Screening, Purification and Characterization of Bioactive Compounds from Marine Bacteria Isolated from Southern Coast of Thailand

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ชื่อวิทยานิพนธ์ การคัดแยก การทำให้บริสุทธิ์ และคุณสมบัติของสารที่มีฤทธิ์ทางชีวภาพจากแบคทีเรียทางทะเลที่แยกจากชายฝั่งทะเลทางภาคใต้ของประเทศไทย

ผู้เขียน นางสาวสมรักษ์ พันธ์ผล

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บทคัดย่อ

การวิจัยนี้ครอบคลุม การแยกและคัดเลือกแบคทีเรียที่ได้จากทะเล เพื่อศึกษาสารที่มีฤทธิ์ต้านจุลินทรีย์และฤทธิ์ทางชีวภาพอื่นๆเช่นการยับยั้งเอนไซม์อะซิติลโคลีนเอสเทอเรส และความเป็นพิษต่อเซลล์มะเร็ง และการแยก ศึกษาโครงสร้างทางเคมีและคุณสมบัติของสารดังกล่าว ผลการศึกษาการแยกแบคทีเรียจากตัวอย่างชนิดต่างๆที่เก็บจากทะเลเช่นน้ำทะเล ตะกอนดิน ฟองน้ำ และอื่นๆ โดยใช้อาหารชนิดต่างๆใช้วิธีการแยกจากตัวอย่างโดยตรง (direct plating method) และแบบการเจือจางตัวอย่าง (serial dilution method) พบว่าการแยกแบคทีเรียโดยวิธีการเจือจางตัวอย่างให้ผลที่ดีเนื่องจากสามารถแยกแบคทีเรียที่มีความหลากหลายได้มากกว่า แต่ทั้งนี้ขึ้นอยู่กับองค์ประกอบของอาหารที่ใช้แยกเชื้อด้วยเช่นกัน แบคทีเรียที่แยกได้ส่วนใหญ่ (39%) ได้มาจากฟองน้ำซึ่งเป็นกลุ่มตัวอย่างหลักที่เก็บได้ทั้งหมด โดยแบ่งแบคทีเรียที่แยกได้ออกเป็น 2 กลุ่มคือแบคทีเรียทั่วไป และแบคทีเรียไกลดิง

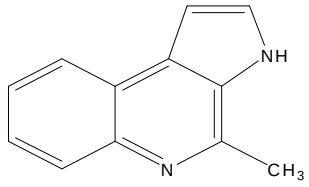
การศึกษาฤทธิ์ต้านจุลินทรีย์โดยใช้วิธี colorimetric microdilution broth assay ที่มี AlamarBlue เป็นตัวอินดิเคเตอร์ ผลการศึกษาพบว่าจำนวนสารสกัดหยาบจากแบคทีเรียทั่วไปจำนวน 199 ตัวอย่างมีฤทธิ์ยับยั้ง *Staphylococcus aureus* TISTR 517, *Candida albicans* TISTR 5239 และ *Saccharomyces cerevisiae* คิดเป็น 3.52, 4.02, และ 3.52% ตามลำดับที่ระดับความเข้มข้น 308.64 μg/ml ในขณะที่สารสกัดหยาบจากแบคทีเรียไกลดิงจำนวน 101 ตัวอย่าง มีจำนวนสารสกัดที่ยับยั้งเชื้อดังกล่าวที่ระดับความเข้มข้น 150 μg/ml คิดเป็น 9.9, 5.94, และ 3.96 % ตามลำดับ เมื่อพิจารณาชนิดของอาหารในการเลี้ยงเชื้อพบว่า สารสกัดหยาบจากแบคทีเรียไกลดิงที่มีฤทธิ์ในการต้านจุลินทรีย์ส่วนใหญ่มาจากอาหารเลี้ยงเชื้อที่มี skim milk เป็นองค์ประกอบ ในขณะที่สารสกัดหยาบจากแบคทีเรียทั่วไปที่มีฤทธิ์ต้านจุลินทรีย์ได้มาจากอาหารชนิด E ซึ่งมีแมนนิทอลเป็นแหล่งคาร์บอนหลัก และเมื่อศึกษาฤทธิ์การยับยั้งเอนไซม์อะซิติลโคลีนเอสเทอเรสของสารสกัดหยาบจากแบคทีเรียไกลดิง 90 ตัวอย่างมีจำนวน 7 ตัวอย่าง (7.78%) ที่สามารถยับยั้งเอนไซม์ดังกล่าวได้ 70 % หรือมากกว่า สำหรับสารสกัดหยาบจากแบคทีเรียทั่วไปไม่มีฤทธิ์ในการ

ยับยั้งเอนไซม์นี้ ผลการทดสอบฤทธิ์การยับยั้งเซลล์มะเร็งทั้ง 4 ชนิดคือ breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human cervix carcinoma (Hela) และ human mouth epidermoid carcinoma (KB) ของสารสกัดหยาบจากแบคทีเรียพบว่าไม่มีสารสกัดหยาบใดที่มีฤทธิ์ในการยับยั้งเซลล์มะเร็งทั้ง 4 ชนิด

จากผลการศึกษาสัณฐานวิทยาและสมบัติโดยทั่วไปของแบคทีเรียไกลดิง สายพันธุ์ GB009 และสายพันธุ์ GB 022 ที่นำมาศึกษาเนื่องจากการผลิตสารออกฤทธิ์ยับยั้งเอนไซม์อะซิติลโคลีนเอสเทอเรสได้ดี พบว่า เป็นแบคทีเรียแกรมลบ รูปร่างเป็นเส้นยาว สร้างเอนไซม์ออกซิเดส ไม่สร้างเอนไซม์คะตาเลส คุณสมบัติอื่นๆของแบคทีเรียทั้งสองพบว่า ต้องการน้ำทะเลหรือโซเดียมคลอไรด์ในการเจริญ ความเข้มข้นที่เหมาะสมของโซเดียมคลอไรด์ สำหรับแบคทีเรียไกลดิงสายพันธุ์ GB 009 และ GB 022 คือ 3 % และ 2 % ตามลำดับ อุณหภูมิที่เหมาะสมในการเจริญคือ 37°C นอกจากนี้ยังพบว่า $MgSO_4 \cdot 7H_2O$ (0.52%), $CaCl_2 \cdot 2H_2O$ (0.12%) และ KCl (0.08 %) มีผลในการเจริญของแบคทีเรียไกลดิงสายพันธุ์ GB 009 ในขณะที่ Ca^{2+} ไม่มีผลต่อการเจริญของแบคทีเรียไกลดิงสายพันธุ์ GB 022 การจัดจำแนกเชื้อแบคทีเรียไกลดิงทั้งสองสายพันธุ์โดยการวิเคราะห์ 16S rRNA gene sequences พบว่าแบคทีเรียไกลดิงสายพันธุ์ GB 009 และ สายพันธุ์ GB 022 มีความคล้ายคลึงทางด้านพันธุกรรมกับ *Rapidithrix thailandica* gen. nov., sp. nov. ที่ 99 % ผลการแยกสารที่มีฤทธิ์ทางชีวภาพจากแบคทีเรียไกลดิงสายพันธุ์ GB 009 ที่เลี้ยงในอาหาร skim milk ที่เตรียมโดยใช้น้ำทะเล โดยมี Amberlite XAD 16 ปริมาณ 2 % และชะสารออกจาก Amberlite XAD 16 resin โดยใช้ เอทิลอะซิเตท ทำการแยกสารโดยวิธีการทางโครมาโตกราฟีชนิดต่างๆโดยใช้การติดตามผลทางฤทธิ์ชีวภาพ(bioassay guided fractionation) และการตกผลึกในระบบของอะซิโตน คลอโรฟอร์ม และเฮกเซน ได้ผลึกใสรูปเข็ม โครงสร้างของสารได้จากวิเคราะห์โดยใช้ข้อมูลทางสเปกโทรสโกปีของสาร และการใช้ข้อมูลทางเอกซ์เรย์ ได้สารชนิดใหม่ 1 ชนิด คือ marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline) ซึ่งมีสูตรโมเลกุลเป็น $C_{12}H_{10}N_2$ ผลการแยกสารที่มีฤทธิ์ชีวภาพจากแบคทีเรียไกลดิงสายพันธุ์ GB 022 โดยใช้เทคนิคโครมาโตกราฟี ได้สารที่มีฤทธิ์ชีวภาพที่มีข้อมูลทางโปรตอนสเปกตรัมคล้ายคลึงกับ marinoquinoline A

การศึกษาฤทธิ์ทางชีวภาพของ marinoquinoline A พบว่ามีฤทธิ์ในการยับยั้งเชื้อจุลินทรีย์ชนิดต่างๆในช่วงแคบ โดยมีฤทธิ์ปานกลางในการยับยั้ง *Candida albicans* TISTR 5239 และ *Saccharomyces cerevisiae* โดยมีค่า MIC ที่ 18.25 และ 9.26 µg/ml ตามลำดับ มีฤทธิ์ในการยับยั้งเชื้อราที่เป็นเส้นใยต่ำ และไม่มีฤทธิ์ในการยับยั้งแบคทีเรีย ฤทธิ์ทางชีวภาพอื่นๆพบว่า

marinoquinoline A มีฤทธิ์ในการยับยั้งเอนไซม์อะซิติลโคลีนเอสเทอเรส โดยมีค่า MIC ที่ 4.9 μM ฤทธิ์ในการยับยั้งเซลล์มะเร็งทั้ง 4 ชนิด มีค่า IC_{50} > 5 μg/ml



Marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline)

Thesis Title	Screening, Purification and Characterization of Bioactive Compounds from Marine Bacteria Isolated from Southern Coast of Thailand
Author	Miss Somrak Panphon
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ABSTRACT

This study covered the isolation and screening of marine bacteria and screening for antimicrobial activity and other biological activities i. e. acetylcholinesterase inhibitory and cytotoxic activity as well as the purification and characterization of bioactive compounds from selected marine bacteria. Marine bacteria used in this study were isolated from various marine specimens for example seawater, sediments, sponges and others using different isolation media and two isolation methods i.e. direct plating and serial dilution methods. Serial dilution method yielded more bacterial diversity than direct plating method. The isolation also depended on the nutrients composition and their concentrations in the isolation media. Most bacterial isolates (39 %) were obtained from sponge samples which were a major group of specimens used for the isolation. The bacterial isolates can be divided into 2 groups i.e. general marine bacteria and gliding bacteria.

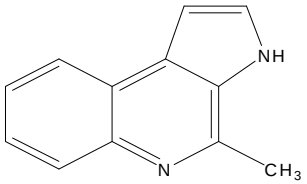
Antimicrobial activity of marine bacterial extracts was investigated by a colorimetric microdilution broth assay with AlamarBlue as a growth indicator. Of 199 general bacterial extracts, the percentages of active extracts against *Staphylococcus aureus* TISTR 517, *Candida albicans* TISTR 5239 and *Saccharomyces cerevisiae* at the concentration of 308.64 µg/ml were 3.52, 4.02 and 3.52, respectively. The crude extracts of marine gliding bacteria had the higher percentages of active extracts against the same tested microorganisms at 9.9%, 5.94% and 3.96%, respectively at the lower concentration (150 µg/ml). Most of active extracts of marine gliding bacteria were obtained from the skim milk medium while active extracts of general marine bacteria were obtained from medium E containing mannitol as a sole carbon source. Approximately 90 marine gliding bacterial extracts were investigated for acetylcholinesterase (AChE) inhibitory activity. It was found that there were 7 extracts (7.78%)

with $\geq$ 70 % acetylcholinesterase inhibition whereas the active extracts of general bacterial extracts had no inhibitory activity. No cytotoxicity against human cancer cell lines including breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human cervix carcinoma (Hela) and human mouth epidermoid carcinoma (KB) was found in all of extracts.

Marine gliding bacterial strains GB 009 and GB 022 were selected for further investigation due to the production of AChE inhibitor compounds. They were identified as Gram-negative bacteria with long filament and positive for oxidase but negative catalase tests. The strains were obligate marine bacteria because of their seawater requirement for natural seawater. The optimal NaCl concentration for growth was 2-3% while the optimal temperature was at 37 °C. It was also found that $MgSO_4 \cdot 7H_2O$ (0.52%), $CaCl_2 \cdot 2H_2O$ (0.12%) and KCl (0.08 %) could support the growth of the two strains whereas the Ca^{2+} had no effect on growth of GB 022. The complete identification was performed based on 16S rRNA gene sequence analysis. Both strains showed 99% similarity to the *Rapidithrix thailandica* gen. nov., sp. nov. The separation of bioactive compound from the marine gliding bacterial strain GB 009, cultured in skim milk broth in seawater containing 2% Amberlite XAD 16 resin, using bioassay guided fractionation led to the isolation of colourless needle-shaped single crystals which were obtained in acetone/chloroform/hexane solution. The structure of compound was elucidated by spectroscopic methods together with an X ray crystallography technique. A novel compound, marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline) with the molecular formula of $C_{12}H_{10}N_2$ was proposed. The isolation of active compounds from the marine bacterial extract of the strain GB 022 was carried out by the same methods. Based on the proton spectrum, the active compound was also identified as marinoquinoline A.

After submission for antimicrobial assay, marinoquinoline A showed *in vitro* activity against a narrow spectrum of tested microorganism including *Candida albicans* TISTR 5239, *Saccharomyces cerevisiae*, *Aspergillus fumigatus* TISTR 3108, *Aspergillus flavus* TISTR 3041, *Staphylococcus aureus* TISTR 517, methicillin-resistant *Staphylococcus aureu*s, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli,* and *Pseudomonas aeruginosa*. It showed moderate antifungal activity against *Candida albicans* TISTR 5239 and *Saccharomyces cerevisiae* with the MIC value of 18.52 and 9.26 µg/ml, respectively. However, it showed only weak antifungal activity against filamentous fungi and even less active in antibacterial assay. Interestingly,

marinoquinoline A showed significant acetylcholinesterase inhibitory activity with an IC_{50} value of 4.9 μM. More importantly, it showed no cytotoxicity against four different human cell lines i.e. breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human cervix carcinoma (Hela) and human mouth epidermoid carcinoma (KB). Based on its biological activity, it could, therefore, potentially be developed as an acetylcholinesterase inhibitor or a drug for treatment of Alzheimer.



Marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline)

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LIST OF ABBREVIATIONS AND SYMBOLS

s	=	singlet
br s	=	broad singlet
d	=	doublet
dd	=	doublet of doublet
J	=	coupling constant
δ	=	chemical shift relative to TMS (tetramethylsilane)
Conc.	=	concentration
M	=	molarity
MHz	=	Megahertz
ppm	=	part per million
rpm	=	revolutions per minute
UV	=	Ultraviolet
NMR	=	Nuclear Magnetic Resonance
1D NMR	=	One Dimensional Nuclear Magnetic Resonance
2D NMR	=	Two Dimensional Nuclear Magnetic Resonance
COSY	=	Correlation Spectroscopy
DEPT	=	Distortionless Enhancement by Polarization Transfer
HMBC	=	Heteronuclear Multiple Bond Correlation
HMQC	=	Hetronuclear Multiple Quantum Coherence
AChE	=	acetylchlolinesterase
$CDCl_3$	=	deuterochloroform
DCM	=	dichloromethane
DMSO	=	dimethylsulfoxide
EtOAc	=	ethyl acetate
MeOH	=	methanol
DI water	=	deionized water

CHAPTER 1

INTRODUCTION

General Introduction

Drug discovery program continues to be an important issue in pharmaceutical industry because of the urgent needs for new drugs to treat emerging human diseases. According to the World Health Organization report in 1998, infectious diseases were the second leading cause of death, contributed to 25% of death worldwide. Infectious diseases, especially in South East Asia and Africa, represented 45% of all death and particularly in the cause of children with less than 4 year old (WHO-Report, 1999). One of factors which contribute to the rise of infectious diseases comes from drug resistant pathogens, which occur due to the improper uses of antimicrobial agents for treatments of human and animal diseases (Mazel and Davies, 1999; Swartz, 2000; Goodyear, 2002; Kümmerer, 2004). Many drug resistant bacteria have been reported worldwide i.e. *Streptococcus pneumonia, Shigella* sp., *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (Clarke *et al*., 2004; Landman *et al*., 2005; Subedi *et al*., 2005; Sivapalasingam *et al*., 2006). Moreover, multidrug-resistant *Acinetobacter* which is the cause of wound infection and osteomyelitis, was also reported (Davis *et al*., 2005). Multidrug-resistance (MDR) has become a difficult problem for treatment due to a long term cure and high risk of death. The treatment, therefore, is considered the second-or third-line drugs which are more expensive and sometimes more toxic. As a consequence, the search for new therapeutic agents with better pharmacological properties or less toxicity or side effects is critically needed. Many approaches in drug discovery have been attempted such as using biosensors, combinatorial chemistry and high-throughput screening methods (Seneci and Miertus, 2000; Keusgen, 2002). In addition, the search of new compounds from natural source, especially marine environment, is of great interest. As a matter of fact the ocean shows the greatest biodiversity, with 34 of 36 phyla of life represented in different marine habitats (Donia and Hamann, 2003), In order to survive in marine environment, which are uniquely different from terrestrial, marine organisms have adapted and evolved to such environments. The adaptation and evaluation led to the

capability of marine organisms to produce unique secondary metabolites showing interesting pharmacological properties (Urban *et al*., 2000; Mayer and Hamann, 2002; Donia and Hamann, 2003; Fenical *et al*., 2003; Newman and Cragg, 2004; Aneiros and Garateix, 2004). Many of bioactive compounds derived from marine sources or synthesized from a prototypical compounds are now in phase II/III clinical trials in cancer therapy, for example, ecteinascidin 743 and squalamine (Newman and Cragg, 2004). Recently, the chemical investigation of marine organisms has expanded to marine microorganisms especially bacteria and fungi. Marine bacteria have been reported to produce compounds with diversity both in chemical structures and biological activities i.e. anticancer, antifungal, antiviral, anti-inflammatory and antimicrobial activities (Fenical and Jensen, 1993; Jensen and Fenical, 1994; Moore *et al*., 1999; Hentschel *et al*., 2001; Kelecom, 2002; Saha *et al*., 2005; Kwon *et al*., 2006). A study by Burgess *et al*. (1999) showed that 35% of over 400 strains of surface-associated bacteria isolated from various specimens of seaweeds and invertebrates collected from the Scottish coastal water, produced antimicrobial compounds. Moreover, Isnansetyo and Kamei (2003a) reported that a new marine bacterium, *Pseudoalteromonas phenolica* sp. nov. O-BC30^{T} produced antibiotic inhibiting methicillin-resistant *Staphylococcus aureus*. These indicate that marine bacteria represent a potential source for new natural bioactive agents.

Although many researches on marine natural products have been carried out worldwide, only a few reports were from Thailand regardless the availability of the country's marine resources. Therefore, this thesis aimed to exploit marine bacteria in Thailand as the sources of new bioactive compounds by developing the screening of antimicrobial activity from marine bacterial extracts using a colorimetric microdilution broth assay with AlmarBlue along with acetylcholinesterase inhibitory and cytotoxic assays. The chemical investigation including the isolation and purification of active compounds were carried out using a bioassay-guided fractionation method followed by the structure elucidation of the active compounds using various spectrometry techniques, i. e. IR, MS, NMR and X-ray.

Literature Review

1. Bacteria in marine environment

The oceans which cover 71% of the earth's surface are ecologically diverse such as salt marshes, mangrove swamps, rocky shores, sandy beaches, coral reefs and volcanic vents in deep sea (Karleskint *et al*., 2006). Moreover, the variations in environmental parameters i.e. pressure, salinity, oxygen diffusion and temperature in oceans have great affects on marine organisms which led to different metabolic pathways and biochemical adaptation of marine organisms.

Marine microorganisms are highly abundant in marine environments and have been isolated from seawater, marine sediments, and from surfaces and tissues of marine algae and marine invertebrates. They are also expected to play many important roles in marine ecosystems such as producer, consumer, decomposer, and parasite. Marine microorganisms belong to all three domains of life including Archaea (or archaebacteria), Bacteria (or eubacteria), and Eucarya (or eukaryotes) (Karleskint *et al*., 2006). Marine prokaryotes can be divided into two groups or domains, the domain Bacteria and the Archaea. Early studies showed that the Archaea were associated to various extreme environments concerning temperature, pH, salinity, and anaerobiosis. However, analyses of PCR-amplified rRNA genes from a variety of habitats revealed that the Archaea were also found in non extreme environments, such as ocean water, lake waters, soil (Chaban, *et al*., 2006) and marine sponges (Hentschel *et al*., 2006).

The domain Bacteria or Eubacteria are a large and heterogenous group of bacteria including most bacteria and the cyanobacteria. Classification of bacteria has been done based on many criteria such as: morphological characteristics and physiological and metabolic characteristics (Prescott, 1993). Currently, nucleic-acid sequencing techniques are a primary tool used for taxonomic and identification of microorganisms. Fenical and Jensen (1993) grouped marine microorganisms based on phylogenetic and further divided according to their obtaining carbon and energy (Table 1). It showed that chemoheterotrophic bacteria represented a large group and diverse group of bacteria. They are different in cell wall and morphology; therefore subgroups can be divided based on morphological characteristics, Gram-positive bacteria and Gram-negative bacteria.

Table 1. Classification of marine microorganisms.

Archaebacteria	Autotrophic eubacteria	Chemoheterotrophic eubacteria	Eukaryotes
Chemoautotrophs	Photoautotrophs	Gram-positive	Photoautotrophs
Methanogen	Anoxygenic photosynthesis	Endospore-forming rods and cocci	Microalgae
Thermoacidophiles [a]	Purple and green photosynthetic	Non-spore-forming rods	Chemoheterotrophs
Chemoherotrophs	bacteria (Order Rhodospirillales)	Non-spore-forming cocci	Protozoa
Halophiles	Oxygenic photosynthesis	(Family Micrococceae)	Flagella
	Cyanobacteria (Order Cyanobacterials)	Actinomycetes	Amoebae
	Prochlorophytes (Order Prochlorales)	(Order Actinomycetales) and	Ciliates
	Chemoautotrophs	related organisms	Fungi
	Nitrifying bacteria	Gram- negative	Higher fungi
	(Family Nitrobacteraceae)	Rods and cocci	Ascomycetes
	Coloeless sulture –oxidizing bacteria [b]	Aerobic (Family Pseudomonadaceae)	Deuteromycetes
	Methane-oxidizing bacteria	Facultative(Family Vibrionaceae)	Basidomycetes
	(Family Methylococcaceae)	Anaerobic (Sulfur- reducing bacteria)	Lower fungi
		Gliding bacteria	(Class Phycomycetes)
		(Order Cytophagales and Beggiatoales)	
		Spirochaetes (Order Spirochaetales)	
		Spiral and curved bacteria	
		(Family Spirillaceae)	
		Budding and/or appendaged bacteria	
		Mycoplasma (Class Mollicutes) [b]	

[A] Includes heterotrophic genera. [b] Only two reports documented.
Souce: Fenical and Jensen (1993).

1.1 Gram-positive bacteria

It was reported that bacteria found in seawater or other marine habitats are mostly unculturable bacteria when using classical media and isolation methods (Ferguson *et al*., 1984). This result was consistent with the recent observation by Simu *et al.* (2005), who investigated marine bacterioplankton in the Baltic Sea and Skagerrak Sea at a 2-m depth and found that non-colony-forming bacteria were the majority of the viable cells. Confirming molecular studies also showed that the dominance of non-colony-forming bacteria was found in clone libraries. These observations suggest that traditional media formulations may be inappropriate for the growth of most marine bacteria.

The distribution of Gram-positive bacteria in marine environments is varied depending on the habitats. They usually represent less than 10% of total seawater population (Fenical and Jensen, 1993). However, the observation on bacteria in the coral reef environments of Belize, Central America, showed that Gram-positive bacteria were a large percentage of cultureable bacteria associated with algal surface (Jensen and Fenical, 1994). It has been shown that Gram-positive bacteria accounted for 14%, 25%, 31%, and 12% of bacterial isolates derived from seawater, sediments, and the surfaces of algae and invertebrates, respectively, and most of them required seawater for growth (Jensen and Fenical, 1995).

Urakawa *et al*. (1999) examined microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan by 16S rRNA gene analysis. The study showed that Gamma *Proteobacteria* and high G+C Gram-positive bacteria were among the dominant groups. Other studies showed that Gram-positive bacteria of the genera *Bacillus* distributed throughout the marine environment including sea water (Yoon *et al*., 2004a), sediment samples (Jaruchoktaweechai *et al*., 2000), deep sea sediments (Rüger *et al*., 2000), Dead Sea (Arahal *et al*., 1999), a shallow marine hydrothermal vent (Caccamo *et al*., 2000; Rusch *et al*., 2005), and marine sponges (Anand *et al*., 2006). The investigation of marine bacilli, isolated from marine invertebrates and seawater from different locations of the Pacific Ocean, revealed that the most of marine bacilli were classified as *Bacillus subtilis* which were able to grow at 0-7 % NaCl but no growth was observed when cultured at 15% NaCl (Ivanova *et al*., 1999). However, alkaliphilic strains of *Bacillus horti*, showed growth in media containing NaCl concentration up to 15% NaCl. In a previous study by the same group, other species of *Bacillus* were reported to

be *B. marinus, B. subtilis, B. pumilus, B. licheniformis, B. cereus* and *B. mycoides* (Ivanova *et al*., 1992 cited by Ivanova *et al*., 1999). Regardless of many reports on the presence of *Bacillus* in the marine environment, *Bacillus marinus* was one of a few obligate marine species due to their sodium and potassium requirement for growth (RÜger and Hentzschel, 1980 cited by RÜger *et al*., 2000) which could be found from tropical Atlantic, Arctic and Antarctic deep-sea sediments (RÜger *et al*., 2000). Some were classified as psychrophilic and psychrotolerant.

Caccamo *et al*. (2001) compared the amplified 16S rDNA restriction patterns of the thermophilic bacilli isolated from shallow marine hydrothermal vents of the Eolian Islands, Italy. The most dominant species was *B. thermodenitrificans*, accounting for 30% of the thermophilic bacilli. In addition, a novel strain of *Bacillus aeolius* was isolated from water sample collected at 15 m depth from the same location (Gugliandolo *et al*., 2003). The strain was thermo-halophilic with the optimal growth temperature at 55°C and 2% NaCl.

Pukall *et al*. (2001) reported the low G+C and high G+C Gram-positive bacteria isolated from surface of bryozoan *Flustra foliacea* collected from the North Sea. Bacterial isolates affiliated to the *Clostridium-Bacillus* and *Actinobacteria* with values of 52.6% and 15.8%, respectively. *Micrococcus luteus*, *Nocardioides*, and *Arthrobacter nicitinovorans* were among the members of high G+C content groups. Additionally, *Arthrobacter*, and *Micrococcus* together with alpha-*Proteobacteria* and gamma-*Proteobacteria* (*Vibrio*, *Pseudoalteromonas)* were also reported from marine sponges (Hentschel *et al*., 2001), as well as *Micrococcus luteus* from sponge *Xestospongia* sp. (Bultel-poncé *et al*., 1998).

List of selected Gram-positive bacteria obtained from marine environments is shown in Table 2.

1.2 Actinomycetes

Actinomycetes are a morphologically diverse group of Gram-positive bacteria with high GC content in excess of 55 mol% (Ensign, 1992). They are the best known as soil bacteria and the sources of valuable bioactive compounds. The actinomycetes are separated into seven major groups according to the oligonucleotide sequences including actinobacteria, nocardioforms, actinoplanetes, thermomonospora, maduromycetes, streptomycetes, and a group with multilocular sporangia (Ensign, 1992).

Table 2. Examples of selected Gram- positive bacteria isolated from marine environments and related marine areas.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
Seawater				
- Seawater of a tidal flat of the Yellow Sea Korea	*Bacillus marisflavi* sp. nov.	0-16% NaCl Optimal growth at 2-5%NaCl	10-47 (Optimal growth at 30-37)	Yoon *et al*., 2003b
	Bacillus aquimaris sp. nov.	Grow up to 18 % NaCl Optimal growth at 2-5% NaCl	10-44 (Optimal growth at 30-37)	
-Seawater, Hwajinpo Beach, East Sea, Korea	*Bacillus hwajinpoensis* sp. nov.	Not occurs without NaCl or in the presence of >20% NaCl Optimal growth at 2-5 % NaCl	10-40 (Optimal growth at 30-35)	Yoon *et al*., 2004a
-Seawater, Baekryung Island, Yellow Sea, Korea	Unnamed *Bacillus* genomospecies	Growth in the presence of 20 % NaCl, but not without NaCl or in the presence of >21% NaCl	10-42 But not at 4 or above 43	Yoon *et al*., 2004a

Table 2. Continued.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Dead Sea water, Jordan	*Bacillus marismortui* sp. nov. Reclassified as *Salibacillus marismortui* comb. nov.	5-25% NaCl Optimal growth at 10% NaCl	15-50 (Optimal growth at 37)	Arahal *et al*., 1999 Arahal *et al*., 2000
-Great Salt Lake, Utah, USA	*Gracilibacillus halotolerans* gen. nov., sp. nov.	0-20 % NaCl Optimal growth at 0 % NaCl	6-50 (Optimal growth at 47)	Wainø *et al*., 1999
-Salt lake near Hwajinpo beach, East Sea, Korea	*Halobacillus salinus* sp. nov.	Optimal growth at 2-10 % NaCl	10-45 (Optimal growth at 30-37)	Yoon *et al*., 2003a
-Water of a shallow sea hot spring at Vulcano Island, Italy, a depth of 15 m	*Bacillus aeolius* sp. nov.	0.5-5 % NaCl Optimal growth at 2 % NaCl	37-65 (Optimal growth at 55)	Gugliandolo *et al*., 2003

Table 2. Continued.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Deep sea Sediment tropical Atlantic, Artic Ocean, and Antarctic Ocean Depth in range of 403-4802 m	*Bacillus marinus*	Optimal growth at 200-400 mM, i. e. 1.2-2.4 % NaCl	Psychrophilic or psychrotolerant, the highest maximum growth temperature found at 30	Rüger *et al*., 2000
- Sediment, a depth of 3 m at hydrothermal vent at Vulcano Island, Italy	*Bacillus vulcani* sp. nov.	0-3% NaCl Optimal growth at 2% NaCl	37-72 (Optimal growth at 60)	Caccamo *et al*., 2000
- Mud sample, Mariana Trench, a depth of 11000 m	*Thermaerobacter marianensis* gen. nov., sp. nov. (G+ in the exponential phase and G- in stationary phase)	0.5-5.0% sea salts optimal growth at 2% sea salts	50-80 (Optimal growth at 74-76)	Takai *et al*., 1999

Table 2. Continued.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Deep sub-seafloor sediment, Nankai Trough, Japan	*Marinilactibacillus piezotolerans* sp. nov.	0-120 g/l NaCl Optimal growth at 10-20 g/l NaCl	4-50 (Optimal growth at 37-40)	Toffin *et al*., 2005
-Sponges, Tuticorin, South East coast of India	*Bacillus* sp.	No growth at 0 % NaCl At 8 % NaCl , growth can occur	No report	Anand *et al*., 2006
-Living sponge, raw Japanese ivory shell, and decomposing alga	*Marinilactibacillus psychrotolerans* gen. nov., sp. nov.	0-17 -20.5%NaCl Optimal growth at 2-3.75 % NaCl	Between -1.8 and 40-45 (Optimal growth at 37-40)	Ishikawa *et al*., 2003
Saltern		0.5-15.0 % NaCl	4-39	Yoon *et al*., 2004b
-Marine solar saltern Korea	*Marinibacillus campisalis* sp. nov.	Optimal growth at 2-3% NaCl	(Optimal growth at 30)	
- Solar saltern Korea	*Pontibacillus chungwhensis* gen. nov., sp. nov.	1-15 % NaCl	15-45	Lim *et al*., 2005

The distribution of actinomycetes in near-shore sediments collected from locations throughout the Bahamas, USA, which was demonstrated by Jensen *et al* in 1991, showed that the low numbers of streptomycetes were found with increasing water depth, with approximately 86% of the streptomycetes from 0 to 1 m and 12% from 1 to 3 m. In contrast with steptomycetes, the high percentage of actinoplanetes was found from samples with increasing water depth, with 41% of the 192 observed colonies found from 15 to 33 m. Moreover, actinoplanetes had a near obligate seawater requirement for growth suggesting the physiological active of actinomycetes in marine environment. A diversity of actinomycetes isolated from sediments from Chesapeake Bay, USA, showed that actinomycete counts were reported from a low of 1.8×10^2 to a high of 1.4×10^5 CFU/ml of sediment and actinoplanetes were the majority of isolates (Takizawa *et al*., 1993). The investigation of actinomycetes, isolated from coastal sediments at 5 to 20 cm under surface at North Beach, Aberystwyth, Wales, United Kingdom, revealed that most isolates belong to the genera *Streptomyces* and *Micromonospora* (Zhao *et al*., 2004). Furthermore, this study also showed that there were no differences between the isolation media with and without seawater. This result suggested that the isolated actinomtcetes were either unobligated halophiles or of terrestrial origin.

