อนุกรมวิธานและเมแทบอไลต์ทุติยภูมิของแอคติโนมัยสีทจากทะเลที่คัดเลือกได้

นายวงศกร พงศ์โสภิตานันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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TAXONOMY AND SECONDARY METABOLITES OF SELECTED MARINE ACTINOMYCETES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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วงศกร พงศ์โสภิตานันท์ : อนุกรมวิธานและเมแทบอไลต์ทุติยภูมิของแอคติโนมัยสีทจากทะเลที่คัดเลือกได้ (TAXONOMY AND SECONDARY METABOLITES OF SELECTED MARINE ACTINOMYCETES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ภก. ดร.คณิต สุวรรณบริรักษ์, ดร.ปัทมา พิทยขจรวุฒิ, 170 หน้า.

การศึกษาอนุกรมวิธานของแอคติโนมัยสีทจำนวน 75 ไอโซเลตที่แยกจากตัวอย่างทางทะเลซึ่งเก็บรวบรวมจากจังหวัดกระบี่ ชุมพร ชลบุรี ตรัง ประจวบคีรีขันธ์ ภูเก็ต และสมุทรสงคราม โดยอาศัยลักษณะทางสัณฐานวิทยา อนุกรมวิธานทางเคมีและการวิเคราะห์ลำดับเบสของยีนในช่วง 16S rRNA พบว่าไอโซเลตที่แยกได้เป็นสมาชิกในวงศ์ *Micromonosporaceae* ได้แก่ สกุล *Jishengella* (1 ไอโซเลต) *Micromonospora* (25 ไอโซเลต) *Salinispora* (13 ไอโซเลต) และ *Verrucosispora* (2 ไอโซเลต) วงศ์ *Nocardiaceae* ได้แก่ สกุล *Nocardia* (2 ไอโซเลต) และ วงศ์ *Streptomycetaceae* ได้แก่ สกุล *Streptomyces* (32 ไอโซเลต) จากผลการศึกษาลักษณะทางฟีโนไทป์และการเข้ากันได้ของ DNA ทำให้สามารถเสนอเป็นแอคติโนมัยสีทสปีชีส์ใหม่ 4 สปีชีส์ คือ *Micromonospora fluostatini* (ไอโซเลต PWB-003^T) *Micromonospora sediminis* (ไอโซเลต CH3-3^T) *Streptomyces chumphonensis* (ไอโซเลต KK1-2^T และ CPB4-7) และ *Streptomyces verrucosisporus* (ไอโซเลต CPB1-1^T CPB2-10 BM1-4 CPB3-1 และ CPB1-18)

จากการวิเคราะห์สารเมแทบอไลต์ทุติยภูมิในน้ำหมักเชื้อโดยเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่าเชื้อสปีชีส์ใหม่ *M. fluostatini* PWB-003^T *S. chumphonensis* KK1-2^T และ เชื้อสปีชีส์เดิม *Streptomyces sanyensis* C10-9-1 ได้แสดงองค์ประกอบทางเคมีที่น่าสนใจและได้ถูกคัดเลือกเพื่อนำไปศึกษาสารเมแทบอไลต์ทุติยภูมิและวิเคราะห์โครงสร้างทางเคมีของสารที่แยกได้โดยเทคนิคนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโตรสโกปี และ แมสสเปกโตรเมทรี จากการศึกษาสามารถแยกไดอะสเตอริโอเมอร์ชนิดใหม่ของ fluostatin C ซึ่งเป็นสารในกลุ่ม fluorenone ได้จาก *M. fluostatini* PWB-003^T โดยสารบริสุทธิ์แสดงความเป็นพิษต่อเซลล์ Vero (IC_{50}, 48.5 µg/ml) แต่ไม่แสดงความเป็นพิษต่อเซลล์ KB และ MCF-7 และไม่แสดงฤทธิ์ยับยั้งจุลชีพต่อ *Bacillus cereus* ATCC 11778 *Mycobacterium tuberculosis* H37Ra และ *Pseudomonas aeruginosa* K2733 ในขณะที่สามารถแยกสาร piericidin A1 ซึ่งเป็นสารในกลุ่ม polysubstituted pyridine alkaloid ได้จากน้ำหมักของ *S. chumphonensis* KK1-2^T โดยสารบริสุทธิ์ไม่แสดงฤทธิ์ต้านจุลชีพต่อ *B. cereus* ATCC 11778 *M. tuberculosis* H37Ra และ *P. aeruginosa* K2733 และ ไม่แสดงความเป็นพิษต่อเซลล์ KB MCF7 และ Vero นอกจากนี้ยังสามารถแยกสารบริสุทธิ์ในกลุ่ม indolocarbazole ทั้งหมดสี่ชนิดจาก *S. sanyensis* C10-9-1 คือ staurosporine staurosporine aglycone K-252D และ 4'-demethylamino-4',5'-dihydroxystaurosporine โดยสารทั้งสี่ชนิดแสดงความเป็นพิษต่อเซลล์ KB, MCF-7 and Vero ในช่วง IC_{50} 0.2 ถึง 45.4 µg/ml นอกจากนี้สาร staurosporine staurosporine aglycone และ K-252D แสดงฤทธิ์ต้าน *M. tuberculosis* H37Ra ในช่วง MIC 6.25 ถึง 12.5 µg/ml และ K-252D แสดงฤทธิ์ยับยั้งเชื้อ *B. cereus* ATCC 11778 โดยมีค่า IC_{50} คือ 25.0 µg/ml

ภาควิชา อาหารและเภสัชเคมี ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: ACTINOMYCETES, MARINE, SECONDARY METABOLITES, TAXONOMY, POLYPHASIC APPROACH

WONGSAKORN PHONGSOPITANUN: TAXONOMY AND SECONDARY METABOLITES OF SELECTED MARINE ACTINOMYCETES. ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., CO-ADVISOR: KHANIT SUWANBORIRUX, Ph.D., PATTAMA PITTAYAKHAJONWUT, Ph.D., 170 pp.

A total of 75 actinomycetes isolated from marine samples collected from Chumphon, Chonburi, Krabi, Phuket, Prachuap-Khiri-Khan, Samut-Songkhram and Trang provinces were taxonomically studied based on their morphology, chemotaxonomy and 16S rRNA gene sequence analyses. The isolates were identified as members of Family *Micromonosporaceae* including *Jishengella* (1 isolate), *Micromonospora* (25 isolates), *Salinispora* (13 isolates) and *Verrucosispora* (2 isolates); Family *Nocardiaceae* including *Nocardia* (2 isolates); and Family *Streptomycetaceae* including *Streptomyces* (32 isolates). On the basis of phenotypic properties and DNA-DNA hybridization, 4 new actinomycete species were proposed including *Streptomyces chumphonensis* (isolates KK1-2^T and CPB4-7), *Streptomyces verrucosisporus* (isolates CPB1-1^T, CPB2-10, BM1-4, CPB3-1 and CPB1-18), *Micromonospora fluostatini* (isolate PWB-003^T) and *Micromonospora sediminis* (isolate CH3-3^T).

Secondary metabolites from culture broth of two new species *M. fluostatini* PWB-003^T, *S. chumphonensis* KK1-2^T and a known species *Streptomyces sanyensis* C10-9-1 were investigated based on their interesting chemical profiles analyzed by an in-house HPLC database. The chemical structures of isolated secondary metabolites were identified by nuclear magnetic resonance spectroscopy and mass spectrometry. A new diastereomer of fluostatin C, fluorenone, was isolated from *M. fluostatini* PWB-003^T. It showed cytotoxicity against Vero cell line (IC_{50}, 48.5 µg/ml) but did not show cytotoxicity against KB and MCF-7 and antimicrobial activity against *Bacillus cereus* ATCC 11778, *Mycobacterium tuberculosis* H37Ra and *Pseudomonas aeruginosa* K2733. Piericidin A1, a polysubstituted pyridine alkaloid, was obtained from *S. chumphonensis* KK1-2^T. However, the compound at concentration 50µg/ml showed no biological activities such as antimicrobial activity against *B. cereus* ATCC 11778, *M. tuberculosis* H37Ra and *P. aeruginosa* K2733 and cytotoxicity against KB, MCF7 and Vero cell lines. Finally, 4 indolocarbazole alkaloids, staurosporine, staurosporine aglycone, K-252D and 4'-demethylamino-4',5'-dihydroxystaurosporine, were isolated from *S. sanyensis* C10-9-1. The four alkaloids exhibited cytotoxicity against KB, MCF-7 and Vero cell lines at IC_{50}s ranged from 0.2-45.4 µg/ml. Furthermore, staurosporine, staurosporine aglycone and K-252D exhibited anti-*M. tuberculosis* H37Ra activity at the MICs ranged from 6.25 to 12.5 µg/ml. In addition, K-252D exhibited anti-*B. cereus* ATCC 11778 activity (IC_{50} 25.0 µg/ml).

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
Field of Study:	Pharmaceutical Chemistry and Natural Products	Advisor's Signature	.....
		Co-Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS AND SYMBOLS

BSA	=	Bovine serum albumin
CFU	=	Colony forming unit
$CDCl_3$	=	Deuterated chloroform
COSY	=	Correlation spectroscopy
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
δ	=	Chemical shift
d	=	Doublet
dd	=	Doublet of doublets
DAP	=	Diaminopimelic acid
DEPT	=	Distortionless enhancement by polarization tranfer
DNase	=	Deoxyribonuclease
dNTP	=	Deoxyribonucleotide triphosphate
DNA	=	Deoxyribonucleic acid
DPG	=	Diphosphatidylglycerol
DON	=	2,7-Dihydroxynapthalene
EDTA	=	Ethylenediaminetetraacetic acid
Ex/Em	=	Excitation and emission wavelengths
F	=	Forward
FAME	=	Fatty acid methyl ester
G+C	=	Guanine-plus-cytosine
h	=	Hour
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation

HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence
^{1}H-NMR	=	Proton nuclear magnetic resonance
HPLC	=	High performance liquid chromatography
Hz	=	Hertz
IC	=	Inhibitory concentration
ISP	=	International *Streptomyces* Project
J	=	Coupling constant
KB	=	Human oral epidermoid carcinoma, ATCC CCL-17
m	=	Multiplet
m/z	=	Mass to charge ratio
MCF-7	=	Human breast cancer, ATCC HTB-22
MEGA	=	Molecular Evolutionary Genetics Analysis
meso-DAP	=	*meso*-Diaminopimelic acid
MHz	=	Megahertz
MIC	=	Minimun Inhibitory Concentration
MK	=	Menaquinone
MS	=	Mass spectrometry
MW	=	Molecular weight
Methyl-PE	=	Methylphosphatidylethanolamine
NA	=	Nutrient agar
NCI-H187	=	Human small-cell lung cancer, ATCC CRL-5804
NPG	=	Ninhydrin-positive glycophospholipid
nov.	=	Novel
NMR	=	Neclear magnetic resonance

NOESY	=	Nuclear Overhouser effect correlation spectroscopy
OD	=	Optical density
OH-PE	=	Hydroxyphosphatidylethanolamine
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PC	=	Phosphatidylcholine
PE	=	Phosphatidylethanolamine
Lyso-PE	=	Lyso-phosphatidylethanolamine
PG	=	Phosphatidylglycerol
PI	=	Phosphatidylinositol
ppm	=	Part per million
rRNA	=	Ribosomal ribonucleic acid
r.p.m.	=	Round per minute
s	=	Singlet
SEM	=	Scanning electron microscope
sp.	=	Species
t	=	Triplet
TAE	=	Tris-acetate EDTA
T_m	=	Melting temperature
TLC	=	Thin layer chromatography
U	=	Units
Vero cells	=	African green monkey kidney fibroblasts; ATCC CCL-81
YS	=	Yeast extract-soluble starch agar

CHAPTER I
INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria having high mol% of the base guanine + cytosine content in their genome. Actinobacteria have been well known as the valuable economically importance microorganisms for a long time because of their ability to produce a large numbers of bioactive secondary metabolites. According to Bèrdy (2005), 45% of known microbial metabolites were produced from the actinomycetes, especially the genus *Streptomyces*. Examples of important acitnomycete-derived bioactive compounds, which have been used in clinical treatments, are amphotericin B, avermectin, chloramphenicol, erythromycin, kanamycin, nystatin, platensimycin, tetracycline and vancomycin.

In general, actinomycetes are widely distributed in terrestrial habitats, mainly in soils and organic materials. Since they have been isolated from these habitats for a century, numerous redundant isolates were obtained. This causes the continuously decreasing rate of the discovery of new bioactive compounds. Moreover, the drug-resistant microorganism crisis seem to be continuously increasing. Thus, to overcome these problems, new unexplored habitats should be considered as new sources for new actinomycetes.

Three quarters of the planet earth covers with the oceans which contains a huge biological diversity. Since the discovery of the true obligate marine actinomycete genus *Salinispora* in the last decade, the ocean has been accepted for the existence of the actinomycetes. These marine actinomycetes produced different types of new secondary metabolites including abyssomicin, arenicolide A, cyanosporasides A, saliniketal A, salinosporamide A, sporolide A, and marinomycin (Bister *et al*., 2004; Kwon *et al*., 2006; Jensen *et al*., 2007). Among these compounds, salinosporamide A exhibited a potent proteasome inhibitor activity against human tumors in mouse models and entered the clinical trial phase I (Feling *et al*., 2003; Solanki *et al*., 2008).

The distribution of actinomycetes in the marine environment are largely unexplored. Thailand is one of many countries having high biological diversity. The coastal part of Thailand is about 2,600 km long which covers 23 provinces. However, the study of marine actinomycetes in Thailand is rarely reported. The hypothesis of this research based on the fact that the unexplored habitats should provide some new actinomycetes species. These novel microorganisms should represent different genomes compared with previously known species and consequencly to produce some new secondary metabolites.

Therefore, the main objectives of this research study are as follows:

1. To isolate and screen marine actinomycete isolates having antimicrobial activities and interesting secondary metabolite profiles
2. To identify the selected marine actinomycete isolates based on phenotypic, chemotaxonomic and genotypic characteristics
3. To separate and elucidate the chemical structures of secondary metabolites from the selected marine actinomycete isolates

CHAPTER II
LITERATURE REVIEW

The actinomycetes are Gram-positive bacteria which have a high percentage of base G+C content (>55%) in their genomes. According to the Bergey's Mannual of Systematic Bacteriology Volume Five (Whitman *et al*., 2012), the actinobacteria encompass six classes (*Actinobacteria*, *Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria* and *Thermoleophilia*), 19 orders, 50 families and 221 genera (Ludwig *et al*., 2012).

Actinomycetes are originally considered to be an intermediate group between fungi and bacteria. The morphology of acitnomycetes usually looks like fungi which have true branching mycelia with septa. Actinomycetes also have many bacterial properties such as: the diameters of their hyphae have only 1 μm within the bacterial size, they do not have nuclear membrane in the cell and they are prokaryotic cytology, which differ from eukaryotic fungi. Moreover actinomycetes are sensitive to numerous phages which are similar in their morphology and in their action to those attacking true bacteria and are not sensitive to fungal phages (H. A. Lechevalier & Lechevalier, 1967). According to Avery & Blank (1954), cell walls of actinomycetes contain peptidoglycan and do not contain chitin or cellulose, strongly suggesting that their cell walls are chemically bacterial type.

These bacteria play an important role in decomposition of nutrient in the environment. Most of them are saprophyte but some are parasitic or mutualistic associations with plants or animals (Goodfellow & Williams, 1983). Because they produce mycelia, the life cycle of them is different from other unicellular bacteria. They form spores for reproduction. The spores of actinomycetes are conidia or arthrospores forming singly or in chain of various lengths or enclosed in sporangia (Lechevalier & Lechevalier, 1967). In the appropriate condition, the spore germinates the germ tube and develops the substrate mycelium toward the solid surface. Upon differentiation the aerial mycelium is formed and later develops to the chain of spores.

Actinomycetes have been well known for the most economically valuable microorganisms. Most of them are able to produce a large number of bioactive compounds. Among the known microbial metabolites today (approximately, 22,500 compounds), 45% were produced by actinomycetes especially by the members of the genus *Steptomyces* (Berdy, 2005). They are widely distributed in various environments, mainly terrestrial soils. Recently, many novel actinomycete speices have been isolated from other habitats including insects (Currie *et al*., 1999), plant materials (Thaechowisan *et al*., 2003) and marine-derived samples especially deep marine sediment (Mincer *et al*., 2002), sponges (Supong *et al*., 2013) and puffer fish (Wu *et al*., 2005). These new habitats have been recognized as potential sources for the isolation of new actinomycetes.

2.1 Diversity of marine actinomycetes

Actinomycetes have been isolated from the marine ecosystem since 1969 but in that time researchers believed that they are not much different from their terrestrial counterparts (Weyland, 1969). The true marine actinomycetes are usually more difficult to culture compared with their terrestrial source because of the special growth requirement such as salt minerals, vitamins, sea water (Zotchev, 2012). In recent years many novel actinomycete genera could be isolated from the marine environment.

Rhodococcus marinonascens is the first novel species of actinomycetes isolated from the marine ecosystem (Helmke & Weyland, 1984). Although sampling for marine actinomycetes began in the late 1960s, it was not until 2005 which the first seawater-obligate marine actinomycetes were described. *Salinispora* was the novel genus of actinomycetes belonging to the family *Micromonosporaceae* which was the first obligate marine actinomycetes, requiring sea water for growth. This genus formed a distinct taxon in the 16S rRNA gene from other members of *Micromonosporaceae*. Up to present, they are 3 species of *Salinispora* including *S. arenicola* and *S. tropica* (Maldonado *et al*, 2005) and *S. pacifica* (Ahmed *et al*., 2013). Accroding to Goodfellow & Fiedler (2010), approximately 50 genera of actinomycetes were isolated from the

marine environment, including 12 novel genera such as *Actinoaurantispora*, *Demequina, Euzebya, Iamia, Marinactinispora, Marisedimenicola, Miniinunas, Phycicola, Salinibacterium, Salinispora, Sciscionella, Serinicoccus*.

Jensen *et al*., (2005) isolated the actinomycetes from 275 marine samples collected from the island of Guam. Totally, 6425 isolates were obtained. Among them, 983 isolates (15%) represented the range of morphological diversity from each samples. Most isolates (58%) required sea water for growth which represented the high degree of marine adaptation. The dominant actinomycetes speices (568 isolates) was found to be the obligate marine actinomycete, *Salinispora*.

In 2006, the marine actinomycete genera including *Dermacoccus*, *Kocuria*, *Micromonospora*, *Streptomyces*, *Tsukamurella* and *Williamsia* were isolated from the sediment samples collected from Mariana Trench (10,898m). More than half of the isolates were detected the non-ribosomal peptide synthetase sequences which corresponding to the ability to produce the secondary metabolites. (Pathom-aree *et al*., 2006). Based on the culture independent techniques, the marine sediments collected from the Canary Basin (3814 m) and the South Pacific Gyre (5126 and 5699 m) revealed the presence of *Salinispora* sequences (Prieto-Davó *et al*., 2013). These providing further support for the occurrence of the actinomycetes in deep sea sediments.

In Thailand, seven new species of marine-derived actinomycetes were described, mostly belonged to the family *Micromonosporaceae* such as *Micromonospora krabiensis* (Jongrungruangchok *et al*., 2007), *M. pattaloongensis* (Thawai *et al*., 2008), *M. marina* (Tanasupawat *et al*., 2010), *M. sediminicola* (Supong *et al*. 2012), *M. maritima* (Songsumanus *et al.* 2012). These species were isolated from the marine sediment except the recent study, *M. spongicola* (Supong *et al*., 2013), *Verrucosispora andamanensis* (Supong *et al*., 2013), which were isolated from the marine sponge, *Xestospongia* sp. collected from the gulf of Thailand. The novel marine acitnomycete species during 2008-2013 are shown in Table 2.1

Table 2.1 Some novel marine-derived actinomycete species discovered during 2008 to 2013

Species	Source of isolation	Isolation media	Reference
Actinomadura sediminis	Mangrove sediment from Dugong Creek, Little Andaman, India	Kuster's agar	He *et al*., 2012
Amycolatopsis marina	Deep ocean sediment from the South China sea	SM1 medium	Bian *et al*., 2009
Nocardiopsis litoralis	Sea anemone	Marine agar	Chen *et al*. 2009
Marinactinospora thermotolerans	Mud from the northern China sea	Raffinose-histidine agar	Tian *et al*., 2009
Micromonospora krabiensis	Marine soil, Krabi province, Thailand	Starch casein nitrate agar	Jongrungruangchok, *et al*. 2008
Micromonospora maritima	Mangrove soil, Samut-sakhon, Thailand	Starch casein nitrate agar	Songsumanus *et al*., 2013
Micromonospora marina	Sea sand, Hua-hin, Thailand	Starch casein nitrate agar	Tanasupawat *et al*., 2010
Micromonospora pattaloongensis	Mangrove soil, Thatien, Pattaloong province, Thailand	Starch casein nitrate agar	Thawai *et al*., 2008
Micromonospora sediminicola	Sediment, Krabi province, Andaman sea, Thailand	Starch casein nitrate agar	Supong *et al*., 2013a
Micromonospora spongicola	Marine sponge, *Xestospongia* sp., Thailand	Unknown	Supong *et al*., 2013b
Salinispora arenicola	Marine sediment, Red sea and the sea of Cortez	M1,M2,M3,M4,M5 agar	Maldonado *et al*., 2005
Salinispora pacifica	Sediment, island of Guam and Palau	Sea water agar	Ahmed *et al*, 2013
Salinispora tropica	Marine sediment, Red sea and the sea of Cortez	M1,M2,M3,M4,M5 agar	Maldonado *et al*., 2005
Sciscionella marina	Sediment from the northern China sea	Gauze No.1 medium	Tian *et al*., 2009a
Streptomyces abyssalis	Marine sediment,Xisha island, South China sea	Unknown	Xu *et al*., 2012
Streptomyces glycovorans	Marine sediment,Xisha island, South China sea	Unknown	Xu *et al*., 2012
Streptomyces haliclonae	Marine sponge, *Haliclona* sp., Japan	Starch casein nitrate agar	Khan *et al*., 2010
Streptomyces marinus	Marine sponge, *Haliclona* sp., Japan	Jewfish extract agar	Khan *et al*., 2010
Streptomyces tateyamensis	Marine sponge, *Haliclona* sp., Japan	Starch casein nitrate agar	Khan *et al*., 2010
Streptomyces xinghaiensis	Marine sediment, Xinghai bay, Chaina	Bennett's agar	Zhao *et al*., 2009
Streptomyces xishnsis	Marine sediment,Xisha island, South China sea	Unknown	Xu et al., 2012
Verrucosispora andamanensis	Marine sponge, *Xestospongia* sp. Thailand	Unknown	Supong et al., 2013c
Verrucosispora fiedleri	Sediment, Raune Fjord, Norway	Starch casein agar	Goodfellow et al., 2013
Verrucosispora maris	Sedimant, Sea of Japan	Colloidal chitin agar	Goodfellow et al., 2012

2.2 Bioactive secondary metabolites from marine actinomycetes

Actinomycetes are known for their capacity to produce of secondary metabolites with diverse biological activities. Approximately 22,500 bioactive metabolites have been isolated from microorganisms and it has been estimated that 10,100 compounds were isolated from actinomycetes (Berdy, 2005; Dharmaraj, 2010). The major of these compounds are derived from the genus *Streptomyces* which is widely distributed in marine and terrestrial habitats and represented in a large number of species among all actinomycete genera (Dharmaraj, 2010). The secondary metabolites of some marine-derived *Streptomyces* are shown in Table 2.2.

Marine-derived actinomycetes have been accepted to be an efficient producer of secondary metabolites. One of the extensive studies of marine actinomycetes is *Salinispora* which is obligate marine actinomycetes. In 2003, Feling *et al.* reported the isolation of Salinosporamide A from *Salinispora tropica*. This compound has a potent proteasome inhibitor activity against human tumors in mouse models and entered the clinical trial phase I (Feling *et al*., 2003; Solanki *et al*., 2008). *S. tropica* produces not only salinosporamide A but also the novel polycyclic macrolide with unknown bioactivity, sporolides A and B. Unlike *S. tropica*, *S. arenicola* produced other novel secondary metabolites such as saliniketals A and B, rifamycin B, cyclomarin A and staurosporine which show cytotoxic, antimicrobial and antiviral activities respectively (Jensen *et al*., 2007).

Marinomycins A-D, the novel antitumor-antibiotics of the unusual macrolides composed of dimeric 2-hydroxy-6-alkenyl-benzoic acid lactones with conjugated tetraene-pentahydroxy polyketide chains, were isolated from the novel marine actinomycete genera '*Marinispora*'. These four new compounds showed significant antimicrobial activities against methicillin resistant *Staphylococcus aureus* (MRSA) and represented the selective cancer cell cytotoxicities against melanoma cell lines (Kwon *et al*., 2006).

Beside *Salinispora* and *Marinospora*, *Verrucosispora* is one of the novel genera of marine-derived actinomycetes which has considerable attention in natural product

chemists. According to the study of Bister *et al*. (2004), *Verrucosispora* strain AB18-032, isolated from a sediment sample collected in the Japan Sea at a depth of 298 m, produced a novel antibiotic abyssomicin C, a polycyclic polyketide, which exhibited the antibacterial activity against gram-positive bacteria as well as multiple resistant and vancomycin resistant *Staphylococcus aureus*. This compound is the first known bacterial secondary metabolite that inhibits the biosynthesis of PABA and be developed as antibacterial agents against drug-resistant pathogen. In addition, a recent study of Fiedler *et al.* (2008) reported that *Verrucosispora* strain MG-37 produced a novel actinofuran antibiotic named as proximicin which showed antibacterial activity but strong cytostatic effect to various human tumor cell lines.

In Thailand, Supong *et al.* (2012) reported the isolation of novel c-glycosylated benz[a]anthraquinones named urdamycinone E, udamycinone G and dehydroxyaquayamycin from *Streptomyces* sp. BCC isolated from Sichang island, Chonburi province at the depth of 5 m. These compounds exhibited potent anti-*Plasmodium falciparum* K1 with IC_{50} values in a range of 0.0534-2.93 µg/ml and anti-*Mycobacterium tuberculosis* with MICs in a range 3.13-12.50 µg/ml.

Table 2.2 Bioactive compounds from marine actinomycetes

Compound	Chemical class	Species	Activity	Reference
Abyssomycin C	Polycyclic polyketide	*Verrucosispora maris*	Antibacterial	Bister *et al*., 2004
Actinofuranone	Polykedide	*Streptomyces* sp.	Cytotoxic	Cho *et al*., 2006
Albidopyrone	α-pyrone	*Streptomyces* sp.	Cytotoxic	Hohmann *et al*., 2009
Aureoverticillactam	Macrocyclic lactam	*Streptomyces aureoverticillaris*	Acticancer	Mitchell *et al*., 2004
Carboxamycin	Benzoxazole	*Streptomyces* sp.	Antibacterial,cytotoxic	Hohmann *et al*., 2009
Cyclomarin A	Cyclic peptide	*Salinispora arenicola*	Anti-inflammatory	Schultz *et al*., 2008
Cyanosporaside A	cyclopenta[a]indene glycosides	*Salinispora pacifica*	Unknown	Oh *et al*., 2006
Dermacozines	*Phenazines*	*Dermacoccus* sp.	Cytotoxic, radical savenging	Abdel-Mageed *et al*., 2010
Diapepinomicin	Farnesylated dibenzodiazepinone	*Micromonospora* sp.	Anticancer	Charan et al., 2004
Daryamides	Polyketide	*Streptomyces* sp.	Cytotoxic, antifungal	Asolkar *et al*., 2006
Enterocin	Polyketide	*Streptomyces maritimus*	Bacteriostatic	Piel *et al*., 2000
Essramycin	Thyazolopyrimidine	*Streptomyces* sp.	Antibacterial	El-Gendy *et al*., 2008
Lynamicins	Bisindole pyrrole	*Marinispora* sp.	Antibacterial	McArthur *et al*., 2008
Mansouramycins	Isoquinolinequinones	*Streptomyces* sp.	Cytotoxic	Hawas *et al*., 2009
Marinopyrroles	Bispyrrole	*Streptomyces* sp.	Actibacterial, cytotoxic	Hughes *et al*., 2008
Merchercharmycin	Peptide	*Thermoactinomyces* sp.	Antitumor	Kanoh *et al*., 2005
Piperrazimycins	Peptide	*Streptomyces* sp.	Cytotoxic	Miller *et al*., 2007
Proximicins	Aminofuran	*Verrucosispora* sp.	Cytostatic	Fiedler *et al*., 2008
Resistoflavine	Quinone	*Streptomyces chibaensis*	Cytotoxic, antibacterial	Kock *et al*., 2005
Salinamides	Bicyclic depsipeptide	*Streptomyces* sp.	Anti-inflammatory	Moore *et al*., 1999
Saliniketal A	Polyketide	*Salinispora arenicola*	Cancer chemoprevention	William *et al*., 2007
Salinipyrone	Polyketide	*Salinispora pacifica*	Cytotoxic	Oh *et al*., 2008
Salinosporamide A	Fused γ-lactam-β-lactone	*Salanispora tropica*	Anticancer	Feling *et al*., 2003
Sporolide A	Halogenated macrolide	*Salinispora tropica*	Unknown	Buchanan *et al*., 2005
Thiocoraline	Thiodepsipeptide	*Micromonospora* sp.	Anticancer	Perez Baz *et al*., 1997
Urukthapelstatin A	Cyclic peptide	*Mechercharimyces* sp.	Anticancer	Matsuo *et al*., 2007

2.3 Taxonomic studies on marine-actinomycetes

In present, the polyphasic approach including phenotypic, chemotaxonomic and genotypic characteristics have been used for the classification and identification of actinomycete.

2.3.1 Phenotypic characteristics

2.3.1.1 Morphological characteristics

Actinomycetes exhibit a unique diverse morphology form the other bacteria. They produce mycelia, which are differentiated to the spores. The difference of spore morphology in particular the position, surface and arrangement of spores, is the key characters to classify the actinomycetes in the genus level (Shirling & Gottlieb, 1966).

2.3.1.2 Phenotypic characteristics

The cultural characteristic, the growth and the appearance of colony on standard media, and the ability to utilize and/or degrade organic material have been accepted for the identification of actinomycetes. The key standard methods as previously proposed by Shirling & Gottlieb (1966) are very useful for the identification of *Streptomyces* species as well as the other rare genera.

2.3.2 Chemotaxonomy of actinomycetes

Chemotaxonomy is the study of the similarity and difference of certain compounds which present among the organisms being classified. Chemotaxonomic properties including the composition of cell wall peptidoglycan, whole-cell sugars, polar lipids composition, isoprenoid quinones and cellular fatty acids are the key markers for the classification of actinomycetes.

2.3.2.1 The composition of cell wall peptidoglycan

All actinomycetes contain cell-wall peptidoglycan. This structure consists of the glycan moiety which is the alternating polymers of *N*-aetylmuramic acid and *N*-acetylglucosamine and peptide chain which links between glycan chains. Three parts of this structure including the variation of peptide, the isomers of diaminopimelic acid and the *N*-acyl types of muramic acid have been used for the classification of actinomycetes. The variation in the peptide moiety are shown in Table 2.3 (Lechevalier & Lechevalier, 1970). The presence of diaminopimelic acid in cell wall peptidoglycan is one of the most important characters for classifying the members of the genus *Streptomyces* and other rare actinomycete genera. All *Streptomyces* species contain only *LL*-diaminopimelic acid isomer in contrast others rare genera contain *meso*-diaminopimelic acid, 3-OH diaminopimelic acid, 3,4 dihydroxydiaminopimelic acid and/or the combination of various isomers (Staneck & Roberts, 1974; Matsumoto *et al*., 2014). Moreover, type of *N*-acyl muramic acid, glycolyl or acetyl type, has been useful for the classification of actinomycetes (Uchida & Aida, 1984).

2.3.2.2 Whole-cell sugars

Lechevalier & Lechevalier (1970) classified the actinomycetes which contain *meso*-diaminopimelic acid in cell wall peptidoglycan in four groups according to the difference of diagnostic sugars in whole cell hydrolysate as shown in Table 2.4.

Table 2.3 Cell wall chemotypes of actinomycetes (Lechevalier & Lechevalier, 1970)

Cell wall type	DAB*	lysine	ornithine	Aspartic acid	glycine	*meso*-DAP	LL-DAP	arabinose	galactose
I	-	-	-	-	+	-	+	-	-
II	-	-	-	-	+	+**	-	-	-
III	-	-	-	-	-	+	-	-	-
IV	-	-	-	-	-	+	-	+	+
V	-	+	+	-	*	-	-	-	-
VI	-	+	-	+	*	-	-	-	-
VII	+	+	-	+	*	-	-	-	-
VIII	-	-	-	+	*	-	-	-	-

*, Glycine is variably present in these groups; **, hydroxyl DAP may be present

+, present; -, absent

Table 2.4 Whole-cell sugar patterns of actinomycetes with *meso*-diaminopimelic acid (Lechevalier & Lechevalier, 1970)

Type	Diagnostic sugar			
	arabinose	galactose	madurose	xylose
A	+	+	-	-
B	-	-	+	-
C	-	-	-	-
D	+	-	-	+

2.3.2.3 Polar lipids composition

Lechevalier *et al*. (1977) surveyed the phospholipid composition of 97 actinomycete isolates representing 20 genera and classified phospholipids composition of actinomycetes in five groups based on the presence and absence of nitrogenous phosphopholipids. Phospholipid type PI exhibit no nitrogenous phophospholipids. Phospholipid type PII contains only one nitrogenous phospholipids. Phospholipid type

PIII represents the phosphatidyl chloline in phospholipids composition. Phospholipid type PIV contains an unidentified phospholipid containing gluocosamine (GluNU). Phospholipid type PV contains phosphatidyl glycerol in addition to GluNU. The difference among phospholipid types is listed in table 2.5.

Table 2.5 Phospholipids type of actinomycetes (Lechevalier *et al*., 1977)

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

Abbreviation: PIMs, phosphatidyl inositol mannosides; PI, phosphatidyl inositol; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PME, phosphatidyl methyl ethanolamine; GluNu, glucosamine containing unknown phospholipids; DPG, diphosphatidyl glycerol; ND, no data; v, variable; -, absent

2.3.2.4 Isoprenoid quinones

Isoprenoid quinones are a component of bacterial cell membrane and play the important role in the electron transport system. Isoprenoid quinones are classified by the difference of core structures as ubiquinone and menaquinone (Figure 2.1). According to the study of Collin & Jones (1981), only menaquinones are observed in the cell membrane of actinobacteria. The difference of the number of isoprene units and the degree of hydrogenation are the important characteristics for the classification of actinomycetes in the genus level.

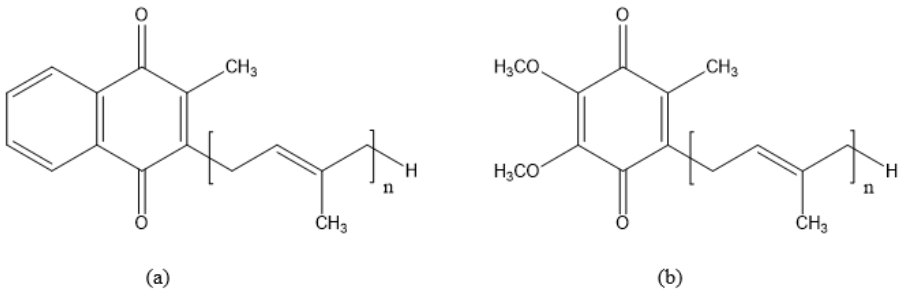

Figure 2.1 Chemical structures of menaquinones (a) and ubiquinones (b)

2.3.2.5 Cellular fatty acids

The difference in cellular fatty acid composition, including the number of carbon atoms in molecule, saturated or monounsatured fatty acids and iso- or anteiso-branched fatty acid, is used to classify actinomycetes in the genus level. In addition, the presence of mycolic acids (2-hydroxy-3-alkyl fatty acids with a long alkyl chain) is a useful key characteristic for identification members of the family *Nocardiaceae*, *Mycobacteriaceae* and *Corynebacteriaceae*. According to Sasser (1990), gas chromatography was found to be the effective and rapid method for the analysis of bacterial cellular fatty acids.

2.3.3 Genotypic characteristics

In present, modern microbial taxonomy have been influenced by the development of molecular genetic techniques (Tindall *et al*., 2010). In the systematic of actinomycetes, 16S rRNA gene sequence analysis, phylogenetic analysis, DNA base composition and DNA-DNA hybridization are often used among the actinobacterial taxonomists.

2.3.3.1 16S rRNA gene sequences and phylogenetic tree analysis

Ribosomal 16S rRNA is a part of 30S small subunit of bacterial ribosome. As it exhibits highly conserved regions in all bacteria and slow rate of evolution, it has served as the primary key for the phylogeny-based identification when compared with

the well-curated 16S rRNA gene sequence databases. In 2012, Kim *et al*. created an effective identification function based on Basic Local Alignment Search Tool (BLAST) searches and pairwise global sequence alignments (http://eztaxon-e.ezbiocloud.net/). This search tool provides an effective taxonomic backbone for bacterial identification in present.

2.3.3.1 DNA base composition

DNA contains four nitrogenous base adenine (A), guanine (G), cytosine (C) and thymine (T). This character is useful for the classification and identification of actinomycetes in the genus and maybe the species levels. In general, the base composition of bacterial genome ranges from 25% - 80% G+C. Because of the high G+C content in actinobacterial genome, this character can distinguished the members of actinobacteria from other bacterial phyla. Moreover, the DNA base compositions of the strains of the same species exhibit narrow range (1 – 3 mole % G+C). Therefore, the difference in DNA base composition can be assumed to the different of the genome and belong to the difference species (Tamaoka, 1994).

2.3.3.2 DNA-DNA hybridization

DNA-DNA hybridization represents the indirect way to compare two genomes. Up to present, the values 70% of DNA-DNA relatedness have been accepted for the threshold for assigning strains to the same species (Wayne *et al*., 1987).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Marine samples collection and isolation

3.1.1 Samples collection and pretreatment

Twenty five Marine samples, including sediment and marine sponges, were collected from Chumphon (Bormao bay, Chumphon beach and Koh Khai), Chonburi (The nature education center for mangrove conservation and ecotourism, Bangsaen beach), Phuket (Phanwa Beach, Laem Phanwa), Trang (Koh Rok Nork; Koh Rok Nai; Koh Mah) *Prachuap* Khiri Khan (Koh Talu), Samut Songkhram (mangrove forest) and Krabi Provinces (mangrove forest) using scuba diving gears.

3.1.2 Isolation method

The marine actinomycetes were isolated using standard dilution-plating method. One gram of samples was suspended in 9 ml of sterile natural seawater to make 10-fold dilution series to 10^{-4}. Each diluted suspension (0.1 ml) was spreaded on M1, M2 (Zhang *et al*., 2006) and seawater-proline media (Inahashi *et al*., 2011; modified with seawater in this study) supplemented with nalidixic acid 25 μg ml^{-1} and cycloheximide 50 μg ml^{-1} (Appendix A). The plates were incubated at 28 °C for 30 days. The colonies of marine actinomycetes were observed using a light microscope and were transferred to ISP2 media agar. The purified cultures were maintained on ISP2 medium at 4 °C. All isolates were preserved using freeze-drying and freezing at -80 °C in 15% (v/v) glycerol solution.

3.2 Identification methods

The selected marine actinomycete isolates were identified using the polyphasic approach including phenotypic, chemotaxonomic and genotypic characteristics. The inoculum of all isolates was obtained from the culture grown in yeast extract-dextrose broth (Appendix A) in a shaking condition at 180 r.p.m. 30 °C for 4-7 days. The cultures (1 ml, each) were washed with sterile 0.85% (w/v) saline solution 3 times to eliminate the culture broth. These inoculums were used for all phenotypic studies.

3.2.1 Morphological and cultural characteristics

Morphological characteristics of the isolates was observed using light and scanning electron microscopes (JEOL, JSM-7610F Tokyo, Japan) on the culture grown on ISP2 medium at 28 °C for 14 days. Cultural characteristics were determined using 14-day cultures grown at 28 °C on various media including yeast extract-malt extract (ISP medium no. 2), oat meal 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) (Shirling & Gottlieb, 1966) and nutrient media (Appendix A). The color designation of colony including aerial mycelia, substrate mycelia and diffusible pigment was determined using the NBS/IBCC color system (Kelly, 1964).

3.2.2 Physiological characteristics

The growth of the isolates was determined after 14-day culture. The isolates were cultured on ISP2 medium agar with various incubation temperatures (15, 20, 30, 37 and 45 °C), pH (4, 5, 6, 7, 8 and 9) and NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7% (w/v)) at 30 °C for 14 days. The maximum NaCl concentration for growth was recorded

3.2.3 Biochemical characteristics

All biochemical characteristics were determined after the cultures were grown on the suitable media at 28 °C for 14 days, as the followings

a. Starch hydrolysis was determined using inorganic-salt starch medium (ISP4) (Appendix A). The clear zone around colonies after flooding with 1% (v/v) of iodine solution indicated the positive result of starch hydrolysis.

b. The ability to liquefy gelatin was observed using bouillon gelatin broth (Appendix A). After incubation, the cultures were placed at 4 °C for 1 hour. The positive would show the liquid solution of gelatin.

c. The ability to peptonize skim milk was observed on the skim milk agar. The clear zone around colony indicated the positive test for skim milk peptonization.

d. The ability to reduce nitrate to nitrite was determined using peptone KNO_3 broth (Appendix A) supplemented with 50% (v/v) artificial seawater. After incubation, the culture broth was added with 0.5 ml each of sulfanilic acid and *N,N*-dimethyl-1-naphtylamine solutions. The pink to red color represented the presence of nitrite (positive). If the color not change, zinc powder would be added to detect the over nitrate reduction. The red to pink color after added zinc powder indicated the negative for nitrate reduction test while, no color change indicated the over nitrate reduction (positive).

e. The carbon utilization was determined on ISP 9 medium (Appendix A) supplemented with 1% (w/v) of carbon sources (Shirling & Gottlieb, 1966). The media containing glucose and no carbon sources were used as positive and negative controls, respectively.

3.2.4 Chemotaxonomic characteristics

All chemotaxonomic characteristics were determined using freeze-dried cells which were obtained from the cultures grown in yeast extract-dextrose broth modified with sea water at 28 °C for 7 days in a shaking condition at 180 r.p.m. The culture broth were washed twice with sterile distilled water before freeze-drying.

3.2.4.1 Isomers of diaminopimelic acids analysis

The isomers of diaminopimelic acid were analyzed using the standard TLC method (Staneck & Robert, 1974). Briefly, 10 mg of freeze-dried cells were hydrolyzed with 1 ml of 6 N HCl at 100 °C for 18 hour. Whole cell hydrolysate were filtered with a filter paper and evaporated the filtrate to dryness. The dried extracts were dissolved with 0.3 ml of distilled water and were applied on the base line of a cellulose TLC plate (20 x 20 cm) which was developed twice with the solvent system: methanol-water-6 N HCL-pyridine (80:26:4:10, v/v). After the second developing, the spots were visualized by spraying with 0.2 % ninhydrin solution (Appendix B) and heated at 100 °C for 5 minutes. DAP isomers appeared as dark-green spots as comparison with a DAP standard solution.

3.2.4.2 Whole-cell sugars analysis

The whole-cell sugars were analyzed using the standard TLC method (Staneck & Robert, 1974). The freeze-dried cells (50mg) were hydrolyzed with 1N H_2SO_4 at 100 °C for 2 hours. pH of the hydrolysates was adjusted to 5.2-5.5 using saturated $Ba(OH)_2$ solution. The precipitate was removed by centrifugation at 4,500 r.p.m. for 10 minutes. The supernatants were transferred to new test tubes and were evaporated to dryness. The dry extracts were dissolved with 200 µl of distilled water and were applied on a cellulose TLC plate (20 x 20 cm). The TLC plates were developed twice with n-butanol-water-pyridine-pyridine-toluene (10:6:6:1, v/v). The sugars were visualized by spraying with acid aniline phthalate solution (Appendix B) and heated at

100 °C for 4 minutes. The mixture sugar solution of galactose, arabinose xylose, rhamnose, mannose, glucose and ribose were used as the standard solution.

3.2.4.3 Mycolic acids analysis

The mycolic acids were extracted following the method as previously described by Tomiyasu (1982). Briefly, 2 ml of 10% KOH-methanol were added into 50-200 mg of freeze-dried cells in a test tube with a screw cap. The cells were hydrolyzed at 100 °C for 2 hours in a heat box and then 0.6 ml of 6N HCl and 2 ml of n-hexane were added into the test tube and shaking well to extract the lipids. After shaking well, the test tube was centrifuged at 3,000 r.p.m. for 10 min and the upper layer was transferred to a new test tube with a cap. The lower layer was extracted again using 2 ml of n-hexane and collected the upper layer. After drying the upper layer with nitrogen gas, 2 ml of benzene-methanol-H_2SO_4 (10:20:1) was added in to this tube and heated at 100 °C for 2 hour. After cooling, 2 ml each of water and hexane was added into the tube and vortexed for 5 minutes, then the tube was centrifuged at 3,000 r.p.m. for 10 minutes. The upper layer was transferred to a new tube and the lower layer was extracted again using 2 ml of hexane. After the upper layer was dried with nitrogen gas, the small amount of n-hexane was added into the tube to dissolve the lipids and then applied the lipid fraction to TLC (silica gel, 10 x 10 cm). The lipid extract of *Nocardia nova* JCM 4044^T was used as a positive control of mycolic acids. The TLC plate was developed with hexane-diethylether (4:1) and was visualized using iodine vapor. Based on this system, mycolic acids appeared (approximately) at R_f 0.47.

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

Freeze-dried cells (10 mg) were hydrolyzed with 0.1 ml of 6N HCl at 100 °C for 3 hours. The hydrolysate was added with 0.1 ml of distilled water and 2 ml of diethyl ether (saturated with distilled water). The solution was vortexed for 1 minute and centrifuged at 2,000 r.p.m. for 5 minutes. The upper phase was discarded and then added 4 ml of diethyl ether, vortexed and centrifuged at 3,000 r.p.m. for 10 minutes.

The upper phase was collected to a new test tube and the lower phase was partitioned twice with diethyl ether (each of upper phases was separately collected in a test tube). Each of upper phases was added with 10 µl of 1N NaOH solution and evaporated to dryness, then added 2 ml of 0.02% 2,7-dihydroxynaphthalene in conc. H_2SO_4. The solutions were heated at 100 ^{o}C for 10 minutes. The reddish purple color indicated the glycolyl type while colorless indicated the acetyl type (Uchida & Aida (1977).

