Chemical and Microbial Cohabitant Profiling of the Sponge *Penares nux*

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Thesis Title	Chemical and Microbial Cohabitant Profiling of the Sponge *Penares nux*
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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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Thesis Title Chemical and Microbial Cohabitant Profiling of the Sponge *Penares nux*

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ABSTRACT

The objective of this dissertation is to investigate the chemical profile among each part of the sponge *Penares nux*, and to study the impact of such profiling on the surrounding biota, namely the bacterial population on the sponge surface. The sponge *Penares nux* has a unique structural feature of protruding gorgonian-like capitums randomly developing from the rock-attached bases. The sponge is also found to develop an appendage extending from its base. Previous work showed the specific allocation of toxic kabiramides towards its capitum. In this study, the contents of three kabiramides (kabiramides B, C, and G) in the three parts (capitum, appendage, and base) as well as the sponge surface-attached bacteria were examined. Using LC-ESI-MS, *P. nux* was found to allocate kabiramides B, C, and G primarily towards the capitum and appendage. The plate counts of the cohabiting bacteria swabbed directly from the sponge's surfaces showed that the bacteria aggregated more densely on the surfaces of the bases. The specific allocation of the toxic macrolides significantly correlated in a reverse manner, i.e., the denser populations of the bacteria on the sponge surfaces, the lower the content of kabiramides. Twenty-two distinct bacterial isolates were subjected to phylogenetic analysis. The sponge *P. nux* yielded a diverse microbial community, including Alphaproteabacteria, Gammaproteobacteria, Actinobacteria and Flavobacteria.

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Oyenike Olufunmi Olatunji

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br	broad (for IR and NMR signals)
CFU	colony forming unit
COSY	correlation spectroscopy
d	doublet (for NMR signals)
ESI	electrospray ionization
HMBC	heteronuclear multiple bond multiple-quantum coherence
HMQC	heteronuclear multiple-quantum coherence
HRESIMS	high-resolution electrospray ionization mass spectroscopy
IC_{50}	inhibitory concentration at 50% of test subject
IC_{99}	inhibitory concentration at 99% of test subject
LC_{50}	lethal concentration at 50% of test subject
m	multiplet (for NMR signals)
q	quartet (for NMR signals)
s	singlet (for NMR signals)
t	Triplet (for NMR signals)

CHAPTER 1

INTRODUCTION

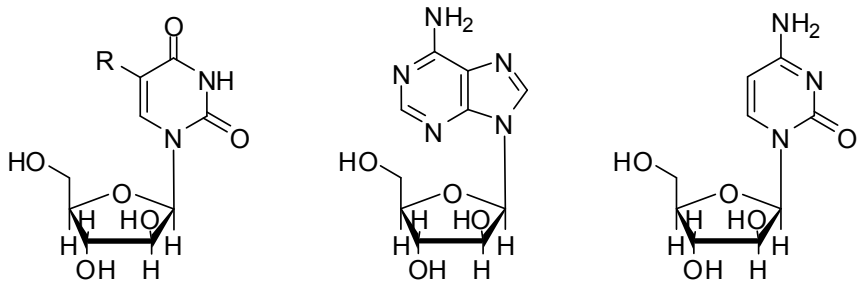
1.1 General introduction

Nature has yielded an extensive range of rich structurally diverse and biologically active compounds that serve as highly effective drug leads to combat a multitude of diseases. Traditionally, terrestrial organisms, plants and microorganisms alike, have represented the richest sources of drugs leads. Some outstanding examples include Pacific yew (*Taxus brevifolia*), which is the source of the most recently approved anticancer drug, paclitaxel (Wani et al., 1971). Paclitaxel (Taxol®) is well known for its use as one of the most recently approved anticancer drugs as well as its unique mode of action by stabilizing microtubules, thus promoting their polymerization, interrupting cell division, and resulting in cell death. This compound was subsequently found to be produced by the endophytic fungus *Taxomyces andreanae* (Stierle et al., 1993). Currently, paclitaxel (Taxol®) is approved to be used for the treatments of ovarian, breast, and small-cell lung cancers (Rowinsky et al., 1992). Also recently, becoming highly attended drug leads were topotecan and irinotecan, a synthetic analogs of the camptothecin derivatives, from *Camptotheca acuminata*. The drugs have been approved for the treatment of ovarian, small-cell lung, and colorectal cancers (Wall et al., 1966; Creemers et al., 1996; Bertino, 1997). The vinca alkaloids, vincristine and vinblastine, isolated from *Catharanthus roseus*, have been approved for the treatments of leukemia, lymphoma, advanced testicular cancer, breast cancer, and Kaposi's sarcoma (Johnson et al., 1963; Cragg and Newman, 2005). Etoposide, a synthetic derivative of epipodophyllotoxin from *Podophyllum peltatum* was another anticancer drug, recently approved for the treatment of lymphoma, bronchial, and testicular cancers (Williams et al., 1987; Cragg and Newman, 2005; Harvey, 1999).

Not until the past four decades have marine natural products emerged as an alternative and promising sources of compounds with unique chemical entities and novel pharmacological properties (Newman et al., 2000). This is due to the extreme and harsh environments, in which marine organisms inhabit. These include high salinity, high pressure,

variable temperatures, low light intensity, and low nutrient availability. Such physical and chemical conditions are distinctively different from those of the terrestrial environment, thus leading to different chemicals produced by its inhabitants (Cragg et al., 1997; Wallace, 1997; Capon, 2001).

The first marine natural products were isolated from the sponge *Cryptotethya crypta* in the 1950's (Bergmann and Feeney, 1950; Bergmann and Feeney, 1951). The discovery of the nucleosides spongothymidine and spongouridine led to the development of chemical derivatives ara-C, the first marine-derived anticancer agent, and the antiviral drug ara-A (Proksch et al., 2002). Ara-C is currently used in the routine treatment of patients with leukemia and lymphoma.

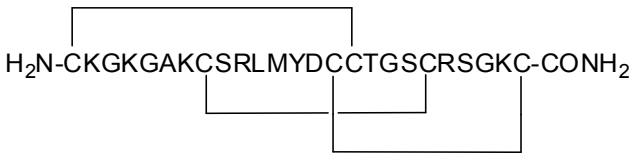


spongothymidine; R = CH_3
spongouridine; R = H

ara-A

ara-C

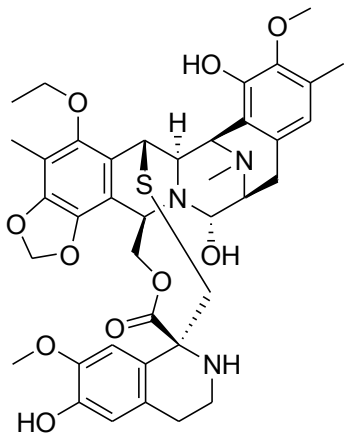
Currently, several marine-derived natural products and their derivatives are available in the market as effective drugs. These included ziconotide (Prialt®), which is synthetic form of ω-conotoxin isolated from the venom of cone snail *Conus magus.* Ziconotide is now available in the market as a potent intrathecal analgesic for severe chronic pain (Myers et al., 1993; Mayer et al., 2010).



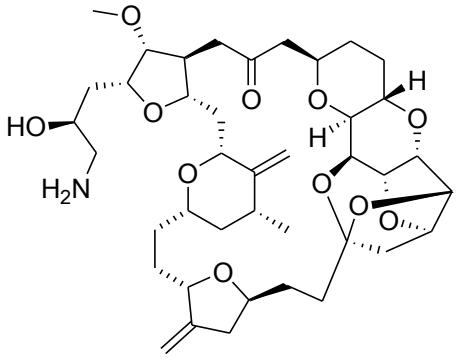
ziconotide

Another example is trabectedin (Yondelis®), the first anticancer drug from the sea, originally isolated from a tropical sea squirt *Ecteinascidia turbinata* (Rinehart et al., 1990).

European Union approved the drug for the treatment of advanced soft tissue sarcoma (Molinski et al., 2009). Other example includes eribulin mesylate (Halaven®), a synthetic macrocyclic ketone analogue of the marine natural product halichondrin B, isolated from the marine sponge *Halichondria okadai* (Hirata and Uemura, 1986). Eribulin interferes with microtubule dynamics, which is a protein component of the cytoskeleton needed to support the rapid growth of cancer cells. The US Food and Drug Administration and European Medicines Agency approved the drug in November 2010 for the treatment of metastatic breast cancer (Mayer et al., 2010; Huyck et al., 2011).

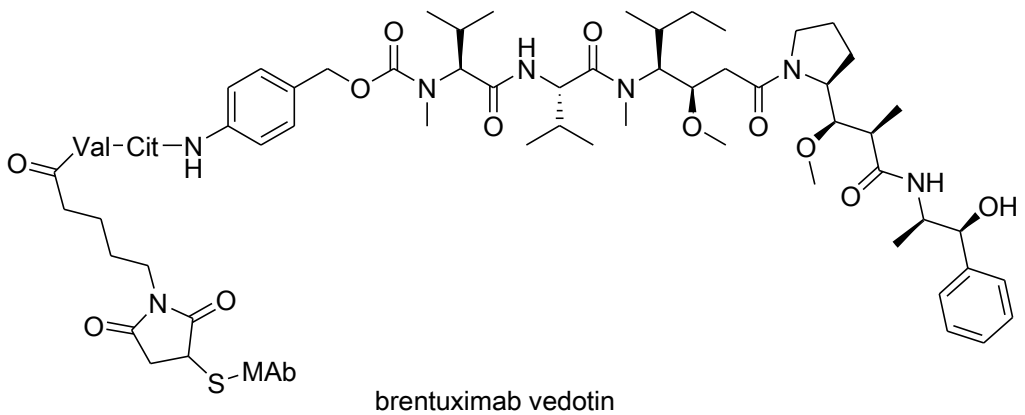


trabectedin



eribulin mesylate

Brentuximab vedotin (Adcetris®) is the latest marine drug to enter into the market. It is based on a fully synthetic analog of dolastatin 10 linked to an anti-CD30 antibody. Dolastatin 10 was first isolated in 1972, from the sea hare *Dolabella auricularia* (Pettit et al., 1987; Pettit et al., 1993). Brentuximab vedotin was approved for the treatment of Hodgkin's and systemic anaplastic large cell lymphoma (Katz et al., 2011; Younes et al., 2012).



brentuximab vedotin

In addition to the currently approved drugs listed above, several other marine-derived natural products are in different stages of clinical trials and are listed in Table 1.

Table1. The marine pharmaceutical clinical pipeline

Compound name	Source	Disease area
Approved		
cytarabine, Ara-C (Cytosar-U®)	*Cryptotethia crypta* (sponge; synthetic derivative of spongothymidine)	antileukemic
vidarabine, Ara-A (Vira-A®)	*Cryptotethia crypta* (sponge; synthetic derivative of spongouridine)	antiviral
Ziconotide (Prialt®)	*Conus magus* (cone snail; synthetic derivative of ω-conotoxin)	chronic pain
trabectedin (Yondelis®)	*Ecteinascidia turbinata* (tunicate)	soft tissue sarcoma
eribulin mesylate (Halaven®)	*Halichondria okadai* (sponge; synthetic derivative of halichondrin B)	metastatic breast cancer
brentuximab vedotin (Adcetris®)	*Dolabella auricularia* (sea hare; synthetic derivative of dolastatin 10)	Hodgkin's lymphoma
Phase III		
plitidepsin (Aplidine®)	*Aplidium albicans* (tunicate; analog of didemnin B)	multiple myeloma
Phase II		
TZT 1027 (Soblidotin)	*Dolabella auricularia* (sea hare; synthetic derivative of dolastatin 10)	soft tissue sarcoma
PM1004 (Zalypsis®)	*Jorunna funebris* (nudibranch; analog of jorumycin)	cervical cancer

Table 1. (cont.)

Compound name	Source	Disease area
elisidepsin (Irvalec®)	*Elysia rufescens* (sea slug; synthetic derivative of kahalalide F)	advanced gastric cancer
plinabulin	*Halimeda lacrimosa* (green algae; analog of halimide)	non-small cell lung cancer
Phase I		
bryostatin 1	*Bugula neritina* (bryozoan)	metastatic solid tumors
hemiasterlin	*Hemiasterella minor* (sponge)	malignant tumors
marizomib (salinosporamide A)	*Salinispora tropica* (marine bacteria)	multiple myeloma
pseudopterosins	*Pseudopterogorgia elisabethae* (soft coral)	wound healing

*Adapted from Gerwick and Moore (2012), Yonghong, L (2012) and Martin et al (2014).

Although many marine natural products have been studied to yield crucial information about their biological activities and mechanism of action, much less is known about their ecological functions in the marine environments. This brought about marine chemical ecology, the study of chemical interactions between marine organisms and their environments (Hay, 1996). Examples of ecological roles of marine natural products include unpalatability that deters predators, antisettlement cues for larvae, and preventing fouling by epiphytes. Marine chemical ecology depends on chemical and ecological experiments to determine the individual component or suite of compounds responsible for the observed interactions. The research allows a better understanding of the diverse role of secondary metabolites produced by marine organisms and their ecological interactions.

An example of marine chemical ecology research is the study of the sponge *Crambe crambe* (Becerro et al., 1995; Turon et al., 1996; Becerro et al., 1997). The study explores an extensive array of parameters, which includes size, age, habitat, reproductive cycle, competitive pressures, and degree of physical protection, and correlated them to the chemical bioactivity of the sponge extracts. The surface of the sponge was found to be without bacteria,

and an extract of the sponge was found to impede growth of bacteria isolated from neighboring seawater. *C. crambe* extract significantly inhibit rejuvenation of damage tissue in coexisting sponge *Scopalina lophyropoda*. Bioactive secondary metabolites from sponge *C. crambe* were found to be in a higher concentration in the outer sponge tissues, especially in sphericulous cells. Marine chemical ecology indicates that secondary metabolites can have numerous roles in a specific organism, and that these metabolites may be crucial for effective adjustment to environmental changes induced by the presence of competitors

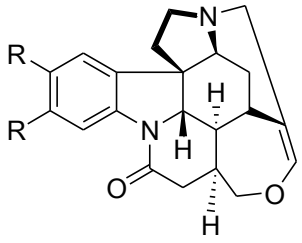
1.2 Chemical profiling of natural products

Chemical profiling is one of the useful methods for assessing the distribution of secondary metabolites and chemical variations. It is an approach of profiling a natural product sample to determine its chemical composition, thus allowing detection of chemically diverse metabolites. In plants, chemical profiling among closely related species has proved useful for quality control and taxonomic purposes. Described below are selected examples in which chemical profiling was employed in certain medicinal plants that had been long known among the most adulterated herbal products by related yet not medically potent species.

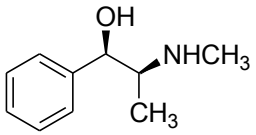
An example is the study of *Strychnos.* Three different species: *Strychnos nux-vomica, S. ignatii*, and *S. icaja,* were discriminated based on different parts of the plants (seeds, roots, leaves, and barks). An NMR-based analytical technique integrated with multivariate analysis was used to discriminate all the three species based on their metabolites such as strychnine, loganin, and brucine (Frédérich et al., 2004).

Another example is the study of *Ephedra*. *Ephedra* is one of the oldest medicinal plants known to humankind. However, three different species, namely *E. sinica*, *E. intermedia,* and *E. equisetina*, are broadly and frequently used as medicinal plant without differentiation as long as there is certain amount of ephedrine alkaloids are found varying from 0.02 to 3.4%. By means of NMR-based chemical profiling, it was possible to discriminate the three species using the benzoic acid analogs apart from ephedrine alkaloids as chemotaxonomic markers (Kim et al., 2005).

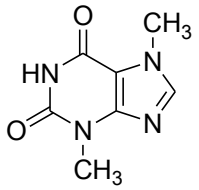
Profiling in 11 species of *Ilex* was carried out and based on their metabolites, and each species could be discriminated from its adulterants. Combination of the NMR technique and multivariate analysis showed that the contributing metabolites were arbutin, caffeine and theobromine (Choi et al., 2005).



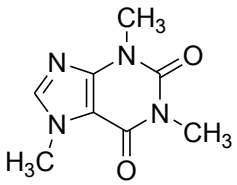
strychnine; R = H
brucine; R = OCH_3



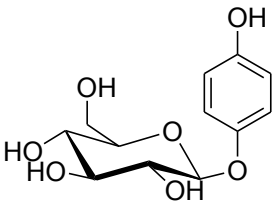
ephedrine



threobromine



caffeine

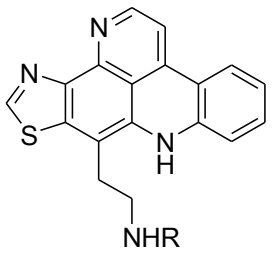


arbutin

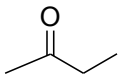
Similar to the investigation in plants, chemical profiling in various marine organisms have been studied. In order to figure out the locations and the ecological roles of these secondary metabolites, there are numerous reports of variation of secondary metabolites in various marine species i.e., seaweeds (Gerwick et al., 1985; Paul and Van Alstyne, 1988; Meyer and Paul, 1992), algae (Paul and Fenical, 1986; Amade and Lemée, 1998), gorgonians (Harvell and Fenical, 1989; Harvell et al., 1993; Dube et al., 2002), nudibranches (Thompson et al., 1982; Kernan et al., 1988; Van Alstyne and Paul, 1990; De Nys et al., 1996), soft corals (Maida et al., 1993; Kelman et al., 2000), brachiopods (Mahon et al., 2003), bryozoans (Peters et al., 2004), and ascidians (López-Legantil et al., 2005; López-Legantil et al., 2006). This variability in the production of secondary metabolites has been reported at both inter-specimen and intra-specimen levels (Turon et al., 1996; Bencerro et al., 1997, Bencerro et al., 1998; Bentancourt-Lozano et al., 1998; Schupp et al., 1999). The inter-specimen variation has been attributed to genetic or environmental factors such as light intensity, location, temperature, depth, and salinity (Becerro et

al., 1995; Uriz et al., 1996b; Turon et al., 1996) whereas intra-specimen variations were caused by symbiosis or chemical allocation. Chemical defense is one of allocation purpose and becomes an important strategy to defend against threats from their environment with respect to resources investment and chemical defense allocation, which brings synergistic effect between chemical and structural defense (Thoms and Schupp, 2007).

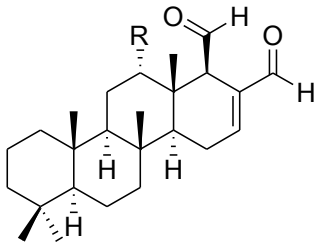
An example of intra-specimen variation is the allocations of kuanoniamines C and D in the different parts of the sponge *Oceanapia* sp. The sponge has an unusual growth form having two different morphs in the same colony. It consists of a turnip-shaped base buried in the sand and a small capitum attached to a fistules. Kuanoniamines C and D showed a sharp increase from the basal (0.4 and 0.1% dry mass respectively) to the capitum (1.2 and 0.4% dry mass). The distribution of kuanoniamines C and D between the different parts supports the optimal defense theory, as the higher content of the two toxic alkaloids are primarily found in the protruding part vulnerable to the attack (Schupp et al., 1999). Another example of intracolonial variation of secondary metabolites was found in the sponge *Cacospongia* sp. The tips and bases showed different level in the amounts of scalaradial and desacetylscalaradial. The tip showed doubled concentration of desacetylscalaradial more than the base. A similar trend was also found for scalaradial (Becerro et al., 1998).



R

kuanoniamine C; 

kuanoniamine D; 



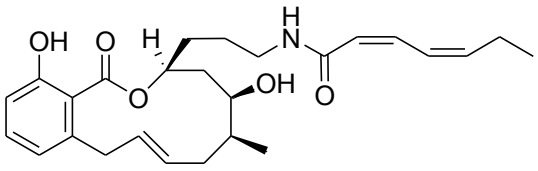
R

scalaradial; 

desacetylscalaradial; OH

On the other hand, an example of inter-specimen variation in chemical profile can be seen in the production of salicylihalamide A by the sponge *Haliclona* sp. Two morphs of the sponge, green and brown, were collected from the same sites. Salicylihalamide A, the major

cytotoxic metabolite, was detected in the green morphs but not in the brown ones (Abdo et al., 2007).



salicylihalamide A

To extend the examples of ecologically important secondary metabolites specifically distributed and employed by sponges, the secondary metabolites from sponges that have been reported intra- and/or inter-specimen variation of contents is compiled in Table 2.

1.3 Sponges and their associated microbes

Sponges are among the oldest of the marine benthic communities. They are sessile, filter feeding organisms, and they possess several tiny pores on their surface, which enable water to penetrate and flow through a series of canals where microorganisms and organic particles are filtered out and ingested (Lee et al., 2001). Sponges have been the focal point of current research interest due to two major reasons: (i) they form a close relationship with a broad range of microorganisms, and (ii) they are the source of biologically active compounds (Taylor et al., 2007).

The abundance and array of microbial assemblage in sponges were first described in the 1970's (Reiswig, 1975; Vacelet and Donadey, 1977; Wilkinson and Fay, 1979). Through the use of electron microscopy, sponges were shown to contain a variety of microorganisms, which account up to 40% of the biomass. There are two different sponge types in respect to their association with bacteria, high microbial abundance and low microbial abundance sponges (Hentschel et al., 2006). High microbial abundance are those sponge specimens with high number of bacteria with densities of 10^8 to 10^{10} bacterial cells counts per gram of sponge body weight. Low microbial abundance sponges have bacterial densities of 10^5 to 10^6 bacterial cells per gram sponge tissue.

Table 2. Sponge secondary metabolites that have been reported in variation of contents

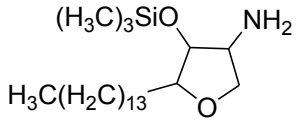
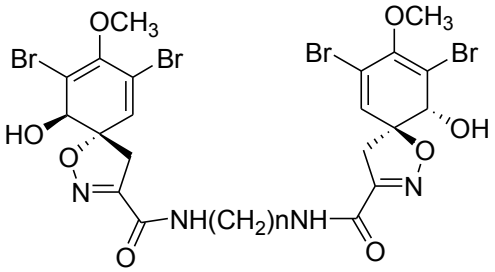
Structure/Name	Source	Types of variation	References
 cerebroside	*Chondrilla nucula*	tissue-specific and geographical variation	Schmitz and McDonald, 1974; Swearingen and Pawlik, 1998
 areothionine; n = 4 homoaerothionin; n = 5	*Aplysina fistularis* (= *Verongia thiona*)	cellular localization and seasonal variation	Thompson et al., 1983; Betancourt-Lozano et al., 1998

Table 2. (cont.)

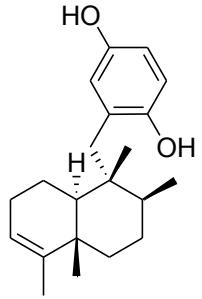
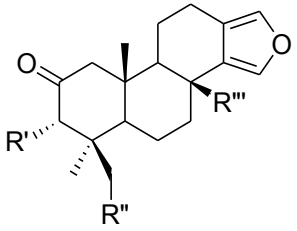
Structure/Name	Source	Types of variation	References
 avarol	*Dysidea avara*	cellular localization, temporal and geographical variation	Müller et al., 1986; Uriz et al., 1996a; Martí et al., 2003; De Caralt et al., 2013
 R' R" R"' spongiadiol; OH OH CH3 spongiadiol diacetate; $OCOCH_3$ $OCOCH_3$ CH_3 spongiatriol; OH OH CH_2OH spongiatriol triacetate; $OCOCH_3$ $OCOCH_3$ CH_2OCOCH_3	*Rhopaloeides odorabile*	tissue-specific, spatial and geographical variation	Thompson et al., 1987

Table 2. (cont.)

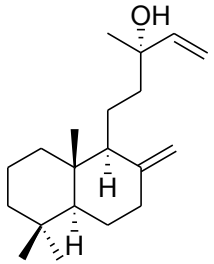
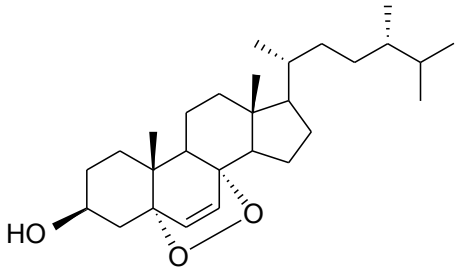
Structure/Name	Source	Types of variation	References
 manoöl	*Aplysilla glacialis*	temporal and spatial variation	Bobzin and Faulkner, 1992
 cholesterol endoperoxide	*A. glacialis*	temporal and spatial variation	Bobzin and Faulkner, 1992

Table 2. (cont.)

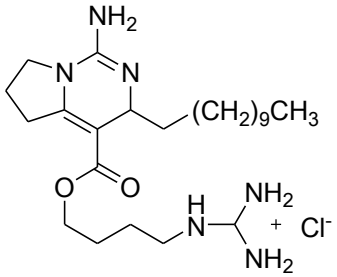
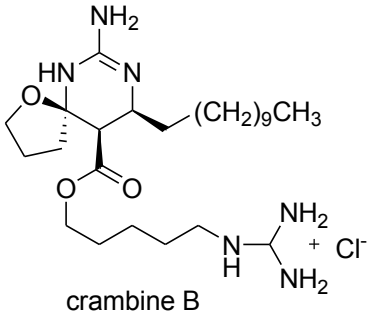
Structure/Name	Source	Types of variation	References
 crambine A	*Crambe crambe*	cellular localization size, structure, and geographical variation	Becerro et al., 1995; Turon et al., 1996
 crambine B	*C. crambe*	cellular localization size, structure, and geographical variation	Becerro et al., 1995; Turon et al., 1996

Table 2. (cont.)

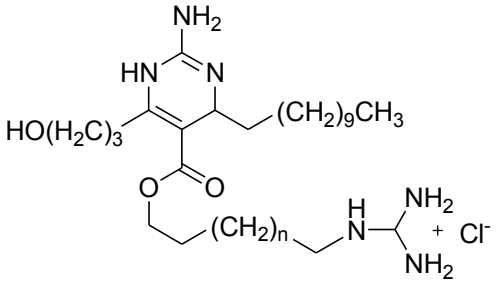
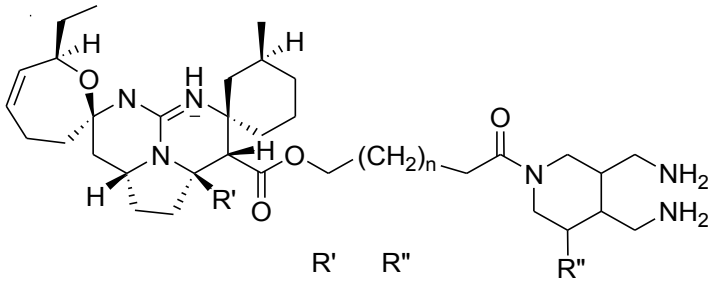
Structure/Name	Source	Types of variation	References
 crambine C1; n = 2 crambine C2; n = 1	*C. crambe*	cell localization, size, structure and geographical variation	Turon et al., 1996
 R' R" crambescidin 816; OH OH n = 13 crambescidin 830; OH OH n = 14 crambescidin 844; OH OH n = 15 crambescidin 800; H OH n = 13	*C. crambe*	cellular localization, size, structure and geographical variation	Turon et al., 1996

Table 2. (cont.)

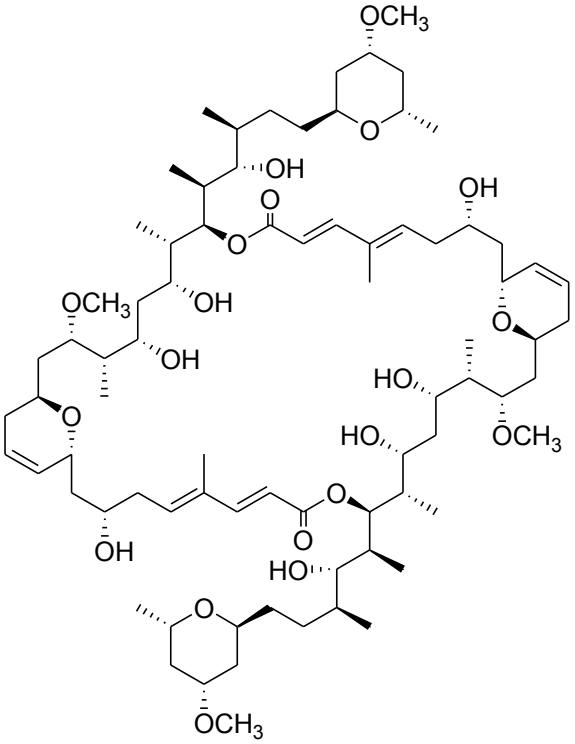
Structure/Name	Source	Types of variation	References
 swinholide A	*Theonella swinhoei*	symbiotic cyanobacterial cell variation	Bewley et al., 1996

Table 2. (cont.)

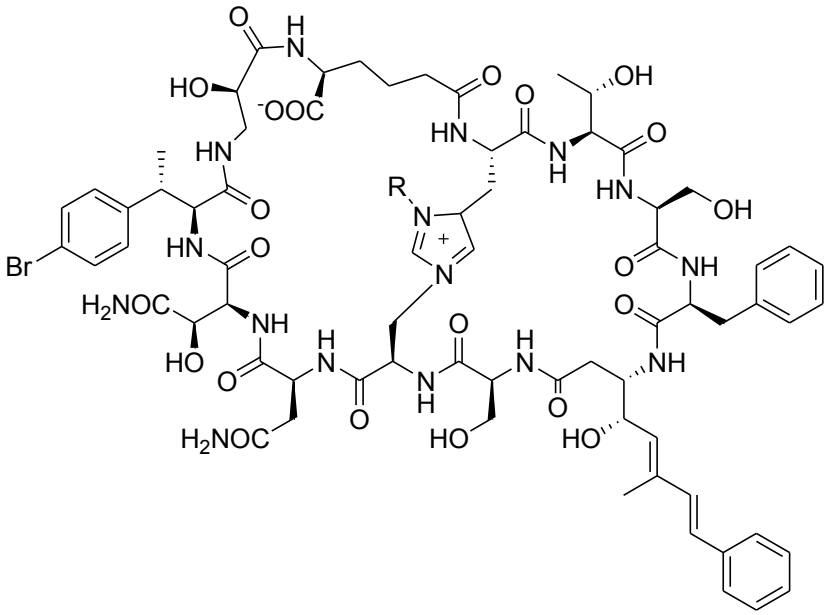
Structure/Name	Source	Types of variation	References
 P951; R = hexose	*T. swinhoei*	symbiotic cyanobacterial cell variation	Bewley et al., 1996

Table 2. (cont.)

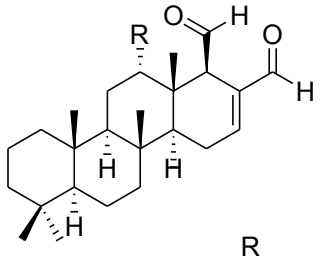
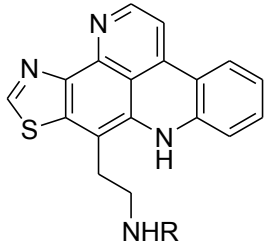
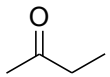
Structure/Name	Source	Types of variation	References
 R  scalaradial; desacetylscalaradial; OH	*Cacospongia* sp.	tissue-specific and geographical variation	Becerro et al., 1998
 R  kuanoniamine C;  kuanoniamine D; *N*-deacetyl-kuanoniamine D; H	*Oceanapia* sp.	tissue-specific variation	Eder et al., 1998; Schupp et al., 1999

Table 2 (cont.)

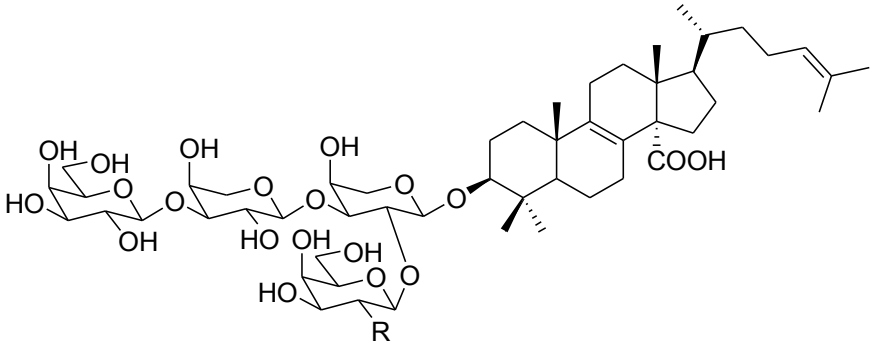
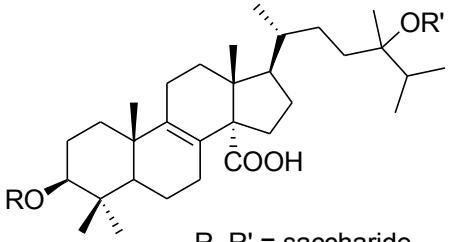
Structure/Name	Source	Types of variation	References
 R formoside; OH formoside B; $NHCOCH_3$  R, R' = saccharide **Mixture A saccharide**: arabinose, galactose, *N*-acetyl glucosamine glusose **Mixture B saccharide**: arabinose, galactose, unknown amino sugar	*Erylus formosus*	tissue-specific variation	Kubanek et al., 2000; Kubanek et al., 2002

Table 2 (cont.)

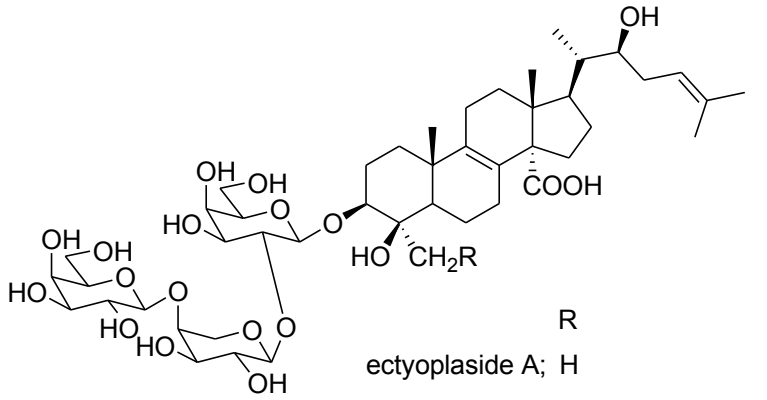
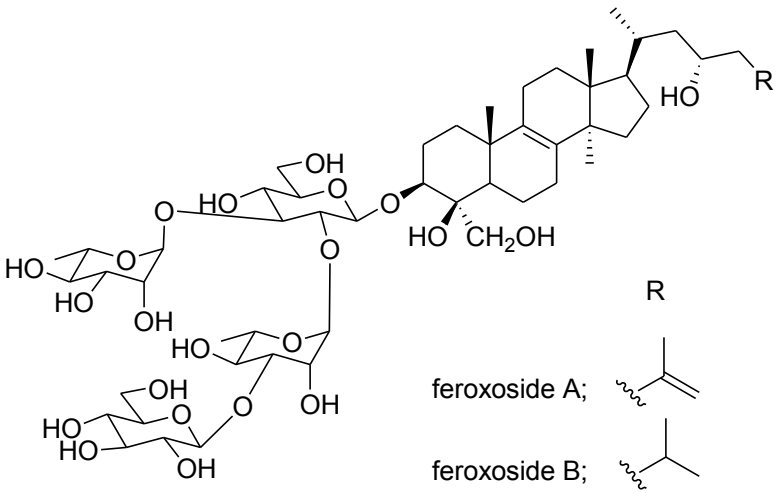
Structure/Name	Source	Types of variation	References
 	*Ectyoplasia ferox*	tissue-specific variation	Kubanek et al., 2002

Table 2 (cont.)

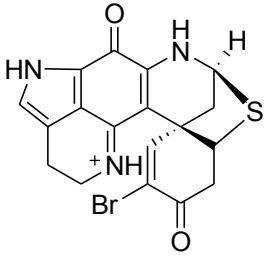
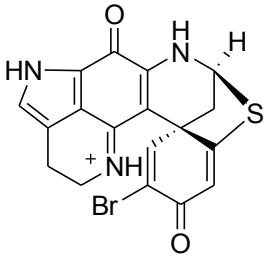
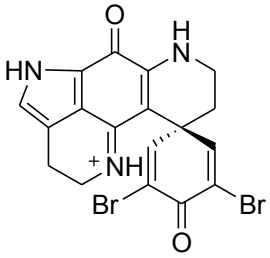
Structure/Name	Source	Types of variation	References
 discorhabdin A	*Latrunculia* sp.	species, geographical and genetic variation	Miller et al., 2001
 discorhabdin B	*Latrunculia* sp.	species, geographical and genetic variation	Miller et al., 2001
 discorhabdin C	*Latrunculia* sp.	species, geographical and genetic variation	Yang and Baker, 1995; Miller et al., 2001

Table 2 (cont.)