Marine sediment was also shown to be a prolific source of unique actinomycetes. Interesting study on the marine-derived actinomycetes came from a report of Mincer *et al*. (2002) which numerous actinomycetes strains were isolated from marine sediments collected from several locations i.e. Bahamus, USA, Red Sea, Egypt and Sea of Cortez, Mexico at a depth of 1-30 m. This study showed that the family *Micromonosporaceae* were the major group in marine environment. Based on the results, MAR 1, an obligated marine actinomycetes, was proposed in a new genus *Salinospora*. Furthermore, approximately 90% of actinomycetes isolated from marine sediments, represented the two new groups within the family *Micromonosporaceae* (Magarvey *et al*., 2004), which formed glistening colonies that were purple or pale-to-bright orange on the isolation medium with species specificity.

Recent study by Jensen *et al*. (2005) from Scripps Institution of Oceanography, USA, showed that 58 % actinomycetes isolated from marine samples collected around the island of Guam, required seawater for growth. This indicated that the majority of actinomycetes had adapted to grow in the presence of seawater (Jensen *et al*., 2005). In addition the dominant

actinomycetes were affiliated to the new genus *Silinospora*. The two new species belonging to this genus, for which the names *Salinispora arenicola* and *Salinispora tropica* were described (Maldonado *et al*., 2005), and the third *Salinispora pacifica* was proposed, based on < 60% interspecies DNA- DNA hybridization (Jensen and Mafnas, 2006). The genus *Salinispora* was described as the first actinomycetes genus required seawater for growth (Jensen and Mafnas, 2006). Recently, *Salinispora* strains were found from the sponge *Pseudoceratina clavata* collected from the Great Barrier Reef (Kim *et al*., 2005). Importantly, members of this group produced the metabolites with biological activities against tumors cell lines and some bacteria (Mincer *et al*., 2002; Kim *et al*., 2005), and also demonstrated as a potential new source of rifamycin antibiotics (Kim *et al*., 2006). The distribution study of *Salinispora* including *S. arenicola*, *S. tropica*, and *S. pacifica* showed that *S. arenicola* represented the large numbers of strains which were cultured from all locations. This study also observed that *Salinispora* strains were able to grow at 10°C but not at 4°C (Jensen and Mafnas, 2006).

Recently, several other novel species of actinomycetes obtained from sediments were reported i. e. *Brevibacterium samyangense*, an opaque, circular, yellow colony from sand sediment, collected from Samyang Beach in Jeju, Korea (Lee, 2006), *Nocardia harenae*, isolated from beach sand on the coast of Jeju Island, Korea (Seo and Lee, 2006), *Williamsia marianensis* sp. nov. (Pathom-aree *et al*., 2006a) and *Dermacoccus abyssi* sp. nov. from Mariana Trench (Pathom-aree *et al*., 2006b).

Beside marine sediment, members of actinomycetes were also isolated from other specimens such as seawater and marine organisms. *Aeromicrobium marinum* sp. nov., member of the family *Nocardioidaceae*, was reported as an obligately salt-requiring bacterium and abundant indigenous bacterium found in the German Wadden Sea (Bruns *et al*., 2003). In addition, the study on bacteria inhabiting the sea surface microlayer from of coastal stations (polluted and oligotrophic) showed that Gram-positive bacteria, *Actinobacteria* and *Firmicutes*, were more abundant in the polluted site (Agogué *et al*., 2005). In marine sponge *Rhopaloeides odorabile,* it was shown that 30 % of the clones clustered within the *Actinobacteria* (Webster *et al*., 2001). This study also indicated that the culture media with addition of sponge extract resulted in an increase in the number of novel cultivated morphotypes. Moreover, a study by

Hentschel *et al*. (2002) showed that 12% of all sponge-derived sequences were related to *Actinobacteria.*

Lampert *et al*. (2006) reported the culturable bacterial community within the mucus of the Red Sea coral *Fungia scutaria*, collected from the coral reef of the Gulf of Eilat, northern Red Sea. *Actinobacteria* and Gram-positive bacteria of BC (*Firmicutes* including *Bacillus-Clostridium*) were found in the mucus coral accounting for 23% and 5%, respectively, of bacterial communities.

Examples of selected actinomycete isolated form marine environments are shown in Table 3.

1.3 Gram-negative bacteria

The Gram-negative bacteria are the largest and most diverse group of marine chemoheterotroph which can be easily isolated and cultivated. Many of them were classified as novel bacteria. For example, a novel specie of marine bacterium, *Psychrobacter nivimaris* could grow in a range of 0-13% NaCl, with optimum at 0-9% NaCl (Heuchert *et al*., 2004) whereas a novel species of *Pseudoalteromonas translucida* isolated from seawater, Japan, grew in the presence of NaCl at 1-8% (Ivanova *et al*., 2002a). *Pseudoalteromonas* are among the good producers of bioactive secondary metabolites (Holmström and Kjelleberge, 1999). The abundance of *Pseudoalteromonas* species in marine samples was demonstrated using a real time quantitative PCR (RTQ-PCR) method (Skovhus *et al*., 2004). It was found that the rDNA fractions of *Pseudoalteromonas* were about 1.55% on the green alga *Ulva lactuca*, 0.10% on the tunicate *Ciona intestinalis*, and 0.06% on the green alga *Ulvaria fusca*.

In marine organisms i.e. sponges, it has been shown that the sponge *Aplysina aerophoba* harbored large amounts of bacteria which contributed to 40% of the sponge biomass (Ahn *et al*., 2003). In some sponges, bacteria can constitute up to 60% of biomass and were abundant in the mesophyl tissue (Friedrich *et al*., 1999; Imhoff and Stöhr, 2003). Most abundant bacterial morphotypes in mesophyl sponge had slime layers, additional sheaths and enlarged periplasms (Friedrich *et al*., 1999). These additional features serve as self-protection within the mesophyl of the sponge. Investigation of culturable epibiotic bacteria from the sponges *Petrosia ficiformis* showed that 58% of 57 aerobic heterotrophic bacterial strains, counted on Marine agar and TCBS agar, were Gram-negative bacteria. In addition, *Pseudomonas* and *Vibrio* were found

Table 3. Examples of selected actinomycetes isolated from different marine environments.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
Seawater -Amursky Bay of the Gulf of Peter the Great, East Sea, 5 m in depth	*Salinibacterium amurskyense* gen. nov., sp. nov.	0-10% NaCl Optimal growth at 1-3% NaCl	4-37 (Optimal growth at 25-28)	Han *et al*., 2003
-Surface water German Wadden Sea	*Aeromicrobium marinum* sp. nov.	Between 0.8-107 ‰ Optimal growth at 53.3‰	Between 4 and 35 (Optimal growth at 25)	Bruns *et al*., 2003
Sediment -Island of Kauai, Hawaii	*Nocardiopsis dassonvillei*	No data available	No data available	Schumacher *et al*., 2001
- A depth of 289 m, Sea of Japan	*Williamsia maris* sp. nov.	No report	10-37 (Optimal growth at 28)	Stach *et al*., 2004
- Troitsa Bay of the Gulf of Peter the great, East Siberian Sea	*Kocuria marina* sp. nov.	No requirement but able to grow of up to 15% NaCl	Growth at 4-43	Kim *et al.,* 2004

Table 3. Continued.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Coastal sediment, 5-20 cm. under surface, North Beach, Aberystwyth, Wales, UK	*Micromonospora* species *Streptomyces* species	No data available	No data available	Zhao *et al*., 2004
-Marine sediments, Bahamas, Red Sea (Egypt) and Sea of Cortez (Mexico)	*Salinospora* species Revised to *Salinispora* gen. nov.	Obligate marine actinomycetes	No data available	Mincer *et al*., 2002 Maldonado *et al*., 2005
-Sediment, a depth of 20 cm. on the seashore of Abu Qir Bay, West of Alexandria, Egypt	*Nocardiopsis aegyptia* sp. nov.	5 % NaCl but was not able to grow in the presence of 10 % NaCl	Grow at 10 and 40 (Optimal growth at 25-28)	Sabry *et al*., 2004
-Sediments, Bay of Bengal Ocean, India	*Streptomyces psammoticus*	NaCl tolerance < 7 %	25-42 (Optimal growth at 30)	Sujatha *et al*., 2005

Table 3. Continued.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Sediment Jiaozhou Bay, China	*Streptomyces* sp.	No data available	Optimal growth ~ 28 Not grow at 45 or at 10	Li *et al*., 2005
-Off-shore sediment, at depth of 56 cm. La Jolla, CA.	*Marinispora* sp.	No data available	No data available	Kwon *et al*., 2006
Marine organisms - Sponge *Pseudoceratina clavata*, Great Barrier Reef	*Salinispora* species	No growth at 0 % Sea salts Grow well in 100 % Sea salts Some can grow well in 150 % sea salts	No data available	Kim *et al*., 2005 Kim *et al*., 2006
-Sponge *Dendrilla nigra*	*Streptomyces* sp.	0-8% NaCl No growth at 10 % NaCl	No growth at 4 Can grow at 40	Selvin *et al*., 2004

from all specimens (Chelossi *et al*., 2004). Recently, the molecular method with specific PCR primers led to discovery of a novel candidate phylum of *Poribacteria* in marine sponges (Fieseler *et al*., 2004). The 16S rRNA gene sequences analysis, using universal bacterial PCR primers, showed that the microbial diversity in marine sponges included *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *Proteobacteria* (Alpha-, Gamma-, Delta-), *Nitrospira*, *Cyanobacteria*, and *Bacteriodetes* as well as the *Gemmatimonadetes* (Hentschel *et al*., 2006). Another study of marine bacteria isolated from marine organisms showed that 82.76% of bacterial isolates from arm homogenates, excised wound tissue, or from swabs of arm stumps of the brittlestar were Gram-negative bacteria (Strahl *et al*., 2002).

Lampert *et al*. (2006) described culturable bacterial community within the mucus of the Red Sea coral *Fungia scutaria* collected from the coral reef of the Gulf of Eilat, northern Red Sea using both culture-based methods and molecular techniques. The results revealed that more than 30% of the isolated bacteria were novel species and a new genus, related to Gamma-*Proteobacteria*, Alpha-*Proteobacteria*, *Actinobacteria*, CFB (*Cytophaga-Flavobacter /Flexibacter-Bacteroides*) and BC (*Firmicutes* including *Bacillus-Clostridium* strains) with a percentage of 35%, 32%, 23%, 5% and 5%, respectively.

Examples of selected Gram-negative bacteria isolated from marine habitats are given in Table 4.

Table 4. Examples of selected Gram-negative bacteria isolated from marine environments.

Habitat/Location	Bacterial strain	Growth condition		Reference
		NaCl/Seawater requirement	Temperature (°C)	
Seawater				
- Shallow coastal seawater, Keelung, Taiwan	*Vibrio ruber* sp. nov.	1-10% NaCl (Optimal growth at 2%NaCl)	20-40 (Optimal growth at 25-30)	Shieh *et al*., 2003
-Coastal seawater, Georgia	*Silicibacter pomeroyi* sp. nov.	Optimal at 100-400 mM at 30°C	10-40	González *et al*., 2003
-Shallow coastal seawater, An-Ping Harbour, Tainan, Taiwan	*Pseudidiomarina taiwanensis* gen. nov., sp. nov.	Optimal growth at1-4 % NaCl	Optimal growth at 30-35	Jean *et al*., 2006
-Surface water, Central Baltic Sea	*Belliella baltica* gen. nov., sp. nov.	0-6 % NaCl (Optimal growth at 0 - 3 % NaCl)	4-37 (Optimal around 25)	Brettar *et al*., 2004
-Seawater associate with red tide, a depth of 1 m, Masan Bay, Korea,	*Kordia algicida* gen. nov., sp. nov.	Optimal growth at 3 % NaCl No grow at 0 and 6 % NaCl	Optimal growth at 30	Sohn *et al*., 2004

Table 4. Continued.

Habitat/Location	Bacterial strain	Growth condition		Reference
		NaCl/Seawater requirement	Temperature (°C)	
- Seawater, Japan At depth of 5-8 m	*Pseudoalteromonas translucida* sp. nov.	1-8% NaCl (Optimal growth at 1-3% NaCl)	4-30 (Optimal growth at 25)	Ivanova *et al*., 2002a
- Deep seawater Hachijo Island, Japan At depth of 6000 m	*Psychrobacter pacificensis* sp. nov.	Optimal growth at 3%NaCl or seawater No growth at 0 or 8% NaCl	Optimal growth at 25 Maximal growth being 38	Maruyama *et al*., 2000
- Hydrothermal vent seawater, Tachibana Bay, Nagasaki Prefecture, Japan Sediment	*Rhodothermus obamensis* sp. nov.	1-5 % NaCl (Optimal growth at 3% NaCl)	Between 50 and 85 (Optimal growth at 80)	Sako *et al*., 1996
- Coastal sediment Reineke Island, Peter the Great Bay, Sea of Japan, Russia	*Reinekea marinisedimentorum* gen. nov., sp. nov.	0.5-5 % NaCl	4-37 not at 40	Romanenko *et al*., 2004

Table 4. Continued.

Habitat/Location	Bacterial strain	Growth condition		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Tidal flat sediment, Ganghwa Island, Korea	*Zooshikella ganghwensis* gen. nov., sp. nov.	1-7 % NaCl (Optimal at 3-4 % NaCl)	Optimal growth at 35	Yi *et al*., 2003
-Sediment , Troitsa Bay, Sea of Japan, a depth of 3 m	*Arenibacter troitsensis* sp. nov.	1-6% NaCl	10-42	Nedashkovskaya *et al*., 2003b
-Deep Sediment, the West African Coast, a depth of 2770 m	*Psychromonas profunda* sp. nov.	No growth in absence of NaCl Growth with half strength seawater	Maximal growth at 3-4	Xu *et al*., 2003
Marine organisms - Gorgonian *Paragorgia arborea*, Pacific Ocean	*Pseudoalteromonas paragorgicola* sp. nov.	1-6 % NaCl	4-30 (Optimal growth at 25)	Ivanova *et al*., 2002a
- Australian sponge *Fascaplysinopsis reticulata*	*Pseudoalteromonas maricaloris* sp. nov.	0.5-10% NaCl (Optimal growth at 1-3% NaCl)	10-37 (Optimal growth at 25-30)	Ivanova *et al*., 2002b

Table 4. Continued.

Habitat/Location	Bacterial strain	Growth conditions		Reference
		NaCl/Seawater requirement	Temperature (°C)	
-Benthic marine worms *Phascolosoma japonicum* Troitsa Bay, Sea of Japan	*Shewanella affinis* sp. nov.	0.5-6.0 % NaCl, some grow at 8% NaCl	10-34 (Optimal growth at 20-25)	Ivanova *et al*., 2004a
- Green alga *Acrosiphonia sonderi* (Kütz) Kornm, Troitsa Bay, Gulf of Peter the Great, Sea of Japan	*Mesonia algae* gen. nov., sp. nov.	1-15 % NaCl	4-34 (Optimal growth at 21-23)	Nedashkovskaya *et al*., 2003a
-Eggs of the sea hare *Aplysia kurodai*, South Sea of Korea	*Photobacterium aplysiae* sp. nov.	Optimal growth at 3 % NaCl	Optimal growth at 25	Seo *et al*., 2005

1.4 Gliding bacteria

Gliding bacteria are a taxonomically heterogeneous group of Gram-negative bacteria with various shapes i. e. rod , spiral and filament that are only motile when adherent to different kinds of solid or a semi-solid surface by a process called gliding motility (Reichenbach, 1992; Siddiqui *et al*., 2001; Mcbride, 2001). The definition of bacterial gliding motility is defined as "smooth translocation of cells over the surfaces by an active process that does not involve flagella (Mcbride, 2004). With gliding motility, the cells move in the direction of their long axes resulting colonies that have thin spreading edges. Nearly all of gliding bacteria produce extracellular polymer and leave a trail of slime or mucilage. Gliding motility is depended on several growth factors such as temperature and nutrient concentrations (Reichenbach, 1992). To observe the gliding motility, agar media with a low peptone contents (0.1% or less) were used. Bacteria with gliding motility are highly diverse group as shown in Table 5. They can be divided into three major groups i. e. the myxobacteria, the cyanobacteria and the *Cytophaga- Flavobacterium* (Mcbride, 2001).

1.4.1 Myxobacteria

Myxobacteria are gliding bacteria with relatively large (0.6-1.2 by 3-15 μm) rod-shaped cells (Reichenbach, 1999). The generation time for growth is between 4 h and 20 h dependening on the genus (Gerth and Müller, 2005). Vegetative cells occur in two different types (Dawid, 2000):

Cell type I: slender, usually tipped flexible rods up to 1 μm in diameter and up to 20 μm in length.

Cell type II : cylindrical rigid rods with rounded ends up to 1μm wide and up to 10 μm long.

Myxobacteria are very common in soils with neutral or slightly alkaline pH (Reichenbach and Dworkin, 1992). They can be isolated from soil, dung of various animals, decaying plant materials and on the bark of living, dead trees as well as fresh water (Reichenbach, 2001). Recently, many were also isolated from marine samples such as seawater, mud, sand and algae (Iizuka *et al*., 1998; Li *et al*., 2002; Iizuka *et al*., 2003a; Iizuka *et al*., 2003b; Zhang *et al*., 2005). It is interesting to note that myxobacteria have the largest genomes of all bacteria (9500-10000 kbp) and a DNA with a G+C content of 66-72 mol% (Reichenbach,1999).

Table 5. Occurrence of gliding bacteria among the eubacteria.

Bacterial group	Representative genera of gliding bacteria
Cyanobateria	*Oscillatoria, Spirulina, Anabaena, Phormidium,* many others
Cytophaga-Flavobacterium group	*Flavobacterium*[a], *Cytophaga*, *Saprospira*, *Flexibacter*, many others
δ Proteobacteria	*Myxococcus*, *Stigmatella*, *Chondromyces*, *Desulfonema*, many others
β Proteobacteria	*Vitreoscilla*, *Simonsiella*
γ Proteobacteria	*Lysobacter*, *Beggiatoa*, *Leucothrix*, *Thiothrix*
Green nonsulfur bacteria	*Chloroflexus*, *Herpetosiphon*, *Heliothrix*
Green sulfur bacteria	*Chloroherpeton*
Planctomyces	*Isophaera*
Gram –positive bacteria [b]	*Heliobacterium*, *Filibacter*, *Mycoplasma* [c]

[a] Many bacteria that were previously classified in the genus *Flavobacterium* do not display gliding motility and have been reclassified into the genus *Chryseobacterium.*

[b] All of the gliding bacteria that have been identified within the "Gram-positive" group lack typical Gram-positive cell structure. Mycoplasma lack a cell wall, *Heliobacterium chlorum* has an outer membrane, an *Filibacter limicola* exhibits Gram-negative staining behavior and has an outer surface layer.

[c] Some, but not all mycoplasma display gliding motility.

Source: Modified from McBride (2001).

Myxobacteria were also believed to be obligated aerobic and mesophilic bacteria with a temperature optimum of 30°C (Dawid, 2000). However, the first taxon in myxobacteria, identified as *Anaeromyxobacter dehalogenans* gen. nov., sp. nov grew in anaerobic condition (Sanford *et al*., 2002). Additionally, pyschrophilic myxobacteria of the genera *Polyangium* and *Nannocystis*, isolated from antarctic soil samples could grow at the temperature in a range of 4-9 °C (Dawid, 2000) and novel moderately thermophilic myxobacteria, isolated from soil samples of semiarid and warm climates, grew very fast at temperature of 42-44 °C (Gerth and Müller, 2005).

Iizuka *et al*. (1998) reported that two marine myxobacteria, isolated from a wet sand sample and a dry Laminariales seaweed sample, were related to the genus *Nannocystis.* Moreover, the strains were also reported as specific marine bacteria because of their NaCl requirement for growth, with the optimum concentration between 2 and 3%. In contrast, the terrestrial strains could not observed growth at NaCl concentrations higher than 2.0% (w/v). In addition, both isolates required a higher concentration of magnesium compared to the terrestrial strains used as reference strains. Later, Iizuka *et al*. (2003a) reported a new genus and species of *Plesiocystis pacifica* from a semi-dried sample of seagrass (*Zostera* sp.) which required seawater for growth with an optimal concentration between 2 and 3%.

Fudou *et al*. (2002) reported the novel genus and species of marine myxobacteria *Haliangium ochraceum* gen. nov., sp. nov. and *Haliangium tepidum* sp. nov. The two strains grew in 0.5-6.0% NaCl with an optimum at 1-3% NaCl but showed difference in temperature range for growth. *Haliangium ochraceum* had optimum temperature at 30-34°C, while *Haliangium tepidum* had optimum temperature at 37-40°C.

Iizuka *et al*. (2003b) reported the isolation of six novel marine myxobacteria obtained from various coastal samples such as mud, sand and algae, collected around Japan. The six strains were proposed into a new taxon of marine myxobacteria with the name of *Enhygromyxa salina* gen. nov., sp. nov. These strains required NaCl for growth with optimum concentration of 1.0-2.0% (w/v). In addition, they also need Mg^{2+}, Ca^{2+} for growth.

Another unique characteristic of myxobacteria is fruiting bodies formation under starvation conditions (Reichenbach, 2001). The fruiting bodies were measured in a range of 20 and 1000 μm in size which can be seen by naked eye. Each fruiting body was formed by

hundreds of thousands of cells, with rod-shaped cells differentiated into myxospores (Karamanos *et al*., 1999). They usually appear in bright yellow, orange, red, brown, or black (Reichenbach, 2001). It was observed that the fruiting body structure of salt-tolerant myxobacteria from marine samples were formed in conditions with lower seawater content i. e. lower than 60% seawater or distilled water supplemented with $MgCl_2$ (Li *et al*., 2002). It was suggested that the myxobacteria do not form fruiting body in natural marine environments (Li *et al*., 2002). Similar results were observed from a recent report by Zhang *et al.* (2005). The halotolerant *Myxococcus fulvus* strain HW-1 changed in morphology in response to seawater concentration. The cells were shorter when increasing the seawater concentration. And the fruiting body structure was formed completely in conditions with low concentrations of seawater or salts i.e. less than 60% seawater; salinity, 2.1%. In contrast, the halophilic *Haliangium ochraceum* strain SMP-2 did not change in cell size and morphology of the fruiting body-like structure in response to different salt concentrations.

1.4.2 Cytophaga-Flavobacterium-Bacteroides (CFB)

This group comprised of diverse bacterial division. Several genera are anaerobic, especially the family *Bacteroidaceae* The family *Flavobacteriaceae* is heterotrophic, motile by gliding or non-motile microorganisms (McCammon and Bowman, 2000; Barbeyron *et al*., 2001; Nedashkovskaya *et al*., 2003a; Yi and Chun, 2004). The order *Cytophagales*, is restricted to gliding bacteria with rod-shaped cells (Reichenbach, 1992). The gliding motility of members of the *Cytophaga-Flavobacterium* group appear to glide by different mechanisms (McBride, 2001). Genetic analyses have provided a number of *gld* genes and proteins required for gliding (McBride, 2004).

Distribution of the *Cytophaga-Flavobacterium* group in marine environment by fluorescence in situ hybridization (FISH) technique demonstrated the abundance of this group in marine water with a median of 18%. Whereas the distribution in the Baltic Sea (depth, 78 m) and the Antarctic Ocean (depth, 0 m) were 2 % and 72 %, respectively (Glöckner *et al*., 1999). Moreover, members of the family *Flavobacteriaceae* were found from many areas i.e. sea ice, sediments, marine algae, sponges, saline lakes (Barbeyron *et al*., 2001; Suzuki *et al*., 2001; Bowman *et al*., 2003; Imhoff and Stöhr, 2003). In marine environments, the *Cytophagales* were obtained from living and dead seaweeds, sediments and on decaying sea animals (Reichenbach,

1992). The *Cytophaga-Flavobacterium-Bacteroides* group is known as decomposers of biomolecules such as cellulose, agar, and chitin indicating that the *Cytophaga-Flavobacter* group plays an important role of carbon cycling in ocean. A gliding bacterium *Cytophaga* sp., isolated from Harima-Nada, eastern Seto Inland Sea, Japan in 1990, was reported to kill marine phytoplankton (Imai *et al*., 1993). Moreover, algicidal activity was also found in a gliding bacterium *Saprospira* sp. SS98-5 (Furusawa *et al*., 2003). This strain, isolated from Kagoshima Bay, Japan, exhibited algicidal activity against the diatom *Chaetoceros ceratosporum*. Beside algicidal activity, some of bacteria in this group have been also reported as fish pathogens. Since the family *Flavobacteriaceae* contains complex group of bacteria, several strains of this group have been reclassified and proposed to new genera (McCammon and Bowman, 2000).

Examples of selected marine gliding bacteria including some characteristics are shown in Table 6.

Table 6. Examples of selected gliding bacteria derived from marine environments.

Habitat/ Location	Bacterial strain	Some characteristics			Reference
		NaCl/ Seawater requirement	Temperature (°C)	Flexirubin pigment	
Seawater -Mediterranean coast of Valencia (Spain)	*Gelidibacter mesophilus* sp. nov. (glide on BM agar added with some carbohydrates)	No growth without NaCl or at 8% NaCl or more	4-37	-	Macián *et al*., 2002
- Gulf of Peter the Great, Sea of Japan, Pacific Ocean	*Reichenbachia agariperforans* gen. nov., sp. nov.	1-6% NaCl	4-35 (Optimum at 25-28)	+	Nedashkovskaya *et al*., 2003c
-Gulf of Peter the Great, Sea of Japan	*Maribacter orientalis* sp. nov.	1-5% NaCl	4-32 (Optimum at 21-23)	-	Nedashkovskaya *et al*., 2004a
-Gulf of Peter the Great, Sea of Japan	*Maribacter aquivivus* sp. nov.	1-7 % NaCl	4-30 (Optimum at 21-23)	-	Nedashkovskaya *et al*., 2004a
- Amursky Bay, Pacific Ocean	*Zobellia amurskyensis* sp. nov.	1-6 % NaCl (Optimum at 2% NaCl)	4-32 (Optimum at 23-25)	+	Nedashkovskaya *et al*., 2004b
Sediment & Sand -Sediment, Auke Bay, USA	*Crocinitomix catalasitica* gen. nov., sp. nov.	0.1-1.0 M NaCl	0-30 (Optimum at 25)	No report	Bowman *et al*., 2003

Table 6. Continued.

Habitat/ Location	Bacterial strain	Some characteristics			Reference
		NaCl/ Seawater requirement	Temperature (°C)	Flexirubin pigment	
- Sediment, Gulf of Peter the Great, Sea of Japan, Pacific Ocean	*Maribacter sedimenticola* sp. nov.	1-6% NaCl	4-33 (Optimum at 22-24)	-	Nedashkovskaya *et al*., 2004a
-Tidal flat sediment, Yellow Sea, Korea	*Gaetbulimicrobium brevivitae* gen. nov., sp. nov.	Optimum at 2-3% NaCl	10-41 (Optimum at 37)	-	Yoon *et al*., 2006
-Particulate material, The Southern Ocean	*Olleya marilimosa* gen. nov., sp. nov.	0.2-0.9 M NaCl Optimum at 0.2-0.5 M NaCl	4-30	-	Nichols *et al*., 2005
Marine eukaryote					
-Brown alga *Fucus serratus* L., Bornholm, at the entrance to Baltic Sea	*Cellulophaga baltica* gen. nov., sp. nov.	10-50 ‰ sea salts optimum at 20 ‰	2-30 (Optimum at 26-30)	-	Johansen *et al*., 1999
- Brown alga *Fucus evanescens*	*Formosa algae* gen. nov., sp. nov.	0-6 % NaCl	(Optimum at 23)	-	Ivanova *et al*., 2004b

Table 6. Continued.

Habitat/ Location	Bacterial strain	Some characteristics			Reference
		NaCl/ Seawater requirement	Temperature (°C)	Flexirubin pigment	
-Brown alga *Laminaria japonica* Troitsa Bay, Sea of Japan	*Zobellia laminariae* sp. nov.	1.5-6 % NaCl Optimum at 2 % NaCl	4-30 (Optimum at 21-23)	+	Nedashkovskaya *et al*., 2004b
-Brown alga *Chorda filum* Troitsa Bay, Sea of Japan	*Winogradskyella thalassocola* gen. nov., sp. nov.	1-8% NaCl	4-33 (Optimum at 21-23)	-	Nedashkovskaya *et al*., 2005
-Brown alga *Laminaria japonica* Troitsa Bay, Sea of Japan	*Winogradskyella eximia* sp. nov.	1-5% NaCl	4-33 (Optimum at 21-23)	-	Nedashkovskaya *et al*., 2005
-Green alga *Avrainvillea riukiuensisi,* Palau	*Tenacibaculum amylolyticum* gen. nov., sp. nov.	Requires at least ½ strength sea water	19-35 (Optimum at 30)	-	Suzuki *et al*., 2001
-Green alga *Acrosiphonia sonderi*, Troitsa Bay, Sea of Japan	*Zobellia russellii* sp. nov.	Between 1 and 10 % NaCl Optimum at 2-3 %NaCl	4-38 (Optimum at 25-28)	+	Nedashkovskaya *et al*., 2004b

Table 6. Continued.

Habitat/ Location	Bacterial strain	Some characteristics			Reference
		NaCl/ Seawater requirement	Temperature (°C)	Flexirubin pigment	
-Green alga *Ulva fenestrata,* Gulf of Peter the Great, Sea of Japan	*Maribacter ulvicola* sp. nov.	1-4 % NaCl	4-32 (Optimum at 21-23)	-	Nedashkovskaya *et al*., 2004a
-Green alga *Acrosiphonia sonderi* Troitsa Bay, Sea of Japan	*Winogradskyella epiphytica* sp. nov.	1-8 % NaCl	4-37 (Optimum at 23-25)	-	Nedashkovskaya *et al*., 2005
-Green alga *Acrosiphonia sonderi* Troitsa Bay, Sea of Japan	*Formosa agariphila* sp. nov.	1-8 % NaCl	4-33 (Optimum at 21-23)	No report	Nedashkovskaya *et al*., 2006a
Green alga *Ulva fenestrata* Pallada Bay, Sea of Japan	*Arenibacter palladensis* sp. nov.	0-10%NaCl	4-38 (Optimum at 23-25)	No report	Nedashkovskaya *et al*., 2006b
-Red alga *Delesseria sanguinea*, Brittany,France	*Zobellia galactanovoran*s gen. nov., sp. nov.	0.5-6 % NaCl Optimum at 2.5% NaCl	13-45 (Optimum at 35)	+	Barbeyron *et al*., 2001

Table 6. Continued.

Habitat/ Location	Bacterial strain	Some characteristics			Reference
		NaCl/ Seawater requirement	Temperature (°C)	Flexirubin pigment	
-Sponge *Halichondria okadai*, Numaza, Japan	*Tenacibaculum mesophilum* gen. nov., sp. nov.	Requires 1/10 strength of sea water or 1 % NaCl	15-40 (Optimum at 28-35)	-	Suzuki *et al*., 2001
-Spong *Lissodendoryx isodictyalis* Bahamas	*Stenothermobacter spongiae* gen. nov., sp. nov.	2-6%NaCl	20.0-36.0 (Optimum at 28-30)	-	Lau *et al*., 2006
-Sea ice algal assemblage, and continental shelf sediment , Antarctica	*Brumimicrobium glaciale* gen. nov., sp. nov.	0.1-1.0 M NaCl	Grow at -2 to 25 (Optimum at 16-19)	No report	Bowman *et al*., 2003

2. Microbial secondary metabolites

2.1 Production of secondary metabolites by microorganisms

Secondary metabolites are low-molecular-weight compounds, which are biosynthesized from primary metabolites: amino acid, acetyl-coenzyme A, mevalonic acid, and intermediates of the shikimic acid pathway (Herbert, 1989). Many of secondary metabolites from microorganisms are useful in medicine. They act as antibiotic agents, antitumor agents, immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, and antiparasitic agents (Demain, 1999). It is revealed that 55% of antibiotics known in 1995 were produced by actinomycetes of the genus *Streptomyces*, 11% from other actinomycetes, 12% from nonfilamentous bacteria and 22% from filamentous fungi (Demain, 1999). The biosynthesis of antibiotics is regulated by many factors. The influences of the medium composition and the culture conditions on the biosynthesis of antibiotics were recognized. It has been shown that nutrient limitation could result in a slow growth rate and the expression of biosynthetic genes simultaneously (Lancini and Lorenzetti, 1993). The production of brominated cyclic depsipeptides by the marine bacterium *Pseudoalteromonas maricaloris* KMM 636 varied depending on the media composition. However, the bacterium produced dibrominated derivatives with slightly lower antimicrobial activities while growing on nutrient rich medium (Sobolevskaya *et al*., 2005). Moreover, it has been reported that the technique of competitive induction could increase antibiotic production by marine bacteria (Trischman *et al*., 2004).

