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RENIERAMYCINS: NONRIBOSOMAL PEPTIDE SYNTHETASE GENE AND CYTOTOXICITY AGAINST LUNG CANCER CELLS

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สารกลุ่มเรเนียรามัยซินเป็นอัลคาลอยด์ที่ได้จากฟองน้ำทะเล ซึ่งเป็นสารที่มีฤทธิ์ความเป็นพิษต่อเซลล์และมีโครงสร้างเป็นบิสเตตราไฮโดรไอโซควิโนลีนควิโนนคล้ายกับสารกลุ่มซาฟรามัยซินและซาฟราซินที่ได้จากจุลินทรีย์ สารกลุ่มนี้จัดเป็นพวกนอนไรโบโซมอลเพปไทด์ จึงได้ทำการตรวจสอบยีนที่เกี่ยวข้องกับชีวสังเคราะห์ของสารกลุ่มเรเนียรามัยซินจากฟองน้ำสีน้ำเงิน *Xestospongia* sp. ของไทยโดยวิธีเมตาจีโนมิกทำให้ได้ข้อมูลบางส่วนของยีนขนาด 764 bp ที่มีความสัมพันธ์กับส่วน A-domain ถึง T-domain ของยีนนอนไรโบโซมอลเพปไทด์ซินเธเทส *saf A* ที่เกี่ยวข้องกับชีวะสังเคราะห์สารซาฟรามัยซินเอ็มเอ๊กซ์1 ได้ใช้วิธี gene walking เพื่อให้ทราบข้อมูลยีนใกล้เคียงโดยการสร้างห้องสมุดดีเอ็นเอชนิดฟอสมิดซึ่งต้องใช้ดีเอ็นเอที่มีขนาดเหมาะสม แต่การสกัดดีเอ็นเอจากฟองน้ำ *Xestospongia* sp. โดยวิธีการต่างๆไม่สามารถได้ดีเอ็นเอที่มีคุณภาพและปริมาณที่เหมาะสมได้ แม้จะมีการกำจัดสารเรเนียรามัยซินก่อนการสกัดดีเอ็นเอที่ทำให้คุณภาพดีขึ้นแล้วก็ยังไม่เพียงพอต่อการนำไปสร้างห้องสมุดดีเอ็นเอชนิดฟอสมิดเพื่อหาข้อมูลเพิ่มเติมของยีนนอนไรโบโซมอลเพปไทด์ซินเธเทสที่เกี่ยวข้องกับชีวสังเคราะห์ของสารเรเนียรามัยซินได้

จากข้อมูลที่พบว่าหมู่ควิโนนของสารเรเนียรามัยซินเอ็มมีผลต่อการทำลายดีเอ็นเอผ่านทางอนุมูลอิสระจึงได้ศึกษาผลดังกล่าวในเซลล์มะเร็งปอด โดยพบว่าสารเรเนียรามัยซินเอ็มทำให้เซลล์มะเร็งตายผ่านขบวนการเนโครซิสในสัดส่วนที่สูงเมื่อเทียบกับการตายผ่านขบวนการอะพอพโตซิส ซึ่งอาจส่งผลให้เกิดความเป็นพิษที่ไม่พึงประสงค์ของสารมากขึ้นโดยการตรวจวัดด้วย dihydroethidium fluorescence probe ที่มีความจำเพาะต่อชนิดอนุมูลอิสระ จึงพบว่าอนุมูลอิสระชนิด superoxide anion เป็นสาเหตุทำให้เกิดการตายของเซลล์มะเร็งแบบเนโครซิส เมื่อเตรียมสาร 5-*O*-อะเซทิลไฮโดรควิโนนเรเนียรามัยซินเอ็มโดยการแทนที่หมู่ควิโนนด้วยหมู่ไฮโดรควิโนน พบว่าสารใหม่นี้สามารถลดขบวนการเนโครซิส ในขณะที่ยังคงทำให้เซลล์มะเร็งตายผ่านทางขบวนการอะพอพโตซิส ดังนั้นสารอนุพันธ์ใหม่นี้จึงมีศักยภาพที่จะพัฒนาเป็นสารต้านมะเร็งต่อไป

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชเวท ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEY WORDS: RENIERAMYCINS / NONRIBOSOMAL PEPTIDE SYNTHETASE GENE / *XESTOSPONGIA* SPONGE / CYTOTOXICITY / APOPTOSIS / NECROSIS / LUNG CANCER CELLS

THANIWAN CHEUN-AROM: RENIERAMYCINS: NONRIBOSOMAL PEPTIDE SYNTHETASE GENE AND CYTOTOXICITY AGAINST LUNG CANCER CELLS ADVISOR: KHANIT SUWANBORIRUX, Ph.D., CO-ADVISOR: TAKSINA CHUANASA, Ph.D., 177 pp.

Renieramycins, the bistetrahydroisoquinolinequinone cytotoxic alkaloids mainly obtained from marine sponges, are structurally related to saframycins and safracins produced by microorganisms. These compounds are biogenetically classified as nonribosomal peptides. To identify nonribosomal peptide synthetase (NRPS) genes involved in biosynthesis of renieramycins produced by the Thai blue sponge *Xestospongia* sp., metagenomic approach was performed. A gene with a size of 764 bp was identified to be related to the NRPS gene *safA,* involved in saframycin Mx1 biosynthesis, localizing at a continuous region of A-domain to T-domain. To further identify neighboring genes, a gene walking approach which required suitable size of DNA for fosmid library construction was used. Several protocols were applied for DNA extraction from the marine sponge *Xestospongia* sp., however the DNA quality and quantity were not satistified. Although removal of renieramycins from the sample was applied prior to DNA extraction, the quality of the obtained DNA was not adequately improved for a good fosmid library construction. Hence, gene walking for more information of NRPS gene involved in biosynthesis of renieramycins was not successful.

According to the DNA damage effect by the quinone moiety, renieramycin M (RM) was studied for its cytotoxic effect on lung cancer cells. RM mediated necrotic cell death in a high proportion compared to apoptotic cell death. This effect might be crutial for the fear of unwanted toxicity. The specific dihydroethidium fluorescence probe was used to reveal that the necrosis mediated by RM was through its ability to generate intracellular superoxide anion from the quinone moiety. Modification by replacing a quinone of RM with a hydroquinone of 5-*O*-acetylhydroquinone renieramycin M (ARM) was demonstrated to abolish necrosis-inducing effect while fully maintaining apoptosis-inducing effect of the parent RM in lung cancer cells. Therefore, ARM is a promising candidate for further development of a new anticancer agent.

Department : Pharmacognosy and Pharmaceutical Botany Student's Signature .....

Field of Study : Pharmacognosy Advisor's Signature .....

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CONTENTS

Page

Page

Page

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

FIGURE Page

LIST OF SCHEME

LIST OF ABBREVIATIONS

%	=	Percent or part per hundred
A, T, C, G	=	Nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively
bp	=	Base pair
^{13}C NMR	=	Carbon-13 Nuclear Magnetic Resonance
ºC	=	Degree Celsius
$CDCl_3$	=	Deuterated chloroform
CTAB	=	Cetyltrimethylammonium bromide
$DCFH_2$-DA	=	2′,7′-dichlorofluorescein diacetate
DHE	=	dihydroethidium
DNA	=	Deoxyribonucleic acid
DEPT	=	Distortionless Enhancement by Polarization Transfer
δ	=	Chemical shift
EDTA	=	Ethylenediaminetetraacetic acid
FABMS	=	Fast Atom Bombardment Mass Spectrometry
EtOAc	=	Ethyl acetate
g	=	Gram
g	=	earth's gravitational field
h	=	Hour
^{1}H-NMR	=	Proton Nuclear Magnetic Resonance
HMBC	=	^{1}H-detected Heteronuclear Multiple Bond Correlation
HMQC	=	^{1}H-detected Heteronuclear Multiple Quantum Coherence
H_2O	=	Water
HPF	=	3′-(*p*-hydroxyphenyl) fluorescein
HPLC-DAD	=	High performance liquid chromatography-diode array detectors
Hz	=	Hertz
IC_{50}	=	Concentration showing 50% inhibition
IR	=	Infrared

J	=	Coupling constant
Kb	=	Kilo base pair
kg	=	Kilogram
l	=	Liter
λmax	=	Wavelength at maximal absorption
M^+	=	Molecular ion
m	=	Multiplet (for NMR spectra)
MeOH	=	Methanol
mg	=	Milligram
MHz	=	Mega Hertz
MIC	=	Minimum inhibitory concentration
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
MS	=	Mass spectrum
MTT	=	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	=	Molecular weight
m/z	=	Mass to charge ratio
nm	=	Nanometer
NAC	=	*N*-acetylcysteine
NCBI	=	National center for biotechnology information
NMR	=	Nuclear Magnetic Resonance
NRPS	=	Nonribosomal peptide synthetase
OC	=	Opened circular DNA form
ppm	=	Part per million
PCR	=	Polymerase chain reaction
q	=	Quartet (for NMR spectra)
quint	=	Quintet (for NMR spectra)
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Revolution per minute
sec	=	Second

sext	=	Sextet (for NMR spectra)
SC	=	Supercoiled DNA form
SOC	=	Super optimal broth with catabolite repression
s	=	Singlet (for NMR spectra)
SDS	=	Sodium dodecyl sulphate
sp.	=	Species (singular)
SD	=	Standard deviation
TAE buffer	=	Tris-acetate and EDTA buffer
t	=	Triplet (for NMR spectra)
TLC	=	Thin layer chromatography
UV-VIS	=	Ultraviolet and visible spectrophotometry
µl	=	Microliter
µg	=	Microgram
µM	=	Micromolar

Nucleotide codes

A	=	Adenine
G	=	Guanine
C	=	Cytosine
T	=	Thymine
U	=	Uracil
R	=	Purine (A or G)
Y	=	Pyrimidine (C or T)
N	=	Any nucleotide
W	=	Weak (Aor T)
S	=	Strong (G or C)
M	=	Amino (A or C)
K	=	Keto (G or T)
B	=	Not A (G or C or T)
H	=	Not G (A or C or T)
D	=	Not C (A or G or T)
V	=	Not T (A or Gor C)

Amino acid codes

A	/	Ala	=	Alanine
C	/	Cys	=	Cysteine
D	/	Asp	=	Asparatic acid
E	/	Glu	=	Glutamic acid
F	/	Phe	=	Phenylalanine
G	/	Gly	=	Glycine
H	/	His	=	Histidine
I	/	Ile	=	Isoleucine
K	/	Lys	=	Lysine
L	/	Leu	=	Leucine
M	/	Met	=	Methionine
N	/	Asn	=	Asparagine
P	/	Pro	=	Proline
Q	/	Gln	=	Glutamine
R	/	Arg	=	Arginine
S	/	Ser	=	Serine
T	/	Thr	=	Threonine
V	/	Val	=	Valine
W	/	Trp	=	Tryptophan
Y	/	Tyr	=	Tyrosine

CHAPTER I

INTRODUCTION

Marine invertebrates especially sponges (phylum Porifera) are one of the oldest filter-feeder animals, which absorb nutrients and remove microorganisms from seawater by pumping thousands of liters of seawater per day through their aquiferous system (Li *et al*., 1998). Sponges are remarkable natural sources for their unmatched diversity of secondary metabolites with promising potentials to become effective drugs for therapeutic applications (Sipkema *et al*., 2005). In the majority of cases, production of compounds derived from sponges is obstructed by inherent difficulties in collecting or culturing large quantity of these sponges (Kennedy *et al*., 2008), although these marine natural products have pronounced anticancer activity (Paleari *et al*. 2006), anti-infective activity (Rao *et al*., 2004), or other bioactivities (Chang *et al*., 2003), making them interesting lead compounds for medical and biotechnological applications. A serious problem for drug development and maintainable production lies in the limited amounts of biomass of most marine invertebrates available from wild stocks. Thus, most pharmacologically active marine natural products can only be isolated in minute yields. A number of total syntheses of pharmacologically active natural products have been successfully established but many cases are not economically feasible due to the complexity of the molecular structures and the low yields (Proksch *et al*., 2003). A possible alternative solution that has yet to be successfully applied on a useful scale is the production of compounds through heterologous expression of their biosyntheses in an easily manageable host. This involves the identification of the biosynthetic gene cluster, the cloning into a suitable expression vector and expression in a host amenable to large-scale fermentation. As the technology becomes more widely available, in vitro gene synthesis to accommodate host codon usage can be employed instead of cloning of the native genes. Consequently, increasing the production of interesting compounds by genetic manipulation would benefit the control of production. In addition, the knowledge cluster could be used for combinatorial creation of novel analogues (Moore, 2005; Konig *et al*., 2006).

However, over the last decade, the application of genomic technology to marine organisms has been opening up new avenues of research (Hofmann *et al*., 2005) at molecular and biochemical levels because large genomic sequence databases are available (Fortman and Sherman, 2005). Biosynthetic pathway and its regulation are needed to be determined if large scale production of a bioactive compound would be feasible for drug development. Further, knowledge about whether the biosynthesis pathway is simple or complex such as the number of genetic elements involved and how the biosynthesis pathway regulation would be very helpful in characterizing bioactive metabolites and prioritizing them for biotechnological production of drugs or drug candidates from the sea (Konig *et al*., 2006).

Renieramycins are a group of the bistetrahydroisoquinoline alkaloids possessing potent cytotoxicity. The compounds have been isolated from marine sponges in several genera, especially the *Xestospongia* sponge. The similarity observed for the structures of renieramycins to saframycins and safracins, the antibiotics produced by microorganisms (Pospiech *et al*., 1995), suggested that renieramycins are likely to be synthesized by a similar nonribosomal peptide synthetase (NRPS) mechanism. This assumption might provide the basis for characterizing the renieramycin biosynthetic pathway, as the general scheme for nonribosomal peptide biosynthesis has been well established (Marahiel *et al*., 1997; Konz and Marahiel, 1999). Therefore, the first aim is to study the nonribosomal peptide synthesis gene involved in biosynthesis of renieramycins from the blue sponge *Xestospongia* sp. This objective consists of two main parts. The first part, an efficient extraction method to prepare DNA from the Thai marine sponge *Xestospongia* sp. was developed. The second part is to study NRPS genes involved in renieramycin biosynthesis by metagenomic approach and Fosmid library construction for gene walking.

As of some reports, the tetrahydroisoquinoline alkaloids family binds to DNA by alkylation of specific nucleotide sequences (Rao and Lown, 1990; Swenberg, *et al*., 1990; Pommier *et al*., 1996; Upton and Swenberg, 1997; Avendano and Menendez, 2008). Most of these alkaloids contain the quinone group and act by reductive alkylation, especially, the isoquinoline nitrogen is vital for DNA alkylation (Ishiguro *et al*., 1981; Hill and Remers, 1991; Pommier *et al*., 1996; Avendano and

Menendez, 2008). The presence of the quinone group in the structure of certain compounds; for example, saframycins, naphthyridinomycin, and bioxalomycin β2 have been reported to enhance the ability of DNA alkylation and to produce DNA strand breaks by generating free radicals and reactive oxygen species (Lown *et al*., 1976; Begleiter, 1983; Begleiter and Blair, 1984; Begleiter, 1985; Williams and Herberich, 1998). Interestingly, quinone agents may correlate with generation of free radicals which play an important role in necrotic cell death causing unwanted inflammatory results such as furylbenzoquinone and naphtoquinone derivatives strongly affecting necrotic cell death on transplantable liver tumor (Liou and Storz, 2010). Therefore, the second aim is to study on necrosis effect of renieramycin M and necrotic abolishing effect of its hydroquinone derivative in human non-small cell lung cancer cells.

CHAPTER II

LITERATURE REVIEW

1. The blue sponge *Xestospongia* sp.

The sponge genus *Xestospongia* belongs to phylum Porifera, class Demospongiae, order Haplosclerida, and family Petrosiidae (Hooper, 2000) and comprises over 8,000 extant species (Montalvo and Hill, 2011). The characterization of the genus *Xestospongia* includes ectosomal skeleton indistinct, choanosomal skeleton confused isotropic reticulation of multispicular tracts, generally lacking spongin and sometimes with single spicules scattered throughout mesohyl between major spicule tracts, stony texture and oxeote spicules in one size category only (Hooper, 2000).

The marine blue sponge *Xestospongia* sp. was collected from Sichang Island, Chonburi Province, Thailand. The sponge was identified by Dr. John N. A. Hooper as *Xestospongia* sp. (family *Petrosiidae*). The voucher specimens have been deposited at the Queensland Museum, South Brisbane, Australia (sample code QMG306998) and at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Its texture is hard, brittle, and easily crumbled. It is light grayish-blue when alive and pinkish in ethanol. Picture of the sponge is shown in Figure 2.1.

Figure 2.1 Picture of the marine blue sponge *Xestospongi*a sp. (photograph by Khanit Suwanborirux)

2. Renieramycin alkaloids and related compounds

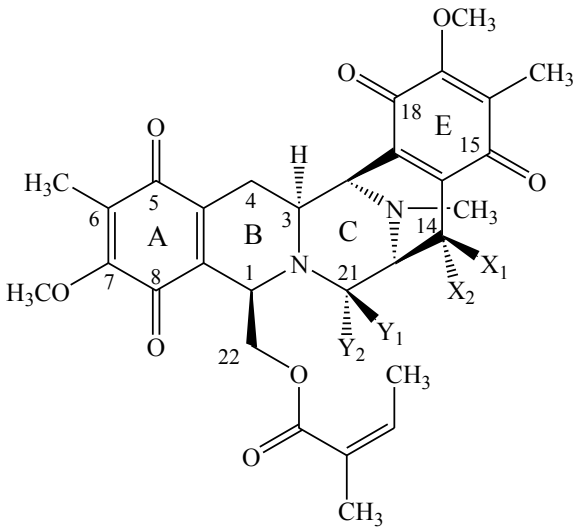
2.1 Natural sources and chemical structures

Renieramycins, belonging to the group of bistetrahydroisoquinolinequinone alkaloids, were isolated from marine sponges of the genera *Reniera* (Frincke and Faulkner, 1982), *Xestospongia* (McKee and Ireland, 1987), *Haliclona,* (Parameswaran *et al*., 1998), *Cribrochalina* (Pettit, 1992) and *Neopetrosia* (Oku, 2003). The first report of renieramycins A-D **[1a-d]** was described from the Maxican sponge *Reniera* sp. in 1982 (Frincke and Faulkner, 1982). Seven years later, renieramycins E **[1e]** and F **[1f]** were isolated from the same genus (He and Faulkner, 1989). In 1992, renieramycins G **[1g]** was reported from the marine sponge *Xestospongia caycedoi* (Davidson, 1992). In 2003, renieramycins J **[1j]**, K **[1k]**, and L **[1l]** were obtained from the Thai blue sponge *Xestospongia* sp. which was collected in the Sichang Island, in the Gulf of Thailand (Suwanborirux *et al.*, 2003). Currently, Suwanborirux and coworkers have succeeded in gram-scale preparation of stabilized renieramycins from the Thai blue sponge *Xestospongia* sp. by pretreatment with potassium cyanide (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Saito *et al*., 2004). The Thai blue sponge *Xestospongia* sp. was pretreated with potassium cyanide to increase the mass-production of renieramycin M **[1m]**, a major renieramycin, and four minor compounds including renieramycins O **[1o]**, Q **[1q]**, R **[1r]** and S **[1s]**. Recently, three

new bistetrahydroisoquinoline marine natural products, renieramycins W **[1w]**, X **[1x]**, and Y **[1y]**, along with two known renieramycins M **[1m]** and T **[1t]**, were isolated from the KCN-pretreated Philippine blue sponge *Xestospongia* sp. (Tatsukawa *et al*., 2012). Structures of renieramycins are shown in Figure 2.2.

The chemical structures of renieramycins from sponges are closely related to natural tetrahydroisoquinoline families from different marine sources such as ecteinascidin-743 (ET-743) isolated from the Caribbean tunicate *Ecteinascidia turbinata* in 1990. The common structural feature of ET-743 consists of three tetrahydroisoquinoline subunits and an active carbinolamine functional group (Reinhart *et al*., 1990). In addition, Fontana *et al*. reported jorumycin **[2a]** from the nudibranch *Jorunna funebris* in 2000 (Fontana *et al*., 2000). The structure of jorumycin is most similar to that of renieramycin F with exception of the acetate group on the alcohol versus the angelate ester of the renieramycins. In 2006, Charupant and coworkers isolated new stabilized renieramycin-type, jorunnamycins A-C **[2b-d]** from the mantles, the visceral organs, and the egg ribbons of the Thai *Jorunna funebris* pretreated by potassium cyanide (Charupant *et al*., 2006). The chemical structures of tetrahydroisoquinolines from other marine natural sources are shown in Figure 2.3.

Subsequently, other related microbial compounds such as saframycins A, B, C, D, and E [**3a-e**] were isolated from *Streptomyces lavendulae* in 1977 (Arai *et al*., 1977). The structure of saframycin C was the first of this family to be determined. In addition, Safracins A and B **[3f, 3g]** were isolated from *Pseudomonas fluorescens* A2-2 (Ikeda *et al*., 1983) and saframycin Mx1 **[3h]** from *Myxococcus xanthus* (Pospiech *et al*., 1995). Structures of bistetrahydroisoquinolines from microorganisms are shown in Figure 2.4.



renieramycin

A **[1a]** : $X_1 = H, X_2 = OH, Y_1 = Y_2 = H$

B **[1b]** : $X_1 = H, X_2 = OC_2H_5, Y_1 = Y_2 = H$

C **[1c]** : $X_1 = H, X_2 = OH, Y_1, Y_2 = O$

D **[1d]** : $X_1 = H, X_2 = OC_2H_5, Y_1, Y_2 = O$

E **[1e]** : $X_1 = X_2 = H, Y_1 = H, Y_2 = OH$

F **[1f]** : $X_1 = H, X_2 = OCH_3, Y_1 = H, Y_2 = OH$

G **[1g]** : $X_1 = X_2 = Y_1 = Y_2 = H$

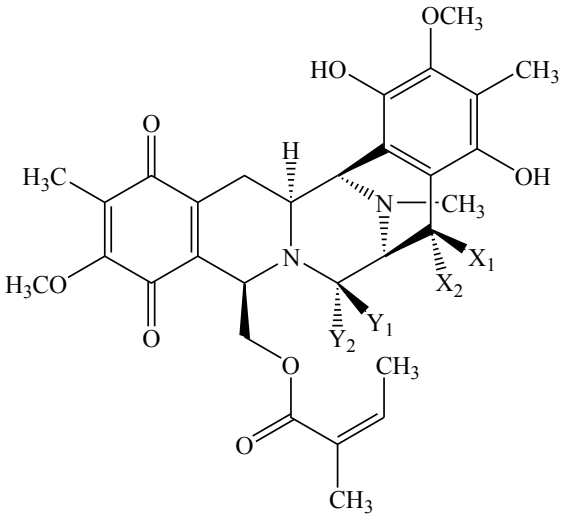
J **[1j]** : $X_1 = X_2 = Y_1 = H, Y_2 = CH_2COCH_3$

K **[1k]** : $X_1 = Y_1 = Y_2 = H, X_2 = OCH_3$

M **[1m]**: $X_1 = X_2 = Y_1 = H, Y_2 = CN$

R **[1r]** : $X_1 = Y_1 = H, X_2 = OCH_3, Y_2 = CN$

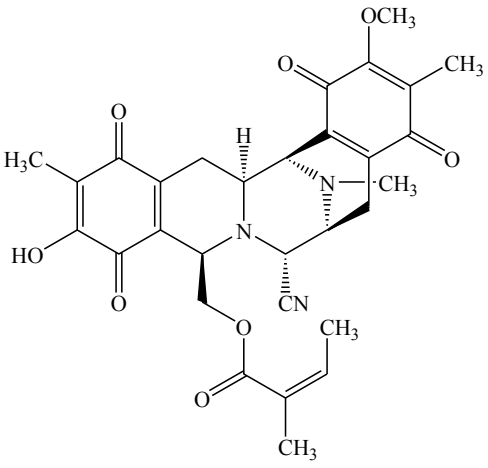
Figure 2.2 The chemical structures of renieramycins from marine sponges.



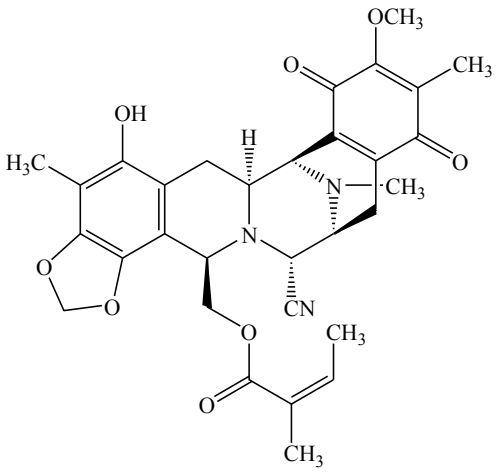
renieramycin

L **[1l]** : $X_1 = X_2 = Y_1 = H$, $Y_2 = CH_2COCH_3$

Q **[1q]** : $X_1, X_2 = O$, $Y_1 = H$, $Y_2 = CN$

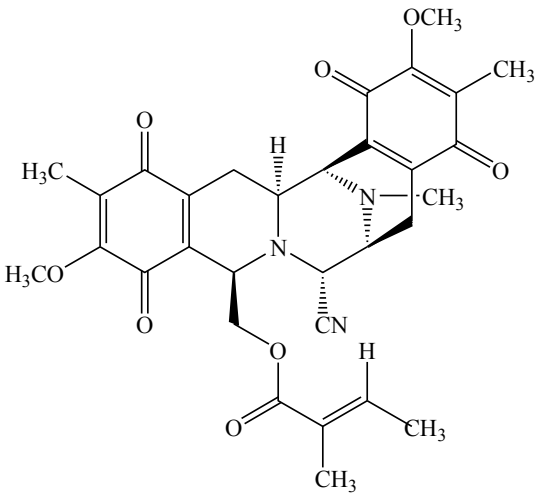


renieramycin S **[1s]**

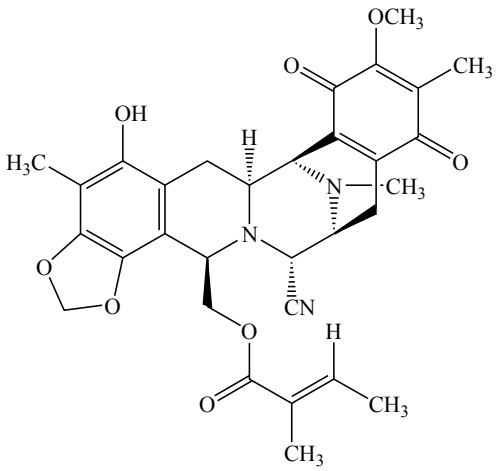


renieramycin T **[1t]**

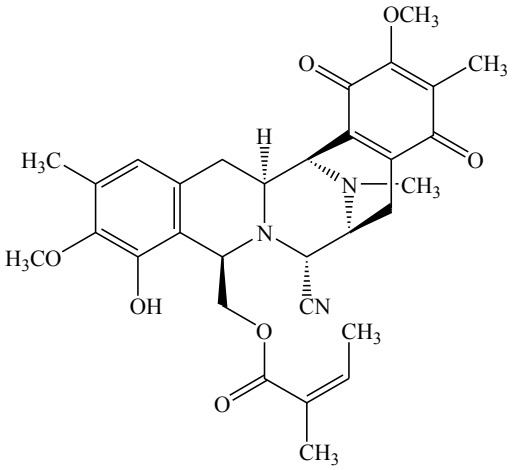
Figure 2.2 (continued).



renieramycin W **[1w]**

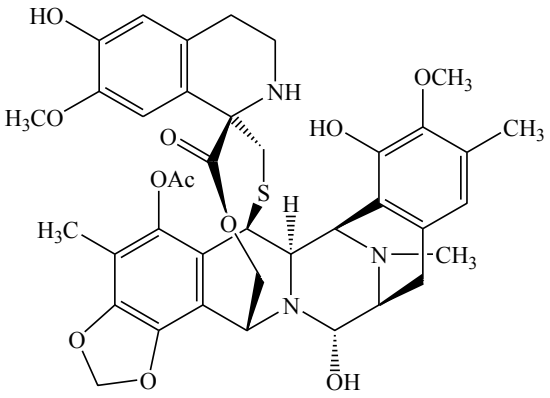


renieramycin X **[1x]**

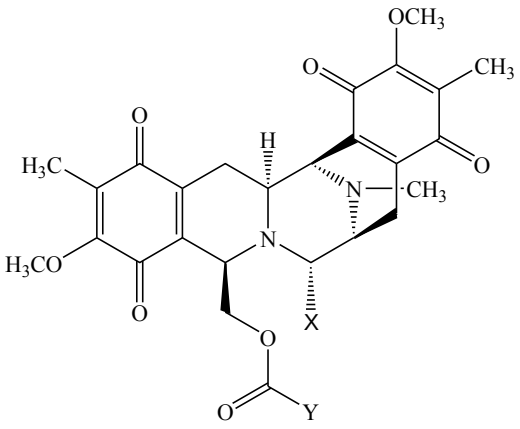


renieramycin Y **[1y]**

Figure 2.2 (continued).

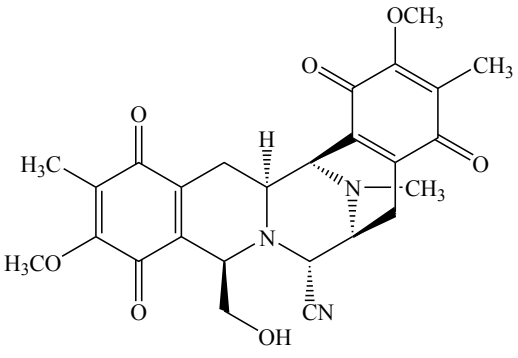


ecteinascidin-743 (ET-743)

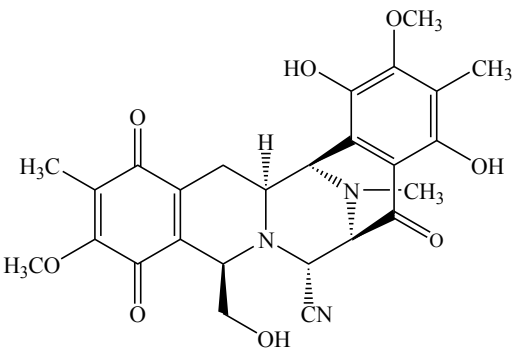


jorumycin **[2a]** : X = OH, Y = CH_3

jorunnamycin C **[2d]** : X = CN, Y = C_2H_5

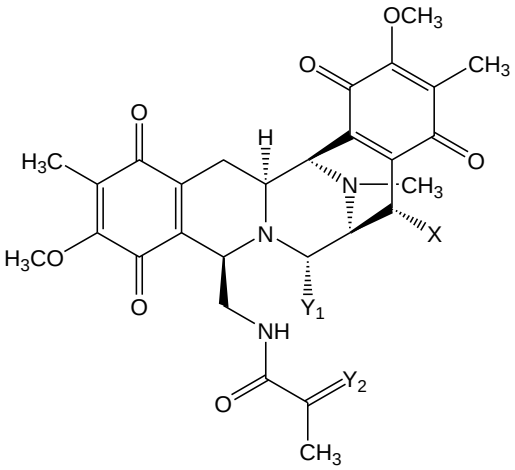


jorunnamycin A **[2b]**



jorunnamycin B **[2c]**

Figure 2.3 The chemical structures of related tetrahydroisoquinolines from other marine natural sources.

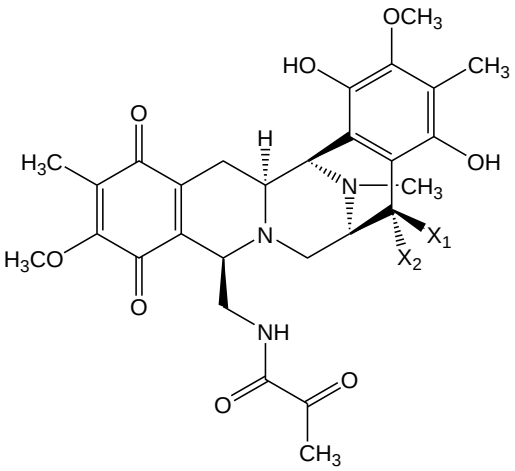


saframycin

A **[3a]** : X = H, Y_1 = CN, Y_2 = O

B **[3b]** : X = H, Y_1 = H, Y_2 = O

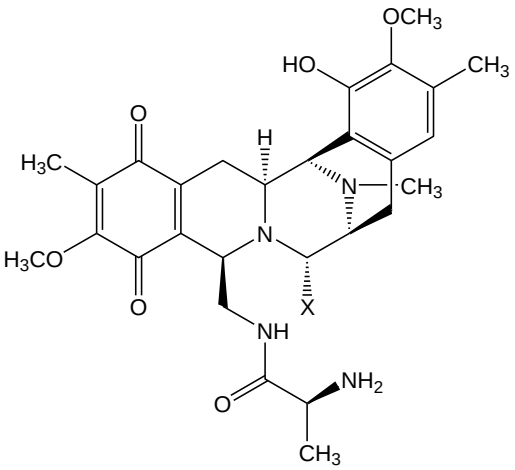
C **[3c]** : X = OCH_3, Y_1 = H, Y_2 = O



saframycin

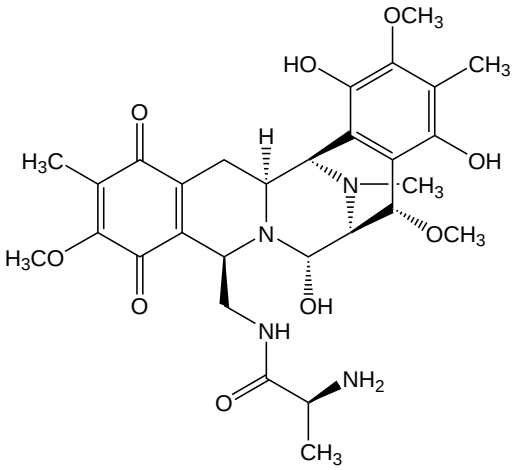
D [**3d]** : X_1, X_2 = O

E [**3e]** : X_1 = H, X_2 = OH



safracin A **[3f]** : X = H

safracin B **[3g]**: X = OH



saframycin Mx1**[3h]**

Figure 2.4 The chemical structures of bistetrahydroisoquinolines from microbial sources.

2.2 Biological activity of tetrahydroisoquinoline alkaloids

Renieramycins A-D (Frincke and Faulkner, 1982) and renieramycins H-I (Parameswaran *et al.,* 1998) showed moderate antimicrobial activity. In 2001, Rashid *et al.* described mimosamycin from the cytotoxic fractions of an aqueous extract of the marine sponge *Haliclona* sp., which was the principle cytotoxin with an IC_{50} 10 μg/ml against melanoma and ovarian human tumor cell lines. Several renieramycins (**1m-1o**, and **1q-1s**) showed very potent cytotoxic activity against two human cell lines, HCT116 (human colon carcinoma), and QG56 (human lung carcinoma) with IC_{50}s in the range of 5.6 nM – 7.1 nM (Suwanborirux *et al.,* 2003; Amnuoypol *et al.,* 2004; Saito *et al.,* 2004). Recently, renieramycin M, a major alkaloid of the *Xestospongia* sponge, has been shown to induce apoptosis through activation of p53-dependent pathway and inhibit metastasis in lung cancer cells (Halim *et al*., 2011). Renieramycin G **[1g]** was isolated from the sponge *Xestospongia caycedoi* by Davidson in 1992 and exhibited cytotoxicity against KB (human epidermoid carcinoma of nasopharynx) and LoVo cell lines (human colon adenocarcinoma cell) with MIC values of 0.5 and 1.0 μg/ml, respectively. The cytotoxicity of renieramycin P against 3Y1, HeLa and P388 cells was reported with IC_{50}s of 5.3, 12.3, and 0.53 nM, respectively (Oku *et al*., 20003).

The related natural tetrahydroisoquinoline from different marine sources such as ecteinascidin-743 (ET-743), is remarkably active and potent antitumor in a variety of in vitro and in vivo systems and has been selected for development as an anticancer agent. Recently, ET-743 (Trabectidin, Yondelis®) has been approved by the European Commission for the treatment of advanced or metastatic soft tissue sarcoma (Cuevas and Francesch, 2009). It has been also marketed for the treatment of relapsed platinum-sensitive ovarian cancer in combination with doxorubicin (Meco *et al*., 2003; Sledge *et al*., 2003).

Consequently, other related microbial compounds such as saframycins have been found to display antitumor and antimicrobial activities. Saframycin A exhibited the most potent antitumor activity against L1210 leukemia with IC_{50} 5.6 nM. It has a nitrile at C-21 which allows the formation of an electrophilic iminium species that alkylates DNA in the minor groove. In addition, saframycins B, C, and D which lack

a leaving group at C-21 has much lower activity (Kishi *et al*., 1984; Scott and Willium, 2002).

3. Nonribosomal Peptides (NRP)

Natural products are important elements of modern therapy. Secondary metabolites can be classified into different groups according to biosynthesis pathways (Table 2.1) (Thomas, 2004). One class of natural products, the nonribosomally produced peptides, is of extraordinary pharmacological importance.

Table 2.1 Ways of assembling secondary metabolites.

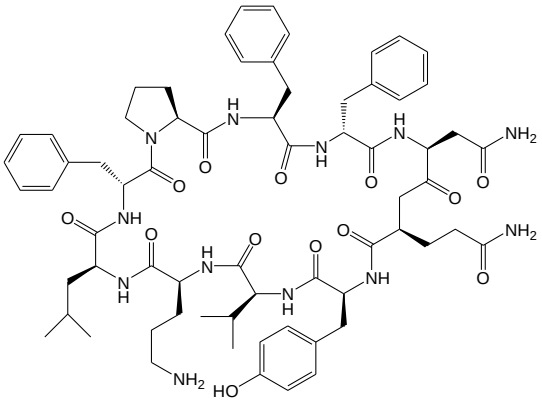
Pathway	Precursors	Groups
Shikimate	Shikimic acid	Alkaloids, Quinones, Flavonoids, Phenylpropanoids etc.
Polyketide or Acetate	Acetyl CoA & Malonyl CoA	Fatty acids, Acetylenes,Aromatic polyketides etc.
Mevalonate & Nonmevalonate	Isoprene Units	Terpenoids & Steroids
Nonribosomal Peptides	Amino acid	Nonribosomal Peptides

3.1 History of nonribosomal peptides

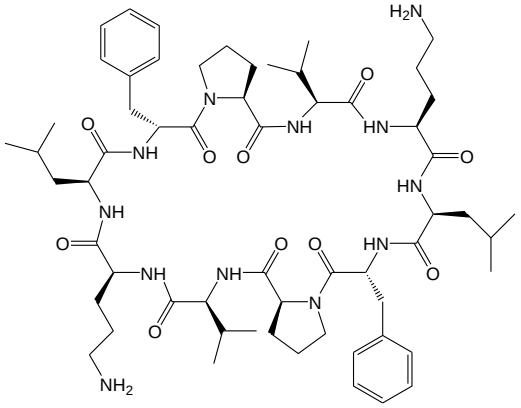
Nonribosomal peptide biosynthesis is carried out by the so-called nonribosomal peptide synthetases (NRPSs). NRPSs catalyze the assembly of a large number of complex peptide natural products, many of which display therapeutically useful activity. Each cycle of chain extension is carried out by a dedicated module of the multifunctional enzymes. NRPSs occur in a wide range of organisms, including bacteria, fungi, plants and marine organisms (Keller and Schauwecker, 2003). Peptide antibiotics represent a large and diverse group of bioactive natural products with a wide range of applications in medicine, agriculture, and biochemical research. These metabolites show functional diversity including antibiotics (e.g. penicillins, vancomycin, bacitracin and gramicidin), immunosuppressive agents (e.g. rapamycin

and cyclosporin), cytostatic agents (e.g. bleomycin and epothilon), and siderophores (e.g. myxochelin and enterobactin) (Challis *et al*., 2000). Examples of the structural diversity of bioactive compounds of nonribosomal origin are shown in Figure 2.5 (Schwarzer *et al*., 2003).

