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Antiparasitic Peptide from the Ocean: Discovery, Synthesis and Structure-Activity Relationship Study

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ANTIPARASITIC PEPTIDE FROM THE OCEAN: DISCOVERY, SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIP STUDY

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ABSTRACT

ANTIPARASITIC PEPTIDE FROM THE OCEAN: DISCOVERY, SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIP STUDY

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Dissertation supervised by Dr. Kevin J. Tidgewell

Millions of people die every year because of infectious diseases, and malaria is among the top five of the deadliest infectious disease. In 2018, malaria took more than four hundred thousand lives, and more than half of them are children under five years of age. Most malaria-affected parts of the world are also the home of the most underprivileged people. Seemingly, antimalarial drug discovery never achieved the attraction that it requires. Like malaria, another infectious disease that is not extensively explored by drug discovery campaigns is American trypanosomiasis or Chagas disease. More than a hundred years have passed since discovering the disease, and surprisingly only two drugs are clinically available; both are far from ideal. It is imperative to say that these disease areas require more attention from drug discovery researchers. Hence, my project

focuses on drug discovery for these neglected diseases utilizing one of the most prolific sources for lead molecule generation, marine cyanobacteria.

This thesis describes the discovery of an *N*-methylated peptide, naranjamide, from a marine cyanobacterium collected in the Portobelo National Park, Panama. The compound inhibited the growth of *Trypanosoma cruzi* and *Plasmodium falciparum* parasites. To confirm the structure and established its antiparasitic potential, I attempted to synthesize the molecule and found the synthetic version to inhibit both *T. cruzi* and *P. falciparum* parasite with IC_{50} values of 9.2 μM and 2.8 μM, respectively. Later, a series of non-methylated analogs were synthesized, which are found to be malaria selective. A more detailed study is required to establish a complete structure-activity relationship.

DEDICATION

Dedicated to my family and all graduate students.

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Chapter I: Introduction

1.1. History of natural product drug-discovery

Bioactive natural products have been used for centuries by humans, with the first recorded example dating to around 2600 BC in Mesopotamia. Some of the earliest examples include cedar oil (*Cedrus* sp.), cypress oil (*Cupressus sempevirens*), licorice oil (*Glycyrrhiza glabra*), myrrh (*Commiphora* sp.) and poppy juice (*Papaver somniferum*), which are still employed for treating different illnesses today. Nearly all human civilizations documented their use of natural products for the remedy of illness. The Egyptian *Ebers Papyrus*, around 3500 years old, included approximately 800 prescriptions. Over 700 natural products are described in it, including Aloe (*Aloe vera*), Frankincense oil (*Boswellia carteri*), and Castor oil (*Ricinus communis*).[1] Some other notable examples of texts on medicine, that registered bioactive natural products, include *Corpus Hippocraticum* by Hippocrates and other physicians, De Materia Medica by Dioscorides, Charaka Samhita from Indian Ayurveda, and Wu Shi Er Bing Fang (in English, *Prescription for fifty-two diseases*), the oldest book on Chinese traditional medicine.[2-5]

The source of knowledge compiled in these texts came from people exploring the use of natural products while searching for answers to disease or sudden death.[6] At that time, most of the researchers did not know that natural products contain bioactive molecules, which was the reason for their medicinal property. Few researchers

hypothesized that these natural products have different compounds responsible for their activity. In 1804, the hypothesis was proven when the German Pharmacist Friedrich Wilhelm Sertürner isolated the first pure natural compound, morphine from opium. In 1827, Heinrich Merck commercialized morphine and made it the first commercialized medicine.[7] Soon after, structure identification allowed scientists to synthesize many of these natural products and open a new research branch of chemistry, total synthesis. Many bioactive compounds of natural product origin were synthesized at that time, and the ability to make them synthetically has allowed their widespread use to be possible. It is worthy of mentioning some of these (**Figure 1.1**) : morphine (**1.1)** from *Papaver somniferum* (opium poppy), salicin (**1.2)** from *Salix alba* (white willow), strychnine (**1.3**) from *Strychnos nux-vomica* (strychnos), quinine (**1.4)** from *Cinchona ledgeriana* (cinchona bark), colchicine (**1.5**) from *Colchicum autumnale* (colchicum), caffeine (**1.6**) from *Coffea arabica*, nicotine (**1.7**) from *Nicotiana tabacum*, atropine (**1.8**) from *Atropa belladonna* and cocaine (**1.9**) from *Erythroxylum coca*. Many of these molecules are still used for treating diseases or for other purposes.[8] For example, colchicine is used for familial Mediterranean fever (FMF) and acute gout flares,[9] and atropine is used as antidote for muscarinic agent (organophosphorus, carbamate, or muscarinic mushroom) poisoning and to treat bradycardia.[10] Access to these natural products synthetically also allowed researchers to

modify their chemical structure in order to alter their pharmacokinetic and/or pharmacodynamic properties.[11]

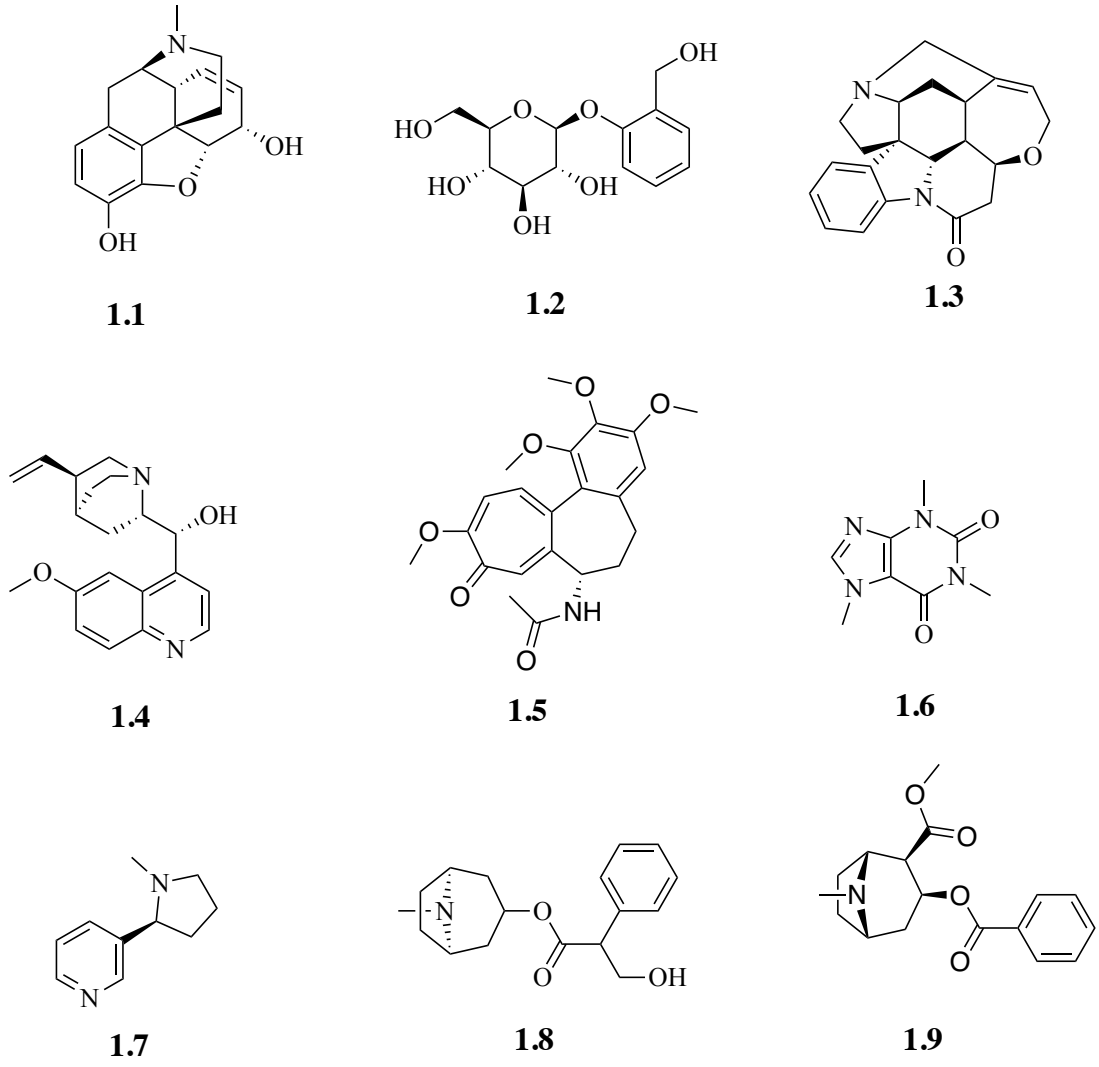


Figure 1.1: Historically significant natural products

Until the 20th century, drug-discovery efforts principally explored terrestrial plants. Researchers started to scout microorganisms as the source for bioactive compounds in the

last century and intensified the exploration of bioactive molecules from nature.[12] Efforts by researchers have led to the discovery of numerous FDA approved new molecular entities (NME), semisynthetic natural compounds, and natural compound inspired synthetic compounds. The global medicine market is about 1.1 trillion USD, natural products and related compounds comprise about 35% of that market.[13] Approximately 60% of small molecule therapeutics approved by the FDA in the last 40 years have origins connected to natural products.[14] The FDA was established in 1906 to ensure the safety and effectiveness of human and veterinary drugs and vaccines and other biological products and medical devices intended for human use.[15] Many natural products currently in use, including those mentioned above, were introduced to the clinic long before the FDA was established, and they became officially approved. For example, the semisynthetic plant derivative guaifenesin from guaiacum resin, got its official approval as a cough suppressant in 1852, though people used it as early as the 16th century. Since the establishment of the FDA, many new or existing natural products and their derivatives have been approved for clinical use.

The most recent analysis by Newman and Cragg[16] on natural products as sources of new drugs over the last four decades identified nearly half (930) of all FDA-approved drugs (1881) are either natural products, natural product derivatives or inspired by natural products. Among these approved natural products, 71 are unmodified natural products, and

356 are natural products derivative. Remaining 503 drugs include natural products and synthetic drugs with natural product pharmacophores or mimic of natural product. An earlier analysis by Patridge *et al.*[17] revealed that 44% of approved were derived from mammals (Bovine, porcine, equine, canine, and human). Among the non-mammalian natural products (NMNP), almost half (45% of NMNPs; 25% overall) of them are plant-derived. Two other significant sources of NMNP include bacteria (29% of NMNPs; 16% overall) and fungi (22% of NMNPs; 12% overall). Unfortunately, rigorous procedures to isolate and identify natural products, complexity of the molecules making total synthesis difficult, advances in both high throughput screening (HTS) and combinatorial synthesis, contribute to the shifting of drug discovery campaigns by many pharmaceutical companies away from natural products to synthetic compounds.[17-18] Nevertheless, natural products and their derivatives are still the most diverse sources for chemical entities to treat diseases or to elucidate disease mechanisms. A cheminformatic analysis on a representative set of natural products, bioactive molecules, and synthetic compounds has found that the chemical diversity possessed by natural products covers a large and diffuse area in the chemical space as compared to the synthetic compounds which are concentrated, hence less diverse.[19]

The history of medicine is full of exciting tales glorifying the impact of natural products chemistry in drug discovery. In general, natural products show high selectivity

and affinity for their specific biological target.[20] Today much of what we know about neurochemistry and the function of the central nervous system (CNS) has been aided by the use of natural products. Many of the receptors within the CNS were discovered by studying their interactions with natural products. For example, research on opium alkaloids from *Papaver somniferum* guided researchers to the discovery of the opioid receptors and has helped to elucidate the mechanism of antinociception mediated by these receptors. In this case, ethnopharmacological knowledge has aided the identification and study of the terrestrial natural product morphine.[21] Another inspiring story for natural product research is the discovery of ivermectin and artemisinin. In 2015, the Nobel prize in Physiology and Medicine was awarded to William C. Campbell together with Satoshi Ōmura "for their discoveries concerning a novel therapy against roundworm infections" and to Tu Youyou "for her discoveries concerning a novel therapy against malaria".[22]

Both ivermectin (**1.11**) and artemisinin (**1.12**) were discovered when researchers were searching nature for the arsenals to fight helminthiasis and malaria, respectively. Ivermectin resulted from a collaborative effort by Campbell and Omura from Merck Research Laboratories and Kitasato Institute, respectively, where they searched microorganisms for compounds with the anthelmintic property. Both Campbell and Omura were motivated by their philosophy about natural product chemistry. Campbell believed in nature's ability to produce compounds beyond human imagination, and Omura considered

microorganisms and microbial compounds as "splendid gifts."[18] During the COVID-19 pandemic, ivermectin draws significant attention from clinicians when researchers from Royal Melbourne Hospital and Monash University published the in vitro efficacy of ivermectin in the inhibition of SARS-CoV-2.[23]

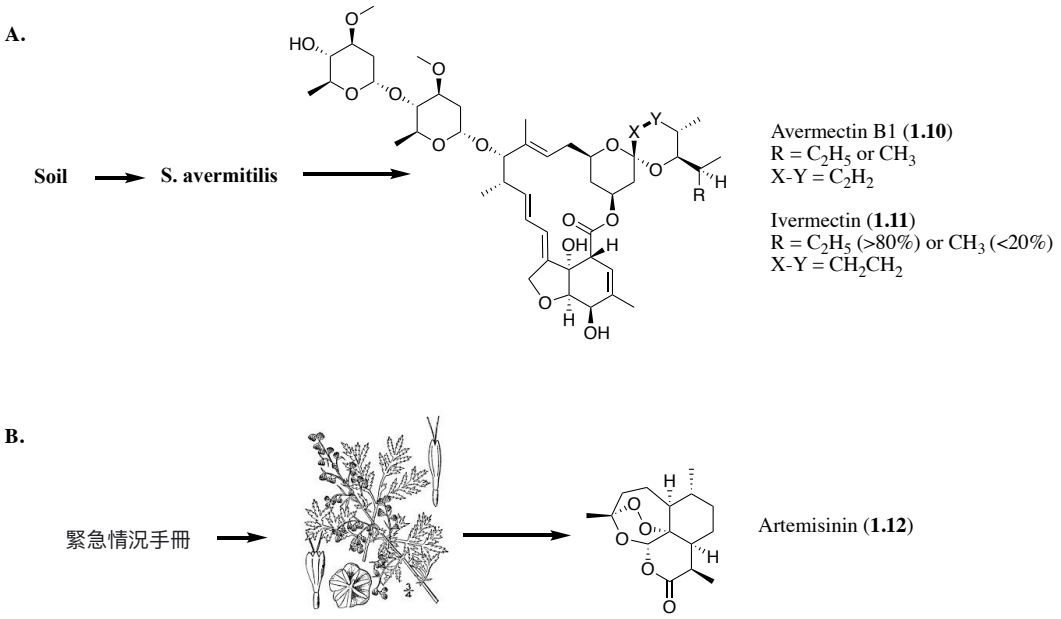


Figure 1.2: Discovery of Avermectin and Artemisinin. (Image recreated and modified from reference 18 by ChemDraw 18.1. (**A**) avermectin-producing *S. avermitilis* was isolated from soil, from which avermectins were discovered and later developed into ivermectin (**1.11**).
(B) The ancient Chinese medicine "Handbook of Prescriptions for Emergencies" inspired the discovery of artemisinin (**1.12**) from *A. annua* L. plants.

Tu Youyou was a phytochemist and the head of the malaria research group at the China Academy of Chinese Medical Sciences. Belief in the wisdom of traditional Chinese medicine inspired her to discover artemisinin. She described artemisinin as "the gift from Chinese medicine." Awarding Nobel prizes to natural product researchers certainly reestablishes the hope among natural product chemists to thrive in this field. With the

advancement in other areas of sciences like genomics, synthetic biology, metabolomics, bioinformatics, and analytical techniques, which could aid in exploring bioactive NP, researchers are now anticipating an imminent golden age of NP drug discovery.[18, 24-25] This will undoubtedly motivate young investigators who want to pursue their research career in natural product chemistry.

1.2. Marine natural products

The marine environment is the sanctuary of numerous organisms. They survive in diverse and hostile surroundings by producing an abundance of secondary metabolites with various bioactivities.[26] In comparison with terrestrial sources, there are very few ethnopharmacological reports about marine source organisms. A few notable examples include red algae *Chondrus crispus and Mastocarpus stellatus* as the remedy for colds, sore throats, chest infections, kidney trouble and burns, and red algae *Porphyra umbilicalis* for relieving indigestion. The species being investigated for bioactive marine natural products (MNPs) include marine microorganisms (bacteria, fungi, cyanobacteria, dinoflagellates), algae, sponges, cnidarians, bryozoans, molluscs, tunicates, echinoderms, mangroves, and other intertidal plants and microorganisms.[27] Despite covering two-thirds of the earth's surface, extensive exploration of the marine environment as a potential source of medicine started only in the mid-1970s aided by the introduction of SCUBA. Thousands of structurally unique bioactive metabolites have been reported from the marine

environment since then.[6] More than 32,000 articles have been indexed in the comprehensive database dedicated to marine natural product (MNP) research, MarinLit, after its establishment in 1970 by Professors John Blunt and Murray Munro at the University of Canterbury, New Zealand.[28] Natural Product Atlas, another online database of microbially derived natural product, lists 9764 compounds from the bacterial origin and the cyanobacterial genus *Lyngbya* is the fourth highest producer in their database of natural products among all bacterial genera.[29] Bioactivities of MNPs cover a diverse range of diseases including antibacterial, antifungal, antiviral, antiparasitic, antitumor, anti-inflammatory, antioxidant, and immunomodulatory activities.[30-32] Apart from providing bioactive molecules, MNPs have started to find their application in therapeutics. As of November 2019, ten MNPs or MNP derived natural products are approved by the FDA (**Figure 1.3**). Of these, two are the original natural product as isolated, and the remaining compounds are synthetic agents with origin related to MNPs.[33]

Cytarabine (Ara-C; **1.13**), an anticancer agent, is the first FDA approved marine derived drug. A similar analog is the antiviral vidarabine (Ara-A; **1.14**) and both are synthetic analogs of a marine sponge derived nucleoside spongouridine. The NP was isolated from the Caribbean sponge *Tethya crypta* (Tethylidae).[34] The compound intrigued researchers when they observed the free form of this nucleoside outside the nucleus, though their natural analogs, purines, and pyrimidines, are bound to DNA and RNA. This

observation led to the idea that the sponge uses this compound for chemical defense. Vidarabine was the first marketed antiviral agents. Though vidarabine is clinically useless now because of poor solubility and toxicity, and the development of safer antivirals,[35] cytarabine is still in the market. In fact, the latter is the best approach (7+3 protocol) currently available to treat acute myelogenous leukemia, where the patient is treated with cytarabine for seven days continuously *via* intravenous administration. The first three days of the treatment also use intravenous daunorubicin, hence the nickname "7+3" protocol.[36]

Twenty years after the approval of those nucleosides, the FDA approved Ziconotide (**1.15**), a synthetic form of ω-conotoxin MVIIA. Ziconotide is one of the three approved analgesics that act on N-type calcium channels ($Ca_V2.2$). This drug is instrumental in managing severe chronic pain associated with cancer and neuropathies. MVIIA was isolated from *Conus magus* in 1979. It is a 25 amino acid linear peptide, containing three disulfide crosslinks, that inhibits $Ca_V2.2$.[21] The inspiration behind this discovery was the findings that cone snail venoms are lethal and that they use their venom for hunting fish. Extensive research led to the discovery of several biologically active conotoxins, most of which potently target ion channels to exert their biological action.[37]

