REVISITING MARINE BIOPROSPECTING OF TROPICAL INDONESIAN MACROALGAE FROM WEST TIMOR

Turupadang, Welem Linggi

Victoria
UNIVERSITY OF WELLINGTON
Te Whare Wānanga
o te Ūpoko o te Ika a Māui
www.vuw.ac.nz

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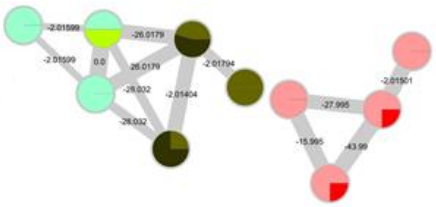
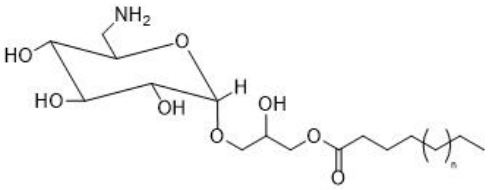
Abstract

Marine algae are an important and historically rich source of new marine-based natural products. This thesis describes the screening of 40 Indonesian macroalgal samples using liquid chromatography-mass spectrometry (LC-MS) based molecular networking, and the subsequent nuclear magnetic resonance (NMR)-guided isolation and structural elucidation of a 6-deoxy-6-aminoglycoglyrecolipid (**60**). Molecular networking was performed using LC-MS/MS data through the online Global Natural Product Social Molecular Networking (GNPS) platform directly from crude extracts. NMR spectroscopy-guided screening was also employed targeting unique peaks detected by ^{1}H NMR to validate any hits from GNPS. Out of 40 macroalgae specimens collected from West Timor waters, six samples were prioritised by the molecular networking screening. Proton NMR revealed three specimens with significantly interesting peaks but only one specimen, *Laurencia snackeyi* was purified further, which yielded compound **60**.





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Glossary

δ	Chemical shift (ppm)
^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	1H NMR Proton nuclear magnetic resonance
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric-Pressure Ionization
C_{18}	Octadecyl derivatised silica
CD_3OD	Deuterated Methanol
COSY	Correlation spectroscopy
***Cox*1**	Mitochondrial marker cytochrome oxidase subunit I
CTAB	Cetyl trimethylammonium bromide
DCM	Dichloromethane
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
DIOL	2,3-dihydroxypropoxy-propyl-derivatised silica
DNA	Deoxyribonucleic acid
ESI	Electrospray Ionization
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
GNPS	Global Natural Product Social Molecular Networking
H_2O	Water
H_2SO_4	Sulfuric acid
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HP20	Copolymer
HP20	PSDVB stationary support
HPLC	High-Performance Liquid Chromatography
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single-quantum correlation spectroscopy
J	Scalar coupling constant (Hz)
LC	Liquid chromatography

LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
m	Multiplet
m/z	Mass-to-Charge Ratio
Me_2CO	Acetone
MeOH	Methanol
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
ppm	Parts per million
PSDVB	Poly-styrene divinylbenzene
QTOF	Quadrupole Time-of-Flight
ROESY	Rotating-frame nuclear Overhauser effect spectroscopy
s	Singlet
SCPS	School of Chemical and Physical Sciences
SCUBA	Self-contained underwater breathing apparatus
t	Triplet
TLC	Thin-layer chromatography
TOF	Time-of-Flight
VUW	Victoria University of Wellington

CHAPTER 1. INTRODUCTION

1.1. History of traditional medicines and natural product discovery

Nature has been a source of traditional remedies, useful for cures and treatment for human civilization throughout history. The ancient Egyptians have recorded the application of traditional medicines as early as 2900 BC, in the form of the Ebers Papyrus, which provides numerous types of remedies based on plants.[1] Another old record dated back to 2600 BC, when a clay-tablet cuneiform from Mesopotamia documented the application of cypress and myrrh as pharmaceutical agents.[2] Two Greek physicians, Hippocrates of Cos (circa 460–377 BC) and Galen (129–200 AD), developed about 400 natural agents and 540 herbal extracts respectively.[3] Hippocrates, for example, prescribed an extract of *Atropa belladonna*, commonly known as deadly nightshade, as an anesthetic and also advised the use of *Ornithogalum caudatum* (squill) juice, which might help diuresis.[3]

Like its European counterpart, natural remedies also flourished in China and India. Wu Shi Er Bing Fang, an ancient medical text, recorded the use of about 247 plant-derived agents as well as 150 notes of drug formulae compiled around 350 BC by the Chinese. Following that, the monograph Shen Nong Ben Cao Jing (Shen Nong Materia Medica) produced during the Eastern Han dynasty (25–220 AD), documented 253 plant-based and 67 of animal derived medicines.[2-3] These include anti-asthmatics from *Ephedra sinica* (ephedra herb), Coptis root (*Coptis chinensis*) as a remedy for diarrhea, and an antihelminthic from chinaberry seed (*Melia azedarach*).

Like the Chinese, the Indian Ayurveda system, dating back to 900 BC, also had a document called Charaka Samhita, which prescribed 341 plant-based remedies.[4] Another record worth mentioning is the Sushruta Samhita (circa 600 BC), which provides around 453 complementary natural medicines alongside primarily surgical procedures.[5] A significant achievement from the Ayurvedic legacy to modern medicine is reserpine (**1**, Figure 1.1), a tranquilizer isolated from the sarpagandha plant (*Rauwolfia serpentina*), which is a therapeutic agent for hypertension, insomnia, and insanity.[6]

The activity of traditional remedies comes from secondary metabolites produced by an organism. These active secondary metabolites produced, by living organisms, are referred as natural products. Natural products are compounds that are not necessarily needed to maintain functionality, differentiated from primary metabolites (such as glucose or DNA) which are essential compounds required for metabolism and for organisms to survive. Secondary metabolites are rare and offer novel structures and functionalities compared to primary metabolites.

Throughout history, traditional remedies have provided a solid ground for discovering modern medicines. The era of modern drugs began in the nineteenth century when a German pharmacist, Friedrich Sertürner, successfully isolated several alkaloids including morphine (**2**, Figure 1.1) from opium poppy *Papaver somniferum* L.[1] Morphine was then commercialized by Merck in 1826 as the first plant-based natural products.[3] Following this progress, more compounds were found including an analgesic, salicin (**3**, Figure 1.1) from willow bark and an antimalarial drug, quinine (**4**, Figure 1.1) from cinchona bark.[6] Salicin was later developed by Bayer in 1900 as a pain reliever, which have commercially known as aspirin (**5**, Figure 1.1).[7]

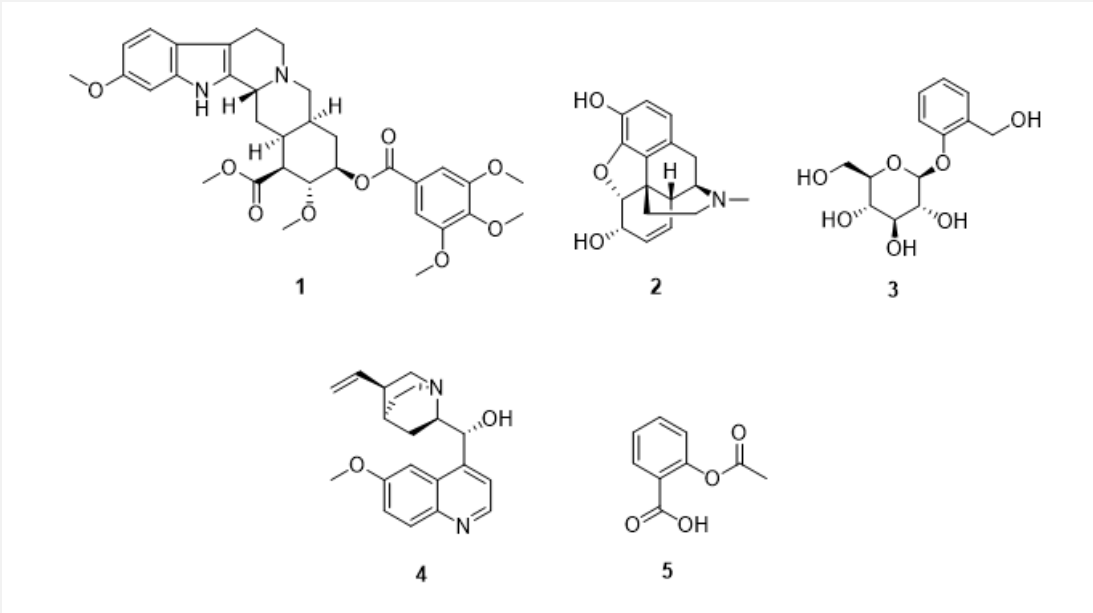


Figure 1.1. Chemical structure of reserpine (1), morphine (2), salicin (3), quinine (4), and aspirin (5)

At the beginning of the twentieth century, natural products continued to make a tremendous contribution to human civilization and provided tools for scientists to

overcome many modern diseases. In 1929, Alexander Fleming continued the positive era of bioactive natural products by discovering the antibacterial properties of penicillin G (**6**, Figure 1.2) from the mold *Penicillium notatum*, which at that time helped physicians to battle infectious diseases.[3] Other key drugs in the modern era are artemisinin (**7**, Figure 1.2) and quinoline (**8**, Figure 1.2), which are both used today as first-line drugs to combat malaria.[8] Artemisinin was discovered by Tu Youyou, a Chinese scientist, in the late 1960s from the sweet wormwood plant (*Artemesia annua*). Later in the late 1970s, Wani *et al.*, discovered paclitaxel (Taxol®; **9**, Figure 1.2) from the plant *Taxus brevifolia*.[9] Taxol underwent lengthy development before it was clinically approved as one of the treatments against ovarian cancer and breast cancer in 1992 and 1994, respectively.[2]

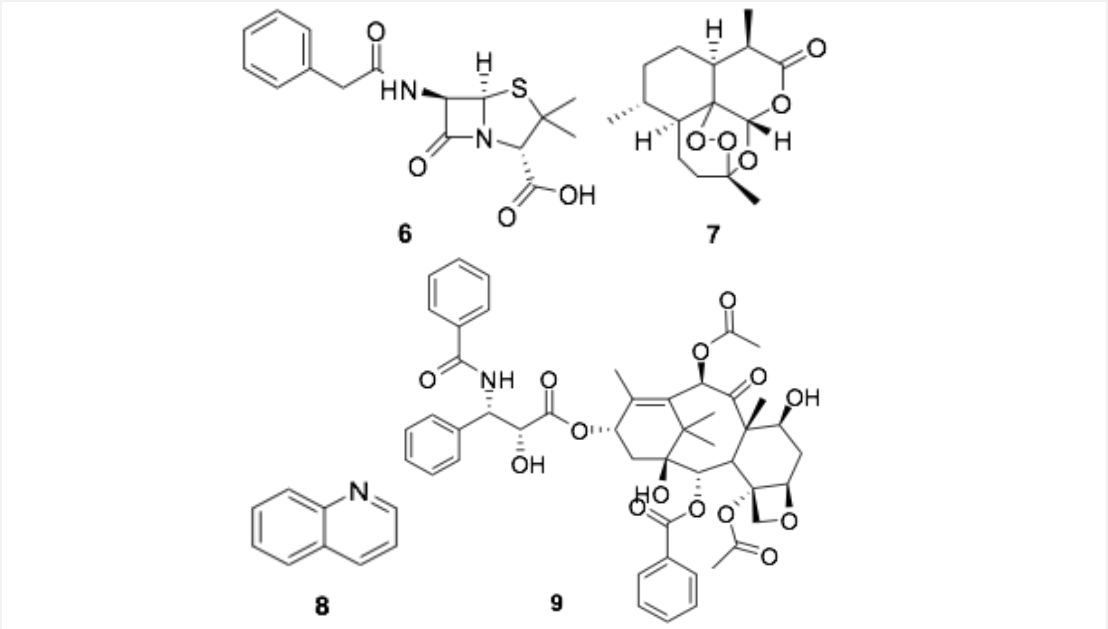


Figure 1.**2.** Chemical structure of penicillin G (**6**), artemisinin (**7**), quinoline (**8**), and paclitaxel (**9**)

However, the slow output of drug research and development (R&D) programs of natural products in many pharmaceutical companies in the late 1980s resulted in the closure of many departments in the industry.[10] After the 1980s, the number of newly discovered molecules plunged from 60 new chemical entities (NCEs) on a yearly basis in the 1980s to 18-34 per year *de novo* discoveries in the decade 2001-2010. The decline happened due to the development of high-throughput screening (HTS), which marginalized the role of natural products in drug discovery by the majority of pharmaceutical industries. [2, 10-11] HTS technology, based on

combinatorial chemistry, continued to be the backbone method of optimizing structures of many compounds.[12] Practically, natural products chemistry was abandoned in this particular period.[1, 13]

In a later development, however, HTS failed to address productivity in drug discovery where fewer compounds made it beyond clinical trials. In fact, there was only one combinatorial chemistry-derived NCE approved by the U.S Food and Drug Administration (FDA),[1, 10] the multi-kinase inhibitor, sorafenib (Nexavar®; **10**, Figure 1.3) from Bayer which is a treatment for renal carcinoma (approved by the FDA in 2005). Natural products once again obtained momentum in the drug design and discovery by its "nature's privileged structure," a structure that can be obtained only from actively perform bioprospecting from the nature.[11] Consequently, there have been recent increases in natural products-based drug discovery.

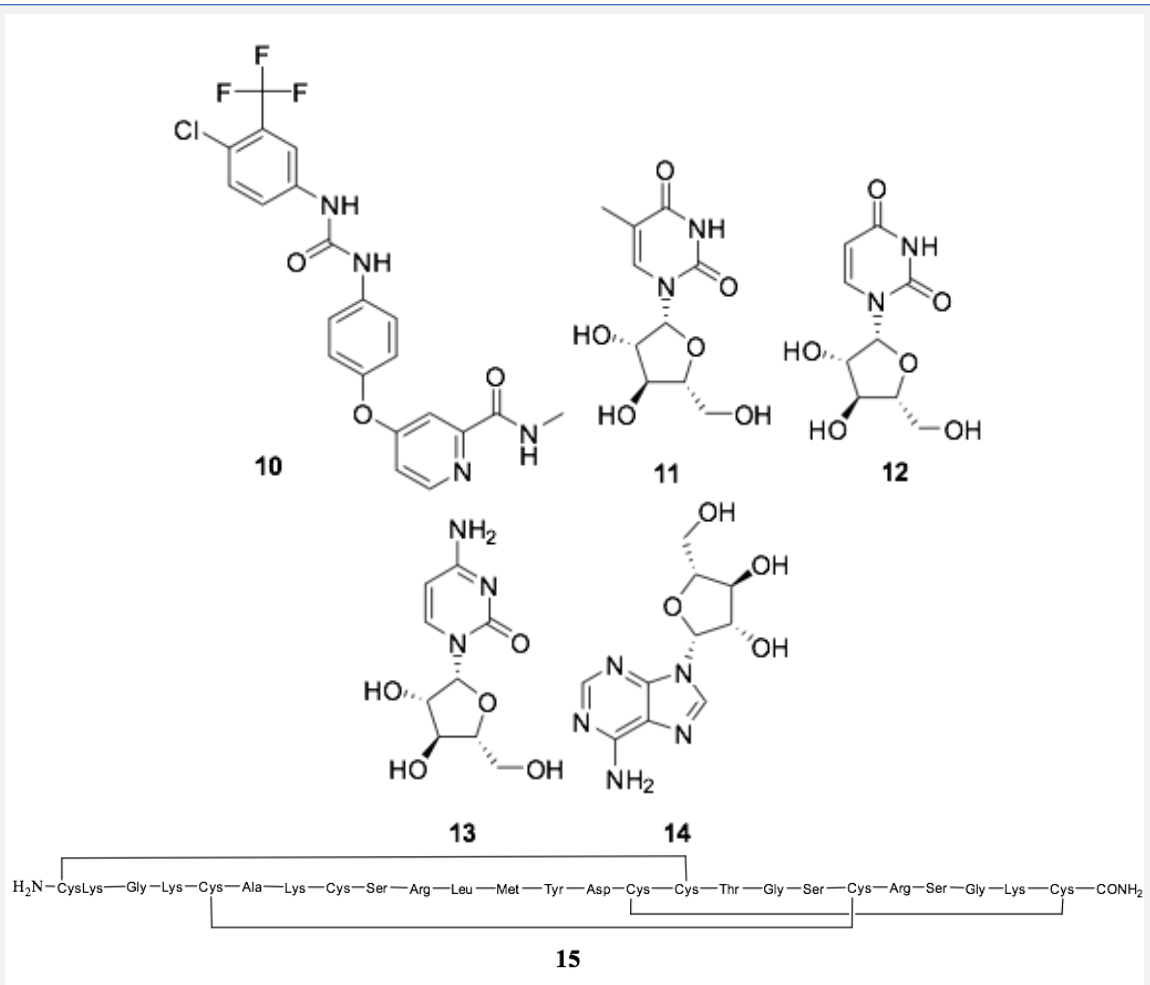


Figure 1.3. Chemical structure of sorafenib (**10**), spongothymidine (**11**), spongouridine (**12**), Ara-C (**13**), Ara-A (**14**), and ziconotide (**15**)

1.2. Marine natural products

Natural products as lead compounds have mainly come from terrestrial plants and microbes as they are widely accessible to collect.[14] An effort to explore the medicinal potential of marine natural products (MNPs) was first detailed at a conference in Rhode Island, USA, in the 1970s. Improved sampling methods, e.g., SCUBA apparatus, has contributed significantly to the progress of discovery new MNPs, not to mention the development of spectroscopy technology and screening protocols.[15] As a result, the number of new marine natural products has increased from 332 in 1984[16] to 1340 in 2016.[17] New compounds have been isolated from marine organisms in subtidal and intertidal zones including micro and macroalgae, littoral plants, various invertebrates (poriferans, anthozoan, bryozoans, mollusks, tunicates, echinoderms, and bacteria).[17]

The first MNPs were reported in 1951 when two nucleosides, spongothymidine (**11**, Figure 1.3) and spongouridine (**12**, Figure 1.3), were extracted from the Caribbean sponge *Tethya crypta*.[18-19] These compounds led to three drugs, which have anti-viral (Ara-A, commonly known as Vidarabine), anticancer (Ara-C also known as Cytosar-U), and anti-HIV activities (azido thymidine-AZT).[20-21] In fact, both Ara-C or Cytarabine (**13**, Figure 1.3) and Ara-A (**14**, Figure 1.3) were the first MNP-derived drugs approved by the FDA in 1969 and 1976, respectively.[22] However, ziconotide (**15**, Figure 1.3), a ω-conotoxin MVIIA (25 amino acid peptide chain) was the first FDA approved drug extracted and isolated directly from its source, the Indo-Pacific marine snail *Conus magus*.[23-24]

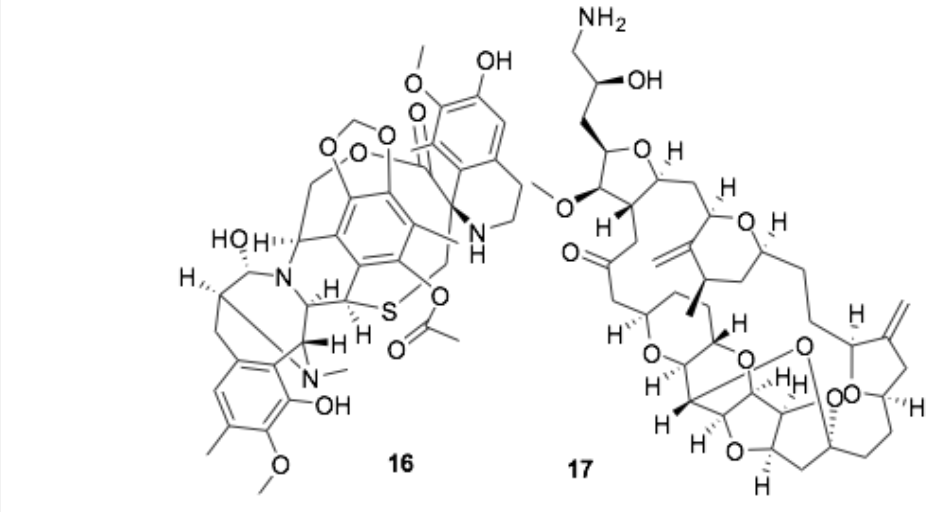


Figure 1.4. Chemical structure of trabectedin (**16**) and eribulin mesylate (**17**)

Currently, there are six FDA approved MNPs drugs including the aforementioned drugs, trabectedin (Yondelis) (**16**, Figure 1.4) and eribulin mesylate (**17**, Figure 1.4).[10, 20] Trabectedin, a tris(tetrahydroisoquinoline) alkaloid, was isolated from *Ecteinascidia turbinata* and is used for the treatment of non-operable soft tissue sarcomas.[24] Eribulin mesylate is a synthetic analogue of halichondrin B, extracted from the Japanese sponge *Halichondria okadai*, and is an FDA-approved treatment for metastatic breast cancer.[25]

Despite only a small number of MNPs drugs being approved, many of them are still in clinical trials,[26] which still provides huge potential for development. At present, there are five compounds in phase III clinical trials, which come from fungi, pufferfish, tunicates, and mollusks. Also, there are ten and six compounds in phase II and I clinical trials respectively, which most of them are coming from mollusks. Most of these compound target a variety of cancers (an updated list of current marine-derived compounds on the drug pipeline is available on the following website: http://marinepharmacology.midwestern.edu/clinical_pipeline.html).