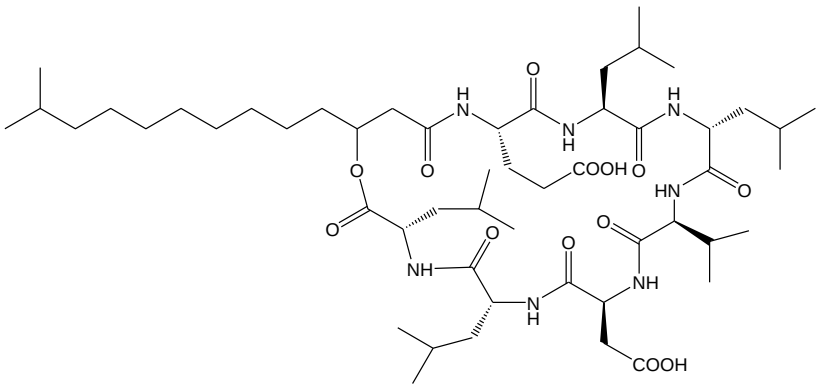
A. antibiotics



Tyrocidin A



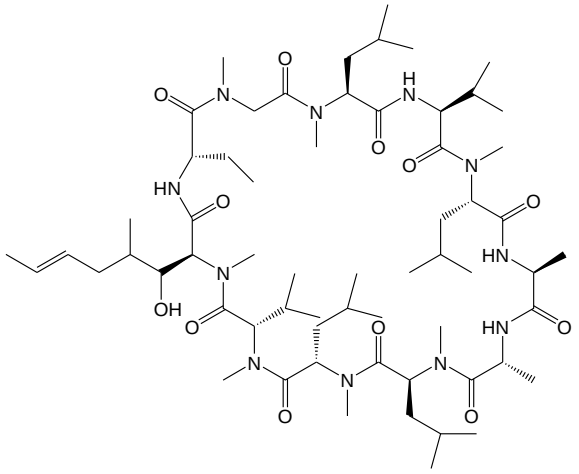
Gramicidin S



Surfactin

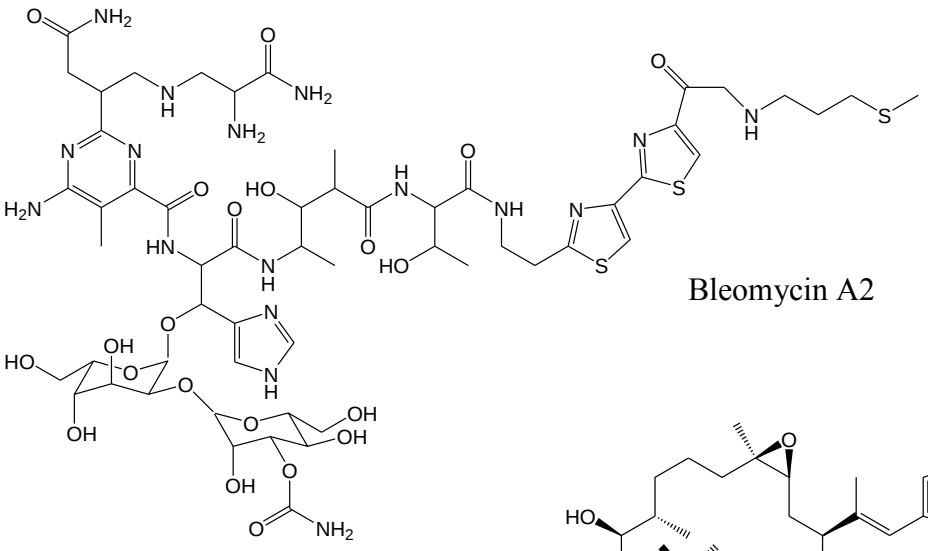
Figure 2.5 Examples of the structural diversity of bioactive compounds of nonribosomal origin.

B. immunosuppressive agent

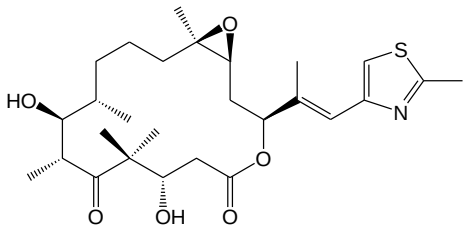


Cyclosporin A

C. cytostatic agents



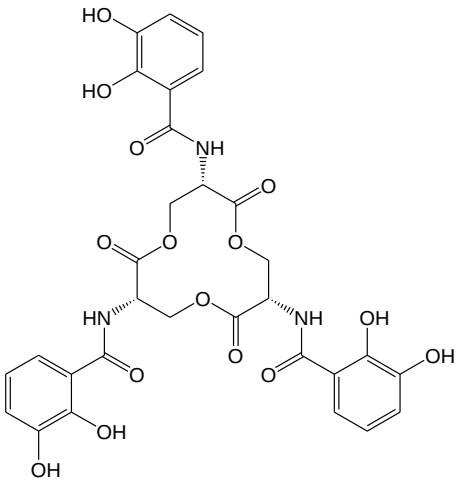
Bleomycin A2



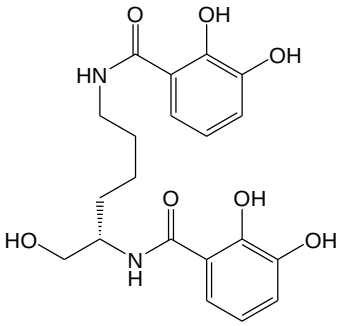
Epothilon

Figure 2.5 (continued).

D. siderophores

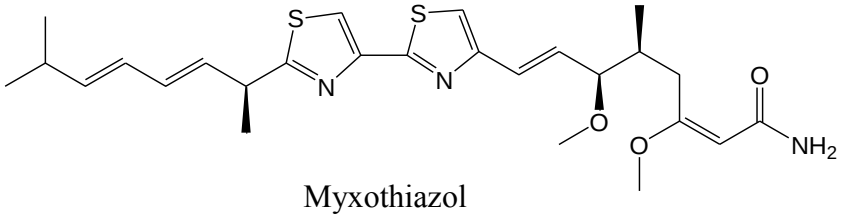


Enterobactin

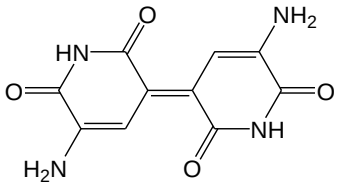


Myxochelin A

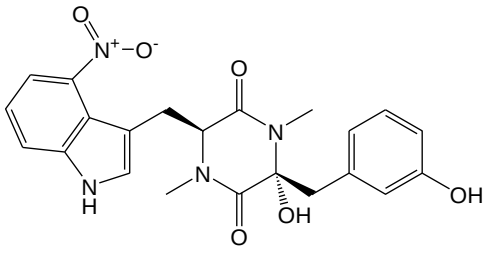
E. others



Myxothiazol



Indigodin



Thaxtomin A

Figure 2.5 (continued).

3.2 Biosynthetic strategies of nonribosomal peptide synthetases

Nonribosomal peptide synthetases (NRPSs) exhibit a modular organization. In this connection, a module is a section of the NRPS's polypeptide chain that is responsible for the incorporation of one amino acid into the final product. Generally, basic modules comprised initiation module, elongation module, and releasing module that are capable of carrying out one cycle of chain extension. The modules can be further subdivided into domains, which represent the enzymatic units that catalyze individual steps of nonribosomal peptide synthesis. The core domains of NRPS on initiation and elongation modules are the adenylation (A), thiolation (T; also referred to as the peptidyl carrier domain, PCP), and condensation (C) domains. The releasing module contains the relreasing domain (RE) or chain-terminating thioesterase (TE) domain for off-loading of the mature peptide chain. The organization of the domains within a module according to this model is $(C\text{-}A\text{-}PCP)_n\text{-}RE$ (Figure 2.6). Domains can be identified on the protein level by characteristic, highly conserved sequence motifs, the so-called "core-motifs". The adenylation domain is composed of 10 core motifs while thiolation domain, condensation domain, and releasing domain contain 1, 7, and 7 core motifs, respectively. The sequences of six highly conserved cores whose order and location within all known domains are from five adenylation domains (cores 1-5) and one thiolation domain (core 6). The sequences of core motifs are shown in Table 2.2. Therefore, the use of degenerate oligonucleotides derived from the conserved cores opens the possibility of amplifying and cloning NRPS from genomic DNA (Borchert *et al*., 1992; Turgay and Marahiel, 1994).

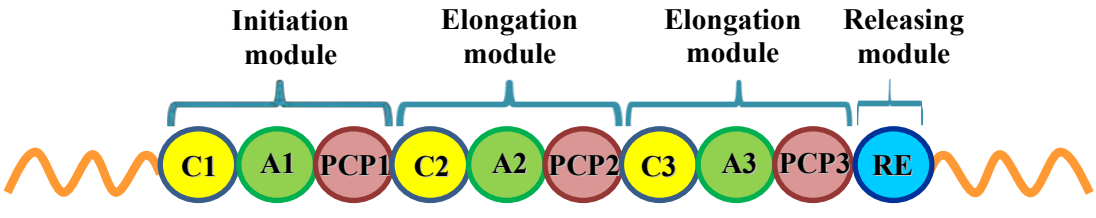


Figure 2.6 The core domains of nonribosomal peptide synthetases.

Table 2.2 The consensus sequence of the conserved motifs of NRPS domains.

Domain	Core motifs	Consensus sequence (Konz and Marahiel, 1999)
Adenylation	A1	L(TS)YxEL
	A2 (core1)	LKAGxAYL(VL)P(LI)D
	A3 (core2)	LAYxxYTSG(ST)TGxPKG
	A4	FDxS
	A5	NxYGPTE
	A6 (core3)	GELxLxGxG(VL)ARGYL
	A7 (core4)	Y(RK)TGDL
	A8 (core5)	GRxDxQVKIRGxRIELGEIE
	A9	LPxYM(IV)P
	A10	NGK(VL)DR
Thiolation	T (core6)	DxFFxLGG(HD)S(LI)
Condensation	C1	SxAQxR(LM)(WY)xL
	C2	RHExLRTxF
	C3	MHHxISDG(WY)xL
	C4	YxD(FY)AVW
	C5	(IV)GxFVNT(QL)(~)xR
	C6	(HN)QD(YV)PFE
	C7	RDxSRNPL
Releasing	RE1	V(LF)(LV)TG(AV)(TN)G(YF)LG
	RE2	VxxxVRA
	RE3	GDL
	RE4	VYPYxxLRx(PL)NVxxT
	RE5	GYxxSKWxx
	RE6	RPG
	RE7	LExx(VI)GFLxxP

Adenylation-(A)-domains control the entry of the substrates into nonribosomal peptide synthesis by selection and activation of the amino (carboxy) acid substrate, as the aminoacyl adenylate by aminoacyl-tRNA synthetases at the expense of ATP (Arnez and Moras, 1997). In the next step, the activated amino acid is transferred onto the thiol moiety of the Ppant prosthetic group attached to the PCP domain. The 4′-phosphopantetheine (Ppant) group of each PCP is posttranslationally introduced onto the side-chain hydroxyl group of a conserved serine residue within the PCPs by a cognate Ppant-transferase (Lambalot *et al*., 1996; Reuter *et al.,* 1999). The PCP represents the transport unit, which enables the activated amino acids and elongation intermediates to move between the catalytic centers. Formation of the peptide bond in nonribosomal peptide synthesis is carried out by the condensation (C) domains which are about 450 amino acids in size and localized between every consecutive pair of A domains and PCPs. The C domains catalyze the formation of the peptide bond between the upstream aminoacyl-or peptidyl-S-PCP moiety and the free amino group of the downstream aminoacyl-S-PCP, thus facilitating the translocation of the growing chain onto the next module. In most NRPS assembly lines a thioesterase-like (Te) domain of about 250 amino acids is found at the C-terminal end of the last module. After transfer of the linear peptidyl intermediate from the last PCP onto the catalytic serine residue of the Te domain (serine is replaced by cysteine in a few examples), this domain catalyzes release of the product by hydrolysis, cyclization, or oligomerization (Mootz *et al*., 2002). The reactions catalyzed by core NRPS domains are shown in Figure 2.7.

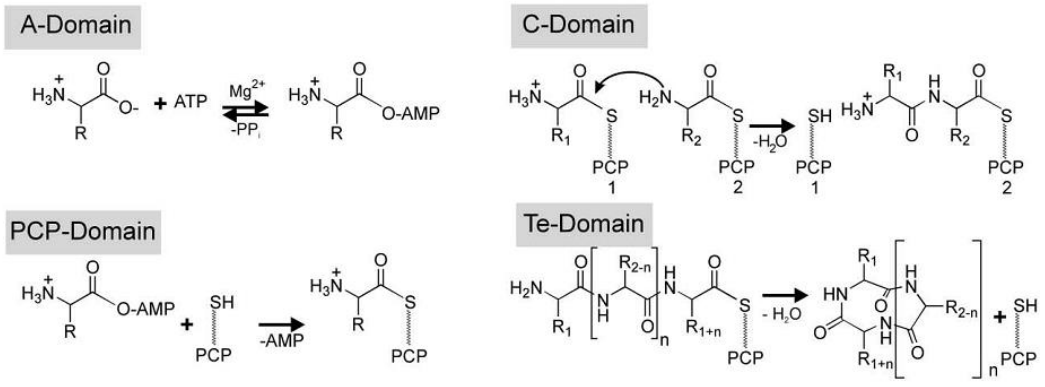


Figure 2.7 Reactions catalyzed by NRPS domains.

In addition, the tailoring enzymes that act on nonribosomal peptides for maturation reactions are crucial for control of biological activity and functional diversity. Some tailoring enzymes are embedded in the NRPS assembly lines and modify the elongating chains (Figure 2.8). The characterization of several types of tailoring enzymatic reactions for NRP was reported including the formation of heterocyclic ring by cyclization (Cy) domains. The formation has been presumed that Cy domains first catalyze peptide bond condensation and then carry out cyclization of the thiol sidechain of cysteine or the hydroxyl sidechain of serine or threonine onto the just-formed peptide bond to form thiohemiaminal or hemiaminal intermediates that are then dehydrated to yield the C=N bond in the thiazoline and oxazoline rings (Gehring *et al*., 1998). Incorporation of thiazole or oxazole rings requires the presence of an additional, oxidation (Ox) domain as part of the accompanying module. Ox-domains are almost exclusively found associated with Cy-domains. Nitrogen atoms of the peptide backbone of nonribosomally synthesized peptides are often *N*-methylated. Methylation of nonribosomal peptides is embedded by methylation (MT) domains using *S*-adenosyl methionine (SAM) as the methyl donor (Haese *et al*., 1994; Burmester *et al*., 1995; Schauwecker *et al*., 2000). In general, the A domains of NRPS assembly lines select the readily available L-amino acids for activation. The presence of D-amino acids in some nonribosomal peptides are operated by epimerization (E) domains via aminoacyl- or peptidyl-*S*-PCP acyl enzyme intermediates (Walsh *et al*., 2001). Formation (F) domains are possibly responsible for the *N*-formylation by means of the cofactor *N*-formyltetrahydrofolate (*N*-formyl- THF). In an alternative mechanism of termination system, the Te-domain is replaced by a reductase-(R)-domain that reduces the C-terminal carboxy group to an aldehyde or even to the corresponding alcohol using NADPH as a cofactor (Silakowski *et al*., 2000; Gaitatzis *et al*., 2001).

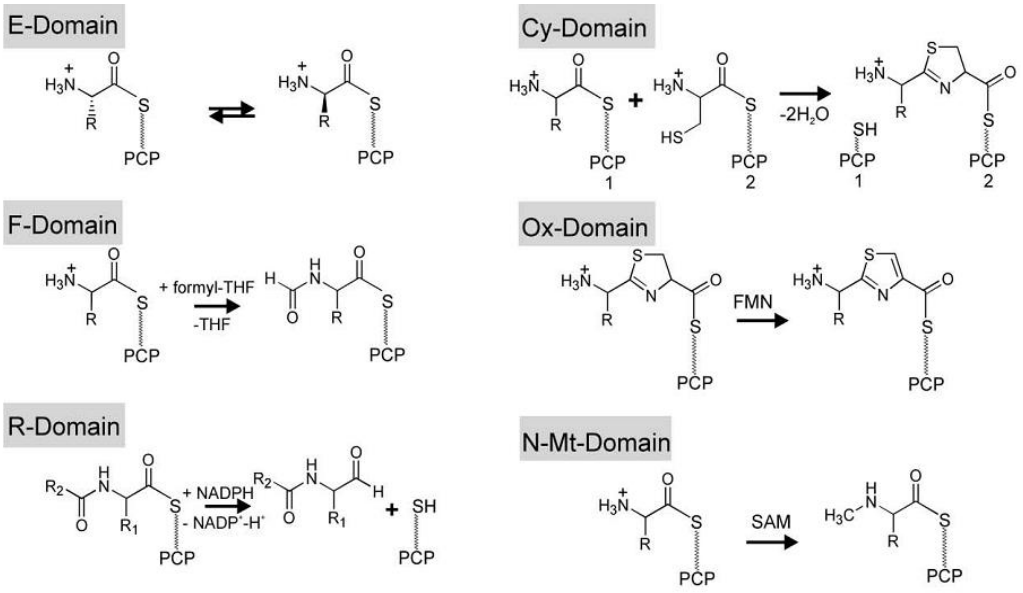
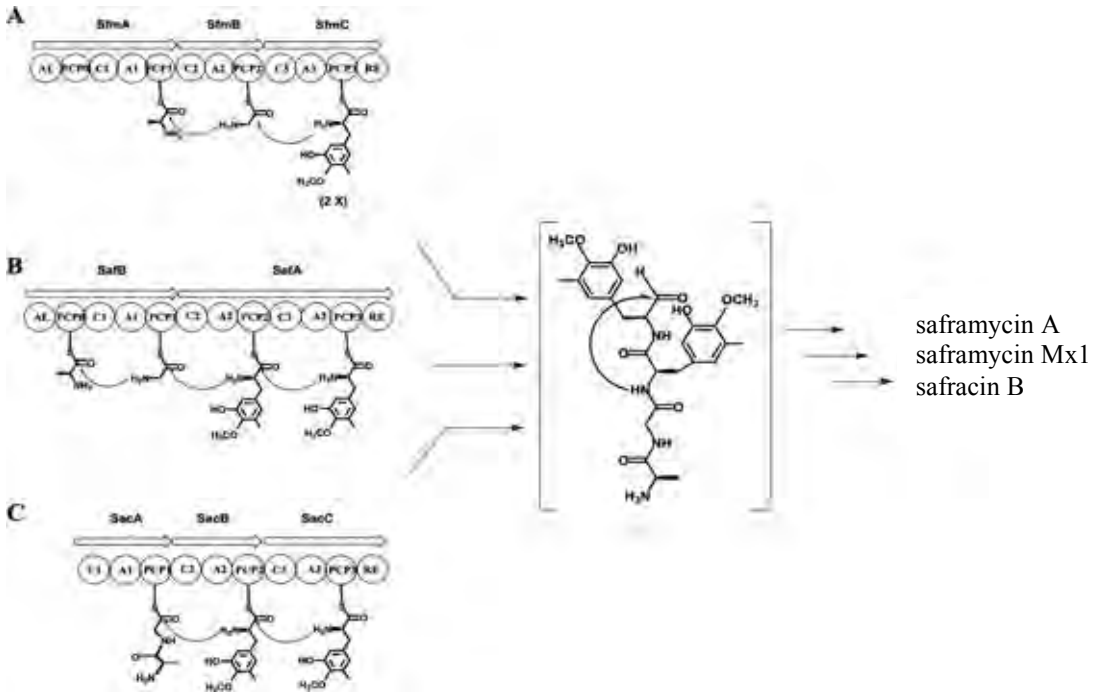


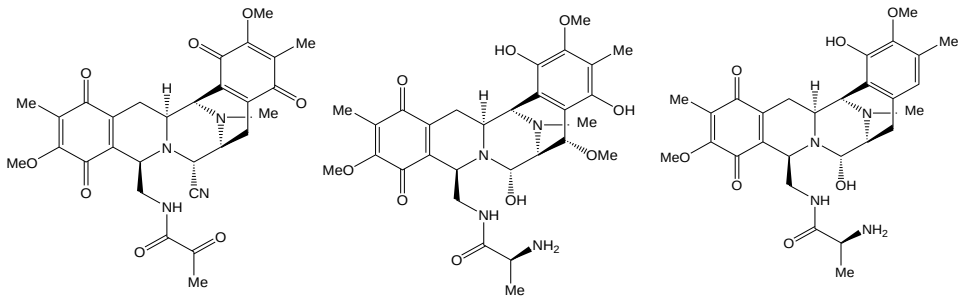
Figure 2.8 The characterization of several types of tailoring enzymatic reactions for NRP natural products.

3.3 Biosynthesis of saframycins and safracins

Saframycins, belonging to the bistetrahydroisoquinoline family of antibiotics, are a group of microbial natural products. There are three NRPS gene clusters for saframycin biosynthesis as shown in Figure 2.9. In 1995, Pospiech *et al*. reported biosynthesis of saframycin Mx1 (SFM-Mx1) produced by *Myxococcus xanthus* (SFM-Mx1). Two large ORFs, named *safA* and *safB* encode the putative SFM-Mx1 NRPSs including SafA 2 modules and SafB 2 modules. In 2005, Velasco *et al*. reported biosynthesis of safracin B (SAC-B) which was isolated from the culture broth of *Pseudomonas fluorescens* strains A2-2 strain obtained from a soil sample in Tagawagun (Fukuoka, Japan) (Ikeda *et al*., 1983). Three large ORFs, named *sacA*, *sacB* and *sacC*, encode the putative safracin NRPSs including SacA, SacB and SacC, respectively. They showed a high similarity to three (Gly, Tyr and Tyr) of the four amino-acid-activating modules found in the saframycin NRPS cluster of *M. xanthus.* In 2008, Li *et al*. reported biosynthesis of saframycin A (SFM-A) produced by *Streptomyces lavendulae* NRRL 11002. SFM-A is likely to be synthesized by a similar NRPS mechanism. SFM-A biosynthesis contains three NRPS genes, including *sfmA*, *sfmB* and *sfmC* within the *sfm* cluster. Previous feeding experiments using

isotope-labeled substrates showed that the backbone of SFM-A is derived from one alanine (Ala), one glycine (Gly), and two tyrosine (Tyr) derivatives, suggesting that it is of tetrapeptide origin (Figure 2.10) (Mikami *et al*., 1985). Strongly supporting results, the SFM-A NRPS system is considered to be three modules including SfmA (C1-A1-PCP1), SfmB (C2-A2-PCP2), and SfmC (C3-A3-PCP3-RE) showing exclusive activities with L-Ala, L-Gly, and L-3h5m*O*mTyr, respectively. The last SfmC module acts twice to incorporate two L-3h5m*O*mTyr residues into the tetrapeptide (Figure 2.9A). SfmA, SfmB, and SfmC constitute an NRPS system that exhibits similarities in domain organization and amino sequence from head to tail to those for SFM-Mx1 (Figure 2.9B) and SAC-B (Figure 2.9C) biosynthesis. Based on the colinearity rule (Marahiel *et al*., 1997), the NRPS module organization parallels the order of the amino acid residues in the resultant polypeptide, sequential incorporation of Ala, Gly, and two Tyr derivatives into the tetrapeptide in SFM-Mx1 biosynthesis was previously considered to be directed by four successive modules including SafB (AL-PCP0), SafB (C1-A1-PCP1), SafA (C2-A2-PCP2), and SafA (C3-A3-PCP3-RE) (Figure 2.9B) (Pospiech *et al*., 1996). Since SacA in SAC-B biosynthesis lacks the first module AL-PCP0, a bifunctional adenylation activation by SacA or direct incorporation of an Ala-Gly dipeptide into the tetrapeptide by SacA was hypothesized (Figure 2.9C) (Velasco *et al*., 2005). In both systems, the last two modules (SafA-C2-A2-PCP2 and SafA-C3-A3-PCP3-RE or SacB-C2-A2-PCP2 and SacC-C3-A3-PCP3-RE) were suggested to be responsible for activation and incorporation of each Tyr derivative, 3-hydroxy-5-methy-*O*-methyltyrosine (3h5m*O*mTyr). We hypothesized that renieramycins are biosynthesized in a similar manner to that of SFM-A, SFM-Mx1 and SAC-B according to their bistetrahydroisoquinoline conserved structure.





Saframycin A Saframycin Mx1 Safracin B

Figure 2.9 Nonribosomal peptide synthetase genes involved in biosynthesis of saframycin A (A), saframycin Mx1 (B), and safracin B (C), respectively.

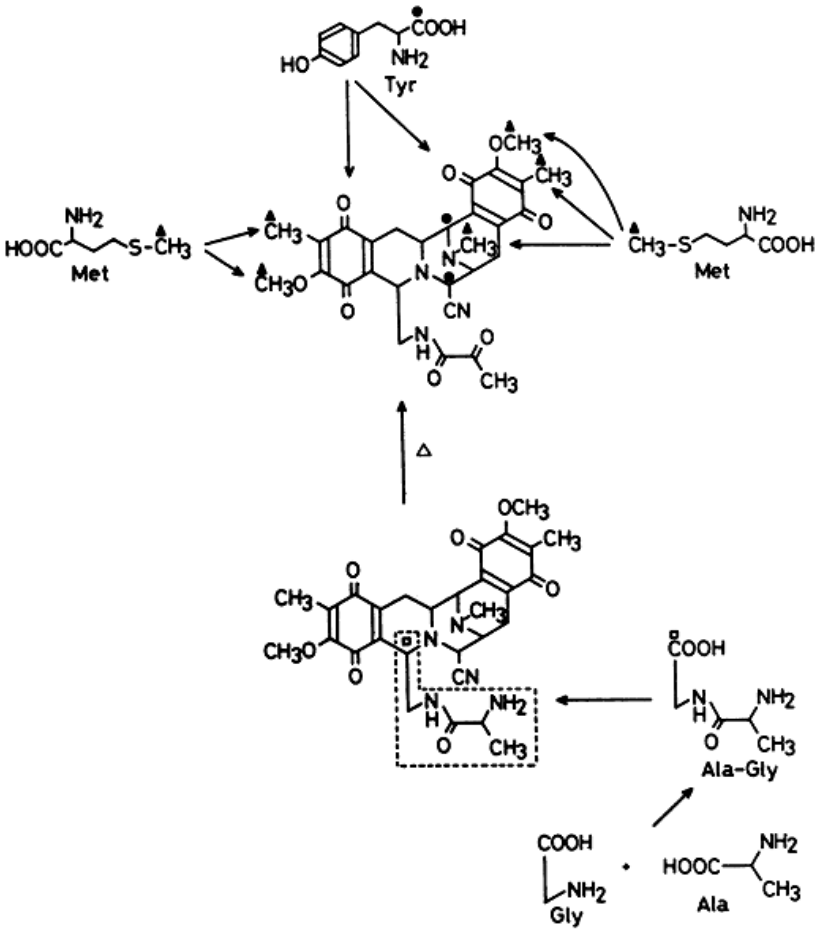


Figure 2.10 Feeding experiments using isotope-labeled substrates of saframycin A.

4. Reactive oxygen species

Reactive Oxygen Species (ROS) are chemically reactive molecules containing oxygen molecules which are normally produced by cellular metabolic process. Although ROS are essential for cellular biological functions, excessive production of ROS or depletion of cellular antioxidant molecules and/or enzymes lead to oxidative stress and subsequent cell damage. Cellular oxydative stress has been widely studied and found to be involved in several diseases such as cancer, neurodegenerative disorders, arteriosclerosis and others (Valko *et al*., 2004). In cancer cells, high levels of ROS can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases (Majno and Joris, 1995; Trump *et al*., 1997). For instance, ROS in cancer are involved in cell cycle progression and proliferation, cell

survival and apoptosis, cell morphology, cell-cell adhesion (Savill and Fadok, 2000; Kurosaka *et al*., 2003; Pelicano *et al*., 2004).

ROS are highly reactive because they contain unpaired electron which generated though oxygen reduction resulting in the production of reactive species which can be descripted as free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$) and non-radicals, specifically hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) (Circu and Aw, 2010).

5. Apoptosis and necrosis

Oxydative stress modulates apoptotic or necrotic cell death (Chandra *et al*., 2000; Miguel, 2007; Lin *et al*., 2010). Apoptosis is one type of cell death responsible for the development and repair process of human body and has been long documented as a distinctive model of programmed cell death (Elmore, 2007). Also, this type of death is the major mechanism of human body for eliminating un-wanted and damaged cells (Budihardjo *et al*., 1999; Norbury and Hickson, 2001). The process of apoptosis involves the activation of several signals and proteins in the well-controlled fashion triggered by the defined stimuli and suppressed by the threshold of the cells. If the trigger is reached the cells, the caspases will be finally activated to cause cell death (Budihardjo *et al*., 1999; Ghobrial *et al*., 2005; Ashkenazi, 2008). At the final step of apoptosis, all cell compartments are wrapped up in the vesicles called apoptotic bodies which will be consumed by the immune cells (Hacker, 2000). Unlike apoptosis, necrosis is considered as a toxic death of unspecifically injured cells by two main mechanisms: intervention with the energy supply of the cells and direct damage to cell membranes (Levin *et al*., 1999). Major morphological changes occurring during necrosis include cell swelling, formation of cytoplasmic vacuoles, distended endoplasmic reticulum, disrupted organelle membranes, swollen and ruptured lysosomes, and eventually disruption of the cell membranes (Kerr *et al*., 1972; Majno and Joris, 1995). Not only is the necrosis cell death unspecific, but also it damages the surrounding cells and tissues by the releasing cytoplasmic components and induces the severe active immune response and inflammation (Trump *et al*., 1997; Savill and Fadok, 2000; Kurosaka *et al*., 2003). Together, even though the cell killing property of substances is of interest for anti-cancer drug development, many

promising candidates are cut off because of these unspecific necrotic responses and related complications. Morphological features of apoptosis and necrosis are shown in Figure 2.11.

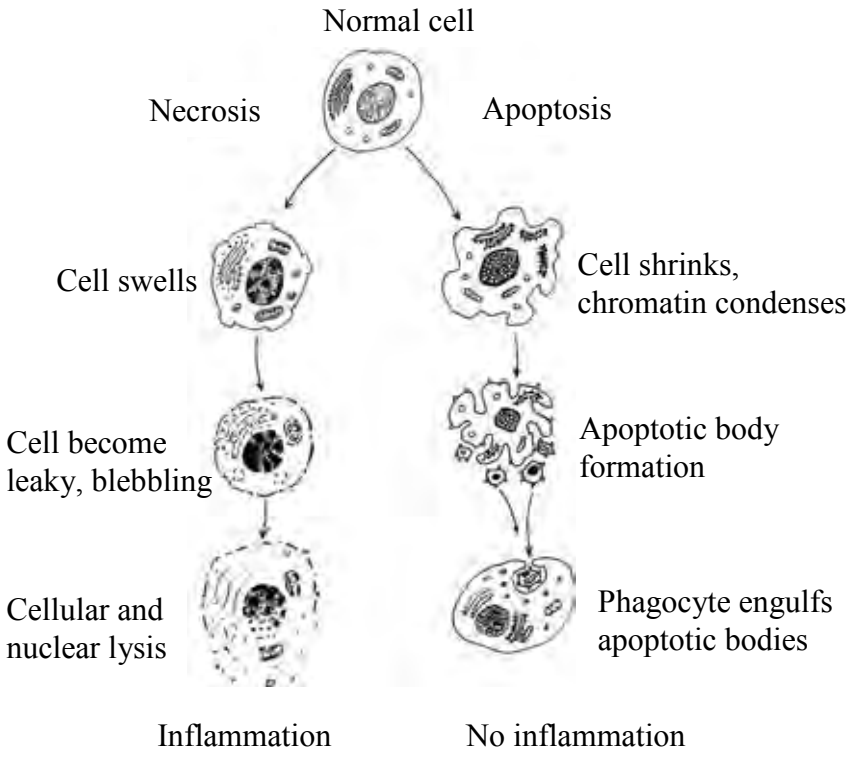


Figure 2.11 Morphological features of apoptosis and necrosis (www.google.com).

CHAPTER III

IDENTIFICATION OF NONRIBOSOMAL PEPTIDE SYNTHETASE GENE INVOLVED IN BIOSYNTHESIS OF RENIERAMYCINS FROM THE BLUE SPONGE *XESTOSPONGIA* SP.

The similarity of the conserved core pentacyclic ring of renieramycin M (RM) to saframycin A (SFM-A), saframycin Mx1 (SFM-Mx1) and safracin B (SAC-B), suggested that these compounds are likely to be synthesized by a similar nonribosomal peptide synthetase (NRPS) mechanism. Along with the previous reports indicated that the pentacyclic backbone of RM may derive from one glycine (Gly), and two tyrosine-derived residues, suggesting that it is of tripeptide origin. However, biosynthesis of RM has not yet been defined. In this study, NRPS gene involved in renieramycins biosynthesis was identified by PCR-based screening. Degenerate primers were designed by amino acid alignment encoded by *SfmC* (for SFM-A), *SafA* (for SFM-Mx1) and *SacC* (for SAC-B). These positions of NRPS genes encoded for the assembly of two units of 3-hydroxy-5-methy-*O*-methyltyrosine (3h5m*O*mTyr), which are the precursors of the conserved pentacyclic ring. However, most DNA preparations based on molecular biology techniques require good DNA in both quantity and quality to provide fine materials for subsequently stages such as PCR, gene cloning, gene library construction and metagenomics (Schmitt *et al*., 2007; Mohamed *et al*., 2008).

In this study, DNA samples obtained as a mixture were isolated from *Xestospongia* sp. sponge and its inescapably-associated microorganisms, resulting in simplicity of sample preparation and avoidance of difficulties associated with culturing environmental microbes (Handelsman *et al*., 1998). The metagenomic approach was primarily utilized to investigate gene(s) involved in renieramycins biosynthesis which might be present either in the marine sponge *Xestospongia* sp. or its associated microorganism community. From our preliminary attempt, several DNA extraction protocols, such as MasterPure® DNA extraction kit, CTAB protocol, SDS/phenol extraction, SDS/CTAB/phenol extraction, and NaOAc salting-out extraction (Maria, 2004; Weising, 2005; Schirmer *et al*., 2005; Aguilera, 2006;

Ferara, 2006; Farrugia *et al*., 2010) were comparatively employed for the *Xestospongia* sponge samples however the obtained DNAs from every protocol were unsatisfied in term of their quality and quantity. Therefore, we hypothesized that the sponge sample might contain highly cytotoxic renieramycins causing a problem for DNA extraction and leading to the low in both DNA quantity and DNA quality. In this work, we developed an efficient extraction method to prepare DNA from the Thai marine sponge *Xestospongia* sp. by preremoval of renieramycins from the sponge samples. The direct DNA-damaging effect of cytotoxic renieramycins was examined by using the double-strand plasmid DNA pBR322.

1. Materials and methods

1.1 Source of the blue sponge *Xestospongia* sp.

The sponge *Xestospongia* sp. (Class: *Demospongiae*, Order: *Haplosclerida*, Family: *Petrosiidae*) is a bluish sponge occurring commonly in the coral reef in the Gulf of Thailand. The marine blue sponge *Xestospongia* sp. was identified by Dr. John N.A. Hooper. The voucher specimens have been deposited at Queensland Museum (serial No. QM G306998), Australia and the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

For this study, the sponge samples were taken from Sichang Island, Cholburi Province, on October 2011 by SCUBA diving at the depth range of 3-5 meters. The living samples were cleaned up the contaminated organisms before being carried to our laboratory and stored at -20°C prior to DNA extraction.

1.2 Reagents and enzymes

1.2.1 Chemical and biological reagents

Absolute ethanol (Wako, USA)
Acetic acid (Merck, Germany)
Agarose (SeaKem®, USA)
Ampicilin antibiotic (Wako, USA)
Chloramphenicol antibiotic (Wako, USA)
Cetyl trimethylaceticbromide: CTAB (Fluka®, USA)

Chloroform/isoamylalcohol (24:1) (Fluka®, USA)

Deoxynucleoside triphosphates (dNTPs) (TaKaRa Bio Inc., Japan)

Ethylacetate, AR grade (Lab scan® Germany)

Ethylenediaminetetraacetic acid (Dojindo Inc., Japan)

Isopropanol (Lab scan® Germany)

LB medium (Macalai tesque, Japan)

LB agar (Macalai tesque, Japan)

Liquid nitrogen

Lysozyme (Vivantis™, Malaysia)

Magnesium chloride (TaKaRa Bio Inc., Japan)

Phenol/chloroform/isoamylalcohol (25:24:1) (Fluka®, USA)

Primers (Operon, Japan)

Proteinase K (Wako, USA)

RNase A (Wako, USA)

Sodium acetate (Macalai tesque, Japan)

Sodium chloride (Wako, USA)

Sodium dodecyl sulphate (Macalai tesque, Japan)

Sodium sulfate, anhydrous (Merck, USA)

Steriled ultrapure water

Taq DNA polymerase (TaKaRa Bio Inc., Japan)

Tris-Hydrochloride (Macalai tesque, Japan)

10x PCR amplification buffer (TaKaRa Bio Inc., Japan)

1.2.2 Enzymes and Kits

CopyControl™ fosmid library production kit (Epicentre®, USA)

DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Switzerland)

DNA marker: 1 kb DNA Extension Ladder (Invitrogen, USA)

DNA marker: Gene Ladder Wide 1; Wide 2 (Wako, USA)

DNA marker: VC Lambda/*Hin*dIII marker (Vivantis™, Malaysia)

KAPATaqExtra (Kapasystems, USA)

pT7-Blue vector (Novagen, USA)

TaKaRa Ex Taq™ (TaKaRa Bio Inc., Japan)

Wizard® Plus SV MiniPreps DNA Purification System (Promega, USA)
Wizard® SV Gel and PCR Clean-Up System (Promega, USA)

1.3 Effect of renieramycins on quality and quantity of DNA from the sponge tissue

1.3.1 Effect of renieramycins on DNA preparation

1.3.1.1 Removal of renieramycins from the sponge tissue

Renieramycin alkaloids were gradually removed from the *Xestospongia* sponge sample by methanol extraction. Each 200 mg wet weight of the sample (50 mg dry weight) was treated with 1 ml of 15 mM potassium cyanide in phosphate buffer solution for 5 hr and then repeatedly macerated with methanol to extract renieramycins at six different times (0, 2, 4, 6, 8, and 10 times; 1 ml methanol for 30 min, each time). The sponge residue after complete extraction was subjected to determine renieramycin amount by HPLC analyses. Each experiment was performed in triplicate.

1.3.1.2 Determination of renieramycins amount in the sponge tissue

After complete extraction, each *Xestospongia* sponge sample was further extracted with methanol (2 ml) overnight and the centrifuged for 5 min (500 × *g*). One ml of the methanol supernatant was mixed with 4 ml of water and subsequently partitioned with 2 ml of ethyl acetate. One-ml portion of the ethyl acetate layer was evaporated to dryness and redissolved in 200 µl of methanol for further HPLC analysis of the remaining renieramycin amount in each sample. The HPLC analysis was performed on a Shimadzu LD-10AD HPLC system equipped with a Shimadzu SPD-10A UV/VIS detector at a wavelength of 270 nm. A LiChrospher 100 RP-18 HPLC column (5 µm, 4.6 × 125 mm) was used with 20 µl injection volume. A mixture of methanol:water (7:3) was used as the mobile phase at a flow rate of 1 ml/min. Quantitative determination of renieramycins was carried out by means of the standard curve of renieramycin M (RM) previously isolated from the sponge *Xestospongia* sp. (Suwanborirux *et al*., 2003) as the standard compound (retention time = 7.5 min) and acenaphthene (30 µg/ml) as the internal standard. Each experiment was performed in triplicate.