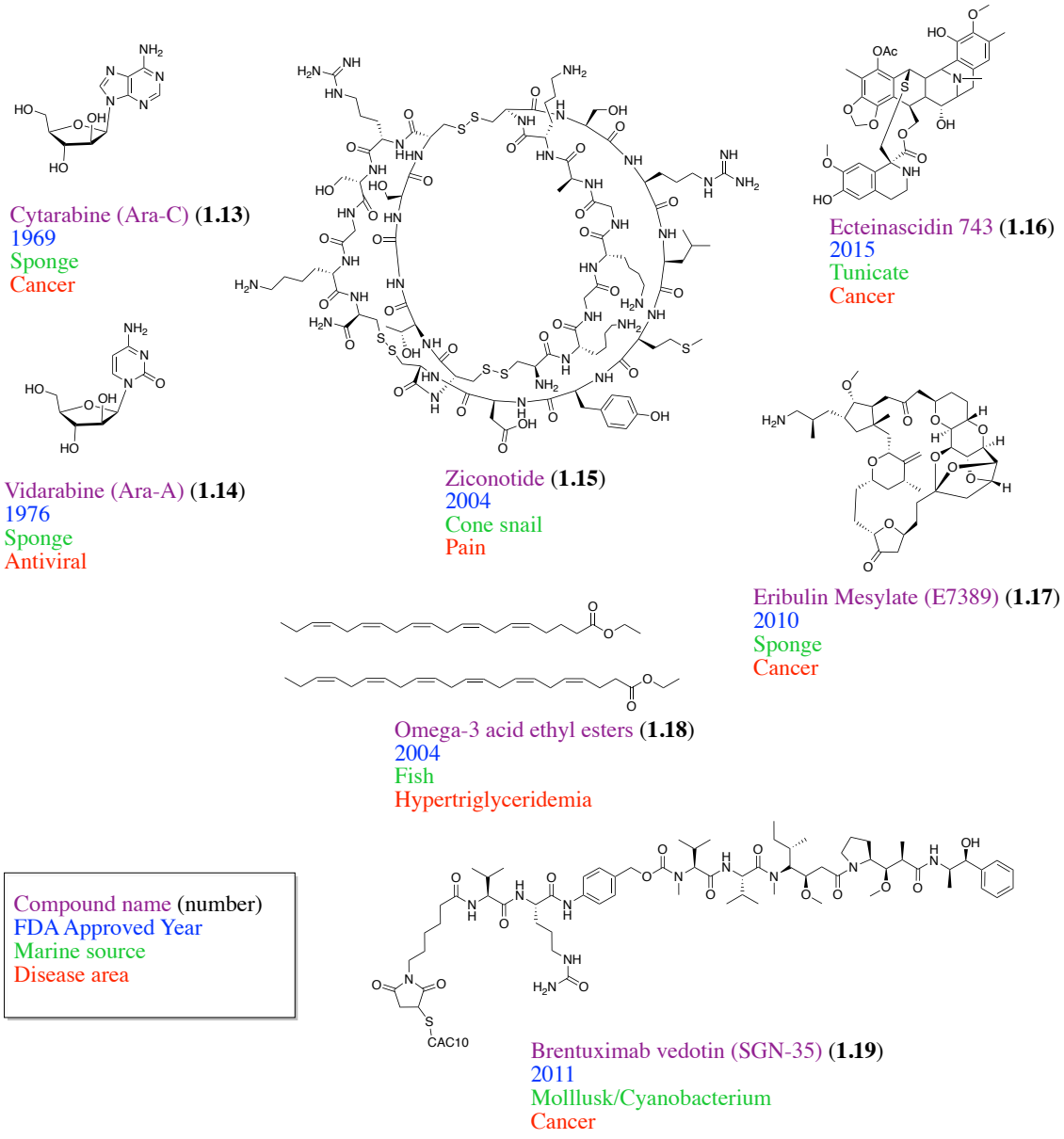


Figure 1.3: FDA approved MNPs or MNP derived natural products

The first European Union approved marine anticancer agent is ecteinascidin 743, also known as trabectedin or ET-743 (**1.16**). It was isolated from the Caribbean tunicate *Ecteinascidia turbinate* and has been used for the treatment of ovarian cancer and soft tissue sarcoma.[38] Ecteinascidin is a tetrahydroisoquinoline alkaloid and is currently produced synthetically. It is a DNA alkylating agent, and unlike traditional alkylating agents, it binds to the minor grove of DNA.[39]

A structurally simpler version of the polyketide halichondrin B resulted in the anticancer agent eribulin mesylate (**1.17**). Halichondrin B was isolated from the marine sponge *Halichondria okada*. The total synthesis of Halichondrin B by the Kishi laboratory allowed researchers to explore many of its analogs, which resulted in the discovery of eribulin.[40] Both eribulin mesylate and halichondrin B act *via* tubulin inhibition by binding at the vinca site of β-tubulin.[41]

Omega-3 fatty acids from marine fish are well known for their lipid-lowering property and are used in combination with diet and/or other lipid-lowering agents.[42] The marketed drug Lovaza contains a combination of the ethyl esters of eicosapentaenoic acid and docosahexaenoic acid (**1.18**). Anchovies, herring, salmon, mackerel, smelts, and jacks from the South Pacific Ocean are commonly the sources of these oils used to make commercial products.[41]

The most recently approved anticancer drug related to MNP is brentuximab vedotin (**1.19**). It is an antibody-drug conjugate, where the drug part is the pentapeptide monomethyl auristatin E (MMAE) (**1.21**), a derivative of the MNP dolastatin 10 (**1.20**). Dolastatin 10 (**1.20**) has potent antitumor activity and was initially isolated from the sea hare *Dolabella auricularia*.[43] Interestingly, the origin of dolastatin 10 was later identified as the sea hare's cyanobacterial diet, *Symploca hydnoides*.[44] A detailed discussion about compound **1.20** and **1.21** is described in section 1.2.1.

In the first 35 years of marine drug discovery, only four products reached the market. However, within the next 15 years, the number of approved drugs reached ten. In addition to these, 27 more compounds are at different stages of clinical trials. The most prevalent indication for these compounds is for cancer, and others have been developed for cognition and schizophrenia, wound healing, and Alzheimer's disease.[33, 36]

1.2.1. Marine cyanobacteria as the source of bioactive compounds

Cyanobacteria are a group of gram-negative prokaryotes that are capable of performing oxygenic photosynthesis, a unique ability among prokaryotes.[45] They are approximately 2.6 to 3.5 billion years old, making them one of the earliest photosynthetic organisms.[46] They can exist in various forms like unicellular, filamentous, planktonic or benthic, and colonial.[45] Cyanobacteria are the most ubiquitous photosynthetic organisms,

and they can be found almost everywhere, i.e., marine, freshwater, and terrestrial environments.[47]

Researchers considered cyanobacteria as a potential source for bioactive NP, when it was discovered that freshwater genera *Nostoc* had been used long ago to treat gout, fistula, and several other diseases. The field of marine cyanobacterial drug discovery became prominent became emerging since the 1990s after the extensive exploration initiated by RE Moore and WH Gerwick.[48] Marine cyanobacteria have drawn the attention of NP chemists because of their ready availability, rapid growth and diversity, their role in the surrounding environment, and their ability to provide highly bioactive secondary metabolites.[49] Marine cyanobacteria achieve this abundance of chemical diversity *via* complex approaches that utilize mainly polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS) as their biosynthetic tools.[50] Apart from potent toxins, marine cyanobacteria produce an abundance of exciting molecules with a wide array of activities like anticancer, antifungal, anti-HIV, antimicrobial, immunosuppressant, anticoagulant, anti-inflammatory, antiprotozoal, antimalarial, antiviral, and antitubercular activities.[51-55] Compounds exhibiting these activities are mostly isolated from the genera *Oscillatoriales, Lyngbya, Moorea*, *Okeania,* and *Caldora*.[56-57]

An analysis of all MNPs and related compounds, approved by the FDA and in clinical trials,[33] revealed that more than half of them (57%) have marine cyanobacteria as

their source organism (**Figure 1.4A**). Among the ten products already approved (**Figure 1.4B**), one-third of them have links associated with marine cyanobacteria. Whereas two-third of the MNPs and related compounds in clinical trials come from marine cyanobacteria. This statistic is quite encouraging as among a multitude of marine sources, a single class of organisms, cyanobacteria, promises a majority of products with the potential to reach the clinic.

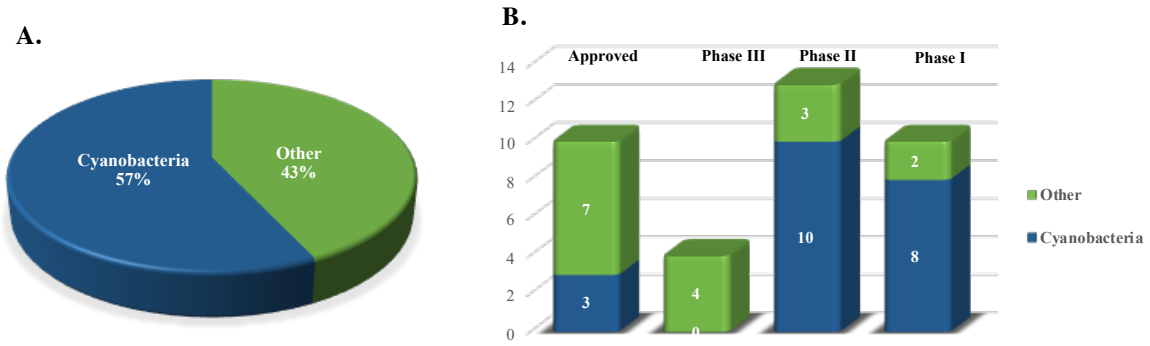


Figure 1.4: FDA approved marine drugs: cyanobacteria vs. others (Plots are generated based on the data given in reference 33)

The marine cyanobacterial compound that has attracted the most attention is dolastatin 10 (**1.20**). Though this compound is not directly used in clinics, it has inspired the development of monomethyl auristatin E (MMAE) (**1.21**) and monomethyl auristatin F (MMAF) (**1.22**) which are the active warheads of several antibody-drug conjugates (**Figure 1.5**). Some of these conjugates are already clinically approved and/or in clinical trials. Dolastatin 10 was fist isolated in 1972 from the hare *Dolabella auricularia* in the

Indian Ocean. Petit *et al.*[58] found that extracts obtained from that marine organism were very effective against the National Cancer Institute's (NCI) murine P388 lymphocytic leukemia cell line. Fifteen years of continuous work led to the isolation and structure determination of the unique linear pentapeptide Dolastatin 10. This extremely potent cytotoxic compound was isolated in an extremely low yield (10^{-6} to 10^{-7}%) from the sea hare. However, Luesch *et al.*[44] discovered dolastatin 10 from the Palauan marine cyanobacterium Symploca sp. VP642, thus building up the rationale that a cyanobacterium is the original producer of this compound. Dolastatin 10 ended up in the sea hare as a result of the consumption of cyanobacteria by the animal as food. Dolastatin 10 exhibits its anti-tumor effect by disrupting the microtubule assembly.[59] It also inhibits BCL-2 protein *via* phosphorylation and induces apoptosis.[60] Attempts to explore the SAR of dolastatin 10 resulted in several useful bioactive analogs, most notably monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF), where the C-terminus dolaphenine moiety of desmethyl dolastatin 10 was replaced with phenethylamine (**Figure 1.5**).[61]

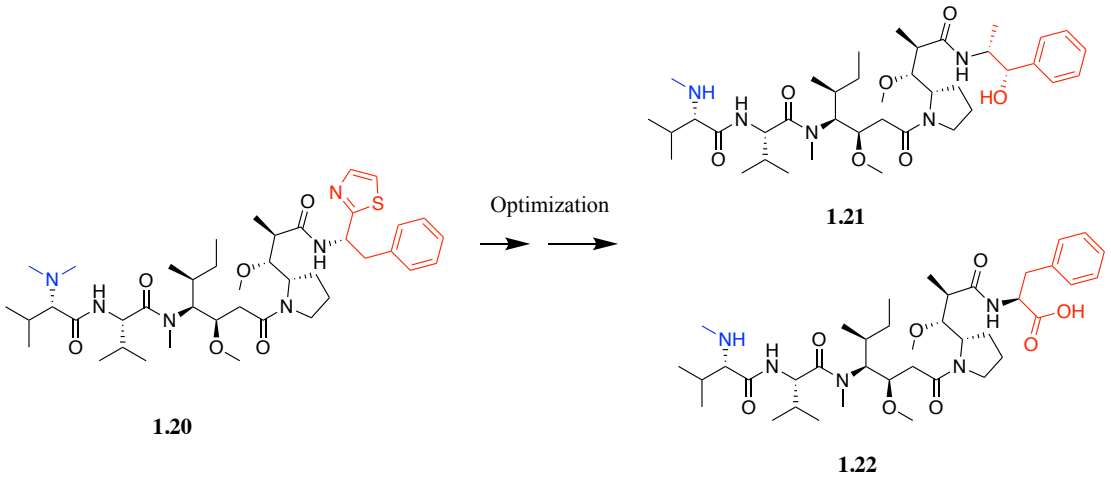


Figure 1.5: Development of MMAE (**1.21**) and MMAF (**1.22**) from dolastatin 10 (**1.20**)

The first mAb-linked auristatin derivative is brentuximab vedotin (**1.13)** by Seattle Genetics where MMAE (**1.21**) is linked to the chimeric anti-CD30 antibody CAC10 (SGN-30) by a valine-citrulline linker and a self-immolative spacer, *p*-aminobenzyloxycarbonyl (PABC).[62] The stable dipepetide, valine-citrulline, is readily cleavable by the lysosomal enzyme cathepsin B. The spacer PABC facilitate the proteolysis by keeping the drug away from the proteolytic site.[63] Once the peptide is cleaved by the cathepsin B, the PABC group rapidly cleaved *via* 1,6-elimination and released the MMAE in the tumor microenvironment (**Figure 1.6**).

Besides anticancer molecules, marine cyanobacteria possess compounds with many other activities ranging from targeting mammalian ion channels and GPCRs to parasitic

macromolecules. Notable antimalarial compounds from marine cyanobacteria are discussed in section 1.3.1.2.

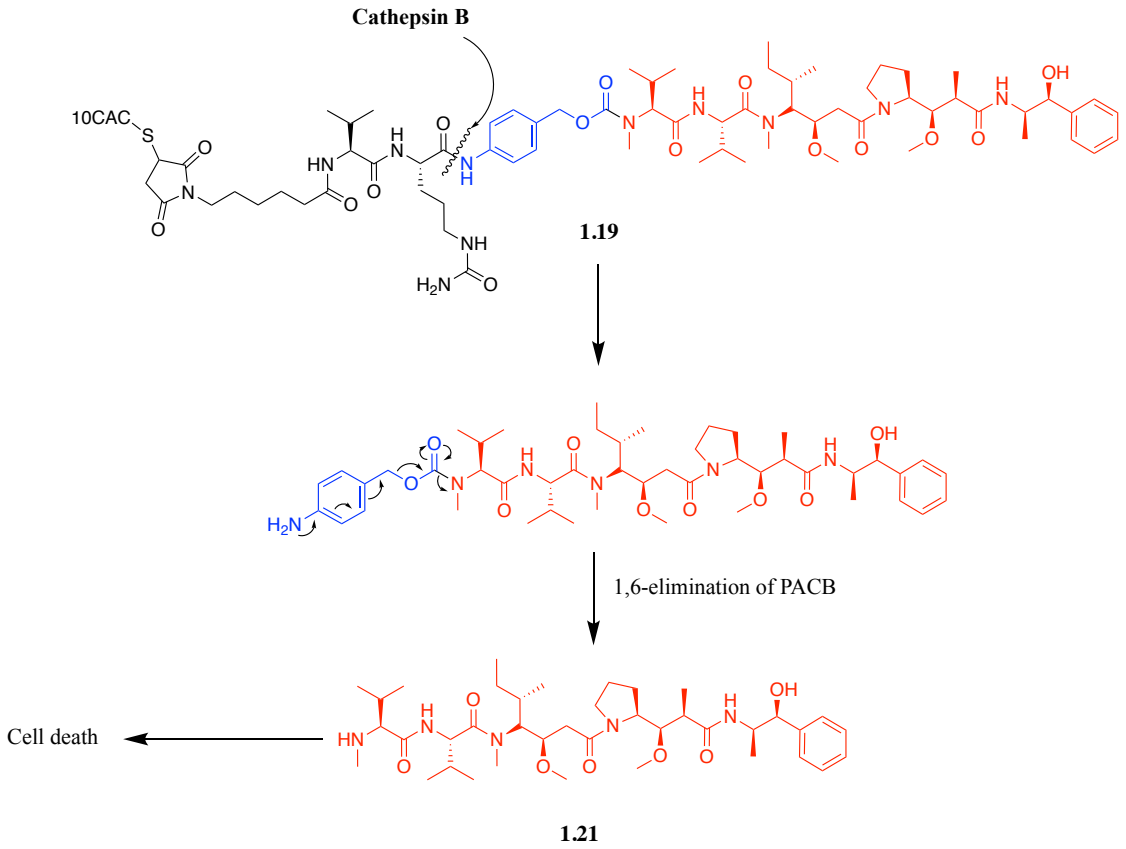


Figure 1.6: Mechanism of Action of Brentuximab vedotin (**1.19**).

1.3. Tropical diseases

According to the World Health Organization (WHO), the diseases that occur mostly in tropical regions are called tropical diseases, which are common infectious diseases that include but not limited to malaria, leishmaniasis, schistosomiasis, onchocerciasis,

lymphatic filariasis, Chagas disease, African trypanosomiasis, and dengue. These diseases generally involve a non-mammalian vector that carries the causative organism (parasite, bacteria, or virus) and transmits the disease to humans upon biting. These diseases affect more than 1 billion people, mainly the most impoverished population of the developing world living in places infested heavily by the infective vectors. Many of these tropical diseases do not draw much attention from the developed world because of the poor socioeconomic condition of the affected population,[64] authentic data unavailability, and difficult to pronounce the name and hence called neglect tropical diseases.[65]

Discussion on all the tropical diseases is beyond the scope of this chapter. I will focus on malaria and Chagas disease (American trypanosomiasis) as our drug discovery effort resulted in the discovery of a marine cyanobacterial metabolite with activity against parasites for those diseases.

1.3.1. Malaria

Malaria is a life-threatening vector-borne disease caused by five species of *Plasmodium* parasites that are transmitted *via* the bites of infected female Anopheles mosquitoes. Among the five species, *P. falciparum* and *P. vivax* are the most dangerous. *P. vivax* is predominant in the Americas, whereas P. falciparum rules the rest of the world.[66]

Malarial patients typically manifest fevers, sometimes associated with chills, headache, and other febrile symptoms. Once an infected female Anopheles mosquito bites

someone, the parasites pass to the human host, reproduce *via* sexual reproduction, and initiate the pre-erythrocytic phase by infecting the liver (**Figure 1.7**). There they develop into schizonts and rupture hepatic parenchymal cells to release merozoites. Then merozoites enter the erythrocytic phase and multiply *via* asexual reproduction to produce and release more merozoites. The clinical manifestations of malaria appear in this erythrocytic stage.[67] It is difficult to recognize the early signs of malaria, which are often mild, and this makes it a difficult disease to treat. The disease usually progresses to severe conditions and may be fatal if not treated within 24 hours. Multiorgan failure is common in severe cases in adults, whereas severe anemia and respiratory distress are common in children.

