Figure A.26**) of **13** indicated the presence of nineteen carbons including one aldehyde group at δ_C 191.7 (C-9), two carbonyl carbons at δ_C 170.4 (C-7′) and 161.3 (C-7), twelve aromatic carbons at δ_C 110-165, two oxygenated carbons at δ_C 62.3 (C-9′) and δ_C 57.3 (9′-OMe), and two methyl groups (δ_C 21.3, C-8 and

δ_C 14.4, C-8′). After comparing the NMR data of **13** with those of **8**, the difference between **8** and **13** were the signals at C-9′. The replacement of one methyl group (C-9′) in compound **8** by a methoxymethylene group in compound **13** was determined by the signal of a methyl group at 9.3 (C-9′ in 8) which was disappeared along with the appearance of two oxygenated carbons at δ_C 62.3 (C-9′) and δ_C 57.3 (9′-OMe). Based on the above data as well as the comparison of the ^{13}C NMR spectrum of 13 with that of 9′-*O*-methylprotocetraric acid [28], **13** was elucidated as 9′-*O*-methylprotocetraric acid.



Compound **13**: 9′-*O*-methylprotocetraric acid

3.3.3.2 Isolation and structural elucidation of compound 14

Fraction **P3.2** was further separated by silica gel column eluting with CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07) to achieve **14**. The results of isolation were displayed in **Table 3.18**.

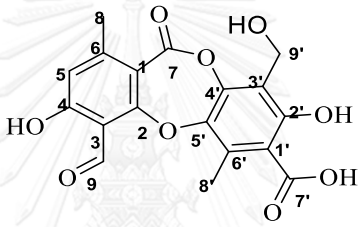
Table 3. 18 The isolation of compound **14**

solvent system	fractions	weight (mg)	remarks
CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07)	**P3.2.1**	1.1 (g)	White power
	P3.2.2	202.2	White powder (Compound 14)
	P3.2.3	1.8 (g)	Brown solid

Compound **14** (202.2 mg, 6.13 %) was isolated as white powder from **P.3.2** with R_f 0.12 in CH_2Cl_2: MeOH: AcOH (9.5: 0.5: 0.07). The ^{1}H NMR spectrum (**Figure A.27**) exhibited one formyl group at δ_H 10.58 (3-CHO), one methylene group at δ_H 4.58 (2H, H-9′), and two signals of methyl groups at δ_H 2.42 (H-8) and δ_H 2.41 (H-8′). Moreover,

the ^{13}C NMR spectrum (**Figure A.28**) of **14** revealed total eighteen carbons including one aldehyde group at δ_C 191.9 (C-9), two carbonyl carbons at δ_C 170.3 (C-7′) and 161.4 (C-7), twelve aromatic carbons at δ_C 110-165, one oxygenated carbon at δ_C 52.9 (C-9′), and two methyl groups at δ_C 21.5 (C-8) and 14.4 (C-8′). Compound 14 was found to be of less one carbon than compound **13**, where the methoxy signal at δ_C 57.2 (9′-OMe) in **13** was disappeared in **14**. Therefore, the substituent at C 9′ (δ_C 57.2) must be hydroxy group.

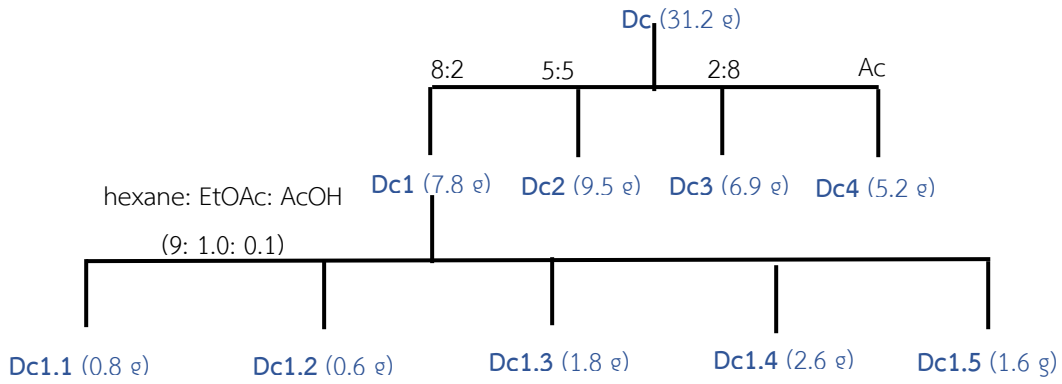
The ^{13}C NMR spectrum of **14** was compared with that of protocetraric acid [28], it was thus assured that the structure of **14** was completely established as protocetraric acid.



Compound **14**: protocetraric acid

3.4 Isolation and structural elucidation of chemical constituents of dichloromethane fraction

Fraction **DC** (31.2 g) was separated by silica gel column with solvent system hexane: EtOAc (8:2, 5:5, 2:8) to obtain four fractions: **DC1** (7.8 g, 25.00 %), **DC2** (9.5 g, 30.45 %), **DC3** (6.9 g, 22.12 %), and **DC4** (5.2 g, 16.17 %). Using CC/S4, **DC1** (7.8 g) was separated into five sub-fractions: **DC1.1** (0.8 g, 10.26 %), **DC1.2** (0.6 g, 7.69 %), **DC1.3** (1.8 g, 23.08 %), **DC1.4** (2.6 g, 33.33 %), and **DC1.5** (1.6 g, 20.51 %). The details of fractionation are presented in **Scheme 3.8** and **Table 3.19**.



Scheme 3. 8 Fractionation and isolation of dichloromethane fraction

Table 3. 19 The separation of **DC1**

solvent system	fractions	weight (gram)	remarks
hexane: EtOAc: AcOH (9: 1.0: 0.1)	**DC1.1**	0.8	Colorless oil
	DC1.2	0.6	Yellow needle
	DC1.3	1.8	Yellow needle
	DC1.4	2.6	Orange needle
	DC1.5	1.6	Dark brown solid

3.4.1 Structural education of compounds yielded from DC1.2

Fraction **DC1.2** was separated by silica gel column eluting by hexane: CH_2Cl_2: AcOH (9.0: 1.0: 0.1). The details of the isolation are displayed in **Table 3.20**.

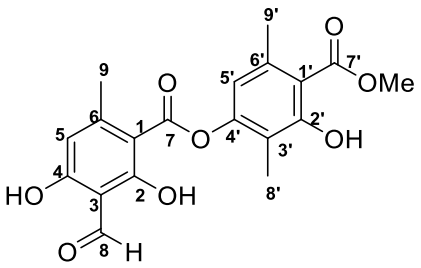
Table 3. 20 The separation of **DC1.2**

solvent system	fractions	weight (mg)	remarks
hexane: CH_2Cl_2: AcOH (9.0: 1.0: 0.1)	**DC1.2.1**	6.2	Colorless needle (compound **15**)
	DC1.2.2	5.0	Colorless needle (compound **16**)
	DC1.2.3	6.5	Colorless needle (compound **17**)
	DC1.2.4	380.4	Colorless needle
	DC1.2.5	3.4	Colorless needle (compound **18**)

3.4.1.1 Structural elucidation of compound 15

Compound **15** (6.2 mg, 1.03 %) was obtained as colorless needle from **DC1.2**. The TLC displayed a single spot with R_f 0.5 [hexane: CH_2Cl_2: MeOH (8.0: 2.0: 0.13)]. The ^{1}H NMR spectrum (**Figure A.29**) indicated all ten singlet resonances including three singlet signals at δ_H 12.54, 12.50 and 11.94 (2-OH, 2′-OH, and 4-OH) as chelated hydroxy group. It also displayed one formyl group at δ_H 10.36 (1H, 3-CHO), two isolated aromatic protons [6.51 and 6.40 (1H each, s, 5-H and 5′-H)], one methoxy group at δ_H 3.97 (3H, -COOMe), and three methyl groups at δ_H 2.69 (3H, H-8), 2.54 (3H, H-8′), and 2.10 (3H, H-9′). The aromatic methyl group at δ_H 2.65 shifted significantly to the low-field characterized of 6-CH_3 position of lichen depsides.

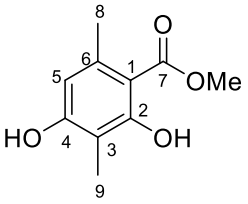
The ^{13}C NMR spectrum (**Figure A.30**) exhibited signals due to nineteen carbons corresponding one aldehyde carbon at δ_C 194.0, two carboxyl signals at δ_C 172.3 (C-7′), 169.8 (C-7), twelve aromatic carbons at δ_C 100-170, one methoxy signal at δ_C 52.4 (-COOMe), and three methyl signals at δ_C 25.7 (C-8), 24.1 (C-8′), and 9.5 (C-9′). It was consistent with the ^{1}H NMR as well as comparison with that of atranorin [8], **15** was established as atranorin.



Compound **15**: atranorin

3.4.1.2 Structural elucidation of compound 16

Compound **16** was a monocyclic compound as white needle from **DC1.2**. Its R_f was 0.10 [hexane: CH_2Cl_2: MeOH (8.0: 2.0: 0.13)] with 0.83 %yield. The ^{1}H NMR spectrum (**Figure 3.31**) showed the signals of one chelated hydroxy group at δ_H 12.03 (1H, 2-OH), two methoxy groups at δ_H 3.92 (3H, COOMe), and two methyl groups at δ_H 2.45 (3H, H8), and δ_H 2.10 (3H, H-9). Moreover, the ^{13}C NMR spectrum (Figure 3.32), ten carbon signals which belonged to one carboxyl signal at δ_C 172.7 (C-7), six aromatic carbons at the range of δ_C 105-165, one methoxy signal at δ_C 51.9 (-COOMe), and two methyl signals at δ_C 24.2 (C-8), and 7.7 (C-9) were indicated. When the spectrum of **16** was compared with that of **10**, it was found that **16** contained one carbon less than in **10**. It also exhibited the disappearance of two signals at δ_C 193.4 and 56.1 along with one signal at δ_C 7.7 (typical signal for methyl group which has two oxygenated aromatic carbon at other position). Based on the NMR data, it was strongly suggested that compound **16** be a mono-aromatic with two hydroxy groups at C-2 and C-4 along with a methyl group at C-3 (replaced for formyl group in **10**). Along with comparison with that of methyl β-orsinolcarboxylate[24]. The structure of **16** was therefore elucidated as methyl β-orsinolcarboxylate.

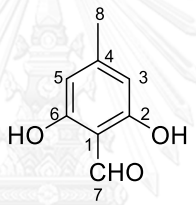


Compound **16**: methyl β-orsinolcarboxylate

3.4.1.3 Structural elucidation of compound 17

Compound **17** (6.5 mg, 1.08 %) was a monocyclic compound obtained as white needle. The red single spot with R_f 0.05 [hexane: CH_2Cl_2: MeOH (8.0: 2.0: 0.13)] was displayed on TCL. The ^{1}H NMR spectrum (Figure 3.33) showed signals of one formyl group at δ_H 10.28 (1H, 3-CHO), two symmetric protons at δ_H 6.25 (2H, H1 and H5) and one methyl group at δ_H 2.25 (3H, H7).

The ^{13}C NMR spectrum (**Figure A.34**) confirmed the resonances of 6 carbon signals including of one aromatic methyl group (δ 22.6, C-8), two methine carbons (δ 108.6, C-3, C-5), one formyl group (δ 193.4, C-7) and two oxygenated aromatic carbons (δ 150.9, C-2, C-6). These spectroscopic data were compatible with the atranol, [30] therefore compound **17** was atranol.



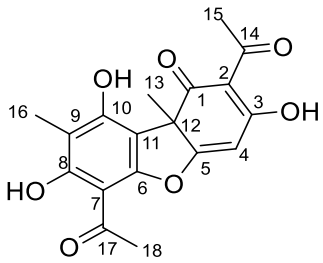
Compound **17**: atranol

3.4.1.4 Structural elucidation of compound 18

Compound **18** (3.4 mg, 0.67 %) was isolated as yellow needle. The TLC showed a single dark-blue spot with R_f 0.90 [CH_2Cl_2: MeOH: AcOH (9.0: 0.2: 0.06)]. The ^{1}H NMR spectrum (**Figure A.35**) exhibited signals for two chelated hydroxyl groups at δ_H 13.31 (8-OH), δ_H 11.03 (10-OH), one aromatic proton at δ_H 5.98 (1 H, H_4), and four methyl groups at δ_H 2.68 (3 H, H-18), δ_H 2.68 (3 H, H-15), δ_H 2.11 (3 H, C-16), and δ_H 1.76 (3 H, H-13). The ^{13}C NMR spectrum (**Figure A.36**) displayed the signals for three carbonyl carbons [δ 201.9 (C-17), 200.5 (C-14) and 198.2 (C-1)], four methyl groups [δ 32.3 (C-18), 31.4 (C-13), 28.0 (C-15) and 7.7 (C-16)]. **Table 3.21** shows the resemblance of NMR data between compound **18** and usnic acid [32]. The structure of **18** was therefore identified as usnic acid.

Table 3. 21 The comparison of NMR data of **18** and usnic acid

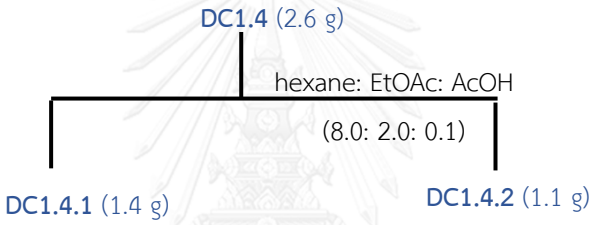
Position	**18** ($CDCl_3$)		Usnic acid ($CDCl_3$)	
	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C
1		198.2		198.7
2		105.4		105.4
3		191.9		191.8
4	5.98 s	98.5	5.92 s	98.1
5		179.6		179.9
6		155.4		155.2
7		101.7		101.9
8		164.1		166.5
9		109.5		107.8
10		157.7		159.7
11		104.1		102.2
12		59.3		59.7
13	1.76 s	31.4	1.75 s	31.8
14		200.5		201.8
15	2.68 s	28.0	2.67 s	28.0
16	2.11 s	7.7	2.10 s	7.6
17		201.9		204.6
18	2.66 s	32.3	2.68 s	33.1
3-OH	-		18.84 s	
8-OH	13.31 s		13.31 s	
10-OH	11.03 s		11.02 s	



Compound **18**: usnic acid

3.4.2 Structural education of compounds yielded from DC1.4

Fraction **DC1.4** (2.6 g) was separated into two sub-fractions**: DC1.4.1** (1.4 g) and **DC1.4.2** (1.1 g) by silica gel column with solvent system hexane: EtOAc: AcOH (8.0: 2.0: 0.1). The details of separation are shown in **Scheme 3.9**.



Scheme 3. 9 Separation of fraction **DC1.4**

Fraction DC1.4.1

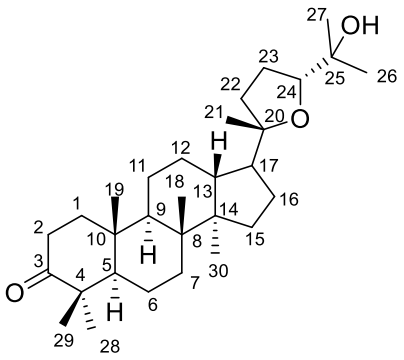
The purification of **DC1.4.1** (1.4 g) using silica gel column and solvent system: hexane: EtOAc: AcOH (9: 1.0: 0.1) led to the isolation of four compounds: **20** (7.5 mg), **21** (10.2 mg), **22** (15.2 mg) and **23** (65.0 mg). The details of separation of **DC1.4.1** are presented in **Table 3.22**.

Table 3. 22 Isolation of compounds **19-22**

solvent system	fractions	weight (mg)	remarks
hexane: EtOAc: AcOH (9.0: 1.0: 0.06)	**DC1.4.1.1**	160.5	Colorless syrup
	DC1.4.1.2	7.5	Colorless solid (**19**)
	DC1.4.1.3	10.2	Colorless solid (**20**)
	DC1.4.1.4	15.8	Colorless solid (**21**)
	DC1.4.1.5	65.0	Colorless needle (**22**)
	DC1.4.1.6	762.5	Brown syrup

3.4.2.1 Structural elucidation of compound 19

Compound **19** was obtained as colorless solid (7.5 mg, 0.54 %) from **DC1.4.1**. The TLC showed a clear spot with R_f 0.38 [hexane: EtOAc: AcOH (8.0:2.0:0.06)]. The ^{1}H NMR spectrum (**Figure A.37**) displayed eight signals of methyl groups of triterpenoid compound at δ_H 1.19 (3 H. s, H-27), 1.14 (3 H, s, H-21), 1.10 (3 H, s, H-26), 1.07 (3 H, s, H-28), 1.03 (3 H, s, H-29), 1.00 (3 H, s, H-18), 0.93 (3 H, s, H-19), and 0.87 (3 H, s, H-30). It also showed one proton connected with oxygenated carbon resonated at δ_H 3.76 (1H, H-24). Moreover, the ^{13}C NMR spectrum (**Figure A.38**) confirmed the presence of thirty carbon. The presence of one carbonyl carbon at δ_C 218.1, three carbons connected with oxygen atom at δ_C 86.6, 84.6, and 71.3 was the typical oxygenated carbon signals of C-3, C-20, C-24, and C-25 in dammarane skeleton. The NMR spectra of **19** were similar to those of (20*S*, 24*R*)-ocotillone [33]. Based on above evidence, the structure of compound 19 was established as (20*S*, 24*R*)-ocotillone.



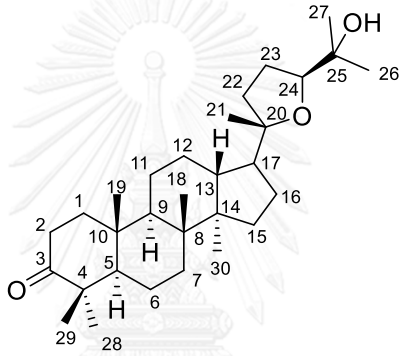
Compound **19**: (20*S*, 24*R*)-ocotillone

Table 3. 23 The ^{1}H and ^{13}C NMR spectroscopic data of **19, 20, 21** ($CDCl_3$)

Position	**19**		**20**		**21**	
	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C
1		40.0		40.0		29.0
2	2.46 m	34.2	2.45 m	34.2	2.41, 2.49, m	34.0
3		218.1		218.1		218.3
4		47.2		47.2		47.5
5		55.1		55.1		54.9
6		19.2		19.2		19.5
7		34.7		34.7		33.0
8		40.4		40.4		40.0
9		50.0		50.0		49.2
10		36.9		36.9		37.1
11		22.0		22.0		21.5
12		25.8		25.8		25.1
13		43.0		43.0		38.4
14		50.0		50.0		42.0
15		31.0		31.0		29.2
16		27.0		27.0		33.0
17		50.2		50.2		56.0
18	1.00 s	16.1	1.00 s	15.3		49.0
19	0.93 s	15.2	0.93 s	16.1	2.98 m	47.0
20		86.6		86.5		29.9
21	1.14 s	27.5	1.13 s	23.7		37.2
22		34.7		34.7	1.07 s	26.0
23		26.7		26.7	0.92 s	21.5
24	3.76 m	84.6	3.74 m	83.5	0.97 s	15.9
25		71.3		71.6	1.01 s	16.0
26	1.10 s	24.1	1.11 s	24.4	0.98 s	14.8
27	1.19 s	27.8	1.21 s	27.6		150.5
28	1.07 s	26.7	1.07 s	26.8		180.7
29	1.03 s	21.0	1.03 s	21.1	4.61, 4.73, s	109.9
30	0.87 s	16.2	0.87 s	16.5	1.69 s	19.8

3.4.2.2 Structural elucidation of compound 20

Compound **20** (10.2 mg, 0.73 %) was obtained as white powder. TLC displayed a single spot with R_f 0.38 [hexane: EtOAc: AcOH (8.0:2.0:0.06)]. The ^{1}H and ^{13}C NMR data (**Figures A.39 and A.40**) exhibited typical single signals of six methyl groups and three oxygenated carbons at δ_C 86.5, 83.5, and 71.6 belonging to dammarane triterpenoid. The NMR spectroscopic data of both compounds were found to be compatible as compared in **Table 3.15**, except for methyl group (C-21). In 20, the methyl group was detected at δ_C 27.5 (C-21) while this was visualized at δ_C 23.7 (C-21) in **19**. Therefore, **20** was proposed as (20*S*, 24*S*)-ocotillone [33].