2.2 Marine bacterial metabolites

Classes of microbial secondary metabolites or natural products have been described based on their biosynthetic origin such as polyketides, terpenes, and alkaloid. However, in this review, secondary metabolites from marine bacteria are reviewed based on biological activities.

2.2.1 Marine bacterial metabolites with antimicrobial activity

Studies of marine bacteria isolated from northern and southern parts of the Pacific Ocean, showed that 26% of 491 strains of marine bacteria produced antimicrobial compounds, which were active against fish pathogens such as *Aeromonas hydrophila* and *Vibrio anquillarum*. In addition, they were especially active against *Staphylococcus epidermidis*, *Proteus vulgaris*, *Enterococcus faecalis* and *Candida albicans* (Ivanova *et al*., 1998). Furthermore, a study by Burgess *et al*. (1999) showed that 35% of over 400 strains of marine epiphytic bacteria, isolated

from the surfaces of seaweed, starfish and nudibranchs produced antimicrobial compounds. These secondary metabolites showed antimicrobial activity against multidrug-resistant pathogens i.e. vancomycin-resistant enterococcus and methicillin-resistant *Staphylocoocus aureus*. Strahl *et al*. (2002) showed that the bacteria, isolated from arm homogenates, excised wound tissue, or from swabs of arm stumps of the brittlestar, *Amphipholis gracillima*, had broad- spectrum antibacterial activity against a number of common bacterial strains. These bacteria were known as subcuticular bacteria (SCB) described by Holland and Nealson in 1978 (Strahl *et al*., 2002). Study of SCB showed quick colonization during tissue regeneration such as damaged ophiuroid arm tips. Anand *et al*. (2006) reported that 21% of the bacterial strains associated with sponges, collected from the water off the coast of South East India, produced antimicrobial compounds and the strain *Bacillus* sp. was a potential producer. Numerous of marine bacterial metabolites have been characterized and many of them are new.

The first documented identification of marine bacterial metabolites was a highly brominated pyrrole antibiotic containing five bromine atoms per molecule **(1),** which was isolated from bacterium obtained from the surface of the caribbean seagrass *Thalassia* described by Burkholder and co-workers in 1966 (Fenical and Jensen, 1993). The metabolites, identified as [2,3,4-tribromo-5(1′hydroxyl, 2′,4′-dibromophenyl) pyrrole] was produced by the bacterium, which was first identified as *Pseudomonas bromoutilis* (Burkholder *et al*., 1966). Later, the strain was found to be related to the genus *Alteromonas* (Fenical and Jensen, 1993). The metabolites showed *in vitro* antimicrobial activity against Gram-positive bacteria, with the minimum inhibitory concentration (MICs) ranging from 0.0063 to 0.2 μg/ml. It was inactive against Gram-negative bacteria and in whole-animal assays (Fenical and Jensen, 1993). Later, the characterization of several antimicrobial brominated pyrroles, tetrabromopyrrole, hexabromo-2,2′-bipyrrole, and several simple phenolic compounds were isolated from the purple-pigmented *Alteromonas* sp., isolated from seawater sample collected from the North Pacific Ocean Gyre. Tetrabromopyrrole **(2)** showed moderate in vitro antimicrobial activity against *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* (Fenical and Jensen, 1993).

A compound MC21-A, identified as 3,3′,5,5′-tetrabromo-2,2′- biphenyldiol **(3)**, isolated from the new marine bacterium *Pseudoalteromonas phenolica* which was isolated from

seawater collected off Ogasawara Island, Tokyo, Japan was reported (Isnansetyo and Kamei, 2003a,b). The compound MC21-A displayed antimicrobial activity against several strains of methicillin-resistant *Staphylococcus aureus* with MIC of 1- 2 µg/ml. This compound was also highly active against *Enterococcus serolicida*, *Enterococcus faecium*, and *Enterococcus faecalis* but was less active against *Streptococcus* spp.

Jayatilake *et al*. (1996) reported the antimicrobial compounds obtained from cultivation of *Pseudomonas aeruginosa*, associated with the sponge *Isodictya setifera* Topsent. The bacterium produced new diketopiperazine (DKP) and two known phenazine alkaloids **(4)** and **(5).** Antibacterial activity of these compounds was demonstrated that diketopiperazine (DKP) were inactive as antibiotics or cytotoxins while two phenazines showed active against *Bacillus cereus* with MIC by disk assay < 0.5 µg/ml. Compound **4** showed more potent than compound **5.** Moreover, the compounds were less active against Gram-positive bacteria such as *M. luteus* and *S. aureus*

Another group of diketopiperazine identified as DD-diketopiperazines with strong antibacterial activity against *Vibrio anguillarum* was reported (Fdhila *et al*., 2003). These compounds included the DD-diketopiperazines cyclo (D)-Pro-(D)-Phe **(6)**, cyclo (D)-Pro-(D)-Leu **(7)**, cyclo (D)-Pro-(D)-Val **(8),** cyclo (D)-Pro-(D)-Ile **(9),** and cyclo *–trans*-4-OH- (D)-Pro (D)-Phe **(10)**. These compounds showed a very strong inhibitory activity against *Vibrio anguillarum* with MIC values in the range 0.03-0.07 µg/ml. The two producing marine bacteria were isolated from cultures of larvae of molluscs *Pecten maximus*.

Kawano *et al*. (1997) reported thiotropocin **(11)** from the culture broth of the marine bacterium *Caulobacter* sp. This bacterium was isolated from the cultivation broth of a microalgal, *Micromonas* sp. (*Prasiophyceae)* collected from seawater in Japan. The compound showed strong antimicrobial activity against *Staphylococcus aureus* and moderate activity against *Enterococcus seriolicida,* the pathogen of yellowtail and eels. Thiotropocin also inhibited the growth of the microalgae i. e. *Skeletonema costatum* and *Heterosigma akashiwo*, a red tide phytoplankton at a concentration of 1 µg/ml. Thiotropocin was previously reported from *Pseudomonas* sp. It showed strong *in vitro* antimicrobial activity under acidic conditions (Tsubotani *et al*., 1984). Later, Kanowa *et al*. (1998) reported that thiotropocin exhibited strong

inhibitory activity against proliferation of HUVEC, ECV304, HT1080, P388D1, and heat-induced hemolysis.

Gerard *et al*. (1997) reported the isolation of massetolides A-H, a novel cyclic depsipetides, and the known compound viscosin from cultures of two *Pseudomonas* sp. isolated from a marine alga and a marine tube worm, respectively. Massetolides A **(12)** and viscosin **(13)** exhibited *in vitro* antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare*.

Gerard *et al*. (1999) reported the compounds loloatins A-D **(14-17)**, a family of new cyclic decapeptide antibiotics. These compounds displayed *in vitro* antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and drug-resistant *Streptococcus pneumoniae.* Loloatins A-D were isolated from the culture medium of a tropical marine bacterium, possibly in the genus *Bacillus*, which was isolated from the Great Barrier Reef off Loloata Island, Papua New Guinea.

Jaruchoktaweechai *et al*. (2000) reported macrolactin metabolites from the marine bacterium *Bacillus* sp. ScO26, isolated from marine sediments (depth 15 m) around Sichang Island, Chonburi, Thailand. This bacterium produced the known compound macrolactin F **(18)** together with the new compounds, 7-*O*-succinyl macrolactin F **(19)** and 7-*O*-succinyl macrolactin A **(20).** The compounds **18-20** exhibited antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. The known macrolactin A-F were reported from the culture broth of an unidenfied Gram-positive deep-sea bacterium which required salt for growth (Fenical and Jensen, 1993). Macrolactin A was a major metabolite which exhibited modest antibacterial activity but was active against B16-F10 murine melanoma *in vitro* an IC_{50} of 3.5 µg/ml. Significantly, this compound inhibited several viruses, including herpes simplex (IC_{50} of 5.0 µg/ml) and human immunodeficiency virus, HIV (IC_{50} of 10.0 µg/ml).

Barsby *et al*. (2002) reported the novel antifungal polyketides basiliskamides A **(21)** and B **(22)**. These metabolites produced by *Bacillus laterosporus* obtained from tropical water off the coast of Papua New Guinea. Basiliskamides A and B exhibited potent *in vitro* activity against *Candida albicans* and *Aspergillus fumigatus*.

Oleinikova *et al*. (2003) described a methyl ester of bacillomycin D **(23).** This compound was isolated from culture of the marine bacterium *Bacillus subtilis* KMM 457 and

was presented as a new representative of iturin antibiotics. The compound inhibited growth of *Candida albicans* but was inactive against Gram-positive and Gram-negative bacteria.

Aassila *et al*. (2003) described the first report of the alkaloid harman **(24)** from a marine bacterium. The bacterium was identified as *Enterococcus faecium* associated with tunicate *Microcosmus australis.* Harman showed significant antibacterial activity against marine bacteria *Vibrio anguillarum, Vibrio alginolyticus, Vibrio harveyi,* and *Vibrio carchariae* with MIC of 3.12, 12.5, 25, and 25 µg/ml, respectively.

An active compound, norharman (a beta-carboline alkaloid) **(25)** was described (Zheng *et al*., 2005). The compound was produced by marine bacterium associated with the sponge *Hymeniacidon perleve*, collected in the intertidal zone on Nanji Island, China. The producing strain was identified as *Pseudoalteromonas piscicida*. Norharman showed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and the plant pathogen *Agrobacterium tumefaciens* with MIC values of 50, 50, and 100 µg/ml, respectively. This report was suggested as the first report of norharman isolated from a marine bacterium associated with the sponge.

Mitova *et al*. (2004) reported the isolation of novel peptides from *Ruegeria* strain of bacteria associated with the sponge *Suberites domuncula*. Compounds **26** and **27** were new peptides and showed moderate activity against *Bacillus subtilis* with MIC values of 25 and 50 µg/ml, respectively. The compounds were not active against *Escherichia coli* and *Saccharomyces cerevisiae*.

The chemical structures of selected marine metabolites with antimicrobial activity from common marine bacteria are given in Table 7. While the selected marine metabolites with antimicrobial activity isolated from marine actinomycetes are shown in Table 8.

In case of bacterial metabolites from gliding bacteria, most metabolites were reported from soil myxobacteria (Reichenbach, 2001; Gerth *et al*., 2003). Most of natural products were produced by strains of *Sorangium cellulosum*, *Myxococcus* and *Chondromyces* while, *Nannocystis* and *Corallococcus* were found to be poor producers (Gerth *et al*., 2003).

Summary of selected metabolites with antimicrobial activity from gliding bacteria are shown in Table 9

Table 7. Summary of selected metabolites with antimicrobial activity from general marine bacteria.

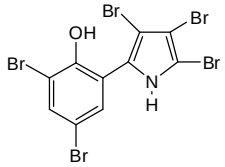
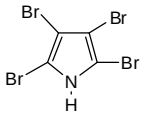
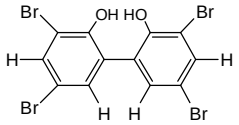
Metabolites/Structure	Producing strain	Source	Reference
[2,3,4-tribromo-5(1′hydroxyl, 2′,4′-dibromophenyl) pyrrole]  **(1)**	-First identified as *Pseudomonas bromoutilis* (Burkholder *et al*., 1966). Later, the strain was related to the genus *Alteromonas*	Seagrass *Thalassia*	Fenical and Jensen, 1993
Tetrabromopyrrole  **(2)**	*Alteromonas* sp.	Seawater	Fenical and Jensen, 1993
3,3′,5,5′-tetrabromo-2,2′- biphenyldiol  **(3)**	*Pseudoalteromonas phenolica*	Seawater	Isnansetyo and Kamei, 2003a

Table 7. Continued.

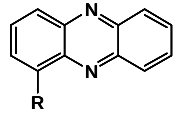
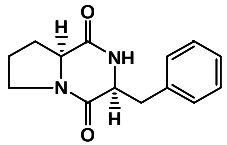
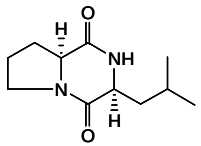
Metabolites/Structure	Producing strain	Source	Reference
Phenazine alkaloids  **(4)** R = $-CO_2H$ **(5)** R = $-CONH_2$	*Pseudomonas aeruginosa*	Sponge *Isodictya setifera* Topsent	Jayatilake *et al*., 1996
DD-diketopiperazines cyclo (D)-Pro-(D)-Phe  **(6)** Cyclo (D)-Pro-(D)-Leu  **(7)**	Unidentified strains	Larvae of mollusc *Pecten maximus*	Fdhila *et al*., 2003

Table 7. Continued.

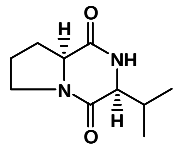
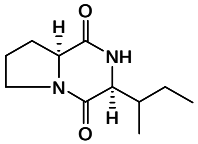
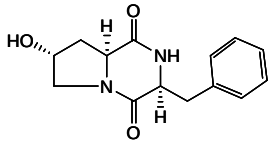
Metabolites/Structure	Producing strain	Source	Reference
Cyclo (D)-Pro-(D)-Val  **(8)**	Unidentified strains	Larvae of mollusc *Pecten maximus*	Fdhila *et al*., 2003
Cyclo (D)-Pro-(D)-Ile  **(9)**			
Cyclo *–trans*-4-OH- (D)-Pro (D)-Phe  **(10)**			

Table 7. Continued.

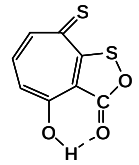
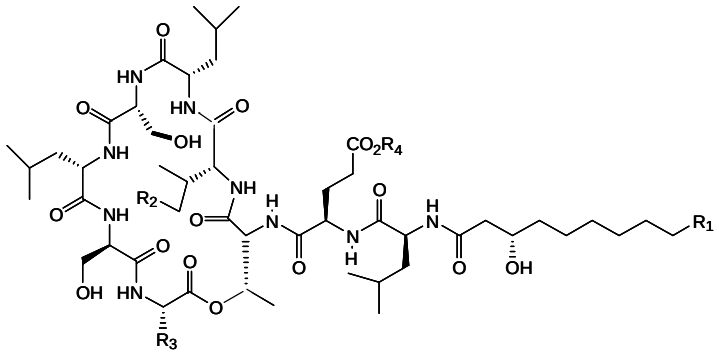
Metabolites/Structure	Producing strain	Source	Reference
Thiotropocin [a]  **(11)**	*Caulobacter* sp.	Microalgal *Micromonas* sp. (*Prasiophyceae)*	Kawano *et al*., 1997
Massetolide A **(12)** , viscosin **(13)**  **(12)** R_1= CH_3-, R_2 = CH_3, R_3 = $CH(CH_3)CH_2CH_3$, R_4 = H **(13)** R_1 = CH_3-, R_2 = H, R_3 = $CH(CH_3)CH_2CH_3$, R_4 = H	*Pseudomonas* sp.	Marine alga and marine tube worm	Gerard *et al*., 1997

Table 7. Continued.

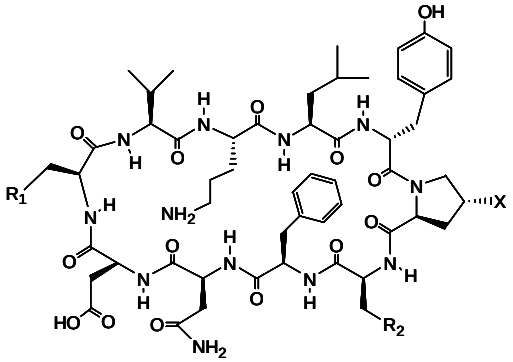
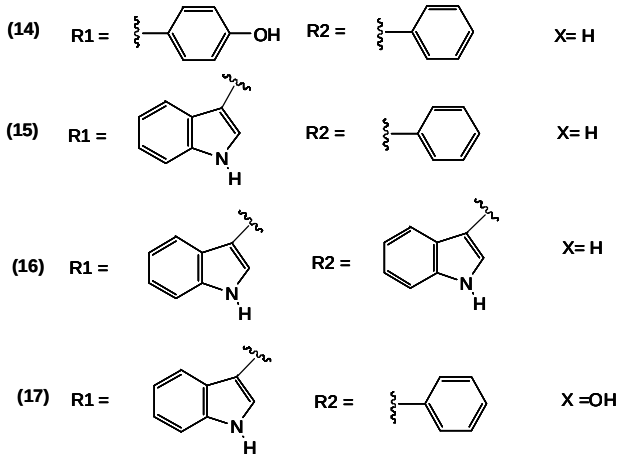
Metabolites/Structure	Producing strain	Source	Reference
Loloatin A**(14)**, B **(15)**, C **(16)** and D **(17)**  	Unidentified species (possibly in the genus *Bacillus*)	Great Barrier Reef off Loloata Island	Gerard *et al*., 1999

Table 7. Continued.

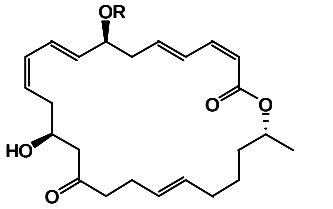
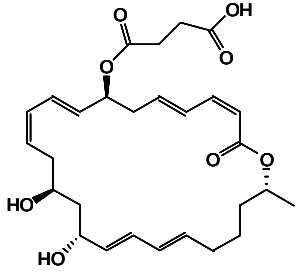
Metabolites/Structure	Producing strain	Source	Reference
Macrolactin F **(18)**	*Bacillus* sp. ScO26	Marine sediments	Jaruchoktaweechai *et al*., 2000
7-*O*-succinyl macrolactin F **(19)**			
 **(18)** R = H **(19)** R =			
7-*O*-succinyl macrolactin A			
 **(20)**			

Table 7. Continued.

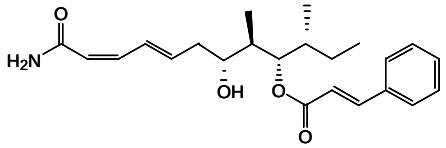
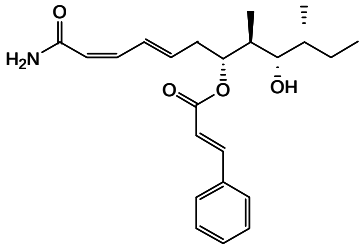
Metabolites/Structure	Producing strain	Source	Reference
Basiliskamide A  (21)	*Bacillus laterosporus*	Coastal water	Barsby *et al*., 2002
Basiliskamide B  (22)			

Table 7. Continued.

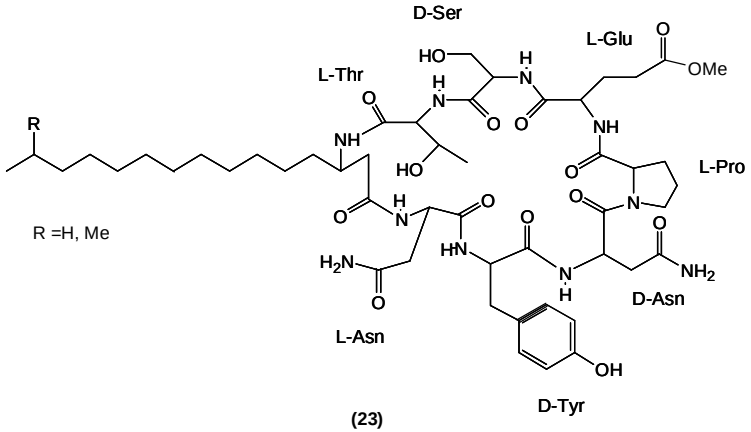
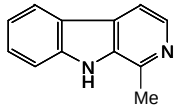
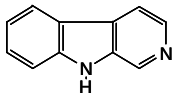
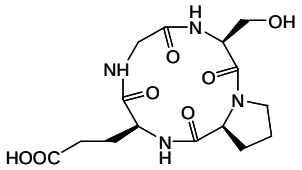
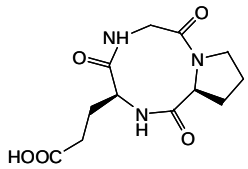
Metabolites/Structure	Producing strain	Source	Reference
Bacillomycin D  (23)	*Bacillus subtilis* KMM 457	No information given	Oleinikova *et al*., 2003
Alkaloid Harman  (24)	*Enterococcus faecium*	Tunicate *Microcosmus australis*	Aassila *et al*., 2003

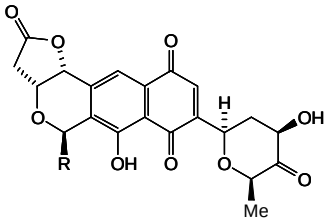
Table 7. Continued.

Metabolites/Structure	Producing strain	Source	Reference
Norharman (a beta-carboline alkaloid)  **(25)**	*Pseudoalteromonas piscicida*	Sponge *Hymeniacidon perleve*	Zheng *et al*., 2005
Cyclopeptide **(26)** and **(27)**  **(26)**  **(27)**	*Ruegeria* sp.	Sponge *Suberites domuncula*	Mitova *et al*., 2004

[a] The compounds also showed antimicroalgae activity and other biological activities i. e. inhibition on proliferation of human umbilical vein endothelial cells, and tumor cells, mast cell degranulation and hyaluronidase activity (Kanawano *et al*., 1998).

Table 8. Summary of selected metabolites with antimicrobial activity from marine actinomycetes.

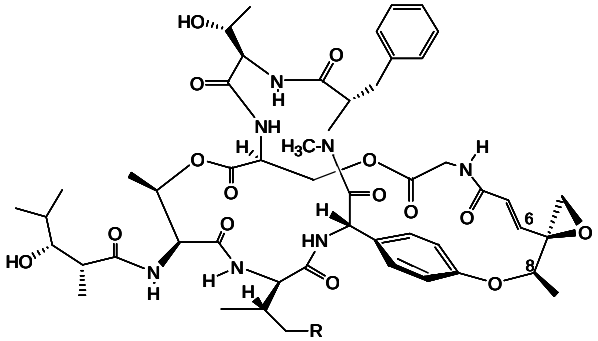
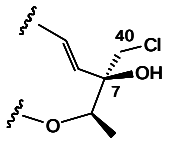
Metabolites/Structure	Producing strain	Source	Reference
Halawanones A-B	*Streptomyces* sp.	Sediment sample	Ford *et al*., 1998



(A) R = CH_2Me

(B) R = Me

Table 8. Continued.

Metabolites/Structure	Producing strain	Source	Reference
Salinamides (A , B) [a]  **(A)** R = Me  **(B)** R = Me	*Streptomyces* sp.	Jellyfish *Cassiopeia xamachana*	Moore *et al*., 1999

[a] The compounds also showed potent anti-inflammatory activity.

Table 8. Continued.

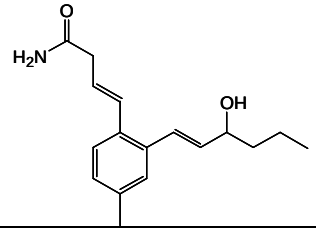
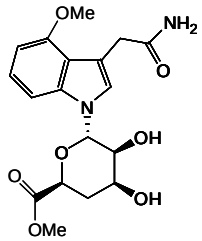
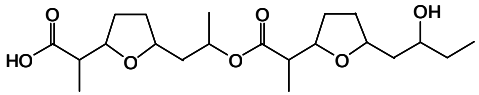
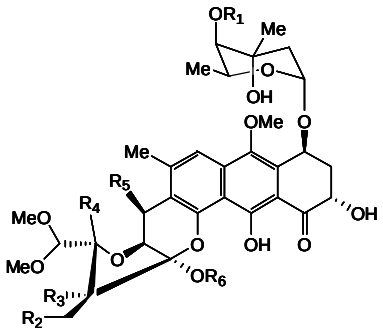
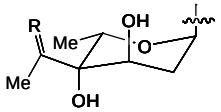
Metabolites/Structure	Producing strain	Source	Reference
Lorneamide A 	*Streptomyces* and related genera	Sample of beach sand	Capon *et al*., 2000
Kahakamide A 	*Nocardiopsis dassonvillei*	Shallow water sediment sample	Schumacher *et al*., 2001
Bonactin 	*Streptomyces* sp. BD21-2	Shallow-water sediment	Schumacher *et al*., 2003

Table 8. Continued.

Metabolites/Structure	Producing strain	Source	Reference
Trioxacarcins (A, B, C, D, E) [b]	*Streptomyces* sp.	No information given	Maskey *et al.* , 2004



a =



(A) R_1 = COMe, R_2-R_3 = R_4-R_5 = O, R_6 =a: R = O

(B) R_1 = COMe, R_2= R_3 = OH, R_4-R_5 = O, R_6 =a: R = O

(C) R_1 = COMe, R_2-R_3 = R_4-R_5 = O, R_6 =a: R = OH, H

(D) R_1 = H, R_2-R_3 = R_4-R_5 = O, R_6 =a: R = O

(E) R_1 = COMe, R_2 = R_3 = R_4 = R_5 = OH, R_6 = H

[b] Some showed high antitumor and antimalaria activity.

Table 8. Continued.

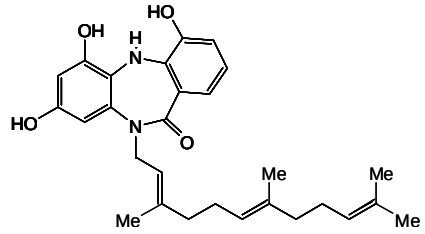
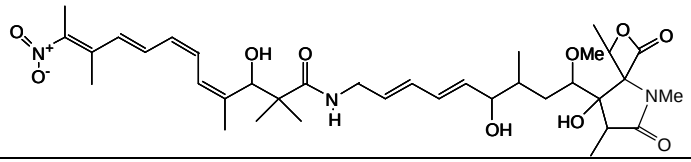
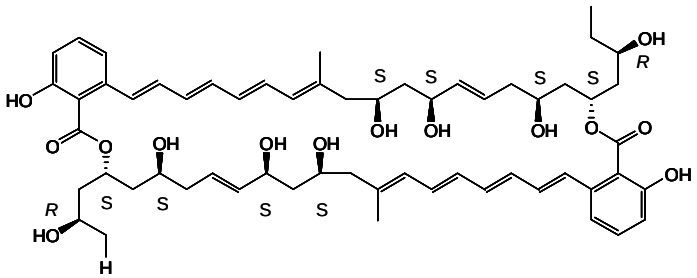
Metabolites/Structure	Producing strain	Source	Reference
Diazepinomicin 	*Micromonospora* sp.	Marine ascidian *Didemnum proliferum* Kott	Charan *et al*., 2004
Lajollamycin [d] 	*Streptomyces nodosus*	Marine sediment	Manam *et al*., 2005
Marinomycin A [d] 	*Marinispora* CNQ-140	Sediment	Kwon *et al*., 2006

Table 8. Continued.

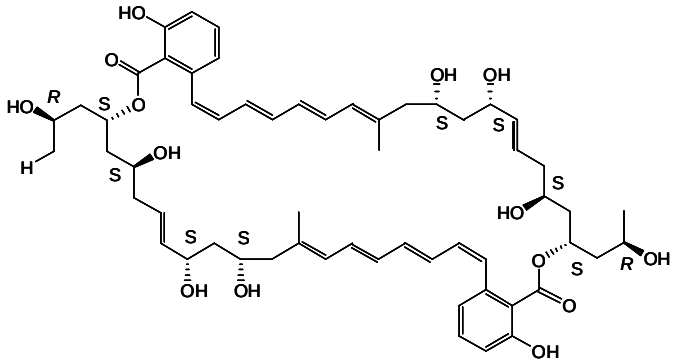
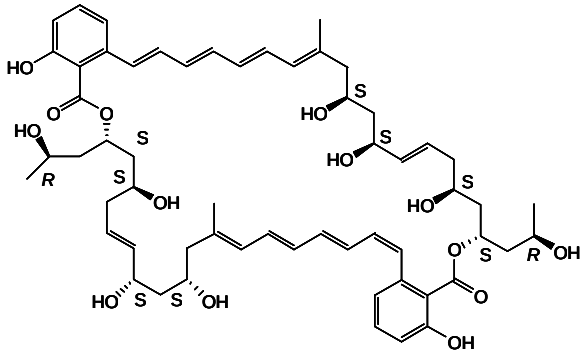
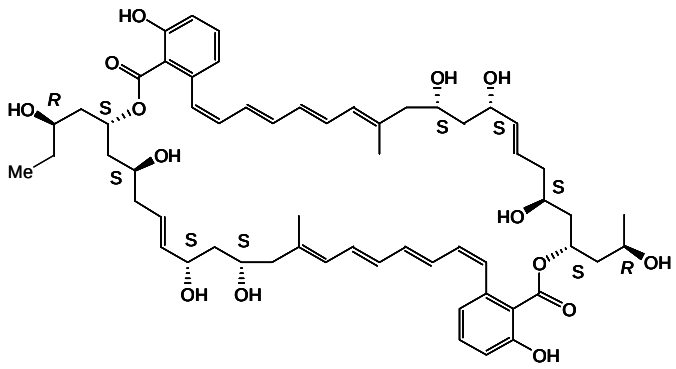
Metabolites/Structure	Producing strain	Source	Reference
Marinomycin B [d] 	*Marinispora* CNQ-140	Sediment	Kwon *et al*., 2006
Marinomycin C [d] 			

Table 8. Continued.

Metabolites/Structure	Producing strain	Source	Reference
Marinomycin D [d] 	*Marinispora* CNQ-140	Sediment	Kwon *et al*., 2006

[d] The compounds also showed antitumor activity.

Table 9. Summary of selected metabolites from gliding bacteria.

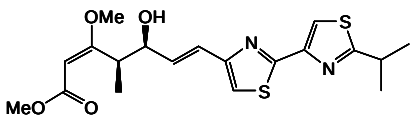
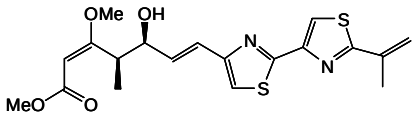
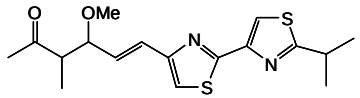
Metabolites/Structure	Producing strain	Biological activity	Reference
Cystothiazole C 	*Cystobacter fuscus* strain AJ-13278	Antifungal activity	Suzuki *et al*., 1998
Cystothiazole D 			
Cystothiazole E 			

Table 9. Continued.

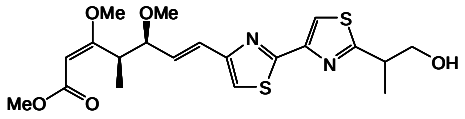
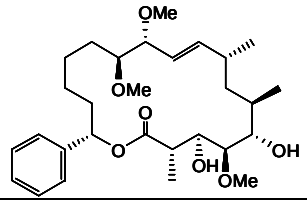
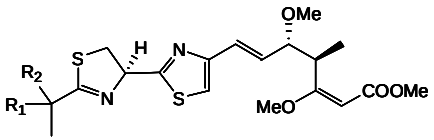
Metabolites/Structure	Producing strain	Biological activity	Reference
Cystothiazole F 	*Cystobacter fuscus* strain AJ-13278	Antifungal activity	Suzuki *et al*., 1998
Soraphen 	*Sorangium cellulosum*	Antifungal activity	Gerth *et al*., 2003
Melithiazols A , D , K and L  **(A)** R_1, R_2 = $-CH_2^-$ **(D)** R_1 = Me, R_2 = H **(K)** R_1, R_2 = $-CH_2O^-$ **(L)** R_1 = CH_2OMe, R_2= H	*Melittangium lichenicola*, *Archangium gephyra* and *Myxococcus stipitatus*	Antifungal activity Most active : Melithiazol A, L Cytotoxic activity Lipophilicities activity	Böhlendorf *et al*., 1999

Table 9. Continued.

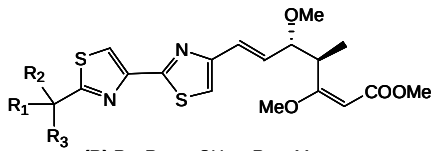
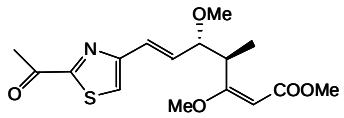
Metabolites/Structure	Producing strain	Biological activity	Reference
Melithiazols B , E , F , G , H , I , M , and N  **(B)** R_1, R_2, = $-CH_2^-$, R_3 = Me **(E)** R_1 = Me, R_2 = Me, R_3 = H **(F)** R_1 = Ph, R_2 = H, R_3 = H **(G)** R_1 = Et, R_2 = Me, R_3 = H **(H)** R_1 = Me, R_2 = H, R_3 = H **(I)** R_1 = iProp, R_2 = H, R_3 = H **(M)** R_1 = Me, R_2, R_3, = $-O^-$ **(N)** R_1, R_2, = $-CH_2O^-$, R_3 = Me	*Melittangium lichenicola*, *Archangium gephyra* and *Myxococcus stipitatus*	Antifungal activity Most active : Melithiazol B, M Cytotoxic activity Lipophilicities activity	Böhlendorf *et al*., 1999
Melithiazol C 			

Table 9. Continued.