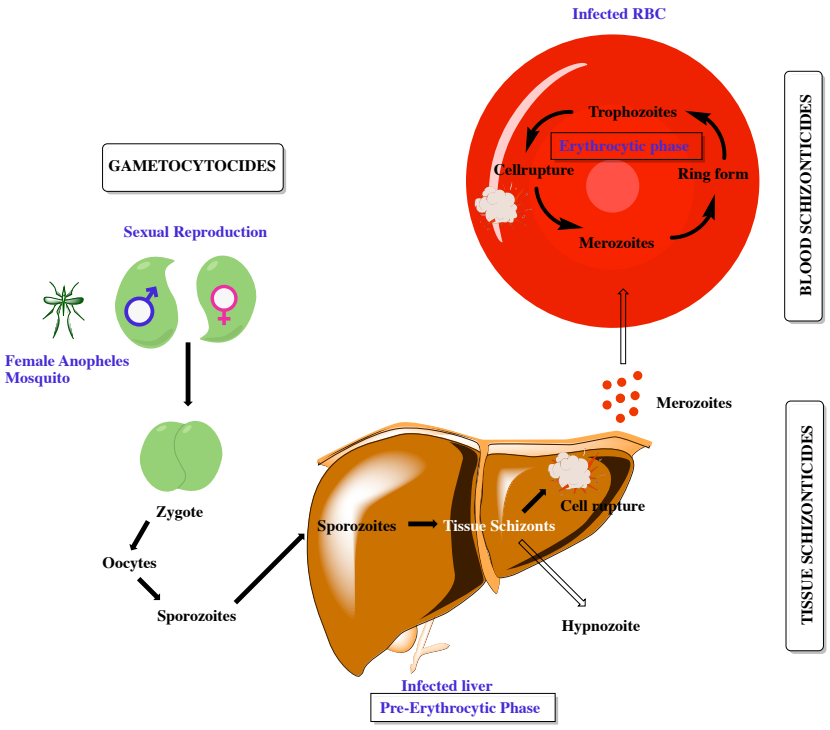


Figure 1.7: The life cycle of malaria parasite (Recreated and modified by ChemDraw 18.0 from Kumar *et al*.[67])

Despite clear understanding of the diseases, vectors, and parasites, malaria is still one of the major fatal illnesses of the world. It is the 6th leading cause of death in low-income countries.[68] The burden of malaria on the US is small, about 2000 cases each year, according to the CDC,[66,] but some parts of the world are suffering heavily, especially Africa. According to the World Malaria Report 2019, published by WHO,[69] every year of the last decade has seen more than 200 million patients worldwide (**Figure 1.8**).

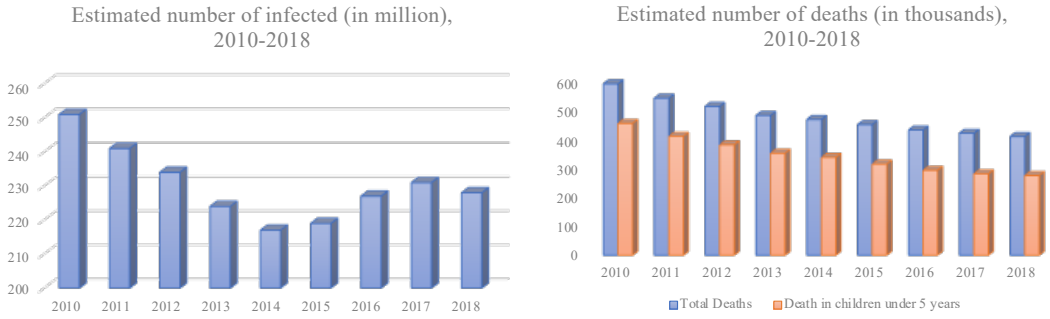


Figure 1.8: Total infected and total deaths due to malaria from 2010-2018. (Plots are generated on the basis of WHO World Malaria Report 2019.[69])

Though mortality due to Malaria is reducing every year, the number is still high, a staggering 405,000 deaths in 2018. Ninety-five percent of those patients belong to Africa and India. Malaria is deadlier for children under five years old, and they constituted 67% (272,000) of total deaths in 2018. What is not visible from the chart is, the primary contributor to child mortality is the premature delivery of babies with low birth weight due to maternal malaria. I belong to the Indian subcontinent, and that region of the world is at

a high risk of malaria. I have seen how poor hygiene, lack of sanitation, and inefficient public health policies make that region an excellent breeding ground for mosquitoes, and aid in spreading of malaria, and other mosquito-borne diseases like Dengue fever and Chikungunya. Approaches to control malaria focuses on managing the vector and also providing effective therapy for patients, which is particularly important because of the high number of deaths worldwide. It is imperative to say death by malaria has reduced with the discovery of artemisinins. Still, researchers are skeptical because of the chance of emergence of resistant strains against artemisinins and occurrences of reinfection because of the inability to eradicate malaria vector in poor countries. It is possible in the near future that the world will be helpless without new agents in hand.[70] It emphasizes the urgency of new antimalarial drug development.

1.3.1.1. Antimalarial agents

Based on their structural class and mechanism of action, commercially available antimalarial drugs (**Figure 1.9**) can be broadly classified into three groups:[71-72]

1. Quinoline derivatives/aryl amino alcohol compounds: quinine (**1.4**), mefloquine (**1.23**), halofantrine (**1.24**), lumefantrine (**1.25**), chloroquine (**1.26**), amodiaquine (**1.27**), cyloquine (**1.28**), primaquine (1.29)
2. Antifolate compounds: proguanil (**1.30**), pyrimethamine (**1.31**), trimethoprim (**1.32**).

3. Artemisinin compounds: artemisinin (**1.12**), artesunate (**1.34**), artemether (**1.35**), arteether (**1.36**)

In section 1.1, a discussion has been made about artemisinin derivatives, which are the most effective antimalarials currently used in several combination therapies. Interestingly, the first chemotherapeutic agent effective against malaria, quinine (**1.4**), was also isolated from a natural source, the bark of the cinchona tree in 1820. The plant cinchona contains other quinoline alkaloids like quinidine, cinchonine, and cinchonidine, which are also effective against malaria. Quinine was the first successful example of a chemical compound to treat an infectious disease.[73] Due to the high toxicity associated with quinine therapy, new molecules were developed to control malaria. The first synthetic antimalarial drug, methylene blue (**1.33**), synthesized by Paul Ehrlich in 1891, was used in the late 19th and early 20th centuries against all types of malaria. Methylene blue was used widely because the then primary medicine, quinine, was in limited supply as it could only be obtained from the natural sources. Methylene blue was later discontinued because of the discovery of safer agents. However, it recently gained interest from malaria researchers because of its effectiveness in combination chemotherapy.[74]

Due to concurrent hepatic and erythrocytic episodes in the same patient and unavailability of a single agent to kill both tissue schizonts and blood schizonts, antimalarial drugs are used in combination. These drugs target different signalling

pathways of the *Plasmodiam* paracite. For example, quinnine and most of the quinoline alkaloids are lethal to blood schizonts only and inhibit them by binding with the toxic heme (ferriprotoporphyrin IX). Heme is the digestive end product of hemoglobin which is sequestered by histidine rich protein and lipids to insoluble, chemically inert hemozoin. Drug binding to heme resulted in the prevention of sequestration. The resulting heme buildup causes oxidative damage to membranes, digestive proteases, or other critical biomolecules of paracites. Drugs like proguanil, pyrimethamine and trimethoprim selectively inhibit *Plasmodium* dihydrofolate reductase (DHFR) and thereby inhibit the biosynthesis of nucleic acid.

Also, some highly potent antimalrial drugs', like primaquine (acts against primary and latent hepatic stage), mechanism of action is poorly unnderstood. One hypothesis about primaquine is that it converts to highly reactive oxidative inntermediates to exert toxicity or may inhibit protozoal electronn transprot chain.[75]

Several natural and synthetic molecules have been developed after the discovery of quinine, but the emergence of resistant parasites makes their use restricted to particular situations. The threat of resistant pathogen emergence will eliminate the success of the malaria control program achieved so far.

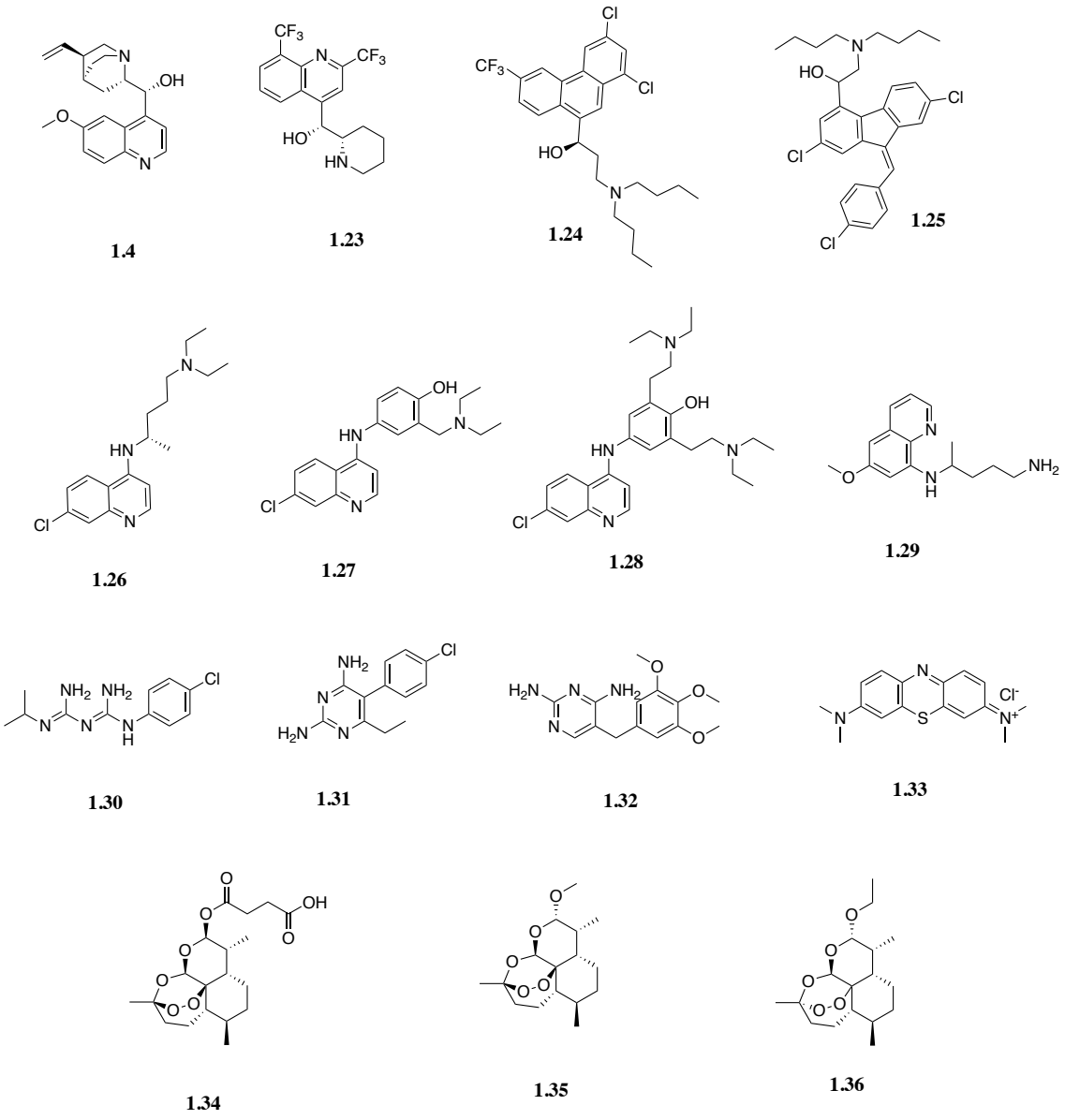
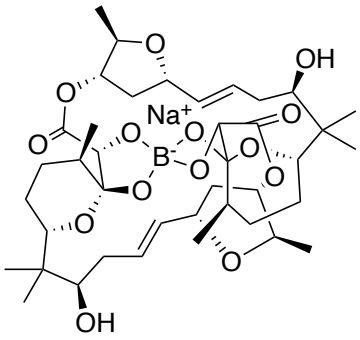


Figure 1.9: Commercially available antimalarial drugs.

1.3.1.2. Antimalarial agents from marine cyanobacteria and other marine sources

All antimalarials isolated from marine sources can be broadly classified into five categories: (i) isonitrile containing derivatives; (ii) alkaloids; (iii) endoperoxides; (iv) peptides, and (v) quinones and phenols.[76] The first marine antimalarial drug discovered was aplasmomycin A (**1.37**). It was isolated from a strain of *Streptomyces griseus* found in shallow sea sediment in Sagami bay. Among marine antimalarial compounds, manzamines (complex polycyclic (7–8 rings or more) alkaloids) are the most potent antimalarials comparable to currently available drugs.[77]



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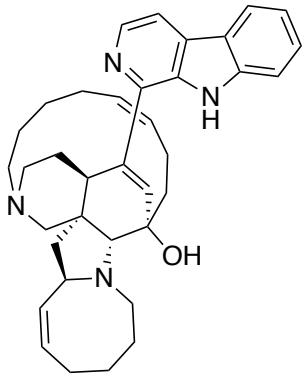


1.38

Figure 1.10: Aplasmomycin A (**1.37**) and Manzamine A(**1.38**)

Since the discovery of manzamine A (**1.38**), the first compound from this class isolated from an Okinawan sponge in 1986, more than 60 manzamines have been reported. They were isolated from taxonomically unrelated sponges of different genera and orders.

This finding leads researchers to speculate that manzamines have a symbiotic origin. Later *Micronosphora* sp was identified as a manzamine producing bacteria.[76] Besides antimalarial activity, manzamines are also known to possess anti-inflammatory, antifungal, antibacterial, and antitubercular activity and activity against AIDS opportunistic pathogens. [78-81]

An analysis of compounds[82-84] isolated from marine cyanobacteria in the 21st century revealed that the majority of the antimalarials isolated from marine cyanobacteria are peptides (**Figure 1.11**). In the linear depsipeptide series, gallinamide A (**1.39**) was isolated from a *Schizothrix* species obtained from rocky reef near Piedras Gallinas near Portobello on the Caribbean coast of Panama.[85] *Moorea producens* is known to produce the antimalarial lipopeptides, named dragonamides A (**1.40**), B (**1.41**) and E (**1.42**), carmabin A (**1.43**) and dragomabin (**1.44**).[86-87] Among cyclic peptides, lagunamides A-C (**1.45 - 1.47**) from *Lyngbya majuscula* and venturamides A-B (**1.48 – 1.49)** from *Oscillatoria* sp. were found to be active against malaria parasites.[88-90] Shao *et al.*[91] discovered a unique polyhydroxy macrolide with a 40 membered lactone ring from *Okeania hirsute*, collected from the Caribbean coast of Panama. The compound, Bastimolide A (**1.50**), showed activity against four resistant strains of *Plasmodium falciparum*.

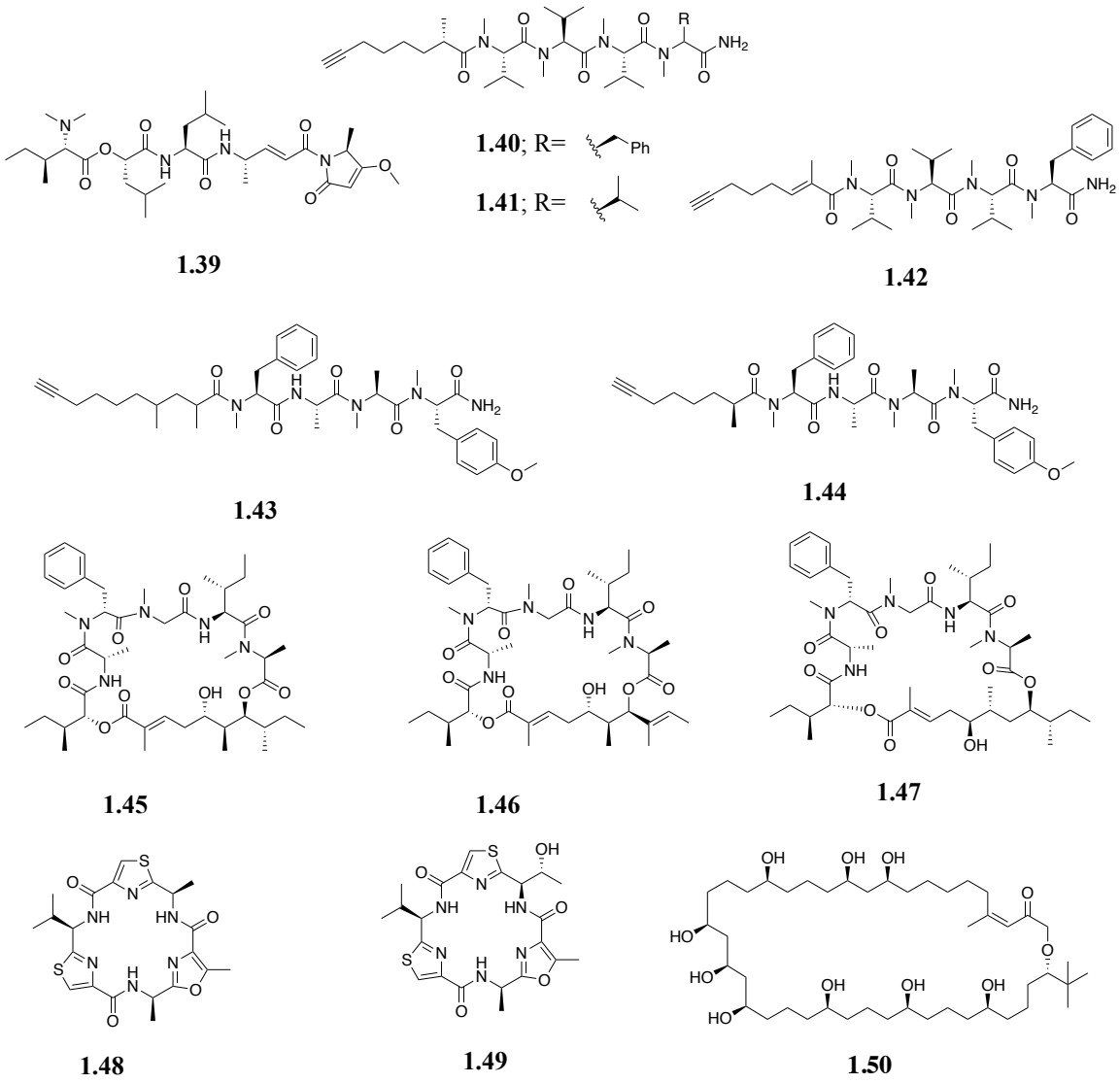


Figure 1.11: Antimalarial drugs from marine sources.

Clearly, antimalarial drug discovery is downplayed in comparison to other drug discovery campaigns. Drug exploration for potential antimalarial lead is extremely important to develop new drugs that could overcome the problem of resistance emergence, the high toxicity of most commonly known drugs, and to provide insights about discovering new mechanisms to target the pathogens. Marine natural products came out as

a promising source of antimalarial lead discovery and provided compounds with nanomolar potency (IC_{50}) against the malaria parasite. For example, manzamine A has IC_{50} values of 8.2 nM and 15 nM against chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains, respectively.[77] Overall, antimalarial drug discovery form marine natural products has potential for eliminating this deadly disease from this world.

1.3.2. Chagas disease

Chagas disease (CD), also known as American trypanosomiasis, is named after the discoverer of the illness Carlos Chagas and is caused by the parasite *Trypanosoma cruzi*. It is transmitted to animals and people by triatomine bugs (or "kissing bug") and is endemic in the poverty-affected rural Latin America.[92] Once an infected bug bites a human, it releases trypomastigotes in its feces which then enters the human host through the injury or mucosal membrane. After transforming into intracellular amastigotes, followed by multiplication by binary fission, they are released from the infected cell into the blood as trypomastigotes. From there, they can infect other cells or may be transferred to another uninfected kissing bug when it takes a blood meal (**Figure 1.12**). Clinical manifestations appear when the trypomastigotes burst out of the cells into the blood.

