Chemical studies on bioactive compounds from Indonesian marine organisms

（インドネシア産海洋生物に含まれる生理活性物質の化学的研究）

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Chapter 1. General Introduction

1. 1. Introduction

Oceans contain more than 80% of diverse plant and animal species in the world. They are a source of bioactive compounds [1]. The first biologically active marine natural product was formally published in late 1950 and it was recognized that marine plants and animals are genetically and biochemically unique in 1970 [2]. The marine ecosystem provides unlimited sources of bioactive compounds with various chemical structures, which are considered as potent sources for drug discovery [3]. A large number of drugs and agrochemicals have been isolated from marine resources. Recently, the study of new natural products discovered from marine organisms has significantly improved because marine represents an interesting source for the finding of novel secondary metabolites as candidates for new drugs [4]. These supported one of the important reasons that the ocean which is called the mother origin of life [5]. Ocean contain huge number of organisms which produce large number of secondary metabolites that different from land. For instance, bromine-containing compounds that only found in ocean organisms so they are very important for life. In recent years, many bioactive compounds have been isolated from various marine organisms like seaweeds, sponges, tunicates, and soft corals [6, 1].

Sponges, sessile metazoans, consist of gelatinous material-mesophyl, and are the simplest form of multicellular animals. Marine sponges are one of the most prolific and important source of new interesting chemicals. A large number of valuable novel compounds have been discovered from the marine sponges, leading to consider them as gold mine for scientists [7]. Chemical and physical conditions in the marine

environment and intensive pressure from competitors, predators, and pathogens have resulted in producing a wide array of chemically diverse secondary metabolites. Studies on marine natural substances for pharmaceutical purposes are still limited due to the lack of ethno-medical history and the difficulties of dealing with collection of marine organisms [8]. Since the improved of new diving methods and remote operated machines, it is possible to collect various marine samples so that during the last ten year over 5,000 new substances have been isolated from shallow waters to 900-m depths of the sea [9].

1.2. Significance of the discovery of bioactive compounds from the Indonesian marine organisms

Indonesia is one of the richest biodiversity and well known as a mega-diversity country. The Indonesian marine resources are abundant but still underexplored, particularly for a search of new bioactive compounds for pharmaceautical and agrochemical purposes. In the past three decades, studies on marine biodiversity including marine natural products chemistry have been actively conducted by collaboration with foreign researchers. The preliminary taxonomy review on the Indonesian marine sponges collected during the Snellius-II Expedition and unpublished data from the Sibolga Expedition (conductied in 1935) was reported in 1989.As many as 830 sponge species were identified which were compared to the species of relatively well-studied in the Indo-Pacific region and to the West Indian sponges, but the sponges appeared to be dissimilar to the Australian sponges [10].

Study on the discovery for new bioactive compounds from the Indonesian marine organisms was an attractive attention of scientists as reported for the first time in

1997. Four new compounds along with two known derivatives, were recognized as polybrominated diphenyl ether derivatives, isolated from the sponge *Dysidea herbacea* in West Sumatera. The compounds were revealed to show bioactivity in the brine shrimp lethality test with LC_{50} ranging from 0.94 to 3.30 μg/ml [11]. Inspired by the important invention, a series of studies on the search of new compounds from the Indonesian sponge *Xestospongia* sp. were published. Two new xestosaprol derivatives, xestosaprols D and E, significantly inhibited pathogenic multiple-resistance *Staphylococcus aureus* but showed a weak cytotoxic effect against SKOV cells [12]. In a further related report, eight new xestosaprol derivatives were identified, but unfortunately their biological activity were not observed yet so far [13].

Researches on this topic significantly increased, followed by the finding of two new cytotoxic candidaspongiolides from a sponge collected in West Timor Sea, which exhibited high cytotoxic activity against NBT-T2 cells with IC_{50} value of 4.7 and 19.0 ng/mL [14]. Hereafter, aaptamin derivatives isolated from the buthanol extract of the sponge *Aaptos suberitoides* collected in Ambon Water showed cytotoxic activity against the murine lymphoma L5178Y cell line with IC_{50} value ranging from 0.9 to 8.3 μM [15]. The ethanolic extract of the sponges *Aaptus suberitoides*, *Callyspongia* sp., and *Xestospongia* sp. collected in East Java Water exhibited a high cytotoxicity againt the tumor cell lines HT-29 and T47D [16]. Moreover, two fractions from crude ethanolic extract of the Indonesian marine sponge *Cinachyrella* sp. possessed a cytotoxic activity against T47D cancer cell line with IC_{50} of 93.8 and 72.9 μg/mL, which showed a similar activity shown by the commercial anticancer drug doxorubicin with IC_{50} of 86.7 μg/mL [17]. Seven new adociaquinone derivatives together with another seven related known compounds were elucidated, which revealed a potential

inhibitory activity against eight different protein kinases involved in cell proliferation, cancer, diabetes and neurodegenerative disorders as well as for their antioxidant and antibacterial activities [18].

A research on a sponge *Panera* sp. collected in South Sulawesi Sea, Samalona island, the same area as the sponge reported in our study, illustrated that a partial pure fraction showed cytotoxic activity against *Arthemia salina* Leach with LC_{50} of 0.1 μg/mL. This finding indicated that the single fraction had potential to be developed as an anticancer agent [19]. The GC-MS and IR profiles of dichloromethan extracts of the sponge *Clathria reinwardtii* collected in Spermonde Islands were obtained, but unfortunately no report on their biological activities was published so far [20]. A related study reported that a hexane extract of the marine sponge *Clathria basilana* collected in East Nusa Tenggara island exhibited an activity against A-549 cancer cells with inhibition more than 50% (MTT test) at a concentration of 125 ppm [21]. The newest report explained that crude methanol extracts of some unidentified sponges collected from Biak Water, the eastern part of Indonesia, possessed a cytotoxic activity against NBT-T2 cells with IC_{50} ranging from 10.0 to 0.1 μg/mL, suggesting that the marine sponges offer a promising anticancer candidates [22].

Studies on the isolation of bioactive compounds from the Indonesian soft coral *Sarcophyton* sp. were also reported. A new cembranoid (2-hydroxy crassocolide E), alongside some related known cembranoids sarcophytoxide, sarcrassin E, 11,12-epoxy sarcophytol A, and sarcophytol A showed a cytotoxic activity against MCF-7 [23]. Moreover, a bacterial symbiont of a soft coral *Sarcophyton* sp. collected from North Java Sea showed an antioxidant activity of pigment extracts against a DPPH solution [24].

1.3. Searching for anti-oomycete natural compounds

Plant diseases, caused by pathogens such as virus, fungi, bacteria, and oomycetes, always affect the people's daily life and health [25, 26]. Since plant pathogens can propagate rapidly via wind, insects, water, and human over great distances, plant diseases caused by the pathogens very easily spread and infect other plants, which are difficult to be protected [27]Song *et al*., 2009]. Tomato mosaic virus (TMV) is known to infect at least 125 plant species, such as tobacco, peppers, and tomatos [27]. The fungi *Fusarium oxysporum* caused banana panama diseases, leading to enermous cost woldwide in the 1950s. The rust fungi *Puccinia graminis* and *Puccinia striiformis* are the most widespread diseases of wheat globally [25].

Phytophthora is a genus of plant-damaging oomycetes (water molds), which are capable of causing enormous economic losses on crops worldwide, as well as environmental damage in natural ecosystem. *Phytophthora* species are well known as pathogens of agrochemical crops. The oomycete *Phytophthora infestans* was the effective agent of the potato blight that caused blight light of crops in the Great Irish Famine in 1845-1849, and still remains the most destructive pathogen of solanaceous crops [28, 29]. *Phytophthora* blight, caused by the phytophatogen *P. capsic*i, is a serious disease in crop production including cucurbit, pepper, tomato, eggplant, and a number of other important vegetable crops worldwide [30-32]. Further reports that *P. Capsci* seriously caused foot rot of black pepper [33, 34] and strowberry fruit rot disease [35].

Increasing of the agricultural productivity for over the half century has been due to control of pests with synthetic pesticides, which continue to be the major tools for protecting foods from agricultural pests [36, 37]. Otherwise, the intensive and uncontrolled use of highly toxic pesticides has caused serious problems to the environment such as pollution [38, 39] and toxicity to users [40]. The most important aspect of using pesticides is that pathogenic organisms have generated resistance to current active substances of pesticides in response to selection pressure due to the high doses and intensive applications of pesticides, causing great economic losses [40, 41]. Regarding to the accurate facts, both industry and academia have increased their efforts in finding the solutions to overcome those problems.

Control of the pathogens requires an alternative strategy instead of handling with synthetic chemicals. Several recomended approaches have been introduced to minimize the negative consequences of the continous pesticide use including application of the biological control of plant diseases. *Phytophthora infestans* causing the late blight on tomato and chili plant was effectively controlled by mycoextracts of *Fusarium graminearum* and *F. culmorum* [42]Carabet *et al*., 2008], by antagonistic actinomycetes *S. Rubrolavendulae* [43], and by rhizobacterial isolates *Bacillus* sp. and *Paenibacillus* sp. [44]. Biological control has many adventages as a pest control method compared with pesticides. One of the most important benefits of using biological control is that it is an environmental friendly method and does not introduce pollutants into environment. Besides biological disease control is an attractive alternative strategy for the control of plant diseases, because it also provides practices compatible with the goal of a sustainable agricultural system [45]. However, biological control agents limit the subsequent use of pesticides, where they are being used against one pest. It is clearly

difficult to continue using pesticides against other pests on the same crops or other diseases vector in the same field [46].

There is a need to search for alternative chemical substances in order to address environmental concern. Natural products and their analogs are particularly attractive due to their adventages, such as complex substances with unique mode of action, absorption by plants, low mammalian toxicity, and short environmental persistance [47, 48]. In order to search for new bioactive natural compounds for agrochemical purposes, secondary metabolites from marine organisms attracted the interest of scientists. Among the marine organisms, macroalgae and sponges are the most diverse species chemically [49, 50]. Several newest alternatives reported to protect plants from diseases caused phytophatogens were by using crude extracts and compounds from macroalgae and sponges.

A number of seaweed extracts were reported to possess antiphytophatogenic activities. The ethanolic extracts of the red algae *Gracilaria chilensis* and the brown algae *Durvillaea antartica* prevented the growth of *Phytophthora cinnamomi* [51]. Extract from seaweed *Turbinaria conoides* showed an activity against root rot pathogen *Fusarium oxysporum* [52]. Moreover, the extract of brown seaweeds *Cystoseria myriophylloides*, *Laminaria digitata*, and *Fucus spiralis* revealed to show a significant inhibition against tomato pathogens *Verticillium dahliae* and *Agrobacterium tumefaciens* [53]. A further report explained that crude methanolic extract *of Sargassum vulgare* possessed a strong activity against *Pythium aphanidermatum* causing pythium leak disease in potato [54]. In addition, crude extracts of browm seaweeds and red seaweeds revealed to show an antifungal activity both *in vitro* and*in vivo* against postharvest pathogens *Botrytis cinerea, Monilina laxa,* and *Penicillium digitatum* [55].

These evidences expressed that macroalgae contain compounds with different chemical properties, which could be considered for controlling specific plant pathogens.

Synthetic topsentin, a sponge-derived natural product, and its analogs revealed inhibition activities against plant pathogens *Corynespora cassiicola, Phytophthora capsici, Sclerotinia sclerotiorum, Rhizoctonia solani,* and *Botrytis cinerea* [56]. Moreover, halenaquinone, xestoquinone analogs, extracted from a sponge *Xestospongia* sp. demonstrated an inhibitory activity against *P. capsisi* at a dose of 25 μg/disc [57].

1.4. Searching for cytotoxic natural compounds

The ability of a certain substance to inhibit the growth of human cancer cell lines *in vitro* is a significant indicator that the substance has a promising activity as an anticancer agent. Cancer is defined as a group of the diseases characterized by the uncontrolled growth and spread of abnormal cells and one of the deadliest diseases globally. Cancer represents the second most common cause of death worldwide after the cardiovascular diseases according to the World Health Organization [58]. The finding for new medicines is an urgent priority in cancer chemotherapy due to the expenditious development of resistance to multiple therapeutic drugs, high toxicity to normal cells, low selectivity, and their negative side effects. The search for novel natural substances to minimize the negative effects and to have high selectivity for cancer cells is urgent to conduct. [59].

Among the organisms of marine origanisms, sponges are the most popular source of the greatest diversity of marine secondary metabolites, which have provided about 30% of all marine natural products [60]. They are considered as an important source of bioactive compounds that are interesting candicates for new drugs,

particularly as anticancer, anti-imflammatory, and analgesic agents [61]. A summary of several natural compounds/extract isolated from marine sponges and their bioactivities in the last five years is shown in Table 1.1.

Table 1.1. Several bioactive compounds/extracts from marine sponges in the last five years and their activities

Compound /Extract	**Species**	**Activity**	**Reference**
Scalaradial and cacospongianolide	*Cacospongia scalaris Fasciospongia cavernosa*	Apoptosis-inducing factors in human carcinoma cell lines	[62]
Crambescidin 816 and crambescin C1	*Crambe crambe*	Cytotoxic against cortical neurons, blok calcium channels in a neuroblastoma X glioma cell line	[63, 64]
Methanol extract	*Haliclona* sp.	Anticancer against A549 cells	[65]
Methanol extract	*Geodia cydonium*	Anti-imflamatory effect on human breast cancer MCF-7 cell line	[66]
Scalarane sestetrterpenoid	*Carteriospongia* sp.	antileukemia	[67]
Crambescidins 800 and 814	*Monanchora* sp	Anti cancer against HCT-116, MDA-435, HL-60, and B16-F10	[68]
Monanchocidin B and Ptilomycalin A	*Monanchora pulchra*	Cytotoxic activity against HeLa cells	[69]
Sesterpene analogs	*Hyrtios erectus*	Antiproliferative activity against breast adenocarcinoma (MCF-7), colorectal carcinoma (HCT-116), and hepatocellular carcinoma cells (HepG2)	[70]
n-hexane extract	*Hyrtios erectus*	Inhibit proliferation of the breast cancer MCF-7 cell	[71]
EtOAc extract	*Pseudoceratina arabica*	Antimicrobial against *Escherichia coli , Enterobacter cloacae, Klebsiella oxytoca*	[72]
Plakortinic acid A and B	*Plakortis halichondrioides - Xestospongia*	Anti cancer against A2058 melanoma and DU-145 prostate cancer	[73]

	deweerdtae	cells	
Unguiculin A and Ptilomycalins E-H	*Monanchora unguiculata*	Cytotoxic against KB cells, and antimalaria against *Plasmodium falciparum*	[74]

1.5. Crambescidin derivatives and their biological activity

Crambescidins are a member of pentacyclic guanidine alkaloids, besides ptilomycalins, monanchocidins, and monanchomycalins. Ptilomycalin A isolated from the sponges *Ptilocaulis spicilifer* and *Hemimycale* sp. is the first member of that group [75]. Crambescidins 800, 816, 830, and 844 were the first crambescidin analogs isolated from the Mediterranean sponge *Crambe crambe*, characterized by pentacyclic guanidine alkaloid possessing an ester with a linear ω-hydroxy fatty acid amide of a hydroxyspermidine. All the crambesdins, except for crambescidin 830, showed antiviral and cytotoxic activities against HSV-1 and L1210 murine leukemia cells, respectively [76]. This finding was followed by discovery of crambescidins 359 and 431 isolated from the sponge *Monanchora unguiculata* [77]. Crambescidin 359 is the simplest member with out substituents at C-13 and C-14, meanwhile crambescidin 431 possessing an ethyl ester at C-14. Moreover, crambescidin 826 isolated from *Monanchora* sp. showed an inhibitory activity against HIV-1 envelop-mediated fusion [78]. The newest members of crambescidins were isolated from a French Polynesian sponge *Monanchora* sp. And named crambescidins 786 and 814 [68]. The summary of crambescidin derivatives and their bioligical properties is described in Table 1.2.

Table 1.2.Summary of crambescidin analogsisolated from marine sponges and their biological activity

Compound	Species	Activity	Reference
Crambescidin 800	*Crambe crambe*	Antivirus HIV-1 Citotoxic against L1210 murine leukemia cells Protected HT22 cells against oxidative tixicity Anti tubercolosis, antimicrobial, and antiprotozoal activity, and cytotoxicity against KB cell line, HCT-116, MDA-435, HL-60, B16-F10	[68, 76, 78-81]
Crambescidin 816	*Crambe crambe*	Antivirus HIV-1 Citotoxic against L1210 murine leukemia cells Ca++ antagonist effect and inhibit acetylcholin-induced contraction Cytotoxic effect against tumour cell HepG2	[76, 78, 79, 82, 83]
Crambescidin 844	*Crambe crambe*	Antivirus HIV-1 Cytotoxic against L1210 murine leukemia cells	[76, 78, 79]
Crambescidin 826	*Monanchora* sp	Antivirus HIV-1 Cytotoxic against KB cell line and B16-F10	[68, 78]
Crambescidin 786	*Monanchora* sp	Cytotoxic against KB cell line, HCT-116, MRC-5, and HL-60	[68]
Crambescidin 814	*Monanchora* sp	Cytotoxic against KB cell line, HCT-116, and HL-60	[68]
Crambescidin 657	*Crambe crambe*	Cytotoxic against tumor cel lines : P-388, A-549, HT-29, MEL-28	[84, 85]

1.6. Objectives

The objectives of this study are to investigate the isolation and structure elucidation of bioactive substances from the Indonesian marine organisms especially the red algae*Laurencia intricata* and the sponge *Clathria bulbotoxa* as well as their biological activities such as anti-oomycete and cytotoxic activities against the plant patogen *Phytophthora capsici* and human epidermoid carcinoma A413 cells, respectively.

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Chapter 2. Anti-oomycete and Cytotoxic Activity of Brominated Sesquiterpenes from the Red Seaweed *Laurencia intricata*

2.1. Introduction

Among various sources of new drugs, natural products are of particular significance. Approximately one third of today's best selling drugs are either natural products or have been developed based on lead structures provided by nature [1]. A large number of marine organisms, including seaweeds and sponges, produce secondary metabolites that have a bioactivity against pathogenic microorganisms or certain physiological states of human diseases. Regarding to drug discovery and develovement, marine organisms started to attract interest from scientists approximately 60 years ago with the discovery of the sponge-derived nucleosides spongothymidine and spongouridine [2]. Meanwhile, scientific researches on therapeutic property of seaweeds began during 1970s, which was successful in isolating compounds from brown seaweeds that show anticancer and antitumor activity [3]. In addition, soft corals are well known as potential sources of various bioactive compounds containing terpenoids and sterols that show pharmacological activities [4].

Seaweeds are classified into three categories based on the presence of specific pigments, Chlorophyceae (green algae), Phaeophyceae (brown algae), and Rhodophyceae (red algae). The color of green algae is due to the presence of chlorophylls *a* and *b*, while fucoxanthin, chlorophylls *a* and *c* are responsible for the greenish brown color of Phaeophyceae. The red color of Rhodophycea is indicated by the presence of phycobilins [5, 6]. Seaweeds are potential sources of bioactive substances with a variety of biological activities leading to significant opportunities in development of new pharmaceauticals, nutraceuticals, and cosmeceuticals [7-9].

Seaweeds are currently being explored as novel and abundant resources of substances for drugs and cosmetic application [10, 11].

Seaweeds produce a large variety of natural compounds. Most of them are halogenated, revealing the availability of chloride and bromide ions in seawater as the main halogens used to enhance biological activity of secondary metabolites, while iodine and fluorine are unusual halogens in natural products [12, 13]. Bromide ion is well known to be used by mostly algae to produce halogeno compounds, although concentration of chloride ion is higher than bromide ion in seawater [14]. On the other hand, iodination is more intensive in the brown algae than red and green seaweeds, for instance, *Laminaria digitata* accumulates and uses iodine in a large concentration representing content about 1% of dry weight [15]. Accordingly, about 90 and 7% secondary metabolites isolated from red and green algae contain bromine or chlorine, respectively, while less than 1% found in brown algal compound [16].

Among the marine macroalgae, the red algae of the genus *Laurencia* are a more diverse group with about 140 species and distribute throughout the world's ocean except in the Arctic and Antarctic ocean [17], and about 500 halogenated metabolites have been isolated from more than 60 species [18]. This genus is considered as the most prolific producers of halogenated secondary metabolites in the marine environment and produces mainly sesquiterpenes and a few diterpenes, triterpenes, and other types [19-21].

