TAXONOMY AND SECONDARY METABOLITES OF SELECTED ENDOPHYTIC ACTINOMYCETE STRAINS

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อนุกรมวิธานและเมแทบอไลต์ทุติยภูมิของสายพันธุ์แอคติโนมัยสีทเอนโดไฟต์ที่คัดเลือก

นายณัฐกร คุณเจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ณัฐกร คุณเจริญ : อนุกรมวิธานและเมแทบอไลต์ทุติยภูมิของสายพันธุ์แอคติโนมัยสีทเอนโดไฟต์ที่คัดเลือก. (TAXONOMY AND SECONDARY METABOLITES OF SELECTED ENDOPHYTIC ACTINOMYCETE STRAINS) อ.ที่ปรึกษาหลัก : ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาร่วม : ดร. ปัทมา พิทยขจรวุฒิ

การศึกษาอนุกรมวิธานของแอคติโนมัยสีทเอนโดไฟต์จำนวน 50 ไอโซเลตที่แยกจากตัวอย่างพืชจำนวน 14 ชนิดซึ่งเก็บรวบรวมจากจังหวัดกรุงเทพมหานคร ฉะเชิงเทรา เชียงใหม่ นครปฐม ปราจีนบุรี และสุพรรณบุรี โดยอาศัยลักษณะทางสัณฐานวิทยา อนุกรมวิธานเคมีทางเคมี และการวิเคราะห์ลำเบสของยีน 16S rRNA พบว่าไอโซเลตที่แยกได้เป็นสมาชิกในวงศ์ *Streptomycetaceae* ได้แก่ สกุล *Streptomyces* (22 ไอโซเลต) และวงศ์ *Micromonosporaceae* ได้แก่ สกุล *Micromonospora* (25 ไอโซเลต) *Plantactinospora* (1 ไอโซเลต) และ *Polymorphospora* (1 ไอโซเลต) จากผลการศึกษาลักษณะทางฟีโนไทป์ อนุกรมวิธานทางเคมี การวิเคราะห์จีโนม และการเข้ากันได้ของ DNA ทำให้สามารถเสนอเป็นแอคติโนมัยสีทสปีชีส์ใหม่จำนวน 5 สปีชีส์ คือ *Micromonospora globbae* (สายพันธุ์ WPS1-2^{T}) *Micromonospora azadirachtae* (สายพันธุ์ AZ1-19^{T}) *Micromonospora radicis* (สายพันธุ์ AZ1-13^{T}) *Micromonospora musae* (สายพันธุ์ MS1-9^{T} และ NGC1-4) และ *Streptomyces endoradicus* (สายพันธุ์ DS1-2^{T} และ AZ1-7)

จากการวิเคราะห์สารเมแทบอไลต์ทุติยภูมิในน้ำหมักเชื้อโดยเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่า *Streptomyces pseudovenezuelae* SKH1-2 และ *Streptomyces aculeolatus* MS1-6 ได้แสดงรายละเอียดทางเคมีที่น่าสนใจและได้ถูกคัดเลือกเพื่อนำไปศึกษาสารเมแทบอไลต์ทุติภูมิและวิเคราะห์โครงสร้างทางเคมีของสารที่แยกได้ด้วยเทคนิคนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโตรสโกปี และแมสสเปกโตรสโกปี จากการศึกษาสามารถแยกสาร chartreusin และ lumichrome ซึ่งเป็นสารในกลุ่ม polyketide ได้จาก *S. pseudovenezuelae* SKH1-2 โดย chartreusin แสดงฤทธิ์ยับยั้ง *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341 และ *Staphylococcus aureus* ATCC 6538p โดยมีค่า MIC คือ 3.1, 1.6 และ 12.5 ug/mL ตามลำดับ ในขณะที่ lumichrome ไม่แสดงฤทธิ์ยับยั้งจุลินทรีย์ นอกจากนี้ยังสามารถแยกสารบริสุทธิ์กลุ่ม naphthoquinone คือ arromycin และสารที่เป็นอนุพันธ์ของกรด salicylic คือ salaceyin A ได้จาก *S. aculeolatus* MS1-6 โดยสาร arromycin มีรายงานว่าเป็นสารต้านแบคทีเรียที่ออกฤทธิ์จำเพาะกับแบคทีเรียแกรมบวก ได้แก่ *B. subtilis*, *S. aureus*, *Listeria ivanovii* และ *Enterococcus faecium* ในขณะที่สาร salaceyin A มีฤทธิ์ต้านราโรคพืช ได้แก่ *Cladosporium cucumerinum*, *Colletotrichum orbiculare* and *Phytophthora capsici*

สาขาวิชา	เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ	ลายมือชื่อนิสิต .....
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A total of 50 endophytic actinomycetes isolated from fourteen plant species collected from six provinces of Thailand including Bangkok, Chachoengsao, Chiang Mai, Nakhon Pathom, Prachinburi and Suphan Buri were taxonomically studied relied on their phenotypic and chemotaxonomic characteristics, and the 16S rRNA gene sequence analysis. The isolates were identified as members of the family *Streptomycetaceae* involving *Streptomyces* (22 isolates), and the family *Micromonosporaceae* including *Micromonospora* (25 isolates), *Plantactinospora* (1 isolate) and *Polymorphospora* (1 isolate). Based on phenotypic characteristics, whole genome sequence analysis and DNA-DNA hybridization, 5 novel actinomycete species were proposed consisting of *Micromonospora globbae* (strain WPS1-2^T), *Micromonospora azadirachtae* (strain AZ1-19^T), *Micromonospora radicis* (strain AZ1-13^T), *Micromonospora musae* (strain MS1-9^T and NGC1-4) and *Streptomyces endoradicus* (strain DS1-2^T and AZ1-7).

Secondary metabolites from culture broth of *Streptomyces pseudovenezuelae* SKH1-2 and *Streptomyces aculeolatus* MS1-6 were studied based on their interested chemical profiles analysed by the in-house HPLC chemical database. The chemical structures of the isolated secondary metabolites were identified using nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy. Chartreusin and lumichrome, polyketide, were isolated from *S. pseudovenezuelae* SKH1-2^T. Chartreusin exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341 and *Staphylococcus aureus* ATCC 6538p with MIC values of 3.1, 1.6 and 12.5 ug/mL, respectively, whilst lumichrome did not show any antimicrobial activity. In addition, a naphthoquinone, arromycin, and one derivative of salicylic acid, salaceyin A, were isolated from *S. aculeolatus* MS1-6. Arromycin has been reported as anti-Gram-positive bacteria such as *B. subtilis*, *S. aureus*, *Listeria ivanovii* and *Enterococcus faecium* while salaceyin A has the anti-phytopathogenic fungi including *Cladosporium cucumerinum*, *Colletotrichum orbiculare* and *Phytophthora capsici*.

Field of Study:	Pharmaceutical Chemistry and Natural Products	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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Nattakorn Kuncharoen

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

AL	=	Aminolipid
ANI	=	Average nucleotide identity
APL	=	Aminophospholipid
A_2pm/DAP	=	Diaminopimelic acid
$CDCl_3$	=	Deuterated chloroform
COSY	=	Correlation spectroscopy
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
δ	=	Chemical shift
d	=	Doublet
dd	=	Doublet of doublets
dDDH	=	Digital DNA-DNA hybridisation
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
DPG	=	Diphosphatidylglycerol
DON	=	2, 7-Dihydroxynaphthalene
EDTA	=	Ethylenediaminetetraacetic acid
F	=	Forward
FAME	=	Fatty acid methyl ester
G+C	=	Guanine plus cytosine
gyrB	=	Gyrase B
h	=	hour
H-NMR	=	Proton nuclear magnetic resonance
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HPLC	=	High Performance Liquid Chromatography
HSQC	=	Heteronuclear single-quantum correlation

Hz	=	Hertz
IC	=	Inhibitory concentration
ISP	=	International *Streptomyces* Project
J	=	Coupling constant
LL-DAP	=	*LL*-Diaminopimelic acid
m	=	Multiplex
m/z	=	Mass to charge ratio
MEGA	=	Molecular Evolutionary Genetics Analysis
meso-DAP	=	*meso*-Diaminopimelic acid
MHz	=	Megahertz
MIC	=	Minimum Inhibitory Concentration
MS	=	Mass spectrometry
MW	=	Molecular weight
NA	=	Nutrient agar
NCI-H187	=	Human small-cell lung cancer, ATCC CRL-5804
nov.	=	novel
NMR	=	Nuclear magnetic resonance
OD	=	Optical density
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PE	=	Phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PI	=	Phosphatidylinositol
PME	=	Phosphatidylinositol mannoside
ppm	=	Part per million
q	=	Quadlet
qd	=	Quadlet of doublets

rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
s	=	Singlet
SEM	=	Scanning electron microscope
sp.	=	Species
t	=	Triplet
T_m	=	Melting temperature
TLC	=	Thin layer chromatography
U	=	Units

CHAPTER I

INTRODUCTION

Actinomycetes are aerobic, Gram-positive, filamentous bacteria which presented true branching hyphae and high mol% Guanine (G) and Cytosine (C) in chromosomal DNA belonged to the phylum *Actinobacteria*. Actinomycetes are saprophytes which played a key role in the recycling of complex organic matters: dead plants, animals, algae and fungi, resulting in humus formation (Lechevalier & Lechevalier, 1967). They are a large group of microorganisms which well known as an important producer of more than half bioactive compounds, particularly antimicrobial drugs: avermectin, chloramphenicol, gentamicin, erythromycin, kanamycin, rifampicin tetracycline and vancomycin (Bérdy, 2005; Bérdy, 2012). The actinomycetes are commonly inhabited in both terrestrial and aquatic ecosystems. Recently, drugs derived from natural sources play an important role in the treatment of human diseases (Joseph & Priya, 2011). The endophytic actinomycetes can produce numerous biologically active compounds such as antimicrobial agents and anti-cancer drugs, because they have a PKS/NRPS (Polyketides synthase/Non-ribosomal peptides synthase) gene, secondary metabolites biosynthetic gene clusters, in their genomic DNA; thus, they are presented as an alternative source of natural compounds, which will be developed, modified, and used as human drugs in the future (Bérdy, 2005; Bérdy, 2012; Masand *et al.*, 2015).

Over the last several decades, many types of bacteria in various phyla such as *Proteobacteria*, *Actinobacteria*, *Bacteroides* and *Verrucomicrobia* have been found to be associated with the internal tissues of the plant as endophytes (Matsumoto & Takahashi, 2017). This term includes the presence of microorganisms inside the infested plant tissues without being recognised as pathogens in host plants. Plant endosphere is a countless of micro-ecosystems where different niches can be

resided by numerous different microorganisms (El-Shatoury *et al.*, 2013). After the first isolation of the genus *Frankia* from non-legume root nodules in early 1886 (Okazaki, 2003), the plant species have been accepted as the great and trustworthy source of actinomycetes (Golinska *et al.*, 2015).

Endophytic actinomycetes are widely distributed in various types of plants such as medicinal plants, crops, woody plants, mangrove plants and indigenous plants (Supong *et al.*, 2016). They were really found to fall within a narrow genera distribution: *Streptomyces* represented a predominant genus, and *Micromonospora*, *Microbispora*, *Nocardia*, *Nocardioides*, *Pseudonocardia* and *Streptosporangium* were also considered as common genera (Qin *et al.*, 2009a). It is well known that the endophytic actinomycetes have been considered as an outstanding source of bioactive secondary metabolites. Numerous types of secondary metabolite have been discovered from endophytic, for instance, alkaloids (Inahashi *et al.*, 2011a), macrolides (Inahashi *et al.*, 2015) and terpenes (Ding *et al.*, 2015). These metabolites presented various biological activities such as antibacterial, antifungal, anti-phytopathogens, immunosuppressant and anticancer (Strobel *et al.*, 2004). Although a lot of genera of endophytic actinomycetes can produce many bioactive compounds, most of the active metabolites were still remarkably obtained from *Streptomyces* strains, for example, bacaryolanes A-C (Ding *et al.*, 2015), kandenols A-E (Ding *et al.*, 2012) and bafilomycins (Yu *et al.*, 2011). Consequently, plant-derived actinomycetes are promising as a source for the discovery of novel bioactive compounds.

Currently, the distribution of actinomycetes in the plant endosphere are considerably unexplored. Thailand is well known as the richest of biodiversity in south-East Asia. It contains approximately 2,819 different plant species. Nevertheless, it has little reports on the investigation of endophytic actinomycetes in Thailand. The assumption of this study relied on the fact that the unexplored habitats could provide some novel actinomycete species. Moreover, endophytic actinomycetes are interesting

sources of biologically active compounds. Therefore, the main purposes of this study are as follows:

1. To isolate and identify the selected endophytic actinomycetes from Thai plant species based on their phenotypic, chemotaxonomic and genotypic characteristics.

2. To screen the endophytic actinomycetes that have antimicrobial activity and interesting secondary metabolite profiles.

3. To isolate, purify and elucidate the chemical structures of secondary metabolites from the selected endophytic actinomycetes.

CHAPTER II

LITERATURE REVIEW

Actinomycetes are initially considered to be a group of microorganisms between fungi and bacteria. The morphological features of actinomycetes commonly like fungi which exhibit true branching mycelia with septa. However, the actinomycetes have many bacterial properties including the diameter of their hyphae is 1 μm within bacterial size and do not have a nuclear envelope in the cell, which is very different from fungi. Furthermore, actinomycetes are sensitively attacked by numerous phages which are similar in their morphology and action to those attack bacteria and are not sensitive to fungal viruses (Lechevalier & Lechevalier, 1967). Composition of cell wall of actinomycetes strongly suggested that actinomycetes were considered as bacteria because their cell walls contained peptidoglycan like Gram-positive bacterial cell wall, and do not have chitin or cellulose (Avery & Blank, 1954).

Actinomycetes are Gram-positive, mycelial bacteria with a high guanine and cytosine (G+C) content (> 55 mol%) in their chromosomal DNA, which classified in the phylum *Actinobacteria* (Lechevalier & Lechevalier, 1967). According to the Bergey's Manual of Systematic Bacteriology Volume five, the phylum *Actinobacteria* contains 6 classes, 23 orders. Class *Actinobacteria* is the largest class containing 16 orders: *Actinomycetales*, *Actinopolysporales*, *Bifidobacteriales*, *Catenulisporales*, *Corynebacteriales*, *Frankiales*, *Glycomycetales*, *Jiangelales*, *Kineosporiales*, *Micrococcales*, *Micromonosporales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales*, *Streptosporangiales*, and *Insertae sedis*. Class *Nitriliruptoria* consists of 2 orders, *Nitriliruptorales* and *Euzebyales*. Class *Thermoleophilia* includes 2 orders such as *Thermoleophilales*, and *Solirubrobacterales*. Each of the class *Acidimibrobiia*, *Coriobacteriia* and *Rubrobacteria* comprises 1 order containing *Acidimicrobiales*,

Coriobacteriales and *Rubrobacterales*, respectively. Currently, the phylum *Actinobacteria* have 50 families and 221 genera (Ludwig *et al.*, 2012).

Actinomycetes are generally believed to have an important role as decomposers which recycle nutrients in the environment. Most of them are saprophytes, but some are parasitic or mutualistic associated with plants and animals (Goodfellow & Williams, 1983). For the last decades, actinomycetes have been well known as key producers of various biologically active metabolites such as antimicrobial drugs, immunosuppressive agents, anticancer drugs, and enzymes (Bérdy, 2005; Matsumoto & Takahashi, 2017). Actinomycetes are extensively distributed in various environments, especially terrestrial soils. Presently, a lot of new actinomycete species have been found in other habitats such as plant tissues (Taechowisan & Lumyong, 2003), root nodules (Trujillo *et al.*, 2006), near-shore sediment (Phongsopitanun *et al.*, 2015) and marine sponge (Supong *et al.*, 2013). These new habitats have been recognised as a reservoir for the isolation of novel actinomycetes.

2.1 Biodiversity of endophytic actinomycetes

The actinomycetes occurring as an endosymbiont in various parts of the plant including roots, stems, and leaves are called “endophytic actinomycetes” (Hallmann *et al.*, 1997; Rosenblueth & Martinez-Romero, 2006). There are approximately 300,000 different plant species in the kingdom Plantae; many species having ethnobotanical use and interesting endemic locations generally produce novel and known endophytic actinomycetes (Strobel & Daisy, 2003). Since the genus *Frankia* was first isolated from non-legume root nodules in early 1886 (Okazaki, 2003), the plant species has been accepted sources for actinomycete research (Golinska *et al.*, 2015).

Minamiyama *et al.*, (2003) observed in scanning electron microscope (SEM) studies, the mycelia of *Streptomyces galbus* was spread on the surface of tissue-culture medium which was growing in *Rhododendron* (Ericaceae) seedling, grew on the

leaf surface and entered leaf tissues via stroma. In addition, the authors noticed that the hyphae of *S. galbus* were present separately or in colonies in intercellular spaces but not inside in epidermal or mesophyll cell within host leaves. Most of the endophytic actinomycetes have been received from roots followed by stems and least in leaves (Qin *et al.*, 2009a). Numerous endophytic actinomycetes reported to date have been isolated from various plant types such as ethnobotanical plants, medicinal plants, tropical plants, aquatic plants and agricultural plants.

Taechowisan & Lumyong, (2003) isolated 59 endophytic actinomycetes from healthy roots of 2 plants species in the family Zingiberaceae; *Zingiber officinale* and *Alpinia galanga* in Chiang Mai, Thailand and all isolates are *Streptomyces* sp. which commonly found in the root.

Bunyoo *et al.*, (2009) reported 11 strains of endophytic actinomycetes isolated from healthy roots of *Acacia auriculiformis* (wattle tree), which contained 5 genera such as *Streptomycetes* sp., *Actinoallomurus* sp., *Amycolatopsis* sp., *Kribbella* sp. and *Microbispora* sp. The authors proposed 1 strain (GMKU 931) as a novel species, *Actinoallomurus acacia*e sp. nov., and published on *International Journal of Systematic and Evolutionary Microbiology* (Thamchaipenet *et al.*, 2010).

Janso & Carter, (2010) studied the diversity of endophytic actinomycetes from native tropical plants; herbs, palms, ferns, club mosses, grasses, and sedges collected from Papua New Guinea (Solomon Island and Mborokua Island), and described that isolates were prevalently found in roots but uncommon in leaves. Tropical climate zones are the greatest biologically diverse terrestrial and aquatic plants on the earth and thus possible to provide some resources for the acquisition of novel endophytic microorganisms.

Qin *et al.*, (2009a) documented the isolation of 2,174 endophytic actinomycetes belong to 32 different genera from medicinal plants collected from Xishuangbanna tropical rainforest of China. The occurrence of endophytic actinomycetes in roots,

leaves, stems and fruits of 26 medicinal plant species collected from Panxi plateau, tropical forest of South-west Sichuan, China, including *Streptomyces*, *Micromonospora*, *Oerskovia*, *Nonomuraea*, and *Promicromonospora* has been reported (Zhao *et al.*, 2011).

Kim *et al.*, (2012b) studied endophytic actinomycetes recovered from roots of eleven native herbaceous plant species of Korea. The study has analysed the isolation of 61 strains, assigned to 15 different genera. The member of genus *Streptomyces* consisted of 45.9 % of total isolates, followed by *Micromonospora* (18.8 %), *Rhodococcus* (6.6 %), *Microbispora* (4.9 %) and *Micrococcus* (4.9 %). Other minor components included *Microbacterium*, *Streptacidiphilus*, *Arthrobacter*, *Dietzia*, *Kitasatospora*, *Herbiconiux, Mycobacterium*, *Nocardia*, *Rathayibacter*, and *Tsukamurella*.

Artemisia annua L. (with a common name Qinghao), a member of Asteraceae family, is the traditional Chinese medicine since 168 B.C., the artemisinin and its derivatives which extracted from Qinghao provided the most effective treatment for malaria. Besides malaria, artemisinin and its derivatives showed effective on viruses and some cancers such as ovarian, oral, and prostate cancer (Arsenault *et al.*, 2008; Woerdenbag *et al.*, 1990). Many researchers believed that endophytes associated with *A. annua* contribute to the production of bioactive compounds. The endophytic actinomycetes in leaves, stems, and roots of *A. annua* comprising *Streptomyces*, *Promicromonospora*, *Pseudonocardia*, *Nocardia*, *Nonomuraea*, *Micromonospora*, *Actinomadura*, *Streptosporangium*, *Amycolatopsis*, *Dactylosporangium*, *Microbispora*, and *Phytomonospora* have been reported (Li *et al.*, 2012).

Kaewkla & Franco, (2013) isolated 576 endophytic actinomycetes from leaves, stems, and roots of two eucalyptus, Grey Box and Red Gum, a native apricot tree and native pine tree. The classification revealed that endophytic actinomycetes isolates belonged to some different genera; *Streptomyces*, *Actinomadura*, *Actinomycetospora*,

Actinopolymorpha, *Amycolatopsis*, *Micromonospora*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Nonomuraea*, *Polymorphospora*, *Promicromonospora*, and *Pseudonocardia*.

Plant endosphere is a prosperous dwelling of endophytic actinomycetes. Now, the discovery of endophytic actinomycetes from different parts of various plant species are affiliated to a wide range genera, for example, *Actinoallomurus* (Li *et al.*, 2015a), *Actinomadura* (Qin *et al.*, 2009a), *Glycomyces* (Qin *et al.*, 2008), *Jiangella* (Qin *et al.*, 2009b), *Micromonospora* (Trujillo *et al.*, 2006), *Microbispora* (Li *et al.*, 2015b), *Phytohabitans* (Inahashi *et al.*, 2010), *Nocardioides* (Qin *et al.*, 2012b), *Plantactinospora* (Zhu *et al.*, 2012), *Promicromonospora* (Qin *et al.*, 2012a), *Saccharopolyspora* (Qin *et al.*, 2010), *Sphaerisporangium* (Xing *et al.*, 2015), *Streptomyces* (Liu *et al.*, 2009), *Streptosporangium* (Inahashi *et al.*, 2011b) and *Wangella* (Jia *et al.*, 2013). In Thailand, some investigators studied endophytic actinomycetes isolated from Thai medicinal and agricultural plant species. They discovered some novel species of plant-derived actinomycetes as shown in Table 2.1.

2.2 Bioactive secondary metabolites from endophytic actinomycetes

The abundance of alternative and complementary medicines has increased popularity in current decades. Drugs derived from natural sources play an important role in the treatment of human diseases (Joseph & Priya, 2011). Actinomycetes are well known for their capability to produce secondary metabolites with diverse biological activities. Recently, 22,500 bioactive compounds have been isolated from microorganisms and estimate of 10,100 bioactive compounds were isolated from actinomycetes, particularly, the genus *Streptomyces* which provides a majority (70%) of bioactive compounds and others (30%) from non-*Streptomyces* species (Bérdy, 2005). The recent report of important secondary metabolites from endophytic actinomycetes with their chemical class and biological activity are shown in Table 2.2.

Table 2.1 Novel species of plant-derived actinomycetes discovered in Thailand

Species	Source of isolation	References
Actinoallomurus oryzae	Roots of *Oryza sativa* L. KDML 105	Indananda *et al.*, (2011)
Actinoallomurus acaciae	Roots of *Acacia auriculiformis*	Thamchaipenet *et al.*, (2010)
Actinomadura syzygii	Roots of *Syzygium cumini* L. Skeels	Rachniyom *et al.*, (2015a)
Actinophytocola oryzae	Roots of *Oryza sativa* L. RD6	Indananda *et al.*, (2010)
Amycolatopsis samaneae	Roots of *Samanea saman* (Jacq.) Merr	Duangmal *et al.*, (2011)
Amycolatopsis stemonae	Stems of *Stemona* sp.	Klykleung *et al.*, (2015)
Actinomycetospora endophytica	Roots of *Podochilus microphyllus* Lindl.	Sakdapetsiri *et al.*, (2018)
Asanoa endophytica	Rhizomes of *Boesenbergia rotunda*	Niemhom *et al.*, (2016a)
Jiangella endophytica	Rhizomes of Kaempferia elegans	Niemhom *et al.*, (2019)
Micromonospora costi	Leaves of *Costus speciosus*	Thawai, (2015)
Micromonospora endophytica	Leaves of *Oryza sativa* L.	Thanaboripat *et al.*, (2015)
Micromonospora oryzae	Roots of *Oryza sativa* L.	Kittiwongwattana *et al.*, (2015)
Nonomuraea syzygii	Roots of *Syzygium cumini* L. Skeels	Rachniyom *et al.*, (2015b)
Phytohabitans kaempferiae	Leaves of *Kaempferia larsenii*	Niemhom *et al.*, (2016b)
Pseudonocardia acaciae	Roots of *Acacia auriculiformis*	Duangmal *et al.*, (2009)
Sphaerisporangium rufum	Roots of *Oryza sativa* L.	Mingma *et al.*, (2014)
Streptomyces oryzae	Stems of *Oryza sativa* L.	Mingma *et al.*, (2015)
Streptomyces phyllanthi	Stems of *Phyllanthus amarus*	Klykleung *et al.*, (2016)

Table 2.2 Bioactive secondary metabolites from endophytic actinomycetes

Compounds	Chemical class	Species	Activity	References
Actinoallolides A-E	Macrolide	*Actinoallomurus flavus*	Anti-trypanosomal	Inahashi *et al.*, (2015)
Antimycin A_{18}	Macrolide	*Streptomyces albidoflavus*	Antifungal	Yan *et al.*, (2010)
Bacaryolanes A-C	Sesquiterpene	*Streptomyces* sp.	Antibacterial	Ding *et al.*, (2015)
Bafilomycins	Macrolide	*Streptomyces* sp.	Cytotoxic	Yu *et al.*, (2011)
Coronamycins	Peptide	*Streptomyces* sp.	Antifungal	Ezra *et al.*, (2014)
Kakadumycin A	Peptide	*Streptomyces* sp.	Antibacterial	Castillo *et al.*, (2003)
Kandenols A-E	Sesquiterpene	*Streptomyces* sp.	Antibacterial Cytotoxic	Ding *et al.*, (2012)
Lupinacidins A-B	Anthraquinone	*Micromonospora lupini*	antitumor	Igarashi *et al.*, (2007)
Pterocidin	Polyketide	*S. hygroscopicus*	Anticancer	Igarashi *et al.*, (2006)
Trehangelins A-C	Polyketide	*Polymorphospora rubra*	Photo-oxidative hemolysis inhibitor	Nakashima *et al.*, (2013)

The plant endosphere comprises a large variety of endophytic microorganisms, which establish a complex of micro-ecosystem. More recently, actinomycetes residing in plant tissues are the rich repertoire of secondary metabolites (El-Shatoury *et al.*, 2013). One of the studies in early 1997, *Streptomyces* sp. DSM 11575 was isolated from root nodules of *Alnus glutinosa*. This strain produced alnumycin, a new naphthoquinone antibiotic, which displays antibacterial property against Gram-positive bacteria such as *Bacillus subtilis*, *Arthrobacter crystallopoites*, *Micrococcus luteus*, and *Rhodococcus* sp. but was inactive against Gram-negative bacteria such as *Escherichia coli*, *Proteus rettgeri*, and *Agrobacterium tumefaciens* (Bieber *et al.*, 1998). Munumbicins A-D were obtained from *Streptomyces* NRRL 30562, which was isolated

from *Kennedia nigriscans*. The munumbicins were described as novel antibiotics with broad spectrum of activity against many human pathogenic bacteria and plant pathogenic fungi. The most admirable biological activity of the munumbicins was munumbicin D, which showed antimalarial activity against *Plasmodium falciparum* with IC_{50} of 4.5±0.07 ng·mL^{-1} (Castillo *et al.*, 2002)

A new benzamide, 2-amino-3, 4-dihydroxy-5-methoxybezamide isolated from the endophytic *Streptomyces* sp. YIM 67086, showed antimicrobial activities against *E. coli* and *Candida albicans* with MICs of 64 and 32 μg·mL^{-1}, respectively. In addition, the strain YIM 67086 also produced 4 known compounds such as 4-hydroxy-3-methoxybenzoic acid, phenylacetic acid, *N*-acetyltyramine, and *p*-hydoxytruxinic acid. 4-Hydroxy-3-methoxybenzoic acid and *p*-hydoxytruxinic acid had inhibit *C. albicans* with MIC of 64 and 32 μg·mL^{-1} (Yang *et al.*, 2015).

Maklamicin, a novel spirotetronate-calss polyketide, was isolated from endophytic *Micromonospora* GMKU 236. The compound showed strong to moderate antimicrobial activities against Gram-positive bacteria such as *Micrococcus luteus*, *B. subtilus*, *B. cereus*, *S. aureus*, and *Enterococcus faecalis* with MICs of 0.2, 1.7, 6.5, 13, and 13 μg·mL^{-1}, respectively, and was slightly active against *C. albicans* (MIC = 50 μg·mL^{-1}). Moreover, maklamicin displayed moderate cancer cell cytotoxicity against human cervical cancer cells (HeLa) and human breast cancer cells (MCF7) with IC_{50} values of 17 and 34 μM (Igarashi *et al*., 2011). *Micromonospora lupini* Lupac 08 was firstly isolated from root nodules of *Lupinus angustifolius* by Trujillo *et al*. (2007). The strain Lupac 08 produced a new anthraquinone derivative, lupinacidin C, which exhibited the strong potent inhibitory effects on the invasion of murine colon carcinoma cells into the reconstituted basement membrane (Igarashi *et al.*, 2011).

The first report of the anti-trypanosomal activity of endophytic actinomycetes has been described by (Zin *et al.*, 2011). Eight endophytic actinomycetes were isolated from stems and roots of medicinal plants based on ethnobotanical properties in

Malaysia. Among these isolates, the crude extract of *Streptomyces* sp. SUK 17, isolated from *Cinnamomum zrylanicum*, had positive anti-trypanosomal activity against *Trypanosoma b. brucei* strain BS221 and vero cell with IC_{50} value of 4.48 and 60.98 $\mu g \cdot mL^{-1}$, respectively. Similarly, *Streptosporangium oxazolinicum* K07-0460, which firstly isolated from an orchid collected in Okinawa prefecture, Japan, produced three novel alkaloid antibiotics, spoxazomicins A-C (Inahashi *et al.*, 2011a). Spoxazomicin A showed the highest anti-trypanosomal activity against *T. b. brucei* strain GUTat 3.1 with IC_{50} value of 0.11 $\mu g \cdot mL^{-1}$, which had 14 to 21-fold more potent activity than those of clinically used for anti-trypanosomal medicines. Spoxazomicin B also showed anti-trypanosomal activity with IC_{50} value of 0.55 $\mu g \cdot mL^{-1}$, but spoxazomicin C showed weakly active against *T. brucei* strain GUTat 3.1 with IC_{50} value around 3 $\mu g \cdot mL^{-1}$.

In Thailand, Indananda *et al.*, (2013) reported the discovery of a new polyketide named linfuranone A from *Microbispora* GMKU 363 isolated from root of Thai medicinal plant "*Clinacanthus siamensis* Bremek" (Lin Ngu Hao). This compound had no antimicrobial and cytotoxic activity, but it displayed antiatherogenic activity. By the treatment of 50 μM linfuranone A in mouse ST-13 pre-adipocytes, 47% of pre-adipocytes were differentiated into the matured adipocytes and accumulated the lipid droplets.

2.3 Taxonomic studies on endophytic actinomycetes

Presently, the taxonomic position of actinomycetes has been classified and identified based on the polyphasic approach which is a combination of phenotypic, chemotaxonomic, and genotypic characteristics.

2.3.1 Phenotypic characteristics

2.3.1.1 Morphological characteristics

Actinomycetes display an outstanding diverse morphology from other bacteria. They produce true branched aerial and substrate hyphae; however, some genera including *Micromonospora*, *Plantactinospora* and *Polymorphospora*, have no aerial mycelia. The spore formation involving its position, surface and arrangement is a key morphological criterion to identify the actinomycetes at the genus level (Shirling & Gottlieb, 1966).

2.3.1.2 Cultural characteristics

The cultural characteristics of actinomycetes refer to the growth and the colonial appearance on various culture media, and the utilisation and/or degradation of organic materials such as sugars and amino acids. The standard methods, which previously proposed by Shirling & Gottlieb, (1966) are practically useful for the identification of *Streptomyces* species included with other genera.

2.3.2 Chemotaxonomic characteristics

Chemotaxonomy is the study of the chemical variant of certain compounds that found among the microbial cells being classified. The chemotaxonomic properties incorporating the composition of cell-wall peptidoglycan, whole-cell sugars, phospholipids composition, isoprenoid quinones, and cellular fatty acids are the key characters to classify the actinomycetes.

2.3.2.1 The composition of cell-wall peptidoglycan

The cell-wall peptidoglycan is one of the chemical components in all actinomycetes and could be used to separate the actinomycetes into board group at

the genus level. The structure of peptidoglycan contains an enormous polymer constituent with many identical subunits including the backbone of β-1,4 linked disaccharides, *N*-acetylglucosamine and *N*-acetylmuramic acid, and different peptide chain of four alternating amino acids which connected to the carboxyl group of *N*-acetylmuramic acid. Three parts of the cell-wall peptidoglycan comprising the isomers of diaminopimelic acid (DAP), the differences in amino acids of the peptide chain, and *N*-acyl types of muramic acid have been importantly used for the classification of actinomycetes. The variety of amino acids in peptide moiety is shown in Table 2.4 (Lechevalier *et al.*, 1971). The isomers of diaminopimelic acid present in the cell-wall peptidoglycan are one of the important keys to classify the members of the genus *Streptomyces* and other genera of actinomycetes. *LL*-diaminopimelic acid isomer is found in all *Streptomyces* species, whilst other actinomycete genera consist of *meso*-diaminopimelic acid, 3-OH-diaminopimelic acid, 3,4-dihydroxydiaminopimelic acid and/or the integration of various isomers (Matsumoto *et al.*, 2014; Staneck & Roberts, 1974). Furthermore, the *N*-acyl type of muramic acid – acetyl or glycolyl type – has been properly used for the classification of actinomycetes (Uchida & Aida, 1984).

2.3.2.2 Whole cell sugars

The analysis of sugars composition in whole-cell hydrolysates is necessary for the classification and identification of actinomycetes which have *meso*-DAP in the cell-wall peptidoglycan. The whole cell sugar patterns are divided into four groups based on the presence of diagnostic sugars as given in Table 2.5 (Lechevalier *et al.*, 1971).

Table 2.3 Chemotypes of cell wall in actinomycetes (Lechevalier *et al*., 1971)

Chemical component	**Cell wall type**							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
Diaminobutyric acid*	-	-	-	-	-	-	+	-
Lysine	-	-	-	-	+	+	+	-
Ornithine	-	-	-	-	+	-	-	-
Aspartic acid	-	-	-	-	-	+	+	+
Glycine	+	+	-	-	*	*	*	*
Meso-DAP	-	+**	+	+	-	-	-	-
LL-DAP	+	-	-	-	-	-	-	-
Arabinose	-	-	-	+	-	-	-	-
Galactose	-	-	-	+	-	-	-	-

*, Glycine variably present in these group; **, hydroxyl-DAP may present

+, present; -, absent

Table 2.4 Whole-cell sugar patterns of actinomycetes with *meso*-DAP (Lechevalier *et al*., 1971)

Type	**Diagnostic sugar**			
	Arabinose	**Galactose**	**Madurose**	**Xylose**
A	+	+	-	-
B	-	-	+	-
C	-	-	-	-
D	+	-	-	+

2.3.2.3 Phospholipids composition

Phospholipids or polar lipids are important components of bacterial cell membranes. It has been reported that the composition of the phospholipids can be separated the actinomycetes into five groups based on the presence and absence of nitrogenous phospholipids (Lechevalier *et al.*, 1977). Phospholipid type PI has no

nitrogenous phospholipids; type PII includes one nitrogenous phospholipid; type PIII contains phosphatidylcholine; type PIV consists of an unidentified phospholipid containing glucosamine (GluNU), and type PV includes diphosphatidylglycerol as well as GluNU. The different phospholipid types are presented in Table 2.6.

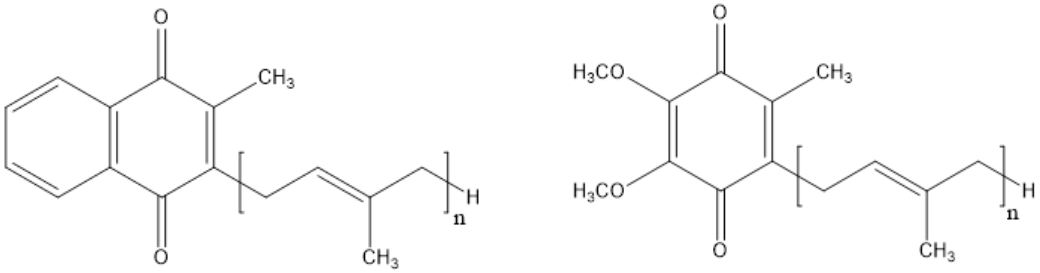
2.3.2.4 Isoprenoid quinones

Isoprenoid quinones are a component of the bacterial cytoplasmic membrane where they play a key role in the electron transport system. The isoprenoid quinones are designed based on the difference of core structures as ubiquinone and menaquinone (Figure 2.1). Menaquinones are generally presented in the plasma membrane of Gram-positive bacteria, especially in actinobacteria whereas ubiquinones are commonly found in Gram-negative bacteria and yeasts (Collins *et al.*, 1977). The different number of the isoprene units and the degree of hydrogenation are the key characters for classifying actinomycetes at the genus level.

Table 2.5 Phospholipid types of actinomycete (Lechevalier *et al.*, 1977)

Phospholipid	**Polar lipid**							
type	**PIMs**	**PI**	**PC**	**PG**	**PE**	**PME**	**GluNU**	**DPG**
PI	+	+	-	v	-	-	-	v
PII	+	+	-	v	+	-	-	+
PIII	v	+	+	v	v	+	-	v
PIV	ND	+	-	-	v	v	+	+
PV	ND	+	-	v	v	-	+	+

Abbreviation: PIMs, phosphatidylinositol mannosides; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidyl methyl ethanolamine; GluNU, glucosamine containing unknown phospholipids; DPG, diphosphatidylglycerol; ND, no data; v, variable; -, absent



(a) (b)

Figure 2.1 Chemical structures of menaquinones (a) and ubiquinones (b)

2.3.2.5 Cellular fatty acids

Fatty acids are generally found in the plasma membrane of all bacteria. The difference of cellular fatty acid components involving the number of carbon atoms in molecule, presence of saturated and unsaturated (*iso*- and *anteiso*-) fatty acids, and occurrence of methyl groups fatty acid, presence of cyclopropane fatty acids, and occurrence of hydroxyl-fatty acids, is used for the classification of actinomycetes at the genus level. Additionally, the presence of mycolic acids, 2-alkyl-3-hydroxy long chain fatty acids, is an important characteristic used to identify the members of the family *Corynebacteriaceae*, *Mycobacteriaceae*, and *Nocardiaceae*. Recently, the cellular fatty acid composition was analysed by gas chromatography which provided effective and reproducible results (Sasser, 1990).

2.3.3 Genotypic characteristics

It is well known that molecular genetic techniques have been currently impacted on modern microbial taxonomy (Tindall *et al.*, 2010). Some of the molecular methods: 16S rRNA gene sequences analysis, phylogenetic analysis, DNA base composition, DNA-DNA hybridisation and whole-genome sequences analysis, are commonly used in the systemic of actinomycetes.

2.3.3.1 16S rRNA gene sequences and phylogenetic analysis

The 16S rRNA gene is a powerful molecular marker for microbial taxonomic purposes because it exhibits highly conserved regions and poorly subject to the horizontal gene transfer in archaea and bacteria. Thus, the sequencing and phylogenetic analysis of the 16S rRNA gene has been recognised as a proper method for studying the bacterial classification at various taxonomic levels (Ramasamy *et al.*, 2014). Kim *et al.*, (2012a) has generated a useful identification database (https://www.ezbiocloud.net/) relied on Basic Local Alignment Search Tool (BLAST) searches and pairwise sequence alignments, and now the database is developed and updated by Yoon *et al.*, (2017). This web-based tool gives an effective taxonomic backbone for the identification of bacteria at present.

2.3.3.2 DNA base composition

Genotypic information is obtained from deoxyribonucleic acid (DNA) present in bacterial cell. Genomic DNA consists of four nitrogenous bases: adenine (A), guanine (G), cytosine (C) and thymine (T). Genomic G+C content varies remarkably in archaea and bacteria ranged from 25-80 % G+C. The actinobacteria have high G+C content in their genomes, so this character can be distinguished the members of the phylum *Actinobacteria* from other phyla. In addition, the mol% G+C range is not more than 3 % within a species and not rather than 10 % within a genus (Vandamme et al., 1996). Consequently, the variation of G+C content (mol%) in bacterial genomes is effectively used to differentiate between bacterial taxa at the genus and maybe species levels (Tamaoka, 1994).

2.3.3.3 DNA-DNA hybridisation

DNA-DNA hybridisation (DDH) is one of the techniques based on an attempt to make comparisons of bacterial whole genomes between different species to calculate their overall genomic similarities. Up to date, the values less than 70 % DNA-

DNA relatedness have been accepted for the thresholds for delineation of the new species (Wayne *et al.*, 1987).