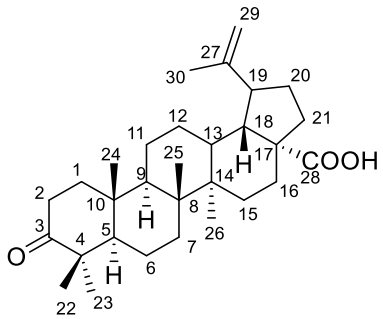


Compound **20**: (20S, 24S)-ocotillone

3.4.2.3 Structural elucidation of compound 21

Compound **21** was obtained as white powder (1.13 % yield). It showed a single spot on TLC with R_f 0.35 in hexane: EtOAc: AcOH (8.0: 2.0: 0.06). In the ^{1}H NMR spectrum (**Figure A.41**), six singlet signals of methyl groups at δ_H 1.69 (3H, s, H-30), 1.07 (3 H, s, H-22), 1.01 (3 H, s, H-25), 0.98 (3 H, s, H-26), 0.97 (3 H, s, H-24), and 0.92 (3 H, s, H-23) were displayed as typical signals of triterpenoid compounds. Moreover, it also showed two signals belonging to two olefinic protons at δ_H 4.73 and 4.61 (2H, d, 5 Hz, H-29). This was strongly suggested that the structure of 21 be lupane-skeleton. In addition to the ^{13}C NMR spectrum (**Figure A.42**), 21 had total thirty carbons including one carbonyl carbon at δ_C 218.3, one carboxyl carbon at δ_C 180.7 and two olefinic carbons at δ_C 150.5, δ_C 109.9 which showed typical signals of C-3, C-28, C-27, C-29 in lupanonic acid skeleton. Based on the above data along with the comparison of spectroscopic

data of **21** with those in literature [34], the structure of **21** was established as betulonic acid.



Compound **21**: betulonic acid

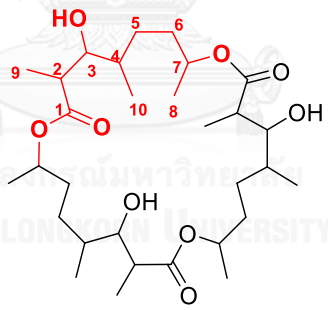
3.4.2.4 Structural elucidation of compound 22

Compound **22** was obtained as white needle (65.0 mg, yield%= 4.64 %) with R_f 0.3 in hexane: EtOAc: AcOH (6.0: 4.0: 0.06). Three doublet signals of methyl groups at δ_H 1.21 (3 H, d, 6.4, H-8), 1.01 (3 H, d, 6.8, H-9), and 0.84 (3 H, d, 6.8, H-10) were displayed in the ^{1}H NMR spectrum (**Figure A.43**). It was suggested that each methyl group connect with one methine carbon which appeared at δ_H 5.02 (1 H, m, H-7), 3.60 (1 H, dd, 9.6, 2.4, H-3)**,** and 1.50 (1 H, m, H-4). Moreover, the ^{13}C NMR spectrum (Figure 3.44) indicated that **22** had total ten carbon signals including one carboxyl carbon at δ_C 176.6, two oxygenated carbons at δ_C 77.2, 70.8, and seven carbons at from 10.0 to 45.0 ppm.

In Table **3.24**, the comparison of the NMR data between **22** and dasypogalactone [35] is displayed. The resemblance of spectroscopic data clearly revealed that **22** was dasypogalactone.

Table 3. 24 The comparison of NMR of **22** with dasypogalactone ($CDCl_3$)

Position	**22**		Dasypogalactone	
	δ_H, J(Hz)	δ_C	δ_H, J(Hz)	δ_C
1		176.6		177.1
2	2.53 m	43.8	2.56 m	44.1
3	3.60 dd, 9.6, 2.4	77.2	3.61 dd, 8.8, 2.4	77.5
4	1.50 m	34.1	1.54 m	34.7
5	1.35- 1.51 m	29.8	1.35- 1.50	29.1
6	1.45- 1.52 m	33.9		34.1
7	5.02 m	70.8		71.1
8	1.21 d, 6.4	20.4	1.26 d, 6.3	20.6
9	1.01 d 6.8	13.8	1.09 d, 7.0	14.4
10	0.82 d, 6.8	11.4	0.87 d, 6.5	12.4



Compound **22**: dasypogalactone

Fraction DC1.4.2 (1.1 g)

Two compounds: **23** (5.2 mg), **24** (7.4 mg) were obtained from the separation of **DC1.4.2** (1.1 g) using silica gel column with solvent system hexane: EtOAc: AcOH (7.5: 2.5: 0.06). The results of the chromatography column was detailed in **Table 3.25**.

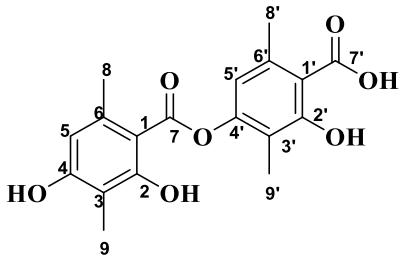
Table 3. 25 Isolation of compound **23** and **24**

solvent system	fractions	weight (mg)	remarks
hexane: EtOAc: AcOH (7.5: 2.5: 0.06)	**DC1.4.1.1**	128.2	Colorless oil
	DC1.4.1.2	5.2	White powder (**23**)
	DC1.4.1.3	7.4	White powder (**24**)
	DC1.4.1.4	782.1	White powder

3.4.2.5 Structural elucidation of compound 23

Compound **23** was obtained as white powder (0.47 %yield) with a dark-pink spot at R_f 0.41 in hexane: EtOAc: AcOH (5.0: 5.0: 0.06). In the ^{1}H NMR spectrum (**Figure A.45**), two singlet signals at δ_H 11.13 and 10.33 (2-OH, and 2′-OH) as chelated hydroxy group were indicated. It also displayed four methyl groups at δ_H 2.44 (6H, H-8 and H-8′), δ_H 1.94 (6H, H-9 and H-9′). The information from the ^{1}H NMR spectrum along with the dark-pink spot on TLC was a strong evidence to propose the structure of **23** as a depside skeleton like **3**.

The ^{13}C NMR spectrum (**Figure A.46**) revealed eighteen carbon signals including two carbonyl carbons at δ_C 173.1 (C-7′), δ_C 169.2 (C-7), twelve aromatic carbons belonging to two phenyl rings at δ_C 108-162, and four methyl carbons at δ_C 23.5 (C-8), δ_C 22.7 (C-8′),δ_C 8.0 (C-9), and δ_C 9.1 (C-9′). The disappearance of methoxy signal at δ_H 3.78 (3H, 4-OMe) was noticed when its spectrum was compared with **3**. Therefore, the substituent at C-4 was a hydroxy group. The resemblance between NMR data of **23** and those of 4-*O*-demethylbabartic acid [36], **23** was elucidated as 4-*O*-demethylbabartic acid.



Compound **23**: 4-*O*-demethylbabartic acid

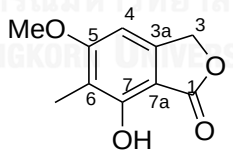
3.4.2.6 Structural elucidation of compound 24

Compound **24** (7.4 mg, 0.67 %) was isolated as white needle. A single spot with R_f 0.15 was displayed on TLC in hexane: EtOAc: AcOH (8.0: 2.0: 0.06). The ^{1}H NMR spectrum (**Figure A.47**) exhibited one hydroxy group at δ_H 7.73 (7-OH), one oxymethylene group at δ_H 5.24 (2 H, H-3), one methoxy group at δ_H 3.90 (3 H, 5-OMe), and one methyl group at δ_H 2.10 (3 H, 6-Me). The chemical shift of H-3 was shifted to the lower field indicating that it was linked to a phenyl and one carboxyl group (-O-C=O).

The ^{13}C NMR spectrum (**Figure 3.48**) reveals 10 carbons: one carboxyl group δ_C 173.0 (C-1), two oxygenated carbons δ_C 154.8 (C-7), δ_C 165.2 (C-5), one methine carbons δ_C 95.8 (C-4), one methyl group δ_C 7.7 (6-CH_3), one methoxy group at δ_C 56.3 (7-OCH_3), one oxymethylene group at δ_C 70.5 (C-3). The two last carbons were at δ_C 145.8 (C-3a) and δ_C 104.2 (C-7a). This NMR spectrum was similar to that of 7-hydroxy-5-methoxy-6-methylphthalide [32], as detailed in **Table 3.26**. Compound **24** was elucidated as 7-hydroxy-5-methoxy-6-methylphthalide.

Table 3. 26 The comparison of NMR of **24** and 7-hydroxy-5-methoxy-6-methylphthalide ($CDCl_3$)

Position	**24**		7-Hydroxy-5-methoxy-6-methylphthalide	
	δ_H, J (Hz)	δ_C	δ_H, J (Hz)	δ_C
1		173.0		173.0
2				
3	5.24 s	70.5	5.23 s	70.5
3a		145.8		145.8
4	6.48 s	95.8	6.47 s	95.9
5		165.2		165.2
6		113.2		113.2
7		154.9		154.9
7a		104.2		104.3
5-OMe	3.90 s	56.3	3.90 s	56.3
6-Me	2.10 s	7.72	2.10 s	7.7
7-OH	7.70 s		7.73 s	



Compound **24**: 7-hydroxy-5-methoxy-6-methylphthalide

3.5 Biological activities

Lichens have long been known to be of high potential for medical application and widely used as traditional medicine. In this work, two biological activities tests namely antibacterial and anti-oxidation were conducted.

3.5.1 Antibacterial test

The antibacterial activity of isolated compounds was investigated by Kirby-Bauer antibiotic testing method [37] against five bacterial pathogens including *P. acnes* KCCM41747, *S. aureus* ATCC25923, *S. sobrinus* KCCM11898, *S. mutans* ATCC25175, and *S. typhi* ATCC5442. *P. acnes* exists in human skin as a commensal as well as a pathogen causing many diseases such as acne vulgaris and medical-device infections [38]. *S. aureus* is also a pathogen found on the skin and in the nose. It is a causative agent of food poisoning, skin infections and hospital-acquired infections [39]. *S. sobrinus* and *S. mutans* belong to mutans group of oral streptococci inducing tooth decay and cavities [40]. Furthermore, a bacterial infection named typhoid fever caused many deaths in the world is from *S. typhi* [41].

The antibacterial activity of nineteen isolated compounds was achieved from measuring the diameter of the observed inhibition zone (mm), **Table 3.27.** Usnic acid (**18**)- one of the most abundant in lichen exhibited excellent activity for all five bacteria. Some researches addressed the high ability of *Usnea* lichen extracts or usnic acid against *P. acnes*, *S. aureus, S. mutans* and *S. typhi* [42, 43]. The antibacterial ability of usnic acid in this study was in good agreement with previous report as 28.00±0.00, 22.00±0.00, 23.00±0.82, and 20.67±1.89, respectively for *P. acnes*, *S. aureus, S. mutans* and *S. typhi*. Especially, the same concentration antibacterial ability of usnic acid against *P. acnes* and *S. typhi* was even higher than the reference antibacterial agent–chloramphenicol. For *S. sobrinus*, to the best of our knowledge, there has been no study using lichen extracts or usnic acid. Usnic acid was found to be very effective against *S. sorbinus* with the inhibition zone of 23.00±0.82. This result embarks the interesting point to develop this bioactive compound as an anti-infectious diseases.

Comparing the antibacterial results from this work of depsidones indicated that depsidones such as virensic acid, 9′-*O*-methylprotocetraric acid, protocetraric acid with methyl group at C-6′ exhibited higher activity than those formed the 5-membering at C-6′. It showed weak to very good activity against *P. acnes*, with inhibition zone 13.33±0.47 (protocetraric acid). They all showed weak activity against *S. aureus, S. sobrinus*, *S. mutans*, and *S. typhi (*excepted stictic acid showed moderate activity

against *S. sobrinus*). The methyl group at C-6′ may play an important role for anti *P. acnes* activity.

Other compounds displayed moderate ability as listed below:

- for *S. sorbinus*: stictic acid (**1**), diffactaic acid (**4**), antranol (**17**), (20*S*,24*R*)-ocotillone (**19**), (20S,4S)- ocotillone (**20**), and betulonic acid (**21**)
- for *S. aureus*: cryptostictic acid (**5**)
- for *S. mutans*: (20*S*,4*S*)-ocotillone (**20**), betulonic acid (**21**)
- for *S. typhi*: diffactaic acid (**4**), 7-hydroxyl-5-methyl phthalide (**24**)

Table 3. 27 Antibacterial activity of isolated compounds from *U. baileyi*

Code of compounds (1 mM)	Inhibition zone average (mm) ± SD				
	P. acnes *KCCM41747*	*S.aureus* *ATCC25923*	*S. sobrinus* *KCCM11898*	*S. mutans* *ATCC25175*	*S. typhi* *ATCC 422*
Stictic acid (**1**)	6.00±0.00	8.00±1.41	8.33±1.25	6.00±0.00	7.00±0.00
Constictic acid (**2**)	6.00±0.00	6.00±0.00	7.00±0.00	6.00±0.00	6.00±0.00
Diffactaic acid (**4**)	6.00±0.00	10.33±0.47	10.00±0.00	11.67±0.94	9.33±0.47
Cryptostictic acid (**5**)	6.00±0.00	8.67±0.94	7.00±0.00	6.00±0.00	6.00±0.00
Hypoconstictic acid (**6**)	6.00±0.00	7.00±0.00	7.00±0.00	6.00±0.00	6.00±0.00
Menegazziaic acid (**7**)	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
Virensic acid (**8**)	7.33±0.47	7.00±0.00	7.00±0.00	7.67±0.47	8.00±0.00
Methylstictic acid (**9**)	7.00±0.00	7.00±0.00	7.00±0.00	7.33±0.47	7.67±0.47
9′-*O*-methylprotocetraric acid (**13**)	7.33±0.47	6.00±0.00	7.00±0.00	6.00±0.00	7.33±0.47
Protocetraric acid (**14**)	13.33±0.47	7.00±0.00	7.00±0.00	6.00±0.00	7.00±0.00
Atranorin (**15**)	6.00±0.00	7.33±0.47	8.00±0.82	7.33±0.47	7.33±0.47
Methyl β-orcinol carboxylate (**16**)	6.00±0.00	7.33±0.47	8.00±0.82	7.33±0.47	7.00±0.00
Antranol (**17**)	6.00±0.00	7.33±0.47	9.33±1.25	7.00±0.00	7.67±0.47
Usnic acid (**18**)	28.00±0.00	22.00±0.00	23.00±0.82	23.00±0.82	20.67±1.89
(20S, 24R)-ocotillone (**19**)	7.00±0.00	7.33±0.47	9.33±0.47	8.00±0.00	7.67±0.47
(20S, 4S)- ocotillone (**20**)	7.00±0.00	7.33±0.47	10.00±0.82	8.67±0.47	7.67±0.47
Betulonic acid (**21**)	7.00±0.00	7.00±0.00	8.33±0.47	8.33±0.47	8.00±0.00
Dasypogalactone (**22**)	7.33±0.47	8.00±0.00	7.67±0.47	7.00±0.00	7.67±0.47
7-hydroxyl-5-methyl phthalide (**24**)	6.00±0.00	7.67±0.47	7.67±0.94	7.33±0.47	8.33±0.47
C*	25.00±0.00	26.00±0.80	26.00±0.00	29.33±0.47	12.00±0.00

C*: Chloramphenicol (positive control)

Key to the inhibition zone activity (mm): inhibition zone > 15.0 excellent, 13.1-15.0: very good, 10.1 – 13.0: good, 8.1-10.0: moderate, 6.1-8.0: weak, <6.0: no activity

3.5.2 Anti-oxidation activity

The antioxidant activities were measured by DPPH radical scavenging assay [22]. The percentage inhibition and IC_{50} of tested compounds were collected in **Table 3.28**. The scavenging effects of all compounds were in the range of 20 – 86%. Among the tested compounds, virensic acid (**8**) exhibited the highest DPPH radical scavenging activity (IC_{50} 0.41 mM). The other compounds, such as stictic acid (**1**), diffractaic acid (**4**), atranorin (**15**), methyl β-orcinorcatboxylate (**16**), dasypogalactone (**22**), (20*S*,24*R*)-ocotillone (**19**), (20*S*,24*S*)-ocotillone (**20**) showed very low scavenging activity. Based on the above results, depsidone displayed higher antioxidant activity than other types of compound present in lichen. In this case, virensic acid (**8**) and potocetraric acid (**14**) were higher active with IC_{50} 0.41 and 0.81 mM than other depsidones. The depsidones without 5-member ring lactone at C-6′ was higher active than those with 5-member ring lactone. The above results demonstrated the important role of methyl group at C-6′ the same as antibacterial activity.

Table 3. 28 DPPH radical scavenging activity of isolatted compounds from lichen *U. baileyi*

Compounds	IC_{50} (mM)	Compounds	IC_{50} (mM)
Stictic acid (**1**)	>2.59	Atranorin (**15**)	>2.67
Constictic acid (**2**)	1.31	Methyl β-orcinorcatboxylate (**16**)	>5.10
Diffractaic acid (**4**)	>2.67	(20*S*,24*R*)-ocotillone (**19**)	>2.18
Menegazziaicacid (**7**)	1.51	20*S*,24*S*)-ocotillone (**20**)	>2.18
Virensic acid (**8**)	0.41	Dasypogalactone (**22**)	>1.79
Methylstictic acid (**9**)	1.02	7-hydroxy-5-methoxy-6-methylphthalide (**24**)	3.88
Protocetraric acid (**14**)	0.81	Ascorbic acid	0.05

CHAPTER 4
CONCLUSIONS

4.1 Chemical constituent of lichen *Usnea baileyi*

The chemical investigation from *U.baileyi* collected in Lam Dong, Vietnam led to isolation of twenty-four known compound including stictic acid (**1**), constictic acid (**2**), babartic acid (**3**), diffactaic acid(**4**), cryptostictic acid (**5**), hypoconstictic acid (**6**), menegazziaic acid (**7**), virensic acid (**8**), methylstictic acid (**9**), methyl 4-*O*-methylhaematomate (**10**), 8′-*O*-methylconstictic acid (**11**), 8′-*O*-methylmenegazziaic acid (**12**), 9′-*O*-methylprotocetraric acid (**13**), protocetraric acid (**14**), atranorin (**15**), methyl β-orsinolcarboxylate (**16**), atranol (**17**), usnic acid (**18**), (20*S*, 24*R*)-ocotillone (**19**), (20*S*, 24*S*)-ocotillone (**20**), betulonic acid (**21**), dasypogalactone (**22**), 4-*O*-demethylbabartic acid (**23**), and 7-hydroxy-5-methoxy-6-methylphthalide (**24**) as shown in **Figure 4.1**. The chemical structure of the isolated compounds were elucidated by NMR, and also compared to NMR data of those in the literatures.

