BIODIVERSITY AND SECONDARY METABOLITES OF RARE ACTINOMYCETES FROM SOILS

Miss Apakorn Songsumanus

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutical Chemistry and Natural Products
Faculty of Pharmaceutical Sciences
Chulalongkorn University
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Thesis Title	BIODIVERSITY AND SECONDARY METABOLITES OF RARE ACTINOMYCETES FROM SOILS
By	Miss Apakorn Songsumanus
Field of Study	Pharmaceutical Chemistry and Natural Products
Thesis Advisor	Associate Professor Somboon Tanasupawat, Ph.D.
Thesis Co-advisor	Khanit Suwanborirux, Ph.D

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

…… Dean of the Faculty of

(Associate Professor Pintip Pongpech, Ph.D.) Pharmaceutical Sciences

THESIS COMMITTEE

…….. Chairman

(Associate Professor Nongluksna Sriubolmas, Ph.D.)

…….……. Thesis Advisor

(Associate Professor Somboon Tanasupawat, Ph.D.)

…….. Thesis Co-advisor

(Khanit Suwanborirux, Ph.D.)

…….. Examiner

(Associate Professor Pintip Pongpech, Ph.D.)

…….. External Examiner

(Assistant Professor Chitti Thawai, Ph.D.)

…….. External Examiner

(Prasat Kittakoop, Ph.D.)

อาภากร ทรงสุมนัส : ความหลากหลายทางชีวภาพและสารทุติยภูมิของแอคติโนมัยซีทส์ที่หายากจากดิน (BIODIVERSITY AND SECONDARY METABOLITES OF RARE ACTINOMYCETES FROM SOILS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สมบูรณ์ ธนาศุภวัฒน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ภก. ดร. คณิต สุวรรณบริรักษ์, 186 หน้า

แอคติโนมัยซีทส์ที่หายากจำนวน 49 ไอโซเลตคัดแยกได้จากดินจำนวน 19 ตัวอย่างซึ่งเก็บจากจังหวัด เลย ชลบุรี พะเยา ชัยภูมิ และสมุทรสาคร โดยใช้อาหารวุ้น humic vitamin และ starch casein nitrate จากการศึกษาลักษณะทางฟีโนไทป์ และอนุกรมวิธานเคมี รวมทั้งการวิเคราะห์ ความคล้ายคลึงของลำดับเบสในช่วง 16S rRNA gene และความคล้ายคลึงของ DNA ของสายพันธุ์ตัวแทน สามารถจำแนกแอคติโนมัยซีทส์ที่แยกได้ เป็น 6 สกุล คือ *Micromonospora* 38 สายพันธุ์ *Microbispora* 2 สายพันธุ์ *Actinomadura* 4 สายพันธุ์ *Nocardia* 3 สายพันธุ์ *Nonomuraea* และ*Pseudonocardia* สกุลละ 1 สายพันธุ์ สามารถจำแนก *Micromonospora* เป็น 12 กลุ่ม และพิสูจน์เอกลักษณ์กลุ่ม III, IV, V, IX, XI และ XII เป็น *M. marina, M. aurantiaca, M. chalcea, M. chokoriensis, M. tulbaghiae* และ *M. chersina* ตามลำดับ ส่วนสายพันธุ์ในกลุ่ม I, II, VI, VII, VIII และ X เป็นชนิดใหม่ และได้เสนอชื่อกลุ่ม VIII เป็น *Micromonospora humi* นอกจากนี้ยังสามารถจำแนกสายพันธุ์ในสกุล *Actinomadura* เป็น 4 กลุ่ม โดยพิสูจน์เอกลักษณ์ กลุ่ม B และ D เป็น *A. glauciflava* และ *A. nitritigenes* ตามลำดับ สายพันธุ์ในสกุล *Nocardia* เป็น 2 กลุ่มโดยกลุ่มที่ 2 เป็น *N. cyriacigeorgica* และสายพันธุ์ในสกุล *Pseudonocardia* เป็น *P. carboxydivorans* และพบว่าสายพันธุ์ที่เหลือในสกุล *Microbispora, Nonomuraea, Actinomadura* และ *Nocardia* เป็นชนิดใหม่

จากการคัดกรองสารทุติยภูมิของตัวแทนแอคติโนมัยซีทส์ที่หายากแต่ละกลุ่ม โดยอาศัยเทคนิค HPLC และการเปรียบเทียบสเปคตรัม UV กับฐานข้อมูล พบว่า *Micromonospora* สายพันธุ์ AL7-5 ในกลุ่ม V ผลิตสาร rakicidin และ BU-4664L เมื่อทำการสกัดสารจากน้ำหมักเชื้อ และวิเคราะห์โครงสร้างทางเคมีด้วยการเปรียบเทียบข้อมูล NMR และ MS พบว่า *Micromonospora* สายพันธุ์ D10-9-5 ในกลุ่ม II ผลิตสาร SEK34 และ SEK34B ในอาหารสูตร A11M และ *Actinomadura* สายพันธุ์ CYP1-5 ในกลุ่ม A ผลิตสาร decatromicin B และ BE-45722C ในอาหารสูตร A3M โดยสาร decatromicin B และ BE-45722C แสดงฤทธิ์ ต้านเชื้อ *B. subtilis* ที่ MIC 0.78 และ 0.39 µg/ml ฤทธิ์ต้านเชื้อมาลาเรีย *Plasmodium falciparum* K1 ที่ IC_{50} 2.07 และ 4.76 µg/ml และฤทธิ์เป็นพิษต่อ Vero cell line ที่ IC_{50} 47.07 และ 32.71 µg/ml ตามลำดับ ส่วนสาร SEK34 และ SEK34B ไม่แสดงฤทธิ์ทางชีวภาพดังกล่าว

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ ลายมือชื่อนิสิต.....

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ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS: RARE ACTINOMECETES / *MICROMONOSPORA* / *MICROBISPORA* / *ACTINOMADURA* / *NOCARDIA* / *NONOMURAEA* / *PSEUDONOCARDIA*

APAKORN SONGSUMANUS: BIODIVERSITY AND SECONDARY METABOLITES OF RARE ACTINOMYCETES FROM SOILS. ADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: KHANIT SUWANBORIRUX, Ph.D., 186 pp.

Isolation of 49 strains of rare actinomycetes from 19 soil samples collected from Loei, Chonburi, Phayao, Chaiyaphum and Samutsakhon provinces was carried out using humic vitamin agar and starch casein nitrate agar. On the basis of their phenotypic and chemotaxonomic properties including the phylogenetic analysis using 16S rRNA gene sequence similarity and level of DNA-DNA relatedness of the representative strains, the isolated actinomycetes were classified into six genera, including *Micromonospora* (38 strains), *Microbispora* (2 strains), *Actinomadura* (4 strains), *Nocardia* (3 strains), *Nonomuraea* (1 strain) and *Pseudonocardia* (1 strain). All *Micromonospora* strains were divided into 12 groups. Groups III, IV, V, IX, XI and XII were identified as *M. marina, M. aurantiaca, M. chalcea, M. chokoriensis, M. tulbaghiae* and *M. chersina,* respectively while Groups I, II, VI, VII, VIII and X as new species. *Micromonospora humi* sp. nov. was proposed for the new species of Group VIII. Furthermore, *Actinomadura* strains were divided into 4 groups and Groups B and D were identified as *A. glauciflava* and *A. nitritigenes,* respectively. *Nocardia* strains were divided into 2 groups and Group 2 was *N. cyriacigeorgica.* The strain of *Pseudonocardia* was identified as *P. carboxydivorans*. The remaining strains of the genera *Microbispora, Nonomuraea, Actinomadura* and *Nocardia* were identified as new species.

In the course of secondary metabolites screening from selected rare actinomycetes using HPLC-UV and comparing the UV spectra with in-house database, compounds rakicidin and BU-4664L were detected from *Micromonospora* sp. AL7-5 of Group V. Isolation of secondary metabolites from cultured broths and chemical structure elucidation by comparison of NMR and MS spectral data revealed that *Micromonospora* sp. D10-9-5 of Group II produced compounds SEK34 and SEK34B in A11M culture medium and *Actinomadura* sp. CYP1-5 of Group A produced compounds decatromicin B and BE-45722C in A3M culture medium. Decatromicin B and BE-45722C exhibited anti-*Bacillus subtilis* with MICs 0.78 and 0.39 µg/ml, antimalarial activity against *Plasmodium falciparum* K1 with IC_{50}s 2.07 and 4.76 µg/ml and cytotoxicity against Vero cell line with IC_{50}s 47.07 and 32.71µg/ml, respectively while SEK34 and SEK34B were inactive.

Field of Study : Pharmaceutical Chemistry and Natural Products

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Student's Signature .....

Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVATIONS

a	=	Anteiso
ATCC	=	American Type Culture Collection, Maryland, U.S.A.
$Ba(OH)_2$	=	Bariumhydroxide
BSA	=	Bovine serum albumin
cm	=	Centimeter
Cz	=	Czapek's sucrose
°C	=	Degree celsius
Ca^{2+}	=	Calcium ion
CH_3OH	=	Methanol
CD_3OD	=	Deuterated methanol
CH_3CN	=	Acetonitrile
$CHCl_3$	=	Chloroform
C_5H_5N	=	Pyridine
CH_3COCH_3	=	Acetone
COSY	=	Correlation spectroscopy
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
δ	=	Chemical shift
d	=	Doublet
dd	=	Doublet of doublet
DAP	=	Diaminopimelic acid
DAB	=	Diaminobutyric acid
DDBJ	=	DNA Data Bank of Japan
DMSO	=	Dimethylsulfoxide
DMSO *d6*	=	Deuterated dimethylsulfoxide
DNase	=	Deoxyribonuclease
dNTP	=	Deoxyribonucleotide triphosphate
DNA	=	Deoxyribonucleic acid
rDNA	=	Ribosomal deoxyribonucleic acid
DPG	=	Diphosphatidylglycerol
DON	=	2,7-Dihydroxynapthalene

DSM	=	Deutsche Sammlung von Mikroorganismen
EDTA	=	Disodiumethylenediaminetetraacetate
Ex/Em	=	Excitation and emission wavelengths
EMBL	=	European Molecular Biology Laboratory
FAME	=	Fatty acid methyl ester
FAB MS	=	Fast atom bombardment mass spectrometry
Fe^{2+}	=	Iron ion
g	=	Gram
µg	=	Microgram
mg	=	Milligram
g	=	Gravity
G+C	=	Guanine-plus-cytosine
GenBank	=	National Institute of Health genetic sequence database
Gly. A.	=	Glycerol-asparagine agar
Glu. A.	=	Glucose-asparagine agar
h	=	Hour
HV	=	Humic-vitamin agar
HCl	=	Hydrochloric acid
HCOOH	=	Formic acid
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
^{1}H-NMR	=	Proton nuclear magnetic resonance
H_2O	=	Water
H_2O_2	=	Hydrogen peroxide
HPLC	=	High performance liquid chromatography
HPTLC	=	High performance thin layer chromatography
HSQC	=	Heteronuclear single quantum coherence
H_2S	=	Hydrogen sulphide
H_2SO_4	=	Sulfuric acid
Hz	=	Hertz
i	=	Iso
IC	=	Inhibitory concentration
I.S.	=	Inorganic salts-starch agar
ISP	=	International Streptomyces Project

J	=	Coupling constant
JCM	=	Japan Collection of Microorganisms
K_2HPO_4	=	Potassium phosphate
KNO_3	=	Potassium nitrate
KOH	=	Potassium hydroxide
L	=	Liter
µl	=	Microliter
m	=	Multiplet
mm	=	Millimeter
µm	=	Micrometer
mm^3	=	Cubic millimeter
ml	=	Milliliter
mM	=	Millimole
M	=	Molar
Max	=	Maximum
MEGA	=	Molecular Evolutionary Genetics Analysis
MeOH	=	Methanol
MK	=	Menaquinone
meso-DAP	=	*meso*-Diaminopimelic acid
min	=	Minute
MIC	=	Minimum Inhibitory Concentration
MW	=	Molecular weight
Mg^{2+}	=	Magnesium ion
m/z	=	Mass to charge ratio
MS	=	Mass spectrometry
Mn^{2+}	=	Manganese ion
Methyl-PE	=	Methylphosphatidylethanolamine
nt	=	nucleotide
N	=	Normal
N.A.	=	Nutrient agar
Na^+	=	Sodium ion
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide

NBRC	=	NITE Biological Resource Center
NPG	=	Ninhydrin-positive glycophospholipids
nm	=	Nanometer
nov.	=	Novel
OD	=	Optical density
ON	=	Oatmeal nitrate agar
O.M.	=	Oatmeal agar
OH-PE	=	Hydroxyphosphatidylethanolamine
%	=	Percent
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PC	=	phosphatidylcholine
PE	=	Phosphatidylethanolamine
lyso-PE	=	lyso-phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PI	=	Phosphatidylinositol
P.I.A.	=	Peptone-yeast extract iron agar
ppm	=	Part per million
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
s	=	Singlet
sec	=	Second
SEM	=	Scanning electron microscope
SDS	=	Sodium dodecyl sulfate
sp.	=	Species
SSC	=	Standard sodium citrate
T.A.	=	Tyrosine agar
TAE	=	Tris-acetate EDTA
Tm	=	Melting temperature
TBE	=	Tris-borate EDTA
TLC	=	Thin layer chromatography
UV	=	Ultraviolet

Y.M. = Yeast extract-malt extract agar

CHAPTER I

INTRODUCTION

The actinomycetes are Gram-positive bacteria which have the formation of branching filaments but distinguish from fungi by having no cell nucleus and single hypha (0.4 to 1.2 μm in diameter) smaller than fungi (3 to 8 μm in diameter). They may produce a single spore, a sporangium and a fragment of hyphae which are the most important morphological criteria used to recognize actinomycetes. Microorganisms belonging to the group of common actinomycetes in soils are mainly divided into two groups, namely, the streptomycetes and rare actinomycetes.

Bacteria belonging to the order *Actinomycetales* are well known as the secondary metabolites producers and many of the metabolites are active against pathogenic microorganisms. Members of the genus *Streptomyces* have long been found and recognized as prolifilic producers of useful bioactive compounds (Tanaka and Omura, 1990). The rate of discovery of new metabolites has declined, however the searching new antibiotics have been continuously studied due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. The filamentous *Actinomycetales* species produce over 10,000 bioactive compounds, 7,600 compounds derived from *Streptomyces* and 2,500 compounds from the so called rare actinomycetes species. These compounds from actinomycetes represented the largest group (45%) of bioactive microbial metabolites (Berdy, 2005) e.g. streptomycin *(Streptomyces griseus)*, hygromycin *(Streptomyces hygroscopicus)*, gentamicin (*Micromonospora* spp.), rifamycin (*Amycolatopsis mediterrane*), erythromycin (*Saccharopolyspora erythraea)* and salinomycin (*Streptomyces albus)*. However, the secondary metabolites which were isolated from rare actinomycetes still a few discovery comparing with *Streptomyces.* Recently, much attention has focused on rare actinomycetes as a new source for searching of new bioactive compounds. The strains in rare actinomycetes that have been reported for antibiotic productions were in genera, *Actinoplanes, Actinokineospora, Acrocarpospora, Actinosynnema, Actinomadura, Amycolatopsis, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kineosporia, Kutzneria, Microbispora, Micromonospora, Microtetraspora, Nocardia, Nonomuraea, Thermomonospora,*

Pseudonocardia, Thermobifida, Saccharomonospora, Spirilliplanes, Streptosporangium and *Virgosporangium* (Lazzarini *et al*., 2000).

Micromonospora strains that have been reported to produce the useful secondary metabolites are interesting. Recently, diketopiperazine Sch 725418 which produced from *Micromonospora* sp. exhibited inhibitory activity against *Saccharomyces cerevisiae* PM503 with MIC values 32 mg/ml (Yang *et al*., 2004). Retymicin, Galtamycin B, Saquayamycin Z and Ribofuranosyllumichrome were produced from *Micromonospora* sp. Tü 6368 (Ströch *et al*., 2005). *Microbispora* strains displayed the ability to produce antibiotics, for example, bispolide (Okujo *et al*. 2007), propeptin (Esumi *et al*., 2002), hibarimicins and hiramibicin-related compound (Cho *et al*., 2002), glucosylquestiomycin (Igarashi *et al*., 1998) and microbiaeratin (Ivanova *et al*., 2007). The other rare actinomycete strains of *Actinomadura* could produce chandrananimycins A-C (Maskey *et al*., 2003), daunomycin (Kim *et al*., 2000) and ZHD-0501 (Han *et al*., 2005).

Generally, actinomycetes have been isolated from terrestrial sources, however the marine-derived actinomycetes have been recognized as a source of novel antibiotics and anti-cancer agents (Jensen *et al*., 2005), for example, *Micromonospora rifamycinica* which was isolated from mangrove sediment produced rifamycin S (Huang *et al*., 2008). Moreover, crytpoendolithic actinomycetes such as *Micromonospora endolithica* sp. nov. and *Micromonospora coerulea* were isolated (Hirsch *et al*., 2004). In Thailand, many rare actinomycete strains have been proposed, for example, *Microbispora corallina* (Nakajima *et al*., 1999), *Microbispora siamensis* (Boondaeng *et al*., 2009), *Micromonospora marina* (Tanasupawat *et al*., 2010), *Micromonospora chaiyaphumensis* (Jongrungruangchok *et al*., 2008a), *Micromonospora pattaloongensis* (Thawai *et al*., 2008), *Planotetraspora thailandica* (Suriyachadkun *et al*., 2009) and *Actinomycetospora chiangmaiensis* (Jiang *et al*., 2008). Therefore, the natural sources of Thailand are the valuable resource for the discovery of novel actinomycetes.

This work aims to isolate and identify the rare actinomycetes as well as ferment the selected rare actinomycete strains, elucidate and evaluate biological activities of the isolated secondary metabolites.

The main objectives of this investigation are as follows:

1. To study the biodiversity of the rare actinomycetes from soils
2. To study the taxonomy of the rare actinomycetes
3. To isolate bioactive secondary metabolites from selected rare actinomycetes

CHAPTER II

LITERATURE REVIEWS

2.1 Taxonomy of actinomycetes

The classification of actinomycetes is based on their morphological and physiological characteristics since the last decade however this does not reflect sometimes the natural relationships among actinomycetes and relate organisms. Recently, chemotaxonomic analyses, comparative analysis of homologous sequences and the phylogenetic branching pattern have been used for classification at the genus and family level. The delineation of order *Actinomycetales* based upon 16S rRNA gene sequence comparison was reported by Stackebrandt and Schumann (2006) as indicated below.

Order: *Actinomycetales*

Suborder *Micromonosporineae*
- Family *Micromonosporaceae*

Suborder *Frankineae*
- Family *Frankiaceae*
- Family *Aciduthermaceae*
- Family *Sporichthyaceae*
- Family *Geodermatophilaceae*
- Family *Cryptasporangiaceae*
- Family *Microsphaeroceae*

Suborder *Pseudonocardineae*
- Family *Actinasynnemaceae*
- Family *Pseudonocardiaceae*

Suborder *Streptomycineae*
- Family *Streptomycetaceae*

Suborder *Corynebacterineae*
- Family *Nocardiaceae*
- Family *Gordoniaceae*
- Family *Mycobacteriaceae*
- Family *Dietziaceae*
- Family *Tsukamurellaceae*
- Family *Corynebacteriaceae*

Suborder *Micrococcineae*
- Family *Intrasporangiaceae*
- Family *Dermabacteraceae*
- Family *Jonesiaceae*
- Family *Brevibacteriaceae*
- Family *Intrasporangiaceae*
- Family *Dermatophiluceae*
- Family *Micrococcaceae*
- Family *Pramicromonosporaceae*
- Family *Cellulamonadaceae*
- Family *Microbacterinaceae*

Suborder *Actinomycineae*
- Family *Actinomycetaceae*

Suborder *Propionibacterineae*
- Family *Propionibacteriaceae*
- Family *Nocardioidaceae*

Suborder *Streptosporangineae*
- Family *Streptosporangiaceae*
- Family *Thermomonosporaceae*
- Family *Noeardiopsaceae*

Suborder *Glycomycineae*
- Family *Glycomycetaceae*

2.1.1 The genera of rare actinomycetes

The rare actinomycetes are separated into different genera via the criterion of phenotype, genotype and chemotype. Most rare actinomycetes possess cell wall type II to IV with peptidoglycans containing *meso*-diaminopimelic acid (DAP) (Goodfellow, 1988). Many rare actinomycetes characteristics have been reported such as *Thermomonospora, Cryptosporangium, Dactylosporangium, Saccharomonospora, Pseudonocardia* and *Thermobifida.* Examples of rare actinomycetes that have been isolated from soils in Thailand are *Micromonospora, Microbispora, Nonomuraea, Actinomadura, Pseudonocardia*, *Nocardia,* etc.

2.1.1.1 *Micromonospora*

The genus *Micromonospora* belonging to the family *Micromonosporaceae* was firstly described by Ørskov (1923). *Micromonospora* was differentiated from other genera in the same family by characteristics as shown in Table 2.1. At present, *Micromonospora* contains 41 species with validly published names. In Thailand, 8 *Micromonospora* species including *M. aurantionigra, M. eburnea, M. siamensis, M. narathiwatensis M. pattaloongensis*, *M. chaiyaphumensis*, *M. krabiensis* and *M. marina* (Thawai *et al*., 2004, 2005a, 2005b, 2007, 2008, Jongrungruangchok *et al*., 2008a, 2008b, Tanasupawat *et al*., 2010) were reported.

Micromonospora strains are Gram-positive, chemoorganotrophic, well developed, generally aerobic, branched, and substrate mycelium. Single conidia are one of the well-defined criteria in the genus *Micromonospora*. They normally lack aerial mycelium, form light yellow-orange to orange-red and yellowish white to grayish yellow colonies (occasionally brown, maroon or blue-green) composed of tightly woven, fine hyphae. The dark brown or black spores are formed at the surface of the colonies which darken as a result of sporulation and usually may become viscid or mucoid. The spore surface appears smooth, rough and nodular, and non-motile. This organism can grow in 1.5 to 5% NaCl, normally can grow in 3% NaCl. The most *Micromonospora* strains are sensitive to pH below 6.0. Their growth occurs normally between 20 °C and 40 °C but not above 50°C (Holt, 1989). All strains of *Micromonospora* show positive results for gelatin liquefaction.

Chemotaxonomically, the cell walls of *Micromonospora* strains have been found to contain *meso*-DAP, glutamic acid, glycine and D-alanine, corresponding to cell wall type II (Lechevalier and Lechevalier, 1970). Muramic acid does not occur as *N*-acetyl, but as the *N*-glycolyl derivative. The whole cell sugar composes of the pentoses, xylose and arabinose and the hexoses, glucose and galactose, corresponding to whole cell sugar pattern D of Lechevalier and Lechevalier (1970). The polar lipid profiles are diphosphotidylglycerol (DPG), phosphotidylinositol (PI), phosphatidylinositolmannosides (PIMs) and phosphatidyl-ethanolamine (PE), but phosphatidylcholine (PC) is not detected. This pattern corresponds to phospholipid type II as reported by Lechevalier *et al*. (1977). The predominant cellular fatty acids are iso- and anteiso-branched fatty acid. Unsaturated or 10-methyl fatty acids may be found in certain strains, that are i-$C_{15:0}$, i-$C_{16:0}$, i-$C_{17:0}$, a-$C_{15:0}$, $C_{17:0}$ and a-$C_{17:0}$. This pattern corresponds to fatty acid type 3d (Kroppenstedt, 1985), but mycolic acid and cyclic fatty acids are not presented. The predominant menaquinone components of *Micromonospora* are MK-9 and MK-10, except MK-12 is found only in *Micromonospora echinospora* subsp. *pallida.* The DNA G + C content ranges from 71 to 73 mol%.

Table 2.1 Differential characteristics of the genera belonging to family *Micromonosporaceae*

Family	Genus	DNA G+C (mol%)	Spores motility	Fatty acid Type[a]	Cell wall Type[b]	Major menaquinone(s)	Phospho lipid Type[c]	whole cell sugar(s)[d]	Diamino acid
Micromonosporaceae	*Longispora*	70	-	2d	II	MK-10(H_4,H_6)	PII	D,A	*meso*-DAP
	Virgosporangium	71	+	2d	II	MK-10(H_4,H_6)	PII	D,A	*meso*-DAP
	Asanoa	71-72	-	2d	II	MK-10(H_6,H_8)	PII	D,A	*meso*-DAP
	Actinoplanes	72-73	+	2d	II	MK-9(H_4), MK -10 (H_4,H_6)	PII	D	*meso*-DAP
	Spirilliplanes	69	+	2d	II	MK-10(H_4)	PII	D,A	*meso*-DAP
	Micromonospora	71-73	-	3d	II	MK-9(H_4,H_6), MK-	PII	D	*meso*-DAP
	Dactylosporangium	72-73	+	3d	II	10(H_4,H_6)	PII	D	*meso*-DAP
	Catellatospora	71-72	-	3d	II	MK-9(H_4,H_6, H_8)	PII	D	*meso*-DAP
	Pillimelia	ND	+	2d	II	MK-9(H_4,H_6), MK-	PII	D	*meso*-DAP
	Verrucosispora	70	-	2b	II	10(H_6,H_8) MK-9(H_2,H_4) MK-9(H_4)	PII	D	*meso*-DAP

[a] According to the classification of Kroppenstedt (1985)
[b] According to the classification of Lechevalier *et al.* (1977)
[c,d] According to the classification of Lechevalier and Lechevalier (1970)

Table 2.2 Differential characteristics of the genera belonging to family *Streptosporangeaceae.*

Family	Genus	DNA G+C (mol%)	Spores motility	Fatty acid Type[a]	Cell wall Type[b]	Major menaquinone(s)	Phospholipid Type[c]	Whole cell sugar(s)[d]	Diamino acid
Streptosporangeaceae	*Acrocapospora*	68-69	-	3c	III	MK-9(H_2, H_4, H_6)	IV, II	B,C	*meso*-DAP
	Herbidospora	69-71	-	3c	III	MK-10(H_4, H_6, H_8)	IV	B	*meso*-DAP
	Microbispora	71-73	-	3c	III	MK-9(H_0, H_2, H_4)	IV	B,C	*meso*-DAP
	Microtetraspora	69-71	-	3c	III	MK-9(H_2, H_4, H_6)	IV	B,C	*meso*-DAP
	Nonomuraea	64-69	-	3c	III	MK-9(H_0, H_2, H_4)	IV	B,C	*meso*-DAP
	Planobispora	70-71	+	3c	III	MK-9(H_2, H_4)	IV	B	*meso*-DAP
	Planomonospora	72	+	3c	III	MK-9(H_2)	IV	B	*meso*-DAP
	Planotetraspora	71	+	ND	III	ND	ND	D,A	*meso*-DAP
	Streptosporangium	ND	-	3c	III	MK-9(H_4)	IV	B	*meso*-DAP

[a] According to the classification of Kroppenstedt (1985)
[b] According to the classification of Lechevalier *et al.* (1977)
[c,d] According to the classification of Lechevalier and Lechevalier (1970)

Table 2.3 Differential characteristics of the genera belonging to family *Thermomonosporaceae*

Family	Genus	DNA G+C (mol%)	Spores motility	Fatty acid Type[a]	Cell wall Type[b]	Major menaquinone(s)	Phospholipid Type[c]	Whole cell sugar(s)[d]	Diamino acid
Thermomonosporaceae	*Actinocorallia*	66-73	-	3a	III	MK-9(H_4, H_6, H_8)	II	B	*meso*-DAP
	Actinomadura	66-72	-	3a	III	MK-9(H_4, H_6, H_8)	I	B	*meso*-DAP
	Spirillospora	71-73	-	3a	III	MK-9(H_4, H_6)	I/II	B	*meso*-DAP
	Thermomonospora	ND	-	3a	III	MK-9(H_4, H_6, H_8)	I	C	*meso*-DAP

[a] According to the classification of Kroppenstedt (1985)
[b] According to the classification of Lechevalier *et al.* (1977)
[c,d] According to the classification of Lechevalier and Lechevalier (1970)

Table 2.4 Differential characteristics of the genera belonging to family *Pseudonocardiaceae*

Family	Genus	DNA G+C (mol%)	Spores Motility	Spore-chains	Fatty acid Type[a]	Cell wall Type[b]	Major menaquinone(s)	Phospholipid Type[c]	whole cell sugar(s) [d]	Diamino acid
Pseudonocardiaceae	*Actinoalloteichus*	73	-	+	2d	III	MK-8(H_4),MK-9(H_2,H_4)	II	C	*meso*-DAP
	Streptoalloteichus	ND	+	-	3e	III	MK-10(H_4, H_6)	II	E	*meso*-DAP
	Amycolatopsis	75	-	+	3f	IV	MK-8(H_4),MK-9(H_2)	II	A	*meso*-DAP
	Pseudonocardia	68-79	-	+	2b	IV	MK-8(H_2, H_4)	II/III	A	*meso*-DAP
	Actinokineospora	68-70	+	+	2d	IV	MK-9(H_4)	II	A	*meso*-DAP
	Kutzneria	69-71	-	-	3c	III	MK-8(H_4),MK, 9(H_2,H_4,H_6), MK-10(H_4)	II	E	*meso*-DAP
	Kibdelosporangium	49.8	-	-	3c	IV	MK-	II	A,B	*meso*-DAP
	Saccharopolyspora	70	-	+	2c	IV	8(H_4,H_6),MK9(H_2,H_4,H_6)	III	A	*meso*-DAP
	Saccharomonospora	ND	-	-	2a	IV	MK-9(H_4)	II	A	*meso*-DAP
	Actinopolyspora	68	-	+	2c	IV	MK-9(H_4)	III	A	*meso*-DAP
	Thermocrispum	69-73	-	-	3f	III	MK-9(H_4), MK-10(H_4) MK-9(H_4)	III	C,A	*meso*-DAP

[a] According to the classification of Kroppenstedt (1985)
[b] According to the classification of Lechevalier *et al.* (1977)
[c,d] According to the classification of Lechevalier and Lechevalier (1970)

Table 2.5 Differential characteristics of the genera belonging to family *Nocardiaceae*

Family	Genus	DNA G+C (mol%)	Spores motility	Fatty acid Type[a]	Cell wall Type[b]	Major menaquinone(s)	Phospholipid Type[c]	Whole cell sugar(s)[d]	Diamino acid
Nocardiaceae	*Nocardia*	64-72	-	1b	IV	MK-9($H_{4cyclic}$)	II	A	*meso*-DAP
	Rhodococcus	67-73	-	1b	IV	MK-8(H_2)	II	A	*meso*-DAP

[a] According to the classification of Kroppenstedt (1985)
[b] According to the classification of Lechevalier *et al.* (1977)
[c,d] According to the classification of Lechevalier and Lechevalier (1970)

2.1.1.2 *Microbispora*

The genus *Microbispora* belonging to the family *Streptosporangiaceae* was proposed by Nonomura and Ohara (1957). *Microbispora* was differentiated from other genera in the same family by the characteristics as shown in Table 2.2. In the past, the species were cited as members of the genus *Microbispora* namely *Mb. thermorosea, Mb. aerate, Mb. thermodiastatica, Mb. parva, Mb. amethystogenes, Mb. chromogenes, Mb. diastatica, Mb. viridis, Mb. karnatakensis*, and *Mb. indica*. In 1990, Miyadoh *et al*. proposed that 10 species of *Microbispora* should be combined into the type species *Mb. rosea* with two subspecies, *Mb. rosea* subsp. *rosea* and *Mb. rosea* subsp. *aerate* based on DNA-DNA hybridization experiment (Wang *et al*., 1996). At the present, In the *List of Prokaryotic names*, *Microbispora* comprises of four species and two subspecies. Recently, two new species of *Microbispora* were isolated from soils in Thailand, namely *Mb. coralline* (Nikajima *et al*., 1999) and *Mb. siamensis* (Boondaeng *et al*. (2009).

Microbispora strains are aerobic, Gram-positive and non-motile which typically form obvious aerial mycelium bearing longitudinal pairs of spores that may be closely arranged either along the aerial hyphae or very short sporophore. *Microbispora* spores are oval to ellipsoidal with smooth surface and usually 1.2 to 1.8 μm in diameter. The substrate mycelium of them is pinkish to brownish red (0.5 to 0.8 μm in diameter). Some species produce soluble pigments.

Chemotaxonomically, the cell walls of *Microbispora* strains contain diaminopimelic acid, in the *meso*-form, glutamic acid, and alanine that indicate the cell wall type III (Lechevalier and Lechevalier, 1970). The *N*-acyl group of muramic acid in peptidoglycan is the *N*-acetyl type. Their whole cell sugars contain madurose, corresponding to whole cell sugar type B (Lechevalier and Lechevalier, 1970). The phospholipids contain in the cells are phosphatidylethanolamine (PE) and ninhydrin-positive glycophospholipids (NPG), corresponding to the phospholipid type IV (Lechevalier *et al*., 1977). Their cellular fatty acid compositions are a-$C_{16:0}$, i-$C_{16:0}$ and 10-Me-$C_{17:0}$ as major components, corresponding to the fatty acid type 3c (Kroppenstedt, 1985). The predominant menaquinone is MK-9. The DNA G+C content of this genus ranges 71 to 73 mol%.

2.1.1.3 *Nonomuraea*

The genus *Nonomuraea* belonging to the family *Streptosporangiaceae* was firstly proposed by Zhang *et al*. (1998). *Nonomuraea* was differentiated from other genera in the same family by characteristics as shown in Table 2.2. In the *List of Prokaryotic names*, *Nonomuraea* comprises of 23 species and two subspecies with validly published names. Recently, *No. antimicrobica* (Qin *et al*. 2009) *No. candida* (Le Roes and Meyers, 2009) *No. rosea* (Kämpfer *et al*. 2010) were validly proposed as new species.

Nonomuraea strains are aerobic, Gram-positive, and non-acid-fast. They typically form extensively branched substrate and aerial mycelium. Aerial hyphae that differentiate into spore chains have various shapes, straight, hooked or spiral. Spores surfaces are smooth, spiny or warty surfaces, and non-motile.

Chemotaxonomically, the cell walls of *Nonomuraea* strains have been found to contain *meso*-DAP, corresponding to cell wall type III (Lechevalier and Lechevalier, 1970). The *N*-acyl group of the muramic acid is the *N*-acetyl type. The whole cell sugar composes of madurose, corresponding to whole cell sugar pattern B (Lechevalier and Lechevalier, 1970). The polar lipid profiles are diphosphotidylglycerol (DPG), hydroxylated phosphotidylethanolamine (OH-PE), glucosamine containing phospholipids (GluNU) and uncharacterized glycolipid, corresponding to phospholipid type IV (Lechevalier *et al*. 1977). The predominant cellular fatty acids are i-$C_{16:0}$, $C_{16:0}$, $C_{17:0}$ and 10-methyl-$C_{17:0}$, corresponding to fatty acid type 3d (Kroppenstedt, 1985). The predominant components of menaquinone are MK-9(H_0), MK-9(H_2) and MK-9(H_4). The DNA G + C content of this genus ranges from 64 to 69 mol%.

2.1.1.4 *Actinomadura*

The genus *Actinomadura* belonging to the family *Thermomonosporaceae* was firstly proposed by Lechevalier and Lechevalier (1970). *Actinomadura* was differentiated from other genera in the same family by characteristics as shown in Table 2.3. In the *List of Prokaryotic names, Actinomadura* comprises of 43 species and 2 subspecies with validly published names. Recently, *A. keratinilytica* (Puhl *et al*., 2009), *A. miaoliensis* (Tseng *et al*., 2009), *A. flavalba* (Qin *et al*., 2009), *A. scrupuli* (Lee and Lee, 2010) and *A. sputi* (Yassin *et al*., 2010) were validly proposed as new species.

Actinomadura strains are aerobic, Gram-positive, and non-acid–alcohol-fast. They typically produce well-developed, extensively branched mycelium, and non-fragmenting vegetative hyphae. Aerial hyphae differentiate into spore chains which have various shapes and ornamentation, for example, straight, hooked or spiral. Spores are oval or short-rod-like with smooth, spiny or warty surfaces and non-motile.

Chemotaxonomically, the cell walls of *Actinomadura* strains have been found to contain *meso*-DAP, glutamic acid, glycine and D-alanine, corresponding to cell wall type III (Lechevalier and Lechevalier, 1970). The *N*-acyl group of muramic acid is the *N*-acetyl type. The whole cell sugar composes of madurose, corresponding to whole cell sugar pattern B (Lechevalier and Lechevalier, 1970). Their polar lipids have no nitrogenous phospholipids, corresponding to phospholipid type I (Lechevalier *et al*., 1977). The predominant cellular fatty acids are i-$C_{16:0}$, $C_{16:0}$, $C_{17:0}$ and 10-methyl-$C_{17:0}$, corresponding to fatty acid type 3d (Kroppenstedt, 1985), except for *A. rubrobrunea* and *A. viridilutea*, which can be distinguished from other species by the relatively high proportions of iso-branched and low proportions of 10-methyl-branched fatty acids. The predominant menaquinone components of *Actinomadura* are MK-9(H_4), MK-9(H_6), and MK-9(H_8). The DNA G + C content of this genus ranges from 65 to 69 mol%.

2.1.1.5 *Pseudonocardia*

The genus *Pseudonocardia* belonging to the family *Pseudonocardiaceae* was proposed originally by Henssen (1957). *Pseudonocardia* was differentiated from other genera in the same family by the characteristics as shown in Table 2.4. In the *List of Prokaryotic names*, the genus *Pseudonocardia* comprises of 35 species with validly published names. Recently, *P. acaciae* (Duangmal *et al*., 2009) *P. endophytica* (Chen *et al*., 2009), *P. adelaidensis* (Kaewkla and Franco, 2010), *P. parietis* (Schäfer *et al*., 2009), *P. babensis* (Sakiyama *et al*., 2010) and *P. tropica* (Qin *et al*., 2010) were validly proposed as new species.

Pseudonocardia strains are Gram-positive aerobic bacteria that form vegetative and aerial mycelium. Spore chains were produced by fragmentation into rod-shaped at the end of mycelium. The substrate mycelium and aerial mycelium appear

yellowish and powdery white, respectively. *Pseudonocardia* strains grow well at 30 °C. They can grow in 0 to 8% NaCl.

Chemotaxonomically, the peptidoglycan contains *meso*- DAP, arabinose and galactose, corresponding to cell wall type IV and whole cell sugar pattern A (Lechevalier and Lechevalier, 1970). Major menaquinone is MK-8. No mycolic acids and phospholipid type II or III are found (Lechevalier *et al*., 1977). The branched compound i-$C_{16:0}$ is the major fatty acid in this genus. DNA G+C content ranges 68 to 79 mol%.

2.1.1.6 *Nocardia*

The genus *Nocardia* belonging to the family *Nocardiaceae* (Stackebrandt *et al*., 1997). *Nocardia* was differentiated from other genera in the same family by the characteristics as shown in Table 2.5. In the *List of Prokaryotic names*, *Nocardia* comprises of 80 species with validly published names, including the recently described species, *N. blacklockiae*, *N. wallacei* (Conville *et al*., 2009), *N. iowensis* (Lamm *et al*., 2009), *N. jinanensis* (Sun *et al*., 2009), *N. mikamii* (Jannat-Khah *et al*., 2010), and *N. niwae* (Moser *et al*., 2011). In Thailand, *N. thailandica* was isolated from clinical specimens by Kageyama *et al*. (2004).

Nocardia strains are aerobic, Gram-positive, and acid alcohol-fast at some stages of the growth cycle. They form extensively branched substrate hyphae that fragment into rod-shaped to cocci, and non-motile. Aerial hyphae always present and confer a powdery or velvety. They usually form carotenoid-like pigments. Morphological colonies are variable, smooth, irregular or wrinkled.

Chemotaxonomically, the peptidoglycan contains *meso*-DAP, arabinose and galactose, corresponding to cell wall type IV and whole cell sugar pattern A (Lechevalier and Lechevalier, 1970). Major menaquinone is MK-8(H_6). They contain mycolic acids and phospholipid type II is found (Lechevalier *et al*., 1977). The saturated or unsaturated such as $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ are the major fatty acid, corresponding to fatty acid type 1b (Kroppenstedt, 1985). The DNA G+C content ranges 64 to 72 mol%.

2.1.2 Source and isolation methods of rare actinomycetes

Actinomycetes are widely distributed in soil, aquatic, marine sediment, and extreme environments. They are primarily saprophytic microorganisms of the soil and contribute significantly to the turnover of complex biopolymers such as lignocellulose, hemicellulose, pectin, keratin and chitin. *Actinopolyspora, Saccharopolyspora, Actinomadura, Micromonospora, Actinomyces* and *Actinoplanes* were found from marine sediment (Vijayakumar *et al*., 2007). *Microbispora, Microtetraspora, Nocardia, Actinomadura* and *Saccharothrix* from desert soil (Takahashi *et al*., 1996). Recently, *Micromonospora coriariae, Micromonospora lupine, Micromonospora saelicesensis* and *Krebbella lupini* were isolated from diverse plant roots (Trujillo *et al*., 2006a, 2007, 2006b).

Many methods for the isolation of rare actinomycetes have been designed to eliminate non-filamentous bacteria from the substrates or to suppress their growth on isolation media. However, the streptomycetes are also desirable eliminate or suppress because they are widely distributed in nature and tend to grow faster than rare actinomycetes (Hayakawa, 2008). In the soil environment, actinomycetes produce spore to promote their survival and dispersal. The differential properties of their spores are advised to utilize for selective isolation (Hayakawa, 2008). The most effective pretreatment that used to isolate rare actinomycetes was wet heat at 70 °C for 15 min and 1.5% phenol treatment using hair hydrolysated vitamin agar and *Micromonospora, Microbispora, Actinoplanes,* and *Streptosporangium* were isolated (Seong *et al*., 2001). Various isolation methods of rare actinomycetes have been developed as shown in Table 2.6 (Hayakawa, 2008).

Table 2.6 Summary of methods developed for the selective isolation of rare actinomycetes from soil (1987–2007) (adapted from Hayakawa, 2008)

Pretreatment	Culture media	Genera selected
Physical:		
None	HV agar with or without nalidixic acid and trimethoprim	*Streptomyces* and other genera
Phenol 1.5%	HV agar with nalidixic acid and tunicamycin	Many rare actinomycete genera including *Spirilliplanes*
Chemical:		
SDS 0.05% and yeast extract 5%	HV agar with nalidixic acid	*Streptomyces* and other genera
Phenol 1.5%	HV agar with nalidixic acid and tunicamycin	*Micromonospora*
Phenol 1.5%	HV agar	*Streptomyces violaceusniger* cluster
Chloramine-T	HV agar with nalidixic acid	*Herbidospora*, *Microbispora*, *Microtetraspora*, *Nonomuraea*, and *Streptosporangium*
Physical and chemical:		
Dry heat at 110 °C for 1 h and phenol 1.0%	HV agar with kanamycin, josamycin, lysosyme, and nalidixic acid	*Actinomadura viridis*
Dry heat at 120 °C for 1 h and phenol 1.5%-CG 0.01%	HV agar with nalidixic acid	*Microbispora*
Dry heat at 120 °C for 1 h and BC 0.01% or 0.03%	HV agar with nalidixic acid and leucomycin (or tunicamycin)	*Streptosporangium* or *Dactylosporangium*
Dry heat at 120 °C for 1 h and BC 0.05%	LSV-SE agar with kanamycin, nalidixic acid and norfloxaxin	*Microtetraspora*
Enrichment:		
Chemotaxis (γ-collidin, vanillin)	HV agar with nalidixic acid	*Actinoplanes*, *Actinokineospora*, *Actinosynnema*, *Virgosporangium*
Enrichment and physical:		
Pollen-baiting and drying	HV agar with nalidixic acid	*Actinoplanes*
Rehydration (30 °C, 90 min) and centrifugation (1,500 x g, 20 min)	HV agar with nalidixic acid and trimethoprim	*Actinoplanes*, *Actinokineospora*, *Actinosynnema*, *Catenuloplanes*, *Cryptosporangium*, *Dactylosporangium*, *Geodermatophilius*, *Kineosporia*, *Sporichthya*
$CaCO_3$, rehydration and centrifugation	HV agar with fradiomycin, kanamycin, nalidixic acid, and trimethoprim	*Actinokineospora*
Sucrose-gradient centrifugation (240 x g, 30 min)	HV agar with nalidixic acid, and chlortetracycline	*Nocardia*
Moist incubation and drying	HV agar with nalidixic acid, and trimethoprim	*Streptomyces* and other genera

2.1.3 Identification techniques of actinomycetes

Phenotypic, genotypic and chemotaxonomic techniques were routine procedure for bacterial characterization. The discriminatory taxonomic power of some techniques describe here.

2.1.3.1 Phenotypic characteristics

Phenotypic characteristics comprise of morphological, cultural, biochemical and physiological characteristics. The growth of colonies, they firstly develop the substrate mycelium or primary mycelium. Some hyphae vertically grow and form aerial mycelium or secondary mycelium which directly contact to the air. The aerial mycelium support a change of the morphological character of colonies e.g. spore production. However, some genera, *Micromonospora and Actinoplanes,* have no aerial mycelium. The formations of spores are the most important morphological criterion to identify actinomycetes at the genus level. For example, the presences of single spores, pair spores and branched sporophore are *Micromonospora*, *Microbispora* and *Microtetraspora*, respectively. The methods for phenotypic studies usually use the method characterization of *Streptomyces* species by Shirling and Gottlieb (1966).

2.1.3.2 Chemotaxonomic characteristics

Chemotaxonomic characteristics are the study of cell wall components and lipid which are synthesized by highly regulated enzymes in the cell. They were used as a criterion for classification and identification because they usually found among microorganisms, and the component should be homologous among the strains within the taxon. Chemotaxonomic markers normally are use to classify at the genus level.

Cell wall composition

Most bacteria have peptidoglycan which is alternating polymer of *N*-acetylglucosamine and *N*-acetylmuramic acid, and a peptide moiety that links the glycan chain. The variation in the peptide moiety holds significant systematic information, especially, in the actinomycetes. The diagnostic of 2, 6-Diaminopimelic acid (DAP) as the key amino acid parameter because bacterial cell generally contains one of the isomers, *LL*-form and *meso*-(*DL*)-form. Both isomers also have been found in members of actinomycetes. *LL*-DAP always found in the hydrolysated cell wall of *Streptomyces* and *Nocardioides* and

meso-DAP found in many rare actinomycetes e.i. *Micromonospora, Microbispora, Actinomadura, Nocardia* and *Nonomuraea*. However, some actinomycete, *Katasatospora,* has both isomers. 3-hydroxy-*meso*-DAP is always found in *Actinoplanes* and *Ampullariella*. Cell wall chemotypes of the actinomycetes are shown in Table 2.7. Paper chromatography (Becker *et al*., 1965), cellulose thin layer chromatography (Staneck *et al*., 1974) and HPLC (Tisdall and Anhal, 1979) have been used to separate DAP isomers in cell wall peptidoglycan. In addition to, the *N*-acetyl group of muramic acid of some actinomycetes is substituted by the *N*-glycolyl group, so, this is also useful for actinomycetes identification which can be determined with whole cell hydrolysated by glycolate test (Uchida *et al.,* 1999).

The sugar composition often presents valuable information on the classification and identification of actinomycetes. The sugar pattern plays a key role in the identification of sporulating actinomycetes which have *meso*-DAP in the cell wall. Whole cell sugar patterns contribute to the cell wall chemotypes of actinomycetes proposed by Lechevalier and Lechevalier (1970). Whole cell sugar patterns of related genera are shown in Table 2.8. In 1970, whole cell sugars are analysed by using cellulose TLC and developed with n-butanol-water-pyridine-toluene (5:3:3:4, v/v). The sugars are visualized by spraying with acid aniline phthalate and then heated at 100 °C for 4 min (Staneck and Robert, 1974).

Isoprenoid quinones

Isoprenoid quinones are constituents of bacterial plasma membrane. Their roles probably are about electron transport system in respiration and oxidative phosphorylation. Isoprenoid quinones typically have 2 types, menaquinone, 2-methyl-3-polyprenyl-I,4-naphthoquinones and ubiquinone, 2,3-dimethoxy-5-methyl-6-polyprenyl-I,4-benzoquinones. Menaquinone normally found as the predominant in plasma membrane of actinomycetes (Collins *et al*., 1977). The number of isoprene units and the degree of hydrogenation of double bonds in the isoprenyl chain are utilized as the important key for identification (Komagata and Suzuki, 1987).