2.3.3.4 Whole genome sequences

At present, bacterial whole genome sequences are publicly available, and they have become a method for differentiating species as they provide a reproducible, reliable, and highly informative means to infer phylogenetic relationship among prokaryotes. The genome sequence similarities are calculated relied on the overall genome-related index (OGRI) including average nucleotide identity (ANI) and digital DNA-DNA Hybridisation (dDDH) values (Chun *et al.*, 2018). Bacterial isolates that have ANI and dDDH values less than 95-96 % (Ritcher *et al.*, 2009) and 70 % (Goris *et al.*, 2007), respectively are considered as novel species.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Plant Samples collection and isolation

3.1.1 Samples collection

Fourteen healthy plant samples, which has ethnobotanical history, little studies, easy to find and grow in various environment, in the families Acanthaceae (1 sample), Asphodelaceae (1 sample), Commelinaceae (1 sample), Euphorbiaceae (1 sample), Meliaceae (1 samples), Musaceae (5 samples), Orchidaceae (1 sample) and Zingiberaceae (1 sample) were collected in Thailand during June to August 2015 (Table 3.1). The plants were carefully dug out to confirm that maximum amounts of root material were completely collected. The samples were placed in plastic bags and processed within 6 h after collection.

3.1.2 Surface-sterilisation and isolation

Each root sample was washed with running tap water to remove soil particles and organic matters, cut into 2 cm in length, and then surface-sterilised according to the modified method of Coombs & Franco, (2003) and Qin *et al.*, (2009a) by soaking in 75 % (v/v) ethanol for 5 minutes, 3 % (v/v) sodium hypochlorite for 10 minutes, washing three times with sterile water to remove sterilizing agents, and finally immersed in 10 % (w/v) sodium bicarbonate for 10 minutes to inhibit fungal growth and disrupt plant tissues. Each of the surface-sterilised roots was ground in a sterile mortar and then pestle with 4 % (w/v) sucrose. The homogenized roots were incubated at 60 °C for 10 min in a water bath, and then 0.1-mL of each sample was spread onto 4 isolation media: 2.5 % (w/v) water agar (WA) (Okazaki, 2003), starch casein gellan gum (SCG) (Küster & Williams, 1964), glycerol arginine gellan gum (GAG) (Arai, 1975), and Gauze mineral medium no. 1 (Gauze *et al.*, 1983). supplemented with 25 $\mu g \cdot mL^{-1}$ of nalidixic acid and 50 $\mu g \cdot mL^{-1}$ of cycloheximide. The isolated plates were incubated at

28±2 °C for 21-30 days. The colonies of endophytic actinomycetes were observed and transferred to yeast extract-malt extract (ISP2) agar for purification. The pure cultures were maintained on ISP2 agar slant at 4 °C. All isolates will be preserved by lyophilized and freezing at 80 °C in 15 % (v/v) glycerol solution for long term preservation.

Table 3.1 List of plant samples in this study

Scientific name	**Common name**	**Family**	**Source (Province)**
Aloe vera L.	Aloe	Asphodelaceae	Chachoengsao
Azadirachta indica var. *siamensis* Valeton.	Neem tree	Meliaceae	Chachoengsao
Clinacanthus nutans (Burm.f) Lindau.	Sabah snake grass	Acanthaceae	Prachinburi
Curcuma aromatica L.	Wan nang kham	Zingiberaceae	Bangkok
Dendrobium scabrilingue Lindl.	Ueang sae hom	Orchidaceae	Chiang Mai
Euphorbia hirta L.	Nom raatchasee	Euphorbiaceae	Chachoengsao
Globba winitii C.H. Wright	Globba	Zingiberaceae	Bangkok
Musa (AAB) Kluai 'Chang'	Plantain	Musaceae	Suphan Buri
Musa (ABB) Kluai 'Namwa'	-	Musaceae	Chachoengsao
Musa (AAB) Kluai 'Nom Sao'	-	Musaceae	Nakhon Pathom
Musa (AAA) Kluai 'Sai Nam Phueng'	-	Musaceae	Suphan Buri
Musa (AA) Kluai 'Sao Kratuep Ho'	-	Musaceae	Suphan Buri
Stahlianthus sp.	Wan khao phansa	Zingiberaceae	Bangkok
Tradescantia spathacea Stearn.	Oyster Lily	Commelinaceae	Prachinburi

Geographic location: Bangkok (13°44′38.2″N/100°31′53.9″E), Chachoengsao (13°39′34.6″N/101°04′33.4″E), Nakhon Pathom (13°48′58.6″N/100°13′19.9″E), Prachinburi (14°11′55.5″ N/101°44′37.1″E), and Suphan Buri (14°54′22.5″N/100°04′50″E) provinces

3.2 Identification methods

The selected endophytic actinomycete strains were identified relied on the polyphasic taxonomic approach including phenotypic, chemotaxonomic and genotypic characteristics. The cell suspension of all strains was received from the culture cultivated in yeast extract-malt extract broth (Shirling & Gottlieb, 1966) under shaking condition at 180 rpm 28±2 °C for 4-7 days. A 0.1-mL of each culture was washed three times with 0.85% (w/v) NaCl solution to eliminate the culture broth and used for all phenotypic studies.

3.2.1 Morphological and cultural characteristics

Morphological characteristics of the isolates were observed after culturing on ISP 2 agar for 14-21 days at 28±2 °C by using the scanning electron microscopy (JEOL, JSM-6610LV, Tokyo, Japan). Cultural characteristics were determined after incubation at 28±2 °C for 14 days on various media (Shirling & Gottlieb, 1966) including yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6), tyrosine agar (ISP 7), nutrient agar and Czapek's solution agar (Appendix A). The names and designations of the colonial colours and soluble pigments were assessed using the *Colour Harmony Manual* (Jacobson *et al.*, 1958).

3.2.2 Physiological characteristics

The growth of the strains at different environmental parameters consisted of temperature (20, 30, 37, 37, 45, 50 and 55 °C), pH (4, 5, 6, 7, 8, 9, 10, 11 and 12) and NaCl (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 %, w/v) concentration were evaluated using ISP 2 agar as the basal medium after incubation at 30 °C for 14 days.

3.2.3 Biochemical characteristics

The utilisation of carbohydrates as sole carbon sources was determined using ISP 9 (Nihon Pharmaceutical) as the basal medium supplemented with a final concentration of 1 % (w/v) of the carbon sources (Shirling & Gottlieb, 1966). Starch hydrolysis, nitrate reduction, milk coagulation and peptonization, gelatin liquefaction and H_2S production were tested on ISP 4 agar (Difco), ISP 8 broth (0.5 % peptone, 0.3 % beef extract, 0.1 % KNO_3, pH 7.0), 10 % (w/v) skimmed milk (Difco), glucose-peptone-gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin pH 7.0) and ISP 6 agar (Nihon Pharmaceutical), respectively. Enzymatic activity was evaluated using the API ZYM (bioMérieux) kit.

3.2.4 Chemotaxonomic characteristics

All chemotaxonomic characteristics were determined using biomass obtained from yeast extract-malt extract broth cultures grown under shaking condition at 180 rpm, 30 °C for 7 days and freeze dried.

3.2.4.1 Diaminopimelic acid (DAP) isomer analysis

The diaminopimelic acid isomers were analysed using thin layer chromatography (TLC) (Staneck & Roberts, 1974). Approximately 10 mg of dried cells were placed into a small screw-capped tube with 1 mL of 6 N HCl and heated for 18 hours at 100 °C. After cooling, the whole cell hydrolysates were filtered through filtered paper (Whatman no. 1) and evaporated to dryness. The dried filtrates were dissolved with 0.3 mL of distilled water and applied on the baseline of a cellulose TLC sheet (20 cm x 20 cm). Ascending the TLC were developed twice with the solvent system methanol – water – 6 N HCl-pyridine (80:26:4:10, v/v). After developing, the chromatograms were visualized by spraying with 0.2 % ninhydrin solution and heated at 100 °C for 3-5 minutes. DAP isomers appeared as dark-green fading to yellow spots as a comparison with a DAP standard solution, which contained both *meso*-DAP and *LL*-DAP.

3.2.4.2 Whole cell sugars analysis

Reducing sugars of whole cell hydrolysates were determined by the HPLC procedure (Mikami & Ishida, 1983). Briefly, 50 mg of the freeze-dried cell was hydrolyzed with 1 mL of 1 N H_2SO_4 at 100 °C for 2 hours in a screw-capped tube. After cooling, the hydrolysate was added with saturated barium hydroxide [$Ba(OH)_2$] solution for adjusting the pH 5.2-5.5. The precipitate was removed by centrifugation at 4,500 rpm for 10 minutes. The supernatant was transferred into a new tube and evaporated to dryness. The residue was dissolved with 400 μL of distilled water and purified using Dowex 50WX8 (200-400 mesh) and Dowex 1X8 (200-400) mesh ion exchange resins. The fraction was collected and filtrate using 0.5 μm nylon membrane filter. The filtrate was analysed by HPLC performing on Shim-pack ISA-07//s2504 (Shimadzu, 4 mm × 250 mm) with a gradient system of 0.1 M boric acid (pH 8.0) and 0.4 M boric acid (pH 9.0) supplemented with 1 % (w/v) arginine at flow rate 0.6 mL/min. A mixture of 0.1 % (w/v) of each sugar including galactose, arabinose and xylose, rhamnose, mannose, glucose and ribose was used as a standard solution.

3.2.4.3 Mycolic acid analysis

The mycolic acids were analysed according to the method of Tomiyasu, (1982). Approximately 50-200 mg of biomass were placed into a screw-capped tube and hydrolyzed with 2 mL of 10 % KOH-methanol (2:1, v/v) at 100 °C for 2 hours. Then, 0.6 mL of 6 N HCl and 2 mL of *n*-hexane were added into the residue and shaking well to extract the lipids. After shaking, the extract was centrifuged at 3,000 rpm for 10 minutes and the upper layer was transferred into a new tube. The lower layer was extracted again with 2 mL of *n*-hexane and collected the upper layer. The upper layer was dried with nitrogen gas, 2 mL of benzene-methanol-H_2SO_4 (10:20:1, v/v) were added to the residue and heated for 2 hours at 100 °C. After cooling, the residue was added with 2 mL each of distilled water and hexane and vortexed for 5 minutes, and then centrifuged at 3,000 rpm for 10 minutes. The upper layer was transferred to a

new tube and the lower layer was extracted again with 2 mL of hexane. The upper layer was evaporated to dryness, then dissolved with a small amount of *n*-hexane and applied on a silica TLC plate (10 cm x 10 cm). The lipids extract of *Nocardia nova* JCM 4044^T was used as a positive control of mycolic acids. The TLC plate was developed with the solvent system of hexane-diethyl ether (4:1, v/v) and visualized the chromatogram by iodine vapour. Based on this system, mycolic acids will appear approximately at R_f 0.47.

3.2.4.4 Cell wall *N*-acyl type of muramic acid

The cell wall *N*-acyl type of muramic acid was determined following the method described by Uchida & Aida, (1984). The freeze-dried cells (10 mg) were placed into a screw-capped tube and hydrolyzed with 0.1 mL of 6 N HCl at 100 °C for 2 hours. The hydrolysates were purified using Dowex 1X8 (200-400) mesh ion exchange resins and eluted with 0.4 mL distilled water to furnish fraction 1, 1.0 mL distilled water to give fractions 2 and 3 and finally eluted with 1 mL of 0.5 N HCl to provide fractions 4, 5 and 6. The standard series were 0, 30, 60 and 90 nmol sodium glycolate. The 0.1-mL of fraction 5 and the standard solutions were added with 2 mL of 0.02 % (w/v) of 2,7-dihydroxynaphthalene in conc. H_2SO_4, then heated at 100 °C for 10 minutes and cooled immediately. The sample and the standards were added with 1.9 mL of 2 N H_2SO_4, mixed well, measured using the colourimetric method at a wavelength of 530 nm and calculated the concentration of glycolate. If the concentration of glycolate is more than 10 nmol/mg cell, it is indicated that glycolyl muramic acid was presented in the cell-wall peptidoglycan.

3.2.4.5 Polar lipids analysis

The polar lipids were analysed by the method of Minnikin *et al.*, (1984). Approximately 100-150 mg of freeze-dried cells were suspended in 3 mL of methanol-0.3 % (w/v) NaCl and 3 mL of petroleum ether in a screw-capped tube, mixed well for 15 minutes. The suspension was centrifuged at 3,000 rpm for 10 minutes, then the

supernatant was discarded, and 1 mL of petroleum ether was added to the cells and mixed well for 15 minutes, centrifuged and removed the supernatant. The lower layer was heated at 100 °C for 5 minutes and immediately cooled by tap water. The residue was added with 2.3 mL of chloroform-methanol-water (90:100:30, v/v) and mixed well for 15 minutes, centrifuged and transferred the upper layer into a new screw-capped tube (no.2). The lower layer was extracted twice with 2.3 mL of chloroform-methanol-water (50:100:40, v/v), mixed well for 15 minutes. The upper layer was collected to the tube from the previous step. The solution in the tube was added with 1.3 mL each of chloroform and water and mixed well for 5 minutes. The lower layer was transferred into a new vial and evaporated to dryness.

The polar lipids were determined using 2-dimensional TLC described by Minnikin *et al.*, (1977). The polar lipids extract was dissolved with 200 μL of chloroform-methanol (2:1, v/v) and applied on the corner of a silica gel TLC sheet (10 cm x 10 cm). The first dimension of TLC developing was performed in the solvent system of chloroform-methanol-distilled water (65:25:4, v/v) and the second dimension was developed in chloroform-acetic acid-methanol-distilled water (40:7.5:6:2, v/v). To visualize and compare the chromatogram patterns, each of TLC sheet was sprayed with five specific reagents consisting of molybdenum blue, ninhydrin, dragendorff's reagent, anisaldehyde and phosphomolybdic acid.

3.2.4.6 Cellular fatty acids analysis

The cellular fatty acid methyl esters were extracted according to the standard method (Sasser, 1990) with slight modification. Four steps to prepare the cellular fatty acid methyl esters were described as follow;

a) Saponification – 1 mL of reagent 1 (45 g of NaOH dissolved in 150 mL each of methanol and distilled water) was added to each screw-capped tube containing 40 mg of the freeze-dried cells, then vortexed and heated at 100 °C for 5

minutes. After heating, the tube was vigorously vortexed for 5-10 seconds, heated again at 100 °C for 25 minutes and immediately cooled.

b) Methylation – The cooled tube was added with 2 mL of reagent 2 (325 mL of 6 N HCl and 275 mL of methanol) and vortex briefly. The tube was heated for 10 minutes at 80±1 °C (This step is critical in time and temperature).

c) Extraction – 1.25 mL of reagent 3 (hexane and methyl *tret*-butyl ether, 1:1, v/v) were added to the cooled tube and vortexed for 5 minutes. The tube was centrifuged at 4,500 rpm for 10 minutes. The upper layer was transferred to a new tube and the lower layer was discarded.

d) Base wash – 3 mL of reagent 4 (10.8 g of NaOH dissolved in 900 mL distilled water) were added to the upper layer, vortexed for 5 minutes. The tube was centrifuged at 4,500 rpm for 10 minutes, 2/3 of the upper phase was collected to a vial for gas chromatography analysis.

The cellular fatty acid methyl esters were analysed by gas chromatography following the instruction of the Microbial Identification System (MIDI) Sherlock system version 6.0.

3.2.4.7 Menaquinones analysis

The menaquinones were extracted according to the method of Collins *et al.*, (1977). The freeze-dried cells (300 mg) were extracted with 20 mL of chloroform-methanol (2:1, v/v) and stirred continuously overnight. The residue was removed by filtration and the extract was evaporated to dryness using rotary evaporator at low temperature (< 37 °C). The extract was applied to a preparative silica gel TLC plate (5 cm x 20 cm). Ascending the TLC plate was performed with benzene. The chromatogram was visualized under UV light at 254 nm, scraped off and extracted with acetone (HPLC grade). The acetone extract was filtered through 0.5 μm nylon membrane filter and analysed by HPLC (Agilent 1100, Agilent, Santa Clara, CA, USA)

and mass spectrometer (JEOL JMS-T, 100LP) equipped with a Pegasil ODS column (Senshu, Tokyo, Japan), according to Tamaoka *et al.*, (1983).

3.2.5 Genotypic characteristics

3.2.5.1 Chromosomal DNA extraction

For 16S rRNA gene amplification, the chromosomal DNA was extracted from the cell grown in yeast extract-malt extract broth under shaking condition at 180 rpm, 30 °C for 4-7 days. The cell suspension (1 mL) of each isolate was washed three times with sterile distilled water. After washing, 300 μL of Tris-EDTA buffer and a small amount of aluminium oxide (Al_2O_3) were added into the cells in a microcentrifuge tube, then lysed the cells using micromixer for 90 seconds. About 300 μL of phenol-chloroform (1:1. v/v) were added to the suspension, then mixed well and centrifuged at 14,000 rpm for 15 minutes. The upper phase was collected to a new tube, then 1/10 volume of 3 mM sodium acetate and 2 volume of cold isopropanol were added. The tube was centrifuged, discarded the supernatant, washed sequentially with 70 % and 95 % ethanol and air-dried at room temperature. The genomic DNA was dissolved with 50 μL of 0.1X standard sodium citrate (SSC) solution (Appendix B) and kept at 4 °C.

The extraction of genomic DNA for the DNA base composition and whole genome sequences analysis and DNA-DNA hybridisation was prepared according to the method of Kudo *et al.*, (1998). The cells were grown in yeast extract-malt extract broth under shaking condition at 180 rpm, 30 °C for 4-7 days were harvested, washed twice with saline-EDTA (Appendix B), and resuspended with the same solution. Egg-white lysozyme, achromopeptidase and *N*-acetylmuramidase were added into the cell suspension, incubated at 37 °C for 1 hour or until the suspension became sticky and frozen at -20 °C. The frozen suspension was added with the 3-fold volume of Tris-SDS (Appendix B) and kept at 60 °C for 10 minutes. The suspension was transferred to a 50-mL conical tube, cooled on ice, added an equal volume of phenol saturated with

water and mixed for 10 minutes by shaking. The solution was centrifuged at 10,000 rpm for 15 minutes and the upper layer was transferred into a new tube. A 2-fold volume of cold ethanol was added to the upper layer to precipitate the chromosomal DNA and spooled with a sterile glass rod. The DNA was washed with 70 %, 90 % and 95 % ethanol, respectively, and air-dried at room temperature. The genomic DNA was dissolved with 0.1X SSC solution (preserved at 4 °C) and purified using the AccuPrep Genomic DNA extraction kit (Bioneer Pacific, Australia) according to the manufacturer's instructions. The chromosomal DNA has measured the purity and concentration by using Nanodrop ND-2000 UV-Vis spectrophotometer. Pure DNA normally yield the absorbance 260/280 ratio of 1.8-2.0 and 260/230 in the range of 1.8-2.2.

3.2.5.2 Amplification of 16S rRNA gene

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using two primers 20F (5´-GAGTTTGATCCTGGCTCAG-3´, positions 9-27) and 1530R (5´-GTTACCTTGTTACGACTT-3´, positions 1509-1492). The PCR mixture (final volume 100 μL) contained 4 μL of each primer (10 pmol·μL^{-1}), 2 μL of dNTP (10 mM), 10 μL of 10X *Taq* buffer, 4 μL of $MgCl_2$ (50 mM), 0.5 μL of *Taq* DNA polymerase, 65.5 μL of ultrapure water, and 10 μL of DNA template. The PCR reaction was performed with an initial denaturation at 94 °C for 3 minutes, followed by 30 cycles with denaturation at 94 °C for 1 minute, annealing at 50 °C for 1 minute and extension at 72 °C for 3 minutes (Suriyachadkun *et al.*, 2009). The PCR product was checked by gel electrophoresis and purified using the PCR purification kit (Gene aid). The nucleotide sequencing was performed using the universal primers: 27F, 518F, 800R, and 1492R (Lane, 1991) at Macrogen, Korea. The sequences were analysed using EzBiocloud server (www.ezbiocloud.net/) (Yoon *et al.*, 2017). The sequences were aligned against the sequences of the closely related type strains, which obtained from the GenBank/DDBJ/EMBL, using Bioedit software (Hall, 1999). A phylogenetic distance matrix was calculated with the Kimura-2-parameter model (Kimura, 1980). Similar tree

topologies were monitored in the phylogeny constructed through the neighbour-joining (Saitou & Nei, 1987), maximum parsimony (Fitch, 1971), and maximum likelihood (Felsenstein, 1981) method using MEGA 7.0 (Kumar *et al.*, 2016). The bootstrap values were calculated based on 1,000 replications (Felsenstein, 1985).

3.2.5.3 Determination of DNA base composition

The determination of DNA base composition was performed according to the method described by Tamaoka & Komagata, (1984). The DNA solution (2 $\mu g \cdot \mu L^{-1}$) was heated at 100 °C for 5 minutes, and immediately cooled in ice. A 10-μL of nuclease P1 solution was added to the denatured DNA and incubated at 50 °C for 1 hour. After incubation, the solution was added with 10 μL of alkaline phosphatase solution and incubated at 37 °C for 1 hour. The nucleosides composition in the sample solution was analysed by HPLC. An equimolar mixture of nucleosides was used as the quantitative standard for DNA base composition analysis as shown in the equation below.

$$\text{Mol\% G+C} = \frac{\frac{G}{Gs}+\frac{C}{Cs}}{\frac{A}{As}+\frac{G}{Gs}+\frac{C}{Cs}+\frac{T}{Ts}}$$

When; A, T, C, and G corresponding to the peak area of adenine, thymine, cytosine, and guanine in the sample solution, respectively.

As, Ts, Cs, and Gs corresponding to the peak area of adenine, thymine, cytosine, and guanine in standard solution, respectively.

3.2.5.4 DNA-DNA hybridisation

DNA-DNA relatedness was determined using the microplate hybridisation method (Ezaki *et al.*, 1989). Four steps of DNA-DNA hybridisation were described as follow;

a) Fixation of DNA samples: the DNA solution (100 μg·mL^{-1} in 0.1X SSC) was added to a microcentrifuge tube, then boiled at 100 °C for 10 minutes and immediately cooled in ice. After cooling, the DNA was diluted to 10 μg·mL^{-1} with distilled water and added with 0.5 mL of 2X phosphate buffer saline (PBS) solution and 0.1 mL of 1 M $MgCl_2$. The DNA solution was dispensed (100 μL) to each well of 96-well plate (totally 1 μg of DNA per well). The plate was tightly sealed and incubated at 37 °C overnight. The calf thymus DNA was used as a control.

b) Preparation of labelling probe: the DNA solution (10 μg·mL^{-1}) was labelled with the photobiotin solution and then exposed to the light (500 watts) for 30 minutes (in an icebox). The labelled DNA solution was added with 124 μL of ultrapure water, 16 μL of 0.1 M Tris-HCl buffer and 160 μL of *n*-butanol. The DNA solution was partitioned twice with *n*-butanol and then the upper layer (*n*-butanol) was removed. The lower layer was boiled at 100 °C for 15 minutes and immediately cooled on ice. After cooling, the DNA solution was diluted into the hybridisation solution (Appendix B) to obtain the final concentration of DNA probe 1 μg·mL^{-1}. The DNA probe was dispensed into each well of 96-well plate (finally contained 0.1 μg of DNA probe).

c) Hybridisation: After fixation the DNA overnight, the solution in the plate was discarded and 100 μL of the hybridisation solution was added to each well. The plate was sealed with aluminium foil and incubated overnight at an optimal hybridisation temperature which calculated from the equation below.

Hybridisation temperature (°C) = (0.41 x GC% of the DNA probe) + 24.3

d) Detection: The hybridisation solution was discarded. The plate was washed three times with 0.2 mL of 0.2X SSC. About 0.2 mL of solution I (Phosphate buffer saline supplemented with 0.5 % (w/v) of bovine serum albumin) was added to each well and incubated at room temperature for 10 minutes. After incubation, the solution I was discarded and 0.1 mL of solution II (streptavidin-peroxidase dissolved in the solution I) were added to each well and incubated at 37 °C for 30 minutes. The solution II was discarded and washed three times with 0.2 mL of 1X PBS solution. The 0.1-mL of 10 mg·mL^{-1} of tetramethylbenzidine – 0.3 % (v/v) of H_2O_2 was added into each well and then incubated at room temperature for 3 minutes (blue colour appeared). After incubation, 0.1 mL of 2 M H_2SO_4 was added to the wells to stop reaction (yellow colour occurred). The absorbance was measured at 450 nm using microplate reader (Microplate reader Wallac 1420, PerkinElmer^{TM}). The DNA-DNA relatedness value (%) was calculated from the equation below.

$$\text{DNA-DNA relatedness} = \frac{\textbf{DNA sample} - \textbf{Calf thymus}}{\textbf{DNA probe} - \textbf{Calf thymus}} \times 100$$

3.2.5.5 Whole genome sequences analysis

Whole genome sequencing of the selected isolates was performed using an Illumina Miseq platform (Illumina, Inc., San Diego, US-CA) by using 2 × 250 bp paired-end reads. A *de novo* assembling the reads to contigs were carried out using SPAdes 3.12 (Bankevich et al., 2012). All genomes were annotated on Prokka software 1.12 (Seemann, 2014) following the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Average nucleotide identity (ANI) values of the selected isolates together with their closely related type strains were pairwise calculated using ANI-Blast (ANIb) and ANI-MUMmer (ANIm) algorithms (Ritcher *et al.*, 2009) implemented within the JspeciesWS web service (Ritcher *et al.*, 2016). The digital DNA-DNA hybridisation (dDDH) was calculated with the Genome-to-Genome Distance Calculator (GGDC 2.1) using the

BLAST+ method (Meier-Kolthoff *et al.*, 2013). Results relied on recommended formula 2 (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genomes. The selected strains that exhibited the ANI and dDDH values less than 95-96 % (Ritcher *et al.*, 2009) and 70 % (Goris *et al.*, 2007), respectively were indicated as new species.

3.3 Screening of antimicrobial activity

An inoculum of the isolates was prepared by cultivation in 10 mL of a medium 301 (Appendix A) for 5 days at 28 °C, 200 rpm. Each inoculum was cultured in 10 mL of four production media including medium 30, medium 51, medium 57 and medium P1 (Appendix A) at 28 °C, 200 rpm for 7 days. After incubation, each culture liquid was extracted with 10 mL of 95 % ethanol for 1 h under shaking at 200 rpm for 1 h, centrifuged at 1,630 *g* for 10 min and the supernatant was collected. The supernatants of thirty-seven isolates (50 μL) were applied to paper disks (6 mm) which were then used in antimicrobial activity tests.

Antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Staphylococcus aureus* ATCC 6538p, *Escherichia coli* NIHJ, *Xanthomonas campestris* pv. *oryzae* KB88, *Candida albicans* ATCC 64548 and *Mucor racemosus* IFO 4581 were determined by a paper disk diffusion method (Mearns-Spragg *et al.*, 1998). The media used for lawn inoculation of bacterial (1×10^8 cell/mL) and fungal test strains (1×10^6 cell/mL) were Muller-Hinton agar (MHA) and Sabouraud Dextrose Agar (SDA), respectively. Inoculated plates were incubated at 37 °C for 24 h for bacteria and at 25 °C for 48 h for fungi. All experiments were performed in triplicate. The inhibition zone (mm) was observed and recorded as an index of antimicrobial activity.

3.4 Secondary metabolites study the selected actinomycete strains

3.4.1 Fermentation and extraction

A seed culture of the selected strains was cultivated in the medium 301 for 4-7 days at 28±2 °C, 200 rpm (up to the nature of the isolates). Approximately 1% of each inoculum was transferred into the production medium and incubated under shaking condition (180 rpm) at 28±2 °C for 6 days. The detail for fermentation and extraction of the selected isolates were explained in the results of each isolate (chapter IV).

3.4.2 Chemical profile analysis

The chemical profile of the crude extracts was analysed using HPLC (UltiMate 3000, DIONEX) performed with a C-18 column (Sunfire™, 5 μm, 4.6 × 150 mm) with a gradient system of 0-100 % CH_3CN in H_2O plus 0.05 % (v/v) formic acid, flow rate 0.5 mL/min for 18 minutes. The UV/UV-VIS was used as a detector. The HPLC chromatograms received from the system were determined by comparing with the in-house database of BIOTEC, NSTDA, Thailand. The LC-UV spectra were measured by an Agilent LC/UV system equipped with Pegasil ODS SP100 column (5 μm, 4.6 × 250 mm; Senshu Scientific Co. Ltd.) with a gradient system of 5-100 % acetonitrile in water plus 0.01 % (v/v) trifluoroacetic acid. The chemical profile, UV absorbance and retention time were compared with the in-house database of Kitasato Institute of Life Science, Tokyo, Japan and the dictionary of Natural Product (http://dnp.chemnetbase.com/).

3.4.3 Purification and chemical structure elucidation of the compounds

The crude extract of each selected strain was firstly purified using open-column chromatography (Sephadex LH-20, ODS, and/or silica gel as a stationary phase) followed by preparative HPLC [Pegasil ODS SP100 column (Shiseido, 20 × 250 mm) or CAPCELL PAK UG-120 column (Shiseido, 20 × 250 mm)]. Details for the isolation process and purification schemes of each isolate were given in chapter IV. HRESI-MS spectra were obtained from a Bruker MicroOTOF mass spectrophotometer. ESI-MS spectra were determined on a QSTAR Elite Hybrid MS/MS spectrometer (AB Sciex Co., MA, USA). UV

spectra were measured with a Hitachi U-2810 spectrophotometer (Hitachi, Tokyo, Japan). NMR spectra were measured using a Bruker Avance III 400 MHz or a Bruker Avance 500 MHz NMR spectrometer.

3.5 Biological activities testing of the isolated compounds

3.5.1 Antibacterial activity

The crude extracts and/or isolated compounds were evaluated the antibacterial activity against *B. cereus* ATCC 11778 and *E. coli* ATCC 25922, which are the representative strains of Gram-positive and Gram-negative bacteria, respectively, using the resazurin microtiter-plate assay (REMA) (Sarker *et al.*, 2007). The tested bacteria were cultivated in 5 mL of tryptic soy broth at 37 °C for 12 hours under shaking condition at 200 rpm to reach the optical density in the range of 0.5-1.0 at 600 nm (approximately 5 x 10^6 CFU/mL), and then 10 μL of the cell suspension was dispensed to each well of 96-well plate to achieve the final concentration of 5 x 10^4 CFU/well, then 15 μL of tested material, 50 μL of 0.25 mM resazurin and 74 μL of Mueller-Hinton broth (MHB) were added. The plate was prepared in triplicate and placed in an incubator set at 37 °C for 3 hours. After incubation, the fluorescent intensity (excitation/emission at 530/590) was measured. The minimum inhibitory concentration (MIC) is the lowest concentration of the compound which inhibits 90 % of the bacterial cell growth. Vancomycin and cefotaxime were used as a positive control for *B. cereus* and *E. coli*, respectively, while 0.5 % (v/v) of DMSO was used as a negative control.

3.5.2 Antifungal activity

The antifungal activity was determined using the modified resazurin microtiter-plate assay (REMA) of Sarker *et al.*, (2007). The yeast, *C. albicans* ATCC 90028, was cultured on potato dextrose agar (PDA) at 30 °C for 3 days and then transferred to RPMI-1640 and adjusted the cell density to 5 x 10^5 CFU/mL. About 45 mL of the cell suspension and 5 μL of tested compounds were added to each well of 96-well plate,

then incubated at 30 °C for 4 hours. After incubation, 10 μL of resazurin solution (62.5 $\mu g \cdot mL^{-1}$) were added to each well and incubated at 37 °C for 30 minutes. The inhibitory concentration (IC_{50}) is the concentration of the tested compound that inhibits 50 % growth of the yeast cells. Amphotericin B and 0.5 % (v/v) DMSO were used as a positive and negative control, respectively.

3.5.3 Anti-Mycobacterium tuberculosis (TB) activity

The anti-*Mycobacterium tuberculosis* activity was evaluated using the green fluorescent protein microplate assay (GFPMA) (Changsen *et al.*, 2003). The minimum inhibitory concentration (MIC) acts for the lowest concentration of the compound which inhibits 90 % of the *M. tuberculosis* cells growth. Some antibiotics; Ethambutol, isoniazid, ofloxacin, rifampicin, and streptomycin were used as the positive control, while 0.5 % (v/v) of DMSO was used as a negative control.

3.5.4 Antimalarial activity

The *in vitro* culture of the asexual erythrocytic stage of *Plasmodium falciparum* (K1, multidrug-resistant strain) was cultivated according to the method of Trager and Jensen (1976). Quantitative assessment of antimalarial activity was determined using the microculture radioisotope technique described by Desjardins *et al.*, (1979). The inhibitory concentration (IC_{50}) represents the concentration that causes 50 % reduction of the growth of parasite as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum* strain K1. Chloroquine diphosphate and 0.5 % (v/v) DMSO were used as positive control and negative control, respectively.

3.5.5 Cytotoxic activity

The crude extracts and/or isolated compounds were determined the cytotoxic activity against KB (human oral cavity cancer), MCF-7 (human breast cancer, ATCC HTC-22), and NCI-H187 (human small-cell lung cancer, ATCC CRL-5804). The cytotoxic activity was tested using the resazurin microplate assay (REMA) as described by Ó Brien

et al., (2002). The inhibitory concentration (IC_{50}) represents the concentration of the tested compound that causes a 50 % reduction of the tested cell lines. Ellipticine and doxorubicin were used as positive controls for KB and NCI-H187 cell lines. Tamoxifen and doxorubicin were used as positive controls for MCF-7. The 0.5 % (v/v) DMSO was used as a negative control for all tests.

3.5.6 Cytotoxicity against Vero cells

The cytotoxicity of the crude extracts and/or isolated compounds against the non-cancerous Vero cell (African green monkey kidney fibroblasts; ATCC CCL-81) was evaluated using the green fluorescent protein microplate assay (GFPMA) (Changsen *et al.*, 2003). The inhibition concentration (IC_{50}) represents the concentration of the compound that causes a 50 % reduction of the Vero cells. Ellipticine and 0.5 % (v/v) were used as the positive and negative controls, respectively.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Plant samples collection and isolation of endophytic actinomycetes

In the present study, 50 actinomycete isolates were isolated from surface-sterilised roots of fourteen plant species collected in six provinces of Thailand including Bangkok, Chachoengsao, Chiang Mai, Nakhon Pathom, Prachinburi and Suphan Buri. Starch casein gellan gum, glycerol arginine gellan gum, 2.5 % water agar, and Gauze mineral medium no.1 yielded 23, 12, 10, and 5 isolates, respectively (Table 4.1).

4.2 Identification of endophytic actinomycetes

The actinomycete isolates were primarily classified using the 16S rRNA gene sequences analysis, morphological and chemotaxonomic characteristics. Based on these findings, they were classified into two families, *Streptomycetaceae* and *Micromonosporaceae*, incorporating with four genera: *Micromonospora* (26 isolates), *Plantactinospora* (1 isolate), *Polymorphospora* (1 isolate) and *Streptomyces* (22 isolates) (Figure 4.1). The type strains which exhibited the highest similarities of the 16S rRNA gene sequence with each isolate are presented in Table 4.1.

Table 4.1 16S rRNA gene sequence similarity of the isolated actinomycetes

Isolate no.	Plant source	% similarity	Nearest species
AZ1-1[a]	*Azadirachta indica* var. *siamensis* Valeton.	100	*M. krabiensis* DSM 45344^T
AZ1-2[a]	*Azadirachta indica* var. *siamensis* Valeton.	100	*M. krabiensis* DSM 45344^T
AZ1-3[b]	*Azadirachta indica* var. *siamensis* Valeton.	99.92	*M. krabiensis* DSM 45344^T
AZ1-4[b]	*Azadirachta indica* var. *siamensis* Valeton.	100	*M. krabiensis* DSM 45344^T
AZ1-5[a]	*Azadirachta indica* var. *siamensis* Valeton.	100	*M. krabiensis* DSM 45344^T
AZ1-11[b]	*Azadirachta indica* var. *siamensis* Valeton.	99.40	*M. avicenniae* 268506^T
AZ1-13[a]	*Azadirachta indica* var. *siamensis* Valeton.	99.72	*M. zingiberis* PLAI1-1^T
AZ1-19[b]	*Azadirachta indica* var. *siamensis* Valeton.	98.75	*M. costi* CS1-12^T
			M. avicenniae 268506^T
AZ1-28[a]	*Azadirachta indica* var. *siamensis* Valeton.	99.55	*M. chokoriensis* 2-19/6^T
DS1-3[b]	*Dendrobium scabrilingue* Lindl.	99.77	*M. maritima* D10-9-5^T
DS1-4[b]	*Dendrobium scabrilingue* Lindl.	99.92	*M. maritima* D10-9-5^T
NGC1-1[a]	*Musa* (AAB) Kluai 'Chang'	99.93	*M. haikouensis* 232617^T
NGC1-4[a]	*Musa* (AAB) Kluai 'Chang'	98.80	*M. chersina* DSM 44151^T
SKH1-5[d]	*Musa* (AA) Kluai 'Sao Kratuep Ho'	99.78	*M. haikouensis* 232617^T
SKH1-6[d]	*Musa* (AA) Kluai 'Sao Kratuep Ho'	99.85	*M. oryzae* CP2R9-1^T
SNP1-4[a]	*Musa* (AAA) Kluai 'Sai Nam Phueng'	99.70	*M. echinospora* ATCC 15837^T
SNP1-7[d]	*Musa* (AAA) Kluai 'Sai Nam Phueng'	99.40	*M. carbonaceae* DSM 43815^T
MS1-11[a]	*Musa* (ABB) Kluai 'Namwa'	99.92	*M. carbonaceae* DSM 43815^T
MS1-15[b]	*Musa* (ABB) Kluai 'Namwa'	99.85	*M. carbonaceae* DSM 43815^T
MS1-8[d]	*Musa* (ABB) Kluai 'Namwa'	99.87	*M. andamanensis* SP03-05^T
MS1-9[a]	*Musa* (ABB) Kluai 'Namwa'	98.85	*M. peucetia* DSM 43363^T
MS1-16[a]	*Musa* (ABB) Kluai 'Namwa'	99.72	*M. zingiberis* PLAI1-1^T
WDT1-1[a]	*Stahlianthus* sp.	99.92	*M. aurantiaca* ATCC 27029^T
WDT1-2[a]	*Stahlianthus* sp.	99.93	*M. tulbaghiae* TUV1^T
WPS1-2[a]	*Globba winitii* C.H. Wright	99.02	*M. costi* CS1-12^T
WCL1-2[c]	*Curcuma aromatica* L.	99.85	*M. maritima* D10-9-5^T
WDT1-3[c]	*Stahlianthus* sp.	99.11	*Pl. mayteni* YIM 61359^T
AZ1-9[a]	*Azadirachta indica* var. *siamensis* Valeton.	99.92	*P. rubra* TT97-42^T
AZ1-7[b]	*Azadirachta indica* var. *siamensis* Valeton.	97.95	*S. specialis* GW41-1564^T
AZ1-15[b]	*Azadirachta indica* var. *siamensis* Valeton.	97.95	*S. specialis* GW41-1564^T
AZ1-29[a]	*Azadirachta indica* var. *siamensis* Valeton.	97.90	*S. specialis* GW41-1564^T
DS1-1[a]	*Dendrobium scabrilingue* Lindl.	97.91	*S. specialis* GW41-1564^T

Table 4.1 (continued)

Isolate no.	Plant source	% similarity	Nearest species
DS1-2[a]	*Dendrobium scabrilingue* Lindl.	97.99	*S. specialis* GW41-1564^T
MS1-3[a]	*Musa* (ABB) Kluai 'Namwa'	97.80	*S. specialis* GW41-1564^T
MS1-14[a]	*Musa* (ABB) Kluai 'Namwa'	97.86	*S. specialis* GW41-1564^T
MS1-6[d]	*Musa* (ABB) Kluai 'Namwa'	99.45	*S. aculeolatus* NBRC 14824^T
MS1-13[d]	*Musa* (ABB) Kluai 'Namwa'	100	*S. lannensis* TA4-8^T
SKH1-1[d]	*Musa* (AA) Kluai 'Sao Kratuep Ho'	99.16	*S. alboniger* NRRL B-1832^T
SKH1-2[d]	*Musa* (AA) Kluai 'Sao Kratuep Ho'	99.16	*S. pseudovenezuelae* DSM 40212^T
SKH1-3[d]	*Musa* (AA) Kluai 'Sao Kratuep Ho'	99.23	*S. alboniger* NRRL B-1832^T
NS1-3[c]	*Musa* (AAB) Kluai 'Nom Sao'	99.93	*S. cellulosae* NBRC 13027^T
NS1-6[c]	*Musa* (AAB) Kluai 'Nom Sao'	99.77	*S. cellulosae* NBRC 13027^T
NS1-7[c]	*Musa* (AAB) Kluai 'Nom Sao'	100	*S. warraensis* NBRC 13404^T
AV1-4[d]	*Aloe vera* L.	100	*S. tendae* ATCC 19812^T
AV1-10[a]	*Aloe vera* L.	99.85	*S. parvulus* NBRC 13193^T
CN1-1[a]	*Clinacanthus nutans* (Burm.f) Lindau.	99.47	*S. bellus* ISP 5185^T
CN1-2[a]	*Clinacanthus nutans* (Burm.f) Lindau.	99.14	*S. bellus* ISP 5185^T
EH1-1[d]	*Euphorbia hirta* L.	99.75	*S. gradneri* NBRC 15865^T
EH1-2[d]	*Euphorbia hirta* L.	99.70	*S. diastaticus* NRRL B-1773
TS1-4[b]	*Tradescantia spathacea* Stearn.	99.61	*S. pratensis* ch24

a, Starch casein gellan gum; b, Water agar (2.5 %); c, Gauze mineral no.1; d, Glycerol arginine gellan gum

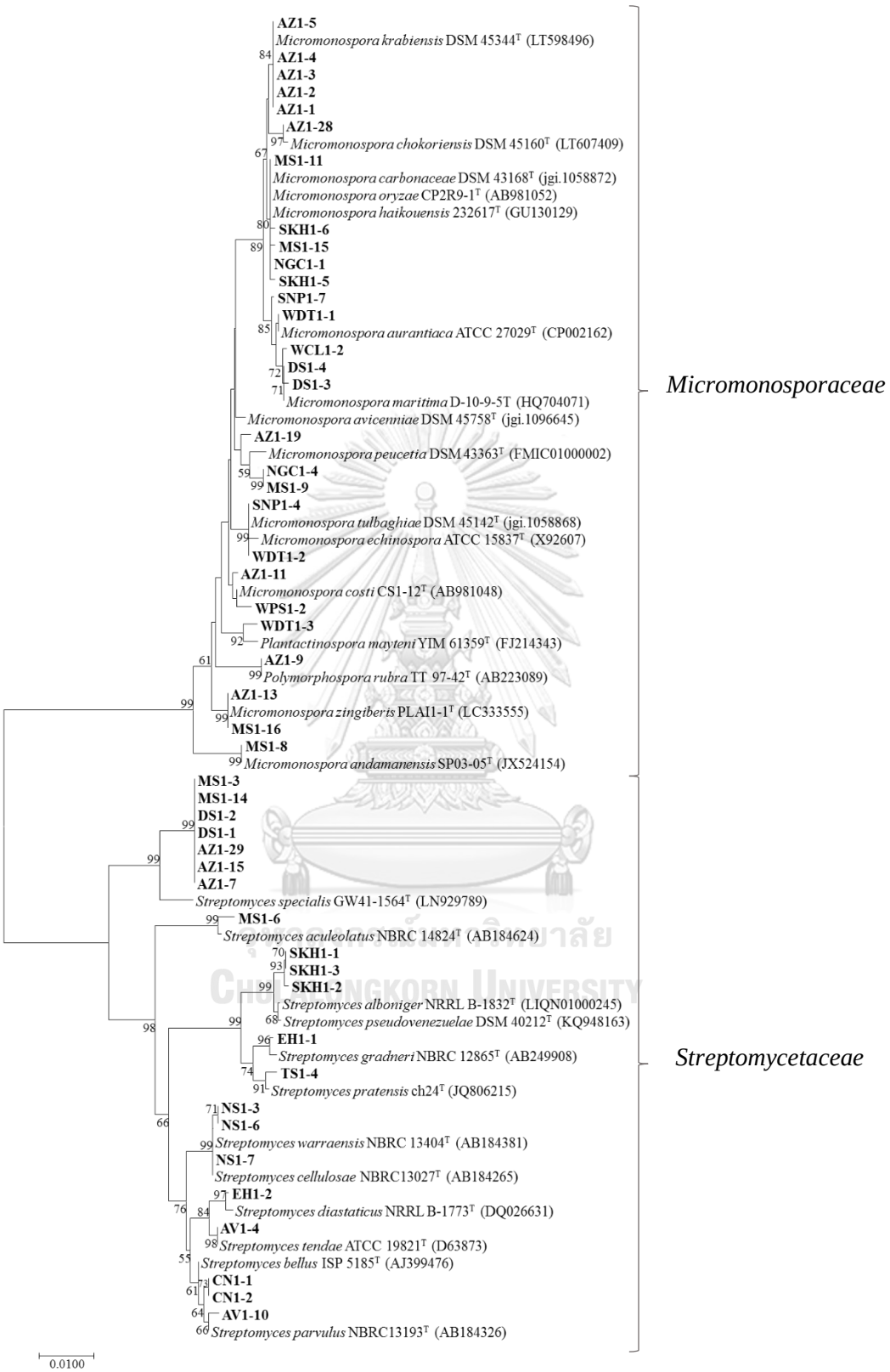


Figure 4.1 A Neighbour-joining phylogenetic relationship based on the 16S rRNA gene sequences of the 50 actinomycete isolates.

