อนุกรมวิธานของแอคติโนแบคทีเรียจากทะเลของประเทศไทย และสารทุติยภูมิของไอโซเลตที่คัดเลือก

TAXONOMY OF THAI MARINE ACTINOBACTERIA AND SECONDARY METABOLITES OF THE SELECTED ISOLATES

คมสันต์ สุป้อง

KHOMSAN SUPONG

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
สาขาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
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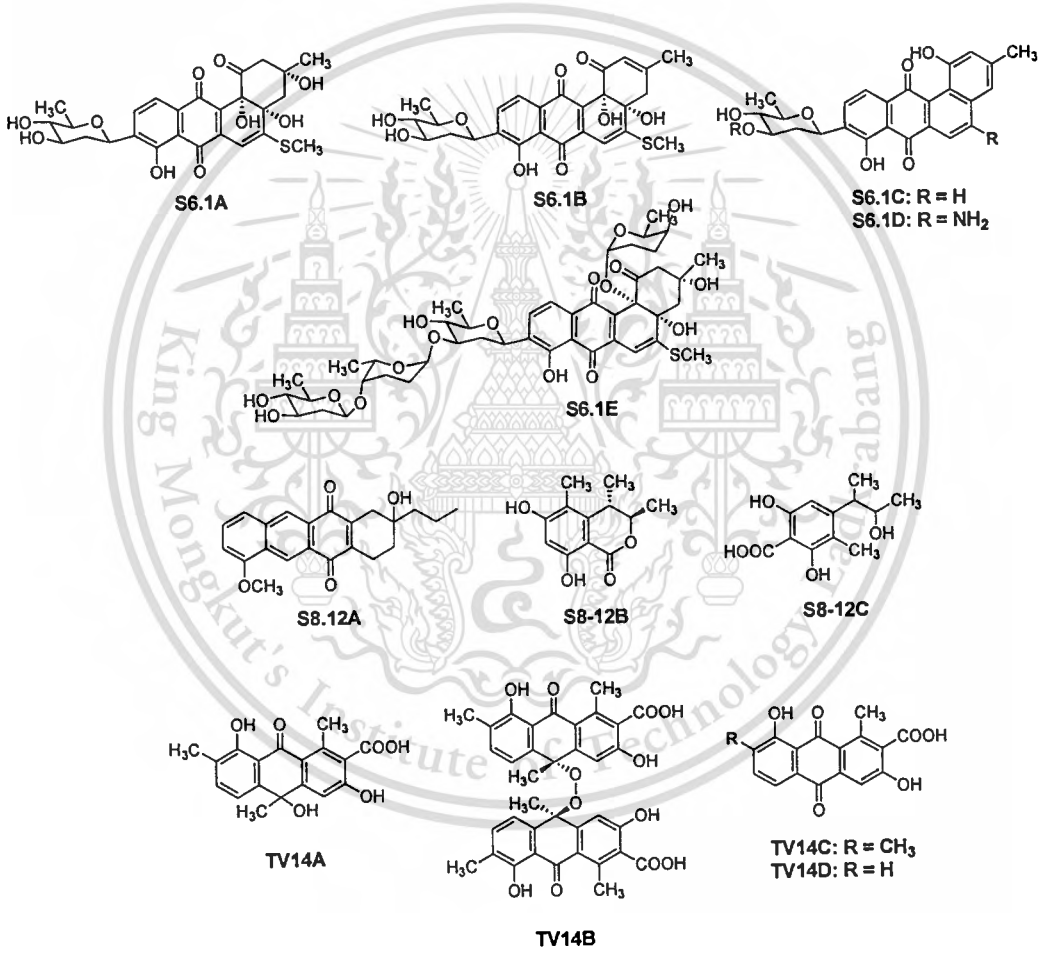
หัวข้อวิทยานิพนธ์	อนุกรมวิธานของแอคติโนแบคทีเรียจากทะเลของประเทศไทย และสารทุติยภูมิของไอโซเลตที่คัดเลือก
นักศึกษา	นายคมสันต์ สุป้อง
รหัสประจำตัว	51067202
ปริญญา	ปรัชญาดุษฎีบัณฑิต
สาขาวิชา	เทคโนโลยีชีวภาพ
พ.ศ.	2556
อาจารย์ที่ปรึกษาวิทยานิพนธ์	ผศ.ดร.จิตติ ท่าไว
อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม	ดร.ปัทมา พิทยขจรวุฒิ

บทคัดย่อ

แอคติโนแบคทีเรียจากทะเลเป็นกลุ่มแบคทีเรียที่มีความน่าสนใจเป็นอย่างมากต่อการศึกษาเพื่อหาเชื้อชนิดใหม่ รวมถึงการค้นหาสารทุติยภูมิที่มีฤทธิ์ทางชีวภาพ ในงานวิจัยนี้ได้คัดแยกแอคติโนแบคทีเรียทั้งหมด 36 สายพันธุ์ จากตะกอนดินใต้ทะเล ฟองน้ำ และสาหร่าย ที่เก็บจากทะเลฝั่งอันดามัน และฝั่งอ่าวไทยของประเทศ จากการศึกษาลักษณะทางอนุกรมวิธานของแอคติโนแบคทีเรีย พบว่าสามารถจัดจำแนกเชื้อได้เป็น 8 สกุล ได้แก่ *Micromonospora* (กลุ่ม I, 18 สายพันธุ์) *Salinispora* (กลุ่ม II, 3 สายพันธุ์) *Verrucosispora* (กลุ่ม III, 4 สายพันธุ์) *Actinomadura* (กลุ่ม IV, 1 สายพันธุ์) *Nocardia* (กลุ่ม V, 1 สายพันธุ์) *Pseudonocardia* (กลุ่ม VI, 1 สายพันธุ์) *Actinomycetospora* (กลุ่ม VII, 3 สายพันธุ์) และ *Streptomyces* (กลุ่ม VIII, 5 สายพันธุ์) นอกจากนี้ยังพบว่ามีสายพันธุ์ที่มีลักษณะทางอนุกรมวิธานที่น่าสนใจได้แก่สายพันธุ์ SH2-13^{T} และ S3-1^{T} ซึ่งจัดอยู่ในสกุล *Micromonospora* ส่วนอีกหนึ่งสายพันธุ์คือ SP03-05^{T} จัดอยู่ในสกุล *Verrucosispora* แอคติโนแบคทีเรียทั้ง 3 สายพันธุ์ มีลักษณะทางฟีโนไทป์และจีโนไทป์ที่แตกต่างจากเชื้อสปีชีส์เดิมที่เคยรายงานมาแล้ว ดังนั้นแอคติโนแบคทีเรียสายพันธุ์ SH2-13^{T} และ S3-1^{T} ถูกรายงานเป็นเชื้อสปีชีส์ใหม่คือ *Micromonospora sediminicola* SH2-13^{T} และ *Micromonospora spongicola* S3-1^{T} ส่วนสายพันธุ์ SP03-05^{T} ถูกรายงานเป็นเชื้อสปีชีส์ใหม่คือ *Verrucosispora andamanensis* SP03-05^{T}

จากการตรวจสอบเบื้องต้นโดยอาศัยฤทธิ์ทางชีวภาพ และรูปแบบของสารที่เชื้อผลิต เพื่อคัดเลือกแอคติโนแบคทีเรียที่มีความน่าสนใจในการสร้างสารทุติยภูมิ พบว่ามีเชื้อที่ถูกคัดเลือกสำหรับการวิเคราะห์องค์ประกอบทางเคมีจำนวน 3 สายพันธุ์ ได้แก่ S6-1 S8-12 และ TV1-14 สารบริสุทธิ์ที่แยกได้ทั้งหมดจากแต่ละสายพันธุ์ เมื่อนำมาวิเคราะห์ลักษณะโครงสร้างทางเคมี โดยอาศัยเทคนิคทางด้านสเปกโตรสโคปี พบว่า *Streptomyces* sp. S6-1 สร้างสารใหม่ 4 ชนิด คือ S6.1A – S6.1D และสารที่มีผู้รายงานแล้ว 1 ชนิด คือ urdamycin E (S6.1E) ซึ่งสารเหล่านี้มีฤทธิ์

ยับยั้งเซลล์มะเร็ง (KB, IC_{50} 0.179 – 33.24 μg/ml; MCF-7, IC_{50} 0.196 – 5.05 μg/ml; NCI-H187, IC_{50} 0.092 – 3.97 μg/ml) ยับยั้งเชื้อมาลาเรีย (IC_{50} 0.053 – 2.93 μg/ml) และยับยั้งเชื้อวัณโรค (MIC 3.13 –12.50 μg/ml) *Micromonospora* sp. S8-12 สร้างสารทั้งหมด 3 ชนิดได้แก่ S8.12A S8.12B และ S8.12C ซึ่งสารทั้งสามชนิดเป็นสารที่เคยมีรายงานแล้ว และยังพบว่าสาร S8.12A มีฤทธิ์เป็นพิษต่อเซลล์มะเร็ง (KB, IC_{50} 30.22 μg/ml; MCF-7, IC_{50} 30.62 μg/ml; NCI-H187, IC_{50} 42.33 μg/ml) และมีฤทธิ์ยับยั้งเชื้อมาลาเรีย ที่ IC_{50} 4.44 μg/ml *Actinomadura* sp. TV1-14 สามารถผลิตสารใหม่ 3 ชนิด ได้แก่ TV14A TV14C และ TV14D รวมถึงสารที่เคยค้นพบมาแล้วคือ oxanthromicin (TV14B) ซึ่งพบว่าสาร TV14A – TV14C มีฤทธิ์ยับยั้งการเจริญของเชื้อรา *Candida albicans* อยู่ในระดับ IC_{50} 17.18 – 29.55 μg/ml



คำสำคัญ: แอคติโนแบคทีเรียจากทะเล อนุกรมวิธาน สารทุติยภูมิ สารออกฤทธิ์ทางชีวภาพ

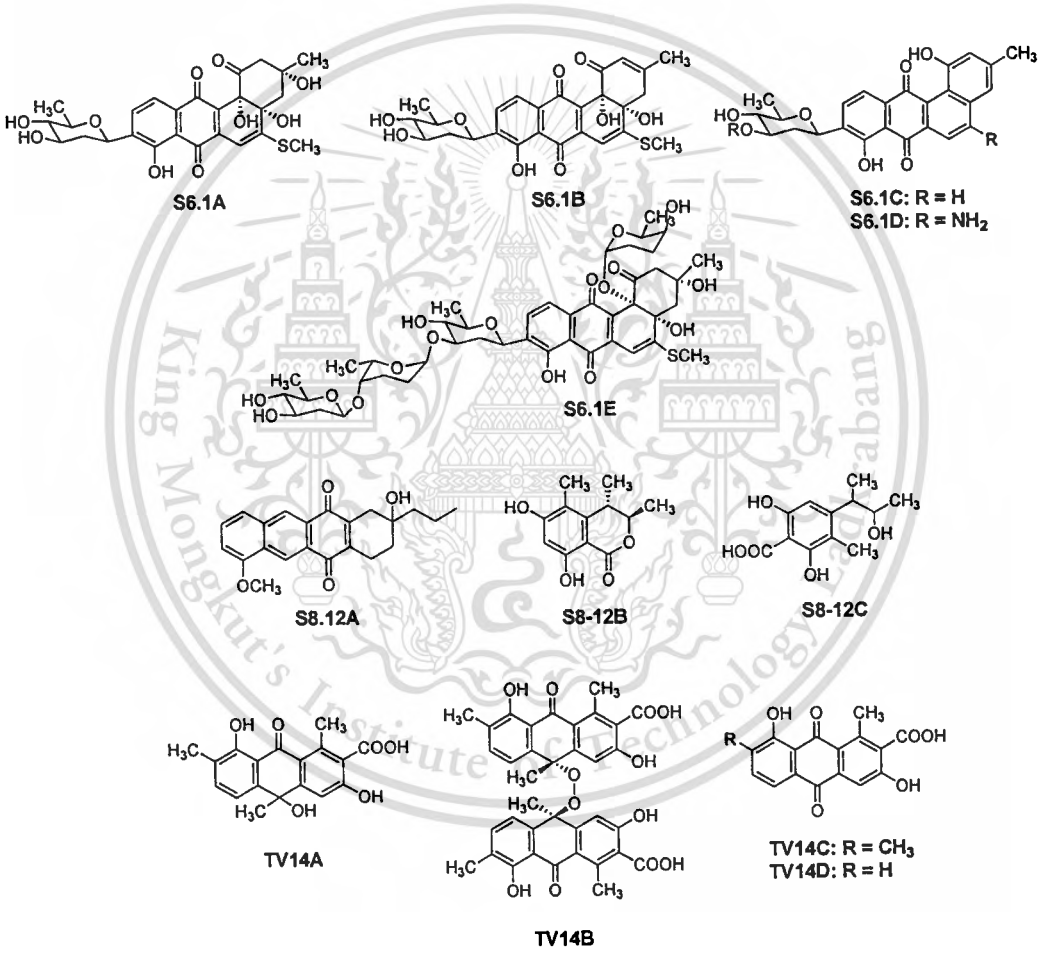
Thesis Title	Taxonomy of Thai marine actinobacteria and secondary metabolites of the selected isolates
Student	Mr. Khomsan Supong
Student ID	51067202
Degree	Ph.D.
Program	Biotechnology
Year	2013
Thesis Advisor	Asst. Prof. Dr. Chitti Thawai
Thesis Co-advisor	Dr. Pattama Pittayakhajonwut

ABSTRACT

Marine actinobacteria are of great interest in the discovery of new species and bioactive secondary metabolites. In the course of investigation for new species and secondary metabolites of Thai marine actinobacteria, a total of thirty-six marine actinobacteria were isolated from the sediment, sponge and algal samples collected at the Andaman Sea and the Gulf of Thailand. All marine actinobacterial strains were studied taxonomically using a polyphasic approach. Actinobacteria belonging to a total of eight genera were identified as *Micromonospora* (group I, 18 strains), *Salinispora* (group II, 3 strains), *Verrucosispora* (group III, 4 strains), *Actinomadura* (group IV, 1 strain), *Nocardia* (group V, 1 strain), *Pseudonocardia* (group VI, 1 strain), *Actinomycetospora* (group VII, 3 strains) and *Streptomyces* (group VIII, 5 strains). Here, we found the strains SH2-13^T, S3-1^T (Group I) belonged to the genus *Micromonospora* and SP03-05^T (Group III) belonged to the genus *Verrucosispora* were phenotypically and genotypically distinguishable from all recognized *Micromonospora* and *Verrucosispora* species. Therefore, the strains SH2-13^T and S3-1^T were proposed to represent the novel species as *Micromonospora sediminicola* and *Micromonospora spongicola*, respectively. In addition, a strain SP03-05^T was proposed to represent a novel species as *Verrucosispora andamanensis*.

The primary screening based on biological activity and chemical profiles revealed that three strains, S6-1, S8-12 and TV1-14 were selected for the study of bioactive secondary metabolites. Secondary metabolites were isolated from these selected strains and their chemical structures were determined by spectroscopic analyses. Four naturally new compounds (S6.1A-S6.1D), and a known urdamycin E (S6.1E) were isolated from *Streptomyces* sp. S6-1. Biological activities of

these compounds showed anticancer (KB, IC_{50} 0.179 – 33.24 μg/ml; MCF-7, IC_{50} 0.196 – 5.05 μg/ml; NCI-H187, IC_{50} 0.092 – 3.97 μg/ml), antimalarial (IC_{50} 0.053 – 2.93 μg/ml), and antitubercular (MIC 3.13 – 12.50 μg/ml) activities. Three known compounds (S8.12A-S8.12C) were isolated from marine *Micromonospora* sp. S8-12. Only compound S8.12A exhibited moderate cytotoxicity (KB, IC_{50} 30.22 μg/ml; MCF-7, IC_{50} 30.62 μg/ml; NCI-H187, IC_{50} 42.33 μg/ml) and antimalarial activity at IC_{50} 4.44 μg/ml. *Actinomadura* sp. TV1-14 produced three naturally new compounds (TV14A, TV14C and TV14D), and a known peroxide oxanthromicin (TV14B). These compounds (TV14A – TV14C) showed antifungal against *Candida albicans* at IC_{50} in a range of 17.18 – 29.55 μg/ml.



Keywords: Marine actinobacteria; Taxonomy; Secondary metabolites; Bioactive compounds

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LIST OF ABBREVIATIONS AND SYMBOLS

A_2pm	Diaminopimelic acid
Aceton-d_6	Deuterated acetone
Ala	Alanine
Ara	Arabnose
ATCC	American Type Culture Collection, Maryland, U.S.A
$Ba(OH)_2$	Barium hydroxide
BSA	Bovine serum albumin
°C	Degree Celsius
^{13}C-NMR	Carbon-13 nuclear magnetic resonance spectroscopy
calcd.	Calculate
CD	Circular dichroism
$CDCl_3$	Chloroform-d_1
CD_3OD	Methanol-d_4
CFU	Colony forming unit
CH_3CN	Acetonitrile
CH_3OH	Methanol
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
cm	Centimeter
cm^{-1}	Wave number unit
COSY	Correlation spectroscopy
Cz. sucrose	Czapek's sucrose
d	Doublet
dd	Doublet of doublet
DDBJ	DNA Data Bank of Japan
DEPT	Distortionless enhancement by polarization transfer
DMSO-d_6	Deuterated dimethylsulphoxide
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

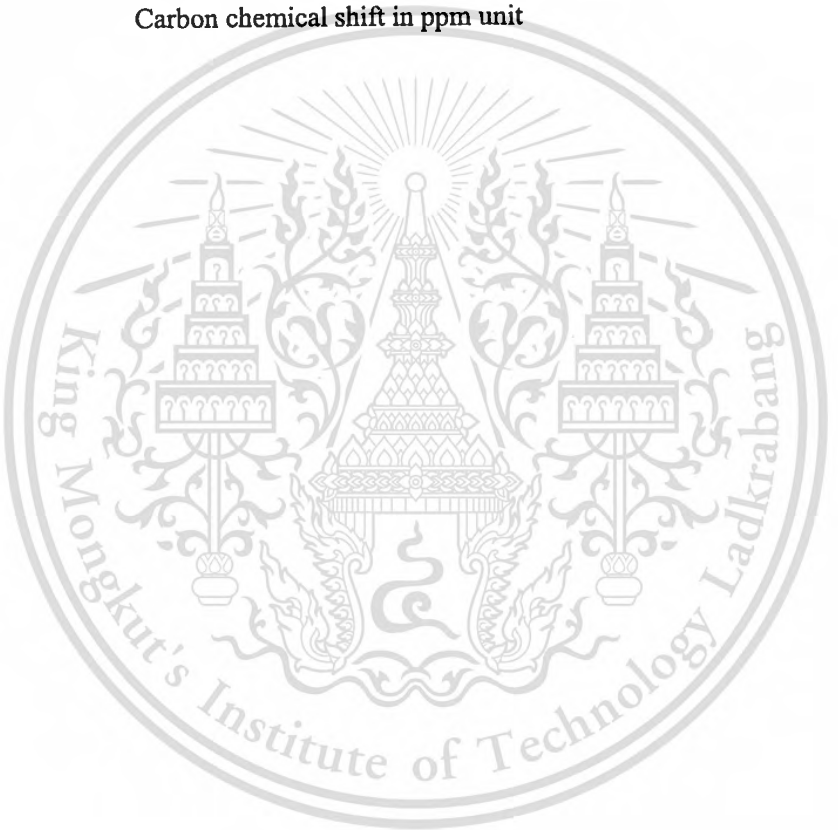
DNA	Deoxyribonucleic acid
DON	2,7-Dhydroxynapthalene
DPG	Dphosphatidylglycerol
DSM	Deutsche Sammlurg von Mikroorganismen
EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
EtOH	Ethanol
g	Gram
G+C	Guanine-plus-cytosine
GenBank	National Institute of Health genetic sequence database
h	Hour
HCOOH	Formic acid
HMBC	Heteronuclear multiple bond corelation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HRESIMS	High resolution electrospray mass spectrometry
HV	Humic acid Vitamin agar
Hz	Hertz
l	Liter
IC_{50}	Half maximal inhibitory concentration
IR	Infrared spectroscopy
ISP	International Streptomyces Project
J	Coupling constant
JCM	Japan Collection of Microorganisms
K_2HPO4	Potassium phosphate
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
LL-DAP	*LL*-Diaminopimelic acid
m	Multiplet

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

M	Molar
MeCN	Acetonitrile
MeOH	Methanol
meso-DAP	*meso*-Diaminopimelic acid
MHz	Megahertz
ml	Milliliter
MK	Menaquinone
min	Minute
mm	Millimeter
mM	Millimolar
MS	Mass spectrometry
m/z	Mass to charge ratio
N.A.	Nutrient agar
NaCl	Sodiumchloride
$NaSO_4$	Sodiumsulphate
NMR	Nuclear magnetic resonance
nm	Nanometer
nM	Nanomolar
nov.	Novel
nt	Nucleotide
ppm	Part per million
PCR	Polymerase chain reaction
s	Singlet
SDS	Sodium Dodecyl Sulfate
sp.	Species
SSC	Saline Sodium Citrate
t	Triplet
UV	Ultraviolet spectrophotometry
μg	Microgram

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

μl	Microliter
μm	Micrometer
ν	Wave number
λ_{max}	Maximum absorption wave length
$[\alpha]_D$	Specific rotation
δ_H	Proton chemical shift in ppm unit
δ_C	Carbon chemical shift in ppm unit



CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problems

Actinobacteria are a group of prokaryotic organisms which are Gram-stain-positive, filamentous bacteria with high G+C ratio of their DNA, and produce asexual spore on aerial or substrate mycelia. These bacteria are primarily saprophyte and abundant in terrestrial soil. In addition, actinobacteria can also distributed in various conditions such as soil, aquatic, plant tissue, marine environments, etc.

Due to 70 % of the earth surface covered by the ocean, it therefore contains an exceptional biological diversity which is a source of marine microorganisms. Discovery of new actinobacteria from different environments that terrestrial source is of a great challenge. However, the isolation and distribution of marine actinobacteria have been described since 1969 (Weyland, 1969), which was believed that they were no different from their terrestrial habitats. Recent evidences suggested that the marine environment is a virtually untapped source of novel actinobacteria compared to the terrestrial environment. A number of researchers have concentrated on isolation and identification of marine actinobacteria from various marine environments. Distribution of marine actinobacteria has been associated with their marine ecology including beach sand, seawater, sediments, seaweed and sponges (Bull and Stach, 2007; Goodfellow and Fiedler, 2010). Up to date, the marine actinobacteria have widely been isolated from sediment and sponge samples (Hames-Kocabas and Uzel, 2012), novel species of *Streptomyces* and *Micromonospora* (Gontang *et al.*, 2007), as well as the novel genera such as *Salinispora*, *Marinispora*, *Marinactinospora, Sciscionella*, *Spinactinospora* and *Solwaraspora* (Maldonado *et al.*, 2005; Kwon *et al.,* 2006; Tian *et al.,* 2009; Chang *et al.*, 2011; Fenical and Jensen, 2006) have been identified. At present, a number of rare actinobacteria from marine sources are still low and it has been difficult to isolate diverse *Streptomyces* spp. and rare actinobacteria from marine conditions. The cultivation methods for marine actinobacteria are needed to be studied (Berdy, 2005). In addition, rare actinobacteria were proved to be difficult to isolate and cultivate, different selection methods and selective isolation media have been reported (Qiu *et al.,* 2008; Hames-Kocabas and Uzel, 2012). The marine actinobacteria that required

seawater was only genus *Salinispora* (Mincer *et al.*, 2002) and it was later classified as obligate marine actinobacteria.

Secondary metabolites isolated from marine actinobacteria are a very important aspect in searching for novel bioactive compounds. Marine actinobacteria have been known as the antibiotic producers which obtained many types of secondary metabolites (Solanki *et al.*, 2008). More than 270 new compounds have been isolated from marine actinobacteria with diverse biological activities (Blunt *et al.*, 2011). The discovery of novel secondary metabolites led to the use in human medicine to fight numerous diseases caused by bacteria, fungi, virus, cancer and immune system disorders (Baltz, 2007; Demain and Sanchez, 2009; Kekuda *et al.*, 2010; Naine *et al.*, 2011; Newman and Cragg, 2007). Many genera of marine actinobacteria have shown to be secondary metabolite producers such as *Streptomyces*, *Nocardia*, *Micromonospora*, *Actinomadura*, *Verrucosispora*, *Saccharopolyspora*, *Marinactinospora*, *Actinoalloteichus* and *Nocardiopsis*. In addition, the obligate marine actinobacteria, *Salinispora* strains, have been reported as a source of structurally diverse secondary metabolites. Members of this genus were found to produce unique compounds, such as salinosporamides from *Salinispora tropica* which are now in clinical trials as potent anticancer agents (Feling *et al.*, 2003; Jensen *et al.*, 2007). The antibacterial agent abyssomicin C was isolated from *Verrucosispora* sp. In addition, the anticancer agents lodopyridone and proximicin isolated from *Saccharomonospora* sp. and *Verrucosispora* sp., respectively (Maloney *et al.*, 2009; Fiedler *et al.*, 2008; Schneider *et al.*, 2008).

Compared to the terrestrial form, the genetic and metabolic diversity of marine actinobacteria showed that the marine environment can be considered as a unique source for the research of novel actinobacteria and secondary metabolites (Mincer *et al.*, 2002; Stach *et al.*, 2003; Maldonado *et al.*, 2005; Pathom-aree *et al.*, 2006). The Andaman Sea and the Gulf of Thailand have rarely been explored for marine actinobacteria in term of a taxonomic study and the secondary metabolites. Therefore, the investigation of marine actinobacteria at the Andaman Sea and the Gulf of Thailand, have been initiated. This tstudy involves the taxonomic study of marine actinobacteria collected from the Andaman Sea and the Gulf of Thailand, and isolation of bioactive compounds from the selected marine actinobacteria. Hence, there is an immense possibility to identify novel marine actinobacteria in the Andaman Sea and the Gulf of Thailand and to discover new bioactive compounds.

1.2 Goal and objectives

1.2.1 To isolate and screen actinobacteria from the marine sources of Thailand.

1.2.2 To identify and characterize the selected marine actinobacterial strains.

1.2.3 To evaluate biological activities and chemical profiles of the crude extracts from marine actinobacterial strains.

1.2.4 To elucidate chemical structures and biological activities of the isolated secondary metabolites.

CHAPTER II

LITERATURE REVIEW

Actinobacteria are Gram-stain-positive or Gram-stain-variable, generally aerobic bacteria and some facultative anaerobes or anaerobes. They have a rigid cell wall that contains muramic acid and some contain wall teichoic acid. Some groups of actinobacteria have a formation of branching filaments that comprise a group of filamentous bacteria. They also form asexual spores such as conidiospore, sporangiospore, and a fragment of hypha (arthrospore), developed on the substrate or aerial mycelium. Filamentous actinobacteria are originally considered as an intermediate group between bacteria and fungi, and they are distinct from fungi by having no cell nucleus and smaller single hypha (0.4 to 1.2 μm in diameter) than of fungi (3 to 8 μm in diameter). Moreover, DNA G+C content ranges from 50 mol% to over 70 mol%. Most of actinobacteria are chemo-organotrophs which grow at neutral pH. They are mainly diverse into two groups, which are streptomycetes and the non-streptomycetes (rare actinobacteria).

2.1 Distribution and isolation of marine actinobacteria

Marine ecosystems contain several unique features that different from other aquatic ecosystems. The main factor is the presence of dissolved components in seawater, particularly sodium chloride (NaCl). The cellular adaptation to high salt content is a fundamental biological process for growth of marine microorganisms. Therefore, the marine environment is a potential source for new secondary metabolites producer.

2.1.1 Distribution of marine actinobacteria

Since oceans cover 70% of the earth surface, there will be many diverse living microorganisms to be discovered. Microorganisms growing in marine environment must have metabolically and physiologically different from terrestrial environment. Marine environment is considered as a source of various microorganisms including fungi, algae, bacteria and filamentous bacteria or actinobacteria. In 1969 (Weyland) the actinobacteria discovered from the sea were officially reported. Actinobacteria are well known as soil bacteria and generally believed to occur in the ocean largely as dormant spores that are washed into the sea (Goodfellow and Haynes, 1984). The distributions of actinobacteria in the sea remain largely undescribed, and even today,

conclusive evidence suggested that these bacteria, which played important role in the marine environment, remained elusive.

Marine actinobacteria are distributed throughout the marine environment from shallow to deep sea, which can be found in seawater (Ramesh *et al.*, 2006), plants (Castillo *et al.*, 2005), animals (Ramesh and Mathivanan, 2009), sponges (Zhang *et al.*, 2006; Zhang *et al.*, 2012; Pimentel-Elardo *et al.*, 2008; Schneemann *et al.*, 2010) and marine sediments (Sabry *et al.*, 2004; Jensen *et al.*, 2005; Das *et al* 2008; Thornburg *et al.*, 2010; Xiao *et al.*, 2011; Fang *et al.*, 2011; Chang *et al.*, 2011). In recent years, numbers of novel actinobacteria have been mostly isolated from marine sediments and sponges. These included novel species of *Streptomyces* and *Micromonospora* are mostly (Gontang *et al.*, 2007; Maldonado *et al.*, 2008), and other rare actinobacteria such as *Actinoalloteichus* (Zhang *et al.*, 2006), *Actinomadura* (He *et al.*, 2012), *Marinactinospora* (Tian *et al.*, 2009), *Nocardiopsis* (Sabry *et al.*, 2004; Chen *et al.*, 2009; Fang *et al.*, 2011), *Saccharopolyspora* (Pimentel-Elardo *et al.*, 2008), *Salinispora* (Maldonado *et al.* 2005), *Solwaraspora* (Mincer *et al.*, 2002), *Spinactinospora* (Chang *et al.*, 2011) and *Verrucosispora* (Dai *et al.*, 2010; Goodfellow *et al.*, 2012), as shown in Table 2.1.

A culture-based evaluation of Gram-stain-positive bacteria in marine sediments from the Republic of Palau showed that 65% of the isolates belonged to the class *Actinobacteria*, and many isolates were identified to the new species (Gontang *et al.* 2007). In addition, Maldonado *et al.* (2009) recently isolated a diverse array of actinobacteria from marine sediments collected in Mexico. On the other hand, marine actinobacteria are common members of sponge-associated microbial communities and have been implicated as the producers of bioactive metabolites that were originally attributed to the host sponge (e.g., Hill *et al.* 2003; Jiang *et al.* 2008; Kim *et al.* 2006).

Table 2.1 Actinobacterial genera distributed in different marine ecology

Actinobacterial genera	Species affiliation	Sources/Locations	References
Actinoalloteichus	*A. hymeniacidonis*	Marine sponge from Dalian, on the Chinese Yellow Sea	Zhang *et al.*, 2006
Actinomadura	*A. formosans,*	Japan Trench, Canary Basin,	Maldonado *et al.*, 2005
	A. fulvescens	fjordsite	Jensen *et al.*, 2005
Amycolatopsis	*Amycolatopsis* sp.	Deep sea sediment (3800 m)	Stach *et al.*, 2003
Frankia	*Frankia sp.*	Deep sea sediment (3800 m)	Stach et al., 2003
Kitasatospora	*Kitasatospora sp.*	Deep sea sediment (3800 m)	Stach et al., 2003
Micromonospora	*M. rhodorangea,*	Sediment Papu New Guinea	Magarvey et al., 2004
	M. halophytica		

Table 2.1 Actinobacterial genera distributed in different marine ecology (continued)

Actinobacterial genera	Species affiliation	Sources/Locations	References
Marinactinospora	*M. thermotolerans*	Sediment in the northern South China Sea	Tian et al., 2009
Nocardioides	*N. jensenii*	Barcelona neuston	Agogue et al., 2005
Nocardiopsis	*N. dassonvillei*	Ovaries of Pufferfish, Bohai Sea of China	Maldonado *et al.*, 2005 Wu *et al.*, 2005
Nonomuraea	*Nonomuraea* sp.	Japan Trench, Canary Basin, fjordsite	Maldonado *et al.*, 2005
Pseudonocardia	*P. alaniniphila,* *P. aurantiaca, P. alnii* *P. antarctica*	Deep sea sediment (3800 m) McMurdo Dry Valleys region of Antarctica	Maldonado *et al.*, 2005 Stach *et al.*, 2003 Prabahar *et al.*, 2004
Saccharopolyspora	*S. cebuensis*	Marine sponge from Cebu, Philippines	Pimentel-Elardo *et al.*, 2008
Salinispora	*S. arenicola, S. tropica*	Sub-tropical sediment	Maldonado *et al.* 2005
Sciscionella	*S. marina*	Sediment in the northern South China Sea	Tian *et al.*, 2009
Spinactinospora	*S. lkalitolerans*	Sediment from the Yellow Sea Cold Water Mass, China	Chang *et al.*, 2011
Streptomyces	*S. xishensis, S. abyssalis* *S. pharmamarensis*	Sediment from the Xisha Island, China Sediment, Mediterranean Sea	Xu *et al.*, 2012 Carro *et al.*, 2012
Streptosporangium	*Streptosporangium* sp.	Japan Trench, Canary Basin, fjord site	Maldonado *et al.*, 2005
Thermoactinomyces	*Thermoactinomyces* sp.	Sea mud at Mecherchar in Republic of Palau (North Pacific Ocean)	Kanoh *et al.*, 2005
Verrucosispora	*V. sediminis* *V. maris*	Deep-sea sediments, South China Sea. Sediment, the Sea of Japan	Dai *et al.*, 2010 Goodfellow *et al.*, 2012

2.1.2 Isolation of marine actinobacteria

Goodfellow and Haynes (1984) reviewed that the source of marine sediment may be valuable for the isolation of novel actinobacteria with a potential to yield useful new products. Sediments and sponges are of the most studied marine samples for marine actinobacterial isolation, the commonly used pre-treatment methods for screening and isolation of marine actinobacteria were shown in Table 2.2. In addition, the novel method for selective enrichment procedure called the cellulose and/or agar-based procedure for isolation of the slow-growing marine actinobacteria from sediment samples was described by Magavey *et al.* (2004), and the method was used to cultivate the obligate marine actinobacteria such as *Salinispora* species.

Table 2.2 Different pre-treatment methods for the selective isolation of marine actinobacteria

Pre-treatment	Sample type	Reference
Mechanic		
Shake with glass beads for 1 h in blood tube rotator	Sediment	Maldonado *et al.*, 2009
Physical		
Wet		
Wet samples diluted in sterile water	Sediment	Bredholdt *et al.*, 2007
Dry		
Dry in laminar flow hood, stamping	Sediment	Mincer *et al.*, 2002; Jensen *et al.*, 2005; Gontang *et al.*, 2007
Dry in laminar flow hood, dilution	Sediment and sponge	Jensen *et al.*, 2005
Heat		
Dilution, incubation in water bath (50 °C, 60 min)	Sediment	Jensen *et al.*, 1991
Incubation at 55 °C, 6 min	Sediment	Mincer *et al.*, 2005
Dilution, incubation in water bath (55 °C, 6 min)	Sponge	Kim *et al.*, 2005
Dilution, incubation at 55 °C for 6 min	Sediment	Jensen *et al.*, 2005
Dilution, incubation at 55 °C for 6 min and at 60 °C 10 min	Sediment	Jensen *et al.*, 2005
Dried sample (speed vac. 30 °C, 16 h), dry heat (120 °C, 60 min)	Sediment	Bredholdt *et al.*, 2008
Dilution, incubation at 40 °C for 60 min	Sponge	Selvin *et al.*, 2009
Freeze		
Freeze (-20 °C, 24 h), thawed, dilution	Sediment	Jensen *et al.*, 2005
Freeze (-20 °C, 24 h), dilution, incubation at room temperature for 48 h	Sediment	Jensen *et al.*, 2005
Freeze at -18 °C	Sediment	Bredholdt *et al.*, 2007
Radiation		
UV irradiation for 30 s (distance 20 cm, 54 nm, 15 W	Sediment	Bredholdt *et al.*, 2007
Super high frequency radiation in microwave oven for 45 s (2460 MHz, 80 W)	Sediment	Bredholdt *et al.*, 2007
Extremely high frequency radiation (1 kHz within wavelength band of 8–11.5 mm)	Sediment	Bredholdt *et al.*, 2007
Heat and radiation		
Air dried (room temperature for 14 day), dry heat (55 °C, 30 min), microwave irradiation (80W, 30 s)	Sediment	Eccleston *et al.*, 2008
Centrifugation		
Dispersion and differential centrifugation	Sediment	Maldonado *et al*, 2005
Physical and/or chemical		
Phenol (1.5%, 30 min at 30 °C) for dried sample (speed vac. 30 °C, 16 h)	Sediment	Bredholdt *et al.*, 2008
Dry heat (120 °C, 60 min) and phenol (1.5%, 30 min at 30 °C) for dried sample (speed vac. 30 °C, 16 h)	Sediment	Bredholdt *et al.*, 2008
Dry heat (120 °C, 60 min) and benzethoniumchloride (0.02%, 30 min at 30 °C) for dried sample (speedvac 30 °C, 16 h)	Sediment	Bredholdt *et al.*, 2008

Isolation of obligate marine actinobacteria, mostly actinobacteria such as member of the genus *Salinispora* was isolated by specific marine nutrient that consisted of sodium chloride (3% NaCl) (Maldonado *et al.,* 2005). On addition, isolation media containing different concentrations of seawater, artificial seawater or deionized water added with 3% NaCl were successfully used for cultivation of marine actinobacteria.

Nitrogen sources including peptone, yeast extract, casein, nitrate, and amino acid, and carbon sources, such as starch, monosaccharide and glycerol, were used for isolation and cultivation of marine actinobacteria. In the other hand, conventional carbon and nitrogen sources including sponge extract, sediment extract, algal extract, and natural sea water were used as the supplements to mimic natural environmental conditions for the selection of the rare marine actinobacteria (Table 2.3). For the isolation of obligate marine actinobacteria from different marine sources including sediment, sponges, algae, and other marine organisms using low nutrient media, were proved to be better than nutrient rich media (Olson *et al.,* 2000; Cho and Giovannoni, 2004; Jensen *et al.,* 2005; Gantang *et al.,* 2007).

Table 2.3 The media which were found successful among several other media for the isolation of marine actinobacteria.

Medium	Formula	Sample	References
M1	Starch,10 g; yeast extract, 4 g; peptone, 2 g; agar, 18 g and 1l of natural seawater	Sponge	Zhang *et al.*, 2006; Abdelmohsen *et al.*, 2010
M2	Glycerol (100%), 6 ml; arginine, 1 g; K_2HPO_4, 1 g; $MgSO_4$, 0.5 g; agar, 18 g and 1l of natural seawater	Sponge	Zhang *et al.*, 2006
M3	K_2HPO_4, 0.466 g; Na_2HPO_4, 0,732 g; KNO_3, 0.10 g; NaCl, 0.29 g; $MgSO_4.7H_2O$, 0.10 g; $CaCO_3$, 0.02 g; sodium propionate, 0.20 g; $FeSO_4.7H_2O$, 200 µg; $ZnSO_4.7H_2O$, 180 µg; $MnSO_4.4H_2O$, 20 µg; agar, 18 g; thiamin-HCl, 4.0 mg, pH 7.0	Sediment	Maldonado *et al.*, 2005
M4	Chitin, 2 g; agar, 18 g and 1l of natural seawater	Sediment	Mincer *et al.*, 2002
M5	Agar,18 g and 1l of natural seawater	Sediment	Mincer *et al.*, 2002
Marine sponge agar (MSA)	Raffinose,10 g; L-histidine, 1 g; ferrous sulphate, 0.01 g; K_2HPO_4, 1 g; $CaCO_3$, 0.5 g; NaCl, 20 g; aqueous host sponge extract, 100 ml; agar, 15 g; double distilled water, 900 ml, pH 7.8	Sponge	Gandhimathi *et al.*, 2008
Medium 2 (NPS)	Noble agar, 8 g; NPS (nutrient poor sediment) extract, 100 ml. NPS extract was prepared by washing (extracting) 900 ml (wet volume) of sand collected from a high-energy beach with 500 ml of sea water.	Sediment	Jensen *et al.*, 2005
Medium 3 (NRS)	Noble agar, 8 g; NRS (nutrient rich sediment) extract, 100 ml. NRS extract was prepared as above using 300 ml (wet volume) of sediment collected at low tide from a mangrove channel.	Sediment	Jensen *et al.*, 2005
Medium 6 (SMP)	Noble agar, 8 g; mannitol, 500 mg; peptone, 100 mg and 1 l of natural seawater	Sediment	Jensen *et al.*, 2005
NaST21Cx agar	Solution A (750 ml of artificial seawater containing K_2HPO_4, 1 g and Bacto Agar, 10 g) and solution B 250 ml of artificial seawater containing KNO_3, 1 g; $MgSO_4$, 1 g; $CaCl_2.2H_2O$, 1 g; $FeCl_3$, 0.2 g and $MnSO_4.7H_2O$, 0.1 g). Solutions A and B are autoclaved separately and mixed and supplemented with 1 ml of trace element solution	Sediment	Magarvey *et al.*, 2004

Table 2.3 The media which were found successful among several other media for the isolation of marine actinobacteria (continued).

Medium	Formula	Sample	References
Raffinose histidine agar (RH)	Raffinose10 g; L-histidine1 g; K_2HPO_4,1 g; $MgSO_4.5H_2O$, 0.5 g; $FeSO_4$, 0.01 g; agar 15 g pH 7.2	Sediment	Maldonado *et al.*, 2009
Sponge agar (SA)	Raffinose, 10 g; L-histidine, 1 g; $FeSO_4.7H_2O$, 0.01 g; K_2HPO_4, 1 g; $CaCO_3$, 0.02 g; $MgSO_4.7H_2O$, 0.5 g; agar, 15 g; NaCl, 20 g and 10% aqueous or organic sponge extract. Aqueous extract of sponge tissue was prepared in phosphate buffered saline (PBS) and was filter sterilized. Organic extract was prepared from the sponge tissue by consecutive extractions with hexane and dichloromethane and methanol (1:1). The combined extract was concentrated in a rotary vacuum evaporator at 40 °C	Sponge	Selvin *et al.*, 2004
Starch-yeast extract-peptone–seawater (SYP-SW) agar	Starch, 10 g; yeast extract, 4 g; peptone, 2 g; artificial seasalts, 33.3 g; agar, 15 g	Sponge	Kennedy *et al.*, 2009

2.2 Taxonomy of actinobacteria

Taxonomy is the science of biological identification and classification. Major characteristics used in taxonomy for the classification and identification of actinobacteria are morphological, physiological, chemotaxonomic and molecular characteristics.

2.2.1 History of actinobacteria taxonomy

In 1918, Buchanan described the order *Actinomycetales* containing a family *Actinomycetaceae*, which comprised of four genera such as *Actinobacillus*, *Leptotrichia*, *Actinomyces* and *Nocardia*. Orskov (1923) classified actinobacteria on the basis of phenotypic characteristics into three large groups that consisted of *Actinomyces, Rhodococcus* and *Nocardia*. In 1943, Waksman and Henrici proposed three families comprised *Microbacteriaceae*, *Actinomycetaceae* and *Streptomycetaceae*. Later, six major groups were recognized and eighteen genera of aerobic actinobacteria on the basis of morphology and chemotaxonomy by Lechavaliers (1970), who introduced chemotaxonomic methods. The initial hierarchical classification system of the *Actinobacteria* by Stackebrandt *et al.*, 1997 embraced 95 genera, belonging to 30 families and 10 suborders. In 2001, Ludwig and Klenk proposed order *Actinomycetales*, class *Actinobacteria*, which constitutes one of the main phyla within domain *Bacteria*, and 219 genera in 48 families accommodated by Zhi *et al.* (2009) in the class *Actinobacteria*, phylum *Actinobacteria*, as shown in Fig. 2.1.

To date, the phylum *Actinobacteria* is well supported by analyses of the 16S and 23S rRNA genes, which contain insertions and deletions in certain proteins, and characteristic gene rearrangements (as reviewed by Goodfellow and Fiedler, 2010). The class *Actinobacteria* now excludes the subclasses *Acidimicrobidae, Coriobacteridae, Nitriliruptoridae*, and *Rubrobacteridae*, with the elevation of these subclasses to classes. In addition, the elevation of suborders to orders, the order *Actinomycetales* is now restricted to member of the family *Actinomycetaceae* and many suborders previously established in the literatures such as *Micrococcineae* and *Pseudonocardineae* are revoked. Nevertheless, the possible confusion that might result from these changes is out-weighed by the advantages of a simpler classification, which resemble to those found in other prokaryotic phyla.

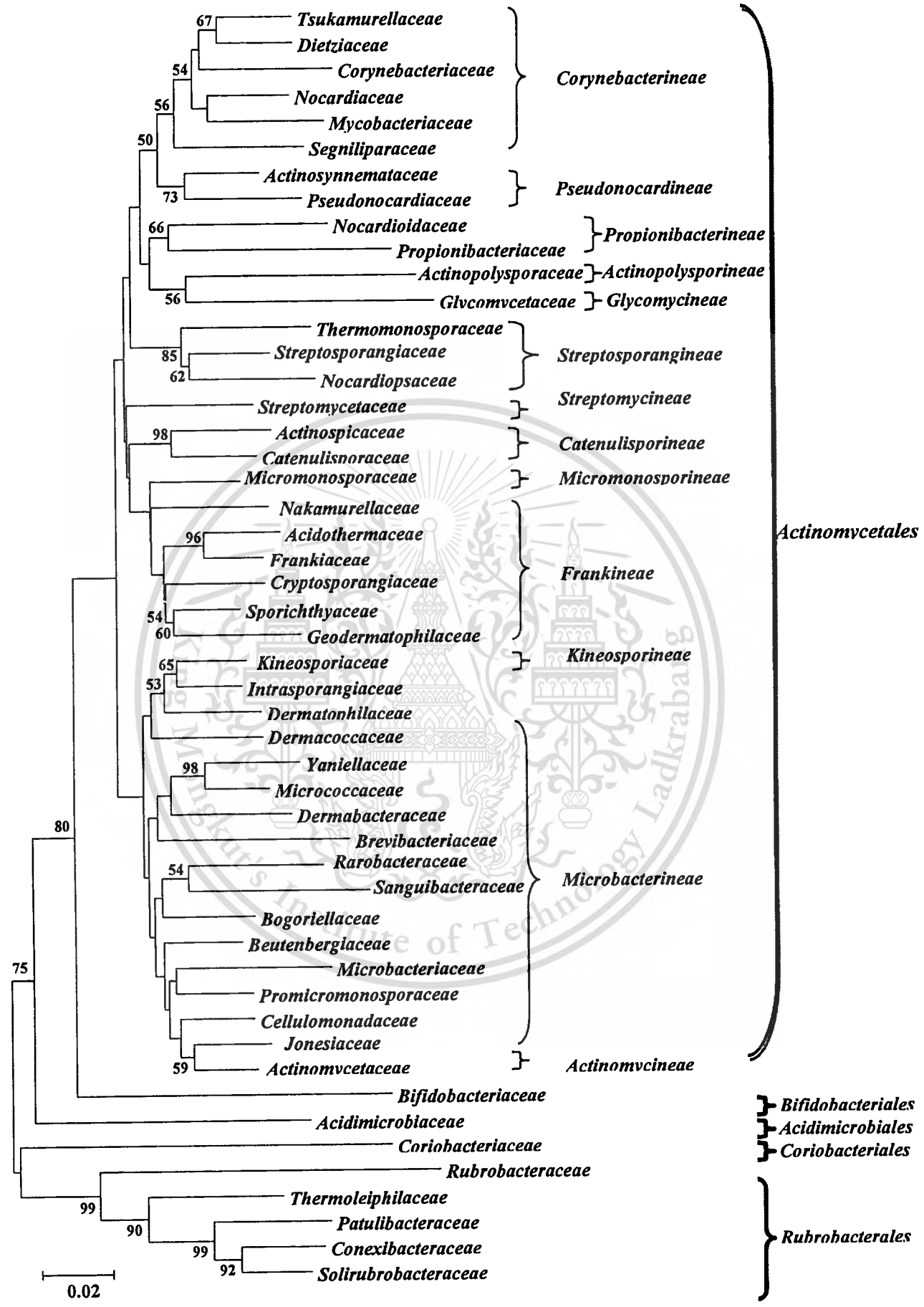


Fig. 2.1 Intraclass relateness of the class *Actinobacteria* showing the presence of 5 orders based on 16S rRNA gene sequence.

With the class *Actinobacteria* defined in this manner, six classes are proposed as shown in Fig 2.2. In addition, the class *Actinobacteria* is now restricted to the clades formerly classified within the subclass *Actinobacteridae*, and the class *Acidimicrobidiia*, *Coriobacteria*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia* are proposed. The order of class *Actinobacteria* is shown in Fig. 2.3.

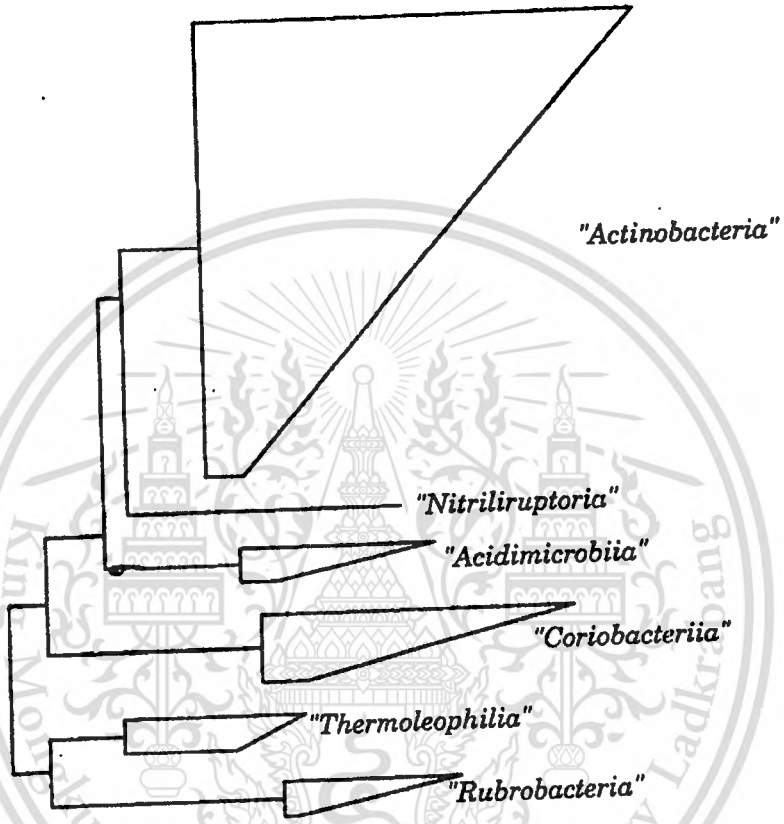


Fig. 2.2 Overview of the classes within the phylum *Actinobacteria*. Consensus dendrogram of the phylogenetic relationships of the 16S rRNA gene was based on various maximum-likelihood and maximum-parsimony analyses and corrected according to the results obtained when applying alternative treeing methods (Whitman *et al.*, 2012).

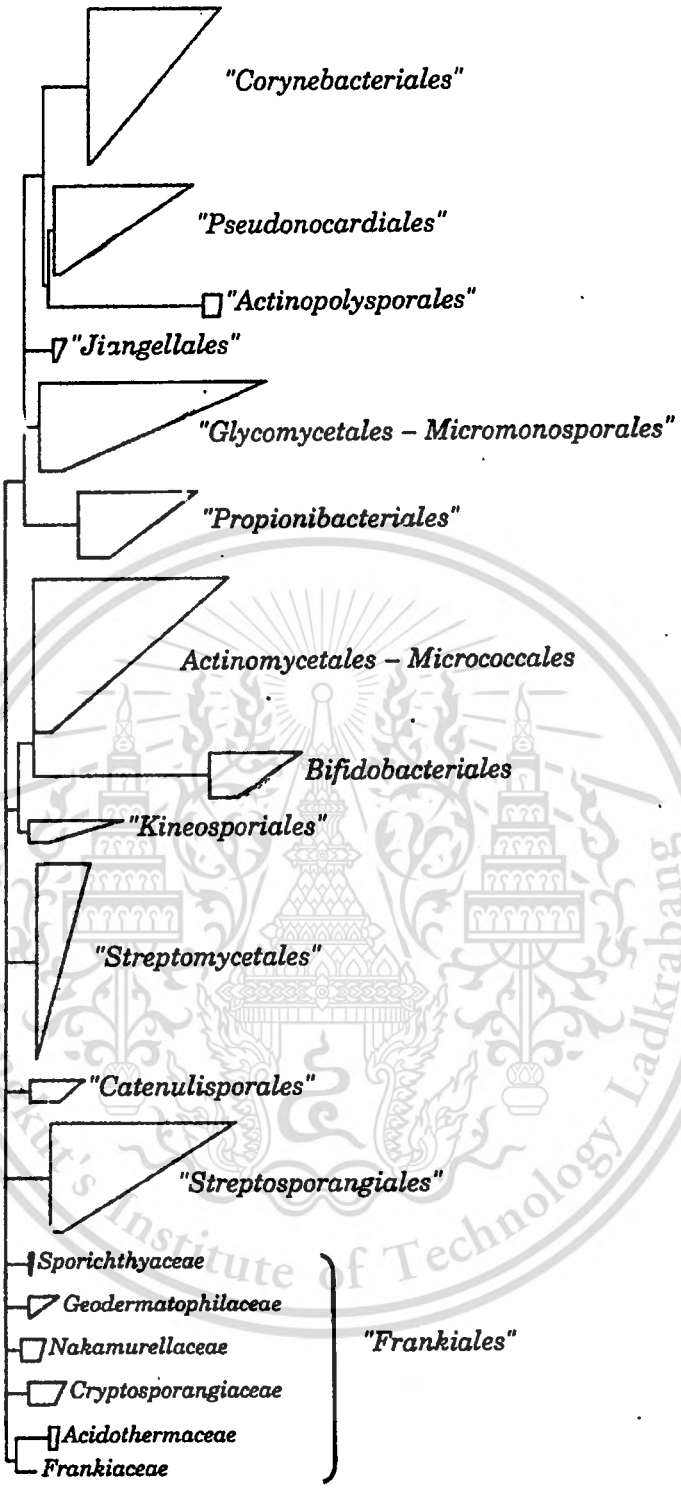


Fig. 2.3 Order of class *Actinobacteria*. Analyses were performed as described in Fig. 2. (Whitman *et al.*, 2012).

Class *Actinobacteria* consisted of fifteen orders which were previously classified by Zhi *et al.* (2009) as suborder within the order *Actinomycetales*, as well as *Bifidobacteriales*, previously classified as an order, and the new order *Jiangellales* is now introduced by Tang *et al.* (2011) below.

Phylum *Actinobacteria*

Class *Actinobacteria*

Order I. *Actinomycetales*
- Family I. *Actinomycetaceae*

Order II. *Actinopolysporales*
- Family I. *Actinopolysporaceae*

Order III. *Bifidobacteriales*
- Family I. *Bifidobacteriaceae*

Order IV. *Catenulisporales*
- Family I. *Catenulisporaceae*
- Family II. *Actinospicaceae*

Order V. *Corynebacteriales*
- Family I. *Corynebacteriaceae*
- Family II. *Dietziaceae*
- Family III. *Mycobacteriaceae*
- Family IV. *Nocardiaceae*
- Family V. *Segniliparaceae*
- Family VI. *Tsukamurellaceae*

Order VI. *Frankiales*
- Family I. *Frankiaceae*
- Family II. *Acidothermaceae*
- Family III. *Cryptosporangiaceae*
- Family IV. *Geodermatophilaceae*
- Family V. *Nakamurellaceae*
- Family VI. *Sporichthyaceae*

Order VII. *Glycomycetales*
- Family I. *Glycomycetaceae*

Order VIII. *Jiangellales*
- Family I. *Jiangellaceae*

Order IX. *Kineosporiales*
- Family I. *Kineosporiaceae*

Order X. *Micrococcales*
- Family I. *Micrococcaceae*
- Family II. *Beutenbergiaceae*
- Family III. *Bogoriellaceae*
- Family IV. *Brevibacteriaceae*
- Family V. *Cellulomonadaceae*
- Family VI. *Dermabacteriaceae*
- Family VII. *Dermacoccaceae*
- Family VIII. *Dermatophilaceae*
- Family IX. *Intrasporangiacea*
- Family X. *Jonesiaceae*
- Family XI. *Microbacteriaceae*
- Family XII. *Promicromonosporaceae*
- Family XIII. *Rarobacteraceae*
- Family XIV. *Ruaniaceae*
- Family XV. *Sanguibacteraceae*

Order XI. *Micromonosporales*
- Family I. *Micromonosporaceae*

Order XII. *Propionibacteriales*
- Family I. *Propionibacteriaceae*
- Family II. *Nocardioidaceae*

Order XIII. *Pseudonocardiales*
- Family I. *Pseudonocardiaceae*

Order XIV. *Streptomycetales*
- Family I. *Streptomycetaceae*

Order XV. *Streptosporangiales*
- Family I. *Streptosporangiales*
- Family II. *Nocardiopsaceae*
- Family III. *Thermomonosporaceae*

2.2.2 Identification technique of actinobacteria

Actinobacteria identification based on phenotypic, chemotaxonomic and genotypic characteristics have been used for classification of actinobacteria and other bacteria.

2.2.2.1 Phenotypic characteristics

Actinobacteria taxonomy formerly based only on morphological characteristic is inadequate in differentiating between different species of many genera. Actinobacteria were morphologically diverse ranging from rod to coccoid, fragmenting hyphal forms to those with a highly differentiated branched mycelium (Trujillo, 2001). The colonial formation of actinobacteria was firstly developed the substrate mycelium or primary mycelium that some hyphae vertically form aerial mycelium or secondary mycelium, directly contacted to the air. However, some genera including *Micromonospora*, *Verrucosispora*, *Salinispora, Actinoplanes*, etc, had no aerial mycelium. Many of these bacteria also produced asexual spores including conidiospore and sporangiospore on the substrate or aerial mycelium. Spore formation of actinobacteria was the most important morphological criterion for identifying at the genus level. For example, single spores, pair spores and branch sporophore was presented in *Micromonospora*, *Microbispora*, and *Microtetraspora*, respectively.

2.2.2.2 Chemotaxonomic characteristics

Chemotaxonomic characteristics are studied on the chemical properties on the cells including type of cell wall, pattern of whole cell sugar, phospholipids, menaquinone profiles, fatty acid, and G+C content in the genomic DNA.

(1) Cell wall composition

The cell wall composition of bacteria is significant for taxonomic value, which differs among the different suborder (Berd, 1973). The peptidoglycan type of Gram-stain-positive bacteria is one of the most important and fundamental indicator of the bacterial classification. Actinobacteria have peptidoglycan which is alternating polymer of *N*-acetylglucosamine and *N*-acetylmuramic acid, the peptide moiety that links glycan chain (Fig. 2.4). Especially, a dibasic amino acid in the tetrapeptide side chain position 3, such as diaminopimelic acid (A_2pm), lysine, ornitine, or 2,4-diaminobutyric acid, have been known as a key of chemotaxonomic analysis (Schleifer and Kandler, 1972). The majority of actinobacteria contains diaminopimelic acid in the peptidoglycan as a cell wall constituent. The diaminopimelic

acid has three stereoisomers (*LL*-, *DD*- and *meso*-A_2pm), and one analog hydroxyl diaminopimelic acid (3-OH-A_2pm), and their relative proportion is distinctive of the genera or species.

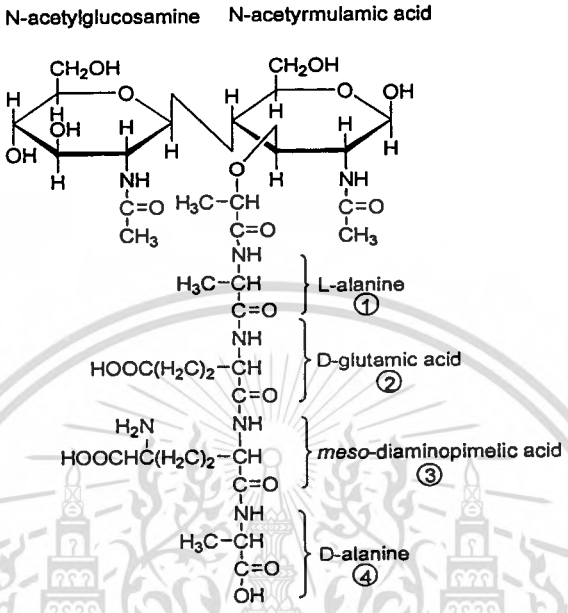


Fig. 2.4 Basal structure of the peptidoglycan in bacterial cell wall.

The cell wall type of actinobacteria has been distinguished based on the characteristics of peptidoglycan composition as shown in Table 2.4.

Table 2.4 Cell wall chemotypes based on peptidoglycan pattern of the actinobacteria

Major constituent	Chemotype							
	I	II	III	IV	V	VI	VII	VIII
LL-Diaminopimelic acid	+							
meso-Diaminopimelic acid		+**	+	+				
Diaminobutyric acid							+	
Aspartic acis						v		
Glycine	+	+					+	
Lysine					+		v	
Ornithine					+			+
Arabinose				+				
Galactose				+		v		

v, variable amounts; **, (3-OH-A_2pm) (may also be present)

(2) Whole cell sugar patterns

The sugar profiles have been determined for identification and classification of actinobacteria in the genus level. Whole cell sugar patterns were contributed to the cell wall chemotypes of actinobacteria proposed by Lechevalier and Lechevalier (1980) (Table 2.5).

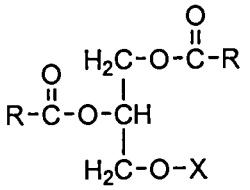
Table 2.5 Whole cell sugar patterns of the actinobacteria

Pattern	Sugar				
	Arabinose	Fuctose	Galactose	Madurose*	Xylose
A	+		+		
B				+	
C	No diagnostic sugars				
D	+				+
E		+			

*Madurose is 3-O-methyl-D-galactose.

(3) Polar lipid composition

Actinobacteria phospholipids are located in cell membrane. Phospholipids are the most common polar lipid types, but glycolipids and amino acid amides are also encountered. Phospholipids are usually derivatives of phosphatidic acid, the most common anes are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), acylphosphatidylglycerol (APG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylinositol (PI). Actinobacteria also have characteristic glycophospholipids, the phosphatidylinositol mannosides (PIMs), that form mono and diacyl dimannosides. Moreover, unknown phospholipids, phosphatidyl glycerol butane-2,3-diol, was isolated from *Streptomyces olivaceus* (Batrakov and Bergelson, 1978). Glucosamine containing phospholipids of unknown structure has been found in member of genera *Promicromonospora*, *Microbispora* and *Streptosporangium* (Lechevalier *et al.*, 1977), as shown in Fig. 2.5. The polar lipid composition is also useful for identification based on the phospholipid types I to V of actinobacteria, as shown in Table 2.6. The phospholipid types of actinobacteria have been used to identify in the genus level shown in Table 2.7.



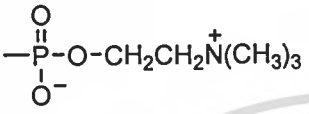
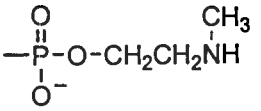
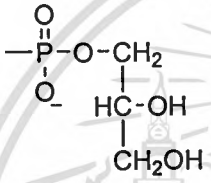
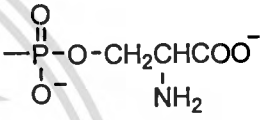
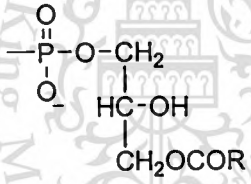
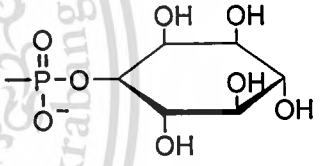
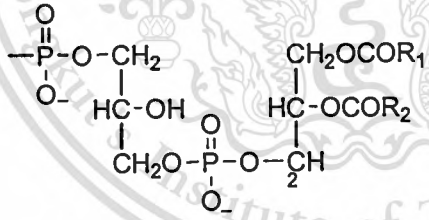
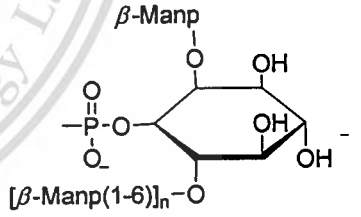
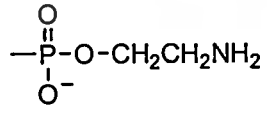
Compounds	X	Compounds	X
PC		PME	
PG		PS	
APG		PI	
DPG		PIMs	
PE			

Fig. 2.5 Structure of phospholipids found in actinobacteria.

Table 2.6 Phospholipid types in actinobacteria

Phospholipid type	Phospholipids								
	PIMs	PI	PC	PG	PE	PME	GluNU	APG	DPG
I	+	+	-	V	-	-	-	V	V
II	+	+	-	V	+	-	-	V	+
III	V	+	+	V	V	+	-	V	V
IV	ND	+	-	-	V	V	+	-	+
V	ND	+	-	+	V	-	+	V	+

V = Variable; + = Present; - = Absent; ND = No data

Table 2.7 Phospholipid types in actinobacteria to genus level identification

Phospholipids type	Diagnostic phospholipids	Genera
I	No nitrogenous phospholipids	*Actinomadura* *Microtetraspora* *Nocardioides* *Spirillospora*
II	Phosphatidyl ethanolamine	*Actinoalloteichus* *Actinoplanes* *Dactylosporangium* *Micromonospora* *Micropolyspora* *Nocardia* *Saccharomonospora* *Salinispora* *Streptomyces* *Verrucosispora*
III	Phosphatidyl choline	*Actinomycetospora* *Kineosporia* *Marinactinospora* *Micropolyspora* *Nocardiopsis* *Nocardia* *Pseudonocardia* *Saccharopolyspora* *Sciscionella* *Spinactinospora*
IV	GluNU (unknown glucosamine-containing phospholipids)	*Actinomadura* *Streptosporangium* *Microbispora* *Microtetraspora* *Planomonospora* *Planobispora*
V	GluNU and phosphatidyl glycerol	*Promicromonospora*

(4) Isoprenoid quinones

Quinones have been widely distributed in bacteria and other microorganisms, and known to have a function as intermediate hydrogen carriers in the electron transport chain. Examples of isoprenoid quinones contain ubiquinone or methaquinone, menaquinone, rhodoquinone, thermoplasmaquinone, caldariellaquinone, methionaquinone, chlorobiumquinone and epoxyubiquinone (Fig. 2.6). Menaquinone was normally found as the predominant in cell membrane of actinobacteria (Collins *et al.*, 1977). The number of isoprene units and the degree of hydrogenation with double bonds in the isoprene chain has been utilized as the important key for identification method (Komakata and Suzuki, 1987).

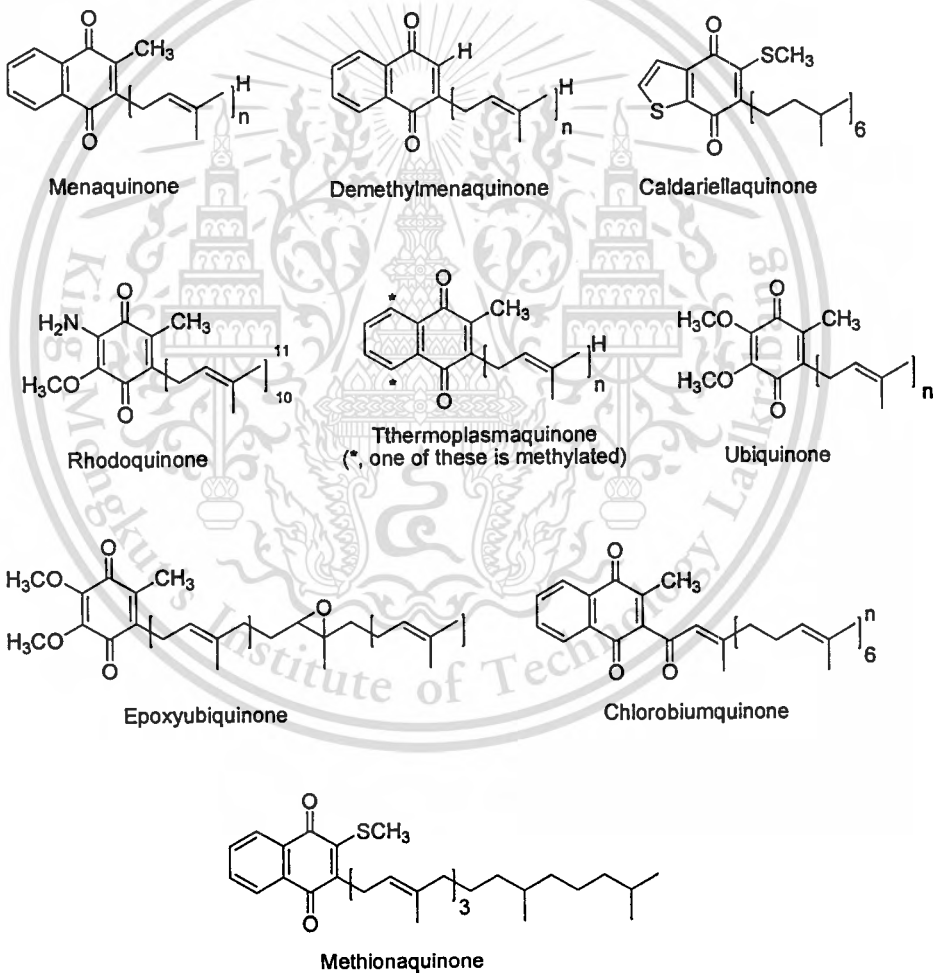


Fig. 2.6 Isoprenoid quinones found in bacterial cells.

(5) Cellular fatty acids

The fatty acids are in a form of lipid bilayer of bacterial cell membranes. Cellular fatty acid composition has been used as a tool for classifying bacteria at the family, genus and species levels. For fatty acid type of actinobacteria, long chain fatty acids may be separated into two broad groups, general fatty acid from ranging in size from 12 to around 20 carbons and the mycolic acids found from 20 up to above 80 carbons. The former groups of acids correspond to a polar portion of phospholipid molecules. The most common type of fatty acids is non-hydroxylated straight chain unsaturated, 10-methyl substituted and *iso*- and *anteiso*- methyl branch fatty acids. The mycolic acids occur as covalent bond with arabinogalactan and also as tretalose dimycolates. The common mycolic acids have no oxygen functions except the 3-hydroxy fatty acids. The mycolic acids have been found in *Mycobacterium*, *Nocardia*, *Corynebacterium* and *Rhodococcus*.

2.2.2.3 Genotypic characteristics

For bacterial identification, 16S rRNA gene sequence technique is usually used for primary screening propose and determining the phylogenetic position. The use of phylogenetic and molecular evolutionary approaches has greatly help the classification methods (Babalola *et al.*, 2009; Hozzein ang Goodfellow, 2011). Phylogenies and species identification have been commonly derived from 16S rRNA gene and the use of polymerase chain reactions (PCR) for sequences analyses (Wood *et al.*, 2007; Zhi *et al.*, 2009).

DNA-DNA hybridization is considered for species delineation, the bacterial species are considered to be group of strains sharing 50 to 70 % DNA reassociation and 5 to 7 % difference in thermal stability between the homologous and heterologuous duplexes (Ursing *et al.*, 1995). The direct sequencing of 16S rRNA gene by PCR technique provides a phylogenetic framework, which serves as the backbone of bacterial taxonomy. However, there was a report that the resolution of 16S rRNA gene analysis between closely related species was generally low and there was no threshold value of 16S rRNA gene sequence similarity for species recognition (Fox *et al.*, 1992; Stackebrandt *et al.*, 1994). It was also noted that actinobacteria with less than 97% similarity of 16S rRNA gene sequence will generally not give DNA association value of more than 60 % (Stackebrandt *et al.*, 1994).

2.3 Secondary metabolites from marine actinobacteria

Secondary metabolites are chemical compounds originated from living organisms such as animals, plants and microorganisms. Traditionally, plants were used as the most prolific sources of drugs from nature and the use of medicinal plants have been well documented throughout human history (Barton and Nakanishi, 1999), although antibiotics from microorganisms were described since late 1800s and early 1900s (Bugni and Ireland, 2004). Later, intensive studies of bacteria and fungi from soil have shown that microorganisms are also a rich source of pharmaceutically bioactive compounds (Fenical, 1993). The potential of marine microorganisms as a source of antibiotics has begun after penicillin was developed as a human antibiotic in the early 1940s. However, modern biotechnology has moved its focus to microbes and encompassed the discovery of new pharmaceutical drugs from marine microorganisms (Mayer *et al.*, 2011; Bhatnagar and Kim 2012).

Secondary metabolites in general play an important role in drug development, 63% of new drugs were classified as naturally derived (unmodified natural products, modified natural products, or synthetic compounds with a natural products as a pharmacophore). There are now approximately 32,500 natural products reported from microbial sources (Antibase data base), which included approximately 1000 compounds derived from marine microorganisms (Singh and Pelaez, 2008). In addition, over two-thirds of the antibiotics isolated from bacteria and fungi were produced by actinobacteria (Challis and Hopwood, 2003) and several antibiotics were derived from marine actinobacteria (Baltz, 2008). Some bioactive secondary metabolites from marine actinobacteria are listed in Table 2.8, with diverse chemical structures such as macrolides, pyrones, lactones, indoles, terpenes and quinones, and exhibited a broad range of biological activities such as antibacterial, antifungal, anticancer, antimalaria, antitubercular and immunosuppressive. The chemical structures are shown in Fig. 2.7.

Many unique structural features, rarely or never found among the compounds from terrestrial actinobacteria, have been isolated from the marine actinobacteria. It is thus not surprising that their molecular modes of action are something also unique and prompting the investigation as potential leads for drug development. For example, abyssomicins, polycyclic polyketide, were isolated from marine *Verrucosispora* sp. (Riedlinger *et al.*, 2004) and showed antibacterial activity, which specific inhibition to *p*-aminobenzoic acid biosynthesis in bacteria. In addition, abyssomicins could hold a promise in treatment of *Mycobacterium tuberculosis* (Freundlich *et al.*, 2010). The alkaloid, diazepinomicin, isolated from marine *Micromonospora* sp. (Charan *et al.*,

2004) was a potent inducer of apoptosis with dual mode of action-binding to the benzodiazepine receptor and inhibition of Ras/MAP kinase pathway (Gourdeau *et al.*, 2008). Salinosporamide A (marizomib) from *Salinispora tropica* (Felling *et al.*, 2003) exhibited anticancer activity, which inhibited the 20S catalytic core subunit of the proteasome that resulted in the disruption of cellular process, induction of apoptosis, and inhibition of tumor growth and angiogenesis.

Table 2.8 Bioactive natural products isolated from marine actinobacteria during the period 2004-2013

Compound	Actinomycete	Source	Activity	Reference
1-Hydroxy-1-norresistomycin (1)	*Streptomyces chibaensis*	Marine sediments	Antibacterial; Cytotoxicity	Kock *et al.*, 2005
3,5,6-trisubstituted2(1*H*)-pyrazinones (2)	*Streptomyces* sp.	Marine sponge	Cytotoxicity	Motohashi *et al.*, 2011
7-demethylSF2415A3 (3)	*Streptomyces aculeolatus*	Marine sediments	Antibacterial	Motohashi *et al.*, 2008
13-N-demethyl-methylpendolmycin (4)	*Marinactinospora thermotolerans*	Marine sediments	Antimalarial	Huang *et al.*, 2011
A80915G-8~-acid (5)	*Streptomyces* sp.	Marine sediments	Antibacterial	Motohashi *et al.*, 2008
Abyssomicin C (6)	*Verrucosispora* sp.	Marine sediments	Antibacterial, Antitubercular	Riedlinger *et al.*, 2004
Actinoramides (7)	*Streptomyces* sp.	Marine sediments	Antibacterial	Nam *et al.*, 2011
Albidopyrone (8)	*Streptomyces* sp.	Marine sediments	Anti-protein-tyrosin phosphatase B	Hohmann *et al.*, 2009
Anthracyclinone (9)	*Micromonospora* sp.	*Eudistoma vannamei*	Anticancer	Sousa *et al.*, 2012
Antimycin A_{19} (10)	*Streptomyces antibioticus*	Marine sediments	Antifungal	Xu *et al.*, 2011
Arenamides (11)	*Salinispora arenicola*	Marine sediments	Aiticancer, Anti-TNF-induced activation, Inhibited nitric oxide	Asolkar *et al.*, 2009
Arenicolides (12)	*Salinispora arenicola*	Marine sediments	Cytotoxicity	Williams *et al.*, 2007
Arenimycin (13)	*Salinispora arenicola*	Marine sediments	Antibacterial, Anticancer	Asolkar *et al.*, 2010
Aureoverticillactam (14)	*Streptomyces aureoverticillactus*	Marine sediments	Anticancer	Mitchell *et al.*, 2004
Azamerone (15)	*Streptomyces* sp.	Marine sediments	Anti T cells, Antimacrophages	Cho *et al.*, 2006
Bahamaolides A (16)	*Streptomyces* sp.	Marine sediments	Antifungal	Kim *et al.*, 2012
Bendigole A (17)	*Actinomadura* sp.	Marine sponge (*Suberites japonicus*)	Anti GR and NF kB nuclear translocation	Simmons *et al.*, 2011
Benzoxacystol (18)	*Streptomyces* sp.	Marine sediments	Antiglycogensynthase kinase enzyme	Nachtigall *et al.*, 2011

Table 2.8 Bioactive natural products isolated from marine actinobacteria during the period 2004-2013 (continued)

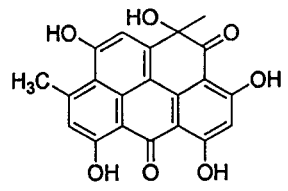
Compound	Actinobacteria	Source	Activity	Reference
Bipyridine alkaloids (19)	*Actinoalloteichus cyanogriseus*	Marine sediments	Antitumor	Fu *et al.*, 2011
Bisanthraquinone (20)	*Streptomyces* sp.	Cyanobacterium	Antibacterial	Socha *et al.*, 2006
Bohemamines (21)	*Streptomyces* sp.	Marine sediments	Antimicrobial	Bugni *et al.*, 2006
Caboxamycin (22)	*Streptomyces* sp.	Marine sediments	Antibacterial, Anticancer	Hohmann *et al.*, 2009
Cebulactams A1 (23)	*Saccharopolyspora* sp.	Marine sponge (*Haliclona* sp.)	Antioxidant	Pimentel-Elardo *et al.*, 2008
Chinikomycin A (24)	*Streptomyces* sp.	Marine sediments	Anticancer	Li *et al.*, 2005
Chlorizidine (25)	*Streptomyces* sp.	Marine sediment	Anticncer	Alvarez-Mico *et al.*, 2013
Chloro-Dihydroquinone (26)	*Streptomycetaceae*	Marine sediments	Anticancer	Soria-Mercado *et al.*, 2005
Chromomycin SA (27)	*Streptomyces* sp.	Marine sediments	Anticancer	Hu *et al.*, 2011
Cyanogrisides A (28)	*Actinoalloeichus cyanogriseus*	Marine sediments	Cytotoxicity	Fu *et al.*, 2011
Cyanosporasides (29)	*Salinispora pacifica*	Marine sediments	Antitumor	Oh *et al.*, 2006
Cyclodysidins (30)	*Streptomyces* sp.	Marine sponge (*Dysidea tupha*)	Cytotoxicity	Abdelmohsen *et al.*, 2012
Cyclo-(L-Pro-L-Met) (31)	*Nocardiopsis* sp.	Seaweed (*Undaria pinnatifida*)	Anti-angiogenesis activity	Shin *et al.*, 2010
Daryamides A (32)	*Streptomyces* sp.	Marine sediments	Antifungal, Anticancer	Asolkar *et al.*, 2006
Dehydroxynocardamine (33)	*Streptomyces* sp.	Marine sponge	Anti-recombinant enzymesortase B	Lee *et al.*, 2005
Diazepinomicin (34)	*Micromonospora* sp.	*Didemnum proliferum*	Antibacterial, Anticancer, Anti-inflammatory	Charan *et al.*, 2004
Fijimycin A (35)	*Streptomyces* sp.	Marine sediments	Antibacterial	Sun *et al.*, 2011
Fijiolides (36)	*Nocardiopsis* sp.	Marine sediments	Anticancer	Nam *et al.*, 2010
Fluostatins (37)	*Micromonospora rosaria.*	Marine sediments	Antibacterial	Zhang *et al.*, 2012
Galvaquinone B (38)	*Streptomyces spinoverrucosus*	Marine sediments	Anticancer	Hu *et al.*, 2012
Glaciapyrrole A (39)	*Streptomyces* sp.	Marine sediments	Antibacterial, Antitumor	Macherla *et al.*, 2005
Glucopiericidin C (40)	*Streptomyces* sp.	Marine sediments	Antibacterial, Antifungal	Shaaban *et al.*, 2011
Indoxamycin A (41)	*Streptomyces* sp.	Marine sediments	Antitumor	Sato *et al.*, 2009
JBIR-65 (diterpene) (42)	*Actinomadura* sp.	Marine sponge	Anticancer, Antioxidant α-tocopherol	Takagi *et al.*, 2010
Lajollamycin (43)	*Streptomyces nodosus*	Marine sediments	Antibacterial	Manan *et al.*, 2005
Lipoxazolidinone A (44)	*Marinispora* sp.	Marine sediments	Antibacterial	Macherla *et al.*, 2007

Table 2.8 Bioactive natural products isolated from marine actinobacteria during the period 2004-2013 (continued)

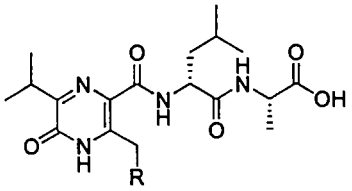
Compound	Actinobacteria	Source	Activity	Reference
Lobophorin F (45)	*Streptomyces* sp.	Marine sediments	Antibacterial, Anticancer	Niu *et al.*, 2011
Lodopyridone (46)	*Saccharomonospora* sp.	Marine sediments	Anticancer	Maloney *et al.*, 2009
Lorneic acid A (47)	*Streptomyces* sp.	Marine sediments	Antiphosphodiesterase	Iwata *et al.*, 2009
Lucentamycin A (48)	*Nocardiopsis lucentensis*	Marine sediments	Antitumor	Cho *et al.*, 2007
Lynamicins (49)	*Marinispora* sp.	Marine sediments	Antibacterial	McArthur *et al.*, 2008
Marinacarbolines (50)	*Marinactinospora thermotolerans*	Marine sediments	Antimalarial	Huang *et al.*, 2011
Marinactinones (51)	*Marinactinospora thermotolerans*	Marine sediments	Cytotoxicity, Topoisomerase II inhibition	Wang *et al.*, 2011
Marineosin A (52)	*Streptomyces* sp.	Marine sediments	Anticancer, Antifungal	Boonlarppradab *et al.*, 2008
Marinisporolide A (53)	*Marinispora* sp.	Marine sediments	Antibacterial, Anticancer, Antifungal	Kwon *et al.*, 2008
Marinomycin A (54)	*Marinispora* sp.	Marine sediments	Antibacterial	Kwon *et al.*, 2006
Marinopyrroles (55)	*Streptomyces* sp.	Marine sediments	Antibacterial	Hughes *et al.*, 2008
Marmycins (56)	*Streptomyces* sp.	Marine sediments	Antibacterial, Antitumor	Martin *et al.*, 2007
Mechercharmycin A (57)	*Thermoactinomyces* sp.	Marine sediments	Anticancer	Kanoh *et al.*, 2005
Methylpendolmy-cin-14-O-α-glucoside (58)	*Marinactinospora thermotolerans*	Marine sediments	Antimalarial	Huang *et al.*, 2011
Nahuoic acid (59)	*Streptomyces* sp.	Marine sediment	Anti-Histone Methyltransferase	Williams *et al.*, 2013
***N*-carboxamido-staurosporine (60)**	*Streptomyces* sp.	Marine sediments	Antibacterial, Antitumor	Wu *et al.*, 2006
Neomaclafungins (61)	*Actinoalloteichus* sp.	Marine sediments	Antifungal	Sato *et al.*, 2012
Nitropyrrolin D (62)	*Streptomyces* sp.	Marine sediments	Anticancer	Kwon *et al.*, 2010
Nocazines (63)	*Nocardiopsis dassonvillei*	Marine sediments	Antibacterial, Anticancer	Fu *et al.*, 2011
Pacificanones A (64)	*Salinispora pacifica*	Marine sediments	Antibacterial	Oh *et al.*, 2008
Phenylpyridine alkaloid (65)	*Actinoalloteichus cyanogriseus*	Marine sediments	Antitumor	Fu *et al.*, 2011
Piericidins (66)	*Streptomyces* sp.	greenish ascidians	Antitumor	Hayakawa *et al.*, 2007
Piperazimycins (67)	*Streptomyces* sp.	Marine sediments	Anticancer	Miller *et al.*, 2006
Proximicin B (68)	*Verrucosispora* sp.	Marine sediments	Antibacterial, Anticacer	Fiedler *et al.*, 2008
Pterocidin (69)	*Streptomyces* sp.	Marine sediments	Antitumor	Igarashi *et al.*, 2012

Table 2.8 Bioactive natural products isolated from marine actinobacteria during the period 2004-2013 (continued)

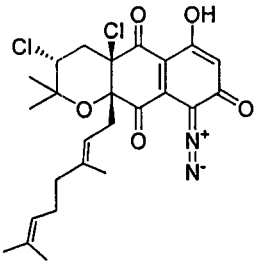
Compound	Actinobacteria	Source	Activity	Reference
Rakicidin D (70)	*Streptomyces* sp.	Marine sediments	Antitumor	Igarashi *et al.*, 2010
Saliniketals (71)	*Salinispora arenicola*	Marine sediments	Anticancer	Williams *et al.*, 2007
Salinipyrone A (72)	*Salinispora pacifica*	Marine sediments	Antibacterial	Oh *et al.*, 2008
Salinosporamide A (73)	*Salinispora tropica*	Marine sediments	Anticancer, Antimalarial	Felling *et al.*, 2003
Salicylamide (JBIR-58) (74)	*Streptomyces* sp.	Marine sponge	Anticancer	Ueda *et al.*, 2010
Spiroindimicins (75)	*Streptomyces* sp.	Marine sediments	Anticancer	Zhang *et al.*, 2012
Streptobactin (76)	*Streptomyces* sp.	Brown alga (*Analipus japonicus*)	Inhibitor of pyroglutamyl peptidase	Matsuo *et al.*, 2011
Streptocarbazoles (77)	*Streptomyces* sp.	Marine sediments	Antitumor	Fu *et al.*, 2012
Streptokordin (78)	*Streptomyces* sp.	Marine sediments	Antitumor	Jeong *et al.*, 2006
Streptopyrrolidine (79)	*Streptomyces* sp.	Marine sediment	Anti-angiogenesis	Shin *et al.*, 2008
Tartrolon D (80)	*Streptomyces* sp.	Marine sediments	Anticancer	Pe'rez *et al.*, 2009
Tetracenoquinocin (81)	*Streptomyces* sp.	Marine sponge (*Haliclona* sp.)	Anticancer	Motohashi *et al.*, 2010
Tetrapeptides (JBIR-34;JBIR-35) (82)	*Streptomyces* sp.	Marin sponge (*Haliclona* sp.)	Antioxidant	Motohashi *et al.*, 2010
Thiocoraline A (83)	*Verrucosispora* sp.	Marine sponge (*Chondrilla caribensis*)	Anticancer	Wyche *et al.*, 2011
Tirandamycin C (84)	*Streptomyces* sp.		Antibacterial	Carlson *et al.*, 2009
Tumescenamides (85)	*Streptomyces tumescens*	Marine sediments	Induced reporter gene expression	Motohashi *et al.*, 2010
Usabamycins (86)	*Streptomyces* sp.	Marine sediments	Antiserotonin (5-hydroxy-tryptamine) receptors	Sato *et al.*, 2011
ZHD-0501 (87)	*Actinomadura* sp.	Marine sediments	Anticancer	Han *et al.*, 2005



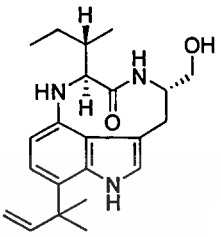
(1) 1-Hydroxy-1-norresistomycin



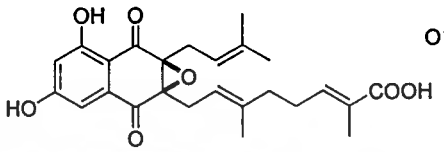
(2) 3,5,6-trisubstituted2(1H)-pyrazinones



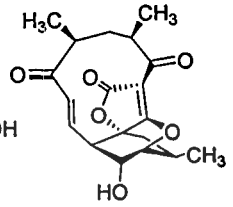
(3) 7-demethyl SF2415A3



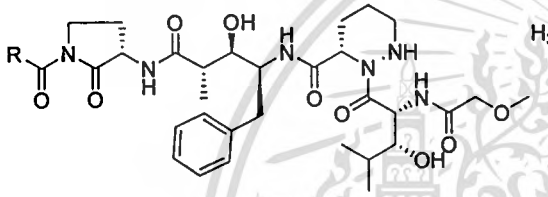
(4) 13-*N*-demethyl-methylpendolmycin



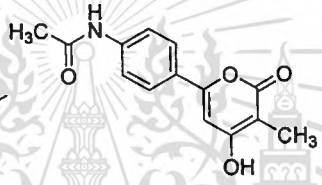
(5) A80915G-8"-acid



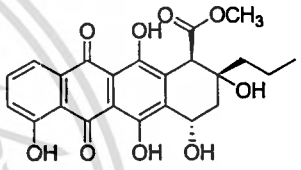
(6) Abyssomicin C



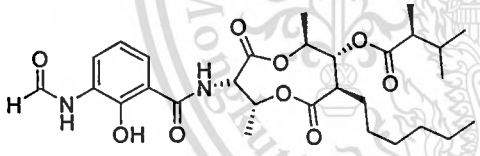
(7) Actinoramides
Actinoramides A: R = NH_2
Actinoramides B: R = CH_3



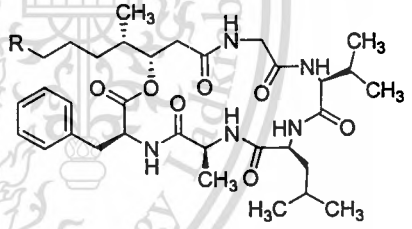
(8) Albidopyrone



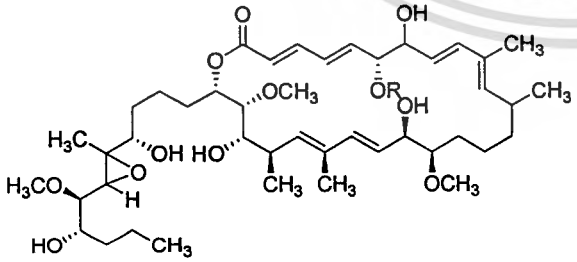
(9) Anthracyclinone



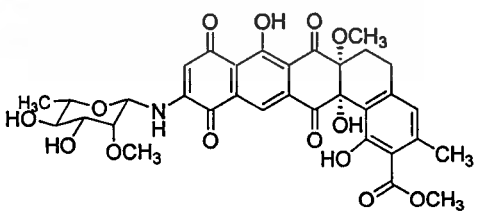
(10) Antimycin A_{19}



(11) Arenamides
Arenamide A: R = $CH_2CH_2CH_3$
Arenamide B: R = CH_3

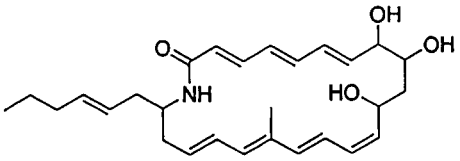


(12) Arenicolides
Arenicolide A: R = CH_3
Arenicolide B: R = H

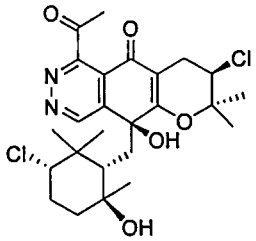


(13) Arenimycin

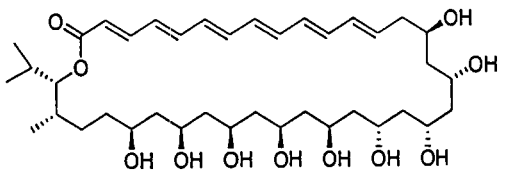
Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria



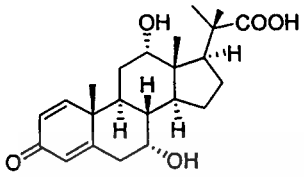
(14) Aureoverticillactam



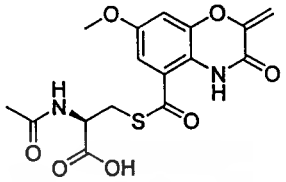
(15) Azamerone



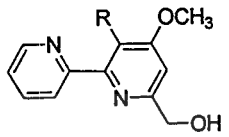
(16) Bahamaolides A



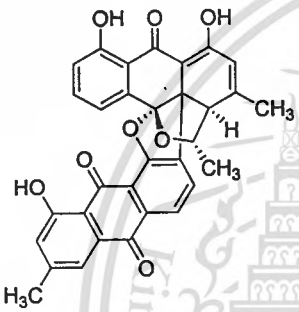
(17) Bendigole A



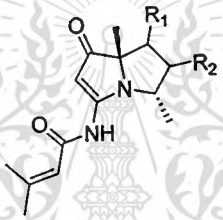
(18) Benzoxacystol



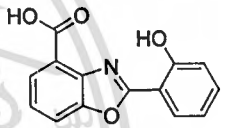
(19) Bipyridine alkaloids
1: R = H; 2: R = OCH_3



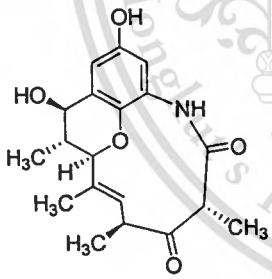
(20) Bisanthraquinone



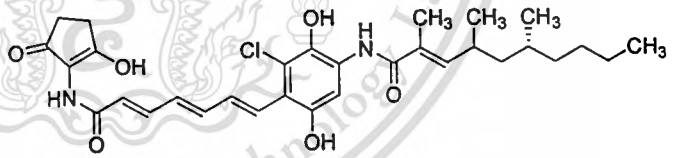
(21) Bohemamines
Bohemamine B: R_1 = H, R_2 = OH
Bohemamine C: R_1 = OH, R_2 = H



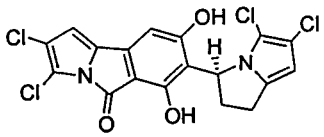
(22) Caboxamycin



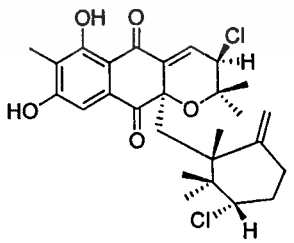
(23) Cebulactams A1



(24) Chinikomycin A

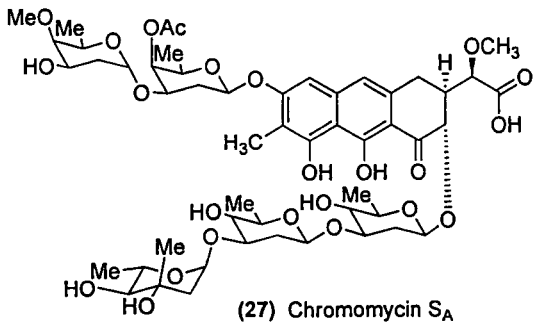


(25) (-)-Chlorizidine

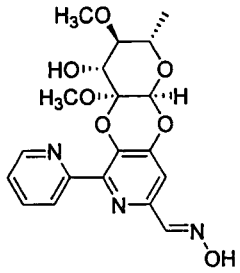


(26) Chloro-Dihydroquinone

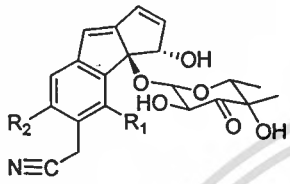
Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria (continued)



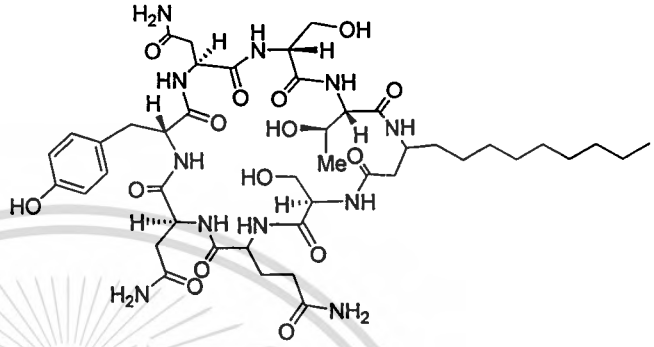
(27) Chromomycin S_A



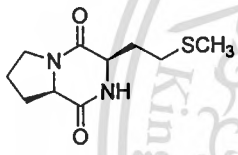
(28) Cyanogriside A



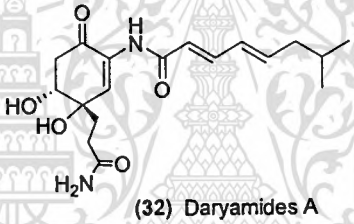
(29) Cyanosporasides
Cyanosporaside A: R_1 =Cl; R_2 = H
Cyanosporaside B: R_1 = H; R_2 = Cl



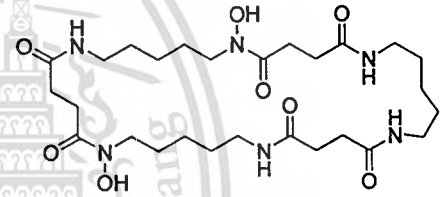
(30) Cyclodysidin A



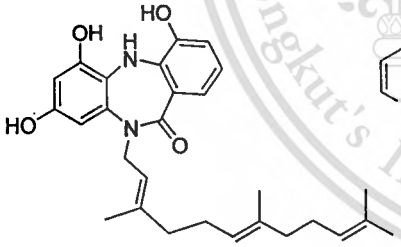
(31) Cyclo-(L-Pro-L-Met)



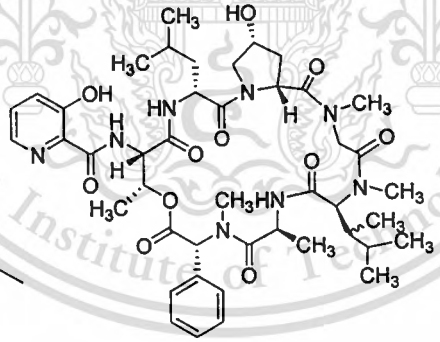
(32) Daryamides A



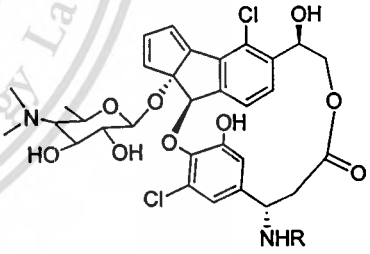
(33) Dehydroxynocardamine



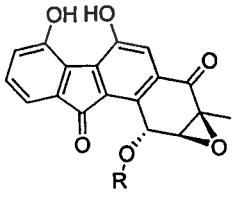
(34) Diazepinomicin



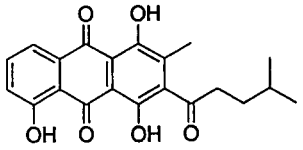
(35) Fijimycin A



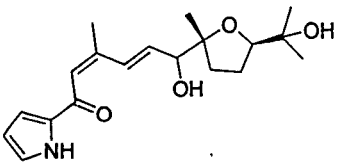
(36) Fijiolides
Fijiolide A: R = $COCH_3$
Fijiolide B: R = H



(37) Fluostatins
Fluostatin C: R = H
Fluostatin F: R = CH_3

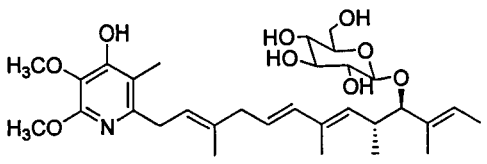


(38) Galvaquinone B

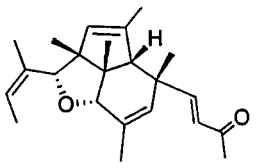


(39) Glaciapyrrole A

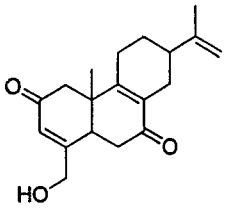
Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria (continued)



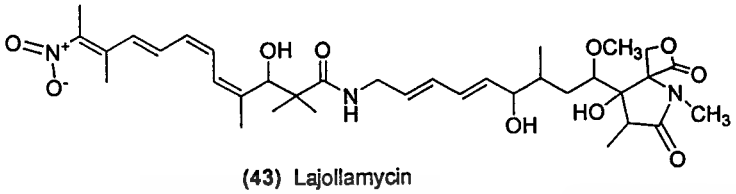
(40) Glucopiericidin C



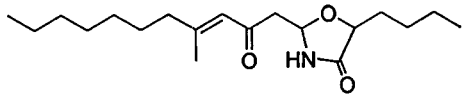
(41) Indoxamycin A



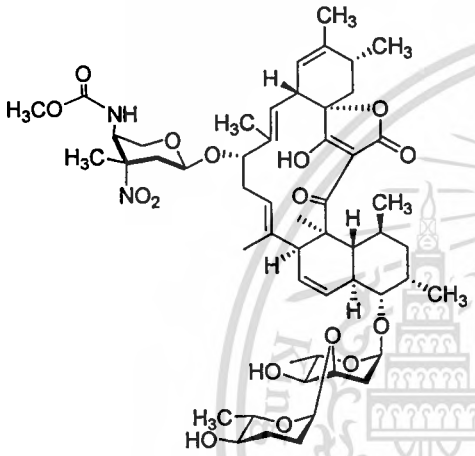
(42) JBIR-65



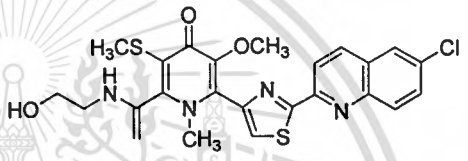
(43) Lajollamycin



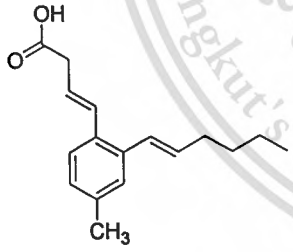
(44) Lipoxazolidinone A



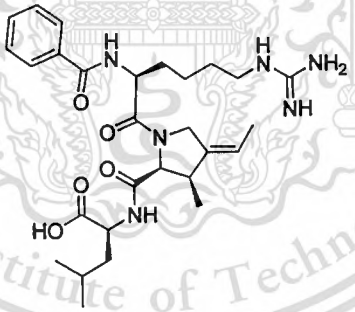
(45) Lobophorin F



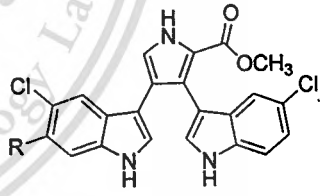
(46) Lodopyridone



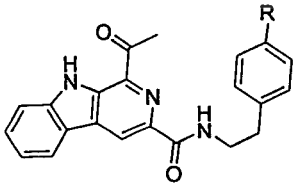
(47) Lorneic acid A



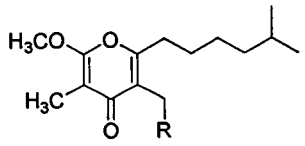
(48) Lucentamycin A



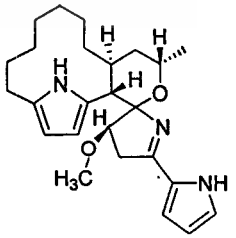
(49) Lynamicins
Lynamicin A: R = H
Lynamicin B: R = Cl



(50) Marinacarbolines
Marinacarboline A: R = OCH3
Marinacarboline B: R = OH
Marinacarboline C: R = H

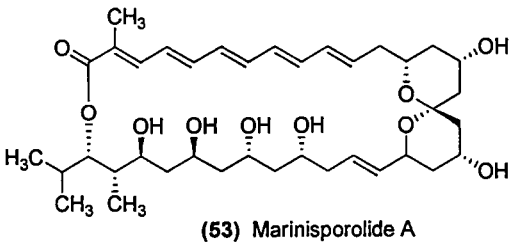


(51) Marinactinones
Marinactinone A: R = H
Marinactinone B: R = CH_3

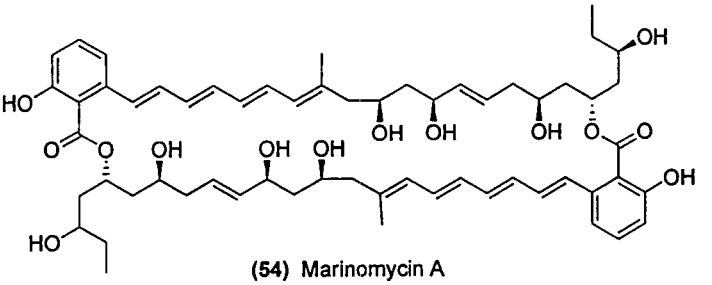


(52) Marineosin A

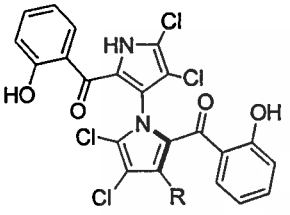
Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria (continued)



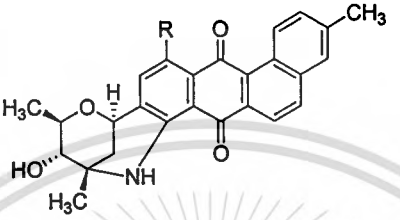
(53) Marinisporolide A



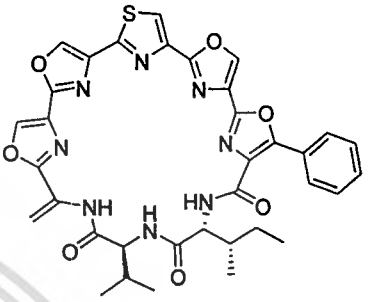
(54) Marinomycin A



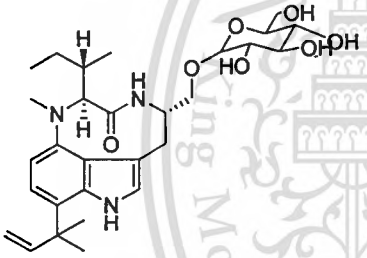
(55) Marinopyrroles
Marinopyrroles A: R = H
Marinopyrroles B: R = Br



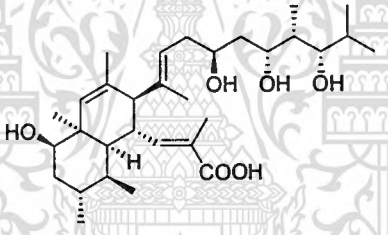
(56) Marmycins
Marmycin A: R = H
Marmycin B: R = Cl



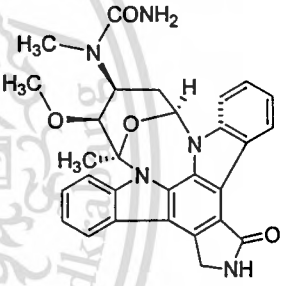
(57) Mechercharmycin A



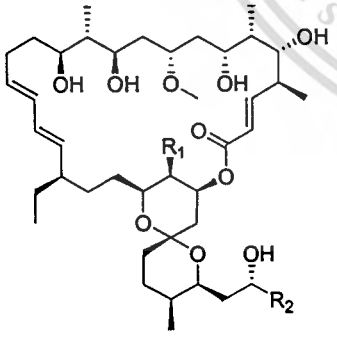
(58) Methylpendolmy-cin-14-*O*-glucoside



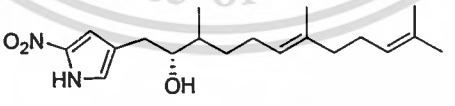
(59) Nahuoic acid



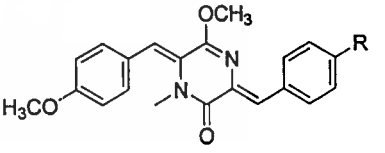
(60) *N*-carboxamido-staurosporine



(61) Neomaclafungins
Neomaclafungin A: $R_1 = CH_2CH_2CH_2OH$; $R_2 = CH_3$
Neomaclafungin B: $R_1 = CH_2CH_2CH(CH_3)OH$; $R_2 = CH_3$
Neomaclafungin C: $R_1 = CH_2CH_2CH_2OH$; $R_2 = CH_2CH_3$

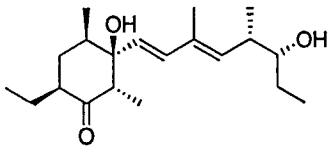


(62) Nitropyrrolin D

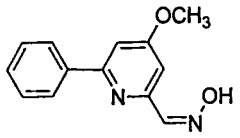


(63) Nocazines
Nocazine A: R = OCH_3
Nocazine B: R = H

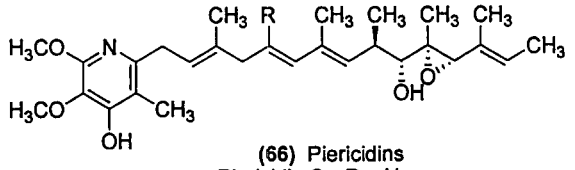
Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria (continued)



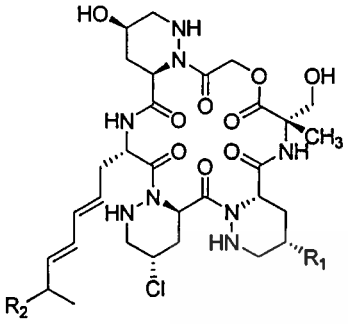
(64) Pacificanone A



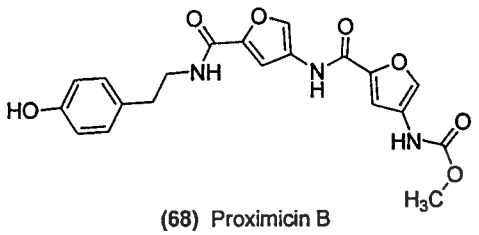
(65) Phenylpyridine alkaloid



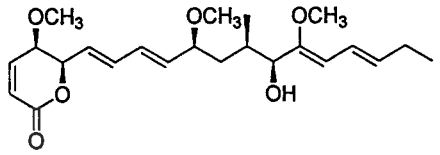
(66) Piericidins
Piericidin C_7: R = H
Piericidin C_8: R = CH_3



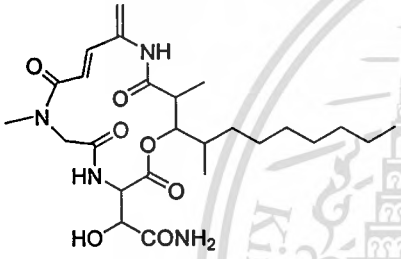
(67) Piperazimycins
Piperazimycin A: R_1 = OH, R_2 = CH_3
Piperazimycin B: R_1 = H, R_2 = CH_3
Piperazimycin C: R_1 = OH, R_2 = CH_2CH_3



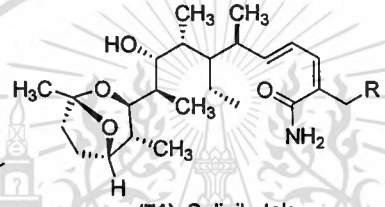
(68) Proximicin B



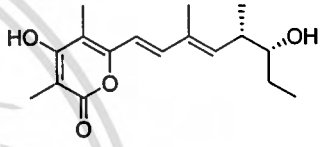
(69) Pterocidin



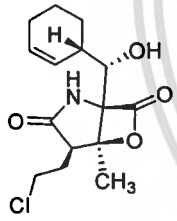
(70) Rakicidin D



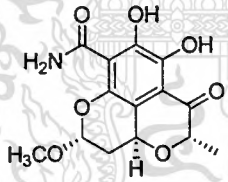
(71) Saliniketals
Saliniketal A: R = H
Saliniketal B: R = OH



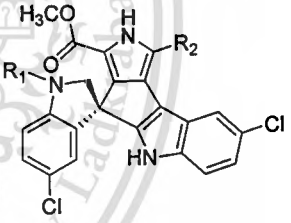
(72) Salinipyrone A



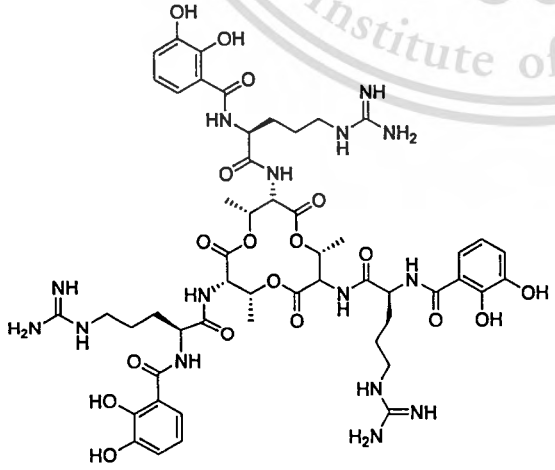
(73) Salinosporamide A



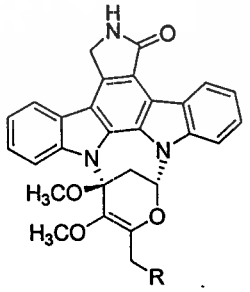
(74) Salicylamide (JBIR-58)



(75) Spiroindimicins
Spiroindimicin B: R_1 = CH_3, R_2 = H
Spiroindimicin C: R_1 = H, R_2 = H
Spiroindimicin D: R_1 = CH_3, R_2 = $COOCH_3$

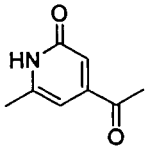


(76) Streptobactin

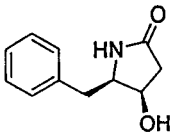


(77) Streptocarbazoles
Streptocarbazole A: R = OH
Streptocarbazole B: R = H

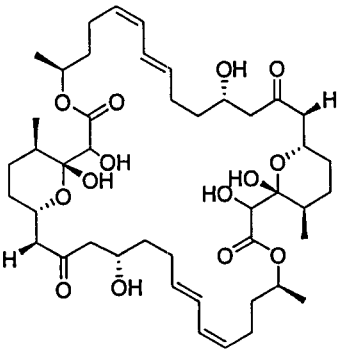
Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria (continued)



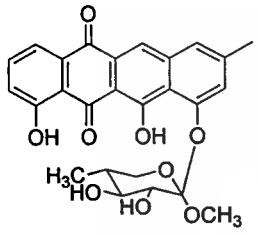
(78) Streptokordin



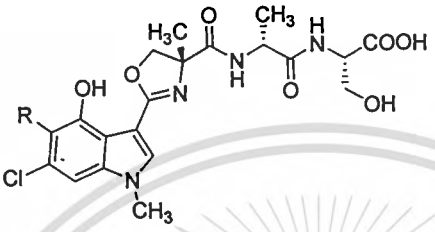
(79) Streptopyrrolidine



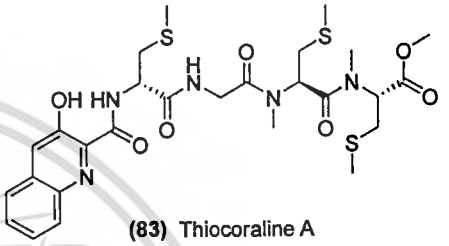
(80) Tartrolon D



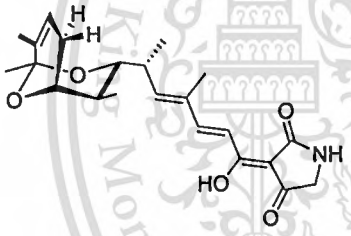
(81) Tetracenoquinocin



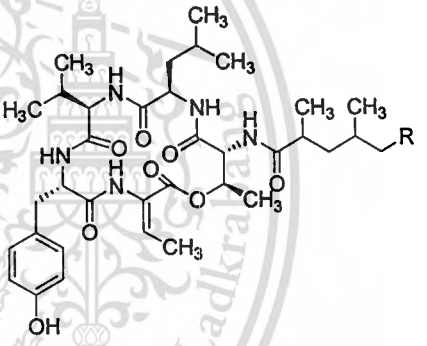
(82) Tetrapeptides
JBIR-34: R = CH_3
JBIR-35: R = H



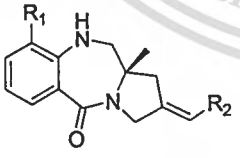
(83) Thiocoraline A



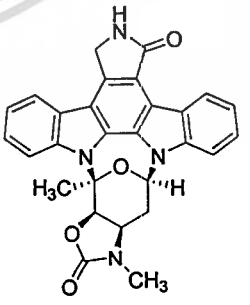
(84) Tirandamycin C



(85) Tumescenamides
Tumescenamide A: R = 
Tumescenamide B: R = 



(86) Usabamycins
Usabamycin A: R_1= OCH_3, R_2 = CH_2CH_3
Usabamycin B: R_1= OCH_3, R_2 = CH_3
Usabamycin C: R_1= H, R_2 = CH_2CH_3



(87) ZHD-0501

Fig. 2.7 Structures of bioactive natural products isolated from marine actinobacteria (continued)

CHAPTER III

RESEARCH METHODOLOGY

3.1 Sample collection and isolation of marine actinobacteria

3.1.1 Sample collection

A total of 18 marine samples were collected from several locations of the Andaman Sea, and the Gulf of Thailand (Table 3.1). The marine samples were collected at the depth of 2-5 m by scuba diving.