Phospholipids

The polar lipids of bacteria are a heterogeneous group of molecules which have amphipathic properties and form the lipid bilayer of bacterial membranes. Phospholipids related to permeability and regulation at the membrane. Phospholipids of actinomycetes are identified into five patterns as shown in Table 2.9 (Lechevalier *et al*., 1977). Diphosphatidylglycerols (DPG), phosphatidylinositiol (PI) and phosphatidylinositol-mannosides (PIMs) widely distributed in the member of actinomycetes, therefore these are not useful for classification and identification. On the contrary, phosphatidylethanolamine (PE), methylphosphatidyl-ethanolamine (PME), phosphatidylcholine (PC) and unknown phospholipids containing glucosamine (GluNU) are relatively uncommon so these are the phospholipids marker to useful for identification (Komagata and Suzuki, 1987). Phospholipid types of related genera are shown in Table 2.10.

Fatty acids

Fatty acids form the lipid bilayer of bacterial membranes. The long chain fatty acids of actinomycetes may be separated into two broad group, ones possess between 12 and 20 carbon atoms and the another have from 20 up to just above 80 carbons, mycolic acid. The occurrence and relative amount of each component reflect the biosynthetics capability of the microorganism and the conditions which it is grown. The carbon chain length, the location of methyl groups e.g. iso- or anteiso- and the position of double bond were used as the key for bacterial characterization (Minnikin and Goodfellow, 1980). Fatty acid types are shown in Table 2.11.

DNA base compositions

DNA molecule consists of two long strands of polydeoxyribonucleotides that form a double helix. Purine and pyrimidine bases are connected by hydrogen bonds. Adenine (A) pairs with thymine (T), and cytosine (C) pairs with guanine (G). A variation in molar ratios of (G+C)/ (G+C+A+T) x 100 is usefulness for classification and identification. DNA base composition values are expressed as G+C content. The determination is changed from Tm method to HPLC method because of accuracy, fast, and small amount of samples (Tamaoka and Komagata, 1984).

Table 2.7 Cell wall chemotypes of the actinomycetes

Chemotypes	Amino acid								
	LL-DAP	meso-DAP	DAB	Aspartic acid	Glycine	Lysine	Omithine	Arabinose	Galactose
I	+				+				
II		+			+				
III		+							
IV		+						+	+
V						+	+		
VI				v					v
VII			+		+	v			
VIII							+		

v, variable

Table 2.8 Whole cell sugar patterns

Patterns	Diagnotic sugars					Genera related
	Arabinose	Fucose	Galactose	Madurose	Xylose	
A	+		+			*Nocardia, Mycobacterium, Rhodococcus*
B				+		*Actinomadura, Streptosporangium, Microbispora*
C	**No diagnostic sugar**					*Nocardiopsis, Saccharothrix*
D	+				+	*Micromonospora, Dactylosporangium Catellatospora, Pillimelia, Verrucosispora*
E		+				

Table 2.9 Phospholipid types

Types	Phospholipid								
	PIMs	**PI**	**PC**	**PG**	**PE**	**PME**	**GluNU**	**APG**	**DPG**
I	+	+		v				v	v
II	+	+		v	+			v	+
III	v	+	+	v	v	+		v	v
IV	ND	+			v	v	+		+
V	ND	+		+	v		+	v	+

v, variable; ND, no data

Table 2.10 Phospholipid types of related genera

Types		**Genera related**
I	No nitrogenous phospholipids*	*Actinomadura, Microtetraspora, Nocardioides, Actinopolymorpha*
II		*Micromonospora, Actinoplanes, Dactilosporangium, Nocardia, Streptomyces, Amycolatopsis, Catellatospora*
III	Phosphatidylcholine	*Nocardiopsis, Pseudonocardia, Kribbella, Catenuloplanes, Saccharopolyspora*
IV	GluNU**	*Microbispora, Streptosporangium, Nonomuraea, Herbidospora*
V	GluNU and phosphatidyl glycerol	*Oerskovia, Promicromonospora*

*, Dianostic phospholipids

**, Unknown phospholipids containing glucosamine

Table 2.11 Fatty acid types

Types 1	Pathway to straight-chain fatty acid
1a	Saturated and unsaturated straight chain
1b	10-Methyl branched fatty acids
1c	Cyclopropane fatty acids
Type 2	Pathway to terminally branched fatty acids
2	*iso*- and *anteiso*-Fatty acid
Type 3	Complex branched fatty acids
3	*iso*- and *anteiso*- fatty acids and 10-methyl branched

2.1.3.3 Genotypic characteristics

Genotypic characterization is the determination of the sequence and the organization of the total bacterial genome. Phylogenetic relationships are often examined using data on nucleotide sequences of ribosomal RNA. Homology indices are the relative values used in comparing microorganisms. DNA homology data reveals not only the relatedness of microorganisms, but also the evaluation of phenotypic characteristics.

16S rRNA gene sequencing

16S rRNA gene sequence technique is usually used for primary screening propose and determine the phylogenetic position of bacterial group because of many reasons i.e. having in almost bacteria, no changing of the function of 16S rRNA gene, and large enough for informatics proposes. 16S rRNA gene sequence begins from DNA extraction, amplification of the gene coding for 16S rRNA, purification of the DNA fragment, and direct sequence in order to determine the order in which the bases are arranged within the length of sample. The sequence is identified by using phylogenetic analysis procedures. However, analysis of 16S rRNA gene sequence generally is used to identify the organisms up to the genus level only (Janda and Abbott, 2007).

Phylogenetic construction

A phylogenetic tree is a diagram that depicts the lines of evolutionary descent of different species and is useful for structuring classifications. The methods of constructing tree can be based on character and distances.

The constructions of phylogeny tree are used to examine all possible topologies. Maximum parsimony is a character-based method which uses a matrix of discrete phylogenic characters to infer one or more optimal phylogenetic trees for a set of species. The tree with the most favorable score is taken as the best estimation of the phylogenetic relationships of the included taxa. The neighbor-joining is a distance-based method. It produces a unique final tree under minimum evolution criterion. This method provides not only the topology but also the branch lengths of the final tree. Bootstrap is used to place confidence intervals on plylogenies (Felsentein, 1985).

DNA-DNA hybridization

DNA-DNA hybridization is the standard method which is studied to classify bacteria at the species level and DNA-DNA relatedness values are the key parameter in the species delineation. DNA-DNA hybridization usually is either a free solution method, S1 nuclease or a solid support method such as nitrocellulose filters and DNA must be labeled with radioactive substance. Later, non-radioactive substances are developed to labelled DNA. Recently, biotinylation of DNA with photobiotin is used for the study of relatedness of bacteria and identification of bacterial strains. A species as a group of strains including the type strain are shared at least 70% total genome DNA–DNA hybridization (Esaki *et al*., 1989).

2.2 Secondary metabolites from rare actinomycetes

Bioactive microbial products are the main source of useful bioactive metabolites. They are produced from eukaryotic microscopic fungi (38%) and prokaryotic bacteria (62%). For prokaryotic bacteria, the secondary metabolites were produced from unicellular bacteria e.g. *Pseudomonas, Bacillus, Cyanobacteria,* and *Myxobacteria* (17%) and the filamentous *Actinomycetales* (45%), *Streptomyces* (34%) and rare actinomycetes (11%) (Berdy, 2005). Therefore, *Actinomycetales* are the mainly source of bioactive metabolites. Many bioactive metabolites are produced by rare actinomycetes as shown on in Table 2.12.

Table 2.12 Bioactive compounds from rare actinomycetes

Compounds	Strains	Activity	References
Anthraquinone	*Micromonospora lupini*	Antitumor activity	Igarashi *et al*. (2007)
Retymicin, Galtamycin B, Saquayamycin Z, Ribofuranosyllumichrome,	*Micromonospora* sp.Tu6368	Cytotoxic activity	Antal *et al*. (2005)
Echinosporamicin	*Micromonospora echinospora*	Antibacterial activity	He *et al*. (2004)
Kosinostatin	*Micromonospora* sp. TP-A0468	Antitumor activity	Furumai *et al*. (2002)
Micromonosporide A	*Micromonospora* sp.	Gastrulation inhibitor	Ohta *et al*. (2001)
IB-96212	*Micromonospora* sp. L-25-ES25-008	Cytotoxic activity	Chimeno *et al*. (2000)
SB-219383	*Micromonospora* sp. SB-219383	Tyrosyl tRNA synthetase inhibitor	Stefansky *et al*. (2000)
Arisostatin A and B	*Micromonospora* sp. TP-A0316	Antibacterial activity	Igarashi *et al*. (2000)
Rusmicin	*Micromonospora* sp.MA7094	Antifungal activity	Sigmund *et al*. (1998)
GTRI-02 1-Hydroxycrisamicin A	*Micromonospora* sp. SA246	Lipid peroxidation inhibitor	Yeo *et al*. (1998)
Thiostepton	*Micromonospora carbonacea*	Antibacterial activity	Puar *et al*. (1997)
YM-47515	*Micromonospora echinospora* subsp. *echinospora*	Antimicrobial activity	Sugawara *et al*. (1997)
Thiocoraline	*Micromonospora* sp. L-13-ACM2-092	Antimicrobial activity	Romero *et al*. (1997)
Pyrrolosporin A	*Micromonospora* sp. C39217-R109-7	Antimicrobial activity	Lam *et al*. (1996)
Antascomicins A, B, C	*Micromonospora chalcea*	Antileckemia cell line P-388	Hochlowski *et al*. (1995)
Cochleamycin			
Cororubicin	*Micromonospora* sp. JY16	Cytotoxic activity	Ishigami *et al*. (1994)
Mycinamicins I and II	*Micromonospora griseorubida* FERM BP-705	Antibacterial activity	Kinoshita *et al*. (1992)
Dynemicins O, P, Q	*Micromonospora chersina* M956-1	Antibacterial activity, Antitumor activity	Saitoh *et al.* (1991)
Sibanomicin	*Micromonospora* sp. SF2364	Antitumor activity	Itoh *et al.* (1990)
Citreamicin	*Micromonospora citrea*	Antibacterial activity	Carter *et al.* (1990)
Microbiaeratin	*Microbispora aerate* IMBAS-11A	Anti-MRSA activity	Ivanova *et al.* (2007)
Bispolides	*Microbispora* sp. A34030	Tyrosine kinase inhibitors	Okujo *et al.* (2007)
Hibarimicins B	*Microbispora rosea* subsp. *hibaria*	Weak antimicrobial activity	Cho *et al.* (2002)
Novel quinolone	*Pseudonocardia* sp. CL38489	Anti-*Helicobacter pyloli*	Dekker *et al*. (1997)
Phenazostatin D	*Pseudonocardia* sp. B6273	Inactive against the tested bacteria and fungi	Maskey *et al*. (2003)

CHAPTER III

EXPERIMENTAL

3.1 Sample preparation and isolation

Nineteen soil samples (top layer of soils, 0-5 cm depth) were collected from the mountainous soils in Loei and Chaiyaphum, from soils in Chonburi, from peat swamp forest soils in Phayao and from mangrove soils near the sea shore in Samutsakhon provinces.

The soil samples were dried at room temperature about 7days and then ground. One gram of air-dried soil sample was suspended in 9 ml of 1.5% phenol for 30 min. Serial dilutions of the suspension were prepared by the 10-fold dilution method (Nonomura and Ohara, 1969). Of the final dilution step (10^{-3}), aliquots of 100 μl were spread onto starch-casein nitrate agar (SCA) plates and humic-vitamin agar (HV) (Appendix A) (Hayakawa and Nonomura, 1987) plates supplemented with antibiotics, nystatin (50 mg/l) and nalidixic acid (20 mg/l) (Ara and Kudo, 2006). The plates were incubated at 30°C for 14 days. The colonies were picked up, streaked for purification on yeast extract-malt extract agar plates (YMA, ISP no. 2) (Appendix A) and incubated at 30°C for 7-21 days. A single colony was transferred into YMA slants and incubated at 30 °C for 14 days. The stock cultures were kept in cold room at 4°C at the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. They were also preserved on 20% glycerol at -20 °C and lyophilized using 10% skim milk as cryoprotectant.

The pH measuring of soil samples was determined using the dried soil samples that were dried under room temperature for 7 days. One gram of dried soil was put into the test tubes containing 2.5 ml of distilled water and shaken for 2 to 3 seconds. The soil sample solutions were measured by pH glass electrode recorder (put the pH glass electrode in a test tube at the 2/3 depth from the surface and hold it for more than 30 second to stabilize electric potential).

3.2 Identification methods

Morphological, cultural, biochemical, and physiological characteristics of microorganisms were determined by the method of Shirling and Gottlieb (1966) and Arai (1975).

3.2.1 Morphological and cultural characteristics

Morphological characteristics of the isolated strains grown on YMA were observed by light and scanning electron microscopy (model JSM-5410LV; JEOL). The scanning electron microscopy was used to determine spore forming and spore surface. The selected strains were streaked on YMA and incubated at 30 °C for 14 days with the exception of the selected strain of *Microbispora* were incubated at 30 °C for 21 days on oatmeal-nitrate agar. The culture agar media were cut to 3-5 mm^3 and the specimens were fixed on 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2 for 1-2 h then washed 3 times with phosphate buffer. They were dehydrated through a gradient ethanol series.

The cultural characteristics were tested using 21-day cultures grown at 30 °C on various agar media such as yeast extract-malt extract (ISP medium no. 2), oatmeal agar (ISP no. 3), inorganic salts-starch agar (ISP no. 4), glycerol-asparagine agar (ISP no. 5), peptone-yeast extract iron agar (ISP no. 6), tyrosine agar (ISP no. 7), glucose-asparagine agar, czapek's sucrose agar and nutrient agar (Appendix A). The colors of the mature aerial mycelium, substrate mycelium and diffusion soluble pigment were determined by the NBS/IBCC color system.

3.2.2 Biochemical and Physiological characteristics

3.2.2.1 Carbon utilization

The basal agar medium (ISP no. 9) (Shirling and Gottlieb, 1966) (Appendix A) supplemented with 0.3% casamino acid was prepared and a carbon source was prepared to give final concentration of approximately 1%. After autoclaving at 110 °C for 10 min, the mixture was agitated and 25 ml of this mixture was poured into 9 cm petri dish. L-arabinose, cellubiose, D- fructose, D- galactose, D-glucose, glycerol, inositol, lactose, D-mannitol, melibiose, raffinose, L-rhamnose, D-ribose, D- salicin, sucrose and D-xylose were used as carbon sources. No carbon source and D-glucose were used as negative and positive control, respectively.

The inoculum of 14-day cultures on YMA was suspended in 5 ml of distilled water and transferred to a sterile test tube. The suspension was precipitated by

centrifugation at 5,000 rpm for 15 min. Cells were collected, washed twice with distilled water, and then were restored at the original volume for using as the inoculum.

The basal agar medium plates were dried by leaving them at room temperature. A loop-full of washed culture was inoculated on the agar surface by streaking straight across the dish in duplicate and incubated at 28-30 °C for 10-14 days.

The results were recorded as follow:

1. Strong positive utilization (++), when growth on tested carbon in basal medium is equal to or greater than positive control.
2. Positive utilization (+), when growth on tested carbon in basal medium is significantly better than negative control, but somewhere less than positive control.
3. Utilization doubtful (±), when growth on tested carbon in basal medium is only slightly better than negative control and significantly less than positive control.
4. Utilization negative (-), when growth on tested carbon in basal medium is similar to or less than negative control.

3.2.2.2 Starch hydrolysis

All strains were streaked on the surface of inorganic salt-starch agar plate (ISP no. 4) (Shirling and Gottlieb, 1966, Appendix A) and incubated at 28-30 °C for 10 days after that Gram's iodine solution was flooded on the surface of the agar plate. A clear zone was indicative of starch hydrolysis.

3.2.2.3 Gelatin liquefaction

All strains were inoculated into bouillon gelatin broth (Arai, 1975, Appendix A) and incubated at 28-30 °C for 21 days. The culture broth was compared with control that was not inoculated when placed at 4 °C for 30 min. The gelatin became liquid if it was hydrolyzed.

3.2.2.4 Nitrate reduction

All strains were inoculated into peptone KNO_3 broth (Appendix A) and incubated at 28-30 °C for 4-6 days. On the forth day, 1 ml of the culture was transferred into a new test tube. Two drops of sulfanilic acid reagent and 3 drops of *N, N*-dimethyl-

1-naphtylamine solution were added and mixed well. If nitrites were present, the mixture would become pink to red.

3.2.2.5 Milk coagulation and milk peptonization

The selected strains were inoculated in 10% skim milk (Appendix A) and incubated at 28-30 °C for 7-14 days. In the case of peptonization, skim milk solution would be clear and in the case of coagulation, skim milk would be precipitated.

3.2.2.6 NaCl tolerance

All strains were streaked on YMA supplement with 0%, 1.5%, 3%, 4%, 5%, 6%, and 7% NaCl and incubated at 28-30 °C for 14 days. The maximum of NaCl concentrations for which the strains can grow, were recorded.

3.2.2.7 pH tolerance

All strains were streaked on YMA adjusted pH at 4, 4.5, 5, 6, 7, and 8 and incubated at 28-30 °C for 14 days. The minimum pH for which the strains can grow, were recorded.

3.2.2.8 Temperature tolerance

All strains were streaked on YMA and incubated at 15, 20, 30, 37, 40, 45, and 50 °C for 14 days. The minimum and maximum of temperature for which the strains can grow, were recorded.

3.2.2.9 Antimicrobial activities

All strains were streaked along the diameter of YMA and grown for 14 days at 30 °C. The test microorganisms were then streaked out perpendicular to selected strains, as close as about 3 mm apart and incubated at 30 °C for 1 day. The tested microorganisms were *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. The clear zone was present if the strains inhibited the growth of test microorganisms.

3.2.3 Chemotaxonomic characteristics

The selected strains were cultivated in yeast extract-malt extract (YM) broth on a rotary shaker 200 rpm at 30 °C. The culture cells were collected by centrifugation at 5,000 rpm for 10 min and then washed 3 times with sterile distilled water. The precipitate was dried by freeze drying. Freeze-dried cells were used for analyses.

3.2.3.1 Isomer of diaminopimelic acid analysis

Freeze-dried cells (10 mg) were hydrolyzed with 1 ml of 6N HCl into a screw cab tube at 100 °C for 18 h. The hydrolyzed solution was filtrated and evaporated until dryness. The extracts were dissolved in 400 µl of distilled water. The solution was applied to cellulose HPTLC plate no. 5787 and developed with methanol: water: 6N hydrochloric acid: pyridine in ratio 80:26:4:10 (v/v). Diaminopimelic acid was visualized by spraying with 0.5% ninhydrin in saturated n-butanol in water and heating at 100 °C for 3 min. One microliter of 0.01 M *DL*- diaminopimelic acid (DAP) which contains *meso*- and *LL*-DAP isomers was used as the standard. The DAP spot were seen as gray green fading to yellow and amino acid spot appeared purple to red color. *LL*-DAP spot appeared above *meso*-DAP spot. (Staneck and Robert, 1974)

3.2.3.2 Cell wall acyl type of muramic acid analysis

To prepare columns, pasteur pipettes were used as columns by loading with dowex (CH_3COO^- form) about 5 mm in height. They were washed with distilled water until pH 6 to7.

Ten milligram of freeze dried cells were hydrolyzed with 100 µl of 6N HCl (Appendix B) into screw cab tube at 100°C for 2 h. The first step, the hydrolyzed solution was loaded to column and eluted with 400 µl of distilled water into a test tube (fraction 1). The second step, the column was transferred to another tube and eluted with 1 ml of distilled water twice (fractions 2 and 3). The third step, the column was transferred to other tube and eluted with 1 ml of 0.5 N HCl in triplicates (fraction 4, 5, and 6). Finally, 100 µl of fraction 5 were transferred to new screw cab tube after that 2 ml of DON reagent (Appendix B) were added and mixed well. The standard series were 0, 30, 60, 90 nmol sodium glycolate. The sample solutions and standard were heated at 100°C for 2 h and immediately cooled by placing tube in water. They were added with 1.9 ml of 2 N H_2SO_4 (Appendix B), mixed well and cooled again. They were measured O.D. at 530 nm

and calculated, if more than 10 nmol/mg cell of glycolate were detected, glycolyl muramic acid was present in peptidoglycan. (Uchida and Aida, 1984)

3.2.3.3 Whole cell sugar analysis

About 50 mg of freeze dried cells were added with 1 ml of 1 N H_2SO_4 and heated at 100 °C for 90 min. The hydrolyzed solution was immediately cooled to room temperature and then adjusted with saturated $Ba(OH)_2$ to pH 5.2 to 5.5. The debris cells were discarded by centrifugation at 3,600 rpm for 10 min, then, the supernatant was transferred and evaporated until dryness. The extract was dissolved with distilled water and transferred to an appendorf tube. The solution was deionized with Dowex 50 (H^+ form) and Dowex 1 (OH^- form). The supernatant was separated by centrifugation for 10 min and filtrated before analyzing by HPLC (Mikami and Ishida, 1983).

3.2.3.4 Cellular fatty acid analysis

Initially, for saponification, about 40 mg of freeze dried cells were added with 1 ml of reagent 1, methanolic base (Appendix B) followed by shaken well, heated at 100 °C for 30 min, and then cooled to room temperature in water. Fatty acids were cleaved from the cell lipids and converted to their sodium salts. For methylation, the solution was added with 2 ml of reagent 2 (Appendix B), mixed well by using a vortex mixer for 5-10 second, heated at 80 °C for 10 min and then quickly cooled to room temperature. The fatty acids (as sodium salts) were converted to fatty acids methyl esters which increased the volatility of the fatty acids for GC analysis. The suspension was added with 1.25 ml of reagent 3 (Appendix B) and mixed end-over-end about 10 min for fatty acid methyl ester extraction from acidic aqueous phase to organic phase. The suspension was added with reagent 4 (Appendix B) and rotated end-over-end for 5 min to remove free fatty acids and residual reagents from the organic extract. Residual reagents can damage the chromatographic system, resulting in tailing and loss of the hydroxyl fatty acid methyl ester. The upper layer was transferred to vial for analyzing using gas chromatography according to the instructions of the Microbial Identification System (MIDI) (Sherlock Microbial Identification System: MIDI Hewlett Packard, Palo Alto, CA, USA (Sasser, 1990; Kämpfer and Kroppenstedt, 1996).

3.2.3.5 Polar lipid analysis

For extraction, about 150 to 300 mg of freeze dried cells were added with 3 ml of methanol: 0.3% NaCl aq. (100:10) and 3 ml of petroleum ether in a screw cab. They were mixed for 15 min and then the upper layer was removed. The lower layer was added with 1 ml of petroleum ether, mixed for 2-5 min. After the upper layer was removed then the lower layer was heated at 100 °C for 5 min and immediately cooled at 37 °C for 5 min. Chloroform: methanol: water (90:100:30) 2.3 ml was added into the lower layer phase and mixed well for 1 h. The upper layer was transferred into another tube. The lower layer was extracted 2 times with chloroform: methanol: water (50:100:40), mixed well for 30 min and centrifuged at 3000 rpm for 10 min and the supernatant was transferred to the upper layer tube. The upper layer fraction was added with 1.3 ml each of chloroform and water, mixed for 30 min then centrifuged at 3000 rpm for 10 min, and the upper layer was removed. The lower layer was dried up with N_2 gas at less than 37 °C.

For analysis, the polar lipid fraction was dissolved in 60 µl of chloroform: methanol (2:1) and applied to two-dimentional silica gel HPTLC no. 1.05633 developed with two solvent system by first solvent system was chloroform: methanol: water (50:100:40) and second system was chloroform: acetic acid: methanol: water (40:7.5:6:2).

For detection, all phospholipids were visualized by using Ditmer and Lester reagent (Appendix B), present blue spot. Phosphotidylethanolamine(PE) and its derivatives, for example, lyso-PE, hydroxy-PE and methyl-PE were detected by spraying with ninhydrin reagent (Appendix B) then heating at 100 °C for 10 min, present purple spot. Glycolipid was presented green yellow spot when sprayed with anisaldehyde reagent (Appendix B) and heated at 100 °C for 10 min. Other lipids were present blue spot. Phosphotidyl choline was present by spraying with dragendroff's reagent (Appendix B). (Minnikin *et al*., 1984)

3.2.3.6 Menaquinone analysis

About 100 to 500 mg of freeze dried cells were extracted with chloroform: methanol (2:1) overnight. The suspension was then filtered and evaporated. The extract was dissolved with small amount of acetone and applied to a silica gel TLC no.1.05744 developed by 100% benzene. The menaquinone band was detected using UV lamp (254 nm), scraped, and put into a test tube. They were dissolved with acetone (HPLC grade) then filtered and dried up with N_2 gas. The menaquinone sample was analyzed by HPLC equipped with a Cosmosil $5C_{18}$ column (4.6 x 150 mm; Nacalai Tesque, Kyoto, Japan). A

mixture of methanol and 2-propanol (2:1, v/v) was used as the elution solvent (Collins *et al*., 1977).

3.2.3.7 DNA base composition analysis

Each strain was cultivated on YM broth containing 1% glycine at 30 °C for 4-5 days on a shaker 200 rpm. Cells were harvested and washed twice with saline-EDTA buffer pH 8.0 (Appendix B) then suspended in saline-EDTA buffer pH 8.0 (Appendix B). The suspension was added with egg-white lysozyme, incubated at 37 °C for 20-40 min for lysing cells and frozen. The frozen suspension was added with tris-SDS and incubated at 60 °C for 10 min. In the case of cells failed to be lysed by enzyme, the cells were freeze dried, manually ground, and dissolved with DNA extraction buffer (Appendix B) as described by Raeder and Broda (1985). Phenol saturated in water was added into the suspension and mixed for 10 min. The suspension was centrifuged at 10,000 rpm for 15 min and the upper layer was transferred to a beaker. Cold absolute ethanol was added and DNA was pooled with sterile glass rod. DNA was respectively washed with 70%, 90%, and 99.5% ethanol. After air-drying, DNA was dissolved in 0.1x SSC (Appendix B) and kept in a refrigerator overnight. DNA was treated with RNase A, RNase T1 and proteinase K (Appendix B) solution at 37 °C for 30 min and the DNA suspension was extracted with phenol saturated with water, pooled, and stored in 0.1x SSC at 4 °C twice. The last purification, after phenol extraction, the upper layer was transferred and added with acetate-EDTA (Appendix B) then precipitated using iso-propanol. DNA was measured at OD_{260nm} and OD_{280nm}. The DNA was suitable if the ratio of OD_{280} /OD_{260} is 0.56-0.59.

DNA solution (100 µl) corresponding to 20 µg was put into an appendorf tube and heated at 100 °C for 10 min then immediately cooled in ice. The denatured DNA was added with 100 µl of nuclease P1 solution (Appendix B) and incubated at 60 °C for 30 min and then the suspension was added with 100 µl of alkaline phosphatase solution (Appendix B) and incubated at 37 °C for 1 h. The nucleoside suspension and the standard were determined using the HPLC method of Tamaoka and Komagata (1984). An equimolar mixture of nucleotides which digested with alkaline phosphatase was used as the quantitative standard.

3.2.4 Genotypic characteristics

3.2.4.1 16S rRNA gene sequence analysis and phylogenetic tree construction

Two-loop full of cells in an appendorf tube were added with 300 µl of extraction buffer and aluminium oxide. Cells were lysed by using a micro mixer for 90 sec. Three hundred microliter of phenol: chloroform (1:1) solution were added and centrifuged at 15,000 rpm at 4 °C for 5 min. The upper layer was transferred and extracted with phenol: chloroform (1:1) solution (Appendix B) again. The upper layer was added with 3M sodium acetate pH 5.2 (Appendix B) and cooled iso-propanol, mixed well and frozen at -80 °C for 15 min. The suspension was centrifuged at 15,000 rpm at 4 °C for 15 min. The upper layer was discarded and the precipitate DNA was washed with 70% and 99% ethanol, respectively. The precipitate DNA was air-dried then dissolved in 20 µl of sterile distilled water and kept at 4 °C.

PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Nakajima *et al.* (1999). Totally, 50 µl of PCR mixture composed of 1 µl of DNA, 0.25 µl of Taq DNA polymerase, 5 µl of 10X polymerase buffer, 4 µl of dNTP mixture, 2.5 µl of each 10 µM primer (20F and 1541R) and 34.75 µl of MilliQ water. DNA thermal cycler was used for 16S rDNA gene amplification by using the temperature profile of initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 15 sec, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were checked by gel electrophoresis. The reaction mixture total 5 µl was run on 0.8% agarose gel in tris-acetate EDTA buffer (Appendix B).

The amplified 16S rRNA gene was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer. Sequencing for each sample was carried out in both forward and reverse directions (Appendix D). The sequencing reaction for each sample was performed in the DNA Thermal Cycler (Gene Amp PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96 °C followed by 25 cycles of denaturing of DNA at 10 sec at 96 °C, primer annealing at 5 sec at 50 °C, and polymerization at 4 min at 60 °C (Ara and Kudo, 2006).

Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/. The sequence was multiple aligned with selected sequences obtained from GenBank database by using the CLUSTAL X version 1.83. The alignment was manually verified and adjusted prior to construct the phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) in the MEGA program version 4.0 (Tamura *et al.*, 2007). The confidence values of branches of the phylogenetic tree were determined using the 1,000 bootstrap replications (Felsenstein, 1985). The values for sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X version 1.83 and gap and ambiguous nucleotides were eliminated from the calculations.

3.2.4.2 DNA-DNA hybridization

DNA-DNA relatedness was measured fluorometrically using the microplate hybridization method (Ezaki *et al*., 1989).

Fifteen microliter of photobiotin and 10 µl of DNA concentration (1 mg/ml) were mixed in an appendorf tube and irradiated with sunlamp (500W) for 25 min. They were added with each 100 µl of 0.1M tris-HCl buffer pH 9.0 (Appendix B) and *n*-butanol then centrifuged at 12,000 rpm for 20 sec. The orange supernatant was discarded and the lower layer was boiled for 15 min, immediately cooled in ice and then sonicated for 3 min. The photobiotinylated DNA solution was mixed with hybridization solution for using as a probe solution.

Ten microgram DNA of an unknown strain, type strains and reference DNA (Calf thymus) were boiled for 10 min and then immediately cooled in ice. They were added with 500 µl of 2x PBS (Appendix B), 100 µl of 0.1 M $MgCl_2$, and filled up to total volume 1 ml with sterile distilled water. One hundred microliter of heated denatured DNA solution were added to microdilution wells (Nunc-Immuno^{TM} Plate: MaxiSorp^{TM} surface) and fixed by incubation at 37 °C for 2 h. After fixing, the solution was discarded. The fixing single strand DNA was added with 100 µl of the photobiotinylated DNA solution and then was incubated for 15-16 h.

The solution in microdilution plate were removed and washed with 200 µl 0.2x SSC for 3 times. One hundred microliter of solution 1 (Appendix B) was added to

each well and incubated at room temperature for 10 min. The solution 1 (Appendix B) was discarded and replaced with 100 µl of solution 2 (Appendix B) then incubated at 37 °C for 30 min. The solution 2 (Appendix B) was discarded and washed with 200 µ1 of 1xPBS for 3 times. One hundred microliter of solution 3 (Appendix B) was added and incubated at 37 °C for 15 min. The enzyme reactions were stopped by adding 100 µl of 2M H_2SO_4. The fluorescence intensity was measured at 450 nm using microplate reader (Microplate Reader Wallac 1420, PerkinElmer^{TM}) and was calculated for the value of percentage DNA homology.

3.4 Fermentation and extraction for preliminary characterization of secondary metabolites

Twenty strains of rare actinomycetes were selected for screening of compounds, including twelve strains of *Micromonospora* sp. AL9-20, P0402, AL1-3, AL4-4, D10-9-5, AL8-2, ASC19-2-1, AL10-3, AL7-5, AL5-1, AL9-13, and AL1-15-2; four strains of *Actinomadura* sp., AL7-14, CYP1-1B, CYP1-5, and AL4-10; two strains of *Nocardia* sp. P1803 and P1605; *Microbispora* sp. AL4-7; and *Nonomureae* sp. P0417. Pure cultures were transferred to the Bn-2 agar slant (Appendix A) and incubated at 30°C for 14 days. One loop full were transferred to V-22 medium (Appendix A) and incubated at 30°C for 3-4 days on a rotary shaker (200 rpm). Three milliliter aliquots of the seed cultures were transferred into 500-ml flasks containing l00 ml of 3 kinds of media such as A3M, A11M, and A16M media (Appendix A). Fermentation was carried out for 5-6 days at 30 °C on a rotary shaker (200 rpm). The culture broth was added with 100 ml of butanol to the flask and shaken for 2 h. Mycelia were removed by centrifugation at 6,000 rpm at 4 °C for 6 min. One milliliter of supernatant was transferred to a small vial and evaporated until dryness. The extract was dissolved with 100 µl of DMSO, filtered, and then analysed by HPLC-UV. The retention time and UV spectra of the peaks were compared by using the in-house database (Biotechnology Research Center, Toyama Prefectural University) containing about 500 reference compounds of microbial origins. *Micromonospora* sp. D10-9-5 on A11M medium and *Actinomadura* sp. CYP1-5 on A3M were selected for further fermentation, extraction, and determination of secondary metabolites because their chromatograms and UV spectra presented interesting unknown peaks.

3.5 Fermentation of the selected strains and isolation of compounds

Micromonospora sp. D10-9-5 was cultivated in a flask containing 100 ml of A11M medium total 2 liters, and *Actinomadura* sp. CYP1-5 was cultured on A3M medium. They were incubated at 30 °C on a rotary shaker (200 rpm) for 5-6 days. Then, each flask was added with 100 ml butanol and shaken for 2 h. They were centrifuged at 6000 rpm for 6 min. The butanol extracts were transferred to another flask and evaporated until dryness. Butanol extract of strains D10-9-5 and CYP1-5 were loaded on a silica gel column and eluted stepwise with 100 ml and 200 ml of $CHCl_3$:CH_3OH, respectively (in ratio 1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1) Fractions were individually analysed by HPLC-UV and the fractions showing the expected peaks were pooled and evaporated. They were further applied to ODS column and eluted with 300 ml of CH_3CN : 0.1% HCOOH (in ratio 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and finally with CH_3OH) at a flow rate of 30 ml/min. Each fraction was analysed by HPLC-UV and the fractions showing the expected peaks were evaporated and then the fractions were dried by lyophilyzation. The conditions for separation of compound were found using HPLC-UV. Finally, each compound was fractionated by preparative HPLC using ODS column (Xterra^{TM}, 7 µm, 19 x 300 mm) and eluted with CH_3CN and 0.1% HCOOH at a flow rate of 15 ml/min.

3.6 Structure elucidation

The pure compounds were elucidated by mass spectroscopy (MS) and nuclear magnetic resonance (NMR).

3.7. Biological activities determination

3.7.1 Antibacterial activity

SEK34, SEK34B, decatromicin B and BE-45722C were tested by resazurin microplate assay (Sarker *et al*., 2007). *Bacillus cereus* ATCC 11778 was inoculated in 5 ml of tryptic soy broth (TSB) at 37 °C for 30 min on shaker 200 rpm (OD600 ~ 0.1). and then diluted 200 folds in 20 ml of TSB, incubated at 37ºC for 3 h on shaker (OD600 ~ 0.45-0.5), and then diluted 30 folds in TSB. To assay in a 384 well-plate, each well containing 5 µl of bacteria ($5x10^4$ CFU/well), 7.5 µl of sample, and 25 µl of 0.25 mM resazurin were cultured in Mueller-Hinton broth (MHB) at the final volume of 75 µl per well and incubated at 37 °C for 3 h. The fluorescent intensity at Ex/Em 530/590 nm was

measured by microplate reader (Molecular device, SpectraMax M5) and data were analyzed. Vancomycin in 10% DMSO was used as positive control and 10% DMSO solution was used as negative control.

3.7.2 Antifungal activity

Candida albicans ATCC 90028 was grown on potato dextrose agar (PDA) at 30 °C for 3 days and then transferred to RPMI-1640 until cell density reaches $5x10^5$ CFU/ml. The yeast cell suspension was added to the 384-well plate; each well containing 45 µl of cell suspension and 5 µl of test sample and then incubated at 37°C for 4 hrs. Thereafter, 10 µl of 62.5 µg/ml resazurin solution was added to each well and incubated at 37 °C for 30 min. Amphotericin B and 0.5 %DMSO were used as a positive and a negative control, respectively (Brien *et al*., 2000).

3.7.3 Antimalarial activity

Plasmodium falciparum (K1, multidrug resistant strain) was cultured according to the method of Trager and Jensen (1976) using continuous cultures (*in vitro*) of asexual erythrocytic stages. Quantitative assessment of antimalarial activity (*in vitro*) was determined by mean of the microculture radioisotope technique based upon the method described by Desjardins *et al.* (1979). Inhibition concentration (IC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H]-Hypoxanthine by *P. falciparum*.

3.7.4 Cytotoxic activity

Cytotoxicity activity against vero cell line (African monkey kidney cell line) was performed by green fluorescent protein (GFP)-based assay (Hunt *et al*., 1999).

Cell suspension ($3.3x10^4$cells/ml) 45 µl was added to each well of 384-well plates containing 5 µl of test compounds previously diluted in 0.5% DMSO, and then incubated at 37 °C for 4 days in incubator with 5% CO_2. Fluorescence signals were measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) at the Ex/Em at 485/535 nm and data were analyzed. Ellipticine and 0.5%DMSO were used as a positive and a negative control, respectively.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Sources and isolate numbers of rare actinomycetes

Nineteen soil samples were collected from 5 provinces, including Loei, Chonburi, Phayao, Chaiyaphum and Samutsakhon. The pH ranges of soil samples were 5.01-9.66, 8.42-8.88, 4.73- 7.52, 6.12 and 7.80-7.82, respectively. Forty-nine strains of rare actinomycetes were isolated by using humic vitamin agar and starch casein nitrate agar (Table 4.1). They were kept in cold room at 4 °C, 20% glycerol at -80 °C and freeze-drying using 10% skim milks as cryoprotectant.

4.2 Identification and characterization of the strains

Forty-nine actinomycete strains contained *meso*-DAP (diaminopimelic acid) in the cell wall. They were divided into 6 genera, 38 strains were identified as *Micromonospora* (Kawamoto, 1989), 2 strains were *Microbispora* (Nonomura and Ohara, 1957), 4 strains were *Actinomadura* (Lechevalier and Lechevalier, 1970), 3 strains were *Nocardia* (Stackebrandt *et al*., 1997) and the last 2 strains were *Nonomuraea* (Zhang *et al*., 1998) and *Pseudonocardia* (Hessen, 1957), respectively, based on their phenotypic (Table 1-3, Appendix C), phylogenetic (Figure 4.1) and chemotaxonomic characteristics, diaminopimelic and *N*-acyl types (Table 4.2), whole cell sugars (Table 4.3), menaquinones (Table 4.4), polar lipid compositions (Table 4.5) and fatty acid compositions (Tables 4.6). The differential chemotaxonomic characteristics of the strains are shown in Table 4.7.

Table 4.1 Sources, Location, Date, pH, and strain numbers

Sources	Locations	Date	pH	Strain No.	No. strain
Mountain soil	Phukradung, Loei	Dec15,2007	7.00	AL1-3, AL1-8, AL1-15-2, AL1-16B, AL1-18A	5
Mountain soil	Phukradung, Loei	Dec15,2007	4.54	AL2-1, AL2-3	2
Mountain soil	Phukradung, Loei	Dec15,2007	5.97	AL3-2, , AL3-9, AL3-12, AL3-13, AL3-14-2, AL3-16	6
Mountain soil	Phukradung, Loei	Dec15,2007	6.05	AL4-4, AL4-5, AL4-7, AL4-8, AL4-10	5
Mountain soil	Phukradung, Loei	Dec15,2007	5.25	AL5-1, AL5-7, AL5-16	3
Mountain soil	Phukradung, Loei	Dec15,2007	5.01	AL7-5, AL7-14	2
Mountain soil	Phukradung, Loei	Dec15,2007	9.66	AL8-2, AL8-7, AL8-8, AL8-16	4
Mountain soil	Phukradung, Loei	Dec15,2007	6.49	AL9-1, AL9-24-1, AL9-13, AL9-22, AL9-20	5
Mountain soil	Phukradung, Loei	Dec15,2007	5.02	AL10-3, AL10-5, AL10-17	3
Mountain Soil	Chaiyaphum	Jan20, 2007	6.12	CYP1-5, CYP1-1B	2
Soil	Nok island, Chonburi	Apr 6, 2007	8.42	ASC13-5-1	1
Soil	Nok island, Chonburi	Apr 6, 2007	8.88	ASC19-2-1	1
Soil	Peat swamp forest, Phayao	Jan24, 2007	4.73	P0402, P0417	2
Soil	Peat swamp forest, Phayao	Jan24, 2007	7.44	P1605	1
Soil	Peat swamp forest, Phayao	Jan24, 2007	7.52	P1803	1
Soil	Peat swamp forest, Phayao	Jan24, 2007	7.04	P1440	1
Mangrove soil	Samutsakhon	Aug18,2006	7.82	D10-9-5	1
Mangrove soil	Samutsakhon	Aug18,2006	7.80	B10-3-3, B10-3-6	2
Mangrove soil	Samutsakhon	Oct12, 2006	7.60	SM1-1, PSM1-8	2
				Total	49

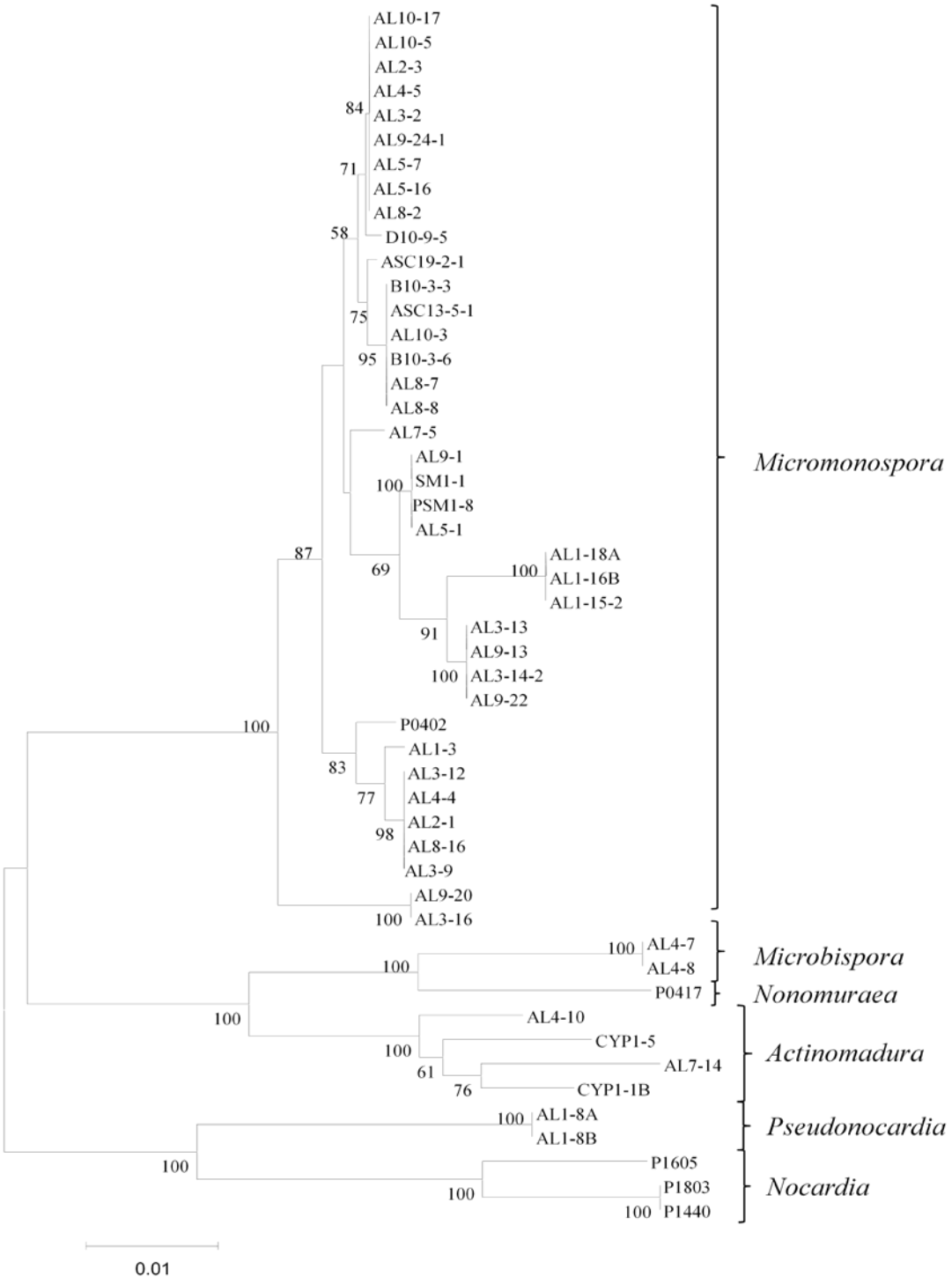

Figure 4.1 Neighbor-joining tree based on the partial 16S rRNA gene sequences of 49 strains

Table 4.2 Diaminopimelic acid types and glycolic analyses of the representative strains

Strain No.	Diaminopimelic types		Glycolic acid (nM)
	meso **-DAP**	***LL*-DAP**	
Micromonospora			
AL8-2	+	-	15.04
AL10-17	+	-	19.46
D10-9-5	+	-	18.90
ASC19-2-1	+	-	13.74
AL8-8	+	-	13.20
AL10-3	+	-	13.37
AL7-5	+	-	13.76
AL3-9	+	-	16.12
AL4-4	+	-	11.81
AL1-3	+	-	22.19
P0402	+	-	14.41
AL3-16	+	-	11.01
AL9-20	+	-	11.84
AL5-1	+	-	16.87
AL9-1	+	-	17.65
AL9-13	+	-	13.36
AL9-22	+	-	15.12
AL1-16B	+	-	14.09
AL1-15-2	+	-	11.08
Microbispora			
AL4-7	+	-	6.92
AL4-8	+	-	8.21
Nonomuraea			
P0417	+	-	7.14
Actinomadura			
CYP1-5	+	-	3.21
AL4-10	+	-	8.18
CYP1-1B	+	-	5.51
AL7-14	+	-	5.55
Nocardia			
P1440	+	-	10.77
P1803	+	-	10.87
P1605	+	-	12.02

Table 4.3 Whole cell sugars of the representative strains

Strain No.	Whole cell sugars								
	Rhamnose	**Ribose**	**Mannose**	**Madurose**	**Fucose**	**Arabinose**	**Galactose**	**Xylose**	**Glucose**
Micromonospora									
AL8-2	-	+	+	-	-	-	+	+	+
AL10-17	-	+	+	-	-	-	+	+	+
D10-9-5	-	+	+	-	-	trace	+	+	+
ASC19-2-1	-	+	+	-	-	trace	+	+	+
AL8-8	-	+	+	-	-	-	+	+	+
AL10-3	-	+	+	-	-	-	+	+	+
AL7-5	trace	+	+	-	-	+	+	+	+
AL3-9	-	+	+	-	-	-	+	+	+
AL4-4	-	+	+	-	-	trace	+	+	+
AL1-3	-	+	+	-	-	-	+	+	+
P0402	trace	+	+	-	-	-	+	+	+
AL3-16	-	+	+	-	-	-	+	+	+
AL9-20	-	+	+	-	-	-	+	+	+
AL5-1	-	+	+	-	-	-	+	+	+
AL9-1	-	+	+	-	-	-	+	+	+
AL9-13	trace	+	+	-	-	-	+	+	+
AL9-22	-	+	+	-	-	-	+	+	+
AL1-16B	-	+	+	-	-	-	+	+	+
AL1-15-2	-	+	+	-	-	trace	+	+	+
Microbispora									
AL4-7	trace	trace	-	+	-	-	+	trace	+
AL4-8	-	+	-	+	-	-	+	trace	+
Nonomuraea									
P0417	-	trace	-	+	-	-	+	+	+
Actinomadura									
CYP1-5	-	+	-	+	-	-	+	-	+
AL4-10	-	+	-	+	-	-	+	+	+
CYP1-1B	+	+	-	+	-	trace	+	-	+
AL7-14	trace	+	-	+	-	trace	+	-	+
Nocardia									
P1440	-	+	+	-	-	+	+	-	+
P1803	-	+	+	-	-	+	+	-	+
P1605	-	+	+	-	-	+	+	-	+