1.3. Indonesia biodiversity

Indonesia has been privileged with the world's third most mega-biodiversity, according to the United Nations Environment Programme-UNEP.[26] It has 47 ecosystem types, ranging from ice fields and alpine meadows to coral-reefs, with approximately 17% of the total number of species in the world found in Indonesia.[27]

Regarding marine biodiversity, Indonesia lies at the heart of the Coral Triangle region (part of Coral Triangle Initiative[†]) which consists of 43,682 square kilometers of coral reef spanning from the Philippines in the north to the Solomon Islands in the south. Nearly 50 percent (19,868 sqkm) of the area is located in Indonesia, providing habitats for 500 species of coral (18 percent of the world's coral reefs) and 5,000 species of fish and mollusca on top of numerous marine plant species.[28] For this reason, it has attracted many researchers to examine the full potential of marine biodiversity in Indonesia.

[†]Coral Triangle Initiatives consist of six nations: Indonesia, Malaysia, the Philippines, Papua New Guinea, the Solomon Islands and Timor Leste

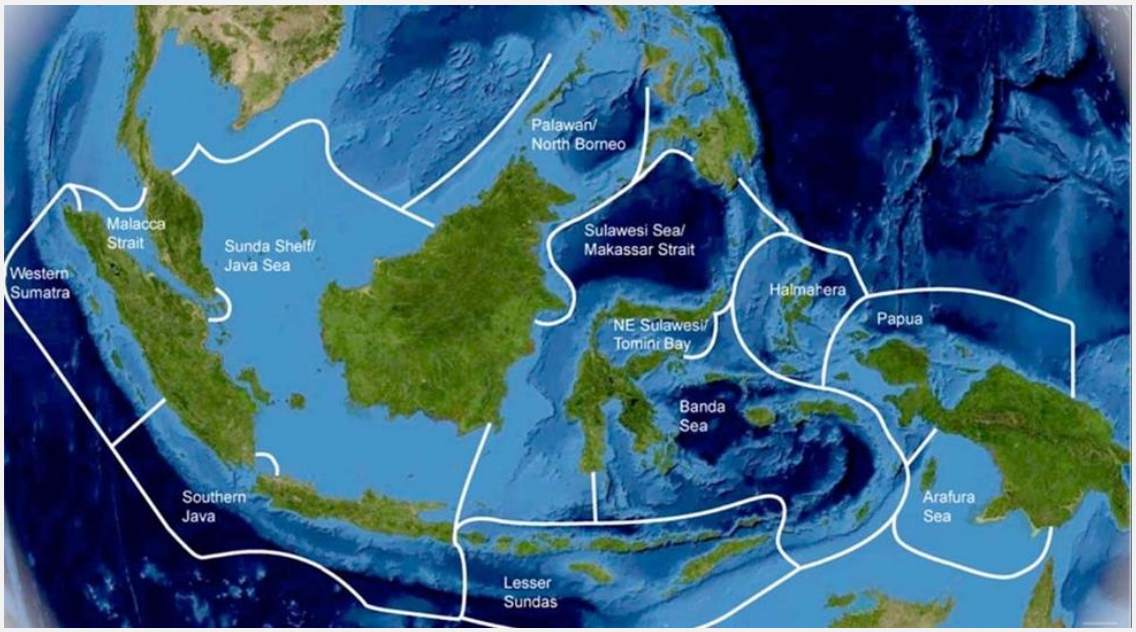


Figure 1.5. Map showing the twelve Indonesia marine ecoregions as defined in the Marine Ecoregions of the World classification scheme[28-30]

1.4. Indonesian marine natural products

Marine natural products research in Indonesia has captured research attention mainly in the period between 2002 and 2003, as well as 2012-2013. However, the first reported paper of MNPs in Indonesia was in 1974 when tricyclic (+)-africanol (**18**, Figure 1.6) was isolated from the soft coral *Lemnalia africana* (Leti Island, Maluku).[31]

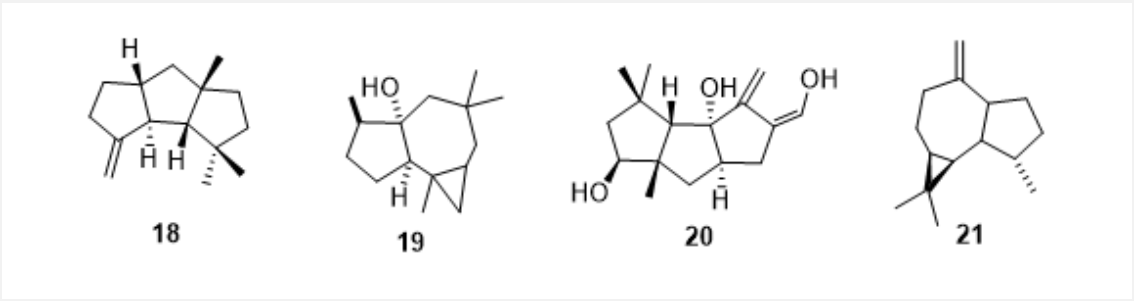


Figure 1.6. Chemical structure of (+)-africanol (**18**), Δ9(12)-Capnellene sesquiterpenes (**19**), (+)-Δ9(12)-capnellene-3β,8β,10α,14β-tetraol (**20**), and aromadendrene (**21**)

Later that decade, a few researchers had also reported their findings from another soft coral genus *Capnella* as sources of Δ9(12)-capnellene sesquiterpenes[32] (**19**, Figure 1.6), while tetraol (+)-Δ9(12)-capnellene-3β,8β,10α,14β-tetraol (**20**, Figure 1.6) was found from a specimen collected at the same location in 1977.[33]

Another group reported that they successfully isolated aromadendrene (**21**, Figure 1.6) from the soft coral *Sinularia mayi* (from Nias Island) in 1978.[34]

Among marine species, sponges have been the richest sources of bioactive compounds from Indonesia, comprising almost 60% of total research covered in this introduction chapter, followed by fungi, tunicates, and other invertebrates (Figure 1.7; see Appendix A). Most of the research has been done in Sulawesi Island especially North and South Sulawesi Provinces which accounted for 22 and 16 published papers, respectively.

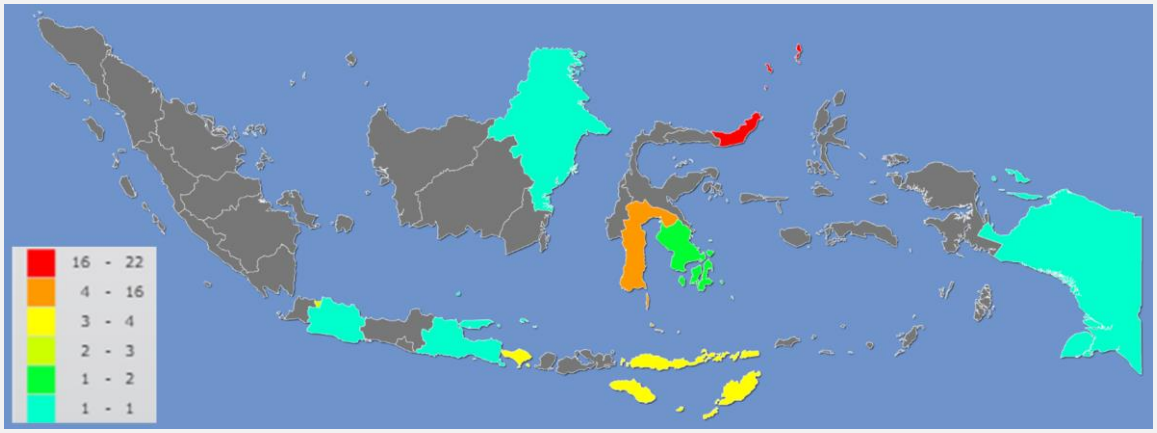


Figure 1.7. Frequency of marine natural products research from new and novel compound from Indonesia based on provinces (see Appendix A for the details); the number indicates total papers published.

Two new sesquiterpenoids, boneratamides-A (**22**) boneratamides-B (**23**, Figure 1.8), have been isolated from the marine sponge *Axinyssa aplysinoides* collected in South Sulawesi.[35] A few secondary metabolites were isolated from North Sulawesi sponges, for example a peptide, microspinosamide (**24**, Figure 1.8), which contains 13 amino acid residues, was isolated from *Sidonops microspinosa*.[36]

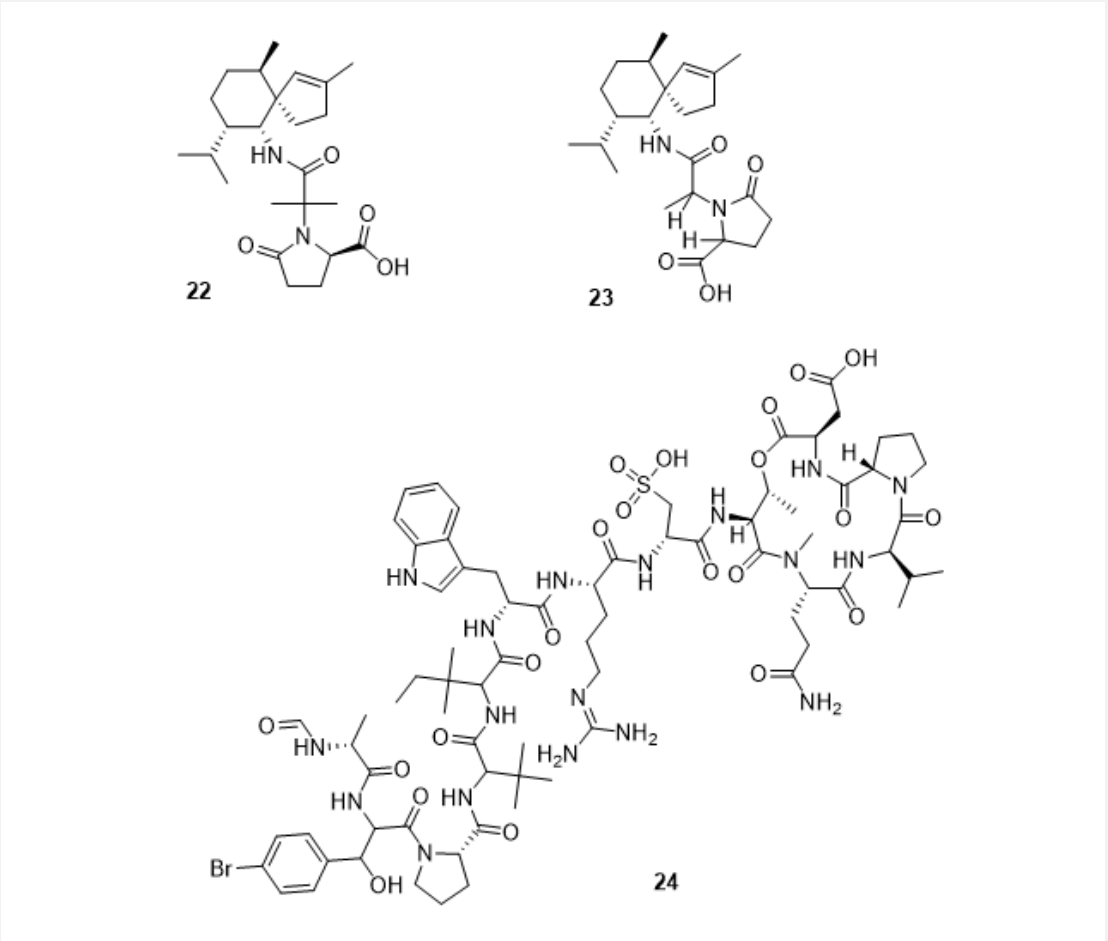


Figure 1.8. Chemical structure of boneratamides-A (**22**), boneratamides-B (**23**), microspinosamide (**24**)

Both organic and aqueous extracts of microspinosamide showed anti-HIV-1 activity at a concentration of 0.12 μM.[36] Other metabolites, manadomanzamines A (**25**) and B (**26**, Figure 1.9) isolated from *Acanthostrongylophora* sp., were also showed activity against HIV-1 with EC_{50} values of 11.5 and 27.0 μM respectively.[37] Manadomanzamines also exhibited strong activity against *Mycobacterium tuberculosis*.[38] Two new imidazole alkaloids, naamidines H (**27**, Figure 1.9) and I (**28**, Figure 1.9), were isolated from the marine sponge *Leucetta chagosensis*[39] (see Table 1-3 in Appendix A for a complete list of MNPs research in Indonesia).

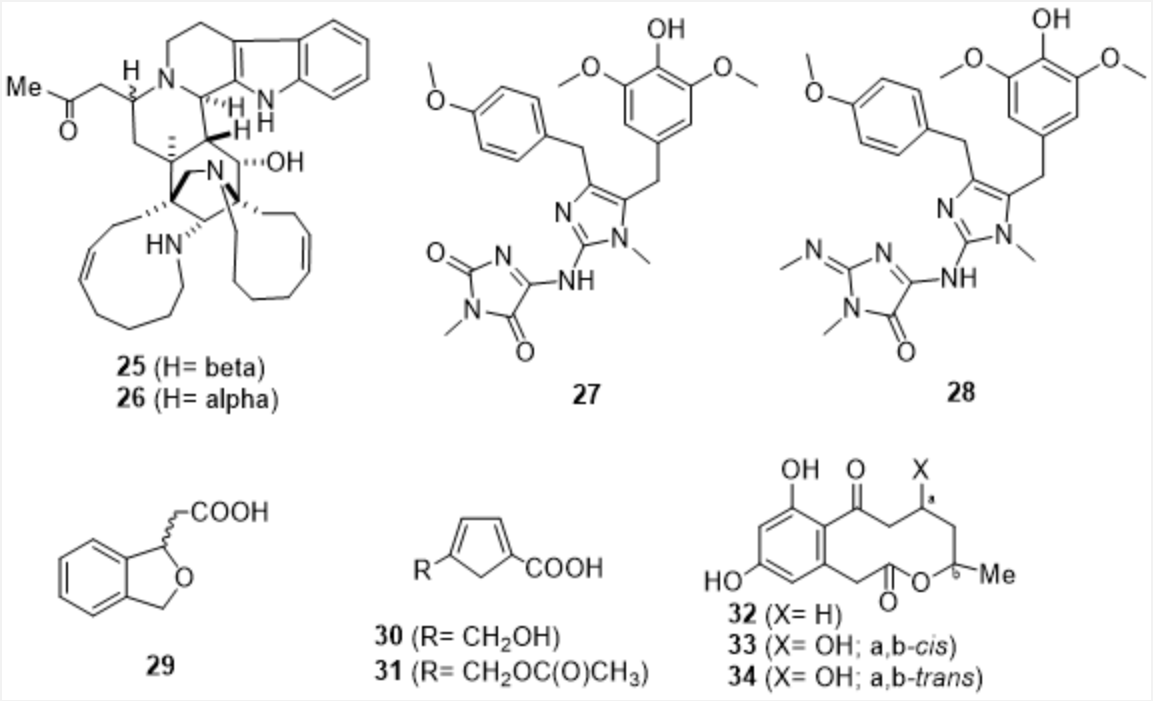


Figure 1.9. Chemical structure of manadomanzamines A (**25**), manadomanzamines B (**26**), naamidines H (**27**), naamidines I (**28**), herbaric acid (**29**), Sumiki's acid (**30**), acetyl Sumiki's acid (**31**), xestodecalactones A, B, and C (**32-34**)

Furthermore, two metabolites were isolated from *Cladosporium herbarum* (symbiont of the sponge *Callyspongia aerizusa*) namely, a new phthalide herbaric acid (**29**, Figure 1.9), which showed no activity, and furan carboxylic acids: Sumiki's acid (**30**) and acetyl Sumiki's acid (**31**, Figure 1.9), which both showed activity against *Bacillus subtilis* and *Staphylococcus aureus*.[40] Another fungus collected from Bali, *Penicillium cf. montanense*, also extracted from a sponge (*Xestospongia exigua*), is a 10-membered macrolides with a fused 1,3-dihydroxybenzene ring xestodecalactones A–C (**32-34**, Figure 1.9), of which only (**33**) was active against *C. albicans*.[41]

1.5. Macroalgae natural products

Commonly known as seaweed, marine macroalgae has historically been sources of edible seaweed[42] and raw materials for primary metabolites including gelatin, gellan, pectin, agar, carrageenan, and alginate.[43] Edible seaweed has been consumed primary by Asian cultures, where species such as brown algae (*Fucus vesiculosus*, Kombu-*Laminaria digitate*, and Wakame-*Undaria pinnatifida*) and red seaweeds (Nori-*Porphyra tenera*) have been commercially produced. Algae have been utilized to more extensive food products, for example jam, cheese, wine,

tea, soup and noodles in Japan.[44] The hydrocolloidal properties of seaweed are exploited as thickening agents and gelling agents in various uses such as salad dressings, sauces and toppings, jelly, marmalade, restructured foods and low sugar/calorie gels.[43, 45]

Macroalgae are found as sessile organisms in intertidal habitats, which is the area between high and low tides. Therefore make marine macroalgae are exposed periodically to both biotic and abiotic stressors.[46-48] The stresses range from herbivorous fish predation, competition, and disease to various environmental conditions (high and low temperature, desiccation, and osmotic stress).[47] Active compound defences are used to fight against pathogens and bio-foulants,[49] colonization/biofilms on seaweeds and bacterial signalling.[50-51] The various ecological situations force macroalgae to develop a chemical defence mechanism through production of bioactive secondary metabolites. This fact along with their ubiquitous and accessible habitats led natural products chemists into study marine macroalgae as the first group amongst other marine organisms.

The classification of secondary metabolits from macroalgae is derived from their biosynthetic origin. Terpenes are the largest and most diverse class of compounds derived from macroalgae. Terpenes, of which the name can be used interchangeably with terpenoids, make up approximately half of the active compounds found from algae.[51] Together with polyketides, amino acid derivatives (including non-ribosomal peptides and simple amino acid derivatives), and alkaloids, they encompass almost a quarter of known algae active compounds. Shikimates, usually found in aromatic natural products, are the next largest group of natural products, and the last group consists of various classes of secondary metabolites that are infrequently found in macroalgae, such as nucleosides and other classes of compounds bound to sugars.

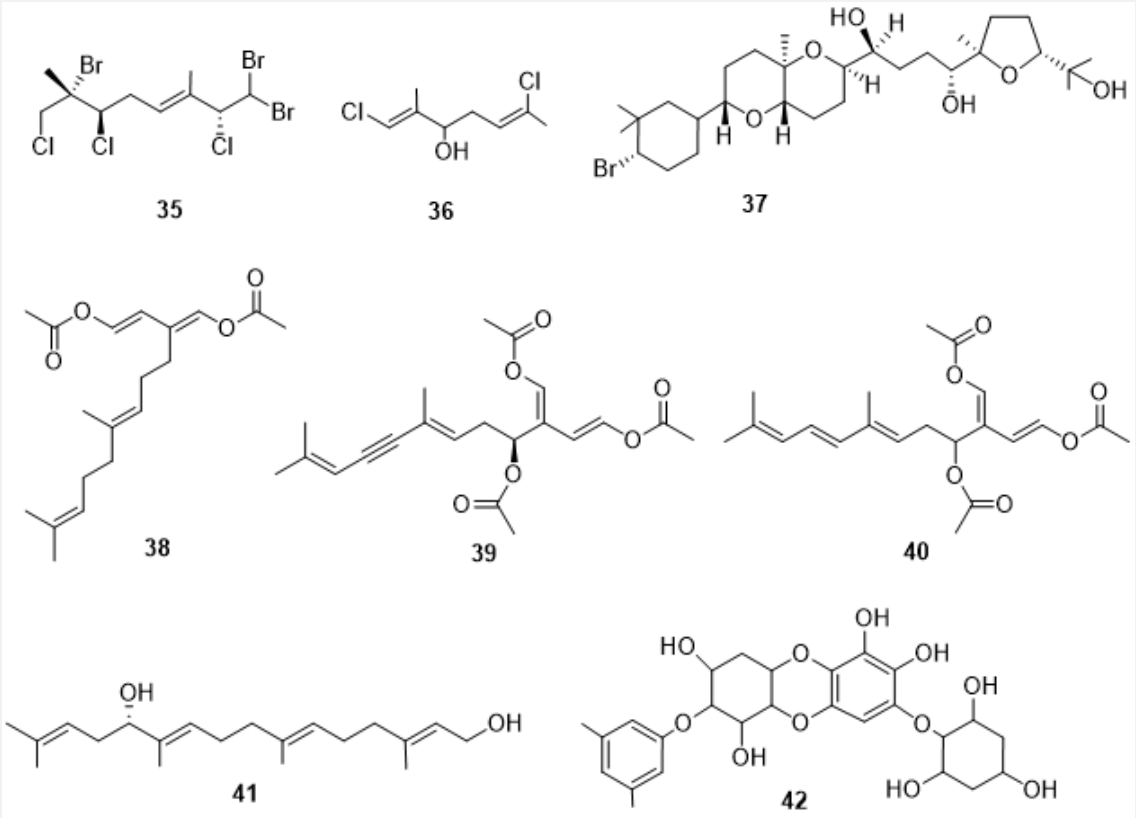


Figure 1.10. Chemical structure of Chemical structure of polyhalogenated monoterpene (**35**), bisnormonoterpenoid (**36**), thyrsiferol (**37**), flexilin (**38**), caulerpenyne (**39**), rhipocephalin (**40**), (S)-12-hydroxygeranylgeraniol (**41**), and diphlorethohydroxycarmalol (**42**)

The first study of red algae was reported by Blunt and co-workers, who isolated a polyhalogenated monoterpene (**35**, Figure 1.10) and a bisnormonoterpenoid (**36**, Figure 1.10) from *Plocamium cruciferum* in 1978.[52] Also, they found the uncommon squalene derived metabolite, thyrsiferol (**37**, Figure 1.10), from the red alga *Laurencia thyrsifera*.[53] Early studies found the simplest form of 1,4-diacetoxybutadiene in a green algae sample, namely flexilin (**38**, Figure 1.10) isolated from *Caulerpa flexilis* in 1978.[54] Two metabolites, caulerpenyne (**39**, Figure 1.10) and rhipocephalin (**40**, Figure 1.10), were isolated from *Caulerpa prolifera* and *Rhipocephalus phoenix*, respectively. Meanwhile, secondary metabolites from brown algae are predominantly terpenes and polyphenols. For example, the diterpene (S)-12-hydroxygeranylgeraniol (**41**, Figure 1.10) was isolated from the brown alga *Bifurcaria bifurcate* collected off the Atlantic coast from Morocco,[55] and diphlorethohydroxycarmalol (DPHC) (**42**, Figure 1.10) was isolated from *Ishige okamurae*, collected along the coast of Jeju Island, Korea.[3] DPHC was shown to be active against postprandial hyperglycemia in diabetic mice, as well as a potent α-glucosidase and α-amylase inhibitor.