3.1.2 Isolation of marine actinobacteria

3.1.2.1 Isolation of marine actinobacteria from sponge and algal samples

The sponge and algal samples were washed three times by sterile artificial seawater. Then, 1 g of each sample was ground and homogenized, and then suspended in 9 ml sterile artificial seawater (Appendix I). After that, 1 ml of the suspension was transferred to another 9 ml sterile artificial seawater tube and this step was repeated to set up a 10-fold dilution series to 10^{-4}. At the final dilution step, aliquots of 0.1 ml were spread onto modified humic acid vitamin (HV) and starch-casein nitrate seawater agars supplemented with antibacterial and antifungal agents.

3.1.2.2 Isolation of marine actinobacteria from sediment samples

(A) Air-dried treatment

The sediment samples were air-dried at room temperature for 7 days and then the dried samples (1 g) were ground and diluted in 9 ml of sterile artificial seawater to dilution series 10^{-2}, 10^{-3} and 10^{-4}. The suspensions were plated on modified humic acid vitamin (HV) and starch-casein nitrate seawater agars supplemented with antibacterial and antifungal agents.

(B) Wet-heat treatment at 70 °C

The sediment samples (1 g) were ground and diluted in 9 ml sterile artificial seawater. After that, the solution were heated at 70 °C in water bath for 15 min, and diluted in 9 ml of sterile artificial seawater to dilution series 10^{-2}, 10^{-3} and 10^{-4}. The suspensions were plated on modified humic acid vitamin (HV) and starch-casein nitrate seawater agars supplemented with antibacterial and antifungal agents.

(C) Dry-heat treatment at 100 °C

The sediment samples were heat at 100 °C for 1 h, and 1 g of samples diluted in 9 ml of sterile artificial seawater to dilution series 10^{-2}, 10^{-3} and 10^{-4}. The suspensions were plated on modified humic acid vitamin (HV) and starch-casein nitrate seawater agars supplemented with antibacterial and antifungal agents.

The isolation plates were incubated at 30°C for 21 days. Actinobacteria colonies were selected under light microscope. Actinobacteria colonies were purified onto yeast extract-malt extract agar (ISP2). The single colony of each strain was transferred to the ISP2 agar slant.

Table 3.1 Marine samples collected from the several location in the Andaman sea and the gulf of Thailand

Type of sample	Sample No.	Depth (m)	Location
Marine sponges	S3	5	Sichang Island, Chonburi Province
	S6	5	Sichang Island, Chonburi Province
	S18	5	Sichang Island, Chonburi Province
	SP206	5	Sichang Island, Chonburi Province
	SP207	5	Koh Pan Yue Island, Chonburi Province
	SP209	5	Sichang Island, Chonburi Province
	SP03	5	Phuket Province
	ST01	3	Phuket Province
	SH4	3	75 million-year shell cemetery (Susan Hoi), Krabi Province
Marine algae	MA3	3	Nangyuan Island, Suratthani Province
	SH3	3	75 million-year shell cemetery (Susan Hoi), Krabi Province
Marine sediments	AN5	2	Ao Nang, Krabi Province
	S8	2	Tao Island, Suratthani Province
	S15	5	Sichang Island, Chonburi Province
	S20	5	Sichang Island, Chonburi Province
	SH2	3	75 million-year shell cemetery (Susan Hoi), Krabi Province
	AN6	3	Ao Nang, Krabi Province
	TV1	3	Talay-Waek, Krabi Province

3.2 Identification methods

Morphological, cultural, physiological and biochemical characteristics of actinobacteria were determined by the methods described by Shirling and Gottlieb (1966), Gordon *et al.* (1974) and Arai (1975).

3.2.1 Morphological and cultural characteristics

The marine actinobacterial strains were grown on modified soil extract seawater agar and ISP 2 seawater agar for 21 days at 30 °C, and observed by light and scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy were prepared by the method of Itoh *et al.* (1989). The cultural characteristics were determined using 14 day-cultures grown at 30 °C on various agar media including yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6), tyrosine agar (ISP 7), Czapek's sucrose agar, glucose-asparagine agar, and nutrient agar (Appendix I). The colour of the mycelium and soluble pigment were determined by NBS/IBCC colour system (Kelly, 1964).

3.2.2 Biochemical characteristics

3.2.2.1 Acid production from carbohydrates

The acid production from carbon sources were examined using the basal inorganic nitrogen medium (Appendix I). The pH value of this medium was adjusted to 7.0 before the addition of 15 ml of 0.04% bromocresol purple solution. After that the agar medium was tubed and sterilized by autoclaving, 0.5 ml of 10% solution of each carbohydrates (autoclaved separately) was added aseptically to the tube (Gordon *et al.*, 1974).

Carbon source and controls required for the test was repeated below :

D-glucose (positive control)	No carbon source (negative control)	
D-cellobiose	D-raffinose	lactose
D-fructose	D-ribose	salicin
D-galactose	D-xylose	sorbose
D-melibiose	L-arabinose	

A few drops of actinobacterial suspention were added into the agar tube, and cultivated at 30 °C for 7-14 days. If actinobacterial strain could produces the acid from carbohydrate that showed the yellow colour of the agar medium.

3.2.2.2 Starch hydrolysis

All strains were streaked on the surface of ISP 4 (inorganic salt-starch agar) (Shirling and Gottlieb, 1966, Appendix I) and incubated at 30 °C for 14 days. After that, Gram's iodine solution was flooded on the surface the agar plate. If the strain could hydrolyze the starch, the clear zone would be observed.

3.2.2.3 Gelatin liquefaction

All strains were incubated into Bouillon gelatin broth (Arai, 1975, Appendix I) and incubated at 30 °C for 7-14 days. The inoculated tube was compared with uninoculated control when observed at 4 °C for 30 min. The gelatin became liquid if it was hydrolyzed.

3.2.2.4 Nitrate reduction

The marine actinobacteria were inoculated into peptone potassium nitrate (KNO_3) broth (Appendix I) and incubated at 30 °C for 7-14 days. The culture broth was tested with two drops of sulfanilic acid and three drops of *N, N*-dimethyl-1-naphtylamine solution and mixed well. If nitrites were present, the culture mixture would become pink to red colour.

3.2.2.5 Coagulation and peptonization of milk

The marine actinobacteria were inoculated in 10% skim milk broth (Appendix I) and incubated at 30 °C for 7-14 days. If milk was peptonized, the broth would become clear, and its coagulated would be converted to the solid form.

3.2.2.6 Sodium chloride tolerance

All strains were streaked on ISP 2 medium agar supplemented with 0-10% sodium chloride and incubated at 30 °C for 14 days. The minimum and maximum of sodium chloride concentrations for growth were recorded.

3.2.2.7 Temperature tolerance

The marine actinobacteria were streaked on ISP2 medium agar and incubated at 10-50 °C for 14 days. The minimum and maximum of temperature for growth of the strain were recorded.

3.2.2.8 pH tolerance

All actinobacterial strains were streaked on ISP 2 medium that adjust pH at 4, 4.5, 5, 6, 7, 8, 9, 10, 11, and 12. The plates were incubated at 30 °C for 14 days. The minimum and maximum of pH for growth were recorded.

3.2.3 Chemotypic characteristics

3.2.3.1 Diaminopimelic acid analysis

Dried cells (10 mg) were hydrolyzed with 6 N HCl at 100 °C for 18 h. The hydrolyzed solution was filtered and evaporated to dryness. The 400 μl of distilled water was added into dried sample. The solution was loaded into cellulose HPTLC plate (no.5716; Merck, Germany) and developed with MeOH: H_2O: 6 N HCl: Pyridine (80:26:4:10). Finally, the cellulose HPTLC plate was sprayed with 0.5% ninhydrin in n-butanol and heated at 100 °C form 3 min. One microlitter of 0.01 M *DL*-diamino pimelic acid (DAP) which contained *meso*-DAP and *LL*-DAP isomers was used as the standard. The DAP spot were seen as gray green fading to yellow and amino acid spot appeared purple to red coulor. *LL*-DAP spot appeared above meso-DAP spot (Staneck and Robert, 1974).

3.2.3.2 Cell wall acyl type

Dried cells (10 mg) were hydrolyzed with 100 μl of 6 N HCl at 100 °C for 2 h. The hydrolyzed solution was then loaded into Dowex (CH_3COO^- form) column (5 cm in height). The column was eluted with 400 μl distilled water and twice of 1 ml distilled water and 0.5 N HCl solution. The final fraction was added with DON reagent (Appendix II). The standard series were 0, 30, 60, and 90 nmol sodium glycolate. The sample solutions and standard were heated at 100 °C for 2 h and immediately cooled by placing tube in water. They were added with 1.9 ml of 2 N H_2SO_4, mixed well and cooled again. They were measured with spectrophotometer at 530 nm ($O.D._{530}$) and calculated. If the value of $O.D._{530}$ more than 10 nM were detected in the sample indicated that contained glycolyl muramic acid in the peptidoglycan (Uchida and Aida, 1984).

3.2.3.3 Whole-cell sugar analysis

Dried cells (50 mg) were hydrolyzed with 1 N H_2SO_4 at 100 °C for 2 h. The pH of hydrolyzed solution was adjusted with saturated $Ba(OH)_2$ into pH 5.2-5.5. The solution was then centrifuged, and the supernatant was evaporated and then added 400 μl of distilled water into the dried sample. The solution was loaded into cellulose HPTLC plate (no.5716; Merck, Germany) and developed with n-butanol: water: toluene: pyridine (10: 6: 6: 1). Finally, the cellulose

sprayed with aniline phthalate and heated at 100 °C for 4 min for detection (Komagata and Suzuki, 1987).

3.2.3.4 Polar lipid analysis

Extraction: Dried cells (150-300 mg) were added with 3 ml of MeOH: 0.3%NaCl aq. (100:10) and 3 ml of petroleum ether and mixed for 15 min. The lower layer was added with 1 ml of petroleum ether and mixed for 2-5 min. The lower layer was heated at 100 °C for 5 min and cooled immediately at 37 °C for 5 min. The suspension was added with $CHCl_3$: MeOH: H_2O (90:100:30) and mixed for 1 h. The upper layer was transferred into another tube. The lower layer was extracted again with $CHCl_3$: MeOH: H_2O (50:100:40) and the supernatant was transferred to the upper layer tube. The upper layer tube was added with 1.3 ml of chloroform and water. The final lower layer was dried with nitrogen gas (<37 °C)(Minnikin*et al.*, 1984).

Analysis of polar lipid: The polar lipid fraction was dissolved with 60 µl of Chloroform: MeOH (2:1) and applied to two-dimentionalTLCsilica-gel plate (no. 1.05633; Merck, Germany) developed with following solvent systems.

The 1st solvent system: $CHCl_3$: MeOH: H_2O (65:25:4)

The 2nd solvent system: $CHCl_3$: CH_3COOH: MeOH: H_2O (40:7.5:6:2)

Detection of the polar lipids:

(1) Dittmer and Lester reagent (Appendix II). For all phospholipids detection (Blue spot)

(2) Ninhydrin reagent (Appendix II). After spraying, heat at 110 °C for 10 min. For phosphatidylethanolamine (PE) and its derivatives (lyso-PE, OH-PE and methyl-PE) detection

(3) Anisaldehyde reagent (Appendix II). After spraying, heat at 110 °C for 10 min. For glycolipids (green-yellow spot) and other lipid (blue spot)

(4) Dragendroff's reagent (Appendix II). For choline-containing phospholipids (phosphatidyl choline) detection

(5) Phosphomolybdic acid reagent (Appendix II). After spraying, heat at 120 °C for 10 min. For all polar lipids detection

3.2.3.5 Cellular fatty acid analysis

Saponification: Dried cells (40 mg) were put into screw-cap tube and added with 1 ml of reagent 1 (Appendix II), and this suspension was shaken well. The suspension was then heated at 100 °C for 30 min and cooled to room temperature in water.

Methylation: The suspension was added with reagent 2 (Appendix II) and mixed for 5 to 10 sec with vortex mixer. The suspension was heated at 80 °C for 10 min and cooled to room temperature in water.

Extraction: The suspension was added with reagent 3 (Appendix II) and mixed for 10 min and then transferred the upper layer to another tube.

Base washing: The reagent 4 (Appendix II) was added into the suspension and mixed for 5 min, if it became to emulsion form, added the reagent 5 (Appendix II) into the suspension. The upper layer was transferred to a new vial. Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI, version 6.0) (Sasser, 1990; Kämpfer and Kroppenstedt) with the ACTIN1 MIDI database.

3.2.3.6 Isoprenoid quinones analysis

Dried cells (100-500 mg) were extracted with $CHCl_3$: MeOH (2:1) on rotary shaker for overnight. The suspension was then filtered and dried under rotary evaporator. The dried sample was dissolved with a small amount of acetone and applied onto a silica gel TLC (no.1.05744, Merck, Germany). The applied TLC was then developed by 100% benzene and the band of menaquinone was detected by using a UV lamp (254 nm). The isoprenoid quinones band was scraped and dissolved with acetone (HPLC grade). The suspension was filtered and dried with nitrogen gas. The Isoprenoid quinone samples were analyzed by HPLC. The HPLC solvent system was eluted with MeOH: 2-propanol (2:1)

3.2.3.7 Analysis of DNA base composition

Genomic DNA extraction and purification: Genomic DNA was isolated from cells grown in ISP 2 artificial seawater broth for 4-5 days according to the modified method of Tamaoka (1994). Cells were harvested and suspended in 10 ml of saline-EDTA buffer pH 8.0 (Appendix II). The cell suspension was inoculated with 20 mg of lysozyme at 37 °C for 3-4 hrs followed by the incubation period of 10 min at 50 °C with 1.0 ml of 10% SDS. The extraction was then carried out by adding an equal volume of phenol: chloroform (1:1) (Appendix II) to the

removal of protein and other debris. The upper layer of the mixture was collected after centrifugation at 10,000 rpm for 20 min. Genomic DNA was precipitated with two volumes of cold absolute ethanol. DNA was dissolved with 0.1x SSC (Appendix II) and treated with RNase A, RNase T_1 and proteinase K solution at 37°C for 1 h for removal of RNA and protein, respectively. Genomic DNA was stored in 0.1x SSC at 4°C.

DNA base composition analysis: The 10 μl of heated DNA (1 mg/ml) was hydrolyzed with 10 μl nuclease P_1 at 50 °C for 1 h and followed by the incubation period of 1 h at 37 °C with 10 μl of alkaline phosphatase. The hydrolyzed DNA was determined using the HPLC method of TamaokaandKomagata (1984). An equimolar mixture of nucleotides (YamasaShoyu, Choshi, Japan) was used as the quantitative standard for analysis of DNA base composition as shown below.

$$\text{Mol\% G+C} = \frac{(G_S/G_R+C_S/C_R)}{(A_S/A_R+G_S/G_R+C_S/C_R+T_S/T_R)}$$

A_R, peak area of adenine (standard)

A_S, peak area of adenine (sample)

C_R, peak area of cytosine (standard)

C_S, peak area of cytosine (sample)

G_R, peak area of guanine (standard)

G_S, peak area of guanine (sample)

T_R, peak area of thymine (standard)

T_S, peak area of thymine (sample)

3.2.4 Genotypic characteristics

3.2.4.1 16S rRNA gene analysis and phylogenetic tree construction

The PCR was performed in a total volume of 100 μl containing 4 μl of DNA sample, 0.5 μl of *Taq* DNA polymerase (5 unit/ml), 10 μl of 10x *Taq* buffer, 10 μl of dNTP mixture, 4 μl of 10 μM forward (20F) and reverse (1500R)primers, 8 μl of 25 mM $MgCl_2$ and 59.5 μl of Milliq water. A DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C (denaturing of DNA), 1 min at 50 °C (primer annealimg), and 2 min at 72 °C (polymerization) and a final

extension for 3 min at 72 °C. The PCR amplified products were analyzed by running 5 μl of the reaction mixture on a 0.85% agarose gel in Tris-acetate EDTA buffer (Appendix II). Agarose gel was stained in an ethidium bromide solution (0.5 mg/ml) and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rDNA band. The PCR mixtures were purified by QIAquick.

The purified 16S rRNA gene was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) with a temperature profile of 3 sec at 96 °C followed by 25 cycles of 10 sec at 96 °C (denaturing of DNA), 5 sec at 50 °C (primer annealimg), and 4 min at 60 °C (polymerization). Sequencing for each sample is carried out in both forward and reverse directions with the following primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), 518F (5′-CCAGCAGCCGCGGTAATACG-3′), 800R (5′-GGYTACCTTGTTACGACTT-3′) and 1492R (5′-TACCAGGGTATCTAATCC-3′).

The values for sequence similarity to all recognized species were first determined using the EzTaxon-e database (Kim *et al.*, 2011) and was performed using the standard BLAST sequence similarity searching program version 2.2.1 that against previously reported sequences at the GenBank/EMBL/DDBJ databases. Then, the sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods (Fitch, 1972) in the MEGA 5 software (Tamura *et al.*, 2011). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

3.2.4.2 DNA-DNA hybridization

DNA-DNA hybridization was conducted in microdilution-well plates (Ezaki *et al.*, 1989). DNA–DNA relatedness (%) was determined using the colorimetric method (Verlander, 1992). Fifteen microliter of photobiotin and 10 μl of genomic DNA (1 mg/ml) were mixed in an

microtube and irradiated with sun lamp (500 W) for 25 min. They were added with each 100 μl of 0.1 M tris-HCl buffer pH 9.0 (Appendix II) and *n*-butanol, after that centrifuged at 12,000 rpm for 20 sec. The supernatant was discarded and the lower layer was boiled for 15 min that immediately cooled on ice and sonicated for 3 min. the photobiotinylated DNA solution was mixed with hybridization solution (Appendix II) for used as a probe solution.

Ten micrograms of DNA from each unknown strains, type strains and reference DNA (Calf thymus) were boiled for 10 min and then immediately cooled on ice. They were added with 500 μl of 2x PBS (Appendix II), 100 μl of 0.1 M $MgCl_2$ and filled up to total volume 1 ml with distilled water. One hundred microliter of denatured DNA solution was added to microdilution wells (Nunc-Immuno™ Plate: MaxiSorp™ surface) and fixed by incubation at 37 °C for 2 h, after that the solution was discarded. The fixed single standard DNA was added with 100 μl of the photobiotinylated DNA solution and incubated for 15-16 hrs.

The solution in microdilution plate was removed and washed with 200 μl 0.02x SSC for 3 times. One hundred microliter of solution 1 (Appendix II) was added to each well and incubated at room temperature for 10 min. The solution 1 (Appendix II) was discarded and replaced with 100 μl of solution 2 (Appendix II) and incubated at 37 °C for 30 min. The solution 2 (Appendix II) was discarded and washed with 200 μl of 1x PBS for 3 times. One hundred microliter of solution 3 (Appendix II) was added and incubated at 37 °C for 15 min. The enzyme reaction was stopped using 100 μl of 2 M H_2SO_4. The fluorescence intensity was measured at 450 nm by microplate reader and was calculated for the value of percentage DNA homology [% DNA similarity = 100 × (sample DNA-calf thymus) / (type strain DNA-calf thymus)].

3.3 Screening for biological activities

The biological activities were conducted by Bioassay Laboratory at National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.

3.3.1 Antibacterial activity

Antibacterial activity against *Bacillus cereus* was tested by resazurin microplate assay (REMA) (Sarker *et al.*, 2007). Minimum inhibitory concentration (MIC) represents the lowest concentration that inhibits the growth of bacteria. Vancomycin and 0.5% DMSO were used as positive and negative controls, respectively.

3.3.2 Antifungal activity

Antifungal activity against *Candida albicans* was tested by resazurinmicroplate assay (REMA) (Sarker*et al.*, 2007). Inhibition concentration (IC_{50}) represents the concentration, which causes 50% growth reduction of the fungus. Amphotericin B and 0.5% DMSO were used as positive and negative controls, respectively.

3.3.3 Antimalarial activity

Antimalarial assay against *Plasmodium falciparum* (K1, multidrug resistant strain) was performed in accordance with the microculture radioisotope technique (Desjardins *et al.*, 1979). Inhibition concentration (IC_{50}) represents the concentration, which causes 50% reduction in parasite growth. Dihydroartemisinin and mefloquine were used as standard references.

3.3.4 Antitubercular activity

Antituberculosis against *Mycobacterium tuberculosis* strain H37Ra was evaluated by green fluorescent protein microplate assay (GFPMA) (Changsen*et al.*, 2003). Minimum inhibitory concentration (MIC) represents the lowest concentration that inhibits the growth of bacteria.Isoniazid, ofloxacin, rifampicin, streptomycin and ethambutol were used as standard references.

3.3.5 Cytotoxicity against cancer cells

Cytotoxicity against KB (humanoral epidermoid carcinoma, ATCC CCL-17), MCF-7 (human breastcancer, ATCC HTB-22), and NCI-H187 cells (human small-cell lungcancer, ATCC CRL-5804) were evaluated by using the resazurinmicroplate assay (REMA) described by O'Brien *et al.* (O'Brien *et al.*, 2000).Inhibition concentration (IC_{50}) represents the concentration, which causes 50% growth reduction of tested cell.Ellipticine was used as a reference for cytotoxicity against KBand NCI-H187 and doxorubicin was used as a reference forcytotoxicity against KB, MCF-7, and NCI-H187. Tamoxifenwas also used as reference for cytotoxicity against MCF-7.

3.3.6 Cytotoxic activity against Vero cell

Cytoxicity against Vero cells (African green monkey kidney fibroblasts; ATCC CCL-81) was evalulated by the green fluorescent protein microplate assay (GFPMA) (Changsen*et al.*, 2003). Inhibition concentration (IC_{50}) represents the concentration, which causes 50% growth reduction of Vero cell. Ellipticine was used as a reference for cytotoxicity against Vero cell assay.

3.4 Chemical profiles analysis

Crude extracts from marine actinobacteria were dissolved in methanol (10 mg/ml) and the solution was analyzed by HPLC (UltiMate 3000, DIONEX), with linear gradient system (CH_3CN/H_2O; 0-100%) at the flow rate 0.5 ml/min for 15 min, using a C-18 column (3 μm), 2×55 mm (Purospher®STAR; Merck).

3.5 Fermentation of the selected strains for secondary metabolites production

For the primary screen, a total of 36 marine actinobacterial strains were cultured in 500 ml Erlenmeyer flasks, each contained 200 ml of medium (ISP 2 artificial seawater). The cultures were activated on a rotary shaker at 200 rpm at 30 °C for 14 days. The cultures were extracted by EtOAc and the crudes were analyzed by HPLC and tested for biological activities.

For the secondary metabolite production, the selected marine actinobacterial strain was cultivated in 250 ml Erlenmeyer flask, each contained 100 ml of a seed medium (ISP 2 artificial seawater). Seed culture was cultivated on a rotary shaker at 200 rpm at 30 °C for 4 days. For production scale (20 l), 10% of seed culture was transferred into 1 l Erlenmeyer flask, contained 250 ml of production medium (ISP 2 artificial seawater) for 80 flasks (20 l). The strain was cultivated on rotary shaker (200 rpm) at 30 °C for 14 days.

3.6 Extraction and isolation of secondary metabolites

The small scale fermentation, culture broth of all strains was separated from the mycelium by centrifugation at 5000 rpm, 10 °C for 15 min. The broth was extracted three times with equal volume of ethyl acetate, the combined ethyl acetate solution were dried over Na_2SO_4 and evaporated under vacuum at 40 °C to dryness (EtOAc extract). The mycelium was macerated in methanol and then the methanol extract were evaporated to dryness (MeOH extract). Ethyl acetate and methanol crude extracts were screened for biological activities and chemical profiles.

Three marine actinobacterial strains (S6-1, S8-12 and TV1-14) were selected by biological activities and HPLC analyses. All selected strains were cultured for the large scale fermentation (20 l), which were extracted with equal volume of EtOAc for three times. The combined EtOAc solution was dried over Na_2SO_4 and evaporated under vacuum at 40 °C to dryness. Crude extract was purified by chromatographic techniques.

Marine actinobacteria, selected for the large scale fermentation, gave the good results for both of biological activities and chemical profiles. The chemical studies including isolation, purification, and structure elucidation of secondary metabolites were shown in Fig. 3.1.

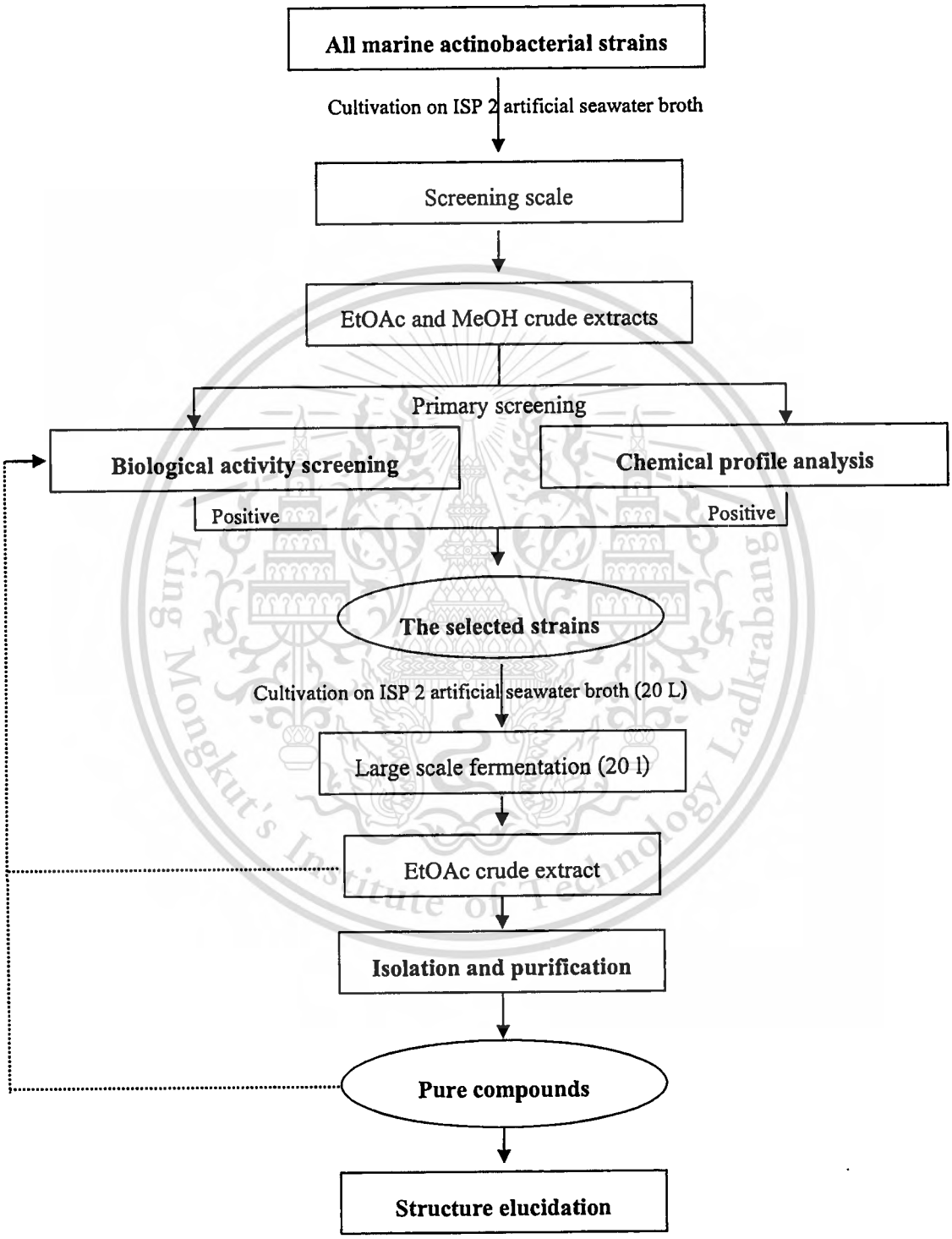


Fig. 3.1 The flow chart of marine actinobacteria selection and secondary metabolite identification process

3.6.1 Extraction, isolation and purification of secondary metabolites from *Streptomyces* sp. S6-1 (BCC 45596)

The EtOAc crude extract (1.58 g) was separated by Sephadex LH-20 column (3.5 cm × 30 cm), eluted with 100 % MeOH to give five fractions (S6F1-S6F5). All fractions were subjected to chemical profiles and ^{1}H NMR analyses, indicating fraction S6F1 and S6F4 did not contain peak at aromatic region. Therefore, no further purification was carried out for S6F1 and S6F4.

Fraction S6F2 was re-separated by Sephadex LH-20 (2.5 cm × 82 cm). The column was eluted with 100% MeOH to yield twelve fractions. All fractions were analyzed by HPLC and ^{1}H NMR spectrum, indicating that fractions S6F2-1 and S6F2-2 contained long chain hydrocarbons and fractions S6F2-5, S6F2-8, S6F2-9, S6F2-10, and S6-2-11 gave low yield with many compounds. Therefore, no further purification was done to these fractions. In addition, the fraction S6F2-3, S6F2-4, S6F2-6, S6F2-7 and S6F2-12 were further investigated. Fraction S6F2-3 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm). The column was eluted with a gradient 37-38% CH_3CN in H_2O for 30 min (at flow rate 10 ml/min) to afford compound S6.1A (3.8 mg) and S6-1B (0.6 mg). Fraction S6F2-4 was separated by Sephadex LH-20 (1.9 cm ×102 cm), eluted with 100% MeOH, to give two fractions (S6F2-4-2 and S6F2-4-3). Fractions S6F2-4-2 and S6F2-4-3 were subjected to semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm), eluted with a gradient 15-50% CH_3CN in H_2O for 30 min at the flow rate 10 ml/min to obtain compounds S6.1A (5.9 mg) and S6.1A (28.1 mg), S6.1B (3.8 mg), respectively. Fraction S6F2-6 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm×150 mm, flow rate 10 ml/min). The column was eluted with a gradient system of 15-85% CH_3CN in H_2O to yield compounds S6.1C (3.5 mg) and S631D (1.8 mg). Fraction S6F2-7 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm×150 mm, flow rate 10 ml/min), eluted with a gradient 50-90% CH_3CN in H_2O for 30 min to furnish compound S6.1C (1.4 mg).

Fraction S6F3 was applied to a silica gel column (2 cm ×15 cm, silica gel 60; Merck), eluted with EtOAc:Hexane (3:2) to give seven fractions (S6F3-1 to S6F3-7). All fractions were analyzed by HPLC and ^{1}H NMR, indicating that all fractions except fraction S6F3-7 gave low yield. Therefore, no further purification was done for fractions S6F3-1 – S6F3-6. Fraction S6F3-7 was further purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm ×150 mm, flow rate 10 ml/min), eluted with gradient system of 40-70% CH_3CN in H_2O for 30 min to furnish compound S6.1C (1.2 mg).

Purification of fraction S6F5 by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm ×150 mm, flow rate 10 ml/min, eluted with 40-70% CH_3CN in H_2O for 30 min) yielded compound S6.1D (3.0 mg). The isolation process of *Streptomyces* sp. S6-1 was summarized in Fig. 3.2.

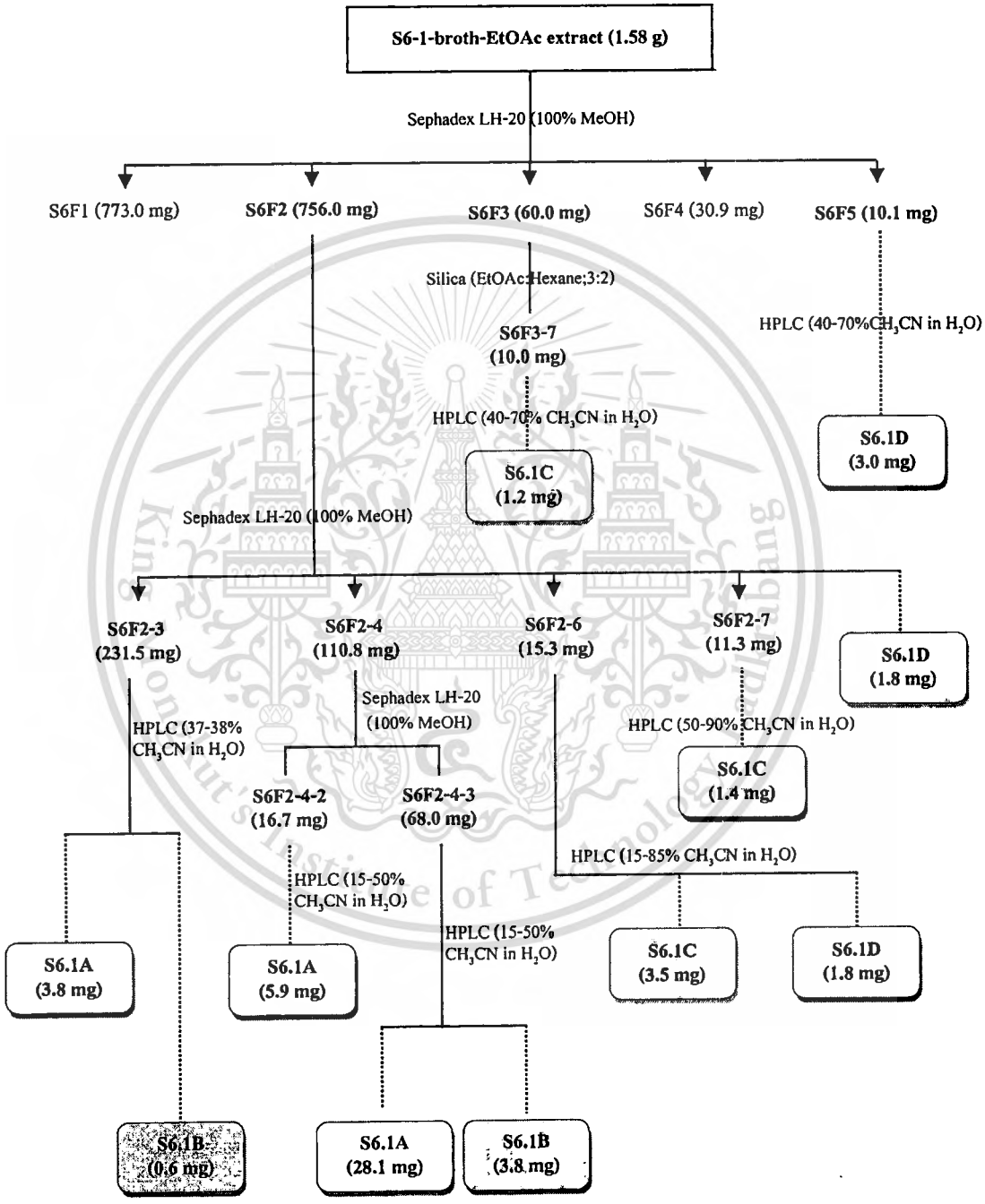


Fig. 3.2 Isolation of secondary metabolites from the crude extract of *Streptomyces* sp. S6-1

For the re-cultivation (20 l) of *Streptomyces* sp. S6-1, the EtOAc crude extract (2.80 g) was separated by using Sephadex LH-20 column (3.5 cm × 49 cm). The column was eluted with 100% MeOH to afford six fractions (RS6F1 to RS6F6). All fractions were analyzed by HPLC and ^{1}H NMR spectrum, indicating that fractions RS6F3 contained long chain hydrocarbons and fractions RS6F1, RS6F4, and RS6F6 gave low yield with many compounds. Therefore, no further purification was done to these fractions. Fraction RS6F2 was re-separated through a Sephadex LH-20 column (100% MeOH as eluent) to provide four fractions (RS6F2-1 to RS6F2-4). Purification of fraction RS6F2-2 by Silica-gel column (3.0 cm × 60 cm, silica gel 60; Merck) was eluted with 5% CH_3OH in CH_2Cl_2 to give compound S6.1E (157.02 mg). Fraction RS6F2-4 was subjected to semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm; flow rate 10 ml/min gradient, eluted with 15-50% CH_3CN in H_2O for 30 min) to provide compound S6.1A (2.85 mg). Fraction RS6F5 was purified by a Sephadex LH-20 column (2.5 cm × 60 cm, eluted with 100% MeOH) to obtain compound S6.1C (7.34 mg). The isolation process of the crude extract of the re-cultivation from *Streptomyces* sp. S6-1 was shown in Fig. 3.3.

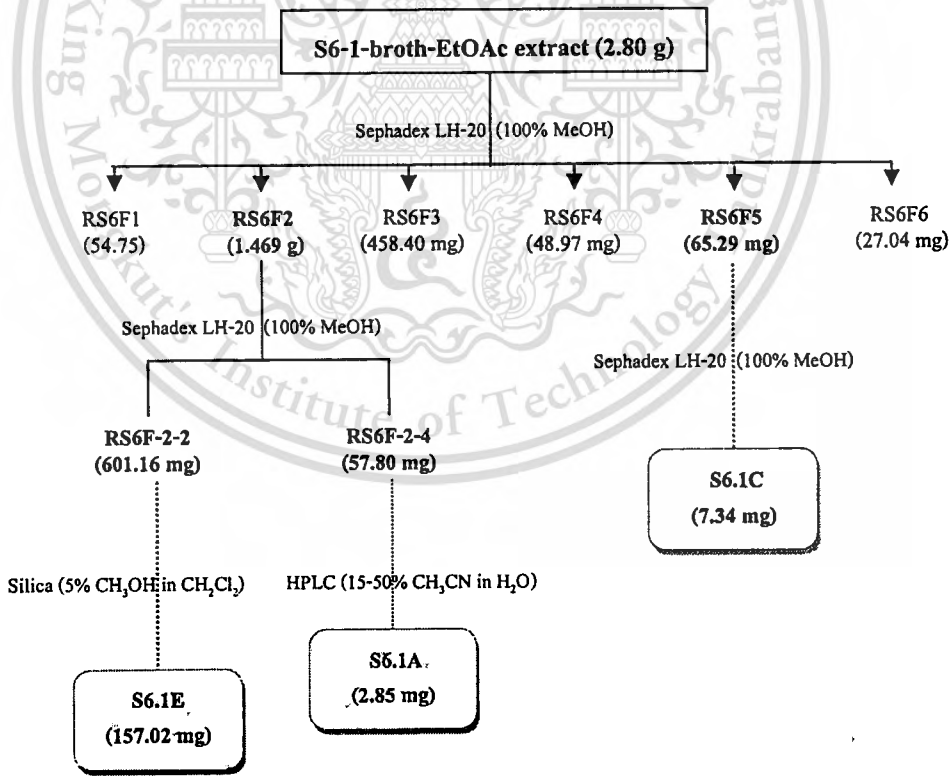


Fig. 3.3 Isolation of secondary metabolites from re-cultivation of *Streptomyces* sp. S6-1

Compound S6.1A (urdamycinone E)

Red amorphous powder

Melting point (°C)	203-205 (dec)
CD λ_{max} ($[\theta]_{25}$)	255 (+13247), 282 (+2036), 296 (+4889), 367 (+1024), 427 (-916), 513 (-981) nm
UV (CH_3OH) λ_{max} (log ε)	231 (4.40), 270 (3.49), 316 (4.24), 428 (3.85) nm
IR, ν_{max} (cm^{-1})	3343, 2920, 1720, 1628, 1579, 1510, 1427, 1286, 1054
HRESIMS *m/z*	555.1299 $[M+Na]^+$(calcd for $C_{26}H_{28}O_{10}SNa$, 555.1295)

Compound S6.1B (urdamycinone G)

Red solid

Melting point (°C)	190-192 (dec)
Optical rotation, $[\alpha]_D^{25}$	-106.16 (c. 0.0250%, $CHCl_3$)
UV (CH_3OH) λ_{max} (log ε)	222 (4.27), 264 (4.18), 292 (4.10), 483 (3.88) nm
IR, ν_{max} (cm^{-1})	3324, 2921, 1627, 1510, 1428, 1253, 1055
HRESIMS *m/z*	537.1191 $[M+Na]^+$ (calcd for $C_{26}H_{26}O_9SNa$, 537.1190)

Compound S6.1C (dehydroxyaquayamycin)

Brown solid

Melting point (°C)	270-272
Optical rotation, $[\alpha]_D^{25}$	+71.24 (c. 0.0200%, $CHCl_3$)
UV (CH_3OH) λ_{max} (log ε)	231(4.40), 270 (3.49), 316 (4.24), 428 (3.85) nm
IR, ν_{max} (cm^{-1})	3372, 2920, 2849, 1619, 1582, 1433, 1265, 1064, 802
HRESIMS *m/z*	457.1264 $[M+Na]^+$ (calcd. for $C_{25}H_{22}O_7Na$, 457.1258)

Compound S6.1D (5-aminodehydroxyaquayamycin)

Green solid

Optical rotation, $[\alpha]_D^{25}$	+98.00 (c. 0.0250%, $CHCl_3$)
UV (CH_3OH), λ_{max} (log ε)	229 (4.35), 288 (4.01), 342 (4.28), 427 (3.84), 584 (3.68) nm
IR, ν_{max} (cm^{-1})	3367, 3233, 2918, 1627, 1502, 1433, 1298, 1278, 1069
HRESIMS *m/z*	448.1400 $[M-H]^-$ (calcd. for $C_{25}H_{22}O_7N$, 448.1402)

Compound S6.1E (urdamycin E)

Red solid

Melting point (°C)	230-232
Optical rotation, $[\alpha]_D^{25}$	+18.9 (c. 0.100%, CH_3OH)
UV (CH_3OH) λ_{maxn} (log ε)	219 (4.18), 270 (3.49), 290 (3.96), 466 (5.12) nm
IR, ν_{max} (cm^{-1})	3401, 2973, 2932, 1729, 1630, 1515, 1431, 1371, 1286, 1255, 1187, 1119, 1059, 975
HRESIMS *m/z*	913.3273 $[M+Na]^+$ (calcd. for $C_{44}H_{58}O_{17}SNa$, 913.3287)

3.6.2 Extraction, isolation and purification of secondary metabolites from *Micromonospora* sp. S8-12 (BCC 45599)

The culture (20 l) of *Micromonospora* sp. S8-12 was extracted with EtOAc, and the EtOAc layer was dried over Na_2SO_4 and evaporated to dryness. The EtOAc crude extract (1.95 g) was fractionated by Sephadex LH-20 column (3.5 cm × 49 cm), eluted with 100% MeOH to provide six fractions (S8F1-S8F6). All were analyzed by HPLC and ^1H NMR, only fraction S8F4 was further investigated due to fractions, S8F2, S8F3, S8F5, and S8F6 contained long chain hydrocarbons and fraction S8F1 gave low yield with many compounds, which was not further purified.

Fraction S8F4 was separated by a Sephadex LH-20 column (2.5 cm ×60 cm). The column was eluted with 100% MeOH to give five fractions (S8F4-1-S8F4-5). On the basis of chemical profiles and ^1H NMR analyses, fractions S8F4-3, S8F4-4, and S8F4-5 were further investigated. Fractions S8F4-1 and S8F4-2 were not further purified due to contained long chain hydrocarbons.

Fraction S8F4-3 was further purified by a Sephadex LH-2 column (2 cm × 90 cm, 100% MeOH as eluent) and followed by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm; eluted with gradient system of 2-45% CH_3CN in H_2O over for 30 min at a flow rate of 10 ml/min) to provide compound S8.12B (1.89 mg). Separation of fraction S8F4-4 using a Sephadex LH-20 column (2 cm × 90 cm, 100% MeOH as eluent) yielded four fractions (S8F4-4-1 - S8F4-4-4). Fractions S8F4-4-1 and S8F4-4-2 were not carried out due to gave low yield with many compounds. Fractions S8F4-4-3 and S8F4-4-4 were further purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm; 10 ml/min gradient elution from 35-80% CH_3CN in H_2O for 30 min) to give compounds S8.12B (1.66 mg) and S8.12A

(2.14 mg), respectively. Fraction S8F4-5 was purified by a Sephadex LH-20 column (2 cm × 90 cm, 100% MeOH as eluent) and followed by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm, eluted with gradient system of 5-40% CH_3CN in H_2O at a flow rate of 10 ml/min) yielded compound S8.12C (5.27 mg). The isolation process of the crude extract from *Micromonospora* sp. S8-12 was shown in Fig. 3.4.

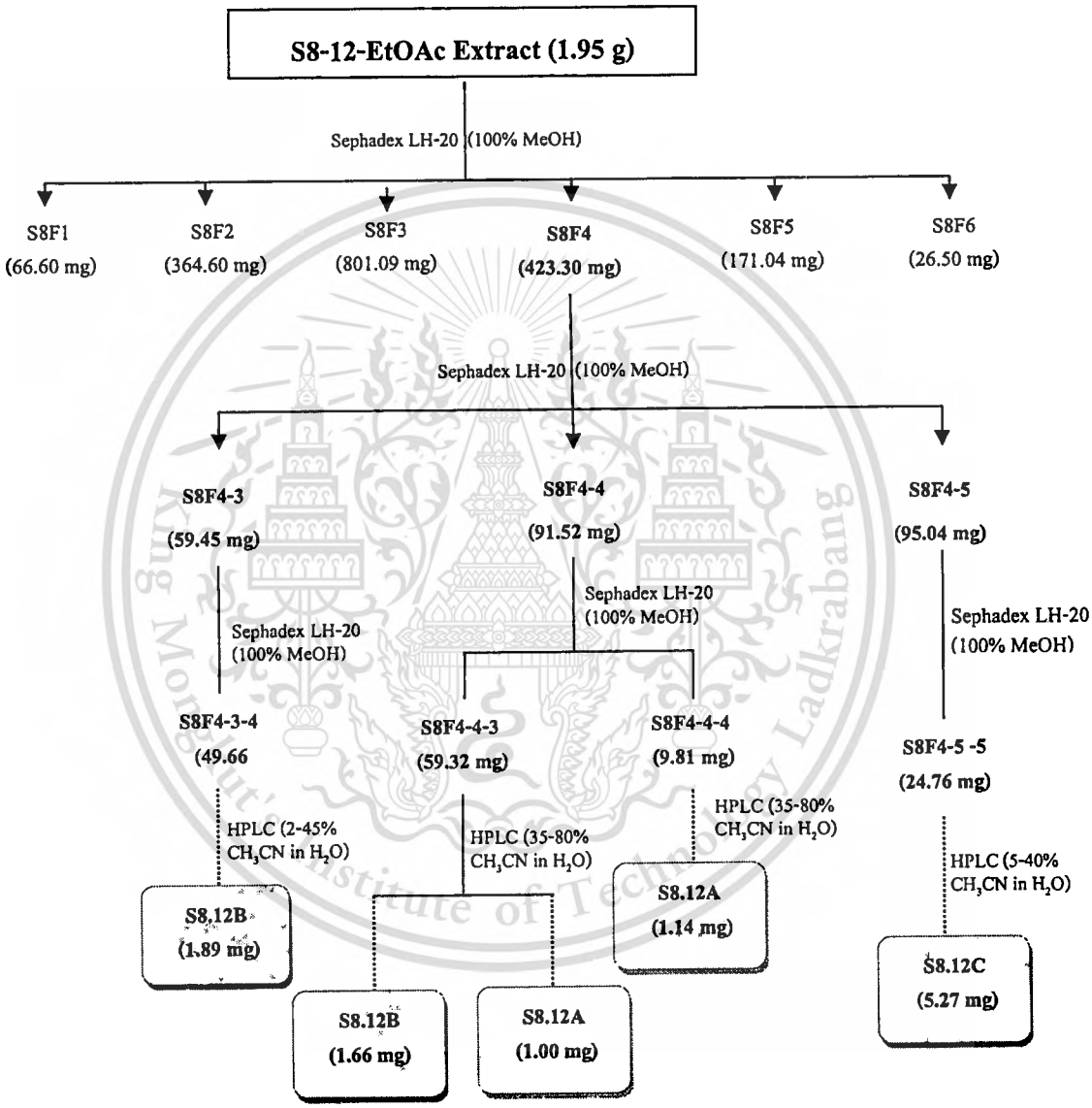


Fig. 3.4 Isolation of secondary metabolites from the crude extract of *Micromonospora* sp. S8-12

Compound S8.12A (7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione)

Yellow powder

Optical rotation, $[\alpha]_D^{25}$	-68.2 (c. 0.1050%, $CHCl_3$)
UV (CH_3OH), λ_{max} (log ε)	251(4.17), 272 (4.02) nm
IR, ν_{max} (cm^{-1})	3488, 2956, 2924, 2852, 1660, 1616, 1465, 1389, 1290, 1069, 794, 752
ESIMS *m/z*	349.2 $[M-H]^-$ ($C_{22}H_{22}O_4$)

Compound S8.12B (6,8-dihydroxy-3,4,5-trimethylisocoumarin)

Reddish brown amorphous solid

Optical rotation, $[\alpha]_D^{25}$	+88.0 (c. 0.1550%, CH_3OH)
UV (CH_3OH), λ_{max} (log ε)	231(3.84), 268 (3.88), 308 (3.71) nm
IR, ν_{max} (cm^{-1})	3292, 2925, 2854, 1644, 1621, 1397, 1318, 1265, 1242, 1163, 1107, 1003, 854, 749, 668
HRESIMS *m/z*	221.0811 $[M-H]^-$(calcd. for $C_{12}H_{13}O_4$, 221.0819)

Compound S8.12C (2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid)

Yellow amorphous solid

Optical rotation, $[\alpha]_D^{25}$	-19.8 (c. 0.1500%, CH_3OH)
UV (CH_3OH), λ_{max} (log ε)	221(3.81), 251 (3.72), 314 (3.29) nm
IR, ν_{max} (cm^{-1})	2956, 2927, 2854, 1740, 1638, 1585, 1462, 1271, 1187, 1054
HRESIMS *m/z*	239.0915 $[M-H]^-$(calcd. for $C_{12}H_{15}O_5$, 239.0925)

3.6.3 Extraction, isolation and purification of secondary metabolites from *Actinomadura* sp. TV1-14 (BCC 45631)

The culture (20 l) of *Actinomadura* sp. TV1-14 was extracted with an equal volume of EtOAc, and EtOAc was combined and evaporated to dryness. The crude extract (0.65 g) was fractionated by using a Sephadex LH-20 column (2.5 cm × 60 cm), eluted with 100% MeOH to give eight fractions (TVF1-TVF8). After ^{1}H NMR and HPLC analyses, the fractions TVF6 and TVF7 were further investigated. Fraction TVF6 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm, flow rate 10 ml/min, eluted with 10-60% CH_3CN in H_2O) to yield compounds TV14A (4.72 mg) and TV14B (3.05 mg). Fraction TVF7 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm, flow rate 10 ml/min, eluted with 10-60% CH_3CN in H_2O) to yield compound TV14C (2.15 mg). The isolation process of the crude extract from *Actinomadura* sp. TV1-14 was shown in Fig.3.5.

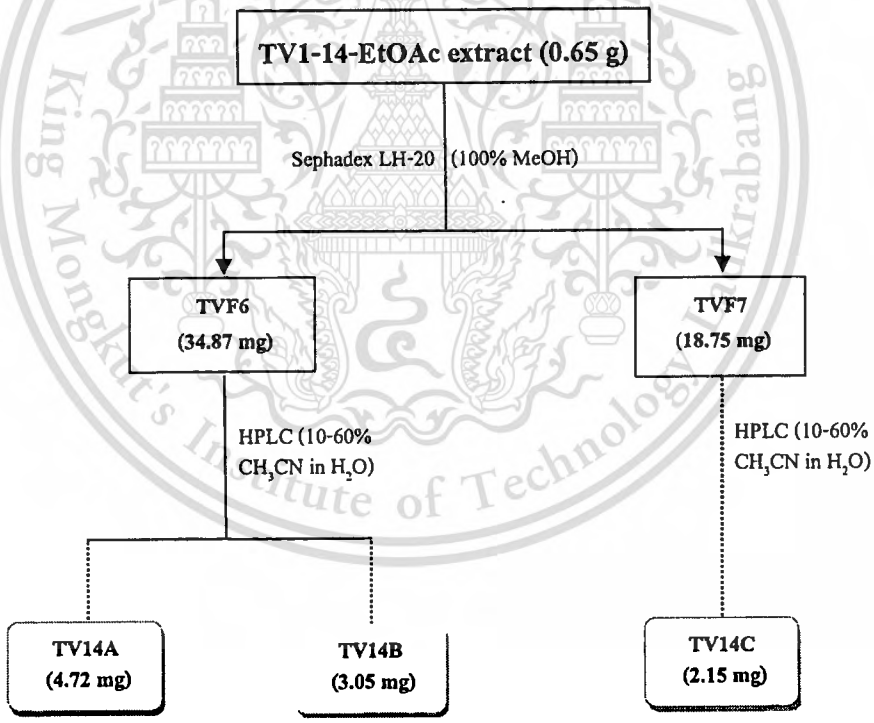


Fig. 3.5 Isolation of secondary metabolites from the crude extract of *Actinomadura* sp. TV1-14

The re-cultivation of *Actinomadura* sp. TV1-14 gave the crude extract (1.60 g), after extraction process. The crude extract was separated by using a Sephadex LH-20 column (3.5 cm × 52 cm). The column was eluted with 20% CH_2Cl_2 in MeOH to give eleven fractions (TVRF1-TVRF11). After HPLC and ^1H NMR analyses, fractions TVRF3, TVRF6, TVRF7, TVRF 8, TVRF9, TVRF10 (contained TV14B), and TVRF11 were further investigated.

Fraction TVRF3 was purified by semi-preparative HPLC (Shiseido C_{18} column, 5μm, 20 mm × 150 mm) , eluted with a gradient system of 30-70% CH_3CN in H_2O with additional 0.05% HCOOH at the flow rate of 10 ml/min. After evaporated to dryness, 11.50 mg of compound TV14B was obtained. Fraction TVRF6 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm, flow rate 10 ml/min, eluted with 30-75% CH_3CN/H_2O in the presence of 0.05% HCOOH) to give compounds TV14A (6.8 mg) and TV14B (2.2 mg). Purification of fraction TVRF7 by using semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm, flow rate 10 ml/min, eluted with 30-70% CH_3CN/H_2O that contained 0.05% HCOOH) yielded compound TV14A (1.9 mg). Fraction TVRF8 was separated by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm). The column was eluted with a gradient system of 30-70% CH_3CN in H_2O, that contain 0.05% HCOOH at the flow rate of 10 ml/min, to yield compounds TV14A (62.1 mg) and TV14C (2.5 mg). Fraction TVRF 9 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm, 10 ml/min, eluted with 30-75% CH_3CN/H_2O, that contained 0.05% HCOOH) to yield compounds TV14A (4.0 mg), TV14B (4.2 mg), TV14C (10.5 mg), and TV14D (3.2 mg). Fraction TVRF11 was purified by semi-preparative HPLC (Shiseido C-18 column, 5 μm, 20 mm × 150 mm). The column was eluted with a gradient system of 30-75% CH_3CN in H_2O that contained 0.05% HCOOH at the flow rate of 10 ml/min to provide compound TV14B (4.0 mg). The isolation process of the crude extract of the re-cultivation from *Actinomadura* sp. TV1-14 was shown in Fig. 3.6.

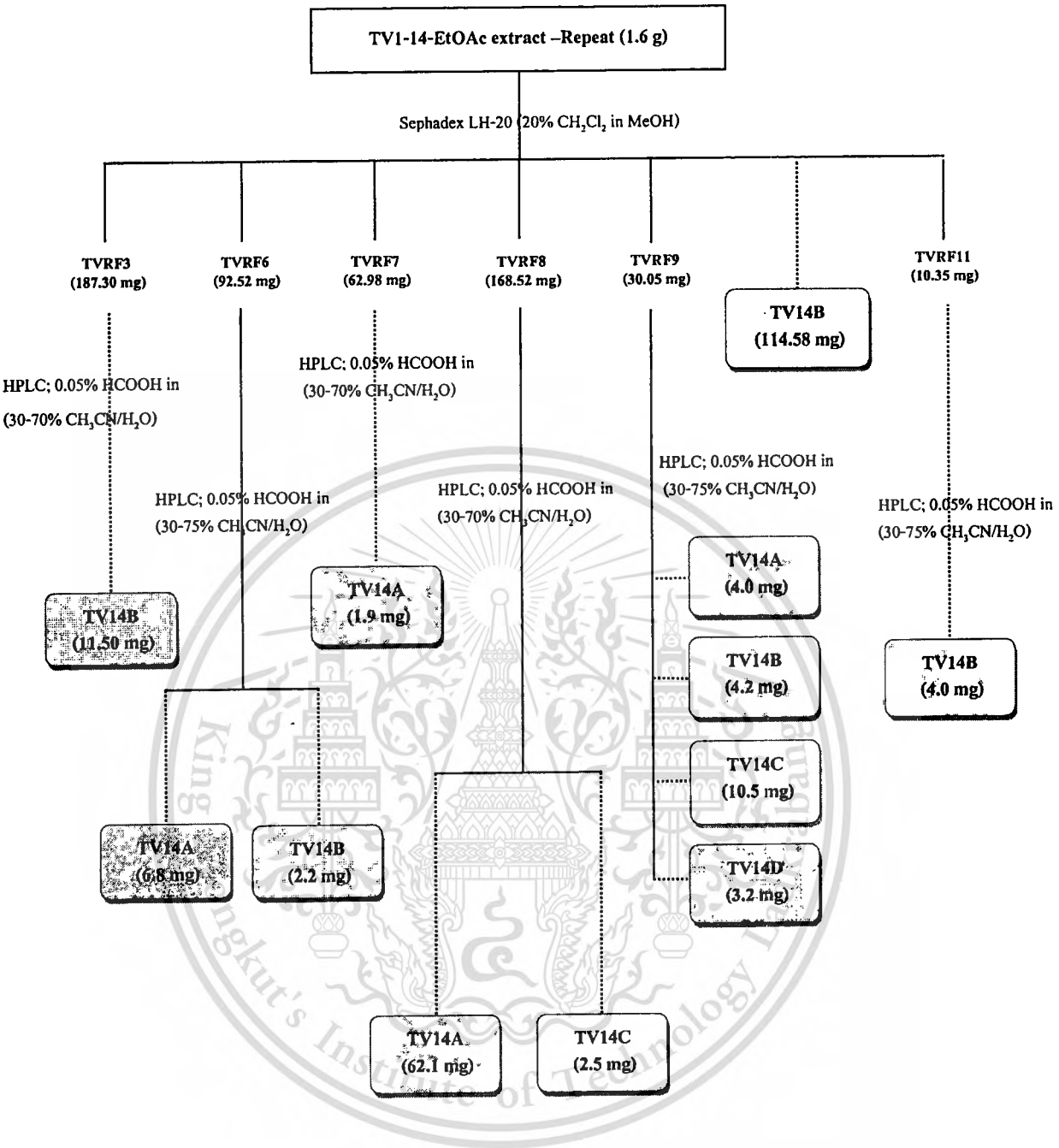


Fig. 3.6 Isolation of secondary metabolites from the re-cultultivation of *Actinomadura* sp. TV1-14

Compound TV14A (9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid)

Brownish yellow amorphous solid

Optical rotation, $[\alpha]_D^{27}$	-4.9 (c. 0.1700%, EtOH)
UV (CH_3OH), λ_{max} (log ε)	220 (4.13), 316 (4.00) nm
IR, ν_{max} (cm^{-1})	3479, 2924, 2854, 1681, 1616, 1571, 1419,1337, 1256, 1184, 1095, 828
HRESIMS *m/z*	351.0831 $[M+Na]^+$ (calcd. for $C_{18}H_{16}O_6Na$, 351.0839)

Compound TV14B (oxanthromicin)

Brownish yellow solid

Melting point (°C)	198-201 (dec)
Optical rotation, $[\alpha]_D^{24}$	-117.7 (c. 0.0975%, EtOH)
UV (CH_3OH), λ_{max} (log ε)	220 (4.37), 314 (4.24) nm
IR, ν_{max} (cm^{-1})	2925, 2854, 1626, 1421, 1335, 1257, 1194, 1078, 887, 827
HRESIMS *m/z*	653.1667 $[M-H]^-$ (calcd. for $C_{36}H_{29}O_{12}$, 653.1664)

Compound TV14C (3,8-dihydroxy-1,7-dimethylanthraquinone-2-carboxylic acid)

Yellow solid

Melting point (°C)	179-182
UV (CH_3OH), λ_{max} (log ε)	222 (3.90), 283 (3.90), 414 (3.36) nm
IR, ν_{max} (cm^{-1})	2924, 2854, 1627, 1583, 1432, 1318, 1268, 1252, 1195, 1021, 754, 670
HRESIMS *m/z*	311.0567 $[M-H]^-$ (calcd. for $C_{17}H_{11}O_6$, 311.0561)

Compound TV14D (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid)

Yellow solid

Melting point (°C)	184-186
UV (CH_3OH), λ_{max} (log ε)	220 (4.14), 281(4.18), 410 (3.69) nm
IR, ν_{max} (cm^{-1})	2924, 2854, 1712, 1454, 1256, 1042
HRESIMS *m/z*	297.0400 $[M-H]^-$ (calcd. for $C_{16}H_9O_6$, 297.0405)

3.7 Instruments for structure elucidation of secondary metabolites

3.7.1 Ultraviolet-Visible (UV-vis) absorption spectroscopy

UV-vis spectra were recorded on a Cary 1E UV–vis spectrophotometer in 100% MeOH or 95% EtOH.

3.7.2 Infrared (IR) absorption spectroscopy

IR spectra were taken on an Alpha FT-IR spectrometer from Bruker.

3.7.3 Optical rotations

Optical rotations were performed on a JASCO model P-1080 polarimeter that equipped with a 1 ml cell.

3.7.4 CD spectropolarimetry

CD spectra were measured on a JASCO model J-810 polarimeter.

3.7.5 Melting points

Melting points were taken on an Electrothermal IA9100 digital melting point apparatus.

3.7.6 Nuclear magnetic resonance spectroscopy

NMR spectra including ^{1}H, ^{13}C, DEPT 135, COSY, NOESY, HMQC and HMBC experiments were recorded on Bruker ADVANCE 500 MHz (^{1}H at 500 MHz and ^{13}C at 125 MHz) and Bruker ADVANCE III 400 MHz (^{1}H at 400 MHz and ^{13}C at 100 MHz) NMR spectrometers.

3.7.7 Mass spectrometry

MicrOTOF from Bruker was employed for electrospray ionization mass spectra (ESIMS) and high resolution electrospray ionization mass spectra (HRESIMS) to determine the mass (*m/z*) of all compounds.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of marine actinobacterial strains

Thirty-six marine actinobacterial strains were isolated from eighteen marine samples collected from the Andaman Sea and the Gulf of Thailand. Sixteen marine actinobacterial strains were isolated from the marine sponges. Three marine actinobacterial strains were isolated from two unknown marine algae. In addition, seventeen strains were isolated from seven marine sediment samples (Table 4.1).

Table 4.1 Actinobacteria from marine sources of Thailand

Nature of samples	Sample No.	Depth (m)	Locations	Strains
Marine sponges	S3	5	Sichang Island, Chonburi Province	S3-1
	S6	5	Sichang Island, Chonburi Province	S6-1
	S18	5	Sichang Island, Chonburi Province	S18-3
	SP206	5	Sichang Island, Chonburi Province	SP206-02, SP206-03, SP206-17
	SP207	5	Koh Pan Yue Island, Chonburi Province	SP207-05, SP207-08
	SP209	5	Sichang Island, Chonburi Province	SP209-09
	SP03	5	Lam Panwa, Phuket Province	SP03-01, SP03-05
	ST01	3	Lam Panwa, Phuket Province	ST01-03, ST01-07, ST01-08, ST01-09
	SH4	3	75 million-year shell cemetery (Susan Hoi), Krabi Province	SH4-3
Marine algae	MA3	3	Nangyuan Island, Suratthani Province	MA3-23
	SH3	3	75 million-year shell cemetery (Susan Hoi), Krabi Province	SH3-2, SH3-3
Marine sediments	AN5	2	Ao Nang, Krabi Province	AN5-1, AN5-12, AN5-16, AN5-55
	S8	2	Tao Island, Suratthani Province	S8-04, S8-07, S8-12
	S15	5	Sichang Island, Chonburi Province	S15-3
	S20	5	Sichang Island, Chonburi Province	S20-7
	SH2	3	75 million-year shell cemetery (Susan Hoi), Krabi Province	SH2-1, SH2-7, SH2-13, SH2-15
	AN6	3	Ao Nang, Krabi Province	AN6-3, AN6-30
	TV1	3	Talay-Wak, Krabi Province	TV1-14, TV1-16

Marine actinobacterial strains were isolated using humic acid vitamin artificial seawater agar (HV) and starch casein nitrate artificial seawater agar (SCN) supplemented with cycloheximide, nystatin and nalidixic acid. Most strains in group I (*Micromonospora*) were isolated by SCN isolation medium. In the group of *Streptomyces* (group VIII), mostly were isolated from HV isolation medium (Table 4.2). All strains were preserved in 15% glycerol (-80 °C) for further study.

Table 4.2 Marine actinobacteria from the differential isolation methods and isolation media

Samples	Treatments	Isolation media	Strains	Genera
Sponges	Wet samples, ground and homogenized in sterile seawater	HV	S6-1	*Streptomyces*
			SP207-05, SP209-09	*Salinispora*
		SCN	S3-1, S18-3, SP206-17, ST01-03, ST01-08, ST01-09, SH4-3	*Micromonospora*
			SP207-08	*Salinispora*
			SP03-01, SP03-05, SP206-02, SP206-03	*Verrucosispora*
			ST01-07	*Nocardia*
Algae	Wet samples, ground and homogenized in sterile seawater	HV	SH3-3	*Actinomycetospora*
		SCN	MA3-23, SH3-2	*Micromonospora*
Sediments	Air-dry at room temperature	HV	S8-04 , S20-7, SH2-1	*Streptomyces*
			S15-3	*Pseudonocardia*
	Incubation the sample at 70 °C for 15 min	HV	AN5-12, AN5-55	*Micromonospora*
		SCN	AN5-1, SH2-7, SH2-13, AN6-3, AN6-30	*Micromonospora*
			SH2-15, TV1-16	*Actinomycetospora*
			TV1-14	*Actinomadura*
			AN5-16	*Streptomyces*
	Dried heat at 100 °C for 1 h	HV	S8-12	*Micromonospora*
		SCN	S8-07	*Micromonospora*

4.2 Identification of marine actinobacterial strains

All actinobacterial strains were subjected to morphological and chemotaxonomic analyses including phylogenetic investigation based on 16S rRNA gene sequences for identification into genus level. Actinobacteria belonging to a total of 8 genera were identified as *Micromonospora*, *Salinispora*, *Verrucosispora*, *Actinomadura*, *Nocardia*, *Pseudonocardia*, *Actinomycetospora* and *Streptomyces* (Fig 4.1).

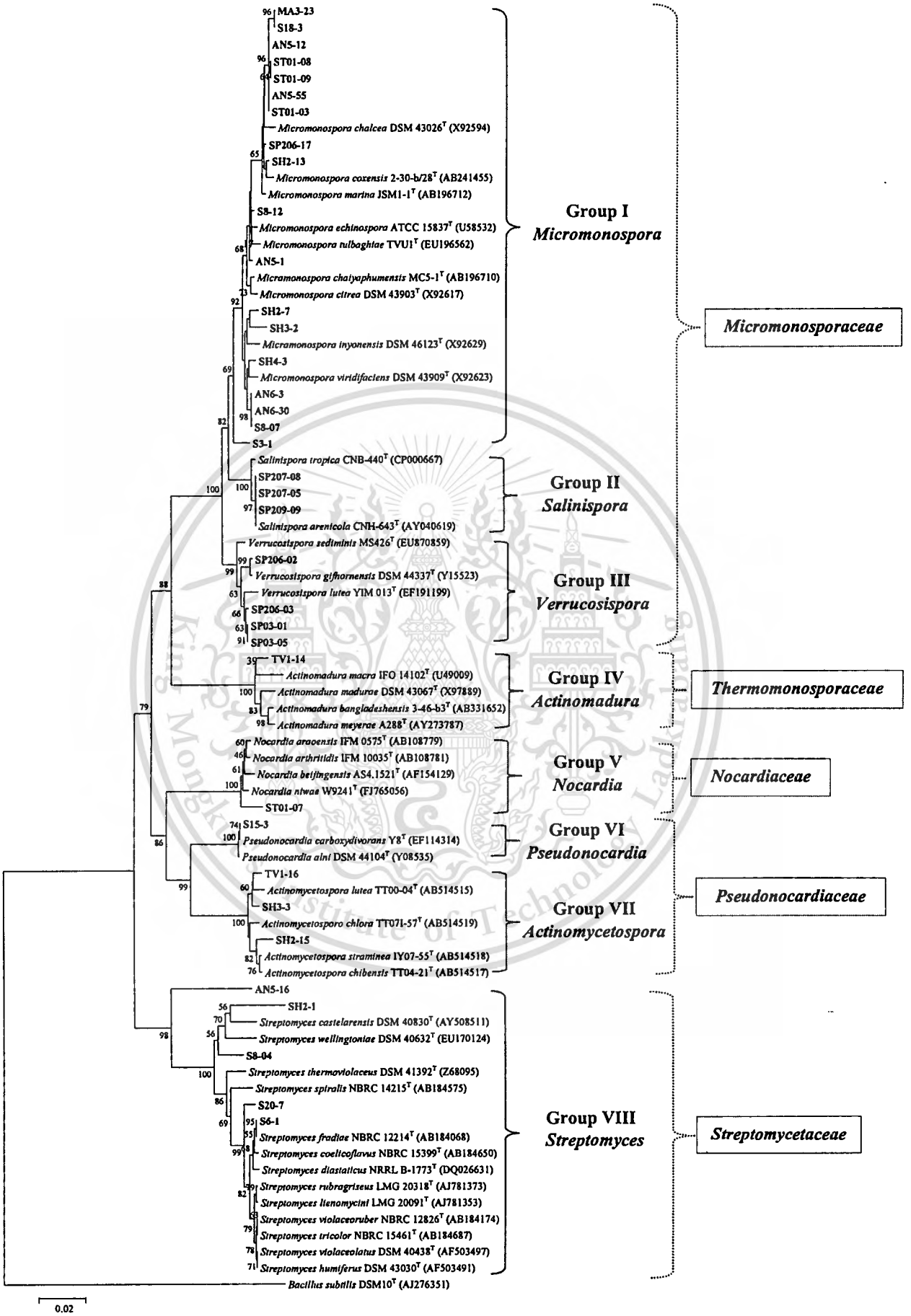


Fig. 4.1 Neighbour-joining tree based on 16S rRNA gene sequences of the marine actinobacterial strains

The marine actinobacteria in group I consisted of eighteen strains, including AN5-1, AN5-12, AN5-55, AN6-3, AN6-30, M3-23, S3-1, S8-07, S8-12, S18-3, SH2-7, SH2-13, SH3-2, SH4-3, SP206-17, ST01-03, ST01-08, and ST01-09. They produced a single spore directly on their substrate hyphae. The spore surfaces were smooth, rough and warty, and non-motile. The colours of the substrate mycelium ranged from yellow to reddish orange, and turned to brownish black to black after sporulation. Some strains produced the soluble pigments on the medium tests. The cultural characteristics of marine actinobacteria in group I are shown in Table 4.3. Most strains grew at 20-40 °C, pH 6-12, and 0-4% NaCl (Table 4.4). Most of these strains peptonized and coagulated protein of milk. Some strains showed positive results of starch hydrolysis and gelatinization. In addition, eight strains reduced nitrate to nitrite. Most strains in this group produced acids from D-cellobiose, sorbose and salicin (Table 4.5).

The chemical profiles of these strains were similar to those of members of the genus *Micromonospora*. Cell wall hydrolysates of the strains contained glutamic acid, glycine, alanine and diaminopimelic acid, and the isomer of diaminopimelic acid was *meso*, some strains contained hydroxyl diaminopimelic acid indicating that these strains had wall chemotype II of Lechevalier and Lechevalier (1970). The acyl type of cell wall muramic acid was glycolyl. The strains contained arabinose, galactose, glucose, ribose and xylose as whole cell sugars (whole-cell sugar pattern D of Lechevalier and Lechevalier, 1970) (Table 4.6). Characteristic phospholipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides, but not phosphatidylcholine (Table 4.7). This pattern corresponds to phospholipid type II of Lechevalier *et al.* (1977). Mycolic acids were absent. The major cellular fatty acids of the strains were iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and $C_{17:0}$. This pattern corresponds to fatty acid type 3b (Kroppenstedt, 1985). Cellular fatty acids of the strains are shown in Table 4.8.

Table 4.3 Cultural characteristics of marine actinobacteria in group I

Media	Strains				
	AN5-1	AN5-12	AN5-55	AN6-3	AN6-30
Yeast extract-malt extract agar (ISP 2)					
Growth	+++	++	+++	++	+++
Colour of reverse surface	Strong orange	Strong orange	Yellowish brown	Vivid orange	Reddish orange
Colour of upper surface	Strong orange	Strong brown	Yellowish brown	Strong orange	Reddish orange
Soluble pigment	-	-	-	-	-
Oatmeal agar (ISP 3)					
Growth	++	+	+++	++	++
Colour of reverse surface	Strong orange	Orange yellow	Orange yellow	Vivid orange	Reddish orange
Colour of upper surface	Strong orange	Orange yellow	Yellowish brown	Vivid orange	Reddish orange
Soluble pigment	-	-	-	-	-
Inorganic salt-starch agar (ISP 4)					
Growth	++	+	+	+	+
Colour of reverse surface	Strong orange yellow	Orange yellow	Yellowish brown	Vivid orange	Reddish orange
Colour of upper surface	Strong orange yellow	Orange yellow	Yellowish brown	Vivid orange	Reddish orange
Soluble pigment	-	-	-	-	-
Glycerol-asparagine agar (ISP 5)					
Growth	+	+	++	+	+
Colour of reverse surface	Yellowish white	Orange yellow	Orange yellow	White	Yellowish pink
Colour of upper surface	Yellowish white	Orange yellow	Yellowish brown	White	Yellowish pink
Soluble pigment	-	-	-	-	-
Peptone-yeast extract iron agar (ISP 6)					
Growth	++	++	++	+	+
Colour of reverse surface	Orange yellow	Deep orange	Orange yellow	Moderate orange	Yellowish pink
Colour of upper surface	Orange yellow	Deep orange	Yellowish brown	Moderate orange	Orange yellow
Soluble pigment	-	-	-	-	-
Tyrosine agar (ISP 7)					
Growth	+	+	++	+	+
Colour of reverse surface	Yellowish brown	Orange yellow	Yellowish brown	Orange yellow	Yellowish pink
Colour of upper surface	Yellowish brown	Orange yellow	Yellowish brown	Orange yellow	Yellowish pink
Soluble pigment	-	-	-	-	-
Czapek's sucrose agar					
Growth	+	+	+	+	+
Colour of reverse surface	Yellowish gray	Orange yellow	Yellowish brown	Colorless	Colorless
Colour of upper surface	Yellowish gray	Orange yellow	Yellowish brown	Colorless	Colorless
Soluble pigment	-	-	-	-	-
Glucose-asparagine agar					
Growth	+	+	++	+	+
Colour of reverse surface	Yellowish white	Orange yellow	Yellowish brown	Colorless	Reddish orange
Colour of upper surface	Yellowish white	Orange yellow	Yellowish brown	Colorless	Yellowish pink
Soluble pigment	-	-	-	-	-
Nutrient agar					
Growth	++	++	+++	+	+
Colour of reverse surface	Yellowish brown	Strong orange	Orange yellow	Orange yellow	Yellowish pink
Colour of upper surface	Yellowish brown	Strong orange	Orange yellow	Orange yellow	Orange yellow
Soluble pigment	-	-	-	-	-

Key: ++++, abundant; +++; good; ++, moderate; + poor.

Table 4.3 Cultural characteristics of marine actinobacteria in group I (continued)

Media	Strains				
	M3-23	S3-1	S8-07	S8-12	S18-3
Yeast extract-malt extract agar (ISP 2)					
Growth	++	+++	+++	+++	+++
Colour of reverse surface	Brownish orange	Moderate orange	Strong orange	Brownish orange	Orange yellow
Colour of upper surface	Strong brown	Strong orange	Deep orange	Deep brown	Orange yellow
Soluble pigment	Brownish orange	Moderate orange	-	Reddish brown	-
Oatmeal agar (ISP 3)					
Growth	++	+	++	++	+++
Colour of reverse surface	Light orange	Moderate orange	Strong orange	Brownish orange	Orange yellow
Colour of upper surface	Light orange	Strong orange	Deep orange	Deep brown	Orange yellow
Soluble pigment	-	Moderate orange	-	-	-
Inorganic salt-starch agar (ISP 4)					
Growth	+	++	+	++	+
Colour of reverse surface	Colorless	Light orange	Strong orange	Light brown	Yellowish gray
Colour of upper surface	Colorless	Light orange	Strong orange	Moderate brown	Orange yellow
Soluble pigment	-	Light orange	-	-	-
Glycerol-asparagine agar (ISP 5)					
Growth	++	++	+	+	+
Colour of reverse surface	Orange yellow	Light orange	Strong orange	Orange yellow	Orange yellow
Colour of upper surface	Yellowish brown	Light orange	Strong orange	Orange yellow	Orange yellow
Soluble pigment	-	Light orange	-	-	-
Peptone-yeast extract iron agar (ISP 6)					
Growth	++	+	+	++	++
Colour of reverse surface	Light orange	Light orange	Deep orange	Brownish orange	Orange yellow
Colour of upper surface	Light orange	Light orange	Deep orange	Brownish orange	Orange yellow
Soluble pigment	-	Light orange	-	-	-
Tyrosine agar (ISP 7)					
Growth	++		+	+	+
Colour of reverse surface	Grayish brown	Reddish orange	Strong orange	Yellowish brown	Orange yellow
Colour of upper surface	Light brown	Reddish orange	Strong orange	Yellowish brown	Orange yellow
Soluble pigment	-	Reddish orange	-	-	-
Czapek's sucrose agar					
Growth	+	+	+	+	+
Colour of reverse surface	Yellowish white	Colorless	Strong orange	Yellowish gray	Deep orange
Colour of upper surface	Grayish brown	Colorless	Strong orange	Yellowish gray	Deep orange
Soluble pigment	-	-	-	-	-
Glucose-asparagine agar					
Growth	++	++	+	+	+
Colour of reverse surface	Orange yellow	Strong orange	Strong orange	Yellowish brown	Orange yellow
Colour of upper surface	Yellowish brown	Strong orange	Strong orange	Yellowish brown	Orange yellow
Soluble pigment	-	Strong orange	-	-	-
Nutrient agar					
Growth	++	++	+	+++	++
Colour of reverse surface	Moderate orange	Moderate orange	Strong orange	Reddish orange	Orange yellow
Colour of upper surface	Moderate orange	Moderate orange	Strong orange	Reddish orange	Orange yellow
Soluble pigment	-	Moderate orange	-	-	-

Key: ++++, abundant; +++; good; ++, moderate; + poor.

Table 4.3 Cultural characteristics of marine actinobacteria in group I (continued)

Media	Strains				
	SH2-7	SH2-13	SH3-2	SH4-3	SP206-17
Yeast extract-malt extract agar (ISP 2)					
Growth	+++	+++	++	++	+++
Colour of reverse surface	Orange yellow	Deep brown	Light yellow	Reddish brown	Moderate orange
Colour of upper surface	Orange yellow	Light orange	Light yellow	Reddish brown	Moderate orange
Soluble pigment	-	Deep brown	-	-	-
Oatmeal agar (ISP 3)					
Growth	+	++	++	++	+++
Colour of reverse surface	Orange yellow	Light orange	Light yellow	Reddish brown	Moderate orange
Colour of upper surface	Orange yellow	Light orange	Light yellow	Reddish brown	Moderate orange
Soluble pigment	-	-	-	-	-
Inorganic salt-starch agar (ISP 4)					
Growth	+	+	+	+++	+
Colour of reverse surface	Orange yellow	Orange yellow	Orange yellow	Light brown	Orange yellow
Colour of upper surface	Orange yellow	Orange yellow	Orange yellow	Reddish brown	Orange yellow
Soluble pigment	-	-	-	-	-
Glycerol-asparagine agar (ISP 5)					
Growth	+	+	+	+	+
Colour of reverse surface	Orange yellow	Yellowish white	Colorless	Orange yellow	Colorless
Colour of upper surface	Orange yellow	Yellowish white	Colorless	Orange yellow	Colorless
Soluble pigment	-	-	-	-	-
Peptone-yeast extract iron agar (ISP 6)					
Growth	+	+	+	+	++
Colour of reverse surface	Orange yellow	Brownish orange	Light yellow	Orange yellow	Moderate orange
Colour of upper surface	Light yellow	Strong orange	Light yellow	Orange yellow	Moderate orange
Soluble pigment	-	-	-	-	-
Tyrosine agar (ISP 7)					
Growth	+	+	+	+	+
Colour of reverse surface	Orange yellow	Yellowish white	Colorless	Orange yellow	Colorless
Colour of upper surface	Light yellow	Yellowish white	Colorless	Orange yellow	Colorless
Soluble pigment	-	-	-	-	-
Czapek's sucrose agar					
Growth	+	+	+	+	+
Colour of reverse surface	Light yellow	Orange yellow	Colorless	Yellowish white	Yellowish white
Colour of upper surface	Light yellow	Orange yellow	Colorless	Yellowish white	Yellowish white
Soluble pigment	-	-	-	-	-
Glucose-asparagine agar					
Growth	+	+	+	+	+
Colour of reverse surface	Light yellow	Yellowish white	Colorless	Yellowish brown	Colorless
Colour of upper surface	Light yellow	Yellowish white	Colorless	Orange yellow	Colorless
Soluble pigment	-	-	-	-	-
Nutrient agar					
Growth	+	+	+	+	+
Colour of reverse surface	Light yellow	Orange yellow	Light yellow	Yellowish brown	Yellowish white
Colour of upper surface	Light yellow	Light orange	Light yellow	Yellowish brown	Yellowish white
Soluble pigment	-	-	-	-	-

Key: ++++, abundant; +++; good; ++, moderate; + poor.

Table 4.3 Cultural characteristics of marine actinobacteria in group I (continued)

Media	Strains		
	ST01-03	ST01-08	ST01-09
Yeast extract-malt extract agar (ISP 2)			
Growth	+++	+++	+++
Colour of reverse surface	Moderate orange	Moderate orange	Strong orange
Colour of upper surface	Moderate orange	Moderate orange	Deep Orange
Soluble pigment	-	-	-
Oatmeal agar (ISP 3)			
Growth	+++	+++	+++
Colour of reverse surface	Moderate orange	Moderate orange	Moderate orange
Colour of upper surface	Moderate orange	Moderate orange	Moderate orange
Soluble pigment	-	-	-
Inorganic salt-starch agar (ISP 4)			
Growth	+	+	+
Colour of reverse surface	Light orange	Light orange	Light orange
Colour of upper surface	Light orange	Light orange	Strong orange
Soluble pigment	-	-	-
Glycerol-asparagine agar (ISP 5)			
Growth	+	+	+
Colour of reverse surface	Yellowish pink	Yellowish pink	Yellowish pink
Colour of upper surface	Yellowish pink	Yellowish pink	Yellowish pink
Soluble pigment	-	-	-
Peptone-yeast extract iron agar (ISP 6)			
Growth	+	+	+
Colour of reverse surface	Light orange	Light orange	Moderate orange
Colour of upper surface	Light orange	Light orange	Light orange
Soluble pigment	-	-	-
Tyrosine agar (ISP 7)			
Growth	+	+	+
Colour of reverse surface	Yellowish pink	Yellowish pink	Yellowish pink
Colour of upper surface	Yellowish pink	Yellowish pink	Yellowish pink
Soluble pigment	-	-	-
Czapek's sucrose agar			
Growth	+	+	+
Colour of reverse surface	Colorless	Colorless	Colorless
Colour of upper surface	Colorless	Colorless	Colorless
Soluble pigment	-	-	-
Glucose-asparagine agar			
Growth	+	+	+
Colour of reverse surface	Orange yellow	Orange yellow	Orange yellow
Colour of upper surface	Orange yellow	Orange yellow	Orange yellow
Soluble pigment	-	-	-
Nutrient agar			
Growth	++	++	++
Colour of reverse surface	Moderate orange	Moderate orange	Moderate orange
Colour of upper surface	Vivid orange	Vivid orange	Vivid orange
Soluble pigment	-	-	-

Key: ++++, abundant; +++; good; ++, moderate; + poor.

Table 4.4 Physiological and biochemical characteristics of marine actinobacteria in group I

Strains	Sodium chloride tolerance (%)										pH tolerance									Growth temperature					Skim milk		Gelatinization	Nitrate reduction	Starch hydrolysis
	1.5	2	3	4	5	6	7	8	9	10	4	4.5	5	6	8	9	10	11	12	20	37	40	45	50	Peptonization	Coagulation			
AN5-1	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
AN5-12	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-
AN5-55	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
AN6-3	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+
AN6-30	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+
M3-23	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+
S3-1	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-	+
S8-07	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-
S8-12	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
S18-3	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-
SH2-7	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-
SH2-13	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+
SH3-2	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+
SH4-3	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
SP206-17	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+
ST01-03	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
ST01-08	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
ST01-09	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+

Almost complete 16S rRNA gene sequences (1,320-1,457 nucleotides) of each strain were used for phylogenetic analysis and compared with 16S rRNA gene sequences of members of the family *Micromonosporaceae*. Phylogenetic analysis based on this large dataset revealed that these strains were placed within the clade of the genus *Micromonospora* (Fig. 4.2). Strain SH3-2 formed the cluster with *Micromonospora fulviviridis* DSM 43906^T, *Micromonospora sagamiensis* DSM 43912^T and *Micromonospora inyonensis* DSM 46123^T in the neighbour-joining tree, the strain was most closely to *Micromonospora fulviviridis* DSM 43906^T with the 16S rRNA gene sequence similarity value of 99.2%. Strain SH2-7 formed a distinct phyletic line related to the recognized *Micromonospora* species in the neighbour-joining tree and showed the highest 16S rRNA gene sequences similarity value with *Micromonospora endolithica* DSM 44398^T (99.5%). Strains AN6-3, AN6-30 and S8-07 formed the cluster with *Micromonospora echinaurantiaca* DSM 43904T, and showed the highest 16S rRNA gene sequence similarity value with *Micromonospora echinaurantiaca* DSM 43904^T at 99.7%, 99.6% and 99.7%, respectively. Strain SH4-3 formed the cluster with *Micromonospora pallida* DSM 43817^T, *Micromonospora viridifaciens* DSM 43909^T and *Micromonospora equina* Y22^T. This strain showed the highest 16S rRNA gene sequence similarity value with *Micromonospora pallida* DSM 43817^T at 99.4%. Strains AN5-1 and S8-12 formed the cluster with *Micromonospora tulbaghiae* TVU1^T and *Micromonospora echinospora* ATCC 15837^T, these strains were most closely to *Micromonospora echinospora* ATCC 15837^T with the 16S rRNA gene sequence similarity value of 99.6%. Strain SP206-07 showed the highest sequence similarity to *Micromonospora marina* JSM1-1^T (99.8%), which formed clade with *Micromonospora marina* JSM1-1^T in neighbour-joining tree. Strains AN5-12, AN5-55, ST01-03, ST01-08, ST01-09, S18-3 and MA3-23 formed the cluster with *Micromonospora aurantiaca* DSM 43813^T in neighbour-joining tree. Strains AN5-12 and ST01-03 showed the highest similarity values with *Micromonospora marina* JSM1-1^T (99.5%). In the contrast, strains AN5-55, ST01-08, ST01-09, S18-3 and MA3-23 exhibited the highest sequence similarity value with *Micromonospora aurantiaca* DSM 43813^T (99.4-99.6%). The morphological, chemotaxonomic characteristics and 16S rRNA gene analyses of these strains were consistent with their classification in the genus *Micromonospora*.

Table 4.5 Acid production of marine actinobacteria in group I

Carbon sources	Strains																	
	AN5-1	AN5-12	AN5-55	AN6-3	AN6-30	M3-23	S3-1	S8-07	S8-12	S18-3	SH2-7	SH2-13	SH3-2	SH4-	SP206-17	ST01-03	ST01-08	ST01-09
D-Cellobiose	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+
D-Fructose	-	+	-	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-
D-Galactose	-	-	-	-	+	-	+	-	+	-	-	+	+	+	+	-	-	-
D-Melibiose	+	+	-	-	-	-	+	-	-	-	-	+	-	+	-	+	-	+
D-Raffinose	-	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+
D-Ribose	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+
D-Xylose	+	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	+	+
L-Arabinose	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	+	+	+	-	+	-	-	-	+	-	+	+	+	+
Salicin	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+
Sorbose	+	+	-	-	+	+	+	-	+	-	-	-	+	+	+	+	+	+

Table 4.6 Whole cell sugar patterns of marine actinobacteria in group I

Strains	sugars								Sugar type
	Ara	Gal	Glc	Mad	Man	Rha	Rib	Xyl	
AN5-1	+	+	+	-	-	+	+	+	D
AN5-12	+	+	+	-	-	+	+	+	D
AN5-55	+	+	+	-	-	-	+	+	D
AN6-3	+	+	+	-	-	+	+	+	D
AN6-30	+	+	+	-	-	+	+	+	D
M3-23	+	+	+	-	-	-	+	+	D
S3-1	+	+	+	-	-	+	+	+	D
S8-07	+	+	+	-	-	+	+	+	D
S8-12	+	+	+	-	-	-	+	+	D
S18-3	+	+	+	-	-	-	+	+	D
SH2-7	+	+	+	-	-	-	+	+	D
SH2-13	+	+	+	-	-	+	+	+	D
SH3-2	+	+	+	-	-	+	+	+	D
SH4-3	+	+	+	-	-	+	+	+	D
SP206-17	+	+	+	-	-	+	+	+	D
ST01-03	+	+	+	-	-	+	+	+	D
ST01-08	+	+	+	-	-	+	+	+	D
ST01-09	+	+	+	-	-	-	+	+	D

Ara, arabinose; Gal, galactose; Glc, glucose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose

Table 4.7 Phospholipid profiles of marine actinobacteria in group I

Strains	Phospholipids									
	APG	DPG	GluNU	PC	PE	PME	PG	PI	PIMs	PS
AN5-1	-	+	-	-	+	-	+	+	+	-
AN5-12	-	+	-	-	+	-	+	+	+	-
AN5-55	-	+	-	-	+	-	+	+	+	-
AN6-3	-	+	-	-	+	-	+	+	+	-
AN6-30	-	+	-	-	+	-	+	+	+	-
M3-23	-	+	-	-	+	-	+	+	+	-
S3-1	-	+	-	-	+	+	+	+	+	-
S8-07	-	+	-	-	+	-	+	+	+	-
S8-12	-	+	-	-	+	-	+	+	+	-
S18-3	-	+	-	-	+	-	+	+	+	-
SH2-7	-	+	-	-	+	-	+	+	+	-
SH2-13	-	+	-	-	+	-	+	+	+	-
SH3-2	-	+	-	-	+	-	+	+	+	-
SH4-3	-	+	-	-	+	-	+	+	+	-
SP206-17	-	+	-	-	+	+	+	+	+	-
ST01-03	-	+	-	-	+	-	+	+	+	-
ST01-08	-	+	-	-	+	-	+	+	+	-
ST01-09	-	+	-	-	+	-	+	+	+	-

APG, acylphosphatidylglycerol; DPG, diphosphatidylglycerol; GluNU, unknown glucosamine-containing phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidylmethylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; PS, phosphatidylserine

Table 4.8 Cellular fatty acid compositions (%) of marine actinobacteria in group I

Fatty acids	Strains																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Saturated fatty acids																		
$C_{14:0}$	0.4	0.6	0.5	0.4	0.5	0.3	0.3	0.9	0.2	0.2	0.3	0.2	0.1	0.5	0.6	0.5	0.4	0.2
$C_{16:0}$	1.3	2.6	2.4	1.9	2.0	2.5	2.4	2.3	1.4	1.4	1.3	1.1	1.0	5.3	4.1	1.7	1.8	0.6
$C_{17:0}$	1.1	1.4	0.6	8.9	9.2	1.6	1.3	13.2	0.7	4.0	1.4	6.4	1.8	1.8	5.4	1.2	1.1	0.4
$C_{18:0}$	3.0	2.6	2.4	0.7	3.5	4.4	7.2	1.2	2.4	1.1	2.4	1.1	1.0	1.9	3.8	1.1	1.3	0.5
$C_{19:0}$	0.2	0.2	0.3	0.4	1.4	0.2	0.3	0.6	-	0.3		0.5	0.2	0.1	0.5	-	-	-
Unsaturated fatty acids																		
$C_{16:1}$ ω7c	0.7	1.6	1.4	0.7	0.6	1.1	1.6	0.8	1.2	0.5	0.3	0.6	0.6	6.0	0.5	3.5	2.5	1.5
$C_{17:1}$ ω8c	3.2	4.5	1.1	16.6	5.6	1.6	2.0	12.9	1.8	11.1	-	8.9	3.7	5.3	7.0	6.6	4.5	3.8
a-$C_{17:1}$ ω9c	-	0.7	0.7	0.3	0.5	0.6	1.3	0.6	0.9	1.2	1.1	1.1	-	1.1	0.8	0.9	1.3	1.8
$C_{18:1}$ ω9c	7.9	15.2	5.2	1.5	2.5	5.0	9.5	1.1	6.6	7.3	8.3	2.7	1.1	4.3	11.7	4.7	5.0	6.4
$C_{19:1}$ ω9c	0.5	-	0.1	0.5	0.4	0.1	0.1	0.3	0.3	1.0	0.6	0.7	0.1	-	0.5	-	0.2	0.2
Branched fatty acids																		
i-$C_{14:0}$	1.7	1.1	1.8	0.3	0.5	0.9	0.3	0.6	0.9	0.4	1.2	0.7	0.5	1.3	0.4	2.5	1.0	2.3
i-$C_{15:0}$	7.1	7.4	10.2	31.8	30.7	12.5	3.0	29.6	17.2	19.8	8.6	27.7	26.1	10.2	13.0	15.5	24.4	11.2
i-$C_{15:1}$	0.2	0.2	0.1	0.5	0.7	-	-	0.8	0.2	0.1	0.2	0.5	0.9	0.1	0.1	0.7	0.7	0.5
n-$C_{15:0}$	1.0	5.9	4.5	6.6	4.0	6.0	0.3	7.8	3.5	7.3	2.3	6.2	12.2	4.1	6.8	6.0	7.2	4.2
i-$C_{16:0}$	53.9	23.4	43.4	3.9	9.2	33.9	38.1	5.5	29.2	10.4	23.9	14.3	11.2	41.7	6.7	37.9	23.3	41.1
i-$C_{16:1}$	-	0.6	1.2	0.3	0.4	1.0	1.5	0.5	1.1	0.2	1.2	1.0	-	2.7	0.2	2.5	1.3	2.2
i-$C_{17:0}$	3.5	4.8	4.2	6.0	12.0	6.0	7.2	5.4	8.7	7.2	5.5	7.6	9.6	2.5	6.0	2.8	5.5	3.1
a-$C_{17:0}$	2.8	9.4	8.9	5.2	7.8	13.2	19.2	6.6	8.1	16.0	27.2	6.9	22.9	7.1	20.6	7.5	6.8	9.0
i-$C_{18:0}$	1.0	0.6	1.4	-	0.3	1.5	2.4	0.1	0.9	0.2	1.0	0.3	0.3	0.5	0.2	0.8	0.3	0.9
10-Methylated fatty acids																		
$C_{16:0}$	4.0	4.5	5.2	3.0	3.1	5.6	0.5	2.4	11.2	6.6	5.3	12.8	3.1	2.4	4.0	4.3	9.1	7.5
$C_{17:0}$	-	-	-	6.3	2.3	-	-	3.8	-	1.7	-	-	1.5	-	0.7	-	-	-
$C_{18:0}$	4.3	0.8	1.6	0.5	1.2	0.6	-	0.3	1.8	0.6	2.4	-	0.3	0.1	0.4	-	0.4	0.5
Hydroxyled fatty acids																		
2-OH-$C_{16:1}$	-	-	-	-	-		-	-	-	-	3.2	-	-	-	-	-	-	-

Strains: 1, AN5-1; 2, AN5-12; 3, AN5-55; 4, AN6-3; 5, AN6-30; 6, M3-23; 7, S3-1; 8, S8-07; 9, S8-12; 10, S18-3; 11, SH2-7; 12, SH2-13; 13, SH3-2; 14, SH4-3; 15, SP206-17; 16, ST01-03; 17, ST01-08; 18, ST01-09.