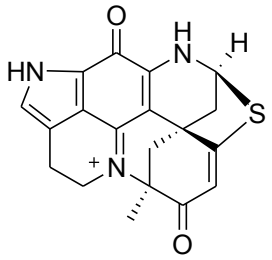
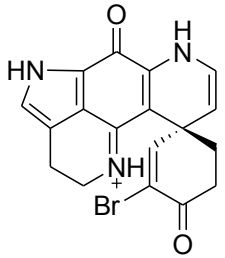
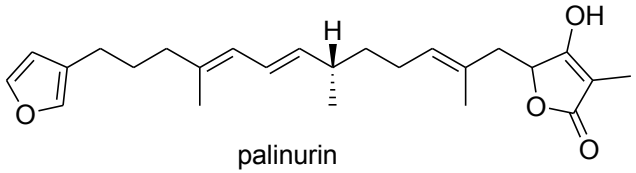
Structure/Name	Source	Types of variation	References
 discorhabdin D	*Latrunculia* sp.	species, geographical and genetic variation	Miller et al., 2001
 discorhabdin G	*Latrunculia* sp.	tissue-specific and species variation	Yang and Baker, 1995; Miller et al., 2001; Furrow et al., 2003
 palinurin	*Ircinia variabilis*	geographical variation	Martí et al., 2003

Table 2 (cont.)

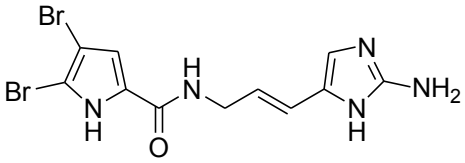
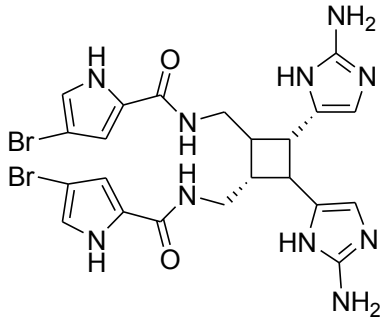
Structure/Name	Source	Types of variation	References
 oroidin	*Agelas conifera*	cellular localization	Richelle-Maurer et al., 2003
 sceptrin	*A. conifera*	cellular localization	Richelle-Maurer et al., 2003

Table 2 (cont.)

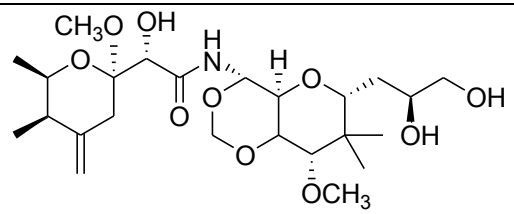
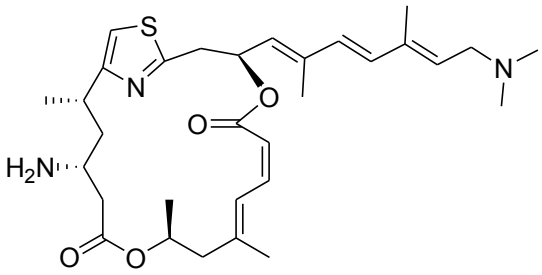
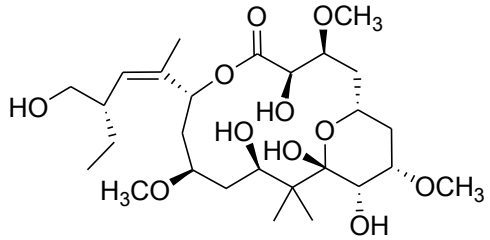
Structure/Name	Source	Types of variation	References
 mycalamide A	*Mycale hentscheli*	geographical variation	Page et al., 2005
 pateamine	*M. hentscheli*	geographical variation	Page et al., 2005
 peloruside A	*M. hentscheli*	geographical variation	Page et al., 2005

Table 2 (cont.)

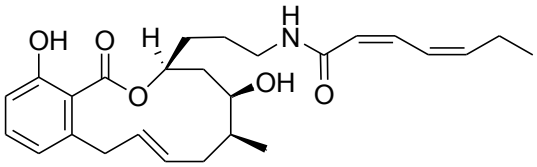
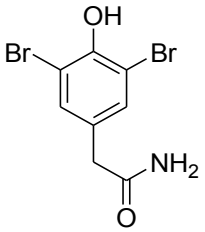
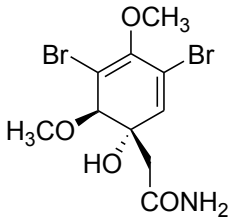
Structure/Name	Source	Types of variation	References
 salicylihalamide A	*Haliclona* sp.	spatial, temporal, color and geographical variation	Abdo et al., 2007
 2-(3',5'-dibromo-4'-hydroxyphenyl)acetamide	*Aplysilla fulva*	geographical variation	Nuñez et al., 2008; Freeman and Gleason, 2010
 aplysinafulvin	*A. fulva*	geographical variation	Nuñez et al., 2008; Freeman and Gleason, 2010

Table 2 (cont.)

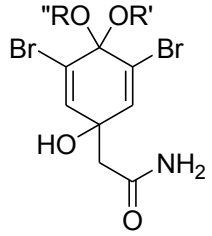
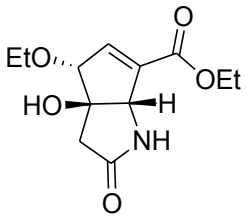
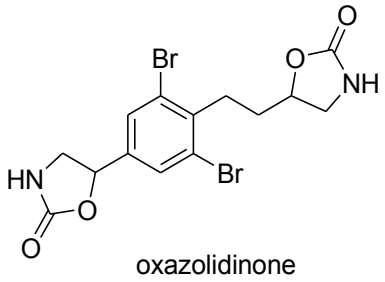
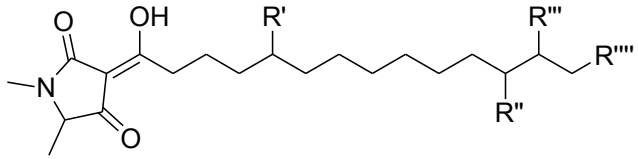
Structure/Name	Source	Types of variation	References
 R' R" 2-(3,5-dibromo-1-hydroxy-4,4-dimethoxycyclohexa-2,5-dienyl)acetamide; CH_3 CH_3 2-(3,5-dibromo-4-ethoxy-1-hydroxy-4-methoxycyclohexa-2,5-dienyl)acetamide; CH_3 Et	*A. fulva*	skeletal and geographical variation	Nuñez et al., 2008; Freeman and Gleason, 2010
 subereatensin	*A. fulva*	structural and geographical variation	Nuñez et al., 2008; Freeman and Gleason, 2010

Table 2 (cont.)

Structure/Name	Source	Types of variation	References
 oxazolidinone	*A. fulva; A. aerophoba*	structural and geographical variation	Turon et al., 2000; Nuñez et al., 2008; Freeman and Gleason, 2010
	*Melophlus sarasinorum*	tissue-specific variation	Aoki et al., 2000; Xu et al., 2006; Rohde and Schupp, 2011

	R'	R''	R'''	R''''
melophin B;	CH_3	H	H	H
melophin P;	H	H	H	CH_2CH_3
melophin Q;	H	H	CH_3	H
melophin R;	H	CH_3	H	H
melophin S;	CH_3	H	H	H

Table 2 (cont.)

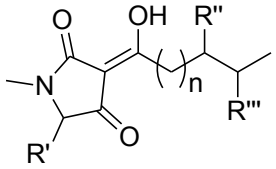
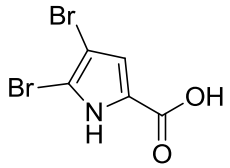
Structure/Name	Source	Types of variation	References
 R' R'' R''' n melophin A; H H H 12 melophin D; H H H 11 melophin E; H H CH_3 11 melophin G; H H H 10 melophin H; H H CH_3 10 melophin I; H CH_3 H 10 melophin O; CH_3 CH_3 H 8	*Melophlus sarasinorum*	tissue-specific variation	Aoki et al., 2000; Xu et al., 2006; Rohde and Schupp, 2011
 4,5-dibromopyrrole-2-carboxylic acid	*Agelas wiedenmayeri*	geographical variation	Assman et al., 2000

Table 2. (cont.)

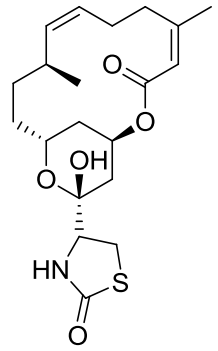
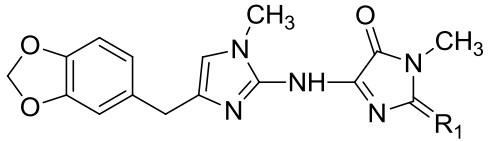
Structure/Name	Source	Types of variation	References
 Latrunculin B	*Neogombata magnifica*	cellular localization	Gillor et al., 2000
 R_1 clathridimine; —NH clathridine; —O	*Clathrina clathrus*	cellular localization	Roué et al., 2010

Table 2 (cont.)

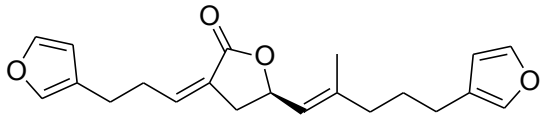
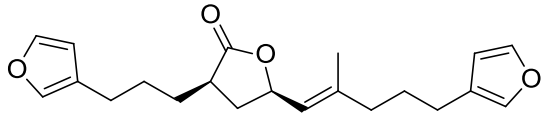
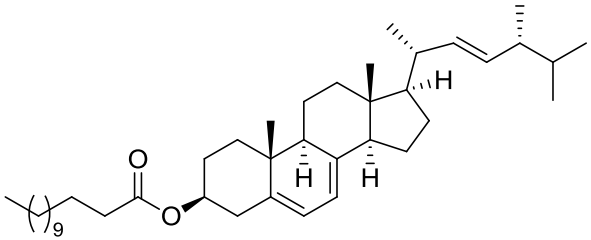
Structure/Name	Source	Types of variation	References
 nitenin	*Spongia lamella*	geographical variation	Noyer et al., 2011
 dihydronitenin	*S. lamella*	geographical variation	Noyer et al., 2011
 ergosteryl myristate	*S. lamella*	geographical variation	Noyer et al., 2011

Table 2 (cont.)

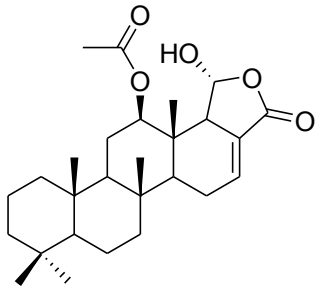
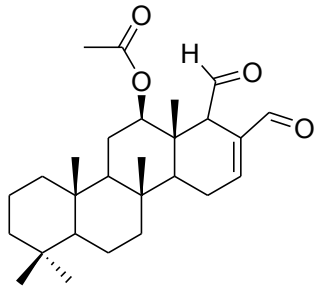
Structure/Name	Source	Types of variation	References
 12-episcalarin	*Spongia lamella*	geographical variation	Noyer et al., 2011
 12-episcalaradial	*S. lamella*	geographical variation	Noyer et al., 2011

Table 2 (cont.)

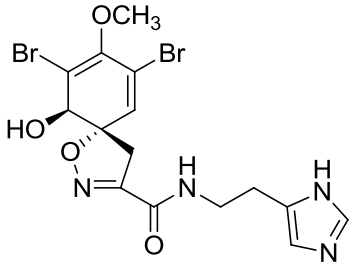
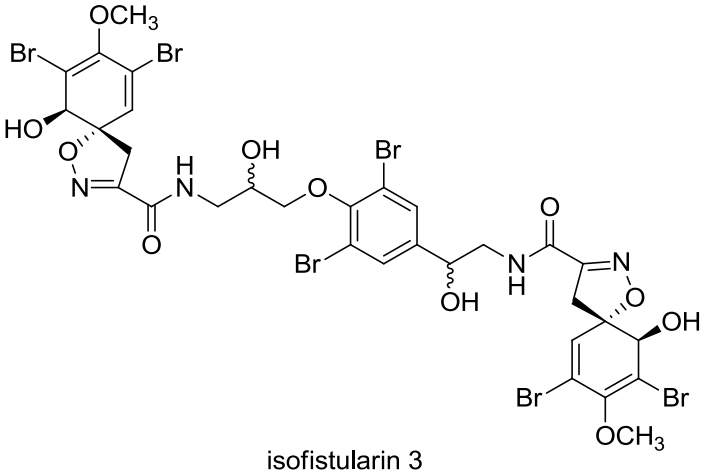
Structure/Name	Source	Types of variation	References
 aerophobin 1	*Aplysina aerophoba*	cellular localization, symbiosis, and activated defense	Turon et al., 2000; Martí et al., 2003; Ebel et al., 2007; Sacristán- Soriano et al., 2011; Sacristán-Soriano et al.,2012
 isofistularin 3	*A. aerophoba*	cellular localization, symbiosis, and activated defense	Turon et al., 2000; Martí et al., 2003; Ebel et al., 2007; Sacristán- Soriano et al., 2011; Sacristán-Soriano et al.,2012

Table 2 (cont.)

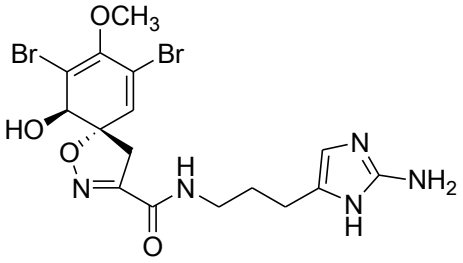
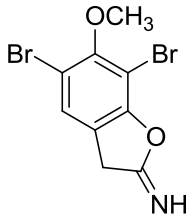
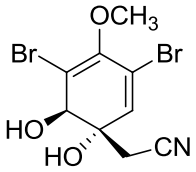
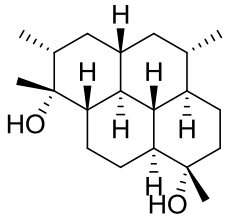
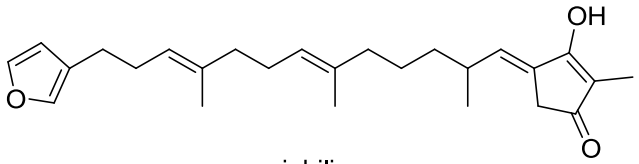
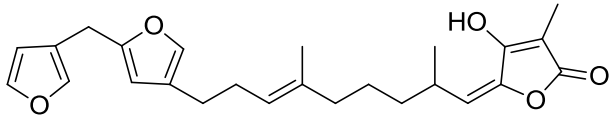
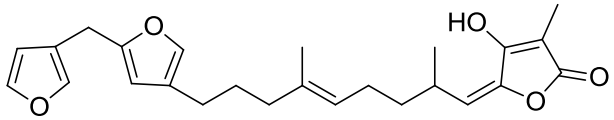
Structure/Name	Source	Types of variation	References
 aerophobin 2	*Aplysina aerophoba*	cellular localization, symbiosis, and activated defense	Turon et al., 2000; Martí et al., 2003; Ebel et al., 2007; Sacristán- Soriano et al., 2011; Sacristán-Soriano et al.,2012
 aplysinimine	*A. aerophoba*	cellular localization, symbiosis, and activated defense	Turon et al., 2000; Martí et al., 2003; Ebel et al., 2007; Sacristán- Soriano et al., 2011; Sacristán-Soriano et al.,2012
 aeroplysinin 1	*A. aerophoba*	cellular localization, symbiosis, and activated defense	Turon et al., 2000; Martí et al., 2003; Ebel et al., 2007; Sacristán- Soriano et al., 2012

Table 2 (cont.)

Structure/Name	Source	Types of variation	References
 diisocyanoadociane	*Amphimedon* sp.	cellular localization and tissue-specific variation	Garson et al., 1992
 variabilin	*Ircina felix, I. campana*	tissue-specific variation	Freeman and Gleason, 2010; Freeman and Gleason, 2012
 ircinin I	*I. felix, I. campana*	tissue-specific variation	Freeman and Gleason, 2010; Freeman and Gleason, 2012
 ircinin II	*I. felix, I. campana*	tissue-specific variation	Freeman and Gleason, 2010, Freeman and Gleason, 2012

The recent developments in molecular cultivation-independent techniques such as 16S rRNA gene sequencing, fluorescent in situ hybridization, and denaturing gradient gel electrophoresis have given better knowledge into the diversity of symbionts in sponges. Sponge associated microbes composed of 31 bacterial phyla; *Acidobacteria, Actinobacteria, Aquifaciae, Bacteroidetes, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Gemmatimonadetes, Lentisphaerae, Nitrospira, Poribacteria, Proteobacteria, Spirochaetes*, and *Verrucomicrobia*, two archaeal phyla (*Crenarchaeota* and *Euryarchaeota*), and some eukaryotic microbes (Taylor et al., 2007; Abdelmohsen et al., 2010; Sipkema et al., 2011; Montalvo and Hill, 2011; Lee et al., 2011). Hentschel et al. (2006) reported that the internal mesohyl layer of sponges is mainly composed of heterotrophic and autotrophic bacteria, whereas the external layer of the sponge that is exposed to sunlight is generally predominated by photosynthetic bacteria such as cyanobacteria (Kennedy et al., 2007). Despite the fact that sponges carry out phagocytosis on bacterial cells in the mesohyl, it was found that sponge-specific microbial communities are abundant in the mesohyl.

Marine sponges acquire their microbial associates in two ways. The first approach is by filter feeding and selective retention of bacteria. Sponges can engulf bacteria in the surrounding seawater when they filter the food particles out of the water column. This process is known as horizontal transmission (Taylor et al., 2007). The second is vertical transmission of bacterial associates from adult sponges to their offspring, i.e., progeny (Sharp et al., 2007; Usher et al., 2001). This transmission is typical among marine sponges, which use such method to maintain stable microbial associations to each sponge generation (Schmitt et al., 2008). Sponge-associated bacteria are assumed to provide their host sponge with a variety of advantages nutrient procurement (Wilkinson and Fay, 1979; Hoffman et al., 2009), stabilization of the sponge skeleton (Wilkinson et al., 1981), processing of metabolic waste (Beer and Ilan, 1998), shielding from UV light (Shick and Dunlap, 2002), and chemical defense (Schmidt et al., 2000).

Previous studies showed that associated bacterial communities in certain species of sponges were highly similar and consistently different from the bacterial communities in the surrounding seawater. Hentschel et al. (2002) confirmed that there were uniform microbial communities in three taxonomically distant marine sponges *Aplysina aerophoba, Rhopaloeides*

odorabile, and *Theonella swinhoei* from different geographic regions that were unique from those in the water column or in the surrounding water. In addition, Friedrich et al. (2001) established that the composition of sponge-associated bacterial communities was unaffected by environmental disturbance resulting from transplantation to different habitats. These studies suggest that there is a stable, distinct, and mutualistic association between the two types of organisms (Taylor et al., 2007). However, some sponge-associated bacteria do not appear to be uniform. For example, Wichels et al. (2006) determined that the bacterial communities associated with the sponge *Halichondria panicea* varied greatly over time and space. Lee et al. (2007) showed that *Callyspongia* and *Mycale* sponges from different biogeographical regions had different bacterial associates. These studies have given awareness into the diversity of the bacterial communities associated with different sponges and the numerous bacterial groups that have so far been found only in marine sponges. In order to determine whether any sponge is associated with a particular microbial community, the bacterial community must be comprehensively analyzed using molecular techniques. These help in elucidating the exact relationships between sponges and their associated microbial communities.

Recently, sponge associated microorganisms have received attention due to their ability to produce bioactive secondary metabolites (Faulkner et al., 2000). There is cumulative evidence that bacteria associated with sponges are the true producers of these compounds. These metabolites provide the chemical defense system to their hosts, since sponges lack the complex immune system of higher animals (Kennedy et al., 2007). Compilation of sponge associated bacteria and their bioactive compounds are given in Table 3.

Table 3. Sponge-associated bacteria and their bioactive compounds

Sponges	Microorganisms	Compounds	Biological activity	References
Acanthella acuta	*Bacillus pumilus* AAS3 (firmicutes)	GG11	antitumor activity (inhibits HM02 and HepG2 cell lines at 50% inhibition)	Ramm et al., 2004
Acantho-strongylophora sp.	*Micromonospora* sp. (actinobacteria)	manzamine A	antimalarial activity (inhibits the growth of malaria parasite *Plasmodium berghei* at 90%) antitumor (IC_{50}s 0.07, 0.05 and 0.15 µg/mL against P388, KB and LoVo cell lines, respectively)	Dunlap et al., 2007; Ang et al., 2000
Aplysina aerophoba	*Bacillus subtilis* A184 (firmicutes)	surfactin	antitumor activity (IC_{50}s 26 and 30 µM against LoVo and Hep2 cell lines, respectively)	Pabel et al., 2003
	Bacillus subtilis A190 (firmicutes)	surfactin		
	Bacillus subtilis A202 (firmicutes)	surfactin		
	Bacillus pumilus A586 (firmicutes)	pumilacidin containing β-hydroxy fatty acid	antiviral activity (IC_{50s} 5.2 and 6.7 µg/mL against HSV-1 strain)	Pabel et al., 2003

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Axinella polypoides	*Streptomyces axinellae* Po1001 (actinobacteria)	tetromycin 1	antiparasitic activity (IC_{50} 32 µM against *Trypanosoma brucei brucei*)	Pimentel-Elardo et al., 2009, 2011
Chondrilla caribensis f	*Verrucosispora* sp. WMMA107 (actinobacteria)	22'-deoxythio-coraline, thiochon-drilline A-C, 12'-sulfoxythio-coraline	cytotoxic activity (EC_{50} 0.13, 2.86 and 1.26 µM against A549 cell lines, respectively)	Wyche et al., 2011
Cinachyra sp.	*Streptomyces* sp. SpC080624SC-11 (actinobacteria)	JBIR-46, JBIR-48	antiproliferative (IC_{50}s 189 and 96 µM against HL-60 cell lines, respectively)	Khan et al., 2010
Cinachyrella australiensis	*Micromonospora* sp. SpC080624GE-05 (actinobacteria)	cyclo-(L-Pro-D-Phe)	antifungal activity (7-mm zone of *Candida albicans*)	Gao et al., 2010
Craniella australiensis	*Streptomyces* sp. DA11 (actinobacteria)	chitinase	antifungal activity (clear zones 11 mm and 10 mm against *Aspergillus niger* and *Candida albicans*, respectively)	Han et al., 2009

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Demospongiae sponge	*Streptomyces* sp.	JBIR-58	cytotoxic activity (IC_{50} 28 µM against HeLa cell lines)	Ueda et al., 2010
Dysidea avara	Unidentified bacterium	2-methylthio-1,4-naphthoquinone	antiangiogenic activity (100% embryo elimination at 1 ng/disc), antimicrobial activity (>2-mm zone of inhibition against *Bacillus subtilis*)	Thakur and Müller, 2005
Dysidea avara	*Bacillus vallismortis* (firmicutes)	bacillamide A	algicidal activity (LC_{50} 3.2 µg/mL against *Cochlodinium polykrikoides*)	Yu et al., 2009
Dysidea sp.	*Vibrio* sp. (Gamma-proteobacteria)	tetrabromo-diphenylethers	antibacterial activity (MIC 0.1 µg/mL against *Klebsiella pneumonia*)	Elyakov et al., 1991
Fascaplysinopsis reticulata	*Pseudoalteromonas maricaloris* KMM636T (Gamma-proteobacteria)	bromo-alterochromide A	cytotoxic on developing eggs of sea urchin (MIC 40 µg/mL)	Speitling et al., 2007

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Halichondria japonica	*Bacillus cereus* QN03323 (firmicutes)	YM-266183	antibacterial activity (MIC 0.025 µg/mL against *Staphylococcus aureus*)	Nagai et al., 2003
Halichondria okadai	*Alteromonas* sp. (Gamma-proteobacteria)	alteramide A	cytotoxic activity (IC_{50}s 0.1 and 1.7 µg/mL against P388 and L1210 cell lines, respectively)	Shigemori et al., 1992
Halichondria okadai	*Rubritalea squalenifasciens* HOact23^{T} (Verrucomicrobiae)	Dia-polycopenedioic acid xylosyl esters A	oxygen suppression activity (IC_{50} 5.1 µM)	Shindo et al., 1992
Halichondria panacea	*Microbacterium* sp. (actinobacteria)	1-*O*-acyl-3-[R-glucopyranosyl-(1-3)-(6-*O*-acyl-R-mannopyranosyl)]glycerol	antitumor activity (50% inhibition HM02 and Hep G2 cell lines at 0.4 – 3 µg/mL)	Wicke et al., 2000
Halichondria panacea	*Norcadiopsis* sp. (actinobacteria)	norcapyrones A-D	cytotoxic activity (IC_{50}s 8.7 – 22.2 µM against MCF-7 cell)	Scheeman et al., 2010a

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Halichondria panacea	*Streptomyces* sp. (actinobacteria)	mayamycin	cytotoxic activity (IC_{50}s 0.2, 0.3, 0.2, and 0.22 µM against Hep G2, HT-29, GXF25IL and NIH-3TS cell lines; respectively) antibacterial activity (IC_{50} 8.0 µM against *Bacillus subtilis*)	Scheeman et al., 2010b
Haliclona sp.	*Streptomyces* sp. (actinobacteria)	JBIR-34, JBIR-35	DPPH antioxidant activity (IC_{50} 1.0 and 2.5 mM, respectively)	Motohashi et al., 2010
Homophymia sp.	*Pseudomonas* sp. (Gamma-proteobacteria)	2-undecyl-4-quinolone	antimalarial activity (IC_{50} 0.001 µg/mL against *Plasmodium falciparum*)	Bultel-Poncé et al., 1999
		2-undecen-1'-yl-4-quinolone	cytotoxic activity (IC_{50} 5 µg/mL against KB cell)	Bultel-Poncé et al., 1999
		2-nonyl-4-hydroxy-quinoline N-oxide	antimicrobial activity (10-mm zone of inhibition against *Staphylococcus aureus*)	Bultel-Poncé et al., 1999

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Hyatella sp.	*Vibrio* sp. M22-1 (Gamma-proteobacteria)	andrimid	antibacterial activity (MICs 0.06 and 1 μg/mL against *Bacillus subtilis* and *Staphylococcus aureus MRSA*, respectively)	Oclarit et al., 1994
Hymeniacidon perlevis	*Pseudo-alteromonas piscicida* NJ6-3-1 (Gamma-proteobacteria)	norhaman	cytotoxic activity (IC_{50} 5 μg/mL against HeLa cell lines)	Zheng et al., 2005
Hymeniacidon sp	*Streptomyces carnosus* AZS17 (actinobacteria)	lobophorin C	cytotoxic activity (IC_{50} 0.6 μg/mL against BEL-7402 cell lines)	Wie et al., 2011
		lobophorin D	cytotoxic activity (IC_{50} 7.5 μM against MDA-MB 435 cell lines)	Wie et al., 2011
Hyrtios altum	*Vibrio* sp. (Gamma-proteobacteria)	trisindoline	cytotoxic activity (IC_{50}s 3.51 and 6.63 μM against MES-SA and HCT 15 cell lines, respectively)	Kobayashi and Kitagawa, 1994; Kobayashi et al., 1994

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Lamellodyside a herbacea	*Oscillatoria spongeliae* (cyanobacteria)	2-(2'-4'-dibromo-phenyl)-4,6-dibromophenol	antibacterial activity (MIC 4 and 6.3 µg/mL against *Staphylococcus aureus* and *Bacillus subtilis*, respectively)	Hinde et al., 1994
Leucetta microraphis	Unidentified bacterium	leucamide A	antitumor activity (GI_{50}s 5.2, 5.9, 5.1 µg/mL against HM02, HepG2, and Huh 7 cell lines, respectively)	König et al., 2005
Mediterranean sponges	*Streptomyces* sp. 11 (actinobacteria)	staurosporine	antiparasitic activity (IC_{50} 5.3 µM against *Leishmania major*; IC_{50} 0.022 µM against *Trypanosoma brucei brucei*)	Pimentel-Elardo et al., 2010
Mediterranean sponge	*Streptomyces* sp. T03 (actinobacteria)	butenolide	antiparasitic activity (IC_{50} 31.77 µM against *T. brucei brucei*)	Pimentel-Elardo et al., 2010

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Mycale plumose	*Saccharopolyspora* sp. nov. (actinobacteria)	metacyclo-prodigiosin	cytotoxic activity (IC_{50}s 0.007, 0.022, and 0.11 μM against P388, HL-60, and A549 cell lines, respectively)	Liu et al., 2005
Ptilocaulis trachys	*Lyngbya majuscule* (cyanobacteria)	majusculamide C	antifungal activity (27-mm zone of inhibition against *Rhizoctonia solani*)	Williams et al., 1993; Dunlap et al., 2007
Stelleta tenuis	*Alcaligenes faecalis* A72 (Beta-proteobacteria)	cyclo-(L-Pro-L-Phe)	antimicrobial activity (MIC 50 μg/mL against *S. aureus*)	Li, 2009
Subrea clavata	*Salinospora* sp. (actinobacteria)	rifamycin	antimicrobial activity (MIC < 0.2 μg/mL against *S. aureus*; MIC 0.01 μg/mL against *Mycobacterium tuberculosis*)	Kim et al., 2006

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Theonella swinhoei	Unidentified bacterium	swinholide A	cytotoxic activity (IC_{50}s 0.03 and 0.04 µg/mL against L1210 and KB cell lines, respectively)	Bewley et al., 1996
	Entotheonella palauensis	theonegramide	antifungal activity (MIC 6.3 µg/mL against *C. albicans*)	Bewley and Faulkner, 1994
	Uncultured bacterium	onnamide A	cytotoxic activity (IC_{50}s 2.4 and 25 nM against P388 and HL-60 cell lines, respectively)	Piel et al., 2004
Xestospongia sp.	*Micrococcus luteus* R-1588-10 (actinobacteria)	triclosan	antimicrobial activity (MICs 0.001, 0.03 and 0.001 *against Staphylococcus aureus, Escherichia coli* and *Vibrio anguillarum*, respectively)	Bultel-Poncé et al., 1998

Table3. (cont.)

Sponges	Microorganisms	Compounds	Biological activity	References
Unidentified sponge	*Actinomadura* sp. (actinobacteria)	JBIR-65	neuroprotective activity (EC_{50} 31 μM against L-glutamate toxicity)	Takagi et al., 2010

1.1 Trisoxazole macrolides

Trisoxazole macrolides are among unique classes of exclusively marine secondary metabolites found in the sponges of the genena *Halichondria*, *Mycale*, *Jaspis*, *Chondrosia*, and *Pachastrissa* (now referred to as *Penares*), as well as certain species of the sponge-feeding nudibranchs such as *Hexabranchus sanguineus*. These metabolites were transferred from the producing sponge to nudibranch and passed to accumulate in their mantle and egg masses to be used as defensive chemicals (Pawlik et al., 1988), thus, showing the distribution and ecological role of trisoxazole macrolides.

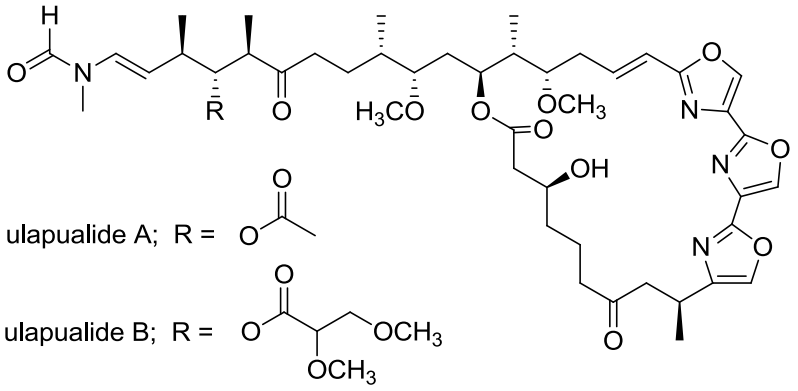
Trisoxazole macrolides are characterized by 25-membered lactone ring containing three consecutive oxazole moieties connected to an 11-carbon panhandle side chain with an *N*-formyl terminal. The differences between the structures are mostly in the oxygenation patterns and alkyl group substitution of the aliphatic chain of the structures. To date, 46 congeners have been reported (vide infra).

The biological activities of trisoxazole macrolides have been studied. The activities include antifungal activity, cytotoxicity, inhibition of cell division in the fertilized sea urchin eggs, ichthyotoxicity, and inhibition of actin filament dynamics (Roesener and Scheuer, 1986; Matsunaga et al., 1986; Kernan and Faulkner, 1987; Kernan et al., 1988; Matsunaga et al., 1989; Fusetani et al., 1989; Pawlik, 1993; Phuwapraisirisan et al., 2002; Mizushina et al., 2002; Tanaka et al., 2003; Shin et al., 2004). Trisoxazole macrolides can be divided into six major classes; ulapualides, kabiramides, halichondramides, mycalolides, jaspisamides and

halishigamides. The classification is based on oxygenation patterns as well as substitution on C-4 – C-7 and on C-30 –C-33.

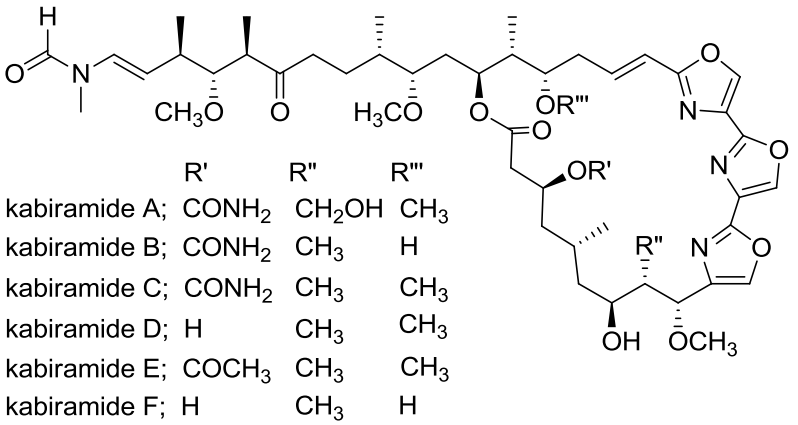
1.4.1 Ulapualides

Ulapualides A and B were the first members of trisoxazole macrolides isolated from the egg masses of the nudibranch *Hexabranchus sanguineus* collected from Pupukea, Hawaii in 1986 (Roesener et al., 1986; Matsunaga et al., 1989). They inhibit L1210 leukemia cell proliferation at IC_{50}s of 0.01-0.03 µg/mL and potent antifungal activity (11-mm zone of *Candida albicans* inhibition at 0.4 nmol/disc of ulapualide B).



1.4.2 Kabiramides

Kabiramides A-E were isolated from the nudibranch *Hexabranchus* eggmasses (Matsunaga et al., 1986; Matsunaga et al., 1989). Kabiramides B and C were also isolated from the sponge *Halichondria* sp. and *Pachastrissa nux* (Kernan and Faulkner, 1987; Petchprayoon et al., 2006; Sirirak et al., 2011b). Kabiramides showed strong antifertilization activity of sea urchin eggs (IC_{99}s 0.2-1.0 µg/mL), as well as cytotoxic activity (IC_{50}s 0.01-0.18 µg/mL) against L1210 cell lines (Matsunaga et al., 1989). Biological activities of kabiramides will be discussed in section 1.5.