Table 4.4 Menaquinones of the representative strains

Strain No.	% Menaquinones													
	2,3- epoxy MK-8 (H_4-ω-cycl)	MK-8 (H_4-ω-cycl)	MK-9(H_8)	MK-9(H_4) or MK-9/III-VII-H_4	MK-9(H_2)	MK-9(H_4)	MK-9(H_6)	MK-9(H_8)	MK-10(H_8)	MK-10(H_2)	MK-10(H_4)	MK-10(H_6)	MK-10(H_8)	MK-11(H_4)
Micromonospora														
AL8-2	-	-	-	-	-	13.09	4.42	-	-	-	43.22	19.40	4.74	-
AL10-17	-	-	-	-	-	8.61	2.57	-	-	-	52.95	21.05	5.34	-
D10-9-5	-	-	-	-	-	-	-	-	-	-	14.82	46.65	27.48	-
ASC19-2-1	-	-	-	-	-	10.13	-	-	-	-	64.98	13.06	1.08	-
AL8-8	-	-	-	-	-	7.53	7.64	2.16	-	-	27.24	26.40	15.01	-
AL10-3	-	-	-	-	-	10.72	16.66	4.85	-	-	17.57	33.26	13.52	-
AL7-5	-	-	-	-	-	18.99	6.56	-	-	-	42.52	17.39	2.91	-
AL3-9	-	-	-	-	-	-	-	-	-	-	59.55	28.87	3.51	-
AL4-4	-	-	-	-	-	6.12	-	-	-	-	60.31	23.42	2.26	-
AL1-3	-	-	-	-	0.52	5.06	2.67	-	-	2.00	46.64	29.76	7.54	-
P0402					-	-	-	-	-	-	64.5	35.6	-	-
AL3-16	-	-	-	-	-	8.70	15.72	6.17	-	-	12.68	33.30	23.44	-
AL9-20	-	-	-	-	-	10.89	9.67	1.50	-	-	30.67	32.08	12.21	-
AL5-1	-	-	-	-	2.32	8.50	4.21	-	-	6.47	47.58	19.17	8.88	-
AL9-1	-	-	-	-	-	12.75	10.42	4.23	-	-	23.50	27.86	18.22	-
AL9-13	-	-	-	-	-	7.96	3.62	-	-	-	48.17	24.97	6.67	-
AL9-22	-	-	-	-	-	11.84	-	-	-	-	72.73	7.39	-	-
AL1-16B	-	-	-	-	-	14.23	30.62	14.52	-	-	6.82	20.20	13.61	-
AL1-15-2	-	-	-	-	-	6.55	22.16	5.03	-	-	9.43	40.33	14.64	-
Microbispora														
AL4-7	-	-	-	66.86	24.04	-	8.09	-	-	-	-	-	-	-
AL4-8	-	-	21.71	31.24	37.24	-	-	-	-	-	-	-		
Nonomuraea														
P0417	-	-	3.90	-	6.39	68.92	17.08	-	-	-	-	-	-	-

Table 4.4 Menaquinones of the representative strains (continued)

Strain No.	% Menaquinones													
	2,3- epoxy MK-8 (H_4-ω-cycl)	MK-8 (H_4-ω-cycl)	MK-9(H_8)	MK-9(H_4) or MK-9/III-VII-H_4	MK-9(H_2)	MK-9(H_4)	MK-9(H_6)	MK-9(H_8)	MK-10(H_0)	MK-10(H_2)	MK-10(H_4)	MK-10(H_6)	MK-10(H_8)	MK-11(H_4)
Actinomadura														
CYP1-5	-	-	0.51	-	3.51	12.10	62.32	16.99	-	-	-	-	-	-
AL4-10	-	-	0.60	-	2.70	8.82	64.06	12.57	-	-	-	-	-	-
AL7-14	-	-	-	-	-	12.67	63.52	14.23	-	-	-	-	-	-
Nocardia														
P1440	17.89	72.89	-	-	-	-	-	-	-	-	-	-	-	-
P1803	33.35	59.87	-	-	-	-	-	-	-	-	-	-	-	-
P1605	33.61	61.37	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.5 Polar lipid compositions of the representative strains

Strain No.	Polar lipids									
	PG	**DPG**	**NPG**	**PIMs**	**PI**	**PE**	**Methyl-PE**	**OH-PE**	**lyso-PE**	**PC**
Micromonospora										
AL8-2	+	+	+	+	-	+	-	-	-	-
D10-9-5	+	-	-	-	-	+	-	-	-	-
ASC19-2-1	-	+	-	-	-	+	+	+	-	-
AL10-3	+	+	+	+	-	+	-	-	-	-
AL7-5	-	+	-	-	+	+	-	-	-	-
AL4-4	-	+	-	+	-	+	+	+	-	-
P0402	+	+	-	+	+	+	-	-	-	-
AL9-20	+	+	-	+	-	+	-	-	-	-
AL5-1	+	+	+	+	-	-	+	-	-	-
AL9-13	+	+	+	+	-	+	-	-	-	-
AL1-15-2	-	+	-	-	-	-	+	-	-	-
Microbispora										
AL4-7	-	+	+	-	-	+	+	+	+	-
Nonomuraea										
P0417	+	-	-	-	+	+	-	+	-	-
Actinomadura										
CYP1-5	+	+	-	+	+	-	-	-	-	-
AL4-10	-	+	-	+	+	-	-	-	-	-
AL7-14	-	+	-	-	+	-	-	-	-	-
Nocardia										
P1440	-	+	-	+	+	+	+	+	-	-
P1803	-	+	-	+	+	+	+	+	-	-

Abbreviations: PG, Phosphatidylglycerol; DPG, Diphosphatidylglycerol; NPG, Ninhydrin positive glycophospholipids; PIMs, Phosphatidylinositolmannosides; PI, Phosphatidyl-inositol; PE, Phosphatidylethanolamine; Methyl-PE, Methylphosphatidylethanolamine; OH-PE, Hydroxyphosphatidylethanolamine; lyso-PE, lyso-Phosphatidylethanolamine; PC, Phosphatidylcholine

Table 4.6 Fatty acid compositions of the representative strains

	----------*M*----------																			--*Mb*--		-*No*-	---------*A*-------				------*N*-----		
Fatty acid	**AL8-2**	**AL10-17**	**D10-9-5**	**ASC19-2-1**	**AL8-8**	**AL10-3**	**AL7-5**	**AL3-9**	**AL4-4**	**AL1-3**	**P0402**	**AL3-16**	**AL9-20**	**AL5-1**	**AL9-1**	**AL9-13**	**AL9-22**	**AL1-16B**	**AL1-15-2**	**AL4-7**	**AL4-8**	**P0417**	**AL7-14**	**CYP1-5**	**AL4-10**	**CYP1-1B**	**P1440**	**P1803**	**P1605**
Saturated																													
C9:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-
C10:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	0.1	-	-	-	-
C12:0	-	-	-	-	-	-	-	-	0.1	-	0.1	0.1	0.1	-	-	-	-	-	0.1	0.1	0.1	-	0.9	0.2	0.3	0.2	-	-	-
C12:0 2OH	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C13:0	-	-	0.1	-	-	0.1	-	-	0.1	-	0.1	0.2	0.1	0.1	0.2	-	-	-	-	0.2	0.3	-	0.1	-	-	-	-	-	-
C14:0	-	-	0.1	0.1	-	-	0.1	-	0.1	0.1	0.2	0.3	0.2	0.1	0.2	-	0.1	0.1	0.3	2.1	0.9	-	6.0	3.1	2.4	5.9	2.7	1.3	1.3
C14:02OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-
C15:0	-	-	1.0	0.2	0.1	-	0.1	2.6	2.7	0.7	2.7	1.7	1.3	1.3	2.5	0.1	0.6	0.5	0.5	3.0	5.6	10.0	1.5	1.8	0.7	1.6	0.8	-	-
C15:0 2OH	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	0.4	-	3.7	-	-	-	-	-	-	-
C16:0	0.4	0.5	0.5	0.8	0.4	1.5	0.9	0.6	1.0	1.0	1.5	3.2	1.9	0.8	1.4	0.3	0.7	0.5	3.5	13.9	8.9	2.3	44.2	39.9	34.3	39.9	38.7	34.2	34.0
C16:0 N alc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-
C16:0 2OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6	-	0.4	6.8	-	-	9.9	-	-	-
C16:0 3OH	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-
C 17:0	0.5	0.4	5.6	2.8	1.1	11.3	1.1	8.7	8.1	2.8	13.6	7.8	9.7	8.8	10.7	1.0	4.7	4.2	6.3	0.6	7.7	3.1	0.7	3.1	1.2	0.4	-	-	-
C17:0cyclo	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	-	-	-	-		-	-
C17:0 2OH	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	0.2	-	-	-
C17:0 3OH	-	-	-	-	-	-	-	-	-	-	-	0.2	0.2	-	0.2	-	0.1	-	-	-	0.1	-	-	-	-	-	-	-	-
C18:0	3.1	3.2	0.7	1.7	1.3	1.3	3.0	1.8	0.5	2.0	1.7	2.0	1.8	1.2	1.9	1.3	1.3	1.7	5.1	0.8	2.3	-	3.5	2.2	5.4	4.4	20.9	32.3	22.2
C18:0 2OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	0.7	-	-	-
C18:0 3OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-
C19:0	-	0.2	-	0.3	-	0.9	0.2	0.4	-	0.2	0.4	0.3	0.4	0.8	0.7	0.2	0.6	0.6	0.6	-	0.1	-	-	-	-	-	-	-	-
C 20:0	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.1	-	-	-

M, Micromonospora; Mb, Microbispora; No, Nonomuraea; A, Actinomadura; N, Nocardia

Table 4.6 Fatty acid compositions of the representative strains (continued)

	----------*M*----------																				*--Mb--*	*-No-*	*---------A-------*				*------N-----*		
Fatty acid	**AL8-2**	**AL10-17**	**D10-9-5**	**ASC19-2-1**	**AL8-8**	**AL10-3**	**AL7-5**	**AL3-9**	**AL4-4**	**AL1-3**	**P0402**	**AL3-16**	**AL9-20**	**AL5-1**	**AL9-1**	**AL9-13**	**AL9-22**	**AL1-16B**	**AL1-15-2**	**AL4-7**	**AL4-8**	**P0417**	**CYP1-5**	**AL4-10**	**CYP1-1B**	**AL7-14**	**P1440**	**P1803**	**P1605**
Unsaturated																													
C15:1 ω6c	-	-	-	0.1	-	-	-	0.2	0.4	0.1	0.1	-	-	-	-	-	0.1	-	-	0.2	0.4	-	-	-	-	-	-	-
C15:1 ω8c	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.1	-	-	0.1	-	-	-	0.2	-	-	-	-	-	-	-	-
C16:1 2OH	-	-	1.1	0.2	0.3	2.9	0.3	1.1	-	0.9	-	-	0.3	0.7	2.2	1.0	0.4	3.7	-	0.1	-	0.1	-	-	-	-	-	-	-
C16:1 ω9c	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	0.4	-	-	0.2	-	0.4	0.3	-	-	-
C17:1 ω5c	-	-	-	0.1	-	-	-	0.1	0.1	-	-	0.2	0.1	0.1	0.1	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-
C17:1 ω6c	-	0.1	-	-	-	-	-	1.4	-	-	-	-	-	-	-	-	-	-	-	0.8	-	7.1	-	-	0.1	-	-	-	-
C17:1 ω8c	0.9	0.6	10.7	6.4	2.6	3.2	2.3	5.3	13.8	3.6	12.5	7.9	9.9	8.7	3.5	3.7	7.1	2.9	5.2	1.6	8.6	5.8	1.0	0.8	0.2	0.3	-	-	-
C17:1 ω9c	5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C18:1 ω9c	1.9	3.5	2.7	3.4	4.3	0.9	5.4	0.5	1.1	1.5	2.4	4.2	3.6	2.5	1.0	2.7	3.2	1.2	1.0	31.6	1.2	0.2	5.9	16.1	12.4	7.7	9.1	8.7	7.1
C18:1 ω7c	0.1	0.1	0.1	0.1	0.2	0.1	0.1	-	0.2	0.1	0.2	0.4	0.3	0.1	0.1	-	0.1	0.1	-	0.9	0.1	-	-	0.3	0.2	-	-	-	-
C18:3 ω6c (6,9,12)	-	-	0.2	-	-	0.2	-	-	-	-	0.2	0.2	0.1	0.2	0.3	-	-	0.2	0.1	-	-	-	0.6	-	-	-	-	-	-
C18:1 2OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	-	-	-	-	-	-	-	-	-
C20:1 ω9c	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	0.6	-	-	-	-	-
C20:2 ω6, 9c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	0.5	0.3	-	-	-
C20:4 ω6, 9, 12, 15c	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

M, Micromonospora; Mb, Microbispora; No, Nonomuraea; A, Actinomadura; N, Nocardia

Table 4.6 Fatty acid compositions of the representative strains (continued)

	----------M----------																				*--Mb--*	*-No-*	*---------A-------*			*------N-----*		
Fatty acid	**AL8-2**	**AL10-17**	**D10-9--5**	**ASC19-2-1**	**AL8-8**	**AL10-3**	**AL7-5**	**AL3-9**	**AL4-4**	**AL1-3**	**P0402**	**AL3-16**	**AL9-20**	**AL5-1**	**AL9-1**	**AL9-13**	**AL9-22**	**AL1-16B**	**AL1-15-2**	**AL4-7**	**AL4-8**	**P0417**	**CYP1-5**	**AL4-10**	**CYP1-1B**	**AL7-14**	**P1440**	**P1803**	**P1605**
Branched																													
i-C10:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
i-C 11:0	-	0.1	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	0.7	-	0.7
a-C11:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
i-C12:0	0.1	0.2	-	-	0.1	-	-	-	-	0.1	-	-	-	-	-	0.1	-	-	-	-	0.2	-	0.1	-	-	-	0.8	-	0.8
i-C13:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1		0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.2	-	0.1	-	-	-	-	-	-	-	-	-
a-C13:0	0.1	0.1	-	-	0.1	-	-	-	-	-	-	-	-	0.1	0.1	-	0.1	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-
i-C14:0	0.9	0.8	0.1	0.3	0.9	0.2	0.6	1.2	2.4	1.5	2.4	0.1	0.1	0.2	0.1	0.8	0.4	0.6	0.7	0.6	2.1	1.1	0.6	0.2	0.1	-	-	-	-
a-C14:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3OH-i-C14:0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
i-C15:0	5.8	6.2	19.5	18.5	6.4	20.7	13.8	20.0	16.6	14.3	12.7	30.5	27.8	21.4	26.4	11.4	20.7	23.6	28.3	0.7	4.4	7.4	-	-	-	-	-	-	-
F-i-C15:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-
a-C15:0	-	2.1	3.6	6.1	3.0	6.1	2.6	4.6	7.1	5.4	10.6	5.4	7.3	7.0	5.5	2.7	-	2.9	3.6	-	-	0.9	-	-	-	-	-	-	-
F-i-C 15:1	-	0.1	0.2	-	0.1	0.2	-	0.1	-	-	-	0.1	-	0.2	0.2	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-
3OH-i-C15:0	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A-a-C15:1	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
a-C15:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.0	-	-	0.1	2.6	-	-	-	-	-	-	-	-
G-i-C15:1	-	-	0.1	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	0.1	-	-	-	0.1	-	-	-	-	-	-	-
i-C16:0	44.1	39.3	7.1	20.1	42.5	5.3	35.6	22.8	27.4	36.1	22.6	4.0	2.6	4.6	3.8	35.8	14.9	20.4	13.9	3.6	27.2	18.6	25.6	3.6	1.4	0.6	-	-	-
3 OH-i-C16:0	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
a-C16:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	0.1	-	-	-	-	-	-	-
H-i-C16:1	1.5	-	0.4	0.7	1.2	-	0.6	0.7	2.0	3.1	0.9	0.1	-	-	0.1	1.0	0.3	0.6	0.6	0.1	0.4	-	-	-	-	-	-	-	-
G-i-C16:1	-	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	-	0.5	-	-	-	-	-
i-C17:0	4.3	6.3	8.5	10.0	4.1	6.8	9.2	3.9	1.7	3.0	2.2	6.1	7.0	10.6	12.1	6.6	10.7	10.9	13.0	-	1.7	0.5	-	-	-	-	-	-	-
a-C17:0	9.9	12.5	15.4	13.2	11.4	12.4	7.4	4.4	3.6	5.1	7.5	8.2	10.9	14.2	11.0	8.9	12.1	7.0	6.5	-	3.1	0.8	-	-	-	-	-	-	-
i-C17:1 ω9c	0.8	4.8	11.0	8.3	6.2	4.1	7.5	1.7	2.5	3.9	1.4	4.4	5.0	6.7	5.7	6.7	6.8	5.5	7.2	-	-		-	-	-	-	-	-	-
a-C17:1 ω9c	-	0.6	1.8	1.1	1.1	0.6	0.4	0.2	0.6	0.8	0.8	0.6	0.7	0.6	0.4	0.6	0.6	0.5	0.8	-	-	0.1	-	-	-	-	-	-	-
i-C18:0	2.8	3.2	0.2	0.9	3.4	0.1	2.8	1.2	0.4	1.7	0.3	-	-	0.1	0.1	3.8	0.7	1.2	0.5	-	0.8	0.2	2.0	0.5	0.1	-	-	-	-
H-i-C18:1	0.4	0.3	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.3	0.2	-	-	0.1	0.2	0.2	0.1	0.2		-	-	-	-	-	-	-	-	-	-
i-19:0	0.1	-	0.1	0.1	-	0.0	0.1	-	-	0.1	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	-	-	-	-	-	-	-	-
I-i- 19:1	0.1	0.2	-	0.1	-	0.1	-	-	-	-	-	-	-	0.1	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.6 Fatty acid compositions of the representative strains (continued)

	----------*M*----------																			--*Mb*--		-*No*-	---------*A*-------				------*N*-----		
Fatty acid	**AL8-2**	**AL10-17**	**D10-9--5**	**ASC19-2-1**	**AL8-8**	**AL10-3**	**AL7-5**	**AL3-9**	**AL4-4**	**AL1-3**	**P0402**	**AL3-16**	**AL9-20**	**AL5-1**	**AL9-1**	**AL9-13**	**AL9-22**	**AL1-16B**	**AL1-15-2**	**AL4-7**	**AL4-8**	**P0417**	**CYP1-5**	**AL4-10**	**CYP1-1B**	**AL7-14**	**P1440**	**P1803**	**P1605**
Branched																													
10-Methyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.0	4.0	2.5	0.8	0.7	1.5	2.1	-	-	-
17:0	11.7	9.3	2.5	1.8	7.0	13.5	2.6	13.4	5.9	9.3	1.6	7.3	5.7	5.6	6.1	8.9	4.3	7.7	1.1	3.1	11.1	27.5	2.3	0.4	0.2	0.4	-	-	-
18:0(TBSA)	2.6	3.7	0.7	0.5	0.8	3.1	1.6	1.0	0.4	1.4	0.3	3.0	1.6	1.7	2.2	1.1	1.5	1.7	0.2	3.1	1.4	0.3	8.7	15.1	15.8	20.2	15.0	17.7	22.2
19:0	-	-	2.0	-	0.2	0.5	-	0.2	-	-	-	0.1	0.1	0.2	0.2	-	0.1	0.2	-	-	-	-	-	-	-	-	-	-	-
Summed feature 1	-	-	0.1	0.2	-	-	0.1	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Summed feature 3	0.1	0.1	0.2	0.9	0.4	-	0.7	0.7	0.8	0.3	0.5	0.4	0.3	0.1	0.1	0.2	0.4	0.1	0.3	2.3	4.4	1.8	1.2	16.3	3.2	3.6	9.7	5.8	9.8
Summed feature 5	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	24.2	-	-	-	-	-	-	-	-	-
Summed feature 6	0.2	0.4	3.3	0.5	0.3	0.8	0.3	-	0.2	0.5	0.3	0.7	0.8	0.9	0.5	0.5	0.9	0.8	0.2	-	-	-	0.2	0.2	-	-	-	-	-

M, Micromonospora; Mb, Microbispora; No, Nonomuraea; A, Actinomadura; N, Nocardia

Summed feature 1 $C_{15:1}$ iso H /$C_{13:0}$ 3OH
Summed feature 3 $C_{16:1\ \omega7c}$/ i-$C_{15:0}$ 2OH
Summed feature 5 $C_{18:2\ \omega6,\ 9c}$/a-$C_{18:0}$
Summed feature 6 $C_{19:1\ \omega9c}$/$C_{19:1\ \omega11c}$

Table 4.7 Differential chemotaxonomic characteristics of the representative strains

***N*-acyl type of Muramic acid**	**Key intermediate sugars**	**Major menaquinones**	**Major fatty acids**	**Strain No.**	**Genus**
N-Glycolyl	Mannose, xylose	MK-9(H_4 ,H_6) MK-10(H_4 ,H_6)	i-$C_{15:0}$, a-$C_{15:0}$, i-$C_{16:0}$, i-$C_{17:0}$, a-$C_{17:0}$, 10-methyl $C_{17:0}$	AL8-2, AL10-17, D10-9-5, ASC19-2-1, AL8-8, AL10-3, AL7-5, AL3-9, AL4-4, AL1-3, P0402, AL3-16, AL9-20, AL5-1, AL9-1, AL9-13, AL9-22, AL1-16B, AL1-15-2	*Micromonospora*
	Mannose, arabinose	MK-8(H_4)	$C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$, 10-methyl $C_{18:0}$	P1803, P1440, P1605	*Nocardia*
N-Acetyl	Madurose	MK-9(H_0 ,H_2,H_4)	$C_{16:0}$, $C_{17:1\omega8c}$, $C_{18:1\omega9c}$, i-$C_{16:0}$, 10-methyl $C_{17:0}$	AL4-7, AL4-8	*Microbispora*
		MK-9(H_4 ,H_6,H_8)	$C_{16:0}$, $C_{18:1\omega9c}$	CYP1-5, CYP1-1B, AL4-10, AL7-14	*Actinomadura*
		MK-9(H_2 ,H_4,H_6)	i-$C_{16:0}$, 10-methyl $C_{17:0}$	P0417	*Nonomuraea*

4.2.1. Characteristics of *Micromonospora*

Thirty-eight strains including AL1-3, AL1-16B, AL1-18A, AL1-15-2, AL2-1, AL2-3, AL3-2, AL3-9, AL3-12, AL3-13, AL3-14-2, AL3-16, AL4-4, AL4-5, AL5-1, AL5-7, AL5-16, AL7-5, AL8-2, AL8-7, AL8-8, AL8-16, AL9-1, AL9-13, AL9-20, AL9-22, AL9-24-1, AL10-3, AL10-17, AL10-5, ASC13-5-1, ASC19-2-1, B10-3-3, B10-3-6, D10-9-5, SM1-1, PSM1-8 and P0402 were assigned in the genus *Micromonospora* based on their phenotypic, genotypic and chemotaxonomic characteristics (Kawamoto, 1989). Twenty-three strains, AL8-2, AL10-17, D10-9-5, ASC19-2-1, AL8-8, AL10-3, AL7-5, AL3-9, AL4-4, AL1-3, P0402, AL3-16, AL9-20, AL5-1, AL9-1, AL9-13, AL9-22, AL5-1, AL9-1, AL9-13, AL9-22, AL1-16B and AL1-15-2 were selected randomly as the representative strains in each group for further chemotaxonomic and genotypic analyses.

For morphological and cultural characteristics, they formed single spores on the substrate mycelium and lack aerial mycelium. The vegetative mycelia were fine, 0.2 to 0.34 μm diameter, and conidial spores were approximately 0.6 to 0.9 μm diameter. The spore surfaces were smooth, rough and warty, and non-motile. The colors of substrate mycelium were moderate yellow to strong orange yellow, and turned to brownish black to black after sporulation. A soluble pigment was not produced. The cultural characteristic of these *Micromonospora* strains on various agar media are shown in Table 1 (Appendix C).

Twenty-three representative strains of *Micromonospora* presented *N*-glycolyl muramic acid in the peptidoglycan. Most of the strains contained ribose, mannose, galactose, xylose, arabinose and glucose as whole cell sugars which corresponded to whole cell sugar pattern D (Lechevalier and Lechevalier, 1970). Their major menaquinones were MK-9(H_4), MK-9(H_6), MK-9(H_8), MK-10(H_4), MK-10(H_6) and MK-10(H_8). Characteristics of phospholipids were diphosphotidylglycerol (DPG), phosphotidylgylcerol (PG), phisphatidylinositol (PI), phosphotidylinositolmannosides (PIMs) and phosphatidylethanolamine (PE) but not phosphotigylcholine (PC) which corresponded to phospholipid pattern II (Lechevalier *et al.,* 1977). The predominants of fatty acids were i-$C_{15:0}$, i-$C_{16:0}$, i-$C_{17:0}$ and a-$C_{17:0}$, corresponding to fatty acid pattern 3b (Kroppenstedt, 1985). The DNA G+C contents ranged from 70.8 to 74.5 mol%.

Phylogenetic tree using the almost complete 16S rRNA gene sequences of the representative strains of *Micromonospora* and the type strains of validly described species.

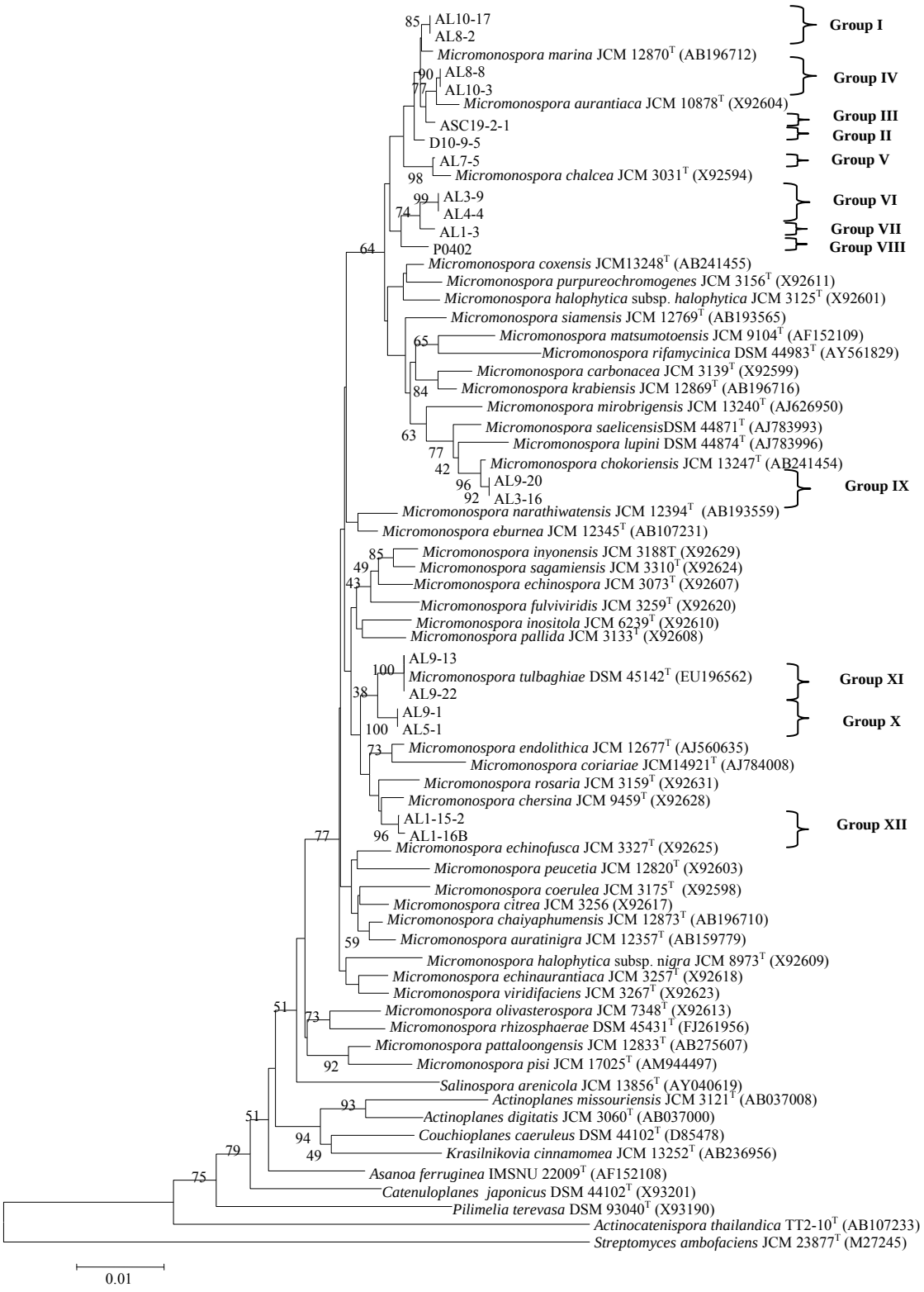

Figure 4.2 Neighbor-joining tree based on almost complete 16S rRNA gene sequences, showing relative among the representative *Micromonospora* strains and the member of genus *Micromonospora*

indicated that 38 strains were divided into 12 groups as shown in Figure 4.2. Groups I, II, III, VI, VII, VIII, IX and X formed distinct phyletic lines in the 16S rRNA gene tree, and the Groups IV, IX, XI, and XII strains formed subclade with *M. aurantiaca* JCM 10878^T, *M. chalcea* JCM 3031^T, *M. chokoriensis* JCM 13247^T and *M. tulbaghiae* DSM 45142^T. The members in each group presented the high values of 16S rRNA gene similarity over 99%.

Group I contained 9 strains, strains AL2-3, AL10-5, AL5-16, AL3-2, AL9-24-1, AL8-2, AL10-17, AL4-5 and AL5-7. Spores of the representative strain were rough. The morphology of strain AL8-2 is shown in Figure 4.3. All strains grew on 1.5 to 5% NaCl, on pH 5 to 8 and at 15 and 40 °C. Production of melanoid pigment was negative. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive. Strains AL3-2, AL8-2 and AL10-17 could reduce nitrate to nitrite (Table 2, Appendix C). All strains utilized D-glucose, lactose, L-arabinose, D-fructose, D-melibiose, D-galactose, cellobiose and sucrose as sole carbon sources but not D-mannitol, D-ribose, glycerol and salicin. Their growths were variable on L-rhamnose, raffinose, inositol and D-xylose (Table 3, Appendix C). Differential characteristics between the representative strains of Group I and closely related *Micromonospora* species are shown in Table 4.8.

Figure 4.3 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL8-2 on YMA

Strains AL8-2 and AL10-17 were selected to be the representative strains in this group for phylogenetic tree analysis using the almost complete 16S rRNA gene sequences. The strains AL8-2 (1484 nt) and AL10-17 (1464 nt) were placed in a monophyletic cluster, and closely related to *M. marina* JCM 12870^T (99.7%), *M. aurantiaca* JCM 10878 ^{T} (99.4%) and *M. chalcea* JCM 3031^T (99.2%). The level of DNA-DNA relatedness among strain AL8-2 and *M. marina* JCM 12870^T was 28.5%

(Table 4.9). These values were obtained from the means of two determinations and were below the threshold value of 70 % for distinguishing genomic species (Wayne *et al*., 1987). It was evident from the phenotypic and genotypic data that strains AL8-2 and AL10-17 should be a new species of the genus *Micromonospora*.

In addition, these strains were closest related to Group II (99.8%) so these possibly were identified as same species with Group II. They should be confirmed by DNA-DNA hybridization.

Table 4.8 Differential characteristics of strains AL8-2, AL10-17 and related *Micromonospora* species. +, positive; w, weakly positive; -, negative; ND, not determined

Characteristics	**AL8-2**	**AL10-17**	**D10-9-5**	**JCM 12870^T**	**JCM 10878^T**	**JCM 3031^T**
Max. NaCl tolerance(%,w/v)	5	5	5	7	4	5
Growth at pH 5	+	+	-	+	+	-
Peptonization of milk	+	+	+	-	+	-
Nitrate reduction	+	+	-	-	+	-
Utilization of:						
Raffinose	+	+	-	-	-	+
Lactose	+	+	w	-	w	+
L-Arabinose	+	+	+	-	+	w
D-Fructose	+	+	-	-	+	-
D-Melibiose	+	+	w	-	-	+
Glycerol	-	-	-	+	+	+
Salicin	-	-	-	+	+	+
D-Galactose	+	+	-	+	+	+
Cellobiose	+	+	-	+	+	+
Sucrose	+	+	+	-	-	-
D-Xylose	+	+	w	-	-	-

Table 4.9 DNA base composition and DNA-DNA relatedness of strains AL8-2, AL10-17 and related *Micromonospora* species

Strain	G+C content (mol%)	DNA-DNA relatedness (%) with labeled strains*
		AL8-2
AL8-2	73.6	100 ±0.11
AL10-17	72.4	105.8±0.11
M.marina JCM 12870^{T}	72.0[§]	28.5±0.06

* Values obtained from two independent determinations.

[§]Data from Tanasupawat *et al*. (2010)

Group II contained strain D10-9-5. Spores at maturity were spherical to oval, appeared to be rough, and non-motile. The color of the vegetative mycelium was dark grayish yellowish brown on YMA as shown in Figure 4.4. Production of melanoid pigment and nitrate reduction were negative. Strain D10-9-5 grew on 1.5 to 5% NaCl, on pH 6 to 8 and at 15 to 40 °C. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive. Strain D10-9-5 inhibited the growth of *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 and *M. luteus* ATCC 9341 on YMA for 14 days (Table 2, Appendix C). They utilized D-glucose, inositol, L-arabinose, D-melibiose, sucrose and D-xylose as sole carbon sources but not D-mannitol, L-rhamnose, raffinose, lactose, D-fructose, D-ribose, glycerol, salicin, D-galactose and cellobiose (Table 3, Appendix C). Differential characteristics between strain D10-9-5 and closely related *Micromonospora* species are shown in Table 4.10

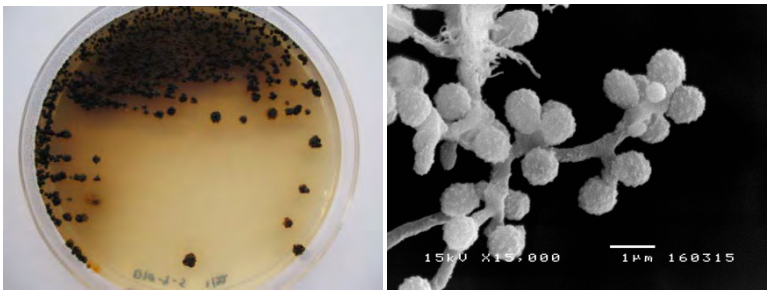

Figure 4.4 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. D10-9-5 on YMA

On the basis of 16S rRNA gene sequence and phylogenetic tree analysis, strain D10-9-5 (1495 nt) was closely related to *M. marina* JCM 12870^{T} (99.6%) followed by *M.*

coxensis JCM 13248^T (99.4%), *M. aurantiaca* JCM 10878^T (99.3%), *M. humi* JCM 15292^T (99.3%) and *M. chalcea* JCM 3031^T (99.1%). The levels of DNA-DNA relatedness between strain D10-9-5 and closely related species were less than 55.8% as shown in Table 4.11. These values were obtained from the means of four determinations and were below the threshold value of 70 % for distinguishing genomic species (Wayne *et al*., 1987). Therefore, it was evident from the genotypic and phenotypic data that strain D10-9-5 should be a new species in the genus *Micromonospora*.

Table 4.10 Differential characteristics of strain D10-9-5 and related *Micromonospora* species. +, positive; w, weakly positive; -, negative; ND, not determined

Characteristics	**D10-9-5**	**JCM 12870^T**	**JCM13248^T**	**JCM 10878^T**	**P0402^T**	**JCM 3031^T**	**JCM 3125^T**
Maximum NaCl tolerance (%)	5	7	3	4	3	5	4
Growth at pH 5	-	+	w	+	+	-	-
Decomposition of tyrosine	-	+	-	+	-	-	-
Peptonization of milk	+	-	-	+	+	-	+
Nitrate reduction	-	-	+	+	-	-	+
Utilization of:							
D-Mannitol	-	-	w	-	-	-	-
L-Arabinose	+	-	+	w	+	w	+
L-Rhamnose	-	-	+	-	-	-	-
Raffinose	-	-	+	-	-	+	+
Inositol	+	-	w	-	-	-	-
D-Fructose	-	-	+	+	-	+	+
Glycerol	-	+	+	-	-	-	+
Salicin	-	+	+	+	+	+	-
D-Galactose	-	+	+	w	+	+	+
Cellobiose	-	+	+	+	+	+	-
Lactose	-	-	+	-	+	+	+
Mellibiose	w	-	+	-	-	+	+

Table 4.11 DNA base composition and DNA-DNA relatedness of strain D10-9-5 and related *Micromonospora* species

Strains	G+C content (mol%)	DNA-DNA relatedness (%) with labeled strains*	
		D10-9-5	JSM 12870^{T}
D10-9-5	73.6	100±0.02	21.8±0.51
M. marina JCM 12870 ^{T}	72.0[§]	40.8±0.2	100±0.03
M. coxensis JCM 13248 ^{T}	73.0[†]	7.9±0.06	6.4±0.09
M. aurantiaca JCM 10878^{T}	72.0[†]	37.8±0.15	55.8±0.35
M. humi P0402^{T}	73.0	48.7±0.09	30.9±0.42
M. halophytica JCM 3125^{T}	72.5[†]	15.6±0.14	8.6±0.12
M. chalcea JCM 3031^{T}	72.0[†]	24.2±0.11	24.9±0.58

*Values obtained from four independent determinations.

[†]Data from Ara and Kudo (2007)

[§]Data from Tanasupawat *et al*. (2010)

Group III contained strain ASC19-2-1. Strain ASC19-2-1 produced well-developed and branch substrate hyphae. Rough spores were borne singly on substrate hyphae. The color of the vegetative mycelium on YMA was brownish gray as shown in Figure 4.5. Strain ASC19-2-1 grew on 1.5 to 4% NaCl, on pH 5 to 8 and at 15 to 40 °C. Milk Peptonization, coagulation, gelatin liquefaction and starch hydrolysis were positive. Nitrate reduction was negative (Table 2, Appendix C). Strain ASC19-2-1 utilized D-glucose, lactose, D-fructose, glycerol, salicin, D-galactose, cellobiose, sucrose and D-xylose as sole carbon sources but not D-mannitol, L-rhamnose, raffinose, inositol, L-arabinose, D-ribose and D-melibiose (Table 3, Appendix C).

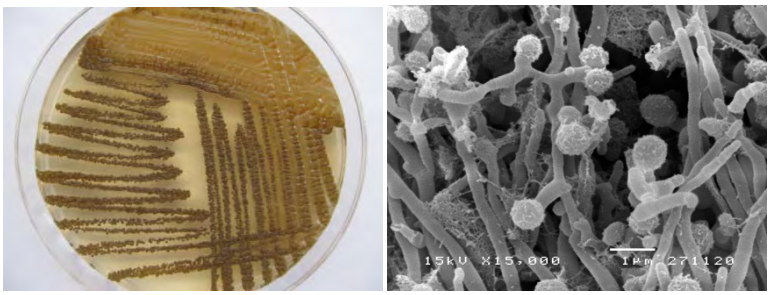

Figure 4.5 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. ASC19-2-1 on YMA

On the basis of 16S rRNA gene sequence and phylogenetic tree construction revealed that strain ASC19-2-1(1493 nt) was closely related with *M. marina* JCM 12870^{T} (99.7%). The level of DNA-DNA relatedness between strain ASC19-2-1 and closely related species, *M. marina* JCM 12870^{T}, was 83.3%. These values were obtained from the means of two determinations and were higher the threshold value of 70 % for distinguishing genomic species (Wayne *et al*., 1987). Therefore, strain ASC19-2-1 was identified as *M. marina* (Tanasupawat *et al*., 2010).

Group IV contained 6 strains, strains AL8-7, AL8-8, AL10-3, B10-3-3, B10-3-6 and ASC13-5-1. Strain AL10-3 produced smooth spores at the end of hyphae. The color of the vegetative mycelium was dark brown. Colonial appearance is shown in Figure 4.6. They grew on 1.5 to 5% NaCl, on pH 6 to 8 and at 15 to 40 °C. Peptonization of milk, gelatin liquefaction, nitrate reduction and starch hydrolysis were positive (Table 2, Appendix C). They utilized D-glucose, L-arabinose, D-fructose, glycerol, D-galactose, cellobiose, sucrose and D-xylose as sole carbon sources but not D-mannitol and D-ribose. Their growths were variable on L-rhamnose, raffinose, inositol, lactose, D-mellibiose and salicin (Table 3, Appendix C).

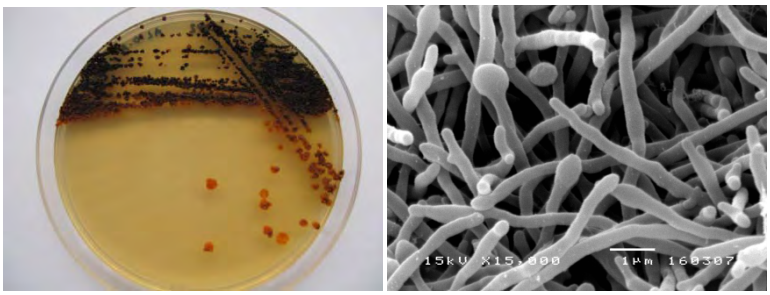

Figure 4.6 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL10-3 on YMA

Strains AL8-8 and AL10-3 were selected to be the representative strains in this group for phylogenetic tree analysis using the almost complete 16S rRNA gene sequences. Strains AL8-8 (1506 nt) and AL10-3 (1501 nt) were closely related to *M. aurantiaca* JCM 10878^{T} (99.7%) followed by *M. marina* JCM 12870^{T} (99.6%), *M. coxensis* JCM 13248 ^{T} (99.4%), *M. chalcea* JCM 3031^{T} (99.1%) and *M. halophytica* JCM 3125^{T} (99.1%). The levels of DNA-DNA relatedness between strain AL8-8 and closest

related species, *M. aurantiaca* JCM 10878^T, were 103.6%. Therefore, they were identified as *M. aurantiaca* (Sveshnikova *et al*., 1969).

Group V contained strain AL7-5. Strain AL7-5 produced many rough singly spores on substrate hyphae. The color of the vegetative mycelium was brownish black on YMA. Colonial appearance is shown in Figure 4.7. This strain grew on 1.5 to 4% NaCl, on pH 6 to 8 and at 20 to 45 °C. Peptonization and coagulation of milk, gelatin liquefaction, nitrate reduction and starch hydrolysis were positive (Table 2, Appendix C). This strain utilized glucose, raffinose, lactose, L-arabinose, D-mellibiose, glycerol, D-galactose, cellobiose, sucrose, D-xylose and weakly on D-fructose but not D-mannitol, inositol, D-ribose and salicin (Table 3, Appendix C).

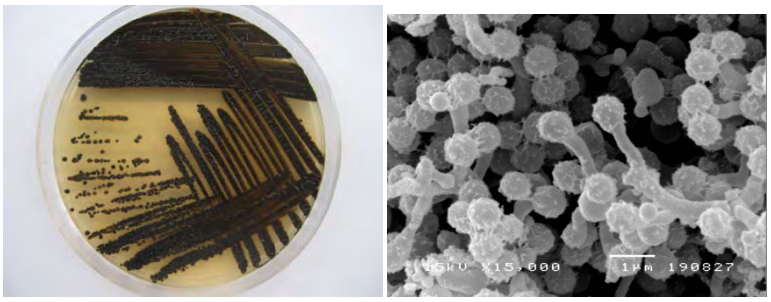

Figure 4.7 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL7-5 on YMA

On the basis of 16S rRNA gene sequence and phylogenetic analysis, strain AL7-5 (1485 nt) was closely related to *M. chalcea* JCM 3031^T (99.7%), which was supported by a high bootstrap value (Figure 4.2). This strain differentiated in the peptonization of milk, nitrate reduction, the maximum NaCl for growth and utilization of carbon from *M. chalcea* JCM 3031^T are shown in Table 4.19 whereas the level of DNA-DNA relatedness between strain AL7-5 and closely related species, *M. chalcea* JCM 3031^T, was 92.6%. Therefore, strain AL7-5 was identified as *M. chalcea* (Skerman *et al*., 1980).

Group VI contained 5 strains, strains AL2-1, AL3-9, AL3-12, AL4-4 and AL8-16. Strains AL3-9 and AL4-4 were the representative strains in this group. Strain AL4-4 produced smooth spores on hyphae. The color of the vegetative mycelium was brownish black on YMA. Colonial appearance is shown in Figure 4.8. All strains grew on 1.5 to 5% NaCl, on pH 5 to 8 and at 20 to 40 °C. Peptonization and coagulation of milk, gelatin

liquefaction and starch hydrolysis were positive. Nitrate reduction was negative (Table 2, Appendix C). They utilized D-glucose, L-rhamnose, lactose, L-arabinose, D-melibiose, glycerol, salicin, D-galactose, and cellobiose as sole carbon sources but not D-mannitol, raffinose, inositol, D-ribose and sucrose. The utilizations of D-fructose and D-xylose were variable (Table 3, Appendix C). Differential characteristics of strain AL4-4 and related *Micromonospora* species are shown in Table 4.12.

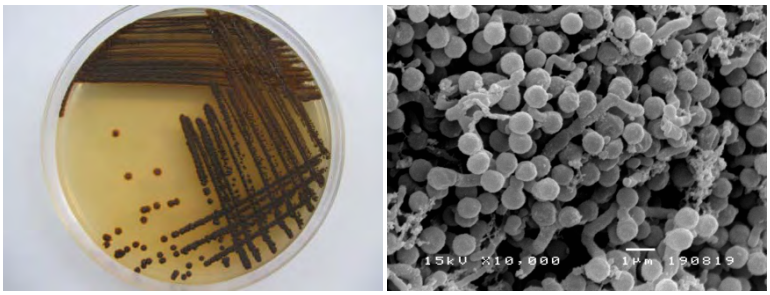

Figure 4.8 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL4-4 on YMA

On the basis of 16S rRNA gene sequence and phylogenetic analysis, strains AL3-9 (1458 nt) and AL4-4 (1459 nt) were closely related to *M. siamensis* JCM 12769^T (99.0%), *M. coxensis* JCM 13248^T (98.9%) and *M. krabiensis* JCM 12869^T (98.9%). In addition, strains AL3-9 and AL4-4 were closely related to Group VII (99.6%), Group II (99.1%) and Group VIII (99.1%). The levels of DNA-DNA relatedness between strain AL4-4 and closely related species were less than 56.8% (Table 4.13). These values were obtained from the means of two determinations and were below the threshold value of 70 % for distinguishing genomic species (Wayne *et al*., 1987). It was evident from the genotypic and phenotypic data that they should be a new species in the genus *Micromonospora*.

Table 4.12 Differential characteristics of strain AL4-4 and related *Micromonospora* species. +, positive ; w, weakly positive ; -, negative

Characteristics	AL4-4	AL3-9	AL1-3 (VII)	D10-9-5 (II)	P0402 (VIII)	JCM 12769^T
Maximum NaCl tolerance (%)	5	5	2	5	5	3
Growth at pH 5	+	+	-	-	+	+
Utilization of:						
L-Rhamnose	w	w	+	-	-	-
Raffinose	+	+	-	+	-	-
Inositol	-	-	-	+	-	-
Lactose	w	w	+	w	+	-
L-Arabinose	w	w	w	+	+	-
D-Fructose	+	+	+	-	-	-
D-Melibiose	+	+	+	w	-	-
D-Galactose	+	+	+	-	+	+
Sucrose	-	-	+	+	-	-
D-Xylose	+	+	-	w	-	+

Table 4.13 DNA base composition and DNA-DNA relatedness of strain AL4-4 and related *Micromonospora* species

Strains	G+C content (mol%)	DNA-DNA relatedness (%) with labeled strains*	
		AL4-4	D10-9-5
AL4-4	74.3	100±0.02	5.2±0.01
AL3-9	74.5	111.5±0.09	6.1±0.03
AL1-3 (VII)	74.1	56.8±0.02	20.2±0.01
D10-9-5 (II)	73.6	41.6±0.30	100±0.19
P0402 (VIII)	73.4	54.1±0.00	56.8±0.01
M. siamensis TT2-4 ^{T}	73.0[§]	24.3±0.02	31.7±0.01
M. coxensis JCM 13248^T	73.0[†]	11.6±0.02	9.5±0.02

*Values obtained from two independent determinations.