4.2.1 Family *Streptomycetaceae*

Twenty-two isolates: AZ1-7, AZ1-15, AZ1-29, DS1-1, DS1-2, MS1-3, MS1-14, MS1-6, MS1-3, SKH1-1, SKH1-2, SKH1-3, NS1-3, NS1-6, NS1-7, AV1-4, AV1-10, CN1-1, CN1-2, EH1-1, EH1-2, TS1-4, were designed into the genus *Streptomyces* belonged to the family *Streptomycetaceae*. They formed well developed pinkish-white-coloured to light-greyish-grey-coloured substrate mycelia which fragmented easily and pale-greenish-yellow-coloured to dark-greyish-coloured aerial mycelia on ISP 2 agar after cultured for 14 days. Almost isolates produced elliptical or short rod spores in spiral chains. Based on the chemotaxonomic analysis, all isolates consisted of *LL*-diaminopimelic acid (A_2pm) as a diagnostic amino acid in cell-wall peptidoglycan, glucose and ribose (no diagnostic sugar) in their cell-wall hydrolysates. *N*-acetyl of muramic acid was presented in cell-wall peptidoglycan, while mycolic acids were absent. Moreover, the analysis of 16S rRNA gene sequences (ranged from 97.70 to 100 % similarity) and phylogeny highlighted that all these isolates formed a clade within the genus *Streptomyces* (Figure 4.2).

4.2.2 Family *Micromonosporaceae*

Members of this genus were aerobic, Gram-positive, filamentous bacteria which exhibited outstanding morphological characteristics involving non-motile single spores borne directly on the tips of non-fragmented substrate hyphae, and aerial mycelia were absence. The mycolic acids were absent. They were assigned into three genera based on the key morphological and chemotaxonomic properties, the 16S rRNA gene sequences and phylogenetic analysis.

Genus *Micromonospora* contained 26 isolates including AZ1-1, AZ1-2, AZ1-3, AZ1-4, AZ1-5, AZ1-11, AZ1-13, AZ1-19, AZ1-28, DS1-3, DS1-4, NGC1-1, NGC1-4, SKH1-5, SKH1-6, SNP1-4, SNP1-7, MS1-11, MS1-15, MS1-8, MS1-9, MS1-16, WDT1-1, WDT1-2, WPS1-2 and WCL1-2. They produced single spores on the tips of the substrate mycelia. The colour of the colonies formed on ISP 2 agar after cultured more than 10 days was

vivid orange to olive grey. According to the 16S rRNA gene sequence analysis, all isolates showed the highest similarities of the 16S rRNA gene sequence with the type strains of the genus *Micromonospora* and formed a cluster with those validly published *Micromonospora* species in the phylogenetic tree (Figure 4.3). They were identified as *Micromonospora*.

Genus *Plantactinospora* consisted of a single isolate, WDT1-3. It formed single spores and short spore chains at the tips of the substrate mycelia. The colonies were pale-orange-yellow coloured on ISP 2 agar after incubated for 14 days. The 16S rRNA gene sequence analysis revealed that the isolate exhibited the highest 16S rRNA gene sequence similarity value of 99.11 % with *Pl. mayteni* YIM 61359^{T}. The phylogenetic tree analysis (Figure 4.3) confirmed that isolate WDT1-3 was identified as *Plantactinospora*.

Genus *Polymorphospora* comprised 1 isolate, AZ1-9, which produced single spores at the tips of the substrate mycelia. The colour of its colonies on ISP 2 agar after cultured for 2 weeks was vivid reddish orange. Based on the analysis of 16S rRNA gene sequence, isolate AZ1-9 was phylogenetically closest to *Polymorphospora rubra* TT97-42^{T} with 99.92% similarity; furthermore, the phylogeny (Figure 4.3) argued that the isolate was identified as *Polymorphospora*.

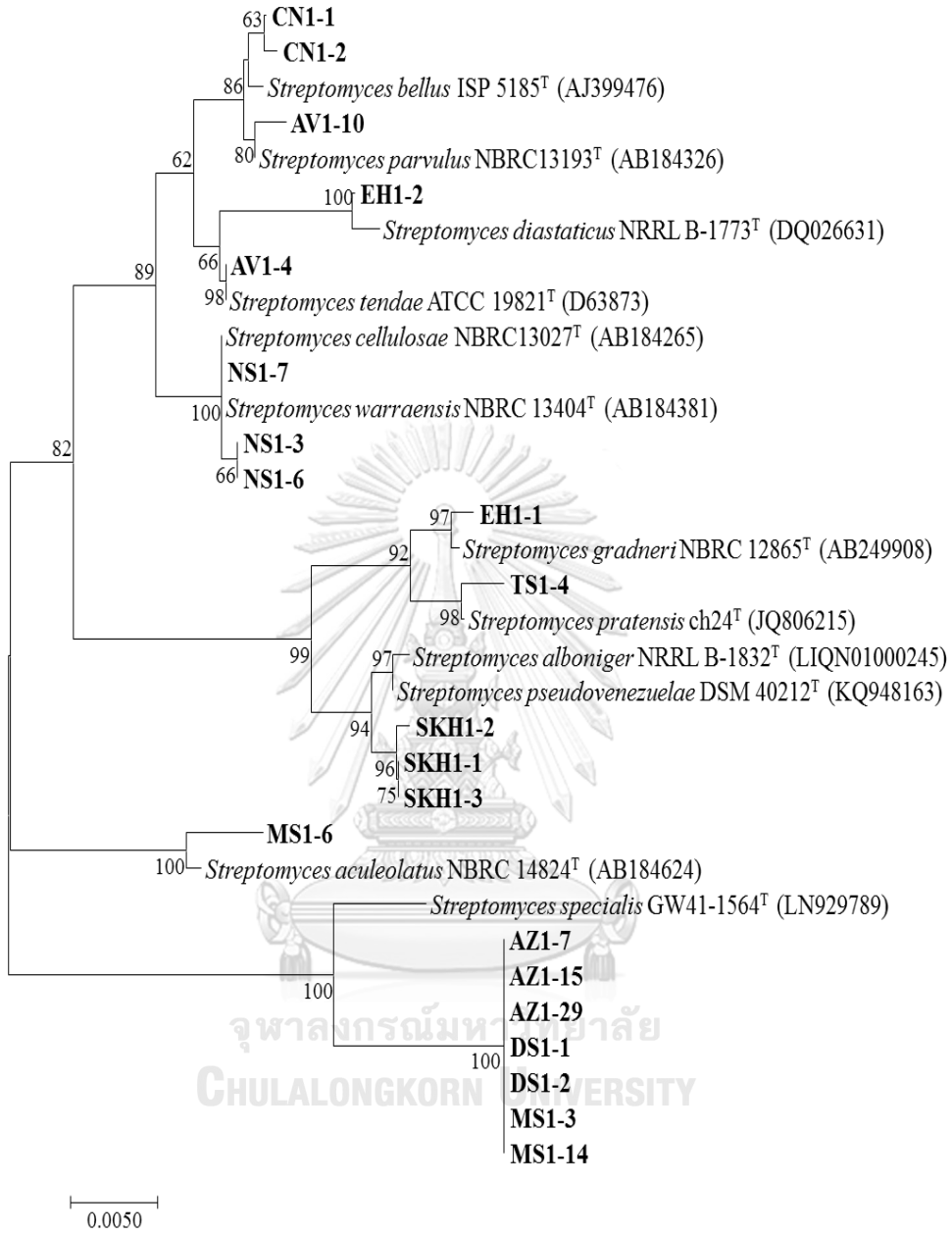


Figure 4.2 Neighbour-joining phylogenetic tree relied on the 16S rRNA gene sequences of the *Streptomyces* isolates and some type strains.

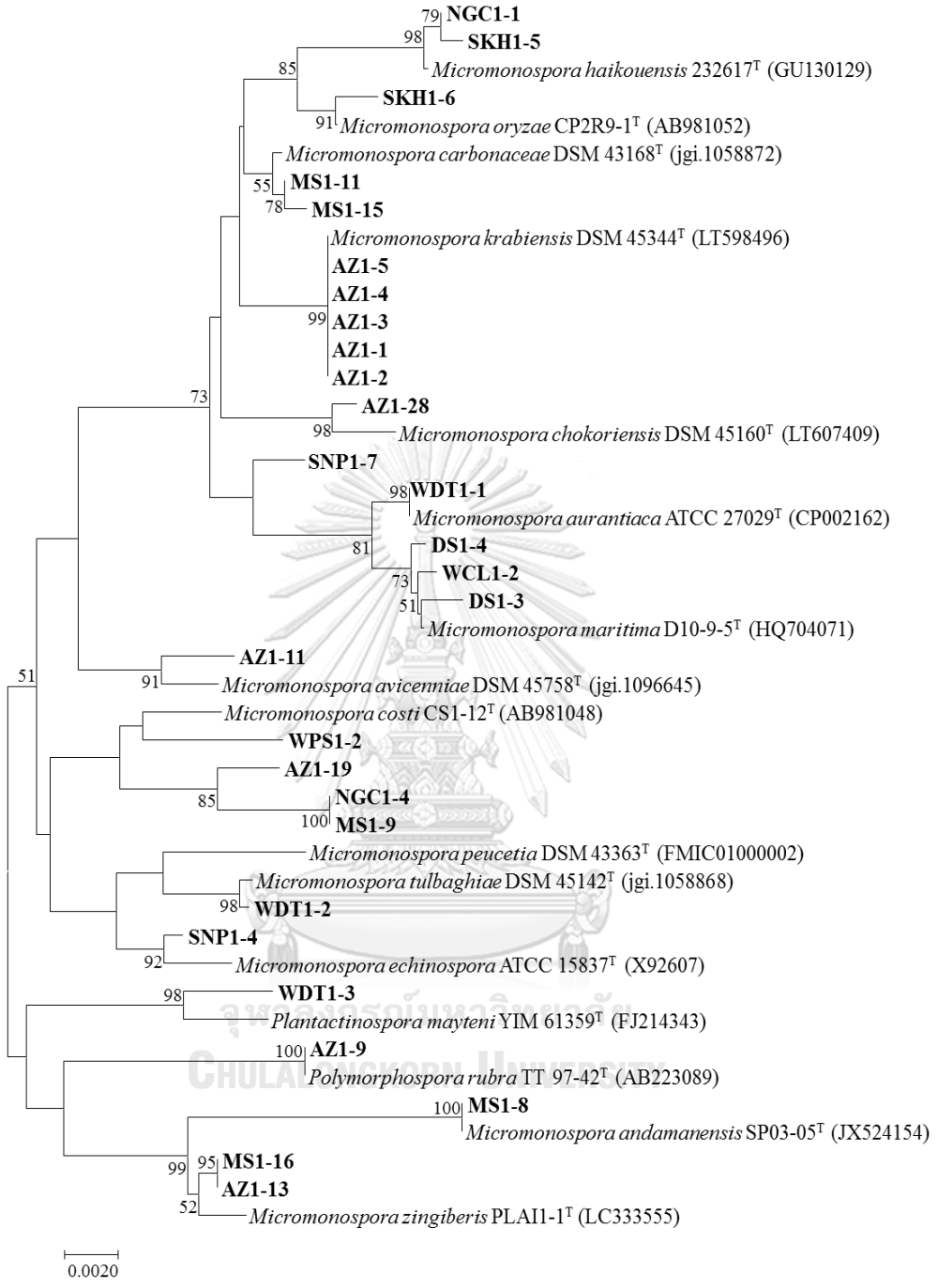


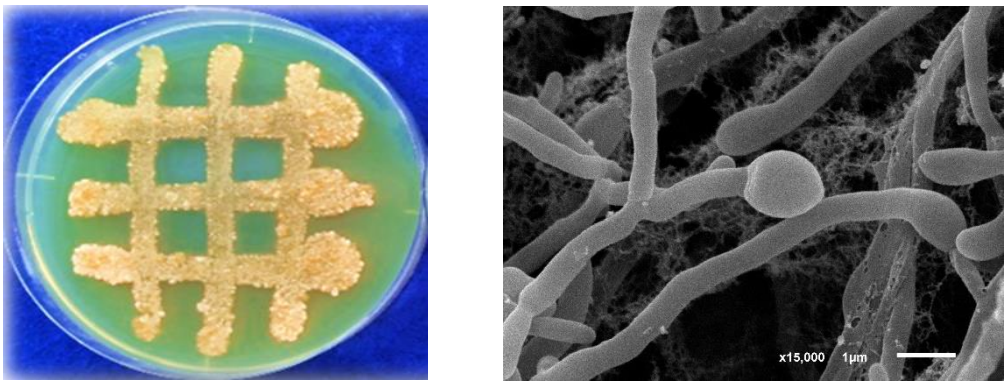
Figure 4.3 Neighbour-joining phylogeny based on the 16S rRNA gene sequences of the isolates in the family *Micromonosporaceae* and some type strains of the genus *Micromonospora*, *Plantactinospora* and *Polymorphospora*.

4.3 Taxonomic studies of novel endophytic actinomycete species

In the present study, seven isolates that exhibited the 16S rRNA gene sequence similarities lower than 99 % in BLASTn analysis including WPS1-2, AZ1-19, AZ1-13, MS1-9, NGC1-4, AZ1-7 and DS1-2 were recruited for a polyphasic taxonomic study.

4.3.1 Characterization of *Micromonospora globbae*

According to the morphological observation, strain WPS1-2^T produced oval-shaped spores with a smooth surface which were borne singly on the tip of the substrate mycelia. The spores were 1.1–1.2 μm in size. Vivid-orange-yellow substrate hyphae were observed on ISP 2 agar medium, whilst aerial mycelia were absent (Figure 4.4). The cultural characteristics of strain WPS1-2^T were described in Table 4.2. The strain grew well at pH 5.0-9.0, but weakly at pH 4.0. The temperature range for growth of strain WPS1-2^T was 20-45 °C (optimally at 30 °C). No growth was observed at 50 °C. Phenotypic characteristics were positive for starch hydrolysis and peptonization and coagulation of skim milk; nonetheless, gelatin liquefaction and nitrate reduction were negative. Strain WPS1-2^T utilised glucose, glycerol, *myo*-inositol, D-mannitol, mannose, raffinose, D-salicin, and sucrose as sole carbon sources but not L-arabinose, cellobiose, D-fructose, galactose, rhamnose, ribose, sorbose, and D-xylose. The strain exhibited enzymatic activities of α-chymotrypsin, α-galactosidase, α-glucosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, leucine arylamidase, and trypsin, but showed weakly activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase. The activity of lipase (C14), cysteine arylamidase, β-glucuronidase, α-mannosidase, and α-fucosidase were not found. The differential phenotypic characteristics of strain WPS1-2^T compared with its related type strains including *Micromonospora costi* CS1-12^T, *Micromonospora fulviviridis* NBRC 14026^T, and *Micromonospora krabiensis* MA-2^T were given in Table 4.3.



(a) (b)

Figure 4.4 The colonial appearance (a) and scanning electron micrograph of the oval-shaped spore with a smooth surface of strain WPS1-2^T after cultured on ISP 2 agar at 30 °C for 14 days.

Chemotaxonomic properties revealed that strain WPS1-2^T consisted of *meso*-diaminopimelic acid and 3-OH-*meso*-diaminopimelic acid in the cell-wall peptidoglycan. Arabinose, glucose, ribose, and xylose were presented as diagnostic sugar in whole-cell hydrolysates. The *N*-acyl type of muramic acid was glycolyl. Mycolic acids were absent. Phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), and phosphatidylglycerol (PG), three unidentified phospholipids (PL), aminophospholipids (APL), unidentified aminolipids (AL), and two unidentified glycolipids (G1 and G2) were detected as polar lipids which corresponding to phospholipid type II (Lechevalier *et al.*, 1977). The major cellular fatty acids (> 10 %) were *iso*-$C_{15:0}$ (27.6 %), *iso*-$C_{16:0}$ (14.1 %) and *anteiso*-$C_{15:0}$ (10.3 %). Although strain WPS1-2^T has a fatty acid profile like *M. costi* CS1-12^T and *M. krabiensis* MA-2^T, the amounts are different were shown in Table 4.4. The major menaquinones were MK-10(H_8) (59.9 %) and MK-10(H_{10}) (40.1 %). The G+C content of DNA was 73.7 mol%.

Table 4.2 Cultural characteristics of strain WPS1-2^T and closely related type strains. Strain: **1**, Strain WPS1-2^T; **2**, *Micromonospora costi* CS1-12^T; **3**, *M. fulviviridis* NBRC 10426; **4**, *M. krabiensis* MA-2^T. All data were determined in this study.

Medium	1	2	3	4
ISP medium 2				
Growth	Good	Good	Good	Good
Colonial colour	Vivid orange yellow	Strong orange yellow	Deep orange yellow	Strong orange
Soluble pigment	None	None	None	Strong yellow
ISP medium 3				
Growth	Moderate	Good	Good	Good
Colonial colour	Moderate orange yellow	Strong orange yellow	Strong orange yellow	Vivid orange
Soluble pigment	None	None	None	None
ISP medium 4				
Growth	Moderate	Moderate	Moderate	Moderate
Colonial colour	Moderate orange yellow	Strong orange yellow	Vivid yellow	Deep yellowish brown
Soluble pigment	None	None	None	None
ISP medium 5				
Growth	Poor	Moderate	Moderate	Moderate
Colonial colour	Moderate orange yellow	Vivid orange yellow	Moderate orange yellow	Dark yellowish brown
Soluble pigment	None	None	None	None
ISP medium 6				
Growth	Good	Good	Moderate	Moderate
Colonial colour	Moderate orange yellow	Dark orange yellow	Brownish grey	Strong yellowish brown
Soluble pigment	None	None	None	None
ISP medium 7				
Growth	Poor	Poor	Poor	Poor
Colonial colour	Moderate orange yellow	Moderate orange yellow	Vivid orange yellow	Strong yellowish brown
Soluble pigment	None	None	None	None
Nutrient agar				
Growth	Good	Moderate	Moderate	Good
Colonial colour	Vivid orange yellow	Vivid orange yellow	Strong orange yellow	Strong orange yellow
Soluble pigment	None	None	None	None

Table 4.3 Differential phenotypic characteristics of strain WPS1-2^{T}and closely related type strains.

Strains: **1**. WPS1-2^T; **2**. *M. costi* CS1-12^T; **3**. *M. fulviviridis* NBRC 14026^T; **4**. *M. krabiensis* MA-2^T. +, Positive; w, weakly positive; -, negative. All data are from this study.

Characteristics	1	2	3	4
Spore morphology				
Shape	Oval	Global	Global	Oval
Spore surface	Smooth	Rough and nodule	Rough	Rough
Maximum NaCl tolerance (%, w/v)	3	4	2	3
Growth at 45 °C	+	+	w	-
Utilisation of:				
Glycerol	w	-	-	-
myo-Inositol	+	-	-	w
Raffinose	+	-	+	-
D-Salicin	+	-	-	-
Sucrose	+	-	+	+
API ZYM				
Acid phosphatase	w	+	+	-
Alkaline phosphatase	w	+	+	+
α-Chymotrypsin	+	+	+	-
Cystine arylamidase	-	-	+	+
Esterase (C 4)	w	+	+	+
Esterase Lipase (C 8)	w	w	+	+
α-Galactosidase	+	+	+	-
β-Galactosidase	+	+	+	w
β-Glucosidase	w	w	+	-
Naphthol-AS-BI-phosphohydrolase	w	+	+	-
Valine arylamidase	w	w	+	+

Table 4.4 Cellular fatty acid compositions (%) of strain WPS1-2^T and closely related type strains.

The amount of fatty acid less than 0.5 % in all strains was omitted; -, not present.

Fatty acid	WPS1-2^T	*M. costi* CS1-12^T	*M. fulviviridis* NBRC 14026^T	*M. krabiensis* MA-2^T
Saturated fatty acids				
$C_{14:0}$	-	0.6	-	0.7
$C_{16:0}$	1.8	3.3	1.4	5.9
$C_{17:0}$	5.5	6.3	2.1	5.4
$C_{18:0}$	1.1	2.0	3.9	6.6
$C_{19:0}$	-	0.5	-	-
Unsaturated fatty acids				
$C_{16:1}$ 2OH	0.5	-	-	0.5
$C_{15:1}\omega6c$	0.6	-	-	-
$C_{17:1}\omega8c$	5.6	2.2	-	0.8
$C_{18:1}\omega9c$	1.0	0.7	1.8	0.9
Unsaturated branched fatty acids				
iso-$C_{15:1}$ G	3.4	1.0	-	2.4
anteiso-$C_{15:1}$ A	-	-	-	0.5
iso-$C_{16:1}$ G	2.3	0.4	-	3.8
iso-$C_{17:1}\omega5c$	-	1.4	-	-
anteiso-$C_{17:1}\omega9c$	1.1	0.6	2.2	1.3
Branched fatty acids				
iso-$C_{14:0}$	1.3	0.9	1.7	2.0
iso-$C_{15:0}$	27.6	30.0	10.9	17.0
anteiso-$C_{15:0}$	10.3	15.0	2.6	14.6
iso-$C_{16:0}$	14.1	10.1	35.8	18.4
iso-$C_{17:0}$	6.1	6.3	5.2	4.6
anteiso-$C_{17:0}$	9.9	15.0	11.4	9.5
iso-$C_{18:0}$	-	-	-	0.7
10-Methyl fatty acids				
10-methyl $C_{17:0}$	1.5	1.3	4.7	0.7
Summed feature 3a	1.3	1.2	0.6	0.6
Summed feature 9[b]	1.4	-	7.2	0.9

[a]Summed feature 3 comprised $C_{16:1}\omega7c$ or $C_{16:1}\omega6c$

[b]Summed feature 9 comprised 10-methyl $C_{16:0}$ or iso-$C_{17:1}\omega9c$

The nearest complete 16S rRNA gene sequence (1457 nt) of strain WPS1-2^T exhibited the highest similarity to *M. costi* CS1-12^T (99.02 %; 14 nt difference at 1438). A subset of the closest phylogenetic neighbours of strain WPS1-2^T based on the neighbour-joining (NJ) method presented in Figure 4.5 emphasized that the strain formed an independent cluster with *M. costi* CS1-12^T. The phylogenetic tree which included all *Micromonospora* species with validly published names is available in Appendix C. Although a clade of strain WPS1-2^T in the NJ-tree could be recovered by the tree analysed by the maximum-likelihood method, it could not be recovered in the maximum-parsimony phylogenetic tree. To clarify the position of strain WPS1-2^T to its closest phylogenetic relatives, the *gyrB* gene sequence (1117 nt) of strain WPS1-2^T and closely related species in the genus *Micromonospora* were compared. The level of *gyrB* gene sequence similarity of strain WPS1-2^T and all available sequences of the genus *Micromonospora* were ranged from 91.0 to 95.0 %. The NJ-phylogenetic tree of all available *Micromonospora* species based on the *gyrB* gene (Figure 4.6) indicated that strain WPS1-2^T was recovered in one cluster with *M. costi* CS1-12^T, supported by a 97 % bootstrap value. The result demonstrated that strain WPS1-2^T was closely related to *M. costi* CS1-12^T.

Based on the highest 16S rRNA gene similarity and phylogenetic tree analysis based on *gyrB* gene, *M. costi* CS1-12^T, *M. fulviviridis* DSM 43906^T and *M. krabiensis* MA-2^T were chosen for DNA-DNA hybridisation experiment to delineate the novel species status of strain WPS1-2^T. The DNA-DNA relatedness values of strain WPS1-2^T between its closely related type strains were described as follows: *M. costi* CS1-12^T (60.0±2.1 %), *M. fulviviridis* NBRC 14026^T (19.0±0.3 %), and *M. krabiensis* MA-2^T (43.1±0.3 %). These values were lower than 70%, the threshold value assigning for the same species (Wayne *et al.*, 1987).

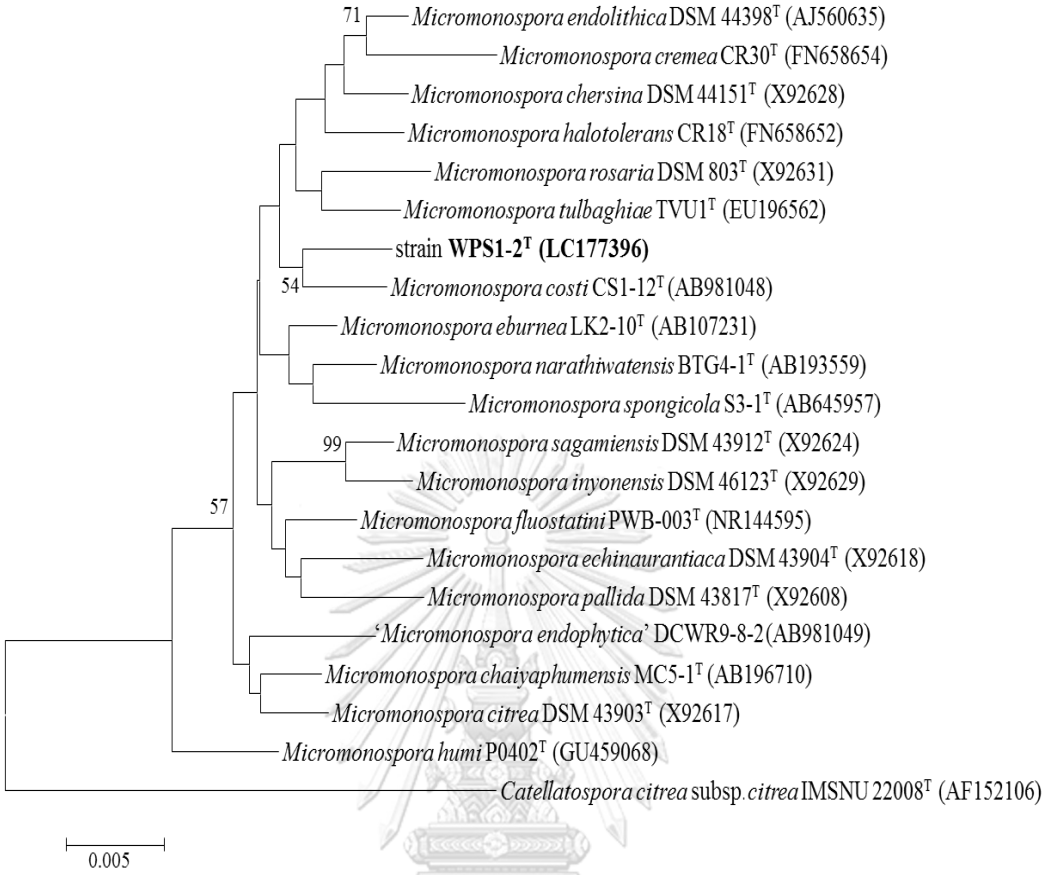


Figure 4.5 A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain WPS1-2^T and the closely related type strains.

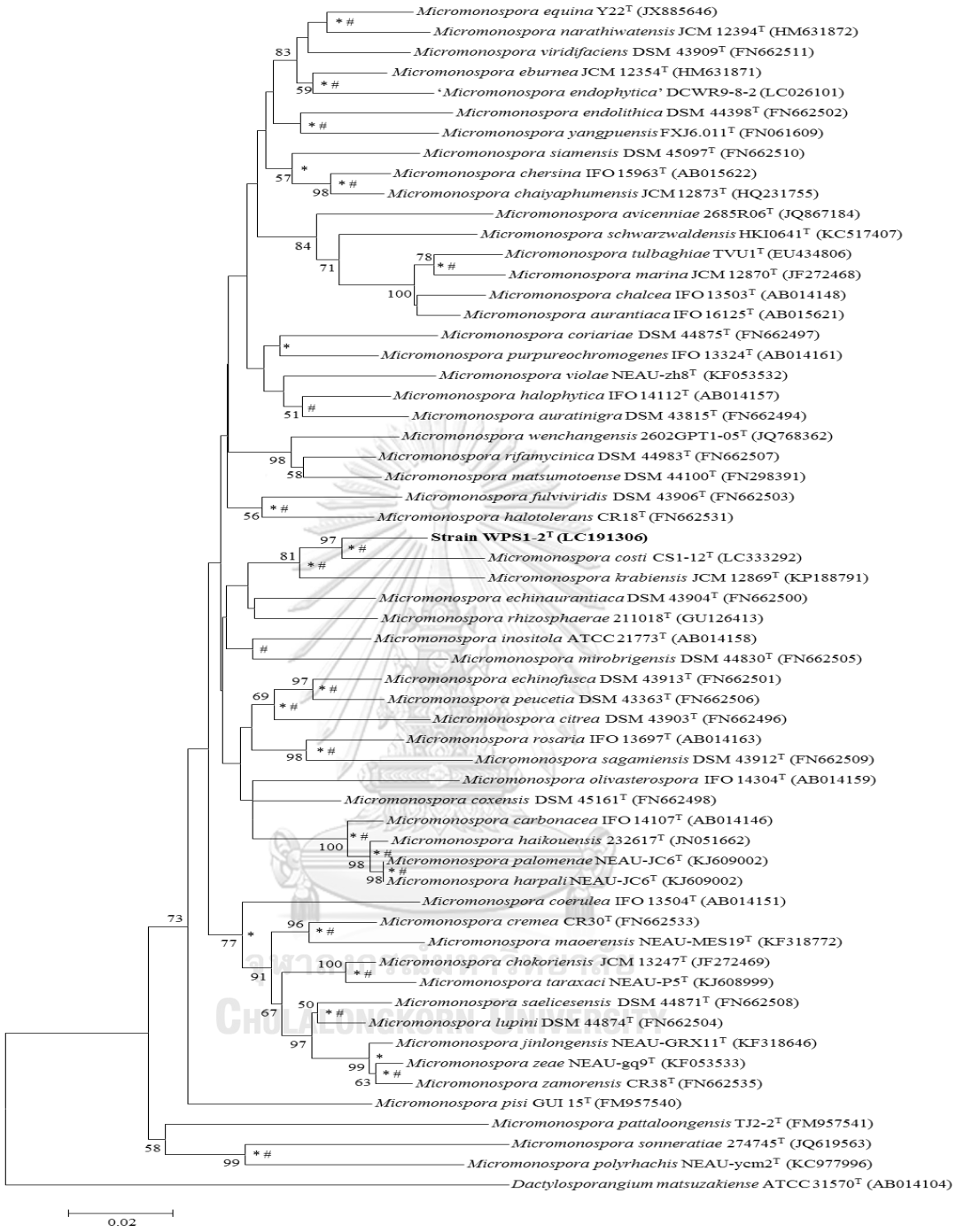


Figure 4.6 NJ-phylogenetic relationships based on the *gyrB* gene sequences (1117 nt) of strain WPS1-2^{T} and closely related type strains.

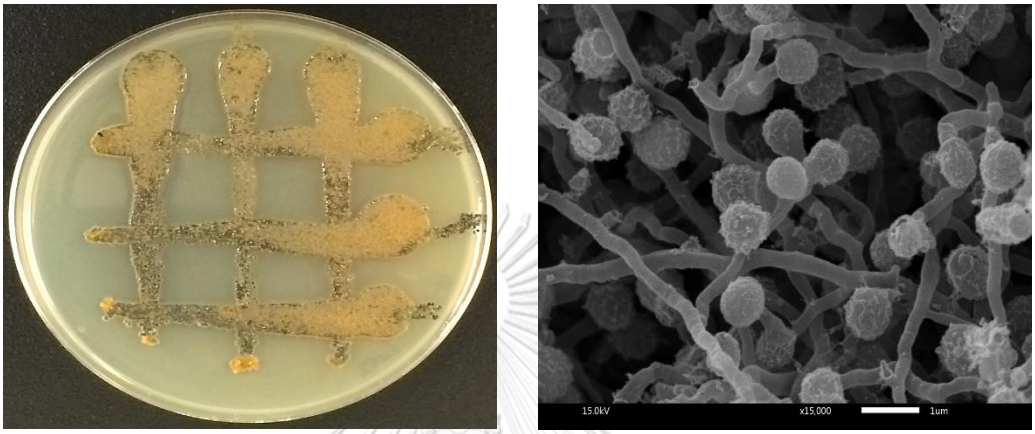
In comparison with the closely related type strains of the genus *Micromonospora*, the strain WPS1-2^T could be distinguished from the type strains *M. costi* CS1-12^T, *M. fulviviridis* NBRC 14026^T, and *M. krabiensis* MA-2^T by the morphology of spore and whole cell sugars pattern including the phenotypic characteristics, especially the utilisation of carbon sources, enzyme activity and NaCl tolerance (Table 4.4).

The integration of taxonomic data based on phenotypic, chemotaxonomic and genotypic characteristics as well as DNA-DNA relatedness, strain WPS1-2 should be classified as a novel species of the genus *Micromonospora*, for which the name *Micromonospora globbae* sp. nov. is proposed. The etymology of *Micromonospora globbae* is glob'bae. N. L. gen. n. *globbae*, of *Globba* botanical genus.

4.3.2 Characterization of *Micromonospora azadirachtae*

Strain AZ1-19^T produced branched substrate mycelia without fragmentation but lacks aerial hyphae on various agar media. The spherical spores (0.6–0.9 μm in size) with slightly rugose surface were observed to be borne singly on the tips of the substrate mycelia (Figure 4.7). The colonial colour was observed to be olive on ISP 2 agar and a clove brown soluble pigment was observed on ISP 7 agar medium. The cultural characteristics of strain AZ1-19^T were illustrated in Table 4.5. The strain grew well on ISP 2 and Czapak's agar, moderately on ISP 3, ISP 4 and ISP 6 media, and poorly on ISP 5, ISP 7 and nutrient agar. Strain AZ1-19^T can tolerate 3 % (w/v) NaCl and grow at 20-37 °C (optimally at 30 °C). The pH range for growth was pH 7.0-10.0. The enzymatic test showed that the strain was found to be positive for esterase (C 4), esterase lipase (C 8), trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, and *N*-acetyl-β-glucosaminidase activities, and to have weak activity for alkaline phosphatase and leucine arylamidase. Activities of lipase (C 14), valine arylamidase, cysteine arylamidase, α-galatosidase,

β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase were not detected. Additional physiological and biochemical data of strain AZ1-19^T compared with closely related type strains were summarized in Table 4.6.



(a) (b)

Figure 4.7 The colonial appearance (a) and scanning electron micrograph of the spherical spores with a slightly rugose surface of strain AZ1-19^T after cultured on ISP 2 agar at 30 °C for 21 days.

The cell-wall peptidoglycan of strain AZ1-19^T composed of *meso*-diaminopimelic. Glucose, xylose, galactose and mannose were presented in whole-cell hydrolysates. *N*- glycolyl was found in the cell-wall peptidoglycan. Mycolic acids were not observed. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), two unidentified phospholipids, an unidentified aminolipids and three unidentified glycolipids were presented as polar lipids which corresponded to phospholipid type II (Lechevalier *et al.*, 1977). The cellular fatty acids were found to compose of large amounts of *iso*-$C_{16:0}$ (35.7 %), *iso*-$C_{15:0}$ (17.2 %) *anteiso*-$C_{15:0}$ (8.2 %), and *anteiso*-$C_{17:0}$ (5.8 %) corresponding to fatty acid type 3b (Kroppenstedt, 1985). Although strain AZ1-19^T exhibited a fatty acid profile similar to those of *M. avicenniae* JCM 31034^T, *M. costi* CS1-12^T, *M. haikouensis* JCM 31036^{T}and *M. siamensis*

JCM 12769^T, the amounts of various components were different as shown in Table 4.7. The predominant menaquinones were MK-9(H_6) (32.6 %), MK-10(H_6) (41.1 %), and MK-10(H_8) (13.2 %) while MK-9(H_4) (2.7 %), MK-9(H_8) (6.9 %) and MK-9(H_4) (3.4 %) were found as minor components. The G+C content of the genomic DNA was 69.8 mol%.

An almost complete 16S rRNA gene sequence (1,449 bp, GenBank accession number LC224297) was obtained from strain AZ1-19^T. Pairwise sequence similarities calculated by the EzBiocloud server (Yoon *et al.*, 2017) between the strain and the type strains of the genus *Micromonospora* with validly published names indicated that strain AZ1-19^T was closely related to *M. avicenniae* 268506^T (98.75 %; 18 nt differences at 1436 positions) and *M. costi* CS1-12^T (98.75 % similarity; 18 nt differences at 1436 positions), *M. haikouensis* 232617^T (98.68 %; 19 nt differences at 1436 positions) and *M. siamensis* TT2-4^T (98.61 %; 20 nt differences at 1400 positions). Although strain AZ1-19^T showed high 16S rRNA gene sequence similarity with *M. costi* CS1-12^T, it shared a tight clade with *M. avicenniae* 268506^T in the 16S rRNA gene analysis based on the neighbour-joining (NJ) algorithm (Figure 4.8) supported by the bootstrap value of 69 %; furthermore, this cluster was also retrieved with the other tree-making methods: maximum-likelihood and maximum-parsimony. In addition, the 16S rRNA sequence of strain AZ1-19^T was pairwise aligned with those of *M. avicenniae* JCM 31034^T, *M. costi* CS1-12^T, *M. haikouensis* JCM 31036^T and *M. siamensis* JCM 12769^T, respectively, using EMBOSS Matcher (https://www.ebi.ac.uk/Tools/psa/emboss_matcher/). Strain AZ1-19^T showed significantly different nucleotides in comparison with *M. avicenniae* 268506^T (23 nt differences at 1446 positions) and *M. costi* CS1-12^T (22 nt differences at 1446 positions).