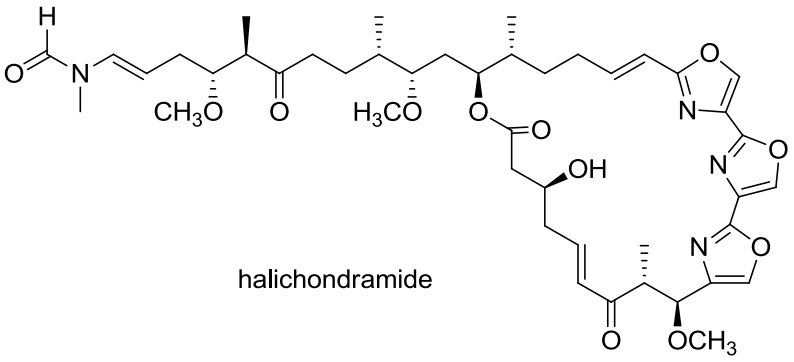


	R'	R"	R'''
kabiramide A;	$CONH_2$	CH_2OH	CH_3
kabiramide B;	$CONH_2$	CH_3	H
kabiramide C;	$CONH_2$	CH_3	CH_3
kabiramide D;	H	CH_3	CH_3
kabiramide E;	$COCH_3$	CH_3	CH_3
kabiramide F;	H	CH_3	H

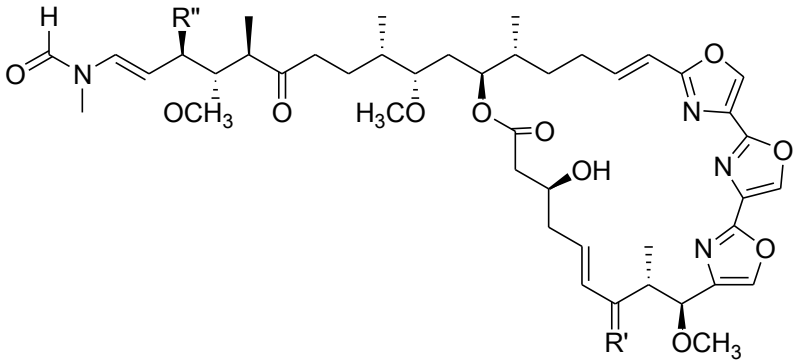
1.4.3 Halichondramides

Halichondramide, halichondramide acid, dihydrohalichondramide, isohalichondramide, halichondramide imide, and halichondramide ester were isolated from the sponge *Halichondria* sp. They showed antifungal activity against *Candida albicans* at IC_{50} values of 0.2-0.5 µg/mL (Kernan et al., 1988b; Pawlik et al., 1988) as well as antifertilization activity against sea urchin eggs (IC_{99} 0.5-4.0 µg/mL). 33-Methyldihydrohalichondramide and tetrahydrohalichondramide were isolated from the nudibranch *Hexabranchus sanguineus*. They displayed strong cytotoxic activity against L-1210 cell lines at IC_{50} values of 0.03 and 0.05 µg/mL (Matsunaga et al., 1989).

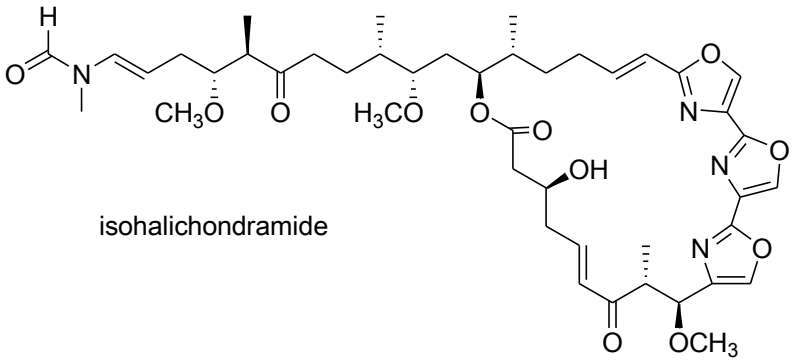
Shin et al. (2004) reported the isolation of neohalichondramide, *19Z*-halichondramide and secohalichondramide from the sponge *Chondrosia corticata*. They all exhibited cytotoxic activity against K562 cell lines with IC_{50}s of 0.38-0.9 µg/mL. They also showed antifungal activity against *Candida albicans* and *Aspergillus niger* (clear zone of inhibition 10-20 mm and 10-15 mm, respectively at concentration of 2 µg/disc). 33-Methyltetrahydrohalichondramide was isolated from the nudibranch *Hexabranchus sanguineus* and showed antifungal activity at MIC values of 0.250, 0.125, and 0.500 µg/mL against *C. albicans*, *C. glabrata*, and *C. neoforman*, respectively (Dalisay et al., 2009).



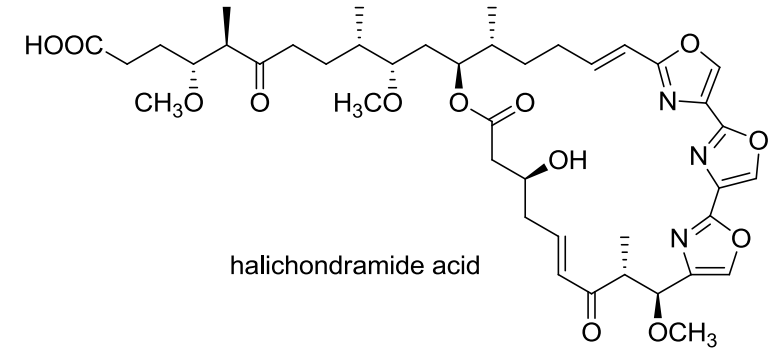
halichondramide



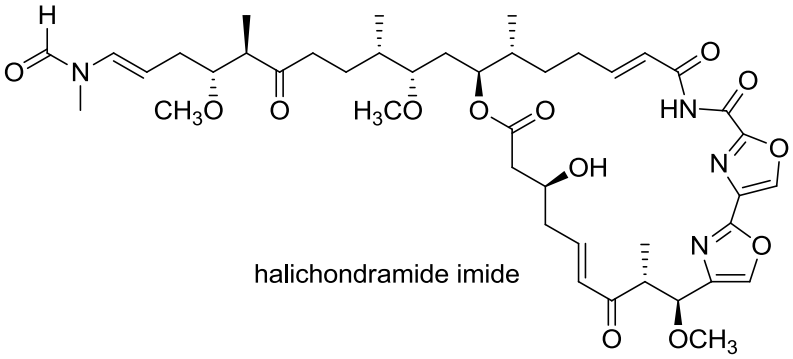
	R'	R"
dihydrohalichondramide;	O	H
tetrahydrohalichondramide;	H, OH	H
33-methyldihydrohalichondramide;	O	CH_3



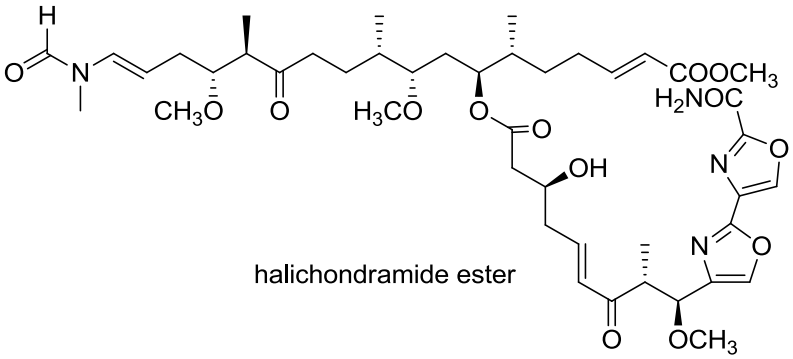
isohalichondramide



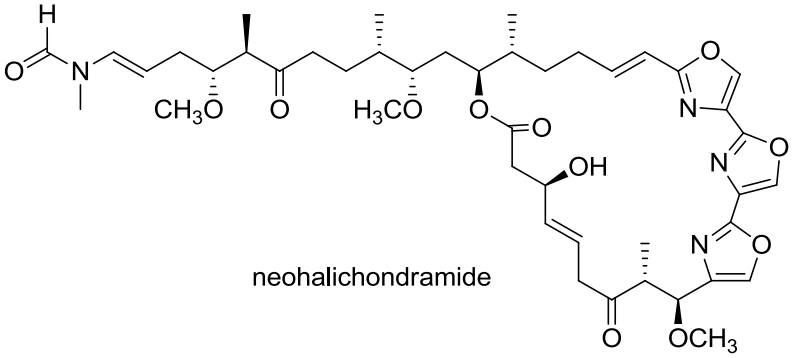
halichondramide acid



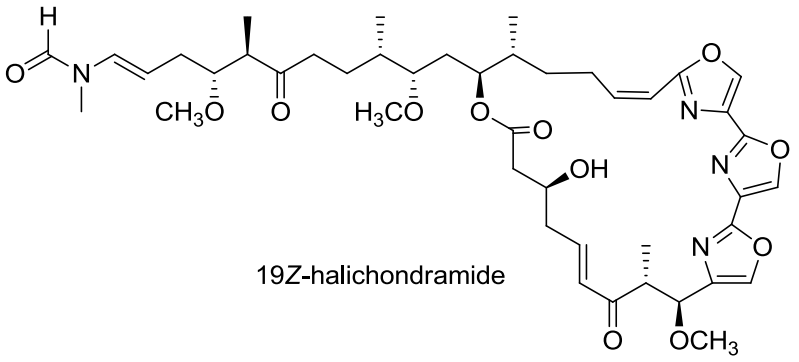
halichondramide imide

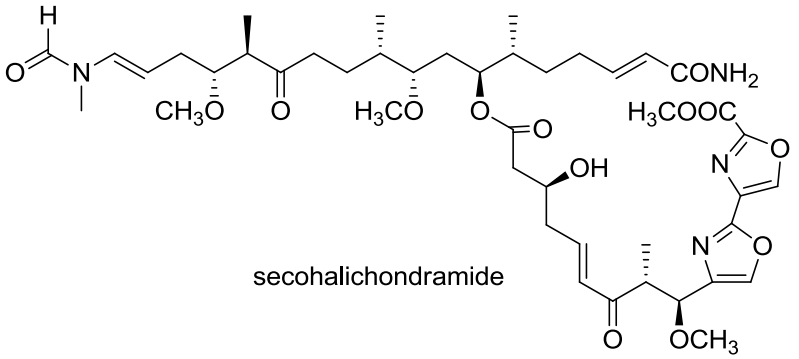


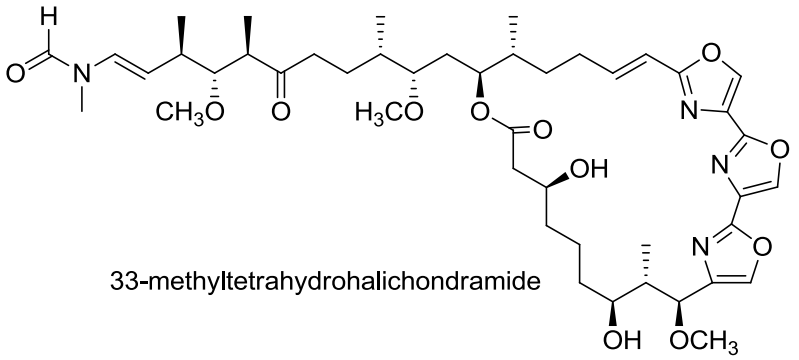
halichondramide ester



neohalichondramide





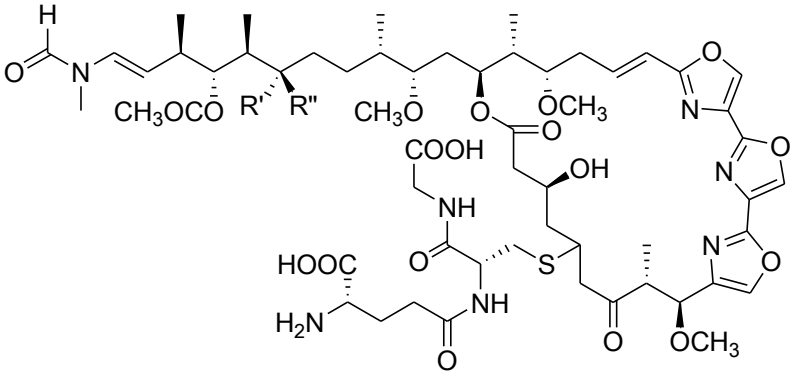


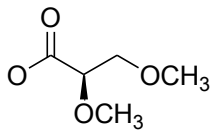
1.4.4 Mycalolides

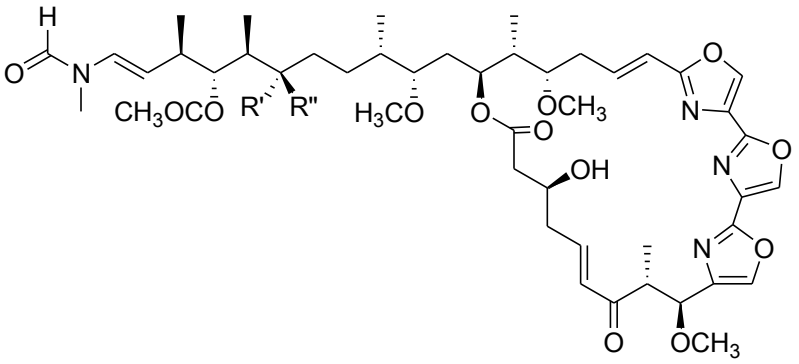
Mycalolides A-C and thiomycalolides A and B were isolated from the sponge *Mycale* sp. Mycalolides A-C showed potent cytotoxic activity against B-16 melanoma cells at IC_{50} values of 0.5-1.0 ng/mL and LC_{50} 2.5 μM against NCI's 60-human tumor cell lines for mycalolide C. They inhibited proteasome activity at IC_{50} value of 30 μg/mL (Fusetani et al., 1989; Fusetani et al., 1991; Saito et al., 1994; Matsunaga et al., 1998a; Matsunaga et al., 1998b; Phuwapraisirisan et al., 2002; Tsukamoto et al., 2005). Thiomycalolides A and B exhibited cytotoxic activity against

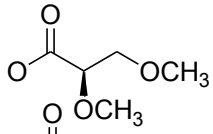
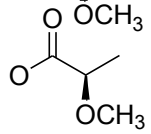
P388 cell lines with IC_{50} value of 18 ng/mL (Matsunaga et al., 1998a). 30-Hydroxymycalolide A, 32-hydroxymycalolide A, and 38-hydroxymycalolide B were isolated from the sponge *Mycale magellanica* and showed cytotoxic activity against L12110 cell lines with IC_{50} values of 19, 13, and 15 ng/mL, respectively (Matsunaga et al., 1998b). 30,32-Dihydroxymycalolide A was obtained from the sponge *Mycale izuensis*. It exhibited cytotoxicity against Hela cell lines with IC_{50} value of 2.6 ng/mL (Phuwapraisirisan et al., 2002).

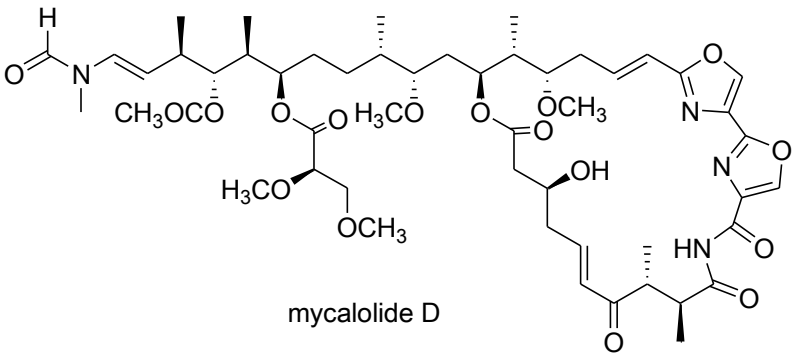
Mycalolides D and E were isolated from the the stony coral *Turbastrea faulkeneri*, along with mycalolide C. Mycalolide D showed cytotoxic activity against NCI's 60-human tumor cell lines at LC_{50}av value of 0.6 µM (Rachid et al., 1995). Secomycalolide A was isolated from the sponge *Mycale* sp. It exhibited proteasome inhibitory activity at IC_{50} value of 11 µg/mL (Tsukamoto et al., 2005).



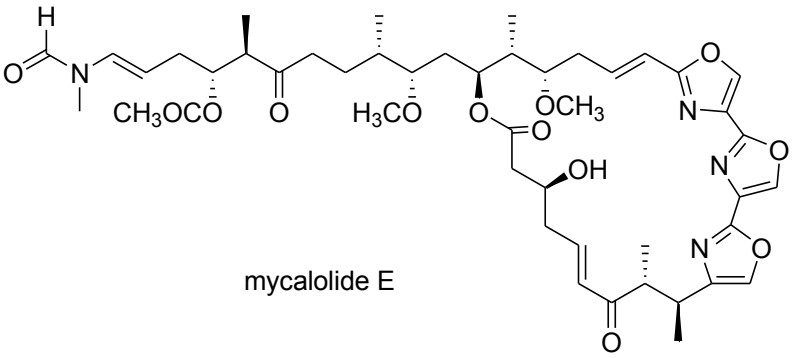
	R'	R"
thiomycalolide A;	=O	
thiomycalolide B;	H	



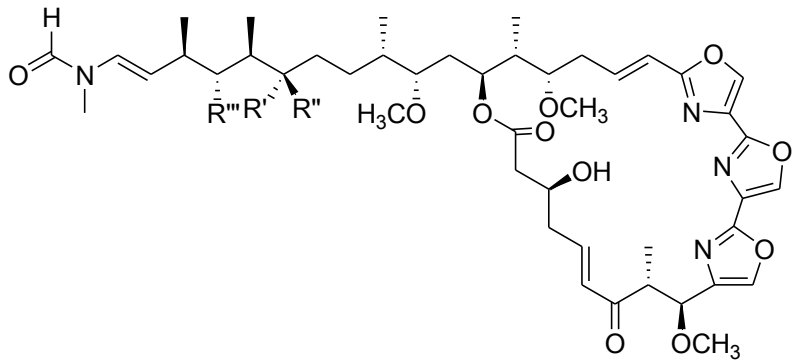
	R'	R"
mycalolide A;		=O
mycalolide B;	H	
mycalolide C;	H	

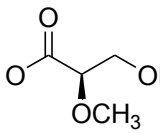


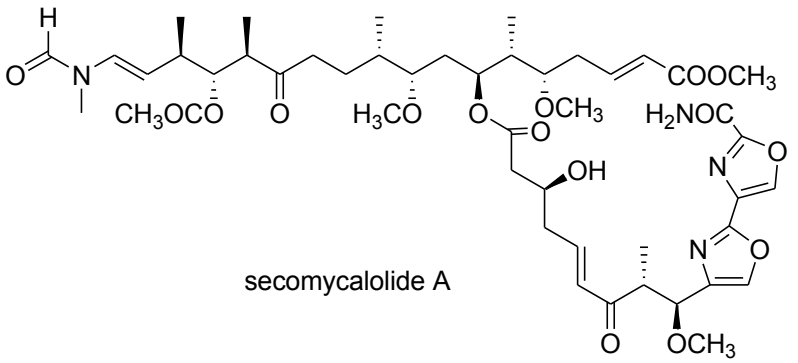
mycalolide D



mycalolide E



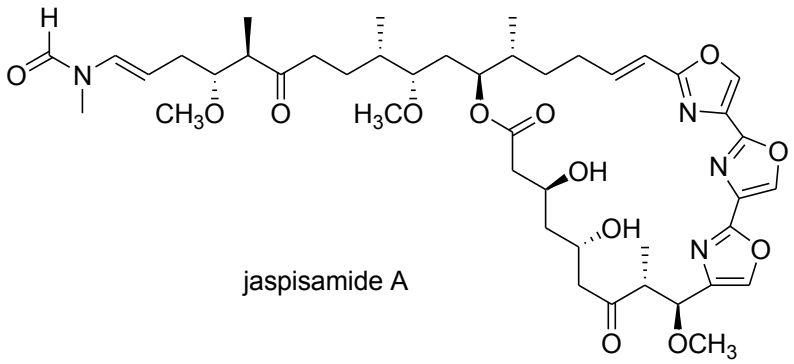
	R'	R"	R"'
30-hydroxymycalolide A;	H	OH	$OCOCH_3$
32-hydroxymycalolide A;	=O		OH
38-hydroxymycalolide B;	H		$OCOCH_3$
30,32-dihydroxymycalolide A;	H	OH	OH



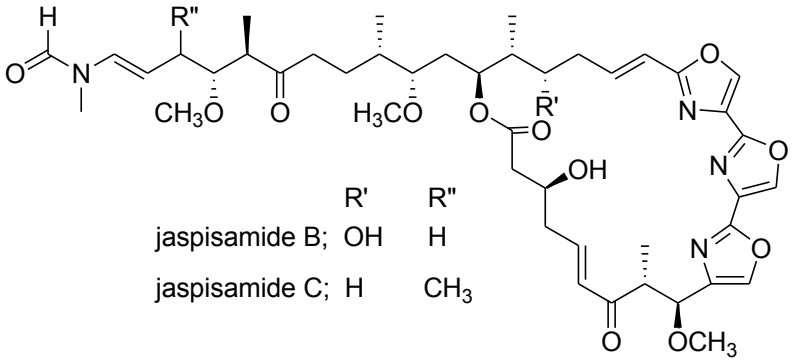
secomycalolide A

1.4.5 Jaspisamides

Jaspisamides A-C were isolated from the sponge *Jaspis* sp. Jaspisamide A exhibited cytotoxic activity against L1210 and KB cell lines at IC_{50} values of < 0.001 and 0.015 µg/mL, respectively, while jaspisamide B and C displayed cytotoxic activity against L1210 and KB cell lines at IC_{50} range < 0.001 -0.013 µg/mL, respectively (Kobayashi et al., 1993).



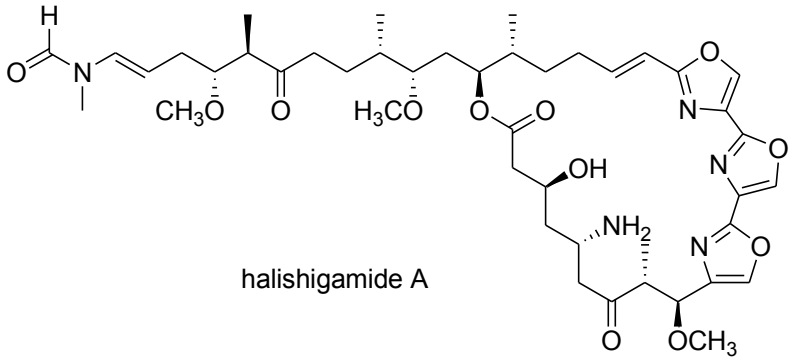
jaspisamide A



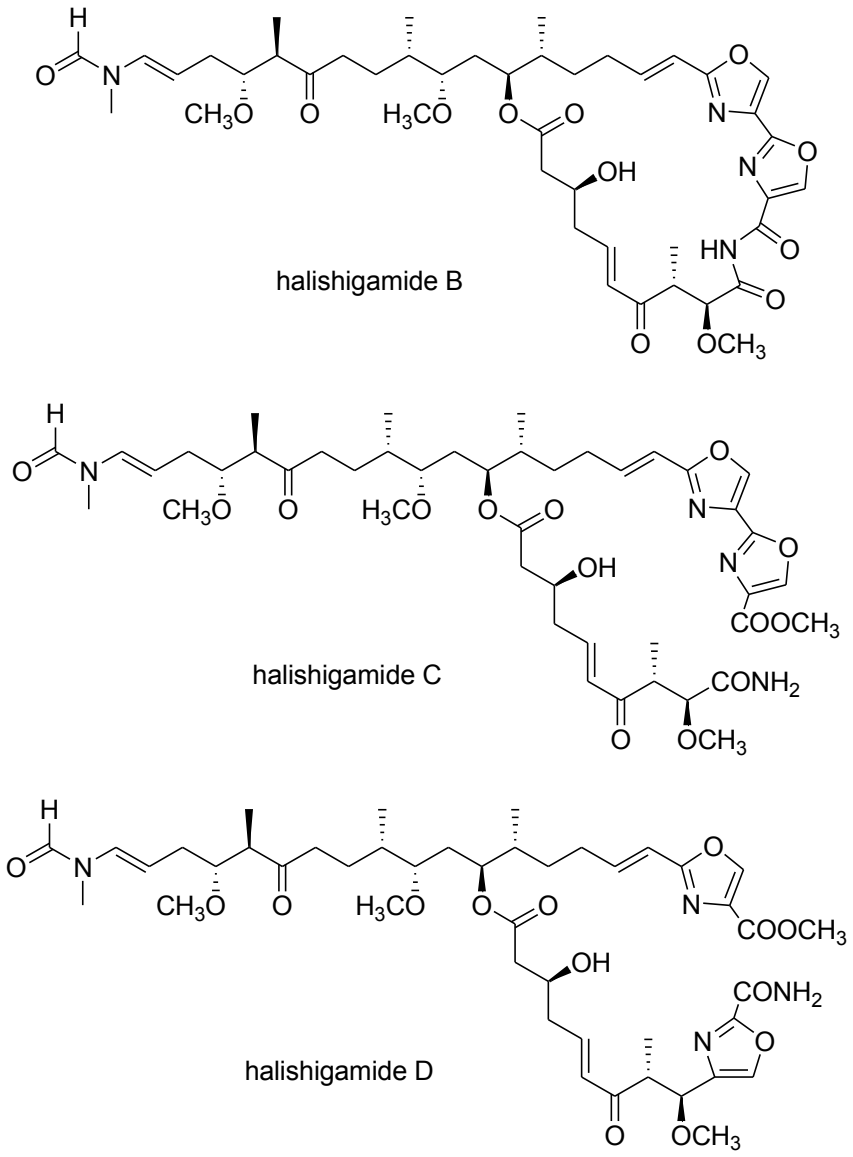
	R'	R"
jaspisamide B;	OH	H
jaspisamide C;	H	CH_3

1.4.6 Halishigamides

Halishigamides A-D were isolated from the sponge *Halichondria* sp. Halishigamide A showed potent cytotoxic activity against L1210 and KB cell lines at IC_{50} values of 0.0036 and 0.012 µg/mL, respectively as well as antifungal activity against *Trichophyton metagrophytes* at MIC value of 0.1 µg/mL (Kobayashi et al., 1997). Halishigamides B-D had weak cytotoxic activity against L1210 and KB cell lines (IC_{50} values of 1.1-5.2 µg/mL and 1.8-7.5 µg/mL, respectively). They also showed moderate antifungal activity against *Trichophyton metagrophytes* at MIC values of 6.5-25 µg/mL (Kobayashi et al., 1997).



halishigamide A



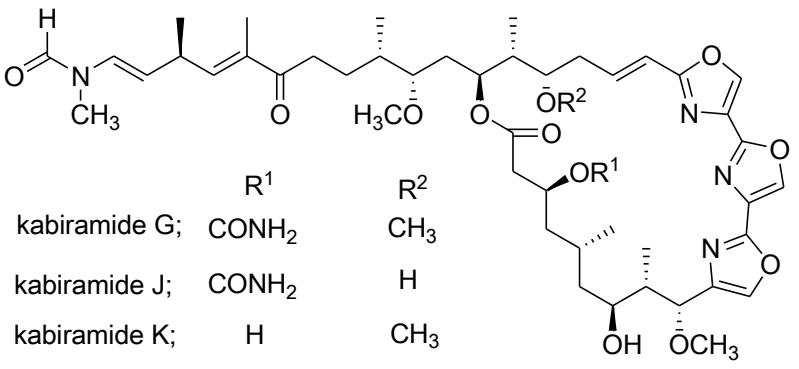
1.2 Chemical constituents from the sponge *Penares nux*

The sponge *Penares nux* (formerly described as *Pachastrissa nux*; Van Soest et al.; 2010) belongs to the family Geodiidae (order Astrophorida). The sponge is massive with thick encrusting form. It is black in color, with a rubbery texture, sticky and easily torn. *P. nux* appears as a grayish-black colony under water with a protruding, gorgonian-like appearance known as the capitum and an irregular shaped base part, which is buried by sediments.

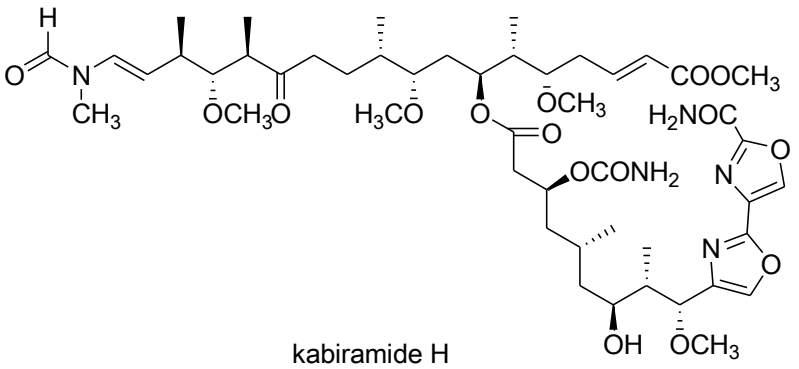
Presuming all the reports regarding the chemical constituents from the sponge *Pachastrissa nux*, which has already been transferred to *Penares nux* as stated, to date, there have been only one major class of chemical constituents ever been reported from the sponges of the

genus *Penares.* The trisoxazole macrolides specifically of the kabiramide family. This included kabiramides B-D, and F-L from *P. nux* collected from Sichang Island, Chonburi, and Koh Tao, Surat-Thani Provinces (Petchprayoon et al., 2006; Sirirak et al., 2011b; Sirirak et al., 2013). All kabiramide analogs were reported to be biologically active.

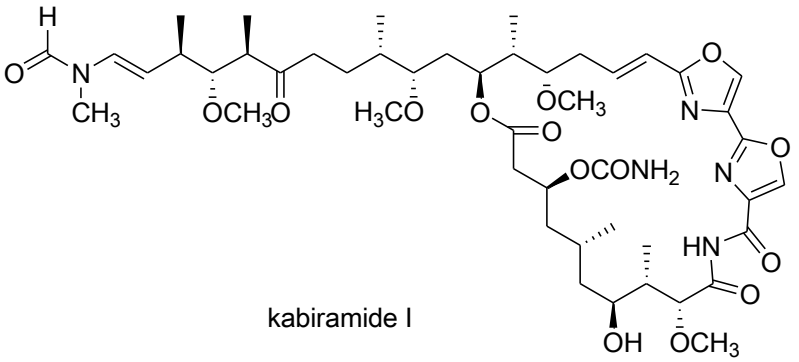
Kabiramides B-F exhibited strong cytotoxic activity against A-549 human lung carcinoma, KB oral human epidermal carcinoma, BC breast cancer, NCI-H187 human small cell lung cancer and HT-29 human colon adenocarcinoma cell lines with IC_{50} values of 0.01-0.18 µg/mL, as well as antimalarial activity against *Plasmodium falciparum* K1 strain with IC_{50} values of 1.67-4.79 µM (Sirirak et al., 2011b; Petchprayoon et al., 2006). Kabiramide G showed cytotoxic activity against A-549 human lung carcinoma and HT-29 human colon adenocarcinoma cell lines with IC_{50} values of 0.05 and 0.1 µg/mL, respectively (Petchprayoon et al., 2006; Sirirak et al., 2011b). Kabiramide I displayed cytotoxicity against A-549 human lung carcinoma and HT-29 human colon adenocarcinoma cell lines with IC_{50} values of 2 and 0.4 µg/mL, respectively (Petchprayoon et al., 2006) as well as antimalarial activity against *Plasmodium falciparum* K1 strain with IC_{50} value of 4.5 µM (Sirirak et al., 2013). Kabiramide J and K showed potent cytotoxic activity against MCF-7 breast adenocarcinoma cell line with IC_{50} values of 0.02 and 0.07 µM respectively. They also displayed strong antimalarial activity against *Plasmodium falciparum* K1 strain with IC_{50} values of 0.31 and 0.39 µM (Sirirak et al., 2011b). Kabiramide L showed antiplasmodial activity against *Plasmodium falciparum* K1 strain with IC_{50} value of 2.6 µM (Sirirak et al., 2013).



	R^1	R^2
kabiramide G;	$CONH_2$	CH_3
kabiramide J;	$CONH_2$	H
kabiramide K;	H	CH_3
kabiramide L;	H	H



kabiramide H



kabiramide I

1.3 Rationale and objectives

The sponge *Penares nux* is among the abundant sponge species found in various locations throughout the Gulf of Thailand. From the previous work by at Marine Natural Products Research Unit, Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University, a series of trisoxazole macrolides were isolated and reported (Sirirak et al., 2011b; Sirirak et al., 2013). In addition, intracolonial allocation of the macrolides between the two differentiated growth forms has been observed and reported. It was found that separate extraction of the two parts yielded two different extracts with significantly different activities; the capitum extract showed greater activity than the base extract. Further investigation on the allocation of kabiramide derivatives, using kabiramides C and G as chemical markers showed significant difference in the accumulation profiles of the two markers between the capitum and the base (Sirirak et al., 2011a).

It is of interest to carry on the results from the previous studies described above and to investigate if the sponge produces this metabolite as a form of chemical defense or

involvement of epibiotic and/or symbiotic microbes, or even fouling bacteria casting an impact on the chemical production and allocations in the *P. nux* sponge. It is important to analyze the specific association of bacteria with sponges in the search for new pharmaceutical drugs and also it gives an insight into sponge specific bacteria as a form of epibacterial defense. The objectives of this dissertation include

i) the determination, both quantitatively and qualitatively, the chemical profiles in the sponge *Penares nux*.

ii) the evaluation of the microbial profiling i.e. culturable bacteria associated with the sponge *Penares nux*.

iii) the correlation of accumulating toxic macrolides and the associated microorganisms in the sponge tissue.

CHAPTER 2

EXPERIMENTAL

2.1 General experimental procedures

Unless stated otherwise, all chromatographic packing materials were used as purchased. Solvents for chromatographic and general purposes were commercial grade and were re-distilled prior to use. All HPLC solvents were HPLC grade, and were filtered through a 0.45-μm membrane filter and degassed in an ultrasonic bath. Thin layer chromatography (TLC) was performed on silica gel 60 F 254 on aluminum supports (0.02 mm layer thickness; Merck®). Visualization was done under UV light (254 nm), and with iodine vapor. Vacuum and flash chromatographies were performed on SiO_2 60 (particle size 0.04-0.06 mm; Scharlau®). Size exclusion chromatography was conducted on Sephadex™ LH-20 (GE Healthcare®), which was saturated in eluting solvents for 24 hours before using. Semi-preparative HPLC was performed either on a Waters 600E System Controller equipped with a Rheodyne 7125 injector port, a Waters 484 tunable absorbance detector, and a Jasco 807-IT integrator, or on a Thermo Finigan Spectra System Controller 1000 equipped with P 4000 quaternary pump, Rheodyne 7725i injector port, and UV 6000 LP diode array detector, operated under ChromQuest 4.2.34 version 3.1.6 software.

IR spectra were recorded on a Perkin-Elmer® Spectrum One FT-IR spectrophotometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University). UV spectra were obtained from a Hewlett Packard 8452A diode array spectrophotometer (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University). NMR spectra were operated on a Varian Unity Inova 500 MHz NMR spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C; Scientific Equipment Center, Prince of Songkla University). The operating solvents were benzene-d_6 (7.15 ppm of residual C_6HD_5 for ^{1}H NMR and 128.0 ppm for ^{13}C NMR). The chemical shifts were referenced the solvent signals as stated for internal standards.

The LC-MS based quantitative analysis was performed on an Agilent 1260 infinity HPLC system (Agilent, USA) equipped with a binary pump (G1312B), an auto sampler (G1367E), a column compartment (G1316A), and a degasser (G4225A). This is coupled to an API 3200™ triple quadrupole mass analyzer system, equipped with an electrospray ionization source (AB Sciex, USA). The ESI interface was operated in positive mode scanning from *m/z* 300-1200. The ion spray voltage was set to 5500V, and the turbo spray temperature was kept at 500°C. Ion source gas 1 and ion source gas 2 pressures were set at 60 psi. The full scan chromatograms and peak area integration were performed using Analyst® 1.6 software.