1.6. Macroalgae natural product in Indonesia: status and potential

Indonesia is one of the richest countries in the world for marine species. About 45% of the world's marine algae species are found in Indonesia, including 196 green algal species, 134 brown algal species, and 452 red algal species.[56] Algae species are mainly spread across the central and eastern parts of Indonesia such as Sulawesi, Bali, Nusa Tenggara, and Maluku. Due to this high diversity of marine macroalgae, eastern parts of Indonesia are commonly referred to as "the barn of seaweed." However, according to the algaebase database (http://www.algaebase.org), less than one percent of marine algae have been reported from Indonesia (out of more than 360,000 records of known algae worldwide).[57]

Owing to this highly abundant resource, the phycocolloid industry produces polysaccharide compounds (primary metabolite) from seaweed and has been established in Indonesia to support many coastal communities around those aforementioned areas. A few important species that have been cultivated, namely *Kappaphycus alvarezii*, *Eucheuma* spp., and *Gracilaria* sp, are the major contributors to dry seaweed production in Indonesia. In fact, Indonesia has been the largest producer of seaweed farming since 2014 when its share of global production increased dramatically from 6.7 percent in 2005, to 36.9 percent.[58] However, little attention has been given to Indonesian algae as a source of pharmacological supply, and only a few studies have been conducted on this topic in Indonesia. Most of the research has conducted focused on red algae species, for example, *Vidalia* sp. and *Gracilaria asiatica* which a phenolic vidalenolone (**43**, Figure 1.11) and a cyclopropyl gracilarioside (**44**, Figure 1.11) respectively, isolated from these algae.[59-60] Gracilarioside was found to be mildly cytotoxic to the human A375-S2 melanoma cell line.

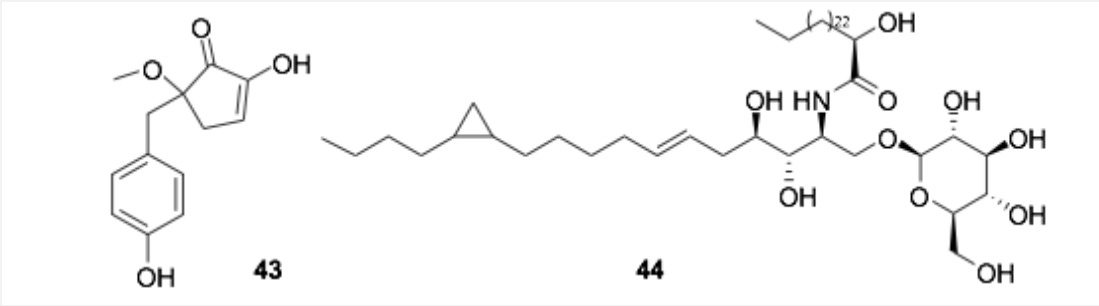


Figure 1.11. Chemical structure of vidalenolone (**42**) and gracilarioside (**44**)

One of the most significant issues in marine pharmacological research is the supply of raw materials. Even production of active metabolits on the gram scale is difficult to achieve from natural sources.[20] Therefore, most of the clinical investigations from marine secondary metabolites are supplied from chemical synthesis.[11] This problem may be addressed by a greater supply of macroalgae. Once a novel compound has been isolated from macroalgae, the abundance of naturally-occurring macroalgae in Indonesia could support the industry, especially for drug discovery purposes. Furthermore, already established seaweed farms in Indonesia may also sustain this industry from the supply side and best farming practices.

1.7. Proposed research

Despite their hugely diverse biogeography and significant potential source for drug development, tropical macroalga natural products have not been extensively studied. This project investigated Indonesian tropical marine macroalgae with the aim of isolating new secondary metabolites.

In Chapter 2, I will discuss about natural product molecular networking may assist the screening of macroalgae.

In Chapter 3, I will focus my research on structure elucidation of a compound resulted from molecular networking screening described in the Chapter 2.

CHAPTER 2. MOLECULAR NETWORKING TO SCREEN MACROALGAL SECONDARY METABOLITES

This chapter describes how molecular networking was used as a screening tool to prioritise the isolation workflow of 40 macroalgae sampled from West Timor waters, Indonesia, in addition to a Nuclear Magnetic Resonance-based (NMR) spectroscopy strategy. I describe first the screening and sampling of macroalga for this study. After that, I explain the use of Mass Spectrometry (MS) to generate spectra that were used as data to produce the molecular network with the Global Natural Product Social Molecular Networking (GNPS) website. Next, I show the screening process using the molecular network which assisted in the selection of six samples of macroalgae out of the 40 samples for further examination. After that, I explain the importance of column chromatography in the screening process. Lastly, the final part of the chapter discusses the screening process using an NMR-based protocol to choose the samples of interest to be investigated further.

2.1. Screening and dereplication of secondary metabolites

After an organism has been chosen as a species of interest, the next step is preliminary solvent extraction which results in crude extracts (these are described more in section 2.4). Once the crude extracts are obtained, various screening methods are employed to highlight those worthy or more in-depth investigation. The traditional approach has been bioassay-guided screening, which will usually lead to the isolation of biologically active compounds.[61-63] Although the bioassay-guided screening eventually produces a potential pharmaceutical compound, the frequent re-isolation of previously known metabolites is a major challenge for this strategy.[62, 64] Alternative approaches namely spectroscopy-guided screening utilising NMR spectroscopy or MS coupled with liquid chromatography (LC-MS) have been introduced as screening tools. This newer strategy combined with high quality spectral databases can help identify known compounds present in the extracts or active substances that have already been studied.[64-65] The procedure is known as dereplication, a term first coined in the CRC Handbook of Antibiotic Compounds published in 1980.[65] Dereplication provides rapid identification of known compound within crude extracts or semi-purified mixtures, facilitating

prioritisation for further elucidation of only potentially new structures or halting an isolation process of known secondary metabolites.[65-66] Hence, dereplication is a substantial and important strategy in natural products screening.

2.2. Isolating secondary metabolites

Secondary metabolites are typically amphiphilic, which allow the compounds to transverse both hydrophilic and hydrophobic environments. Their wide polarity and solubility make it difficult to analyse and handle the compounds, which may be present in the initial purification of a crude extract. In particular, finding single solvents that will dissolve all the components of a mixture is difficult, if not impossible without some level of pre-fractionation. Preliminary purification steps have commonly been done via liquid/liquid partitioning. However, as some extracts may form stable emulsions,[67] leading to the incomplete partitioning of the extract and target compounds, as well as the requirement of large volumes of solvent, this straightforward technique has been changed to column chromatography. The widely used reversed-phase chromatography procedure that was developed by Blunt and Munro has been used for many years to overcome problems associated with standard flash column chromatography (normal phase).[67]

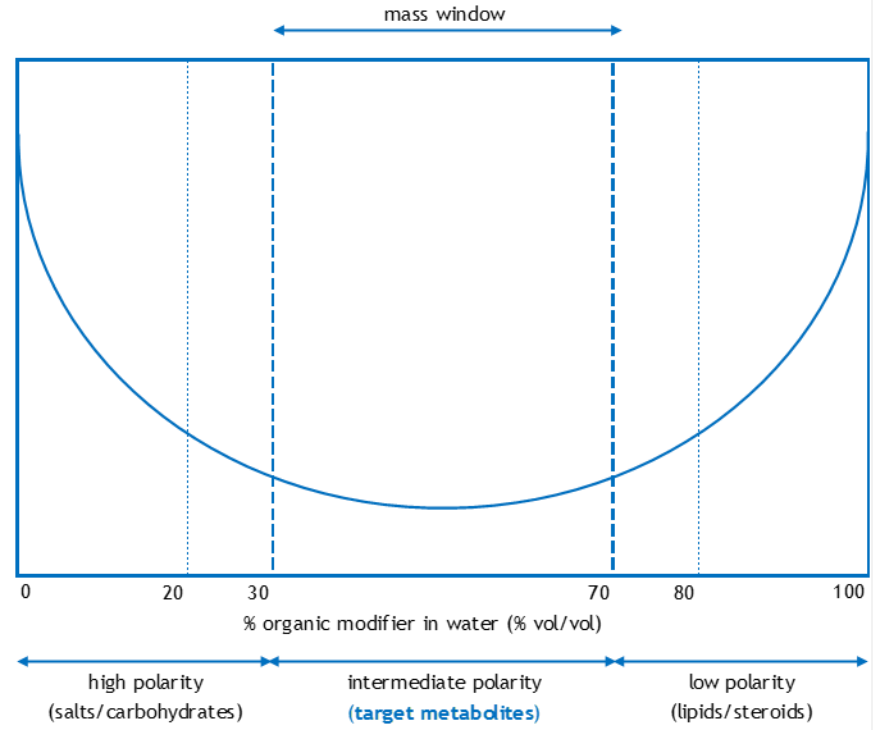


Figure 2.1. Schematic showing the mass distribution in a typical marine macroalgae extracts (adapted from Woolner, 2017[68])

In living systems, most organisms have molecules that are either very non-polar (lipids/steroids) or very polar (proteins/sugars). However, most interesting (amphiphilic) natural products are frequently not discovered with these molecules but in the intermediate "mass window" (Figure 2.1).[68]

At Victoria University of Wellington (VUW), the reversed-phase poly-styrene divinylbenzene (PSDVB) copolymer (HP20) was found to have the advantage of being a relatively inexpensive adsorbent, stable throughout the pH range, and reusable unlike silica gel (normal phase). Also, acetone and methanol, environmentally friendly solvents, are widely used for this technique which gives an additional advantage. This method is known as cyclic loading and has benefited the natural product research group at VUW for quite some time now. The cyclic loading system enables crude extracts to be loaded directly onto the PSDVB column without the need for preconcentration.

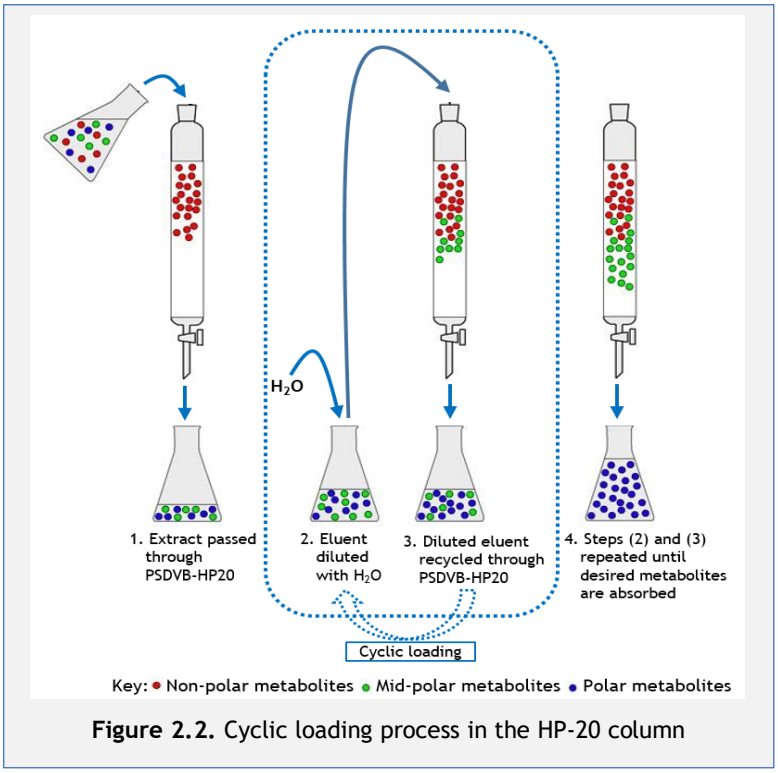


Figure 2.2. Cyclic loading process in the HP-20 column

The cyclic loading starts by firstly passing the crude extracts through the PSDVB-HP20 column (Figure 2.2). The next step is to dilute the eluent with water and recycle back onto the column. Normally dilution with water would cause irreversible precipitation of extracted non-polar materials, but these compounds will already have adsorbed to the column and are no longer present. The dilution and recycling is repeated three to four times. By adding the water to eluent,

typically an equal volume of H_2O (100% v/v), it gradually increases the polarity of eluent which leads to more mid-polar compounds having greater affinity and adsorption onto the PSDVB.

Once the cycle is completed, the last step is to wash the column with H_2O to remove salts. The next phase is complete elution using an organic solvent (usually acetone, Me_2CO). Previous research at VUW has suggested that the target metabolites elute mostly in the intermediate 30-75% acetone in water fractions. In this study a series of solutions was used as follows: (1) 30% Me_2CO/H_2O, (2) 75% Me_2CO/H_2O, and (3) 100% Me_2CO. While the 100% Me_2CO fraction can be immediately concentrated under reduced pressure, but the 30% and 75% fractions contain too much water for concentration, some H_2O/organic modifier mixtures froth vigorously under vacuum. These fractions must be further processed via back loading.

Back loading involves cyclic loading the eluted fraction by diluting with water and passing it through a small PSDVB-column. This causes the eluted compounds to re-adsorb to the column. The column can then be stripped with a pure organic solvent, thereby removing the water from the sample and generally also concentrating the fraction as well. Both the 30% and 75% Me_2CO fractions are back loaded onto separate PSDVB columns. After the first back loading, the eluent is diluted with water (100% v/v) and back loaded into the column (the step is repeated thrice). Before the final 100% Me_2CO is used to elute the PSDVB column, the column is blown dry to reduce the amount of water present in the column. Then, the fractions are converted to only Me_2CO and the eluents can be concentrated under reduced pressure.

Popplewell,[69] who previously studied 34 temperate New Zealand red algae in the marine natural products group at VUW, developed a method for algal screening that was modified from VUW in-house protocols designed for the screening of sponge extracts.[69-70] The protocol involves the extraction from 2 g or more (wet weight) algal material because macroalgae have a richer fraction of secondary metabolites, compared to sponge extracts which typically require ~ 100 g wet weight.[69] Since the mid-1990s, the MNPs group at VUW has been utilising NMR-guided isolation of secondary metabolites.

2.3. Liquid chromatography-mass spectrometry

MS has become a standard procedure for investigating complex mixtures and molecules. Liquid chromatography coupled with mass spectrometry (LC-MS) is a hyphenated analytical technique that synergises the ability to perform fractionation via liquid chromatography with the mass analysis capability of MS.[71] This particular technique works by ionisation of a molecule that is then smashed and turned into charged fragments, which then would be quantitated based on their mass-to-charge (m/z) ratio.[72-73] An LC-MS system includes elements such as high-performance liquid chromatography (HPLC) system; the ionization source (which interfaces the LC to the MS); and the mass spectrometer. A computer system is used to control all these elements (Figure 2.3).[71]

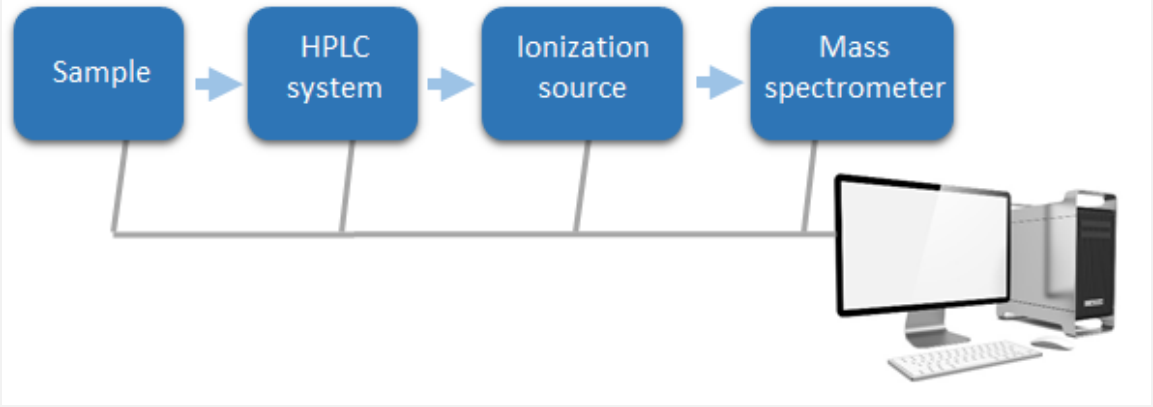


Figure 2.3. Component of an LC-MS system (adapted from Korfmacher, 2005[71])

A mixture of water and common hydrophilic solvents (isopropanol, methanol, or acetonitrile) as the mobile phase is prepared by the HPLC system. A pumping mechanism in the HPLC system allows pressurised liquid and a sample mixture to pass through a column filled with the adsorbent stationary phase. The adsorbent is typically a granular material (1–50 μm) made of solid particles (typically a silica-based bonded phase e.g. C18).[74] The ionization source is used as the interface between the HPLC eluent and the mass spectrometer. Two common atmospheric-pressure ionisation (API) technique sources are electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI). The eluent from the LC is nebulised so that ions are produced from the evaporating droplets. Nebulisation is achieved either pneumatically via APCI or by a strong electrical field in ESI.[74] A number of mass analysers are available but nowadays time-of-flight (TOF) MS is the most widely used system in drug discovery research. In a quadrupole time-of-

fight (QTOF) instrument, as used in this study, the quadrupole is used to select precursor ions which will be fragmented later in a collision cell. These generated ions are then spearated by the TOF and detected by a photo-electron multiplier plate.[75]

In natural products discovery, the data produced from tandem MS can be employed as a screening method. It is a robust approach because even a crude extract sample is enough for the screening process (due to the higher sensitivity of the instrument compared to other analytical techniques).[73] In this process a molecular network is produced to organise mass spectra into groups based on similarities in their fragmentation patterns with the expectation that structurally related molecules will yield similar spectra.[76] Therefore, molecular networking using MS can be applied as a data-rich approach that represents an important advance for the field of natural product research.

2.4. Algal screening through Global Natural Product Social Molecular Networking

Although a single LC-MS experiment is already a powerful technique that can collect thousands of spectra in a relatively brief time, most of the data is only sitting in the researchers' drawers or computer. Moreover, most natural products databases such as the Dictionary of Natural Products (http://dnp.chemnetbase.com/) and MarinLit (http://pubs.rsc.org/marinlit) only provide services to their subscribers. Recognising this need, the University of California San Diego (UCSD) Centre for Computational Mass Spectrometry (http://proteomics.ucsd.edu) developed Global Natural Product Social Molecular Networking (GNPS) to accommodate the demand for robust dereplication of natural products that is freely available to the global research community.[77] GNPS is an open database that provides the ability to analyse, organise, and create networks from tandem mass spectra data.[77] Moreover, the publicly available GNPS database (known as MassIVE) is used to compare experiment data with the known spectra library which is useful for dereplication in natural products.

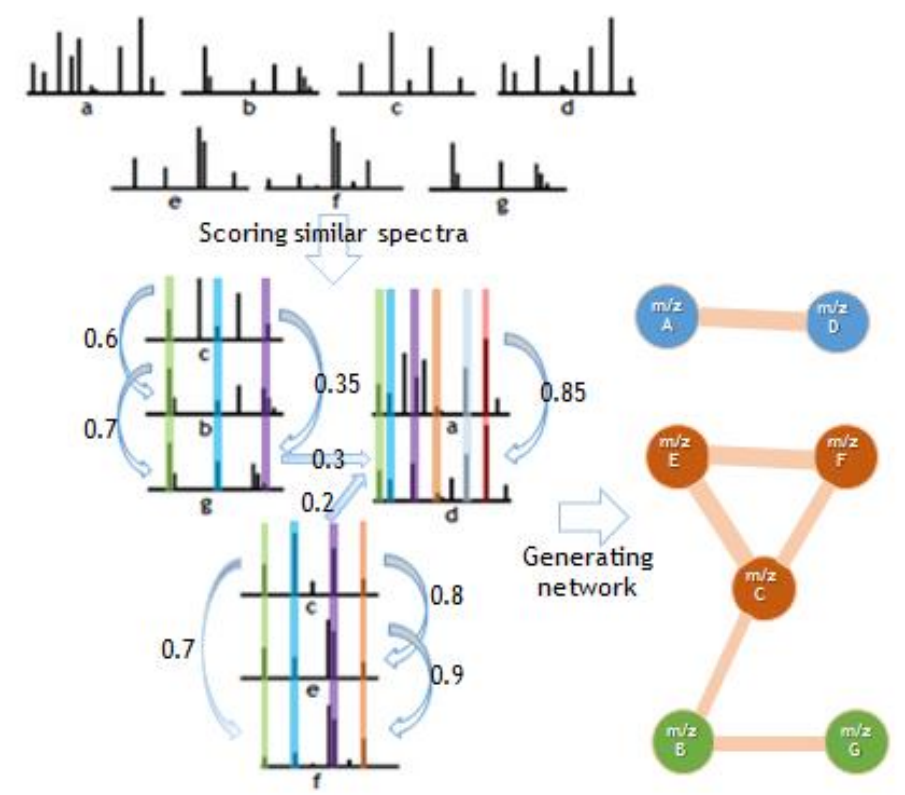


Figure 2.4. A scheme shows how molecular networks are created from LC-MS data (Modified from Watrous, et al., used by permission)[78]

Instead of a linear comparison, GNPS uses a vector-based approach to match two or more different MS/MS spectra (see Figure 2.4 for details). Experimental mass spectra with unique fragmentation patterns are combined into multidimensional vectors. Vectorisation in GNPS happens by taking not only the intensity of the peak but also the mass-to-charge ratio of the ion (m/z). An overall vector is generated by plotting the m/z ratio of multiple peaks (*n*) in *n*-dimensional space. Each overall vector represents a compound or potentially isomers and will be shown as nodes. The overall vectors of different compounds can be aligned and compared with each other, thus the cosine of the angle between two or more vectors can be used to measure their similarity. The cosine score represents how closely related two nodes (hence compounds) are, which varies from 0 (completely unrelated) to 1 (identical spectra). In the network, the cosine score is usually represented by the thickness of a line (edge) that connects two nodes.