[†]Data from Ara&Kudo (2007); [§]Data from Tanasupawat *et al*. (2010)

Group VII contained strain AL1-3. Strain AL1-3 produced rough spores on hyphae. The color of the vegetative mycelium on YMA was brownish black and the colonial appearance is shown in Figure 4.9. Strain AL1-3 grew on 1.5 to 2% NaCl, on pH 6 to 8 and at 15 to 37 °C. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive. Nitrate reduction was negative (Table 2, Appendix C). This strain utilized D-glucose, L-rhamnose, lactose, L-arabinose, D-fructose, D-melibiose, glycerol, salicin, D-galactose, cellobiose and sucrose as sole carbon sources but not D-mannitol, raffinose, inositol, D-ribose and D-xylose (Table 3, Appendix C). Differential characteristics of strain AL1-3 and related *Micromonospora* species are shown in Table 4.14

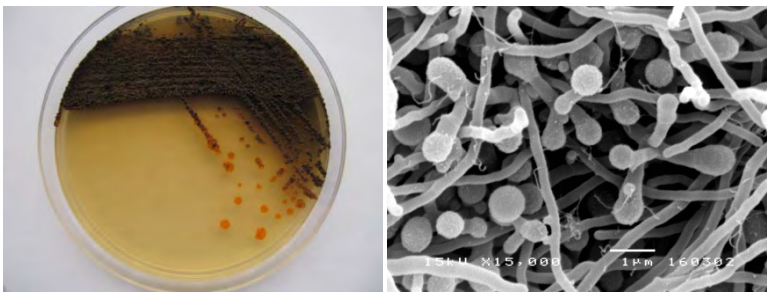

Figure 4.9 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL1-3 on YMA

On the basis of 16S rRNA gene sequence and phylogenetic analysis, strain AL1-3 (1487 nt) was closely related to Group VI (99.6%), Group VIII (99.4%), Group II (99.1%) and *M. coxensis* JCM 13248^{T} (99.1%). The levels of DNA-DNA relatedness between strain AL1-3 and closely related species were less than 56.8% (Table 4.15). These values were obtained from the means of two determinations and were below the threshold value of 70 % for distinguishing genomic species (Wayne *et al*., 1987). It was evident from the genotypic and phenotypic data that strain AL1-3 should be a new species in the genus *Micromonospora*.

Table 4.14 Differential characteristics of strain AL1-3 and related *Micromonospora* species. +, positive ; w, weakly positive ; -, negative

Characteristics	**AL1-3**	**AL4-4 (VI)**	**P0402 (VIII)**	**D10-9-5 (II)**	**JCM 13248^T**
Maximum NaCl tolerance (%)	2	5	5	5	4
Growth at pH 5	-	+	+	-	w
Peptonization of milk	+	+	+	+	-
Nitrate reduction	-	-	-	-	+
Utilization of:					
L-Rhamnose	+	w	-	-	-
D-Mannitol	-	-	-	-	w
Raffinose	-	-	-	+	+
Lactose	+	w	+	w	+
L-Arabinose	w	w	+	+	+
D-Fructose	+	+	-	-	+
D-Melibiose	+	+	-	w	+
Glycerol	+	+	-	-	+
Salicin	+	+	+	-	+
D-Galactose	+	+	+	-	+
Cellobiose	+	+	-	-	+
Sucrose	+	-	-	+	+
D-Xylose	-	+	-	w	+

Table 4.15 DNA base composition and DNA-DNA relatedness of strain AL1-3 and related *Micromonospora* species

Strains	G+C content (mol%)	DNA-DNA relatedness (%) with labeled strains*		
		AL1-3	AL4-4	P0402
AL1-3	74.1	100±0.03	56.8 ±0.02	54.4±0.04
AL4-4 (VI)	74.5	55.5±0.04	100±0.10	26.1±0.00
P0402 (VIII)	73.0	52.2±0.02	54.1±0.03	100±0.01
D10-9-5 (VII)	73.6	20.2±0.01	41.6±0.03	56.8±0.01
M. coxensis JCM 13248^{T}	73.0†	21.6±0.00	11.6±0.02	13.7±0.02

*Values obtained from two independent determinations.

†Data from Ara and Kudo (2007)

Group VIII contained strain P0402. The color of the vegetative mycelium was brownish black on YMA. Spores were spherical to oval which appeared to be smooth and non-motile as shown in Figure 4.10. Strain P0402 grew on 1.5 to 6% NaCl, on pH 5 to 8 and at 15 to 37 °C. Peptonization of milk and gelatin liquefaction was positive. Nitrate reduction and starch hydrolysis were negative (Table 2, Appendix C). This strain utilized L-arabinose, D-galactose, D-glucose and lactose as sole carbon sources but not D-fructose, glycerol, raffinose, D-mannitol, L-rhamnose, inositol, D-melibiose, D-cellobiose, D-ribose and salicin (Table 3, Appendix C). Differential characteristics of strain P0402 and related *Micromonospora* species are shown in Table 4.16.

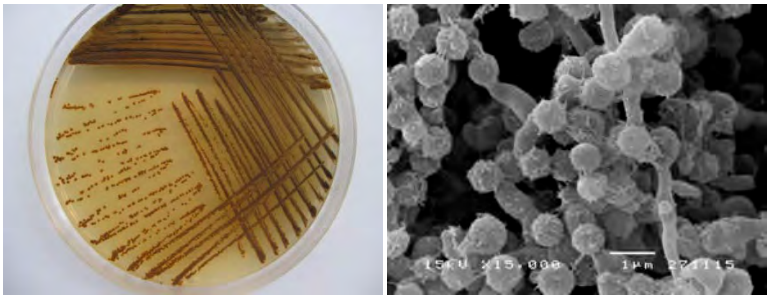

Figure 4.10 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. P0402 on YMA

Phylogenetic tree analysis using 16S rRNA gene sequences revealed that a close relationship between strain P0402 (1480 nt) and *M. coxensis* JCM 13248 ^{T} (99.0 %), *M. eburnea* JCM 12345^{T} (99.0%), *M. marina* JCM 12870^{T} (98.9%), *M. halophytica* JCM 3125 ^{T} (98.7%), *M. chalcea* JCM 3031^{T} (98.7%), *M. purpureochromogenes* JCM 3156 ^{T} (98.6%) and *M. aurantiaca* JCM 10878^{T} (98.5%). The levels of DNA-DNA relatedness between strain P0402 and closely related species were less than 15.19% (Table 4.17). It was evident from the genotypic and phenotypic data that strain P0402 should be a new species in the genus *Micromonospora*.

Table 4.16 Differential characteristics of strain P0402 and related *Micromonospora* species. +, positive; w, weakly positive; -, negative

Characteristics	**P0402**	**JCM 13248^{T}**	**JCM 12345^{T}**	**JCM 12870^{T}**	**JCM 3125^{T}**	**JCM 3031^{T}**	**JCM 3156^{T}**	**JCM 10878^{T}**
Maximum NaCl tolerance (%)	5	3	4	7	4	5	1.5	4
Growth at pH 5	+	w	+	+	-	-	-	+
Decomposition of tyrosine	-	-	-	+	-	-	-	+
Utilization of:								
L-Arabinose	+	+	-	+	+	w	-	w
D-Fructose	-	+	-	-	+	w	+	+
Glycerol	-	+	+	w	w	-	-	-
Lactose	+	+	+	-	+	+	w	-
D-Melibiose	-	+	+	+	+	+	+	+
Raffinose	-	+	+	+	+	+	+	-
D-Ribose	-	-	-	+	-	-	-	-

Table 4.17 DNA base composition and DNA-DNA relatedness of strain P0402 and related *Micromonospora* species

Strains	G+C content (mol%)	DNA-DNA relatedness (%) with labelled strains*
		P0402
P0402	73.0	100 ± 0.03
M. coxensis JCM 13248 ^{T}	73.0†	6.33 ± 0.01
M. eburnea JCM 12345 ^{T}	71.5‡	7.17 ± 0.00
M. marina JCM 12870^{T}	72.0§	15.19 ± 0.01
M. halophytica JCM 3125^{T}	72.5†	10.13 ± 0.01
M. chalcea JCM 3031^{T}	72.0†	10.13 ± 0.01
M. purpureochromogenes JCM 3156^{T}	73.0†	4.64 ± 0.01
M. aurantiaca JCM 10878^{T}	72.0†	6.75 ± 0.01

*Values are expressed as the means of three determinations.

†Data from Ara & Kudo (2007); ‡Data from Thawai *et al.* (2005a); §Data from Tanasupawat *et al*. (2010)

Group IX contained 2 strains, strains AL3-16 and AL9-20. Strain AL9-20 produced single spores on hyphae. The color of the vegetative mycelium was strong orange yellow on YMA. Colonial apperarance is shown in Figure 4.11. They grew on 1.5 to 5% NaCl, on pH 6 to 8 and at 15 to 37 °C. Gelatin liquefaction and starch hydrolysis were positive but peptonization of milk and nitrate reduction were negative (Table 2, Appendix C). They utilized D-glucose, D-mannitol, raffinose, lactose, L-arabinose, D-fructose, sucrose and weakly D-melibiose but not L-rhamnose, inositol, D-ribose, glycerol, salicin, D-galactose and cellobiose (Table 3, Appendix C).

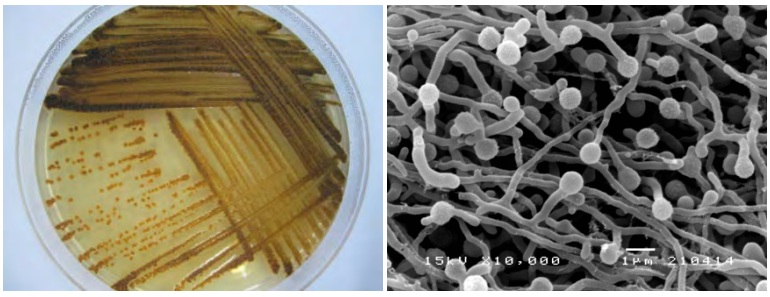

Figure 4.11 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL9-20 on YMA

Phylogenetic analysis using 16S rRNA gene sequences revealed that strains AL3-16 (1496 nt) and AL9-20 (1482 nt) were closely related to *M. chokoriensis* JCM 13247^{T} (99.8%) and *M. saelicensis* JCM 44871^{T} (99.3%). On the basis of phenotypic and 16S rRNA gene similarity, these strains were identified as *M. chokoriensis* (Ara and Kudo, 2007).

Group X contained 4 strains, strains AL5-1, AL9-1, SM1-1 and PSM1-8. Strains AL5-1 and AL9-1 were the representative strains in this group. Strain AL5-1 produced single spores on hyphae. The color of the vegetative mycelium was black on YMA. Colonial apperarance is shown in Figure 4.12. They grew on 1.5 to 4% NaCl, on pH 6 to 8 and at 15 to 45 °C. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive. Nitrate reduction was negative (Table 2, Appendix C). They utilized D-glucose, D-mannitol, L-rhamnose, raffinose, inositol, lactose, L-arabinose, D-fructose, D-mellibiose, glycerol, salicin, D-galactose, cellobiose and sucrose as sole carbon sources but not D-ribose and D-xylose (Table 3, Appendix C).

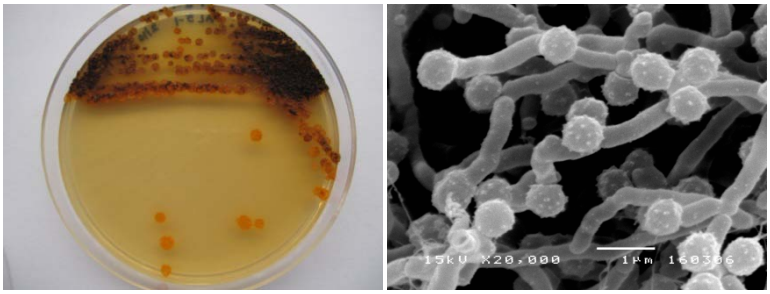

Figure 4.12 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL5-1 on YMA

Phylogenetic analysis using 16S rRNA gene sequences revealed that strains AL5-1(1486 nt) and AL9-1 (1457 nt) were closely related to *M. tulbaghiae* DSM 45142 ^{T} (99.4%), *M. marina* JCM 12870 ^{T} (99.3%) and *M. endolithica* JCM 12677^{T} (99.3%). They were possibly identified as new species in the genus *Micromonospora*, however they should be confirmed by DNA-DNA hybridization.

Group XI contained 4 strains, strains AL9-13, AL9-22, AL3-13 and AL3-14-2. Strains AL9-13 and AL9-22 were the representative strains in this group. Strain AL9-13 produced rough spores and the vegetative mycelium color was black. Colonial appearance

and spore morphology are shown in Figure 4.13. They grew on 1.5 to 4% NaCl, on pH 5 to 8 and at 15 to 45 °C. Peptonization and coagulation of milk, gelatin liquefaction, nitrate reduction and starch hydrolysis were positive (Table 2, Appendix C). They utilized D-glucose, L-arabinose, D-fructose, salicin, cellobiose, sucrose, D-xylose, and weakly L-rhamnose, glycerol and D-galactose but not D-mannitol, raffinose, inositol, lactose, D-ribose and D-melibiose (Table 3, Appendix C).

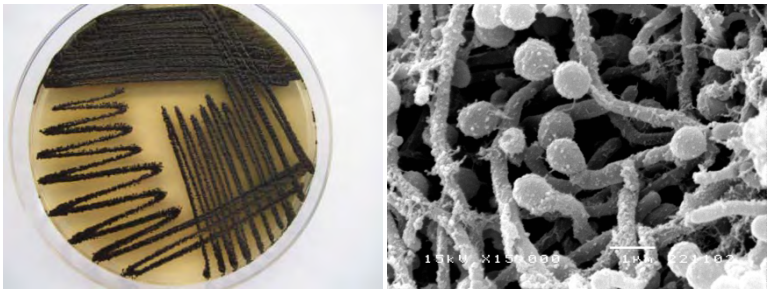

Figure 4.13 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL9-13 on YMA

Phylogenetic analysis using 16S rRNA gene sequences revealed that strains AL9-13 (1460 nt) and AL9-20 (1482 nt) were *M. tulbaghiae* DSM 45142 ^{T} by showing 100% similarity. Therefore, they were identified as *M. tulbaghiae* (Kirby and Meyers, 2010).

Group XII comprised of 3 strains, strains AL1-15-2, AL1-16B and AL1-18A. Strains AL1-15-2 and AL1-16B were the representative strains in this group. The strain AL1-15-2 produced warty spores on the substrate hyphae and the vegetative mycelium color was black. The colonial appearance and spore morphology are shown in Figure 4.14. They grew on 1.5 to 3% NaCl, on pH 5 to 8 and at 20 to 37 °C. Milk peptonization, gelatin liquefaction, nitrate reduction and starch hydrolysis were negative (Table 2, Appendix C). They utilized D-glucose, raffinose, L-arabinose, D-fructose, salicin, D-galactose, cellobiose, sucrose and D-xylose as sole carbon sources but not D-mannitol, inositol, lactose, D-ribose and D-melibiose (Table 3, Appendix C).

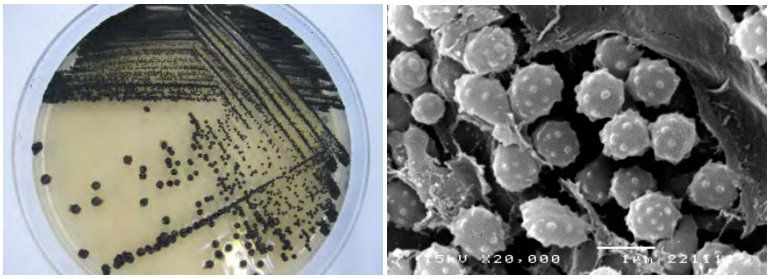

Figure 4.14 The colonial appearance and scanning electron micrograph of *Micromonospora* sp. AL1-15-2 on YMA

Phylogenetic analysis using 16S rRNA gene sequences revealed that strains AL1-15-2 (1479 nt) and AL1-16B (1505 nt) were closely related to *M. chersina* JCM 9459^T (99.5, 99.4%), *M. rosaria* JCM 3159^T (99.3, 99.2%) and *M. endolithica* JCM 12677^T (99.1, 99.1%). The levels of DNA-DNA relatedness between strain AL1-15-2 and closest related species were higher than 70% as shown in Table 4.18. Therefore, strains AL1-15-2 and AL1-16B were identified as *M. chersina* (Tomita *et al*., 1992).

Table 4.18 DNA base composition and DNA-DNA relatedness of strain AL1-15-2 and related *Micromonospora* species

Strains	**G+C content (mol%)**	**DNA-DNA relatedness (%) with labelled strains***
		M. chersina JCM 9459^T
AL1-15-2	72.1	87.8 ±0.13
AL1-16B	72.0	78.4 ±0.12
M. chersina JCM 9459^T	72.9[a]	100±0.01
M. rosaria JCM 3159^T	72.9 [a]	21.6±0.08

*Values are expressed as the means of two determinations.

[a]Data from Thawai *et al.* (2005b)

Table 4.19 Differential characteristics of 12 Groups of *Micromonospora* strains and related species

Strains 1, Group I; 2, Group II; 3, Group III; 4, Group IV; 5, Group V; 6, Group VI; 7, Group VII; 8, Group VIII; 9, Group IX; 10, Group X; 11, Group XI; 12, Group XII; 13, *M. marina* JCM 12870^T ; 14, *M. aurantiaca* JCM 10878^T ; 15, *M. chalcea* JCM 3031^T ; 16, *M. halophytica* JCM 3125; 17, *M. coxensis* JCM 13248^T ; 18, *M. purpureochromogenes* JCM 3156^T ; 19, *M. chokoriensis* JCM 13247^T ; 20, *M. siamensis* JCM 12769^T; 21, *M. chaiyaphumensis* JCM 12873^T; 22, *M. auratinigra* JCM 12357^T; 23, *M. endolithica* JCM 12677^T; 24, *M. coriariae* JCM 14921^T; 25, *M. rosaria* JCM 3159^T; 26, *M. chersina* JCM 9459^T; 27, *M. mastumotoensis* JCM 9104^T; 28, *M. krabiensis* JCM 12869^T; 29, *M. eburnea* JCM 12345^T. +, positive; w, weakly positive; -, negative; nd, no data

characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**
Max. NaCl(%,w/v)	**5**	**5**	**4**	**5**	**4**	**5**	**2**	**5**	**5**	**4**	**4**	**3**	**5**	**3**	**3**	**4**	**4**	**1.5**	**5**	**5**	**4**	**2**	**2**	**7**	**2**	**4**	**3**	**4**	**4**
Growth at pH 5	+	-	+	+	-	+	-	+	-	+	+	+	+	+	-	-	+		-	+	+	+	+	+	-	-		+	+
Growth at 40 °C	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+
Growth at 45 °C	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-	-	+	-	-	-	+	-	-	-	+	+	-	+	+
Peptonization of milk	+	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	-	+	-	+	+	**w**	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	-	+	+	-	-	-	-	-	+	-	-	+	-	+	+	**w**	-	-	-	-	-	+	-	+	+	-	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	**w**	-	+	+	+	+	+	+	+	+	+	+	+
Utilization of:																													
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	-	-	-	-	-	-	-	-	+	**w**	-	-	-	-	**w**	-	-	-	+	-	-	-	**w**	-	-	-	-	+	**w**
L-Rhamnose	**+(-3)**	-	-	**w(-2)**	-	**w**	+	-	-	**w**	**w**	**+(-1)**	-	-	-	-	-	-	**w**	**w**	-	-	-	-	-	-	-	**w**	+
L-Raffinose	**-(+3)**	-	-	**+(-1)**	+	-	-	-	+	+	-	+	-	-	+	+	+	+	+	+	**w**	+	-	-	-	-	+	+	+
Inositol	**-(+2)**	+	-	+	-	-	-	-	-	**w(-2)**	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-		
Lactose	+	-	**w**	**w(-2)**	+	+	+	-	+	+	-	-	-	**w**	+	**w**	+	**w**	+	+	+	+	+	-	-	+	-	**w**	+
L-Arabinose	+	+	+	+	+	**w**	**w**	**w**	+	+	+	+	-	+	**w**	+	+	+	+	+	+	+	+	-	**w**	-	-	+	+	-
D-Fructose	+	-	**w**	+	**w**	**+(-2)**	+	-	+	+	+	-	-	+	-	+	+	+	+	**w**	+	+	-	-	-	**w**	+	+	-
D-Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	**w**	-	-
D-Melibiose	+	**w**	-	**+(-2)**	+	+	+	-	**w**	+	-	-	-	-	+	**w**	+	+	**w**	+	+	+	-	-	-	+	-	+	+
Glycerol	-	-	+	**w**	+	+	+	+	-	**w**	**w**	**+(-1)**	+	+	+	+	+	-	-	-	**w**	-	+	+	**w**	-	-	-	+
Salicin	-	-	+	**w(-3)**	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	**w**	**w**	**w**	**w**	-	+	+	+
D-Galactose	**+(w4)**	-	+	+	+	+	+	+	-	+	**w**	+	+	+	+	+	+	+	-	+	+	+		-	**w**	+	+	+	**w**
Cellobiose	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	**nd**	+	**w**	**w**	+	+	+	-	+
Sucrose	+	+	**w**	+	+	-	+	-	+	+	+	+	-	-	-	+	+	+	+	**nd**	**nd**	**nd**	-	-	-	+	**nd**	**nd**	**nd**
D-Xylose	**-(+4)**	**W**	**w**	**+(w3)**	+	**-(w1)**	-	-	-	**-(w1)**	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-	**nd**	**nd**	+	+

4.2.2. Characteristics of *Microbispora*

Strains AL4-7 and AL4-8 were assigned in the genus *Microbispora* based on their phenotypic, genotypic and chemotaxonomic characteristics (Nonomura and Ohara, 1957).

They produced longitudinally paired spores on aerial hyphae when cultured on oatmeal nitrate agar for 21 days. The sporophores with paired spores were branched alternately from the vegetative mycelium. The substrate mycelia were 0.34 to 0.39 µm in diameter. Each spore was oval and their size was approximately 0.95 to 1.15 µm in diameter. The spore surfaces were smooth and non-motile. The colors of substrate mycelium were deep yellowish pink on YMA agar as shown in Figure 4.15. The cultural characteristics are shown in Table 1(Appendix C). Strains AL4-7 and AL4-8 grew on 1.5 to 2% NaCl, on pH 6 to 8 and at 20 to 40 °C. Peptonization of milk, gelatin liquefaction, starch hydrolysis and nitrate reduction were negative (Table 2, Appendix C). They utilized D-mannitol, L-rhamnose, raffinose, inositol, L-arabinose, D-fructose, glycerol, cellobiose, sucrose and D-xylose as sole carbon sources but not lactose, D-ribose, D-melibiose, salicin and D-galactose (Table 3, Appendix C). Differential characteristics of strains AL4-7, AL4-8 and related *Microbispora* species are shown in Table 4.20.

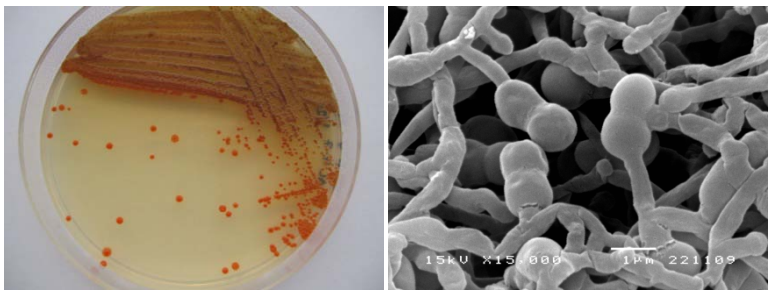

Figure 4.15 The colonial appearance on YMA and scanning electron micrograph of *Microbispora* sp. AL4-7 on ON

Strains AL4-7 and AL4-8 contained *N*-acetyl type of muramic acid in the peptidoglycan in the cell wall. An analysis of whole cell hydrolysates offered ribose, madurose, galactose and glucose which corresponded to whole cell sugar pattern B (Lechevalier and Lechevalier, 1970). Predominant menaquinones were MK-9(H_2), MK-9(III, VII H_4) and MK-9(H_6). The polar lipid profile was DPG, PE, PME, OH-PE, lyso-PE and NPG, corresponded to phospholipid pattern IV (Lechevalier *et al.,* 1977). The fatty acid profile was characterized by major amounts of $C_{16:0}$, iso-$C_{16:0}$, $C_{17:1\omega8c}$, $C_{18:1\omega9c}$

and 10-methyl $C_{17:0}$, corresponded to fatty acid pattern 3c (Kroppenstedt, 1985). The DNA G+C content was 70.2 mol%.

Phylogenetic tree construction indicated that strains AL4-7 (1484 nt) and AL4-8 (1513 nt) were closely related to *Microbispora rosea* subsp. *rosea* (*Mb. karnatakensis*) JCM 8972^T and *Microbispora rosea* subsp. *rosea* (*Mb. amethystogenes*) JCM 3021^T by showing 99.3% similarity (Figure 4.16). They were possibly identified as new species in the genus *Microbispora* however they should be confirmed by DNA-DNA hybridization.

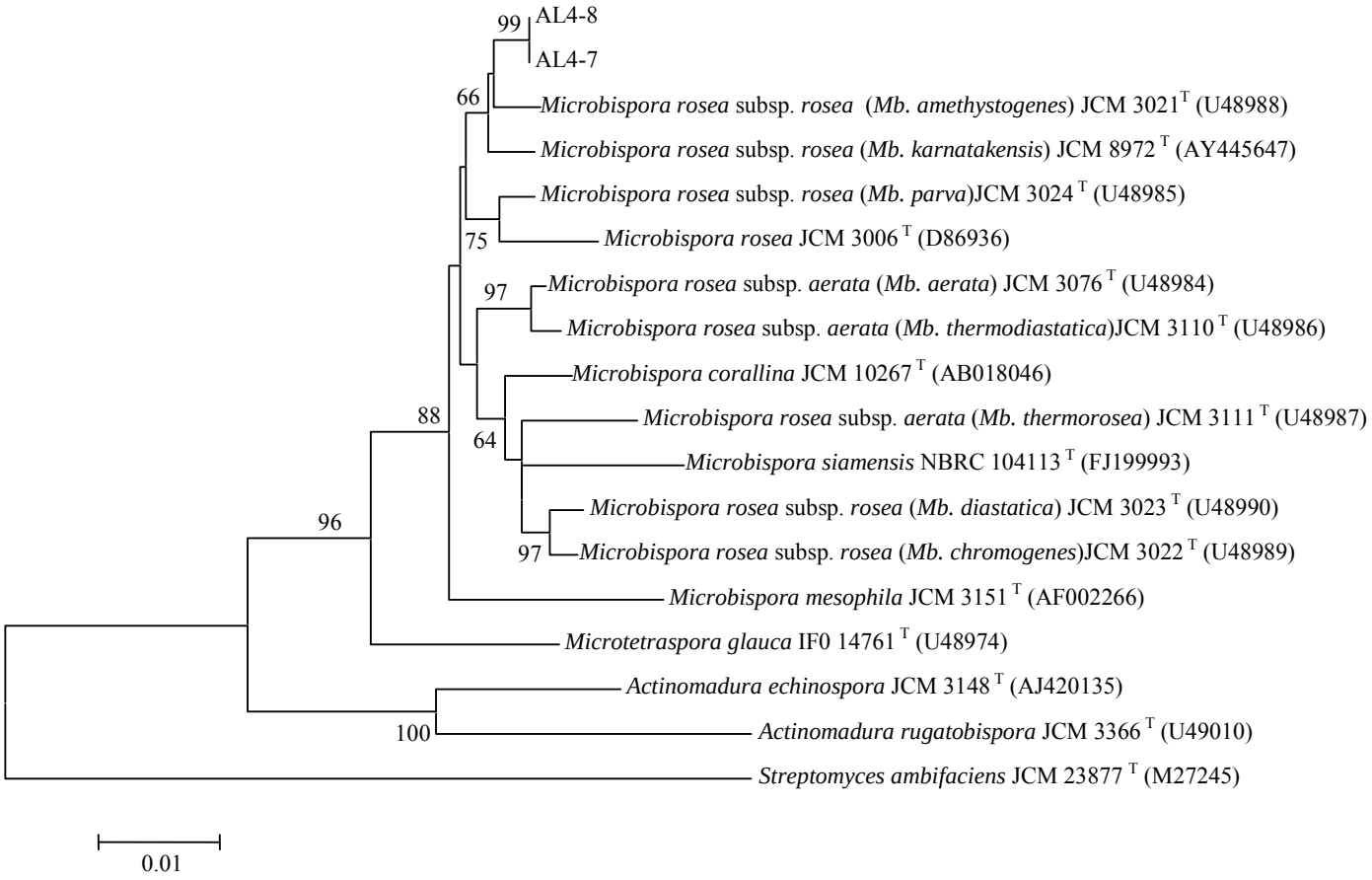

Figure 4.16 Neighbor-joining tree based on almost complete 16S rRNA gene sequences, showing relative among strains AL4-7, AL4-8 and the member of genus *Microbispora*

Table 4.20 Differential characteristics of strains AL4-7 and AL4-8 and related *Microbispora* species. +, positive; w, weakly positive; -, negative; ND, not determined

characteristics	**AL4-7**	**AL4-8**	**JCM 8972^T**	**JCM 3021^T**
Temperature range (°C)	20-40	20-40	ND	ND
Peptonization of milk	-	-	ND	ND
Gelatin liquefaction	w	-	ND	ND
Nitrate reduction	-	-	+	+
Starch hydrolysis	-	-	ND	ND
Utilization of:				
D-Mannitol	+	+	-	+
L-Raffinose	+	+	-	+
Inositol	-	-	+	+
Lactose	+	+	-	+
L-Arabinose	+	+	-	+
D-Fructose	+	+	-	+
D-Melibiose	-	-	+	-
Salicin	-	-	+	+
D-Galactose	-	-	+	+
Cellobiose	+	+	-	-
Sucrose	+	+	-	+
D-Xylose	+	+	-	+

4.2.3 Characteristics of *Nonomuraea*

Strain P0417 was isolated from peat swamp forest and it was assigned in the genus *Nonomuraea* based on phenotypic, genotypic and chemotaxonomic characteristics (Zhang *et al*., 1998).

Strain P0417 formed extensively branch substrate and aerial mycelia. The color of substrate mycelium was grayish yellow. The colonial appearance and spore morphology are shown in Figure 4.17. This strain produced conidial spores on the substrate hyphae. They grew on 1.5 to 2 % NaCl, on pH 4.5 to 8 and at 20 to 37 °C. Peptonization of milk, gelatin liquefaction, starch hydrolysis and nitrate reduction were negative (Table 2, Appendix C). This strain utilized D-mannitol, L-rhamnose, inositol, L-arabinose, D-fructose, D-melibiose and glycerol but not raffinose, lactose, D-ribose, salicin, D-galactose, cellobiose, sucrose and D-xylose (Table 3, Appendix C). Differential characteristics of strain P0417 and related *Nonomuraea* species are shown in Table 4.21.

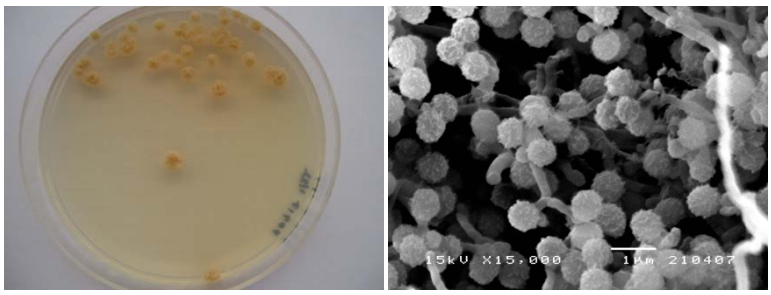

Figure 4.17 The colonial appearance and scanning electron micrograph of *Nonomuraea* sp. P0417 on YMA

Strain P0417 contained *N*-acetyl type of muramic acid in the peptidoglycan in the cell wall. An analysis of whole cell hydrolysates offered madurose, galactose, xylose and glucose, corresponded to whole cell sugar pattern B (Lechevalier and Lechevalier, 1970). Major menaquinones of strain P0417 were MK-9(H_0), MK-9(H_2), MK-9(H_4) and MK-9(H_6). A polar lipid profile was PG, PI, PE and OH-PE, corresponded to phospholipid pattern IV (Lechevalier *et al.,* 1977). The fatty acid profile was characterized by major amounts of i-$C_{15:0}$, $C_{17:1\omega6c}$, $C_{17:1\omega8c}$, i-$C_{16:0}$ and 10-methyl $C_{17:0}$. The DNA G+C content of strain P0417 was 68.4 mol%.

Phylogenetic tree analysis indicated that strain P0417 (1491 nt) formed distinct phyletic line in the genus *Nonomuraea* as shown in Figure 4.18. This strain was closely related to *No. helvata* JCM 3143^T (98.6%), *No. turkmeniaca* JCM 6836^T (98.5%) and

No. kuesteri JCM 13854^T (98.3%). The level of DNA-DNA relatedness between strain P0417 and closest related species was 38.6% as shown in Table 4.22. It was evident from the genotypic and phenotypic data that strain P0417 should be a new species in the genus *Nonomuraea*.

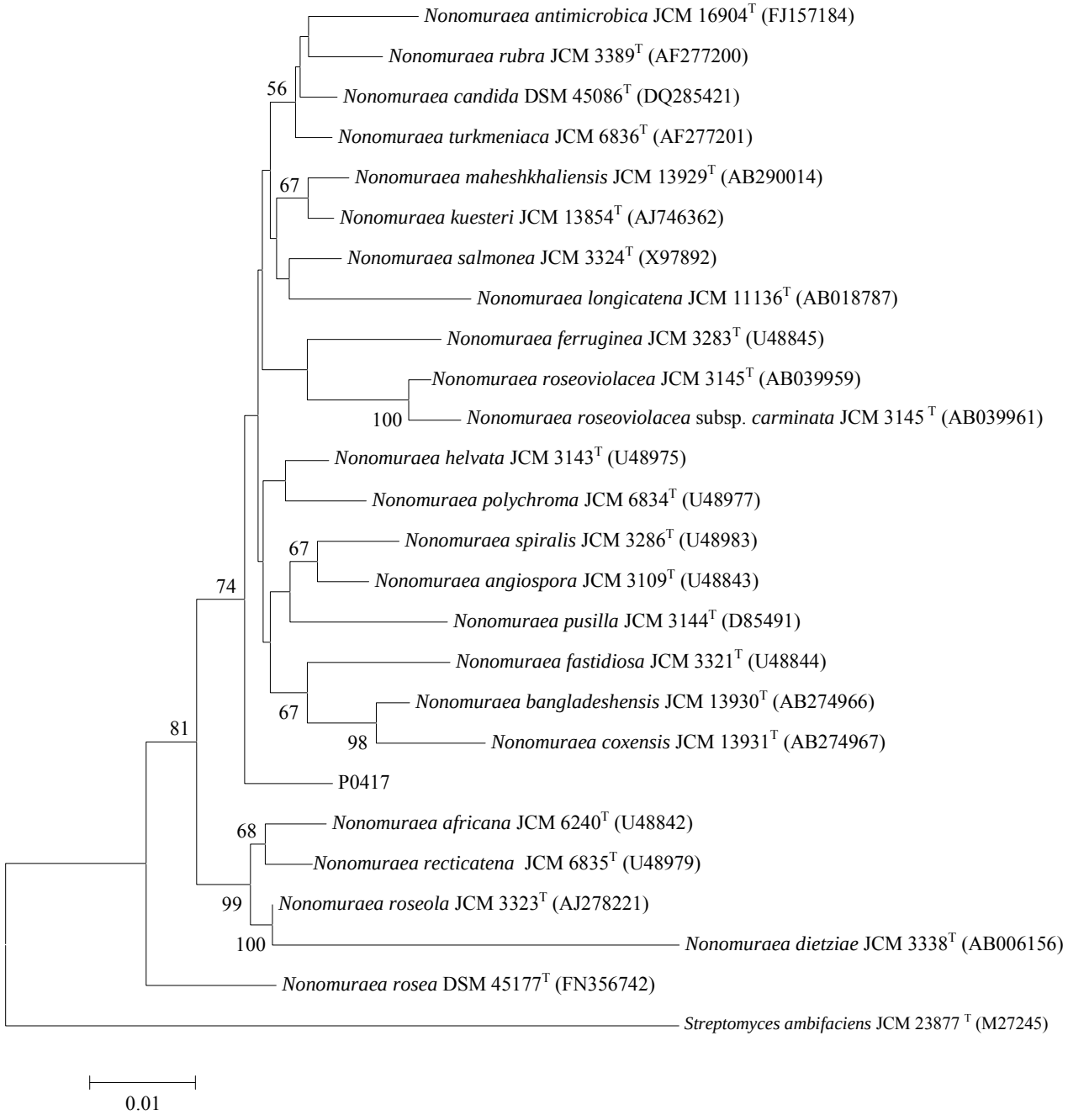

Figure 4.18 Neighbor-joining tree based on almost complete 16S rRNA gene sequences, showing relative among strains P0417 and the member of genus *Nonomuraea*

Table 4.21 Differential characteristics of strain P0417 and related *Nonomuraea* species
+, positive; w, weakly positive; -, negative ; ND, not determined

Characteristics	**P0417**	**JCM3143^T**	**JCM6836^T**	**JCM13854^T**
Maximum NaCl tolerance(%,w/v)	2	5	5	ND
Growth at pH 5	+	+	-	ND
Temperature range (°C)	20-37	ND	ND	ND
Peptonization of milk	-	ND	ND	ND
Gelatin liquefaction	w	-[a]	+[a]	-[a]
Nitrate reduction	-	+[a]	+[a]	ND
Starch hydrolysis	-	-[a]	+[a]	-[a]
Utilization of:				
D-Mannitol	+	+	ND	ND
L-Rhamnose	+	-[a]	+[a]	+[a]
L-Raffinose	-	+[a]	+[a]	ND
Inositol	+	+[a]	+	+[a]
Lactose	w	w	ND	
L-Arabinose	+	-	+[a]	+[a]
D-Fructose	+	-	+[a]	+[a]
D-Ribose	-	-	-[a]	ND
D-Melibiose	+	-	ND	ND
Glycerol	+	+	ND	ND
Salicin	w	+	ND	ND
Cellobiose	w	+	+[a]	+[a]
Sucrose	-	+[a]	+[a]	+[a]
D-Xylose	-	+[a]	+[a]	+[a]

[a] Data from Kamfer *et al*. (2005)

Table 4.22 DNA base composition and DNA-DNA relatedness of strain P0417 and related *Nonomuraea* species

Strains	**G+C content (mol%)**	**DNA-DNA relatedness (%) with labelled strain***
		Nn. helvata JCM 3143^T
P0417	68.4	38.6 ±0.07
Nn. helvata JCM 3143^T	ND	100 ±0.01

*Values are expressed as the means of two determinations.

4.2.4 Characteristics of *Actinomadura*

Four isolates, strains CYP1-5, AL 4-10, CYP1-1B and AL7-14 were assigned in the genus *Actinomadura* based on their phenotypic, genotypic and chemotaxonomic characteristics (Lechevalier and Lechevalier, 1970).

They formed non fragment substrate mycelium and aerial hyphae differentiated into spore chain. Spore chains were spiral. The spore of each strain was different shape and non-motile as shown in Figure 4.20-4.23. The cultural characteristics of 4 strains are shown in Table 1(Appendix C).

For chemotaxonomic characteristics, they contained *N*-acetyl type of muramic acid in peptidoglycan in the cell wall. The reducing sugars of whole cell hydrolysates were ribose, madurose, galactose and glucose, corresponded to whole cell sugar pattern B (Lechevalier and Lechevalier, 1970). The predominant of isoprenoid quinones were MK-9(H_4), MK-9(H_6) and MK-9(H_8). Polar lipid composition was PG, DPG, PIMs and PI, corresponded to phospholipid pattern I (Lechevalier *et al.,* 1977). The predominant fatty acid was $C_{16:0}$, i-$C_{16:0}$ and 10-methyl $C_{18:0}$, corresponded to fatty acid pattern 3a (Kroppenstedt, 1985). The DNA G+C content ranges were 71.7 to 74.3 mol%.

Phylogenetic tree analysis using 16S rRNA gene sequences indicated that these 4 strains were divided into 4 groups, Groups A to D as shown in Figure 4.19.

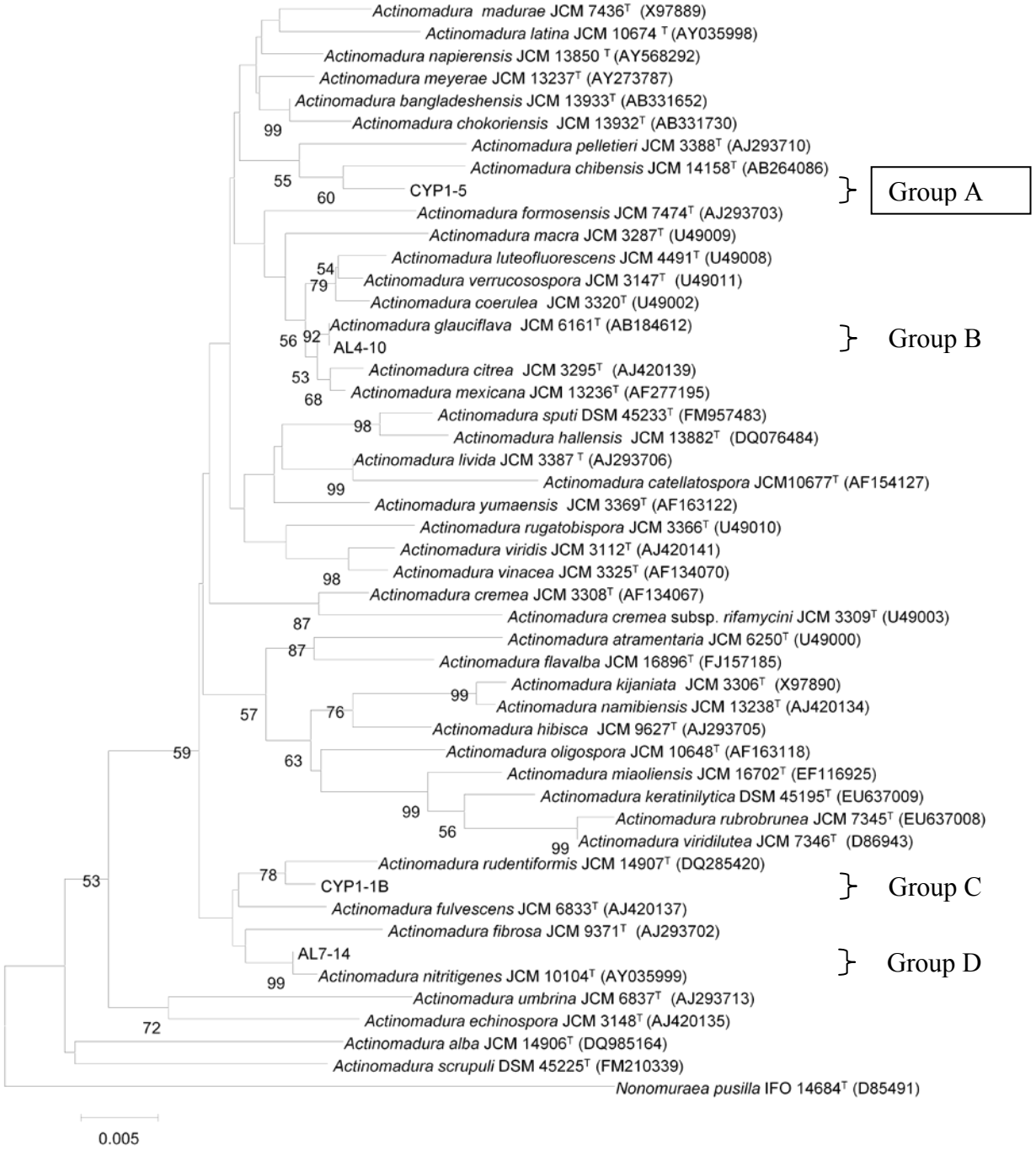

Figure 4.19 Neighbor-joining tree based on almost complete 16S rRNA gene sequences, showing relative among strains CYP1-5, AL4-10, CYP1-1B, AL7-14 and the member of genus *Actinomadura*

Group A contained strain CYP1-5 which isolated from soil at Chaiyaphum. They produced non fragment substrate mycelium and aerial hyphae differentiated into spore chain. Spore chain was spiral. Spores were non-motile and short-rod-like with warty surfaces. Colonial appearances were brownish pink on YMA as shown in Figure 4.20. Strain CYP1-5 grew at 1.5- 3% NaCl, on pH 6-8 and at 20 and 45 °C. Gelatin liquefaction and melanoid production were positive. Peptonization of milk, nitrate reduction and starch hydrolysis were negative. Strain CYP1-5 produced secondary metabolites that could inhibited the growth of *Bacilllus subtilis* ATCC 6633, *Pseudomonas aerugenosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* ATCC 9341 (Table 2, Appendix C). This strain utilized L-rhamnose and glucose as sole carbon sources but not D-mannitol, raffinose, inositol, lactose, L-arabinose, D-fructose, D-ribose, D-melibiose, glycerol, salicin, D-galactose, cellobiose, sucrose and D-xylose (Table 3, Appendix C). Differential characteristics of strain CYP1-5 and related *Actinomadura* species are shown in Table 4.23.

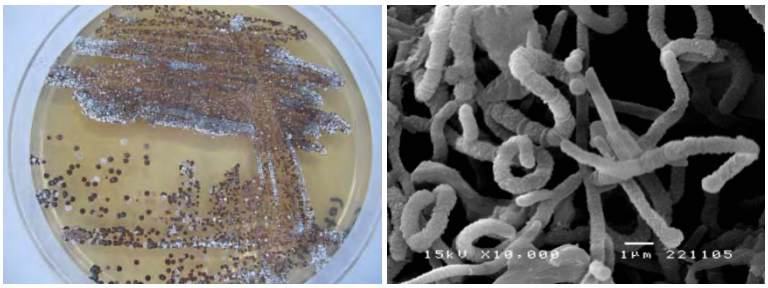

Figure 4.20 The colonial appearance and scanning electron micrograph of *Actinomadura* sp. CYP1-5 on YMA

Phylogenetic tree analysis using 16S rRNA gene sequences indicated that strain CYP1-5 (1513 nt) was closely related to *Actinomadura chibensis* JCM 14158^T (98.8%) and *Actinomadura pelletieri* JCM 3388^T (98.5%). The levels of DNA-DNA relatedness between strain CYP1-5 and closest related species were less than 20.3% (Table 4.24). It was evident from the genotypic and phenotypic data that strain CYP1-5 should be a new species in the genus *Actinomadura*.

Table 4.23 Differential characteristics of strains CYP1-5, AL4-10, CYP1-1B, AL7-14 and related *Actinomadura* species

+, positive; w, weakly positive; -, negative; ND, not determined

Characteristics	**CYP1-5**	**AL4-10**	**CYP1-1B**	**AL7-14**	**JCM 14158^T**	**JCM 3388^T**	**JCM 14907^T**	**JCM 10104^T**	**JCM 6161^T**
Max.NaCl tolerance(%,w/v)	3	5	5	7	2	2	3	7	5
Growth at pH 5	-	+	+	+	+	-	+	-	+
Growth at 40 °C	+	-	+	+	ND	+	+	+	+
Growth at 45 °C	+	-	+	+	ND	+	+	+	-
Peptonization of milk	-	+	-	+	+	ND	+	+	+
Gelatin liquefaction	+	+	-	+	+	ND	+	+	+
Nitrate reduction	-	+	-	+	ND	ND	-	+	+
Starch hydrolysis	-	+	+	+	ND	ND	w	+	+
Utilization of:									
D-Mannitol	-	+	+	+	-	-	+	+	+
L-Rhamnose	+	+	+	+	-	-	w	+	+
L-Raffinose	-	+	-	+	-	-	+	w	-
Inositol	-	+	-	+	-	-	+	-	w
Lactose	-	-	+	+	w	-	+	+	-
L-Arabinose	-	+	+	-	-	-	+	+	+
D-Fructose	-	-	w	+	-	-	+	+	-
D-Ribose	-	-	-	-	-	-	w	w	-
D-Melibiose	-	-	-	+	-	-	+	+	-
Glycerol	-	-	-	+	+	w	+	+	-
Salicin	-	-	-	+	+	w	+	w	-
D-Galactose	-	-	-	+	ND	ND	+	+	-
Cellobiose	-	+	+	+	w	w	+	+	+
Sucrose	-	+	+	+	-	-	+	+	w
D-Xylose	-	+	+	+	-	-	w	+	+

Table 4.24 DNA base composition and DNA-DNA relatedness of strain CYP1-5 and related *Actinomadura* species

Strain	G+C content (mol%)	DNA-DNA relatedness (%) with labelled strains*
		CYP1-5
CYP1-5	73.5	100 ±0.12
A. chibensis JCM 14158^T	ND	10.6 ±0.00
A. pelletieri JCM 3388^T	66.0$^€$	20.3 ±0.04

*Values are expressed as the means of two determinations.

$^€$ Data from Fitcher *et al*. (1983)

Group B contained strain AL4-10. The substrate mycelium was strong greenish yellow. Colonial appearance is shown in Figure 4.21. They grew on 1.5 to 5% NaCl, on pH 5 to 8 and at 20 to 37 °C. Peptonization of milk, gelatin liquefaction, starch hydrolysis and nitrate reduction were positive. Strain AL4-10 produced secondary metabolites that could inhibited the growth of *Bacilllus subtilis* ATCC 6633 (Table 2, Appendix C). This strain utilized D-glucose, D-mannitol, L-rhamnose, raffinose, inositol, L-arabinose, cellobiose, sucrose and D-xylose as sole carbon sources but not lactose, D-fructose, D-ribose, D-melibiose, glycerol, salicin and D-galactose (Table 3, Appendix C).