A variety of structures of secondary metabolites have been characterized from the genus *Laurencia* collected in various parts of the world since 1960s, and are mostly halogenated with bromine and chlorine atoms [22, 23]. Halogenated substances have been reported to exhibit various biological properties such as antioxidant, antimicrobia

[24, 25], antifeedant [26], anthelmintic [27, 28], and cytotoxic activities [29, 30]. The summary of biological activity of compounds isolated from the genus *Laurencia* is summarized in Table 2.1.

Table 2.1. Biological activity of secondary metabolites from the genus *Laurencia*

Compound	Species	Activity	Reference
Scopariol, isorigidol,	*L. scoparia*	Anthelmintic against parasit *Nippostrongylus brasiliensis* (L4)	[31]
8-bromo-10-epi-β-snyderol	*L. obtusa*	Antimalaria against *Plasmodium falciparum*	[28]
Chamigrene-type sesquiterpeniods	*L.chondrioides*	Inhibit fish and human pathogen bacteria	[32]
Laurebiphenyl	*L. tristicha*	Cytotoxic against A549, BGC-823, Bel 7402, HCT-8, and HeLa cell lines	[33]
Hexadecanoic acid, octadecanoic acid	*L. brandenii*	Antimicrobial against human pathogens, antipest against *Sitopzhilus oryzae*, meggoticidal activity against *Sarcophage albiceps* larvae, termicidal activity against *Microtermes obesi*	[34]
Elatol	*L. microcladia*	Anticancer against B16F10, A 549), DU145, MCF-7, and L292	[35]
Laurene-type sesquiterpenoid	*L. obtusa*	Cytotoxic against Ehrlich ascites carcinoma	[36]
Ethanol extract	*L. pacifica*	Cytotoxic against cancer cell lines HT29, SJ-G2, MCF-7, A2780, A431, DU145, BE2-C, MIA, ADM, and MCF10A	[37]
β–Amyrin	*L. microcladia*	Antimicrobial against *Staphylococcus aureus* and *Salmonella thypi*	[38]
Elatol and obtusol	*L. dendroidea*	Larvacidal activity against *Aedes aegypti*	[39]
Ethanol/chloroform extract	*L. papillosa*	Cytotoxic against jurkat cancer cell line (acute lymphoblastic leukemia)	[40]
Laurinterol	*L. nidifica*	Cytotoxic against brine shrimp larvae, repellent activity against maize weevil *Sioiphiluszeamais*, insecticidal activity against *Reticulitermessperatus*, and acetylcholinesterase inhibitory	[41]

effect

A549: human lung carcinoma; BGC-823: stomach cancer; Bel 7402: hepatoma; HCT-8: colon cancer; B16F10: melanoma murine, DU145: carcinoma of human prostate; MCF-7: human breast adenocarcinoma; L292: mouse fibroblast; HT29: colon carcinoma; SJ-G2: glicoblastoma; A2780: ovary carcinoma; A431: skin carcinoma; BE2-C: neuroblastoma; MIA: pancreas; ADM: murine glioblastoma; MCF10A: normal breast cell line

Studies on the isolation and biological activity investigation of the halogenated sesquiterpenes from the genus *Laurencia* have been continously conducted besides diterpenes and triperpenes [42]. Aplysistatin, a brominated sesquiterpene, was discovered for the first time from an acetone-hexane extract of the South Pacific Ocean sea hare (*Aplysia angasi*), revealing significant inhibition of murine lymphomatic leukemia P-388 [43]. The structural features including an oxepane ring and the interesting biological activity have inspired other chemists to research total synthesis of the aplysistatin [44-47] and related bioactive brominated sesquiterpenoids palisadins A and B [48]. A further study revealed that aplysistatin isolated from *Laurencia snackeyi* possessed anti inflammatory activity, was identified along with other halogenated metabolites of palisadin A, and palisol [49].

Two new brominated sesqueterpenes oxachamigrene and 5-acetoxyoxachamigrene were isolated from *L. obtusa* but no biological activity was reported so far [50]. Tiomanene and acetylmajopolene derivatives were found from a Malaysian *Laurencia* sp. showing a moderate antimicrobial activity against some marine bacteria [51]. Chamigrane sesquiterpenes, laurokamins A–C were isolated from *L. okamurae* [52] Furthermore, brominated diterpenes glandulaurencianols A–C were isolated from *L. glandulifera* and the sea hare *Aplysia punctata* [53], antifungal laurane sesquiterpene derivatives from *L. okamurai* [54], and cuparane-derived sesquiterpenes purified from *L. tristicha* [55].

As a part of a search for bioactive compounds from the Indonesian marine organisms, I report the results of examination of 21 species of seaweeds, 6 species of sponges, and 2 species of soft corals, collected from 5 different islands in the area of South Sulawesi Sea. The aim of the present work described in this chapter is to select promising crude extracts showing significant activity as anti-oomycete and cytotoxic agents against the plant pathogen *Phytophthora capsici* and the human epidermoid carcinoma A431 cells, respectively. Other important points are to isolate and characterize anti-oomycete and cytotoxic compounds from the seaweed *Laurencia intricata* and then to evaluate their biological activities.

2.2. Results and discussion

2.2.1. Screening of biological activities

As many as 29 marine organisms were collected in three different periods in six small islands in South Sulawesi Waters. The organisms were categorized and identified in three groups and as seaweeds, sponges, and soft corals (Table 2.2, Figure 2.1). The freeze dried organisms were separately homogenated then partitioned in two different solvent polarity to obtain crude hexane and methanol extracts for each species. Screening was performed as preliminary assessment to investigate the activity of crude extracts as antiphytopathogenic and cytotoxic candidates against the plant pathogen *Phytophthora capsici* and A431 human cell cancer lines. All potential crude extracts were selected based on their activity to inhibit colony growth with inhibition zone > 1.0 mm at dose 50 μg/disk, and 50% inhibition of cancer cell population with IC_{50} value < 0.01 μg/mL.

Table 2.2. The marine organism species collected in different small islands in three periods of collection

Organism	Species	Sampling site (island)	Date of collection
Seaweed	*Amphiroa fragilissima*	Libukkang	March 16, 2014
	Acanthophora muscoides	Lae Lae	March 21, 2014
	Dictyota dichotoma	Lae Lae	March 21, 2014
	Halimeda opuntia	Barrang Lompo	March, 8, 2014
	Halimeda macroloba	Barrang Lompo	March, 8, 2014
	Halimeda discoidea	Lae Lae	March 21, 2014
	Hypnea cervicornis	Libukkang	March 16, 2014
	Halymenia durviallei	Lae Lae	March 21, 2014
	Hormophysa triquetra	Barrang Lompo	March, 8, 2014
	Laurencia intricata	Barrang Lompo	March, 8, 2014
	Sargassum cinereum	Barrang Lompo	March, 8, 2014
	Turbinaria conoides	Lae Lae	March 21, 2014
	Dictyospheria cavernosa	Kodingareng Keke	Aug 1, 2014
	Chlorodermis fastigiata	Samalona	Aug 10, 2014
	Chaetomorpha crassa	Lae Lae	Aug 8, 2015
	Padina australis	Lae Lae	Aug 8, 2015
	Ulva reticulate	Lae Lae	Aug 8, 2015
	Gracilaria coronopifolia	Barrang Lompo	Aug 2, 2015
	Gracilaria eucheumatoides	Barrang Lompo	Aug 2, 2015
	Codium fragile	Lae Lae	Aug 8, 2015
	Coulerva sertularioides	Lae Lae	Aug 8, 2015
Sponge	*Clathria reinwardti*	Libukkang	March 16, 2014
	Carteriospongia foliascens	Libukkang	March 16, 2014
	Tedania ignis	Kodingareng Keke	Aug 1, 2014
	Haliclona koromella	Kodingareng Keke	Aug 2, 2014
	Agelas conifera	Kodingareng Keke	Aug 2, 2014
	Clathria bulbotoxa	Samalona	Aug 15, 2015
Soft coral	*Lobophytum pauciflorum*	Libukkang	March 16, 2014
	Sarcophyton glaucum	Libukkang	March 16, 2014

Figure. 2.1. Twenty nine marine organisms collected in South Sulawesi Sea

Table 2.3. Yield of crude hexane and metanol extracts of the twenty nine marine organisms

Species	Wet weight (g)	Weight of extract (mg)	
		Hexane	90% Methanol
Amphiroa fragilissima	780	460	130
Acanthophora muscoides	340	180	100
Dictyota dichotoma	538	380	200
Halimeda opuntia	558	310	210
Halimeda macroloba	663	340	330
Halimeda discoidea	681	490	330
Hypnea cervicornis	434	100	90
Halymenia durviallei	1,300	900	430
Hormophysa triquetra	109	110	60
Laurencia intricata	500	340	260
Sargassum cinereum	1,000	130	110
Turbinaria conoides	333	700	370
Dictyospheria cavernosa	178	130	60
Chlorodermis fastigiata	409	980	740
Chaetomorpha crassa	850	920	987
Padina australis	1,200	2,417	2,845
Ulva reticulata	1,300	1,538	1,285
Gracilaria coronopifolia	900	283	278
Gracilaria eucheumatoides	1,300	299	599
Codium fragile	1,200	578	489
Coulerva sertularioides	1,300	787	1,073
Clathria reinwardti	983	1,980	450
Carteriospongia foliascens	250	590	720
Tedania ignis	145	280	70
Haliclona koromella	70	40	20
Agelas conifera	320	610	230
Clathria bulbotoxa	750	2,540	3,815
Lobophytum pauciflarum	1,250	3,720	2,871
Sarcophyton glaucum	765	6,831	4,472

This preliminary assessment revealed that a few species of the collected Indonesian marine organisms showing potential as sources for specified agrochemical and anticancer substances. The species mentioned are the sponges *T. ignis* and *C. bulbotoxa,* along the seaweeds *L. intricata, G. coronopifolia* and *G. eucheumatoides*. Moreover, the crude methanol and hexane extracts of the red seaweed *L. intricata* indicated remarkable activity against the plant patogent *P. capsici* (inhibition zone = 1.0

and 2.0 mm) and the A431 cancer cells (IC_{50} = 0.034 μg/mL), respectively, while the methanol extract of the sponge *C. bulbotoxa* showed significant activity for both tests (inhibition zone = 2.0 mm, IC_{50} = 0.046 μg/mL). The most anti-oomycete methanol extract of *T. ignis* was separated and purified, unfortunately, the amount of active purified compound was not sufficient for structural analyses. Accordingly, methanol and hexane extracts of *L. intricata* and methanol extract of *C. bulbotoxa* were selected as the most promising candidates (Figures 2.2 and 2.3) then promoted to further bioactivity-guided purification in order to search for new active compounds.

Table 2.4. Result of screening of anti-oomycete and cytotoxic activity of crude extracts

Species	Anti-oomycete (IZ)[a]		Cytotoxicity (IC_{50})[b]	
	Hexane	Methanol	Hexane	Methanol
Amphiroa fragilissima	0	0	0.28	1.98
Acanthophora muscoides	0	0	0.39	1.69
Dictyota dichotoma	0	0	1.42	1.71
Halimeda opuntia	0	0	0.52	2.69
Halimeda macroloba	0	0	1.71	2.22
Halimeda discoidea	0	1.0	0.21	2.91
Hypnea cervicornis	0	0	0.81	1.65
Halymenia durviallei	0	0	1.37	0.94
Hormophysa triquetra	0	0	0.52	1.79
Laurencia intricata	0	2.0	0.034	0.73
Sargassum cinereum	0	0	1.40	2.09
Turbinaria conoides	0	0	1.14	0.49
Dictyospheria cavernosa	0	1.0	2.49	2.1
Chlorodermis fastigiata	0	0	2.12	1.8
Chaetomorpha crassa	0	0	1.99	0.74
Padina australis	0	0	1.57	0.57
Ulva reticulata	0	0	1.95	1.66
Gracilaria coronopifolia	0	1.5	0.23	0.29
Gracilaria eucheumatoides	0	0	0.092	0.17
Codium fragile	0	0	0.61	0.36
Coulerva sertularioides	0	1.0	0.54	0.36
Clathria reinwardti	0	1.0	0.32	2.5
Carteriospongia foliascens	0	0	0.14	0.31
Tedania ignis	0	4.0	3.05	0.036
Haliclona koromella	0	0	2.43	1.40
Agelas conifera	0	0	2.87	0.22

Clathria bulbotoxa	1.0	2.0	1.86	0.046
Lobophytum pauciflarum	0	0	2.24	0.31
Sarcophyton glaucum	0	0	1.89	1.52

[a] Inhibition zone (IZ, mm) against plant pathogen *Phytophthora capsici* at a dose of 50 μg/disk
[b] against human-vulval-derived epidermoid carcinoma A431 cells at concentrations of 0, 0.1, 1, and 10 μg/mL

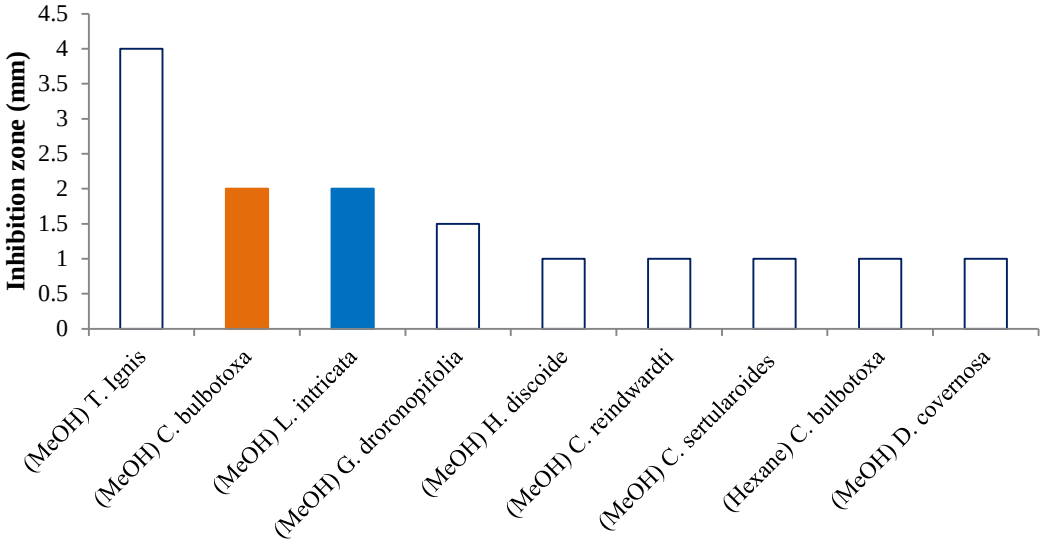


Figure 2.2. Anti-oomycete activity of the best nine crude extracts against *Phytophthora capsici* at a dose of 50 μg/disk

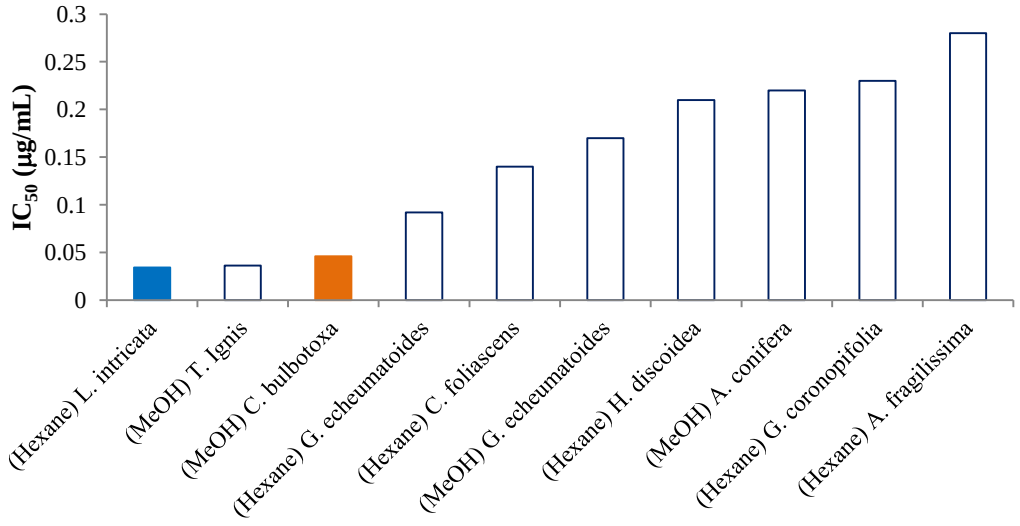


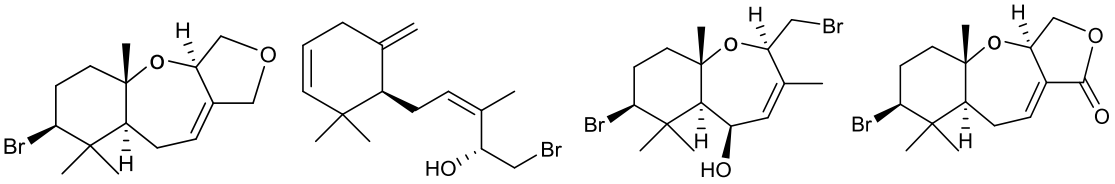
Figure 2.3. Cytotoxic activity of the best ten crude extracts against human-vulval epidermoid carcinoma A431 cells

Screening of biological activity of the crude hexane and methanol extracts was conducted to evaluate the potency of the marine organisms as anti-oomycete and cytotoxic agents against the plant pathogen *Phytophthora capsici* and human epidermoid carcinoma A431 cells, respectively. Crude extracts showing significant activity against the two assays were selected as candidates and subjected to next bioassay-guided fractionation.

2.2.2. Bioactive compounds from the seaweed *Laurencia intricata*

Description of the selected red seaweed has been depicted with ecological notes and referred to its systematical group as follows, Division: Rhodophyta; Class: Florideophyceae; Order: Ceramiales; Famili: Rhodomelaceae; Genus: *Laurencia*; Species: *Laurencia intricata* (Figure 2.4). The crude hexane (340 mg) and methanol (260 mg) extracts were found to be active as anti-oomycete against the plant pathogen *Phytophthora capsici* (inhibition zone = 2 mm at 50 μg/disk) and cytotoxic against the human vulval epidermoid A431 cells (IC_{50} = 0.034 μg/mL), respectively. Those extracts were combined then subjected to boiassay-guided separation followed by the final reversed-phase HPLC to obtain four active substances. These substances were found to be known brominated sesquiterpenes namely palisadin A, palisol, 5β-hydroxypalisadin B, and aplysistatin (Figure 2.5) respectively, by comparison with reported spectroscopic data. The four metabolites were evaluated for the cytotoxic and anti-oomycete activities.

Figure 2.4. *Laurencia intricata*



palisadin A palisol 5β-hydroxypalisadin B aplysistatin

Figure 2.5. Chemical structures of the known brominated sesquiterpenes

The ^{1}H and ^{13}C NMR spectral data of each fraction were compared to those of reported compounds, which indicated that fraction 2.3.2 was as palisadin A (Figures 2.6 ans 2.7, Table 2.5) [49, 56], fraction 2.3.4 was identical with palisol (Figure 2.9, Table 2.6) [49], fraction 2.5.11 was found as 5β-hydroxypalisadin B (Figures 2.11 and 2.12, Table 2.7) [57], and fraction 2.6.6 was assigned as aplysistatin (Figures 2.14 and 2.15, Table 2.8) [49].