The *gyrB* gene sequence of strain AZ1-19^T with its phylogenetic neighbours was pairwise calculated on GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results emphasized that strain AZ1-19^T was closely related to *M. avicenniae* 268506^T (97.10 % similarity) and formed an independent cluster with that type strain in the NJ-

tree of *gyrB* gene sequences (Figure 4.9). Additionally, the affiliation of that cluster was very similar to that in the phylogenetic trees analysed by other tree-making methods. On the controversy, other studies have reported that the topology of phylogenetic trees based on *gyrB* gene sequences can differ from those based on 16S rRNA gene sequences (Carro *et al.*, 2012; Kirby & Meyers, 2010; Wang *et al.*, 2011).

Based on the high 16S rRNA gene sequence similarities and the phylogenetic tree based on *gyrB* gene sequences, *M. avicenniae* 268506^T, *M. costi* CS1-12^T, *M. haikouensis* 232617^T and *M. siamensis* TT2-4^T were recruited for comparing DNA-DNA homology. DNA-DNA relatedness values between strain AZ1-19^T and the related type strains *M. avicenniae* 268506^T, *M. costi* CS1-12^T, *M. haikouensis* 232617^T and *M. siamensis* TT2-4^T were 52.0±4.2 %, 39.3±1.1 %, 39.3±0.1 % and 40.3±0.4 %, respectively. These values were lower than 70%, which highlighted that strain AZ1-19^T represents a novel genomic species according to the currently accepted value for bacterial species delineation (Wayne *et al.*, 1987).

In summary, the data obtained from this study clearly indicated that strain AZ1-19^T represents a novel species of the genus *Micromonospora* for which we propose the name *Micromonospora azadirachtae* sp. nov. The etymology of *Micromonospora azadirachtae* is a.za.di.rach'tae. N. L. fem. *azadirachtae*, of *Azadirachta*, the botanical source from which the type strain was isolated.

Table 4.5 Cultural characteristics of strain AZ1-19^T and closely related type strains.

Strain: **1**. Strain AZ1-19^T; **2**. *M. costi* CS1-12^T; **3**. *M. haikouensis* JCM 31036^T; **4**. *M. avicenniae* JCM 31034^T; **5**. *M. siamensis* JCM 12769^T. All data are obtained from the study.

Medium	1	2	3	4	5
ISP medium 2					
Growth	Good	Good	Good	Good	Good
Colonial colour	Olive Grey	Russet Orange	Lt Copper Brown	Orange	Melon Yellow
Soluble pigment	None	None	None	None	None
ISP medium 3					
Growth	Moderate	Good	Moderate	Moderate	Moderate
Colonial colour	Mustard Tan	Orange	Cocoa Brown	Mustard	Lt Melon Yellow
Soluble pigment	None	None	None	None	None
ISP medium 4					
Growth	Moderate	Good	Good	Moderate	Moderate
Colonial colour	Oatmeal Sand	Orange	Salmon Pink	Melon Yellow	Melon Yellow
Soluble pigment	None	None	None	None	None
ISP medium 5					
Growth	Poor	Poor	Poor	Poor	Poor
Colonial colour	Lt Mustard Tan	Melon Yellow	Peach Pink	Lt Melon Yellow	Lt Melon Yellow
Soluble pigment	None	None	None	None	None
ISP medium 6					
Growth	Moderate	Good	Moderate	Moderate	Moderate
Colonial colour	Lt Amber	Bright Marigold	Tile Red	Apricot	Brite Maize
Soluble pigment	None	None	None	None	None
ISP medium 7					
Growth	Moderate	Poor	Poor	Poor	Poor
Colonial colour	Camel Tan	Light Tan	Lt Spice Brown	Lt Melon Yellow	Apricot
Soluble pigment	Clove Brown	None	None	None	Deep Brown
Nutrient agar					
Growth	Poor	Moderate	Poor	Poor	Poor
Colonial colour	Topaz, Butterscotch	Light Amber	Copper Brown	Brite Maize	Melon Yellow
Soluble pigment	None	None	None	None	None
Czapek Solution agar					
Growth	Good	Poor	Good	Good	Poor
Colonial colour	Lt Melon Yellow	Brite Melon Yellow	Dk Olive	Melon Yellow	Lt Melon Yellow
Soluble pigment	None	None	None	None	None

Table 4.6 Phenotypic characteristics of strain AZ1-19^T and closely related type strains. Strains: **1**. AZ1-19^T; **2**. *M. costi* CS1-12^T; **3**. *M. haikouensis* JCM 31036^T; **4**. *M. avicenniae* JCM 31034^T; **5**. *M. siamensis* JCM 12769^T. All data were obtained in this study. +, Positive reaction; -, negative reaction; w, weak positive reaction.

Characteristics	1	2	3	4	5
Spore morphology					
Shape	Spherical	Spherical[a]	Spherical[b]	Spherical[c]	Spherical[d]
Number of spores	Single	Single[a]	Single[a]	Single and cluster[c]	Single[d]
Spore surface	Slightly rugose	Rough and nodule[a]	Smooth[b]	Smooth[c]	Smooth[d]
Starch hydrolysis	-	+	+	-	+
Growth at 45 °C	-	+	-	-	-
Growth at 4 % (w/v) NaCl	-	w	w	w	w
Growth at pH 6.0	-	+	+	+	+
Utilisation of carbon sources					
L-Arabinose	+	+	w	+	-
Sucrose	+	-	+	+	+
D-Fructose	-	-	w	w	w
Rhamnose	+	-	+	+	-
D-Mannitol	+	-	+	-	-
D-Mannose	+	-	+	+	+
Salicin	+	-	-	+	-
D-Galactose	+	-	+	+	-
α-Cyclodextrin	-	-	-	+	-
API ZYM					
Alkaline phosphatase	w	+	+	+	-
Esterase (C 4)	+	w	+	w	+
Esterase lipase (C 8)	+	w	w	w	+
Leucine arylamidase	w	+	+	+	+
Valine arylamidase	-	w	+	w	-
α-Chymotrypsin	+	+	+	+	-
Naphthol-AS-BI-phosphohydrolase	+	w	-	+	+
α-Galactosidase	-	w	+	+	+
β-Glucosidase	-	w	+	-	+
N-Acetyl-β-glucosaminidase	+	+	+	+	-

[a]Data obtained from Thawai, (2015); [b]Data obtained from Xie *et al.*, (2012); [c]Data obtained from Li *et al.*, (2013); [d]Data obtained from Thawai *et al.*, (2005).

Table 4.7 Cellular fatty acid compositions (%) of strain AZ1-19^T and closely related type strains.

Strains: **1**. AZ1-19^T; **2**. *M. costi* CS1-12^T; **3**. *M. haikouensis* JCM 31036^T; **4**. *M. avicenniae* JCM 31034^T; **5**. *M. siamensis* JCM 12769^T. All data were obtained in this study.

Fatty acid	1	2	3	4	5
Saturated fatty acids					
$C_{12:0}$	0.6	-	0.5	-	-
$C_{14:0}$	-	-	2.1	-	-
$C_{16:0}$	-	0.8	6.3	2.3	0.8
$C_{17:0}$	3.7	5.6	6.5	4.5	7.4
$C_{18:0}$	-	0.8	5.8	1.7	1.5
$C_{19:0}$	0.8	0.5	-	0.6	2.1
Unsaturated fatty acids					
$C_{17:1}\omega$8c	0.7	1.82	8.7	1.2	11.3
$C_{18:1}\omega$9c	-	-	-	-	2.5
Unsaturated branched fatty acids					
iso-$C_{15:1}$ G	1.1	-	-	-	-
iso-$C_{16:1}$ G	2.4	-	-	-	-
iso-$C_{16:1}$ H	-	0.6	1.6	1.2	0.5
iso-$C_{17:1}$ 3-OH	2.9	-	0.8	1.3	-
anteiso-$C_{17:1}\omega$9c	0.8	-	0.5	-	0.8
Branched fatty acids					
iso- $C_{10:0}$	4.0	-	-	2.5	-
iso-$C_{14:0}$	3.6	0.6	1.3	0.8	-
anteiso-$C_{14:0}$	0.6	-	-	-	-
iso-$C_{15:0}$	17.2	20.3	6.0	25.6	19.5
anteiso-$C_{15:0}$	8.2	11.5	5.3	6.5	3.0
iso-$C_{16:0}$	35.7	15.9	21.4	19.3	17.9
iso-$C_{17:0}$	3.1	6.4	1.1	6.0	6.8
anteiso-$C_{17:0}$	5.8	21.3	3.0	9.8	10.1
iso-$C_{18:0}$	-	0.6	0.7	-	-
10-Methyl fatty acids					
10-methyl $C_{17:0}$	3.8	8.6	16.4	9.3	5.6
10-methyl $C_{18:0}$	-	1.0	3.7	0.9	0.9
Summed feature 9[a]	3.2	1.9	1.6	3.3	7.1

[a]Summed feature 9 comprises 10-methyl $C_{16:0}$ or *iso*-$C_{17:1}\omega$9c

The amounts of fatty acids less than 0.5% in all strains were omitted; -, not present.

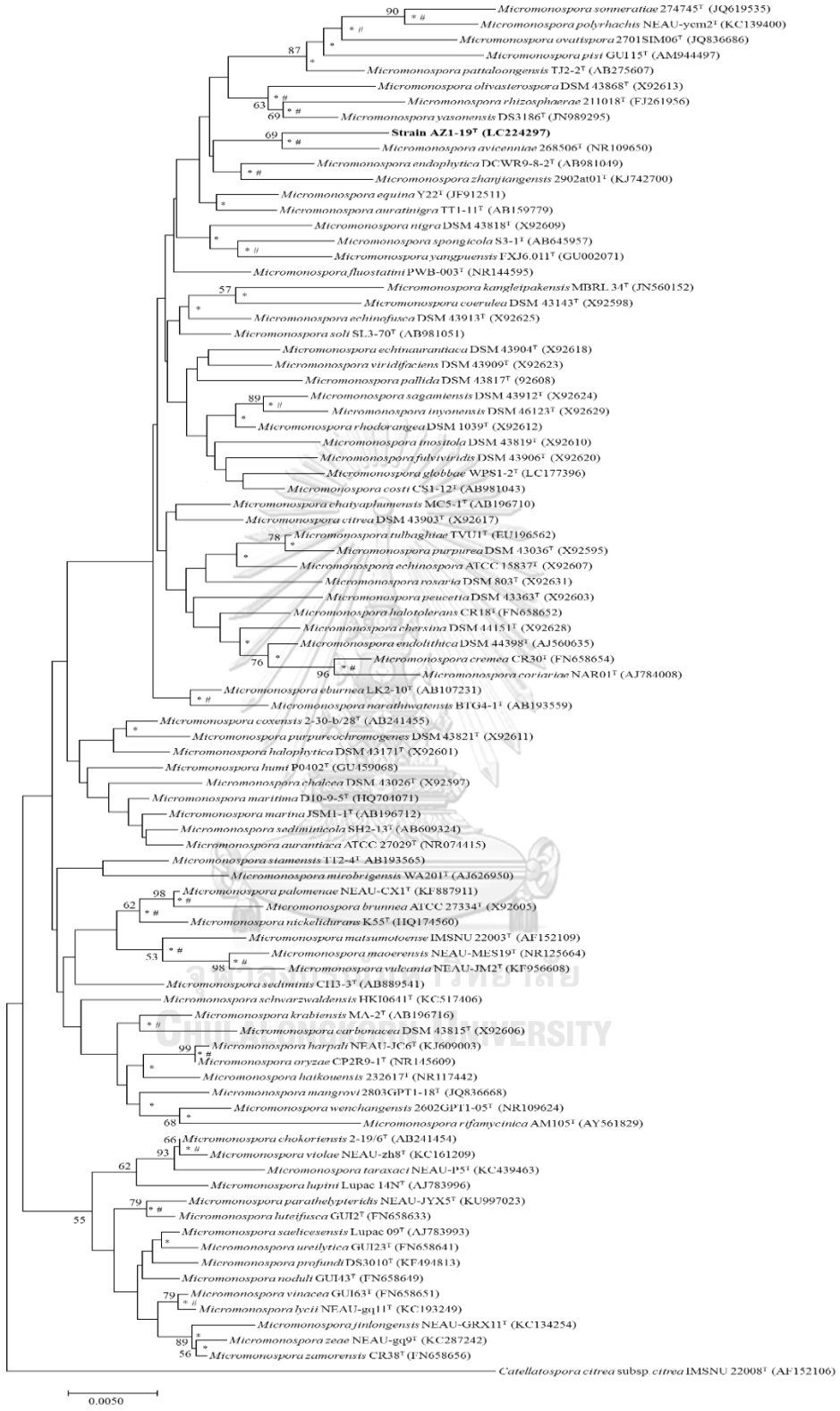


Figure 4.8 Phylogenetic tree based on neighbour-joining analysis of 16S rRNA gene sequences of strain AZ1-19^{T} and members of the genus *Micromonospora*.

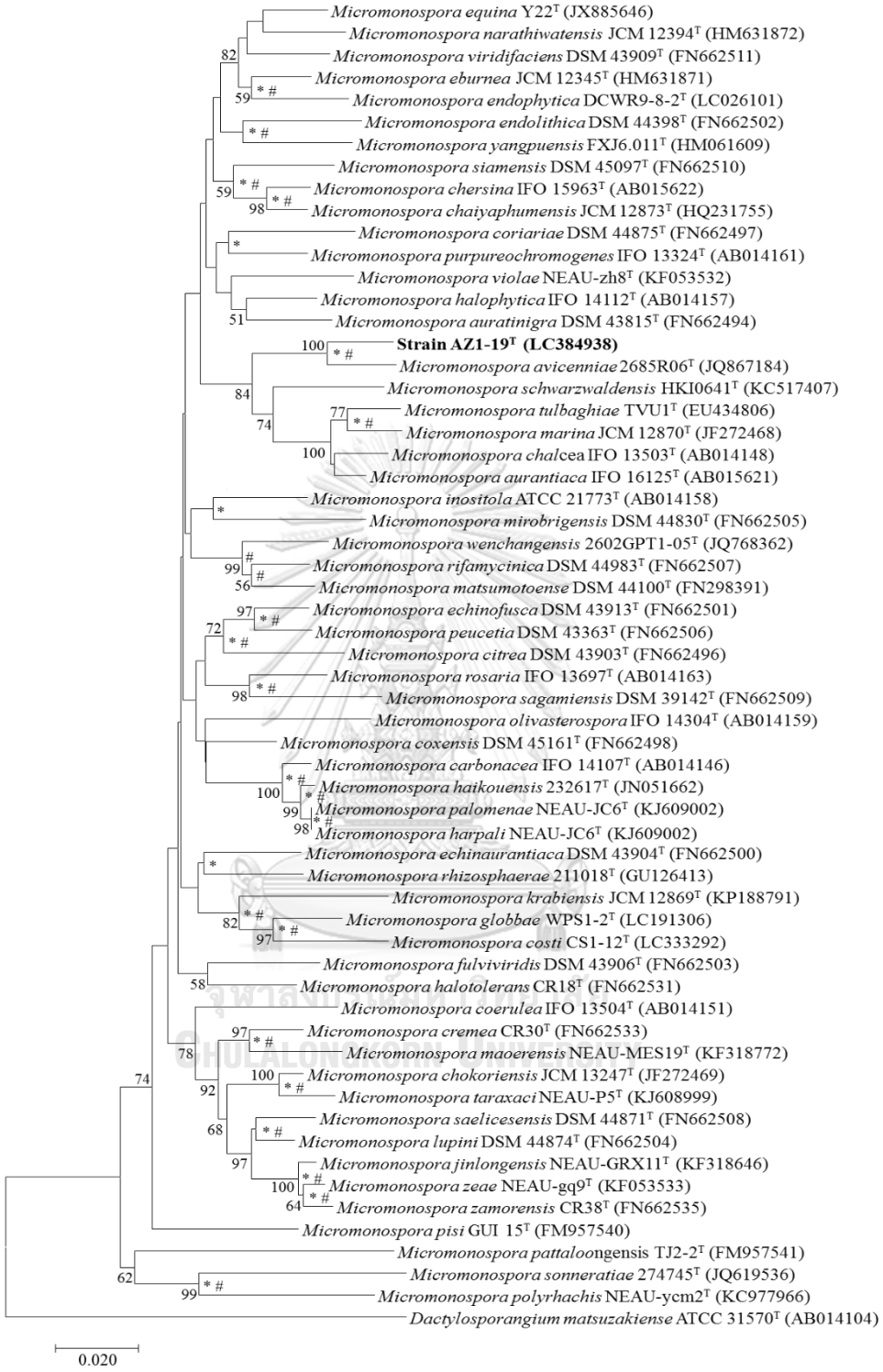
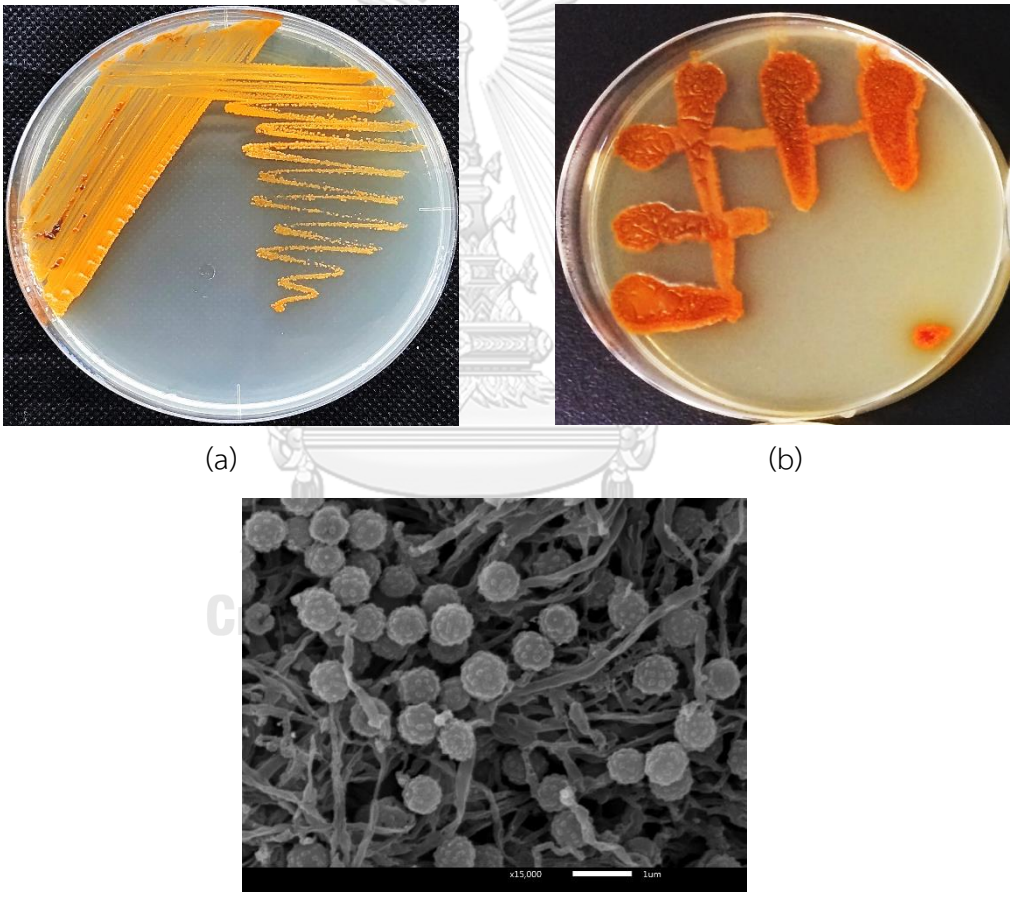


Figure 4.9 Phylogeny based on neighbour-joining analysis of *gyrB* gene sequences of strain AZ1-19^{T} and the type strains of the genus *Micromonospora*.

4.3.3 Characterization of *Micromonospora radicis*

Strain AZ1-13^{T} was found to form well-developed branched substrate mycelia without fragmentation. Aerial mycelia were not detected on all tested agar media. The non-motile spherical spores (0.6-0.8 μm in size) with a warty surface were borne singly on the tips of substrate hyphae (Figure 4.10). The colour of colonies was burnt orange in younger states and became orange rust with older age on ISP 2 agar plate. Strain AZ1-13^{T} grew well on ISP 2, ISP 4 and Czapek's solution agar; moderately on ISP 6, and poorly on ISP 3, ISP 5, ISP 7 and nutrient agar.



(a) (b)

(C)

Figure 4.10 The colonial appearance (a) after cultivated on ISP 2 agar at 30 °C for 7 days. The appearance of colonies (b) and scanning electron micrograph of the spherical spores with a slightly rugose surface of strain AZ1-13^{T} (C) grown on ISP 2 agar at 30 °C for 21 days.

The cultural characteristics of strain AZ1-13^T were summarized in Table 4.8. The pH range for growth was from pH 6.0 to 10.0 (optimally at 8.0). The strain grew well between 20 and 37 °C with an optimum at 30 °C whereas no growth was observed below 20 °C. Milk peptonization and coagulation and starch hydrolysis of strain AZ1-13^T were positive, but gelatin liquefaction and nitrate reduction were negative. The strain utilised L-arabinose, cellobiose, α-cyclodextrin, D-fructose, D-glucose, D-mannitol, D-mannose, salicin, sucrose and D-xylose as sole carbon sources, but did not adonitol, dextran, dulcitol, D-galactose, *myo*-inositol, raffinose and rhamnose. Alkaline phosphatase, α-chymotrypsin, esterase (C 4), esterase lipase (C 8), α-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucosidase, leucine arylamidase and trypsin are positive enzymic reactions whilst acid phosphatase, cysteine arylamidase, lipase (C 14) and valine arylamidase are weakly positives. The activities of α-fucosidase, α-galatosidase, β-galactosidase, β-glucuronidase, α-mannosidase and naphthol-AS-BI-phosphohydrolase are not detected. Further physiological and biochemical data of strain AZ1-13^T compared with the closely related type strains were described in Table 4.9.

According to chemotaxonomic properties of strain AZ1-13^T, the *meso*-diaminopimelic acid was presented as the diagnostic amino acid in the cell-wall peptidoglycan. Glucose, xylose, mannose, and ribose were found in whole-cell hydrolysates. The acyl type of peptidoglycan was *N*-glycolyl. Mycolic acids were absence. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), an unidentified phospholipid (PL), an unidentified aminolipid (AL), two unidentified glycolipids (GL1 and GL2) and two unidentified lipids (L1 and L2) were presented as polar lipid profile corresponding to phospholipid type II of Lechevalier *et al.*, (1977). The cellular fatty acids contained large amount of *iso*-$C_{15:0}$ (24.6 %), 10-methyl $C_{17:0}$ (15.9 %), $C_{17:0}$ (9.9 %), *anteiso*-$C_{17:0}$ (7.5%) and *iso*-$C_{17:1}\omega$8c (5.5%) assigning to fatty acid type 3a as described by

Kroppenstedt, (1985). Although strain AZ1-13^T showed a fatty acid profile like *J. zingiberis* PLAI1-1^T and *M. endophytica* JCM 18317^T, the amounts are different were listed in Table 4.10. The predominant menaquinones were MK-9(H_4) (76.3 %) and MK-9(H_6) (16.7 %) whereas MK-9(H_8) (2.8 %), MK-10(H_4) (3.2 %) and MK-10(H_6) (0.9 %) were minor components.

Nearest complete 16S rRNA gene sequence (1,478 bp) showed that strain AZ1-13^T was a member of the genus *Micromonospora* of the family *Micromonosporaceae*. The pairwise analysis of 16S rRNA gene sequence similarities indicated that the strain AZ1-13^T was most closely related to *J. zingiberis* PLAI1-1^T (99.72 % similarity; 4 nt difference at 1406) and *M. endophytic* 202201^T (99.23 % similarity; 11 nt difference at 1437). A neighbour-joining (NJ) phylogenetic tree of the family *Micromonosporaceae* based on 16S rRNA gene sequences (Figure 4.11) argued that the strain AZ1-13^T and *J. zingiberis* PLAI1-1^T located within the genus *Micromonospora* close to the clade of the former genera *Jishengella*, *Verrucosispora* and *Xiangella*. Their affiliations to the genus *Micromonospora* were supported by maximum-likelihood and the maximum-parsimony phylogenetic tree. Moreover, the cluster involved *Verrucosispora*, *Xiangella* and *Jishengella* were combined into the genus *Micromonospora* for close phylogeny (Li *et al.*, 2019; Nouioui *et al.*, 2018). Therefore, strain AZ1-13^T have a close relationship with the genus *Micromonospora*.

Genome sequences of strain AZ1-13^T, *J. zingiberis* PLAI1-1^T and *M. endophytic* JCM 18317^T were 5.96, 6.51 and 6.53 Mb in size, respectively, with average *in silico* DNA G+C contents of 71.9, 71.2 and 70.9 mol%, respectively. The ANIb and ANIm values of the draft genomes between strain AZ1-13^T and *J. zingiberis* PLAI1-1^T were 87.4 and 89.3 %, respectively, and between *M. endophytic* JCM 18317^T were 85.1 and 87.9 %, respectively (Table 4.11). Both ANI values were distinctly lower than 95-96 % for species delineation (Ritcher *et al.*, 2009). The digital DNA-DNA hybridisation between the genomes of the strain AZ1-13^T and its closest species, *J. zingiberis* PLAI1-1^T and *M.*

endophytica JCM 18317^T, were 34.8 and 30.9 %, respectively (Table 4.11), which clearly below 70 % threshold used to confirm the species strata of new strains (Goris *et al.*, 2007), so supporting the strain AZ1-13^T could be represented as a novel species within the genus *Micromonospora*. The draft genomic features of the strain AZ1-13^T and its closest type strains were summarized in Table 4.12.

In conclusion, the combination of genomic, chemotaxonomic, morphological and phenotypic data significantly highlighted that strain AZ1-13^T represents a novel *Micromonospora* species and propose the name *Micromonospora radicis* sp. nov. to accommodate this strain. The etymology of *Micromonospora radicis* is ra'di.cis. N.L. gen. n. *radicis* of a root.

Table 4.8 Cultural characteristics of strain AZ1-13^T and closely related type strains. Strain: **1**, Strain AZ1-13^T; **2**, *Jishengella zingiberis* PLAI1-1^T; **3**, *M. endophytica* JCM 18317^T. All data were determined in this study.

Medium	1	2	3
ISP medium 2			
Growth	Good	Good	Good
Colonial colour	Orange Rust	Orange Rust	Brite Orange
Soluble pigment	None	None	None
ISP medium 3			
Growth	Poor	None	Moderate
Colonial colour	Amber Topaz	None	Brite Orange
Soluble pigment	None	None	None
ISP medium 4			
Growth	Good	Moderate	Good
Colonial colour	Sum Orange	Brite Orange	Brite Orange
Soluble pigment	None	None	None
ISP medium 5			
Growth	Poor	Poor	Poor
Colonial colour	Melon Yellow	Brite Orange	Brite Melon Yellow
Soluble pigment	None	None	None
ISP medium 6			
Growth	Moderate	None	Moderate
Colonial colour	Amber	None	Brite Melon Yellow
Soluble pigment	None	None	None
ISP medium 7			
Growth	Poor	None	Moderate
Colonial colour	Butterscotch	None	Orange
Soluble pigment	None	None	None
Nutrient agar			
Growth	Poor	Poor	Poor
Colonial colour	Marigold Sunflower	Brite Orange	Brite Yellow
Soluble pigment	None	None	None
Czapek Solution agar			
Growth	Good	Moderate	Good
Colonial colour	Brite Melon Yellow	Brite Orange	Brite Melon Yellow
Soluble pigment	None	None	None

Table 4.9 Differential phenotypic characteristics between strain AZ1-13^T and its closely related type strains.

Strains: **1**. AZ1-13^T; **2**. *J. zingiberis* PLAI1-1^T; **3**. *M. endophytica* JCM 18317^T. +, Positive reaction; w, weak positive reaction; -, negative reaction. All data obtained from this study.

Characteristics	**1**	**2**	**3**
Coagulation	+	-	+
Gelatin liquefaction	-	+	+
Maximum NaCl (% w/v) tolerance	4	4	3
pH range for growth	4-10	4-11	4-11
Temperature range for growth (°C)	20-37	15-40	20-37
Utilisation of			
α-Cyclodextrin	w	-	-
Dextran	-	-	+
D-Galactose	-	-	w
API ZYM			
Acid phosphatase	w	+	+
Cystine arylamidase	w	-	-
Esterase lipase (C 8)	+	+	w
β-Galactosidase	-	w	+
N-acetyl-β-Glucosaminidase	+	-	w
Lipase (C 14)	w	-	-

Table 4.10 Cellular fatty acid compositions (%) of strain AZ1-13^T and its closely related type strains.

Fatty acid	AZ1-13^T	*M. endophytica* JCM 18317^T	*J. zingiberis* PLAI1-1^T
Saturated fatty acids			
$C_{12:0}$	0.5	1.0	-
$C_{16:0}$	2.9	2.9	2.1
$C_{17:0}$	9.9	6.6	15.1
$C_{18:0}$	2.0	2.8	0.6
$C_{19:0}$	1.2	0.5	-
Unsaturated fatty acids			
$C_{11:0}$ 2-OH	-	-	0.7
$C_{15:1}\omega6c$	-	-	0.5
$C_{17:1}\omega8c$	-	10.3	-
Unsaturated branched fatty acids			
iso-$C_{17:1}\omega8c$	5.5	-	19.3
anteiso-$C_{17:1}\omega9c$	0.5	-	-
Branched fatty acids			
iso-$C_{10:0}$	3.0	-	-
iso-$C_{14:0}$	-	0.2	1.5
iso-$C_{15:0}$	24.6	38.0	18.5
anteiso-$C_{15:0}$	3.4	7.8	2.9
iso-$C_{16:0}$	5.4	4.0	25.4
iso-$C_{17:0}$	4.7	6.2	2.2
iso-$C_{17:0}$ 3-OH	2.0	-	1.3
anteiso-$C_{17:0}$	7.5	8.6	-
anteiso-$C_{19:0}$	-	-	0.9
10-Methyl fatty acids			
10-methyl $C_{17:0}$	15.9	1.7	1.2
10-methyl $C_{18:0}$	2.9	0.8	-
Summed feature 3[a]	0.6	-	0.8
Summed feature 9[b]	4.7	-	1.9

[a]Summed feature 3 contained $C_{16:1}\omega7c$ or $C_{16:1}\omega6c$

[b]Summed feature 9 comprised 10-methyl $C_{16:0}$

The amount of fatty acid less than 0.5% in all strains was omitted; -, not present.

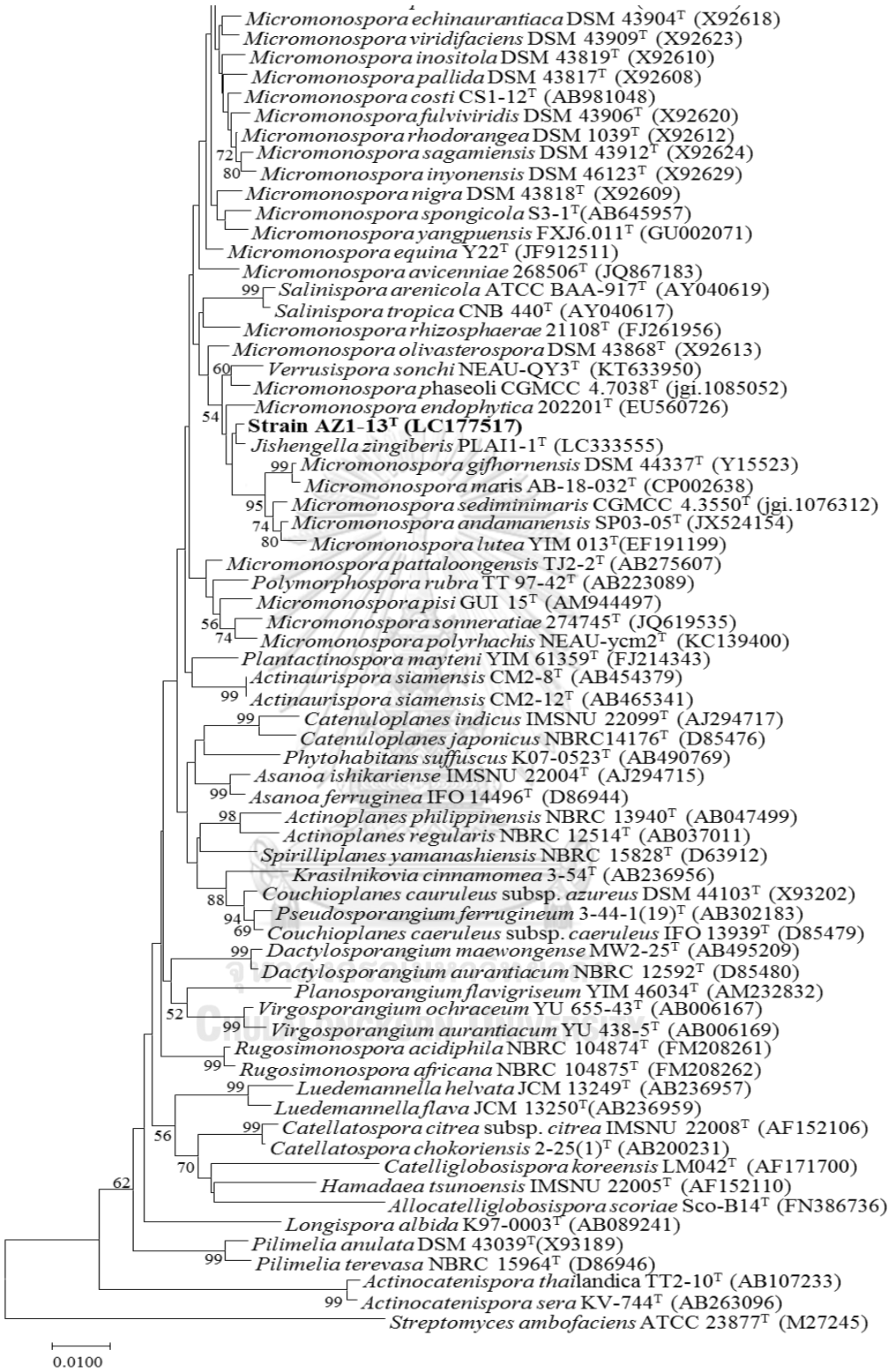


Figure 4.11 A neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain AZ1-13^{T} and the valid type species of recognised genera of the family *Micromonosporaceae*.

Table 4.11 The ANIb, ANIm and digital DNA-DNA hybridisation (dDDH) values between the draft genomes of strain AZ1-13^T and its closely related type strains.
Draft genomes: 1, Strain AZ1-13^T; 2, *J. zingiberis* PLAI1-1^T; 3, *M. endophytica* JCM 18317^T.
All data were determined in this study.

Query genome	Reference genome	ANIb (%)	ANIm (%)	% dDDH (Formula 2*)	Model C.I.
1	2	87.4	89.3	34.8	[32.4 - 37.4%]
1	3	85.1	87.9	30.9	[28.5 - 33.4%]

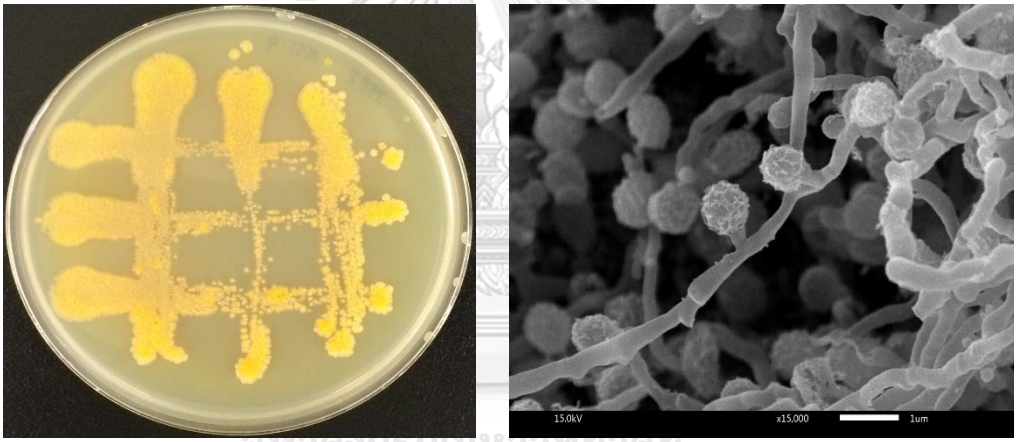
*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genomes.

Table 4.12 Genomic features of strain AZ1-13^T (QXEC00000000), *J. zingiberis* PLAI1-1^T (SJJR00000000) and *M. endophytica* JCM 18317^T (QXIQ00000000).
All data obtained from this study.

Attribute	AZ1-13^T	PLAI1-1^T	JCM 18317^T
Genome sizes (bp)	5,963,322	6,506,206	6,538,203
G+C content (%)	71.9	71.2	70.9
N50	205084	275109	110271
No. of contigs	83	45	148
Total genes	5397	5907	6065
No. of coding sequences	5344	5837	6007
RNA genes	53	69	58

4.3.4 Characterization of *Micromonospora musae*

Strains MS1-9^T and NGC1-4 produced extensively branched, non-fragmenting substrate hyphae on various agar media. The colonial appearance of both strains was brite-melon-yellow coloured when grew on ISP 2 agar for 14 days. Good growth was observed on ISP 2 and ISP 4; moderate growth was noticed on ISP3 and ISP6, and poor growth was detected on ISP 5, ISP 7 and Czapek's solution agar media. Strains MS1-9^T and NGC1-4 produced clove-brown coloured soluble pigment on ISP 7 agar. Single, non-motile, and spherical spores (0.8–0.9 μm in size) were borne singly on the tips of the substrate mycelia. The spore surface was rugose (Figure 4.12).



(a) (b)

Figure 4.12 The colonial appearance (a) and scanning electron micrograph (b) of strain MS1-9^T grown on yeast extract-malt extract agar medium at 30 °C for 21 days. Bars represent 1 μm.

Cultural characteristics of strains MS1-9^T and NGC1-4 as well as the related type strains were shown in Table 4.13. Both strains grew well at 20-37 °C (optimally at 30 °C). Strains MS1-9^T and NGC1-4 grew well from 0-3% (w/v) NaCl, moderately on 4% (w/v), but failed to grow above 4% (w/v) NaCl. The growth was observed between pH 6.0 to 10.0 with an optimum at 8.0. Strains MS1-9^T and NGC1-4 utilised adonitol,

L-arabinose, D-cellobiose, α-cyclodextrin, D-fructose, D-galactose, D-glucose, D-mannose, D-mannitol, D-rhamnose, salicin, sucrose and D-xylose, but not dextran, dulcitol, *myo*-inositol and D-raffinose. Both strains exhibited positive enzymatic activity for acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C 4), esterase lipase (C 8), α-galactosidase, β-galactosidase, β-glucosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase, but negative for α-fucosidase, β-glucuronidase, lipase (C 14) and α-mannosidase. Additional physiological and biochemical characteristics of strains MS1-9^T and NGC1-4 compared with the closely related type strains were explained in Table 4.14.

The chemotaxonomic data revealed that *meso*-diaminopimelic acid was noted as the diagnostic amino acid in cell-wall peptidoglycan of strains MS1-9^T and NGC1-4. Glucose, xylose, mannose, and ribose were presented in the whole-cell hydrolysates. The acyl type of peptidoglycan was glycolyl. Mycolic acid methyl esters were absence. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), three unidentified phospholipids (PL1-PL3), two unidentified glycolipids (GL1-GL2), an aminophospholipid (APL) and three unidentified lipids (L1-L3) were identified as the phospholipids profile, corresponding to phospholipid type II (Lechevalier *et al.*, 1977). Both strains contained *iso*-$C_{15:0}$ (29.9, 31.2 %), *anteiso*-$C_{15:0}$ (15.0, 14.9 %), *anteiso*-$C_{17:0}$ (16.5, 12.2 %), *iso*-$C_{17:0}$ (11.5, 9.0 %), and $C_{17:0}$ (5.3, 10.7 %) as major fatty acids according to fatty acid type 3b (Kroppenstedt, 1985). However, their quantities varied among the strains (Table 4.15). Strains MS1-9^T and NGC1-4 presented *iso*-$C_{10:0}$ (1.2, 2.6 %) and *iso*-$C_{17:0}$ 3-OH (0.9, 1.2 %) which are absent in the related type strains. The predominant menaquinones were of strains MS1-9^T and NGC1-4 were MK-10(H_6) (31.5, 34.5 %), MK-9(H_6) (22.9, 31.3 %), MK-10(H_8) (19.1, 15.1 %), and MK-9(H_8) (10.1, 9.3 %) whereas MK-9(H_4) (9.0, 7.2 %) and MK-10(H_4) (7.3, 2.7 %) were minor components.