A cluster is formed when nodes are connected by edges and comprises a unique set of molecules which are related as structurally similar compounds tend to have similar properties and belong to the same group.[79-80] GNPS also enables annotation

of putative nodes, therefore the mass difference can be used to annotate other nodes.[81]

GNPS provides network visualisation on their website (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash2.jsp). However, a more sophisticated third-party software, called Cytoscape, is available to visualise the network (http://www.cytoscape.org).[82-83] In this open-source freeware, visualisation of key defining attribute values in the network are shown in different shapes, colours, and sizes.[84] Cytoscape is a reliable tool for displaying large data sets in other areas such as metabolomics, biochemical pathways, population networks and even social science research.[85-88] The current version of the freeware is Cytoscape v3.7 (released in October 2018).

2.5. Sampling of Indonesian macroalgal from West Timor

In this study sampling of Indonesian macroalga was done in two different islands; Timor Island and Semau Island of West Timor waters, Indonesia (Figure 2.5, see also section 5.1). A total of 40 species were collected during low tides (Table 2.1 and Appendix B) and dried under the sun for further extraction. The number of green alga (Chlorophyta) and red alga (Rhodophyta) collected were the same while less brown alga (Phaeophyta) were sampled. Interestingly, Akle Beach in Semau island provided more species of species compared to the other three beaches.

Table 2.1. Number of macroalgal collected on each site based on phylum

Location	Number of Phaeophyta (brown)	Number of Rhodophyta (red)	Number of Chlorophyta (green)	Total
Sulamu Beach	4	3	2	9
Pasir Panjang Beach	-	4	2	6
Akle Beach	4	6	10	20
Tablolong Beach	2	2	1	5
Total	10	15	15	**40**

Figure 2.5. Sampling locations of macroalgae from West Timor waters, Indonesia (insert is the approximate sampling location from New Zealand). The map retrieved from https://www.google.com/maps/@-10.1345414,123.5736125,70500m/data=!3m1!1e3 (used by permission)

A selected number of specimens were identified using deoxyribonucleic acid (DNA) barcoding to ensure if the species were known or relatively new sources of macroalgae natural products. During sampling, clean apices from algae were rapidly dried and stored in silica gel. DNA extraction was performed at Phycology research group at School of Biology Sciences Victoria University of Wellington with the help of Assoc. Professor Joe Zuccarello. The result showed that the specimen of interest is grouped with only *Laurencia snakeyi* (Figure 2.6).

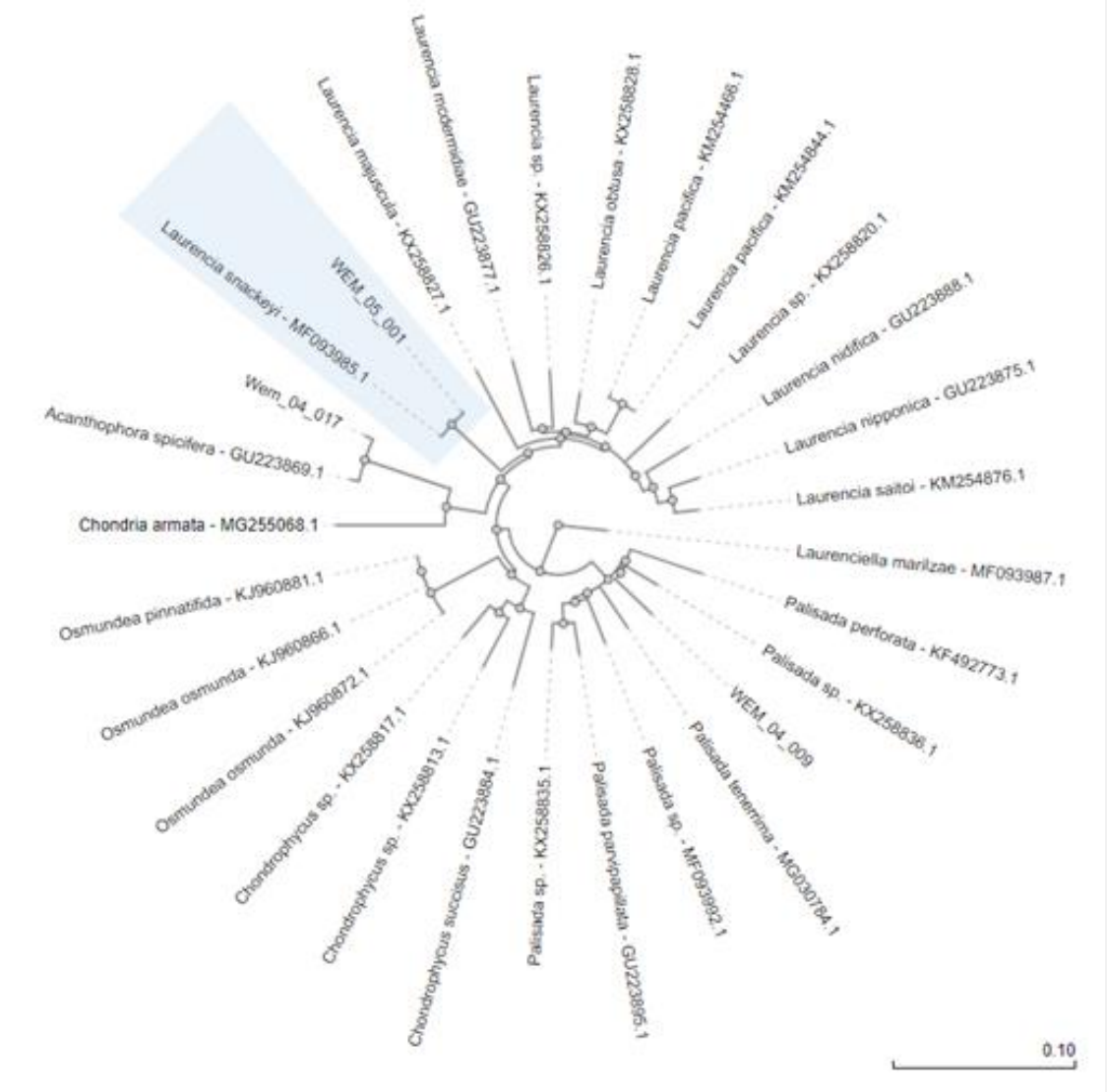


Figure 2.6. Phylogram displaying clustering of the included species *Laurencia snackeyi*

2.6. LC-MS Screening results of West Timor macroalgal

The 40 macroalgae samples collected from West Timor waters, in Indonesia, (see Appendix B) were extracted using methanol (MeOH) at room temperature and were dried afterward to obtain a crude extract weight. Prior to LC-MS analysis, all samples were diluted in MeOH to a set of concentration (0.1 µg μL^{-1}). In the LC-MS, both positive and negative modes were employed to get fragments of the samples based on their m/z ratio, in order to maximise the amount of data obtained since some compounds only ionised under one mode.

Spectra data from LC-MS/MS were converted and exported as mgf files to the GNPS website along with text files of meta-data attributes to produce the network. On

the GNPS website, for each dataset from positive or negative ion mode was run separately. To create consensus spectra, all key parameters were set: parent mass tolerance was set to 0.02 Da and MS/MS fragment ion tolerance was set at 0.02 Da. Also, the consens5us spectra that contained less than two spectra were discarded. The Minimum Pairs Cosines score was set to 0.7 and the minimum fragment ions matched was set to six data to produce the network. The results were then exported and later visualised in the Cytoscape application (version 3.7) and displayed in "preferred layout" settings. Positive mode data showed 574 nodes and 857 edges (Figure 2.7a) while negative mode showed fewer nodes and edges, 120 and 182, respectively (Figure 2.7b).

The positive ion GNPS network shows that macroalgae collected from Akle Beach have potential for further study (Figure 2.7a). The network is dominated by clusters formed from algae collected at Akle Beach (more than 10 clusters), followed by two clusters from Sulamu Beach and Tablolong Beach (which connect to a Sulamu Beach cluster), with no clusters from Pasir Panjang Beach. Akle Beach has the most clusters since 20 out of 40 macroalgae samples were collected from this site. Conversely, in the negative ion mode, no single cluster was formed from algae collected from a specific location (Figure 2.7b), as they have nodes that also belong connect to extracts from to other sites.

However, the negative ion network also confirms that potential macroalgae for screening come from similar beaches as shown in the positive ion mode (i.e., Akle Beach, Sulamu Beach, and Tablolong Beach). This may occur due to the production of secondary metabolites from macroalgae as chemical defences to herbivores. As Pasir Panjang Beach is located in an urban areas, therefore, the absence of herbivory fish in this site might slightly alter the function of secondary metabolites in this particular beach.[89-90]

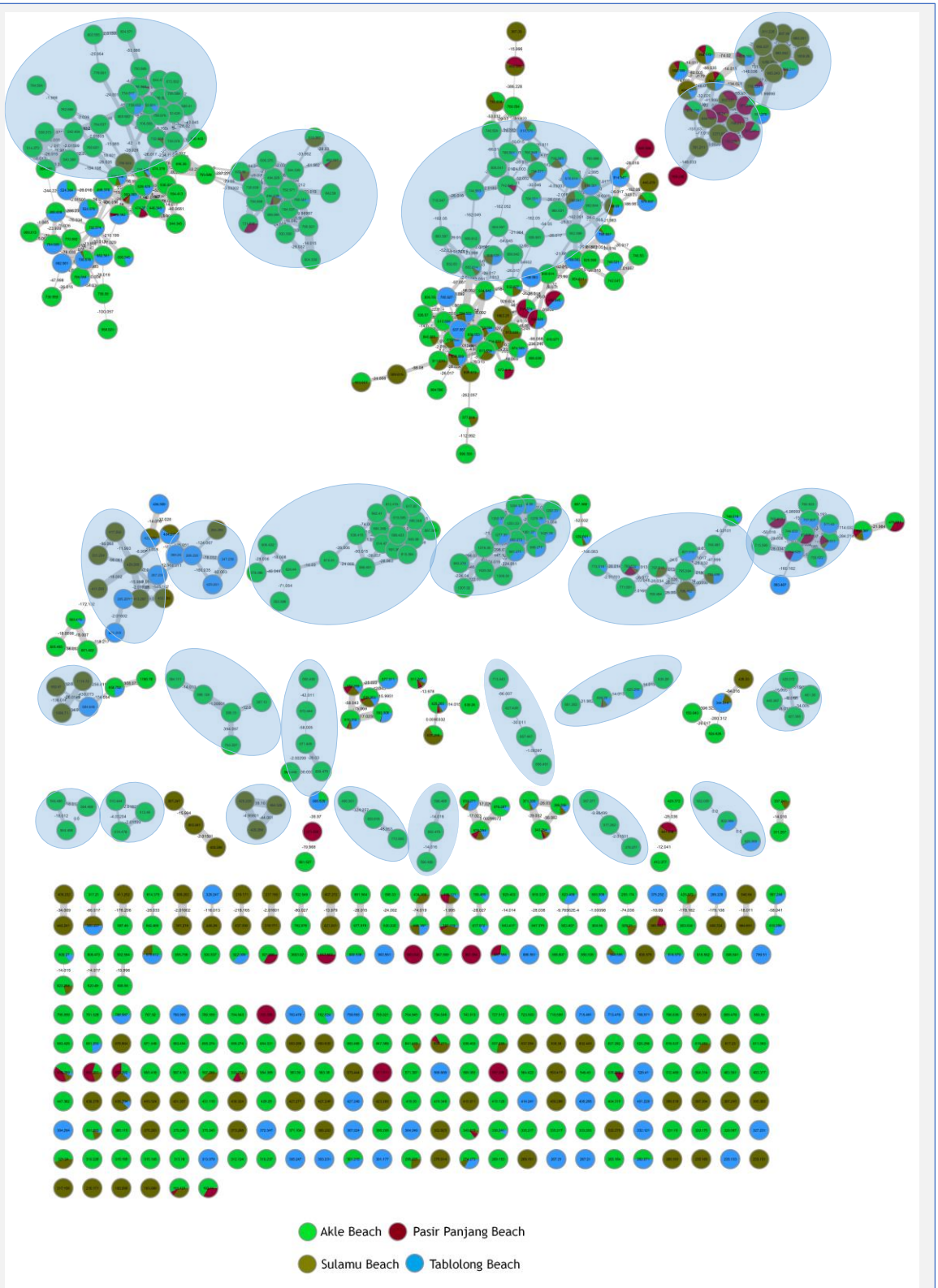


Figure 2.7a. GNPS network of macroalgae based upon sampling locations in positive ion mode. Colors are arbitrarily assigned to collection site

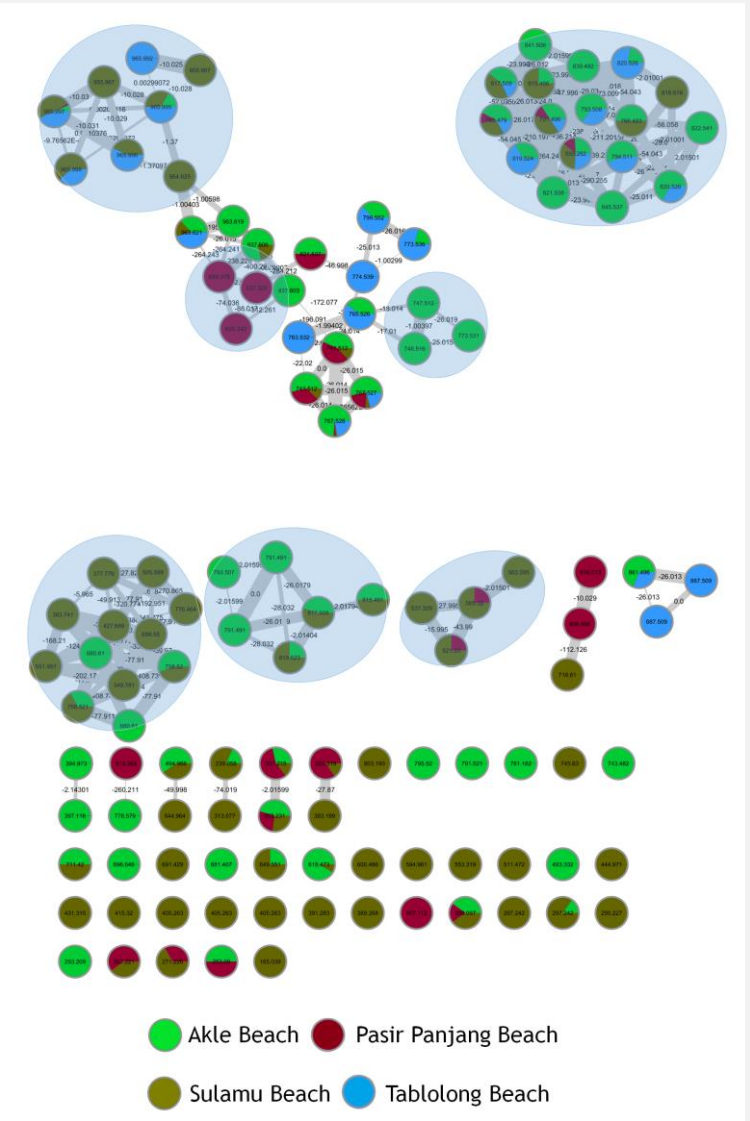


Figure 2.7b. GNPS network of macroalgae based upon sampling locations in negative ion mode. Colors are arbitrarily assigned to collection site

When the phylum of macroalgae is considered for grouping the GNPS network (Figure 2.8), green macroalgae (Chlorophyta) were shown as the most prolific source of compounds followed by brown (Phaeophyta) and red macroalgae (Rhodophyta). Thirteen clusters were formed from green algae while brown and red algae generated nine and seven clusters, respectively. These results contradict the trend in macroalgae secondary metabolites research since the most prolific source of macroalga natural products is red algae which account for more than 50%, followed by brown for almost 40% and the rest are from green seaweed.[16, 91-96]

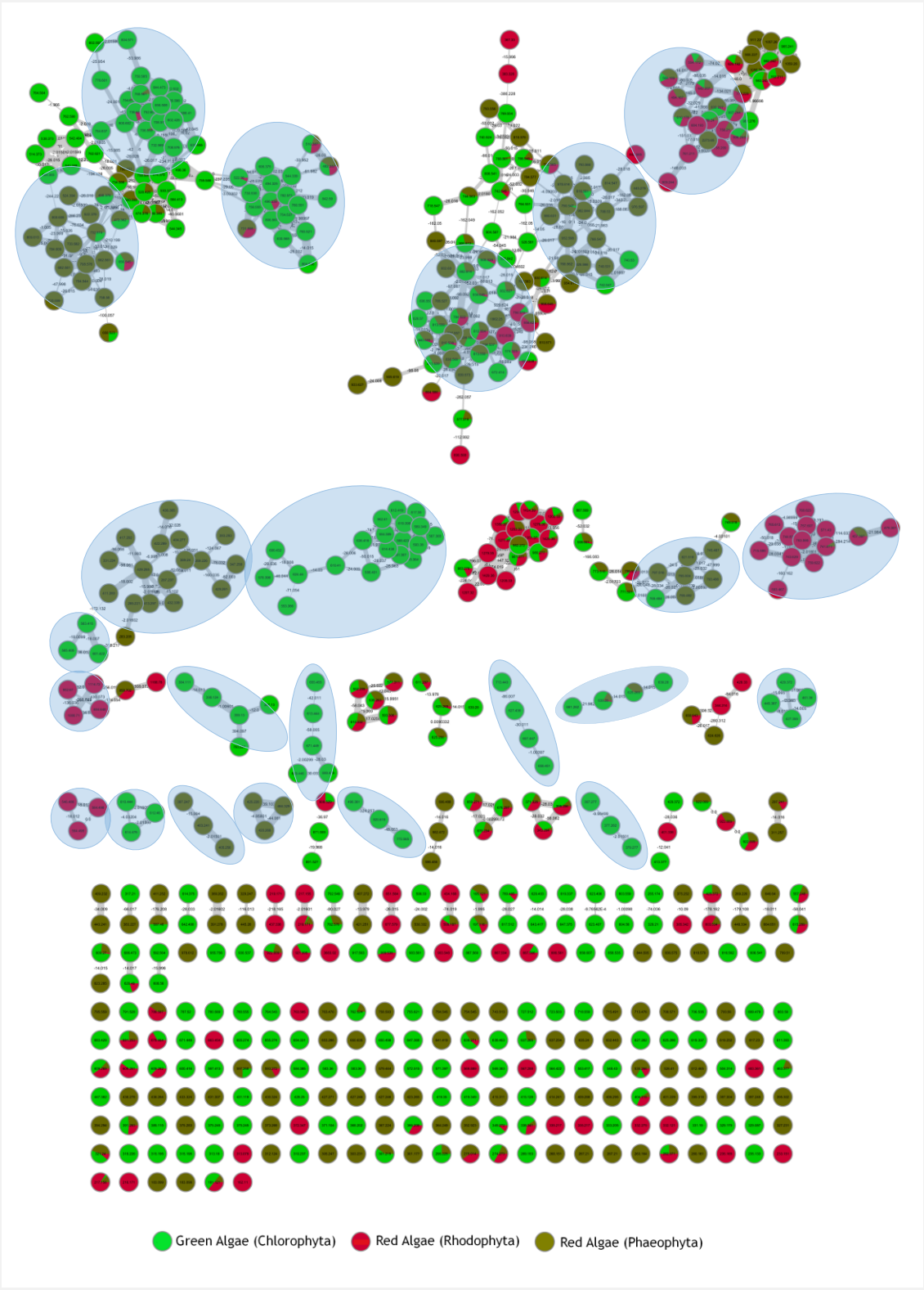


Figure 2.8a. GNPS network of macroalgae based upon phylum in positive ion mode. Colors are arbitrarily assigned to collection site

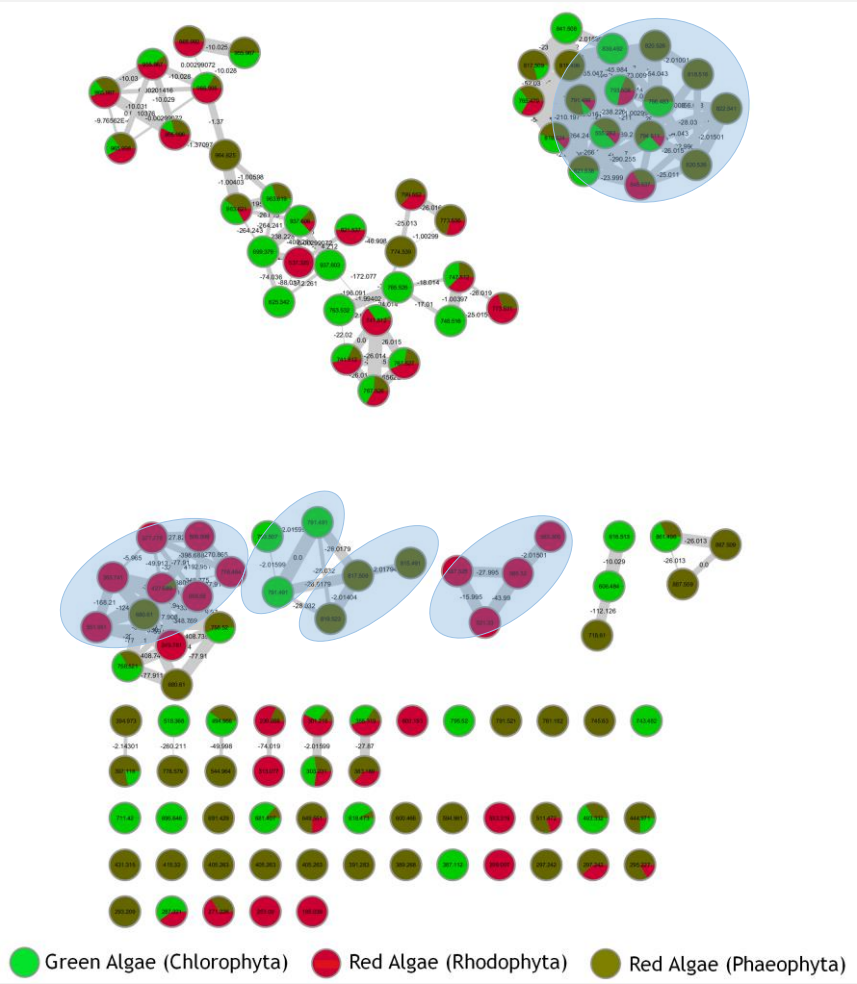


Figure 2.8b. GNPS network of macroalgae based upon phylum in negative ion mode. Colors are arbitrarily assigned to collection site

However, seasonal assemblage of intertidal macroalgae in the tropics usually shift to more green and red algae in late of summer/start of the rainy season (mostly between Oct-Dec each year).[97] The samples collected from West Timor waters found more green and red macroalgal species compared to brown during the Oct-Nov sampling (Appendix B). This result suggested brown algae may be an under studied resource for finding new marine natural products.