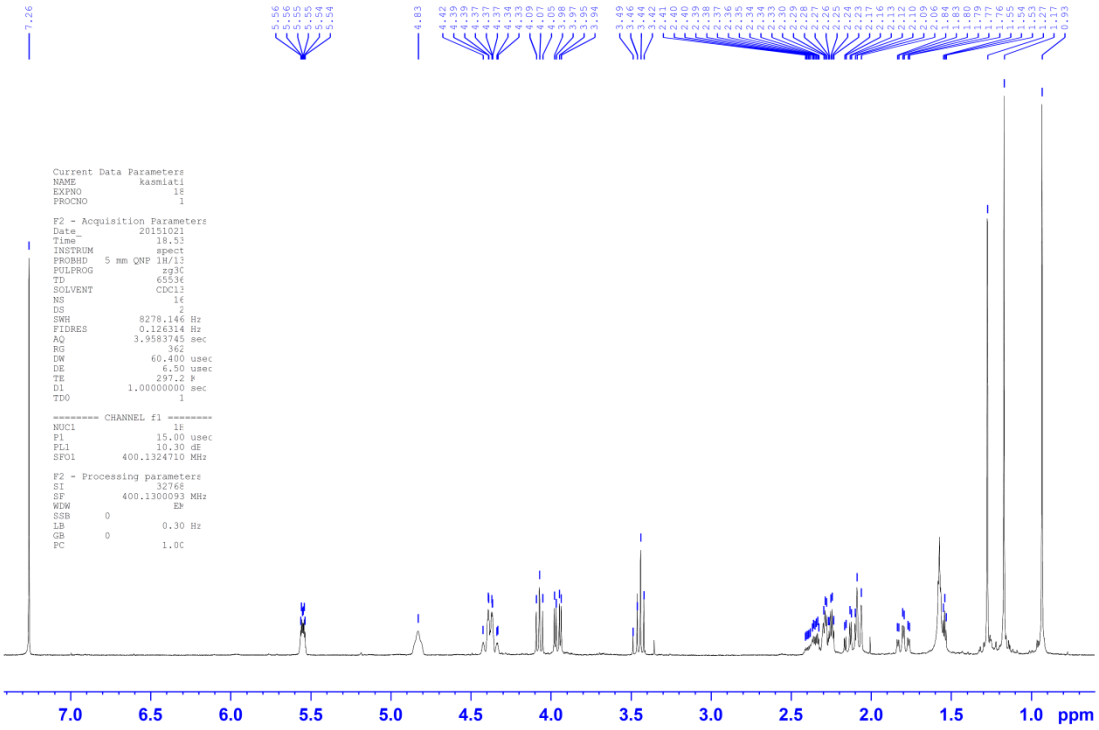


Figure 2.6. ^{1}H NMR spectra of palisadin A ($CDCl_3$, 400 MHz)

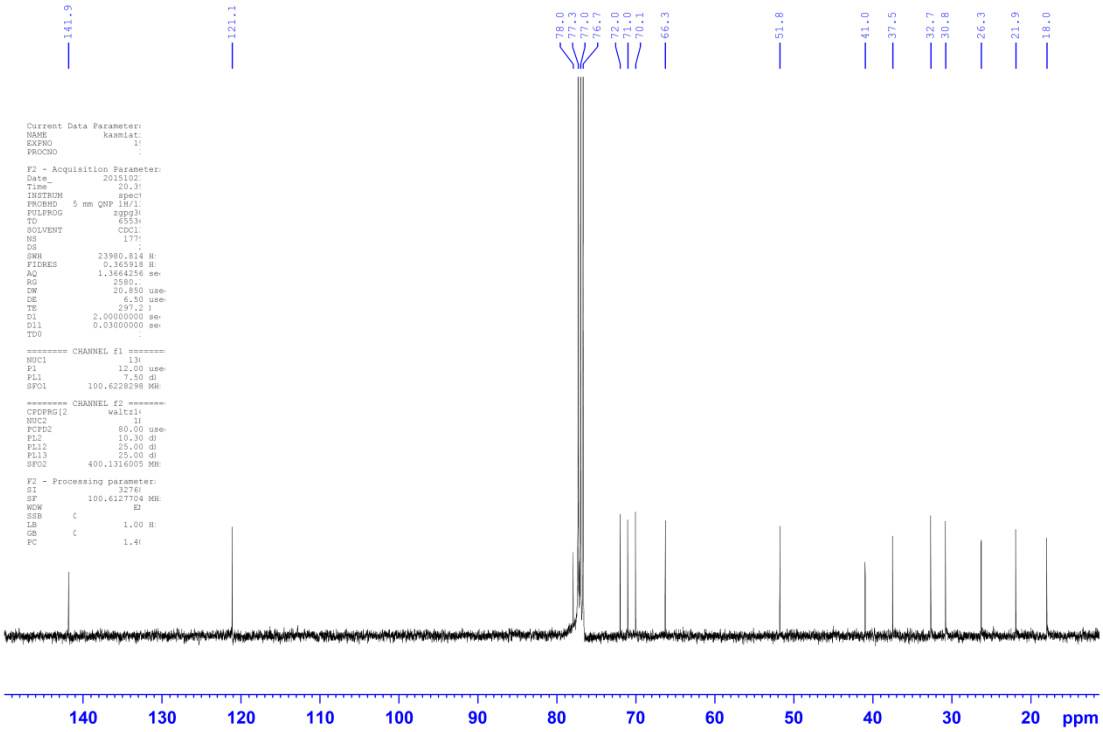


Figure 2.7. ^{13}C NMR spectra of palisadin A ($CDCl_3$, 100 MHz)

Table 2.5. NMR data for palisadin A and reference data ($CDCl_3$)

Position	palisadin A (400 MHz)		reference data [49] (600 MHz)	
	δ_C (ppm)	δ_H (ppm, m, *J* in Hz)	δ_C (ppm)	δ_H (ppm, m, *J* in Hz)
1a	72.0	3.45, t (8.4)	72.7	3.44, dd (8.0, 8.0)
1b		4.07, t (8.0)		4.07, dd (8.0, 8.0)
2	70.1	4.83, m	70.7	4.83, br s
3	141.9	-	142.5	-
4	121.2	5.55, m	121.8	5.55, br s
5a	26.3	2.29, m	27.0	2.36, m
5b		2.37, m		
6	51.8	2.08, m	52.5	2.05, m
7	78.0	-	78.7	-
8a	37.5	1.55, dt (12.0, 4.0)	38.2	1.55, ddd (3.0, 3.0, 13.0)
8b		1.80, td (16.0, 4.0)		1.80, ddd (3.0, 3.0, 13.0)
9a	32.7	2.15, dd (13.6, 3.6)	33.4	2.26, m
9b		2.26, m		
10	66.3	3.96, dd (12.8, 4.0)	67.0	3.96, dd (5.0, 12.0)
11	41.0	-	41.7	-
12	70.0	4.37, m	71.7	4.38, dd (13.0, 13.0)
13	22.0	1.27, s	22.6	1.27, s
14	18.0	0.93, s	18.7	0.93, s
15	30.8	1.17, s	31.5	1.17, s

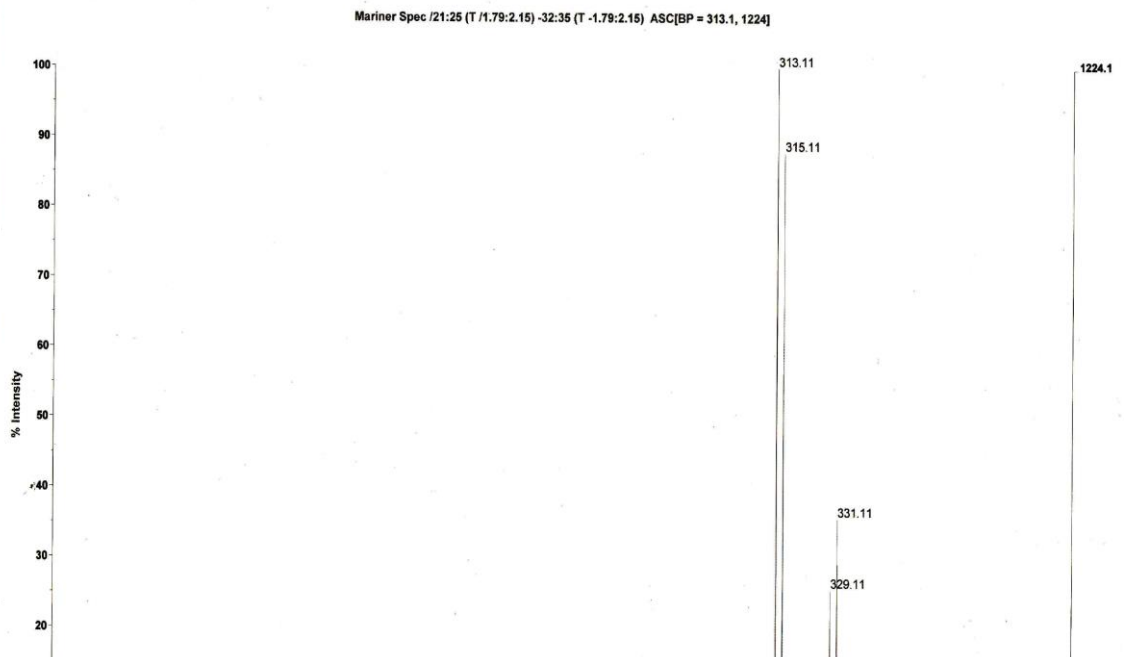


Figure 2.8. ESI-MS spectra of palisadin A

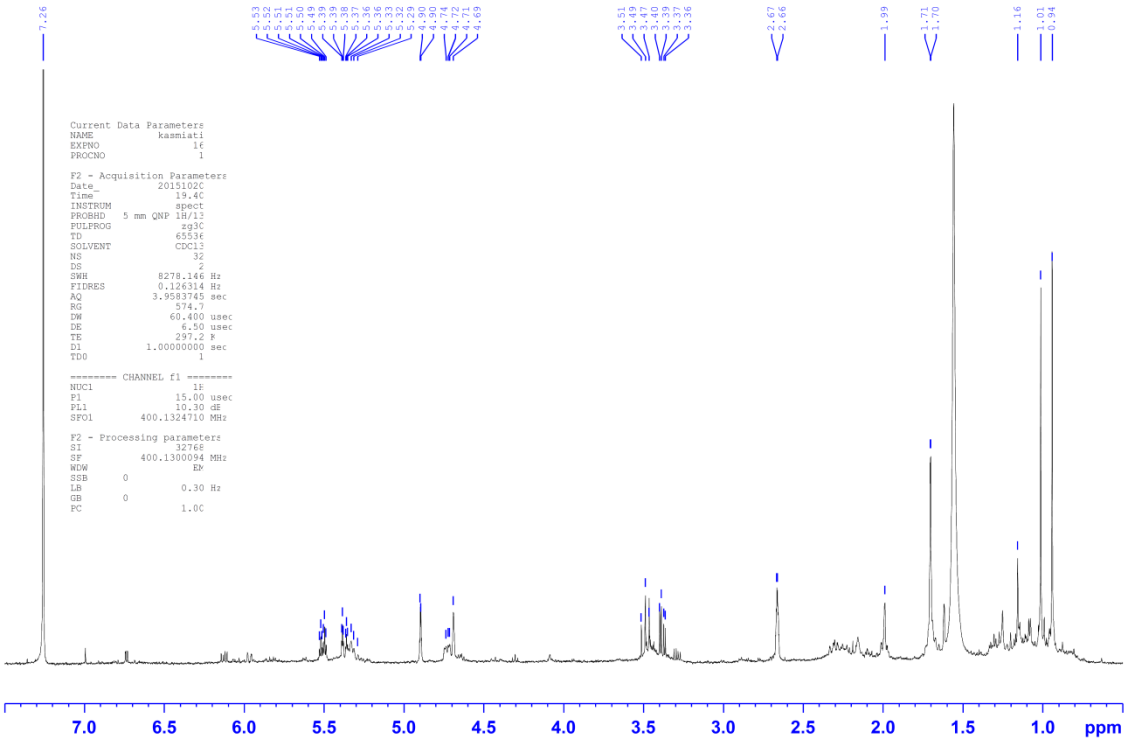


Figure 2.9. ^{1}H NMR spectra of palisol ($CDCl_3$, 400 MHz)

Table 2.6. ^{1}H NMR data for palisol and reference data ($CDCl_3$)

Position	palisol (400 MHz)	reference data [49] (600 MHz)
	δ_H (ppm, m, *J* in Hz)	δ_H (ppm, m, *J* in Hz)
1a	3.38, dd (10.0, 4.0)	3.38, dd (5.0, 9.0)
1b	3.49, t (10.4)	3.49, dd (9.0, 9.0)
2	4.72, m	4.73, ddd (1.0, 5.0, 9.0)
3		
4	5.32, m	5.33, m
5a	1.99, m	2.00, m
5b	2.30, m	2.31, m
6	1.99, m	2.00, m
7		
8	2.66, br s	2.66, br s
9	5.51, dt (9.9, 3.6)	5.51, ddd (4.0, 4.0, 10.0)
10	5.36, dt (10.0, 2.0)	5.38, ddd (1.0, 1.0, 10.0)
11		
12	1.71, s	1.70, s
13a	4.69, s	4.69, s
13b	4.90, s	4.90, s
14	0.94, s	0.94, s
15	1.01, s	1.01, s

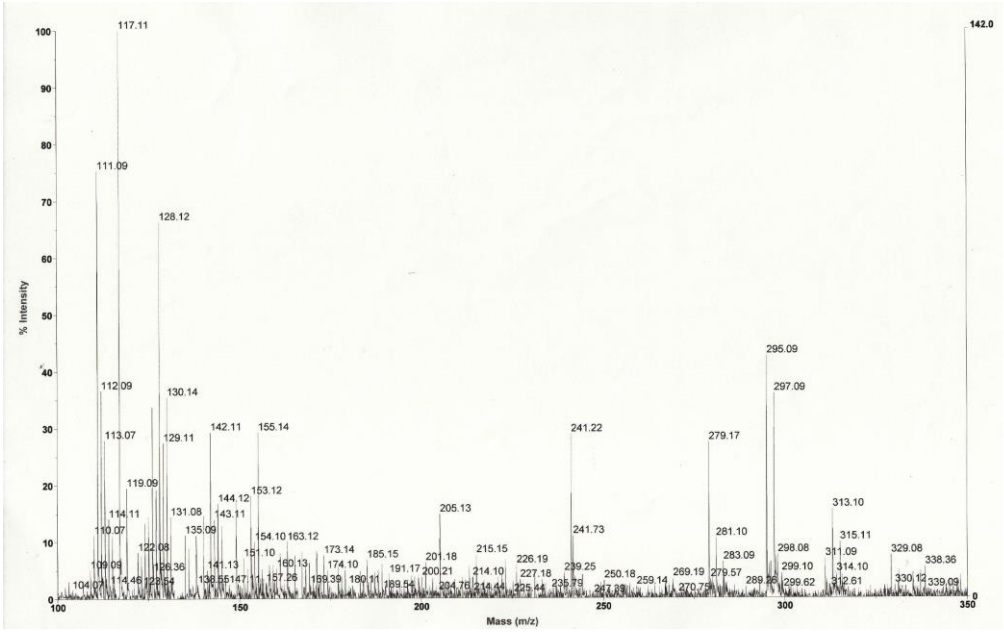


Figure 2.10. ESI-MS spectra of palisol

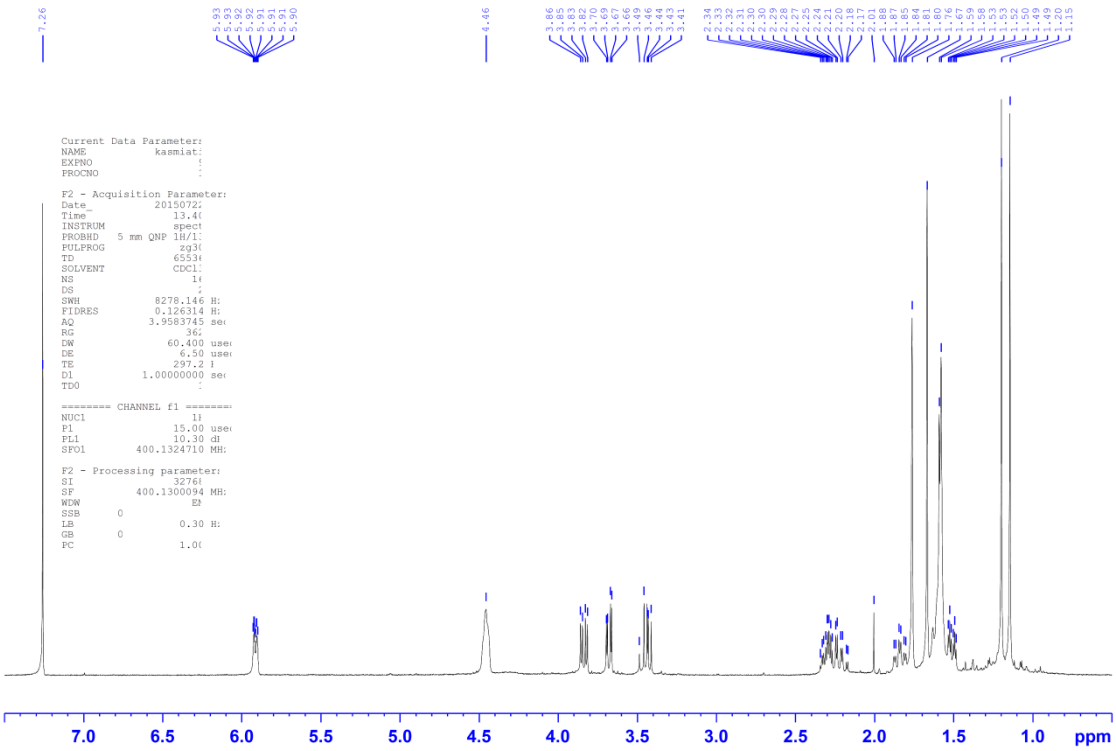


Figure 2.11. ^{1}H NMR spectra of 5β-hydroxypalisadin B ($CDCl_3$, 400 MHz)

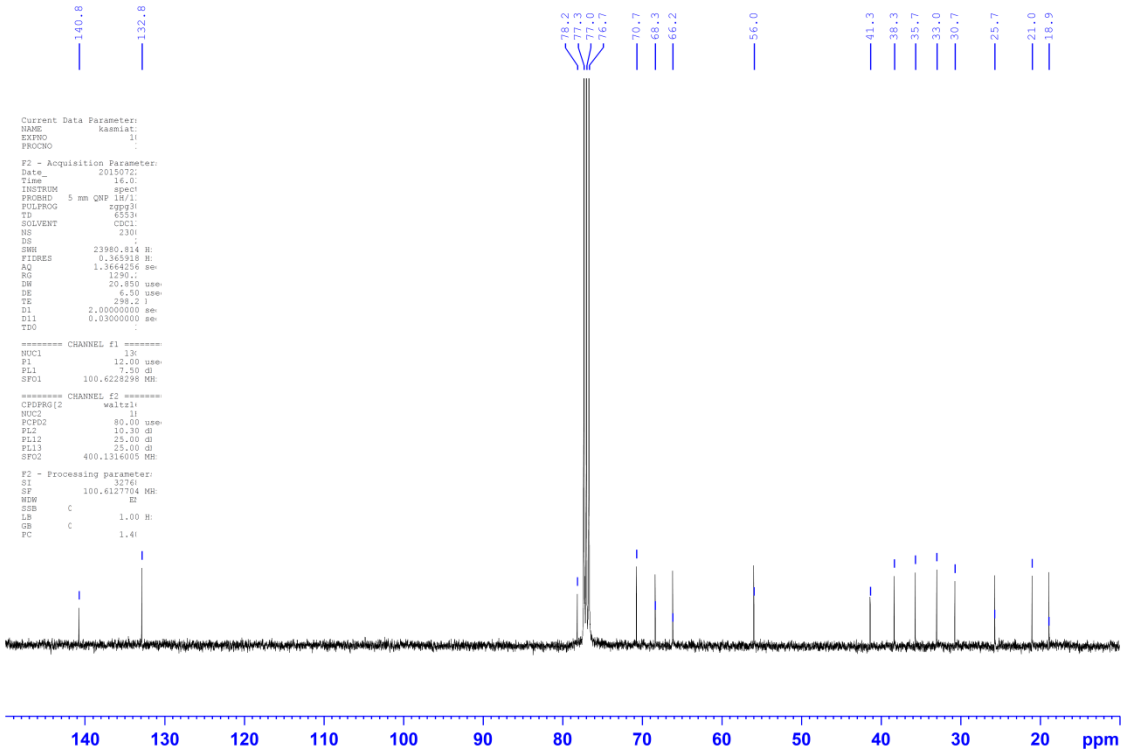


Figure 2.12. ^{13}C NMR spectra of 5β-hydroxypalisadin B ($CDCl_3$, 400 MHz)

Table 2.7. NMR data for 5β-hydroxypalisadin B and reference data ($CDCl_3$)

Position	5β-hydroxypalisadin B (400 MHz)		Reference data [57] (600 MHz)	
	δ_C (ppm)	δ_H (ppm, m, *J* in Hz)	δ_C (ppm)	δ_H (ppm, m, *J* in Hz)
1a	35.7	3.43, dd (10.4, 7.6)	35.6	3.43, ddd (12.0, 8.0, 4.0)
1b		3.67, dd (10.4, 2.8)		3.68, dd, (10.5, 3.0)
2	70.7	4.45, br s	70.8	4.46, br s
3	140.8	-	140.8	-
4	132.9	5.91, d (6.3)	132.9	5.91, d (6.3)
5	68.3	4.45, br s	68.4	4.46, br s
6	56.0	1.61, s	56.0	1.60, s
7	78.2	-	78.2	-
8a	38.3	1.51, dt (12.6, 4.4)	38.4	1.52, ddd (12.6, 3.6, 3.6)
8b		1.83, td (12.8, 4.4)		1.84, ddd, (12.6, 12.6. 4.5)
9a	33.0	2.22, td (12.8, 4.0)	33.0	2.24, m
9b		2.29, m		2.28, m
10	66.2	3.83, dd (12.0, 4.0)	66.2	3.84, dd (12.0, 5.1)
11	41.4	-	41.4	-
12	21.0	1.76, s	21.0	1.78, s
13	25.7	1.66, s	25.7	1.67, s
14	18.9	1.19, s	18.9	1.20, s
15	30.7	1.14, s	30.7	1,14, s