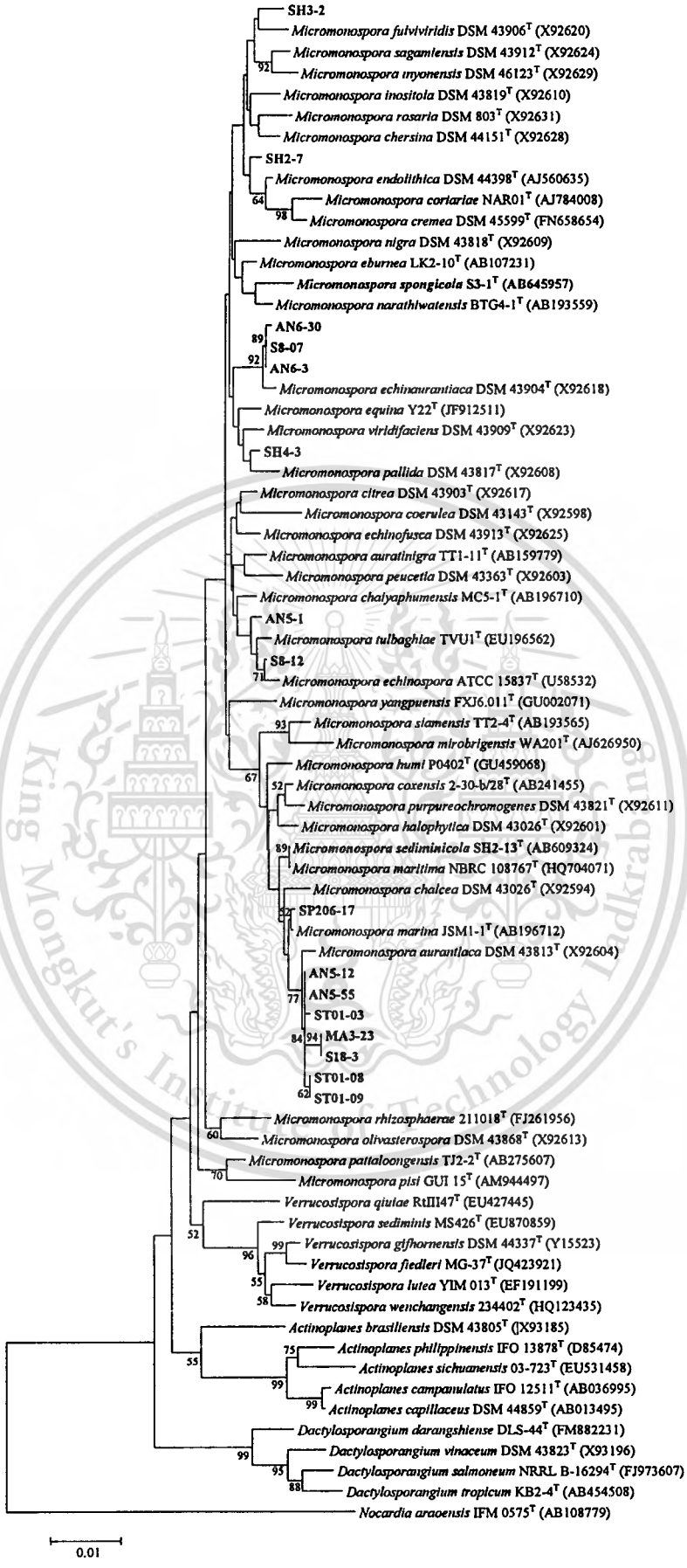


Fig. 4.2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of *Micromonospora* strains

The outstanding strains, SH2-13 and S3-1 were selected to complete for the taxonomic characterization. Strain SH2-13 was aerobic, Gram-stain-positive, mesophilic marine actinobacteria that well-developed and extensively branched substrate hyphae. Aerial mycelium was not formed. The colour of the vegetative mycelium on ISP 2 medium was deep brown. A deep brown soluble pigment was produced on ISP 2 medium. Single spores with warty were produced on the substrate mycelium (Fig. 4.3). Nitrate reduction was negative. Starch hydrolysis, gelatin liquefaction and peptonization of milk were positive. Strain SH2-13 produced acids from cellobiose, D-galactose, D-fructose, D-melibiose, D-raffinose and D-xylose. Optimal temperature for growth was between 25-37°C. The strain grew at 20-40°C and pH 6-12. The optimum NaCl concentration for growth was 3%. The maximum NaCl concentration for growth was 4%.

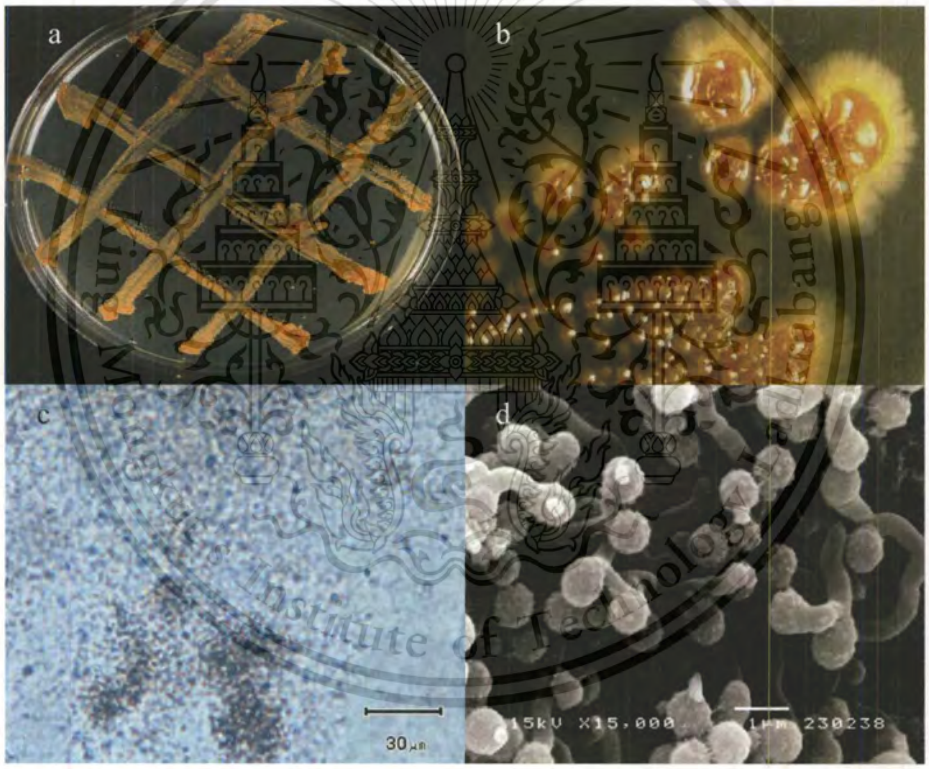


Fig. 4.3 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Micromonospora sediminicola* SH2-13^{T}

The cell wall peptidoglycan contained *meso*-diaminopimelic acid. The acyl type of the cell-wall muramic acid was glycolyl. The phospholipid profiles comprised phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The predominant menaquinones were MK-10(H_2), MK-10(H_4) and MK-10(H_6). The whole-cell sugars were arabinose, galactose, glucose, rhamnose, ribose and xylose. The fatty acid pattern consisted of iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:1}$ ω9c, $C_{17:1}$ ω8c, iso-$C_{17:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, and $C_{17:0}$. Differential cellular fatty acid of *Micromonospora sediminicola* SH2-13^T and the related *Micromonospora* species are shown in Table 4.10. The DNA G+C content of strain SH2-13 was 74.8 mol%.

An almost-complete 16S rRNA gene sequence (1,416 nucleotides) was obtained for strain SH2-13^T and compared with those deposited in the public databases. The highest levels of 16S rRNA gene sequence similarity were with *Micromonospora marina* JSM1-1^T (99.1 %), *Micromonospora coxensis* 2-30-b/28^T (99.1 %), *Micromonospora aurantiaca* DSM 43813^T (98.8 %) and *Micromonospora chalcea* DSM 43026^T (98.7 %). Phylogenetic relationship of strain SH2-13^T based on the neighbour-joining method showed that the strain formed a distinct phyletic line related to the recognized *Micromonospora* species and other members of the family *Micromonosporaceae* (Fig. 4.4). Strain SH2-13^T showed some different physiological and biochemical characteristics compared to the related *Micromonospora* species distinguished on the basis of acid production from D-cellobiose, D-fructose, D-galactose, D-melibiose, D-raffinose, D-xylose and L-arabinose, nitrate reduction, the growth at pH 5, at 45 °C and hydrolysis of starch (Table 4.9).

A low level of DNA–DNA relatedness values between SH2-13^T and the related strains *Micromonospora marina* JSM1-1^T, *Micromonospora coxensis* 2-30-b/28^T, *Micromonospora aurantiaca* JCM 10878^T and *Micromonospora chalcea* JCM 3031^T ranged from 10.9 ± 0.6 – 27.5 ± 0.2 which is well below the 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987), as shown in Table 4.11. Furthermore, the chemotaxonomic result indicated that strain SH2-13^T did not contain mannose in cell hydrolysates while the two clostest relatives, *Micromonospora marina* JCM12870^T (Tanasupawat *et al.*, 2010) and *Micromonospora coxensis* 2-30-b/28^T (Ara and Kudo, 2007) presented mannose in their cell-hydrolysates. It is evident from the phenotypic, chemotypic and genotypic data presented above that strain SH2-13^T is distinguishable from previously described *Micromonospora* species.

Strain SH2-13^T represents a novel species of the genus *Micromonospora* for which proposed the name *Micromonospora sediminicola* sp. nov. The type strain, SH2-13^T (=NBRC 107934^T =BCC 45601^T) was isolated from marine sediment sample collected from the Andaman Sea, Krabi province, Thailand.

Table 4.9 Differential characteristics of strain SH2-13^T and the type strains of the most closely related *Micromonospora* species.

Characteristics	Strains				
	1	2	3	4	5
Starch hydrolysis	+	+	+	-	+
Nitrate reduction	-	-	+	+	+
Growth at 45 °C	-	-	+	+	+
Growth at pH 5	-	+	-	+	-
Acid production from :					
D-Cellobiose	+	-	-	-	-
D-Fructose	+	-	-	-	-
D-Galactose	+	-	-	-	-
D-Melibiose	+	-	-	-	-
D-Raffinose	+	-	-	-	-
D-Xylose	+	-	+	-	-
L-Arabinose	-	-	+	-	-

Strains: 1, SH2-13^T; 2, *M. marina* JSM1-1^T; 3, *M. coxensis* 2-30-b/28^T; 4, *M. aurantiaca* JCM 10878^T; 5, *M. chalcea* JCM 3031^T. All phenotypic data were determined in this study.

Table 4.10 Cellular fatty acids compositions (%) of strain SH2-13^T and its closest relatives

Fatty acids	Strains				
	1	2	3	4	5
Saturated fatty acids					
$C_{14:0}$	0.2	1.1	0.3	-	0.2
$C_{16:0}$	1.1	5.1	1.0	0.7	0.7
$C_{17:0}$	4.7	6.4	1.0	7.7	1.8
$C_{18:0}$	1.1	2.5	0.9	0.5	0.4
$C_{19:0}$	0.5	0.4	-	0.6	0.2
Unsaturated fatty acids					
$C_{17:1}$ ω8c	8.9	12.6	3.6	18.2	7.5
$C_{18:1}$ ω9c	2.7	10.4	5.3	3.0	3.9
Branched fatty acids					
i-$C_{13:0}$	0.3	-	-	-	0.3
i-$C_{14:0}$	0.7	0.7	1.7	0.8	1.3
i-$C_{15:0}$	27.7	19.4	9.8	20.0	17.5
i-$C_{15:1}$	0.5	0.1	0.3	-	0.8
a-$C_{15:0}$	6.2	5.9	2.1	6.5	7.3
i-$C_{16:0}$	14.3	16.5	40.0	20.6	20.9
i-$C_{16:1}$ H	1.0	0.5	7.7	0.5	1.1
i-$C_{17:0}$	7.6	4.8	2.1	3.6	3.9
i-$C_{17:1}$	-	-	2.8	-	-
i-$C_{17:1}$ ω9c	12.8	4.7	10.4	4.4	9.2
a-$C_{17:1}$ ω9c	1.1	0.4	-	0.5	1.8
a-$C_{17:0}$	6.9	5.2	6.1	7.1	10.7
i-$C_{18:0}$	0.3	0.3	0.5	-	0.4
10-Methyl fatty acids					
10-Methyl $C_{17:0}$	-	-	-	2.7	4.6

Strains: 1, SH2-13^T; 2, *M. marina* JSM1-1^T; 3, *M. coxensis* 2-30-b/28^T; 4, *M. aurantiaca* JCM 10878^T; 5, *M. chalcea* JCM 3031^T.

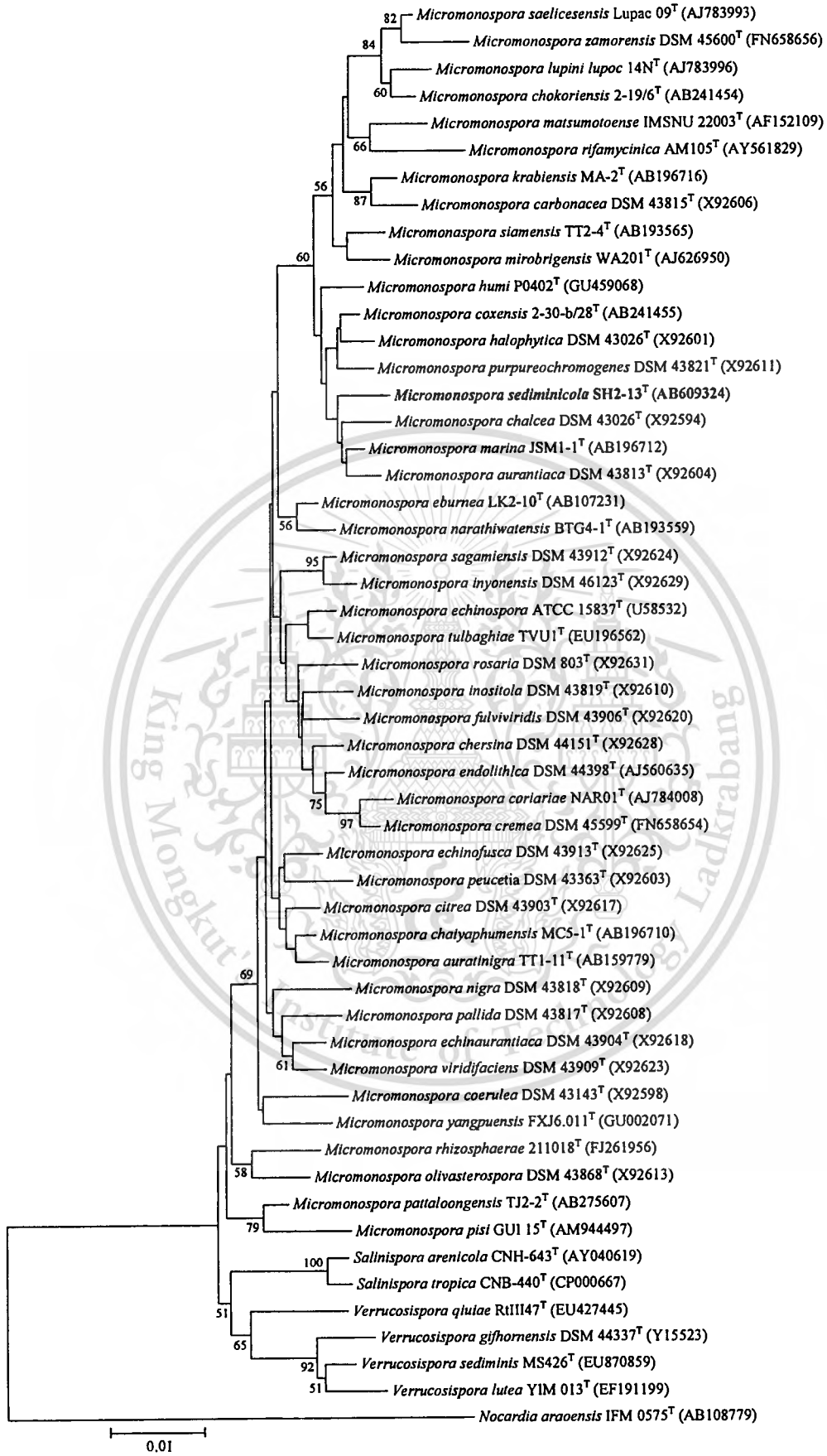


Fig. 4.4 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain SH2-13^T and other members of the genus *Micromonospora*

Table 4.11 DNA–DNA relatedness among the strain SH2-13^T and the related *Micromonospora* species

Strains	DNA–DNA binding (%) with labelled DNA from:				
	1	2	3	4	5
1. SH2-13^T	100	27.5 ± 0.2	18.4 ± 0.6	16.9 ± 0.7	15.5 ± 0.7
2. *M. marina* JSM1-1^T	24.2 ± 0.2	100	25.8 ± 1.5	20.4 ± 0.9	17.2 ± 0.3
3. *M. coxensis* 2-30-b/28^T	16.5 ± 0.3	27.2 ± 0.7	100	18.1 ± 1.7	14.1 ± 0.2
4. *M. aurantiaca* JCM 10878^T	15.8 ± 0.4	22.6 ± 1.2	19.2 ± 1.2	100	28.2 ± 0.8
5. *M. chalcea* JCM 3031^T	10.9 ± 0.6	12.5 ± 1.4	10.7 ± 0.9	32.1 ± 1.4	100

The strain S3-1 was aerobic, Gram-stain-positive which formed oval spores on the substrate mycelium, but aerial mycelium was not formed (Fig. 4.5). The colour of the substrate mycelium on ISP 2, ISP 3 and glucose-asparagine medium agar was strong orange. Moderate orange soluble pigments were produced on ISP 2, ISP 3, ISP 7 and nutrient medium agar. The maximum temperature for growth was 40 °C. The strain grew on the pH ranged from 6-12. The maximum NaCl tolerance was 4%. Strain S3-1^T produced acids from D-cellobiose, D-fructose, D-galactose, lactose, D-melibiose, salicin, sorbose and D-xylose. Starch hydrolysis, gelatin liquefaction and milk peptonization were positive. Nitrate was not reduced.

The diagnostic diamino acid of the peptidoglycan was *meso*-diaminopimelic acid. Whole-cell sugars were arabinose, galactose, glucose, rhamnose, ribose and xylose. Major polar lipids were phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The fatty acid pattern consisted of iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{18:1}$ ω9c, iso-$C_{17:0}$ and $C_{18:0}$. The predominant menaquinones were MK-10(H_4) and MK-10(H_6). The DNA G+C contented of the strain was 72.7 mol%.

The almost-complete 16S rRNA gene sequence (1,457 nucleotides) was obtained for strain S3-1^T and compared with those deposited in the public databases. The results indicated that this strain belonged to the genus *Micromonospora* and the highest similarity value was observed with *Micromonospora nigra* DSM 43818^T (98.8%) follow by *Micromonospora yangpuensis* FXJ6.011^T (98.7%) and *Micromonospora narathiwatensis* BTG4-1^T (98.6%). The phylogenetic tree constructed with 16S rRNA gene sequences data of all members of the genus *Micromonospora* also indicated that strain S3-1^T formed a clade with *Micromonospora nigra* DSM 43818^T and *Micromonospora yangpuensis* FXJ6.011^T in the neighbour-joining tree

(Fig. 4.6). Furthermore, the level of DNA–DNA relatedness between strain S3-1^T and the related strains *Micromonospora nigra* NBRC 16103^T, *Micromonospora yangpuensis* NBRC 107727^T and *Micromonospora narathiwatensis* BTG4-1^T were 25.0 ± 0.7%, 19.4 ± 1.5% and 14.8 ± 0.9% respectively.

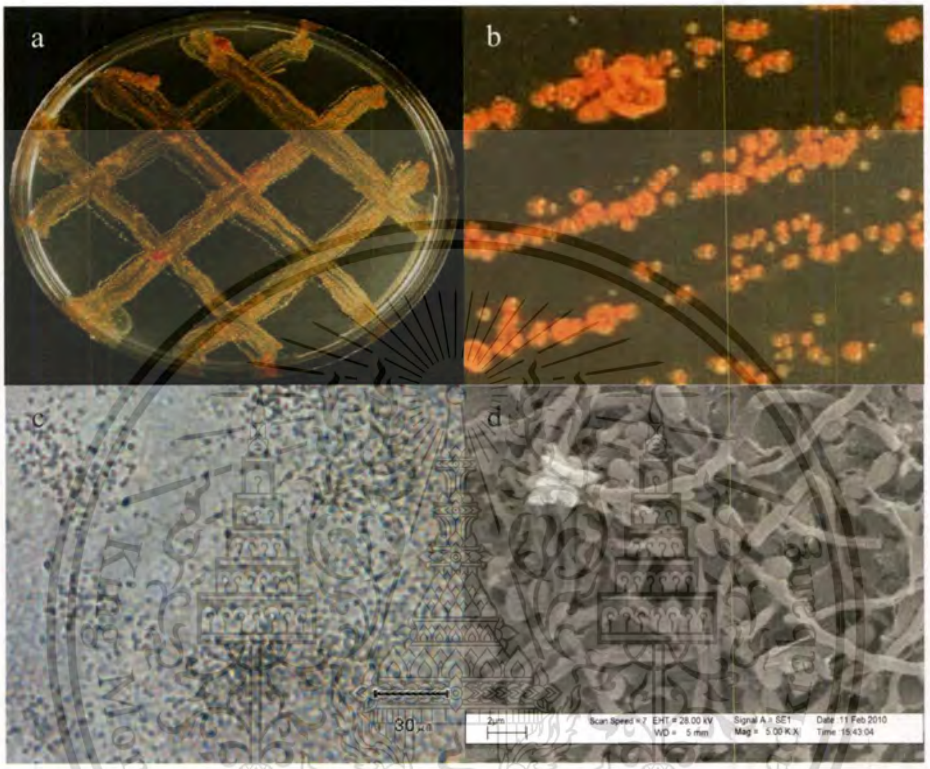


Fig. 4.5 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Micromonospora spongicola* S3-1^T

On the basis of morphological and chemotaxonomic analyses, it was indicated that strain S3-1^T was identified as a member of the genus *Micromonospora.* However, the 16S rRNA gene sequence similarity values are low (98.6-98.8%) and DNA–DNA relatedness values between strain S3-1^T and the closest strains were below 25.0% less than the value of 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987).

In addition, the strain S3-1^T was distinguished from related strains by differences in the acid production from D-cellobiose, D-fructose, D-galactose, D-melibiose, D-raffinose, D-xylose, L-arabinose, D-sucrose, lactose, sorbose and salicin (Table 4.12). Furthermore, 10-methyl-$C_{18:0}$,

$C_{14:0}$ and iso-$C_{14:0}$ were not detected in strain S3-1^T which differentiating properties from these related species (Table 4.13). These results supported that strain S3-1^T represents a novel species in the genus *Micromonospora*, for which the name *Micromonospora spongicola* sp. nov., was proposed. The type strain was S3-1^T (=BCC 45595^T =NBRC 108779^T), isolated from the marine sponge collected from the Gulf of Thailand.

Table 4.12 Differential characteristics of strain S3-1^T and type strains of the related *Micromonospora* species.

Characteristics	Strains			
	1	2	3	4
Starch hydrolysis	+	+	+	-
Gelatin liquefaction	+	-	+	+
Growth at 45 °C	-	+	-	-
Growth at pH 6	-	+	+	+
Acid production from :				
D-Cellobiose	+	+	+	-
D-Fructose	+	+	+	-
D-Galactose	+	-	+	-
D-Melibiose	+	+	+	-
D-Raffinose	-	+	-	-
D-Xylose	+	-	+	-
D-Sucrose	-	-	+	-
L-Arabinose	-	-	+	+
Lactose	+	-	+	-
Salicin	+	+	+	-
Sorbose	+	+	+	-

Strains: 1, S3-1^T; 2, *M. nigra* NBRC 16103^T; 3, *M. yangpuensis* NBRC 107727^T and 4, *M. narathiwatensis* BTG4-1^T. All phenotypic data was determined in this study.

Table 4.13 Cellular fatty acid compositions (%) of strain S3-1^T and type strains of the related *Micromonospora* species.

Fatty acids	Strains			
	1	2	3	4
Saturated fatty acids				
$C_{12:0}$	-	-	0.3	0.4
$C_{14:0}$	-	1.4	1.1	0.7
$C_{16:0}$	2.4	2.9	11.5	1.1
$C_{17:0}$	1.3	1.1	4.1	6.2
$C_{18:0}$	7.2	2.2	3.9	0.6
$C_{19:0}$	0.3	-	0.4	0.5
Unsaturated fatty acids				
$C_{16:1}$ ω7c	1.6	1.3	2.8	0.3
$C_{17:1}$ ω5c	0.2	-	-	-
$C_{17:1}$ ω8c	2.0	2.5	12.2	11.0
i-$C_{17:1}$ ω9c	0.5	7.7	1.3	-
a-$C_{17:1}$ ω9c	1.3	0.7	0.3	-
$C_{18:1}$ ω7c	0.3	0.7	1.1	0.4
$C_{18:1}$ ω9c	9.5	15.8	18.1	1.4
Branched fatty acids				
i-$C_{14:0}$	-	2.4	0.5	0.7
i-$C_{15:0}$	3.0	5.6	6.3	41.8
i-$C_{15:1}$	-	0.4	-	0.8
a-$C_{15:0}$	0.3	1.3	0.8	4.6
i-$C_{16:0}$	38.1	39.3	26.8	9.3
i-$C_{16:1}$	1.5	2.5	0.5	0.7
i-$C_{17:0}$	7.2	3.3	3.2	3.8
3-OH -i-$C_{17:0}$	0.2	-	-	-
a-$C_{17:0}$	19.2	3.3	2.8	3.9
i-$C_{18:0}$	2.4	1.2	0.6	-
10-Methylated				
$C_{17:0}$	-	-	-	3.0
$C_{18:0}$	-	1.7	1.1	0.5

Strains: 1, S3-1^T; 2, *M. nigra* NBRC 16103^T; 3, *M. yangpuensis* NBRC107727^T and 4, *M. narathiwatensis* BTG4-1^T.

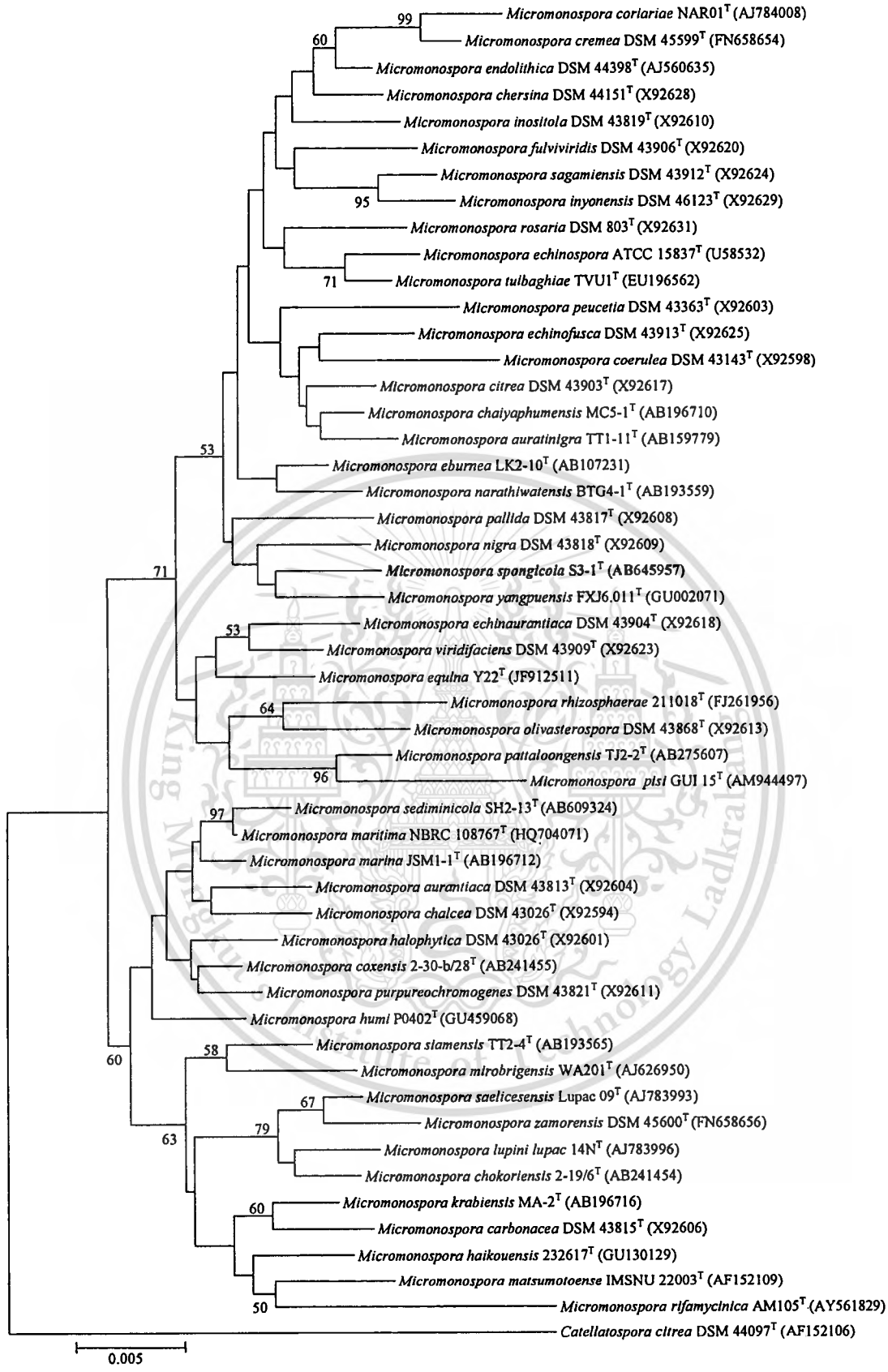


Fig. 4.6 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain S3-1 and other members of the genus *Micromonospora*

The strain S8-12 was selected for investigation of bioactive secondary metabolites from the crude analyse due to the strain exhibited the interested biological activities and chemical profiles. Strain S8-12 formed single spores on the substrate mycelium but aerial mycelium was absent (Fig. 4.7). Deep brown of the substrate mycelium were produced on ISP 2, and ISP 3 agars. Strong reddish brown soluble pigments were produced on the ISP 2 agar. The maximum temperature for growth was 45°C. The strain grew on the pH ranged from 6-12. The maximum NaCl tolerance was 6%. Strain S8-12 produced acid from D-cellobiose, D-fructose, D-galactose, lactose, D-melibiose, salicin, sorbose and D-xylose. Starch hydrolysis, gelatin liquefaction, nitrate reduction and peptonization of milk were positive.

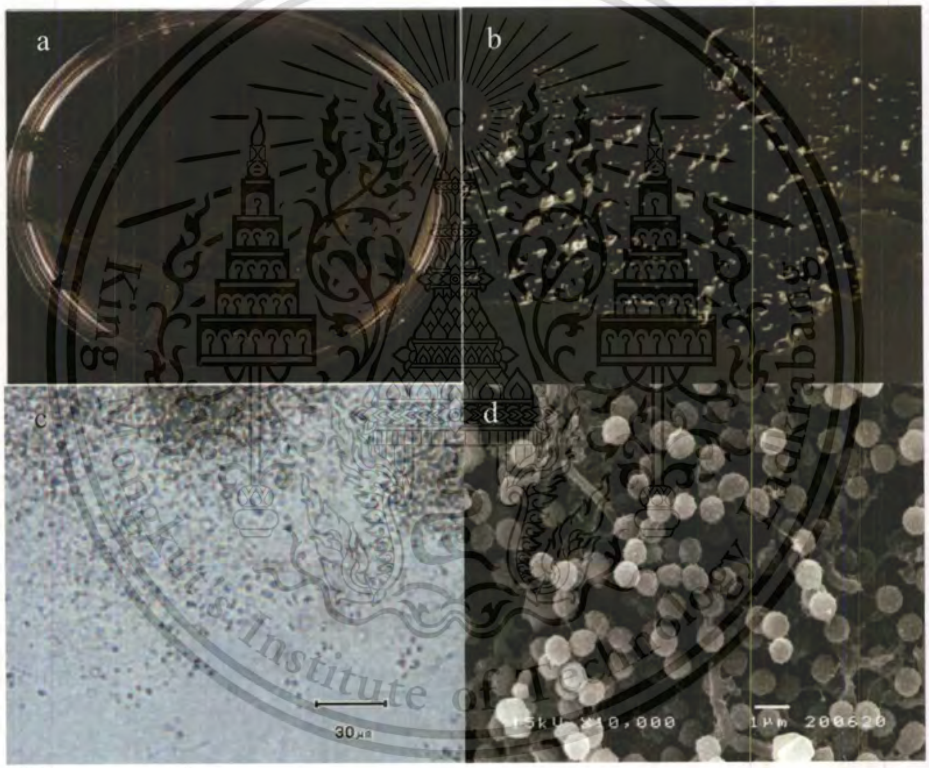


Fig. 4.7 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Micromonospora* sp. S8-12^{T}

The diagnostic diamino acid of the peptidoglycan was *meso*-diaminopimelic acid. Whole-cell sugars were arabinose, galactose, glucose, ribose and xylose. Major polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. Major fatty acids of strain S8-12 comprised iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{18:1}$ ω9c, iso-$C_{17:0}$ and $C_{18:0}$. The predominant menaquinones were MK-10(H_4) and MK-10(H_6). The DNA G+C content of the strain was 72.3 mol%.

An almost-complete 16S rRNA gene sequence (1,409 nucleotides) was obtained for strain S8-12. Strain S8-12 showed highest 16S rRNA gene sequences similarity value with *Micromonospora echinospora* ATCC 15837^T (99.6%). The phylogenetic tree base on 16S rRNA gene sequences showed that strain S8-12 formed a clade with *Micromonospora echinospora* ATCC 15837^T (Fig. 4.8).

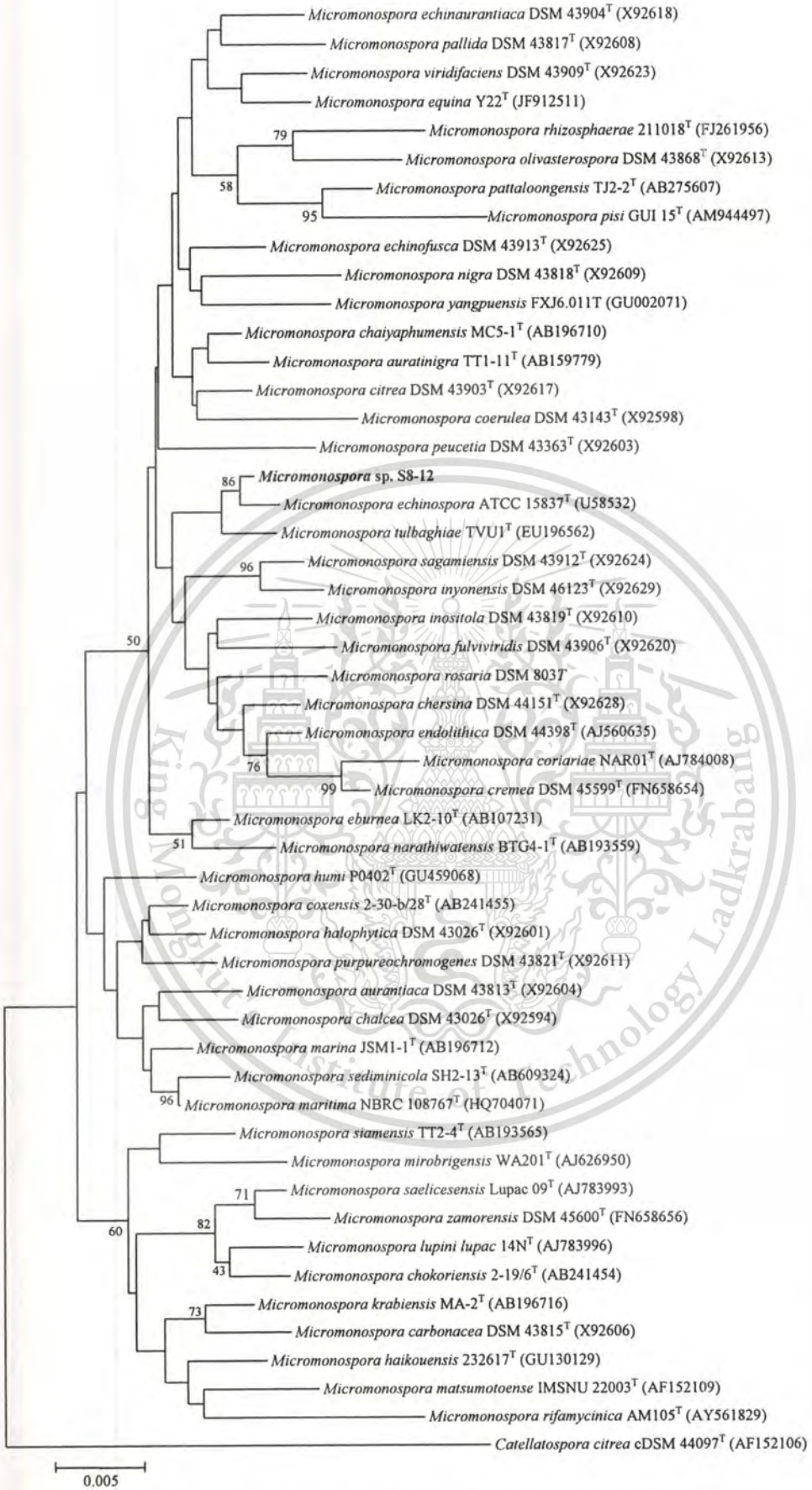


Fig. 4.8 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain S8-12 and other members of the genus *Micromonospora*

The marine actinobacteria in group II consisted of three strains, SP207-05, SP207-08, and SP209-09, which formed single spores on their substrate mycelium. Vegetative hyphae extensively branched but not fragmented (Fig. 4.9). Aerial mycelium and soluble pigment were not produced. The substrate mycelium formed colour of moderate orange to vivid orange (Table 4.14). These strains grew on 3-4% NaCl, pH 6-9, and 20-37 °C. Starch hydrolysis, gelatin liquefaction, and nitrate reductase were positive. Coagulation and peptonization of milk were negative (Table 4.15). In addition, these strains produced acids from D-cellobiose, D-fructose, lactose, salicin and sorbose. Marine actinobactria in this group required sea water for growth and well grown on sodium-supplemented medium (3 % NaCl) indicated that this group was assigned as the obligated marine actinobacteria (Jensen *et al.*, 2005).

The strains contained *meso*-diaminopimelic acid in the peptidoglycan and had wall chemotype II (Lechevalier & Lechevalier, 1970). The acyl type of cell wall muramic acid was glycolyl. The whole cell sugars were arabinose, galactose, glucose, mannose, ribose and xylose which corresponded to whole cell sugar pattern D (Lechevalier and Lechevalier, 1970). The predominant polar lipids comprised phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. Major fatty acid comprised $C_{17:0}$, $C_{17:1}$ ω8c, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$(Table 4.16). On the basis of morphological and chemotaxonomic data, it was shown that marine actinobacteria in this group had the properties that consistent with their assignment to the genus *Salinispora* (Jensen *et al.*, 2005).

Fig. 4.9 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c) of *Salinispora* sp. SP207-08

16S rRNA gene sequences analyses confirmed that these strains were classified to the genus *Salinispor*. In addition, phylogenetic tree based on 16S rRNA gene sequences showed that these strains formed a clade with *Salinispora arenicola* CNH-643^T (Fig. 4.10) and showed the highest similarity value with *Salinispora arenicola* CNH-643^T (100%).

Table 4.14 Cultural characteristics of marine actinobacteria in group II

Media	Strains		
	SP207-05	SP207-08	SP209-09
Yeast extract-malt extract agar (ISP 2)			
Growth	+++	+++	+++
Colour of reverse surface	Moderate orange	Moderate orange	Brownish orange
Colour of upper surface	Moderate orange	Moderate orange	Moderate orange
Oatmeal agar (ISP 3)			
Growth	++	++	+
Colour of reverse surface	Moderate orange	Moderate orange	Light orange yellow
Colour of upper surface	Moderate orange	Light orange yellow	Light orange yellow
Inorganic salt-starch agar (ISP 4)			
Growth	+	+	+
Colour of reverse surface	Vivid orange	Vivid orange	Light orange yellow
Colour of upper surface	Vivid orange	Vivid orange	Light orange yellow
Glycerol-asparagine agar (ISP 5)			
Growth	+	+	++
Colour of reverse surface	Moderate orange	Moderate orange	Moderate orange
Colour of upper surface	Moderate orange	Moderate orange	Moderate orange
Peptone-yeast extract iron agar (ISP 6)			
Growth	+	+	+
Colour of reverse surface	Deep orange	Brownish orange	Moderate orange
Colour of upper surface	Deep orange	Brownish orange	Moderate orange
Tyrosine agar (ISP 7)			
Growth	+	+	+
Colour of reverse surface	Moderate orange	Moderate orange	Moderate orange
Colour of upper surface	Vivid orange	Moderate orange	Moderate orange
Czapek's sucrose agar			
Growth	+	+	+
Colour of reverse surface	Light orange	Light orange	Moderate orange
Colour of upper surface	Light orange	Light orange	Moderate orange
Glucose-asparagine agar			
Growth	+	+	++
Colour of reverse surface	Light orange	Light orange	Light Orange
Colour of upper surface	Light orange	Light orange	Light orange
Nutrient agar			
Growth	+	+	+
Colour of reverse surface	Light orange	Light orange	Light orange
Colour of upper surface	Light orange	Light orange	Light orange

None of the strains formed aerial hyphae or produced diffusible pigments

Key: +++, good; ++, moderate; + poor.

Table 4.15 Physiological and biochemical characteristics of marine actinobacteria in group II

Characteristics	Strains		
	SP207-05	SP207-08	SP209-09
Starch hydrolysis	+	+	+
Nitrate reduction	+	+	+
Gelatinization	+	+	+
Coagulation of milk	-	-	-
Peptonization of milk	-	-	-
NaCl tolerance (%W/V)	3-4	3-4	3-4
Growth at 40 °C	-	-	-
pH tolerance	6-12	6-12	6-12
Acid production from :			
D-Cellobiose	+	+	+
D-Fructose	+	+	+
D-Galactose	+	-	+
D-Melibiose	-	-	-
D-Raffinose	-	-	-
D-Ribose	-	+	-
D-Xylose	-	+	-
L-Arabinose	-	+	-
Lactose	+	+	+
Salicin	+	+	+
Sorbose	+	+	+

Table 4.16 Cellular fatty acid compositions (%) of marine actinobacteria in group II

Fatty acids	Strains		
	SP03-05	SP207-08	SP209-09
Saturated fatty acids			
$C_{13:0}$	0.6	0.3	0.6
$C_{14:0}$	0.6	0.3	0.7
$C_{16:0}$	1.0	1.2	1.0
$C_{17:0}$	5.5	5.3	5.4
$C_{18:0}$	0.9	1.0	0.8
Unsaturated fatty acids			
$C_{15:1}$ ω6c	0.6	-	0.3
$C_{16:1}$ ω7c	0.4	0.3	0.4
$C_{17:1}$ ω8c	12.7	11.1	14.9
$C_{18:1}$ ω7c	0.2	0.2	0.3
$C_{18:1}$ ω9c	2.4	2.8	2.6
$C_{19:1}$ ω6c	0.2	0.1	2.3
$C_{19:1}$ ω9c	1.6	2.1	1.6
Branched fatty acids			
i-$C_{14:0}$	2.7	1.4	1.3
i-$C_{15:0}$	7.0	7.2	15.0
i-$C_{15:1}$	-	-	
a-$C_{15:0}$	5.8	2.8	6.0
i-$C_{16:0}$	38.9	50.4	28.2
i-$C_{16:1}$	1.7	2.2	1.2
i-$C_{17:0}$	1.6	2.7	4.2
a-$C_{17:0}$	4.2	3.5	6.1
a-$C_{17:1}$	0.6	0.3	0.6
i-$C_{18:0}$	0.4	0.5	0.3
10-Methylated			
$C_{16:0}$	1.5	2.4	2.7
$C_{17:0}$	6.5	-	-
$C_{18:0}$	1.0	1.2	0.9

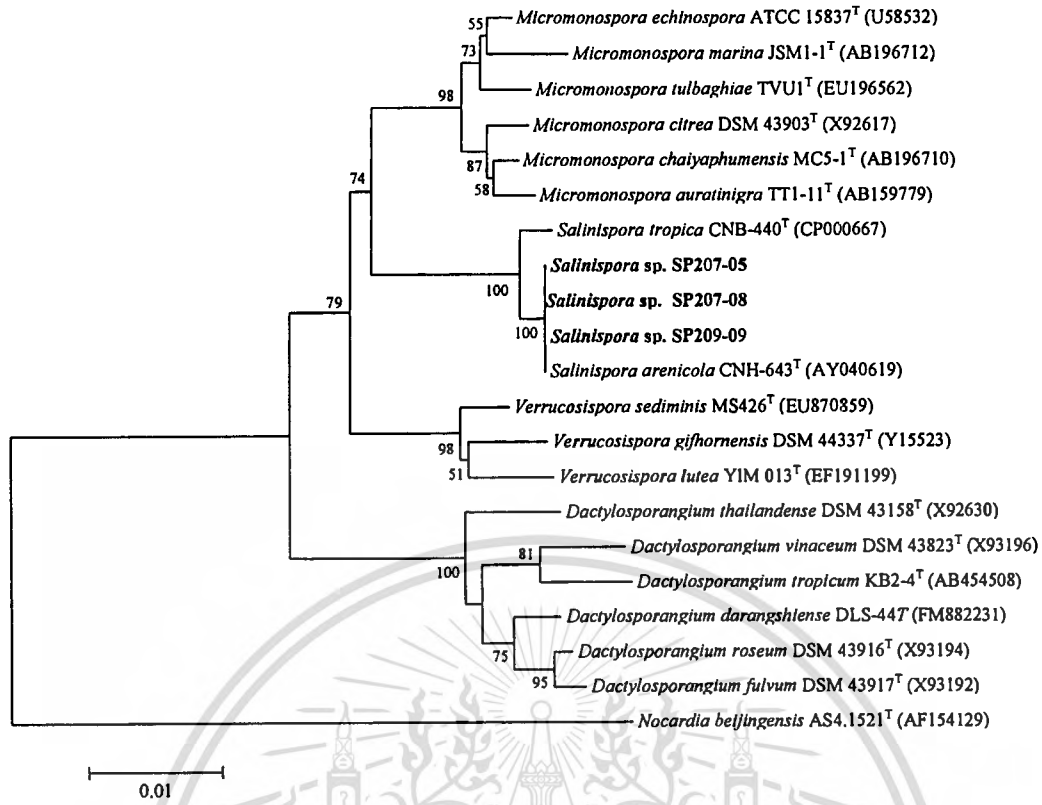


Fig. 4.10 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains SP207-05, SP207-08, SP209-09 and other members of the family *Micromonosporaceae*

The marine actinobacteria in group III contained four strains such as SP03-01, SP03-05, SP206-02 and SP206-03. In this group, strain SP206-02 formed the clustered spores on the substrate mycelium (Fig. 4.11) but strains SP03-01, SP03-05, and SP206-03 produced single spores on the substrate mycelium (Fig. 4.13). The spore surfaces were smooth to warty and non-motile. Aerial mycelium was absent on any medium tests. They grew well on ISP 2 agar. The colours of the substrate mycelium ranged from light orange to strong orange (Table 4.17). These strains grew at 0-4% NaCl, on pH 6-12, and at 20-40 °C (Table 4.18). In addition, most strains produced acids from D-cellobiose, D-fructose, lactose, salicin and sorbose.

The chemical profiles of these strains were similar to those of members of the genus *Verrucosispora*. Cell wall hydrolysates of the strains consisted of *meso*-diaminopimelic acid indicating that these strains had wall chemotype II (Lechevalier and Lechevalier, 1970). The acyl type of cell wall muramic acid was glycolyl. The whole cell sugars were arabinose, galactose glucose, mannose, ribose and xylose which corresponded to whole-cell sugar pattern D

(Lechevalier and Lechevalier, 1970). Characteristic phospholipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. This pattern corresponds to phospholipid type II (Lechevalier *et al.*, 1977). The major menaquinones were MK-9(H_4) and MK-9(H_6). The major cellular fatty acids of the strains were iso-$C_{15:0}$, $C_{16:0}$, and anteiso-$C_{17:0}$ (Table 4.19), this pattern corresponds to fatty acid type 2d (Kroppenstedt, 1985).

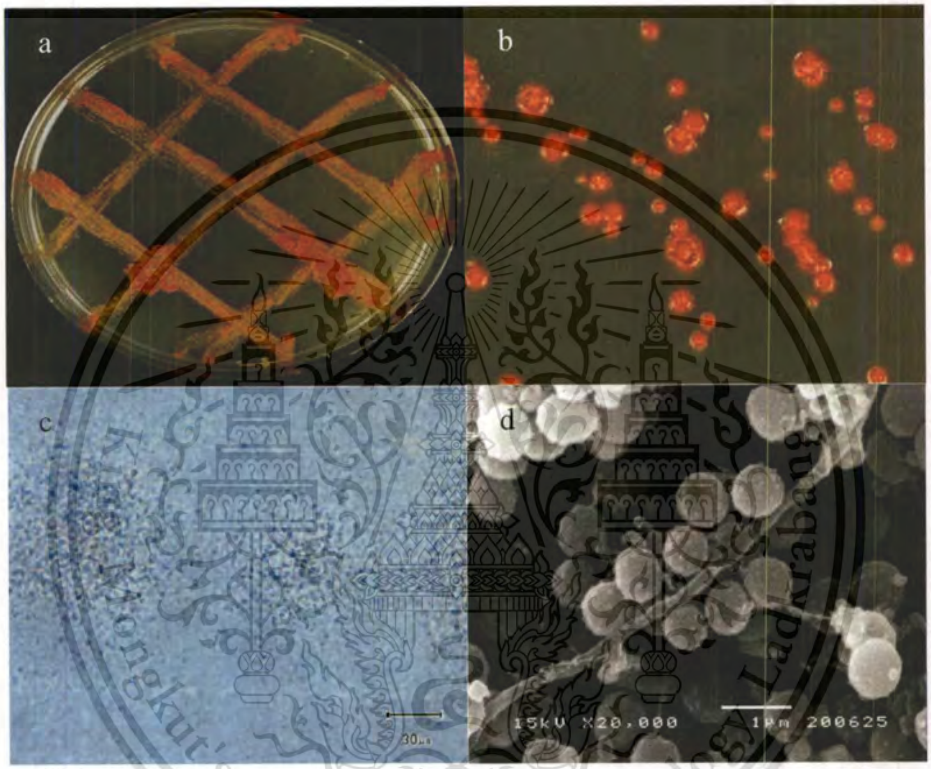


Fig. 4.11 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Verrucosispora* sp. SP206-02

Almost complete 16S rRNA gene sequences (1,401-1,447 nucleotides) of each strain were used for phylogenetic analysis and compared against 16S rRNA gene sequences of members of the family *Micromonosporaceae*. Phylogenetic analysis based on this large dataset revealed that these strains were placed within the clade of the genus *Verrucosispora* (Fig. 4.12). The morphological, chemotaxonomic characteristics and 16S rRNA gene analyses of these strains were consistent with their classification in the genus *Verrucosispora* (Rheims *et al.*, 1998). 16S rRNA gene sequence similarity indicated that strain SP206-02 showed highest similarity value

with *Verrucosispora gifhornensis* DSM 44337^T (99.8%). Strains SP03-01 and SP206-03 located within a clade of *Verrucosispora wenchangensis* and showed highest similarity values with *Verrucosispora wenchangensis* 234402^T at 99.6% and 99.7%, respectively. In contrast, the strain SP03-05 exhibited the low 16S rRNA gene sequences similarity value (96.7-98.3%) with all *Verrucosispora* species.

Table 4.17 Cultural characteristics of marine actinobacteria in group III

Media	Strains			
	SP03-05	SP03-01	SP206-03	SP206-02
Yeast extract-malt extract agar (ISP 2)				
Growth	+++	+++	+++	+++
Colour of reverse surface	Strong orange	Strong orange	Strong orange	Strong orange
Colour of upper surface	Deep orange	Deep orange	Deep orange	Vivid orange
Oatmeal agar (ISP 3)				
Growth	+	++	+	+++
Colour of reverse surface	Strong orange	Strong orange	Strong orange	Vivid orange
Colour of upper surface	Strong orange	Strong orange	Strong orange	Vivid orange
Inorganic salt-starch agar (ISP 4)				
Growth	+	++	+	++
Colour of reverse surface	Strong orange	Strong orange	Strong orange	Vivid orange
Colour of upper surface	Vivid orange	Vivid orange	Vivid orange	Vivid orange
Glycerol-asparagine agar (ISP 5)				
Growth	+	++	+	++
Colour of reverse surface	Light orange	Brownish orange	Light orange	Vivid orange
Colour of upper surface	Light orange	Brownish orange	Light orange	Vivid orange
Peptone-yeast extract iron agar (ISP 6)				
Growth	+	+	+	+
Colour of reverse surface	Light orange	Moderate orange	Light orange	Moderate orange
Colour of upper surface	Light orange	Moderate orange	Light orange	Moderate orange
Tyrosine agar (ISP 7)				
Growth	+	+	+	++
Colour of reverse surface	Yellowish white	Moderate orange	Yellowish white	Vivid orange
Colour of upper surface	Yellowish white	Moderate orange	Yellowish white	Vivid orange
Czapek's sucrose agar				
Growth	+	+	+	+
Colour of reverse surface	Colorless	Moderate orange	Moderate orange	Moderate orange
Colour of upper surface	Colorless	Moderate orange	Moderate orange	Moderate orange
Glucose-asparagine agar				
Growth	+	++	+	++
Colour of reverse surface	Light orange	Strong orange	Light orange	Moderate orange
Colour of upper surface	Light orange	Strong orange	Light orange	Moderate orange
Nutrient agar				
Growth	+	+	+	++
Colour of reverse surface	Strong orange	Moderate orange	Strong orange	Vivid orange
Colour of upper surface	Strong orange	Moderate orange	Strong orange	Vivid orange

None of the strains produced diffusible pigments

Key: +++, good; ++, moderate; + poor.

Table 4.18 Physiological and biochemical characteristic of marine actinobacteria in group III

Characteristics	Strains			
	SP03-05	**SP03-01**	**SP206-03**	**SP206-02**
Nitrate reduction	-	-	-	-
Starch hydrolysis	+	+	+	+
Gelatinization	-	-	+	+
Coagulation of milk	-	-	+	+
Peptonization of milk	+	-	+	-
NaCl tolerance (%W/V)	0-4	0-4	0-4	0-4
Growth at 40 °C	+	+	+	+
pH range for growth	6-12	6-12	6-12	6-12
Acid production from :				
D-Cellobiose	+	+	+	-
D-Fructose	+	+	+	-
D-Galactose	+	-	+	-
D-Melibiose	-	-	-	+
D-Raffinose	-	-	-	+
D-Ribose	-	+	-	+
D-Xylose	-	+	+	+
L-Arabinose	-	+	-	-
Lactose	+	+	+	+
Salicin	+	+	+	-
Sorbose	+	+	+	-

Table 4.19 Cellular fatty acid compositions (%) of marine actinobacteria in group III

Fatty acids	Strains			
	SP03-05	SP03-01	SP206-03	SP206-02
Saturated fatty acids				
$C_{14:0}$	1.4	-	0.4	0.6
$C_{16:0}$	4.0	5.6	1.5	4.2
$C_{17:0}$	9.0	12.2	7.0	14.6
$C_{18:0}$	7.2	7.4	1.6	3.8
Unsaturated fatty acids				
$C_{16:1}$ ω7c	1.3		1.3	
$C_{17:1}$ ω7c	-			
$C_{17:1}$ ω8c	5.1	6.0	15.0	12.5
$C_{18:1}$ ω9c	4.2	3.8	4.1	2.7
$C_{19:1}$ ω9c	0.2	-	0.7	0.4
$C_{20:1}$ ω7c	1.1	-	-	-
Branched fatty acids				
i-$C_{14:0}$	1.0	4.3	0.9	1.4
i-$C_{15:0}$	25.9	25.0	21.6	11.5
i-$C_{15:1}$	-	-	0.1	0.1
a-$C_{15:0}$	10.8	11.1	8.3	3.0
i-$C_{16:0}$	8.8	8.9	19.0	26.6
i-$C_{16:1}$	0.1	-	0.5	-
i-$C_{17:0}$	7.3	5.1	5.4	2.2
a-$C_{17:0}$	5.5	6.0	4.9	4.3
i-$C_{18:0}$	-	-	0.3	0.8
10-Methylated fatty acids				
$C_{16:0}$	2.0	-	3.3	-
$C_{17:0}$	0.3	-	1.6	5.2
$C_{18:0}$	0.2	-	0.3	0.9

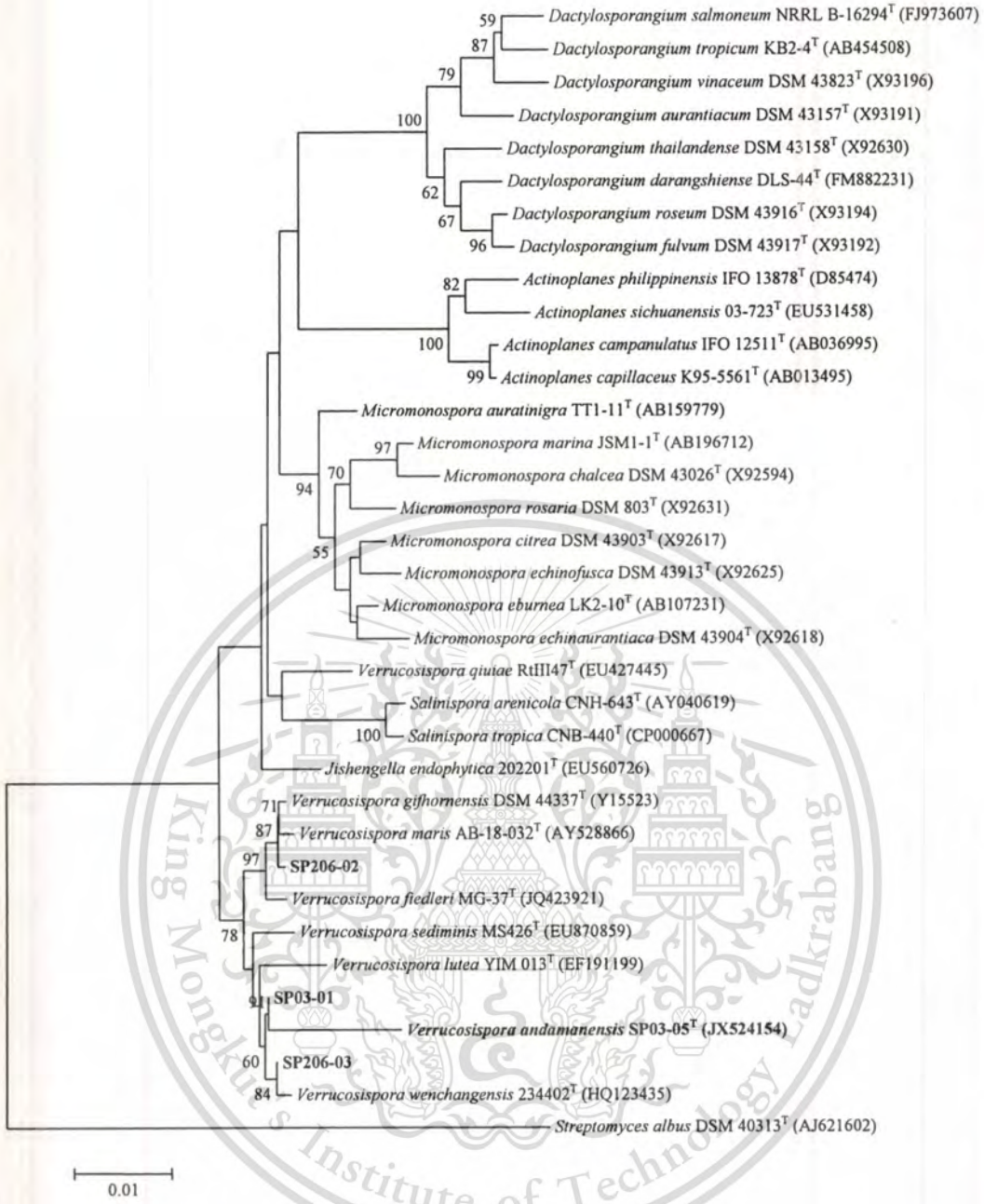


Fig. 4.12 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains SP03-01, SP03-05, SP206-02, SP206-03, and other members of the genus *Verrucosispora*

Strain SP03-05 was selected to complete for the taxonomic characterization. The strain was aerobic, Gram-stain-positive bacteria which formed spherical spores on the substrate mycelium, but aerial mycelium was absent (Fig. 4.13). The strain grew well on ISP 2 and ISP 4 media agar and produced vivid orange to strong orange of the substrate mycelium. No soluble pigment was produced on medium tests. The temperature for growth ranged from 20-40°C, at pH 6-12 and in the presence of 4% NaCl. In addition, strain SP03-05^{T} produced acids from D-cellobiose, D-fructose, D-galactose, D-sucrose, lactose, salicin and sorbose. Starch hydrolysis and milk peptonization were positive. Nitrate reduction, coagulation of milk, and gelatin liquefaction were negative.

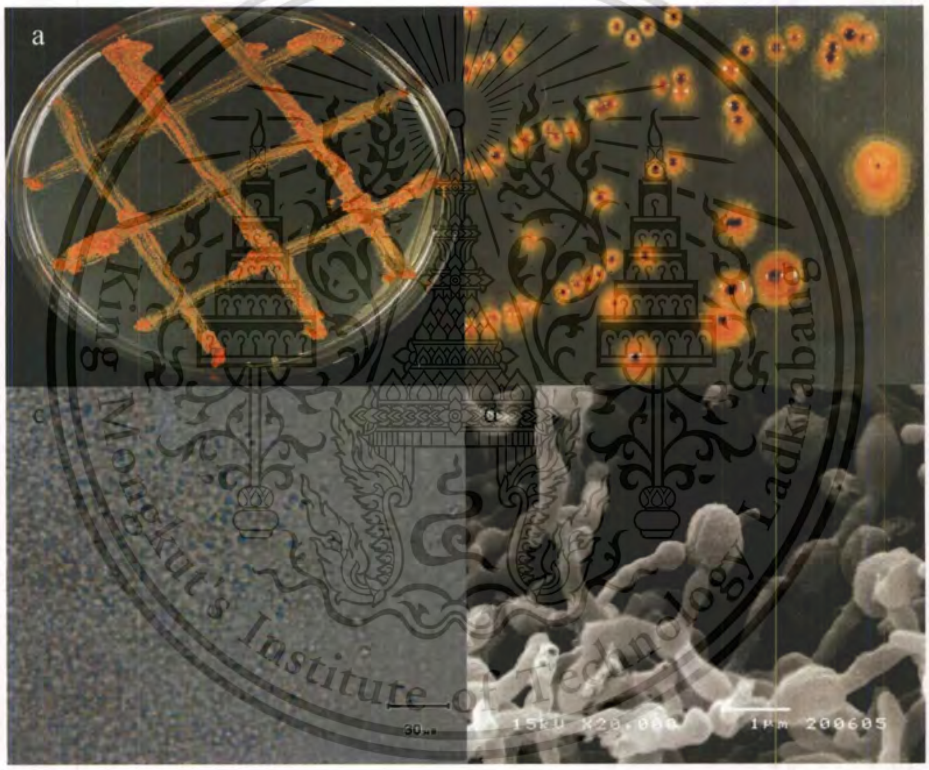


Fig. 4.13 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Verrucosispora andamanensis* SP03-05^{T}

The cell wall peptidoglycan consisted of *meso*-diaminopimelic acid. The acyl type of the muramyl residue was glycolyl. Whole-cell sugars were arabinose, galactose, glucose, rhamnose, ribose and xylose. Major phospholipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositolmannosides. The major fatty acid pattern consisted

of iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{17:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, $C_{18:0}$, anteiso-$C_{17:0}$, $C_{17:1}$ ω8c, $C_{18:1}$ ω9c and $C_{16:0}$. The predominant menaquinones were MK-9(H_4), MK-9(H_6) and MK-9(H_2). The DNA G+C content of the strain was 72.4 mol%.

An almost-complete 16S rRNA gene sequence (1,401 nucleotides) was obtained for strain SP03-05^T and compared with those deposited in the public databases. The highest levels of 16S rRNA gene sequence similarity were with *Verrucosispora lutea* YIM 013^T (96.90%), *Verrucosispora sediminis* MS426^T (96.90%), *Verrucosispora gifhornensis* DSM 44337^T (96.80%), *Verrucosispora maris* AB-18-032^T (96.80%) and *Verrucosispora qiuiae* RtIII47^T (95.40%). The neighbour-joining tree of strain SP03-05^T showed that the strain formed a cluster with *Verrucosispora lutea* YIM 013^T, *Verrucosispora sediminis* MS426^T, *Verrucosispora gifhornensis* DSM 44337^T and *Verrucosispora maris* AB-18-032^T (Fig. 4.14). A low level of DNA–DNA relatedness values between strain SP03-05^T and the type strain of *Verrucosispora* species ranged from 10.1 ± 0.2 to 25.4 ± 0.4, which is well below the 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987) (Table 4.22). Furthermore, on the basis of morphological, chemotaxonomic and genotypic analyses indicated that strain SP03-05^T was classified to the genus *Verrucosispora*. The characteristics shown in Table 4.20 indicated that strain SP03-05^T showed some different physiological and biochemical characteristics compared to the recognized *Verrucosispora* species distinguished on the basis of acid production from D-cellobiose, D-fructose, D-galactose and D-melibiose, nitrate reduction, the growth at 40°C, coagulation of milk and gelatin liquefaction. In addition, fatty acid profile of strain SP03-05^T comprised of unsaturated fatty acid $C_{20:1}$ ω7c that were not detected in the other strains (Table 4.21). Therefore, strain SP03-05^T represents a novel species of the genus *Verrucosispora*, for which the name *Verrucosispora andamanensis* sp. nov., was proposed. The type strain was SP03-05^T (=BCC 45620^T =NBRC 109075^T), isolated from the marine sponge (*Xestospongia* sp.) collected from the Andaman Sea of Thailand.

Table 4.20 Differential characteristics of strain SP03-05^T and type strains of the genus *Verrucosispora*.

Characteristics	Strains					
	1	2	3	4	5	6
Nitrate reduction	-	+	-	-	-	-
Gelatinization	-	-	+	-	-	-
Coagulation of milk	-	+	+	+	+	-
Maximum NaCl tolerance (%W/V)	4	2	4	3	4	5
Growth at 40 °C	+	-	-	+	-	+
pH range for growth	6-12	6-12	6-12	7-12	7-12	6-12
Acid production from :						
D-Cellobiose	+	-	+	+	+	+
D-Fructose	+	+	+	-	-	-
D-Galactose	+	-	+	+	-	+
D-Melibiose	-	-	-	-	-	+
D-Raffinose	-	-	-	-	-	-
D-Ribose	-	+	-	-	-	+
D-Xylose	-	+	-	-	+	+
L-Arabinose	-	+	-	-	-	+
Lactose	+	+	+	+	-	+
Salicin	+	+	+	+	-	+
Sorbose	+	+	+	+	-	+

Strain: 1, SP03-05^T; 2, *Verrucosispora lutea* NBRC 106530^T; 3, *Verrucosispora sediminis* NBRC 107745^T; 4, *Verrucosispora gifhornensis* DSM 44991^T; 5, *Verrucosispora maris* DSM45365^T and 6, *Verrucosispora qiuiae* NBRC 106684^T. All phenotypic data were determined in this study. +, Positive; -, Negative.

Table 4.21 Cellular fatty acid compositions (%) of strain SP03-05^T compared with type strains of the genus *Verrucosispora*.

Fatty acids[a]	Strains					
	1	2	3	4	5	6
Saturated fatty acids						
$C_{14:0}$	1.4	0.3	0.2	0.2	0.6	0.4
$C_{16:0}$	4.0	0.6	0.9	1.0	3.8	1.9
$C_{17:0}$	9.0	5.7	3.8	5.0	11.8	9.8
$C_{18:0}$	7.2	0.2	0.6	0.2	2.1	0.8
Unsaturated fatty acids						
$C_{16:1}$ ω7c	1.3	0.3	0.3	0.3	0.9	0.3
$C_{17:1}$ ω7c	-	-	-	-	-	23.0
$C_{17:1}$ ω8c	5.1	23.2	9.9	24.7	14.1	-
$C_{18:1}$ ω9c	4.2	1.5	1.8	1.2	2.1	1.5
$C_{19:1}$ ω9c	0.2	1.2	1.0	0.4	0.3	0.6
$C_{20:1}$ ω7c	1.1	-	-	-	-	-
Branched fatty acids						
i-$C_{14:0}$	1.0	2.5	0.7	1.7	1.9	1.5
i-$C_{15:0}$	25.9	9.0	23.0	18.5	15.7	17.0
a-$C_{15:0}$	10.8	6.1	5.8	3.8	4.0	5.5
i-$C_{16:0}$	8.8	31.1	19.0	26.0	30.0	20.5
i-$C_{16:1}$	0.1	1.8	1.8	-	-	0.2
i-$C_{17:0}$	7.3	1.0	5.8	1.6	1.9	1.1
a-$C_{17:0}$	5.5	1.9	4.9	3.5	3.8	4.9
10-Methylated						
$C_{16:0}$	2.0	1.4	4.3	1.0	0.7	0.7
$C_{17:0}$	0.3	8.4	11.6	7.3	3.7	5.1
$C_{18:0}$	0.2	0.4	1.6	0.3	0.5	0.3

Strain: 1, SP03-05^T; 2, *Verrucosispora lutea* NBRC 106530^T; 3, *Verrucosispora sediminis* NBRC 107745^T; 4, *Verrucosispora gifhornensis* DSM 44991^T; 5, *Verrucosispora maris* DSM45365^T and 6, *Verrucosispora qiuiae* NBRC 106684^T.

Table 4.22 DNA–DNA relatedness among the strain SP03-05^T and *Verrucosispora* species

Strains	DNA–DNA binding (%) with labelled DNA from:					
	1	2	3	4	5	6
1. SP03-05^T	100	25.4 ± 0.4	18.4 ± 0.6	16.9 ± 0.7	15.5 ± 0.7	11.5 ± 0.7
2. *V. lutea* NBRC 106530^T	20.2 ± 0.2	100	21.2 ± 1.5	20.4 ± 0.9	14.2 ± 0.3	12.2 ± 0.3
3. *V. sediminis* NBRC 107745^T	18.5 ± 0.3	22.3 ± 0.7	100	18.1 ± 1.7	14.1 ± 2.2	10.1 ± 0.2
4. *V. gifhornensis* DSM 44991^T	15.8 ± 0.4	18.7 ± 1.2	19.2 ± 1.2	100	25.2 ± 0.8	14.2 ± 0.8
5. *V. maris* DSM45365^T	13.5 ± 0.6	12.5 ± 1.4	10.7 ± 0.9	22.1 ± 1.4	100	16.2 ± 0.8
6. *V. qiuiae* NBRC 106684^T	11.2 ± 0.8	13.3 ± 1.2	14.5 ± 1.3	18.3 ± 2.1	19.2 ± 0.9	100

Fig. 4.14 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain SP03-05 and other members of the family *Micromonosporaceae*

The marine actinobacteria in group IV consisted of strain TV1-14 that formed an extensively branched and non fragmented substrate mycelium but the aerial mycelium was not found on the medium tests (Fig. 4.15). The strain produced single spore on the tip of substrate mycelium. Light pink to strong yellowish pink of the mycelium was appeared on ISP 2, ISP 3, and ISP 4 agars. Strain TV1-14 grew well on ISP 2 agar, but weakly on ISP 4, ISP 5, ISP 6, ISP 7, czapek's sucrose, glucose-asparagine and nutrient agars. Strong orange soluble pigments were produced on the ISP 2 agar (Table 4.23). The strain grew on 0-5% NaCl, on pH 6-12, and at 20-37 ºC. Peptonization of milk was positive but starch hydrolysis, gelatin liquefaction, and nitrate reduction were negative. The strain produced acids from D-cellobiose, D-fructose and sorbose.

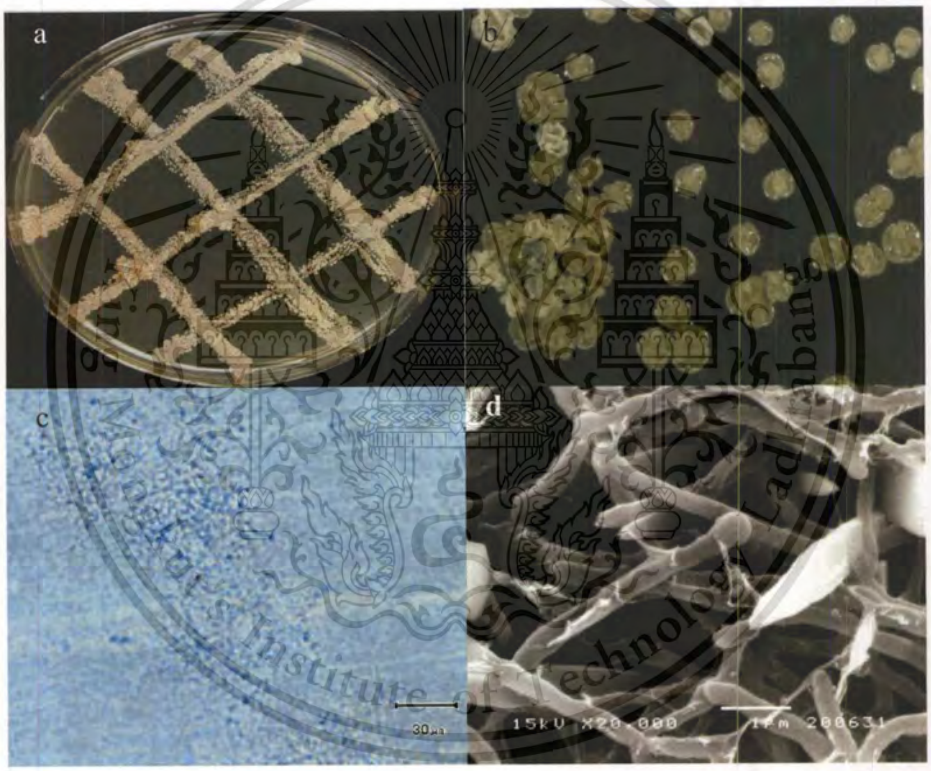


Fig. 4.15 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Actinomadura* sp. TV1-14

Strain TV1-14 contained *meso*-diaminipimelic acid in the peptidoglycan of cell wall. The whole cell sugar pattern comprised arabinose, galactose, glucose, madurose, mannose, and rhamnose which corresponded to whole-cell sugar pattern B (Lechevalier and Lechevalier, 1970). Major polar lipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol,

and phosphatidylinositol mannosides. Cellular fatty acids were $C_{14:0}$ (9.6%), i-$C_{16:0}$ (4.5%), $C_{16:1}$ ω7c (14.7%), 10-methyl-$C_{16:0}$ (8.4%), $C_{17:0}$ (4.9%), $C_{17:1}$ ω8c (2.1%), 10-methyl-$C_{17:0}$ (3.7%), $C_{18:1}$ ω7c (26.4%) and $C_{18:0}$(16.5%). The predominant menaquinones were MK-9(H_4), MK-9(H_6) and MK-9(H_8). The DNA G+C content of the strain was 73.5 mol%.

An almost complete 16S rRNA gene sequence (1,397 nucleotides) was obtained from strain TV1-14 and 16S rRNA gene sequence analysis revealed that strain TV1-14 was closely related to species of the genus *Actinomadura*. However, the phylogenetic tree based on the 16S rRNA gene sequence showed that the strain formed clade with the genus *Actinomadura* (Fig. 4.16). The highest levels of 16S rRNA gene sequence similarity was found with *Actinomadura macra* IFO 14102^T (97.7%). The DNA–DNA relatedness between strain TV1-14 and *Actinomadura macra* NBRC 14102^T was 23.8±1.0, which is well below the 70% cut-off point for recognition of genomic species (Wayne *et al.*, 1987), thus suggesting that the test strain should be considered as a separate species.

In addition, the differential phenotypic characteristics (Table 4.24) showed that strain TV1-14 can be readily distinguished from the related species *Actinomadura macra*, including acid production from D-cellobiose, D-galactose, D-xylose, and D-sucrose, and tolerance on 5% NaCl. On the basis of phenotypic and genotypic characteristics presented that strain TV1-14 is a member of the genus *Actinomadura*, and is distinguishable prom previously described *Actinomadura* species. So, this strain should be judged to be a new species of the genus *Actinomadura*.

Table 4.23 Cultural characteristics of *Actinomadura* sp. TV1-14

Media	Growth	Colour of colony		Soluble
		upper surface	reverse surface	pigment
Yeast extract-malt extract agar (ISP 2)	Good	Light pink	Strong yellowish pink	Strong orange
Oatmeal agar (ISP 3)	Moderate	Light pink	Strong yellowish pink	None
Inorganic salt-starch agar (ISP 4)	Poor	Light pink	Moderate yellowish pink	None
Glycerol-asparagine agar (ISP 5)	Poor	Pinkish white	Pinkish white	None
Peptone-yeast extract iron agar (ISP 6)	Poor	Vivid yellowish pink	Moderate yellowish pink	None
Tyrosine agar (ISP 7)	Poor	Vivid orange	Vivid orange	None
Czapek's sucrose agar	Poor	Moderate yellowish pink	Moderate yellowish pink	None
Glucose-asparagine agar	Poor	Pinkish white	Pinkish white	None
Nutrient agar	Poor	Yellowish white	Yellowish white	None

Table 4.24 Differntial characteristics of strain TV1-14 and *Actinomadura macra* NBRC 14102^T

Characteristics	Strains	
	TV1-14	***A. macra***
Hydrolysis of starch	-	-
Nitrate reduction	-	+
Gelatinization	-	+
Maximum NaCl tolerance (%)	5	2
Growth at 40 °C	+	-
pH tolerance	5-12	6-12
Acid production from :		
D-Cellobiose	+	-
D-Fructose	-	+
D-Galactose	-	+
D-Mellibiose	+	-
D-Raffinose	+	-
D-Xylose	-	+
D-Sucrose	-	+

+, Positive; -, Negative.

Fig. 4.16 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain TV1-14 and other members of genus *Actinomadura*

The marine actinobacteria in group V contained only one strain, ST01-07. This strain isolated from the marine sponge collected from the Andaman Sea of Thailand. Strain ST01-07 formed extensively branched substrate hyphae and aerial hyphae weakly formed. The strain formed the globose bodies directly on the substrate mycelium and produced longitudinally rod-shaped pairs of spore (Fig. 4.17). The colour of colonies was grayish yellow to dark grayish yellow on the ISP 2 agar. Light orange to moderated orange substrate mycelium were observed on ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, czapek's sucrose, glucose-asparagine, and nutrient agars. Yellowish brown soluble pigment was produced on the ISP 2 agar (Table 4.25). The strain grew on 0-4% NaCl, at pH 6-12, and 20-40 °C. Nitrate reduction and coagulation of milk were positive but hydrolysis of starch, gelatin liquefaction, and peptonization of starch were negative. The strain produced acid from D-cellobiose, D-fructose, D-galactose, D-ribose, D-sucrose and sorbose.

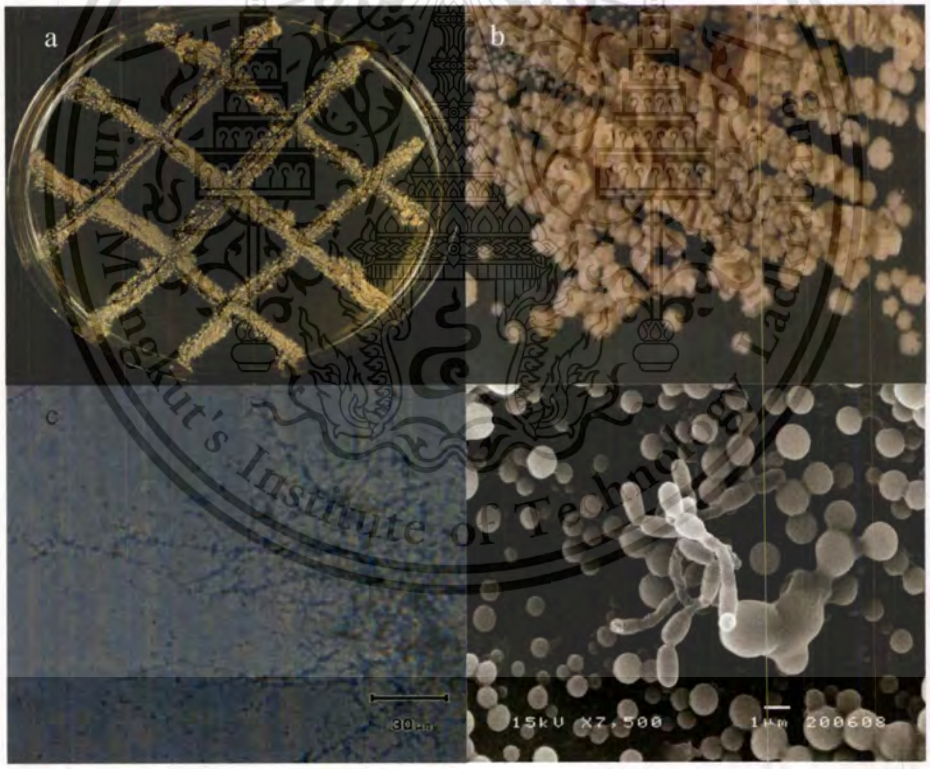


Fig. 4.17 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Nocardia* sp. ST01-07

Strain ST01-07 contained *meso*-diaminopimelic acid in the peptidoglycan of the cell wall. Whole cell sugars were arabinose, galactose, glucose, mannose, and ribose which corresponded to whole cell sugar pattern A (Lechevalier and Lechevalier, 1970). The major polar lipids comprised diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides. Predominant menaquinones were MK-8($H_{4\omega\text{-cycl}}$) and 2, 3-epoxy- MK-8($H_{4\omega\text{-cycl}}$). Cellular fatty acids were $C_{15:1}$ ω5c (3.4%), $C_{16:0}$ (19.5%), $C_{16:1}$ ω7c (8.2%), $C_{17:0}$ (13.2%), $C_{17:1}$ ω8c (19.8%), 10-methyl-$C_{17:0}$ (7.7%), $C_{18:1}$ ω9c (7.8%), $C_{18:0}$ (5.6%) and 10-methyl-$C_{18:0}$(3.4%). The G+C content of the strain was 70.2 mol%.

Table 4.25 Cultural characteristics of *Nocardia* sp. ST01-07

Media	Growth	Colour of colony		Soluble pigment
		upper surface	reverse surface	
Yeast extract-malt extract agar (ISP 2)	Moderate	Grayish yellow	Dark grayish yellow	Yellowish brown
Oatmeal agar (ISP 3)	Poor	Light orange yellow	Light orange yellow	None
Inorganic salt-starch agar (ISP 4)	Poor	Light orange yellow	Light orange yellow	None
Glycerol-asparagine agar (ISP 5)	Poor	Light orange yellow	Pale orange yellow	None
Peptone-yeast extract iron agar (ISP 6)	Poor	Light orange yellow	Light orange yellow	None
Tyrosine agar (ISP 7)	Poor	Light orange yellow	Light orange yellow	None
Czapek's sucrose agar	Poor	Moderate orange yellow	Light orange yellow	None
Glucose-asparagine agar	Poor	Moderate orange yellow	Light orange yellow	None
Nutrient agar	Moderate	Light orange yellow	Pale orange yellow	None

An almost-complete 16S rRNA gene sequence (1,399 nucleotides) was obtained from strain ST01-07. The highest levels of 16S rRNA gene sequences similarity were with *Nocardia araoensis* IFM 0575^T (98.8%), *Nocardia beijingensis* AS4.1521^T (98.5%), *Nocardia niwae* W9241^T (98.5%) and *Nocardia arthritidis* IFM 10035^T (98.4%). The neighbour-joining tree showed that the strain formed a distinct phyletic line of *Nocardia araoensis* IFM 0575^T, *Nocardia beijingensis* AS4.1521^T, *Nocardia niwae* W9241^T and *Nocardia arthritidis* IFM 10035^T (Fig. 4.18). A low level of DNA–DNA relatedness values between strain ST01-07 and the related *Nocardia* species ranged from 11.9±0.8 to 24.4±0.6 (Table 4.27) which is well below the 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987). Furthermore, based on morphological, chemotaxonomic and genotypic analyzes indicated that strain ST01-07 belonged to the members of the genus *Nocardia*. The

differential characteristics shown in Table 4.26 indicated that strain ST01-07 showed some different physiological and biochemical characteristics compared to the related *Nocardia* species that distinguished on the basis of acid production from D-cellobiose, D-fructose, D-galactose, and D-sucrose, nitrate reduction, the growth on 4% NaCl. Therefore, based on phenotypic, chemotypic and genotypic characteristics showed that strain ST01-07 represents a novel species of the genus *Nocardia*.

Table 4.26 Differential characteristics of strain ST01-07^T and related *Nocardia* species

Characteristics	Strains				
	ST01-07	**2**	**3**	**4**	**5**
Starch hydrolysis	-	+	+	+	-
Nitrate reduction	+	-	+	+	+
Gelatinization	-	+	+	+	+
Peptonization	-	+	+	+	+
Maximum NaCl tolerance (%W/V)	7	6	6	7	6
Growth at 45 °C	-	+	+	+	+
Growth at pH 5	-	+	+	+	+
Acid production from :					
D-Cellobiose	+	+	+	+	-
D-Fructose	+	-	-	+	-
D-Galactose	+	-	-	+	-
D-Sucrose	+	-	-	-	-
D-Xylose	-	+	+	+	+
Salicin	-	-	-	-	+

Strain: 1, ST01-07; 2, *Nocardia araoensis* NBRC 100135^T; 3, *Nocardia beijingensis* NBRC 16342^T; 4, *Nocardia arthritidis* NBRC NBRC 100137^T; and 5, *Nocardia niwae* DSM 45340^T. +, Positive; -, Negative.

Table 4.27 DNA–DNA relatedness among the strain ST0-07^T and related *Nocardia* species

Strains	DNA–DNA binding (%) with labelled DNA from:				
	1	2	3	4	5
1 ST01-07	100	24.4± 0.6	18.4 ± 0.6	11.9 ± 0.8	13.5 ± 0.4
2 *N. araoensis* NBRC 100135^T	22.2 ± 0.4	100	20.2 ± 1.2	21.4 ± 0.7	17.2 ± 0.3
3 *N. beijingensis* NBRC 16342^T	21.5 ± 0.3	18.3 ± 0.9	100	14.1 ± 1.3	14.1 ± 2.2
4 *N. arthritidis* NBRC NBRC 100137^T	14.1 ± 0.4	14.7 ± 1.3	18.2 ± 0.2	100	23.2 ± 0.8
5 *N. niwae* DSM 45340^T	10.3 ± 0.6	12.8 ± 0.4	12.7 ± 0.5	23.1 ± 1.1	100

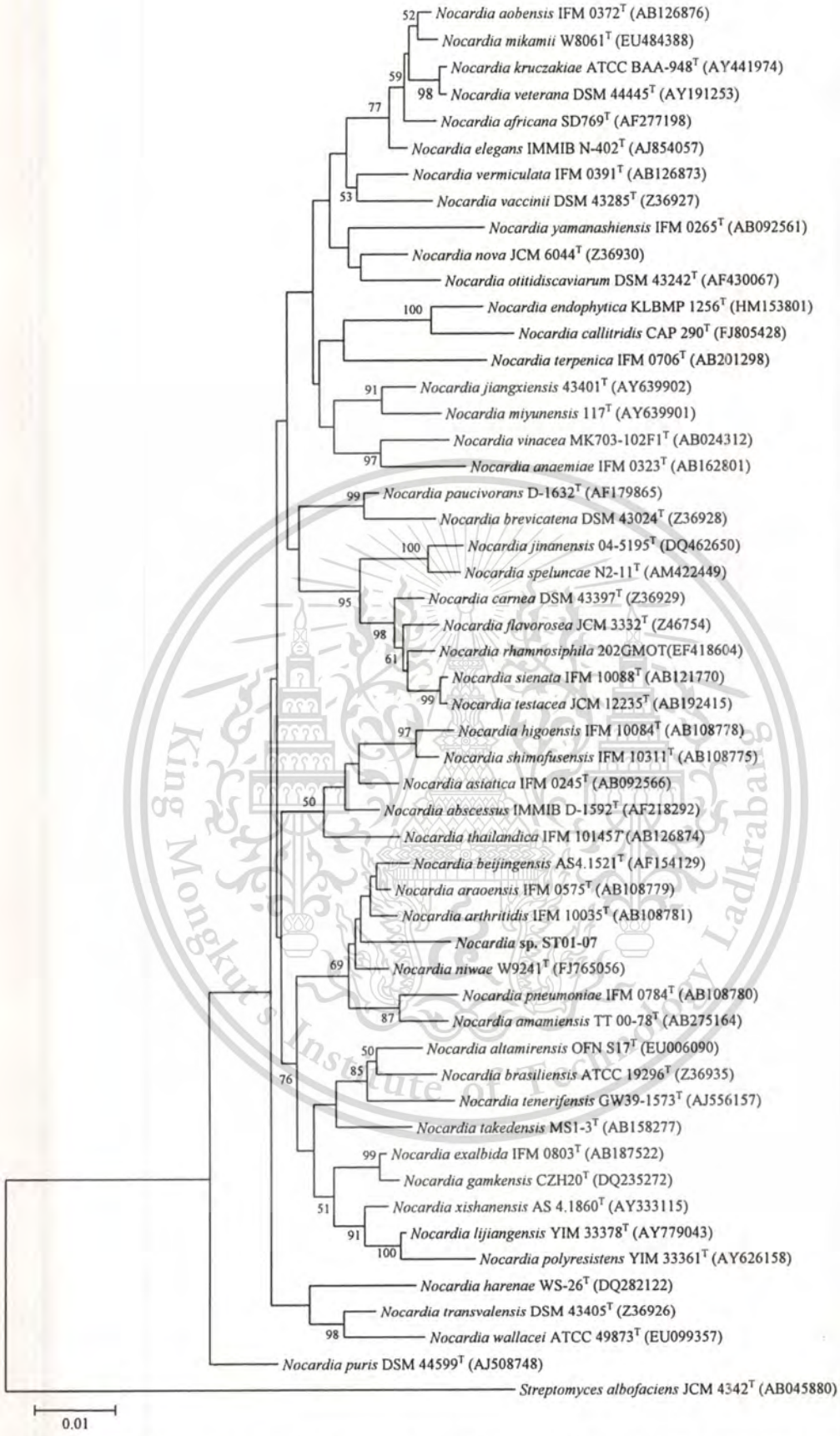


Fig. 4.18 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain ST01-07 and other members of the genus *Nocardia*

The marine actinobacteria in group VI consisted of one strain, S15-3. This strain produced the chain of spores directly on the branching substrate mycelium (Fig. 4.19). The substrate mycelium could fragment to the rod-shaped elements. The strain grew well on ISP 2, ISP 3, ISP 4, ISP 5, czapek's sucrose and glucose asparagine agars, and moderately grew on ISP 6, ISP 7 and nutrient agars (Table 4.28). Strain S15-3 grew on 0-4% NaCl, at pH 4-12, and 20-40 °C. Starch hydrolysis and gelatin liquefaction were positive. Peptonization and coagulation of milk, and nitrate reduction were negative. In addition, the strain produced acids from D-cellobiose, D-fructose, D-galactose, and D-ribose.

Strain S15-3 contained *meso*-diaminopimelic acid in the cell wall. Whole cell sugars comprised arabinose, galactose, glucose, ribose and xylose. Characteristic hospholipids contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The cellular fatty acids of strain S15-3 were iso-$C_{14:0}$ (1.9%), iso-$C_{15:0}$ (2.6%), $C_{16:0}$ (7.4%), iso-$C_{16:0}$ (51.3%), iso-$C_{16:1}$ (7.0%), $C_{16:1}$ ω7c (6.2%), 10-methyl-$C_{16:0}$ (3.8%), $C_{17:0}$ (2.0%), iso-$C_{17:0}$ (2.4%), anteiso-$C_{17:0}$ (3.6%), $C_{17:1}$ ω8c (3.3%), 10-methyl-$C_{17:0}$ (2.0%), $C_{18:0}$ (1.4%) and $C_{18:1}$ ω9c (1.1%).

A database search based on 16S rRNA gene sequence analysis demonstrated that strain S15-3 belongs to the family *Pseudonocardiaceae* (Stackebrandt *et al.*, 1997). Phylogenetic study was performed with 16S rRNA gene sequences of type strains of *Pseudonocardia* species with validly published names, which were combined into the genus *Pseudonocardia* (Fig. 4.20). In addition, strain S15-3 showed the highest similarity value with *Pseudonocardia antarctica* DVS5a1^T (99.9%).

Fig. 4.19 Colonial appearance on ISP 2 seawater agar (a and b), and light micrograph (c) of *Pseudonocardia* sp. S15-3

Table 4.28 Cultural characteristics of strain S15-3

Media	Growth	Colour of colony		Soluble
		upper surface	reverse surface	pigment
Yeast extract-malt extract agar (ISP 2)	Good	Pale orange yellow	Dark orange yellow	None
Oatmeal agar (ISP 3)	Good	Pale orange yellow	Dark orange yellow	None
Inorganic salt-starch agar (ISP 4)	Good	Pale orange yellow	Moderate yellowish brown	None
Glycerol-asparagine agar (ISP 5)	Good	Pale orange yellow	Dark orange yellow	None
Peptone-yeast extract iron agar (ISP 6)	Moderate	Yellowish white	Light olive brown	None
Tyrosine agar (ISP 7)	Moderate	Pale orange yellow	Strong yellowish brown	None
Czapek's sucrose agar	Good	Pale orange yellow	Strong yellowish brown	None
Glucose-asparagine agar	Good	Pale orange yellow	Strong yellowish brown	None
Nutrient agar	Moderate	Yellowish white	Light olive brown	None

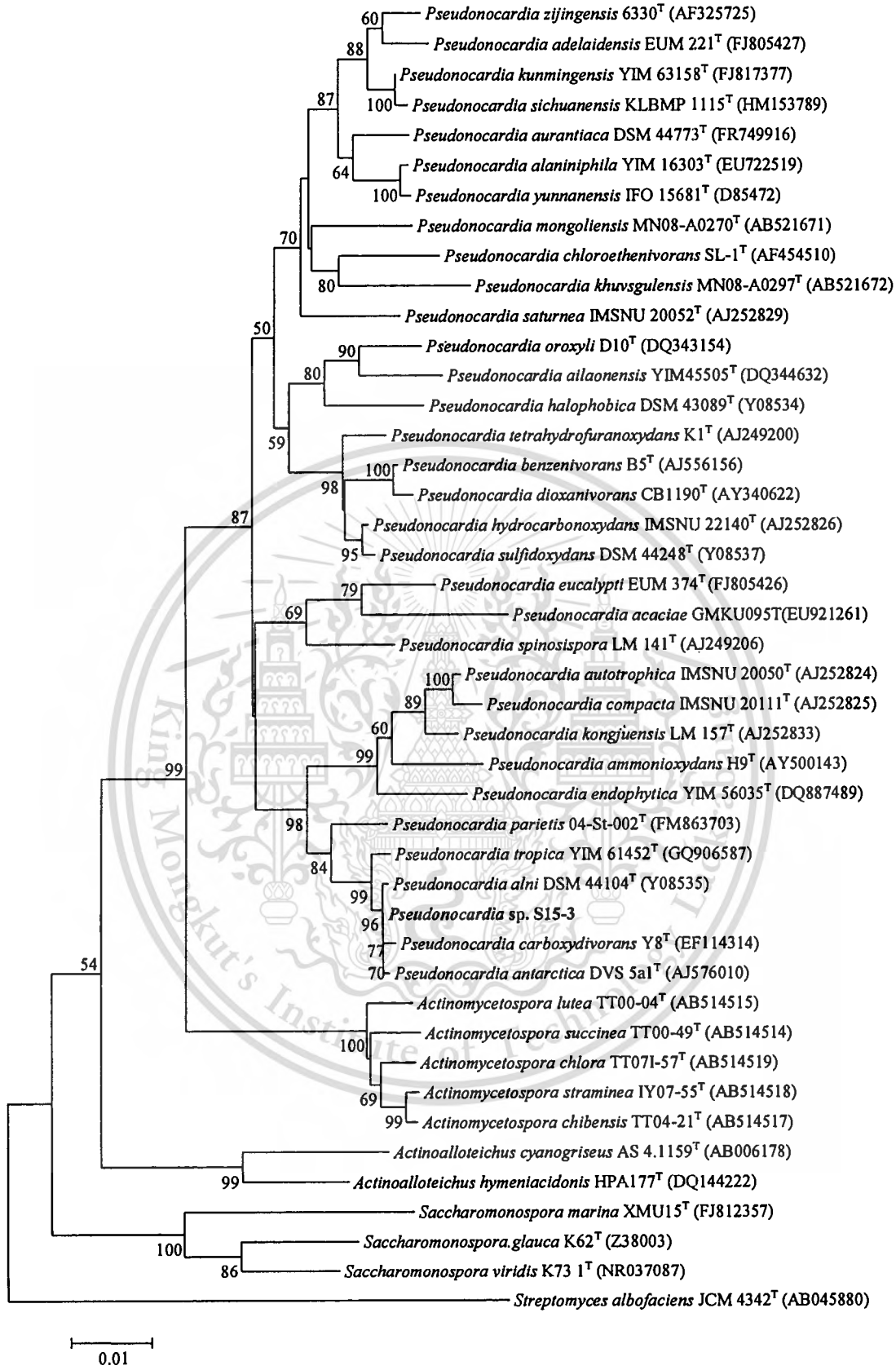


Fig. 4.20 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain S15-3 and other members of the family *Pseudonocardiaceae*

Marine actinobacteria in group VII consisted of three strains, SH3-3, SH2-15 and TV1-16. These strains produced long, well-developed, branched substrate mycelium and could fragment into rod-shaped. Colonies had a powdery surface (Fig. 4.22). Soluble pigments were not observed on the medium tests. Cultural characteristics are shown in Table 4.29. All members in this group grew at 0-6% NaCl, pH 6-12. Strains TV1-16 and SH2-15 grew at 20-40 °C, but strain SH3-3 grew at 20-37 °C (Table 4.30). All strains in this group produced acids from D-cellobiose, D-melibiose, D-ribose and L-arabinose.

The chemotypic characteristics of this group were similar to those of members of the genus *Actinomycetospora* that contained *meso*-diaminopimelic acid in the peptidoglycan of cell wall. Whole cell sugars comprised arabinose, galactose, glucose, ribose and xylose (sugar type D; Lechevalier and Lechevalier, 1970). The predominant phospholipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. MK-8 (H_4) was the major menaquinone. Major fatty acids were $C_{16:0}$, $C_{18:0}$, $C_{16:1}$ ω7c, $C_{17:1}$ ω8c, $C_{18:1}$ ω9c, iso-$C_{15:0}$, iso-$C_{16:1}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, and 10-methyl-$C_{16:0}$. Cellular fatty acids of all strains are shown in Table 4.31. The strains displayed morphological properties typical of the genus *Actinomycetospora* (Jiang *et al.*, 2008), and were confirmed by chemotaxonomic and 16S rRNA gene sequences analyses. Genotypic characteristic based on 16S rRNA gene sequences exhibited that strain SH2-15, SH3-3 and TV1-16 showed highest similarity values with *Actinomycetospora straminea* IY07-55^T (98.9%), *Actinomycetospora chlora* TT07I-57^T (99.2%) and *Actinomycetospora lutea* TT00-04^T (99.0%), respectively. Phylogenetic tree analysis using 16S rRNA gene sequences of the strains indicated that the strains fell within the cluster of the genus *Actinomycetospora* (Fig. 4.21). On the basis of 16S rRNA gene sequences similarity and phylogenetic tree analyses revealed that these strains were possibly identified as new species in the genus *Actinomycetospora*, however, the DNA-DNA hybridization experiment should be performed before considering as a new taxa.