1.3.1.3 Genomic DNA extraction from the sponge tissue

Each new 200 mg wet weight from the same *Xestospongia* sponge sample (50 mg dry weight) was repeatedly macerated with methanol to remove renieramycins prior to DNA extraction as previous described in 1.3.1.1. The sponge sample after final methanol maceration was dried and DNA from each sample was extracted by modified sodium acetate salting-out method (Farrugia *et al*., 2010). The sponge sample was ground under liquid nitrogen and dispersed in 750 µl of lysis buffer (20% SDS, 5 mM EDTA, and 10 mM Tris-HCl). After centrifugation for 5 min (500 × *g* at 4°C), the supernatant was transferred to a new tube and incubated with proteinase K (10 µg/µl) at 56°C for 3 h and further mixed with RNase A (10 µg/µl) at 37°C for 1 h. The debris was precipitated with 250 µl of 5 M sodium acetate. The aqueous phase containing nucleic acids was centrifuged for 5 min (5,000 × *g* at 4°C). The supernatant was transferred to a new tube, added 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of cold absolute ethanol and kept at -20°C for 30 min. DNA was carefully spooled from the aqueous phase which was further precipitated by centrifuging for 30 min (13,000 × *g* at 4°C). DNA pellet was collected and combined with the spooled DNA to redissolve in 50 µl of sterile ultrapure water. DNA was further purified by 5 µl of cetyltrimethylammonium bromide (CTAB) solution (0.3% CTAB and 5 M NaCl) and incubated at 60°C for 1 h. After centrifugation for 10 min (13,000 × *g* at 4°C), the supernatant was transferred to a new tube. The DNA was finally precipitated by adding absolute ethanol and centrifuged for DNA pellet collection. DNA extraction for each sample was made in triplicate. The extracted DNA was used for comparating the quality and quantity in 1.3.1.4.

1.3.1.4 Evaluation of the extracted DNA

The extracted DNA was examined for its purity and quantity by spectrophotometry. To prepare a sample solution, 1 µl of DNA sample was mixed thoroughly with 49 µl of ultrapure water into a 50 µl disposable cuvette (Hitachi High-Tech™, Japan). The UV maximal absorptions of the DNA solution were measured on a UV1700 spectrophotometer, Shimadzu, Japan at the wavelengths of 260 nm (A_{260}) and 28 nm (A_{280}). The ratio of the absorptions at 260 nm to 280 nm is

commonly used to calculate the purity of DNA with respect to protein contamination, since proteins (in particular, the aromatic amino acids) regularly absorb at 280 nm. The acceptable purity level of the extracted DNA was estimated by calculating A_{260} to A_{280} ratio with an expected value range of 1.8-2.0 (Sambrook and Russell, 2001). The yield of the obtained DNA was quatified from A_{260}. The concentration of double-stranded DNA can be comparatively calculated from the below equation (Sambrook and Russell, 2001).

1 OD_{260} unit = 50 μg/ml double-stranded DNA

In addition, the quantity of the extracted DNA was determined by PCR amplification. The several dilution of extracted DNA obtainable from with or without RM elimination sponge samples were used for PCR template. Each PCR reaction contained universal bacterial primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T) designed to amplify the conserved 16S rRNA fragment of bacteria (Frank *et al*., 2008; Rani *et al*., 2009). Fifty μl of the PCR mixtures contained 1 μl of the extracted DNA solution, 4 μl of dNTP mixture (2.5 mM each of the four deoxynucleoside triphosphates; dTTP, dCTP, dGTP, and dATP), 4 μl of 25 mM $MgCl_2$, 0.2-1.0 μM of each primer (final concentration), 5 μl of 10X *Ex Taq* buffer, 0.25 μl of 5 U of *TaKaRa Ex Taq* DNA polymerase and sterile water adjusted to 50 μl (TaKaRa Bio Inc., Japan). All PCR reactions were run by a thermocycler (Labnet, Edison, USA) as follows: 94°C for 2 min, followed by 35 cycles of 94°C (30 sec), 52.5°C (30 sec) and 72°C (2 min), and a final elongation step of 72°C for 10 min. The PCR product (approximately 1,400 bp) was electrophoresed on a 1% agarose gel, run in Tris-acetic acid-EDTA (TAE) buffer at 100 V for 30 min and stained with ethidium bromide. The intensity of DNA band was visualized and compared between different template DNAs obtained from with or without renieramycins elimination.

1.3.2 Effect of quinone-generated free radicals from renieramycins on DNA damage

1.3.2.1 Synthesis of 5,8,15,18-tetra-*O*-acetylbishydroquinone renieramycin M

A solution of renieramycin M (RM) (10 mg, 0.0173 mmol) in ethyl acetate (3 ml) was hydrogenated over 10% Pd/C (6.0 mg) at 1 atm for 4 h. The catalyst was removed by filtration and washed with ethyl acetate. The combined filtrate was evaporated to dryness to obtain the hydroquinone RM. This crude material was used in the next step without further purification. The hydroquinone RM was acetylated with 4.5 equivalents of acetic anhydride (8 µl) in pyridine (0.5 ml) and the reaction mixture was stirred for 4 h at room temperature under argon atmosphere. The reaction was quenched with 5 ml distilled water and the mixture was extracted with dichloromethane (3×5 ml). The combined dichloromethane extract was evaporated to dryness and subjected to chromatographic purification on a silica gel column using a mixture solution of ethylacetate:hexane (2:8) as the eluent to afford 5,8,15,18-tetra-*O*-acetylbishydroquinone renieramycin M (8 mg, 80% yield) as a colorless solid. The compound was identified by comparing the NMR spectral data with the literature (Amnuoypol *et al*., 2004).

^{1}H NMR (300 MHz) spectrum was obtained with a Bruker Avance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University). Solvent for NMR measurement was deuterated chloroform ($CDCl_3$). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal. The spectral data are shown in Table 3.1.

Table 3.1 ^{1}H-NMR spectral data of 5,8,15,18-tetra-*O*-acetylbishydroquinone renieramycin M in $CDCl_3$.

proton	**5,8,15,18-tetra-*O*-acetyl bishydroquinone RM**	**5,8,15,18-tetra-*O*-acetyl bishydroquinone RM (Amnuoypol *et al*., 2004)**
1-H	4.07 (1H, m)	4.10 (1H, m)
3-H	3.21 (1H, d, 11.6)	3.22 (1H, ddd, 11.6, 2.6, 2.3)
4-Hβ	1.84 (1H, dd, 15.4, 11.6)	1.89 (1H, dd, 15.5, 11.6)
4-Hα	2.66 (1H, d, 15.4)	2.71 (1H, dd, 15.5, 2.6)
11-H	3.68 (1H, d, 2.3)	3.68 (1H, d, 2.3)
13-H	3.34 (1H, overlap)	3.33 (1H, ddd, 7.9, 2.6, 1.3)
14-Hβ	2.06 (1H, overlap)	2.27 (1H, d, 17.8)
14-Hα	2.71 (1H, overlap)	2.82 (1H, dd, 17.8, 7.9)
21-H	4.00 (1H, d, 2.6)	4.00 (1H, d, 2.6)
22-Ha	3.69 (1H, m)	3.68 (1H, m)
22-Hb	4.07 (1H, m)	4.10 (1H, m)
6-CH_3	2.06 (3H, s)	2.06 (3H, s)
16-CH_3	2.04 (3H, s)	2.04 (3H, s)
7-OCH_3	3.71 (3H, s)	3.71 (3H, s)
17-OCH_3	3.75 (3H, s)	3.74 (3H, s)
12-NCH_3	2.06 (3H, s)	2.23 (3H, s)
$OCO\underline{CH_3}$	2.35 (3H,s)	2.38 (3H,s)
	2.31 (3H,s)	2.37 (3H,s)
	2.26 (3H,s)	2.33 (3H,s)
	2.24 (3H,s)	2.28 (3H,s)
26-H	6.01 (1H, overlap)	6.05 (1H, qq, 7.3, 1.7)
27-H_3	1.94 (3H, overlap)	1.96 (3H, dq, 7.3, 1.7)
28-H_3	1.68 (3H, overlap)	1.70 (3H, dq, 1.7, 1.5)

1.3.2.2 Effect of renieramycins on pBR322 plasmid DNA

The solutions of renieramycin derivatives, including RM and 5,8,15,18-tetra-*O*-acetylbishydroquinone renieramycin M, were prepared by dissolving in methanol and potassium phosphate buffer to achieve indicated concentrations (0.006, 0.06, 0.15, 0.30, and 1.5 mM) containing less than 10% methanol. Twenty µl of each sample solution was mixed with 1 µl (100 ng) of pBR322 plasmid DNA (Vivantis, Malaysia) and incubated at 56ºC for 3 h. The reaction mixture was electrophoresed on a 1% agarose gel, run in TAE buffer at 100 V for 30 min. After electrophoresis, the gel was stained with ethidium bromide and visualized under ultraviolet light. The band intensities of the supercoiled DNA form (SC) and opened circular DNA form (OC) of pBR322 plasmid DNA were measured with MiniBIS Gel Documentation and analyzed with Gel Quant Analysis (DNR BioImaging Systems, Jerusalem, Israel). All experiments were run in triplicate.

1.3.2.3 Determination of free radicals affecting on pBR322 plasmid DNA damage

Trolox was used as a free radical scavenger. Each reaction mixture (21 µl) contained 10 µl of 0.15 mM RM (dissolved in potassium phosphate buffer containing less than 10% methanol), 10 µl of Trolox in final concentrations (1.25 or 2.5 mM), and 1 µl of 100 ng pBR322 plasmid DNA. After incubation at 56ºC for 3 h, the mixture was added with 5 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose in water) and then loaded onto a 1% agarose gel and visualized as previous described in 1.3.2.2.

1.4 General techniques in genetic engineering

1.4.1 DNA preparation

The 200 mg wet weight of the *Xestospongia* sponge sample (50 mg dry weight) was repeatedly macerated with methanol to remove renieramycins prior to DNA extraction at optimal 8 times (1 ml methanol for 30 min, each time). The sponge sample after final methanol maceration was dried and DNA from each sample was extracted by modified sodium acetate salting-out method as previous described in 1.3.1.3.

1.4.2 Degenerate primer design

Several degenerate primers were generally designed to obtain gene sequence information ranged from A domain through RE domain in an NRPS system. The degenerate primers in this study were designed by CLC Sequences Viewer version 6.3 from the conserved regions of amino acid alignment of genes *SfmC* for saframycin A from *Streptomyces lavendulae* (accession number: DQ838002) (Li *et al*., 2008), *SafA* for saframycin Mx1 from *Myxococcus xanthus* (accession number: MXU24657) (Pospiech *et al*., 1995) and *SfcC* (or *SacC*) for safracin B from *Pseudomonas fluorescens* (accession number: AY061859) (Velasco *et al*., 2005) that were retrieved from National Center for Biotechnology Information (NCBI). The designed primers were synthesized by Eurofins Operon, Japan.

1.4.3 PCR amplification

The genomic DNA extracted from the *Xestospongia* sponge was used as templates for PCR amplification of NRPS genes involved in renieramycin biosynthesis using TaKaRa Ex Taq^{TM} Polymerase (TaKaRa Bio Inc., Japan), following the manufacturer's protocol as pevious described in 1.3.1.4. PCR reaction conditions were started with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 30 sec, extension at 72°C for 1.30 min, and a final extension at 72°C for 5 min, then held at 4°C. PCR amplification was carried out in a MultiGene Thermalcycler (TaKaRa Bio Inc., Japan). The PCR products were run on a 1% agarose gel with Lambda marker. An expected size of the PCR product was approximately 770 bp. Following electrophoresis, PCR product band was excised from gel based on DNA marker bands and gel slice was placed in a 1.5 ml microcentrifuge tube. Ten µl per 10 mg of gel slice of membrane binding solution was added, vortexed and incubated at 60-70°C until gel slice is complete dissolved. The dissolved gel mixture was transferred to minicolumn assembly (insert minicolumn into a collection tube) and then centrifuged at 15,000 × *g* for 1 min. The flowthrough supernatant was discarded from the collection tube and reinserted the minicolumn into a collection tube. The washing step started by adding 700 µl of membrane wash solution to the column and centrifuging at 15,000 × *g* for 1 min (repeat 2 times). The collection tube was

discarded and the column assembly was recentrifuged for 1 min with the microcentrifuge lid open to evaporate residual ethanol and then carefully transferred the minicolumn to a 1.5 ml microcentrifuge tube. The expected PCR product was eluted from the column by 50 µl of nuclease-free water, incubated at room temperature for 1 min and centrifuged at 15,000 × *g* for 1 min. The PCR product was then cloned as described in 1.4.4

1.4.4 Cloning of a partial NRPS gene encoding for the assembly of tyrosine derivatives

1.4.4.1 Preparation of *E. coli* competent cells

The overnight culture (200 µl) of *E. coli* DH5α was inoculated into 100 ml of LB medium, the grown at 37°C, 250 rpm until OD_{600} reached 0.3-0.4 (usually 2-3 h). The culture was placed on ice for 30 min and centrifuged at 5000 × *g*, 4°C for 10 min. The medium was removed and the cell pellet was gently resuspended with 10 ml of 100 mM cold $CaCl_2$ on ice. After incubation on ice for 30 min and centrifugation at 5000 × *g*, 4°C for 10 min, the supernatant was discarded and then the pellet was resuspended in 1.8 ml of cold 100 mM $CaCl_2$ and 0.4 ml of sterile glycerol. The cell suspension was aliquot to 100 µl per tube on ice prior to immediately keeping in liquid nitrogen. The competent cells were finally stored at -80°C until use.

1.4.4.2 Ligation and transformation of NRPS gene

The PCR products of NRPS fragments already added adenine at 3′ end by Taq DNA polymerase were ligated into pT7Blue vector, T/A linearized vector (Promega, USA), prior to transforming to *Escherichia coli* DH5α competent cells. Each PCR product (3 µl) was ligated with pT7Blue vector (1 µl) using 1 µl of T4 DNA ligase enzyme in 5 µl of 2× ligation buffer. The ligation mixture was incubated at 16°C for 3 h and the ligation products were transformed to *E. coli* DH5α competent cells by heat shock method. The competent cells were removed from the -80°C freezer and thawed on ice. After thawing, 100 µl of the cells was mixed with 5 µl of the ligation mixture and incubated on ice for 30 min. The cell mixture was heat shocked at 42°C for 90 sec and rapidly placed on ice for 5 min. The cell mixture was then added with 300 µl of LB (Luria-Bertani) medium (1% w/v tryptone, 0.5% w/v yeast extract and 0.5% w/v NaCl) and incubated at37°C for 1 h.

1.4.4.3 Selection of recombinant clones

The recombinant clones were selected using blue/white selection technique. The LB-Amp (LB medium with ampicillin) plates were prepared earlier by adding ampicillin, 50 μg ml^{-1} (25 μl). The 200 μl of cell culture was spreaded with 2% 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide (DMF) (40 μl) and 100 mM isopropylthio-β-galactoside (IPTG) (40 μl) on a LB-Amp plate. The recombinant *E. coli* cell mixture was plated onto the X-gal plate and incubated at 37°C overnight. White colonies were randomly chosen from the overnight plates and checked for correct size of inserts by colony PCR. The PCR cycling program was the same as that of PCR product amplification. The size of colony PCR products was determined by gel agarose electrophoresis. The colonies inserted with expected-size PCR products were cultured in LB-Amp broth at 37°C overnight for plasmid extraction.

1.4.5 Plasmid extraction

Plasmids containing expected inserts were isolated from *E. coli* DH5α transformed cells using Wizard® Plus SV MiniPreps DNA Purification System (Promega, USA). Cells were grown in 5 ml of LB-Amp medium at 37°C overnight and collected by centrifugation at 8,000 × *g* for 5 min at room temperatre. The cell pellet were resuspended in a 1.5 ml microcentrifuge tube with 250 μl of cell resuspension solution and added 250 μl of cell lysis solution to each sample, inverted 4 times to mix. Alkaline protease solution (10 μl) was added, inverted 4 times to mix and incubated 5 min at room temperatre. Neutralization solution (350 μl) was then added and inverted 4 times to mix. The supernatant was collected by centrifugation at 15,000 × *g* for 10 min at room temperature. The plasmid solution was purified through spin column with 750 μl of wash solution. The plasmid DNA was finally eluted from the column with 50 μl of nuclease-free water and kept in -20°C. The purified plasmids were used as templates for nucleotide sequencing by an ABI Prism 3100 genetic analyzer (Applied Biosystems Hitachi, USA).

1.4.6 Nucleotide sequence analysis

The purified plasmids containing expected inserts were subjected to cycle sequencing by ABI PRISM® dGTP BigDye™ Terminators v3.0 Ready Reaction

Cycle Sequencing Kit using 96-well MicroAmp® reaction plate. Twenty µl of the PCR mixtures contained template as described in Table 3.2, 1 µl of 3.2 pmol/µl primer, 8 µl of Terminator Ready Reaction Mix (ABI PRISM® DNA polymerase), and denionized water adjusted to 20 µl (ABI Applied Biosystems, USA). The cycle sequencing was started with an initial denaturation step at 96°C for 2 min, followed by 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, extension at 60°C for 4 min, and then held at 4°C. The PCR products were purified by ethanol/sodium acetate precipitation in 96-well reaction plate. The ethanol/sodium acetate solution was prepared by combining the following for 20 µl of each PCR product: 3 µl of 3 M sodium acetate (NaOAc) pH 4.6, 62.5 µl of non-denatured 95% ethanol, and 14.5 µl of denionized water. The 96-well reaction plate was sealed with strip caps prior to inverting the 96-well plate for a few times and left at room temperature for 15 min to precipitate the extension products. The 96-well reaction plate was centrifuged at 4,000 × *g* for 30 min and then removed the supernatant by inverting the plate on the paper towel prior to adding 150 µl of 70% ethanol and centrifuged at 13,000 × *g* for 30 min. After removing supernatant, the sample was dried by centrifuged at 13,000 × *g* for 10 min and then added 10 µl of Hi-Di formamide. The sample was carried out in a MultiGene Thermalcycler (TaKaRa Bio Inc., Japan) for denaturing and then subjected to an ABI Prism 3100 genetic analyzer (Hitachi). The nucleotide sequences were assembled and analyzed by DNA Star software (DNA Star Inc, USA). Homology search analysis and gene alignment were performed using BLAST (http://www.ncbi.nlm.gov/BLAST/) and CLUSTAL W (hpp://www.ebi.ac.uk).

Table 3.2 The amount of template used in a cycle sequencing reaction.

Template	Quantity
PCR product:	
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
1000-2000 bp	10-40 ng
>2000 bp	40-100 ng
Single-stranded	50-100 ng
Double-stranded	200-500 ng
Fosmid, Cosmid, BAC	0.5-1.0 µg
Bacterial genomic DNA	2-3 µg

1.4.7 Construction of genomic library

1.4.7.1 Construction of genomic library by TaKaRa Bio Inc.

Check the titer of genomic DNA transformation

TaKaRa Bio Inc. used three different cloning vectors including pSTV28/HincII-BAP, pUC118/EcoRI-BAP, and Lambda ZAPII/EcoRI to make 9 ligation mixtures. The ligation DNA mixture information is showed in Table 3.3. The ligation DNA mixture (1 µl) was added into *E. coli* HST08 cells (50 µl) and then kept on ice for 30 min prior to heat shock at 42°C for 45 sec and kept on ice for 2 min. The sterile 450 ul of super optimal broth catabolite repression (SOC) medium (2% w/v tryptone, 0.5% w/v Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose) was added to the mixture which was further incubated at 37°C, 200 rpm for 1 h. The transformed cells were spreaded onto the LB agar plate containing appropriate antibiotics (ampicillin, 50 µg/ml, chloramphenicol, 50 µg/ml) after added 2% X-gal in DMF (40 µl), 100 mM IPTG (40 µl). The plate was incubated at 37°C

overnight. The white colonies were checked by colony PCR using vector primers as described in 1.4.4.

Table 3.3 The information of ligation DNA mixture provided by TaKaRa Bio Inc.

<納品物>
サンプル(納品済)

Ligation no. Vector

種類	検討番号	ベクター	状態	液量	チューブ容量
供与検体	-	-	カイメン(生体)	-	50mL
ライブラリー溶液	検討 1	pSTV28/HincII・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL
	検討 2	pSTV28/HincII・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL
	検討 3	pSTV28/HincII・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL
	検討 4	pUC118/HincII・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL
	検討 5	pUC118/EcoRI・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 20μL	自立型 0.5mL
	検討 2-2	pSTV28/HincII・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL
	検討 2-3	pSTV28/HincII・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL
	検討 2-4	Lambda ZAP II/EcoRI	Pharge Packagin 溶液 (-80℃保存)	約 400μL	自立型 2mL
	検討 2-5	pUC118/EcoRI・BAP	ライゲーション DNA 溶液 (-20℃保存)	約 30μL	自立型 0.5mL

1.4.7.2 Construction of genomic library by CopyControl™ Fosmid Library Production Kits

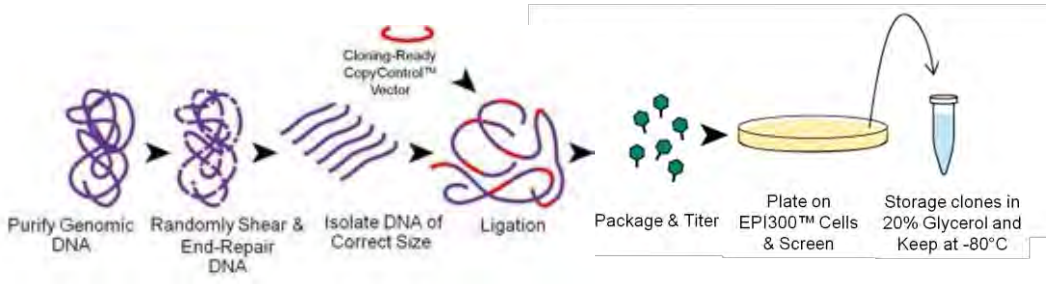


Figure 3.1 Outline of fosmid library construction.

Shearing the insert DNA

The extracted DNA as previously prepared in 1.4.1 was used for fosmid library construction. The genomic DNA was randomly sheared into approximately 40 Kb fragments by passing it through a 200 µl of small bore pipette tip 50-100 times. This process led to the highly random generation of DNA

fragments in contrast to the DNA libraries obtained from partial digestion of the DNA by restriction endonucleases. The shearing extent of DNA was determined by electrophoresis on a 20 cm agarose gel (1%) run in Tris-acetic acid-EDTA (TAE) buffer at 50 V overnight and T7 DNA size marker (100 ng) was used.

End-repairing of the sheared DNA

This step generated blunt-ended, 5'-phosphorylated DNA. The end-repair reaction can be scaled up or scaled down as dictated by the amount of DNA available. All of the reagents listed below were thawed and thoroughly mixed on ice before dispensing. The reactions were incubated at room temperature for 45 min and then added gel loading buffer (10 µl) prior to incubating at 70°C for 10 min to inactivate the end-repair enzyme mixture.

8 µl, 10× End-Repair Buffer
8 µl, 2.5 mM dNTP Mixture
8 µl, 10 mM ATP
X µl, up to 20 µg, sheared insert DNA (approximately 0.5 µg/ml)
4 µl, End-Repair Enzyme Mixture
Y µl, sterile water
80 µl, Total reaction volume

Size selection of the end-repaired DNA

The inactivated end-repaired DNA mixture was subjected to electrophoresis on a 20 cm agarose gel (1%) at 50 V overnight by low melting point (LMP) agarose gel electrophoresis. The end-repaired DNA was visualized and excised by the T7 DNA size marker. The T7 DNA marker lane was stained with ethidium bromide and visualized with UV light. The position of the T7 DNA was marked in the gel using a pasteur pipet. The excised 2-4 mm wide gel was transferd to a sterile 1.5 ml microcentrifuge tube.

Recovery of the size-fractionated DNA

The weight of the gel slices was measured. One mg of solidified agarose was assumed to approximately yield 1 µl of molten agarose. The LMP agarose was melted by incubating the tube at 70°C for 10-15 min and quickly

transfered to 45°C. The appropriate volume of 45°C warmed GELase 50× buffer was added to make 1× final concentration of the buffer. One unit (1 µl) of GELase enzyme preparation was carefully added to the tube for each 100 µl of the molten agarose and incubated at 45°C for 3 h. The reaction was inactivated at 70°C for 10 min. Aliquots (500 µl) of the agarose solution were transferred into sterile 1.5 ml microcentrifuge tubes, chilled in an ice bath for 5 min, and then centrifuged at 10,000 × *g* for 20 min to precipitate insoluble oligosaccharides. The supernatant (~90-95% of the volume) was carefully transferred to a 1.5 ml microcentrifuge tube, added with 1/10 volume of 3 M sodium acetate pH 7 and 2.5 volumes of cold absolute ethanol, mixed by gentle inversion, and kept at -20°C for 30 min. DNA was carefully spooled from the aqueous phase and gently resuspended in 10 µl of sterile water. The concentration of the DNA was estimated by running an aliquot of the DNA on an agarose gel using dilutions of known amounts of the T7 DNA size marker as the standard.

Ligation Reaction

The following reagents were combined in the order listed and mixed thoroughly after each addition. A 10:1 molar ratio of CopyControl pCC1FOS Vector to the insert DNA is optimal. (0.5 µg CopyControl pCC1FOS Vector ~ 0.09 pmoles vector and 0.25 µg of 40 Kb insert DNA ~ 0.009 pmoles insert DNA). The ligation mixture was incubated at room temperature for 2 h and at 70°C for 10 min to inactivate the Fast-Link DNA Ligase.

1 µl, 10X Fast-Link Ligation Buffer
1 µl, 10 mM ATP
1 µl, CopyControl pCC1FOS Vector (0.5 µg/µl)
X µl, concentrated insert DNA (0.25 µg of ~ 40 Kb DNA)
1 µl, Fast-Link DNA Ligase
Y µl, sterile water
---
10 µl, Total reaction volume

Packaging the CopyControl Fosmid Clones

Ten µl of the ligation mixture were added to each 25 µl of the thawed MaxPlax Lambda packaging extracts and incubated at 30°C for 90 min. Additional 25 µl of the MaxPlax Lambda packaging extracts was added to the mixture which was further incubated at 30°C for 90 min. Phage dilution buffer (PDB) was added to make 1 ml final volume. Finally, 25 µl of chloroform was added and gently mixed prior to storing at 4°C.

Titering the Packaged CopyControl Fosmid Clones

Five ml of the *E. coli* EPI300 overnight culture were inoculated with 50 ml of LB broth containing 10 mM $MgSO_4$ and shaked at 37°C until the titer of cells reached OD_{600} = 0.8-1.0. The cells were stored at 4°C unit use.

A serial dilution of the packaged phage particles (1 ml) from the previous step was prepared with PDB in sterile 1.5 ml microcentrifuge tubes.

A) The packaged phage particles solution.
B) 1:10 dilution, 10 µl of the packaged phage particles solution with 90 µl of PDB.
C) $1:10^2$ dilution, 10 µl of the 1:10 dilution with 90 µl of PDB.
D) $1:10^3$ dilution, 10 µl of the $1:10^2$ dilutions with 90 µl of PDB.

Ten µl of each dilution was added with 100 µl of the prepared *E. coli* EPI300 host cells and incubated at 37°C for 20 min. The infected *E. coli* EPI300 cells (110 µl) were spreaded on a LB plate containing 12.5 µg/ml of chloramphenicol and incubated at 37°C overnight to select the CopyControl Fosmid clones. Colonies were counted and calculated the titer of the packaged phage particles.

$$\text{Titer} = \frac{(\text{No. of colonies})\ (\text{dilution factor})\ (1000\ \mu\text{l/ml})}{(\text{volume of phage plated } (\mu\text{l}))}$$

Plating and Selecting the CopyControl Fosmid Library

The desired dilution of the phage particle was selected by the optimal titer. The transformation of the packaged phage particles to *E. coli* EPI300 as described in the previous step. The colonies were washed with minimum volumes of

LB broth. The LB broth was added with 20% final concentration of glycerol pior to storing the clones at -80°C. The fosmid clones were used for screening by colony hybridization and PCR analysis of liquid gel pools.

1.4.8 Screening of NRPS gene by colony hybridization

Preparing colony lifts

The fosmid clones were spreaded onto the LB agar plate containing 50 µg/ml chloramphenicol after added 2% X-gal in DMF (40 µl), 100 mM IPTG (40 µl). The plate was incubated at 37°C overnight. Agar plate containing colonies was pre-cooled at 4°C for 30 min and a nylon membrane disc was placed onto the surface of each pre-cooled plate. The membrane was left on the plates for 1 min and the orientations of the membranes were marked relative to the plates (Figure 3.2). Each membrane disc was removed from the plate and bloted on following solutions; denaturation solution for 15 min, neutralization solution for 15 min and 2X SSC for 10 min. The nylon membrane was driped onto a dry Whatman 3MM paper between steps for excessive liquid. The DNA was fixed to the membrane by bake at 110ºC 1.5 h and removed cell debris by treating the membrane with proteinase K and incubated at 37ºC for 1 h. The fixed membrane was used for hybridizing with labeled DNA probe.

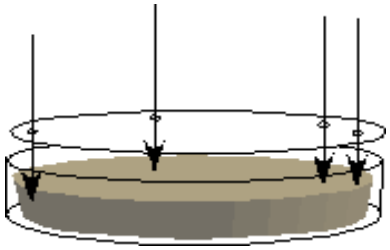


Figure 3.2 Marking the orientation of the membrane relative to the plate. Using needle poke holes through the filter and agar in an asymmetric pattern.

DIG-DNA labeling and quantification of labeling efficiency

The NRPS probe was prepared by using the DIG High Prime DNA Labeling and Detection Starter Kit. Digoxigenin (DIG), a steroid hapten, was used to label DNA probes for hybridization and subsequent color detection by enzyme immunoassay. DNA is randomly labeled with Digoxigenin-11-dUTP using DIG-High Prime. One µg of DNA product was added with sterile double distilled water to a final volume of 16 µl. The DNA was denatured by heating in a boiling water bath for 10 min and quickly chilled in an ice/water bath. Four µl of DIG-High Prime were mixed thoroughly to the denatured DNA, centrifuged briefly before incubation at 37°C for 1 h or overnight. The reaction was stoped by adding 2 µl of 0.2 M EDTA (pH 8.0) and/or by heating to 65°C for 10 min.

Yield determination of the DIG-labeled DNA is important for optimal and reproducible hybridization results. Too high of a probe concentration in the hybridization mix causes background, while too low of a concentration leads to weak signals. The recommended concentration of the labeled probes was 1 ng/µl. A dilution series of the labeled probes and the control DNA (1 ng/µl) were prepared as described in Table 3.4. One µl spot of the labeled probes and the control probe was applied onto the marked squares of a nylon membrane and air dry for 2 min. The membrane was diped in 2 ml of following solutions for approximate times. Between steps, the nylon membrane was driped onto a dry Whatman 3 MM paper for excessive liquid.

1) blocking solution for 2 min
2) anti-dioxigenin-alkaline phosphatase for 3 min
3) blocking solution for 1 min
4) maleic acid buffer for 1 min
5) detection buffer for 1 min

Two ml of the freshly prepared color substrate, nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP), were added to the nylon membrane and visualized under dark condition for 5-30 min. The color reaction was stoped after a

maximum of 30 min by briefly rinsing in water. The quality of labeled DNA was determined by comparing violet color intensity with control DNA.

Table 3.4 A dilution series of the labeled probe and the control DNA.

Tube	DNA (µl)	From tube no.	DNA dilution buffer	Dilution	Final concentration of the control DNA
1		Diluted original			1 ng/µl
2	2	1	198	$1:10^2$	10 pg/µl
3	15	2	35	$1:3.3\times10^2$	3 pg/µl
4	5	2	45	$1:10^3$	1 pg/µl
5	5	3	45	$1:3.3\times10^3$	0.3 pg/µl
6	5	4	45	$1:10^4$	0.1 pg/µl
7	5	5	45	$1:3.3\times10^4$	0.03 pg/µl
8	5	6	45	$1:10^5$	0.01 pg/µl
9	0	-	50	-	0

Hybridizing a DIG-labeled DNA probe to colony lifts

An appropriate volume (10 ml/100 cm^2 nylon membrane) of the hybridization solution (DIG Easy Hyb) was pre-heated to hybridization temperature at 42°C. The nylon membrane was prehybridized using heated hybridization solution in the plastic bag at 42ºC for 1 h with gentle agitation and the hybridization solution was removed. The DIG-labeled DNA probe (~25 ng/ml) was denatured by boiling for 5 min and rapidly cooling in ice/water. The denatured DIG-labeled DNA probe was added to pre-heated hybridization solution (3.5 ml/100 cm^2 nylon membrane) and mixed well but avoid foaming (bubbles may lead to background). The DIG-labeled DNA probe solution was added to prehybridized nylon membrane and incubated for 4 h to overnight with gentle agitation. Post-hybridization, the nylon membrane was washed by low stringency wash buffer at room temperature for 5 min (2 times) and high stringency wash buffer at 68ºC for 15 min (2 times).

Detecting probe-target hybrids with a chromogenic assay

The membrane was washed in washing buffer for 5 min and subsequently incubated in 30 ml of blocking solution for 30 min and in 10 ml of anti-dioxigenin-alkaline phosphatase solution (antibody) for 30 min. The membrane was washed twice by washing buffer to remove unbound antibody for 15 min and finally equilibrated in 20 ml of detection buffer for 2-5 min prior to incubating membrane with NBT/BCIP solution in the dark until hybridization signals detected the desired intensity for 15-30 min. The reaction was stopped by incubating membrane in TE buffer for 5 min.

1.4.9 Screening of NRPS gene by PCR analysis of liquid gel pools

The genomic library clones in LB culture broth were measured for cell density by spectrophotometry as described in 1.3.1.4 prior to diluting as 10^2 cfu/ml/tube final concentration. The clone mixture was added to appropiate volume of LB medium containing a supplement of 5 g/l SeaPrep agarose (Cambrex) and appropiate antibiotic for selection. Aliquot (0.5-1 ml) of the cell cultures was transferred to a 1.5 ml microcentrifuge tube and stored on wet ice for 1 h to complete the gelling process and incubated at 37 °C overnight. After suspended colonies, each tube was vortexed shortly to homogenize the mixture and 0.5 µl of the cell culture were aliquoted for PCR and subsequently added the 20% glycerol final concentration to the remaining cells for storage at -80 °C. Clone mixtures generating PCR products were subjected to the next round of liquid gel cultivation and PCR screening at lower cfu numbers until single clones can be isolated. Outline of the screening procedure of genomic libraries is showed in Figure 3.3.

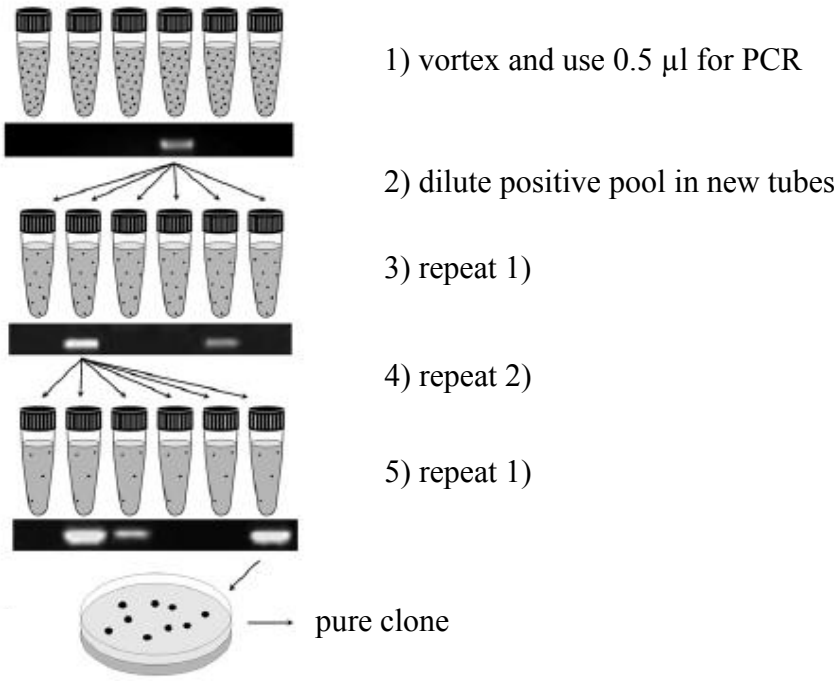


Figure 3.3 Outline of the screening procedure of of genomic libraries. (Hrvatin and Piel, 2007).

2. Results

2.1 Effect of renieramycins on quality and quantity of DNA from sponge tissue

The *Xestospongia* sponges with different cycles of renieramycins removal by methanol extraction were measured for the remaining renieramycins in samples by HPLC using amount of renieramycin M (RM), the major renieramycin, to represent all renieramycins in the sponge tissue as shown in Figure 3.4. The DNA from the sponge samples were obtained by a modified NaOAc salting-out method. DNA yield and purity were determined by spectrophotometry as presented in Figure 3.4 and Table 3.5. The original amount of RM in the sponge tissue was 41.2 μg (0.02%) while the DNA yield from the sponge without removal of renieramycins was 88.8 μg/200 mg sponge tissues (0.04%). The results showed that RM content is dramatically decrease at the 4-time elimination cycle and completely removed at 8-time elimination cycle. The DNA yield and purity were significantly increased related to the decreased amount of renieramycins. The highest DNA yield (251.2 μg/200 mg sponge tissues) and DNA purity (A_{260}/A_{280} 1.83) were obtained at 8-time elimination cycle. Interestingly, the total DNA yield and purity were decreased at 10-time elimination cycle due to the possible DNA degradation. The DNA quality and quantity were confirmed by gel electrophoresis as shown in Figure 3.5. The intensities of the obtained DNA from the sponge samples as visualized in agarose gel were increased in a renieramycin elimination cycles-dependent manner as well as the increasing of the DNA yield.

In addition, the total expressed DNA in μg per 50 mg dry weight sponge was quantified by 16S rRNA gene amplification. As a filter feeder, sponge is able to filter thousands of liters of water 1 day, which make sponge harbor large numbers of diverse bacteria in its tissue. The 16S rRNA gene sequences has been the most common target for housekeeping genetic marker because of its presence in almost all bacteria and the function of the 16S rRNA gene over time has not changed (Janda and Abbott, 2007). Hence, the 16S rRNA gene was performed for amplifying the obtainable DNA mixture from the *Xestospongia* sponge tissue.

Close to full-length 16S rRNA gene fragments were amplified from several dilutions of the total extracted DNA. PCR products consequence from DNA were analyzed on gel electrophoresis (Figure 3.6). The intensity of the PCR product was visualized and compared between different template DNAs from the sponge tissues with or without renieramycins elimination. The DNA from renieramycin-eliminated sponge samples not only has an advantage of higher yield and purity than that of non renieramycin-eliminated samples, but also a number of PCR reaction to amplify 16S rRNA gene. Therefore, the obtainable DNA from optimal 8 times of renieramycins elimination was appropriately performed for amplifying the 16S rRNA gene to further study bacreial genetic and taxonomy.