The disease propagates in two phases. The acute phase may be asymptomatic in most cases. However, some patients may show acute symptoms like fever, headache, skin lesions, unilateral palpebral edema, lymphadenopathy, hepatosplenomegaly, pallor,

breathing difficulty, and abdominal or chest pain.[93-94] The acute phase resolves spontaneously within 4-8 weeks, and at this point, the number of circulating parasites in the blood drops at a level undetectable by microscopy. If untreated, the patient enters the chronic phase without any sign or symptoms. Decades after the onset of acute infection, an average of 20% of patients suffer severely due to cardiomyopathy, or megaesophagus, or megacolon. The cardiac involvement is more frequent than gastrointestinal involvement. CD is the principal cause of infectious cardiomyopathy.[95]

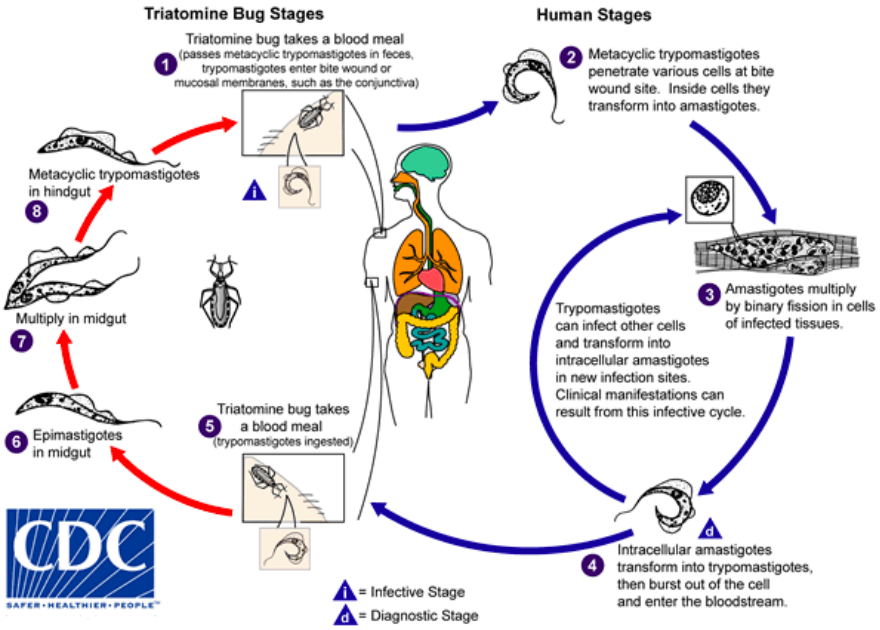


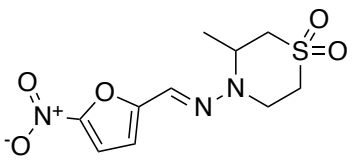
Figure 1.12: The life cycle of *T. cruzi* (obtained from CDC; labeled for non-commercial reuse).[92]

CD is prevalent over a wide geographical area of the Americas, covering the southern USA, Central America to the north of Argentina, and Chile. The CD control

programs took several initiatives like vector control programs, blood bank screening, and in some countries, congenital CD screening programs that lower the burden effectively.[96] There was a three-fold decrease in the total number of infected people from the 1980s to 2010. Simultaneously, the death rate decreased as well. Though the overall picture is a success story, there are pockets in these regions where transmission has increased.[94] Drug discovery research for CD seriously lacks the attention of the scientific community. CD is one of the most neglected among NTDs. It is evident from the fact that there are only two drugs in the market, and people have to rely on them despite their toxicity.

1.3.2.1. Drugs for Chagas Disease and marine natural products:

Nifurtimox (**1.51**) and Benznidazole (**1.52**) are the currently available medicines used to treat CD. Nifurtimox was introduced in the late 1960s to treat CD. In the US, it is regulated by the CDC only under investigational protocol and is not approved by the FDA. Whereas benznidazole received FDA approval in 2017.



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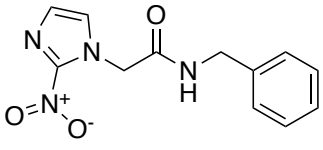
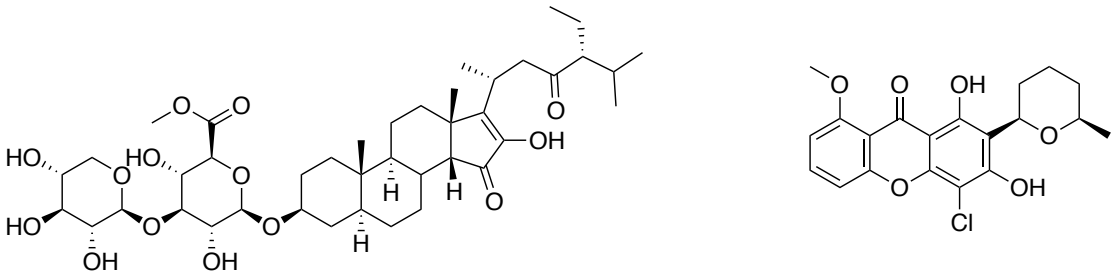


1.52

Figure 1.13: Clinically approved CD drug

The mechanisms of action of these drugs are different. Nifurtimox inhibits the pyruvic acid synthesis and thus kills *T. cruzi* by inhibiting carbohydrate metabolism. Benznidazole is a prodrug, which becomes activated by the parasite enzymes and the active metabolites inhibit glutathione and trypanothione of *T. cruzi*.[96]



1.53 **1.54**

Figure 1.14: Pandaroside G (**1.53**) and Chaetoxanthones C (**1.54**)

A review on trypanocidal marine natural products published by Jones *et al.*[97] classified anti-CD compounds into four subclasses: (i) terpenes; (ii) polyketides and xanthones; (iii) alkaloids and (iv) peptides. The most potent marine natural product reported was pandaroside G (**1.53**; *T. cruzi* IC_{50}=0.77 μM), which was isolated from the Caribbean sponge *Pandaros acanthifolium.*[98] Unfortunately, the molecule lacks selectivity since it also inhibited mammalian L6 cells (IC_{50}= 0.22 μM). The most active and selective marine compound reported against *T. cruzi* was chaetoxanthones C (**1.54**; IC_{50}= 38 μM; selectivity index of 31), a heterocyclic-substituted xanthone analog, isolated from the

marine-derived fungus *Chaetomium* sp.[97] Considering the need for new and safer therapeutic agents for CD, a more extensive search should be conducted exploring all viable sources. Marine sources are very promising in this regard, as they are known to produce a multitude of compounds having antiprotozoal activity.

1.4. Conclusion

The most innovative and efficient synthetic chemistry laboratory is not located in any academic or industrial setting; it is in nature. With that inspiration, we explore oceans in search of the cure for diseases that do not draw much attention from the pharmaceutical industry. Many drug discovery stories start with the gift from nature, and then the human brain takes that lead to find cures for diseases. Lead discovery is the main objective of our natural product chemistry research. This lead molecule may help us developing a cure for neglected tropical diseases or may aid in elucidating information about a potential target.

Chapter II: Isolation, Structure Elucidation and Total Synthesis of Naranjamide

2.1 Introduction

The field of marine drug discovery came forth as an alternate source of bioactive natural products besides terrestrial compounds in the 1940s and, on an average, has provided more than 28,000 novel compounds each year.[99] From 2007 to 2016, more than 400 new natural products have been isolated from marine cyanobacteria, with 126 peptides and peptide-related structures, of which 39 are linear peptides.[56] Unlike other marine sources, harvesting cyanobacteria does not represent a threat to the environment since cyanobacteria combine the ability of rapid growth, genetic tractability, and cultivability, which make them a sustainable source of natural products.[56] Their wide range of

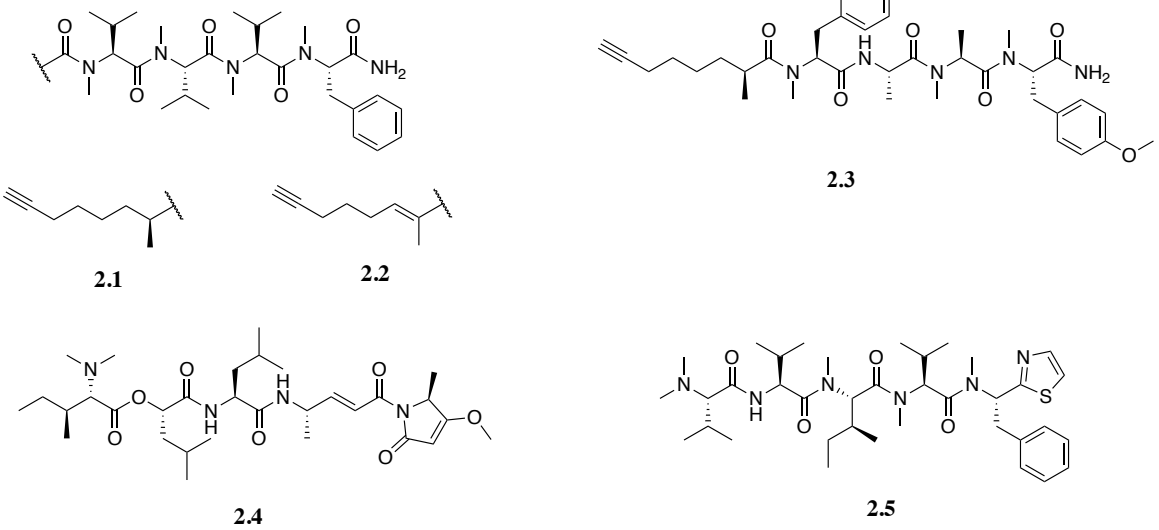


Figure 2.1: Antiparasitic compounds isolated from Panamanian cyanobacteria.

bioactivities includes, but is not limited to, anticancer, antibacterial, antifungal, antiprotozoal, antiviral, immunomodulation, and cytotoxic activities.[48]

Every year neglected tropical diseases (NTDs) impose billions of dollars of economic burden on developing countries and affect more than one billion people in 149 countries.[100] Populations living in poverty with poor hygiene as well as exposure to infectious vectors, domestic animals, and livestock are the most affected by NTDs.[101] Effectiveness of available medications is restrained by the emergence of resistant pathogens and severe side effects associated with therapy. Since these diseases do not possess a significant threat to the population of developed countries, and financial return is minimal, the pharmaceutical industry is less focused on developing new agents for these diseases.[102] In recent years, several marine cyanobacterial metabolites with antiprotozoal activity have been reported. Lipopeptides like dragonamide A (**2.1**), dragonamide E (**2.2**), and carbamin A (**2.3**), isolated from *Lyngbya majuscula*,[86-87] and the linear peptide gallinamide A (**2.4**), isolated from *Schizothrix* sp.,[85] (**Figure 2.1**) are some notable examples with antiparasitic activity. We aimed to explore Panamanian marine cyanobacteria as a potential source of bioactive compounds that can produce molecules **with** activity against NTDs.

In 2012, an orange cyanobacterial mat was collected from Portobelo, Panama. Bioactivity-guided fractionation of this cyanobacterial mat resulted in the discovery of an

N-methylated pentapeptide (**2.5**), which inhibited the growth of *T. cruzi* and *P. falciparum* parasites. Using 1D and 2D NMR (Section **4.6.3**), the structure was determined to be a linear peptide where the *N*-terminus contains an *N*, *N*-dimethyl-valine moiety, followed by a valine, *N*-methyl isoleucine, *N*-methyl valine and *N*-methyl dolaphenine at the C-terminus. MS-MS analysis confirmed the amino acid sequence of the structure. The compound was given the trivial name Naranjamide since the crude cyanobacterial mat was orange in color, and the Spanish name for the color orange is Naranja. My contribution to this project was to conduct a total synthesis of naranjamide, which also showed growth inhibition of *T. cruzi* and *P. falciparum,* and confirmed the antiparasitic potential of the compound.

This chapter describes the discovery, structure elucidation, and attempts at the total synthesis of the molecule and studies to confirm its potential as an antiparasitic compound.

2.2 Discovery of naranjamide

An orange cyanobacterium collected by hand using SCUBA in Portobelo, Panama, was extracted using a 2:1 dichloromethane: methanol mixture to yield an extract (4.2 g) and given the in-house extract code A2002 (**Figure 2.2)**. The extract was tested for its antiprotozoal activity and was found to inhibit P. falciparum growth (82.6%). The extract was then fractionated (**Figure 2.2**) by silica vacuum chromatography and the fraction

(2002H) obtained with 1:1 ethyl acetate: methanol inhibited the growth of both P. falciparum (97.5%) and T. cruzi (75%).

Further fractionation of 2002H by C18 Sep-Pak yielded the fraction 2002H4, eluting with 80% methanol in water, which showed an increment of T. cruzi growth inhibition (81.5%) while retaining P. falciparum growth-inhibiting (79.6%) activity. Fraction 2002H4 was then purified with reverse-phase HPLC to yield 0.8 mg of pure compound **2.5**.

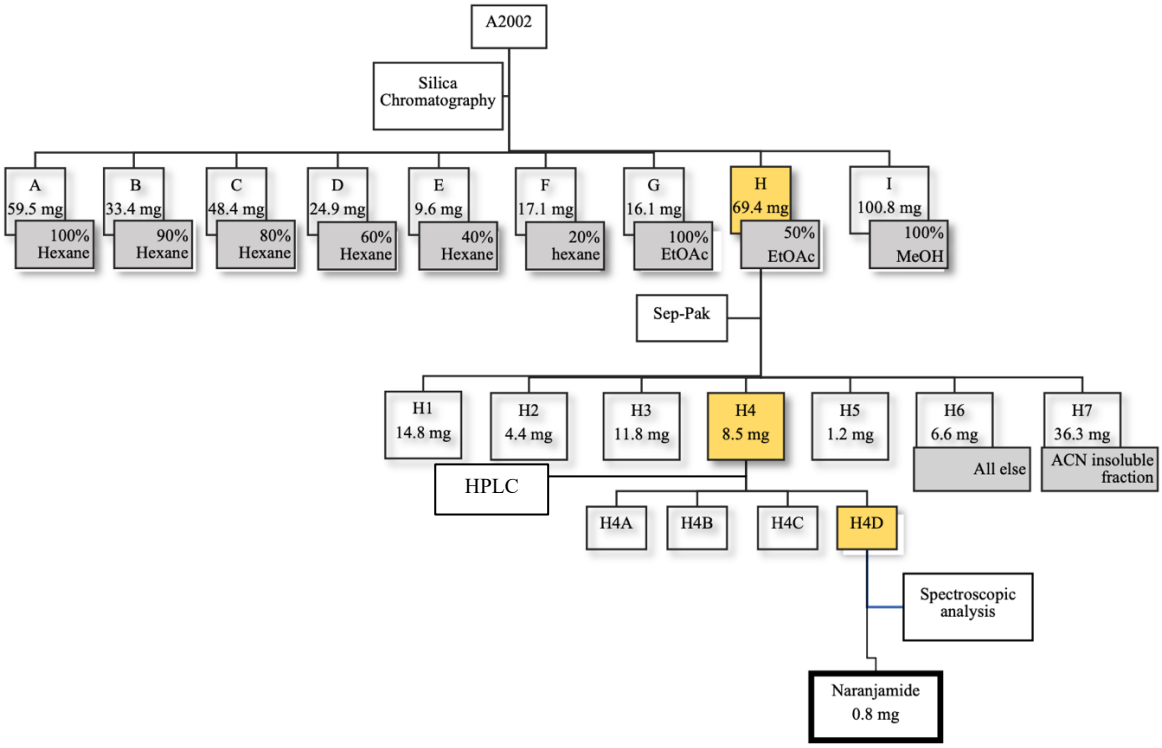


Figure 2.2: Bioactivity-guided fractionation of A2002

Naranjamide (**2.5**) was isolated as a clear solid. Its molecular formula was determined as $C_{37}H_{60}N_6O_4S$ by HRESIMS (*m/z* 685.44576 [M+H$^+$], calcd. 684.9751] (**Figure 4.1**), indicating the presence of 11 degrees of unsaturation. Following the interpretation of 2D NMR experiments (Table **2.1**), ^{1}H and ^{13}C NMR were assigned to the subunits constituting naranjamide, namely dolavaline (C-17 to C-21), valine (C-14 to N-16), *N*-methyl isoleucine (C-11 to N-13), *N*-methyl valine (C-8 to N-10) and dolaphenine (N-1 to N-7). The amino acid sequence was later confirmed by tandem MS-MS analysis. Naranjamide (**2.5**) is a linear peptide with a dolaphenine residue at the N-terminus, a common privileged structural unit of bioactive natural peptides, where the peptide chain ends at a des-carboxy thiazole functionality.[103] COSY spectrum clearly showed the correlation between the alpha proton (C-6; δ_H = 6.60) and two benzylic protons (C-6a; δ_H = 3.30 and 3.69) of the dolaphenine moiety. Corresponding HSQC correlations identified the alpha carbon (C-6, δ_C = 54.34) and the benzylic carbon (C-6a, δ_C = 36.05). Proton signals between 7.25 to 7.77 represent the phenyl and thiazole moiety of dolaphenine. Both HMBC and NOESY data correlate H_3-7a (δ_H = 2.88) with C-6 and C-8 (δ_C = 169.91) and with H-6 and H-6a, respectively. These correlations indicate an *N*-methyl amide adjacent to C-6. Multiple other *N*-methyl amide functionalities have also been observed and identified naranjamide (**2.5**) as an *N*-methylated peptide. There is only one non-methylated amide where NH-16 (δ_H = 6.95) belongs to a valine moiety.

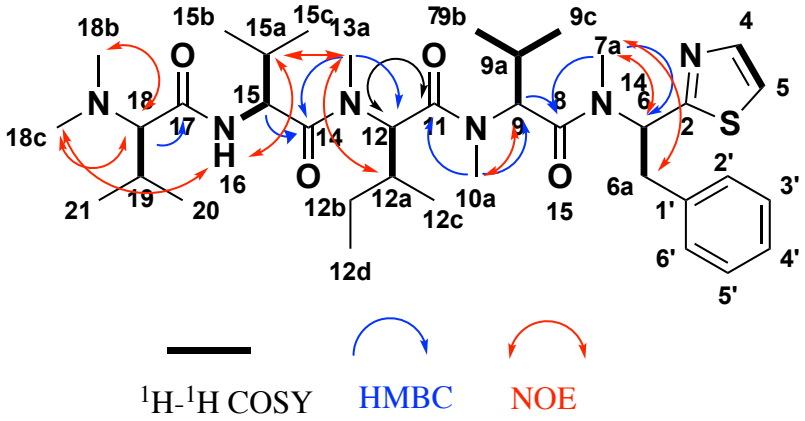


Figure 2.3: Key 2D correlations for determining the structure of naranjamide (**2.5**)

COSY and NOESY correlations (**Figure 2.3**) with NH-16 identified the alpha proton H-15 (δ_H = 4.85, δ_C = 53.32) and the beta proton H-15a (δ_H = 1.94, δ_C = 30.71), and the HMBC spectra linked H-15 and C-14 (δ_C = 172.60). This valine was identified as the penultimate residue as it was adjacent to the *N, N*-dimethyl valine residue on its N-terminal.

Proton peaks of *N, N*-dimethyl (C-18bc) were not distinguishable as a singlet because of overlapping with beta proton (9a) signals. The HSQC (C-18bc, δ_H = 2.30, δ_C = 42.79) suggested the presence of *N*-alkyl amine. From NOE data, it was seen that these alkyl protons are correlated with an alpha proton (C-18, δ_H = 2.49, δ_C = 76.50) and NH-16, thus suggesting the connection between an *N*- alkyl valine and valine moieties. The presence of *N, N*-dimethyl valine was later confirmed from fragment mass analysis (**Figure 2.4**). HMBC correlation of H-13a (δ_H = 3.04) with C-14 (δ_C = 172.60) and C-12 (δ_C = 56.34), and NOESY correlation of H_3-13a (δ_H = 3.04) with H-12a (δ_H = 2.07) and H-15a

(δ_H = 1.94) connects the penultimate valine with an *N*-methylisoleucine on the other side. The later residue was followed by *N*-methylvaline, as HMBC correlations were seen between H_3-10a (δ_H = 2.46) and C-9 (δ_C = 58.29) and C-11 (δ_C = 169.74), and NOE showed correlations of H_3-10a with H-9 (δ_H = 5.06). The HMBC correlation between H-9 and C-8 connects this *N*-methylvaline with the terminal *N*-methyldolaphenine moiety and thus completes the sequence. The alkyl side chains of amino acid residues were not distinguishable as they resonate inn close proximity. Thus MS-MS analysis (**Figure** 2.4) aided in the confirmation of the individual residues and their sequences.