Molecular networking based on individual species enabled a selection of samples to be prioritised in the isolation workflow. From both positive and negative mode networks, six samples were selected to be processed further for NMR screening (Figure 2.9). The samples are: *Amphiroa* sp1 (WEM_01_005) from Sulamu Beach; *Amphiroa* sp2 (WEM_03_002), *Ulva* sp1 (WEM_03_004), and *Ulva* sp2 (WEM_03_005) from Pasir Panjang Beach; *Padina* sp2 (WEM_04_019) from Semau Island, *Laurencia snackeyi* (WEM_05_001) from Tablolong Beach. These samples were selected based on clusters formed where the clusters were dominated by one of the six specimens.

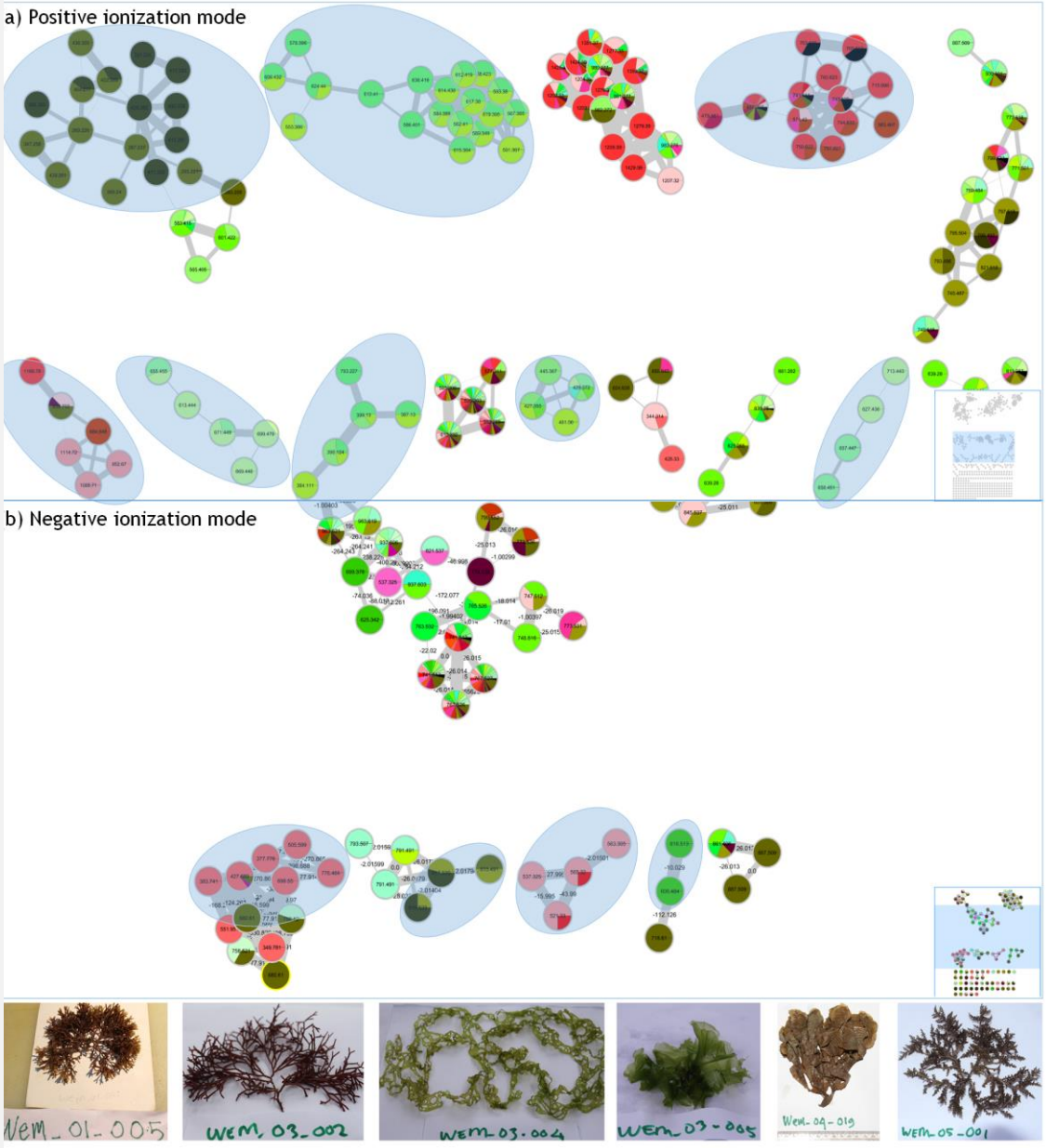


Figure 2.9. GNPS network of macroalgae based on specimens. Colors are arbitrarily assigned to 40 different samples of species

2.7. NMR-guided screening

Six extracts that had been prioritised through molecular networking (previous section), were then fractionated before using ^{1}H NMR to verify the presence of interesting secondary metabolites. Although NMR spectroscopy does not offer biological information, it is perceived that novel structures often lead to interesting biological activity.[70] As argued before, the intermediate 75% fraction (mass window) showed the most interesting peaks compared to the 30% and 100% Me_2CO fractions.

Out of six samples, three were initially chosen as having interesting peaks between 3.5-5 ppm (Figure 2.10); namely WEM_01_005 (*Amphiroa* sp1), WEM_04_019 (*Padina* sp2), and WEM_05_001 (*Laurencia snackeyi*). The resonances in these downfield correspond to oxymethine protons, predominantly sugars signals. Because of the small amount available, two of the samples were not processed further, and only WEM_05_001 (*Laurencia snackeyi*) was purified further and will be discussed in the following chapter.

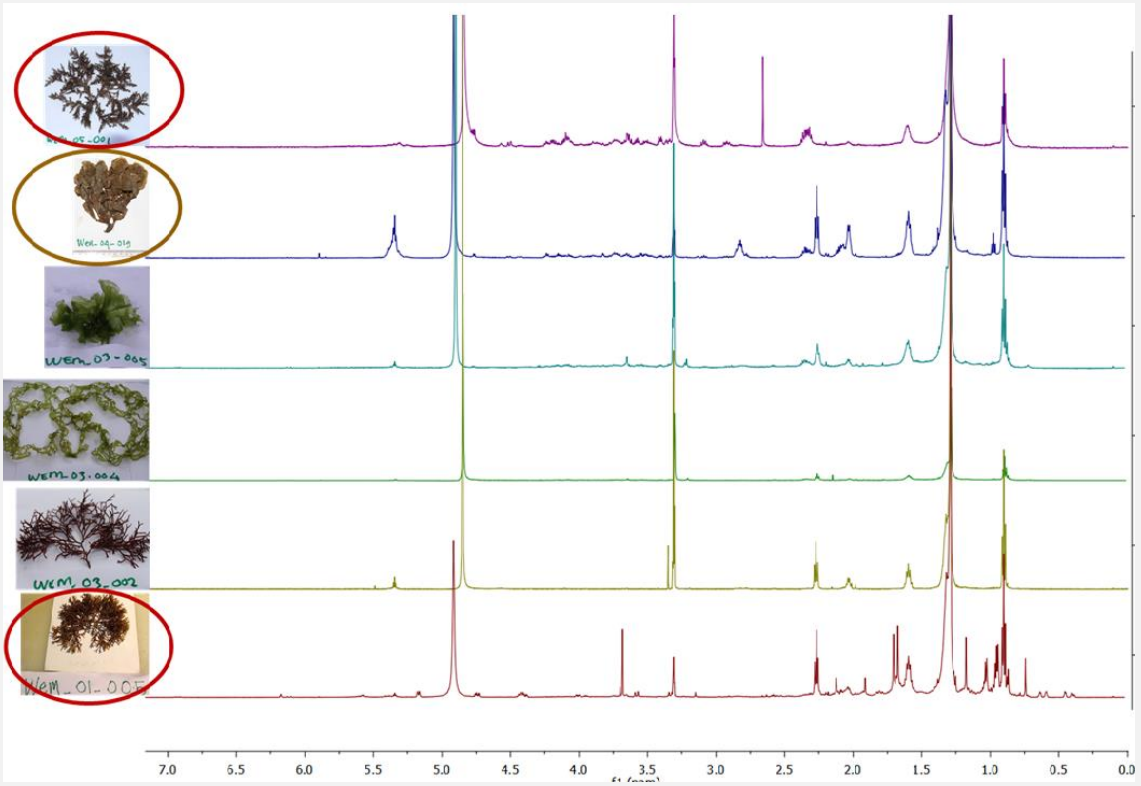


Figure 2.10. ^{1}H NMR spectra from 75% semi-purified fractions of six prioritized samples of macroalgae (600 MHz, CD_3OD)

CHAPTER 3. WEST TIMOR MACROALGAE MARINE NATURAL PRODUCTS: *Laurencia snackeyi*

3.1. Introduction

In Chapter 2, the sample "WEM_05_001" was prioritised for further investigation based upon liquid chromatography-mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) screening. This alga was identified as *Laurencia snackeyi*. Specimen of genus *Laurencia* are found mainly in tropical, subtropical, and temperate coastal waters[98] and belong to phylum Rhodophyta, order Ceramiles and Family Rhodomelaceae (see Table 3.1 for the complete classification).[99]

Figure 3.1. Specimen *Laurencia snackeyi* collected from Tablolong Beach

According to Algaebase, this species was first found in Semau Island, Indonesia, during the expedition by Weber-van Bosse in 1923 (the island next to where WEM_05_001 was collected from).[99-101] *L. snackeyi* is also found in various locations such as New Caledonia, eastern Australia, Palau Island, Micronesia, the Philippines, Malaysia and Vietnam.[101] This species grows on the fringing reefs, limestone and dead corals.[101]

Table 3.1. Taxonomic classification of *Laurencia snackeyi*

Empire: Eukaryota
Kingdom: Plantae
Subkingdom: Biliphyta
Phylum: Rhodophyta
Subphylum: Eurhodophytina
Class: Florideophyceae
Subclass: Rhodymeniophycidae
Order: Ceramiales
Family: Rhodomelaceae
Tribe: Laurencieae
Genus: *Laurencia*
Species: *snackeyi*

3.2. Chemical investigations of *Laurencia* species

Genus *Laurencia* has been the source of 1,076 reported compounds worldwide (Marinlit MNPs database, accessed November 2018), which are mainly in sesquiterpenes, diterpenes, triterpenes, and C15-acetogenins.[98, 102] Regarding *L. snackeyi* specifically, a number of halogenated and non-halogenated sesquiterpene secondary metabolites have been reported so far.[103-105] Most of the reported metabolites came from South East Asia. For example, palisadin A (**45**), aplysistatin (**46**), 5-acetoxypalisadin B (**47**), and palisol (**48**) were isolated from *L. snackeyi* which was collected from Kota Kinabalu, Sabah, Malaysia. Aplysistatin (**46**) inhibited nitric oxide (NO) and prostaglandin-E2 (PGE2) release which may suggest it's use as an anti-inflammatory agent.[103] A few other metabolites such as 5β-hydroxypalisadin B (**49**), palisadin B (**50**), palisol (**48**), and pacifigorgiol (**51**) also came from *L. snackeyi* collected from Malaysia. 5β-Hydroxypalisadin B (**49**) significantly suppressed PGE2 production and reduced nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in Lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.[104] This anti-inflammatory effect was tested further in a zebrafish (*Danio rerio*) embryo *in vivo* model and the results were comparable to the well-known anti-inflammatory agent dexamethasone.[106] Meanwhile, other research done in Malaysia and Vietnam resulted in the isolation of a few non-halogenated sesquiterpenes, i.e., snakeol (**52**), snakediol (**53**); and nine known sesquiterpenes

such as: (*R*,*Z*)-33-dimethyl-5-methylene-4-(3-methylpenta-24-dien-1-yl)cyclohex-1-ene (**54**), palisol (**48**), pacifigorgiol (**55**), palisadin D (**56**), palisadin A (**45**), palisadin B (**50**), 5-acetoxypalisadin B (**57**), debromolaurinterol (**58**) and α-bromocuparane (**59**).[101] Snakeol (**52**) and snakediol (**52**) showed strong antibacterial activity against two human clinical bacterial pathogens, *Salmonella typhi* and *Escherichia coli*.

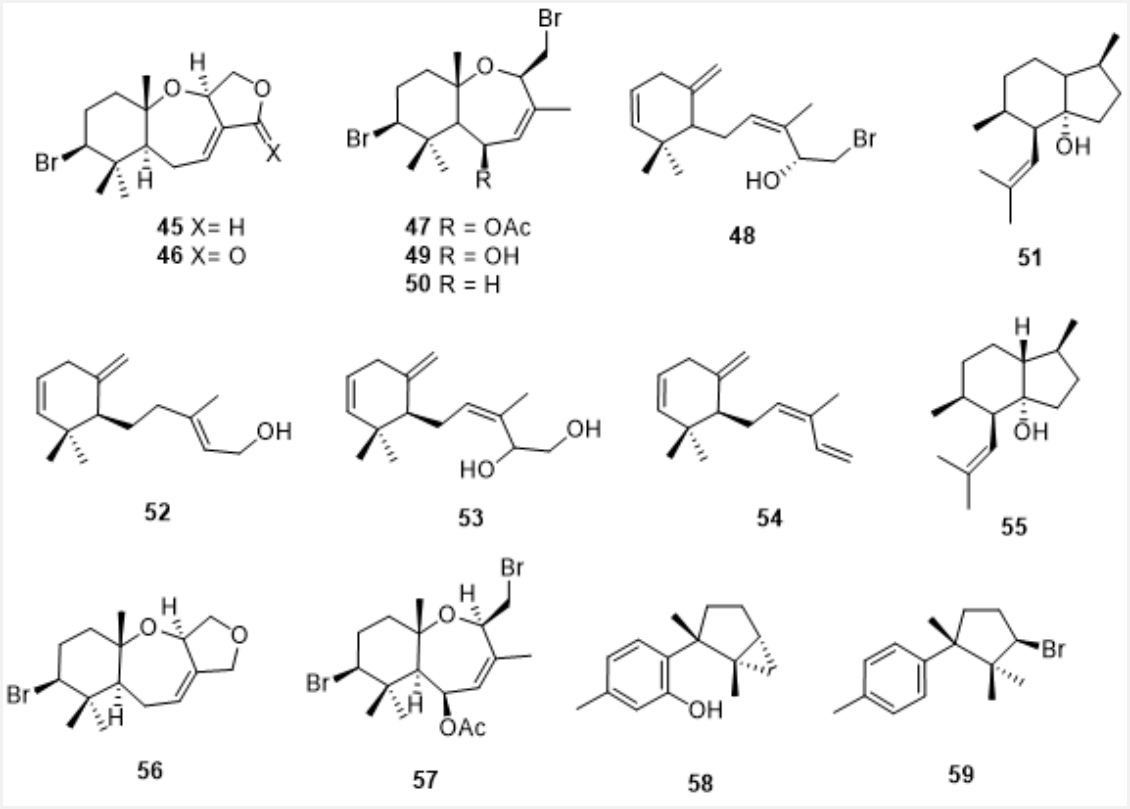
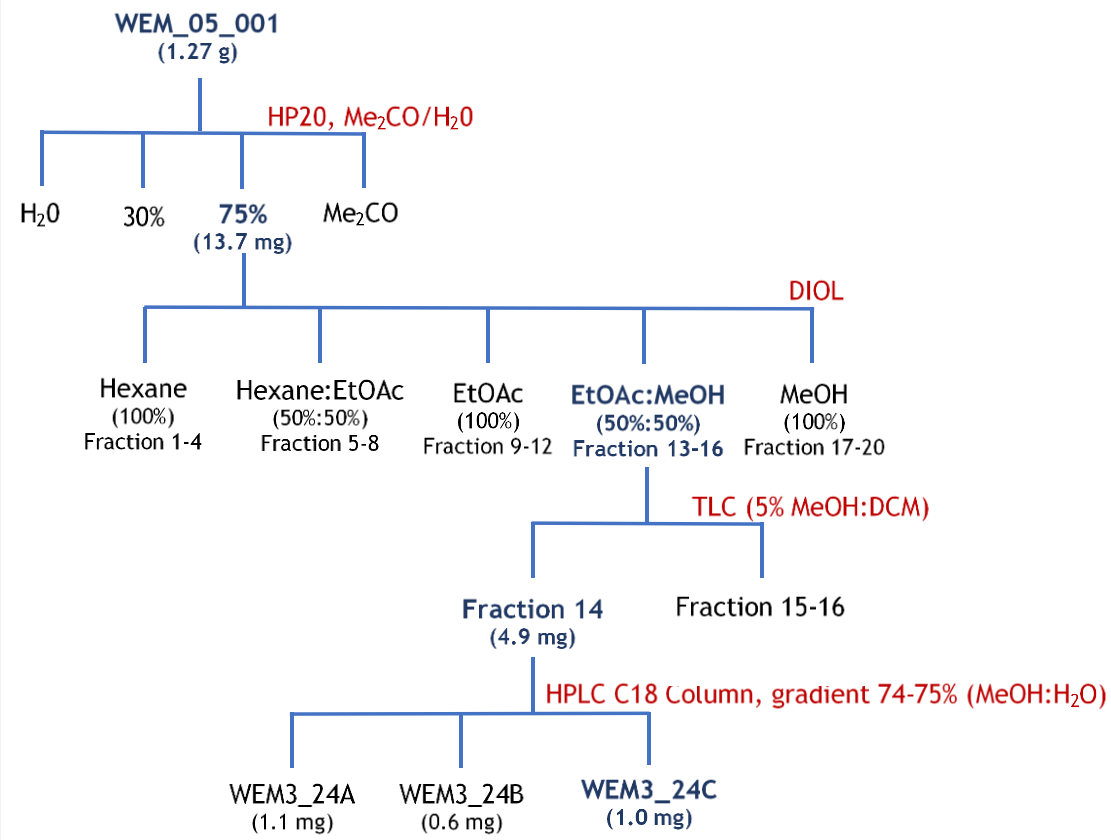


Figure 3.2. Previous secondary metabolites extracted from *Laurencia snackeyi*

3.3. Isolation and purification procedure

Specimen of WEM_05_001 was collected from Tablolong Beach, West Timor, Indonesia, in October 2017. The sample was dried under the sun and the thallus of the alga was cut into smaller portion before being subjected to extraction. Dried WEM_05_001 (1.27 g) was extracted with MeOH twice at the Laboratory of Integrated Science Research of Universitas Nusa Cendana in Kupang, West Timor, Indonesia. The crude extract was then dried under vacuum before being sent to Victoria University of Wellington, New Zealand, for screening and an isolation/purification process.

The extracts were resuspended in MeOH that was cyclic-loaded onto PSDVB then eluted with 30% Me_2CO/H_2O, 75% Me_2CO/H_2O and Me_2CO. The 75% Me_2CO/H_2O (13.7 mg) fraction contained interesting oxymethine peaks (3-5 ppm), detected by ^1H NMR spectroscopy. Further purification involved normal phase chromatography using a DIOL column with stepwise elution from hexane, through EtOAc to MeOH and collected in test tubes which yielded 4.9 mg in fraction 14. Further purification using C_{18} HPLC led to the isolation of aminoglyceroglycolipid (**60**) (1.0 mg) (Scheme 3.1).