Monoaromatic rings

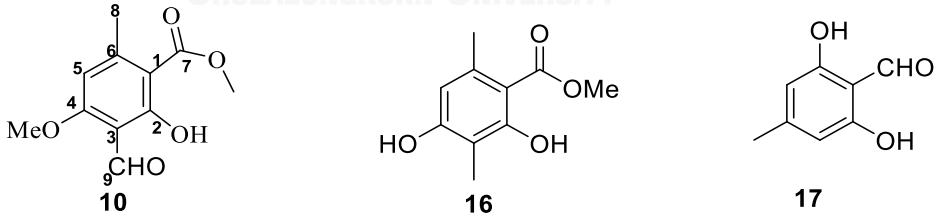
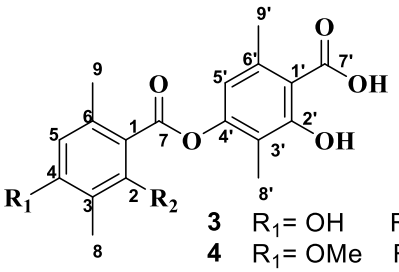
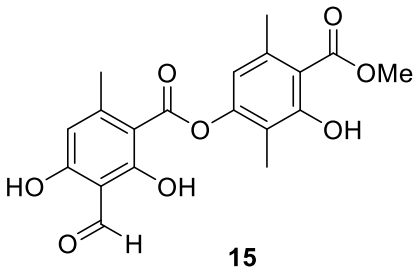


Figure 4. 1 Chemical structure of secondary metabolites from *U. baileyi*

Depsides

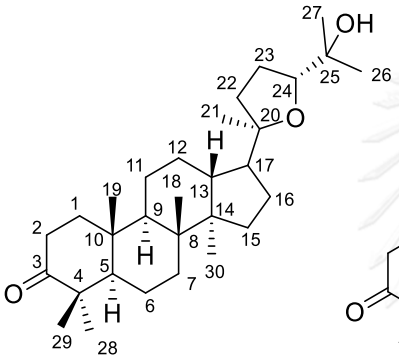


3 R_1= OH R_2= OMe
4 R_1= OMe R_2= OMe
23 R_1= OH R_2= OH



15

Triterpenoids



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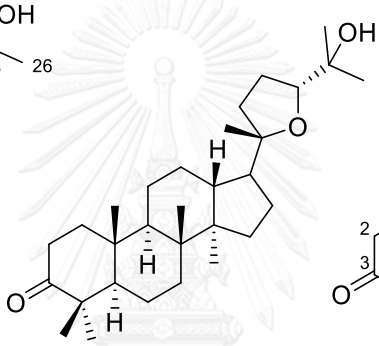

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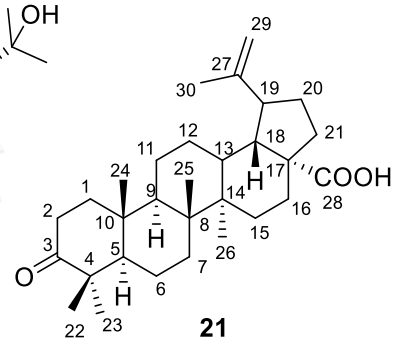

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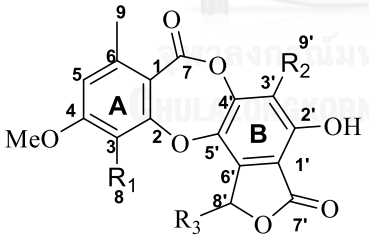
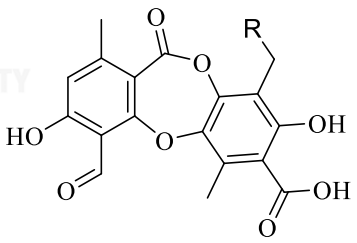
Depsidones



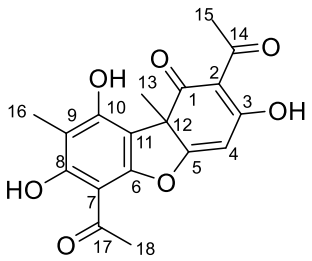
1 R_1: CHO R_2: Me R_3: OH
2 R_1: CHO R_2: CH_2OH R_3: OH
5 R_1: CH_2OH R_2: Me R_3: OH
6 R_1: Me R_2: CH_2OH R_3:OH
7 R_1: OH R_2: Me R_3:OH
9 R_1: CHO R_2: CH_2OH R_3: OMe
11 R_1: CHO R_2: Me R_3:OMe
12 R_1: OH R_2: Me R_3: OMe



8 R: H
13 R: OMe
14 R: OH

Figure 4.1 (continues)

Other skeleton



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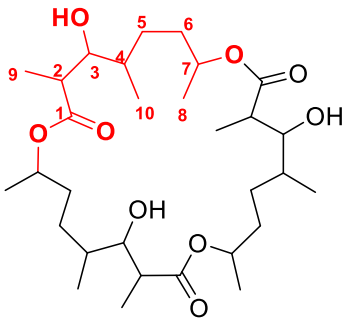

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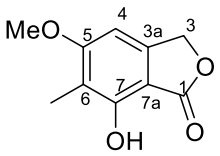

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Figure 4.1 (continued)

4.2 Biological activities

The antibacterial activities of nineteen compounds (**1**, **2**, **4**-**9**, **13**-**22**, **24**) were studied against *Propionibacterium acnes*, *Staphylococcus aureus, Streptococcus sobrinus, Streptococcus mutans* and *Salmonella typhi*. The results indicated that usnic acid (**18**) showed the highest activity against all bacteria with inhibition zone average in a range of 20.67- 28.00 (mm). Some of other compounds showed good or moderate or weak abilities.

Moreover, the extracted lichens were measured by DPPH radical scavenging assay. The scavenging abilities of **13** compounds were in the range of 19.79% - 85.76%. Among the tested extracts, virensic acid exhibited highest DPPH radical scavenging activity (IC_{50} 0.41 mM).

4.3 Suggestions for future work

The possible future research would be related to the chemical constituent from the remaining dichloromethane fraction of *U.baileyi*. Furthermore, preparation some derivatives from the major compounds as stictic acid and protocetraric acid. Then, some biological activities such as cancer cell line and some inhibitory activities examined on isolated or derivatives compounds.

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APPENDIX

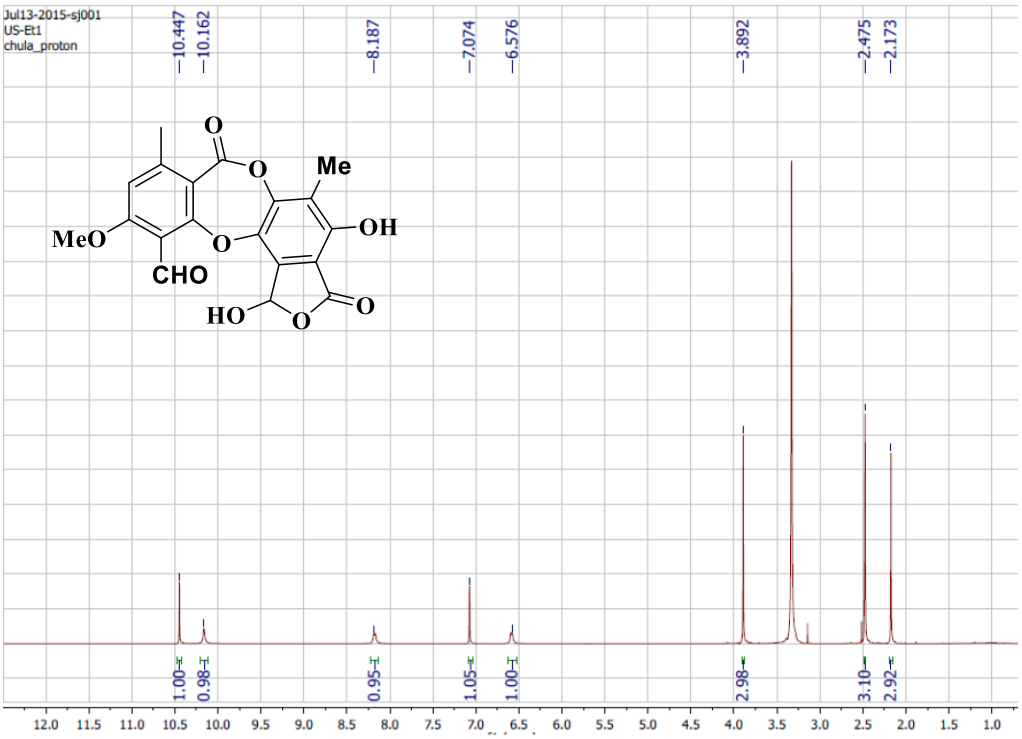

Figure A. 1 ^{1}H NMR (400 MHz) spectrum of compound **1** (DMSO-d6)

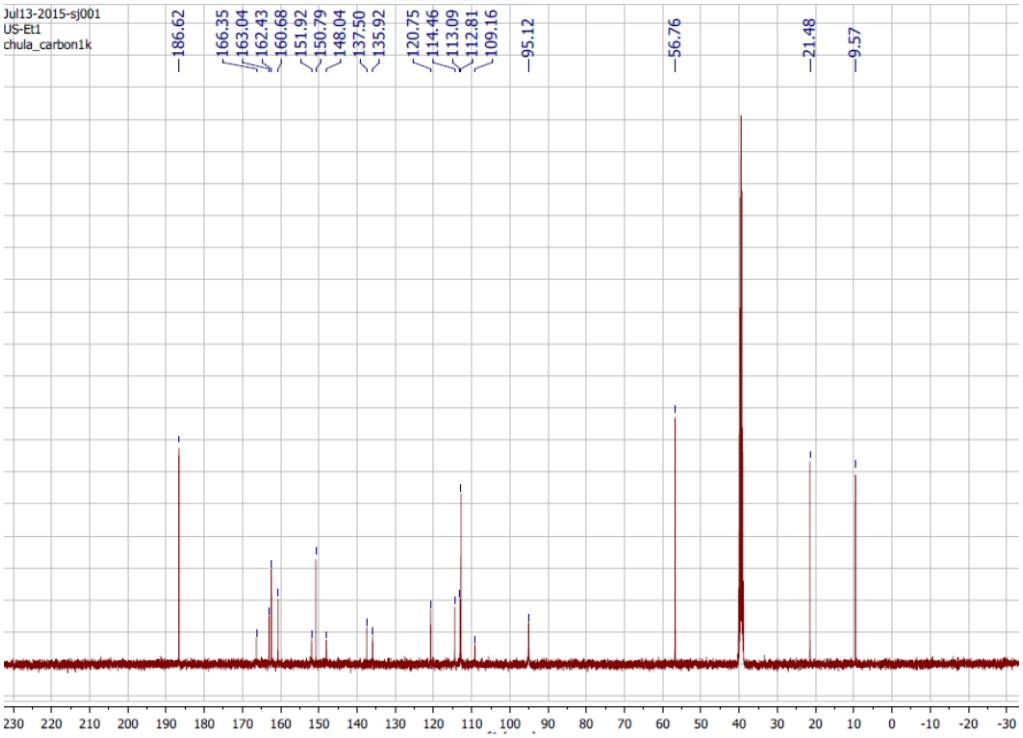

Figure A. 2 ^{13}C NMR (100 MHz) spectrum of compound **1** (DMSO-d_6)

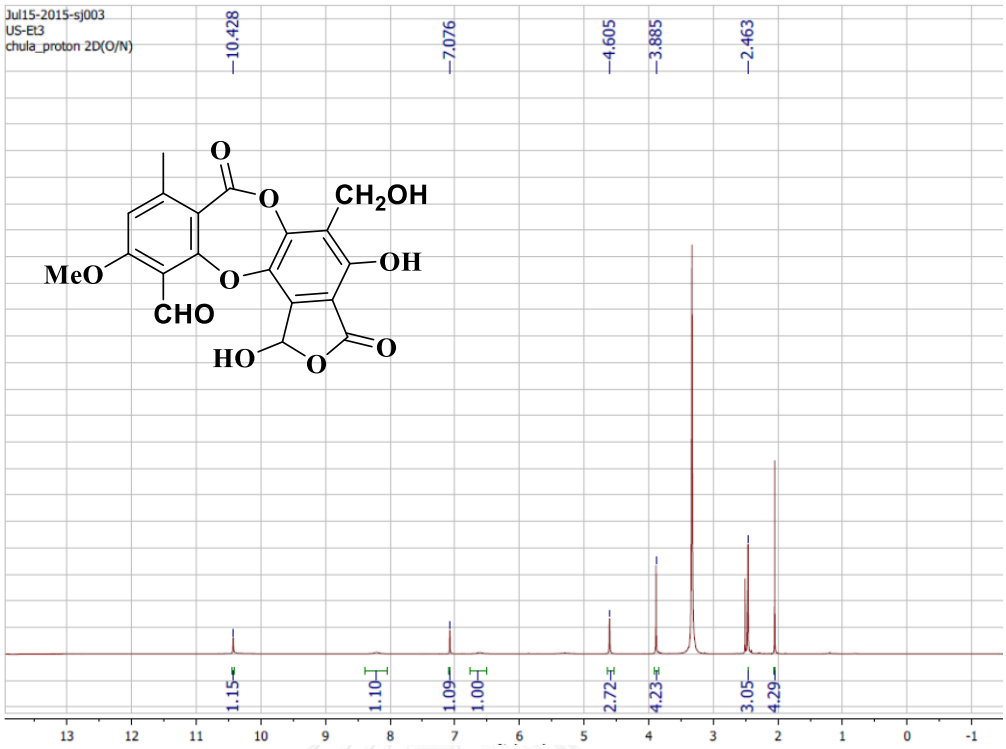

Figure A. 3 ^{1}H NMR (400 MHz) spectrum of compound **2** (DMSO-d_6)

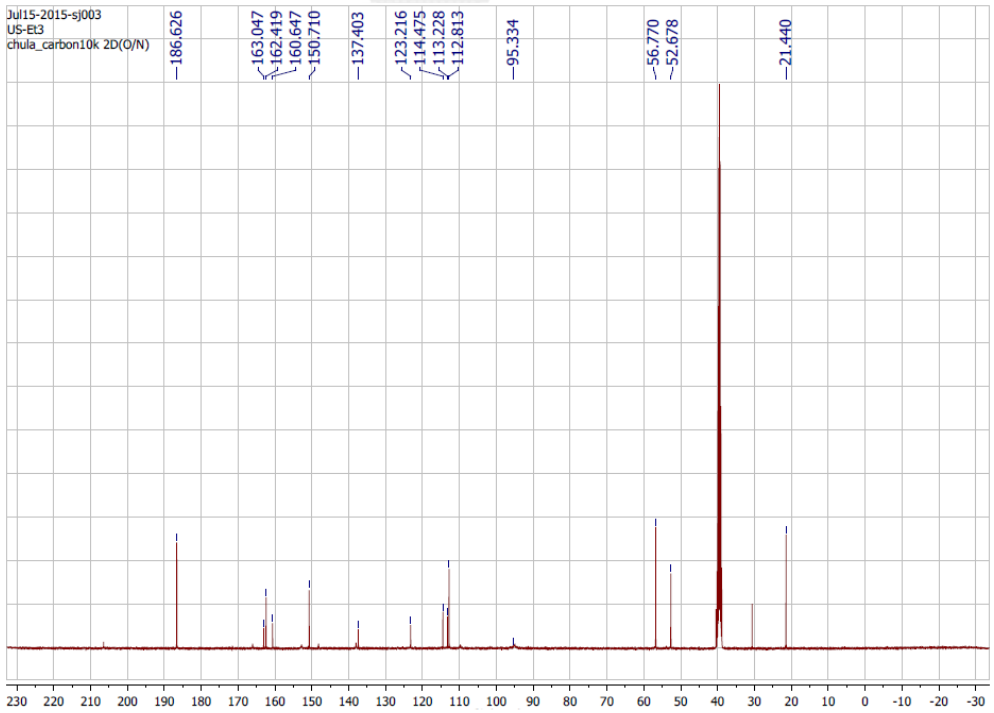

Figure A. 4 ^{13}C NMR (100 MHz) spectrum of compound **2** (DMSO-d_6)

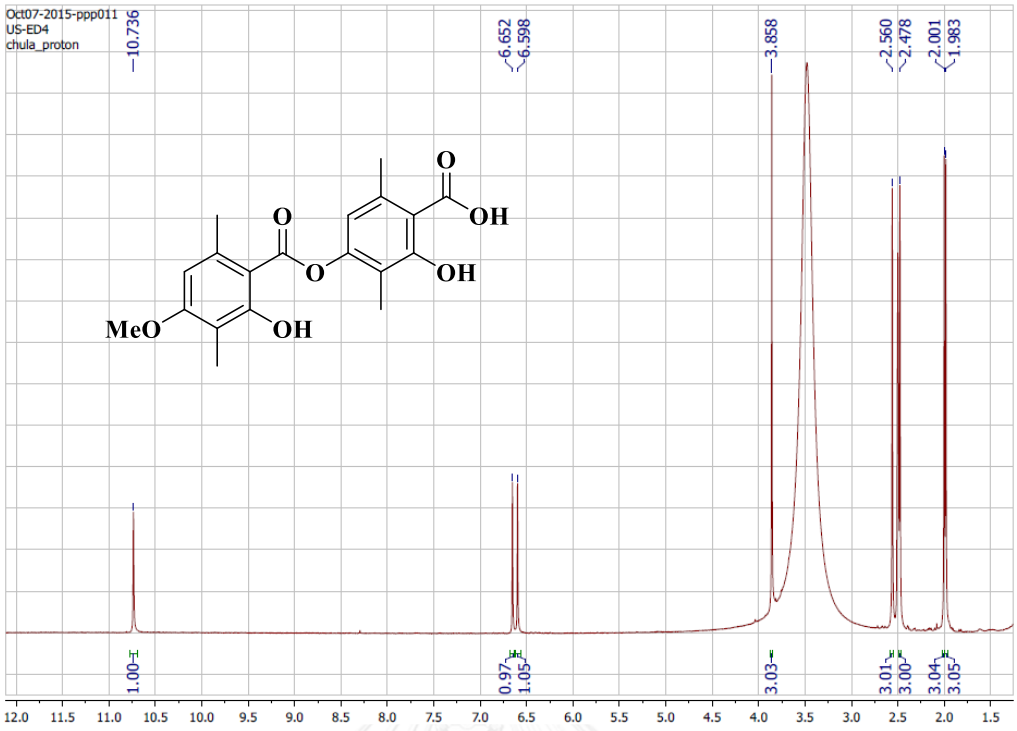

Figure A. 5 ^{1}H NMR (400 MHz) spectrum of compound **3** (DMSO-d_6)

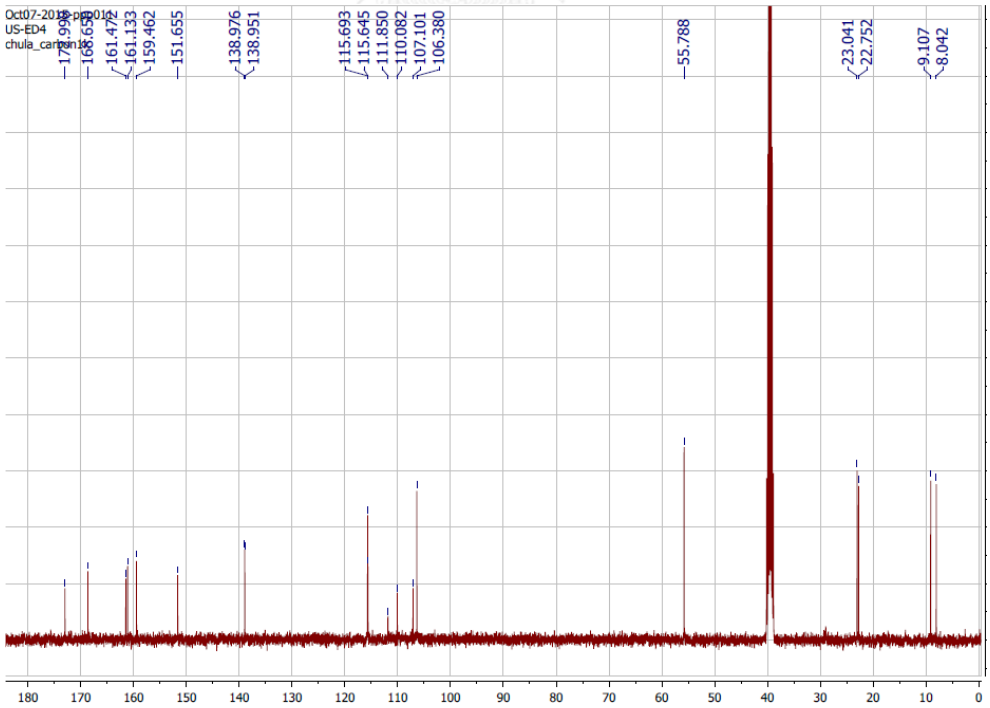

Figure A. 6 ^{13}C NMR (100 MHz) spectrum of compound **3** (DMSO-d_6)

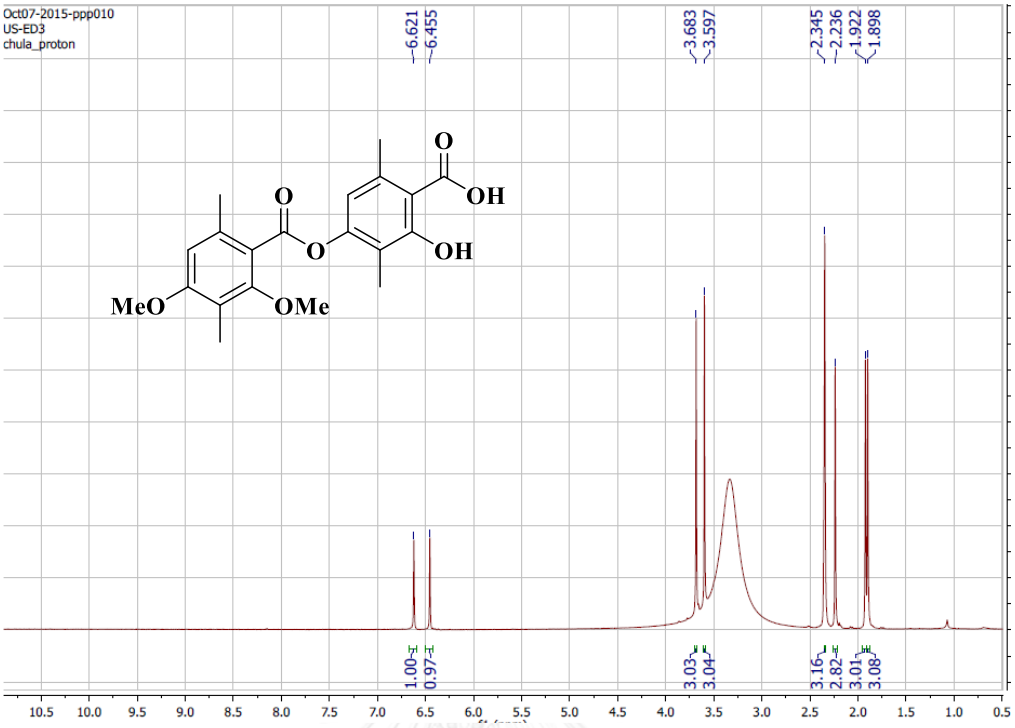

Figure A. 7 ^{1}H NMR (400 MHz) spectrum of compound **4** (DMSO-d_6)