3.2.4.5 Polar lipids analysis

Freeze-dried cells (150 mg) were suspended in 3 ml of methanol-0.3% NaCl (100:1) and 3 ml of petroleum ether and mixed for 15 minutes. The cell suspensions were centrifuged at 3,000 r.p.m. for 10 minutes. The supernatants were discard and then added 1 ml of petroleum ether to the cells and mixed again for 15 minutes, centrifuged at 3,000 r.p.m. for 10 minutes and removed the supernatant. The lower layers (cells) were heated at 100 ^{o}C for 5 min and immediately cooled using tap water. After cooling, added 2.3 ml of chloroform-methanol-water (90:100:30) to the solution, mixed well for 15 minutes, centrifuged at 3,000 r.p.m. for 10 minutes and transferred the upper layer to a new test tube (tube no. 2). The lower layer was extracted twice with 2.3 ml of chloroform-methanol-water (50:100:40), mixed well for 15 minutes and the upper layer was transferred to the tube in the previous step (tube no. 2). The solution in tube no.2 was added with 1.3 ml each of chloroform and water, mixed well for 5 minutes. The lower layer was collected to a new vial and dried with nitrogen gas (Minnikin *et al*., 1984).

The polar lipids were identified using two dimensional TLC method (Minnikin *et al*., 1977). The polar lipid extract was dissolved with 200 µl of chloroform-methanol (2:1 v/v) and was applied on the corner of a silica-gel TLC plate (10 x 10 cm). The first dimension of TLC developing was performed in chloroform-methanol-water (65:25:4, v/v) and the second dimension was developed in chloroform-acetic acid-methanol-water (40:7.5:6:2, v/v). To compare the chromatogram patterns, each TLC plate was sprayed with five specific reagents including; molybdenum blue,

ninhydrin, Dragendorff's reagent, anisaldehyde and phosphomolybdic acid. All reagents used are shown in Appendix B.

3.2.4.6 Cellular fatty acids analysis

Cellular fatty methyl ester were prepared followed the method of Sasser (1990) with slight modification. Four steps to prepare the cellular fatty acid methyl esters were as the followings

a. Saponification: 1.0 ml of fatty acid reagent 1 (Appendix B) was added to 40 mg of the freeze-dried cells in the capped test tube. The tube was heated at 100 °C for 5 minutes and vortex for 5-10 seconds and then was returned to complete 30 minutes heating.

b. Methylation: The cooled tube was added with 2.0 ml of fatty acid reagent 2 (Appendix B) and was briefly vortexed. After vortexing, the tube were heated at 80±1 °C for 10 minutes. (This step is strictly in time and temperature.)

c. Extraction: The tube was uncapped and added 1.25 ml of fatty acid reagent 3 (Appendix B) and was vortexed for 5 minutes. After vortexing, the tube was centrifuged at 4,500 r.p.m. for 10 minutes. The upper layer was transferred to a new tube and the lower layer was discarded.

d. Base wash: 3 ml of fatty acid reagent 4 (Appendix B) was added to the upper layer and vortexed for 5 minutes. The tube was centrifuged at 4,500 r.p.m. for 10 minutes, 2/3 of the upper layer was pipetted into a vial for gas chromatography.

The cellular fatty acid methyl ester samples were analyzed using gas chromatography (25 m x 0.2 mm phenyl methyl silicone fused silica capillary column; FID detector, 170 – 250 °C at 5 °C/min; hydrogen and nitrogen are carrier and makeup gas, respectively) according to the instruction of the Microbial Identification System (MIDI) Sherlock system version (6.0).

3.2.4.7 Menaquinones analysis

Freeze-dried cells (300 mg) were extracted with 20 ml of chloroform-methanol (2:1, v/v) and stirred overnight. The cell debris was removed using filter paper and the filtrates were evaporated to dryness (temperature should not over 37 °C). The dry extracts were dissolved with small amount of acetone and applied on a preparative silica-gel TLC plate (5 x 20 cm). The TLC plate was developed using benzene. The menaquinones were visualized using UV light (254 nm), scraped off and extracted with acetone (HPLC grade). The acetone extract was filtered through 0.5 µm membrane and analyzed by HPLC [Cosmosil 5 C18 4.6 x 150 mm, Nacalai Tesque; Methanol-2-propanol (2:1) or LC/MS (CAPCELL PAK C18 UG120, Shiseido, Tokyo, Japan; Methanol-2-propanol (7:3)] (Collin *et al*., 1977).

3.2.5 Genotypic characteristics

3.2.5.1 Extraction of genomic DNA

The genomic DNA for 16S rRNA gene amplification was extracted from the cell grown in yeast extract-dextrose broth supplemented with 50% (v/v) artificial sea water in a shaking condition at 180 r.p.m. 30 °C for 4-7 days. The cell suspension of each strain (1ml) was collected in each micro centrifuge tube and was washed twice with sterile distilled water. After washing, 300 µl of TE-buffer and small amount of aluminium oxide (full micro spatula) was added in to the tube. The cells were lysed using a micro-mixer for 90 sec, then 300 µl of phenol : chloroform (1:1) were added and centrifuged at 14,000 r.p.m. for 15 minutes. The upper layer was transferred to a new tube and then 3 mM sodium acetate (1/10 volume) and cold ethanol (2 volumes) were added. The tube was centrifuged at 14,000 r.p.m. for 10 minutes and discarded the supernatant. The tube was respectively washed with 70% and 95% ethanol and wait for air-dryness. 50 µl of sterile ultrapure water were added to dissolve the DNA. This DNA was kept at 4 °C.

The genomic DNA for DNA base composition analysis and DNA-DNA hybridization were extracted from the freeze-dried cells obtained from the culture grown in yeast extract-dextrose broth (Appendix A) using a shaking condition at 180 r.p.m. 30 °C for 4-7 days (Raeder & Broda (1985). Freeze-drying cells (500 mg) were ground using a mortar. The cell powder was added with 5 ml of DNA extraction buffer (Appendix B), 3.5 ml of phenol saturated with water and 1.5 ml of chloroform and mixed the solution by inverting the tube. The tube was centrifuged at 8000 r.p.m. for 15 minutes and the upper layer were transferred to a new tube. The genomic DNA in the upper layer was precipitated using iso-propanol and was spooled using a clean glass rod. The DNA was respectively washed with 70% and 95% ethanol (v/v) and was air-dried. The DNA was dissolved in 0.1X saline sodium citrate (SSC) solution (Appendix B) and preserved at 4 °C.

3.2.5.2 Amplification of 16S rRNA gene

The amplification of 16S rRNA gene was carried out using two primers 20F (5′ -GAGTTTGATCCTGGCTCAG-3′, positions 9-27) and 1500R (5′-GTTACCTTGTTACGACTT-3′, positions 1492-1509). The PCR mixture (final volume 100 µl) contained 4 µl each of primers (10 pmol/µl), 2 µl of dNTP (10 mM), 10 µl of 10x *Taq* buffer, 8 µl of MgCl (25 mM), 0.5 µl of *Taq* DNA polymerase, 61.5 µl of dH_2O and 10 µl of template DNA. The amplification was performed with an initial denaturation at 94 °C for 3 minutes, followed by 30 cycles with denaturation at 94 °C for 1 minute, annealing at 50 °C for 1 minute and extension at 72 °C for 2 minutes, followed by the last step at 72 °C for 3 minutes (Suriyachadkun *et al*. 2009). The PCR product was purified using the PCR purification kit (Gene aid). The sequencing of nucleotides was performed using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), 518F (5′-CCAGCAGCCGCGGTAATACG-3′), 800R (5′-TACCAGGGTATCTAATCC-3′), 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991) (Macrogen; Seoul, Korea).

3.2.5.3 Amplification of *gyrB* gene

The amplification of enzyme gyrase subunit B gene (*gryB*) was carried out according to the method as previously described by Garcia *et al*. (2010). The two PCR products which were overlapped, were amplified to obtain the final sequence of 1,100 nucleotides. The first amplification, 500-bp fragment, was carried out using two primers GYF1 (5′-TCCGGYGGYCTGCACGGCGT-3′; position 19–38) and GYR1B (5′-CGGAAGCCC TCYTCGTGSGT-3′; position 548–567). The PCR mixture (final volume 100 µl) contained 4 µl each of primers (10 pmol/µl), 2 µl of dNTP (10 mM), 10 µl of 10x *Taq* buffer, 8 µl of MgCl (25mM), 0.5 µl of *Taq* DNA polymerase, 61.5 µl of dH_2O and 10 µl of template DNA. The amplification was performed with an initial denaturation at 95 °C for 9 minutes, followed by 35 cycles with denaturation at 95 °C for 1 minute, annealing at 62 °C for 1 minute and extension at 72 °C for 2 minutes, followed by the last step at 72 °C for 7 minutes. The second amplification, 900-bp fragment, was carried out using the primers GYF3 (5′-ACSGTCGACTTCGACTTCCA-3′, position 220–239) and GYR3B (5′-CAGCACSAYCTTGTGGTA-3′, position 1210–1226). The amplification was performed with an initial denaturation at 95 °C for 9 minutes, followed by 35 cycles with denaturation at 95 °C for 1 minute, annealing at 54 °C for 1 minute and extension at 72 °C for 2 minutes, followed by the last step at 72 °C for 7 minutes. Both PCR products were purified using the PCR purification kit (Gene aid) and were sequenced by Macrogen (Seoul, Korea). The first PCR product was sequenced using primers GYF1 and GYR1B while the second PCR product was sequenced using primers GYF3, GYR3B and GYF4 (5′-ACCCACGAGGAGGGCTTCCG-3′, position 548–567)

3.2.5.4 BLASTn and Phylogenetic tree analysis

BLASTn search was performed using EzTaxon-e server (Kim *et al*., 2012). The sequences were aligned against the selected type strains sequences which obtained from the GenBank/DDBJ/EMBL by using BioEdit software (Hall, 1999). The phylogenetic trees [neighbor-joining (Saitou & Nei, 1987), maximum parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981)] were constructed using MEGA 6.0 (Tamura *et al*., 2013). The topology of nodes was evaluated using the bootstrap resampling method with 1,000 replications (Felsenstein, 1985).

3.2.5.5 DNA base composition analysis

The DNA solution (2 µg/µl) was heated at 100 °C for 5 min and immediately cooled in ice. Then 10 µl of nuclease P1 solution (Appendix B), was added to the denatured DNA and incubated at 50 °C for 1 h. After incubation, added 10 µl of the alkaline phosphatase (Appendix B) solution and incubated at 37 °C for 1 h. The composition of the nucleosides in the sample was analyzed by HPLC (Nakarai Cosmosil 5C18 (150 x 4.6 mm; eluted with 0.2 M $NH_4H_2PO_4$-acetonitrile (20:1); detector wave length 270 nm). An equimolar mixture of nucleosides was used as the quantitative standard for DNA based composition analysis as shown in the equation below.

$$\text{Mol\% G+C} = \frac{\left(\frac{\mathbf{G}}{\mathbf{GS}}+\frac{\mathbf{C}}{\mathbf{Cs}}\right)}{\left(\frac{\mathbf{A}}{\mathbf{As}}+\frac{\mathbf{G}}{\mathbf{Gs}}+\frac{\mathbf{C}}{\mathbf{Cs}}+\frac{\mathbf{T}}{\mathbf{Ts}}\right)}$$

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

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

3.2.5.6 DNA-DNA hybridization

DNA-DNA relatedness was determined using the microplate hybridization method (Ezaki *et al*., 1989). The purity of DNA should be 1.8 – 2.0 of the absorbance ratio at 260/280

The fixation of DNA samples: DNA solutions (100 µg/ml in 0.1 x SSC) were boiled at 100 °C for 10 minutes and were immediately cooled in ice. After cooling, the DNA solutions were diluted to 10 µg/ml and were dispensed (100 µl) to each well of the 96-wells plate (totally 1 µg of DNA per well). The plate was tightly sealed and incubated at 37 °C overnight. The calf thymus DNA was used as a control.

Preparation of labeling probe: The DNA solution (100 µg/ml) 10 µl was sonicated for 2 minutes and then 10 µl of photobiotin solution was added into the solution. The DNA solution was exposed to the light (500 watt) for 30 minutes (in an ice box). Then, 127 µl of milli Q water, 16 µl of 0.1 M Tris-HCl buffer and 160 µl was added into the DNA solution. This solution was partitioned twice with 160 µl of *n*-butanol and then the butanol-layer (upper layer) was removed. The lower layer (water phase) was boiled at 100 °C for 10 minutes and immediately cooled on ice. After cooling, the DNA solution was diluted into the hybridization solution (Appendix B) to obtain the final concentration of DNA probe 1 µg/ml. The DNA probe was dispensed into each microplate well (100 µl). Finally, each well contained 0.1 µg of the DNA probe.

Hybridization: After incubation the plate overnight, the solution in the plate was discarded and 100 µl of the hybridization solution were added to each well. The plate was tightly sealed with the plastic sticker and was incubated overnight at optimal hybridization temperature as calculated from the equation below.

Hybridization temperature = 0.41 x GC% of the DNA probe + 24.3

Detection: The hybridization solutions were discarded and the plate was washed with 0.2 ml of 0.2 X SSC three times. 0.2 ml of PBS-BSA-Triton solution was added to each well and incubated at room temperature for 10 minutes. After incubation, PBS-BSA-Triton solution was discarded and 0.1 ml of streptavidin-β-

galactosidase solution was added into each well and then incubated at 37 °C for 30 minutes. Discarded solution 2 in the microplate well and washed wells with 0.2 ml of PBS buffer solution three times. Added 0.2 ml of 0.1 mg/ml of 4-methylumbelliferyl-β-D-galactopyranoside (Appendix B). The intensity of fluorescence was measured using a microplate reader (E_x/E_m, 350/460 nm) (Microplate reader Wallac 1420, PerkinElmer™). The DNA-DNA relatedness values were calculated as the equation below.

$$\text{DNA-DNA relatedness (\%)} = \frac{\textbf{DNA sample} - \textbf{Calf thymus}}{\textbf{Labelled type strain} - \textbf{Calf thymus}} \text{ X } 100$$

3.3 Screening of antimicrobial activities

The culture broth library was prepared by using four different production media including 301 medium, 54 medium, 51 medium and yeast-dextrose broth (Appendix A). The inoculum of each strain was cultured in yeast extract-dextrose broth for 4-7 days. Then, 0.1 ml of the inoculum was transferred to 10 ml of the screening production media and incubated in a shaking condition at 180 r.p.m. 30 °C for 7-14 days. After incubation, 10 ml of 95% ethanol were added into the culture broth and shaked at 180 r.p.m. for 2 hours. The extract solution was centrifuged at 3,400 r.p.m. for 15 minutes and preserved at -20 °C. The production medium without the culture were used as a negative control.

The screening of antimicrobial activities was performed using agar disc diffusion method (Qin *et al*., 2009). Each of paper disc (8 mm) was soaked into the extract solution and air-dried. After drying, the discs were put onto the surface of the agar plate containing a tested microorganism and cooled at 4 °C for 30 minutes before incubation. The bacterial plates were incubated at 37 °C for 24 hours while yeast and filamentous fungi were incubated at 30 °C for 48 hours. The inhibition zones (mm) were measured using a vernier caliper. Three bacteria, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* NIHJ KB213, one yeast, *Candida*

albicans KF1, and one filamentous fungi *Mucor racemosus* IFO 4581^{T} were used as the tested microorganisms.

3.4 Secondary metabolites study of the selected actinomycete isolates

3.4.1 Fermentation and extraction

The inoculum of selected actinomycete isolates was cultured in yeast extract-dextrose broth in a shaking condition (180-200 r.p.m.) at 30 ^{o}C, for 5 - 7 days (up to the isolates). Each of the inoculum (1%) was transferred into the production medium and incubated in a shaking condition (180-200 r.p.m.) at 30 ^{o}C. The incubation period was varied depended on the isolates (7 - 14 days). The details for fermentation and extraction of selected strains were described in the result of each isolate (chapter IV).

3.4.3 Chemical profile analysis

The chemical profile (UV and retention time) of the crude extracts was analyzed by using HPLC (UltiMate 3000, DIONEX) equipped with a C-18 column (Puropher®Star; Merck, 5 µm, 2.1 x 50 mm) with the linear gradient system (0 - 100% CH_3CN in H_2O + 0.05% formic acid), flow rate 0.5 ml/min for 18 minutes. The UV/UV-VIS was used as a detector. The HPLC chromatograms obtained from this system were analyzed compared with the in-house database of BIOTEC, NSTDA. The LC-ESI-MS spectra were measured using an AB Sciex QSTAR Hybrid LC/MS/MS system (AB Sciex, Framinghm, MA, USA) equipped with Inertsil ODS-4 column (3 x 250 mm, 5 µm (Tokyo, Japan)) with the linear gradient of acetonitrile and water (5 - 100%) plus 2 mM ammonium acetate. The chemical profile (retention time, UV absorbance and pseudomolecular ion) was compared with the reported chemical profile using both Kitasato Institute of Life Science's in-house database (Tokyo, Japan) and the Dictionary of Natural Product database (http://dnp. chemnetbase.com).

3.4.4 Isolation and chemical structure elucidation of the compounds

The crude extract of each selected isolate was isolated by column chromatography (Sephadex LH-20 and/or ODS as a stationary phase) and preparative high performance liquid chromatography [Pegasil ODS sp100 column (20 x 250 mm) or Pegasil silica gel SP100 column (20 x 250 mm) or Sunfire C18 column (10μM, 19 x 250mm)]. Details in isolation process and isolation schemes of each selected isolate were shown in Chapter IV. HRESIMS data were obtained from a Bruker MicroOTOF mass spectrometer. NMR spectra were measured using a Bruker Avance 500 MHz or a Bruker Avance III 400 MHz NMR spectrometer.

3.5 Biological activities screening of the isolated compounds.

3.5.1 Antibacterial activity

The crude extracts and/or isolated compounds were determined the antibacterial activity against *Bacillus cereus* ATCC 11778 by using the resazurin microplate assay (REMA) (Sarker *et al*., 2007). The tested bacterium was cultured in 5 ml of tryptic soy broth at 37 ^{o}C for 30 minutes on a shaking condition at 200 r.p.m. to reach the OD_{600} approximately 0.1 and then this cell suspension was 30-fold diluted in the same medium. The antibacterial activity was determined using the 384-well plate, each well contained 5 μl of *B. cereus* cells ($5x10^4$ CFU/well), 7.5 μl of tested sample, 25 μl of 0.25 mM rezazurin and 37 μl of Mueller-Hinton broth (MHB). The plate was incubated at 37 ^{o}C for 3 hour and then fluorescent intensity (excitation/emission at 530/590) was measured. The minimum inhibitory concentration (MIC) is the lowest concentration of the compound that inhibits the growth of the bacterial cells. Vancomycin and 0.5% DMSO were used as the positive and negative control, respectively.

3.5.2 Antifungal activity

The antifungal activity against *Candida albicans* ATCC 90028 of the crude extracts and isolated compound were determined using the resazurin microplate assay (REMA) (Sarker *et al*., 2007). The yeast was grown on potato dextrose agar (PDA) at 30 ^{o}C for 3 days. Then the yeast cells were transferred to RPMI-1640 and adjusted the cell density to 5 x 10^5 CFU/ml. 45 µl of the yeast cell suspension were added into each well of the 96-well plate; each well contained 5 µl of the test compounds. The plate was incubated at 37 ^{o}C for 4 h and then 10 µl of of resazurin solution (62.5 µg/ml) was added to each well and incubated at 37 ^{o}C for 30 minutes. The inhibition concentration (IC_{50}) represents the concentration of the test compound that causes 50% growth reduction of the yeast cells. Amphotericin B and 0.5% (v/v) of DMSO were used as the positive and negative controls, respectively.

3.5.3 Anti-*Mycobacterium tuberculosis* activity

The anti-*Mycobacterium tuberculosis* activity was determined by using the green fluorescent protein microplate assay (GFPMA) (Changsen *et al*., 2003). The frozen *M. tuberculosis* H37Ra *gfp* were thaw and cultured in 7H9GTw-kanamycin until the optical density at 550 nm reached 0.4-0.5. The cells were washed with PBS and suspended in 20 ml of PBS buffer. The assay was performed in black-clear buttom, 96-well plate. The test compounds were prepared in DMSO and twofold dilution were prepared in 100 µl of 7H9GC broth (without tween 80). 100 µl of the cultures were added to each well. The final volume was 200 µl and the final bacterial density was 5 x 10^5 CFU/ml. The plate was incubated at 37 ^{o}C. The fluorescence was measured daily for 8 days using 485 and 508 for excitation and emission, respectively. The minimum inhibitory concentration (MIC) represents the lowest concentration of the compound that inhibits the growth of *Mycobacterium tuberculosis* H37Ra. Ethambutol, isoniazid, ofloxacin, rifampicin, and streptomycin were used as positive controls and 0.5% (v/v) of DMSO was used as a negative control.

3.5.4 Cytotoxic activities against cancer cell lines

The crude extracts and/or isolated compounds were determined the cytotoxic activity against KB (human oral cavity cancer), MCF-7 (human breast cancer, ATCC HTC-22) and NCI-H187 cell lines (human small-cell lung cancer, ATCC CRL-5804). The cytotoxicity tests were performed by using the resazurin microplate assay (REMA) (O'Brien *et al*., 2000). The inhibition concentration (IC_{50}) indicates the concentration of the test compound that causes 50% reduction of the tested cell-lines. Ellipticine and doxorubicin were used as positive controls for anti-KB and anti-NCI-H187 activities. Tamoxifen and doxorubicin were used as positive controls for anti-MCF-7 activity. The 0.5% (v/v) DMSO was used as a negative control for all tests.

3.5.5 Cytotoxicity against Vero cells

The cytotoxicity of the isolated compounds against the Vero cells (African green monkey kidney fibroblasts; ATCC CCL-81) was determined by the green fluorescent protein microplate technique (GFPMA) (Changsen *et al*., 2003). The inhibition concentration (IC_{50}) exhibits the concentration of the compound which causes 50% reduction of the Vero cells. Ellipticin and 0.5% (v/v) DMSO were used as the positive and negative control, respectively.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Marine sample collection and Isolation of marine actinomycetes

Total, 75 actinomycetes were isolated from marine samples collected from 7 provinces of Thailand by using M1, M2 and sea water proline agar. 40 isolates were isolated from the samples collected from Thai Gulf and 35 isolates were isolated from the samples collected from Andaman Sea (Table 4.1).

4.2 Identification of marine actinomycetes

All actinomycete isolates were preliminary classified using the 16S rRNA gene sequence analysis, chemotaxonomic and morphological characteristics. On the basis of these results, they were classified in 3 families (*Streptomycetaceae*, *Micromonosporaceae* and *Nocardiaceae*) including 6 genera *Jishengella* (1 isolate), *Nocardia* (2 isolates), *Micromonospora* (25 isolates), *Salinispora* (13 isolates), *Streptomyces* (32 isolates) and *Verrucosispora* (2 isolates) (Figure 4.1). The type strains which showed the highest 16S rRNA gene sequence similarity of each isolate are shown in Table 4.2

Table 4.1 Location of marine samples and code of the actinomycete isolates

Location	Province	Area	Source	Depth (m)	Isolation code	No. of isolate
Bomao bay	Chumphon	Thai Gulf	Sediment	0	BM1-1, BM1-4, BM2-1, BM2-4, BM2-6	5
Chumphon beach			Sediment	0	CPB1-1, CPB1-11, CPB1-12, CPB1-13, CPB1-14, CPB1-18, CPB1-21, CPB1-3, CPB2-10, CPB3-1, CPB4-7	11
Koh Khai			Sediment	7-8	KK1-10, KK1-17, KK1-2, KK2-1, KK4-14, KK4-8, KK5-10	7
The nature education center for mangrove conservation and ecotourism	Chonburi		Mangrove sediment, Mud	0-1	CH3-1, CH3-2, CH3-3, CH3-9, CH3-14, CH4-1, CH7-4m, CH7-4S	8
Bangsaen beach			Sand	2-3	BS-002, BS-003, BS-007	3
Koh Talu	Prachuap Khiri Khan		Sediment	8-10	KT2-1, KT2-3	2
Mangrove forest	Samut Songkhram		Mangrove sediment, Mud	0	A2-1, D2-1, D2-2, C10-9-1	4
Phanwa beach	Phuket	Andaman Sea	Sand	0	PWB-002, PWB-003, PWB-005, PWB-010, PWB-011, PWB-012, PWB-016, PWB-020	8
Laem phanwa			Sediment	10	PW-002, PW-004, PW-006, PW-007	4
Kho Rok Nork	Trang		Sponge	6-10	SRK1-1, SRK1-2, SRK1-3, SRK2-1, SRK2-3	5
Koh Rok Nai			Sediment Sponge	8-10 6-10	KRN1-1, KRN2-1 SRN1-1, SRN1-2	4
Koh Mah			Sponge	6-10	SPM3-5, SPM3-1, SPM3-3, SPM3-6, SPM3-7, SPM3-8, SPM9-1, SPM9-2	8
Mangrove forest	Krabi		Mangrove sediment, Mud	0	LKB1-1, LKB1-14, LKB1-4, LKB1-5, LKB1-6, LKB1-7	6
Total						**75**

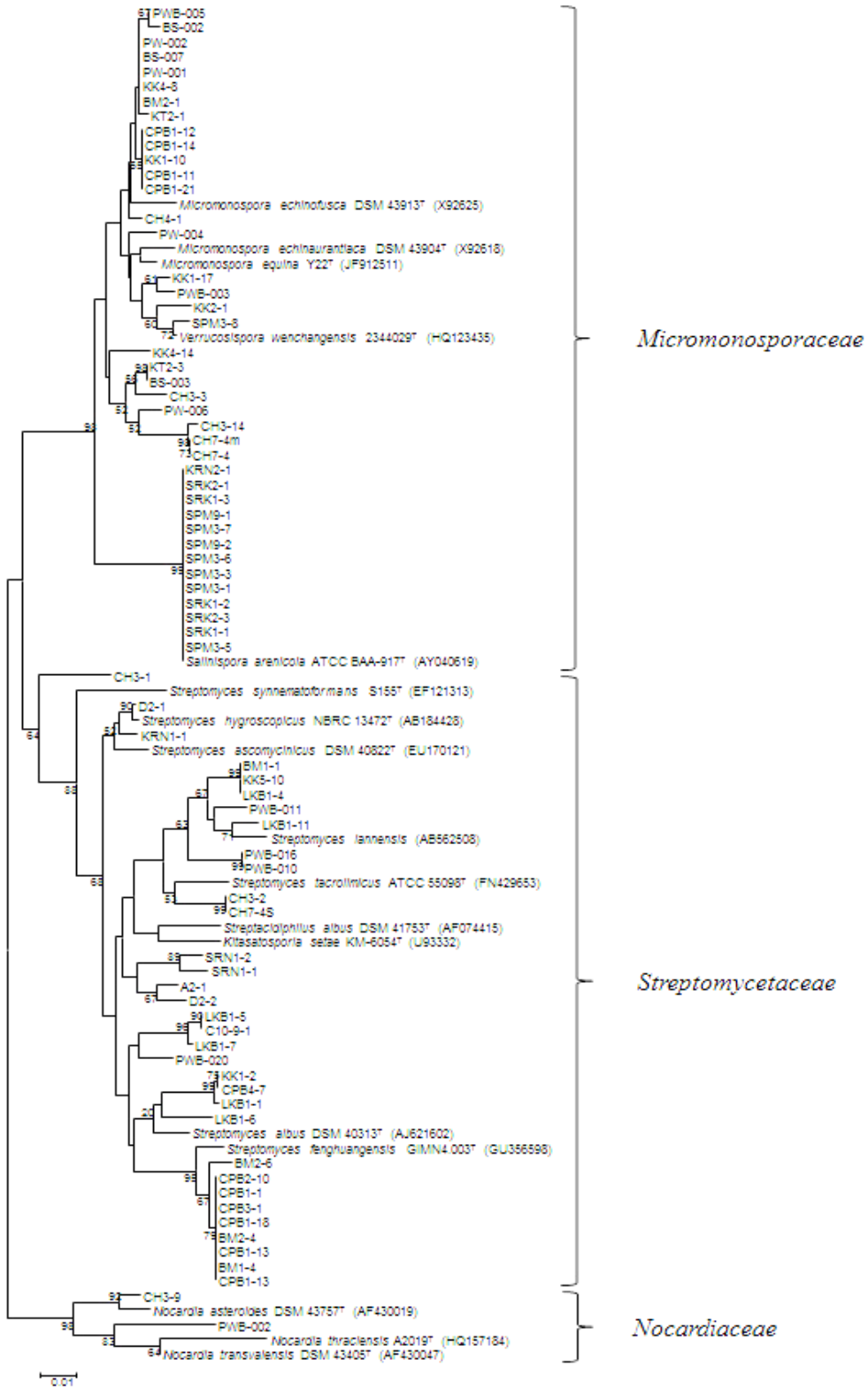

Figure 4.1 Phylogenetic relationship based on neighbor-joining analysis of the 16S rRNA gene sequences of the 75 actinomycete isolates

Table 4.2 16S rRNA gene sequence similarity of the actinomycete isolates

Isolate code.	Closely related species	Similarity (%)	Length of nucleotide sequence	Accession No.
KK1-17	*Jishengella endophytica* 202201^T	99.65	1445	LC158546
BM2-1		99.13	1400	LC158511
BS-002		99.82	1321	LC158515
BS-007		100	600	LC158517
KK4-8	*Micromonospora aurantiaca* ATCC 27029^T	99.86	1412	LC158512
KT2-1		99.67	1364	LC158513
PW-001		100	560	LC158516
PW-002		99.66	580	LC158518
PWB-005		99.50	1315	LC158514
BS-003		99.81	530	LC158520
CH7-4m	*Micromonospora chalcea* DSM 43026^T	99.46	559	LC158521
CH3-14		99.71	1363	LC158522
KT2-3		99.90	1444	LC158519
PWB-003*	*Micromonospora eburnea* LK2-10^T	99.38	1462	LC033898
KK4-14	*Micromonospora humi* P0402^T	99.57	1389	LC158523
CPB1-3		100	1408	LC158525
CPB1-12		100	1355	LC158526
CPB1-14	*Micromonospora marina* JSM 1-1^T	100	1411	LC158527
CPB1-11		100	549	LC158529
CPB1-21		100	600	LC158530
KK1-10		100	579	LC158528
CH4-1	*Micromonospora maritima* D10-9-5^T	100	1379	LC158524
PW-004	*Micromonospora olivasterospora* DSM 43868^T	98.30	599	LC158531
CH3-3*	*Micromonospora palomenae* NEAU-CX1^T	98.97	1469	AB889541
PW-006		100	569	LC158532
PWB-012	*Micromonospora tulbaghiae* TVU1^T	99.82	569	LC158510
CH3-9	*Nocardia higoensis* NBRC 100133^T	99.47	1322	LC158549
PWB-002	*Nocardia testacea* NBRC 100365^T	99.21	509	LC158550
KRN2-1		100	650	LC158533
SRK1-2		100	609	LC158542
SRK1-3		100	640	LC158535
SRK2-1		100	660	LC158534
SRK2-3	*Salinispora arenicola* CNH-643^T	100	600	LC158543
SPM3-1		100	630	LC158541
SPM3-3		100	609	LC158540
SPM3-6		100	649	LC158539
SPM3-7		100	639	LC158537
SPM9-1		100	679	LC158536
SPM9-2		100	650	LC158538

Asterisk (*) was the selected candidate of novel actinomycete species

Table 4.2 (Continued)

Isolate no.	Closely related species	Similarity (%)	Length of nucleotide sequence	Accession No.
SRK1-1	*Salinispora arenicola* CNH-643^T	100	550	LC158544
SPM 3-5		100	640	LC158545
BM2-6	*Streptomyces barkulensis* RC1831^T	98.86	1475	LC158559
LKB1-4	*Streptomyces coelicoflavus* NBRC 15399^T	99.57	470	LC158552
KK5-10		99.46	589	LC158554
BM1-1	*Streptomyces diastaticus* subsp. *ardeciacus* NRRL-B 1773^T	99.63	1369	LC158553
SRN1-2	*Streptomyces gulbargensis* DAS131^T	98.25	630	LC158551
BM1-4*		98.57	1411	LC069043
CPB1-1*		98.63	1403	LC069041
CPB1-18*	*Streptomyces fenghuangensis* GIMN 4.003^T	98.74	1448	LC069045
CPB2-10*		98.57	1398	LC069044
CPB3-1*		98.67	1452	LC069042
SRN1-1	*Streptomyces gulbargensis* DAS131^T	98.25	630	LC158563
CH7-4S	*Streptomyces hydrogenans* NBRC 13475^T	99.93	1436	LC158571
D2-1	*Streptomyces iranensi* HM 35^T	99.57	1388	LC158573
CH3-1	*Streptomyces mayteni* YIM 60475^T	99.35	1413	LC158557
PWB-011	*Streptomyces olivaceoviridis* NBRC 13066^T	99.02	410	LC158558
KRN1-1	*Streptomyces platensis* JCM 4662^T	99.18	610	LC158556
BM2-4	*Streptomyces radiopugnans* R97^T	99.07	535	LC158560
CPB1-13		99.17	600	LC158561
LKB1-6	*Streptomyces rimosus* subsp. *paromomycinus* DSM 41429^T	97.68	560	LC158562
A2-1	*Streptomyces sanglieri* NBRC 100784^T	99.35	1394	LC158572
C10-9-1		99.93	1487	LC158575
LKB1-5	*Streptomyces sanyensis* 219820^T	99.84	619	LC158564
LKB1-7		99.80	509	LC158565
PWB-016	*Streptomyces somaliensis* DSM 40738^T	98.64	589	LC158567
PWB-010		98.15	597	LC158566
D2-2	*Streptomyces sundarbansensis* MS1/7^T	99.78	1365	LC158574
LKB1-11	*Streptomyces tritolerans* DAS 165^T	99.84	630	LC158568
KK1-2*		98.36	1475	AB738400
CPB4-7*	*Streptomyces xianghaiensis* S187^T	98.34	1391	AB738401
LKB1-1		98.21	1286	LC158555
CH3-2	*Streptomyces violascens* ISP 5183^T	99.86	1424	LC158569
PWB-020	*Streptomyces wuyuanensis* FX61^T	99.43	1478	LC158570
KK2-1	*Verrucosispora gifhornensis* DSM 44337^T	99.68	630	LC158547
SPM3-8	*Verrucosispora sediminis* MS426^T	99.51	609	LC158548

Asterisk (*) was the selected candidate of novel actinomycete species

4.2.1 Family *Streptomycetaceae*

Thirty two isolates including A2-1, BM1-1, BM1-4, BM2-4, BM2-6, C10-9-1, CH3-1, CH3-2, CH7-4S, CPB1-1, CPB1-13, CPB1-18, CPB2-10, CPB3-1, CPB4-7, D2-1, D2-2, KK1-2, KK5-10, KRN1-1, LKB1-1, LKB1-4, LKB1-5, LKB1-6, LKB1-7, LKB1-11, PWB-010, PWB-011, PWB-016, PWB-020, SRN1-1 and SRN1-2 were identified as *Streptomyces* which belonged to the family *Streptomycetaceae*. They produced extensively branch aerial and substrate mycelia. Almost all isolates produced spiral spore chains while long straight spore chains were occasionally observed (Figure 4.2). The chemotaxonomic analysis revealed that all of these isolates contained LL-diaminopimelic acids and glucose and ribose (no diagnostic sugars) in their whole-cell hydrolysates. The *N*-acyl type of muramic acid was acetyl type. The mycolic acids were absent. In addition, the 16S rRNA gene sequence analysis (ranged from 98.2 to 99.9 % similarity) and phylogenetic tree analysis represented that these isolates shared the clade within the genus *Streptomyces* (Figure 4.3).

Among them, 12 isolates including BM1-4, BM2-6, BM2-4, CH3-1, CPB1-1, CPB1-13, CPB1-18, CPB2-10, CPB3-1, CPB4-7, KK1-2 and LKB1-6 required sea water for their growth. The salt requirement of these isolates may represent a good adaptation of the marine isolates to their ecosystems.

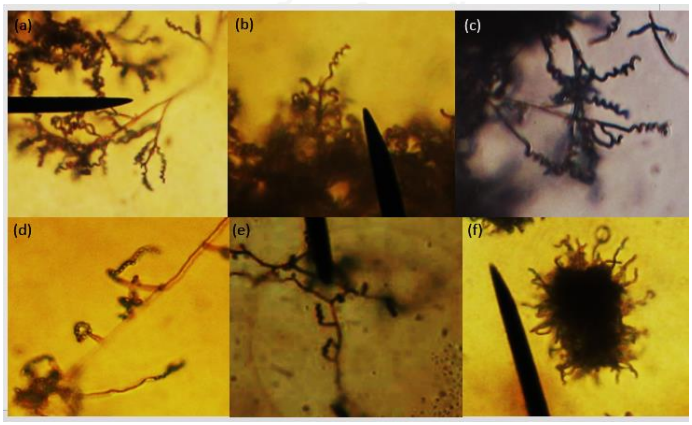

Figure 4.2 Light micrograph showing the spore morphology of the representative *Streptomyces* isolates after incubation on ISP2 agar at 30 °C for 14 days [(a) to (f) are the isolates BM1-1, CH3-1, KK5-10, CPB3-1, BM2-6 and CH3-2, respectively]. (a) – (e) represented the spiral spore chains (Spiral type) while (f) represented the straight spore chains (*Rectiflexibiles* type). Magnification, x 400.

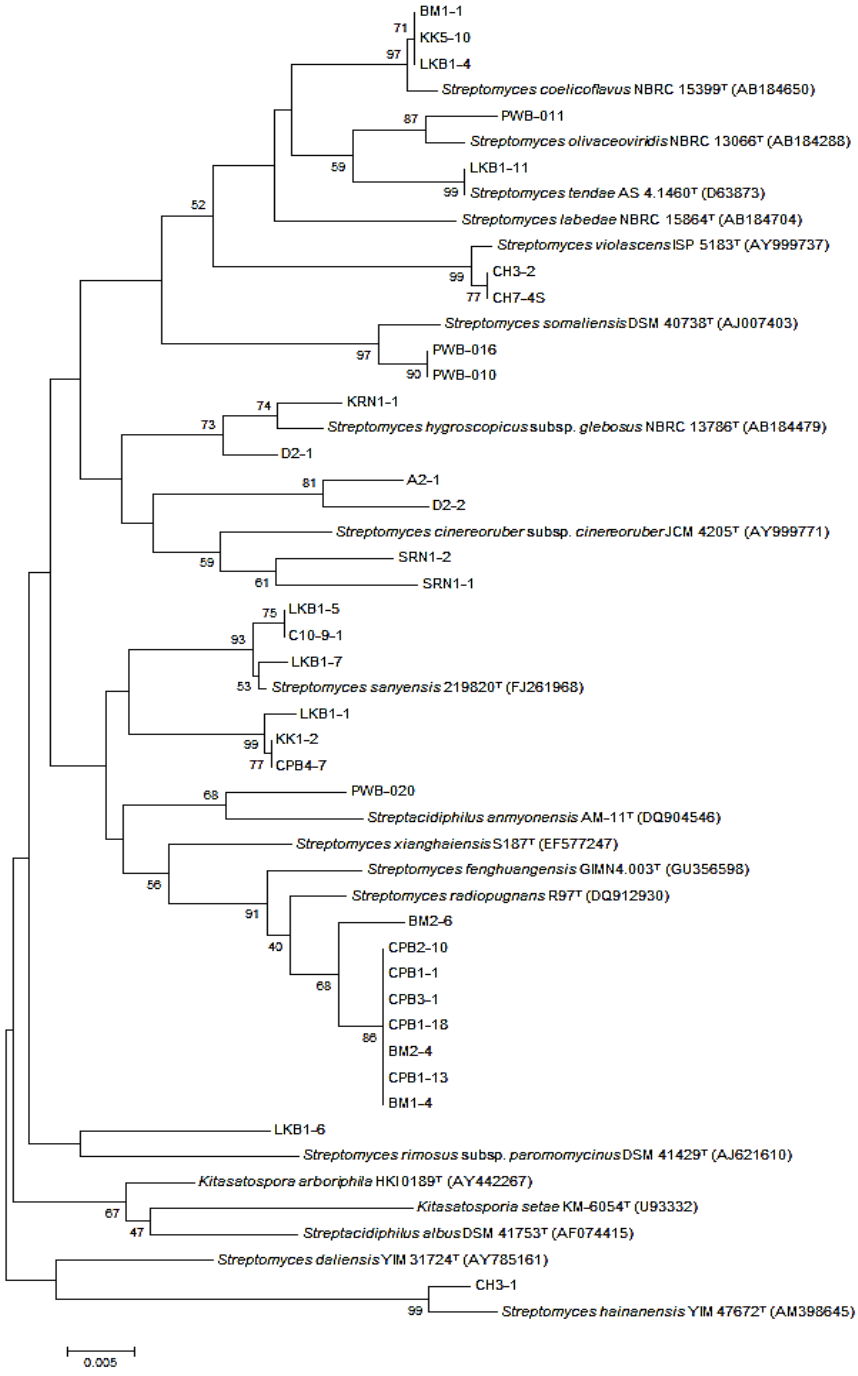

Figure 4.3 Phylogenetic relationship based on neightbour-joining analysis of the 16S rRNA gene sequences of *Streptomyces* isolates and some type strains of the genus *Streptomyces*, *Kitasatospora* and *Streptacidiphilus*. Numbers at branch nodes indicate bootstrap percentage obtained from 1,000 replications (only values > 50 are shown). Bar, 0.005 substitutions per nucleotide position.

4.2.2 Family *Micromonosporaceae*

The members of this group exhibited a monomeric spore on the substrate mycelia but lacked of aerial mycelia. The mycolic acids were absent. They were divided into four genera based on the key morphological and chemotaxonomic characteristics and16S rRNA gene sequence analysis including phylogenetic tree relationship.

Genus *Micromonospora* consisted of 25 isolates (BM2-1, BS-002, BS-003, BS-007, CH3-14, CH3-3, CH4-1, CH7-4m, CPB1-11, CPB1-12, CPB1-14, CPB1-21, CPB1-3, KK1-10, KK4-14, KK4-8, KT2-1, KT2-3, PW-001, PW-002, PW-004, PW-006, PWB-003, PWB-005 and PWB-012). Almost all isolates showed orange to brown substrate mycelia which changed in to dark brown or black color when cultured more than 10 days (Figure 4.4). Based on BLASTn and phylogenetic analyses, these strains showed the highest 16S rRNA gene sequence similarities with type strains of the members of the genus *Micromonospora* and formed clade with those validly published *Micromonospora* species in the phylogenetic trees (Figure 4.5). These 25 isolates were classified as *Micromonospora*.

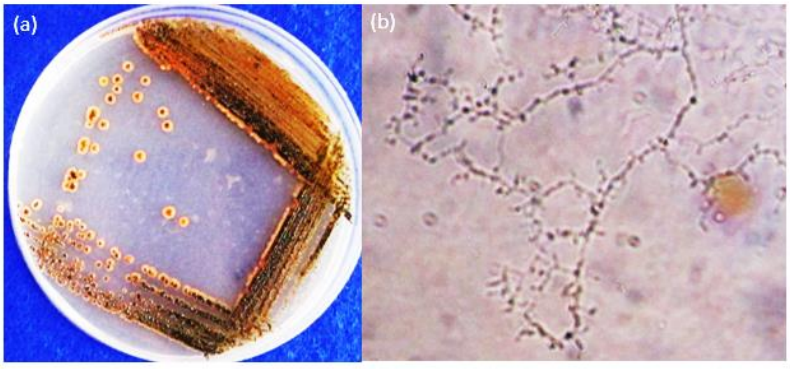

Figure 4.4 The colonial appearance (a) and light micrograph (b) (magnification, x 400) of the representative *Micromonospora* sp. KK4-8 showing the monomeric spore on substrate mycelia and the absence of aerial mycelia after the culture grown on ISP2 agar at 30 °C for 14 days.

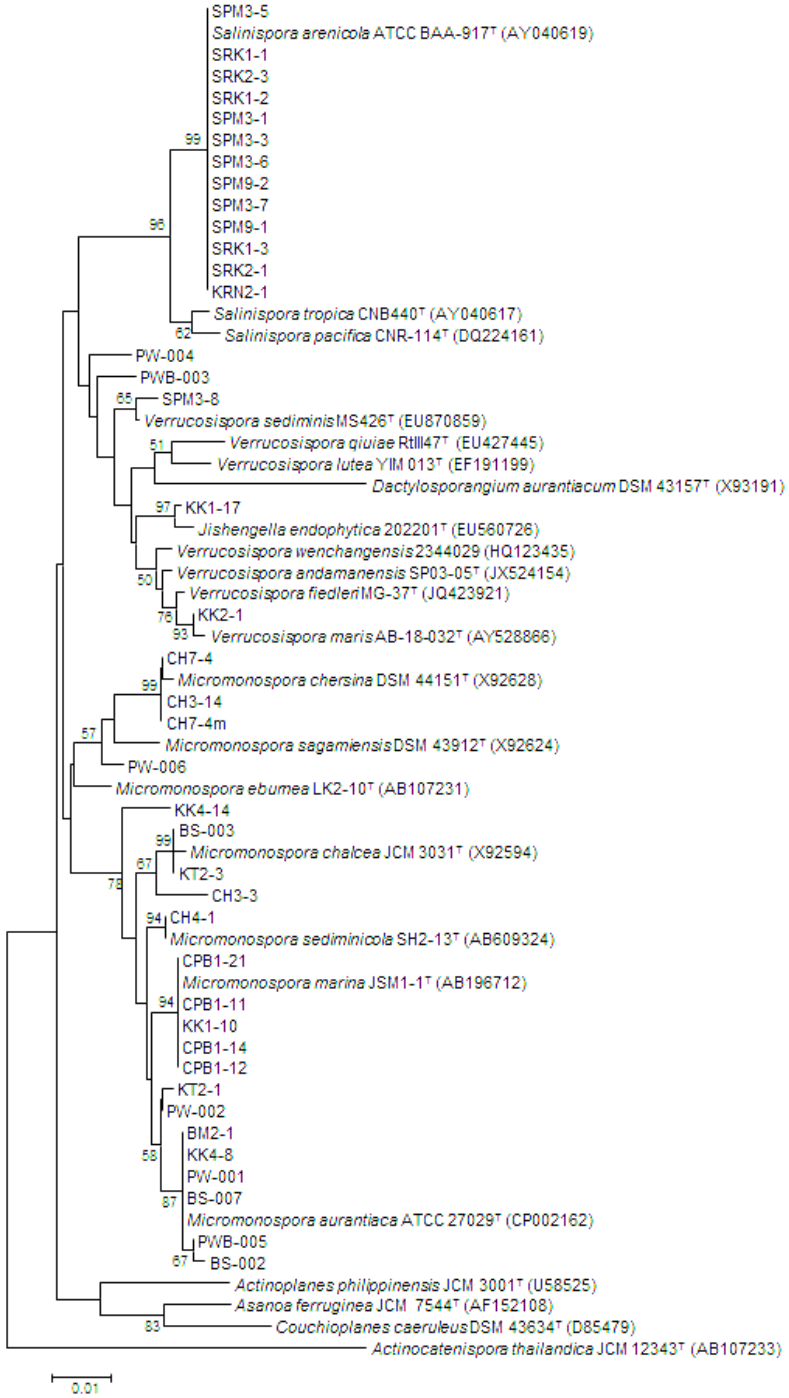

Figure 4.5 Phylogenetic relationship based on neightbour-joining analysis of the 16S rRNA gene sequences of the 40 actinomycete isolates which classified in the family *Micromonosporaceae*. Numbers at branch nodes indicate bootstrap percentage obtained from 1,000 replications (only values > 50 are shown). Bar, 0.01 substitutions per nucleotide position.