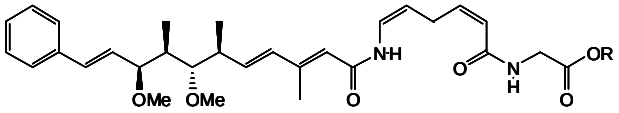
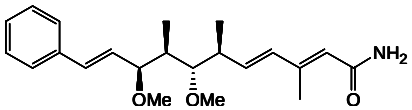
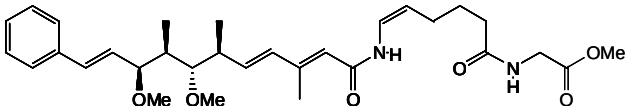
Metabolites/Structure	Producing strain	Biological activity	Reference
Crocacins A, B  **(A)** R = Me **(B)** R = H	*Chondromyces crocatus*	Antifungal activity and cytotoxicity activity	Jansen *et al*., 1999
Crocacin C 			
Crocacin D 	*Chondromyces pediculatus*		

Table 9. Continued.

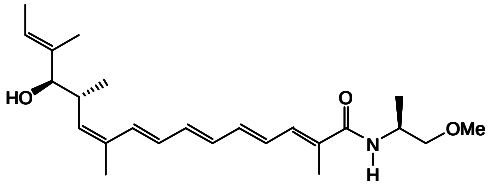
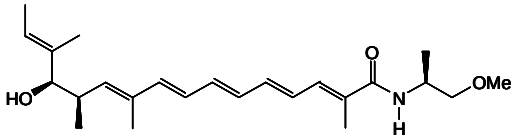
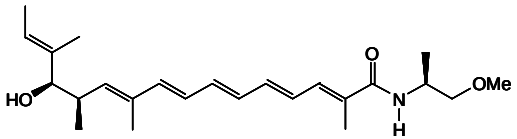
Metabolites/Structure	Producing strain	Biological activity	Reference
(6E, 10z)-2′-*O*-methylmyxalamide D 	*Cystobacter fuscus*	Antifungal activity	Kundim *et al*., 2004
2′-*O*-methylmyxalamide D 			
(6E)- 2′-*O*-methylmyxalamide D 			

Table 9. Continued.

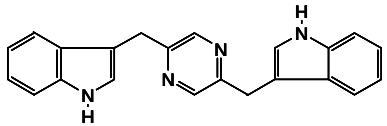
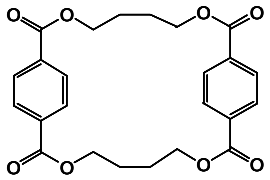
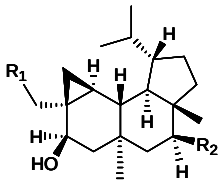
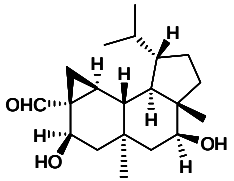
Metabolites/Structure	Producing strain	Biological activity	Reference
2,5- bis(3-indolymethyl)pyrazine 	*Cytophaga* sp. strain AM13.1	No antimicrobial activity	Shaaban *et al*., 2002
Pharacine 			

Table 9. Continued.

Metabolites/Structure	Producing strain	Biological activity	Reference
Diterpenoids of the neoverrucosane class  **(1)** R_1 = H, R_2 = OH **(2)** R_1 = OH, R_2 = H **(3)** R_1 = R_2 = OH  **(4)**	Marine gliding bacterium *Saprospira grandis* ATCC 23116	No biological activities	Spyere *et al*., 2003

2.2.2 Marine bacterial metabolites with cytotoxicity

Many bacterial metabolites had cytotoxicity. Results of the cytotoxicity are expressed in terms of the dose which inhibits cell growth to 50 % of the control growth (ED_{50} = ID_{50} = IC_{50}), usually expressed in μg/ml. An active extract is one with an ED_{50} of <20 μg/ml and a pure compound is active if the ED_{50} < 4 μg/ml (Schmitz *et al*., 1993). Many cytotoxic compounds from marine bacteria have been reported and selected metabolites are summarized in Table 10

2.2.3 Marine bacterial metabolites with other biological activities

Marine bacteria can produce secondary metabolites with a wide variety of biological activities. For example, the marine bacterium, *Aeromonas caviae*, isolated from the gastrointestinal tracts of marine fishes, produced immunosuppressive substances, a 70 kDa polysaccharide containing mannose: glucose (4:1) (Ohmori *et al*., 1998). Some selected marine bacterial metabolites are shown in Table 11.

Table 10. Summary of selected marine metabolites with cytotoxicity.

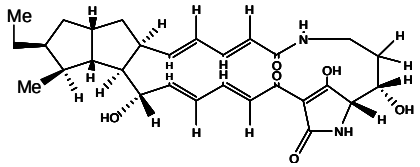
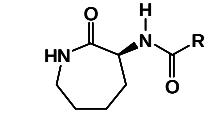
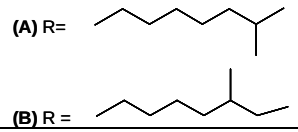
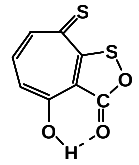
Metabolites/Structure	Producing strain	Source	Reference
Alteramide A 	*Alteromonas* sp.	Sponge *Halichondria okadai*	Shigemori *et al*., 1992
Caprolactins A and B  	Unidentified Gram-positive bacterium	Deep-ocean sediment	Davidson *et al*., 1993
Thiotropocin 	*Caulobacter* sp.	Microalgal *Micromonas* sp. (*Prasiophyceae)*	Kawano *et al*., 1997

Table 10. Continued.

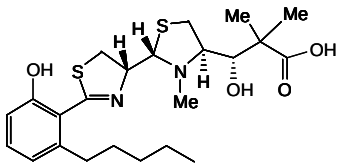
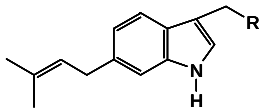
Metabolites/Structure	Producing strain	Source	Reference
Agrochelin 	*Agrobacterium* sp.	No information given	Cañedo *et al*., 1999
6-prenyltrptophol **(1)** Aldoxime mixture **(2)**  **(1)** R = -CH_2OH **(2)** R = -CH=NOH	*Streptomyces* sp.	Marine invertebrate	López *et al*., 2003

Table 10. Continued.

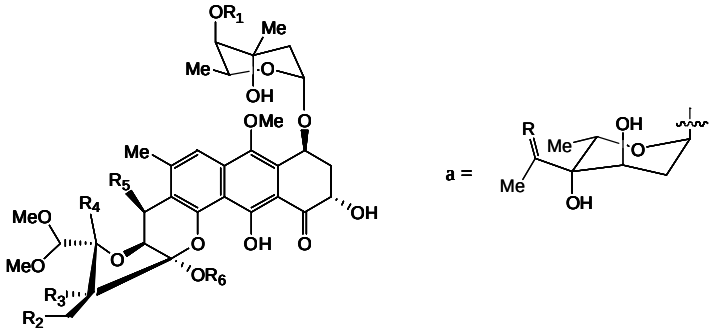
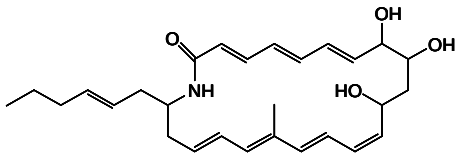
Metabolites/Structure	Producing strain	Source	Reference
Trioxacarcins (A, B, C, D)  **(A)** R_1 = COMe, R_2-R_3 = R_4-R_5 = O, R_6 =a: R = O **(B)** R_1 = COMe, R_2= R_3 = OH, R_4-R_5 = O, R_6 =a: R = O **(C)** R_1 = COMe, R_2-R_3 = R_4-R_5 = O, R_6 =a: R = OH, H **(D)** R_1 = H, R_2-R_3 = R_4-R_5 = O, R_6 =a: R = O	*Streptomyces* sp.	No information given	Maskey *et al*., 2004
Aureoverticillactam 	*Streptomyces aureoverticillatus*	Sediment	Mitchell *et al*., 2004

Table 10. Continued.

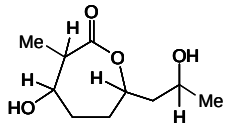
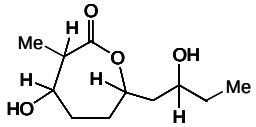
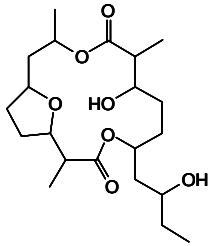
Metabolites/Structure	Producing strain	Source	Reference
Feigrisolide A 	*Streptomyces* sp.	No information given	Sobolevskaya *et al*., 2004
Feigrisolide B 			
Feigrisolide C 			

Table 10. Continued.

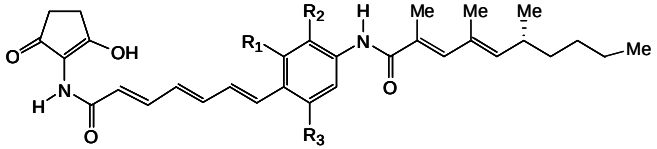
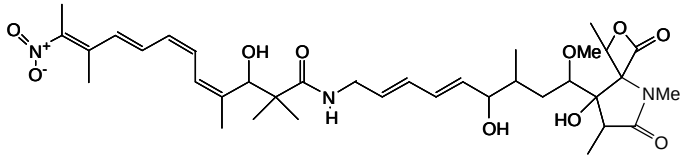
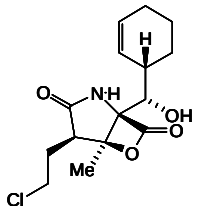
Metabolites/Structure	Producing strain	Source	Reference
Chinikomycins A and B  **(A)** R_1 = Cl, R_2 = R_3 = OH **(B)** quinone from of **(A)**	*Streptomyces* sp. M045	Sediment	Li *et al*., 2005
Lajollamycin 	*Streptomyces nodosus*	Marine sediment	Manam *et al*., 2005
Salinosporamide A 	*Salinispora tropica* strain CNB-392	Sediment	Feling *et al*., 2003 cited by Williams *et al*., 2005

Table 10. Continued.

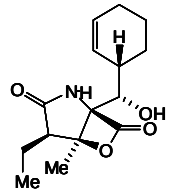
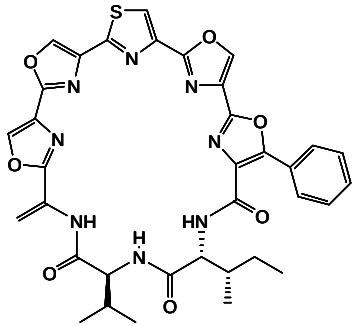
Metabolites/Structure	Producing strain	Source	Reference
Salinosporamide B 	*Salinispora tropica* strain CNB-392	Sediment	Williams *et al*., 2005
Mechercharmycin A 	*Thermoactinomyces* sp. YM3-251	Sea mud	Kanoh *et al*., 2005

Table 11. Selected marine metabolites with other biological activities.

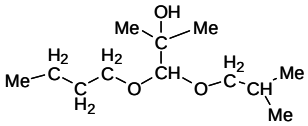
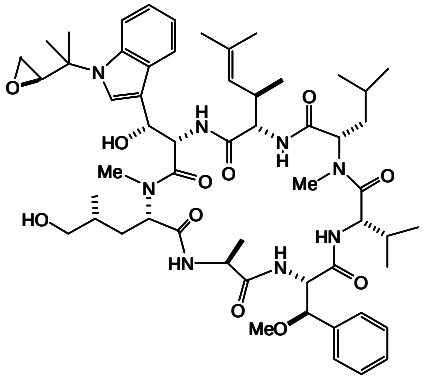
Metabolites/Structure	Producing strain	Biological activity	Reference
70 kDa Polysaccharide (mannose: glucose (4:1)	*Aeromonas caviae*	Immunosuppressive activity	Ohmori *et al*., 1998
[1-(2′-methylpropoxy)-2-hydroxy-2-methylpropoxy] butane 	*Vibrio angustum* S14	Inducing the acylated homoserine lactone (AHL) reporter system in *Agrobacterium tumefaciens* and bioluminescence in *Vibrio harveyi*	De Nys *et al*., 2001
Cyclomarine A 	*Streptomyces* sp.	Anti-inflammatory activity Cytotoxicity activity	Renner *et al*., 1999

Table 11. Continuted.

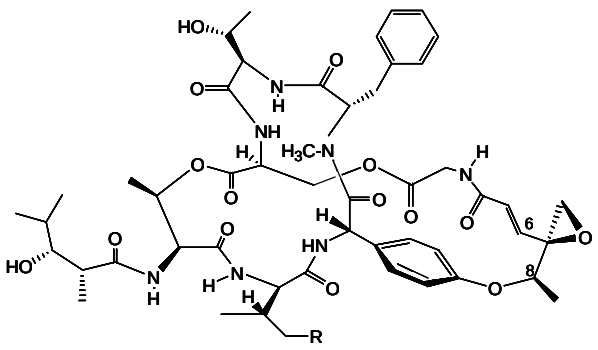
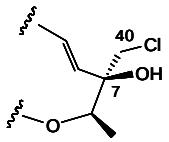
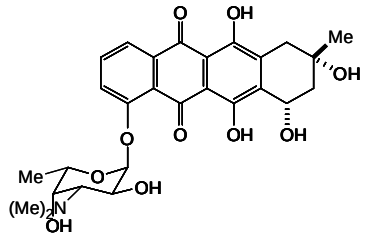
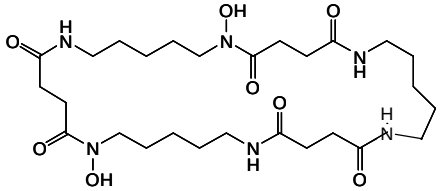
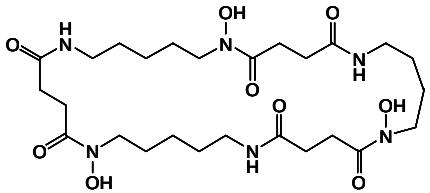
Metabolites/Structure	Producing strain	Biological activity	Reference
Salinamides A and B  **(A)** R = Me  **(B)** R = Me	*Streptomyces* sp.	Anti-inflammatory activity Cytotoxicity activity	Moore *et al*., 1999
Komodoquinone A 	*Streptomyces* sp. KS3	Neuritogenic activity against the neuroblastoma cell line (Neuro 2A)	Itoh *et al*., 2003

Table 11. Continuted.

Metabolites/Structure	Producing strain	Biological activity	Reference
Organic extract / No structure given	*Streptomyces* sp.173	Insecticidal activities	Xiong *et al*., 2004
Low-molecular weight compounds/ No structure given	*Pseudoalteromonas* spp.	Surface activity Hemolytic activity	Kalinovskaya *et al.,* 2004
Cyclic peptides Dehydroxynocardamine  Desmethylenylnocardamine 	*Streptomyces* sp.	Sortase B inhibitory activity All showed weak activity	Lee *et al*., 2005

3. Antimicrobial assay with AlamarBlue

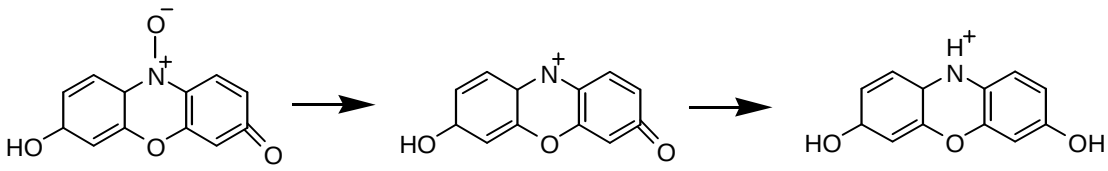
Various methods have been used for antimicrobial activity determination. These methods included disc or paper diffusion methods, bioautographic methods and dilution methods (Rios *et al*., 1988; Hadacek and Greger, 2000). Diffusion method is a common method for studying antimicrobial activity (Encarnación *et al*., 2000; Aassila *et al*., 2003; Kalinovskava *et al*., 2004). This technique used disk or well as reservoir containing the extracts solution to be tested. The result of antimicrobial activity is indicated by a clear zone around the reservoir due to the inhibition of the growth of microorganisms (Rios *et al*., 1988). Since these methods are based on the diffusivity of the compounds in agar, the high polar compounds can diffuse greater or better than non-polar compounds, a larger zone of inhibition regardless of potency can be produced. Therefore, there is no strong relation between clear zone diameter and antimicrobial activity (Rios *et al*., 1988). Moreover, this method requires long time for incubation before the result can be observed. Several methods therefore were developed in order to rapidly test for antimicrobial activities i.e. luminescent methods (Hattori *et al*., 1998; Simon *et al*., 2001;Vesterlund *et al*., 2004), flow cytometry (Ramani *et al*., 1997; Gökahmetoğlu *et al*., 2003), green fluorescent protein reporter microplate assay (Collins *et al*., 1998), and colorimetric microdilution broth assay with redox dye (Kanjana-Opas, 2002).

Colorimetric microdilution broth assay with AlamarBlue

The AlamarBlue (resazurin), an oxidation reduction dye, is an indicator of cellular growth and viability of cells, which can be applied to different types of biological assays ranging from cell proliferation (Kwack and Lynch, 2000) to a detection of viability of *Haemophilus influenzae* in serum bactericidal activity (SBA) assay (Romero-Steiner *et al*., 2004) and determining the abundance of contaminant degrading microorganisms (Guerin *et al*., 2001). AlamarBlue (resazurin) has also been used widely for antimicrobial susceptibility testing of several bacteria i.e. *Staphylococcus aureus*, *Streptococcus* spp., *Enterococcus faecalis, Escherichia coli, Klepsiella pneumoniae, Enterobacter cloacae,* and *Pseudomonas aeruginosa* (Jorgensen *et al*., 1997), *Leptospira* spp. (Murray and Hospenthal, 2004), fungi (Espinel-Ingroff *et al*., 1997; Kanjana-Opas, 2002) and protozoa (McBride *et al*., 2005, Rolón *et al*, 2006).

The AlamarBlue is reduced in response to the chemical reduction of medium resulting from cell growth or by the mitochondrial enzymes. The reduction of resazurin involves

two stages (Tratnyek *et al*., 2001; Guerin *et al*., 2001). The first stage, resazurin (blue color /oxidized form /nonfluororescent) is reduced to resorufin (pink color, reduced form / fluororescent). This step is not reversible by atmospheric oxygen. Secondly, resorufin is reduced to dihydroresorufin with colorless and non fluorescent. The second stage of reduction is reversible by atmospheric oxygen. The reduction reaction of resazurin is shown in Figure 1. The reduced product of resazurin, resorufin, can be detected by either colorimetric or fluorometric methods. The absorption peaks for resazurin and resorufin in butanol extracted were found at 610 nm and 575 nm, respectively (Zrimšek *et al*., 2004).



Blue Pink Colorless

Figure 1. Reduction of resazurin.

Source: Guerin *et al*. (2001).

Objective

To isolate, purify and characterize novel bioactive compounds from marine bacteria.

Scope of research works:

1. Isolation of marine bacteria from specimens collected from the Southern coasts of Thailand.
2. Screening of marine bacterial extracts for biological activities.
3. Purification and characterization of bioactive compounds.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Microorganisms

Microorganisms used for antimicrobial assay were obtained from the culture collection at Bangkok MIRCEN, Thailand Institute of Scientific and Technological Research and other organizations (Table 12). Stock cultures of these microorganisms were maintained in 50 % glycerol and kept at –80 °C.

Table 12. Microorganisms used for antimicrobial testing.

Microorganisms	Source
Staphylococcus aureus TISTR 517	MIRCEN, Thailand
Candida albicans TISTR 5239	
Aspergillus fumigatus TISTR 3108	
Aspergillus flavus TISTR 3041	
Bacillus subtilis, Bacillus cereus, and	Microbiology Laboratory, Faculty of
Escherichia coli	Agro-Industry, PSU
Pseudomonas aeruginosa	Pharmacognosy and Pharmaceutical
Methicillin-resistant *Staphylococcus aureus*	Botany Laboratory, Faculty of
	Pharmaceutical Science, PSU
Saccharomyces cerevisiae	Microbiology Laboratory, Faculty of
	Agro-Industry, PSU

2. Culture media and chemicals

Yeast extracts (Merck, Germany).

Peptone (Merck, Germany).

Agar (Merck, Germany).

Nutreint agar (Merck, Germany).

Tryptone (Merck,Germany).

Potato dextrose agar (PDA)(Merck, Germany).

Skim milk (commercial grade).

Galactose (Fluka, Switzerland).

Mannitol (Fluka, Switzerland).

RPMI 1640 medium with L-glutamate without sodium bicarbonate (GIBCO).

Methanol AR grade (LAB-SCAN, Thailand).

Dichrolomethane AR grade(LAB-SCAN,Thailand).

Dimethyl sulfoxide (DMSO) AR grade (Fisher Scientific, UK).

Acetone AR grade (Merck, Germany).

Chloroform AR grade (Merck, Germany).

Streptomycin sulfate (Fluka, Switzerland).

Chloramphenicol (Sigma, Switzerland).

Amphicillin (Fluka, Switerzerland).

Oxytetracyclin (Fluka, Switzerland).

Amphotericin B (Fluka, Switzerland).

Vancomycin hydrochloride (Fluka, Switzerland).

Resazurin (Sigma, Switzerland).

Normal phase silica gel 60 (0.040-0.063 mm) (Merck, Germany).

Normal phase silica gel 60 GF254 (Merck, Germany).

Reversed phase silica gel C_{18} (Fluka, Switzerland).

Normal phase silica gel 60 F254 alumimium sheets, (Merck, Germany).

Sephadex LH 20 (Fluka, Switzerland).

Amberlite XAD 16 (Fluka, Switzerland).

3. Isolation and cultivation media

Isolation media for marine bacteria were media A, B, C, D, E and F, which were modified from a ZoBell medium (Kjelleberg and Hákansson, 1977) by supplementing with galactose, mannitol, seaweed and chitinous material as shown in Table 13. Cultivation media used for screening of secondary metabolites production were media B, E, G and an original isolation medium (Table 14), except for the isolates which were isolated from medium B and E, media A was chosen as cultivation medium. In case of marine gliding bacteria, sodium glutamate medium (Glu), modified from a mineral medium and SAP II agar (Reichenbach, 1992), was used for the isolation whereas glycerol medium (Gly), sodium glutamate medium (Glu) and skim milk medium (SK) were used for cultivation of marine gliding bacteria (Table 15).

4. Instruments

Vortex mixer (GENIE -2, Lab line instruments).

Incubator shaker (Model KMC-8480 SR-L, LMS co, LTD).

Incubator (Model BE 500, Memmert).

Homogenizer (Model T 25, IKa libortechnik).

Evaporator (Model R-200, BÜCHI).

Microflow advance biosafety cabinet class 2 (Model ABS 1200, Astec microflow).

Spectrophotometer (UV/VIS spectrophotometer Model V-530, Jasco).

Microscope (Model YS 100, Nikon).

Multichanel pipette (transferpette®-12).

Sterile 96 U-shaped well plate (Nunc, Denmark).

Hemacytometer (BOECO, Germany).

NMR (Fourier Transform NMR spectrometer 500 MHz, model UNITY INOVA, Varian).

UV-Visible spectrophotometer (Model UV-1601).

Scanning Electron Microscope (JSM-5800LV, JEOL).

Table 13. Marine-based media used for isolation of marine bacteria.

Media	Composition	
A	Yeast extract	1.0 g
	Peptone	1.0 g
	Galactose	10.0 g
	Seawater	1.0 l
	Agar	15.0 g
B	Yeast extract	5.0 g
	Peptone	5.0 g
	Seaweed meal	5.0 g
	Seawater	1.0 l
	Agar	15.0 g
C	NH_4NO_3	0.01 g
	$K_2HPO_4 \cdot 3H_2O$	0.01 g
	Sodium glutamate	1.0 g
	Seawater	1.0 l
	Agar	13.0 g
D	Yeast extract	1.0 g
	Peptone	5.0 g
	$FeSO_4 \cdot 7H_2O$	0.1 g
	Seawater	1.0 l
	Agar	15.0 g
E	Yeast extract	5.0 g
	Peptone	10.0 g

Table 13. Continued.

Media	Composition	
E	Mannitol	10.0 g
	Seawater	1.0 l
	Agar	15.0 g
F	Yeast extract	0.5 g
	Peptone	0.5 g
	Chitinous material	5.0 g
	Seawater	1.0 l
	Agar	15.0 g

Table 14. Marine-based fermentation broth for cultivation of marine bacteria.

Media	Composition	
B	Yeast extract	5.0 g
	Peptone	5.0 g
	Seaweed meal	5.0 g
	Seawater	1.0 l
E	Yeast extract	5.0 g
	Peptone	5.0 g
	Seaweed meal	5.0 g
	Seawater	1.0 l
G	Chitinous material	10.0 g
	Fish meal	10.0 g
	Seawater	1.0 l
Original isolation media	Media A, B, C, D, E, and F. In case of isolates which were obtained from media B and E, medium A was chosen	

Table 15. Marine-based media for isolation and cultivation of marine gliding bacteria.

Media	Composition	
Glycerol medium*	NH_4NO_3	0.01 g
(Gly)	$K_2HPO_4 \cdot 3H_2O$	0.01 g
	Glycerol	1.0 g
	Seawater	1.0 l
	Agar	15.0 g
Sodium glutamate medium*	NH_4NO_3	0.01 g
(Glu)	$K_2HPO_4 \cdot 3H_2O$	0.01 g
	Sodium glutamate	1.0 g
	Seawater	1.0 l
	Agar	15.0 g
SAP II agar	Tryptone	1.0 g
	Yeast extract	1.0 g
	Seawater	1.0 l
	Agar	15.0 g
Skim milk medium	Skim milk	5.0 g
(SK)	Yeast extract	3.0 g
	$MgSO_4 \cdot 7H_2O$	1.0 g
	Seawater	1.0 l

* In case of glycerol and sodium glutamate broth, agar was not added.

Methods

Part I : Screening of Marine Bacteria with Biological Activity

1. Sample collection

Marine specimens including sediments, algae, invertebrates and seawater were collected either with the aid of scuba diving or snorkeling. Each specimen was kept in a plastic bag and stored in an ice box until isolation process took place.

2. Isolation of marine bacteria

2.1 Isolation of bacteria

Marine bacteria were isolated from the specimens either by using a direct plating or a serial dilution methods on different marine-based media. Direct plating method began with rinsing of samples with sterile seawater, cutting sample into small pieces and putting on different isolation media. The plates were incubated at room temperature for 2-3 days. Bacteria surrounding the specimen were chosen by using a sterile needle loop and then streaked on the fresh agar plate. Single colonies were picked and streaked on the new agar plates until pure colony was obtained. For serial dilution method, samples were rinsed with sterile seawater several times prior to cutting and were homogenized in sterile seawater at 9500 rpm for 10 minutes. For sediment samples, about 1 g of the sediment was suspended in 9 ml of sterile seawater, followed by a serial dilution with sterile seawater to the dilutions of 10^{-2}-10^{-4} before plating on the isolation media by spread plate technique. After 2-3 days of incubation at room temperature, bacterial colonies were observed and transferred onto new agar plates. Single colonies were picked and transferred on to new agar plates. The procedure was repeated until pure culture was obtained. The marine bacterial isolates were assigned as the culture numbers of bacteria as “CNB” (culture number of bacteria) for further references. The bacterial isolates were cultured on seawater nutrient agar slant and kept at 4 °C as working strains. For culture collection, the solution of bacterial isolates were preserved with 50% glycerol and stored at -80 °C.

2.2 Isolation of gliding bacteria

Marine gliding bacteria were isolated using a direct plating technique. The samples were rinsed with sterile seawater to reduce the contamination of other bacteria. Samples were cut into small pieces by a sterile sharp blade and then placed on the Na-glutamate agar medium. The plates were incubated at room temperature and observed daily by naked eye and/or under a stereomicroscope. The spreading appearance surrounding specimens was considered for the occurrence of gliding bacteria and then was selected for further isolation. The area of swarm colony of gliding bacteria was cut into a small block of agar culture (0.5 x 0.5 cm) using a steriled sharp blade and then transferred to a fresh medium (SAP II agar). The swarm colony of gliding bacteria was observed daily. The procedure was repeated until a pure isolate was obtained. The culture numbers of gliding bacteria were assigned as GB for further references.

3. Preparation of marine bacterial extracts

The marine bacterial isolates were grown on nutrient agar slant, prepared using seawater as diluent, and incubated at 30 °C for 24 h. The starter cultures were prepared. A loopful of the bacterial colony was inoculated in a 250 ml Erlenmeyer flask containing 100 ml of seawater nutrient broth. The flasks were incubated on a shaker (200 rpm) at 30 °C for18- 20 h. Cultivation process was carried out by inoculating 5-10% of the starter into a 250 ml Erlenmeyer flask containing 100 ml of different sterile cultivation media and incubated on a shaker (200 rpm) at 30 °C for 4-5 days.

For gliding bacteria, the edges of swarm colonies of gliding bacteria grown on SAP II agar were cut into small pieces and then transferred into a 250 ml Erlenmeyer flask containing 100 ml cultivating broth. The flasks were incubated on a shaker (180-200 rpm) at 30 °C for 5-6 days. The bacterial extracts were prepared by solvent extraction of cultivation broth with ethyl acetate with equal volume of cultivation broth. The extraction was carried out twice. The extracts were dried with a rotary evaporator and dissolved with small amount of ethyl acetate and transferred into scintillation vials and dried with a vacuum evaporator before weighing. The extracts were kept at -20 °C until testing for antimicrobial activity.

4. Screening of marine bacteria producing antimicrobial compounds

4.1 Optimal conditions for antimicrobial assay in a primary screening

The colorimetric microdilution broth antimicrobial assay was developed in order to use as a rapid screening method with AlamarBlue as growth indicator. *Staphylococcus aureus* TISTR 517, *Pseudomonas aeruginosa*, *Candida albicans* TISTR 5239 and *Aspergillus fumigatus* TISTR 3108, were used as test microorganisms. Effects of inoculum size, dimethylsulfoxide (DMSO) on growth of test microorganisms and the susceptibility of test strains to antibiotics, used as positive control, were also carried out.

Bacteria were grown on nutrient agar at 37 °C for 24 h. A loopful of colony was inoculated to a test tube containing 5 ml of RPMI 1640 medium. The test tubes were incubated on a shaker at 37 °C for 12-15 h. The culture broth were diluted and optical densities was measured at 600 nm. The AlamarBlue solution (0.8% w/v resazurin solution) was added to each inoculum suspension of tested bacteria at a concentration of 1% and mixed well using a vortex mixer before transferring 100 µl of inoculum suspension to the 96-well plate using a multichannel pipette. At each dilution, the effect of DMSO and antibacterial agent (vancomycin, 0.5 mg/ml) were studied as negative and positive controls, respectively. Ten microlitters of sterile DMSO and antimicrobial agent solution were added to the well in row A containing 190 µl of bacterial suspension. By using a multichannel pipette, a half-fold serial dilution was performed in these two columns by mixing from the two first wells before transferring 100 µl of the mixture solution to the second well. The same practice was continued through the columns, and 100 µl of excess mixture solution was discarded from the last wells (row H). The plates were incubated at 37 °C and bacterial growth was observed by visual inspection of AlamarBlue color changing from blue to pink at every 3 h. An innoculum size that gave AlamarBlue reduction time within 10-12 h was considered optimal.

For antifungal assay, a loopful of *Candida albicans* TISTR 5239 grown on PDA at 37 °C for 24 h was inoculated to a test tube containing 5 ml of RPMI 1640 medium and then incubated at 37 °C for 12-15 h. The cells in the culture broth were counted using a hemacytometer. The cell suspension were diluted with RPMI 1640 medium to obtain different concentrations of yeast cells ranging from 5 x 10^4 to 1 x10^6 cells/ml The AlamarBlue solution was added to the inoculum suspension at a concentration of 1%. The growth of yeast was

determined by observing the change in color of AlamarBlue in 96-well plate at 37 °C every 3 h. The inoculum size that changed color of AlamarBlue within 10-15 h was chosen. In the case of filamentous fungi, *Aspergillus fumigatus* TISTR 3108, a six days-old PDA plate of fungus was rinsed with a 0.1% sterile tween 80. The fungal spores were washed out with a sterile loop and then filtered through the sterile coarse cloth. The amount of spores in the suspension were counted using a hemacytometer and diluted with RPMI 1640 medium to obtain different inculum concentrations ranging from $1x10^5$ to $5x10^6$spores/ml. AlamarBlue solution was added to the inoculum suspension at a concentration of 1%. The change of AlamarBlue from blue to pink was observed to indicate the growth of fungi. The inoculum suspension that changed the color of AlamarBlue within 20-24 h was chosen.

4.2 Screening of marine bacterial extracts for antimicrobial activity

Primary screening for antimicrobial activity of bacterial extracts was performed at a concentration of 150 µg/ml, against test microorganisms. The optimal inoculum suspension was prepared by the method described above and mixed well with AlamarBlue. The 1.25 µl of the bacterial extracts solution (12.5 mg/ml) was added to the well containing 100 µl of inoculum suspension. The 10 µl of amphotericin B (0.5 mg/ml) and vancomycin (0.5 mg/ml), a positive control for antifungal testing and antibacterial testing, respectively were added to the top well containing 190 µl of inoculum suspension. The 10 µl of DMSO, a negative control, was added into the top wells containing 100 of µl of inoculum suspension. The half- fold serial dilution was performed in these two columns by mixing and transferring 100 µl of mixture solution from the top well to the bottom well. Finally, the 100 µl mixture solution at the bottom row of the column was discarded. In an assay, the extracts obtained from each fermentation media were used as media control. The plates were incubated at 37 °C for appropriate incubation times selected. The antimicrobial activity was observed by visual inspection of AlamarBlue color changing. The extracts in the wells that appeared blue color were interpreted as active when they were compared with the column used as growth control and media control.