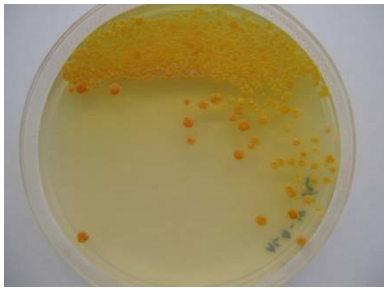

Figure 4.21 The colonial appearance of *Actinomadura* sp. AL4-10 on YMA

Strain AL4-10 (1507 nt) exhibited the highest 16S rRNA gene sequence similarity 100% to *A. glauciflava* JCM 6161^T (Figure 4.19). Based on phenotypic and genotypic characteristics, the data revealed that strain AL4-10 was identified as *A. glauciflava* (Lu *et al*., 2003).

Group C contained strain CYP1-1B. They produced an extensively branched substrate mycelium. The colonial appearance was strong brown and substrate mycelium color was dark orange yellow on YMA. Strain CYP1-1B produced conidial spores (Figure 4.22). This strain grew on 1.5-5% NaCl, on pH 4.5 to 8 and at 20 to 45 °C. Starch hydrolysis was positive. Peptonization of milk, gelatin liquefaction and nitrate reduction were negative. Strain CYP 1-1B produced secondary metabolites that could inhibited the growth of *Bacilllus subtilis* ATCC 6633, *Pseudomonas aerugenosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 (Table 2, Appendix C). They utilized D-glucose, D-mannitol, L-rhamnose, lactose, L-arabinose, D-fructose, cellobiose, sucrose and D-xylose (Table 3, Appendix C). Differential characteristics of strain CYP1-1B and related *Actinomadura* species are shown in Table 4.23.

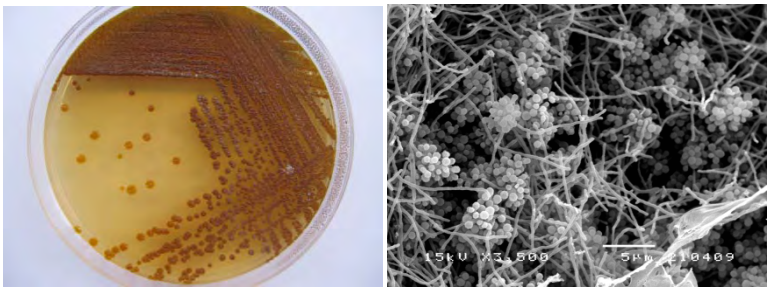

Figure 4.22 The colonial appearance and scanning electron micrograph of *Actinomadura* sp. CYP1-1B on YMA

Strain CYP1-1B (1488 nt) exhibited the highest 16S rRNA gene sequence similarity to *A. rudentiformis* JCM 14907^T (99.2%) followed by *A. nitritigenes* JCM 10104^T (99.1 %). The levels of DNA-DNA relatedness between strain CYP1-1B and closely related species were less than 30.3% (Table 4.25). These values were below the threshold value of 70 % for distinguishing genomic species (Wayne *et al*., 1987). It was evident from the genotypic and phenotypic data that strain CYP1-1B should be a new species in the genus *Actinomadura*.

Table 4.25 DNA base composition and DNA-DNA relatedness of strain CYP1-1B and related *Actinomadura* species

Strain	G+C content (mol%)	DNA-DNA relatedness (%) with labeled strains*	
		CYP1-1B	JCM 14907^T
CYP1-1B	74.3	100±0.02	10±0.02
A. rudentiformis JCM 14907^T	ND	8.8±0.02	100±0.06
A. nitritigenes JCM 10104^T	74.0[a]	30.3±0.01	2.8±0.00

*Values obtained from two independent determinations.

[a], Data from Lipski and Altendorf (1995)

Group D contained strain AL7-14. This strain produced an extensively branched substrate mycelium and aerial hyphae differentiated into spiral chains of spore. The substrate mycelium was strong yellowish white. This strain produced oval to ellipsoidal spores that had rough surfaces and were 0.73 to 0.89 µm. Spore chains were 4 spores long and straight to hook-like. The morphology of strain AL7-14 is shown in Figure 4.23. Strain AL7-14 grew at 1.5-7% NaCl, on pH 4 to 8 and at 15 to 45 °C. Peptonization of milk, gelatin liquefaction and starch hydrolysis were positive. Nitrate reduction was negative. Strain AL7-14 produced secondary metabolites that could inhibit the growth of *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* ATCC 9341(Table 2, Appendix C). This strain utilized D-glucose, D-mannitol, L-rhamnose, raffinose, inositol, lactose, D-fructose, D-melibiose, glycerol, salicin, D-galactose, cellobiose, sucrose and D-xylose as sole carbon sources (Table 3, Appendix C). Differential characteristics of strain AL7-14 and related *Actinomadura* species are shown in Table 4.23.

Figure 4.23 The colonial appearance and scanning electron micrograph of *Actinomadura* sp. AL7-14 on YMA

Strain AL7-14 (1490 nt) exhibited the highest 16S rRNA gene sequence similarity 99.8% to *A. nitritigenes* JCM 10104^{T}. Therefore, this strain was identified as *A. nitritigenes* (Lipski and Altendorf, 1995).

4.2.5 Characteristics of *Pseudonocardia*

Strain AL1-8 was assigned in the genus *Pseudonocardia* based on its phenotypic and genotypic properties (Hessen, 1957). Strain AL1-8 produced chains of spores formed by budding from substrate mycelium. The rod-shaped substrate mycelium was strong brown and the aerial mycelium was yellowish white (Figure 4.24). Strain AL1-8 grew on 1.5 to 7% NaCl, on pH 4.5 to 8 and at 15 to 37 °C. Milk peptonization, nitrate reduction and starch hydrolysis were negative. Gelatin liquefaction was positive (Table 2, Appendix C). This strain utilized D-glucose, D-mannitol, L-rhamnose, raffinose, inositol, lactose, L-arabinose, D-fructose, D-melibiose, glycerol, salicin, D-galactose, cellobiose, sucrose and D-xylose as sole carbon sources but not D-ribose (Table 3, Appendix C).

Figure 4.24 The colonial appearance and scanning electron micrograph of *Pseudonocardia* sp. AL1-8 on YMA

Strain AL1-8 (1490 nt) exhibited the highest 16S rRNA gene sequence similarity to *Pseudonocardia carboxydivorans* JCM 14827 ^{T} (100%) (Figure 4.25). Therefore, this strain was identified as *Pseudonocardia carboxydivorans* (Park *et al*., 2008).

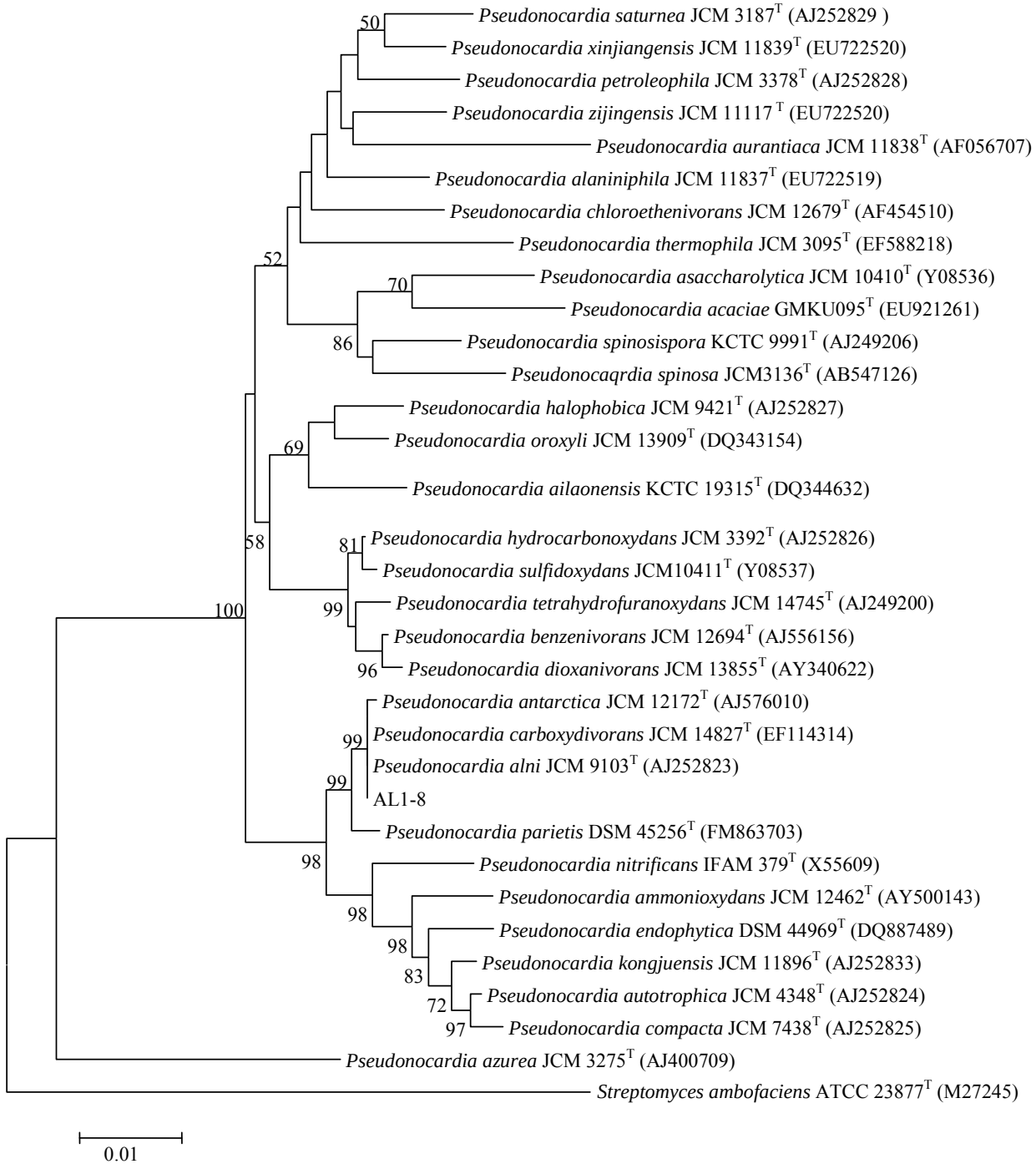

Figure 4.25 Neighbor-joining tree based on almost complete 16S rRNA gene sequences, showing relative among strain AL1-8 and the member of genus *Pseudonocardia*

4.2.6 Characteristics of *Nocardia*

Three strains, strains P1440, P1803 and P1605 were isolated from peat swamp forest soils and were assigned in the genus *Nocardia* based on their phenotypic, genotypic and chemotaxonomic characteristics (Stackebrandt *et al*., 1997).

They produced extensively branched substrate hyphae that fragmented into rod-shaped and non-motile spores. Aerial hyphae were powdery and yellowish white (Figure 4.26- 4.27). The substrate mycelia were light orange yellow to brilliant yellow on YMA. The cultural characteristics are shown in Table 1 (Appendix C).

These strains contained *N*-glycolyl type of muramic acid. The reducing sugars of whole cell hydrolysates were ribose, mannose, arabinose, galactose and glucose which corresponded to whole cell sugar pattern A (Lechevalier and Lechevalier, 1970). The predominants of isoprenoid quinone were 2,3-epoxy MK-8(H_4-ωcycl) and MK-8(H_4-ωcycl). Polar lipid composition was DPG, PIMs, PI, PE, Methyl-PE and OH-PE, corresponded to phospholipid pattern II (Lechevalier *et al.,* 1977). The predominants fatty acid were $C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$ and 10-methyl $C_{18:0}$, corresponded to fatty acid pattern 1b of Kroppenstedt (1985). The DNA G+C content ranges were 69.9 to 70.9 mol%.

Phylogenetic tree analysis using 16S rRNA gene sequences indicated that these 3 strains were divided into 2 groups, Group 1 and 2 as shown in Figure 4.28.

Group 1 contained strains P1440 and P1803. They grew on 1.5 to 5% NaCl, on pH 5 to 8 and at 20 to 40 °C. Peptonization of milk and gelatin liquefaction were weakly positive. Nitrate reduction was positive and starch hydrolysis was negative (Table 2, Appendix C). They utilized D-glucose, L-rhamnose, lactose, glycerol, salicin, D-galactose, cellobiose and D-xylose as sole carbon sources but not D-mannitol, raffinose, inositol, L-arabinose, D-fructose, D-ribose, D-melibiose and sucrose (Table 3, Appendix C). Differential characteristics of strain P1440, P1803 and related *Nocardia* species are shown in Table 4.26.

Strains P1440 (1502 nt) and P1803 (1496 nt) were closely related to *N. arthritidis* JCM 12120^T (99.4%) and *N. araoensis* JCM 12118^T (99.4%). The levels of DNA-DNA relatedness between strain P1803 and closely related species were less than 32.4% (Table 4.27). These values were below the threshold value of 70 % for distinguishing genomic

species (Wayne *et al*., 1987). It was evident from the genotypic and phenotypic data that they should be new species in the genus *Nocardia.*

Figure 4.26 The colonial appearance and scanning electron micrograph of *Nocardia* sp. P1803 on YMA

Group 2 contained strain P1605. This strain grew on 1.5 to 7% NaCl, on pH 5 to 8 and at 15 to 45 °C. Milk peptonization and starch hydrolysis were negative. Gelatin liquefaction and nitrate reduction were positive (Table 2, Appendix C). This strain utilized D-glucose, D-mannitol, L-rhamnose, inositol, D-fructose, glycerol, salicin, galactose, cellobiose, sucrose and weakly raffinose, lactose, D-mellibiose and xylose (Table 3, Appendix C).

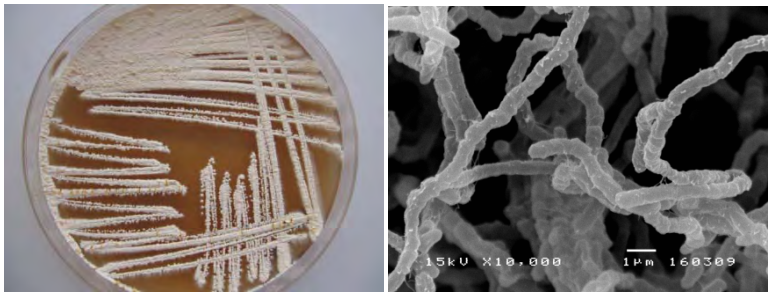

Figure 4.27 The colonial appearance and scanning electron micrograph of *Nocardia* sp. P1605 on YMA

Strain P1605 (1506 nt) exhibited the highest 16S rRNA gene sequence similarity to *N. cyriacigeorgica* JCM 11763^{T} (99.9%). This strain was identified as *N. cyriacigeorgica* (Yassin *et al*., 2001).

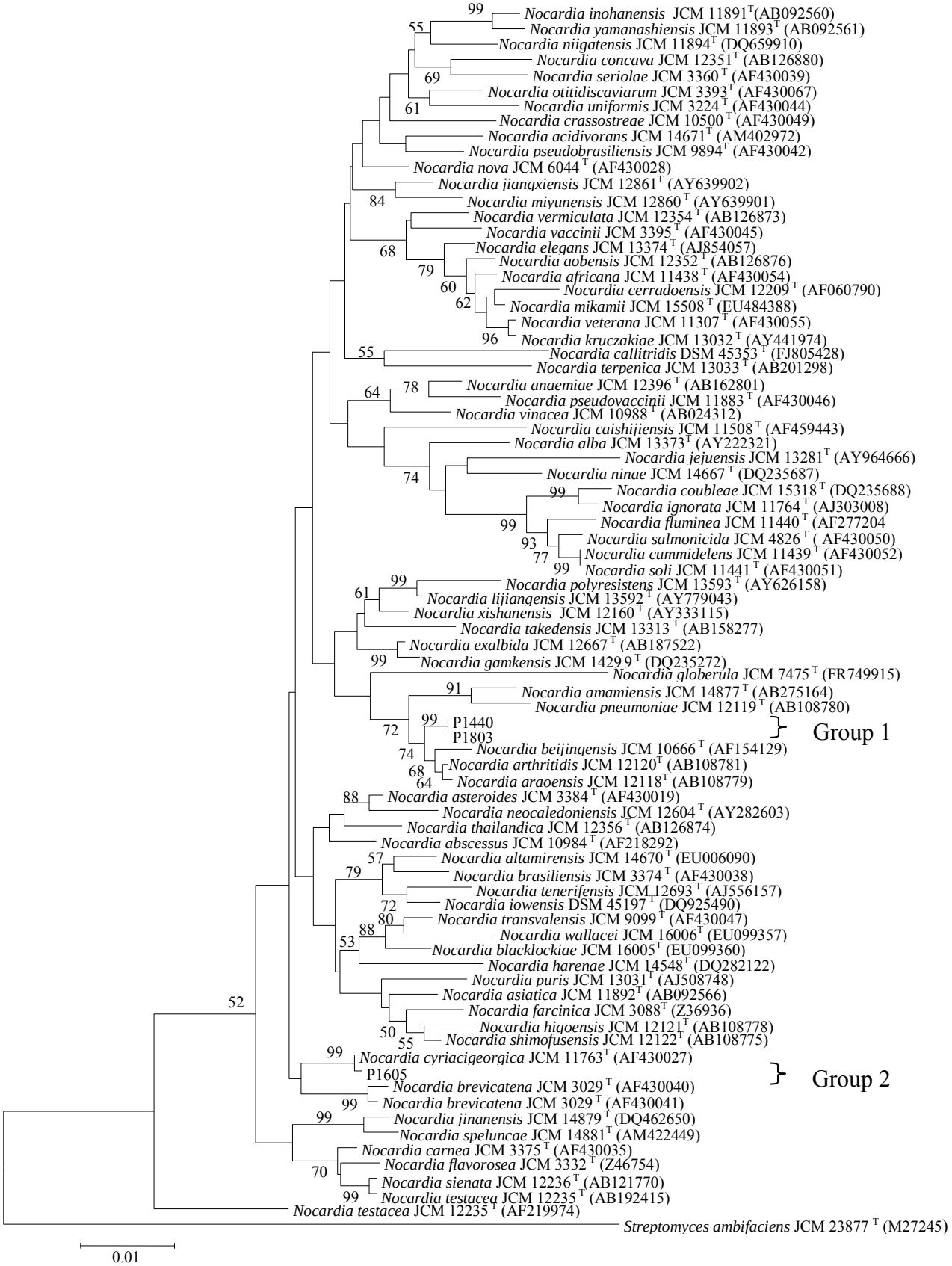

Figure 4.28 Neighbor-joining tree based on almost complete 16S rRNA gene sequences, showing relative among the representative *Nocardia* and the member of genus *Nocardia*

Table 4.26 Differential characteristics of strain P1440, P1803 and related *Nocardia* species. +, positive; w, weakly positive; -, negative, ND, not determined

Characteristics	**P1440**	**P1803**	**JCM 12120^T**	**JCM 12118^T**
Maximum NaCl tolerance(%,w/v)	5	5	5	6
Growth at pH 5	+	+	+	+
Growth at 40 °C	+	+	ND	ND
Peptonization of milk	w	w	ND	ND
Gelatin liquefaction	w	w	+	w
Nitrate reduction	+	+	ND	ND
Starch hydrolysis	-	-	ND	ND
Utilization of:				
L-Rhamnose	w	w	-	-
Lactose	w	w	+	+
L-Arabinose	-	-	+	-
D-Fructose	-	-	+	w
D-Ribose	-	-	+	+
D-Melibiose	-	-	+	+
Salicin	w	w	+	+
D-Galactose	w	w	+	+
D-Xylose	+	+	+	w

Table 4.27 DNA base composition and DNA-DNA relatedness of strain P1803 and related *Nocardia* species

Strain	**G+C content (mol%)**	**DNA-DNA relatedness (%) with labeled strains***
		P1803
P1803	69.9	100±0.04
N. arthritidis JCM 12120^T	68.0[a]	30.0±0.06
N. araoensis JCM 12118^T	69.0[a]	32.4±0.07

*Values obtained from two independent determinations.

[a], Data from Kageyama *et al*. (2004)

4.3 Preliminary characterization of secondary metabolites using HPLC-UV

In order to primarily screen for secondary metabolites**,** the extracts of culture broths were subjected to compare HPLC patterns and UV spectra with in-house database. Twenty strains of rare actinomycetes were selected for screening of secondary metabolites on 3 different media (A3M, A11M and A16), including 12 strains of *Micromonospora* sp. (AL8-2, D10-9-5, ASC19-2-1, AL10-3, AL7-5, AL4-4, AL1-3, P0402, AL9-20, AL5-1, AL9-13 and AL1-15-2), 4 strains of *Actinomadura* sp. (AL7-14, CYP1-1B, CYP1-5 and AL4-10), 2 strains of *Nocardia* sp. (P1803 and P1605), *Microbispora* sp. (AL4-7) and *Nonomureae* sp. (P0417). The results of screening are shown in Table 4.28. We found strain AL7-5, which was identified as *Micromonospora chalcea* might produce rakicidin (Figure 4.29) when it was cultivated on A3M, A11M and A16 media and produced compound BU-4664L (Figure 4.30) only on A16 medium. Rakicidin was firstly isolated from *Micromonospora* sp. R385-2 (McBrien *et al*., 1995). Compound BU-4664L was originally isolated from *Micromonospora* sp. M990-6 as a lipoxygenase inhibitor and was shown to have potent *in vitro* and *in vivo* antitumor activity (Okumura and Kobaru, 1996). In 2005, the structure of BU-4664L was revised on the basis of NMR analysis (Igarashi *et al*., 2005).

Two strains of rare actinomycetes, *Micromonospora* sp. D10-9-5 and *Actinomadura* sp. CYP1-5 produced the unknown peaks after comparing HPLC patterns and UV spectra with in-house database. The HPLC pattern of compounds A and C was derived from strain D10-9-5 cultured on A11M as shown in Figure 4.31. *Actinomadura* sp. CYP1-5 was cultured on A3M and produced compounds D, E, F, G and H as shown in Figure 4.32. Compound D was also a common compound found from *Actinomadura* sp. AL7-14, CYP1-1B and AL4-10. Therefore, we paid our attention to isolate compounds A, C, F, G and H.

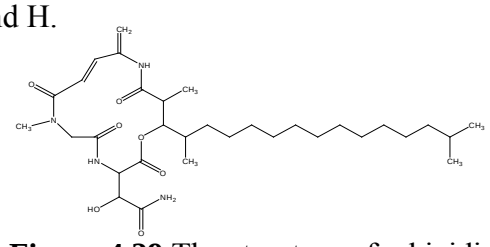

Figure 4.29 The structure of rakicidin

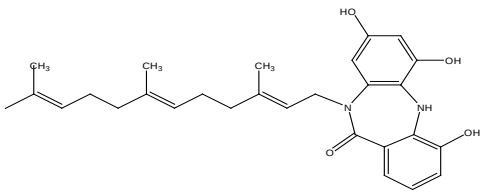

Figure 4.30 The structure of BU-4664L

Table 4.28 The secondary metabolites of the selected strains detected by HPLC-UV

Strain no.	Produced compound on each media (retention time)		
	A3M	**A11M**	**A16**
AL9-20	-	-	-
P0402	-	-	-
AL1-3	-	-	-
AL4-4	-	-	-
D10-9-5	-	Unknown (6.7, 7.5)	-
AL8-2	-	-	-
ASC19-2-1	-	-	-
AL10-3	-	-	-
AL7-5	Rakicidin (24.8)	Rakicidin (24.8)	BU-4664L, Rakicidin (24.1, 24.8)
AL5-1	-	-	-
AL9-13	-	-	-
AL1-15-2	-	-	-
AL4-7	-	-	-
AL7-14	Unknown (6.4)	-	-
CYP1-1B	Unknown (6.4)	-	Unknown (6.4)
CYP1-5	Unknown (6.4, 21.1,23.1, 23.8, 24.2, 29.8)	-	Unknown (6.4, 21.1, 22.0)
AL4-10	Unknown (6.5)	Unknown (6.4)	-
P0417	-	-	-
P1803	-	-	-
P1605	-	-	-

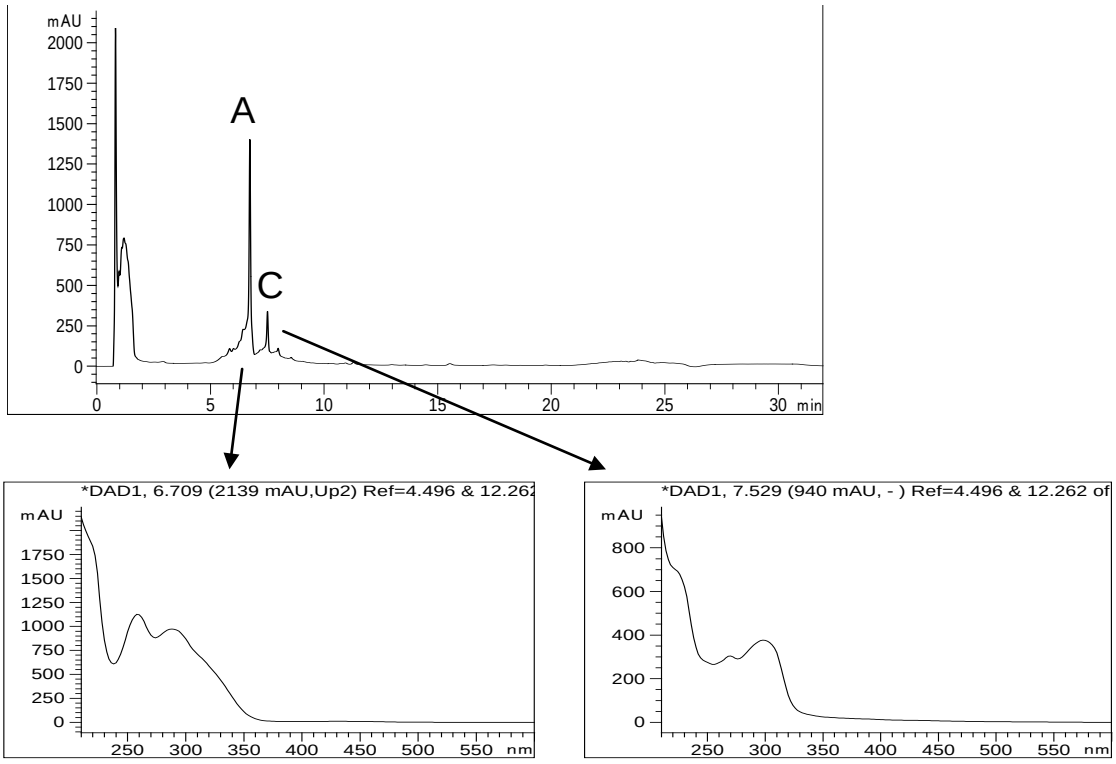

Figure 4.31 The HPLC chromatogram and UV spectra of compounds A and C produced by *Micromonospora* sp. D10-9-5 on A11M medium

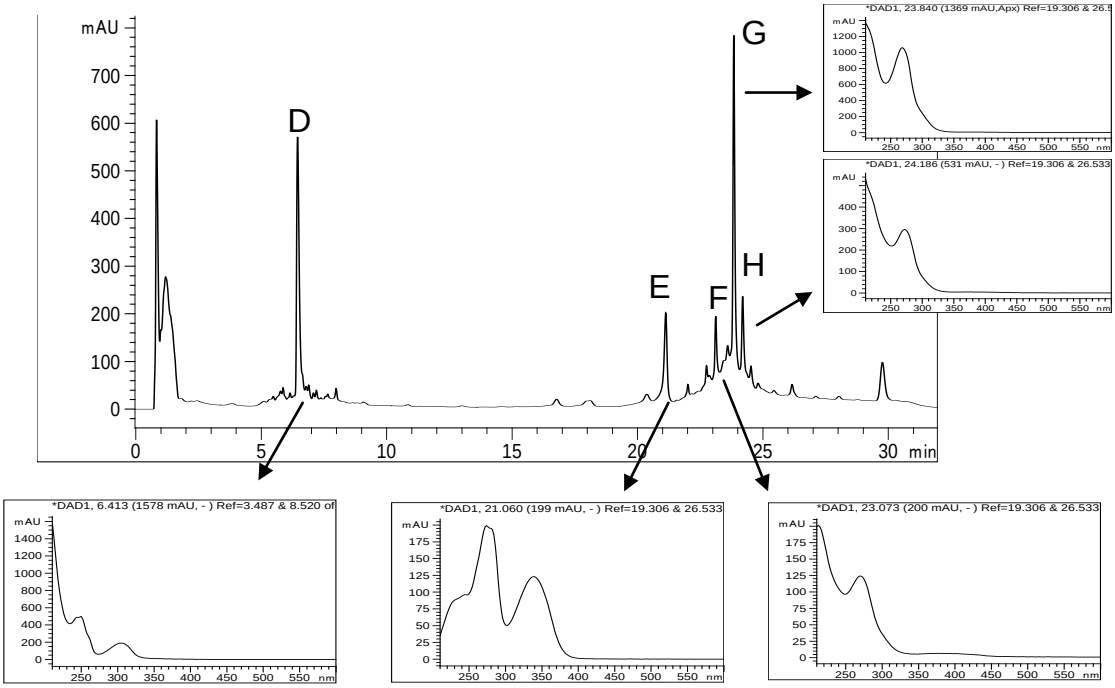

Figure 4.32 The HPLC chromatogram and UV spectra of compounds D, E, F, G and H produced by *Actinomadura* sp. CYP1-5 on A3M medium

4.4 Fermentation, isolation and structure determination of the compounds from *Micromonospora* sp. D10-9-5

The isolation procedure of compounds A and C from the culture broth of *Micromonospora* sp. D10-9-5 on A11M medium is shown in Figure 4.33. The butanol extract (2.54 g) was fractionated by a silica gel column (3 x 15 cm) eluted with $CHCl_3$ and CH_3OH (1:0 to 0:1 gradient) and then analyzed by HPLC-UV. The 4:1 and 2:1 fractions were pooled, evaporated and weighed. The pooled fraction was further applied to an ODS flash column (5 x 15 cm) and eluted with CH_3CN and 0.1% HCOOH (2:8 to 8:2 gradient) at a flow rate of 30 ml/min. The 4:6 fraction showing the expected peaks was further seperated by preparative HPLC using ODS column (Xterra^{TM}, 7 µm, 19 x 300 mm), eluted with CH_3CN and 0.1% HCOOH at a flow rate of 15 ml/min, and detected by UV at 254 nm, to give two compounds at following retention times (minute); compound A (11 min) and compound C (13 min). The weights of compounds A and C were 39.1 and 18.4 mg, respectively.

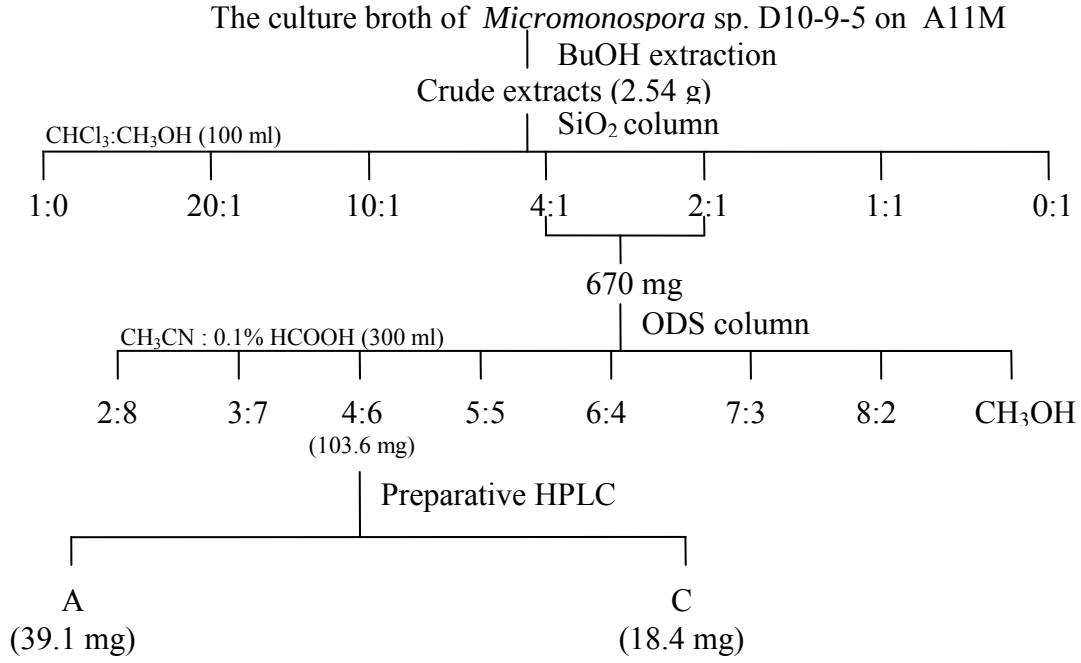

Figure 4.33 The isolation procedure for compounds A and C

Compound A was colorless amorphous solid. The ^{1}H NMR (500 MHz), ^{13}C NMR (100 MHz), and 2D NMR spectral data of compound A (Table 4.29) revealed that compound A was similar to a polyketide, SEK34. The molecular weight of compound A

was determined as 302 by FABMS showing the $[M + Na]^+$ ion at *m/z* 325 in the positive mode and the $[M – H]^-$ ion at *m/z* 301 in the negative mode.

Compound C was colorless amorphous solid. The ^1H NMR spectrum of compound C is almost similar to that of compound A. The absence of methylene proton signals H_2-14 and the presence of an olefinic proton (H-14) at δ_H 6.16 (s) ppm indicated that compound C was a dehydrated derivative of compound A, SEK34B. The structures of SEK34 and SEK34B are shown in Figure 4.34. Compounds SEK34 and SEK34B were firstly isolated from the transformants of *Streptomyces coelicolor* CH999 in which contained only the *act* genes (McDaniel *et al*., 1994).

Table 4.29 Comparison of ^1H (500 MHz) and ^{13}C (100 MHz) NMR data in DMSO-*d6* of SEK34, compounds A and C

	SEK34		**Compound A**		**Compound C**
carbon	**^{13}C(ppm)**	**^1H(ppm) (m, J in Hz)**	**^{13}C(ppm)**	**^1H(ppm) (m, J in Hz)**	**^1H(ppm) (m, J in Hz)**
1	163.8	11.58(s)	163.9	-	-
2	88.2	5.18(s)	88.2	5.16 (s)	5.06(s, 1H)
3	170.4	-	170.7	-	-
4	99.5	5.54(s,)	99.8	5.53(s)	5.42(s)
5	165.6	-	165.5	-	-
6	37.5	4.12 (d,16.2)	37.5	4.12 (d,16.2)	4.42(s)
-	-	4.21(d,15.8)	-	4.21 (d,15.8)	-
7	136.1	-	136.2	-	-
8	124.5	6.97 (d, 8.4)	124.5	6.97 (d, 8.4)	7.55(d, 7.6)
9	135.0	7.50 (dd,7.8,7.9)	135.0	7.50 (dd,7.8,7.9)	7.66(dd,7.5, 8.4)
10	118.0	6.90 (d, 7.4)	118.0	6.90 (d, 7.4)	7.29(d, 7.3)
11	159.8	-	159.8	-	-
12	118.5	-	118.5	-	-
13	193.1	-	193.1	-	-
14	49.5	2.63 (d,15.9)	49.6	2.64 (d,15.9)	6.16(s)
-	-	3.06 (d, 15.8)	-	3.06 (d, 15.8)	-
15	100.8	-	100.9	-	-
15-OH	-	6.99 (s)	-	6.99 (s)	-
16	27.5	1.59 (s)	27.5	1.60 (s)	2.35(s)

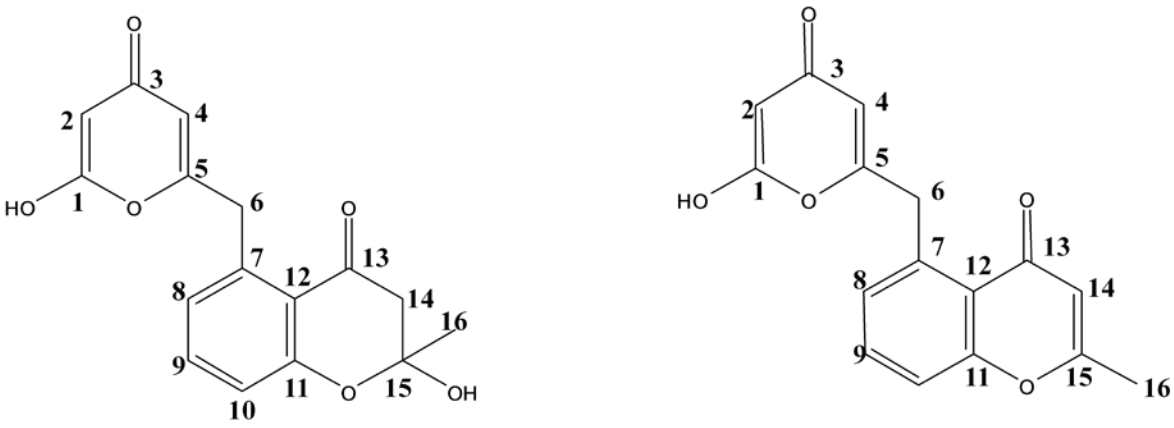

Figure 4.34 The structures of SEK34 (A) and SEK34B (B)

4.5 Fermentation, isolation and structure determination of *Actinomadura* sp.CYP1-5

The isolation procedure of compounds F, G and H from the culture broth of *Actinomadura* sp. CYP1-5 on A3M medium is shown in Figure 4.35. The butanol extract (5.33 g) was fractionated by a silica gel column (4 x 15 cm) eluted with the gradient mixture of $CHCl_3$ and CH_3OH (1:0 to 0:1). The 4:1 and 2:1 fractions were pooled and evaporated. The pooled fraction was further applied to an ODS flash column (5 x 15 cm) and eluted with CH_3CN and 0.1% HCOOH (2:8 to 8:2 gradient). Compound G was found in the 8:2 and CH_3OH fractions and compound H in CH_3OH fractions only. Each fraction was evaporated and extracted by partitioning with ethyl acetate. The dried ethyl acetate extracts were further seperated by preparative HPLC (Xterra^{TM}, 7 µm, 19 x 300 mm) using gradient mixture of CH_3CN and 0.1% HCOOH as the mobile phase, and detected by UV at 254 nm, to give two compounds at following retention times (minute); compound G (19 min) and compound H (23 min). Each fraction was extracted with ethyl acetate and then evaporated. The weights of compounds G and H were 17.9 and 9.4 mg. respectively.

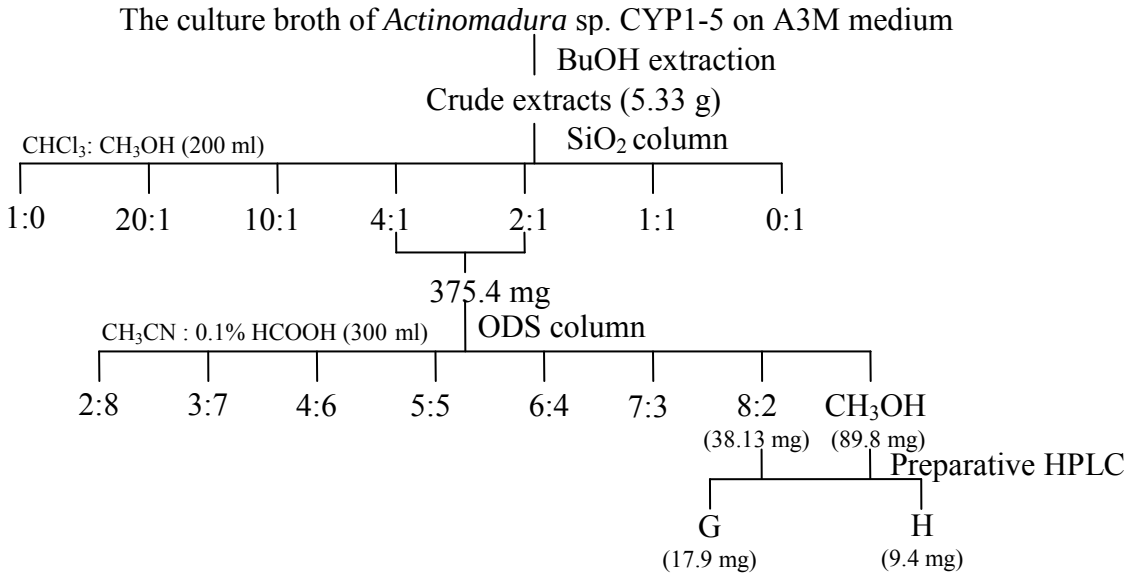

Figure 4.35 The isolation procedure for compounds G and H

Compound G was colorless amorphous solids. The molecular weight of compound G was determined as 854 by FABMS showing the $(M+Na)^+$ ion at *m/z* 877 in the positive mode and the $(M-H)^-$ ion at *m/z* 853 in the negative mode. Careful analyses of its ^1H NMR (Figure 10, Appendix C), ^{13}C NMR (Figure 11, Appendix C), and 2D NMR (Figures 12-14, Appendix C) spectra revealed that compound G was similar to decatromicin B ($C_{45}H_{56}Cl_2N_2O_{10}$). Decatromicin B was isolated from the culture broth of *Actinomadura* sp. MK73-NF4 and inhibited growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (Momose *et al*., 1999). The structure of decatromicin B is shown in Figure 4.36.

Compound H was colorless amorphous solids. The ^1H NMR of compound H (Figure 9, Appendix C) was almost identical to that of compound G. The absence of an olefinic proton at 6.13 ppm indicated that the substitution of one additional chlorine atom at C4''. Therefore, compound H was identified as BE-45722C. Compound BE-45722C was obtained by culture broth of *Actinomadura* sp. A 45722 (Torigoei *et al*., 1997). The structure of BE-45722C is shown in Figure 4.37.

Table 4.30 Comparison of ^{1}H (500 MHz) and ^{13}C (100MHz) NMR data in CD_3OD of decatromicin B and compound G

	Decatromicin B		Compound G	
carbon	**^{13}C(ppm)**	**(m, J in Hz)**	**^{13}C(ppm)**	**(m, J in Hz)**
1	169.99	-	170.63	-
2	103.94	-	104.42	-
3	205.29	-	206.03	-
4	55.97	-	56.22	-
5	41.78	1.82(m)	41.86	1.87(m)
6	24.00	1.33(m), 1.80(m)	24.15	1.30(m), 1.82(m)
7	33.24	1.61(m), 1.61(m)	33.40	1.59(m), 1.59(m)
8	35.47	2.38(m)	35.63	2.37(m)
9	87.40	3.38(dd, 5.2, 11.2)	87.59	3.40
10	39.78	2.15(br dd, 10.0, 11.2)	39.90	2.17(m)
11	125.43	5.63(br d, 10.4)	125.71	5.68(br t, 10.5)
12	132.69	5.69(ddd, 2.2, 5.8, 10.4)	132.69	5.71(m)
13	44.00	2.89(m)	44.26	2.88(m)
14	38.01	1.99(m), 1.99(m)	38.18	2.01(m), 2.01(m)
15	132.65	5.21(m)	132.69	5.20(ddd, 6.5, 7.25, 14.75)
16	129.10	5.42(ddd, 4.8, 10.0, 16.0)	129.40	5.43(ddd, 5.0, 9.5, 14.75)
17	45.62	2.36(4.8, 12.0), 2.48(10.0, 12.0)	45.92	2.37(m), 2.49(m)
18	140.16	-	140.49	-
19	126.86	4.99(br s)	126.88	4.99(br s)
20	43.89	-	44.03	-
21	144.10	7.05(d, 1.6)	143.97	7.06(d, 1.5)
22	132.83	-	133.05	-
23	37.23	2.68(m)	37.34	2.72(m)
24	30.98	1.82(d, 14.2), 2.43(dd, 9.0, 14.2)	31.20	1.87(14.5) , 2.49 (m)
25	86.86	-	87.14	-
26	200.45	-	200.58	-
27	24.13	1.84(m), 2.72(m)	24.32	1.87(m), 2.72(m)
28	12.28	0.91(t, 7.6)	12.4	0.92
29	13.51	1.02(d, 7.2)	13.70	1.04 (d, 7.0)
30	19.12	1.79(d, 1.0)	19.31	1.79
31	26.86	1.30(s)	27.06	1.31(s)
32	170.57	-	170.63	-
33	27.33	1.59(m), 1.72(m)	27.51	1.59(m), 1.75(m)
34	13.26	0.91(t, 7.9)	13.38	0.91(t, 7.5)
1’	102.86	4.55(dd, 1.8, 9.8)	103.09	4.55 (dd, 1.5, 8.0)
2’	41.13	1.57(m), 2.26(ddd, 1.6, 5.0, 12.6)	41.41	1.55(m), 2.28(ddd,1.5, 5.0, 12.0)
3’	70.30	3.72(ddd, 5.0, 10.0, 12.0)	70.53	3.72 (ddd, 5.0, 10.0, 10.5)
4’	59.74	3.58(t, 10.0)	58.53	3.60 (m)
5’	72.44	3.45(qd6, 6.6, 10.0)	72.61	3.47 (m)
6’	18.79	1.23(d, 6.6)	18.97	1.24 (d, 6.0)
2’’	122.56	-	122.79	-
3’’	119.58	-	119.83	-
4’’	109.33	6.11(s)	109.53	6.13(s)
5’’	114.67	-	114.76	-
6’’	161.68	-	161.86	-

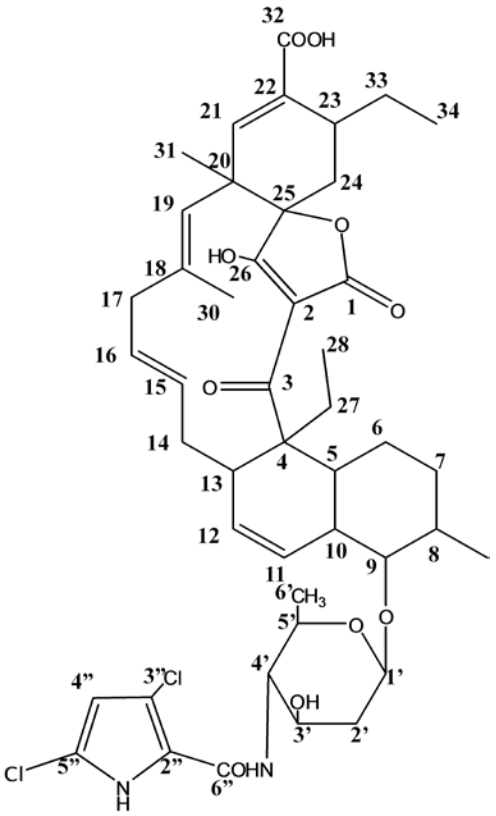

Figure 4.36 The structure of decatromicin B

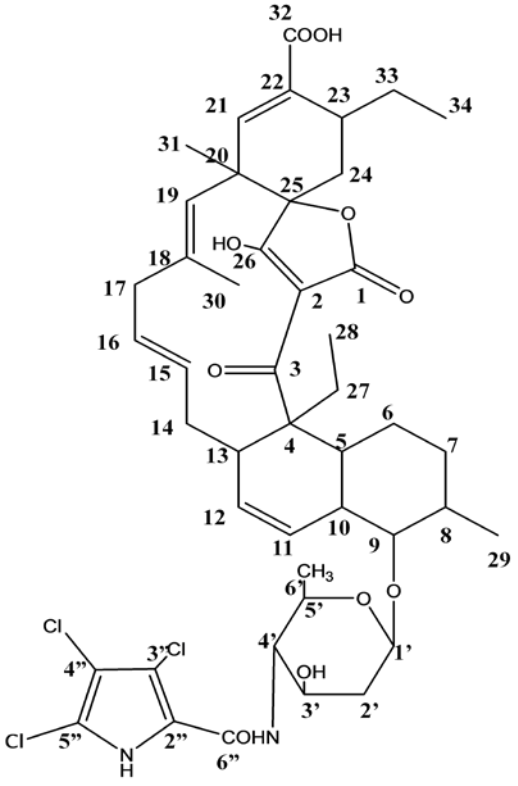

Figure 4.37 The structure of BE-45722C

4.6 Biological activities of compounds SEK34, SEK34B, decatromicin B and BE-45722C

Biological activities of four secondary metabolites were evaluated. Decatromicin B and BE-45722C displayed anti-*B. subtilis* activity, antimalarial activity and cytotoxicity. Decatromicin B exhibited a growth inhibition of *B. subtilis* with MIC of 0.781 µg/ml. Decatromicin B possess high *in vitro* antiplasmodial activity, with a mean 50% inhibitory dose of 2.07 µg/ml. Cytotoxic activity of decatromicin B was tested against vero cell line (African monkey kidney cell line) comparing with ellipticine and IC_{50} of decatromicin B was at 47.07 µg/ml. BE-45722C displayed anti-*B. subtilis* activity at MIC of 0.391 µg/ml, antimalarial activity at IC_{50} of 4.76 µg/ml and cytotoxicity at IC_{50} of 2.71µg/ml. Decatromicin B and BE-45722C did not display anti-*C. albicans*. Compounds SEK34 and SEK34B did not exhibit anti-*B. subtilis*, anti-*C. albicans,* antimalarial and cytotoxic activities.