2.2 Animal material

The sponge specimens were collected using SCUBA at the depth of 15-20 m, from Koh Tao, in May 2012. The second collection was from another location Saiburi, Pattani Province, in October 2013. The specimens were preserved in an ice chest (0°C) upon surfacing, and at -20°C once arriving at the lab until the time of extraction. The sponge was identified as *Penares nux* (family Geodiidae) by Dr. Sumaitt Putchakarn, Institute of Marine Science, Burapha University. Recently, reallocation of *Pachastrissa nux* to the genus *Penares* was published. The sponge appears as a grayish-black colony under water with a protruding, gorgonian-like appearance known as the capitum and an irregular shaped base part, which is buried in the sediments (Figure 1). The collection was performed per capitum and appendage basis, i.e., for any capitum and appendage collected an associated and attached mass of the base was sampled. A voucher specimen (AP13-011-10) was deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

2.3 Isolation of standard kabiramide markers from the sponge *Penares nux*

The freeze-dried sponge (312 g) was chopped and macerated exhaustively (500 mL × 5) in CH_3OH. The dried CH_3OH-extract was partitioned with a series of solvents to yield hexane-, CH_2Cl_2-, and *n*-BuOH-extracts (3.2, 9.4, and 2.1 g, respectively). The CH_2Cl_2-extract was selected for further purification because it is the fraction that contains the trisoxazole

macrolides. The CH_2Cl_2-extract was fractionated over a SiO_2 column (5% CH_3OH in CH_2Cl_2), and fractions with similar chromatographic patterns were combined to yield two major fractions.

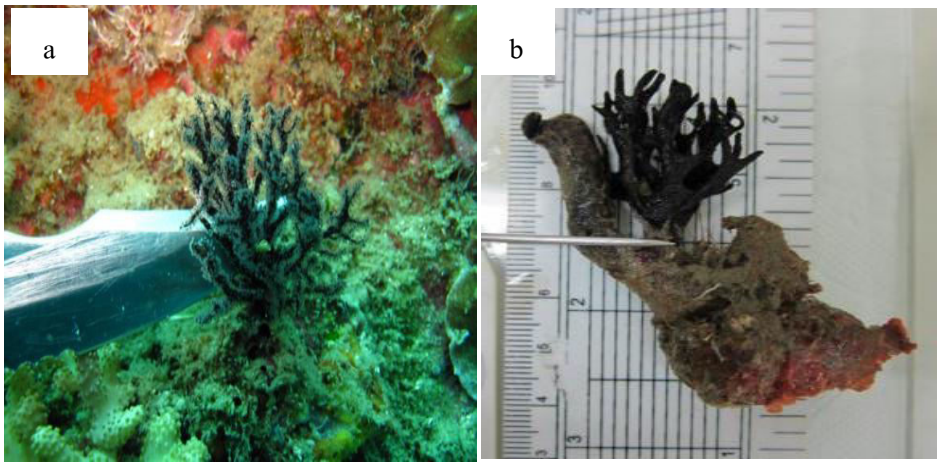


Figure 1. The sponge *Penares nux:* underwater (a) surface (b)

The first fraction (434.3 mg) was chromatographed over Sephadex™ LH-20 (CH_3OH), then with RP-C18 HPLC (VertiSep™, 10 µm, 250 × 10 mm; H_2O/CH_3CN 55:45, flow rate 3.5 mL/min) to yield kabiramide B (82.8 mg), kabiramide C (190.0 mg), and kabiramide G (60.2 mg). The second fraction (217.1 mg) was purified using Sephadex™ LH-20 (CH_3OH), then RP-C18 HPLC (VertiSep™, 10 µm, 250 × 10 mm; H_2O/CH_3CN 55:45, flow rate 3.5 mL/min) to yield additional kabiramide C (30.3 mg) and kabiramide G (15.1 mg).

Kabiramide B; pale yellow amorphous solid; UV (CH_3OH) λ_{max} (log ε) 249 (4.73) nm; IR (neat) ν_{max} 3367, 2945, 2832, 2595, 2228, 2044, 1654, 1449, 1423, 1293, 1115, 1030, 667 cm^{-1}; ^1H NMR (C_6D_6, 500 MHz) δ 7.91 (7.57) (1H, s, 35-NC*H*O), 7.56 (1H, ddd, *J* = 15.5, 10.0, 5.0 Hz, H-20), 7.26 (1H, s, H-14), 7.15 (1H, s, H-17), 7.12 (1H,s, H-11), 6.02 (1H, d, *J* = 16.5 Hz, H-19), 5.82 (7.36) (1H, d, *J* = 14.1 Hz, H-35), 5.74 (1H, ddd, *J* = 10.0, 10.0, 4.5 Hz, H-24), 5.31 (1H, br dd, *J* = 12.0, 6.0 Hz, H-3), 4.94 (1H, br s, H-9), 4.87 (5.0) (1H, dd, *J* = 14.1, 9.5 Hz, H-34), 4.50 (1H, br d, *J* = 9.0 Hz, H-22), 3.89 (1H, d, *J* = 5.5 Hz, H-7), 3.42 (3H, s, 26-OCH_3), 3.28 (3H, s, 32-OCH_3), 3.31 (1H, dd, *J* = 9.0, 3.0 Hz, H-32), 3.30 (1H, dd, *J* = 6.0, 3.0 Hz, H-26), 3.02 (3H, s, 9-OCH_3), 2.73 (2H, dd, *J* = 6.0, 2.0 Hz, H-2), 2.67 (1H, dd, *J* = 9.0, 7.0 Hz, H-31), 2.64 (3H, s, 35-NCH_3), 2.50 (2H, m, H-29), 2.45 (2H, ddd, *J* = 6.5, 6.0, 2.5 Hz, H-21), 2.18 (1H, dd, 6.5, 6.0

Hz, H-8), 2.11 (2H, m, H-28), 2.07 (2H, m, H-4), 2.04 (1H, m, H-33), 2.00 (2H, ddd, *J* = 9.5, 2.5, 2.5 Hz, H-25), 1.92 (1H, m, H-5), 1.80 (1H, m, H-27), 1.70 (2H, m, H-6), 1.50 (1H, m, H-23), 1.05 (3H, d, *J* = 7.1 Hz, 23-CH_3), 1.01 (3H, d, *J* = 6.8 Hz, 33-CH_3), 0.97 (3H, d, *J* = 7.1 Hz, 8-CH_3), 0.91 (3H, d, *J* = 6.5 Hz, 27-CH_3), 0.78 (0.75) (3H, d, *J* = 7.0 Hz, 31-CH_3), 0.71 (3H, d, *J* = 6.0 Hz, 5-CH_3); ^{13}C NMR (C_6D_6, 125 MHz) δ 212.6 (C, C-30), 171.6 (C, C-1), 164.1 (C, C-18), 161.5 (160.5) (CH, C-35-NC*H*O), 158.4 (C, C-3-OCONH_2), 156.5 (C, C-15), 155.7 (C, C-12), 149.9 (CH, C-20), 142.8 (C, C-10), 136.9 (CH, C-14), 136.6 (CH, C-17), 135.6 (CH, C-11), 131.4 (C, C-13), 129.9 (C, C-16), 129.1 (125.3) (CH, C-35), 114.9 (CH, C-19), 110.2 (112.4) (CH, C-34), 87.6 (CH, C-32), 82.4 (CH, C-26), 78.3 (CH, C-9), 74.3 (CH, C-24), 73.3 (CH, C-7), 71.3 (CH, C-3), 68.3 (CH, C-22), 61.2 (CH_3, C-32-OCH_3), 57.8 (CH_3, C-26-OCH_3), 57.4 (CH_3, C-9-OCH_3), 49.2 (49.3) (C, C-31), 45.7 (CH_2, C-4), 43.5 (CH_2, C-2), 43.4 (CH_2, C-6), 43.3 (CH, C-23), 42.3 (CH_2, C-29), 38.5 (CH, C-8), 38.4 (CH_2, C-21), 37.7 (38.0) (CH, C-33), 35.4 (CH_2, C-25), 35.3 (CH, C-27), 26.9 (31.9) (CH_3, C-35-NCH_3), 25.8 (CH_2, C-28), 25.6 (CH, C-5), 19.7 (19.8) (CH_3, C-33-CH_3), 19.3 (CH_3, C-5-CH_3), 15.8 (15.7) (CH_3, C-27-CH_3), 13.7 (CH_3, C-31-CH_3), 10.7 (CH_3, C-8-CH_3), 9.9 (CH_3, C-23-CH_3); HRESI-MS *m/z*: $[M+Na]^+$ 950.4589 (calcd. for $C_{47}H_{69}N_5O_{14}Na$, 950.4841).

Kabiramide C; pale yellow amorphous solid; UV (CH_3OH) λ_{max} (log ε) 234 (4.81) nm; IR (neat) ν_{max} 3435, 2090, 1651, 1645, 1497, 1463, 1423, 1319, 1292, 1016, 667, 500 cm^{-1}; ^{1}H NMR (C_6D_6, 500 MHz) δ 8.01 (1H, ddd, *J* = 16.0, 9.1, 5.1 Hz, H-20), 7.91 (7.58) (1H, s, 35-NC*H*O), 7.26 (1H, s, H-14), 7.15 (1H, s, H-17), 7.12 (1H,s, H-11), 5.96 (1H, d, *J* = 16.0 Hz, H-19), 5.85 (7.36) (1H, m, H-24), 5.80 (7.34) (1H, d, *J* = 14.5 Hz, H-35), 5.46 (1H, dd, *J* = 10.0, 10.0 Hz, H-3), 4.90 (1H, br s, H-9), 4.87 (5.0) (1H, dd, *J* = 14.5, 10.0 Hz, H-34), 4.18 (1H, ddd, *J* = 9.6, 4.9, 2.9 Hz, H-22), 3.93 (1H, dt, *J* = 7.5, 3.5 Hz, H-7), 3.50 (3H, s, 22-OCH_3), 3.39 (3.38) (3H, s, 26-OCH_3), 3.26 (3H, s, 32-OCH_3), 3.24 (1H, dd, *J* = 9.1, 2.4 Hz, H-32), 3.20 (1H, ddd, *J* = 9.0, 6.0, 2.5 Hz, H-26), 3.07 (3.08) (3H, s, 9-OCH_3), 2.71 (2H, dddd, *J* = 15.9, 5.1, 4.9, 2.4 Hz, H-21), 2.64 (2.17) (3H, s, 35-NCH_3), 2.63 (2H, overlapped, H-2), 2.62 (1H, m, H-31), 2.50 (2H, ddd, *J* = 19.3, 9.3, 2.9 Hz, H-29), 2.32 (1H, overlapped, H-8), 2.11 (1H, m, H-5), 2.10 (1H, overlapped, H-23), 2.02 (1H, m, H-33), 2.00 (2H, m, H-28), 1.99 (2H, dd, *J* = 11.7, 10.0 Hz, H-4), 1.91 (2H, ddd, *J* = 12.5, 9.3, 1.0 Hz, H-25), 1.77 (1H, m, H-27), 1.72 (2H, m, H-6), 1.01 (3H, d, *J* = 7.6 Hz, 33-CH_3), 1.00 (3H, d,

J = 6.8 Hz, CH_3-8), 0.99 (3H, d, *J* = 6.2 Hz, 23-CH_3), 0.86 (0.87) (3H, d, *J* = 6.8 Hz, 27-CH_3), 0.78 (0.73) (3H, d, *J* = 6.8 Hz, 31-CH_3), 0.77 (3H, d, *J* = 6.3 Hz, 5-CH_3); ^{13}C NMR (C_6D_6, 125 MHz) δ 212.5 (212.6) (C, C-30), 171.6 (C, C-1), 163.5 (C, C-18), 161.6 (160.5) (CH, C-35-NC*H*O), 160.4 (C, C-3-$OCONH_2$), 157.9 (C, C-15), 156.4 (C, C-12), 144.2 (CH, C-20), 142.8 (C, C-10), 136.9 (CH, C-14), 136.5 (CH, C-17), 135.6 (CH, C-11), 131.4 (C, C-13), 130.1 (C, C-16), 129.0 (125.2) (CH, C-35), 114.8 (CH, C-19), 110.2 (112.4) (CH, C-34), 87.6 (87.5) (CH, C-32), 82.3 (CH, C-26), 78.7 (CH, C-22), 78.5 (CH, C-9), 74.1 (CH, C-24), 73.2 (CH, C-7), 70.0 (CH, C-3), 61.1 (61.2) (CH_3, C-32-OCH_3), 57.8 (57.9) (CH_3, C-26-OCH_3), 57.6 (CH_3, C-9-OCH_3), 57.3 (CH_3, C-22-OCH_3), 49.1 (C, C-31), 45.3 (CH_2, C-4), 43.9 (CH_2, C-2), 43.7 (CH_2, C-6), 42.4 (CH_2, C-29), 41.5 (CH, C-23), 37.9 (CH, C-8), 37.7 (38.0) (CH, C-33), 35.2 (CH, C-27), 34.7 (CH_2, C-21), 33.9 (CH_2, C-25), 26.9 (32.0) (CH_3, C-35-NCH_3), 25.8 (CH_2, C-28), 25.3 (CH, C-5), 19.6 (19.8) (CH_3, C-33-CH_3), 19.1 (CH_3, C-5-CH_3), 15.8 (15.7) (CH_3, C-27-CH_3), 13.6 (CH_3, C-31-CH_3), 10.8 (CH_3, C-8-CH_3), 8.9 (CH_3, C-23-CH_3); HRESI-MS *m/z*: $[M+Na]^+$ 964.5458 (calcd. for $C_{48}H_{72}N_5O_{14}Na$, 964.4998).

Kabiramide G; pale yellow amorphous solid; UV (CH_3OH) λ_{max} (log ε) 247 (4.60) nm; IR (neat) ν_{max} 3367, 2945, 2833, 2595, 2227, 2044, 1651, 1449, 1417, 1115, 1031, 667 cm^{-1}; ^{1}H NMR (C_6D_6, 500 MHz) δ 8.01 (1H, dddd, *J* = 16.0, 9.2, 5.1, 2.3 Hz, H-20), 7.89 (7.56) (1H, s, 35-NC*H*O), 7.26 (1H, s, H-14), 7.15 (1H, s, H-17), 7.12 (1H,s, H-11), 6.30 (6.33) (1H, dq, *J* = 8.1, 1.4 Hz, H-32), 5.99 (1H, br d, *J* = 16.0 Hz, H-19), 5.83 (7.39) (1H, d, *J* =14.4 Hz, H-35), 5.82 (1H, ddd, *J* =9.4, 4.8, 1.8 Hz, H-24), 5.46 (1H, br dd, *J* = 9.6, 9.6 Hz, H-3), 4.87 (4.90) (1H, d, *J* = 1.3 Hz, H-9), 4.46 (4.60) (1H, dd, *J* = 14.4, 7.2 Hz, H-34), 4.16 (1H, ddd, *J* = 10.1, 4.6, 2.8 Hz, H-22), 3.92 (1H, ddd, *J* = 12.4, 4.6, 4.6 Hz, H-7), 3.49 (3H, s, 22-OCH_3), 3.34 (3H, s, 26-OCH_3), 3.19 (1H, m, H-26), 3.06 (3.07) (3H, s, 9-OCH_3), 2.90 (2.93) (1H, ddq, *J* = 8.1, 7.2, 6.6 Hz, H-33), 2.70 (2H, ddd, *J* = 15.3, 5.5, 1.8 Hz, H-21), 2.65 (2H, dd, *J* = 8.0, 7.3 Hz, H-29), 2.62 (2H, br d, *J* = 14.0 Hz, H-2), 2.60 (1H, br d, *J* = 8.7 Hz, H-27), 2.57 (3H, s, 35-NCH_3), 2.30 (1H, dqd, *J* = 12.4, 6.2, 1.3 Hz, H-8), 2.03 (1H, d, *J* = 7.0, 1.5 Hz, H-5), 2.02 (1H, br d, *J* = 7.3, 3.0 Hz, H-23), 2.00 (2H, br d, *J* = 12.8 Hz, H-4), 1.89 (2H, ddd, *J* = 15.3, 9.4, 1.8 Hz, H-25), 1.86 (1.79) (3H, d, *J* = 1.4 Hz, 31-CH_3), 1.81 (2H, m, H-28), 1.71 (2H, br d, *J* = 8.7, 4.6 Hz, H-6), 0.99 (3H, d, *J* = 6.2 Hz, 8-CH_3), 0.98 (3H, d, *J* = 7.3 Hz, 23-CH_3), 0.91 (0.95) (3H, d, *J* = 6.6 Hz, 33-CH_3), 0.87

(0.86) (3H, d, J = 7.0 Hz, 27-CH_3), 0.76 (0.77) (3H, d, J = 6.7 Hz, 5-CH_3); ^{13}C NMR (C_6D_6, 125 MHz) δ 200.9 (C, C-30), 172.6 (C, C-1), 163.5 (C, C-18), 161.6 (160.5) (CH, C-35-NC*H*O), 157.9 (C, C-3-OCONH_2), 156.4 (C, C-15), 155.7 (C, C-12), 144.1 (CH, C-20), 143.9 (CH, C-20), 142.8 (C, C-10), 136.9 (CH, C-17), 136.6 (CH, C-14), 135.9 (C, C-31), 135.6 (CH, C-11), 131.4 (C, C-13), 130.1 (C, C-16), 128.6 (124.7) (CH, C-35), 114.8 (CH, C-19), 111.7 (113.9) (CH, C-34), 82.4 (CH, C-26), 78.7 (CH, C-22), 78.6 (CH, C-9), 74.1 (CH, C-24), 73.2 (CH, C-7), 70.0 (CH, C-3), 57.9 (CH_3, C-26-OCH_3), 57.7 (CH_3, C-9-OCH_3), 57.3 (CH_3, C-22-OCH_3), 49.1 (C, C-31), 45.4 (CH_2, C-4), 43.9 (CH_2, C-6), 43.7 (CH_2, C-2), 41.5 (CH, C-23), 38.5 (CH, C-8), 35.7 (CH_2, C-29), 35.0 (CH, C-27), 34.7 (CH_2, C-21), 34.5 (34.6) (CH, C-33), 33.7 (CH_2, C-25), 27.4 (CH_2, C-28), 26.9 (33.7) (CH_3, C-35-NCH_3), 25.3 (CH, C-5), 20.9 (20.6) (CH_3, C-33-CH_3), 19.1 (CH_3, C-5-CH_3), 15.9 (15.6) (CH_3, C-27-CH_3), 11.8 (11.7) (CH_3, C-31-CH_3), 10.8 (CH_3, C-8-CH_3), 8.9 (CH_3, C-23-CH_3); HRESI-MS *m/z*: $[M+Na]^+$ 932.5438 (calcd. for $C_{47}H_{68}N_5O_{13}Na$, 932.4735).

Note: Chemical shifts of the minor conformers are represented in parentheses.

2.4 Chemical profiling in *Penares nux*

2.4.1 Sample preparations

Each sponge colony was cut into different parts (Figure 2) and all covering organisms on the surface were removed before freeze-drying. Each part from each colony was extracted and quantified independently and separately. The dry sponge tissues were refluxed with 15 mL of THF for 1 hour and rinsed with 3 × 5 mL THF. The extract was evaporated and stored at -20°C until determination of the major chemical markers. Each dry extract was dissolved in CH_3CN and diluted to get a concentration of 500 µg/mL. This was filtered through a 0.45-µm membrane filter, and subjected to the quantification without further pre-chromatographic treatment.

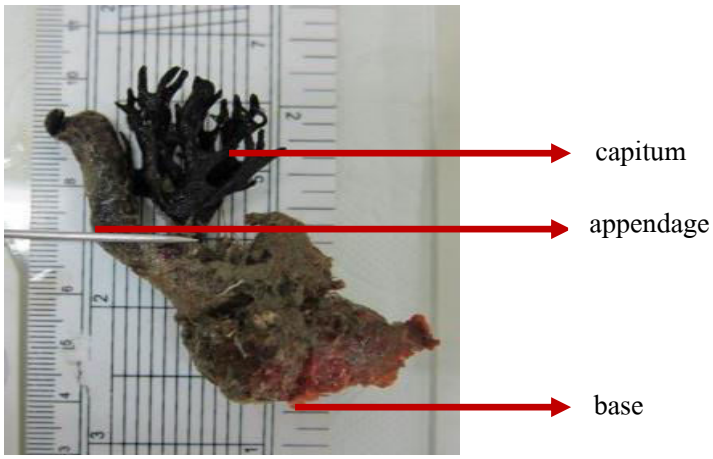


Figure 2. Upon surfacing appearance of sponge *Penares nux* showing capitum, appendage and base parts.

2.4.2 Standard preparations

Kabiramides B, C, and G were selected as chemical markers throughout this investigation due to the abundance, structural variation, and resolution within an appropriate chromatographic condition. The standards were obtained in-house according to the isolation protocol described in 2.3. The purity of each chemical marker was referred to spectroscopic authentication from which the signals of possible impurities are unable to be observed. The stock solution of reference standard was prepared by accurately weighed and dissolved in 1% (v/v) aqueous AcOH/CH_3CN to give a concentration of 100 µg/mL. Stock solutions were further diluted with 1% (v/v) aqueous AcOH/CH_3CN to prepare different concentrations of kabiramide standard solutions. 30 – 0.01 µg/mL for kabiramide C, and 5 – 0.03 µg/mL for kabiramides B and G, respectively. LOQ for kabiramides B, C and G were 0.03, 0.01, and 0.03 µg/mL, respectively.

2.4.3 LC-MS based quantification of kabiramide contents

The LC-ESI-MS/MS based quantification was performed on an RP-C18 column (Vertical VertiSep^{TM}UPS, 5 µm, 150 × 2.1 mm). The chromatographic conditions were as followed; 1% aqueous AcOH/CH_3CN 45:55, flow rate 0.8 mL/min, 45 °C, 20-µL injection, and ESI detection. Determination of kabiramides B, C, and G content was referred to the peak area (t_R = 5.3, 6.8, 8.3 min, respectively) obtained directly from each total ion chromatograms and MS base peaks at *m/z* (928.6, 942.7, 899.6) respectively. Each sample was subjected to the devised

chromatographic condition (all triplicates). The concentration was determined from a calibration curve plotted between peak area and concentration. The contents were calculated on sponge dry weight basis (mg/g).

2.4.4 Validation of analytical method

Prior to the quantification, the devised analytical method was examined and validated for the linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) according to the guideline by the International Conference on Harmonization (ICH, 2005).

2.4.4.1 Linearity

Standard kabiramide C was diluted with 1% aqueous AcOH/CH_3CN to a range of concentrations 30 – LOQ µg/mL. Standard kabiramides B and G were performed at a concentration range of 5 – LOQ µg/mL (all in triplicate). The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

2.4.4.2 Precision

Intra-day precision was performed by injecting in triplicates, on the same day, three different concentrations of kabiramide C (0.5, 10, and 30 µg/mL) and kabiramides B and G (0.06, 1, and 5 µg/mL). Inter-day was determined by analyzing in triplicate, the same solutions employed in intra-day precision on three consecutive days. Precision was expressed as relative standard deviation (RSD) of retention times and peaks areas for kabiramides B, C and G.

2.4.4.3 Accuracy

Kabiramide C at the concentrations of 0.5, 10, and 30 µg/mL, and kabiramides B and G at the concentrations of 0.06, 1, and 5 µg/mL were spiked into sample solutions. Prior to analysis, the background levels of kabiramides B, C and G in the extract were determined in order to calculate actual recoveries. The amount of each analyte was determined in triplicate.

2.4.4.4 Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by means of signal to noise ratio of 3:1 and 10.1 respectively. All calculated LOD and LOQ concentrations were confirmed by subjecting prepared standard solution to devised method.

2.5 Microbial profiling in *P. nux*

2.5.1 Animal material

The specimens of the sponge *P. nux* were separately collected on a single colony basis from Koh Tao, Surat-Thani Province, in May 2012. The sponge was identified and authenticated as described in 2.2. The same specimens used for chemical profiling was also used for the microbial profiling. The specimens are preserved in an ice chest (0°C) upon surfacing, and immediately processed in the laboratory for bacterial isolation.

2.5.2 Isolation, enumeration, and culture of sponge associated bacteria

Prior to analysis, each sponge specimen was thoroughly rinsed with sterile seawater to remove loosely attached bacteria. Swabbed surface method was used for the isolation and enumeration of marine bacteria. The surface of each sponge specimen was swabbed with a sterile cotton swab by way of placing a sterile plastic film having a hole of 1×1 cm^2 on the sponge surface. The cotton swab was then soaked in 2 mL of sterile seawater, and vortexed. Ten-fold serial dilutions of each solution were prepared and an aliquots of 100 µL from each dilution was plated on marine agar 2216 (Difco) (triplicate). Plates were incubated for 7 days at 25^oC. Colony-forming units (CFU) were counted and the average number of bacteria referred to the swabbed area (CFU/cm^2).

All colonies with different pigmentation and morphology were picked out. Single colony of each isolate were inoculated on marine agar slant and glycerol stocks and stored at 4˚C and -80˚C, respectively, as stock cultures (Chelossi et al., 2004). The purpose of this experiment is to cultivate surface microbial community from the sponge.

2.5.3 DNA extraction

Pure colonies were picked using sterilized inoculating loop and suspended in 50 µL sterile distilled water. The suspension was heated for 20 min at 110˚C, followed by centrifugation at 4000 *g* for 2 min. The DNA in the resulting lysate was purified by extracting with 600 µL chloroform-isoamyl alcohol (24:1). The aqueous phase was precipitated with 0.6 volume of isopropyl alcohol and centrifuged at 15,000 *g* for 10 min. The pellet was washed with 70% ethanol, air dried and resuspended in 30 µL ultrapure DNase/RNase-free distilled water. DNA integrity was assessed by agarose gel electrophoresis (Lee et al., 2002; Dupont et al., 2013).

2.5.4 PCR amplification

The PCR was performed in a total volume of 100 µL containing 2 µL of DNA mixture, 5 µL of 10 µM forward (20F) and reverse (1540R) primers, 8 µL of 25 mM $MgCl_2$, 10 µL of 10mM dNTP, 0.5 µL 5 unit Taq polymerase, 10 µL of 10mM PCR buffer, and 64.5 µL of MilliQ water. A DNA Thermal cycler (Biometra) was used with a temperature profile of 3 min at 94^{o}C followed by 30 cycles of 1 min at 94^{o}C (denaturing of DNA), 1 min at 50^{o}C (primer annealing), and 2 min at 72^{o}C (polymerization) and a final extention for 3 min at 72^{o}C. The PCR amplified products were analyzed by running 5 µL of the reaction mixture on a 0.8% agarose gel electrophoresis in Tris-borate EDTA buffer. Agarose gel was stained with SYBR® safe DNA gel stain and examined under UV-transilluminator (Syngene bio-imaging) to visualize the amplified 16S rDNA band. The PCR mixtures were purified using favor Prep™ PCR kit (Favorgen Biotech Corp). The expected base pair was 1500bp.

2.5.5 16S rRNA sequencing and phylogenetic analysis

The purified 16S rDNA was used as the template for sequencing with big dye terminator sequencing ready reaction kit (Applied Biosystem) and analysed by ABI 377 automated DNA sequencer (Perkin Elmer)**.** The sequencing reaction for each sample was performed in a DNA Thermal cycler followed by 25 cycles of 10 secs at 96^{o}C (denaturing of DNA), 5 sec at 50^{o}C (primer annealing) and 4 min at 60^{o}C (polymerization). Sequencing for each

sample was carried out in both forward and reverse direction with the following primers: 27F (5´AGAGTTTGATCCTGGCTCAG 3´) and 1492R (5' GGTTACCTTGTTACGACTT 3').

The values for sequence similarity to all recognized species were first defined using Ez Taxon-e database (Chun et al., 2007) and was performed using the standard BLAST sequence similarity program version 2.2.1 against previously reported sequences at the Genbank/EMBL databases (Thompson et al., 2011). The sequence was then aligned with the selected sequences obtained from the databases using CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods in the MEGA 5 software (Taimura et al., 2011). The confidence values of branches of the phylogenetic tree were determined using the boost strap analyses which are based on 1000 resamplings.

2.5.6 Preparation of the bacteria crude extracts

The isolated surfaced-attached bacteria were randomly selected and eight of the isolates were cultured in 300 mL marine broth (Difco) using 500 mL Erlenmeyer flasks. Flasks were incubated on a rotatory shaker at 220 rpm for 7 days at 25°C. The broth was first centrifuged at 5000 *g* for 30 min to remove the cells, and then extracted 3 times with 100 mL EtOAc. After solvent removal under vacuum by using a rotary evaporator, the extracts were used as the crude samples for antimicrobial activity.

2.5.7 Screening of antimicrobial activity of isolated bacteria crude extracts

Eight of the isolated marine bacteria (PR03, PR04, PR07, PR08, PR09, PR10, PR16, and PR17) were screened for antimicrobial activity, using four pathogenic microorganisms such as *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* TISTR 517, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 10145. These strains were kindly provided by Dr. Sukanya Dej-adisai, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Antimicrobial activity was assayed in triplicate using agar disc diffusion method. The discs (6 mm in diameter) were

prepared using 10 μL of crude extract diluted in EtOAc to a concentration of 50 mg/mL; thus each disc contained 0.5 mg of crude extract (500 μg/disc). The discs were placed onto the Mueller Hinton agar (MHA) surface containing the test microorganisms, and incubated at 37°C for 24 hours. Oxacillin (1 μg/disc), vancomycin (30 μg/disc), norfloxacin (10 μg/disc), and tetracycline (30 μg/disc) were used as positive control. The diameters of any inhibition zones formed around the paper discs were then measured using vernier caliper.

2.6 Statistics

The comparison of the trisoxazole macrolide content (mg/g sponge dry weight) among the different parts of the sponge colony was determined using Kruskal-Wallis test (SPSS16.0 software). Kruskal-Wallis test was also used to compare the bacteria populations between the different parts of the sponge. In order to establish if there is any qualitative relationship between the trisoxazole macrolides and the microbial populations, Spearman's rank correlation coefficient was employed to test correlations between the concentrations of kabiramides and the bacterial CFU counts.

CHAPTER 3

RESULTS

The sponge *Penares nux* has two growth forms, a protruding gorgonian-like capitum and an irregular-shaped base buried in sediment. Occasionally, the sponge is also found to grow an appendage extending from its base part. Previous studies revealed the significant differences in the accumulation profiles of the toxic kabiramides between the capitum and the base parts (Sirirak et al., 2011a). In this study, the relationship between the microbial population and the production and allocation of trisoxazole macrolides in the sponge *P. nux* using LC-MS-MS and swabbed-surface CFU counts was investigated. In this chapter, the results are divided into two parts. The first part involves the chemical profiling of the toxic trisoxazole macrolides between two geographical sites using the LC-MS-MS analytical protocol. The second one consists of the microbial profiling in *P. nux*, the relationship between the microbial population and the chemical profiles of the macrolides in the sponge *P. nux*, and the microbial diversity on the sponge surface.

3.1 Isolation of chemical markers from the sponge *Penares nux*

The sponge *Penares nux* was collected in May 2012 from Koh-Tao, Surat-Thani Province. The freeze-dried sponge (312 g) was macerated exhaustively (500 mL × 5) in CH_3OH. The dried CH_3OH extract was partitioned in hexane, CH_2Cl_2, and *n*-BuOH to yield extract from each solvent weighing 3.2, 9.4, and 2.1 g, respectively. The CH_2Cl_2-extract was fractionated using a protocol reported by Sirirak et al (2011b) to yield kabiramides B, C, and G (83, 220, and 75 mg, respectively). The purities of the isolated compounds were confirmed and referred to the NMR spectra, in which no remarkable impurity was observed and the authenticity of the isolated markers was compared with the published data (Figures 3-8; Matsunaga et al., 1986; Matsunaga et al., 1989; Petchprayoon et al., 2006).

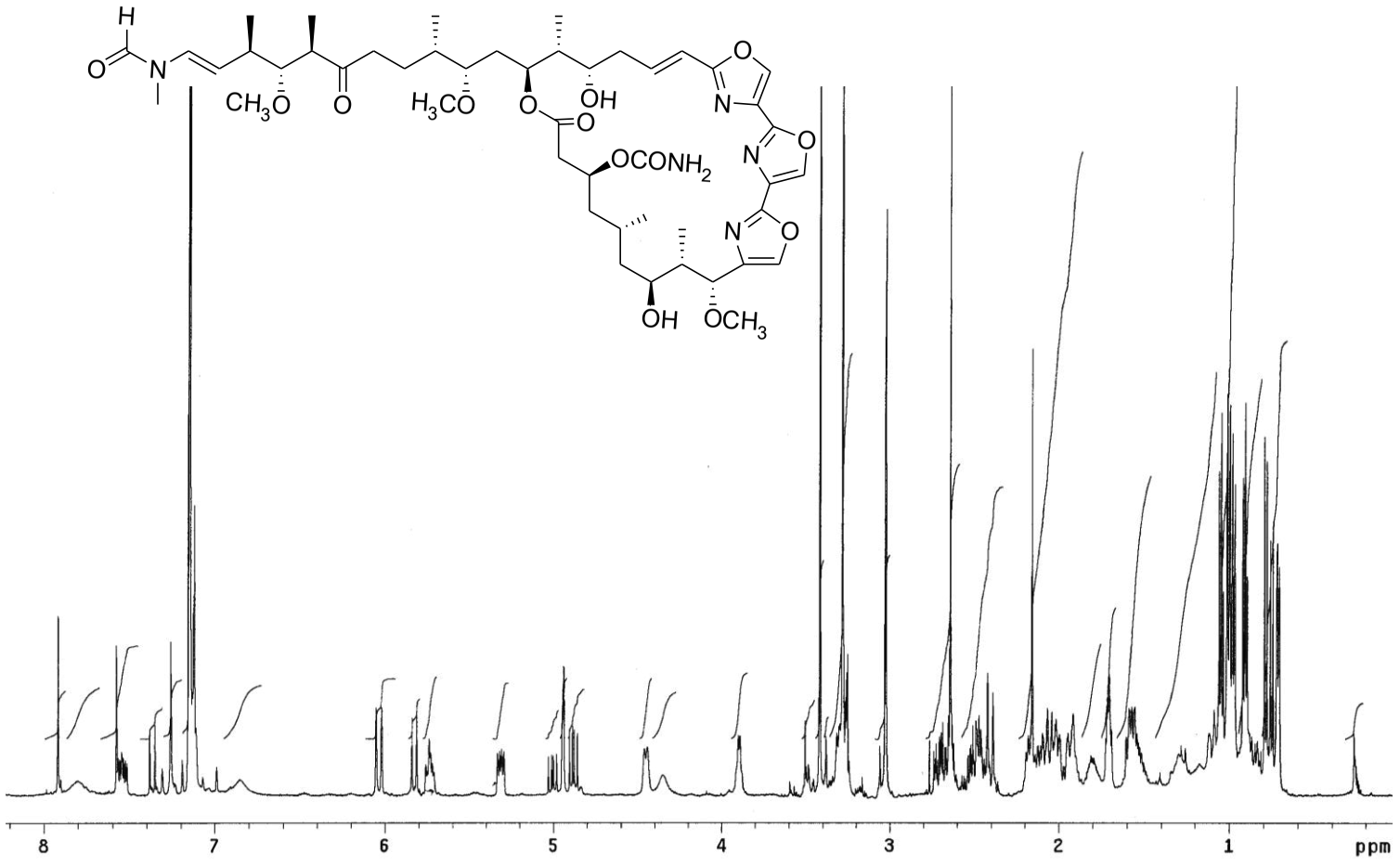


Figure 3. ^{1}H NMR spectrum of kabiramide B (500 MHz, C_6D_6).