Scheme 3.1. The procedure leading to the isolation of WEM3_24C from WEM_05-001

3.4. Structure elucidation of aminoglyceroglycolipid - WEM3_24C

Detailed NMR analysis identified two substructures. Analysis of the 1D NMR spectra of WEM3_24C (600 MHz, CD_3OD) showed the presence of resonances attributable to a number of oxymethine protons (δ_C 67-75; δ_H 3-5). These resonances consisted of one acetal (CH-1 δ_C 100.1, δ_H 4.76), four oxymethines (CH-2 δ_C 73.5, δ_H 3.40; CH-3 δ_C 74.9, δ_H 3.63; CH-4 δ_C 75.1, δ_H 3.09; CH-5 δ_C 69.9, δ_H 4.07) and one deshielded methylene (CH_2-6 δ_C 54.3, δ_H 3.36, 2.92).

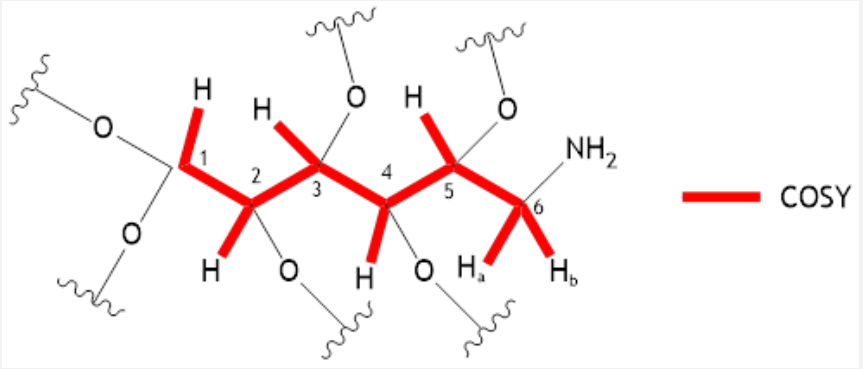


Figure 3.3. COSY correlations establishing a carbon chain consistent with a sugar moiety

Furthermore, a contiguous carbon chain typical of a sugar moiety was revealed from a series of vicinal correlation spectroscopy (COSY) correlations (couplings H-1 (δ 4.76) to H-2 (δ 3.40), H-2 to H-3 (δ 3.63), H-3 to H-4 (δ 3.09), H-4 to H-5 (δ 4.07), H-5 to H-6_a (δ 2.92) and to H-6_b (δ 3.36); and a geminal between H-6_a and H-6_b). However, the more shielded chemical shift of C-6 was not consistent with a common carbohydrate, which indicates the presence of an amino functionality (Figure 3.3). There were no further correlations with H-6 suggesting the absence of amide functionality at this carbon position.

A second spin system was revealed from the 2D NMR spectra that contained two oxymethylenes (CH2-3' δ_C 67.1, δ_H 4.11, 3.57; CH2-1' δ_C 64.3, δ_H 4.18, 4.50) and an oxymethine CH-2' (δ_C 71.7, δ_H 5.31). COSY correlations showed vicinal couplings from H-1'a,b to oxymethine H-2' and from H-2' to H-3'a,b. Geminal couplings between H-1'a (δ_H 4.18) to H-1'b (δ_H 4.50) and also H-3'a (δ_H 4.11) to H-3'b (δ_H 3.57) were also observed (Figure 3.4). No further COSY correlations were observed which indicated that C-1', C-2', and C-3' made up a glycerol subunit. The HMBC experiment revealed correlations from H-3'a, b to C-1', and between

H-3'b and H-1'a to C2 further strengthening this assignment. Additionally, an HMBC correlation between H-1'a to C-1" (δ_C 175.1) indicated attachment of an ester unit to the glycerol.

Furthermore, next to the glycerol unit, a lipid chain was also established through the COSY experiment, with connections between the methylene protons (H-2" (δ_H 2.31) to H-3" (δ_H 1.60)) and from H-3" to a large methylene envelope (δ_H 1.29). The methylene envelope then showed further COSY correlations to methyl CH3-7" (δ_H 0.90). Based on the integration values, the lipid chain was estimated as containing 14 methylene units in total, making it a 20-fatty acid. A Heteronuclear Multiple Bond Correlation (HMBC) correlation from methylene H2-2" to ester glycerol C-1" connected the lipid chain and glycerol unit.

Finally, another HMBC experiment connected the glycerol unit (H-3'a, b) to C-1 of the sugar moiety, which combined the two structures together (Figure 3.4). The summation of the NMR data indicated that the compound purified was an amino-glycero-glycolipid (Figure 3.4).

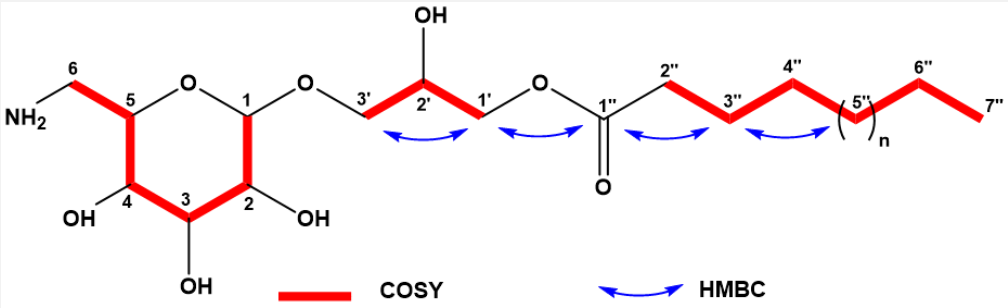


Figure 3.4. COSY and HMBC correlations establishing a connection between a sugar moiety, a glycerol, and lipid unit

The next step was to establish the relative configuration of the carbohydrate system by utilising the coupling constants of the protons around the sugar moiety. As pointed out by Angyal,[107] a large coupling constant (~9 Hz) is found in an axial to axial proton relationship, whereas smaller coupling constants are found in the axial to equatorial or equatorial to equatorial proton relationship (both ~3.5 Hz). Starting with the anomeric proton, H-1 was observed as a doublet with a coupling constant of 3.8 Hz, which indicated an equatorial-axial relationship with proton H-2. Proton H-2 was shown as a doublet of doublets (J = 9.7 and 3.8 Hz), indicative of a 1,2-trans diaxial relationship between H-2 and H-3 (large coupling constant).

This was followed by H-3 which has an apparent triplet multiplicity and a large coupling constant (9.3 Hz). These three interactions in the ring system close to the anomeric position, proved that anomeric proton H-1 must be equatorial given the smaller coupling constant (Figure 3.5). A 1,2-trans diaxial vicinal coupling was also found in the interaction of H-3 to H-4 (triplet) with a 9.8 Hz coupling constant, as well as H-4 to H-5. H-5 presented as a doublet of doublets (dd) instead of a doublet of doublet of doublets (ddd), being a doublet to H-4 and doublets to each H-6a and H-6b). As it is a dd, where one of its coupling constants was larger than ~9 Hz (9.6 and 2.2 Hz), one coupling must be close to 0 Hz indicating an approximate 90° dihedral angle between H-5 and H-6b. This established the sugar as 6-deoxy-6-amino-glucose and in addition indicated an α-link to the glycerol unit.

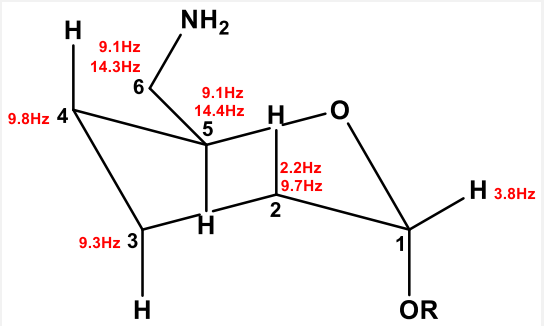
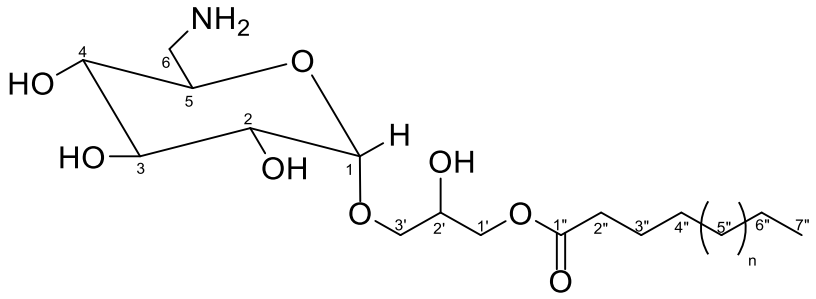


Figure 3.5. Coupling constants determination of the relative amino-sugar moiety

Also, the glycerol linkage was supported using a 2D ROESY (rotating-frame overhauser effect spectroscopy) experiment, confirming the connection between the glucose moiety and the glycerol unit (Figure 3.6).

Unfortunately, due to inconclusive mass spectrometry data, we could not able to find any ions using ESI, APCI OR MALDI-MS ion sources that was consistent with the NMR data. In this case, the length of the lipid chain could not be confirmed exactly by MS or detected in the GNPS molecular network. Unfortunately, due to time constraints, the absolute configuration of the sugar and the biological activity of the compound could not be determined.

Table 3.2. Tabulated NMR of WEM3_24C for 6-deoxy-6-aminoglycoglyrecolipid (**60**)



60

Position	^{13}C		^{1}H			COSY	HMBC (^{1}H to ^{13}C)
	δ (ppm)	HSQC (mult)	δ (ppm)	mult	J (Hz)		
1	100.1	CH	4.76	d	3.8	2	3'a, 3'b
2	73.5	CH	3.40	dd	3.8, 9.7	1	-
3	74.9	CH	3.63	t	9.3	4, 2	1
4	75	CH	3.09	t	9.8	3	-
5	69.9	CH	4.07	dd	2.1, 9.6	6a, 6b, 4	1
6a	54.3	CH_2	2.92	dd	9.1, 14.3	5	-
6b	-	-	3.36	dd	2.2, 14.4	5	-
1'a	64.3	CH_2	4.18	dd	6.9, 12.0	1'b, 2'	3'a, 3'b
1'b	-	-	4.50	-	-	1'a, 2'	-
2'	71.7	CH	5.31	dd	2.7, 5.9	1'a, 1'b, 3'a	1'a, 3'b
3'a	67.1	CH_2	4.11	dd	5.2, 10.9	2', 3'a	-
3'b	-	-	3.57	-	-	2', 3'b	-
1"	175.1	C	-	-	-	-	1'a
2"	35.2	CH_2	2.31	s	-	3"	3"
3"	26	CH_2	1.60	d	3.77	2", 4"	2"
4"	30.8	CH_2	1.29	s	-	7"	5"
5"	33.1	CH_2	1.29	s	-	7"	7"
6"	23.8	CH_2	1.29	s	-	7"	7"
7"	14.5	CH_3	0.90	s	-	6"	-

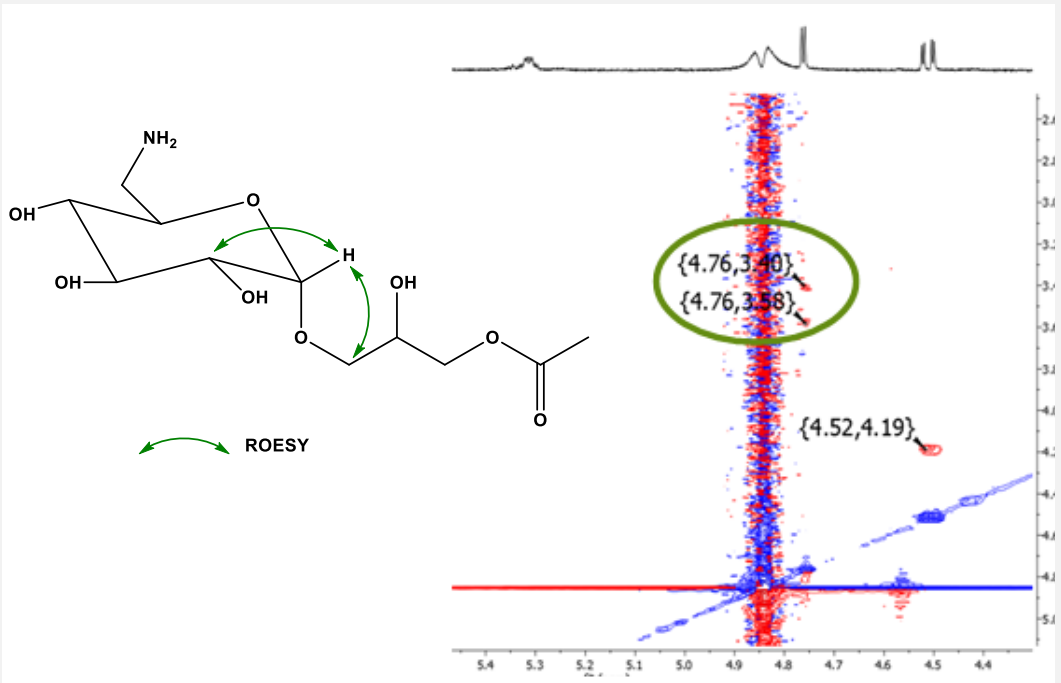


Figure 3.6. ROESY correlations establishing connection between the sugar moiety and glycerol functionality

3.5. Deoxyaminoglycoglycerolipids

Glycolipids are carbohydrate-based lipids, which mainly store energy for the producing organism but may also play various biological roles, such as anti-inflammatory, anti-tumour, and anti-viral activities.[108] They are mostly found in higher plants, marine algae, and cyanobacteria.[109] Based on the a glycosidic fragment linked to a lipidic molecule, there are three main groups of glycoconjugates such as glycosphingolipids, glycoglycerolipids, and atypical glycolipids.[110] (See Barnathan and coworkers who had reviewed marine invertebrate glycolipids).[111]

6-Deoxy-6-aminoglycoglyrecolipids are glycolipids that have an amine replacing the terminal C-6 alcohol residue in the carbohydrate moiety;[112] there are a number of compounds in this category (Figure 3.7). The crude extract of an undescribed algal species led to the isolation of two bioactive aminoglycoglycerolipids (**61**, **62**) which showed strong anti-tumour activity against the enzyme Myt1 kinase.[113] Another aminoglycoglycerolipid avrainvilloside (**63**) which carries the rare 6-deoxy-6-aminoglucose moiety, was obtained from the green alga *Avrainvillea*

nigricans collected from the Carribean.[114] A structurally similar compound, ishigoside (**64**) was isolated from the methanolic extract of the brown algae *Ishige okamurae* collected from the coast of Busan, Korea.[115] Ishigoside has one less methylene chain compared to avrainvilloside, and the researchers claim that ishigoside was a potential free-radical scavenger activity.[115] Although the number of lipid link could not be determined, **60** appeared to be missing an ester link at C-2' and hence is likely to be new compound and possibly has some kind of similar activity as well. So, future work could be done to isolate or synthesize the compound for testing.

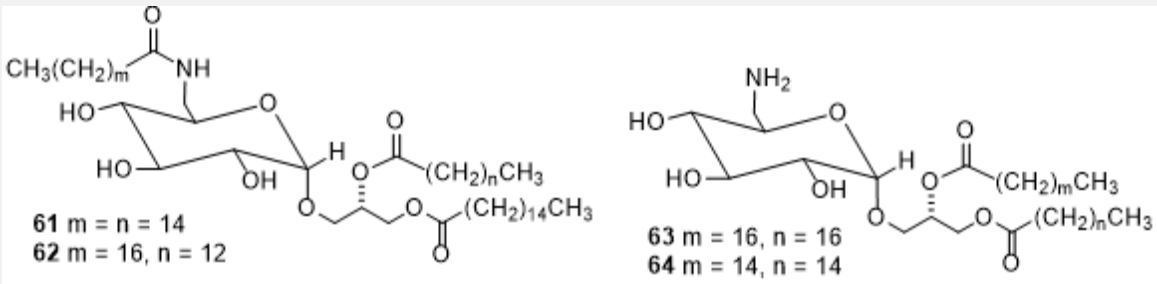


Figure 3.7. Metabolites from deoxyaminoglycoglycerolipids marine algae

CHAPTER 4. CONCLUDING REMARKS

Indonesia has a strong macroalgal primary metabolites industry and is the top supplier of phycocolloid sources in the world. Despite the value of these algae, the untapped potential economic importance of the secondary metabolites of Indonesian macroalgae has not been extensively reported. Macroalgae, ecologically sessile organisms, have had to develop non-physical defence mechanisms to survive in the harsh coastal environment, therefore many produce bioactive defensive chemical metabolites.

This study began with the sampling of 40 specimens of tropical Indonesian macroalgae from West Timor waters. A molecular networking screening was employed to prioritise the isolation workflow. The crude extracts of the samples were analysed using liquid chromatography-mass spectrometry (LC-MS)/mass spectrometry (MS) to generate data for creating the molecular network through the Global Natural Product Social Molecular Networking (GNPS) platform. Based on the clusters formed, six samples were prioritised from the molecular network. The suggestions from the screening process were then validated with ^{1}H NMR spectroscopy, which finally prioritised one sample (the other two specimens with interesting ^{1}H NMR spectra did not have enough extracted mass to process further). The structural elucidation of the pure compound isolated highlighted a known 6-deoxy-6-aminoglycoglyrecolipid (**60**). The presence of the 6-deoxy-6-amino motif in the sugar moiety is rare, and literature studies have shown such compounds to have strong anti-tumour activity as well as a potential free-radical scavenger capacity.

Molecular networking through the GNPS platform is rapid and has cut the time to be more efficient in the isolation workflow when compared to bioassays or nuclear magnetic resonance (NMR) screening. However, further optimization can be done to improve the screening procedure. First, optimisation of both LC-MS/MS methods and GNPS settings. This is to maximise the dereplication power through utilisation of the database available from the GNPS website. Secondly, the biomass for extraction should be increased to a minimum 5 g dry weight of macroalgae to improve the amount of extracted secondary metabolites.

Finally, the effort to explore marine natural products from tropical Indonesian macroalgae should be continued not only to support the local industry and coastal communities around Indonesia, but also to find novel secondary metabolites for further work.

CHAPTER 5. EXPERIMENTAL

All nuclear magnetic resonance (NMR) spectra were obtained using a 600 MHz Varian DirectDrive spectrometer equipped with a cryogenic probe, operating at 600 MHz (for ^{1}H) and 150 MHz (^{13}C nuclei). The residual solvent peak was used as an internal reference for ^{1}H (δ_H 7.26, $CDCl_3$; 3.31, CD_3OD; 2.50, DMSO–d_6) and ^{13}C (δ_C 49.00, CD_3OD) chemical shifts. Mass spectrometry (MS) data was collected using an Agilent 6530 quadrupole time-of-flight (QTOF) liquid chromatography-mass spectrometry (LC-MS) instrument. High performance liquid chromatography (HPLC) purifications were carried out using a Rainin Dynamax SD-200 solvent delivery system with 25 mL pump heads. Reversed phase HPLC was carried out with Phenomenex Prodigy octadecyl-derivatised silica gel (C_{18}) (analytical: 4.6 × 250 mm, 5 μm; semi-preparative: 10 × 250 mm,5 μm). Semipreparative HPLC was performed at a flowrate of 5 mL/min. All solvents used for column chromatography were of HPLC grade, and the H_2O was glass distilled.

Thin layer chromatography (TLC) analysis was performed using Macherey-Nagel plastic Polygram SIL G/UV_{254} plates. Developed TLC plates were visualised under a UV lamp source (λ= 254 nm), then analysed by dipping in a solution of 5% H_2SO_4 (conc.)/MeOH, followed by a solution of 0.1% vanillin/EtOH (% wt/vol) and heating. The mobile phase used for TLC was 5% MeOH/DCM (dichloromethane). Normal-phase column chromatography was carried out using silica (SiO_2) gels. Reversed-phase column chromatography was achieved using Supelco Dianion PSDVB HP20 chromatographic resin.

5.1. Sampling location

The research was carried out in Timor Island and Semau Island coastal waters, East Nusa Tenggara Province, Indonesia. Four locations were visited to collect fresh samples of macroalgae (see Figure 2.5). Three of the sites are in Timor Island i.e., Sulamu Beach (10° 3' 5.463" S and 123° 36' 52.29" E), Pasir Panjang Beach (10° 8' 55.464" S and 123° 36' 13.356" E), and Tablolong Beach (10° 19' 2.136" S and 123° 28' 13.728" E). While another collection site, Akle Beach, is on Semau Island (10° 19' 20.7048" S and 123° 20' 12.444" E). Sampling of the fresh macroalgae was done in the intertidal area during the low tide period. Samples were collected in water no deeper than knee height. A total of 40 samples of different species of

brown, red, and green macroalgae were collected from all four sites (Appendix B).

5.2. Deoxyribonucleic acid barcoding

Deoxyribonucleic acid (DNA) barcoding began with extraction of dry tissue from alga (stored in silica gel) using a modified Cetyl trimethylammonium bromide (CTAB) buffer procedure.[116] A 615 bp fragment from the 5′ end of the mitochondrial marker cytochrome oxidase subunit I (*cox*1) gene was amplified using primer pairs GazF1 and GazR1 (GazF1 5′ TCAACAAATCATAAAGATATTGG 3′ and GazR1 5′ ACTTCTGGATGTCCAAAAAAYCA 3’).[117] The Polymerase chain reaction (PCR) products were cleaned with ExoSAP-IT (USB, Cleveland, Ohio) enzymes and sequenced commercially (Macrogen Inc., Seoul, Korea).

5.3. Sample extraction

Prior to extraction, samples were dried under the sun for two to three days, labelled and stored. The extraction was done in the Integrated Science Laboratory of Universitas Nusa Cendana, Kupang, Indonesia. The samples were macerated and extracted twice in methanol for 24-hour periods. Both extracts were then combined. The methanol extract was subsequently concentrated in vacuo to dry samples completely or remove the methanol. As the last step, the remaining solution (presumably water) was removed in a freeze dryer at -100oC to -121oC. The dried samples were finally obtained and sent to Victoria University of Wellington for further analysis (see Appendix B).