CHAPTER IV

CONCLUSION

In the course of investigation of rare actinomycetes distributed in soils in Thailand, 49 strains were identified using polyphasic taxonomic approaches. The phenotypic, genotypic and chemotaxonimic characterisatics revealed that 38 strains were placed within the clade of the genus *Micromonospora* , 4 strains of *Actinomadura*, 3 strains of *Nocardia*, 2 strains of *Microbispora*, 1 strain of *Pseudonocardia* and 1 strain of *Nonomuraea*.

Thirty-eight strains identified as *Micromonospora*. All contained *meso*-DAP and *N*-glycolyl muramic acid in the peptidoglycan. Most of all strains contained ribose, mannose, galactose, xylose, arabinose and glucose as whole cell sugars. Their major menaquinones were MK-9(H_4, H_6, H_8) and MK-10(H_4, H_6, H_8). Characteristic phospholipids were DPG, PG, PI, PIMs and PE but not PC. The predominant fatty acids were i-$C_{15:0}$, i-$C_{16:0}$, i-$C_{17:0}$ and a-$C_{17:0}$. The DNA G+C contents were 70.8 to 74.5 mol%. All isolated strains were divided into 12 groups as shown in the phylogenetic tree (Figure 4.2). Groups III (strain ASC19-2-1), IV (strains AL8-8, AL10-3), V (strain AL7-5), IX (strains AL3-16, AL9-20), XI (strains AL9-13, AL9-22) and XII (strains AL1-15-2, AL1-16B) were identified as *M. marina, M. aurantiaca, M. chalcea, M. chokoriensis, M. tulbaghiae* and *M. chersina,* respectively, based on phenotypic and chemotaxonomic properties together with the phylogenetic analysis using 16S rRNA gene sequences. The differential of phenotypic and chemotaxonomic characteristics together with the phylogenetic tree revealed that Groups I, II, VI, VII, VIII and X of *Micromonospora* should be the novel species based on a low levels of DNA-DNA relatedness (21.6-40.8%) and 16S rRNA gene sequences (99.0-99.6% similarity). *Micromonospora humi* sp. nov. was proposed for the novel species of Group VIII.

Four strains were identified as *Actinomadura*, including strains CYP1-5 and CYP1-1B from soil in Chaiyaphum and strains AL4-10 and AL7-14 from Loei soils. They contained the *N*-acetyl type of muramic acid in the peptidoglycan and ribose, madurose, galactose and glucose as whole cell sugars. The predominant isoprenoid

quinones were MK-9(H_4, H_6, H_8). The polar lipids were PG, DPG, PIMs and PI. The predominant fatty acids were $C_{16:0}$, i-$C_{16:0}$ and 10-methyl $C_{18:0}$. The DNA G+C contents were 71.7 to 74.3 mol%. All isolated *Actinomadura* were divided into 4 groups. The strains AL4-10 and AL7-14 of Groups B and D were identified as *A. glauciflava* JCM 6161^T (100% similarity) and *A. nitritigenes* JCM 10104^T (99.8% similarity), respectively, based on phenotypic and chemotaxonomic characteristics together with 16S rRNA gene analysis. The strain CYP1-5 of Group A was closely related to *A. chibensis* JCM 14158^T (98.8% similarity) and *A. pelletieri* JCM 3388^T (98.5% similarity). The level of DNA-DNA relatedness between strain CYP1-5 and closest related species was less than 20.3%. The evidence of phenotypic, chemotaxonomic and genotypic properties revealed that strain CYP1-5 should be a novel species in the genus *Actinomadura*. The strain CYP1-1B of Group C was closely related to *A. rudentiformis* JCM 14907^T (99.2% similarity) and *A. nitritigenes* JCM10104^T (99.1 % similarity). The level of DNA-DNA relatedness between strain CYP1-1B and closely related species was less than 30.3%. It was evident that strain CYP1-1B was a novel species in the genus *Actinomadura*.

Three strains (P1440, P1803 and P1605) isolated from peat swamp forest soils, Phayao province were identified as *Nocardia*. The peptidoglycan of the cell wall contained the *N*-glycolyl type of muramic acid. The reducing sugars of whole cell hydrolysates were ribose, mannose, arabinose, galactose and glucose. The predominant isoprenoid quinones were 2, 3-epoxy MK-8(H_4-ωcycl) and MK-8(H_4-ωcycl). The polar lipid composition was DPG, PIMs, PI, PE, Methyl-PE and OH-PE. The predominant fatty acids were $C_{16:0}$, $C_{18:0}$, $C_{18:1\omega9c}$ and 10-methyl $C_{18:0}$. The DNA G+C content was 69.9 to 70.9 mol%. They were divided into 2 groups, Group 1 (P1440, P1803) and Group 2 (P1605). Group 1, strains P1440 and P1803 exhibited the highest 16S rRNA gene sequence similarity to *N. arthritidis* JCM 12120^T (99.4% similarity) and *N. araoensis* JCM 12118^T (99.4% similarity). The level of DNA-DNA relatedness between strain P1803 and closest related species was less than 32.4%. It was evident that strains P1440 and P1803 should be a novel species in the genus *Nocardia*. Furthermore, strain P1605 was identified as *N. cyriacigeorgica* based on 99.9% similarity.

Two strains AL4-7 and AL4-8, which were obtained from Loei soils, were identified as *Microbispora.* The peptidoglycan in the cell wall contained *N*-acetyl type

of muramic acid. These strains contained whole cell hydrolysates of ribose, madurose, galactose and glucose. The major menaquinones were MK-9 (III, VII H_4), MK-9(H_2), MK-9(H_6) and MK-9(H_0). The polar lipids were PG, PE, PME, OH-PE, lyso-PE and NPG. The major fatty acids were $C_{16:0}$, $C_{17:1\omega8c}$, $C_{18:1\omega9c}$, iso-$C_{16:0}$ and 10-methyl $C_{17:0}$. The DNA G+C content of strain AL4-7 was 70.2 mol%. They were closely related to *Microbispora rosea* subsp. *rosea* (*Mb. karnatakensis*) JCM 8972^T and *Microbispora rosea* subsp. *rosea* (*Mb. amethystogenes*) JCM 3021^T with 99.3% similarity of 16S rRNA gene sequence. They should be a novel species in the genus *Microbispora.*

Strain AL1-8 was identified as *Pseudonocardia* based on phenotypic characteristics and 16S rRNA gene analysis. Strain AL1-8 was closely related to *P. carboxydivorans* JCM 14827^T (100% similarity), therefore strain AL1-8 was identified as *P. carboxydivorans.*

Strain P0417 was identified as *Nonomuraea* based on phenotypic and chemotaxonomic properties together with 16S rRNA gene analysis. The peptidoglycan in the cell wall contained *N*-acetyl type of muramic acid. The whole cell sugars included mannose, xylose and glucose. The major menaquinones were MK-9(H_0, H_2, H_4, H_6) and polar lipid profiles were PG, PI, PE and OH-PE. The major fatty acids were i-$C_{15:0}$, $C_{17:1\omega6c}$, $C_{17:1\omega8c}$, i-$C_{16:0}$ and 10-methyl $C_{17:0}$. The DNA G+C content was 68.4 mol%. Strain P0417 was closely related to *No. helvata* JCM 3143^T (98.6%), *No. turkmeniaca* JCM 6836^T (98.5%) and *No. kuesteri* JCM 13854^T (98.3 %). The level of DNA-DNA relatedness between strain P0417 and closest related species, *No. helvata* JCM 3143^T, was 38.6%, therefore strain P0417 should be a novel species of the genus *Nonomuraea*.

In the conclusion, the rare actinomycetes isolated from mountain soils in Phukradung, Loei and Chaiyaphum provinces were various species of *M. aurantiaca, M. chalcea, M. chokoriensis, M. tulbaghiae, M. chersina*, *A. glauciflavain, A. nitritigenes*, and *P. carboxydivorans.* Moreover, 4 novel species of *Micromonospora*, 2 novel species of *Actinomadura* and 1 species of *Microbispora* were also found. In peat swamp forest soils collected from Phayao province, *M. humi and N. cyriacigeorgica* and each of the novel species of *Nonomuraea* and *Nocardia* were isolated. In mangrove soil from Samutsakhon province, *M. aurantiaca* and 2 novel

species of *Micromonospora* were isolated whereas *M. marina* and *M. aurantiaca* were isolated from soil collected in Chonburi province.

The primary screening of secondary metabolites revealed that 3 strains produced the secondary metabolites when were cultivated on A3M, A11M, and A16 media. *Micromonospora* sp. AL7-5 produced rakicidin when was cultivated on 3 kinds of media and produced compound BU-4664L only on A16.

SEK34 and SEK34B were isolated from the fermentation of A11M broth of *Micromonospora* sp. D10-9-5. These compounds were previously isolated as an 'unnatural' natural product from a transformant of *Streptomyces coelicolor* harboring recombinant biosynthetic genes for actinorhodin. This is the first report on the isolation of SEK34 and SEK34B as natural products.

In addition, decatromicin B and BE-45722C were produced from *Actinomadura* sp. CYP1-5 on A3M medium. Decatromicin B was previously isolated from the culture broth of *Actinomadura* sp. MK73-NF4 and inhibited growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). In this study, we also found dacatromicin B and BE45722C displayed anti-*B. subtilis* activity, antimalarial activity, and cytotoxicity.

As the results of this research, the diverse genera and species of the rare actinomycetes were distributed in many soil samples however there are still a lot of strains waiting for the discovery on their new taxon and new secondary metabolites.

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APPENDICES

APPENDIX A

Culture media

All media were dispended and sterilized in autoclave for 15 min at 15 pounds pressure (121°C) for media except for carbon utilization test which was sterilized at 110 pounds for 110°C for 10 min. All media were prepared in 100 ml of distilled water.

1. Sodium-caseinate agar (SCN)

Sodium caseinate	0.2	g
Glucose	0.1	g
K_2HPO_4	0.02	g
$MgSO_4$	0.02	g
$FeSO_4$	trace amount	
Agar	1.5	g

2. Humic-vitamin agar (HV)

Humic acid	0.1	g
(dissolved in 10 ml of 0.2 N NaOH solution)		
Na_2HPO_4	0.05	g
KCl	0.171	g
$MgSO_4.7H_2O$	5.0	mg
$FeSO_4.7H_2O$	1.0	mg
$CaCO_3$	2.0	mg
Vitamin B solution (see below)	1.0	ml
Agar	1.5	g
<u>Vitamin B solution:</u>		
Thiamine –HCl	5.0	mg
Riboflavin	5.0	mg
Nicothinate	5.0	mg
Pyridoxine-HCl	5.0	mg
Inositol	5.0	mg
Ca-pantothenate	5.0	mg
p- Amino benzoate	5.0	mg
d-Biotin	2.5	mg

3. Yeast extract-malt extract agar (YMA), ISP no.2

Bacto- Yeast Extract (Difco)	0.4	g
Bacto- Malt Extract (Difco)	1.0	g
Glucose	0.4	g
Agar	1.5	g

pH 7.3

4. Oatmeal agar, (OMA), ISP no. 3

Oatmeal	20.0	g
Agar	18.0	g

Cook or steam 20 g of oatmeal in 1,000 ml distilled water for 20 minutes. Filter through cheese cloth and add distilled water to restore volume of filtrate to 1,000 ml. Add 1 ml of trace salts solution (A) and adjust to pH 7.2 with NaOH and finally, add 18 g of agar, liquefy by steaming at 100°C for 15-20 min.

5. Inorganic salts-starch agar, (IS), ISP no. 4

Soluble starch	1.0	g
K_2HPO_4	0.1	g
$MgSO_4.7H_2O$	0.1	g
NaCl	0.1	g
$(NH_4)_2SO_4$	0.2	g
$CaCO_3$	0.2	g
Trace salts solution (A)	0.1	ml

pH 7.0-7.4

6. Glycerol-asparagine agar, (GlyA), ISP no.5

Glycerol	1.0	g
L-Asparagine	0.1	g
K_2HPO_4	0.1	g
Trace salts solution (A)	0.1	ml
Agar	2.0	g

7. Peptone-yeast extract iron agar, (PIA), ISP no. 6

Bacto-Peptone Iron, dehydrated (Difco)	3.6	g
Bacto-Yeast Extract (Difco)	0.1	g
pH 7.0-7.2		

8. Tyrosine agar, (TA), ISP no. 7

Glycerol	1.5	g
L-tyrosine (Difco)	0.05	g
L-Asparagin (Difco)	0.1	g
K_2HPO_4	0.05	g
$MgSO_4.7H_2O$	0.05	g
NaCl	0.05	g
$FeSO_4.7H_2O$	0.01	g
Trace salts solution (A)	0.1	ml
Agar	2.0	g
pH 7.2-7.4		

Trace salt solution (A)

$FeSO_4.7H_2O$	0.1	g
$MnCl_2.4H_2O$	0.1	g
$ZnSO_4.7H_2O$	0.1	g

9. Glucose asparagine agar, (GluA)

Glucose	1	g
Asparagine	0.05	g
K_2HPO_4	0.05	g
Bacto-agar	1.5	g

10. Nutrient agar (NA)

Meat extract	1	g
Peptone	1	g
NaCl	0.1-0.2	g
Agar	1.5	g

11. Czapek's sucrose agar

Sucrose	3	g
K_2HPO_4	0.1	g
$MgSO_4$	0.05	g
KCl	0.05	g
$FeSO_4$	0.001	g
Agar	1.5-1.7	g

pH 7.0-7.2

12. Carbon utilization medium, ISP medium no.9

Carbohydrate	1.0	g
$(NH_4)_2SO_4$	0.264	g
$K_2HPO_4.3H_2O$	0.565	g
KH_2PO_4 anhydrous	0.238	g
$MgSO_4.7H_2O$	0.1	g
Pridham and Gottlieb trace salts (B)	0.1	ml

pH 6.8-7.0

Trace salts solution (B)

$CuSO_4.5H_2O$	0.64	g
$Fe_4.7H_2O$	0.11	g
$MnCl_2.4H_2O$	0.79	g
$ZnSO_4.7H_2O$	15.0	g

13. Peptone KNO_3 broth

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g

pH 7.0

14. Boullion gelatin broth

Peptone	1.0	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15.0	g

pH 7.0-7.2

15. Peptonization and Coagulation test medium

Skim milk (Difco)	10.0	g

16. Sabouraud's dextrose agar (Difco)

Neopeptone	1.0	g
Dextrose	4.0	g
Agar	1.5	g

17. Oatmeal nitrate agar

Oatmeal powder	0.3	g
KNO_3	0.02	g
K_2HPO_4	0.05	g
$MgSO_4.7H_2O$	0.02	g
Agar	1.5	g

18. Bn-2 medium

Glucose	0.5	g
Soluble starch	0.5	g
Meat extract	0.1	g
Yeast extract	0.1	g
NZ-case	0.2	g
NaCl	0.2	g
$CaCO_3$	0.1	g
Agar	1.5	g

pH 7.0

19. V-22 medium

Soluble starch	1	g
Glucose	0.5	g
NZ-case	0.3	g
Yeast extract	0.2	g
Tryptone	0.5	g

K_2HPO_4	0.1	g
$MgSO_4.7H_2O$	0.05	g
$CaCO_3$	0.3	g
pH 7.0		

20. A3M medium

Glucose	0.5	g
Glycerol	2.0	g
Soluble starch	2.0	g
Pharmamedia	1.5	g
Yeast extract	0.3	g
HP-20	1.0	g
pH 7.0		

21. A11M medium

Glucose	0.2	g
Soluble starch	2.5	g
Yeast extract	0.5	g
Polypeptone	0.5	g
NZ-amine	0.5	g
$CaCO_3$	0.3	g
HP-20	1.0	g
pH 7.0		

22. A16M medium

Glucose	2.0	g
Pharmamedia	1.0	g
$CaCO_3$	0.5	g
HP-20	1.0	g

APPENDIX B

Reagents and Buffers

1. DON Reagent

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

Add conc. H_2SO_4 in 2,7-dihydroxynapthalene (DON) wait until the yellow solution become colorless (24 h). Keep this solution in refrigerator.

2. 6N HCl

Conc. HCl	60	ml
Distiller water	60	ml

Add. conc. HCl into the distilled water.

3. 2N H_2SO_4

Conc. H_2SO_4	2	ml
Distilled water	34	ml

Add conc. HCl into the distilled water

4. Ninhydrin solution

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

5. 5% Trichloro-acetic acid

Trichloro acetic acid	5	g
Distilled water	100	ml

Add conc. HCl into the distilled water

6. Reagent 1 for fatty acid analysis (Saponification reagent)

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

Dissolve NaOH pellets in Milli-Q water and add MeOH.

7. Reagent 2 for fatty acid analysis (Methylation reagent)

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

pH must be below 1.5

8. Reagent 3 for fatty acid analysis (Extraction solvent)

n-Hexane (HPLC grade or n-Hexane 1000)	50	ml
Methyl-tert-Butyl Ether (HPLC grade)	50	ml

9. Reagent 4 for fatty acid analysis (base wash reagent)

Sodium hydroxide	1.2	g
Mili-Q water	100	ml

10. Reagent 5 for fatty acid analysis (Saturated sodium chloride)

11. Dittmer & Lester reagent

Solution A

MoO_3	4.011	g
25 N H_2SO_4	100	ml

Dissolve 4.011 g of MoO_3 in 100 mL of 25 N H_2SO_4 by heating.

Solution B

Molybdenum powder	0.178	g
Solution A	50	ml

Add 0.178 g of Molybdenum powder to 50 mL of solution A, and boil it for 15 minutes. After cooling, remove the precipitate by decantation, Before spraying, mix solution A (50 mL) plus solution B (50 mL) plus water (100 mL)

12. Anisaldehyde reagent

Ethanol	90.0	ml
H_2SO_4	5.0	ml
p-Anisaldehyde	5.0	ml
Acetic acid	1.0	ml

13. Dragendroff's reagent

Solution A

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

Solution B

KI	40	g
Distilled water	100	ml

Before spraying, mix solution A (10 ml) plus solution B (10 ml) plus acetic acid (10 ml).

14. Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	ml

Dissolve by gentle heating in a fume hood.

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

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

Dissolve by gentle heating in a fume hood

Two drops of sulphanilic acid solution and three drops of *N*,*N*-dimethyl-1-naphthylamine into peptone nitrate broth inoculing with the test microorgnisms.

15. Phenol : Chloroform (1 : 1 v/v)

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

16. 100xDenhardt solution

Bovine serum albumin	2%
Polyvinylpyrrolidone	2%

Ficoll 400 2%

17. 0.5M EDTA (pH 8.0)

800 mL of distilled water, 186.1 g of disodium ethylenediaminetetraacetate.$2H_2O$ was added and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH (20 g of NaOH pellets). The volume was adjusted to 1 litre. The solution was dispensed into aliquots and sterilized by autoclaving for 15 minutes at 15 lb/in^2.

18. 5M NaCl

292.2 g of sodium chloride was added 800 ml of distilled water and adjusted the volume to 1 l with distilled water. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2

19. 8mM $Na_{2H}PO_4$

1.5mM KH_2PO_4

137 mM NaCl

2.7 mM KCl

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

20. 10 mg/mL Salmon sperm DNA

A 10 mg of Salmon sperm DNA was dissolved in 1 mL of 10 mM TE buffer pH 7.6. Boiling for 10 minutes, immediately cooling in ice and sonication for 3 minutes.

21. 3M Sodium acetate pH 5.2

408.1 g of sodium acetate was added to 800 ml of distilled water and then adjusted the pH to 5.2 with glacial acetic acid. The volume was adjusted to 1 litre. The solution was sterilized by autoclaving for 15 minutes at 15lb/in^2.

22. 10% Sodium dodecyl sulphate (SDS)

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

23. 20xSSC

3M NaCl

0.1 M Tri-sodiumcitrate

The 20xSSC was adjusted the pH to 7.0 with 1N NaOH. The solution was sterilized by autoclaving for 15 minutes at 15lb/in^2.

24. 1M Tris-HCl pH 8.0

The 1M Tris was prepared by dissolving 121.1 g of Tris base in 800 ml of distilled water. The pH was adjusted to the desired value by adding conc. HCl (pH 8.0, 42 mL of HCl). The solution was cooled to room temperature before making final adjustment to the desired pH. The volume of the solution was adjusted to 1 litter with distilled water and sterilized by autoclving.

25. RNase A solution

RNase A	20	mg
0.15 M NaCl	10	ml

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

26. RNase T_1 solution

RNase T_1	80	µl
0.1 M Tris-HCl (pH 7.5)	10	ml

Mix 80 µL of RNase T_1 in 10 mL of 0.1 M Tris-HCl (pH 7.5) and heat at 95°C for 5 minutes. Keep RNase T_1 solution in –20°C.

27. Proteinase K

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

Use freshly prepared solution

28. Nuclease P1 solution

Nuclease P1	0.1	mg

40 mM CH_3COONa+12mM $ZnSO_4$ (pH 5.3)	1	ml
Store at 4°C		

29. Alkaline phosphatase solution

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

30. 0.1 M Tris-HCl buffer, pH 9

Tris	1.21	mg
Distilled water	100	ml
Adjust the pH to 9 with HCl		

31. TE buffer

10 mM Tris HCl (pH 8.0)

1 m M Na_2-EDTA (pH 8.0)

32. TE buffer + RNase

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

33. Saline-Na_2 EDTA

0.1 M NaCl

50 M EDTA.2Na (pH 8.0)

34. Reagent and buffer for DNA-DNA hybridization

34.1 Prehybridization solution

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

34.2 Hybridization solution

Prehybridization solution	100	ml
Dextran-sulfate	5	g

34.3 Solution I

Bovine serum albumin (Fraction V)	0.25	g
Titron X-100	50	µl
PBS	50	ml

34.4 Solution II

Streptavidin-POD	1	µl
Solution I	4	ml

34.5 Solution III

3,3',5,5'-Tetramethylbenzidine (TMB) (10 mg/mL in DMSO)	100	µl
0.3% H_2O_2	100	µl
0.4 M Citric acid + 0.2 M Na_2HPO_4 buffer pH 6.2 in 10% DMSO	100	µl

34.6 2M H_2SO_4

H_2SO_4	22	ml
Distilled water	178	ml

The solution was sterilized by autoclaving.

35. Ethidium bromide solution (10 mg/mL)

The ethidium bromide solution was prepared by dissolved 1 g of ethidium bromide in 100 ml of distilled water. The solution was stored in light-tight container at room temperature.

36. Gel loading buffer

0.25 g of bromophenol blue was dissolved in 20 ml of 15% glycerol.

37. Tris-acetate EDTA (TAE) buffer

1xTBE buffer was used an electrophoresis buffer throughout the study. The working solution of 1xTBE buffer was prepared from stock solution of 5xTAE buffer, as followed.

Tris-base	5.4	g
Boric acid	2.75	g
Na_2-EDTA	0.47	g
Distilled water	100	ml

38. Agarose gel

Agarose	1.6	g
1xTBE buffer	200	ml

39. DNA extract buffer

0.2 M Tris-HCl buffer (pH8.5)

0.25 M NaCl

0.025 M EDTA

0.5% SDS

APPENDIX C

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL1-3	Y.M.	abundant	brownish black	brownish black	-
	O.M.	good	strong brown	brownish orange	-
	I.S.	good	deep orange	deep orange	-
	Gly.A.	good	strong brown	brownish orange	-
	P.I.A	moderate	dark grayish brown	dark grayish brown	-
	T.A.	abundant	brownish black	grayish brown	-
	Glu.A	moderate	deep yellowish brown	grayish yellowish brown	-
	Cz.	poor	light brown	grayish brown	-
	N.A.	good	strong brown	strong brown	-
AL1-8	Y.M.	abundant	yellowish white	strong brown	-
	O.M.	abundant	yellowish white	dark orange yellow	-
	I.S.	abundant	yellowish white	light orange yellow	-
	Gly.A.	abundant	yellowish white	brownish orange	-
	P.I.A	abundant	yellowish white	strong brown	-
	T.A.	abundant	yellowish white	brilliant orange yellow	-
	Glu.A	abundant	yellowish white	brilliant orange yellow	-
	Cz.	abundant	yellowish white	light orange yellow	-
	N.A.	abundant	yellowish white	strong brown	-
AL1-15-2	Y.M.	abundant	black	black	-
	O.M.	good	black	dark gray	-
	I.S.	good	brownish black	brownish black	-
	Gly.A.	abundant	brownish black	brownish black	-
	P.I.A	moderate	dark orange yellow	strong yellow	-
	T.A.	good	black	black	-
	Glu.A	good	dark gray	dark gray	-
	Cz.	poor	dark gray	dark gray	-
	N.A.	abundant	dark gray	brownish black	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL1-16B	Y.M.	abundant	black	black	-
	O.M.	good	black	dark gray	-
	I.S.	good	brownish black	brownish black	-
	Gly.A.	good	dark gray	dark gray	-
	P.I.A	moderate	dark orange yellow	strong yellow	-
	T.A.	good	dark gray	dark gray	-
	Glu.A	moderate	dark gray	dark gray	-
	Cz.	poor	dark gray	dark gray	-
	N.A.	abundant	brownish black	brownish black	-
AL1-18	Y.M.	abundant	black	black	-
	O.M.	good	black	dark gray	-
	I.S.	good	brownish black	brownish black	-
	Gly.A.	good	brownish black	brownish black	-
	P.I.A	moderate	dark orange yellow	strong yellow	-
	T.A.	good	black	black	-
	Glu.A	moderate	dark grayish yellowish brown	moderate olive brown	-
	Cz.	poor	dark gray	dark gray	-
	N.A.	abundant	dark gray	brownish black	-
AL2-1	Y.M.	abundant	black	dark yellowish brown	-
	O.M.	abundant	dark brown	moderate brown	-
	I.S.	good	strong yellowish brown	strong yellowish brown	-
	Gly.A.	good	black	dark grayish yellowish brown	-
	P.I.A	good	strong yellowish brown	strong yellowish brown	-
	T.A.	good	dark grayish brown	dark grayish yellowish brown	-
	Glu.A	moderate	dark grayish yellowish brown	dark grayish yellowish brown	-
	Cz.	poor	yellowish gray	dark grayish yellowish brown	-
	N.A.	abundant	black	dark grayish yellowish brown	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL2-3	Y.M.	abundant	deep orange yellow	brilliant orange yellow	-
	O.M.	abundant	dark brown	moderate brown	-
	I.S.	good	brownish black	dark orange yellow	-
	Gly.A.	moderate	dark grayish brown	moderate olive brown	-
	P.I.A	good	strong orange yellow	strong orange yellow	-
	T.A.	moderate	deep yellowish brown	dark yellowish brown	-
	Glu.A	moderate	brownish black	brownish black	-
	Cz.	poor	brownish black	brownish black	-
	N.A.	moderate	strong yellowish brown	strong yellowish brown	-
AL3-2	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	dark brown	moderate brown	-
	I.S.	good	brownish black	brownish black	-
	Gly.A.	abundant	dark grayish brown	dark grayish yellowish brown	-
	P.I.A	good	yellowish gray	yellowish gray	-
	T.A.	moderate	moderate olive brown	grayish yellowish brown	-
	Glu.A	good	brownish black	dark grayish brown	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	poor	grayish yellowish brown	grayish yellowish brown	-
AL3-9	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	brownish black	brownish black	-
	P.I.A	moderate	brownish black	brownish black	-
	T.A.	abundant	brownish black	dark grayish brown	-
	Glu.A	moderate	brownish black	brownish black	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	abundant	brownish black	brownish black	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL3-12	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	brownish black	brownish black	-
	P.I.A	moderate	brownish black	brownish black	-
	T.A.	abundant	brownish black	dark grayish brown	-
	Glu.A	moderate	brownish black	brownish black	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	abundant	brownish black	brownish black	-
AL3-13	Y.M.	abundant	black	black	-
	O.M.	moderate	dark grayish yellowish brown	dark grayish yellowish brown	-
	I.S.	poor	light grayish yellowish brown	light grayish yellowish brown	-
	Gly.A.	poor	pale orange yellow	pale orange yellow	-
	P.I.A	good	moderate orange yellow	light olive brown	-
	T.A.	moderate	light grayish yellowish brown	light orange yellow	-
	Glu.A	poor	yellowish gray	yellowish gray	-
	Cz.	poor	dark grayish brown	dark gray	-
	N.A.	moderate	moderate olive brown	moderate olive brown	-
AL3-14-2	Y.M.	abundant	black	black	-
	O.M.	moderate	strong yellowish brown	dark grayish yellowish brown	-
	I.S.	moderate	moderate brown	strong yellowish brown	-
	Gly.A.	poor	moderate yellowish brown	moderate yellowish brown	-
	P.I.A	moderate	light grayish yellowish brown	strong orange yellow	-
	T.A.	moderate	grayish brown	moderate yellowish brown	-
	Glu.A	moderate	brownish black	dark gray	-
	Cz.	poor	light grayish brown	dark gray	-
	N.A.	moderate	moderate olive brown	moderate olive brown	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL3-16	Y.M.	abundant	deep orange yellow	strong orange yellow	-
	O.M.	moderate	brilliant orange yellow	light orange yellow	-
	I.S.	abundant	brownish black	moderate orange yellow	-
	Gly.A.	good	strong orange yellow	strong orange yellow	-
	P.I.A	good	deep orange yellow	deep orange yellow	-
	T.A.	good	deep orange yellow	deep orange yellow	-
	Glu.A	moderate	light orange yellow	moderate orange yellow	-
	Cz.	poor	pale orange yellow	pale orange yellow	-
	N.A.	moderate	deep orange yellow	strong orange yellow	-
AL4-4	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	strong brown	brownish black	-
	P.I.A	moderate	brownish black	deep brown	-
	T.A.	moderate	brownish black	deep grayish brown	-
	Glu.A	moderate	brownish black	brownish black	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	abundant	brownish black	brownish black	-
AL4-5	Y.M.	abundant	brownish black	dark brown	-
	O.M.	good	moderate orange yellow	moderate orange yellow	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	strong brown	brownish black	-
	P.I.A	moderate	brownish black	deep brown	-
	T.A.	moderate	brownish black	deep grayish brown	-
	Glu.A	good	deep orange yellow	deep orange yellow	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	abundant	brownish black	brownish black	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL4-7	Y.M.	good	deep pink	deep yellowish pink	-
	O.M.	good	moderate pink	moderate pink	-
	I.S.	abundant	pinkish white	pinkish white	-
	Gly.A.	poor	pinkish white	pinkish white	-
	P.I.A	poor	dark yellowish pink	dark yellowish pink	-
	T.A.	good	pinkish white	pinkish white	-
	Glu.A	abundant	pinkish white	pinkish white	-
	Cz.	good	pinkish white	pinkish white	-
	N.A.	good	dark pink	vivid yellowish pink	-
AL4-8	Y.M.	good	deep pink	deep yellowish pink	-
	O.M.	good	moderate pink	moderate pink	-
	I.S.	abundant	pinkish white	pinkish white	-
	Gly.A.	poor	pinkish white	pinkish white	-
	P.I.A	poor	dark yellowish pink	dark yellowish pink	-
	T.A.	good	pinkish white	pinkish white	-
	Glu.A	abundant	pinkish white	pinkish white	-
	Cz.	good	pinkish white	pinkish white	-
	N.A.	good	dark pink	vivid yellowish pink	-
AL4-10	Y.M.	abundant	strong greenish yellow	strong greenish yellow	+
	O.M.	abundant	yellowish white	yellowish white	-
	I.S.	good	brownish orange	deep orange yellow	+
	Gly.A.	abundant	vivid orange yellow	strong yellow	+
	P.I.A	abundant	strong greenish yellow	dark yellow	-
	T.A.	poor	yellowish white	strong greenish yellow	+
	Glu.A	good	brilliant greenish yellow	brilliant greenish yellow	+
	Cz.	poor	yellowish white	yellowish white	+
	N.A.	abundant	strong greenish yellow	strong greenish yellow	+

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL5-1	Y.M.	abundant	black	black	-
	O.M.	moderate	dark grayish yellowish brown	dark grayish yellowish brow	-
	I.S.	moderate	strong yellowish brown	moderate olive brown	-
	Gly.A.	moderate	light olive brown	moderate olive brown	-
	P.I.A	good	deep orange yellow	deep orange yellow	-
	T.A.	good	dark brown	dark brown	-
	Glu.A	good	strong yellowish brown	moderate yellowish brown	-
	Cz.	moderate	moderate yellowish brown	dark orange yellow	-
	N.A.	moderate	deep orange yellow	brownish orange	-
AL5-7	Y.M.	abundant	deep orange	strong orange	
	O.M.	good	deep orange	deep orange	-
	I.S.	good	deep orange yellow	deep orange yellow	-
	Gly.A.	abundant	strong brown	brownish black	-
	P.I.A	moderate	strong orange yellow	deep orange yellow	-
	T.A.	good	strong orange yellow	strong orange yellow	-
	Glu.A	good	moderate yellowish brown	deep yellowish brown	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	moderate	strong orange yellow	strong orange yellow	-
AL5-16	Y.M.	abundant	moderate olive brown	moderate olive brown	-
	O.M.	abundant	strong brown	brownish black	-
	I.S.	good	deep orange yellow	moderate orange yellow	-
	Gly.A.	moderate	light orange yellow	moderate orange yellow	-
	P.I.A	moderate	strong orange yellow	strong orange yellow	-
	T.A.	moderate	strong orange yellow	deep orange yellow	-
	Glu.A	moderate	dark orange yellow	light orange	-
	Cz.	moderate	grayish yellowish brown	light orange	-
	N.A.	moderate	moderate orange yellow	moderate orange yellow	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL7-5	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	dark grayish reddish brown	dark grayish reddish brown	-
	I.S.	good	brownish black	brownish black	-
	Gly.A.	good	strong yellowish brown	dark orange yellow	-
	P.I.A	good	light grayish yellowish brown	dark orange yellow	-
	T.A.	good	grayish yellowish brown	light grayish reddish brown	-
	Glu.A	poor	moderate orange yellow	pale orange yellow	-
	Cz.	poor	dark grayish yellow	strong yellowish brown	-
	N.A.	good	dark orange yellow	light yellowish brown	-
AL7-14	Y.M.	abundant	greenish white	strong yellowish white	-
	O.M.	abundant	greenish white	deep yellow	-
	I.S.	abundant	greenish white	yellowish white	-
	Gly.A.	abundant	greenish white	light yellowish brown	-
	P.I.A	abundant	greenish white	deep orange yellow	-
	T.A.	abundant	greenish white	dark orange yellow	-
	Glu.A	good	greenish white	strong brown	-
	Cz.	good	greenish white	moderate yellow	-
	N.A.	abundant	greenish white	brilliant orange yellow	-
AL8-2	Y.M.	abundant	dark yellow	dark yellow	-
	O.M.	abundant	dark brown	moderate brown	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	dark grayish brown	dark gray	-
	P.I.A	good	yellowish gray	yellowish gray	-
	T.A.	good	moderate olive brown	dark grayish yellowish brown	-
	Glu.A	good	dark grayish brown	dark grayish brown	-
	Cz.	good	dark grayish brown	brown gray	-
	N.A.	good	grayish yellowish brown	grayish yellowish brown	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL8-7	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	dark brown	dark yellowish brown	-
	I.S.	good	strong orange yellow	light orange yellow	-
	Gly.A.	moderate	deep yellow	dark grayish yellow	-
	P.I.A	good	strong brown	moderate yellowish brown	-
	T.A.	good	moderate olive brown	dark grayish brown	-
	Glu.A	good	dark olive brown	dark olive brown	-
	Cz.	moderate	grayish yellowish brown	grayish yellowish brown	-
	N.A.	abundant	dark brown	dark brown	-
AL8-8	Y.M.	abundant	brownish black	brownish black	-
	O.M.	moderate	strong yellowish brown	strong yellowish brown	-
	I.S.	moderate	strong orange yellow	light orange yellow	-
	Gly.A.	moderate	deep yellow	grayish yellow	-
	P.I.A	good	strong brown	moderate yellowish brown	-
	T.A.	moderate	strong yellowish brown	moderate yellowish brown	-
	Glu.A	moderate	moderate olive brown	moderate olive brown	-
	Cz.	moderate	grayish yellowish brown	grayish yellowish brown	-
	N.A.	good	dark brown	dark brown	-
AL8-16	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	good	deep orange	deep orange	-
	Gly.A.	good	brownish black	brownish black	-
	P.I.A	moderate	deep orange	strong brown	-
	T.A.	moderate	brownish black	dark grayish brown	-
	Glu.A	poor	dark yellowish brown	brownish gray	-
	Cz.	poor	brownish black	brownish gray	-
	N.A.	abundant	brownish black	brownish black	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL9-1	Y.M.	good	strong yellowish brown	strong yellowish brown	-
	O.M.	moderate	dark orange yellow	dark orange yellow	-
	I.S.	moderate	dark orange yellow	dark orange yellow	-
	Gly.A.	moderate	moderate olive brown	moderate olive brown	-
	P.I.A	moderate	strong orange yellow	strong orange yellow	-
	T.A.	good	strong yellowish brown	strong yellowish brown	-
	Glu.A	good	dark yellowish brown	dark gray	-
	Cz.	moderate	moderate yellowish brown	dark orange yellow	-
	N.A.	moderate	deep orange yellow	deep yellow	-
AL9-13	Y.M.	abundant	black	black	-
	O.M.	moderate	dark grayish yellowish brown	dark grayish yellowish brown	-
	I.S.	moderate	strong yellowish brown	strong yellowish brown	-
	Gly.A.	moderate	brownish black	brownish black	-
	P.I.A	good	light brown	deep orange yellow	-
	T.A.	moderate	moderate olive brown	moderate yellowish brown	-
	Glu.A	good	brownish black	dark gray	-
	Cz.	moderate	dark grayish brown	dark gray	-
	N.A.	moderate	moderate olive brown	dark yellow	-
AL9-20	Y.M.	abundant	deep orange yellow	strong orange yellow	-
	O.M.	moderate	brilliant orange yellow	light orange yellow	-
	I.S.	abundant	brownish black	dark grayish yellowish brown	-
	Gly.A.	good	moderate olive brown	dark olive brown	-
	P.I.A	good	deep orange yellow	deep orange yellow	-
	T.A.	good	deep yellowish brown	grayish yellowish brown	-
	Glu.A	moderate	light orange yellow	moderate orange yellow	-
	Cz.	poor	pale orange yellow	pale orange yellow	-
	N.A.	moderate	deep orange yellow	deep orange yellow	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL9-22	Y.M.	abundant	black	black	-
	O.M.	moderate	dark grayish yellowish brown	dark grayish yellowish brown	-
	I.S.	poor	strong yellowish brown	strong yellowish brown	-
	Gly.A.	poor	pale orange yellow	pale orange yellow	-
	P.I.A	good	light brown	deep orange yellow	-
	T.A.	moderate	moderate olive brown	moderate yellowish brown	-
	Glu.A	moderate	brownish black	dark gray	-
	Cz.	moderate	dark grayish brown	dark gray	-
	N.A.	abundant	black	black	-
AL9-24-1	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	deep brown	dark brown	-
	I.S.	good	deep orange yellow	deep orange yellow	-
	Gly.A.	good	deep orange yellow	strong orange yellow	-
	P.I.A	abundant	brownish orange	brownish orange	-
	T.A.	good	strong orange yellow	strong orange yellow	-
	Glu.A	good	dark orange yellow	light yellowish brown	-
	Cz.	good	dark grayish brown	brownish gray	-
	N.A.	good	grayish yellowish brown	grayish yellowish brown	-
AL10-3	Y.M.	abundant	brownish black	dark brown	-
	O.M.	abundant	deep brown	dark brown	-
	I.S.	good	deep orange yellow	deep orange yellow	-
	Gly.A.	good	dark yellowish brown	dark yellowish brown	-
	P.I.A	good	strong brown	strong brown	-
	T.A.	good	strong orange yellow	strong orange yellow	-
	Glu.A	abundant	dark grayish yellowish brown	dark yellowish brown	-
	Cz.	moderate	grayish yellowish brown	grayish yellowish brown	-
	N.A.	abundant	dark yellowish brown	dark yellowish brown	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
AL10-5	Y.M.	abundant	brownish black	dark brown	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	abundant	dark grayish yellowish brown	light olive brown	-
	Gly.A.	abundant	dark grayish yellow	moderate yellowish brown	-
	P.I.A	good	strong orange yellow	strong orange yellow	-
	T.A.	moderate	strong brown	deep yellow	-
	Glu.A	abundant	brownish black	dark grayish brown	-
	Cz.	good	brownish black	light brown	-
	N.A.	moderate	strong orange yellow	strong orange yellow	-
AL10-17	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	dark grayish brown	brownish black	-
	P.I.A	good	yellowish gray	yellowish gray	-
	T.A.	moderate	moderate olive brown	dark grayish yellowish brown	-
	Glu.A	abundant	brownish black	brownish black	-
	Cz.	good	brownish black	brownish black	-
	N.A.	good	grayish yellowish brown	grayish yellowish brown	-
ASC13-5-1	Y.M.	abundant	brownish black	brownish black	-
	O.M.	abundant	brownish black	brownish black	-
	I.S.	good	moderate orange yellow	moderate orange yellow	-
	Gly.A.	good	dark grayish yellowish brown	dark yellowish brown	-
	P.I.A	good	dark grayish yellowish brown	dark grayish yellowish brown	-
	T.A.	good	moderate orange yellow	strong orange	-
	Glu.A	good	brownish black	dark grayish yellowish brown	-
	Cz.	poor	brownish black	dark grayish yellowish brown	-
	N.A.	good	dark grayish yellowish brown	dark grayish yellowish brown	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
ASC19-2-1	Y.M.	abundant	brownish gray	brownish gray	-
	O.M.	abundant	moderate brown	moderate brown	-
	I.S.	abundant	brownish black	brownish black	-
	Gly.A.	abundant	brownish black	brownish black	-
	P.I.A	good	deep orange yellow	deep orange yellow	-
	T.A.	abundant	brownish black	brownish gray	-
	Glu.A	good	brownish gray	brownish gray	-
	Cz.	moderate	brownish black	brownish gray	-
	N.A.	good	brownish black	brownish gray	-
P0402	Y.M.	abundant	brownish black	brownish black	-
	O.M.	poor	pale yellowish pink	pale yellowish pink	-
	I.S.	good	deep orange	deep orange	-
	Gly.A.	poor	strong brown	moderate orange	-
	P.I.A	moderate	dark orange yellow	strong yellowish brown	-
	T.A.	poor	moderate orange yellow	dark orange yellow	-
	Glu.A	poor	moderate orange	dark orange yellow	-
	Cz.	poor	light brown	grayish brown	-
	N.A.	good	deep brown	deep brown	-
P0417	Y.M.	abundant	yellowish gray	grayish yellow	-
	O.M.	abundant	yellowish white	dark yellow	-
	I.S.	good	yellowish white	yellowish white	-
	Gly.A.	abundant	yellowish white	yellowish white	-
	P.I.A	moderate	yellowish gray	light orange yellow	-
	T.A.	abundant	yellowish gray	light grayish brown	-
	Glu.A	poor	yellowish white	pale yellow	-
	Cz.	good	yellowish white	pale yellow	-
	N.A.	moderate	yellowish gray	deep yellow	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
P0415	Y.M.	good	yellowish gray	grayish yellow	-
	O.M.	good	yellowish white	yellowish white	-
	I.S.	good	yellowish white	yellowish white	-
	Gly.A.	abundant	yellowish white	yellowish white	-
	P.I.A	good	yellowish gray	light orange yellow	-
	T.A.	good	yellowish gray	light grayish yellow	-
	Glu.A	good	yellowish white	pale yellow	-
	Cz.	good	yellowish white	pale yellow	-
	N.A.	good	yellowish gray	deep yellow	-
P1605	Y.M.	abundant	greenish white	brilliant orange yellow	-
	O.M.	good	greenish white	yellowish white	-
	I.S.	good	greenish white	yellowish white	-
	Gly.A.	abundant	greenish white	brilliant orange yellow	-
	P.I.A	good	greenish white	deep orange yellow	-
	T.A.	abundant	greenish white	light orange yellow	-
	Glu.A	abundant	greenish white	light orange yellow	-
	Cz.	good	greenish white	yellowish white	-
	N.A.	good	greenish white	moderate orange yellow	-
P1803	Y.M.	good	yellowish white	light orange yellow	-
	O.M.	poor	yellowish white	yellowish white	-
	I.S.	good	yellowish white	yellowish white	-
	Gly.A.	moderate	yellowish white	yellowish gray	-
	P.I.A	moderate	yellowish gray	light orange yellow	-
	T.A.	moderate	yellowish gray	light grayish brown	-
	Glu.A	abundant	yellowish white	yellowish white	-
	Cz.	poor	yellowish white	dark grayish yellow	-
	N.A.	poor	yellowish white	dark grayish yellow	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
P1440	Y.M.	good	yellowish white	light orange yellow	-
	O.M.	poor	yellowish white	yellowish white	-
	I.S.	good	yellowish white	yellowish white	-
	Gly.A.	moderate	yellowish white	yellowish gray	-
	P.I.A	moderate	yellowish gray	light orange yellow	-
	T.A.	moderate	yellowish gray	light grayish brown	-
	Glu.A	abundant	yellowish white	yellowish white	-
	Cz.	poor	yellowish white	dark grayish yellow	-
	N.A.	poor	yellowish white	dark grayish yellow	-
CYP1-5	Y.M.	abundant	brownish pink	dark orange yellow	-
	O.M.	moderate	yellowish white	yellowish white	-
	I.S.	poor	pinkish white	pinkish white	-
	Gly.A.	poor	yellowish white	yellowish white	-
	P.I.A	poor	yellowish gray	yellowish gray	-
	T.A.	good	deep yellowish brown	dark reddish purple	+
	Glu.A	good	light gray	light gray	-
	Cz.	poor	pinkish white	pinkish white	-
	N.A.	abundant	light gray	dark orange yellow	-
CYP1-1B	Y.M.	abundant	strong brown	dark orange yellow	-
	O.M.	good	strong reddish brown	brownish orange	-
	I.S.	abundant	yellowish white	dark yellow	-
	Gly.A.	abundant	strong greenish yellow	strong greenish yellow	-
	P.I.A	good	dark yellow	dark yellow	-
	T.A.	abundant	yellowish gray	brownish black	+
	Glu.A	good	grayish yellowish brown	dark grayish yellowish brown	-
	Cz.	abundant	yellowish white	yellowish white	-
	N.A.	abundant	yellowish white	yellowish white	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
D10-9-5	Y.M.	abundant	dark grayish brown	dark grayish brown	-
	O.M.	good	grayish reddish orange	moderate reddish orange	-
	I.S.	moderate	moderate yellowish brown	moderate yellow	-
	Gly.A.	poor	dark yellow	moderate yellow	-
	P.I.A	moderate	strong orange yellow	strong orange yellow	-
	T.A.	moderate	dark reddish brown	deep brown	-
	Glu.A	moderate	dark yellow	dark yellow	-
	Cz.	moderate	dark gray	dark yellow	-
	N.A.	good	moderate olive brown	moderate olive brown	-
B10-3-3	Y.M.	abundant	brownish black	brownish black	-
	O.M.	good	strong brown	brownish orange	-
	I.S.	moderate	moderate orange yellow	moderate orange yellow	-
	Gly.A.	moderate	deep yellow	dark yellow	-
	P.I.A	abundant	strong yellowish brown	strong yellowish brown	-
	T.A.	moderate	moderate yellowish brown	grayish yellowish brown	-
	Glu.A	good	deep yellowish brown	light yellowish brown	-
	Cz.	moderate	dark grayish yellow	grayish yellowish brown	-
	N.A.	moderate	dark orange yellow	dark orange yellow	-
B10-3-6	Y.M.	abundant	brownish black	brownish black	-
	O.M.	moderate	strong yellowish brown	strong yellowish brown	-
	I.S.	moderate	strong orange yellow	strong orange yellow	-
	Gly.A.	moderate	deep yellow	dark yellow	-
	P.I.A	moderate	deep orange yellow	deep orange yellow	-
	T.A.	moderate	moderate yellowish brown	grayish yellowish brown	-
	Glu.A	moderate	dark yellowish brown	light grayish yellowish brown	-
	Cz.	moderate	dark grayish yellow	grayish yellowish brown	-
	N.A.	moderate	dark orange yellow	dark orange yellow	-