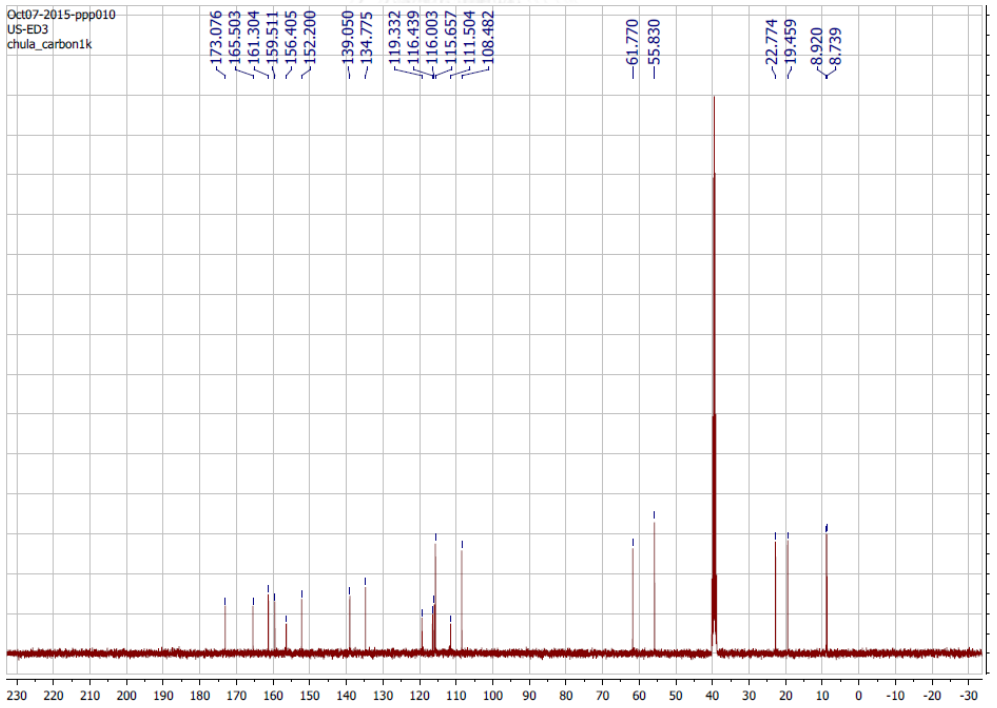

Figure A. 8 ^{13}C NMR (100 MHz) spectrum of compound **4** (DMSO-d_6)

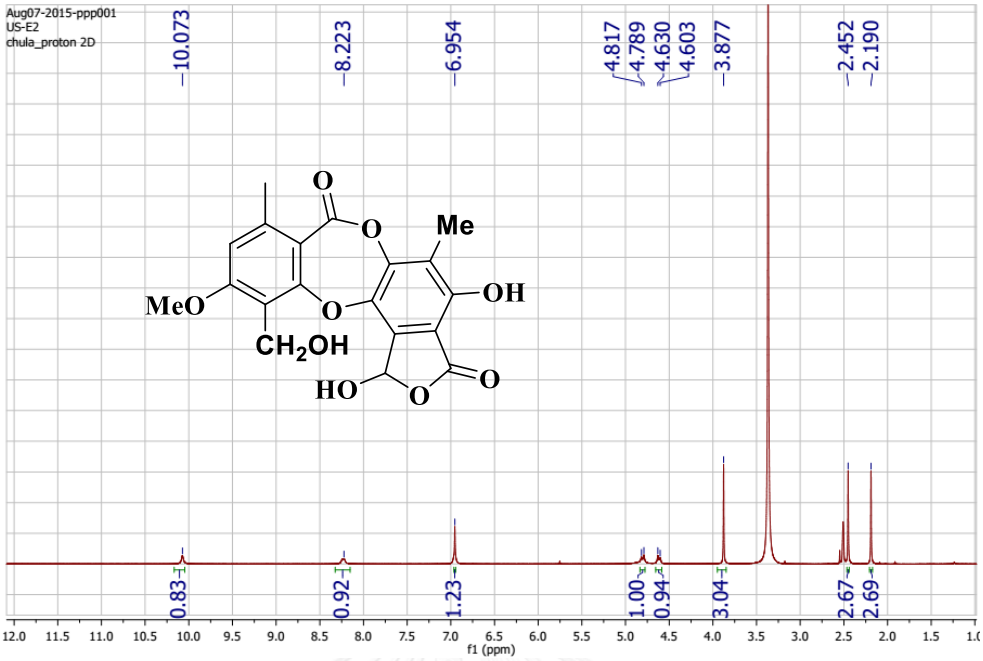

Figure A. 9 ^{1}H NMR (400 MHz) spectrum of compound **5** (DMSO-d_6)

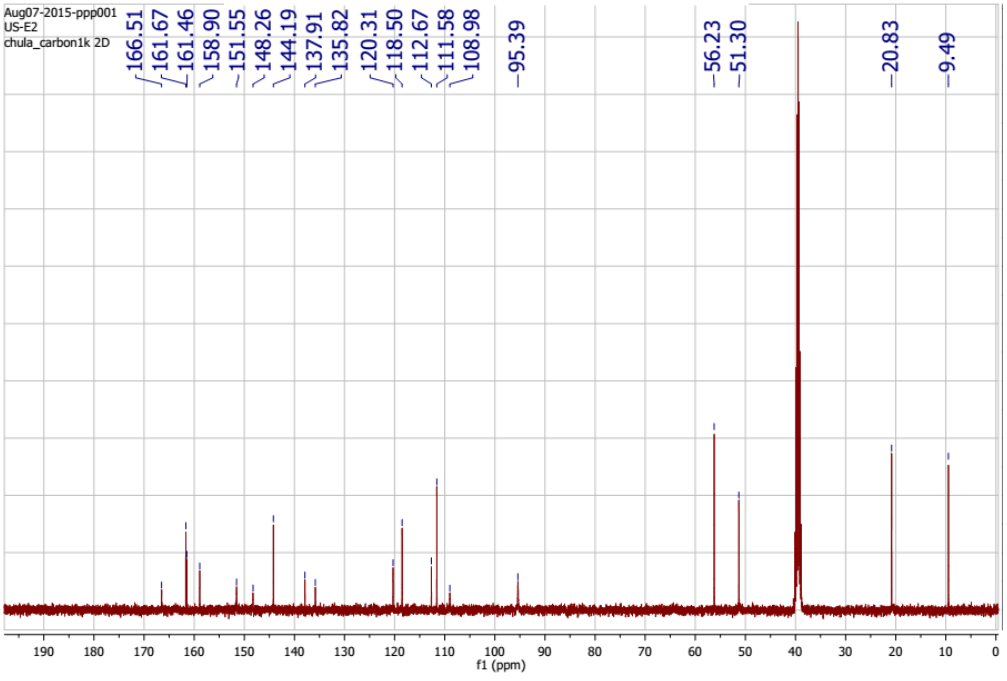

Figure A. 10 ^{13}C NMR (100 MHz) spectrum of compound **5** (DMSO-d_6)

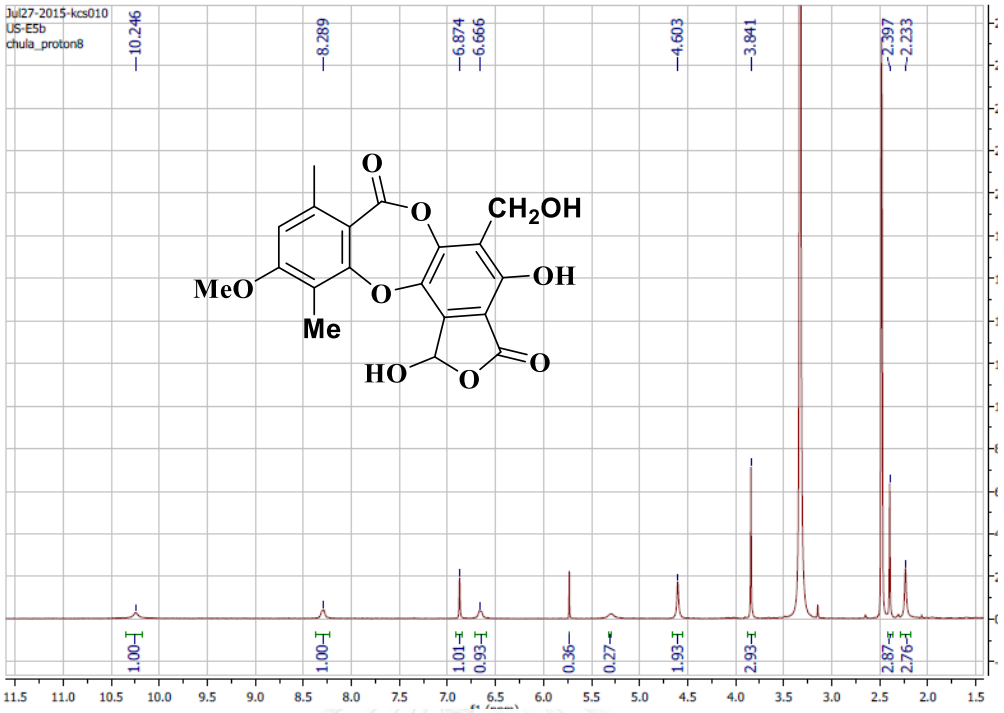

Figure A. 11 ^{1}H NMR (400 MHz) spectrum of compound **6** (DMSO-d_6)

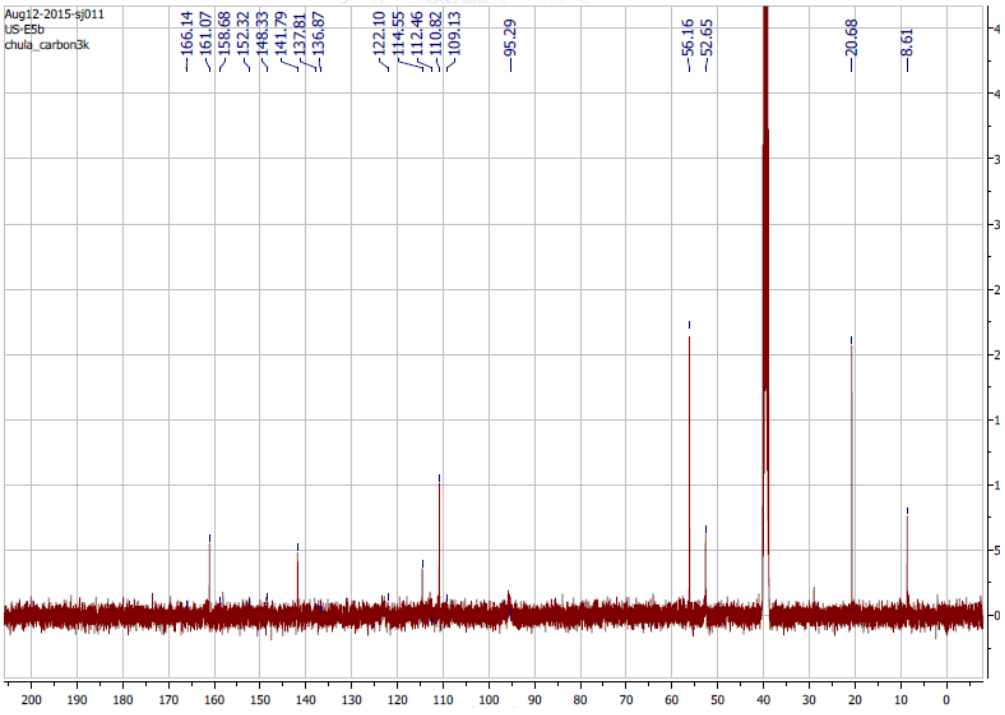

Figure A. 12 ^{13}C NMR (100 MHz) spectrum of compound **6** (DMSO-d_6)

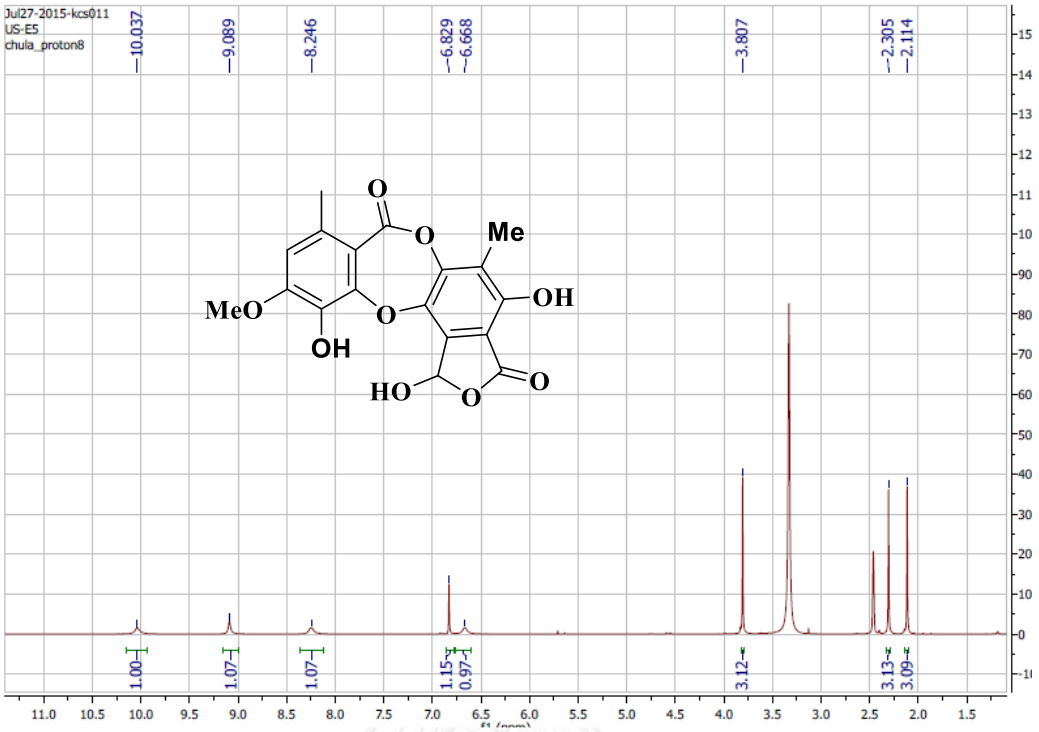

Figure A. 13 ^{1}H NMR (400 MHz) spectrum of compound **7** (DMSO-d_6)

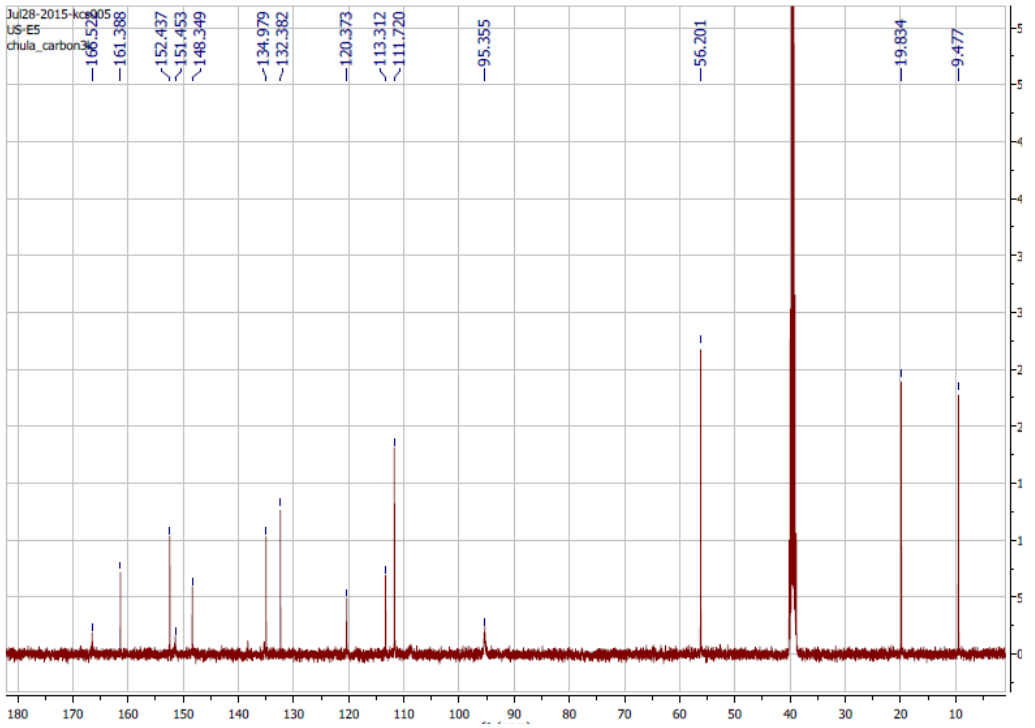

Figure A. 14 ^{13}C NMR (100 MHz) spectrum of compound **7** (DMSO-d_6)

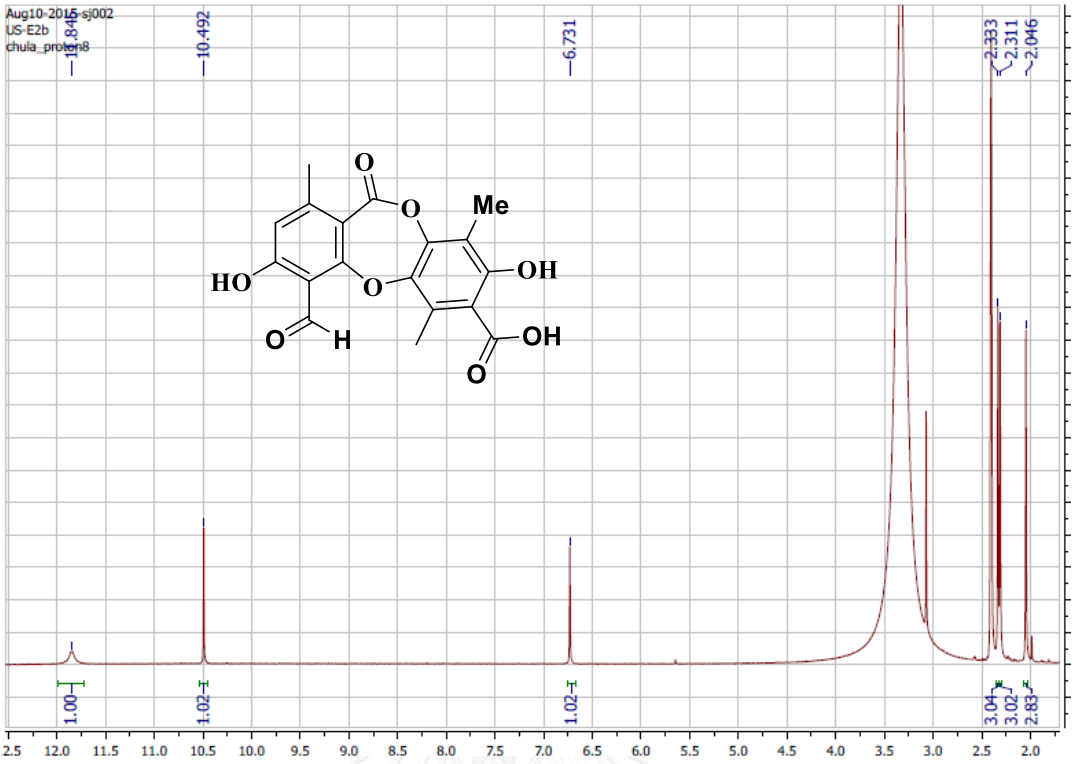

Figure A. 15 ^{1}H NMR (400 MHz) spectrum of compound **8** (DMSO-d_6)