Genus *Salinispora* comprised 13 isolates (KRN2-1, SPM3-5, SPM3-1, SPM3-3, SPM3-6, SPM3-7, SPM9-1, SPM9-2, SRK1-1, SRK1-2, SRK1-3, SRK2-1 and SRK2-3). All strains required sea water for growth. The 16S rRNA gene analysis revealed that these isolates showed the highest similarity with *Salinispora arenicola* CNH-643^T (100% similarity). This was confirmed by the phylogenetic analysis that these isolates form the same clade with *S. arenicola* (Figure 4.5). They were classified as *Salinispora*.

Genus *Verrucosispora* comprised 2 isolates including KK2-1 and SPM3-8. The representative isolate KK2-1 produced hairly spores (Figure 4.6) which born singly on the substrate mycelia. It contained *meso*-diaminopimelic acids, xylose, mannose, and small amount of ribose in whole-cell hydrolysate. Its phospholipid profiles were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol mannosides and phosphatidylinositol (type PII). BLASTn analysis of 16S rRNA gene sequences revealed that the isolates KK2-1 and SPM3-8 were the closest similar to *Verrucosispora gifhornensis* DSM 44337^T (99.7%) and *Verrucosispora sediminis* MS426^T (99.5%), respectively. On the basis of 16S rRNA gene sequence and phylogenetic tree analysis (Figure 4.5), they were classified as *Verrucosispora*.

Genus *Jishengella* comprised one isolate, KK1-17. It produced nodular warty spores on the substrate mycelia (Figure 4.7). Cell wall peptidoglycan contained *meso*-diaminopimelic acids. Arabinose, glucose, mannose, ribose and xylose were detected in whole-cell hydrolysate. The polar lipid profiles were diphosphatidyl glycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositolmannosides, one unidentified aminolipid, two unidentified phospholipids and six unidentified lipids (type PII). The isolate KK1-17 exhibited the highest 16S rRNA gene sequence similarity to *Jishengella endophytica* 202201^T (99.65%). In addition, phylogenetic analysis revealed that isolate KK1-17 shared the same node with *Jishengella endophytica* 202201^T (Figure 4.5). Therefore, isolate KK1-17 was classified as *Jishengella*.

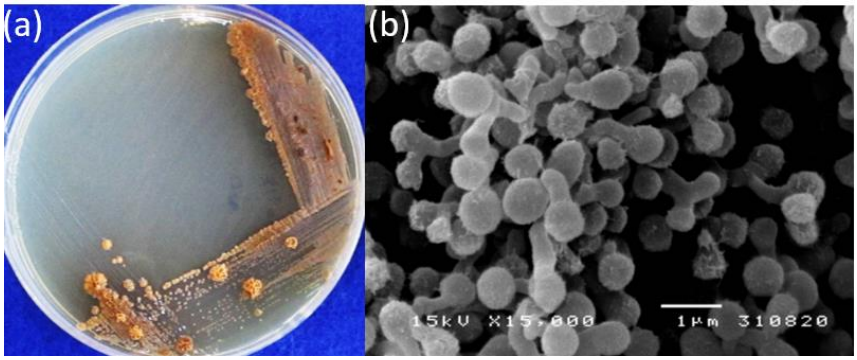

Figure 4.6 Colonial appearance (a) and scanning electron micrograph (b) of *Verrucosispora* sp. KK2-1 after the culture grown on the ISP2 medium supplemented with artificial sea water at 30 °C for 14 days representing the brown colony on agar medium and hairy spores on the substrate mycelia.

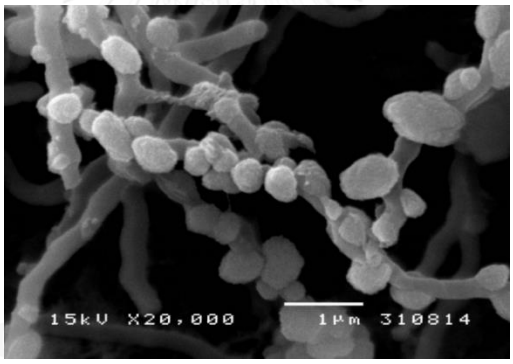

Figure 4.7 Electron micrograph showed the nodular spore on the substrate mycelia of *Jishengella* sp. KK1-17 after the culture grown on ISP2 agar at 30 °C for 14 days.

4.2.3 Family *Nocardiaceae*

The members of this group consisted of two isolates including CH3-9 and PWB-002. Both of them showed the fragmentation on the substrate mycelia (Figure 4.8). The representative isolate, CH3-9 contained *meso*-diaminopimelic acid in cell-wall peptidoglycan. Its polar lipid profiles were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositolmannosides and two unidentified phosphoglycolipids (type PIV). The mycolic acids were observed in whole-cell extract (R_f value approximately 0.47) which co-migration with the extract obtained from the reference strain, *Nocardia nova* JCM 6044^T. The 16S rRNA gene sequence analysis revealed that the isolates CH3-9 and PWB-002 showed the highest similarities to *Nocardia higoensis* NBRC 100133^T (99.47%) and *Nocardia testacea* NBRC 100365^T (99.21%), respectively. Furthermore, both isolates showed the phylogenetic relationship within the genus *Nocardia* (Figure 4.9). Based on the fragmentation of substrate mycelia, the presence of mycolic acids and 16S rRNA gene sequence analysis, these isolates were identified as *Nocardia*.

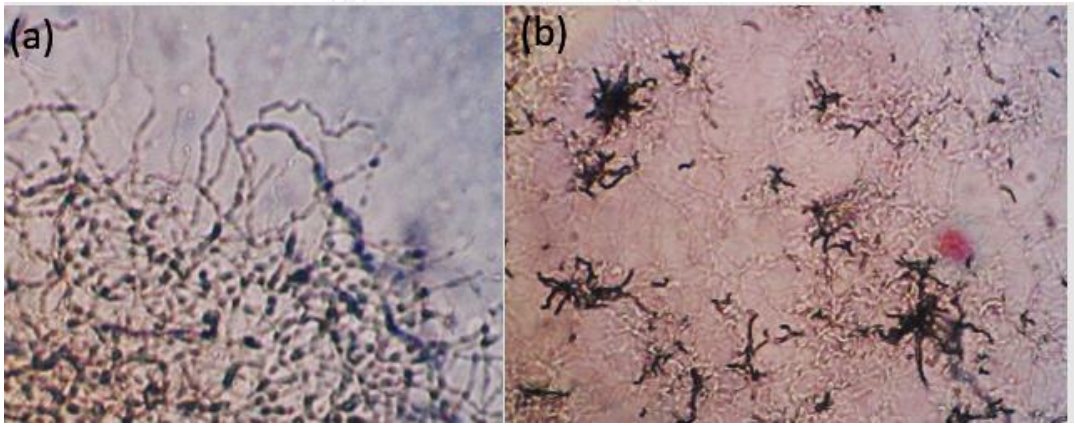

Figure 4.8 Light micrograph showed the fragmentation of the substrate mycelia of the isolates CH3-9 and PWB-002 after the cultures grown on ISP2 agar at 30 °C for 14 days. Magnification, x 400

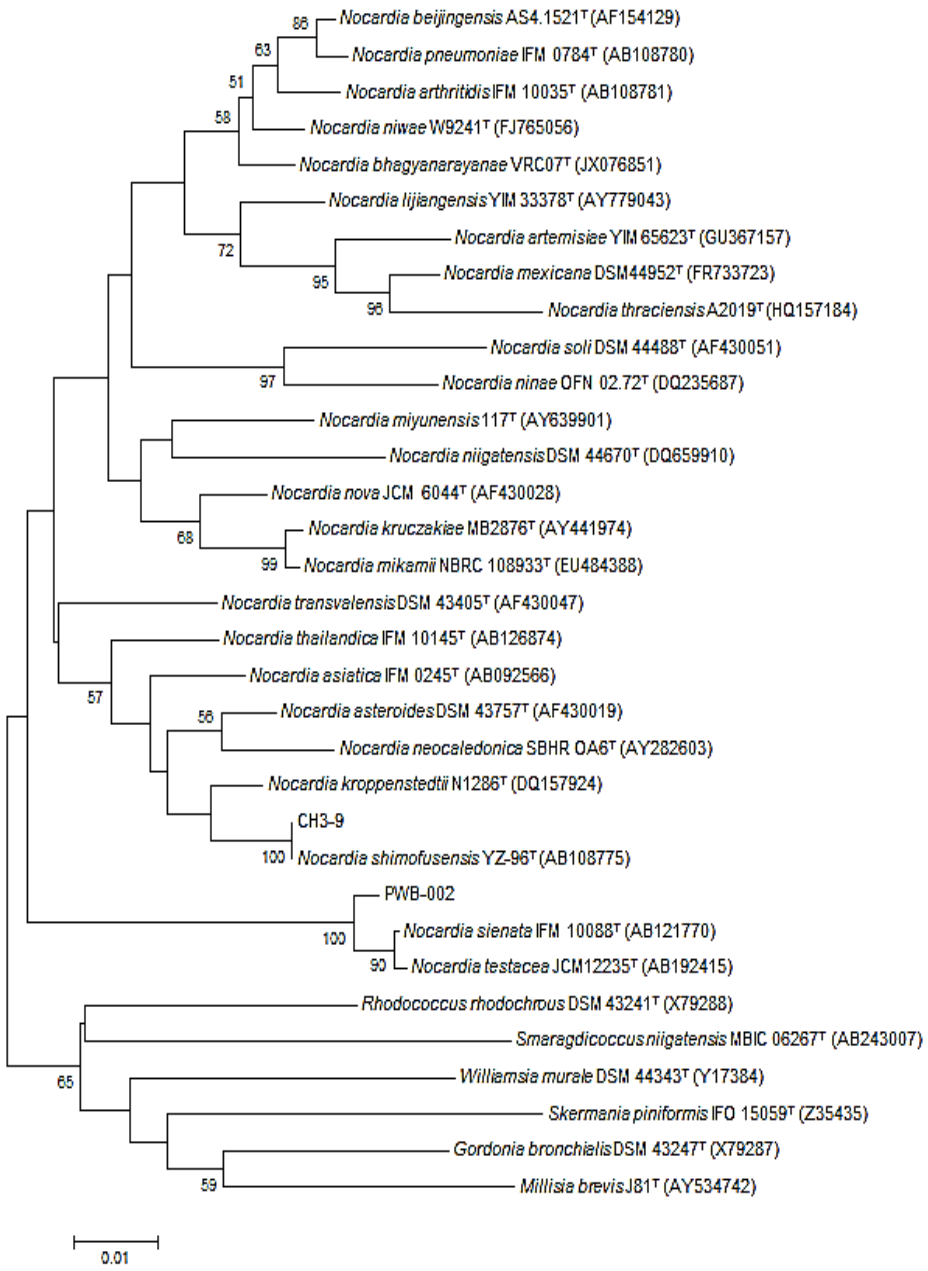

Figure 4.9 Neightbour-joining analysis based on the 16S rRNA gene sequences of isolate CH3-9, PWB-002 and members of the genus *Nocardia* plus type species of the family *Nocardiaceae*. Numbers at branch nodes indicate bootstrap percentage obtained from 1,000 replications (only values > 50 are shown). Bar, 0.01 substitutions per nucleotide position.

4.3 Taxonomic studies of novel marine actinomycetes species

In this study, 8 isolates (KK1-2, CPB4-7, CPB1-1, CPB2-10, CPB3-1, BM1-4, CPB1-18 and CH3-3) which showed 16S rRNA gene sequence similarities lower than 99% in BLASTn analysis and one isolate (PWB-003), which exhibited the unique phospholipid profiles, were selected for a polyphasic study.

4.3.1 Characterization of *Streptomyces chumphonensis* strains KK1-2^T and CPB4-7

The morphological observation found that strains KK1-2^T and CPB4-7 produced long straight chain of rough short-rod spores. Spores were 0.5 x 0.8-1.0 μM in size. White aerial masses and light- yellow to deep yellow substrate mycelia were observed on all ISP agar media (Figure 4.10). Both strains produced grayish to greenish yellow and light olive-brown pigments on ISP2 and nutrient agar media, respectively (Table 4.3). Phenotypic characteristics were positive for nitrate reduction, while weakly positive for skim milk peptonization, but negative for coagulation of skim milk and starch hydrolysis. However, the liquefaction of gelatin varied between isolates. Both strains utilized D-glucose, D-xylose, melezitose, sorbitol, D-mannitol, *myo*-inositol and L-rhamnose as carbon sources. According to API ZYM system, both strains showed positive activities of alkaline phosphatase and α-glucosidase, while esterase (C4), esterase lipase (C8), lipase (C14), valine arylmidase, cysteine arylamidase and acid phosphatse were weakly positive but the enzyme activities of trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were negative (Table 4.4).

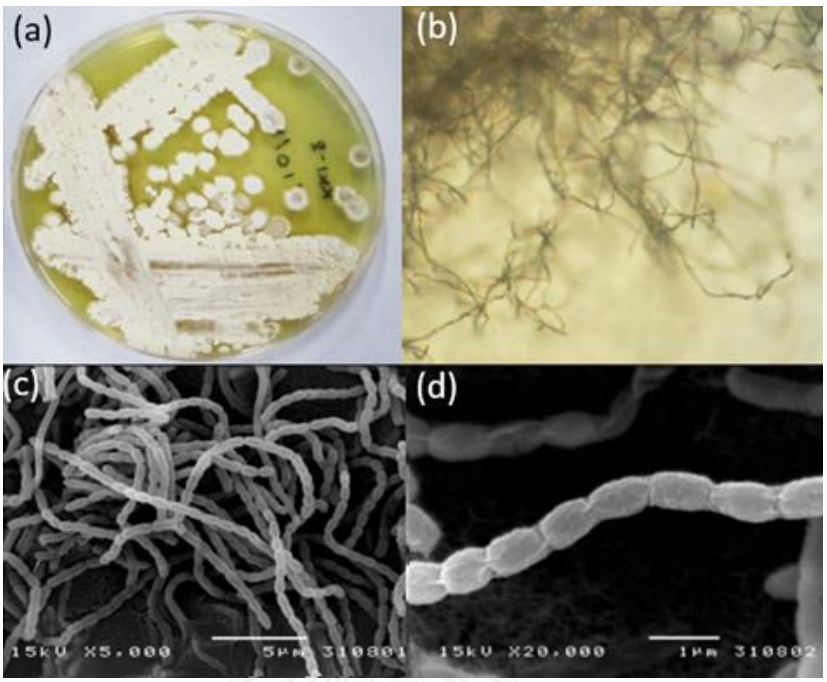

Figure 4.10 The colonial appearance (a) and light micrograph (b) (magnification, x 400) of *Streptomyces chumphonensis* KK1-2^{T} after the culture grown on ISP2 agar at 30 °C for 14 days. The scanning electron micrograph showed the long chain of spores (c) and the slightly rough surface of spores (d).

Both isolates contained LL-diaminopimelic acid, glucose and ribose in their whole-cell hydrolysate. The acyl type of muramic acids is aetyl. The polar lipid profiles were phosphatidyl ethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglygerol (PG), phosphatidylinositol mannosides (PIMs). However, the unidentified aminolipids, glycolipids, ninhydrin-positive glycolipid and unidentified lipids were also observed. The presence of phosphatidylethanolamine could classify this polar lipids pattern in group PII (Lethecalier et al., 1977). The isoprenoid quinones found in the cells of these isolates (KK1-2 and CPB4-7) were MK-9(H6) and MK-9(H8). The major cellular fatty acids were anteiso-C15:0 (19.7-22.0%), iso-C16:0 (19.9-22.7%) and iso-C15:0 (14.2-19.0%) (Table 4.5). The genomic DNA G+C content of isolate KK1-2 and CPB4-7 were 73.3 and 74.2 mol%, respectively.

Almost complete 16S rRNA gene sequences of the strains KK1-2 (1475 nt) and CPB4-7 (1391 nt) showed the highest similarity to *S. xinghaiensis* JCM 16958^T (98.2%), *S. rimosus* subsp. *paromomycinus* JCM 4541^T (98.1%), *S. sclerotialus* JCM 4828^T (98.1%) and *S. flocculus* JCM 4476^T (98.0%). The phylogenetic analysis of the 16S rRNA gene of these both strains was shown in Figure 4.11. The DNA-DNA hybridization study revealed that strain KK1-2^T showed low levels of DNA-DNA relatedness to *S. xinghaiensis* JCM 16958^T (58.3±9.6%), *S. rimosus* subsp. *paromomycinus* JCM 4541^T (33.3 ± 1.4%), *S. sclerotialus* JCM 4828^T (48.7 ± 1.9%) and *S. flocculus* JCM 4476^T (55.6 ± 5.7%). These values were lower than 70%, the cutoff level for assigning strains to the same species (Wayne *et al*., 1987) and indicated that the strains KK1-2^T and CPB4-7 are the member of a novel species. In addition, strains KK1-2^T and CPB4-7 exhibited DNA-DNA relatedness values of 100 ± 0.5% each other. This indicated that both strains were the same species.

On the basis of phenotypic and chemotaxonomic and genotypic data, strains KK1-2^T and CPB4-7 are distinguished from previously described *Streptomyces* species. Therefore, these two isolates should be classified as a novel species of the genus *Streptomyces* for which the name *Streptomyces chumphonensis* sp. nov. is proposed

The etymology of the *Streptomyces chumphonensis* is chum.phon.en´sis. N.L. masc. adj, chumphonensis pertaining to Chumphon Province in the southern part of Thailand, where the type strain was isolated.

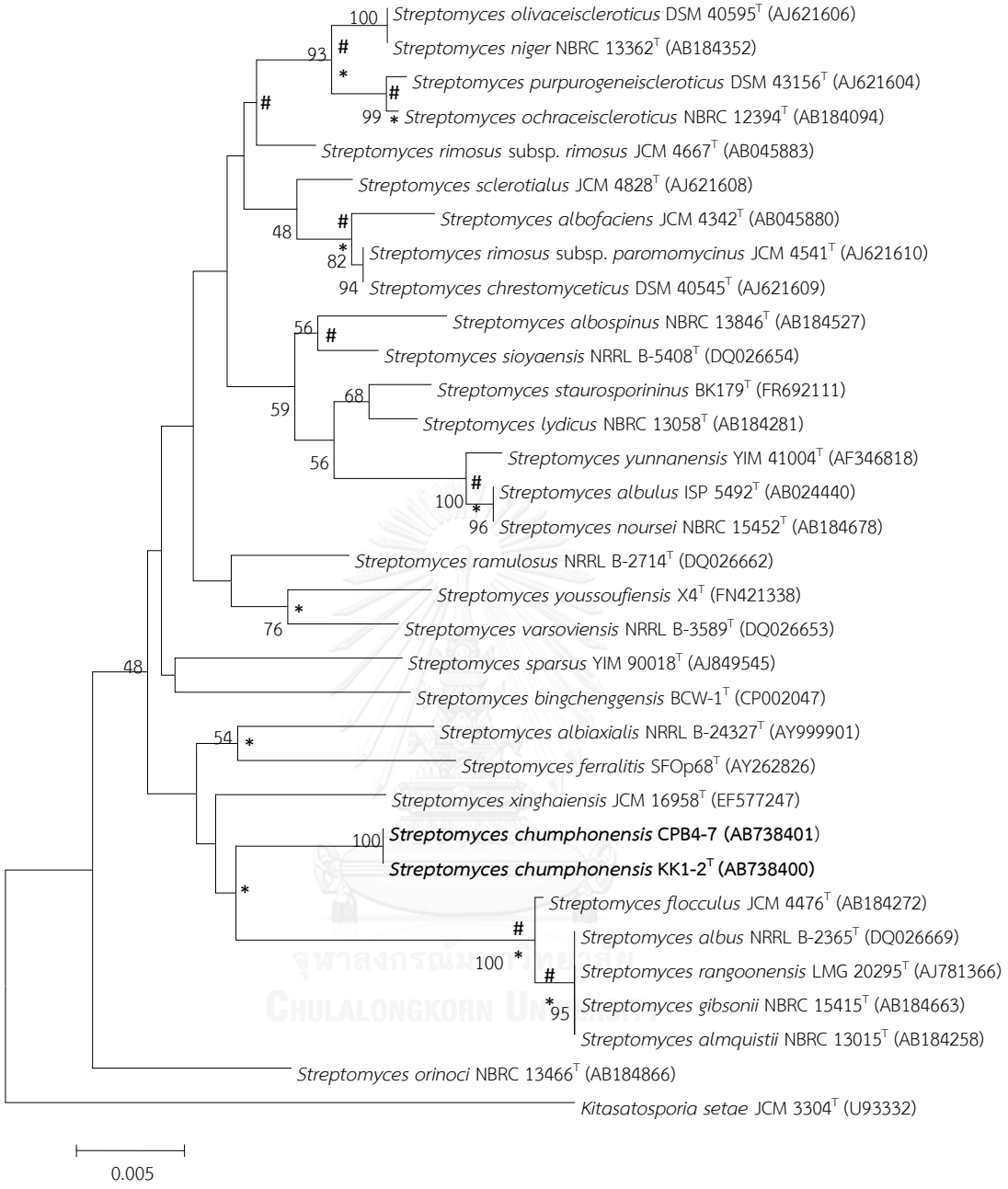

Figure 4.11 Phylogenetic relationships based on neighbor-joining analysis of 16S rRNA gene sequences of *Streptomyces chumphonensis* strains KK1-2^T and CPB4-7 and related *Streptomyces* species. *Kitasatospora satae* JCM 3304^T was used as an out group. Asterisk (*, #) indicated the branches were recovered in the maximum-likelihood and maximum-parsimony tree respectively. The number at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown) Bar, 0.005 substitutions per nucleotide position.

Table 4.3 Cultural characteristics of *Streptomyces chumphonensis* strains KK1-2^T and CPB4-7 incubated at 30 ^oC for 14 days

Medium	KK1-2^T			CPB4-7		
	Growth	**Color of colony**	**Soluble pigment**	**Growth**	**Color of colony**	**Soluble pigment**
Yeast extract-malt extract agar (ISP2)	Good	Yellowish white	Grayish greenish yellow	Good	Yellowish white	Grayish greenish yellow
Oat meal agar (ISP3)	Good	Yellowish white	-	Good	Yellowish white	-
Inorganic salt-starch agar (ISP4)	Good	White	-	Good	White	-
Glycerol-asparagines agar (ISP5)	Good	Yellowish white	-	Good	Yellowish white	-
Peptone-yeast extract iron agar (ISP6)	Good	Yellowish white	-	Good	Yellowish white	-
Tyrosine agar (ISP7)	Good	white	-	Good	White	-
Nutrient agar	Good	Yellowish white	Light olive brown	Good	Yellowish white	Light olive brown

Table 4.4 Differential characteristics between *Streptomyces chumphonensis* strains KK1-2^T, CPB4-7 and closely related *Streptomyces* species

Strains: 1, KK1-2^T; 2, CPB4-7; 3, *S. xinghaiensis* JCM 16958^T; 4, *S. flocculus* JCM 4476^T; 5, *S. rimosus* subsp. *paromomycinus* JCM 4541^T; 6, *S. sclerotialus* JCM 4828^T

Characteristics	1	2	3	4	5	6
Gelatin liquefaction	±	-	-	+	+	-
Starch hydrolysis	+	+	-	-	+	+
Nitrate reduction	+	+	+	+	+	-
Skimmed milk peptonization	±	±	-	+	+	-
Skimmed milk coagulation	-	-	-	+	+	-
Growth at 45 °C	-	-	+	+	-	+
Utilization of:						
L-Arabinose	-	-	+	-	-	+
Fructose	-	-	+	±	+	+
myo-Inositol	-	-	+	-	+	+
D-Manitol	-	-	+	+	+	+
Melibiose	-	-	-	-	±	+
D-Melezitose	-	-	+	-	-	+
Raffinose	-	-	+	-	±	+
L-Rhamnose	-	-	-	-	-	+
Sorbitol	-	-	-	-	+	+
Sucrose	-	-	+	+	-	+
D-Xylose	-	-	+	+	-	+
Enzyme activity of:						
Esterase (C4)	±	±	±	±	±	-
Esterase lipase (C8)	±	±	±	±	±	-
Lipase (C14)	±	±	-	+	±	-
Valine allylamidase	±	±	-	+	-	+
Cystine allylamidase	±	±	-	±	±	±
Acid phosphatase	±	±	-	+	+	+
Napthol-AS-BI-phospohydrase	-	-	-	+	+	+
β-Galactosidase	-	-	-	-	±	+
α-Glucosidase	+	+	±	-	+	+
β-Glucosidase	-	-	-	-	+	+
N-acetyl-β-glucosaminidase	-	-	+	+	+	-
α-Mannosidase	-	-	-	+	±	-

Table 4.5 Cellular fatty acid compositions (%) of *Streptomyces chumphonensis* KK1-2^T and closely related type strains

Strains: 1, KK1-2^T; 2, CPB4-7; 3, *S. xinghaiensis* JCM 16958 ^{T}; 4, *S. flocculus* JCM 4476 ^{T}; 5, *S. rimosus* subsp. *paromomycinus* JCM 4541^T; 6, *S. sclerotialus* JCM 4828^T

Fatty acid	1	2	3	4	5	6
Saturated fatty acids						
$C_{14:0}$	0.6	0.7	0.6	0.6	1.4	0.5
$C_{16:0}$	8.1	7.4	12.3	12.3	10.5	5.5
$C_{17:0}$ cyclic	-	-	0.5	9.1	1.8	1.1
$C_{17:0}$	0.8	1.1	2.5	0.2	0.8	0.7
$C_{18:0}$	0.3	0.9	2.0	0.7	0.7	0.3
$C_{20:0}$	-	-	-	2.5	-	-
Unsaturated fatty acids						
$C_{17:1}$ **ω**8c	0.4	0.2	1.8	-	0.1	0.3
$C_{18:1}$ **ω**9c	-	0.3	1.4	-	0.1	0.1
Branched fatty acids						
iso-$C_{14:0}$	3.5	3.1	2.7	2.8	3.7	5.2
iso-$C_{15:0}$	14.2	19.0	3.4	3.9	14.0	9.1
anteiso-$C_{15:0}$	22.0	19.7	13.8	12.6	15.8	19.2
iso-$C_{16:0}$	22.7	19.9	18.7	25.3	19.8	29.3
iso-$C_{16:1}$ H	0.8	0.8	1.1	4.3	1.5	1.6
iso-$C_{17:0}$	9.2	12.0	5.2	2.3	7.0	4.1
anteiso-$C_{17:0}$	11.2	9.4	19.2	10.0	13.3	12.6
anteiso-$C_{17:1}$ A	-	0.6	-	-	-	-
anteiso-$C_{17:1}$ **ω**9*c*	0.6	-	3.1	5.6	1.7	2.5
iso-$C_{18:0}$	0.5	0.4	2.2	1.9	0.9	0.9
iso-$C_{18:1}$ H	-	-	0.5	-	-	0.4
anteiso-$C_{19:0}$	-	-	0.5	-	-	0.1

4.3.2 Characterization of *Streptomyces verrucosisporus* strains CPB1-1^T, CPB2-10, CPB3-1, CPB1-18 and BM1-4

All five strains grew well on ISP2, ISP4, ISP5, ISP6, ISP7 and marine media but poorly on the ISP3 medium. A white to greenish gray aerial mass was observed after the culture was grown on most ISP and marine media except for on ISP3 medium where no aerial mycelia formed. A light to strong yellow green pigment was observed on various ISP media (except ISP3, where no pigment was present) and marine agar. The cultural characteristics of all the strains are summarized in Table 4.6. The strains produced an open loop of spiral spore chain (retinaculum-apertum type) on ISP2 medium. The spiral type of spore chain could be observed for immature spores. Each chains of spores contained 13-15 warty spores of 0.7-1.0 x 0.8-1.3 µm in size and ellipsoid to ovule in shape (Figure 4.12). Growth was found to occur at 20-45 °C (optimum 30-37 °C) and pH 6.0 - 9.0 (optimum pH 8.0 – 9.0) with a maximum NaCl tolerance of 6 % (w/v).

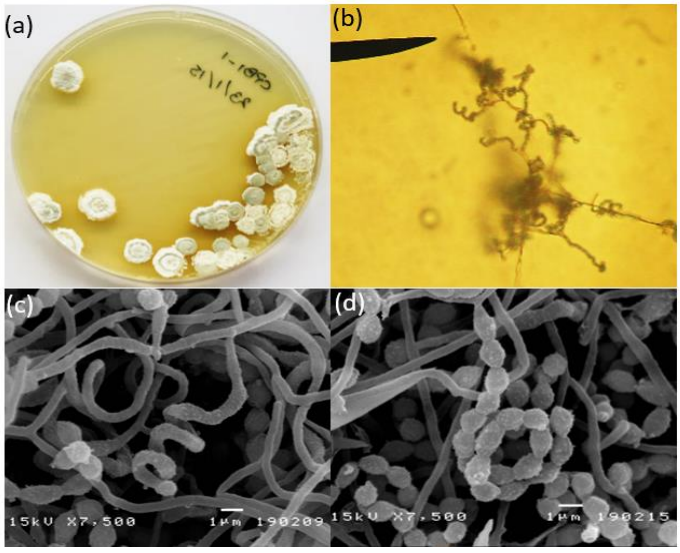

Figure 4.12 Colonial appearance (a) and light micrograph (b) (magnification, x 400) of *Streptomyces verrucosisporus* CPB1-1^T after incubation on ISP2 agar at 30 °C for 14 days. The scanning electron micrograph showing the immature (c) and mature spore chain (d) of the strain.

The almost complete 16S rRNA gene of these five strains (CPB1-1^T, CPB2-10, CPB3-1, CPB1-18 and BM1-4) ranged from 1403 to 1452 nt. They showed the highest 16S rRNA gene sequence similarity to *S. mangrovicola* GY1^T (NCIMB 14980^T) (99.0%), *S. fenghuangensis* GIMN4.003^T (98.6%), *S. barkulensis* RC1831^T (JCM 18754^T) (98.5%) and *S. radiopugnans* R97^T (JCM 15480^T) (98.3%). Due to the large number of members in the genus *Streptomyces*, the 16S rRNA gene sequences from the 46 species with the highest sequence similarity to strain CPB1-1^T were selected for phylogenetic analysis. In all the phylogenetic tree analysis methods (NJ, MP and ML), these strains clustered separately to the related *Streptomyces* species found in adjacent clusters such as *S. macrosporus* NBRC 14748^T, *S. megasporus* NBRC 14749^T, *S. glaucosporus* NBRC 15416^T, *S. mangrovi* HA11110^T, *S. mangrovicola* GY1^T, *S. barkulensis* RC1831^T, *S. fenghuangensis* GIMN 4.003^T, *S. nanhaiensis* SCSIO 01248^T, *S. radiopugnans* R97^T and *S. atacamensis* C60^T. This result was supported by high bootstrap values and was also recovered in the MP and ML phylogenetic trees (Figure 4.13).

All strains five strains contained LL-diaminopimelic acid in the cell wall peptidoglycan. Whole cell sugars of these strains contained glucose and ribose, but no diagnostic sugar pattern was present (Lechevalier & Lechevalier, 1970). Mycolic acids were absent. *N*-acyl muramic acids were acetyl. The menaquinones of strain CPB1-1^T were MK-9(H_6) (43%), MK-9(H_8) (26%), MK-10(H_6) (20%) and MK-10(H_8) (11%), while the major cellular fatty were anteiso-$C_{15:0}$ (37.3%), anteiso-$C_{17:0}$ (20.8%) and iso-$C_{16:0}$ (18.5%). The cellular fatty acid profile of strain CPB1-1^T and related *Streptomyces* species were similar, but the proportions of some components were different (Table 4.7). The polar lipids of strain CPB1-1^T were diphosphatidylglycerol, lyso-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositolmannoside, phosphatidylinositol, two unidentified aminolipids, two unidentified phospholipids and an unidentified glycolipid (type PII). In addition, *Streptomyces fenghuangensis* NRRL B-24801^T contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol mannoside, phosphatidylinositol, unidentified aminolipid, two unidentified phospholipids and an unidentified glycolipid, which is similar to the polar lipids of *Streptomyces mangrovicola* GY1^T (NCIMB 14980^T)

(Yousif *et al*., 2015), *Streptomyces barkulensis* RC1831^T (JCM 18754^T) (Ray *et al*., 2014) and *Streptomyces radiopugnans* R97^T (Mao *et al*., 2007). The presence of lyso-phosphatidylethanolamine was useful to distinguish strain CPB1-1^T from its closely related *Streptomyces* species as mentioned above.

Based on the spore morphology, chemotaxonomic characteristics and phylogenetic trees, the five strains of this study (CPB1-1^T, CPB2-10, CPB3-1, BM1-4 and CPB1-18) had consistent characteristics with members of the genus *Streptomyces* (Kämpfer, 2012) and so were classified in this genus. To confirm the novel species status of these five strains (CPB1-1^T, CPB2-10, CPB3-1, BM1-4 and CPB1-18), they were compared for DNA-DNA relatedness (Ezaki *et al*., 1989) to each other and to *S. mangrovicola* GY1^T (=NCIMB 14980^T), *S. fenghuangensis* GIMN4.003^T (=NRRL B-24801^T), *S. barkulensis* RC1831^T (=JCM 18754^T) and *S. radiopugnans* R97^T (=JCM 15480^T). These latter four species were selected based on the recommendation by Stackebrandt & Ebers (2006) that a 16S rRNA gene sequence similarity range above 98.7-99.0% is required for testing the genomic uniqueness of a novel isolate. The DNA-DNA relatedness between the five strains CPB1-1^T, CPB2-10, CPB3-1, CPB1-18 and BM1-4 was 81-94%, while CPB1-1^T (as a representative of the five strains) showed only low levels of DNA-DNA relatedness to *S. fenghuangensis* NRRL B-24801^T (49.8 ± 7.0%), *S. mangrovicola* NCIMB 14980^T (20.5 ± 2.7%), *S. barkulensis* JCM 18754^T (51.9 ± 5.4%) and *S. radiopugnans* JCM 15480^T (54.3 ± 4.4%). These values were lower than the 70% cut-off level for assigning strains to the same species (Wayne *et al*., 1987), and so strain CPB1-1^T is representative of the other four strains, all as a single novel species.

Examining strain CPB1-1^T further as a representative example of the other four and type strain, it showed typical characteristics consistent with CPB2-10, CPB3-1, BM1-4 and CPB1-18. However, several characteristics varied between those strains, such as the utilization of D-mannose, L-arabinose and D-xylose, and the enzyme activities of lipase (C14), valinearylamidase, acid phosphatase and α-glucosidase (Table 4.8). These results showed the variable phenotypic properties between strains in the same species. Compared with the closely related *Streptomyces* species, the selected CPB1-1^T strain could be distinguished by its spore chain morphology, cultural characteristics

on agar media, maximum temperature for growth, tolerance of NaCl, nitrate reduction and utilization of D-mannose, L-arabinose, D-fructose, D-xylose and D-cellobiose (Table 4.6 and 4.8). In addition, the presence of *lyso*-phosphatidylethanolamine in the phospholipid profile was distinctive from *S. fenghuangensis* NRRL B-24801^{T} and related type strains (Yousif *et al*., 2015; Ray *et al*., 2014; Mao *et al*., 2007). It is evident from the phenotypic, chemotaxonomic and genotypic data mentioned above that strains CPB1-1^{T}, CPB2-10, BM1-4, CPB3-1 and CPB1-18 should be classified as representing a novel species of the genus *Streptomyces*, for which the name *Streptomyces verrucosisporus* sp. nov. is herein proposed.

The etymology of the *Streptomyces verrucosisporus* is ver.ru.co.si.spo'rus. L. adj. *verruscosus* covered with warts; Gr. fem. n. *spora* a seed and, in biology, a spore; N.L. masc. adj. *verrucosisporus* with warty spores.

Table 4.6 Cultural characteristics of *S. verrucosisporus* strains CPB1-1^{T}, CPB2-10, CPB1-18, CPB3-1, BM1-4 and closely related *Streptomyces* species

Media/Characteristics	Strains								
	CPB1-1 ^{T}	CPB2-10	CPB1-18	CPB3-1	BM1-4	*S. mangrovicola* NCIMB 14980 ^{T}	*S. fenghuangensis* NRRL B-24801^{T}	*S. barkulensis* JCM 18754 ^{T}	*S. radiopugnans* JCM 15480 ^{T}
Yeast extract-malt extract agar (ISP medium 2)									
Growth	Very good	Good	Very good	Good	Good	Good	Good	Good	Good
Aerial mycelia	Abundance	Abundance	Abundance	Abundance	Abundance	Poor	Absent	Moderate	Moderate
Color of aerial mass	White/Light greenish gray	Light greenish gray	Light greenish gray	Light greenish gray	White/ Light greenish gray	Light greenish gray/ Greenish white	-	Light greenish gray	Light greenish gray
Reverse color	Strong greenish yellow	Strong greenish yellow	Moderate greenish yellow	Strong greenish yellow	Moderate greenish yellow	Moderate greenish yellow	Light yellow green	Moderate greenish yellow	Light greenish yellow
Soluble pigment	Light yellow green	-	Light yellow green	-	Light yellow green	Grayish yellow	-	Yellow gray	-
Oat meal agar (ISP medium 3)									
Growth	Poor	Poor	Poor	No growth	No growth	Moderate	Good	Poor	Poor
Aerial mycelia	Absent	Absent	Absent			Absent	Absent	Absent	Absent
Color of aerial mass	-	-	-			-	-	-	-
Reverse color	Pale greenish yellow	Pale greenish yellow	Pale greenish yellow			Pale greenish yellow	Light yellow green	Pale greenish yellow	Pale greenish yellow
Soluble pigment	-	-	-			-			
Inorganic-salt starch agar (ISP medium 4)									
Growth	Very good	Good	Very good	Good	Good	Moderate	Good	Good	Good
Aerial mycelia	Abundance	Abundance	Abundance	Abundance	Abundance	Moderate	Poor	Abundance	Moderate
Color of aerial mass	Light greenish gray	White/ Light greenish yellow	Light greenish gray	White/ Light greenish gray	White/ Light greenish gray	Light greenish gray	White	Greenish gray	White/ Light greenish gray
Reverse color	Grayish yellow	Light greenish yellow	Grayish yellow	Grayish yellow	Grayish yellow	Moderate greenish yellow	Light yellow green	Grayish greenish yellow	Light yellow green
Soluble pigment	Light yellow green	-	Light yellow green	Light yellow green	Light yellow green	Grayish yellow	-	Yellowish gray	-

Table 4.6 (Continued)

Media/Characteristics	Strains								
	CPB1-1 T	CPB2-10	CPB1-18	CPB3-1	BM1-4	*S. mangrovicola* NCIMB 14980 T	*S. fenghuangensis* NRRL B-24801T	*S. barkulensis* JCM 18754 T	*S. radiopugnans* JCM 15480 T
Peptone-yeast extract iron agar (ISP medium 6)									
Growth	Very good	Very good	Very good	Good	Good	Moderate	Good	Good	Good
Aerial mycelia	Abundance	Abundance	Abundance	Abundance	Abundance	Poor	Moderate	Abundance	Abundance
Color of aerial mass	Light greenish gray	White/ Light greenish gray	Light greenish gray	White/ Light greenish gray	White/ greenish gray	Light greenish gray	Light greenish gray	White/ Light greenish gray	White/ Light greenish gray
Reverse color	Light greenish yellow	Dark greenish yellow	Deep greenish yellow	Moderate olive brown	Dark grayish yellow	Colorless	Deep yellow	Moderate greenish yellow	Strong greenish yellow
Soluble pigment	Light yellow green	-	Dark greenish yellow	Light olive	Dark greenish yellow	Grayish yellow	-	-	
Tyrosine agar (ISP medium 7)									
Growth	Very good	Good	Very good	Very good	Good	Good	Good	Good	Good
Aerial mycelia	Abundance	Abundance	Abundance	Abundance	Abundance	Poor	Poor	Abundance	Moderate
Color of aerial mass	White/ Light greenish gray	White	White/ Light greenish gray	Light greenish gray	White/ Light greenish gray	White	White	Light greenish gray	White
Reverse color	Light greenish yellow	Light greenish yellow	Moderate greenish yellow	Moderate greenish yellow	Moderate greenish yellow	Pale greenish yellow	Light yellow green	Grayish greenish yellow	Light greenish yellow
Soluble pigment	Light yellow green	Moderate greenish yellow	Light yellow green	Light yellow green	Light yellow green	Grayish yellow	-	Yellowish gray	-
Marine agar									
Growth	Good	Good	Good	Good	Good	Poor	Good	Very good	Good
Aerial mycelia	Abundance	Abundance	Abundance	Abundance	Abundance	Poor	Moderate	Abundance	Abundance
Color of aerial mass	White/ Light greenish gray	Greenish gray	Greenish gray	Greenish gray	Greenish gray	Light greenish gray	Light greenish gray	Pale green	White/ medium gray
Reverse color	Strong yellow green	Moderate yellow green	Strong yellow green	Strong yellow green	Strong yellow green	Colorless	Moderate yellow green	Very pale green	Light yellow green
Soluble pigment	Strong yellow green	Strong yellow green	Strong yellow green	Strong yellow green	Strong yellow green	Grayish yellow	-	-	-

Table 4.7 Cellular fatty acid compositions (%) of *Streptomyces verrucosisporus* CPB1-1^T and related *Streptomyces* species

Strains: 1, Strain CPB1-1^T; 2, *S. mangrovicola* NCIMB 14980 ^{T}; 3, *S. fenghuangensis* NRRL B-24801^T; 4, *S. barkulensis* JCM 18754^T; 5, *S. radiopugnans* JCM 15480^T

Fatty acid	1	2	3	4	5
Saturated fatty acids					
$C_{16:0}$	2.3	3.2	4.7	1.3	3.4
Unsaturated branched fatty acids					
iso-$C_{16:1}$ H	2.3	1.5	1.9	4.8	3.1
anteiso-$C_{17:1}$ω9c	2.6	0.8	1.0	1.1	2.3
Branched fatty acids					
iso-$C_{14:0}$	2.8	7.5	6.2	10.5	8.1
iso-$C_{15:0}$	3.7	6.8	6.6	3.8	7.5
anteiso-$C_{15:0}$	37.3	26.4	23.5	30.0	26.0
iso-$C_{16:0}$	18.5	27.1	25.6	31.3	27.2
iso-$C_{17:0}$	1.9	4.3	4.4	1.4	3.9
anteiso-$C_{17:0}$	20.8	12.4	14.4	8.3	9.9
*Summed feature 3	-	2.9	2.1	-	-

Fatty acids comprising less than 2.0% in all strains are omitted.

* Summed feature 3 comprised $C_{16:1}$ω7C and/or $C_{16:1}$ω6C.

Table 4.8 Differential characteristics between *S. verrucosisporus* strains CPB1-1^T, CPB2-10, CPB1-18, CPB3-1, BM1-4 and closely related type strains of *Streptomyces* species

Characteristics	CPB1-1^T	CPB2-10	CPB1-18	CPB3-1	BM1-4	*S. mangrovicola* NCIMB 14980^T	*S. fenghuangensis* NRRL B-24801^T	*S. barkulensis* JCM 18754^T	*S. radiopugnans* JCM 15480^T
Growth on marine agar:	Good	Good	Good	Good	Good	Poor	Good	Very good	Good
Aerial mass color	Greenish gray	Greenish gray	Greenish gray	Greenish gray	Greenish gray	Light greenish gray	Light greenish gray	Pale green	White/ Medium gray
Substrate mycelia color	Strong yellow green	Moderate yellow green	Strong yellow green	Strong yellow green	Strong yellow green	Colorless	Moderate yellow green	Very pale green	Light yellow green
Soluble pigment	Strong yellow green	Strong yellow green	Strong yellow green	Strong yellow green	Strong yellow green	-	-	-	-
Growth at 50 °C	-	-	-	-	-	ND	ND	-	+
Nitrate reduction	+	+	+	+	+	+	+	+	-
Utilization of:									
L-Arabinose	w	w	w	w	-	-	+	-	+
D-Cellobiose	+	w	w	w	+	+	w	w	+
D-Fructose	-	-	-	-	-	-	w	-	w
D-Mannose	w	w	-	-	-	+	+	w	+
D-Xylose	w	-	w	w	-	+	+	+	+
Enzyme activity of:									
Esterase (C4)	w	+	+	+	w	+	+	+	+
Esterase Lipase (C8)	w	w	+	+	w	w	+	+	+
Lipase (C14)	-	-	+	+	-	-	-	w	+
Valinearylamidase	w	w	w	w	-	+	+	+	+
Cystinearylamidase	-	-	-	-	-	w	w	w	-

Table 4.8 (Continued)

Characteristics	CPB1-1^T	CPB2-10	CPB1-18	CPB3-1	BM1-4	*S. mangrovicola* NCIMB 14980^T	*S. fenghuangensis* NRRL B-24801^T	*S. barkulensis* JCM 18754^T	*S. radiopugnans* JCM 15480^T
Enzyme activity of:									
α-Chymotrypsin	-	-	-	-	-	w	w	w	-
Acid phosphatase	-	-	w	w	-	-	w	w	w
Napthol-AS-BI-phosphohydrolase	w	w	w	-	w	w	+	+	w
α-Galactosidase	-	-	-	w	-	-	+	w	w
β-Galactosidase	-	-	-	-	-	-	-	w	-
α-Glucosidase	-	-	+	-	w	-	w	w	+
β-Glucosidase	+	w	+	w	+	-	-	-	w

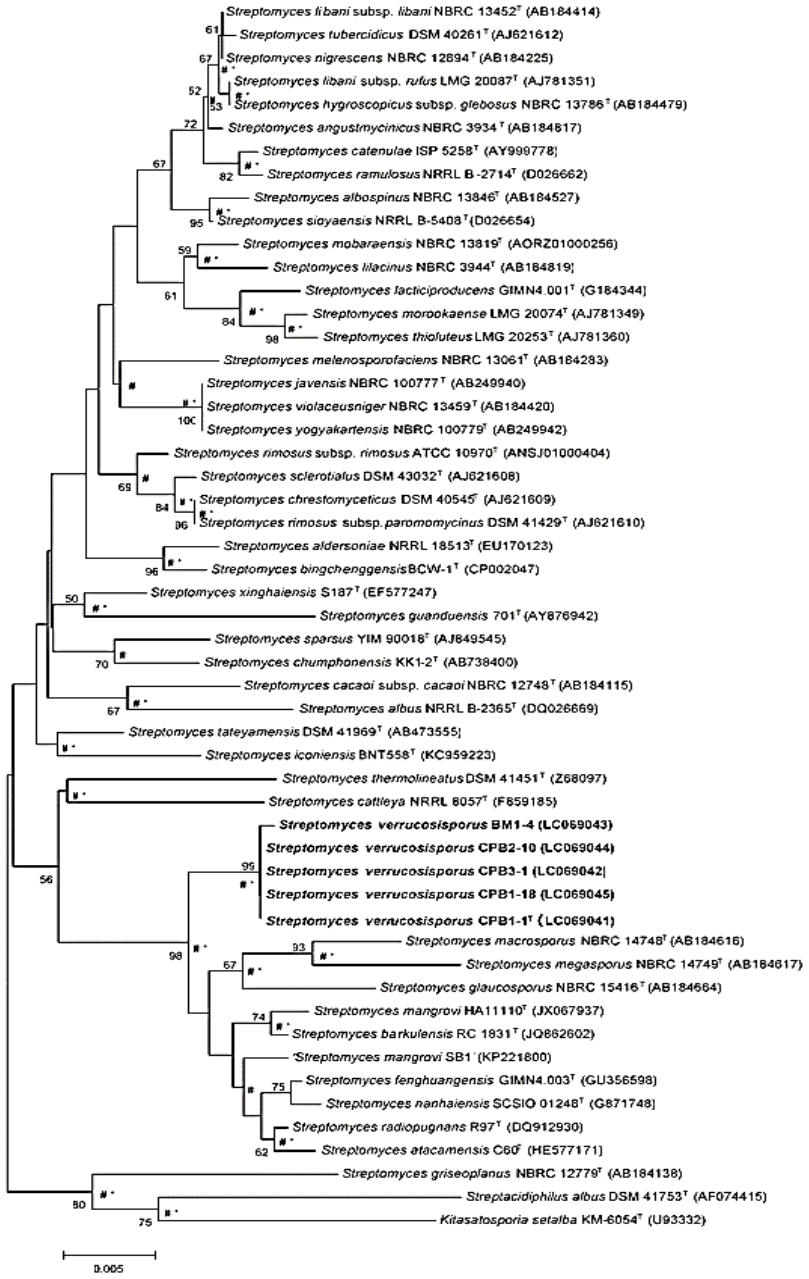

Figure 4.13 Phylogenetic relationships based on neighbor-joining analysis of 16S rRNA gene sequences of *Streptomyces verrucosisporus* CPB1-1^{T}, CPB2-10, CPB3-1, CPB1-18 and BM1-4, plus the 46 most related species of the genus *Streptomyces*. Type species of the family *Streptomycetaceae*, *Kitasatosporia setae* KM-6054^{T} and *Streptacidiphilus albus* DSM 41753^{T} were used as out groups. Asterisk ($^{\#,*}$) indicate that the branch was also recovered in the maximum-likelihood and maximum-parsimony analyses, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only values of > 50% are shown). Bar: 0.005 substitutions per nucleotide position.