5. Acetylcholinesterase inhibitory activity

Acetylcholinesterase inhibitory activity of bacterial extracts was determined at a concentration of 0.1 mg/ml by applying a modified Ellman's method in a 96-well microplate assay (Ingkaninan *et al*., 2003). Briefly, 125 µl of 3 mM DTNB (5,5′-dithiobis[2-nitrobenzoic

acid], 25 µl of 15 mM ATCI (acetylthiocholine iodide), and 50 µl of buffer (50 mM Tris-HCl pH 8.0), 25 µl of sample dissolved in buffer containing no more than 10% methanol were added to the wells followed by 25 µl of 0.28 U/ml AChE (acetylcholinesterase). The optical density of the solution were then read at 405 nm every 5s for 2 min by a CERES UV 900C microplate reader (Bio-Tek Instrument, USA). Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. Every experiment was done in triplicate. In principle, the enzyme hydrolyzes the substrate acetylthiocholine (acetylthiocholin iodide) to produce thiocholine. This product reacts with Ellman's reagent (5,5´-dithiobis[2-nitrobenzoic acid] to yield 5-thio-2-nitrobenzoate and the yellow colored 2-nitrobenzoate-5-mercaptothiocholine, which was quantitatively measured by microplate reader (Rollinger *et al*., 2004).

6. Cytotoxicity activity

Cell preparation

The cells used for the experiments were the human cell lines including breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human cervix carcinoma (Hela) and human mouth epidermoid carcinoma (KB). The cells were grown as momolayer culture in T-75 flasks and were subcultured twice a week at 37 °C in an atmosphere containing 5% CO_2 in air and 100% relative humidity for 3-4 days. Culture medium used was Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL, USA), supplemented with 10% Fetal Bovine Serum (FBS, Gibco, BRL, USA), 50 IU/ml penicillin G, 50 IU/ml streptomycin and 0.125 µg/ml amphotericin B. Adherent cells at a logarithmic growth phase were detached by addition of 2-3 ml of a 0.05% trypsin (Gibco, 1:250) and 0.02% EDTA mixture and incubation for 2-5 min at 37°C. Cells were plated (100 µl per well) in 96-well flat-bottom microtiter plate at densities of 5 x 10^3 cells/well (Papazisis *et al*., 1998). Cells were left for 24 h at 37°C to resume exponential growth.

Cytotoxicity assay

The 100 µl of either complete culture medium (control wells), or the bacterial extract (50 µg/ml) diluted in complete culture medium, was added in the 96-well plate containing cell lines. Six replicate wells for bacterial extract testing were used. Background control wells (*n* =

8), were also used in the experiment. Cell growth was evaluated 96 h later by means of the sulforhodamine B (SRB) assay described by Skehan *et al.* (1990) with some modifications. In brief, the culture medium was aspirated prior to fixation. The 50 µl of 10% cold (4°C) trichloroacetic acid were gently added to the wells. Microplates were left for 30 min at 4°C, washed five times with deionized water, and left to dry at room temperature for at least 24 h. Subsequently, 70 µl 0.4% (w/v) SRB, in 1% acetic acid solution were added to each well and left at room temperature for 20 min. SRB was removed and the plates were washed five times with 1% acetic acid before air drying. Bound SRB was solubilized with 200 µl 10 mM unbuffered Tris-based solution and plates were left on a plate shaker for at least 10 min. Absornance was read in a 96- well plate reader (A-5022, Anthos labteck, Ins.) at a wavelength 492 nm subtracting the background measurement at 620 nm. The test optical density (OD) value was defined as the absorbance of each individual well, minus the blank value ("blank" is the mean optical density of the background control wells, $n = 8$). Mean values and coefficients of variation from six replicate wells were calculated automatically. Dose-response curves were plotted (values expressed as percentage of control optical density). Curves and statistical analysis were performed using Excel 7.0 software for windows 95. Cytotoxicity was expressed as percentage of control optical density. The IC_{50} was calculated from dose- response curve.

7. Morphological and some physiological properties of selected marine gliding bacteria

7.1 Characterization and identification of the marine gliding bacterial strains GB 009 and GB 022

Marine gliding bacteria, the strains GB 009 and GB 022, were studied for morphological and some physiological properties including Gram staining, oxidase test, catalase test, and cell morphology under scanning electron microscope (SEM). Determination of oxidase and catalase were performed according to method described by MacFaddin (2000). The bacterial strains grown in SAP II agar at 30°C for 48 h were used for SEM sample preparation. The scanning electron microscopy of the strains was carried out as described by Shi and Xia (2003) with minor modification. Briefly, bacteria were fixed by immersion in 2.5 % glutaraldehyde with 0.1 M sodium cacodylate buffer for 2 h and were washed with 0.1 M sodium cacodylate buffer three times. This was followed by post-fixing in 1% OsO_4 in the same buffer for 1 h and rinsing with distilled water three times. The samples were dehydrated by an ethanol series of 50,

70, 80, 90, and 100% ethanol and stored in 100% ethanol. Specimens in 100% ethanol were critical-point dried in a CO_2 atmosphere using Critical Point Dryer and mounted on aluminium stubs and gold-coated for 5 min in a Sputter Coater. The specimens were observed under a scanning electron microscope (JSM-5800LV, JEOL).

Identification of the selected strains was determined by analysis of the complete 16S rRNA gene sequences using previously described procedures for DNA extraction, PCR amplification, sequence of the 16 S rRNA gene and phylogenetic analyses (Srisukchayakul *et al*. , 2007). Briefly, the genomic DNA was extracted with a Wizard® genomic DNA purification kit (Promega) and determined using an agarose gel electrophoresis (0.8 % agarose in TAE buffer). 16S rRNA fragment was amplified by PCR (GeneAmp® PCR System 9700, Applied Biosystem, Foster City, CA, USA and GeneAmp PCR System 2400, PERKIN ELMER, Norwalk CT, USA) with the universal primer sets of BF1 (5'-GAGTTTGATCATGGCTCAG-3') and BR1 (5'-CGGTTACCTTGTTACGACTT-3'). Amplified product was purified by GFX PCR DNA and gel band purification kit (Amersham Biosciences, Buckinghamshire, England) and examined for quality and quantity on an agarose gel electrophoresis (1% agarose in TAE buffer). Sequencing reactions were performed by using a ABI PRISM™ BigDye terminator cycle sequencing kit version 3.1 (Applied Biosystem, Foster City, CA, USA). The internal oligonucleotide primers used for full length sequencing of amplified 16S rRNA fragment were BF1 and BR1 (see above), 339F (5'- CTCCTACGGGAGGCAGCAG-3'), 785F (5'-GGATTAGATACCCTGGTAGTC-3'), 1099F (5'-GCAACGAGCGCAACCC-3'), 536R (5'-GTATTACCGCGGCTGCTG-3'), 802R (5'-TACCAGGGTATCTAATCC-3') and 1115R (5'-AGGGTTGCGCTCGTTG-3') (Nakagawa *et al*. 2001). Electrophoresis of sequence reaction products was performed by using an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The sequences were compared to the compilation of 16S rRNA genes available in the GeneBank nucleotide database by Blast (Basic Local Alignment Search Tool) search program of National Center for Biotechnology Information.

7.2 Natural seawater requirement

Marine bacteria were grown on SAP II agar using 100% natural seawater as the diluent at 30 °C for 24 h. The swarm colony was cut into a small circular piece with a diameter of 0.6 cm by a sterile cork borer. Each piece was placed on SAP II agar containing different

concentrations of seawater (0, 20, 40, 60, 80, and 100%). Each experiment was carried out in triplicate. After incubation at 30 °C for 24 and 48 h, the growth of marine gliding bacteria was observed.

7.3 NaCl requirement

Marine bacteria were grown on SAP II agar using 100% natural seawater at 30 °C for 24 h. The swarm colony was cut into a small circular piece with a diameter of 0.6 cm by a sterile cork borer before placing on SAP II agar containing different concentration of NaCl (0, 1, 2, 3, 4, 6, 8, and 10%). All experiments were done in triplicate. After incubation at 30°C for 24 and 48 h**,** the growth of marine gliding bacteria was observed.

7.4 Effect of Mg^{2+}, Ca^{2+}, and K^{+} on growth of marine gliding bacteria

Marine bacteria were grown on SAP II agar using 100% natural seawater at 30 °C for 24 h. The swarm colony was cut into a small circular piece with a diameter of 0.6 cm by a sterile cork borer before placing on SAP II agar containing three different concentrations of $MgSO_4 \cdot 7H_2O$ (0.13, 0.26, and 0.52 %), $CaCl_2 \cdot 2H_2O$ (0.03, 0.06, and 0.12 %) and KCl (0.02, 0.04 and 0.08 %). All experiments were done in triplicate. After incubation at 30 °C for 24 and 48 h**,** the growth of marine gliding bacteria was observed.

7.5 Effect of temperature on growth of marine gliding bacteria

Marine bacteria were grown on SAP II agar using 100% natural seawater at 30 °C for 24 h. The swarm colony was cut into a small circular piece with a diameter of 0.6 cm by a sterile cork borer before placing on SAP II agar containing 100% seawater. The plates were incubated at different temperatures of 4, 25, 30, 37, and 45 °C. After incubation for 24 and 48 h**,** the growth of marine gliding bacteria from triplicate experiments was examined.

7.6 Growth measurement

The growth of marine gliding bacteria was measured by the length of the radius of the swarm colony at three different points.

7.7 Statistical analysis

Test for significant differences between means at $P< 0.05$ level were performed by one-way ANOVA using software SPSS 10.0 for window.

Part II: Purification and Chemical Structure Elucidation of Bioactive Compounds from the Selected Marine Gliding Bacteria

1. Isolation of bioactive compounds from the marine gliding bacteria strain GB 009

1.1 Preparation of marine bacterial extracts

The swarm colony of 24 h-old culture of marine gliding bacteria grown on SAP II agar was cut into small pieces. A total of 5 pieces of agar culture were inoculated into a 250 ml Erlenmeyer flask containing 100 ml of starter medium (0.3 g yeast extract, 0.1 g Mg $SO_4 \cdot 7H_2O$, 0.01 g skim milk and 100 ml seawater) and then incubated on a rotary shaker (180-200 rpm) at 30-34°C for 18-20 h. Ten milliliters of the active starter culture was inoculated in each 250 ml Erlenmeyer flasks containing 100 ml of skim milk medium. The culture flasks were incubated on a rotary shaker (180-200 rpm) at 30-34 °C. After 2 days of cultivation, 2 g of an autoclaved Amberlite XAD 16 resin was added to the culture in each flask to absorb organic compounds and then returned to the shaker for 4 days. The resin was filtered and washed with 100 ml of deionized water to remove salts. The collected resins were soaked in 100 ml of ethyl acetate for 8-10 h at room temperature. During time of soaking, the resins were occasionally stirred. The ethyl acetate was filtered and resins were soaked again in 100 ml of methanol for 8-10 h. Both the ethyl acetate (F1) and the methanolic (F2) fractions were evaporated with a rotary evaporator. The dried extract F1 was dissolved with small amount of ethyl acetate to give a fraction designed as F1.1 and remained crude extract was eluted from a round evaporator flask by dissolving with methanol. This was designed as F1.2. Each fraction was dried with a rotary evaporator to give a crude extract.

The methanolic F2 was dried with a rotary evaporator and dissolved with methanol.

1.2 Purification of bioactive compound

1.2.1 Sephadex LH 20 column chromatography

The dried extract was dissolved with small amount of methanol and applied to a sephadex LH 20 column (gel length 95 cm, 2 cm diameter), which was equilibrated with MeOH. The column was eluted with MeOH at a flow rate of 0.5 ml/min. Fractions were collected at the volume of 10 ml of each fraction. TLC analysis was performed on a normal phase silica gel 60

F254 with 9:1 DCM-MeOH as the developing solvent and visualized under UV light. Color reaction was developed by spraying with Dragendroff's reagent for detecting nitrogenous base compound. The fractions which have similar pattern of TLC were combined, dried and weighed before testing for antimicrobial activity against *Staphylococcus aureus* TISTR 517, *Candida albicans* TISTR 5239 and *Saccharomyces cerevisiae* at a concentration of 308.64, 154.32 and 77.16 µg/ml and acetylcholinesterase inhibitory activity at a concentration of 0.1 mg/ml, respectively.

1.2.2 Flash column chromatography

Flash column chromatography was performed using normal phase silica gel 60 (0.040-0.063 mm) column (gel length 14.0 cm, 1.0 cm diameter). The compounds were eluted with 100 ml of DCM (dichloromethane), EtOAc, DCM-MeOH (9:1), and MeOH, respectively.

1.3 Crystallization

Crystallization of bioactive compound, marinoquinoline A, was done by using a mixture solvent system of acetone-chloroform-hexane (1:1.5:1.5).

2. Chemical structure elucidation of bioactive compound

The chemical structure of the bioactive compound was elucidated by spectroscopic methods including 1D NMR, 2D NMR, and by an X-ray crystallographic technique. 1D NMR including proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR) and 2 D NMR spectra were recorded on a Varian Unity 500 spectrometer, operating at 500 MHz for proton NMR and 125 MHz for carbon NMR. Chemical shifts (δ) were expressed in ppm with reference to internal TMS in $CDCl_3$. The crystal data and measurement conditions determined by an X-ray crystallographic technique were carried out as described by Kanjana-Opas *et al.* (2006).

CHAPTER 3

RESULTS AND DISCUSSION

Part I: Screening of Marine Bacteria with Biological Activity

1. Sample collection

Sites for samples collection in this study are shown in Table 16 and location map of sample collections are shown in Figure 2. Types of specimens collected during each trip are shown in Table 17. From the results, a total of 166 various specimens collected from five sampling sites were used for isolation of common marine bacteria and 114 specimens were used for isolation of marine gliding bacteria at MIRCEN, Thailand. The distribution of collected specimens showed that sponge samples were a major group of specimens (47%) followed by sediment/sand (16%) (Figure 3). Sponge samples were mainly focused because they are known to be good sources of bioactive compounds and harbor large numbers of microorganisms (Faulkner, 2000; Ahn *et al*., 2003). The bacterial population densities may reach 10^8-10^9 bacteria per gram of sponge wet weight (Hentschel *et al*., 2006). In addition, many bacteria associated with sponges have been reported to produce secondary metabolites such as antimicrobial compounds (Mitova *et al*., 2004; Zheng *et al*., 2005) and cytotoxic compounds (Shigemori *et al*., 1992).

2. Isolation of marine bacteria

2.1 Isolation of bacteria

Approximately 300 marine bacterial isolates were isolated from different media by two isolation methods. Fifty nine isolates were obtained from direct plating method whereas 241 were obtained from serial dilution method. Most of the bacterial isolates (39%) were obtained from sponge samples as shown in Figure 4. In addition, the correlation between numbers of specimens and numbers of isolates was also observed in the sediment/sand samples which represented the second largest group of specimens whereas such correlation was not found in the

Table 16. Sites for sample collection during April-July 2002.

Sampling sites	Collection Date	Method	Depth (ft)	Location map
Ko Tao, Ko Nang Yuan, Surat Tani	25-28 April 2002	Scuba diving	20-70	1
		Snorkeling	Beach& Intertidal zone	
Thepa estury, Songkhla	8 May 2002	Snorkeling	Beach & Intertidal zone	2
Ko Tao Surat Tani	22 May 2002	Scuba diving	50	3
		Snorkeling	Beach & Intertidal zone	
Losin pinnacle, Pattani	15 June 2002	Scuba diving	30-70	4
Pak meng ,Trung, 75 million year shell fossil Krabi, Beach at Phuket	8-10 July 2002	Snorkeling	Beach & Intertidal zone	5

Figure 2. Map of southern coasts of Thailand depicting the sites of sample collection during April-July 2002.

Table 17. Types of specimens from five locations during April- July 2002.

Specimen	Sampling site					Total number of specimens
	1	2	3	4	5	
Sponges	20	-	14	38	22	77
Corals	5	-	2	9	7	17
Algae	-	-	-	9	31	15
Sediments/sand	-	1	6	6	14	26
Shells	4	4	2	2	16	14
Biofilm	-	1	4	-	-	5
Others	2	3	-	4	54	12
Total of specimens	31	9	28	68	144*	280

* Of 144 specimens, 114 were used for isolation of gliding bacteria.

Sampling site: 1: Ko Tao, Ko Nang Yuan, Surat Tani.

2: Thepa estury, Songkhla.

3: Ko Tao, Surat Tani.

4: Losin pinnacle, Pattani.

5: Trung, Krabi, Phuket.

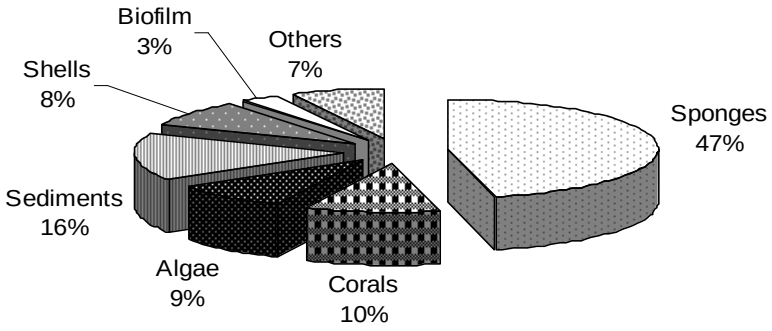


Figure 3. Percentage of specimens collected from five locations during April-July 2002 for isolation of general bacteria.

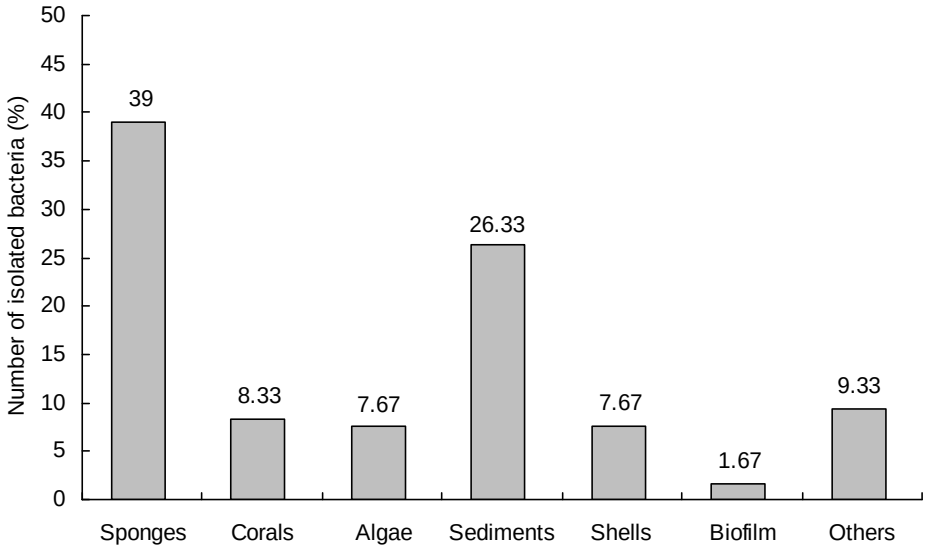


Figure 4. Percentage of isolated bacteria from marine specimens.

other groups of specimens. Numbers of all bacterial isolates obtained from different media and their isolating sources are shown in Figure 5. From the results, most of these isolates (91) were isolated from medium D. This might be explained due to the addition of $FeSO_4 \cdot 7H_2O$ in the medium, which was not found in the other media. Because the iron is known to be limiting factor in seawater and it is also an essential element required for growth of microbial cells (Tortell *et al*., 1999). The majority of the cellular Fe was found in the respiratory chain associated with many enzymes such as NADH-Q reductase, succinate-Q dehydrogenase, cytochrome b1, and cytochrome oxidase complexs. It was well known that many marine bacteria produced Fe-biding siderophores for iron acquisition systems, which was transported to the bacterial cells via specific cell-surface receptor. The medium such as marine agar 2216 (Difco) that has been widely used for cultivation of marine bacteria contained Fe.

It can be concluded that the serial dilution technique was the most suitable method for isolation of general bacteria. By means of a serial dilution method, the bacteria found in specimens were dispersed and suspended in broth medium. When the bacterial suspension was placed on the culture medium, numerous bacterial colonies were formed on agar plate. In contrast, direct plating technique only bacteria that found on the edge of specimens and moved fast could form the colonies. Therefore, a low numbers of bacteria could be seen on the culture media.

2.2 Isolation of gliding bacteria

Gliding bacteria, total of 56 isolates were obtained from various samples as shown in Figure 6. It can be concluded that seaweed was the good source of gliding bacteria.

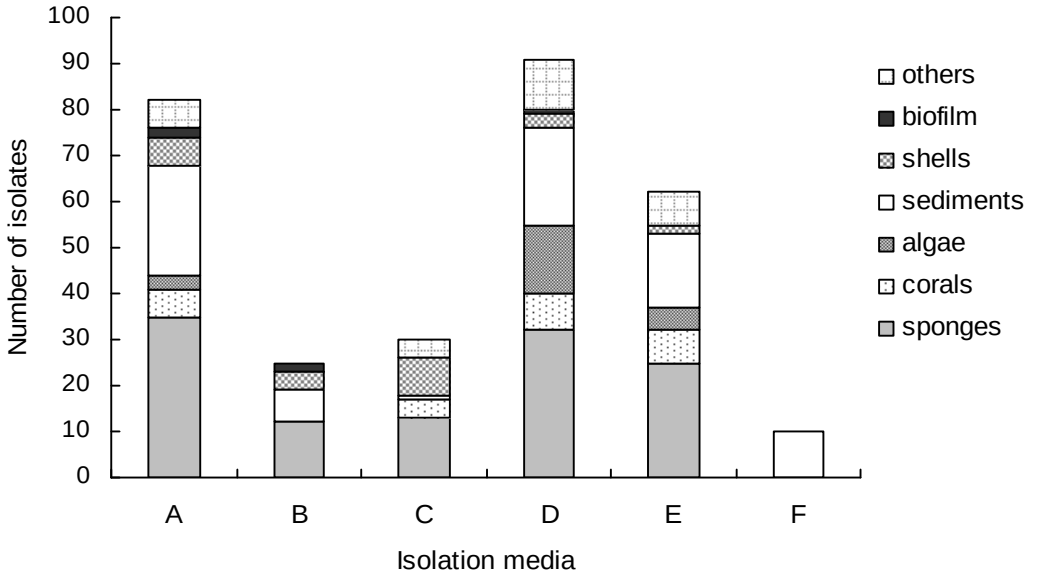


Figure 5. Numbers of marine bacterial isolates obtained from different media compositions and their sources.

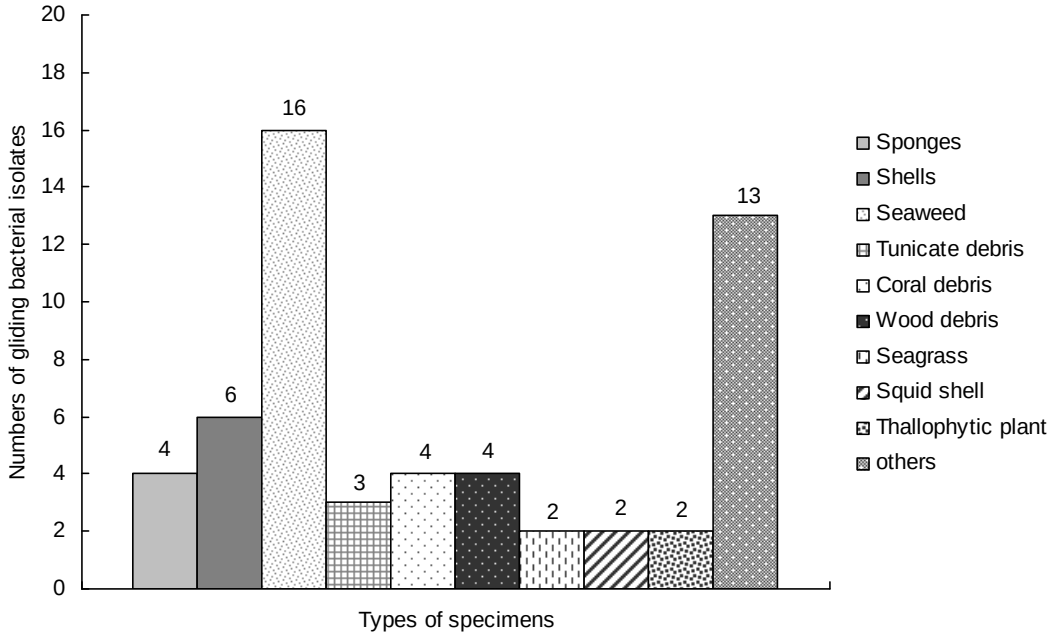


Figure 6. Numbers of gliding bacterial isolates obtained from marine specimens.

3. Preparation of marine bacterial extracts

Approximately 199 extracts were obtained from general marine bacteria of 50 isolates. The numbers of extracts that obtained from media A, B, C, D, E, and G were 28, 50, 17, 5, 50, and 49 extracts, respectively. In case of marine gliding bacteria, a total of 101 extracts were obtained from marine gliding bacteria of 56 isolates. Among the extracts, 55 extracts were obtained from skim milk media, 25 extracts obtained from glutamate media and 21 extracts obtained from glycerol media.

4. Screening of marine bacteria producing antimicrobial compounds

4.1 Optimal conditions for antimicrobial assay in a primary screening

Using appropriate cutoff level of reduction time for selecting optimal microbial inocula, the results are summarized in Table 18.

The results showed that the maximal concentration of DMSO (5%) could affect the growth of test strains. However, the effects of DMSO would decrease as incubation progress. This study found that at a level of 2.5 % DMSO no inhibitory effect on growth of test microorganisms was found. Therefore, the suitable amount of DMSO used for sample preparation should not exceed 2.5% .

4.2 Screening of marine bacterial extracts for antimicrobial activity

A total of 300 bacterial extracts were tested for antimicrobial activity against *Staphylococcus aureus* TISTR 517, *Pseudomonas aeruginosa*, *Aspergillus fumigatus* TISTR 3108, *Candida albicans* TISTR 5239 and *Sacharomyces cerevisiae*. Antimicrobial activity of active extracts are shown in Figure 7 (A). The results showed that the percentage of marine gliding bacterial extracts active against *S. aureus* TISTR 517, *C. albicans* TISTR 5239 and *S. cerevisiae* were 9.9, 5.94 and 3.96 %, respectively. In case of general bacterial extracts, the extracts showed inhibitory effect on growth of test strains against *S. aureus* TISTR 517, *C. albicans* TISTR 5239 and *S. cerevisiae* as 3.52, 4.02 and 3.52%, respectively. In addition, all extracts (300 extracts) had no inhibitory activity against *Pseudomonas aeruginosa* and *Aspergillus fumigatus* TISTR 3108 at the concentration tested (300 µg/ml for general bacterial extracts and 150 µg/ml for gliding bacterial extracts). It was found that the percentage of active marine gliding bacterial extracts against prokaryotic cells, eukaryote cells and both were 9.90, 5.94 and 4.95, respectively (Figure 7 B) while the general bacterial extracts were 3.52, 7.54 and

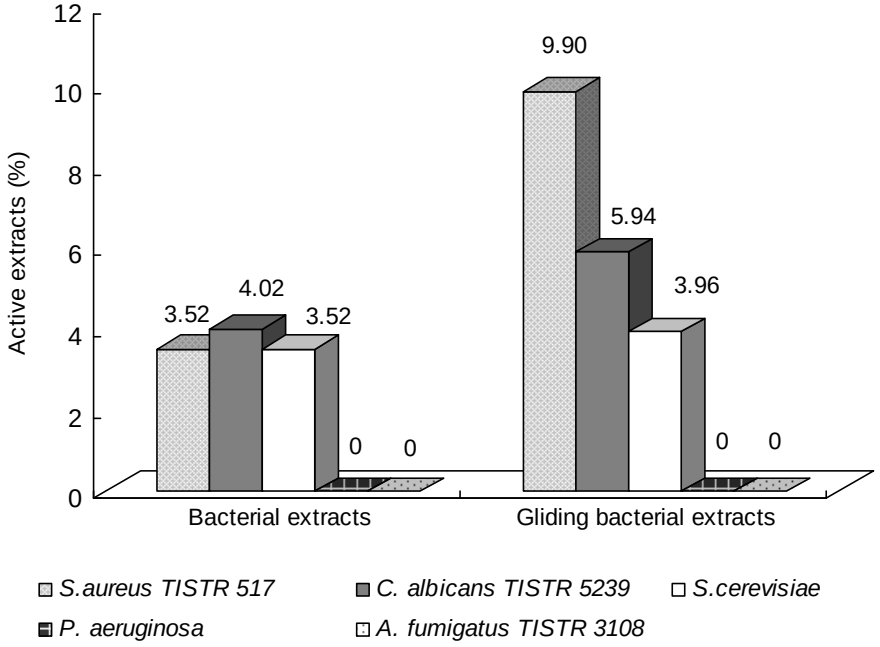
Table 18. Summary of AlamarBlue reduction time of test microorganisms in RPMI 1640 medium at 37 °C and effect of DMSO on their growth and antimicrobial susceptibility.

Test microorganisms	Inoculum size (cells,spores/ml)	Reduction time of AlamarBlue (h)	DMSO conc. (%)[a]	Antimicrobial susceptibility [b] (μg/ml)
S. aureus TISTR 517	4.5×10^5	8-10	5	0.78
P. aeruginosa	4.0×10^6	18-20	5	25
C. albicans TISTR 5239	1.0×10^5	10	5	0.39
S. cerevisiae	5.0×10^5	18-20	5	1.56
A. fumigatus TISTR3108	1.0×10^6	24	5	1.56

[a] Inhibitory concentration of DMSO on growth of microorganisms.

[b] Antimicrobial compounds used for antimicrobial susceptibility testing : Vancomycin for bacteria and amphotericin B for yeast and fungi.

A



B

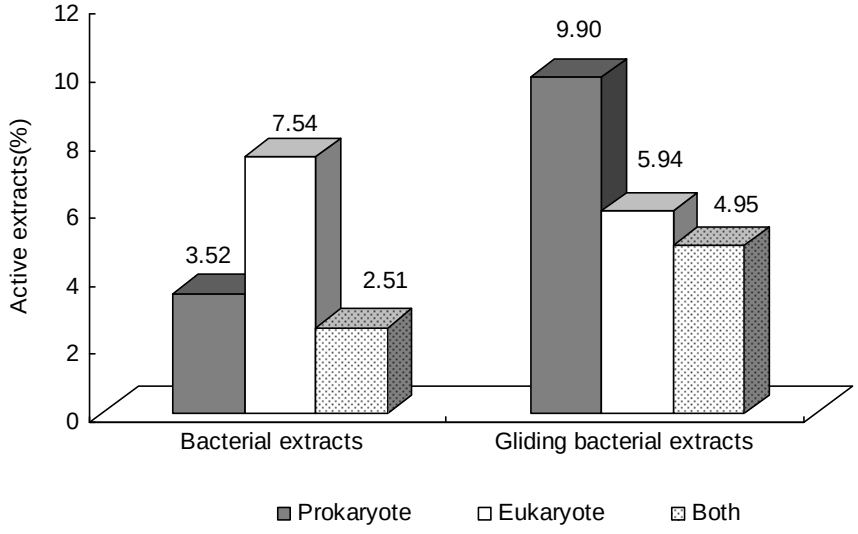


Figure 7. **A** : Percentage of active extracts against test microorganisms.

B : Percentage of active extracts classified by type of tested microorganisms.

2.51%, respectively. This indicated that the marine gliding bacteria represented potential sources of antimicrobial compounds.

4.2.1 Effect of different media on production of antimicrobial compounds

Most of gliding bacterial extracts were active against *S. aureus* with a percentage of 16.36. Based on the results shown in Table 19, skim milk medium gave good results for antimicrobial production medium by marine gliding bacteria whereas other media such as glycerol medium and glutamate medium were not suitable.

For general bacteria, it was found that medium E yielded the highest percentage of active extracts as shown in Table 20.

In this study, the low nutrient media were not successful for the production of antimicrobial compounds by both marine gliding bacteria and general bacteria. This may be explained by the fact that types of nutrients were not suitable and/ or culture conditions. Many factors influenced the production of antimicrobial compounds by marine bacteria. For example, Saha *et al.* (2005) found that natural seawater stimulated antibiotic production of a marine Gram-positive bacterium belonging to *Actinobacteria.* For a novel marine bacterium *Streptomyces psammoticus*, it was found that the best carbon and nitrogen source for growth and antibiotic production were glucose and ammonium nitrate, respectively (Sujatha *et al*., 2005). Moreover, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ concentrations could affect antibiotic production by this strain. Trischman *et al*., (2004) found that competitive conditions influenced the production of antibiotic by marine bacteria.