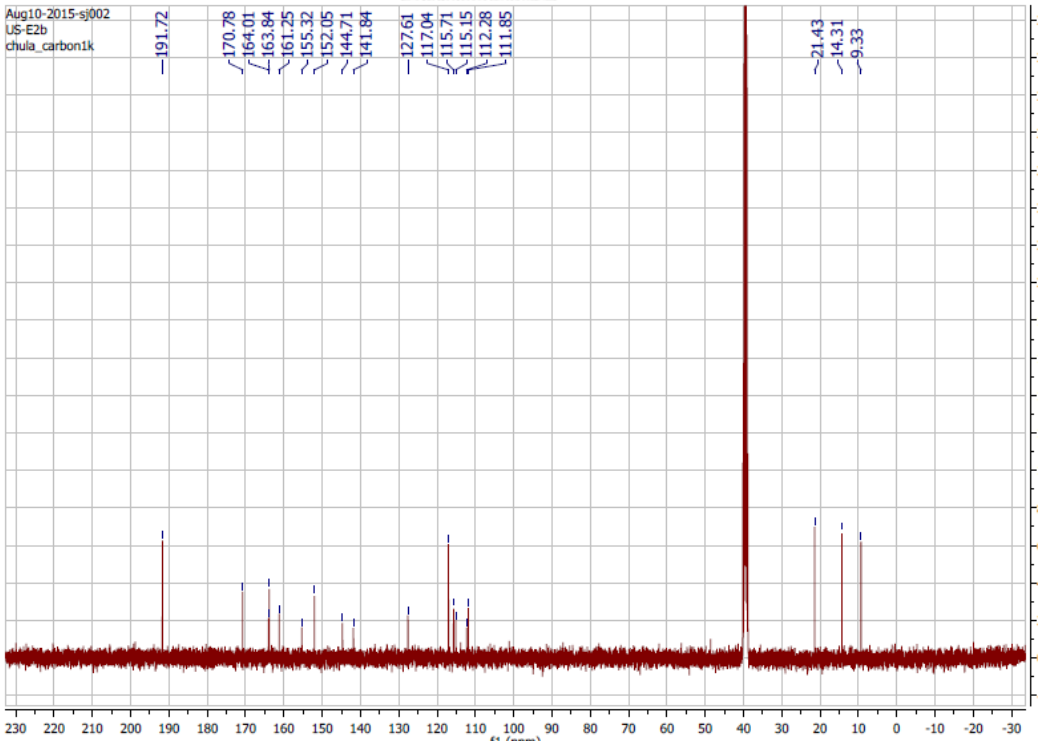

Figure A. 16 ^{13}C NMR (100 MHz) spectrum of compound **8** (DMSO-d_6)

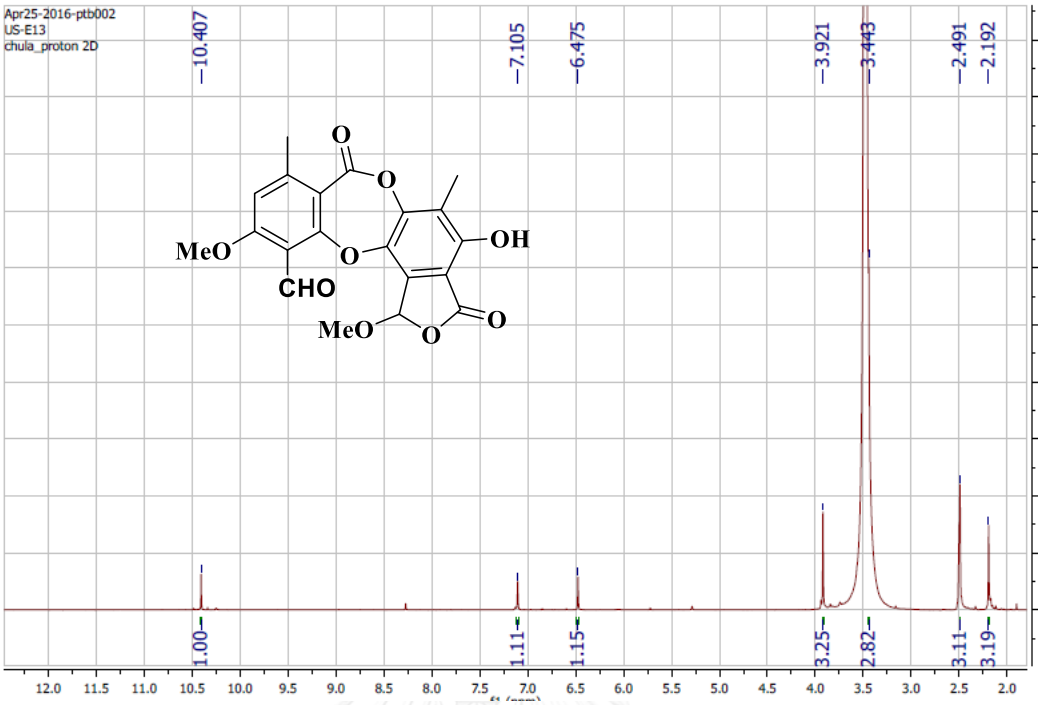

Figure A. 17 ^{1}H NMR (400 MHz) spectrum of compound **9** (DMSO-d_6)

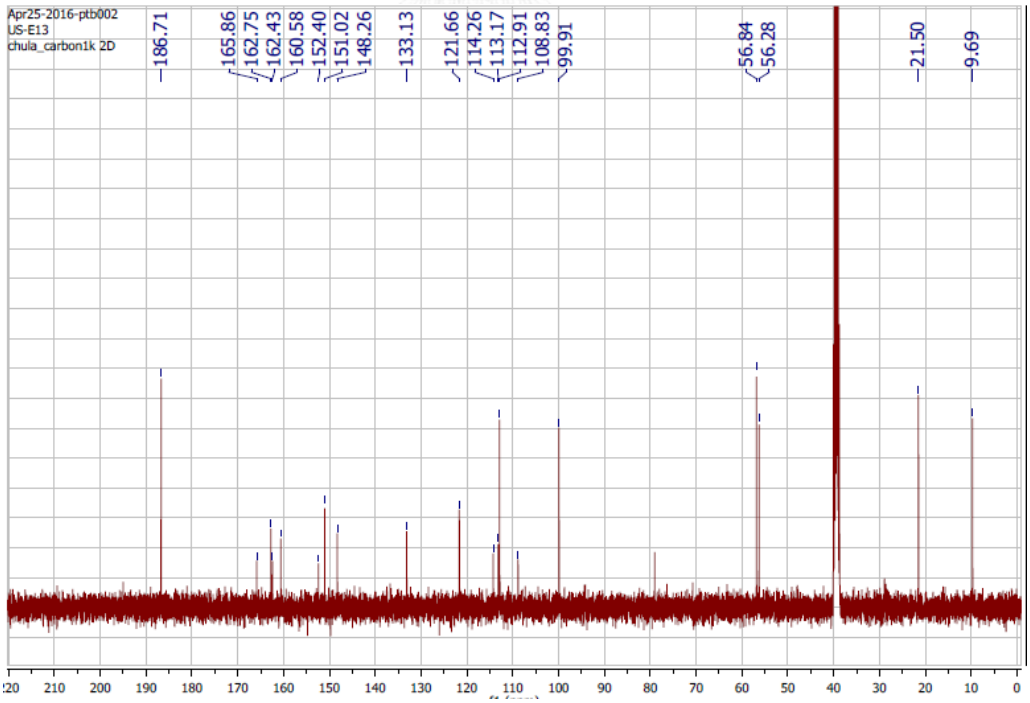

Figure A. 18 ^{13}C NMR (100 MHz) spectrum of compound **9** (DMSO-d_6)

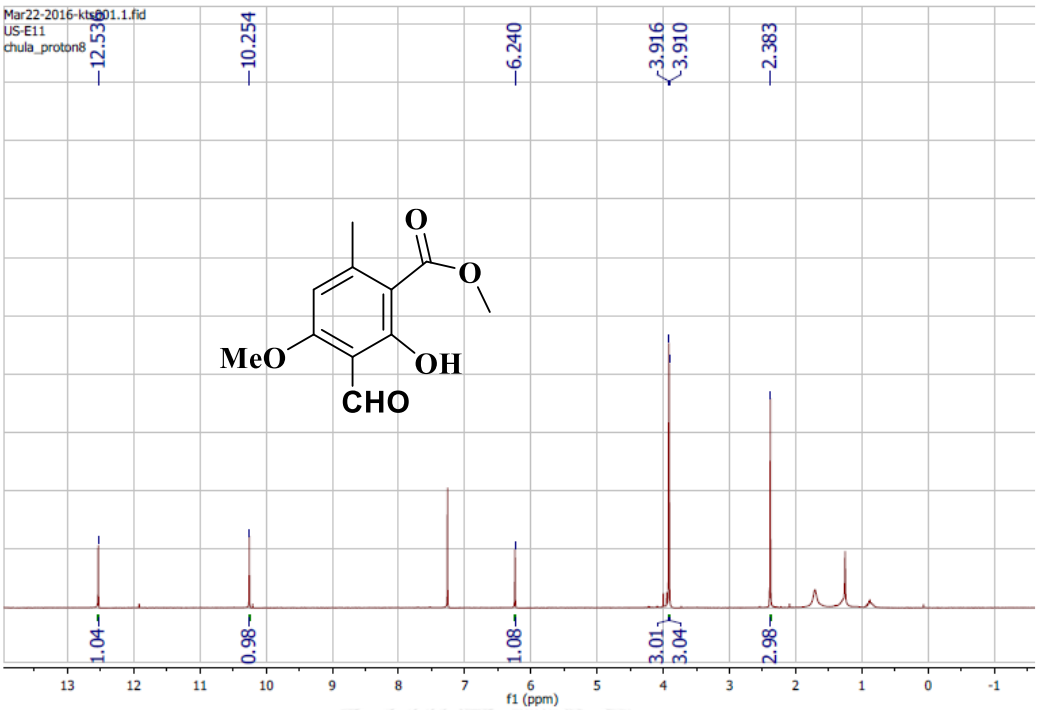

Figure A. 19 ^{1}H NMR (400 MHz) spectrum of compound **10** ($CDCl_3$)

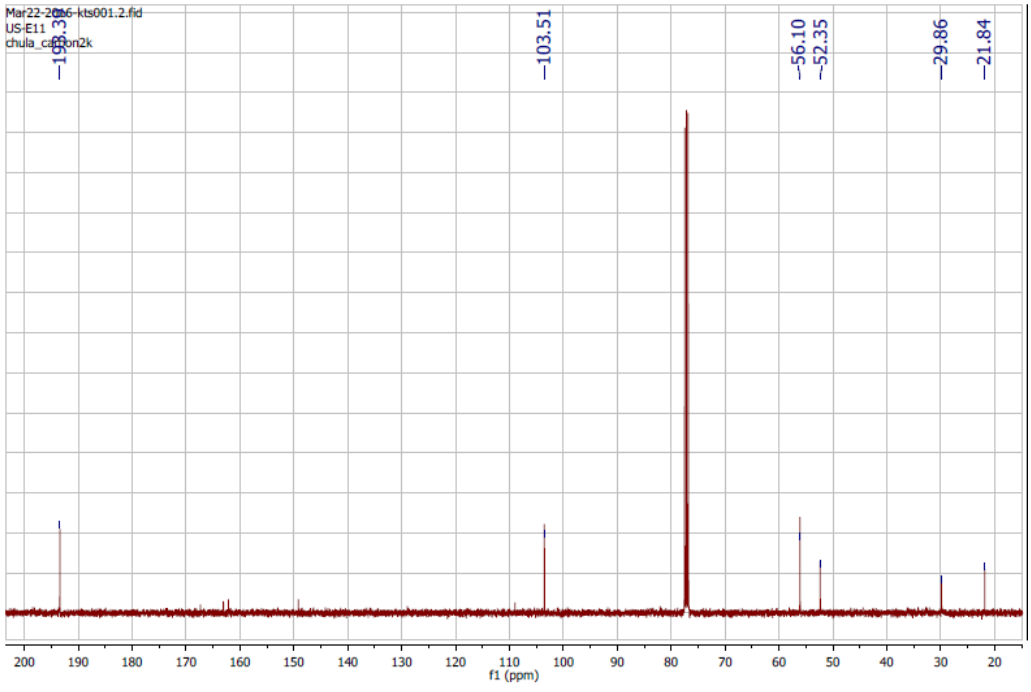

Figure A. 20 ^{13}C NMR (100 MHz) spectrum of compound **10** ($CDCl_3$)

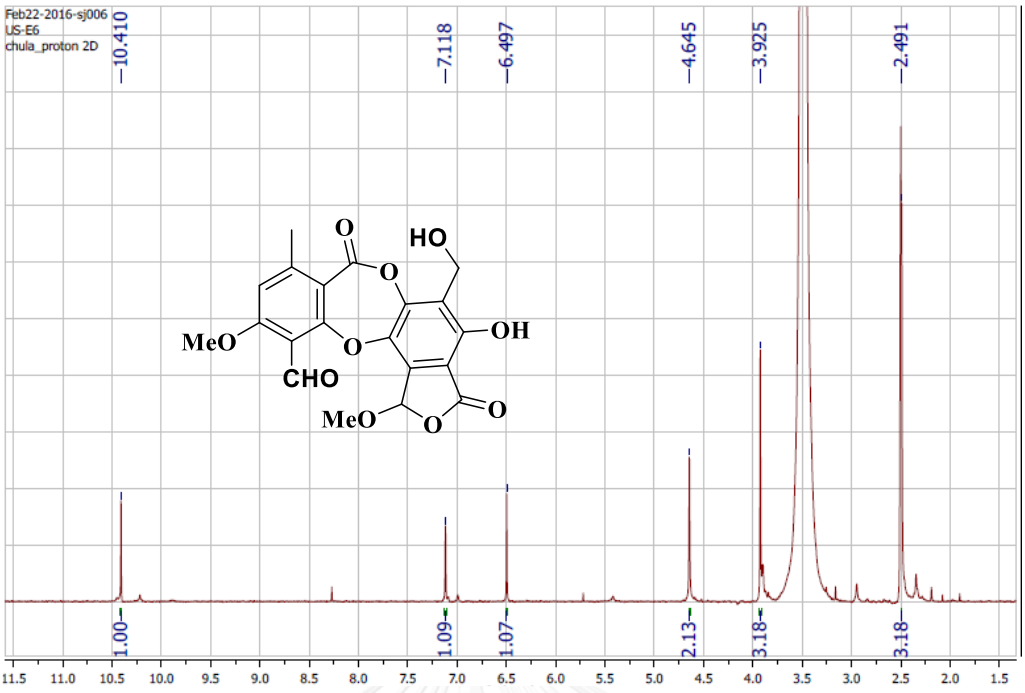

Figure A. 21 ^{1}H NMR (400 MHz) spectrum of compound **11** (DMSO-d_6)

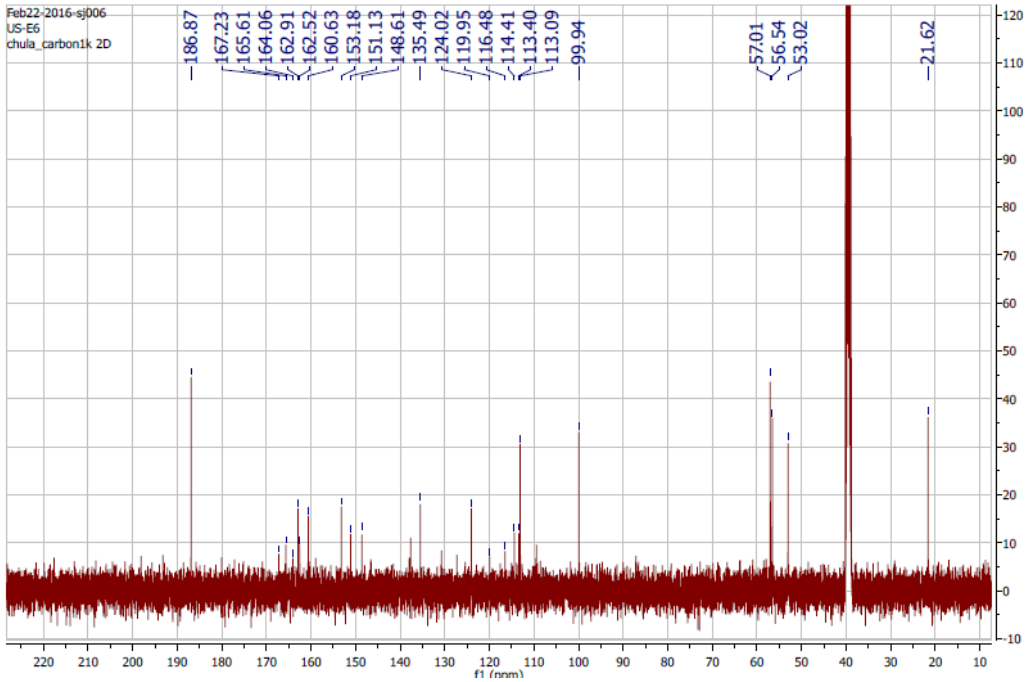

Figure A. 22 ^{13}C NMR (100 MHz) spectrum of compound **11** (DMSO-d_6)

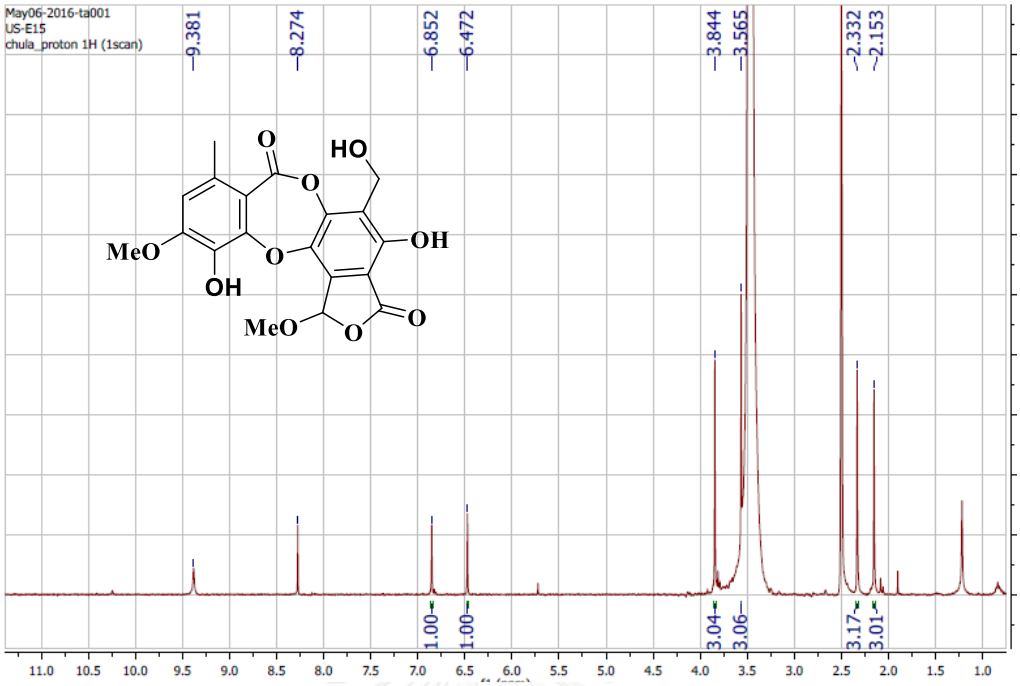

Figure A. 23 ^{1}H NMR (400 MHz) spectrum of compound **12** (DMSO-d_6)

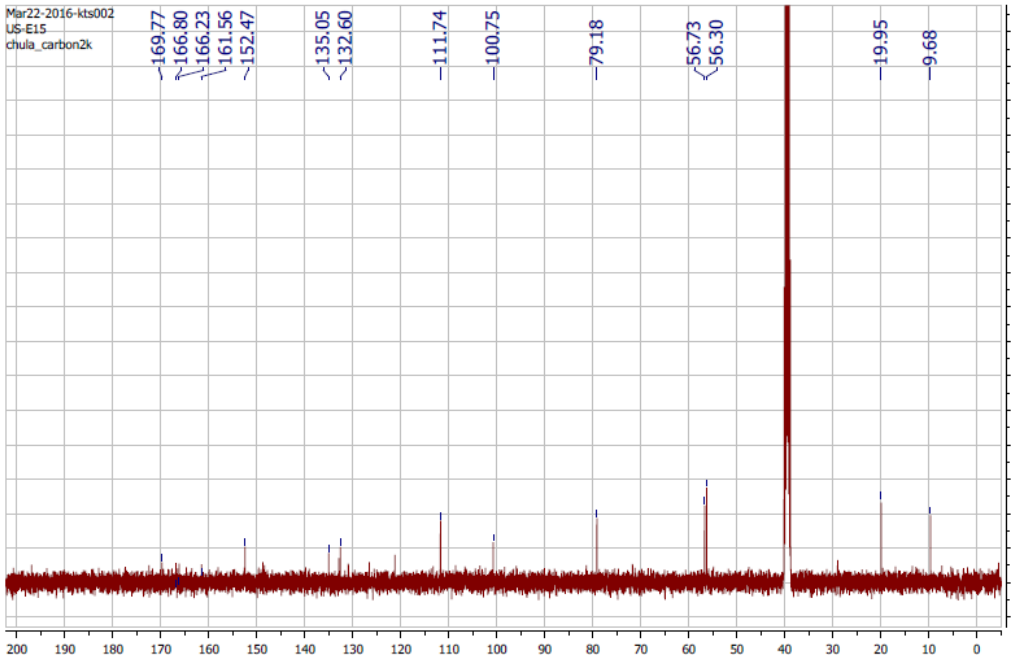

Figure A. 24 ^{13}C NMR (100 MHz) spectrum of compound **12** (DMSO-d_6)