In general, linear peptides with 2-10 amino acid residues are flexible.[104] N-methylation on peptide backbones confers restriction on their structure.[105] Thus, depending on the methylation pattern, part of a liner peptide can be partially flexible. The ^{1}H NMR showed a set of minor peaks along the larger peaks, especially the *N*-methyl and benzylic proton peaks were split to generate minor peaks (δ_H = 2.85, 2.92, 2.95, 3.10, 3.87). This observation made us believe in the presence of another conformer of naranjamide. Presence of a major and a minor conformer is possible since naranjamide has a flexible and a rigid portion. The flexible part, the freely rotating benzyl moiety at the C-terminal, may spatially interact with the other part of the molecule and result in a major and minor conformer. Overlapping peaks and intricate splitting patterns made it difficult to determine the multiplicity, calculate coupling constants for correlated protons, and integrate peaks to

identify the actual number of protons. However, COSY, HSQC, HMBC, and NOESY spectra helped to build correlations in each spin system, and connections were made from each spin system. To confirm the configuration of each stereocenter, a total synthesis of naranjamide (**2.5**) was attempted to compare the NMRs of both natural and synthetic naranjamide and validate its bioactivity.

Table 2.1: NMR data of Naranjamide (**2.5**).

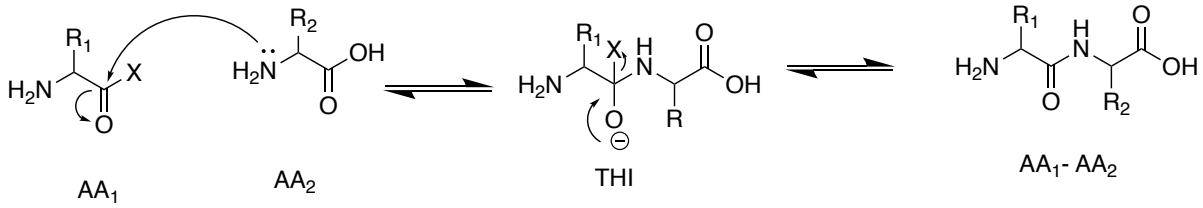
C/H No.	δ_C[a]	δ_H (multiplicity, *J* in Hz)	COSY	HMBC	NOE
2	169.18				
4	143.02	7.77 (d, 3.3)	5		
5	119.73	7.36 (d, 3.3)	4		
6	54.34	6.60 (dd, 11.5, 5)	6a	2, 6a, 7a	6a, 6b2', 7a
6a	36.05	3.69 (dd, 15, 5) 3.30 (dd, 15, 11.5)	6	6, 6b1′, 6b2′	
6b1′	136.92				
6b2′,6b6′	128.89	7.27	6b3′, 6b5′		
6b3′, 6b5′	129.24	7.39	6b2′, 6b6′		
6b4′					
7a	29.75	2.88		6, 8	6, 6a, 9, 0.69
8	169.91				
9	58.29	5.06 (d, 2.7)	9a	8, 10a	
9a	27.10	2.32 (m)	9, 9b, 9c		
9b, 9c	15.41, 17.47,	0.69, 0.93 (m)	9a		

10a	29.56	2.46 (s)		9, 11	9
11	169.74				
12	56.34	5.08 (d, 10.5)	12a	13a	
12a	32.44	2.07 (m)	12, 0.68		13
12b					
12c					
12d					
13a	30.16	3.04 (s)	12	12, 14	0.68, 0.84, 12, 15, 15a, 2.07
14	172.60				
15	53.32	4.85 (dd, 5.4)	16, 15a	14	15a, 13a, 15a, 16,
15a	30.71	1.94 (m)	15, 15bc		15
15bc	13.98, 20.05	0.92, 1.01 (m)	15a		
16 NH		6.95	15		18, 20
17	169.77				
18	76.50	2.49		18	
18bc	42.79	2.30 s, 6H			16, 18, 19
19	27.68	2.12	20, 21	18	20, 21
20	17.45	0.95		19, 20	19, 21
21	20.05	1.03		20, 19	19, 20

[a]C-13 data are extracted from HSQC spectra.

2.3. Peptide synthesis

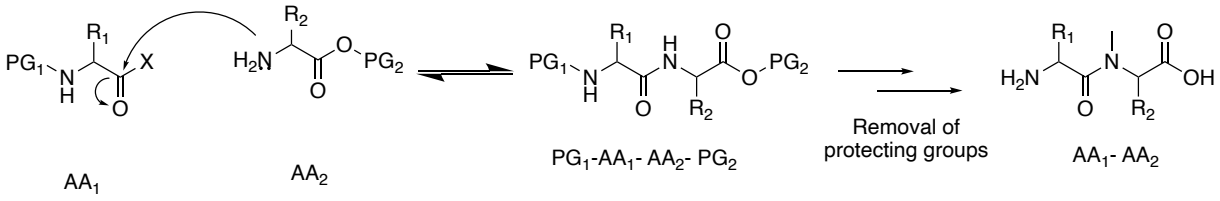
Re-creating the work of nature has always been a well-perceived challenge among organic chemists. The challenge of imitating nature inspired the first simple peptide bond formation by Theodor Curtius in 1881 and later by Emil Fischer in 1902, when they synthesized benzoylglycyl-glycine and ethoxycarbonylglycyl-glycine ethyl ester respectively.[106-108] Since then, peptide synthesis became one of the most sought synthetic targets, but difficulties emerged in the synthesis of even simple and well-defined peptides. On the bright side, obstacles brought a surge in efforts to methodology development for peptide synthesis.[108] The isolation of oxytocic pituitary peptide hormone, oxytocin, its structure determination and, its total synthesis was inspirational in the collective approach towards the development of peptide synthetic procedures.[109-112] Though the early scientists were motivated by the philosophy of recreating what nature makes, methodology and strategic development in peptide synthesis have enabled researchers to tweak natures design synthesize modified peptides with various biological activity.



Scheme **2.1**

Peptide synthesis is principally the formation of an amide bond between a carboxyl group and an amine group of two amino acids. The bond formation requires activation of the carboxylic acid group of an amino acid (Scheme **2.1**), where an electron-withdrawing atom or group (**X**) will be attached to the acid ($\mathbf{AA_1}$). Without the activation, this bond formation is not possible spontaneously at room temperature. Because elevated temperature is required to facilitate the dehydration during the amide bond formation at which the stability of the synthesized compound might be compromised.[113] The activating group improves the electrophilicity of the $\mathbf{AA_1}$ carbonyl group and enables the incoming nucleophile, which is the amine of a second amino acid ($\mathbf{AA_2}$), to attack efficiently. Good leaving group ability of the **X** facilitates the collapse of the tetrahedral intermediate (**THI**) and thus forms the peptide bond.

Peptide coupling reagents serve the role of the activating group. A large number of various coupling reagents have been developed for this purpose.[114] However, peptide coupling reaction to synthesize a simple dipeptide ($\mathbf{AA_1}$- $\mathbf{AA_2}$) following Scheme **2.1** will end up in a reaction mixture with multiple products with a different number of amino acids arranged randomly. The reaction mixture may or may not have the dipeptide $\mathbf{AA_1}$- $\mathbf{AA_2}$ in the mix. This random coupling happens because of a lack of any control over the reaction, and there are numerous combinations of the activated electrophile and incoming nucleophile possible.



Scheme **2.2**

For example, the first amino acid **AA_1** may self-condensed and gives the dipeptide **AA_1-AA_1**. Besides, they may be further activated on their free C-terminus, or they may act as the nucleophile on their free N-terminus and form another peptide bond with either **AA_1**, **AA_2**, or **AA_1-AA_1** again. Such a polymerization reaction may go on and on. Researchers developed the strategic use of easily cleavable protecting groups to eliminate this problem of uncontrolled polymerization. So, the reaction is designed in a way that, N-terminus of **AA_1**, C-terminus of **AA_2**, and any other reactive group in the side chain will be masked with protecting groups. Once the desired bond is formed, the protecting groups will be cleaved to get the target molecule (Scheme **2.2**).

2.3.1. Amide bond in Medicinal Chemistry

Amide bond formation is not only crucial for protein, and peptide synthesis, but also various compounds of therapeutic importance like peptoids, oligocarbamates, oligoamides, polyenamides, benzodiazepines, diketopiperazines, and hydantoins possess amide functionality.[115] Amide bonds have both hydrogen bond accepting and hydrogen

bond donating properties. Also, the reactivity of an amide can be modified by several factors like steric and electronic effect by neighboring groups, and strain on the bond. Overall, the amide is an attractive functional group in drug design. Perhaps, one of the well-known example (**Figure 2.4**) is the inhibition of cell wall transpeptidase enzyme of gram-positive bacteria by penicillins.[116-117]

Transpeptidase enzyme is a serine protease that catalyzes the last step of bacterial cell wall peptidoglycan synthesis, where two polysaccharide chains are crosslinked by a short peptide composed of unnatural (R)- (or D-) amino acids. The crosslinking happens when *N*-terminus glycine of a pentaglycyl moiety forms a peptide bond with the D-Ala-D-Ala sequence of other chain and replace the C-terminal D-Ala to form the peptide.

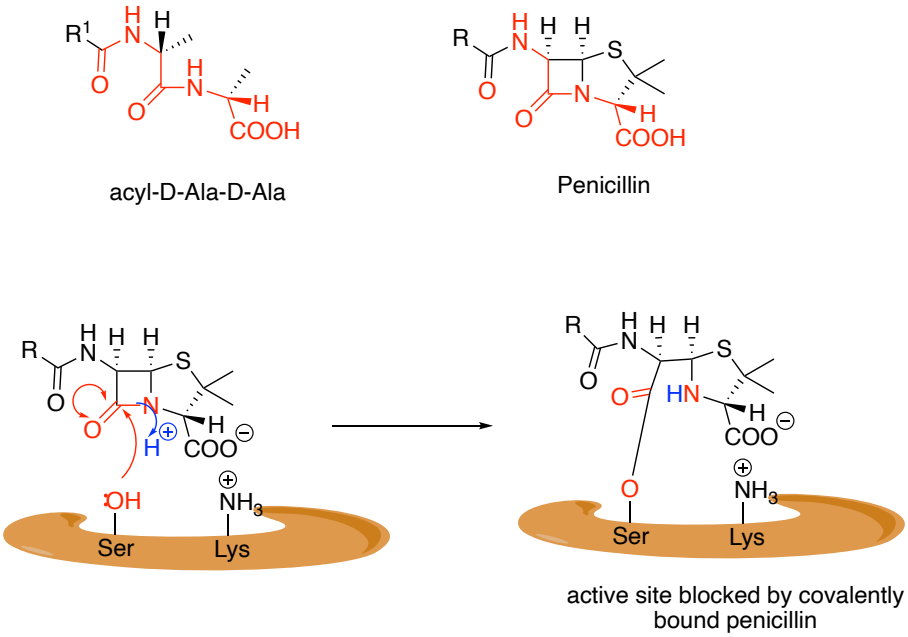
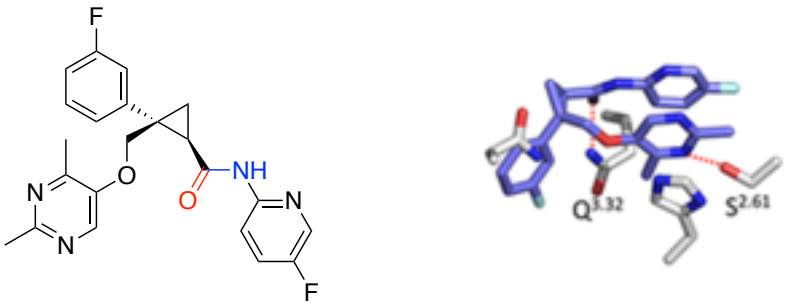


Figure 2.4: Mechanism of action of penicillin (modified).[118-119]

Penicillin acts in this step by mimicking the structure of D-Ala-D-Ala. So, the transpeptidase enzyme mistakenly takes penicillin as a substrate. Penicillin irreversibly inhibits the enzyme when the Serine OH group of the active site covalently attacks the highly reactive ß-lactam ring. ß-lactams are highly strained cyclic amide that acts as a good electrophile, where nucleophilic attack opens the lactam ring to relieve the strain. Since penicillin possesses the cyclic structure, it does not split into two parts and leaves the active site. Instead, it masks the newly formed ester bond between serine and penicillin at the active site so that water or any other nucleophile cannot cause hydrolysis. Once the enzyme is covalently linked with penicillin, i.e., acylated, it cannot catalyze the crosslinking further, resulted in leaky cell wall.[118-119]

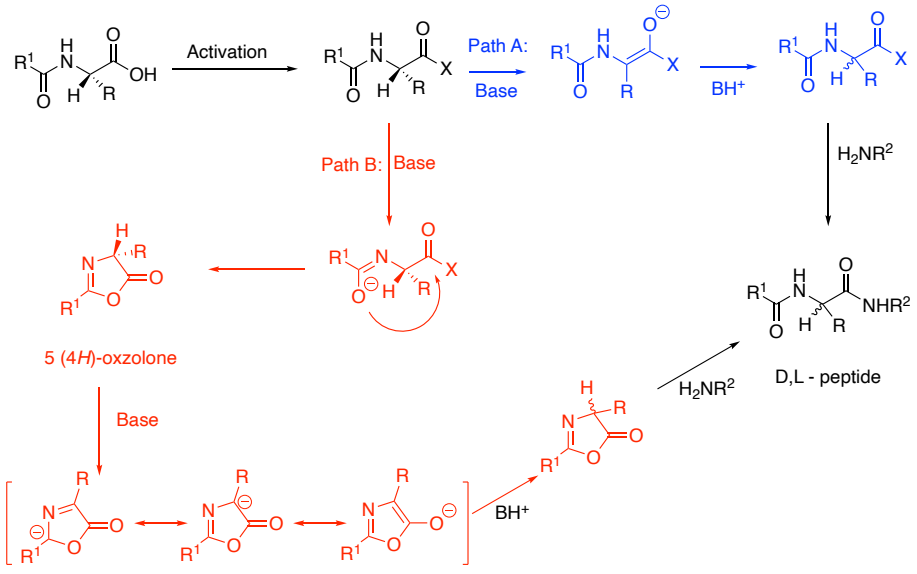
One recent example (**Figure 2.5**) of incorporating amide in chemical structure to play a role in target binding is the dual orexin receptor antagonist Lemborexant (**2.6**), approved to treat insomnia.[120-121] Two aromatic portions (amidopyridine and pyrimidine) of the molecule are in *cis*-configuration from the central cyclopropyl ring and occupy a hydrophobic pocket in the receptor. The role of the amide here is to form the H-bond with $Q126^{3.32}$.[122]



2.6

Figure 2.5: Lemborexant (2.6) and H-bonding with Glutamine (Use of this image is permitted by the American Chemical Society; Incident no: 3754512).[122]

The ubiquity of the amide bond is apparent in clinically approved drugs. In 2019, the FDA approved 48 drugs in various therapeutic categories.[120] After studying the structures of these approved drugs, 37 are small molecules (including six small molecules for two combination drugs Trikafta and Recarbrio). One-third of them contain at least one



Scheme **2.3**

amide bond, and are approved for treating various types of cancer, cystic fibrosis, migraines, insomnia, and urinary tract infection

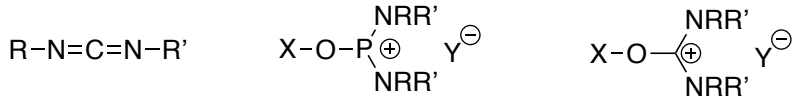
A review of chemical transformations used by three major pharmaceutical companies (GSK, Pfizer, and AstraZeneca) identified amide bond formation is the most ubiquitous reaction used by medicinal chemists.[123] The reviewers found that amide bond formation accounted for 16% of all reactions pursued in the pharmaceutical industry. And they recognized the availability of a plethora of amide formation methodologies, which resulted from the development in the field of peptide synthesis, as one of the main reasons for amide formation being at the top among all reaction categories.

2.3.2. Peptide coupling reagents

Peptide coupling can be carried out in the solution or the solid-phase (SPPS). In both processes, peptide coupling reagents facilitate the peptide formation by activating the carboxylic acid. Based on the activation method, two strategies are applied: (i) *in situ* activation and (ii) an activated species can be prepared and isolated before the coupling step. However, activation comes with the limitations of chirality loss of the activated amino acid and generation of unwanted side products. Two significant pathways (Scheme **2.3**) have been identified for the loss of chirality: (i) direct enolization (path A) and (ii) oxazolone formation (path B).[124]

Major side products generated in peptide coupling reactions are *N*-carboxy anhydride, diketopiperazine, and guanidine. The latter is particularly generated with uronium coupling reagents.[125] Thus, strategies like using additives, careful selection of base, and using appropriate protecting groups are employed to eliminate or minimize these obstacles while designing a peptide coupling reaction. A comprehensive chemical review El-Faham and Albericio[124] has described various collection coupling reagents, including carbodiimides, phosphonium salts, aminium/uronium salts, anhydrides, active esters, acylazoles, acyl azides, acyl halides, organophosphorus reagents, organosulfur reagents, triazines, pyridinium coupling reagents, and polymer-supported reagents. Discussing all of them is beyond the scope of this dissertation. Thus, the following section will discuss the most commonly used peptide coupling reagents, i.e., carbodiimides (**2.7**), phosphonium (**2.8**), and ammonium salts (**2.9**), which I also used practically in my synthetic approaches.

3.2.1. Carbodiimides

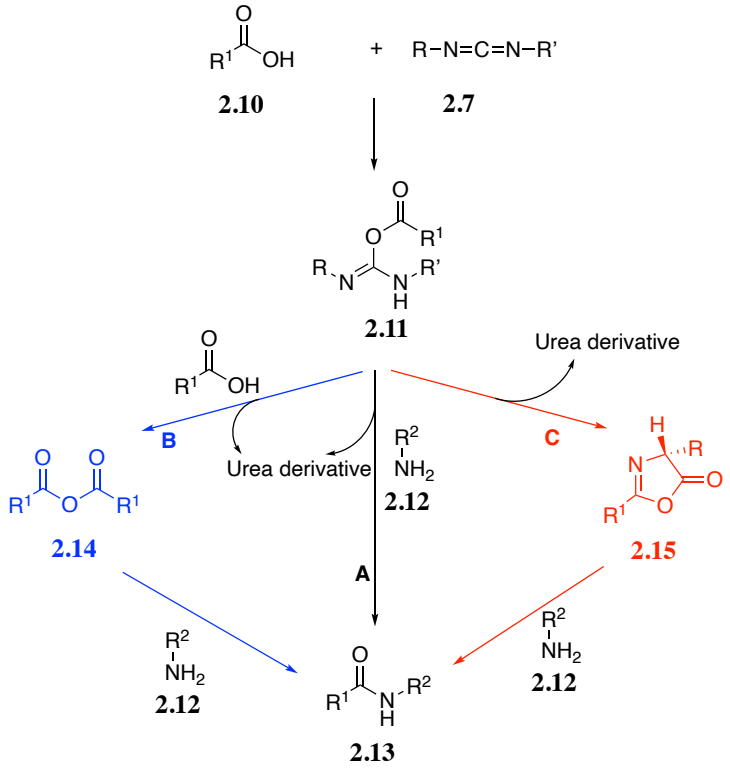


2.7 **2.8** **2.9**

Figure 2.6: Commonly used peptide coupling reagents.