Table 19. Effect of different media on numbers of active gliding bacterial extracts prepared from 56 isolates.

Cultivating media	Total of extracts tested	No. of active extracts	% of active extracts [a]	No. of active extracts against microorganisms [b]		
				SA	CA	SC
Skim milk medium*	55	10	18.18	9	6	4
Glutamate medium**	25	0	0	0	0	0
Glycerol medium***	21	1	4.76	1	0	0
Total	101	11	-	10	6	4

[a] Percentage of active extracts per total extracts obtained from each media.

[b] No activity against *Pseudomonas aeruginosa* and *Aspergillus fumigatus* TISTR 3108.

SA: *Staphylococcus aureus* TISTR 517, CA: *Candida albicans* TISTR 5239,

SC: *Saccharomyces cerevisiae.*

* 1 isolate did not grow.

** 31 isolates did not grow.

*** 35 isolates did not grow.

Table 20. Effect of different media on numbers of active general bacterial extracts.

Fermentation media	Total of extracts tested	No. of active extracts	% of active extracts [a]	No. of active extracts against microorganisms [b]		
				SA	CA	SC
A	28	0	0	0	0	0
B	50	5	10	2	3	2
C	17	1	5.9	0	1	0
D	5	0	0	0	0	0
E	50	10	20	4	4	5
G	49	1	2.0	1	0	0

[a] Percentage of active extracts per total extracts.

[b] No activity against *Pseudomonas aeruginosa* and *Aspergillus fumigatus* TISTR 3108.

SA: *Staphylococcus aureus* TISTR 517, CA: *Candida albicans* TISTR 5239,

SC: *Saccharomyces cerevisiae.*

4.2.2 Characteristics of antimicrobial producing strains

General marine bacteria used in primary screening are shown in Table 21. In this study, it was found that antimicrobial compound producing strains were Gram- negative bacteria (Table 22).

Table 21. Numbers of marine bacteria used in primary screening.

Source	No. of isolates tested	No. of isolates	
		Gram-negative	Gram-positive
Sponges	27	23	4
Corals	6	6	0
Shells	6	6	0
Sediments	4	2	2
Algae	1	0	1
Others	6	5	1
Total	50	42	8

Table 22. Characteristics of antimicrobial producing marine bacterial strains.

Source	No. of active isolates	Shape	Gram 's staining
Sponge	6	Rod/short rod, Coccus	Negative
Coral	4	Rod/short rod, Coccus	Negative
Shell	4	Rod/short rod	Negative

5. Acetylcholinesterase inhibitory activity

It was found that there were 7 active extracts (Table 23) showed strong AChE inhibitory activity. The biological activities of marine gliding bacterial extracts are summarized in Table 24.

Table 23. Screening for acetylcholinesterase inhibitory activity of crude marine bacterial extracts

Fermentation media	No. of extract tested	No.(%) of active extract [a]
Skim milk medium	55	7 (12.73)
Glutamate medium	16	0
Glycerol medium	19	0

[a] Extract with percent acetylcholinesterase inhibition of $\geq$ 70 (for details see Table 24).

Acetylcholinesterase is a regulatory enzyme that plays critical roles in cholinergic transmission by hydrolyzing the excitatory transmitter acetylcholine. Therefore, Acetylcholinesterase inhibitors are the main target for the treatment of Alzheimer's disease (AD) Currently; the drugs used for the treatment of this disease have not been decided. Therefore, the search of acetylcholinesterase inhibitor is of great interest. Most of the studies focused on screening and isolation of acetylcholinesterase inhibitors from plant materials (Ingkaninan *et al*., 2003; Rollinger *et al*., 2006).

6. Cytotoxicity activity

The cytotoxicity of marine bacterial extracts was evaluated. It is interesting to note that non of the extracts showed cytotoxicity. This may due to the fact that the media used for culture were not suitable for the production of cytotoxic compounds.

Table 24. Antimicrobial activity and acetylcholinesterase inhibitory activity of active marine gliding bacterial extracts.

Sample code	Antimicrobial activity [a]			Acetylcholinesterase inhibitory activity (%) [b]
	SA	CA	SC	
GB 005 sk	+	+		74.14 ± 3.18
GB005 gly	+			16.07 ± 6.47
GB008 sk	+			63.95 ± 3.04
GB 009* sk	**+**	**+**		**81.32 ± 2.56**
GB010 sk	+	+	+	79.58 ± 4.76
GB011 sk	+			69.40 ± 5.61
GB014 sk				77.13 ± 0.93
GB016 sk		+	+	77.49 ± 0.88
GB 022* sk	**+**	**+**	**+**	**90.70 ± 2.13**
GB 023 sk	+	+	+	76.24 ± 7.06
GB 032 sk	+			50.07 ± 4.17
GB 036 sk	+			63.85 ± 3.41

[a] Antimicrobial activity of extracts was determined at concentration of 150 µg/ml.

SA: *Staphylococcus aureus* TISTR 517, CA: *Candida albicans* TISTR 5239,

SC: *Saccharomyces cerevisiae.*

[b] Values are expressed as mean ± S.D. (*n*=3).

Sk : Skim milk medium; gly : glycerol medium . * Selected strains for further study.

7. Morphological and some physiological properties of selected marine gliding bacteria

7.1 Characterization and identification of the marine gliding bacterial strains GB 009 and GB 022

Morphological and physiological characterizations of marine gliding bacteria producing marinoquinoline A were determined by cultivation on SAP II agar using seawater. A swarm colony was observed, indicating gliding motility (Figure 8). The morphology of the strains GB 009 and GB 022 under scanning microscope (SEM) are shown in Figure 9. It was shown that the marine gliding bacteria were filament-shaped cells and had rounded ends. The cell size of the strain GB 009 was 0.4-0.5 µm in width and 10-15 µm in length while the cell size of the strain GB 022 was 0.3-0.4 µm in width and 10-15 µm in length. Some characteristics of both strains were determined. It was found that both strains were Gram-negative, oxidase–positive and catalase-negative. In addition, they required seawater and NaCl for growth. Details of seawater and NaCl requirement of both strains were given below.

The identification of the marine gliding bacteria strain GB 009 and GB 022 was performed on the basis of 16S rRNA gene sequences analyses. The strains GB 009 and GB 022 showed 99 % 16 S rRNA gene sequences (1400 bp/1406 bp, and 1401 bp/ 1406 bp, for the strain GB 009 and GB 022 , respectively) similarity to the strains of *Rapidithrix thailandica* gen. nov., sp. nov. (Srisukchayakul *et al*., 2007). The full length of DNA sequences of the strain GB 009 and GB 022 shown in Appendix 1 and 2, repectively.

7.2 Natural seawater requirement

Effect of natural seawater (32 ppt) at different concentrations prepared in SAP II agar media on the growth of the selected marine gliding bacteria was observed. The growth of gliding bacteria was evaluated on the basis of the spreading of colony on agar media. The radius of swarm colony was measured after incubation at 30 °C for 24 and 48 h. The results are shown in Figure 10. The strain GB 009 showed the fastest growth on the medium containing 100 % seawater in comparison with the others. On the medium with deionized water, a small radius of swarm colony of the strain GB 009 was observed. This may be explained by the fact that the seawater salts might diffuse from the piece of culture agar due to the strain grown on SAP II agar prepared by 100 % natural seawater before it was cut and placed on the SAP II agar with DI water. There was no difference on the growth of the strain on media prepared from 60 %

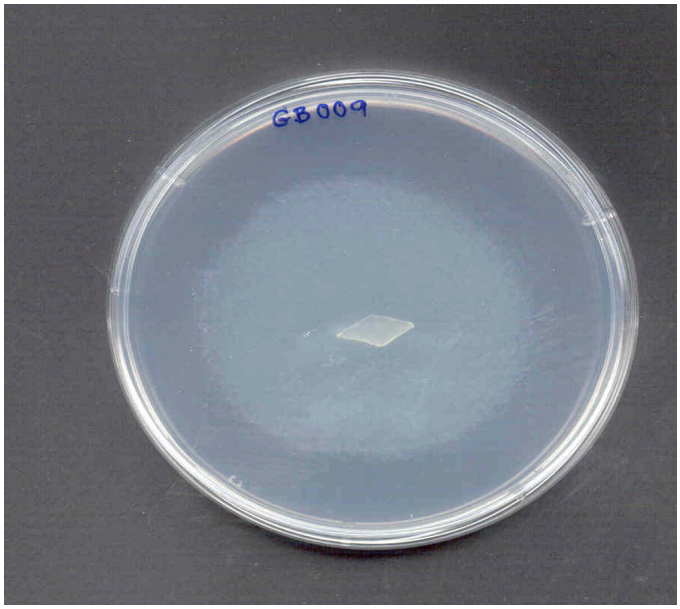


Figure 8. Growth of marine gliding bacteria on SAP II agar prepared using seawater at 30°C for 48 h. The strain produced swarm colony during growth.

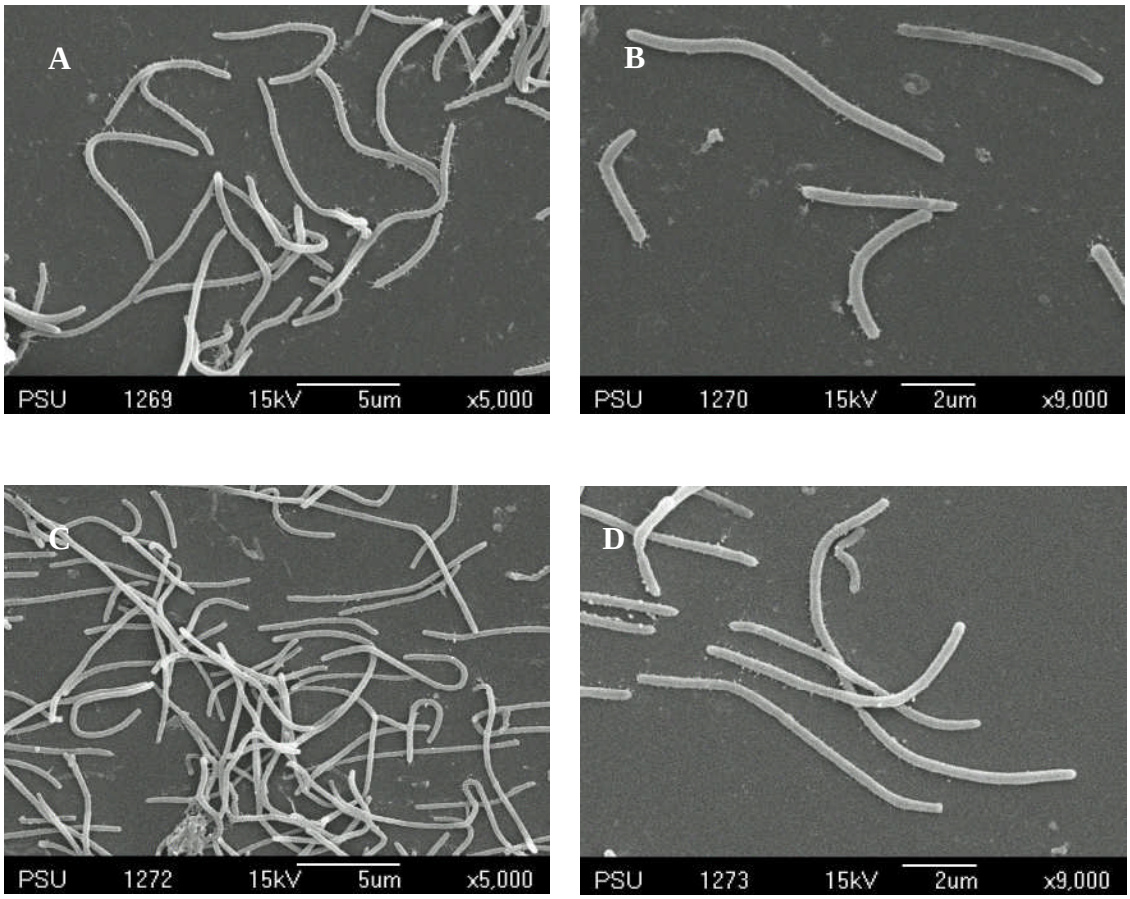
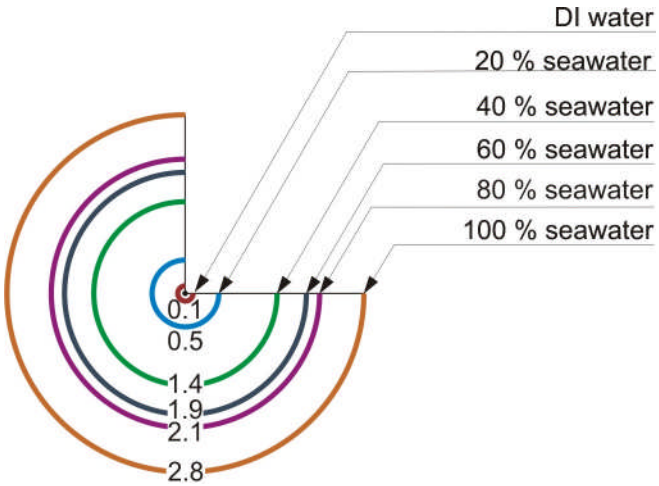


Figure 9. Scanning electron micrographs of marine gliding bacteria GB 009 (A, B) and GB 022 (C, D) aged 48 h on SAP II agar prepared using seawater at 30 °C.

A



B

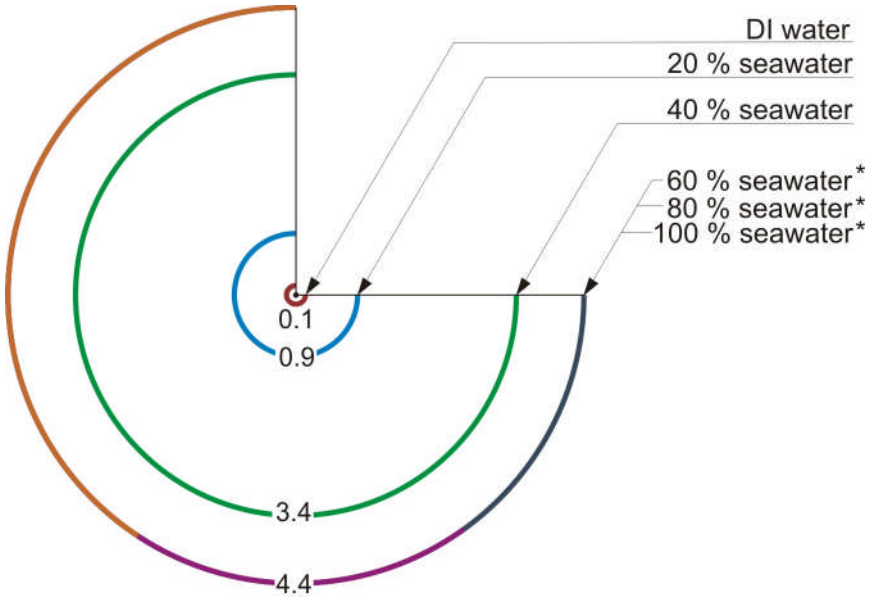


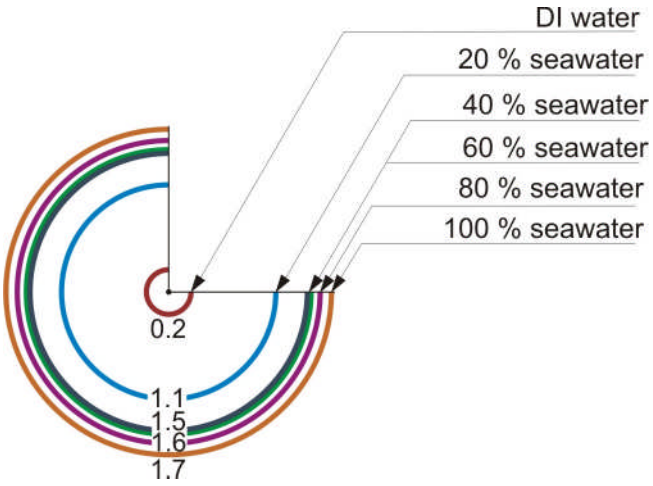
Figure 10. Effect of seawater on growth of the marine gliding bacterial strain GB 009 on SAP II agar at room temperature after 24 h (A) and 48 h (B). Values are expressed as average data from triplicate experiments (cm.). * Swarm colony ends at the edge of agar plate.

seawater or 80% seawater after 24 h of incubation. After 48 h of incubation, the radius of swarm colony of the strain GB 009 on the media with seawater in a range of 60%-100% was not measured since the swarm colony covered the entire plate. The strain GB 022 also required seawater for growth (Figure 11). Moreover, it showed that the strain GB 022 grew well on the media with 40 % seawater after 48 h of incubation. However, based on the statistical analysis, there was no difference in the growth of the strain GB 022 on SAP II agar prepared with seawater in a range of 40% - 100%.

7.3 NaCl requirement

The growth of the two strains was also observed on SAP II agar containing different concentrations of NaCl after 24 and 48 h of incubation at 30 °C. The NaCl requirements of the strains GB 009 and GB 022 on SAP II agar are shown in Figure 12 and 13, respectively. From the results, the growth of strain GB 009 increased with the increased NaCl concentration up to 3 %, and then decreased until the NaCl concentration was 6%. No growth was observed on the media with NaCl concentration at 8 % or more. This observation was consistent with the seawater requirement of the strain GB 009 showing the optimal growth on the medium with 100 % seawater (32 ppt). In case of the strain GB 022, maximum growth was found on medium containing 2% NaCl. Slight growth of the strain GB 022 was observed on the medium with 6 % NaCl. In addition, no growth occurred on medium with NaCl at 8% or more. However, the statistical analysis showed that there was no difference in growth of the strain GB 022 on the media with 6 % NaCl and 8 % NaCl. The pattern of NaCl requirement of the strain GB 022 showed that this strain required lower NaCl concentration for growth than the strain GB 009. The NaCl requirement of the strain GB 022 was consistent with its seawater requirement observed on the media with the seawater concentration of at least 40%. The NaCl requirement of the two strains provided supportive evidence of the seawater requirement of both because the NaCl was the main salt of seawater. Obviously, the growth of the strains on media with seawater was faster than that on media with NaCl, indicating that the substitution of seawater by the addition of NaCl could not entirely replace seawater for cultivation of marine gliding bacteria strains GB009 and GB 022. Based on these observations, it was concluded that both strains were halophilic marine gliding bacteria.

A



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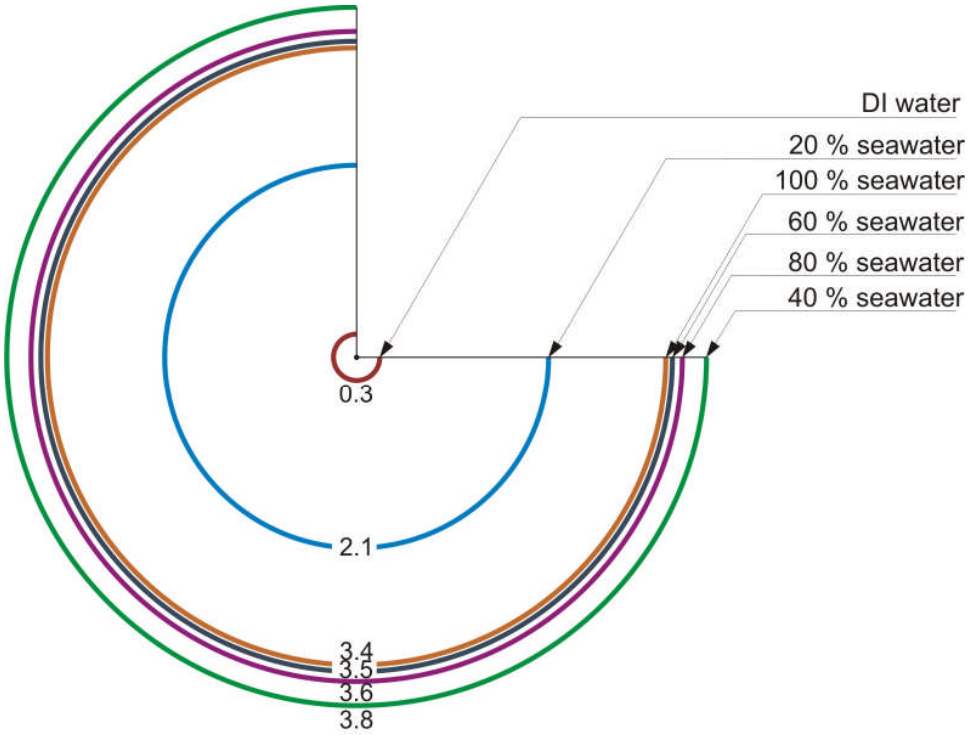
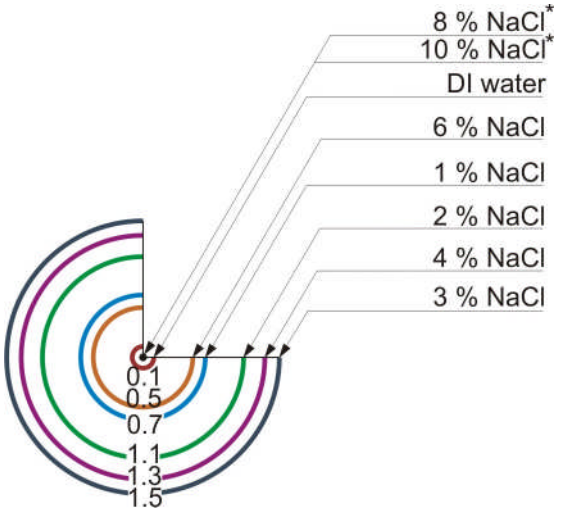


Figure 11. Effect of seawater on growth of the marine gliding bacterial strain GB 022 on SAP II agar at room temperature after 24 h (A) and 48 h (B). Values are expressed as average data from triplicate experiments (cm.).

A



B

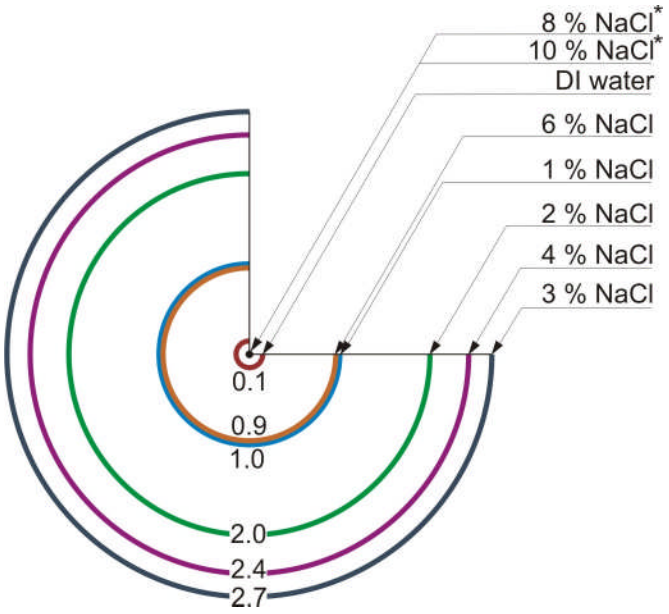
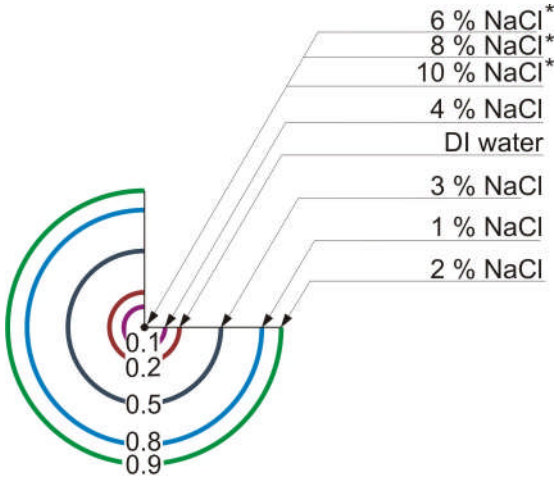


Figure 12. Effect of NaCl on growth of the marine gliding bacterial strain GB 009 on SAP II agar at room temperature after 24 h (A) and 48 h (B). Values are expressed as average data from triplicate experiments (cm.). * No growth was observed.

A



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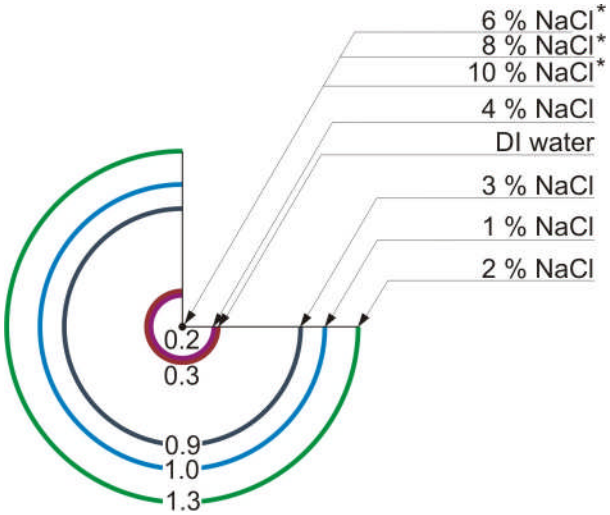


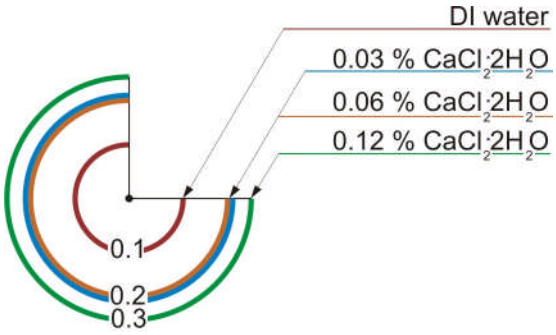
Figure 13. Effect of NaCl on growth of the marine gliding bacterial strain GB 022 on SAP II agar at room temperature after 24 h (A) and 48 h (B). Values are expressed as average data from triplicate experiments (cm.). * No growth was observed.

In other work on marine gliding bacteria, the growth of a new marine gliding bacterium, *Zobellia amurskyensis*, obtained from seawater occurred at 1-6 % NaCl with optimum at 2 % NaCl (Nedashkovskaya *et al*., 2004b). Similar observations were found on a new marine gliding bacterium *Algibacter lectus*, which was isolated from the green algae, the growth occurred at 1-6 % NaCl (Nedashkovskaya *et al*., 2004c) and a new genus and species of gliding bacterium, *Reichenbachia agariperforans* isolated from seawater sample (Nedashkovskaya *et al*., 2003c). In contrast, the growth of a novel genus and species of marine gliding bacterium *Formosa algae*, obtained from the thallus of the brown algae, occurred at 0-5 % NaCl (Ivanova *et al*., 2004b). Since bacteria which were isolated from marine environments could be classified as halophilic bacteria or halotolerant bacteria, therefore, the NaCl or seawater requirement was variable among the different species. In moderate halophilic bacteria, these properties depended on growth temperature and the composition of nutrients (Ventosa *et al*., 1998). In complex media, some organisms were presented as halotolerant rather than halophilic. In minimal media some required salt for growth like a halophile.

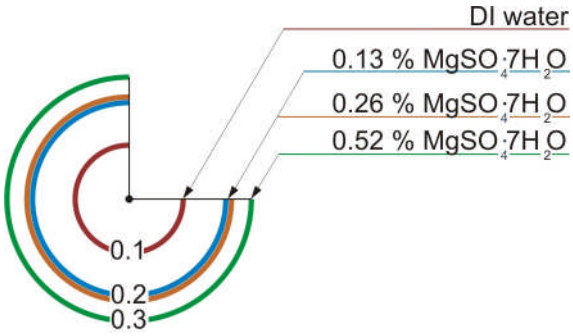
7.4 Effect of Mg^{2+}, Ca^{2+}, and K^{+} on growth of marine gliding bacteria

Besides NaCl, the main salts of seawater also contained Mg^{2+}, Ca^{2+}, and K^{+}. Therefore, the effect of these main salts on growth of marine gliding bacteria GB 009 and GB 022 was observed. The growth of the strains was observed on SAP II agar containing $MgSO_4 \cdot 7H_2O$ in a range of 0.13-0.52%, $CaCl_2 \cdot 2H_2O$ in a range of 0.03-0.12 % and KCl in a range of 0.02-0.08 %. The growth of the strains on media containing different concentrations of Ca^{2+}, Mg^{2+}, and K^{+} are shown in Figure 14, for the strain GB 009 and Figure 15 for the strain GB 022. It was found that the strains showed slight growth on media containing all of these ions. However, Ca^{2+}, Mg^{2+}, and K^{+} ions at the maximal concentration could support the growth of the strain GB 009. There was no significant difference in the growth of this bacterium on media containing three level concentrations of all elements in this study. In case of marine gliding bacterium strain GB 022, this bacterium showed no difference in growth on the media with three level different concentrations of Ca^{2+}, compared with the growth on media with deionized water after 48 h of incubation. It was suggested that Ca^{2+} ($CaCl_2 \cdot 2H_2O$) at a high concentration of 0.12 % could not support the growth of the strain GB 022. $MgSO_4 \cdot 7H_2O$ concentration with a range of 0.13-0.52 % could support growth while KCl could support growth at a concentration of

A



B



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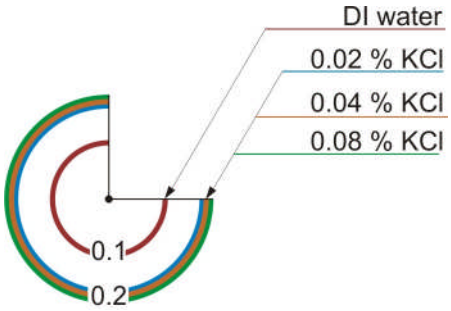
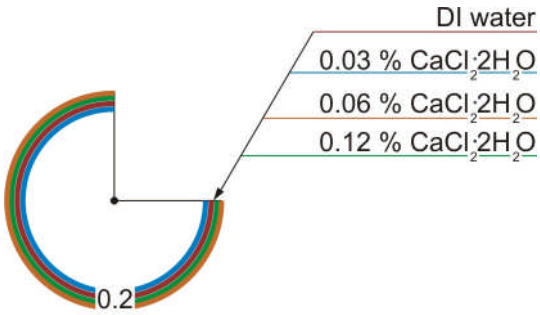


Figure 14. Effect of $CaCl_2 \cdot 2H_2O$ (A), $MgSO_4 \cdot 7H_2O$ (B) and KCl (C) on growth of the marine gliding bacterial strain GB 009 on SAP II agar at room temperature after 48 h. Values are expressed as average data from triplicate experiments (cm.).

A



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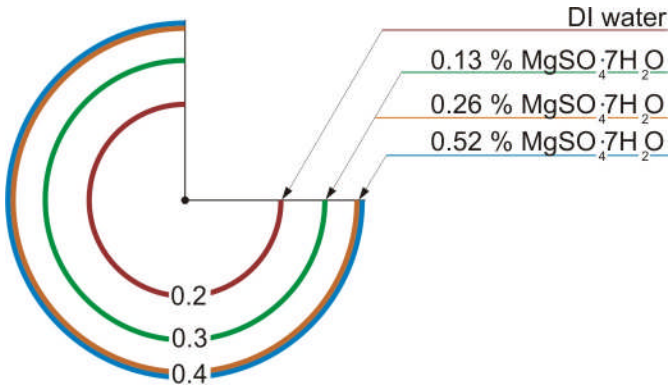

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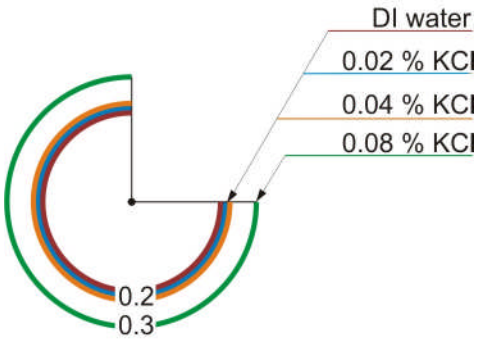


Figure 15. Effect of $CaCl_2 \cdot 2H_2O$ (A), $MgSO_4 \cdot 7H_2O$ (B) and KCl (C) on growth of the marine gliding bacterial strain GB 022 on SAP II agar at room temperature after 48 h. Values are expressed as average data from triplicate experiments(cm.).