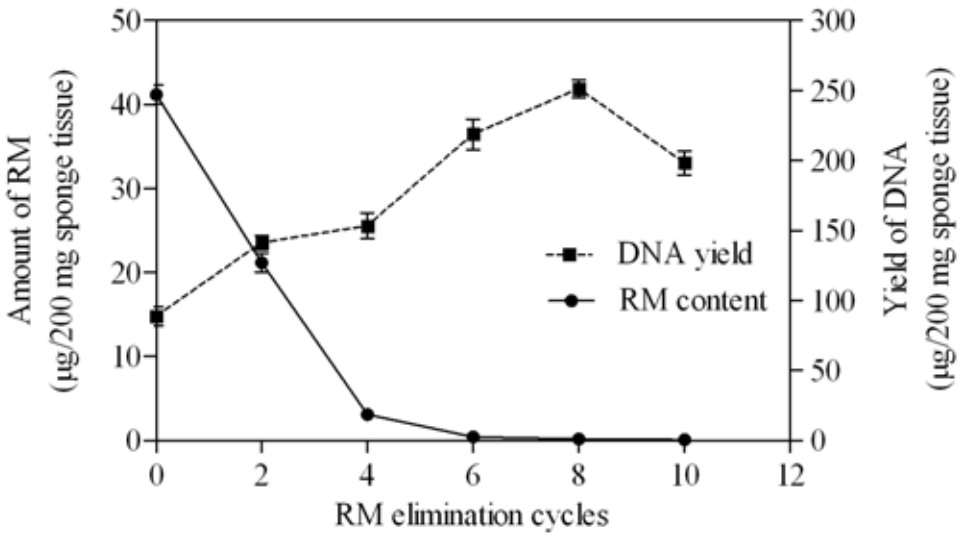


Figure 3.4 Determination of renieramycin M (RM) content and DNA yield in *Xestospongia* sponge samples with different cycles of the renieramycins elimination. Data represent the means of triplicated measurements±SD.

Table 3.5 Influence of numbers of renieramycins elimination cycles on purity and yield of DNA from *Xestospongia* sponge.

renieramycins elimination cycles	**DNA purity A_{260}/A_{280}**	**Yield of DNA extracted (µg/200 mg of sponge)**
0	1.49±0.03	88.83±6.18
2	1.50±0.05	141.25±4.47
4	1.53±0.07	153.41±8.17
6	1.81±0.03	218.75±9.69
8	1.83±0.01	251.25±5.64
10	1.79±0.02	198.33±7.57

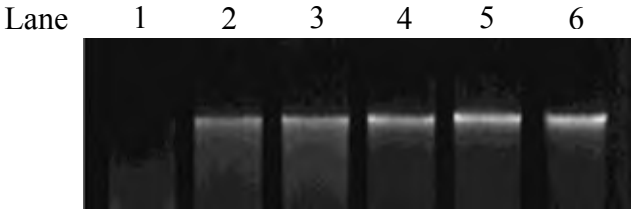


Figure 3.5 Electrophoresis pattern of extracted DNAs from renieramycin-eliminated *Xestospongia* sponge. Lane 1 = genomic DNA without renieramycin elimination; lanes 2-6 = genomic DNAs obtained from samples with different cycles of renieramycin elimination for 2, 4, 6, 8 and 10 times, respectively.

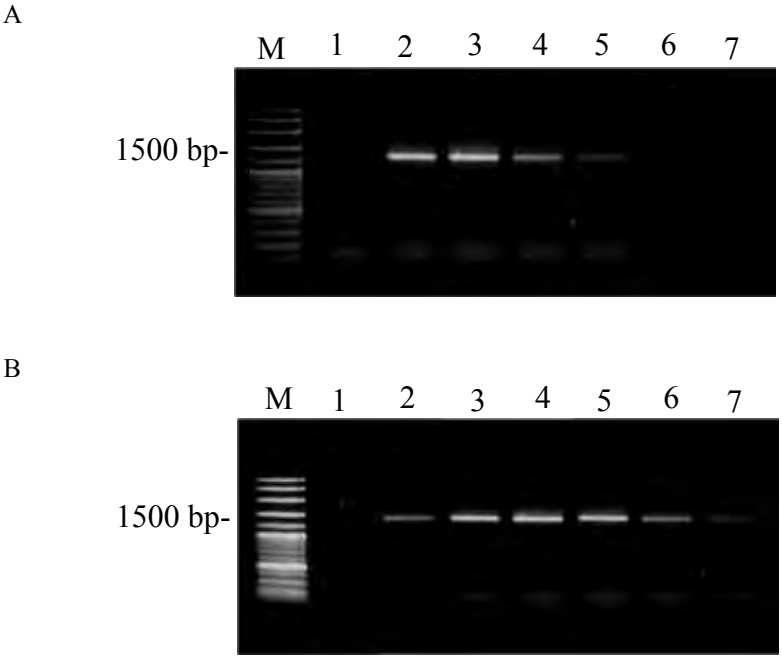


Figure 3.6 Sponge associated bacterial 16S rRNA product amplified using several DNA dilutions. (A): genomic DNA from the sponge without renieramycins elimination. (B): genomic DNA from the sponge with 8-time renieramycins elimination. Lane 1 = no dilution; lane 2 = 1:50; lane 3 = 1:100; lane 4 = 1:500; lane 5 = $1:10^3$; lane 6 = $1:10^4$ and lane 7 = $1:10^5$ dilution.

2.2 Effect of renieramycins on DNA damage

Early works on the study of the mechanisms of quinone have shown that the presence of a quinone group in the compounds such as adriamycin, daunorubicin, mitomycin C, trenimon, and aziridinylbenzoquinone can result in the induction of DNA strand breaks (Lown *et al*., 1976; Lown *et al*., 1977; Vig, 1977; Begleiter, 1983; Begleiter and Leith, 1990). The investigation of the quinone role was designed to prove a direct involvement in the DNA damage of RM. Hence, a reduction of the quinone moiety to the hydroquinone has been designed as a model for non-quinone agent. Therefore, the 5,8,15,18-tetra-*O*-acetylbishydroquinone renieramycin M (TRM) was synthesized by successive hydrogenation and acetylation of RM. Its structure was elucidated by comparative interpretation of ^{1}H-NMR with the reported spectral data (Amnouypol *et al*., 2004). The structure of TRM is shown in Figure 3.7.

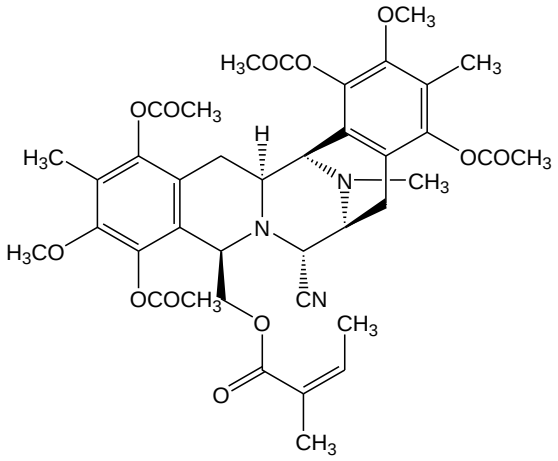


Figure 3.7 Structure of 5,8,15,18-tetra-*O*-acetylbishydroquinone renieramycin M.

2.2.1 Effect of renieramycin M and 5,8,15,18-tetra-*O*-acetylbishydro-quinone renieramycin M on pBR322 plasmid DNA

RM and TRM were used to comparatively examine their DNA damage effects on pBR322 plasmid DNA in cell-free system by gel electrophoresis as shown in Figure 3.8. In general, the common conformation of double-strand pBR322 plasmid DNA in a standard condition is mostly in the supercoiled form rather than the nicked DNA form, (or open circular DNA). In various stress conditions, it is damaged mostly by nicking and immensely turned into the nicked or relaxed DNA

form. The two different conformations are able to be characterized based on their mobility on gel electrophoresis. The fast mobile band on the agarose gel represents the supercoiled DNA whereas the slow mobile band corresponds to the nicked DNA. The results showed that the quinone alkylating RM induced DNA double-strand breaks in contrast to the non-quinone alkylating TRM. The pBR322 plasmid DNA of RM-treatment was changed the supercoiled form to the nicked DNA form in a RM-concentration dependent manner. The relative percentage of DNA breakage and the degree of nicked DNA increment with a correlation to the reduction of supercoiled DNA in RM-treatment are shown in Figure 3.8A and 3.8B, respectively. The DNA damage effect of RM is a RM-concentration dependent manner. Meanswhile, pBR322 plasmid DNA conformations were completely unchanged in every TRM-treatment conditions as illustrated in Figure 3.8C. This suggested that the quinone moiety must be an important part in DNA strand breaks.

2.2.2 Determination of quinone-generated free radicals effecting on pBR322 plasmid DNA damage

The oxygen molecule is changed into superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) by environmental pollutants, radiolysis, UV and the reaction pathway to H_2O in living tissues (Dasgupta and De, 2004). These oxygen radicals induce some oxidative damages to biomolecules, such as carbohydrates, proteins, lipids and DNA (Kellog and Fridovich, 1975; Lai and Piette, 1977; Wiseman and Halliwell, 1996). Among them, $OH^{\bullet}$ is the most reactive radical that induces cruel damage to biomolecules (Gutteridge, 1984). The quinone compounds have been reported as one of the free radical producing chemicals (Lown *et al*., 1976; Begleiter, 1983; Begleiter and Blair, 1984; Begleiter, 1985; Rao and Lown, 1990; Pommier *et al*., 1996; Scott and Williams, 2002). In order to support the free radicals produced by the RM quinone causing DNA damage, a free radical scavenger Trolox was used with RM to interact pBR322 plasmid DNA. The results showed that 1.25 and 2.5 mM of Trolox reduced double-strand DNA breaks by RM, respectively (Figure 3.9). Therefore, the quinone moiety of the RM structure generated free radicals leading to pBR322 plasmid DNA breakage.

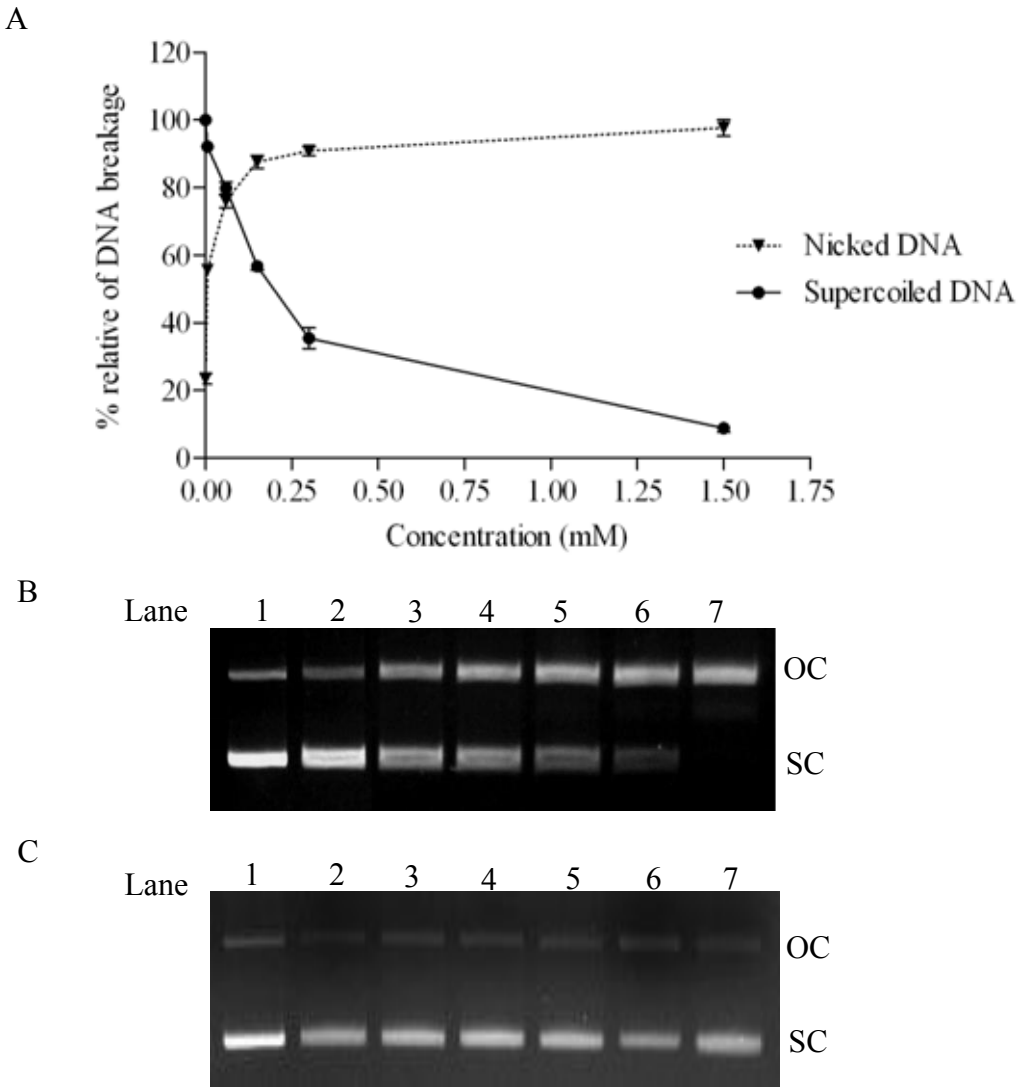


Figure 3.8 Effect of renieramycin M (RM) and 5,8,15,18-tetra-*O*-acetyl bishydroquinone renieramycin M (TRM) on pBR322 plasmid DNA. (A): Relative percentage of DNA breakage in RM-treatment. (B) and (C): Gel electrophoresis of pBR322 plasmid DNA form in RM- and TRM-treatments, respectively; Lane 1 = pBR322 DNA in the normal condition; Lane 2 = pBR322 DNA without test compound in the treatment condition; Lanes 3-7 = pBR322 DNA with test compound at 0.006, 0.06, 0.15, 0.30, and 1.5 mM, respectively. OC = open circular form or nicked DNA (▼), SC = close circular form or supercoiled DNA (●). Data represent the means of three triplicates±SD.

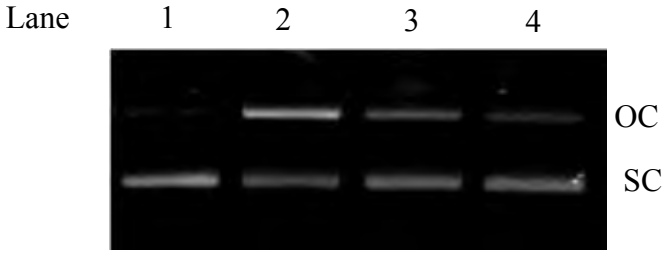


Figure 3.9 Effect of Trolox for protecting DNA breakage by renieramycin M (RM). Lane 1 = pBR322 DNA with 2.5 mM Trolox in the treatment condition (negative control); Lane 2 = pBR322 DNA with 0.15 mM RM in the treatment condition (positive control); Lanes 3 and 4 = pBR322 DNA with 0.15 mM RM and Trolox at 1.25 and 2.5 mM in the treatment condition, respectively. OC = open circular form or nicked DNA, SC = close circular form or supercoiled DNA.

2.3 Identification of the partial NRPS gene

2.3.1 Degenerate primer design

Renieramycins are a group of bistetrahydroisoquinoline alkaloids which are closely related to saframycins and safracins produced by microorganisms. The core structures of these alkaloids commonly compose of the fused pentacyclic ring derived from one glycine (Gly) and two tyrosine (Tyr) residues, suggesting the tripeptide origin as shown in Figure 3.10.

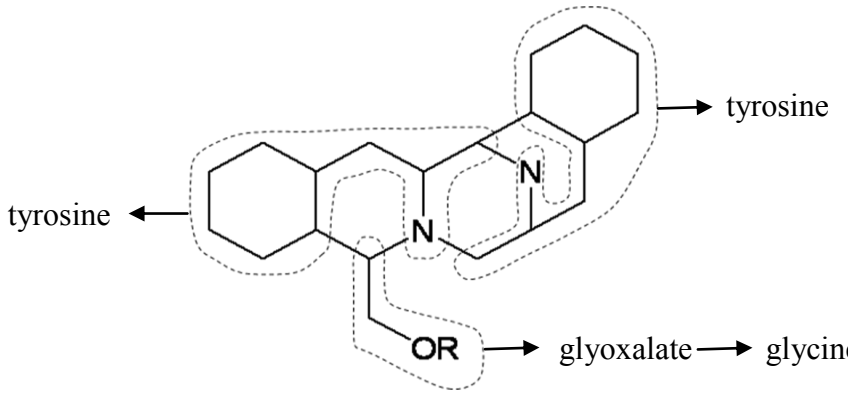


Figure 3.10 The common core structures of renieramycins to saframycins and safracins.

The similarity of the conserved core pentacyclic ring of renieramycins to saframycins and safracins suggested that these compounds are likely to be synthesized by a similar nonribosomal peptide synthetase (NRPS) mechanism. Three NRPS gene clusters have been previously reported for saframycin A, saframycin Mx1, and safracin B (Ikeda *et al*., 1983; Pospiech *et al*., 1995; Li *et al*., 2008) (as shown in Figure 3.11) of which *SfmC* (for saframycin A), *SacC* (for safracin B), and *SafA* (for saframycin Mx1) encode for the assembly of two units of 3-hydroxy-5-methy-*O*-methyltyrosine, the precursors of the conserved pentacyclic ring. A partial alignment (A domain to RE domain) from the full amino acid sequences (Appendix D) encoded by *SfmC, SacC,* and *SafA* genes is illustrated in Figure 3.12. The total amino acid sequences of *SfmC, SacC,* and *SafA* genes were 1485, 2605 and 1432 base pairs (bp), reaspectively. These alignments of amino acid sequences were shown the rangs of A-domain (amino acid at 1,582-2,121 bp), T-domain (amino acid at 2,124-2,239 bp) and RE-domain (amino acid at 2,241-2,628 bp). The core motifs (A1-A10, T1, and RE1-RE4) of the domains in Figure 3.12 were derived from the consensus sequences of the NRPS conserved motifs as previously described in Table 2.2. The core motifs were used to deduce nucleotide sequences of the degenerative primers of NRPS gene as listed in Table 3.6 and the relative positions of the designed degenerate primers are shown in Figure 3.13. The designed degenerate primers for a partial NRPS gene of renieramycins biosynthesis containing 5 forward and 6 reverse primers which were possibly maked 30 primer pairs. Its primer was used for PCR amplification of extracted DNA from the *Xestospongia* sponge tissue.

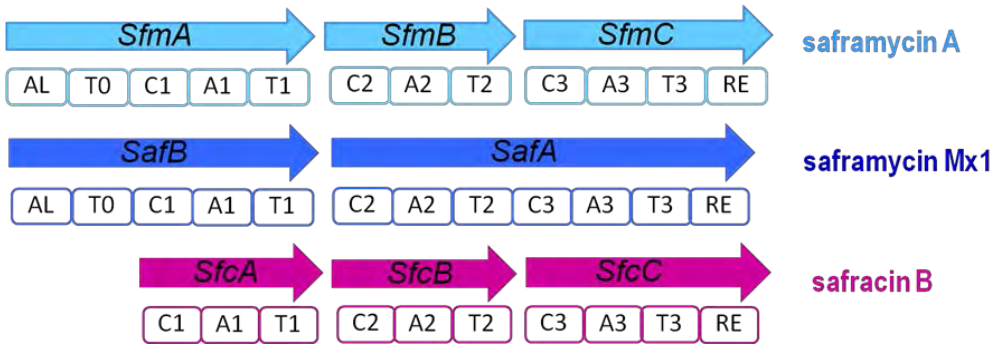


Figure 3.11 Constitute NRPS domains of saframycin A and Mx1 and safracin B.

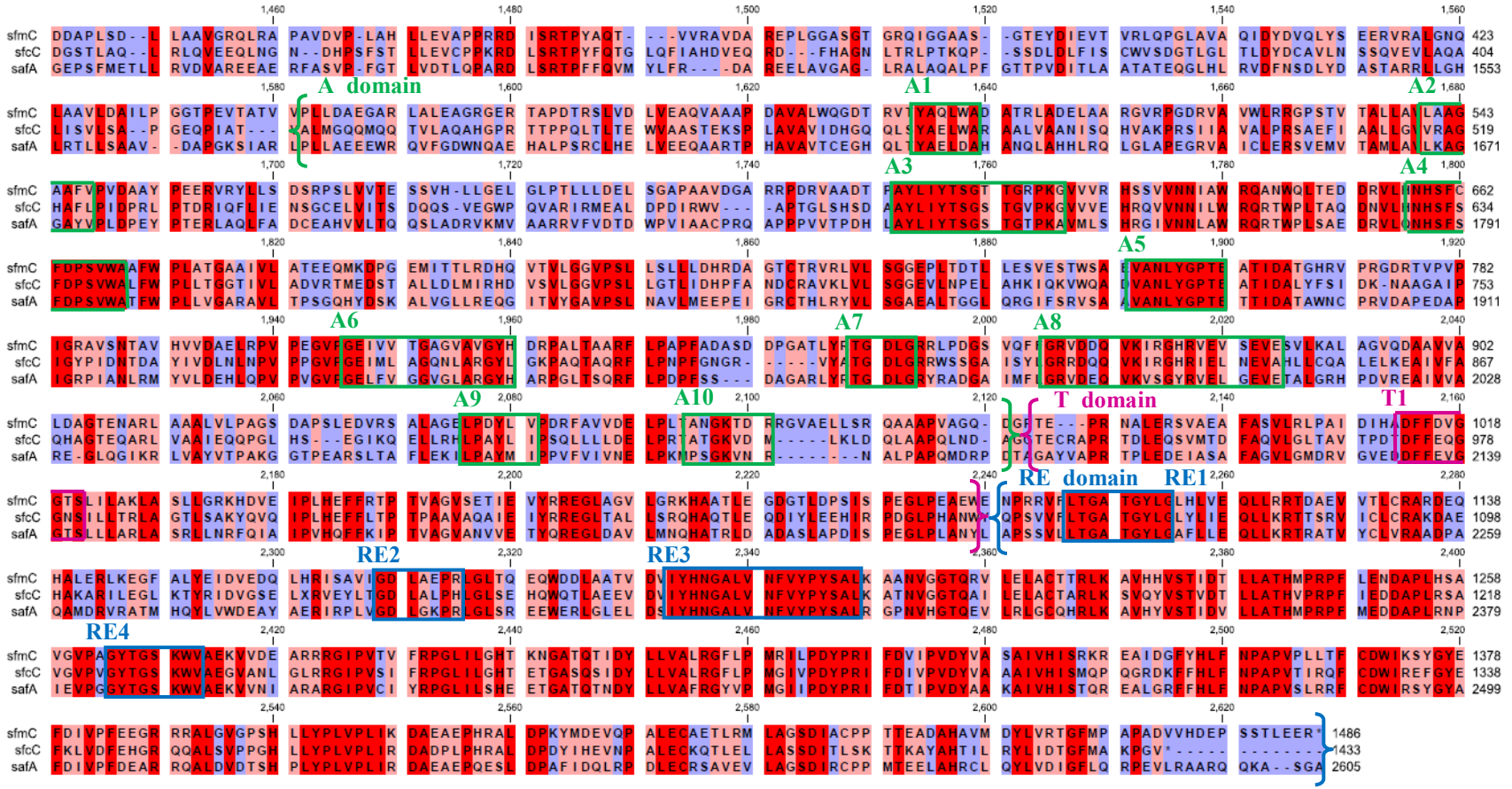


Figure 3.12 A partial alignment (A-domain to RE-domain) of amino acid sequences encoded by *sfmC*, *sfcC*, and *safA* genes.

Table 3.6 Degenerate primers designed for NRPS gene of renieramycins biosynthesis.

Primer name	Primer nucleotide sequences (5´ to 3´)	Direction
XSF A3	TAY ACB TCH GGN WCN AAR GC	Forward
XSF A4	TTY WSN TTY GAY CCN WSN GTN TGG	Forward
XSF A5	GTN GCN AAY YTN TAY GGN CCN ACN GA	Forward
XSF T	GAY TTY TTY GAI SWI GGI GG	Forward
XSF RE1	GTW BYW YTW ACW GGD GCW ACW GG	Forward
XSR A5	YTC NGT NGG BCC RTA KGC	Reverse
XSR T	KTN CCN CCN WSN TCR AAR AAR TC	Reverse
XSR RE1	CCN ARR TAN CCN GTN GCN CCN GT	Reverse
XSR RE2	AAR TTN CAN ARN GCN CCR TTR TGR TAD AT	Reverse
XSR RE3	RTA IAC RAA RTT IAC IAR IGC ICC	Reverse
XSR RE4	IAC CCA YTT ISW ICC IGT RTA ICC	Reverse

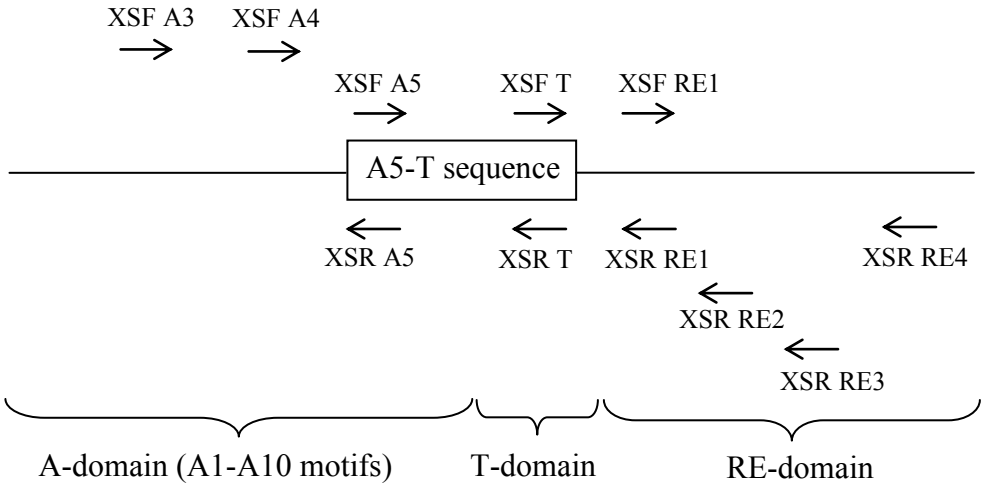


Figure 3.13 Relative positions of the designed degenerate primers on NRPS gene of renieramycin biosynthesis. (→) represents forward primers and (←) represents reverse primers.

2.3.2 Cloning and sequencing of a partial NRPS gene encoding for the assembly of tyrosine derivative

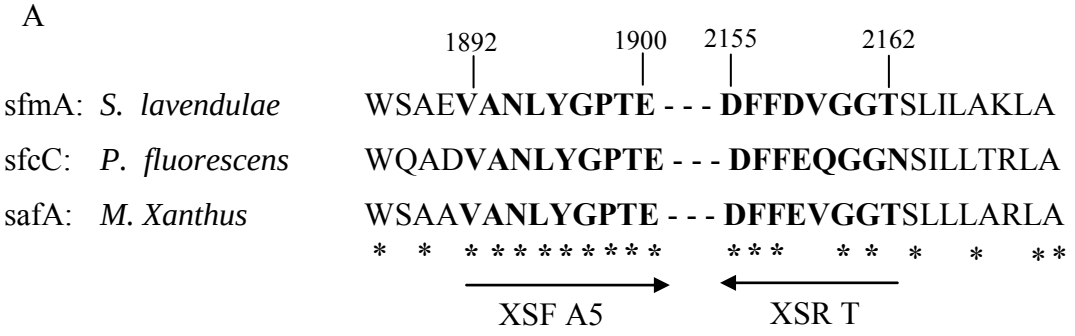
The extracted DNA from *Xestospongia* sponge was performed for PCR amplification using designed degenerate primers. The 30 possibilities of the primer pairs were tried to amplify NRPS gene of renieramycins biosynthesis. However, only a primer pair of XSF A5 and XSR T was successful to amplify a partial NRPS gene. The amino acid sequences of XSF A5 and XSR T primers obtainable from core motifs A5 and T are shown in Figure 3.14A. The amino acid sequences of XSF A5 forward primer started to amplify from position 1,900 while XSR T reverse primer from position 2,155. Hence, an expected size of the PCR product was approximately 255 amino acids or 770 bp measured on gel electrophoresis as shown in Figure 3.14B. The obtained PCR products were cloned into the pT7Blue vector (Novagen, USA) and the vector map is shown in Figure 3.14C. The PCR products were usually amplified by Taq DNA polymerase which was preferentially added adenines to the 3′ end of the product. The PCR products of NRPS fragments were cloned into the pT7Blue vector as a T/A linearized vector that has complementary 3′ thymine overhangs. The ligated mixture was transformed into *E. coli* DH5α competent cells prior to selecting the clones by blue-white colony selection method and then confirmed by colony PCR using a primer pair of XSF A5 and XSR T. The PCR positive clones were subjected to cycle sequencing by ABI PRISM® dGTP BigDye™ Terminators v3.0 Ready Reaction Cycle Sequencing Kit using 96-well MicroAmp® reaction plate prior to sequencing by an ABI Prism 3100 genetic analyzer (Hitachi). The sequences results of 13 positive clones were aligned to *safA* (saframycin Mx1), *sfcC* (safracin B), and *sfmC* (saframycin A) genes using for reference genes as shown in Figure 3.15. The almost clones showed high similarity to the reference genes and complete similarity was presented in deep red color while low similarity was presented in blue color. Comparing among 13 clones, the amino acid sequence of the clone number 13 expressed glycine (G) at position 7 while other clones expressed threonine or alanine similar to the reference genes. Addition, all amino acid sequences of 13 clones contained asparagine (N), serine (S), and threonine (T) at position 67 that were completely different from amino acid sequences of the reference which were alanine (A), glycine (G), and asparatic acid (D). Although, some of

amino acid sequences of 13 positive clones were differed from the reference genes, its amplicons were closely related to a partial sequence (A5 to T domains) of *safA*, *sfcC*, and *sfmC*. Homology search analysis of 13 positive clones was measured using NCBI blast (Appendix E). Comparing results showed that these amplicons were closely related to a partial sequence (A5 to T domains) of *safA*, *sfcC*, and *sfmC* with 53.3%, 46.5%, and 42.0% identity at nucleotide level, respectively and 55.9%, 45.7%, and 45.4% identity at amino acid, respectively. The results are shown in Figure 3.16. The total 764 nucleotide sequences data obtainable from 13 positive clones (Figure 3.17) were considered to design specific primers for genomic library screening. The specific primers were used in this study as shown in Table 3.7. The gene specific primer (GSP) contained 3 forward and 2 reverse primers. A primer pair of GSP-74F and GSP-604R was designed for screening genomic library while the other primers were used for gene walking.

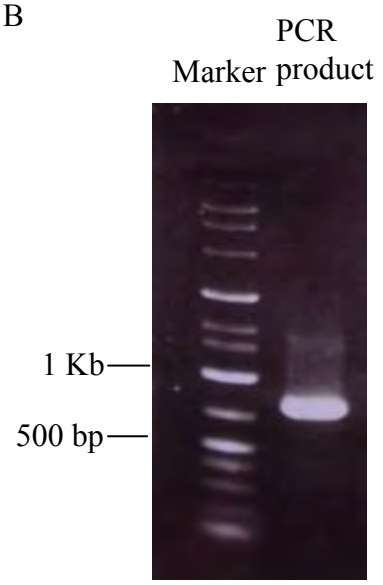
2.4 Labeling a partial NRPS gene as a probe for colony hybridization

The GSP-74F and GSP-604R primers were prepared as the NRPS probe by using the DIG High Prime DNA Labeling and Detection Starter Kit I for colony hybridization screening of genomic library. The PCR products obtainable from this primer pair were randomly labeled with Digoxigenin-11-dUTP using DIG-High Prime. The quantification of labeling efficiency was performed prior to using probe as shown in Figure 3.18. The results showed that the lowest concentration of the labeled NRPS probe dilution was visualized at concentration 0.3 pg/µl whereas the labeled control DNA was visualized at concentration 0.1 pg/µl (the original dilution 1 ng/µl). Therefore, the labeled NRPS probe was 3-time diluted less than the labeled control DNA and was used in DIG-DNA labeling and quantification of labeling efficiency step. The Digoxigenin-11-dUTP labeled NRPS probe was further performed for colony hybridization of TaKaRa Bio Inc. genomic library screening

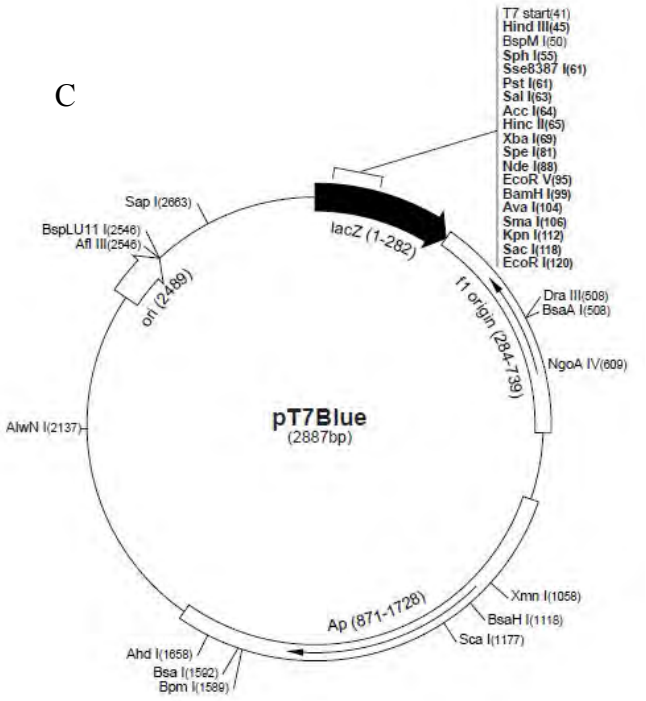
A



B



C



Blunt cloning site

↓

Nde I *Eco*R V *Bam*H I

TACTAGTCATATGGAT - - - ATCGGATCCCCGGG

Figure 3.14 Cloning of partial NRPS genes. (A): Amino acid sequences of degenerate primer design from the core motifs A5 and T. (B): Gel electrophoresis of the PCR product amplified from primers XSF A5 and XSR T (approximate size 770 bp). (C): The pT7Blue cloning vector map.

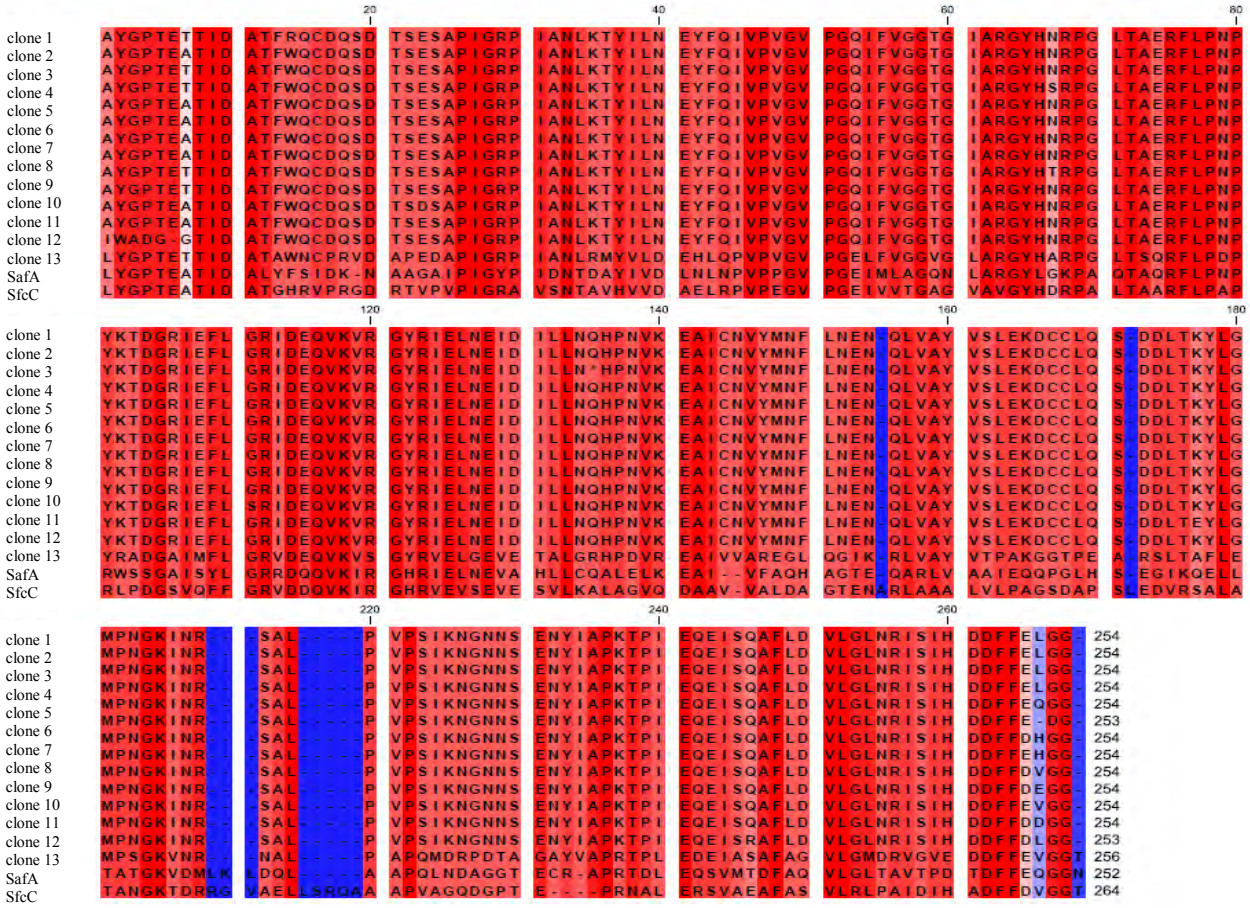


Figure 3.15 The alignment of amino acid sequencing results of motifs A5 and T in the NRPS genes from sponge genome comparing with safA, sfcC, and sfmC as a reference amino acid sequences.

DNA sequences

Amino acid sequences

	Fragment	SfmC	SafA	SacC
Fragment		42.0	53.3	46.5
SfmC	39.0		56.2	58.8
SafA	55.9	45.7		50.1
SacC	43.3	46.0	45.4	

Figure 3.16 DNA sequences and amino acid sequences of a PCR gene fragment comparing with *sfmC, safA*, and *sfcC* NRPS gene.

```
  1 GCATATGGAC CTACTGAGGC CACGATCGAT GCAACGTTTT GGCAATGCGA TCAATCTGAC
 61 ACTTCAGATA GTGCTCCGAT CGGAAGGCCA ATAGCAAATC TGAAAACATA TATTTTAAAT
121 GAATACTTTC AAATCGTTCC CGTTGGTGTC CCAGGACAAA TATTTGTTGG TGGGACTGGT
181 ATTGCACGTG GGTATCACAA CCGCCCAGGA TTAACAGCTG AACGATTTCT TCCGAATCCA
241 TTTTCTAATA CTGGAGAGCG GATTTATAAA ACAGGTGATT TAGGAAGATA TAAAACAGAC
301 GGAAGGATTG AATTTCTCAG CCGCATTGAT GAGCAAGTAA AAGTGAGGGG TTATAGAATA
361 GAGCTGAACG AAATAGATAT CTTATTAAAT CAACATCCAA ATGTAAAGGA AGCCATTTGT
421 AATGTTTATA TGAATTTCTT GAATGAAAAT CAGCTGGTTG CATATGTATC CTTAGAAAAA
481 GATTGTTGCT TACAATCAGA TGATTTAACT AAATATCTTG GAGAAAGACT GCCATCTTAT
541 ATGATACCTT CGTTCTTGAT GATTCTTGAT AAATTGCCAA AAATGCCAAA TGGAAAAATT
601 AACAGGAGTG CTCTACCTGT TCCATCAATA AAAAATGGGA ACAATTCAGA AAACTATATT
661 GCCCCAAAGA CACCTATTGA ACAAGAAATA AGTCAAGCGT TTTTAGATGT TTTGGGTTTA
721 AATCGAATTA GTATCCATGA TGATTTCTTC GAGGTGGGGG GCAA
```

Figure 3.17 The total 764 nucleotide sequences data obtainable from the positive 13 clones.

Table 3.7 Specific PCR amplification primers for screening of NRPS gene used in this study.