4.3.3 Characterization of *Micromonospora fluostatini* strain PWB-003^T

Strain PWB-003^T produced non-fragmented branched substrate mycelia but not produced aerial mycelia on various agar media. Rough spores were borne singly on substrate mycelia and were 0.2-1.0 µm in size (Figure 4.14). The cultural characteristics of strain PWB-003^T were determined and summarized in Table 4.9. The strain grew well on yeast-starch and JCM 47 media but not grew on ISP 3 and ISP 6 media. It produced light grayish yellowish brown to brownish orange pigment on ISP 7, yeast-starch agar, nutrient agar and JCM 47 agar. The strain grew at pH 6-10. The optimum temperature for growth of strain PWB-003^T was 30-37 °C. No growth were observed at 20 and 45 °C after incubated for 7 days.

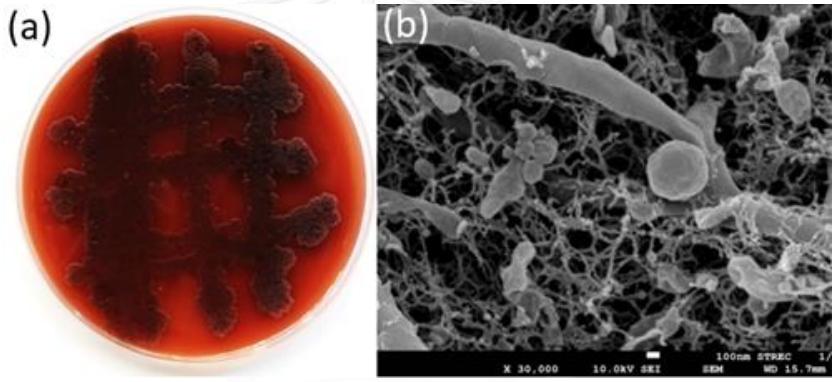

Figure 4.14 Colonial appearance (a) and scanning electron micrograph (b) of *Micromonospora fluostatini* PWB-003^T after incubation on JCM 47 agar at 30 °C for 30 days.

Strain PWB-003^T showed the identical chemotaxonomic characteristics to those members of the genus *Micromonospora*. It contained *meso*-diaminopimelic acid in cell wall peptidoglycan and contained ribose, xylose, arabinose, mannose and glucose in whole-cell hydrolysates. According to the classification of Lechevalier & Lechevalier (1970), the presence of xylose and arabinose as diagnostic sugar were classified as sugar pattern D. The *N*-acyl group of muramic acid was glycolyl. The mycolic acids were absent. The fatty acid profile contained amount of $C_{18:1}\omega9c$ (22.3%), iso-$C_{16:0}$ (25.9%),

anteiso-$C_{17:0}$ (12.4%), iso-$C_{15:0}$ (7.7%) and iso-$C_{17:0}$ (6.2%) (Table 4.10). The difference of fatty acid profile between strain PWB-003^T and related type strains are showed in Table 2. The menaquinones were MK-10 (H_4) (91.4%) and small amount of MK-10 (H_0) (1.6%), MK-10 (H_2) (1.9%), and MK-10 (H_6) (4.9%). The G+C content of DNA was 74.5 mol %. The phospholipids were phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), phosphatidylinositol (PI), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol mannoside (PIM). Unknown phospholipids, unknown phosphoglycolipid, unknown ninhydrin-positive glycolipid and unknown lipids were also detected. The containing of both PE and PME in phospholipids profile was unique when compared with other related *Micromonospora* species which contained only one nitrogenous phospholipids. (Kroppenstedt *et al*., 2005; Jongrungruangchok *et al*., 2008; Thawai *et al.*, 2004; Thawai *et al*., 2005; Zhang *et al*., 2012).

Almost complete 16S rRNA gene sequence (1462nt) of strain PWB-003^T was closely related to *Micromonospora eburnea* LK2-10^T (99.38%), *Micromonospora chaiyaphumensis* MC5-1^T (99.16%), *Micromonospora yangpuensis* JCM 18319^T (98.97%), *Micromonospora echinaurantiaca* JCM 3257^T (98.97%), *Micromonospora pallida* JCM 3133^T (98.97%), *Micromonospora sagamiensis* JCM 3310^T and *Micromonospora auratinigra* TT1-11^T (98.97%). The phylogenetic trees based on 16S rRNA gene indicated that strain PWB-003^T shared a clade with *M. yangpuensis* FXJ6.011^T (Figure 4.15). Although the bootstrap values of this clade is lower than 50, however this association was supported by maximum-parsimony and maximum-likelihood algorithm employed. In addition, the *gyrB* gene similarity between strain PWB-003^T and other species of the genus *Micromonospora* ranged from 82.7% to 98.6%. The phylogenetic tree analysis based on *gyrB* gene revealed that strain PWB-003^T shared a clade with *Micromonospora rosaria* IFO 13697^T with high bootstrap value (Figure 4.10). However, the 16S rRNA gene sequence of strain PWB-003^T showed low similarity (98.5%) to *M. rosaria* IFO 13697^T. This similarity was below the recommended value (98.7–99%) for the DNA-DNA reassociation experiment (Stackebrandt & Ebers, 2006). Furthermore, these two species could be distinguished using the morphology of spores and whole

cells sugars pattern (Horan & Brodsky, 1986). On the basis of morphological and chemotaxonomic characteristics including16S rRNA gene and *gyrB* gene analysis as mentioned above, the strain PWB-003^{T}could be classified in the genus *Micromonospora*.

In comparison with the closely related *Micromonospora* species, the strain PWB-003^T could be distinguished from them by phenotypic properties (Table 4.11) in particular, starch hydrolysis, gelatin liquefaction, nitrate reduction, skim milk peptonization/coagulation, acid production from carbon source, enzyme activity, tolerant of NaCl, the presence of both phosphatidyl methylethanolamine and phosphatidylethanolamine in polar lipid profile. Moreover, strain PWB-003^T showed low DNA-DNA relatedness values with related type strains (11.3±1.3% to 38.8±1.1%) which were significantly lower than 70%, the threshold value for assigning strain for the same species (Wayne *et al*., 1987).

Based on phenotypic characteristics, chemotaxonomic characteristics, genotypic characteristics together with DNA-DNA relatedness, strain PWB-003^T should be classified as a novel species of the genus *Micromonospora*, for which the name *Micromonospora fluostatini* sp. nov. is proposed.

The etymology of the *Micromonospora fluostatini* is flu.o.sta.ti'ni. N.L. gen. n. fluostatini of fluostatin, referring to the ability to produce fluostatin antibiotics.

Figure 4.15 Phylogenetic relationships based on neighbor-joining analysis of 16S rRNA gene sequences (1462 nt) of *M. fluostatini* PWB-003^{T} and all members of the genus *Micromonospora*. *Actinoplanes regularis* DSM 43151^{T} was used as an out group. Asterisk ($^{*, \#}$) indicated the branches were recovered in the maximum-likelihood and maximum-parsimony, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown).

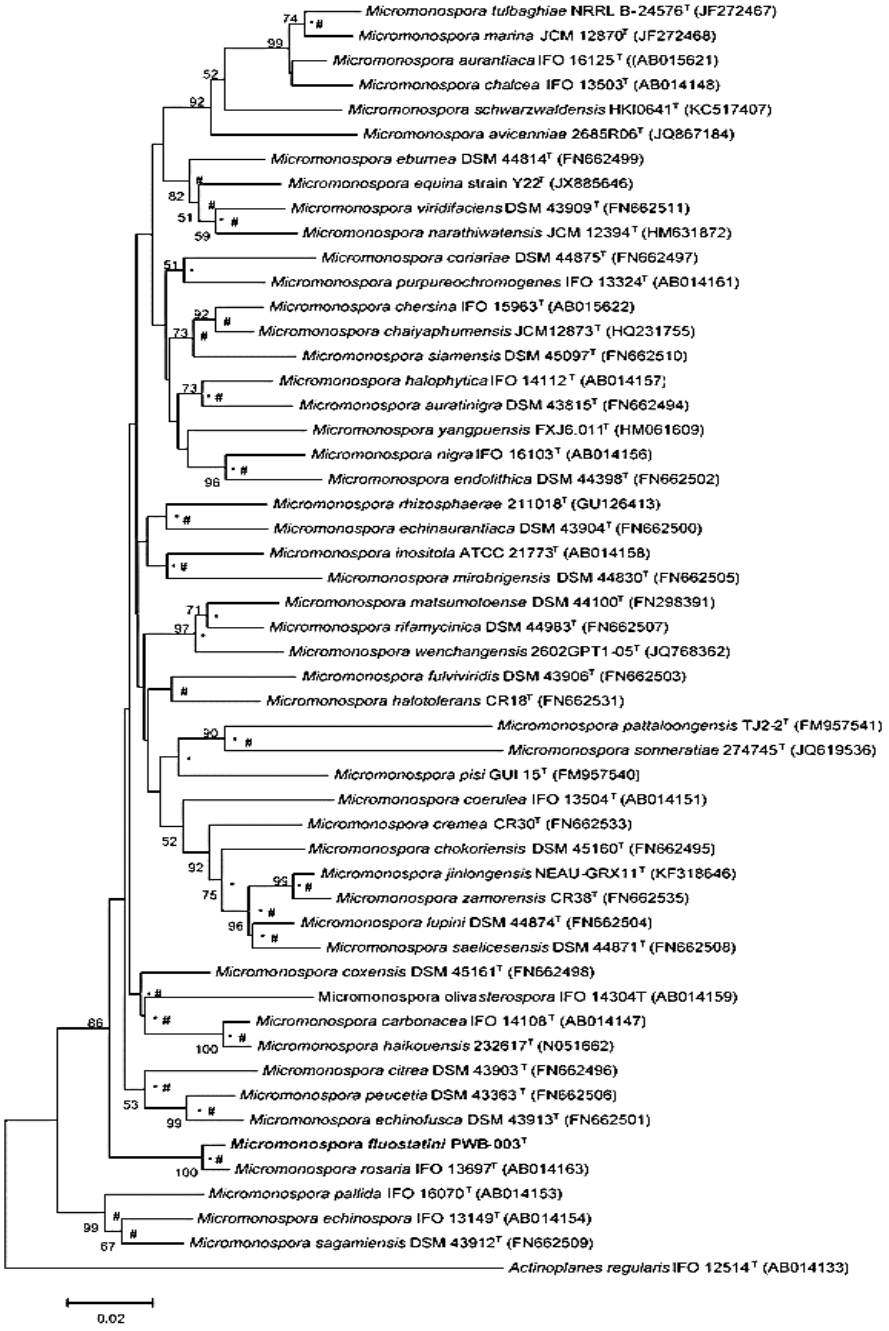

Figure 4.16 Phylogenetic relationships based on neighbor-joining analysis of *gryB* gene sequences (1048 nt) of *M. fluostatini* PWB-003^{T} and members of the genus *Micromonospora*. *Actinoplanes regularis* IFO 12514^{T} was used as an out group. Asterisk ($^{*, \#}$) indicated the branches were recovered in the maximum-likelihood and maximum-parsimony, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown). Bar, 0.002 substitutions per nucleotide position.

Table 4.9 Cultural characteristics of strain PWB-003^{T} and related *Micromonospora* species

Strains: 1, Strain PWB-003^{T}; 2, *Micromonospora eburnea* LK2-10^{T}; 3, *M. chaiyaphumensis* MC5-1^{T}; 4, *M. yangpuensis* JCM 18319^{T}; 5, *M. sagamiensis* JCM 3310^{T}; 6. *M. echinaurantiaca* JCM 3257^{T}; 7. *M. pallida* JCM 3133^{T}; 8; *M. auratinigra* TT1-11^{T}

Medium	1	2	3	4	5	6	7	8
ISP medium 2								
Growth	Poor	Moderate	Moderate	Moderate	Poor	Good	Moderate	Good
Colony color	Light yellow (86), Pale yellow (89)	Pale greenish yellow (104), Deep greenish yellow (100)	Deep orange yellow (69)	Brilliant orange yellow (67), Deep orange yellow (69)	Grayish yellow (90)	Strong orange yellow (68)	Light yellow (86)	Brownish black (65)
Soluble pigment	None	Light yellow green (119)	None	None	None	Brilliant orange yellow (67)	None	Moderate olive (107)
ISP medium 3								
Growth	No growth	Good	Very good	Poor	Moderate	Very good	Poor	Good
Colony color		Dark orange yellow (72)	Strong yellowish brown (74), Dark olive brown (96)	Brilliant orange yellow (67)	Strong yellow (84)	Vivid orange (48)	Strong yellow (84)	Strong orange yellow (68), Dark grayish yellowish brown (81)
Soluble pigment		None	None	None	None	Brilliant orange yellow (67)	None	None
ISP medium 4								
Growth	Moderate	No growth	Moderate	Good	Good	Good	Moderate	Good
Colony color	Light orange yellow (70)		Strong yellow (84), Light orange yellow (70)	Dark grayish yellow (91), Moderate olive brown (95)	Strong brown (55)	Strong orange yellow (68)	Light orange yellow (70)	Brilliant yellow (49)
Soluble pigment	None		None	None	None	None	None	Blight yellow green (119)

Table 4.9 (Continued)

Strains: 1, Strain PWB-003^{T}; 2, *Micromonospora eburnea* LK2-10^{T}; 3, *M. chaiyaphumensis* MC5-1^{T}; 4, *M. yangpuensis* JCM 18319^{T}; 5, *M. sagamiensis* JCM 3310^{T}; 6. *M. echinaurantiaca* JCM 3257^{T}; 7. *M. pallida* JCM 3133^{T}; 8; *M. auratinigra* TT1-11^{T}

Medium	1	2	3	4	5	6	7	8
ISP medium 7								
Growth	Moderate	Poor	Good	Good	Moderate	Moderate	Poor	Good
Colony color	Light yellowish brown (76)	Colorless	Brownish black (65)	Dark grayish yellow (91), Light orange yellow (70)	Brownish orange (54)	Dark orange yellow (72)	Dark grayish yellow (91)	Brownish black (65)
Soluble pigment	Light grayish yellowish brown	None	None	Dark orange yellow (72)	Moderate yellow (87)	Light orange yellow (70)	Light yellowish brown (76)	Light greenish gray (154)
Nutrient agar								
Growth	Poor	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Colony color	Strong yellowish brown (74)	Moderate yellow green (120), Pale greenish yellow (104)	Strong yellowish brown (74), Deep yellowish brown (75)	Brilliant orange (49)	Grayish red (19)	Brilliant orange yellow (67)	Grayish yellowish green (122)	Dark grayish olive (111)
Soluble pigment	Light yellowish brown (76)	None	None	None	Light yellowish brown (76)	None	Moderate greenish yellow (102)	None
YS agar								
Growth	Good	Good	Good	Moderate	Very good	Very good	Moderate	Good
Colony color	Light orange yellow (70), Dark yellow (87)	Grayish olive green (127), Moderate yellow green (120)	Deep orange yellow (69), Dark olive brown (96)	Moderate olive brown (95), Dark olive brown (96)	Pale yellow (89)	Strong orange yellow (68)	Pale greenish yellow (104)	Dark olive brown (90)
Soluble pigment	None	Light yellow green (119)	None	Dark orange yellow (72)	Moderate greenish yellow (102)	Brilliant orange yellow (66)	None	Light yellow green (119)
JCM 47 medium								
Growth	Very good	Very good	Moderate	Moderate	Very good	Very good	Very good	Good
Colony color	Purplish gray (233)	Dark grayish olive green (128)	Dark grayish yellow (91), Light olive brown (94)	Brilliant orange yellow (67), Dark orange yellow (72)	Dark reddish purple (242)	Deep orange yellow (69)	Pale yellow (89)	Light orange yellow (70), Light grayish olive (109)
Soluble pigment	Brownish orange (54)	None	None	None	Light yellowish brown (76)	Grayish yellow (90)	None	None

Table 4.10 Cellular fatty acid compositions (%) of *Micromonospora fluostatini* PWB-003^T and related *Micromonospora* species

Strains: 1, Strain PWB-003^T; 2, *Micromonospora eburnea* LK2-10^T; 3, *M. chaiyaphumensis* MC5-1^T; 4, *M. yangpuensis* JCM 18319^T; 5, *M. sagamiensis* JCM 3310^T; 6, *M. echinaurantiaca* JCM 3257^T; 7, *M. pallida* JCM 3133^T

Fatty acid	1	2	3	4	5	6	7
Saturated fatty acids							
$C_{16:0}$	2.7	0.7	1.0	4.8	2.7	1.6	4.3
$C_{17:0}$	1.4	4.6	2.0	9.8	2.8	18.1	1.2
$C_{18:0}$	2.7	1.1	2.2	3.6	4.9	1.4	2.6
Unsaturated fatty acids							
$C_{16:1}$ 2OH	-	1.1	1.3	0.5	-	-	-
$C_{17:1}$**ω**8c	5.7	2.1	1.7	14.8	4.8	8.1	1.6
$C_{18:1}$**ω**9c	22.3	-	-	6.3	4.4	0.6	1.8
Branched fatty acids							
anteiso-$C_{11:0}$	1.6	1.2	0.9	1.5	1.7	2.0	1.3
iso-$C_{14:0}$	0.5	1.0	0.6	0.7	0.5	1.5	0.4
iso-$C_{15:0}$	7.7	31.1	19.4	9.2	29.3	32.7	50.8
anteiso-$C_{15:0}$	2.0	5.0	2.4	0.9	1.4	4.4	2.7
iso-$C_{16:0}$	25.9	20.1	31.9	31.7	12.9	11.2	5.7
iso-$C_{17:0}$	6.2	8.6	11.8	4.0	14.0	4.8	12.4
anteiso-$C_{17:0}$	12.4	6.2	8.0	3.6	5.4	3.4	4.4
iso-$C_{18:0}$	0.7	0.8	3.9	0.5	0.5	0.2	0.1
10-Methyl fatty acids							
10-methyl $C_{17:0}$	1.2	8.2	3.5	0.6	0.3	2.7	0.3
10-methyl $C_{18:0}$ TSBA	0.5	1.2	0.6	0.1	-	0.1	0.2
Summed feature 3[a]	0.5	0.1	0.2	2.1	1.7	0.3	2.7
Summed feature 9[e]	1.2	2.0	5.1	0.7	7.0	0.8	3.9

The amount of fatty acid less than 1% in all strains was omitted.

[a] Summed feature 3 comprised $C_{16:1}$**ω**7c or $C_{16:1}$**ω**6c

[e] Summed feature 9 comprised iso-$C_{17:1}$**ω**9c.

Table 4.11 Differential characteristics of *Micromonospora fluostatini* PWB-003^T and related *Micromonospora* species

Strains: 1, Strain PWB-003^T; 2, *Micromonospora eburnea* LK2-10^T; 3, *M. chaiyaphumensis* MC5-1^T; 4, *M. yangpuensis* JCM 18319^T; 5, *M. sagamiensis* JCM 3310^T; 6, *M. echinaurantiaca* JCM 3257^T; 7, *M. pallida* JCM 3133^T; 8, *M. auratinigra* TT1-11^T

Characteristics	Strain							
	1	2	3	4	5	6	7	8
Hydrolysis of starch	+	-	+	+	+	+	+	+
Gelatin liquefying	-	+	+	-	-	+	-	+
Nitrate reduction	-	+	-	-	+	-	-	-
Skimmed milk peptonization	+	+	+	-	-	-	-	-
Skimmed milk coagulation	-	+	-	-	w	-	-	w
NaCl tolerance (% w/v)	≤3	≤4	≤4	≤3	≤2	≤5	≤3	≤3
Growth at pH 5	-	+	+	-	-	-	-	+
Growth at 37 °C	+	+	+	-	+	+	+	+
Acid production from								
L-Arabinose	-	-	+	-	w	+	+	+
D-Cellobiose	w	-	+	+	+	+	+	+
Fructose	-	-	+	-	-	+	+	+
D-Mannose	w	-	+	+	w	+	+	-
D-Melibiose	w	-	+	+	w	+	+	+
D-Raffinose	w	-	+	+	w	+	+	+
L-Rhamnose	w	-	+	+	w	w	+	+
Salicin	w	-	+	-	+	w	+	-
D-Sorbitol	-	-	+	+	-	+	+	-
Xylitol	-	-	w	w	w	w	+	+
Enzyme activity of								
Acid phosphatase	-	+	+	w	-	+	-	w
N-Acetyl-β-glucosaminidase	-	+	+	+	-	+	+	-
Alkaline phosphatase	+	-	w	+	w	+	w	-
α-Chymotrypsin	+	-	+	w	-	-	w	+
Cystine arylamidase	-	-	w	w	-	-	w	-
α-Galactosidase	-	+	+	-	-	w	-	-
β-Galactosidase	-	+	+	+	w	+	+	w
α-Glucosidase	+	+	+	+	w	+	+	+
β-Glucosidase	+	+	+	w	w	-	+	w
Lipase (C14)	w	-	w	w	w	w	w	-
α-Mannosidase	-	-	w	-	-	-	-	-
Napthol-AS-BI-Phosphohydrolase	w	+	-	w	w	w	w	w
Trypsin	+	w	+	+	w	+	+	w
Valine arylamidase	-	-	w	w	-	-	w	-

+, positive; w, weakly positive; -, negative

4.3.4 Characterization of *Micromonospora sediminis* strain CH3-3^T

Strain CH3-3^T grew well on marine agar; moderately grew on ISP2, ISP6 and ISP 7 media; poorly grew on ISP3, ISP4, ISP5 and nutrient media. It produced single spore on substrate mycelia while aerial mycelia were absent. The strain appears dark grayish yellow on ISP2, ISP4 and marine agar media; light yellow on ISP7; pale yellow on ISP3 and nutrient agar; moderate yellow on ISP5 and grayish yellow on ISP6. Dark greenish yellow and light yellow green pigment were observed on ISP2 and marine agar, respectively (Table 4.12). The comparison of cultural characteristics between strain CH3-3^T and related *Micromonospora* species were summarized in Supplementary Table S1. The strain formed the monomeric spore on substrate mycelia (Figure 4.17). The spores were smooth on surface and were 0.32 to 0.35 µm in size. The strain utilized L-arabinose, cellobiose, galactose, D-mannitol, L-rhamnose and sucrose; weakly utilized salicin and D-xylose but did not utilize fructose*, myo*-inositol, D-raffinose and ribose. Growth was observed at pH 7-9 and at 28-37 °C. The maximum concentration of NaCl for growth was 2% (w/v). Others details for phenotypic characteristics were showed in Table 1 and description of the species.

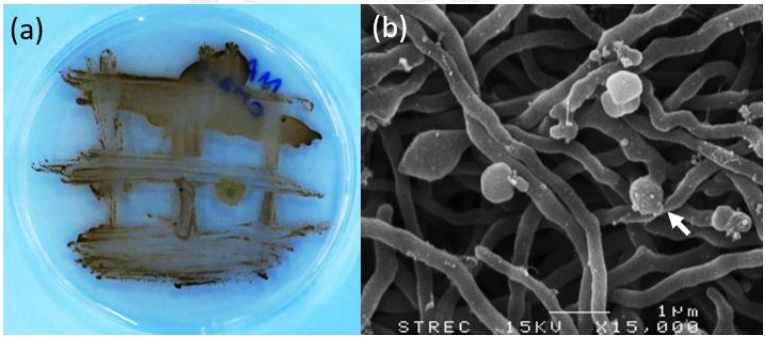

Figure 4.17 The colonial appearance (a) and scanning electron micrograph (b) of *Micromonospora sediminis* CH3-3^T after incubation on marine agar at 30 °C for 14 days.

The chemotaxonomic results revealed that strain CH3-3^T exhibited typical characteristics consistent with members of the genus *Micromonospora*. *Meso*-diaminopimelic acid was detected in whole-cell hydrolysates. The strain contained glucose, mannose, xylose, ribose and small amount of rhamnose as whole-cell sugars. The acyl type of cell-wall muramic acid was glycolyl type. Mycolic acids were not present. The polar lipids were phosphatidyl ethanolamine (PE), phosphatidylinositol (PI), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), three unidentified glycolipids, an unidentified phospholipid and two unidentified lipids (Supplementary Figure S1). Menaquinones were MK-10(H_4) (53.4%), MK-10(H_6) (33.5%) and MK-10(H_8) (13.1%). Major cellular fatty acids (> 10%) were *iso*-$C_{15:0}$ (30.2%), *iso*-$C_{16:0}$ (27.9%) and *iso*-$C_{17:0}$ (14.8%) (Table 4.13). The DNA G+C content was 73.8 mol%.

BLAST analysis of 16S rRNA gene revealed that strain CH3-3^T showed highest similarity with *Micromonospora palomenae* NEAU-CX1^T (98.97%) and *Micromonospora coxensis* 2-30-b/28^T (98.97%). According to the Figure 2, It can be seen that the strain shared the same node with *M. palomenae* NEAU-CX1^T as well as formed the subclade with *Micromonospora halophytica* DSM 43171^T, *M. coxensis* 2-30-b/28^T and *Micromonospora purpureochromogenes* DSM 43821^T. However, the topologies of this subclade were extremely low (Figure 4.18). To clarify the phylogenetic relationship, *gyrB* genes of the members of genus *Micromonospora* were obtained and compared. Figure 4.19 revealed that the strain formed node with *M. coxensis* DSM 45161^T but showed low bootstrap value. However, this node was recovered in the tree calculating from maximum-likelihood. The level of *gyrB* gene similarity among stain CH3-3^T related *Micromonospora* species range from 87.3 to 93.8%. Among them, *M. coxensis* DSM 45161^T showed the highest *gyrB* gene similarity with strain CH3-3^T (93.8%).

Based on both highest 16S rRNA gene and *gyrB* gene similarity as well as phylogenetic relationship of 16S rRNA gene and *gyrB* gene, *M. palomenae* JCM 30056^T, *M. halophytica* JCM 3125^T, *M. coxensis* JCM 13248^T and *M. purpureochromogenes* JCM 3156^T were selected for DNA-DNA hybridization experiment to confirm the novel species status of strain CH3-3^T. Levels of DNA-DNA relatedness between strain CH3-3^T and closely related *Micromonospora* species were as follows: *M. palomenae* JCM

30056^T (61.5 ± 2.0 %); *M. halophytica* JCM 3125^T (35.2 ± 10.3%); *M. coxensis* JCM 13248^T (42.9 ± 5.8%) and *M. purpureochromogenes* JCM 3156^T (47.9 ± 3.2%). These values were lower than 70%, cut off point recommended by Wayne *et al*. (1987) for the same species.

According to the results of morphological and chemotaxonomic properties including 16S rRNA gene and *gyrB* gene analysis mentioned above, strain CH3-3^T could be classified in the genus *Micromonospora* (Genilloud, 2012). In addition, this strain exhibited different phenotypic characteristics from its closely related taxa in particular; cultural characteristics; liquefaction of gelatin; utilization of L-arabinose, cellobiose, *myo*-inositol, D-mannitol, D-raffinose, L-rhamnose and sucrose as sole carbon source; enzyme activity of acid phosphatase, *N*-acetyl-β-glucosaminidase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase lipase (C8), α-galactosidase, β-galactosidase, β-glucuronidase, lipase (C14), napthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase (Table 4.14).

On the basis of phenotypic and genotypic properties as well as DNA-DNA relatedness, it is evident that the strain CH3-3^T could be clearly distinguished from previously described *Micromonospora* species. Therefore, strain CH3-3^T represents the novel species of the genus *Micromonospora* for which the name *Micromonospora sediminis* sp. nov., is proposed. The etymology of *Micromonospora sediminis* is se.di'mi.nis. L. gen. n. sediminis of sediment.

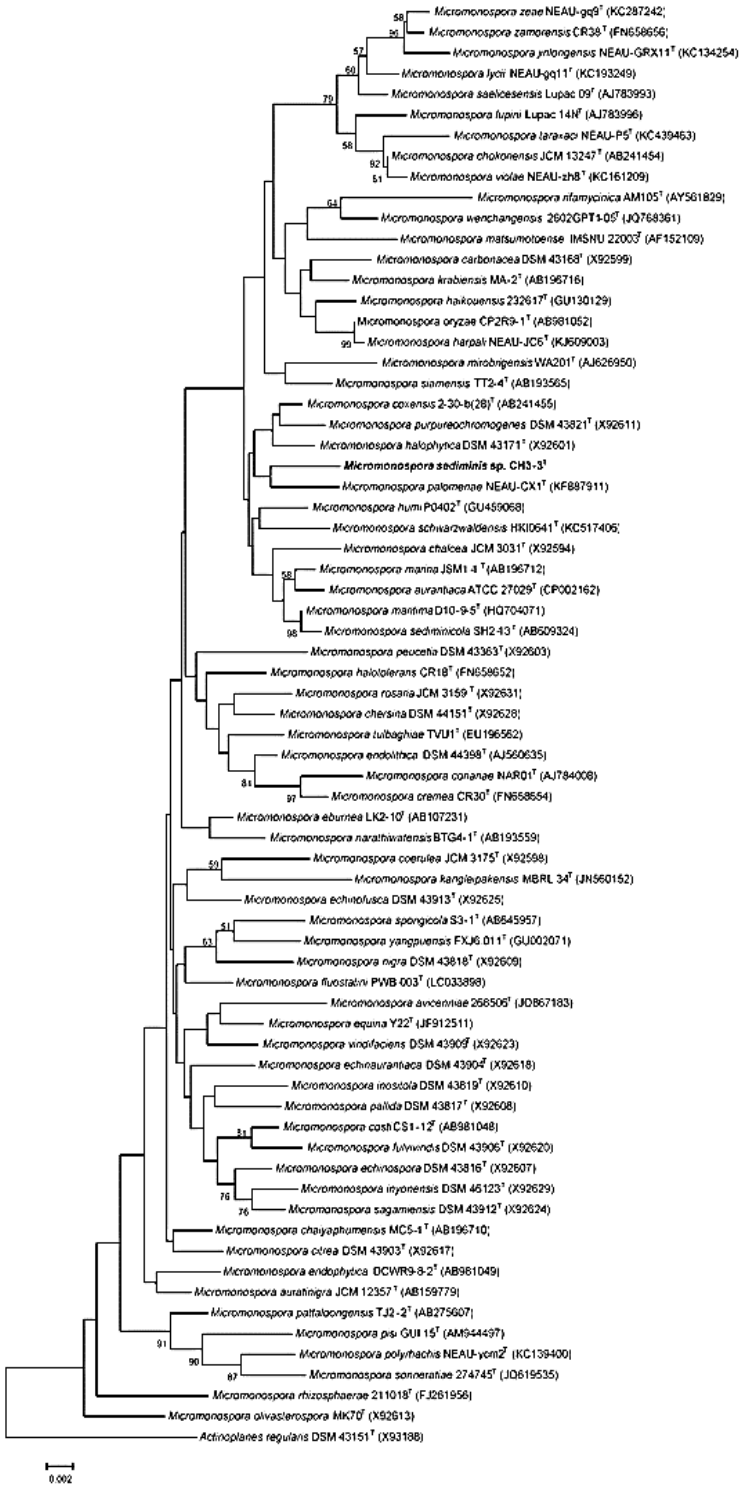

Figure 4.18 Phylogenetic relationships based on neighbor-joining analysis (Saitou & Nei, 1987) of 16S rRNA gene sequences of *Micromonospora sediminis* CH3-3^{T} and related *Micromonospora* species. *Actinoplanes regularis* DSM 43151^{T} was used as an out group. Asterisks ($^{*, \#}$) indicate the branches which were recovered in the Maximum Likelihood tree and Maximum Parsimony tree respectively. The number at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown) Bar, 0.002 substitutions per nucleotide position.

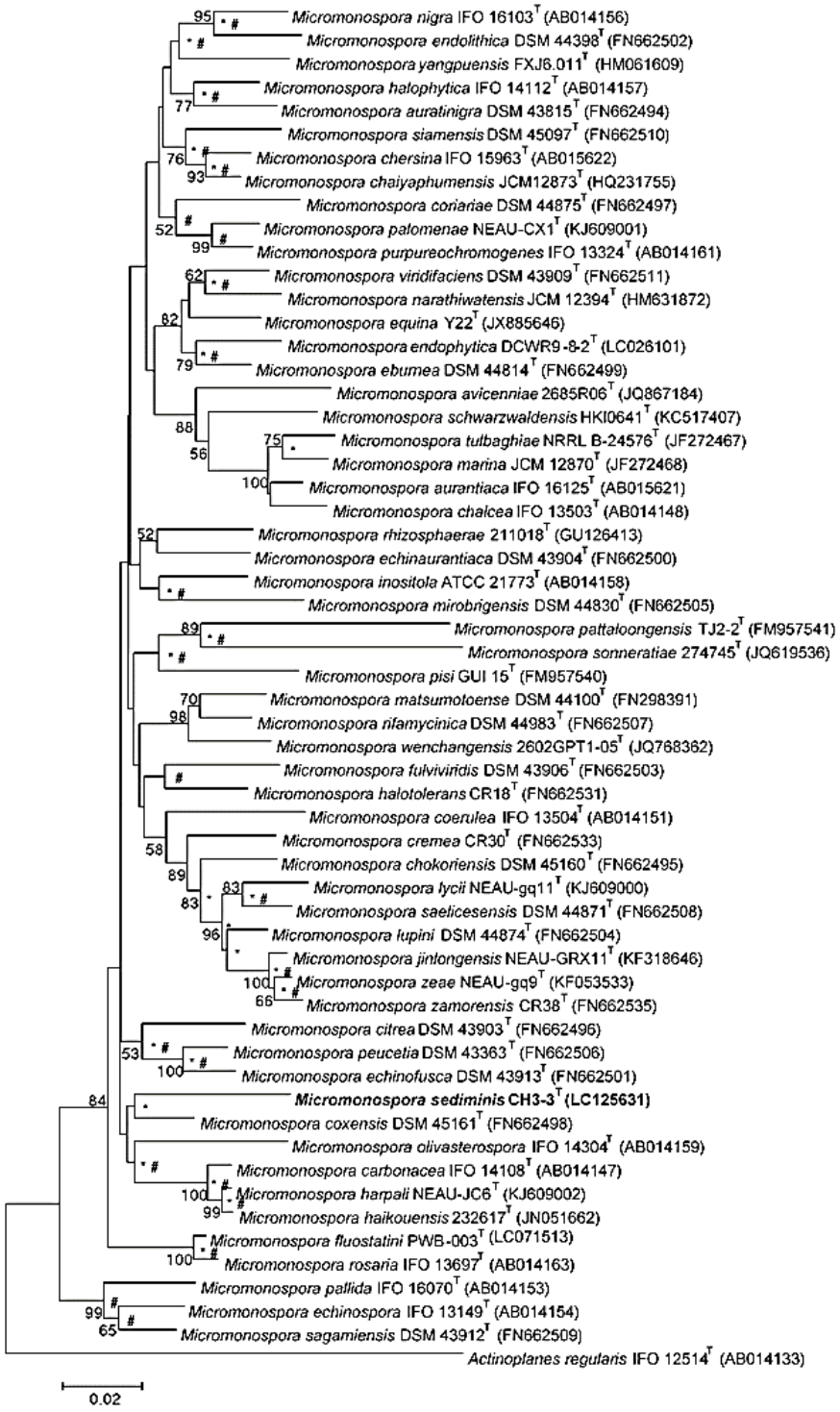

Figure 4.19 Phylogenetic relationships based on neighbor-joining analysis of *gyrB* gene sequences of *Micromonospora sediminis* CH3-3^T and *Micromonospora* species. *Actinoplanes regularis* IFO 12514^T was used as an out group. Asterisks ($^{*, \#}$) indicate the branches which were recovered in the maximum- likelihood tree and maximum-parsimony tree respectively. The number at branch nodes indicate bootstrap percentages derived from 1000 replications (only value > 50% are shown) Bar, 0.02 substitutions per nucleotide position.

Table 4.12 Cultural characteristics of *Micromonospora sediminis* CH3-3^T and closely related type strains

Medium	CH3-3^T	JCM 30056^T	JCM 3125^T	JCM 3156^T	JCM 13248^T
ISP medium 2					
Growth	Moderate	Good	Moderate	Good	Good
Colony color	Dark grayish yellow	Light yellowish brown	Strong orange yellow	Strong orange yellow	Moderate orange yellow
Reverse	Dark grayish yellow	Strong yellowish brown	Strong orange yellow	Strong orange yellow	Strong orange yellow
Soluble pigment	Dark greenish yellow	Strong yellowish brown	None	Pale yellow	None
ISP medium 3					
Growth	Poor	Very good	Moderate	Good	Good
Colony color	Pale yellow	Grayish yellowish brown	Moderate orange yellow	Dark orange yellow	Moderate orange yellow
Reverse color	Pale yellow	Dark grayish yellowish brown	Moderate orange yellow	Dark orange yellow	Moderate orange yellow
Soluble pigment	None		Pale orange yellow	Light orange yellow	None
ISP medium 4					
Growth	Poor	Good	Good	Moderate	Good
Colony color	Dark grayish yellow	Deep yellowish brown	Deep orange yellow	Strong yellowish brown to Dark yellowish brown	Moderate orange yellow
Reverse color	Dark grayish yellow	Dark yellowish brown	Deep orange yellow	Strong yellowish brown to Dark yellowish brown	Strong orange yellow
Soluble pigment	None	Moderate yellowish brown	None	Moderate orange yellow	None
ISP medium 5					
Growth	Poor	Moderate	Moderate	Moderate	Good
Colony color	Moderate yellow	Deep yellowish brown	Moderate orange yellow	Light yellowish brown	Moderate orange yellow
Reverse color	Moderate yellow	Dark yellowish brown	Moderate orange yellow	Light yellowish brown	Light orange
Soluble pigment	None	Strong yellowish brown	Pale yellow	Light yellowish brown	Pale yellow

Table 4.12 (Continued)

Medium	CH3-3^T	JCM 30056^T	JCM 3125^T	JCM 3156^T	JCM 13248^T
ISP medium 6					
Growth	Moderate	Poor	Moderate	Moderate	Moderate
Aerial mycelium	Grayish yellow	Moderate yellowish brown	Light orange yellow	Strong orange yellow	Vivid orange yellow
Reverse color	Grayish yellow	Moderate yellowish brown	Moderate orange yellow	Strong orange yellow	Vivid orange yellow
Soluble pigment	None	Strong yellowish brown	None	None	None
ISP medium 7					
Growth	Moderate	Good	Moderate	Moderate	Good
Colony color	Light yellow	Deep yellowish brown	Moderate yellow	Strong brown	Moderate orange yellow
Reverse color	Light yellow	Dark yellowish brown	Moderate yellow	Strong brown	Strong orange yellow
Soluble pigment	None	Strong yellowish brown	None	Light yellowish brown	None
Nutrient agar					
Growth	Poor	Good	Moderate	Good	Good
Colony color	Pale yellow	Dark yellowish brown	Vivid orange yellow	Strong yellowish brown	Strong orange yellow
Reverse color	Pale yellow	Dark yellowish brown	Moderate orange yellow	Strong yellowish brown	Strong orange yellow
Soluble pigment	None	Strong yellowish brown	None	Dark orange yellow	None

Table 4.13 Cellular fatty acid compositions (%) of *Micromonospora sediminis* CH3-3^T and closely related *Micromonospora* species. Fatty acids comprising less than 0.4% were omitted. –, not present

Fatty acid	CH3-3^T	*M. palomenae* JCM 30056^T	*M. coxensis* JCM 13248^T
Saturated fatty acids			
$C_{14:0}$	-	0.9	-
$C_{15:0}$	1.4	-	-
$C_{16:0}$	0.7	1.8	0.6
$C_{17:0}$	4.9	1.4	1.0
$C_{18:0}$	1.2	4.0	0.8
$C_{18:0}$ 10-methyl TBSA	-	0.9	-
$C_{19:0}$	0.4	0.5	-
$C_{20:0}$			
Unsaturated fatty acids			
$C_{17:1}$ **ω**8c	7.0	3.2	5.3
$C_{16:1}$ **ω**7c	0.6	-	1.0
$C_{18:1}$ **ω**9c	1.3	8.1	3.5
Unsaturated Branched fatty acids			
iso-$C_{15:1}$ G	-	0.7	-
iso-$C_{16:1}$ G	-	16.2	-
anteiso-$C_{17:1}$ **ω**9c	-	5.2	-
Branched fatty acids			
iso-$C_{14:0}$	0.7	2.6	1.4
iso-$C_{15:0}$	30.2	5.0	11.3
anteiso-$C_{15:0}$	3.2	5.9	3.4
iso-$C_{16:0}$	27.9	20.2	59.1
iso-$C_{17:0}$	14.8	2.5	2.6
iso-$C_{17:0}$ 3-OH	-	-	0.4
anteiso-$C_{17:0}$	5.0	7.8	7.4
iso-$C_{18:0}$	0.9	1.0	0.9
[a]Summed feature 3	-	1.4	-
[b]Summed feature 6	-	0.8	-
[c]Summed feature 9	-	5.9	-

[a]Summed feature 3 comprised $C_{16:1}$ **ω**6c and/or $C_{16:1}$ **ω**7c

[b]Summed feature 6 comprised $C_{19:1}$ **ω**11c and/or $C_{19:1}$ **ω**9c

[c]Summed feature 9 comprised $C_{16:0}$ 10-methyl and/or iso-$C_{17:1}$ **ω** 9c

Table 4.14 Differential characteristics between *Micromonospora sediminis* CH3-3^T and closely related *Micromonospora* species

Strain: 1, strain CH3-3^T; 2, *Micromonospora palomenae* JCM 30056^T 3, *M. halophytica* JCM 3125^T; 4, *M. purpureochromogenes* JCM 3156^T; 5, *M. coxensis* JCM 13248^T

Characteristics	1	2	3	4	5
Growth on ISP2 medium	Moderate	Good	Moderate	Good	Good
Color of colony	Dark grayish yellow	Light brown	Strong orange yellow	Strong orange yellow	Moderate orange yellow
Soluble pigment	Dark greenish yellow	Strong yellowish brown	none	Pale yellow	none
Gelatin liquefaction	-	+	+	w	+
Nitrate reduction	+	-	+	+	+
Utilization of					
L-arabinose	+	-	-	-	-
myo-inositol	-	w	+	-	-
cellobiose	+	+	-	+	+
D-mannitol	+	w	+	-	-
D-raffinose	-	-	+	+	+
L-rhamnose	+	+	+	-	-
sucrose	+	w	+	+	+
Enzyme activities of					
Alkaline phosphatase	-	+	-	+	+
Esterase Lipase (C8)	w	-	+	+	+
Lipase (C14)	-	-	+	-	-
Valine arylamidase	-	+	-	-	+
Cystine arylamidase	-	-	-	-	w
Trypsin	w	+	+	+	+
α-Chymotrypsin	+	+	-	+	+
Acid phosphatase	w	w	-	+	+
Napthol-AS-BI- phosphohydrolase	w	w	w	+	-
α-Galactosidase	-	-	-	+	+
β-Galactosidase	w	+	+	+	+
β-Glucuronidase	-	-	-	+	-
β-Glucosidase	w	-	-	-	w
N-acetyl-β-glucosaminidase	-	+	-	+	+

+, positive; w, weakly positive; -, negative

4.4 Characterization of selected *Streptomyces sanyensis* C10-9-1^T

Strain C10-9-1 produced long chain spores with smooth spore surface on aerial mycelia (Figure 4.20). The strain contained *LL*-diaminopimelic acid, glucose and ribose in its whole-cell hydrolysate. Based on these characteristics, it could be classified in the genus *Streptomyces*.

The almost complete 16S rRNA gene sequences (1487 nt) analysis revealed that it showed the highest 16S rRNA gene sequences similarity with *Streptomyces sanyensis* 219820^T (99.93 %). According to the phylogenetic analysis, the strain C10-9-1 shared the same node with *S. sanyensis* 219820^T (Figure 4.21). This was supported by the high bootstrap value of 100. Moreover, the morphology and phenotypic characteristics of the strain was similar to the previously described *S. sanyensis* 219820^T (Table 4.15; Table 4.16). However, they showed some different in cultural characteristics. The isolate C10-9-1 produced dark greenigh yellow pigment but *S. sanyensis* 219820^T did not produce any pigments (Sui *et al*., 2011). This might be influenced by the variable of organic materials as well as the presence or absence of sea water in the culture media. Based on the results mentioned, the isolate C10-9-1 was identified as *S. sanyensis* C10-9-1.

S. sanyensis was first proposed by Sui *et al*. (2011). At that time, they reported the cytotoxic activity against the human colon tumor cell line HCT-116 of the type strain of this species. However, the active compounds have not determined yet. According to the isolation of staurosporine and its derivatives in this study, it might be assumed that the anti-cancer activity in previous report came from these compounds.