The 16S rRNA gene sequences analysis revealed that the strains MS1-9^T (1,400 nt) and NGC1-4 (1,418 nt) are members of the genus *Micromonospora* of the family *Micromonosporaceae*. The pairwise analysis of the 16S rRNA gene sequence showed 100 % similarity between the strain MS1-9^T and NGC1-4. Both exhibited 16S rRNA gene similarity value of 98.1 % with *Micromonospora peucetia* DSM43363^T, *M. endolithica* DSM 44398^T and *M. krabiensis* DSM 45344^T (= MA-2^T = JCM 12869^T), subsequent by *M. costi* CS1-12^T (97.9 %) and *M. chersina* DSM 44151^T (97.8 %). A neighbour-joining (NJ) phylogenetic analysis based on 16S rRNA gene sequences (Figure 4.13) emphasized that the strains MS1-9^T and NGC1-4 formed an independent cluster with *M. peucetia* DSM43363^T; moreover, the affiliation of this clade was relatively similar with the phylogenies analysed by maximum-likelihood and maximum-parsimony methods. The findings claimed by the NJ-tree of the *gyrB* gene sequence, in which a well-distinct branch including the two strains was also developed and supported 99 % of a bootstrap value (Figure 4.14); nonetheless, showing closely related with *M. krabiensis* JCM 12869^T. Based on the integration of 16S rRNA gene and *gyrB* gene phylogenetic trees, *M. peucetia* DSM 43363^T (= JCM 12820^T), *M. endolithica* DSM 44398^T (= JCM 12677^T), *M. krabiensis* DSM 45344^T (= MA-2^T = JCM 12869^T), *M. costi* CS1-12^T and *M. chersina* DSM 44151^T (= JCM 9459^T) were considerably selected for the comparison of genetic profiles.

Draft genome sequences of strains MS1-9^T and NGC1-4, *M. endolithica* JCM 12677^T, and *M. costi* CS1-12^T, obtained from this study, were 7.06, 7.05, 6.97 and 7.18 Mb in size, respectively. The genome sequences of *M. peucetia* DSM 43363^T (FMIC01000002), *M. krabiensis* DSM 45344^T (LT598496), and *M. chersina* DSM 44151^T (FMIB00000000) were received from the GenBank and were 7.36, 7.07, and 6.68 Mb in size. *In silico* DNA G+C contents (mol%) of both novel strains, *M. peucetia* DSM43363^T, *M. endolithica* JCM 12677^T, *M. krabiensis* DSM 45344^T, *M. costi* CS1-12^T and *M. chersina* DSM 44151^T were 72.2, 72.3, 72.5, 72.8, 73.0 and 73.6 mol%, respectively. The genomic

statistics of strains MS1-9^T and NGC1-4 and their related type strains were described in Table 4.16. Average nucleotide identity values, ANIb and ANIm, of the genomes among both new isolates and all closest species were clearly lower than 95-96 % (Table 4.17) for the species delineation (Ritcher *et al.*, 2009). Strain MS1-9^T showed digital DNA-DNA hybridisation (dDDH) values of 26.8 % (C. I. model 24.4-29.3 %), 25.7 % (C. I. model 23.4-28.2 %), 28.9 % (C. I. model 26.6-31.4 %), 31.0 % (C. I. model 28.6-35.5 %), and 25.9 % (C. I. model 23.5-28.3 %) related with *M. peucetia* DSM43363^T, *M. endolithica* JCM 12677^T, *M. krabiensis* DSM 45344^T, *M. costi* CS1-12^T and *M. chersina* DSM 44151^T, respectively (Table 4.17). The dDDH values were lower to the recommended threshold of 70% for species delineation (Goris *et al.*, 2007) supports strains MS1-9^T and NGC1-4 representing a new species within the genus *Micromonospora*.

The data of the phenotypic, chemotaxonomic, phylogenetic and genomic characteristics of the strains MS1-9^T and NGC1-4 confirmed that both strains were significantly different from their closely related type strains. Thus, strains MS1-9^T and NGC1-4 represent a novel species of the genus *Micromonospora*, for which the name of *Micromonospora musae* sp. nov. is proposed. The etymology of *Micromonospora musae* is mu'sae. N. L. gen. n. *musae*, of *Musa* botanical genus.

Table 4.13 Cultural characteristics of strains MS1-9^T and NGC1-4 and their closely related type strains. Strains: **1,** MS1-9^T; **2,** NGC1-4; **3,** *M. peucetia* JCM 12820^T; **4,** *M. endolithica* JCM 12677^T; **5,** *M. krabiensis* MA-2^T; **6**, *M. costi* CS1-12^T; **7**, *M. chersina* JCM 9459^T. All data were obtained from this study.

Medium	1	2	3	4	5	6	7
ISP medium 2							
Growth	Good	Good	Good	Poor	Good	Good	Good
Colonial colour	Brite Melon Yellow	Brite Melon Yellow	Dark Brown	Russet Orange	Russet Orange	Russet Orange	Ebony Teak
Soluble pigment	None	None	None	None	Cinnamon Brown	None	Olive Grey
ISP medium 3							
Growth	Moderate	Moderate	Moderate	Moderate	Good	Good	Good
Colonial colour	Lt Melon Yellow	Lt Melon Yellow	Cinnamon Brown	Russet Orange	Amber	Orange	Dark Brown
Soluble pigment	None	None	None	None	None	None	None
ISP medium 4							
Growth	Good	Good	Good	Good	Moderate	Good	Good
Colonial colour	Melon Yellow	Melon Yellow	Melon Yellow	Light Orange	Bright Orange	Orange	Mustard Tan
Soluble pigment	None	None	None	None	None	None	None
ISP medium 5							
Growth	Poor	Poor	Poor	Poor	Moderate	Poor	Poor
Colonial colour	Lt Melon Yellow	Lt Melon Yellow	Oatmeal Sand	Melon Yellow	Bright Orange	Melon Yellow	Lt Mustard Tan
Soluble pigment	None	None	None	None	None	None	None
ISP medium 6							
Growth	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Colonial colour	Lt Melon Yellow	Lt Melon Yellow	Lt Melon Yellow	Orange	Bright Orange	Bright Marigold	Dark Brown
Soluble pigment	None	None	None	None	None	None	None
ISP medium 7							
Growth	Poor	Poor	Moderate	Poor	Poor	Poor	Moderate
Colonial colour	Camel	Camel	Clove Brown	Lt Melon Yellow	Bright Orange	Light Tan	Convert Brown
Soluble pigment	Clove Brown	Clove Brown	Cinnamon Brown	None	None	None	None
Nutrient agar							
Growth	Poor	Poor	Moderate	Poor	Good	Moderate	Poor
Colonial colour	Brite Maize	Brize Maize	Lt Melon Yellow	Melon Yellow	Russet Orange	Brite Maize	Bamboo
Soluble pigment	None	None	None	None	None	None	None
Czapek Solution agar							
Growth	Poor	Poor	Good	Poor	Moderate	Poor	Moderate
Colonial colour	Lt Melon Yellow	Lt Melon Yellow	Mustard Tan	Lt Melon Yellow	Bright Orange	Brite Melon Yellow	Camel
Soluble pigment	None	None	None	None	None	None	None

Table 4.14 Differential phenotypic characteristics between strains MS1-9^T and NGC1-4 and their closely related type strains.

Strains: **1,** MS1-9^T; **2,** NGC1-4; **3,** *M. peucetia* JCM 12820^T; **4,** *M. endolithica* JCM 12677^T; **5,** *M. krabiensis* MA-2^T; **6**, *M. costi* CS1-12^T; **7**, *M. chersina* JCM 9459^T. All data were obtained from this study. +, Positive reaction; w, weak positive reaction; -, negative reaction.

Characteristics	1	2	3	4	5	6	7
Starch hydrolysis	-	-	+	-	+	+	+
Peptonization	+	+	+	+	+	+	-
Coagulation	+	+	+	+	+	+	-
Gelatin liquefaction	-	-	-	-	-	-	+
Growth at 45 °C	-	-	-	-	-	+	+
Growth at pH 10.0	+	+	+	+	-	+	+
Utilisation of							
Adonitol	w	w	-	-	-	-	-
L-Arabinose	+	+	-	+	+	+	+
D-Cellobiose	+	+	+	+	w	+	-
α-Cyclodextrin	+	+	+	+	-	+	+
D-Fructose	+	+	w	+	+	w	w
D-Galactose	+	+	-	+	+	-	+
myo-Inositol	-	-	-	-	w	-	w
D-Mannose	+	+	+	+	+	-	+
D-Mannitol	w	w	-	-	w	-	-
D-Raffinose	-	-	-	+	-	-	+
D-Rhamnose	w	w	-	-	w	-	-
Salicin	+	+	-	-	-	-	w
Sucrose	+	+	+	+	+	-	+
API ZYM							
Acid phosphatase	+	+	+	-	+	+	+
Alkaline phosphatase	+	+	w	-	+	+	+
α-Chymotrypsin	+	+	+	-	+	+	+
Cystine arylamidase	w	w	-	-	-	-	w
Esterase (C 4)	+	+	+	+	+	w	w
Esterase Lipase (C 8)	w	w	+	+	w	w	w
α-galactosidase	+	+	-	w	+	w	+
β-galactosidase	+	+	-	+	+	+	+
β-glucosidase	+	+	w	-	+	w	+
N-acetyl-β-glucosaminidase	+	+	-	-	+	+	-
Lipase (C 14)	-	-	-	-	-	-	w
Naphthol-AS-BI-phosphohydrolase	+	+	+	w	-	w	w
Trypsin	+	+	+	w	+	+	+
Valine arylamidase	+	+	w	w	+	w	w

Table 4.15 Cellular fatty acids profiles (%) of strains MS1-9^T and NGC1-4 and their closely related type strains.

Strains: **1,** MS1-9^T; **2,** NGC1-4; **3,** *M. peucetia* JCM 12820^T; **4,** *M. endolithica* JCM 12677^T; **5,** *M. krabiensis* MA-2^T; **6**, *M. costi* CS1-12^T; **7**, *M. chersina* JCM 9459^T. All data were obtained from this study.

Fatty acid	1	2	3	4	5	6	7
Saturated fatty acids							
$C_{16:0}$	3.0	3.1	3.1	6.3	0.8	3.1	5.9
$C_{17:0}$	5.3	10.7	-	4.9	5.6	5.5	5.4
$C_{18:0}$	1.7	1.6	1.7	3.8	0.8	4.2	6.6
$C_{19:0}$	0.5	0.6	-	0.9	0.5	-	-
Unsaturated fatty acids							
$C_{17:1}\omega$8c	1.4	1.4	2.2	13.1	1.8	2.3	0.8
$C_{18:1}\omega$9c	0.7	0.7	5.5	7.6	-	1.7	0.9
Unsaturated branched fatty acids							
iso-$C_{15:1}$G	1.1	1.1	2.2	1.0	-	-	2.4
anteiso-$C_{17:1}\omega$9c	0.7	0.5	1.1	1.0	-	0.9	1.3
Branched fatty acids							
iso-$C_{10:0}$	1.2	2.6	-	-	-	-	-
iso-$C_{14:0}$	0.5	0.7	0.7	-	0.6	0.8	2.0
iso-$C_{15:0}$	29.9	31.2	20.1	13.8	20.3	15.0	17.0
anteiso-$C_{15:0}$	15.0	14.9	2.1	2.8	11.5	3.4	14.6
iso-$C_{16:0}$	6.5	4.1	26.8	7.4	15.9	27.1	18.4
iso-$C_{17:0}$	11.5	9.0	4.5	6.8	6.4	5.8	4.6
iso-$C_{17:0}$ 3-OH	0.9	1.2	-	-	-	-	-
anteiso-$C_{17:0}$	16.5	12.2	3.3	5.7	21.3	10.7	9.5
iso-$C_{18:0}$	-	-	-	-	0.6	1.1	0.7
iso-$C_{19:0}$	-	-	-	0.6	-	-	-
10-Methyl fatty acids							
10-methyl $C_{17:0}$	0.8	0.9	4.6	7.8	8.6	6.7	0.7
10-methyl $C_{18:0}$	-	-	6.7	4.2	1.0	1.1	-
Summed feature 3[a]	1.0	0.5	1.9	1.1	-	1.6	0.6
Summed feature 9[b]	1.8	1.5	7.3	8.1	1.9	2.7	0.9

The amount of fatty acid less than 0.5% in all strains was omitted.

[a]Summed feature 3 contained $C_{16:1}\omega$6c or $C_{16:1}\omega$7c

[b]Summed feature 9 comprised iso-$C_{17:1}\omega$9c

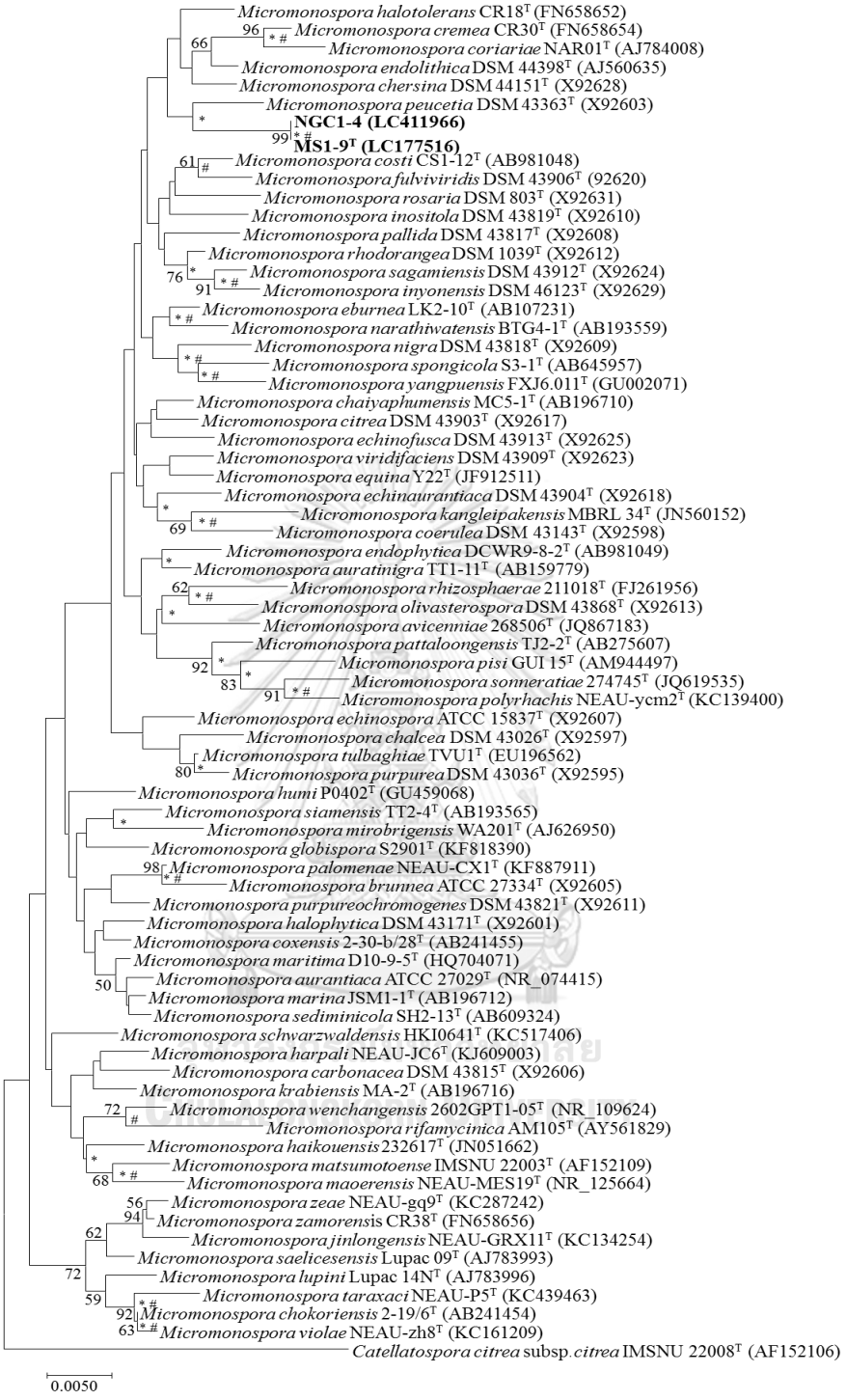


Figure 4.13 A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain MS1-9^T and NGC1-4 and related type strains.

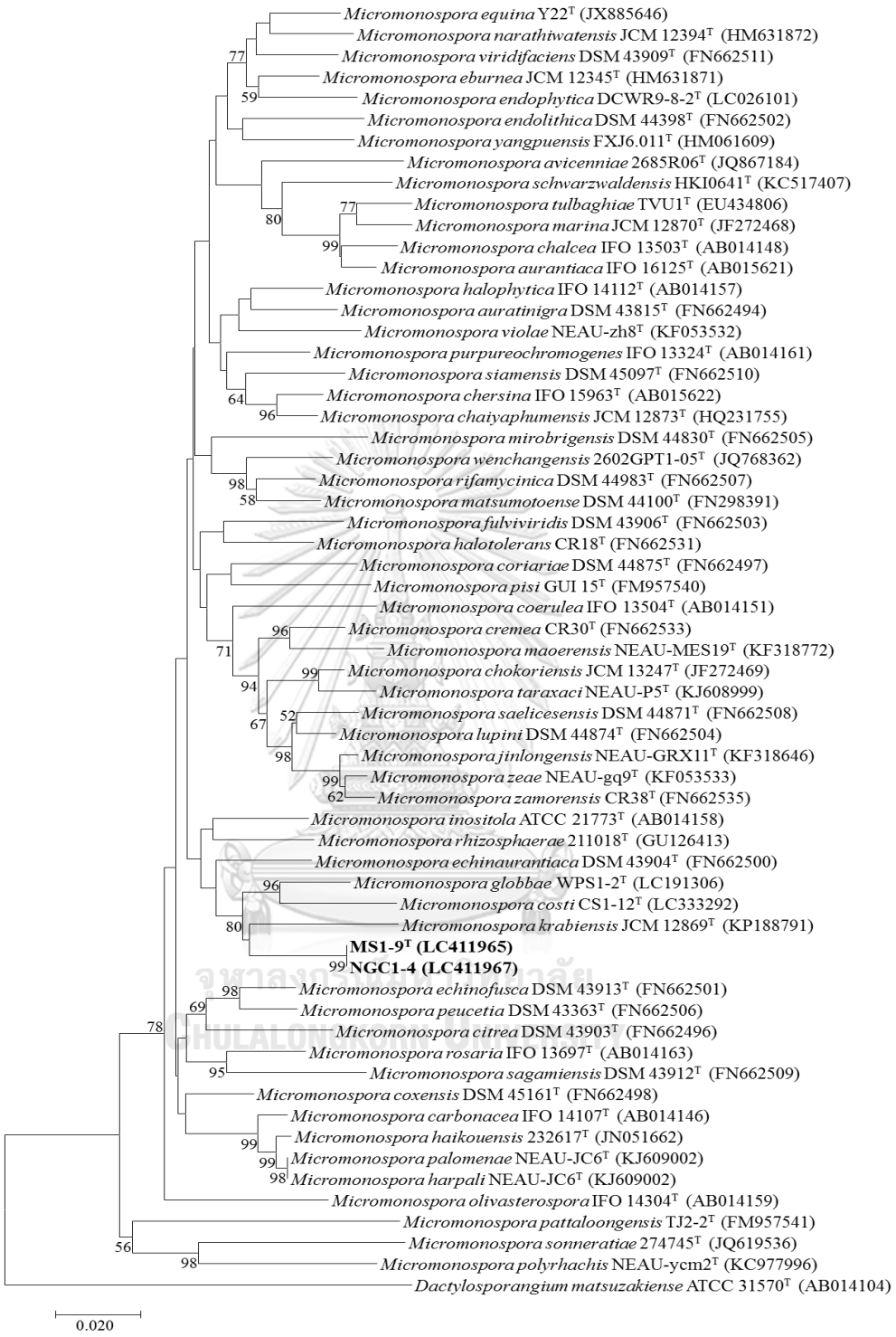


Figure 4.14 A neighbour-joining phylogeny based on *gyrB* gene sequences of strain MS1-9^{T} (1,120 nt) and NGC1-4 (1,060 nt) and their closest type strains.

Table 4.16 Genome statistics of strains MS1-9^T and NGC1-4 and other closely related type strains.

Genome: **1,** MS1-9^T; **2,** NGC1-4; **3,** *M. peucetia* DSM 43363^T (= JCM 12820^T); **4,** *M. endolithica* JCM 12677^T; **5,** *M. krabiensis* DSM 45844^T (= MA-2^T); **6**, *M. costi* CS1-12^T; **7**, *M. chersina* DSM 44151^T (= JCM 9459^T).

Feature	1	2	3*	4	5*	6	7*
Accession no.	RAZT00000000	RAZS00000000	FMIC00000000	RBAK00000000	LT598496	RBAN00000000	FMIB00000000
Genome size (bp)	7,069,771	7,058,875	7,365,767	6,970,905	7,074,838	7,182,425	6,683,691
G+C content (%)	72.2	72.2	72.3	72.5	72.8	73.0	73.6
No. of Contigs	34	28	1	40	1	20	1
Total genes	6,859	6,851	6,354	6,859	6,498	7,080	6,207
Protein coding genes	6,126	6,144	6,062	6,164	6,211	6,344	5,994
RNA genes	100	70	64	63	87	93	68
tRNAs	90	62	49	55	75	83	56

*****Data obtained from GenBank

Table 4.17 The ANIb, ANIm and digital DNA-DNA hybridisation (dDDH) values between the draft genomes of strains MS1-9^T and its closely related type strains.

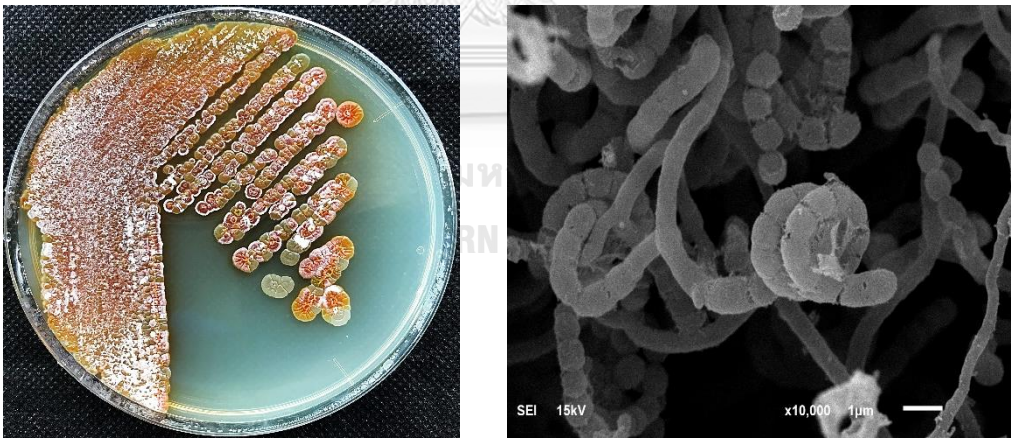
Genomes: 1, MS1-9^T; 2, NGC1-4; 3, *M. peucetia* JCM 12820^T; 4, *M. endolithica* JCM 12677^T; 5, *M. krabiensis* MA-2^T; 6, *M. costi* CS1-12^T; 7, *M. chersina* JCM 9459^T. All data were obtained from this study.

Query genome	Reference genome	ANIb (%)	ANIm (%)	% dDDH (Formula 2*)	Model C.I.
1	2	99.13	99.15	92.9	[91 - 94.5%]
1	3	81.46	86.35	26.8	[24.4 - 29.3%]
1	4	80.99	86.08	25.7	[23.4 - 28.2%]
1	5	83.96	87.15	28.9	[26.6 - 31.4%]
1	6	85.47	87.77	31.0	[28.6 - 33.5%]
1	7	81.57	86.21	25.9	[23.5 - 28.3%]

*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genomes

4.3.5 Characterization of *Streptomyces endoradicus*

It is visible that strains DS1-2^T and AZ1-7 belong to the genus *Streptomyces*. The strains produced elliptical or short rod spores (0.6 × 0.8 μm in size) with a smooth surface and no whirls, sporangia or flagellate spores were observed. The arrangement of spores was a spiral chain and contained more than 20 spores per chain (Figure 4.15). Both DS1-2^T and AZ1-7 produced well-developed straw-wheat-coloured substrate mycelia which fragmented easily and pearl-shell-tint-coloured aerial hyphae on ISP 2 agar, but not formed the aerial mass on ISP 5 and ISP 6 agar media. These two strains produced golden-brown and camel-coloured diffusible pigment on ISP 2 and ISP 7, respectively. Good growth was detected on ISP 4, ISP 5, ISP 7 and Czapek's solution agar; moderate growth was observed on ISP 2, ISP 3 and ISP 6, and no growth on nutrient agar. The aerial mass of both strains DS1-2^T and AZ1-7 was not found on ISP 5 and ISP 6 agar plate. Cultural characteristics of strains DS1-2^T and AZ1-7 together with their closely related type strains were listed in Table 4.18.



(a) (b)

Figure 4.15 The colonial appearance of strain DS1-2^T (a) after culture on Sucrose Bennett's agar at 30 °C for 14 days and scanning electron micrograph of strain DS1-2^T (b) grown on 10-fold diluted yeast extract-malt extract agar medium at 30 °C for 28 days, Bars represent 1 μm.

The growth was observed from pH 6.0 to 10.0 (optimally at 8.0). Both strains grew well at 30 °C, but not below 20 °C and over 30 °C. Strain DS1-2^T and AZ1-7 utilised L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, D-mannose, D-mannitol, D-raffinose, D-rhamnose, sucrose and D-xylose but not adonitol, α-cyclodextrin, dextran, dulcitol and salicin. Enzymatic activity tests of these two strains revealed that alkaline phosphatase, α-chymotrypsin, esterase (C 4), esterase lipase (C 8), α-glucosidase, *N*-acetyl-β-glucosaminidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase were positive, while acid phosphatase, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase and lipase (C 14) were negative. Further physiological and biochemical results of strain DS1-2^T and AZ1-7 compared with the closely related type strains are described in Table 4.19.

According to the chemotaxonomic results, strains DS1-2^T and AZ1-7 contained *LL*-diaminopimelic acid as the diagnostic amino acid in the cell-wall peptidoglycan. Glucose was detected in the whole-cell hydrolysates. *N*-acetyl was presented in cell-wall peptidoglycan. Mycolic acid methyl esters were absence. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol mannoside (PIM), two unidentified phospholipids (PL1-PL2), a ninhydrin-positive glycolipid (NPG) and seven unidentified lipids (L1-L7) were observed as the phospholipids profile, harmonizing with phospholipid type II (Lechevalier *et al.*, 1977). Both strains showed *iso*-$C_{16:0}$ (30.2 %) and *iso*-$C_{16:1}$G (15.5 %) as major fatty acids, the typical fatty acids found in the genus *Streptomyces* (Kroppenstedt, 1985). Nonetheless, their quantities varied among the strains (Table 4.20). Both strains DS1-2^T and AZ1-7 exhibited *anteiso*-$C_{17:0}$ (11.1 %) and *anteiso*-$C_{17:1}$A (8.8 %) which are absent in their related type strains. The major menaquinones were MK-9(H_6) (26.5 %), MK-10(H_6) (19.6 %), MK-9(H_8) (19.2 %), MK-10(H_8) (14.7 %) and MK-10(H_4) (11.2 %) whilst MK-9(H_4) (5.2 %) and MK-10(H_{10}) (3.5 %) were minor components.

Table 4.18 Cultural characteristics of strains DS1-2^T and AZ1-7 and their closely related type strains.

Strains: **1**, DS1-2^T; **2**, AZ1-7; **3**, *S. specialis* GW 41-1546^T; **4**, *S. hoynatensis* KCTC 29097^T; **5**, *S. klenkii* KCTC 29202^T. All data were obtained from this study.

Medium	1	2	3	4	5
ISP medium 2					
Growth	Moderate	Moderate	Good	Moderate	Good
Aerial-mass colour	Pearl Shell Tint	Pearl Shell Tint	Pink Tint	None	Pearl Shell Tint
Reverse-phase colour	Straw Wheat	Straw Wheat	Slate	Bamboo	Clove Brown
Soluble pigment	Golden Brown	Golden Brown	Black Olive	None	None
ISP medium 3					
Growth	Moderate	Moderate	Good	Poor	Good
Aerial-mass colour	Natural	Natural	Pink Tint	Pearl Shell Tint	Natural
Reverse-phase colour	Sand	Sand	Slate	Yellow Tint	Yellow Tint
Soluble pigment	None	None	Black Olive	None	None
ISP medium 4					
Growth	Good	Good	Good	Moderate	Good
Aerial-mass colour	Shell Pink	Shell Pink	Pink Tint	Pearl Shell Tint	Shell Pink
Reverse-phase colour	Straw Wheat	Straw Wheat	Slate	Bamboo	Yellow Maple
Soluble pigment	None	None	Black Olive	None	None
ISP medium 5					
Growth	Good	Good	Good	Poor	Poor
Aerial-mass colour	None	None	Pink Tint	None	Pearl Shell Tint
Reverse-phase colour	Parchment	Parchment	Slate	Yellow Tint	Yellow Tint
Soluble pigment	None	None	Black Olive	None	None
ISP medium 6					
Growth	Moderate	Moderate	Moderate	None	Poor
Aerial-mass colour	None	None	None	None	None
Reverse-phase colour	Sand	Sand	Dark Orchid Taupe	None	Yellow Tint
Soluble pigment	None	None	Black Olive	None	None
ISP medium 7					
Growth	Good	Good	Moderate	Moderate	Moderate
Aerial-mass colour	Pearl Shell Tint	Pearl Shell Tint	Pink Tint	None	Pearl Shell Tint
Reverse-phase colour	Sand	Sand	Slate	Bamboo	Yellow Tint
Soluble pigment	Camel	Camel	Black Olive	None	None
Nutrient agar					
Growth	None	None	Poor	None	Poor
Aerial-mass colour	None	None	None	None	None
Reverse-phase colour	None	None	Yellow Tint	None	Yellow Tint
Soluble pigment	None	None	None	None	None
Czapek Solution agar					
Growth	Good	Good	Poor	Poor	Moderate
Aerial-mass colour	Shell Pink	Shell Pink	Citron Grey	None	Pearl Shell Tint
Reverse-phase colour	Sand	Sand	Yellow Tint	Yellow Tint	Yellow Tint
Soluble pigment	None	None	None	None	None

Table 4.19 Differential phenotypic characteristics between strains DS1-2^T and AZ1-7 and their closely related type strains.

Strains: **1,** DS1-2^T; **2,** AZ1-7; **3,** *S. specialis* GW 41-1546^T; **4,** *S. hoynatensis* KCTC 29097^T; **5,** *S. klenkii* KCTC 29202^T. All data were obtained from this study. +, Positive reaction; w, weak positive reaction; -, negative reaction.

Characteristics	1	2	3	4	5
Peptonization	+	+	+	+	-
Coagulation	+	+	+	+	-
Gelatin liquefaction	-	-	+	-	-
Growth at 45 °C	-	-	-	+	+
Growth at pH 11.0	-	-	-	-	+
Utilisation of					
Adonitol	-	-	+	-	+
L-Arabinose	+	+	-	+	-
D-Cellobiose	+	+	-	+	-
Dextran	-	-	-	+	-
D-Fructose	+	+	+	+	-
D-Galactose	w	w	-	+	-
myo-Inositol	+	+	+	-	-
D-Mannose	+	+	-	+	-
D-Mannitol	+	+	-	+	+
D-Raffinose	+	+	-	+	-
D-Rhamnose	+	+	-	+	-
Salicin	-	-	-	w	-
Sucrose	+	+	+	+	w
D-Xylose	+	+	-	+	-
API ZYM					
Acid phosphatase	-	-	+	+	w
α-Chymotrypsin	+	+	-	-	w
Cystine arylamidase	-	-	w	-	+
Esterase lipase (C 8)	+	+	+	w	+
α-Galactosidase	-	-	+	-	-
β-Galactosidase	-	-	-	+	-
β-Glucosidase	-	-	+	-	-
Lipase (C 14)	-	-	w	-	-
α-Mannosidase	+	+	-	+	-
Naphthol-AS-BI-Phosphohydrolase	w	w	+	+	-
Valine arylamidase	+	+	w	+	+

Table 4.20 Cellular fatty acids profiles (%) of strains DS1-2^T and AZ1-7 and their closely related type strains.

Strains: **1,** DS1-2^T; **2,** AZ1-7; **3,** *S. specialis* GW 41-1546^T; **4,** *S. hoynatensis* KCTC 29097^T; **5,** *S. klenkii* KCTC 29202^T. All data were obtained from this study.

Fatty acid	1	2	3	4	5
Saturated fatty acids					
$C_{10:0}$	-	-	0.8	-	-
$C_{14:0}$	0.6	0.6	0.8	-	-
$C_{16:0}$	3.9	3.8	4.4	4.0	0.9
$C_{17:0}$	0.5	0.5	0.5	1.1	-
$C_{19:0}$	-	-	0.5	-	-
Unsaturated fatty acids					
$C_{11:0}$ 2-OH	-	-	1.0	-	-
$C_{17:0}$ 2-OH	0.5	0.5	-	-	-
$C_{17:0}$ cyclo	-	-	1.7	1.7	0.7
$C_{19:0}\omega$6c cyclo	-	-	0.7	-	-
$C_{14:1}\omega$5c	1.0	0.9	-	-	-
$C_{15:1}\omega$8c	-	-	0.8	-	-
$C_{16:1}$ 2-OH	-	-	0.5	-	0.5
$C_{17:1}\omega$5c	1.0	0.9	-	0.6	0.6
$C_{17:1}\omega$8c	1.3	1.3	1.6	1.3	-
$C_{18:1}$ 2-OH	0.7	0.7	0.9	-	-
$C_{18:1}\omega$9c	0.8	0.8	-	-	-
$C_{18:3}\omega$6c	0.5	0.6	-	-	-
Unsaturated branched fatty acids					
iso-$C_{15:1}$F	-	-	0.5	-	-
iso-$C_{15:1}$G	0.9	0.8	-	-	-
anteiso-$C_{15:1}$A	1.1	1.0	-	-	-
iso-$C_{16:1}$G	15.5	15.4	-	-	-
iso-$C_{16:1}$H	-	-	4.9	10.0	9.6
iso-$C_{17:1}\omega$5c	-	-	-	0.6	0.6
anteiso-$C_{17:1}\omega$9c	-	-	8.9	3.4	4.3
anteiso-$C_{17:1}$A	8.8	8.9	-	-	-
iso-$C_{18:1}$H	0.6	0.6	-	1.3	-
iso-$C_{19:1}$I	-	-	0.7	-	-
Branched fatty acids					
iso-$C_{10:0}$	0.7	0.6	-	-	-
anteiso-$C_{11:0}$	-	-	0.5	-	-

Table 4.21 (continued)

Fatty acid	1	2	3	4	5
anteiso-$C_{13:0}$	0.5	0.5	0.6	-	-
iso-$C_{14:0}$	-	-	-	0.6	0.5
anteiso-$C_{14:0}$	0.5	0.5	0.6	-	-
iso-$C_{15:0}$	1.5	1.4	1.9	0.8	2.4
anteiso-$C_{15:0}$	2.7	2.8	2.5	1.7	6.9
anteiso-$C_{15:0}$A	1.1	1.0	-	-	-
iso-$C_{16:0}$ 3-OH	-	-	0.6	1.3	-
anteiso-$C_{16:0}$	-	-	0.6	-	-
iso-$C_{17:0}$	2.2	2.1	2.8	1.0	1.3
iso-$C_{17:0}$ 3-OH	-	-	2.2	0.9	0.8
anteiso-$C_{17:0}$	11.1	11.1	27.7	8.1	19.6
iso-$C_{18:0}$	0.5	0.5	-	-	-
iso-$C_{19:0}$	1.8	1.8	-	-	-
anteiso-$C_{19:0}$	-	-	0.6	-	-
10-Methyl fatty acids					
10-methyl $C_{17:0}$	-	-	1.0	-	-
Summed feature 3[a]	2.6	2.7	3.4	1.1	0.9
Summed feature 6[b]	0.9	0.8	-	-	-
Summed feature 7[c]	1.1	1.0	-	-	-
Summed feature 8[d]	0.8	0.7	-	-	-
Summed feature 9[e]	3.5	3.4	2.9	0.8	0.6

[a]Summed feature 3 contained $C_{16:1}\omega6c$ or $C_{16:1}\omega7c$

[b]Summed feature 6 contained $C_{19:1}\omega9c$ or $C_{19:1}\omega11c$

[c]Summed feature 7 contained $C_{19:1}\omega6c$ or $C_{19:1}\omega7c$ or $C_{19:1}$cyclo

[d]Summed feature 8 included $C_{18:1}\omega6c$

[e]Summed feature 9 comprised iso-$C_{17:1}\omega9c$

The amount of fatty acid less than 0.5 % in all strains was omitted; -, not present.

The results of 16S rRNA gene sequences analysis revealed that the strains DS1-2^T (1,429 nt) and AZ1-7 (1,428 nt) were members of the genus *Streptomyces*. The pairwise 16S rRNA gene sequence analysis showed 100 % similarity between the strains DS1-2^T and AZ1-7. The strain DS1-2^T exhibited 97.9 % 16S rRNA similarity with *Streptomyces specialis* GW41-1564^T, followed by 97.2 % with *S. hoynatensis* S 1412^T,

97.1 % with *S. hainanensis* YIM 47672^T, 96.9 % with *S. avicenniae* NRRL B-24776^T and 96.8 % with *S. klenkii* S 2704^T. A neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences (Figure 4.16) highlighted that the strains DS1-2^T and AZ1-7 formed an independent clade within the cluster consisting of *S. specialis* GW41-1564^T, *S. hoynatensis* S 1412^T and *S. klenkii* S 2704^T with high bootstrap values; in addition, the position of this clade was slightly similar to the phylogenetic trees analysed by maximum-likelihood and maximum-parsimony algorithms. Based on the combination of 16S rRNA gene sequence similarities and the phylogeny, *S. specialis* GW41-1564^T, *S. hoynatensis* S 1412^T and *S. klenkii* S 2704^T were considerably designed into the comparison of genetic profiles.

Genome sequences of strains DS1-2^T, AZ1-7, *Streptomyces hoynatensis* KCTC 29097^T and *Streptomyces klenkii* KCTC 29202^T, receiving from this study, were 7.56 Mb, 7.60 Mb, 7.26 Mb and 8.23 Mb in size, respectively, and the genome sequence of *Streptomyces specialis* GW 41-1564^T (FAXE00000000) was obtained from the GenBank and was 5.85 Mb in size (Loucif *et al.*, 2017). Average *in silico* DNA G+C contents of strains DS1-2^T and AZ1-7, *S. specialis* GW 41-1564^T, S. *hoynatensis* KCTC 29097^T and *S. klenkii* KCTC 29202^T were 73.2, 72.6, 74.4 and 72.4 mol%, respectively. The genomic features of the strains DS1-2T, AZ1-7 and their type strains were described in Table 4.21. The ANIb and ANIm values of the draft genomes among the strain DS1-2^T and its closest related species: *S. specialis* GW 41-1564^T, S. *hoynatensis* KCTC 29097^T and *S. klenkii* KCTC 29202^T, were significantly lower than 95-96 % (Table 4.23) for the species delineation (Ritcher *et al.*, 2009). The results of digital DNA-DNA hybridisation (dDDH) revealed 23.1 % (C. I. model 20.9-25.6 %), 22.0 % (C. I. model 19.8-24.5 %) and 20.2 % (C. I. model 18.0-22.6 %) DNA-DNA relatedness by strain DS1-2^T, *Streptomyces specialis* GW 41-1546^T, *Streptomyces hoynatensis* KCTC 29097^T and *Streptomyces klenkii* KCTC 29202^T, respectively (Table 4.22). The dDDH values were distinctly lower to the recommended threshold of 70 % for species delineation (Goris *et al.*, 2007) supports

the strains DS1-2^{T} and AZ1-7 representing to a novel species within the genus *Streptomyces*.