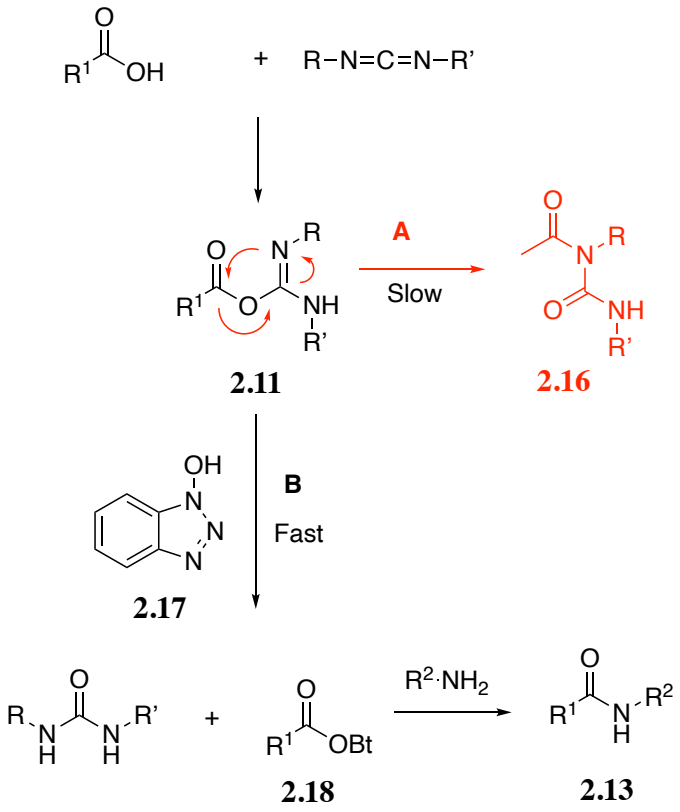
Dicyclohexylcarbodiimde (DCC) (**2.25; Figure 2.9**) is the most commonly used carbodiimide, which was first introduced by Sheehan in 1955.[126] The carbodiimide (**2.7**)

activates (Scheme **2.**4) the corresponding carboxylic acid (**2.10**) by forming *O*-acylisourea (**2.11**), which is highly reactive to execute the coupling reaction with the corresponding amine (**2.12**) to create the peptide (**2.13**) (Pathway A). There two other pathways that the *O*-acylisourea can follow to form the peptide: (i) forming a symmetrical anhydride (**2.14**) by reacting with a second molecule of carboxylic acid when excess carboxylic acid is used (Pathway B); (ii) forming an oxazolone (**2.15**), which is less reactive and can undergo racemization (Pathway C).[124]



Scheme **2.4**

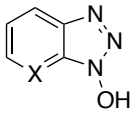
The high reactivity of *O*-acylisourea (**2.11**) also leads to an undesirable side reaction by forming *N*-acylurea (**2.16**) via rearrangement (Pathway A; Scheme **2.5**). The formed urea is stable and thus lower the reaction yield by depleting more carboxylic acid.



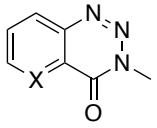
Scheme **2.5**

Cleverly, Kong, and Geiger have solved this problem by introducing *N*-hydroxyderivatives (HOXt) as additives.[127] They used hydroxy benzotriazole (HOBt, **2.12**) in their seminal paper of using additives to suppress side reactions HOBt (**2.17**) is a

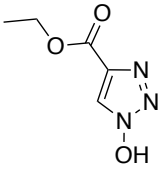
nucleophile and forms (Path B; Scheme **2.**5) the corresponding active Bt ester (**2.18**) with the highly reactive *O*-acylisourea (**2.11**) by a reaction faster than the rearrangement reaction to form *N*-acylurea **(2.16**). The formed ester (**2.18**) is reactive enough to undergo the aminolysis and formed the peptide (**2.13**, Pathway B, Scheme **2.5**). Also, the use of HOXt additives prevent the formation of oxazolone mediated peptide coupling as well and thus retain the stereo configuration of the alpha carbon as well.[128]



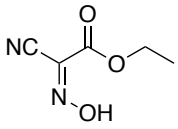
2.17; X= CH
2.19; X= N



2.20; X= CH
2.21; X= N



2.22



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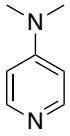
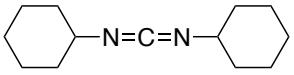


2.24

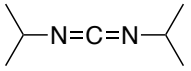
Figure 2.7: Additives used in peptide couplings

Introduction of DCC/HOBt combination enhances the scope of carbodiimide reagents and various other HOXt additives (**Figure 2.7**) like 1-hydroxy-7-azabenzotriazole (HOAT; **2.19**),[129] 3,4-dihydro-3-hydroxy-4-oxo-1,2,3,-benzotriazole (HODhbt; **2.20**),[127] 3-hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3,-triazine (HODhat; **2.21**),[130] ethyl-1-

hydroxy-1*H*1,2,3 triazole-4-carboxylate (HOCt; **2.22**),[131] and ethyl-2-cyano-2-(hydroxyimi-no)acetate (Oxyma; **2.23**).[132] Similar nucleophilic additive role can also be performed by DMAP (**2.24**).[133]



2.25



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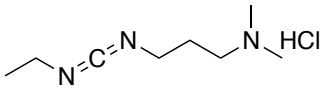

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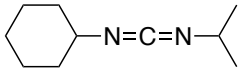


2.28

Figure 2.8: Commonly used carbodiimides.

Among the carbodiimides, DCC (**2.25**) is the most commonly used reagent for SPPS using *tert*-butyloxycarbonyl (Boc) chemistry. The urea byproduct, *N, N*-dicyclohexylurea (DCU), is only soluble in trifluoroacetic acid and thus makes the removal of DCU easy while removing the Boc protection using TFA. However, the same reason makes DCC useless in SPPS using fluorenylmethyloxycarbonyl (Fmoc) since it cannot be filtered off from the reaction medium or washed off with common organic solvents. In solution-phase synthesis, traces of DCU retains in the system even after extensive chromatographic separation. Depending on the synthetic strategy, better coupling reagents have substituted DCC.[114, 114] *N, N'*-diisopropylcarbodiimide (DIC; **2.26**),[134] *N*-ethyl-*N'*-(3-

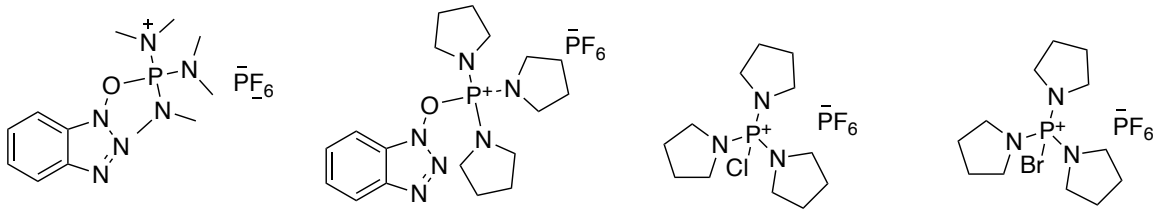
dimethyl aminopropyl)carbodiimide (EDC; **2.27**),[135] and *N*-cyclohexyl-*N'* - isopropylcarbodiimide (CIC; **2.28**)[136] are among the most popular. EDC is preferred for solution-phase synthesis since both the reagent and the urea byproduct are water-soluble and thus can be easily removed by an aqueous workup.

Carbodiimide/HOXt mediated and many other coupling reagents often require the use of nonnucleophilic base like triethylamine (TEA), diisopropylethylamine (DIPEA), and *N*-methylmorpholine (NMM) which could enhance the preactivation step.[124] In my synthetic approach to naranjamide, most of the peptide coupling reactions utilized the EDC/HOBt system because of its superiority in solution phase synthesis.

2.3.2.2. Phosphonium salt

The application of alkyl phosphonium salts as a coupling reagent was first introduced by Gawne *et al.* in 1969.[137] But their widespread application started with the introduction of CloP and BroP by Castro.[135] These reagents suffered the obstacle of racemization. When HOBt (**2.17**) found its application in peptide chemistry, Clop-HOBt fused coupling reagent, (benzotriazole-1-acyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP; **2.29**), also known as Castro's reagent, was developed in 1975.[138] The coupling reaction (Scheme **2.6**) proceed via the deprotonation of the desired acid first by a hindered base like TEA or DIPEA. The deprotonated acid then reacts with the **2.29** to generate an activated acyl phosphonium intermediate (**2.30)** and HOBt.

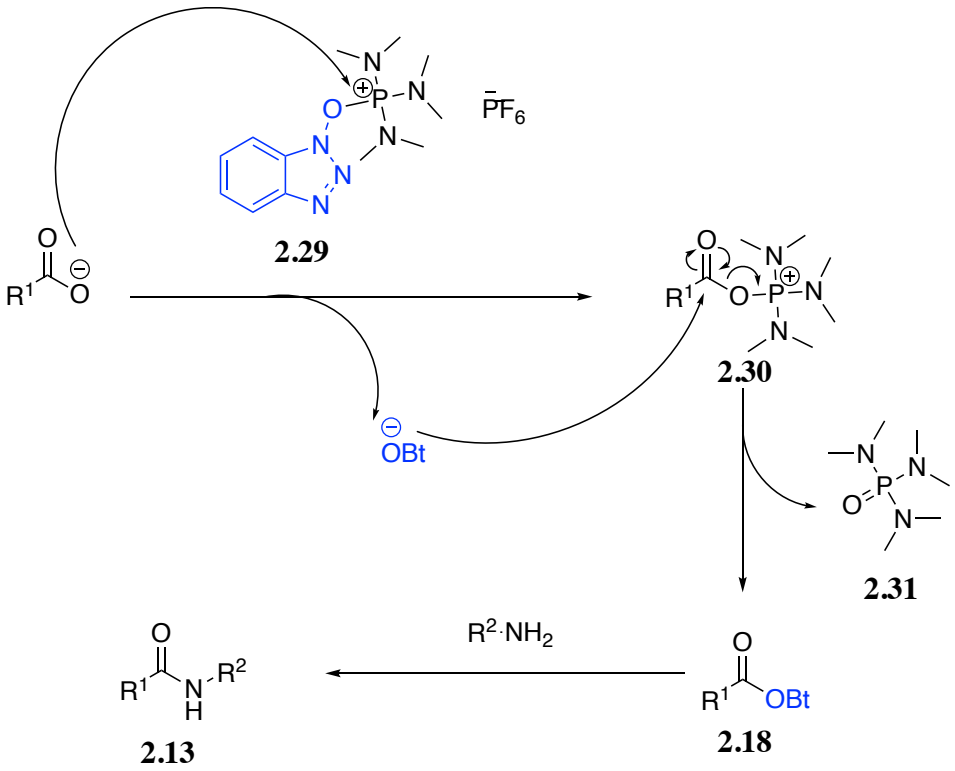
Activated **2.30** is highly reactive, like *O*-acylisourea (**2.11**) generated in carbodiimides mediated reaction (Scheme **2.4** or **2.5**). So, the *in situ* generated HOBt readily reacts with the intermediate **2.30** to form the activated Bt ester (**2.18**; Scheme **2.6**), which then coupled with the corresponding amine. Generation of the corresponding phosphine oxide (**2.31**; hexamethylphosphoric triamide, HMPA) facilitate the reaction to move forward.[139]



2.29 **2.32** **2.33** **2.34**

Figure 2.9: Commonly used phosphonium salts

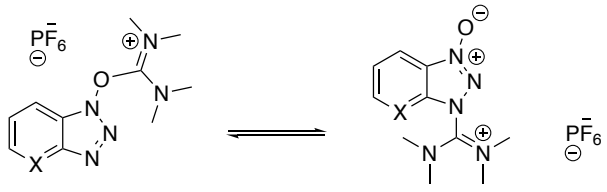
However, **2.31** is exceptionally toxic. The substitution of all three dimethyl substituents of **2.29** was replaced with pyrrolidine to get PyBop (**2.32**), which is equally effective but produce a less toxic byproduct. Both **2.29** and **2.32** are not useful for coupling to *N*-methyl amino acid. Replacing the HOBt part of **2.32** with chloride or bromide generates PyClop (**2.33**) and PyBrop (**2.34**), respectively. These reagents produce the corresponding acyl halide instead of Bt ester and allow the incoming secondary amino group to attack the highly electrophilic acyl halide.[139]



Scheme **2.6**

2.3.2.3. Aminium/Uronium salts

The first aminium reagent, *O*-(1*H*-benzotriazole-1-yl)-*N*,*N*,N',*N'*-tetramethyluronium hexafluorophosphate (HBTU; **2.35**), was introduced in 1978.[140]

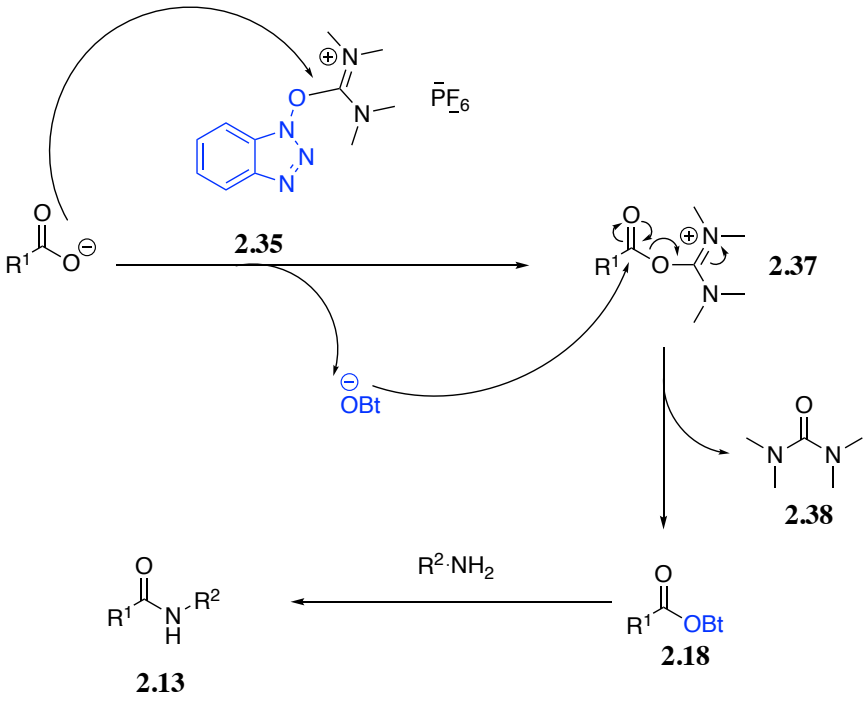


2.35; X = CH
2.36; X = N

Figure 2.10: Equilibrium between the uranium salt and guanidium salt

Like the phosphonium salts, aminium salts were considered initially as uranium type structure where aminium salts possess a positively charged carbon atom instead of the charged phosphorus atom. However, X-ray crystallography found these salts to exist as an aminium type (guadinium *N*-oxides) structures. In solution, both uranium and aminium form exist in equilibrium (**Figure 2.10**).

Their mechanism of activation (Scheme **2.7**) is similar to that of phosphonium reagents as well, and proceeds via generation of carboxyl uranium salts (**2.37**), followed by active Bt/At ester generation (**2.18**). The driving force for this type of reaction is the generation of urea byproduct (**2.38**).



Scheme **2.7**

The azabenzotriazol derivative, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU; **2.36**), was developed later which is very efficient in sterically hindered coupling and minimize racemization.[139] When the nonnucleophilic counterion is tetrafluoroborate, **2.35** and **2.36** are called TBTU and TATU, respectively. But the counterion does not influence the rate of reaction or racemization.[125]

2.3.3. Synthetic approaches to naranjamide

The total synthesis of naranjamide (**2.5**) was envisioned through a convergent route utilizing solution-phase peptide synthesis, where two peptide segments (left/red and right/blue in **Figure** 2.4) were coupled at the final step to generate the pentapeptide. The convergent route was selected because of partial methylation of peptide bonds in naranjamide, sparing one of the non-methylated amides. So, I aimed to synthesize the right peptide segment, execute the methylation of it, and then complete the final coupling through the non-methylated amide bond.

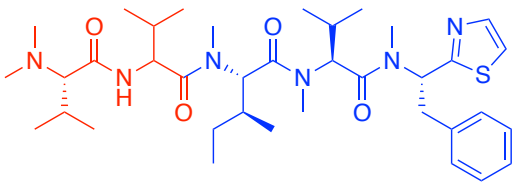


Figure 2.11: Two segments of naranjamide (**2.5**)

Synthesis of the left segment (Scheme **2.8**) started with the coupling of (*S*)-dolavaline **2.39** and the methyl ester of *l*-valine **2.40** using EDC/HOBt. I choose EDC/HOBt method because of its advantages, like better solubility and easy workup, over other coupling reagents in solution phase synthesis. The synthesized dipeptide **2.41** was then hydrolyzed under basic conditions to cleave the ester and reveal the carboxylic acid **58**. Proton NMR of **2.42** showed a singlet at 3.3 ppm, which integrated for a value of 0.25. Since this is the resonance region for methoxy (-OCH_3) protons, we can rationalize that the 3.3 ppm peak was originated from the methoxy protons of remaining starting material. However, this starting material has no way to undergo the subsequent reaction, i.e., coupling with the right segment. So, compound **2.42** was taken to the next step.

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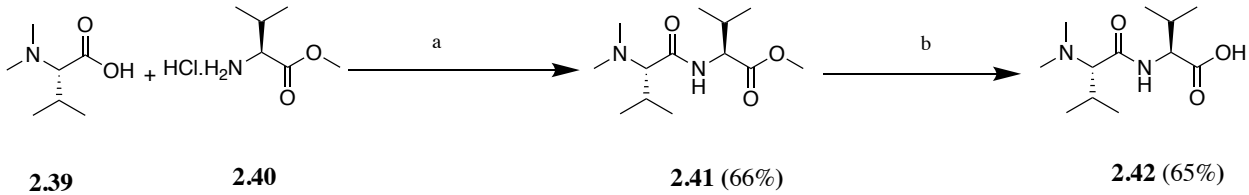
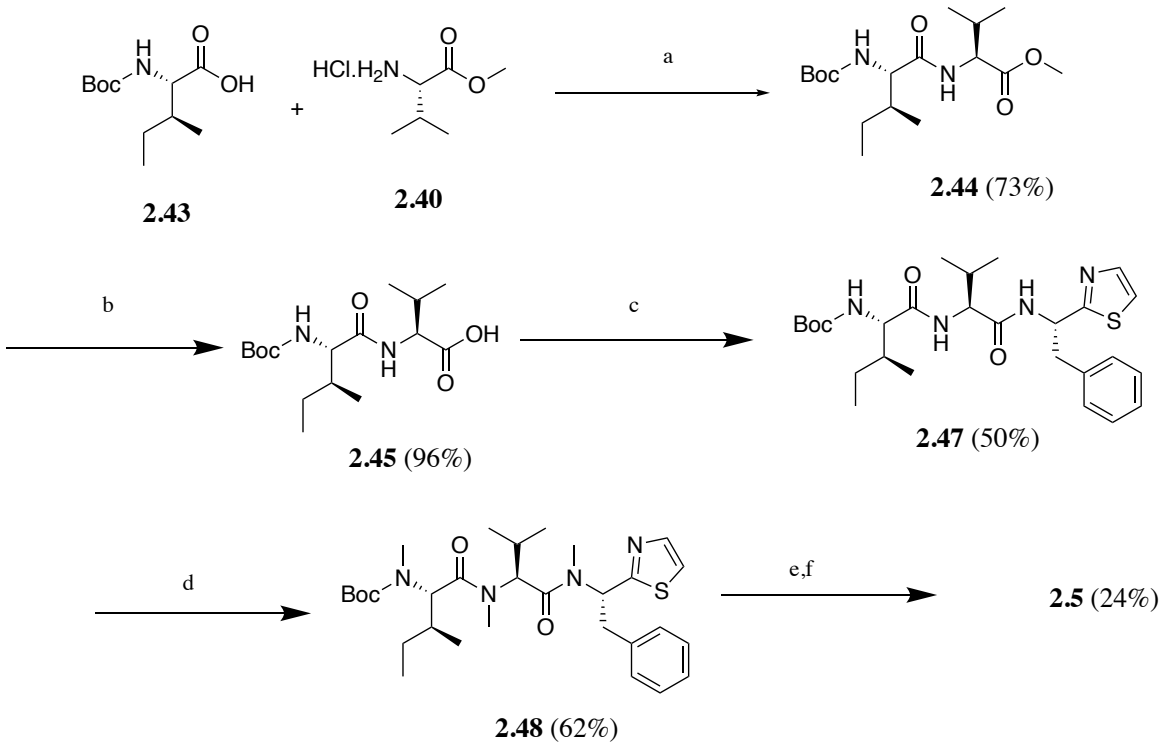


2.39 **2.40** **2.41** (66%) **2.42** (65%)

Scheme **2.8**

Conditions: (a) 1.1 equiv EDC.HCl, 1.1 equiv HOBt, 3 equiv DIPEA, CH_2Cl_2, 0°C to rt, Overnight; (b) 3 equiv NaOH, MeOH, 3h, reflux.