Table 4.29 Cultural characteristics of marine actinobacteria in group VII

Media	Strains		
	TV1-16	SH3-3	SH2-15
Yeast extract-malt extract agar (ISP 2)			
Growth	+++	++	+++
Colour of reverse surface	Yellowish white	Brilliant orange yellow	Brilliant orange yellow
Colour of upper surface	Yellowish white	Strong orange yellow	Strong orange yellow
Soluble pigment	-	-	-
Oatmeal agar (ISP 3)			
Growth	++	+	+
Colour of reverse surface	Pale yellow	Pale yellow	Pale yellow
Colour of upper surface	Yellowish white	Grayish yellowish brown	Yellowish white
Soluble pigment	-	-	-
Inorganic salt-starch agar (ISP 4)			
Growth	++	++	++
Colour of reverse surface	Vivid orange	Yellowish white	Yellowish white
Colour of upper surface	Yellowish white	Yellowish white	Yellowish white
Soluble pigment	-	-	-
Glycerol-asparagine agar (ISP 5)			
Growth	+	+	+
Colour of reverse surface	Yellowish white	Yellowish white	Yellowish white
Colour of upper surface	Yellowish white	Pale yellow	Pale yellow
Soluble pigment	-	-	-
Peptone-yeast extract iron agar (ISP 6)			
Growth	+	+	+
Colour of reverse surface	Light yellow	Light yellow	Light yellow
Colour of upper surface	Light yellow	Light yellow	Light yellow
Soluble pigment	-	-	-
Tyrosine agar (ISP 7)			
Growth	+	+	+
Colour of reverse surface	Pale yellow	Pale yellow	Pale yellow
Colour of upper surface	Yellowish white	Light yellow	Yellowish white
Soluble pigment	-	-	-
Czapek's sucrose agar			
Growth	+	++	+
Colour of reverse surface	Yellowish white	Yellowish white	Yellowish white
Colour of upper surface	Yellowish white	Pale yellow	Yellowish white
Soluble pigment	-	-	-
Glucose-asparagine agar			
Growth	+	+	++
Colour of reverse surface	Yellowish white	Yellowish white	Yellowish white
Colour of upper surface	Yellowish white	Pale yellow	Yellowish white
Soluble pigment	-	-	-
Nutrient agar			
Growth	+++	+	++
Colour of reverse surface	Light orange	Light yellow	Yellowish white
Colour of upper surface	Yellowish white	Vivid yellow	Yellowish white
Soluble pigment	-	-	-

Key: ++++, abundant; +++, good; ++, moderate; + poor.

Table 4.30 Physiological and biochemical characteristics of marine actinobacteria in group VII

Characteristics	Strains		
	TV1-16	SH3-3	SH2-15
Starch hydrolysis	-	-	-
Nitrate reduction	-	+	+
Gelatinization	-	-	-
Coagulation of milk	-	-	-
Peptonization of milk	+	-	-
NaCl tolerance (%)	0-6	0-6	0-6
Growth at 40 °C	+	-	+
pH tolerance	6-12	6-12	6-12
Acid production from :			
D-Cellobiose	+	+	+
D-Fructose	+	+	-
D-Galactose	-	-	-
D-Melibiose	+	+	+
D-Raffinose	-	-	-
D-Ribose	+	+	+
D-Xylose	-	-	+
L-Arabinose	+	+	+
Lactose	-	+	-
Salicin	-	+	+
Sorbose	-	-	+

+, Positive; -, Negative.

Table 4.31 Cellular fatty acid compositions (%) of marine actinobacteria in group VII

Fatty acids	Strains		
	TV1-16	SH3-3	SH2-15
Saturated fatty acids			
$C_{14:0}$	0.5	1.1	0.5
$C_{16:0}$	5.9	6.4	3.1
$C_{17:0}$	2.3	3.7	1.3
$C_{18:0}$	4.6	1.0	1.1
Unsaturated fatty acids			
$C_{15:1}$ ω6c	1.1	3.9	2.1
$C_{16:1}$ ω7c	14.2	7.9	5.8
a-$C_{17:1}$ ω9c	1.2	-	-
$C_{17:1}$ ω8c	10.5	12.3	7.9
$C_{18:1}$ ω9c	5.7	1.1	1.2
Branched fatty acids			
i-$C_{14:0}$	2.2	3.2	4.6
i-$C_{15:0}$	7.9	2.0	2.3
i-$C_{15:1}$	0.1	-	0.1
a-$C_{15:0}$	2.0	2.2	0.3
i-$C_{16:0}$	-	34.8	53.9
a-$C_{16:0}$	0.2	0.1	0.04
i-$C_{16:1}$	10.3	5.5	8.8
i-$C_{17:0}$	4.2	0.7	0.5
a-$C_{17:0}$	12.2	3.9	0.1
i-$C_{18:0}$	1.0		0.4
10-Methylated			
$C_{16:0}$	7.9	2.3	1.3
$C_{17:0}$	3.4	3.2	1.7
$C_{18:0}$	0.5	0.2	0.1

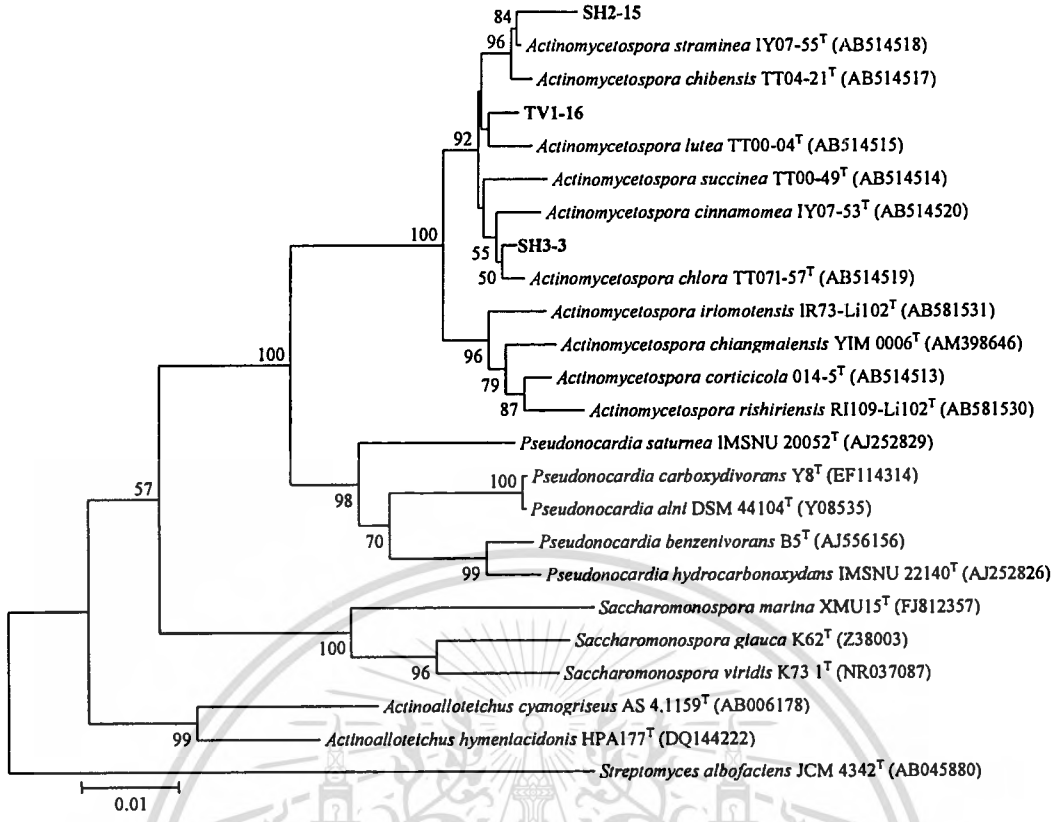


Fig. 4.21 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains SH2-15, SH3-3, TV1-14, and other members of the family *Pseudonocardiaceae*

Strain TV1-16 was selected to complete for the taxonomic characterization. This strain produced well-developed branched substrate mycelium that fragmented into rod-shaped elements (Fig. 4.22). The strain grew well on ISP 2 and nutrient agars, but poor growth was observed on ISP 5, ISP 6, ISP 7, czapek's sucrose, and glucose-asparagine agars. Colonies formed the colour of yellowish white and had a powder surface on the ISP 2 agar. The soluble pigments were not found on the medium tests. The strain grew at 20-37 °C, pH 6-12, and in the presence of 6 % NaCl. The peptonization of milk was positive but starch hydrolysis, gelatinization, and coagulation of milk were negative. Nitrate was not reduced to nitrite.

The cell wall contained *meso*-diaminopimelic acid in the peptidoglycan. Arabinose, galactose, glucose, ribose and xylose were detected as whole-cell sugar. The predominant phospholipids were phosphatidylglycerol, diphosphatidylglycerol, phasphatidylinositol, phophatidylethanolamine, and phosphatidylcholine. The major menaquinone was MK-9(H_4).

The major fatty acids were iso-$C_{15:0}$, iso-$C_{16:1}$, $C_{16:1}$ ω7c, $C_{16:0}$, 10-methyl-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, $C_{17:1}$ ω8c, $C_{18:1}$ ω9c and $C_{18:0}$. The DNA G+C content of strain TV1-16 was 73.5 mol%.

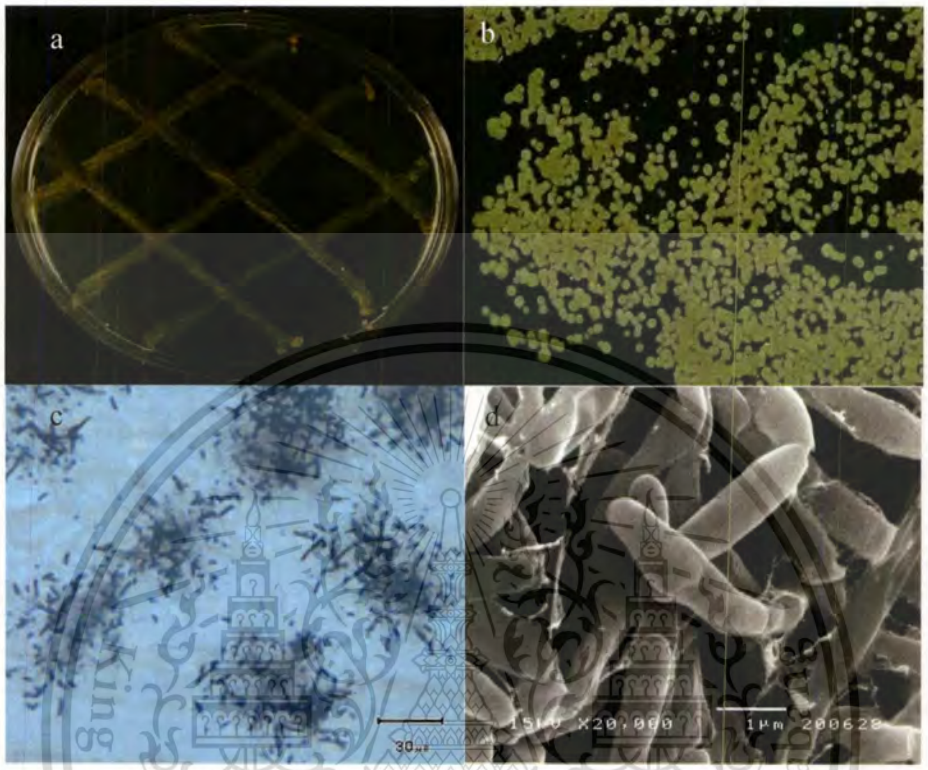


Fig. 4.22 Colonial appearance on ISP 2 seawater agar (a and b) light micrograph (c), and scanning electron micrograph (d) of *Actinomycetospora* sp. TV16

The almost-complete 16S rRNA gene sequence (1,447 nucleotides) was obtained from strain TV1-16. The genotypic characteristics indicated that this strain was assigned to the genus *Actinomycetospora* and exhibited the 16s rRNA gene sequence highest similarity value with *Actinomycetospora lutea* TT00-04^T (99.0%). The phylogenetic tree constructed with 16S rRNA gene sequences data of all members of the genus *Actinomycetospora* using the neighbor-joining method also indicated that strain TV1-16 formed a clade with *Actinomycetospora lutea* TT00-04^T (Figure 4.23). DNA–DNA hybridization study performed between strain TV1-16 and *Actinomycetospora lutea* NBRC 103690^T. The result displayed a DNA-DNA relatedness value below 21.3±0.8, less than the value of 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987).

In addition, the strain TV1-16 was discriminated from related strain by the differences in the acid production from D-cellobiose, D-fructose, D-galactose, D-melibiose, D-raffinose, D-xylose, L-arabinose, D-ribose, D-sucrose, lactose, sorbose and salicin (Table 4.32). Thus, these results supported that strain TV1-16 represents a novel species of the genus *Actinomycetospora*.

Table 4.32 Differential characteristics of strain TV1-16 and *Actinomycetospora lutea* NBRC 103690^T

Characteristics	TV1-16	NBRC 103690^T
Starch hydrolysis	-	-
Nitrate reduction	-	-
Gelatinization	-	-
Coagulation of milk	-	-
Peptonization of milk	+	-
Maximum NaCl tolerance (%)	6	2
Growth at 40 °C	-	+
pH tolerance	6-12	5-8
Acid production from :		
D-Cellobiose	+	+
D-Fructose	+	+
D-Galactose	-	-
D-Melibiose	+	-
D-Raffinose	-	+
D-Ribose	+	-
D-Xylose	-	+
D-Sucrose	-	+
L-Arabinose	+	-
Lactose	-	-
Salicin	-	+
Sorbose	-	+

+, Positive; -, Negative.

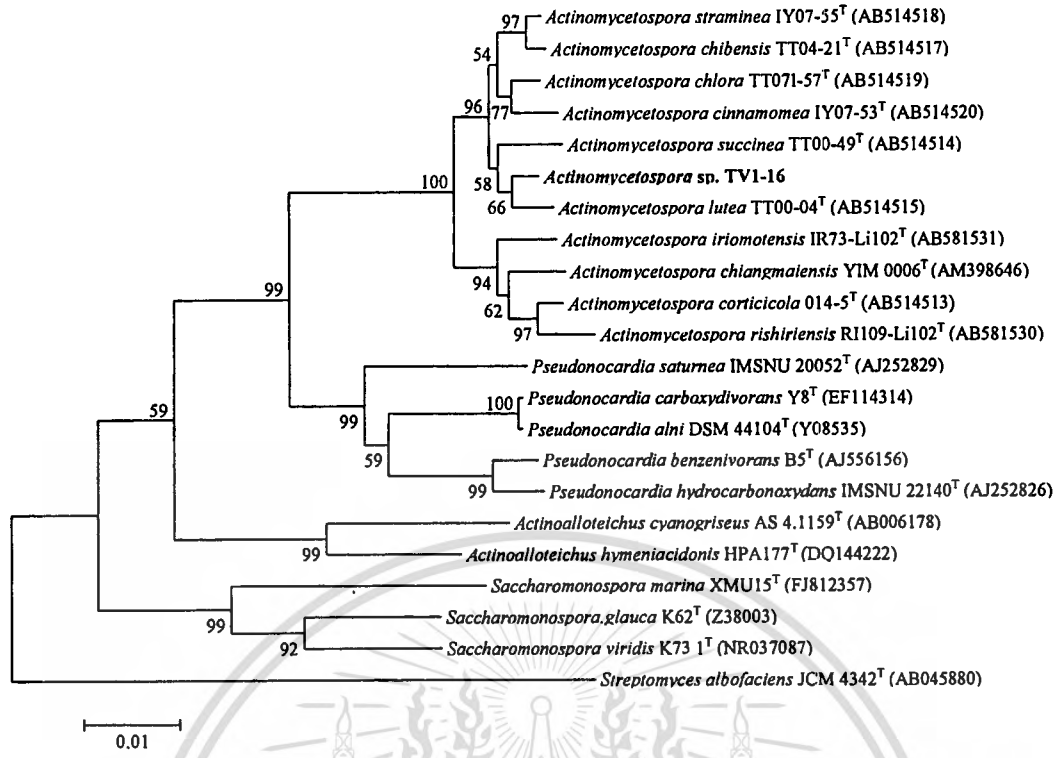


Fig. 4.23 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain TV1-16 and other members of the genus *Actinomadura*

Marine actinobacteria in group VIII comprised five strains including S6-1, S20-7, S8-04, SH2-1, and AN5-16. All strains in this group produced the straight to long spiral chain of spores on their aerial hyphae. The spore surface was smooth to rough and non-motile. The colours of the substrate mycelium ranged from yellow to red, some strains produced soluble pigments on the medium tests. The cultural characteristics of strains are shown in Table 4.33. Growth of strains S8-04 and AN5-16 was observed at pH range of 6-12. Strains S6-1 and S20-7 grew at pH 4-12, these strains showed positive results of starch hydrolysis, nitrate reduction, gelatinization and coagulation of milk. Maximum NaCl tolerance of the strains S20-7 and AN5-16 were at 4%. Strains S8-04 and SH2-1 showed negative results of nitrate reduction, and these strains grew at 10% and 8% NaCl, respectively. Only strain S6-1 could grow at 50 °C. In addition, most strains produced acids from D-cellobiose, D-fructose, D-galactose, lactose, salicin and sorbose (Table 4.34).

The marine actinobacterial strains in this group had the chemical profiles that similar to those of members of genus *Streptomyces*. They exhibited *LL*-diaminopimelic acid in peptidoglycan of cell wall. The whole cell sugars of strains S20-7 and SH2-1 comprised galactose, glucose, ribose

and xylose. Strain S8-04 contained galactose and xylose. Strains S6-1 and AN5-16 consisted of mannose and galactose as the characteristic whole-cell sugar (Table 4.35). The predominant phospholipids of these strains were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides (Table 4.36). In addition, cellular fatty acids of marine acinobacterial in group VIII are shown in Table 4.37.

Comparison of the almost complete 16S rRNA gene sequence obtained for these strains (1310-1428 nucleotides) with corresponding sequences of the type strains in EzTaxon database indicated that these strains formed a phyletic line related to the recognized *Streptomyces* species in neighbour-joining tree (Fig. 4.24). Strain S6-1 formed clade with *Streptomyces fradiae* NBRC 12214^T that showed the highest 16S rRNA gene sequence similarity value at 100%. Strain S20-7 formed a clade with *Streptomyces hyderabadensis* OU-40^T in neighbor-joining tree. This strain showed 99.5% 16S rRNA gene sequence similarity with *Streptomyces hyderabadensis* OU-40^T. Strain SH2-1 formed the cluster with *Streptomyces spongiae* Sp080513SC-24^T and *Streptomyces neopeptinius* KNF 2047^T in neighbour-joining tree. This strain showed closely related 16S rRNA gene sequence similarity value with *Streptomyces spongiae* Sp080513SC-24^T (99.3%). Strain S8.04 formed the cluster with *Streptomyces qinglanensis* 172205^T, *Streptomyces abikoensis* NBRC 13860^T and *Streptomyces varsoviensis* NRRL B-3589^T which had the highest 16S rRNA gene sequence similarity value with *Streptomyces varsoviensis* NRRL B-3589^T (98.9%). In addition, strain AN5-16 formed a distinct monophyletic line at the periphery of recognized member of the genus *Streptomyces*. This strain showed low levels of 16S rRNA gene sequence similarity (92.2-93.5%) to all recognized *Streptomyces* species. On the basis of morphological, chemotaxonomic and phylogenetic data, these strains should be classified to the genus *Streptomyces*.

Table 4.33 Cultural characteristics of marine actinobacteria in group VIII

Media	Strains				
	S6-1	S20-7	S8-04	SH2-1	AN5-16
Yeast extract-malt extract agar (ISP 2)					
Growth	++++	++++	+++	+++	+++
Colour of reverse surface	Dark red	Greenish yellow	Yellowish brown	Yellowish white	Greenish white
Colour of upper surface	Grayish pink	Yellowish gray	Orange yellow	Yellowish white	Greenish white
Soluble pigment	Grayish red	-	Yellowish brown	-	Blue green
Oatmeal agar (ISP 3)					
Growth	++++	++++	++	+++	++
Colour of reverse surface	Dark red	Greenish yellow	Grayish yellow	Yellowish white	White
Colour of upper surface	Grayish pink	Yellowish gray	Yellowish White	Yellowish white	White
Soluble pigment	Grayish red	-	-	-	-
Inorganic salt-starch agar (ISP 4)					
Growth	++	+++	+++	+	+
Colour of reverse surface	Grayish pink	Yellowish gray	Pale yellow	White	White
Colour of upper surface	Pinkish gray	Yellowish gray	Yellowish white	White	White
Soluble pigment	-	-	-	-	-
Glycerol-asparagine agar (ISP 5)					
Growth	+++	+++	++	++	+
Colour of reverse surface	Moderate red	Yellowish gray	Pale yellow	Greenish white	White
Colour of upper surface	Pinkish gray	Yellowish gray	Yellowish white	Greenish white	White
Soluble pigment	Grayish red	-	-	-	-
Peptone-yeast extract iron agar (ISP 6)					
Growth	+++	+++	+	+	+
Colour of reverse surface	Grayish red	Pale yellow	Pale yellow	White	White
Colour of upper surface	Reddish gray	Yellowish white	Yellowish white	White	White
Soluble pigment	Grayish red	-	-	-	-
Tyrosine agar (ISP 7)					
Growth	++	+++	++	+++	+
Colour of reverse surface	Grayish red	Yellowish gray	Grayish yellow	Yellowish white	White
Colour of upper surface	Reddish gray	Yellowish gray	White	Pale yellow	White
Soluble pigment	-	-	-	-	-
Czapek's sucrose agar					
Growth	++	+++	+	+	+
Colour of reverse surface	Pinkish white	Yellowish gray	Yellowish white	White	White
Colour of upper surface	Pinkish gray	Yellowish gray	Yellowish white	White	White
Soluble pigment	-	-	-	-	-
Glucose-asparagine agar					
Growth	++	+++	++	+	+
Colour of reverse surface	Dark pink	Yellowish gray	Pale yellow	White	White
Colour of upper surface	Pinkish gray	Yellowish gray	Yellowish white	White	White
Soluble pigment	-	-	-	-	-
Nutrient agar					
Growth	+++	+++	+++	+	++
Colour of reverse surface	Dark red	Yellowish gray	Light olive brown	White	White
Colour of upper surface	Pinkish white	Yellowish gray	Yellowish gray	White	White
Soluble pigment	Deep red	-	-	-	-

Key: ++++, abundant; +++; good; ++, moderate; + poor.

Table 4.34 Physiological and biochemical characteristics of marine actinobacteria in group VIII

Characteristics	Strains				
	S6-1	S20-7	S8-04	SH2-1	AN5-16
Starch hydrolysis	+	+	+	-	+
Nitrate reduction	+	+	-	-	+
Gelatinization	+	+	+	+	+
Coagulation of milk	+	+	+	-	-
Peptonization of milk	+	-	+	+	-
Maximum NaCl tolerance (%)	10	4	10	8	4
Growth at 50 °C	+	-	-	-	-
pH range for growth	4-12	4-12	6-12	5-12	6-12
Acid production from :					
D-Cellobiose	+	+	+	+	+
D-Fructose	+	+	+	+	-
D-Galactose	+	+	+	+	-
D-Melibiose	+	-	-	+	-
D-Raffinose	-	-	-	-	-
D-Ribose	+	+	+	-	-
D-Xylose	+	+	-	+	+
L-Arabinose	+	+	-	-	-
Lactose	+	+	+	+	-
Salicin	+	+	+	+	-
Sorbose	+	+	+	+	-

+, Positive; -, Negative.

Table 4.35 Whole cell sugar patterns of marine actinobacteria in group VIII

Strains	sugars							
	Ara	Gal	Glc	Mad	Man	Rha	Rib	Xyl
S6-1	-	+	+	-	+	-	+	+
S20-7	-	+	+	-	-	-	+	+
S8-04	-	+	-	-	-	-	-	+
SH2-1	-	+	+	-	-	-	+	+
AN5-16	-	+	+	-	+	-	+	-

Ara, arabinose; Gal, galactose; Glc, glucose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose

Table 4.36 Phospholipid profiles of marine actinobacteria in group VIII

Strains	Phospholipids									
	APG	DPG	GluNU	PC	PE	PME	PG	PI	PIMs	PS
S6-1	-	+	-	-	+	-	+	+	+	-
S20-7	-	+	-	-	+	-	+	+	+	-
S8-04	-	+	-	-	+	-	+	+	+	-
SH2-1	-	+	-	-	+	-	+	+	+	-
AN5-16	-	+	-	-	+	-	+	+	+	-

APG, acylphosphatidylglycerol; DPG, diphosphatidylglycerol; GluNU, unknown glucosamine-containing phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidylmethylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; PS, phosphatidylserine

Table 4.37 Cellular fatty acid compositions (%) of marine actinobacteria in group VIII

Fatty acids	Strains				
	S6-1	S20-7	S8-04	SH2-1	AN5-16
Saturated fatty acids					
$C_{12:0}$	0.7	0.5	0.2	0.5	0.2
$C_{14:0}$	2.6	3.2	2.8	4.3	
$C_{16:0}$	20.2	25.2	14.1	17.5	2.6
$C_{17:0}$	1.9	1.0	0.7	0.7	1.5
$C_{18:0}$	0.2	0.4	0.1	0.3	
Unsaturated fatty acids					
$C_{16:1}$ ω7c	1.2	1.3	2.2	2.2	1.4
$C_{17:1}$ ω7c	-	-	-	-	0.7
$C_{17:1}$ ω8c	0.1	0.8	1.1	1.7	-
a-$C_{17:1}$ ω9c	0.4	0.6	0.9	0.5	-
$C_{18:1}$ ω9c	0.2	0.2	0.1	0.1	0.1
Branched fatty acids					
i-$C_{13:0}$	0.8	0.7	0.3	0.4	-
a-$C_{13:0}$	0.4	0.3	0.7	0.7	0.2
i-$C_{14:0}$	8.1	12.5	14.1	11.9	0.4
i-$C_{15:0}$	12.9	9.2	10.2	8.4	2.5
i-$C_{15:1}$	-	0.2	0.3	0.1	0.9
a-$C_{15:0}$	23.1	18.5	22.1	13.5	6.9
a-$C_{15:1}$					1.9
i-$C_{16:0}$	15.3	14.2	15.1	27.3	26.6
i-$C_{16:1}$	0.2	2.2	1.3	1.4	9.8
i-$C_{17:0}$	3.2	3.0	2.7	1.0	4.3
a-$C_{17:0}$	4.9	4.4	3.3	3.7	25.7
a-$C_{17:1}$	-	-	-	-	10.0
i-$C_{18:0}$	0.3	0.4	0.6	0.1	1.6
10-Methylated					
$C_{16:0}$	0.4	0.2	0.4	0.5	-

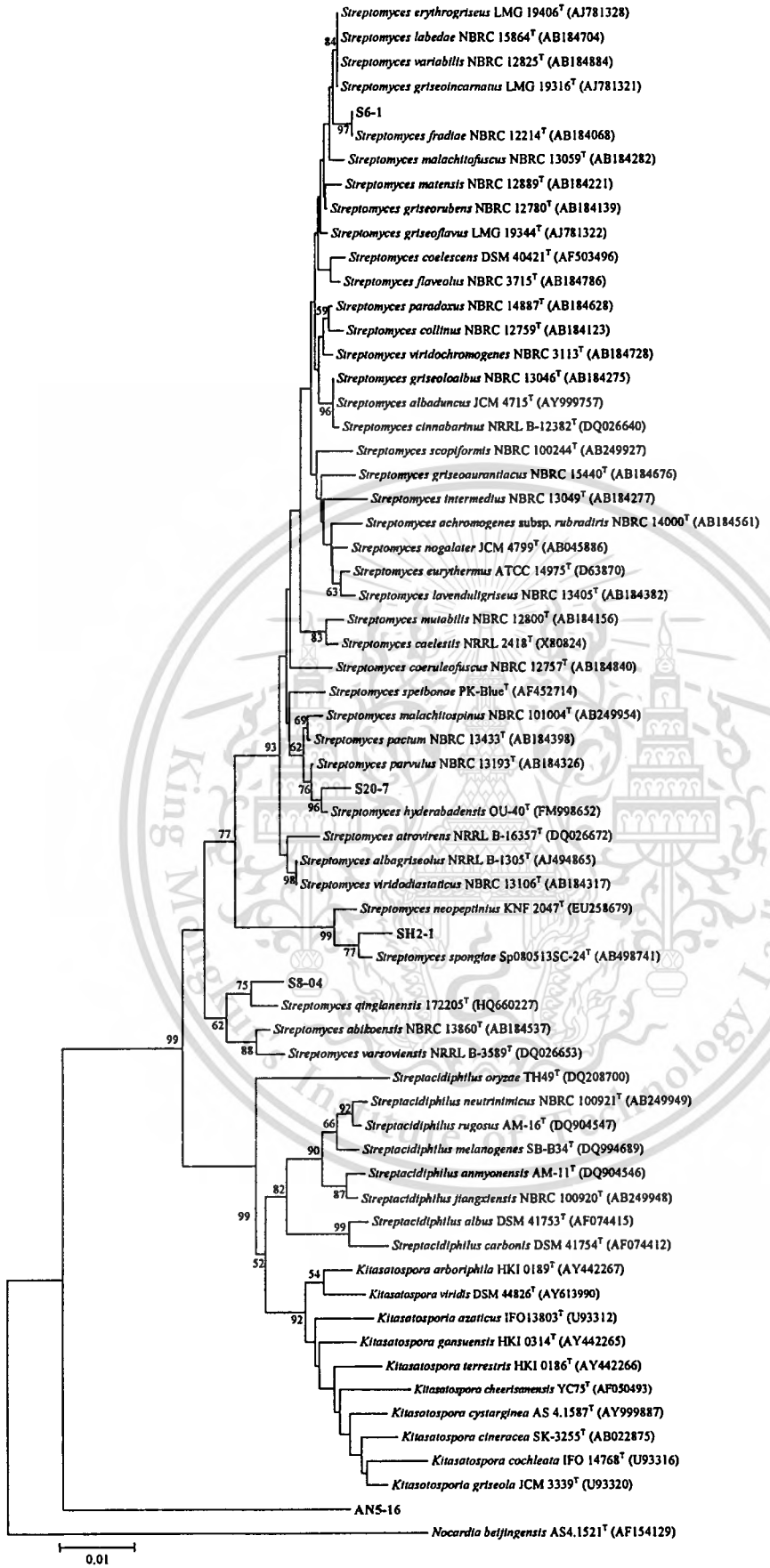


Fig. 4.24 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the correlation of the genus *Streptomyces*

The strains S6-1 and AN5-16 were selected for the taxonomic studies because they displayed several phenotypic and genotypic characteristics that differed from other strains in this group. Strain S6-1 was Gram-strain-positive, aerobic actinobacteria, and formed dark red substrate mycelium and grayish pink aerial mycelium on the ISP 2 agar. Strain S6-1 produced spiral conidiospore chains on the aerial mycelium (Fig. 4.25). Grayish red to deep red soluble pigments were produced on the ISP 2, ISP 3, ISP 5, ISP 6 and nutrient agar, but not on the ISP 4, ISP 7, czapek's sucrose, and glucose-asparagine agars. The strain grew well on ISP 2, ISP 3, ISP 5, ISP 6, and nutrient agars; moderately on ISP 4, ISP 7, Czapek's sucrose and Glucose-asparagine agars. The range of temperature for growth was 10-50 °C, on pH 4-12 and in the presence of 10 % NaCl. In addition, strain S6-1 produced acids from D-cellobiose, D-fructose, D-galactose, D-sucrose, lactose, salicin and sorbose. Starch hydrolysis, peptonization and coagulation of milk, nitrate reduction, and gelatin liquefaction were positive.

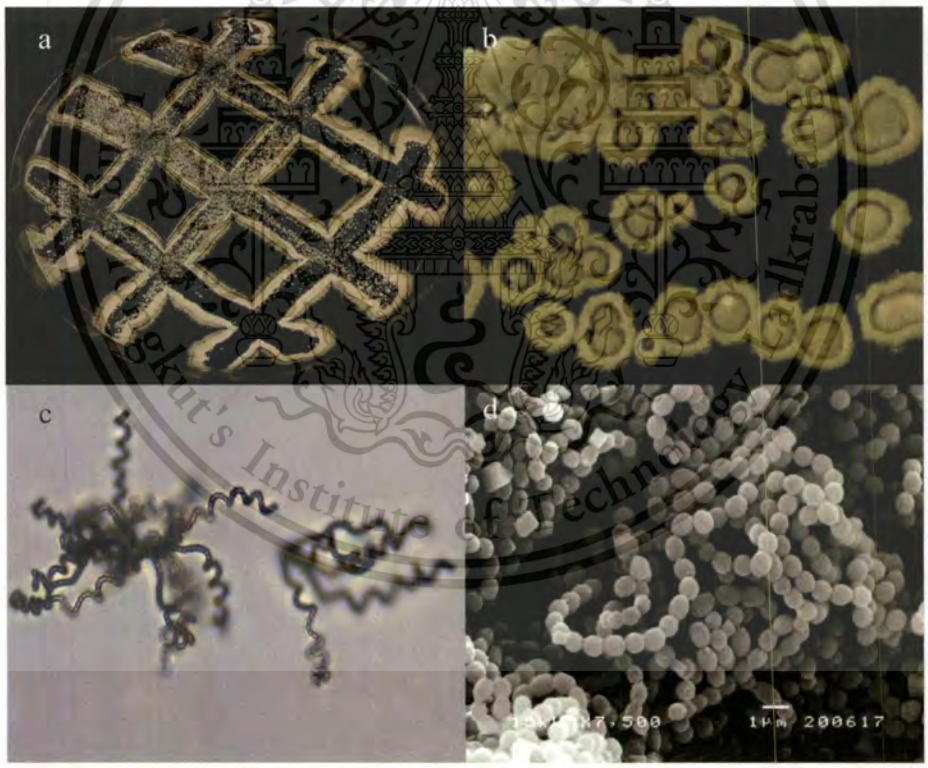


Fig. 4.25 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of *Streptomyces* sp. S6-1

Strain S6-1 contained *LL*-diaminopimelic acid in peptidoglycan of the cell wall. The whole cell sugars comprised galactose, glucose, mannose, ribose and xylose. Major polar lipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major fatty acids comprised iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$. The predominant menaquinones were MK-9(H_2), MK-9(H_4) and MK-9(H_6). The DNA G+C content of strain S6-1 was 75.2 mol%.

An almost-complete 16S rRNA gene sequence (1,428 nucleotides) was obtained from strain S6-1. The highest levels of 16S rRNA gene sequence similarity were with *Streptomyces fradiae* NBRC 12214^T (100%). In addition, phylogenetic tree analyses based on 16S rRNA gene sequences showed that the strain was the member of the genus *Streptomyces* and formed a clade with *Streptomyces fradiae* NBRC 12214^T (Fig. 4.26). Furthermore, strain S6-1 exhibited the antimicrobial activities against bacteria and fungi on the agar medium. EtOAc crude extract of the strain showed the interesting biological activities and HPLC profiles that strain S6-1 might be a source of bioactive secondary metabolites. Thus, strain S6-1 was selected for the natural product studies that would be described in a part of structure elucidation of secondary metabolites.

Strain AN5-16 formed white substrate mycelium on the the medium tests. Spiral-spore chains were directly produced on the aerial mycelium (Fig. 4.27). The strain grew well on ISP 2 agar, but weakly on ISP 4, ISP 5, ISP 6, ISP 7, czapek's sucrose, and glucose-asparagine agars. Strain AN5-16 grew on the presence of 0-4 % NaCl, pH 6-12, and at 20-45 °C. The strain produced acids from D-cellobiose and D-xylose. Starch hydrolysis, nitrate reduction, and gelatin liquefaction were positive. In addition, the strain showed negative results of peptonization and coagulation of milk.

Strain AN5-16 contained *LL*-diaminopimelic acid in peptidoglycan of the cell wall. The whole cell sugars consisted of galactose, glucose and ribose. Major polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major cellular fatty acids consisted of iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$, iso-$C_{16:1}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$ and anteiso-$C_{17:1}$. The DNA G+C content of strain AN5-16 was 70.7 mol%.

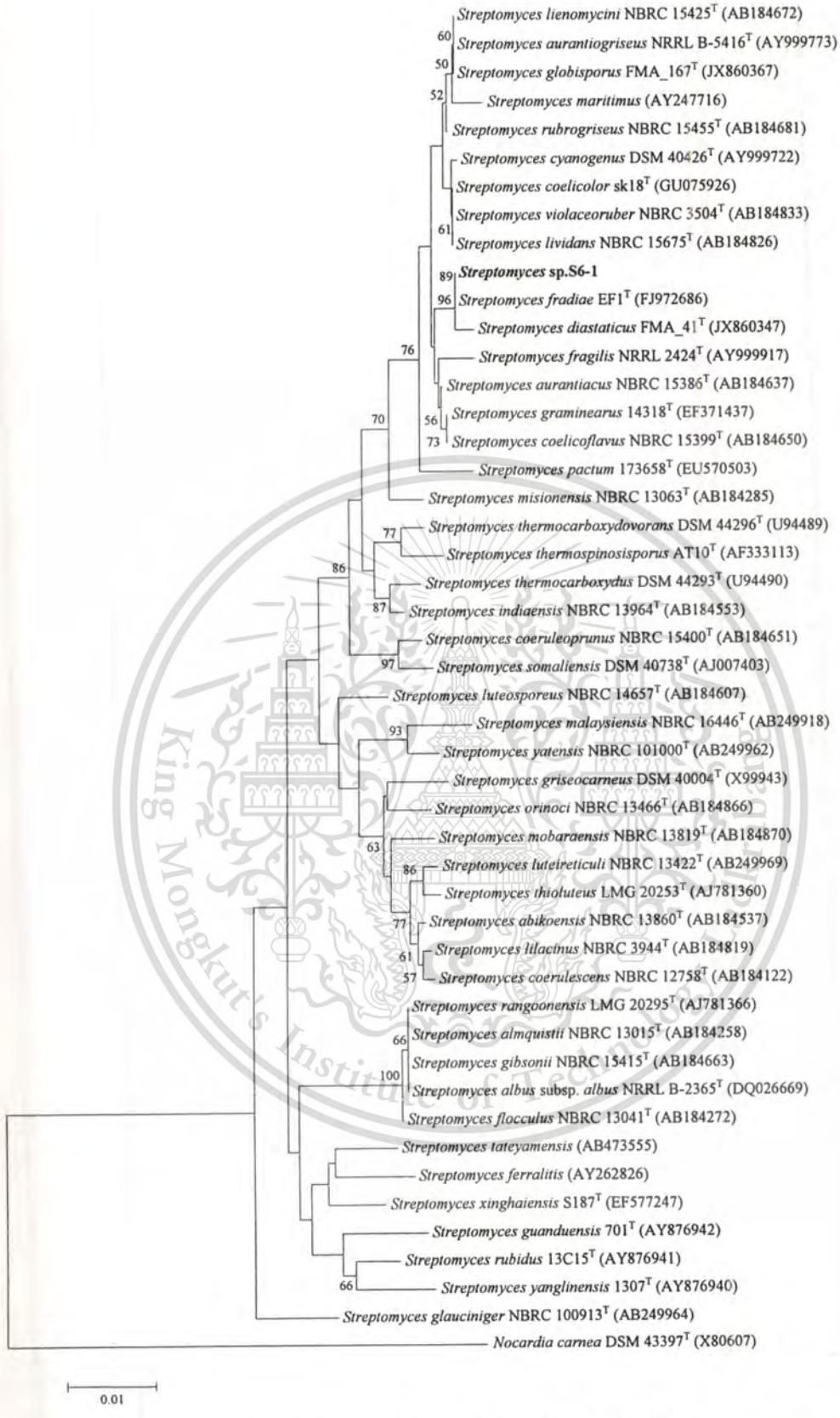


Fig. 4.26 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the correlation of strain S6-1 and the related species of the genus *Streptomyces*

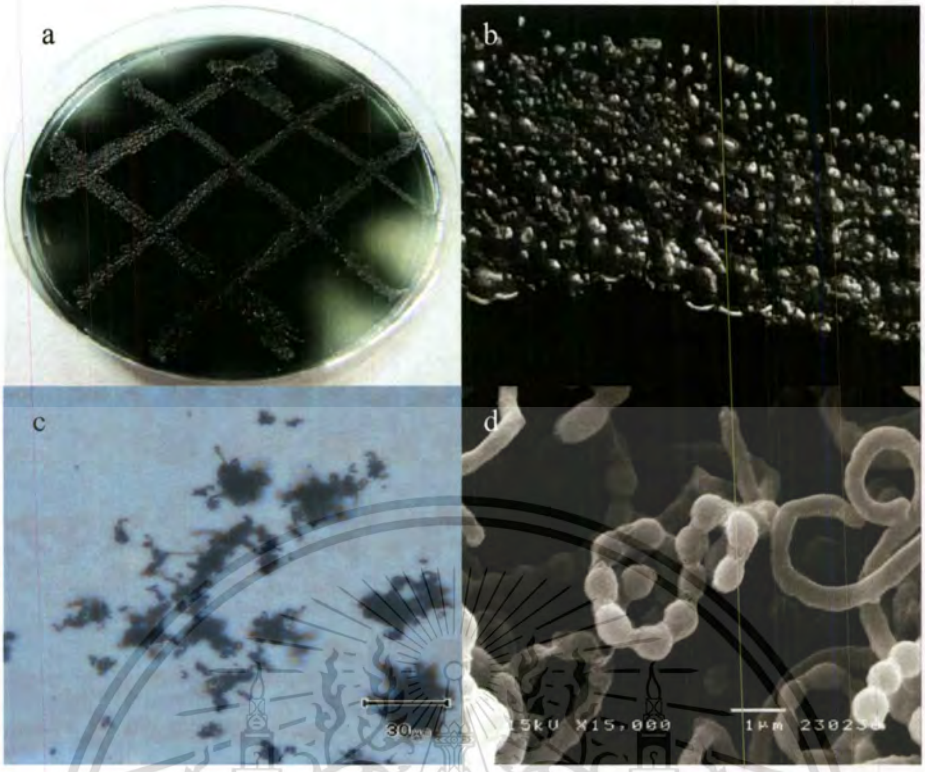


Fig. 4.27 Colonial appearance on ISP 2 seawater agar (a and b) and light micrograph (c), and scanning electron micrograph (d) of strain AN5-16

An almost-complete 16S rRNA gene sequence (1,419 nucleotides) was obtained from strain AN5-16. The strain showed low levels of 16S rRNA gene sequence similarity to *Streptomyces* species ranging from 92.2-93.5%, and may represent a new genus within the family *Streptomycetaceae*. Phylogenetic relationship of strain AN5-16 based on 16S rRNA gene sequence showed that the strain formed a distinct monophyletic line at the periphery of recognized members of the genus S*treptomyces* (Fig. 4.28).

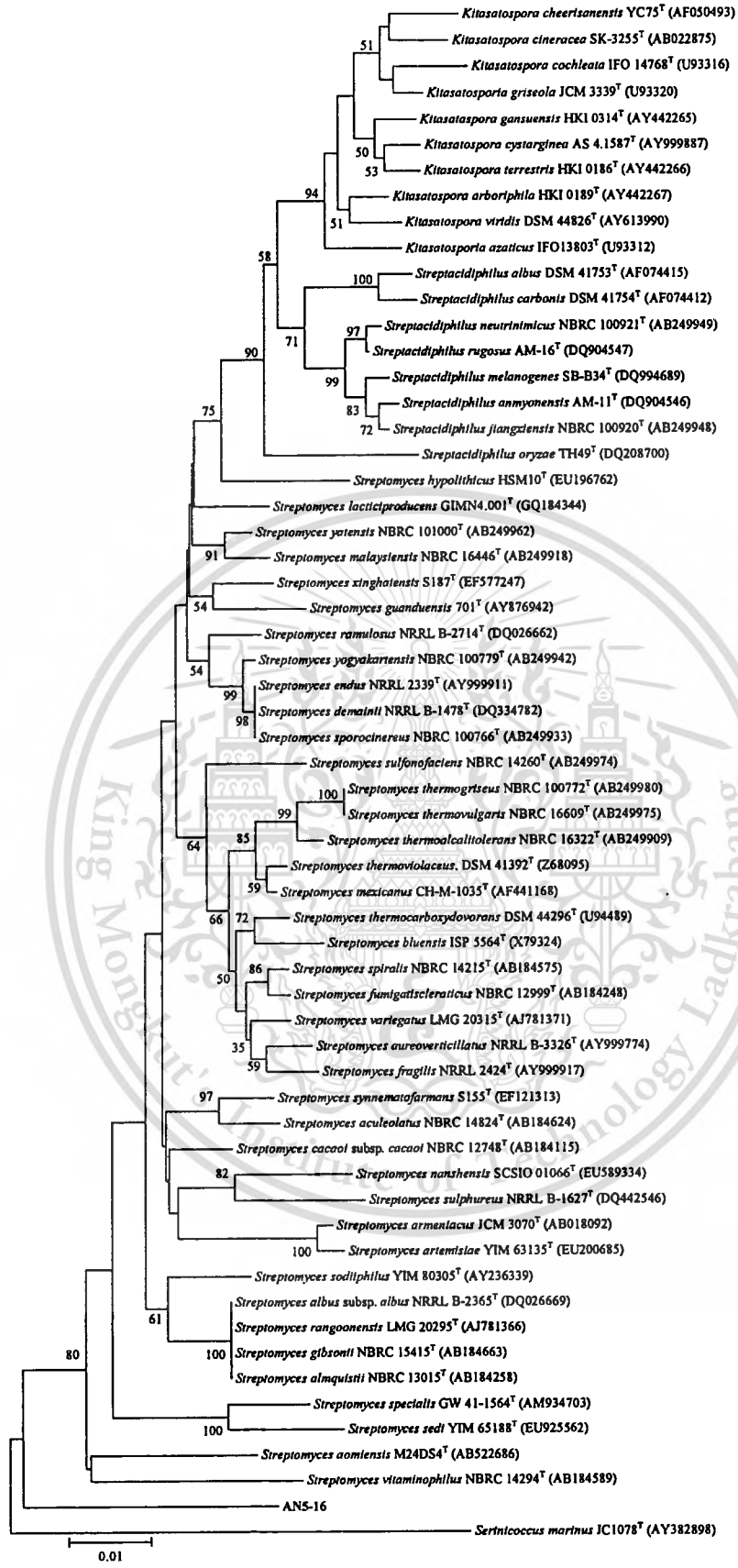


Fig. 4.28 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the correlation of strain AN5-16 and other members of the family *Streptomycetaceae*

4.3 Secondary metabolites of the selected marine actinobacterial strains

4.3.1 Biological activities and chemical screening

Thirty-six strains belonging to eight actinobacterial genera were cultivated on ISP 2 supplemented with artificial seawater, and the crude extracts from these cultures were tested for biological activities against *Bacillus cereus*, *Candida albicans*, *Plasmodium falciparum*, *Mycobacterium tuberculosis* and cancerous cells. These crude extracts from thirty-six strains were also analyzed by HPLC analysis. These strains including *Streptomyces* sp. S6-1, *Micromonospora* sp. S8-12, and *Actinomadura* sp. TV1-14 were chosen based on both biological activities and chemical profiles for large scale fermentation (20 l). *Streptomyces* sp. S6-1 showed biological activities against *Plasmodium falciparum* and cancerous cells (KB, MCF-7, NCI-H187). In addition, two selected strains, *Micromonospora* sp. S8-12 and *Actinomadura* sp. TV1-14, exhibited biological activities against cancer cells and *Bacillus cereus*, respectively (Table 4.38).

Table 4.38 Biological activities of the selected marine actinobacterial strains

Crude extracts	Anti *B. cereus* MIC (μg/ml)	Anti *C. albicans* IC_{50} (μg/ml)	Anti-TB MIC (μg/ml)	Antimalaria IC_{50}±SD (μg/ml)	Anticancer IC_{50} (μg/ml)		
					KB	MCF-7	NCI-H187
***Streptomyces* sp. S6-1**							
Large scale 20 l	Inactive	Inactive	Inactive	1.45	18.81	13.18	12.33
***Micromonospora* sp. S8-12**							
Large scale 20 l	Inactive	Inactive	Inactive	Inactive	35.63	Inactive	45.72
***Actinomadura* sp. TV1-14**							
Large scale 20 l	12.50	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive

4.3.2 Isolation and structure elucidation of secondary metabolites from the selected marine actinobacterial strains

4.3.2.1 Isolation and structure elucidation of secondary metabolites from the marine *Streptomyces* sp. S6-1 (BCC 45596)

HPLC profile of crude EtOAc extract of *Streptomyces* sp. S6-1 (Fig. 4.29(a)) comprised a major peak at RT 4.53 (peak A), and the minor peaks at RTs 4.94 (peak B), 6.38 (peak D) and 7.66 (peak C). These peaks gave the interesting UV profiles, compared to BIOTEC's database. In addition, the known small molecules including 3-hydroxy-2-methyl-4-pyrone and diketopiperazines showed at RTs 2.23-3.72. The re-cultivation of *Streptomyces* sp. S6-1 (Fig.

4.29(b)) showed a major peak at RT 5.21 (peak E), which gave a similar UV profiles to peak A in the first cultivation. However, the peaks A and C were minor products, and peaks B and D were not found in the re-cultivation.

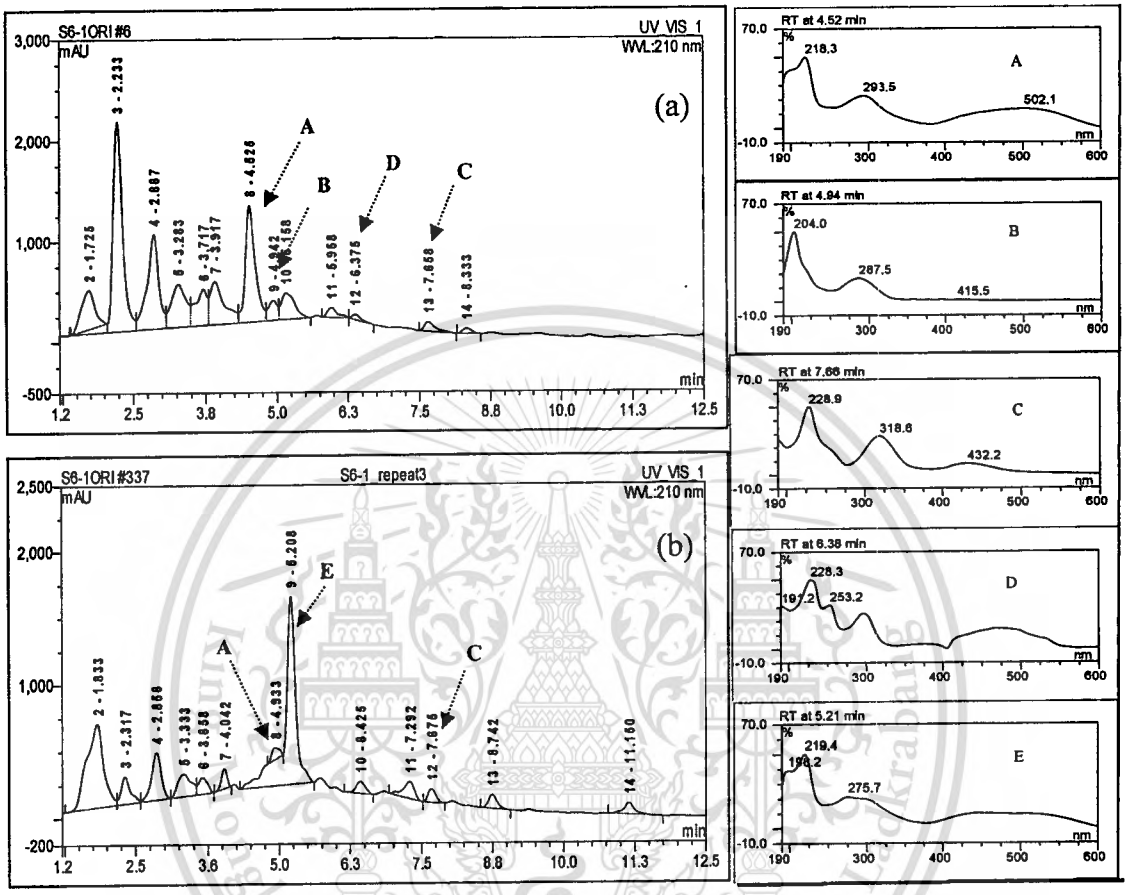


Fig. 4.29 HPLC profiles of first cultivation (a), and re-cultivation (b) of *Streptomyces* sp. S6-1

EtOAc crude extract (20 l) of *Streptomyces* sp. S6-1 was purified by chromatographic techniques to give four compounds that showed the HPLC peaks at RTs 4.53 (compound S6.1A), 4.94 (compound S6.1B), 7.66 (compound S6.1C), and 6.38 (compound S6.1D). In addition, compound S6.1 E at RT 5.21 was only isolated from the crude of the re-cultivation.

Chemical structures of secondary metabolites (S6.1A-S6.1H) were elucidated by spectral information. Five compounds were identified as the angucycline antibiotics (compounds S6.1A-S6.1E), four of which are naturally new compounds including compound S6.1A (urdamycinone E), S6.1B (urdamycinone G), S6.1C (dehydroxyaquayamycin) and S6.1D (5-aminodehydroxyaquayamycin). The known angucycline compound S6.1E (urdamycin E)together

together with two diketopiperazines (S6.1F and S6.1G), and 3-hydroxy-2-methyl-4-pyrone (S6.1H) were also determined (Fig. 4.30).

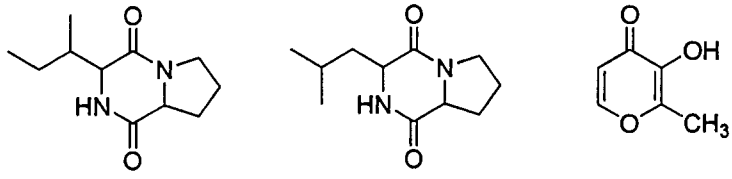


Fig. 4.30 Two known diketopiperazines (S6.1F and S6.1G), and 3-hydroxy-2-methyl-4-pyrone (S6.1H)

Compound S6.1A (Fig. 4.31) was obtained as red amorphous powder with melting point 203-205 °C (dec.). The UV (MeOH) spectrum displayed the absorption bands at λ_{max} (log ε) 231 (4.40), 270 (3.49), 316 (4.24) and 428 (3.85) nm. The CD spectrum showed the absorption peaks at λ_{max} ($[\theta]_{25}$) 255 (+13247), 282 (+2036), 296 (+4889), 367 (+1024), 427 (-916), 513 (-981) nm. IR spectrum (Appendix IV, Fig. 1) showed the absorption peaks at ν_{max} 3343 (OH), 2920 (CH), 1720 (C=O of ketone), and 1510 (C=C) cm^{-1}. Compound S6.1A gave the molecular ion peak at *m/z* 555.1299 $[M+Na]^+$ in HRESIMS spectrum, suggesting the molecular formula of $C_{26}H_{28}O_{10}S$.

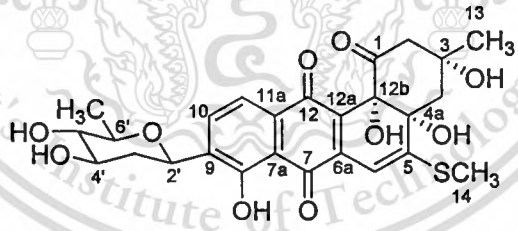


Fig. 4.31 Chemical structure of compound S6.1A (urdamycinone E)

The ^1H NMR spectrum of compound S6.1A (Appendix IV, Figs. 2-3) showed two singlet methyl protons at δ_H 1.26 and 2.51, and a doublet methyl group at δ_H 1.35 (J = 6.15 Hz). Three aromatic/olefinic protons comprised the singlet methine proton at δ_H 6.42 (H-6), and two doublet protons at δ_H 7.55 (H-11) and 7.88 (H-10).

The ^{13}C NMR (Appendix IV, Fig. 4) and DEPT (Appendix IV, Fig. 5) spectroscopic data of compound S6.1A showed the presence of 26 signals, which were three methyl, three methylene,

seven methine, and thirteen quaternary carbons. In HMQC (Appendix IV, Fig. 9) spectrum, three aromatic/olefinic protons at δ_H 6.42, 7.55, and 7.88 correlated to the carbons at δ_C 105.1 (C-6), 118.4 (C-11), and 133.3 (C-10), respectively. In HMBC spectrum (Appendix IV, Fig. 10-12), the singlet methine proton at δ_H 6.42 (H-6) showed correlations to the quaternary carbons at δ_C 83.0 (C-4a), 134.0 (C-12a), 164.0 (C-5), and 189.5 (C-7). Two doublet protons at δ_H 7.55 (H-11) and 7.88 (H-10) gave HMBC correlations to the carbons at δ_C 114.2 (C-7a), 138.1 (C-9), 181.6 (C-12) and 71.1 (C-2′), 131.2 (C-11a), 157.5 (C-8), respectively. The chelated hydroxyl proton at δ_H 12.41 correlated to the quaternary carbons at C-8, C-9, and C-7a. The spectral information suggested a quinone structure, which was supported by the IR absorption at ν_{max} 1628 cm^{-1}. In addition, the methyl at δ_H 1.26 (H_3-13) showed HMBC correlations to two methylene carbons at δ_C 45.5 (C-4) and 51.9 (C-2), and to a quaternary carbon attached to oxygen at 76.2 (C-3). The nonequivalent methylene proton at δ_H 2.13 (H_a-4) and 2.39 (H_b-4) gave the HMBC correlations to two quaternary carbons at δ_C 77.8 (C-12b) and 83.0 (C-4a). Also the nonequivalent methylene protons at δ_H 2.67 (H_a-2) and 3.03 (H_b-2) showed cross-peak correlations in HMBC spectrum to δ_C 205.03 (C-1), C-3, C-4, and C-12b. Two hydroxyl protons at δ_H 4.75 (3-OH) and 5.43 (4a-OH) showed HMBC correlations to the carbons at C-3, C-4, C-13 and C-4, C-4a, C-5, respectively. The methyl at δ_H 2.51, showing correlations in HMQC spectrum to the carbon at δ_C 13.5 (C-14) and in HMBC spectrum to the carbon at C-5 indicated attachment on a sulfur atom. The remaining signals, supported by COSY and HMBC spectral data, suggested that to be those of the glycosidic moiety. The COSY spectrum (Appendix IV, Fig. 6) revealed the spin system extending from H-2′ to H-6′ and HMBC spectrum showed correlations from the sp^3 methine proton at δ_H 4.87 (H-2′) to two carbons at δ_C 138.1 (C-9) and 133.3 (C-10). The latter suggested a C-glycosidic linkage. The triplet at δ_H 3.07 (H-5′) gave a coupling constant of 8.83 Hz, indicating the axial position. Hence, the methyl at C-6′ and two hydroxyl protons at C-4′ and C-5′ are in equatorial positions. The NOESY spectrum (Appendix IV, Fig. 7-8), showing the cross-peak correlations from H_{ax}-2′ to H_{ax}-4′ and H_{ax}-6′, also confirmed the above assumption. In addition, hydroxyl protons (at δ_H 4.20, 4.75 and 5.43) on C-12b, C-3, and C-4a showed cross-peak correlation in the NOESY spectrum, suggesting that they are on the same face of the molecule. Compound S6.1A (urdamycinone E) was previously obtained from hydrolysis of urdamycin E, but has never been isolated from natural sources (Rohr and Zeeck, 1987). The completed NMR spectroscopic data of compound S6.1A (urdamycinone E) is shown in Table 4.39.

Table 4.39 ^{1}H, ^{13}C, COSY, and HMBC spectral data (500 MHz, acetone-d_6) of compound S6.1A (urdamycinone E)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	COSY	HMBC (H to C)
1	205.0	-	-	-
2	51.9	2.67, dd (12.82, 3.05)	4, 13	1, 3, 4, 12b, 13
		3.02, d (12.82)	-	1, 3
3	76.2	-	-	-
4	45.5	2.13, dd (14.92, 3.05)	-	2, 4a
		2.39, d (14.92)	2	2, 13
4a	83.0	-	-	-
5	164.0	-	-	-
6	105.1	6.42, s	-	4a, 5, 7, 12a
6a	138.1	-	-	-
7	189.5	-	-	-
7a	114.2	-	-	-
8	157.5	-	-	-
9	138.1	-	-	-
10	133.3	7.88, d (7.82)	11	8, 11a, 2′
11	118.4	7.55, d (7.82)	10	7a, 9, 12
11a	131.2	-	-	-
12	181.6	-	-	-
12a	134.0	-	-	-
12b	77.8	-	-	-
13	29.5	1.26, s	-	2, 4
14	13.5	2.51, s	-	5, 6
2′	71.1	4.87, dd (10.08, 1.02)	3′	9, 10
3′	40.0	1.28-1.38, m	2′, 4′	4′, 5′, 6′
		2.37-2.41, m	-	4′, 5′
4′	76.3	3.44-3.49, m	3′, 5′	2′, 6′
5′	77.9	3.07, dd (8.83, 8.83)	4′, 6′	4′ 6′ 7′
6′	72.5	3.70-3.75, m	5′, 7′	-
7′	17.7	1.35, d (6.15)	6′	5′ 6′
3-OH	-	4.75, s	-	3, 4, 13
4a-OH	-	5.43, s	-	4, 4a, 5
8-OH	-	12.41, s	-	7a, 8, 9
12b-OH	-	4.70, s	-	1, 12a, 12b
4′-OH	-	4.25, br s	-	-
5′-OH	-	4.20, br s	-	-

Compound S6.1B (Fig. 4.32) was obtained as a red solid with the melting point 190-196° C (dec.). The UV spectrum showed the absorption peaks at λ_{max} (log ε) 222 (4.27), 264 (4.18), 292 (4.10) and 483 (3.88) nm. The IR spectrum (Appendix IV, Fig. 13) showed the absorption peaks at ν_{max} 3324 (OH stretching), 2921(CH stretching), 1627 (C=O stretching of aromatic ketone) cm^{-1}. Compound S6.1B revealed the molecular ion at *m/z* 537.1191 $[M+Na]^+$ in HRESIMS spectrum, suggesting the molecular formula of $C_{26}H_{26}O_9S$.

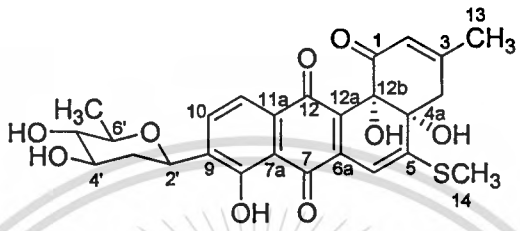


Fig. 4.32 Chemical structure of compound S6.1B (urdamycinone G)

The ^{1}H NMR spectrum (Appendix IV, Fig. 14-15) of compound S6.1B showed three methyl protons, four aromatic/olefinic protons that similar to compound S6.1A, except the additional methine proton at δ_H 6.07 (H-2), the low field methyl proton at δ_H 1.86 (H_3-13), and the absence of a methylene signal.

The ^{13}C NMR spectrum (Appendix IV, Fig. 16) of compound S6.1B exhibited 26 signals, which were classified by DEPT (Appendix IV, Fig. 17) and HMQC spectra analyses as three methyl carbons at δ_C 156.8 (C-3), 13.4 (C-14) and 17.7 (C-7′), two methylene carbons at δ_C 43.4 (C-4) and 40.0 (C-3′), four aromatic/olefinic methine carbons at δ_C 122.9 (C-2), 105.1 (C-6), 133.2 (C-10) and 118.4 (C-11), four sp^3 methine carbons at δ_C 71.0 (C-2′), 76.2 (C-4′), 77.9 (C-5′) and 72.6 (C-6′), and thirteen quaternary carbons at δ_C 195.9 (C-1), 156.8 (C-3), 80.0 (C-4a), 165.1 (C-5), 138.8 (C-6a), 189.3 (C-7), 114.2 (C-7a), 157.4 (C-8), 137.6 (C-9), 131.2 (C-11a), 181.0 (C-12), 134.1 (C-12a) and 76.3 (C-12b). The ^{13}C NMR spectral data of compound S6.1B is also similar to compound S6.1A, except the absence of the methylene carbon and an additional methine carbon at δ_C 122.9 (C-2), together with a higher field carbonyl carbon at δ_C 195.9 (C-1). ^{1}H and ^{13}C NMR spectra indicated the existence of a double bond at C-2. In addition, the 2D NMR spectra (Appendix IV, Figs. 18-23) of compound S6.1B confirmed the existent of quinone core-structure similar to the compoundd S6.1A. The completed NMR spectroscopic data of compound S6.1B is shown in Table 4.40.

Table 4.40 ^{1}H, ^{13}C, COSY, and HMBC spectral data (500 MHz, acetone-d_6) of compound S6.1B (urdamycinone G)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	COSY	HMBC (H to C)
1	195.9	-	-	-
2	122.9	6.07, s	4, 13	1, 13, 4
3	156.8	-	-	-
4	43.4	2.60-2.70, m	-	4a
		2.80-2.95, m	2	2, 3
4a	80.0	-	-	-
5	165.1	-	-	-
6	105.1	6.43, s	-	5, 7, 4a, 12a
6a	138.8	-	-	-
7	189.3	-	-	-
7a	114.2	-	-	-
8	157.4	-	-	-
9	137.6	-	-	-
10	133.2	7.85, d (7.82)	11	8, 11a, 2′
11	118.4	7.53, d (7.82)	10	7a, 9, 12
11a	131.2	-	-	-
12	181.0	-	-	-
12a	134.1	-	-	-
12b	76.3	-	-	-
13	22.7	1.86, s	-	2, 3, 4
14	13.4	2.51, s	-	5
2′	71.0	4.87,d (10.06)	3′	8, 10, 4′, 6′
3′	40.0	1.26-1.40, m	2′, 4′	4′, 5′
		2.36-2.41, m	2′, 4′	3′
4′	76.2	3.43-3.48, m	3′, 5′	5′
5′	77.9	3.05, d (11.15, 11.15)	4′, 6′	7′, 4′, 6′
6′	72.6	3.69-3.74, m	5', 7′	5′
7′	17.7	1.35, d (6.94)	6′	4′, 5′, 6′
4a-OH	-	4.66, br s	-	-
8-OH	-	12.41, br s	-	-
12b-OH	-	4.96, s	-	-
4′-OH	-	4.21, br s	-	-
5′-OH	-	4.15, br s	-	-

Compound S6.1B should be derived from the same biosynthesis as that of compound S6.1A, therefore, the stereochemistries at C-4a and C-12a are assigned to be the same as urdamycinone E (S6.1A). Compound S6.1B could be depicted as the dehydrated product of compound S6.1A (Fig. 4.36), hence, it might be an artifact. Therefore, compound S6.1A was treated with dehydration condition (e.g. 30% CH_3COOH or 8% HCl in MeOH), and found unchanged. The result indicated that compound S6.1A is not an artifact.

Compound S6.1C (Fig. 4.33) was obtained as a brownish green solid with melting point 270-271 °C. The HRESIMS spectrum of compound S6.1C gave a molecular ion peak at *m/z* 457.1264 $[M+Na]^+$, suggesting the molecular formula of $C_{25}H_{22}O_7$. The UV (MeOH) spectrum showed the absorption peaks at λ_{max} (log ε) 231 (4.40), 270 (3.49), 316 (4.24), 428 (3.85) nm. The IR spectrum (Appendix IV, Fig. 24) showed absorption peak at ν_{max} 3372 (OH stretching), 2920, 2849 (CH stretching) and a chelated quinone carbonyl at ν_{max} 1619 cm^{-1}.

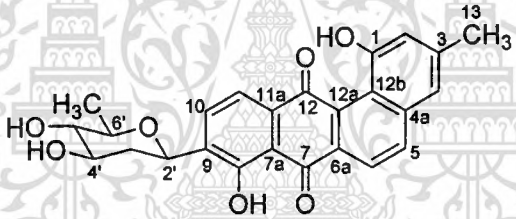


Fig. 4.33 Chemical structure of compound S6.1C (dehydroxyaquayamycin)

The ^1H NMR spectrum (Appendix IV, Fig. 25) of compound S6.1C showed two methyl signals as a singlet at δ_H 1.99 (H-13) and a doublet at δ_H 0.82 (H-7′), one methylene proton at δ_H 1.81 (H-3′), six aromatic proton signals that comprised two singlet protons at δ_H 6.53 (H-2) and 6.91 (H-4), and four doublet protons at δ_H 7.77 (H-5), 7.70 (H-6), 7.39 (H-10) and 7.20 (H-11), four methine protons at δ_H 4.35 (H-2′), 3.10 (H-4′), 2.44 (H-5′) and 2.05 (H-6′), and four hydroxyl protons at δ_H 4.62 (4′-OH), 4.54 (5′-OH), 10.38 (1-OH) and 12.04 (8-OH).

The ^{13}C NMR (Appendix IV, Figs. 26-28) and DEPT (Appendix IV, Fig. 29) spectral data showed 25 signals, suggesting that two methyl carbons at δ_C 21.5 (C-13) and 18.9 (C-7′), one methylene carbons at δ_C 40.0 (C-3′), six aromatic methine carbons at δ_C 117.6 (C-2), 120.2 (C-4), 135.7 (C-5), 121.8 (C-6), 133.6 (C-10) and 119.8 (C-11), four sp^3 methine carbons at δ_C 70.2 (C-2′), 72.1 (C-4′), 77.5 (C-5′) and 76.6 (C-6′), and twelve quaternary carbons at δ_C 155.5 (C-1),

141.8 (C-3), 138.9 (C-4a), 133.6 (C-6a), 188.4 (C-7), 114.8 (C-7a), 157.3 (C-8), 137.5 (C-9), 134.7 (C-11a), 187.0 (C-12), 135.0 (C-12a) and 119.1 (C-12b).

In HMQC spectrum, two singlet protons at δ_H 6.53 (H-2) and 6.91 (H-4) correlated to the carbons at δ_C 117.6 (C-2), 120.2 (C-4), respectively. In HMBC spectrum two singlet methine protons at H-2 and H-4 showed correlations to the carbons at δ_C 155.5 (C-1), 21.5 (C-13), 120.2 (C-4) and 138.9 (C-4a), 135.7 (C-5), 119.1 (C-12b), 21.5 (C-13), respectively. Four doublet protons at δ_H 7.77 (H-5), 7.70 (H-6), 7.39 (H-10) and 7.20 (H-11), correlated in HMQC to the carbons at δ_C 135.7 (C-5), 121.8 (C-6), 133.6 (C-10) and 119.8 (C-11), respectively. In HMBC spectrum, two douplet methine protons at H-5 and H-6 showed correlations to the carbons at C-6a, C-12b and C-4a, C-7, C-12a, respectively. Two doublet methine protons at H-10 and H-11 gave HMBC correlation to the carbons at δ_C 157.3 (C-8), 134.7 (C-11a), 70.2 (C-2′) and 114.8 (C-7a), 137.5 (C-9), 187.0 (C-12), respectively. The chelated hydroxyl proton at δ_H 12.04 correlated to the quaternary carbons at C-7a and C-9. The spectral information suggested a quinone structure, supported by the IR absorption at ν_{max} 1619 cm^{-1}. The 2D NMR spectra (Appendix IV, Figs. 30-33) confirmed that the core structure (quinone skeleton) of compound S6.1C has C-glycosidic linkage at C-9, similar to the compound S6.1A and S6.1B.

Compound S6.1C (dehydroxyaquayamycin) was similar to that of tetrangulol (Kuntsmann and Mitscher, 1966) and also displayed similar ^1H NMR spectral data to that of the dehydrated product of dihydroaquayamycin, originately derived from the reduction of aquayamycin (Sezaki *et al.*, 1970). Therefore, the structure of compound S6.1C is depicted as shown. The completed NMR spectral data of compound S6.1C is shown in Table 4.41.

Table 4.41 ^{1}H, ^{13}C, COSY, and HMBC spectral data (500 MHz, DMSO-d_6) of compound S6.1C (dehydroxyaquayamycin)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	COSY	HMBC (H to C)
1	155.5	-	-	-
2	117.6	6.53, s	-	1, 13, 4
3	141.8	-	-	-
4	120.2	6.91, s	-	4a, 5, 12b, 13
4a	138.9	-	-	-
5	135.7	7.77, d, (8.70)	6	6a, 12b,
6	121.8	7.70, d, (8.70)	5	4a, 7, 12a
6a	133.6	-	-	-
7	188.4	-	-	-
7a	114.8	-	-	-
8	157.3	-	-	-
9	137.5	-	-	-
10	133.6	7.39, d. (7.80)	11	8, 11a, 2′
11	119.8	7.20, d, (7.80)	10	7a, 9, 12
11a	134.7	-	-	-
12	187.0	-	-	-
12a	135.0	-	-	-
12b	119.1	-	-	-
13	21.5	1.99, s	-	3
2′	70.2	4.35, d, (11.30)	3′	9, 10, 6′
3′	40.0	1.81, m	4′	4′, 5′
		0.82, m	4′, 2′	2′, 5′
4′	72.1	3.10, m	3′, 5′, 5′-OH	-
5′	77.5	2.44, m	4′, 4′-OH	-
6′	76.6	2.05	-	3′
7′	18.9	0.82, d (6.00)	6′	5′
1-OH	-	10.38, s	-	1, 2
8-OH	-	12.04, s	-	7a, 9
4′-OH	-	4.62, d (5.15)	4′	4′, 5′
5′-OH	-	4.54, d (4.63)	5′	4′, 5′

Compound S6.1D (Fig. 4.34) was obtained as a green solid. The UV (MeOH) spectrum showed the peak at λ_{max} (log ε) 229 (4.35), 288 (4.01), 342 (4.28), 427 (3.84) and 584 (3.68) nm. The IR spectrum (Appendix IV, Fig. 34) displayed the peak at ν_{max} 3367 (OH stretching), 2918 (CH stretching), and a chelated quinone carbonyl at ν_{max} 1627cm^{-1}. The HRESIMS spectrum of compound S6.1D gave a molecular ion peak at *m/z* 448.1400 $[M-H]^-$, suggesting the molecular formula of $C_{25}H_{23}O_7N$.

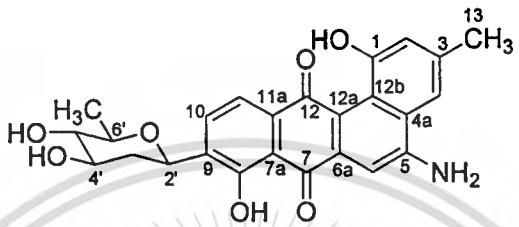


Fig. 4.34 Chemical structure of compound S6.1D (5-aminodehydroxyaquayamycin)

The ^{1}H NMR spectrum (Appendix IV, Fig. 35) of compound S6.1D exhibited two methyl signals at a singlet at δ_H 2.44 and a doublet at δ_H 1.36 (J = 6.10). Five aromatic proton signals, which were two singlet protons at δ_H 7.58 and 7.63, and three doublet protons at δ_H 7.05 (J = 1.30), 7.89 (J = 7.80), 7.82 (J = 7.80). The ^{1}H NMR spectrum of compound S6.1D showed similar to that of compound S6.1C, except the additional broad proton at δ_H 7.31 (5-NH_2) and the singlet proton at δ_H 7.63 (H-6).

The ^{13}C NMR spectrum (Appendix IV, Fig. 36) of compound S6.1D revealed 25 signals, which were classified by DEPT (Appendix IV, Fig. 37) and HMQC spectra as two methyl carbons at δ_C 20.4 (C-13) and 17.8 (C-7′), one methylene carbon at δ_C 40.0 (C-3′), five aromatic carbons at δ_C 120.2 (C-2), 113.4 (C-4), 105.1 (C-6), 133.5 (C-10) and 120.1 (C-11), four sp^3 methine carbons at 71.1 (C-2′), 72.6 (C-4′), 77.9 (C-5′) and 76.3 (C-6′), and thirteen quaternary carbons at δ_C 156.8 (C-1), 139.5 (C-3), 126.2 (C-4a), 154.0 (C-5), 138.1 (C-6a), 189.5 (C-7), 114.5 (C-7a), 157.7 (C-8), 136.9 (C-9), 134.7 (C-11a), 183.9 (C-12), 118.4 (C-12a) and 123.0 (C-12b). The ^{13}C NMR spectrum of compound S6.1D is also similar to that of compound S6.1C, except the absence the methine carbon and an additional quaternary carbon at δ_C 154.0 (C-5) together with higher field methine carbon at δ_C 105.1 (C-6). In HMBC spectrum, the broad proton peak at δ_H 7.31 (5-NH_2) showed correlation to the quaternary carbon at δ_C 126.2 (C-4a) and methine carbon at δ_C 105.1 (C-6). On the basis of the 2D NMR spectral analysis

(Appendix IV, Figs. 38-43), the chemical structure of compound S6.1D was 5-aminodehydroxyaquayamycin. The completed NMR spectral data of compound S6.1D is shown in Table 4.42.

Table 4.42 ^{1}H, ^{13}C, COSY, and HMBC spectral data (500 MHz, acetone-d_6) of compound S6.1D (5-aminodehydroxyaquayamycin)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	COSY	HMBC (H to C)
1	156.8	-	-	-
2	120.2	7.03, d, (1.30)		1, 4, 13, 12b
3	139.49	-	-	-
4	113.4	7.58, s		2, 5, 13, 4a, 12b
4a	126.2	-	-	-
5	154.0	-	-	-
6	105.1	7.63, s	-	4a, 7, 12a,
6a	138.1	-	-	-
7	189.5	-	-	-
7a	114.5	-	-	-
8	157.7	-	-	-
9	136.9	-	-	-
10	133.5	7.89, d, (7.80)		2', 8, 11a
11	120.1	7.82, d, (7.80)		7a, 9, 12
11a	134.7	-	-	-
12	183.9	-	-	-
12a	118.4	-	-	-
12b	123.0	-	-	-
13	20.4	2.44, s		2, 3, 4
2′	71.1	4.88, d, (10.50)	3′	9
3′	40.0	1.42, m	2′, 4′	2′, 4′
		2.44, m	2′, 4′	4′, 5′
4′	72.6	3.73, m	3′, 5′, 7′	-
5′	77.9	3.08, m	4′, 6′	7′, 4′, 6′
6′	76.3	3.48, m	5′, 7′	-
7′	17.8	1.36, d, (6.10)	6′	5′
1-OH	-	12.38, s	-	1, 2
5-NH_2	-	7.31, br, s	-	4a, 6

Aquayamycin containing amino group have never been reported from actinobacteria or other microorganisms. In this study, 5-aminodehydroxyaquayamycin was isolated from the marine *Streptomyces* sp. S6-1 in the Gulf of Thailand. Unfortunately, compound S6.1D could not be isolated in the re-cultivation. This compound might be derived from the dehydroxyaquayamycin in the biosynthetic pathway of aquayamycin (Fig. 4.36).

Compound S6.1E (Fig. 4.35) was isolated after re-cultivation of *Streptomyces* sp. S6-1, the compound was obtained as red solid with the melting point 230-233 °C. The UV (MeOH) spectrum showed the absorption peaks at λ_{max} (log ε) 219 (4.18), 270 (3.49), 290 (3.96) and 466 (5.12) nm. The IR spectrum (Appendix IV, Fig. 44) displayed characteristic absorption of hydroxyl (ν_{max} 3401 cm^{-1}), carbonyl (ν_{max} 1729 and 1630 cm^{-1}), and benzene ring (ν_{max} 1515 cm^{-1}). The molecular formula of compound S6.1E was determined by HRESIMS as $C_{44}H_{58}O_{17}SNa$ by giving the molecular ion peak at *m/z* 913.3273 $[M+Na]^+$.

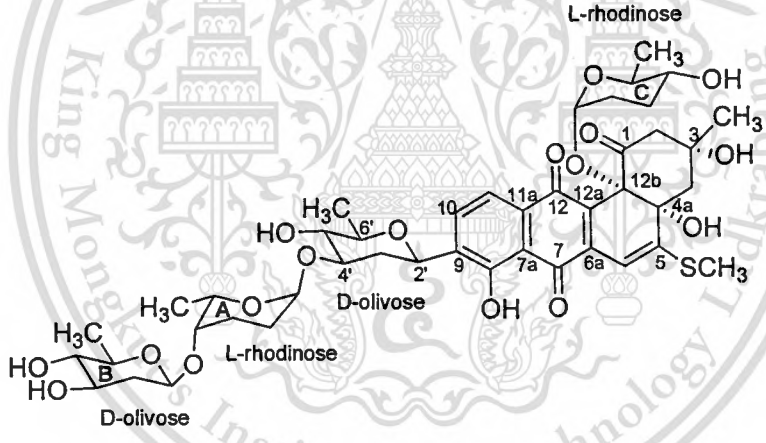


Fig. 4.35 Chemical structure of compound S6.1E (urdamycin E)

The ^1H NMR spectrum (Appendix IV, Figs. 45-49) of compound S6.1E showed signals representing an angucycline glycoside, which comprised one chelated hydroxyl proton at δ_H 12.45 (8-OH); three oromatic/olefinic protons [two doublets at δ_H 7.89 (H-10) and 7.59 (H-11), and one singlet at δ_H 6.47 (H-6)]; fourteen sp^3 methine protons at δ_H 4.87 (H-2′), 3.78 (H-4′), 2.83 (H-5′), 4.87 (H-6′), 4.97 (H-1A), 3.51 (H-4A), 4.20 (H-5A), 4.59 (H-1B), 3.78 (H-3B), 3.15 (H-4B), 3.20 (H-5B), 5.29 (H-1C), 3.30 (H-4C) and 3.65 (H-5C); eight methylene protons at δ_H 2.91 (H-2), 2.42 (H-4), 1.86 (H-3′), 1.95-2.05 (H-2A, H-3A, H-2C and H-3C) and 2.52 (H-2B);

six methyl protons [two singlets at δ_H 1.17 (H-13), 2.52 (H-14), and four doublets at δ_H 1.36 (H-7′), 1.22 (5A-CH_3), 1.14 (5B-CH_3) and 0.52 (5C-CH_3).

The ^{13}C NMR (Appendix IV, Figs. 50-52) and DEPT (Appendix IV, Fig. 53) spectral data of compound S6.1E revealed the presence of 44 signals, which were six methyl, eight methylene, seventeen methine and thirteen quaternary carbons. The methyl at δ_H 2.52 (H-14) showed correlation in HMQC spectrum to the carbons at δ_C 13.6 (C-14) and in HMBC spectrum to the carbons at δ_C 164.2 (C-5) indicating the attachment to a sulfur atom. Spectral analysis of 2D NMR spectra (Appendix IV, Figs. 54-65) of compound S6.1E confirmed the existent of quinone core-structure similar to the compound S6.1A. Compound S6.1E differed from compound S6.1A by consisting of three *O*-glycossidic bonds. Compound S6.1E was identified as urdamycin E, isolated from *Streptomyces fradiae* (Rohr and Zeeck, 1987). ^{1}H and ^{13}C NMR spectral data of compound S6.1E and urdamycin E are shown in Table 4.43.

Treatment of urdamycin E (S6.1E) with conc. H_2SO_4 in H_2O–MeOH solution at room temperature for 2 h gave urdamycinone E (Rohr and Zeeck, 1987). The result suggested that urdamycin E (S6.1E) could possibly be a precursor of urdamycinone E (S6.1A), and urdamycinone G (S6.1B), which have benz[*a*]anthraquinone as its core skeleton (Fig. 4.36).

The biosynthetic pathway of these compounds was suggested to be acetyl-CoA (polyketide pathway) via rearrangement of a linear tetraclyclic intermediated (Gould and Cheng, 1994). In addition, core structure (benz[*a*]anthraquinone) showing a unique *C*-linkaged of glycone to the *β*-*C*-glycosidic bond (Mittler *et al.*, 2007). For the anglucycline antibiotics, *C*-glycosidic sugar was D-olivose (2, 6-dideoxy-D-glucose) that transferd at C-9 of aglycone in the glycosylation of the biosynthetic pathway (Hoffmiester *et al.*, 2000). In addition, three other *O*-glycosidically linked sugars in compound S6.1E (urdamycin E) were identified as one *β*-D-olivose (sugar B) and two *α*-L-rhodinose (sugars A and C) (Rohr and Zeeck, 1987).

Table 4.43 ^{1}H and ^{13}C NMR data of compounds S6.1E and urdymicin E (50 MHz, acetone-d_6)

Position	Compounds			
	S6.1E		Urdamycin E (Rohr and Zeeck, 1987)	
	δ_C (ppm)[a]	δ_H (ppm), multiplicity, (J in Hz)[a]	δ_C (ppm)[b]	δ_H (ppm), multiplicity, (J in Hz)[c]
1	201.5	-	202.2	-
2	54.1	2.91, d (12.60)	54.7	2.92, d (13)
3	75.8	-	75.7	-
4	45.1	2.42, dd (1.98, 14.80)	45.6	1.9~2.2 (complex)
4a	81.7	-	82.3	-
5	164.2	-	164.8	-
6	104.7	6.47, s	105.3	6.49, s
6a	114.3	-	114.8	-
7	189.5	-	190.0	-
7a	131.5	-	132.0	-
8	157.4	-	158.0	-
9	137.1	-	137.6	-
10	133.4	7.89, d (8.30)	134.0	7.93, d (8)
11	118.8	7.59, d (8.30)	119.4	7.61, d (8)
11a	134.5	-	135.0	-
12	182.2	-	182.2	-
12a	137.4	-	138.0	-
12b	83.6	-	84.2	-
13	29.4	1.17, s	28~32	1.17, s
14	13.6	2.52, s	14.3	2.54, s
2′	71.4	4.87, s	71.9	4.90, dd (10, 1)
3′	39.6	1.86, m	40.2	2.18, ddd (13, 5, 2)
4′	78.6	3.78, m	78.2	3.80, ddd (12, 9, 5)
5′	77.6	2.83, m	79.0	2.92, dd (9, 9)
6′	71.4	4.87, m	72.4	3.51, dq (9, 6)
7′	17.9	1.36, d (6.10)	18.6	1.37, d (6)
1A	95.1	4.97, s	94.9	4.99, br, s
2A	25.5	1.95-2.05	25.3	-
3A	24.7	1.95-2.05	25.0	-
4A	75.7	3.51, s	76.3	3.56, br, s
5A	66.7	4.20, m	67.2	4.21, dq (1.5, 6.5)
5A-CH_3	17.5	1.22, d (6.14)	17.2	1.15, d (6.5)
1B	101.5	4.59, m	102.1	4.60, dd (10, 1.5)
2B	37.0	2.52, s	37.1	2.54, s
3B	78.6	3.78, m	76.3	3.5, m
4B	76.3	3.15, m	76.9	3.16, dd (9, 9)
5B	71.8	3.20, m	71.5	3.2, m
5B-CH_3	16.4	1.14, d (6.10)	18.2	1.23, d (6)
1C	94.3	5.29, d (2.80)	95.6	5.32, br, s
2C	22.9	1.95-2.05	26.1	-
3C	24.3	1.95-2.05	23.5	-
4C	66.3	3.30, br, s	66.9	3.32, br, s
5C	67.1	3.65, m	67.7	3.4~3.7 (complex)
5C-CH_3	16.2	0.52, d (6.57)	16.9	0.53, d (6.5)
	-	4.01,br, s	-	3.98, s
	-	4.07, br, s	-	4.04, s
3-OH	-	4.37, s	-	4.35, s
4a-OH		5.44		
8-OH	-	12.45, s	-	12.38, br,s

[a] 500 MHz, acetone-d_6; [b] 50.3 MHz, acetone-d_6; [c] 200 MHz, acetone-d_6

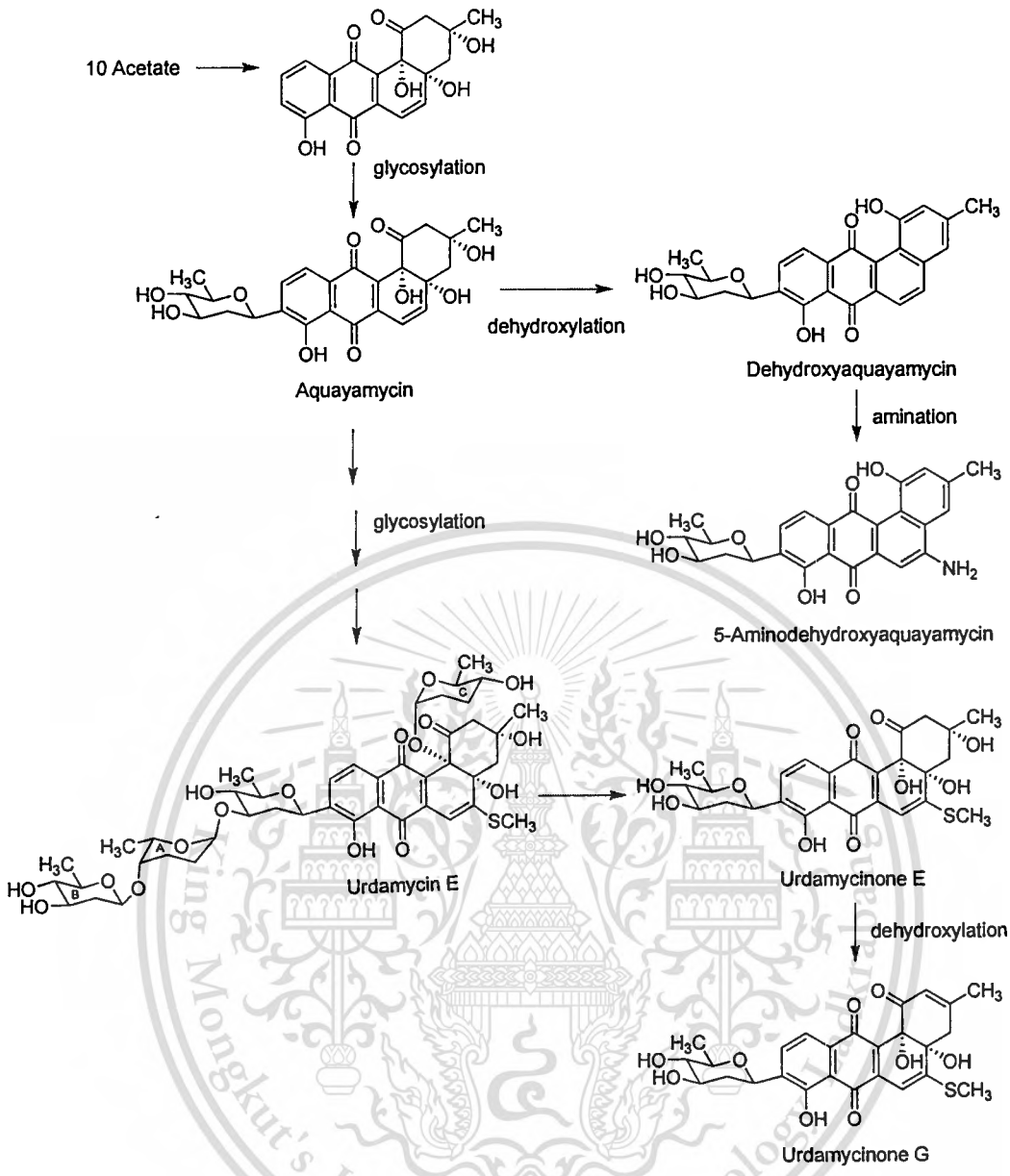


Fig. 4.36 Possible biosynthesis of urdamycinone E (S6.1A), urdamycinone G (S6.1B), dehydroxylaquayamycin (S6.1C), 5-aminodehydroxylaquayamycin (S6.1D), and urdamycin E (S6.1E) isolated from marine *Streptomyces* sp. S6-1 (Gould and Cheng, 1994; Homeister *et al.*, 2001)

Benz[α]anthraquinones derivatives have been found in many *Streptomyces* (Gould and Cheng, 1994; Sawa *et al.*, 1991; Taniguchi *et al.*, 2002). Aquayamycin, a benz[α]anthracene with the presence of C-glycosidic linkage, showed dopamine β-hydroxylase inhibition (Nagatsu *et al.*, 1968). Five compounds from *Streptomyce* sp. S6-1 (S6.1A-S6-.1E) showed strong cytotoxicity against Vero, KB, MCF-7 and NCI-H187 cell lines. Compound S6.1A, S6.1B, S6.1C and S6.1E exhibited biological activities against *Plasmodium falciparum* and

Mycobacterium tuberculosis. Compounds S6.1B, S6.1D and S6.1E showed activity against *Candida albicans*. In addition, the antibacterial activity was found in the sulfur containing angucycline antibiotics (S6.1A, S6.1B and S6.1E). Biological activities of the isolated compounds are shown in Table 4.44.

Table 4.44 Biological activities of pure compounds isolated from *Streptomyces* sp. S6-1

Compound	Anti *B. cereus* MIC (μg/ml)	Anti *C. albicans* IC_{50} (μg/ml)	Anti-TB MIC (μg/ml)	Anti-malaria IC_{50}±SD (μg/ml)	Cytotoxicity IC_{50}±SD (μg/ml)	Anti-Cancer IC_{50} (μg/ml)		
						KB	MCF-7	NCI-H187
S6.1A	50	Inactive	3.13	0.0534±0.029	1.71±0.040	0.179±0.056	0.196±0.038	0.092±0.006
S6.1B	25	9.67	12.50	0.142±0.091	3.05±0.284	0.324±0.108	0.450±0.104	0.242±0.035
S6.1C	Inactive	Inactive	6.25	2.93±0.54	10.07±0.781	6.96±1.68	3.41±0.057	3.97±0.572
S6.1D	Inactive	45.62	Inactive	Inactive	26.89±5.96	33.24±7.00	5.05±0.010	3.38
S6.1E	50	41.07	12.50	0.173	15.46	1.39	0.757	0.289

4.3.2.2 Isolation and structure elucidation of secondary metabolites from the marine *Micromonospora* sp. S8-12 (BCC 45599)

The EtOAc crude extract from *Micromonospora* sp. S8-12 composed of three interesting peaks, which were determined by HPLC analysis at RTs 7.82 (peak A), 5.37 (peak B) and 3.73 (peak C). The UV profiles of these peaks were interesting compared with BIOTEC's database. The chemical profiles of the crude are shown in Fig. 4.37.

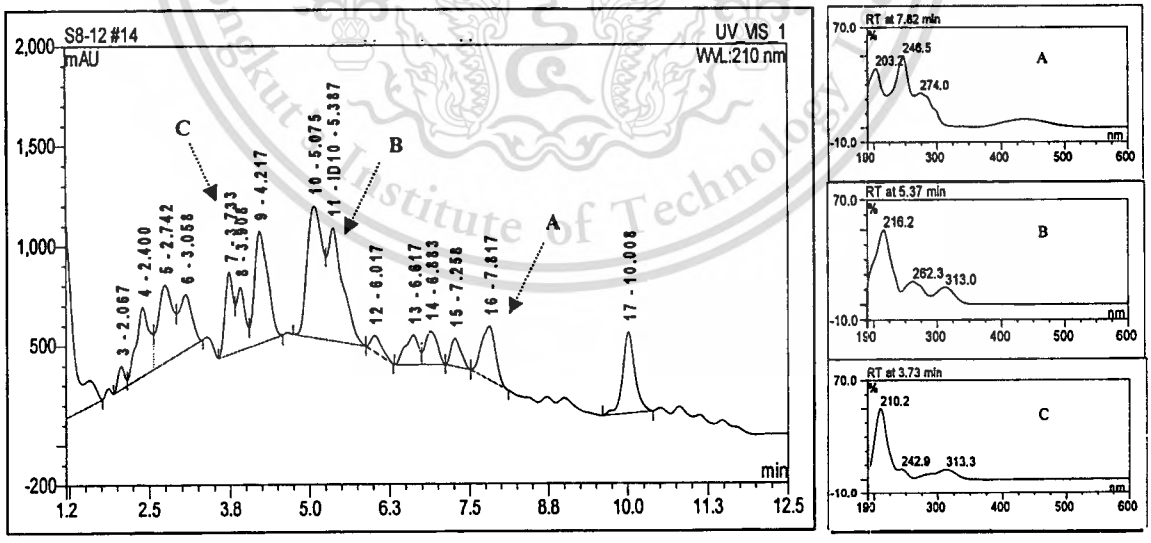


Fig. 4.37 HPLC profiles of the EtOAc crude extract (20 l) from *Micromonospora* sp. S8-12

The EtOAc crude extract from *Micromonospora* sp. S8-12 exhibited biological activities against KB and NCI-H187 cells with IC_{50} values at 35.63 and 45.75 µg/ml, respectively. The crude was purified by Sephadex LH-20 column and semi-preparative HPLC to give three pure compounds, which were 7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione (S8.12A), 6,8-dihydroxy-3,4,5-trimethylisocoumarin (S8.12B) and 2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid (S8.12C). Their chemical structures were elucidated by spectral analyses.

Compound S8.12A (Fig. 4.38) was obtained as yellow powder. The UV (MeOH) spectrum showed the absorption peaks at λ_{max} (log ε) 251 (4.17), 272 (4.02) nm. The IR spectrum (Appendix IV, Fig. 66) displayed absorption bands at ν_{max} 3488 (hydroxyl stretching), 2956 (CH), 1660 (conjugated ketone), and 1616 (C=C) cm^{-1}. The ESIMS spectrum established the molecular formula of compound S8.12A as $C_{22}H_{23}O_4$, showing the mass ion peak at *m/z* 349.2 $[M-H]^-$.

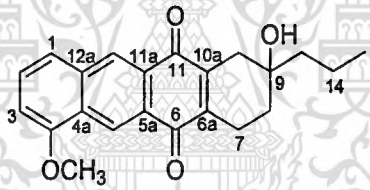


Fig. 4.38 Chemical structure of compound S8.12A (7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione)

The ^1H NMR spectrum (Appendix IV, Fig. 67) of compound S8.12A showed the presence of twelve proton signals, which were two methyl protons at δ_H 0.99 (H-15) and 4.05 (4-OCH_3), two singlet protons at δ_H 9.05 (H-5), 8.53 (H-12), and aromatic signals at δ_H 7.58 (H-1), 7.58 (H-2), and 6.91 (H-3). Moreover, ^1H NMR spectrum showed signals of five methylene protons (see Table 55).

Analyses of ^{13}C NMR (Appendix IV, Fig. 68) and DEPT (Appendix IV, Fig. 69) spectroscopic data of compound S8.12A revealed 22 signals, which comprised one methyl carbon at δ_C 14.8 (C-15), one methoxyl carbon at δ_C 56.0 (4-OCH_3), five methylene carbons at δ_C 16.7 (C-14), 21.3 (C-7), 32.1 (C-8), 36.1 (C-10), 45.0 (C-13), five sp^2 methine carbons at δ_C 122.2 (C-1), 130.0 (C-2), 107.2 (C-3), 123.6 (C-5), 128.1 (C-12), and ten quaternary carbons at δ_C 157.4

(C-4), 127.3 (C-4a), 129.4 (C-5a), 143.7 (C-6a), 70.1 (C-9), 146.1 (C-10a), 129.4 (C-11a), 136.1 (C-12a), 184.3 (C-6) and 184.9 (C-11). Two low field carbons at δ_C 184.3 (C-6) and 184.9 (C-11) indicated the presence of conjugated carbonyl carbons.

In HMQC spectrum, five aromatic protons at δ_H 7.58 (H-1), 7.58 (H-2), 6.91 (H-3), 9.05 (H-5) and 8.53 (H-12) correlated to the carbons at δ_C 122.2 (C-1), 130.0 (C-2), 107.2 (C-3), 123.6 (C-5), and 128.1 (C-12), respectively. The HMBC spectrum showed correlation from the singlet aromatic protons at δ_H 9.05 (H-5), 8.53 (H-12) and 6.91 (H-3) to the carbonyl at δ_C 184.3 (C-6), 184.9 (C-11) and 122.2 (C-1), 127.3 (C-4a), respectively; two doublet aromatic protons at δ_H 7.58 (H-1) and 7.58 (H-2) to the carbons at δ_C 107.2 (C-3), 127.3 (C-4a) and 157.4 (C-4), 136.1 (C-12a), respectively. The methyl protons at δ_H 0.99 and 4.05 correlated in HMQC to the carbons at δ_C 14.8 (C-15), and 56.0 (4-OCH_3), respectively, and in the HMBC spectrum to the carbons at δ_C 45.0 (C-13), 16.7 (C-14) and 157.4 (C-4), respectively. The non-equivalent methylene protons at δ_H 2.63/2.79 (H_2-10) correlated in the HMQC spectrum to the carbon at δ_C 36.1 (C-10), and in the HMBC spectrum to the carbons at δ_C 143.7 (C-6a), 32.1 (C-8), and 70.1 (C-9). Two methylene protons at δ_H 1.60 (H-13) and 1.60 (H-14) correlated in the HMQC spectrum to the carbons at δ_C 16.7 (C-14) and 70.1 (C-9), respectively. On the basis of the 2D NMR spectral analysis (Appendix IV, Figs. 70-73) of compound S8.12A was identical to the known 7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione, which was previously reported from the marine *Micromonospora* sp. (Sousa *et al.*, 2012). However, the stereochemistry of C-9 can not be determined. The ^{1}H and ^{13}C NMR spectroscopic data of compound S8.12A are shown in Table 4.45.

Table 4.45 ^{1}H and ^{13}C NMR data of compound S8-12A and 7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione (500 MHz, in pyridine-d_5)

Position	S8.12A		7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione (Sousa *et al.*, 2012)	
	δ_C (ppm)[a]	δ_H (ppm), multiplicity, (J in Hz)[a]	δ_C (ppm)[b]	δ_H (ppm), multiplicity, (J in Hz)[b]
1	122.2	7.58, d (8.20)	122.9	7.65, d (8.0)
2	130.0	7.58, d (8.20)	131.0	7.56, t (8.0)
3	107.2	6.91, s	108.4	6.96, d (8.0)
4	157.4	-	157.9	-
4a	127.3	-	127.8	-
5	123.6	9.05, s	123.6	9.30, s
5a	129.4	-	129.4	-
6	184.3	-	184.9	-
6a	143.7	-	147.2	-
7	21.3	2.81-2.90, m	22.6	3.12, m
				3.01, br, d (18.7)
8	32.1	1.90, m	33.2	2.03, m
9	70.1	-	69.2	-
10	36.1	2.63, d (20.10)	37.2	3.24, d (19.1)
		2.79, d (20.10)		2.78, d (19.1)
10a	146.1	-	145.4	-
11	184.9	-	185.5	-
11a	129.4	-	132.2	-
12	128.1	8.53, s	128.6	8.96, s
12a	136.1	-	136.8	-
13	45.0	1.60, m	46.3	1.70, m
14	16.7	1.60, m	17.6	1.67, m
15	14.8	0.99, t (6.40)	15.7	0.95, t (6.8)
4-OCH_3	56.0	4.05, s	56.5	3.84, s

[a] 400 MHz , $CDCl_3$; [b] 500 MHz, pyridine-d_5

Compound S8.12B (Fig. 4.39) was obtained as brown solid. HRESIMS spectrum gave the mass peak ion at *m/z* 221.0811 $[M-H]^-$, suggesting the molecular formula of $C_{12}H_{14}O_4$. The UV spectrum showed absorption bands at λ_{max}(log ε) 231 (3.84), 268 (3.88), 308 (3.71) nm. The IR spectrum (Appendix IV, Fig. 74) showed the absorption peaks at ν_{max} 3292, 2925, 2854, 1644, 1621, 1397, 1318, 1265, 1242, 1163, 1107, 1003, 854, 749, 668 cm^{-1}.

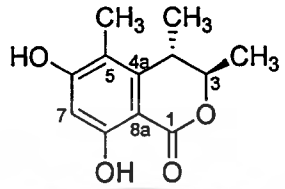


Fig. 4.39 Chemical structure of compound S8.12B (6,8-dihydroxy-3,4,5-trimethylisocoumarin)

The ^{1}H NMR spectrum (Appendix IV, Fig. 75) of compound S8.12B showed seven proton signals belonging to three methyl at δ_H 1.24 (3-CH_3), 1.24 (4-CH_3) and 2.05 (5-CH_3), two methine at δ_H 3.11 (H-4) and 4.67 (H-3), one aromatic proton at δ_H 6.29 (H-7), and a hydroxyl proton at δ_H 11.40 (8-OH).

The ^{13}C NMR (Appendix IV, Fig. 76) and DEPT (Appendix IV, Fig. 77) spectroscopic data showed twelve carbon signals, which comprised three methyl at δ_C 19.0 (3-CH_3), 19.1 (4-CH_3) and 9.1 (5-CH_3), two methine at δ_C 34.3 (C-4) and 79.7 (C-3), one aromatic at δ_C 100.5 (C-7), and six quaternary carbons at δ_C 99.2 (C-8a), 113.9 (C-5), 143.2 (C-4a), 162.4 (C-6), 168.5 (C-8) and 168.8 (C-1).