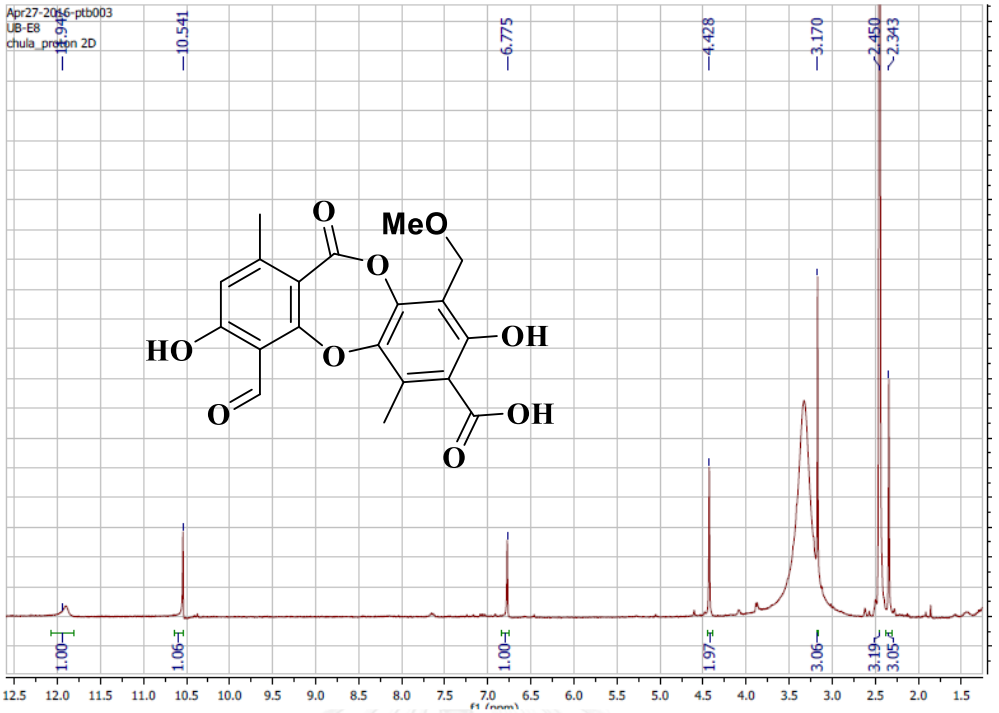

Figure A. 25 ^{1}H NMR (400 MHz) spectrum of compound **13** (DMSO-d_6)

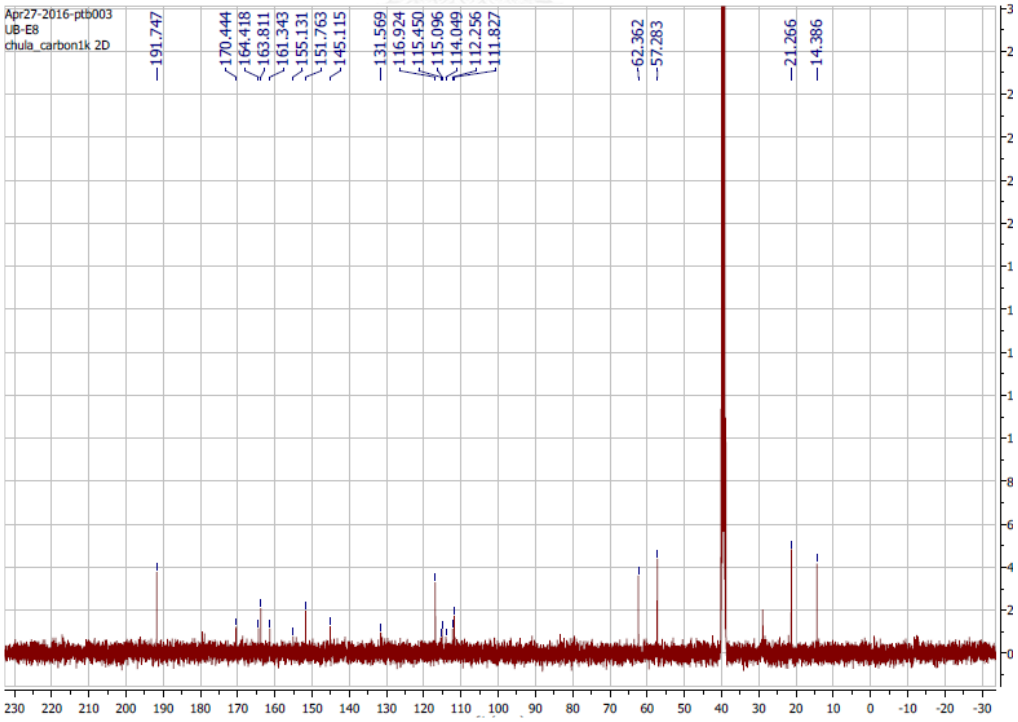

Figure A. 26 ^{13}C NMR (100 MHz) spectrum of compound **13** (DMSO-d_6)

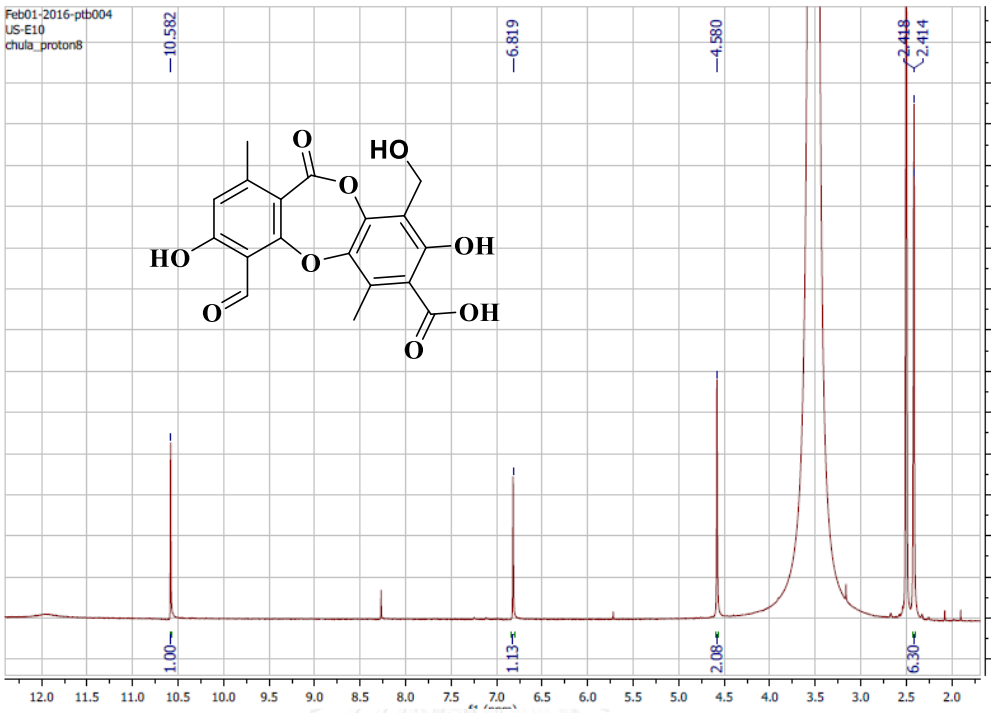

Figure A. 27 ^{1}H NMR (400 MHz) spectrum of compound **14** (DMSO-d_6)

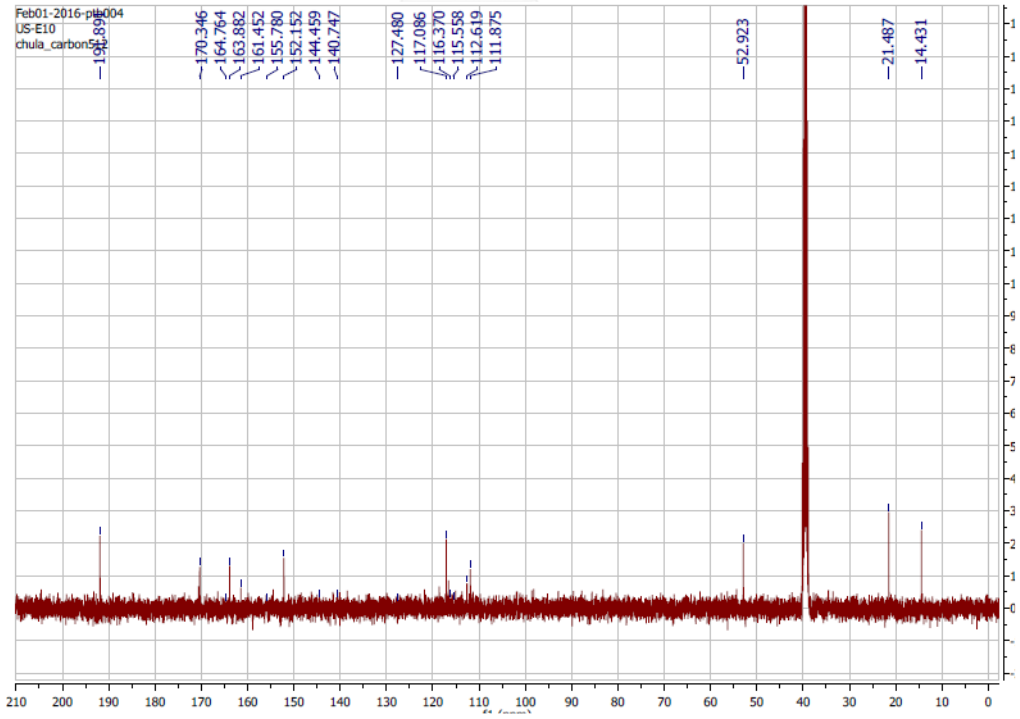

Figure A. 28 ^{13}C NMR (100 MHz) spectrum of compound **14** (DMSO-d_6)

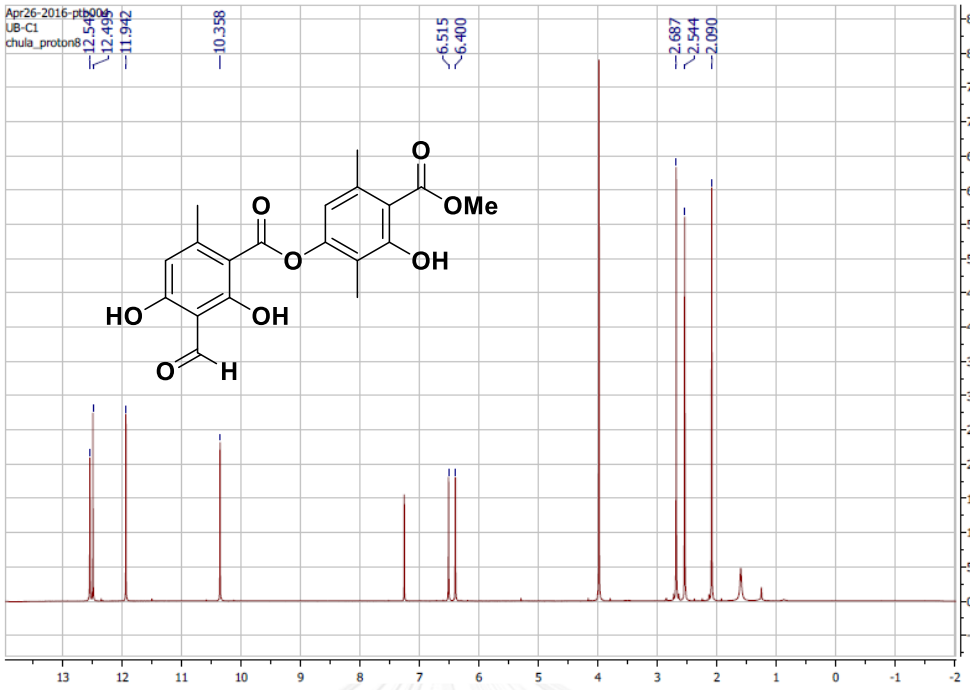

Figure A. 29 ^{1}H NMR (400 MHz) spectrum of compound **15** ($CDCl_3$)

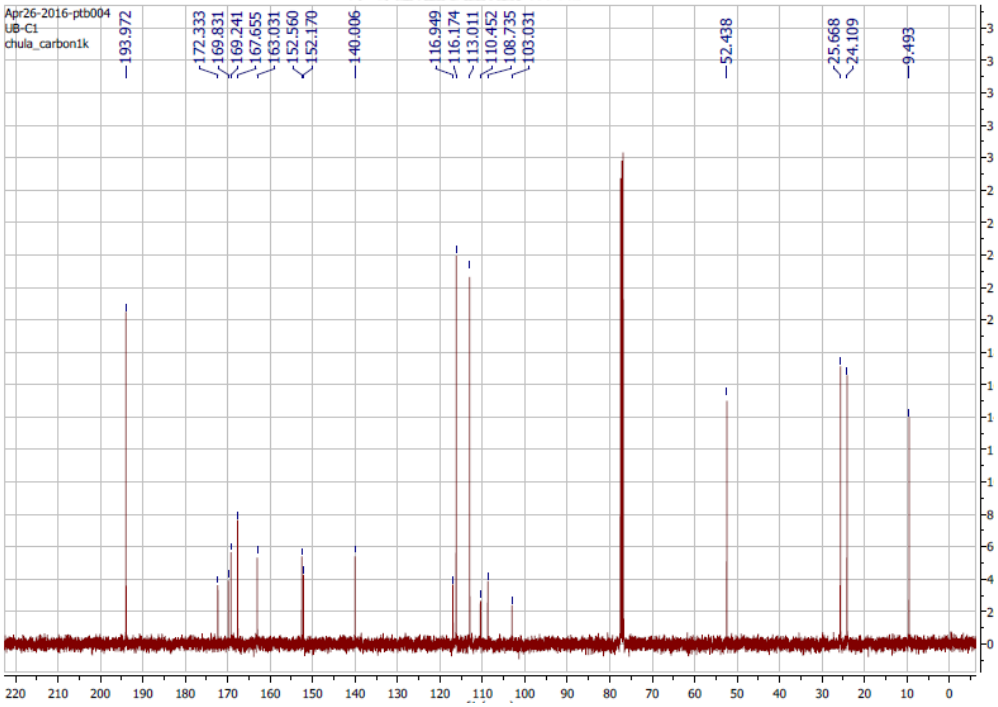

Figure A. 30 ^{13}C NMR (100 MHz) spectrum of compound **15** ($CDCl_3$)

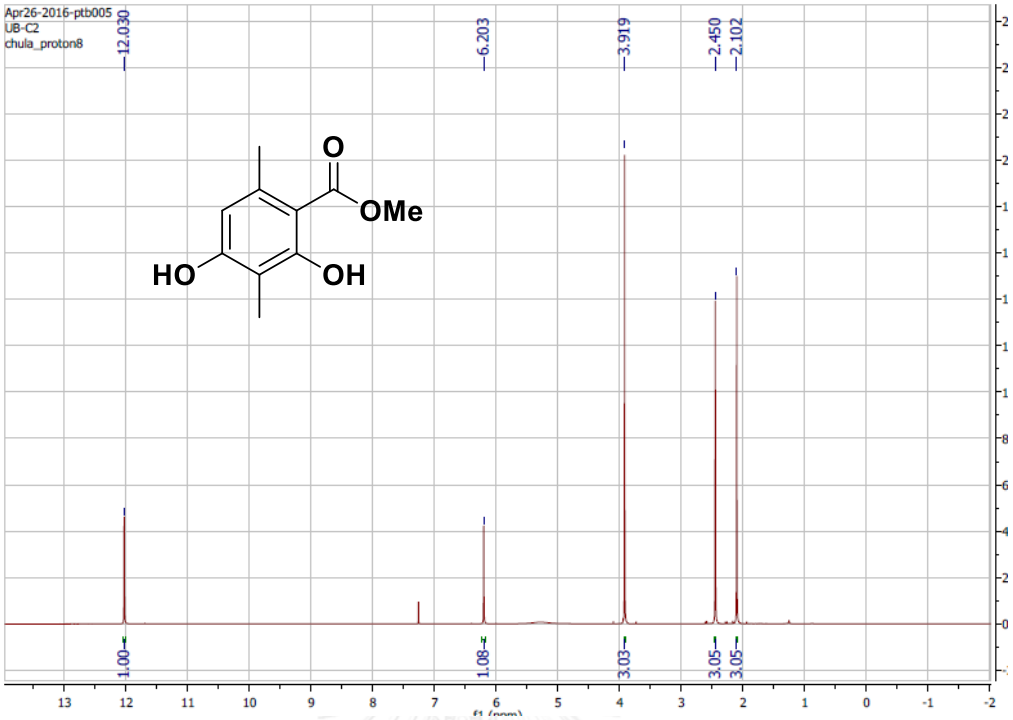

Figure A. 31 ^{1}H NMR (400 MHz) spectrum of compound **16** ($CDCl_3$)

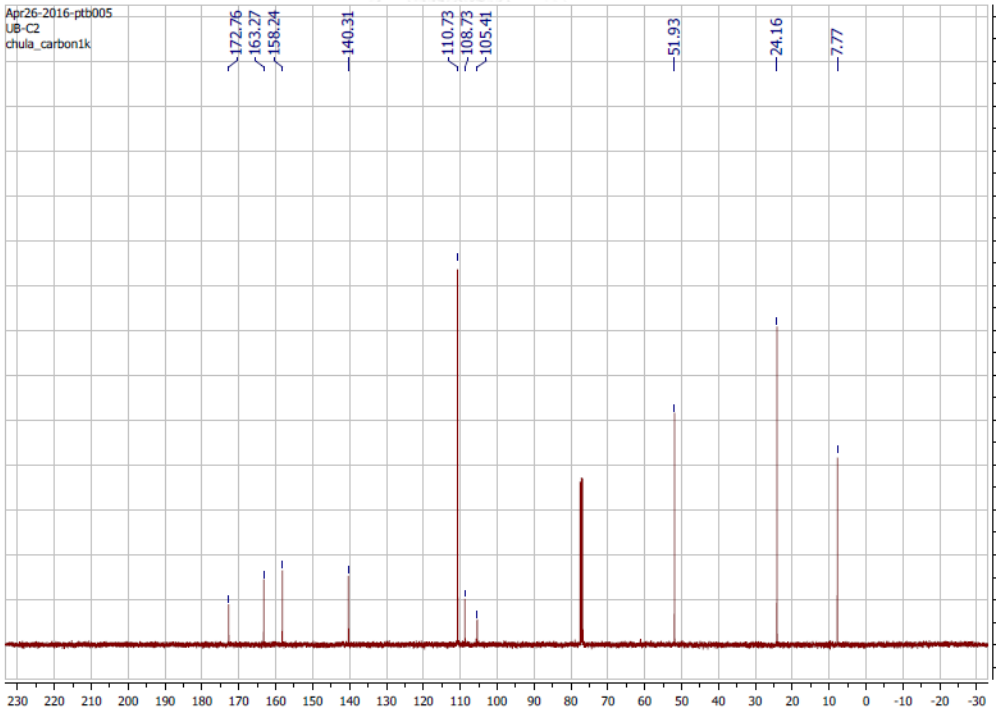

Figure A. 32 ^{13}C NMR (100 MHz) spectrum of compound **16** ($CDCl_3$)