Table 1 Cultural characteristics of the isolates incubated at 30 ºC for 21 days (continued)

Strain No.	Media	Growth	Color of upper surface	Color of reverse surface	Soluble pigment
SM1-1	Y.M.	moderate	brownish black	dark brown	-
	O.M.	moderate	dark grayish yellowish brown	dark grayish yellowish brown	-
	I.S.	good	deep yellowish brown	deep yellowish brown	-
	Gly.A.	moderate	dark orange yellow	dark orange yellow	-
	P.I.A	good	strong orange yellow	deep orange yellow	-
	T.A.	good	strong brown	brownish orange	-
	Glu.A	good	dark yellowish brown	dark yellowish brown	-
	Cz.	moderate	dark yellowish brown	grayish yellowish brown	-
	N.A.	moderate	deep orange yellow	deep yellow	-
PSM1-8	Y.M.	moderate	moderate olive brown	light olive brown	-
	O.M.	moderate	dark grayish yellowish brown	dark grayish yellowish brown	-
	I.S.	poor	light grayish yellowish brown	light grayish yellowish brown	-
	Gly.A.	good	dark yellowish brown	dark yellowish brown	-
	P.I.A	good	strong orange yellow	strong orange yellow	-
	T.A.	moderate	strong yellowish brown	dark orange yellow	-
	Glu.A	poor	dark orange yellow	dark orange yellow	-
	Cz.	moderate	dark yellowish brown	grayish yellowish brown	-
	N.A.	moderate	deep orange yellow	dark yellow	-

Table 2 The physiological and biochemical characteristic of strains in *Micromonospora*, *Microbispora*, *Nonomuraea*, *Actinomadura*, *Pseudonocardia* and *Nocardia.* +++; Abundant, ++; Good, +; Moderate; w, Week

Group	Strain No.	NaCl tolerance							pH tolerance					Growth at (°C)							Skim milk		Gelatin liquefaction	Nitrate reduction	Starch hydrolysis	Antimicrobial activities
		1.5	2	3	4	5	6	7	4	4.5	5	6	8	15	20	30	37	40	45	50	peptonization	Coagulation				
I	AL2-3	+++	+++	+++	++	w	-	-	-	-	+++	+++	+++	+	+	+++	+++	+	-	-	+++	w	+	-	+	-
	AL3-2	+++	+++	+++	++	w	-	-	-	-	++	+++	+++	+	+	+++	++	++	-	-	++	-	+	+	+	
	AL4-5	+++	+++	++	++	-	-	-	-	-	++	+++	+++	+	+	+++	+++	++	-	-	++	-	+	-	+	
	AL5-7	+++	++	++	+	-	-	-	-	-	++	+++	+++	+	+	+++	++	++	-	-	++	-	+	-	+	
	AL5-16	+++	+++	++	++	-	-	-	-	-	+++	+++	+++	+	+	+++	++	++	-	-	++	-	+	-	+	
	AL8-2	+++	++	++	++	w	-	-	-	-	+++	+++	+++	+	+	+++	+++	++	-	-	++	w	+	+	+	
	AL9-24-1	+++	+++	++	++	w	-	-	-	-	+++	+++	+++	+	+	+++	+++	++	-	-	++	-	+	-	+	
	AL10-5	+++	+++	++	+	-	-	-	-	-	++	+++	+++	+	+	+++	+++	++	-	-	++	-	+	-	+	
	AL10-17	+++	+++	++	+	w	-	-	-	-	++	+++	+++	+	+	+++	+++	++	-	-	+	-	+	+	+	
II	D10-9-5	++	++	+	+	+	-	-	-	-	-	+++	+++	w	+++	+++	+++	w	-	-	+	-	+	-	+	*B. subtilis, S.aureus, M. luteus,*
III	ASC19-2-1	+++	++	++	++	-	-	-	-	-	+++	+++	+++	w	+++	+++	+++	w	-	-	++	+	+	-	+	-
IV	AL8-7	+++	++	++	+	+	-	-	-	-	+++	+++	+++	+	+	+++	+++	++	-	-	+++	-	+	+	+	
	AL8-8	++	++	++	+	+	-	-	-	-	+++	+++	+++	+	+++	+++	+++	+	-	-	++	-	+	+	+	-
	AL10-3	+++	++	++	+	+	-	-	-	-	+++	+++	+++	+	+++	+++	+++	++	-	-	++	-	+	+	+	
	ASC13-5-1	+++	+++	+++	+	+	-	-	-	-	+++	+++	+++	+	+	+++	+++	++	-		++	-	+	+	+	
	B10-3-3	+++	+++	++	+	-	-	-	-	-	+++	+++	+++	+	+	+++	++	++	-	-	+++	-	+	+	+	
	B10-3-6	++	++	++	+	-	-	-	-	-	++	+++	+++	+	+	+++	++	+	-	-	++	-	+	+	+	
V	AL7-5	++	++	+	+	-	-	-	-	-	-	+++	+++	w	+++	+++	+++	+++	+++	-	++	+	+	+	+	*M. luteus*
VI	AL2-1	+++	+++	+	+	w	-	-	-	-	+++	+++	+++	+	+	+++	++	++	-	-	++	+	+	-	+	-
	AL3-9	+++	+++	+	w	w	-	-	-	+	+++	+++	+++	+	++	+++	+++	w	-	-	+	+	+	-	+	
	AL3-12	+++	+++	+	w	w	-	-	-	++	+++	+++	+++	+	+	+++	+++	w	-	-	++	+	+	-	+	
	AL4-4	+++	+++	+	w	w	-	-	-	++	+++	+++	+++	+	+	++	+++	w	-	-	+	++	+	-	+	
	AL8-16	+++	+++	+	w	w	-	-	-	+	+++	+++	+++	+	+	+++	+++	w	-	-	+	+	+	-	+	
VII	AL1-3	++	++	-	-	-	-	-	-	-	-	+++	+++	-	++	+++	+++	w	-	-	+	-	+	-	+	-
VIII	P0402	++	++	++	++	+	-	-	-	-	+++	+++	+++	-	+	++	++	-	-	-	++	-	+	-	-	-
IX	AL3-16	+++	++	+	+	w	-	-	-	-	-	+++	+++	-	+++	+++	+++	-	-	-	-	-	+	-	+	-
	AL9-20	+++	++	+	+	w	-	-	-	-	-	+++	+++	-	+++	+++	+++	-	-	-	w	-	+	-	+	

Table 2 The physiological and biochemical characteristic of strains in *Micromonospora*, *Microbispora*, *Nonomuraea*, *Actinomadura*, *Pseudonocardia* and *Nocardia* (continued). +++; Abundant, ++; Good, +; Moderate, w; Week

Group	Strain No.	NaCl tolerance							pH tolerance					Growth at (°C)							Skim milk		Gelatin liquefaction	Nitrate reduction	Starch hydrolysis	Antimicrobial activities
		1.5	2	3	4	5	6	7	4	4.5	5	6	8	15	20	30	37	40	45	50	peptonization	Coagulation				
X	AL5-1	+++	+++	++	++	w	-	-	-	-	-	+++	+++	w	+	+++	+++	+	+	-	+	-	+	-	+	-
	AL9-1	++	++	++	++	-	-	-	-	-	-	+++	+++	+	+	+++	+++	+	+	-	++	-	+	-	+	
	SM1-1	+++	+++	++	++	-	-	-	-	-	+	+++	+++	+	+	+++	++	+	+	-	++	-	+	-	+	
	PSM1-8	++	++	++	+	-	-	-	-	-	+	+++	+++	+	+	+++	++	+	+	-	++	-	+	-	+	
XI	AL3-13	+++	+++	+++	++	-	-	-	-	-	++	+++	+++	+	+	+++	+++	+	+	-	++	+++	+	+	+	-
	AL3-14-2	+++	+++	++	++	-	-	-	-	-	++	++	++	+	+	+++	+++	w	+	-	++	-	+	+	+	
	AL9-13	+++	++	++	+	-	-	-	-	-	++	+++	+++	+	+	+++	++	+	+	-	++	+	+	+	+	
	AL9-22	+++	++	++	+	-	-	-	-	-	++	+++	+++	+	+	+++	++	+	+	-	++	+	+	+	+	
XII	AL1-15-2	+++	+++	++	-	-	-	-	-	-	+++	+++	+++	-	+	+++	+++	-	-	-	-	-	+	-	-	-
	AL1-16B	+++	++	++	-	-	-	-	-	-	++	++	+++	-	+	+++	+++	w	-	-	-	-	+	-	-	
	AL4-7	+	+	-	-	-	-	-	-	-	-	+++	+++	-	++	++	++	w	-	-	-	-	w	-	-	-
	AL4-8	+	+	-	-	-	-	-	-	-	-	+++	+++	-	++	++	++	w	-	-	-	-	-	-	-	
	P0417	++	+	-	-	-	-	-	-	+	++	+++	+++	-	+	++	++	-	-	-	-	-	w	-	-	-
A	CYP1-5	+	+	w	-	-	-	-	-	-	-	+++	+++	-	w	++	++	++	w	-	-	-	+	-	-	*B. subtilis, P. aerugenosa, S.aureus, M. luteus,*
B	AL4-10	++	++	+	+	w	-	-	-	-	w	+++	+++	-	++	++	++	-	-	-	+	-	+	+	+	*B. subtilis*
C	CYP1-1B	+++	++	+	+	w	-	-	-	++	++	+++	+++	-	++	++	++	++	++	-	-	-	-	-	++	*B. subtilis, P. aerugenosa, S.aureus*
D	AL7-14	+++	++	++	+++	+++	+++	+++	++	+++	+++	+++	+++	w	+	+++	+++	+++	+++	-	+	-	+	-	+	*S.aureus, M. luteus,*
	AL1-8A	+++	++	++	++	++	++	++	-	w	+++	+++	+++	++	+++	+++	+++	-	-	-	-	-	+	-	-	-
	AL1-8B	+++	++	++	++	++	++	++	-	w	+++	+++	+++	++	+++	+++	+++	-	-	-	-	-	+	-	-	
1	P1440	++	+	+	w	w	-	-	-	-	+++	+++	+++	-	+++	+++	+++	+++	-	-	w	w	w	+	-	-
	P1803	++	+	+	w	w	-	-	-	-	+++	+++	+++	-	+++	+++	+++	+++	-	-	w	w	w	+	-	
2	P1605	+++	+++	+++	+++	+++	+	+	-	-	+++	+++	+++	+	++	+++	+++	+++	+++	-	-	w	+	+	-	-

Table 3 Carbon utilization of strains in *Micromonospora*, *Microbispora*, *Nonomuraea*, *Actinomadura*, *Pseudonocardia* and *Nocardia*

Group	Strain No.	Carbon utilization															
		Glucose	D-mannitol	L-rhamnose	Raffinose	Inositol	Lactose	L-arabinose	D-fructose	D-ribose	D-melibiose	Glycerol	Salicin	D-galactose	Cellubiose	Sucrose	D-xylose
I	AL2-3	++	-	+	-	-	++	+	+	-	++	-	-	±	-	+	-
	AL3-2	++	-	+	-	-	++	+	+	-	++	-	-	±	-	-	-
	AL4-5	++	-	+	-	-	++	++	++	-	++	-	-	±	+	+	++
	AL5-7	++	-	+	+	+	+	++	++	-	++	-	-	++	++	-	-
	AL5-16	++	-	-	-	+	+	+	-	-	+	-	-	±	++	++	-
	AL8-2	++	-	-	+	-	++	++	+	-	++	-	-	++	+	++	+
	AL9-24-1	++	-	+	-	-	++	++	+	-	++	-	-	++	++	+	+
	AL10-5	++	-	+	-	-	+	++	-	-	++	-	-	++	++	+	-
	AL10-17	++	-	-	+	-	++	+	+	-	++	-	-	++	++	+	+
II	D10-9-5	++	-	-	-	+	-	++	-	-	±	-	-	-	-	+	±
III	ASC19-2-1	++	-	-	-	-	±	-	±	-	-	++	++	++	++	±	±
IV	AL8-7	++	-	±	-	±	+	+	+	-	++	++	++	++	++	+	±
	AL8-8	++	-	-	++	-	+	+	+	-	-	++	±	++	++	+	+
	AL10-3	++	-	-	++	-	-	++	++	-	-	±	-	++	++	++	++
	ASC13-5-1	++	-	±	+	±	++	+	+	-	+	±	-	+	+	+	+
	B10-3-3	++	-	±	+	±	+	+	+	-	++	±	-	+	±	+	±
	B10-3-6	++	-	±	+	±	+	+	+	-	++	±	-	+	±	+	±
V	AL7-5	++	-	-	+	-	++	+	±	-	++	++	-	++	++	+	+
VI	AL2-1	++	-	±	-	-	+	±	-	-	+	+	+	++	+	-	-
	AL3-9-1	++	-	±	-	-	++	±	++	-	++	++	++	++	++	-	-
	AL3-12	++	-	±	-	-	++	±	+	-	++	++	++	++	++	-	±
	AL4-4	++	-	±	-	-	++	±	+	-	++	++	+	++	++	-	-
	AL8-16	++	-	+	-	-	++	±	-	-	++	+	+	++	++	-	-
VII	AL1-3	++	-	+	-	-	++	±	+	-	++	+	+	++	++	+	-
VIII	P0402	++	-	-	-	-	+	±	-	-	-	-	+	+	-	-	-
IX	AL3-16	++	+	-	++	-	+	++	+	-	±	-	-	-	-	++	-
	AL9-20	++	+	-	++	-	+	++	+	-	±	-	-	-	-	++	-
X	AL5-1	++	±	±	++	±	+	+	+	-	++	±	+	++	++	++	-
	AL9-1	++	±	±	++	±	+	++	+	-	++	±	+	++	++	++	-
	SM1-1	++	±	±	+	-	+	+	++	-	++	±	+	++	++	++	±
	PSM1-8	++	±	±	+	-	+	+	+	-	++	±	+	+	+	++	-
XI	AL3-13	++	-	±	-	-	-	++	++	-	-	±	+	±	++	++	++
	AL3-14-2	++	-	±	-	-	-	++	++	-	-	±	++	±	++	++	++
	AL9-13	++	-	±	-	-	-	++	++	-	-	±	+	±	++	++	++
	AL9-22	++	-	±	-	-	-	++	++	-	-	±	+	±	++	++	++
XII	AL1-15-2	++	-	-	+	-	-	+	+	-	-	+	+	+	++	++	++
	AL1-16B	++	-	++	++	-	-	+	++	-	-	-	+	+	++	++	++
	AL4-7	+	++	+	+	+	-	+	+	-	-	+	-	-	+	+	+
	AL4-8	+	+	+	+	+	-	+	+	-	-	+	-	-	+	+	+
	P0417	+	+	+	-	+	-	+	+	-	+	+	-	-	-	-	-
A	CYP1-5	+	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
B	AL4-10	+	++	++	++	++	-	++	-	-	-	-	-	-	++	++	++
C	CYP1-1B	++	++	++	-	-	+	++	±	-	-	-	-	-	+	++	++
D	AL7-14	+	++	++	++	++	++	-	+	-	++	++	++	++	++	+	++
	AL1-8A	++	++	++	++	++	++	+	++	-	++	++	++	++	+	++	++
	AL1-8B	++	++	++	++	++	++	+	++	-	++	++	++	++	+	++	++
1	P1440	++	-	±	-	-	±	-	-	-	-	+	±	±	+	-	+
	P1803	++	-	±	-	-	±	-	-	-	-	+	±	±	+	-	+
2	P1605	++	+	+	±	++	±	-	+	-	±	++	+	+	+	+	±

Table 4 Similarity percentage of the representative *Micromonospora* strains

	% Similarity																		
Strains	**AL10-17**	**AL8-2**	**D10-9-5**	**ASC19-2-1**	**AL8-8**	**AL10-3**	**AL7-5**	**AL3-9**	**AL4-4**	**AL1-3**	**P0402**	**AL9-20**	**AL3-16**	**AL9-1**	**AL5-1**	**AL9-13**	**AL9-22**	**AL1-15-2**	**AL1-16B**
AB037008	95.7	95.7	95.8	95.8	95.7	95.7	95.7	96.1	96.1	96.1	95.7	95.6	95.6	96.1	96.1	96.1	96.1	96.4	96.5
AB037000	96.4	96.4	96.5	96.4	96.2	96.2	96.5	96.9	96.9	96.9	96.5	96.2	96.2	96.7	96.7	96.8	96.8	97.3	97.3
D85478	96.7	96.7	96.8	96.6	96.5	96.5	96.5	96.8	96.8	96.8	96.5	96.2	96.2	97.0	97.0	96.8	96.8	97.4	97.3
AB236956	96.4	96.4	96.4	96.7	96.4	96.4	96.2	96.5	96.5	96.5	96.3	96.5	96.5	96.7	96.7	96.5	96.5	96.6	96.5
X92613	97.5	97.5	97.6	97.7	97.5	97.5	97.2	98.0	98.0	97.6	97.5	98.1	98.1	97.9	97.9	97.6	97.6	97.7	97.8
FJ261956	97.6	97.6	97.8	97.9	97.6	97.6	97.3	97.5	97.5	97.2	97.5	97.4	97.4	98.2	98.2	97.9	97.9	98.0	97.9
AB275607	97.9	97.9	98.1	98.2	97.9	97.9	97.6	98.1	98.1	98.1	98.0	97.6	97.6	98.2	98.2	97.9	97.9	97.9	97.8
AL3-9	98.9	98.9	99.1	98.8	98.7	98.7	99.0	100	100	99.6	99.1	98.5	98.5	98.3	98.3	98.4	98.4	98.2	98.2
AL4-4	98.9	98.9	99.1	98.8	98.7	98.7	99.0	100	100	99.6	99.1	98.5	98.5	98.3	98.3	98.4	98.4	98.2	98.2
AL1-3	99.0	99.0	99.1	98.8	98.8	98.8	99.1	99.6	99.6	100	99.4	98.2	98.2	98.4	98.4	98.5	98.5	98.4	98.3
P0402	99.1	99.1	99.2	98.9	98.8	98.8	99.0	99.1	99.1	99.4	100	98.0	98.0	98.5	98.5	98.4	98.4	98.5	98.5
AL10-17	100	100	99.8	99.7	99.7	99.7	99.4	98.9	98.9	99.0	99.1	98.3	98.3	99.4	99.4	98.8	98.8	98.2	98.1
AL8-2	100	100	99.8	99.7	99.7	99.7	99.4	98.9	98.9	99.0	99.1	98.3	98.3	99.4	99.4	98.8	98.8	98.2	98.1
AL7-5	99.4	99.4	99.3	99.4	99.4	99.4	100	99.0	99.0	99.1	99.0	97.9	97.9	98.8	98.8	99.4	99.4	98.5	98.5
ASC19-2-1	99.7	99.7	99.6	100	99.7	99.7	99.4	98.8	98.8	98.8	98.9	98.4	98.4	99.1	99.1	98.8	98.8	98.2	98.1
AB196712	99.7	99.7	99.6	99.7	99.6	99.6	99.3	98.8	98.8	98.8	98.9	98.5	98.5	99.3	99.3	98.8	98.8	98.2	98.1
AL8-8	99.7	99.7	99.5	99.7	100	100	99.4	98.7	98.7	98.8	98.8	98.5	98.5	99.1	99.1	98.8	98.8	98.1	98.0
AL10-3	99.7	99.7	99.5	99.7	100	100	99.4	98.7	98.7	98.8	98.8	98.5	98.5	99.1	99.1	98.8	98.8	98.1	98.0
X92604	99.4	99.4	99.2	99.4	99.7	99.7	99.1	98.4	98.4	98.5	98.5	98.2	98.2	98.8	98.8	98.5	98.5	97.8	97.7
X92594	99.2	99.2	99.1	99.2	99.1	99.1	99.7	98.8	98.8	98.8	98.8	97.6	97.6	98.6	98.6	99.1	99.1	98.3	98.2
D10-9-5	99.8	99.8	100	99.6	99.5	99.5	99.3	99.1	99.1	99.1	99.2	98.2	98.2	99.2	99.2	98.7	98.7	98.3	98.2
AL9-20	98.3	98.3	98.2	98.4	98.5	98.5	97.9	98.5	98.5	98.2	98.0	100	100	97.9	97.9	97.4	97.4	97.2	97.1
AL3-16	98.3	98.3	98.2	98.4	98.5	98.5	97.9	98.5	98.5	98.2	98.0	100	100	97.9	97.9	97.4	97.4	97.2	97.1
AB241454	98.5	98.5	98.3	98.5	98.6	98.6	98.0	98.7	98.7	98.3	98.2	99.8	99.8	97.9	97.9	97.4	97.4	97.2	97.1
AJ783993	98.5	98.5	98.4	98.8	98.8	98.8	98.2	98.3	98.3	98.5	98.2	99.3	99.3	97.9	97.9	97.6	97.6	97.7	97.6
AJ626950	98.3	98.3	98.5	98.5	98.5	98.5	97.9	98.4	98.4	98.0	98.3	98.9	98.9	97.9	97.9	97.5	97.5	97.6	97.5
AB193565	98.5	98.5	98.6	98.4	98.6	98.6	98.8	99.0	99.0	98.6	98.7	98.6	98.6	98.0	98.0	98.3	98.3	98.5	98.4
AF152109	97.9	97.9	98.1	97.9	98.0	98.0	98.2	98.5	98.5	98.8	98.5	98.2	98.2	97.3	97.3	97.6	97.6	98.2	98.1
AY561829	97.7	97.7	97.9	97.6	97.9	97.9	97.7	98.1	98.1	98.0	97.8	97.6	97.6	97.1	97.1	97.1	97.1	97.2	97.1
X92599	98.3	98.3	98.5	98.2	98.5	98.5	98.0	98.8	98.8	98.5	98.2	98.8	98.8	97.7	97.7	97.4	97.4	97.6	97.5
AB196716	98.5	98.5	98.7	98.4	98.6	98.6	98.2	98.9	98.9	98.8	98.6	98.6	98.6	97.9	97.9	97.6	97.6	97.9	97.9
X92601	99.1	99.1	99.2	98.8	99.1	99.1	98.5	98.7	98.7	98.7	98.8	98.2	98.2	98.5	98.5	97.9	97.9	97.8	97.7
AB241455	99.4	99.4	99.5	99.1	99.4	99.4	98.8	98.9	98.9	99.1	99.2	98.3	98.3	98.8	98.8	98.2	98.2	98.4	98.3
X92611	98.8	98.8	99.0	99.1	99.1	99.1	98.5	98.5	98.5	98.6	98.8	98.2	98.2	98.2	98.2	97.9	97.9	98.5	98.4
X92625	98.2	98.2	98.3	98.2	98.2	98.2	97.9	98.2	98.2	98.1	98.2	97.8	97.8	98.8	98.8	98.5	98.5	98.5	98.5
X92598	97.9	97.9	98.0	97.9	97.7	97.7	97.4	97.5	97.5	97.6	97.9	97.7	97.7	98.5	98.5	98.0	98.0	98.4	98.3
X92617	98.6	98.6	98.8	98.4	98.3	98.3	98.1	98.2	98.2	98.4	98.5	97.4	97.4	99.2	99.2	98.7	98.7	98.8	98.8
X92603	97.7	97.7	97.9	97.7	97.6	97.6	98.1	98.2	98.2	98.4	98.2	97.3	97.3	98.3	98.3	98.7	98.7	98.8	98.9
X92610	97.9	97.9	98.1	97.8	97.7	97.7	98.0	97.9	97.9	97.9	97.9	97.1	97.1	98.5	98.5	98.6	98.6	98.9	98.8
X92608	98.2	98.2	98.2	98.0	97.9	97.9	97.9	97.9	97.9	98.0	98.2	97.8	97.8	98.7	98.7	98.4	98.4	98.5	98.4
X92618	98.5	98.5	98.6	98.4	98.3	98.3	98.1	98.2	98.2	98.2	98.6	97.7	97.7	98.8	98.8	98.4	98.4	98.4	98.3
X92623	98.2	98.2	98.4	98.2	98.1	98.1	97.8	98.2	98.2	98.2	98.4	97.8	97.8	98.5	98.5	98.1	98.1	98.5	98.5
X92629	98.2	98.2	98.4	98.0	97.9	97.9	98.1	97.9	97.9	97.9	98.1	97.4	97.4	98.8	98.8	98.7	98.7	98.5	98.4
X92624	98.2	98.2	98.4	98.0	97.9	97.9	98.1	97.9	97.9	97.9	98.1	97.4	97.4	98.8	98.8	98.7	98.7	98.5	98.4
X92607	98.2	98.2	98.4	98.0	97.9	97.9	98.0	97.9	97.9	97.9	98.1	97.6	97.6	98.8	98.8	98.6	98.6	98.5	98.5
X92620	97.9	97.9	98.1	97.9	97.8	97.8	98.2	98.1	98.1	98.1	98.2	97.1	97.1	98.5	98.5	98.8	98.8	98.9	99.0
X92609	97.8	97.8	97.6	98.0	97.8	97.8	97.7	97.6	97.6	97.6	97.8	97.5	97.5	98.4	98.4	98.3	98.3	98.3	98.2
AL1-15-2	98.2	98.2	98.3	98.2	98.1	98.1	98.5	98.2	98.2	98.4	98.5	97.2	97.2	98.8	98.8	99.1	99.1	100	99.9
AL1-16B	98.1	98.1	98.2	98.1	98.0	98.0	98.5	98.2	98.2	98.3	98.5	97.1	97.1	98.7	98.7	99.1	99.1	99.9	100
X92631	98.3	98.3	98.5	98.3	98.2	98.2	98.7	98.1	98.1	98.2	98.3	97.2	97.2	98.8	98.8	99.1	99.1	99.3	99.2
X92628	98.0	98.0	98.2	97.9	97.8	97.8	98.3	98.2	98.2	98.4	98.4	97.0	97.0	98.6	98.6	98.9	98.9	99.5	99.4
AL9-1	99.4	99.4	99.2	99.1	99.1	99.1	98.8	98.3	98.3	98.4	98.5	97.9	97.9	100	100	99.4	99.4	98.8	98.7
AL5-1	99.4	99.4	99.2	99.1	99.1	99.1	98.8	98.3	98.3	98.4	98.5	97.9	97.9	100	100	99.4	99.4	98.8	98.7
AL9-13	98.8	98.8	98.7	98.8	98.8	98.8	99.4	98.4	98.4	98.5	98.4	97.4	97.4	99.4	99.4	100	100	99.1	99.1
AL9-22	98.8	98.8	98.7	98.8	98.8	98.8	99.4	98.4	98.4	98.5	98.4	97.4	97.4	99.4	99.4	100	100	99.1	99.1
EU196562	98.8	98.8	98.7	98.8	98.8	98.8	99.4	98.4	98.4	98.5	98.4	97.4	97.4	99.4	99.4	100	100	99.1	99.1
AJ560635	98.4	98.4	98.2	98.2	98.2	98.2	98.6	98.2	98.2	98.4	98.4	97.6	97.6	99.0	99.0	99.2	99.2	99.3	99.2
AB193559	98.2	98.2	98.3	98.2	98.1	98.1	98.6	98.7	98.7	98.6	98.5	97.3	97.3	98.5	98.5	98.9	98.9	99.1	99.1
AB107231	98.6	98.6	98.8	98.4	98.3	98.3	98.3	98.8	98.8	98.7	99.0	97.8	97.8	98.9	98.9	98.6	98.6	98.9	98.8
AB196710	98.5	98.5	98.7	98.8	98.5	98.5	98.2	98.2	98.2	98.4	98.5	97.7	97.7	99.1	99.1	98.8	98.8	99.1	99.1
AJ783996	98.2	98.2	98.1	98.5	98.5	98.5	97.9	98.0	98.0	97.6	97.9	99.0	99.0	97.6	97.6	97.3	97.3	97.3	97.2
AM944497	97.4	97.4	97.4	97.6	97.4	97.4	97.1	97.4	97.4	97.4	97.2	97.8	97.8	97.7	97.7	97.4	97.4	97.4	97.3
AJ784008	97.6	97.6	97.4	97.4	97.3	97.3	97.9	97.4	97.4	97.6	97.6	97.2	97.2	98.2	98.2	98.5	98.5	98.8	98.7
AB159779	98.5	98.5	98.6	98.5	98.3	98.3	98.0	98.5	98.5	98.8	98.5	98.1	98.1	99.1	99.1	98.6	98.6	98.8	98.7
AY040619	97.3	97.3	97.1	97.0	97.0	97.0	97.1	97.0	97.0	96.7	97.2	96.8	96.8	97.6	97.6	97.5	97.5	97.0	97.0
AF152108	97.0	97.0	97.2	97.0	96.9	96.9	97.2	97.6	97.6	97.5	97.1	97.0	97.0	97.4	97.4	97.6	97.6	97.6	97.5
X93201	96.4	96.4	96.6	96.5	96.3	96.3	96.1	97.0	97.0	96.7	96.5	97.0	97.0	96.8	96.8	96.4	96.4	96.6	96.5
X93190	94.8	94.8	94.9	94.9	94.8	94.8	94.7	95.1	95.1	95.2	94.8	94.9	94.9	95.2	95.2	95.2	95.2	95.3	95.2
AB107233	92.7	92.7	92.7	92.6	92.5	92.5	92.5	92.7	92.7	92.6	92.8	92.5	92.5	93.1	93.1	92.9	92.9	93.0	92.9
M27245	89.5	89.5	89.6	89.4	89.3	89.3	89.5	89.6	89.6	89.4	89.5	89.0	89.0	89.5	89.5	89.5	89.5	89.5	89.5

Table 5 Similarity percentage of the representative *Microbispora* strains

	% similarity	
Strains	**AL4-7**	**AL4-8**
AL4-7	100	100
AL4-8	100	100
Microbispora rosea subsp. *rosea* (*Mb. karnatakensis*) JCM 8972 ^{T} (AY445647)	99.3	99.3
Microbispora rosea subsp. *rosea* (*Mb. amethystogenes*) JCM 3021^{T} (U48988)	99.3	99.3
Microbispora rosea subsp. *rosea* (*Mb. parva*)JCM 3024 ^{T} (U48985)	98.6	98.6
Microbispora rosea JCM 3006 ^{T} (D86936)	98.4	98.4
Microbispora rosea subsp. *aerata* (*Mb. aerata*) JCM 3076 ^{T} (U48984)	98.5	98.5
Microbispora rosea subsp. *aerata* (*Mb. thermodiastatica*)JCM 3110 ^{T} (U48986)	98.1	98.1
Microbispora rosea subsp. *aerata* (*Mb. thermorosea*) JCM 3111 ^{T} (U48987)	97.3	97.3
Microbispora corallina JCM 10267 ^{T} (AB018046)	98.4	98.4
Microbispora rosea subsp. *rosea* (*Mb. chromogenes*)JCM 3022 ^{T} (U48989)	98.5	98.5
Microbispora siamensis NBRC 104113 ^{T} (FJ199993)	97.4	97.4
Microbispora rosea subsp. *rosea* (*Mb. diastatica*) JCM 3023 ^{T} (U48990)	98.3	98.3
Microbispora mesophila JCM 3151 ^{T} (AF002266)	97.5	97.5

Table 6 Similarity percentage of the representative *Nonomuraea* strains

% similarity	
Strains	**P0417**
Nonomuraea dietziae JCM 3338(AB006156)	94.1
Nonomuraea roseola JCM 3323 (AJ278221)	97.8
Nonomuraea recticatena JCM 6835 ^{T} (U48979)	97.1
Nonomuraea africana JCM 6240 ^{T} (U48842)	97.1
Nonomuraea ferruginea JCM 3283 ^{T} (U48845)	96.9
Nonomuraea spiralis JCM 3286 ^{T} (U48983)	97.4
Nonomuraea angiospora JCM 3109^{T} (U48843)	97.9
Nonomuraea antimicrobica JCM 16904 (FJ157184)	97.5
Nonomuraea fastidiosa JCM 3321 ^{T} (U48844)	97.4
Nonomuraea helvata JCM 3143 ^{T} (U48975)	98.6
Nonomuraea polychroma JCM 6834 ^{T} (U48977)	97.8
Nonomuraea rubra JCM 3389 ^{T} (AF277200)	98.1
Nonomuraea turkmeniaca JCM 6836 ^{T} (AF277201)	98.5
Nonomuraea bangladeshensis JCM 13930 ^{T} (AB274966)	97.7
Nonomuraea coxensis JCM 13931 (AB274967)	96.8
Nonomuraea candida DSM 45086 ^{T} (DQ285421)	98.2
Nonomuraea maheshkhaliensis JCM 13929 ^{T} (AB290014)	98.2
Nonomuraea kuesteri JCM 13854 ^{T} (AJ746362)	98.3
Nonomuraea salmonea JCM 3324 (X97892)	98.2
Nonomuraea roseoviolacea JCM 3145 ^{T} (AB039959)	97.1
Nonomuraea roseoviolacea subsp. *carminata* JCM 3145 ^{T} (AB039961)	97.4
Nonomuraea longicatena JCM 11136 ^{T} (AB018787)	97.2
Nonomuraea rosea DSM 45177 ^{T} (FN356742)	97.8
P0417	100
Nonomuraea pusilla JCM 3144 (D85491)	97.3
Streptomyces ambifaciens JCM 23877 ^{T} (M27245)	91.1

Table 7 Similarity percentage of the representative *Actinomadura* strains

Strains	% Similarity			
	CYP1-5	**AL4-10**	**CYP1-1B**	**AL7-14**
Actinomadura alba JCM 14906^{T} (DQ985164)	95.6	96.4	96.6	96.9
Actinomadura scrupuli DSM 45225^{T} (FM210339)	96.0	96.5	96.9	96.9
Actinomadura chibensis JCM 14158^{T} (AB264086)	98.8	97.6	97.4	97.6
CYP1-5	100	98.1	97.8	98.0
Actinomadura pelletieri JCM 3388^{T} (AJ293710)	98.5	97.9	97.6	97.3
Actinomadura macra JCM 3287^{T} (U49009)	98.0	98.5	97.5	97.7
Actinomadura glauciflava JCM 6161^{T} (AB184612)	98.1	100	98.1	98.7
AL4-10	98.1	100	98.1	98.7
Actinomadura citrea JCM 3295^{T} (AJ420139)	97.9	99.6	97.7	98.3
Actinomadura mexicana JCM 13236^{T} (AF277195)	97.9	99.7	97.9	98.4
Actinomadura luteofluorescens JCM 4491^{T} (U49008)	97.7	99.4	97.7	98.3
Actinomadura verrucosospora JCM 3147^{T} (U49011)	97.8	99.4	97.8	98.4
Actinomadura coerulea JCM 3320^{T} (U49002)	97.7	99.5	97.7	98.4
Actinomadura madurae JCM 7436^{T} (X97889)	97.9	98.5	98.9	98.2
Actinomadura bangladeshensis JCM 13933^{T} (AB331652)	98.5	98.8	98.7	98.8
Actinomadura chokoriensis JCM 13932^{T} (AB331730)	98.1	98.4	98.3	98.4
Actinomadura catellatospora JCM10677^{T} (AF154127)	96.8	97.5	96.7	97.0
Actinomadura livida JCM 3387^{T} (AJ293706)	98.0	98.7	97.9	98.2
Actinomadura vinacea JCM 3325^{T} (AF134070)	97.8	98.1	97.7	98.2
Actinomadura viridis JCM 3112^{T} (AJ420141)	97.5	98.3	97.5	98.0
Actinomadura rugatobispora JCM 3366^{T} (U49010)	97.7	97.9	97.8	97.8
Actinomadura atramentaria JCM 6250^{T} (U49000)	96.7	97.1	96.5	96.9
Actinomadura flavalba JCM 16896^{T} (FJ157185)	96.9	97.8	97.3	97.5
Actinomadura keratinilytica DSM 45195^{T} (EU637009)	96.9	96.7	97.3	97.6
Actinomadura miaoliensis JCM 16702^{T} (EF116925)	97.3	96.9	97.7	98.1
Actinomadura rubrobrunea JCM 7345^{T} (EU637008)	96.3	96.1	97.0	96.9
Actinomadura viridilutea JCM 7345^{T} (D86943)	96.5	96.3	97.3	97.2
Actinomadura kijaniata JCM 3306^{T} (X97890)	96.5	96.9	97.7	97.7
Actinomadura namibiensis JCM 13238^{T} (AJ420134)	96.5	97.0	97.9	97.7
Actinomadura oligospora JCM 10648^{T} (AF163118)	97.0	97.5	97.7	98.0
Actinomadura hibisca JCM 9627^{T} (AJ293705)	96.9	97.7	98.1	98.5
Actinomadura rudentiformis JCM 14907^{T} (DQ285420)	97.3	97.6	99.2	98.4
Actinomadura fulvescens JCM 6833^{T} (AJ420137)	97.6	98.2	98.6	99.0
CYP1-1B	97.8	98.1	100	99.2
AL7-14	98.0	98.7	99.2	100
Actinomadura cremea JCM 3308^{T} (AF134067)	97.9	98.6	97.9	97.8
Actinomadura cremea subsp. *rifamycini* JCM 3309^{T} (U49003)	96.6	97.3	97.7	97.6
Actinomadura fibrosa JCM 9371^{T} (AJ293702)	97.0	98.5	98.3	98.8
Actinomadura nitritigenes JCM 10104^{T} (AY035999)	97.8	98.5	99.1	99.8
Actinomadura formosensis JCM 7474^{T} (AJ293703)	97.4	98.6	97.5	97.8
Actinomadura latina JCM 10674^{T} (AY035998)	98.0	97.8	98.1	98.0
Actinomadura napierensis JCM 13850^{T} (AY568292)	98.3	98.8	98.7	98.7
Actinomadura meyerae JCM 13237^{T} (AY273787)	98.4	98.9	98.4	98.8
Actinomadura yumaensis JCM 3369^{T} (AF163122)	97.9	98.8	98.0	98.5
Actinomadura hallensis JCM 13882^{T} (DQ076484)	97.3	97.7	97.3	97.7
Actinomadura sputi DSM 45233^{T} (FM957483)	97.4	97.6	97.7	97.7
Actinomadura umbrina JCM 6837^{T} (AJ293713)	96.9	96.5	96.2	96.4
Actinomadura echinospora JCM 3148^{T} (AJ420135)	97.1	96.7	97.0	97.3
Nonomuraea pusilla IFO 14684^{T} (D85491)	94.2	93.9	94.4	94.7

Table 8 Similarity percentage of the representative *Nocardia* strains

	% similarity		
Strains	**P1605**	**P1440**	**P1803**
AB121770	98.1	96.3	96.3
AB192415	98.1	96.3	96.3
Z46754	98.3	96.2	96.2
AF430035	98.4	96.6	96.6
DQ462650	97.1	96.4	96.4
AM422449	97	96.3	96.3
P1605	100	97.1	97.1
AF430027	99.9	97.2	97.2
AB092566	97.7	98.4	98.4
AJ508748	97.7	97.9	97.9
AB108778	97	98	98
AB108775	97.8	97.7	97.7
Z36936	97.7	98	98
AF218292	98.8	97.9	97.9
AF430019	98.4	97.3	97.3
AY282603	97.8	96.8	96.8
AB126874	98.1	97.7	97.7
EU006090	97.7	97.7	97.7
AJ556157	97	97.3	97.3
DQ925490	97.5	97.7	97.7
AF430038	97.7	97.4	97.4
AF430047	97.7	97.2	97.2
EU099357	96.9	96.5	96.5
EU099360	97.6	97.4	97.4
DQ282122	97.4	96.9	96.9
P1440	97.1	100	100
P1803	97.1	100	100
AB108781	97.2	99.4	99.4
AB108779	97.1	99.4	99.4
AF154129	96.8	99.2	99.2
AB275164	96	98.3	98.3
AB108780	95.6	98.1	98.1
FR749915	95.1	96.4	96.4
AF430040	98.4	97.7	97.7
AF430041	98.5	97.9	97.9
AB187522	98	98.3	98.3
DQ235272	97.8	98.1	98.1
AY333115	97.8	98.1	98.1
AY626158	96.6	97	97
AY779043	97.4	97.8	97.8
AB158277	96.8	98.1	98.1
FJ805428	96.6	96.2	96.2
AB201298	96.5	95.9	95.9
AY639902	97.8	96.7	96.7
AY639901	97.3	96.6	96.6
AM402972	97.7	96.3	96.3
DQ659910	97.2	96.2	96.2
AB126880	96.7	96.2	96.2
AY964666	95.7	95	95
AB092560	96.9	96.8	96.8
AB092561	96.8	96.9	96.9
AF060790	96.4	96.8	96.8
AF430055	96.9	97.2	97.2
AF430054	96.9	97.5	97.5
AJ854057	97.3	97.8	97.8
AY441974	96.9	97.3	97.3
EU484388	96.9	97.4	97.4
AB126876	97	97.6	97.6
AB126873	97.6	97.4	97.4
AF430045	96.9	97.3	97.3
AF430028	97.9	96.7	96.7
AF430067	97.7	96.3	96.3
AF430044	97.1	96	96
AF430039	96.9	96.2	96.2
AF430049	97.5	95.8	95.8
AF430042	97.8	97	97
AY222321	97.1	96.6	96.6
DQ235687	96.8	96.2	96.2
DQ235688	95.9	95.1	95.1
AJ303008	95.9	95.3	95.3
AF430052	96.4	95.8	95.8
AF430051	96.4	95.8	95.8
AF430050	96.6	96	96
AF277204	96.5	95.5	95.5
AB162801	97	96.2	96.2
AF430046	96.3	95.8	95.8
AB024312	97.2	96.6	96.6
AF459443	96.9	96	96
AF219974	96.9	95.1	95.1
M27245	89.8	89.8	89.8