In HMQC spectrum, three methyl protons at δ_H 1.24 (3-CH_3), 1.24 (4-CH_3), and 2.05 (5-CH_3) correlated to the carbons at δ_C 19.0, 19.1, and 9.1, respectively. The HMBC spectrum gave the correlations from the singlet methyl proton at δ_H 2.05 (5-CH_3) to the quaternary carbons at δ_C 143.2 (C-4a), 113.9 (C-5), and 162.4 (C-6); two methyl protons at δ_H 1.24 (3-CH_3 and 4-CH_3) to the carbons at δ_C 34.34 (C-4), 79.72 (C-3), and 143.2 (C-4a); the singlet aromatic proton at δ_H 6.29 (H-7) to the carbons at 113.9 (C-5), 162.4 (C-6), 168.5 (C-8) and 99.2 (C-8a); two quartet methine protons at δ_H 4.67 (H-3) and 3.11 (H-4) to the carbons at δ_C 168.8 (C-1), 34.3 (C-4), 143.2 (C-4a), 19.1 (4-CH_3) and 143.2 (C-4a), 19.1 (4-CH_3), 113.9 (C-5), 168.5 (C-8), respectively. The chelated hydroxyl proton at δ_H 11.40 (8-OH) correlated in the HMBC spectrum to the carbons at δ_C 162.4 (C-6), 100.5 (C-7) and 99.2 (C-8a). Base on 2D NMR spectral analysis (Appendix IV, Figs. 78-81), the chemical structure of compound S8.12B was 6,8-dihydroxy-

3,4,5-trimethylisocoumarin. The completed NMR spectral data of compound S8.12B (6,8-dihydroxy-3,4,5-trimethylisocoumarin) is shown in Table 4.46.

The relative stereochemistry of 3-CH_3 and 4-CH_3, suggested by the NOE spectrum that was the *trans*-configulation (Xin *et al.*, 2007), and assigned as the 3*R*, 4*S*- configuration (Hassall and Jones, 1962). Therefore, the compound S8.12B gave $[\alpha]_D^{25}$ +88.0 (MeOH) that similar to the (3*R*, 4*S*)-6,8-dihydroxy-3,4,5-trimethylisochromarin.

The citrinin derivatives, closely related to 6,8-dihydroxy-3,4,5-trimethylisocoumarin, have been produced by several fungal species, which belonged mainly to the genus *Penicillium* (Chen *et al.* 2011). Recently, 6,8-dihydroxy-3,4,5-trimethylisocoumarin was also isolated from the marine-derived fungus *Penicillium* (Xin *et al.*, 2007; Chen *et al.* 2011; Zhou *et al.* 2013). In addition, the compound was derived from the citrinin biosynthetic pathway (Brown *et al.*, 1949) (Fig. 4.41).

Table 4.46 ^1H, ^{13}C, COSY, and HMBC spectral data (500 MHz, acetone-d_6) of compound S8.12B (6,8-dihydroxy-3,4,5-trimethylisocoumarin)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	COSY	HMBC (H to C)
1	168.8	-	-	-
3	79.7	4.67, q	4-CH_3	1, 4, 4a, 4-CH_3
4	34.3	3.11, q	4-CH_3	4a, 4-CH_3, 5, 8a
4a	143.2	-	-	-
5	113.9	-	-	-
6	162.4	-	-	-
7	100.5	6.29, s	-	5, 6, 8, 8a
8	168.5	-	-	-
8a	99.2	-	-	-
3-CH_3	19.0	1.24, m	-	3, 4
4-CH_3	19.1	1.24, m	3, 4	3, 4a
5-CH_3	9.1	2.05, s	-	4a, 5, 6
8-OH	-	11.40, s	-	6, 7, 8a
6-OH	-	-	-	-

Compound S8.12C (Fig. 4.40) was obtained as a yellow solid with the molecular formula of $C_{12}H_{16}O_5$, determined by HRESIMS at *m/z* 239.0915 $[M-H]^-$. The IR spectrum (Appendix IV, Fig. 82) exhibited the absorption peaks at ν_{max} 2956, 2927, 2854, 1740, 1638, 1585, 1462, 1271, 1187, 1054 cm^{-1}. The UV spectrum showed the absorption bands at λ_{max} (log ε) 221 (3.81), 251 (3.72), 314 (3.29) nm.

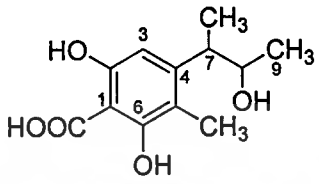


Fig. 4.40 Chemical structure of compound S8.12C [2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid]

The ^1H NMR spectrum (Appendix IV, Fig. 83) of compound S8.12C showed seven signals, which were three methyl at δ_H 0.95 (H_3-9), 1.96 (5-CH_3), 1.04 (7-CH_3), two sp^3 methine at δ_H 2.92 (H-7), 3.70 (H-8), and one singlet proton at δ_H 5.98 (H-3).

The ^{13}C NMR (Appendix IV, Fig. 84) and DEPT spectroscopic data (Appendix IV, Fig. 85) of compound S8.12D revealed twelve carbon signals that comprised three methyl at δ_C 20.1 (C-9), 11.0 (5-CH_3), 15.9 (7-CH_3), three methine at δ_C 103.3 (C-3), 42.2 (C-7), 69.8 (C-8), and six quaternary carbons at δ_C 102.8 (C-1), 160.1 (C-2), 147.3 (C-4), 111.4 (C-5), 160.5 (C-6) and 176.3 (1-COOH). The proton at δ_H 5.98 (H-3) having a carbon at δ_C 103.3 (C-3) in HMQC spectrum, which showed correlations in HMBC spectrum to the quaternary carbons at δ_C 102.8 (C-1), 160.1 (C-2), and 111.4 (C-5). Two methine protons at δ_H 2.92 (H-7) and 3.70 (H-8) showed HMBC correlation to the methyl carbons at δ_C 103.3 (C-3), 111.4 (C-5), 15.9 (7-CH_3), 69.8 (C-8), 20.1 (C-9), and 15.9 (7-CH_3), respectively. Three methyl protons at δ_H 0.95 (H-9), 1.96 (5-CH_3), and 1.04 (7-CH_3) correlated in the HMQC to the carbons at δ_C 20.1 (C-9), 11.0 (5-CH_3), and 15.9 (7-CH_3), respectively. The HMBC spectrum gave the correlations from the singlet methyl proton at δ_H 1.96 (5-CH_3) to the carbons at δ_C 147.3 (C-4), 111.4 (C-5) and 160.5 (C-6); two doublet methyl protons at δ_H 0.95 and 1.04 to the carbons at δ_C 42.2 (C-7), 69.8 (C-8) and 147.3 (C-4), 42.2 (C-7), 69.8 (C-8), respectively. The hydroxyl proton at δ_H 4.32 (8-OH) showed HMBC correlations to the carbons at δ_C 42.2 (C-7), 69.8 (C-8) and 20.1 (C-9). . On the basis of the 2D NMR spectral analysis (Appendix IV, Figs. 86-89), the chemical structure of compound

S8.12C was 2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid. The completed NMR spectral data of compound S8.12C [2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid] is shown in Table 4.47.

Compound S8.12C was produced by *Micromonospora* sp. S8-12 as a minor product, which could be derived from the intermediate of compound S8.12B (Fig. 4.41). This is the first report of its isolation from the marine *Micromonospora* sp.

Table 4.47 ^{1}H, ^{13}C, COSY, and HMBC spectral data (400 MHz, DMSO-d_6) of compound S8.12 C (2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	COSY	HMBC (H to C)
1	102.8	-	-	-
2	160.1	-	-	-
3	103.3	5.98, s	-	1, 2, 5
4	147.3	-	-	-
5	111.4	-	-	-
6	160.5	-	-	-
7	42.2	2.92, m	8, 7-CH_3	3, 5, 7-CH_3, 8, 9
8	69.8	3.70, m	7, 9	7-CH_3
9	20.1	0.95, d (6.20)	8	7, 8
1-COOH	176.3	-	-	-
5-CH_3	11.0	1.96, s		4, 5, 6
7-CH_3	15.9	1.04, d (7.00)	7	4, 7, 8
2-OH	-	-	-	-
6-OH	-	-	-	-
8-OH	-	4.32, d (4.40)	-	7, 8, 9

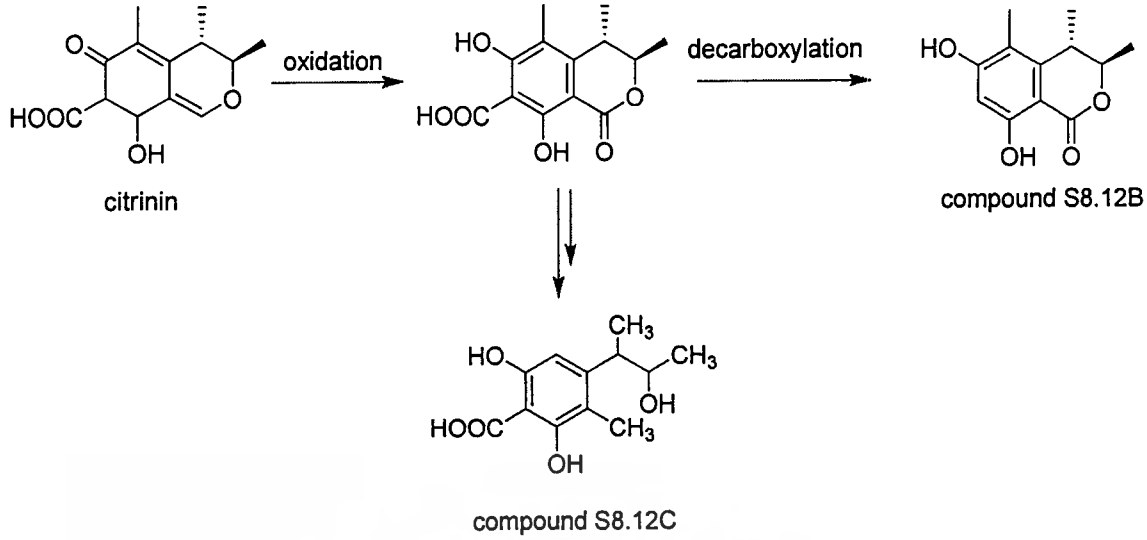


Fig. 4.41 Possible biosynthetic pathway of compounds S8.12B and S8.12C derived from citrinin (Chen *et al.*, 2011)

Biological activities of metabolites from *Micromonospora* sp. S8-12, compound S8.12A exhibited antimalarial activity at IC_{50} value of 4.44 µg/ml and showed cytotoxicity against Vero cell at IC_{50} value of 18.19 µg/ml. Howover, compound S8.12A showed biological activity against KB, MCF-7, and NCI-H187 cells at IC_{50} values of 30.22, 30.62, and 42.33 µg/ml, respectively. On a contrary, compounds S8.12B and S8.12C were inactive for all tests (Table 4.48).

Table 4.48 Biological activities of metabolites from *Micromonospora* sp. S8-12

Compounds	Anti *B. cereus* MIC (µg/ml)	Anti *C. albicans* IC_{50}(µg/ml)	Anti-TB MIC (µg/ml)	Anti-malarial IC_{50} (µg/ml)	Cytotoxicity IC_{50} (µg/ml)	Anti-Cancer IC_{50} (µg/ml)		
						KB	MCF-7	NCI-H187
S8.12A	Inactive	Inactive	Inactive	4.44	18.19	30.22	30.62	42.33
S8.12B	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
S8.12C	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive

4.3.2.3 Isolation and structure elucidation of secondary metabolites from the marine *Actinomadura* sp. TV1-14 (BCC 45631)

Large scale fermentation (20 l) of marine *Actinomadura* sp. TV1-14 was carried out to study chemical ingediants and biological activities. The crude extract exhibited antibacterial activity against *Bacillus cereus* with MIC value of 12.50 µg/ml, and showed antiphytopathogen against both *Colletotrichum capsici* and *Colletotrichum gloeosporioides* at MIC value of 25 µg/ml.

The chemical profile of the crude was analyzed by HPLC, which composed of two major peaks at RT 5.68 (peak A) and 8.63 (peak B), and the minor peaks at RTs 6.29, 7.26, 7.67 (peak D, peak C and peak E). These peaks showed the interesting UV profiles, compared with the BIOTEC's database. Moreover, peaks A and B gave similar UV profiles as shown in the fig. 4.42.

The re-cultivation of *Actinomadura* sp. TV1-14 showed similarly chemical profiles to that of the original culture. The chemical profiles of *Actinomadura* sp. TV1-14 is shown in Fig. 4.42.

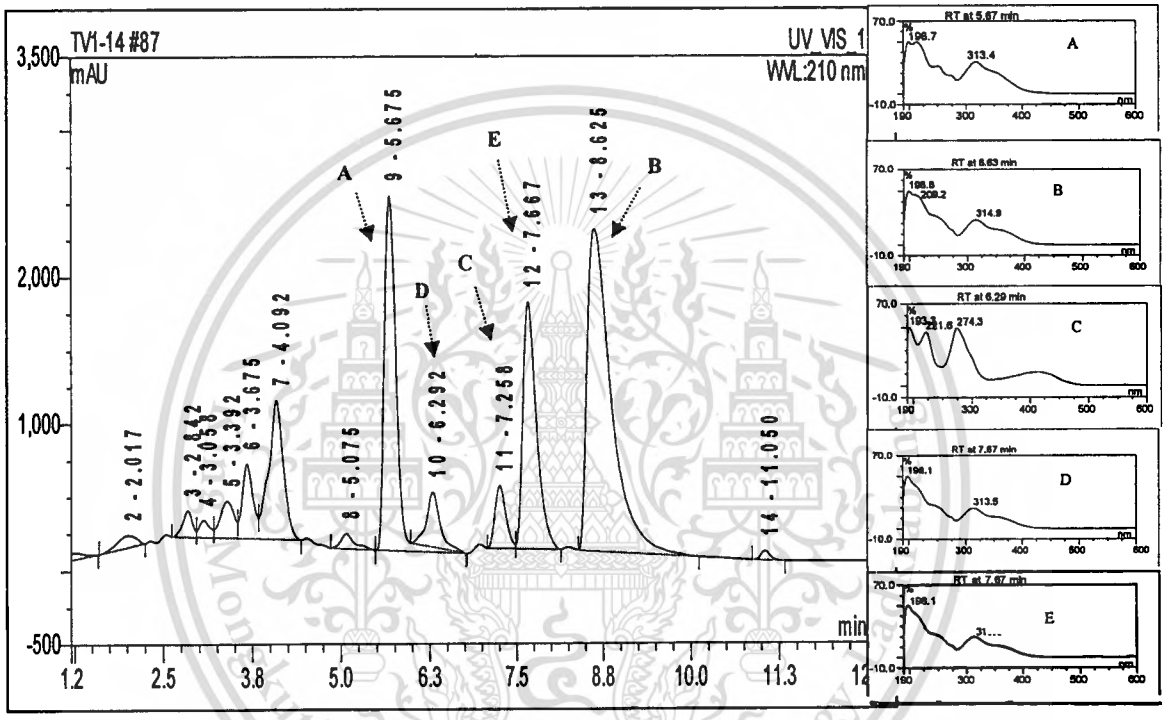
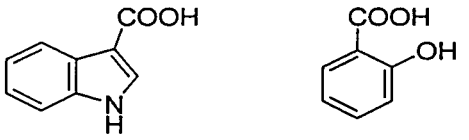


Fig. 4.42 HPLC profile of the EtOAc crude extract (20 l) from *Actinomadura* sp. TV1-14

The crude was separated by Sephadex LH-20 and followed by semi-preparative HPLC to give six metabolites, two of which (TV14A, TV14B) are major compounds together with TV14C, TV14D, TV14E (indole-3-carboxylic acid) and TV14F (2-hydroxy-benzoic acid). Chemical structures of these compounds were elucidated by NMR spectral analyses.



TV14E TV14F

Fig. 4.43 Chemical structures of TV14E and TV14F isolated from *Actinomadura* sp. TV1-14

Compound TV14A (Fig. 4.44) was obtained as a brown powder. HRESIMS spectrum gave the mass peak ion at *m/z* 351.0831 $[M+Na]^+$, suggesting the molecular formula of $C_{18}H_{16}O_6$. The UV spectrum showed the absorption bands at λ_{max} (log ε) 220 (4.13), 316 (4.00) nm. The IR spectrum (Appendix IV, Fig. 90) displayed the absorption peaks at ν_{max} 3479 (OH stretching), 2924 (CH stretching) and 1616 (C=O stretching of aromatic ketone) cm^{-1}.

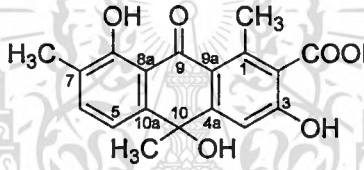


Fig. 4.44 Chemical structure of compound TV14A (9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid)

The ^1H NMR spectrum (Appendix IV, Fig. 91) of compound TV14A showed seven signals, which were one hydroxyl proton at δ_H 13.15 (8-OH), three methyl signals at δ_H 2.86 (1-CH_3), 2.22 (7-CH_3) and 1.55 (10-CH_3), three aromatic protons at δ_H 7.30 (H-5), 7.42 (H-6), and 7.50 (H-4).

The ^{13}C NMR (Appendix IV, Fig. 92) and DEPT (Appendix IV, Fig. 93) spectral data of compound TV14A showed eighteen carbons, including three methyl carbons at δ_C 20.1 (1-CH_3), 14.5 (7-CH_3) and 38.6 (10-CH_3), three methine carbons at δ_C 111.0 (C-4), 114.7 (C-5) and 136.1 (C-6), and twelve quaternary carbons at δ_C 143.2 (C-1), 120.6 (C-2), 160.6 (C-3), 156.1 (C-4a), 124.5 (C-7), 159.9 (C-8), 115.4 (C-8a), 190.0 (C-9), 122.3 (C-9a), 70.1 (C-10), 147.5 (C-10a) and 169.8 (2-COOH).

In HMQC spectrum, three aromatic protons at δ_H 7.50 (H-4), 7.30 (H-5), and 7.42 (H-6) showed correlations to the carbons at C-4, C-5, and C-6, respectively. In HMBC spectrum, the singlet methine proton at δ_H 7.50 (H-4) showed correlations to the quaternary carbons at δ_C 120.6 (C-2), 122.3 (C-9a) and 70.1 (C-10); two doublet protons at 7.30 (H-5) and 7.42 (H-6) to the

carbons at δ_C 124.5 (C-7), 115.4 (C-8a), 70.1 (C-10) and 159.9 (C-8), 147.5 (C-10a), 14.5 (7-CH_3), respectively. In addition, the methyl protons at δ_H 2.86 (1-CH_3) showed HMBC correlations to the carbon at δ_C 143.2 (C-1), 120.6 (C-2) and 122.3 (C-9a); two methyl at δ_H 2.22 (7-CH_3) and 1.55 (10-CH_3) showed HMBC correlations to the quaternary carbons at δ_C 136.1 (C-6), 124.5 (C-7), 159.9 (C-8) and 156.1 (C-4a), 70.1 (C-10), 147.5 (C-10a), respectively. The presence of carbonyl carbon at δ_C 190.0 (C-9) was also supported by the IR absorption at ν_{max} 1616 cm^{-1}. On the basis of the 2D NMR spectral analysis (Appendix IV, Figs. 94-97), the chemical structure of compound TV14A was 9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid. The completed NMR spectral data of compound TV14A (9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid) is shown in Table 4.49.

Reaction of compound TV14A with diazomethane gave a mixture of TV14AM1 and TV14AM2 (Fig. 4.45), which purified by semi-preparative HPLC. Compounds TV14AM1 and TV14AM2 gave molecular ion peaks at *m/z* 357.1331$[M+H]^+$, and 371.1481 $[M+H]^+$, respectively. The ^1H NMR spectrum of compound TV14M1 (Appendix IV, Fig. 122) showed two additional methyl signals at δ_H 3.95 (2-COO**CH**$_3$) and 3.93 (3-O**CH**$_3$), while compound TV14M2 (Appendix IV, Fig. 123) showed three additional methyl signals at δ_H 3.96 (2-COO**CH**$_3$), 3.97 (3-O**CH**$_3$), and 2.93 (10-O**CH**$_3$). The completed NMR spectral data of compounds TV14AM1 and TV14AM2 are shown in Table 4.50.

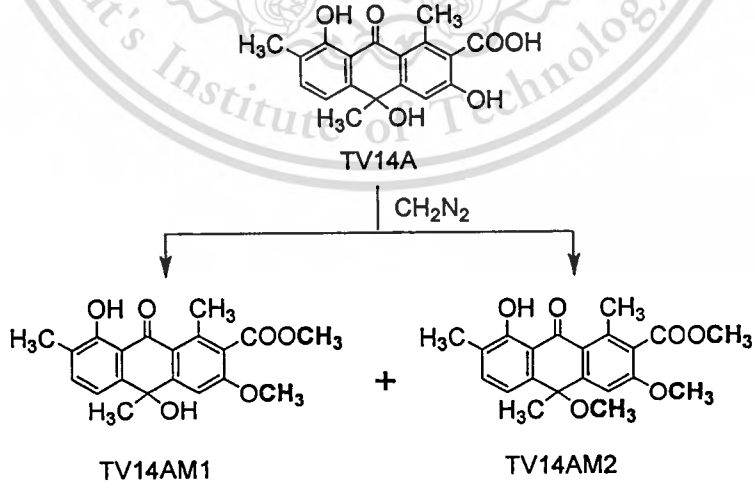


Fig. 4.45 Reaction of compound TV14A with diazomethane

Table 4.49 ^{1}H, ^{13}C, and HMBC spectral data (500 MHz, acetone-d_6) of compound TV14A (9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	HMBC (H to C)
1	143.2	-	-
2	120.6	-	-
3	160.6	-	-
4	111.0	7.50, s	2, 3, 9a,10
4a	156.1	-	-
5	114.7	7.30, d (7.74)	7, 8a, 10
6	136.1	7.42, d (7.74)	8, 10a, 7-CH_3
7	124.5	-	-
8	159.9	-	-
8a	115.4	-	-
9	190.0	-	-
9a	122.3	-	-
10	70.1	-	-
10a	147.5	-	-
1-CH_3	20.1	2.86, s	1, 2, 9a
2-COOH	169.8	-	-
7-CH_3	14.5	2.22, s	6, 7, 8
10-CH_3	38.6	1.55, s	4a, 10, 10a
8-OH	-	13.15	-

Table 4.50 ^{1}H, ^{13}C, and HMBC spectral data (500 MHz, $CDCl_3$) of compounds TV14AM1 and TV14AM2

Position	TV14AM1		TV14AM2		HMBC (H to C)
	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	
1	140.8	-	141.2	-	-
2	121.6	-	126.6	-	-
3	159.6	-	159.8	-	-
4	105.4	7.39, s	105.8	7.22, s	9a, 10
4a	154.9	-	151.7	-	-
5	114.3	7.24, d (7.73)	115.4	7.09, d (7.73)	7, 8a, 10
6	136.5	7.37, d (7.73)	136.6	7.42, d (7.73)	8, 10a, 7-CH_3
7	114.2	-	115.8	-	-
8	160.7	-	161.2	-	-
8a	126.0	-	125.7	-	-
9	189.5	-	189.8	-	-
9a	126.2	-	123.0	-	-
10	71.3	-	77.0	-	-
10a	146.0	-	142.4	-	-
1-CH_3	20.2	2.63, s	20.6	2.73, s	1, 2, 9a
2-$COOCH_3$	168.3	-	168.3	-	-
7-CH_3	15.4	2.27, s	15.4	2.30, s	6, 8
10-CH_3	38.5	1.59	38.0	1.63, s	4a, 10, 10a
2-$COOCH_3$	52.5	3.95, s	52.6	3.96, s	2-$COOCH_3$
3-OCH_3	56.2	3.93, s	56.2	3.97, s	3
10-OCH_3			52.5	2.93, s	10

Compound TV14B (Fig. 4.46), the major metabolite, was obtained as a yellow solid. The UV spectrum showed absorption bands at λ_{max} (log ε) 220 (4.37) and 314 (4.24) nm. The IR spectra (Appendix IV, Fig. 98) indicated the presence of hydroxyl (ν_{max} 2925-2854 cm^{-1}) and carbonyl (ν_{max} 1626 cm^{-1}). The molecular formula of the compound TV14B was deduced as $C_{36}H_{30}O_{12}$, giving a mass ion peak at *m/z* 653.1667 $[M-H]^{\cdot}$ in HRESIMS spectrum.

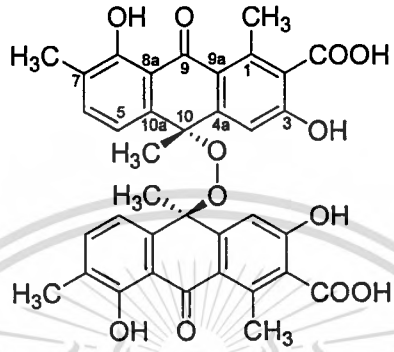


Fig. 4.46 Chemical structure of compound TV14B (oxanthromicin)

The ^{1}H NMR spectrum (Appendix IV, Fig. 99) of compound TV14B revealed the presence of seven signals, one chelated phenolic proton at δ_H 13.69, three singlet methyl signals at δ_H 1.35, 2.30, 2.90, two doublet aromatic signals at δ_H 6.75 (J = 7.70), 7.45 (J = 7.70), and one singlet aromatic proton at δ_H 7.00.

The ^{13}C NMR (Appendix IV, Fig. 100) and DEPT (Appendix IV, Fig. 101) spectroscopic data of compound TV14B revealed eighteen carbons, consisting of three methyl [at δ_C 20.6 (1-CH_3), 14.7 (7-CH_3) and 33.2 (10-CH_3)], three methine [at δ_C 113.6 (C-4), 117.4 (C-5) and 135.8 (C-6)], and twelve quaternary carbons [δ_C 143.2 (C-1), 121.1 (C-2), 160.9 (C-3), 150.5 (C-4a), 125.3 (C-7), 160.5 (C-8), 115.3 (C-8a), 189.9 (C-9), 123.8 (C-9a), 80.0 (C-10), 141.7 (C-10a) and 169.2 (2-COOH)]. IR spectrum showed the absorption peak at ν_{max} 1626 cm^{-1}. In HMQC spectrum, three aromatic protons at δ_H 7.00 (H-4), 6.75 (H-5), and 7.45 (H-6) attached to the carbons at 113.6 (C-4), 117.4 (C-5), and 135.8 (C-6), respectively. In HMBC spectrum, the singlet aromatic proton at δ_H 7.00 (H-4) showed correlations to the quaternary carbons at δ_C 121.1 (C-2), 123.8 (C-9a) and 80.0 (C-10). Two doublet protons at δ_H 6.75 (H-5), and 7.45 (H-6) showed HMBC correlations to the quaternary carbons at δ_C 125.3 (C-7), 115.3 (C-8a), 80.0 (C-10) and 160.5 (C-8), 141.7 (C-10a), respectively. In addition, the methyl protons at δ_H 2.90 (1-CH_3) showed HMBC correlations to the carbons at δ_C 143.2 (C-1), 121.1 (C-2) and 123.8 (C-9a);

two methyl protons at δ_H 2.30 (7-CH_3) and 1.35 (10-CH_3) to the carbons at δ_C 135.8 (C-6), 125.3 (C-7), 160.5 (C-8) and 150.5 (C-4a), 141.7 (C-10a), respectively. The chelated hydroxyl proton at δ_H 13.69 correlated in HMBC spectrum to the carbons at δ_C 125.3 (C-7), 160.5 (C-8) and 115.3 (C-8a). The NMR spectral information led to the compound TV14A but HRESIMS indicated that the molecule must be a dimer. Therefore, compound TV14B was suggested to be the known oxanthromicin. The NMR spectral data (Appendix IV, Figs. 102-105) of compound TV14B was compared to oxanthromicin (Wright *et al.*, 1984) as shown in Table 4.51.

Table 4.51 ^1H, ^{13}C, and HMBC spectral data of compound TV14B and oxanthromicin

Position	TV14B		Oxanthromicin (Wright *et al.*, 1984)		HMBC (H to C)
	δ_C (ppm)[a]	δ_H (ppm), multiplicity, (J in (Hz)[a]	δ_C (ppm)[b]	δ_H (ppm), multiplicity, (J in Hz)[c]	
1	143.2	-	141.1	-	-
2	121.1	-	119.9	-	-
3	160.9	-	159.5	-	-
4	113.6	7.00, s	112.1	6.70, s	2, 3, 9a, 10
4a	150.5	-	148.9	-	-
5	117.4	6.75, d (7.70)	116.7	6.55, d (8.00)	7, 8a, 10
6	135.8	7.45, d (7.70)	135.8	7.25, d (8.00)	8, 10a
7	125.3	-	127.1	-	-
8	160.5	-	158.2	-	-
8a	115.3	-	114.5	-	-
9	189.9	-	189.3	-	-
9a	123.8	-	124.8	-	-
10	80.0	-	79.7	-	-
10a	141.7	-	139.4	-	-
1-CH_3	20.6	2.90, s	20.5	2.85, s	1, 2, 9a
2-COOH	169.2	-	168.7	-	-
7-CH_3	14.7	2.30, s	15.1	2.24, s	6, 7, 8
10-CH_3	33.2	1.35, s	32.9	1.20, s	4a, 10a
8-OH	-	13.69	-	-	7, 8, 8a

[a] 500 MHz, acetone-d_6; [b] 80 MHz, MeOD; [c] 25.2 MHz, MeOD

Moreover, reduction of compound TV14B with palladium on carbon in the presence of hydrogen gas gave compound TV14BH, which was the monomer form of TV14B (Fig. 4.47).

The ^{1}H, ^{13}C NMR and DEPT spectra (Appendix IV, Figs. 124-126) of compound TV14BH were similar to compound TV14A. In addition, the monomeric form (TV14BH) gave $[\alpha]_D^{27}$ −66.5 (EtOH), similar to the monomer of oxanthromicin. The completed NMR spectral data of compound TV14BH is shown in Table 4.52.

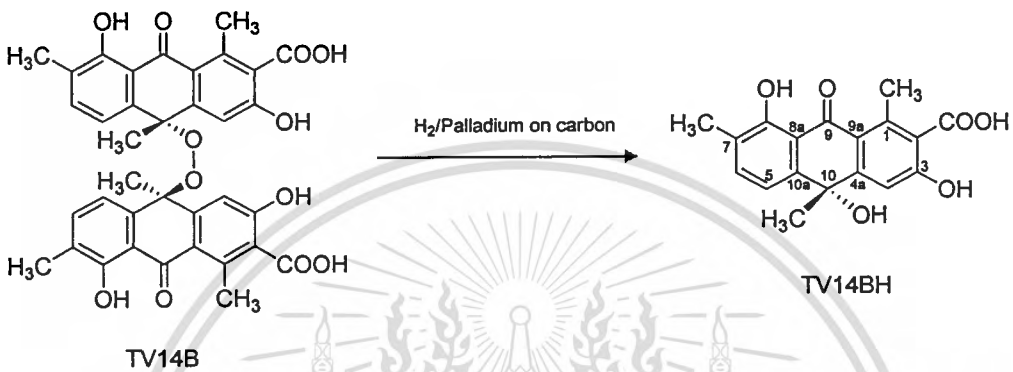


Fig 4.47 Hydrogenation of compound TV14B

Oxanthromicin (TV14B) was the first dimeric anthrone peroxide reported from actinobacterial group (Wright, *et al.*, 1984), its biosynthesis was involved polyketide pathway (Puar *et al.*, 1985). It showed antifungal against *Candida* spp. and dermatophytes (Patel *et al.*, 1984). In this study, compound TV14BH was obtained from the hydrogenolysis of oxanthromicin (Wrighr *et al.*, 1984), which might be derived from the precursor oxanthromicin (compound TV14B). It was suggested that dimeric anthrone peroxide was converted by alkylation, reduction and dehydration to give the monomer into the natural product (Puar *et al.*, 1985). In addition, compound TV14A, a racemic form of the monomer, could slowly decompose at room temperature, and degrade in a presence of acid at 40 °C. Compound TV14A gave $[\alpha]_D^{27}$ -4.9 (EtOH).

Table 4.52 ^{1}H, ^{13}C and HMBC spectral data (400 MHz, acetone-d_6) of compound TV14BH

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	HMBC (H to C)
1	148.5	-	-
2	120.9	-	-
3	168.8	-	-
4	111.7	7.25, s	2, 3, 9a, 10
4a	155.3	-	-
5	114.4	7.27, d (7.77)	7, 8a, 10
6	135.6	7.35, d (7.77)	8, 10a, 7-CH_3
7	124.4	-	-
8	160.2	-	-
8a	115.6	-	-
9	190.5	-	-
9a	119.2	-	-
10	70.4	-	-
10a	147.7	-	-
1-CH_3	20.5	3.11, s	1, 2, 9a
2-COOH	174.0	-	-
7-CH_3	14.8	2.20, s	6, 7, 8
10-CH_3	39.0	1.52, s	4a, 10, 10a
8-OH	-	13.44	7, 8, 8a

Compound TV14C (Fig. 4.48) was obtained as a yellow solid with melting point at 179-182 °C. The UV spectrum exhibited the absorption bands at λ_{max} (log ε) 220 (4.14), 281(4.18), 410 (3.69) nm. The IR spectrum (Appendix IV, Fig. 106) indicated the presence of hydroxyl (ν_{max} 2924 cm^{-1}) and carbonyl (ν_{max} 1627 cm^{-1}). The molecular formula of compound TV14C was established as $C_{17}H_{12}O_6$ by giving a mass ion peak at *m/z* 311.0567 $[M-H]^-$ in the HRESIMS spectrum.

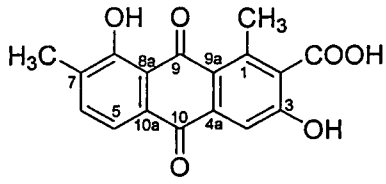


Fig. 4.48 Chemical structure of compound TV14C (3,8-dihydroxy-1,7-dimethylanthraquinone-2-carboxylic acid)

The ^{1}H NMR spectral data (Appendix IV, Fig. 107) of compound TV14C revealed five proton signals, comprising two singlet methyl groups (δ_H 2.24, 2.28) and three aromatic protons (δ_H 7.44, 7.55 and 7.61).

The ^{13}C NMR (Appendix IV, Fig. 108) and DEPT (Appendix IV, Fig. 109) spectroscopic data showed seventeen carbon signals, which comprised two methyl at δ_C 23.1 (1-CH_3), 18.8 (7-CH_3), three methine at δ_C 116.3 (C-4), 120.6 (C-5) and 139.0 (C-6), and twelve quaternary carbons at δ_C 147.5 (C-1), 131.2 (C-2), 152.5 (C-3), 139.4 (C-4a), 137.5 (C-7), 162.8 (C-8), 119.1 (C-8a), 192.5 (C-9), 123.6 (C-9a), 185.1 (C-10), 133.3 (C-10a) and 171.6 (2-COOH).

In HMQC spectrum, three aromatic protons at δ_H 7.44 (H-4), 7.55 (H-5), and 7.61 (H-6) correlated to the carbons at δ_C 116.3 (C-4), 120.6 (C-5), and 139.0 (C-6), respectively. In HMBC spectrum, the singlet methine proton at H-4 showed correlations to the quaternary carbons at δ_C 131.2 (C-2), 123.6 (C-9a), and 185.1 (C-10); two doublet methine protons at H-5 and H-6 to the carbons at δ_C 137.5 (C-7), 119.1 (C-8a), 185.1 (C-10) and 162.8 (C-8), 133.3 (C-10a), 18.8 (7-CH_3), respectively. The 2D NMR spectral information (Appendix IV, Figs. 110-113) indicated a quinone skeleton, supported by the IR absorption peak at ν_{max} 1627 cm^{-1}. In addition, the methyl protons at δ_H 2.24 (1-CH_3) and 2.28 (7-CH_3) correlated in HMQC spectrum to the carbons at δ_C 23.1 and 18.8, respectively and showed HMBC correlations to δ_C 147.5 (C-1), 131.2 (C-2), 123.6 (C-9a) and 139.0 (C-6), 137.5 (C-7), 162.8 (C-8), respectively. The NMR spectral data indicated that TV14C is 3,8-dihydroxy-1,7-dimethylanthraquinone-2-carboxylic acid, shown in Table 4.53.

Table 4.53 ^{1}H, ^{13}C, and HMBC spectral data (400 MHz, DMSO-d_6) of compound TV14C (3,8-dihydroxy-1,7-dimethylanthraquinone-2-carboxylic acid)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	HMBC (H to C)
1	147.5	-	-
2	131.2	-	-
3	152.5	-	-
4	116.3	7.44, s	2, 9a, 10
4a	139.4	-	-
5	120.6	7.55, d (7.69)	7, 8a, 10
6	139.0	7.61, d (7.69)	8, 10a, 7-CH_3
7	137.5	-	-
8	162.8	-	-
8a	119.1	-	-
9	192.5	-	-
9a	123.6	-	-
10	185.1	-	-
10a	133.3	-	-
1-CH_3	23.1	2.24, s	1, 2, 9a
2-COOH	171.6	-	-
7-CH_3	18.8	2.28, s	6, 7, 8

Compound TV14D (Fig 4.49) was obtained as a yellow solid with the melting point at 184-186 °C. The molecular formula was established as $C_{16}H_{10}O_6$ by giving a mass peak at *m/z* 297.0400 $[M-H]^-$ in HRESIMS spectrum. The UV spectrum showed the absorption peaks at λ_{max} (log ε) 220 (4.14), 281(4.18), 410 (3.69) nm. The IR spectrum (Appendix IV, Fig. 114) displayed absorption bands at ν_{max} 1042, 1256, 1454, 1712, 2854, 2924 cm^{-1}.

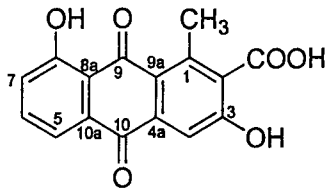


Fig. 4.49 Chemical structure of compound TV14D (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid)

The ^{1}H NMR spectrum (Appendix IV, Fig. 115) of compound TV14D gave six signals, which were four aromatic at δ_H 7.45 (H-4), 7.62 (H-5), 7.71 (H-6), and 7.32 (H-7), one methyl at δ_H 2.85 (1-CH_3), and one chelated phenolic hydroxyl signal at δ_H 13.11 (8-OH).

The ^{13}C spectrum (Appendix IV, Fig. 116) showed sixteen carbon signals, which comprised one methyl at δ_C 20.5 (1-CH_3), four methine at δ_C 114.0 (C-4), 118.5 (C-5), 136.2 (C-6) and 124.8 (C-7), and eleven quaternary carbons at δ_C 145.1 (C-1), 128.5 (C-2), 161.9 (C-9), 136.9 (C-4a), 161.9 (C-8), 117.7 (C-8a), 189.7 (C-9), 121.5 (C-9a), 182.9 (C-10), 133.0 (C-10a) and 169.2 (2-COOH).

The ^{1}H, ^{13}C and DEPT (Appendix IV, Fig. 117) spectral data of compound TV14D were similar to those of compound TV14C. It indicated that the two compounds have the same molecular skeleton, except the substituted methyl group at C-7 was absent for compound TV14D. The 2D NMR spectral analysis (Appendix IV, Figs. 118-121), the chemical structure of compound TV14D was 23,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid. The NMR spectroscopic data suggested that TV14D is 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid, shown in Table 4.54.

Compound TV14D (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid) was a minor metabolite and was an intermediate of the anthraquinone biosynthetic pathway (Bringmann *et al.*, 2009; Fitzgerald *et al.*, 2011) (Fig. 4.50). In *Streptomyces* sp., decarboxylation of 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid gave the production of aloesaponarin II (Bartel *et al.*, 1990). In addition, 3, 8-dihydroxy-1-methylanthraquinone-2-carboxylic acid was an intermediate, which obtained from the synthesis of compound aloesaponarin-I and –II (Cameron *et al.*, 1981).

Table 4.54 ^{1}H, ^{13}C, and HMBC spectral data (500 MHz, DMSO-d_6) of compound TV14D (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid)

Position	δ_C (ppm)	δ_H (ppm), multiplicity, (J in Hz)	HMBC (H to C)
1	145.1	-	-
2	128.5	-	-
3	161.9	-	-
4	114.0	7.45, s	2, 9a, 10
4a	136.9	-	-
5	118.6	7.62, dd (7.89, 1.03)	7, 8a, 10
6	136.2	7.71, t (7.89)	8, 10a
7	124.8	7.32 dd (7.89, 1.03)	5, 8
8	161.9	-	-
8a	117.7	-	-
9	189.7	-	-
9a	121.5	-	-
10	182.9	-	-
10a	133.0	-	-
1-CH_3	20.5	2.85, s	1, 2, 9a
2-COOH	169.2	-	-
8-OH	-	13.11	7, 8, 8a

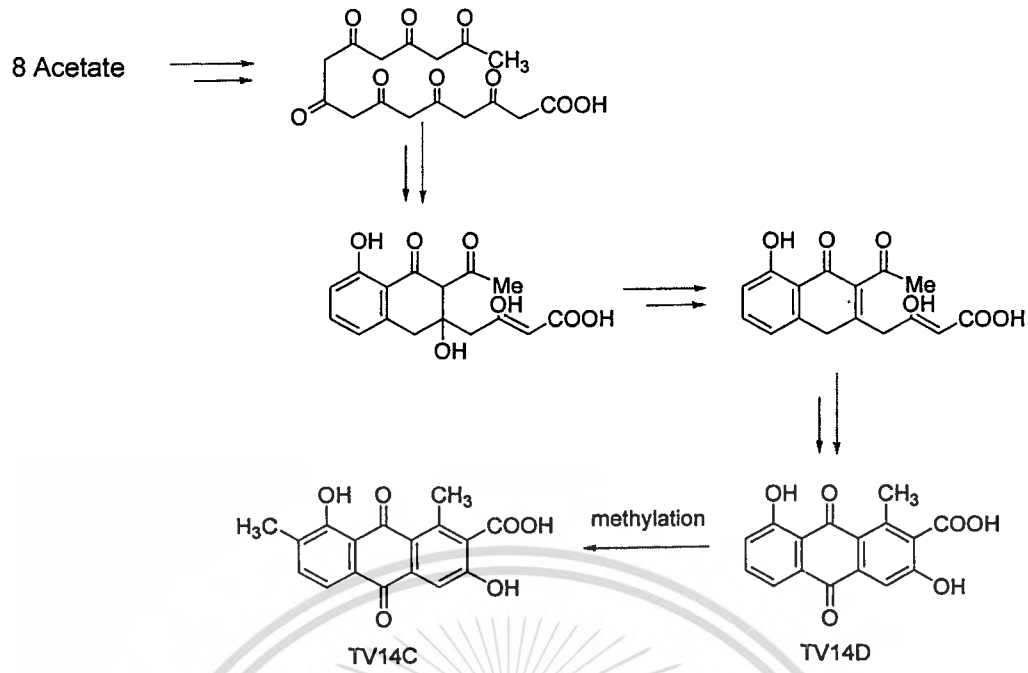


Fig. 4.50 Possible biosynthetic pathway of compounds TV14C and TV14D (Ma *et al.*, 2007; *Bringmann et al.*, 2009; Fizgerald *et al.*, 2011)

Biological activities of compounds TV14A, TV14B and TV14C showed moderate antifungal activity against *Candida albicans* with the IC_{50} values of 29.55, 17.18 and 18.54 μg/ml, respectively. Compound TV14B showed antiphytopathgenic fungal activity against both *Colletotrichum capsici* and *Colletotrichum gloeosporiodes* at the MIC value of 6.25 μg/ml. In addition, compound TV14B showed antibacterial activity against *Bacillus cereus* at MIC value of 3.13 μg/ml. Compound TV14C showed cytoxicity against Vero cell (IC_{50} 18.57 μg/ml) and cancerous (KB, NCI-H187) cells (see Table 4.55).

Table 4.55 Biological activities of secondary metabolites from *Actinomadura* sp. TV1-14

Compound	Anti *B. cereus* MIC (μg/ml)	Anti *C. albicans* IC_{50} (μg/ml)	Antiphytopathogen MIC (μg/ml)		Cytotoxicity IC_{50} (μg/ml)	Anticancer IC_{50}(μg/ml)		
			C. capsici	*C. gloeosporiodes*		KB	MCF-7	NCI-H187
TV14A	Inactive	29.55	Inactive	Inactive	Non-cytotoxic	Inactive	Inactive	Inactive
TV14B	3.13	17.18	6.25	6.25	Non-cytotoxic	Inactive	Inactive	Inactive
TV14C	Inactive	18.54	Inactive	Inactive	18.57	11.47	Inactive	6.94
TV14D	NT	NT	NT	NT	NT	NT	NT	NT

NT; Not being tested

CHAPTER V

CONCLUSIONS

In the research of marine actinobacteria from Thai marine natural sources, thirty-six actinobacterial strains were isolated from sediment, sponge and algal samples that collected from the Andaman Sea and the Gulf of Thailand. On the basis of phenotypic, chemotaxonomic and genotypic characteristics, they were classified to the eight genera such as *Micromonospora*, *Salinispora*, *Verrucosispora Actinomadura*, *Nocardia*, *Pseudonocardia*, *Actinomycetospora* and *Streptomyces*.

Marine actinobacteria in group I consisted of twenty-six strains. They produced a single spore directly on their substrate hyphae. The strains contained *meso*-diaminopimelic acid in the peptidoglycan. Some strains presented hydroxy-diaminopimelic acid. The acyl type of cell wall muramic acid was glycolyl. The strains contained arabinose, galactose, glucose, ribose and xylose as whole cell sugars. Characteristic phospholipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major cellular fatty acids were iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and $C_{17:0}$. The result of 16S rRNA gene sequences analysis confirmed that they were classified to the genus *Micromonospora*. Based on polyphasic taxonomy data, two strains, SH2-13^T and S3-1^T, were described as the novel species including *Micromonospora sediminicola* SH2-13^T sp. nov. and *Micromonospora spongicola* S3-1^T sp. nov.

Marine actinobacteria in group II contained three obligate marine actinobacterial strains (SP207-05, SP207-08 and SP209-09), which produced single spores on the substrate mycelium, but not formed aerial mycelium. Marine actinobactria in this group required sea water for growth and well grew on sodium chloride supplemented medium (3 % NaCl). Cell wall hydrolysates contained *meso*-diaminopimelic acid. The whole cell sugars were arabinose, galactose, glucose, mannose, ribose and xylose. The predominant phospholipids comprised phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. Major fatty acid comprised $C_{17:0}$, $C_{17:1}$ ω8c, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$. 16S rRNA gene sequences analyses confirmed that these strains were identified as the member of genus *Salinispora* and were closely related to *Salinispora aenicola* CNH-643^T.

Marine actinobacteria in group III, consisted of strains SP03-01, SP03-05, SP206-02 and SP206-03 which formed single or clusters of non-motile spores on the substrate mycelium but aerial mycelium was not produced. They contained *meso*-diaminopimelic acid in the peptidoglycan. The acyl type of cell wall muramic acid was glycolyl. The whole cell sugars were arabinose, galactose glucose, mannose, ribose and xylose. Characteristic phospholipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. Major menaquinones were MK-9(H_4) and MK-9(H_6). The major cellular fatty acids comprised iso-$C_{15:0}$, $C_{16:0}$, and anteiso-$C_{17:0}$. Genotypic characteristics based on 16S rRNA gene sequences indicated that this group was classified as the genus *Verrrucosispora*. In addition, strain SP03-05 was published to the novel species, *Verrucosispora andamanensis* SP03-05^T sp. nov.

Marine actinobacteria in group IV contained strain TV1-14. This strain formed non fragment substrate mycelium, but the aerial mycelium was not found. The strain contained *meso*-diaminipimelic acid in the peptidoglycan. The whole cell sugar pattern comprised arabinose, galactose, glucose, madurose, mannose, and rhamnose. Predominant phospholipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, and phosphatidylinositol mannosides. Cellular fatty acids were $C_{14:0}$, i-$C_{16:0}$, $C_{16:1}$ ω7c, 10-methyl-$C_{16:0}$, $C_{17:0}$, $C_{18:1}$ ω7c and $C_{18:0}$. The major menaquinones were MK-9(H_4), MK-9(H_6) and MK-9(H_8). Neighbour-joining tree based on 16S rRNA gene sequence showed that the strain formed the cluster within the genus *Actinomadura*. The highest level of 16S rRNA gene sequence similarity was found with *Actinomadura macra* IFO 14102^T (97.70%). In addition, the low DNA–DNA relatedness value (23.8%) between strain TV1-14 and *Actinomadura macra* NBRC 14102^T confirmed that strain TV1-14 represents a novel species of the genus *Actinomadura*.

Marine actinobacteria in group V consisted of strain ST01-07 which formed extensively branched substrate hyphae, but aerial hyphae was weakly produced. The strain produced spores directly on the substrate mycelium. Strain ST01-07 contained *meso*-diaminopimelic acid in the peptidoglycan. Whole cell sugars were arabinose, galactose, glucose, mannose, and ribose. The major phospholipids comprised diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides. Major menaquinones were MK-8($H_{4\omega\text{-cycl}}$) and 2, 3-epoxy- MK-8($H_{4\omega\text{-cycl}}$). The major fatty acids were $C_{16:0}$, $C_{16:1}$ ω7c, $C_{17:0}$, $C_{17:1}$ ω8c, 10-methyl-$C_{17:0}$, $C_{18:1}$ ω9c and $C_{18:0}$. The result of 16S rRNA gene sequences analysis and a low level of DNA–DNA relatedness values between strain ST01-07 and phylogenetically

closest *Nocardia* species, confirmed that strain ST01-07 represents a novel species of the genus *Nocardia*.

Strain S15-3 was a member of marine actinobacteria in group VI. This strain produced a long chain of spore on the branched substrate mycelium. The substrate mycelium could fragment to the rod-shaped elements. The strain contained *meso*-diaminopimelic acid in the cell wall. Whole cell sugars comprised arabinose, galactose, glucose, ribose and xylose. Phospholipids in the membrane contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The major fatty acids were $C_{16:0}$, iso-$C_{16:0}$, iso-$C_{16:1}$ and $C_{16:1}$ ω7c. Morphological and chemical profiles showed that strain S15-3 was classified to the genus *Pseudonocardia*. In addition, neighbour-joining tree base on 16S rRNA gene sequences confirmed that the strain classified to the genus *Pseudonocardia*, and showed the highest similarity value with *Pseudonocardia antarctica* DVS 5a1^T (99.9%).

Three strains, SH3-3, SH2-15, and TV1-16, were assigned to be the member of the group VII. They produced long, well-developed, branched, and fragmented into rod-shaped substrate mycelium. Colonies had a powdery surface that displayed morphological properties typical of the genus *Actinomycetospora*. The results of chemotaxonomic study and 16S rRNA gene sequences analysis also confirmed that these strain belonged to a member of the genus *Actinomycetospora*. The cell wall contains *meso*-diaminopimelic acid. Whole cell sugars comprised arabinose, galactose, glucose, ribose and xylose. The predominant polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. MK-8 (H_4) was found as the major menaquinone. Major fatty acids were $C_{16:0}$, $C_{18:0}$, $C_{16:1}$ ω7c, $C_{17:1}$ ω8c, $C_{18:1}$ ω9c, iso-$C_{15:0}$, iso-$C_{16:1}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, and 10-methyl-$C_{16:0}$. Strain TV1-16 was closely related to *Actinomycetospora lutea* NBRC 103690^T in the neighbour-joining tree. This strain also presented the phenotypic and genotypic characteristics that differed from all validly described *Actinomycetospora* species. Thus, this strain was tentatively identified as a novel species of the genus *Actinomycetospora*.

Marine actinobacteria in group VIII consisted of five strains, S6-1, S20-7, S8-04, SH2-1, and AN5-16. They formed aerial hyphae with non-motile conidiospores. All strains in this group contained *LL*-diaminopimelic acid in the peptidoglycan. Phylogenetic tree analysis base on 16S rRNA gene sequences indicated that this group formed the cluster within the genus *Streptomyces*. Moreover, phylogenetic relationship of strain AN5-16 indicated that strain AN5-16 formed a distinct phyletic line related to the member of the family *Streptomycetaceae,* and showed the low

levels of 16S rRNA gene sequence similarity value (92.2-93.5%) with the other members of genus *Streptomyces* species. These genotypic results indicated that this strain was possibly represented as a new genus of the family *Streptomycetaceae.*

Three strains such as *Streptomyces* sp. S6-1, *Micromonospora* sp. S8-12 and *Actinomadura* sp. TV1-14 selected based on biological activity and their chemical profiles, were isolated and identified the secondary metabolites.

Streptomyces sp. S6-1 was closely related to *Streptomyces fradiae* NBRC 12214^{T} (99.7%). The strain produced four naturally new compounds, which were S6.1A (urdamycinone E), S6.1B (urdamycinone G), S6.1C (dehydroxyaquayamycin) and S6.1D (5-aminodehydroxyaquayamycin), and a known compound S6.1E (urdamycin E). Biological activities of these compounds displayed strong cytotoxicity against Vero, KB, MCF-7, and NCI-H187 cell lines. Compounds S6.1A, S6.1B, S6.1C and S6.1E exhibited antimalarial and antitubercular activities. The biological activity against *Candida albicans* was found also in the compounds S6.1B, S6.1D and S6.1E. In addition, compounds S6.1A, S6.1B, S6.1E showed antibacterial activity.

Three known compounds were isolated from the marine *Micromonospora* sp. S8-12, including 7,8,9,10-tetrahydro-9-hydroxy-1-methoxy-9-propyltetracene-6,11-dione (S8.12A), 6,8-dihydroxy-3,4,5-trimethylisocoumarin (S8.12B), and 2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid (S8.12C). Compound S8.12A showed moderate cytotoxicity against Vero, KB, MCF-7, and NCI-H187 cell lines.

Actinomadura sp. TV1-14 produced three naturally new compounds, which were TV14A (9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid), TV14C (3,8-dihydroxy-1,7-dimethylanthraquinone-2-carboxylic acid) and TV14D (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid). Moreover, the known peroxide oxanthromicin (TV14B) was isolated as a major metabolite of the marine *Actinomadura* sp. TV1-14. Three compounds TV14A-TV14C displayed moderate biological activity against *Candida albicans.* Compound TV14B showed antibacterial (*Bacillus cereus*) and antipytophatogenic (*Colletrotrichum capsici* and *Colletrotrichum gloeosporiodes*) activities. Only compound TV14C showed cytotoxicity against Vero, KB, and NCI-H187 cell lines.

The results indicated the diversity of marine actinobacteria in the Andaman Sea and the Gulf of Thailand, in term of biological variation and diversed secondary metabolites. It can also be used as sources of candidates in drug discovery.

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APPENDIX I

CULTURE MEDIA

Artificial seawater (Marinium Reef Sea Salt)

Main composition: (units in part per million or ppm)

Chloride	1.9×10^{4}
Sodium	1.05×10^{4}
Magnesium	1.41×10^{3}
Sodium	8.85×10^{2}
Calcium	4.20×10^{2}
Potassium	3.80×10^{2}
Bromine	6.50×10^{1}
Boron	8.10×10^{0}
Strontium	4.60×10^{0}
Fluorine	1.30×10^{0}
Lithium	1.80×10^{-1}
Rubidium	1.30×10^{-1}
Iodine	6.02×10^{-2}
Barium	3.30×10^{-2}
Aluminium	1.03×10^{-2}
Iron	1.10×10^{-2}
Molybdenum	1.05×10^{-2}
Zinc	1.00×10^{-2}
Manganese	2.10×10^{-3}
Vanadium	1.75×10^{-3}
Cobalt	4.65×10^{-4}
pH	7.8-8.3

Basal inorganic nitrogen medium

$(NH_4)_2HPO_4$	1.0	g
KCl	0.2	g
$MgSO_4.7H_2O$	0.2	g
Agar	18	g
Artificial seawater	1	l
pH	7.0	

Humic acid viamin agar with seawater (HV)

Humic acid (dissolved in 10 ml of 2 N NaOH)	0.1	g
Na_2HPO_4	0.05	g
KCl	0.171	g
$MgSO_4.7H_2O$	5.0	mg
$FeSO_4.7H_2O$	1.0	mg
$CaCO_3$	2.0	mg
Vitamin B solution	1.0	ml
Agar	18	g
Artificial seawater	1	l
pH	7.8-8.3	
Vitamin B solution		
Thiamine-HCl	5.0	mg
Riboflavin	5.0	mg
Nicothinate	5.0	mg
Pyridoxine-HCl	5.0	mg
Inositol	5.0	mg
Ca-pantothenate	5.0	mg
p-Amino benzoate	5.0	mg
d-Biotin	2.5	mg

Starch-casein nitrate agar with seawater

Soluble starch	10	g
Sodium caseinate	1	g
KNO_3	2	g
KH_2PO	0.5	g
$MgSO_4$	0.5	g
Agar	18	g
Artificial seawater	1	l
pH	7.8-8.3	

Yeast extract-malt extract agar, ISP medium no.2

Yeast extract	4	g
Malt extract	10	g
Glucose	4	g
Agar	15	g
Artificial seawater	1	l
pH	7.5-8.3	

Oatmeal agar, ISP medium no. 3

Oatmeal	20.0	g
Agar	18.0	g
Artificial seawater	1	l
pH	7.55-8.3	

Inorganic salts-starch agar, ISP medium no. 4

Soluble starch	10	g
K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	1.0	g
NaCl	1.0	g

$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
Trace salts solution (A)	1.0	ml
Agar	20	g
Artificial seawater	1	l
pH	7.5-8.3	

Glycerol-asparagine agar, ISP medium no.5

Glycerol	10	g
L-Asparagine	1.0	g
K_2HPO_4	1.0	g
Trace salts solution (A)	1.0	ml
Agar	20	g
Artificial seawater	1	l
pH	7.5-8.3	

Peptone-yeast extract iron agar, ISP medium no.6

Bacto-Peptone Iron Agar, dehydrated (Difco)	3.6	g
Bacto-Yeast Extract (Difco)	0.1	g
Distilled water	1	l
Agar	20	g
Artificial seawater	1	l
pH	7.5-8.3	

Tyrosine agar, ISP medium no. 7

Glycerol	15	g
L-Tyrosine (Difco)	0.5	g

อนุกรมวิธานของแอคติโนแบคทีเรียจากทะเลของประเทศไทย และสารทุติยภูมิของไอโซเลตที่คัดเลือก

TAXONOMY OF THAI MARINE ACTINOBACTERIA AND SECONDARY METABOLITES OF THE SELECTED ISOLATES

คมสันต์ สุป้อง

KHOMSAN SUPONG

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต

สาขาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์

สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

พ.ศ. 2556

KMITL-2013-SC-D-020-044

L-Asparagine (Difco)	1.0	g
K_2HPO_4	0.5	g
$MgSO_4.7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4.7H_2O$	0.1	g
Trace salts solution (A)	1.0	ml
Agar	20	g
Artificial seawater	1	l
pH	7.5-8.3	

Trace salt solution (A)

$FeSO_4.7H_2O$	0.1	g
$MnCl_2.4H_2O$	0.1	g
$ZnSO_4.7H_2O$	0.1	g
Distilled water	100	ml

Glucose asparagines agar

Glucose	10	g
Asparagine	0.5	g
K_2HPO_4	0.5	g
Agar	15	g
Artificial seawater	1	l
pH	7.5-8.3	

Nutrient agar

Meat extract	10	g
Peptone	10	g

NaCl	1.-2	g
Agar	15	g
Artificial seawater	1	l
pH	7.5-8.3	

Czapek's sucrose agar

Sucrose	30	g
K_2HPO_4	1.0	g
$MgSO_4$	0.5	g
KCl	0.5	g
$FeSO_4$	0.01	g
Agar	18	g
Artificial seawater	1	l
pH	7.0-7.2	

Carbon utilization medium

Carbohydrate	10	g
$(NH_4)_2.SO_4$	2.64	g
$K_2HPO_4.3H_2O$	5.65	g
KH_2PO_4 anhydrous	2.38	g
$MgSO_4.7H_2O$	1.0	g
Pridham and Gottlieb trace salts (B)	1.0	ml
Agar	15	g
Artificial seawater	1	l
pH	7.5-8.3	

Trace salts solution (B)

$CuSO_4.5H_2O$	0.64	g
$FeSO_4.7H_2O$	0.11	g
$MnCl_2.4H_2O$	0.79	g
$ZnSO_4.7H_2O$	0.15	g
Distilled water	100	ml

Bouillon gelatin broth

Peptone	1.0	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15	g
Artificial seawater	100	ml
pH	7.5-8.3	

Peptone KNO_3 broth

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g
Artificial seawater	100	ml
pH	7.5-8.3	

Peptonization and Coagulation test medium

Skim milk (Difco)	10.0	g
Artificial seawater	100	ml

The solution was sterilized at 110 °C for 10 min.

Mueller-Hinton agar (Difco)

Beef infusion from	30	g
Casamino acid, Technical	17.5	g
Starch	1.5	g
Agar	17	g
Distilled water	1	l
pH	7.5-8.3	

Sabouraud's dextrose agar (Difco)

Neopeptone	10	g
Dextrose	40	g
Agar	15	g
Distilled water	1	l
pH	5.6-5.8	

APPENDIX II

REAGENTS AND BUFFER

2 N H_2SO_4

Conc. H_2SO_4	2	ml
Distilled water	34	ml

Add conc. HCl into the distilled water.

6 N HCl

Conc. HCl	60	ml
Distiller water	60	ml

Add conc. HCl into the distilled water.

5% trichloro-acetic acid

Trichloro acetic acid	5	g
Distilled water	100	ml

Add conc. HCl into the distilled water.

DON Reagent

2,7-Dihydroxynapthalene	10	mg
Conc. H_2SO_4	50	ml

Add Conc. H_2SO_4 in 2,7-Dihydroxynapthalene (DON) wait until the yellow solution to colorless (24 h). Keep this solution in refrigerator.

Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	ml

***N,N*-dimethyl-1-naphthylamine solution**

N,N-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	ml

Ninhydrin solution

Ninhydrin	0.3	g
n-Butanol	100	ml
Glacial acetic acid	3	ml

Reagents for fatty acid analysis

Reagent 1 (Sponification reagent)

Sodium hydroxide	15	g
Methanol (HPLC grade)	50	ml
Mili-Q water	50	ml

Dissolve sodium hydroxide pellets in mili-Q water and add methanol.

Reagent 2 (Methylation reagent)

6 N HCl	65	ml
Methanol (HPLC grade)	55	ml

pH below 1.5.

Reagent 3 (Extraction solvent)

n-Hexane (HPLC grade)	50	ml

Methyl-3-Buthyl ether (HPLC grade)	50	ml

Reagent 4 (Base wash reagent)

Sodium hydroxide	1.2	g
Mili-Q water	100	ml

Reagent 5 (Saturated solution chloride)

Sodium hydroxide saturated in mili-Q water

Reagents for polar lipid analysis

Dittmwe & Lester reagent

Solution A

MoO_3	4.011	g
25 N H_2SO_4	100	ml

Dissolve 4.011 g of MoO_3 in 100 ml of 25 N H_2SO_4 with heating.

Solution B

Molybdenum powder	0.178	g
Solution A	50	ml

Add 0.178 g molybdenum powder to 50 ml of solution A, and boil it for 15 min. After cooling, remove the precipitation by decantation. Before spraying, mix solution A (50 ml) plus solution B (50 ml) plus water (100 ml).

Anisaldehyde reagent

Ethanol	90	ml
Conc. H_2SO_4	5	ml
p-Anisaldehyde	5	ml
Acetic acid	1	ml

Dragendorff's reagent

Solution A

Basic bismuth nitrate	1.7	g
Acetic acid	20	ml
Distilled water	80	ml

Solution B

KI	40	g
Distilled water	100	ml

Before spraying, solution A (10 ml) plus solution B (10 ml) and acetic acid (10 ml), were mixed.

0.1 M Tris-HCl buffer, pH 9

Tris	12.11	g
Distilled water	900	ml

Adjust the pH to 9 with HCl.

The 0.1 M Tris was prepared by dissolving 12.11 g of Tris base in 97.89 l of distilled water. The pH was adjusted to the desired value by adding conc. HCl (pH 9.0). The solution was cooled to room temperature before making final adjustment to the desired pH. The volume of solution was adjusted to 1 litter with distilled water and sterilized by autoclaving for 15 min at 15 lb/in^2.

1 M Tris-HCl pH 8.0

Tris	121.1	g
Distilled water	800	ml

The 1 M Tris was prepared by dissolving 121.1 g of Tris base in 800 ml of distilled water. The pH was adjusted to the desired value by adding conc. HCl (pH 8.0). The solution was cooled to room temperature before making final adjustment to the desired pH. The volume of the

solution was adjusted to 1 litter with distilled water and sterilized by autoclaving for 15 min at 15 lb/in^2.

10% Sodium dodecyl sulphate (SDS)

The stock solution of 10% SDS was prepared by dissolved 10 g of sodium dodecyl sulphate in 100 mL sterilized distilled water. Streilization is not required for the preparation of this stock solution.

TE buffer

10 mM Tris-HCl (pH 8)	10	ml
1 mM Na_2-EDTA (pH 8)	10	ml
Distilled water	980	ml

The solution was sterilized by autoclaving for 15 min at 15 lb/in^2.

1 mM Na_2-EDTA (pH 8)

EDTA	292.24	mg
Distilled water	700	ml

292.24 mg of EDTA was dissolved in distilled water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets. The volume was adjusted to 1 litter and sterilized by autoclaving for 15 min at 15 lb/in^2.

TE buffer + RNase

TE buffer	960	ml
RNase A (2 mg/ml)	100	μl

Alkaline phosphatase solution

Alkaline phosphatase	2.4	units
0.1 M Tris-HCl (pH 8.1)	1	ml

Nuclease P_1 solution

Nuclease P1	0.1	mg
40 mM CH_3COONa	500	μl
12 mM $ZnSO_4$ (pH 5.3)	500	μl

The solution was store at 4 °C.

40 mM CH_3COONa

CH_3COONa	3.2812	g
Distilled water	1	l

12 mM $ZnSO_4$

$ZnSO_4$ (anhydrous)	1.9376	g
Distilled water	1	l

Proteinase K

Proteinase K (Sigma)	4	mg
50 mM Tris-HCl (pH 7.5)	1	ml

Use freshly prepared solution.

RNase A solution

RNase A	20	mg
0.15 M NaCl	10	ml

Dissolve 20 mg of RNase A in 10 ml 0.15 M NaCl and heat at 95 °C for 5-10 minutes. Keep RNase A solution in -20 °C.

Phenol:Chloroform (1:1 v/v)

Crystalline phenol was liquidified in distilled water at 65 °C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle at 4 °C.

Ethidium bromide solution (10 mg/ml)

The ethidium bromide solution was prepared by dissolved 1 g of ethidium bromide in 100 ml distilled water.

Agarose gel

Agarose	0.8	g
TE buffer	100	ml

Reagents and buffer for DNA-DNA hybridization

Prehybridization solution

100xDenhardt solution	5	ml
10 mg/ml Salmon sperm DNA	1	ml
20xSSC	10	ml
Formamide	50	ml
Distilled water	34	ml

The solution was stored at 4 °C.

Hybridization solution

Prehybridization solution	100	ml
Dextran-sulfate	5	g

Solution I

Bovine serum albumin	0.25	g
Tritron X-100	50	μl
1xPBS	50	ml

Solution II

Streptavidin-POD	1	μl
Solution I	4	ml

Solution III

3,3',5,5'-Tetramethylbenzidine (TMB)	100	μl
(10 mg/ml in 10% DMSO)		
0.3% H_2O_2	100	μl
0.1 M Citric acid in 10% DMFO	2.5	ml
0.2 M Na_2HPO_4 in 10% DMFO	2.5	ml

100xDenhardt solution

Bovine serum albumin	2	ml
Polyvinylpyrrolidone	2	ml
Ficoll 400	2	ml
Distilled water	94	ml

10 mg/ml Salmon sperm DNA

Salmon sperm DNA (10 mg) was dissoved in 1 ml of 10 mM TE buffer pH 7.6. Then, the solution boiled for 10 min, immediately cooled in ice and sonicated for 3 min.

20xSSC

NaCl	175.3	g
Tri-sodium citrate.$2H_2O$	88.2	g
Distilled water	1	l

The 20xSSC was adjusted the pH to 7.0 with 10 M NaOH. The solution was sterilized by autoclaving for 15 min at 15 lb/in^2.

1xPBS

Na_2HPO_4	0.57	g
KH_2PO_4	0.11	g
NaCl	4.01	g
KCl	0.10	g

Distilled water	1	l

The 2xPBS was adjusted the pH to 7.0 with 1 N NaOH or 1 N HCl. The solution was sterilized by autoclaving for 15 min at 15 lb/in^2.

10 mg/ml TMB in 10% DMFO

TMB	0.001	g
DMFO	10	μl
Distilled water	90	μl

0.3% H_2O_2

3%	10	μl
Distilled water	90	μl

0.1 M Citric acid in 10% DMFO

Citric acid	0.05	g
DMFO	250	μl
Distilled water	2.25	ml

0.2 M Na_2HPO_4 in 10% DMFO

Na_2HPO_4	0.07	g
DMFO	250	μl
Distilled water	2.25	ml

2 M H_2SO_4

Conc. H_2SO_4	22	ml
Distilled water	178	ml

0.1 M $MgCl_2$ in 1xPBS (PBSM)

$MgCl_2$ (anhydrous)	0.95211	mg
10xPBS	10	ml
Distilled water	90	ml

$MgCl_2$ (0.95211 g) was dissolved in 10 ml of 10xPBS and adjusted the volume to 100 ml with sterilized distilled water.

10xPBS

Na_2HPO_4	5.6785	g
KH_2PO_4	1.0205	g
NaCl	40.0725	g
KCl	1.0065	g
Distilled water	1	l

The 10xPBS was adjusted the pH to 7.0 with 1 N NaOH or 1 N HCl. The solution was sterilized by autoclaving for 15 min at 15 lb/in^2.

3 M NaCl

NaCl	17.55	g
Distilled water	100	ml

17.55 g of NaCl was dissolved in distilled water and adjusted the volume to 100 ml. The solution was sterilized by autoclaving for 15 min at 15 lb/in^2.

APPENDIX III

16S rRNA GENE SEQUENCE SIMILARITY VALUES

Table 1 Similarity values (%) of marine actinobacteria in group I and *Micromonospora* species

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13
1 SH2-13	100.00	99.50	99.50	99.20	99.20	99.50	99.40	99.40	99.80	99.60	99.20	99.50	100.00
2 AN5-12	99.50	100.00	100.00	99.70	99.70	100.00	99.90	99.90	99.60	99.60	99.60	99.30	99.50
3 AN5-55	99.50	100.00	100.00	99.70	99.70	100.00	99.90	99.90	99.60	99.60	99.60	99.30	99.50
4 MA3-23	99.20	99.70	99.70	100.00	100.00	99.70	99.60	99.60	99.40	99.30	99.40	99.10	99.20
5 S18-3	99.20	99.70	99.70	100.00	100.00	99.70	99.60	99.60	99.40	99.30	99.40	99.10	99.20
6 ST01-03	99.50	100.00	100.00	99.70	99.70	100.00	99.90	99.90	99.60	99.60	99.60	99.30	99.50
7 ST01-08	99.40	99.90	99.90	99.60	99.60	99.90	100.00	100.00	99.60	99.50	99.60	99.20	99.40
8 ST01-09	99.40	99.90	99.90	99.60	99.60	99.90	100.00	100.00	99.60	99.50	99.60	99.20	99.40
9 SP206-17T	99.80	99.60	99.60	99.40	99.40	99.60	99.60	99.60	100.00	99.70	99.30	99.30	99.80
10 *M. marina* JSM1-1^{T}	99.60	99.60	99.60	99.30	99.30	99.60	99.50	99.50	99.70	100.00	99.20	99.10	99.60
11 *M. aurantiaca* DSM 43813^{T}	99.20	99.60	99.60	99.40	99.40	99.60	99.60	99.60	99.30	99.20	100.00	99.00	99.20
12 *M. coxensis* 2-30-b/28^{T}	99.50	99.30	99.30	99.10	99.10	99.30	99.20	99.20	99.30	99.10	99.00	100.00	99.50
13 *M. maritima* NBRC 108767^{T}	100.00	99.50	99.50	99.20	99.20	99.50	99.40	99.40	99.80	99.60	99.20	99.50	100.00

Table 1 Similarity values (%) of marine actinobacteria in group I and *Micromonospora* species (continued)

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 S3-1	100.00	98.40	98.20	98.20	98.50	98.20	98.40	98.30	98.50	98.40	98.00	98.90	98.50	98.80	98.20
2 AN5-1	98.40	100.00	99.00	98.90	98.80	99.00	99.60	98.60	98.70	99.60	98.80	98.50	98.70	98.50	98.40
3 AN6-3	98.20	99.00	100.00	99.90	98.90	100.00	98.90	99.00	99.10	98.70	99.70	98.60	98.50	98.50	98.80
4 AN6-30	98.20	98.90	99.90	100.00	98.80	99.90	98.80	98.90	99.00	98.60	99.60	98.50	98.40	98.40	98.70
5 SH2-7	98.50	98.80	98.90	98.80	100.00	98.90	98.70	99.20	99.20	98.50	98.60	98.90	99.50	98.50	98.90
6 S8-07	98.20	99.00	100.00	99.90	98.90	100.00	98.90	99.00	99.10	98.70	99.70	98.60	98.50	98.50	98.80
7 S8-12	98.40	99.60	98.90	98.80	98.70	98.90	100.00	98.50	98.70	99.60	98.60	98.30	98.90	98.50	98.50
8 SH3-2	98.30	98.60	99.00	98.90	99.20	99.00	98.50	100.00	99.10	98.30	98.80	98.50	98.80	98.20	98.80
9 SH4-3	98.50	98.70	99.10	99.00	99.20	99.10	98.70	99.10	100.00	98.40	98.90	98.90	98.80	98.60	99.40
10 *M. echinospora* ATCC 15837^T	98.40	99.60	98.70	98.60	98.50	98.70	99.60	98.30	98.40	100.00	98.50	98.20	98.70	98.50	98.20
11 *M. echinaurantiaca* DSM 43904^T	98.00	98.80	99.70	99.60	98.60	99.70	98.60	98.80	98.90	98.50	100.00	98.40	98.20	98.50	98.90
12 *M. narathiwatensis* BTG4-1^T	98.90	98.50	98.60	98.50	98.90	98.60	98.30	98.50	98.90	98.20	98.40	100.00	98.50	98.50	98.40
13 *M. endolithica* DSM 44398^T	98.50	98.70	98.50	98.40	99.50	98.50	98.90	98.80	98.80	98.70	98.20	98.50	100.00	98.40	98.50
14 *M. nigra* DSM 43818^T	98.80	98.50	98.50	98.40	98.50	98.50	98.50	98.20	98.60	98.50	98.50	98.50	98.40	100.00	98.50
15 *M. pallida* DSM 43817^T	98.20	98.40	98.80	98.70	98.90	98.80	98.50	98.80	99.40	98.20	98.90	98.40	98.50	98.50	100.00

Table 2 Similarity values (%) of marine actinobacteria in group II and *Salinispora* species

Taxa	1	2	3	4	5
1 SP207-05	100.00	100.00	100.00	100.00	99.40
2 SP207-08	100.00	100.00	100.00	100.00	99.40
3 SP209-09	100.00	100.00	100.00	100.00	99.40
4 *S. arenicola* CNH-643^T	100.00	100.00	100.00	100.00	99.40
5 *S. tropica* CNB-440^T	99.40	99.40	99.40	99.40	100.00

Table 3 Similarity values (%) of marine actinobacteria in group III and *Verrucosispora* species

Taxa	1	2	3	4	5	6	7	8	9	10	11
1 SP03-01	100.00	99.30	99.80	98.60	99.40	99.30	99.20	98.00	99.20	99.60	99.10
2 SP206-02	99.30	100.00	99.10	98.00	98.80	99.80	98.60	97.40	99.40	98.90	99.50
3 SP206-03	99.80	99.10	100.00	98.50	99.40	99.10	99.10	98.20	99.10	99.70	99.00
4 SP03-05	98.60	98.00	98.50	100.00	98.00	98.00	97.90	96.70	97.90	98.30	97.80
5 *V. sediminis* MS426^T	99.40	98.80	99.40	98.00	100.00	98.80	98.80	97.70	98.80	99.10	98.70
6 *V. gifhornensis* DSM 44337^T	99.30	99.80	99.10	98.00	98.80	100.00	98.60	97.40	99.30	98.90	99.50
7 *V. lutea* YIM 013^T	99.20	98.60	99.10	97.90	98.80	98.60	100.00	98.40	98.80	98.80	98.70
8 *V. qiuiae* RtIII47^T	98.00	97.40	98.20	96.70	97.70	97.40	98.40	100.00	97.60	98.00	97.50
9 *V. fiedleri* MG-37^T	99.20	99.40	99.10	97.90	98.80	99.30	98.80	97.60	100.00	98.80	99.40
10 *V. wenchangensis* 234402^T	99.60	98.90	99.70	98.30	99.10	98.90	98.80	98.00	98.80	100.00	98.80
11 *V. maris* AB-18-032^T	99.10	99.50	99.00	97.80	98.70	99.50	98.70	97.50	99.40	98.80	100.00

Table 4 Similarity values (%) of marine actinobacteria in group IV and *Actinomadura* species

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
1 TV1-14	100.00	98.90	98.20	98.50	97.50	97.80	98.20	97.40	97.60	97.60	97.20	97.50
2 *A. macra* IFO 14102^T	98.90	100.00	98.00	98.20	97.20	97.40	98.30	97.30	97.50	97.90	97.60	97.80
3 *A. meyerii* A288^T	98.20	98.00	100.00	98.80	97.70	97.90	98.10	97.30	97.30	97.90	97.70	97.90
4 *A. bangladeshensis* 3-46-b3^T	98.50	98.20	98.80	100.00	98.50	98.20	98.30	97.60	97.60	97.90	97.60	97.90
5 *A. chokoriensis* 3-45-a/11^T	97.50	97.20	97.70	98.50	100.00	97.20	97.20	96.70	96.40	96.70	96.40	96.70
6 *A. napierensis* B60^T	97.80	97.40	97.90	98.20	97.20	100.00	97.70	97.70	96.80	97.60	97.30	97.30
7 *A. madurae* DSM 43067^T	98.20	98.30	98.10	98.30	97.20	97.70	100.00	97.50	97.30	97.90	97.70	97.60
8 *A. latina* DSM 43382^T	97.40	97.30	97.30	97.60	96.70	97.70	97.50	100.00	96.70	97.10	96.70	96.40
9 *A. pelletieri* JCM 3388^T	97.60	97.50	97.30	97.60	96.40	96.80	97.30	96.70	100.00	97.70	97.50	97.70
10 *A. formosensis* JCM 7474^T	97.60	97.90	97.90	97.90	96.70	97.60	97.90	97.10	97.70	100.00	98.00	98.20
11 *A. mexicana* A290^T	97.20	97.60	97.70	97.60	96.40	97.30	97.70	96.70	97.50	98.00	100.00	99.10
12 *A. glauciflava* AS 4.1202^T	97.50	97.80	97.90	97.90	96.70	97.30	97.60	96.40	97.70	98.20	99.10	100.00

Table 5 Similarity values (%) of marine actinobacteria in group V and *Nocardia* species

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
1 ST01-07	100.00	98.80	98.50	98.50	98.40	97.80	97.60	96.80	97.10	97.10	97.30	97.20
2 *N. araoensis* IFM 0575^T	98.80	100.00	99.30	99.20	99.50	98.30	98.20	96.40	98.20	98.10	97.00	98.40
3 *N. beijingensis* AS4.1521^T	98.50	99.30	100.00	99.00	99.00	98.60	97.70	96.20	97.90	97.90	97.10	98.50
4 *N. niwae* W9241^T	98.50	99.20	99.00	100.00	99.10	97.90	98.50	96.80	98.00	97.80	97.10	98.00
5 *N. arthritidis* IFM 10035^T	98.40	99.50	99.00	99.10	100.00	98.00	97.90	96.10	98.70	98.50	96.70	98.70
6 *N. pneumoniae* IFM 0784^T	97.80	98.30	98.60	97.90	98.00	100.00	98.60	96.50	96.80	96.80	96.00	97.40
7 *N. amamiensis* TT 00-78^T	97.60	98.20	97.70	98.50	97.90	98.60	100.00	96.90	96.70	96.50	95.80	96.70
8 *N. endophytica* KLBMP 1256^T	96.80	96.40	96.20	96.80	96.10	96.50	96.90	100.00	96.80	96.70	97.00	95.10
9 *N. exalbida* IFM 0803^T	97.10	98.20	97.90	98.00	98.70	96.80	96.70	96.80	100.00	99.60	96.40	97.80
10 *N. gamkensis* CZH20^T	97.10	98.10	97.90	97.80	98.50	96.80	96.50	96.70	99.60	100.00	96.30	97.50
11 *N. harenae* WS-26^T	97.30	97.00	97.10	97.10	96.70	96.00	95.80	97.00	96.40	96.30	100.00	97.50
12 *N. asiatica* IFM 0245^T	97.20	98.40	98.50	98.00	98.70	97.40	96.70	95.10	97.80	97.50	97.50	100.00

Table 6 Similarity values (%) of marine actinobacteria in group VI and *Pseudonocardia* species

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
1 S15-3	100.00	99.80	99.80	99.90	99.60	98.50	97.80	97.40	97.10	97.50	97.00	97.20
2 *P. carboxydivorans* Y8^T	99.80	100.00	99.60	99.70	99.50	98.40	97.60	97.20	96.90	97.30	96.80	97.10
3 *P. alni* DSM 44104^T	99.80	99.60	100.00	99.70	99.50	98.40	97.60	97.20	96.90	97.30	96.80	97.10
4 *P. antarctica* DVS 5a1^T	99.90	99.70	99.70	100.00	99.60	98.40	97.70	97.30	97.00	97.40	96.90	97.10
5 *P. tropica* YIM 61452^T	99.60	99.50	99.50	99.60	100.00	98.40	97.50	97.10	96.90	97.30	97.10	97.20
6 *P. parietis* 04-St-002^T	98.50	98.40	98.40	98.40	98.40	100.00	96.80	96.40	96.70	97.10	96.50	96.50
7 *P. autotrophica* IMSNU 20050^T	97.80	97.60	97.60	97.70	97.50	96.80	100.00	99.60	99.00	97.90	96.20	95.20
8 *P. compacta* IMSNU 20111^T	97.40	97.20	97.20	97.30	97.10	96.40	99.60	100.00	98.60	97.50	96.00	95.00
9 *P. kongjuensis* LM 157^T	97.10	96.90	96.90	97.00	96.90	96.70	99.00	98.60	100.00	97.90	96.20	95.60
10 *P. ammonioxydans* H9^T	97.50	97.30	97.30	97.40	97.30	97.10	97.90	97.50	97.90	100.00	95.30	95.20
11 *P. eucalypti* EUM 374^T	97.00	96.80	96.80	96.90	97.10	96.50	96.20	96.00	96.20	95.30	100.00	96.80
12 *P. benzenivorans* B5^T	97.20	97.10	97.10	97.10	97.20	96.50	95.20	95.00	95.60	95.20	96.80	100.00

Table 7 Similarity values (%) of marine actinobacteria in group VII and *Actinomycetospora* species

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
1 TV1-16	100.00	98.20	99.10	99.00	98.90	98.90	98.70	98.30	98.30	97.80	97.80	97.50
2 SH2-15	98.20	100.00	98.00	97.90	98.90	98.00	98.70	97.80	98.20	96.90	96.90	96.70
3 SH3-3	99.10	98.00	100.00	99.10	98.50	99.20	98.30	98.60	98.70	97.40	97.60	97.30
4 *A.* TT00-04^T	99.00	97.90	99.10	100.00	98.40	98.40	98.30	98.30	98.30	97.60	97.80	97.20
5 *A. straminea* IY07-55^T	98.90	98.90	98.50	98.40	100.00	99.00	99.70	98.70	98.50	97.40	97.50	97.30
6 *A. chlora* TT07I-57^T	98.90	98.00	99.20	98.40	99.00	100.00	99.00	98.80	98.80	97.40	97.60	97.30
7 *A. chibensis* TT04-21^T	98.70	98.70	98.30	98.30	99.70	99.00	100.00	98.60	98.40	97.40	97.40	97.20
8 *A. succinea* TT00-49^T	98.30	97.80	98.60	98.30	98.70	98.80	98.60	100.00	98.30	96.90	97.00	96.70
9 *A. cinnamomea* IY07-53^T	98.30	98.20	98.70	98.30	98.50	98.80	98.40	98.30	100.00	97.20	97.20	97.00
10 *A. chiangmaiensis* YIM 0006^T	97.80	96.90	97.40	97.60	97.40	97.40	97.40	96.90	97.20	100.00	98.90	98.50
11 *A. corticicola* 014-5^T	97.80	96.90	97.60	97.80	97.50	97.60	97.40	97.00	97.20	98.90	100.00	98.90
12 *A. rishiriensis* RI109-Li102^T	97.50	96.70	97.30	97.20	97.30	97.30	97.20	96.70	97.00	98.50	98.90	100.00

Table 8 Similarity values (%) of marine actinobacteria in group VIII and *Streptomyces* species

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13
1 S6-1	100.00	93.20	98.80	97.10	96.00	100.00	98.90	97.20	99.40	97.40	97.20	96.30	98.60
2 AN5-16	93.20	100.00	92.80	93.50	91.70	93.20	92.90	93.30	93.20	93.10	93.00	92.20	93.50
3 S20-7	98.80	92.80	100.00	97.20	96.40	98.80	99.50	96.70	98.70	97.50	97.20	96.80	98.60
4 S8-04	97.10	93.50	97.20	100.00	96.40	97.10	97.30	99.10	97.10	98.80	98.90	96.70	97.50
5 SH2-1	96.00	91.70	96.40	96.40	100.00	96.00	96.50	96.50	96.50	96.90	97.00	99.30	96.50
6 *S. fradiae* NBRC 12214^T	100.00	93.20	98.80	97.10	96.00	100.00	98.90	97.20	99.40	97.40	97.20	96.30	98.60
7 *S. hyderabadensis* OU-40^T	98.90	92.90	99.50	97.30	96.50	98.90	100.00	96.80	98.90	97.60	97.40	96.80	98.80
8 *S. qinglanensis* 172205^T	97.20	93.30	96.70	99.10	96.50	97.20	96.80	100.00	97.10	98.20	98.30	96.80	97.10
9 *S. coelescens* DSM 40421^T	99.40	93.20	98.70	97.10	96.50	99.40	98.90	97.10	100.00	97.40	97.20	96.80	98.70
10 *S. abikoensis* NBRC 13860^T	97.40	93.10	97.50	98.80	96.90	97.40	97.60	98.20	97.40	100.00	99.40	97.20	97.60
11 *S. varsoviensis* NRRL B-3589^T	97.20	93.00	97.20	98.90	97.00	97.20	97.40	98.30	97.20	99.40	100.00	97.30	97.50
12 *S. spongiae* Sp080513SC-24^T	96.30	92.20	96.80	96.70	99.30	96.30	96.80	96.80	96.80	97.20	97.30	100.00	96.80
13 *S. coeruleofuscus* NBRC 12757^T	98.60	93.50	98.60	97.50	96.50	98.60	98.80	97.10	98.70	97.60	97.50	96.80	100.00

APPENDIX IV

IR AND NMR SPECTRAL DATA

Compound S6.1A (urdamycinone E)

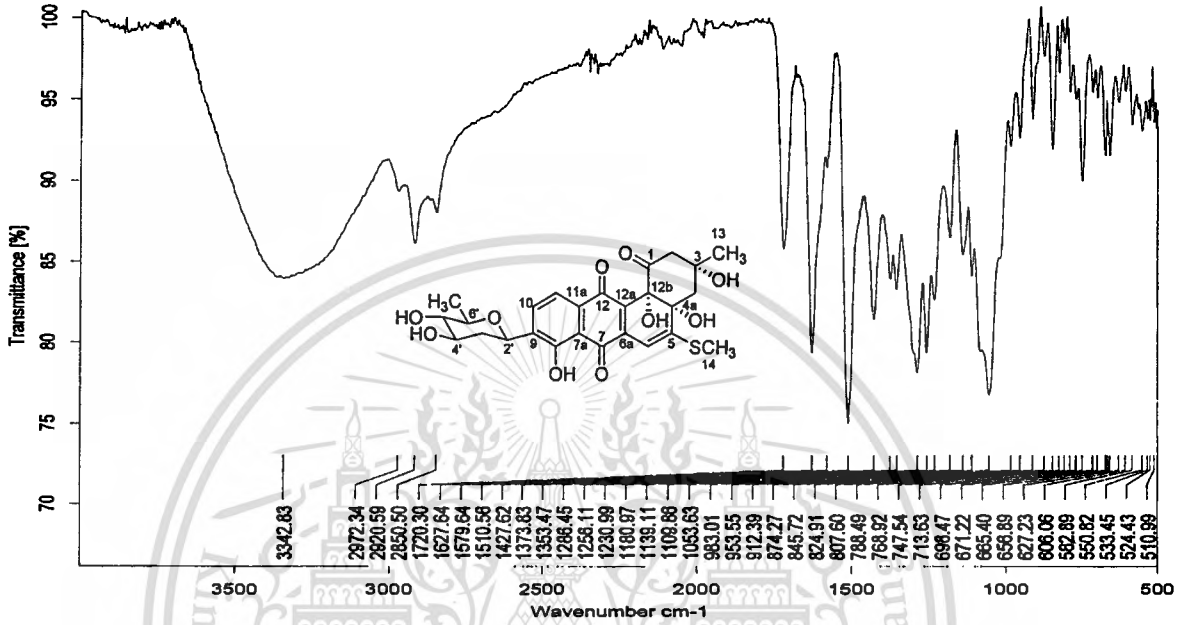


Fig. 1 IR spectrum of compound S6.1A

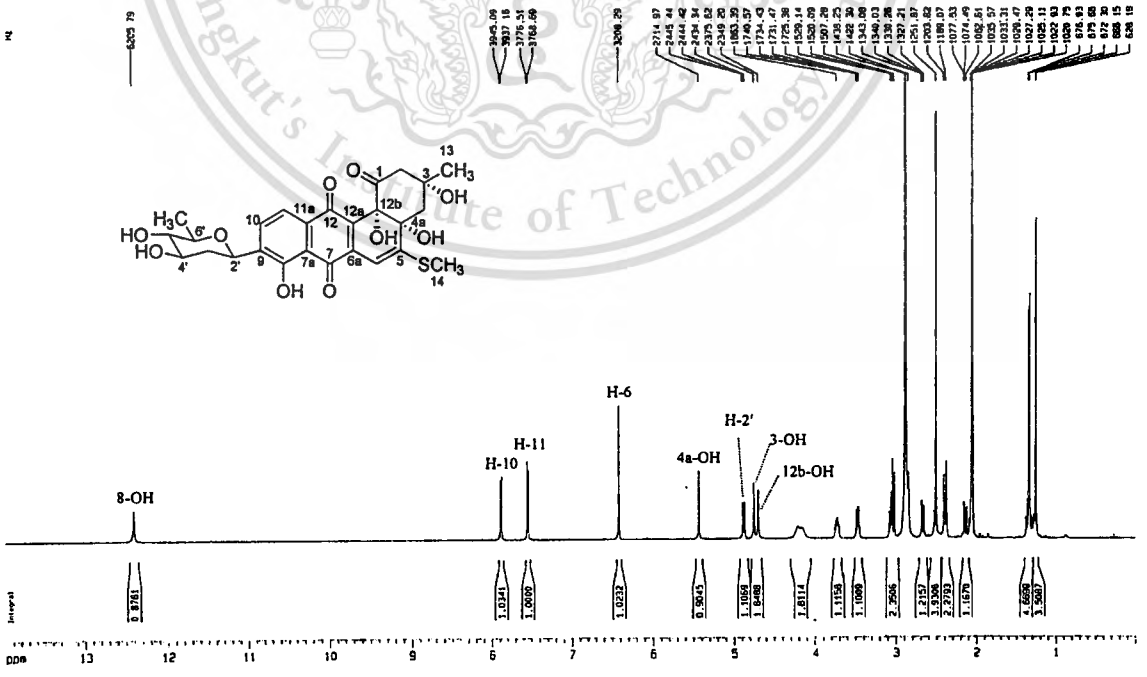


Fig. 2 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1A

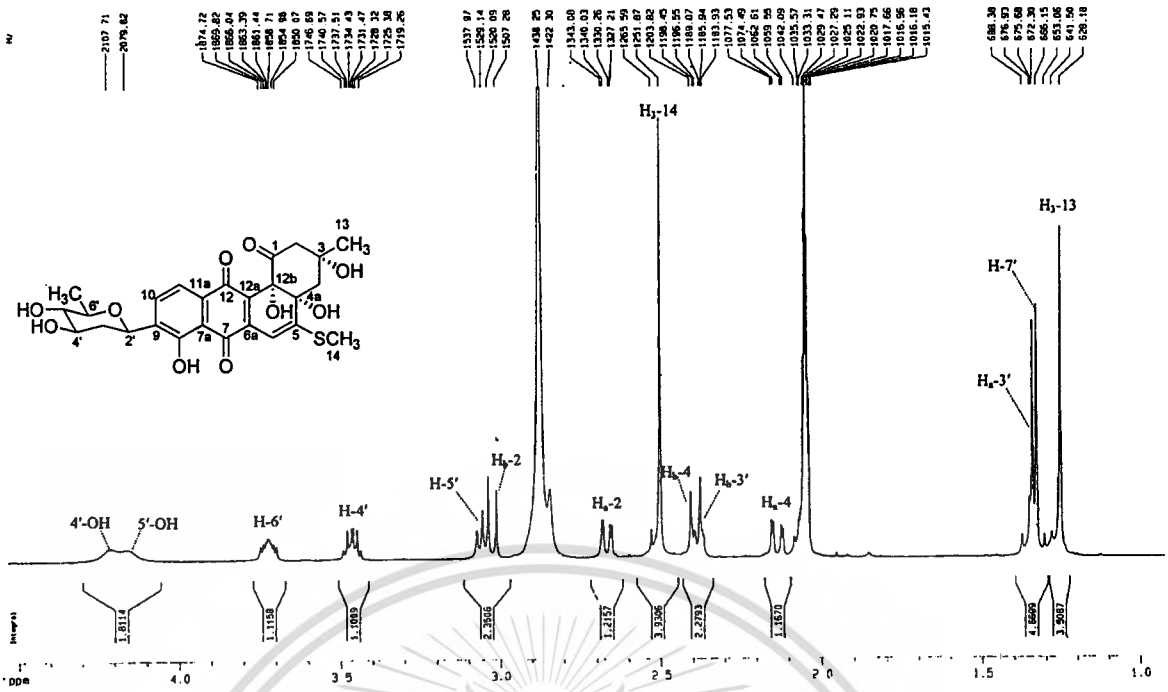


Fig. 3 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1A (expansion of Fig. 2)

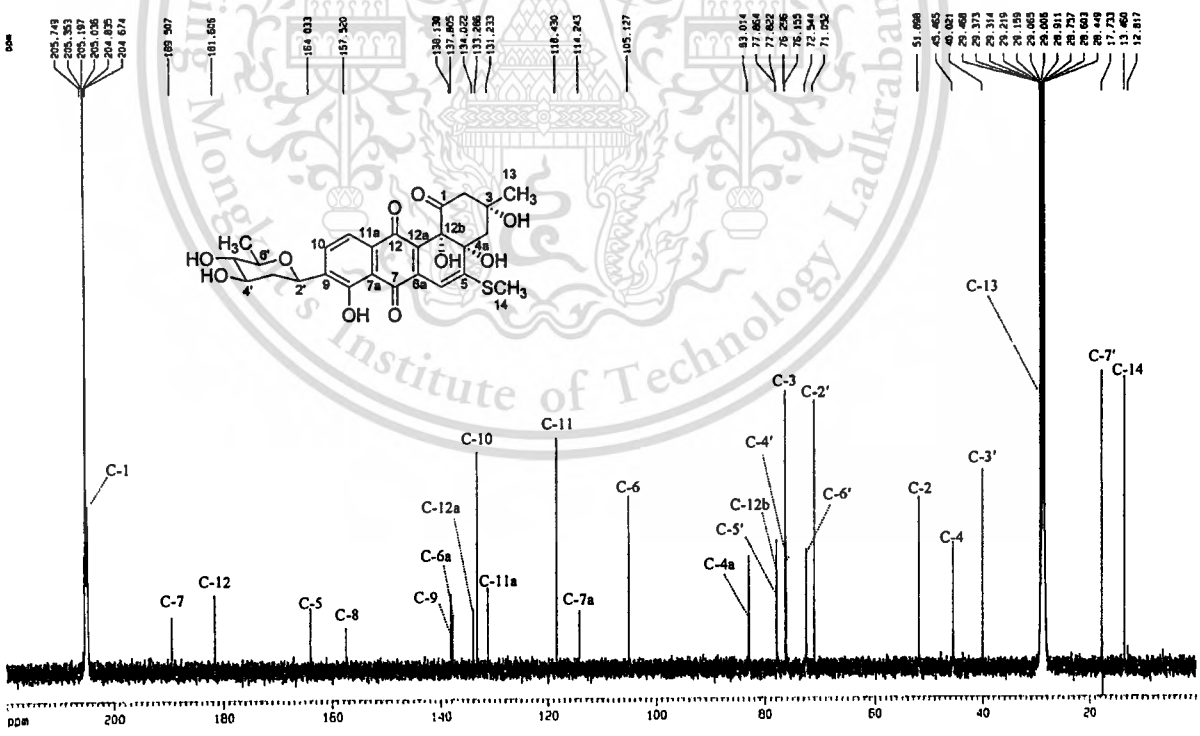


Fig. 4 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S6.1A

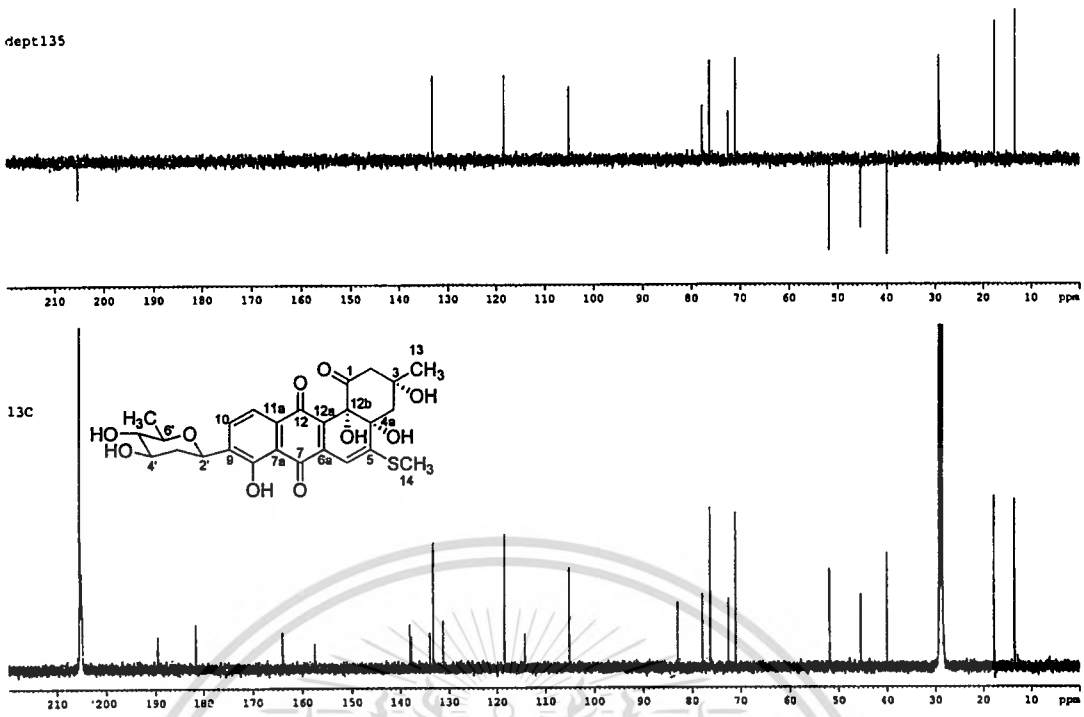


Fig. 5 Dept 135 spectrum (500 MHz, acetone-d_6) of compound S6.1A

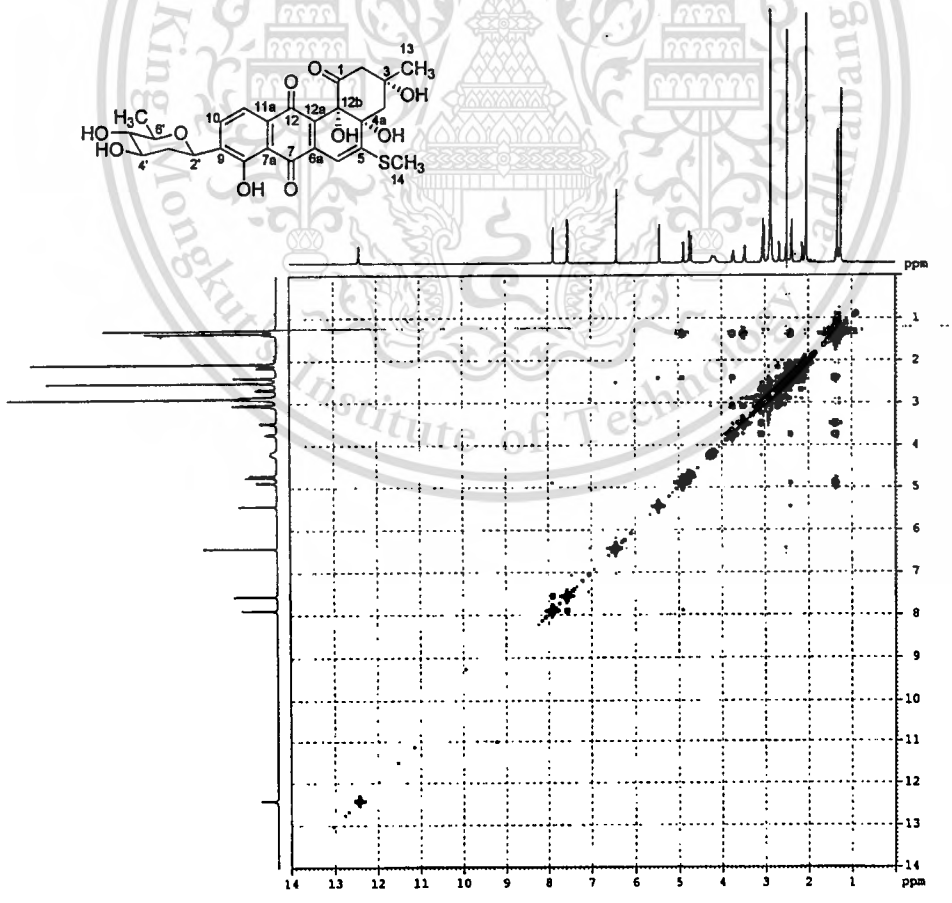


Fig. 6 COSY spectrum (500 MHz, acetone-d_6) of compound S6.1

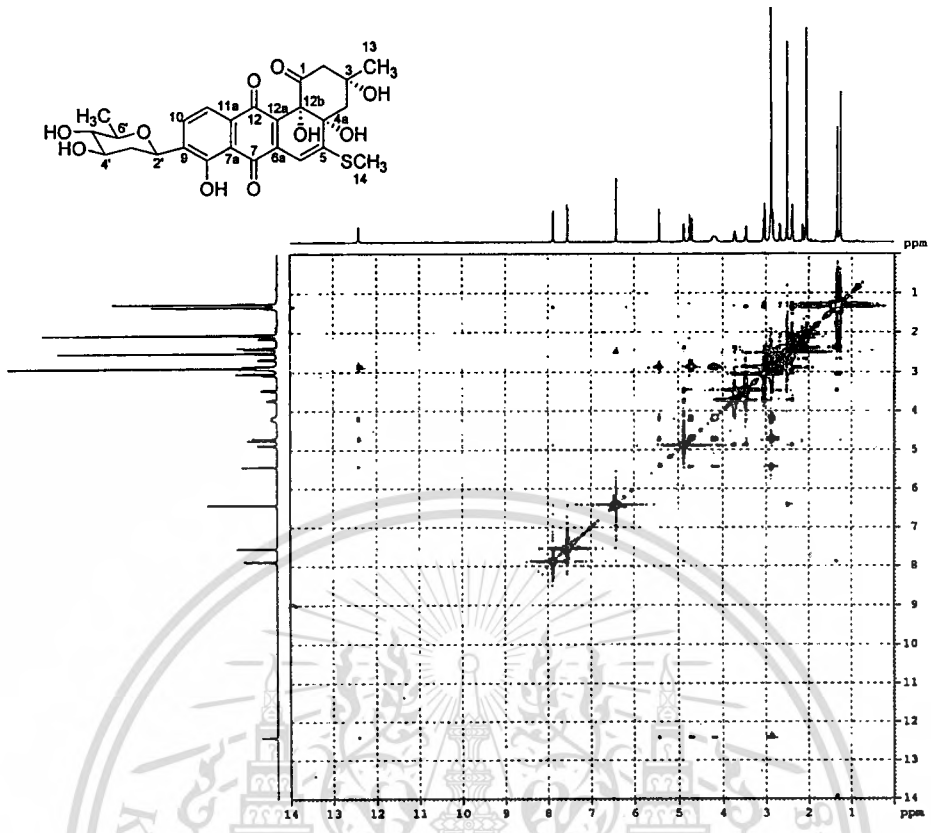


Fig. 7 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1A

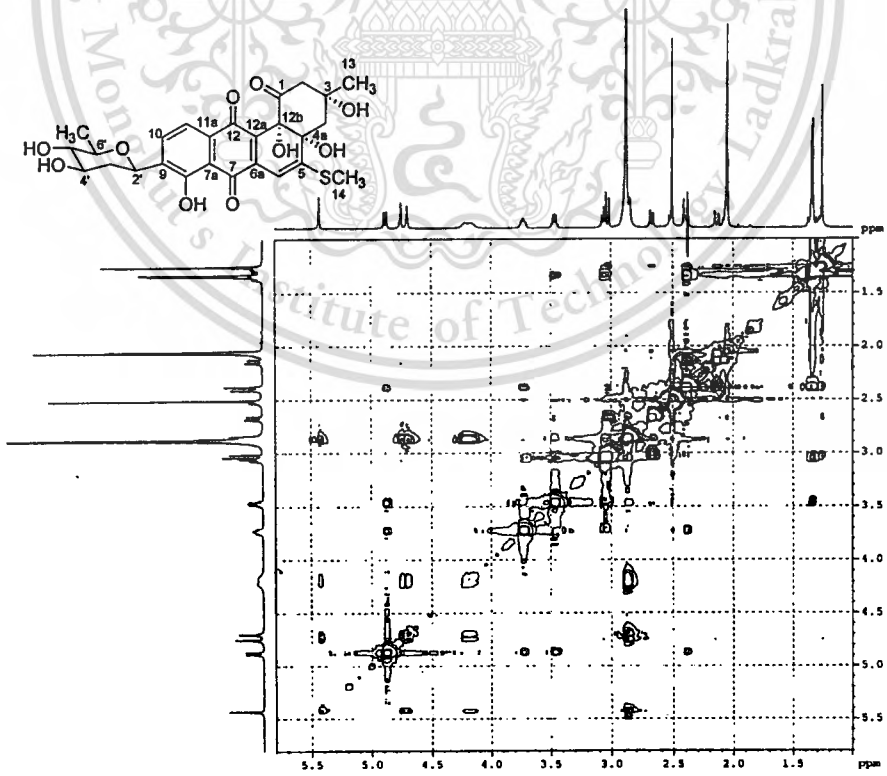


Fig. 8 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1A (expansion of Fig. 7)