Primer name	Primer sequences (5´ to 3´)	Direction
GSP-74F	CTC CGA TCG GAA GGC CAA TAG CAA	Forward
GSP-542F	TGA TAC CTT CGT TCT TGA TGA	Forward
GSP-621F	TCC ATC AAT AAA AAA TGG GAA C	Forward
GSP-64R	CCT TCC GAT CGG AGC ACT TGA	Reverse
GSP-604R	TGA TGG AAC AGG TAG AGC ACT CCT	Reverse

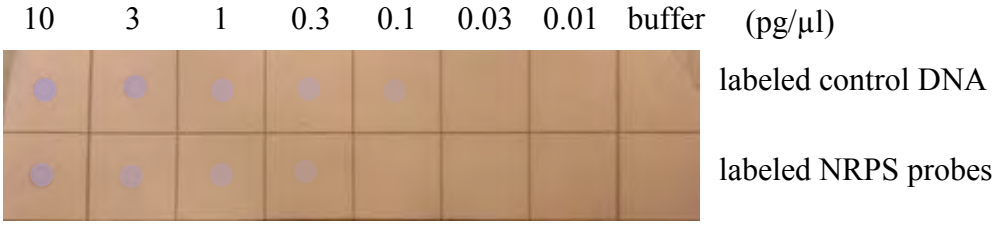


Figure 3.18 Determination of labeling efficiency.

2.5 Genomic library construction

2.5.1 Genomic library from TaKaRa Bio Inc.

A 764 bp partial NRPS gene of A5 core motif to T core motif was obtained from the amplified DNA of the *Xestospongia* sponge using XSF A5 and XSR T primers. A primer pair of GSP-74F and GSP-604R was designed and labeled as a probe for screening of TaKaRa Bio Inc. library. A genomic library from TaKaRa Bio Inc. was obtained from the cloning method using three kinds of vectors including pSTV28/HincII-BAP, pUC118/EcoRI-BAP, and Lambda ZAPII/EcoRI which accept for small insert DNA 1-10 Kb size prior to transforming into *E. coli* HST08 cells by heat shock method. The titer information of the recombinant clones was reported by TaKaRa Bio Inc. as shown in Table 3.8A. Comparatively, the transformed clones of the interested ligation mixture from TaKaRa Bio Inc. reported were rechecked for their titers by blue-white colony counts. The low titer of DNA transformation of the genomic library numbers G2 and G2-5 were presented by counted numbers of the blue and white colonies as 205/98 and 1,250/2,550, respectively as shown in Table 3.8B. The titer results indicated that genomic library G2-5, which was ligated into pUC118/EcoRI.BAP vector, was possibly performed for screening positive clones containing NRPS target gene. The pUC118/EcoRI.BAP vector map is shown in Figure 3.19. The labeled NRPS probe was used to screen genomic library G2-5 by colony hybridization. After colony lefts, denatured DNA of clones was hybridized with the DIG labeled probe on the nylon membrane prior to detecting by immunoassay. For colorimetric detection, the nitro blue tetrazolium conjugated with 5-bromo-4-chloro-3-indoyl phosphate (NBT-BCIP) was used as a substrate of anti-dioxigenin-alkaline phosphatase antibody. The DNA complementary with probe can be changed the colorless substrate solution to blue color. Twenty matched clones

from several thounsand clones were comfirmed by colony PCR using specific GSP-74F and GSP-604R primers, but its amplified products were not obtained. The falsed positive clones which are usually a potential disadvantage of all immunological methods may obtain because DNA probe can be complementary to region of other genes presented in *E. coli* (Telford *et al*., 1977). The genomic library G2-5 was unsuccessful to obtain NRPS gene by colony hybridization. Hence, new genomic library was constructed by CopyControl™ Fosmid Library Production Kits which accept high insert DNA 40-50 Kb and its vector can be induced to high copy number of elevated yields of DNA for sequencing. The semiliquid gel pool method was further performed to screen genomic library obtainable from CopyControl™ Fosmid Library Production Kits.

Table 3.8 Titer results of genomic DNA transformation.

A

Samples ID	**cfu/1 µl library**	
	Blue colony	**White colony**
G2	1,240	300
G2-5	1,200	4,000

B

Samples ID	**cfu/1 µl library**	
	Blue colony	**White colony**
G2	205	98
G2-5	1,250	2,550

Titer calculation

$$\text{Titer} = \frac{[\text{average number of colonies} \times \text{total volume (500 µl)}]}{\text{spread plate volume (µl)}}$$

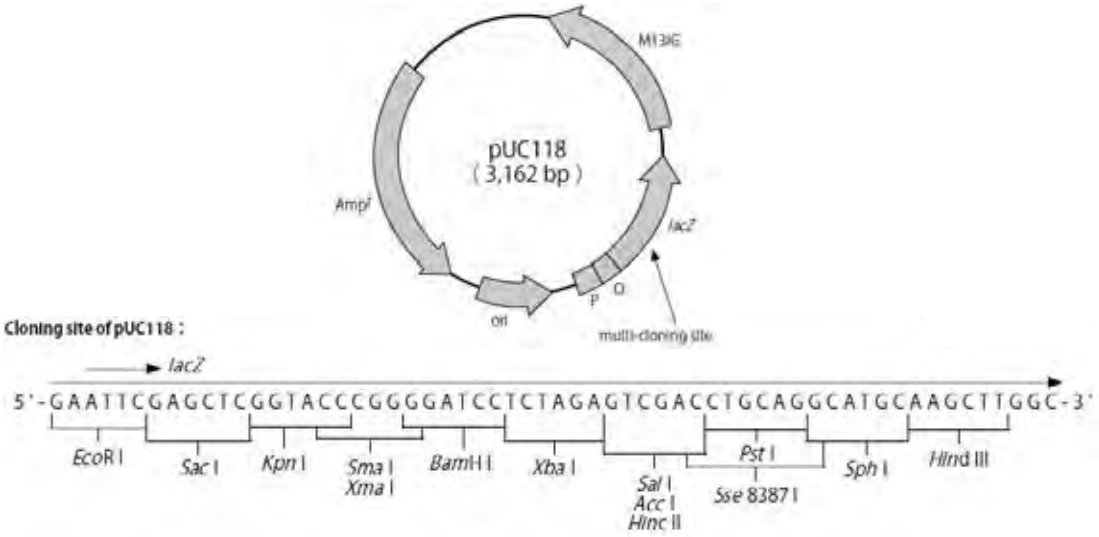
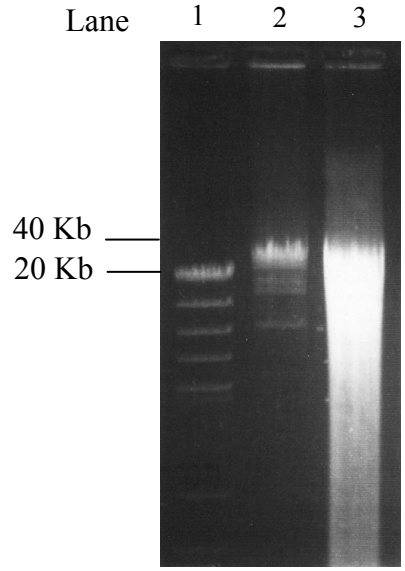


Figure 3.19 Multiple cloning site of pUC118.

2.5.2 Genomic library construction by CopyControl™ Fosmid Library Production Kits

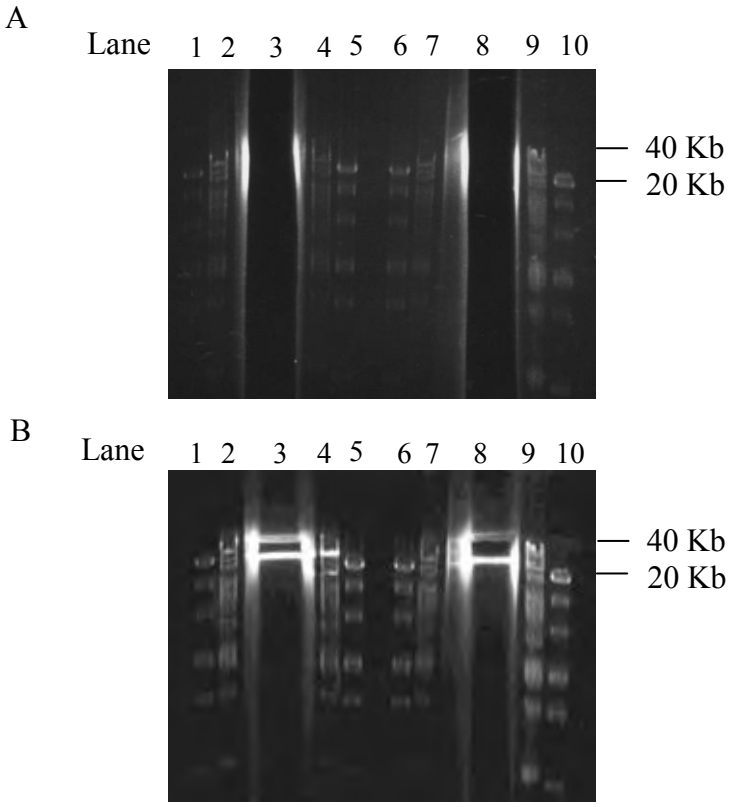
New libraries were constructed by CopyControl™ Fosmid Library Production Kits. The fosmid library construction usually requires the suitable size of DNA. Therefore, the genomic DNA was randomly sheared into approximate 40 Kb fragments by passing through a 200 µl of small bore pipette tip 50-100 times. This method led to the highly random generation of DNA fragments in contrast to the libraries obtained from partial digestion of the DNA by restriction endonuclease. The obtainable DNA from the *Xestospongia* sponge on the gel electrophoresis is shown in Figure 3.20. The extracted genomic DNA from the *Xestospongia* sponge by the modified RM elimination method showed a smear band from 40 Kb which was an appropriate size for fosmid vector. The DNA with appropriate size was added with end-repaired enzyme for generating blunt-ended DNA (5′-phosphorelated DNA) prior to selecting the end-repaired DNA by low melting point (LMP) agarose gel electrophoresis. The expected size of the DNA was detected and marked under UV-light comparable to T7DNA marker. The main gel column contain the DNA was excised prior to UV exposure as shown in Figure 3.21A. UV irridation can decrease cloning efficiencies by 100-1,000 folds. After removal of the DNA, the gel was

revisualizing under UV light as shown in Figure 3.21B. The gel electrophoresis results showed that the corrected size of the end-repaired DNA was obtained and then ligated with pCC1FOS vector prior to packaging the lambda phage and transforming into *E. coli* EPI300. The positive clones were selected by chloramphenicol antibiotic selection plates. The titer of the packaged CopyControl fosmid clones was $3.5x10^3$ cfu/ml. Finally, the 10 fosmid libraries (F1-F10) were obtained for screening with the specific GSP-74F and GSP-604R primers.



Lane 1 = 1 Kb DNA Extension Ladder
Lane 2 = T7 DNA marker
Lane 3 = Genomic DNA from *Xestospongia* sponge

Figure 3.20 Gel electrophoresis of the extracted genomic DNA from *Xestospongia* sponges by modified RM elimination method.



Lanes 1, 5, 6, 10 = Gene Ladder Wide 1

Lanes 2, 4, 7, 9 = T7 DNA marker

Lanes 3, 8 = Recovery end-repaired DNA

Figure 3.21 Gel electrophoresis of recovery end-repaired DNA from low melting point agarose. (A): The expected size of the excised DNA on the gel compared with T7 DNA markers under UV light. (B): After removal of the 40 Kb DNA, the gel was revisualized under UV light.

2.5.2.1 Screening of fosmid mixture clones

All 10 fosmid libraries (F1-F10) were screened by the specific NRPS primers GSP-74F and GSP-604R as shown in Table 3.7. An expected size of the PCR product was approximately 530 bp. The results showed that three libraries including F3, F4, and F5 could be amplified (Figure 3.22). The mixture of fosmid library number 4 (F4) showing bright amplified band may contain NRPS gene. The fosmid libraries F3-F5 were subjected to screen for single colony by cell dilution in semiliquid gel.

Lane 1 = VC Lambda/*Hin*dIII marker
Lane 2 = A partial NRPS gene from the *Xestospongia* sponge using as a control
Lanes 3-12 = Fosmid libraries F1-F10, respectively

Figure 3.22 Gel electrophoresis of fosmid libraries (F1-F10) by PCR using the specific NRPS primers GSP-74F and GSP-604R.

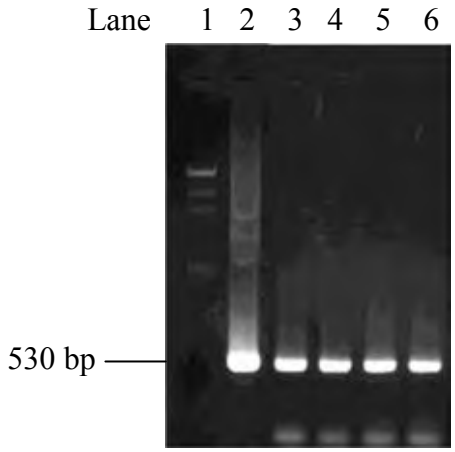
2.5.2.2 Isolation of a single clone using a semiliquid gel

A semiliquid medium was employed to screen genomic DNA libraries for a single clone by PCR using the specific NRPS primers GSP-74F and GSP-604R. A common task in molecular biology is the isolation of clones with known sequences from DNA libraries. For small to medium-sized libraries, several efficient isolation protocols are based on colony hybridization or PCR exist for this

purpose. With increasing clone numbers, however, substantial labor and financial resources have to be invested that render standard procedures impractical. Analyzing larger clone number requires numerous plates to be generated and scraped off, which is both labor-intensive and space consuming. Liquid cultures instead of plates for cultivation are limited. Three-dimensional growth requires less space for the same number of clones, and homogenous PCR samples can be generated by simple vortexing. An inherent danger of growing clone mixtures in liquid media is the domination of fast-growing members after few doubling periods, possibly resulting in a loss of target clones. A semiliquid medium containing ultralow gelling agarose has been used to increase cell mass in libraries in an unbiased way (Elsaesser and Paysan, 2004; Hrvatin and Piel, 2007).

The clone mixture of the fosmid library in a glycerol stock was measured to determine cell density by spectrophotometry and then diluted to 10^2 cfu/ml/tube in semiliquid gel. After incubation overnight, the semiliquid gel culture was mixed by vortex for generating the PCR products and the PCR positive tube was subjected to the next round of liquid gel cultivation and PCR screening at lower cfu numbers until single clones can be isolated. Four single colonies from libraries F3 and F4 (two colonies each) can be amplified with the specific NRPS primers GSP-74F and GSP-604R (Figure 3.23) and the PCR products were subsequently cloned into pT7Blue vector to confirm sequences by sequencing with specific GSP-74F and GSP-604R primers. The sequence results showed that 4 amplified clones were closely related to a partial sequence (A5 to T domains) of *safA* gene (saframycin Mx1) with 90% and 84% identity at the nucleotide level of GSP-74F and GSP-604R primers, respectively (Table 3.9). The positive plasmid clones were extracted by Wizard® Plus SV MiniPreps DNA Purification System (Promega, USA) and checked size insertion by digestion with *Bam*HI. It showed that the insert size was about 40 Kb as shown in Figures 3.24A and B. The final 4 clones were sequenced with two primer pairs of general M13F / M13R vector primers and pCC1FOSFW / pCC1FOSRE vector primers as shown in Table 3.10. All 4 clones were sequenced for gene walking by the designed fragment primers. The results showed that the sequences have no relation to NRPS gene or other target genes (Table 3.11). After gene walking and subcloning,

the final 4 clones were proved not to contain NRPS target involved in renieramycin biosynthesis. Therefore, gene walking of NRPS gene involved in biosynthesis of renieramycins was not successful based on low titer fosmid library.



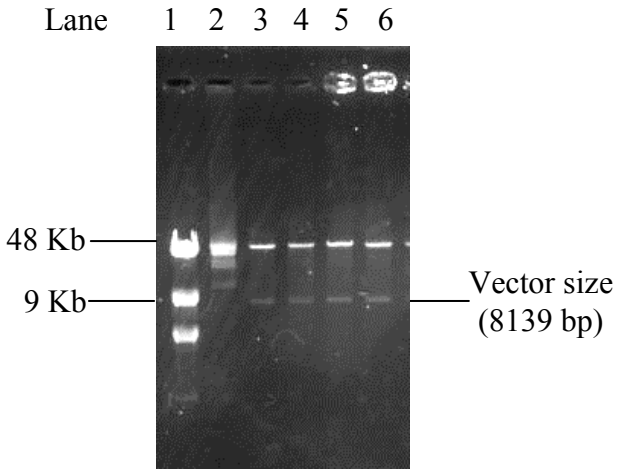
Lane 1 = VC Lambda/*Hin*dIII marker
Lane 2 = A partial NRPS gene using as control
Lanes 3, 4 = Positive PCR product of two single clone from F3
Lanes 5, 6 = Positive PCR product of two single clone from F4

Figure 3.23 Gel electrophoresis of the PCR product of single clones from fosmid libraries F3 and F4.

Table 3.9 NCBI blast results of 4 positive clones comparing to the core NRPS fragment (A5-T).

Clone no.	**GSP-74 forward primer**		**GSP-604 reverse primer**	
	Homologous protein	**Identities**	**Homologous protein**	**Identities**
F3,F4 (4 clones)	*saf A* gene; saframycin Mx1(*Myxococcus xanthus*)	90%	*saf A* gene; saframycin Mx1(*Myxococcus xanthus*)	84%

A



Lane 1 = Gene Ladder Wide 1

Lane 2 = Extracted pCC1FOS plasmid DNA

Lanes 3, 4 = pCC1FOS plasmid DNA from F3 digestion with *Bam*HI

Lanes 5, 6 = pCC1FOS plasmid DNA from F4 digestion with *Bam*HI

B

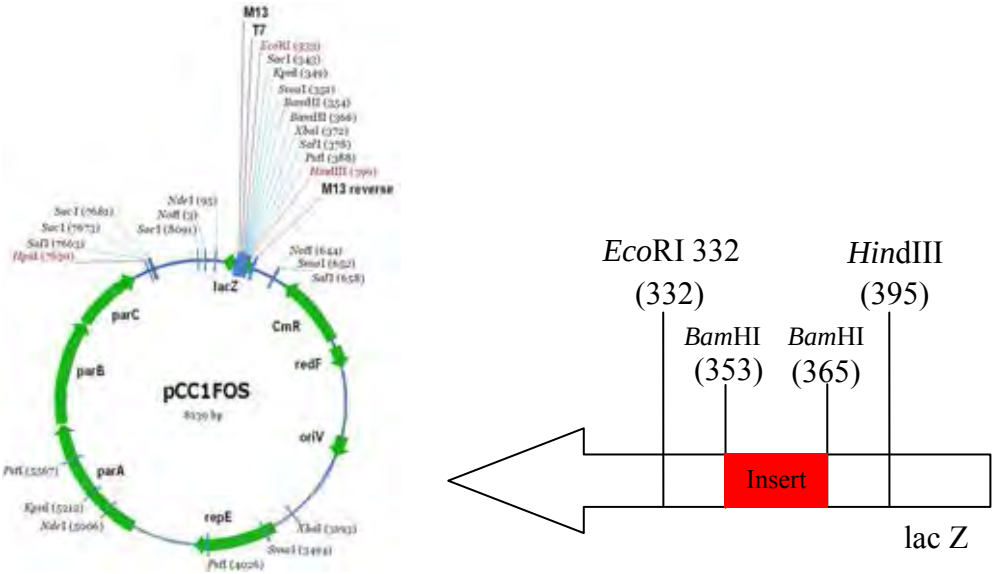


Figure 3.24 Check size of insert DNA. (A): Gel electrophoresis of pCC1FOS plasmid digestion by *Bam*HI of F3 and F4 fosmid libraries. (B): Multiple cloning site of pCC1FOS fosmid vector using for fosmid library construction and restriction enzyme map.

Table 3.10 General PCR amplification primers and fosmid vector primers for gene walking.

Primer name	Primer sequences (5´ to 3´)	Direction
M13F	CGC CAG GGT TTT CCC AGT CAC GAC	Forward
M13R	TCA CAC AGG AAA CAG CTA TGA C	Reverse
pCC1FOSFW	GGA TGT GCT GCA AGG CGT TAA GTT GG	Forward
pCC1FOSRE	CTC GTA TGT TGT GTG GAA TTG TGA GC	Reverse

Table 3.11 Homology search analysis of clones F3 and F4 by NCBI blast.

Clone no./ walking time	Vector forward primer		Vector reverse primer	
	Homologous protein	**Identity**	**Homologous protein**	**Identity**
F3/1	Hypothetic protein (*Lactobacillus casei*)	26%	Hypothetic protein (*Turicibacter sp.*)	30%
F3/2	Hypothetic protein (*Yersinia pestis*)	64%	Hypothetic protein (*Bacillus cereus*)	77%
F4/1	Hypothetic protein (*Methylobacterium nodulans*	49%	Hypothetic protein (*Yersinia pestis*)	64%
F4/2	Histidine kinase (*Thermobispora bispora*)	36%	-	-
F4/3	Hypothetic protein (*Escherichia coli*)	90%	-	-

3. Discussion

The blue sponge *Xestospongia* sp. has been chemically investigated for accumulation of cytotoxic substances, reniermycins (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Charupant *et al*., 2009). To date, there has no report of these compounds having effects on biomolecules including DNA, RNA and proteins, of their living hosts. The chemical removal from sponge samples prior to DNA extraction was therefore not to any concern at the beginning of experiments. However, there were difficulties for subsequent research to work with the non-chemical eliminated DNA due to both low quantity and unsatisfied quality of extracted DNA. One finding is that quinone moieties in renieramycins caused DNA damage. This phenomenon may be either directly or indirectly due to the quinone producing free radicals as reported for other chemicals (Lown *et al*., 1976; Lown *et al*., 1977; Vig, 1977; Begleiter, 1983; Begleiter and Leith, 1990). As a result, the free radicals in the mixture of DNA preparation possibly cause DNA breakage. The other discovery from DNA extraction development is that numbers of renieramycins removal cycle have to be optimized. The greater number of renieramycins removal cycles is not generally suitable for the sponge sample which is subsequently proceeded for molecular biology research. Excessive chemical elimination suspiciously caused DNA damage through organic solvent (methanol) treatment. Hence, organic solvent usage should be concerned when samples have to be operated in molecular research.

The study of biosynthesis of a particular secondary metabolite has been reported to be beneficial for manipulating its production in laboratory (Fortman and Sherman, 2005; Moore, 2005; Konig *et al*., 2006). There are several studies accomplishing biosynthetic information for NRP including safracins and saframycins but not for renieramycins. Since, these compounds share structure similarity, genes encoding enzymes involved in safracin and saframycin biosyntheses were used to propose architecture of enzymes responsible for renieramycins production. The experiment was performed to genetically isolate promising regions which were considered to be conserved for core structure production of these compounds. A conserved motif of adynylation to thiolation domains (A5-T) was found to be similar to that of *safA*, a gene invoved in saframycin Mx1 biosynthesis, at 53.3% of nucleotide sequence and

55.9% of amino acid sequence. Thus, this fragment was then cloned for further experiments. Fosmid library was constructed for NRPS screening using A5-T fragment as a probe. Unfortunately, upstream and downstream sequences which flank A5-T DNA fragment were not obtained in this study.

There were reports of NRPS gene clusters composed of several genes which were scattered through the region and other addiotional genes for complete NRP biosynthesis located between NSPS genes (Walsh *et al*., 2001; Schwarzer *et al*., 2003). This may be one of problems of primer design and sequence verification of renieramycins biosynthetic gene(s). The other problem of this unsucess incident may be caused by insufficient quality of high molecular DNA used for fosmid library construction. Size of DNA suitable for creating library is about 40 kb otherwise DNA with smaller size than 40 kb can be combined to yield 40 kb fragment and accidentally cloned into the library. Although the area of DNA band collection from the electrophoresis gel was selected with the finest estimation, there might be unexpected size of DNA introducing to the library and resulting in incorrect arrangement of DNA sequences. At this point, the probe of NRPS gene for renieramycins bisosynthesis is acquired. Other techniques including RACE PCR should be considered to identify interested genes.

CHAPTER IV

NECROSIS ABOLISHING EFFECT OF 5-*O*-ACETYLHYDROQUINONE RENIERAMYCIN M IN LUNG CANCER CELLS

A novel drugs as well as strategies that possesses high efficacy are of the most interest for cancer research nowadays. Indeed, the main action, in general, of anti-cancer agents is to kill the cancerous cells but preserves the normal cells (Plescia *et al*., 2005). Thus, most drugs are designed to mediate cancer cells through apoptosis rather than necrosis since the former is more controllable (Kroemer *et al*., 1998). Apoptosis is one type of cell death that is responsible for the development and repair process of human body and has been long documented as a distinctive model of programmed cell death (Elmore, 2007). Also, this type of death is the major mechanism of human body for eliminating un-wanted and damaged cells (Elmore, 2007; Norbury and Hickson, 2001). Unlike apoptosis, necrosis is considered as a toxic cell death that the cells are unspecific injured by two main mechanisms; intervention with the energy supply of the cells and direct damage to cell membranes (Levin *et al*., 1999). Not only is the necrosis cell death unspecific, but also it damages the surrounding cells and tissues by the releasing cytoplasmic components and induces the severe active immune response and inflammation (Trump *et al*., 1997; Savill and Fadok, 2000; Kurosaka *et al*., 2003). Together, even though the cell killing property of substances is of interest for anti-cancer drug development, many promising candidates are cut off because of these unspecific necrotic responses and related complications.

One mediator that has been garnered increasing attention in pharmacological field is reactive oxygen species (ROS). Moreover, ROS are shown to be implicated in many anti-cancer drug actions including cisplatin (Pelicano *et al*., 2004; Wang and Lippard, 2005; Wang *et al*., 2008) and doxorubicin (Tsang *et al*., 2003; Wang *et al*., 2004; Chen *et al*., 2007; Luanpitpong *et al*., 2012). In fact, ROS are important cellular mediators that generated continuously along with the electron transport chain (Nishikawa, 2008; Circu and Aw, 2010). ROS are generated though oxygen reduction resulting in the production of reactive species, such as superoxide anion

($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2) (Weinberg and Chandel, 2009; Liou and Storz, 2010). In particular, some specific ROS including hydroxyl radical and superoxide anion were shown in certain studies that mediated necrosis by interacting with the plasma membrane (Malorni *et al*., 1993; Silverberg *et al*., 2011).

As part of our continuing investigation on cytotoxic natural products from Thai marine organisms, we have reported a series of renieramycin alkaloids isolated from the blue sponge, *Xestospongia* sp. distributed around Sichang Island, the Gulf of Thailand (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Saito *et al*., 2012). Renieramycins are a group of bistetrahydroisoquinoline quinone marine alkaloids possessing potent cytotoxicity with IC_{50}s in the range of nM concentrations against several human cancer cell lines (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Charupant *et al*., 2009). Renieramycin M (RM), a major alkaloid of the *Xestospongia* sponge, has been shown to induce apoptosis in lung cancer cells through activation of p53-dependent pathway (Halim *et al*., 2011). The presence of the quinone moiety in RM has been hypothesized to induce necrotic cell death. This property might prevent further development of RM as an effective anticancer agent without toxic complications due to necrosis. To investigate this hypothesis, we used 5-*O*-acetylhydroquinone renieramycin M (ARM), a monoacetyl derivative of partially-reduced RM, to treat with non-small cell lung cancer H23 cells. In this present study, we demonstrated that the partially-reduced ARM significantly reduced necrosis-inducing effect while still fully preserving apoptosis-inducing effect of the parent RM. We also found that necrotic effect of RM was a ROS-dependent mechanism by generating intracellular superoxide anions.

1. Materials and Methods

1.1 Chemical transformation of renieramycin M to hydroquinone renieramycin M

1.1.1 Synthesis of ARM

ARM was synthesized by exploiting RM (10.2 mg, 0.018 mmol) as the starting material. Hydrogenation of RM with 10% Pd/C (6 mg) in EtOAc (3 ml) was conducted at 1 atm of H_2 for 4 h. The catalyst was removed by filtration and washing

with EtOAc. The combined filtrates were concentrated in vacuo to obtain the residue, which was used in the next step without further purification. The bishydroquinone RM was acetylated with 1.5 equivalent of acetic anhydride (2.65 µl) in of pyridine (0.5 ml) and then stirred for 3 h at room temperature under argon atmosphere. The reaction was quenched by addition of water (5 ml) and the resulting mixture was extracted with dichloromethane (3 ml×3). The combined dichloromethane extracts were evaporated to dryness in vacuo and subjected to chromatographic purification on a silica gel column using hexane-ethyl acetate (3:2) as the eluting solvent. ARM (4.1 mg, 40 %) was afforded as a pale yellow solid.

1.1.2 General techniques

1.1.2.1 Analytical thin-layer chromatography (TLC)

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 F254 (E. Merck) precoated plate
Layer thickness	:	0.25 mm
Distance	:	6 cm
Detection	:	Ultraviolet light at wavelengths of 254 and 365 nm.

1.1.2.2 Column chromatography

Adsorbent	:	Silica gel 60 particle size 0.040-0.063 mm (230-400 mesh ASTM) (E. Merck)
Packing method	:	Dry packing
Sample loading	:	The sample was dissolved in a small amount of organic solvent, and then loaded gently on top of the column.
Detection	:	Fractions were examined by TLC under UV light at the wavelengths of 254 and 365 nm.

1.1.2.3 Spectroscopy

HRESIMS	:	HREIMS *m/z* 619.2529 $[M]^+$ (calcd. for $C_{33}H_{37}N_3O_9$, 619.2530); Figure G3
IR	:	υ_{max} (KBr) 3459 (br), 1714, 1651, 1616, Figure G4
^{1}H MMR	:	δ ppm, 300 MHz, in $CDCl_3$; see Table 4.1, Figure G5
^{1}H-^{1}H COSY	:	300 MHz, in $CDCl_3$; Figure G6
^{13}C MMR	:	δ ppm, 75 MHz, in $CDCl_3$; see Table 4.2, Figure G7

DEPT 135 : 300 MHz, in $CDCl_3$; Figure G8

HMQC : 300 MHz, in $CDCl_3$; Figure G9

HMBC : 300 MHz, in $CDCl_3$; Figure G10

1.2 Cell culture

1.2.1 Cells and reagents

Human non-small cell lung cancer H23 cells were obtained from the American Type Culture Collection, ATCC (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium containing 5% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml penicillin-streptomycin in a 5% CO_2 environment at 37°C. *N*-acetylcysteine (NAC), sodium pyruvate, propidium iodide (PI), Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) and dihydroethidium (DHE) were obtained from Sigma Chemical (St. Louis, MO, USA); 3′-(*p*-hydroxyphenyl) fluorescein (HPF) were obtained from Daiichi Pure Chemicals (Invitrogen, CA, USA).

1.2.2 Determination of cytotoxic activity

Cell viability was examined by MTT assay. The H23 cells (1.5×10^5 cells/ml) were cultured in RPMI 1640 medium in a 96-well plate for 24 h at 37°C and then further treated with RM or ARM at various concentrations for 24 h at 37°C. The treated cells were finally incubated with 500 μg/ml of MTT for 4 h at 37°C. The supernatant was carefully removed and dimethyl sulfoxide was then added to dissolve the formazan product, giving a purple color. The color intensity was spectrophotometrically measured at 570 nm using a microplate reader (VICTOR™ X3 Multilabel Plate Reader, Perkin Elmer, USA). All the analyses were repeated at least three different replicate cultures. The cell viability was calculated from optical density (OD) readings and represented as percentage to the non-treated control value.

1.2.3 Apoptosis and necrosis assay

Apoptosis and necrosis cell deaths were determined by Hoechst 33342 and PI co-staining. The H23 cells were treated by RM and ARM at the indicated concentrations for 24 h at 37°C. The cells were stained with 10 μM Hoechst 33342 and 10 μM PI for 30 min at 37°C. Hoechst 33342 stained the nuclei of all cells. The

apoptotic cells displayed condensed chromatin and/or fragmented nuclei. PI stained only the DNA of cell membrane-damaged cells which were considered as necrotic cells. The fluorescent dye stained in cells was visualized and scored under a fluorescence microscope (OLYMPUS IX51, USA).

1.2.4 Determination of sub-G_0 fraction by flow cytometry

The relative cellular DNA content and the cell distribution were investigated during various phases of the cell cycle. The apoptotic cell death induced by either RM or ARM was analyzed by flow cytometry (FACSort, BD Biosciences) using PI buffer dye and visualized the peak of sub-G_0 cells. Attached H23 cells (3.5×10^5 cells/ml) were collected and resuspended in 1 ml of 3% FBS in 1× phosphate buffer saline (PBS). The suspension cells were fixed with 2.5 ml of absolute ethanol by vortex and then kept for 24 h at -20°C. For staining, the collected cells were suspended in 500 µl of PI buffer (10× PBS, 1 ml; 1 mg/ml RNaseA, 1 ml; TritonX-100, 10 µl; 10 mg/ml PI, 200 µl; FBS, 1 ml; and H_2O adjusted to 10 ml) and incubated for 40 min at 37°C prior to analysis. The DNA histogram indicating cell death through sub-G_0 phase was calculated by the percentages of cells occupying the different phases of the cell cycle. All at once, the collected cells for cell cycle analysis were stained by trypan blue dye and subsequently morphologically visualized under an automated cell counter (TC10™ Automated Cell Counter, BIORAD).

1.2.5 Measurement of intracellular specific ROS induced by RM and ARM

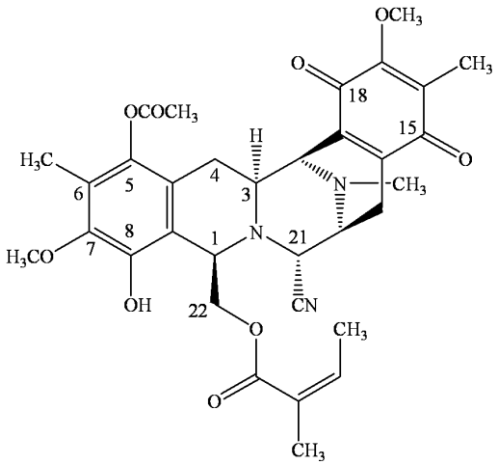
Intracellular ROS were determined using fluorescent probes. The ROS specific probe utilizes dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) as a general oxidative probe, dihydroethidium (DHE) as a superoxide anion probe and 3'-(*p*-hydroxyphenyl) fluorescein (HPF) as a hydroxyl radical probe. To characterize ROS generation was used 1 mM of *N*-acetylcysteine (NAC) and sodium pyruvate. The H23 cells were seeded onto a 96-well plate for 24 h that removed culture media and replaced with a final concentration of 10 µM each fluorescent probe in phosphate buffer saline (PBS), 1 mM ROS scavenger and incubated for 30 min at 4°C. The dye solution was added with 20 µM of test compounds and incubated for different time

periods (0 to 6 h) at 37°C. The fluorescence intensity was analyzed by fluorescence microplate reader (DTX 880 Multimode Detector, Beckman Coulter Inc., USA) using a 480-nm excitation beam and a 530-nm band–pass filter for detecting DCF fluorescence, using a 490-nm excitation beam and a 515-nm band–pass filter for HPF and using a 488-nm excitation beam and a 610-nm band–pass filter for DHE.

2. Results

2.1 Structure determination of ARM

ARM was obtained as a pale yellow solid by reductive acetylation of RM in 40% yield. Its molecular formula, $C_{33}H_{37}N_3O_9$, was established by HRQTOF-MS. The observed molecular ion at *m/z* 619.2528 was close to the calculated value for $C_{33}H_{37}N_3O_9$ 619.2530. Most of the signals in 1D and 2D-NMR spectra of ARM were closely related to those of RM, except for the presence of two quinone carbonyl carbons at δ_C 186.0 (C-15) and 182.8 (C-18) and two oxygenated aromatic carbons at δ_C 143.0 (C-8) and 139.1 (C-5) in ARM instead of four quinone carbonyl carbons in RM. These data suggested that the chemical structure of ARM differs from that of RM by the presence of one quinone and one hydroquinone. The absence of the unique homoallylic coupling (~2 Hz) between 1-H and 4-H confirmed that a quinone moiety at ring A of RM was reduced to a hydroquinone ring of ARM. In addition, the presence of a phenolic hydroxyl group and an acetoxy group was supported by the NMR signals at δ_H 5.81 and at δ_H 2.29/δ_C 20.2 and 168.6, respectively. The placement of the hydroxyl group at C-8 was assured by HMBC correlations of the hydroxyl proton at δ_H 5.81 to C-7 (δ_C 143.8) and C-9 (δ_C 117.1). This information readily assigned the acetoxy group at C-5. All proton and carbon assignments of ARM were completed after extensive NMR measurements using COSY, HMQC, and HMBC techniques. Thus, the structure of ARM was confirmed to be 5-*O*-acetylhydroquinone renieramycin M.



ARM

Table 4.1 ^{1}H-NMR spectral data of renieramycin M and 5-*O*-acetylhydroquinone renieramycin M in $CDCl_3$

proton	Renieramycin M	5-*O*-acetylhydroquinone renieramycin M
1-H	3.99 (1H, m)	4.29 (1H, br s)
3-H	3.11 (1H, ddd, 11.3, 3.1, 2.8)	3.17 (1H, br d, 12.2)
4-Hβ	1.36 (1H, ddd, 17.4, 11.3, 2.7)	1.57 (1H, overlap)
4-Hα	2.89 (1H, dd, 17.4, 2.8)	2.53 (1H, br d, 13.7)
11-H	4.01 (1H, d, 3.1)	3.95 (1H, overlap)
13-H	3.40 (1H, ddd, 7.6, 2.5, 1.8)	3.34 (1H, br d, 7.3)
14-Hβ	2.30 (1H, d, 20.6)	2.32 (1H, overlap)
14-Hα	2.76 (1H, dd, 20.6, 7.6)	2.72 (1H, dd, 20.9, 7.3)
21-H	4.07 (1H, d, 2.5)	4.09 (1H, d, 2.0)
22-Ha	4.10 (1H, dd, 11.6, 2.5)	4.03 (1H, dd, 11.1, 4.7)
22-Hb	4.53 (1H, dd, 11.6, 3.1)	4.48 (1H, br d, 11.1)
6-CH_3	1.90 (3H, s)	2.06 (3H, s)
16-CH_3	1.94 (3H, s)	1.89 (3H, s)
7-OCH_3	3.99 (3H, s)	3.74 (3H, s)
17-OCH_3	4.02 (3H, s)	3.94 (3H, s)
12-NCH_3	2.28 (3H, s)	2.24 (3H, s)
8-OH	-	5.81 (1H, s)
5-OCO$\underline{CH_3}$	-	2.29 (3H,s)
26-H	5.96 (1H, qq, 7.3, 1.5)	5.98 (1H, br q, 7.2)
27-H_3	1.82 (3H, dq, 7.3, 1.5)	1.84 (3H, br d, 7.2)
28-H_3	1.58 (3H, dq, 1.5, 1.2)	1.66 (3H, br s)

Table 4.2 ^{13}C-NMR spectral data of renieramycin M and 5-*O*-acetylhydroquinone renieramycin M in $CDCl_3$.

carbon	Renieramycin M	5-*O*-acetylhydroquinone renieramycin M
1	56.3 CH	56.5 CH
3	54.1 CH	55.2 CH
4	25.4 CH_2	27.8 CH_2
5	185.4 C	139.1 C
6	128.6 C	122.3 C
7	155.8 C	143.8 C
8	180.9 C	143.0 C
9	135.7 C	117.1 C
10	141.3 C	124.3 C
11	54.2 CH	54.6 CH
13	54.6 CH	54.8 CH
14	21.3 CH_2	21.1 CH_2
15	185.9 C	186.0 C
16	128.4 C	128.9 C
17	155.2 C	155.4 C
18	182.5 C	182.8 C
19	135.0 C	135.6 C
20	142.0 C	141.8 C
21	58.5 CH	59.4 CH
22	62.0 CH_2	64.4 CH_2
6-CH_3	8.5 CH_3	8.6 CH_3
16-CH_3	8.7 CH_3	9.9 CH_3
7-OCH_3	60.9 CH_3	60.6 CH_3
17-OCH_3	61.0 CH_3	61.1 CH_3
12-NCH_3	41.5 CH_3	41.4 CH_3
21-CN	116.9 C	117.5 C

Table 4.2 (Continued).