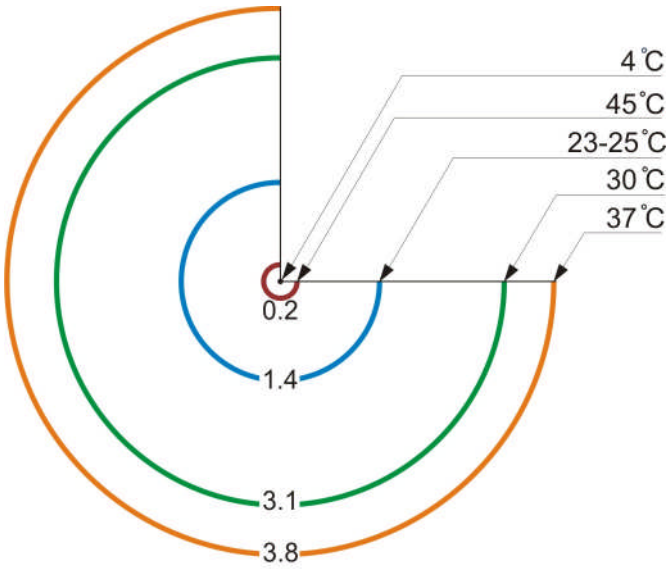
0.08 %. However, small radius was found from the edge of colony in the experiments. These observations indicated that the marine gliding bacteria required more concentrations of these ions for supporting their optimal growth. These were more than 0.52 % $MgSO_4 \cdot 7H_2O$, 0.12 % $CaCl_2 \cdot 2H_2O$ and 0.08 % KCl. Normally, Mg^{2+}, Ca^{2+}, and K^+ are present in seawater at a concentration of 0.129, 0.041, and 0.039 g/100 g of seawater (Karleskint *et al*., 2006). However, the main reason was probably due to the lack of NaCl in medium. Compared with the growth of the strains on media with NaCl or seawater after 24-48 h of incubation, the growth of both strains on media with these factors was obvious. These results indicated that NaCl was the most effective factor in seawater to support the growth of marine gliding bacteria. Moreover, seawater contained many kind of dissolved organic compounds, elements and others that necessary for the growth of marine bacteria.

No growth occurred in the absence of NaCl in the medium. The important role of Na^+ of marine bacteria was found in various cellular functions. For example, in the transport processes in the cell membrane, solute retention, flagella motility, and primary Na^+ pump (MacLeod, 1986; Kogure, 1998). Among the three type of the primary Na^+ pump that have been identified i. e. respiration dependent, decarboxylase-driven and Na^+ ATP synthase, the respiration-dependent primary Na^+ pump played a major role in the marine bacteria (Kogure, 1998). In addition, the marine bacterium *V. angustum* S14, isolated from coastal seawater , was demonstrated that it required Na^+ for growth and starvation survival whereas the *V. anguillarum* M93 which was isolated from diseased fish, *Plecoglossus altivelis* (Salmoniforms) in Lake Biwa, Japan, required Na^+ for only starvation survival (Fujiwara-Nagata and Eguchi, 2004).

7.5 Effect of temperature on growth of marine gliding bacteria

The growth of the marine gliding bacteria GB 009 and GB 022 was observed on SAP II agar medium with 100 % natural seawater (32 ppt) at different temperatures. After 24 and 48 h of incubation, the radius of swarm colony was measured. The results of experiments for the strains GB 009 and GB 022 are given in Figure 16 and 17, respectively. From the results, it can be concluded that the GB 009 had an optimal growth at 37 °C. The growth was not observed after 48 h incubation since there was vast growth of the GB 009 at 30 and 37°C. The slight growth was observed at 45°C while the growth did not occur at 4°C. The similar results were observed in the marine gliding bacterium GB 022 where optimal growth was found at 37°C,

A



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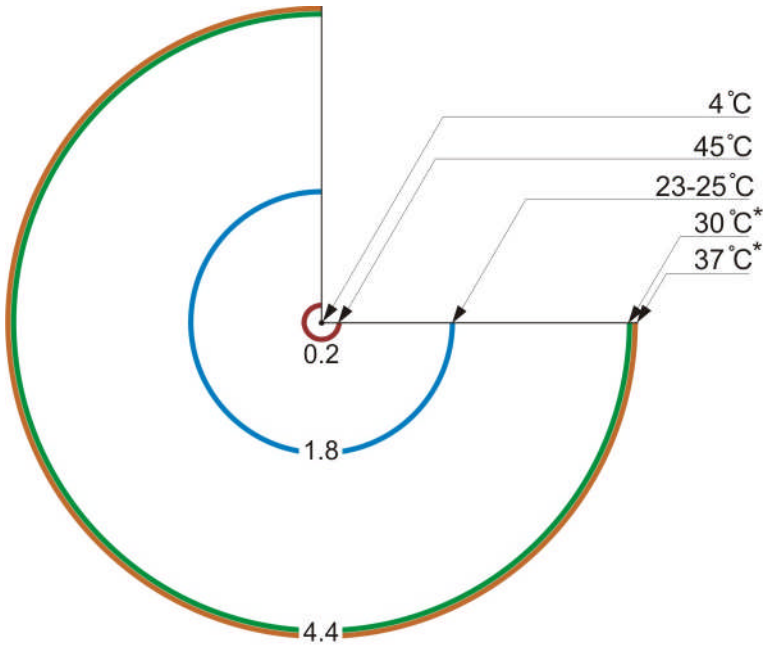
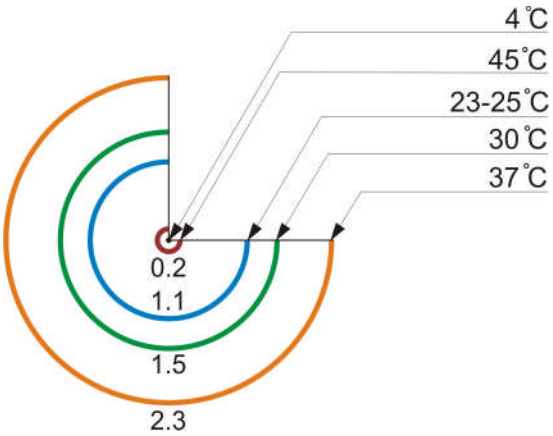


Figure 16. Effect of temperature on growth of the marine gliding bacterial strain GB 009 on SAP II agar at room temperature after 24 h (A) and 48 h (B). Values are expressed as average data from triplicate experiments (cm.). * Swarm colony ends at the edge of agar plate.

A



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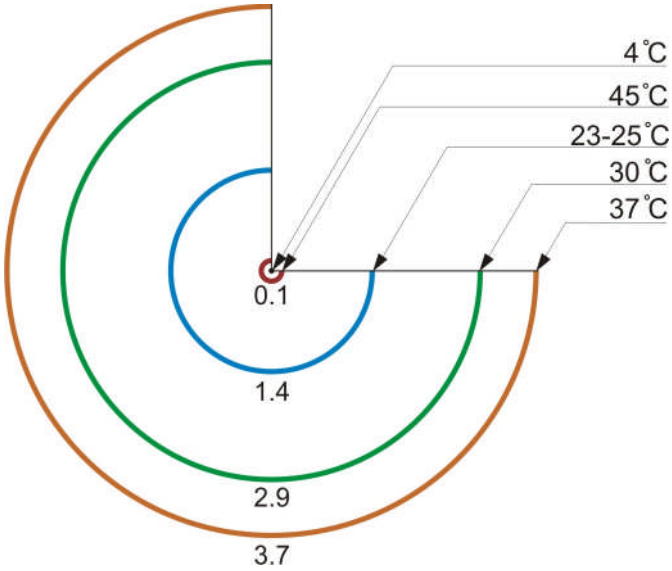


Figure 17. Effect of temperature on growth of the marine gliding bacterial strain GB 022 on SAP II agar at room temperature after 24 h (A) and 48 h (B). Values are expressed as average data from triplicate experiments (cm.).

no growth at 4°C and very slight growth at 45°C, on the medium with 100% natural seawater (~3.2% NaCl). Data obtained from several studies demonstrated that temperature for optimal growth of marine gliding bacteria varied from strain to strain. The *Algibacter lectus*, which was isolated from green algae collected from Sea of Japan, had an optimal temperature at 21-23 °C , while it grew at 4-35°C (Nedashkovskaya *et al*., 2004c). The marine gliding bacterium *Reichenbachia agariperforans*, isolated from seawater collected from Sea of Japan, grew at temperature ranging 4-35 °C and had optimal growth at 28°C (Nedashkovskaya *et al*., 2003c). The *Zobellia galactanovorans*, isolated from a red alga on the sea-shore of Roscoff, Brittany, France, grew between 13 and 45°C, with an optimal around 35°C, while no growth was observed at 10 or 46°C (Barbeyron *et al*., 2001). This difference probably depended on their sources or habitats.

In this study, some main characteristics of the marine gliding bacteria GB 009, isolated from unidentified seaweed collected from Yong Ling beach, Trang and the strain GB 022, isolated from unidentified shell collected from Jaosamran beach, Pechaburi, could be concluded as follow: the strains were Gram-negative bacteria filament-shaped, oxidase positive but negative for catalase. The strains were moderate halophilic bacteria since they required seawater and /or NaCl for growth. Optimal NaCl concentration of growth was 3 % for GB 009 and 2 % for GB 002. Growth occurred well at 37°C on medium with seawater (32 ppt). K^{+}, Mg^{2+} and Ca^{2+} could support growth of the strain GB 009, while K^{+}, Mg^{2+} support growth of the strain GB 022. Summary of some main characteristics of both strains are given in Table 25.

Table 25. Some main characteristics of marine gliding bacterial strains GB 009 and GB 022.

Feature	GB 009	GB 022
Information		
Isolation source	unidentified seaweed	unidentified shell
Location	Yong Ling beach/ Trang	Jaosamran beach/ Pechaburi
Characteristics		
Gram stain	negative	negative
Morphology	filament	filament
Size (μm) (aged 48 h on SAP II agar)	0.4-0.5x10-15	0.3-0.4x10-15
Gliding motility	+	+
Colony mass	cream-colored	cream -colored
Oxidase test	+	+
Catalase test	-	-
Sea water requirement	+	+
NaCl requirement	+	+
NaCl optimum (%)	3	2
No growth at NaCl (%)	8	6*
Temperature growth (°C) (on medium with seawater 32 ppt)		
4 °C	No growth	No growth
37 °C	Optimal	Optimal
45 °C	Slight growth	Slight growth
Phylogenetic analyses (16S rRNA gene sequences)	99% similarity to *Rapidithrix thilandica* gen. nov., sp. nov.	99% similarity to *Rapidithrix thilandica* gen. nov., sp. nov.

* Very slight growth with no significant difference in growth compared at 8% NaCl concentration, which no growth was observed.

Part II: Purification and Chemical Structure Elucidation of Bioactive Compounds from the Selected Marine Gliding Bacteria

1. Isolation of bioactive compound from the marine gliding bacterial strain GB 009

1.1 Preparation of bacterial extracts

The preliminary test was performed for preparing bacterial extracts obtained from the fermentation broth of the bacteria by using Amberlite XAD 16 resin (Fluka). Amberlite XAD 16 is a polystylene adsorbent supplied as insoluble white beads. It is a nonionic, hydrophobic, cross linked polymer. Therefore, the resin is appropriate for the adsorption of organic substances from polar fermentation broth. The ethyl acetate-soluble extract (F1) was removed from resin. The resin was then soaked in methanol (F2). The dired extract of ethyl acetate fraction was dissolved with small amount of ethyl acetate to give a fraction designed as F1.1 and the remaining extract was eluted with methanol. This fraction was designed as F1.2. Each fraction was dried with a rotary evaporator to give a red brown crud extract. From the results (Table 26), of 3600 ml of fermentation broth yielded 283 mg for F1.1, 31.3 mg for F1.2; 676 mg for F2.1 and 452.5 for F2.2. Biological activity testing demonstrated that F1.1 and F1.2 were the active fractions with antimicrobial activity and acetylcholinesterase inhibitory activity. The fraction F1.1 exhibited acetylcholinesterase inhibition of 82.50 % while the fraction F1.2 showed activity of 64.72 %. These results revealed that Amberlite XAD 16 resin was an efficient adsorbent for recovering compounds from the fermentation broth of marine gliding bacteria. In addition, the elution of the harvested resin with EtOAc gave good results in biological activity.

The use of methanol as eluent in soaking process yielded higher amount of crude extracts since it can elute more polar compounds from resins. No biological activity was found in the fractions of F 2. Therefore, the fraction F1.1 and F1.2 were the fractions of interest for further chemical investigation. Since small amount of dried extract of fraction F2 and the main compound of F1.2 was also found in F1.1, based on TLC background, therefore, fraction F1.2 could be combined with F1.1 before the isolation process was carried out.

The technique using XAD 16 resins as adsorbent for recovering metabolites compounds from the bacterial fermentation broth have been used, for example, in work of myxobacteria (Böhlendorf *et al*., 1999; Jansen *et al*., 2000) and a marine gliding bacterium

Table 26. Biological activity of fractions obtained from fermentation broth (3600 ml) of marine gliding bacterial strain GB 009.

Fraction	Weight	Antimicrobial activity[a]			AChE inhibition (%)
	(mg)	SA	CA	SC	
F1.1	283	+/+	+/+	$+^{w}/+^{w}$	82.50
F1.2	31.3	+/+	+/-	+/-	64.72
F2.1	676	-	-	-	18.41
F2.2	452.5	-	-	-	20.42

[a] Antimicrobial activity in each fraction were tested at a concentration of 308.64 /154.32 μg/ml, respectively.

SA: *Staphylococcus aureus* TISTR 517; CA: *Candida albicans* TISTR 5239;

SC: *Saccharomyces cerevisiae.*

Saprospira grandis (Spyere *et al*., 2003). In addition, it has been cited that the addition of XAD 16 resin can increase the yield of soraphane and epothilone produced by a myxobacterium *Sorangium cellulosum* Soce 26 (Gerth *et al*., 2003). The presence of the resin in the broth of the cultures resulted in a continuous accumulation of the excreted metabolites because it prevents feed back inhibitions. Moreover, it was also suggested that XAD 16 resins could reduce the cost of down-stream processing (Gerth *et al*., 2003).

1.2 Purification of bioactive compound

The isolation and purification of the crude extract is shown in Figure 18, 19 and 20.

2. Chemical structure elucidation of bioactive compound

The structure of marinoquinoline A was elucidated by an analysis of ^{1}H NMR (Figure 21) ,^{13}C NMR (Figure 22) as well as of 2D NMR data, including COSY, HMBC, HMQC as shown in Figure 23, 24 and 25, respectively. In addition, the numbers of attached protons on

GB 009 crude extract

1070.6 mg

SA +/+/- CA +/+/- SC +/+/+ PA -/-/- AF -/-/-

AChE inhibition 85.42 %

Sephadex LH 20 : eluted with MeOH

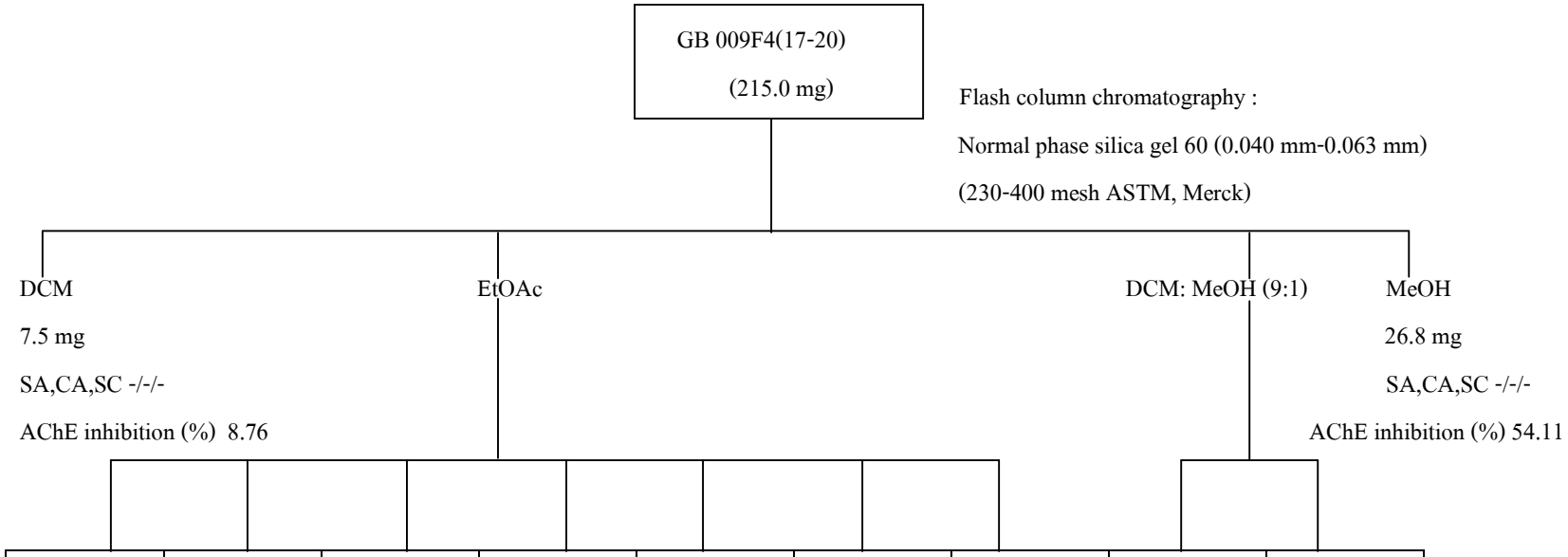
Flow rate 0.5 ml/ min, 10 ml/fraction

F 1 (1-6)	F 2 (7-14)	F 3 (15-16)	F 4 (17-20)	F 5 (21-29)	F 6 (30-35)	F 7 (36-63)	F 8 (64-74)	F 9 (75-85)	F 10 (86-99)	F 11 (100-113)	F 12 (114)
172.7 mg	155.0 mg	122.6 mg	217.7 mg	150.2 mg	45.6 mg	90.7 mg	3.4 mg	4.9 mg	4.1 mg	1.9 mg	3.5 mg
SA -/-/-	$+^{w}$/-/-	$+^{w}$/-/-	+/+/+	+/+/+	+/+/+	$+^{w}$/-/-	$+^{w}/+^{w}$/-	-/-/-	-/-/-	-/-/-	-/-/-
CA -/-/-	-/-/-	-/-/-	+/+/+	$+/+/+^{w}$	$+/+^{w}$/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
SC -/-/-	-/-/-	$+^{w}$/-/-	+/+/+	+/+/+	$+/+^{w}$/-	-/-/-	$+^{w}$/-/-	-/-/-	-/-/-	-/-/-	-/-/-
AChE inhibition (%)											
18.04	54.63	71.50	85.41	90.07	79.04	38.30	34.19	26.10	31.01	44.43	24.43

: SA: *Staphylococcus aureus* TISTR 517, CA: *Candida albicans* TISTR 5239, SC: *Saccharomyces cerevisiae*, PA: *Pseudomonas aeruginosa*, AF : *Aspergillus fumigatus* TISTR 3018

:Antimicrobial activity; + : positive result, + : negative result

Figure 18. Isolation scheme of crude extract from marine gliding bacterial strain GB 009.



F4 (17-20)/1	F4 (17-20)/2	F4 (17-20)/3	F4 (17-20)/4	F4 (17-20)/5	F4 (17-20)/6	F4 (17-20)/7	F4 (17-20)/8	F4 (17-20)/9
14.1 mg	18.2 mg	8.7 mg	6.6 mg	5.4 mg	48.4 mg	19.5 mg	0.4 mg	6.3 mg
SA $+^{w}$/-/-	-/-/-	-/-/-	-/-/-	+/+/$+^{w}$	+/+/+	+/$+^{w}$/-	-/-/-	-/-/-
CA -/-/-	-/-/-	-/-/-	-/-/-	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
SC -/-/-	-/-/-	-/-/-	-/-/	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
AChE inhibition (%)								
47.01	45.58	57.67	74.17	98.76	99.06	95.01	82.38	79.3

Figure 19. Isolation scheme of fraction F4(17-20).

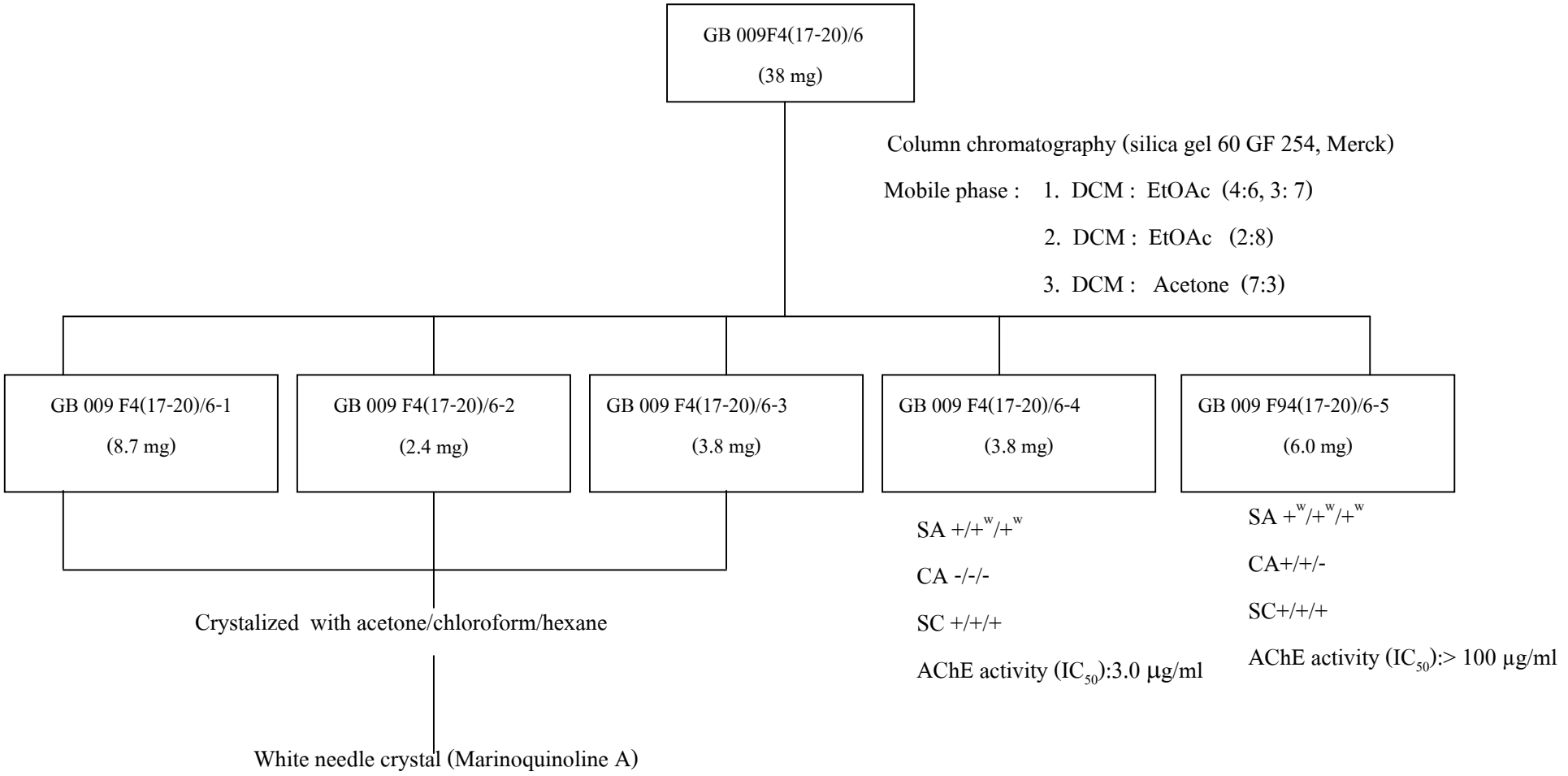


Figure 20. Isolation scheme of marinoquinoline A from the fraction F4 (17-20)/6.

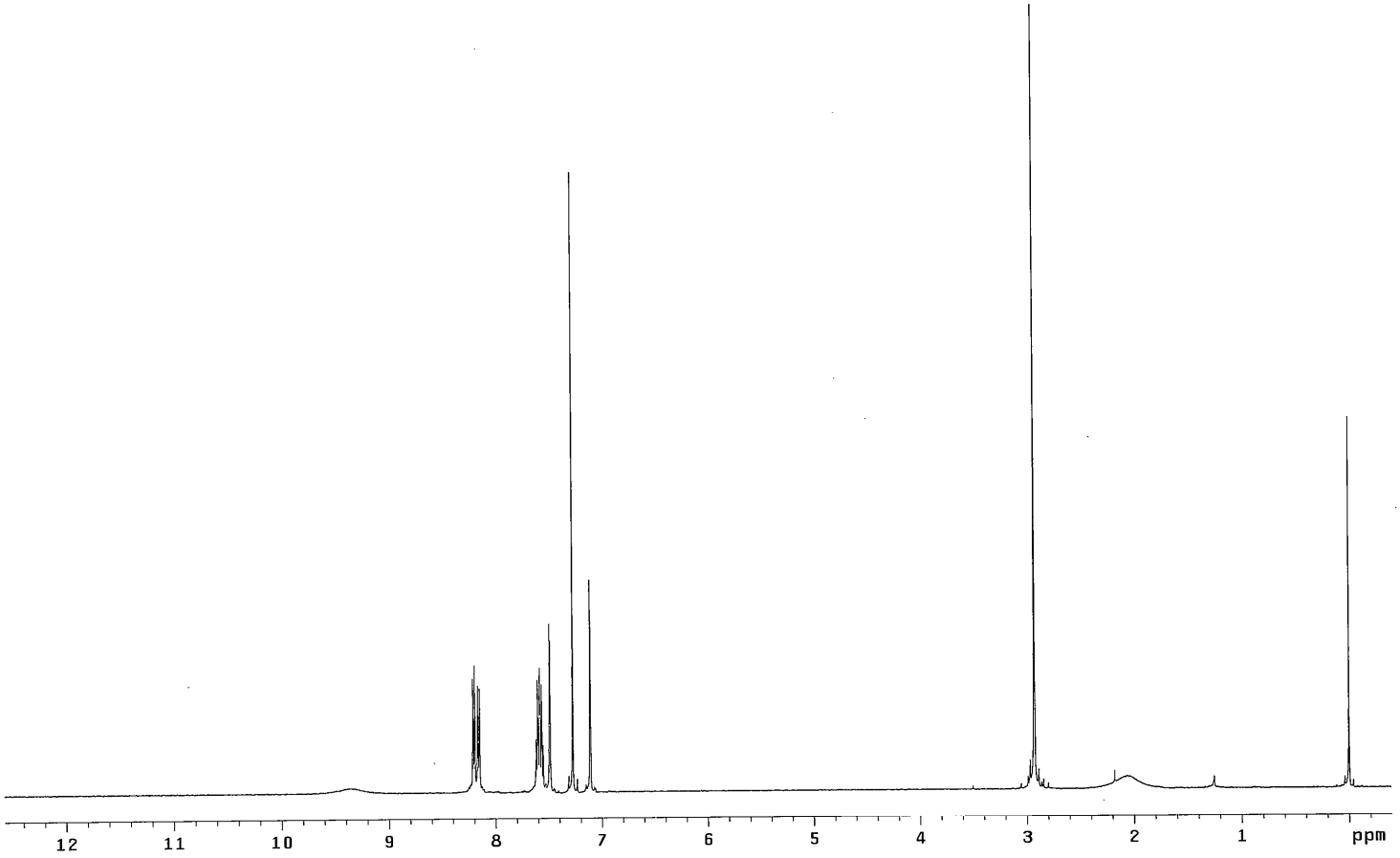


Figure 21. ^{1}H NMR spectrum of marinoquinoline A (500 MHz in $CDCl_3$).

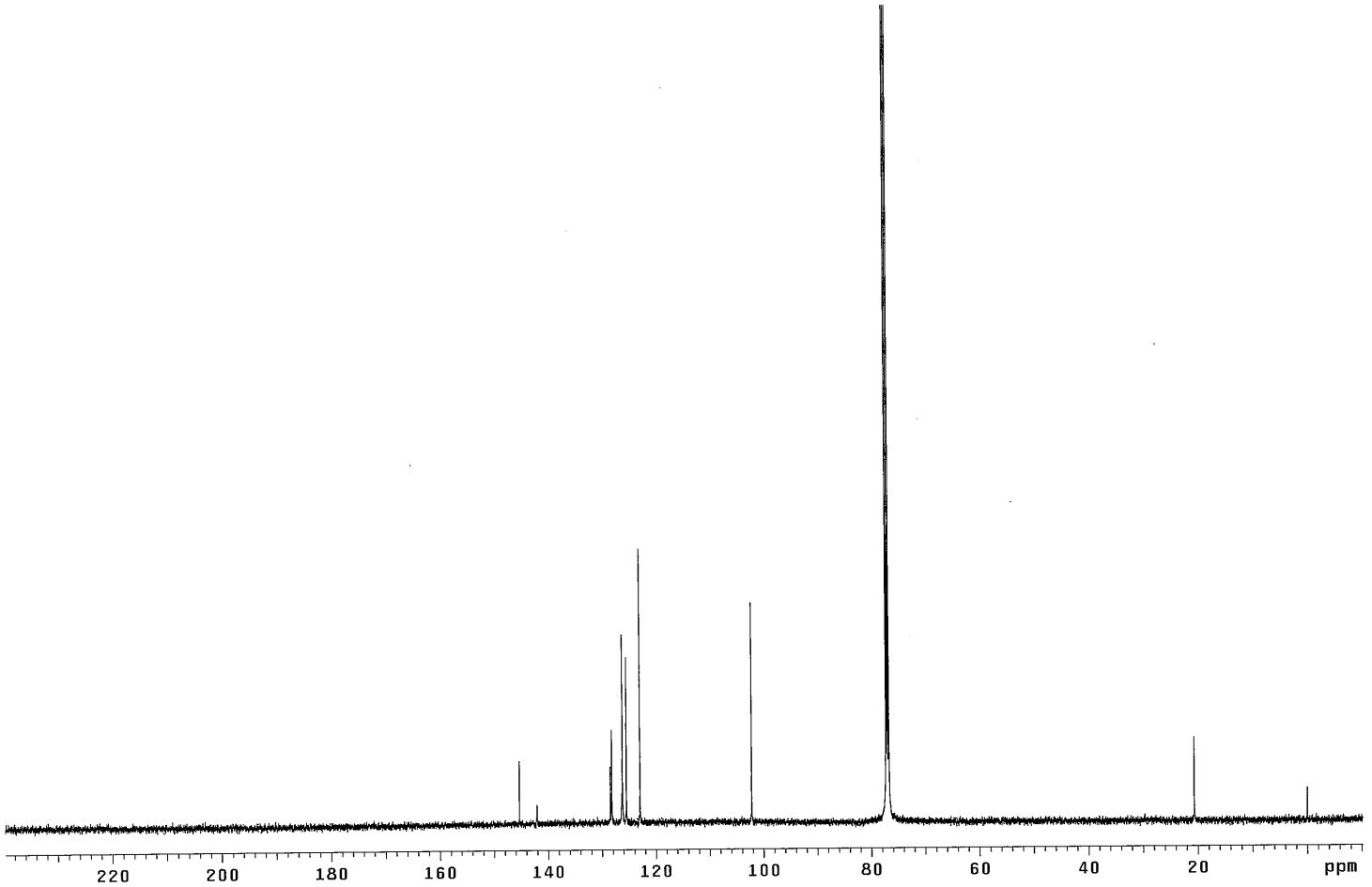


Figure 22. ^{13}C NMR spectrum of marinoquinoline A (125 MHz in $CDCl_3$).