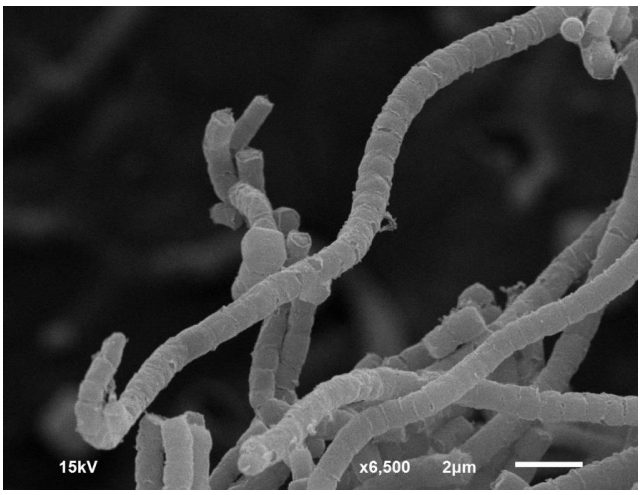

Figure 4.20 Scanning electron micrograph showing spore chain of *Streptomyces sanyensis* C10-9-1 after incubation at 30 °C 14 days on ISP 2 agar.

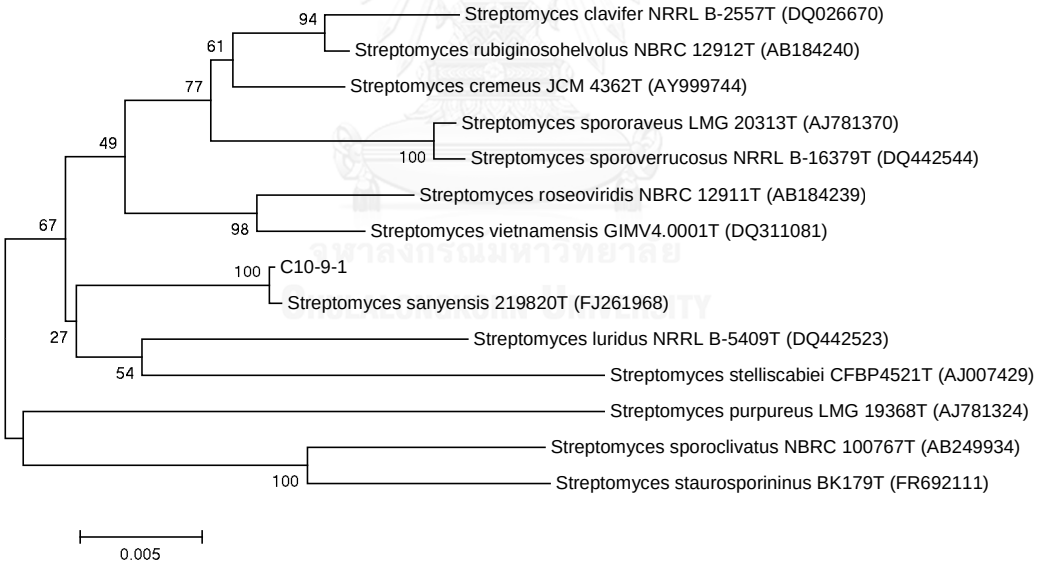

Figure 4.21 Phylogenetic relationship based on almost complete 16S rRNA gene of *Streptomyces sanyensis* C10-9-1 and closely related *Streptomyces* species.

Table 4.15 Cultural characteristics of the *Streptomyces sanyensis* C10-9-1 and *Streptomyces sanyensis* 219820^T

Media/ Characteristics	C10-9-1	*Streptomyces sanyensis* 219820^T (Sui *et al*., 2011)
Yeast extract-malt extract agar (ISP medium 2)		
Growth	Good	Good
Aerial mycelia	White/Light greenish yellow	White/ Gray
Reverse color	Light olive/ Grayish greenish yellow	Light yellowish brown
Soluble pigment	Dark greenish yellow	-
Oat meal agar (ISP medium 3)		
Growth	Good	Good
Aerial mycelia	White/ Light greenish gray/	White/ Gray
Reverse color	Moderate greenish yellow/ Dark greenish yellow	White
Soluble pigment	Dark greenish yellow	-
Inorganic-salt starch agar (ISP medium 4)		
Growth	Good	Good
Aerial mycelia	White/ Greenish gray	White/ Gray
Reverse color	Dark greenish yellow	Pale yellow
Soluble pigment	Light yellow green	-
Glycerol-asparagine agar (ISP medium 5)		
Growth	Moderate	Poor
Aerial mycelia	White	-
Reverse color	Light yellow green	Light yellowish brown
Soluble pigment	-	-
Peptone-yeast extract iron agar (ISP medium 6)		
Growth	White	Moderate
Aerial mycelia	Greenish gray	White
Reverse color	Dark grayish olive	Dark yellowish brown
Soluble pigment	Dark olive	-
Tyrosine agar (ISP medium 7)		
Growth	Good	Poor
Aerial mycelia	White	White
Reverse color	Moderate olive	Pale yellow
Soluble pigment	Dark greenish yellow	-
Nutrient agar		
Growth	Very good	Good
Aerial mycelia	Bluish gray	White
Reverse color	Dark greenish yellow	Dark yellowish brown
Soluble pigment	Dark greenish yellow	-

Table 4.16 Phenotypic comparison between *Streptomyces sanyensis* C10-9-1 and *Streptomyces sanyensis* 219820^T

Characteristics	**C10-9-1**	***Streptomyces sanyensis* 219820^T** (Sui *et al*., 2011)
Type of spore chain	*Rectiflexibile*	*Rectiflexibile*
Spore surface	Smooth	Smooth
Utilization of		
Glucose	+	+
Xylose	-	-
Arabinose	-	-
Mannitol	-	-
Sucrose	-	-
Ribose	w	+
Inositol	-	-
Raffinose	-	-
Fructose	w	-
Rhamnose	-	-
Galactose	-	-

+, positive; w, weakly positive; -, negative

4.5 Antimicrobial activities screening of marine actinomycete isolates

On the basis of preliminary screening for the antimicrobial activities by using four different broth media including YD, 301, 51 and 54 media, totally 44 isolates exhibited the activities against tested microorganism.

Twenty-seven *Streptomyces* isolates showed the antimicrobial activities against tested microorganisms. However, six isolates (SRN1-2, KK5-10, KRN1-1, CH3-1, SRN1-1 and LKB1-11) did not show any antimicrobial activities when cultured in four different media. Among the active isolates, anti-Gram-positive bacteria activity could be observed in most isolates while, anti-Gram-negative bacteria, anti-yeast and anti-mold activities were observed only 5, 8 and 4 isolates, respectively. The detailed for antimicrobial activities of the *Streptomyces* isolates are shown in Table 4.17 and 4.18.

Seventeen isolates of the family *Micromonosporaceae* showed antimicrobial activities against tested microorganisms. 11 isolates from genus *Salinispora* including the isolates SRK2-1, SRK1-3, SPM9-1, SPM3-7, SPM9-2, SPM3-6, SPM3-1, SRK1-2, SRK2-3, SRK1-1 an SPM 3-5 showed antimicrobial activities against *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341 and *Bacillus subtilis* ATCC 6633 while one isolates KRN2-1 showed activities against *Staphylococcus aureus* ATCC 25923 but, the isolate SPM3-3 were not observed for all activities (Table 4.19 and Table 4.20). All of these isolates were identified as *Salinispora arenicola* CNH-643^{T}.

Five isolates of the genus *Micromonospora* including the isolate PWB-003, CH7-4m, CH3-14, CPB1-2 and PW-004 showed antimicrobial activities against tested microorganisms. The isolate PWB-003, which identified as *Micromonospora fluostatini* sp. nov., showed antimicrobial activities against *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ KC213 and *Candida albicans* KF1. The isolate CH7-4m and CH3-14, identified as *Micromonospora chersina* ATCC 27029^{T}, exhibited activities against *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633. In addition, the isolates CPB1-12 and PWB-004 (identified as *Micromonospora marina* JSM 1-1^{T} and *Micromonospora olivasterospora* DSM 43868^{T}, respectively) exhibited

activities against *Kocuria rhizophila* ATCC 9341 and *Bacillus subtilis* ATCC 6633, respectively (Table 4.21 and Table 4.22). No activities were observed for the members of the genus *Nocardia*, *Jishengella* and *Verrucosispora*.

According to the preliminary screening for antimicrobial activities mentioned, the production media seem to be the important factor to observe the antimicrobial activities. The good example could be observed in the members of the *Salinispora* group (Table 4.19 and Table 4.20). No activities were observed when they were grown in 301 medium but, almost all isolates showed the activities against tested Gram-positive bacteria when they were grown in 51 medium. Beside this, the different between strains, same species but different isolate, are other factor for the determination of the antimicrobial activity. For example, based on the 16S rRNA gene sequences analysis, both of the isolates SPM3-3 and SRK1-2 were identified as the same species, *Salinispora arenicola* CNH-643^{T}. However, the isolate SPM3-3 did not show any activities in all tested media in contrasted the isolate SRK1-2 showed the activities in YD, 54, and 51 media. On the basis of this study, it suggested that the preliminary screening should be determined using various different media. Moreover, the same species of the isolates does not mean the same activities will be observed.

Table 4.17 Antimicrobial activities of the *Streptomyces* isolates when cultured in 301 and YD media

Isolates	Inhibition zone (mm)											
	Cultured in 301 medium						Cultured in YD medium					
	S	K	B	E	C	M	S	K	B	E	C	M
SRN1-2	-	-	-	-	-	-	-	-	-	-	-	-
LKB1-4	-	-	-	-	-	-	-	-	-	-	-	-
BM1-1	12.7	17.0	15, 21	19.9	15.7		-	9.8	9.7	11.0	-	-
KK5-10	-	-	-	-	-	-	-	-	-	-	-	-
LKB1-1	21.0	10.1	17.8	15.2	17.0	12.7	-	-	15.6	14.0	14.0	13.0
KRN1-1	-	-	-	-	-	-	-	-	-	-	-	-
CH3-1	-	-	-	-	-	-	-	-	-	-	-	-
D2-1	-	-	-	-	11.9	-	10.4	14.55	10.0		12.2	11.3
PWB-012	20.5	20.2	15.5	-	-	-	17.0	16.7	12.0	-	-	-
PWB-011	-	-	-	-	-	-	-	-	-	-	-	-
CPB2-10	16.3	19.4	11.8	-	-	-	17.3	19.4	12.5	-	-	-
BM1-4	16.9	18.7	12.0	-	-	-	16.1	19.1	11.6	-	-	-
BM2-6	14.9	-	-	-	-	-	18.0	-	-	-	-	-
CPB1-1	16.4	19.4	12.4	-	-	-	16.7	19.7	12.5	-	-	-
CPB1-18	16.1	16.7	11.4	-	-	-	16.5	17.0	11.4	-	-	-
CPB3-1	15.7	16.7	10.9	-	-	-	13.9	19.6	10.9	-	-	-
BM2-4	15.5	17.5	11.0	-	-	-	17.0	17.6	12.5	-	-	-
CPB1-13	14.4	14.9	9.75	-	-	-	22.9	17.15	11.6	-	-	-
LKB1-6	-	-	-	-	-	-	-	-	-	-	-	-
SRN1-1	-	-	-	-	-	-	-	-	-	-	-	-
A2-1	10.4	14.4	9.3	11.3	27.0		-	10.0	-	-	19.8	-
LKB1-5	-	-	-	-	-	-	-	-	-	-	-	-
LKB1-7	-	-	-	-	-	-	-	-	-	-	-	-
C10-9-1			14.8	-	-	-	-	-	-	-	-	-
PWB-010	-	9.6	8.2	-	-	-	-	10.7	10.8	11.3	-	-
PWB-016	-	11.4	8.3	-	-	-		12.0	9.0			
D2-2	12.1	11.0	19.0	-	-	-	-	-	-	-	-	-
LKB1-11	-	-	-	-	-	-	-	-	-	-	-	-
CH3-2	-	-	-	-	17.4	9.7	-	-	-	-	10.0	12.35
PWB-020	11.0	11.6	8.7	-	-	-	-	-	-	-	-	-
CPB4-7	-	-	-	-	10.7	-	-	-	-	-	-	-
KK1-2	-	-	-	-	13.0	-	-	-	-	-	-	-
CH7-4S	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: -, no activity; S, *Staphylococcus aureus* ATCC 25923; K, *Kocuria rhizophila* ATCC 9341; B, *Bacillus subtilis* ATCC 6633; E, *Escherichia coli* NIHJ KC213; C, *Candida albicans* KF1; M, *Mucor racemosus* IFO 4581.

Table 4.18 Antimicrobial activities of the *Streptomyces* isolates when cultured in 54 and 51 media

Isolates	Inhibition zone (mm)											
	Cultured in 54 medium						Cultured in 51 medium					
	S	K	B	E	C	M	S	K	B	E	C	M
SRN1-2	-	-	-	-	-	-	-	-	-	-	-	-
LKB1-4	-	-	-	-	-	-	8.5	-	-	-	-	-
BM1-1	14.0	15.9	21.3 , 10.0	19.12	15.4	-	-	-	-	-	-	-
KK5-10	-	-	-	-	-	-	-	-	-	-	-	-
LKB1-1	-	9.5	9.6	-	14.0	-	-	10.2	-	-	13.3	-
KRN1-1	-	-	-	-	-	-	-	-	-	-	-	-
CH3-1	-	-	-	-	-	-	-	-	-	-	-	-
D2-1	11.3	13.0	12.0	-	11.8	14.4	10.75	14.7	10.5	-	12.4	15.5
PWB-012	28.6	27.9	12.4	-	-	-	31.0	29.7	16.5	9.8	-	-
PWB-011	-	-	-	-	-	-	9.5	13.6	9.7	-	-	-
CPB2-10	18.8	19.3	12.3	-	-	-	18.6	20.3	10.7	-	-	-
BM1-4	18.5	19.5	11.6	-	-	-	22.0	21.4	11.8	-	-	-
BM2-6	16.4	-	-	-	12.0	-	11.5	-	-	-	10.0	-
CPB1-1	18.3	20.0	11.3	-	-	-	20.0	21.1	9.4	-	-	-
CPB1-18	16.3	18.3	9.9	-	-	-	17.2	19.4	10.5	-	-	-
CPB3-1	13.7	17.9	10.9	-	-	-	15.4	-	9.9	-	-	-
BM2-4	16.3	20.8	12.0	-	-	-	19.5	21.4	12.1	-	-	-
CPB1-13	22.7	18.6	13.3	-	-	-	15.5	17.8	12.8	-	-	-
LKB1-6	9.0	-	-	-	-	-	-	-	-	-	-	-
SRN1-1	-	-	-	-	-	-	-	-	-	-	-	-
A2-1	11.7	16.0	13.0	-	12.5	-	9.6	15.5	11.0	-	24.8	-
LKB1-5	-	9.3	13.9	-	-	-	-	-	-	-	-	-
LKB1-7	-	-	-	-	-	-	-	9.3	-	-	-	-
C10-9-1	-	10.2	15.4	-	-	-	-	-	-	-	-	-
PWB-010	-	-	-	-	-	-	-	8.1	15.0	11.9	-	-
PWB-016	-	-	-	9.3 (d)	-	-	-	-	12.3	10.2	-	-
D2-2	-	-	-	-	-	-	-	-	-	-	-	-
LKB1-11	-	-	-	-	-	-	-	-	-	-	-	-
CH3-2	-	-	-	-	12.0	-	-	-	-	-	-	-
PWB-020	14.0	14.65	9.8	-	-	-	12.7	16.2	10.4	-	-	-
CPB4-7	-	11.1	-	-	11.35	-	-	9.7	-	-	12.5	-
KK1-2	15.6	13.3	10.6	-	15.9	18.0	-	-	-	16.4	-	-
CH7-4S	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: -, no activity; S, *Staphylococcus aureus* ATCC 25923; K, *Kocuria rhizophila* ATCC 9341; B, *Bacillus subtilis* ATCC 6633; E, *Escherichia coli* NIHJ KC213; C, *Candida albicans* KF1; M, *Mucor racemosus* IFO 4581.

Table 4.19 Antimicrobial activities of the *Salinispora*, *Verrucosispora* and *Nocardia* isolates when cultured in 301 and YD media

Isolates	Inhibition zone (mm)											
	Cultured in 301 medium						Cultured in YD medium					
	S	K	B	E	C	M	S	K	B	E	C	M
Genus *Salinispora*												
KRN2-1	-	-	-	-	-	-	-	-	-	-	-	-
SRK2-1	-	-	-	-	-	-	-	-	-	-	-	-
SRK1-3	-	-	-	-	-	-	-	-	-	-	-	-
SPM9-1	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-7	-	-	-	-	-	-	-	9.5	-	-	-	-
SPM9-2	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-6	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-3	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-1	-	-	-	-	-	-	-	8.4	-	-	-	-
SRK1-2	-	-	-	-	-	-	-	19.9	-	-	-	-
SRK2-3	-	-	-	-	-	-	-	-	-	-	-	-
SRK1-1	-	-	-	-	-	-	-	-	-	-	-	-
SPM 3-5	-	-	-	-	-	-	-	-	-	-	-	-
Genus *Verrucosispora*												
KK2-1	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-8	-	-	-	-	-	-	-	-	-	-	-	-
Genus *Nocardia*												
CH3-9	-	-	-	-	-	-	-	-	-	-	-	-
PWB-002	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: -, no activity; S, *Staphylococcus aureus* ATCC 25923; K, *Kocuria rhizophila* ATCC 9341; B, *Bacillus subtilis* ATCC 6633; E, *Escherichia coli* NIHJ KC213; C, *Candida albicans* KF1; M, *Mucor racemosus* IFO 4581.

Table 4.20 Antimicrobial activities of the *Salinispora*, *Verrucosispora* and *Nocardia* isolates when cultured in 54 and 51 media

Isolates	Inhibition zone (mm)											
	Cultured in 54 medium						Cultured in 51 medium					
	S	K	B	E	C	M	S	K	B	E	C	M
Genus *Salinispora*												
KRN2-1	-	-	-	-	-	-	11.7	-	-	-	-	-
SRK2-1	14.5	-	-	-	-	-	15.6	24.8	8.9	-	-	-
SRK1-3	8.7	9.5	-	-	-	-	15.6	23.8	10.2	-	-	-
SPM9-1	10.9	-	-	-	-	-	15.1	21.1	8.1	-	-	-
SPM3-7	-	-	-	-	-	-	16.8	26.2	10.2	-	-	-
SPM9-2	8.7	-	-	-	-	-	15.0	22.5	10.2	-	-	-
SPM3-6	10.0	-	-	-	-	-	17.3	22.0	12.35	-	-	-
SPM3-3	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-1	13.3	-	-	-	-	-	16.8	23.5	9.2	-	-	-
SRK1-2	17.6	-	-	-	-	-	17.9	23.6	8.7	-	-	-
SRK2-3	11.1	-	-	-	-	-	15.6	18.5	11.0	-	-	-
SRK1-1	9.6	-	-	-	-	-	13.8	20.4	8.7	-	-	-
SPM 3-5	9.2	-	-	-	-	-	15.7	23.0	10.7	-	-	-
Genus *Verrucosispora*												
KK2-1	-	-	-	-	-	-	-	-	-	-	-	-
SPM3-8	-	-	-	-	-	-	-	-	-	-	-	-
Genus *Nocardia*												
CH3-9	-	-	-	-	-	-	-	-	-	-	-	-
PWB-002	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: -, no activity; S, *Staphylococcus aureus* ATCC 25923; K, *Kocuria rhizophila* ATCC 9341; B, *Bacillus subtilis* ATCC 6633; E, *Escherichia coli* NIHJ KC213; C, *Candida albicans* KF1; M, *Mucor racemosus* IFO 4581.

Table 4.21 Antimicrobial activities of the *Jishenge*lla and *Micromonospora* isolates when cultured in 301 and YD media

Isolates	Inhibition zone (mm)											
	Cultured in 301 medium						Cultured in YD medium					
	S	K	B	E	C	M	S	K	B	E	C	M
Genus *Jishengella*												
KK1-17	-	-	-	-	-	-	-	-	-	-	-	-
Genus *Micromonospora*												
CH3-3	-	-	-	-	-	-	-	-	-	-	-	-
PWB-003	18.0	40.0	15	15	13.0	-	22.6	23.55	16.4	13.0	-	-
BM2-1	-	-	-	-	-	-	-	-	-	-	-	-
KK4-8	-	-	-	-	-	-	-	-	-	-	-	-
KT2-1	-	-	-	-	-	-	-	-	-	-	-	-
PWB-005	-	-	-	-	-	-	-	-	-	-	-	-
BS-002	-	-	-	-	-	-	-	-	-	-	-	-
PW-001	-	-	-	-	-	-	-	-	-	-	-	-
BS-007	-	-	-	-	-	-	-	-	-	-	-	-
KT2-3	-	-	-	-	-	-	-	-	-	-	-	-
BS-003	-	-	-	-	-	-	-	-	-	-	-	-
CH7-4m	9.1	16.7	22.7	-	-	-	15.7	13.3	14.8	-	-	-
CH3-14	9.0	16.2	21.8	-	-	-	15.3	13.6	15.5	-	-	-
KK4-14	-	-	-	-	-	-	-	-	-	-	-	-
CH4-1	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-3	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-12	-	11.0	-	-	-	-	-	-	-	-	-	-
CPB1-14	-	-	-	-	-	-	-	-	-	-	-	-
KK1-10	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-11	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-21	-	-	-	-	-	-	-	-	-	-	-	-
PW-004	-	-	-	-	-	-	-	-	13.4	-	-	-
PW-002	-	-	-	-	-	-	-	-	-	-	-	-
PW-006	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: -, no activity; S, *Staphylococcus aureus* ATCC 25923; K, *Kocuria rhizophila* ATCC 9341; B, *Bacillus subtilis* ATCC 6633; E, *Escherichia coli* NIHJ KC213; C, *Candida albicans* KF1; M, *Mucor racemosus* IFO 4581.

Table 4.22 Antimicrobial activities of the *Jishengella* and *Micromonospora* isolates when cultured in 54 and 51 media

Isolates	Inhibition zone (mm)											
	Cultured in 54 medium						Cultured in 51 medium					
	S	K	B	E	C	M	S	K	B	E	C	M
Genus *Jishengella*												
KK1-17	-	-	-	-	-	-	-	-	-	-	-	-
Genus *Micromonospora*												
CH3-3	-	-	-	-	-	-	-	-	-	-	-	-
PWB-003	21.0	25.4	17.5	10.4	-	-	18.6	26.0	18.9	15.1	-	-
BM2-1	-	-	-	-	-	-	-	-	-	-	-	-
KK4-8	-	-	-	-	-	-	-	-	-	-	-	-
KT2-1	-	-	-	-	-	-	-	-	-	-	-	-
PWB-005	-	-	-	-	-	-	-	-	-	-	-	-
BS-002	-	-	-	-	-	-	-	-	-	-	-	-
PW-001	-	-	-	-	-	-	-	-	-	-	-	-
BS-007	-	-	-	-	-	-	-	-	-	-	-	-
KT2-3	-	-	-	-	-	-	-	-	-	-	-	-
BS-003	-	-	-	-	-	-	-	-	-	-	-	-
CH7-4m	-	-	15.4	-	-	-	-	9.1	9.6	-	-	-
CH3-14	-	-	14.8	-	-	-	-	8.0	8.8	-	-	-
KK4-14	-	-	-	11.8	-	-	-	-	-	-	-	-
CH4-1	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-3	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-12	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-14	-	-	-	-	-	-	-	-	-	-	-	-
KK1-10	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-11	-	-	-	-	-	-	-	-	-	-	-	-
CPB1-21	-	-	-	-	-	-	-	-	-	-	-	-
PW-004	-	-	-	-	-	-	-	-	-	-	-	-
PW-002	-	-	-	-	-	-	-	-	-	-	-	-
PW-006	-	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: -, no activity; S, *Staphylococcus aureus* ATCC 25923; K, *Kocuria rhizophila* ATCC 9341; B, *Bacillus subtilis* ATCC 6633; E, *Escherichia coli* NIHJ KC213; C, *Candida albicans* KF1; M, *Mucor racemosus* IFO 4581.

4.6 Secondary metabolites of three selected marine actinomycetes

Based on the taxonomic studies and HPLC profile analysis of the culture broth, two novel actinomycete species, *Micromonospora fluostatini* PWB-003^T and *Streptomyces chumphonensis* KK1-2^T, and a known *Streptomyces sanyensis* C10-9-1 showed good LC/UV chromatograms and were then selected for secondary metabolites study.

4.6.1 Isolation and structure elucidation of secondary metabolite from the *Micromonospora fluostatini* PWB-003^T

Micromonospora fluostatini PWB-003^T was cultured in 54 medium broth at 30 ^oC, 200 r.p.m. for 7 days. After incubation, the culture broth was centrifuged at 3,000 r.p.m. for 5 minutes to elimitate the cells. The HPLC analysis of the culture broth showed an interesting compound at RT 12.17 minutes (Figure 4.22). To isolate this compound, the culture broth was passed through the octadecylsilane (ODS) column, equilibrated with H_2O, and eluted with the stepwise gradient of H_2O – CH_3CN (100:0, 90:10, 70:30, 60:40, 30:70 and 0:100). The elute fraction containing the target compound (70:30) was evaporated to dryness. The extract was added with 100 ml water and was extracted with ethyl acetate three times. The ethyl acetate layer, containing the target compound, was concentrated using evaporator to yield a deep red solid (52.6 mg). This material was dissolved with small amount of MeOH and purified by HPLC (Pegasil ODS SP100; 20x250 mm; Senshu Sciencetific) with 55% MeOH in water at a flow rate of 15 ml/min. The target compound was obtained (PWB-003 P2, 20 mg). The isolation procedure of compound PWB-003 P2 was summarized in Figure 4.23.

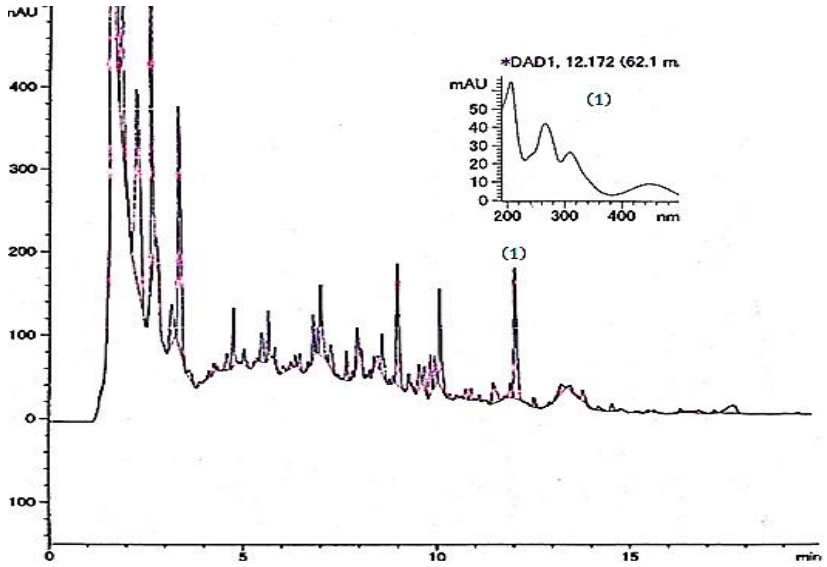

Figure 4.22 HPLC chromatogram of a culture broth of *M. fluostatini* PWB-003^{T} and UV-visible spectrum of the target compound (1)

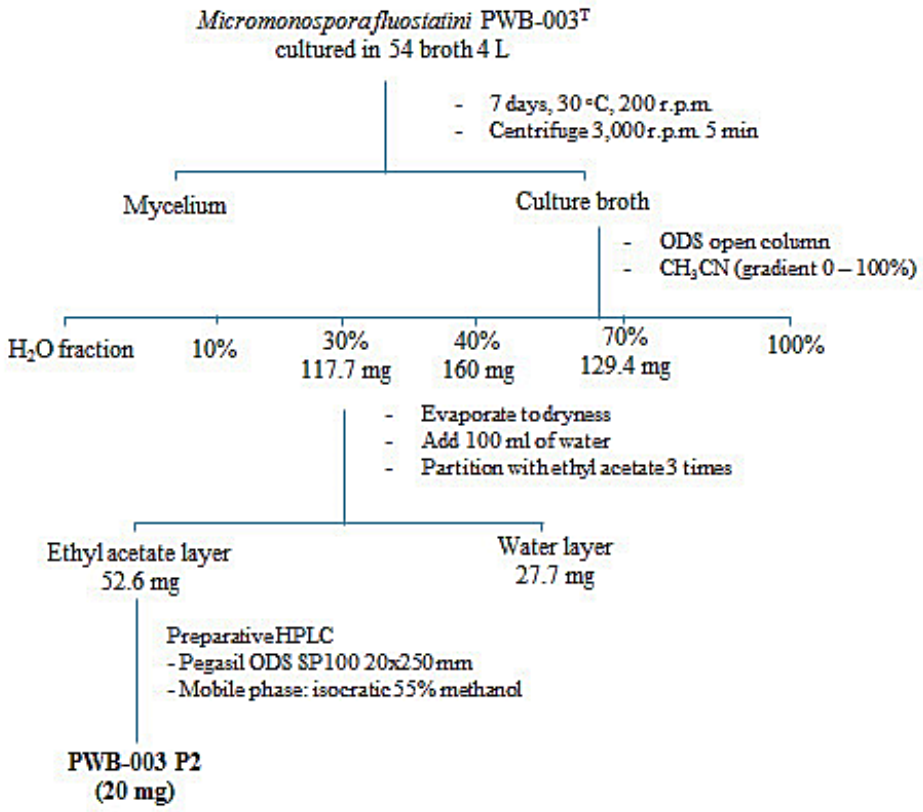

Figure 4.23 The isolation scheme showing the purification process of the selected secondary metabolite from *M. fluostatini* PWB-003^{T}

Compound PWB-003 P2 was obtained as a deep orange solid (20 mg). It showed maximum absorption at λ 215, 271, 348 and 539 nm in the UV spectrum. The LC-ESI-MS spectra indicated a pseudo-molecular ion of $[M+H]^+$ m/z 325.0717, suggesting the molecular formula $C_{18}H_{12}O_6$.

The ^1H NMR spectrum (in DMSO-d_6) indicated the characteristics signals of four aromatic protons at [δ_H 7.3 (s), 7.0 (d, *J* = 7.6 Hz), 7.27 (dd, *J* = 6.8, 7.6 Hz) and 7.1 (d, *J* = 6.8 Hz)], one oxy-methine [at δ_H 5.8 (d, *J* = 2.6 Hz)], one epoxy-methine [at δ_H 3.8 (d, *J* = 2.6 Hz)] and one methyl [at δ_H 1.5 (s)]. The ^{13}C NMR spectrum showed eighteen signals which corresponding to one methyl at δ_C 14.9 (C-12), four aromatic methine at δ_C 120.59 (C-5), 124.18 (C-8), 132.22 (C-9) and 116.20 (C-10), eight aromatic quaternary carbons at δ_C 131.56 (C-4a), 150.33 (C-6), 134.49 (C-6a), 125.60 (C-6b), 135.17 (C-10a), 151.18 (C-7), 131.56 (C-11a) and 131.88 (C-11b), two oxymethine carbons at δ_C 58.9 (C-1) and δ_C 62.2 (C-2), one quaternary oxymethine carbon at 58.1 (C-3), and two carbonyl carbons at δ_C 192.8 (C-4) and 192.16 (C-11).

The 2D NMR spectra (HSQC, COSY and HMBC) (Appendix D) indicated that the compound showed the chemical structure corresponding to the fluostatin C (Table 4.23). Interestingly, the coupling constant of protons at positions H-1 and H-2 of the compound PWB-003 P2 was 2.6 Hz, compared to that of fluostatin C 8.9 Hz (Schneider *et al*., 2006). In a previous report, the absolute stereochemistry of fluostatin C was determined by using Helmchen method and X-ray crystallography (Figure 4.24) and assigned as 1R, 2S and 3S. Based on the coupling constant velue, the compound PWB-003 P2 had the relative configuration between H-1 and H-2 as cis- (Figure 4.24). Therefore, the compound PWB-003 P2 sould be a new diastereoisomer of fluostatin C. However, the further experiments, such as nuclear overhauser effect spectroscopy (NOESY), optical rotatory dispersion, X-ray crystallography or the use of chiral shift reagents in proton NMR, must be dertermined to cinfirm this configulation.

Fluostatin C was first isolated from *Streptomyces* sp. Acta 1383 and showed cytotoxicity against human cancer cell lines including HMO2 (human gastric adenocarcinoma; $IG_{50,}$ 3.2 µg/ml), HepG2 (human hepatocellular carcinoma; IG_{50}, 3.1 µg/ml) and MCF7 (human breast carcinom.a; IG_{50}, 6.0 µg/ml) (Baur *et al*., 2006).

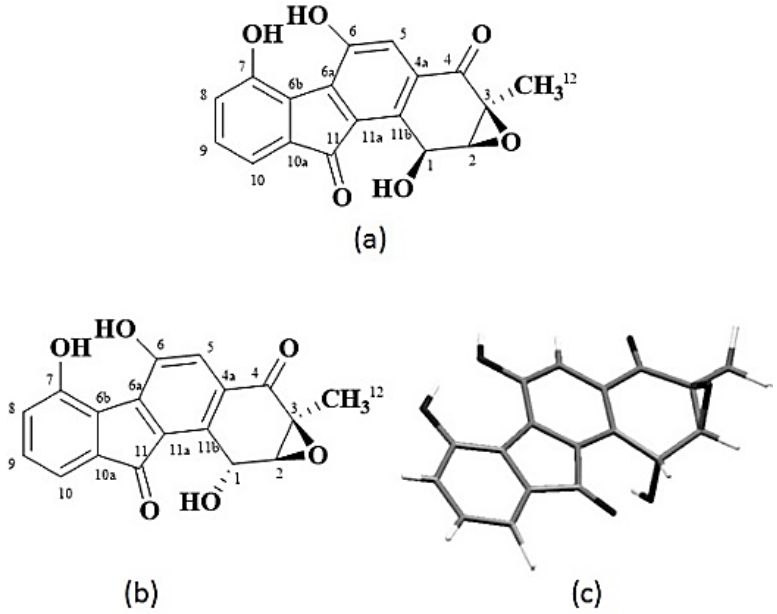

Figure 4.24 Chemical structure of compound PWB-003 P2 (a), fluostatin C (b) and X-ray structure of fluostatin C (c) (Schneider *et al*., 2006)

Table 4.23 The ^{1}H, ^{13}C, COSY and HMBC spectra data of the compound PWB-003P2 and fluostatin C

Position	PWB-003 P2 (in DMSO-d_6)				Fluostatin C (in DMSO-d_6) (Schneider *et al*., 2006)	
	δ_C (ppm)	δ_H (ppm) multiplicity (*J* in Hz)	COSY	HMBC (^{1}H to ^{13}C)	δ_C (ppm)	δ_H (ppm) multiplicity (*J* in Hz)
1	58.9	5.8 (d, 2.6)	2, 5*, 12	2, 3, 4a, 11a	59.2	5.79
2	62.2	3.8 (d, 2.6)	1, 12	1, 3, 11b, 12	62.2	3.78 (d, 8.51)
3	58.1	-	-	-	57.8	-
4	192.8	-	-	-	193.6	-
4a	131.6	-	-	-	130.7	-
5	120.6	7.3 (s)	1*	4, 6, 6a, 11b, 11a	121.0	7.12 (s)
6	150.3	-	-	-	155.4	-
6a	134.49	-	-	-	137.1	-
6b	125.60	-	-	-	127.7	-
7	151.18	-	-	-	155.6	-
8	124.18	7.0 (d, 7.6)	9, 10*	6b, 7, 10	124.7	6.79 (d, 8.05)
9	132.22	7.27 (dd, 6.8, 7.6)	8, 10	7, 8, 10a, 11	130.7	7.07 (dd, 7.00, 8.05)
10	116.20	7.1 (d, 6.8)	8*, 9	8, 6b, 7, 11	113.2	6.88 (d, 7.00)
10a	135.17	-	-	-	134.8	-
11	192.16	-	-	-	193.7	-
11a	131.56	-	-	-	130.7	-
11b	131.88	-	-	-	129.7	-
12	14.92	1.5 (s)	1*, 2*	1, 2, 3, 4	15.1	1.49 (s)

*, weak signal

4.6.2 Isolation and structure elucidation of secondary metabolite from the *Streptomyces chumphonensis* KK1-2^T

The fermentation and isolation procedures of the major secondary metabolite of *S. chumphonensis* KK1-2^T was summarized in Figure 4.21. The strain KK1-2^T was cultured in 54 medium broth (6 L) at 180 r.p.m. at 30 ^oC for 8 days. The 6-L volume of cultured broth was centrifuged at 3,000 r.p.m. for 5 minutes. Cell mycelia cake was extracted with ethanol for 5 hours and then was filtered. The filtrate was evaporated to dryness and resuspended with 300 ml of distilled water. The aqueous suspension was partitioned with ethyl acetate three times (300 ml, each), then the combined ethyl acetate layer was concentrated by evaporation to yield a yellow gum (1.15 g).

The HPLC analysis of the mycelial extract showed a main compound at RT 19.81 minutes (Figure 4.25). To purify this compound, the crude extract was applied to an octadesylsilane (ODS) column, equilibrated with water, and eluted using a stepwise gradient of H_2O-MeOH (100:0, 90:10, 60:40, 40:60, 20:80 and 0:100). Two fractions containing the target compound were eluted with H_2O-MeOH (20:80 and 0:100) and were concentrated to yield 220.6 and 703.9 mg, respectively. Both fractions were dissolved with small amount of MeOH and purified by HPLC (Pegasil ODS SP100; 20x250 mm; Senshu Sciencetific) with isocratic 85% MeOH at 15 ml/min. The amount of the target compound was 170.4 mg (14.82 % yield of the crude extract) and coded as KK1-2 P1 (Figure 4.26).

Compound KK1-2 P1 was obtained as colorless oil (170.4 mg). It showed maximum absorption at λ 235 and 267 nm in the UV spectrum. The HRESIMS showed a pseudo-molecular ion $[M+H]^+$ at *m/z* 416.2795, suggesting the molecular formula $C_{25}H_{37}NO_4$.

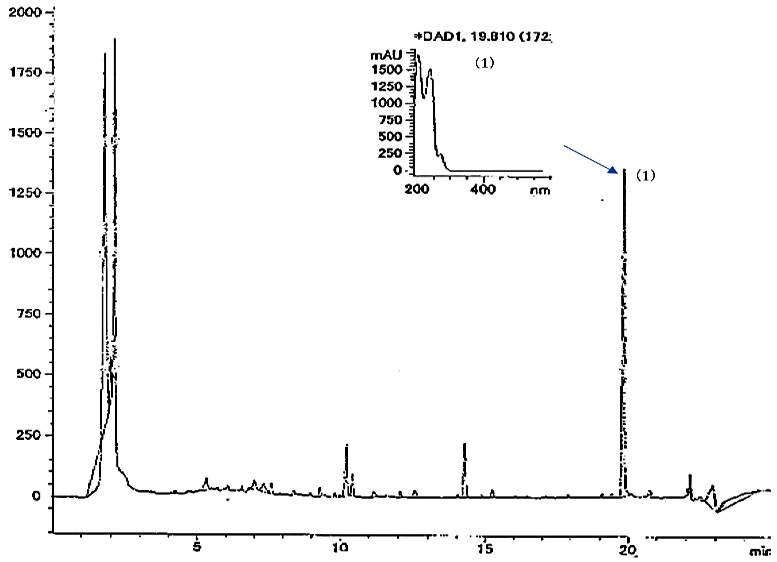

Figure 4.25 HPLC chromatogram of the mycelial extract of *S. chumphonensis* KK1-2^T and the UV-visible spectrum of the target compound **(1)**

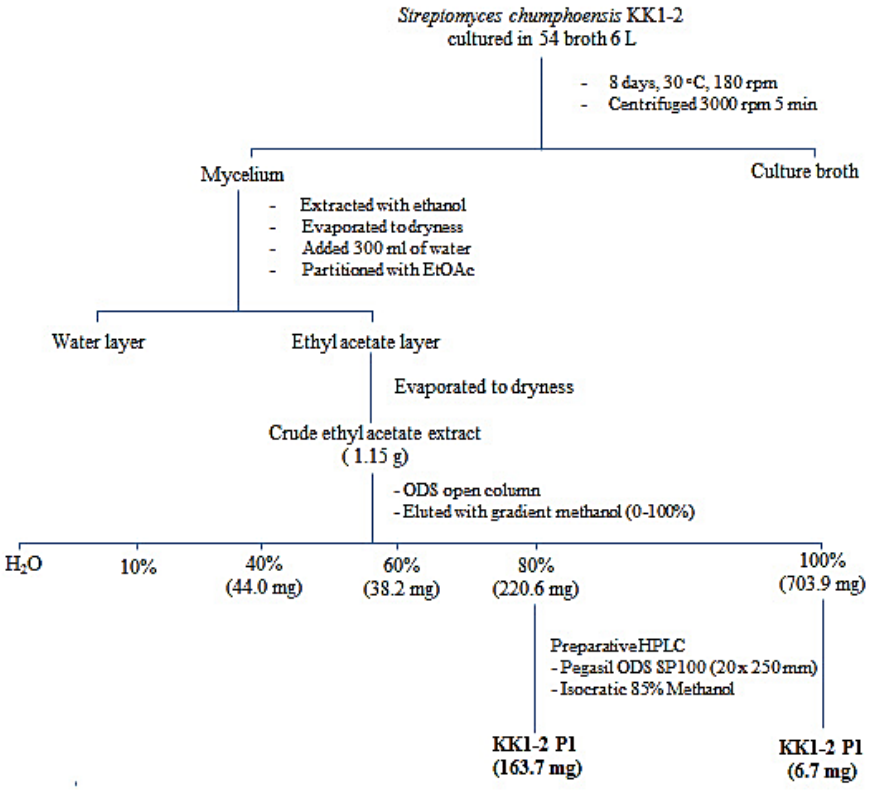

Figure 4.26 The isolation scheme showing the purification process of the secondary metabolites from *S. chumphonensis* KK1-2^T

The ^{1}H NMR spectrum of compound KK1-2 P1 (in $CDCl_3$) displayed characteristic proton signals of six methyls [at δ_H 1.60 (d, J = 6.3 Hz), 1.73 (s), 1.79 (s), 0.78 (d, J = 6.6 Hz), 1.62 (s) and 2.20 (s)], two methoxys [at δ_H 3.81 (s) and 3.92 (s)], two methylenes [at δ_H 3.35 (d, *J* = 6.9 Hz) and 2.77 (d, *J* = 6.9 Hz)], two methines [at δ_H 2.66 (m) and 3.61 (d, *J* = 9.1 Hz] and five olefinic methines [at δ_H 5.39 (t, *J* = 6.8, 13.6 Hz), 5.58 (m), 6.07 (d, *J* = 15.6 Hz), 5.2 (d, *J* = 9.71 Hz) and 5.46 (m)]. The ^{13}C NMR and DEPT 135 spectra gave twenty five carbon signals which corresponding to six methyls [at δ_C 10.5 (C-6′), 13.0 (C-13), 16.5 (C-14), 12.9 (C-15), 17.3 (C-16) and 10.4 (C-17)], two methoxys [at δ_C 60.5 (C-7′) and 53.0 (C-8′)], two methylenes [at δ_C 34.3 (C-1) and 43.0 (C-4)], one methine [at δ_C 36.8 (C-9)] one oxymethine [at δ_C 82.8 (C-10)], five olefinic methines [at δ_C 122.2 (C-2), 126.6 (C-5), 135.6 (C-6), 133.0 (C-8), 123.3 (C-12)], three quaternary olefinics at [δ_C 134.7 (C-3), 135.8 (C-7) and 135.5 (C-11)] and five quaternary aromatics [at δ_C 150.7 (C-1′), 112.1 (C-2′), 154.1 (C-3′), 127.9 (C-4′) and 153.5 (C-5′)].

According to the 2D NMR spectra (COSY, HMQC, HMBC), compound KK1-2 P1 showed identical NMR data as previously described piericidin A1 (Table 4.24) (Liu *et al*., 2012). On the basis of NMR and mass spectra data, compound KK1-2 P1 was identified as piericidin A1 (Figure 4.27).

Piericidin A1 was first isolated from the type strain of *Streptomyces mobaraensis* (Tamura *et al*., 1963). It was found to be a potent mitochondrial electron transport inhibitor (Jeng *et al*., 1968).

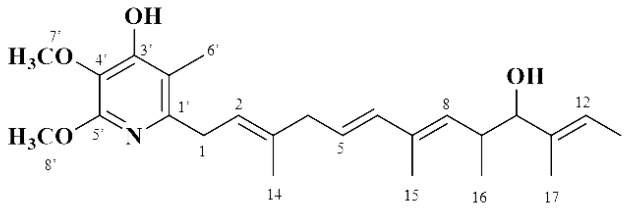

Figure 4.27 The chemical structure of compound KK1-2 P1 (piericidin A1)

Table 4.24 The selected ^{1}H, ^{13}C, COSY and HMBC spectra data of compound KK1-2P1 and piericidin A1

Position	KK1-2 P1 (in $CDCl_3$)				Piericidin A1(in $CDCl_3$) (Liu *et al*., 2012)	
	δ_C (ppm)	δ_H (ppm) multiplicity (*J* in Hz)	COSY	HMBC (^{1}H to ^{13}C)	δ_C (ppm)	δ_H (ppm) multiplicity (*J* in Hz)
1	34.3	3.35 (d, 6.9)	2, 4*, 14*, 6′*	1′, 2′, 2, 3	33.4	3.36 (d, 6.9)
2	122.2	5.39 (t, 13.6, 6.8)	1, 4*, 14*	4, 14	122.2	5.43 (t, 13.1, 6.9)
3	134.7	-	-	-	134.8	-
4	43.0	2.77 (d, 6.9)	2*, 5* ,6*	2, 5, 14	43.1	2.78 (d, 6.9)
5	126.6	5.58 (m)	4, 6	4, 7	126.8	5.65 (m)
6	135.6	6.07 (d, 15.6)	4*, 5	4, 7, 8, 15	135.7	6.08 (d, 15.6)
7	135.8	-	-	-	136.0	-
8	133.0	5.20 (d, 9.7)	9, 15*	6, 9, 10, 15, 16	133.1	5.23 (d, 9.6)
9	36.8	2.66 (m)	8, 10, 16	7, 8 , 10, 16	36.3	2.68 (m)
10	82.8	3.61 (d, 9.1)	9	9, 12, 16, 17	82.8	3.62 (d, 9.1)
11	135.5	-	-	-	135.6	-
12	123.3	5.46 (m)	13	10, 13, 17	123.5	5.5 (m)
13	13.0	1.60 (d, 6.3)	12	11, 12	13.2	1.60 (d, 5.3)
14	16.5	1.73 (s)	1*, 2*, 4*	2, 3	16.6	1.75 (s)
15	12.9	1.79 (s)	8*	6, 8	13.1	1.73 (s)
16	17.3	0.78 (d, 6.6)	9	8, 9, 10	17.3	0.79 (d, 6.7)
17	10.4	1.62 (s)	-	10, 11, 13	10.5	1.59 (s)
1′	150.7	-	-	-	150.8	-
2′	112.1	-	-	-	112.0	-
3′	154.1	-	-	-	154.0	-
4′	127.9	-	-	-	127.8	-
5′	153.5	-	-	-	153.5	-
6′	10.5	2.20 (s)	-	1′, 2′, 3′	10.5	2.28 (s)
7′	60.5	3.81 (s)	-	4′	60.6	3.85 (s)
8′	53.0	3.92 (s)	-	5′	53.0	3.95 (s)

*, weak signal

4.6.3 Isolation and structure elucidation of secondary metabolites from *Streptomyces sanyensis* C10-9-1.

Streptomyces isolate C10-9-1 was cultured in 20L of the 54 medium broth at 30 °C, 200 r.p.m. for 14 days. After incubation, the culture broth was extracted with ethyl acetate three times and then the ethyl acetate layer was evaporated to dryness to yield a gum (4.14 g). The crude ethyl acetate extract showed the anticancer activity against KB (oral cavity epidermal cancer, MIC 38.92 µg/ml), MCF7 (breast cancer, MIC 32.26 µg/ml) and NCI-H187 (small cell lung cancer, MIC 4.53 µg/ml) and showed antifungal activity against *Candida albicans* (IC_{50} 30.47 µg/ml). The chemical profile of the crude extract is shown in Figure 4.28.