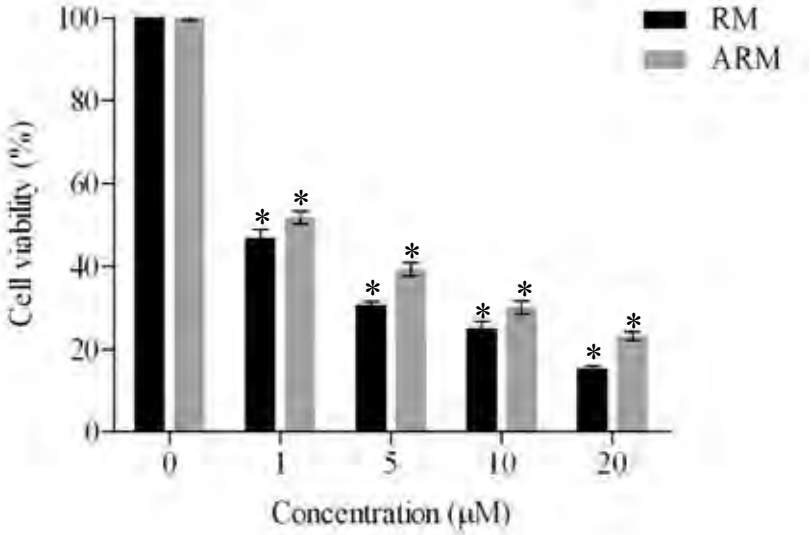
carbon	Renieramycin M	5-*O*-acetylhydroquinone renieramycin M
5-O<u>CO</u>CH_3	-	168.6 C
5-OCO<u>CH_3</u>	-	20.2 CH_3
24	166.5 C	167.1 C
25	126.3 C	126.8 C
26	140.5 CH	139.9 CH
27	15.7 CH_3	15.8 CH_3
28	20.4 CH_3	20.5 CH_3

2.2 Cytotoxic effect of RM and ARM on human lung cancer H23 cells

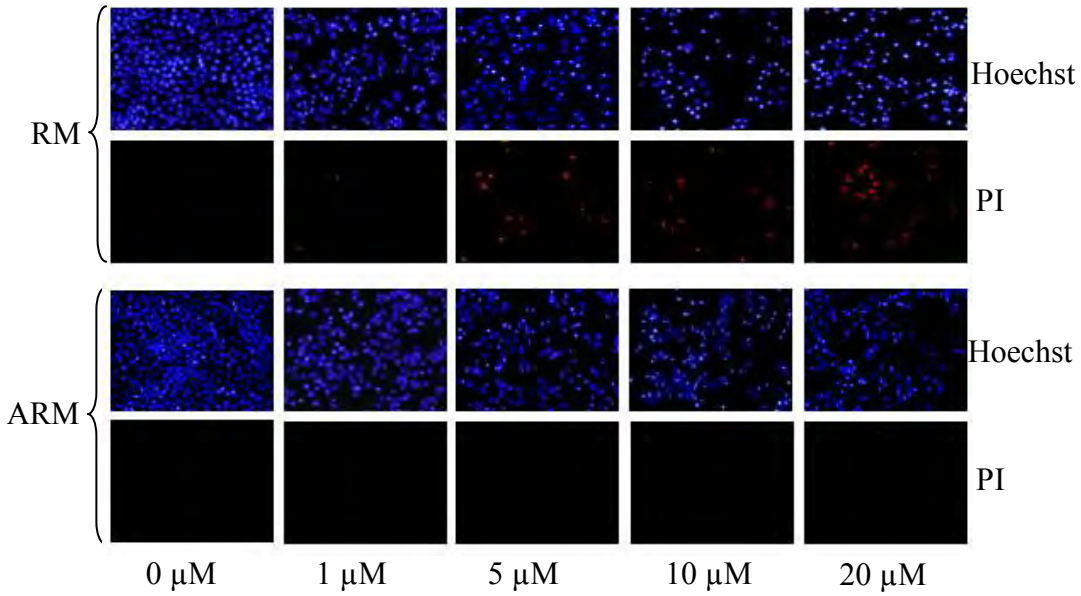
The cytotoxicity of RM and ARM against human non-small cell lung cancer H23 cells was determined using MTT assay. The H23 cells were incubated in the presence or absence of RM and ARM at the concentrations of 0-20 μM for 24 h. The result indicated that the cell viability was significantly decreased in a concentration-dependent manner in response to RM and ARM (Figure 4.1A).

In order to identify modes of cell death in such treatments, cells were treated with various concentrations (0-20 μM) of RM and ARM for 24 h to determine apoptosis and necrosis by Hoechst 33342 and PI assays. Fluorescence microscopy was used to visualize nuclei and other DNA containing organelles in either apoptotic or necrotic cells. The results showed a concentration-dependent increase in a number of apoptosis and necrosis in the RM-treated cells; however, only apoptosis death was observed in the ARM-treated cells (Figure 4.1B, C). Morphology analyses of apoptotic cells and necrotic cells exhibiting condensed and/or fragmented nuclei with intense nuclear fluorescence of Hoechst 33342 and PI-positive were respectively shown in Figure 4.1B. Surprisingly, the percentages of apoptotic cell death in RM- and ARM-treated experiments were comparatively equal at the same concentrations (Figure 4.1C). Together, these findings suggested that the modification done to RM structure could be able to attenuate necrosis-mediating action of RM.

A


RM
ARM
Cell viability (%)
100
80
60
40
20
0
0
1
5
10
20
Concentration (μM)

B


RM
ARM
Hoechst
PI
Hoechst
PI
0 μM
1 μM
5 μM
10 μM
20 μM

C

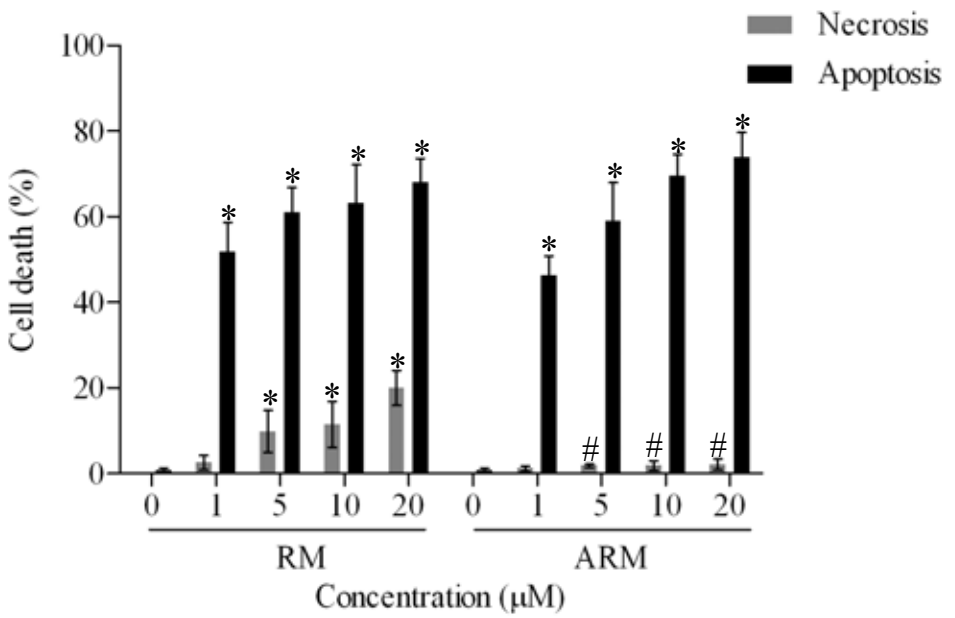


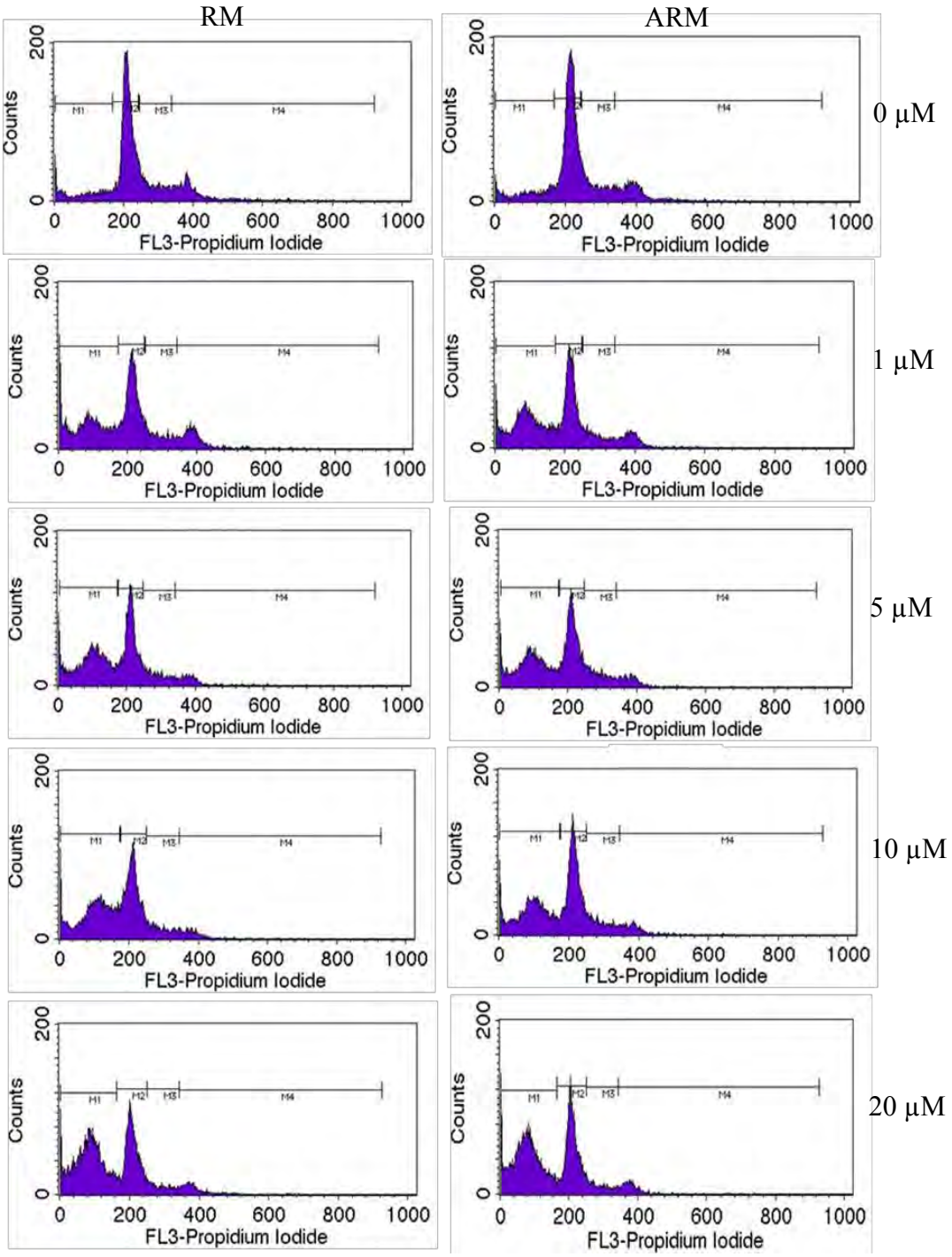
Figure 4.1 RM and ARM cause the concentration-dependent cell death. H23 cells were treated with various concentrations of each compound for 24 h. (A): Cell viability was analyzed by MTT assay. (B): Morphology of apoptosis and necrosis cell deaths were determined by Hoechst 33342 and PI dyes, respectively. (C): Percentage of apoptosis and necrosis cell deaths. * $p <$ 0.05 versus non-treated control in each group. $^{\#}$ $p < 0.05$ versus RM-treated groups.

2.3 Sub-G_0 fraction and membrane integrity analysis

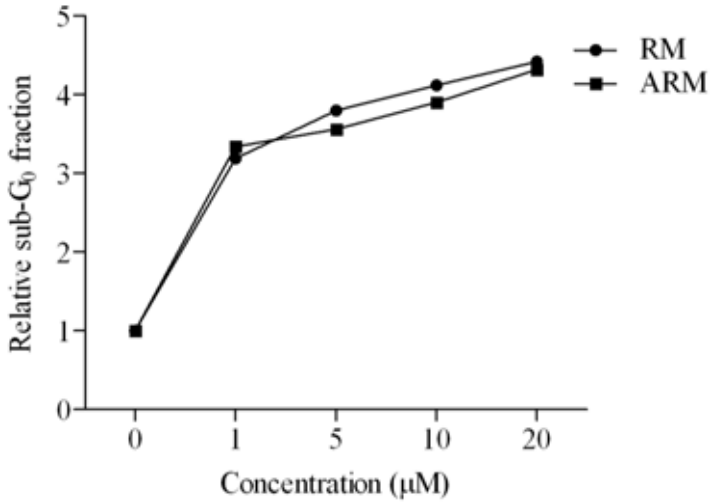
To confirm apoptotic cell death as well as necrosis in response to such compounds, the specific detection methods for apoptosis and necrosis were performed. As an increase in sub-G_0 fraction of the cells indicating apoptosis, the cellular DNA content analysis was used to determine apoptosis responses in both RM and ARM treatments. Cells treated with RM and ARM (0-20 μM) for 24 h were stained with PI buffer and the sub-G_0 fraction was then quantified by flow cytometry. The histograms confirmed comparable increase of apoptosis portions in both RM- and ARM-treated cells in a concentration-dependent manner (Figure 4.2A, B). These results in combination with the above Hoechst staining results suggested that RM and ARM induced apoptosis cell death of H23 cells in a very proximate potency.

For necrosis, since loss of membrane integrity was long known to be a marker of necrosis, the present study thus performed trypan blue exclusion assay to quantify the necrotic cells by an automated cell counter. Trypan blue was not absorbed by living cells and apoptotic cells but it traversed only the damaged membranes in dead cells that were considered as necrosis cells. Automated cell counter indicated living cells with green label and necrosis cells with red label (Figure 4.2C). Clearly, the results indicated the consistent necrosis dead response as above reported and a concentration-dependent increase of high necrosis percentages (ranging from 10-20% at concentrations 5-20 μM of RM) was detected in the RM-treated group only. Interestingly, minute relative percentages (1-3%) of necrotic cell deaths were observed at the same concentration range of ARM (Figure 4.2D).

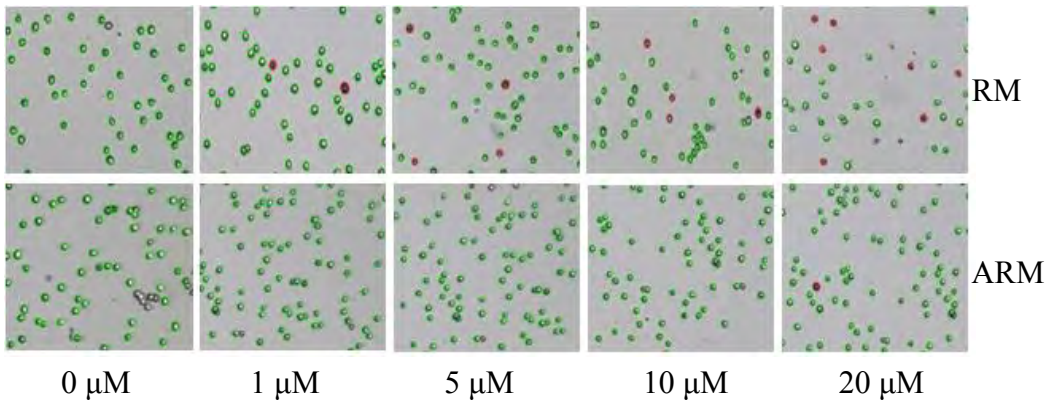
A


RM
ARM
0 µM
1 µM
5 µM
10 µM
20 µM
Counts
FL3-Propidium Iodide
M1
M2
M3
M4
0
200
400
600
800
1000

B


Relative sub-G0 fraction
0
1
2
3
4
5
0
1
5
10
20
Concentration (μM)
RM
ARM

C


RM
ARM
0 μM
1 μM
5 μM
10 μM
20 μM

D

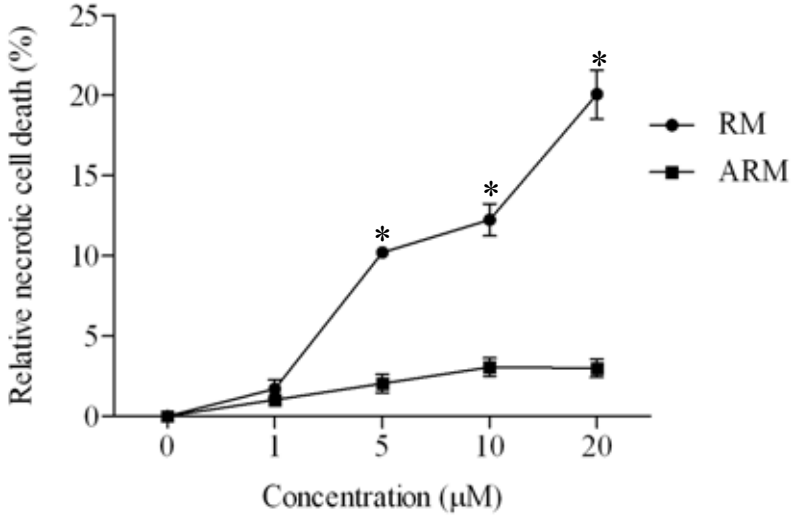
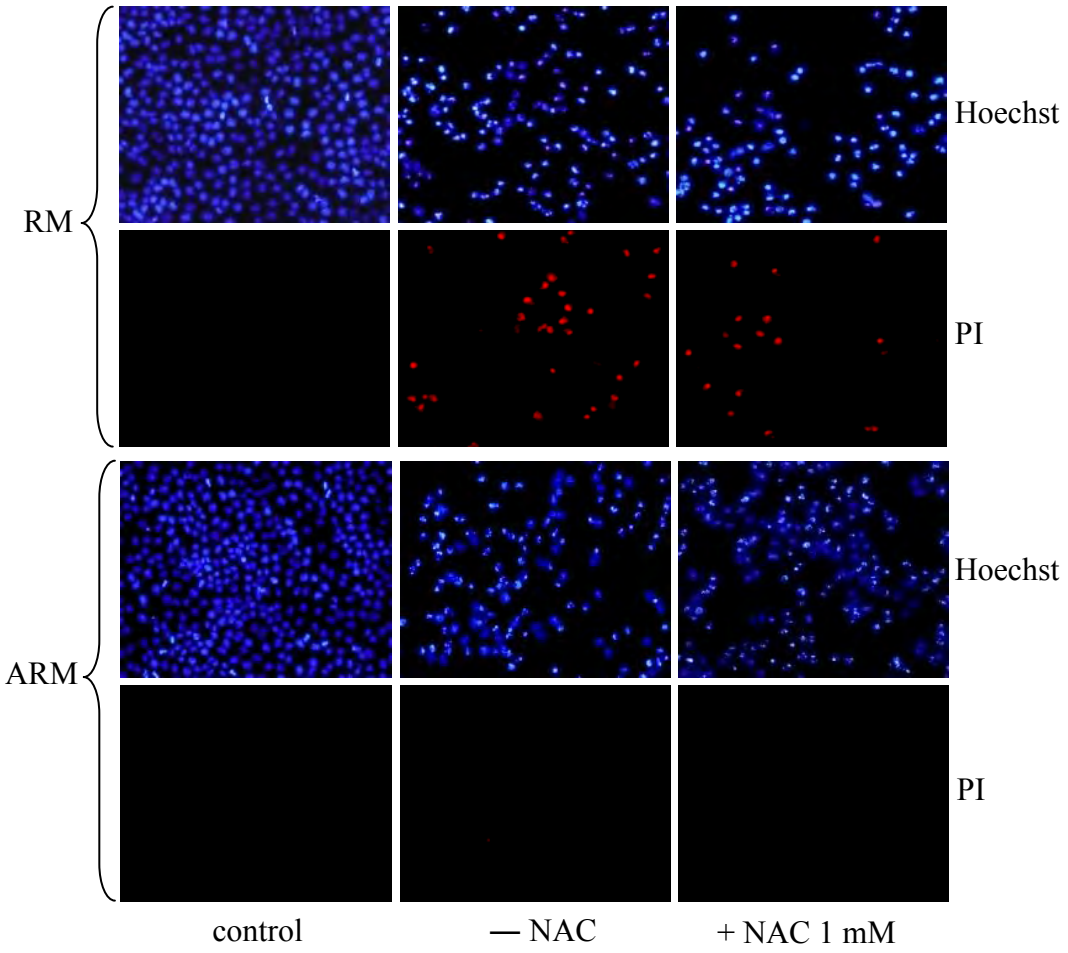

Relative necrotic cell death (%)
0
5
10
15
20
25
0
1
5
10
20
Concentration (μM)
RM
ARM
*
*
*

Figure 4.2 Sub-G_0 analysis by flow cytometry using PI buffer and cell morphology characterization by trypan blue dye. H23 cells were treated with various concentrations of RM and ARM for 24 h. (A): The histogram of DNA content in each population. (B): Relative of DNA contents in sub-G_0 fraction. (C): Morphology of the treated H23 cells was visualized by an automated cell counter, showing live and apoptotic cells with green circles and dead cells with red circles. (D): Relative percentage of necrotic cell death was measured by an automated cell counter. $*p < 0.05$ versus non-treated control in each group.

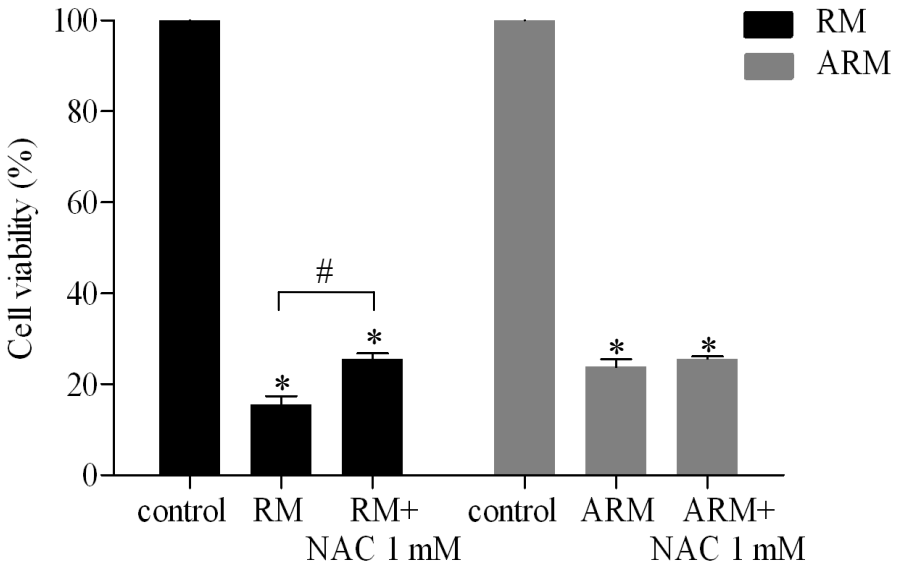
2.4 RM generated ROS-induced necrosis in human lung cancer H23 cells

As mentioned earlier, ROS are hypothesized as one of the key factors for necrotic cell death in response to several stimuli and the quinone moiety has been recognized as a radical generator. To test whether ROS played a role on RM and ARM-mediated cell deaths in our experiments, the known strong anti-oxidant *N*-acetylcysteine (NAC 1 mM) was used. The cells were pre-treated with NAC prior to RM and modified RM treatments and cell viability at 24 h was determined. Modes of death were clarified by nuclear morphology analysis using Hoechst 33342 and PI dyes as shown in Figure 4.3A. The results indicated that NAC significantly prevented cytotoxicity induced by RM but not by ARM (Figure 43B). Also, the results indicated that ROS play a role in regulating necrotic response caused by RM since addition of the anti-oxidant NAC significantly decreased necrotic cells in the RM-treated group whereas such antioxidant did not alter apoptotic death induced by both RM and ARM (Figure 43C). These data supported that the replacement of a quinone group by a monoacetylhydroquinone group in the structure of RM was able to extraordinarily decrease its ROS-dependent necrosis effect.

A


RM
ARM
Hoechst
PI
Hoechst
PI
control
— NAC
+ NAC 1 mM

B


RM
ARM
Cell viability (%)
100
80
60
40
20
0
#
*
control
RM
RM+
NAC 1 mM
control
ARM
ARM+
NAC 1 mM

C

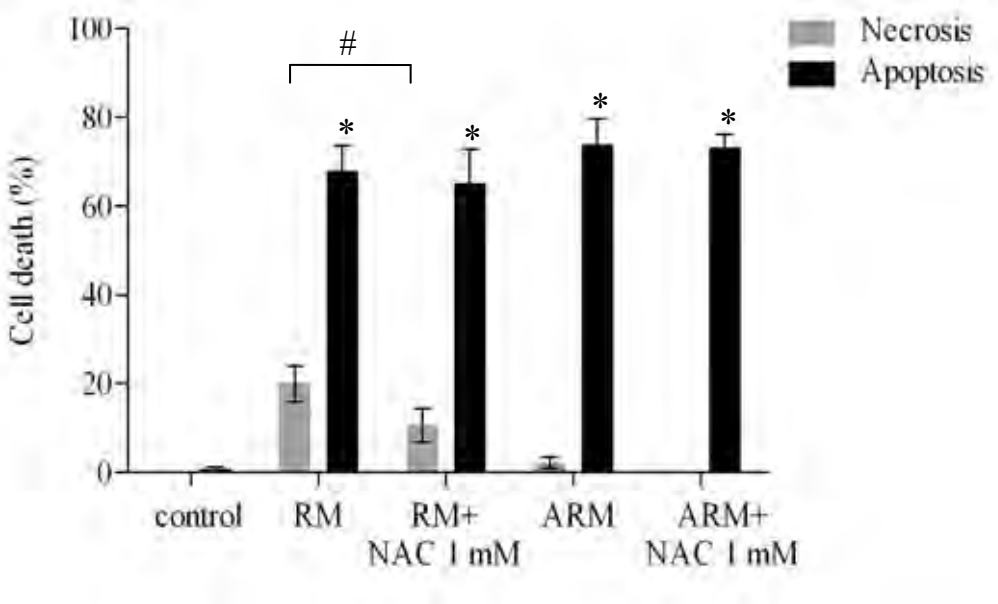


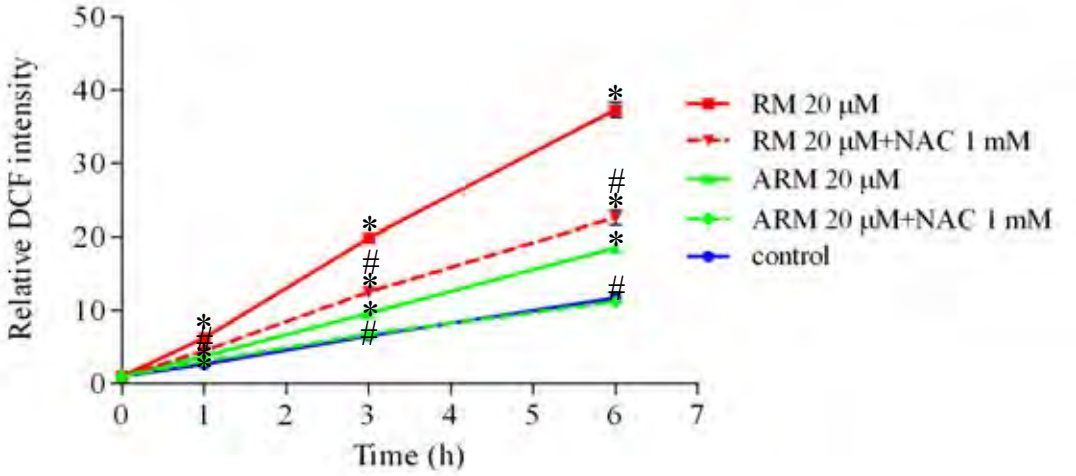
Figure 4.3 Effect of ROS scavengers on ROS-induced cell deaths by RM and ARM on H23 cells. H23 cells were pre-treated with or without 1 mM NAC and incubated with 20 μM of each compound for 24 h. (A): Morphology of apoptosis and necrosis cells was determined by Hoechst and PI. (B): Percentage of cell viability. (C): Percentage of apoptosis and necrosis cell deaths. *$p < 0.05$ versus non-treated control in each group. $^{\#}$ $p < 0.05$ versus RM-treated groups.

2.5 RM generated superoxide anion responsible for its necrosis induction

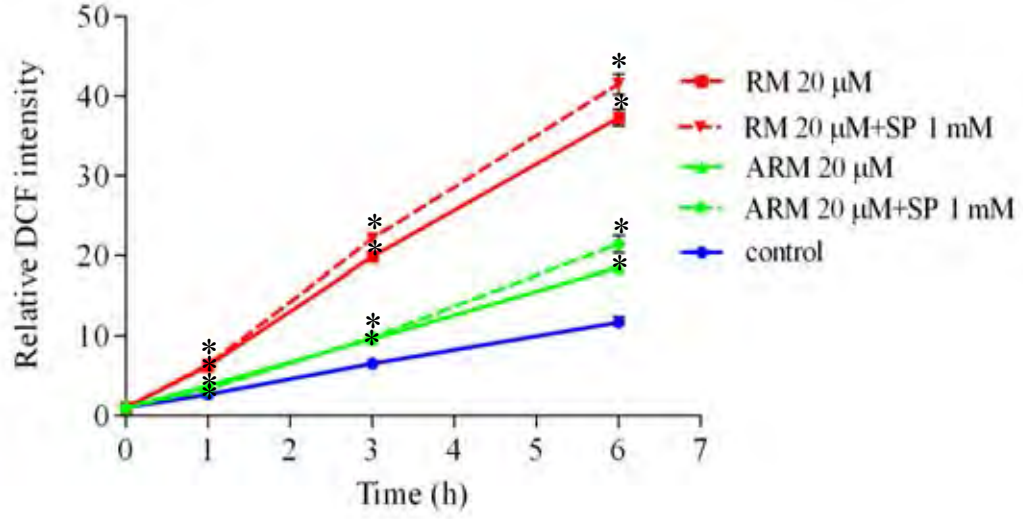
Having shown that ROS played a role in the necrotic mode of action of RM, we further identified the key specific ROS responding in such a necrosis induction. Cellular specific ROS levels after RM and ARM treatments were measured at 0-6 h intervals by specific ROS fluorescence probes which are excellent sensors of ROS due to their high sensitivity (Gomes *et al.*, 2005). $DCFH_2$-DA was used as an oxidative fluorescence probe to show that the increased ROS level by RM was two times higher than that by ARM. The ROS levels were dramatically decreased by addition of NAC, confirming the presence of ROS in the systems caused by both compounds (Figure 4.4A). To identify the presence of hydrogen peroxide in these systems, the cells were pre-treated with sodium pyruvate, a specific hydrogen

peroxide scavenger, and then treated with RM and ARM. The result showed that the hydrogen peroxide inhibitor caused only slight alteration in the ROS signals in both treatments, suggesting that hydrogen peroxide was not the principle ROS presenting in such conditions (Figure 4.4B). HPF fluorescence probe was used as a highly specific probe for hydroxyl radical detection. The experiment further revealed that there was no significant change of intracellular hydroxyl radical level in all treated groups, indicating that this species may be not generated in response to both treatments (Figure 4.4C). Finally, the specific DHE probe for superoxide anion clearly indicated that superoxide anion was significantly up-regulated in response to RM treatment whereas superoxide anion level in the ARM-treated group was comparable to that of un-treated control cells. This information suggested that the modification of RM by quinone replacement could be able to modify the ROS induction property of the compound and attenuate the un-desired necrotic effect of RM by omitting its ability to generate superoxide anion (Figure 4.4D).

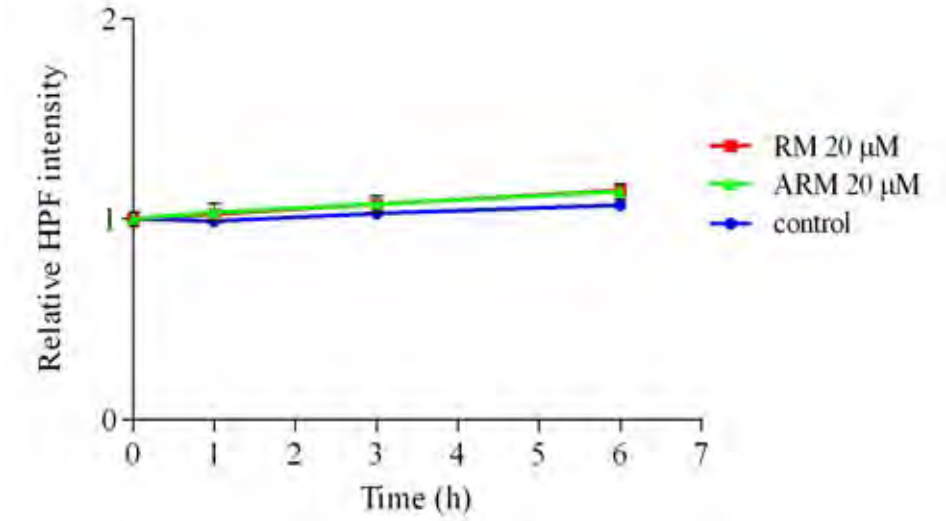
A


Relative DCF intensity
Time (h)
RM 20 μM
RM 20 μM+NAC 1 mM
ARM 20 μM
ARM 20 μM+NAC 1 mM
control

B


Relative DCF intensity
Time (h)
RM 20 μM
RM 20 μM+SP 1 mM
ARM 20 μM
ARM 20 μM+SP 1 mM
control

C



D

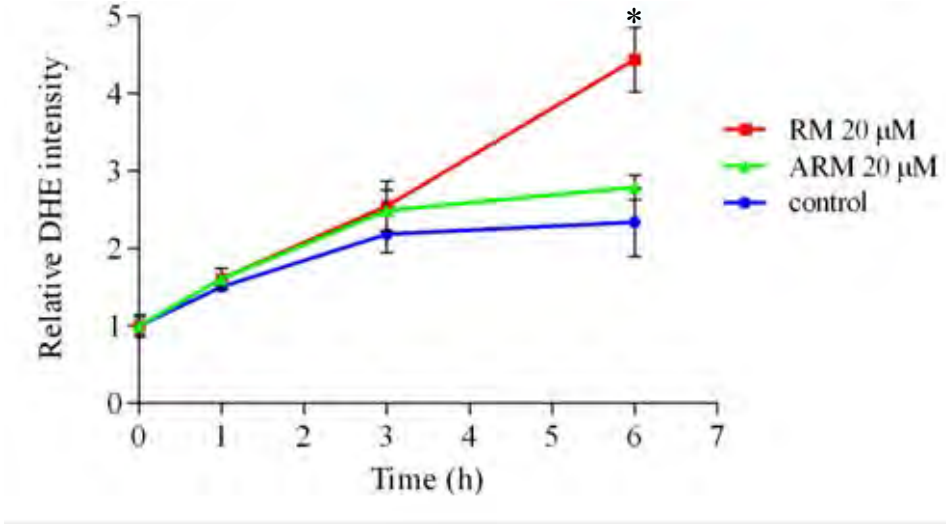


Figure 4.4 Characterization of specific intracellular ROS induced by RM and ARM on H23 cells. H23 cells were incubated with 20 μM of each compound for different time periods. (A): H23 cells were pretreated under the presence or absence of NAC. General intracellular ROS level was measured by $DCFH_2$-DA probe. (B): H23 cells were pretreated under the presence or absence of sodium pyruvate (SP). H_2O_2 level was measured by $DCFH_2$-DA probe. (C): Hydroxyl radical level was measured by HPF probe. (D): Superoxide anion level was measured by DHE probe. Values are means of triplicate samples±SD. *$p < 0.05$ versus non-treated control. $^{\#}$ $p < 0.05$ versus treatment in each group.

3. Discussion

Advanced chemotherapy and strategy are among the most interesting field of cancer research so far. The marine-derived compounds have been demonstrated to have potent anti-cancer activities and recognized as an important source of pharmacologically active substances; however, the unspecific modes of action as well as toxicity to the patient's body have limited their development and use. Recently, the marine organism-derived alkaloid, ecteinascidin 743 (ET-743, Trabectidin, Yondelis®), has been approved by the European Commission for the treatment of advanced or metastatic soft tissue sarcoma (Cuevas and Francesch, 2009). It has been also marketed for the treatment of relapsed platinum-sensitive ovarian cancer in combination with doxorubicin (Meco *et al*., 2003; Sledge *et al*., 2003).

Likewise, RM, a major bistetrahydroisoquinolinequinone isolated from pretreating a Thai blue sponge, *Xestospongia* sp. with KCN has been shown to possess cytotoxicity against a variety of cancer cells (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Charupant *et al*., 2009) and have a potent anti-metastasis activity (Halim *et al*., 2011). However, we found that such a promising effect of RM especially the cytotoxic effect is partially involved with its ability to induce necrosis (Figure 4.1B, C). Unlike apoptosis, necrosis is an undesired mechanism of death for anti-cancer agents since necrosis was intensively shown to induce inflammatory response and its unspecific modes of action may lead to unwanted toxicities in chemotherapy (Majno and Joris, 1995; Trump *et al*., 1997).

The accumulative insight regarding chemical structure and related pharmacological activity lead us to the hypothesis that modification of certain moiety on the molecule of RM may abolish its necrotic effect. Several studies suggested that the quinone group presenting in the structure of compounds is responsible for their ROS induction action (Lown *et al*., 1976; Begleiter, 1983; Begleiter and Blair, 1984; Begleiter, 1985). Accordingly, many anti-tumor agents, for example adriamycin, daunorubicin, actinomycin D, mitomycin C, and treimon, containing such an active moiety were previously shown to mediate DNA strand breaks and cell death via ROS-dependent mechanism (Lown *et al*., 1976; Lown *et al*., 1977; Vig, 1977; Begleiter, 1983; Begleiter and Leith, 1990). Free radical and highly reactive molecules

including semiquinone, superoxide radical, hydrogen peroxide and hydroxyl radical were found in the cells treated with quinone-containing compounds and these molecules were proved to induce necrotic cell death (Malorni *et al*., 1993). Indeed, menadion, a redox active naphtoquinone, was shown to induce necrotic cell death by damaging the integrity of the plasma membrane via free radical-mediated process (Benites *et al*., 2007; Verrax *et al*., 2011). In addition, furylquinones could undergo an activation process by a redox mechanism causing necrotic cell death on TLT hepatoma cells (Benites *et al*., 2007; Benites *et al*., 2011).

RM is structurally related to other alkaloids, including saframycins, naphthyridinomycins, quinocarcins, and ecteinascidins (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Charupant *et al*., 2009; Saito *et al*., 2012). Most of these alkaloids except ecteinascidins contain the quinone group which is able to generate free radicals and ROS associated with antitumor activity and host toxicity (Lown *et al*., 1976; Begleiter, 1983; Begleiter and Blair, 1984; Begleiter, 1985; Rao and Lown, 1990; Pommier *et al*., 1996; Scott and Williams, 2002). Therefore, we attempted to prepare ARM as a monoacetyl-hydroquinone derivative of RM because the related ET-743 contains such a similar functional group and has been approved as a new anticancer drug, Hydrogenation of RM with 10% Pd/C in EtOAc for 4 h gave the bishydroquinone RM in a quantitative yield. Stoichiometric acetylation of the bishydroquinone compound with acetic anhydride (1.5 equiv.) in dry pyridine gave ARM in 40% yield. The 5-*O*-acetyl group in ARM is useful to prevent the reformation of the quinone ring by oxidation and the 8-hydroxyl group is expected to improve better solubility of ARM than that of RM.