Scheme **2.9**

Conditions: (a) 1.1 equiv EDC.HCl, 1.1 equiv HOBt, 3 equiv DIPEA, CH_2Cl_2, 0°C to rt, Overnight; (b) 3 equiv NaOH, MeOH, 3h, reflux; (c) (*S*)-dolaphenine (**2.46**), 1.1 equiv EDC.HCl, 1.1 equiv HOBt, 3 equiv DIPEA, CH_2Cl_2, 0°C to rt, 16 h; (d) (i) 25 equiv NaH, N_2, THF, 0°C, 2h; (ii) excess MeI, N_2, 0°C to rt, 22 h; (e) Trifluoro acetic acid: CH_2Cl_2 (1:1); (f) **2.42**, 1.5 equiv HATU, 5 equiv DIPEA, DMF, rt, N_2, 2h.

Synthesis of the right segment (Scheme **2.9**) started with the coupling of *t*-Boc protected *l*-isoleucine **2.43** with methyl ester *l*-valine **2.40** using EDC/ HOBt. The synthesized dipeptide **2.44** was then hydrolyzed under basic conditions to cleave the ester protection. The generated carboxylic acid **2.45** was next coupled with the hydrochloride salt of *(S)*-dolaphenine **2.46** using EDC/HOBt. The tripeptide **2.47** was then permethylated

according to the method described by Snchez *et al.*[48] in the synthesis of almiramide derivatives. Using methyl iodide and sodium hydride as the base, **2.48** was synthesized. The proton NMR of **2.48** became complex and revealed a similar pattern that exhibited in the proton NMR of **2.5**. Characteristic *N*-methyl peaks appeared in between 2.4-3 ppm. Also, the presence of more than one conformer was evident from the COSY of **2.48**, where we can see the correlation of an alpha proton at 6.6 ppm with two sets of benzylic protons located in between 3-4 ppm. MS data confirmed the structure of **2.48** (**Figure 4.2**), where I found the [M+Na^+] peak at 581.8 (Target mass: 558.32). At the final step in a one-pot reaction, the *t*-Boc protecting group of permethylated peptide **2.48** was removed with TFA to reveal the secondary amine followed by coupling with **2.42.**

In the final coupling step, several attempts (Table **2.2**) were made to couple deprotected **2.48** and **2.42.** Coupling reactions using EDC/HOBt at varying conditions were found unsuccessful. Peptide coupling reactions with a hindered secondary amine are less favorable compared to a primary amine, and both PyBrop and HATU have been found to act efficiently in sterically hindered coupling.[139] I tried both of them, but a successful coupling reaction proceeded with HATU (24% yield**)**.

Table 2.2: Conditions for the final coupling step (Scheme **2.9**)

Coupling reagent (eq)	Base (eq)	Solvent	Time	Result
EDC+HOBt (1:1)	DIPEA (1)	DCM	20 hrs	NPF
EDC+HOBt (1.1:1.1)	DIPEA (1.1)	DCM	20 hrs	NPF
EDC+HOBt (3:3)	DIPEA (1.1)	DCM	20 hrs	NPF
PyBrop (1)	DIPEA (4)	DMF+DCM	1.5 hrs	NPF
PyBrop (1.1)	DIPEA (3)	DCM	48 hrs	NPF
PyBrop (1.5)*	DIPEA (1.8)	DCM	48 hrs	NPF
HATU (1.2)**	DIPEA (4.8)	DMF	2 hrs	24% yield

*NPF = No product found

The ^{1}H NMR data of synthetic and natural naranjamide was compared (**Figure 4.9, Table 4.1**), and the mass spectrum showed the [M+H^{+}] at 684.44 **(Figure 4.10)**. Though, proton NMR and MS data matched with that of naranjamide, the chiral purity of the synthetic compound was not determined. Loss of chirality of any of the alpha carbon can be traced back to the *N*-methylation reaction, where 25 equivalents of strong base was used. A strong base can destroy the chiral integrity of any of the alpha carbon and generate more than one stereoisomer. The yield of the final coupling step was 24%, which is very low as compared to the common HATU mediated coupling.

2.4. Testing for antiparasitic activity

Synthetic **2.5** was tested against *T. cruzi* and *P. falciparum* and was found to inhibit the growth of both parasites with an IC_{50} of 9.2 μM and 2.8 μM, respectively (table 2.3). In contrast, the positive controls chloroquine and nifurtimox have IC_{50} of 19 μM and 3.1 μM, respectively. Also, naranjamide (**6**) was found inactive against *Leishmania donovani*, the causative agent for leishmaniasis, but showed growth inhibition of 90.3% of the MCF-7 cell line.

Table 2.3: Bioactivity of cyanobacterial fractions and naranjamide

Fraction	***L. donovani***		***P. falciparum***		***T. cruzi***		**MCF-7**	
	%GI	**IC_{50}**	**%GI**	**IC_{50}**	**%GI**	**IC_{50}**	**%GI**	**IC_{50}**
Crude (2002)	24.7	-	82.6	-	16.7	-	28.2	-
2002H	13.7	-	97.5	-	75.0	-	13.6	-
2002H4	-	-	79.6	-	81.5	-	-	-
Synthetic Naranjamide (**2.5)**	-	-	-	2.80	-	9.20	90.3	-

2.5. Discussion

In our biological system, *N*-methylation plays a crucial role in controlling biological function, particularly in epigenetic modification. Because of *N*-methylation, identical genotype can result in many different phenotypes.[142] Similarly, the *N*-methylation

of the peptide is also found in nature to promote various physical and biological functions. In general, peptide drugs suffer poor pharmacokinetic profiles, i.e., short *in vivo* half-life and oral bioavailability. But *N*-methylation is known to improve peptide's pharmacokinetic profile (resistance against protease, enhanced lipophilicity, and bioavailability).[143] Multiple *N*-methylation will possibly allow naranjamide to have a better pharmacokinetic profile. One such example is cyclosporin, which is a natural, multiply *N*-methylated cyclic peptide, and can be given orally.[144] **Figure 2.1** listed some marine cyanobacterial compounds with antiparasitic activity, and all of them are either backbone amide methylated (**2.1**- **2.3**) or terminal *N*-alpha methylated (**2.4**). Naranjamide is both backbone amide, and *N*-alpha methylated. Thus *N*-methylation possibly helps in naranjamide's antiparasitic property. It will be beneficial to see if non-methylated analogs still retain the antiparasitic property.

Interestingly, naranjamide also inhibits the growth of MCF-7 cells. The dolaphenine moiety of **2.5** possess a thiazole ring, which is considered as the pharmacologically privileged structure with activities like antimicrobial, antiviral, anticancer, antifungal, antihistaminic and antithyroid.[145-146] Thiazole containing dolaphenine has also been found in many bioactive marine compounds. Naranjamide's structure closely resembles the structure of Dolastatin 10 (**1.20**), mentioned in Chapter 1, which also possesses the dolaphenine moiety and a peptide framework. Dolastatin 10's cytotoxicity is mediated by

the inhibition of microtubule assembly.[103] Thus, naranjamide's cytotoxicity can be possibly correlated with microtubule assembly inhibition and demands further study. This hypothesis also broadens naranjamide's scope as a potential anticancer compound.

We can hypothesize naranjamide's possible biosynthetic origin as well considering the common metabolic pathways used by cyaonobacteria. They produce peptides or metabolites with peptidic substructure by using their non-ribosomal peptide synthetase (NRPS) or hybrid NRPS-polyketide synthase (PKS) machinery. Also, the *N*-methylation are executed by the *N*-methyl transferase (NMT).[147] We can anticipate that cyanobacteria utilized both NRPS and NMT biosynthetic pathways to synthesize naranjamide.

2.6. Conclusion

In summary, a potent antiparasitic *N*-methylated peptide was discovered from a marine cyanobacterium, and a total synthesis was conducted. Naranjamide has activity against both *T. cruzi* and *P. falciparum*. Treatment options against *T. cruzi* are limited. Benznidazole and nifurtimox are the only drugs available on the market that treat only the early stage of Chagas disease, with the penalty of severe side effects and resistance development.[148] Considering the necessity to develop anti-trypanosomal compounds, naranjamide can be used as the lead compound to develop well-tolerated agents for Chagas disease treatment. Naranjamide is more potent than nifurtimox *in vitro*, and future research will examine its *in vivo* efficacy, as well as its mechanism of action. The rational approach

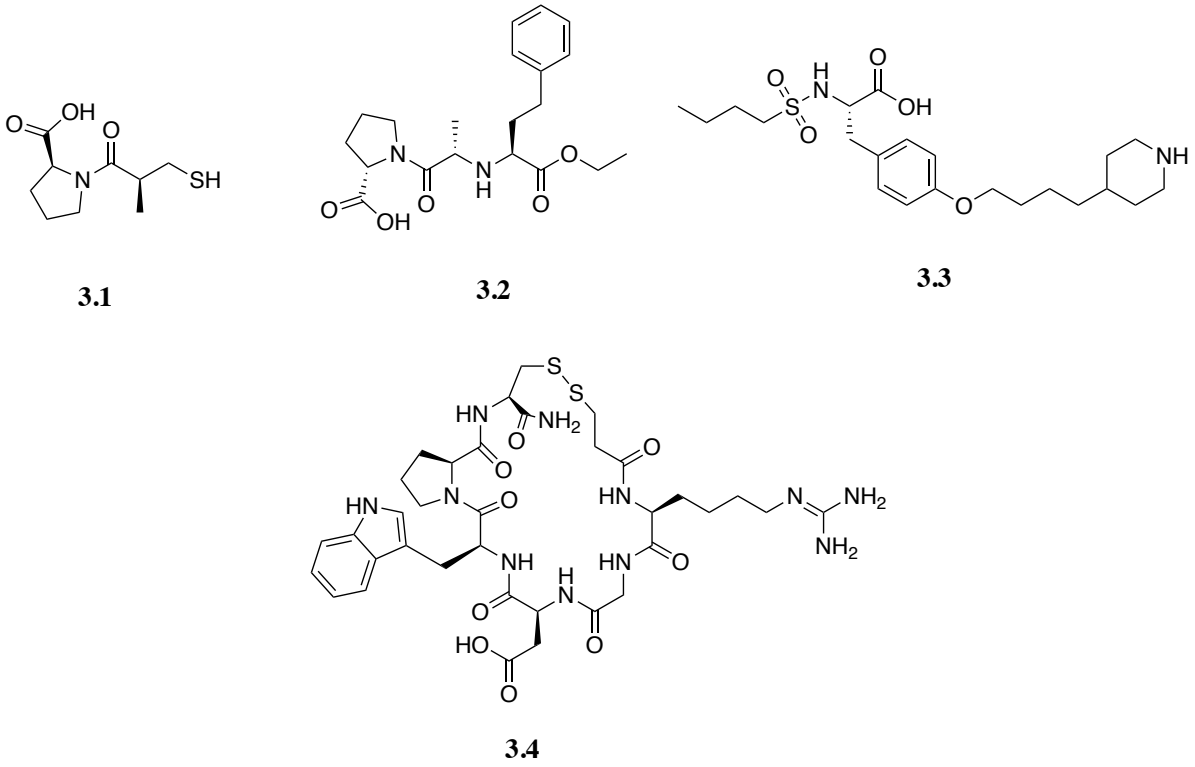
to design more potent analogs against resistant strains will be the next goal to achieve. Also, naranjamide showed cytotoxicity and thus required to be evaluated for its anticancer potential.

Chapter III: Analog synthesis

3.1. Introduction

The twentieth century has seen many significant successes in drug discovery for treating a wide range of diseases. These discovery campaigns approached via screening or rational design, including ligand-based, mechanism-based, or target-based design. Analysis of these compounds helped in developing design principals for small molecular weight compounds. Previously, most pharmaceutical companies had strictly followed these guidelines as a rule of thumb and triaged drug candidates.[149] For example, the very well-known design principle, "Lipinsky's rule of five," considers small molecules with a molecular weight of <500 Da as most likely to be orally active.[150] There was an emphasis on developing orally bioavailable drugs because of patient compliance, and it was logical for pharmaceutical companies to exclude compounds with molecular weight >500 Da while screening a library of millions of molecules. Peptides easily exceed this molecular weight cut-off with just five amino acids. And, were generally considered as less drug-like. However, there are drugs in the market that violate the rule-of-five and are still orally active. One such molecule is cyclosporin, which is orally active despite violating all criteria of "rule-of-five".[144] Excluding oral bioavailability in the selection criteria resulted in the exploration of chemical spaces by researchers that were previously less searched for drug

discovery. Many peptide-based drugs like captopril (**3.1**), enalapril (**3.2**), tirofiban (**3.3**) and Epifibatide have reached the market (**Figure 3.1**) and a resurgence of interest in peptide drug discovery has been observed among pharmaceutical industries.[151]



3.1 **3.2** **3.3**

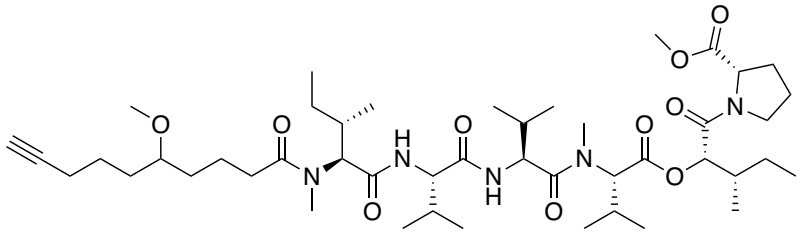
3.4

Figure 3.1: Marketed peptide drugs

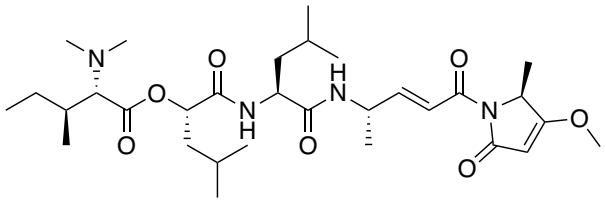
The activity of naranjamide against neglected tropical diseases has brought the necessity to explore its structure-activity relationship. Using the medicinal chemistry approach for peptide drug design, analogs were synthesized to elucidate more information about the molecule and its prospective target.

3.2. Analog design for naranjamide

Researchers have discovered many antiprotozoal peptides from Panamanian marine cyanobacteria. Viridamide A (**3.5**), isolated from *Oscillatoria nigro-viridi*, is an *N*-methylated peptide with a methyl ester at the C-terminus and a methoxylated fatty acid with a terminal acetylene functionality at the N-terminus. Compound **3.5** exhibited an IC_{50} of 5.8 μM to chloroquine-resistant *P. falciparum*, an IC_{50} of 1.37 μM to *L. donovani*, and an IC_{50} of 1.0 μM to *T. cruzi*.[56, 152] Another peptide, Gallinamide A (**3.6**) sowed an IC_{50} of 8.4 μM to chloroquine-resistant *P. falciparum.* It was isolated from a *Schizothrix* sp.[85] Compound **3.6** does not have methylation on the amide nitrogen. But, its *N*-terminus possesses the *N*, *N* – dimethyl isoleucine moiety, and the C-terminus amide nitrogen is part of the modified proline residue. Naranjamide is a pentapeptide with a molecular weight of



3.5



3.6

Figure 3.2: Panamanian cyanobacterial peptide with antiprotozoal activity.

684 Da. A review on peptide drugs by Craik *et al.*[149] highlighted that majority of the approved peptide-based drugs are smaller in size (less than ten amino acid).

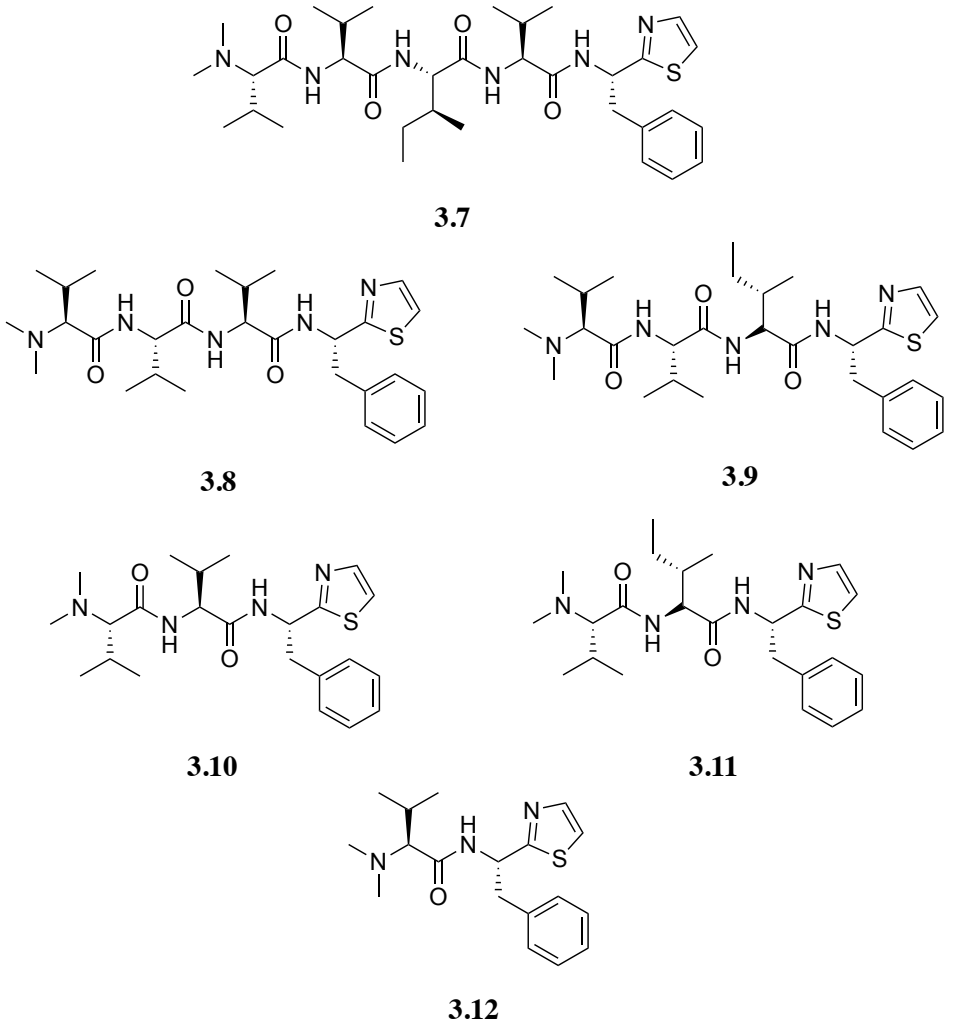
This observation, together with the importance of antimalarial and antichagasic drug discovery and potential of Panamanian cyanobacterial peptides as antiparasitic agents, makes our lead molecule an attractive candidate for medicinal chemistry study.