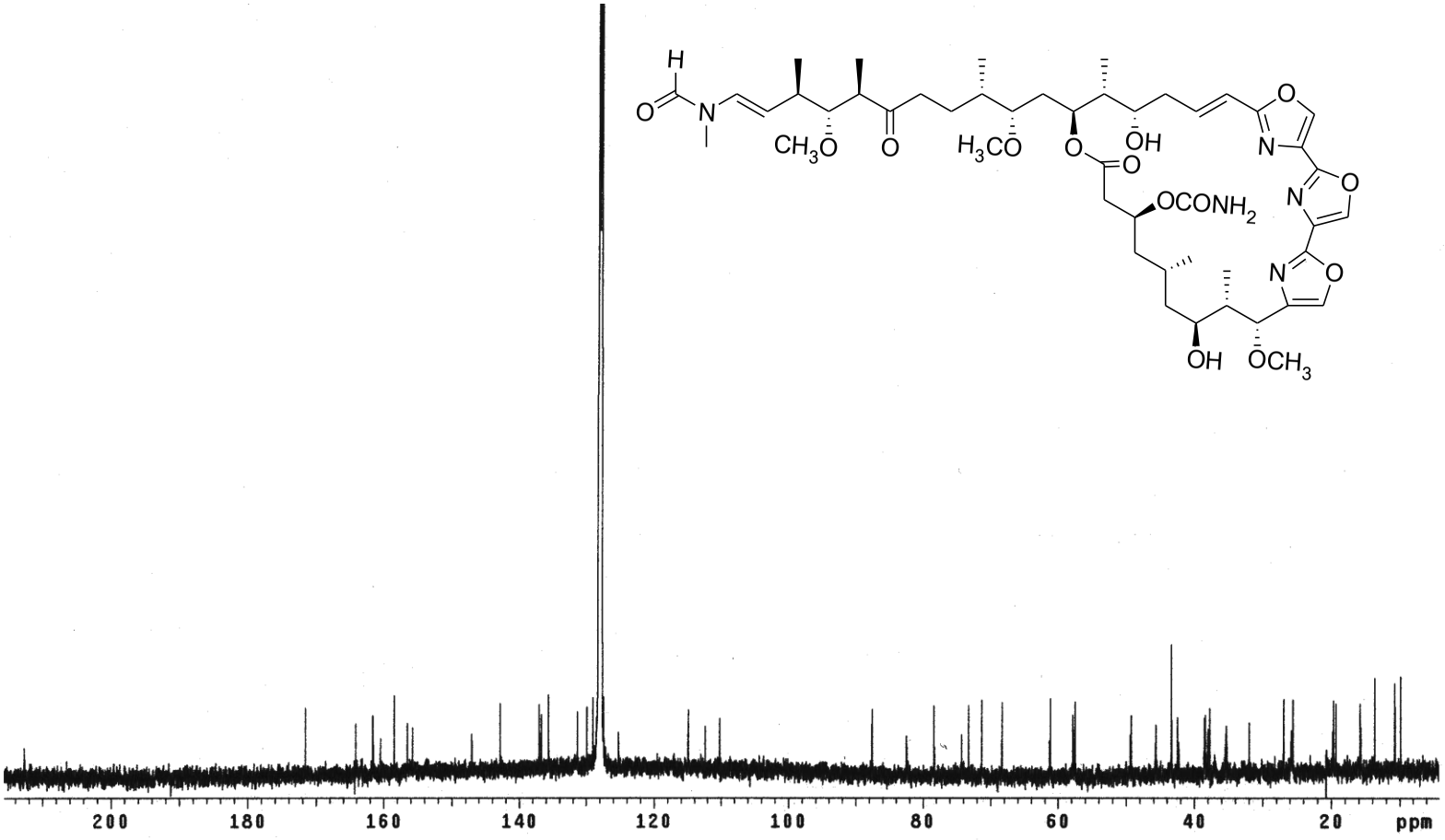


Figure 4. ^{13}C NMR spectrum of kabiramide B (125 MHz, C_6D_6).

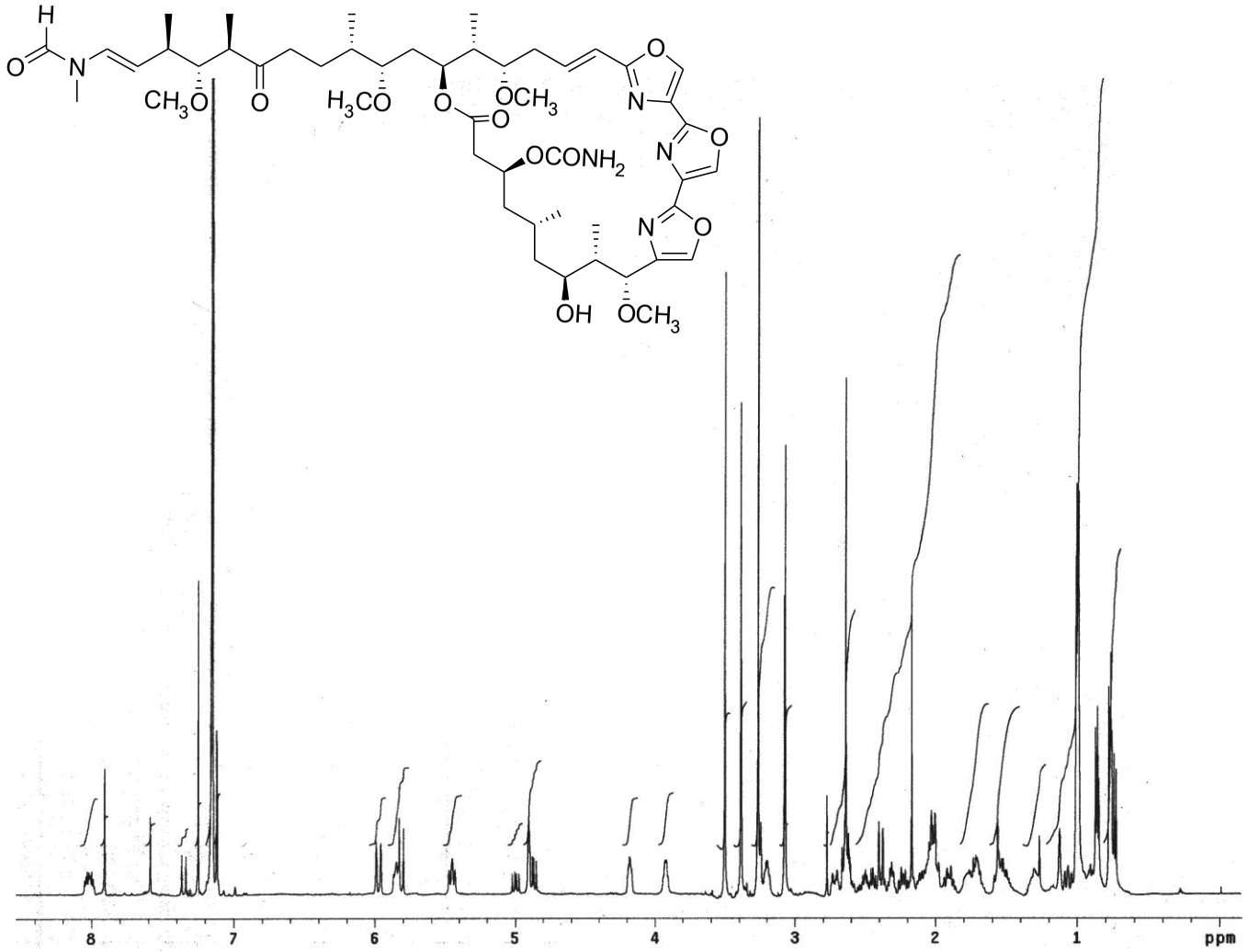


Figure 5. ^{1}H NMR spectrum of kabiramide C (500 MHz, C_6D_6).

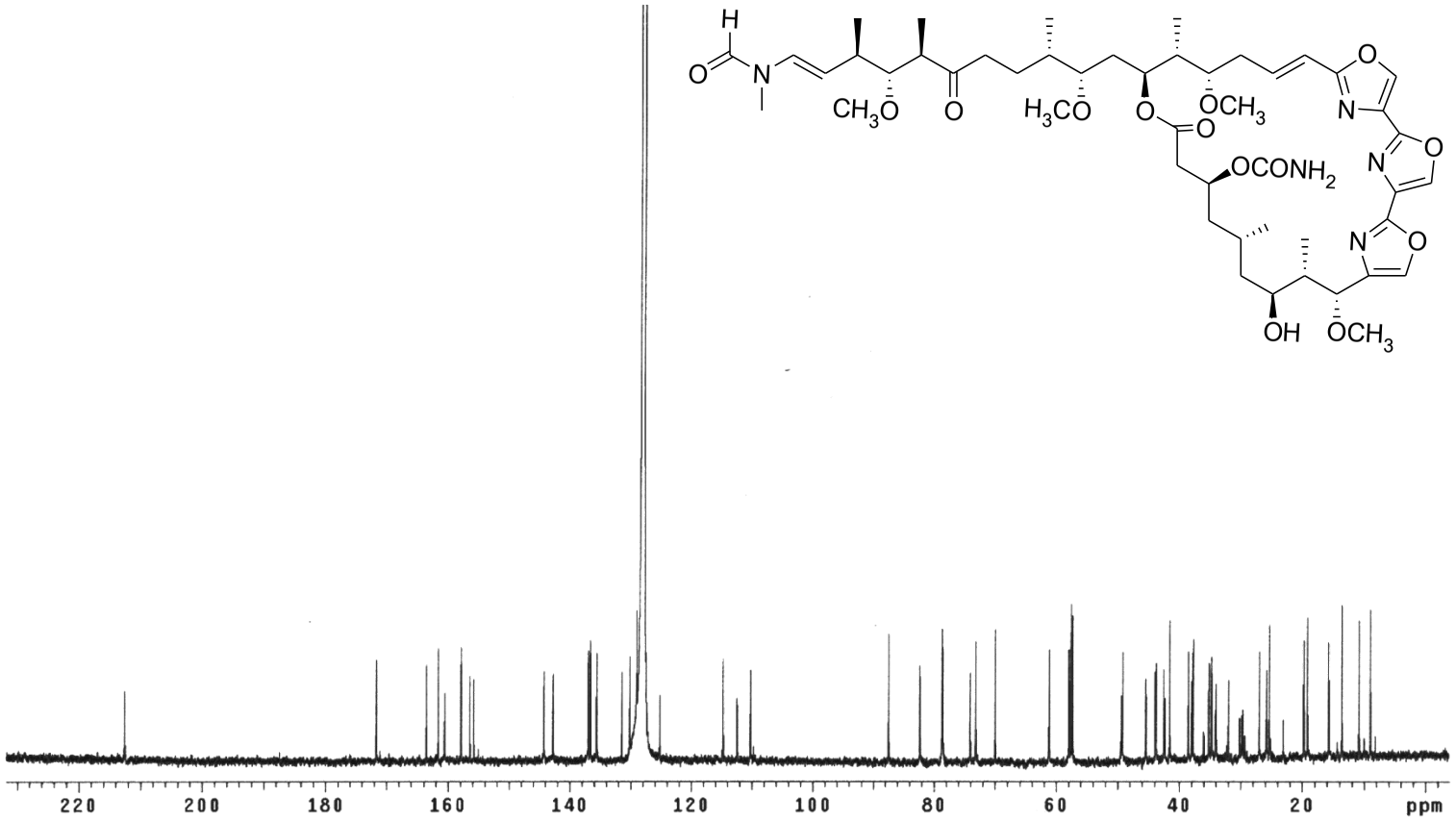


Figure 6. ^{13}C NMR spectrum of kabiramide C (125 MHz, C_6D_6).

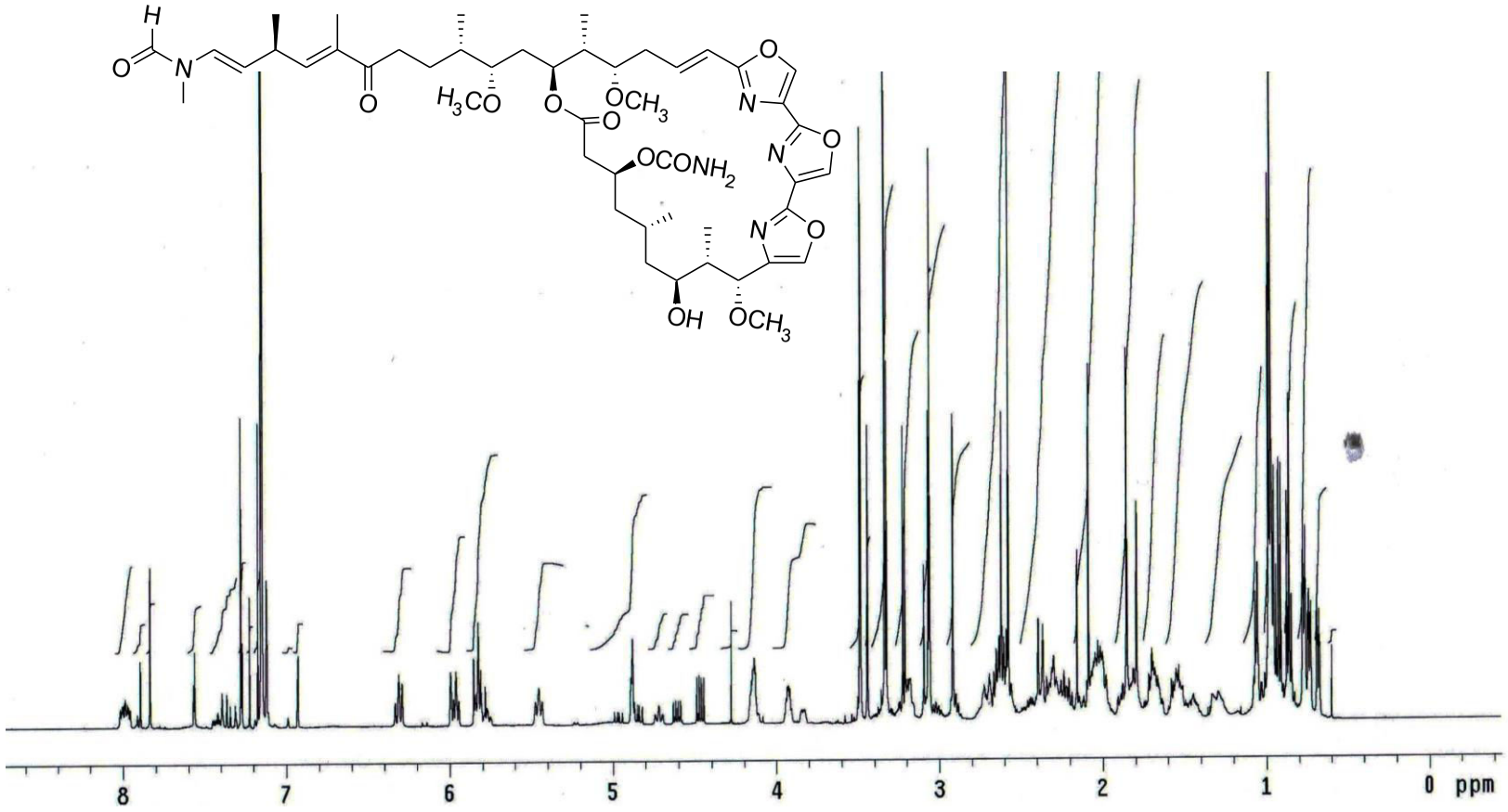


Figure 7. ^{1}H NMR spectrum of kabiramide G (500 MHz, C_6D_6).

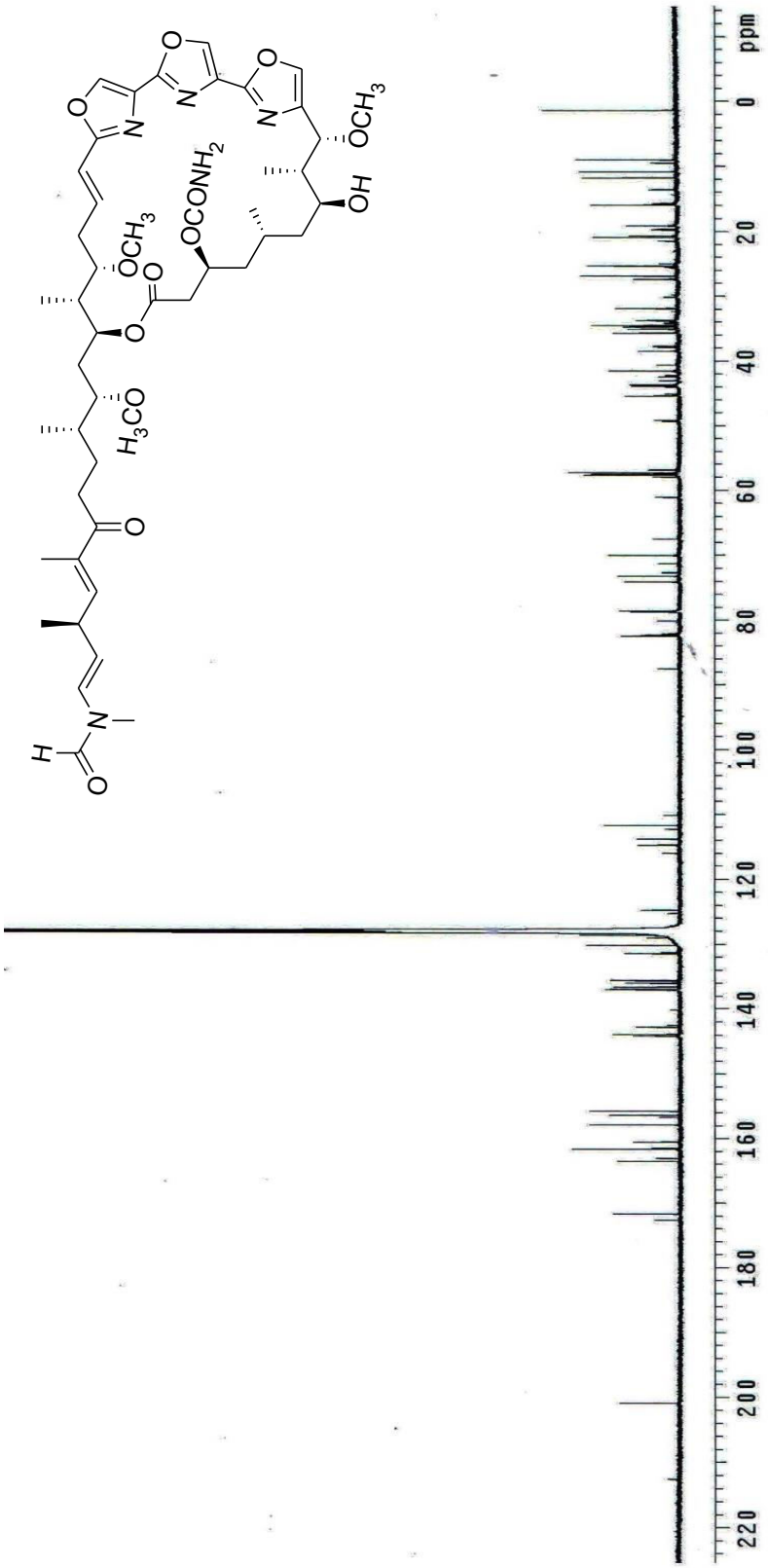


Figure 8. ^{13}C NMR spectrum of kabiramide G (125 MHz, C_6D_6).

3.2 Quantification of kabiramides contents in the sponge *P. nux*

An LC-MS-MS method was modified from a protocol by Sirirak et al (2011a) for the quantification of kabiramides B, C, and G in *P. nux* crude extract. The chromatographic conditions were as followed; 1% aq. AcOH/CH_3CN 45:55, flow rate 0.8 mL/min, 45°C, ESI positive mode detection. Determination of kabiramides B, C and G content was referred to the peak area (t_R = 5.3, 6.8, and 8.3 min, respectively) obtained directly from each total ion chromatograms and MS base peaks at *m/z* 928.6 $[MH]^+$, 942.7 $[MH]^+$, and *m/z* 899.6 $[M + Na - CH_4O]^+$ for kabiramide B, C and G, respectively. Notice that, for kabiramide G, the pseudomolecular peaks either at *m/z* 910 $[MH]^+$ or 932 $[M + Na]^+$ were not pronounced, and the mass of 899.6 was chosen as base peak instead. The modified analytical protocol gave a satisfactory resolution and chromatographic clarity with reasonable retention times and well separated peaks (Figure 9a).

Upon achieving the newly modified quantification protocol, it was validated for linearity, precision, accuracy, LOD and LOQ according to the ICH guidelines.

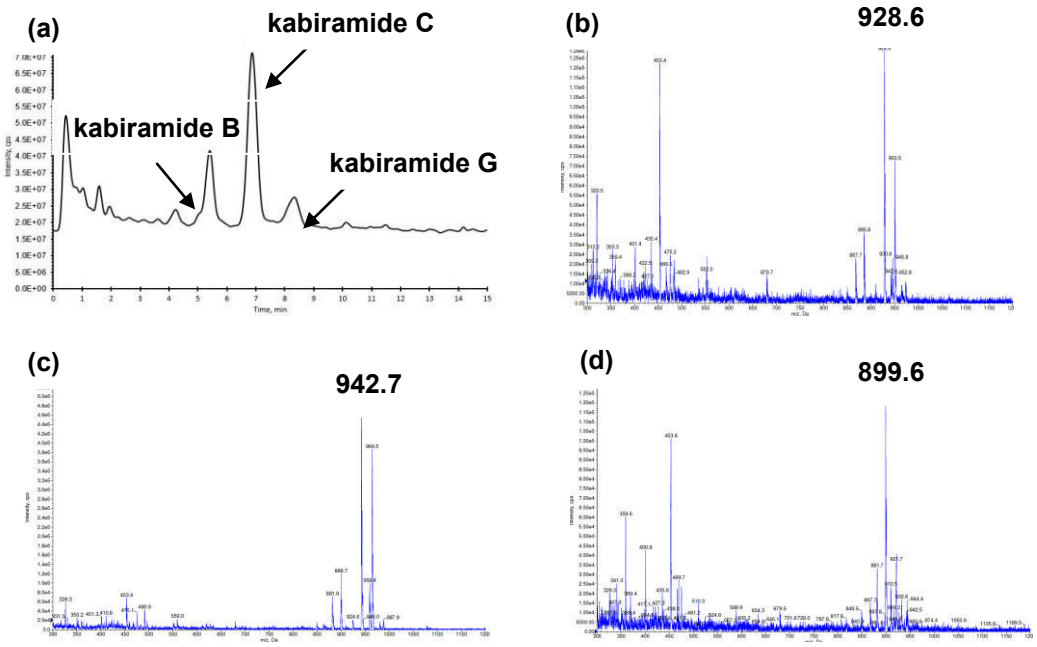


Figure 9. (a) LC-MS-MS chromatograms of *P. nux* crude extracts at a concentration of 500 µg/mL (b) mass spectrum of kabiramide B, (c) mass spectrum of kabiramide C, (d) mass spectrum of kabiramide G.

3.2.1 Linearity

The linearity of the three chemical markers was evaluated over a range of 0.01 – 30 µg/mL (kabiramide C), and 0.03 – 5 µg/mL (kabiramides B and G). The lower limits of linearity as shown were referencing the calculated LOQ (*vide infra*). Good linearity was met for all compounds over the evaluated ranges. The regression plots were expressed as $A = (2.6 \times 10^6)c + 48743$ ($r^2 = 0.9999$), $A = (3.88 \times 10^6)c + 72636$ ($r^2 = 0.9999$), and $A = (7.27 \times 10^6)c + 19003$ ($r^2 = 0.9998$) for kabiramides B, C and G, (where A = peak area and c = concentration in µg/mL), respectively.

3.2.2 Precision

The precision was assessed at three concentration levels that cover the linearity range of the devised analytical protocol. An excellent reproducibility for retention time and peak area was met for all the three kabiramides (Table 4). The intra-day and inter-day % RSD values were less than 5%. The results met the acceptance criterion for % RSD specified by the ICH (ICH, 2005).

Table 4. Intra-day and inter-day precision data

Compounds	concentration (µg/mL)	% RSD intra-day (n=3)		% RSD inter-day (n=9)	
		t_R	peak area	t_R	peak area
kabiramide B	0.06	0.30	0.95	1.04	4.03
	1	0.37	2.00	1.94	3.40
	5	2.38	1.49	2.72	1.73
kabiramide C	0.5	1.55	0.04	0.75	3.60
	10	1.80	0.07	0.77	2.08
	30	0.98	0.13	0.68	3.44
kabiramide G	0.06	0.04	0.65	2.26	0.79
	1	0.01	1.85	1.91	1.48
	5	0.12	1.03	2.63	0.89

Note: t_Rs= 5.3, 6.8, and 8.3 min, for kabiramides B, C, and G, respectively.

3.2.3 Accuracy

The accuracy of the analytical method was assessed by spiking technique over three different concentration levels covering the target range. Kabiramides B, C, and G were spiked into *P. nux* crude extract sample to evaluate recoveries of the standard compounds. The recovery data obtained showed a good accuracy with the recovery percentages in the ranges of 99.3-100.3, 98.7-100.6, and 98.7-101.8% for kabiramides B, C, and G, respectively (Table 5).

Table 5. Recovery of kabiramides B, C and G

compounds	spiked concentration (µg/mL)	% recovery (n=3)
kabiramide B	0.06	100.31±0.61
	1	99.36±1.01
	5	99.46±1.08
kabiramide C	0.5	98.70±1.3
	10	99.62±2.2
	30	100.65±1.2
kabiramide G	0.06	98.75±1.79
	1	100.95±0.79
	5	101.87±0.92

3.2.4 LOD and LOQ

LOD and LOQ were determined by means of signal-to-noise ratios of 3:1 and 10:1 respectively. In this study, the LOD values were 0.008, 0.004, and 0.01 µg/mL for kabiramides B, C and G respectively, whereas the LOQ values were 0.03, 0.01, and 0.03 µg/mL for kabiramides B, C and G, respectively.

3.3 The extraction and quantification of kabiramides in the sponge *P. nux*

As stated previously, the sponge specimens investigated in this study were collected from two collecting locations, Koh-Tao, Surat-Thani Province, and Saiburi District, Pattani Province. The specimens from both locations can be divided according to the growth forms into capitum, appendage, and base parts. The dry weights and extraction yields of the sponge specimens in each part from each location are summarized in Table 6.

Table 6. Specimen dry weights and extraction yields of the capitum, appendage, and base parts of the sponge *P. nux* collected from Koh-Tao and Saiburi

	dry weight range (mean±SD; mg)	extraction yield range (mean±SD; mg/g dry sponge)
# Koh-Tao		
capitums	22 - 365	0.03 - 0.20
(N = 27)	(148.7±103.39)	(0.08±0.04)
appendages	274 - 645	0.01 - 0.07
(N = 26)	(400.06±97.41)	(0.04±0.01)
bases	453 - 2,011	0.01 - 0.03
(N = 42)	(1049.84±374.11)	(0.013±0.005)
# Saiburi		
capitums	379 - 755	0.01 - 0.22
(N = 15)	(567±95.16)	(0.02±0.002)
appendages	423 - 693	0.01 - 0.03
(N = 5)	(511.56±149.73)	(0.02±0.004)
bases	837 - 1,506	0.006 - 0.01
(N = 20)	(1173.41±202.16)	(0.008±0.001)

It must be noted here that the sponge specimens studied in this investigation do not necessarily have all the three growth forms developed in each individual colony. For the sponges collected from Koh-Tao, 16 colonies have only capitum and base parts, 15 have appendage and base, and 11 colonies have all the three parts. As for the samples from Saiburi, 15 specimens have capitum and base, and five have appendage and base. The colonies that developed all three parts were scarce in this location and the collection failed to yield such colonies. The sizes of sponge parts from all the collected specimens range widely on the borderline of being non-parametric distribution. However, the extraction yields from each part, calculated per location basis, were consistent, and were not different statistically (p = 0.106, 0.264, 0.124 for capitum, appendage and base parts, respectively).

Upon obtaining the extracts from each part of the sponge *P. nux*, the quantification of the three markers, kabiramides B, C, and G, in each part of the sponge were carried out (Table 7). Note that, due to the non-parametric nature of the data, range and median were used to present the kabiramide contents, and Kruskal-Wallis *H* analysis was used to determine the differences within the data sets.

Table 7. Kabiramide contents in the sponge *P. nux*.

sponge specimens	ranges and medians of kabiramide contents (mg/g dry sponge)		
	kabiramide B	kabiramide C	kabiramide G
# Koh-Tao			
capitums	0.06 - 1.93	0.04 - 4.26	0.07 - 2.50
(N = 27)	(0.48)	(0.97)	(0.23)
appendages	0.04 - 1.24	0.07 - 1.87	0.02 - 0.49
(N = 26)	(0.16)	(0.29)	(0.08)
bases	0.001 - 0.18	0.001 - 0.28	0.001 - 0.12
(N = 42)	(0.02)	(0.04)	(0.01)
Kruskal-Wallis *H*	62.645	65.298	63.671
	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)
# Saiburi			
capitums	0.04 - 0.39	0.02 - 0.51	0.03 - 0.26
(N = 15)	(0.15)	(0.21)	(0.04)
appendages	0.06 - 0.17	0.03 - 0.13	0.01 - 0.06
(N = 5)	(0.09)	(0.1)	(0.04)
bases	0.008 - 0.04	0.002 - 0.07	0.002 - 0.04
(N = 20)	(0.02)	(0.02)	(0.001)
Kruskal-Wallis *H*	28.768	23.099	24.502
	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)

Throughout the investigated specimens, the contents of each kabiramide marker among the three parts of the sponge are significantly different. Specifically, the capitum part accumulates the highest amount of each kabiramide analog, followed by the appendage and

base parts, respectively. The trend that kabiramide contents are highest in the capital part, then appendage and base, is consistent and parallel among the specimens from both collecting locations. However, when the contents of each kabiramide from two locations are compared, certain variation exists. With an exception of the contents of kabiramide B in the appendage and base parts, the sponge specimens collected from Koh-Tao have the higher contents of most kabiramide markers in each part (Table 8).

Table 8. Site comparison of the kabiramide contents (Kruskal-Wallis test *H*)

	capitum		appendage		base	
Compounds	*H*	*P* value	*H*	*P* value	*H*	*P* value
kabiramide B	11.915	0.001	3.141	0.076	0.018	0.892
kabiramide C	22.202	< 0.001	8.103	0.004	5.206	0.023
kabiramide G	20.982	< 0.001	6.104	0.013	4.042	0.044

3.4 Microbial profiling

This part of investigation consists of the bacterial enumeration based on the CFU count of the surface-attached bacteria from each part of the sponge *P. nux*, and the diversity of the culturable bacteria associated with the sponge. Due to the logistic limitation, the specimens from Saiburi location were preserved in such a way that the integrity and viability of the sponge-associated bacteria from this location were unable to be maintained. Only the enumeration and diversity of bacteria associated with the specimens from Koh-Tao (N = 95) are reported here.

3.4.1 Enumeration of sponge attached bacteria from *P. nux*

All the 95 specimens of the sponge *P. nux* from Koh-Tao expedition were first subjected to the bacterial enumeration experiment prior to transferring to the kabiramide content determination reported in section 3.2. The bacterial enumeration was done using swabbed-surface CFU counts (Chelossi et al., 2004). The results were also categorized based on the parts of the sponge colonies parallel to those reported in section 3.3 (Table 6). Also similar to the results on kabiramide contents, the bacterial counts on the surfaces of the sponge specimens were non-parametric, and Kruskal-Wallis test *H* statistics was used here. A significant difference among the

bacterial counts from the surfaces of the three parts of the sponge was observed. The bacterial counts from the surfaces of base part were higher than those from the appendage and capitum.

Table 9. CFU counts from the three parts of the sponge *P.nux*.

sponge specimens	ranges and medians of CFU counts (CFU/cm^2) $\times 10^6$
capitums	6.67 – 115.33
(N = 27)	(9.93)
appendages	6.60 – 23.83
(N = 26)	(9.03)
bases	10.17 – 161.67
(N = 42)	(24.85)
Kruskal-Wallis *H*	52.584
	($P < 0.001$)

3.4.2 Phylogenetic analysis of bacteria isolated from the surfaces of *P. nux*

Upon achieving the enumeration of sponge surface-associated bacteria, 22 bacterial isolates were selected based on the distinctive colonial morphology. All were subjected to 16S rRNA sequencing and phylogenetic analysis (Figure 10). The 16S rRNA gene sequence of all the 22 isolates were deposited to GenBank/EMBL/DDBJ under accession numbers LC020211-LC020232. The identification of all the isolates (Table 10) indicated that they fall into 13 distinctive bacterial species. Among these, two are of the division Actinobacteria, belonging to the families Micrococcaceae and Dermacoccaceae; seven are Gammaproteobacteria, belonging to the families Halomonadaceae, Idiomarinaceae, Pseuldoalteromonadaceae, Alcanivoracaceae, and Moraxellaceae; three are Alphaproteobacteria, belonging to the families of Sphingomonadaceae, Rhodobacteraceae, and Aurantimonadaceae; and one is Flavobacteria, belonging to the family Flavobacteriaceae.

Table 10. 16S rRNA gene sequence analysis of sponge surface-attached bacteria from *P. nux*

Isolates	Deposited accession number	Closest relative in database	% Identity	Division (Family)
PR01	LC020212	*Salinicola salarius* M27	99.5	Gammaproteobacteria (Halomonadaceae)
PR02	LC020213	*Erythrobacter citreus* RE35F/1	99.1	Alphaproteobacteria (Sphingomonadaceae)
PR03	LC020214	*Erythrobacter citreus* RE35F/1	100	Alphaproteobacteria (Sphingomonadaceae)
PR04	LC020211	*Paracoccus zeaxanthinifaciens* ATCC 21588	98.2	Alphaproteobacteria (Rhodobacteraceae)
PR05	LC020215	*Idiomarina donghaiensis* 908033	99.7	Gammaproteobacteria (Idiomarinaceae)
PR06	LC020216	*Psychrobacter pacificensis* NIBH P2K6	98.7	Gammaproteobacteria (Moraxellaceae)
PR07	LC020217	*Halomonas aquamarina* DSM30161	100	Gammaproteobacteria (Halomonadaceae)
PR08	LC020218	*Pseudoalteromonas paragorgicola* KMM3548	100	Gammaproteobacteria (Pseudo-alteromonadaceae)
PR09	LC020219	*Kocuria palustris* DSM 11925	100	Actinobacteria (Micrococcaceae)
PR10	LC020220	*Vitellibacter vladivostokensis* KMM 3516	99.7	Flavobacteria (Flavobacteriaceae)
PR11	LC020221	*Alcanivorax dieselolei* B-5	99.8	Gammaproteobacteria (Alcanivoracaceae)

Table 10. (cont.)