We have demonstrated herein that RM which contains the quinone moiety in its structure induced ROS specifically superoxide anion radical and such specific ROS was responsible for its necrotic induction (Figure 4.3, 4.4). In addition, modification of the RM structure by replacing a quinone ring with a monoacetylhydroquinone ring in ARM could be able to abolish its ability to induce superoxide anion and attenuate the necrosis inducing effect. It is also interesting that the apoptosis mediating effect of ARM was comparable to that of RM (Figure 4.2A, B). The closely related DNA alkylating alkaloids, such as saframycins and ecteinascidins, were respectively

proposed to exert their alkylation by interaction of thier α-cyanoamine and α-carbinolamine functional groups via the iminium intermediates with the exocyclic 2-amino group of guanine located in the DNA minor groove (Rao and Lown, 1990; Pommier *et al*., 1996). Since the equivalent α-cyanoamine functional group was also found in the structures of RM and ARM, it is possible that both agents could interact with cellular DNA and mediated apoptosis in the similar manner. In accordingly, our previous work demonstrated that RM induced lung cancer cell apoptosis though p53 induction, which in turn down-regulated anti-apoptotic BCL-2 and MCL-1 proteins (Halim *et al*., 2011).

CHAPTER V

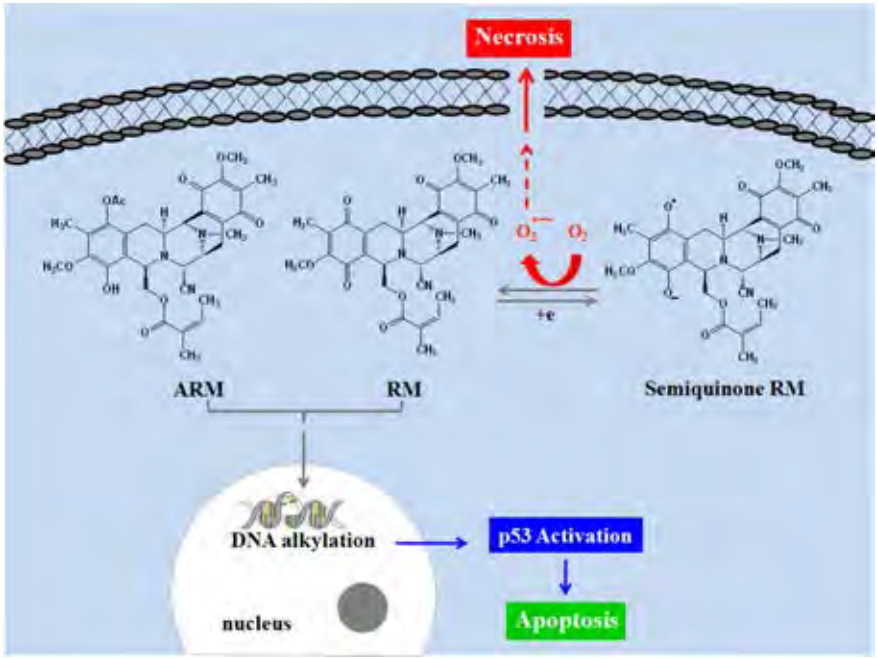
CONCLUSION

1. Identification of nonribosomal peptide synthetase gene involved in biosynthesis of renieramycins from the blue sponge *Xestospongia* sp.

Gene identification based on metagenomic approach was performed in this study to investigation of nonribosomal peptide synthetase (NRPS) genes involved in biosynthesis of renieramycins produced by the Thai blue sponge *Xestospongia* sp. With limited gene information of this sponge, NRPS responsible for renieramycins biosynthesis was proposed by means of other NRPS sequences including *safA*, *sfmC* and *sfcC* involved in diketopeperazine core structure production. PCR product with the size of 764 bp amplified by using degenerate primers contains the partial conserved region of adenylation to thiolation domains. Alignment of the 4 sequences showed that the PCR amplicon was the most similar to *safA*. Neighboring sequences of the 764 bp fragment have to be futher investigated to obtain more information. Additionally, the development of sponge DNA extraction was created due to the problematic operation with non-chemical eliminated DNA. Optimization of removal cycles for renieramycins by organic solvent was found to be critical for quality and quantity of extracted DNA. Finally, quinone functional groups in the RM structure were discovered to generate free radicals in the mixture of DNA preparation, resulting in DNA degradation

2. Necrosis abolishing effect of 5-*O*-acetylhydroquinone renieramycin M in lung cancer cells

In summary, the quinone moiety of renieramycin M (RM) induced ROS production specifically the superoxide anion radical and such a specific ROS was responsible for its necrotic effect as illustrated in Scheme 5.1. The present study provides the first evidence that deletion of a quinone group in the structure of RM could be able to completely abolish necrosis induction and fully preserve apoptotic effect for its cytotoxic activity. The knowledge may have important inferences for structural modification of analogs with similar functional groups and further development of the more effective anti-cancer drugs with the reduction of unwanted toxicity.



Scheme 5.1 The scheme represents the cell death effects of RM and ARM on lung cancer H23 cells. The present study reveals that replacing a quinone of RM by a 5-*O*-acetylhydroquinone of ARM reduces necrosis cell death while fully maintaining apoptosis cell death. The necrosis mediated by RM is proposed as the result of its ability to generate intracellular superoxide anion radicals from the interconversion of a semiquinone and a quinone moiety.

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APPENDICES

APPENDIX A

Purity and yield of DNA extracted from renieramycin-eliminated sponge samples

Table A1 DNA purity and DNA yield extracted from renieramycin-eliminated sponge samples.

	No MeOH extraction				**2 times MeOH extraction**			
	1	**2**	**3**	**Average±SD**	**1**	**2**	**3**	**Average±SD**
OD 260	0.34	0.33	0.38	0.35±0.02	0.54	0.56	0.58	0.56±0.01
OD 280	0.22	0.23	0.25	0.23±0.01	0.34	0.37	0.40	0.37±0.02
OD260/OD280ratio	1.53	1.46	1.50	1.49±0.03	1.57	1.50	1.45	1.50±0.05
Yield (μg)	85.75	84.00	96.75	88.83±6.18	136.25	141.25	146.25	141.25±4.47
	4 times MeOH extraction				**6 times MeOH extraction**			
	1	**2**	**3**	**Average±SD**	**1**	**2**	**3**	**Average±SD**
OD 260	0.64	0.62	0.57	0.61±0.03	0.84	0.85	0.92	0.87±0.03
OD 280	0.39	0.47	0.39	0.42±0.04	0.46	0.48	0.50	0.50±0.01
OD260/OD280ratio	1.63	1.52	1.46	1.53±0.07	1.83	1.77	1.83	1.81±0.03
Yield (μg)	161.50	155.25	143.5	153.41±8.17	212.00	213.00	231.25	218.75±9.69

Table A1 (continued).

	8 times MeOH extraction				10 times MeOH extraction			
	1	**2**	**3**	**Average±SD**	**1**	**2**	**3**	**Average±SD**
OD 260	0.98	1.03	1.00	1.00±0.02	0.76	0.77	0.83	0.79±0.03
OD 280	0.53	0.56	0.54	0.58±0.01	0.42	0.44	0.46	0.48±0.01
OD260/OD280ratio	1.82	1.84	1.83	1.83±0.01	1.81	1.75	1.80	1.79±0.02
Yield (μg)	245.50	258.00	250.25	251.25±5.64	192.25	194.75	208.00	198.33±7.57

APPENDIX B

Standard curve of renieramycin M and HPLC-DAD chromatograms of the remaining renieramycins in sponge samples with a various (0-10 times) renieramycin elimination cycles

Table B1 HPLC analysis of standard renieramycin M (RM).

RM conc. (µg/ml)	RM weight (µg) in 20 µl	AUC1	AUC2	AUC3	Average (AUC)
0.78	0.0156	15022	15216	15073	15103.66
1.56	0.0312	20941	20202	20523	20555.33
3.125	0.0625	44541	44860	45553	44984.66
6.25	0.125	231593	233545	233584	232907.33
12.5	0.25	531029	529474	522737	527746.66
25	0.5	1153990	1179273	1192033	1175098.66
50	1	2300568	2305856	2311866	2306096.66
100	2	4137726	4087816	3991386	4072309.33
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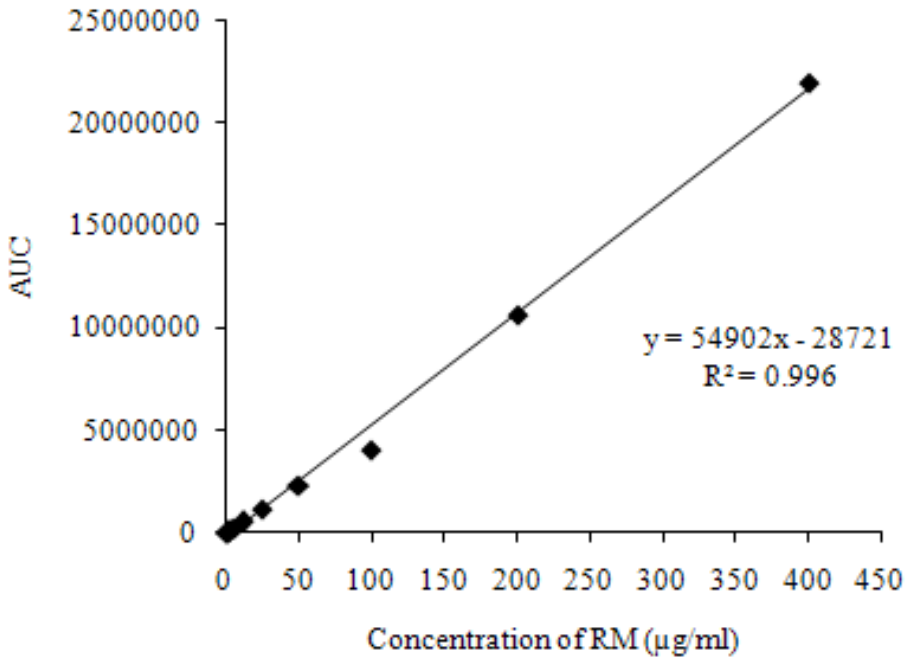

Figure B1 Standard curve of renieramycin M (RM)

Table B2 HPLC analysis of the remaining renieramycin M in sponge samples with different cycles of renieramycins elimination.

MeOH extraction times	Renieramycin M			Average (AUC)	Acenaphthene Average (AUC)	*Area of RM	RM conc. (µg/µl)	RM weight (µg) in total vol. 200 µl
	AUC1	AUC2	AUC3					
0	12107613	12408898	11747250	12087920.33	4195566.00	11290725.07	206.17	41.2±5.63
2	6068766	5513853	6050320	5877646.33	4434691.33	5802919.33	106.21	21.2±5.66
4	677117	834487	829296	780300.00	4769767.00	828587.65	15.61	3.1±1.72
6	106424	102579	120420	109807.66	4530538.00	110754.69	2.54	0.5±0.17
8	36043	34610	39739	36797.33	4556636.00	37328.48	1.20	0.2±0.04
10	9287	10944	10153	10128.00	4463595.66	10064.40	0.70	0.1±0.01
				Average	4491799.00			

*Area of renieramycin M = (Average AUC of RM × Average AUC of Acenaphthene in each time)/ Average AUC of Acenaphthene

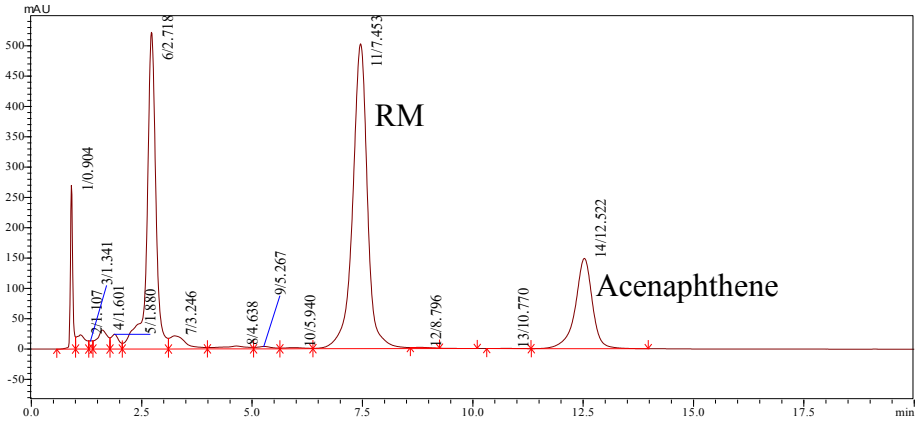

Figure B2 HPLC-DAD chromatograms of RM in no MeOH extraction samples.

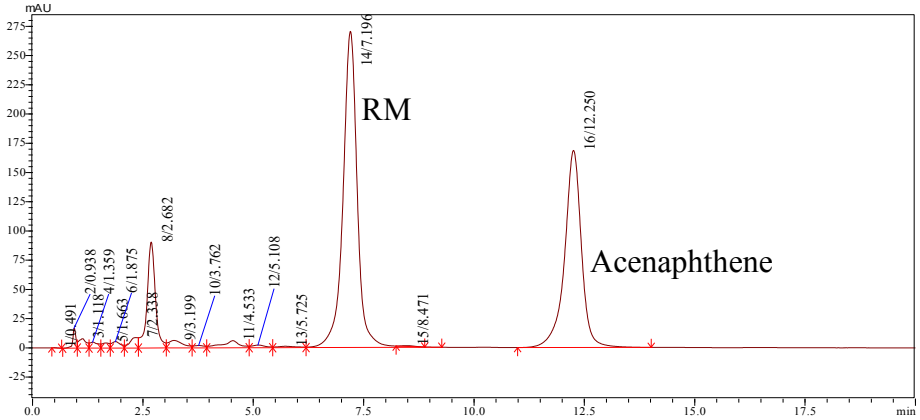

Figure B3 HPLC-DAD chromatograms of RM in 2 times MeOH extraction samples.

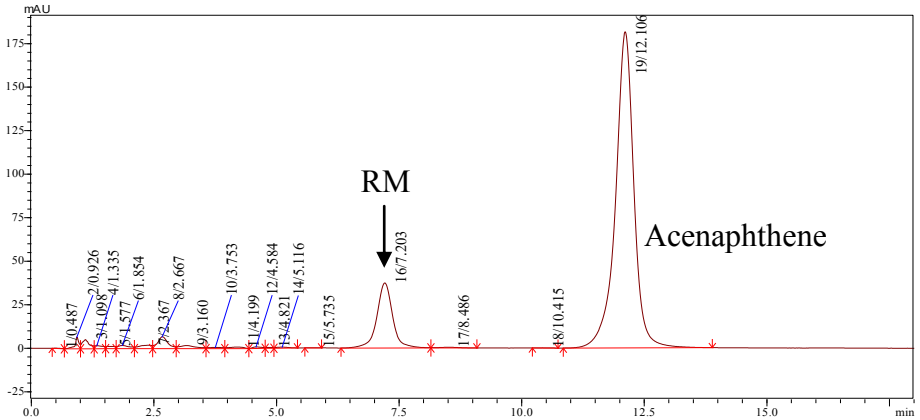

Figure B4 HPLC-DAD chromatograms of RM in 4 times MeOH extraction samples.

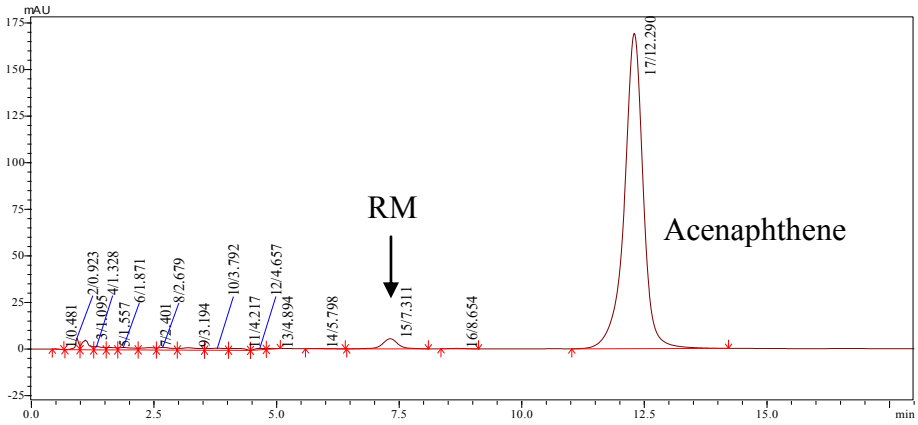

Figure B5 HPLC-DAD chromatograms of RM in 6 times MeOH extraction samples.

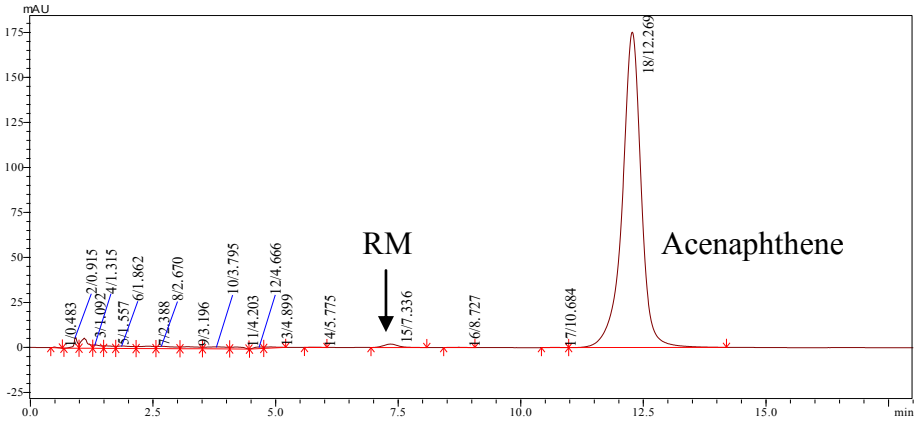

Figure B6 HPLC-DAD chromatograms of RM in 8 times MeOH extraction samples.

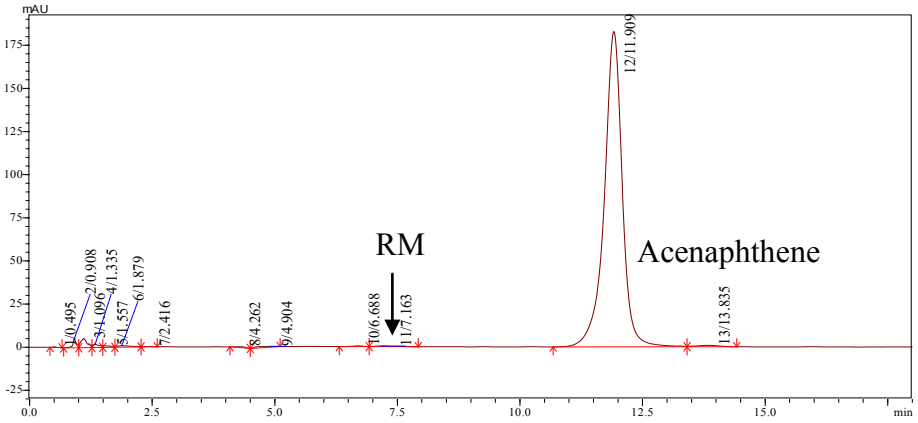

Figure B7 HPLC-DAD chromatograms of RM in 10 times MeOH extraction samples.

APPENDIX C

NRPS nucleotide sequences of saframycin A, saframycin Mx1, and safracin B from Genbank Database

LOCUS DQ838002_sfmC_4458 bp

DEFINITION *Streptomyces lavendulae* strain NRRL 11002 tyrosinase, tyrosinase co-factor, putative translation initiation inhibitor, putative transcriptional regulator, hypothetical protein, putative methyltransferase, hypotheticalprotein, and glyoxalase/bleomycin resistance protein/dioxygenase genes, complete cds; and saframycin A biosynthetic gene cluster, complete sequence.

ACCESSION DQ838002

VERSION DQ838002.1 GI:146446759

KEYWORDS .

SOURCE *Streptomyces lavendulae* (unknown)

ORGANISM *Streptomyces lavendulae*
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.

REFERENCE 1 (bases 1 to 62804)

AUTHORS Li,L., Deng,W., Song,J., Ding,W., Zhao,Q.F., Peng,C., Song,W.W., Tang,G.L. and Liu,W.

TITLE Characterization of the saframycin A gene cluster from *Streptomyces lavendulae* NRRL 11002 revealing a nonribosomal peptide synthetase system for assembling the unusual tetrapeptidyl skeleton in an iterative manner

JOURNAL J. Bacteriol. 190 (1), 251-263 (2008)

PUBMED 17981978

REFERENCE 2 (bases 1 to 62804)

AUTHORS Li,L., Tang,G. and Liu,W.

TITLE Direct Submission

JOURNAL Submitted (06-JUL-2006) State Key Laboratory of Bioorganic and Natural Product Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Fenglin Rd., Shanghai 200032, PRC

FEATURES Location/Qualifiers

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LOCUS MXU24657_safA_7818 bp

DEFINITION *Myxococcus xanthus* saframycin Mx1 synthetase B (safB), saframycin Mx1 synthetase A (safA), and safC genes, complete cds.

ACCESSION U24657

VERSION U24657.1 GI:1171127

KEYWORDS .

SOURCE *Myxococcus xanthus* (unknown)

ORGANISM *Myxococcus xanthus*
Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Cystobacterineae; Myxococcaceae; Myxococcus.

REFERENCE 1 (sites)

AUTHORS Pospiech, A., Cluzel, B., Bietenhader, J. and Schupp, T.

TITLE A new *Myxococcus xanthus* gene cluster for the biosynthesis of the antibiotic saframycin Mx1 encoding a peptide synthetase

JOURNAL Microbiology 141 (Pt 8), 1793-1803 (1995)

PUBMED 7551044

REFERENCE 2 (bases 1 to 14100)

AUTHORS Pospiech, A., Bietenhader, J. and Schupp, T.

TITLE Two multifunctional peptide synthetases and an O-methyltransferase are involved in the biosynthesis of the DNA-binding antibiotic and antitumour agent saframycin Mx1 from *Myxococcus xanthus*

JOURNAL Microbiology 142 (Pt 4), 741-746 (1996)

PUBMED 8936303

REFERENCE 3 (bases 1 to 14100)

AUTHORS Pospiech, A.

TITLE Direct Submission
are involved in the biosynthesis of the DNA-binding antibiotic and antitumour agent saframycin Mx1 from *Myxococcus xanthus*

JOURNAL Submitted (12-APR-1995) Andreas Pospiech, Core Drug Discovery Technologies, Ciba Geigy-AG, Basel, CH-4002, Switzerland

FEATURES Location/Qualifiers

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LOCUS AY061859_sfcC_4299 bp
DEFINITION *Pseudomonas fluorescens* safracin biosynthesis gene cluster, complete sequence.
ACCESSION AY061859
VERSION AY061859.1 GI:37542630
KEYWORDS .
SOURCE *Pseudomonas fluorescens* (unknown)
ORGANISM *Pseudomonas fluorescens*
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 17974)
AUTHORS Velasco,A., Acebo,P., Gomez,A., Schleissner,C., Rodriguez,P., Aparicio,T., Conde,S., Munoz,R., de la Calle,F., Garcia,J.L. and Sanchez-Puelles,J.M.
TITLE Molecular characterization of the safracin biosynthetic pathway from *Pseudomonas fluorescens* A2-2: designing new cytotoxic compounds
JOURNAL Mol. Microbiol. 56 (1), 144-154 (2005)
PUBMED 15773985
REFERENCE 2 (bases 1 to 17974)
AUTHORS Velasco,A., Munoz,R., Henriquez,R. and Sanchez-Puelles,J.M.
TITLE Direct Submission
JOURNAL Submitted (05-NOV-2001) Biotechnology, PharmaMar, c/ de la Calera n. 3, Tres Cantos, Madrid 28760, Spain
FEATURES Location/Qualifiers
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181 cttgctgtcg tgcagcagaa cctgagcgcg tggatcgcac acagcgagtc cttacgcagc
241 cttttcgtcg aagtactgga acgccccgtc aggcttctga tgcctacggg cctggtgaaa
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APPENDIX D

NRPS amino acid sequences of *sfmC*, *safA*, and *sfcC* genes encoding tyrosine of saframycin A, saframycin Mx1, and safracin B, respectively. Underline character represent A-domain to T-domain

LOCUS Saframycin A_SfmC_1485 aa

ORIGIN

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121 rhlveeefsl ghgpllrall lrsadggaae lvlvghrlvl datsldllva ellgedaphg
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1321 ilpdyprifd vipvdyvasa ivhisrkrea idgfyhlfnp apvplltfcd wiksygyefd
1381 ivpfeegrrr algvgpshll yplvplikda eaephraldp kymdevqpal ecaetlrmla
1441 gsdiacpptt eadahavmdy lvrtgfmpap advvhdepss tleer

LOCUS Saframycin Mx1_SafA_2605 aa

ORIGIN

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1201 stfkdmggtp lrvarpvmel alavteaagt glddalqrla rqelgerlep atgpllrlhl
1261 vrlgaeehll lvkahelisd epslealire vlatygallr geqsaassvs ayaeyaareq
1321 awlsgpvcgq qlehwrrklt dlpllrlftd rprpvikthr cdsvstllpl elsrrmeala
1381 srngglraav lagfqvllsr ytgqsdvtvg vradlrseae rgrllgpast alvlrtdlrg
1441 epsfmetllr vdvareeaer fasvpfgtlv dtlqpardls rtpffqvmyl frdareelav
1501 gaglralaqa lpfgttpvdi tlaatateqg lhlrvdfnsd lydastarrl lghlrtllsa
1561 avdapgksia rlpllaeeew rqvfgdwnqa ehalpsrclh elveeqaart phavavtceg
1621 hqltyaelda hanqlahhlr qlglapegrv aiclersvem vtamlavlka ggayvpldpe
1681 ypterlaqlf adceahvvlt qqsladrvkm vaarrvfvdt dwpviaacpr qapppvvtpd
1741 hlayliytsg stgtpkavml shrgivnnla wrqrtwplsa edrvlqnhsf sfdpsvwatf

1801 wpllvgarav ltpsgqhyds kalvgllreq gitvygavps lnavlmeepe igrcthlryv
1861 lsgaealtgg lqrgifsrvs aavanlygpt ettidatawn cprvdapeda pigrpianlr
1921 myvldehlqp vpvgvpgelf vggvglargy harpgltsqr flpdpfssda garlyrtgdl
1981 gryradgaim flgrvdeqvk vsgyrvelge vetalgrhpd vreaivvare glqgikrlva
2041 yvtpakggtp earsltafle kilpaymipp vfvivnelpk mpsgkvnrna lpapqmdrpd
2101 tagayvaprt pledeiasaf agvlgmdrvg veddffevgg tslllarlas rllnrfqiai
2161 pvhqffkipt vagvanvvet yqregldavl mnqhatrlda daslapdisp eglplanyla
2221 pssvlltgat gylgaflleq llkrtratvy clvraadpaq amdrvratmh qylvwdeaya
2281 erirplvgdl gkprlglsre ewerlgleld siyhngalvn fvypysalrg pnvhgtqevl
2341 rlgcqhrlka vhyvstidvl lathmprpfm eddaplrnpi evpggytgsk wvaekvvnia
2401 rargipvciy rpglilshee tgatqtndyl lvafrgyvpm giipdyprif dtipvdyaak
2461 aivhistqre algrffhlfn papvslrrfc dwirsygyaf divpfdearr qaldvdtshp
2521 lyplvplird aeaepqesld pafidqlrpd lecrsavevl agsdircppm teelahrclq
2581 ylvdigflqr pevlraarqq kasga

LOCUS SafracinB_SfcC_1432 aa

ORIGIN

1 mhsptidtfe aalrslpaar dalgayplss eqkrlwllaq lagtatlpvt vryaftgtvd
61 lavvqqnlsa wiahseslrs lfvevlerpv rllmptglvk leyfdrppsd admaeligaa
121 feldkgpllr afitrtaaqq helhlvghpi vvdepslqri aqtlfqtepd hqypavgaia
181 evfqreqtla qdaqiteqwq qwgiglqapa ateiptenpr paikgsdrqv healtawgdq
241 pvaeaeivss wltvlmrwqg sqsalcaikv rdkahanlig plqtylpvrv dmpdgstlaq
301 lrlqveeqln gndhpsfstl levcppkrdl srtpyfqtgl qfiahdveqr dfhagnltrl
361 ptkqpssdld lfiscwvsdg tlgltldydc avlnssqvev laqalisvls apgeqpiatv
421 almgqqmqqt vlaqahgprt tppqltltew vaastekspl avavidhgqq lsyaelwara
481 alvaanisqh vakprsiiav alprsaefia allgvvragh aflpidprlp tdriqflien
541 sgcelvitsd qqsvegwpqv arirmealdp dirwvaptgl shsdaayliy tsgstgvpkg
601 vvvehrqvvn nilwrqrtwp ltaqdnvlhn hsfsfdpsvw alfwplltgg tivladvrtm
661 edstalldlm irhdvsvlgg vpsllgtlid hpfandcrav klvlsggevl npelahkiqk
721 vwqadvanly gpteatidal yfsidknaag aipigypidn tdayivdlnl npvppgvpge
781 imlagqnlar gylgkpaqta qrflpnpfgn grvyatgdlg rrwssgaisy lgrrdqqvki
841 rghrielnev ahllcqalel keaivfaqha gteqarlvaa ieqqpglhse gikqellrhl
901 paylipsqll lldelprtat gkvdmlkldq laapqlndag gtecraprtd leqsvmtdfa
961 qvlgltavtp dtdffeqggn silltrlagt lsakyqvqip lheffltptp aavaqaieiy
1021 rregltalls rqhaqtleqd iyleehirpd glphanwyqp svvfltgatg ylglylieql
1081 lkrttsrvic lcrakdaeha karileglkt yridvgselx rveyltgdla lphlglsehq
1141 wqtlaeevdv iyhngalvnf vypysalkat nvggtqaile lactarlksv qyvstvdtll
1201 athvprpfie ddaplrsavg vpvgytgskw vaegvanlgl rrgipvsifr pglilghtet
1261 gasqsidyll valrgflpmg ivpdyprifd ivpvdyvaaa ivhismqpqg rdkffhlfnp
1321 apvtirqfcd wirefgyefk lvdfehgrqq alsvppghll yplvplirda dplphraldp
1381 dyihevnpal eckqtlella ssditlsktt kayahtilry lidtgfmakp gv

APPENDIX E

Amino acid sequences of 13 positive clones which amplified by A5-T degenerate primer and NCBI blast results

LOCUS clone 1

ORIGIN

```
   1 aygpteatid atfwqcdqsd tsdsapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflsrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff evgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	320	528	99%	5e-96	56%	AAC44129.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	280	503	99%	3e-82	50%	YP_007121014.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	278	525	100%	6e-82	50%	YP_007317765.1
non-ribosomal peptide synthase [Nostoc sp. 'Peltigera membranacea cyanobiont']	276	513	99%	7e-81	53%	ADL59764.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	263	263	99%	7e-81	49%	CAC60249.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	274	274	99%	8e-81	49%	ZP_06711154.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
320 bits(819)	5e-96()	Composition-based stats.	142/254(56%)	190/254(74%)	1/254(0%)	

Features:

```
Query  2     YGPTEATIDATFWQCDQSDTSDSAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTE TIDAT W C + D  + APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLSRIDEQVKVRGYRI  120
            ARGYH RPGLT ++RFLP+PFS + G R+Y+TGDLGRY+ DG I FL R+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
            EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
            MIP   +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFEVGG  254
            +R+ + DDFFEVGG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 2

ORIGIN

```
   1 aygptettid atfrqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff elgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	318	526	99%	2e-95	56%	AAC44129.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	279	504	99%	5e-82	50%	YP_007121014.1
nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] >gb|EJW15724.1| nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29]	277	765	99%	3e-81	49%	ZP_10866681.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	276	527	100%	5e-81	50%	YP_007317765.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	274	274	99%	1e-80	50%	ZP_06711154.1
putative PAS/PAC sensor protein [Microcoleus vaginatus FGP-2] >gb|EGK86185.1| putative PAS/PAC sensor protein [Microcoleus vaginatus FGP-2]	273	542	99%	7e-80	54%	ZP_08494005.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
318 bits(814)	2e-95()	Composition-based stats.	143/254(56%)	191/254(75%)	1/254(0%)	

Features:

```
Query  2     YGPTETTIDATFRQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTETTIDAT   C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH RPGLT++RFLP+PFS  + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP   +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFELGG  254
              +R+ + DDFFE+GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 3

ORIGIN

```
   1 iwadggtida tfwqcdqsdt sesapigrpi anlktyilne yfqivpvgvp gqifvggtgi
  61 argyhnrpgl taerflpnpf sntgeriykt gdlgryktdg rieflgride qvkvrgyrie
 121 lneidillnq hpnvkeaicn vymnflnenq lvayvslekd cclqsddltk ylgerlpsym
 181 ipsflmildk lpkmpngkin rsalpvpsik ngnnsenyia pktpieqeis rafldvlgln
 241 risihddffd lgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	310	516	100%	1e-92	55%	AAC44129.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	269	511	98%	8e-79	50%	YP_007317765.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	269	491	98%	2e-78	49%	YP_007121014.1
nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] >gb|EJW15724.1| nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29]	267	730	97%	1e-77	49%	ZP_10866681.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	265	265	97%	2e-77	49%	ZP_06711154.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	253	253	98%	6e-77	48%	CAC60249.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1893 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
310 bits(793)	1e-92()	Composition-based stats.	137/248(55%)	186/248(75%)	1/248(0%)	

Features:

```
Query  7     TIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGIARGYHN  66
            TIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+ARGYH
Sbjct  1893  TIDATAWNCPRVDAPEDAPIGRPIANLRMYYVLDEHLQPVPVGVPGELFVGGVGLARGYHA  1952

Query  67    RPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRIELNEID  125
             RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+EL E++
Sbjct  1953  RPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRVELGEVE  2012

Query  126   ILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSYMIPSFL  185
              L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+YMIP
Sbjct  2013  TALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAYMIPPVF  2072

Query  186   MILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISRAFLDVLGLNRISIH  245
               +I+++LPKMP+GK+NR+ALP P +  + + Y+AP+TP+E EI+ AF  VLG++R+ +
Sbjct  2073  VIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGMDRVGVE  2132

Query  246   DDFFDLGG  253
             DDFF++GG
Sbjct  2133  DDFFEVGG  2140
```

LOCUS clone 4

ORIGIN

```
   1 aygptettid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhtrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff dvgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	324	533	99%	9e-98	57%	AAC44129.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	278	278	99%	4e-82	50%	ZP_06711154.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	277	522	100%	1e-81	50%	YP_007317765.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	277	499	99%	2e-81	49%	YP_007121014.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	260	260	99%	7e-80	48%	CAC60249.1
nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] >gb|EJW15724.1| nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29]	273	747	99%	1e-79	47%	ZP_10866681.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
324 bits(831)	9e-98()	Composition-based stats.	144/254(57%)	192/254(75%)	1/254(0%)	

Features:

```
Query  2     YGPTETTIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTETTIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHTRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
              ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP  +I+++LPKMP+GK+NR+ALP P +  + + Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFDVGG  254
              +R+ + DDFF+VGG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 5

ORIGIN

```
   1 aygpteatid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff eqgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	320	528	99%	2e-96	56%	AAC44129.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	281	506	99%	9e-83	50%	YP_007121014.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	280	526	100%	2e-82	51%	YP_007317765.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	278	278	99%	4e-82	50%	ZP_06711154.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	265	265	99%	2e-81	49%	CAC60249.1
non-ribosomal peptide synthase [Nostoc sp. 'Peltigera membranacea cyanobiont']	274	512	99%	3e-80	53%	ADL59764.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
320 bits(821)	2e-96()	Composition-based stats.	143/254(56%)	190/254(74%)	1/254(0%)	

Features:

```
Query  2     YGPTEATIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTE TIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP   +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFEQGG  254
             +R+ + DDFFE GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 6

ORIGIN

```
      1 aygptettid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
     61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
    121 elneidilln *hpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
    181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
    241 nrisihddff elgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	322	532	99%	7e-97	57%	AAC44129.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	278	278	99%	2e-82	50%	ZP_06711154.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	279	525	100%	2e-82	51%	YP_007317765.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	279	503	99%	8e-82	50%	YP_007121014.1
nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] >gb|EJW15724.1| nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29]	275	754	99%	2e-80	48%	ZP_10866681.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	261	261	99%	3e-80	49%	CAC60249.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
322 bits(825)	7e-97()	Composition-based stats.	144/254(57%)	191/254(75%)	1/254(0%)	

Features:

```
Query  2     YGPTETTIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTETTIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLN*HPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L  HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP   +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFELGG  254
              +R+ + DDFFE+GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 7

ORIGIN

```
   1 aygptettid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff degg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	322	530	99%	1e-96	56%	AAC44129.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	278	278	99%	3e-82	50%	ZP_06711154.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	278	524	100%	7e-82	50%	YP_007317765.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	278	499	99%	1e-81	49%	YP_007121014.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	261	261	99%	3e-80	49%	CAC60249.1
nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] >gb|EJW15724.1| nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29]	272	748	99%	2e-79	47%	ZP_10866681.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
322 bits(824)	1e-96()	Composition-based stats.	143/254(56%)	191/254(75%)	1/254(0%)	

Features:

```
Query  2     YGPTETTIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTETTIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP   +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFDEGG  254
             +R+ + DDFF+ GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 8

ORIGIN

```
   1 aygpteatid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff dhgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	319	525	99%	7e-96	56%	AAC44129.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	280	525	100%	2e-82	51%	YP_007317765.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	280	503	99%	2e-82	50%	YP_007121014.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	276	276	99%	1e-81	49%	ZP_06711154.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	263	263	99%	8e-81	49%	CAC60249.1
non-ribosomal peptide synthase [Nostoc sp. 'Peltigera membranacea cyanobiont']	273	510	99%	8e-80	52%	ADL59764.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
319 bits(818)	7e-96()	Composition-based stats.	142/254(56%)	190/254(74%)	1/254(0%)	

Features:

```
Query  2     YGPTEATIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTE TIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP   +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFDHGG  254
             +R+ + DDFF+ GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 9

ORIGIN

```
   1 aygpteatid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff elgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	322	532	99%	8e-97	56%	AAC44129.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	284	511	99%	1e-83	50%	YP_007121014.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	283	532	100%	2e-83	51%	YP_007317765.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	267	267	99%	2e-82	50%	CAC60249.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	278	278	99%	5e-82	50%	ZP_06711154.1
non-ribosomal peptide synthase [Nostoc sp. 'Peltigera membranacea cyanobiont']	276	516	99%	7e-81	53%	ADL59764.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
322 bits(825)	8e-97()	Composition-based stats.	143/254(56%)	191/254(75%)	1/254(0%)	

Features:

```
Query  2     YGPTEATIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
            YGPTE TIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP   +I+++LPKMP+GK+NR+ALP P +   + + Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFELGG  254
              +R+ + DDFFE+GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 10

ORIGIN

```
        1 aygpteatid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
       61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
      121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
      181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
      241 nrisihddff edg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	318	523	99%	1e-95	56%	AAC44129.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	279	501	99%	6e-82	50%	YP_007121014.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	277	521	100%	2e-81	51%	YP_007317765.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	275	275	99%	4e-81	50%	ZP_06711154.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	262	262	99%	2e-80	49%	CAC60249.1
nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] >gb|EJW15724.1| nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29]	273	744	99%	2e-79	48%	ZP_10866681.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2139