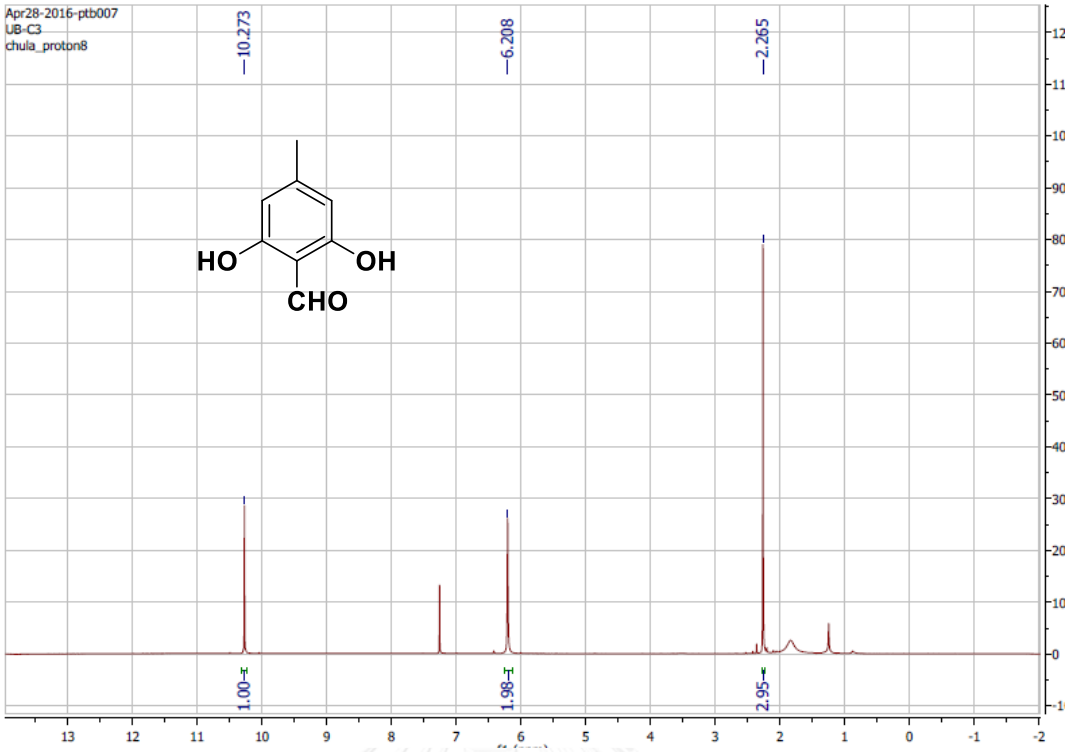

Figure A. 33 ^{1}H NMR (400 MHz) spectrum of compound **17** ($CDCl_3$)

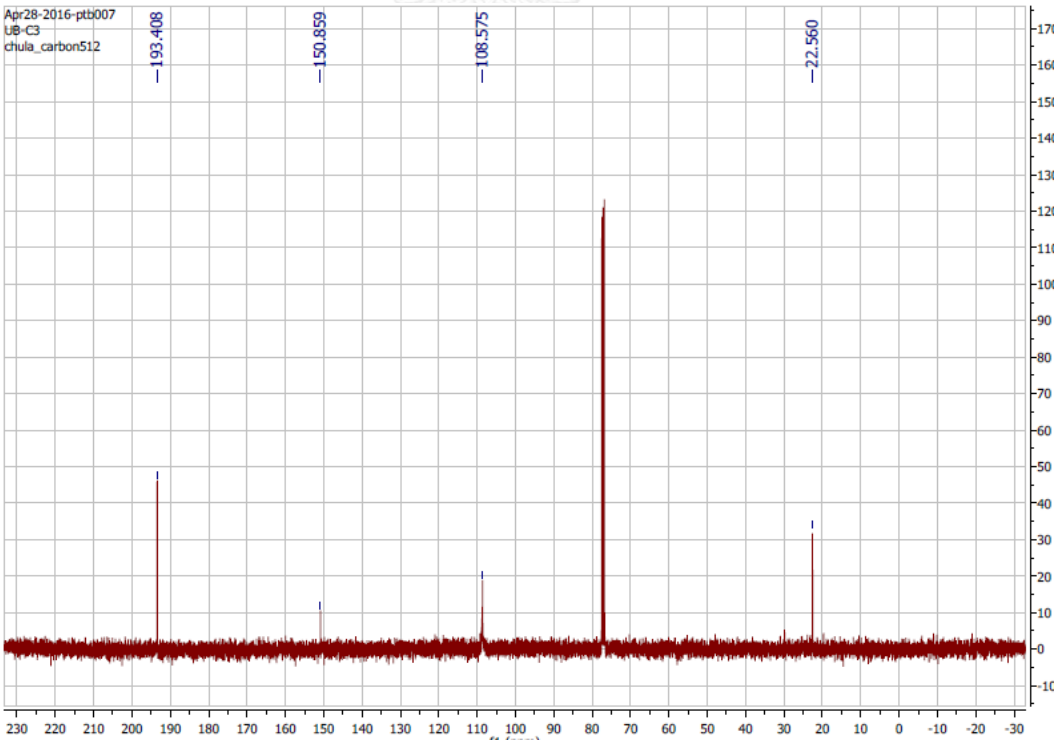

Figure A. 34 ^{13}C NMR (100 MHz) spectrum of compound **17** ($CDCl_3$)

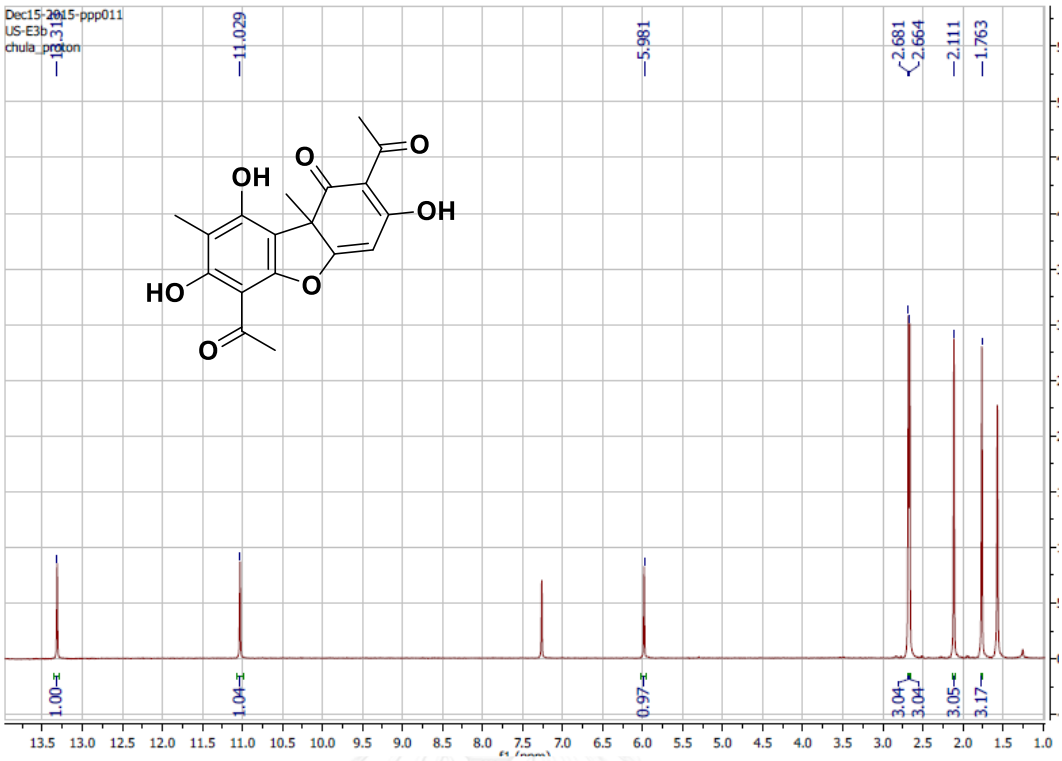

Figure A. 35 ^{1}H NMR (400 MHz) spectrum of compound **18** ($CDCl_3$)

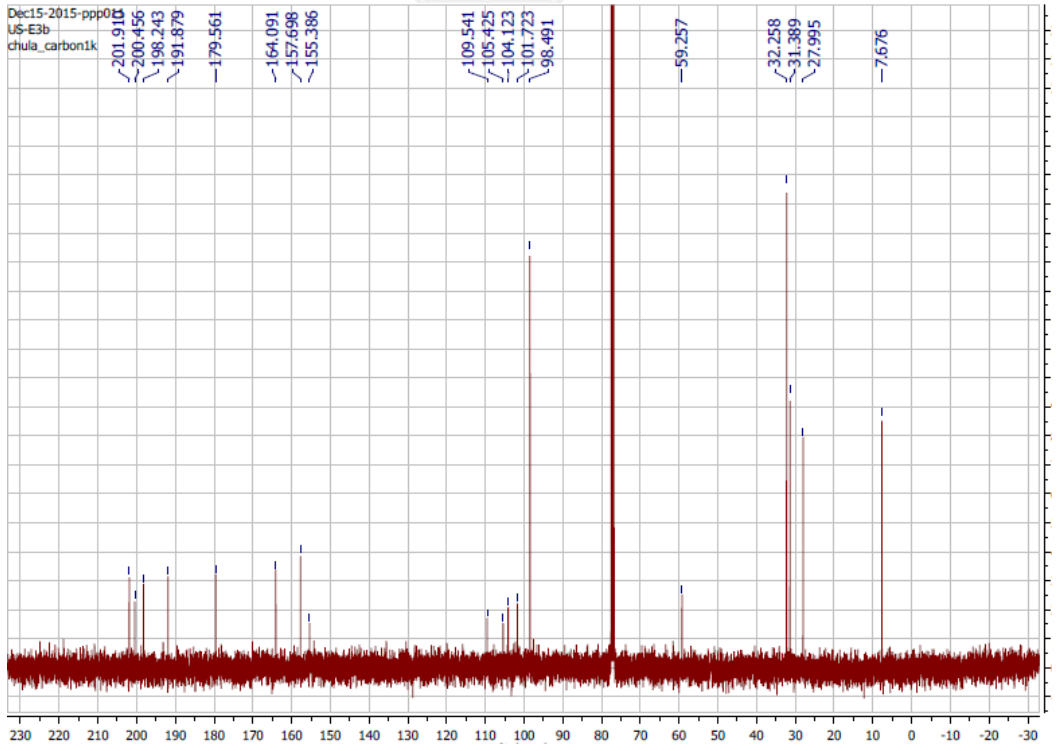

Figure A. 36 ^{13}C NMR (100 MHz) spectrum of compound **18** ($CDCl_3$)

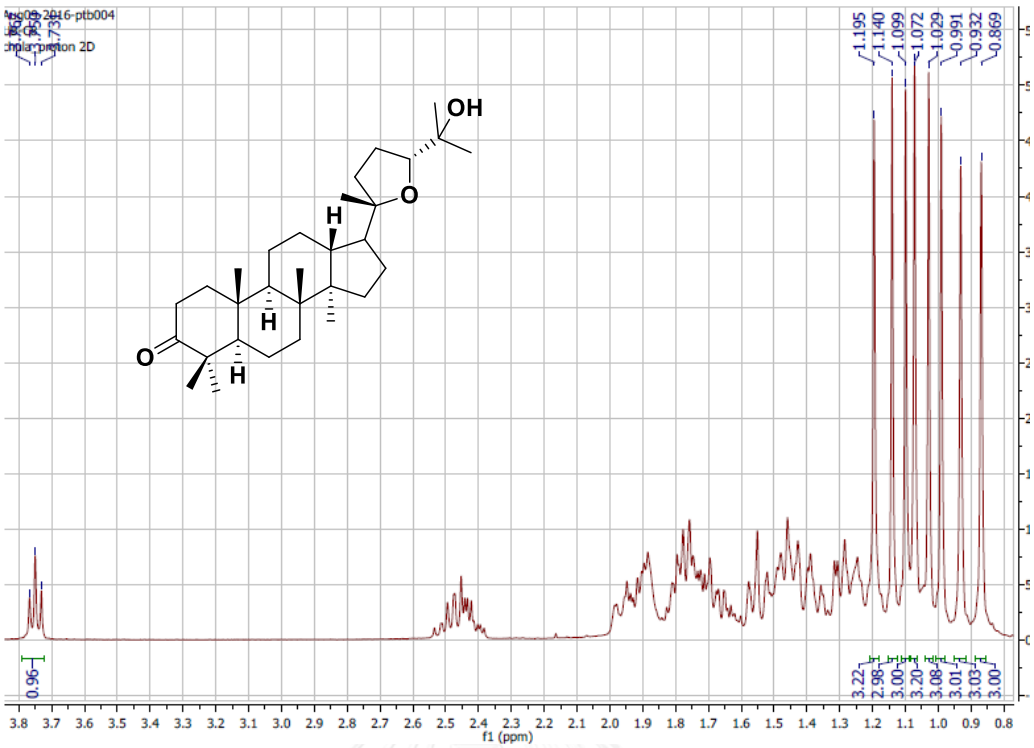

Figure A. 37 ^{1}H NMR (400 MHz) spectrum of compound **19** ($CDCl_3$)

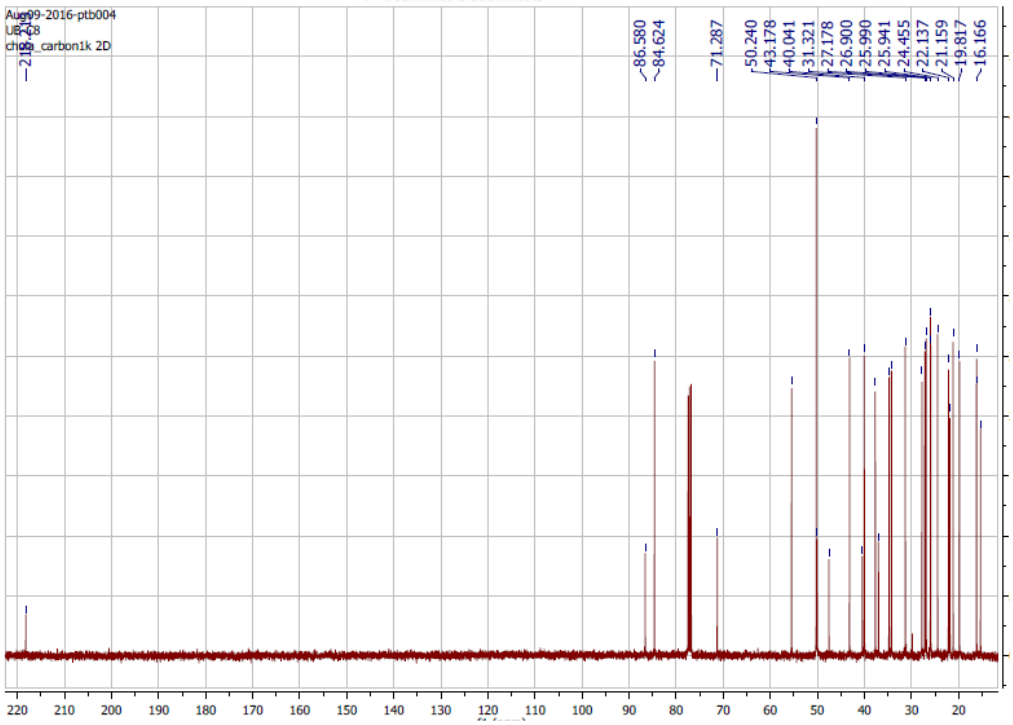

Figure A. 38 ^{13}C NMR (100 MHz) spectrum of compound **19** ($CDCl_3$)

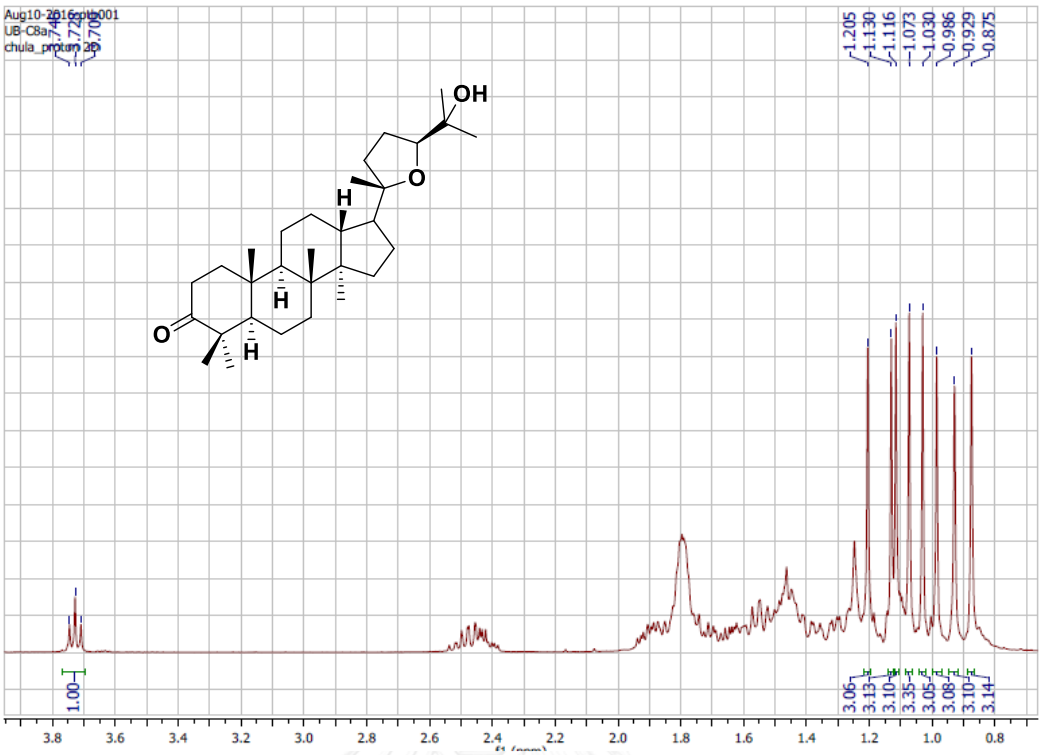

Figure A. 39 ^{1}H NMR (400 MHz) spectrum of compound **20** ($CDCl_3$)

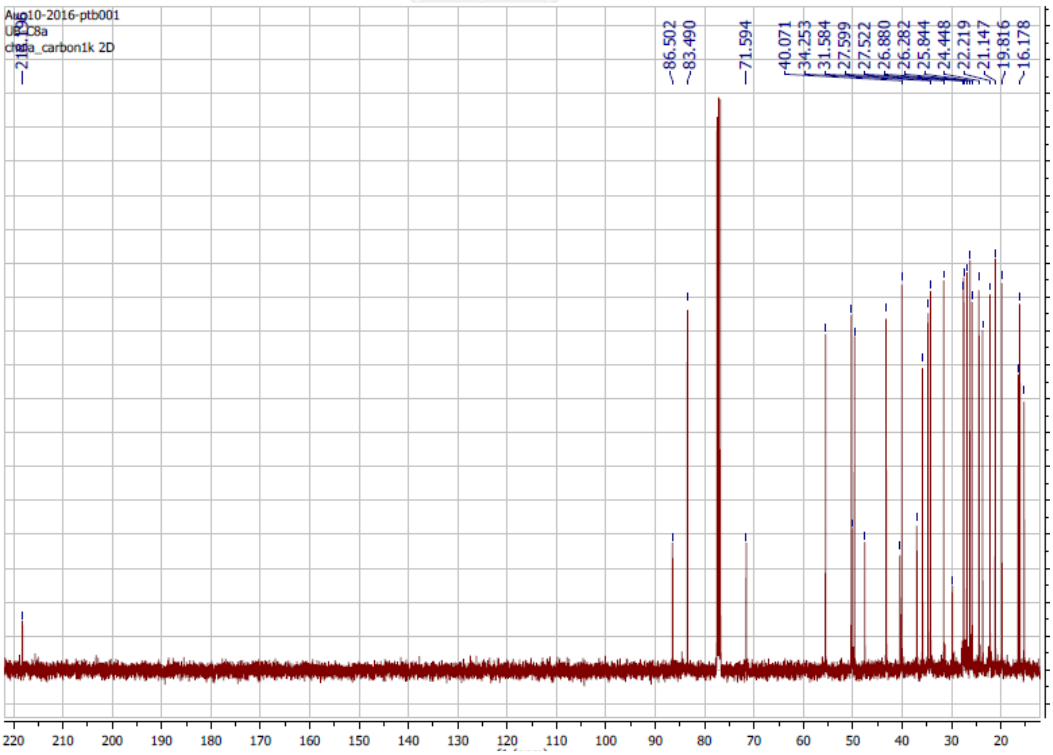

Figure A. 40 ^{13}C NMR (100 MHz) spectrum of compound **20** ($CDCl_3$)