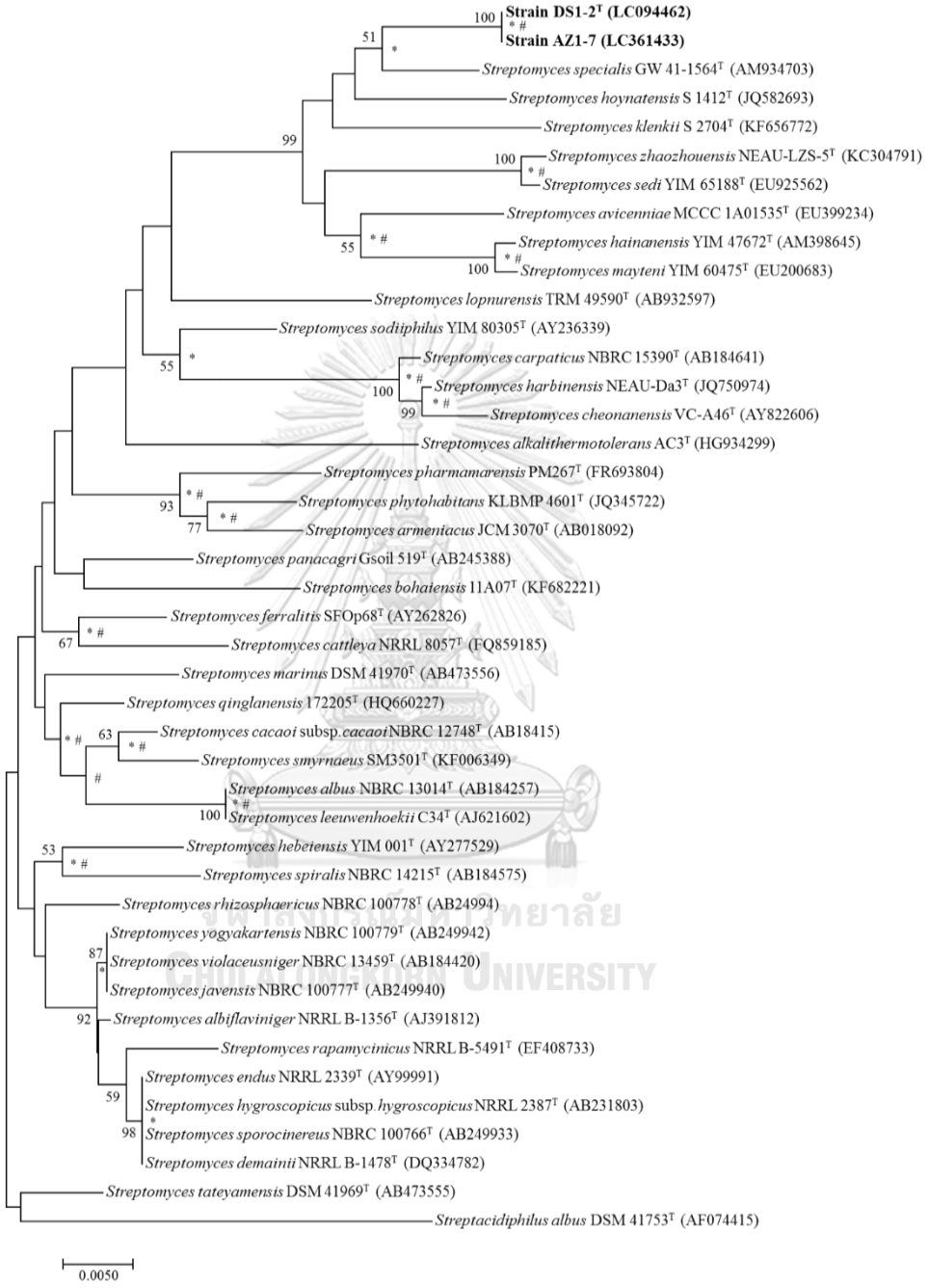


Figure 4.16 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strains DS1-2^{T} (1,429 nt) and AZ1-7 (1,428 nt) and closely related type strains.

In conclusion, the phenotypic, chemotaxonomic, phylogenetic and draft genomic data of strains DS1-2^T and AZ1-7 confirmed that they are significantly different from all closely related type strains. Therefore, they represent a new species of the genus *Streptomyces*, for the name *Streptomyces endoradicus* sp. nov. is proposed. The etymology of *Streptomyces endoradicus* is en.do.ra'di.cus. Gr. pref. endo within; Gr. n. radic plant root; L. masc. suff. -cus adjectival suffix used with the sense of belonging to; N. L. masc. adj. *endoradicus* within plant root, pertaining to the isolation from plant roots.

Table 4.21 Genome statistics of strains DS1-2^T and AZ1-7 and other closely related type strains.

Draft genomes: **1,** DS1-2^T; **2,** AZ1-7; **3,** *S. specialis* GW 41-1546^T; **4,** *S. hoynatensis* KCTC 29097^T; **5,** *S. klenkii* KCTC 29202^T. All data were obtained from this study.

Feature	1	2	3*	4	5
Accession no.	RBDY00000000	RBDX00000000	FAXE00000000	RBAL00000000	RBAM00000000
Genome size (bp)	7,569,598	7,608,576	5,859,524	7,260,715	8,230,710
G+C content (%)	73.2	73.2	72.6	74.4	72.4
No. of Contigs	82	84	168	53	247
Total genes	6,467	6,497	5,550	6,296	7,221
Protein coding genes	6,128	6,165	5,486	5,961	6,868
RNA genes	69	68	64	67	98
tRNAs	54	54	53	54	81

*Data obtained from Loucif *et al.*, (2017).

Table 4.22 The ANIb, ANIm and digital DNA-DNA hybridisation (dDDH) values between the genomes of strains DS1-2^T and AZ1-7 and their closely related type strains. Genomes: 1, DS1-2^T; 2, AZ1-7; 3, *S. specialis* GW 41-1546^T; 4, *S. hoynatensis* KCTC 29097^T; 5, *S. klenkii* KCTC 29202^T. All data were obtained from this study.

Query genome	Reference genome	ANIb (%)	ANIm (%)	% dDDH (Formula 2*)	Model C.I.
1	1	100	100	100	[100 - 100%]
1	2	100	100	100	[100 - 100%]
1	3	80.71	85.34	23.1	[20.9 - 25.6%]
1	4	78.08	84.79	22.0	[19.8 – 24.5%]
1	5	74.28	84.25	20.2	[18.0 – 22.6%]

*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genomes

4.4 Antimicrobial activity screening of endophytic actinomycete isolates

Based on the primary screening of antimicrobial activity using four different production media: 30, 51, 54 and P1, twenty-three isolates showed antimicrobial activity against tested microorganisms.

Fifteen isolates were active against three Gram-positive bacteria, *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341 and *S. aureus* ATCC 6538p. Among these isolates, 7 isolates: NGC1-4, SKH1-5, SKH1-6, SNP1-4, SNP1-7, MS1-11 and MS1-15 belonged to the genus *Micromonospora*, relied on the 16S rRNA gene sequence analyses. The isolate SNP1-7 identified as *M. carbonacea* DSM 43815^T, grown in all production media, showed antimicrobial activity against *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341 and *S. aureus* ATCC 6538p, whereas the isolate NGC1-4 identified as *M. haikouensis* 232617^T was active against *K. rhizophila* ATCC 9341 and *S. aureus* ATCC 6538p only when grown in media 30 and P1 (Table 4.23).

From twenty-three isolates with antimicrobial activity, 16 isolates including AZ1-7, AZ1-15, AZ1-29, AV1-10, DS1-1, DS1-2, MS1-3, MS1-6, MS1-14, SKH1-1, SKH1-2, SKH1-3, NS1-3, NS1-6, NS1-7 and TS1-4 were phylogenetically related to the genus *Streptomyces* according to the analysis of 16S rRNA gene sequences. The isolate AV1-10 identified as *S. parvulus* NBRC 13193^{T}, cultivated in all the production media, exhibited strong antibacterial activity against three tested Gram-positive bacteria, *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341, and *S. aureus* ATCC 6538p, while the isolate NS1-3 identified as *S. cellulosae* NBRC 13027T exhibited antimicrobial activity against only *K. rhizophila* ATCC 9341 (Table 4.23).

Although one-third of the isolates possessed antimicrobial activity, some consisting of *Streptomyces* isolates EH1-1, EH1-2, CN1-1 and CN1-2; *Micromonospora* isolates AZ1-1, AZ1-2, AZ1-3, AZ1-4, AZ1-5, AZ1-11, AZ1-13, AZ1-19, AZ1-28, DS1-3, DS1-4, NGC1-4, MS1-8, MS1-9, MS1-16, WDT1-1, WDT1-2, WPS1-2 and WCL1-2; *Polymorphospora* isolate AZ1-9; and *Plantactinospora* isolate WDT1-3 were not active against any of the tested microorganisms when cultured in all production media.

As mentioned above, the production media tend to be a key factor to determine the antimicrobial activity. Hence, it could be assumed that the preliminary screening for the antimicrobial activity should be evaluated using various production media. In addition, the same species of the isolates did not mean the same activities will be detected.

Table 4.23 Antimicrobial activity of the active isolates cultured in various production media

Isolate no.	Inhibition zone (mm)											
	medium 30[a]			medium 51[a]			medium 57[a]			medium P1[a]		
	B	*K*	*S*	*B*	*K*	*S*	*B*	*K*	*S*	*B*	*K*	*S*
Genus *Micromonospora*												
NGC1-1	11.12	13.83	10.86	-	-	-	-	-	-	13.60	17.12	10.16
SKH1-5	12.36	15.19	9.94	-	-	-	-	-	-	-	-	-
SKH1-6	-	-	-	-	-	-	-	-	-	13.40	17.97	10.86
SNP1-4	-	-	-	10.52	10.27	10.21	10.83	10.97	10.60	9.26	9.97	9.10
SNP1-7	15.16	16.13	15.09	17.20	20.94	16.14	14.23	19.47	14.67	-	-	-
MS1-11	11.27	12.85	10.26	-	-	-	-	-	-	13.60	15.77	12.52
MS1-15	-	-	-	15.60	16.85	15.42	13.80	14.17	12.46	-	-	-
Genus *Streptomyces*												
AZ1-7	13.70	14.66	10.08	-	9.72	-	10.20	12.57	10.10	12.10	13.78	-
AZ1-15	11.70	12.69	6.46	-	7.77	-	10.10	10.58	-	11.40	11.64	-
AZ1-29	12.10	12.12	6.99	-	-	-	13.21	13.46	-	11.20	11.57	-
DS1-1	13.20	14.53	7.12	-	-	-	11.20	11.35	-	10.19	11.90	-
DS1-2	15.24	15.89	9.21	-	-	-	-	7.93	-	11.12	11.87	8.27
MS1-3	10.24	10.82	-	-	-	-	-	-	-	-	9.81	-
MS1-14	11.90	12.29	7.10	-	-	-	-	9.53	-	13.46	14.48	6.90
MS1-6	9.12	9.17	10.05	-	-	-	9.81	9.70	10.39	9.80	9.91	8.91
SKH1-1	-	-	-	12.60	14.50	11.60	8.86	9.24	8.05	-	-	-
SKH1-2	-	-	-	20.42	21.12	8.05	20.21	21.80	6.65	-	-	-
SKH1-3	-	-	-	12.12	14.23	11.03	9.10	9.16	9.08	-	-	-
NS1-3	10.60	12.77	9.35	-	-	-	10.60	11.80	10.62	-	-	-
NS1-6	10.21	11.65	9.17	-	-	-	10.58	10.60	9.65	-	-	-
NS1-7	9.90	12.20	9.81	11.24	11.57	10.35	-	-	-	-	-	-
AV1-10	20.33	22.16	19.60	19.24	20.60	18.33	20.46	21.80	18.25	-	-	-
TS1-4	9.60	10.20	8.10	-	-	-	9.00	11.40	8.62	-	-	-

B, *B. subtilis* ATCC 6633; ***K***, *K. rhizophila* ATCC 9341 and ***S***, *S. aureus* ATCC 6538p.

All active isolates had no activity against *E. coli* NIHJ, *X. campestris* pv. *oryzae* KB88, *C. albicans* ATCC 64548 and *M. racemosus* IFO 4581.

[a]see Appendix A (pages 134-136)

4.5 Secondary metabolites of two selected endophytic actinomycetes

Based on the results from taxonomic studies and chemical analysis of the crude extract from the fermented broth by using HPLC, two endophytic actinomycete species, which were *Streptomyces pseudovenezuelae* SKH1-2 and *Streptomyces aculeolatus* MS1-6, have been selected for secondary metabolites study.

4.5.1 Isolation and structure elucidation of secondary metabolites from *Streptomyces pseudovenezuelae* SKH1-2

S. pseudovenezuelae SKH1-2 was cultivated in 6 L of medium 51 broth under shaking condition (200 rpm) at 28 °C for 6 days. After cultivation, the culture broth was centrifuged at 3,000 rpm for 5 minutes and discarded the cells. The HPLC analysis of the supernatant exhibited two interesting peaks at the retention times (RT) of 15.58 and 20.08 minutes (Figure 4.17).

For the isolation of these compounds, the 6-day culture broth of *Streptomyces pseudovenezuelae* SKH1-2 was firstly extracted with an equivalent volume of 95% (v/v) ethanol and further extracted twice with an equal volume of *n*-butanol and then evaporated to dryness. The *n*-butanol crude extract (3.17 g) was firstly separated through a silica gel open-column chromatography eluting with a gradient system of 0-100 % $CHCl_3$-CH_3OH to yield 7 fractions. The active fraction, $CHCl_3$-CH_3OH (9:1) was purified continuously using octadecylsilyl silica gel (ODS) open-column chromatography and eluted with 10-50 % of CH_3CN in H_2O. Obtained 40 % and 50 % CH_3CN fractions were concentrated *in vacuo* and dryness to afford pure SKH9/1-1 (71.3 mg and 4.9 mg, respectively). The 20 % CH_3CN fraction was further purified by preparative HPLC equipped with CAPCELL PAK UG-120 column (Osaka Soda Co., 20 mm × 250 mm) and eluted with an isocratic system of 20 % CH_3CN in H_2O at a flow rate of 7 mL/min to furnish pure SKH9/1-2 (20.0 mg). The isolation procedure of the compounds was presented in Figure 4.18.

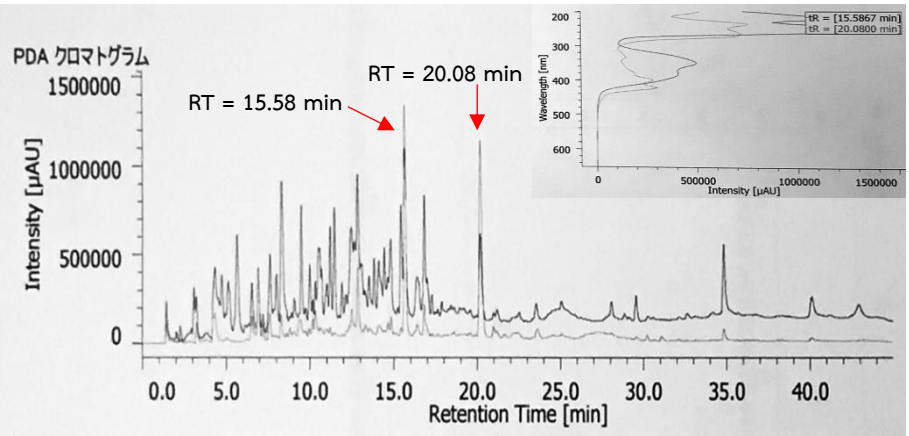


Figure 4.17 HPLC chromatogram of the crude extract of *S. pseudovenezuelae* SKH1-2 and the UV-visible spectra of the target compounds, RT 20.08 min and RT 15.58 min.

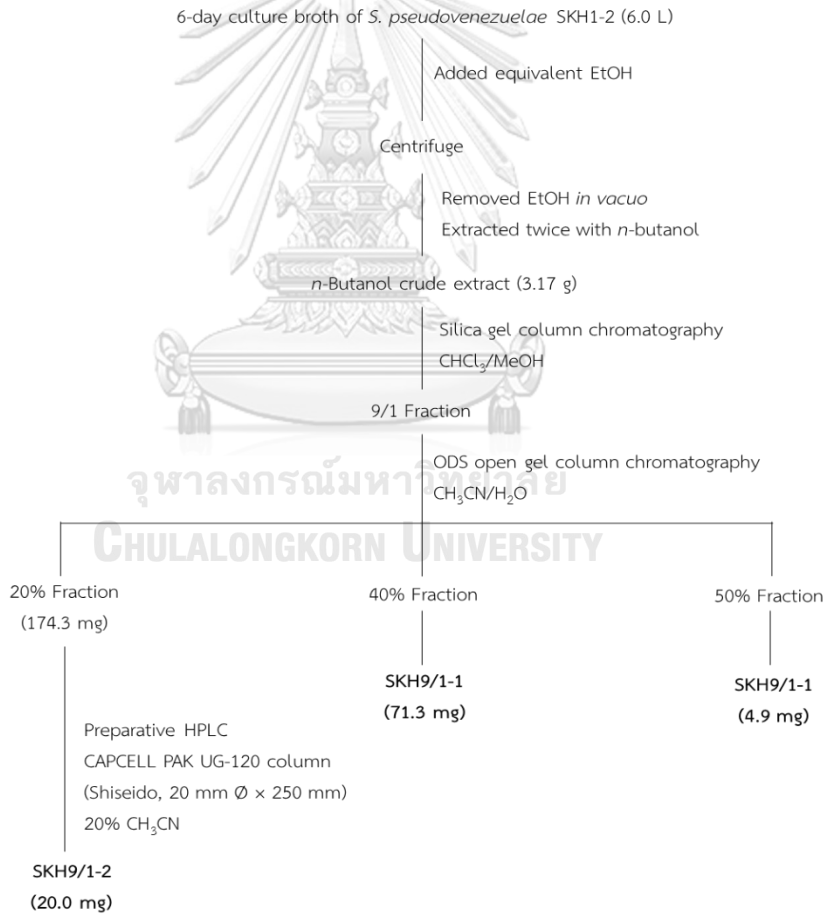


Figure 4.18 The purification scheme of the compounds SKH9/1-1 and SKH9/1-2 isolated from *Streptomyces pseudovenezuelae* SKH1-2

Compound SKH9/1-1 was obtained as greenish-yellow solid. ESI-MS analyses indicated a molecular formula $C_{32}H_{32}O_{14}$ (m/z found 663.1740 $[M+Na]^+$). Its spectral was presented in Table 4.24, respectively.

The ^{13}C-NMR spectrum (in DMSO-d_6) provided one methoxy [at δ 55.8 ppm (3″-OCH_3)], two carbonyl carbons [at δ 158.5 ppm (C-12) and δ 163.7 ppm (C-5)], ten aromatic quaternaries [at δ 138.5 ppm (C-1), δ 146.1 ppm (C-3a), δ 96.8 ppm (C-5a), δ 154.1 ppm (C-6), δ 128.4 ppm (C-6a), δ 155.5 ppm (C-10), δ 119.6 ppm (C-10a), δ 138.5 ppm (C-10b), δ 117.9 ppm (C-12a), δ 114.3 ppm (C-12c)], fifteen methines [at δ 133.0 ppm (C-2), δ 120.7 ppm (C-3), δ 116.9 ppm (C-7), δ 128.4 ppm (C-8), δ 116.3 ppm (C-9), δ 99.3 ppm (C-1′), δ 79.8 ppm (C-2′), δ 77.8 ppm (C-3′), δ 72.0 ppm (C-4′), δ 71.2 ppm (C-5′), δ 99.7 ppm (C-1″), δ 70.2 ppm (C-2″), δ 96.8 ppm (C-3″), δ 108.4 ppm (C-4″) and δ 67.4 ppm (C-5″)] and three methyls [at δ 21.7 ppm (1-CH_3), δ 16.4 ppm (5′-CH_3) and δ 16.5 ppm (5″-CH_3)] and.

The ^{1}H-NMR spectrum indicated one methoxy proton [at δ 3.32 ppm (s)], three methyl protons [at δ 2.72 ppm (s), 1.22 ppm (s) and 8.04 ppm (d, *J* = 6.3 Hz)] and fourteen methine protons [at δ 7.5 ppm (d, *J* = 8.3 Hz), δ 7.52 ppm (d, *J* = 8.3 Hz), δ 8.07 ppm (d, *J* = 8.3 Hz), δ 7.61 ppm (dd, *J* = 8.3, 7.8 Hz), δ 7.72 ppm (d, *J* = 8.0 Hz), δ 5.40 ppm (d, *J* = 7.7 Hz), δ 4.88 ppm (dd, *J* = 9.7, 7.7 Hz), δ 4.21 ppm (dd, *J* = 9.6, 3.6 Hz), δ 4.19 ppm (d, *J* = 3.6 Hz), δ 3.96 ppm (q, *J* = 6.4 Hz), δ 5.42 ppm (d, *J* = 4.1), δ 4.55 ppm (dd, *J* = 10.1, 4.1), δ 3.93 ppm (dd, *J* = 10.1, 3.1 Hz), δ 3.98 ppm (dd, *J* = 3.1, 1.3 Hz) and δ 5.36 ppm (qd, *J* = 6.3, 1.3 Hz)].

Based on the ^{1}H and ^{13}C NMR spectral information (Appendix D), the compound SKH9/1-1 was assigned to be chartreusin (Figure 4.19) which firstly isolated from *Streptomyces chartreusis* (Leach *et al.*, 1953).

Table 4.24 The ^{1}H and ^{13}C assignments of compound SKH9/1-1 (chartreusin)

Position	SKH9/1-1 in DMSO-d_6[a]		Chartreusin in DMSO-d_6[b] (Leach *et al.*, 1953)	
	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)
1	138.5, C	-	139.5, C	-
2	133.0, CH	7.50, d, (8.3)	133.0, CH	7.30, d, (8.3)
3	120.7, CH	7.52, d, (8.3)	120.7, CH	7.42, d, (8.3)
3a	146.1, C	-	146.8, C	-
5	163.7, C	-	164.7, C	-
5a	96.8, C	-	97.4, C	-
6	154.1, C	-	156.9, C	-
6a	128.4, C	-	128.5, C	-
7	116.9, CH	8.07, d, (8.3)	117.5, CH	8.31, d, (8.3)
8	128.4, CH	7.61, dd, (8.3, 7.8)	128.5, CH	7.62, dd, (8.3, 7.8)
9	116.3, CH	7.72, d, (8.0)	115.1, CH	7.73, d, (7.8)
10	155.5, C	-	155.1, C	-
10a	119.6, C	-	119.2, C	-
10b	138.5, C	-	139.5, C	-
12	158.5, C	-	159.1, C	-
12a	117.9, C	-	118.0, C	-
12c	114.3, C	-	109.0, C	-
1-CH_3	21.7, CH_3	2.72, s	22.1, CH_3	2.70, s
1′	99.3, CH	5.40, d, (7.7)	101.1, CH	5.79, d, (7.7)
2′	79.8, CH	4.88, dd, (9.6, 7.7)	80.3, CH	5.04, dd, (9.6, 7.7)
3′	77.8, CH	4.21, dd, (9.6, 3.6)	74.2, CH	4.31, dd, (9.6, 3.6)
4′	72.0, CH	4.19, d, (3.6)	72.2, CH	4.19, d, (3.6)
5′	71.2, CH	3.96, q, (6.4)	71.9, CH	4.09, q, (6.4)
5′-CH_3	16.4, CH_3	1.22, d, (6.4)	17.2, CH_3	1.55, d, (6.4)
1″	99.7, CH	5.42, d, (4.1)	101.9, CH	6.52, d, (4.1)
2″	70.2, CH	4.55, dd, (10.1, 4.1)	69.0, CH	4.53, dd, (10.1, 4.1)
3″	96.8, CH	3.93, dd, (10.1, 3.1)	81.6, CH	3.83, dd, (10.1, 3.1)
4″	108.4, CH	3.98, dd, (3.1, 1.3)	107.3, CH	4.12, dd, (3.1, 1.3)
5″	67.4, CH	5.36, qd, (6.3, 1.3)	67.4, CH	5.00, qd, (6.3,1.3)
3″-OCH_3	55.8, CH_3	3.32, s	56.3, CH_3	3.30, s
5″-CH_3	16.5, CH_3	1.20, d, (6.3)	17.1, CH_3	1.55, d, (6.3)

[a]400 MHz NMR; [b]600 MHz NMR

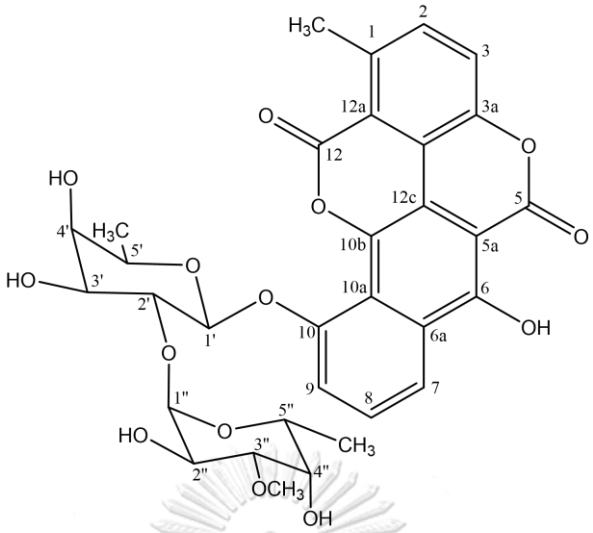


Figure 4.19 The chemical structure of compound SKH9/1-1 (chartreusin)

Compound SKH9/1-2 was obtained as a yellow solid. The molecular formula $C_{12}H_{10}N_4O_2$ was confirmed by ESI-MS analysis, giving the mass ion at m/z found 265.0759 $[M+Na]^+$. Its spectral data of the compound SKH9/1-2 were given in Table 4.25.

The ^{1}H-NMR spectrum in pyridine-d_6 revealed two singlet methyl protons (δ 2.33 and 2.36 ppm), two singlet aromatic protons (δ 7.85 and 8.04 ppm), two singlet NH protons (δ 8.05 and 8.73 ppm). The ^{13}C-NMR spectrum showed 12 carbons including two carbonyl signals [δ 162.2 ppm (C-4) and δ 151.9 ppm (C-2)], six aromatic quaternary signals [δ 147.8 ppm (C-10a), δ 145.1 ppm (C-7), δ 143.2 ppm (C-9a), δ 139.4 ppm (C-8) and δ 139.9 ppm (C-5a), δ 131.1 ppm (C-4a)], two methine signals [δ 127.3 ppm (C-6) and δ 129.8 ppm (C-9)] and two methyl signals [δ 20.6 ppm (C-11) and δ 20.0 ppm (C-12)].

According to the ^{1}H and ^{13}C NMR spectral data (Appendix D), the compound SKH9/1-2 was identical to the previously published data for lumichrome (Tsukamoto *et al.*, 1999), which shown in Figure 4.20.

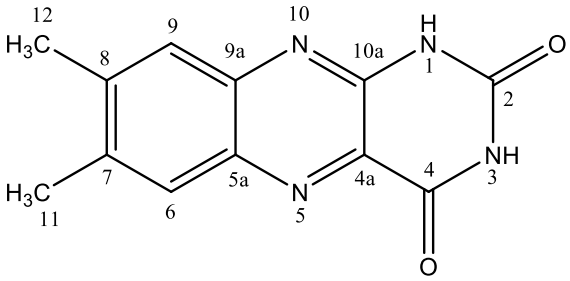


Figure 4.20 The chemical structure of compound SKH9/1-2 (lumichrome)

Table 4.25 The ^{1}H and ^{13}C assignments of compound SKH9/1-2 (lumichrome)

Position	SKH9/1-2 in Pyridine-d_5[a]		Lumichrome in Pyridine-d_5[a] (Tsukamoto *et al.*, 1999)	
	δ_C (ppm)	δ_H (ppm), multiplexity (*J* in Hz)	δ_C (ppm)	δ_H (ppm), multiplexity (*J* in Hz)
1-NH	-	8.05, br. s	-	14.01, br. s
2	151.9, C	-	151.9, C	-
3-NH	-	8.73, br. s	-	14.24, br. s
4	162.2, C	-	162.1, C	-
4a	131.1, C	-	131.0, C	-
5-N	-	-	-	-
5a	139.9, C	-	139.1, C	-
6	127.3, CH	7.85, s	127.1, CH	7.86, s
7	145.1, C	-	144.8, C	-
8	139.4, C	-	139.8, C	-
9	129.8, CH	8.04, s	129.7, CH	8.04, s
9a	143.2, C	-	143.0, C	-
10-N	-	-	-	-
10a	147.8, C	-	147.7, C	-
11	20.6, CH_3	2.33, s	20.4, CH_3	2.28, s
12	20.0, CH_3	2.26, s	19.8, CH_3	2.21, s

[a]400 MHz NMR

4.5.2 Isolation and structure elucidation of secondary metabolites from *Streptomyces aculeolatus* MS1-6

Streptomyces aculeolatus MS1-6 was cultured in medium 30 broth (20 L) under shaking condition at 200 rpm for 7 days at 30 °C. After incubation, the whole culture broth was extracted three times with ethyl acetate and then the layer of ethyl acetate was evaporated to dryness. The chemical profile of the crude extract was given in Figure 4.21.

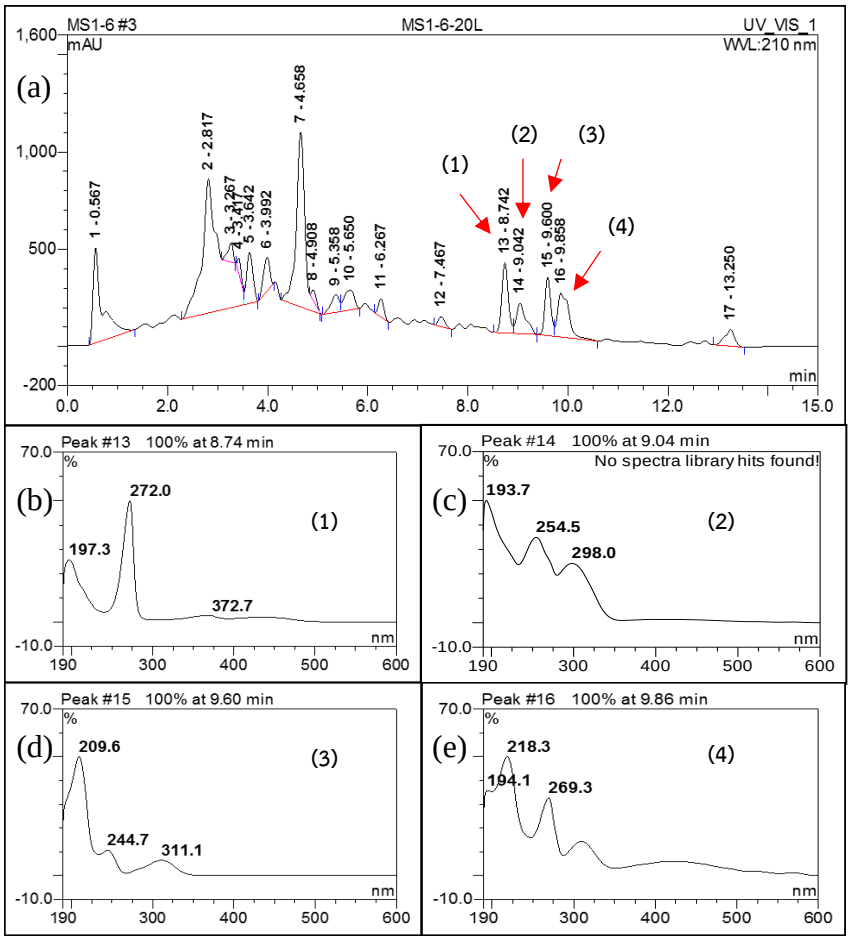


Figure 4.21 The HPLC chromatogram of the crude EtOAc extract of *Streptomyces aculeolatus* MS1-6 (a), UV profile of the peak at RT 8.74 (b), 9.04 (c), 9.60 (d) and 9.86 (e). HPLC condition: Shiseido C18 (5 μm, 2.1 × 50 mm) column; eluted with 0-100% acetonitrile in water plus 0.05% (v/v) formic acid; flow rate 0.5 mL/min.

The crude EtOAc extract (2.96 g) was initially separated through a sephadex LH-20 column (diam. 7 cm × 35 cm) chromatography eluting with 100% CH_3OH to yield 7 fractions. The fraction MS1-6-F5 was further purified using preparative HPLC performed with Shiseido C18 CAPCELL PAK column (5 μm, 20 mm × 250 mm) and eluted with a gradient system of 45-100% CH_3CN in H_2O plus 0.05% (v/v) formic acid at a flow rate of 12 mL/min to furnish two pure compounds, MS1-6-F5-H12 (2.79 mg) and MS1-6-F5-H13 (24.4 mg). The purification scheme of the compounds was shown in Figure 4.22.

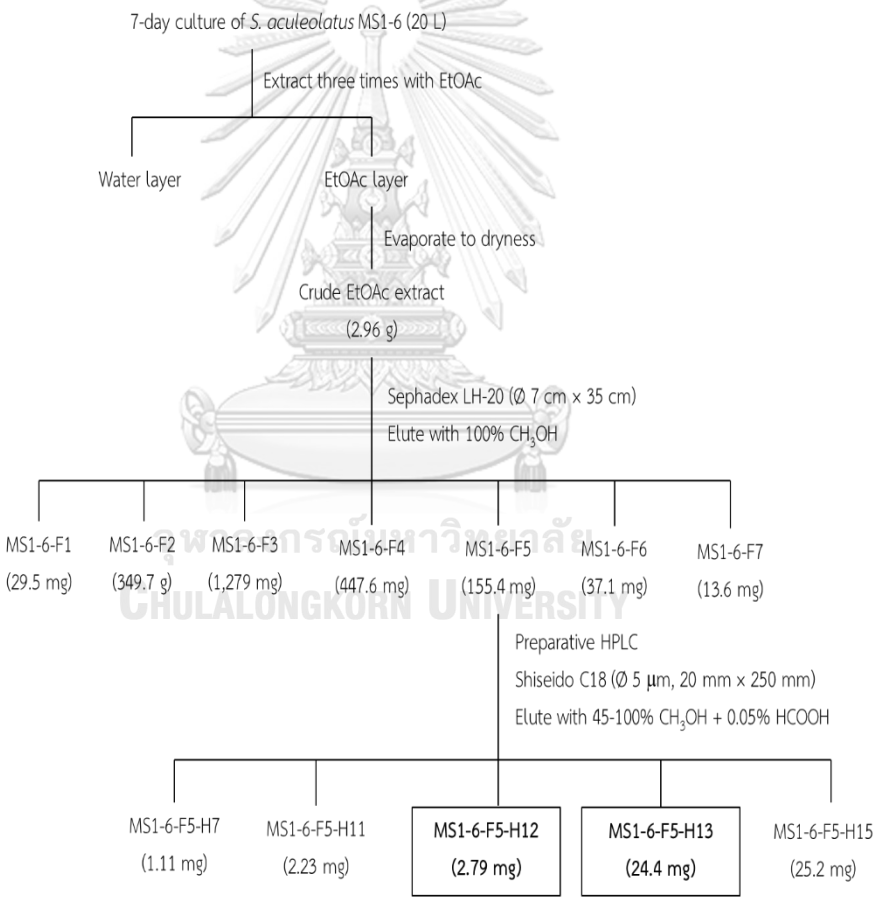


Figure 4.22 The purification scheme of the compounds MS1-6-F5-H12 (arromycin) and MS1-6-F5-H13 (salaceyin A) isolated from the *Streptomyces aculeolatus* MS1-6

Compound MS1-6-F5-H12 was obtained as a brown solid. The HRESIMS illustrated a pseudo-molecular ion at *m/z* 379.1516 $[M+Na]^+$, indicating the molecular formula $C_{21}H_{24}O_5$. the spectral data was given in Table 4.26, respectively.

The ^{13}C-NMR spectrum (in DMSO-d_6) gave the characteristic carbon signals of one methine [δ 106.83 ppm (C-7)], two carbonyls [at δ 189.83 ppm (C-2) and δ 180.41 ppm (C-9)], four methylenes [at δ 25.44 ppm (C-11), δ 45.90 ppm (C-14), δ 35.34 ppm (C-16) and δ 26.11 ppm (C-17)], four methyls [at δ 19.61 ppm (C-18), δ 27.96 ppm (C-19, C-20) and δ 8.13 ppm (C-21)] and eight aromatic quaternaries [at δ 128.54 ppm (C-3), δ 160.74 ppm (C-4), δ 117.94 ppm (C-5), δ 160.71 ppm (C-6), δ 156.40 ppm (C-10), δ 125.22 ppm (C-12), δ 121.32 ppm (C-13) and δ 28.75 ppm (C-15].

The ^{1}H-NMR spectrum emphasized one methine proton [at δ 7.05 ppm (s)], four methylene protons [at δ 3.21 ppm (s), δ 1.68 ppm (s), δ 1.20 ppm (t, *J* = 6.2 Hz) and δ 1.87 ppm (t, *J* = 6.2 Hz)] and four methyl protons [at δ 1.70 ppm (s), 0.80 ppm (6H, s) and 2.04 ppm] and

The 2-dimensional (2D) NMR spectral data: HSQC, HMBC and COSY (Appendix D), gave the chemical structure of compound MS1-6-F5-H12 (Figure 4.23), which was in accordance with the previous report for arromycin (Wong *et al.*, 2012) (Table 4.26).

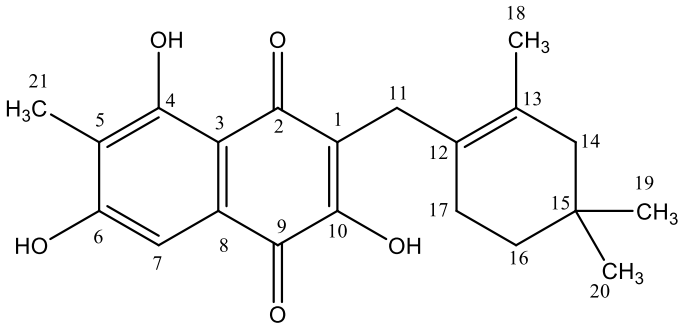


Figure 4.23 The chemical structure of compound MS1-6-F5-H12 (arromycin)

Table 4.26 The ^{1}H and ^{13}C assignments of compound MS1-6-F5-H12 and arromycin

Position	MS1-6-F5-H12 in DMSO-d_6[a]		Arromycin in DMSO-d_6[b]	
	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)
1	-*	-	-*	-
2	189.83, C	-	190.0, C	-
3	128.54, C	-	128.5, C	-
4	160.74, C	-	160.7, C	-
5	117.94, C	-	117.9, C	-
6	160.71, C	-	160.8, C	-
7	106.83, CH	7.05, s	106.8, CH	7.07, s
8	-*	-	-*	-
9	180.41, C	-	180.3, C	-
10	156.40, C	-	156.0, C	-
11	25.44, CH_2	3.20, s	25.4, CH_2	3.21, s
12	125.22, C	-	125.1, C	-
13	121.32, C	-	121.5, C	-
14	45.90, CH_2	1.68, s	45.9, CH_2	1.69, s
15	28.75, C	-	28.8, C	-
16	35.34, CH_2	1.20, t (6.2)	35.3, CH_2	1.20, t (6.5)
17	26.11, CH_2	1.87, t (6.2)	26.1, CH_2	1.85, t (6.5)
18	19.61, CH_3	1.70, s	19.6, CH_3	1.70, s
19	27.96, CH_3	0.80, s	28.0, CH_3	0.80, s
20	27.96, CH_3	0.80, s	28.0, CH_3	0.80, s
21	8.13, CH_3	2.04, s	8.1, CH_3	2.04, s
2-OH	-	12.35	-	10.98
3-OH	-	10.89	-	10.84
5-OH	-	13.10	-	13.11

-*, Resonance not observed; [a]400 MHz NMR; [b]600 MHz NMR

Compound MS1-6-F5-H13 was obtained as a brown solid. The HRESIMS indicated a pseudo-molecular ion at m/z 315.1931 $[M+Na]^+$, suggesting the molecular formula $C_{18}H_{28}O_3$. Its spectral data was shown in Table 4.27, respectively.

The ^{13}C-NMR spectrum (in Acetone-d_6) displayed characteristic carbon signals of one carboxyl at δ 173.58 ppm, two methyls [at δ 22.90 ppm (C-10′, C-11′)], three aromatic quaternary carbons [at δ 113.38 ppm (C-1), δ 163.32 ppm (C-2), δ 147.18 ppm (C-6)], four methines [at δ 36.74 ppm (C-1′), δ 32.95 ppm (C-2′), δ 30.51 ppm (C-5) and δ 28.65 ppm (C-9′)] and eight methylenes [at δ 36.74 ppm (C-1′), δ 32.95 ppm (C-2′), δ 30.51 ppm (C-3′), δ 30.61 ppm (C-4′), δ 30.68 ppm (C-5′), δ 30.74 ppm (C-6′), δ 39.76 ppm (C-7′) and δ 28.09 ppm (C-8′)].

The ^{1}H-NMR spectrum provided three methine protons [at δ 7.05 ppm (s)], three methyl protons [at δ 6.78 ppm (d, *J* = 7.96 Hz), 7.33 ppm (t, *J* = 7.89 Hz) and 1.51 ppm (t, *J* = 6.6 Hz)], five methylene protons [at δ 2.98 (dd, *J* = 7.81 Hz), 1.60 (m), 1.34 ppm (m), 1.15 ppm (m) and 1.28 ppm (m)] and two methyl protons at δ 0.86 ppm (d, *J* = 6.6 Hz).

The 2D NMR (2D) NMR spectral information including HSQC, HMBC and COSY (Appendix D) indicated the chemical structure of compound MS1-6-F5-H13 (Figure 4.23), which previously described as salaceyin A (Kim *et al.*, 2006) (Table 4.27).