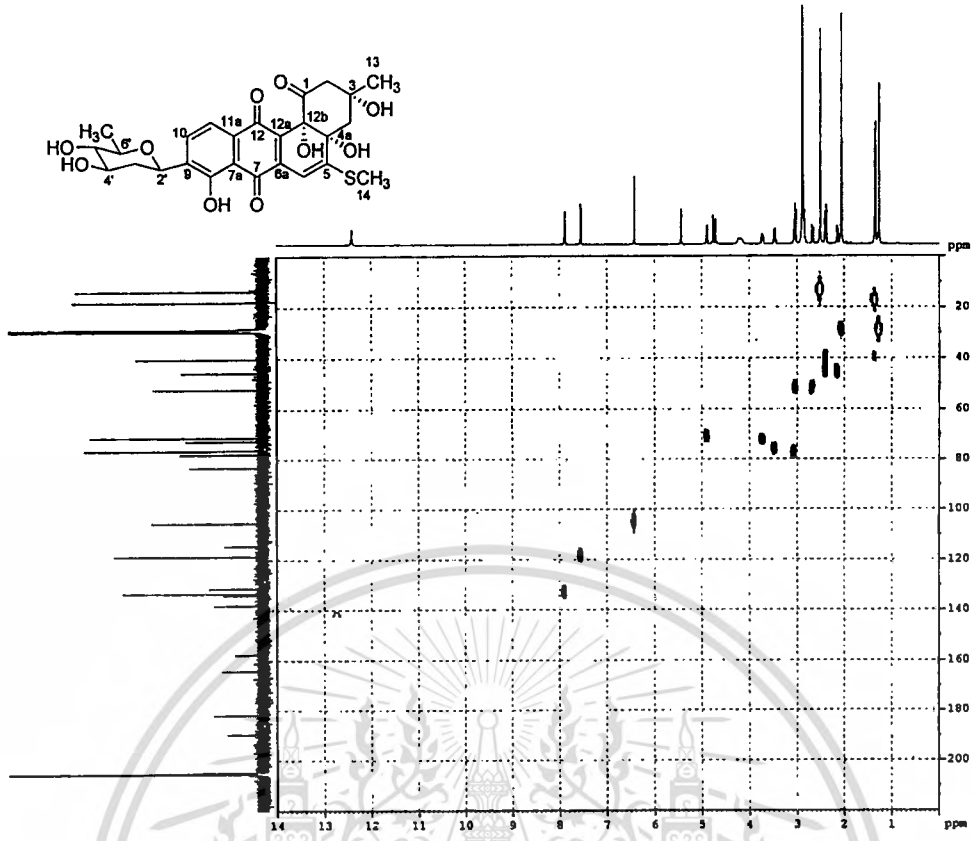
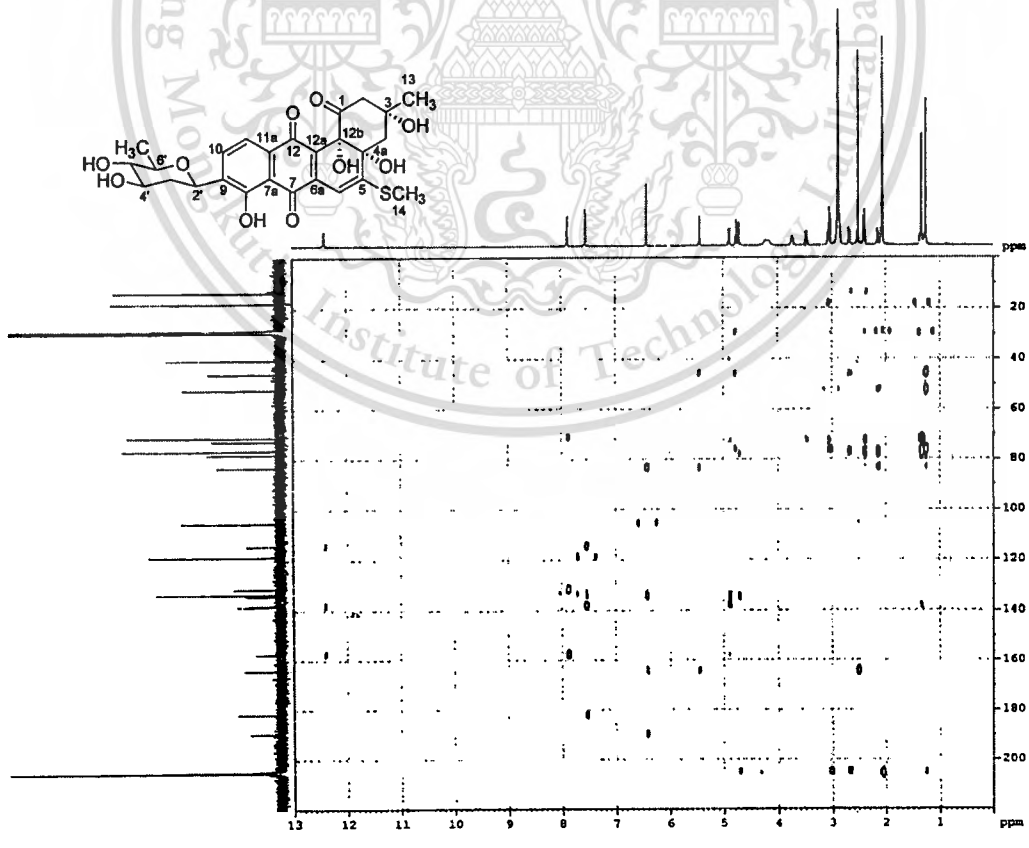


Fig. 9 HMQC spectrum (500 MHz, acetone-d_6) of compound S6.1A



H

Fig. 10 MBC spectrum (500 MHz, acetone-d_6) of compound S6.1A

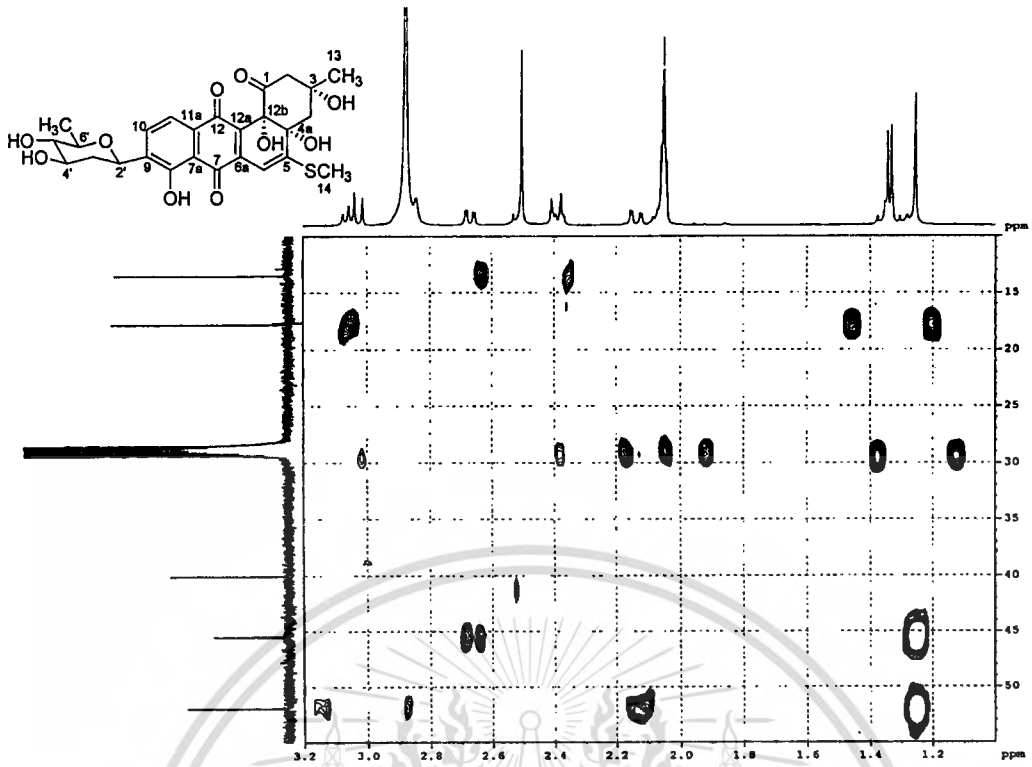


Fig. 11 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1A (expansion of Fig. 10)

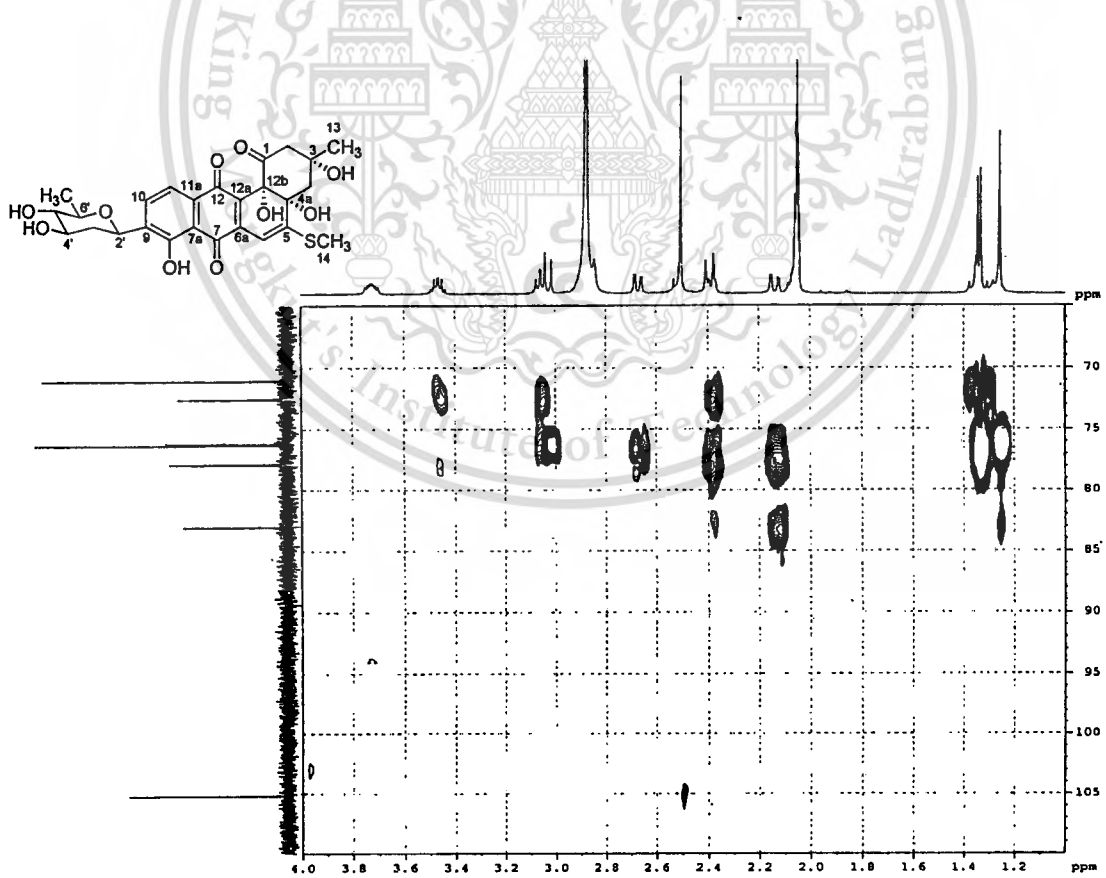


Fig. 12 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1A (expansion of Fig. 10)

Compound S6.1B (urdamycinone G)

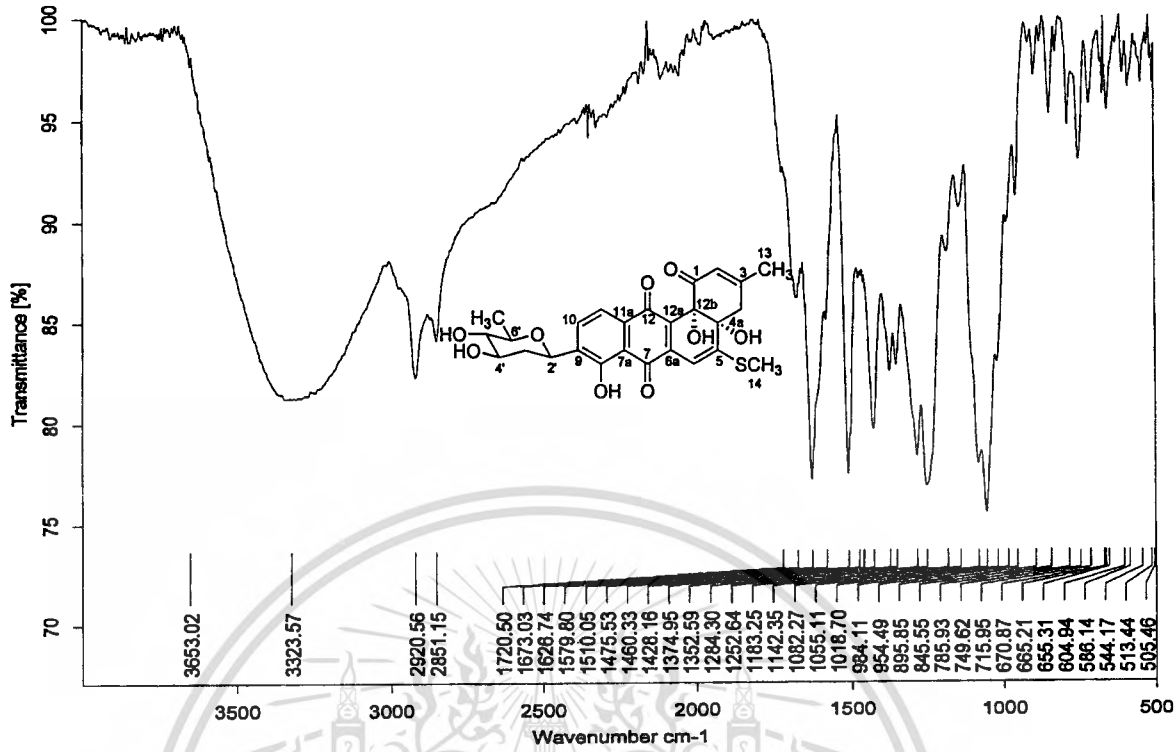


Fig. 13 IR spectrum of compound S6.1B

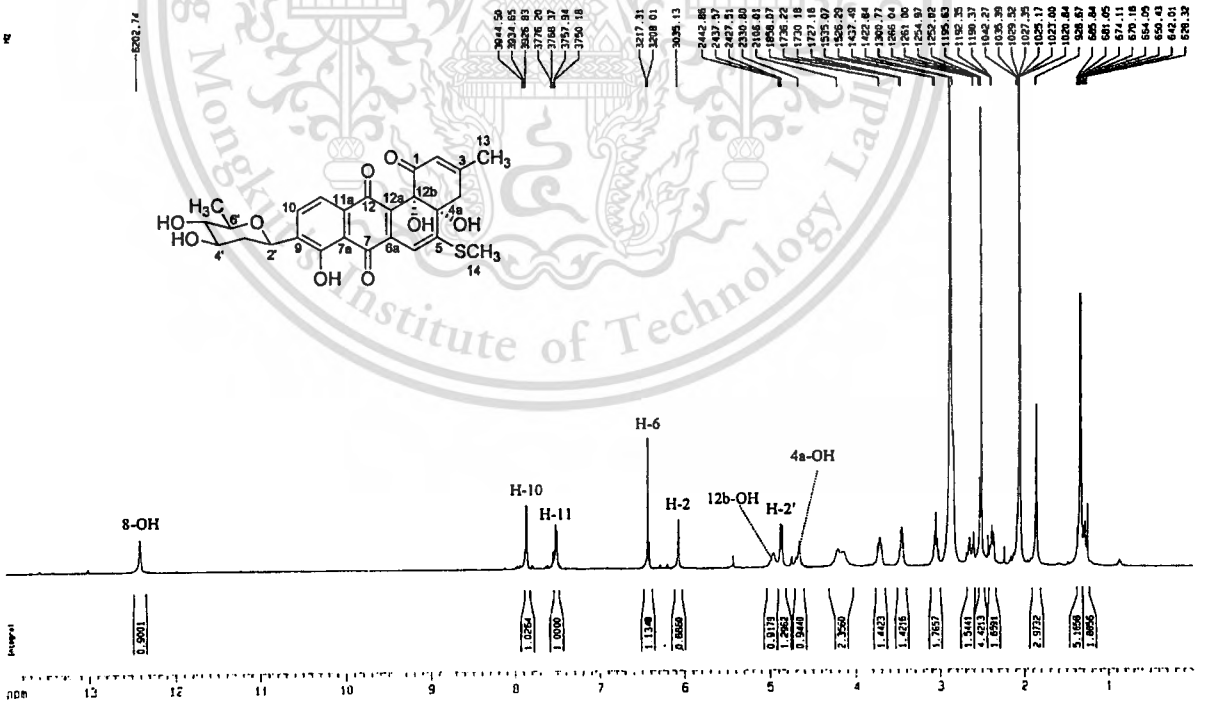


Fig. 14 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1B

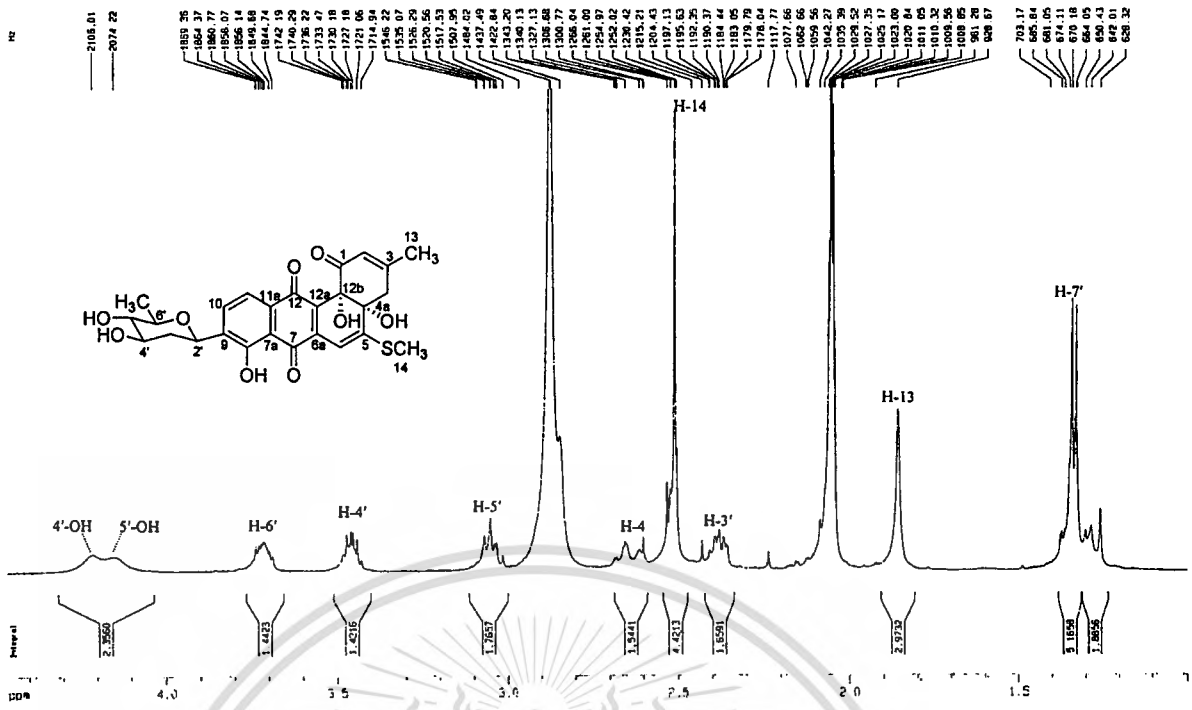


Fig. 15 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1B (expansion of Fig. 14)

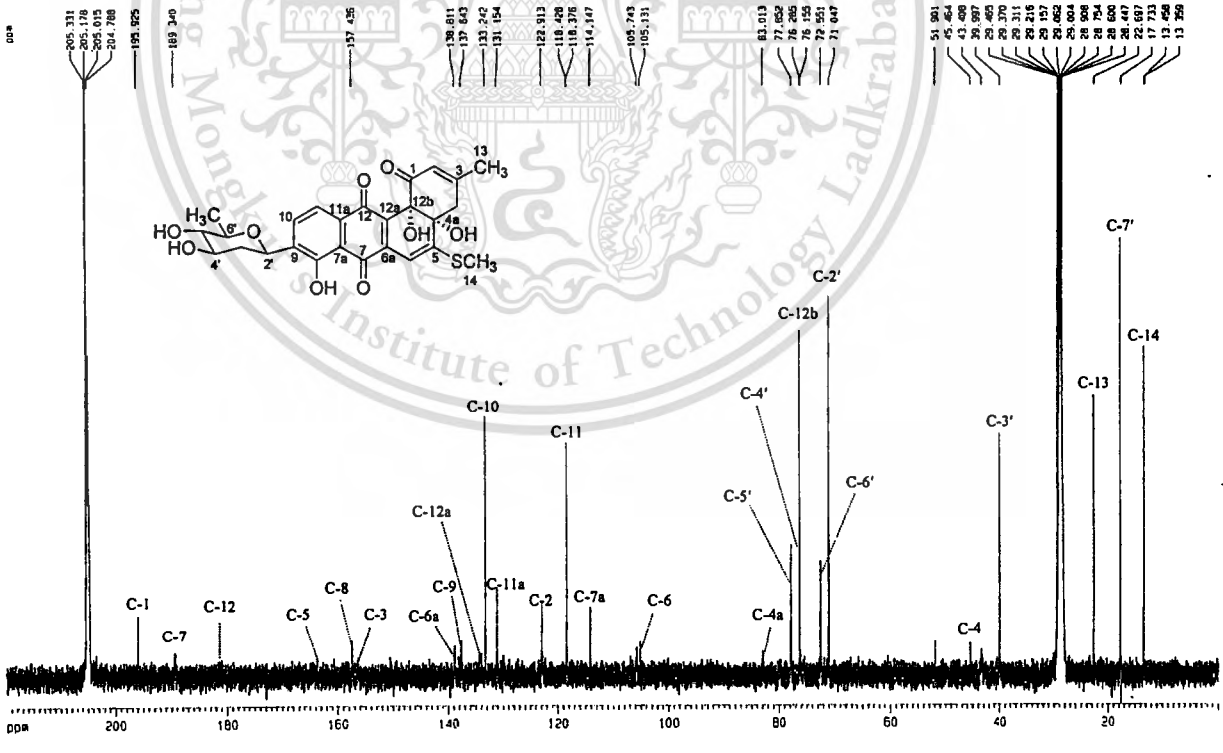


Fig. 16 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S6.1B

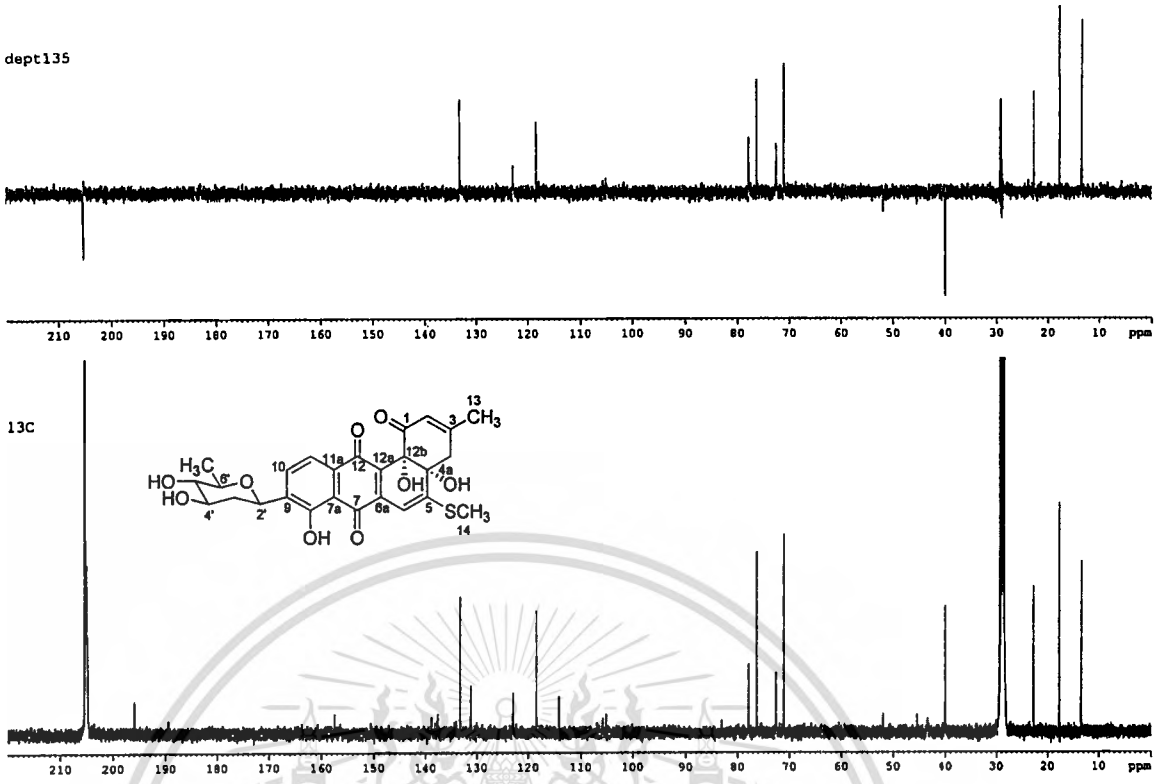


Fig. 17 DEPT spectrum (500 MHz, acetone-d_6) of compound S6.1B

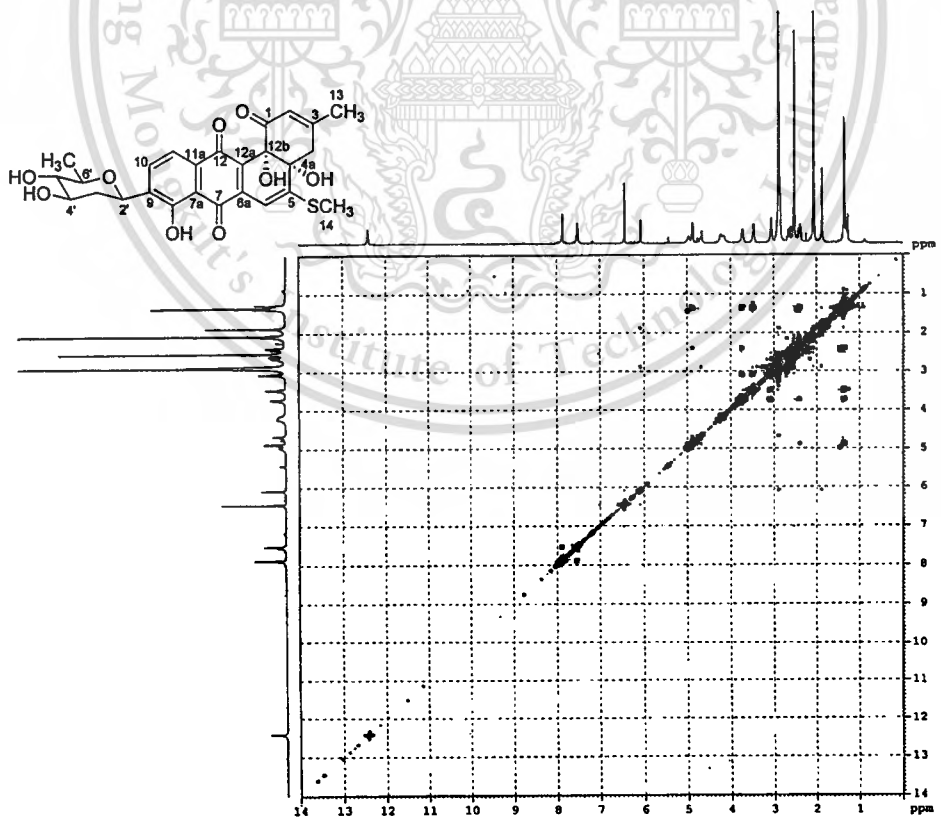


Fig. 18 COSY spectrum (500 MHz, acetone-d_6) of compound S6.1B

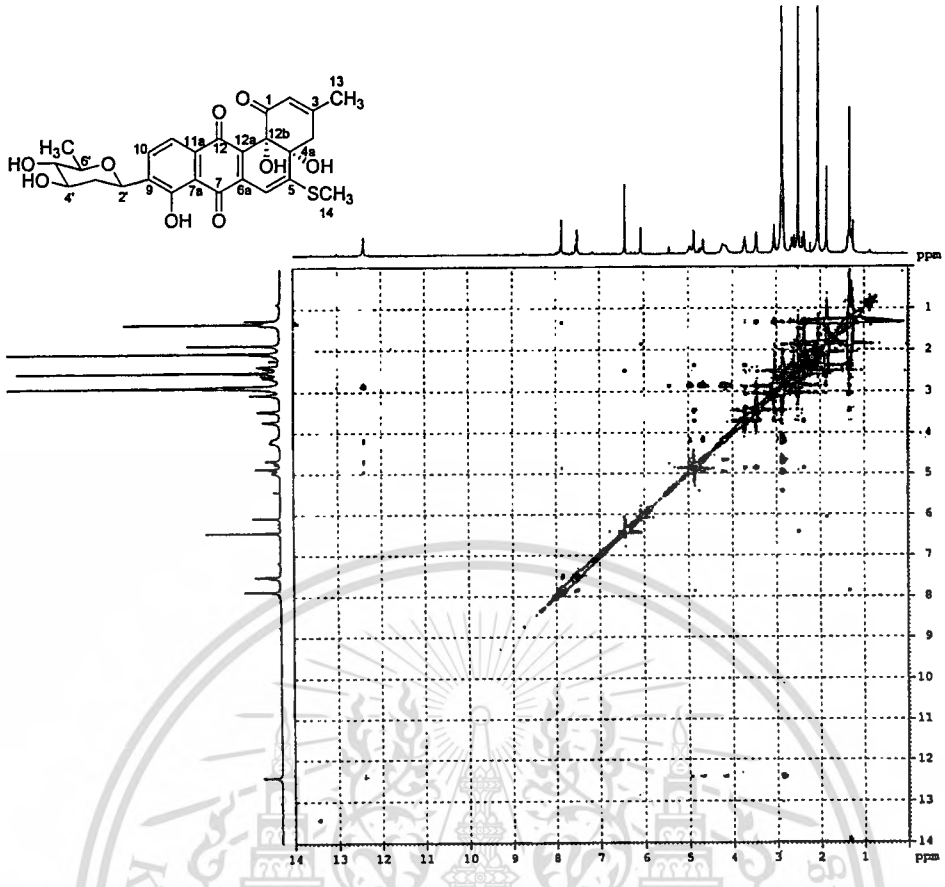


Fig. 19 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1B

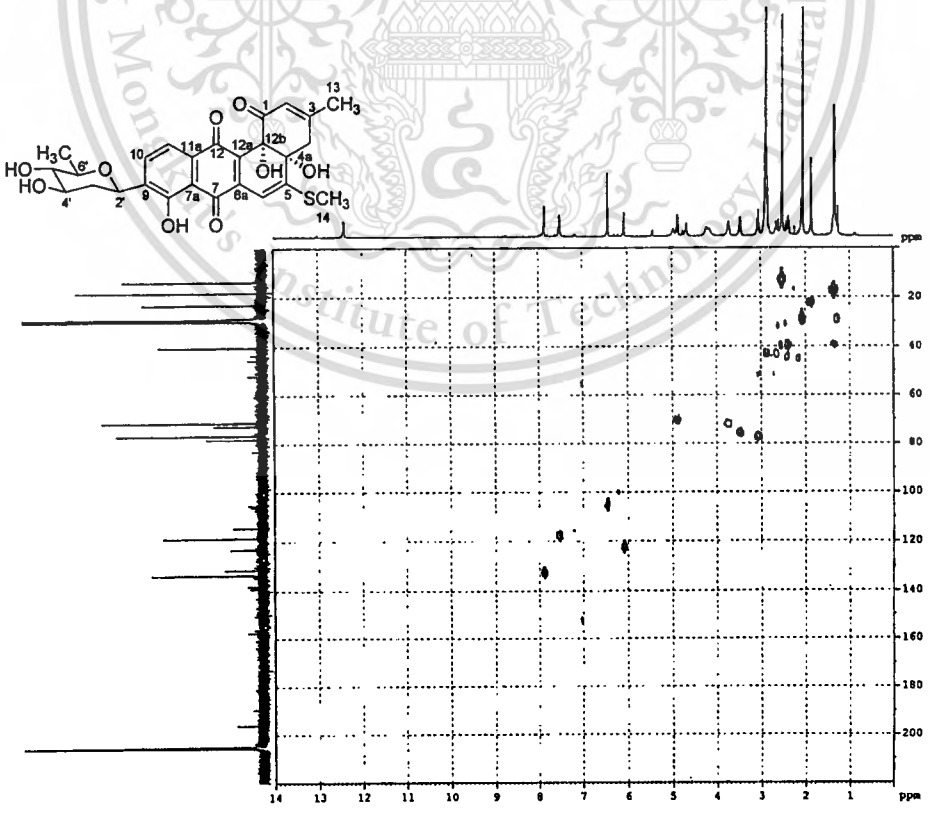


Fig. 20 HMQC spectrum (500 MHz, acetone-d_6) of compound S6.1B

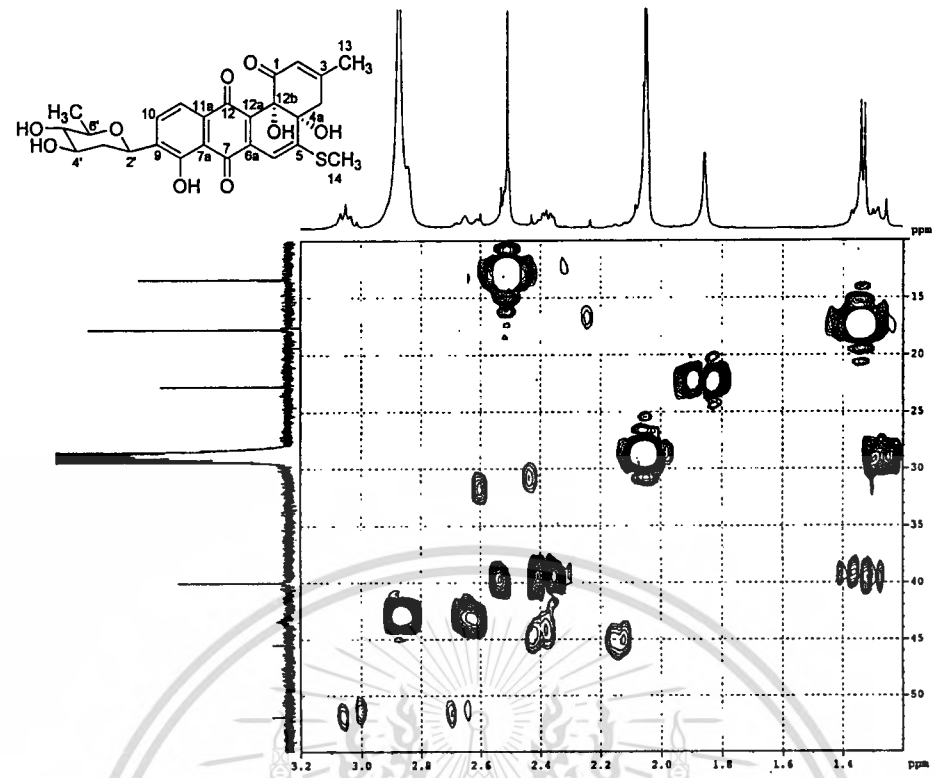


Fig. 21 HMQC spectrum (500 MHz, acetone-d_6) of compound S6.1B (expansion of Fig. 20)

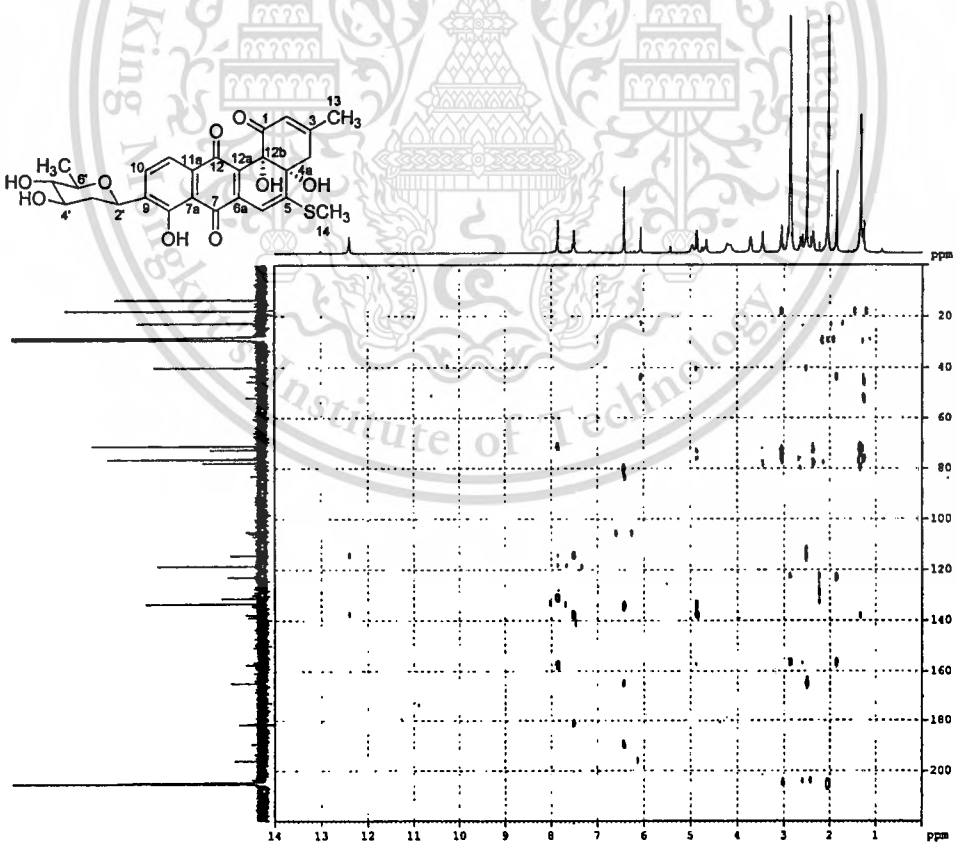


Fig. 22 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1B

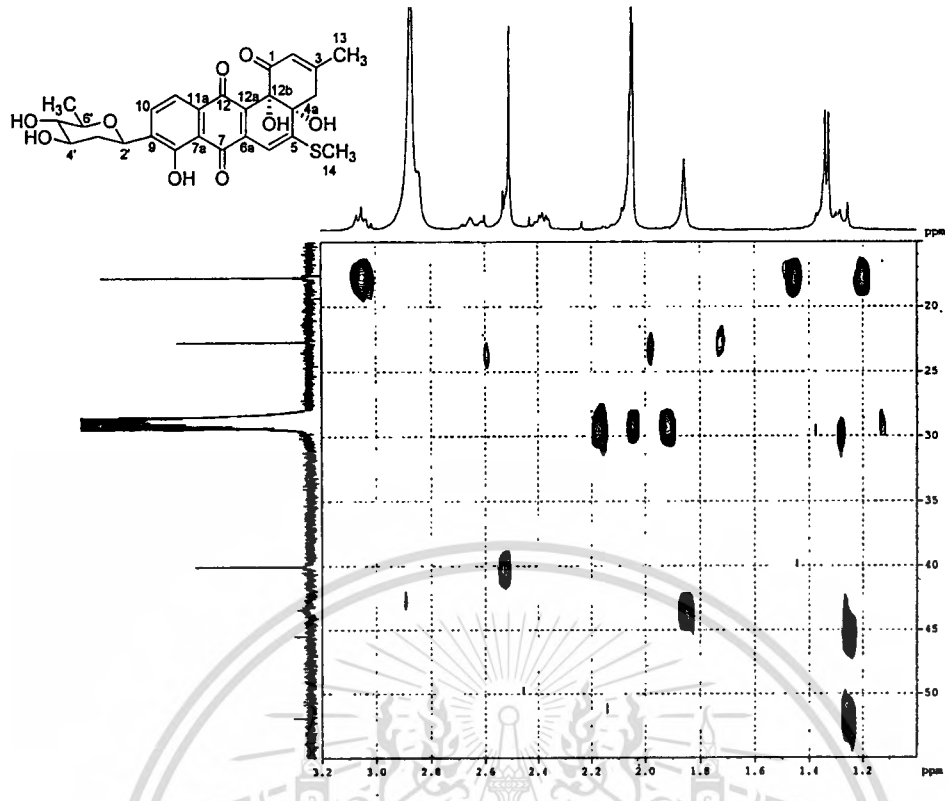


Fig. 23 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1B (expansion of Fig. 22)

Compound S6.1C (dehydroxyaquayamycin)

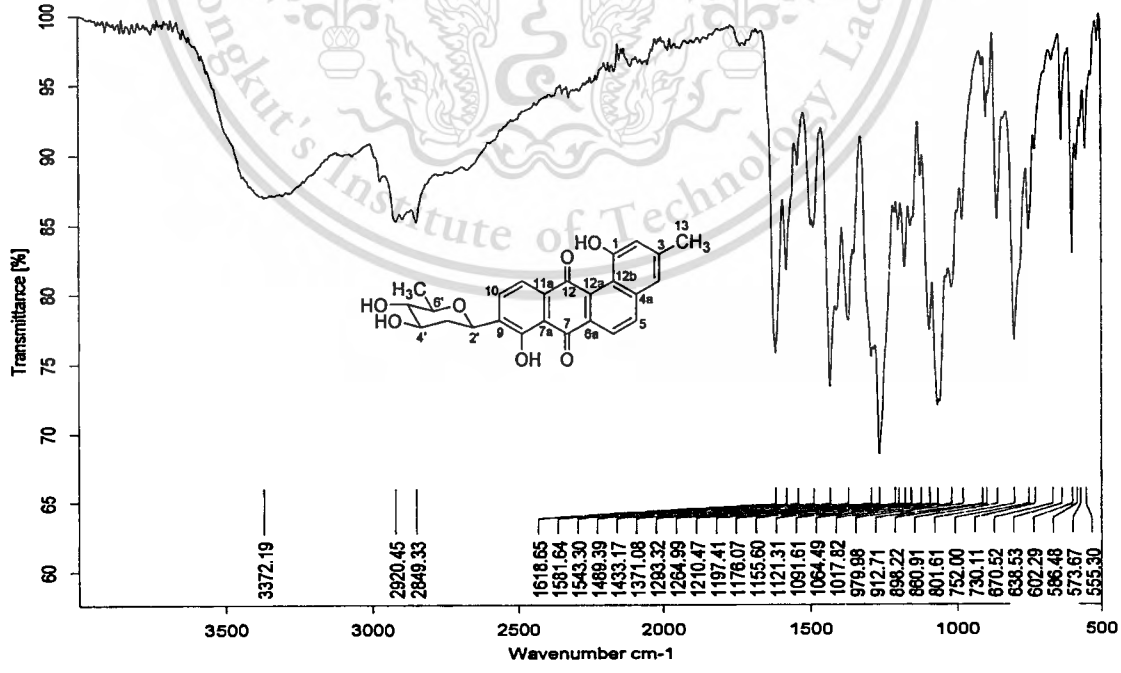


Fig. 24 IR spectrum of compound S6.1C

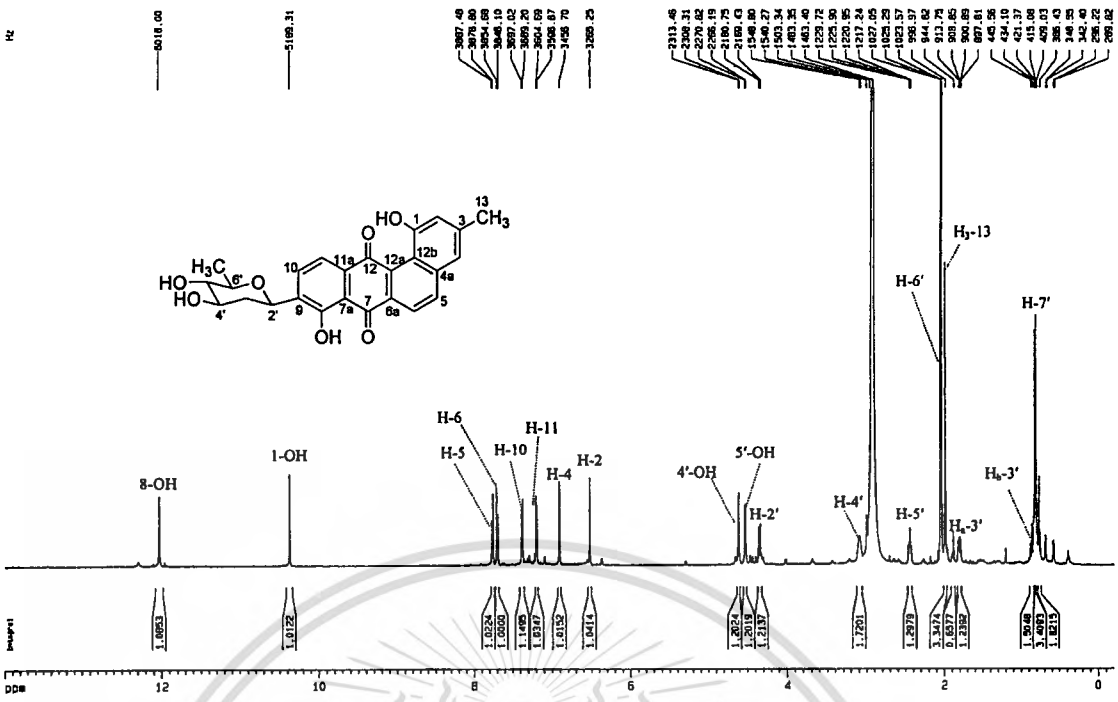


Fig. 25 ^{1}H NMR spectrum (500 MHz, DMSO-d_6) of compound S6.1C

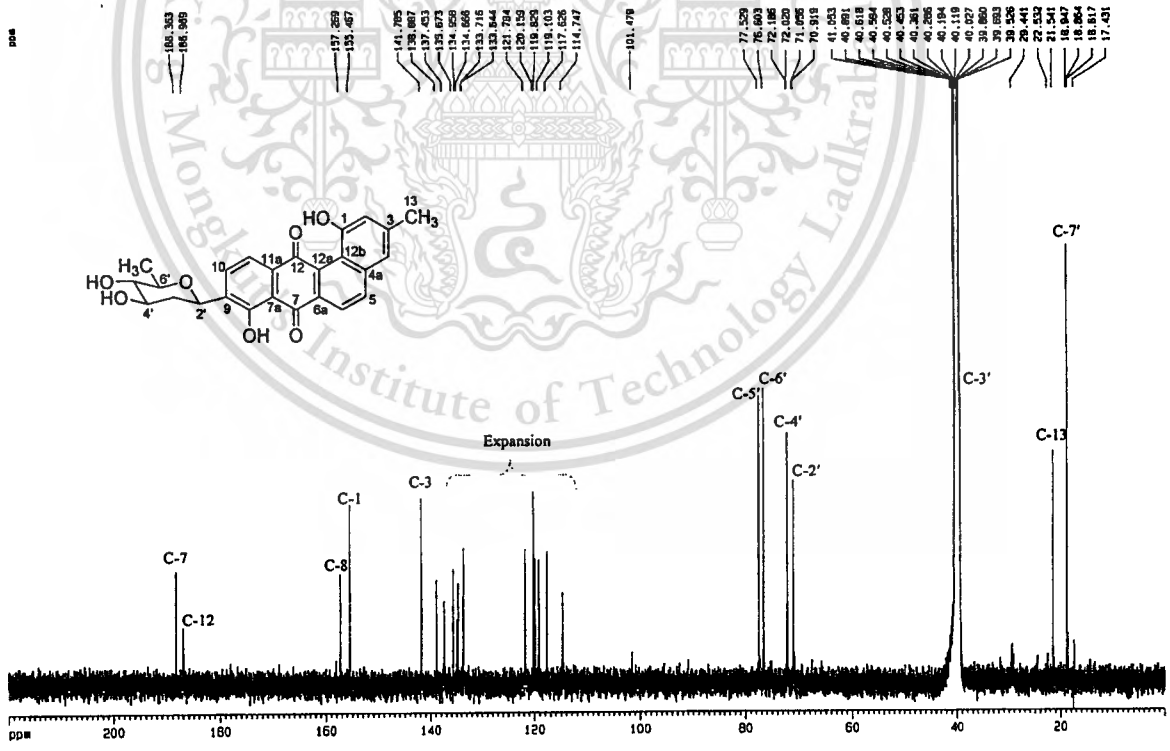


Fig. 26 ^{13}C NMR spectrum (500 MHz, DMSO-d_6) of compound S6.1C

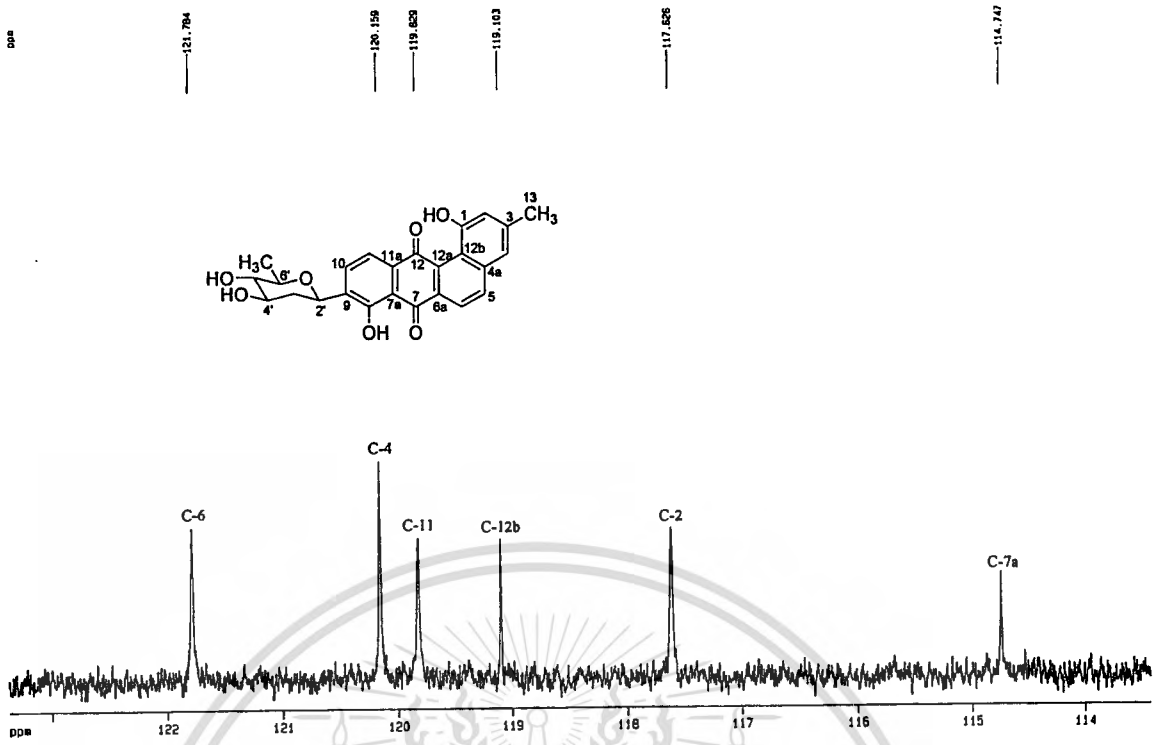


Fig. 27 ^{13}C NMR spectrum (500 MHz, DMSO-d_6) of compound S6.1C (expansion of Fig. 26)

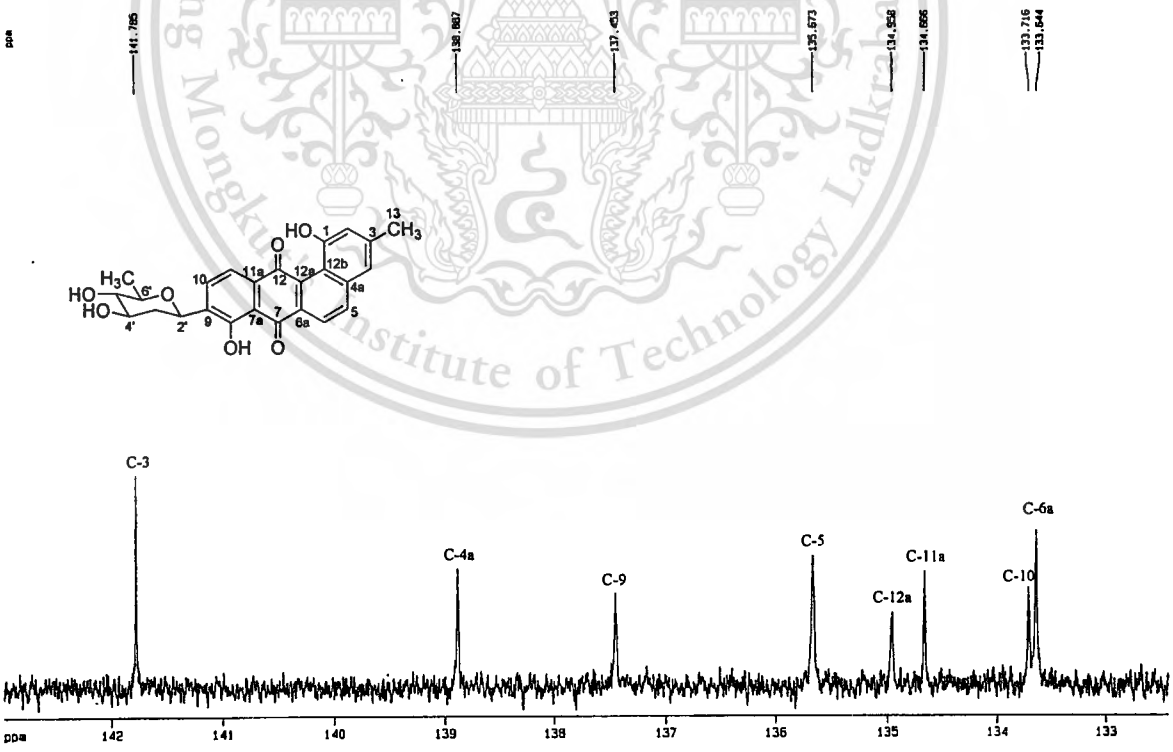


Fig. 28 ^{13}C NMR spectrum (500 MHz, DMSO-d_6) of compound S6.1C (expansion of Fig. 26)

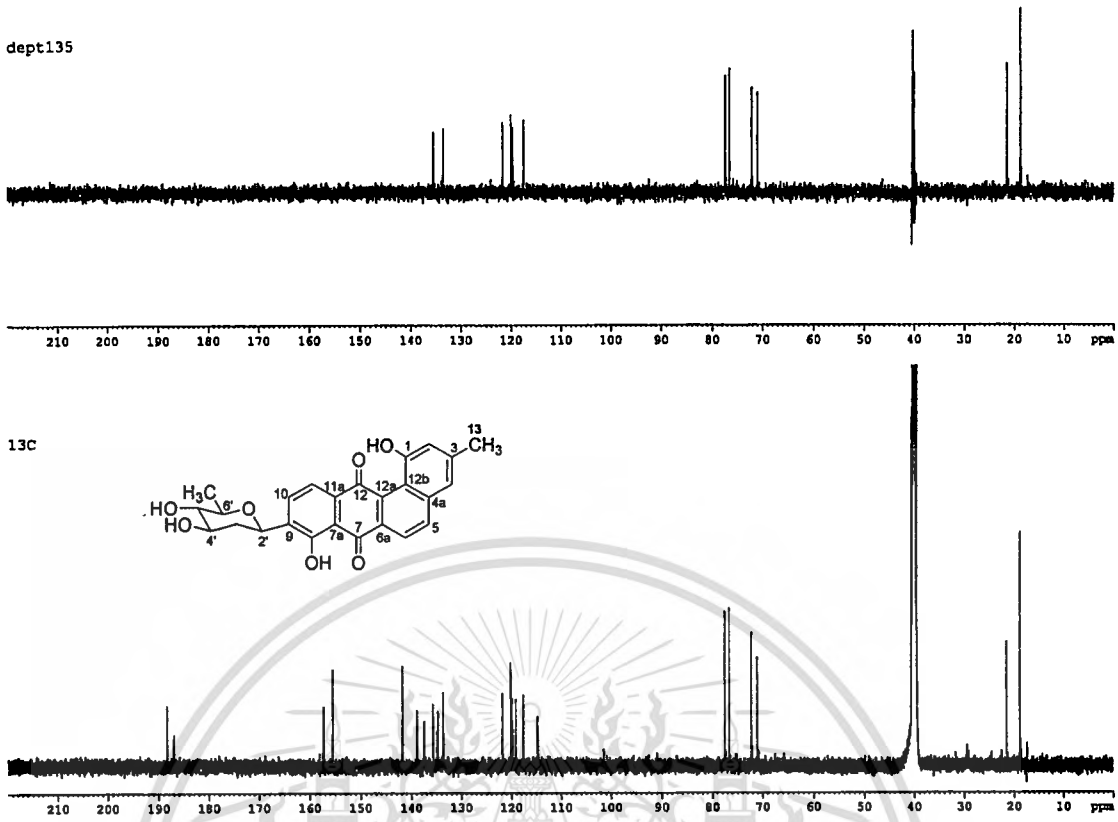


Fig. 29 DEPT 135 spectrum (500 MHz, DMSO-d_6) of compound S6.1C

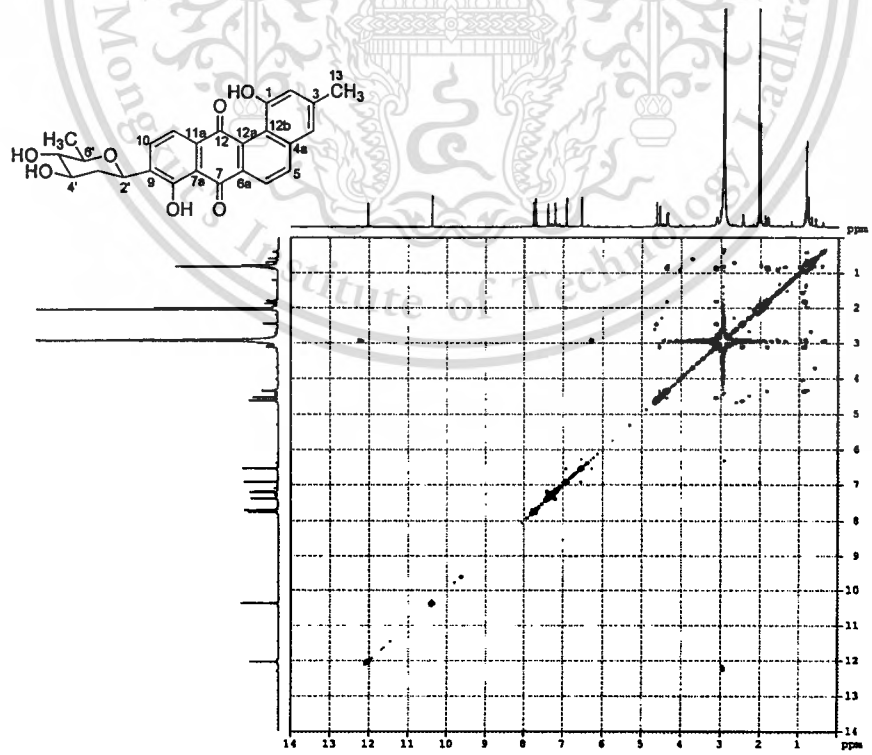


Fig. 30 COSY spectrum (500 MHz, DMSO-d_6) of compound S6.1C

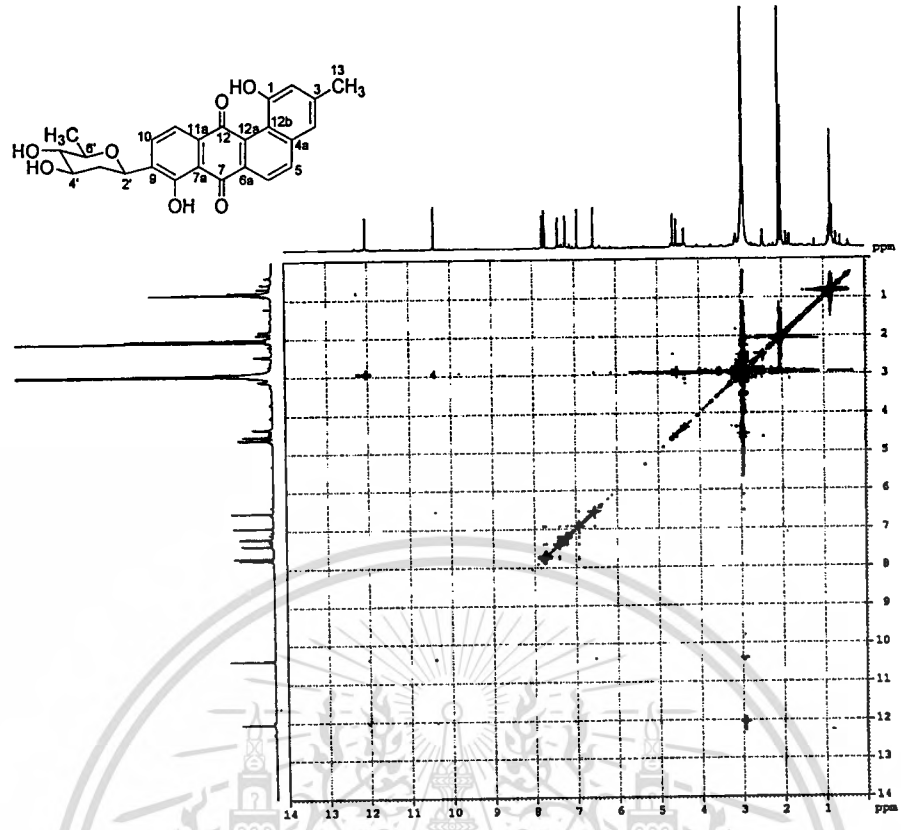


Fig. 31 NOESY spectrum (500 MHz, DMSO-d_6) of compound S6.1C

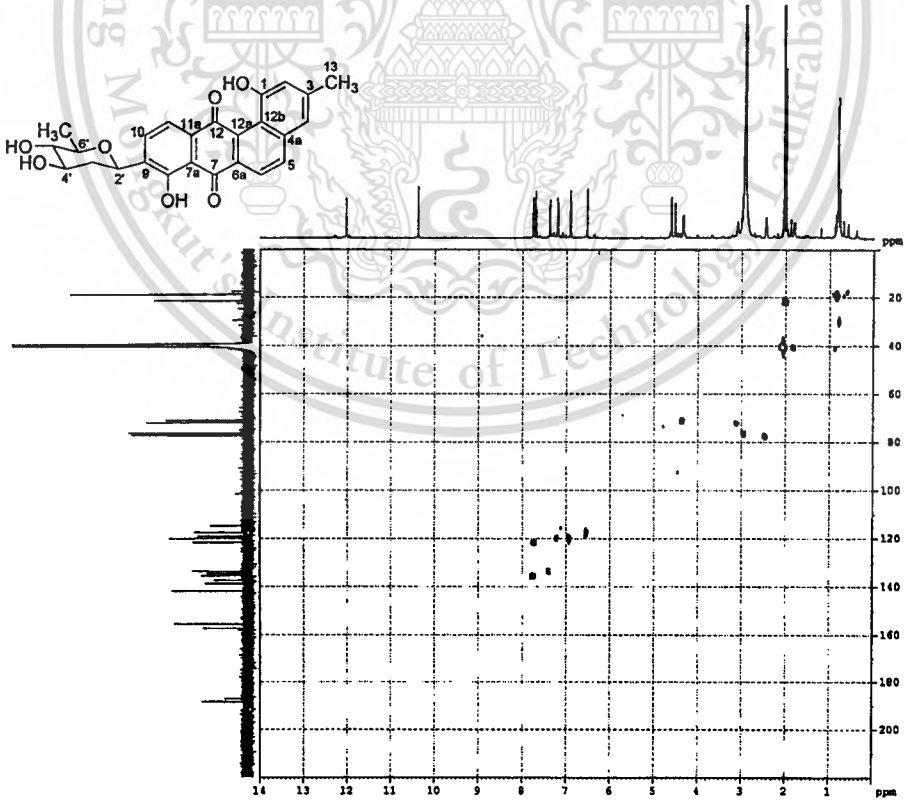


Fig. 32 HMQC spectrum (500 MHz, DMSO-d_6) of compound S6.1C

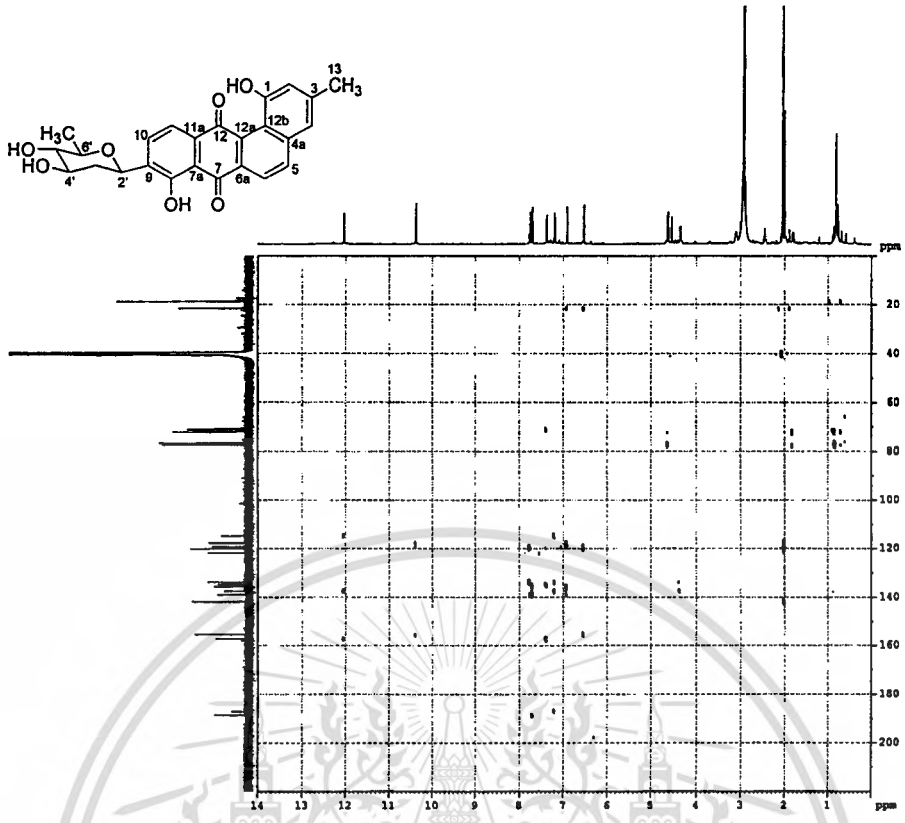


Fig. 33 HMBC spectrum (500 MHz, DMSO-d_6) of compound S6.1C

Compound S6.1D (5-aminodehydroxyaquayamycin)

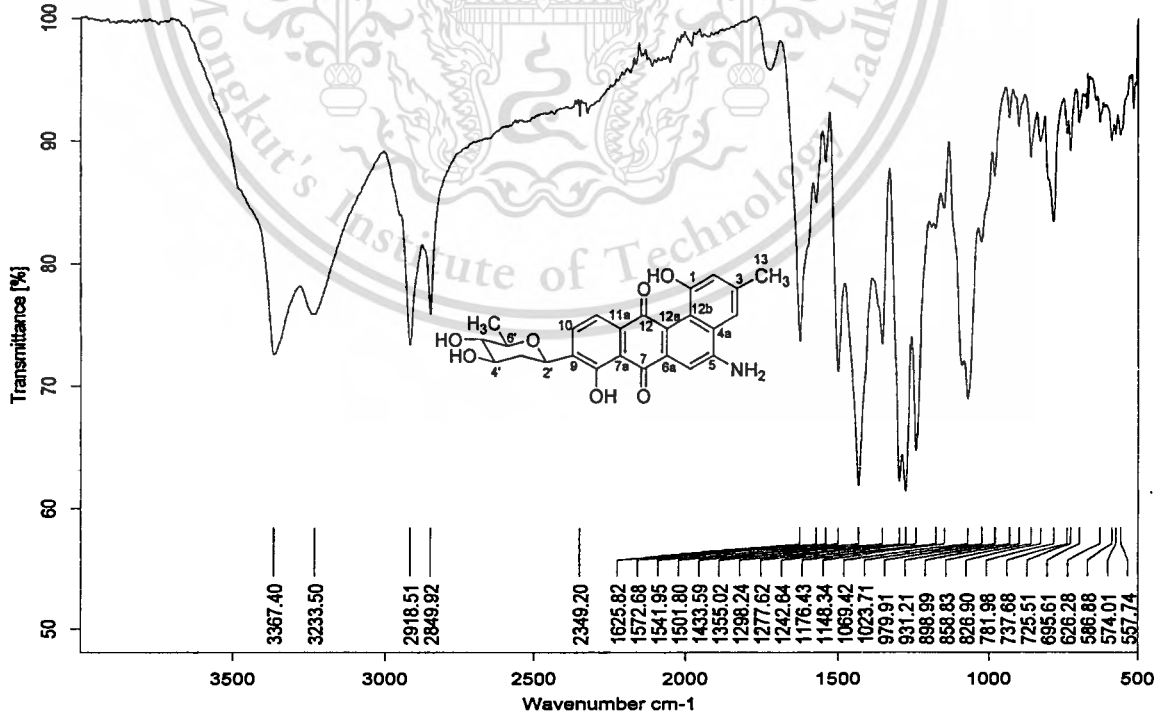


Fig. 34 IR spectrum of compound S6.1D

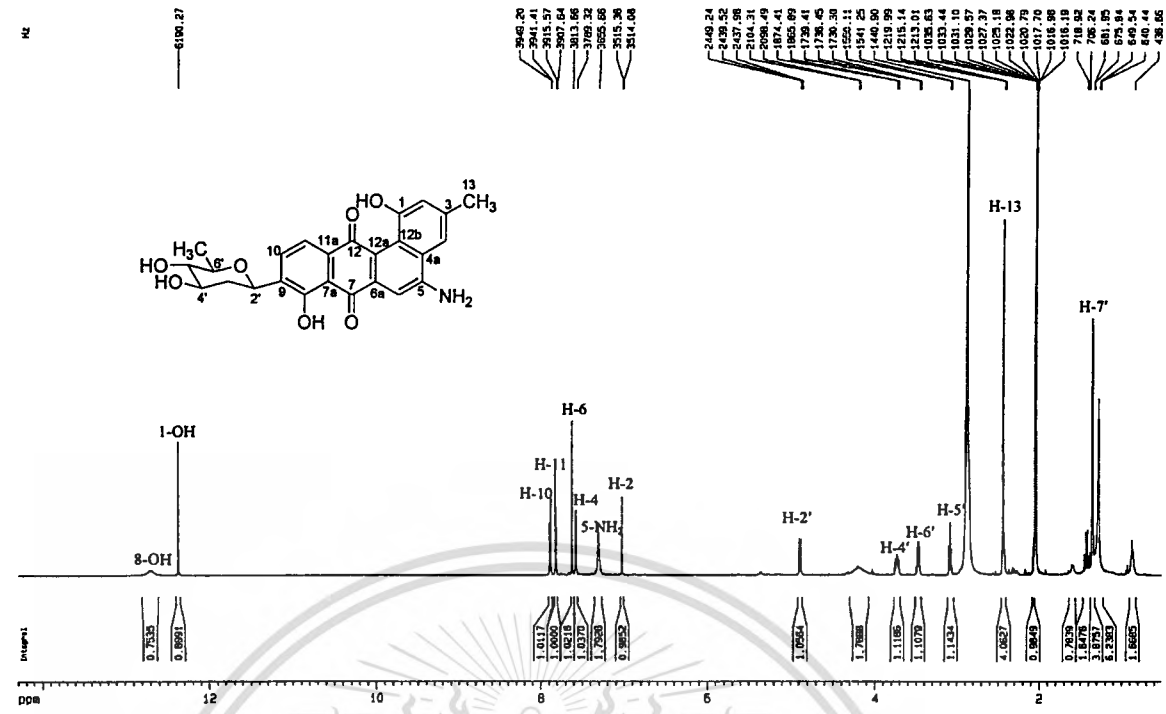


Fig. 35 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1D

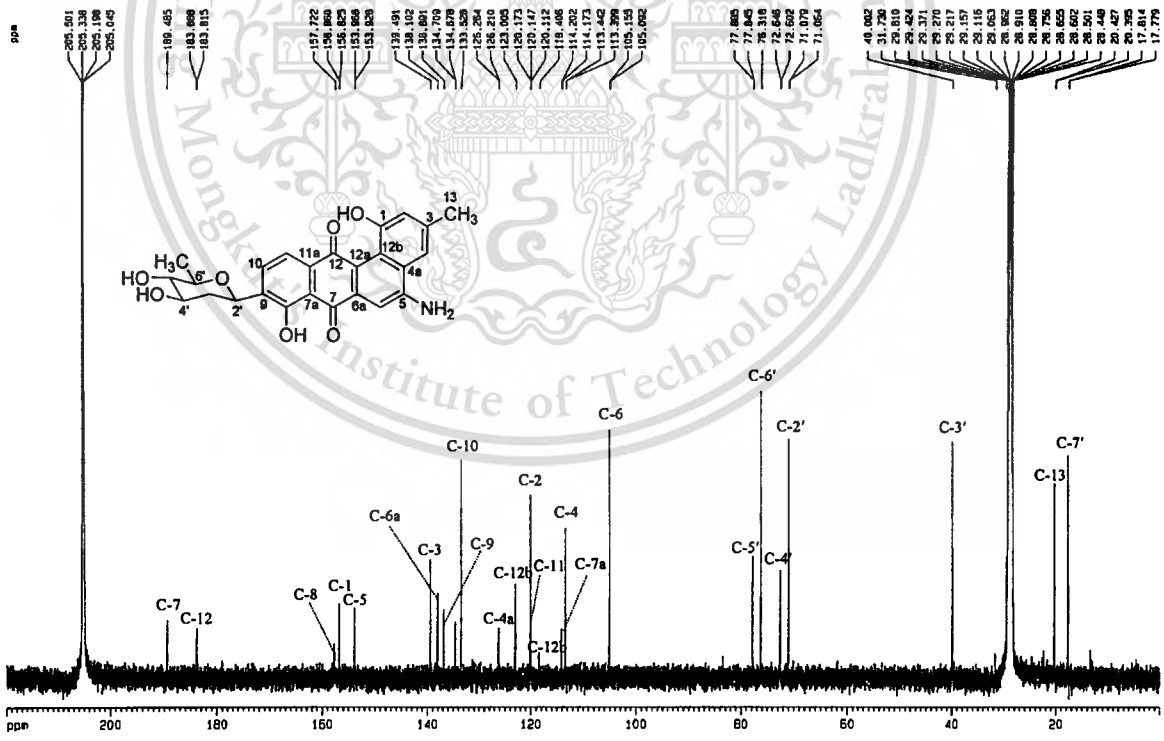


Fig. 36 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S6.1D

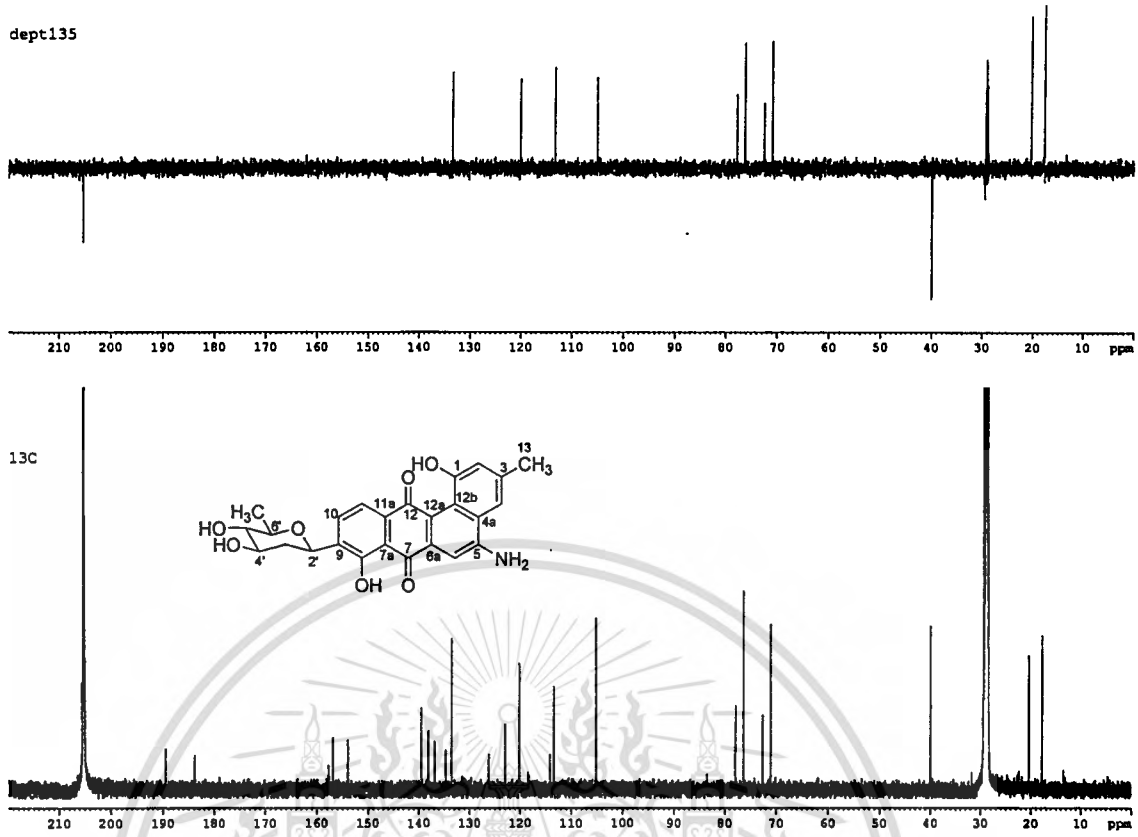


Fig. 37 DEPT 135 spectrum (500 MHz, acetone-d_6) of compound S6.1D

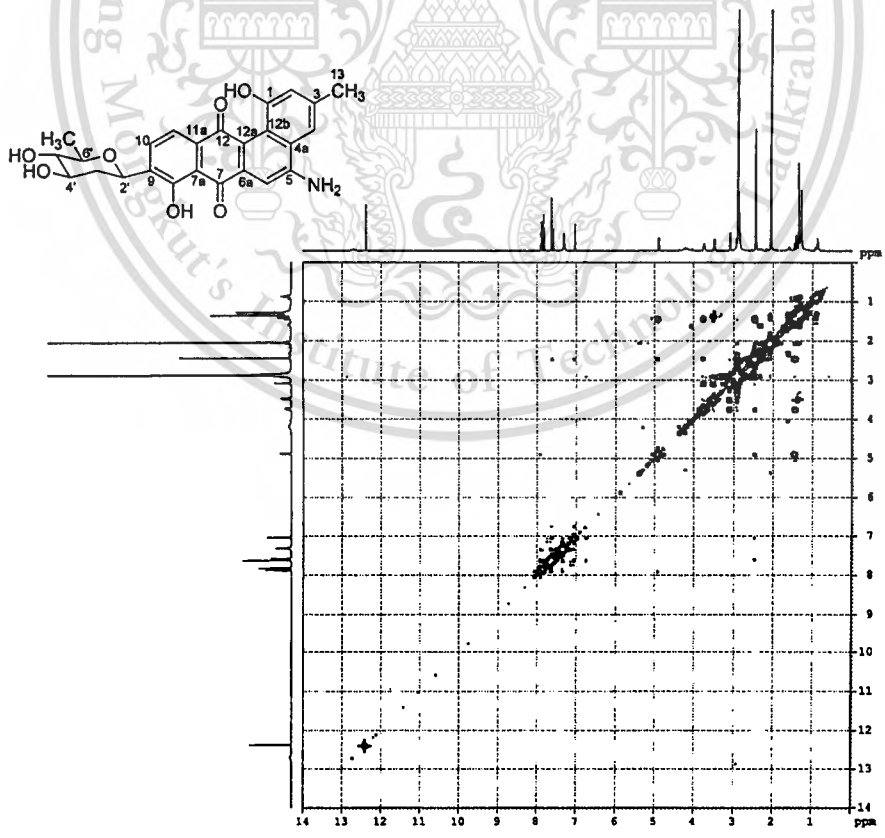


Fig. 38 COSY spectrum (500 MHz, acetone-d_6) of compound S6.1D

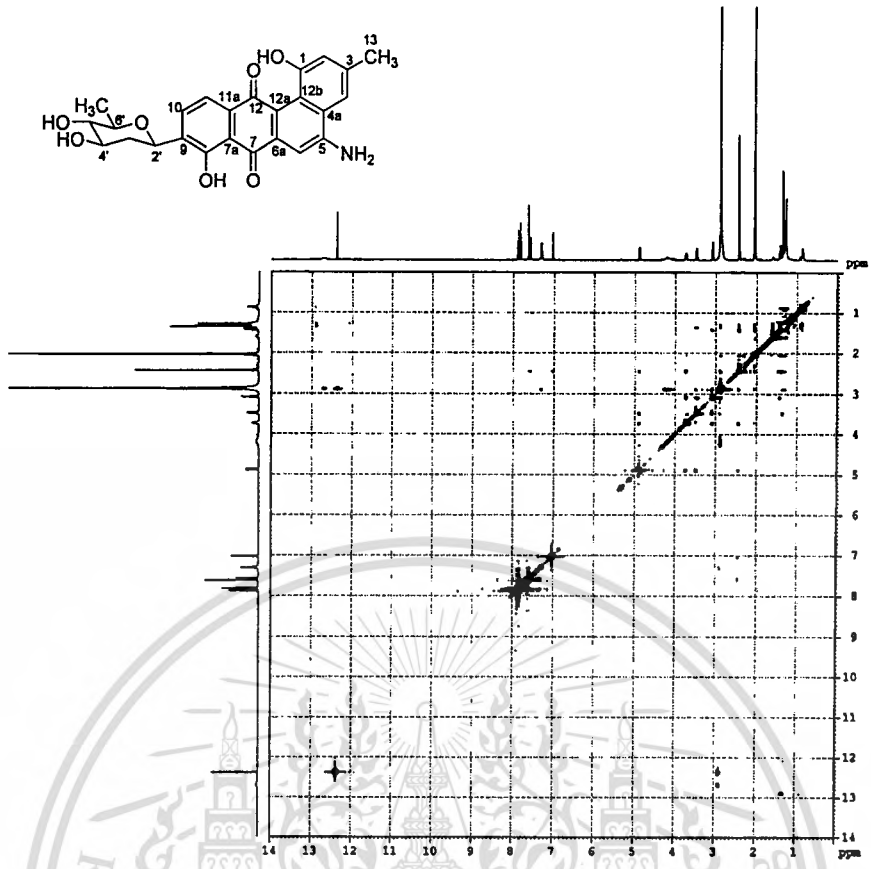


Fig. 39 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1D

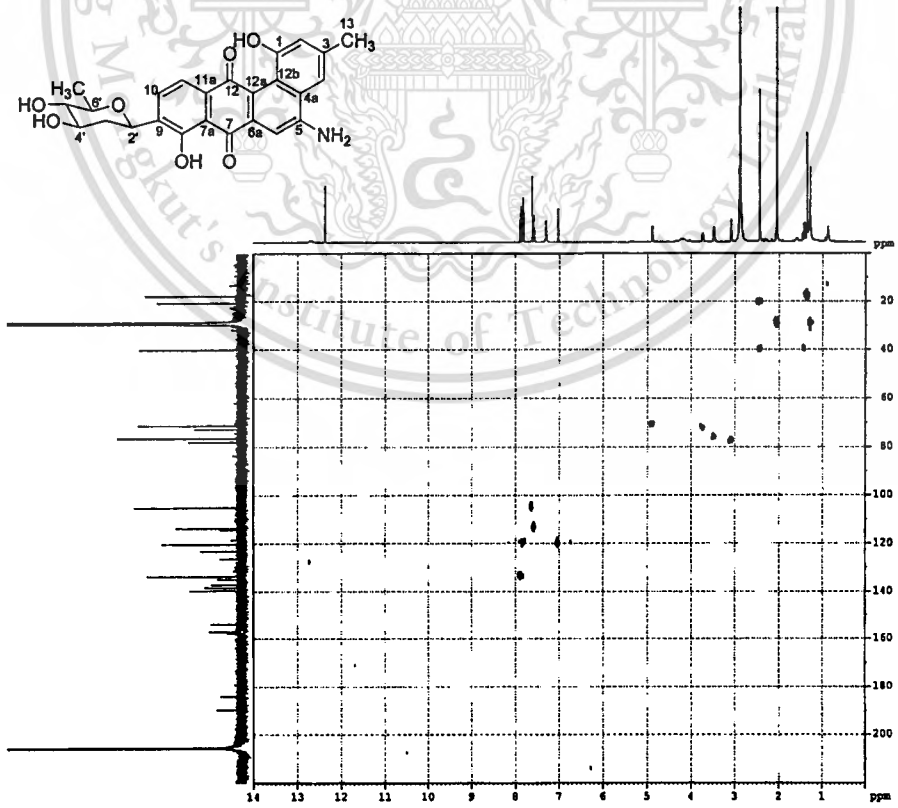


Fig. 40 HMQC spectrum (500 MHz, acetone-d_6) of compound S6.1D

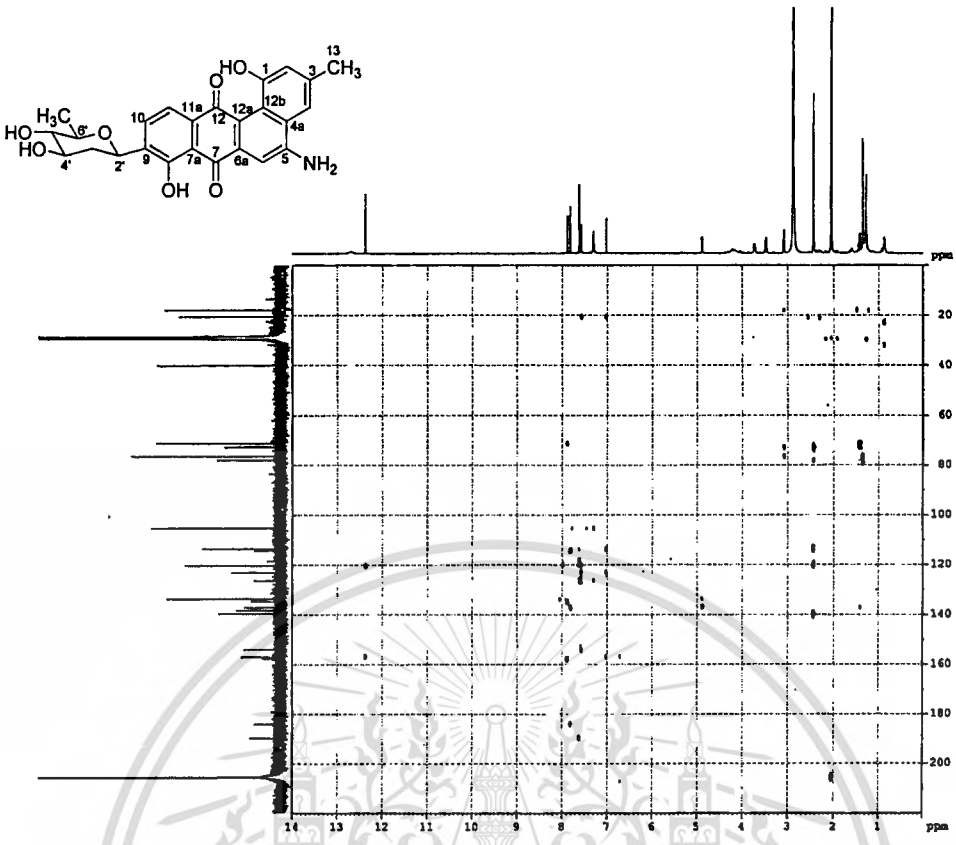


Fig. 41 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1D

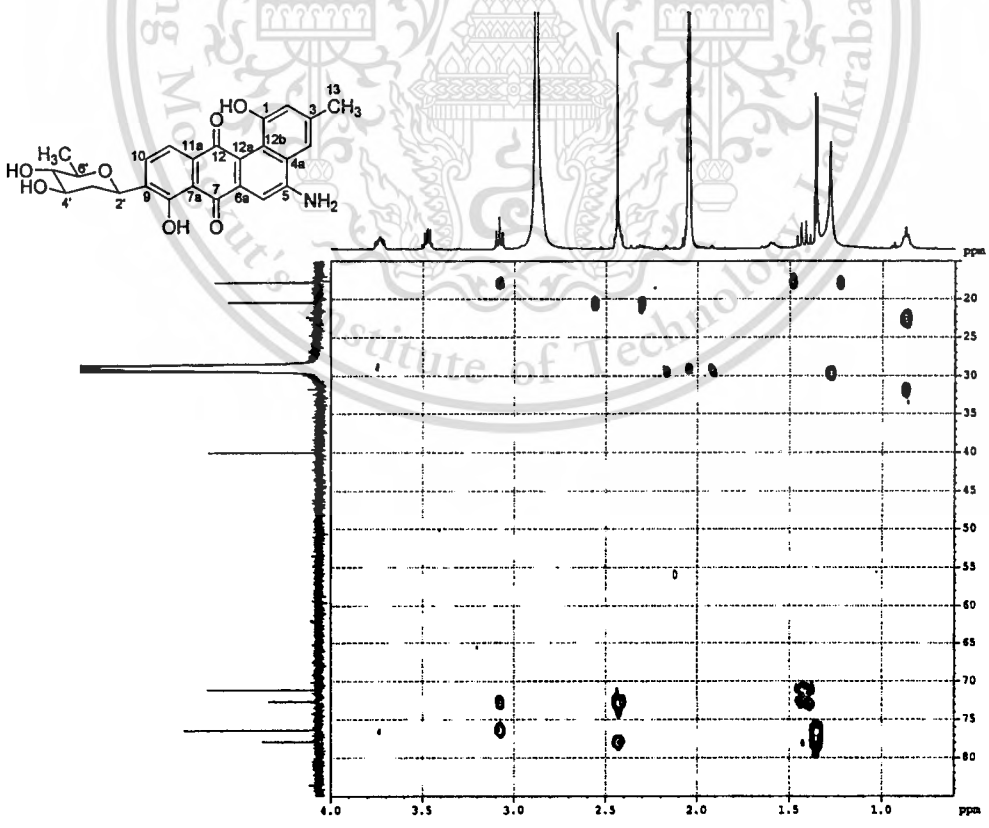


Fig. 42 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1D (expansion of Fig. 41)

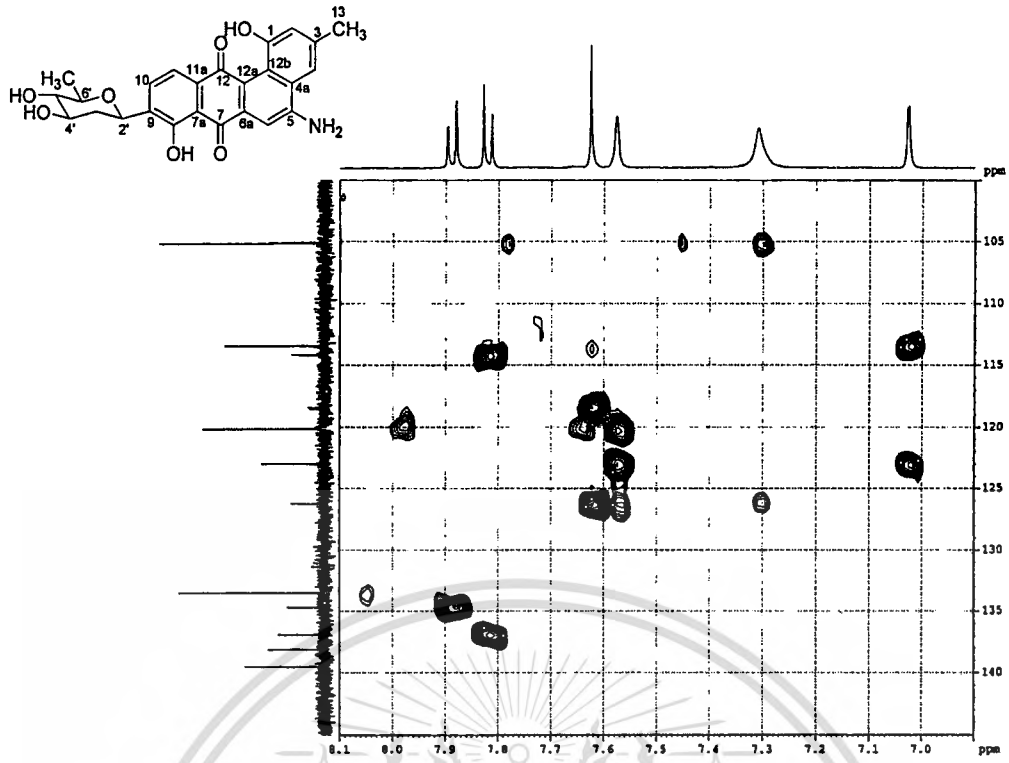


Fig. 43 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1D (expansion of Fig. 41)

Compound S6.1E (urdamycin E)

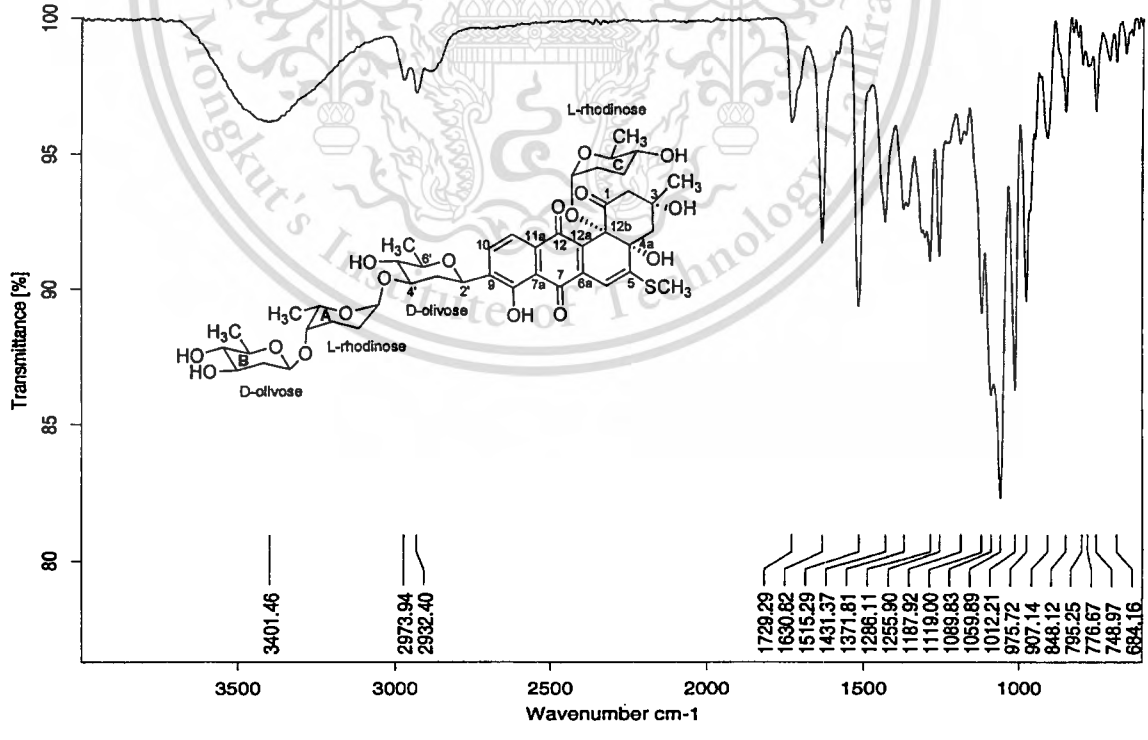


Fig. 44 IR spectrum of compound S6.1E

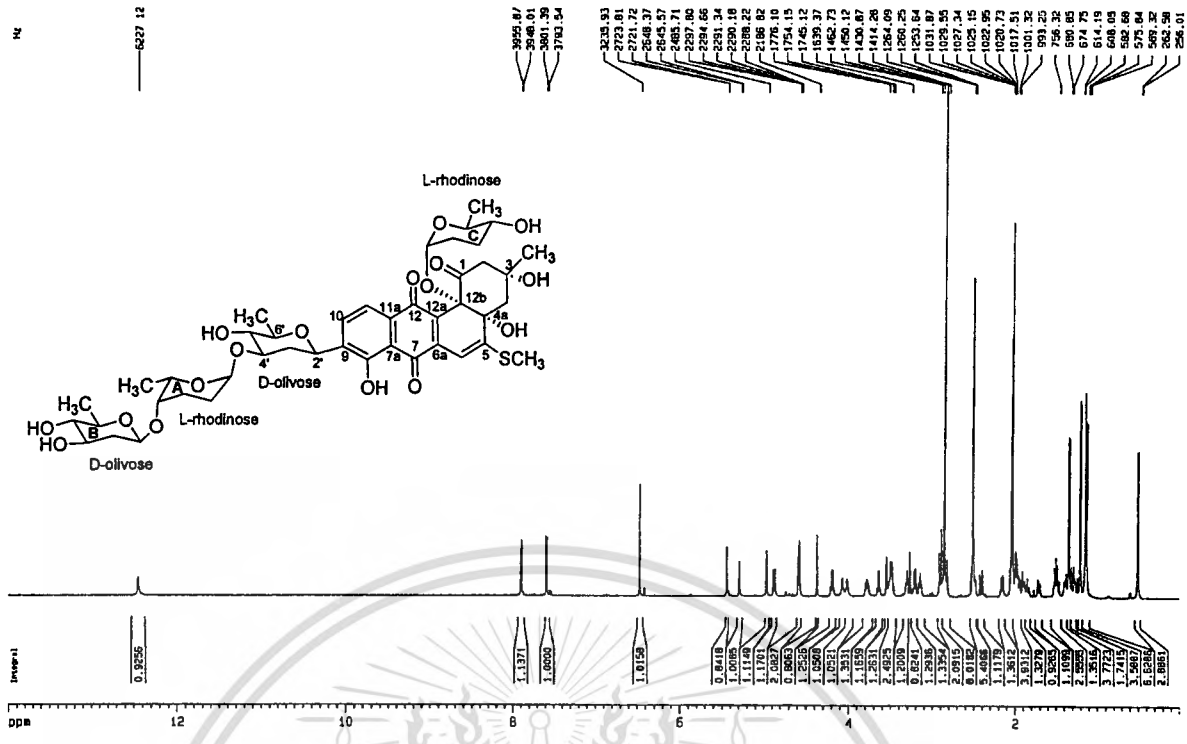


Fig. 45 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E

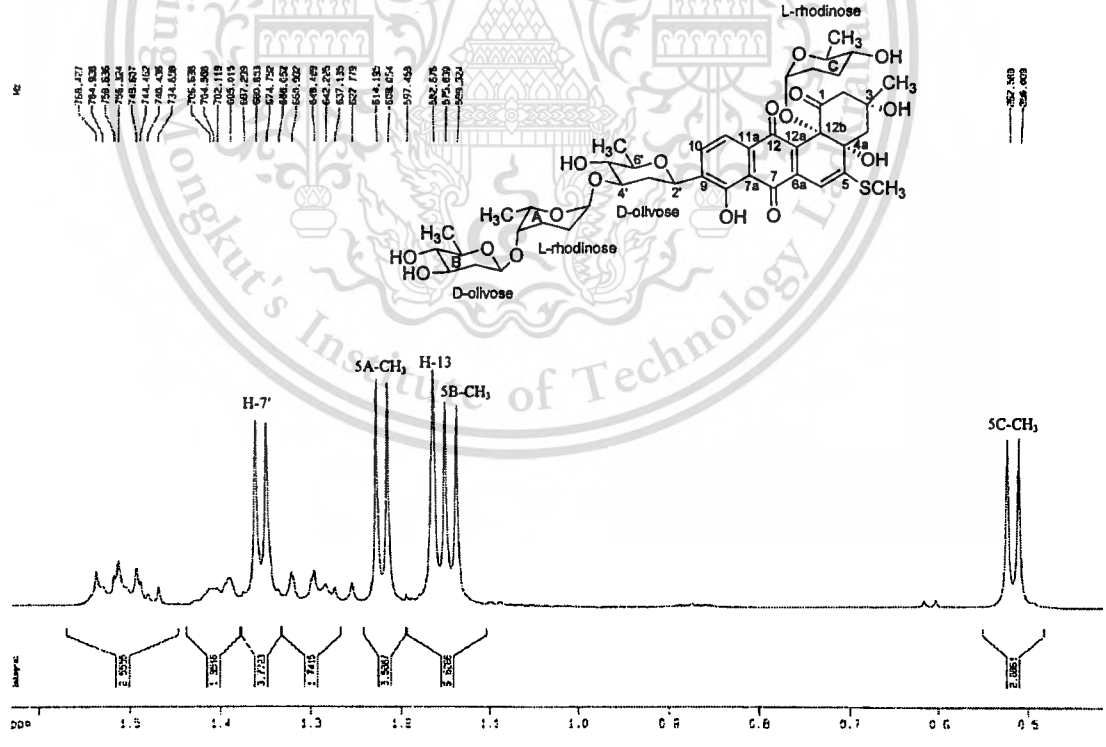


Fig. 46 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 45)