Isolates	Deposited accession number	Closest relative in database	% Identity	Division (Family)
PR12	LC020222	*Kytococcus sedentarius* DSM 20547	99.6	Actinobacteria (Dermacoccaceae)
PR13	LC020223	*Aurantimonas coralicida* DSM 14790	100	Alphaproteobacteria (Aurantimonadaceae)
PR14	LC020224	*Salinicola salarius* M27	99.5	Gammaproteobacteria (Halomonadaceae)
PR15	LC020225	*Salinicola salarius* M27	100	Gammaproteobacteria (Halomonadaceae)
PR16	LC020226	*Idiomarina donghaiensis* 908033	100	Gammaproteobacteria (Idiomarinaceae)
PR17	LC020227	*Halomonas taeanensis* BH 539	98.2	Gammaproteobacteria (Halomonadaceae)
PR18	LC020228	*Psychrobacter pacificensis* NIBH P2K6	99.8	Gammaproteobacteria (Moraxellaceae)
PR19	LC020229	*Erythrobacter citreus* RE35F/1	99.1	Alphaproteobacteria (Sphingomonadaceae)
PR20	LC020230	*Pseudoalteromonas paragorgicola* KMM3548	100	Gammaproteobacteria (Pseudo-alteromonadaceae)
PR21	LC020231	*Salinicola salarius* M27	99.6	Gammaproteobacteria (Halomonadaceae)
PR22	LC020232	*Salinicola salarius* M27	99.8	Gammaproteobacteria (Halomonadaceae)

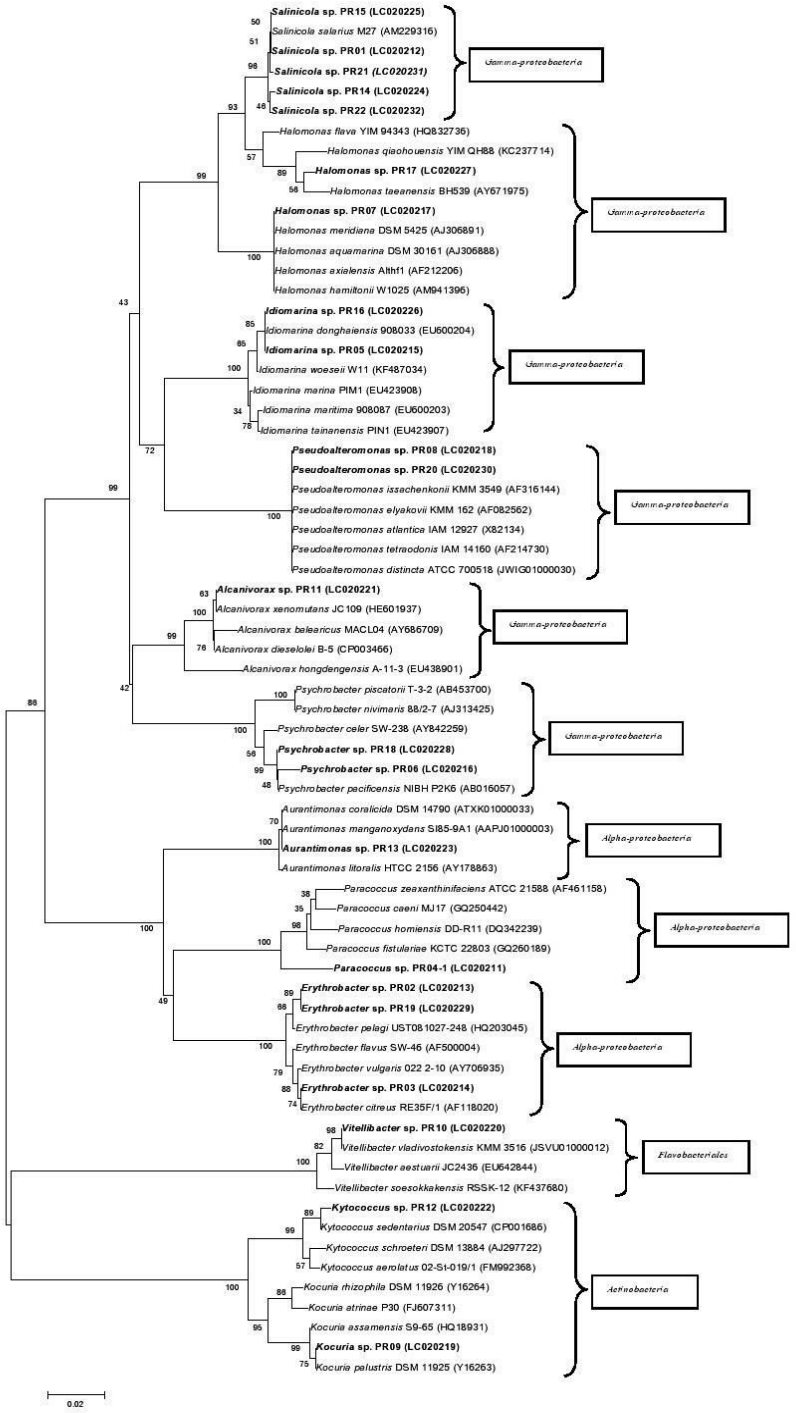


Figure 10. Phylogenetic tree of all the 22 isolated bacteria from the sponge *P. n*ux

3.4.3 The antibacterial activities of selected sponge surface-attached bacterial isolates

Eight isolates obtained from the previously described experiments (PR03, PR04, PR07, PR08, PR09, PR10, PR16, and PR17) were selected and subjected to a larger scale fermentation and extraction. The crude EtOAc extract from each isolate was examined for the antimicrobial activity against pathogenic organisms. However, only two isolates (PR04 and PR17) yielded the extracts that show a weak activity. Isolate PR04 showed the inhibition zones of 9.1 mm and 8.8 mm wide against *S. aureus* and *S. epidermidis*, respectively, whereas isolate PR17 had the inhibition zones of 9.4 mm and 10.1 mm wide against those two targeted bacteria. The positive control antibiotics oxacillin showed the inhibition zones of 22.8 mm and 28.9 mm against *S. aureus* and *S. epidermidis*, respectively and vancomycin had the inhibition zones of 16.2 mm and 20.8 mm wide against those two pathogenic bacteria.

CHAPTER 4

DISCUSSION

The sponge *Penares nux* is one of the common reef-dwelling sponge widely abundant throughout the Gulf of Thailand. The distribution of *P. nux* ranges from the inner part of the Gulf, as in Chonburi Province, all the way down to the lower part as in Pattani and Narathiwat Provinces. The sponge has a distinctive feature of differentiating into a gorgonian-like capitum, which is a rare characteristic among most sponge species. Also the sponge is found to develop a finger-like appendage extending from the core colony. The aim of this research is to investigate the chemical profile among each part of the sponge, and to study the impact of such profiling on the surrounding biota, namely the bacterial population on the sponge surface. The sponge *P. nux* was collected from two collecting sites, Koh-Tao and Saiburi, and was individually subjected to the determination for the contents of three kabiramide markers, kabiramides B, C, and G. The specimens were categorized morphologically into three groups, capitum, appendage, and base growth forms. Enumeration and taxonomic identification of the sponge surface-associated bacteria were performed. Specifically, the bacterial enumeration was carried out in parallel with the kabiramide content analysis to determine the correlation within each specimen. The following discussion is focused first on the quantitation of the kabiramide, followed by the allocation of the kabiramide content in the sponge, and the assessment of bacterial association to the sponge.

4.1 Quantification of the kabiramide analogs

The analytical protocol devised in this investigation was adopted and modified from those described in Sirirak et al (2011a). The MS-MS in positive ESI mode was chosen as detecting device, hence allowing the detection of each kabiramide marker individually within one single chromatographic run without additional pre-chromatographic purification. Upon validation, the recovery percentage and relative standard deviation indicate an excellent precision and accuracy of the devised protocol. This permits the quantification of the three kabiramide analogs to be performed in a routine, high throughput manner.

4.2 Allocation of kabiramides in the sponge *P. nux*

The contents of kabiramides B, C, and G in three parts; capitums, appendages, and bases of the sponge *P. nux*, were examined on an individual part, individual colony basis. The results showed that the sponge was able to allocate the toxic kabiramide analogs to deposit predominantly in the protruding capitum parts, followed by the extending appendages, and bases, respectively. The results on the predominant accumulation of kabiramides in the capitum over the bases are consistent to those reported by Sirirak et al (2011a), hence suggesting such a specific allocation of the toxic macrolides not be a single isolated incidence, but consistent over a span of time. The investigation was extended to examine the kabiramide contents in the appendage part, which could be described as a stretching part of the colony from the core base, and showed that, although not as high as in the capitums, the contents of kabiramide analogs in the appendages were significantly higher than that in the bases. To demonstrate the differences in the contents of each kabiramide markers pictorially, the contents of each kabiramide analog in the different parts of the sponge are shown in Figure 11-15.

On a separated note, it could be argued whether such a variation in kabiramide contents is resulted from fluctuations in extraction process. However, the extraction yields of the crude extracts from each part of the sponge (calculated per sponge dried weight basis) was uniform, and indicated that the chemical allocation of the kabiramides are caused by an inherent influence genuine within each sponge colony, and not from the experimental errors. On the other hand, within each part of the sponge, the level of each kabiramide are not statistically different (p = 0.53, 0.42, 0.43 for the capitum, appendage, and base, respectively). That is, within each part of the sponge, the contents of kabiramides B, C, and G are independent and non-proportionate to each other. The sponge allocates each kabiramide in an independent manner without any specific influences on other analogs.

Location-wise, the predominant allocation of the kabiramides towards the capitums over the appendages and bases is parallel between the specimens from Koh-Tao and Saiburi. This indicates a consistent and universal trend of such specific allocation of the toxic macrolides toward the protruding capitum, and extending appendage parts. However, for each

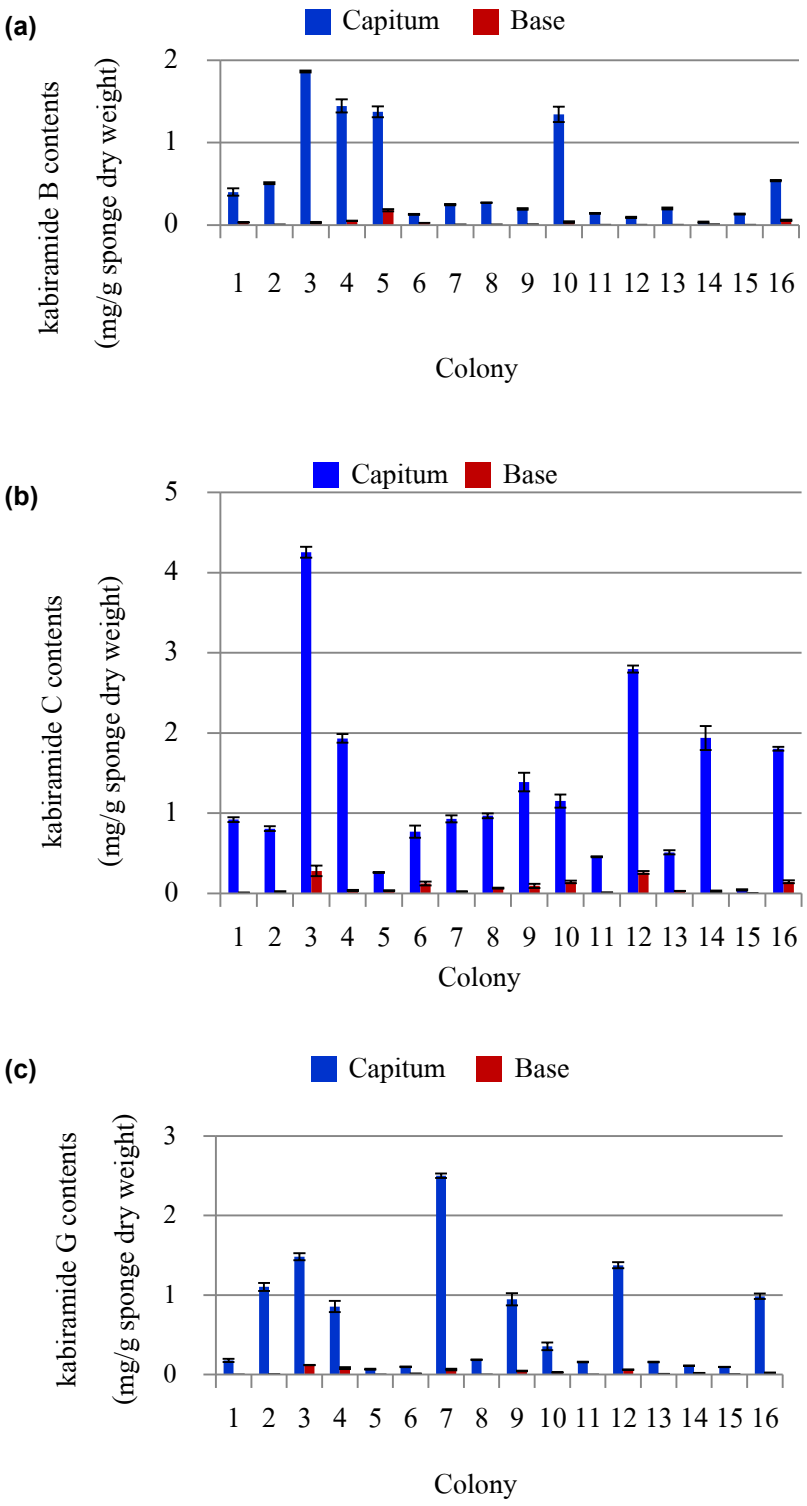


Figure 11. Kabiramide contents in capitum and base parts (a) kabiramide B contents (mg/g), (b) kabiramide C contents (mg/g), (c) kabiramide G contents (mg/g) for Koh Tao location

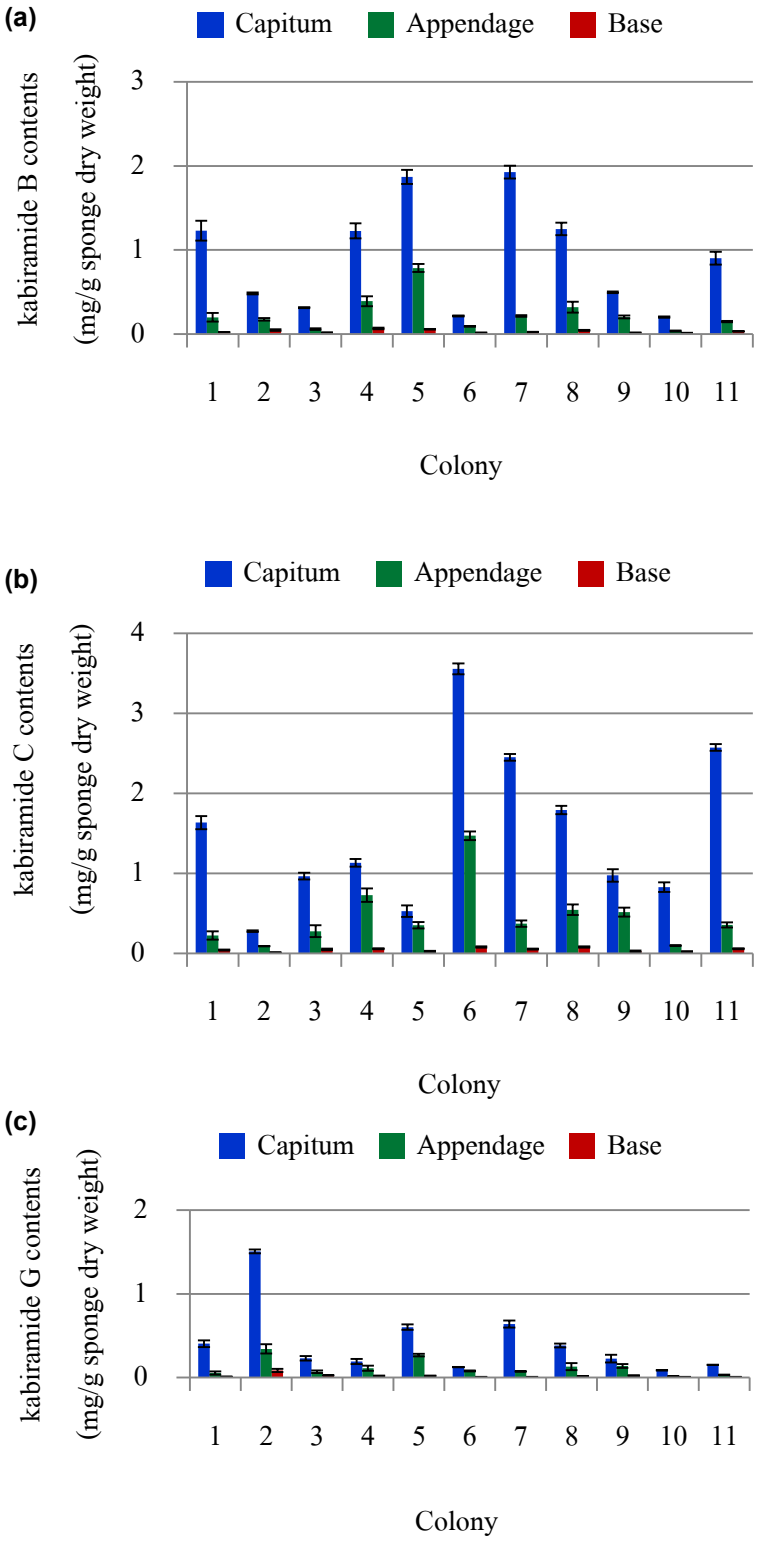


Figure 12. Kabiramide contents in capitum, appendage, and base parts (a) kabiramide B contents (mg/g), (b) kabiramide C contents (mg/g), (c) kabiramide G contents (mg/g) for Koh Tao location

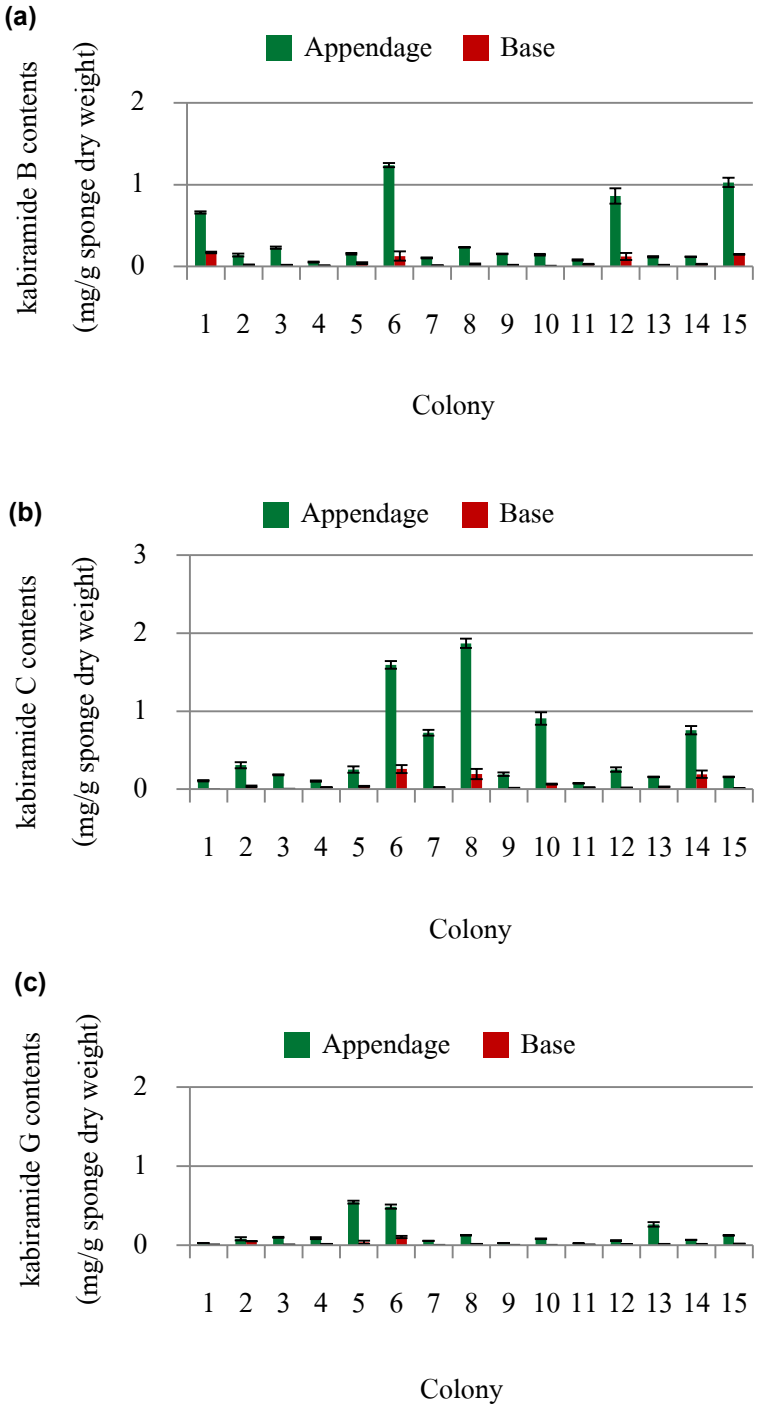


Figure 13. Kabiramide contents in appendage and base parts (a) kabiramide B contents (mg/g), (b) kabiramide C contents (mg/g), (c) kabiramide G contents (mg/g) for Koh Tao location

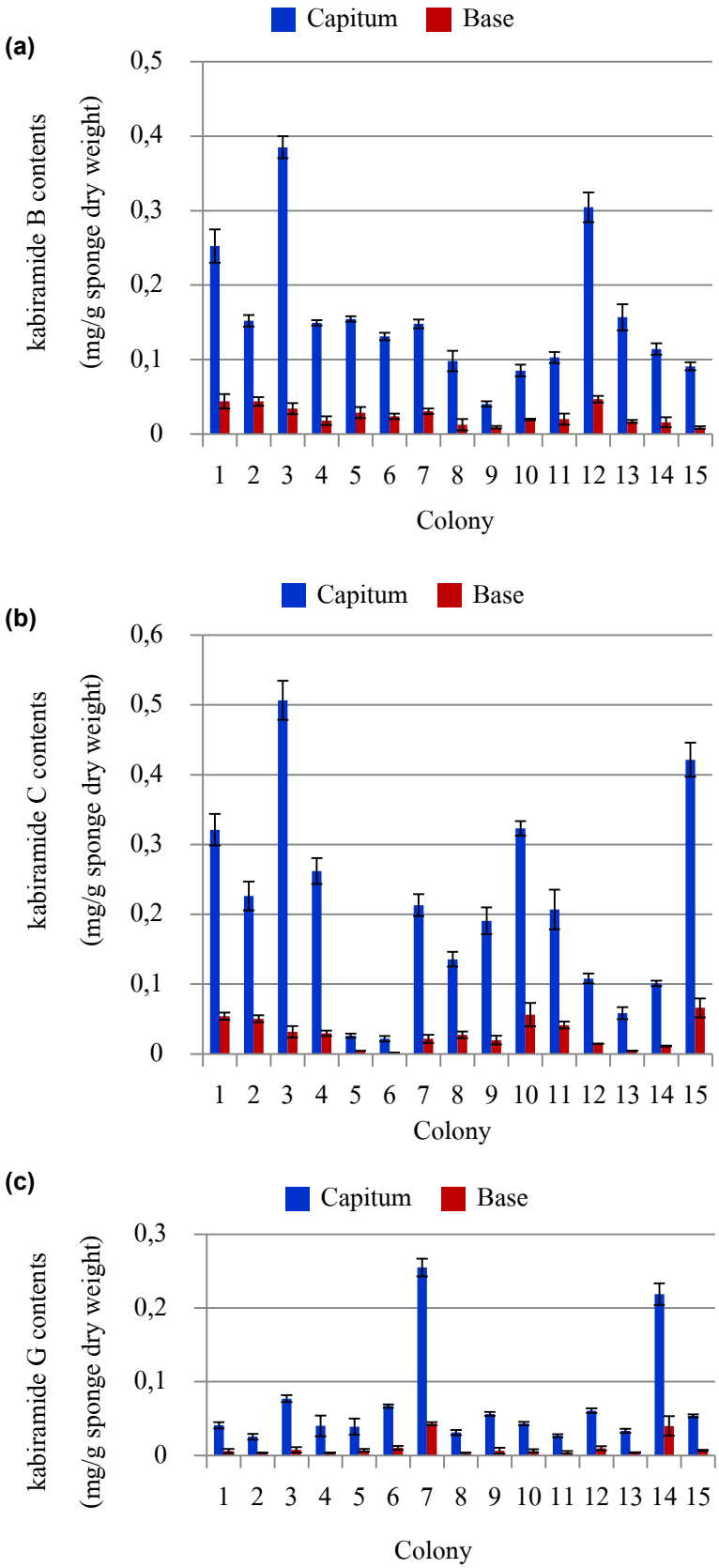


Figure 14. Kabiramide contents in capitum and base parts (a) kabiramide B contents (mg/g), (b) kabiramide C contents (mg/g), (c) kabiramide G contents (mg/g) for Saiburi location

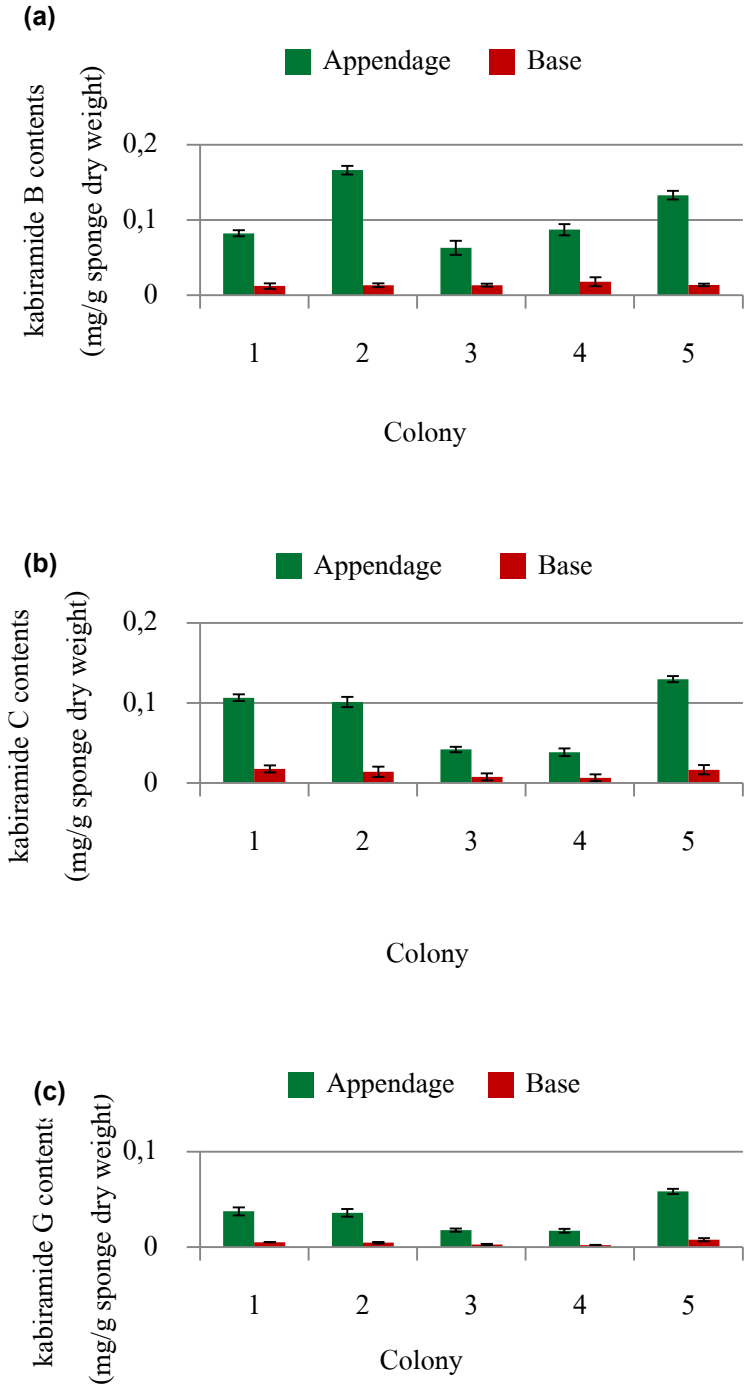


Figure 15. Kabiramide contents in appendage and base parts (a) kabiramide B contents (mg/g), (b) kabiramide C contents (mg/g), (c) kabiramide G contents (mg/g) for Saiburi location

kabiramide analog that is accumulated in each part, with the exception of kabiramide B from the appendage part, the contents of the macrolide are significantly higher in the specimens from Koh-Tao than in those from Saiburi (Table 8, page 85).

Although the impacts from the physical and biological environments have not been investigated in this study, it can be clearly seen that there are geographical influences on the producing and/or accumulating levels of kabiramides in the sponge *P. nux*. Such geographical impact on the metabolites has been observed in various sponge species. For example, the sponge *Alplysina aerophoba* showed significant differences in the concentration of brominated compounds from different locations (Sacristán-Soriano et al., 2011a). Similarly, the sponge *Spongia lamella* showed remarkable variation in the contents of terpenes and ergosterol derivatives from different geographical locations (Noyer et al., 2011). Another example is the sponge *Haliclona* sp. which showed variation in the concentration of salicylihalamide A from different locations (Abdo et al., 2007).

In the previous study on the allocation of the kabiramides in the sponge *P. nux* (Sirirak et al., 2011a), the phenomenon was compared to that in the sponge *Oceanapia* sp. (Schupp et al., 1999), in which both species are able to allocate their toxic components specifically in the more protuding and vulnerable parts. The accumulation of the kabiramides in the appendage, which is an extension from the central core of the sponge colony, can also be comparable to the incidences in the sponge *Cacospongia* sp. (Becerro et al., 1998), in which the sponge can concentrate scalaradial and desacetylscalaradial metabolites highly in the tip of the colony.

Although the ecological impacts of the kabiramides have not been specifically studied, other trisoxazole macrolides, namely halichondramide, dihydrohalichondramide, and tetrahydrohalichondramide, have been reported to have a fish feeding deterrent effects, and have been proposed to act as chemical defense in their accumulating organisms (Pawlik et al., 1988). Such defensive roles could therefore reasonably be extended to account for the role of kabiramides in the sponge *P. nux*.

4.3 Microbiota on the surfaces of the sponge *P. nux*

The antipredatory effects of trisoxazole macrolides as described above (Pawlik et al., 1988) are well known and widely perceived. Although a fish feeding repellent activity of the kabiramides has never been reported, the similarity in the structures and other biological activities may allow the antipredatory effects to be extended for the kabiramides. To avoid the redundancy, the determinations of such activity have therefore not been conducted here. Instead, the attention has been paid on the microbiota, particularly on the bacterial community attached to the sponge's surface. This is conducted in the light of the studies on sponge-microbe interaction, which have highly gained attention over the past decade (Becerro et al., 1994; Wahl et al., 1994; Slattery et al., 1995; Newbold et al., 1999; Kelly et al., 2003; Becerro et al., 2008),

The enumeration of sponge surface-associated bacteria was investigated by mean of swabbed-surface CFU count technique (Chelossi et al., 2004). All the 95 sponge specimens from Koh-Tao expedition, the same as those that were investigated for the kabiramide contents, were subjected to the bacterial enumeration. The results were found in an inverse manner to those of the kabiramide contents; i.e., the base parts of the sponge have a higher population density than the appendages and capitums. The results from the different parts that were previously depicted in section 4.2 are shown here to demonstrate the opposite trends between the contents of the kabiramides and the CFU counts of the bacteria on each part of the sponge (Figure 16).

Primary examination on the correlation between the content of each kabiramide analog and the CFU counts of the bacteria throughout the investigated specimens showed a very weak correlation between each compound and the bacterial population. However, a close observation of the correlation plots (Figure 17) revealed certain severe outliers. Upon dismissal of CFU counts outliers (75% percentile, using the upper quartile of 3.6×10^7 CFU/cm^{2}as the outlier threshold; i.e., any values greater than these were dismissed), the Spearman's correlation r_s of each pair of data improved significantly. A negative correlation was observed between CFU counts vs the kabiramide contents (r_s = -0.594, p = < 0.001; r_s = -0.614, p = < 0.001; r_s = -0.517, p = < 0.001, for kabiramides B, C, and G, respectively). Although such correlations still fell in a range of moderate correlation, they all were significant, and indicated negative correlation between the CFU counts of sponge-surface associated bacteria and the contents of each kabiramide marker, all in a parallel manner.

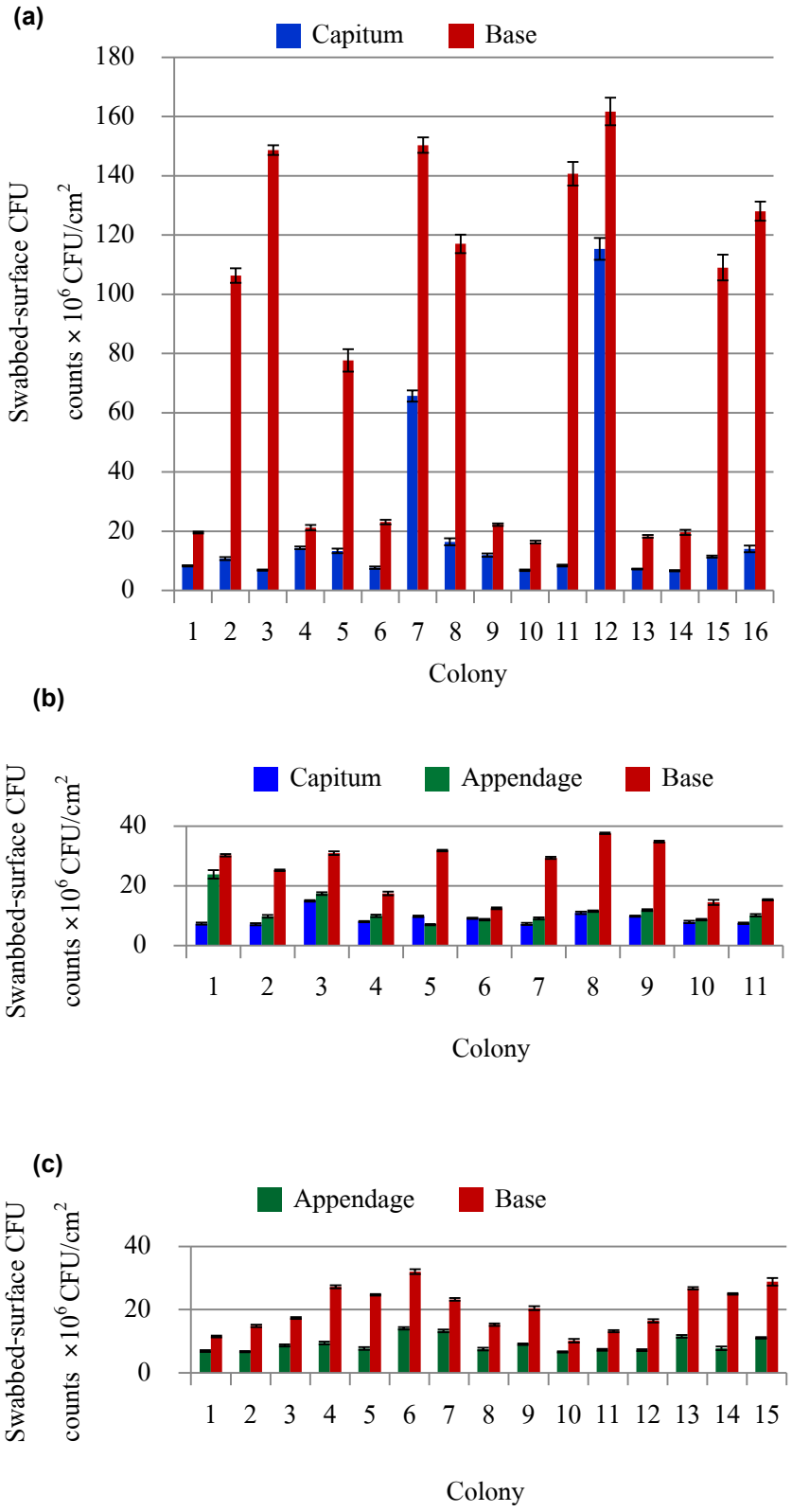


Figure 16. Swabbed-surface CFU counts for the different parts of the sponge (a) capitum and base parts (b) capitum, appendage and base (c) appendage and base parts

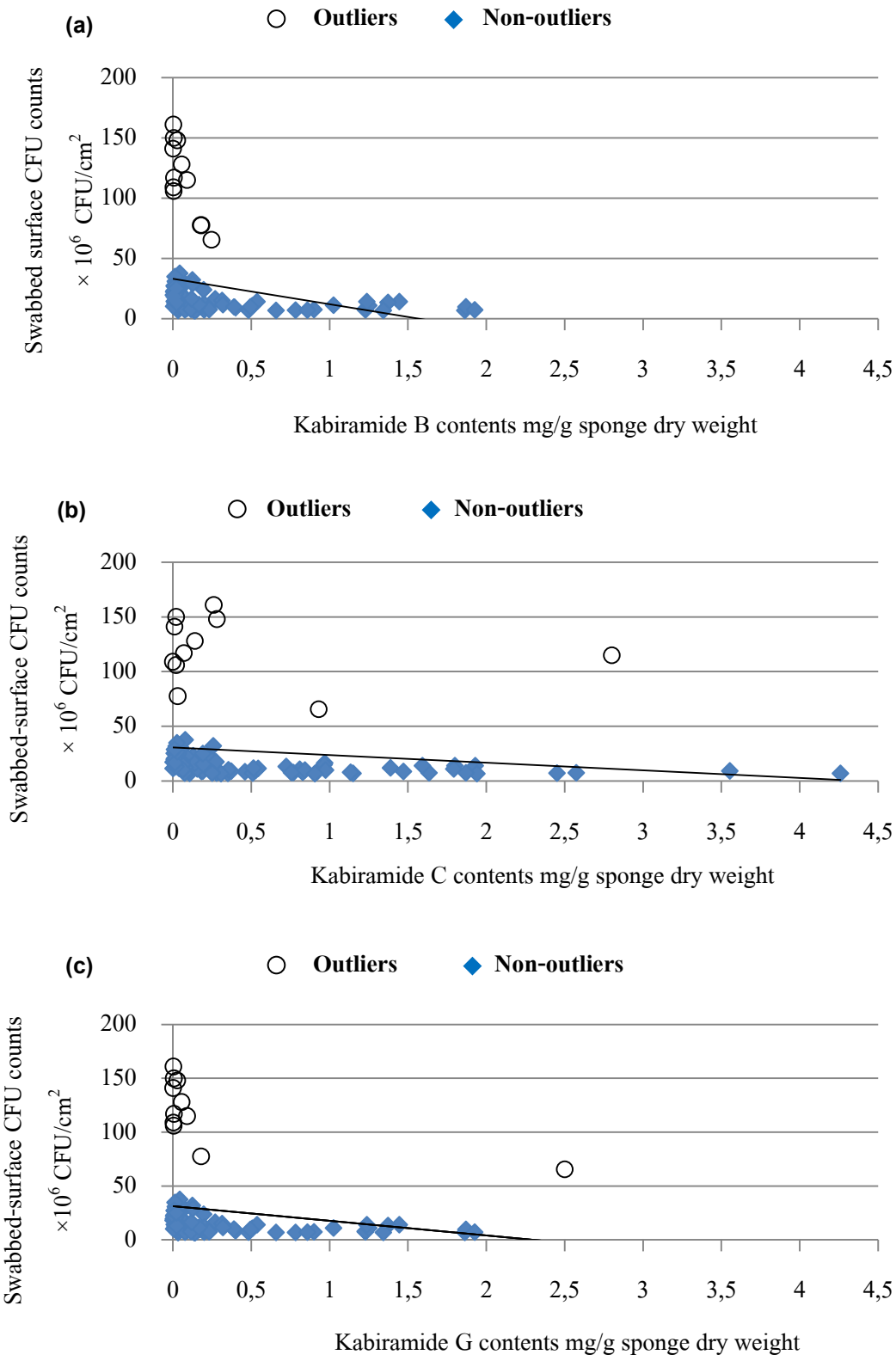


Figure 17. Correlation plots showing the outliers (a) CFU vs kabiramide B, (b) CFU vs kabiramide C, (c) CFU vs kabiramide G (Empty circles are the outliers from CFU counts, dark circles are the non-outliers).