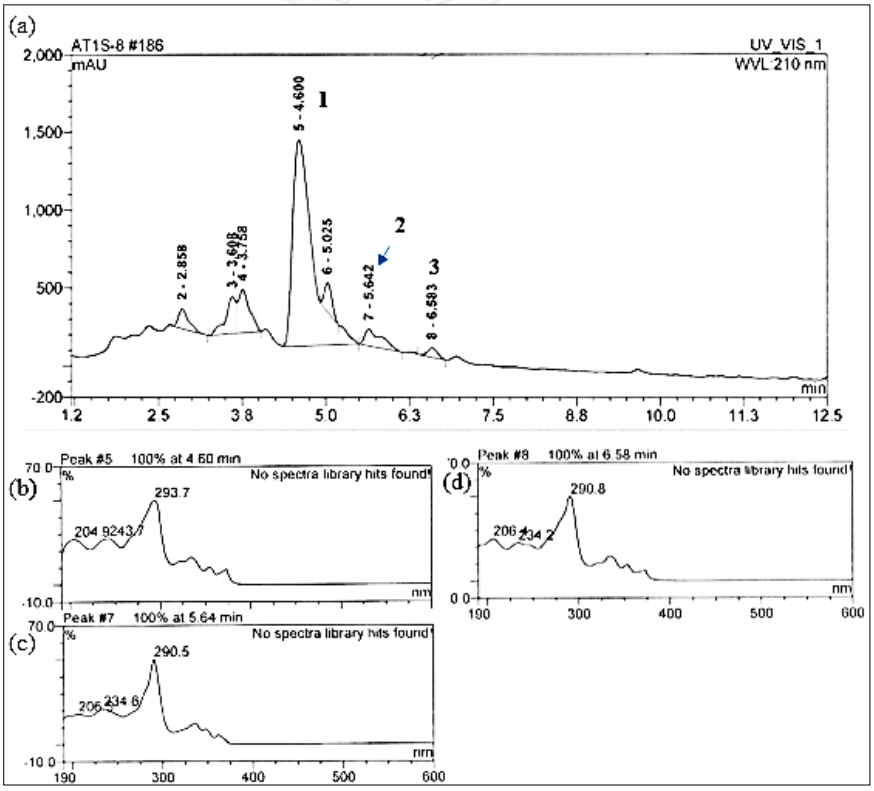

Figure 4.28 The HPLC chromatogram of the crude EtOAc extract from *Streptomyces sanyensis* C10-9-1 (a), UV profile of the peak at RT 4.60 (b), 5.64 (c) and 6.58 (d) minutes, respectively. HPLC condition (column: C18 (5 µm, 2.1 × 50 mm), mobile phase: 0-100% CH_3CN in water + 0.05% formic acid; flow rate: 0.5 ml/min.

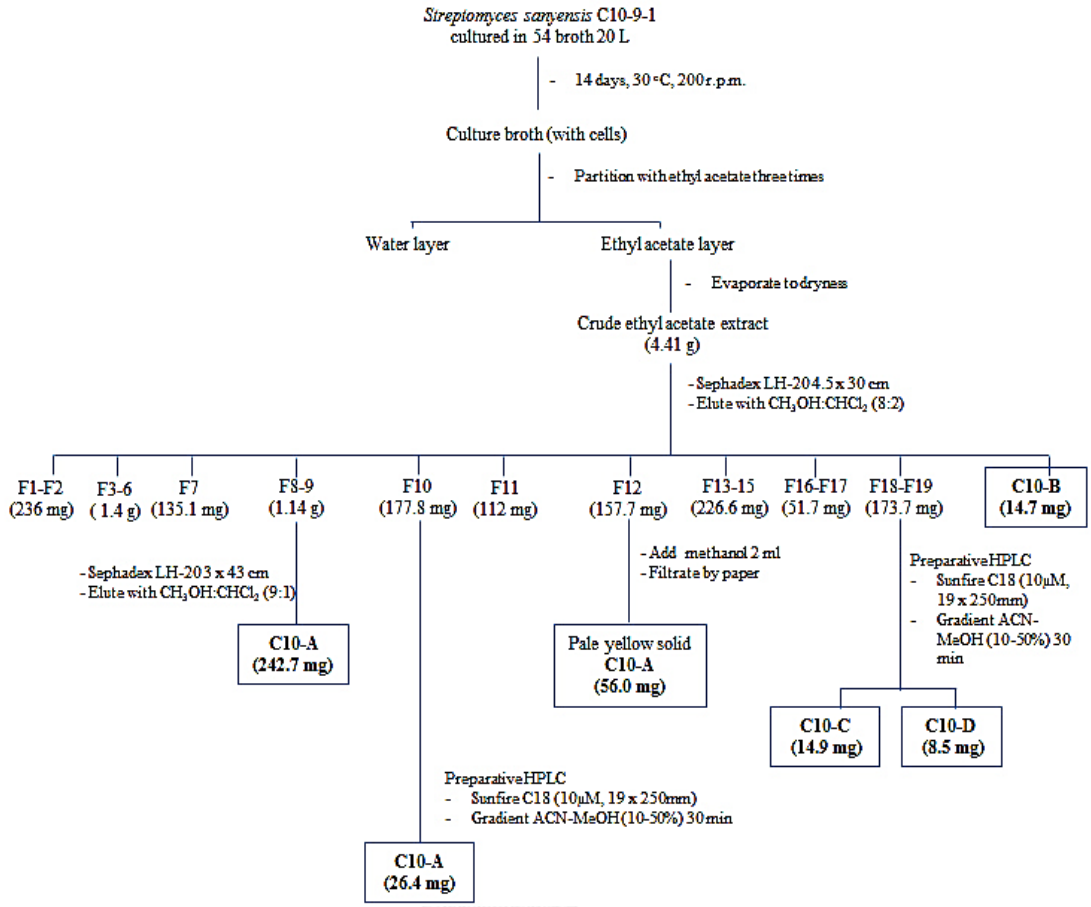

Figure 4.29 The isolation scheme showing the purification process of the secondary metabolites from *Streptomyces sanyensis* C10-9-1

The crude extract was purified by using chromatographic techniques. Four compounds, including C10-A, C10-B, C10-C and C10-D, were isolated. The isolation procedure of these four compounds was summarized in Figure 4.29.

Compound C10-A was obtained as a pale yellow solid (325.1 mg). It showed maximum absorption at λ 214, 238, 292, 320, 334, 353, 370, 418 nm in the UV spectrum. The optical rotation was $[\alpha]^{25}_{D}$ +71.2° (c 0.10%, MeOH). The HRESIMS spectrum showed a pseudo-molecular ion $[M+H]^+$ at *m/z* 467.2078, suggesting the molecular formula $C_{28}H_{27}N_4O_3$.

The ^1H NMR spectrum of compound C10-A (in DMSO-d_6) displayed signals of two methyl [at δ_H 1.45 (s) and 2.30 (s)], one methoxy [at δ_H 3.35 (s)], two methylenes

[at δ_H 4.94 (s) and 2.50 (m)], three methines [at 3.27 (q, *J* = 3.4 Hz), 4.07 (d, *J* = 3.4 Hz) and 6.70 (t, *J* = 3.5 Hz)], eight aromatic methines [at δ_H 7.26 (dd, *J* = 7.6, 7.9 Hz), 7.28 (dd, *J* = 7.1, 8.2 Hz), 7.41 (dd, *J* = 7.1, 8.6 Hz), 7.45 (dd, *J* = 7.6, 8.3 Hz), 7.59 (d, *J* = 8.3 Hz), 7.96 (d, *J* = 8.1 Hz), 7.98 (d, *J* = 8.6 Hz), and 9.27 (d, *J* = 7.9 Hz)], and NH proton [at 8.52 (br s)]. The ^{13}C NMR spectrum (Appendix D) gave 28 signals, which was differentiated by DEPT-135 spectrum (Appendix D), consisting of three methyls [at δ_C 30.2 (2′-CH_3), 33.8 (4′-NCH_3), and 57.8 (3′-OCH_3)], two methylenes [at δ_C 30.0 (C-5′) and 45.9 (C-7)], eleven methines [at δ_C 50.8 (C-4′), 80.5 (C-6′), 83.4 (C-3′), 108.1 (C-1), 115.6 (C-11), 118.7 (C-3), 120.2 (C-9), 121.3 (C-8), 124.6 (C-2), 124.8 (C-10), and 125.4 (C-4)], twelve quaternary carbons [at δ_C 91.7 (C-2′), 114.0 (C-7b), 114.7 (C-4b), 119.3 (C-4c), 123.06 (C-4a), 124.4 (C-7c), 127.2 (C-12b), 130.5 (C-12a), 132.5 (C-7a), 136.9 (C-13a), 139.9 (C-11a), and 172.7 (C-5)].

The 2D NMR spectral data including COSY, HMQC, HMBC data (Appendix D) confirmed the chemical structure of compound C10-A (Figure 4.30), which was consistent with the previously described staurosporine (Meksuriyen & Cordell, 1988) (Table 4.25).

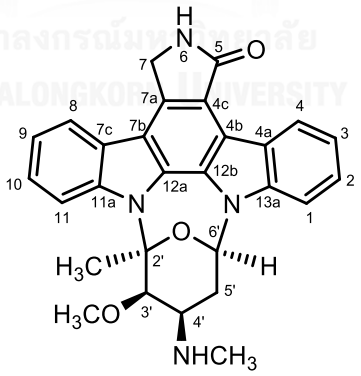

Figure 4.30 The chemical structure of compound C10-A (staurosporine)

Table 4.25 The ^{1}H and ^{13}C spectra data of the compound C10-A and staurosporine

Position	C10-A (in DMSO-d_6)		Staurosporine (in $CDCl_3$) (Meksuriyen & Cordell, 1988)	
	δ_C (ppm)	δ_H (ppm) multiplicity (*J* in Hz)	δ_C (ppm)	δ_H (ppm) multiplicity (*J* in Hz)
1	108.1, CH	7.59 (d, 8.3)	106.9	7.26 (t, 7.6)
2	124.6, CH	7.45 (dd, 7.6, 8.3)	124.9	7.46 (t, 7.6)
3	118.7, CH	7.26 (dd, 7.6, 7.9)	119.6	7.35 (t, 7.6)
4	125.4, CH	9.27 (d, 7.9)	127.0	9.42 (t, 7.6)
4a	123.1, C	-	123.3	-
4b	114.7, C	-	115.3	-
4c	119.3, C	-	132.1	-
5	172.7, C	-	173.6	-
6 -NH	-	8.52 (br s)	-	6.81 (brs)
7	45.9, CH_2	4.94 (s)	45.9	4.99 (AB)
7a	132.5, C	-	118.4	-
7b	114.0, C	-	114.0	-
7c	124.4, C	-	124.5	-
8	121.3, CH	7.96 (d, 8.1)	120.5	7.87 (d, 7.8)
9	120.2, CH	7.28 (dd, 7.1, 8.1)	119.9	7.30 (t, 7.8)
10	124.8, CH	7.41 (dd, 7.1, 8.6)	124.1	7.41 (t, 7.8)
11	115.6, CH	7.98 (d, 8.6)	115.1	7.91 (t, 7.8)
11a	139.9, C	-	139.6	-
12a	130.5, C	-	130.6	-
12b	127.2, C	-	128.2	-
13a	136.9, C	-	136.5	-
2′	91.7, C	-	91.0	-
3′	83.4, CH	4.07 (d, 3.4)	84.0	3.86 (d, 3.6)
4′	50.8, CH	3.27 (q, 3.4)	50.4	3.33 (t, 3.6)
5′	30.0, CH_2	2.50 (superimposed on DMSO-d_6)	30.1	2.71 (dd, 14.7, 3.6) 2.39 (ddd, 14.7, 5.2, 3.6)
6′	80.5, CH	6.70 (t, 3.5)	80.1	6.5 (d, 5.2)
2′- Me	30.2, CH_3	2.30 (s)	30.0	2.3 (s)
3′-OMe	57.8, CH_3	3.33 (s)	57.2	3.3 (s)
4′-NMe	33.8, CH_3	1.44 (s)	33.2	1.5 (s)

Compound C10-B was obtained as a pale yellow solid (14.7 mg). Its molecular formula was determined to be $C_{20}H_{13}N_3O$, based on the HRESIMS data, showing a pseudo-molecular ion at *m/z* 310.0986 $[M-H]^-$. The chemical structure of this compound was determined by the spectroscopic analyses of ^1H, ^{13}C NMR, COSY, HMQC and HMBC data (Appendix D).

The ^1H NMR spectrum of compound C10-B (in DMSO-d_6) showed signals of one methylene [at δ_H 4.9 (s)], eight aromatic methines [at δ_H 7.22 (dd, *J* = 7.2, 7.9 Hz), 7.31 (dd, *J* = 7.3, 7.7 Hz), 7.47 (dd, *J* = 7.3, 8.0 Hz), 7.42 (dd, *J* = 7.2, 8.1 Hz), 7.71 (d, *J* = 8.1 Hz), 7.78 (d, *J* = 8.0 Hz), 8.04 (d, *J* = 7.7 Hz) and 9.2 (d, *J* = 7.9 Hz)], and three NH protons [at δ_H 8.48 (s), 11.47 (s) and 11.64 (s)]. The ^{13}C and DEPT-135 spectra of compound C10-B (Appendix D) gave 20 signals corresponding to one methylene [at δ_C 45.3 (C-7)], eight methines [δ_C 111.3 (C-1), 111.9 (C-11), 118.9 (C-3), 119.9 (C-9), 121.1, (C-8), 125.0 (C-2), 125.0 (C-10) and 125.2 (C-4)] and eleven quaternary carbons [δ_C 112.8 (C-4a), 114.1 (C-7b), 115.6 (C-4b), 118.9 (C-4c), 122.6 (C-7c), 125.4 (C-12b), 127.9 (C-12a), 132.9 (C-7a), 139.1 (C-11a), 139.3 (C-13a) and 172.5 (C-5)] (Table 4.26).

The 1D and 2D NMR spectral information of compound C10-B gave the similar assignments to the previously described K252C (aglycone moiety of staurosporine) (Meksuriyen & Cordell, 1988). The structure of compound C10-B is shown in Figure 4.31.

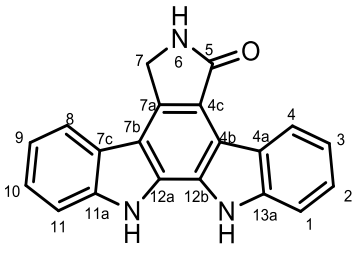

Figure 4.31 The chemical structure of compound C10-B (K232C, aglycone moiety of staurosporine)

Table 4.26 The ^{1}H and ^{13}C spectra data of the compound C10-B and K252C (aglycone moiety of staurosporine)

Position	C10-B (in DMSO-d_6)		K252C (in DMSO-d_6) (Meksuriyen & Cordell, 1988)	
	δ_C (ppm)	δ_H (ppm) multiplicity, J in Hz	δ_C (ppm)	δ_H (ppm) multiplicity, J in Hz
1	111.3, CH	7.71 (d, 8.1)	112.0, CH	7.73 (d, 8.3)
2	125.0(2), CH	7.42 (dd, 7.2, 8.1)	125.1, CH	7.44 (br t)
3	118.9, CH	7.22 (dd, 7.2, 7.9)	119.0, CH	7.24 (br t)
4	125.2, CH	9.20 (d, 7.9)	125.4, CH	9.24 (d, 7.9)
4a	122.8, C	-	123.0, C	-
4b	115.6, C	-	115.7, C	-
4c	118.9, C	-	120.0, C	-
5	172.5, C	-	172.6, C	-
6 -NH	-	8.48 (s)	-	8.49 (br s)
7	45.3, CH_2	4.96 (s)	45.4, CH_2	4.98 (s)
7a	132.9, C	-	133.0, C	-
7b	114.1, C	-	114.2, C	-
7c	122.6, C	-	122.7, C	-
8	121.1, CH	8.04 (d, 7.7)	121.2, CH	8.05 (d, 7.8)
9	119.9, CH	7.31 (dd, 7.3, 7.7)	120.0, CH	7.31 (br t)
10	125.0, CH	7.47 (dd, 7.3, 8.0)	125.1, CH	7.48 (br t)
11	111.9, CH	7.78 (d, 8.0)	111.4, CH	7.79 (d, 8.1)
11a	139.1, C	-	139.2, C	-
12-NH	-	11.64 (s)	-	11.56 (br s)
12a	127.9, C	-	128.0, C	-
12b	125.4, C	-	125.2, C	-
13-NH	-	11.47 (s)	-	11.38 (br s)
13a	139.3, C	-	139.3, C	-

Compound C10-C was obtained as a yellow solid (14.9 mg). It showed maximum absorption at λ 214, 234, 289, 319, 333, 345, 363, 421 nm in UV spectrum. The optical rotation was $[\alpha]^{25}_{D}$ +26.1° (c 0.09%, MeOH). The HRESIMS showed a pseudo-molecular ion $[M+H]^+$ at *m/z* 458.1710, suggesting the molecular formula of $C_{26}H_{23}N_3O_5$. This compound had a consistent spectral data as staurosporine but different in an aglycone moiety. The ^{1}H spectrum of C10-C (in DMSO-d_6) gave signals of one methyl [at δ_H 1.70 (d, *J* = 7.3 Hz)], one methylene [at δ_H 5.0 (d, *J* = 17.5 Hz)], five methines [at 4.05 (d, 3.1 Hz), 4.18 (br s), 4.48 (2H, m) and 6.39 (d, *J* = 9.5 Hz)], eight aromatic protons [at δ_H 7.27 (t, *J* = 7.6 Hz), 7.31 (t, *J* = 7.5 Hz), 7.49 (2H, m), 7.6 (d, *J* = 8.1 Hz), 7.69 (d, *J* = 8.5 Hz), 8.07 (d, *J* = 8.5 Hz) and 9.47 (d, *J* = 7.6 Hz)], three -OH [at δ_H 5.06 (d, *J* = 7.3 Hz), 5.46 (bs) and 6.76 (bs)] and two NH protons [at δ_H 8.56 (s) and 11.70 (s)]. The ^{13}C NMR and DEPT-135 (Appendix D) gave 26 carbons including one methyl [at δ_C 16.5 (C-6′)], one methylene [at δ_C 46.3 (C-7)], thirteen methines [at δ_C 68.2 (C-2′), 72.7 (C-3′), 72.9 (C-4′), 77.7 (C-5′), 78.4 (C-1′), 110.0 (C-1), 112.4 (C-11), 120.4 (C-3), 121.0 (C-9), 122.3 (C-8), 126.2 (C-2), 126.3 (C-10) and 126.8 (C-4)], and eleven quaternary carbons [at δ_C 116.1 (C-7b), 118.8 (C-4b), 119.8 (C-4c), 123.1 (C-7c), 123.5 (C-4a), 125.8 (C-12b), 128.8 (C-12a), 135.1 (C-7a), 140.3 (C-11a), 141.5 (C-13a), and 173.4 (C-5)] (Table 4.27). According to the ^{1}H, ^{13}C and 2D NMR (COSY, HMQC and HMBC) spectral information (Appendix D), compound C10-C had the chemical structure (Figure 4.32) the same as the previously described staurosporine derivative, K232D (Li *et al*., 2013).

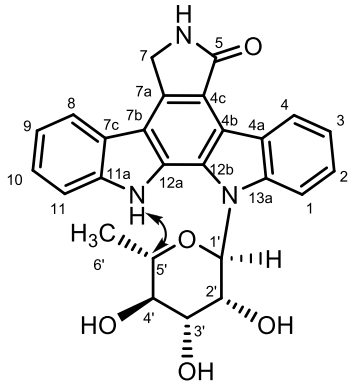

Figure 4.32 The chemical structure of compound C10-C. The arrow (↔) indicated the significant NOESY correlation.

Table 4.27 The ^{1}H and ^{13}C data of compound C10-C and K232D

Position	C10-C (in DMSO-d_6)		K232D (in DMSO-d_6) (Li *et al*., 2013)	
	δ_C (ppm)	δ_H (ppm) multiplicity, *J* in Hz	δ_C (ppm)	δ_H (ppm) multiplicity, *J* in Hz
1	110.0, CH	7.69 (d, 8.5)	110.3, CH	7.69 (d, 8.5)
2	126.2, CH	7.49 (m)	125.4, CH	7.48 (m)
3	120.4, CH	7.27 (t, 7.6)	119.7, CH	7.27 (dt, 7.5, 0.8)
4	126.8, CH	9.47 (d, 7.6)	125.8, CH	9.45 (d, 7.8)
4a	123.5, C	-	122.9, C	-
4b	118.8, C	-	Nd	-
4c	119.8, C	-	118.8, C	-
5	173.4, C	-	172.4, C	-
6 -NH	-	8.56 (s)	-	8.53 (br s)
7	46.3, CH_2	5.00 (d, 17.5)	45.6, CH_2	5.00 (AB, 17.5)
7a	135.1, C	-	134.4, C	-
7b	116.1, C	-	115.4, C	-
7c	123.1, C	-	122.5, C	-
8	122.3, CH	8.07 (d, 8.5)	121.5, CH	8.06 (d, 7.8)
9	121.0, CH	7.31 (t, 7.5)	120.2, CH	7.31 (dt, 7.4, 0.9)
10	126.3, CH	7.49 (m)	125.4, CH	7.50 (m)
11	112.4, CH	7.60 (d, 8.1)	111.8, CH	7.60 (d, 8.1)
11a	140.3, C	-	139.4, C	-
12 -NH	-	11.70 (s)	-	11.68 (br s)
12a	128.8, C	-	Nd	-
12b	125.8, C	-	124.9, C	-
13a	141.5, C	-	140.5, C	-
1′	78.4, CH	6.39 (d, 9.5)	77.3, CH	6.39 (d, 9.6)
2′	68.2, CH	4.48 (m)	67.2, CH	4.48 (m)
3′	72.7, CH	4.05 (d, 3.1)	72.1, CH	4.17 (dd, 3.6, 5.9)
4′	72.9, CH	4.18 (br s)	71.9, CH	4.04 (dd, 1.0, 3.6)
5′	77.7, CH	4.48 (m)	76.7, CH	4.47 (m)
6′	16.5, CH_3	1.70 (d, 7.3)	15.8, CH_3	1.69 (d, 7.3)
2′-OH	-	5.06 (d, 7.3)	-	-
3′-OH	-	6.76 (s)	-	-
4′-OH	-	5.46 (s)	-	-

The compound C10-D was obtained as yellowed solid (8.8 mg). The optical rotation was $[\alpha]^{25}_{D}$ +37.0° (c 0.25%, MeOH). The HRESIMS showing a pseudo-molecular ion at *m/z* 492.1520 $[M+Na]^+$ (Cal for $C_{27}H_{23}N_3NaO_5$).

The ^{1}H NMR spectra gave signals of one methyl [at δ_H 2.4 (s)], one methoxy [at δ_H 3.6 (s)], one methylene [at δ_H 4.96 (d, *J* = 17.9 Hz) and 4.99 (d, *J* = 17.9 Hz)], eight aromatic protons [at δ_H 7.33 (dd, *J* = 7.5, 7.9 Hz), 7.34 (dd, *J* = 7.5, 7.8 Hz), 7.46 (dd, *J* = 7.5, 8.7 Hz), 7.54 (dd, *J* = 7.5, 8.2 Hz), 7.64 (d, *J* = 8.2 Hz), 7.97 (d, *J* = 8.7 Hz), 8.00 (d, *J* = 7.8 Hz) and 9.32 (d, 7.9 Hz)], four methines [at δ_H 3.56 (br d, *J* = 8.6 Hz), 4.13 (d, *J* = 9.7 Hz), 4.14 (brs) and 6.6 (brs)], and one NH proton [at δ_H 8.62 (brs)].

The ^{13}C NMR and DEPT-135 spectra of compound C10-D showed 26 carbons including one methyl [at δ_C 28.7 (2′-CH_3)], one methoxy [at δ_C 61.0 (3′-OCH_3)], one methylene [at δ_C 45.1 (C-7)], twelve methines [at δ_C 65.6 (C-4′), 71.5 (C-5′), 83.0 (C-3′), 87.0 (C-6′), 108.2 (C-1), 115.1 (C-11), 119.4 (C-3), 119.8 (C-9), 120.5 (C-8), 124.4 (C-10), 125.1 (C-2) and 125.5 (C-4)] and eleven quaternary carbons [at δ_C 95.3 (C-2′), 111.4 (C-4b), 115.3 (C-7b), 119.0 (C-4c), 123.5 (C-7c), 124.4 (C-12b), 124.5 (C-4a), 132.3 (C-7a), 136.3 (C-13a), 140.0 (C-11a) and 171.5 (C-5)].

The 2D NMR spectral data (COSY, HMQC and HMBC) (Appendix D) indicated that compound C10-D had the same chemical structure (Figure 4.33) as the previously described staurosporine derivative MLR-52 (or 4'-demethylamino-4',5'-dihydroxystaurosporine) (Table 4.28).

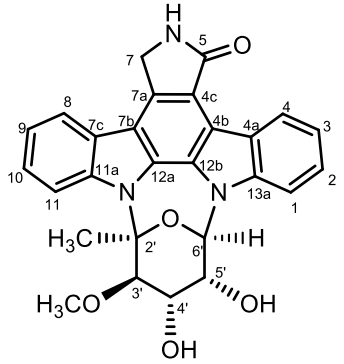

Figure 4.33 The chemical structure of compound C10-D (4'-demethylamino-4',5'-dihydroxystaurosporine)

Table 4.28 The ^{1}H and ^{13}C NMR data of the compound C10-D and MLR-52

Position	C10-0-1 F18 HPLC2 (in DMSO-d_6)		MLR-52 (in DMSO-d_6) (Mcalpine *et al*., 1993)	
	δ_C (ppm)	δ_H (ppm) multiplicity, *J* in Hz	δ_C (ppm)	δ_H (ppm) multiplicity, *J* in Hz
1	108.2, CH	7.64 (d, 8.2)	108.7, CH	7.64 (br d, 8.4)
2	125.1, CH	7.54 (dd, 7.5, 8.2)	125.5, CH	7.54 (br dd, 8.4, 7.0)
3	119.4, CH	7.33 (dd, 7.5, 7.9)	119.7, CH	7.29 (br dd, 8.1, 7.0)
4	125.5, CH	9.32 (d, 7.9)	125.8, CH	9.31 (br d, 8.1)
4a	124.5, C	-	122.8, C	-
4b	114.1, C	-	114.9, C	-
4c	119.0, C	-	119.2, C	-
5	171.5, C	-	171.8, C	-
6 -NH	-	8.62 (br s)	-	-
7	45.1, CH_2	4.96 (d, 17.9) 4.99 (d, 17.9)	45.4, CH_2	4.99 (d, 17.9) 4.95 (d, 17.9)
7a	132.3, C	-	132.6, C	-
7b	115.3, C	-	114.3, C	-
7c	123.5, C	-	123.6, C	-
8	120.5, CH	8.00 (d, 7.8)	120.9, CH	8.01 (br d, 7.7)
9	119.8, CH	7.34 (dd, 7.5, 7.8)	120.1, CH	7.27 (br dd, 7.7, 7.0)
10	124.4, CH	7.46 (dd, 7.5, 8.7)	124.8, CH	7.45 (br dd, 8.8, 7.0)
11	115.1, CH	7.97 (d, 8.7)	115.5, CH	7.98 (br d, 8.8)
11a	140.0, C	-	140.2, C	-
12a	nd	-	127.8, C	-
12b	124.4, C	-	124.6, C	-
13a	136.3, C	-	136.4, C	-
2′	95.3, C	-	95.6, C	-
3′	83.0, CH	4.13 (d, 9.7)	83.1, CH	4.14 (d, 10.3)
4′	65.6, CH	3.56 (br d, 8.0)	65.6, CH	3.57 (dd, 10.3, 2.6)
5′	71.5, CH	4.14 (br s)	71.7, CH	4.16 (dd, 2.6, 1.8)
6′	87.0, CH	6.60 (br s)	87.3, CH	6.61 (d, 1.8)
2′-CH_3	28.7, CH_3	2.39 (s)	29.0, CH_3	2.38 (s)
3′-OCH_3	61.0, CH_3	3.63 (s)	61.6, CH_3	3.62 (s)

nd = The carbon was not seen in the ^{13}C NMR spectrum.

In this dissertation, the secondary metabolites of other two new actinomycete species, *Streptomyces verrucosisporus* and *Micromonospora sediminis*, have not been determined yet. The growth of *M. sediminis* was slow. Moreover, it did not show any antimicrobial activities against tested microorganisms. In addition, its cultured broth did not show the interesting LC/UV profile. *S. verrucosisporus* exhibited the potent antimicrobial activity against tested microorganisms in the preliminary screening. The crude ethyl acetate extract of the strain CPB1-1^{T} showed antimicrobial activity against *Bacillus cereus* (MIC 1.56 µg/ml), *Enterococcus faecium* (MIC 1.56 µg/ml) and *Mycobacterium tuberculosis* H37Ra (MIC 6.25 µg/ml). Although the crude extract showed good biological activity, the bioactive compounds have not successfully identified yet because of the small amount of the active compound in the crude extract.

4.7 Biological activities of the isolated compounds

In this study, the six pure compounds (Figure 4.34) were evaluated the biological activity including anti-*Bacillus cereus*, Anti-*Mycobacterium tuberculosis*, Anti-*Pseudomonas aeruginosa*, cytotoxicity against KB (human oral cavity cancer), MCF-7 (human breast-cancer) and Vero cell lines (African green monkey kidney). The biological activities of these pure compounds as well as the positive controls are summarized in Table 4.29.

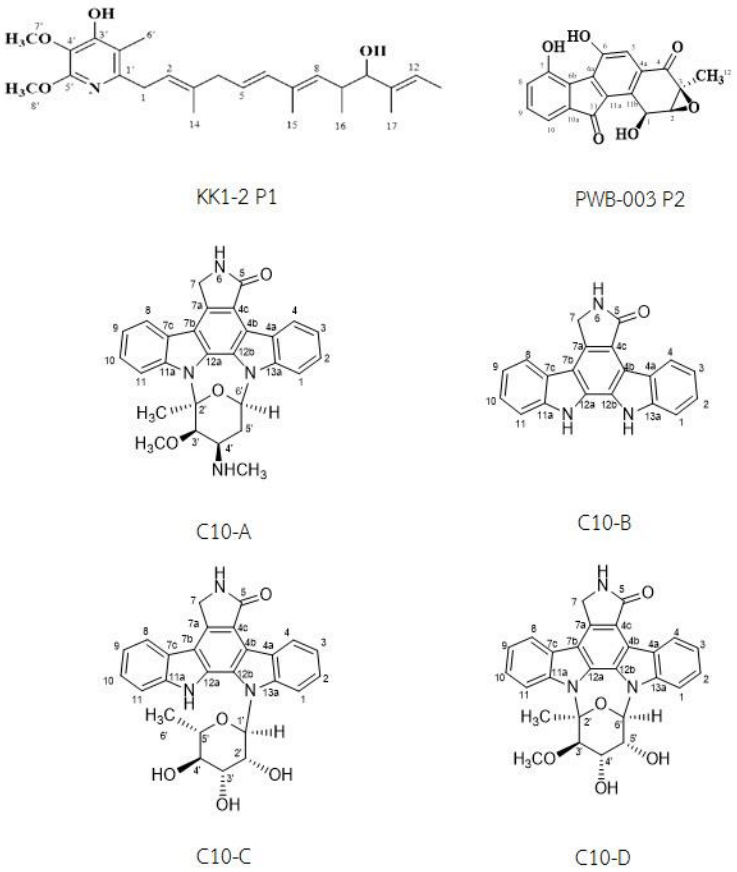

Figure 4.34 The chemical structures of the isolated compounds obtained from this study

Table 4.29 Biological activities of the isolated compounds

Compound	Biological activity					
	Antimicrobial activity MIC (μg/ml)			Cytotoxicity IC_{50} (μg/ml)		
	B. cereus	*M. tuberculosis*	*P. aeruginosa*	KB	MCF-7	Vero
KK1-2 P1	-	-	-	-	-	-
PWB-003 P2	-	-	-	-	-	48.45
C10-A	-	12.5	-	0.38	3.06	nd
C10-B	-	12.5	-	32.81	45.40	1.18
C10-C	25.00	6.25	-	2.17	8.54	0.22
C10-D	-	-	-	3.81	9.77	nd
Vancomycin	2.00	nd	nd	nd	nd	nd
Tamoxifen	nd	nd	nd	nd	7.45	nd
Doxorubicin	nd	nd	nd	0.68	9.56	nd
Rifampicin	nd	0.03	nd	nd	nd	nd
Ofloxacin	nd	0.39	nd	nd	nd	nd
Streptomycin	nd	0.31	nd	nd	nd	nd
Isoniazid	nd	0.05	nd	nd	nd	nd
Ethambutol	nd	0.47	nd	nd	nd	nd
Chloramphenicol	nd	nd	1.0-2.0	nd	nd	nd
Ellipticine	nd	nd	nd	3.19	nd	0.96

-, no activity; nd, not determine

According to the biological assay results of this study, the compound KK1-2 P1 (piericidin A1) did not show both antimicrobial activity and cytotoxicity against tested models. However, it has been reported as a potent mitochondrial electron transport inhibitor (Jeng *et al*., 1968).

Compound PWB-003 P2, a new diastereoisomer of fluostatin C, did not show antimicrobial activity against *B. cereus, M. tuberculosis* and *P. aerugina* but exhibited cytotoxicity against Vero cell line (IC_{50}, 48.45 μg/ml). As previously report, fluostatin C showed the cytotoxicity against HMO2 (human gastric adenocarcinoma; $IG_{50,}$ 3.2 μg/ml), HepG2 (human hepatocellular carcinoma; IG_{50}, 3.1 μg/ml) and MCF7 (human breast carcinoma; IG_{50}, 6.0 μg/ml) (Buar *et al*., 2006). However, the compound PWB-003 P2 did not show the cytotoxicity against MCF7 cell line in this study. This might be influence by the difference of the chemical structure as mentioned above.

Compound C10-A, staurosporine, exhibited antimicrobial activity against *M. tuberculosis* (MIC, 12.5 µg/ml) but did not show activity against *B. cereus* and *P. aeruginosa*. It exhibited cytotoxicity against KB (IC_{50} 0.38 µg/ml) and MCF-7 (IC_{50} 3.06 µg/ml).

Compound C10-B, staurosporine aglycone, showed antimicrobial activity against *M. tuberculosis* (MIC, 12.5 µg/ml) but did not show activity against *B. cereus* and *P. aeruginosa*. It showed cytotoxicity against KB, MCF-7 and Vero cell lines at IC_{50} 32.81, 45.40 and 1.18 µg/ml, respectively. These results are closely similar to the activities of stauorsporine. This indicated that the aglycone moiety of stautosporine played the important role of the biological activities.

Compound C10-C, K-252D, exhibited antimicrobial activity against *B. cereus* (MIC 25.00 µg/ml) and *M. tuberculosis* (MIC 6.25 µg/ml) but did not exhibit activity against *P. aeruginosa*. In addition, it showed cytotoxicity against KB, MCF-7 and Vero cell lines at IC_{50} 2.17, 8.54 and 0.22 µg/ml, respectively.

Compound C10-D, 4'-demethylamino-4',5'-dihydroxystaurosporine, showed cytotoxicity against KB and MCF-7 cell lines at IC_{50} 3.81 and 9.77 µg/ml, respectively. However, it did not show any antimicrobial activity against tested microorganisms.

Staurosporine was first isolated from the culture broth of *Saccharothrix aerocolonigenes* AM-2282 (Omura *et al*., 1977). Staurosporine and its derivatives showed various biological activities including potent protein kinase inhibitor (Tamaoki *et al*., 1986), potent platelet aggregation inhibitor (Oka *et al*., 1986), anti-bacterial (Sancleme *et al*., 1994), anti-fungal (Omura *et al*., 1977) and anti-parasitic activities (Pimentel-Elardo *et al*., 2010), vasorelaxant (Buchholz *et al*., 1991), neurotropic (Rasouly & Lazarovici, 1994), and cell cycle progression inhibitor (Gong *et al*., 1994).

CHAPTER V
CONCLUSION

Thai marine environments found to be the interesting source for the actinomycete isolation. In this study, total 75 actinomycetes were isolated from marine samples collected from 8 provinces of Thailand. On the basis of morphology, chemotaxonomy and 16S rRNA gene sequence analysis, they could classified in 3 families (Family *Streptomycetaceae*, *Micromonosporaceae* and *Nocardiaceae*) including 6 genera *Jishengella* (1 isolate), *Micromonospora* (25 isolates), *Nocardia* (2 isolates), *Salinispora* (13 isolates), *Streptomyces* (32 isolates) and *Verrucosispora* (2 isolates). Among them, 40 isolates were isolated from the samples collected from Thai Gulf and 35 isolates were isolated from the samples collected from Andaman Sea. On the basis of polyphasic approach, four new actinomycetes species including *Streptomyces chumphonensis* (strain KK1-2^T and CPB4-7), *Streptomyces verrucosisporus* (CPB1-1^T, CPB2-10, BM1-4, CPB3-1 and CPB1-18), *Micromonospora fluostatini* (PWB-003^T) and *Micromonospora mangrovi* (CH3-3^T) were proposed.

The primary antimicrobial activity screening revealed that twenty-seven *Streptomyces* isolates, twelve *Salinispora* isolates and five *Micromonospora* isolates showed antimicrobial activity against tested microorganisms. Based on this study, the production media and strains were the main factor that influenced the antimicrobial activity. In this study, three representative strains including *S. chumphonensis* KK1-2^T, *M. fluostatini* PWB-003^T and *S. sanyensis* C10-9-1 were selected for the secondary metabolites isolation. Piericidin A1 was isolated from *S. chumphonensis* KK1-2^T. It did not show any biological activity tested in this study. Meanwhile, a new diastereoisomer of fluostatin C was isolated from *M. fluostatini* PWB-003^T. It exhibited cytotoxicity against Vero cell line (IC_{50}, 48.45 µg/ml). In addition, four compounds including staurosporine, staurosporine aglycone, K232D and 4'-demethylamino-4',5'-dihydroxystaurosporine were isolated from the crude ethyl acetate extract of *S. sanyensis* C10-9-1. Staurosporine exhibited antimicrobial activity against *M. tuberculosis* (MIC, 12.5 µg/ml) and cytotoxicity against KB (IC_{50} 0.38 µg/ml) and MCF-7

(IC_{50} 3.06 µg/ml). Staurosporine agycone exhibited antimicrobial activity against *M. tuberculosis* (MIC, 12.5 µg/ml) and cytotoxicity against KB (IC_{50} 32.81), MCF-7 (IC_{50} 45.40 µg/ml) and Vero cell lines (IC_{50} 1.18 µg/ml). The K232D exhibited antimicrobial activity against *B. cereus* (MIC 25.00 µg/ml) and *M. tuberculosis* (MIC 6.25 µg/ml) and cytotoxicity against KB (IC_{50} 2.17 µg/ml), MCF-7 (IC_{50} 8.54 µg/ml) and Vero cell lines (IC_{50} 0.22 µg/ml). 4'-demethylamino-4',5'-dihydroxystaurosporine showed cytotoxicity against KB (IC_{50} 3.81 µg/ml) and MCF-7 (IC_{50} 9.77 µg/ml).

Based on this study, Thai marine environment is a promising habitat to obtain a new actinomycete speices. Unfortunately, only a new diastereoisomer of fluostatin C was isolated in this work. In future, the new screening technology such as physicochemical screening by LC/MS might be helpful to obtain some useful bioactive secondary metabolites from these bio-resources.

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APPENDICES

APPENDIX A

Culture media

M1 agar (isolation medium)

Starch	10.0	g
Yeast extract	5.0	g
Peptone	2.0	g
Agar	18.0	g
Natural seawater	1	l

M2 agar (isolation medium)

Glycerol	6	ml
Arginine	1.0	g
K_2HPO_4	1.0	g
Mg2SO4.7H2O	0.5	g
Agar	18.0	g
Natural seawater	1	l

Seawater proline agar (isolation medium)

Proline	10.0	g
Agar	15.0	g
Natural sea water	1	l

Yeast extract-malt extract agar (ISP medium 2, ISP 2)

Yeast extract	4.0	g
Malt extract	10.0	g
Glucose	4.0	g
Agar	15.0	g
Distilled water	0.5	l
Artificial seawater	0.5	l

pH 7.2 - 7.4

Oatmeal agar (ISP medium 3, ISP 3)

Oatmeal	20.0	g
Trace salt solution	1.0	ml
Agar	18.0	g
Distilled water	0.5	l
Artificial seawater	0.5	l
pH 7.2 - 7.4		

Inorganic salts-starch agar (ISP medium 4, ISP 4)

Soluble starch	10.0	g
K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	1.0	g
NaCl	1.0	g
$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
Trace salt solution	1.0	ml
Agar	20.0	g
Distilled water	1	l
pH 7.2 - 7.4		

Glycerol-asparagine agar (ISP medium 5, ISP 5)

L-asparagine	1.0	g
Glycerol	10.0	g
K_2HPO_4	1.0	g
Trace salt solution	1.0	ml
Agar	20.0	g
Distilled water	0.5	l
Artificial seawater	0.5	l
pH 7.2 - 7.4		

Peptone-yeast extract iron agar (ISP medium 6, ISP 6)

Bacto-peptone iron agar, dehydrated (Difco)	36.0	g
Yeast extract	1.0	g
Distilled water	0.5	l
Artificial seawater	0.5	l
pH 7.2 - 7.4		

Tyrosine agar (ISP medium 7, ISP 7)

Glycerol	15.0	g
L-Tyrosine (Difco)	0.5	g
L-asparagine (Difco)	1.0	g
K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4.7H_2O$	0.01	g
Trace salt solution	1.0	ml
Agar	20.0	g
50% (v/v) Artificial seawater	1	l
pH 7.2 - 7.4		

Carbon utilization medium (ISP medium 9, ISP 9)

$(NH_4)_2SO_4$	2.64	g
KH_2PO_4	2.38	g
K_2HPO_4	5.65	g
$MgSO_4.7H_2O$	1.0	g
*Solution	1.0	ml
Agar	20.0	g
Distilled water	0.5	l
Artificial seawater	0.5	l
pH 7.0 - 7.4		

***Solution**

$CuSO_4.5H_2O$	0.64	g
$FeSO_4.7H_2O$	0.11	g
$MnCl2._4H_2O$	0.79	g
$ZnSO.7H_2O$	0.15	g
Distilled water	100	ml

Nutrient agar (NA)

Meat extract	10.0	g
Peptone	10.0	g
Agar	15.0	g
Distilled water	0.5	l
Artificial seawater	0.5	L
pH 7.2 - 7.4		

Yeast extract-dextrose broth

Yeast extract	10.0	g
Glucose	10.0	g
Distilled water	0.5	l
Artificial seawater	0.5	L
pH 7.4 - 7.8		

Production medium no. 51

Glucose	5.0	g
Corn steep powder	5.0	g
Oat meal	10.0	g
Pharma media	10.0	g
K_2HPO_4	5.0	g
$MgSO_4.7H_2O$	5.0	g
Trace metal solution	1.0	ml
Tap water	0.5	l

Artificial seawater	0.5	l
pH 7.4 - 7.8		

Production medium no. 54

Soluble starch	20.0	g
Glycerol	5.0	g
Defatted wheat germ	10.0	g
Meat extract	3.0	g
Yeast extract	3.0	g
$CaCO_3$	3.0	g
Tap water	0.5	l
Artificial seawater	0.5	l
pH 7.4 - 7.8		

301 Seed medium

Soluble starch	24.0	g
Glucose	1.0	g
Peptone	3.0	g
Meat extract	3.0	g
Yeast extract	5.0	g
$CaCO_3$	4.0	g
Distilled water	500	ml
Artificial seawater	500	ml
pH 7.0		

Bouillon gelatin broth

Peptone	1.0	g
Meat extract	0.5	g
NaCl	0.5	g

Gelatin	15.0	g
Artificial seawater	50	ml
Distilled water	50	ml
pH 7.2 - 7.4		

Potassium nitrate Broth

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g
Agar	15.0	g
Artificial seawater	50	ml
Distilled water	50	ml
pH 7.2 - 7.4		

Skim milk agar

Skim milk	10.0	g
Agar	15.0	g
Artificial seawater	50	ml
Distilled water	50	ml

* Skim milk must be dissolve in distilled water and separate sterile at 110 ^{o}C for 10 minutes. After cooled down to 55 ^{o}C, mix the skim milk with sterile agar.

APPENDIX B

Reagents and buffers

6N HCl

Conc. HCl	60	ml
Distilled water	60	ml

Add conc. HCl into the distilled water.

2N H_2SO_4

Conc. H_2SO_4	2	ml
Distilled water	34	ml

Add conc. H_2SO_4 into the distilled water.

Aniline-butanol-phthalate reagent

Aniline	2	ml
Phthalic acid	3.25	g
Water-saturated *n*-butanol	100	ml

DON reagent

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

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

Nitrate reduction test reagents

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5N Acetic acid	100	ml

_N,N_-dimethyl-1-naphthylamine solution

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

Reagents for fatty acid analysis

Sponification reagent

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

Dissolve NaOH in Milli-Q water and add methanol.

Methylation reagent

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

Adjust pH to below 1.5.

Extraction solvent

_n_-Hexane (HPLC grade)	50	ml
Methyl-3-butyl ether (HPLC grade)	50	ml

Base washing reagent

Sodium hydroxide	1.2	g
Milli-Q water	100	ml

Saturated sodium chloride solution

Sodium chloride saturated in Milli-Q

Reagents for polar lipid analysis

Anisaldehyde reagent

Ethanol	90	ml
Conc. H_2SO_4	5.0	ml
_p_-Anisaldehyde	5.0	ml
Acetic acid	1.0	ml

Dragendorff's reagent

Solution A

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

Solution B

KI	40	g
Distilled water	100	ml

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

Phosphomolybdic acid reagent

Absolute ethanol	100	ml
Phosphomolybdic acid	5.0	G

Molybdenum blue reagent

Solution A

MoO_3	4.011	g
25N H_2SO_4	100	ml

Dissolve MoO_3 into 25N H_2SO_4 and heat.