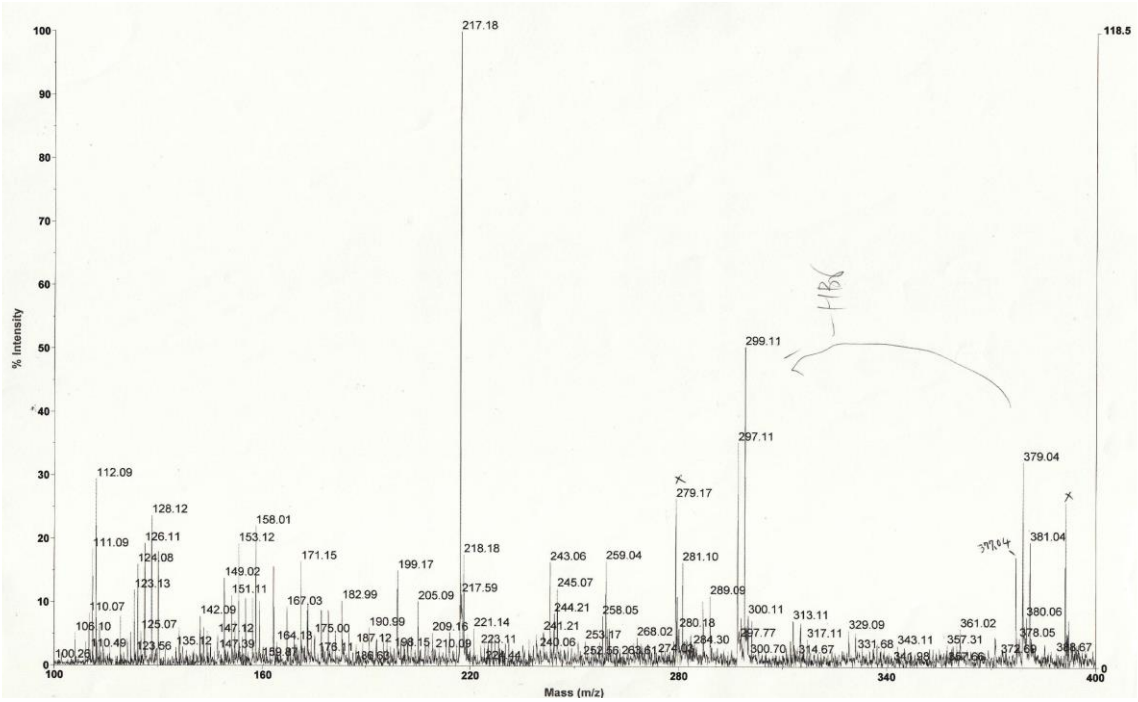


Figure 2.13. ESI-MS spectra of 5β-hydroxypalisadin B

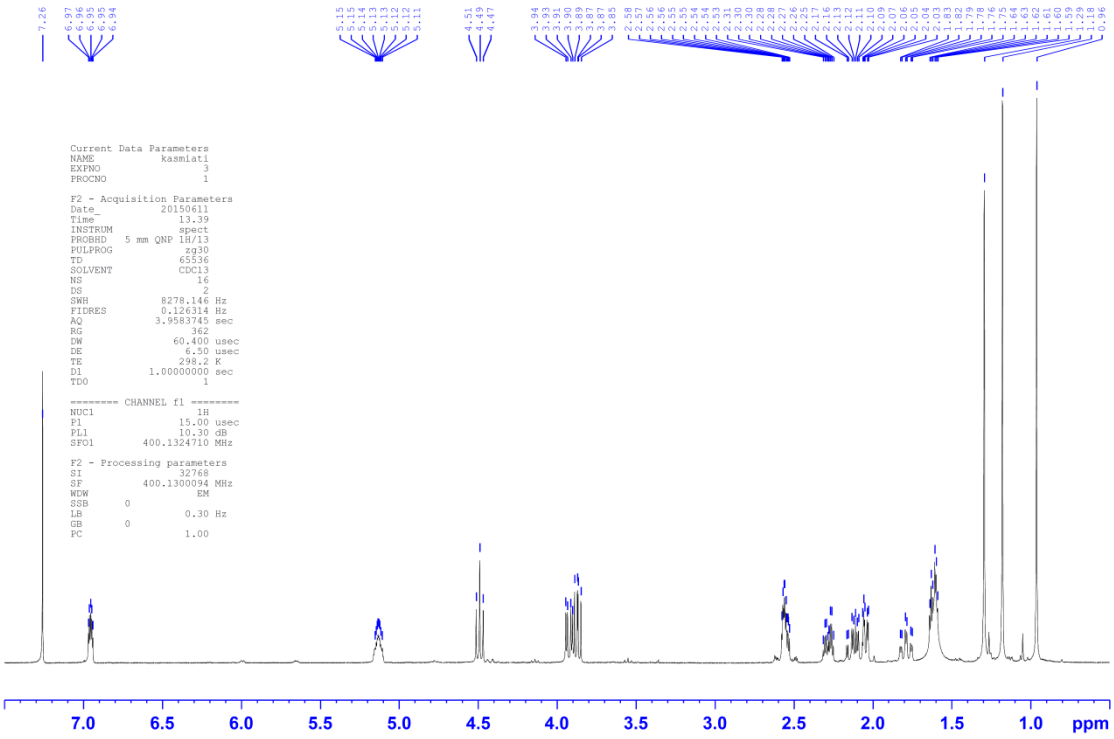


Figure 2.14. ^{1}H NMR spectra of aplysistatin ($CDCl_3$, 400 MHz)

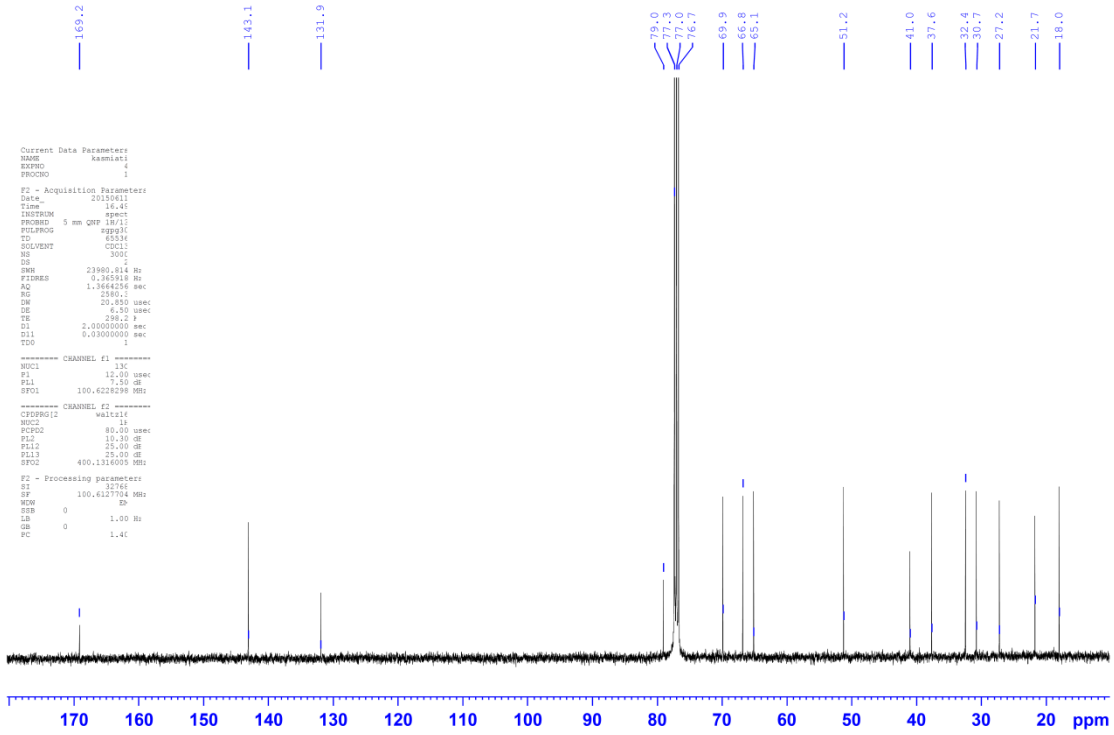


Figure 2.15. ^{13}C NMR spectra of aplysistatin ($CDCl_3$, 100 MHz)

Table 2.8. NMR data for aplysistatin and reference data ($CDCl_3$)

Position	Aplysistatin (400 MHz)		Reference data [49] (600 MHz)	
	δ_C (ppm)	δ_H (ppm, m, *J* in Hz)	δ_C (ppm)	δ_H (ppm, m, *J* in Hz)
1a	69.9	3.87, dd (9.2, 7.2)	69.9	3.86, dd (8.0, 8.0)
1b		4.49, t (8.8)		4.49, dd (8.0, 8.0)
2	66.8	5.13, m	66.8	5.13, br s
3	131.9		131.9	-
4	143.1	6.95, m	143.1	6.96, br s
5	27.2	2.55, m	27.2	2.55, m
6	51.2	2.05, m	51.2	2.04, m
7	79.0		79.0	-
8a	37.6	1.61, dd (12.8, 3.6)	37.7	1.62, ddd (13.0, 3.0, 3.0)
8b		1.79,ddd (13.2, 13.2, 4.0)		1.79, ddd (13.0, 13.0, 3.0)
9a	32.4	2.12, m	32.4	2.10, m
9b		2.28, m		2.28, m
10	65.1	3.93, dd (12.8, 4.4)	65.1	3.93, dd (14.0, 4.0)
11	41.0		41.0	
12	169.1		169.1	
13	21.7	1.29, s	21.7	1.30, s
14	18.0	0.97, s	18.0	0.97, s
15	30.7	1.19, s	30.7	1.19, s

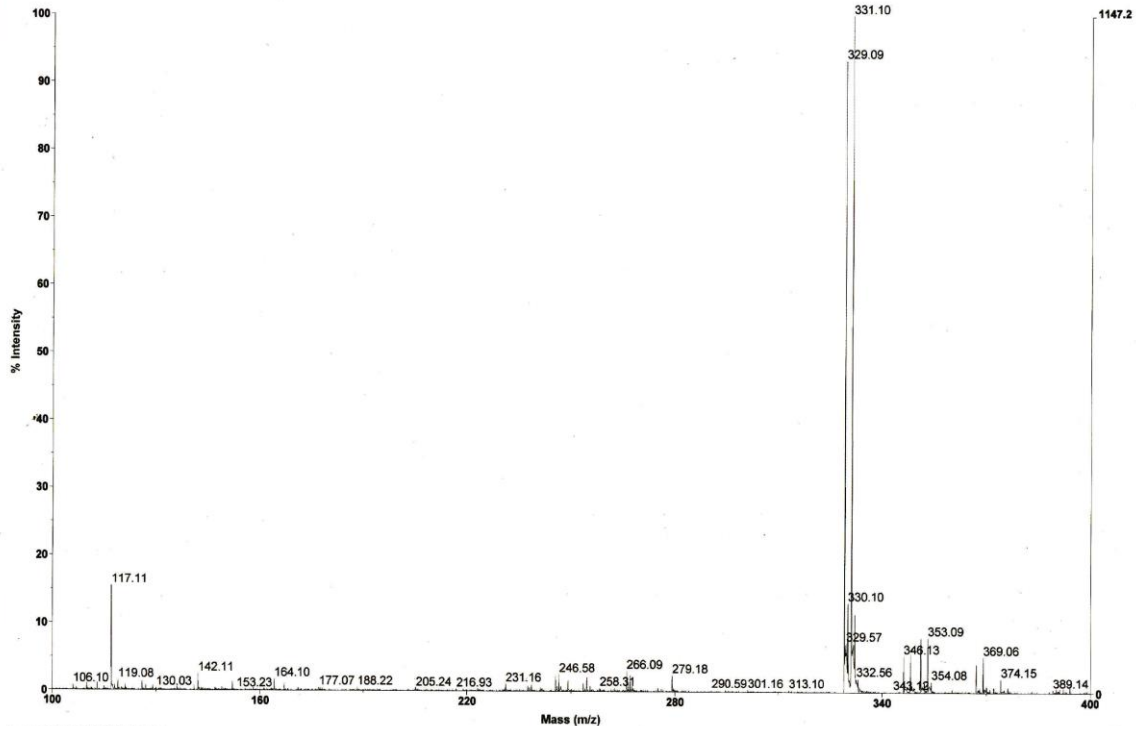


Figure 2.16. ESI-MS spectra of aplysistatin

2.2.3. Biological activities

The *in vitro* activities of palisadin A, palisol, 5β-hydroxypalisadin B, and aplysistatin were evaluated as cytotoxic and anti-oomycete agents against the human epidermoid carcinoma A431 cells and the fungus-like plant pathogent *Phytophthora capsici*, respectively. The results showed that moderate cytotoxic activity was exhibited by aplysistatin, palisol, and 5β-hydroxypalisadin B with the IC_{50} values of 0.15, 0.59, and 0.61 μg/mL respectively. On the other hand, apllysistatin exhibited significant an anti-oomycete activity at a dose of 100 μg/disk, while anti-oomycete activity of other compounds was shown at 300 μg/disk (Table 2.9).

Table 2.9. Cytotoxicity and anti-oomycete activity of the brominated sesquiterpenes

Compound	Cytotoxicity[a]	Anti-oomycete[b]	
	IC_{50} (μg/mL)	Dose (μg/disk)	Inhibition zone (mm)
Palisadin A	1.44	300	2.5
Palisol	0.59	300	2.0
5β-Hydroxypalisadin B	0.61	300	3.0
Aplysistatin	0.15	100	2.5

[a] against human epidermoid carcinoma A431 cells, [b] against the plant pathogen *Phytophthora capsici*

2.3. Materials and Methods

2.3.1. Collection of marine organisms

Twenty-nine marine organisms were collected in five small islands (Barranglompo, Samalona, Kodingareng Keke, Libukang, and Lae Lae) in the area of South Sulawesi Sea (Figure 2.17) in three periods of collection, March and August 2014, and August 2015. Seaweed samples were easily collected by hand along the coast at depth ranging from 0.5 to 3 meters from the sea level, while sponges and soft corals were collected at 0.5 to 4 meters by using snorkling equipment. The collected specimens were immediately cleaned to remove epiphytes and other undesired materials then placed in sepatared plastic bags under 4^{o}C in a cool box and brought to the Laboratory of Marine Biology, Hasanuddin University.

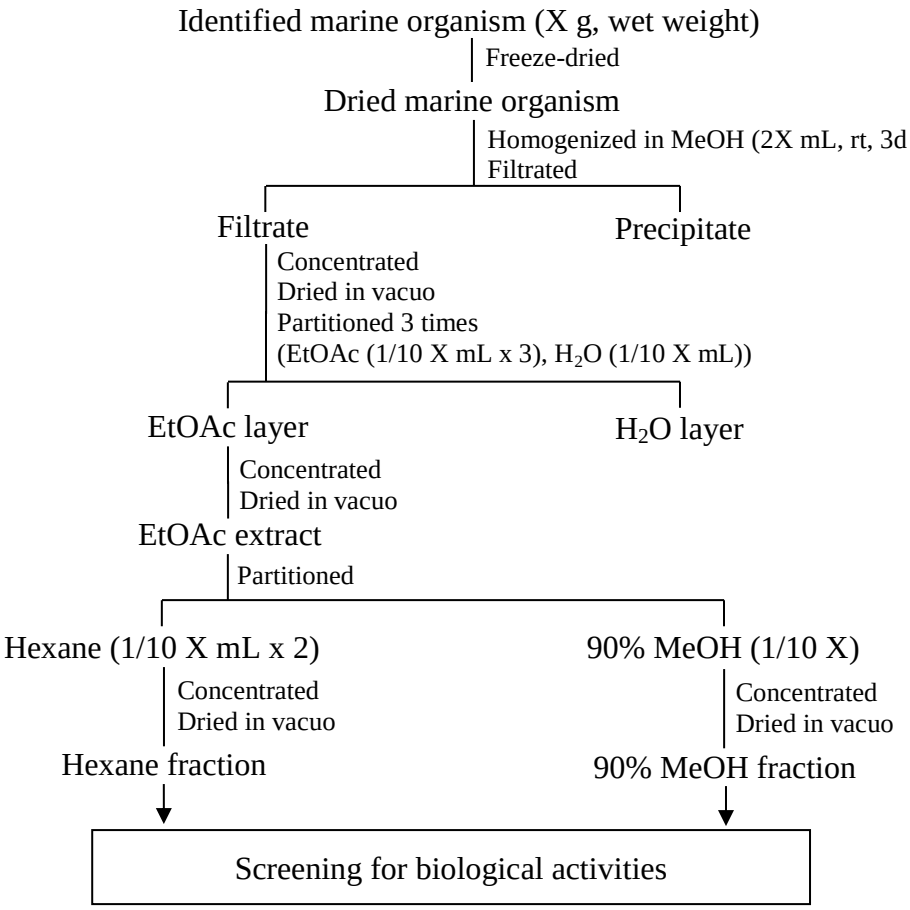
A small amount of individual sample was labelled and prepared for the identification process by my collaborators. Seaweeds, sponges, and soft corals were identified based on their physical features and microscopic characteristics and verified according to literatures [56-59]. Twenty one organisms were recognized as seaweeds, six sponges and two soft corals (Table 2.2). All specimens were preserved at -40^{o}C. Each part of specimens was rinsed with tap water to remove remain impurities, then followed by rinsing by distilated water and subjected to air-drying at room temperature.

The organisms were weighed as wet weight of each sample then freeze-dried overnight before transfering to Nagoya University, Japan.

Figure 2.17. The Indonesian archipelagic map and the five sample sites in the South Sulawesi Sea (highlighted by black square)

2.3.2. Extraction and screening of bioactivities

The freeze-dried specimens were homogenized in MeOH (1:2, wet weight (mg)/volume (mL)) and stood at room temperature for 3 days with regularly shaking. After filtration to separate the aquous organic part and precipitates, the aquous organic filtrate was concentrated using a rotary vacuum evaporator to obtain solid materials. Partition into EtOAc and water fractions was done to obtain EtOAc layers which were then concentrated and dried in vacuo. Finally, the dried EtOAc fraction was subjected to the next partition to collect hexane and 90% methanol fractions. The general protocol of extraction of bioactive compounds from marine organism and screening of anti-oomycete as well as cytotoxic activities is described at Scheme 2.1.



Scheme 2.1. General procedure of extraction and screening for biological activities

2.3.2.1. Cytotoxicity assay

A431 human vulva-derived epidermoid carcinoma cells were used to evaluate the cytotoxicity of the compounds under the conditions reported previously [60]. Briefly, the cells at a density of 1.0 x 10^4 cells/well were cultured in a 24-well plate (BD Falco) in DF6F medium with various concentrations of crude extract (none, 0.1, 1, and 10 μg/mL) or purified compound (none, 0.1, 0.3, 1, 3, and 10 μg/mL) at 37 °C in a humidified 95% air/5% CO_2 condition in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA), followed by cell counting with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL, USA) on day 5. The DF6F medium was composed of a

1:1 ratio of DMEM and Ham F-12 medium (DF) supplemented with six factors, i.e., insulin (10 μg/ml), transferrin (5 μg/ml), 2-aminoethanol (10 μM), sodium selenite (10 nM), 2-mercaptoethanol (10 μM), and oleic acid conjugated with fatty acid-free bovine serum albumin (9.4 μg/ml) (all chemicals were from Sigma-Aldrich, St. Louis, MO, USA). IC_{50} values are shown by the means of two replicates.