5.4. Global natural product social molecular networking

The crude extracts were diluted in 30 mL MeOH and subsampled for untargeted LC-MS/MS (0.1 mg mL^{-1}) to get data for Global Natural Product Social Molecular Networking (GNPS) molecular molecular networking. A molecular network was created using the online workflow at GNPS. The data were filtered by removing all MS/MS peaks within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50Da window throughout the spectrum. The data was then clustered using MS-Cluster with a parent mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further edges

between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against the GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks.

5.5. Isolation of compound from *Laurencia snackeyi*

The crude extract was diluted in 30 mL MeOH passing through a 20 mL column of HP20 beads (see Scheme 3.1). The resulting eluent was diluted with 30 mL of distilled H_2O (50% MeOH in H_2O solution) and passed back through the HP20 column. The eluent was diluted again with 60 mL of distilled H_2O (25% MeOH in H_2O solution) and the resulting 120 mL eluent passed back through the column. Lastly, the eluent was diluted again with 120 mL H_2O (12.5% MeOH solution) then passed back through the column. The column was washed with 60 mL portions of 1) H_2O, 2) 30% Me_2CO/H_2O, 3) 75% Me_2CO/H_2O, and 4) 100% Me_2CO. The 100% Me_2CO fraction was concentrated to dryness under reduced pressure to provide 9.9 mg of material, while the 30% and 75% Me_2CO/H_2O fractions were each backloaded onto 20 mL of individual HP20 resin columns in order to remove the water. Each column was then eluted with Me_2CO to give 0.9 mg of the 30% fraction and 13.7 mg of the 75% fraction.

The 75% fraction was purified further using a 10 mL normal-phase diol column then eluted with a series of solvents, 30 mL each, which were collected in test tubes. The solvents were 100% Hexane (fractions 1-4), 50% Hexane/EtOAc (fractions 5-8), 100% EtOAc (fractions 9-12), 50% EtOAc/MeOH (fractions 13-16), and 100% MeOH (fractions 17-20). All fractions were dried under reduced pressure before TLC analysis was used to combined fractions. Fraction 14 (from 50% EtOAc/MeOH elution) was chromatographed on a semi-preparative C_{18} reversed-phase HPLC column, with a gradient 74-75% MeOH in H_2O as the mobile phase, at a flow rate of 5 mL/min. The compound of interest was the third fraction collected and had a retention time of approximately 1320 s. No further purification was observed with a mass of 1.0 mg recovered (WEM3_24C) which was a 6-deoxy-6-aminoglycoglyrecolipid (**60**).

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APPENDIX A: MARINE NATURAL PRODUCTS RESEARCH IN INDONESIA

Table 1. Marine natural product from sponges

No	Species	Compound and bioactive	Location	Literature
1.	*Sidonops microspinosa*	Microspinosamide: inhibits cytopathic effect of HIV-1 infection	South East Sulawesi	[36]
2.	Family Petrosiidae	Manzamine: 8-hydroxymanzamine A, manzamine F, along with the unprecedented manzamine dimer, *neo*-kauluamine. They show antimaria activity against *Plasmodium berghei*	North Sulawesi	[118]
3.	*Theonella swinhoei*	Bitungolides A–F; Dual-specificity against phosphatase VHR	North Sulawesi	[119]
		Aurantoside F-J are a new compound and showed a detectable antifungal activity	North Sulawesi	[120]
4.	*Haliclona* sp.	Brominated fatty acid showed moderate cytotoxicity against rat bladder epithelial cells)	Alor Island, East Nusa Tenggara	[121] [122] [123]
		Acetylene alcohols: lembehynes B and C. It showed neuritogenic activity against neuroblastoma cells	South Sulawesi	
		Halioxepine showed moderate cytotoxicity against NBT-T2 cells and antioxidant activity	Buton Island, Southeast Sulawesi	
5.	*Hyrtios reticulatus* and *Hyrtios erectus*	1,6-dihydroxy-1,2,3,4-tetrahydro-ß-carboline; hyrtiosulawesine	South Sulawesi	[124]
6.	*Hippospongia* sp.	Sesterterpenoid: barangcadoic acid A and rhopaloic acids A	South Sulawesi	[125]
7.	*Phyllospongia* sp.	Scalarane sesterterpenoids	South Sulawesi	[126]
8.	*Petrosia strongylata*	Sulfated sterols: lembehsterols A-B show inhibitory activity against thymidine phosphorylase (angiogenesis in solid tumors)	North Sulawesi	[127]
9.	*Callyspongia pseudoreticulata*	Diyne which is toxic to brine shrimp assay	South Sulawesi	[128]
10.	*Melophlus sarassinorum*	Tetramic acid: melophlin C is an antimicrobial active against *Bacillus subtilis* and *Staphylococcus aureus*, also antifungal against *Candida albicans*, while melophlins D–O (less active)	South Sulawesi	[129]
11.	*Sigmadocia symbiotica* (symbiont with alga *Ceratodictyon spongiosum*)	*Ceratospongamide: cis,cis-* and *trans,trans-* isomers antiinflammation and inhibit the expression of a human-sPLA$_2$promoter-based reporter	Biaro Island, South Sulawesi	[130]
12.	NA	Manzamine alkaloids which has bioactivity against malaria, TB, and leishmaniasis	South Sulawesi	[131]

No	Species	Compound and bioactive	Location	Literature
13.	*Fascaplysinopsis reticulate*	3-bromofascaplysin, 14-bromoreticulatine, and 14-bromoreticulatate	Indonesia	[132]
14.	*Biemna fortis*	Labuanine is a neuronal differentiation inducer against neuroblastoma	West Flores, East Nusa Tenggara	[133]
15.	*Xestospongia* sp.	Aaptamine antibacterial against *S. aureus*, *E. coli*, *V. anguillarum*; also antifungal against *C. tropicalis*)	Jakarta	[134]
16.	*Stylissa carteri*	Oroidin: latonduines A-B	Latondu Island, South Sulawesi	[135]
17.	*Hyrtios* sp.	Merosesquiterpenes: puupehenone	North Sulawesi	[136]
18.	*Axinyssa aculeate* (also its mollusk nudibranch predator *Phyllidia varicose*)	Sesquiterpenoids: 9-thiocyanatopupukeanane which weakly and moderately antifungal against *B. subtilis* and *C. albicans*	Thousands Island, Jakarta	[137]
19.	*Plakortis* cfr. *lita*	Plakortin, manadoperoxides A–D and peroxyplakoric ester B3. Show antiprotozoal activity against *Trypanosoma brucei rhodesiense*	North Sulawesi	[138]
20.	*Stylissa* sp.	Octapeptide stylissamide which inhibit HeLa cell migration	Biak, Papua	[139]
21.	*Acanthostrongylophora* sp.	Acantholactone	North Sulawesi	[140]
22.	*Hyrtios reticulatus*	Hyrtioreticulins A against the formation of an E1-ubiquitin activating enzyme inhibitor	North Sulawesi	[141]
23.	*Stylissa* sp.	Stevesines cytotoxicity against mouse lymphoma cell line and debromolatonduines	Derawan Island, East Kalimantan	[142]
24.	*Aplysinella strongylata*	19-Hydroxypsammaplysin E showed modest inhibition of chloroquine-sensitive *P falciparum*	Bali	[143]
25.	*Lissodendryx fibrosa*	Sterols: manadosterols A and B, both showed potential as anticancer agents	North Sulawesi	[144]
26.	*Plakortis lita*	hopanoid glycoside: plakohopanoid	North Sulawesi	[145]
27.	*Acanthostrongylophora* sp.	Manzamine-type alkaloids: 12,28-oxamanzamine E, 12,34-oxa-6-hydroxymanzamine E, 8-hydroxymanzamine B and 12,28-oxaircinal. They are showed significant inhibitory enzyme implicated in Alzheimer's disease pathology	North Sulawesi	[146]
28.	*Coelocarteria cfr.singaporensis*	*Ent*-isocopalane diterpenes: coelodiol and coeloic acid. Inhibit human gastric adenocarcinoma	North Sulawesi	[147]
29.	*Corticium simplex*	Steroidal Alkaloids: Cortistatins A-D, an anti-angiogenic activity	Flores Island, East Nusa Tenggara	[148]
30.	*Dactylospongia elegans*	Furanosesterterpene: Furospinosulin-1, an antiproliferative activity against human prostate cancer and antitumor activity	Indonesia	[149]

No	Species	Compound and bioactive	Location	Literature
31.	*Rhabdastrella globostellata*	Globostellatic acids A and D and stelliferin riboside; new natural products. They show selectively active against mouse lymphoma cell	Kapoposang Island, South Sulawesi	[150]
32.	*Xestospongia cf. vansoesti*	Salsolinol and its derivates norsalsolinol, cis-4-hydroxysalsolinol, and trans-4-hydroxysalsolinol. Show inhibition activity against chymotrypsin	North Sulawesi	[151]
33.	*Dasychalina* sp.	Desulfohaplosamate that is a selective cannabinoid CB2-receptor ligand	South Sulawesi	[152]

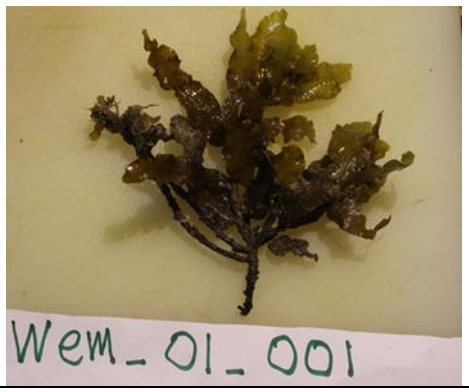
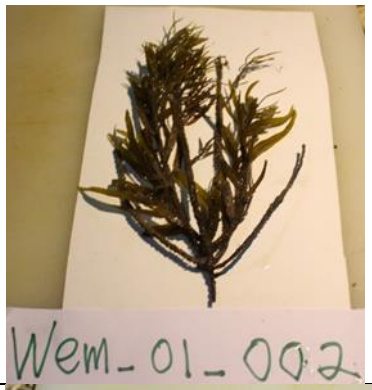
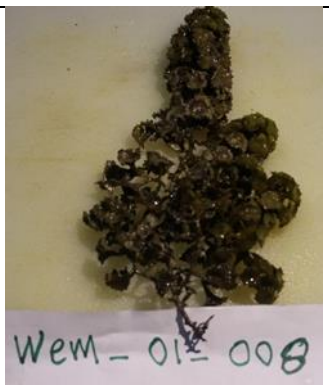
Table 2. Marine natural products from fungi

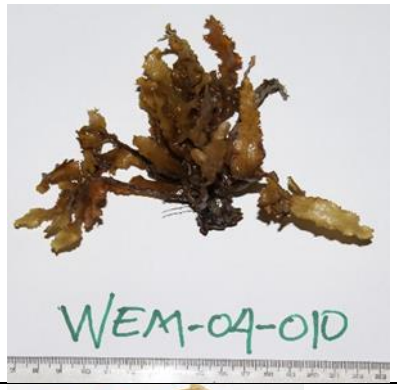
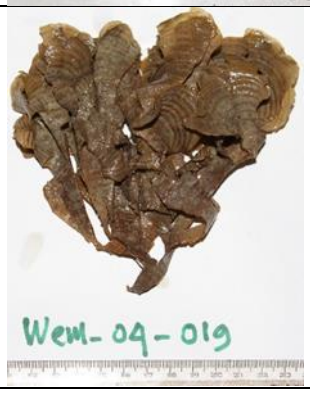
No	Species	Compound and bioactive	Location	Literature
1.	*Curvularia lunata* (symbiont with sponge *Niphates olemda*)	Lunatin antibacterial against *S. aureus*, *E. coli* and *B. subtilis* but inactive against *C. albicans*	Bali	[153]
2.	*Cladosporium herbarum (*symbiont with sponge *Callyspongia aerizusa)*	Phthalide herbaric acid show no activity; furan show activity against *Bacillus subtilis* and *Staphylococcus aureus*	Bali	[153], [40]
3.	*Penicillium cf. montanense* from sponge *Xestospongia exigua*	Xestodecalactones A–C but only xestodecalactones B active against *C. albicans*	Bali	[41]
4.	*Myrothecium* sp. From unidentified sponge	Trichothecenes: roridin R cytotoxic to L1210 cells	North Sulawesi	[154]
5.	*Aspergillus sp.* from unidentified alga	Hexahydroanthrones: tetrahydrobostrycin and 1-deoxytetrahydrobostrycin. Both show weak antibacterial activity against *Staphylococcus aureus* and 1-deoxytetrahydrobostrycin also against *Escherichia coli*	North Sulawesi	[155]
6.	Endophytic *Daldinia eschscholzii* from alga *Gracilaria sp.*	Lactone, antifungal against *Cladosporium cucumerinum*	South Sulawesi	[156]
7.	Unidentified fungi from unknown alga	Naphthalene, fungicidal against *Cladosporium cucumerinum*	East and West Java, and North Jakarta	[157]
8.	Unidentified fungi from unknown sponge	Hexaketide: *iso*-cladospolide B, *seco*-patulolide C; Macrolides: pandangolide 1 and pandangolide 2, cladospolide B	South Sulawesi	[158]

Table 3. Other group of marine natural products from invertebrate (dinoflagellate, cyanobacteria bacteria, coelenterates, tunicates, cnidarian)

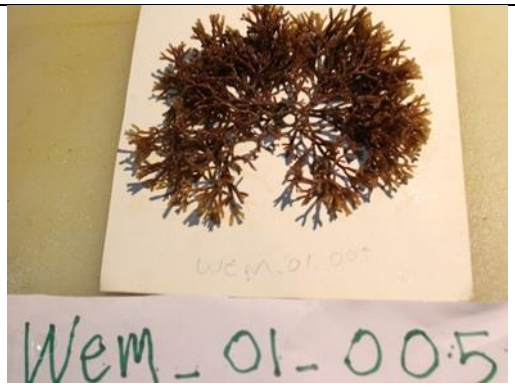
No	Species	Compound and bioactive	Location	Literature
1.	*Amphidinium* sp. as symbiont of marine flatworm	Polyols: karatungiols A and B howed antifungal activity against *Aspergillus niger* and antiprotozoan activity against *Trichomonas foetus*	North Sulawesi	[159]
2.	*Phormidium* sp.	Phormidolide, show activity against brine shrimp tocix	Sulawesi Island	[160]
3.	*Streptomyces* sp.	komodoquinone A dose-dependent neuritogenic activity against the neuroblastoma cell and B	Komodo Island, East Nusa Tenggara	[161]
4.	Xenia sp.	xeniolide F and 9-hydroxyxeniolide F	North Sulawesi	[162]
5.	*Pachyclavularia violacea*	Sterols: ecosterol	North Sulawesi	[163]
6.	*Isis hippuris*	polyoxygenated sterols	Sulawesi Island	[164]
7.	*Didemnum* sp.	(+)-didemniserinolipid B then revised as 31-sulfate	South Sulawesi	[165], [166]
8.	*Eusynstyela latericius*	Hydroxylpyridoacridine alkaloid: styelsamine C	South Sulawesi	[167]
9.	*Leptoclinides dubius*	Leptoclinidamide and (*R*)-leptoclinidamine B	North Sulawesi	[168]
10.	*Cladiella* sp.	6-hydroxyeunicellin diterpenoids, cladieunicellin G and 6-epi-cladieunicellin F	Indonesia	[169]
11.	*Sinularia* sp.	Llkaloids: sinulasulfoxide and sinulasulfone. Sinulasulfoxide proved to moderately inhibit LPS-induced NO release	North Sulawesi	[170]
		Sterols: gorgosterol	North Sulawesi	[171]
		norcembranes chloroscabrolide A and B	North Sulawesi	[172]

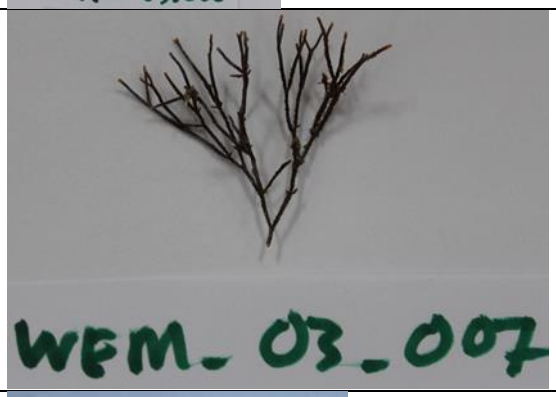
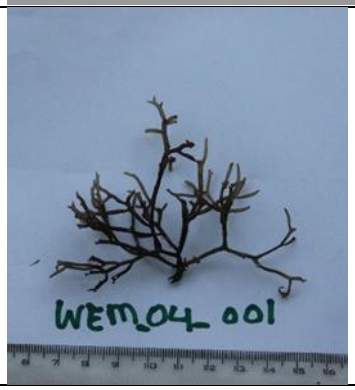
APPENDIX B: MACROALGAE COLLECTED FROM WEST TIMOR WATERS, INDONESIA

1.	WEM_01_001 *Sargassum* sp1 (Brown algae)		Sulamu Beach
2	WEM_01_002 *Sargassum* sp2 (Brown algae)		Sulamu Beach
3	WEM_01_007 *Padina* sp1 (Brown algae)		Sulamu Beach
4	WEM_01_008 *Turbinaria* sp1 (Brown algae)		Sulamu Beach

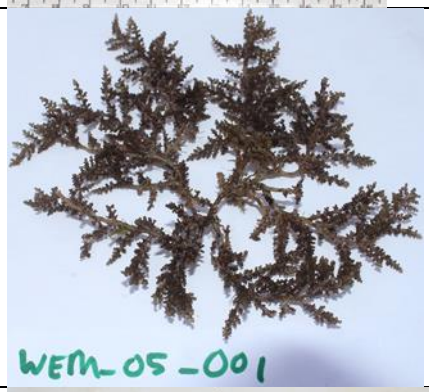
5	WEM_04_003 (*Turbinaria* sp2) Brown algae		Akle Beach
6	WEM_04_006 *Sargassum* sp3 (Brown algae)		Akle Beach
7	WEM_04_010 *Sargassum* sp4 (Brown algae)		Akle Beach
8	WEM_04_019 *Padina* sp2 (Brown algae)		Akle Beach

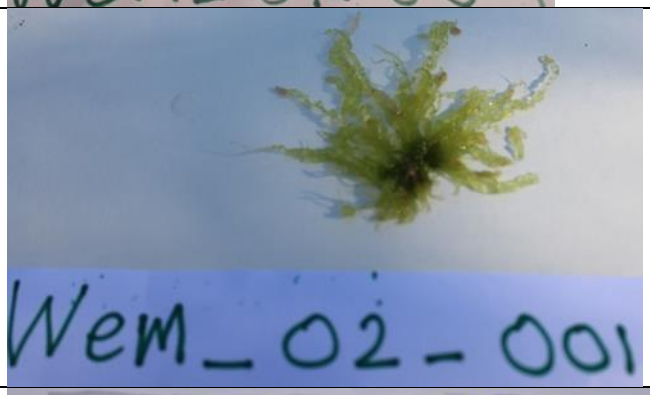
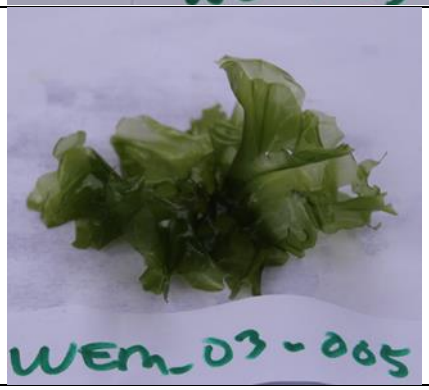
9	WEM_05_002 *Dictyota* sp (Brown algae)		Tablolong Beach
10	WEM_05_005 *Sargassum* sp5 (Brown algae)		Tablolong Beach

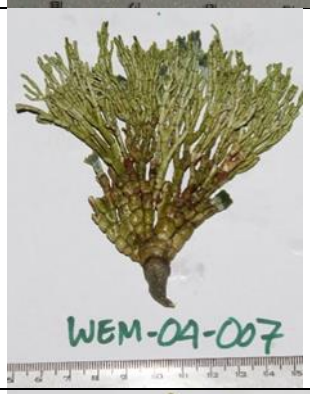
1	WEM_01_003 *Gracilaria* sp1 (Red algae)		Sulamu Beach
2	WEM_01_005 *Amphiroa* sp1 (Red algae)		Sulamu Beach
3	WEM_01_006 *Acanthophora* sp1 (Red algae)		Sulamu Beach
4	WEM_03_001 *Gracilaria* sp2 (Red algae)		Pasir Panjang Beach

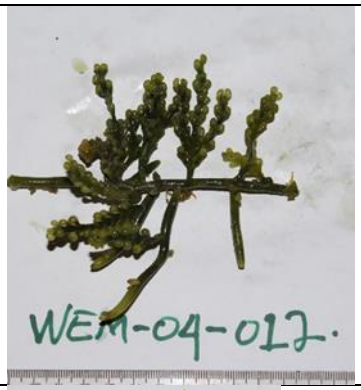
5	WEM_03_002 *Amphiroa* sp2 (Red algae)		Pasir Panjang Beach
6	WEM_03_006 *Halymenia* sp1 (Red algae)		Pasir Panjang Beach
7	WEM_03_007 *Amphiroa* sp3 (Red algae)		Pasir Panjang Beach
8	WEM_04_001 *Gracilaria* sp3 (Red algae)		Akle Beach