Solution B

Molybdenum powder	0.178	g
Solution A	50	ml

Add molybdenum powder into solution A and boil it for 15 minutes. After cooling, remove the precipitation by decantation. Before spraying, mix solution A (50 ml) plus solution B (50 ml) and plus distilled water (50 ml)

Phosphomolybdic acid reagent

Abslute ethanol	100	ml
Phosphomolybdic acid	5.0	g

RNase A solution

RNase A	20	ng
0.15 M NaCl, pH 5.0	10	ml

Dissolve RNase A in 0.15 M NaCl, pH 5.0 and heat at 95 ºC for 5 - 10 minutes. Keep RNase A solution at -20 ºC

RNase T solution

RNase T	800	U
0.1 M Tris-HCl (pH 7.2)	1	ml

Mix RNase T in 0.1M Tris-HCl (pH 7.2) and heat at 95 ºC for 5 minutes. Keep RNase T solution at -20 ºC

Nuclease P1 solution

Nuclease P1	0.1	mg
40 mM CH_3COONa	0.5	ml
12 mM $ZuSO_4$ (pH 5.3)	0.5	ml

Keep at 4 ºC

Alkaline phosphatase solution

Alkaline phosphatase	2.4	U
0.1M Tris-HCl (pH 8.0)	1	ml

DNA extraction buffer

Tris-HCl buffer pH 8.5	0.2	M
NaCl	0.25	M
EDTA	0.025	M
SDS	0.5%	

0.1 M Tris-HCl buffer, pH 9.0

Tris base	12.1	mg

Dissolve Tris base in distilled water. Stir solution and monitor the pH with a pH probe while adding conc. HCl to adjust the pH 9.0. Make up the solution to 1 l with distilled water and autoclave. Store it at room temperature.

1 M Tris-HCl buffer, pH 8.0

Tris base	121.1	mg

Dissolve Tris base in distilled water. Stir solution and monitor the pH with a pH probe while adding conc. HCl to adjust the pH 8.0. Make up the solution to 1 l with distilled water and autoclave. Store it at room temperature.

1 mM Saline-EDTA (Na_2-EDTA) pH 8.0

EDTA	0.29	g

Dissolve EDTA in 900 ml of distilled water. Stir solution and monitor the pH with a pH probe while adding NaOH pellets to adjust the pH 8.0. Make up the solution to 1 l with distilled water and autoclave.

3 M Sodium acetate

Sodium acetate trihydrate ($CH_3COONa.3H_2O$)	408.0	g

Dissolve $CH_3COONa.3H_2O$ in 400 ml of distilled water. Stir solution and monitor the pH with a pH probe while adding glacial acetic acid to adjust the pH 5.2. Make up the solution to 1 l with distilled water and autoclave.

TE buffer

10 mM Tris-HCl (pH 8.0)	10	ml
1 mM Na_2-EDTA (pH 8.0)	10	ml
Distilled water	980	ml

Sterilize the solution by autoclaving

1 mM Na_2-EDTA (pH 8.0)

EDTA	292.24	g

Dissolve EDTA in 700 ml of distilled water. Stir solution and monitor the pH with a pH probe while adding NaOH pellets to adjust the pH 8.0. Dilute the solution to 1 l with distilled water and autoclave. Store it at room temperature.

0.5 M EDTA (pH 8.0)

EDTA	186.1	g

Dissolve EDTA in 800 ml of distilled water. Stir solution and monitor the pH with a pH probe while adding NaOH pellets to adjust the pH 8.0. Dilute the solution to one liter with distilled water and autoclave. Store it at room temperature.

50X Tris-acetate (TAE) buffer

Tris Base	242.28	g
Glacial acetic acid	57.1	ml
0.5M EDTA (pH 8.0)	100	ml

Dissolve Tris Base in 600 ml of distilled water. Stir solution and add glacial acetic acid and 0.5M EDTA (pH 8.0) solution. Make up the volume to 1000 ml with distilled water. Autoclave and store at room temperature.

1X Tris-acetate (TAE) buffer

50X Tris-acetate (TAE) buffer	20	ml
Distilled water	980	ml

0.8% Agarose gel

Agar rose	0.8	g
Distilled water	100	ml

Mix agarose and distilled water and melt the mixture with the microwave.

Reagents and buffers for DNA-DNA hybridization

Pre-hybridization solution (10 ml)

20X SSC	1	ml
50X Denhardt's solution	1	ml
Formamide	5	ml
Sonicated salmon sperm DNA (10 mg/ml)	0.1	ml
Distilled water	2.9	ml

Hybridization solution (10 ml)

20X SSC	1	ml
50X Denhardt's solution	1	ml
Formamide	5	ml
Sonicated salmon sperm DNA (10 mg/ml)	0.1	ml
50% Dextran sulphate solution	0.5	ml
Distilled water	2.4	Ml

PBS-BSA-Triton solution (10 ml)

BSA (Bovine serum albumin)	0.05	g
Triton X	10	µl
20X PBS	0.5	ml
Distilled water	9.5	ml

SABG (Streptoavidin-β-galactosidase) solution (10 ml)

PBS-BSA-Triton solution	10	ml
SABG	10	µl

4-MUF (4 methyllumbelliferyl-*β*-D-galactoside) solution

4-MUF (10 mg/ml)	100	µl
1X PBS	10	ml
Freshly prepare		

4-MUF (10 mg/ml)

4-MUF	1	mg
N-N-dimethylformamide	100	Ml

20X Phosphate buffered saline (PBS)

Na_2HPO_4	28.8	g
NaCl	160.0	g
KH_2PO_4	4.0	g
KCl	4.0	g

Dissolve Na_2HPO_4, NaCl, KH_2PO_4 and KCl in 800 ml of distilled water. Adjust to pH 7.2 - 7.4 with NaOH and volume to 1000 ml. Autoclave and store at room temperature.

1X Phosphate buffered saline (PBS)

20X PBS	50	ml
Sterile distilled water	950	ml

1M Magnesium chloride ($MgCl_2$)

$MgCl_2$	92.5	g

Dissolve $MgCl_2$ in distilled water and adjust volume to 1 l

Phosphate buffered saline-magnesium chloride (PBSMG) solution (10 ml)

20X PBS	0.5	ml
1M $MgCl_2$	1	ml
Distilled water	8.5	ml

Salmon sperm DNA (10 mg/ml)

Salmon sperm DNA	10	mg
TE buffer	1	ml

Dissolve salmon sperm DNA in TE buffer, boil the solution for 10 minutes, immediately cool in ice and sonicate for 3 minutes.

20X Saline sodium citrate (SSC)

NaCl	175.3	g
Sodium citrate	88.2	g
Distilled water	1	l

Dissolve NaCl and Sodium citrate in 700 ml of distilled water. Adjust pH to 7.0 with NaOH, adjust volume to 1000 ml and sterilize by autoclaving.

1X Saline sodium citrate (SSC)