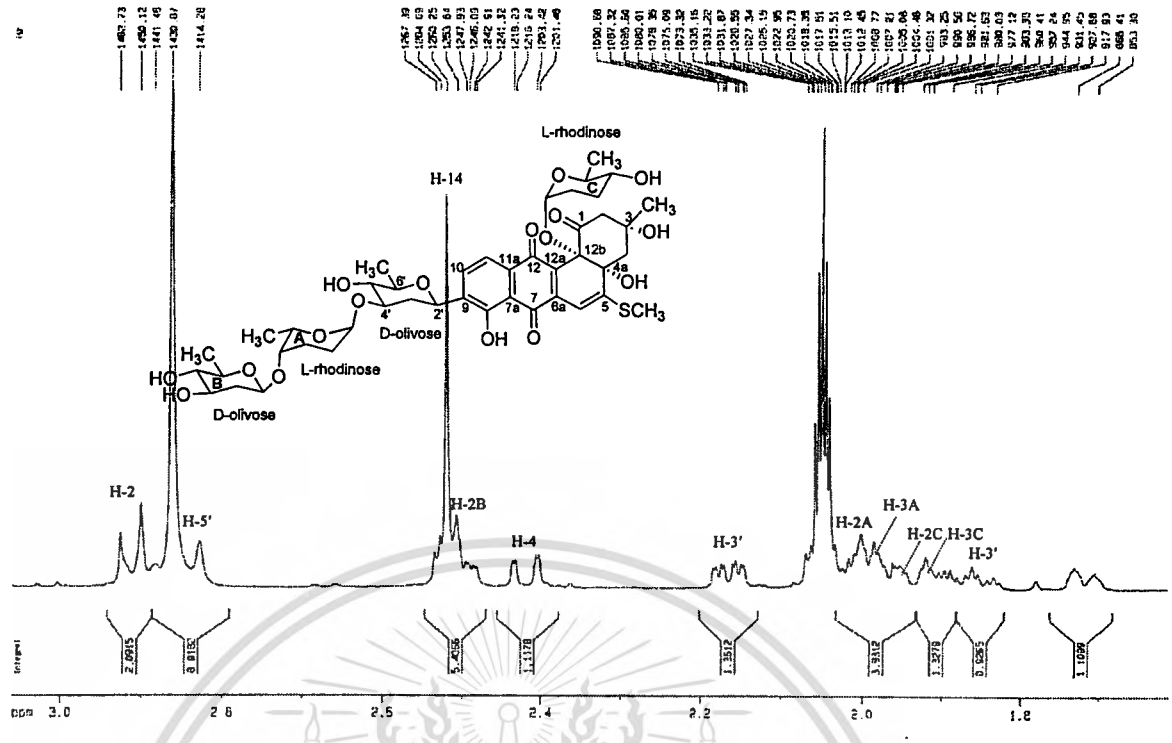


Fig. 47 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 45)

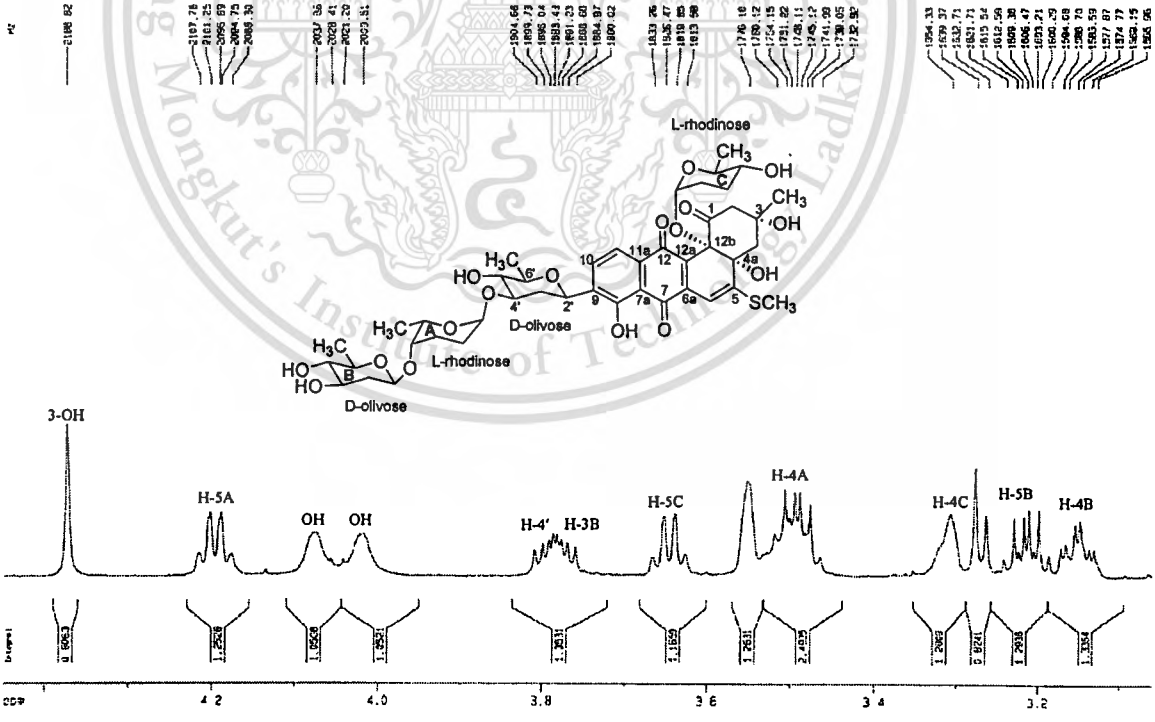


Fig. 48 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 45)

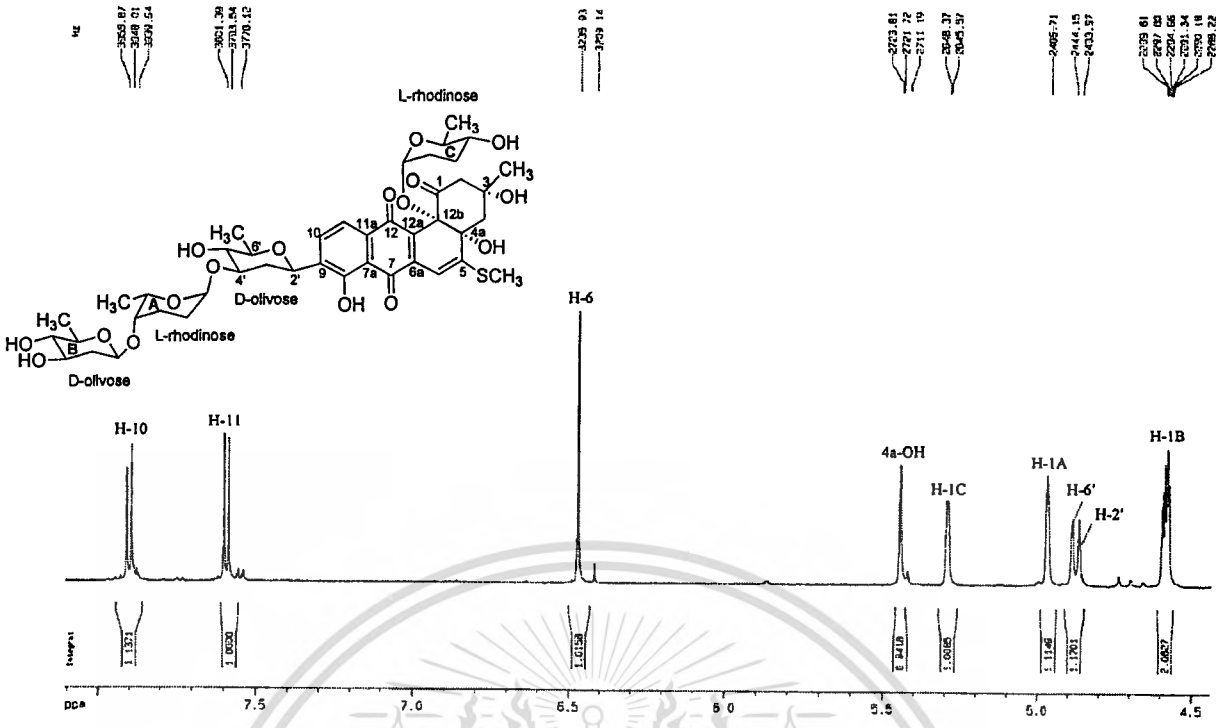


Fig. 49 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 45)

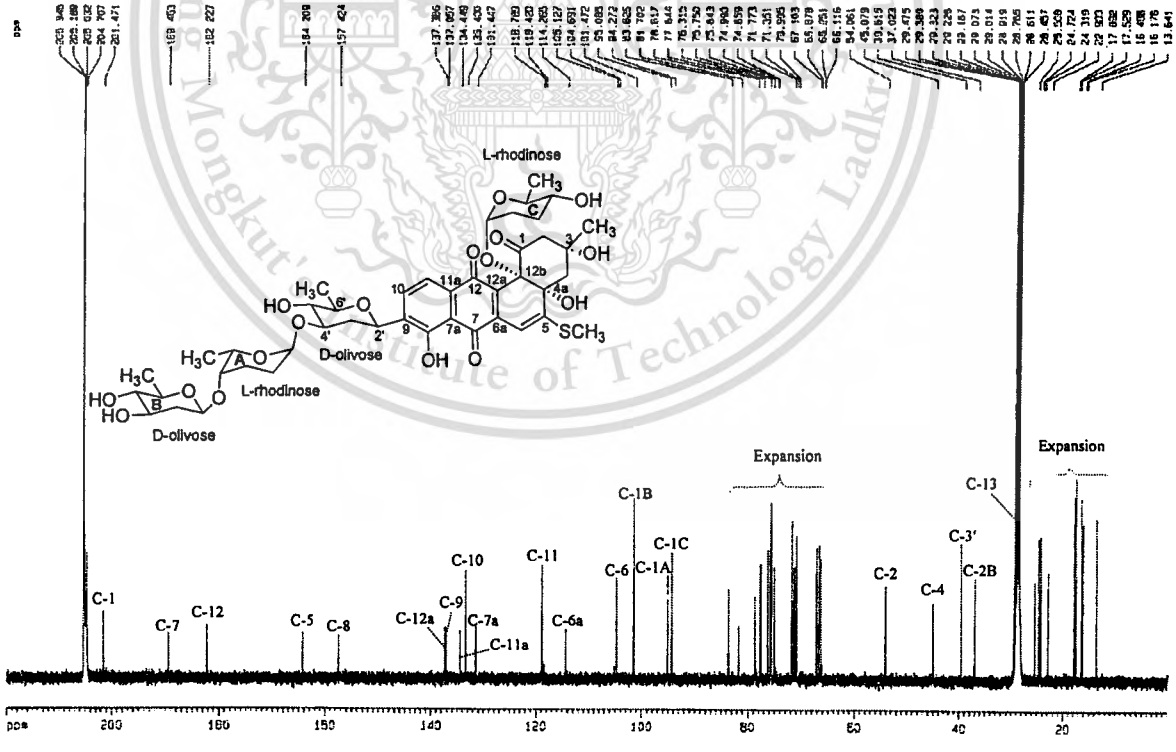


Fig. 50 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E

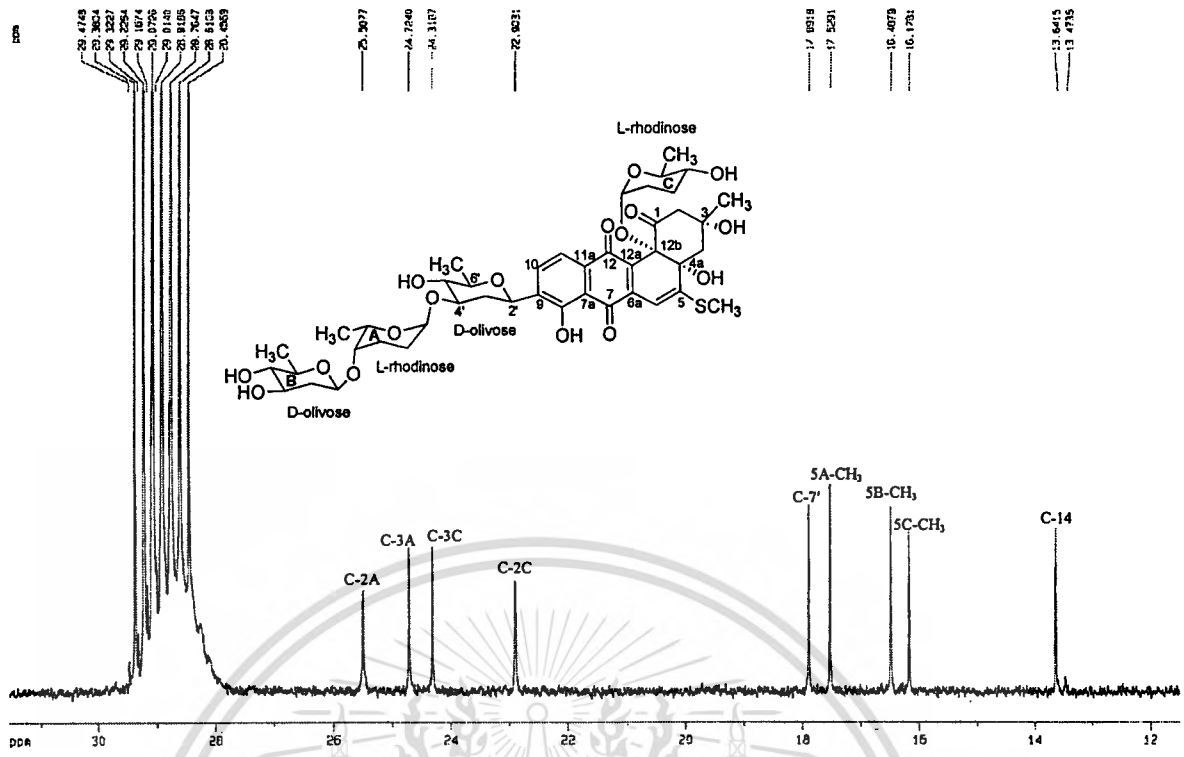


Fig. 51 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 50)

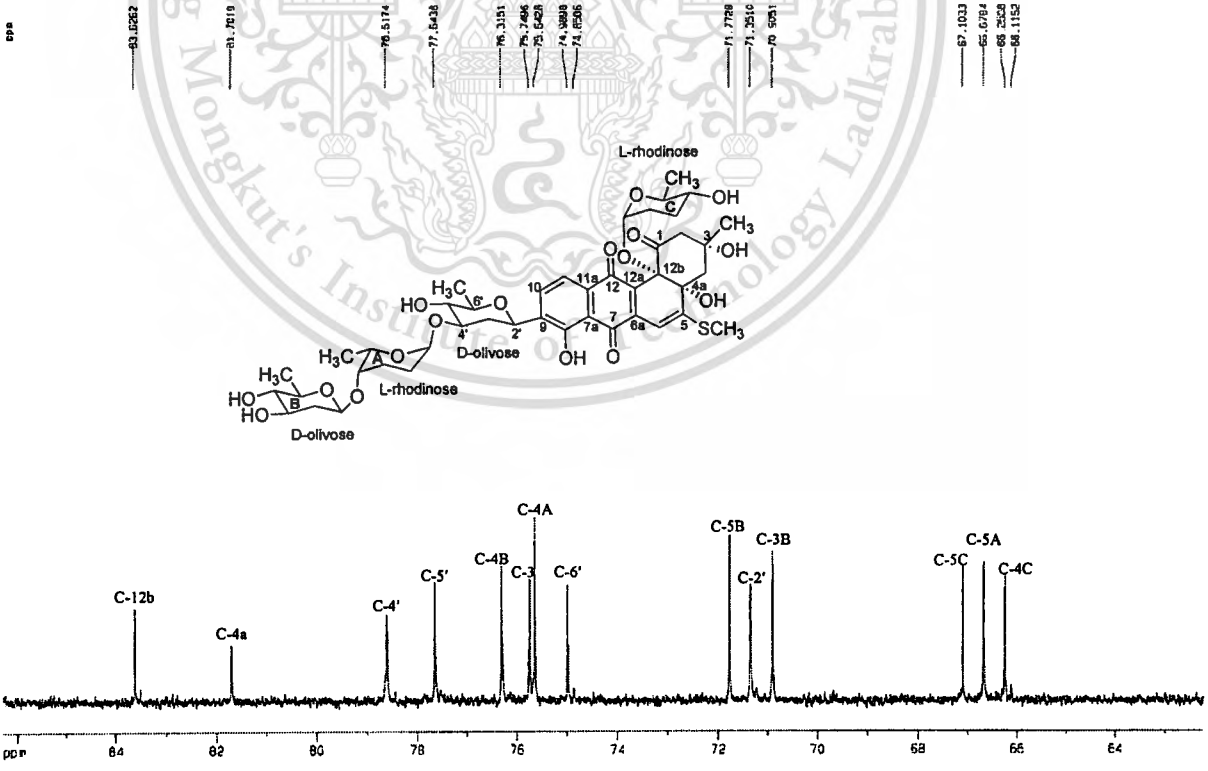


Fig. 52 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 50)

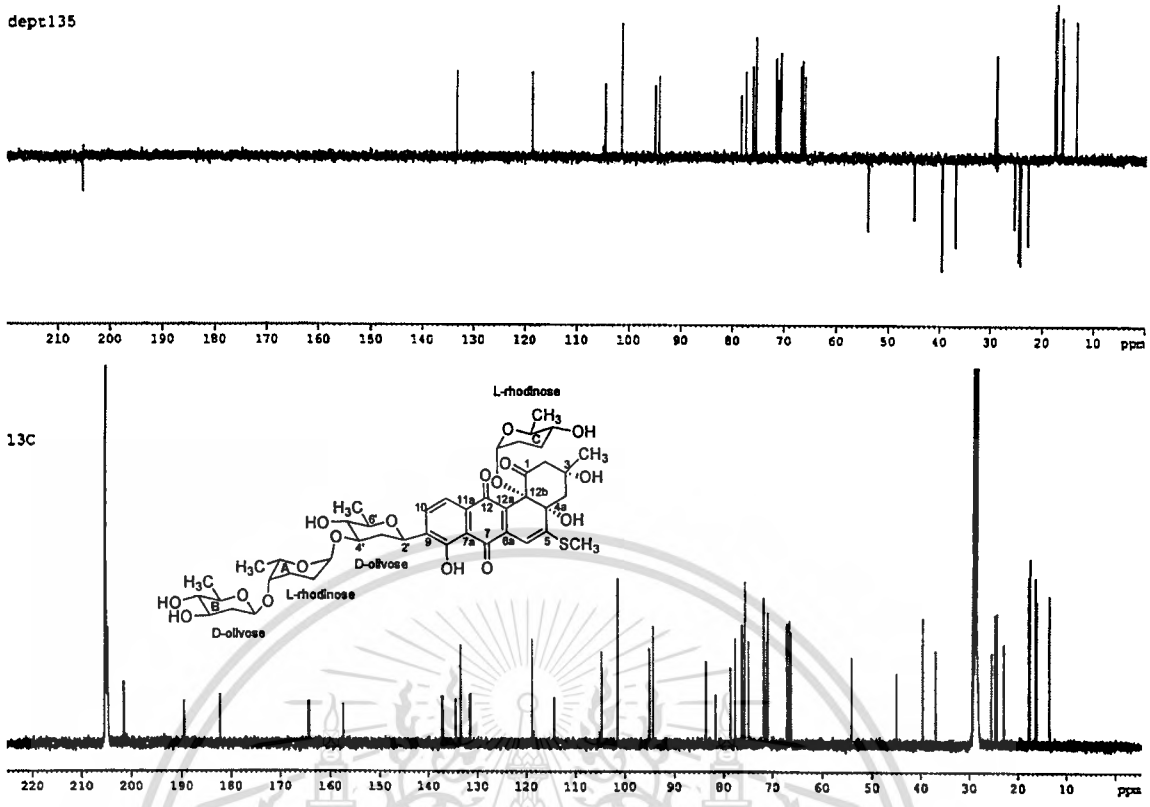


Fig. 53 DEPT 135 (500 MHz, acetone-d_6) of compound S6.1E

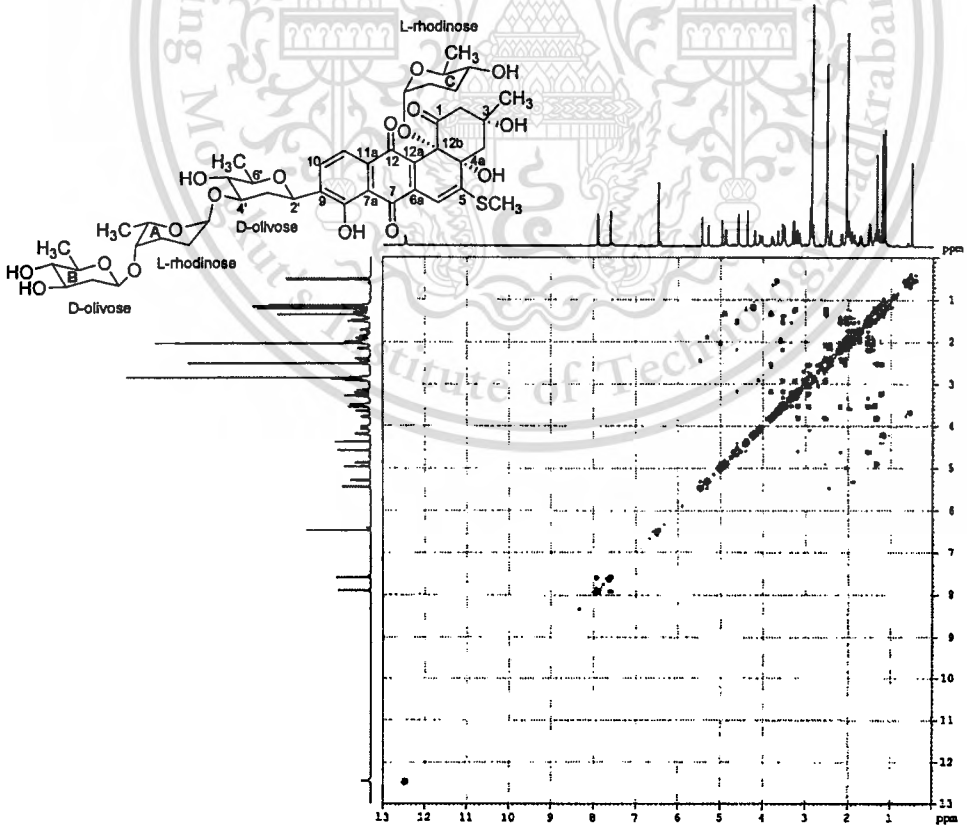


Fig. 54 COSY spectrum (500 MHz, acetone-d_6) of compound S6.1E

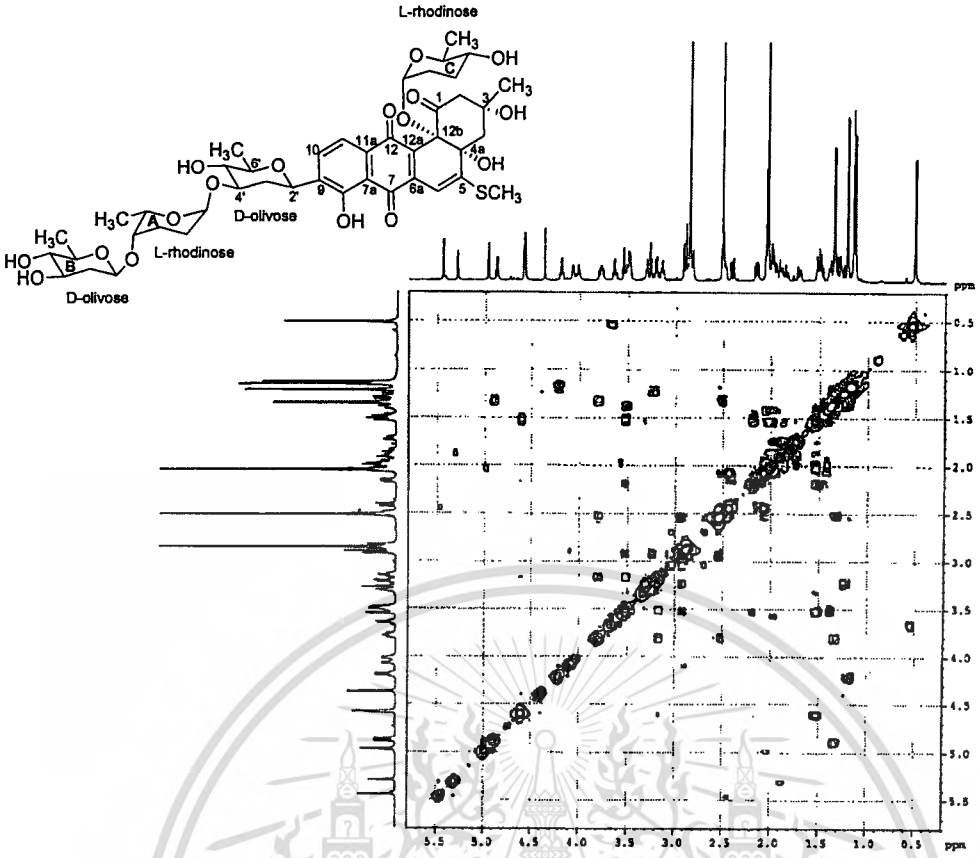


Fig. 55 COSY spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 54)

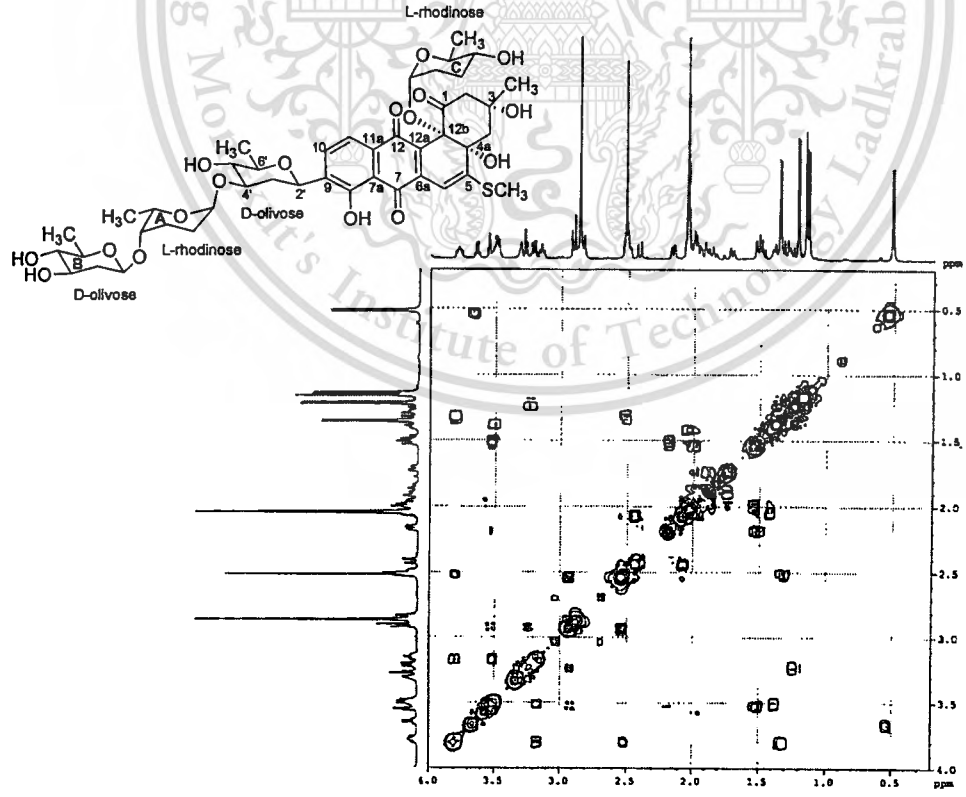


Fig. 56 COSY spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 54)

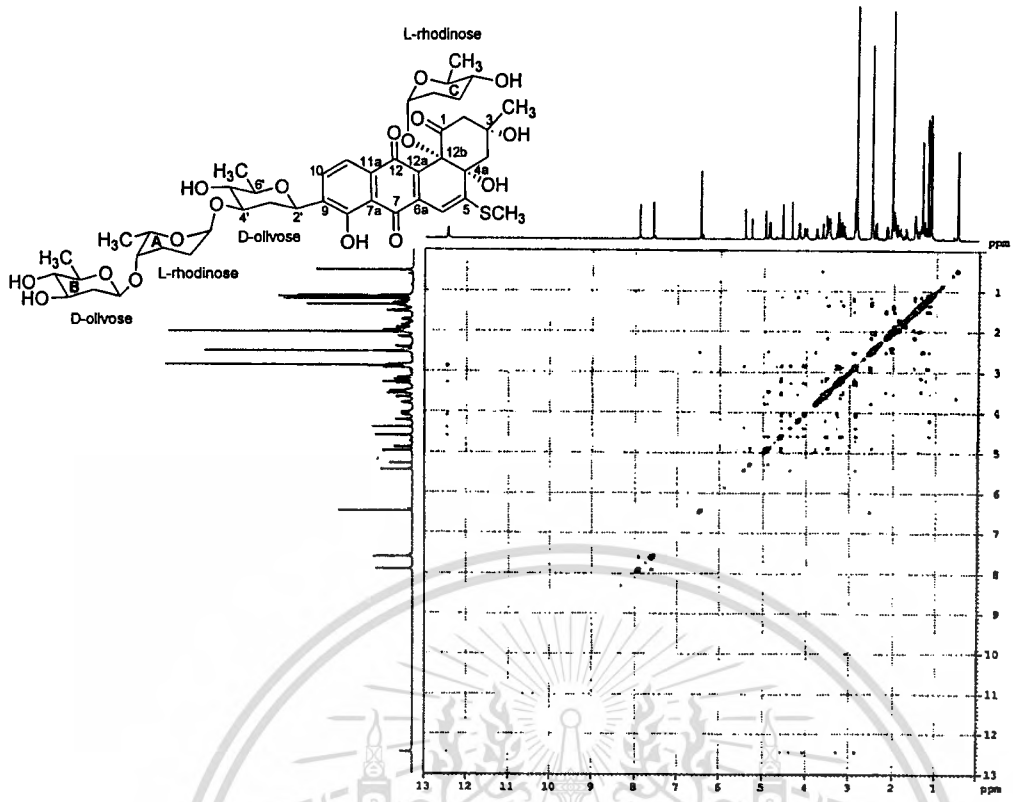


Fig. 57 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1E

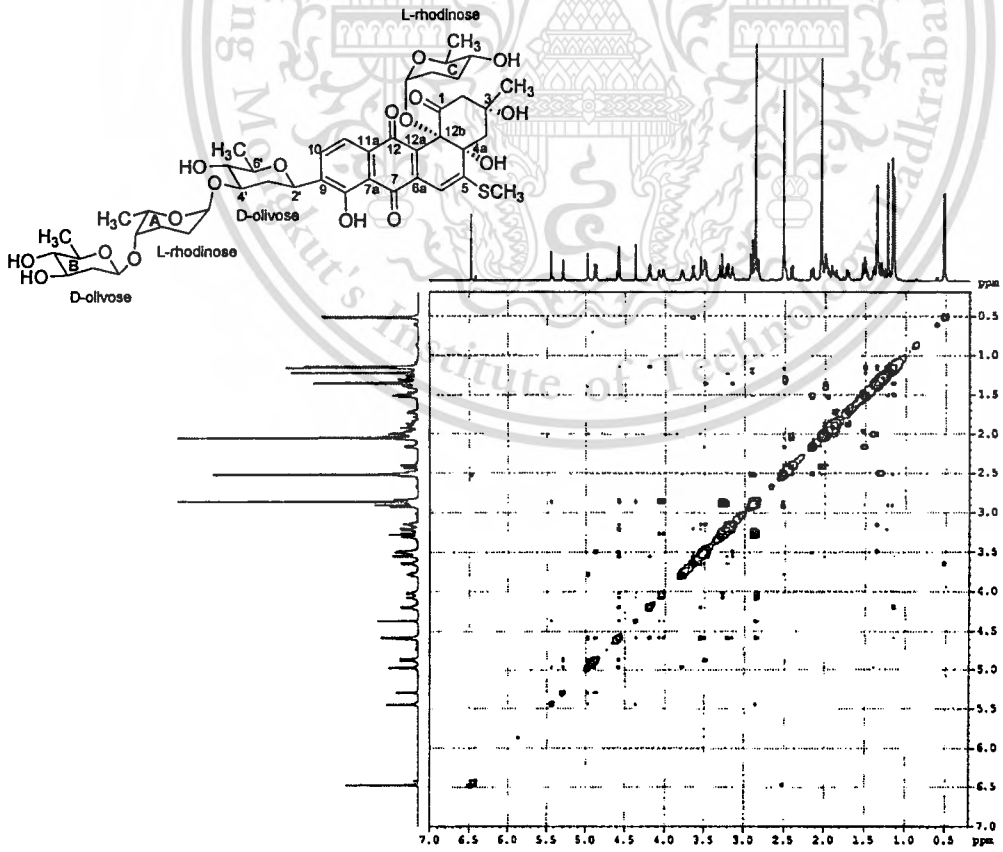


Fig. 58 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 57)

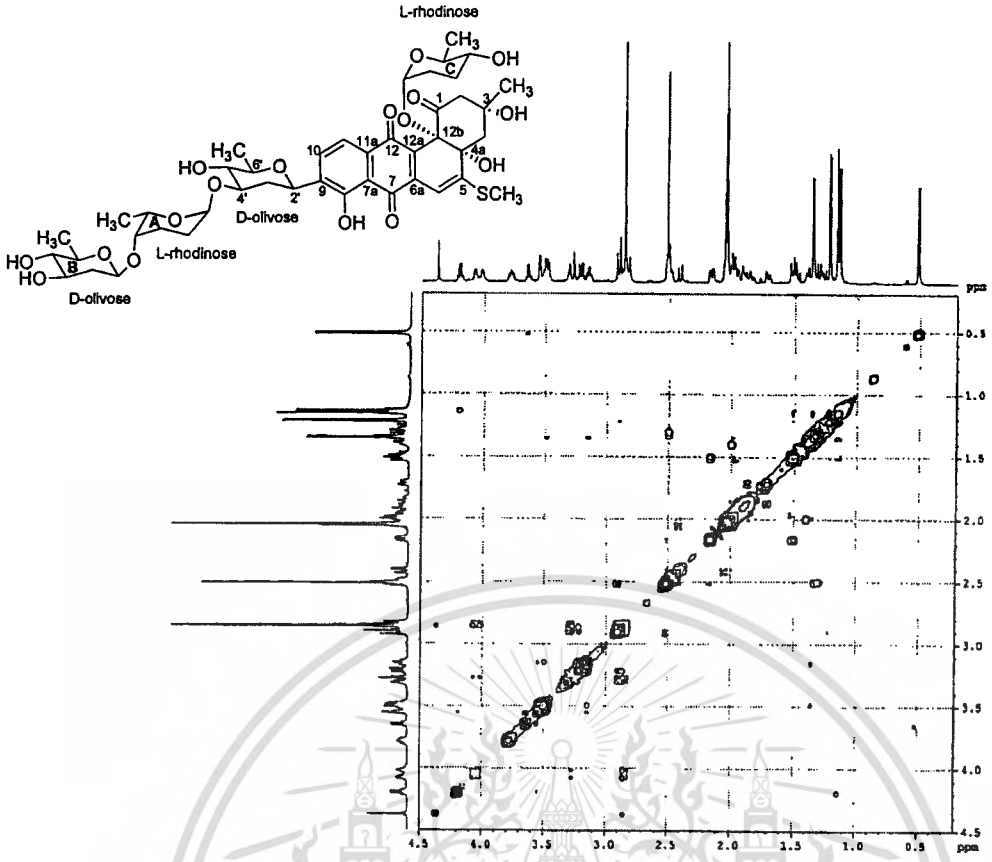


Fig. 59 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 57)

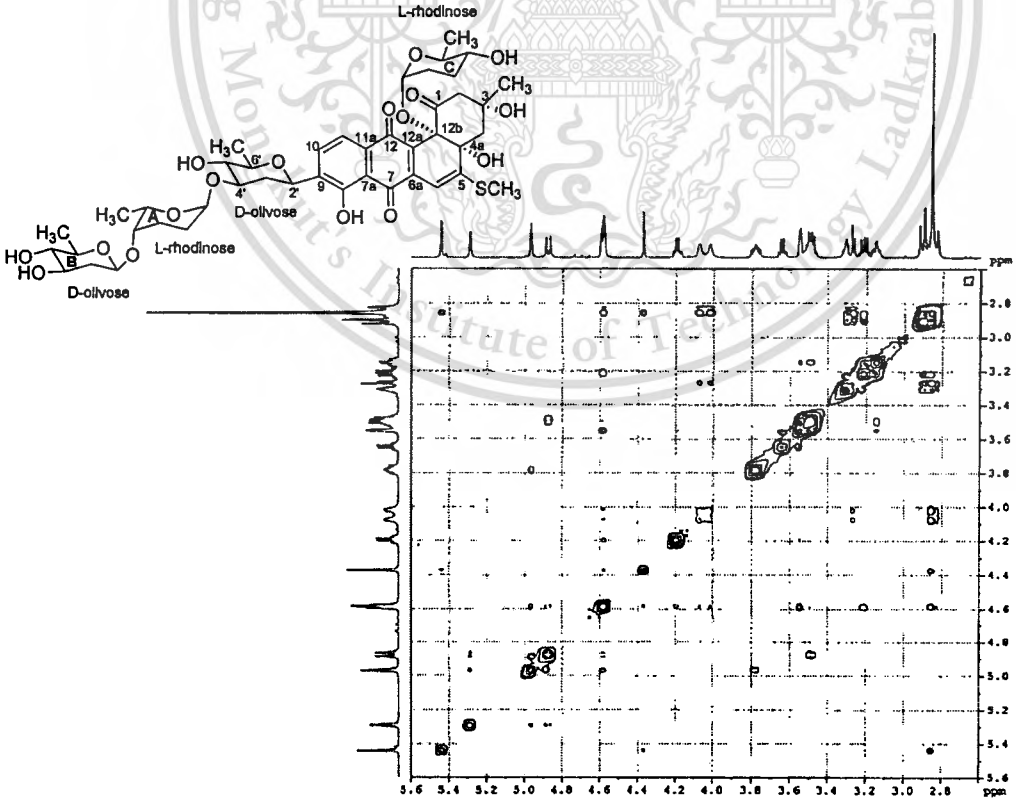


Fig. 60 NOESY spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 57)

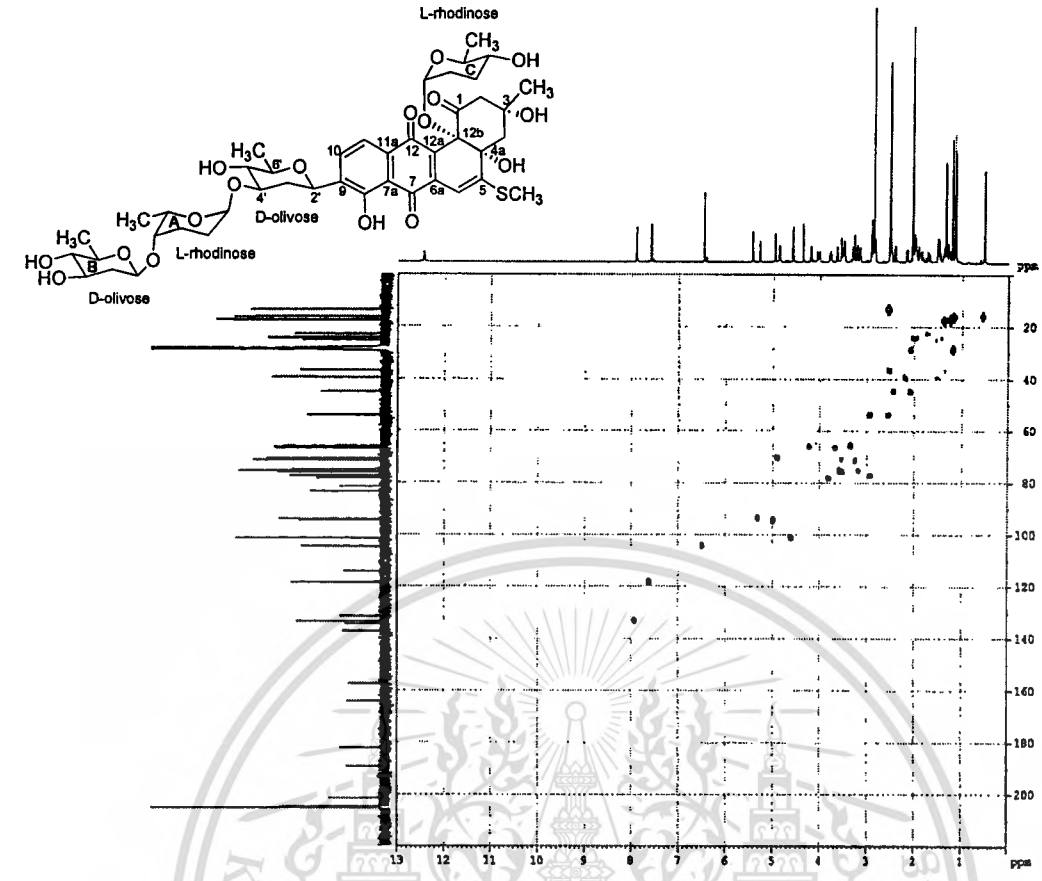


Fig. 61 HMQC spectrum (500 MHz, acetone-d_6) of compound S6.1E

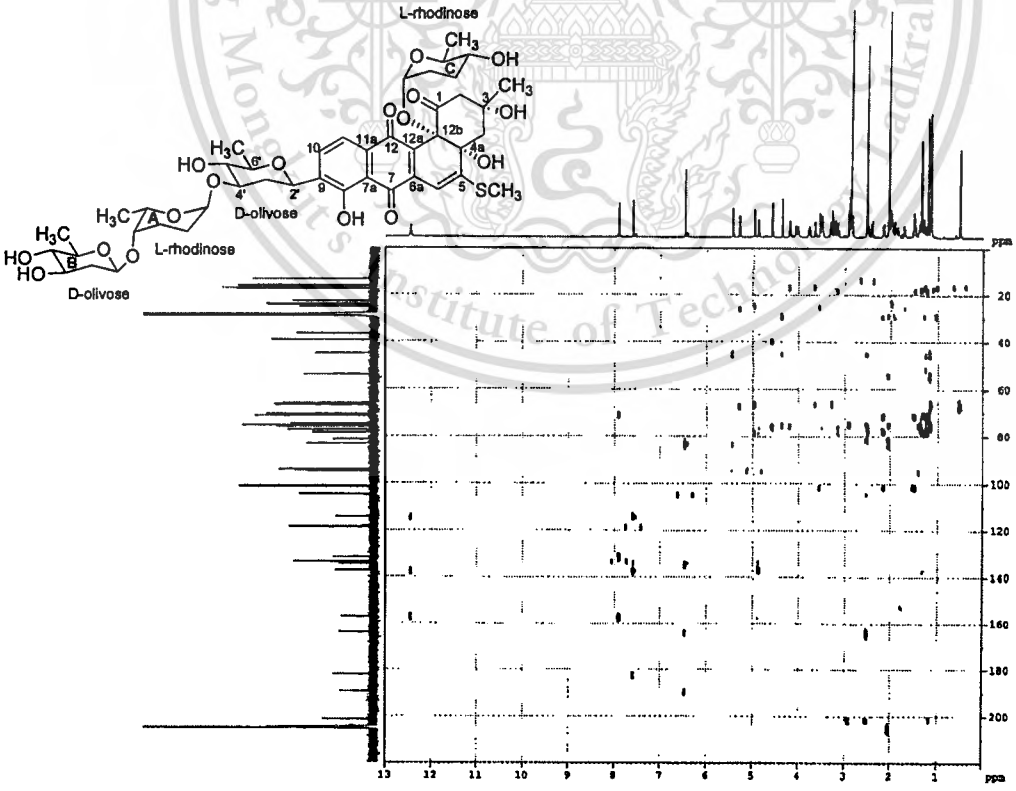


Fig. 62 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1E

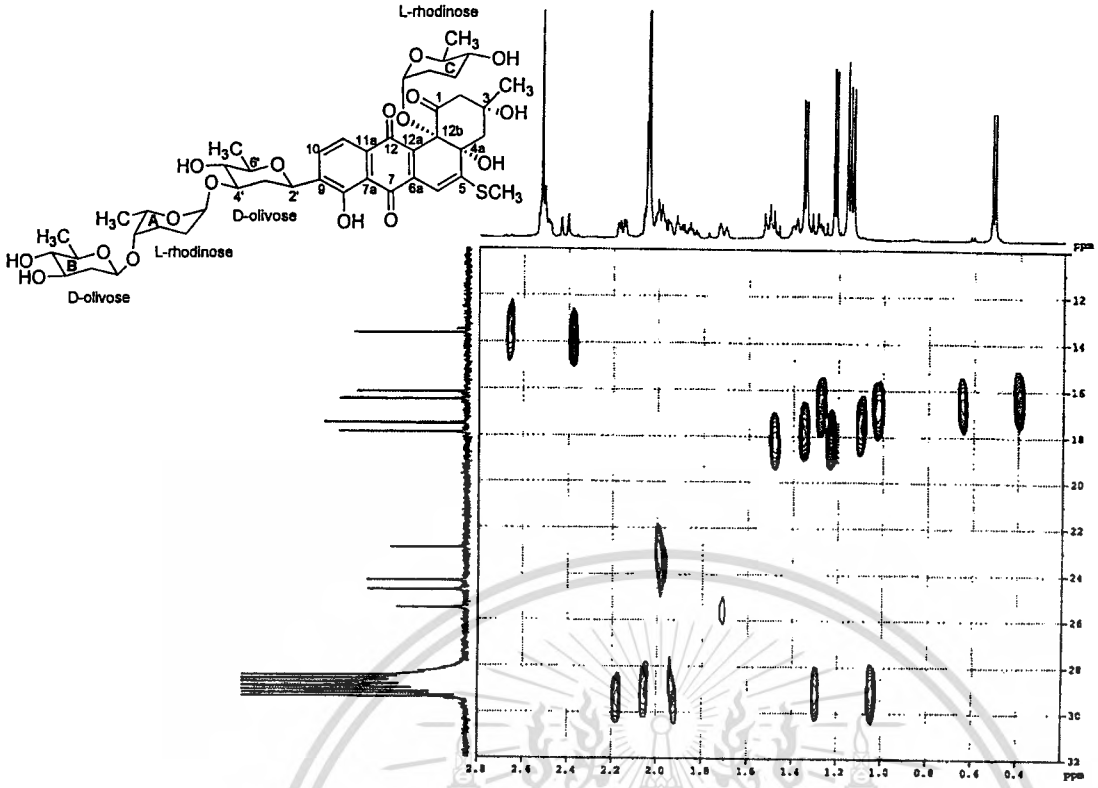


Fig. 63 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 62)

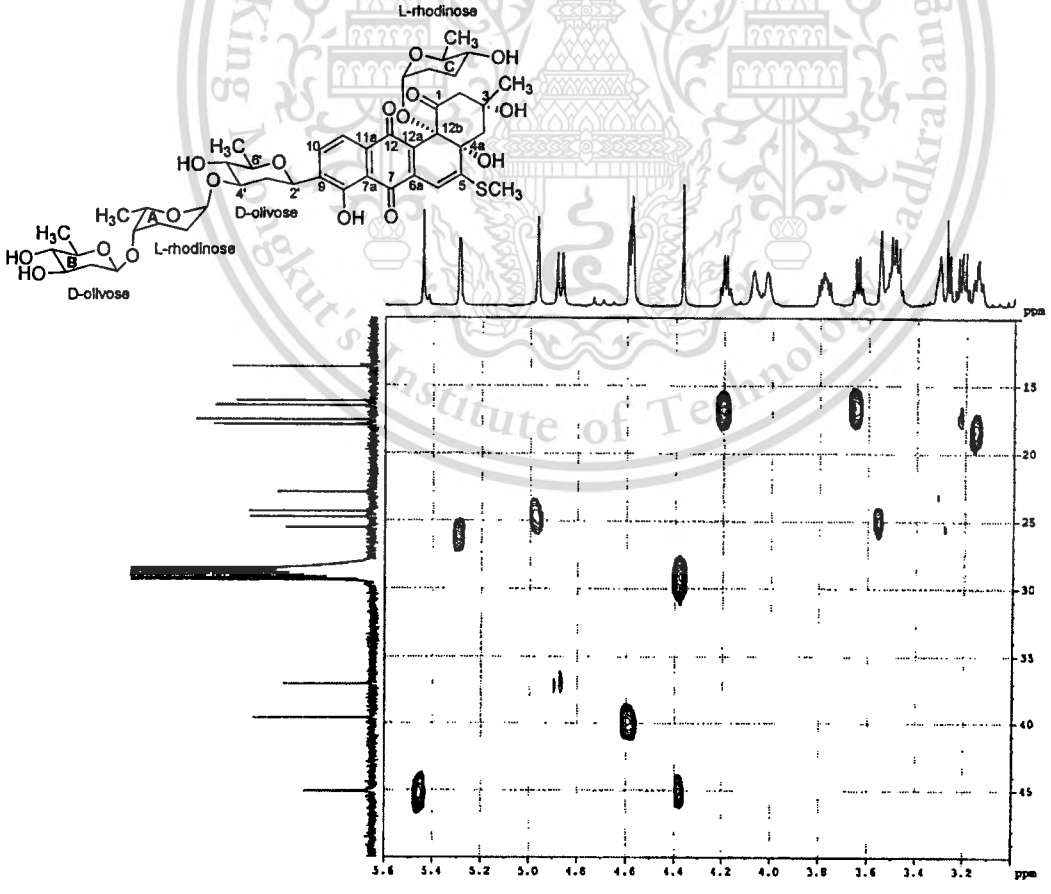


Fig. 64 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 62)

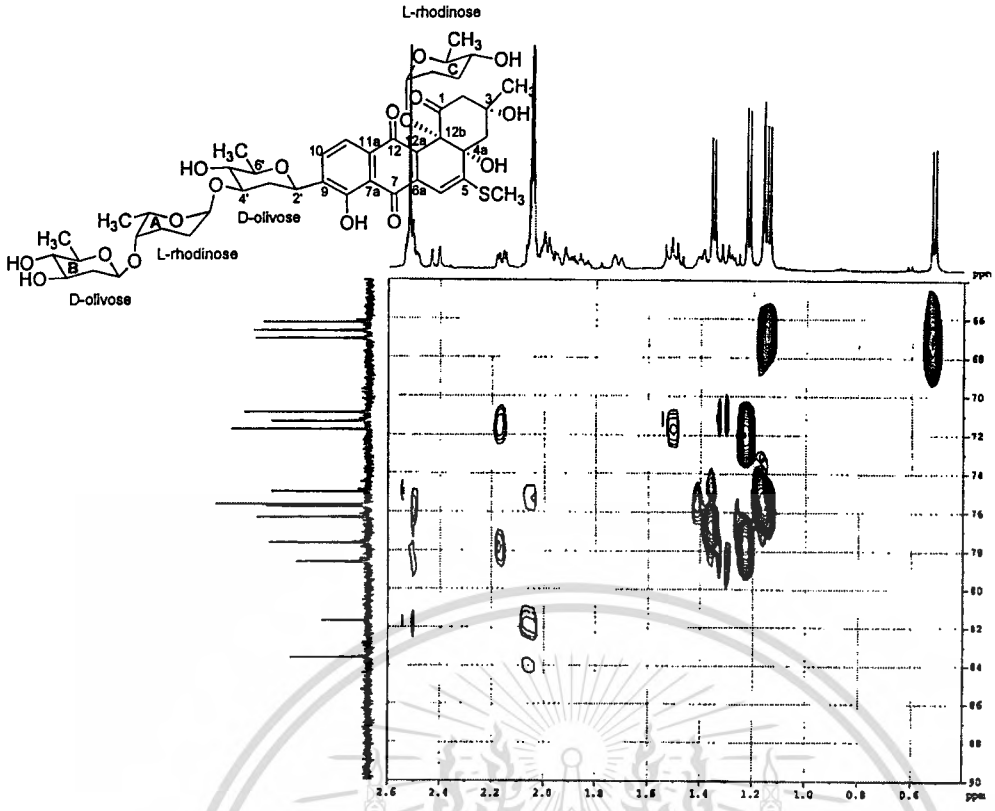


Fig. 65 HMBC spectrum (500 MHz, acetone-d_6) of compound S6.1E (expansion of Fig. 62)

Compound S8.12A (7,8,9,10-tetrahydro-hydroxy-1-methoxy-9-propyltetracene-6,11-dione)

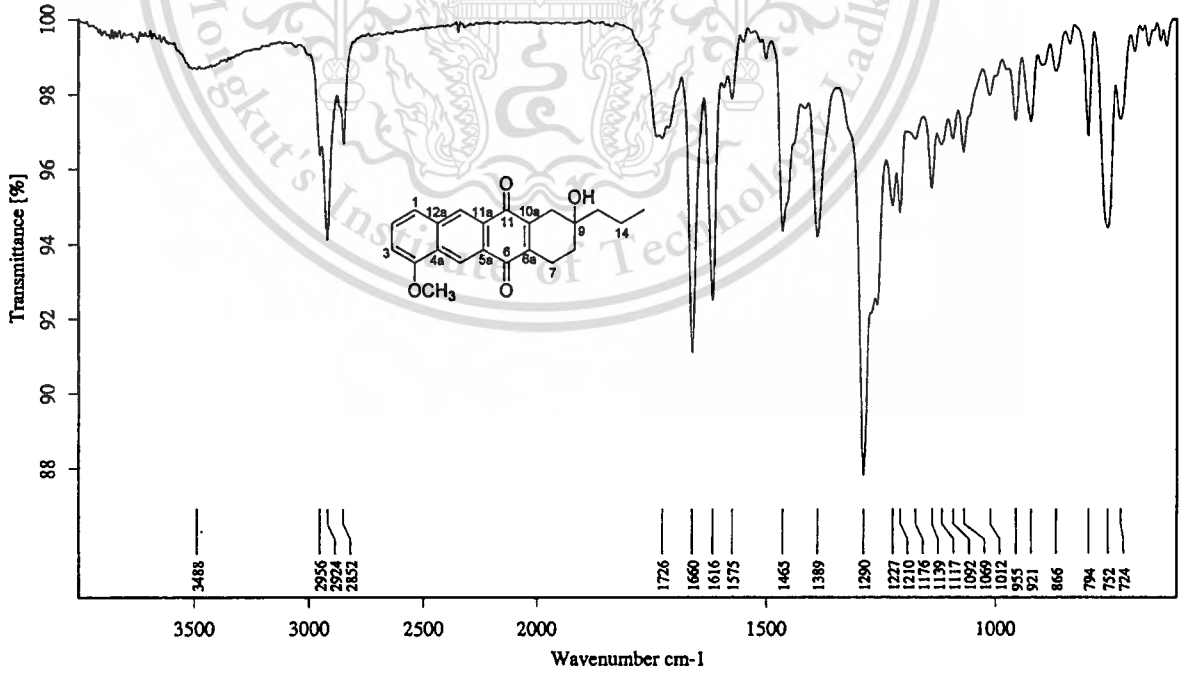


Fig. 66 IR spectrum of compound S8.12A

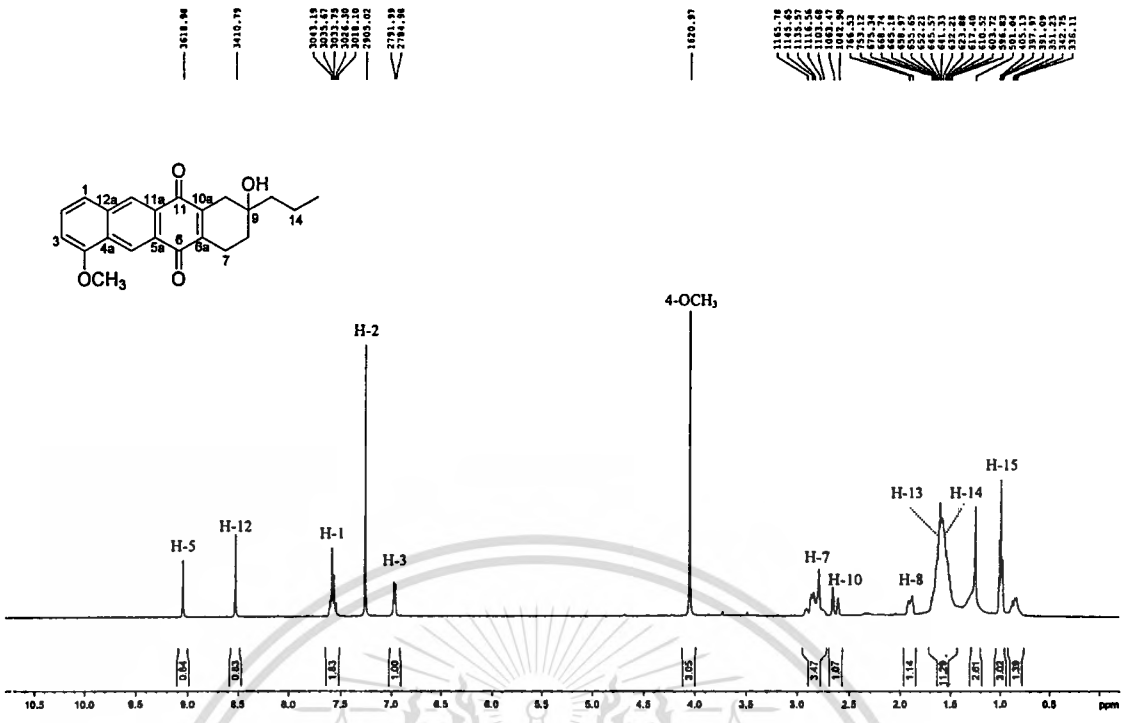


Fig. 67 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound S8.12A

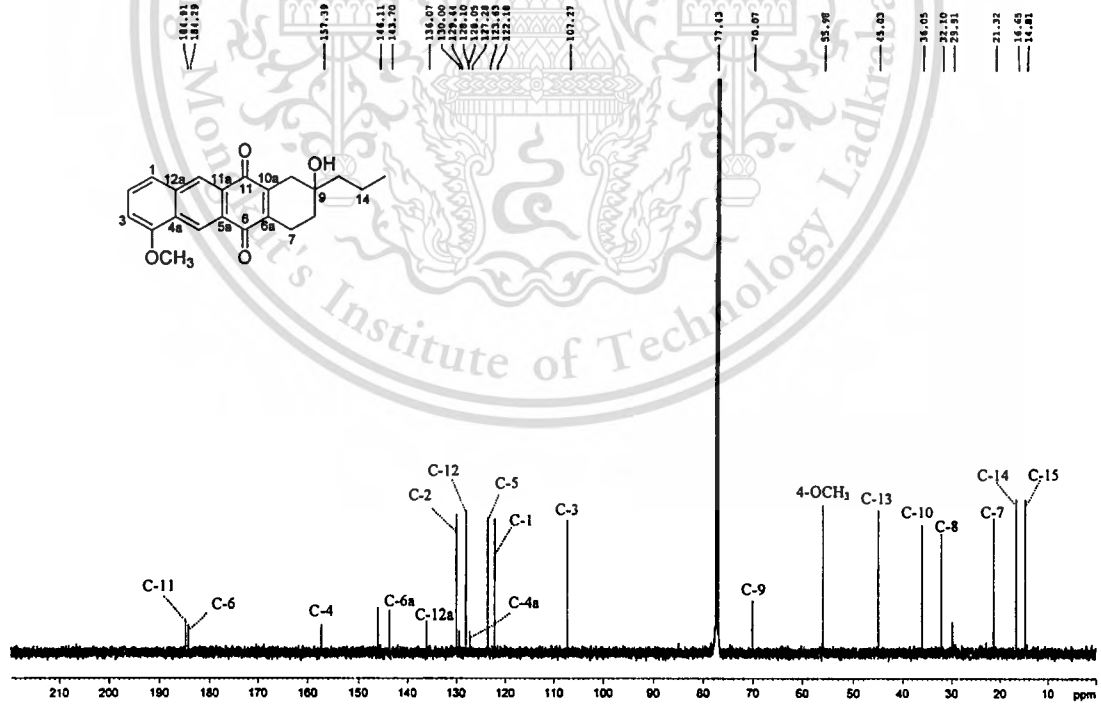


Fig. 68 ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of compound S8.12A

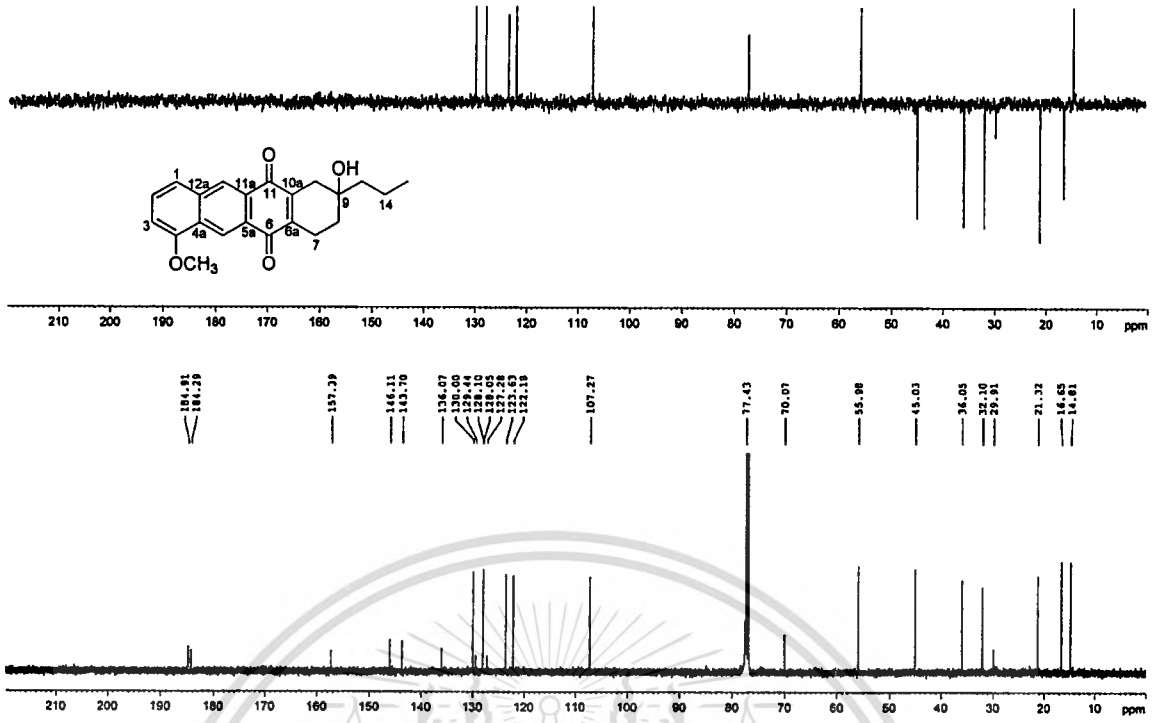


Fig. 69 DEPT 135 spectrum (400 MHz, $CDCl_3$) of compound S8.12A

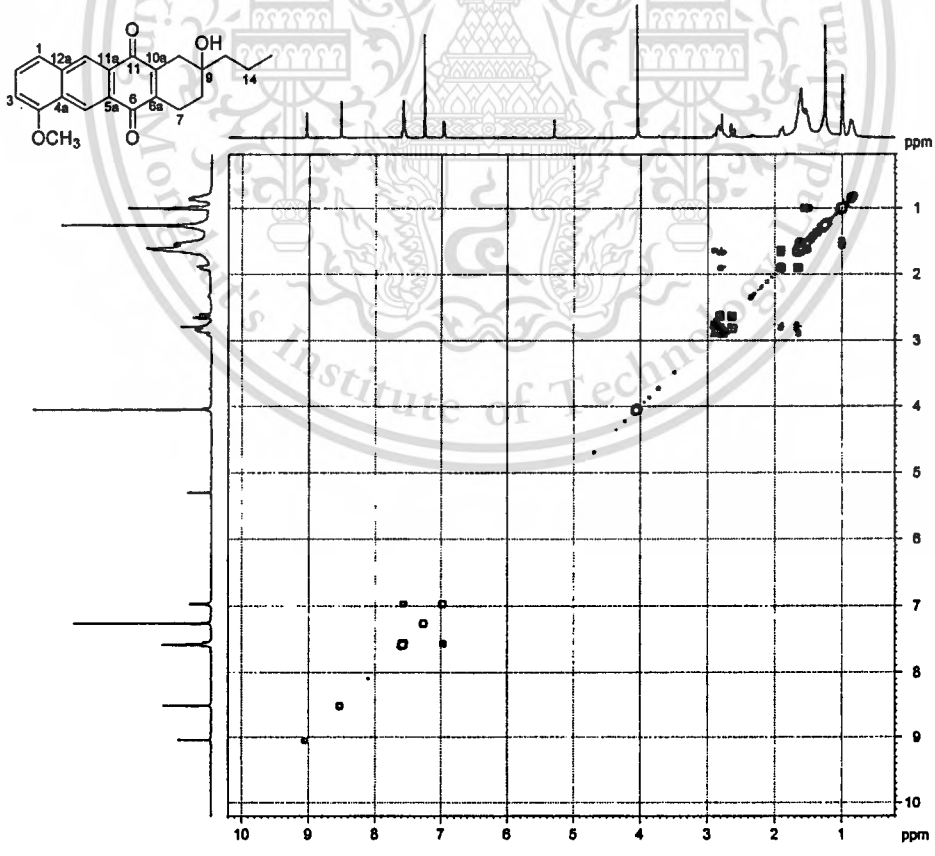


Fig. 70 COSY spectrum (400 MHz, $CDCl_3$) of compound S8.12A

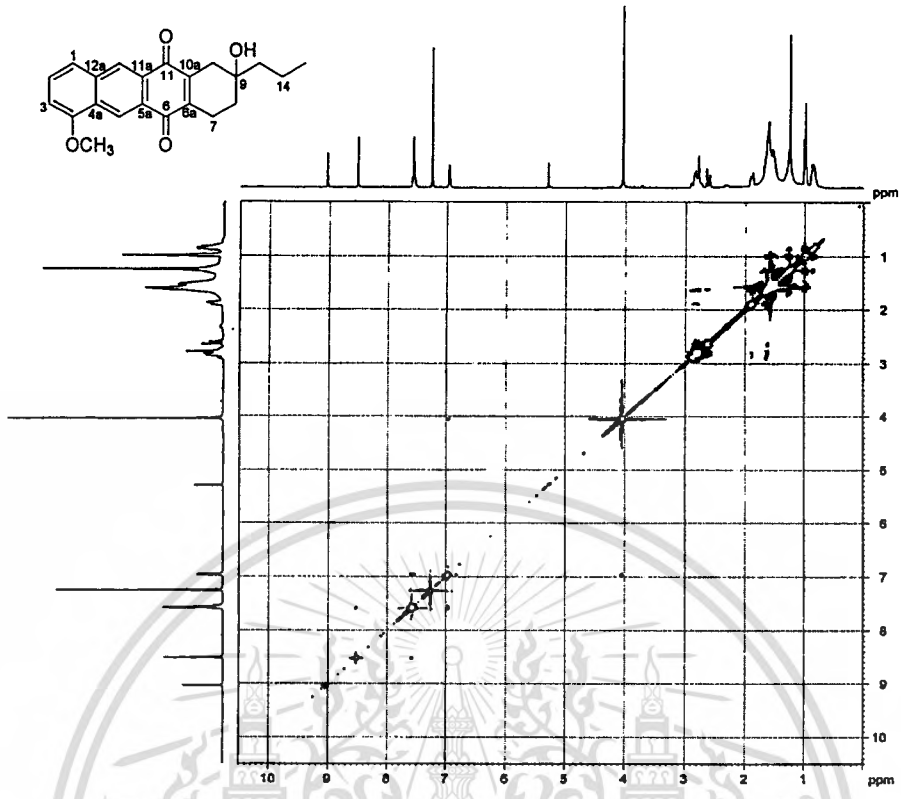


Fig. 71 NOESY spectrum (400 MHz, $CDCl_3$) of compound S8.12A

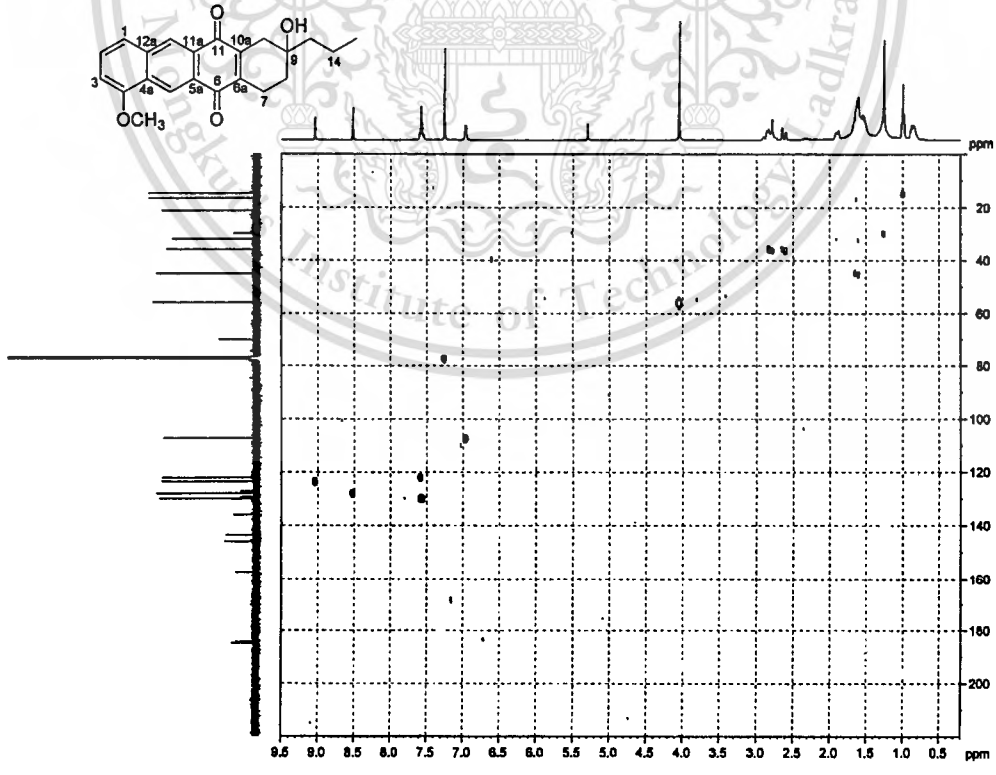


Fig. 72 HMQC spectrum (400 MHz, $CDCl_3$) of compound S8.12A

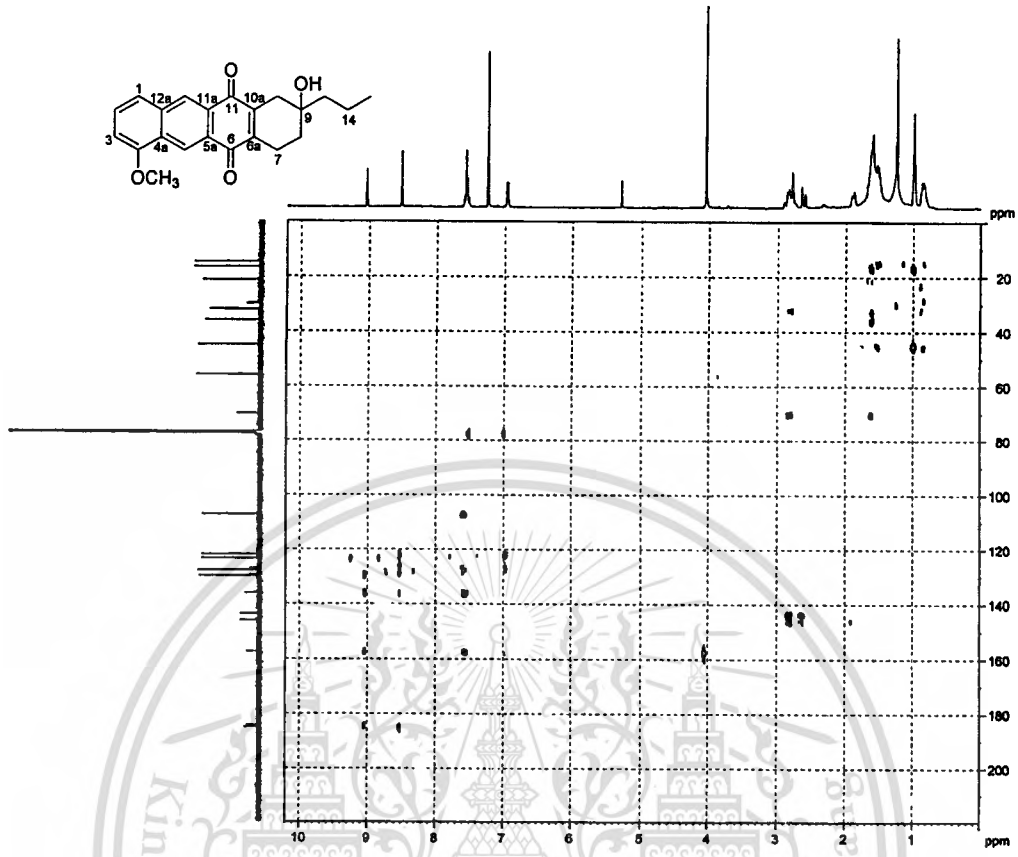


Fig. 73 HMBC spectrum (400 MHz, $CDCl_3$) of compound S8.12A

Compound S8.12B (6,8-dihydroxy-3,4,5-trimethylisocoumarin)

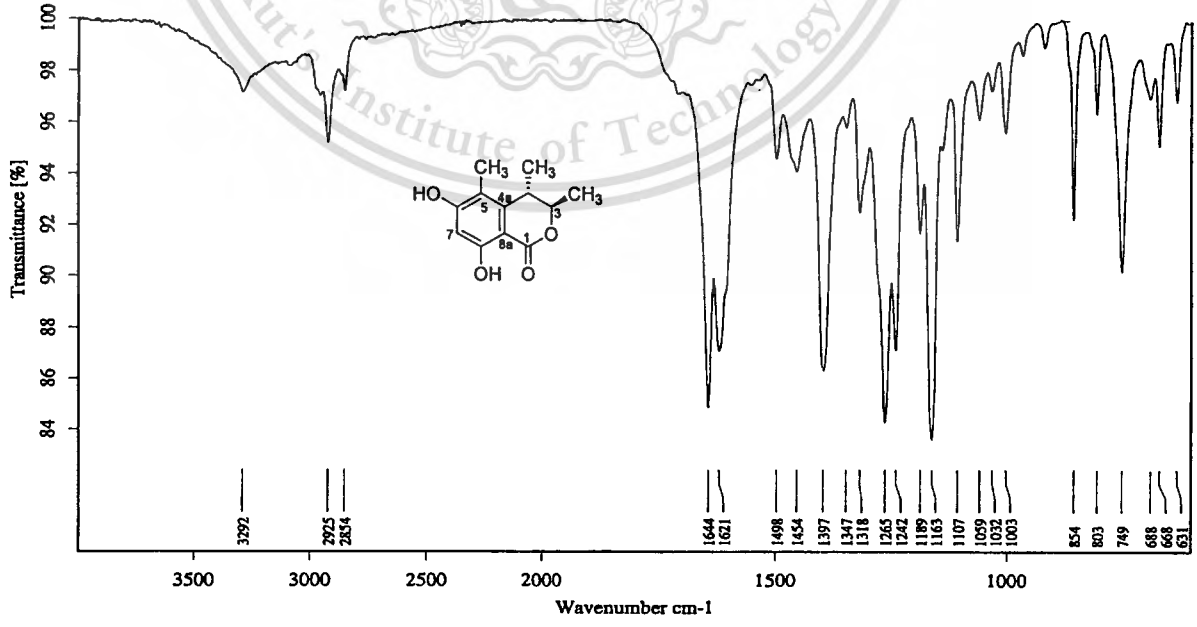


Fig. 74 IR spectrum of compound S8.12B

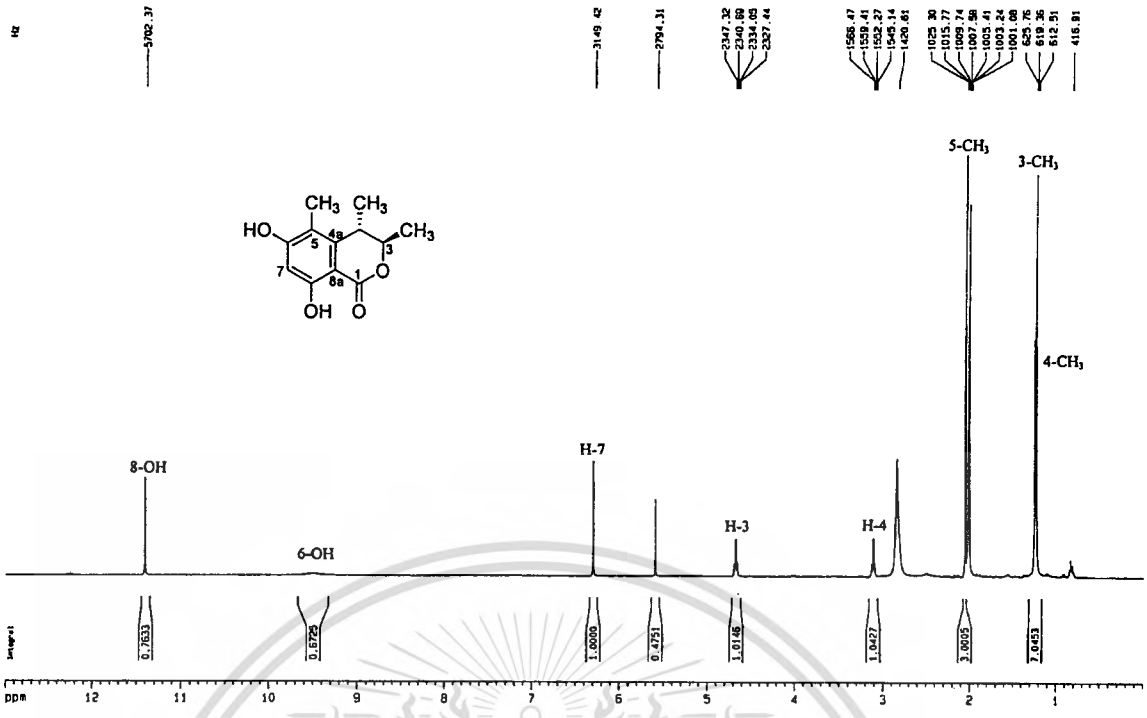


Fig. 75 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound S8.12B

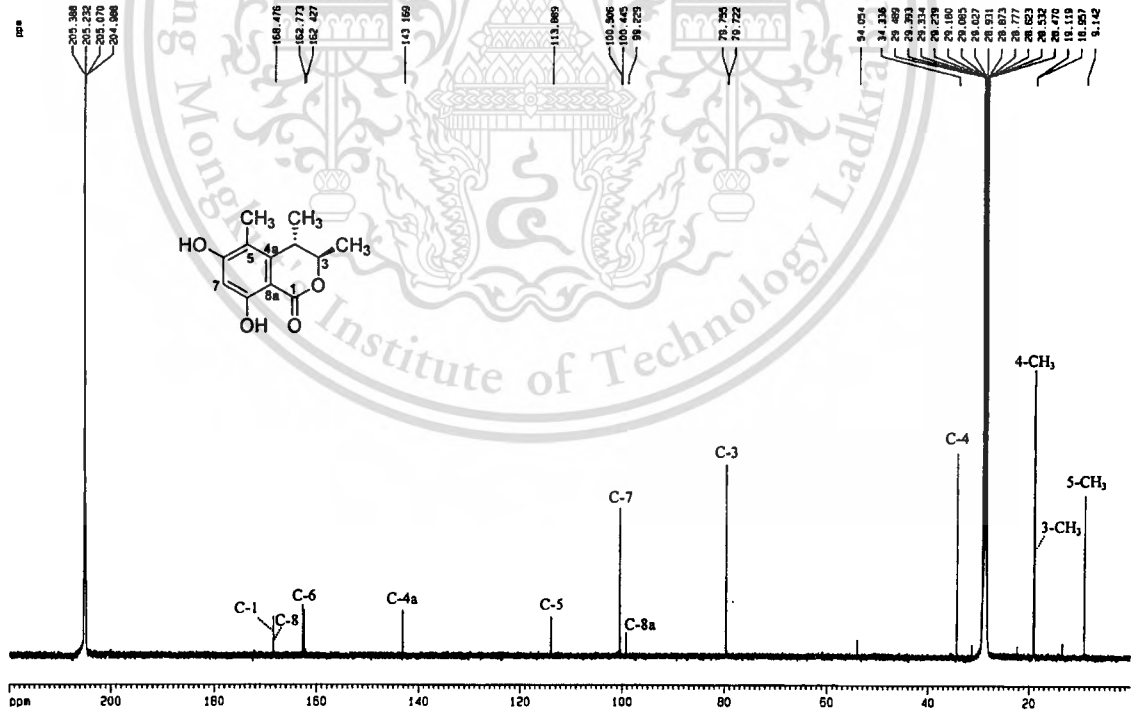


Fig. 76 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound S8.12B

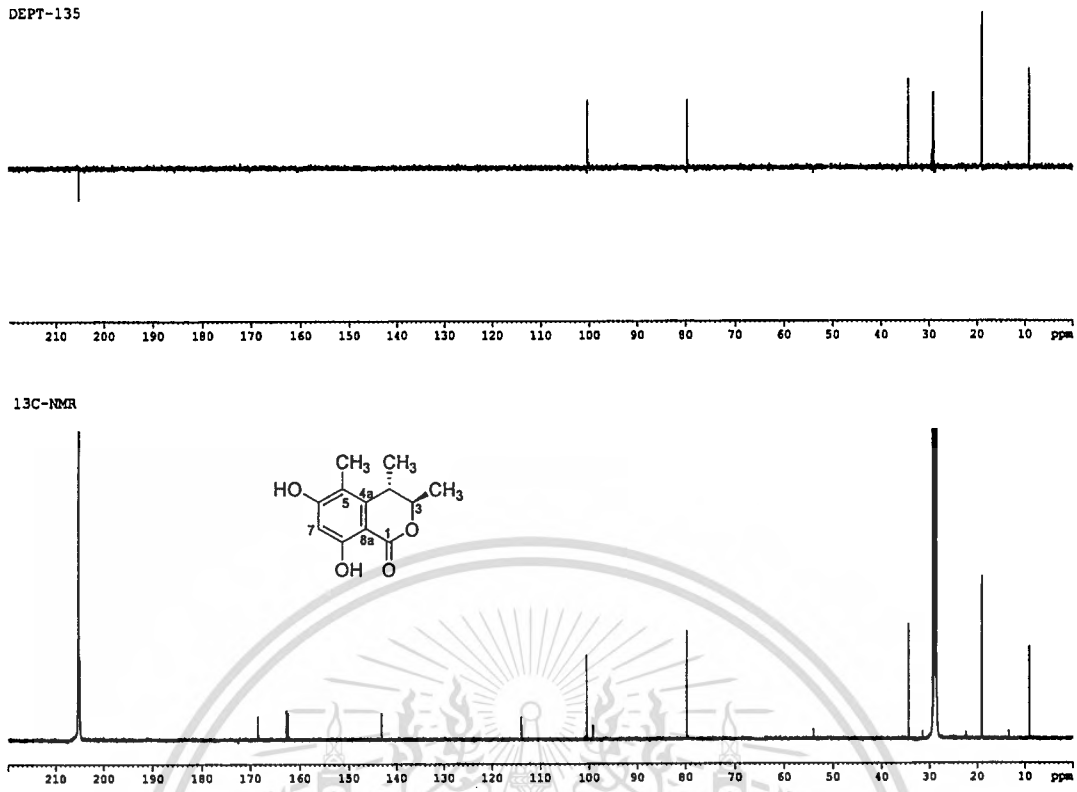


Fig. 77 DEPT 135 spectrum (500 MHz, acetone-d_6) of compound S8.12B

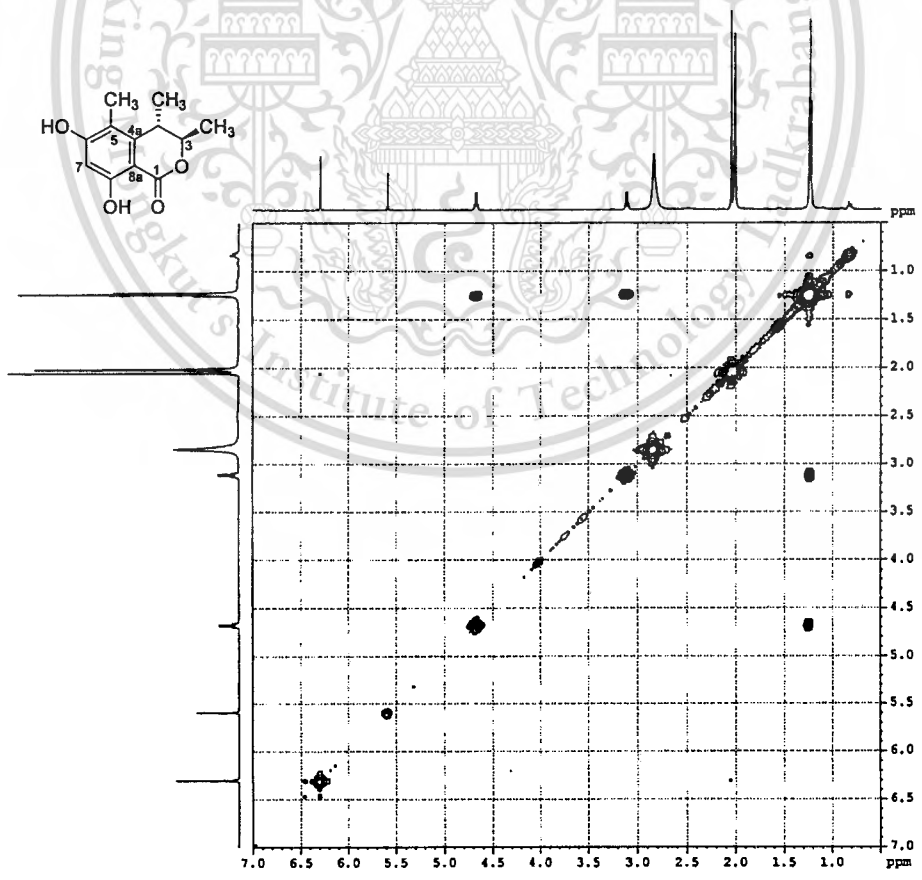


Fig. 78 COSY spectrum (500 MHz, acetone-d_6) of compound S8.12B

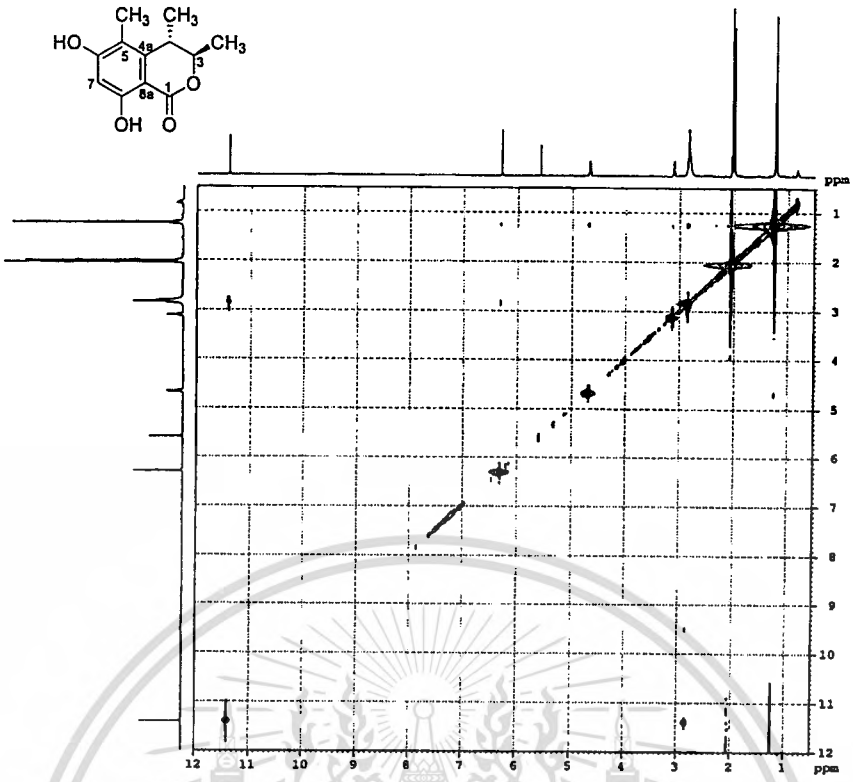


Fig. 79 NOESY spectrum (500 MHz, acetone-d_6) of compound S8.12B

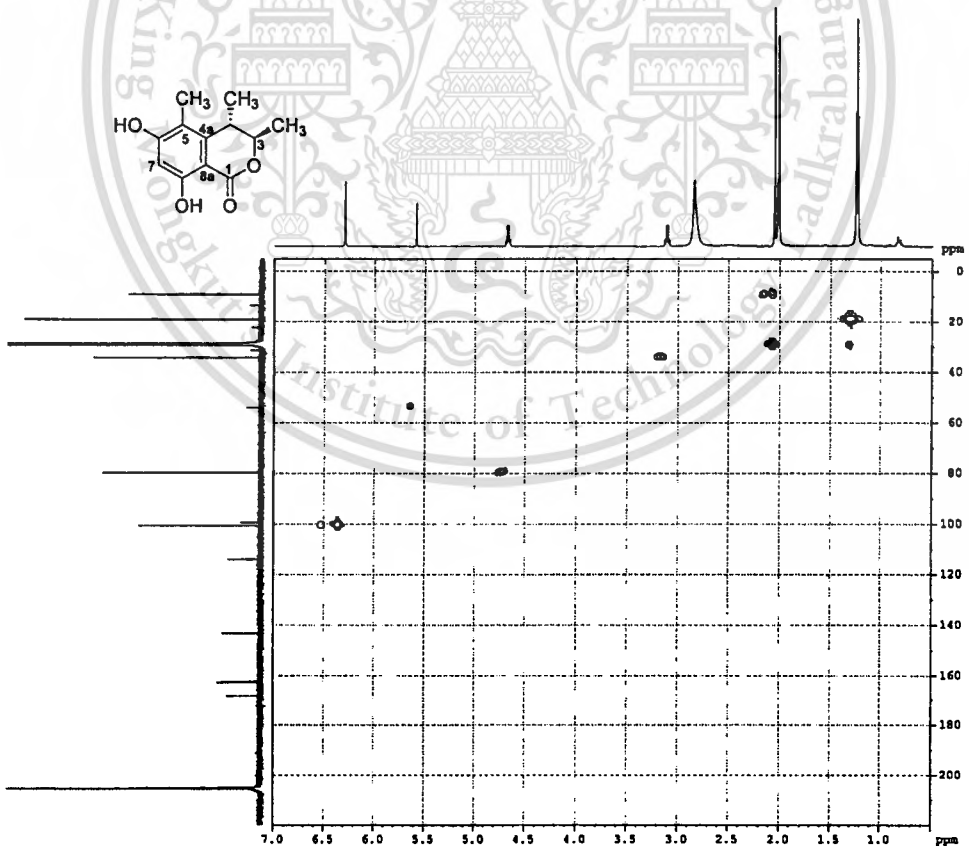


Fig. 80 HMQC spectrum (500 MHz, acetone-d_6) of compound S8.12B

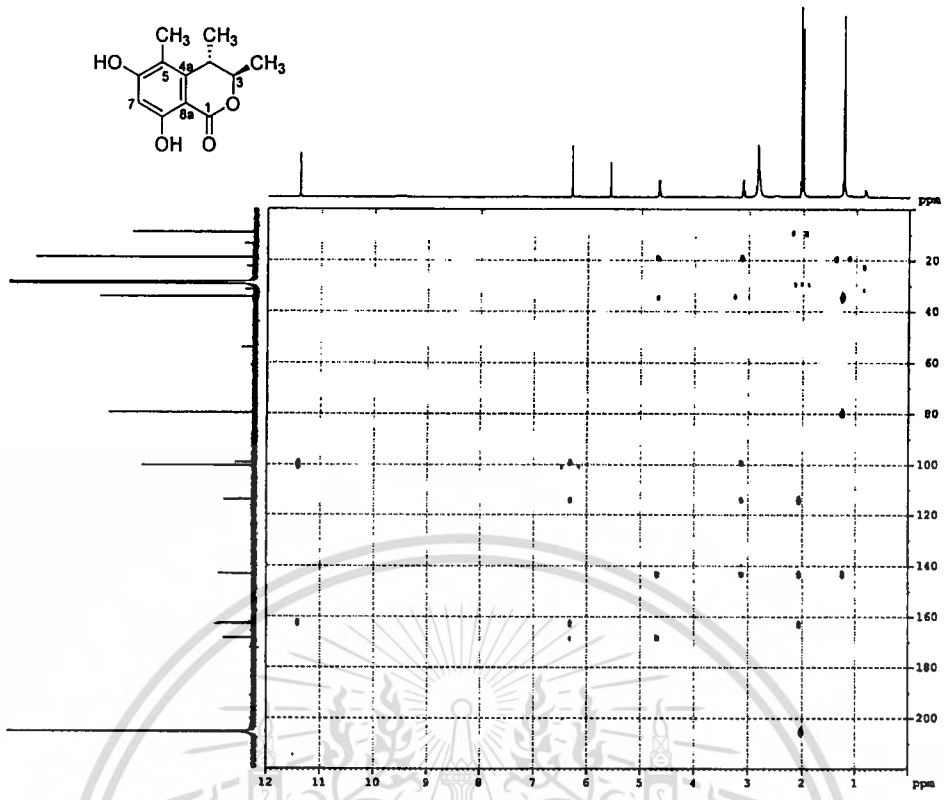


Fig. 81 HMBC spectrum (500 MHz, acetone-d_6) of compound S8.12B

Compound S8.12C (2,6-dihydroxy-4-(2-hydroxy-1-methylpropyl)-5-methylbenzoic acid)

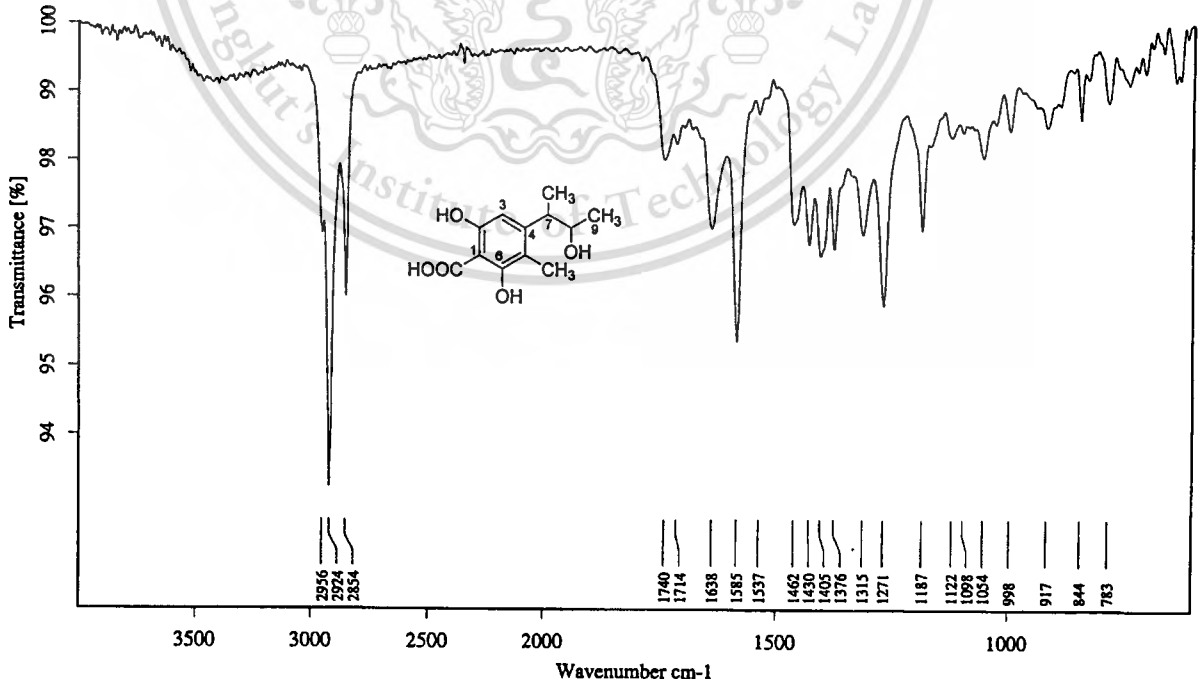


Fig. 82 IR spectrum of compound S8.12C

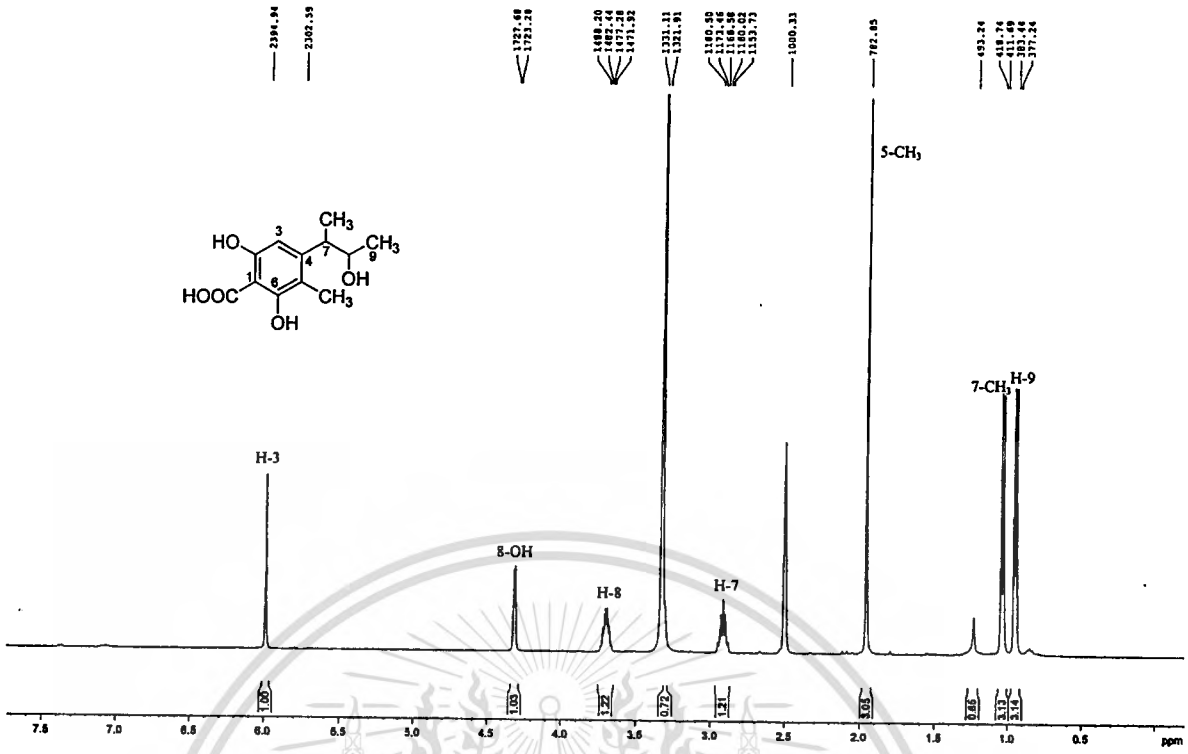


Fig. 83 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound S8.12C

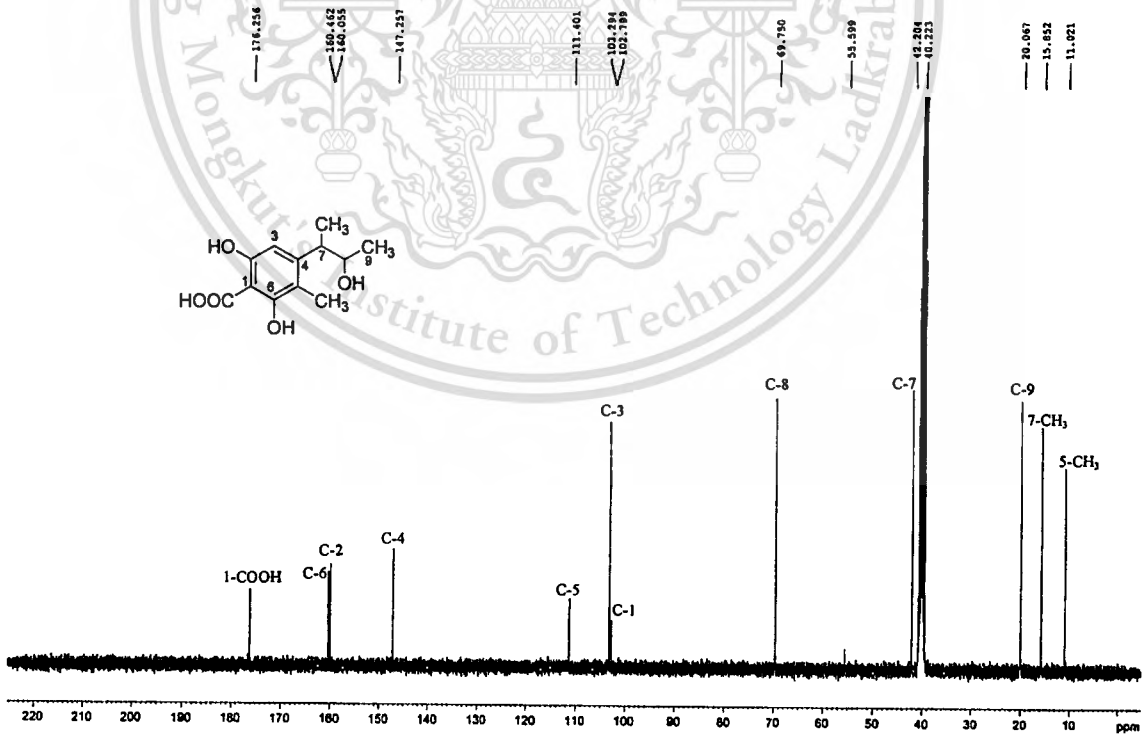


Fig. 84 ^{13}C NMR spectrum (400 MHz, DMSO-d_6) of compound S8.12C

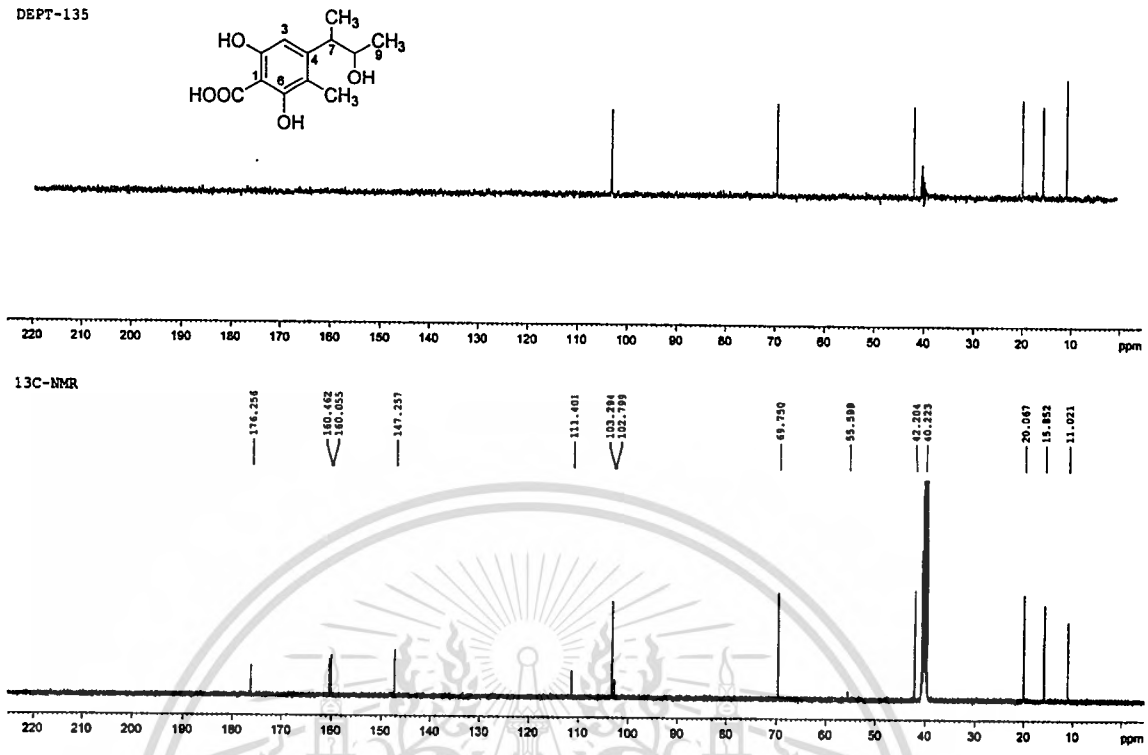


Fig. 85 DEPT 135 spectrum (400 MHz, DMSO-d_6) of compound S8.12C

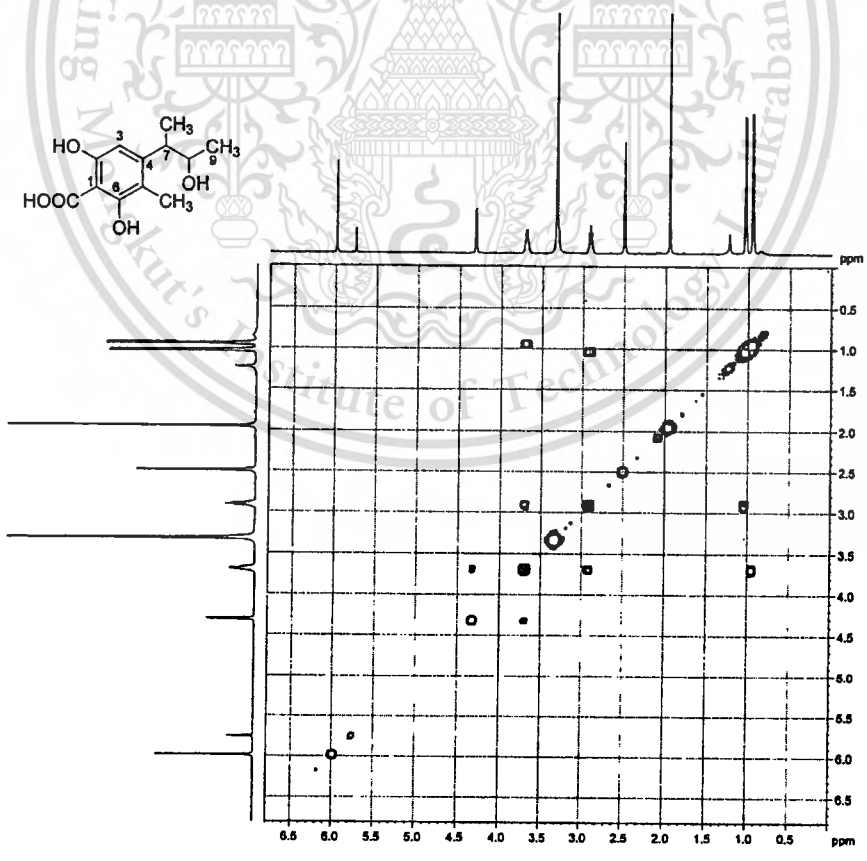


Fig. 86 COSY spectrum (400 MHz, DMSO-d_6) of compound S8.12C

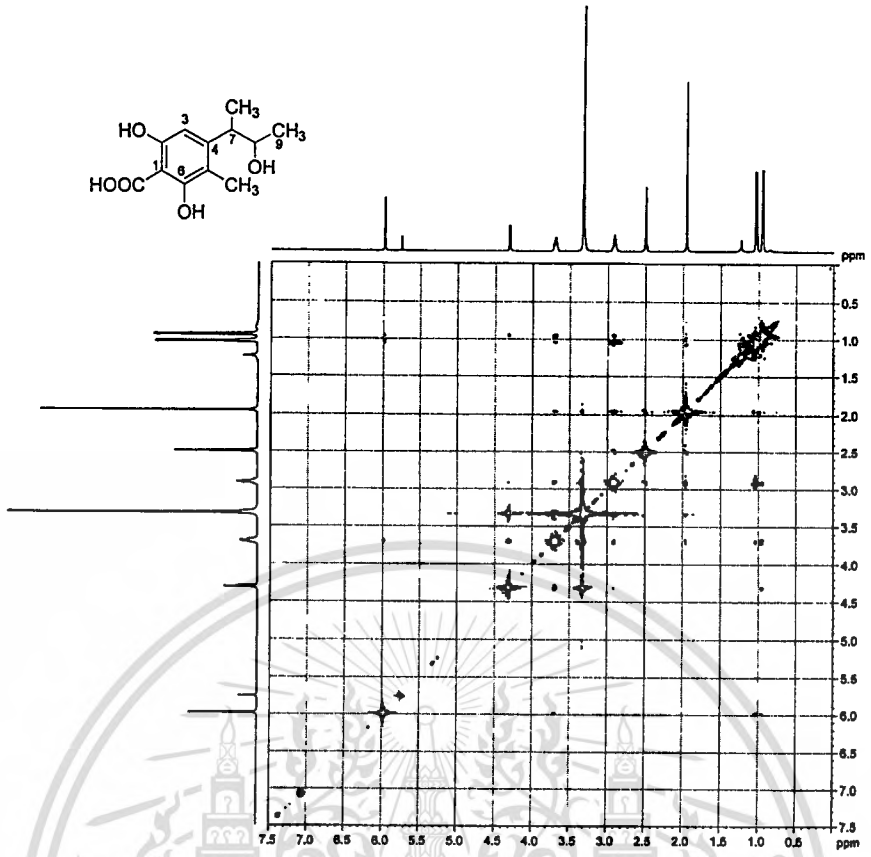


Fig. 87 NOESY spectrum (400 MHz, DMSO-d_6) of compound S8.12C

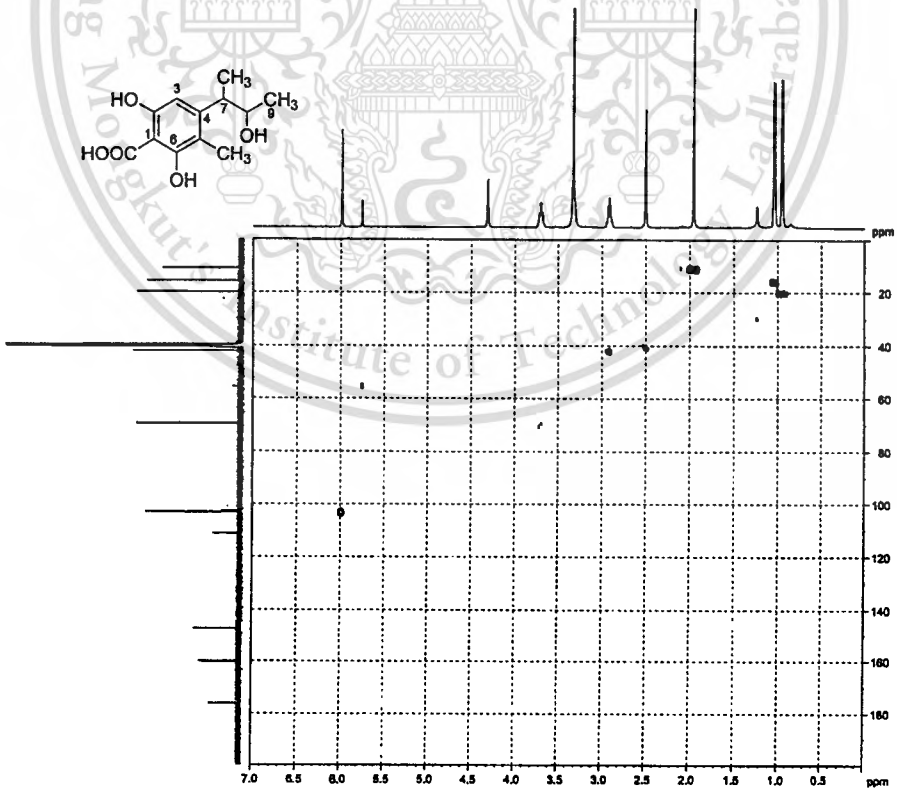


Fig. 88 HMQC spectrum (400 MHz, DMSO-d_6) of compound S8.12C

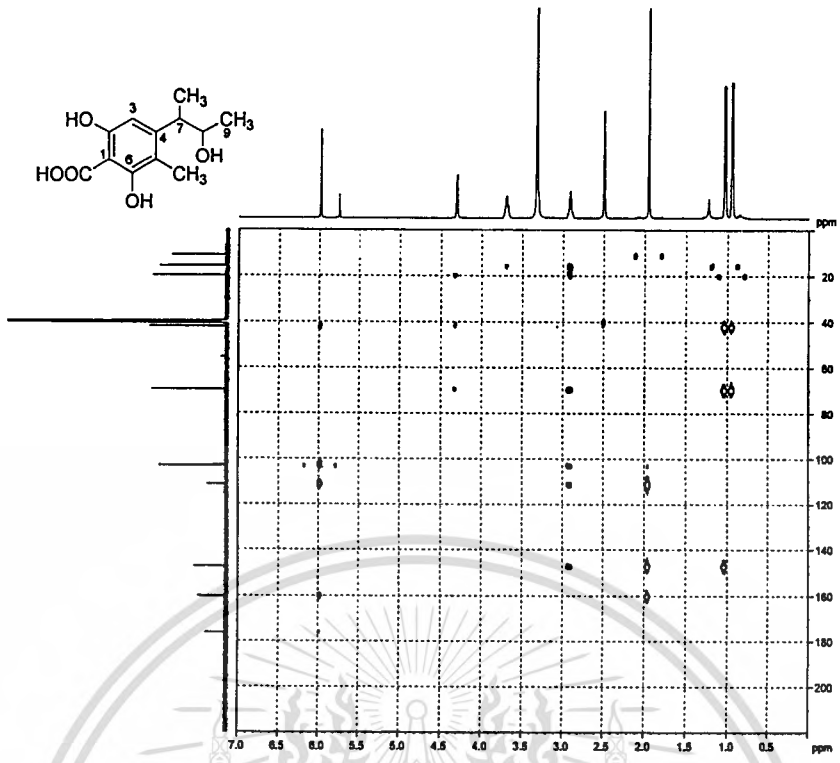


Fig. 89 HMBC spectrum (400 MHz, DMSO-d_6) of compound S8.12C

Compound TV14A (9,10-dihydro-3,8,10-trihydroxy-1,7,10-trimethyl-9-oxo-2-anthracenecarboxylic acid)