9	WEM_04_004 *Galaxaura* sp1 (Red algae)		Akle Beach
10	WEM_04_005 *Amphiroa* sp4 (Red algae)		Akle Beach
11	WEM_04_009 Acanthophora sp2 (Red algae)		Akle Beach
12	WEM_04_017 Acanthophora sp3 (Red algae)		Akle Beach

13	WEM_04_018 *Hypnea* sp1 (Red algae)		Akle Beach
14	WEM_05_001 *Laurencia snackeyi* (Red algae)		Tablolong Beach
15	WEM_05_004 *Hypnea* sp2 (Red algae)		Tablolong Beach

1	WEM_01_004 *Halimeda* sp1 (Green Algae)		Sulamu Beach
2	WEM_02_001 *Ulva* sp1 (Green algae)		Sulamu Beach
3	WEM_03_004 (*Ulva* sp2) Green algae		Pasir Panjang Beach
4	WEM_03_005 (*Ulva* sp3) Green algae		Pasir Panjang Beach

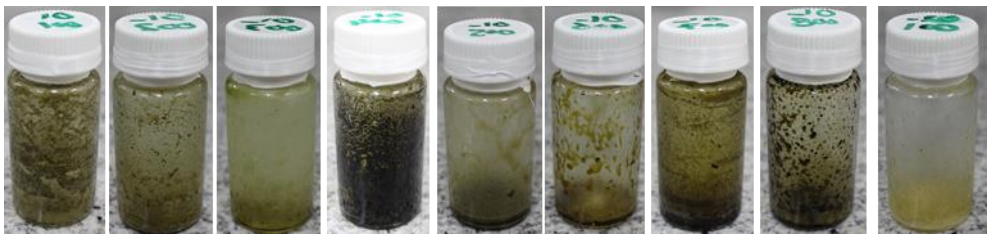
5	WEM_04_002 (*Caulerpa* sp1) Green algae		Akle Beach
6	WEM_04_007 (*Halimeda* sp2) Green algae		Akle Beach
7	WEM_04_008 *Halimeda* sp3 (Green algae)		Akle Beach
8	WEM_04_011 (Caulerpa sp2) Green algae Edible		Akle Beach

9	WEM_04_012 (*Caulerpa* sp3) Green algae		Akle Beach
10	WEM_04_013 *Halimeda* sp4 (Green algae)		Akle Beach
11	WEM_04_014 (*Halimeda* sp5) Green algae		Akle Beach
12	WEM_04_015 *Codium* sp1 (Green algae)		Akle Beach

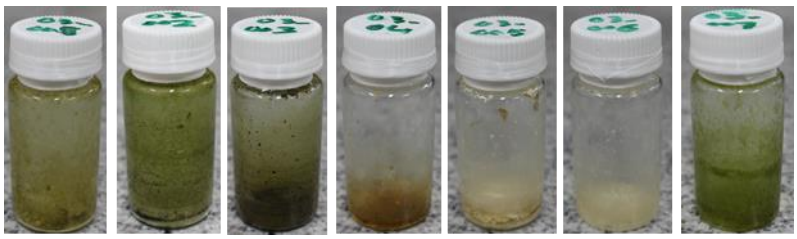
13	WEM_04_016 *Caulerpa* sp4 (Green algae)		Akle Beach
14	WEM_04_020 *Halimeda* sp5 (Green algae)		Akle Beach
15	WEM_05_003 *Codium* sp2 (Green algae)		Tablolong Beach

APPENDIX C: MACROALGAE EXTRACTS AFTER FREZE-DRY AND READY TO BE SENT TO VUW

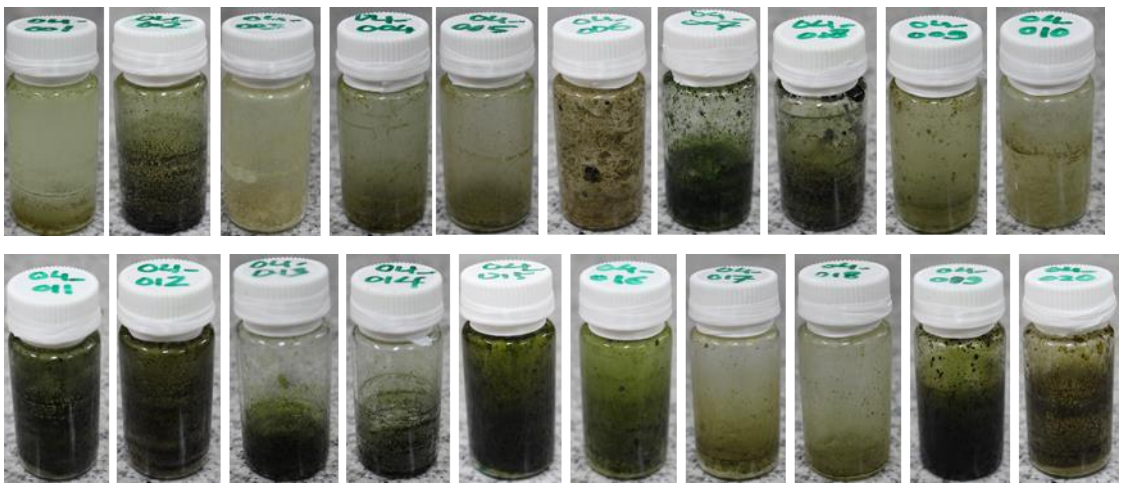
Sulamu Beach



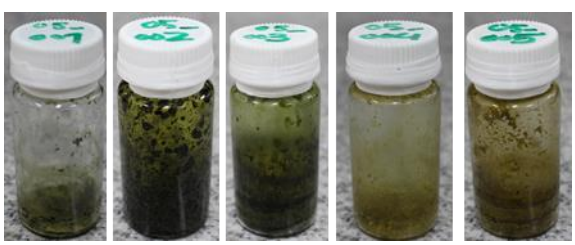
Pasir Panjang Beach



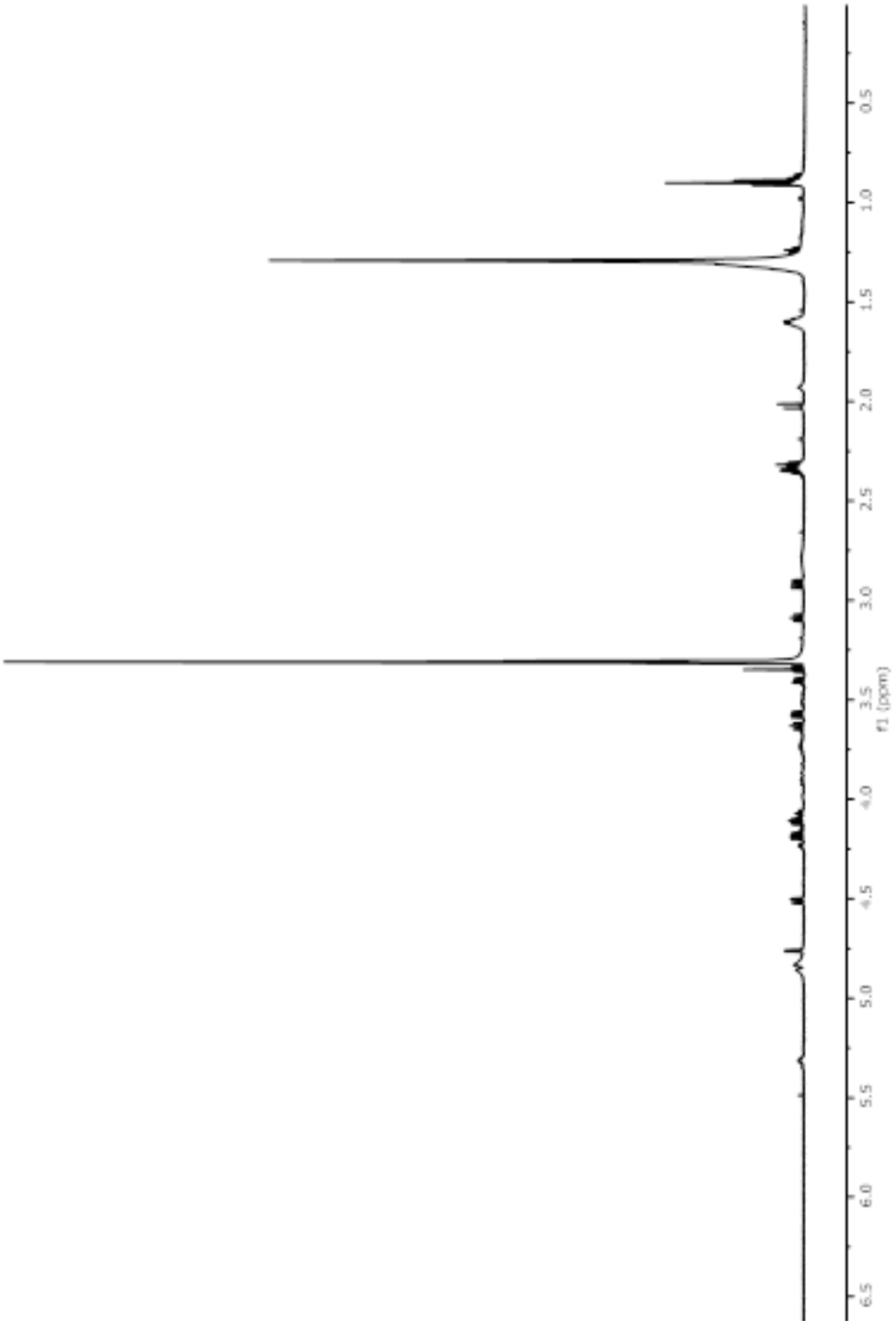
Akle Beach



Tablolong Beach

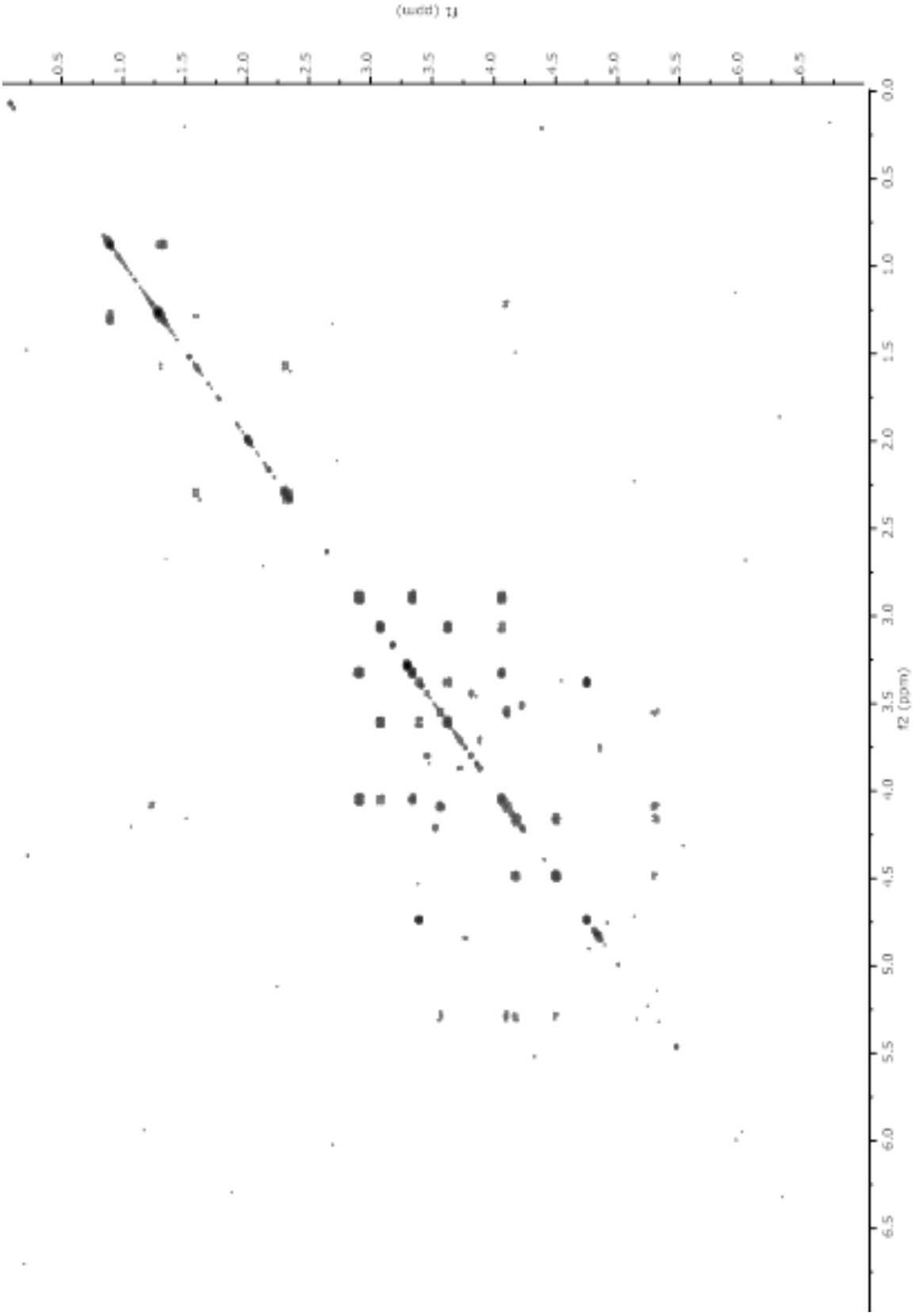


APPENDIX D: 6-deoxy-6-aminoglycoglyrecolipid SPECTRA

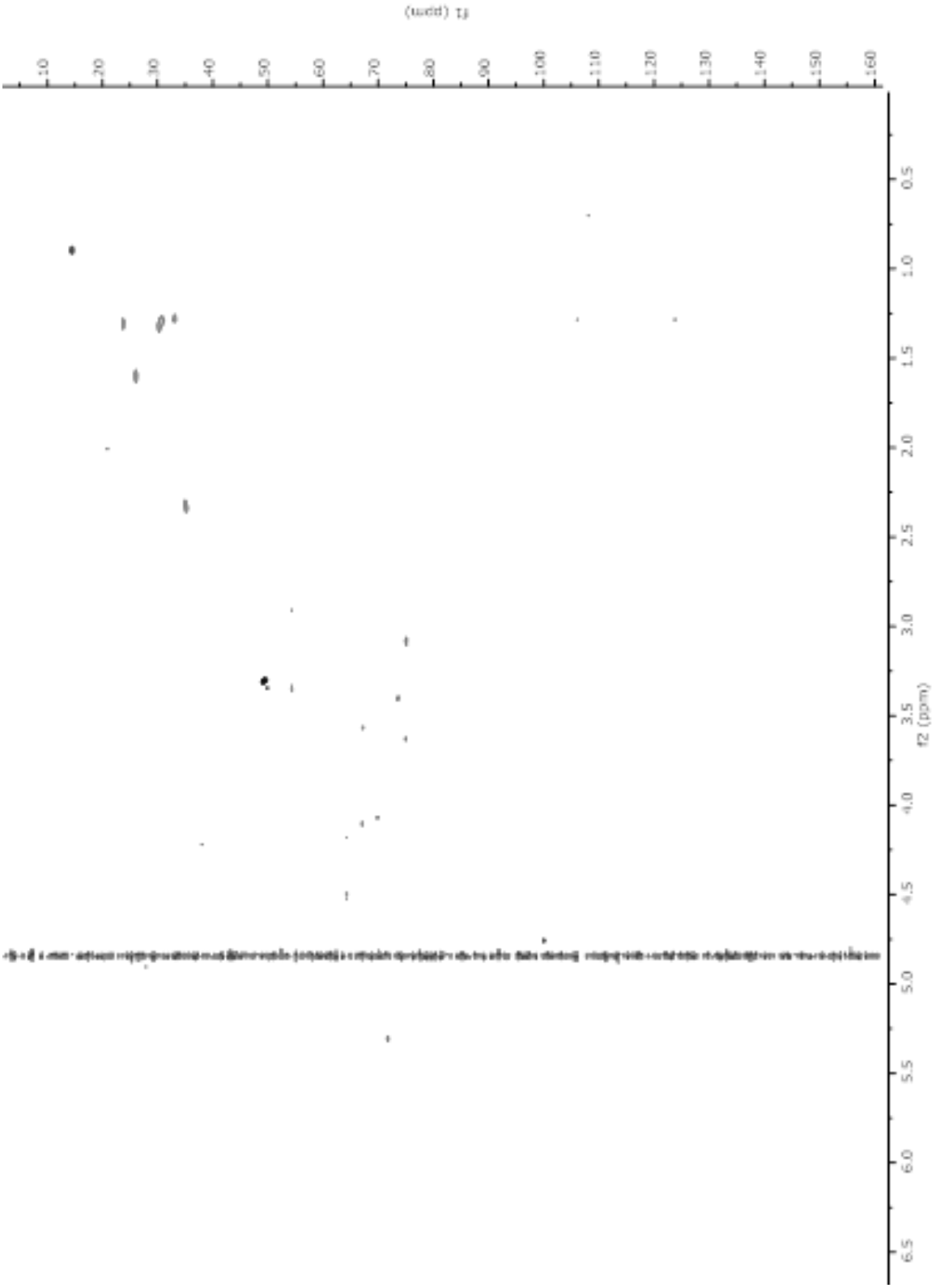


^{1}H NMR spectrum of 6-deoxy-6-aminoglycoglyrecolipid (**60**) (600 MHz, CD_3OD)

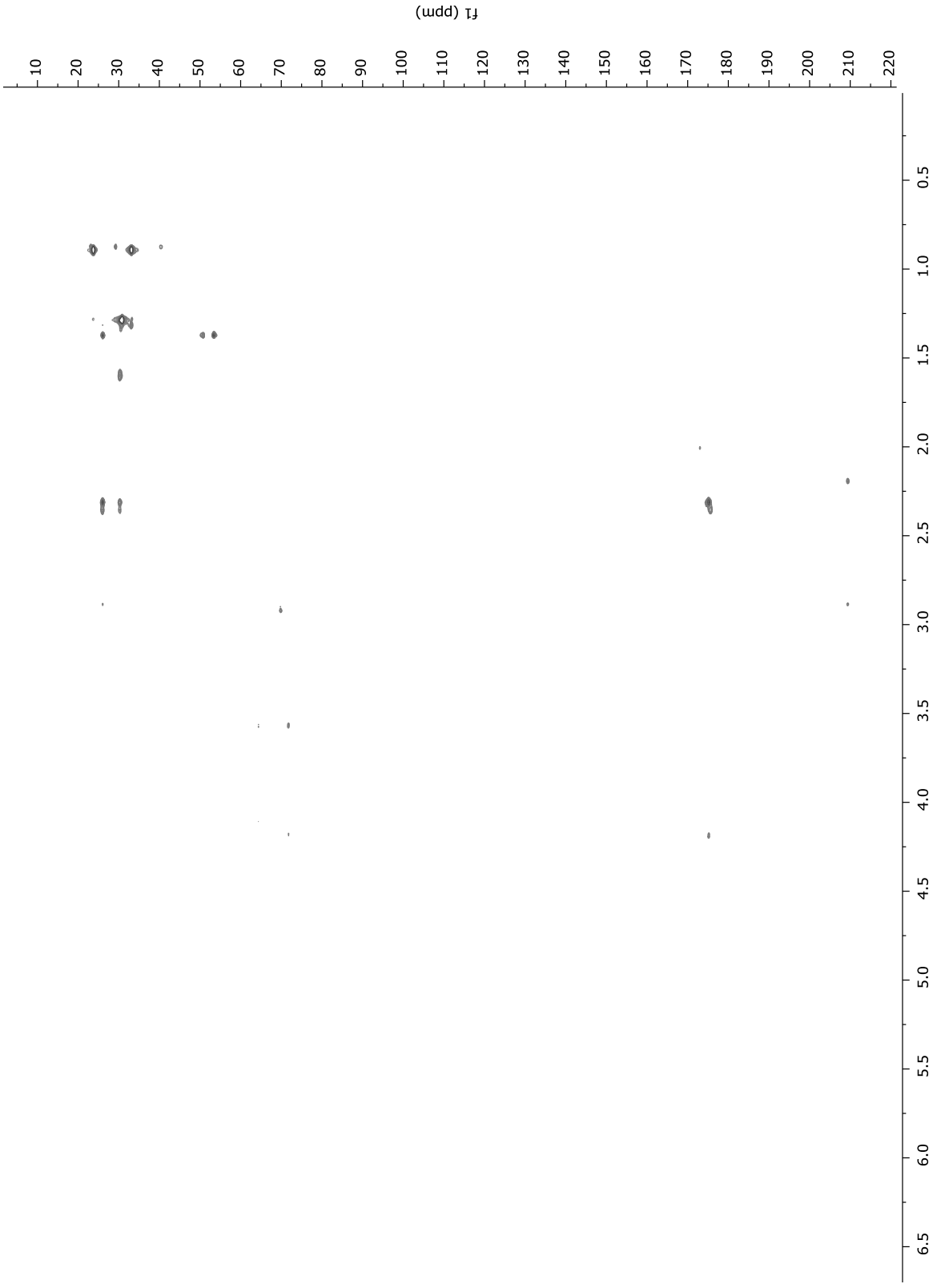
^{13}C NMR spectrum of 6-deoxy-6-aminoglycoglyrecolipid (**60**) (150 MHz, CD_3OD)



COSY spectrum of 6-deoxy-6-aminoglycoglyrecolipid (**60**) (600 MHz, CD_3OD)



HSQC spectrum of 6-deoxy-6-aminoglycoglyrecolipid (**60**) (600 MHz, CD_3OD)



HMBC spectrum of 6-deoxy-6-aminoglycoglyrecolipid (**60**) (600 MHz, CD_3OD)