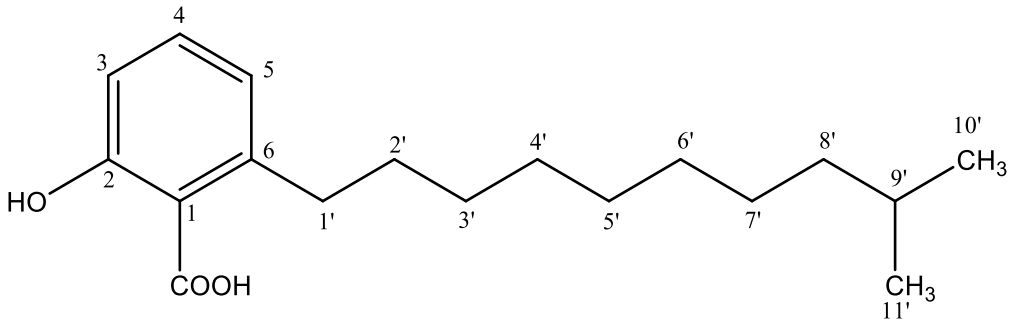


Figure 4.24 The chemical structure of compound MS1-6-F5-H13 (salaceyin A)

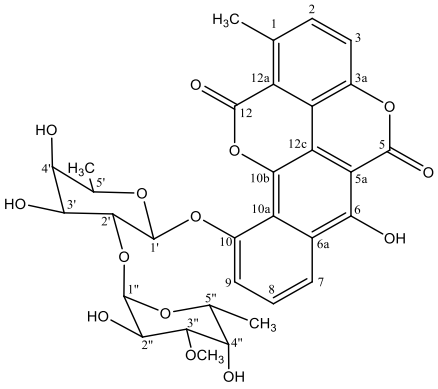
Table 4.27 The ^{1}H and ^{13}C assignments of compound MS1-6-F5-H13 and salaceyin A

Position	MS1-6-F5-H13 in Acetone-d_6[a]		Salaceyin A in $CDCl_3$[a]	
	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)
1	113.38, C	-	110.36, C	-
2	163.32, C	-	163.63, C	-
3	115.89, CH	6.78, d (7.96)	115.85, CH	6.85, d (8.0)
4	134.68, CH	7.33, t (7.89)	135.40, CH	7.34, t (7.6, 8.0)
5	122.92, CH	6.78, d (7.96)	122.75, CH	6.75, d (7.6)
6	147.18, C	-	147.77, C	-
COOH	173.58	-	175.64	-
1′	36.74, CH_2	2.98, dd (7.81)	36.48, CH_2	2.96, t (7.6)
2′	32.95, CH_2	1.60, m	32.02, CH_2	1.58, m
3′	30.51, CH_2	1.34, m	29.92, CH_2	1.23~1.35
4′	30.61, CH_2	1.34, m	29.81, CH_2	1.23~1.35
5′	30.68, CH_2	1.34, m	29.65, CH_2	1.23~1.35
6′	30.74, CH_2	1.34, m	29.49, CH_2	1.23~1.35
7′	39.76, CH_2	1.15, m	27.41, CH_2	1.23~1.35
8′	28.09, CH_2	1.28, m	39.03, CH_2	1.14, m
9′	28.65, CH	1.51, t (6.6)	27.95, CH	1.49, octet
10′	22.90, CH_3	0.86, d (6.6)	22.64, CH_3	0.83, d (6.8)
11′	22.90, CH_3	0.86, d (6.6)	22.64, CH_3	0.83, d (6.8)
2-OH	-	-*	-	10.98, br. s

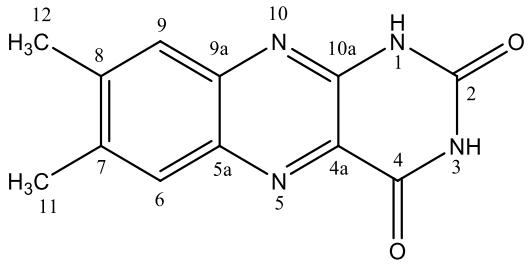
-*, Resonance not observed; [a]400 MHz NMR

4.6 Biological activities of the isolated compounds

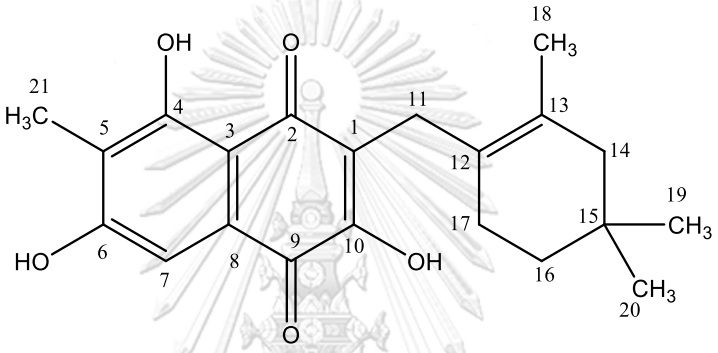
In this study, two compounds, SKH1-2-1 and SKH1-2-2 (Figure 4.25), isolated from *S. pseudovenezuelae* SKH1-2 have evaluated the biological activities consisting of anti-Bacillus subtilis ATCC 6633, anti-*Kocuria rhizophila* ATCC 9341, anti-*Staphylococcus aureus* ATCC 6538p, anti-*Escherichia coli* NIHJ, anti-*Pseudomonas aeruginosa* ATCC 27853 and anti-*Xanthomonas campestris* pv. *oryzae* KB88. The results of the biological activities of the compounds were concluded in Table 4.28.



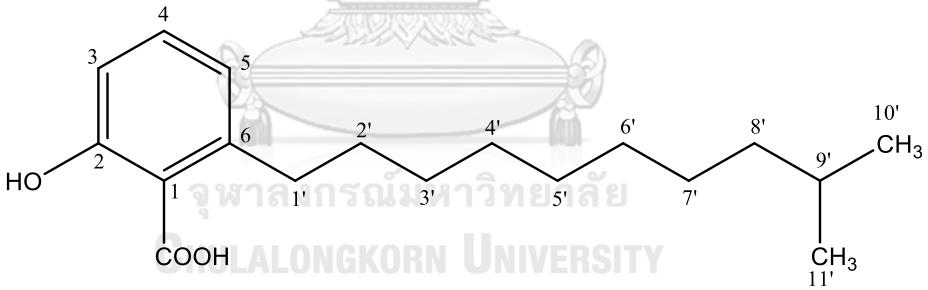
SKH1-2-1



SKH1-2-2



MS1-6-F5-H12



MS1-6-F5-H13

Figure 4.25 The chemical structures of all isolated compounds

Table 4.28 Biological activities of SKH9/1-1 and SKH9/1-2

Tested organism	MIC of compound (μg/mL)			
	SKH9/1-1	SKH9/1-2	Vancomycin	Chloramphenicol
B. subtilis ATCC 6633	3.1±0.04	-	1.0±0.06	-
K. rhizophila ATCC 9341	1.6±0.01	-	0.5±0.02	-
S. aureus ATCC 6538p	12.5±0.02	-	2.0±0.07	-
E. coli NIHJ	> 50	-	-	2.0±0.04
P. aeruginosa ATCC 27853	> 50	-	-	4.0±0.01
X. campestris pv. *oryzae* KB88	-	-	-	4.0±0.02

-, inactive

Compound SKH9/1-1 (known as chartreusin) showed strong antimicrobial activity against Gram-positive bacteria: *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341 and *S. aureus* ATCC 6538p with MIC values of 3.1, 1.6 and 12.5 μg/mL, respectively, but weakly inhibited Gram-negative bacteria. In addition, chartreusin exhibited the activity of anti-cancer by inhibiting RNA synthesis by causing single-strand scission of DNA via the free radicals' formation (Portugal, 2003).

Compound SKH9/1-2 assigned as lumichrome has no activity against all test microorganisms; nevertheless, other studies highlighted that lumichrome has been affected the plants growth by increasing cell differentiation and expansion with a consequential increase in biomass (Pholo *et al.*, 2018), enhanced the osteoblast differentiation at early stage and reduced bone loss by inhibiting osteoclastogenesis in patients who suffered from osteolytic diseases (Liu *et al.*, 2018).

In this dissertation two isolated compounds, MS1-6-F5-H12 (arromycin) and MS1-6-F5-H13 (salaceyin A) from *Streptomyces aculeolatus* MS1-6 (Figure 4.25), have not been determined the biological activities yet because they have just successfully isolated and identified the chemical structures. However, the crude ethyl acetate

extract of the isolate MS1-6 exhibited various biological activities, including anti-*Enterococcus faecium* (MIC 1.56 μg/mL), anti-*Staphylococcus aureus* (MIC 3.13 μg/mL), anti-*Bacillus cereus* (MIC 1.56 μg/mL), anti-*Plasmodium falciparum* K1 (IC_{50} 1.97 μg/mL) and anti-lung cancer (NCI-H187) (IC_{50} 6.82 μg/mL).

It has been reported that compound MS1-6-F5-H12 (arromycin) was naphthoquinone antibiotics, which displayed narrow-spectrum antibacterial activity against Gram-positive bacteria including *Bacillus subtilis*, *Enterococcus faecium*, *Listeria ivanovii*, methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus* and *S. epidermis* with the MIC values of 25, 50, 50, 25, 12.5 and 12.5 μM, respectively (Wong *et al.*, 2012). This previous study was consistent with the biological activities of the crude EtOAc extract of *S. aculeolatus* MS1-6.

Compound MS1-6-F5-H13 was designed as salaceyin A, which is a derivative of salicylic acid and firstly isolated from the endophytic *Streptomyces laceyi* MS53. Salaceyin A showed modest cytotoxic activity against a human breast cancer cell line (SKBR3) with IC_{50} of 3.0 μg/mL (Kim *et al.*, 2006). Moreover, the compound exhibited antifungal activity against *Cladosporium cucumerinum*, *Colletotrichum orbiculare* and *Phytophthora capsici* at the same MIC value of 64 μg/mL (Park *et al.*, 2007).

CHAPTER V

CONCLUSION

Endophytic actinomycetes associated with plant roots are a relatively untapped source of novel species and potential bioactive metabolites. There are numerous plant species which has little studies on the isolation of plant-derived actinomycetes in Thailand. In this study, 50 actinomycete strains were isolated from 14 plant species collected from Bangkok, Chachoengsao, Chiang Mai, Nakhon Pathom, Prachinburi and Suphan Buri provinces. Based on morphological and chemotaxonomic characteristics and the 16S rRNA gene sequence analysis, 45 actinomycetes were identified as *Micromonospora krabiensis* (5 isolates), *M. maritima* (3 isolates), *M. carbonaceae* (3 isolates), *M. avicenniae* (2 isolates), *M. haikouensis* (2 isolates), *M. chokoriensis* (1 isolate), *M. oryzae* (1 isolate), *M. echinospora* (1 isolate), *M. andamanensis* (1 isolate), *M. zingiberis* (1 isolate), *M. aurantiaca* (1 isolate), *M. tulbaghiae* (1 isolates), *Plantactinospora mayteni* (1 isolate), *Polymorphospora rubra* (1 isolate), *Streptomyces specialis* (5 isolates), *S. alboniger* (2 isolates), *S. cellulosae* (2 isolates), *S. bellus* (2 isolates), *S. aculeolatus* (1 isolate), *S. lannensis* (1 isolate), *S. pseudovenezuelae* (1 isolate), *S. warraensis* (1 isolate), *S. tendae* (1 isolate), *S. parvulus* (1 isolate), *S. gradneri* (1 isolate), *S. diastaticus* (1 isolate) and *S. pratensis* (1 isolate). According to the polyphasic taxonomic approaches, five novel actinomycete species including *Micromonospora globbae* WPS1-2^{T}, *Micromonospora azadirachtae* AZ1-19^{T}, *Micromonospora radicis* AZ1-13^{T}, *Micromonospora musae* MS1-9^{T} and NGC1-4 and *Streptomyces endoradicus* DS1-2^{T} and AZ1-7 were proposed.

The preliminary antimicrobial activity screening revealed that seven isolates of the genus *Micromonospora* and sixteen isolates of the genus *Streptomyces* exhibited antimicrobial activity against the test microorganisms. It was found that the production media and the strains were a key factor which influenced the antimicrobial activity.

In the present study, two representative isolates involving *S. pseudovenezuelae* SKH1-2 and *S. aculeolatus* MS1-6 were selected for the isolation of secondary metabolites. Chartreusin isolated from *S. pseudovenezuelae* SKH1-2 was significantly active against Gram-positive bacteria including *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341 and *S. aureus* ATCC 6538p with the MIC values of 3.1, 1.6 and 12.5 μg/mL, respectively whilst lumichrome isolated from the same strain did not exhibit the antimicrobial activity against all test bacteria. Arromycin and salaceyin A were isolated from *S. aculeolatus* MS1-6. The arromycin was known as a narrow-spectrum anti-Gram-positive bacterial agent against *B.* subtilis, *S. aureus*, *L. ivanovii* and *E. faecium* whereas the salaceyin A has been used as anti-phytopathogenic fungi such as *Cladosporium cucumerinum*, *Colletotrichum orbiculare* and *Phytophthora capsici*.

Based on this study, Thai plant species including Aloe, Neem tree, Sabah snake grass, Wan nang kham, Ueang sae hom, Nom raatchasee, Globba, Kluai Chang, Kluai Namwa, Kluai Nom Sao, Kluai Sai Nam Phueng, Kluai Sao Kratuep Ho, Wan khao phansa and Oyster Lily have proven to be invaluable reservoirs of interesting endophytic actinomycetes producing various interesting biologically active compounds. Presently, whole genome sequences analysis is easily carried out and numerous biosynthetic gene clusters of functional metabolites have been identified; hence, the integration of genome analysis and the new screening method such as physicochemical screening by LC/MS might be helpful to obtain some useful secondary bioactive compounds from these bio-resources.

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APPENDICES

APPENDIX A

Culture media

Yeast extract-Malt extract agar (ISP 2 medium)

Yeast extract	0.4	g
Malt extract	1.0	g
Glucose	0.4	g
Agar	1.8	g
Distilled water	100	mL
pH 7.2-7.4		

Oatmeal agar (ISP 3 medium)

Oatmeal	2.0	g
Trace salt solution	0.1	mL
Agar	1.8	g
Distilled water	100	mL
pH 7.2-7.4		

Inorganic salts-starch agar (ISP 4 medium)

Soluble starch	1.0	g
K_2HPO_4	0.1	g
$MgSO_4 \cdot 7H_2O$	0.1	g
NaCl	0.1	g
$(NH_4)_2SO_4$	0.2	g
$CaCO_3$	0.2	g
Trace salt solution	0.1	mL
Agar	1.8	g

Distilled water	100	mL
pH 7.2-7.4		

Glycerol-asparagine agar (ISP 5 medium)

L-asparagine	0.1	g
Glycerol	1.0	g
K_2HPO_4	0.1	g
Trace salt solution	0.1	mL
Agar	1.8	g
Distilled water	100	mL
pH 7.2-7.4		

Peptone-yeast extract iron agar (ISP 6 medium)

Bacto-peptone iron agar, dehydrate (Difco)	3.6	g
Yeast extract	0.1	g
Distilled water	100	mL
pH 7.2-7.4		

Tyrosine agar (ISP 7 medium)

Glycerol	1.5	g
L-asparagine	0.05	g
K_2HPO_4	0.1	g
$MgSO_4 \cdot 7H_2O$	0.05	g
NaCl	0.05	g
$FeSO_4 \cdot 7H_2O$	0.001	g
Trace salt solution	0.1	mL

Agar	2.0	g
Distilled water	100	mL
pH 7.2-7.4		

Carbon utilisation based-medium (ISP 9 medium)

$(NH_4)_2SO_4$	0.264	g
KH_2PO4	0.238	g
K_2HPO_4	0.565	g
$MgSO_4 \cdot 7H_2O$	0.1	g
Solution*	0.1	mL
Agar	2.0	g
Distilled water	100	mL
pH 7.2-7.4		

*Solution contained 0.64 g $CuSO_4 \cdot 5H_2O$, 0.11 g $FeSO_4 \cdot 7H_2O$, 0.79 g $MnCl_2 \cdot 4H_2O$ and 0.15 g $ZnSO_4 \cdot 7H_2O$ per 100 mL

Nutrient agar (NA)

Meat extract	1.0	g
Peptone	1.0	g
Agar	1.5	g
Distilled water	100	mL
pH 7.2-7.4		

Czapek solution agar

Sucrose	3.0	g
$NaNO_3$	0.2	g
K_2HPO_4	0.1	g

$MgSO_4 \cdot 7H_2O$	0.05	g
KCl	0.05	g
$FeSO_4 \cdot 7H_2O$	0.1	mg
Agar	1.5	g
Distilled water	100	mL
pH 7.2-7.4		

301 seed medium

Starch	2.4	g
Glucose	0.1	g
Peptone	0.3	g
Meat extract	0.3	g
Yeast extract	0.5	g
$CaCO_3$	0.4	g
Distilled water	100	mL
pH 7.0		

Medium 30

Starch	2.4	g
Glucose	0.1	g
Peptone	0.3	g
Meat extract	0.3	g
Yeast extract	0.5	g
$CaCO_3$	0.4	g
(4) Trace element*	5	mL/L
Tap water	100	mL
pH 7.0		

Medium 51

Glucose	0.5	g
Corn Steep powder	0.5	g
Oatmeal	1.0	g
Pharmamedia	1.0	g
K_2HPO_4	0.5	g
$MgSO_4 \cdot 7H_2O$	0.5	g
(4) Trace element*	1	mL/L
Tap water	100	mL

pH 7.0

*(4) Trace metal solution contained each 0.1 % (w/v) of $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$.

Medium 57

Glucose	2.0	g
Peptone	0.5	g
Dry yeast	0.3	g
Meat extract	0.5	g
NaCl	0.5	g
$CaCO_3$	0.3	g
Tap water	100	mL

pH 7.0

Medium P1

Sucrose	2.0	g
Cellulose powder**	0.5	g
V8 juice (Campbell)	5 (%v/v)	

Yeast extract	0.5	g
Peptone	0.3	g
$CaCO_3$	0.4	g
$Na_2SiO_3 \cdot 9H_2O$	0.1	g
Tap water	95	mL

pH 7.0

APPENDIX B

Reagents and buffers

DON reagent

2, 7-Dihydroxynaphthalene	10	mg
Conc. H_2SO_4	50	mL

Dissolve 2, 7-dihydroxynaphthalene (DON) in conc. H_2SO_4 and then keep the solution in the darkness until the yellow solution to colourless (24 h) before used. This solution should be freshly prepared.

Reagents for fatty acids extraction

Saponification reagent

NaOH	15	g
Methanol (HPLC grade)	50	mL
Milli-Q water	50	mL

Dissolve NaOH in Milli-Q water and add methanol.

Methylation reagent

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

Adjust pH to below 1.5.

Extraction reagent

n-Hexane (LC grade)	50	mL
Methyl-3-butyl ether (LC grade)	50	mL

Base washing reagent

NaOH	1.2	g
Milli-Q water	100	mL

Saturated sodium chloride solution

NaCl saturated in Milli-Q water

Reagents for the analysis of polar lipids

Anisaldehyde reagent

Ethanol	90	mL
Conc. H_2SO_4	5.0	mL
p-Anisaldehyde	5.0	mL
Acetic acid	1.0	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 detection, mix each 10 mL of solution A, solution B and acetic acid.

Phosphomolybdic acid reagent

Phosphomolybdic acid	5.0	g
Absolute ethanol	100	mL

Molybdenum blue reagent

Solution A

MoO_3	4.011	g
25N H_2SO_4	100	mL

Dissolve MoO_3 with 25N H_2SO_4 and heat.

Solution B

Molybdenum powder	0.178 g
Solution A	50 mL

Add the molybdenum powder into solution A and heat at 100 °C for 15 min. After cooling, remove the precipitation by decantation. Mix each 50 mL of solution A, solution B and distilled water before spraying.

Saline-EDTA (pH 8.0)

0.15 M NaCl	(NaCl: 8.77 g)
0.1 M EDTA	(EDTA·2Na·2H_2O: 37.22 g)
	(Distilled water: 900 mL)
	(Adjust pH to 8.0 with conc. HCl)
	(Fill up to 1000 mL)

Tris-SDS (pH 9.0)

0.1 M Tris-HCl (pH 9.0)	(Tris: 12.11 g, distilled water: 700 mL)
	(Adjust pH to 9.0 with conc. HCl)
1% (w/v) SDS	(Sodium dodecyl sulfate: 10.0 g)
	(Fill up to 1000 mL)

10x Sodium chloride sodium citrate solution

1.5 M NaCl	(NaCl: 87.66 g)
0.15 M Trisodium citrate	(Citrate·3Na·2H_2O: 44.12 g)
	(Distilled water: 700 mL)
	(Adjust pH to 7.0±0.2 with conc. HCl)
	(Fill up to 1000 mL)

APPENDIX C

Polar lipids chromatograms of the novel endophytic actinomycete species and the *gyrB* gene phylogenies of the new *Micromonospora* species

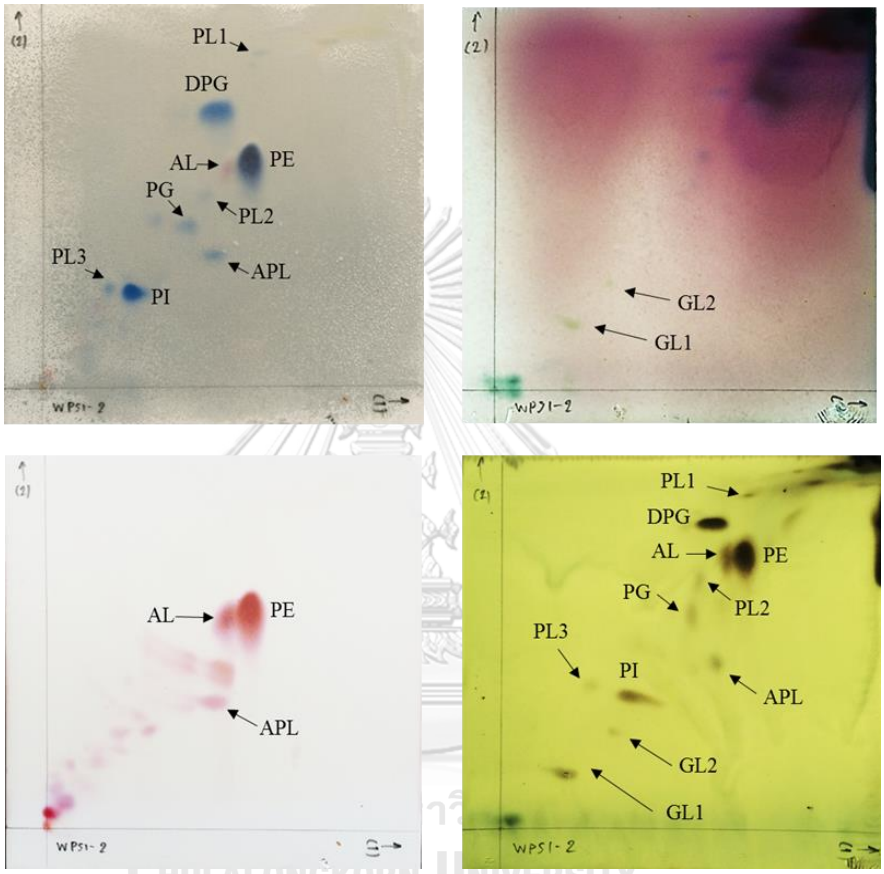

Figure C1 Polar lipid profiles of *Micromonospora globbae* WPS1-2^{T}

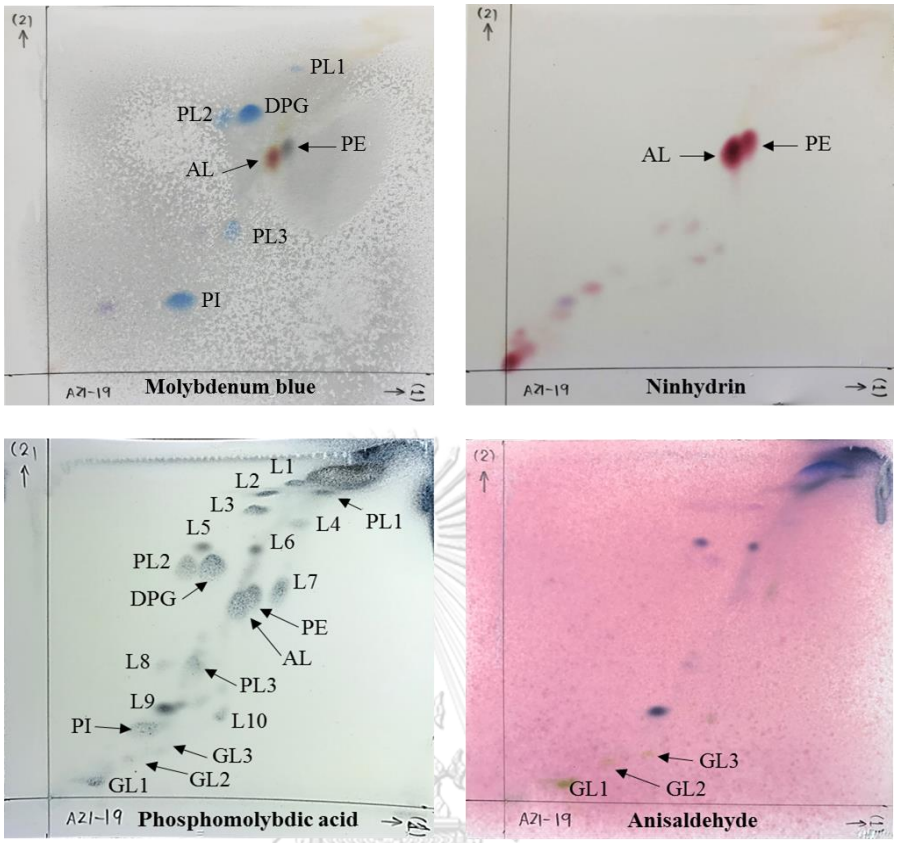

Figure C2 Polar lipid profiles of *Micromonospora azadirachtae* AZ1-19^{T}

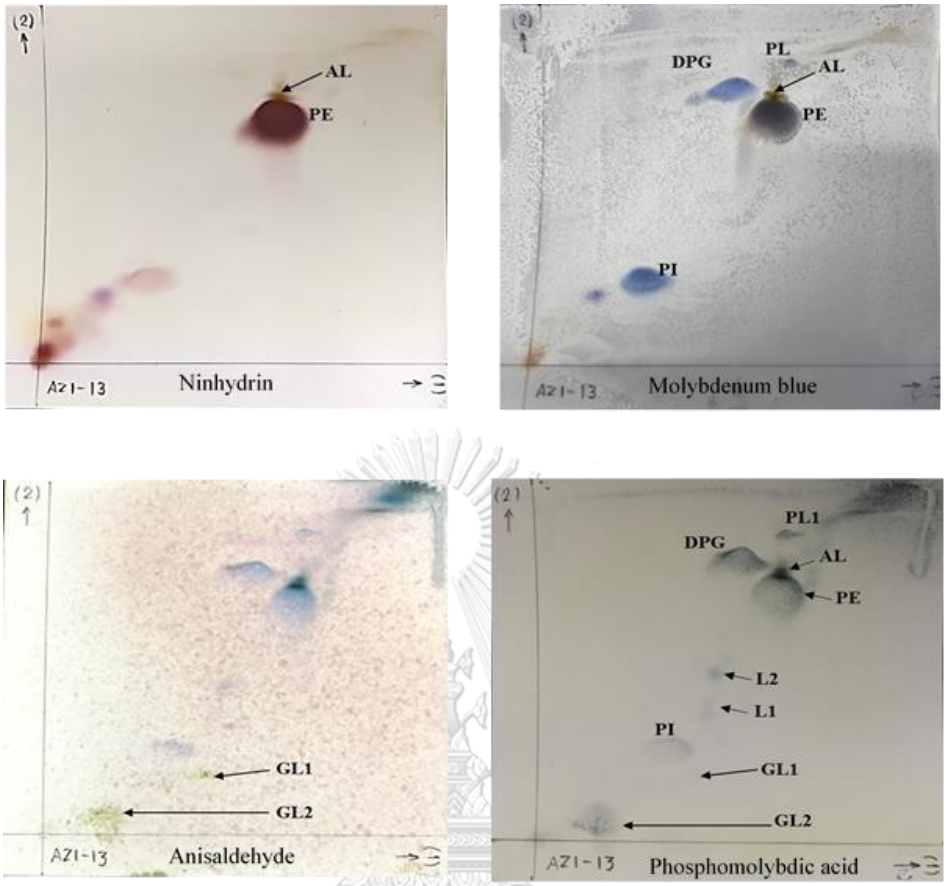

Figure C3 Polar lipid profiles of *Micromonospora phytorhiza* AZ1-13^{T}

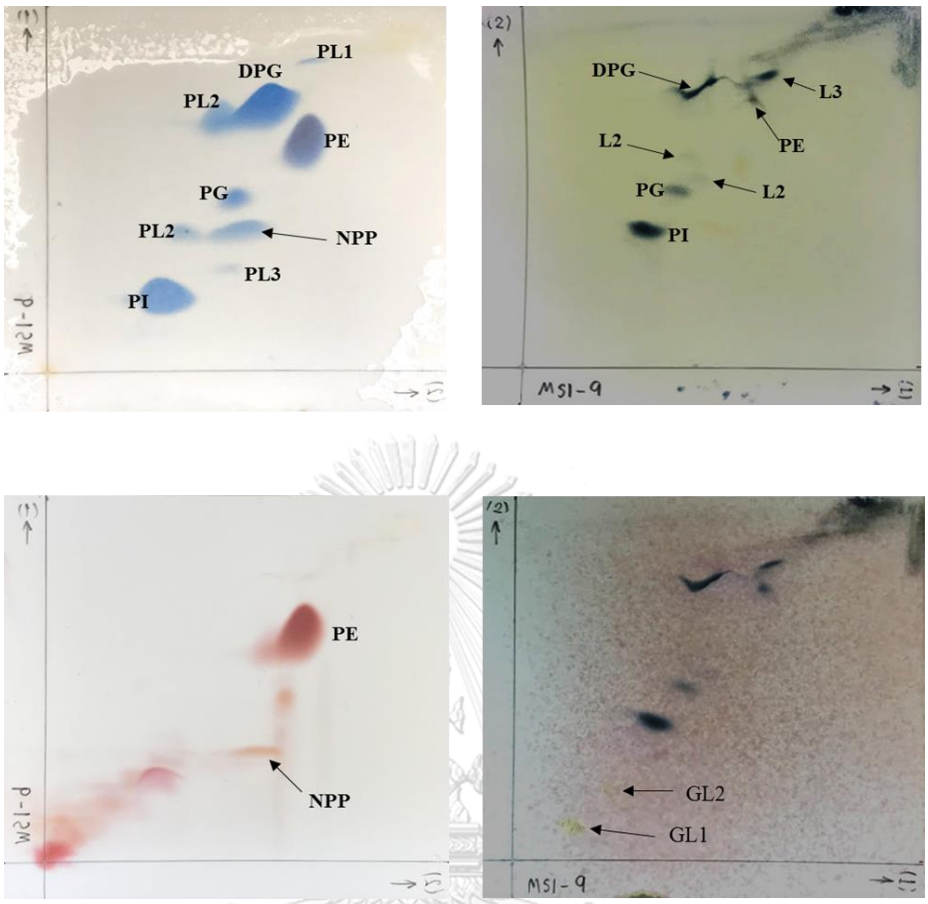

Figure C4 Polar lipid profiles of *Micromonospora musae* MS1-9^{T}

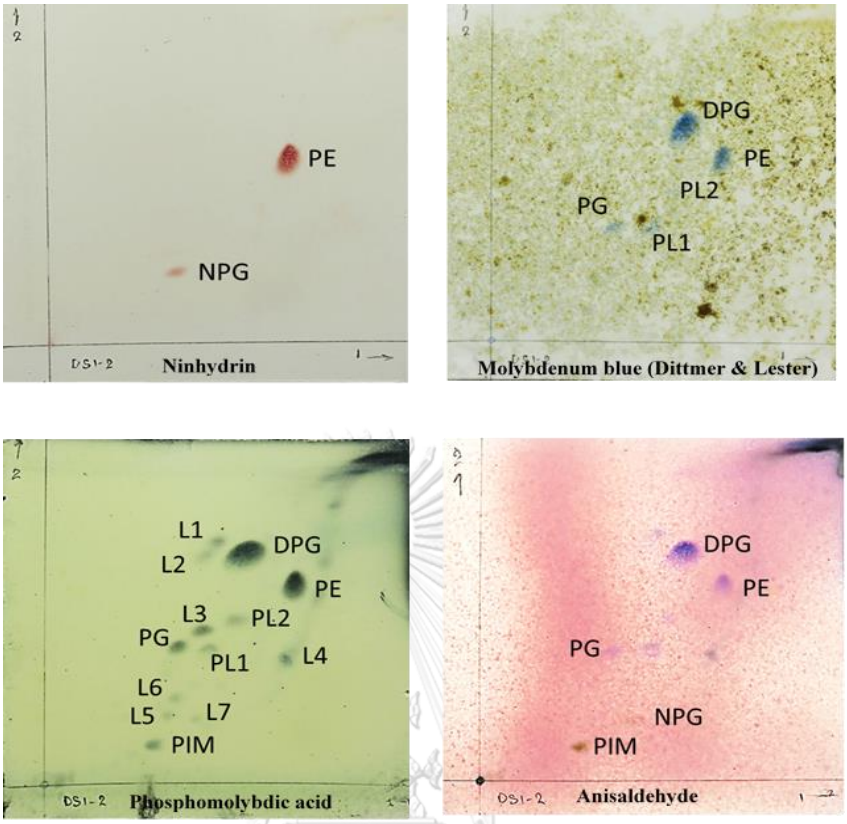

Figure C5 Polar lipid profiles of *Streptomyces endoradicus* DS1-2^{T}

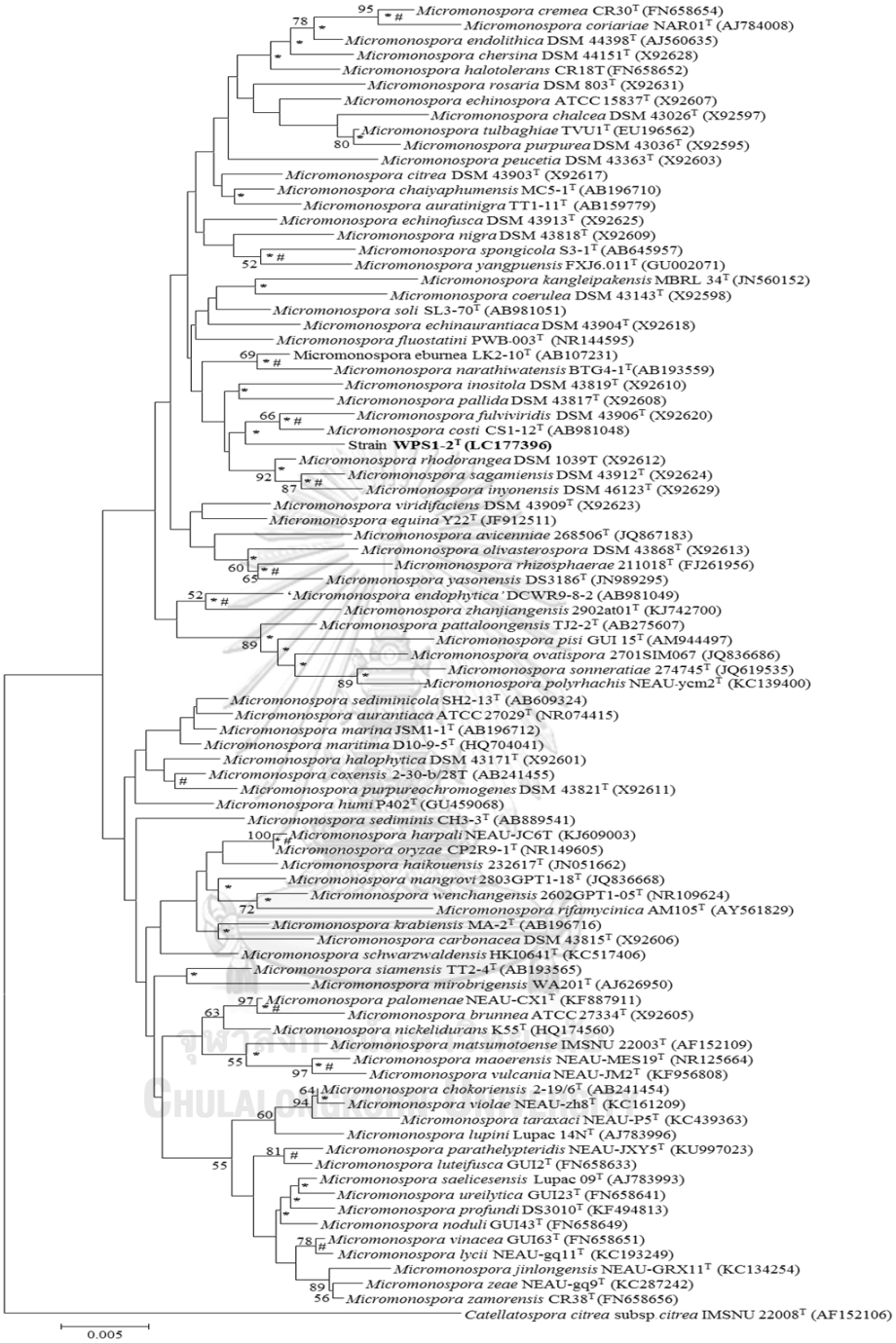

Figure C6 Extended version of phylogenetic relationships based on neighbour-joining analysis of 16S rRNA gene sequences of strain WPS1-2^T and related *Micromonospora* species. *Catellatospora citrea* subsp. *citrea* IMSNU 22008^T was used as an outgroup. Asterisk (*, #) indicated the branches which were recovered in the maximum-likelihood and maximum-parsimony, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown). Bar, 0.005 substitutions per nucleotide position.

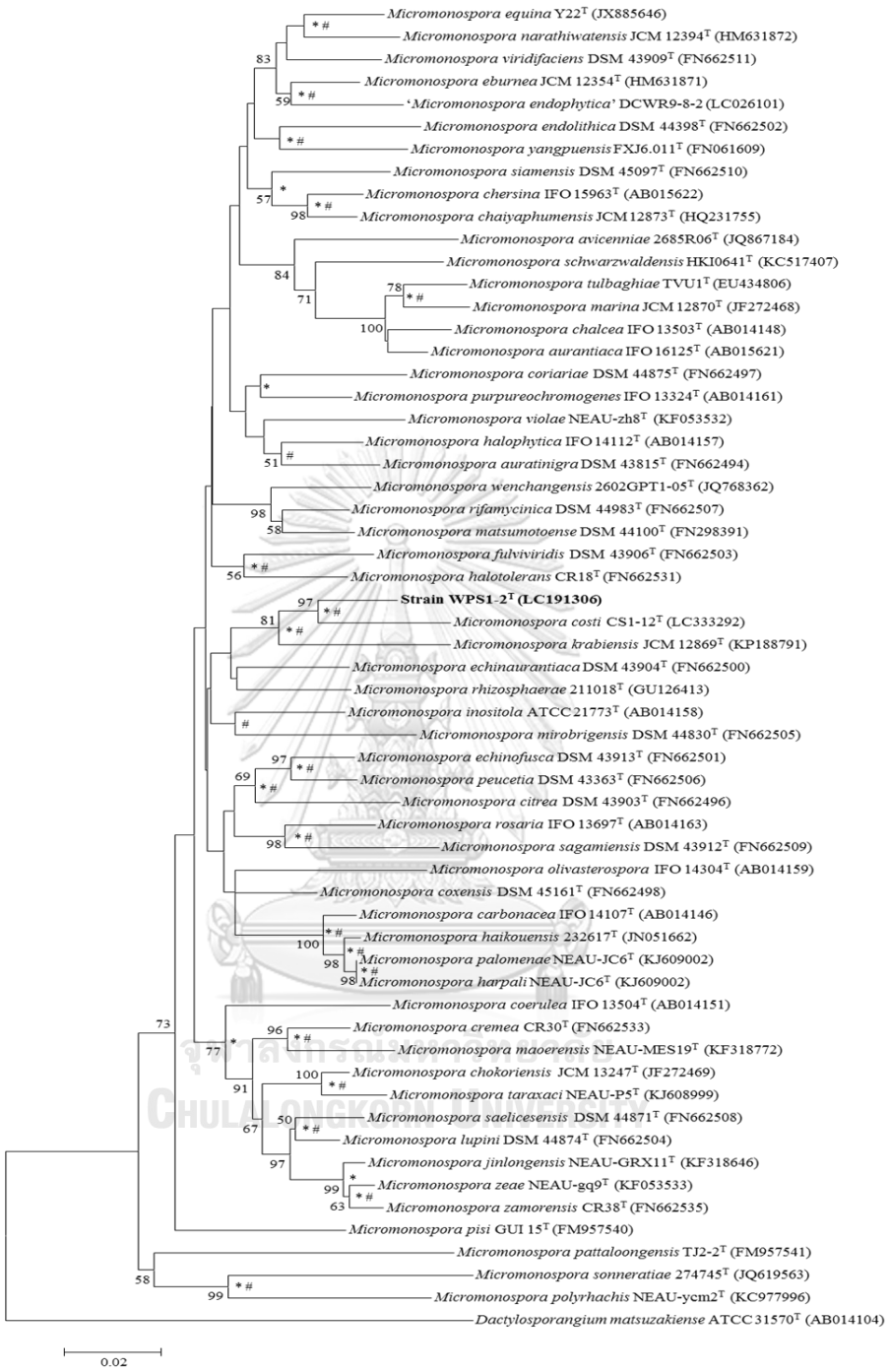

Figure C7 Phylogenetic relationships based on neighbour-joining analysis of *gyrB* gene sequences (1117 nt) of strain WPS1-2^T and closely related species of the genus *Micromonospora*. *Dactylosporangium matsuzakiensis* ATCC 31570^T was used as an outgroup. Asterisk (*, #) indicated the branches were recovered in the maximum-likelihood and maximum-parsimony, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown). Bar, 0.02 substitutions per nucleotide position.