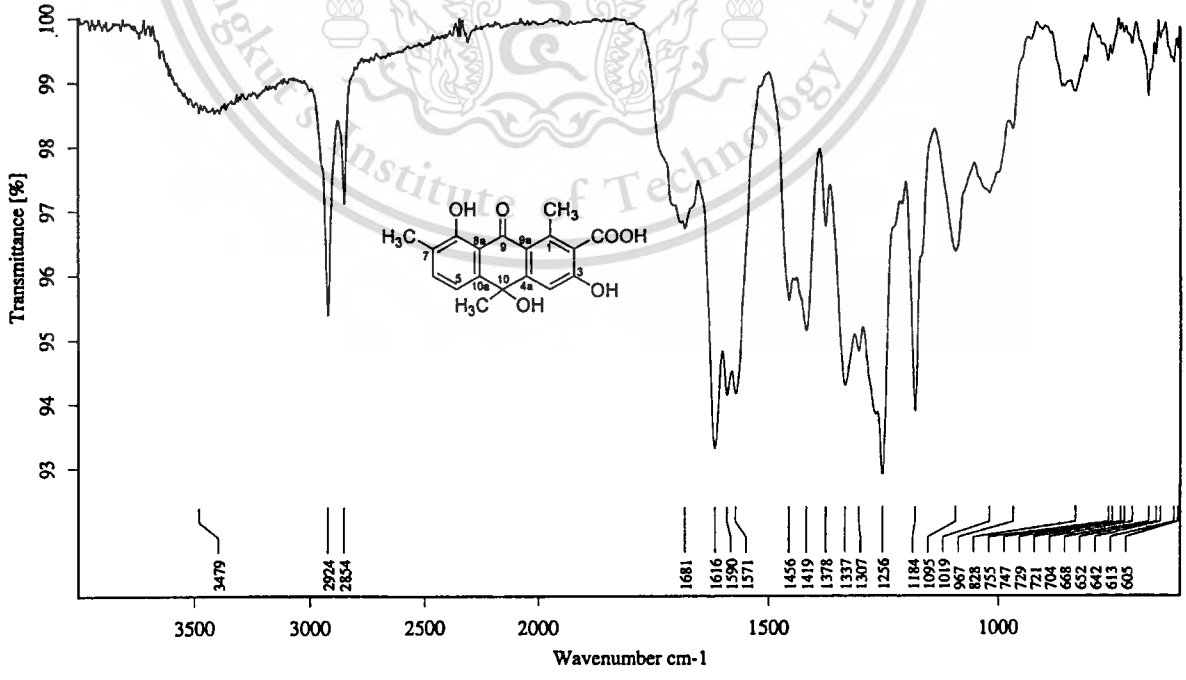


Fig. 90 IR spectrum of compound TV14A

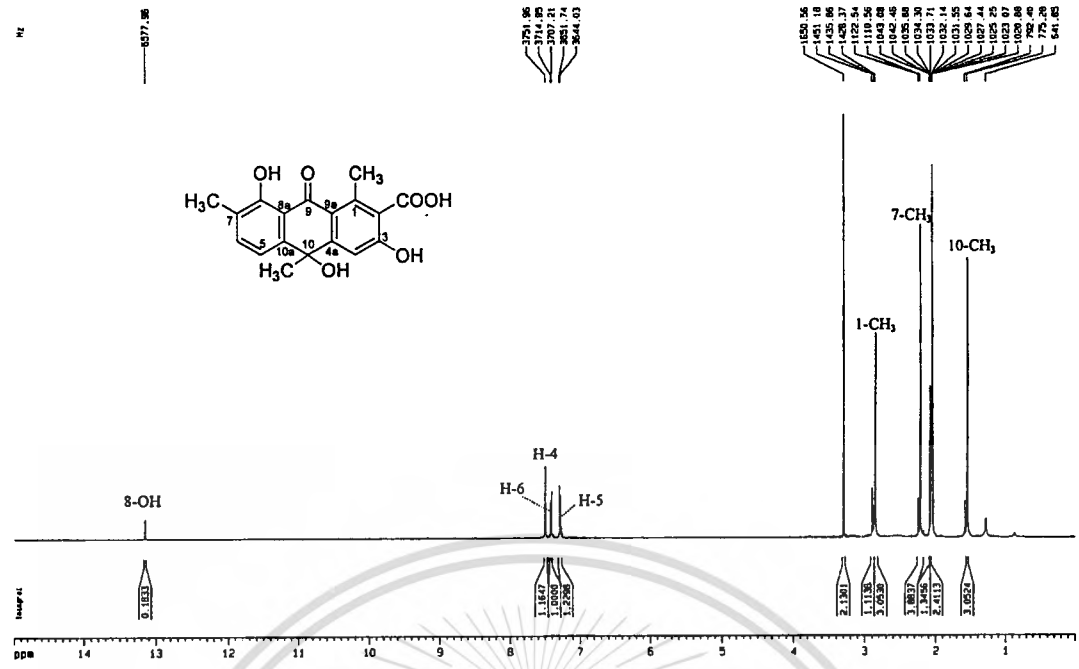


Fig. 91 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound TV14A

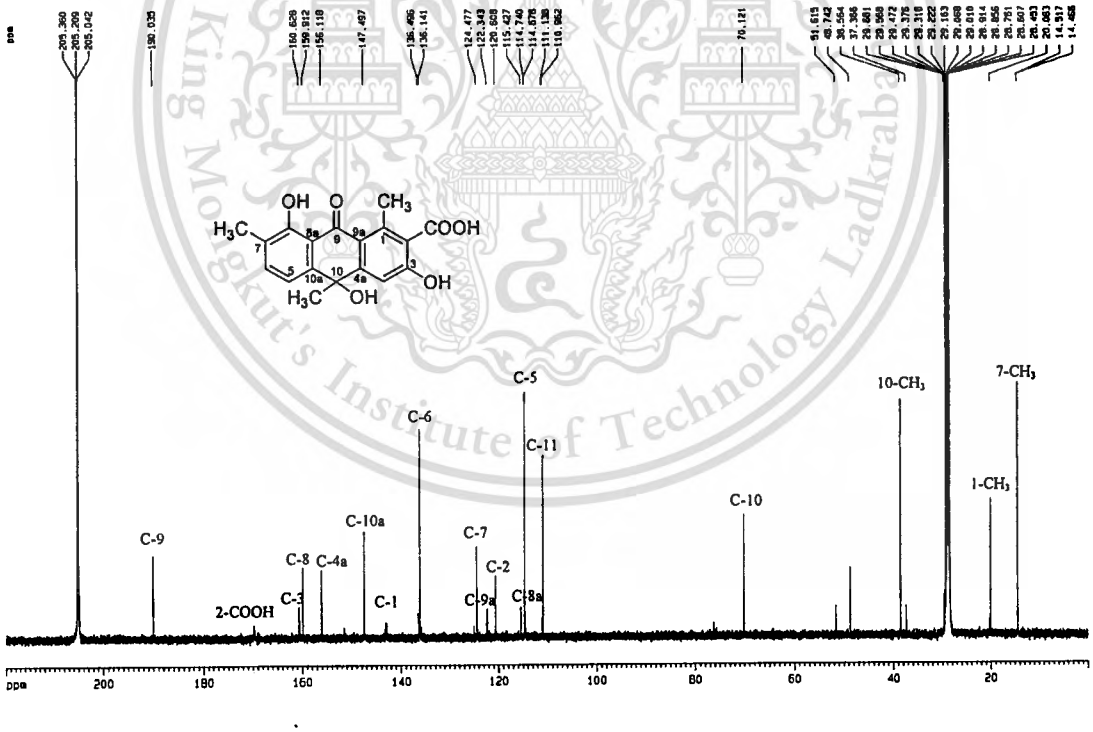


Fig. 92 ^{13}C NMR spectrum (500 MHz, acetone-d_6) of compound TV14A

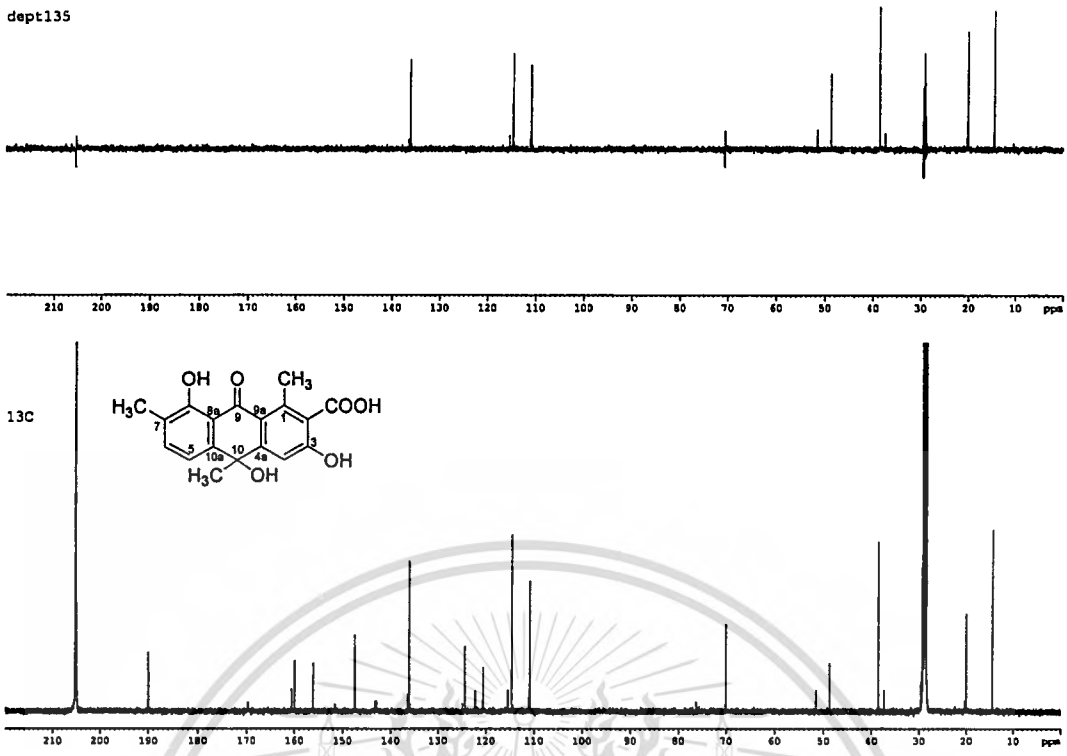


Fig. 93 DEPT 135 spectrum (500 MHz, acetone-d_6) of compound TV14A

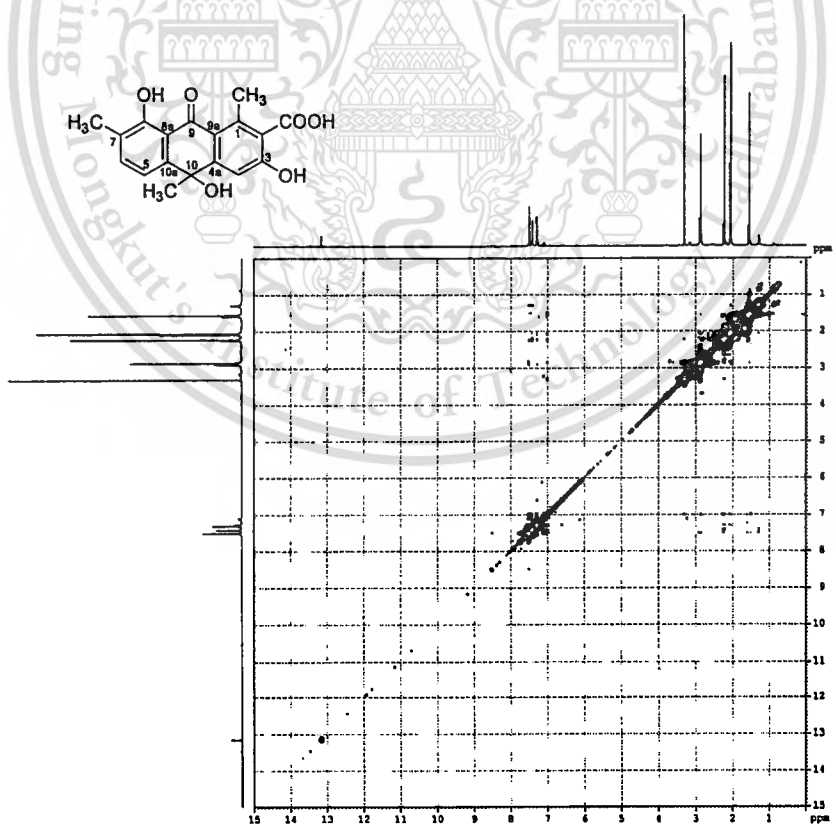


Fig. 94 COSY spectrum (500 MHz, acetone-d_6) of compound TV14A

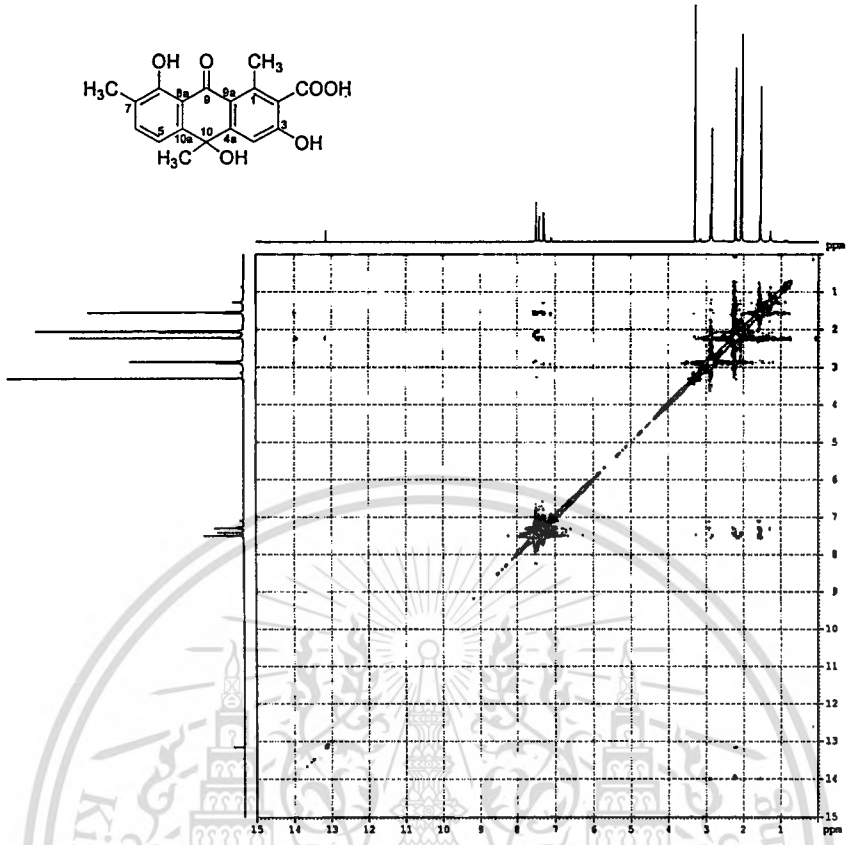


Fig. 95 NOESY spectrum (500 MHz, acetone-d_6) of compound TV14A

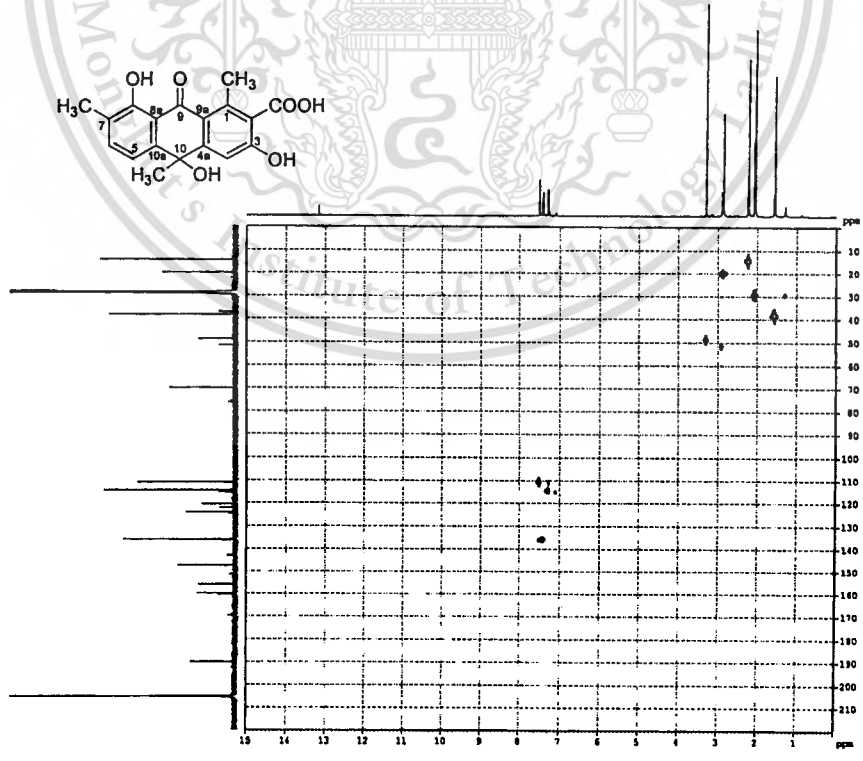


Fig. 96 HMQC spectrum (500 MHz, acetone-d_6) of compound TV14A

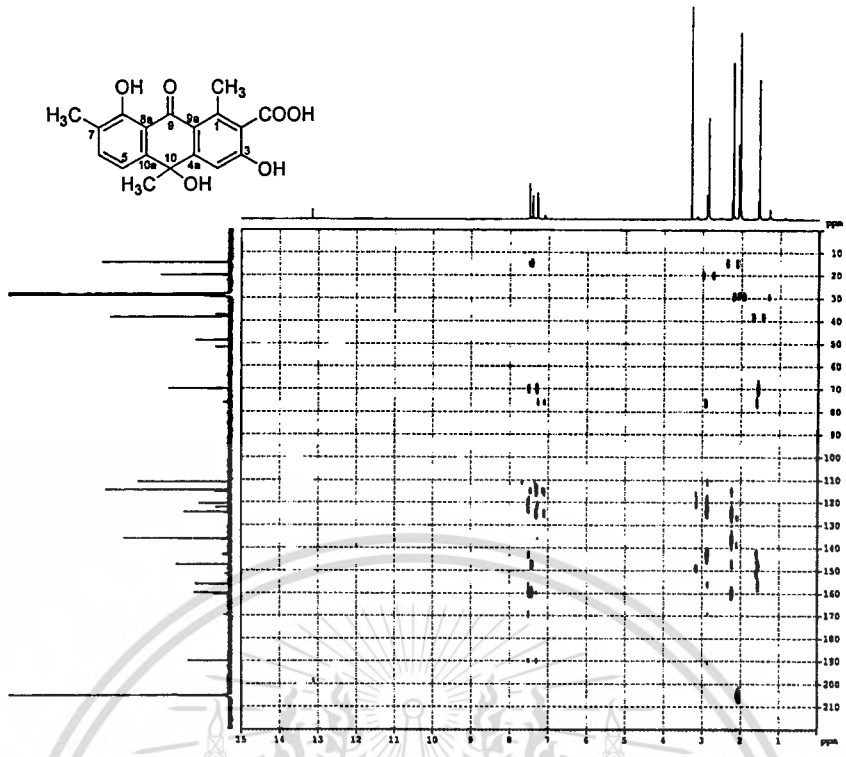


Fig. 97 HMBC spectrum (500 MHz, acetone-d_6) of compound TV14A

Compound TV14B (oxanthromicin)

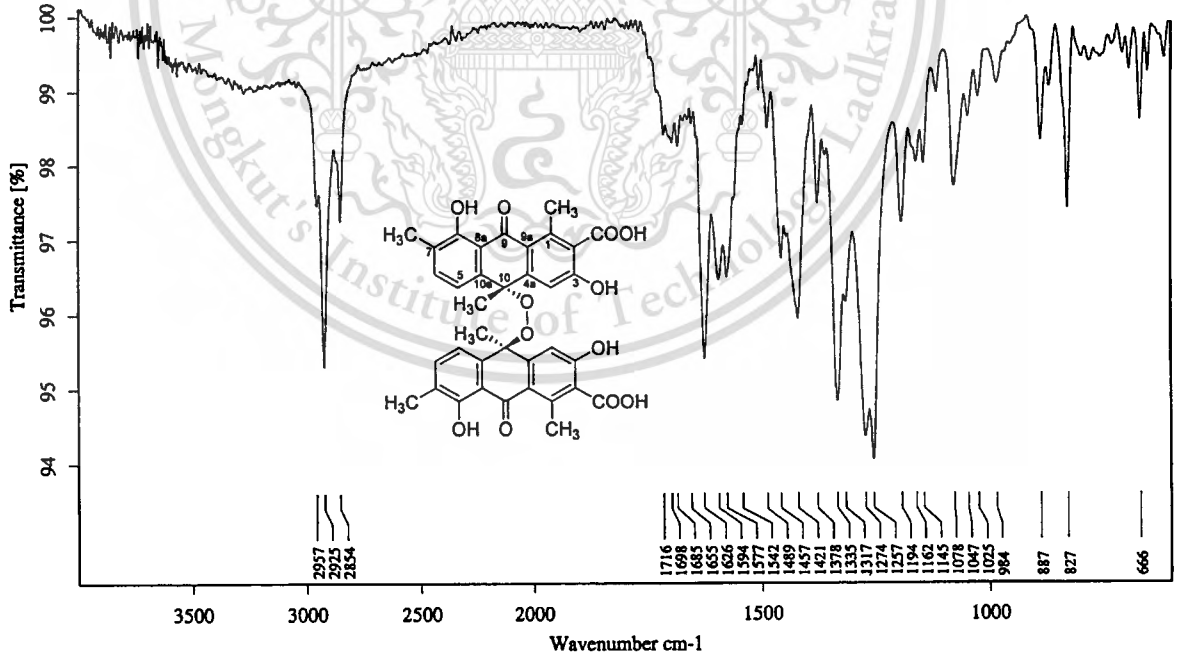


Fig. 98 IR spectrum of compound TV14B

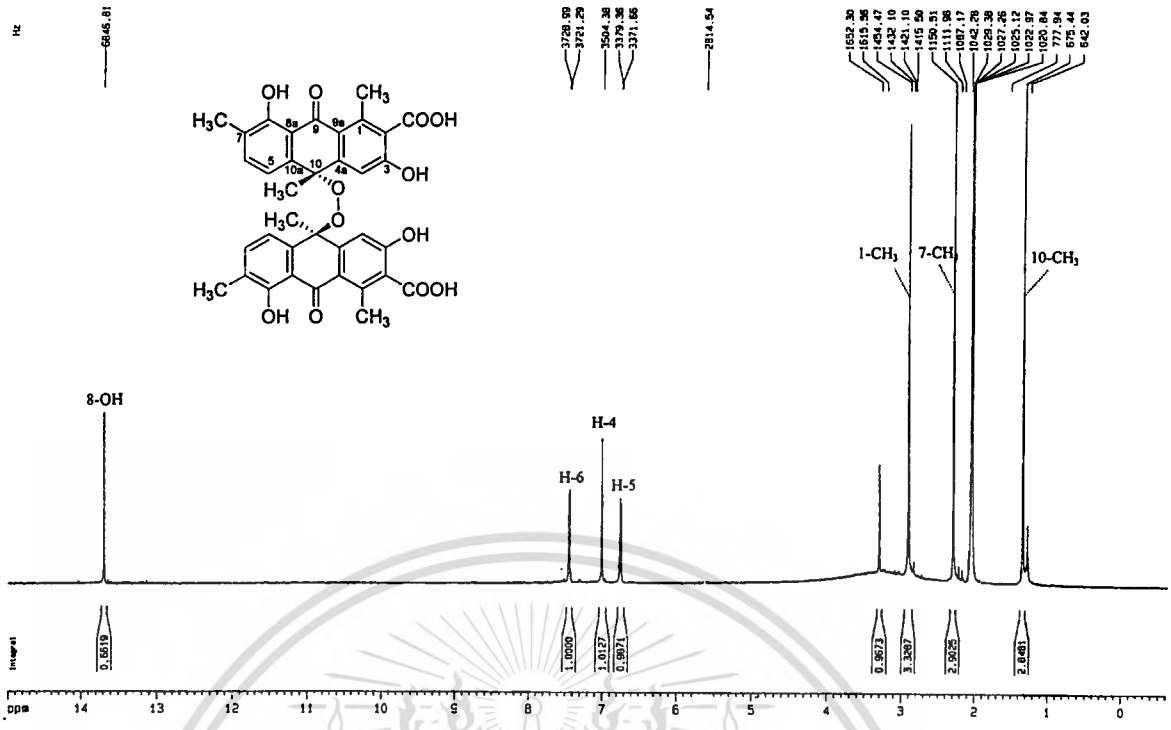


Fig. 99 ^{1}H NMR spectrum (500 MHz, acetone-d_6) of compound TV14B

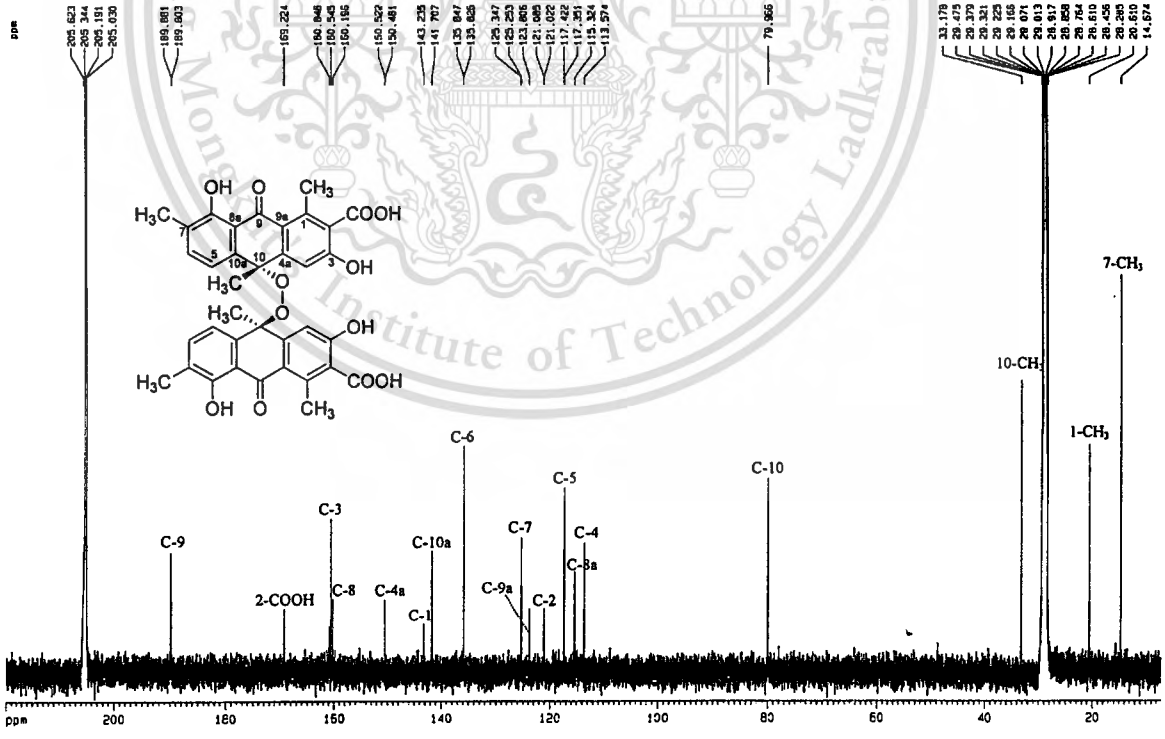


Fig. 100 ^{13}C NMR (500 MHz, acetone-d_6) of compound TV14B

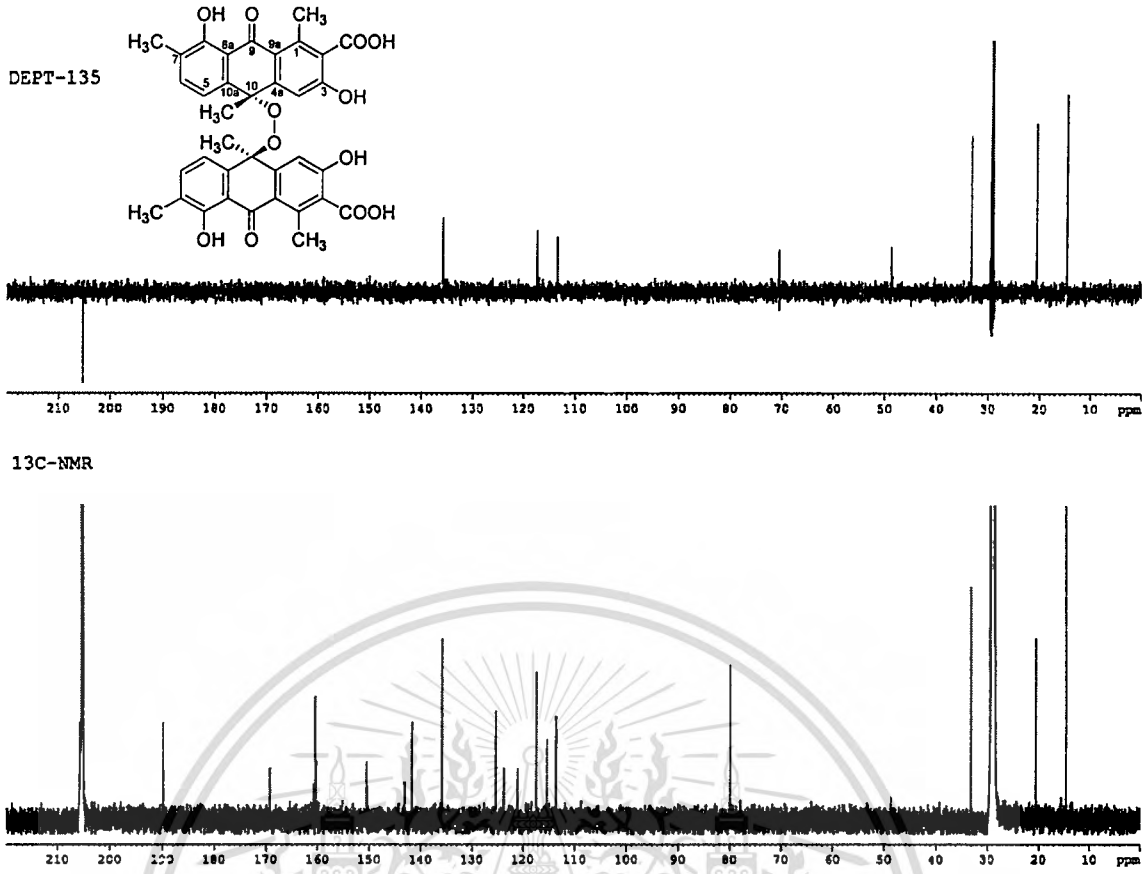


Fig. 101 DEPT 135 spectrum (500 MHz, acetone-d_6) of compound TV14B

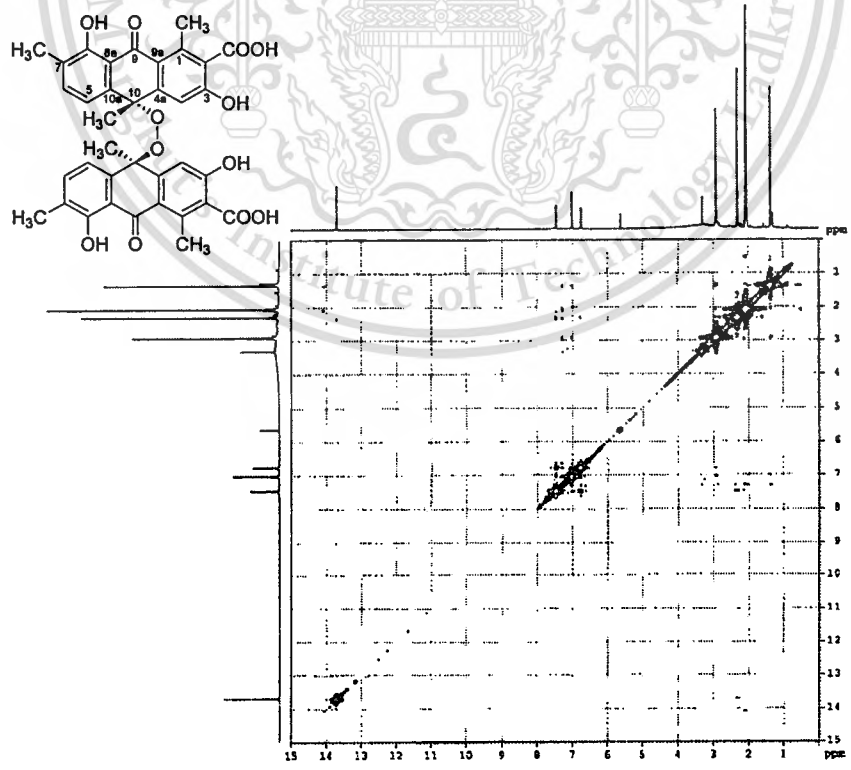


Fig. 102 COSY spectrum (500 MHz, acetone-d_6) of compound TV14B

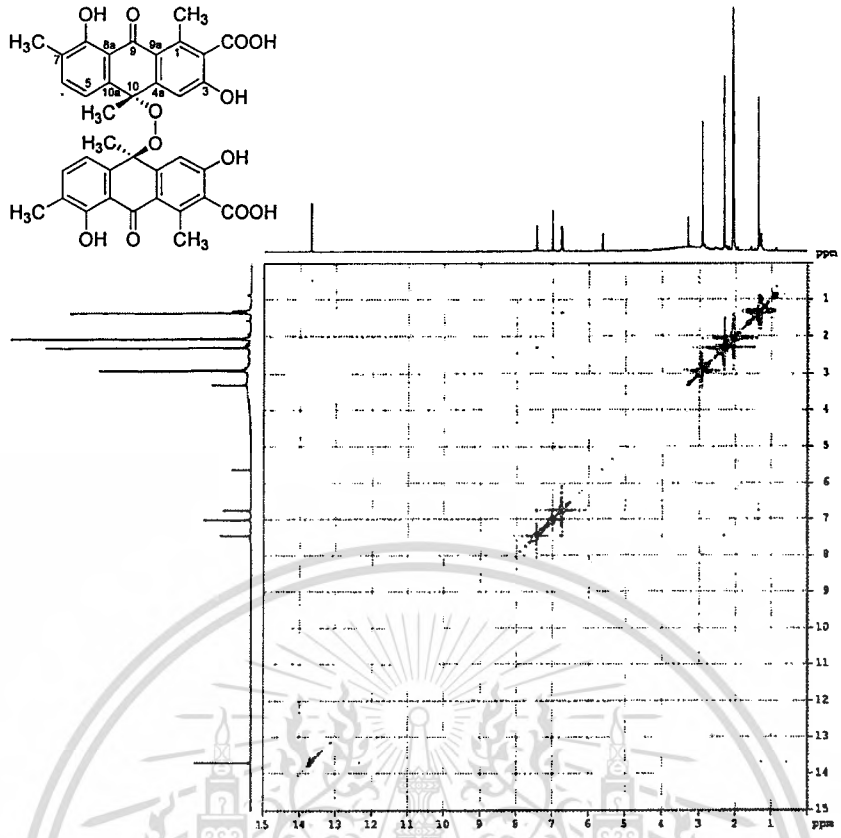


Fig. 103 NOESY spectrum (500 MHz, acetone-d_6) of compound TV14B

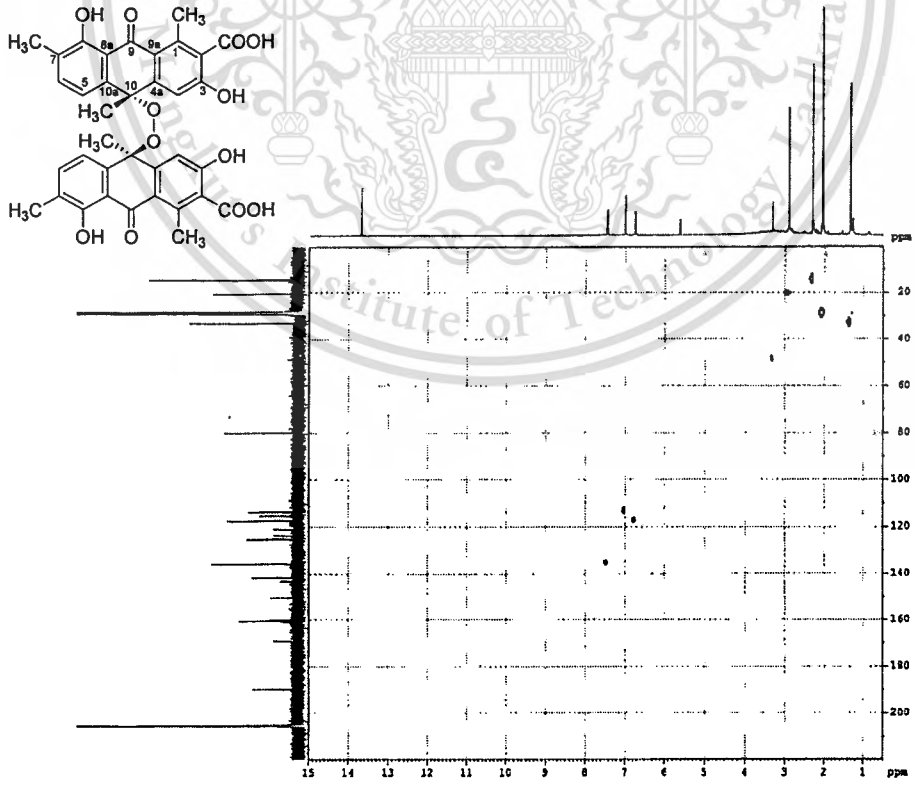


Fig. 104 HMQC spectrum (500 MHz, acetone-d_6) of compound TV14B

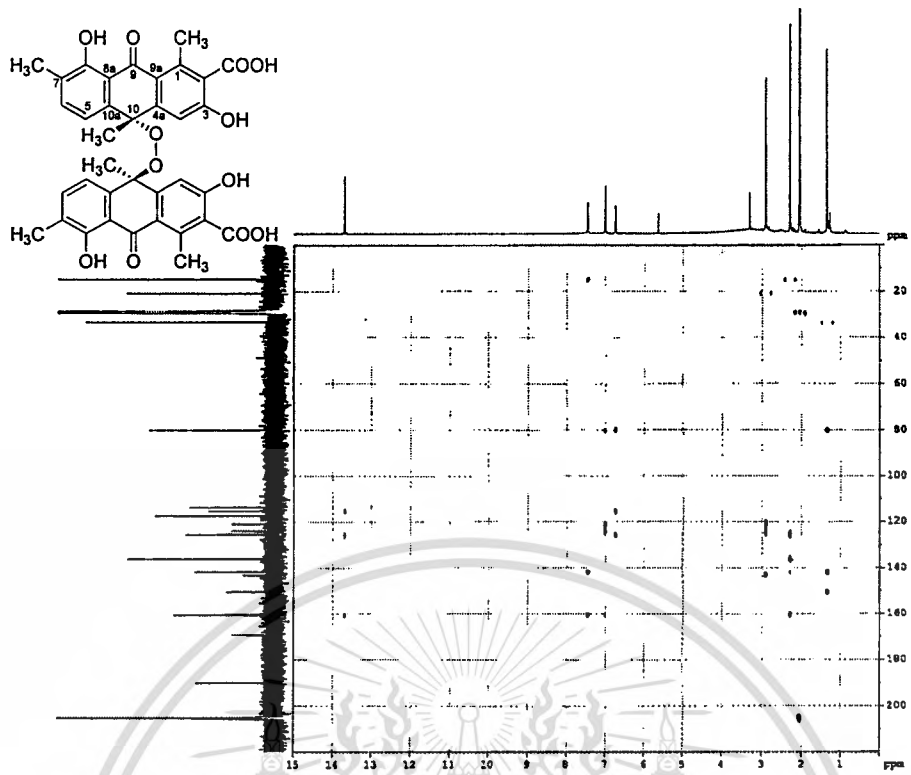


Fig. 105 HMBC spectrum (500 MHz, acetone-d_6) of compound TV14B

Compound TV14C (3,8-dihydroxy-1,7-dimethylanthraquinone-2-carboxylic acid)

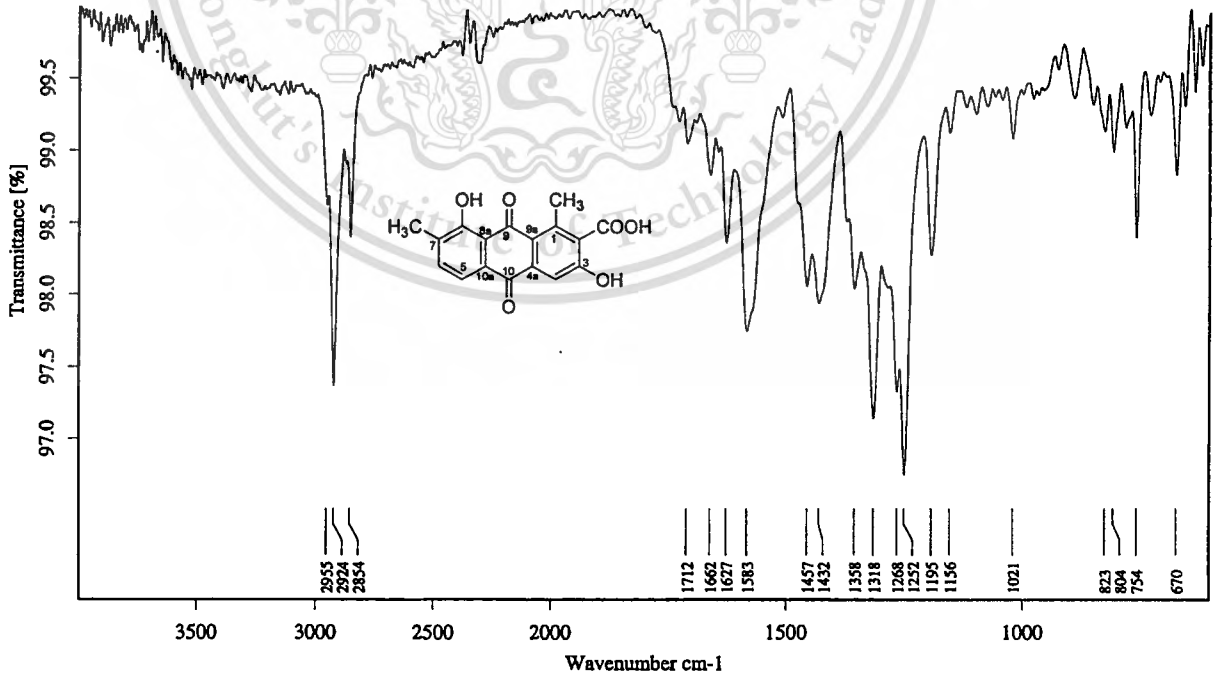


Fig. 106 IR spectrum of compound TV14C

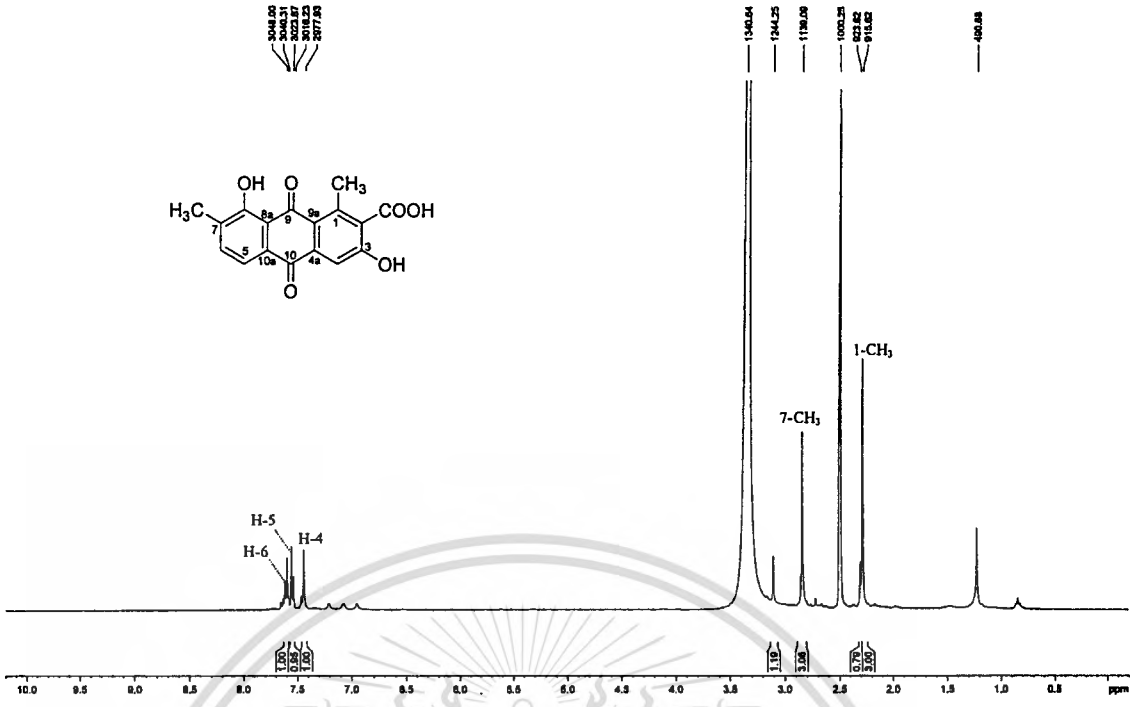


Fig. 107 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound TV14C

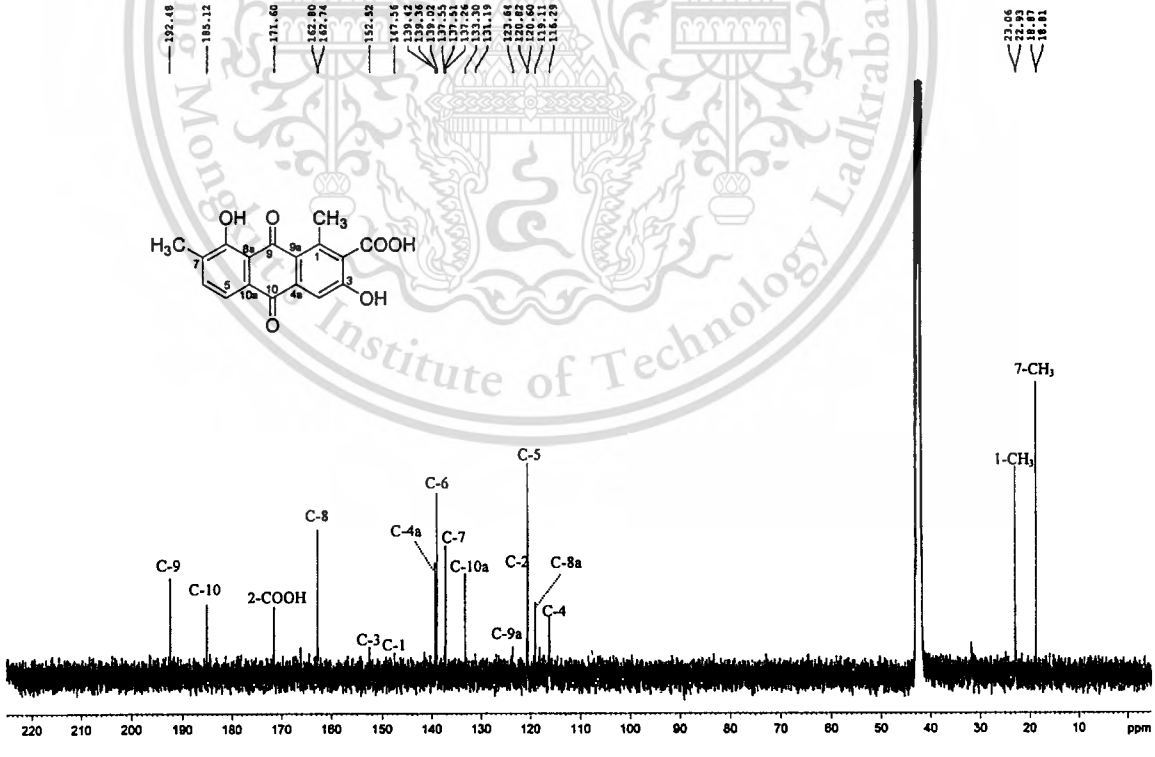


Fig. 108 ^{13}C NMR spectrum (400 MHz, DMSO-d_6) of compound TV14C

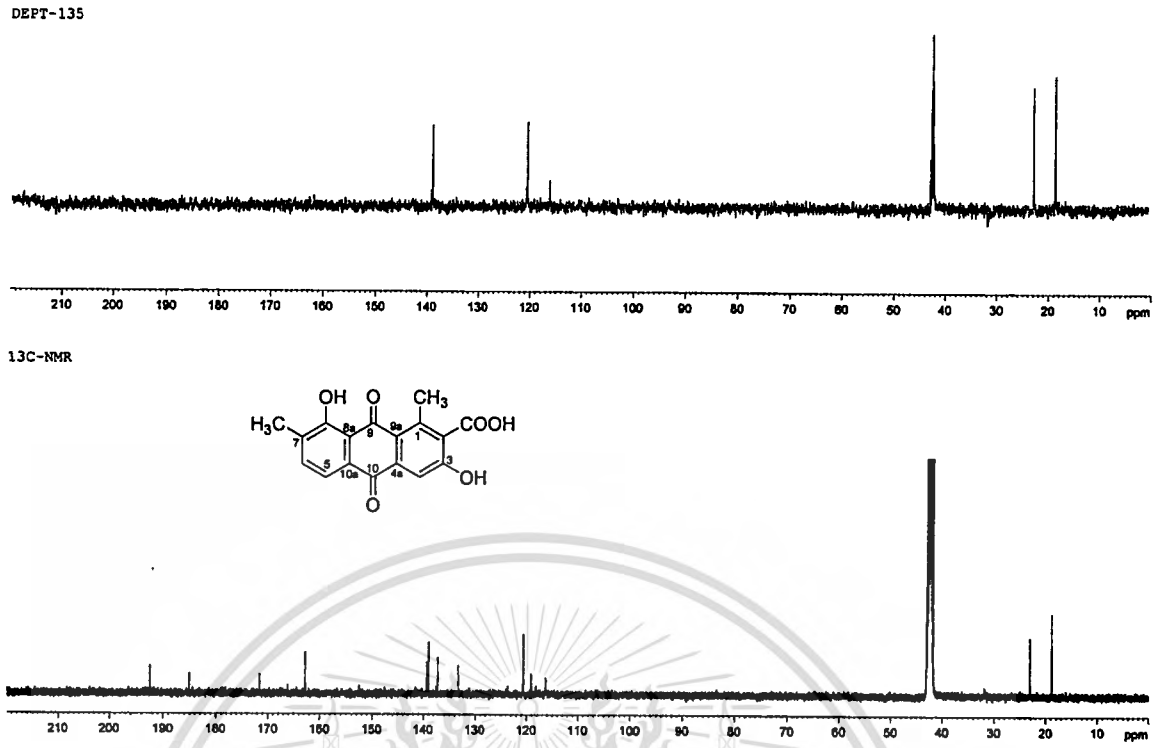


Fig. 109 DEPT 135 (400 MHz, DMSO-d_6) of compound TV14C

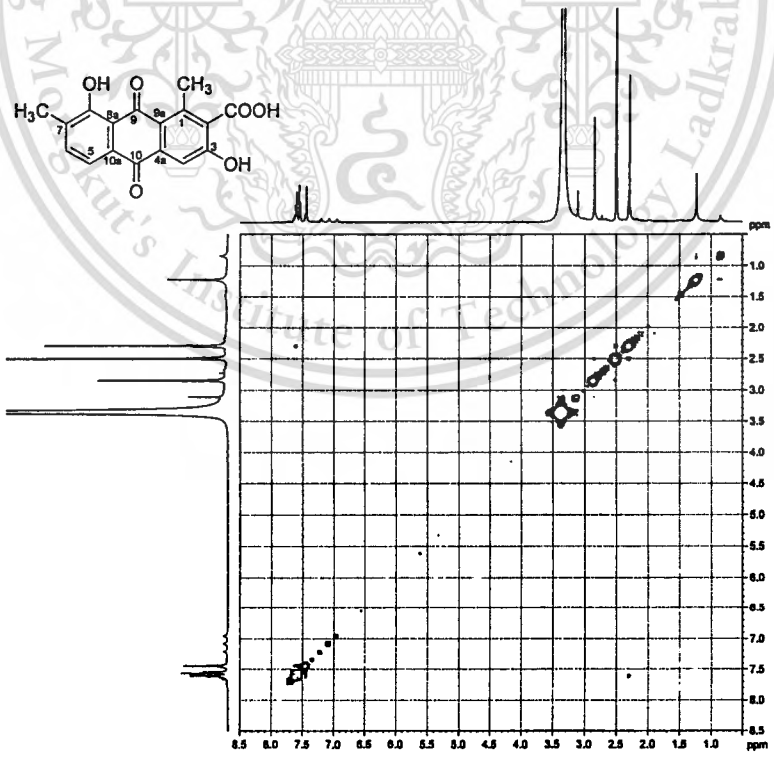


Fig. 110 COSY spectrum (400 MHz, DMSO-d_6) of compound TV14C

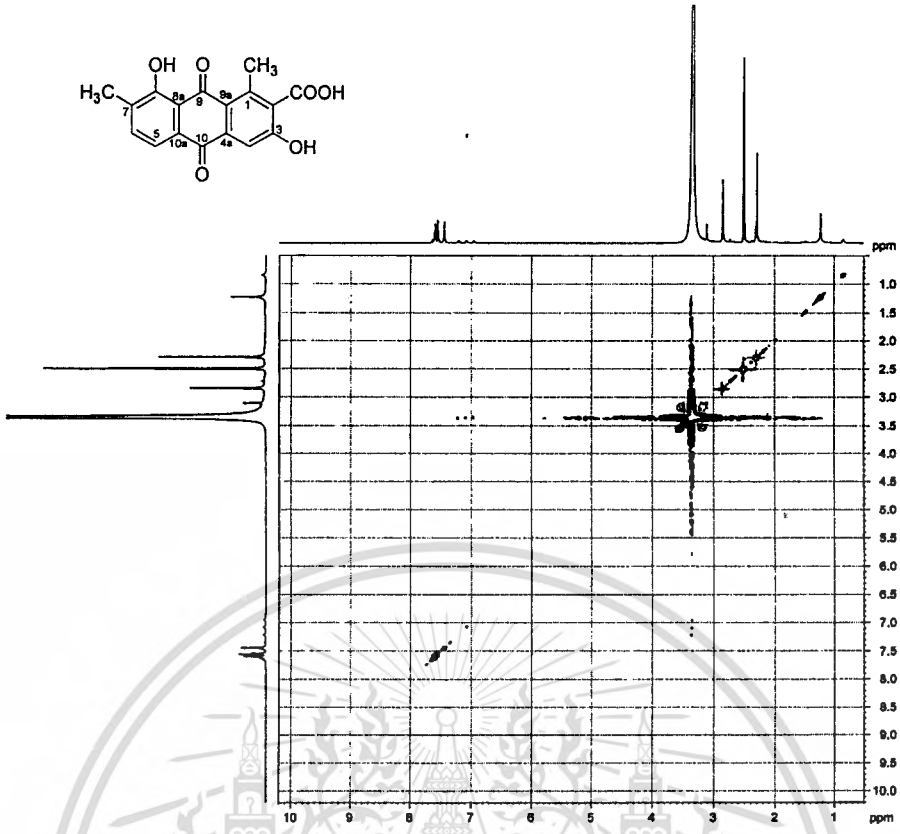


Fig. 111 NOESY spectrum (400 MHz, DMSO-d_6) of compound TV14C

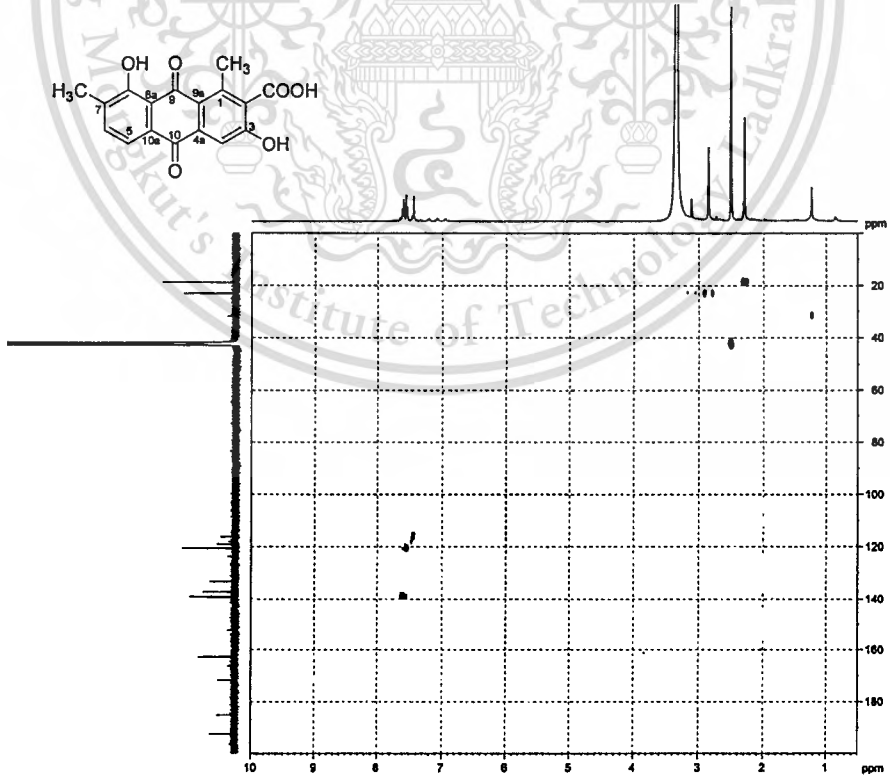


Fig. 112 HMQC spectrum (400 MHz, DMSO-d_6) of compound TV14C

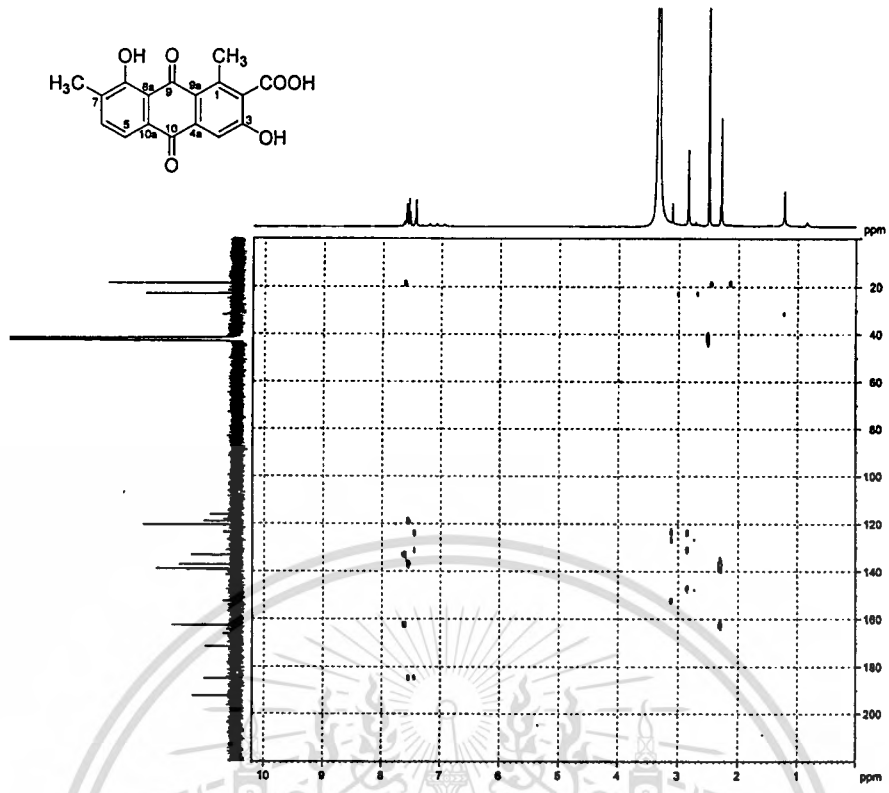


Fig. 113 HMBC spectrum (400 MHz, DMSO-d_6) of compound TV14C

Compound TV14D (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid)

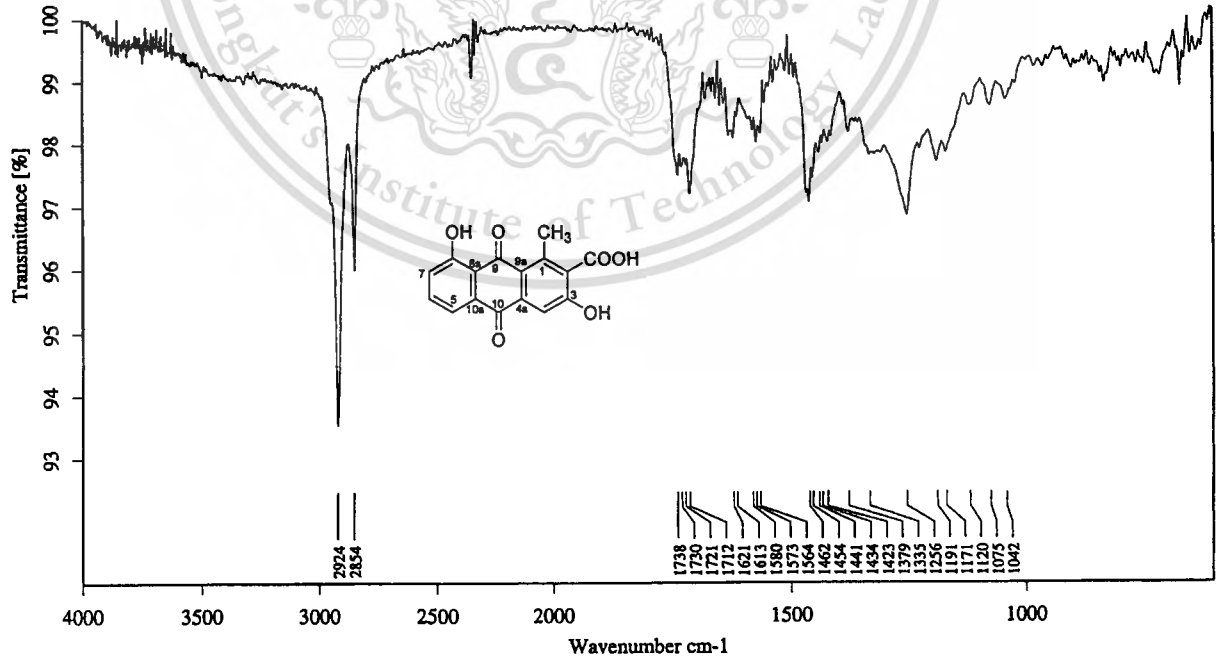


Fig. 114 IR spectrum of compound TV14D

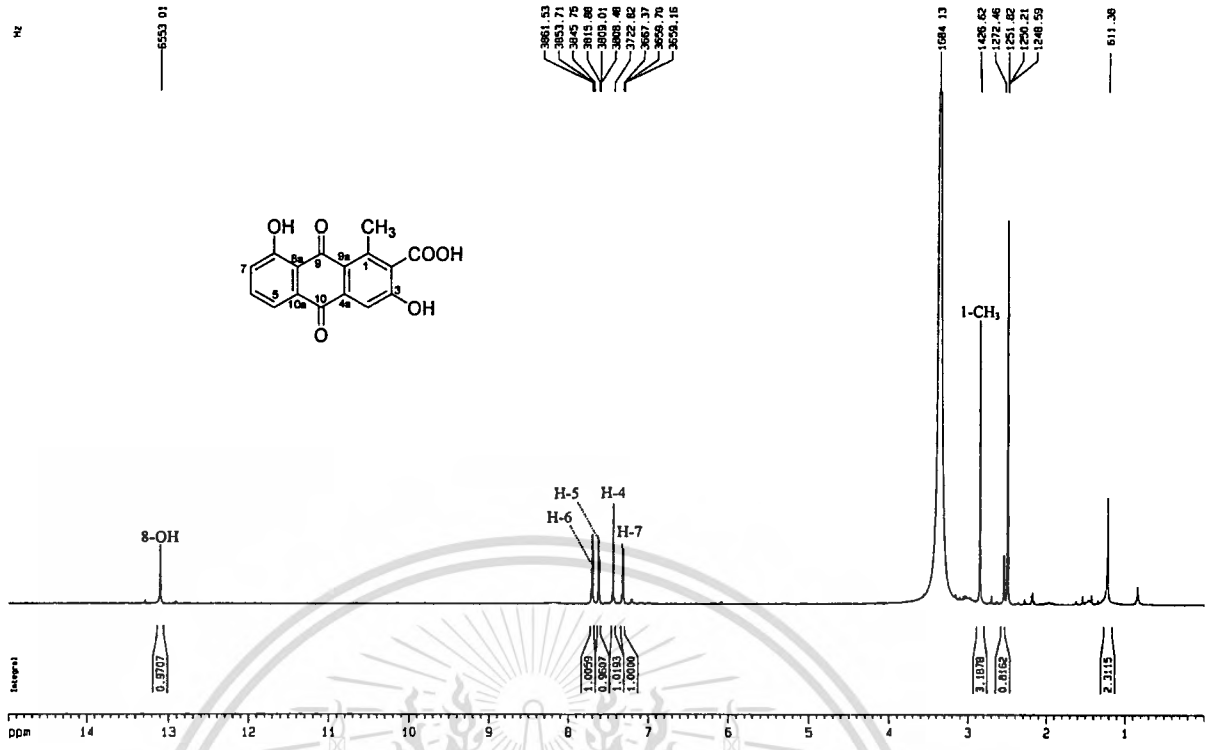


Fig. 115 ^{1}H NMR spectrum (500 MHz, DMSO-d_6) of compound TV14D

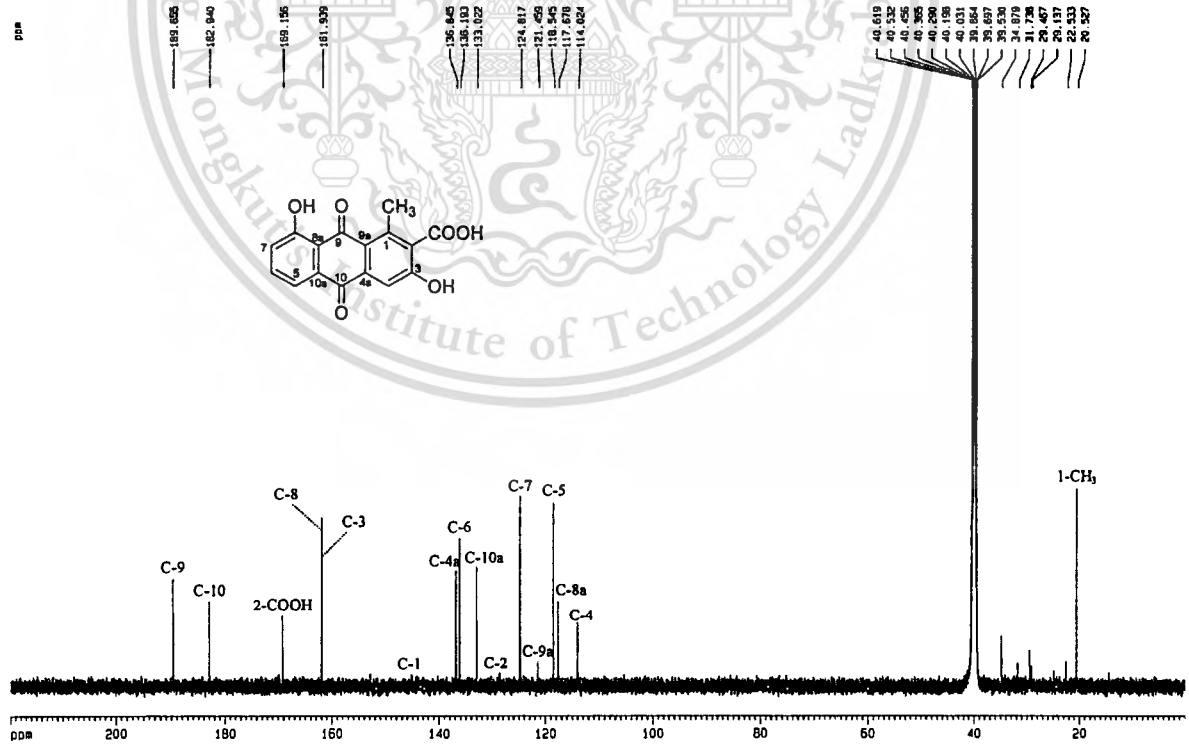


Fig. 116 ^{13}C NMR spectrum (500 MHz, DMSO-d_6) of compound TV14D

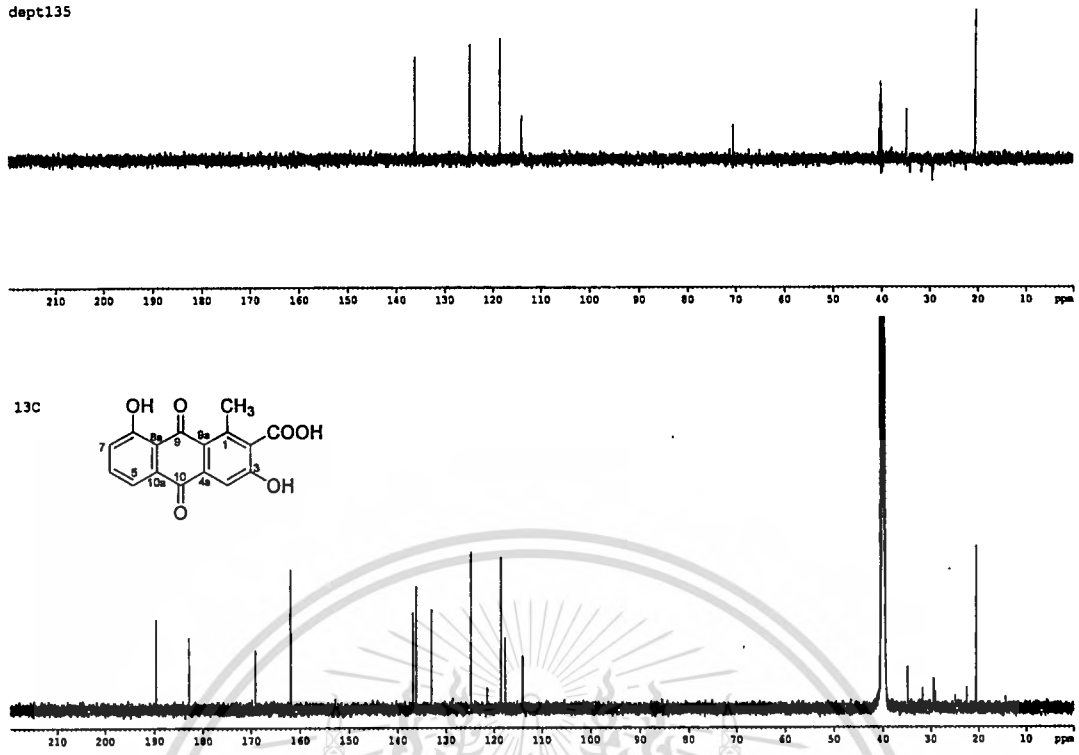


Fig. 117 DEPT 135 spectrum (500 MHz, DMSO-d_6) of compound TV14D

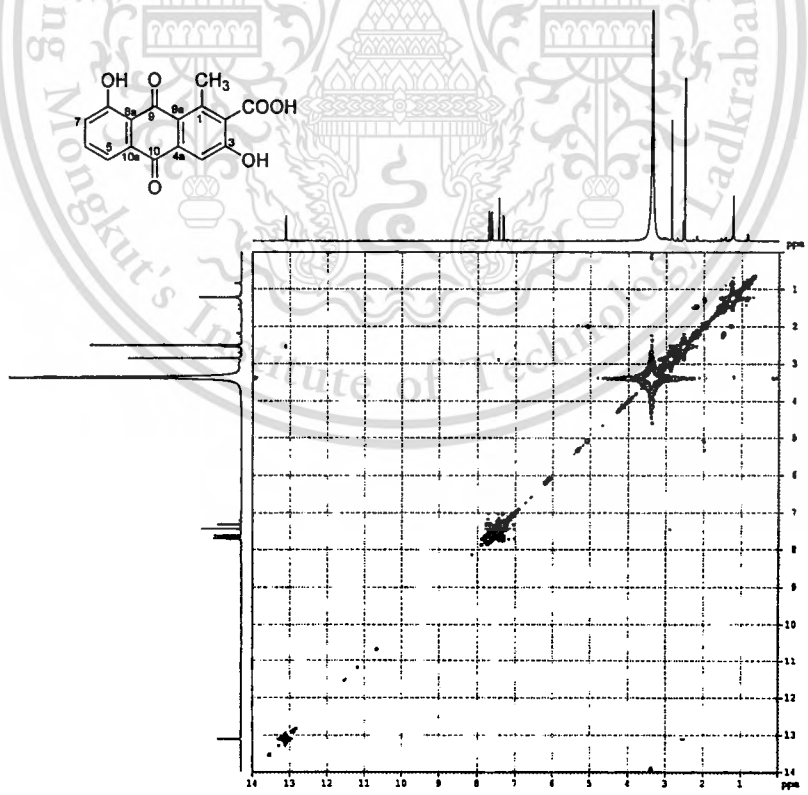


Fig. 118 COSY spectrum (500 MHz, DMSO-d_6) of compound TV14D

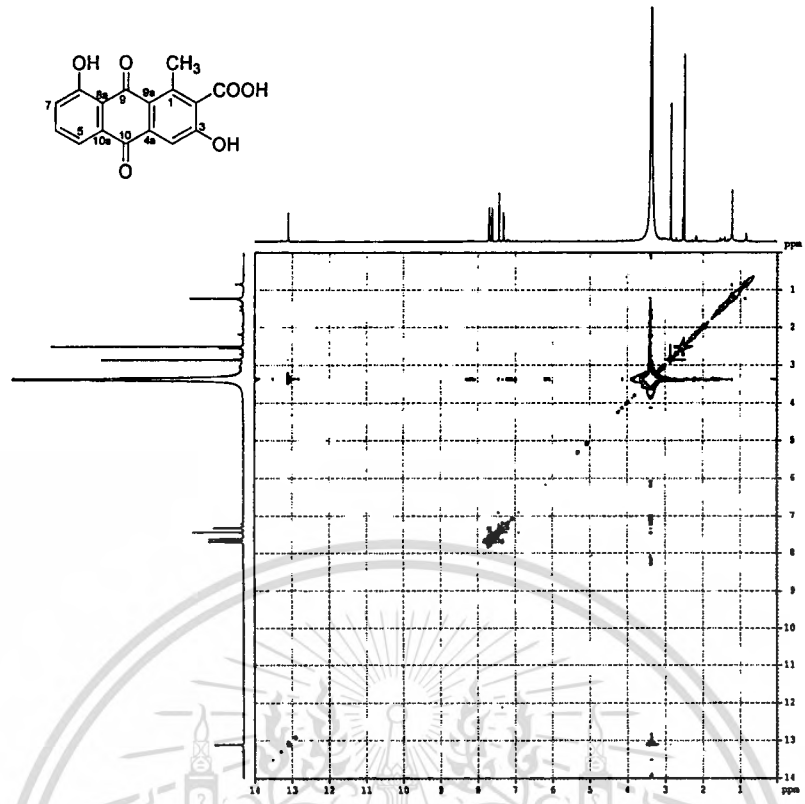


Fig. 119 NOESY spectrum (500 MHz, DMSO-d_6) of compound TV14D

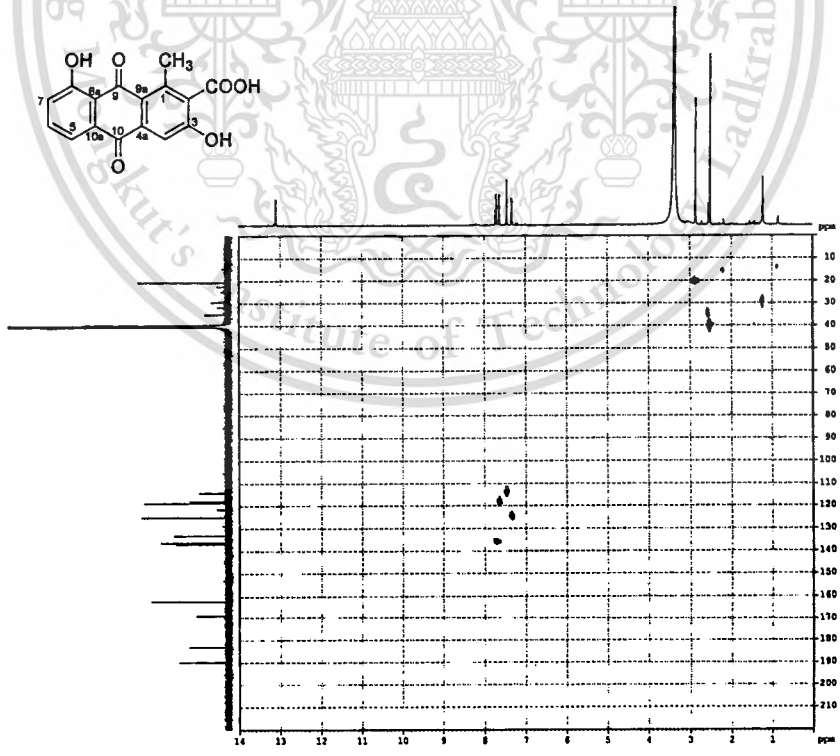


Fig. 120 HMQC spectrum (500 MHz, DMSO-d_6) of compound TV14D

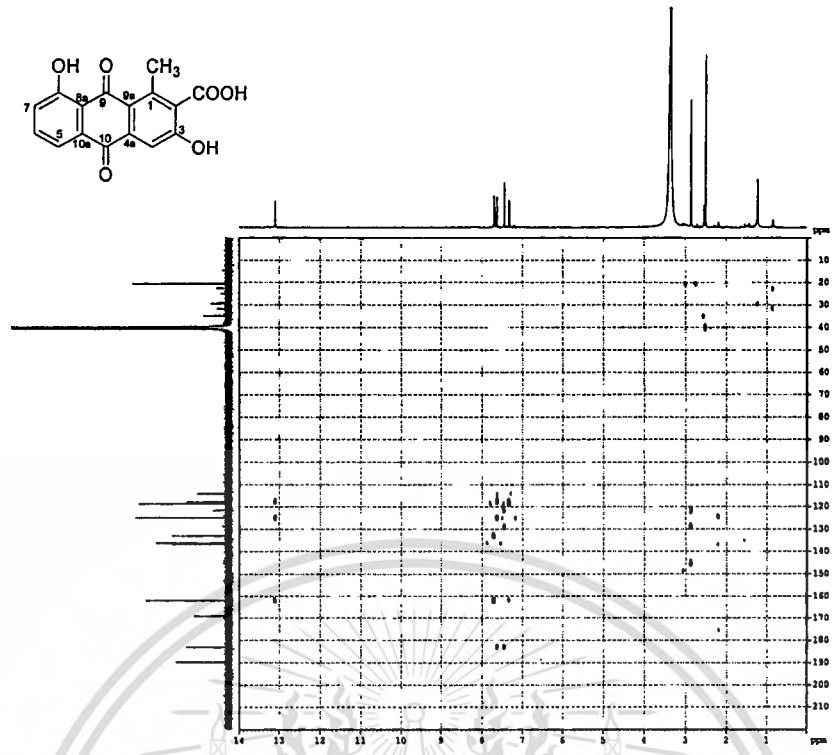


Fig. 121 HMBC (500 MHz, DMSO-d_6) of compound TV14D

Compound TV14AM1

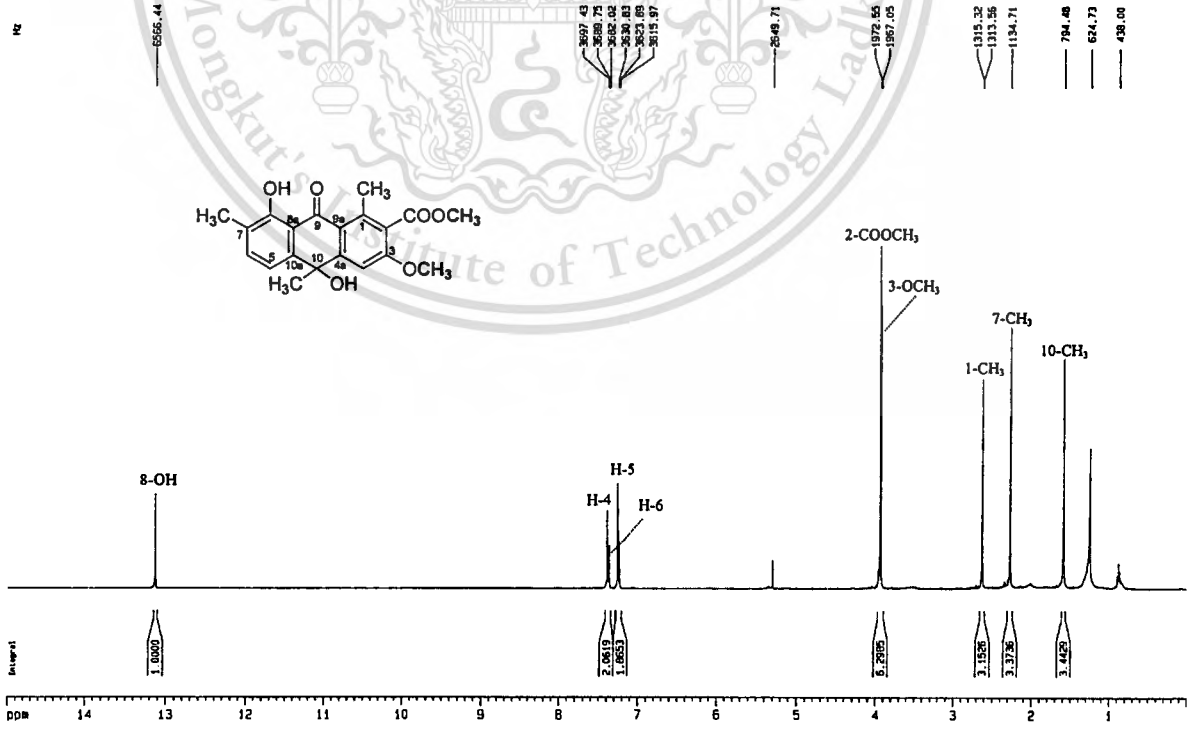


Fig. 122 ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound TV14AM1

Compound TV14AM2

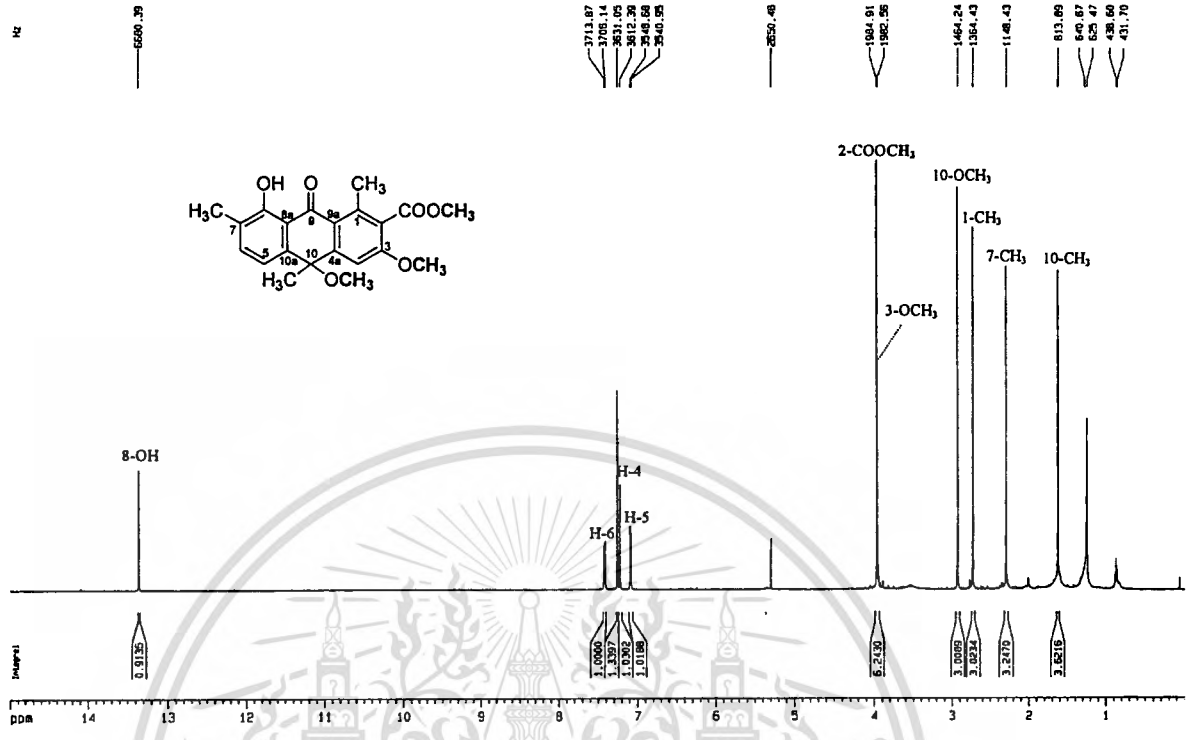


Fig. 123 ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound TV14AM2

Compound TV14BH

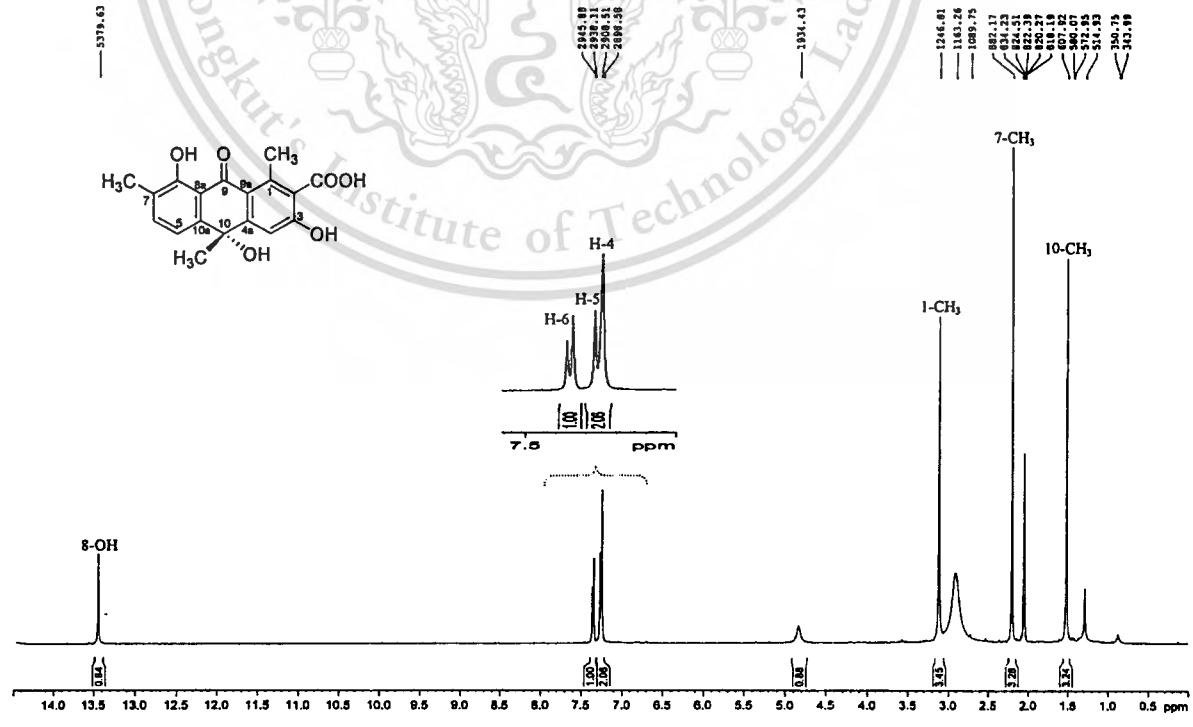


Fig. 124 ^{1}H NMR spectrum (400 MHz, acetone-d_6) of compound TV14BH

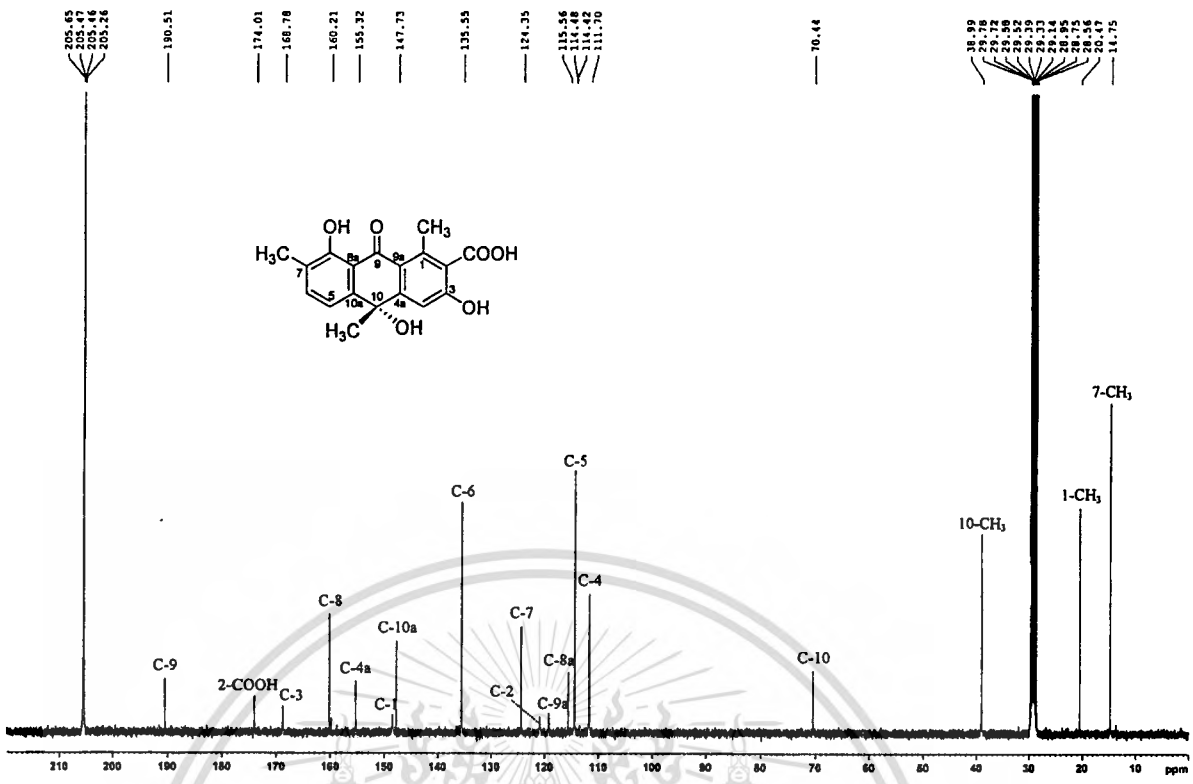


Fig. 125 ^{13}C NMR spectrum (400 MHz, acetone-d_6) of compound TV14BH

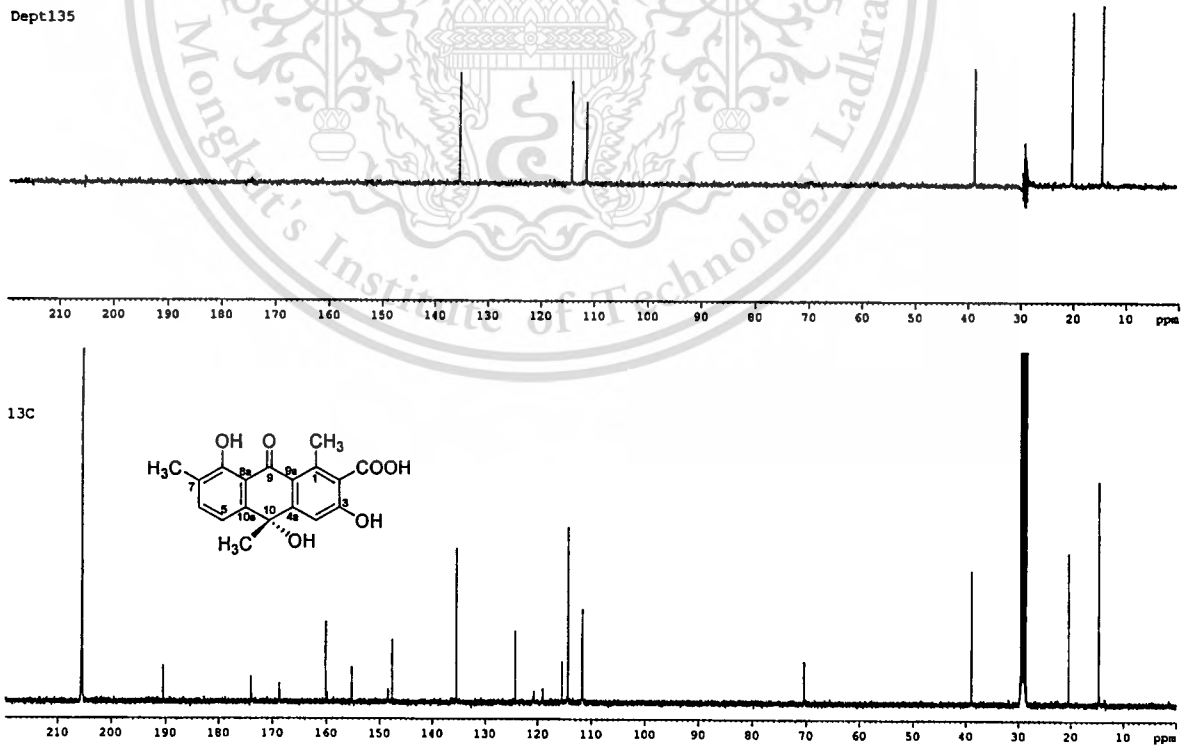


Fig. 126 DEPT 135 spectrum (400 MHz, acetone-d_6) of compound TV14BH

BIOGRAPHY

NAME	Mr. Khomsan Supong
DATE OF BIRTH	May 11th 1985
PLACE OF BIRTH	Petchaboon, Thailand
ADDRESS	64 Mu 11, Ban Nongpheu, Tambon Nong kae, Armpour Si Bun Rueang, Nong Bua Lamphu, 39180, Thailand
INSTITUTION ATTENDED	King Mongkut's Institute of Technology Ladkrabang, 2004-2007: Bachelor of Science (Biotechnology) King Mongkut's Institute of Technology Ladkrabang, 2008-2013: Doctor of Philosophy (Biotechnology)
RESEARCH GRANTS	Thailand Graduate Institute of Science and Technology (TGIST, TG-22-22-52-003D), 2009-2011

PUBLICATIONS:

Supong, K., Thawai, C., Suwanborirux, K., Choowong, W., Supothina, S. and Pittayakhajonwut, P. 2012. "Antimalarial and antitubercular C-glycosylated benz[α]anthraquinones from the marine-derived *Streptomyces* sp. BCC45596." **Phytochemistry Letters**. 5: 651-656.

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