2.3.2.2. Anti-oomycete assay

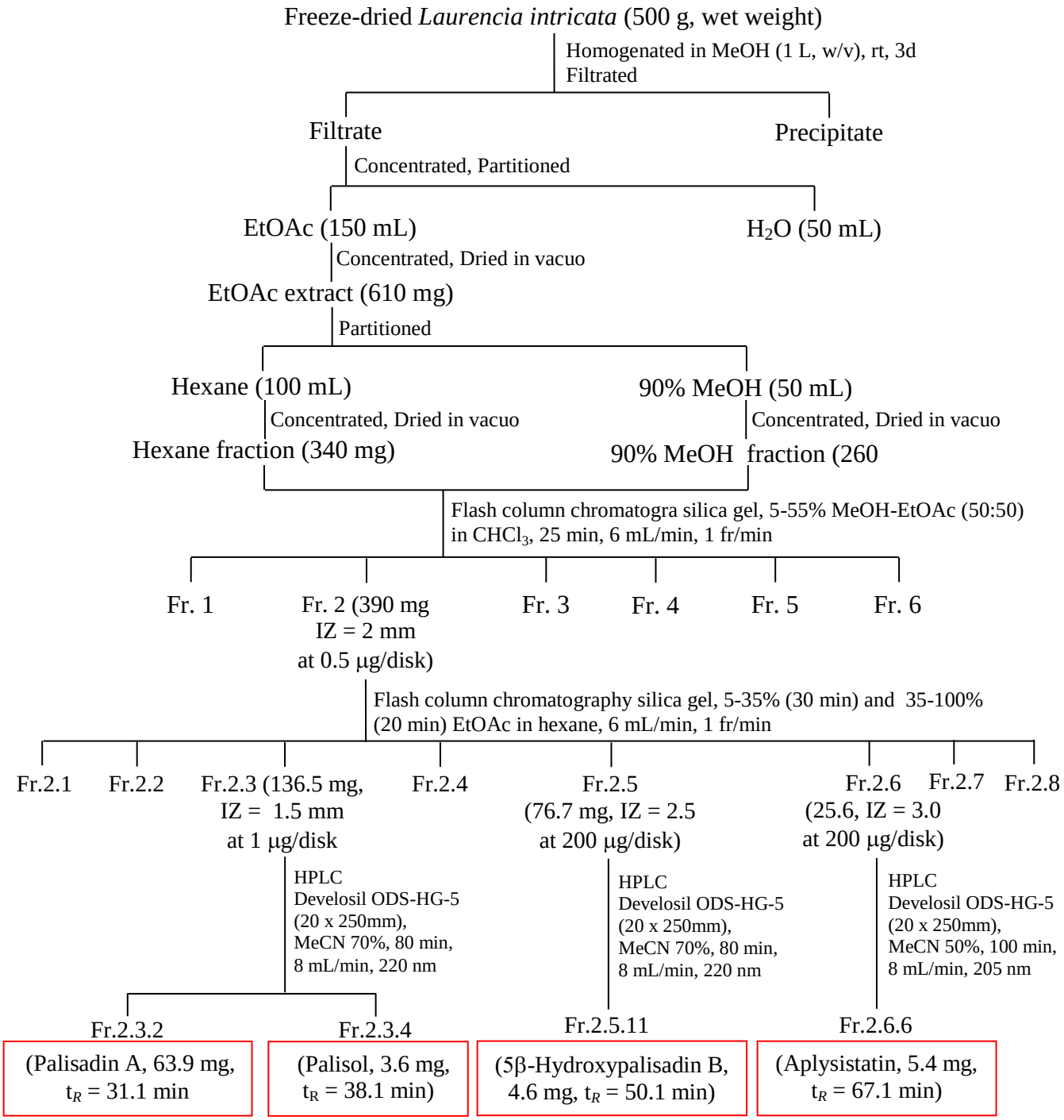
The test was performed by the paper disk diffusion method [61]. Briefly, apiece of the mycelia of the plant pathogen *Phytophthora capsici* NBRC 30696 was pre-cultured on a potato-glucose-agar medium in a 9-cm dish at 25°C and 60% humidity for 7 days in the dark. A piece (5 x 5 mm) of the colony was then inoculated on the center of a 5% V8 juice-1.5%-agar medium in a 9-cm dish and incubated for 48 h at 25°C and 60% humidity. A paper disk (8mm in diameter) containing a crude extract at an appropriate dose of 50 μg/disk or 10, 50, 100, and 300 μg/disk (DMSO as negative control) was placed at 1 cm away from the colony front. After incubation for another 22–24 h, the inhibition zone formed around the sample disk was measured. The activity was represented by the minimum dose that expressed an obvious inhibition zone (usually 0.5 mm or wider).

2.3.3. Extraction and bioassay-guided fractionation of crude extract from *Laurencia intricata*

The seaweed *L.intricata* (500 g, wet weight) was freeze dried, homogenized in MeOH (1 L) and stood at room temperature for 3 days. The homogenate was filtrated and the filtrate was concentrated then extracted three times with EtOAc (150 mL). The

combination organic layers concentrated to obtain EtOAc extract (610 mg) were dissolved in 90% MeOH (50 mL) and extracted twice with hexane (100 mL). Both layers were concentrated to yield hexane (340 mg) and 90% MeOH extracts (240 mg).

The active crude methanol extract (inhibition zone = 2.0 mm at a dose 50 μg/disk) was combined with hexane extract because they have similar thin layer chromatography (TLC) profiles. The combination extract was then fractionated on silica gel (HI-FLAS^{TM} Size M, 14 g, Yamazen Co., Osaka, japan) with 5–55% mixture of MeOH-EtOAc (50:50) in $CHCl_3$(25 min) at a flow rate 6 mL/min to give six fractions (fr.1 – fr.6), where the only fr.2 (390 mg) showed anti-oomycet activity with inhibition zone of 2 mm at a dose 500 μg/disk. This fraction was further chromatographed on silica gel (HI-FLASH^{TM} M size, eluted with two stage of 5–35% (30 min) and 35–100% (20 min) EtOAc in hexane system at a flow rate of 6 mL/min to yield eight partial pure fractions (fr.2.1 – fr.2.8).The anti-oomycete fr.2.3 (136.5 mg, inhibition zone = 2 mm at 1 mg/disk) was subjected to HPLC [Develosil ODS-HG-5 (20 x 250 mm), 70% MeCN (70 min), 8 mL/min, monitored at 220 nm] to give palisadin A (fr.2.3.2, 63.9 mg, t_R = 31.1 min) and palisol (fr.2.3.4, 3.6 mg, t_R = 38.1 min). The active fr.2.5 (76.7 mg, inhibition zone = 2.5 mm at 200 μg/disk) was purified by HPLC [Develosil ODS-HG-5 (20 x 250 mm), 60% MeCN (80min), 8 mL/min, detected at 210 nm] to yield 5β-hydroxypalisadin B (fr.2.5.11, 4.6 mg, t_R = 50.1 min). The last anti-oomycete fr.2.6 (25.6 mg, inhibition zone 2 mm at 50 μg/disk) was purified by HPLC [Develosil ODS-HG-5 (20 x 250 mm), 50% MeCN (100 min), 8 mL/min, detected at 205 nm] to obtaine aplysistatin (fr.2.6.6, 5.4 mg, t_R = 67.1 min). General procedure of extraction and purification of bioactive compounds from the seaweed *L. intricata* is given on Scheme 2.2.



Scheme 2.2. Extraction and bioassay-guided purification of *L. intricata*

IZ: inhibition zone (mm) in anti-oomycete test

2.3.4. Structure elucidation of bioactive compounds

The chemical structures of the active compounds were confirmed by comparison of the ^{1}H and ^{13}C NMR data between the isolated and reported compounds. The 1D NMR spectra were recorded by a Bruker ARX400 (400 MHz) instrument in a $CDCL_3$ solution (99.9% atom enriched). NMR chemical shifts are referenced to the residual $CDCl_3$ peak of δ_H 7.26 and δ_C 77.0.

2.4. Conclusion

The Indonesian red seaweed *Laurencia intricata* was found to be a rich source of the brominated sesquiterpenes such as palisadin A, palisol, 5β-hydroxypalisadin B, and aplysistatin. They showed a weak inhibition against the plant pathogen *P. capsici*. On the other hand, they showed a moderate cytotoxic activity against the human epidermoid carcinoma A431 cells at the IC_{50} values of 1.44, 0.59, 0.61, and 0.15 μg/ml, respectively. These compounds may be candidates or leads for anti-cancer agents.

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Chapter 3. New Crambescidin-type Alkaloids from the Sponge *Clathria bulbotoxa*

3.1. Introduction

Marine sponges (phyla Porifera) are one of the most prolific and the largest sources of novel bioactive compounds among marine organisms [1, 2], with more than 200 new compounds reported each year [3]. During the last decade from 2001 to 2010, approximately 2400 new natural products had been discovered from 671 species of sponges, contributing 29% of the marine natural products reported within the period [4]. Ecological studies reported that sponges produce a wide array of secondary metabolites for defensive purposes to protect them from threats of competitors, predators, and pathogens [5, 6]. Furthermore, sponges are frequently a host for microbial symbionts, which are regarded as one of the most important sources of bioactive molecules [7].

Studies on Indonesian marine sponges are interesting because Indonesia is the largest archipelagic country in the world, encompassing approximately 86,700 square kilometers of coral reef ecosystems [8], which are important for sponges as the most dominant benthic inhabiting coral reefs [9]. The Indonesian coral reefs are located in the coral triangle area, which is the global center of marine biodiversity and recognized as the richest region on the earth [10]. Therefore, sponges from the coral ecosystems produce metabolites with various biological properties and become a target of continuing searching for new bioactive compounds [11, 12].

The biological activities of extracts from Indonesian marine organisms including six sponge species (*Tedania ignis*, *Agelas conifera*, *Clathria bulbotoxa*, *Clatria reinwardti*, *Haliclona koromella*, and *Carteriospongia foliascens*) were recently investigated and found that an extract of the sponge *Clathria bulbotoxa* was highly cytotoxic. The sponges of the genus *Clathria* are widely distributed in the tropical

shallow waters and temperate regions, especially along the cost of the southern hemisphere [13, 14]. This genus has been recognized as an excellent producer of novel secondary metabolites exhibiting diverse chemical structures including alkaloids [15-20], carotenoids [21, 22], peptides [23], sugars [24], terpenoids [25, 26] and sterols [27-29].

For the above reasons as well as the abundant population of the *Clathria bulbotoxa* in the Samalona Island, South Sulawesi Sea, I was interested in the investigation of bioactive compounds from this sponge, leading to the isolation of three new crambescidin-type guanidine alkaloids **1**–**3** as well as three known related products **4–6**. Herein, I present the isolation, structure elucidation, and biological characterization of these guanidine alkaloids.

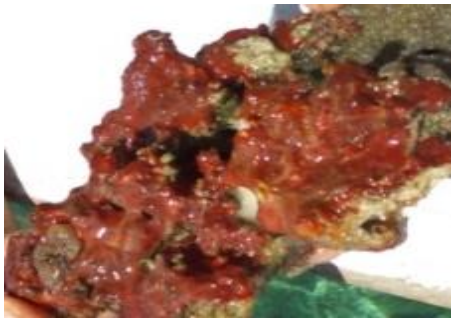

Figure 3.1. *Clathria bulbotoxa*

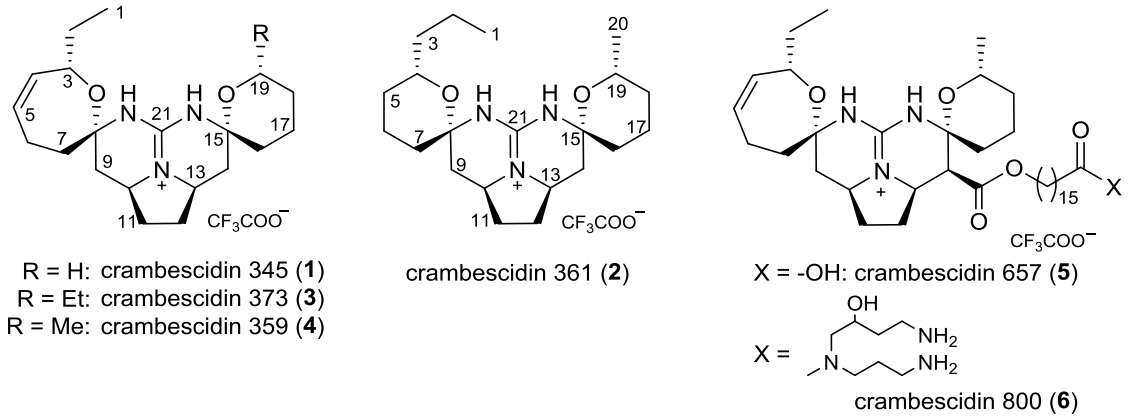

Figure 3.2. Chemical structures of **1–6**

3.2. Results and discussion

3.2.1. Isolation and structural elucidation

The MeOH extract of the freeze-dried sponge was found to be higly cytotoxic against the human epidermoid carcinoma cell line A431 at an IC_{50} value of 0.046 μg/mL and to exhibit anti-oomycete activity against the fungus-like plant pathogen *Phytophthora capsici* at a dose of 50 μg/disk. The active extract was subjected to bioassay-guided fractionation followed by the final reversed-phase HPLC to yield six compounds **1**–**6**. The compounds **4**–**6** were identified as crambescidins 359 [30], 657 [31], and 800 [32] (Figure 3.2), respectively, by comparison with published spectroscopic data, whereas the compounds **1**–**3** were found to be new crambescidin analogs.

Crambescidin 345 (**1**) possesses the molecular formula of $C_{20}H_{31}N_3O_2$ deduced from a high resolution electrospry ionization mass spectrum (HR-ESIMS) using the pseudo-molecular ion at *m/z* 346.2495 $[M + H]^+$ (calculated for $C_{20}H_{32}N_3O_2$ 346.2489) (Figure 3.7). The IR spectrum of **1** exhibited a characteristic absorption at 3109 cm^{-1} (Figure 3.6), which was also observed for **2** (3107 cm^{-1}) (Figure 3.13), **3** (3111 cm^{-1})

(Figure 3.19), and the other known crambescidin-type analogs [31, 33, 34, 35]. It was reported that this absorption was due to the NH streching mode of the cyclic guanidin structure [36].

The ^{1}H and ^{13}C NMR spectra (Tables 3.1) showed that **1** consisted of 30 hydrogen and 20 carbon atoms. A hetero-nuclear single quantum coherence (HSQC) experiment indicated that all hydrogen atoms were attached to carbons, revealing the presence of five CH, eleven CH_2, one CH_3, and three C. Two additional protons were observed at δ_H 10.20 and 10.28 in $CDCl_3$ (Figure 3.5), supporting the presence of the above-mentioned guanidine moiety characteristic of the crambescidin alkaloids. The signals of the three quaternary carbons were found at δ_C 85.1 (C-8), 81.3 (C-15) and 149.4 (C-20). Other NMR signals were characterized as an olefinic bond [δ_C 134.2/δ_H 5.50 (C-4) and δ_C 131.4/δ_H 5.71 (C-5)], one methyl [δ_C 10.8/δ_H 0.84 (C-1)], one oxymethylene [δ_C 62.6/δ_H 3.69 (C-19)], one oxymethine [δ_C 72.1/δ_H 4.35 (C-3)], two *N*-subtituted methines [δ_C 54.9/δ_H 4.03 (C-10) and δ_C 53.5/δ_H 3.96 (C-13)], and ten methylenes (δ_C 19.5–39.1/δ_H 1.45–2.59) based on their chemical shifts. These NMR data exhibited a close similarity to those for crambescidin 359 (**4**) [30], except for the CH_2-19 in **1**, which is replaced with an ethylidene (CH_3-CH<) in **4**.

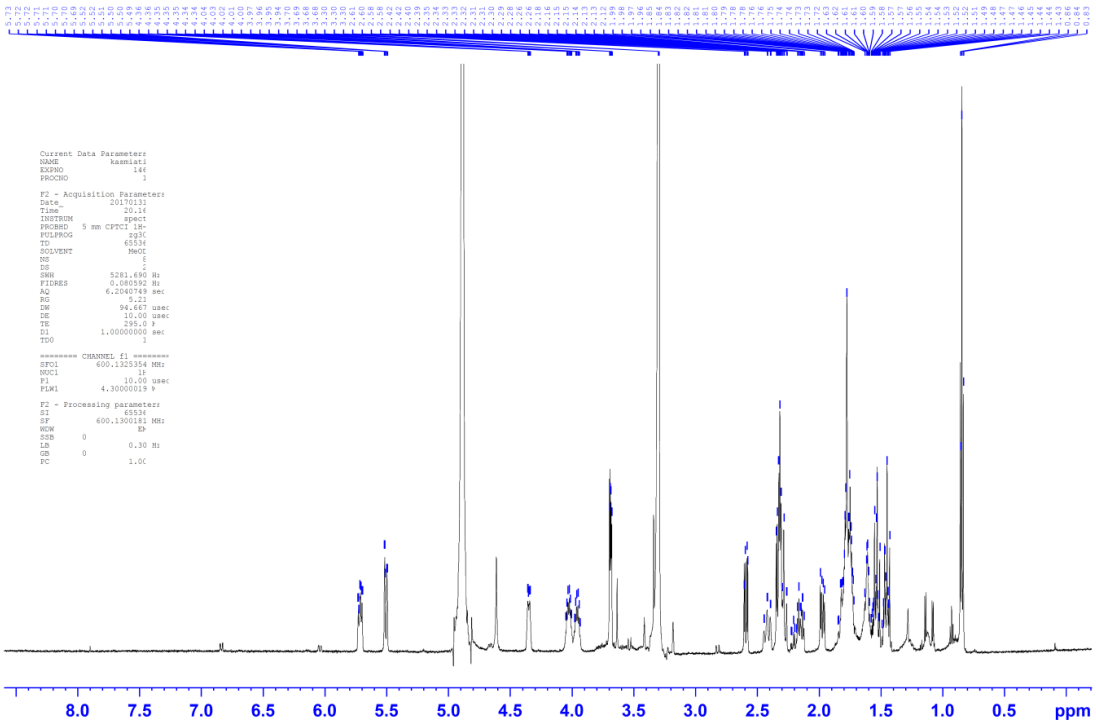

Figure 3.3. ^{1}H NMR spectrum of **1** (600 MHz, CD_3OD)

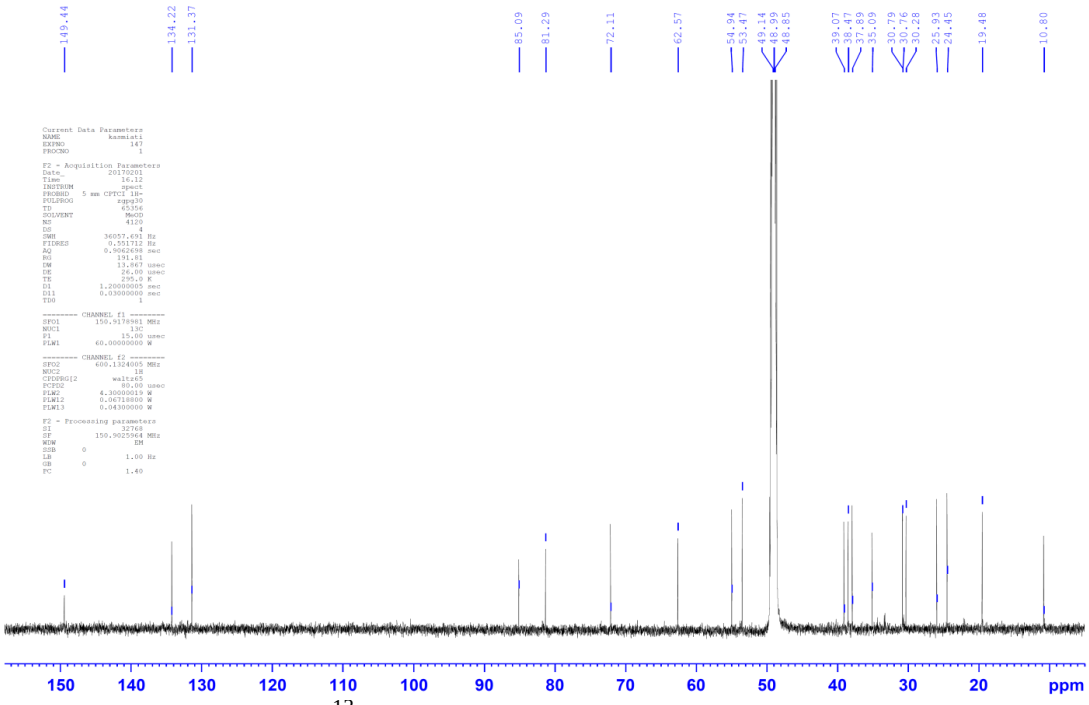

Figure 3.4. ^{13}C NMR spectrum of **1** (150 MHz, CD_3OD)

Table 3.1. ^{1}H and ^{13}C NMR data for **1** (CD_3OD)[a]