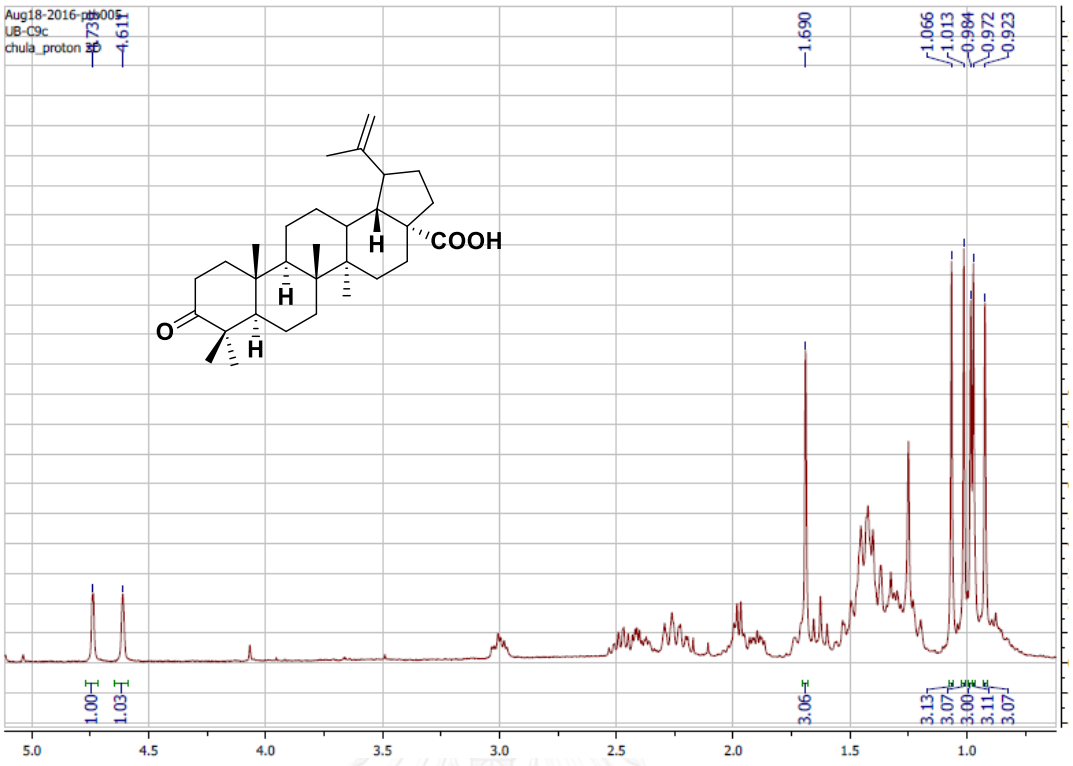

Figure A. 41 ^{1}H NMR (400 MHz) spectrum of compound **21** ($CDCl_3$)

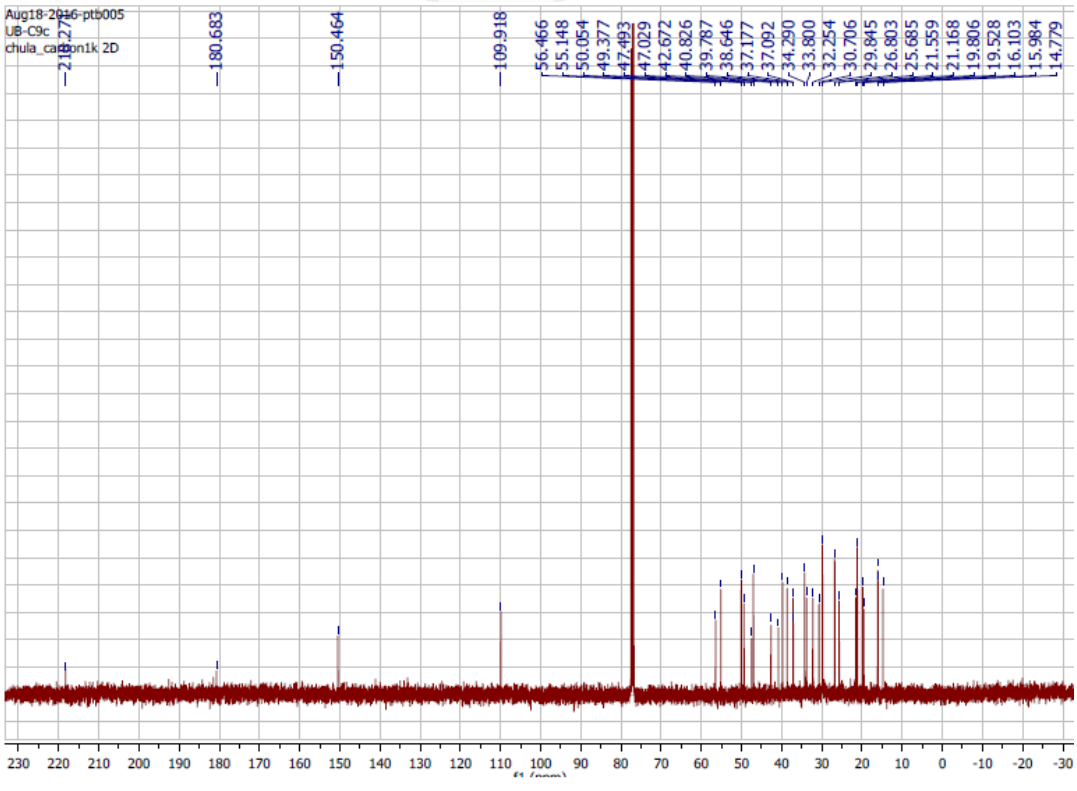

Figure A. 42 ^{13}C NMR (100 MHz) spectrum of compound **21** ($CDCl_3$)

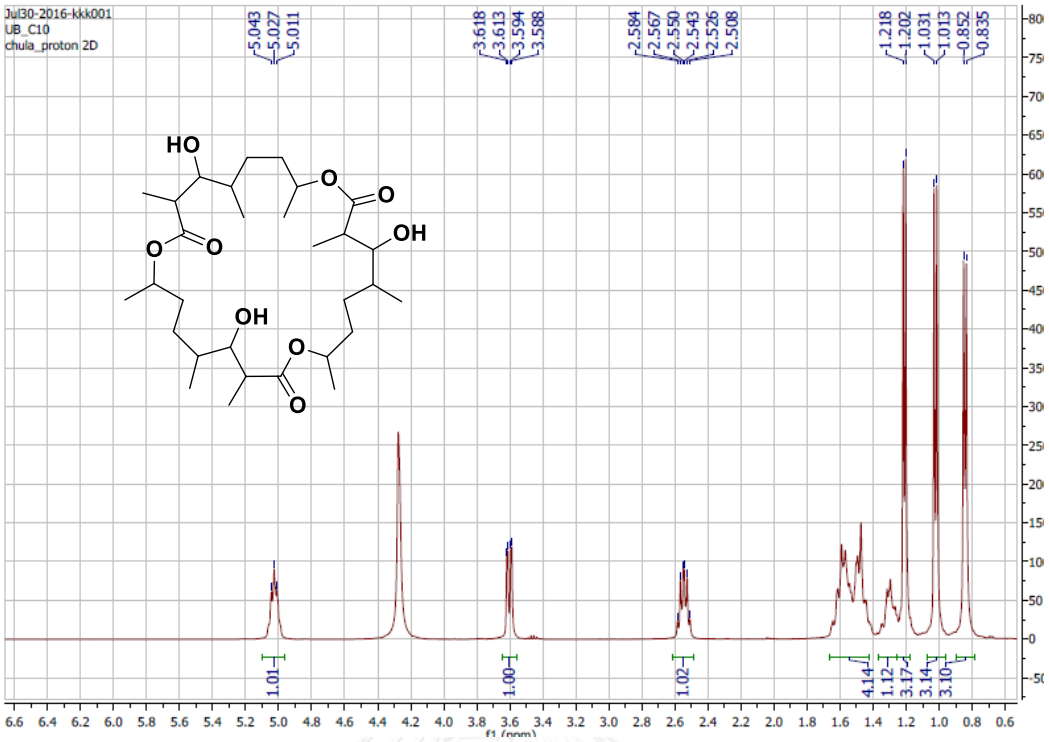

Figure A. 43 ^{1}H NMR (400 MHz) spectrum of compound **22** ($CDCl_3$)

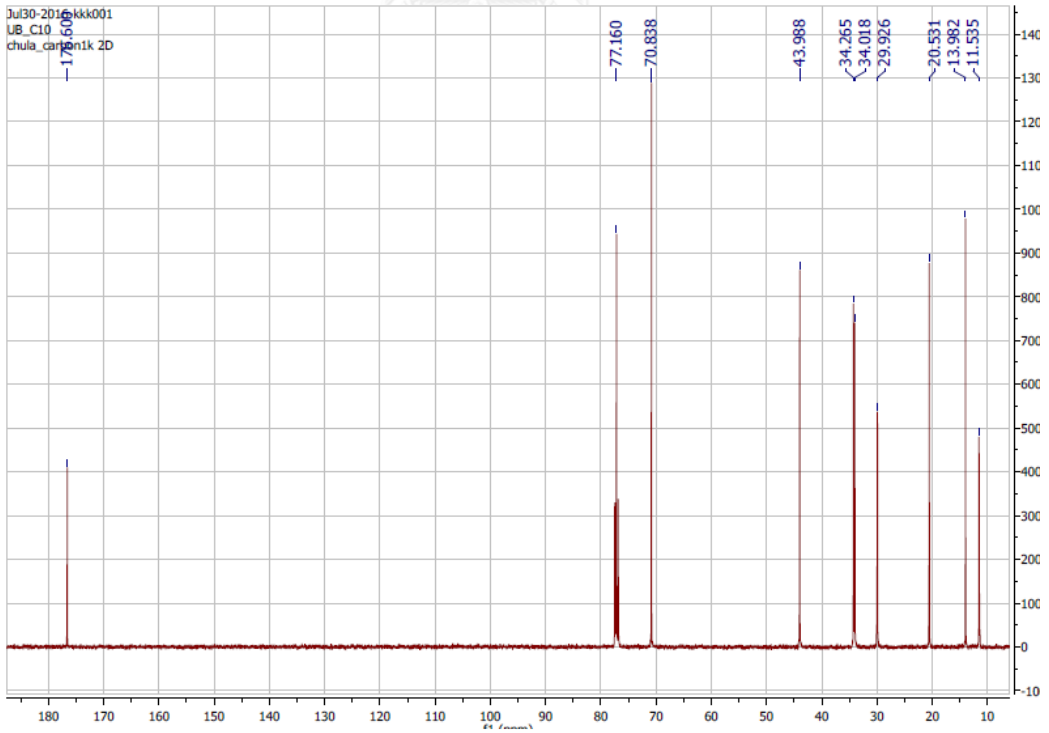

Figure A. 44 ^{13}C NMR (100 MHz) spectrum of compound **22** ($CDCl_3$)

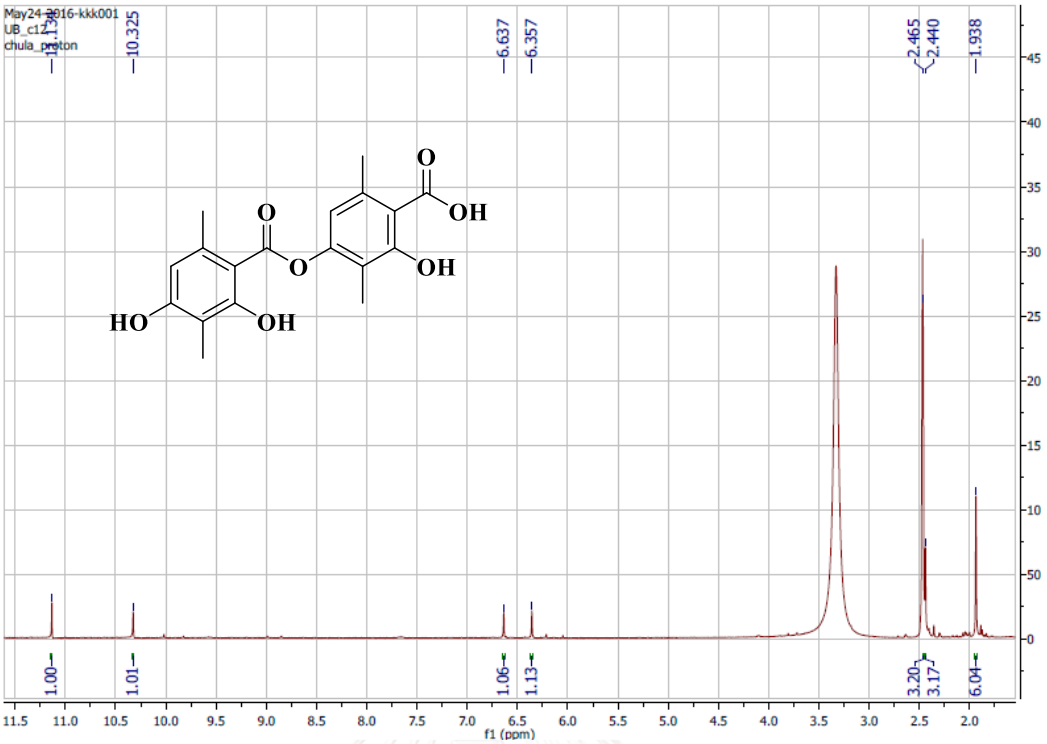

Figure A. 45 1H NMR (400 MHz) spectrum of compound **23** (DMSO-d_6)

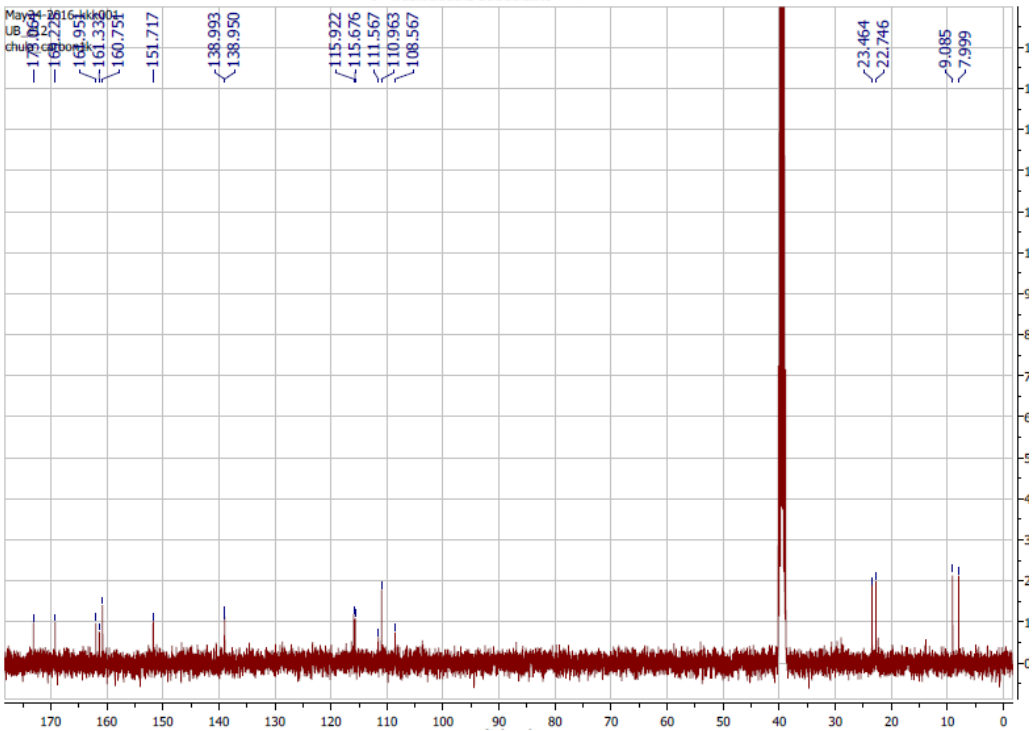

Figure A. 46 ^{13}C NMR (100 MHz) spectrum of compound **23** (DMSO-d_6)

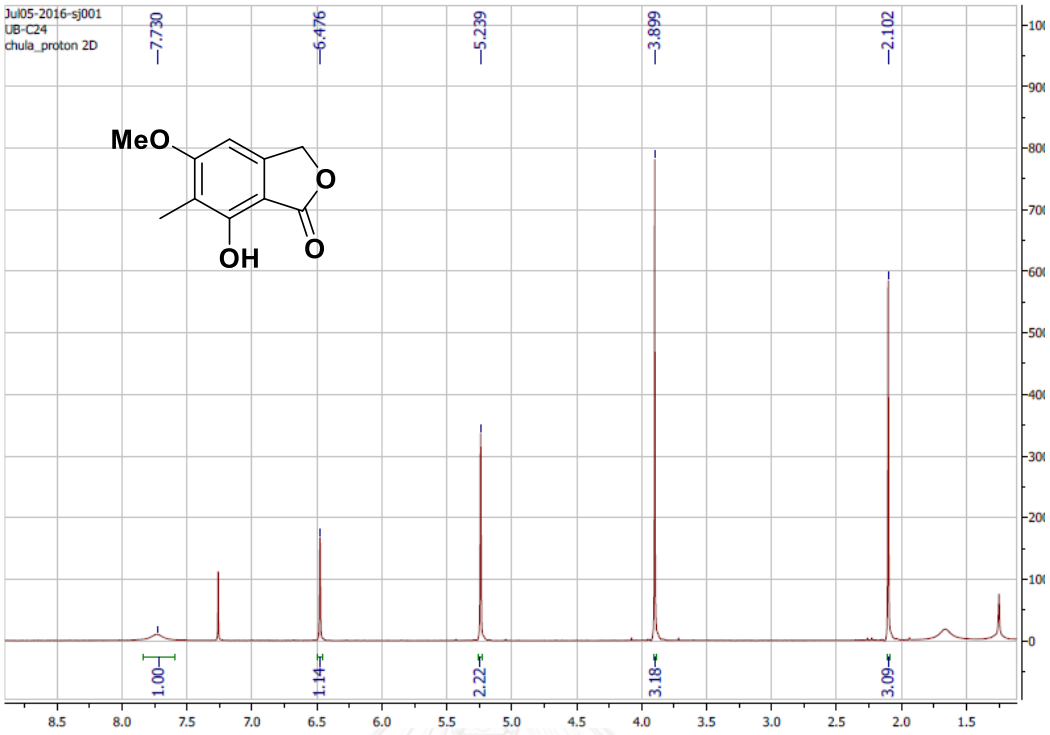

Figure A. 47 ^{1}H NMR (400 MHz) spectrum of compound **24** ($CDCl_3$)

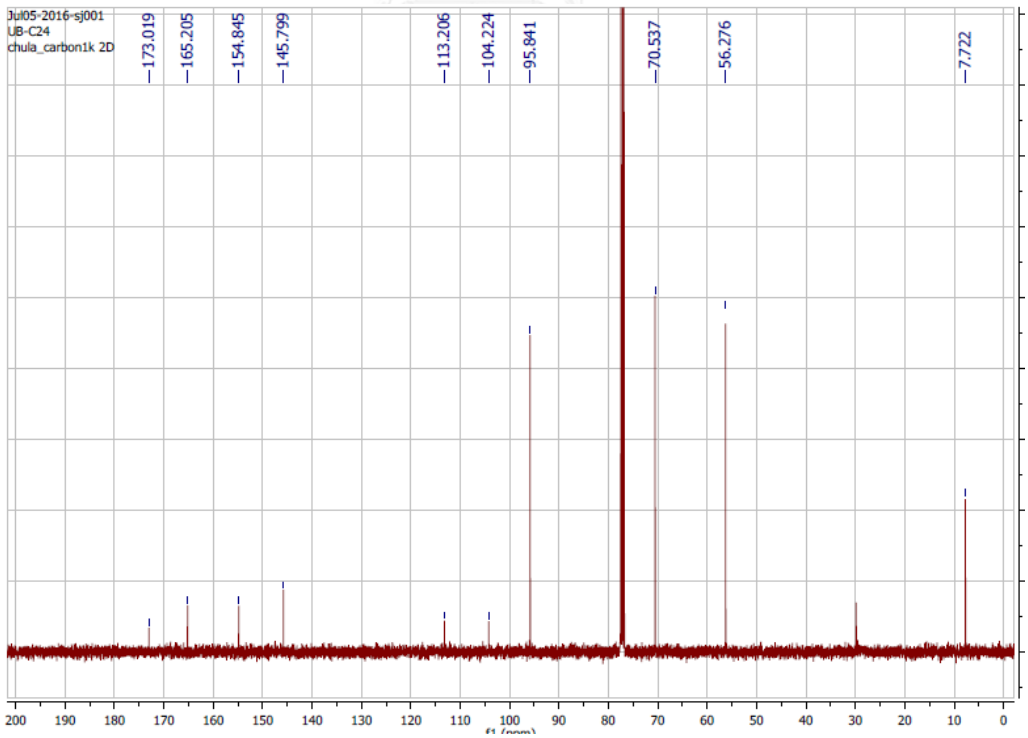

Figure A. 48 ^{13}C NMR (100 MHz) spectrum of compound **24** ($CDCl_3$)

VITA

Mr. Kieu Van Nguyen was born on October 10th, 1991 in Binh Dinh province, Vietnam. I graduated with Bachelor degree of Science in University of Science, Vietnam National University- Hochiminh City in 2013. Then, I continued my Master Degree in program of Organic Chemistry, Faculty of Science, Chulalongkorn University in 2014.

The oral presentation "Depsides and Depsidones from lichen Usnea baileyi (Stirt.) Zahlbr." Will be presented in the 42nd Congress on Science and Technology of Thailand, Central Plaza Ladprao, Bangkok, Thailand on November 29th – December 2nd, 2016.

My present address is Room 1008, CU iHouse, 268 Chulalongkorn Soi9, Charasmuang road, Wangmai, Pathumwan, Bangkok.