Score	Expect	Method	Identities	Positives	Gaps	Frame
318 bits(815)	1e-95()	Composition based stats.	142/253(56%)	189/253(74%)	1/253(0%)	

Features:

```
Query  2     YGPTEATIDATFWQCDQSDTSESAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
             YGPTE TIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
              ARGYH RPGLT++RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V +EAI          +LVAYV+  K    ++ LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP  +I+++LPKMP+GK+NR+ALP P +   + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFEDG  253
              +R + + DDFFE G
Sbjct  2127  DRVGVEDDFFEVG  2139
```

LOCUS clone 11

ORIGIN

```
   1 aygptettid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
  61 iargyhsrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
 121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
 181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
 241 nrisihddff elgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	325	536	99%	7e-98	57%	AAC44129.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	279	279	99%	1e-82	50%	ZP_06711154.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	280	505	99%	2e-82	50%	YP_007121014.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	278	526	100%	4e-82	50%	YP_007317765.1
peptide synthetase [Chloroglocopsis fritschii PCC 6912]	263	263	99%	6e-81	49%	CAC60249.1
putative PAS/PAC sensor protein [Microcoleus vaginatus FGP-2] >gb|EGK86185.1| putative PAS/PAC sensor protein [Microcoleus vaginatus FGP-2]	272	539	99%	1e-79	53%	ZP_08494005.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
325 bits(832)	7e-98()	Composition-based stats.	144/254(57%)	193/254(75%)	1/254(0%)	

Features:

```
Query  2     YGPTETTIDATFWQCDQSDTSFSAPIGRPIANLKTYILNEYFQIVPVGVPGQIFVGGTGI  61
             YGPTETTIDAT W C + D  F APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL  1946

Query  62    ARGYHSRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI  120
             ARGYH+RPGLT+ RFLP+PFS + G R+Y+TGDLGRY+ DG I FLGR+DEQVKV GYR+
Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V +AI          +LVAYV+  K    +I  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

Query  181   MIPSFLMILDKLPKMPNGKINRSALPVPSIKNGNNSENYIAPKTPIEQEISQAFLDVLGL  240
              MIP  +I+++LPKMP+GK+NR+ALP P +  + +  Y+AP+TP+E EI+ AF  VLG+
Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIAGAFAGVLGM  2126

Query  241   NRISIHDDFFELGG  254
             +R+ + DDFFE+GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 12

ORIGIN

```
      1 aygpteatid atfwqcdqsd tsesapigrp ianlktyiln eyfqivpvgv pgqifvggtg
     61 iargyhnrpg ltaerflpnp fsntgeriyk tgdlgryktd grieflgrid eqvkvrgyri
    121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt kylgerlpsy
    181 mipsflmild klpkmpngki nrsalpvpsi kngnnsenyi apktpieqei sqafldvlgl
    241 nrisihddff ehgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	320	528	99%	2e-96	56%	AAC44129.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	281	506	99%	8e-83	50%	YP_007121014.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	280	526	100%	2e-82	51%	YP_007317765.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	278	278	99%	5e-82	50%	ZP_06711154.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	264	264	99%	3e-81	49%	CAC60249.1
non-ribosomal peptide synthase [Nostoc sp. 'Peltigera membranacea cyanobiont']	275	513	99%	3e-80	53%	ADL59764.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
320 bits(821)	2e-96()	Composition-based stats.	143/254(56%)	190/254(74%)	1/254(0%)	

Features:

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            YGPTE TIDAT W C + D  E APIGRPIANL+ Y+L+E+ Q VPVGVPG++FVGG G+
Sbjct  1887  YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYYVLDEHLQPVPVGVPGELFVGGVGL  1946

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Sbjct  1947  ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV  2006

Query  121   ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTKYLGERLPSY  180
              EL E++  L +HP+V+EAI          +LVAYV+  K    ++  LT +L + LP+Y
Sbjct  2007  ELGEVETALGRHPDVREAIVVAREGLQGIKRLVAYVTPAKGGTPEARSLTAFLEKILPAY  2066

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Sbjct  2067  MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM  2126

Query  241   NRISIHDDFFEHGG  254
             +R+ + DDFFE GG
Sbjct  2127  DRVGVEDDFFEVGG  2140
```

LOCUS clone 13

ORIGIN

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      121 elneidilln qhpnvkeaic nvymnflnen qlvayvslek dcclqsddlt eylgerlpsy
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      241 nrisihddff ddgg
```

Description	Max score	Total score	Query cover	E value	Max ident	Accession
saframycin Mx1 synthetase A [Myxococcus xanthus]	319	524	99%	9e-96	56%	AAC44129.1
amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417] >gb|AFZ28247.1| amino acid adenylation enzyme/thioester reductase family protein [Cylindrospermum stagnale PCC 7417]	281	526	100%	7e-83	51%	YP_007317765.1
non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113] >gb|AFZ17608.1| non-ribosomal peptide synthase/amino acid adenylation enzyme [Microcoleus sp. PCC 7113]	279	503	99%	8e-82	50%	YP_007121014.1
non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14] >gb|EFF94276.1| non-ribosomal peptide synthetase MxaA [Streptomyces sp. e14]	276	276	99%	2e-81	49%	ZP_06711154.1
peptide synthetase [Chlorogloeopsis fritschii PCC 6912]	263	263	99%	4e-81	49%	CAC60249.1
non-ribosomal peptide synthase [Nostoc sp. 'Peltigera membranacea cyanobiont']	273	509	99%	8e-80	52%	ADL59764.1

Alignments

saframycin Mx1 synthetase A [Myxococcus xanthus]

Sequence ID: **gb|AAC44129.1|** Length: 2605 Number of Matches: 2

Range 1: 1887 to 2140

Score	Expect	Method	Identities	Positives	Gaps	Frame
319 bits(817)	9e-96()	Composition-based stats.	142/254(56%)	190/254(74%)	1/254(0%)	

Features:

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Sbjct 1887 YGPTETTIDATAWNCPRVDAPEDAPIGRPIANLRMYVLDEHLQPVPVGVPGELFVGGVGL 1946

Query 62   ARGYHNRPGLTAERFLPNPFS-NTGERIYKTGDLGRYKTDGRIEFLGRIDEQVKVRGYRI 120
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Sbjct 1947 ARGYHARPGLTSQRFLPDPFSSDAGARLYRTGDLGRYRADGAIMFLGRVDEQVKVSGYRV 2006

Query 121  ELNEIDILLNQHPNVKEAICNVYMNFLNENQLVAYVSLEKDCCLQSDDLTEYLGERLPSY 180
            EL E++ L +HP+V+EAI        +LVAYV+ K   ++ LT +L + LP+Y
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Sbjct 2067 MIPPVFVIVNELPKMPSGKVNRNALPAPQMDRPDTAGAYVAPRTPLEDEIASAFAGVLGM 2126

Query 241  NRISIHDDFFDDGG 254
            +R+ + DDFF GG
Sbjct 2127 DRVGVEDDFFEVGG 2140
```

APPENDIX F

Reagents for fosmid library construction and screening by colony hybridization

The reagents using for fosmid library construction

CopyControl™ Fosmid library Production Kit containing

1) CopyControl™ pCC1FOS™ Fosmid Vector: 0.5 μg/μl
   (Cloning-Ready; linearized at the unique *Eco*72 I site and dephosphorylated)
2) End-Repaire 10× Buffer
   (330 mM Tris-acetate [pH7.8], 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT)
3) dNTP Mix (2.5 mM each of dATP, dCTP, dGTP, dTTP)
4) End-Repair Enzyme Mix (T4 DNA polymerase and T4 polynucleotide kinase)
5) T7 DNA siza marker: 100 ng/μl
6) ATP solution: 10 mM
7) Fast-Link™ DNA Ligase: 40U or 2U/μl
8) Fast-Link™ 10× Ligation Buffer
9) GELase™ Enzyme Preparation: 25U or 1U/μl
10) GELase™ 50× Buffer (2 M Bis-Tris [pH6.0], 2 M NaCl)
11) EPI300™ *E. coli* strain: glycerol stock
    [(F^- mcrA Δ (*mrr-hsd*RMS-*mcr*BC) ϕ 80d*lacZ* ΔM15Δ*lac*X74 *rec*A1*end*A1 *ara*D139 Δ (*ara*, *leu*) 7697 *gal*U *gal*K λ^- rpsL nupG trfA)]
12) MaxPlax™ Lambda Packaging Extracts

Additionally required reagents

1) LB broth + 10 mM $MgSO_4$
2) LB plate + 12.5 μg/ml chloramphenicol
3) Low melting point (LMP) agarose
4) 3M sodium acetate (pH7.0)
5) Ethanol
6) Phage dilution buffer
   (10 mM Tris-HCl [pH8.3], 100 mM NaCl, 10 mM $MgCl_2$,)
7) TE buffer (10 mM Tris-HCl [pH7.5],1 mM EDTA)

The reagents for colony hybridization screening

Table F1 Reagents of DIG High Prime DNA Labeling and Detection Starter Kit I.

Bottle/Cap	Label	Content including function
1	DIG-High Prime	5× conc. labeling mixture containing optimal conc. of random primers, nucleotides, DIG-dUTP (alkali-labile), klenow enzyme and buffer
2	DIG-labeled control DNA	5 µg/ml pBR328 DNA (linearized with *Bam*HI)
3	DNA dilution buffer	50 µg/ml fish sperm DNA in 10 mM Tris-HCl, 1 mM EDTA [pH8.0]
4	Anti-Digoxigenin-AP Conjugate	750 U/ml from sheep, Fab-fragments, conjugated to alkaline phosphatase
5	NBT/BCIP	5× conc. stock solution [18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indodyl-phosphate in 67% (v/v) DMSO]
6	Blocking solution	10× conc.
7	DIG Easy Hyb Granules	Hybridization solution

Step I: Preparing colony lifts

1) Denaturation solution: 0.5 N NaOH, 1.5 M NaCl
2) neutralization solution: 0.5 M Tris-HCl, 1.5 M NaCl
3) 2× saline sodium citrate (SSC): 20× SSC stock solution containing 3 M sodium acetate and 300 mM trisodium citrate adjust pH 7.0 with HCl

Step II: DIG-DNA labeling and quantification of labeling efficiency

1) DIG-High Prime
2) Blocking solution: a 1× solution was prepared by diluting 10× blocking solution (Bottle 6) 1:10 with maleic acid buffer
4) Anti-dioxigenin-alkaline phosphatase: dilute Anti-dioxigenin-AP 1:5000 in blocking solution
4) Maleic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl adjust with NaOH to pH 7.5
5) Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl [pH 9.5]
6) Tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP): 40 µl of NBT/BCIP stock solution were diluted with 2 ml of detection buffer

Step III: Hybridization

1) Hybridization solution: 64 ml of sterile double distilled water were carefully added to the DIG Easy Hyb Granules and dissolved by stirreing immediately at 37°C for 5 min.
2) Low stringency wash buffer: 2×SSC, 0.1% SDS
3) High stringency wash buffer: 0.5×SSC, 0.1% SDS

Step IV: Immunological detection

1) Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl [pH7.5], 0.3% (v/v) Tween 20
2) Blocking solution: as described in step II
3) Anti-dioxigenin-alkaline phosphatase: as described in step II
4) Detection buffer: as described in step II
5) TE buffer: 10 mM Tris-HCl, 1 mM EDTA [pH8.0]

APPENDIX G

Spectral data; HREIMS mass, IR, ^{1}H-NMR, ^{1}H-^{1}H COSY, ^{13}C-NMR, DEPT135, HMQC, and HMBC

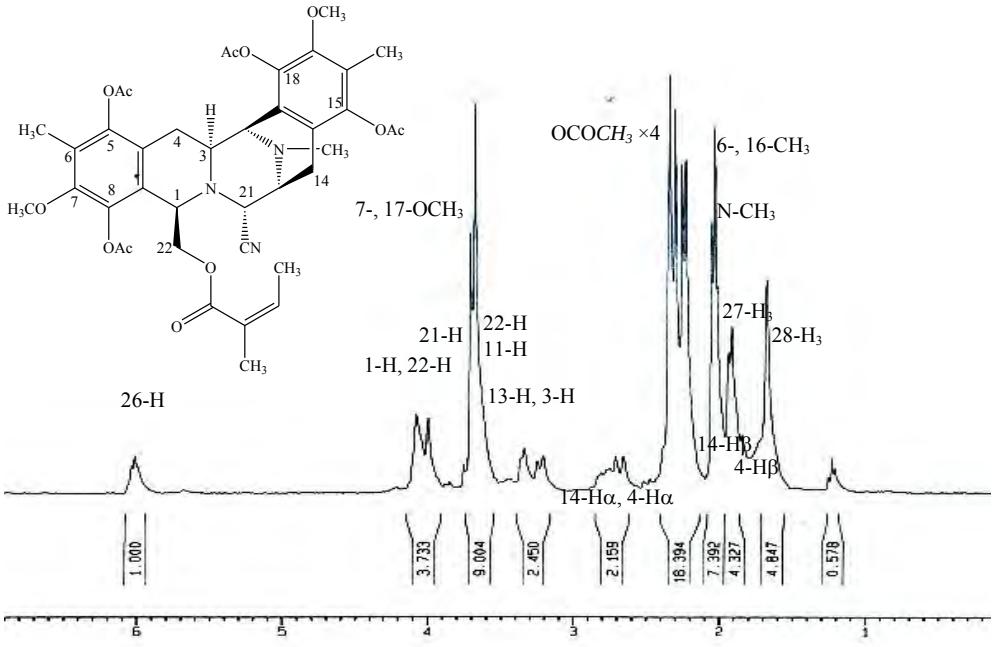

Figure G1 The 300 MHz ^{1}H-NMR spectrum of 5,8,15,18-tetra-*O*-acetylbishydro quinone RM ($CDCl_3$)

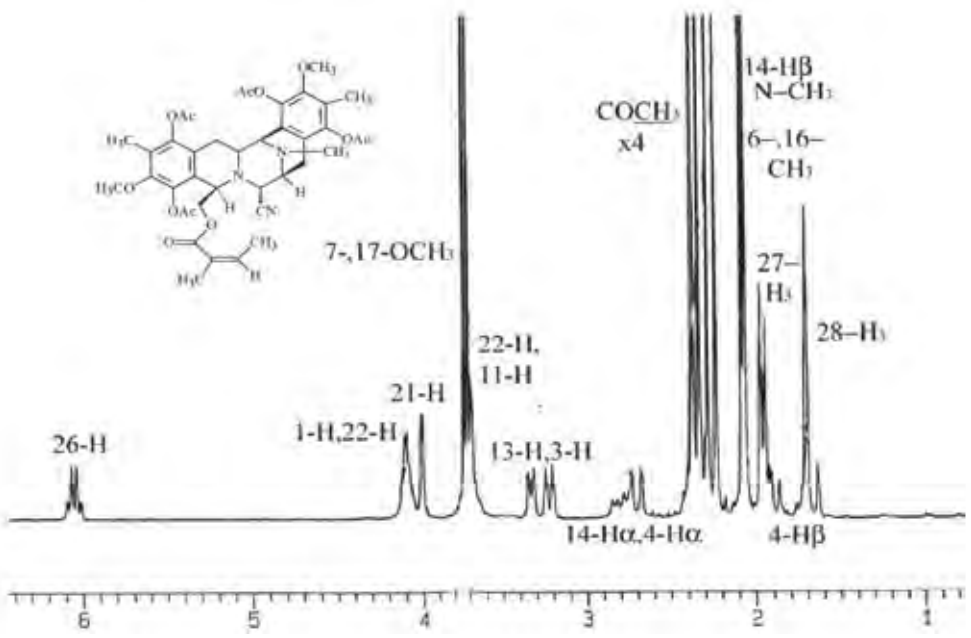

Figure G2 The 300 MHz ^{1}H-NMR spectrum of 5,8,15,18-tetra-*O*-acetylbishydro quinone RM ($CDCl_3$) (Amnuoypol *et al*., 2004.

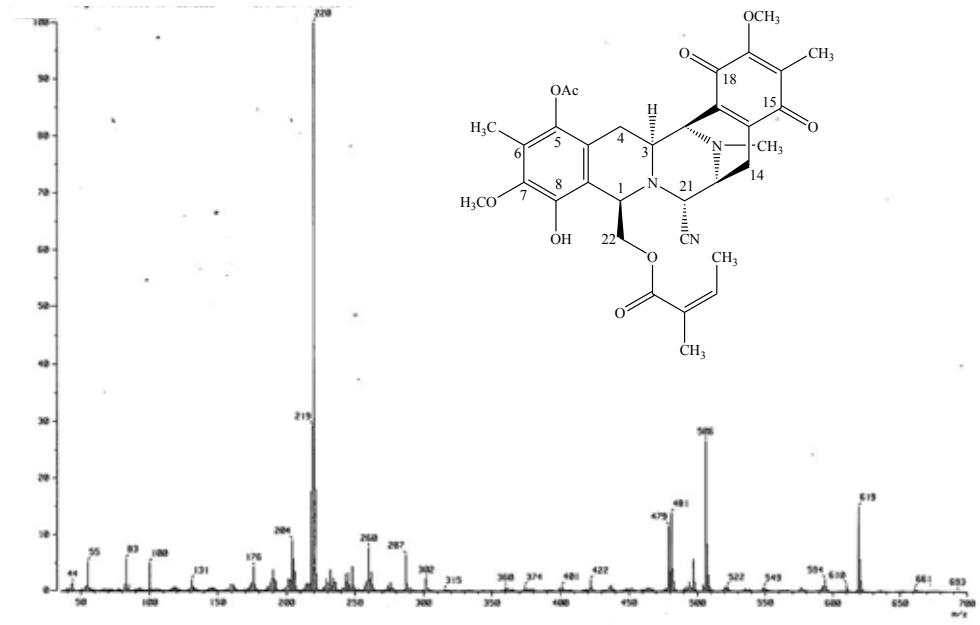

Figure G3 HREIMS mass spectrum of ARM.

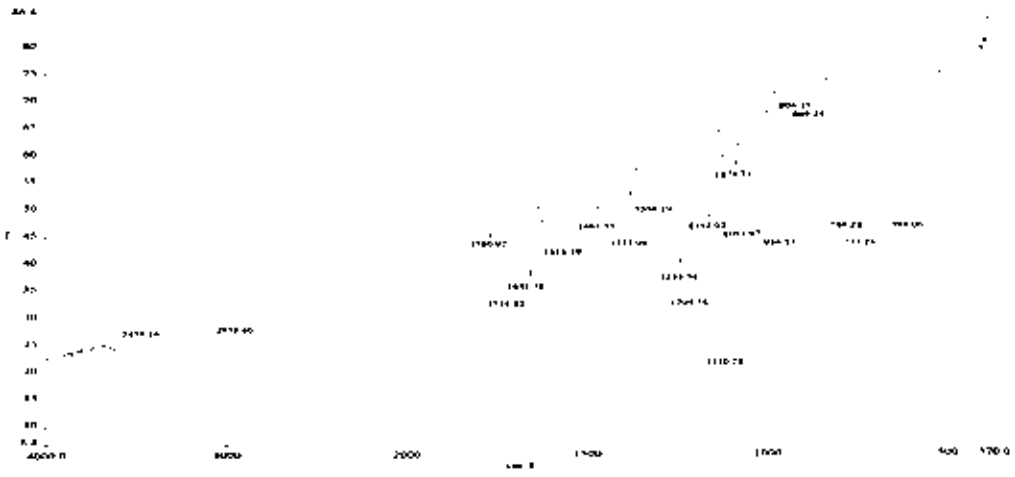

Figure G4 IR spectrum of ARM (KBr).

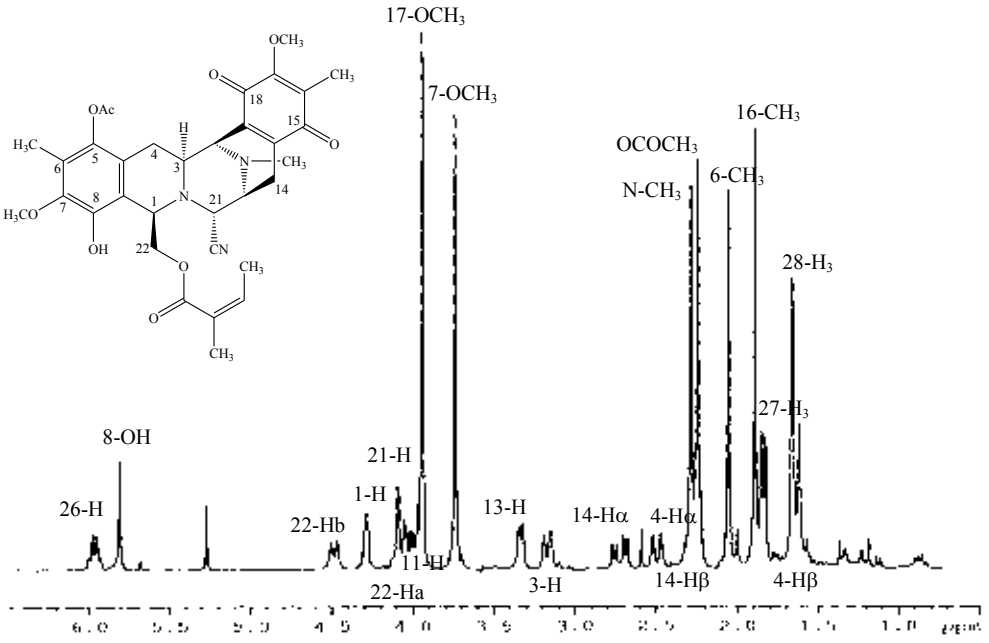

Figure G5 The 300 MHz ^{1}H-NMR spectrum of ARM ($CDCl_3$).

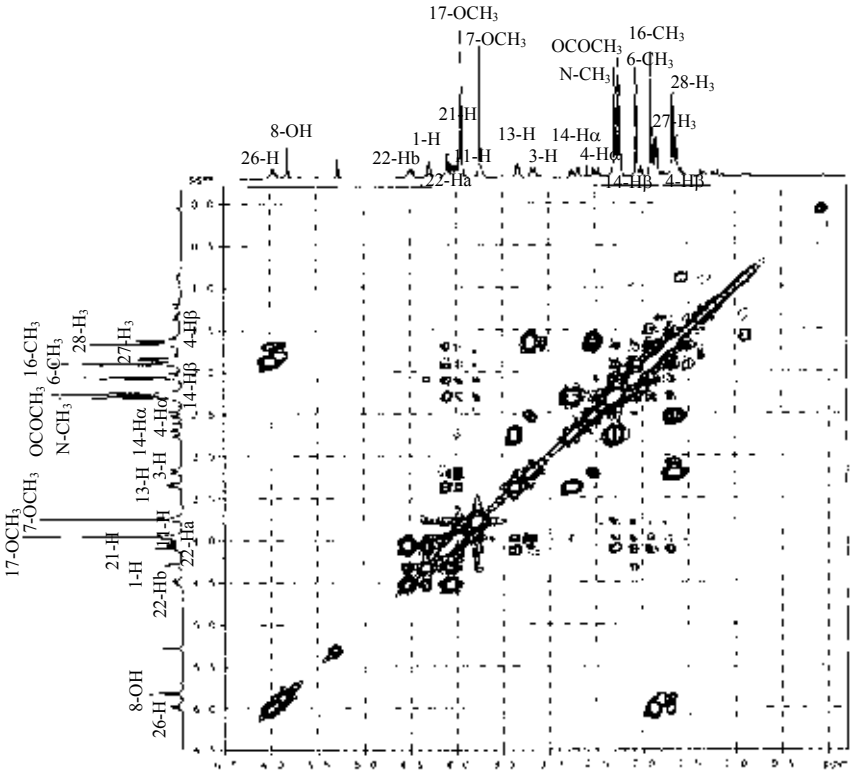

Figure G6 The 300 MHz ^{1}H-^{1}H COSY spectrum of ARM.

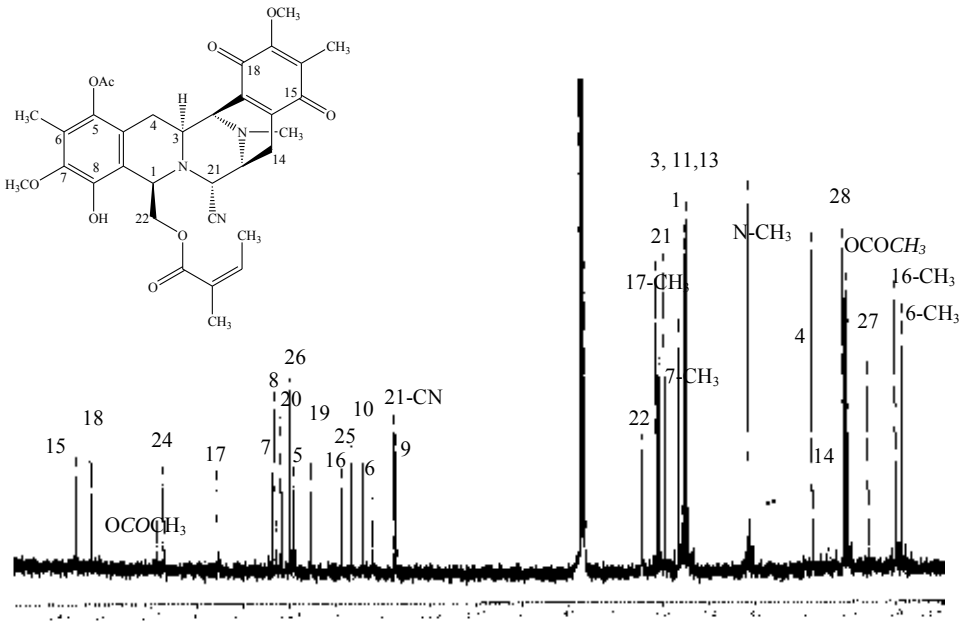

Figure G7 The 75 MHz ^{13}C-NMR spectrum of ARM ($CDCl_3$).

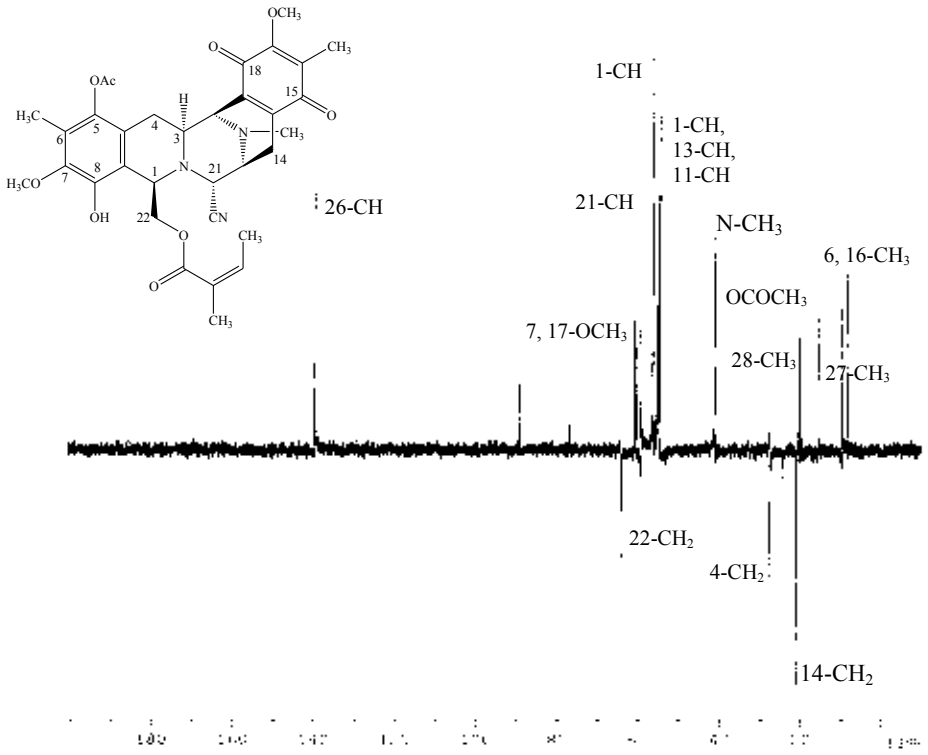

Figure G8 DEPT 135 spectrum of ARM ($CDCl_3$).

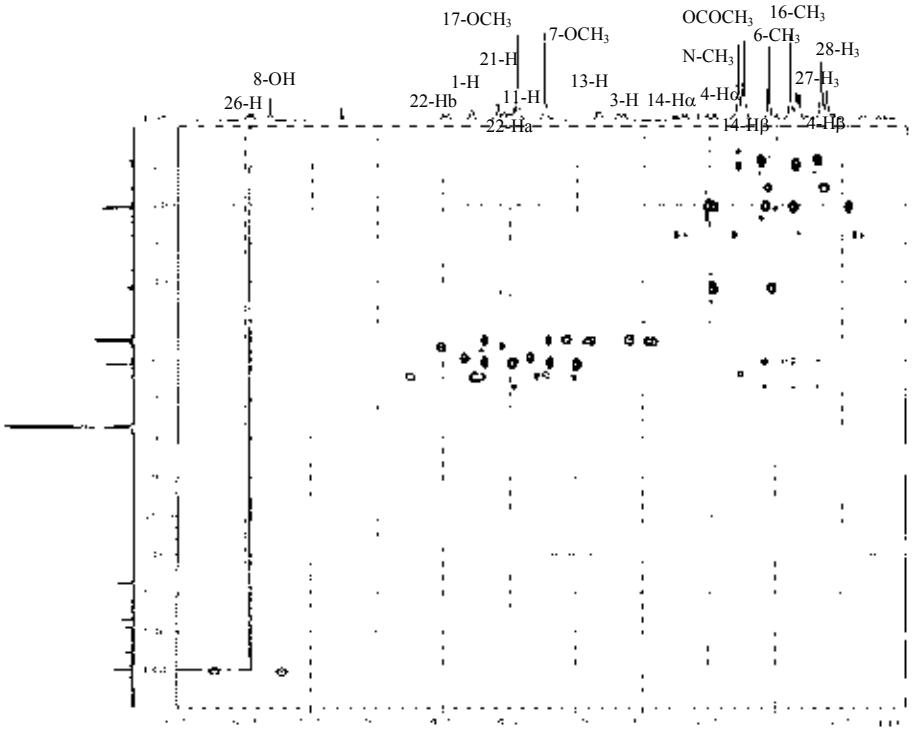

Figure G9 The 300 MHz HMQC spectrum of ARM ($CDCl_3$).

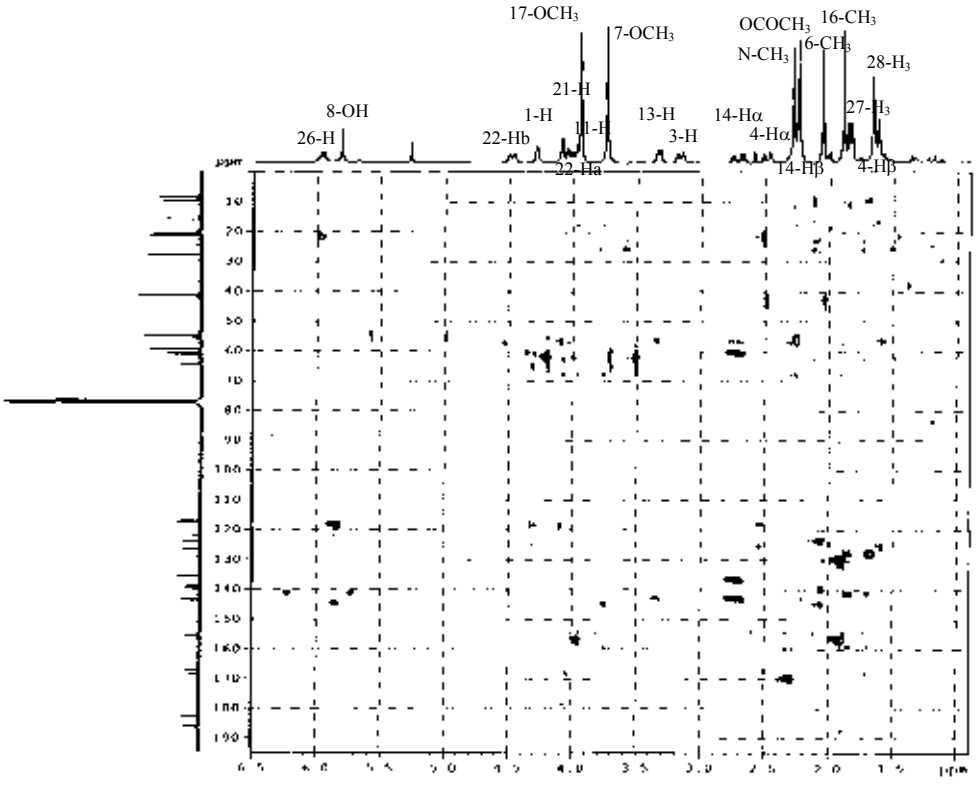

Figure G10 The 300 MHz HMBC spectrum of ARM ($CDCl_3$).

APPENDIX H

Raw data of cell culture in “Necrosis abolishing effect of 5-*O*-acetylhydroquinone renieramycin M in lung cancer cells”

Table H1 Cell viability was analyzed by MTT assay.

Concentration (µM)	Renieramycin M (RM)		5-*O* acetylhydroquinone renieramycin M (ARM)	
	% cell viability (Mean)	SD	% cell viability (Mean)	SD
0	100.00	0.34	100.00	0.55
1	46.80	2.04	51.82	1.56
5	30.69	0.70	39.29	1.67
10	25.05	1.59	30.03	1.53
20	15.25	0.76	23.15	0.99

Table H2 Percentage of apoptosis and necrosis cell deaths.

Concentration (µM)	Renieramycin M				5-*O* acetylhydroquinone renieramycin M			
	% Necrosis		% Apoptosis		% Necrosis		% Apoptosis	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0.00	0.00	0.68	0.62	0.00	0.00	0.68	0.62
1	2.55	1.68	51.78	6.83	1.19	0.61	46.18	4.64
5	9.86	4.90	60.95	5.85	1.84	0.37	59.00	8.94
10	11.52	5.38	63.17	8.96	1.84	1.08	69.50	5.08
20	20.01	4.02	67.99	5.60	2.12	1.22	73.86	5.81

Table H3 Relative of DNA contents in sub-G_0 analysis by flow cytometry.

Concentration (µM)	**Renieramycin M**		**5-*O* acetylhydroquinone renieramycin M**	
	% Gate	**Relative**	**% Gate**	**Relative**
0	12.25	1.00	12.44	1.00
1	38.48	3.19	36.82	3.34
5	39.14	3.80	41.50	3.56
10	46.46	4.12	44.28	3.90
20	50.51	4.42	48.53	4.32

Table H4 Relative percentage of necrotic cell death was measured by automated cell counter.

Concentration (µM)	**Renieramycin M**		**5-*O* acetylhydroquinone renieramycin M**	
	% Necrosis		**% Necrosis**	
	Mean	**SD**	**Mean**	**SD**
0	0.00	0.00	0.00	0.00
1	1.70	0.57	1.03	0.00
5	10.20	0.00	2.05	0.57
10	12.24	1.00	3.07	0.57
20	20.06	1.52	3.00	0.57

Table H5 Percentage of cell viability in the presence or absence of NAC.

Condition	Renieramycin M		Condition	5-*O*-acetylhydroquinone renieramycin M	
	Mean	SD		Mean	SD
control	100.00	0.10	control	100.00	0.10
20 μM RM	15.58	1.75	20 μM ARM	23.81	1.69
20 μM RM + 1 mM NAC	25.53	1.26	20 μM ARM + 1 mM NAC	25.65	0.43

Table H6 Percentage of apoptosis and necrosis cell deaths in the presence or absence of NAC.

Condition	% Necrosis		% Apoptosis	
	Mean	SD	Mean	SD
control	0.00	0.00	0.68	0.61
20 μM RM	20.01	4.02	67.99	5.60
20 μM RM + 1 mM NAC	10.64	3.82	65.13	7.70
20 μM ARM	2.12	1.22	73.86	5.81
20 μM ARM + 1 mM NAC	0.00	0.00	73.24	2.97

Table H7 H23 cells were pretreated under the presence or absence of NAC and measured for general ROS signals by $DCFH_2$-DA.

Condition	0 h		1 h		3 h		6 h	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
control	1.00	0.01	2.69	0.11	6.56	0.28	11.74	0.72
20 μM RM	1.00	0.03	6.27	0.32	19.89	0.44	37.32	1.01
20 μM RM + 1 mM NAC	1.00	0.10	4.48	0.34	12.50	0.52	22.61	0.97
20 μM ARM	1.00	0.02	3.76	0.06	9.62	0.04	18.55	0.39
20 μM ARM + 1 mM NAC	1.00	0.02	3.04	0.01	6.78	0.12	11.16	0.25

Table H8 H23 cells were pretreated under the presence or absence of sodium pyruvate (SP) and measured for H_2O_2 signals by $DCFH_2$-DA.

Condition	0 h		1 h		3 h		6 h	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
control	1.00	0.01	2.69	0.11	6.56	0.28	11.74	0.72
20 μM RM	1.00	0.03	6.27	0.32	19.89	0.44	37.32	1.01
20 μM RM + 1 mM SP	1.00	0.06	6.36	0.09	22.09	0.60	41.52	1.30
20 μM ARM	1.00	0.02	3.76	0.06	9.62	0.04	18.55	0.39
20 μM ARM + 1 mM SP	1.00	0.00	3.30	0.09	9.79	0.38	21.42	1.08

Table H9 Hydroxyl radical signals were measured by HPF.

Condition	0 h		1 h		3 h		6 h	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
control	1.00	0.03	0.98	0.02	1.02	0.00	1.06	0.00
20 μM RM	1.00	0.00	1.02	0.00	1.07	0.00	1.14	0.00
20 μM ARM	1.00	0.00	1.03	0.04	1.07	0.04	1.13	0.03

Table H10 Superoxide anion signals were measured by DHE.

Condition	0 h		1 h		3 h		6 h	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
control	1.00	0.06	1.50	0.03	2.18	0.24	2.33	0.44
20 μM RM	1.00	0.14	1.61	0.13	2.54	0.31	4.43	0.41
20 μM ARM	1.00	0.11	1.60	0.00	2.48	0.26	2.78	0.16

VITA

Miss Thaniwan Cheun-Arom was born on February 14, 1983 in Satun, Thailand. She received her Bachelor's Degree of Science (Microbiology) in 2005 from the Faculty of Science, Prince of Songkla University, Thailand and Master's Degree of Science in Biotechnology in 2007 from the Faculty of Agro-Industry, Prince of Songkla University, Thailand. She was awarded a 2007 Royal Golden Jubilee Scholarship from the Thailand Research Fund (grant No PHD/0248/2550), Japan Student Services Organization (JASSO) and The University of Tokyo Global COE.

Publication

1. Cheun-Arom, T., Chanvorachote, P., Sirimangkalakitti, N., Chuanasa, T., Saito, N., Abe, I., and Suwanborirux, K. 2013. Replacement of a quinone by a 5-*O*-acetylhydroquinone abolishes necrosis-inducing effect while preserving apoptosis-inducing effect of renieramycin M. J. Nat. Prod., submitted.

Poster presentations

1. Cheun-Arom, T., Chuanasa, T., Wakimoto, T., Abe, I., and Suwanborirux, K. Screening for NRPS genes involved in biosynthesis of renieramycins from blue sponge *Xestospongia* sp. CMSI Workshop: Basic Science of Novel Quantum States and New Materials, 11-12 November 2010, Okazaki Conference Center, Tokyo, Japan.
2. Cheun-Arom, T., Chuanasa, T., Wakimoto, T., Abe, I., and Suwanborirux, K. Metagenomic screening for NRPS genes involved in biosynthesis of the marine alkaloid, renieramycins. RGJ-Ph.D. Congress XIII, 6-8 April 2012, Pattaya, Chonburi, Thailand.