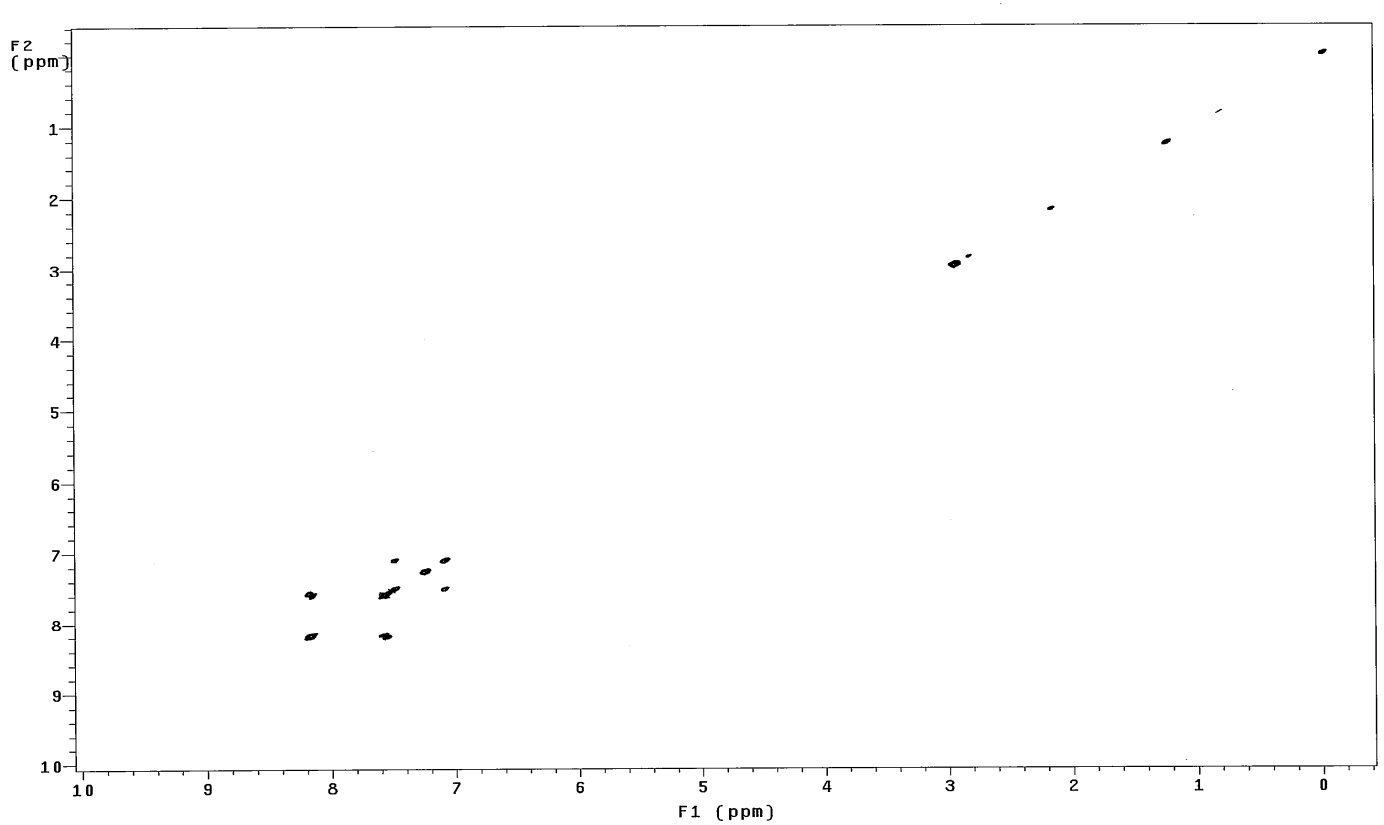


Figure 23. ^{1}H-^{1}H COSY spectrum of marinoquinoline A (500MHz in $CDCl_3$).

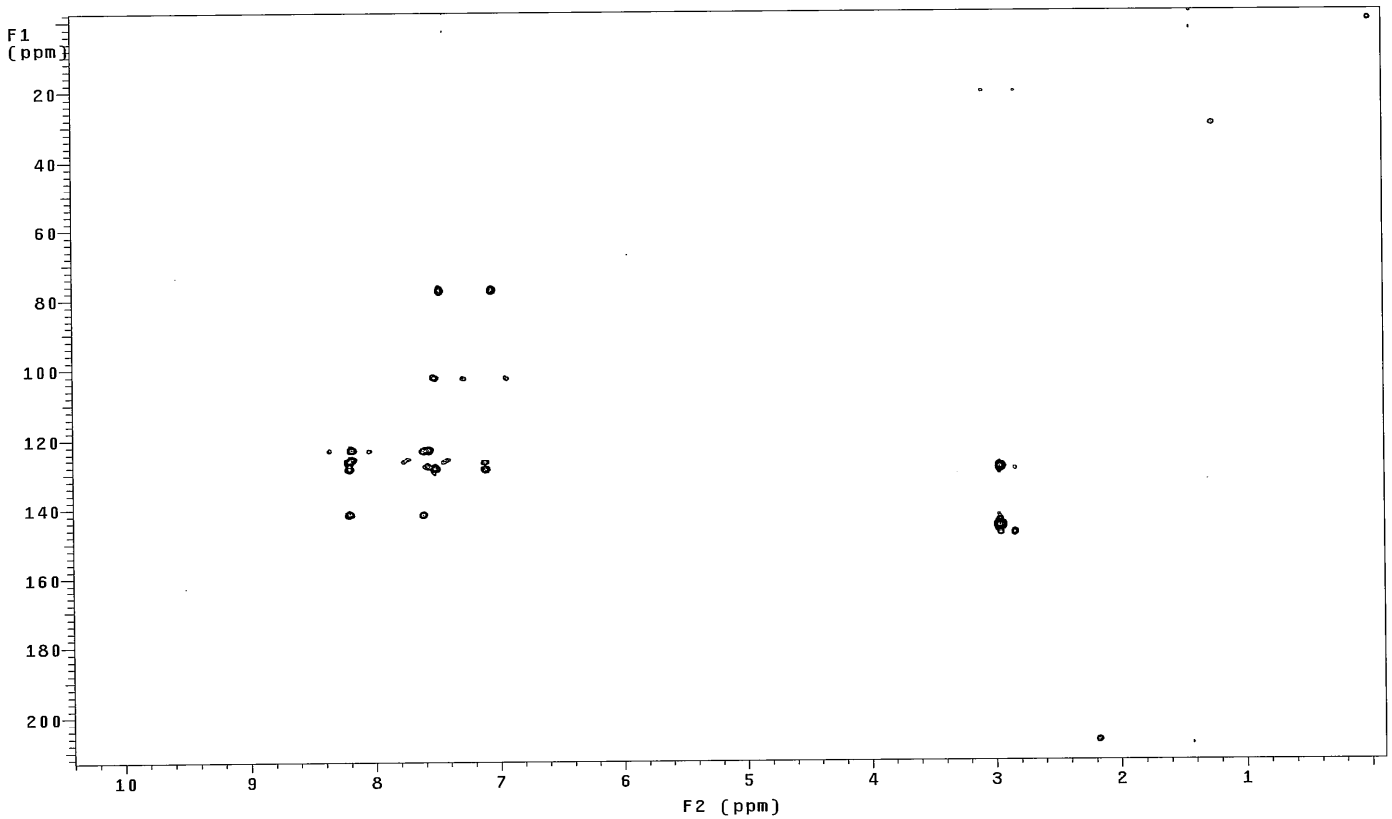


Figure 24. HMBC spectrum of marinoquinoline A (500MHz in $CDCl_3$).

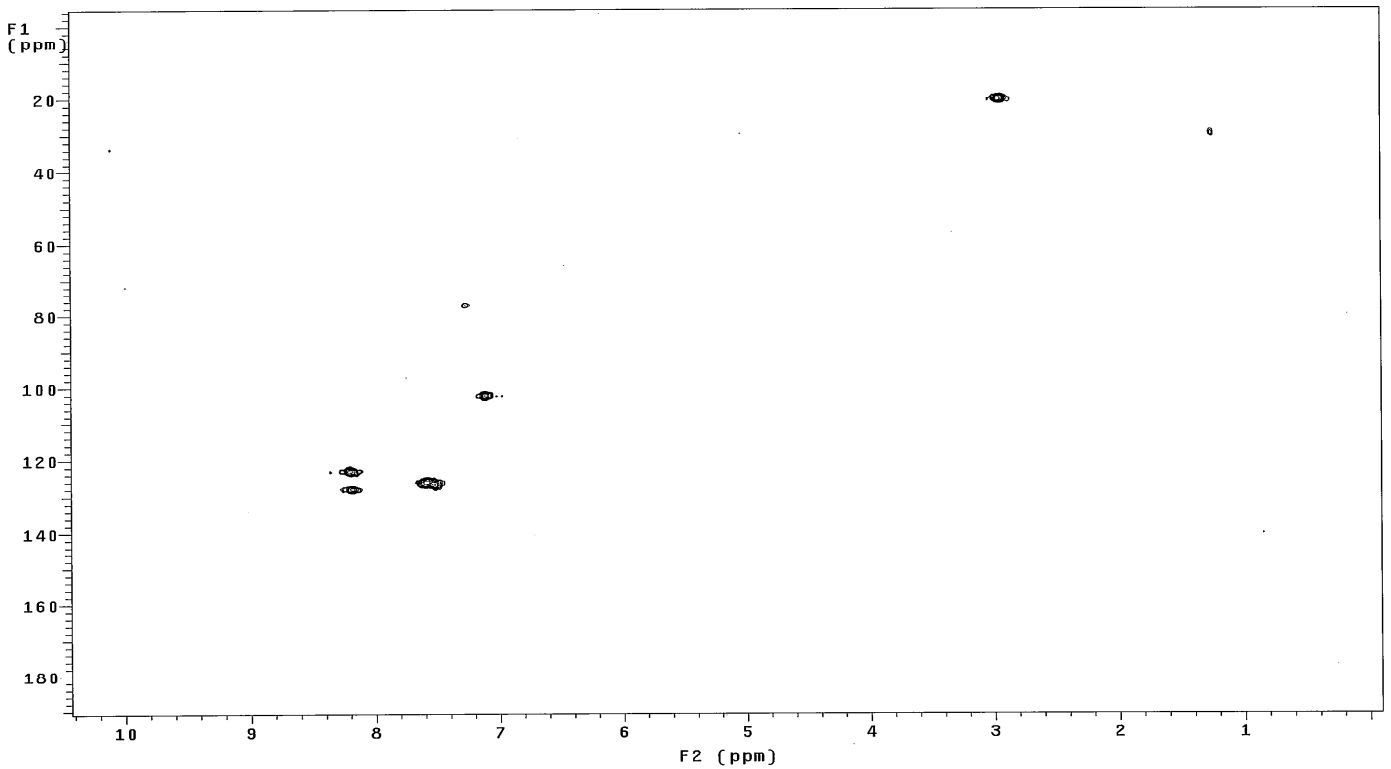


Figure 25. HMQC spectrum of marinoquinoline A (500MHz in $CDCl_3$).

carbon atoms were determined through DEPT experiments. Finally, an X ray crystallographic technique was used for the confirmation of the structure. (Kanjana-Opas, 2006). The X ray derived structure for marinoquinoline A is shown in Figure 26. The structure of the 4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline (marinoquinoline A) is shown in Figure 27.

The ^{1}H NMR spectrum of marinoquinoline A showed 10 proton. The one broad singlet at δ 9.3(1H) was observed indicating that the hydrogen could be attached to nitrogen. The ^{13}C NMR spectrum and HMQC experiment as well as DEPT experiment revealed 12 carbons which included five quaternary carbons, six proton attached carbons and one methyl group. Results of the ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC correlations of marinoquinoline A is shown in Figure 28. In the ^{1}H-^{1}H COSY connectivity recorded, the hydrogen signals at δ 8.20 (dd, J = 8.0 and 1.5 Hz) was coupled to the hydrogen at δ 7.56 and the hydrogen at δ 7.61 was coupled to the hydrogen at δ 8.16 (dd, J = 8.0 and 1.0 Hz). Moreover, the hydrogen at δ 7.48 (d, J = 3.0 Hz) was coupled to the hydrogen at δ 7.10 (d, J = 3.0 Hz). DEPT 90 and DEPT 135 experiments of the compound are shown in appendix 3 and 4, respectively. The UV spectrum of marinoquinoline A is shown in Figure 29. The spectrum displayed maximal short wave length absorption at 203.5 nm and maximal long wave length absorption at 239.5 and 300.5 nm.

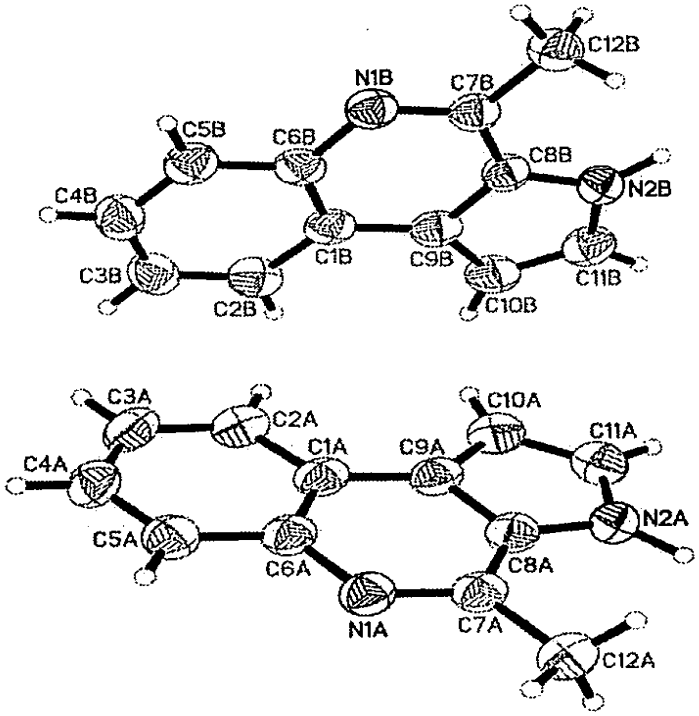


Figure 26. Crystal structure of marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline), hydrogen atoms have been omitted for clarity.

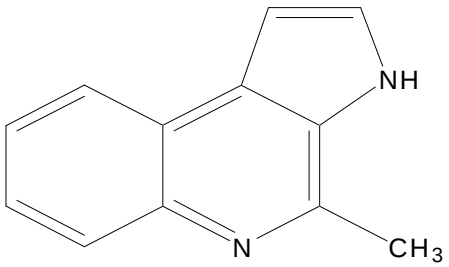


Figure 27. Structure of marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline).

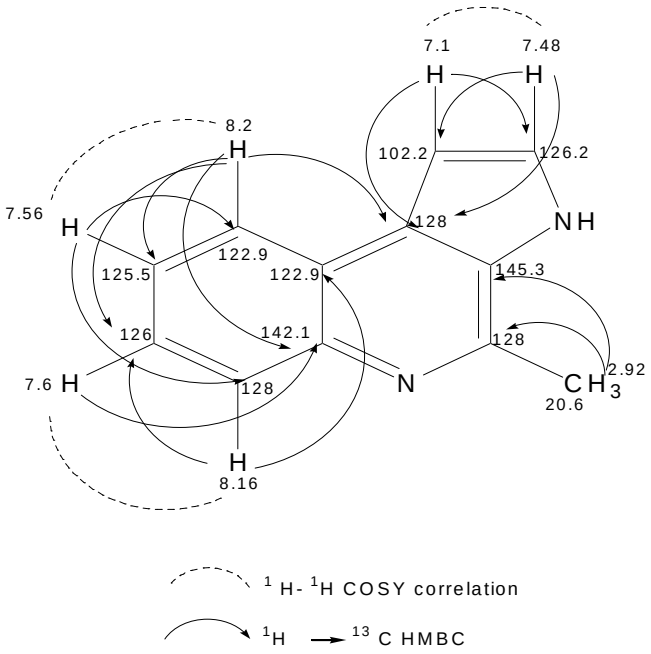


Figure 28. ^{1}H-^{1}H COSY and ^{1}H -^{13}C HMBC correlations of marinoquinoline A (4- methyl-3 *H* –pyrrolo [2,3-*c*] quinoline).

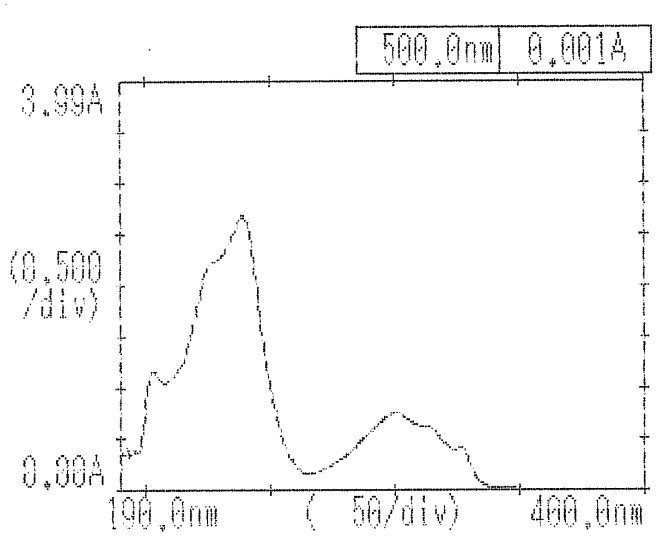


Figure 29. UV spectrum of marinoquinoline A (4- methyl-3 *H* – pyrrolo[2,3-*c*] quinoline) in methanol.

3. Biological activity of marinoquinoline A

Marinoquinoline A was evaluated for biological activities including antimicrobial activity, AChE inhibitory activity, and cytotoxicity as shown in Table 27 and 28.

The susceptibilities of tested fungi and bacteria to antimicrobial agents are shown in Table 29 and 30, respectively (appendix 5).

In summary, biological activities of the new compound, marinoquinoline A cloud be concluded that this compound was significant acetylcholinesterase inhibitor with moderate antifungal activity and no significant cytotoxicity.

Table 27. Antimicrobial activity of marinoquinoline A (4-methyl-3*H*-pyrrolo[2,3-*c*] quinoline).

Microorganisms	MIC (µg/ml)
Fungi	
Candida albicans TISTR 5239	18.52
Saccharomyces cerevisiae	9.26
Aspergillus fumigatus TISTR 3108	74.07
Aspergillus flavus TISTR 3041	74.07
Bacteria	
Staphylococcus aureus TISTR 517	74.07
Methicillin-resistant *Staphylococcus aureus*	74.07
Bacillus subtilis	74.07
Bacillus cereus	74.07
Escherichia coli	148.15
Pseudomonas aeruginosa	No activity at 148.15

Table 28. Acetylcholinesterase inhibitory activity and cytotoxicity of marinoquinoline A (4-methyl-3*H*-pyrrolo[2,3-*c*] quinoline).

Compound	AChE inhibition (IC_{50}, μM)	Cytotoxicity against human cancer cell lines (IC_{50}, μg/ml)			
		MCF-7	HT-29	Hela	KB
Marinoquinoline A	4.9[a]	>5	>5	>5	>5
Galanthamine	0.59 ± 0.14[b]	ND	ND	ND	ND

[a] Value are expressed as average data (n = 2).

[b] Value are expressed as mean ±S.D. (n = 3).

ND : Not determine.

CHAPTER 4

CONCLUSION

The isolation of marine bacteria from various specimens on different media, most isolates (39 %) were obtained from sponge samples, a major group of specimens. Serial dilution method was appropriate method for isolation of marine bacteria

Antimicrobial activity of marine bacterial extracts was investigated using a colorimetric microdilution broth assay with AlamarBlue. The percentage of active extracts from common bacteria exhibited inhibitory activities against *Staphylococcus aureus* TISTR 517, *Candida albicans* TISTR 5239 and *Saccharomyces cerevisiae* at the concentration of 308.64 µg/ml was 3.52, 4.02 and 3.52%, respectively, while the crude extracts of gliding bacteria (150 µg/ml) had the higher percentages of active extracts of 9.9%, 5.94% and 3.96%, respectively. The media composition and their concentrations influenced the production of antimicrobial compounds by marine bacteria. Skim milk medium yielded good results for the production of bioactive compounds by gliding bacteria, especially an inhibitor of AChE. In case of general marine bacteria, most of active extracts were obtained from mannitol containing medium (Medium E).

It was found that marine gliding bacterial extracts had acetylcholinesterase (AChE) inhibitory activity with the percentage value of 7.78%. The crude extracts from the strains GB 009 and GB 022 had high AChE inhibitory activity with percent inhibition of 81.32 ± 2.56 and 90.70 ± 2.13, respectively. Cytotoxicity activity of marine bacterial extracts was determined. It showed that they were not active against four human cell lines including breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human cervix carcinoma (Hela) and human mouth epidermoid carcinoma (KB).

This study revealed that marine gliding bacteria represented an important source of active compounds. Therefore, the marine gliding bacteria strain GB 009 and the strain GB 022 were selected for characterization and their chemical investigation. The gliding bacterial strains GB 009 and GB 022 were Gram-negative bacteria with filamentous-shape, oxidase positive but

negative for catalase. The two strains were obligate marine bacteria since they required seawater and /or NaCl for growth. Optimal NaCl concentrations of growth were found at 3 % for the strain GB 009 and 2 % for the strain GB 022. Growth occurred well at 37 °C on media with seawater (32 ppt). The presence of $MgSO_4 \cdot 7H_2O$ (0.52%), $CaCl_2 \cdot 2H_2O$ (0.12 %) and KCl (0.08 %) supported growth of the GB 009, whereas K^+, Mg^{2+} supported growth of the strain GB 022. The 16S rRNA gene sequences of the strains GB 009 and GB 022 was found to be closely related to *Rapidithrix thailandica* gen. nov., sp. nov. with sequences identity of 99 % (1400 bp/1406 bp and 1401 bp/1406 bp , for the strain GB 009 and GB 022 , respectively).

Bioassay guided fractionation of the ethyl acetate-soluble crude extract of the gliding bacterial strain GB 009 was performed by means of chromatographic techniques and crystallization. The bioactive compound was obtained as colourless needle-shaped single crystals in acetone/chloroform/hexane (1:1.5:1.5) mixture. The structure of bioactive compound was elucidated by spectroscopic methods and an X ray crystallographic technique. By means of the techniques, the structure of marinoquinoline A (4- methyl-3 *H* – pyrrolo [2,3-*c*] quinoline) was proposed with the molecular formula of $C_{12}H_{10}N_2$.

Isolation of the bioactive compound from marine gliding bacterial strain GB 022 was performed. The ethyl acetate soluble crude extract purified by means of chromatography techniques led to an active compound. The main proton spectrum was similar with the proton spectrum of marinoquinoline A. Therefore, the active compound from marine gliding bacterium GB 022 was considered as marinoquinoline A.

Biological activities of marinoquinoline A was investigated and showed that the compound possessed *in vitro* activity against a narrow spectrum of microorganism species. It showed moderate antifungal activity against *Candida albicans* TISTR 5239 and *Saccharomyces cerevisiae* with the MIC value of 18.52 and 9.26 µg/ml, respectively. Weak antifungal activity against filamentous fungi was detected. Moreover, this substance was made less active in antibacterial activity against different pathogens. It was found that marinoquinoline A showed significant inhibitory activity against acetylcholinesterase with an IC_{50} value of 4.9 µM. Marinoquinoline A showed no cytotoxicity against human cell lines including breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human cervix carcinoma (Hela) and human mouth epidermoid carcinoma (KB).

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APPENDIX 1

:GCTCCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGTCGAACGGCAGCACGATCGCTTCGGTGATTGGTGGCGAGTGGCGCACGGGTGCGTAACGCGT
ATGCAATCTGNCCACAACAGGGGGATAGCCCAGAGAAATCTGGATTAATACCCCATAACACAGGGGTTCCGCATGGGACTATTGGTTAAAGCTTCGGCGGTTGT
GGATGAGCATGCGTCCCATTAGCTAGTAGGCGAGGTAACGGCTCACCTAGGCGATGATGGGTAGGGGTTCTGAGAGGAAGGTCCCCCACACTGGTACTGAGATA
CGGACCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCCATGCCGCGTGCAGGAAGACGGCCCTCGGGTTGTAAA
CTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTTGACGGTACCGTACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTACGTAGGCGGTTCGGTAAGTCAGTGGTGAAATTTTACAGCTCAACTGTAAGCGTGCCATTGATACTGTCG
AACTTGAGTCCTGTAGAGGTAGGCGGAATTCCGCATGTAGCGGTGAAATGCATAGATATGCGGAGGAACTCCAAAAGCGAAGGCAGCTTACTGGACAGGAACT
GACGCTGAGGTACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGTTACTAGGTGTGCGCGACATACAGTGCGTGCC
CAAGCGAAAGCGATAAGTAACCCACCTGGGGAGTACGCTCGCAAGAGTGAAACNCAAAGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGATGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGTGACGGATCTAGAGATAGATCTTCCTTCGGGCACAAAGCAAGGTGCTGCATGGCTGTCGTCAG
CTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTACCCTTAGTTGCCATCAGATAATGCTGGGGACTCTAAGGGGACTGCCTACGCAAGT
AGAGAGGAAGGAGGGGACGATGTCAAGTCATCATGGCCCTTACGCCCAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGTAGCTACCTGGCAACAGGA
TGCGAATCTCAAAAAGTGCGTCTCAGTTCGGATTGTGGTCTGCAACTCGACCACATGAAGTTGGAATCGCTAGTAATCGCGCATCAGCAATGGCGCGGTGAATA
CGTTCCCGGACCTTGTACACACCGCCCGTCAAGCCATGGGAGTTGGGTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTTAGGGTCAAACCAGCGACTGGGG
CTAAGTCGTAACAAGGTAACCGA :

Figure 30. The full length of 16S rDNA nucleotide sequence of the marine gliding bacterial strain GB 009.

APPENDIX 2

:CTCCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGTCGAACGGCAGCACGATCGCTTCGGTGATTGGTGGCGAGTGGCGCACGGGTGCGTAACGCGTA
GGCAATCTGCCCACAACAGGGGGATAGCCCAGAGAAATCTGGATTAATACCCCATAACACAGGGGTTCCGCATGGGACTATTGGTTAAAGCTTCGGCGGTTGTG
GATGAGCATGCGTCCCATTAGCTAGTAGGCGAGGTAACGGCTCACCTAGGCGATGATGGGTAGGGGTTCTGAGAGGAAGGTCCCCCACACTGGTACTGAGATAC
GGACCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCCATGCCGCGTGCAGGAAGACGGCCCTCGGGTTGTAAAC
TGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTTGACGGTACCGTACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTACGTAGGCGGTTCGGTAAGTCAGTGGTGAAATTTTACAGCTCAACTGTAAGCGTGCCATTGATACTGTCGA
ACTTGAGTCCTGTAGAGGTAGGCGGAATTCCGCATGTAGCGGTGAAATGCATAGATATGCGGAGGAACTCCAAAAGCGAAGGCAGCTTACTGGACAGGAACTG
ACGCTGAGGTACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGTTACTAGGTGTGCGCGACATACAGTGCGTGCCC
AAGCGAAAGCGATAAGTAACCCACCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGTGACGGATCTAGAGATAGATCTTCCTTCGGGCACAAAGCAAGGTGCTGCATGGCTGTCGTCAGC
TCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTACCCTTAGTTGCCATCAGATAATGCTGGGGACTCTAAGGGGACTGCCTACGCAAGTA
GAGAGGAAGGAGGGGACGATGTCAAGTCATCATGGCCCTTACGCCCAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGTAGCTACCTGGCAACAGGAT
GCGAATCTCAAAAAGTGCGTCTCAGTTCGGATTGTGGTCTGCAACTCGACCACATGAAGTTGGAATCGCTAGTAATCGCGCATCAGCAATGGCGCGGTGAATAC
GTTCCCGGACCTTGTACACACCGCCCGTCAAGCCATGGGAGTTGGGTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTTAGGGTCAAACCAG
CGACTGGGGCTAAGTCGTAACAAGGTAACCGA :

Figure 31. The full length of 16S rDNA nucleotide sequence of the marine gliding bacterial strain GB 022.

APPENDIX 3

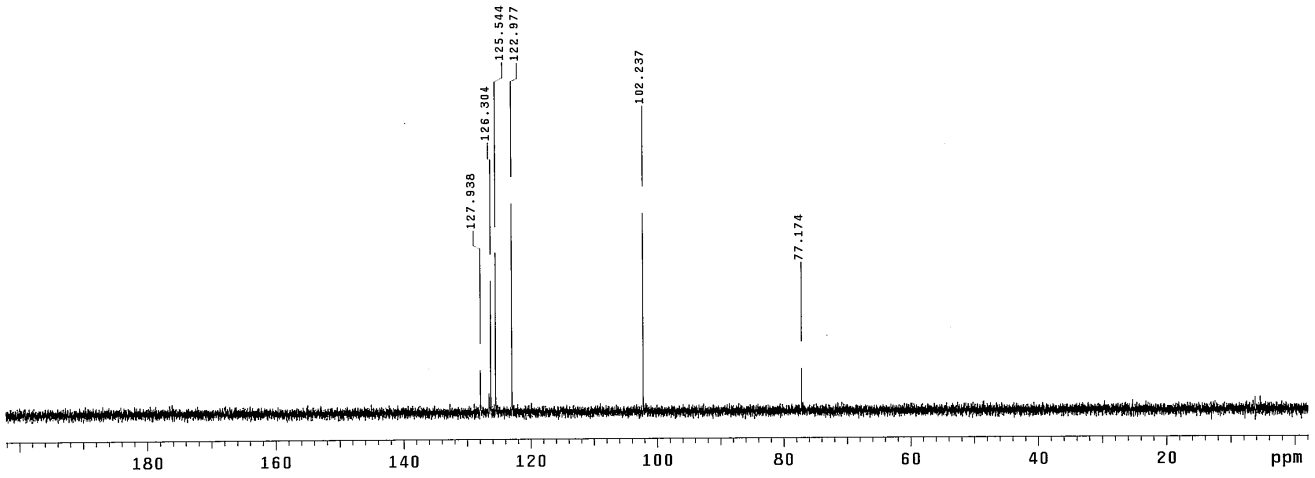

Figure 32. DEPT 90 spectrum of marinoquinoline A (4-methyl-3 *H*-pyrrolo [2,3-c] quinoline)**.**

APPENDIX 4

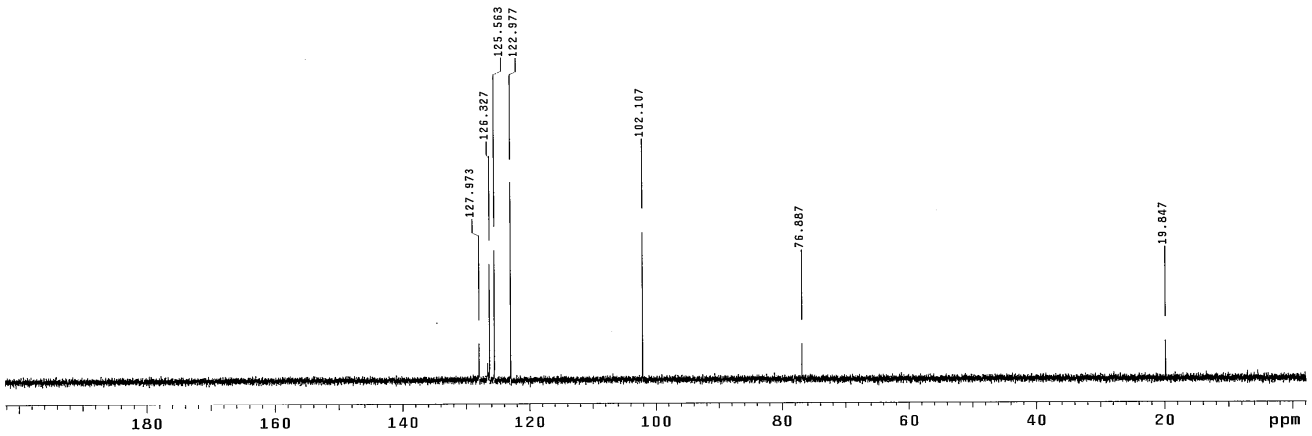

Figure 33. DEPT 135 spectrum of marinoquinoline A (4-methyl-3 *H*-pyrrolo [2,3-c] quinoline).

APPENDIX 5

Table 29. Antifungal susceptibility of fungi ($1x10^3$ spore/ml) determined by colorimetric microdilution broth assay with AlamarBlue.

Fungi	Amphotericin B MIC (µg/ml)
Candida albicans TISTR 5239	0.39
Saccharomycs cerevisiae	1.56
Aspergillus fumigatus TISTR 3108	1.56
Aspergillus flavus TISTR 3041	3.13

Table 30. Antibacterial susceptibility of bacteria ($1x10^5$ CFU/ml) determined by colorimetric microdilution broth assay with AlamarBlue.

Microorganisms	Antibiotic MIC (µg/ml)				
	A	C	O	S	V
Staphylococcus aureus TISTR 517	0.05	1.56	0.39	0.20	0.78
Methicillin-resistant *Staphylococcus aureus*	0.05	1.56	0.39	0.20	1.25
Bacillus subtilis	0.20	0.39	0.39	<0.02	1.56
Bacillus cereus	6.25	1.56	25	0.78	1.56
Escherichia coli	>25	0.78	25	0.39	>25
Pseudomonas aeruginosa	25	25	25	3.13	25

Note : Antibiotic: A: amphicillin; C:chloramphenicol; O: oxytetracyclin; S:streptomycin sulfate; V: vancomycin hydrochloride.

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List of Publication and Proceeding

Kanjana-Opas, A., Panphon, S., Fun, H.-K. and Chantrapromma, S. 2006. 4-Methyl-3*H*-pyrrolo [2, 3-*c*] quinoline. *Acta Cryst*. E. 62 : o2728-o2730.

Phanpol, S., Kanjana-Opas, A., Ingkaninan, K. and Arunpairojana, V. 2005. Screening of acetylcholinesterase inhibitors from marine gliding bacterial extracts. Frontiers in Bioorganic and Natural Products chemistry. 46th Annual Meeting of the American Society of Pharmacognosy, Oregon State University, USA, July 23-27, 2005. (Poster presentation).

Kanjana-Opas, A., Phanpol, S., Sae-Lim, S., Suwanachart, C. and Arunpairojana, V. 2005. Screening for antimicrobial, cytoxic and acetylcholinesterase inhibitory compounds from marine microorganism. Frontiers in Bioorganic and Natural Products chemistry. 46th Annual Meeting of the American Society of Pharmacognosy, Oregon State University, USA, July 23-27, 2005. (Poster presentation).