Figure 1 ^{1}H NMR Spectrum (500 MHz) of compound A in DMSO-*d6*

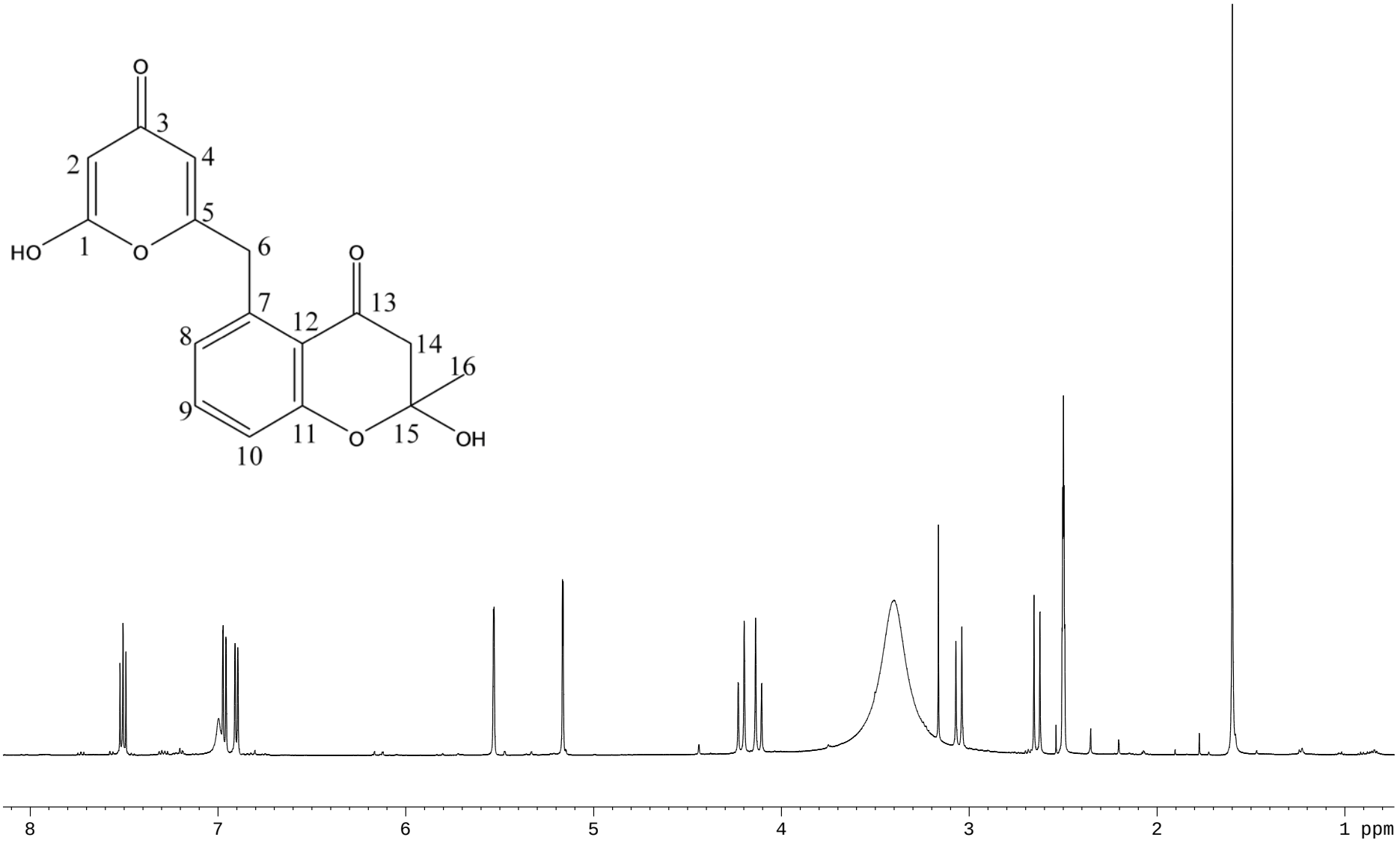


Figure 2 ^{1}H NMR Spectrum (500 MHz) of compound A in CD_3OD

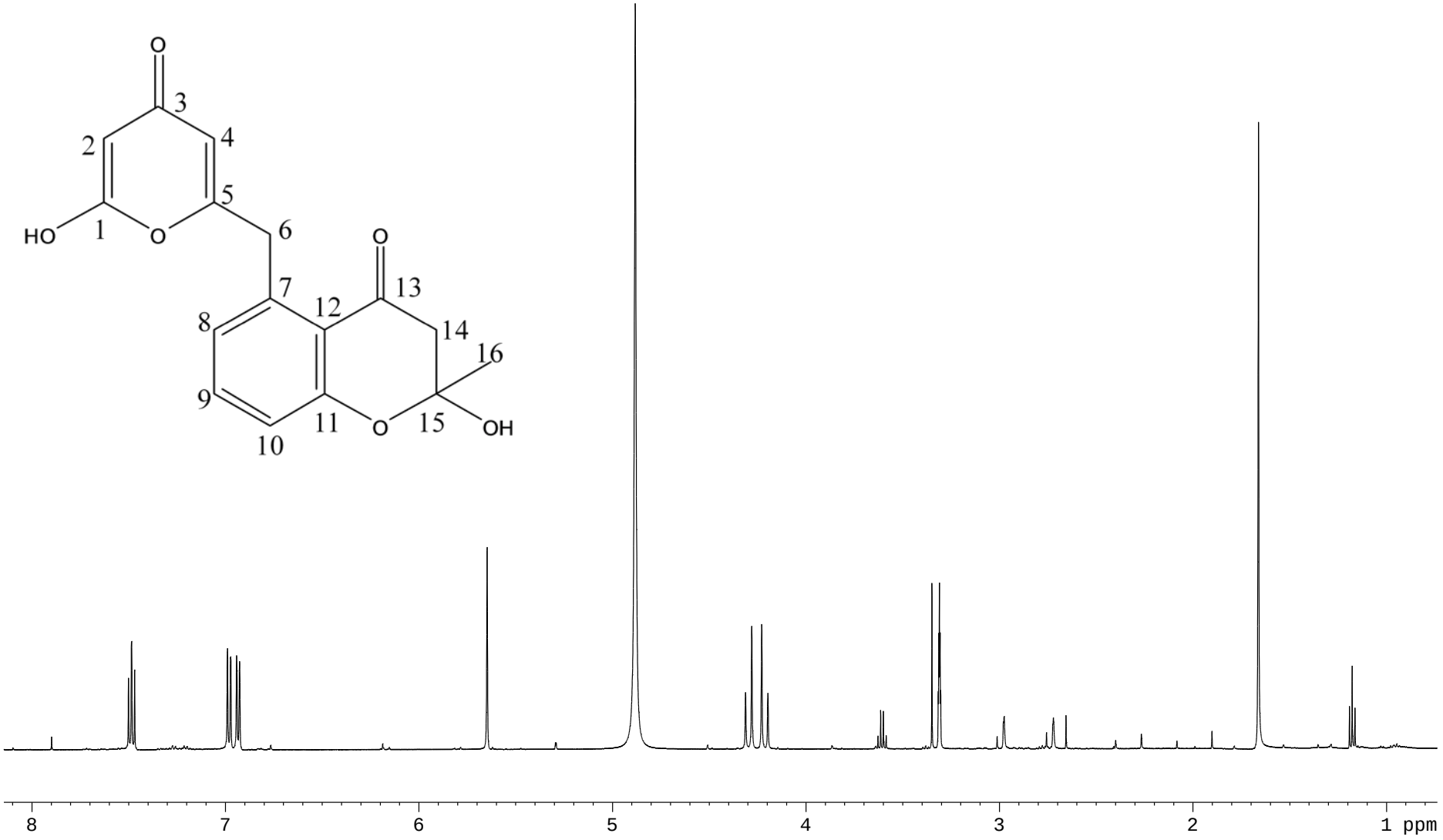


Figure 3 ^{13}C NMR Spectrum (100 MHz) of compound A in DMSO-*d6*

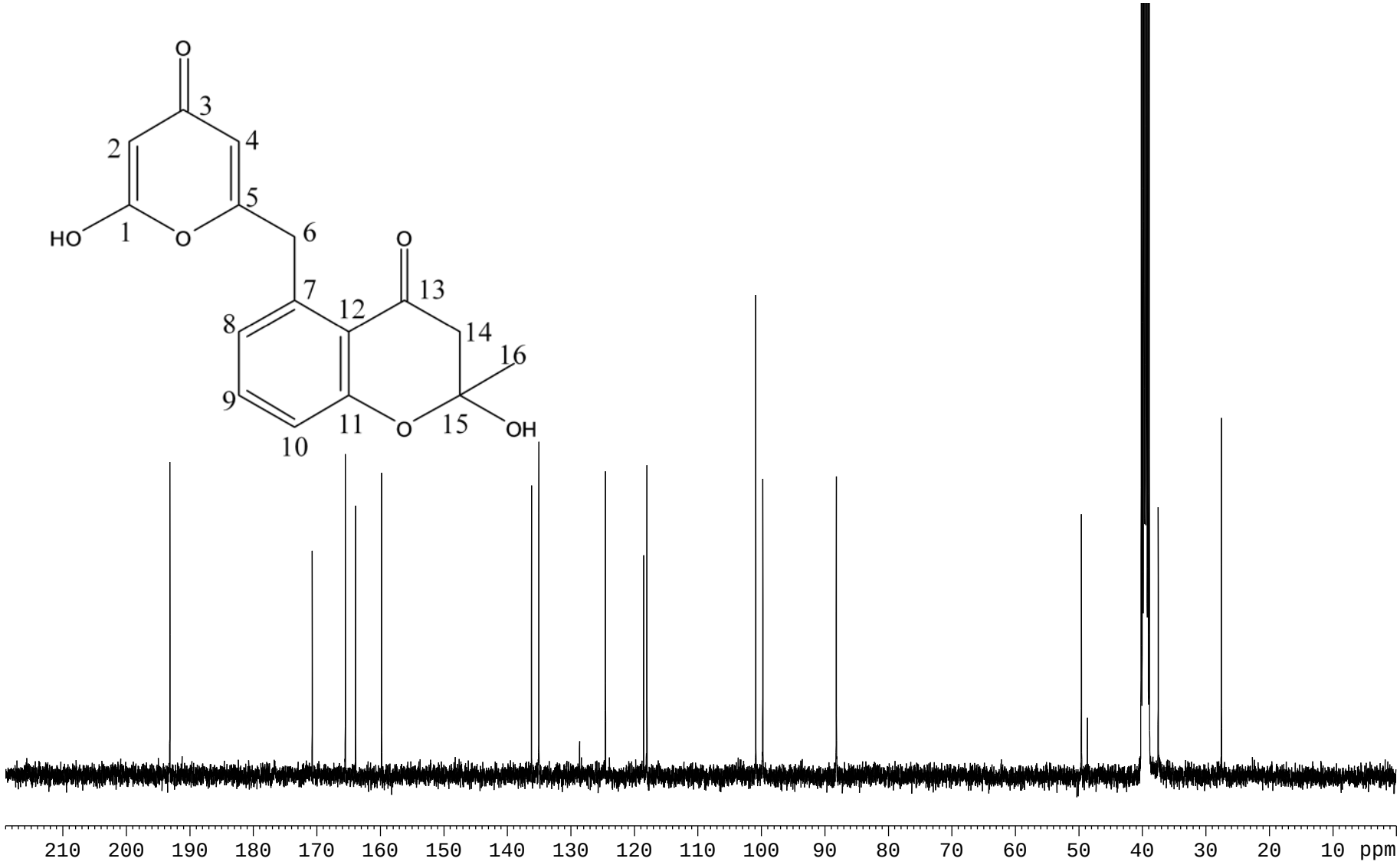


Figure 4 ^{13}C NMR Spectrum (100 MHz) of compound A in CD_3OD

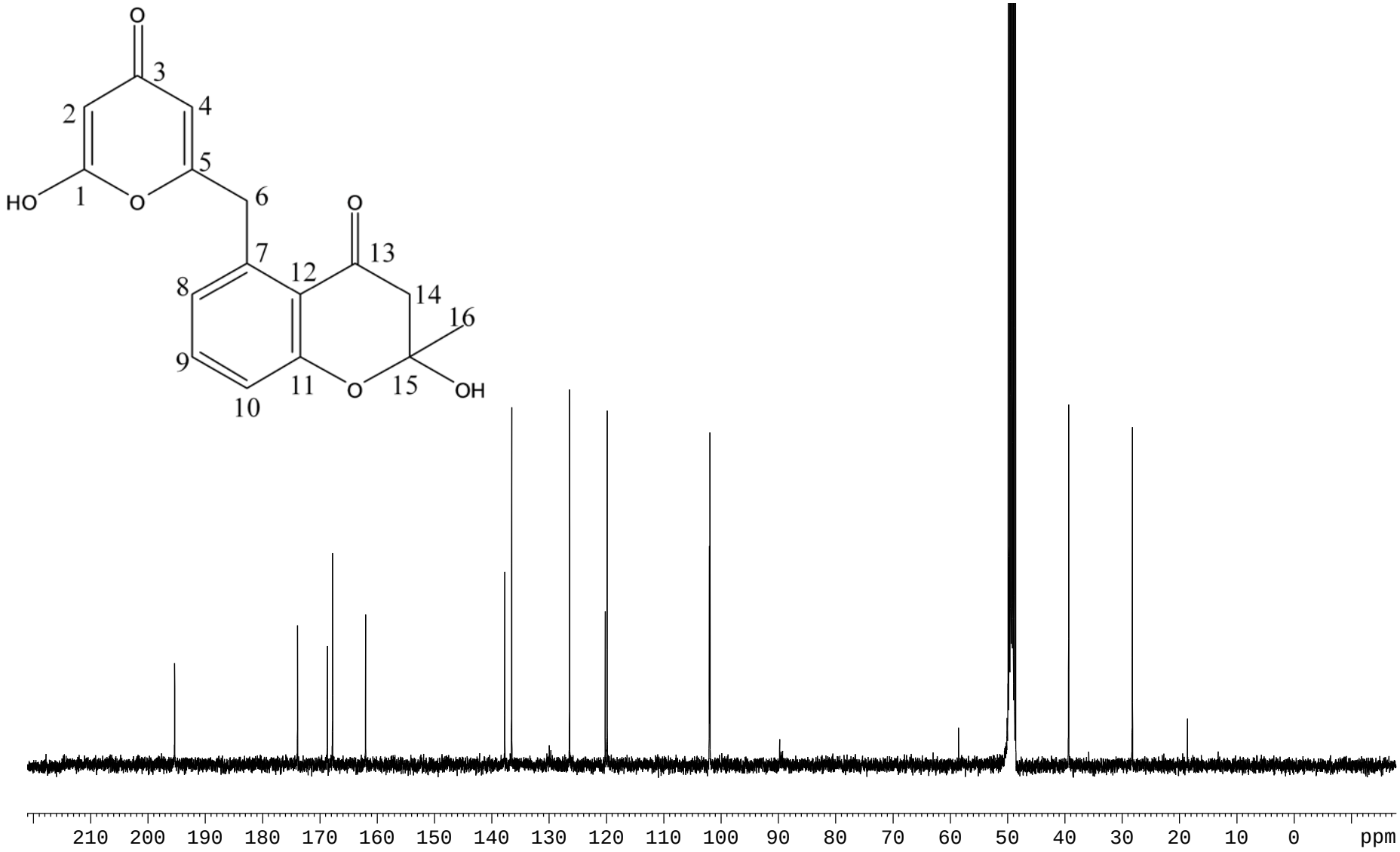


Figure 5 COSY Spectrum (500 MHz) of compound A in CD_3OD

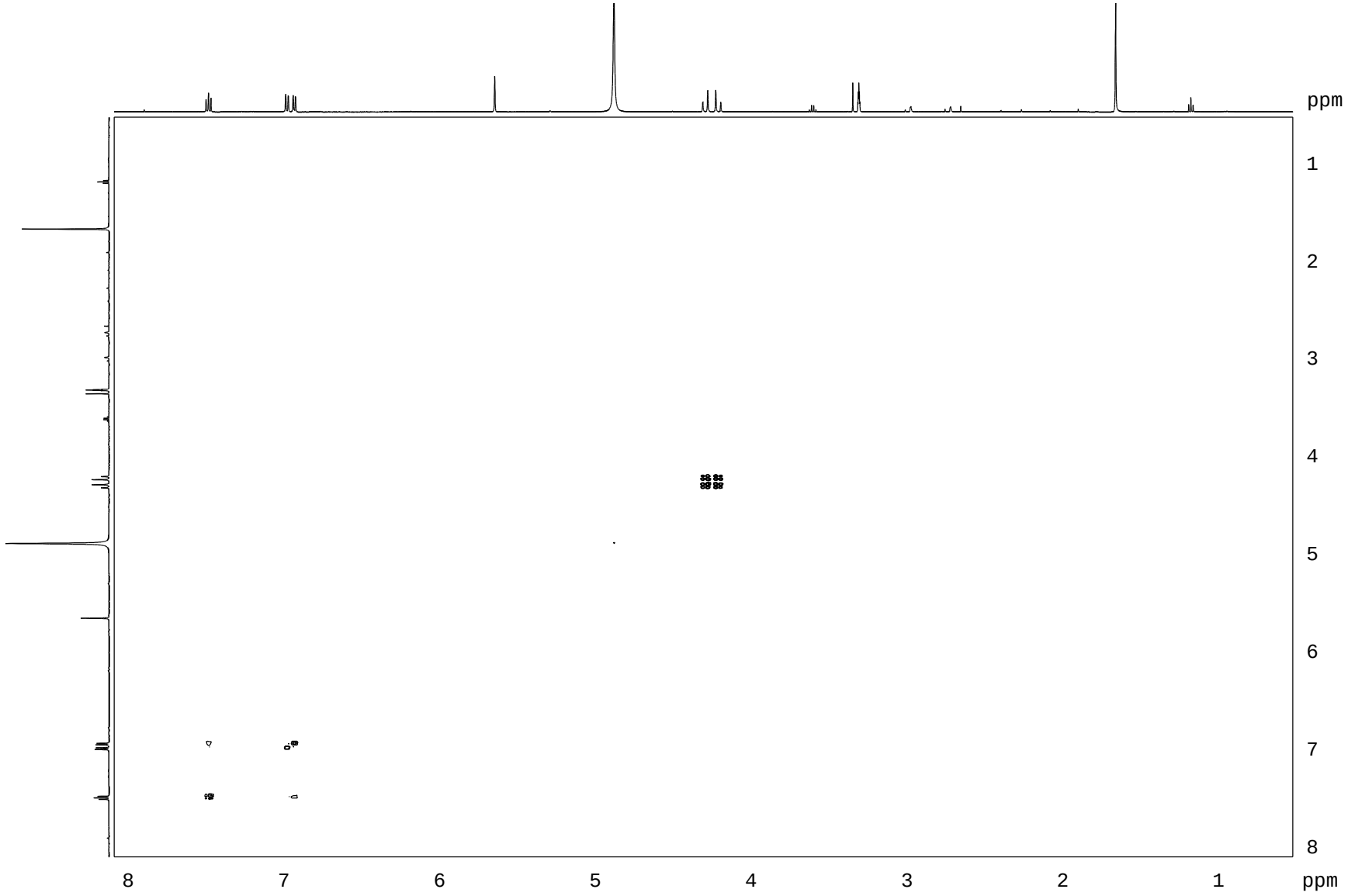


Figure 6 HSQC Spectrum (500 MHz) of compound A in CD_3OD

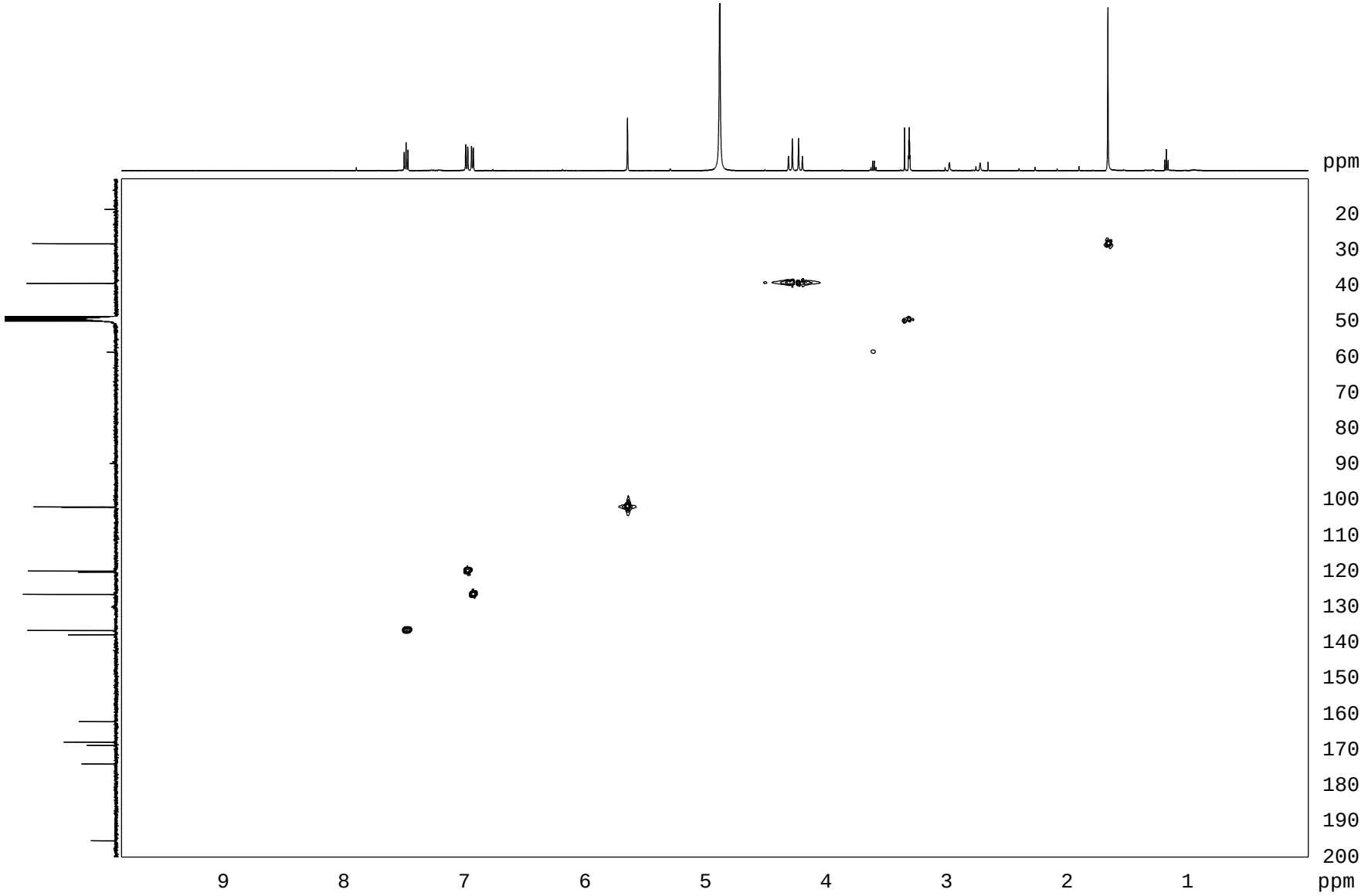


Figure 7 HMBC Spectrum (500 MHz) of compound A in CD_3OD

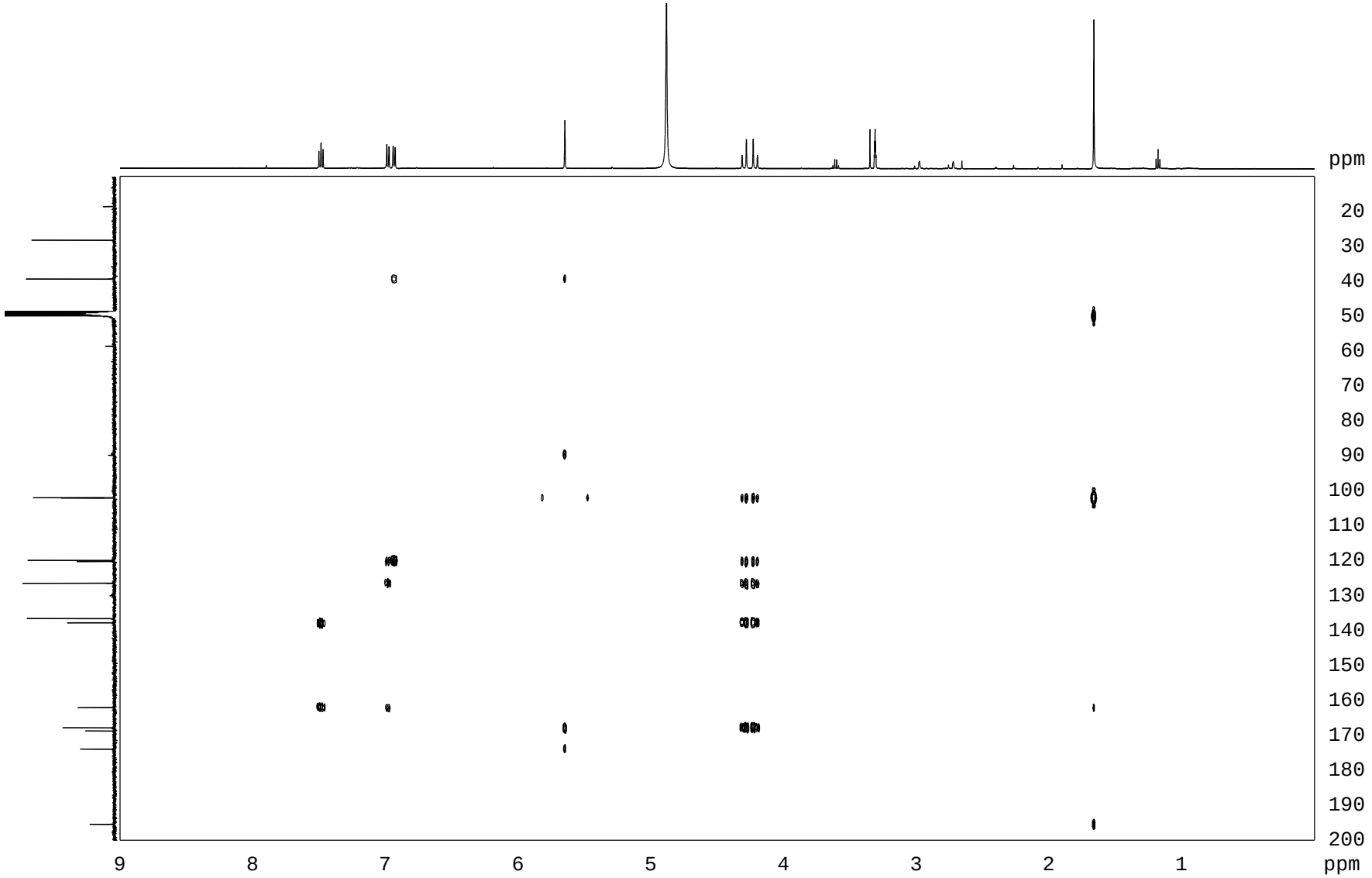


Figure 8 ^{1}H NMR Spectrum (500 MHz) of compound C in DMSO-*d6*

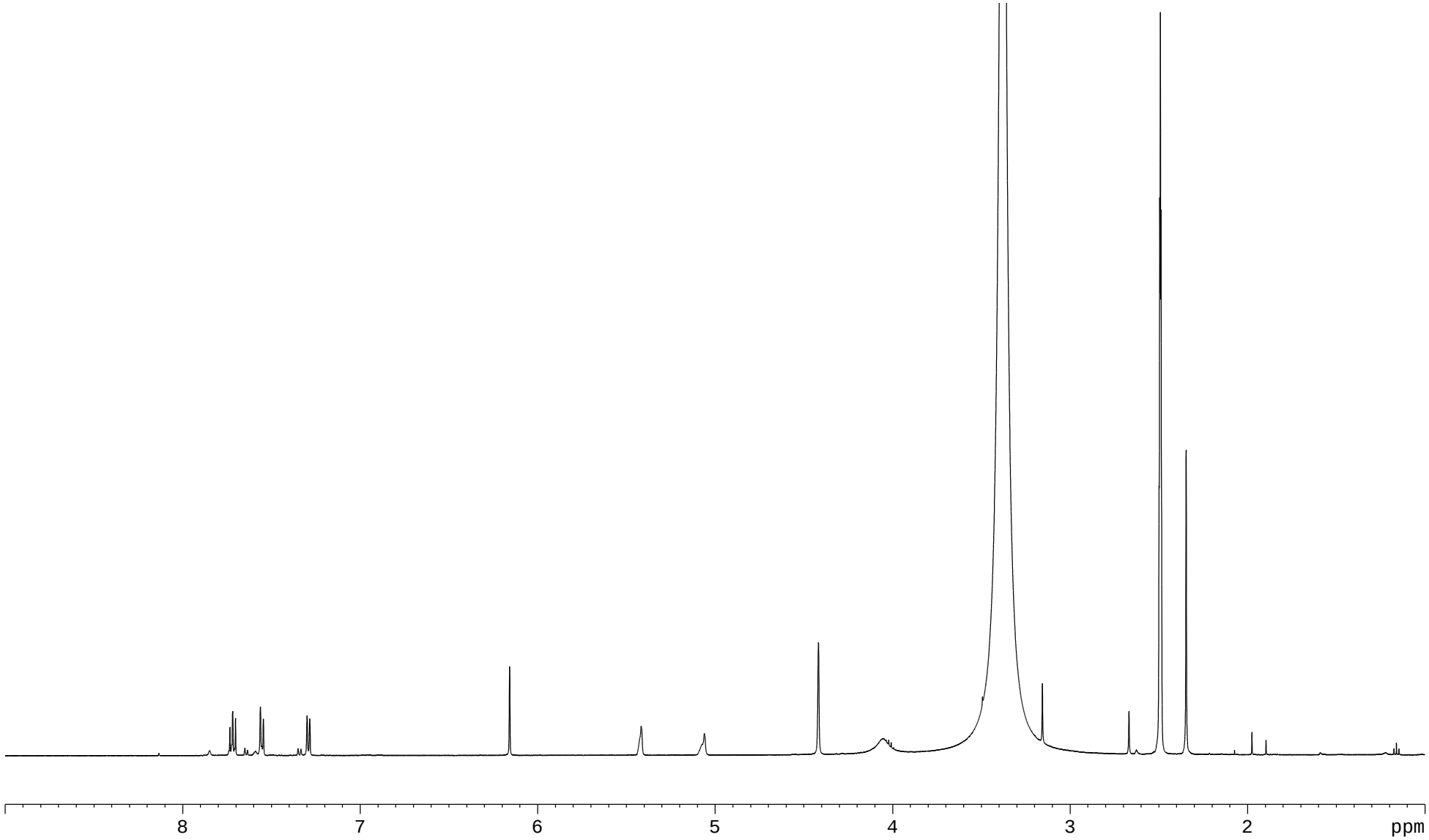


Figure 9 ^{1}H NMR spectral of compounds G and H

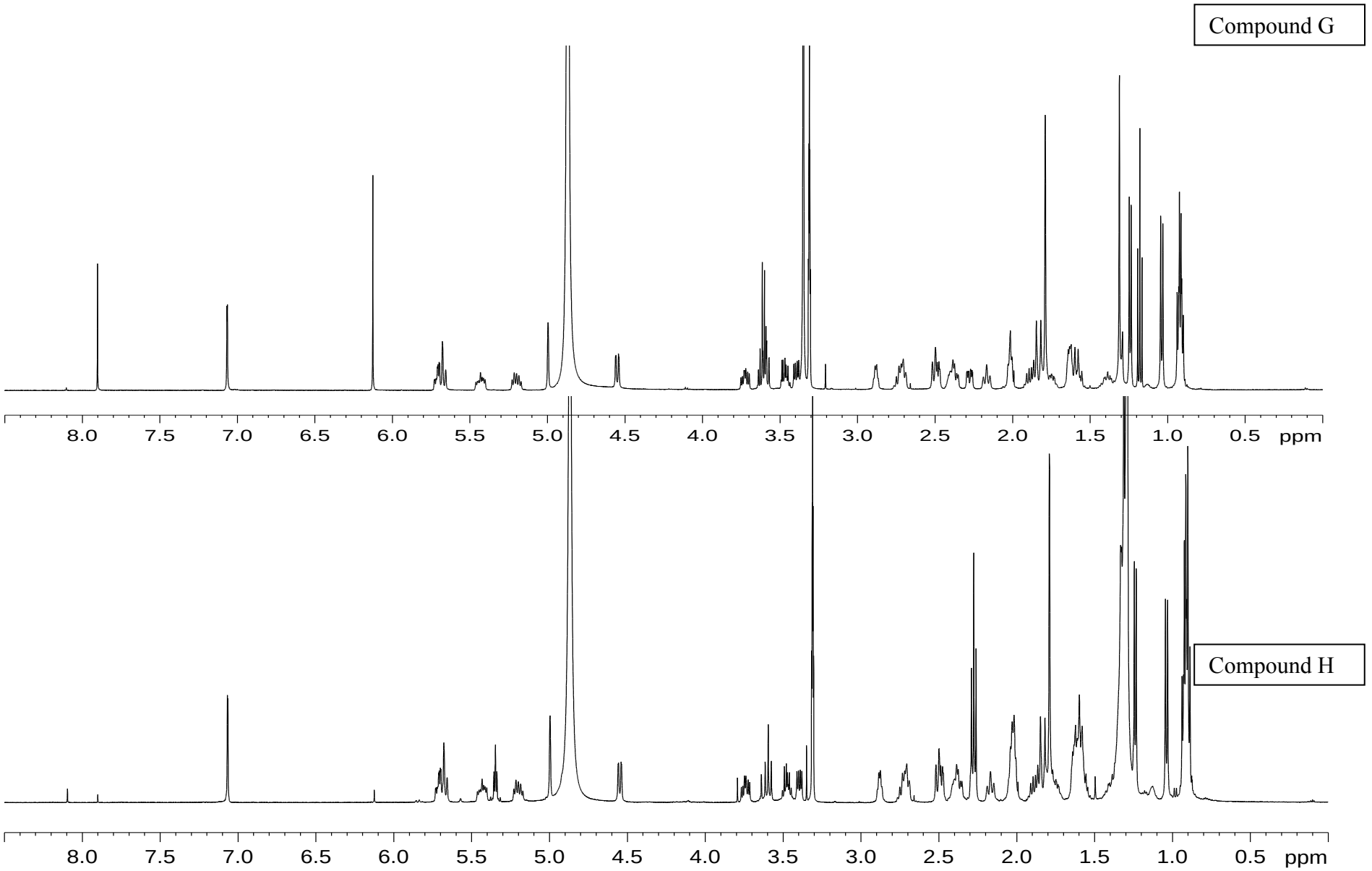


Figure 10 ^{1}H NMR Spectrum (500 MHz) of compound G in CD_3OD

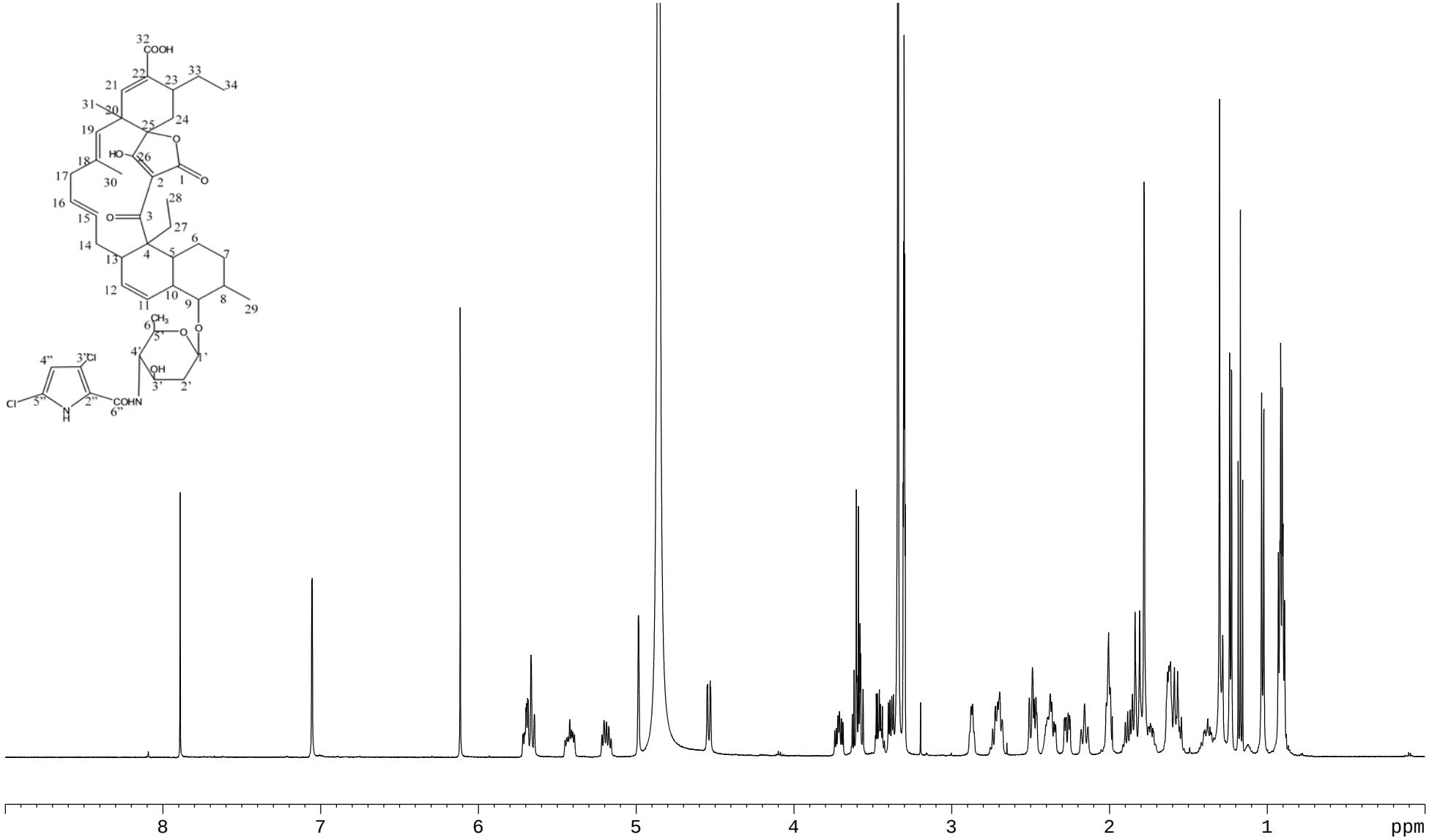


Figure 11 ^{13}C NMR Spectrum (100 MHz) of compound G in CD_3OD

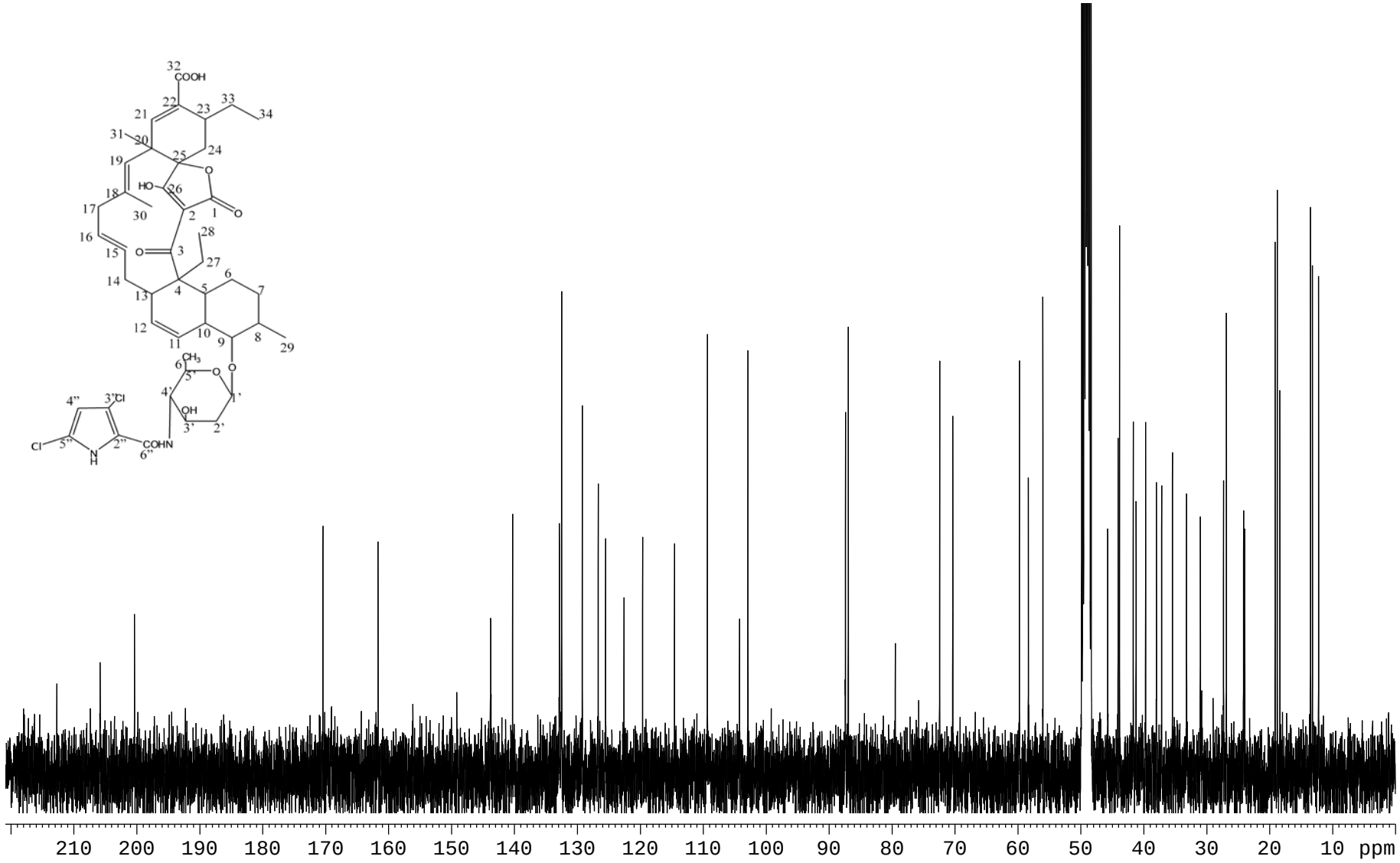


Figure 12 COSY Spectrum (500 MHz) of compound G in CD_3OD

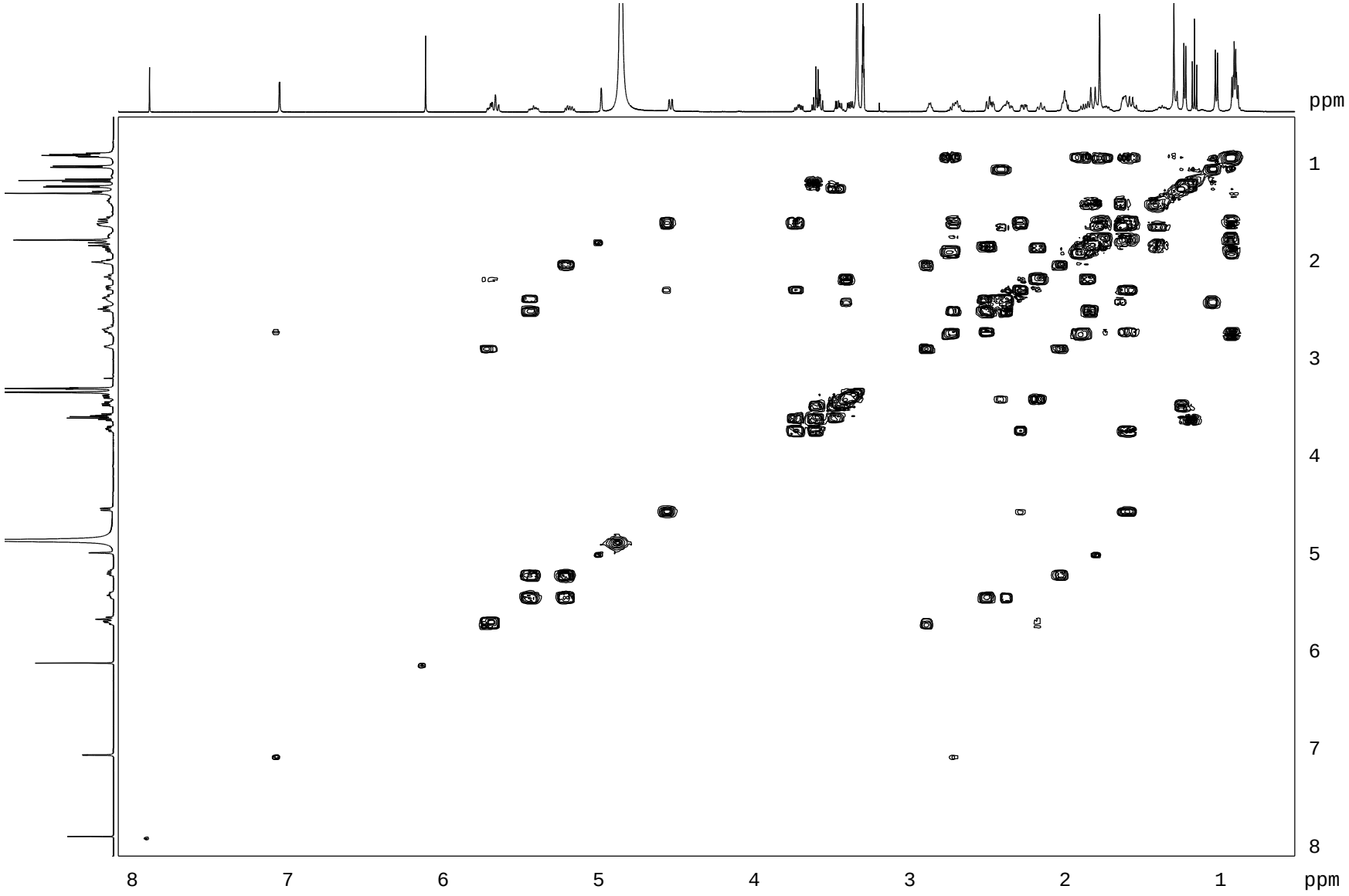


Figure 13 HSQC Spectrum (500 MHz) of compound G in CD_3OD

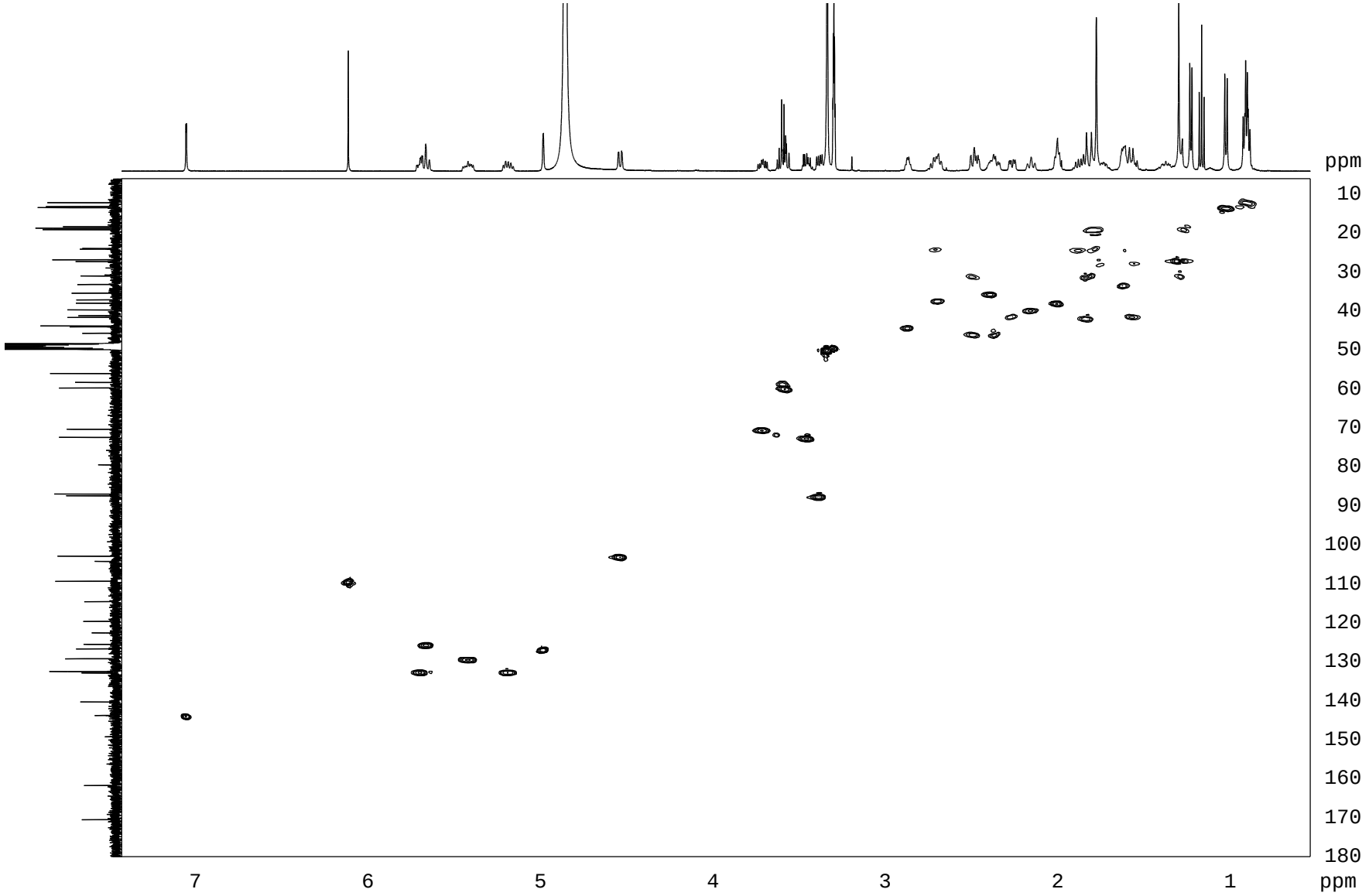
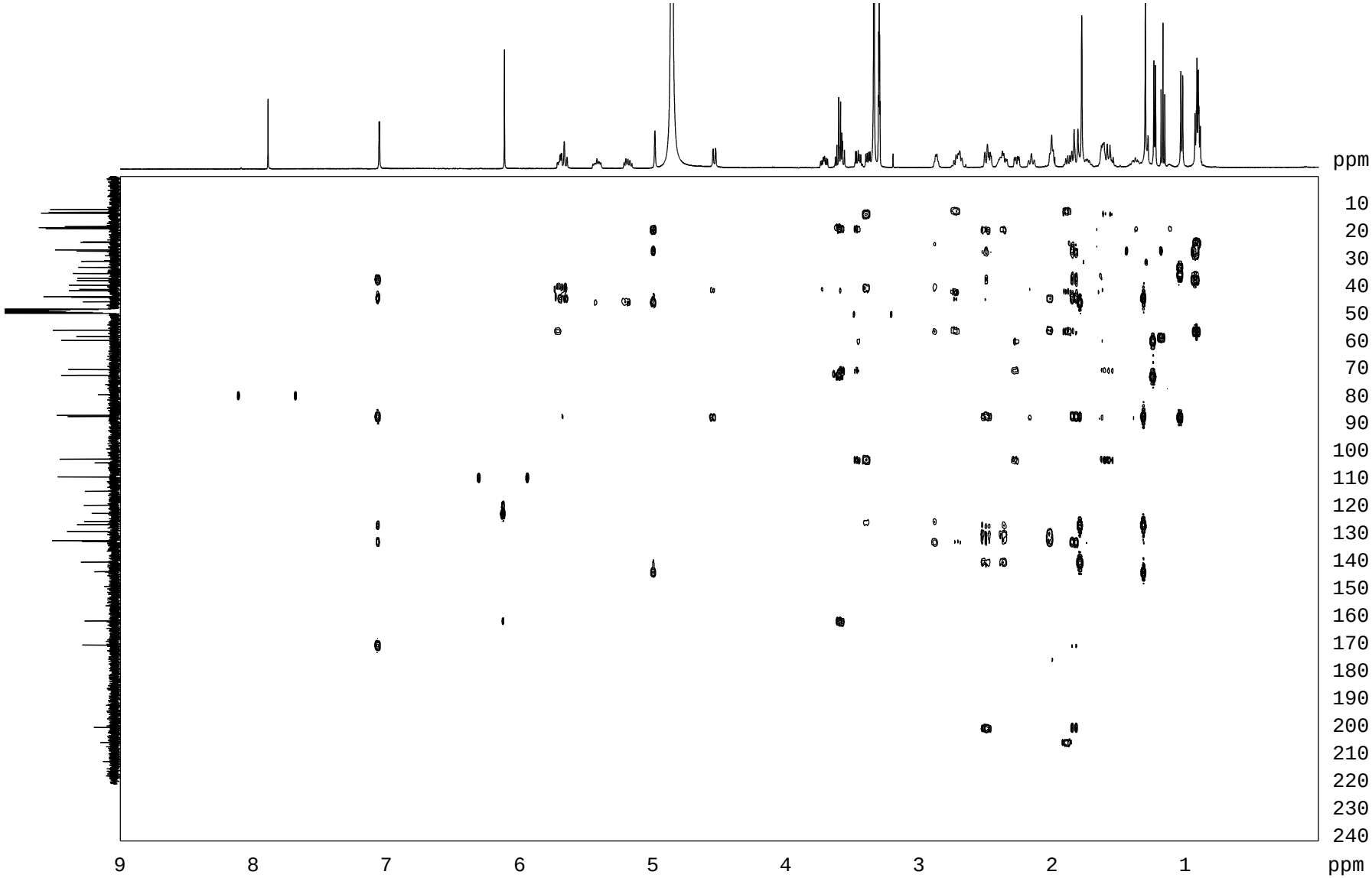


Figure 14 HMBC Spectrum (500 MHz) of compound G in CD_3OD



APPENDIX D

PRIMER and 16S rRNA GENE SEQUENCES

1. Primers

20F	5’-AGTTTGATCCTGGCTC-3’
1541	5’-AAGGAGGTGATCCAGCC-3’
27F	5’-GTTTGATCCTGGCTCAG-3’
350F	5’-TACGGGAGGCAGCAG-3’
350R	5’-CTGCTGCCTCCCGTAG-3’
780F	5’-GATTAGATACCCTGGTAG-3’
780R	5’-CTACCAGGGTATCTAATCC-3’
1100F	5’-GCAACGAGCGCAACCC-3’
1100R	5’-AGGGTTGCGCTCGTTG-3’
1492R	5’-GGTTACCTTGTTACGACTT-3’

2. 16S rRNA gene nucleotide sequence of strains

AL8-2

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D10-9-5

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ASC19-2-1

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AL8-8

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AL10-3

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AL7-5

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AL3-9

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AL4-4

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AL1-3

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P0402

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AL3-16

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AL9-20

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AL5-1

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AL9-1

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AL9-13

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AL9-22

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AL1-16B

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AL1-15-2

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AL4-7

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AL4-8

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CYP1-5

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AL4-10

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CYP1-1B

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AL7-14

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P1440

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VITA

Miss Apakorn Songsumanus was born on April 12th, 1985 in Samutsakhon, Thailand. She received her Bachelor's Degree of Sciences in Biotechnology (Second Class Honors) in 2005 from the Faculty of Science, King Mongkut's Institute of Technology. She has received the scholarship from the Royal Golden Jubilee Ph.D. Program since 2006 for her Ph.D. study in Pharmarceutical Chemistry and Natural Products Program, Faculty of Pharmaceutical Science, Chulalongkorn University.

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