Position	δ_H (ppm), m (*J* in Hz)	δ_C (ppm)[b]
1	0.84, t (7.2)	10.8, CH_3
2a	1.46, m	30.3, CH_2
2b	1.54, m	
3	4.35, brd (10.8)	72.1, CH
4	5.50, dt (10.8, 2.1)	134.2, CH
5a	5.71, m	131.4, CH
6a	2.15, dt (15.3, 7.2)	24.4, CH_2
6b	2.42, brt (15.3)	
7a	1.97, dd (13.5, 6.0)	38.5, CH_2
7b	2.27,t (13.5)	
8		85.1, C
9a	1.45, t (12.7)	37.9, CH_2
9b	2.59, dd (12.7, 4.8)	
10	4.03, m	54.9, CH
11a	1.75, m	30.8, CH_2
11b	2.31, m	
12a	1.75, m	30.8, CH_2
12b	2.31, m	
13	3.96, m	53.5, CH
14a	1.53, t (13.0)	39.1, CH_2
14b	2.33, dd (13.0, 4.5)	
15		81.3, C
16a	1.77, m	35.1, CH_2
17a	1.80, m	19.5, CH_2
18a	1.61, m	25.9, CH_2
19	3.69, m	62.6, CH_2
20		149.4, C

[a] measured at 600 MHz for ^{1}H and 150 MHz for ^{13}C

[b] The number of hydrogens on a carbon was determined by HSQC

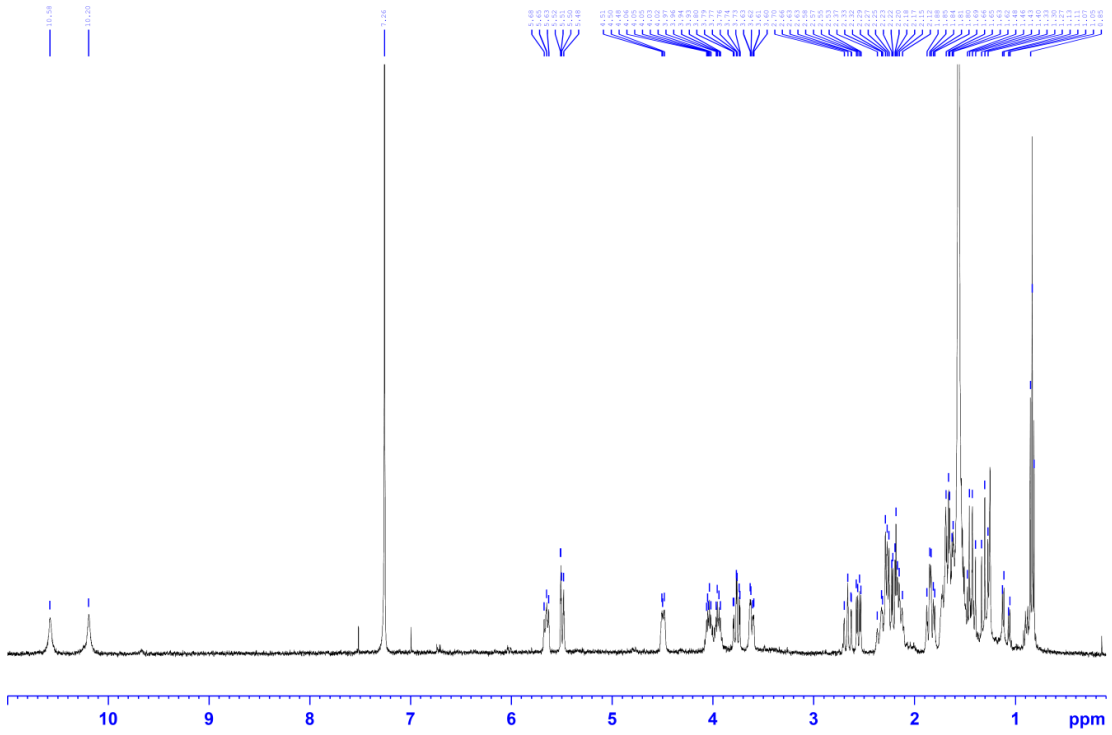

Figure 3.5. ^{1}H NMR spectrum of **1** (400 MHz, $CDCl_3$)

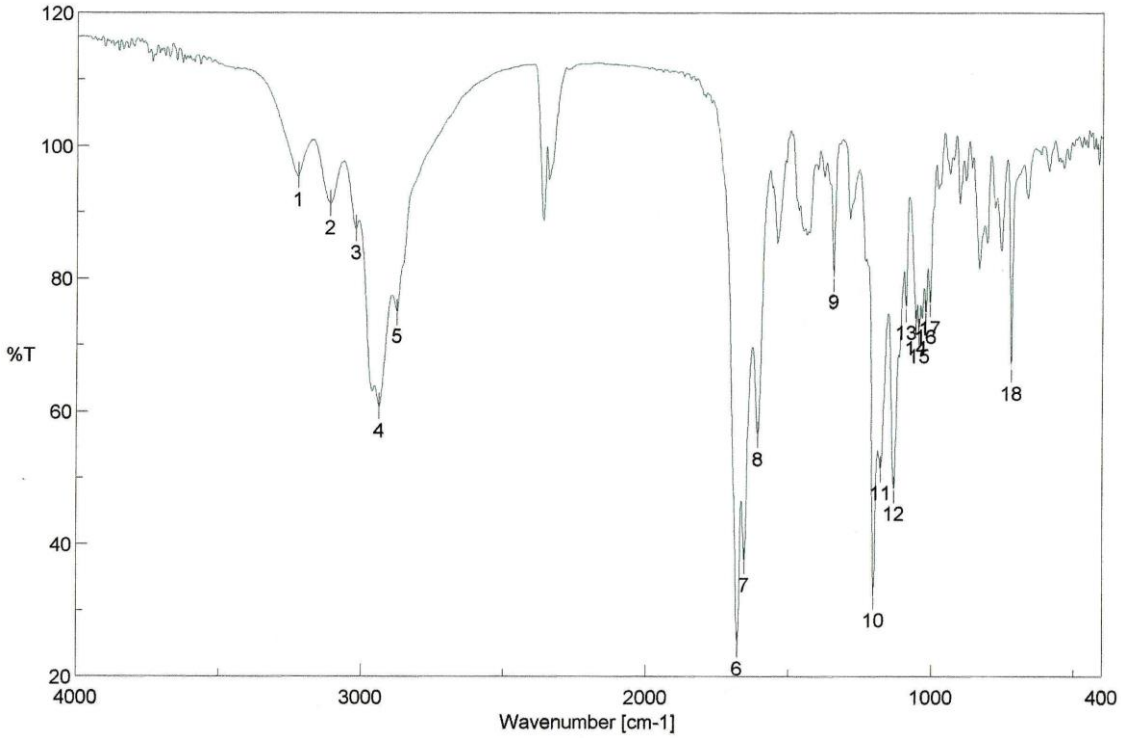

Figure 3.6. IR spectrum of **1**

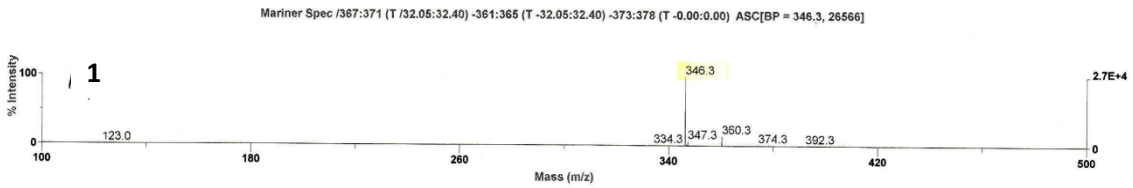

Figure 3.7. ESIMS (+) spectrum of **1**

A correlation spectroscopy (DQF-COSY) experiment was performed to determine the connectivity of the proton-bearing carbons described above, suggesting the presence of four substructures, CH_3-1–CH_2-2–CH-3–CH-4=CH-5–CH_2-6–CH_2-7, CH_2-9–CH-10–CH_2-11, CH_2-12–CH-13–CH_2-14, and CH_2-18–CH_2-19 (Figure 3.8a). A hetero-nuclear multiple-bond connectivity (HMBC) experiment was conducted to connect these substructures and the quaternary carbons. The HMBC correlations from H_2-6, H_2-7, and H-9 to C-8, and from H-9 to C-7 revealed the position of the quaternary carbon C-8, and the correlations from H_2-14, H_2-16, and H_2-19 to C-15 confirmed the position of the quaternary carbon C-15. The other important HMBC signals were found from H_2-14, H_2-17, and H_2-18 to C-16, from H_2-16 to C-14, and from H_2-19, H_2-18, and H_2-16 to C-17, supporting the position of the methylene carbons C-16 and C-17. Although the HMBC signal from H-3 to C-8 was not found, the chemical shift of the C-3 (δ_C 72.1) indicated that this carbon had an oxygen functionality. Based on these analyses, crambescidin 345 (**1**) was deduced as a new crambescidin analog lacking the alkyl group at C-19, which is the second example among the crambescidins reported previously [35].

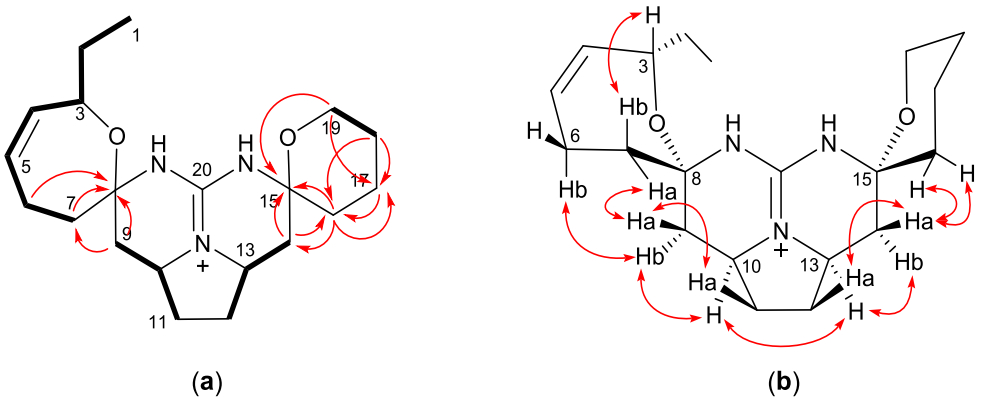

Figure 3.8. Two dimensional NMR correlations for **1**. (**a**) Key DQF-COSY (bold bonds), and HMBC (solid arrows) correlations; (**b**) Key NOESY correlations

The relative configuration for **1** was assigned by NOESY experiments (Figure 3.8b). The NOE correlations of δ_H 4.35 (H-3)/2.27 (H-7b), δ_H 1.97 (H-7a)/1.45 (H-9a), and δ_H 2.42 (H-6b)/2.59 (H-9b) indicated the relative configuration between C-3 and C-8. The relative stereochemistry between C-10 and C-13 was confirmed by the NOE correlations of H-9a/δ_H 1.75 (H-11a), δ_H 1.75 (H-12a)/1.53 (H-14a), H-9b/δ_H 4.03 (H-10), H-10/δ_H 3.96 (H-13), and H-13/δ_H 2.33 (H-14b). An additional NOE correlation of δ_H 1.77 (H-16)/H-14a determined the relative configuration between C-15 and other positions. Furthermore, the large coupling constants of H-9a/H-10 (J = 12.7 Hz) and H-14a/H-13 (J = 13.0 Hz) confirmed the 1,2-diaxial orientation of these hydrogen pairs, concluding the relative stereochemistry of **1** as described in Figure 3.8b. The specific rotation value of **1** (-7.1) indicated a close similarity to that of the structurally similar analog **4** (-8.9), suggesting that **1** possesses the same absolute configuration as that of **4** (Figure 3.2).

Crambescidin 361 (**2**) possesses the molecular formula of $C_{21}H_{35}N_3O_2$ deduced from a HR-ESIMS using the pseudo-molecular ion at *m/z* 362.2765 $[M + H]^+$ (calculated for $C_{21}H_{36}N_3O_2$, 362.2802) (Figure 3.14). Detailed analysis of the ^1H and ^{13}C

NMR spectral data (Figures 3.9 and 3.10, Table 3.2) indicated that **2** consisted of 34 hydrogene and 21 carbon atoms, and HSQC experiments implied that all protons were connected to carbons (four CH, twelve CH_2, two CH_3, and three C). In addition, two exchangeable proton signals were observed at δ_H 10.13, and 10.16 in acetone-d_6 (Figure 3.11). The signals of the three quaternary carbons were observed at δ_C 81.6 (C-8 and C-15) and 149.0 (C-21). Other important NMR signals were recognized as two methyls [δ_C 13.8/δ_H 0.87 (C-1) and δ_C 22.0/δ_H 1.11 (C-20)], two oxymethines [δ_C 71.1/δ_H 3.63 (C-4) and δ_C 68.2/δ_H 3.74 (C-19)], two *N*-subtituted methines [δ_C 53.7/δ_H 4.00 (C-10) and δ_C 53.4/δ_H 4.00 (C-13)], and twelve methylenes [δ_C 19.4–40.4/δ_H 1.26–3.30]. Although these data were characteristic of the crambescidin alkaloids, they showed the absence of the olefinic function that was found in most of the reported crambescidin-type alkaloids. In addition, the pentacyclic guanidine core is symmetrical as indicated by highly overlapping chemical shifts of the corresponding proton and carbon signals (Tables 3.2).

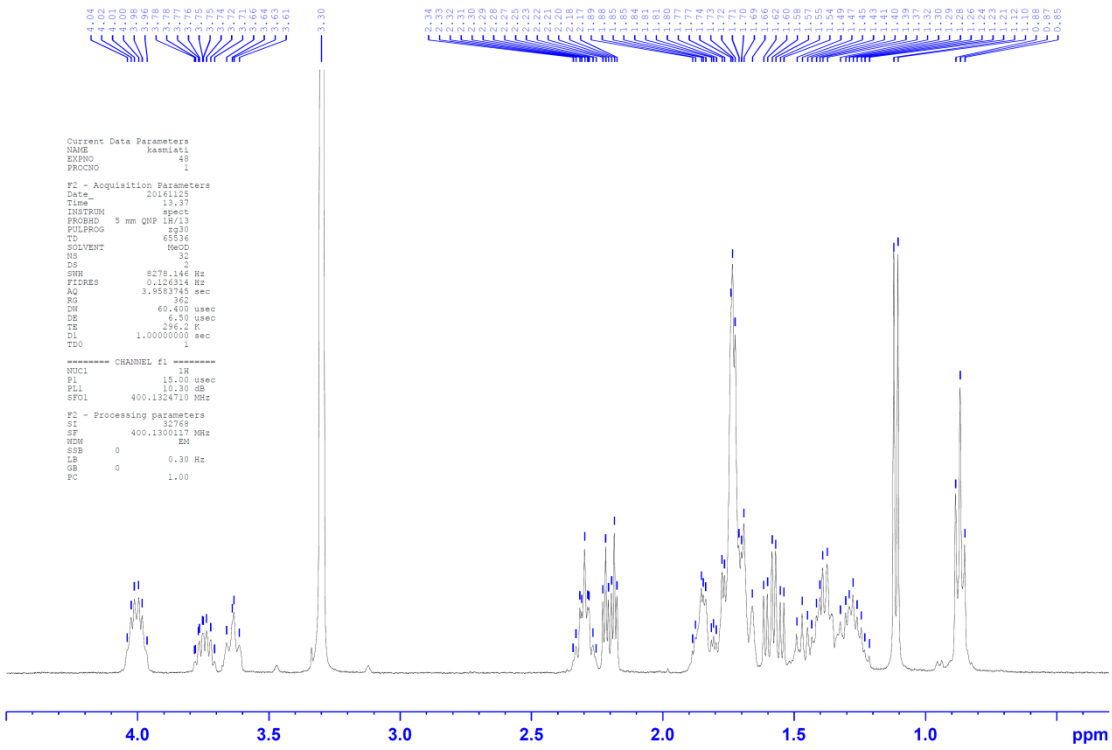

Figure 3.9. ^{1}H NMR spectrum of **2** (400 MHz, CD_3OD)

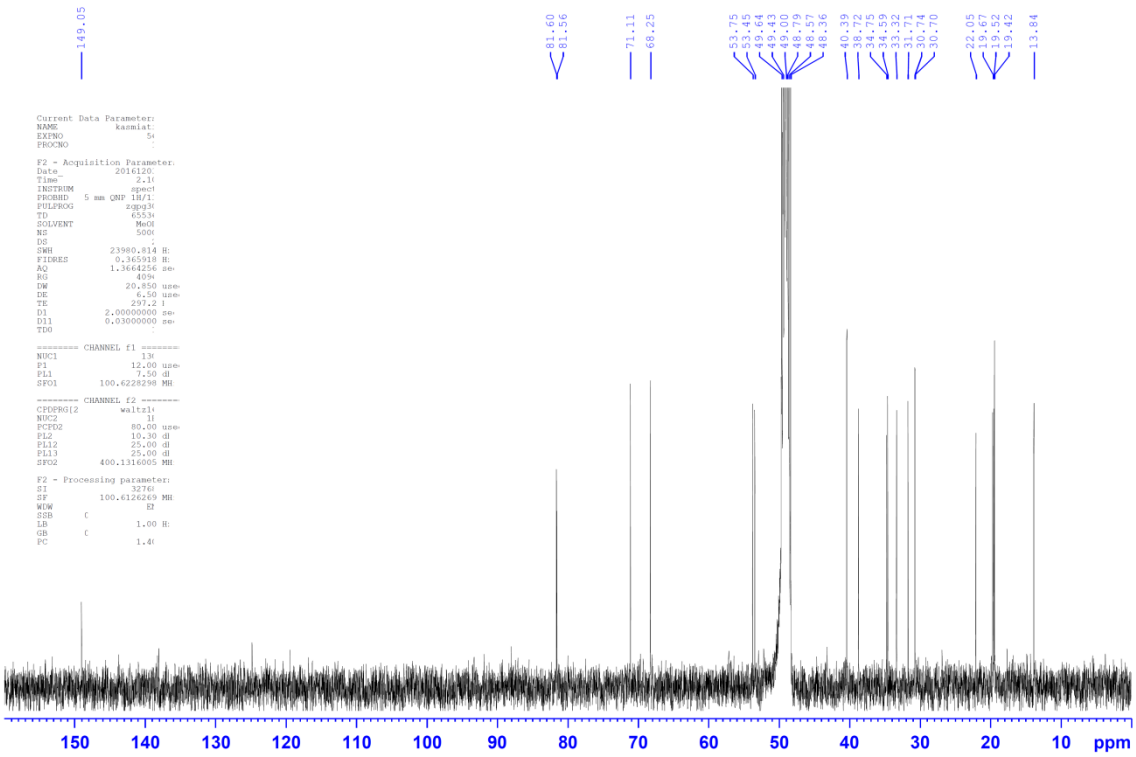

Figure 3.10. ^{13}C NMR spectrum of **2** (100 MHz, CD_3OD)

Table 3.2. ^{1}H and ^{13}C NMR data for **2** (CD_3OD)[a]

Position	δ_H (ppm), m (*J* in Hz)	δ_C (ppm)[b]
1	0.87, t (6.8)	13.8, CH_3
2a	1.38, m	19.4, CH_2
3	1.41, m	38.7, CH_2
	1.47, m	
4	3.63, brt (12.6)	71.1, CH
5a	1.29, m	31.7, CH_2
5b	1.67, m	
6a	1.74, m	19.5, CH_2[e]
6b	1.85, m	
7a	1.74, m	34.7, CH_2[f]
8		81.6, C
9a	1.57, t (12.8)[c]	40.4, CH_2
9b	2.19, dd (12.8, 4.2)[d]	
10	4.00, m	53.7, CH[g]
11a	1.73, m	30.7, CH_2
11b	2.30, m	
12a	1.73, m	30.7, CH_2
12b	2.30, m	
13	4.00, m	53.4, CH[g]
14a	1.59, t (12.8)[c]	40.4, CH_2
14b	2.21, dd (12.8, 4.2)[d]	
15		81.6, C
16a	1.74, m	34.6, CH_2[f]
17a	1.74, m	19.7, CH_2[e]
17b	1.85, m	
18a	1.26, m	33.3, CH_2
18b	1.70, m	
19	3.74, m	68.2, CH
20	1.11, d (9.0)	22,0 CH_3
21		149.0, C

[a] Measured at 400 MHz for ^{1}H and 100 MHz for ^{13}C

[b] The number of hydrogens on a carbon was determined by HSQC. [c-g] Interchangeable signals within the same marks