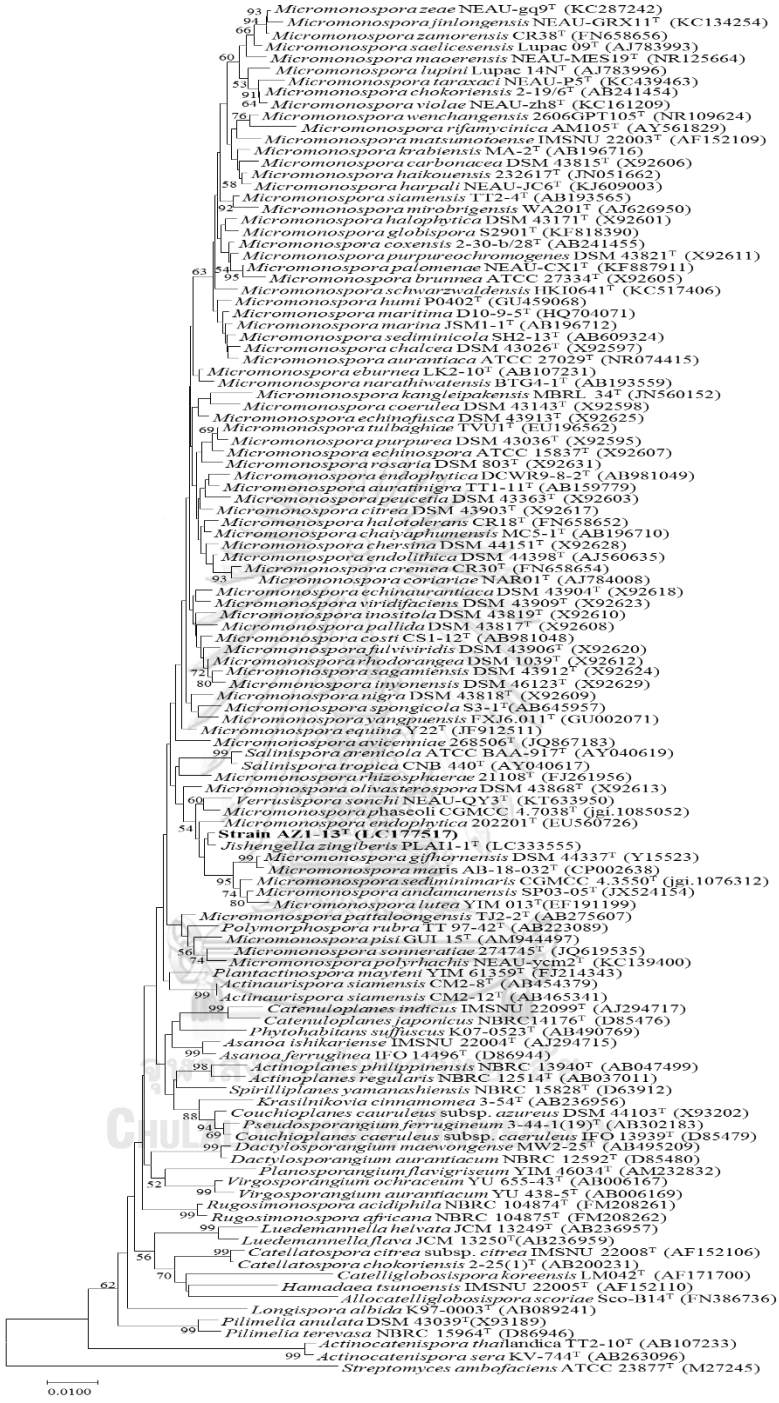

Figure C8 Extended phylogenetic tree based on neighbour-joining analysis of 16S rRNA gene sequences of strain AZ1-13^{T} and the valid type species of recognised genera of the family *Micromonosporaceae*. *Streptomyces ambofaciens* ATCC 23877^{T} was used as an outgroup. Asterisk (*, #) indicated the branches which were recovered in the ML and MP tree, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown at the node). Bar, 0.0100 substitutions per nucleotide position.

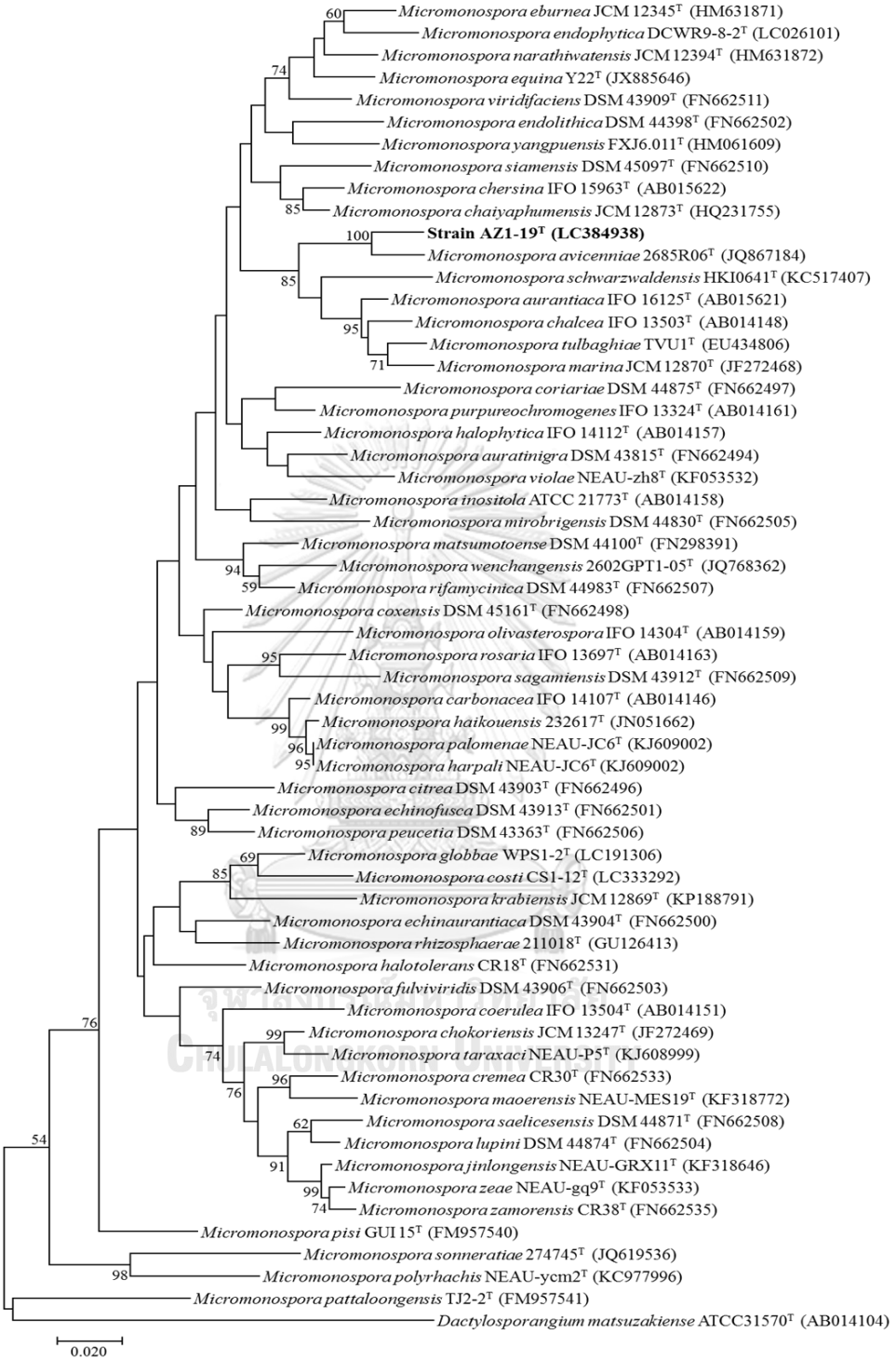

Figure C9 Phylogeny based on maximum-likelihood analysis of *gyrB* gene sequences of the strain AZ1-19^{T} and the closely related available *Micromonospora* species. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown at the node). Bar, 0.020 substitutions per nucleotide position.

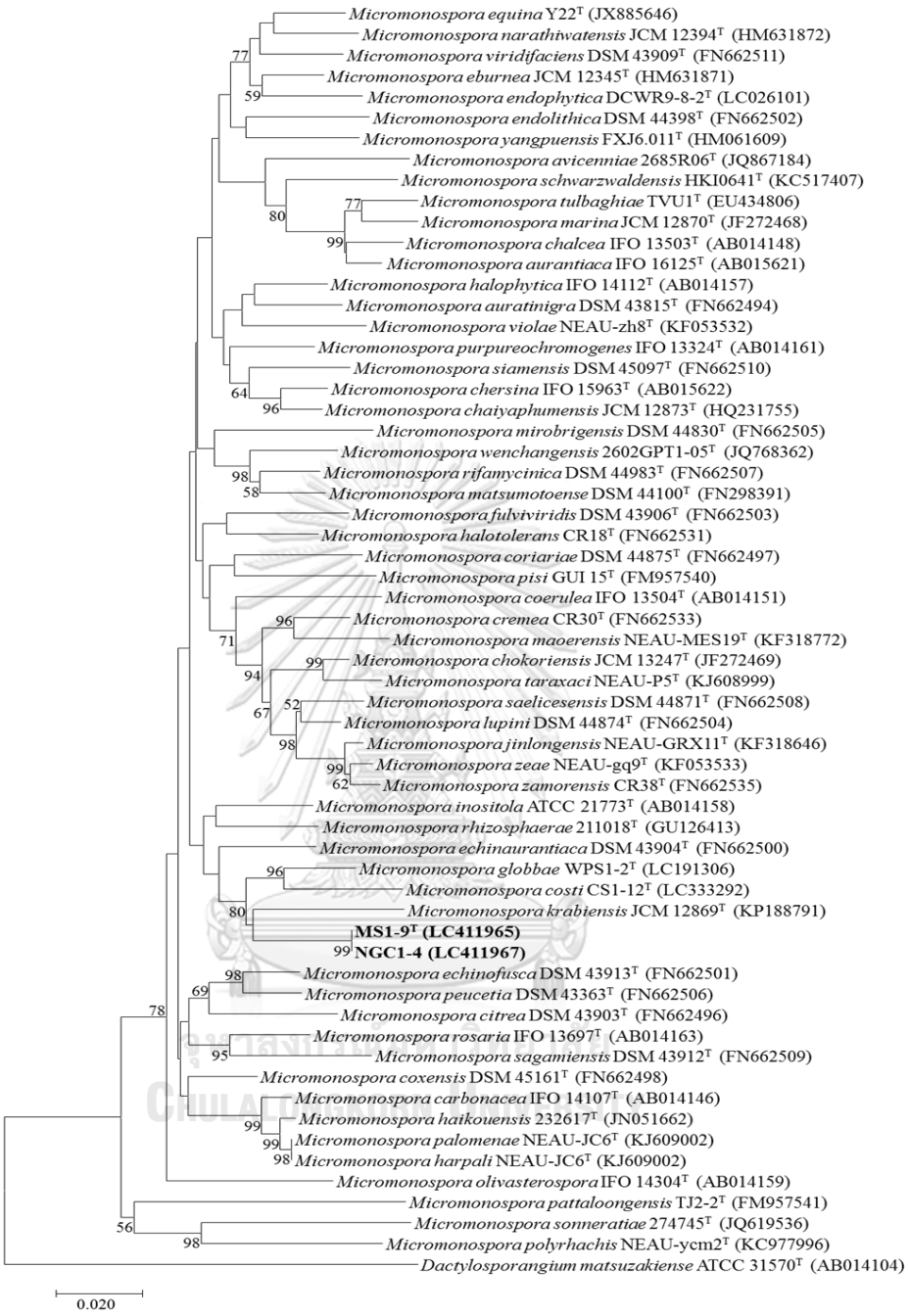

Figure C10 A neighbour-joining phylogenetic relationship based on *gyrB* gene sequences of strain MS1-9^{T} (1,120 nt) and NGC1-4 (1,060 nt) and their closest type strains. *Dactylosporangium matsuzakiense* ATCC 31570^{T} was used as an outgroup. Numbers at branch nodes indicate levels of bootstrap support (%) derived from 1000 replications (only value over 50% are shown at). Bar, 0.02 substitutions per nucleotide position.

APPENDIX D

NMR spectra of the isolated compounds

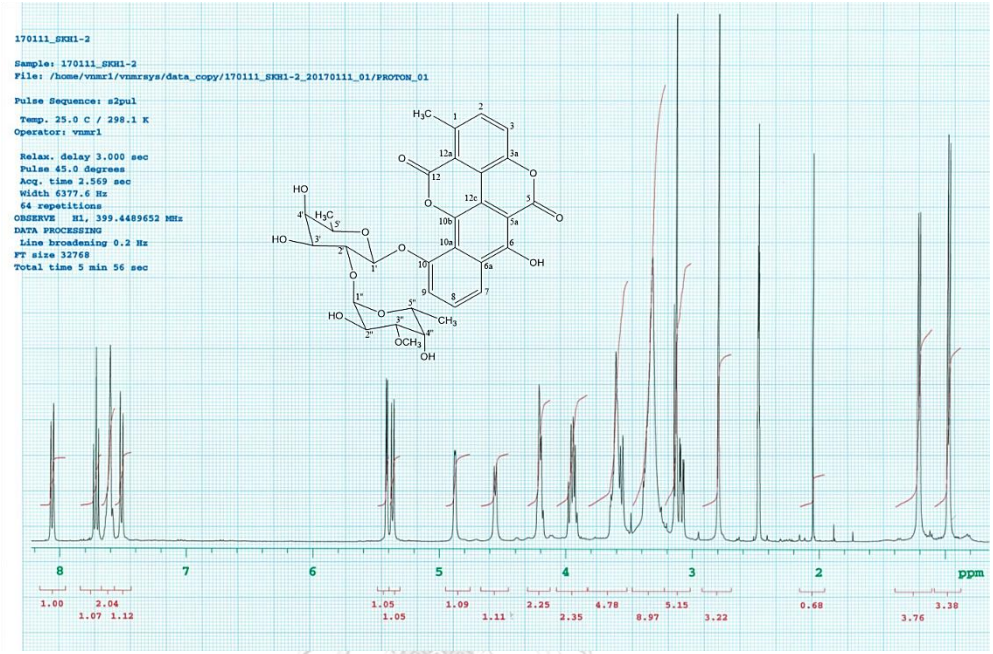

Figure D1 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of SKH9/1-1

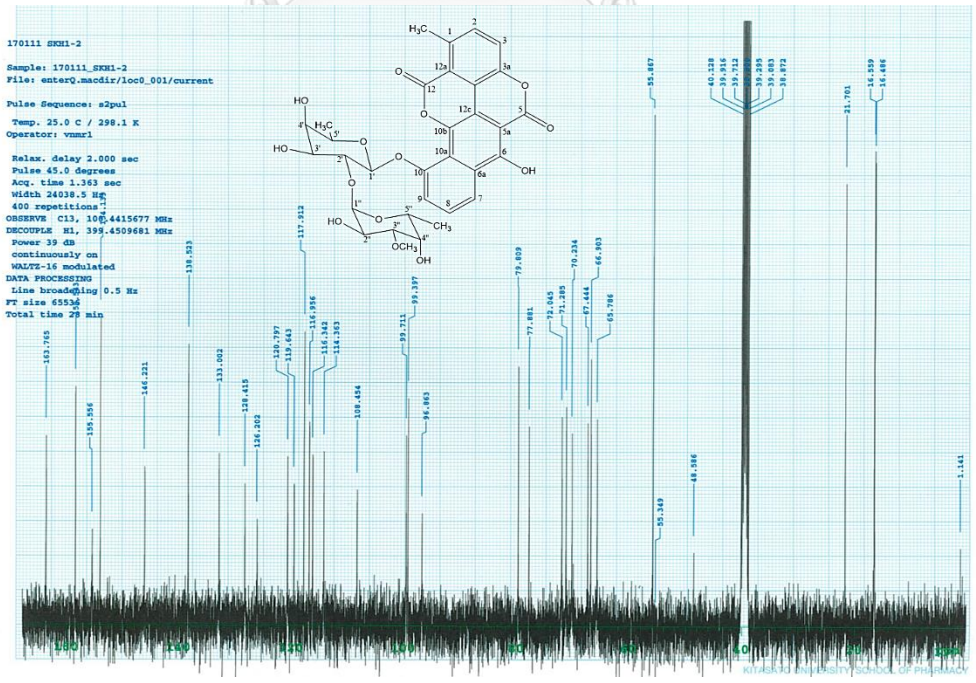

Figure D2 ^{13}C NMR spectrum (400 MHz, DMSO-d_6) of SKH9/1-1

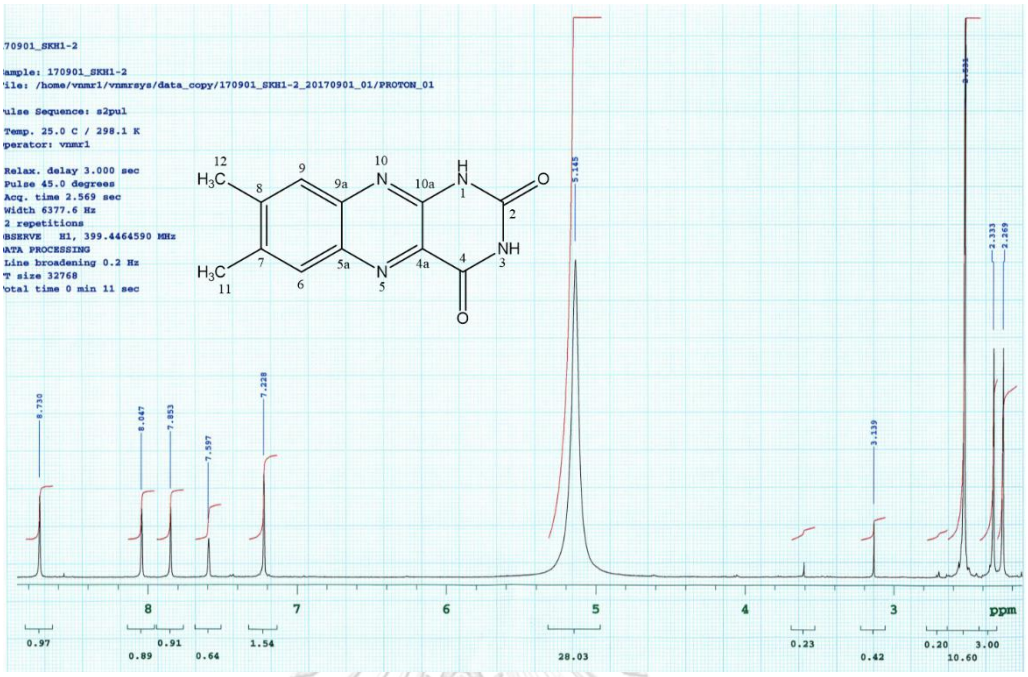

Figure D3 ^{1}H NMR spectrum (400 MHz, Pyridine-d_5) of SKH9/1-2

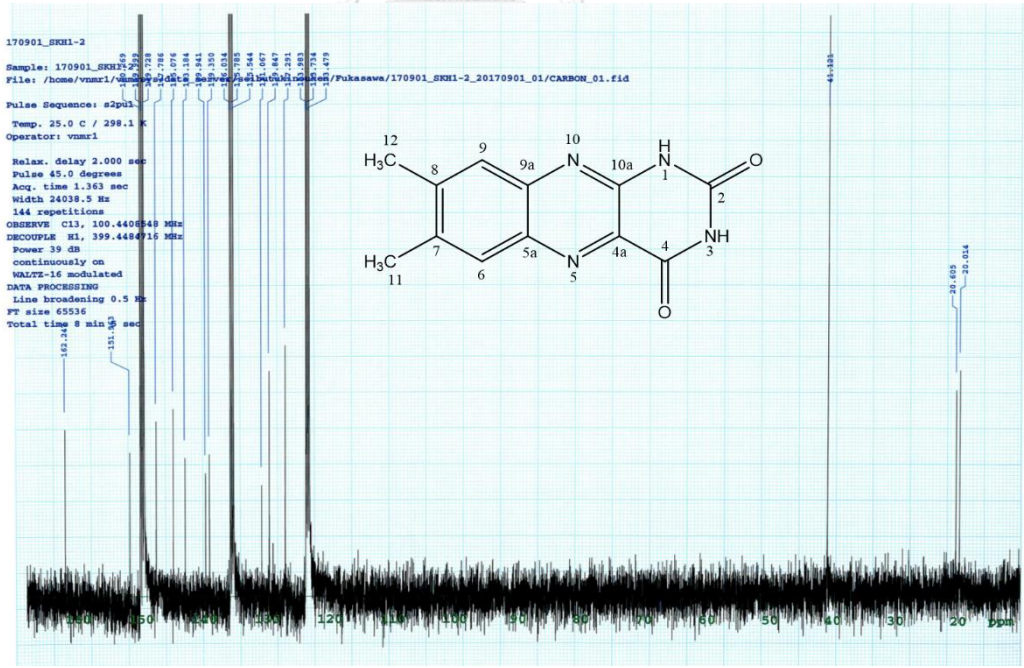

Figure D4 ^{13}C NMR spectrum (400 MHz, Pyridine-d_5) of SKH9/1-2

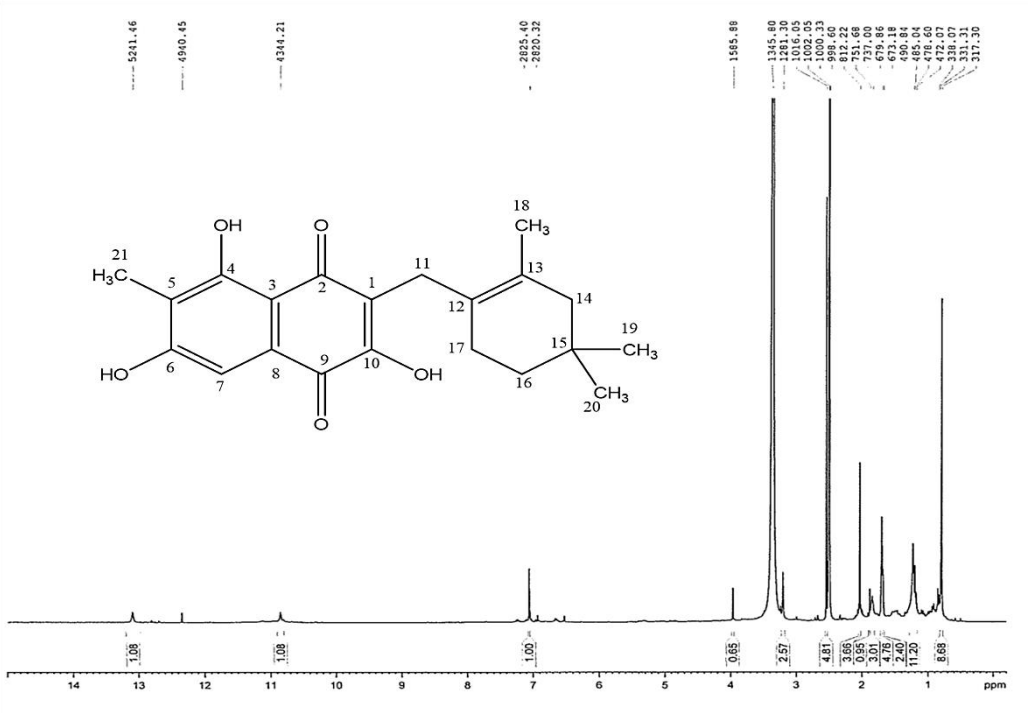

Figure D5 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of MS1-6-F5-H12

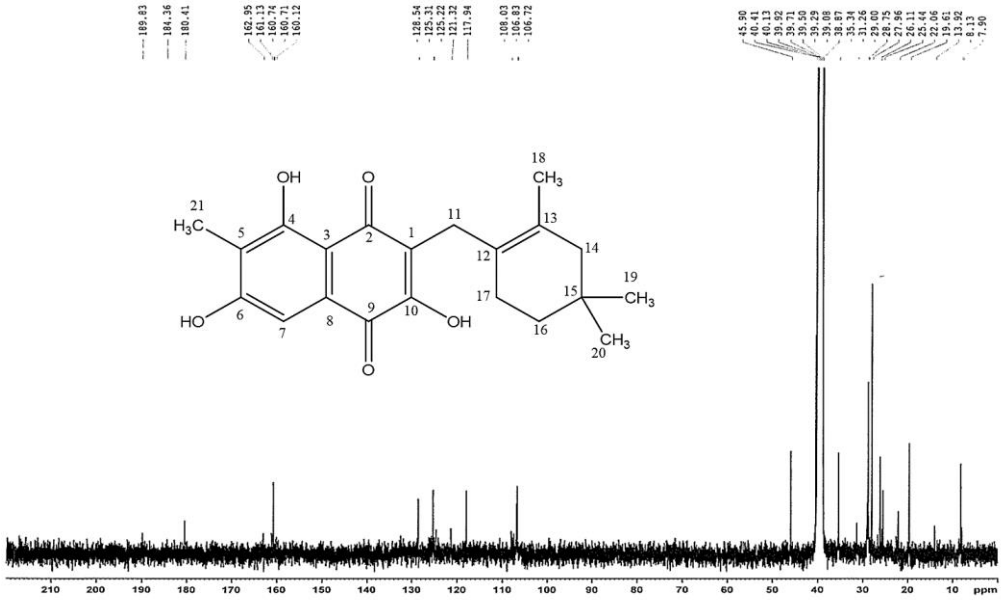

Figure D6 ^{13}C NMR spectrum (400 MHz, DMSO-d_6) of MS1-6-F5-H12

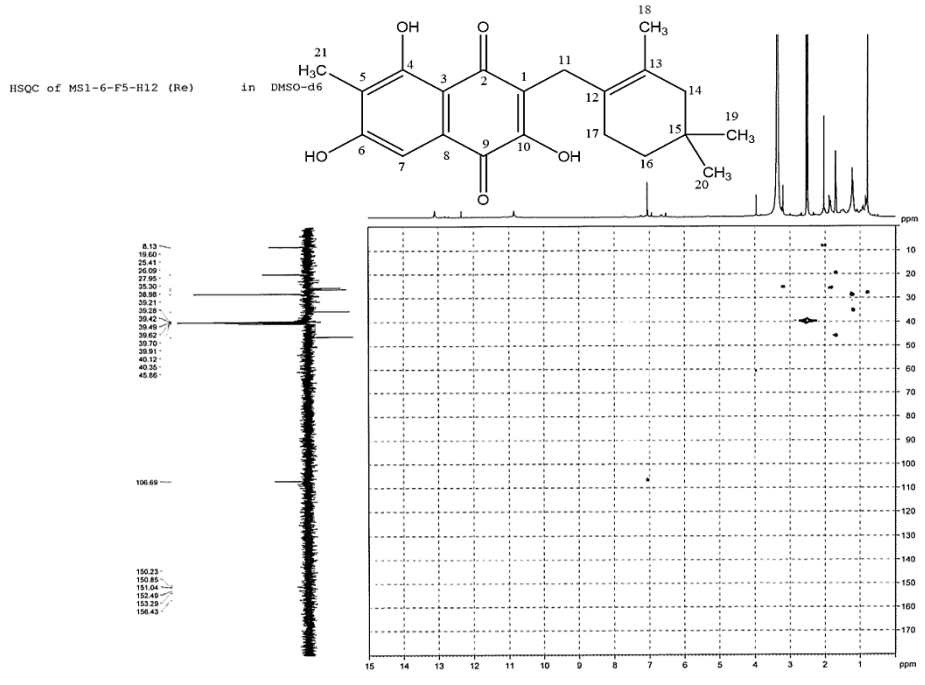

Figure D7 HSQC spectrum (400 MHz, DMSO-d_6) of MS1-6-F5-H12

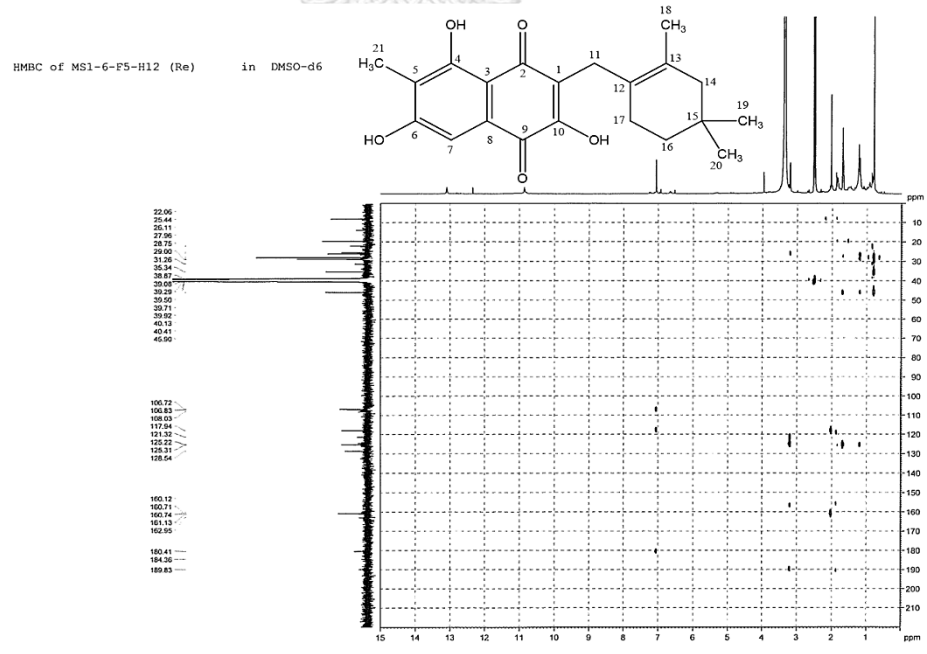

Figure D8 HMBC spectrum (400 MHz, DMSO-d_6) of MS1-6-F5-H12

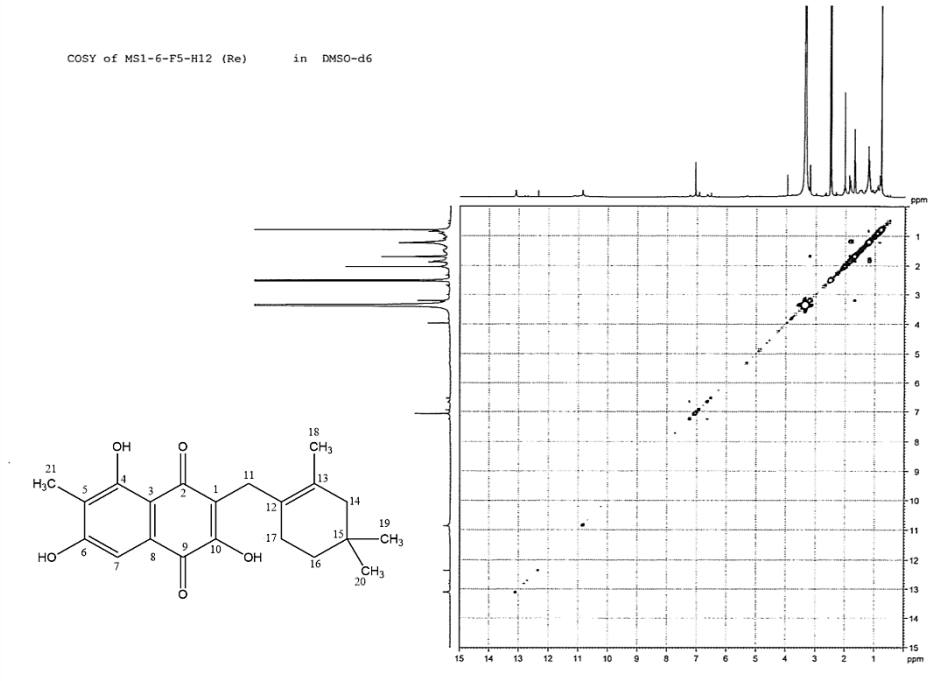

Figure D9 ^{1}H-^{1}H COSY NMR spectrum (400 MHz, DMSO-d_6) of MS1-6-F5-H12

Figure D10 ^{1}H NMR spectrum (400 MHz, Acetone-d_6) of MS1-6-F5-H13

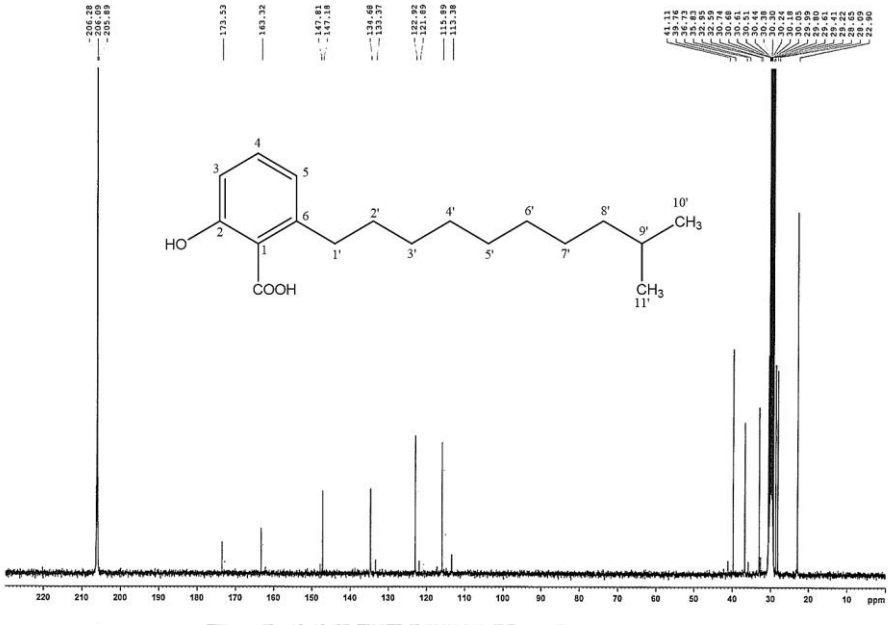

Figure D11 ^{13}C NMR spectrum (400 MHz, Acetone-d_6) of MS1-6-F5-H13

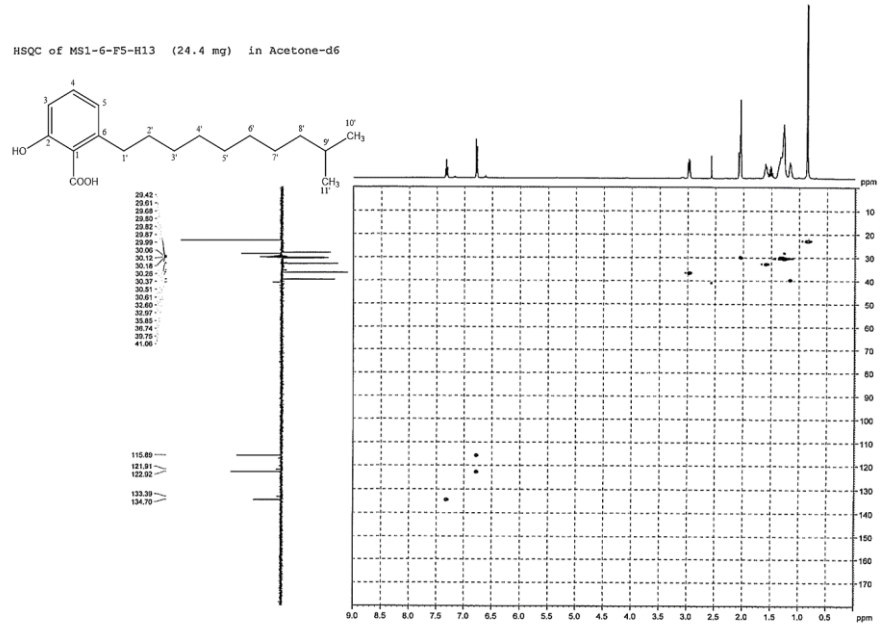

Figure D12 HSQC spectrum (400 MHz, Acetone-d_6) of MS1-6-F5-H13

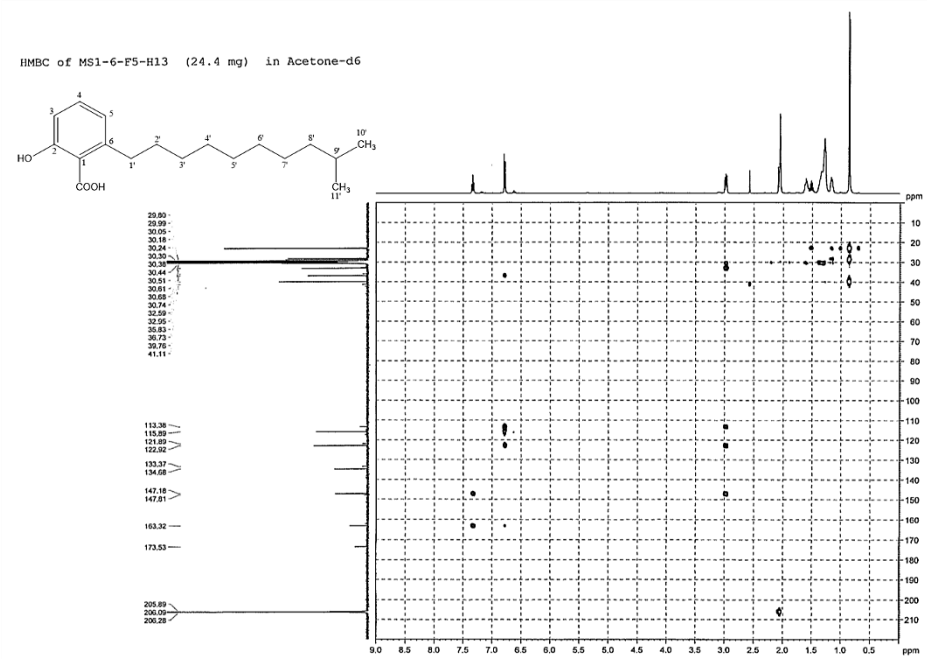

Figure D13 HMBC spectrum (400 MHz, Acetone-d_6) of MS1-6-F5-H13

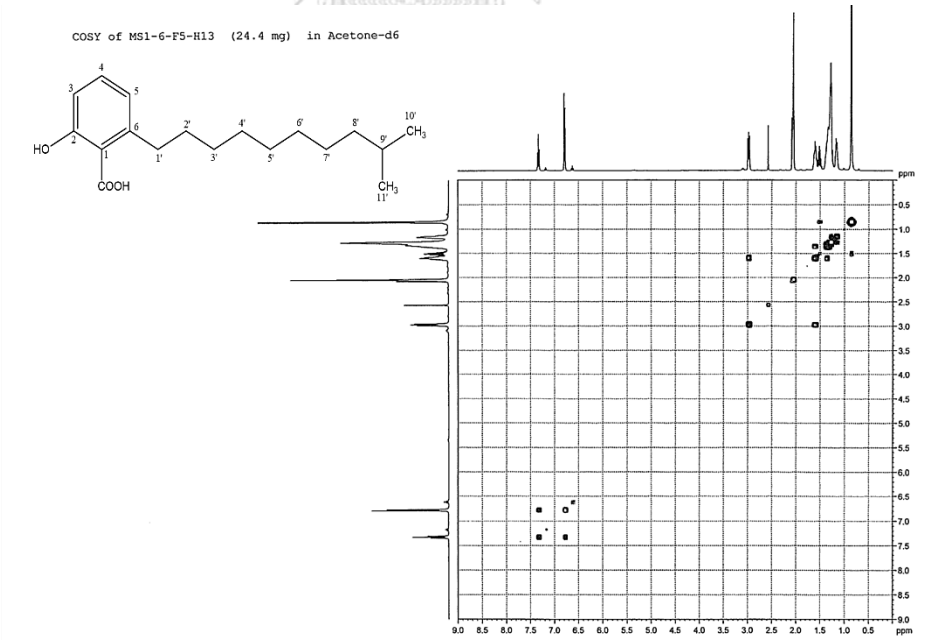

Figure D14 ^{1}H-^{1}H COSY NMR spectrum (400 MHz, Acetone-d_6) of MS1-6-F5-H13

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