Whereas it is clear that the contents of kabiramides on the sponge surface have an impact on the density of the bacterial population associating to the sponge (or vice versa), it must be pointed out that this might not be correlated directly to the antibacterial activities. In fact, despite being reported to be toxic against a vast range of cells and organisms, trisoxazole macrolides are void of antibacterial activities (Matsunaga et al., 1986). On one hand, this may suggest the impact of kabiramides as the compounds might not cast a direct effect to fend off the bacteria on the surface, but rather influence the microhabitat on the sponge surface in such a way that the sponge may use the compounds to defend itself from other types of pathogens and foulers. On the other, it could be possible to consider the reverse influences from the bacteria on the production/accumulation of the macrolides to be allocated specifically as seen.

Upon achieving the plate count experiment with all the culturable bacteria in hand, 22 morphologically distinct colonies were selected and subjected to the taxonomic identification using 16S rRNA sequencing and phylogenetic analysis. The identification indicated the closest relatives of all the 22 isolates to fall into 13 bacterial species. Among these, three species are halotolerant; namely *Salinicola salarius*, *Halomonas taeanensis*, and *Halomonas aquamarina* (Lee et al., 2005; Kim et al., 2007; Guzman et al., 2010), and the rest are obligate halophiles (Maruyama et al., 2000; Denner at al., 2002; Ivanova et al., 2002; Denner et al., 2003; Berry et al., 2003; Liu Chenli and Shao Zongze, 2005; Wu et al., 2009). All have been previously reported from various submerged substrates, from inorganic substrate (Kovács et al., 1999; Maruyama et al., 2000; Denner at al., 2002; Berry et al., 2003; Liu Chenli and Shao Zongze, 2005; Wu et al., 2009), to various marine organisms (Ivanova et al., 2002; Denner et al., 2003; Nedashkovskaya et al., 2003). However, no specific association with the sponge *P. nux* or any taxonomically related sponges has been documented. Some of the identified bacteria have been reported to have potentials for commercialized applications. For examples, *Paracoccus zeaxanthinifaciens* contains zeaxanthin a yellow carotenoid used in poultry pigmentation and in the prevention of age-related macular degeneration (Berry et al., 2003). Another example is *Alcanivorax dieselolei*, which is useful as a bioremediative intervention in polluted marine and coastal system (Liu Chenli and Shao Zongze, 2005). Nonetheless, none has been reported as potential sources of biologically active secondary metabolites.

The investigation toward possible biological activities in any of the bacterial isolates obtained here has also been attempted. Eight bacterial isolates were selected for the antibacterial activity screening. However only two isolates (PR04 and PR17), show a weak activity.

CHAPTER 5

CONCLUSION

This dissertation is focusing on the chemical allocations in the different parts of the sponge *P. nux*, and the impacts of accumulation of trisoxazole macrolides on the microbial community. The newly modified method gave an excellent selectivity, sensitivity, and reproducibility, and allowed a routine analysis to be accomplished. The concentration of the kabiramide derivatives varied greatly among the capitum, appendage, and base parts, and showed that the capitum part has the highest amount of the kabiramides. This study encourages evaluation of the prediction of optimal defense theory. With the defenses in the sponge *P. nux* may be assumed strongest in capitum and appendage parts, and associate closely with the higher accumulating amounts of the toxic kabiramides. This thesis also provided evidence that, although the trisoxazole macrolides contents may vary among different geographical locations, the trends in the macrolide allocation are consistent.

The sponge *P. nux* yielded a diverse microbial community, including Alphaproteabacteria, Gammaproteobacteria, Actinobacteria and Flavobacteria. The microbial community density of surface-attached bacteria on the capitum and appendage part was significantly less than on the base part. The roles of kabiramide and how they influence the sponge to adjusted in its distinct environment and how it interacts with the microbial cohabitants are still the topic to be debated. Chemical compositions of each bacteria isolate showed that none is potential to be either a producer or inducer of the kabiramides. A further study of bacteria associated with the sponge *P. nux* using modern molecular techniques is needed to improve the understanding of the nature of the sponge bacterial association. Such efforts will contribute to the exploration of unique secondary metabolites of sponge-derived microorganisms and to ecology of sponge microbial symbionts.

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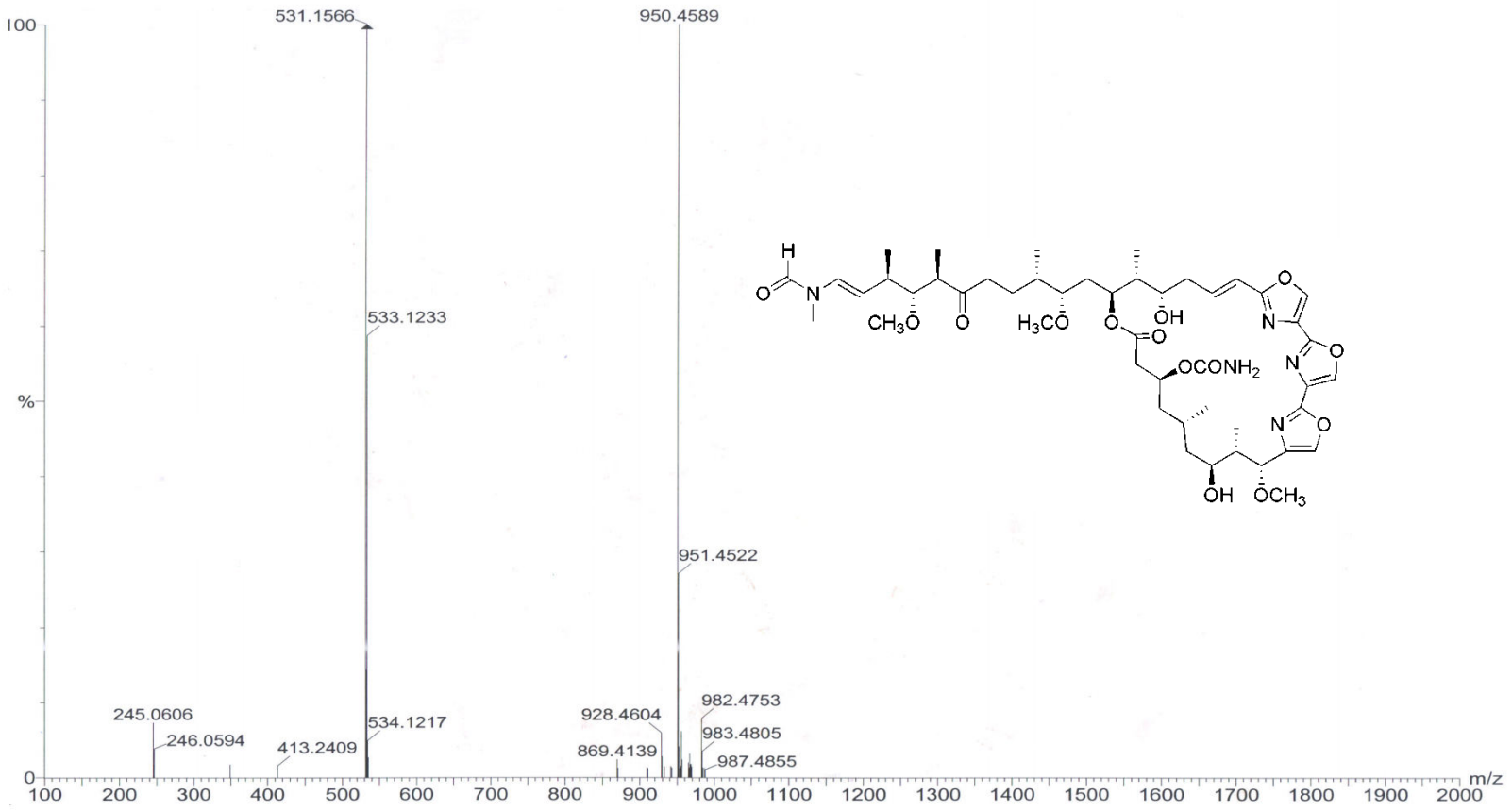
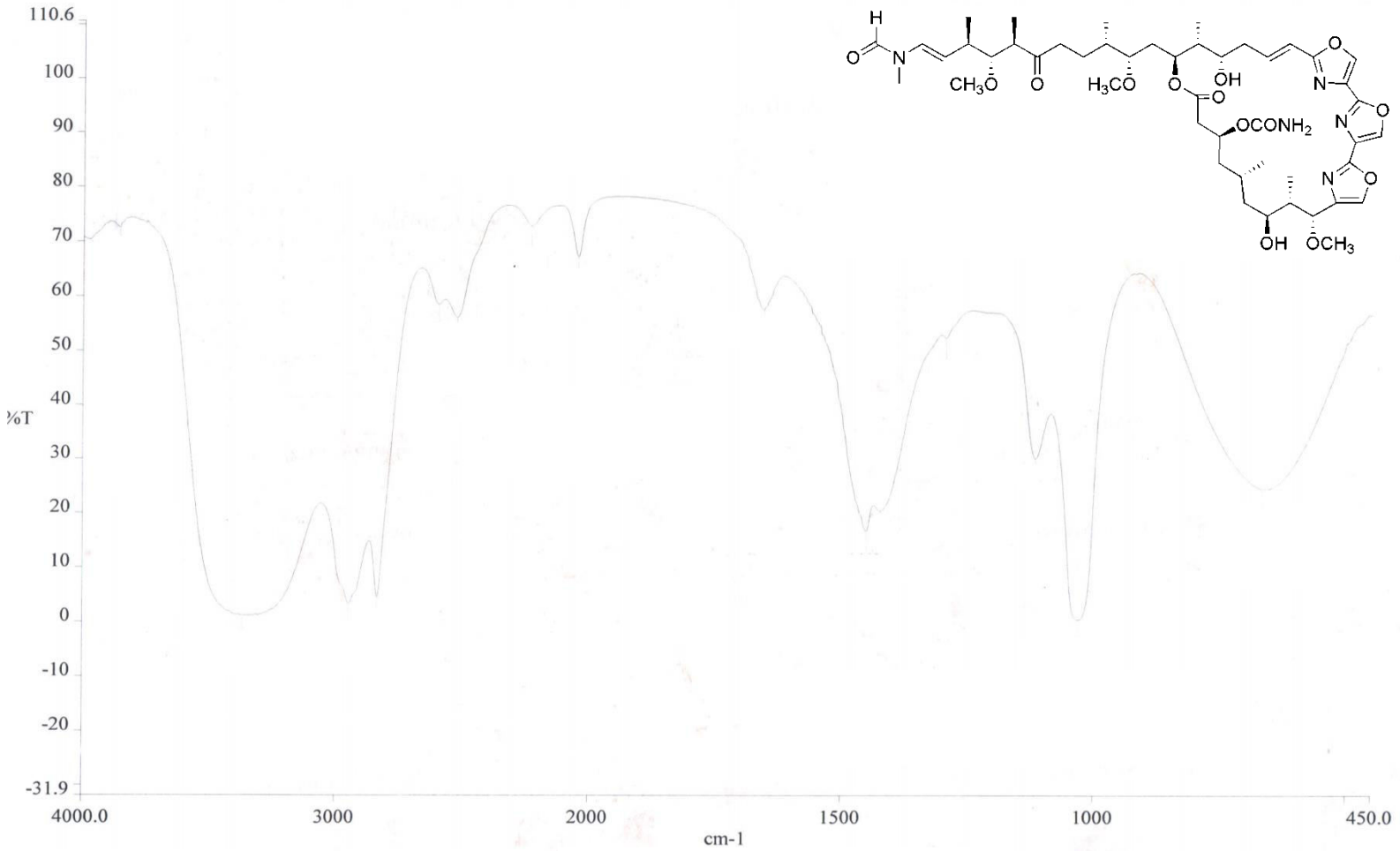
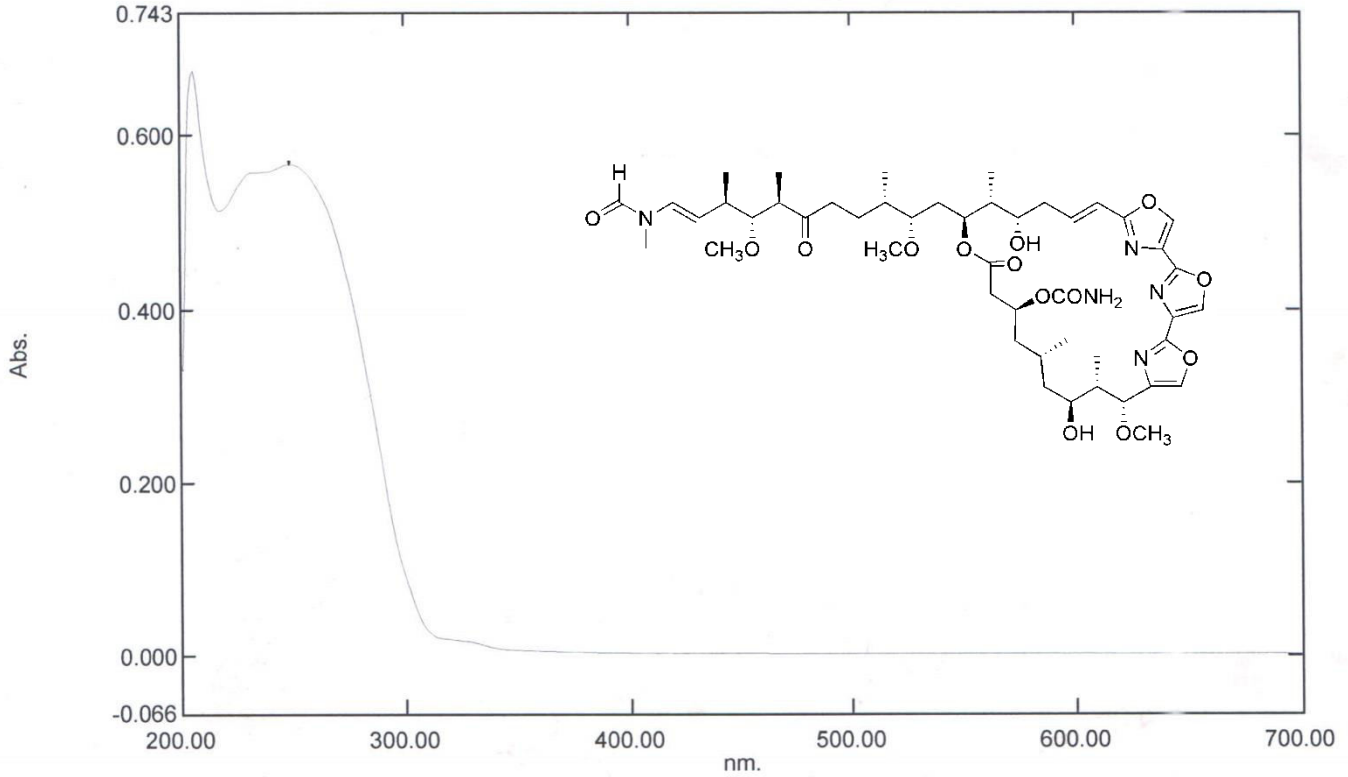
APPENDIX



ESI mass kabiramide B



IR spectrum of kabiramide B (neat)



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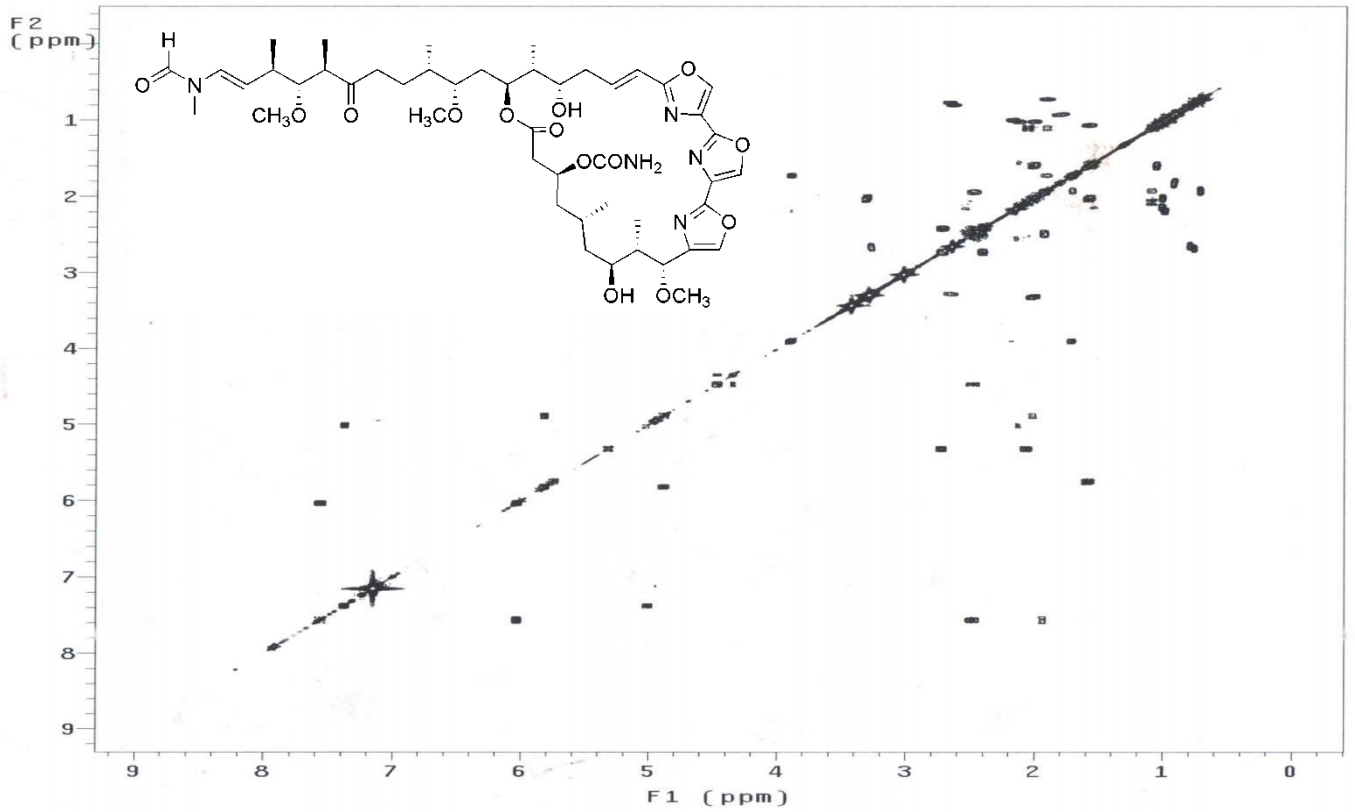
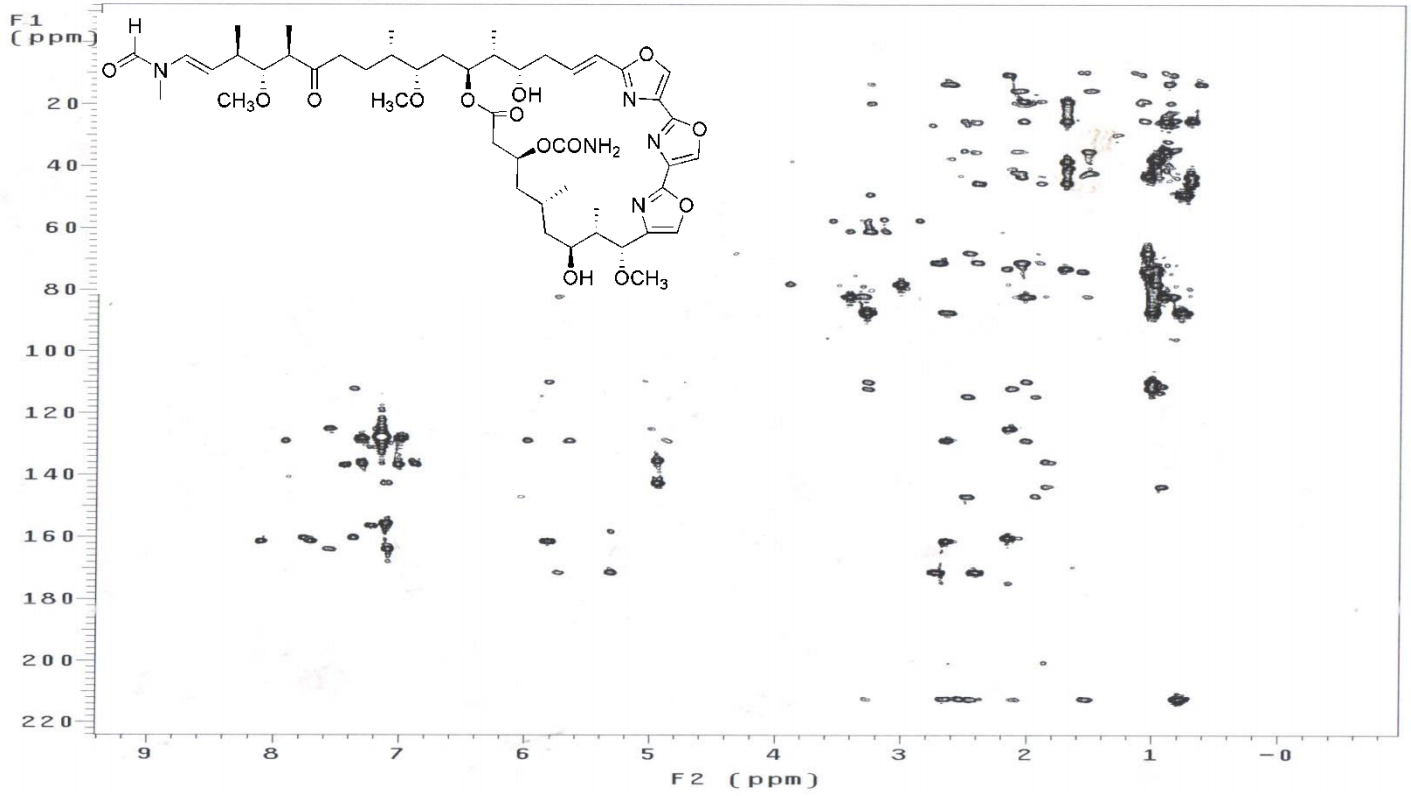


^{1}H-^{1}H COSY spectrum of kabiramide B (500 MHz, C_6D_6)



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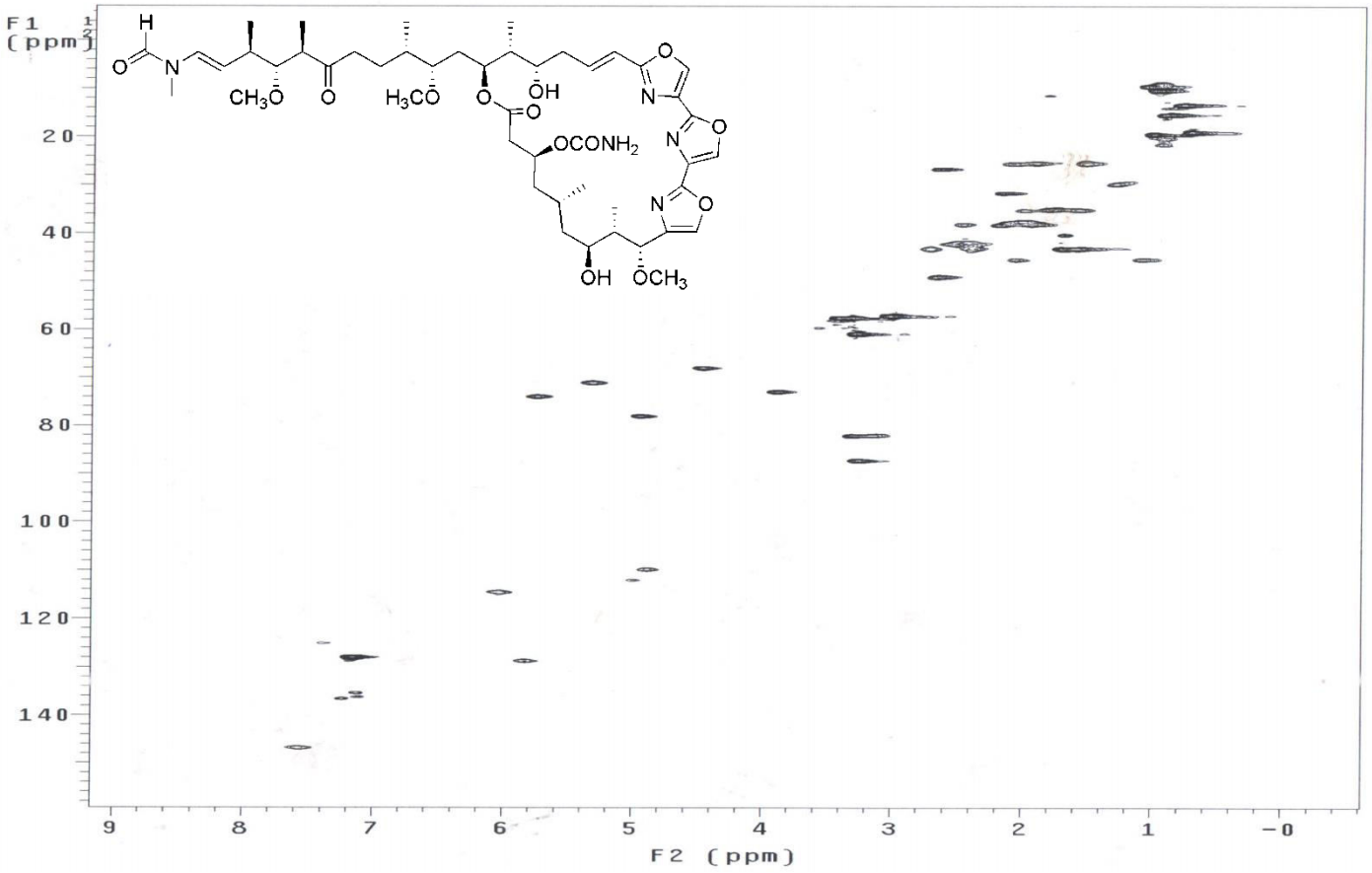
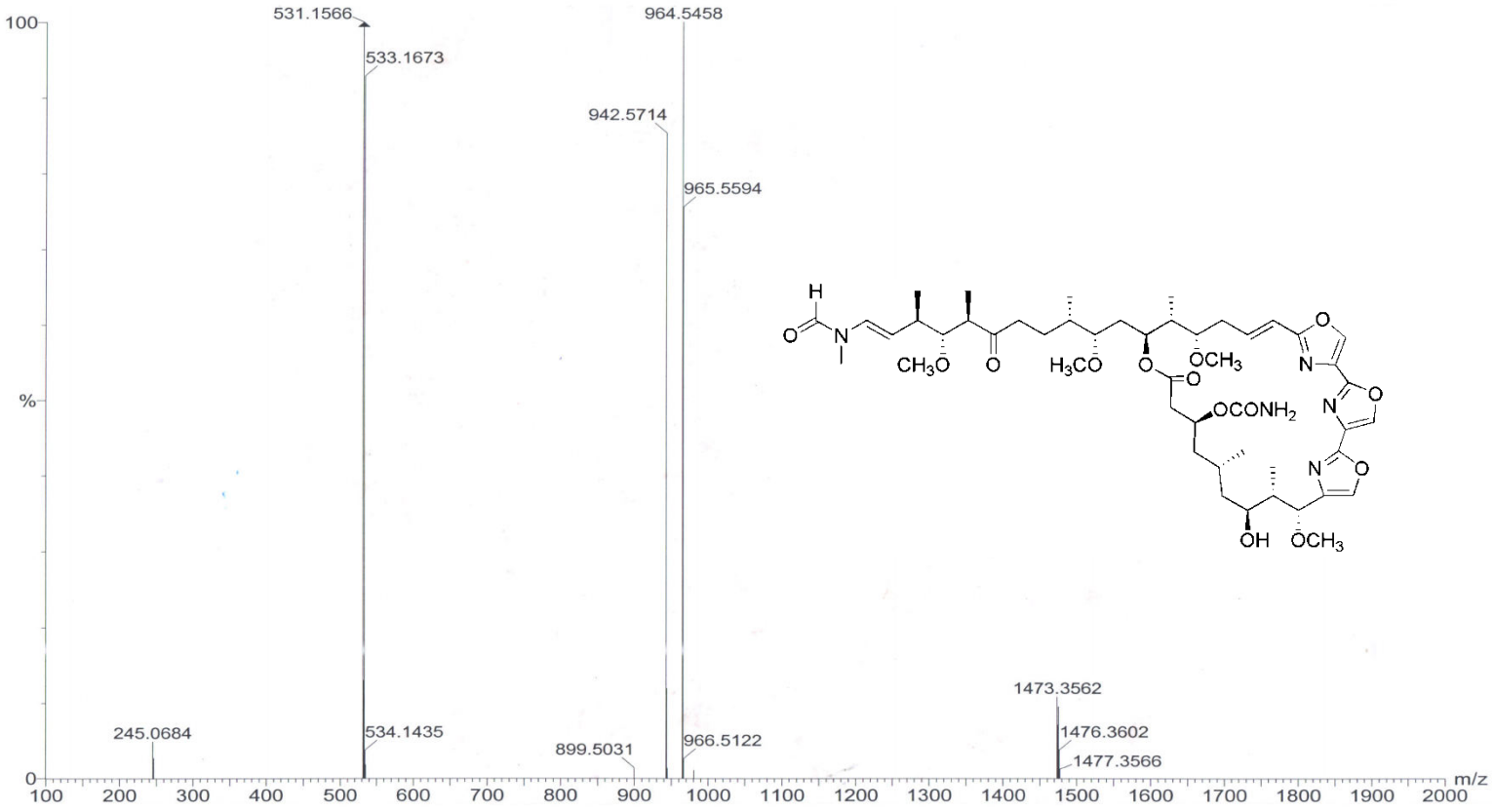
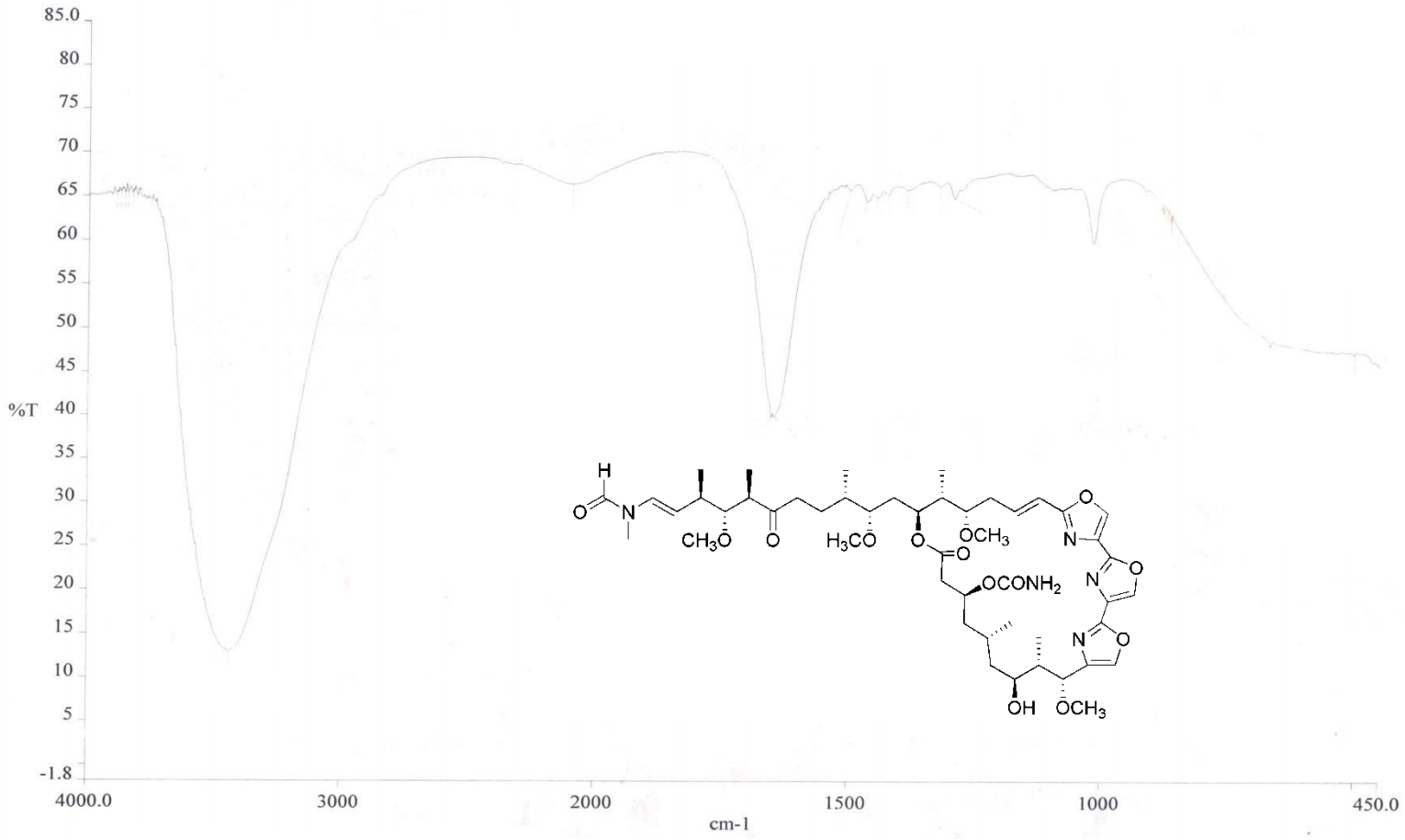


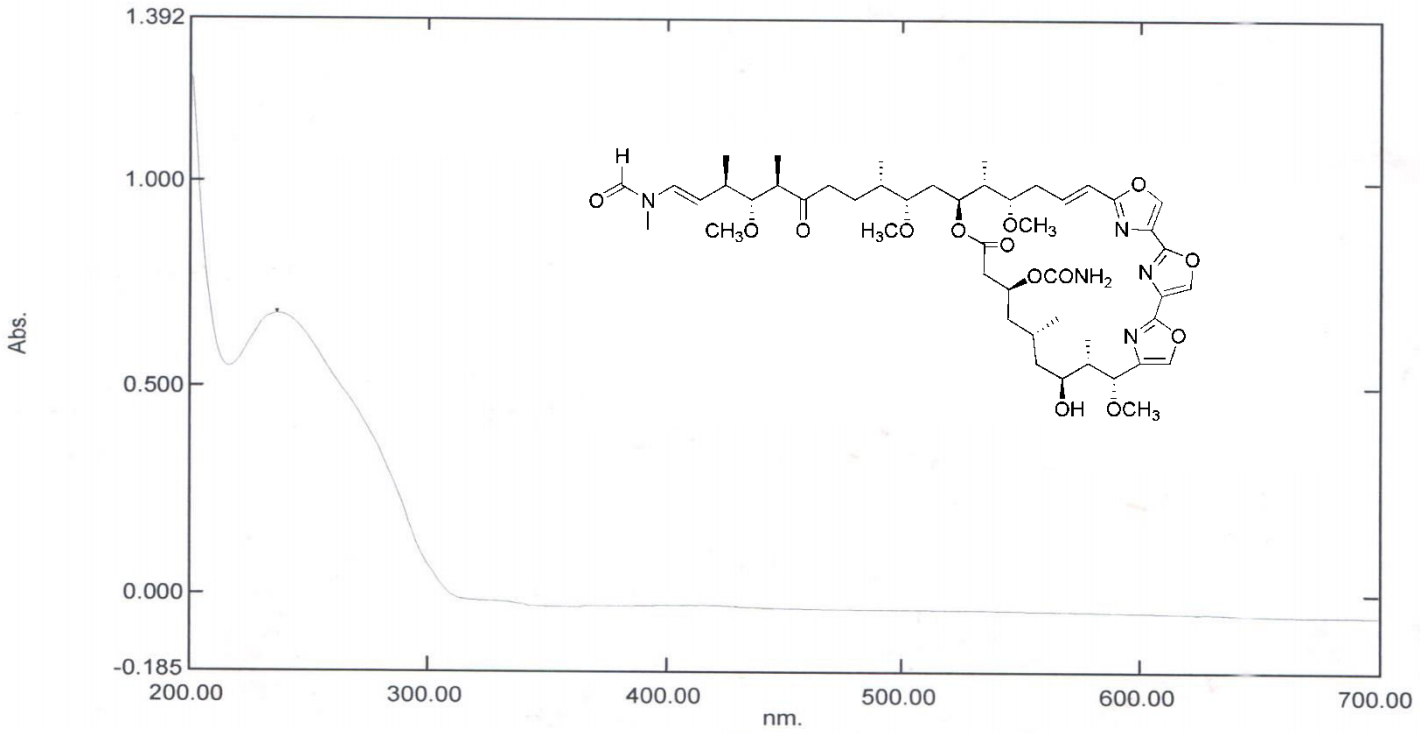
HMQC spectrum of kabiramide B (500 MHz, C_6D_6)