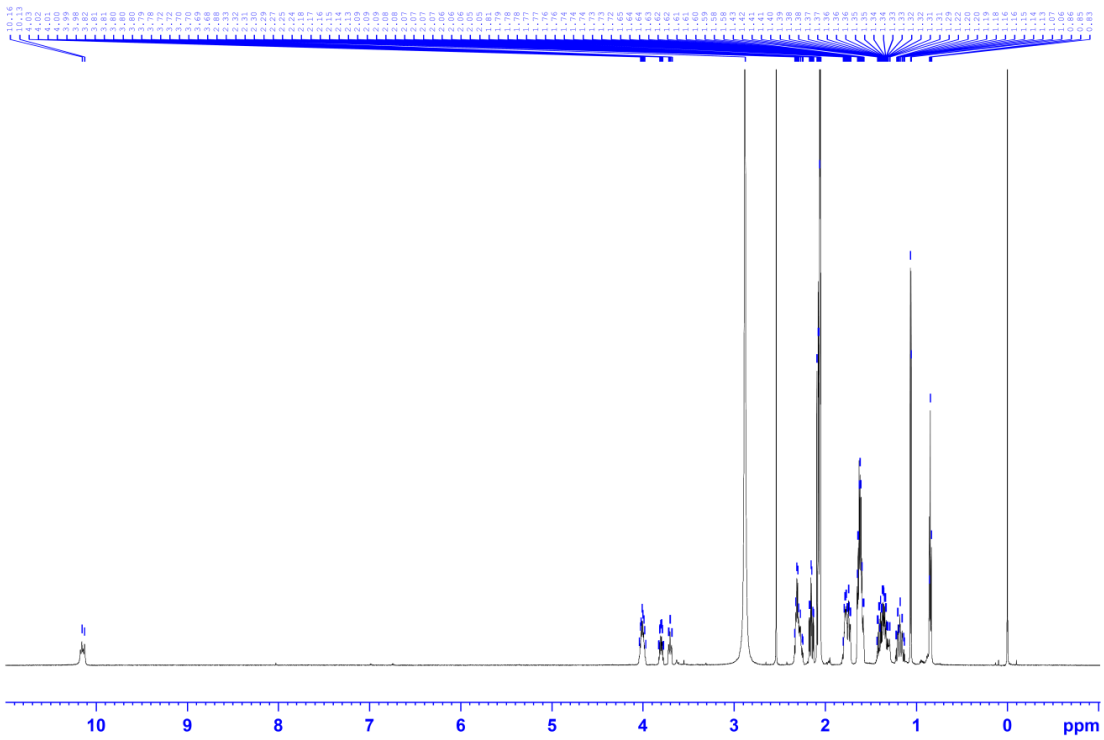

Figure 3.11. ^{1}H NMR spectrum of **2** (600 MHz, acetone-d_6)

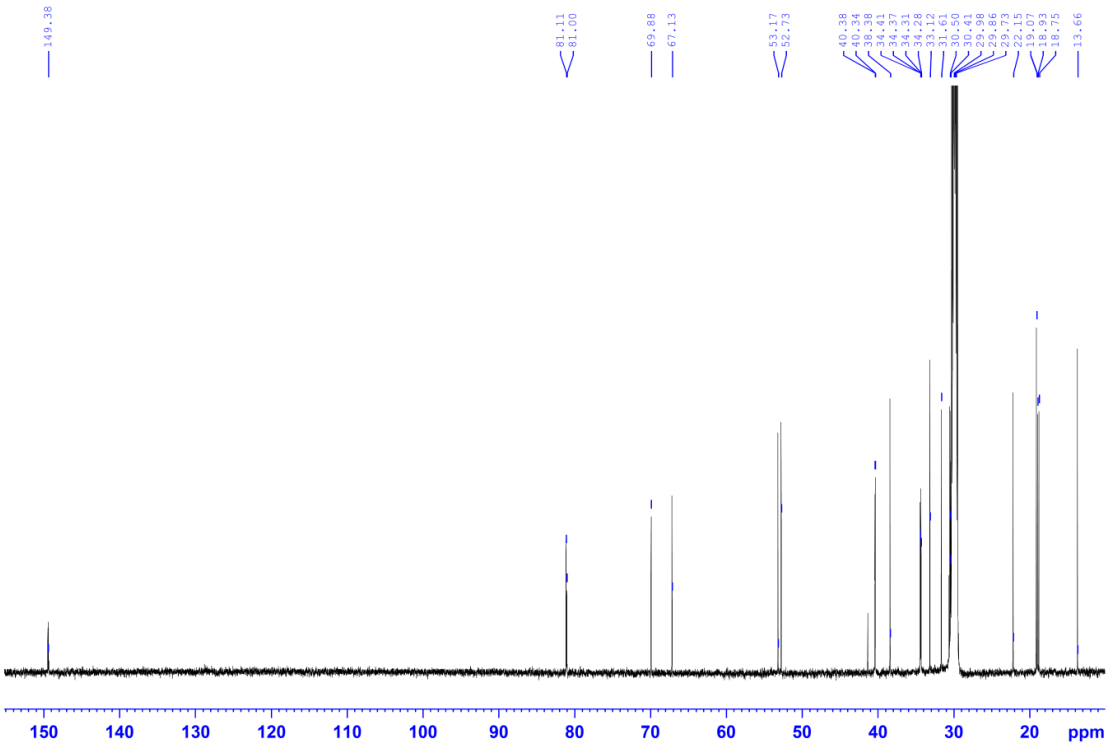

Figure 3.12. ^{13}C NMR spectrum of **2** (150 MHz, acetone-d_6)

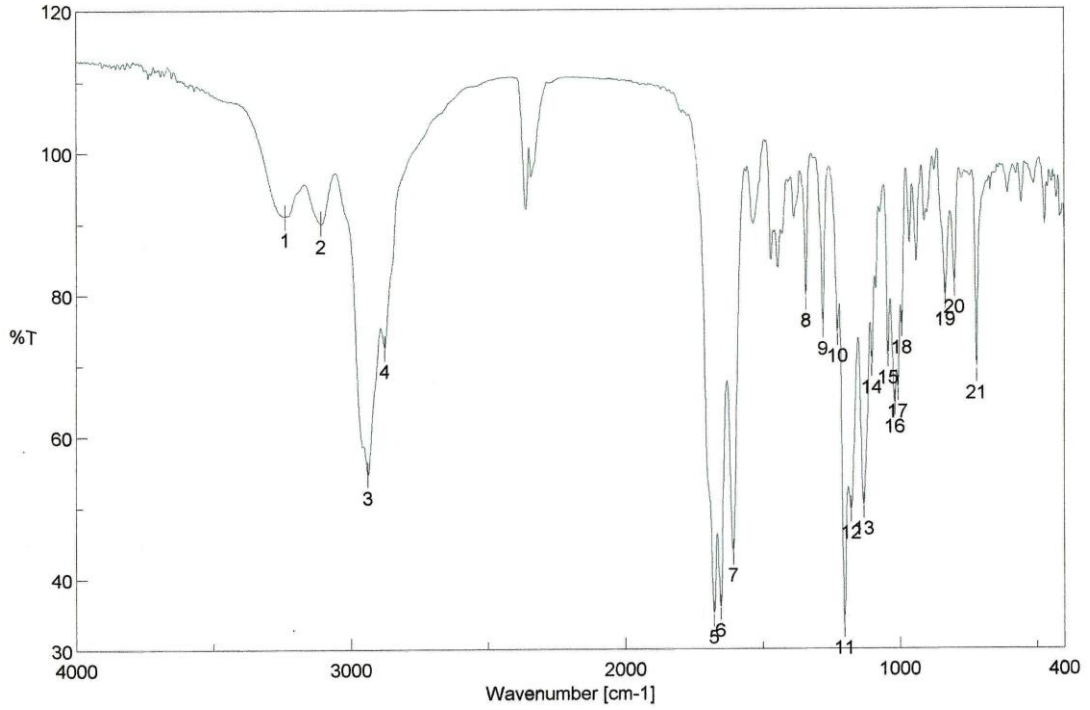

Figure 3.13. IR spectrum of **2**

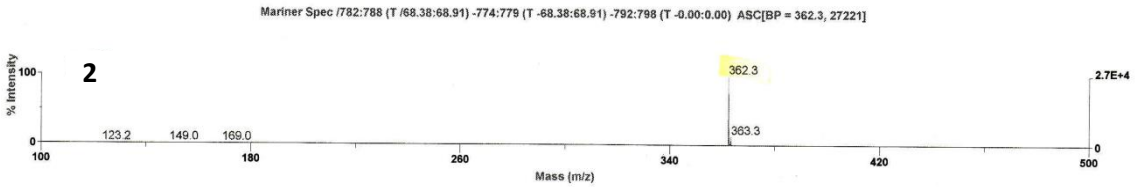

Figure 3.14. ESIMS (+) spectrum of **2**

The DQF-COSY correlations for **2** indicated the five partial structures CH_3-1–CH_2-2, CH_2-3–CH-4–CH_2-5–CH_2-6, CH_2-9–CH-10–CH_2-11, CH_2-12–CH-13–CH_2-14, and CH_2-17–CH_2-18–CH-19–CH_3-20 (Figure 3.15a). The HMBC correlations from H_3-1 to C-3, and from H_2-3 to C-2 and C-1 confirmed the presence of a propyl group on C-4. Furthermore, the correlations from H_2-6 to C-8, from H_2-7 to C-6, from H_2-17 to C-15, and from H_2-16 to C-17 confirmed the connectivities of C-6–C-7 and C-16–C-17. An HMBC spectrum was obtained in acetone-d_6 (Figure 3.15a, dotted arrows) indicating the following important correlations: from NHb to C-7, C-8, C-9, and C-21

and from NHa to C-14, C-15, and C-16, revealing not only the presence of guanidine moiety but also the position of C-7, C-8, C-15, and C-16. Based on these analyses, crambescidin 361 (**2**) was deduced as a new crambescidin analog with two tetrahydropyrane rings instead of the combination of the left-side unsaturated seven membered ring and the right-side tetrahydropyrane ring as found in **1** and most of the crambescidin analogs. Another structural feature of **2** is the presence of a propyl group as an alkyl substituent, which is quite rare in the crambescidin-type alkaloids [37, 38].

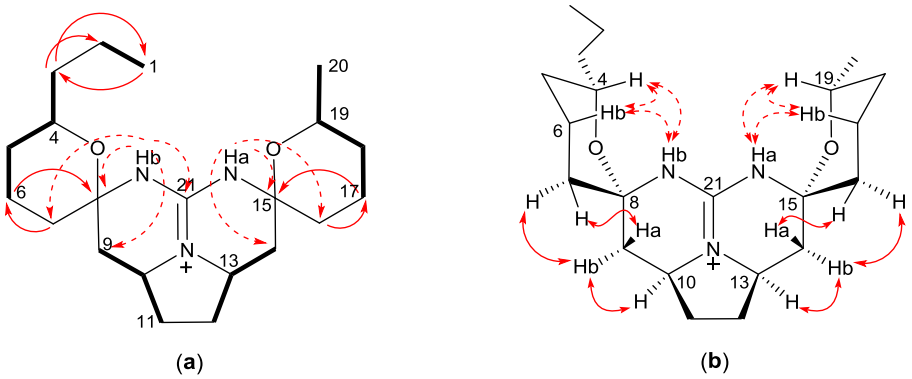

Figure 3.15. Two dimensional NMR correlations for **2**. (**a**) Key DQF-COSY (bold bonds) and HMBC correlations (solid arrows in CD_3OD and dotted arrows in acetone-d_6); (**b**) Key NOESY correlations in CD_3OD (solid arrows) and acetone-d_6 (dotted arrows). The position of the propyl and methyl groups at C-4 and C-19 are tentative

The relative configuration of **2** was examined by the interpretation of NOESY correlations (Figure 3.15b). The chair conformation of both the tetrahydropyrane rings was determined by the 1,3-diaxial correlations of δ_H 3.63 (H-4)/1.85 (H-6b) and δ_H 3.74 (H-19)/1.85 (H-17b) (Figure 3.15b, dotted double arrows). The NOESY correlations of δ_H 10.16 (NHb)/H-4, NHb/H-6b, δ_H 10.13 (NHa)/H-19, and NHa/H-17b indicated that both the guanidine NHs were also in the axial orientation about the tetrahydropyrane rings. The additional NOESY correlations of δ_H 1.74 (H-7)/1.57 (H-9a), H-7/2.19 (H-

9b), H-9b/δ_H 4.00 (H-10), δ_H 4.00 (H-13)/2.21 (H-14b), δ_H 1.59 (H-14a)/1.74 (H-16), and H14b/H-16 suggested the relative configuration between C-8 and C-10 and between C-13 and C-15. Although the NOESY correlation of H-10/H-13 was not obtained due to their identical chemical shifts, the large coupling constants of 12.8 Hz between H-9a and H-10 and between H-14a and H-13 described that both H-10 and H-13 were in the axial-like α orientation. These findings support the relative stereochemistry of **2** as shown in Figure 3.15b. Since it was difficult to determine the position of two alkyl groups due to the highly symmetrical nature of **2**, the alkyl position was tentatively assigned as shown becasue most of the related guanidine alkaloids possess a methylated tetrahydropyrane ring at the right side of the molecule. The specific rotation of **2** (-7.9) similar to that of **1** suggests the identical absolute configuration of **1** and **2**.

Crambescidin 373 (**3**) possesses the molecular formula $C_{22}H_{35}N_3O_2$ as determined by the pseudo-molecular ion at *m/z* 374.2786 $[M+H]^+$ (calculated for $C_{22}H_{36}N_3O_2$, 374.2802) in HR-ESIMS (Figure 3.20). The NMR data (Figures 3.16 and 3.17, Table 3.3) were found to be similar to those for **1** and **4**, indicating that **3** was another crambescidin analog with an ethyl group as supported by the signals at δ_H 1.42/δ_C 30.0 (CH_2-20) and δ_H 0.85/δ_C 10.2 (CH_3-21). This ethyl group was found to be connected to C-19 by a DQF-COSY experiment (Figure 3.21). The exchangeable protons of **3** were observed at δ_H 10.49 and 10.06 in $CDCl_3$ (Figure 3.18), revealing that **3** is the 19-ethyl homolog of **1**. Although satisfactory NOESY data for **3** was not obtained due to the lack of the sample, the close similarity of the NMR data and the specific rotation (-8.0 for **3** and -8.9 for **4**) to those for **4** suggests that the absolute configuration of **3** is the same as that of **4**.

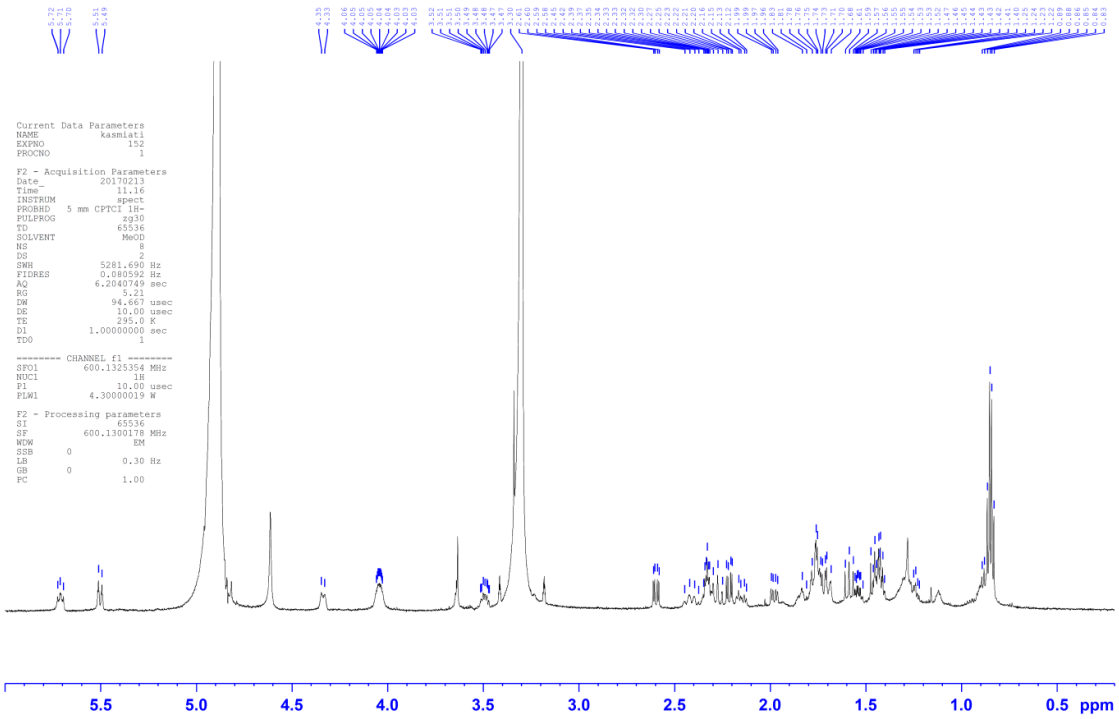

Figure 3.16. ^{1}H NMR spectrum of **3** (600 MHz, CD_3OD)

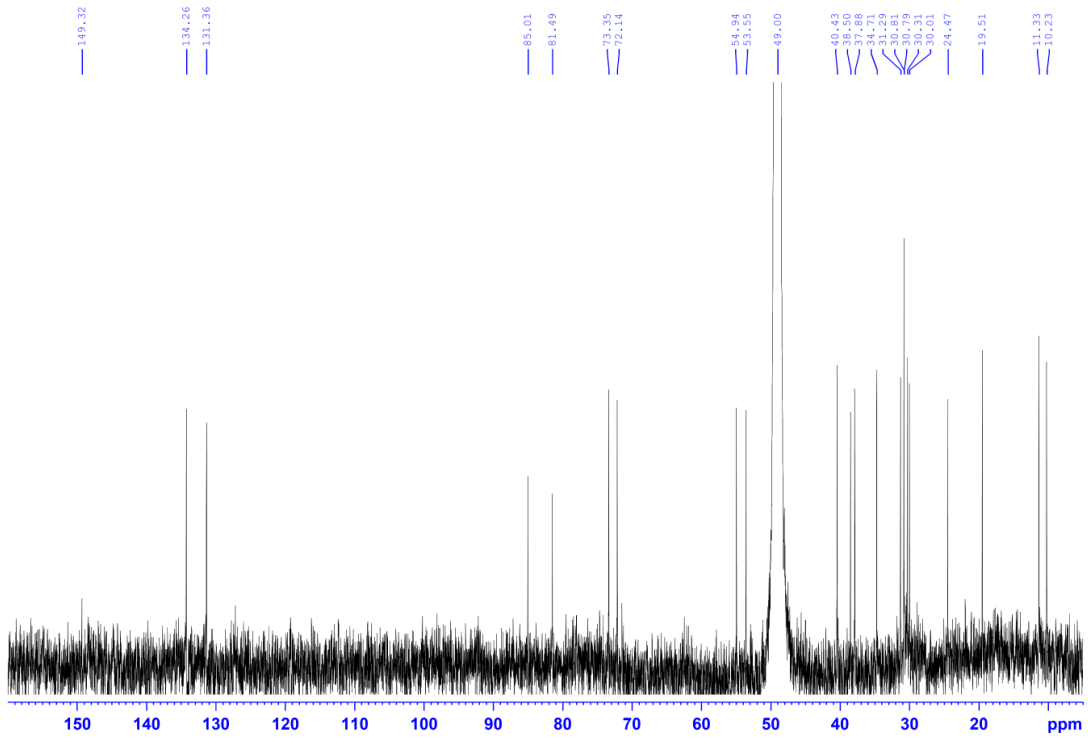

Figure 3.17. ^{13}C NMR spectrum of **3** (150 MHz, CD_3OD)