20X SSC	50	ml
Sterile distilled water	950	ml

APPENDIX C

Phospholipid chromatograms of the novel actinomycete species

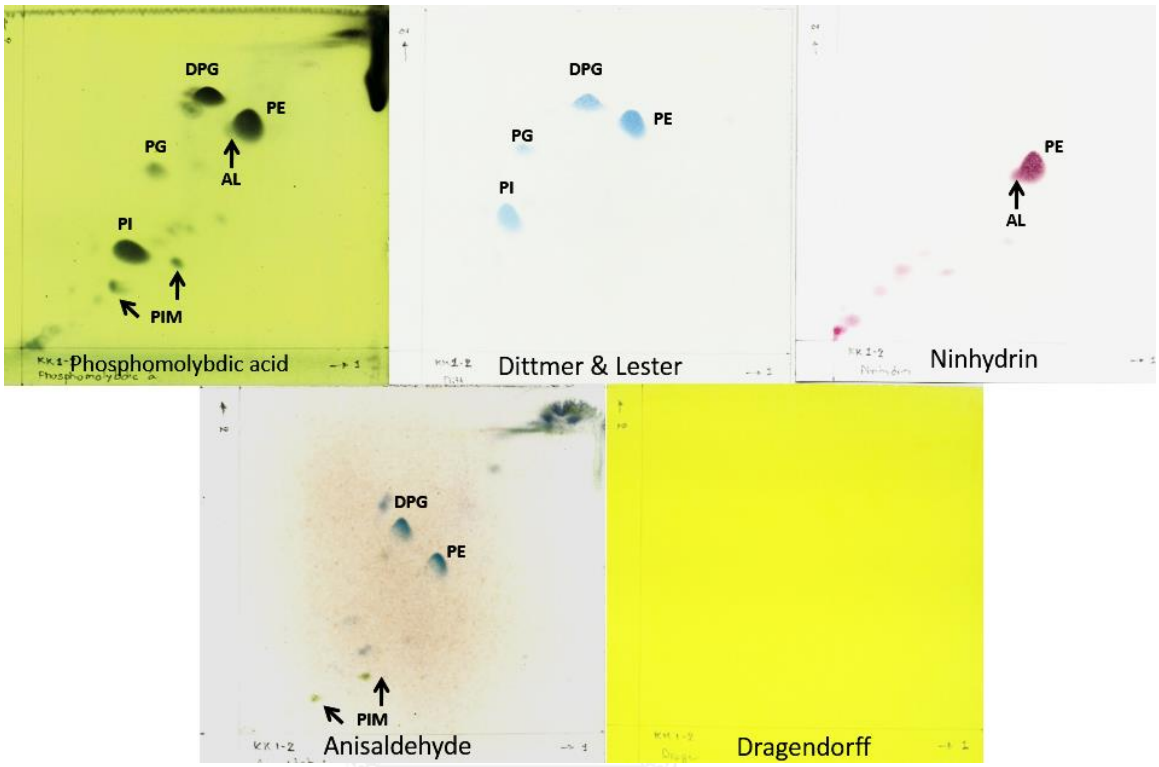

Figure C1 Polar lipid profiles of *Streptomyces chumphonensis* KK1-2^{T}

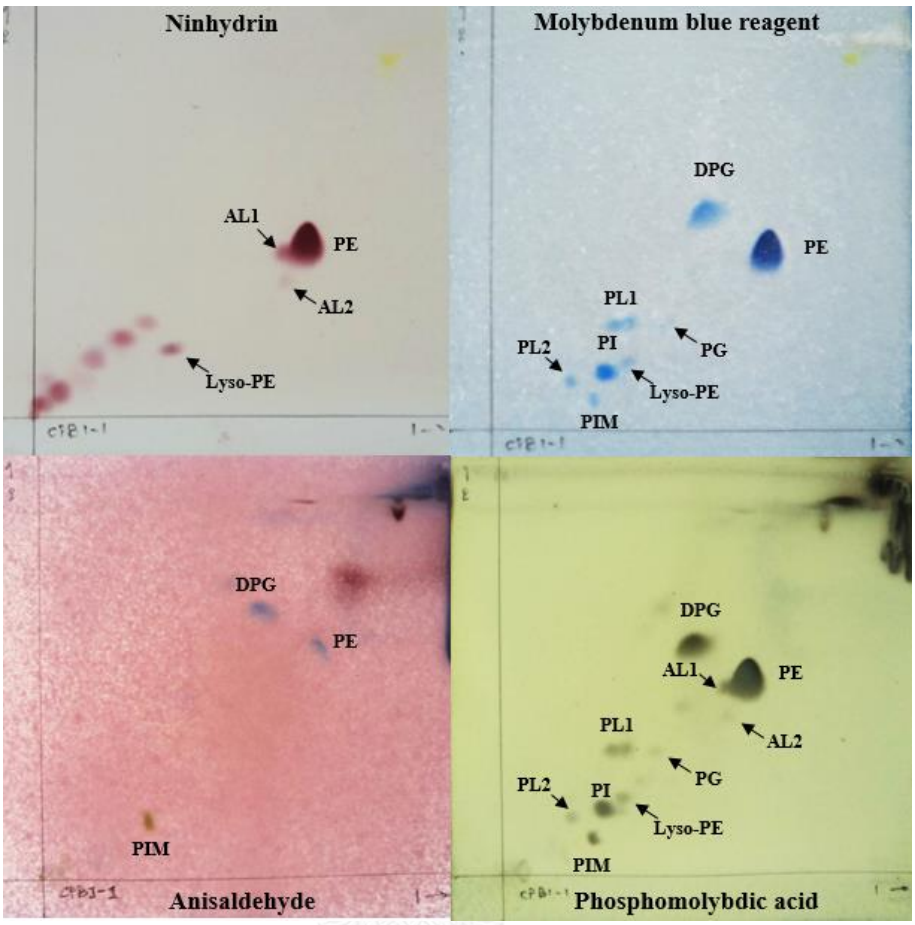

Figure C2 Polar lipid profiles of *Streptomyces verrucosisporus* CPB1-1^{T}

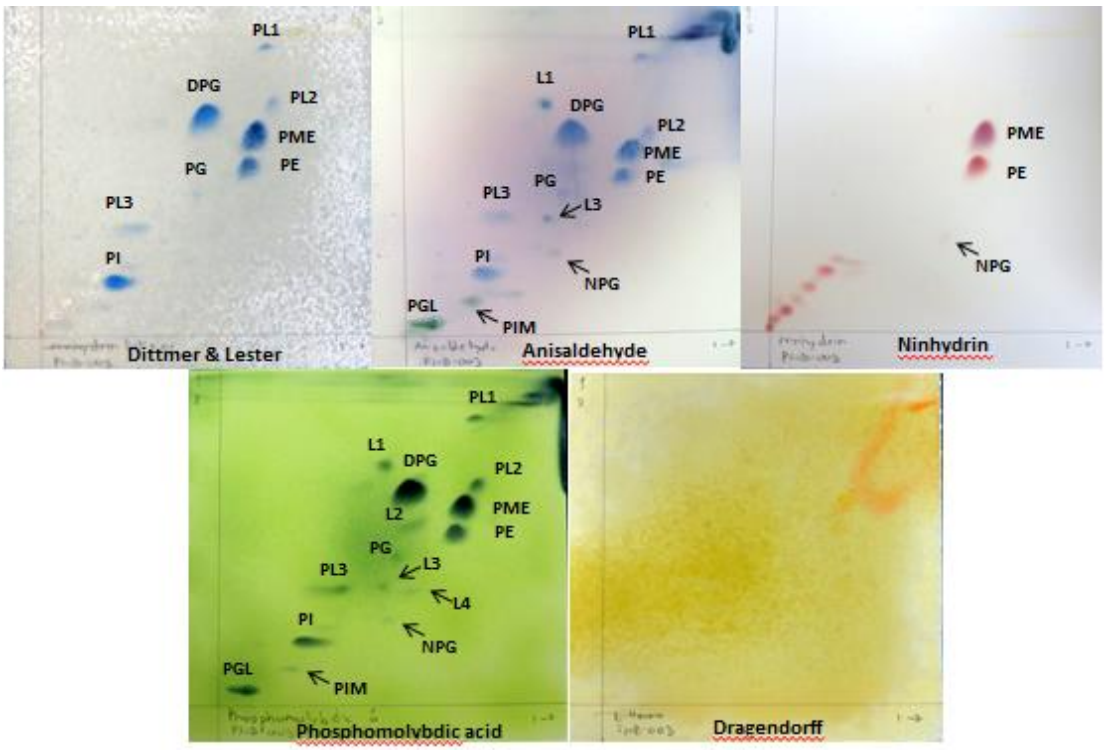

Figure C3 Polar lipid profiles of *Micromonospora fluostatini* PWB-003^{T}

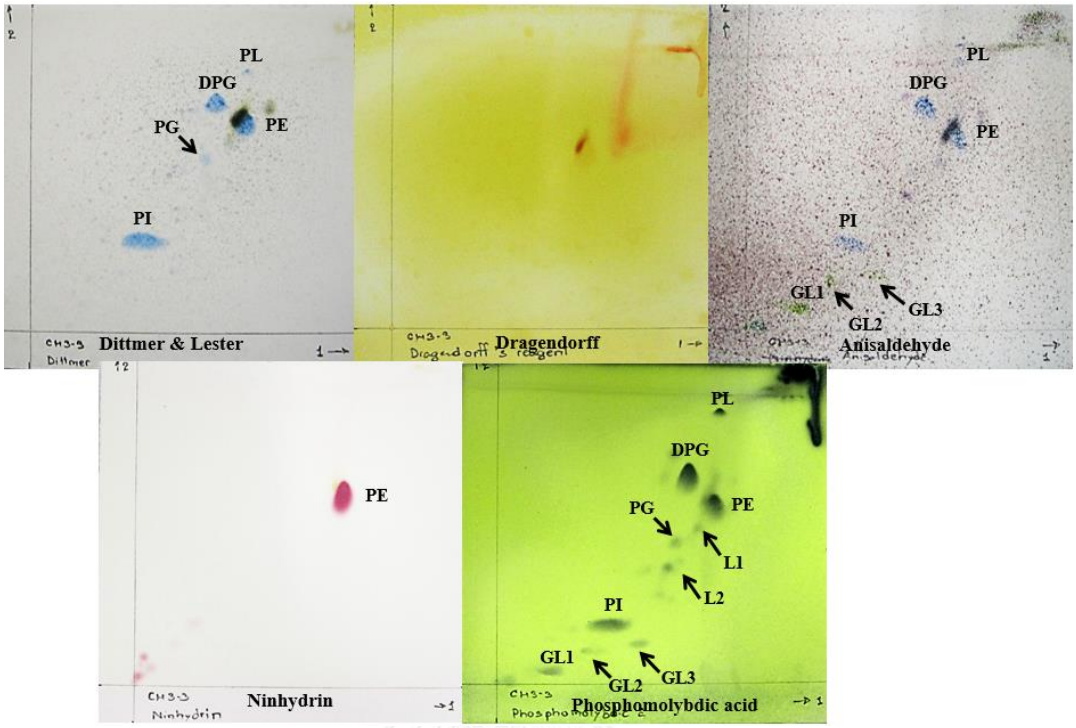

Figure C4 Polar lipid profiles of *Micromonospora sediminis* CH3-3^{T}

APPENDIX D

NMR spectra of the isolated compounds

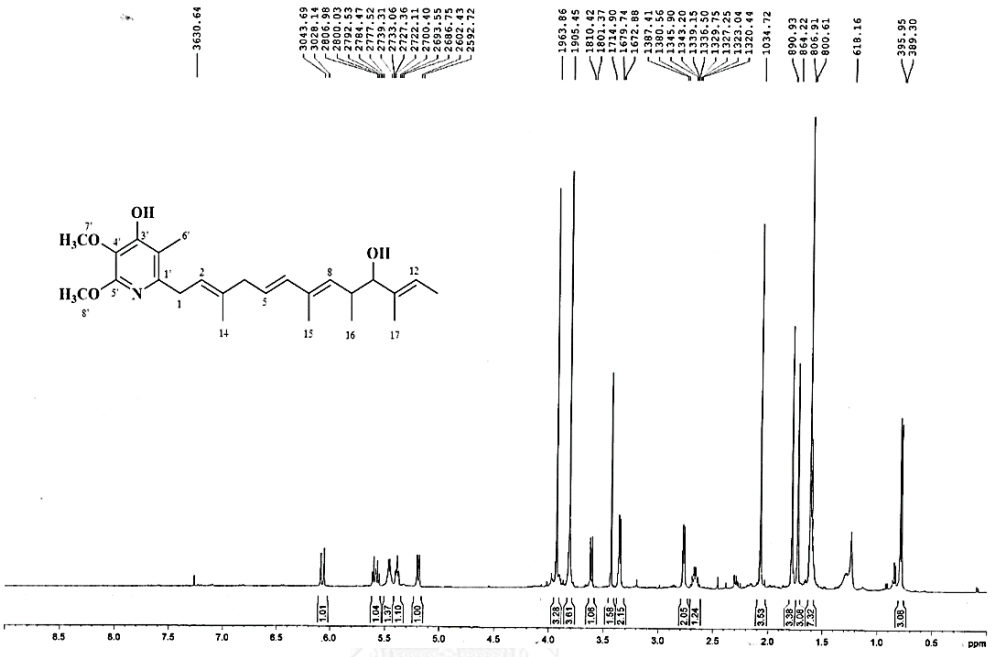

Figure D1 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of KK1-2 P1

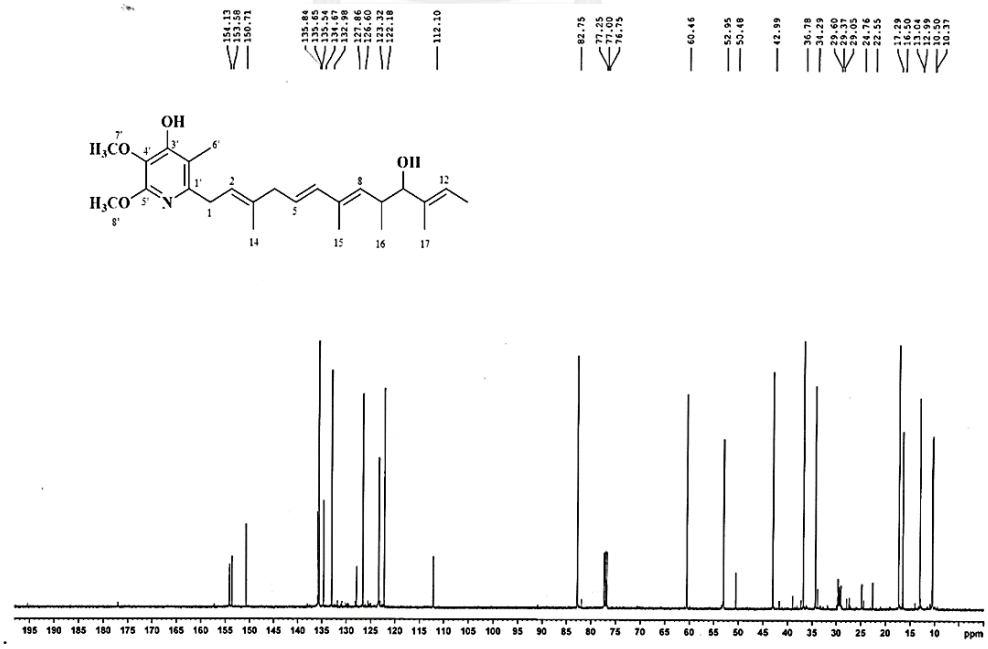

Figure D2 ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of KK1-2 P1

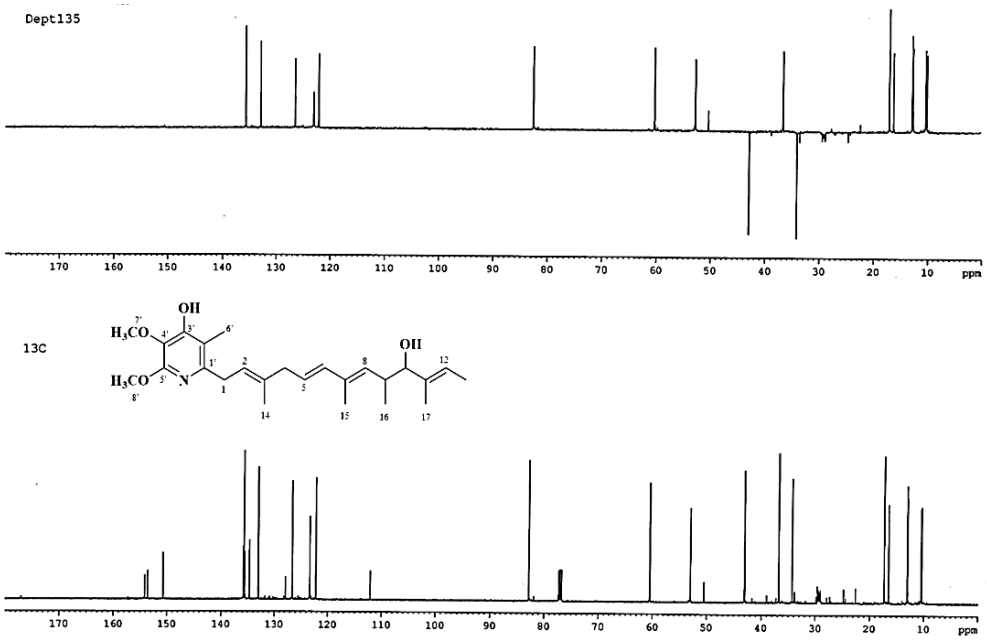

Figure D3 DEPT 135 spectrum (400 MHz, $CDCl_3$) of KK1-2P1

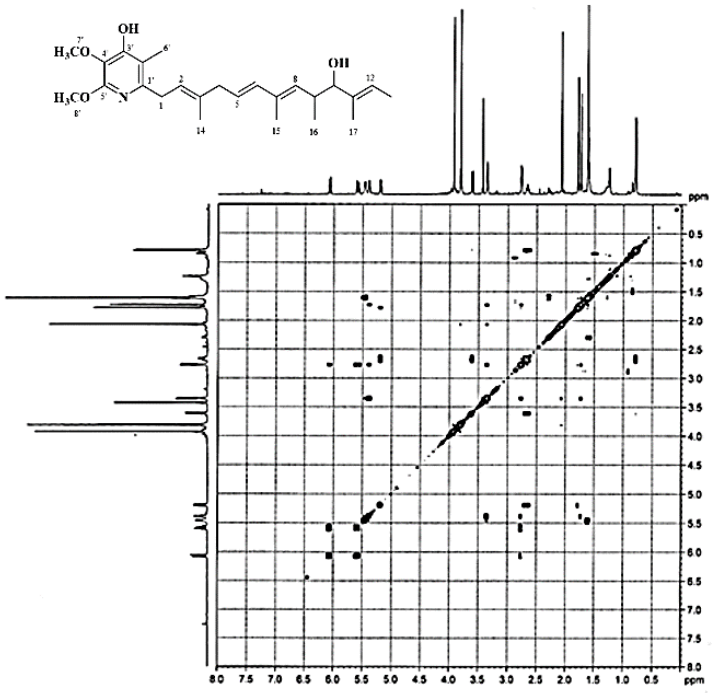

Figure D4 ^{1}H-^{1}H COSY spectrum (400 MHz, $CDCl_3$) of KK1-2P1

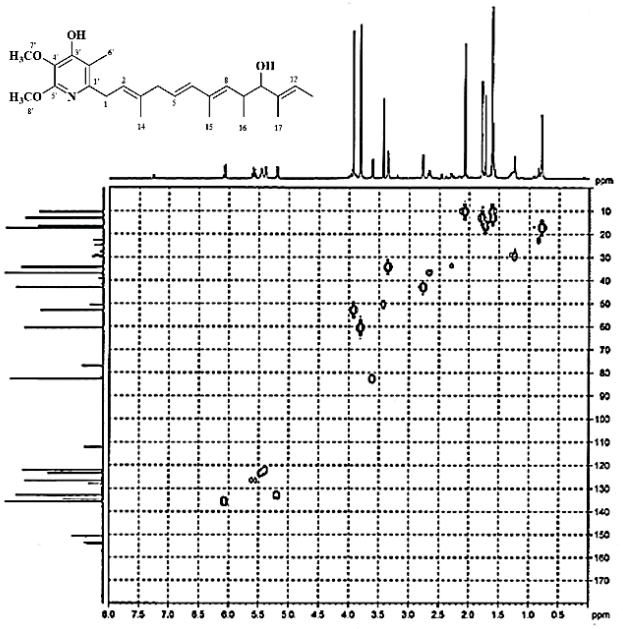

Figure D5 HMQC spectrum (400 MHz, $CDCl_3$) of KK1-2P1

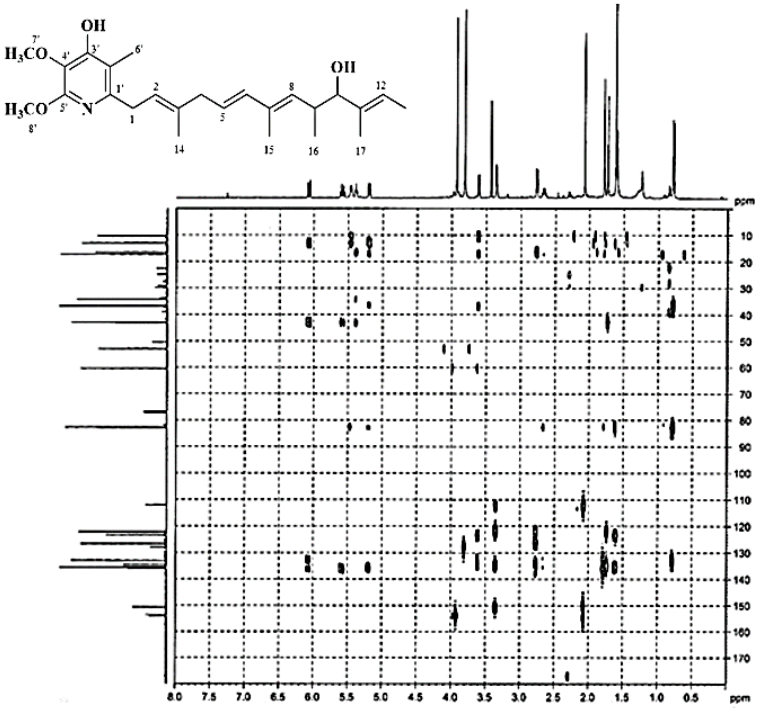

Figure D6 HMBC spectrum (400 MHz, $CDCl_3$) of KK1-2P1

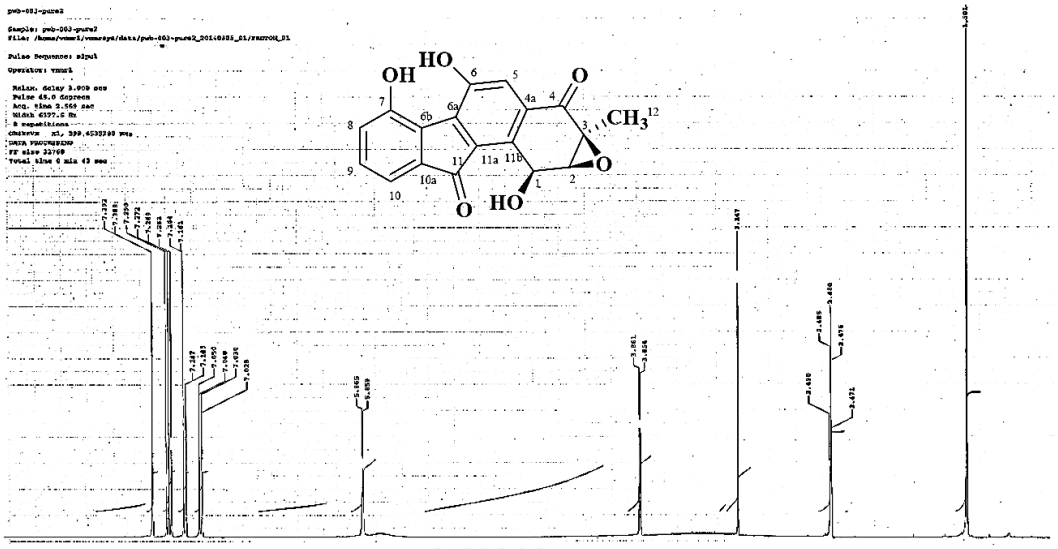

Figure D7 ^{1}H spectrum (400 MHz, DMSO-d_6) of PWB-003 P2

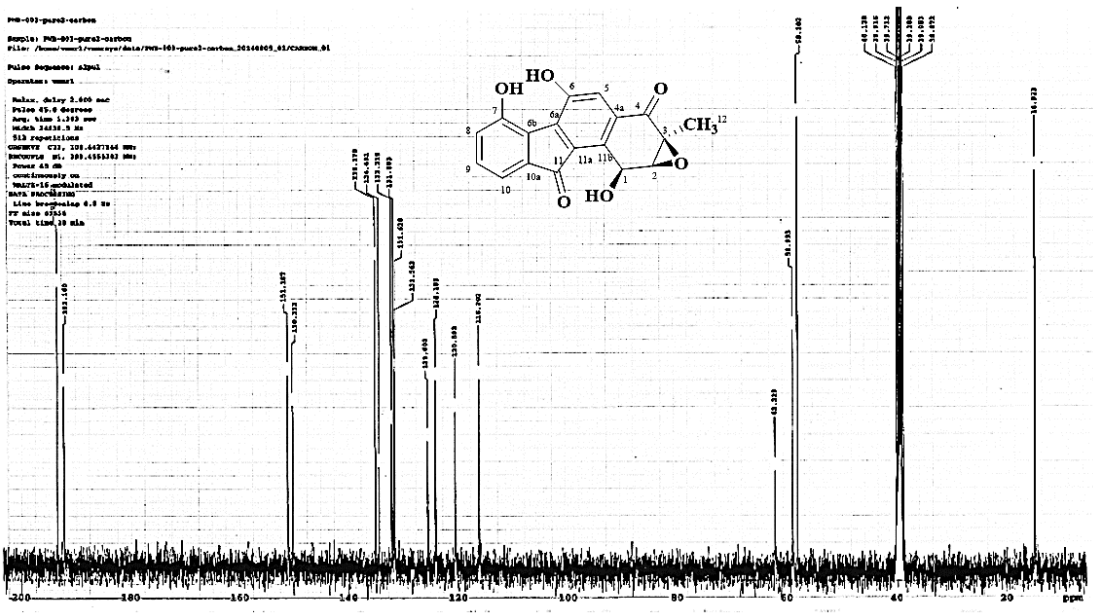

Figure D8 ^{13}C spectrum (400 MHz, DMSO-d_6) of PWB-003 P2

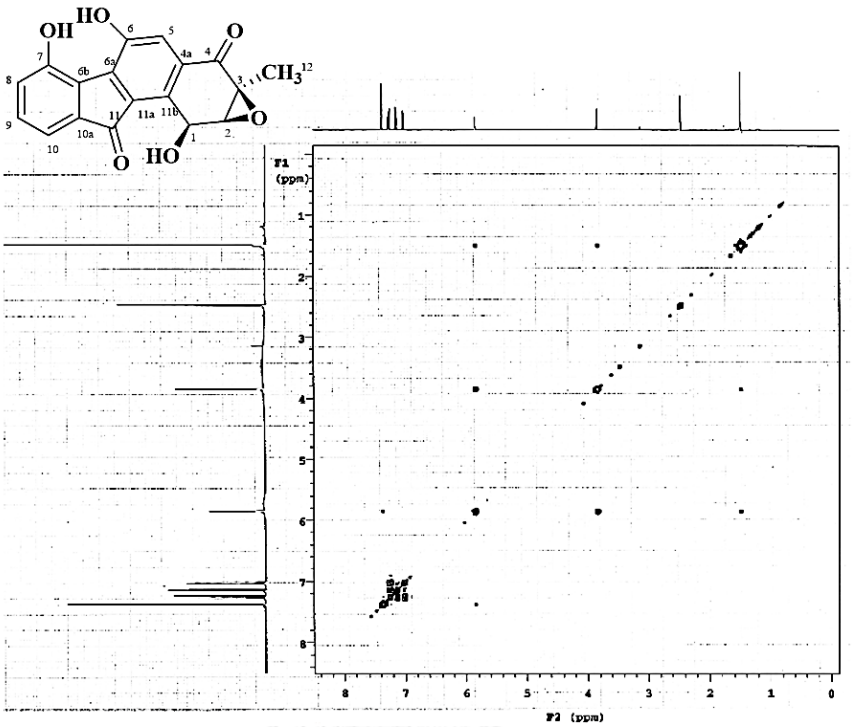

Figure D9 COSY spectrum (400 MHz, DMSO-d_6) of PWB-003 P2

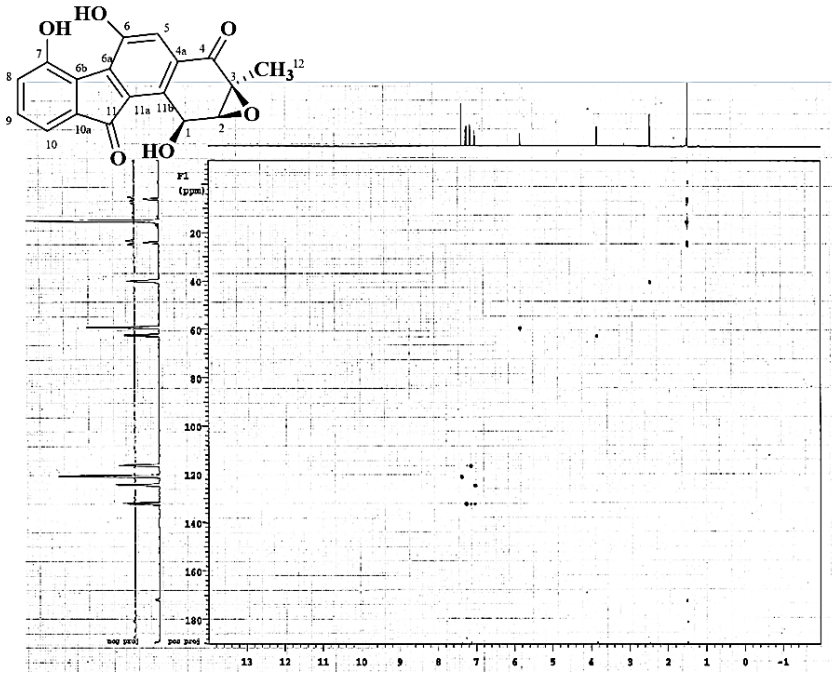

Figure D10 HSQC spectrum (400 MHz, DMSO-d_6) of PWB-003 P2

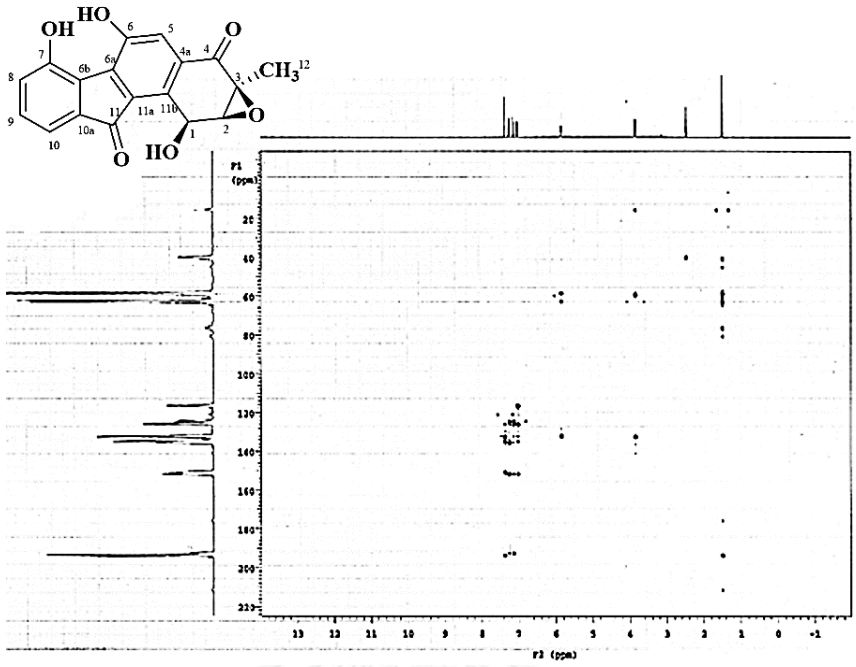

Figure D11 HMBC spectrum (400 MHz, DMSO-d_6) of PWB-003 P2

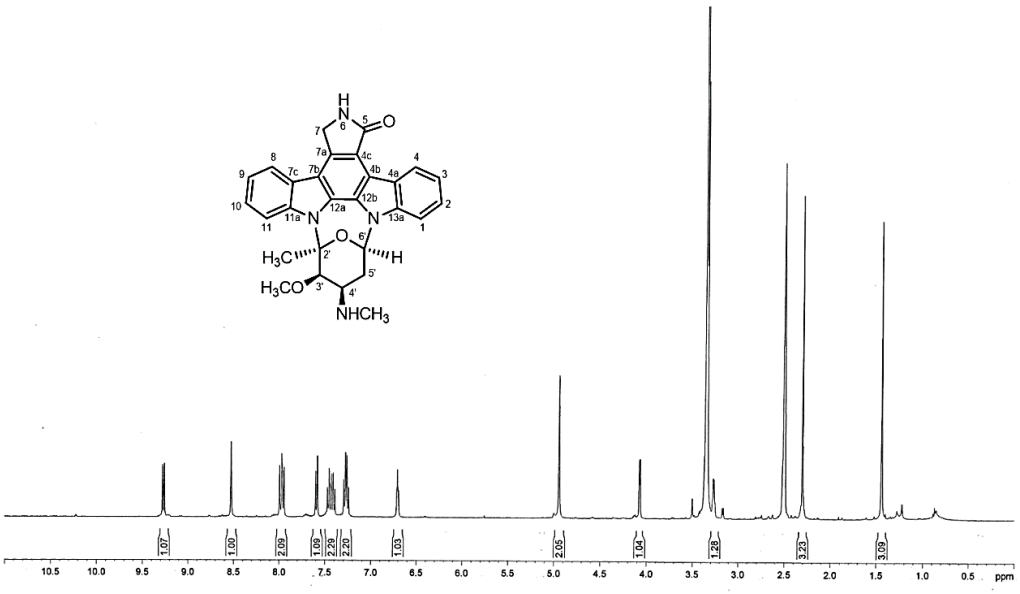

Figure D12 ^{1}H spectrum (400 MHz, DMSO-d_6) of C10-A

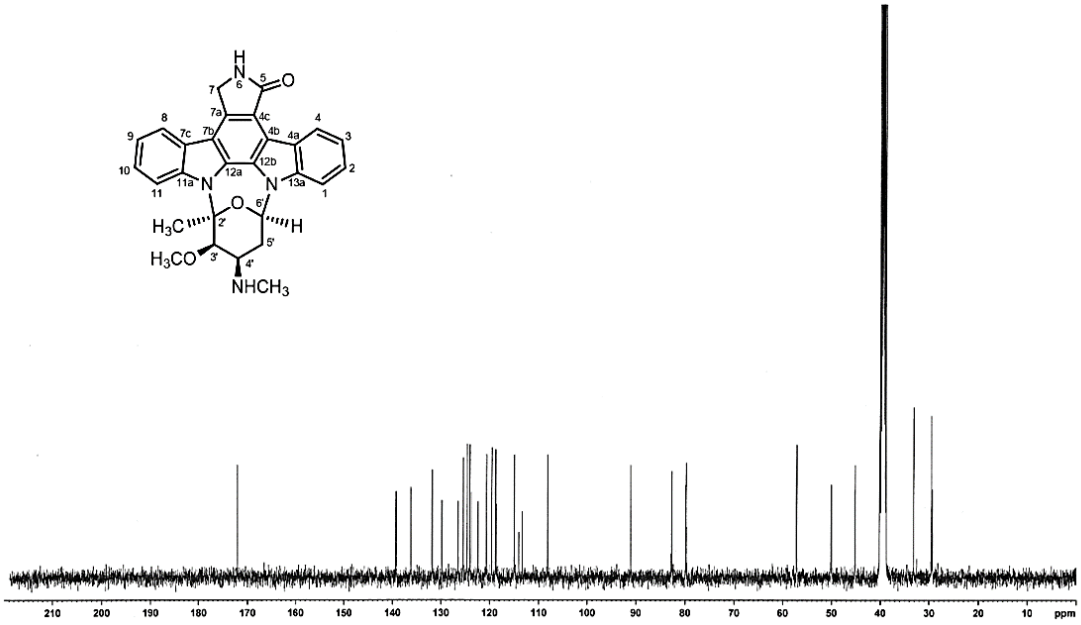

Figure D13 ^{13}C spectrum (400 MHz, DMSO-d_6) of C10-A

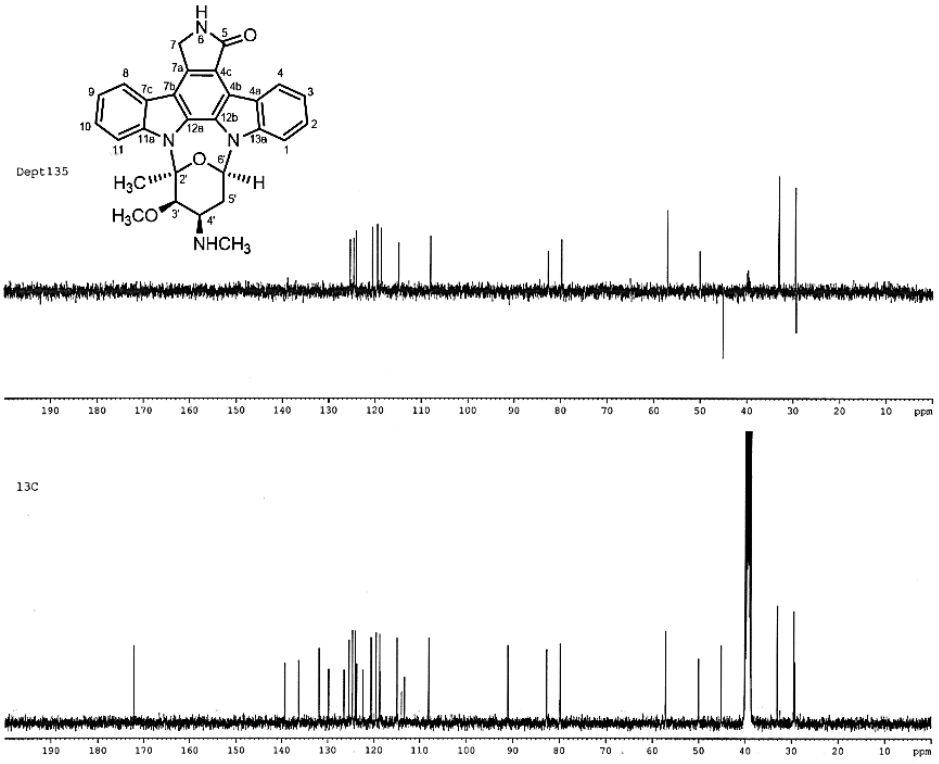

Figure D14 DEPT 135 spectrum (400 MHz, DMSO-d_6) of C10-A

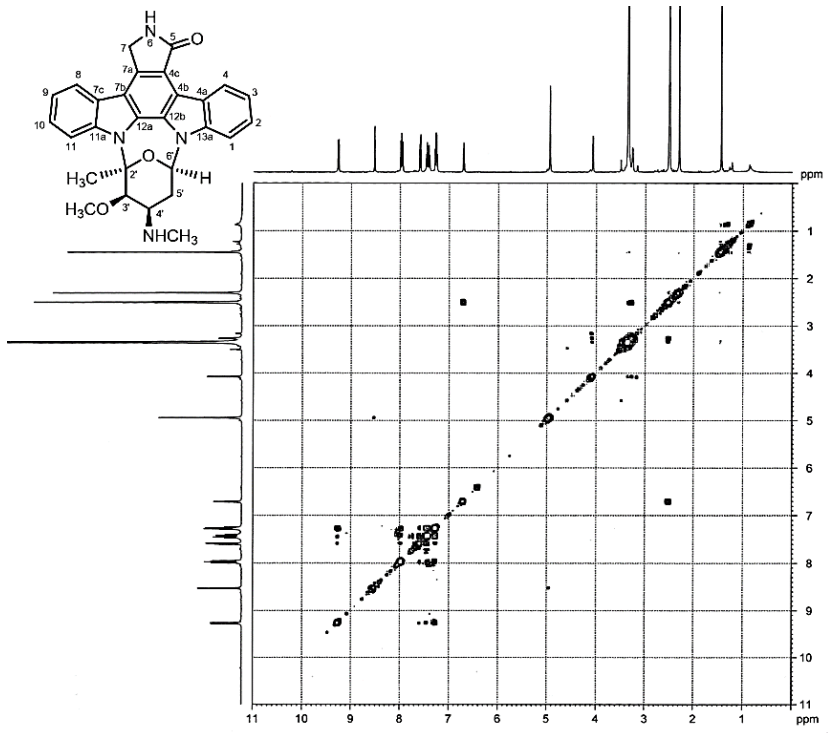

Figure D15 ^{1}H-^{1}H COSY spectrum (400 MHz, DMSO-d_6) of C10-A

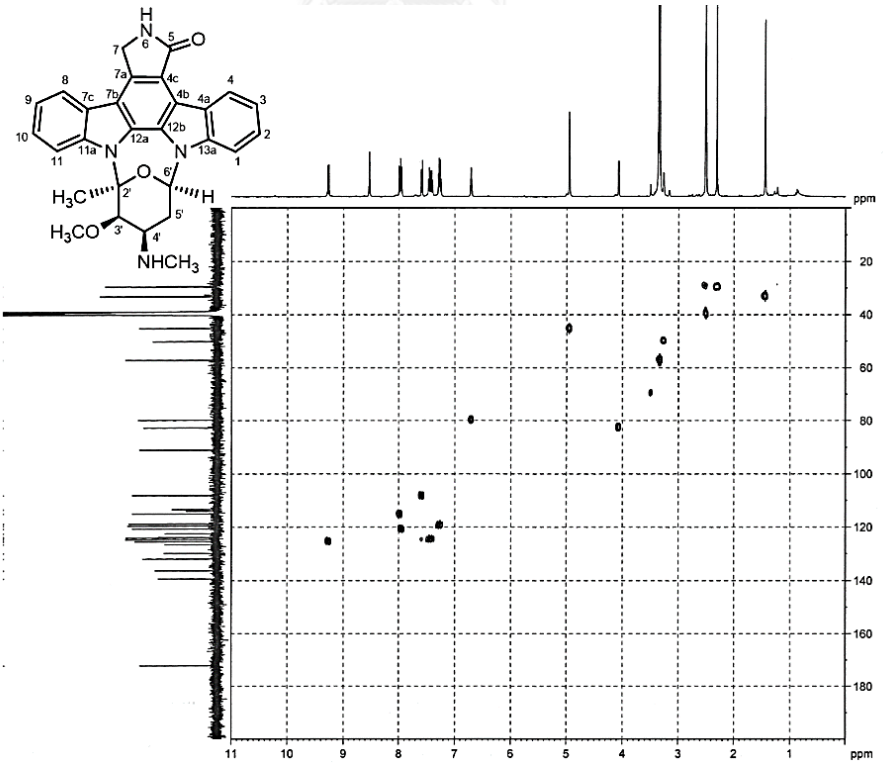

Figure D16 HMQC spectrum (400 MHz, DMSO-d_6) of C10-A

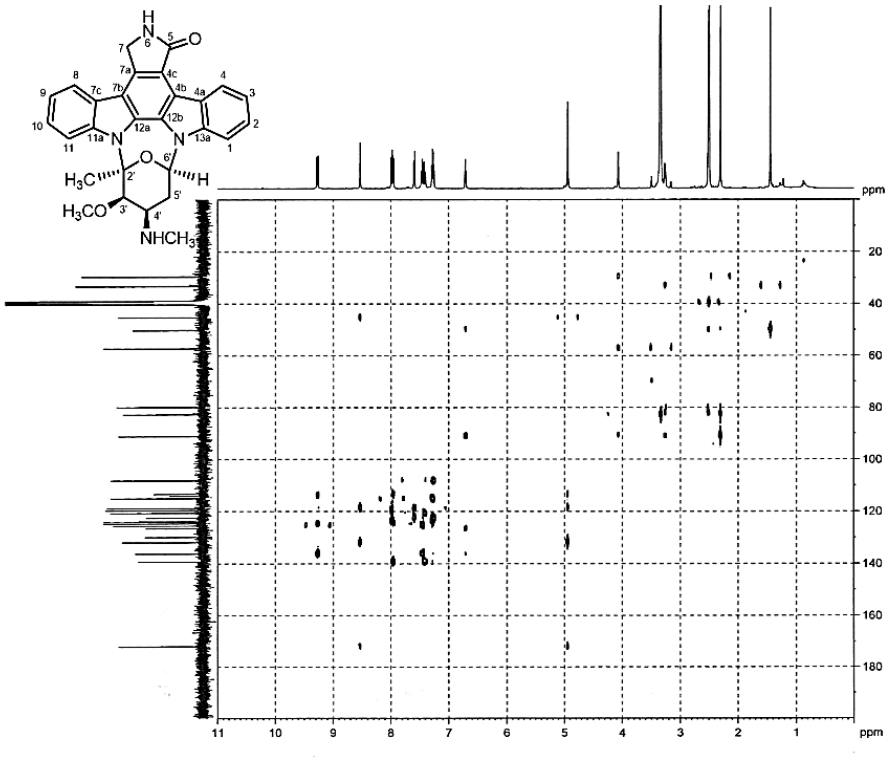

Figure D17 HMBC spectrum (400 MHz, DMSO-d_6) of C10-A

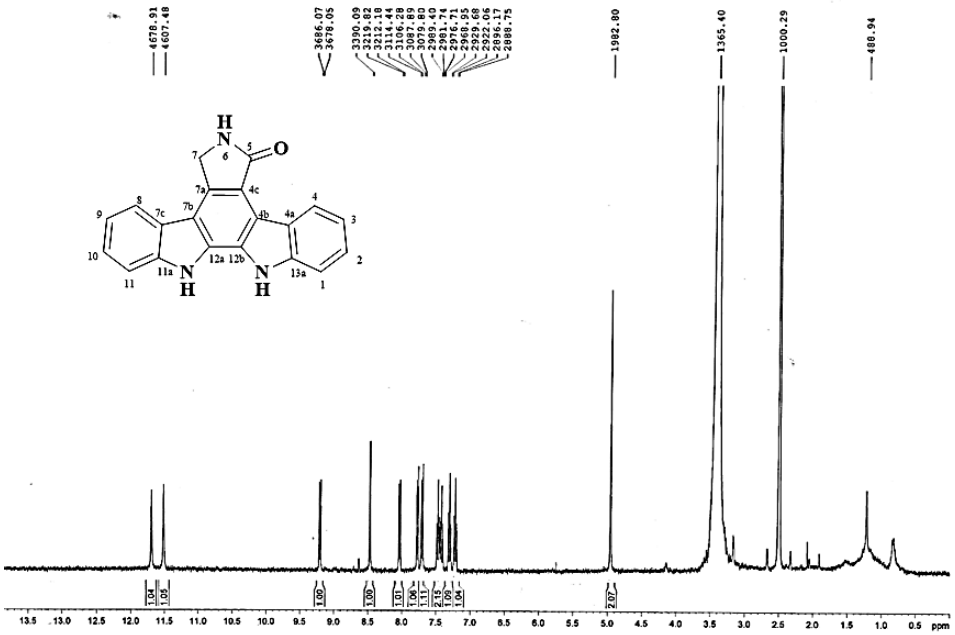

Figure D18 ^{1}H spectrum (400 MHz, DMSO-d_6) of C10-B

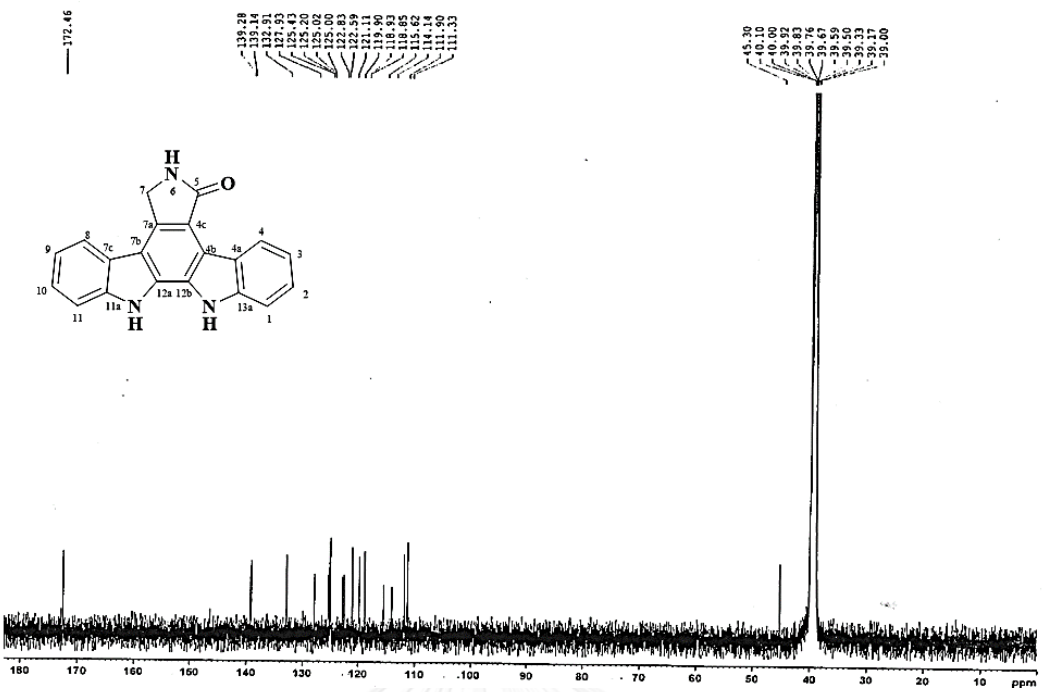

Figure D19 ^{13}C spectrum (400 MHz, DMSO-d_6) of C10-B

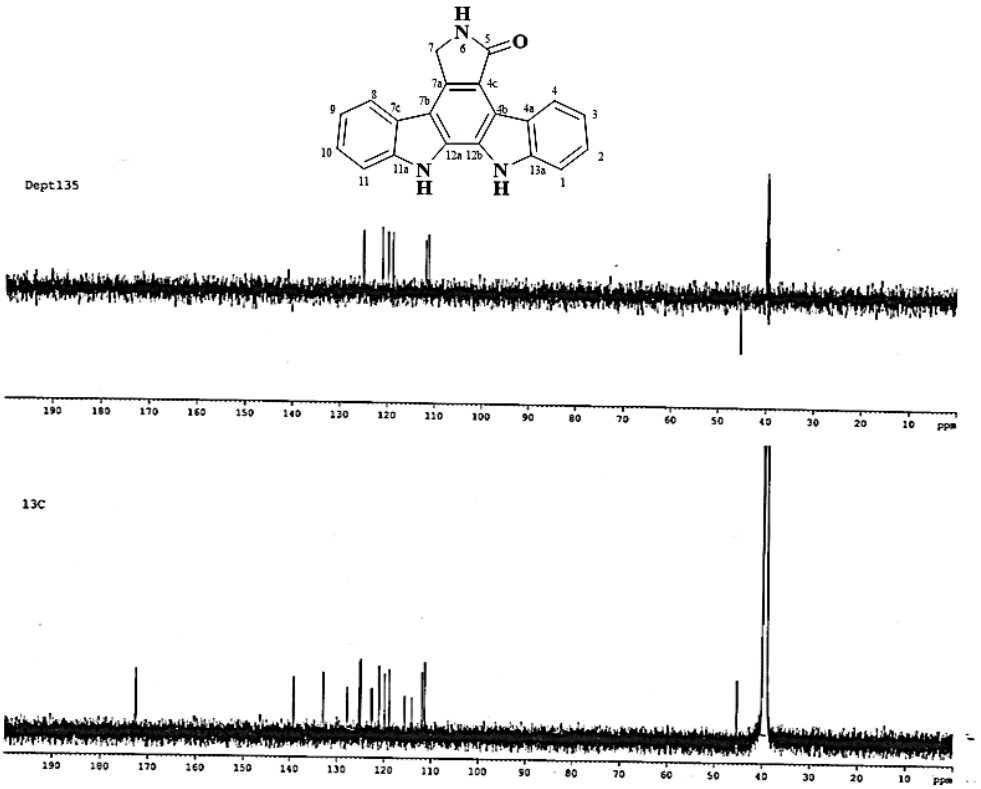

Figure D20 DEPT 135 spectrum (400 MHz, DMSO-d_6) of C10-B

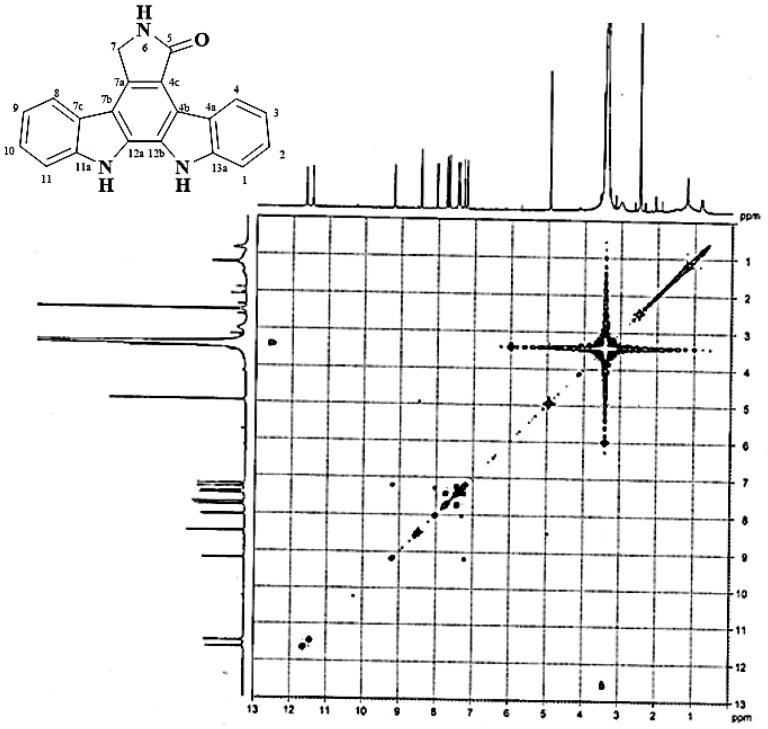

Figure D21 ^{1}H-^{1}H COSY spectrum (400 MHz, DMSO-d_6) of C10-B

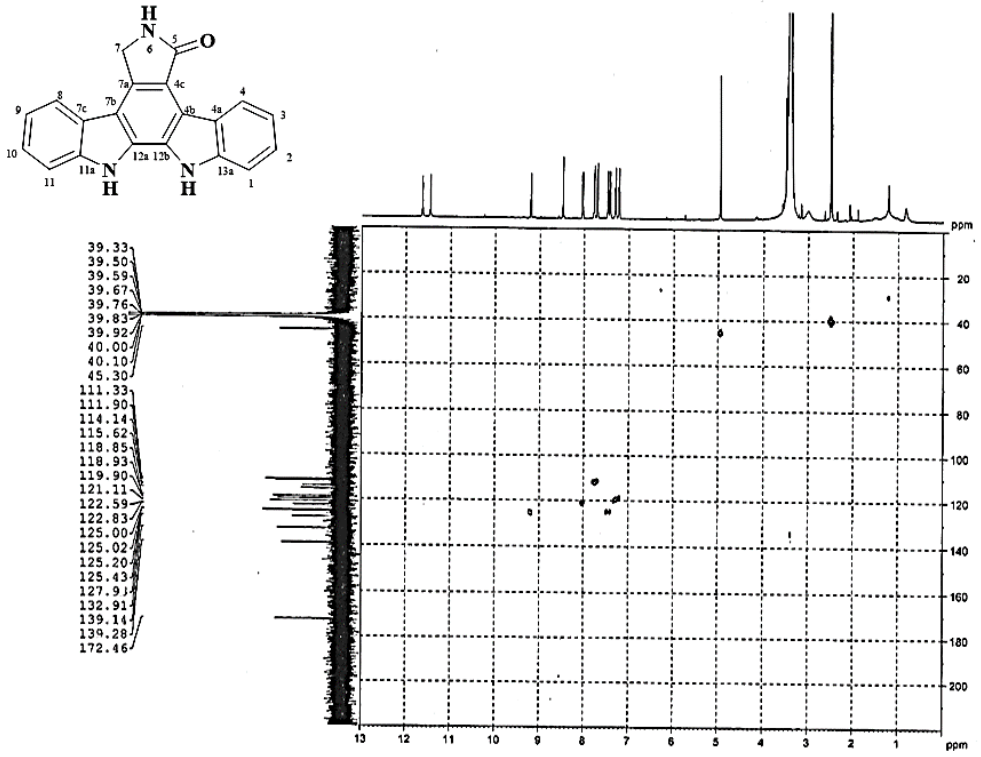

Figure D22 HMQC spectrum (400 MHz, DMSO-d_6) of C10-B

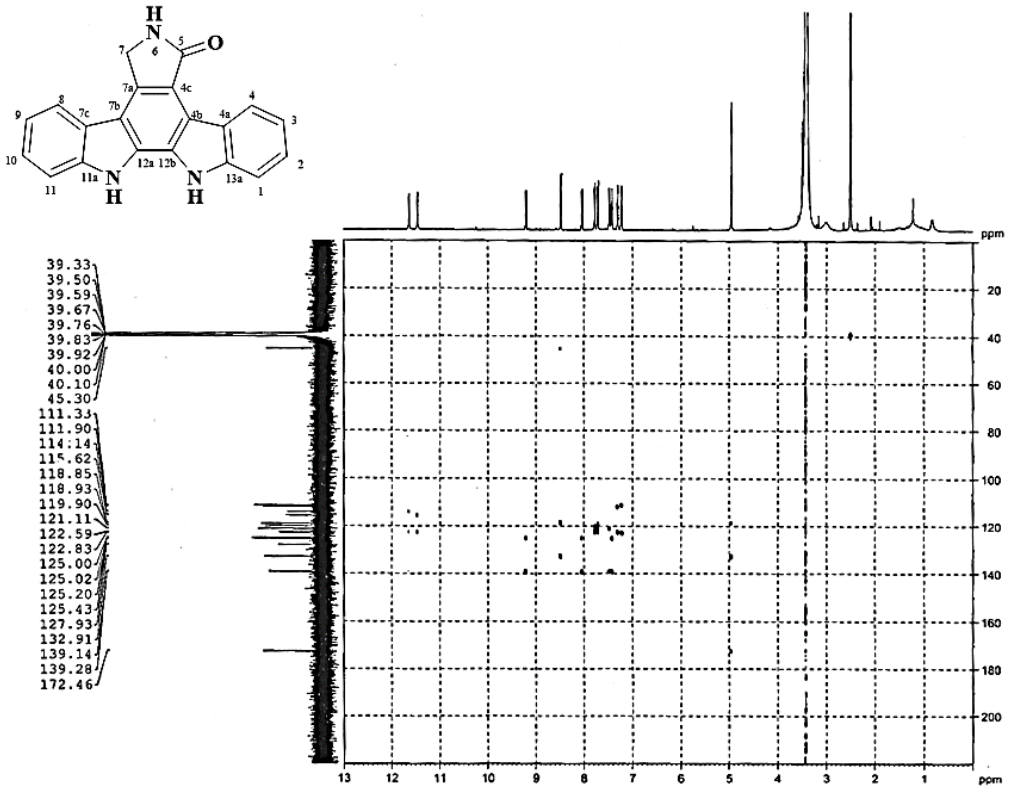

Figure D23 HMBC spectrum (400 MHz, DMSO-d_6) of C10-B

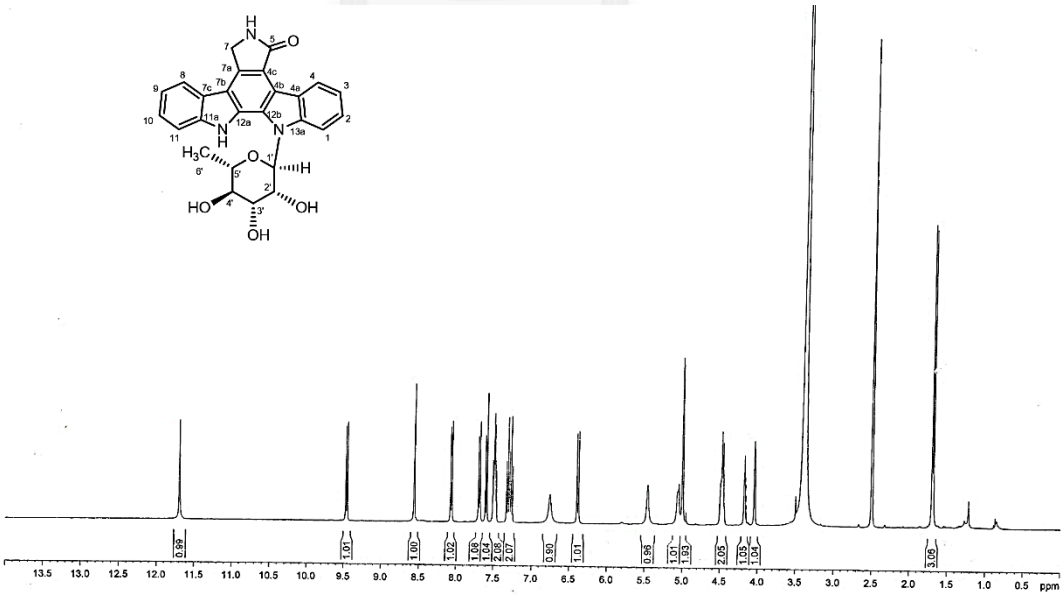

Figure D24 ^{1}H spectrum (400 MHz, DMSO-d_6) of C10-C

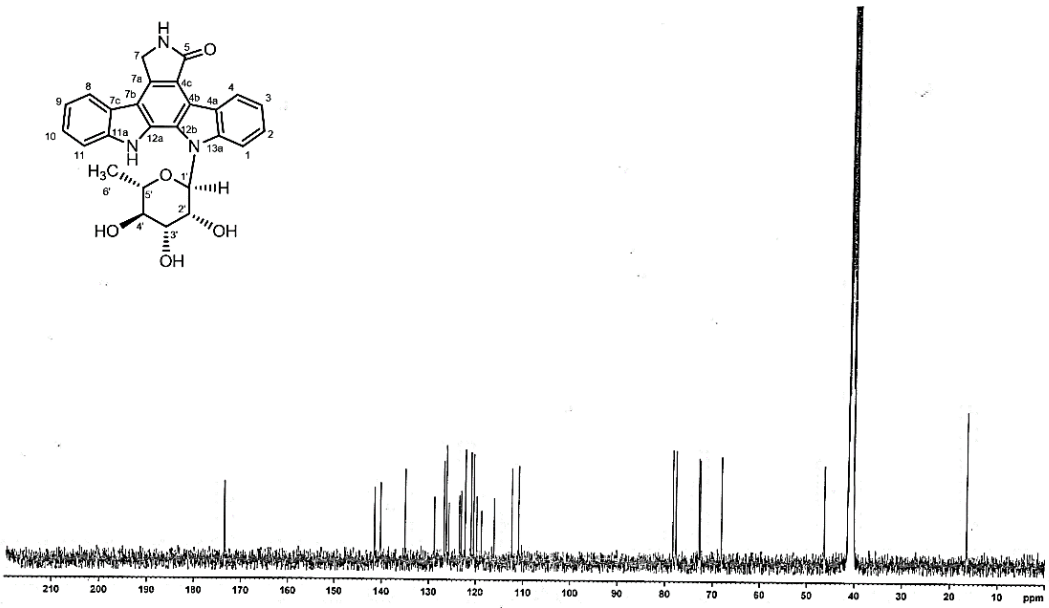

Figure D25 ^{13}C spectrum (400 MHz, DMSO-d_6) of C10-C

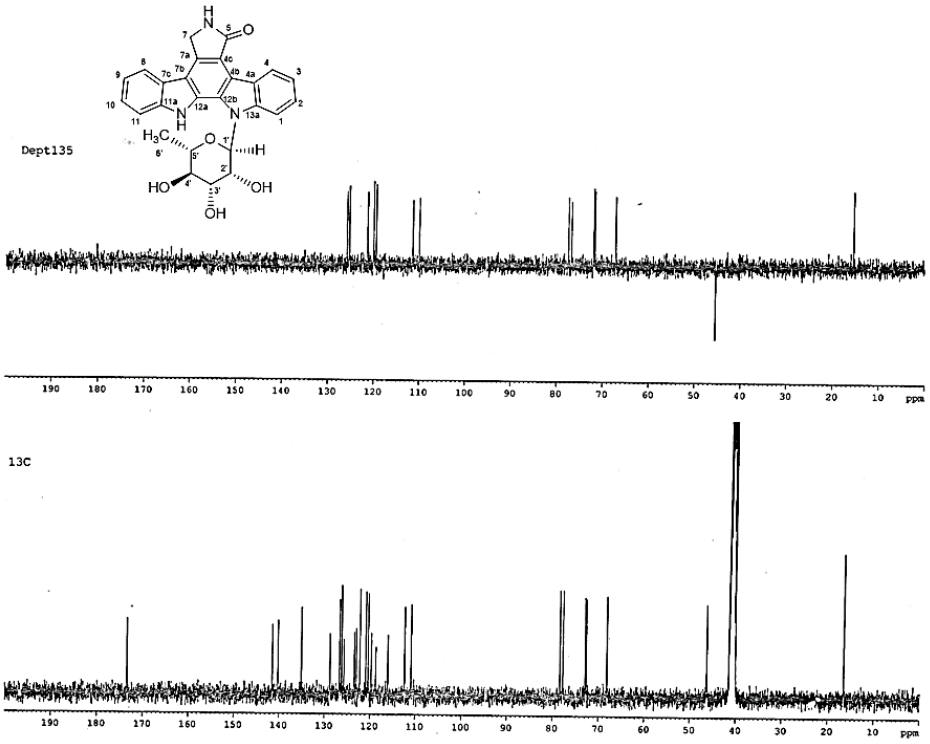

Figure D26 ^{13}C spectrum (400 MHz, DMSO-d_6) of C10-C

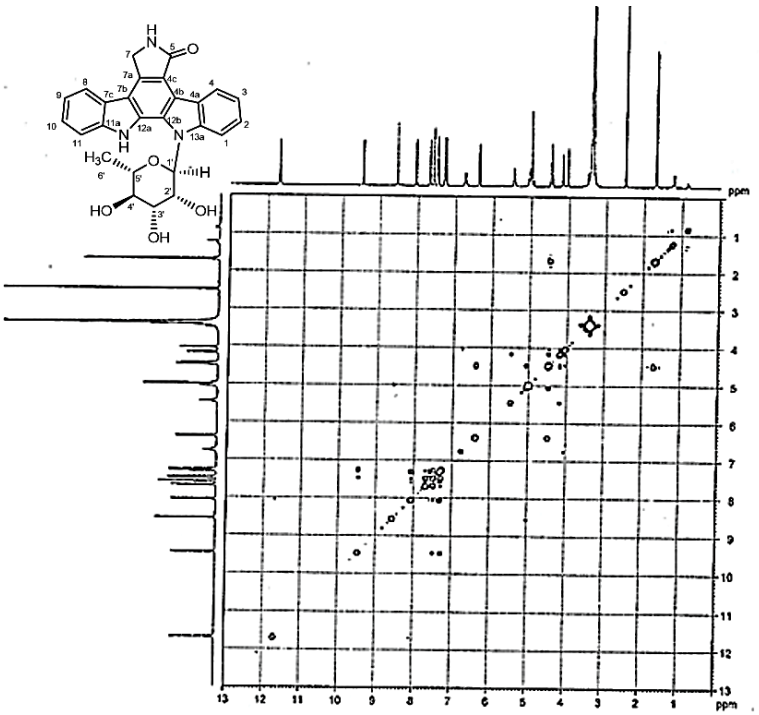

Figure D27 ^{1}H-^{1}H COSY spectrum (400 MHz, DMSO-d_6) of C10-C

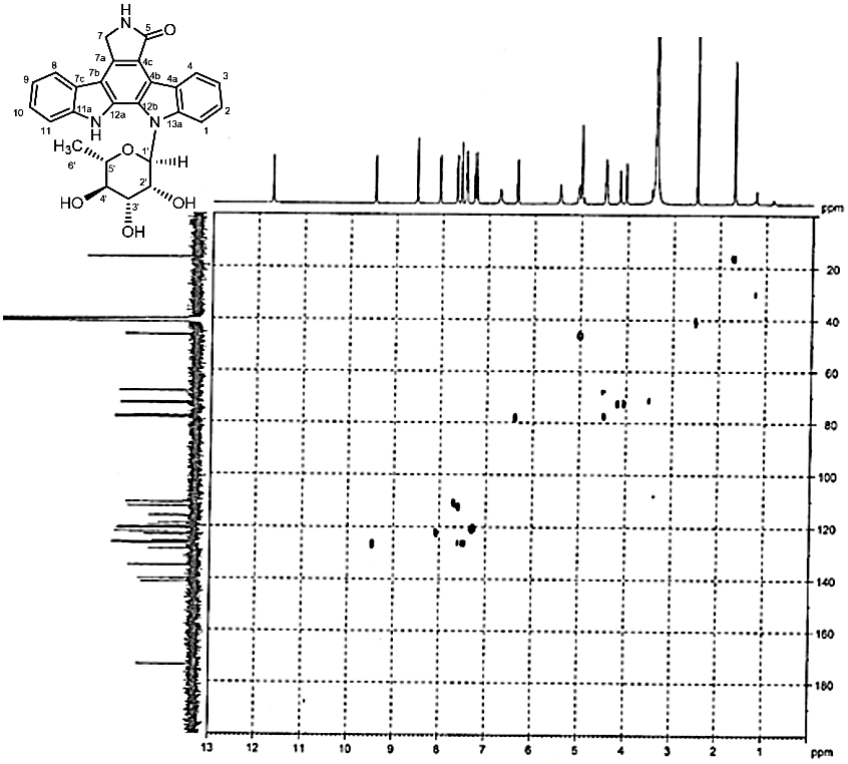

Figure D28 HMQC spectrum (400 MHz, DMSO-d_6) of C10-C

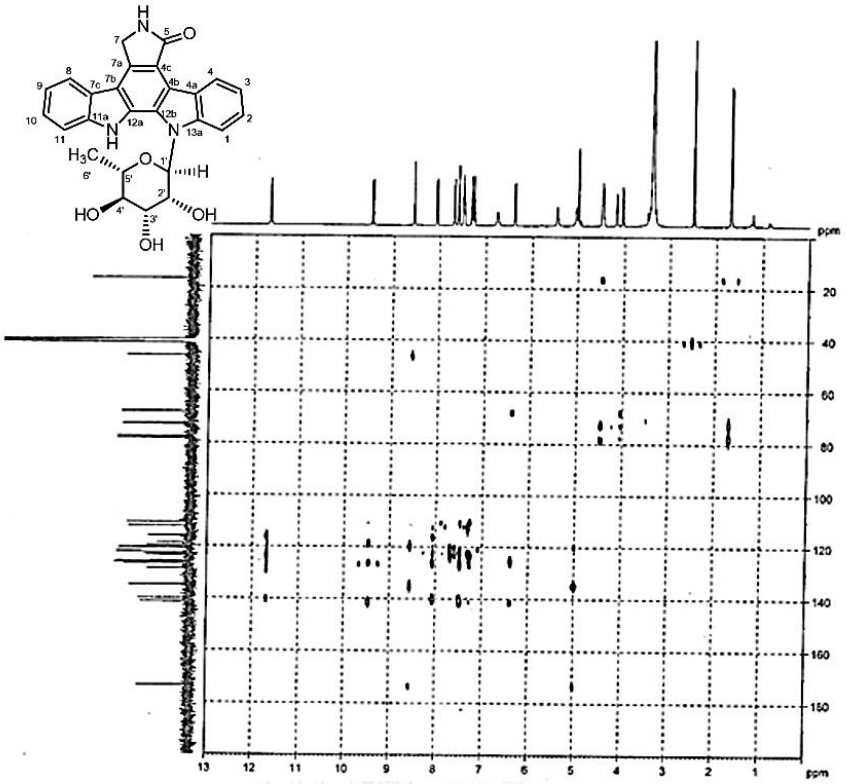

Figure D29 HMBC spectrum (400 MHz, DMSO-d_6) of C10-C

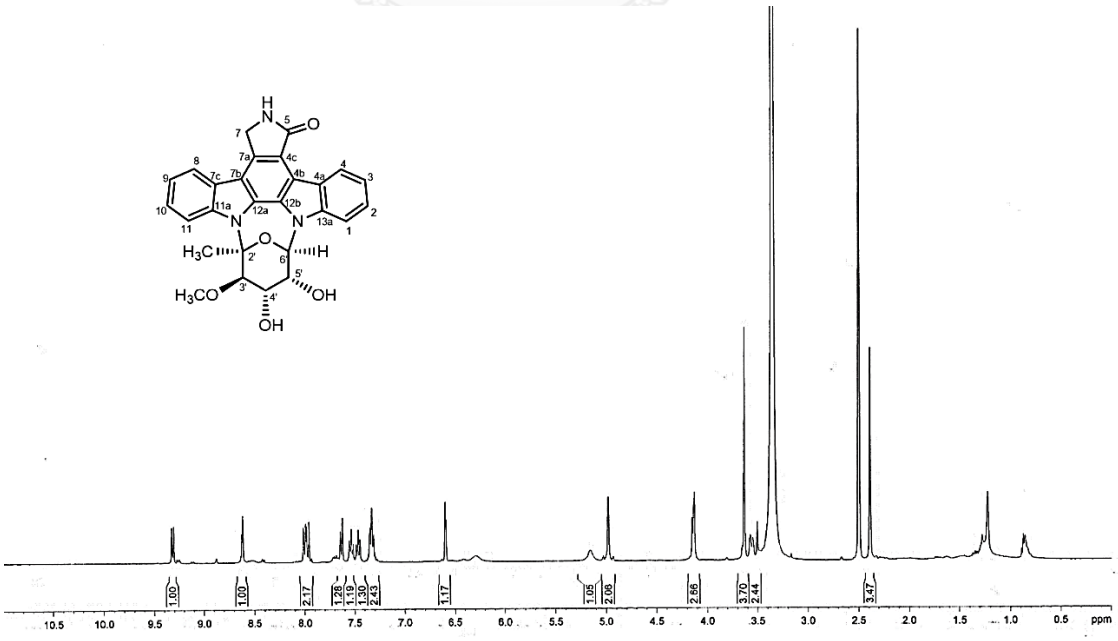

Figure D30 ^{1}H spectrum (400 MHz, DMSO-d_6) of C10-D

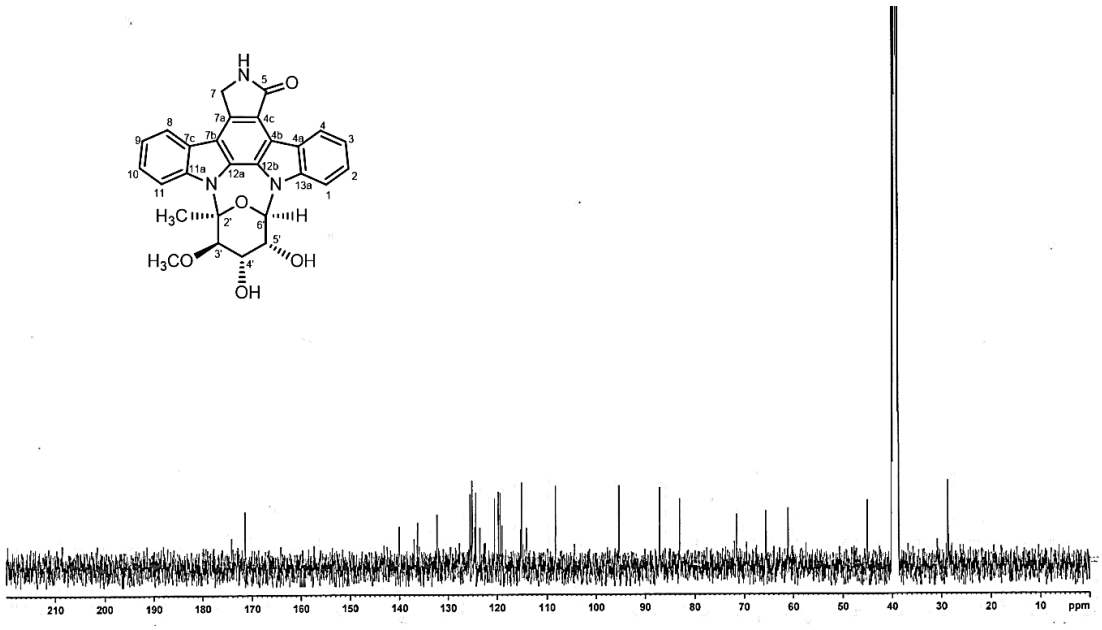

Figure D31 ^{13}C spectrum (400 MHz, DMSO-d_6) of C10-D

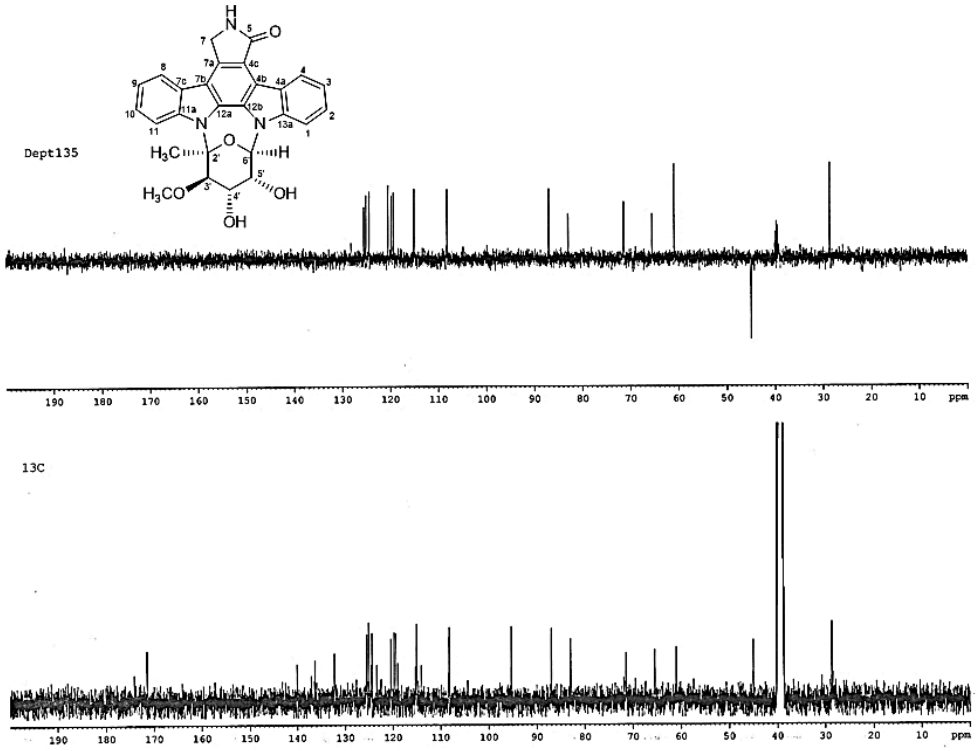

Figure D32 ^{13}C spectrum (400 MHz, DMSO-d_6) of C10-D

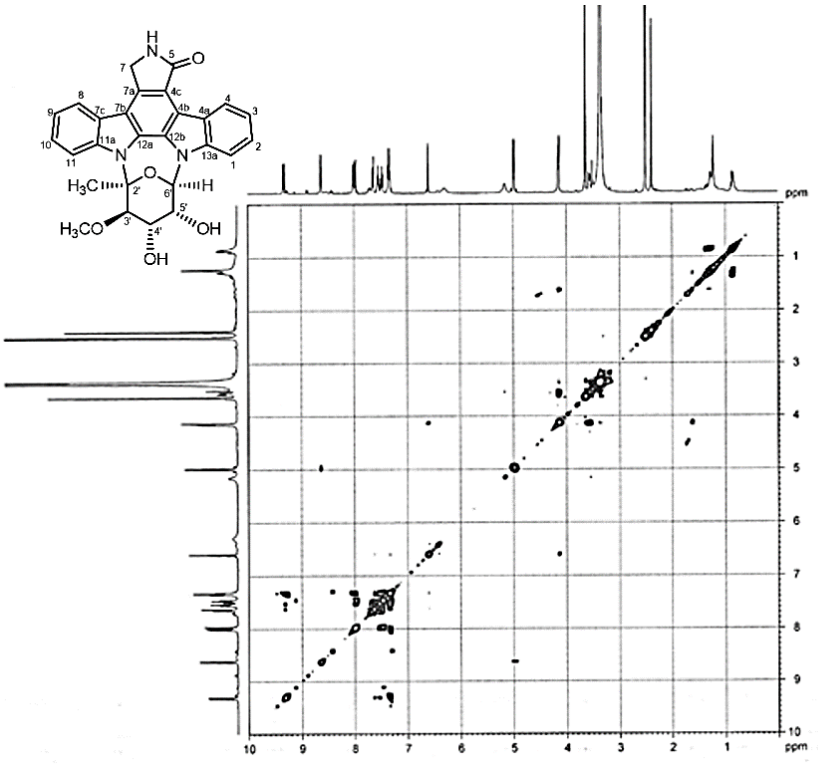

Figure D33 ^{1}H-^{1}H COSY spectrum (400 MHz, DMSO-d_6) of C10-D

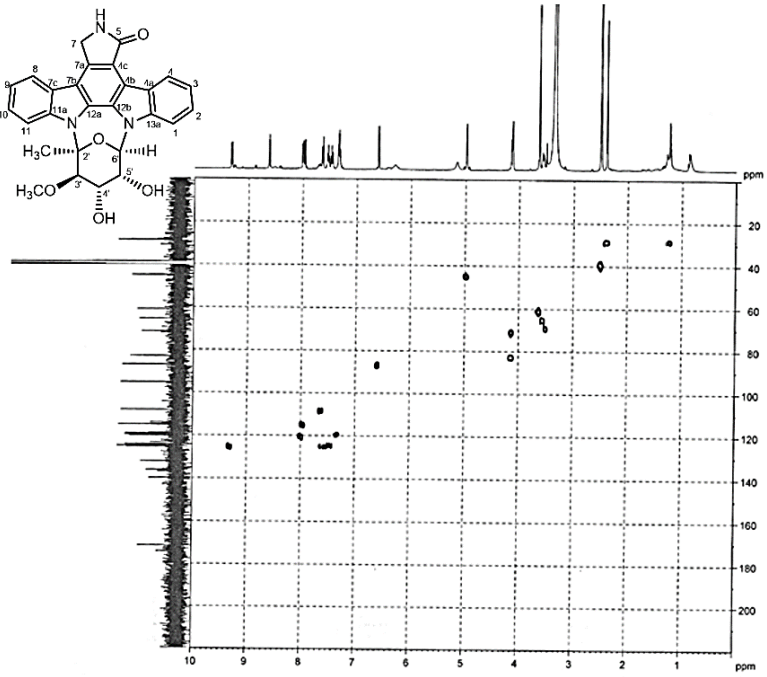

Figure D34 HMQC spectrum (400 MHz, DMSO-d_6) of C10-D

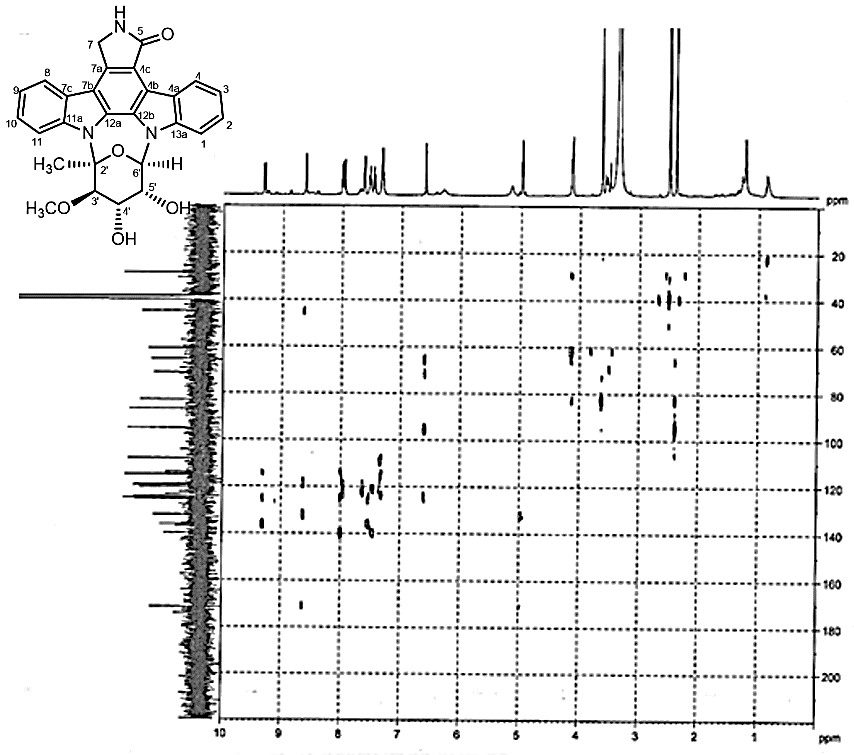

Figure D35 HMBC spectrum (400 MHz, DMSO-d_6) of C10-D

VITA

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Publications:

1. Phongsopitanun, W., Suwanborirux, K. and Tanasupawat, S. (2014). Indentification and antimicrobial activity of Streptomyces strains from Thai mangrove sediment. Thai J Pharm Sci 38, 49-56.

2. Phongsopitanun, W., Thawai, C., Suwanborirux, K., Kudo, T., Ohkuma, M. and Tanasuwawat, S. (2014). Streptomyces chumphonensis sp. nov., isolated from marine sediments. Int J Syst Evol Microbiol 64, 2605-2610.

3. Phongsopitanun, W., Kudo, T., Mori, M., Shiomi, K., Pittayakhajonwut, P., Suwanborirux, K. and Tanawupawat, S.(2015). Micromonospora fluostatini sp. nov., isolated from marine sediment. Int J Syst Evol Microbiol 65, 4417-4423.

4. Phongsopitanun, W., Kudo, T., Ohkuma, M., Pittayakhajonwut, P., Suwanborirux, K. & Tanasupawat, (2016). Micromonospora sediminis sp. nov., isolated from mangrove sediment. . Int J Syst Evol Microbiol (in press) doi: 10.1099/ijsem.0.001175.

5. Phongsopitanun, W., Kudo, T., Ohkuma, M., Pittayakhajonwut, P., Suwanborirux, K. & Tanasupawat, (2016). Streptomyces verrucosisporus sp. nov., isolated from marine sediments. Int J Syst Evol Microbiol (in press) doi: 10.1099/ijsem.0.001240