ESI mass spectrum of kabiramide C



IR spectrum of kabiramide C (neat)



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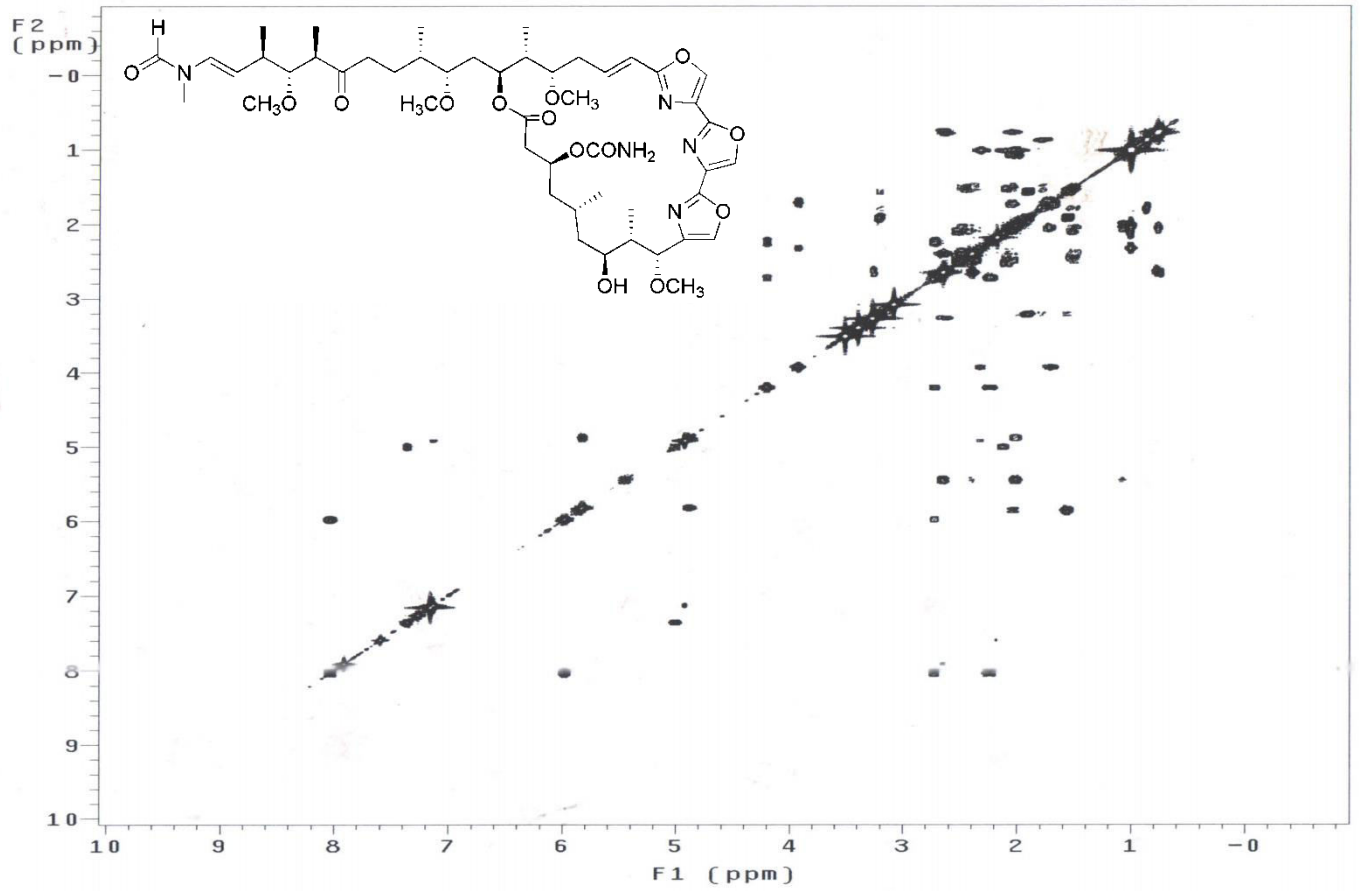
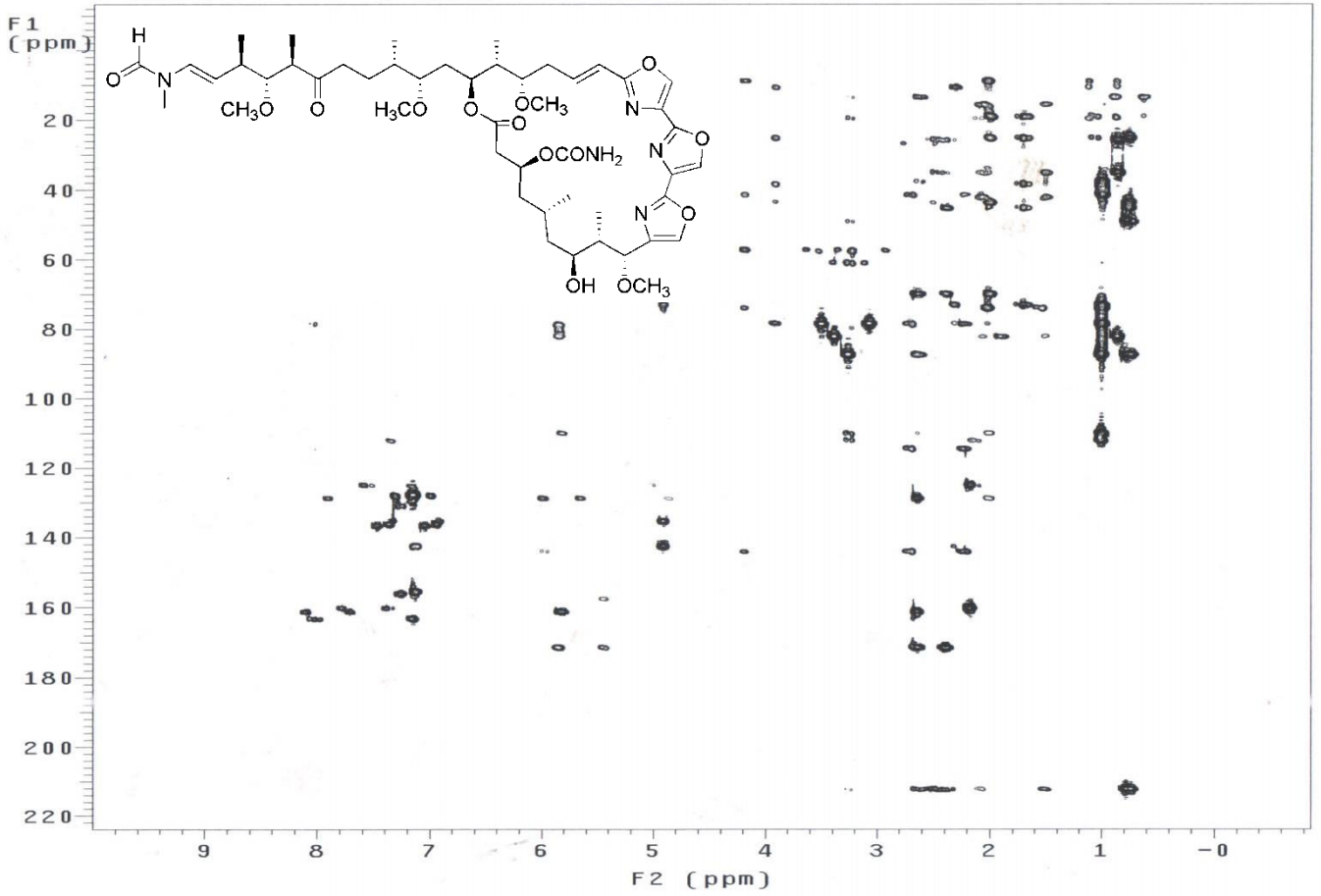
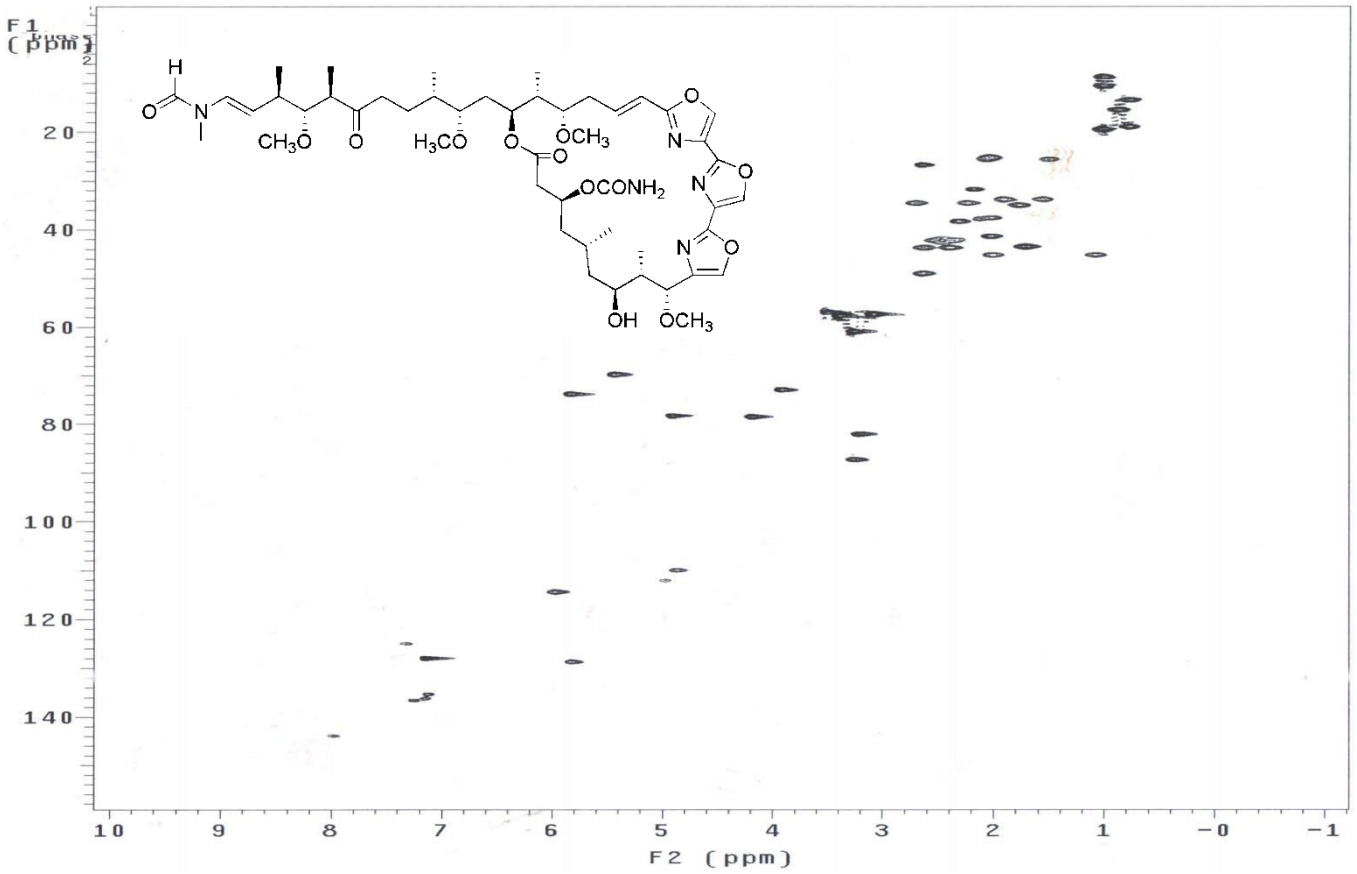


^{1}H-^{1}H COSY spectrum of kabiramide C (500 MHz, C_6D_6)



HMBC spectrum of kabiramide C (500 MHz, C_6D_6)



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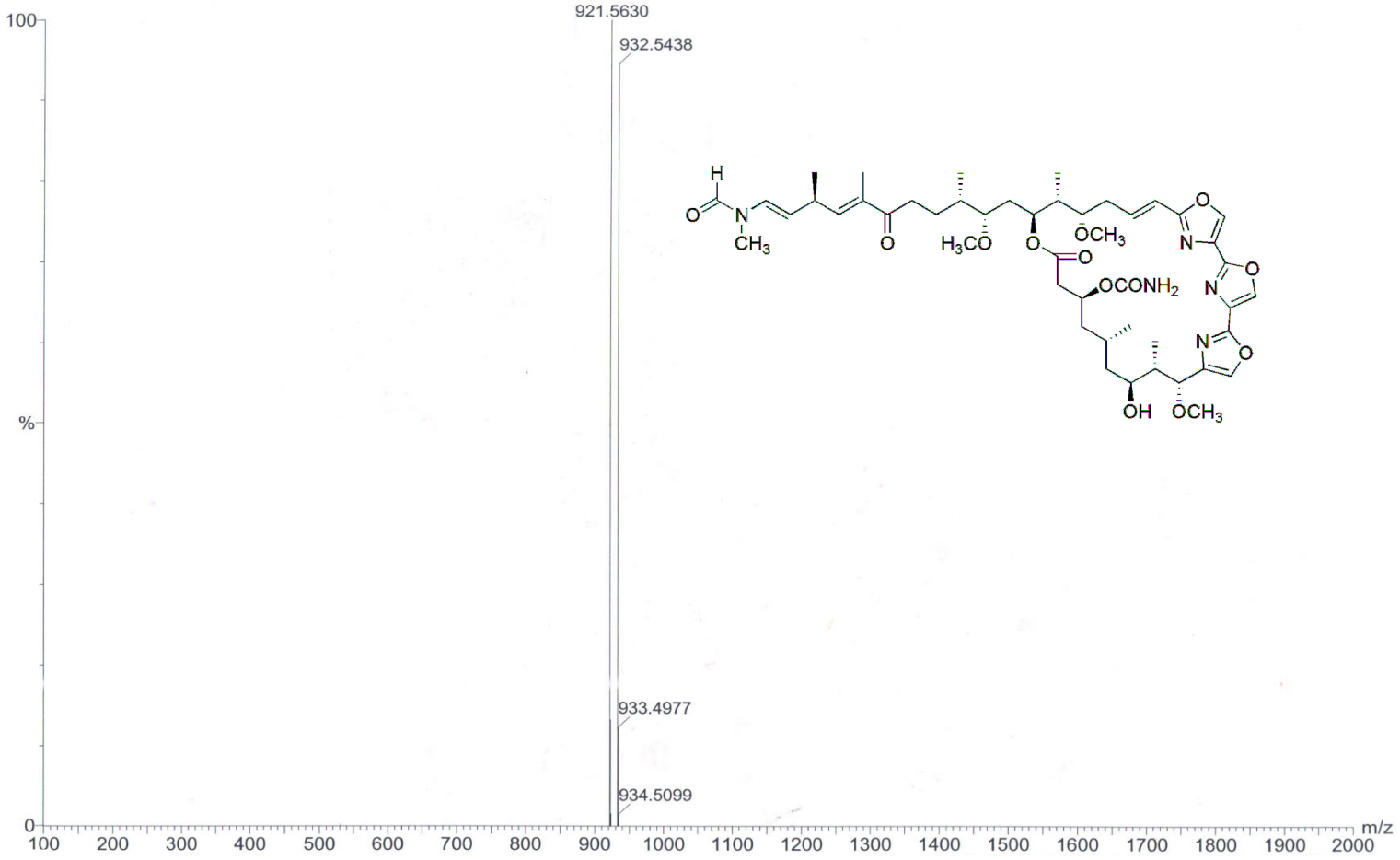
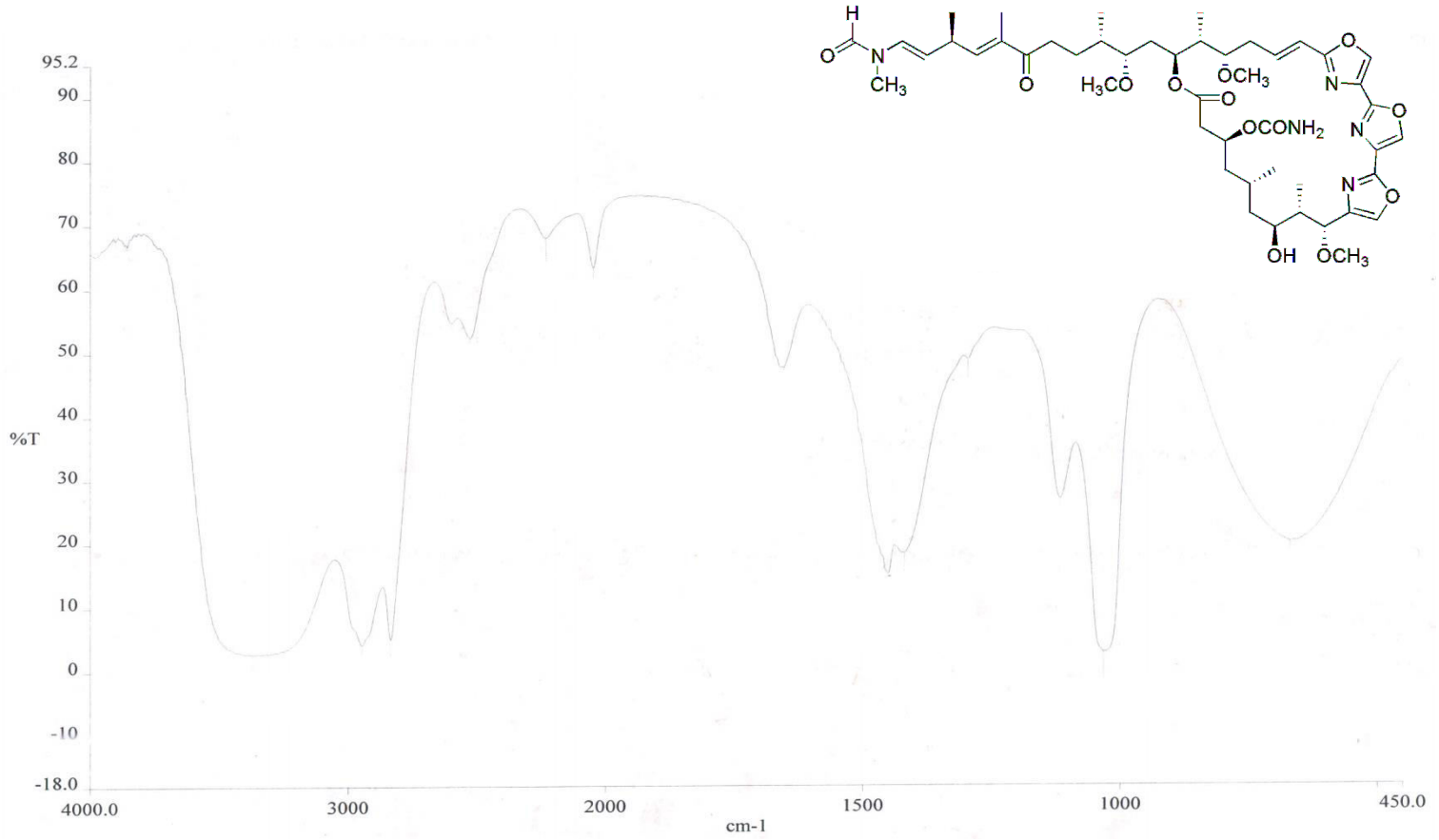
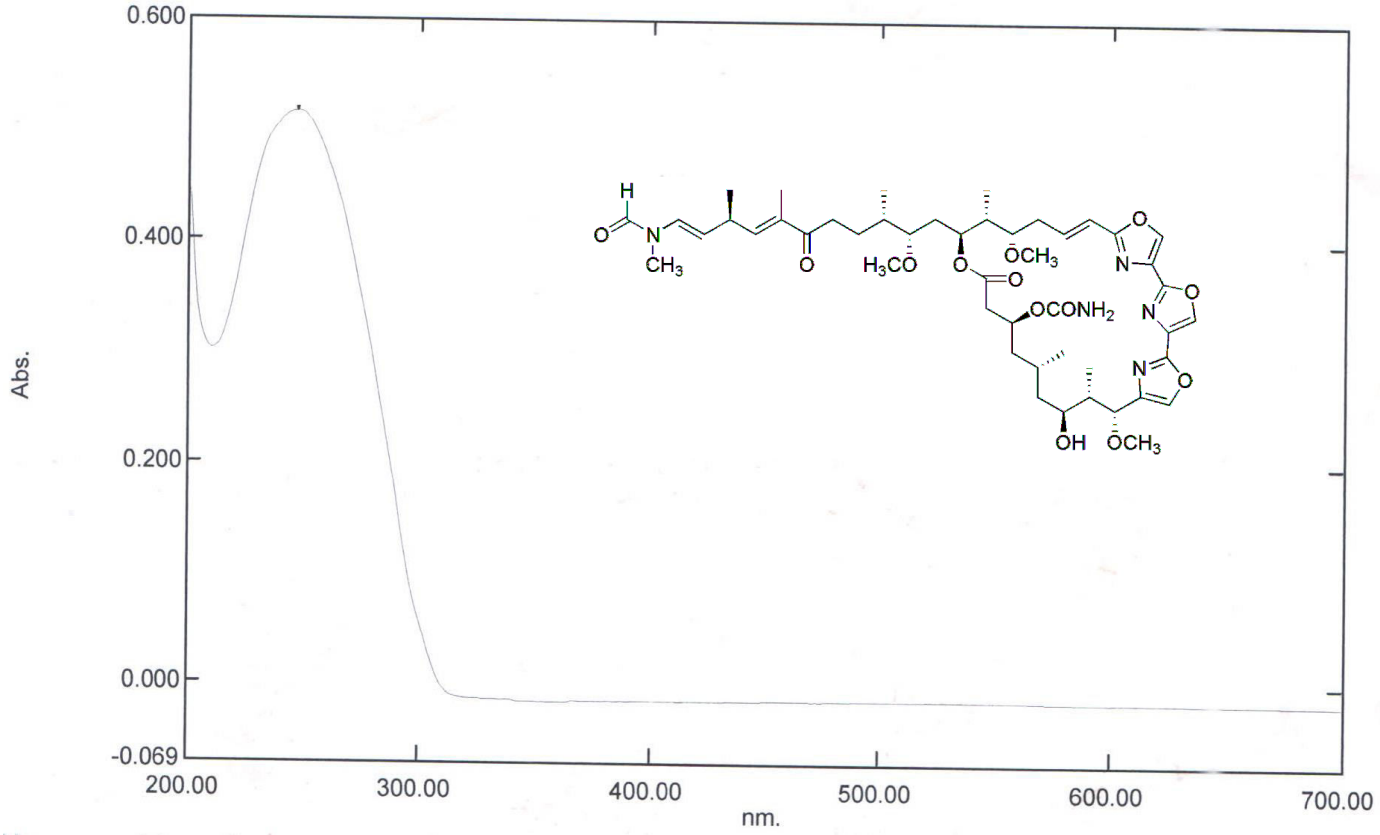


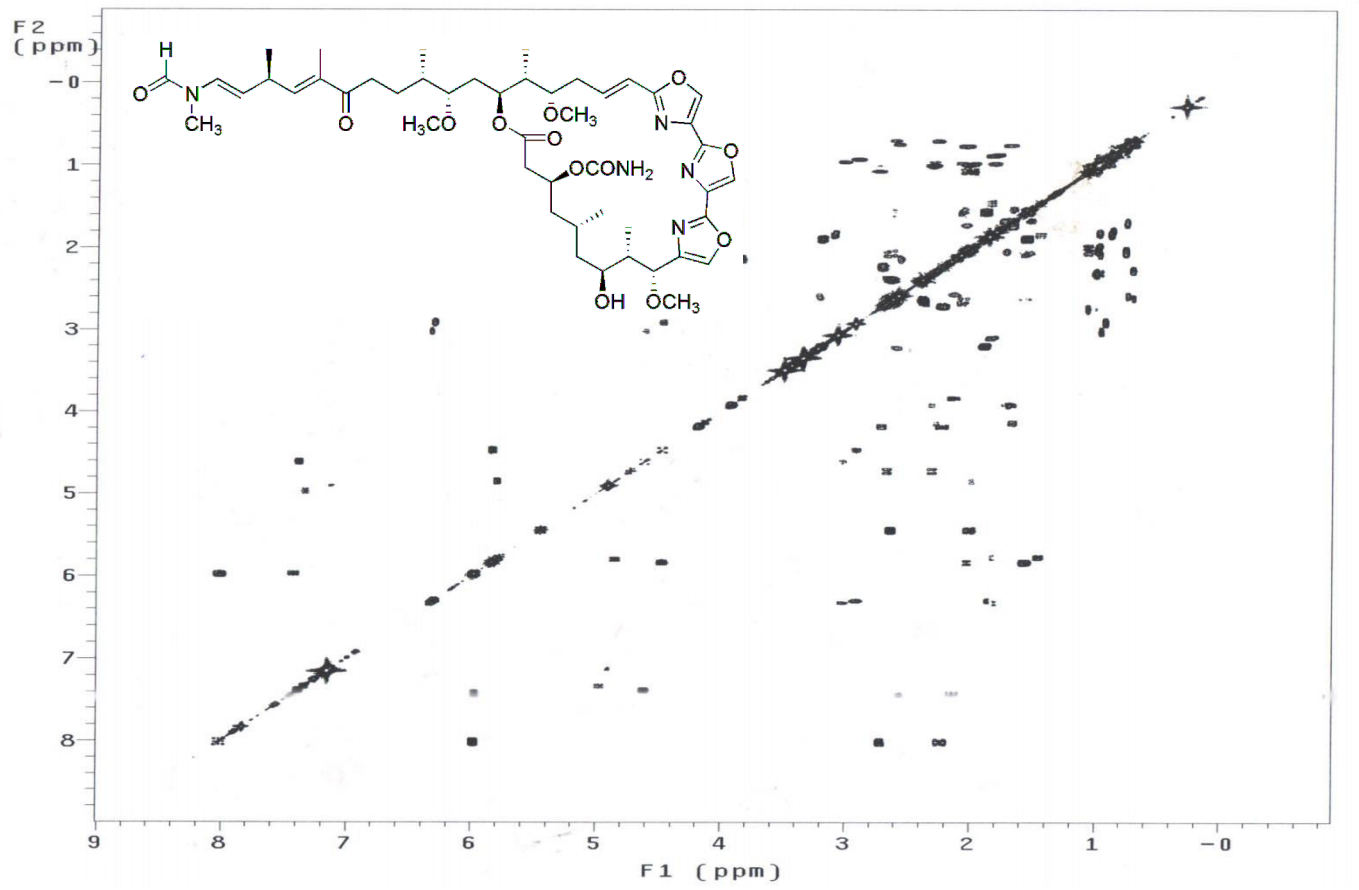
ESI mass kabiramide G



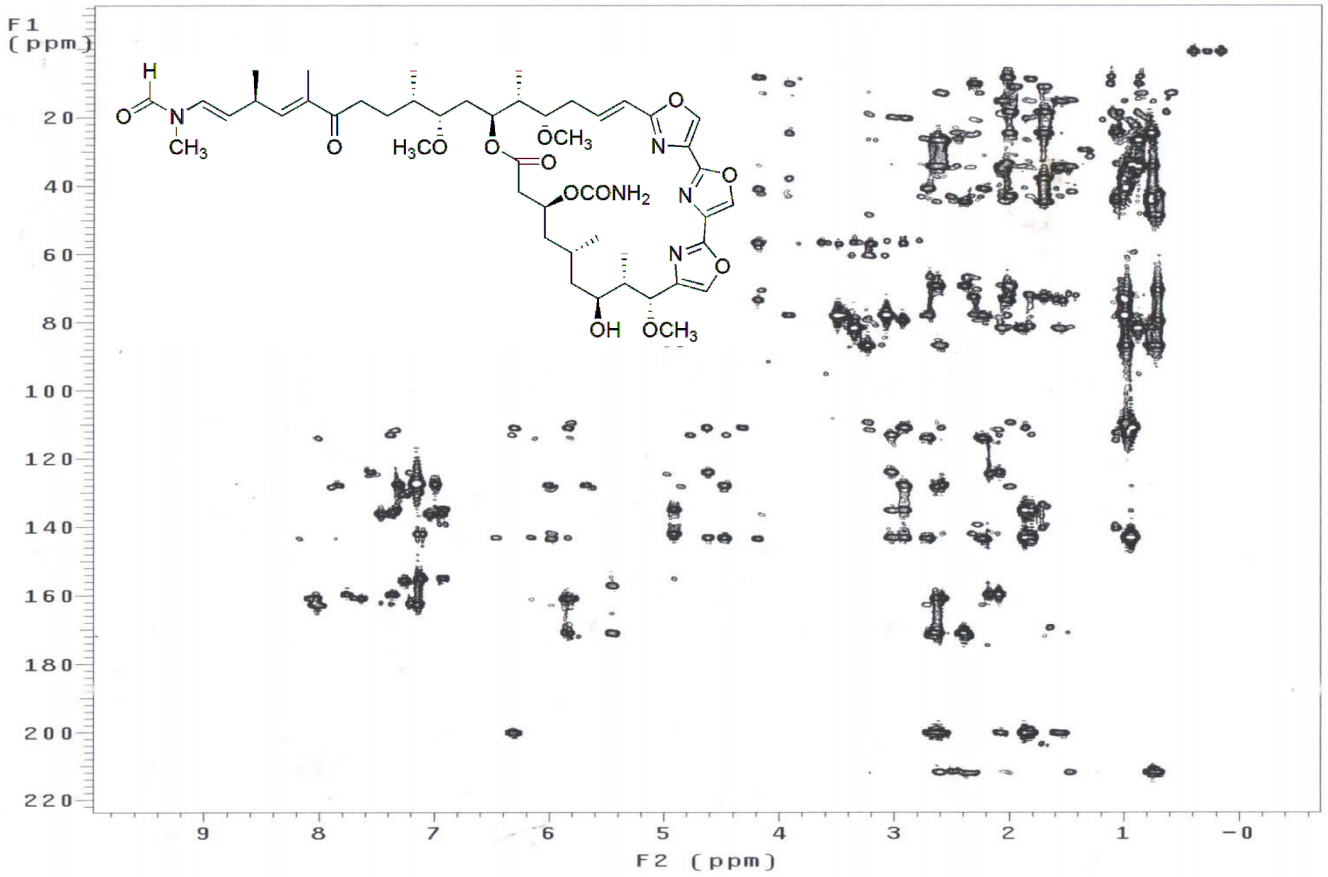
IR spectrum of kabiramide G (neat)



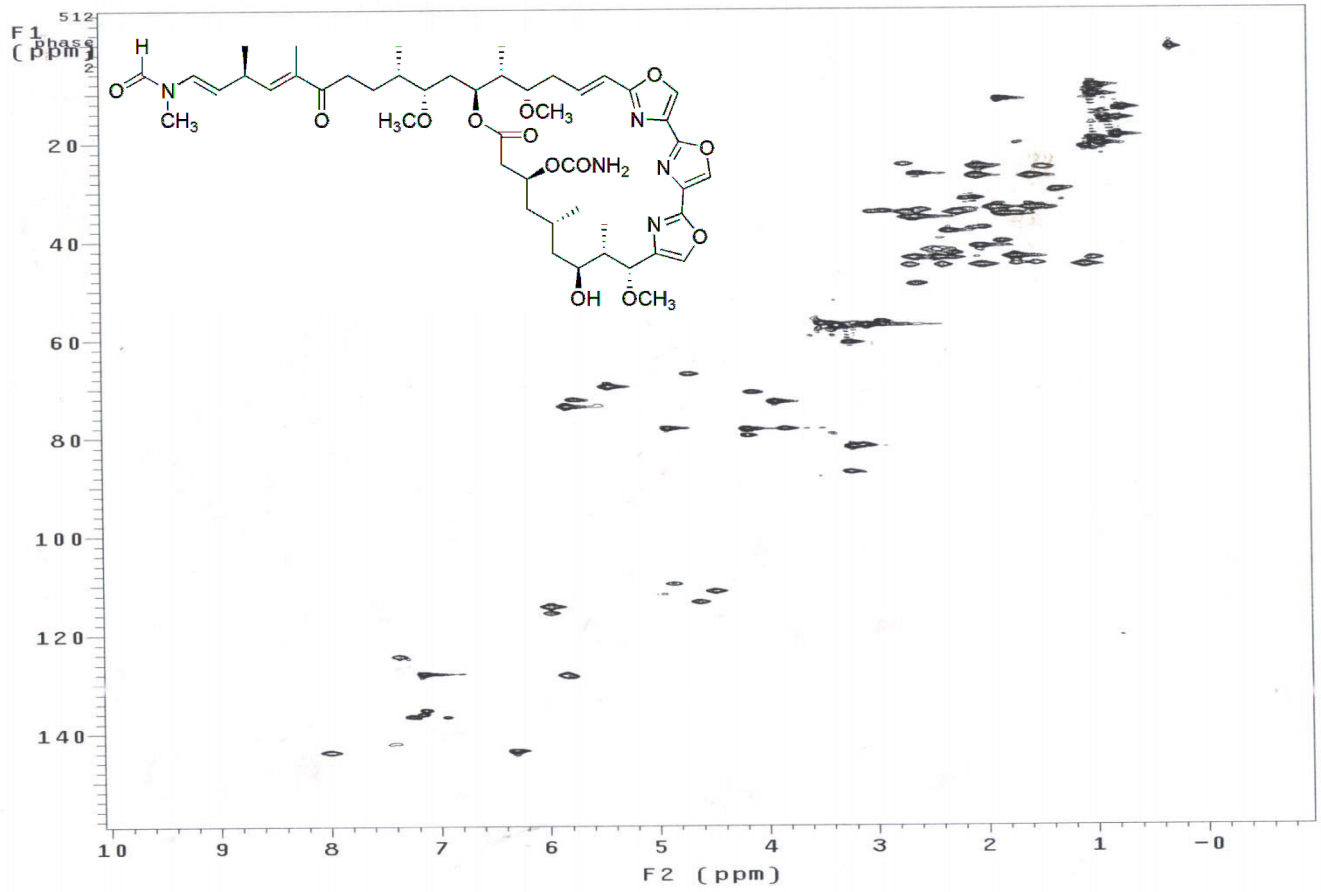
UV spectrum of kabiramide G



^{1}H-^{1}H COSY kabiramide G (500 MHz, C_6D_6)



HMBC spectrum of kabiramide G (500 MHz, C_6D_6)



HMQC spectrum of kabiramide G (500 MHz, C_6D_6)

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2013 Faculty of Pharmaceutical Sciences Thesis Supporting Grant, Prince of Songkla University.

List of Publication and Proceeding

Oyenike O. Olatunji and Anuchit Plubrukarn. Localization of kabiramides in the sponge *Pachastrissa nux* and sponge surface-attached bacteria. In proceeding of Annual meeting of American Society of Pharmacognosy. Copper Mountain, USA, 25-29 July, 2015.

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