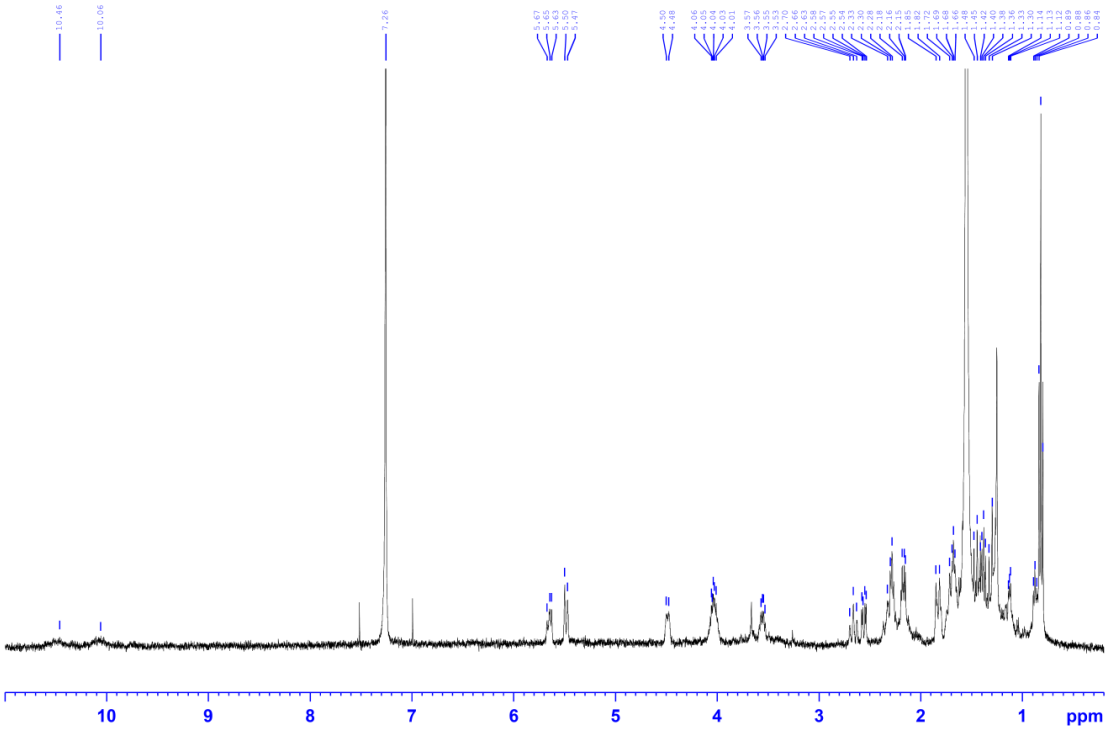

Figure 3.18. ^{1}H NMR spectrum of **3** (400 MHz, $CDCl_3$)

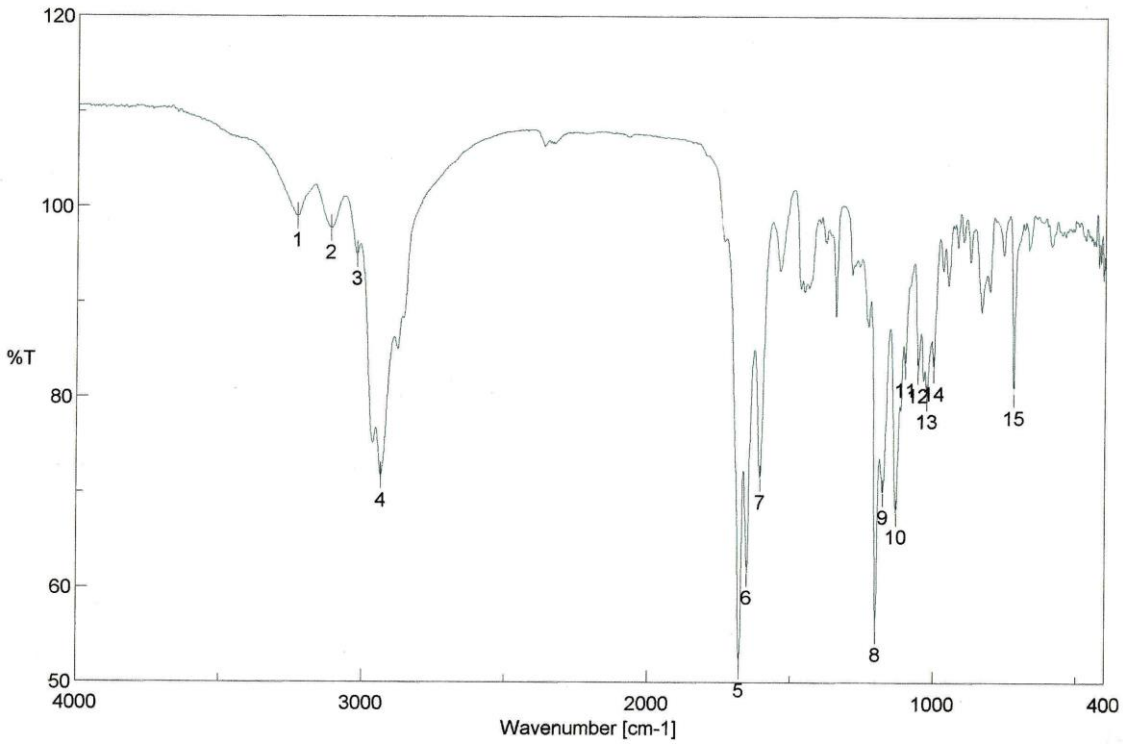

Figure 3.19. IR spectrum of **3**

Table 3.3. ^{1}H and ^{13}C NMR data for **3** (CD_3OD)[a]

Position	δ_H (ppm), m (*J* in Hz)	δ_C (ppm)[b]
1	0.84, t (7.2)	11.3, CH_3
2a	1.46, m	30.3, CH_2
2b	1.54, m	
3	4.33, brd (10.2)	72.1, CH
4	5.50, dt (11.2, 2.1)	134.3, CH
5a	5.71, m	131.4, CH
6a	2.15, dt (15.3, 7.2)	24.5, CH_2
6b	2.42, brt (15.3)	
7a	1.97, dd (13.5, 6.0)	38.5, CH_2
7b	2.27, t (13.5)	
8		85.1, C
9a	1.45, t (12.9)	37.9, CH_2
9b	2.59, dd (12.9, 4.8)	
10	4.05, m	54.9, CH
11a	1.75, m	30.8, CH_2
11b	2.32, m	
12a	1.75, m	30.8, CH_2
12b	2.32, m	
13	4.03, m	53.5, CH
14a	1.59, t (13.0)	40.4, CH_2
14b	2.21, dd (13.0, 4.8)	
15		81.5, C
16a	1.73, m	34.7, CH_2
16b	1.77, m	
17a	1.77,m	19.5, CH_2
17b	1.85, m	
18a	1.26, m	31.3, CH_2
18b	1.70, m	
19	3.50, m	73.3, CH
20	1.42, m	30.0, CH_2
21	0.85, t (7.2)	10.2, CH_3
22		149.4, C

[a] Measured at 600 MHz for ^{1}H and 150 MHz for ^{13}C

[b] The number of hydrogens on a carbon was determined by HSQC

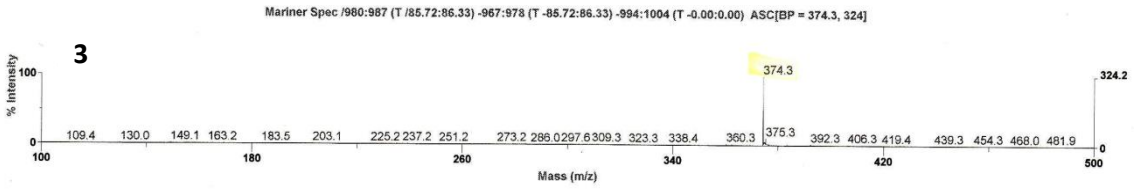

Figure 3.20. ESI-MS (+) spectrum of **3**

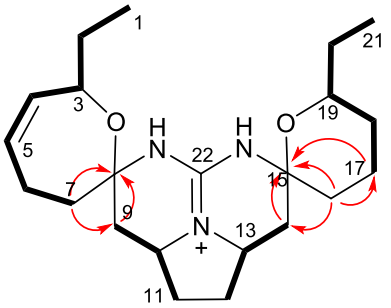

Figure 3.21. Two dimensional NMR correlations of **3**. DQF-COSY and HMBC correlations are indicated by bold bonds and arrows, respectively

Guanidine compounds were mostly reported from marine organisms [39]. They have intriguing structures and wide range of biological activities and have attracted much attention of chemists and pharmacologists for their potential as drug leads [40]. Due to the strongest organic bases, guanidines are fully protonated under physiological conditions to form guanidinium cation, which can interact with biopolymers such as DNA and proteins through hydrogen bonds and/or electrostatic interactions [41, 42]. Since the first pentacyclic guanidine alkaloid ptilomycalin A was isolated from the Caribbean sponge *Ptilocaulis spiculifer* and the Red Sea sponge *Hemimycale* sp. in 1989 [43], an array of cyclic guanidine alkaloids have been reported to date, including ptilomycalins [44, 45], crambescidins [30, 32, 33, 35, 46, 47], monanchocidins [48, 49], and monanchomycalins [37, 38]. Particularly, a number of metabolites of these types have been isolated mainly from marine sponges of the genera *Monanchora* and *Crambe*. The crambescidins and related alkaloids are characterized by a pentacyclic guanidine skeleton (vessel part) with two alkyl groups (ethyl at C-3 and methyl at C-19 in most cases) and a long aliphatic chain with a terminal carboxylate or a terminal spermidine amide. Crambescidins 359 (**4**) and 431 were reported in 2000 as the first crambescidin analogs lacking the long aliphatic chain (at C-14 in **5** and **6**), which is replaced by a hydrogen atom and an ethyl ester group, respectively [30]. Our compounds **1–3** are

additional analogs of this type and structurally characteristic in the following points. Crambescidin 345 (**1**) is the first analog with a non-alkylated tetrahydropyrane ring. Crambescidin 361 (**2**) possesses a rare propyl substituent as well as two tetrahydropyrane rings instead of the combination of one unsaturated oxepane and one tetrahydropyrane rings, which are found in most of the crambescidin-type alkaloids. Crambescidin 373 (**3**) is the first analog with an ethyl group at the right-side tetrahydropyrane ring, which possesses a methyl group in most of the reported crambescidin-type alkaloids.

3.2.2. Biological activity

Biological activities of the isolated crambescidins **1**–**6** were evaluated as cytotoxic and anti-oomycete agents against the human epidermoid carcinoma cell line A431 and the oomycete plant pathogen *Phytophthora capsici*, respectively. All compounds showed cytotoxicity with an IC_{50} value lower than 10 μM (Figure 3.22a). The strongest cytotoxicity was observed for the long side chain-bearing crambescidins **5** and **6** with IC_{50}'s of 12 and 48 nM, respectively. Meanwhile, other crambescidins (**1–4**) without the long side chain part indicated a moderate cytotoxicity with IC_{50}'s of 7.0, 2.5, 0.94, and 3.1 μM, respectively. On the contrary, all new crambescidins **1**–**3** and the known analog **4** showed a higher anti-oomycete activity [minimum inhibitory dose (MID) of 50 μg/disk] than that for **5** and **6** (MID of 100 μg/disk or higher) (Figure 3.22b). It is interesting to note that the highly cytotoxic crambescidins (**5** and **6**) with a long side chain showed a lower anti-oomycete activity than the others. Especially, the most cytotoxic compound **5** showed no anti-oomycete activity even at 500 μg/disk (not

indicated in Figure 3.22b). Consequently, these biological activities are approximately in an inverse relationship (Figure 3.22c).

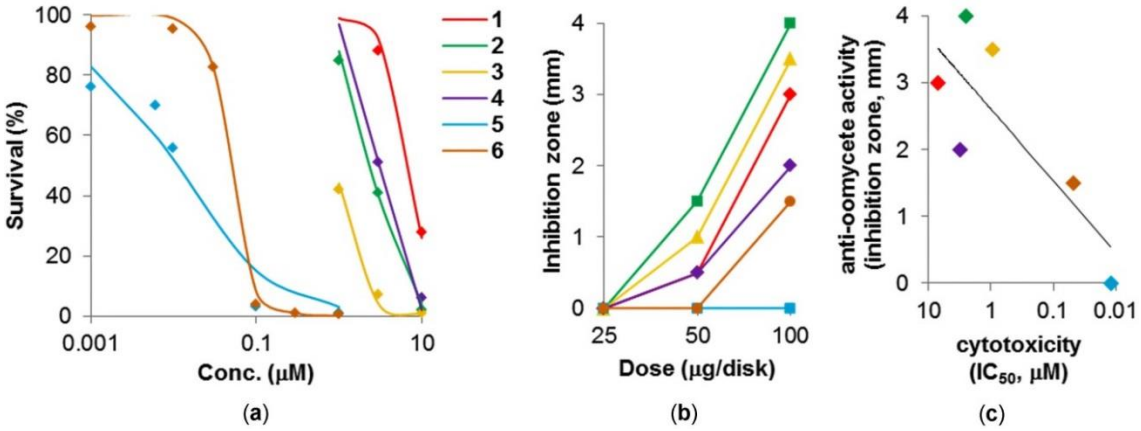

Figure 3.22. Biological activity of **1–6**. (**a**) Cytotoxicity against the human epidermoid carcinoma A431 cells. The curves were generated by sigmoid fitting; (**b**) Anti-oomycete activity against the plant pathogen *P. capsici*. The same colors are used as in Figure 3.22a. (**c**) Inverse relationship between the cytotoxicity and anti-oomycete activities of **1–6**. The data for anti-oomycete activity were observed at a dose of 100 μg/disk.

Previous SAR studies on the crambescidins and their analogs reported that the presence of the long aliphatic side chain enhanced the cytotoxic effect of the guanidine core [50□53]. Our cytotoxicity data indicating the significant effect of the long aliphatic side chain are in good agreement with the previous reports. The long aliphatic side chain could affect the permeability of the guanidine alkaloid into animal cells. In contrast, it did not affect or rather diminished the anti-oomycete activity, which might be attributable to a low permeability through the microbial cell wall (mainly □-glucan) or/and into the hydrophilic agar medium used in the test.

3.3 Materials and methods

3.3.1. Isolation of bioactive compounds

A reddish Indonesian sponge was collected by hand using a snorkeling equipment at a depth between 0.5 and 3 meter in Samalona Island (S5°8'16.4" – E119°23'22.60"), South Sulawesi Sea, in August 2015. The species was identified as *Clathria bulbotoxa* based on the observation of its morphology and spicule elements under a microscope. The sponge possesses bulbous toxa spicule bulging toward the center which distinguishes it from other species [54]. The organism (750 g, wet weight) was lyophilized, homogenized in MeOH (1.5 L) and stand at room temperature for 3 days. The mixture was filtrated and the filtrate was concentrated to give an aqueous residue, which was extracted three times with EtOAc (225 mL). The combined organic layers were concentrated to yield EtOAc extract (6.4 g). This extract was dissolved in 90% MeOH (75 mL) and extracted twice with hexane (150 mL). Both layers were concentrated to obtain 90% MeOH (3.1 g) and hexane (2.5 g) fractions.

The 90% MeOH fraction, which showed a cytotoxicity (IC_{50} = 0.046 μg/mL), was separated by open column chromatography (silica gel, 100 g) eluted with 2, 5, 10, 100% of MeOH in $CHCl_3$ (600 mL each). The fractions were collected by every 100 mL, and appropriately combined to give six fractions (fr.1 – fr.6), where fr.3 (222 mg) eluted with 2-5% MeOH and fr.6 (2.1 g) eluted with 100% MeOH showed significant cytotoxic activity of IC_{50} = 0.013 and 0.092 μg/mL, respectively. Fr.3 was further chromatographed on silica gel (HI-FLASH^{TM} Size L, 30 g, Yamazen Co., Osaka, Japan] with linear gradient of 10–80% $CHCl_3$-MeOH-H_2O (90:9:1) in EtOAc (40 min) at a flow rate of 10 mL/min to yield eight fractions (fr.3.1 – fr.3.8). The cytotoxic fr.3-5

(33.1 mg, IC_{50} = 0.8 ng/mL) was purified by HPLC [Develosil ODS-HG-5 (20 x 250 mm, Nomura Chemical Co., Ltd., Seto, Aichi, Japan), 80–100% MeOH-20 mM NH_4CH_3COO (40 min), 6 mL/min, detected at 215 nm] to give crambescidin 657 (**5**, 16.2 mg, t_R = 48.0 min). The three fractions, fr.3.6 – fr.3.8 (110 mg in total, IC_{50} = 0.081 μg/mL) were combined and subjected to HPLC [Develosil ODS-HG-5 (20 x 250 mm), 50–80% MeOH-0.1% TFA (60 min), flow rate 6 mL/min, monitored at 205 nm] to obtain a **1**-containing fraction (1.5 mg, t_R = 40.8 min), crambescidin 359 (**4**, 26.0 mg, t_R = 45.6 min), crambescidin 361 (**2**, 2.4 mg, t_R = 55.4 min), and crambescidin 373 (**3**, 1.1 mg, t_R = 62.0 min). The **1**-containing fraction was further purified by HPLC [Develosil ODS-UG-5 (10 x 250 mm), 60% MeOH-0.1% TFA, 2 mL/min, detected at 215 nm] to obtain pure crambescidin 345 (**1,** 0.6 mg, t_R = 19.7 min). The fr.6 (2.1 g, IC_{50} = 0.092 μg/mL) was fractionated through a silica gel (50 g) open column, eluted with 10, 20, 40, 60, 100% MeOH-H_2O (90:10) in $CHCl_3$ to afford four fractions (fr.6-1 – fr.6-4). The active fr.6-3 (500 mg, IC_{50} = 0.017 μg/mL) eluted with 40% MeOH-H_2O (90:10) in $CHCl_3$ was then chromatographed on silica gel (HI-FLASH^{TM}, Size L, 30 g) with gradient elution of 10–100% MeOH in $CHCl_3$ for 40 min at a flow rate of 10 mL/min to give four fractions (fr.6.3.1 – fr.6.3.4). The active fr. 6.3.3 (180 mg, IC_{50} = 0.019 μg/mL) eluted with 54-86% MeOH was subjected to HPLC [Develosil ODS-HG-5 (20 x 250 mm), 40–60% MeCN-0.1% TFA (60 min), 8 mL/min, detected at 230 nm] to obtain crambescidin 800 (**6**, 21.7 mg, t_R = 36.3 min).

*3.3.1.1. Crambescidin 345 (**1**)*

Colorless powder; $[\alpha]_D^{26}$-7.1 (0.051, MeOH); IR (film) ν_{max} 3222, 3109, 3019, 1678, 1654, 1607, 1201, 1177, 1131, and 720 cm^{-1}; HR ESIMS *m/z* 346.2495 $[M+H]^+$; calcd. for $C_{20}H_{32}N_3O_2$ 346.2489.

*3.3.1.2. Crambescidin 361 (**2**)*

Pale yellow solid; $[\alpha]_D^{26}$-7.9 (0.13, MeOH); IR (film) ν_{max} 3236, 3107, 1676, 1652, 1606, 1201, 1176, 1131, 1017, and 719 cm^{-1}; HR ESIMS *m/z* 362.2765 $[M+H]^+$; calcd. for $C_{21}H_{36}N_3O_2$ 362.2802.

*3.3.1.3. Crambescidin 373 (**3**)*

Pale yellow solid; $[\alpha]_D^{26}$-8.8 (0.025, MeOH); IR (film) ν_{max} 3228, 3111, 3019, 1678, 1652, 1606, 1201, 1177, 1131, and 720 cm^{-1}; HR ESIMS *m/z* 374.2786 $[M+H]^+$; calcd. for $C_{22}H_{36}N_3O_2$ 374.2802.

*3.3.1.4. Crambescidin 359 (**4**)*

Pale yellow solid; $[\alpha]_D^{26}$-8.9 (0.23, MeOH) (reference [45]: $[\alpha]_D^{26}$-12.7 (0.4, MeOH))

*3.3.1.5. Crambescidin 657 (**5**)*

Yellowish solid; $[\alpha]_D^{26}$-11.0 (0.18, MeOH) (reference [55]: $[\alpha]_D^{25}$ -12.1 (0.34, MeOH))

*3.3.1.6. Crambescidin 800 (**6**)*

Yellowish solid; $[\alpha]_D^{26}$ -8.7 (1.6, MeOH) (reference [45]: $[\alpha]_D^{20}$ -7.8 (4.1, MeOH))

3.3.2. Cytotoxicity assay

Cytotoxic activity was done by following the procedure describe in section 2.2.2.1 with various concentrations of compounds**:** none, 1, 3, 10 μM (**1**–**4)**; none, 0.001, 0.01, 0.1, and 1.0 μM (**5** and **6**)

3.3.3. Anti-oomycetete assay

Anti-oomycete activity for pure compounds was performed by the above-mentioned method in section 2.2.2.2 with doses of none, 25, 50, and 100 μg/disk.

3.4. Conclusions

In the present study, we discovered three new guanidine alkaloids, crambescidins 345 (**1**), 361 (**2**), and 373 (**3**), together with three known crambescidins **4–6** from the Indonesian sponge *Clathria bulbotoxa*. The structures of **1–3** with absolute stereochemistry were determined by spectroscopic analysis including two-dimensional NMR and specific rotation. Although the pentacyclic guanidine core has been found in a number of the crambescidins and related natural compounds, a high diversity in the alkyl substituents (methyl, ethyl, propyl) on the cyclic ether rings of our compounds was observed for the first time, whereas most related products possess ethyl group at C-3 and methyl group at C-19. The biological assays revealed that the long aliphatic side chain in compounds **5** and **6** plays a quite important role for the cytotoxicity against cancer cells (possibly due to the increase of permeability through cell membrane) but conversely not for the inhibition of an oomycete plant pathogen.

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