At this stage of the project, naranjamide's mechanism of action or any information about its target is unknown. So, while synthesizing naranjamide analogs, the ligand-based approach was pursued, and a few considerations were made: (1) search for minimum active sequence of amino acids; (2) role of *N*-methylation; (3) role of the two terminal residues and (4) identify structural features that determine the selectivity between the malaria and Chagas parasites. Within the capacity of synthesis, the first analog synthesized was a non-methylated version (**3.5**) of naranjamide, where all the amide nitrogens were non-methylated. The importance of *N*-methylation in controlling drugs pharmacokinetic property is mentioned in chapter 2. So, my aim for this non-methylated version was to see the role of these *N*-methyl groups in altering bioactivity. Removing the *N*-methyls exposes the amide protons to form hydrogen bonds. However, these protons may or may not be accessible depending on the conformation of the peptide and propensity to form intramolecular hydrogen bonds. Early-stage peptide drug development generally starts with the simplification of the structure (e.g., size reduction).[153] So, subsequent analogs (**3.7**

- **3.12**) were synthesized with reduced chain length by decreasing one subunit at a time. Also, within the same chain length, valine and isoleucine substitution impact on the activity was explored. The simplest analog synthesized was a dipeptide containing the *N*,*N*-dimethyl valine coupled to the dolaphenine moiety. Thiazole containing dolaphenine is a pharmacologically privileged structure, and many cyanobacterial antiprotozoal compounds are known to carry the *N*,*N*-dimethyl moiety at the *N*-terminus.[85, 87, 141 152, 154- 155] Thus, the main focus was to combine the role of *N*,*N*-dimethyl in antiprotozoal inhibition together with the inherent efficacy of dolaphenine. This design will be effective only if both *N*,*N*-dimethyl, and dolaphenine binding sites are in close proximity.

The scope of peptide lead modification is broad. Apart from *N*-methylation and chain length reduction, one common approach is alanine scanning, where each subunit is replaced with an alanine residue. Alanine replacement eliminates the side chain past the β-carbon but does not change the peptide conformation. Alanine scan is a widely used technique in identifying the catalytic site of enzymes or functional roles of proteins/peptide.[156] Another meaningful change that can be brought in naranjamide's structure is changing its conformation. Naranjamide is a partial rigid structure because of *N*-methylation. Thus altering the *N*-methylation pattern will result in analogs with different conformation. Another effective way to rigidify linear peptide conformation is cyclization

by joining side chains or head to tail. Also, short-range cyclization (or a kink in the structure) can be achieved by substitution of a subunit with a proline residue.[157]



3.7

3.8

3.9

3.10

3.11

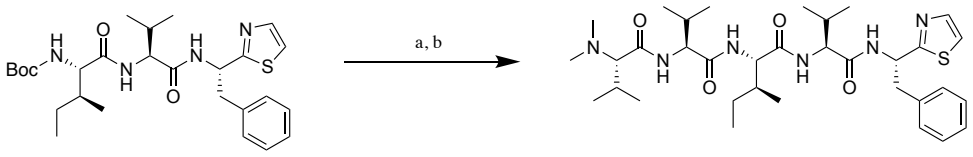
3.12

Figure 3.3: Synthesized naranjamide analogs

Cyclic peptides offer several other advantages over linear structures. One key benefit is the avoidance of proteolysis by protease in the blood, thereby increasing their bioavailability. Cyclization also improves permeability through the cell membrane and

thus make peptide drug able to target both cell surface and intracellular targets.[158-159] The smallest cyclic peptides known to exist are 2,5-diketopiperazine derivatives, which are obtained by condensation of two amino acids.[160] Naranjamide's pentapeptide size is ideal for synthesizing cyclic analogs. So, naranjamide carries a considerable potential to develop a wide range of analogs using ligand-based drug design, which may result in compounds with higher activity against malaria and/or Chagas parasite.

3.3. Synthesis of naranjamide analogs

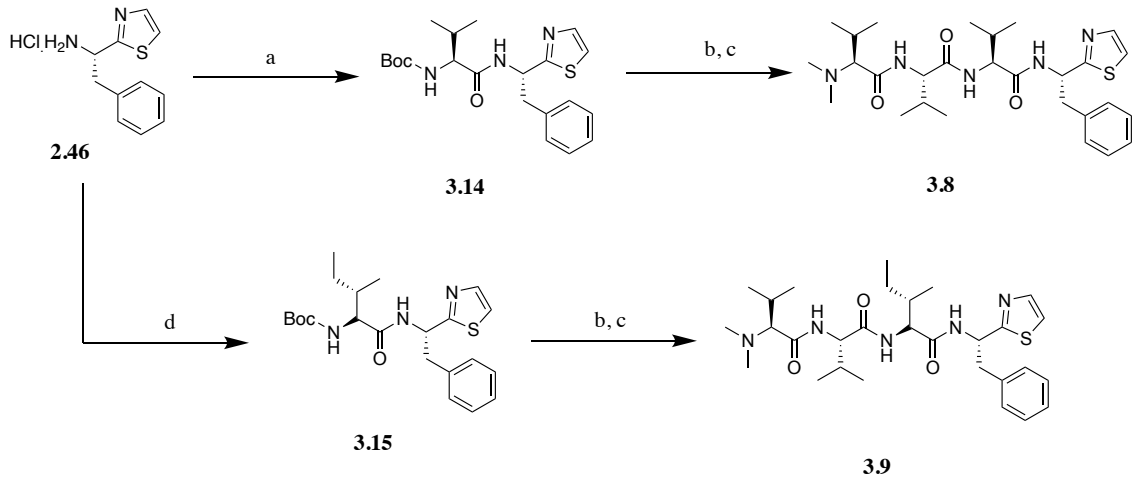


2.47 **3.7**

Scheme **3.1**

Conditions: (a) Trifluoro acetic acid: CH_2Cl_2 (1:1); (b) **2.42**, 1.5 equiv HATU, 5 equiv DIPEA, DMF, rt, N_2, 2h.

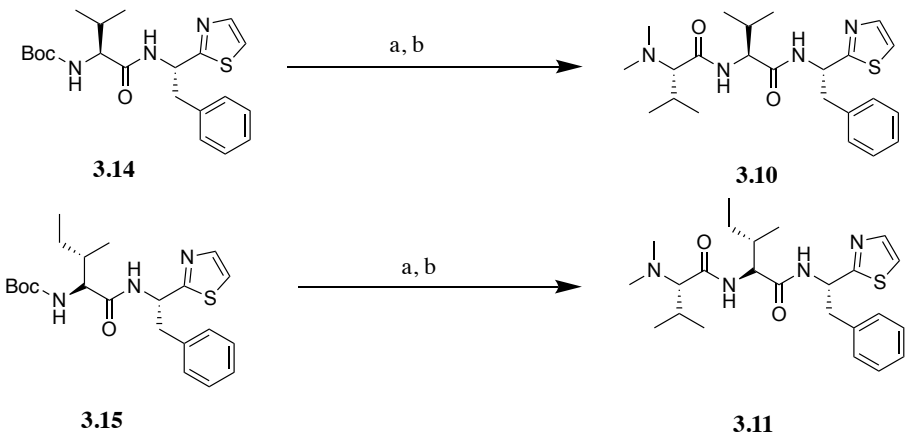
Synthesis of the proposed analogs utilized the similar peptide synthesis schemes described in chapter two for naranjamide. Also, intermediate **2.47** used in naranjamide synthesis was used as the starting point for compound **3.7** (Scheme **3.1**). Synthesis of compound **3.7** started with the deprotection of the *t*-Boc protecting group of **2.47** using TFA, followed by coupling with the dipeptide **2.42** using EDC/HOBt.



Scheme **3.2**

Conditions: (a) **3.13**, 1.1 equiv EDC.HCl, 1.1 equiv HOBt, 3 equiv DIPEA, CH_2Cl_2, 0°C to rt, 16 h; (b) Trifluoro acetic acid: CH_2Cl_2 (1:1); (c) **2.42**, 1.5 equiv HATU, 5 equiv DIPEA, DMF, rt, N_2, 2h. (d) **2.43**, 1.1 equiv EDC.HCl, 1.1 equiv HOBt, 3 equiv DIPEA, CH_2Cl_2,

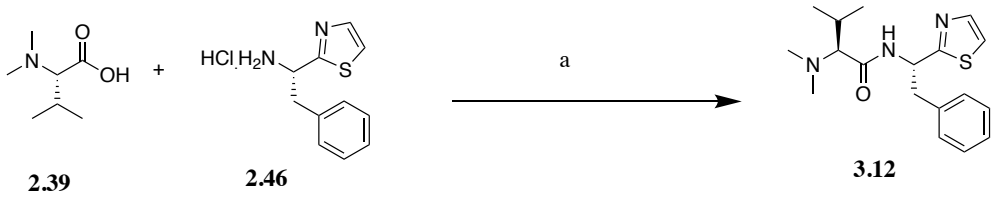
Synthesis of the two tetrapeptides (**3.8** and **3.9;** Scheme **3.2**) started from the same starting material, (*S*)-dolaphenine hydrochloride (**2.46**). Compound **2.46** was first coupled with *t*-Boc protected *l*-valine (**3.13**) to synthesize the *t*-Boc protected dipeptide **3.14**. *t*-Boc group of **3.14** was then deprotected using TFA, followed by the final coupling with the dipeptide **2.42** using EDC/HOBt to get **3.8**. Compound **3.9** followed the same scheme, except the first coupling was performed using *t*-Boc protected *l*-isoleucine (**2.43**).



Scheme **3.3**

Conditions: (a) Trifluoro acetic acid: CH_2Cl_2 (1:1); (b) **2.39**, 1.5 equiv HATU, 5 equiv DIPEA, DMF, rt, N_2, 2h.

Tripeptides **3.10** and **3.12** were synthesized from the *t*-Boc protected dipeptides **3.14** and **3.15** (Scheme **3.3)**. In both routes, the *t*-Boc group was first removed by using TFA, and then subsequent coupling with *N, N*-dimethyl *l*-valine (**2.39**) yielded **3.10** and **3.12,** respectively



Scheme **3.4**

Conditions: (a) **3.13**, 1.1 equiv EDC.HCl, 1.1 equiv HOBt, 3 equiv DIPEA, CH_2Cl_2, 0°C to rt, 16 h;

The dipeptide **3.12** was synthesized by coupling *N, N*-dimethyl *l*-valine (**2.39**) with (*S*)-dolaphenine hydrochloride (**2.46**) using EDC/HOBt peptide coupling reagent (Scheme **3.4**).

3.4. Bioactivity of naranjamide analogs:

Synthesized analogs (**3.7** to **3.12**) were tested against *T. cruzi*, *P. falciparum,* and MCF-7 cell line, and their activities are compared in Table **3.1.** We found that all analogs lack activity against the Chagas parasite and MCF-7 cell line. Also, their potency against the malaria parasite was lower in comparison to synthetic naranjamide and the positive standard chloroquine.

Table 3.1: Bioactivity of naranjamide analogs

Compound	***P. falciparum***	***T. cruzi***		**MCF-7**
	IC_{50} (μM)	**%GI**	**IC_{50}** (μM)	**%GI**
Synthetic naranjamide **(2.5)**	2.80	-	9.20	90.3
3.7	14.46	12.23	-	0
3.8	217.03	9.7	-	0
3.9	49.44	3.68	-	26.9
3.10	43.99	6.16	-	2.8
3.11	23.99	10.53		0
3.12				
Chloroquine	0.063	-	-	-
Benznidazole	-	-	3.8	

3.5 Discussion

The result obtained from the bioassay of compound **3.7** to **3.12** revealed that despite losing potency (IC_{50}: 14.46 to 217.03 μM), all compounds become selective for the malaria parasite. Thus *N*-methylation of the amide is an essential feature for *T. cruzi* inhibition. Also, there is a possibility that the *N*-methylated version will be MCF-7 inhibitor as well. To thoroughly explore the role of *N*-methyl groups, it is required to test the *N*-methylated version.

The highest antimalarial potency among the analogs was obtained for the pentapeptide **3.7** (IC_{50} = 217.03 μM). Antimalarial potency decreased when the peptide length is shortened in general. This lowering was maximum (15x) for the tetrapeptide **3.8** (IC_{50} = 217.03), penultimate moiety at the C-terminus is a valine. Replacement of the penultimate valine with an isoleucine (**3.9**) increased the potency (4x; IC_{50} = 49.44 μM) in comparison to **3.8**. A similar trend was observed in the tripeptide series as well. Compound **3.10** and **3.11** contain valine and isoleucine, respectively, as the penultimate residue at the C-terminus. And, the potency increased by 2-fold in **3.11 (**IC_{50} = 23.99 μM). Though all synthesized compounds were less potent in comparison to naranjamide, we saw a trend of comparative potency improvement when the penultimate residue is isoleucine instead of valine at the C-terminus. It can be hypothesized that, if dolaphenine binds at a specific region at the binding pocket, then there is a hydrophobic region in close proximity to the

dolaphenine binding site that can be accessed with isoleucine. Replacing this penultimate residue with alanine, a polar side chain, and isoleucine homolog will provide more information about this binding site. Besides, if such a hydrophobic site exists, a naranjamide analog with isoleucine at the second last position will increase the antimalarial potency.

3.6 Conclusion

The information obtained from naranjamide analogs can act as the starting point for further structure-activity relationship study. Non-methylated analogs seem to be malaria selective compounds; however, the Chagas selective features are yet to be determined. Also, other medicinal chemistry approaches like alanine scanning and cyclization could elucidate more information about the lead compounds pharmacophore and required features for selectivity and potency.

Chapter IV: Experimental

4.1. General

All evaporations were carried out in vacuum with Heidolph Hei-Vap Advantage Rotary Evaporator. All compounds and reaction intermediates, but molecules with *N, N*-dimethyl moiety, were dehydrated using $MgSO_4$. For reaction intermediates or compounds with *N, N*-dimethyl moiety was dehydrated using Na_2SO_4. Labconco FreeZone benchtop freeze dryer was used for concentrating samples. All NMR spectra were recorded at 400/500/600 MHz (Bruker Ultrashield™ 400, 500, and Ascend™ 600), respectively. The spectra were recorded in either deuterochloroform ($CDCl_3$) or deuteromethanol (CD_3OD) as solvent at room temperature. The following abbreviations are used to describe the peak patterns where appropriate: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, and b = broad signal. All coupling constants (*J*) are given in Hz. Mass spectra: HRMS: Thermo Scientific LTQ-XL linear ion-trap MS in ESI mode and Advion Expression compact MS. Accelerated chromatographic isolation was carried out on a Biotage Isolera One system with UV detection using HPLC grade solvent. Column chromatography was performed using Sorbent Technologies silica gel (230-400 mesh). Analytical TLC was performed using Merck silica gel 60 F_{254s} foil plates. Analytical HPLC was performed on a Dionex Ultimate 3000 system using Phenomenox Kinetex C-18 HPLC column. HPLC grade solvents were used for column chromatography and all reactions.

All starting materials and HPLC grade solvents were purchased from Fisher Scientific or Sigma and used without further treatment.

4.2. Field sampling, extraction, and isolation

An orange cyanobacterial mat was collected in July 2010 (collection code PAP-17Jul10-1A) by hand using SCUBA at Dos Hermanas in the Portobelo National Park (on the Caribbean side), Panama (GPS coordinates: N 9 35.860 W 79 40.106). A voucher sample was taken for storage at the Smithsonian Tropical Research Institute in Panama. It was given a field identification of Lyngbia penificiliformis. Unfortunately, phylogenetic analysis was not possible because of the degradation of the sample preserved for 16s-rRNA extraction. A total of 800 mL of cyanobacterial samples was collected. The collected biomass was then stored in the sea-water/ethanol mixture at -20°C until extraction.

The cyanobacterial mat was extracted repeatedly with a 2:1 mixture of dichloromethane: methanol (5 x 500 mL for each extraction) and filtered through cheesecloth. Organic layers obtained from each extraction were combined and concentrated by rotary evaporation at 25°C to obtain 4.2 g of the crude extract (2002). This crude extract was then subjected to flash silica gel column chromatography and separated into nine fractions with hexane:ethyl acetate (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100) and ethyl acetate:methanol (75:25, 50:50, and 0:100). Fraction 2002H was further

fractionated into four fractions by using a Sep-Pak. Fraction 2002H4, obtained from the Sep-Pak separation, yielded the pure compound naranjamide (**6**) upon further purification in HPLC as fraction 2002H4D.

4.3. Synthetic procedures

4.3.1. Procedure for peptide coupling using EDC·HCl/HOBt

To a round bottom flask/glass vial the amine protected carboxylic acid (1 eq), the carboxy protected amine (1 eq), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrogen chloride (EDC·HCl, 1.1 eq), hydroxybenzotriazole hydrate (HOBt·xH_2O, 1.1 eq) was added in dichloromethane (0.1 to 0.2 M) at 0°C, which resulted in a heterogeneous suspension. After 5 min, 3 eq of *N, N*-diisopropylethylamine (DIPEA), or triethylamine (TEA) was added to the reaction mixture, which turned it into a homogeneous solution. The reaction was run for 14-48 hours at room temperature, after which the reaction mixture was diluted with dichloromethane. Except for compound **2.41** and naranjamide analogs, the reaction mixture was then successively washed with 10% hydrochloric acid (3x), saturated sodium bicarbonate (3x), and brine (1x). The organic part was dried over anhydrous magnesium sulfate, filtered, concentrated under reduced pressure, and purified by flash silica column chromatography. For compound **2.41**, the reaction mixture was washed 10% hydrochloric acid. Aqueous parts were then combined, basified with 10%

NaOH and extracted with dichloromethane. The pure compound was isolated using flash column chromatography. All the analogs (**3.7** to **3.12**) were purified by using flash chromatography described in section 4.4.

4.3.2. Procedure for ester hydrolysis

To a 100 mL round bottom flask, the carboxy protected peptide (1 eq) was dissolved in methanol (0.2 M). Sodium hydroxide (3-5 eq) was added, and the reaction was run for 3 hours under reflux condition at 90-95° C. After that, the reaction mixture was concentrated under reduced pressure to evaporate all methanol, dissolved in water and acidified with hydrochloric acid. For compound **2.45**, the hydrolyzed product was then extracted with ethyl acetate (3x15 mL). The organic part was dried over anhydrous magnesium sulfate, was filtered, and was concentrated under reduced pressure. Due to the zwitterionic nature of compound **2.42**, the acidified solution was dried completely. The solid residue was then suspended in acetone and filtered to remove undissolved NaCl, which was generated due to acidic workup. The acetone soluble fraction was then dried and purified by column chromatography.

4.3.3. Procedure for t-Boc deprotection

The *t*-Boc-protected amine (1 eq) was treated with a mixture of trifluoracetic acid and dichloromethane (1:1; 0.01 M) for 2 hours. The solvent was removed in vacuo, and the crude compound was then directly transferred for the next step.

4.3.4. Procedure for methylation

To a solution of the *t*-Boc -protected peptide (1 equiv) in dry THF (0.15 M) was added NaH (60% dispersion in mineral oil, 25 eq), and the solution was stirred under nitrogen at 0° C for 2 h. Methyl iodide (50 eq) was added dropwise, and the resulting solution was stirred to room temperature for a further 22 h. The reaction mixture was quenched with dropwise addition of water and extracted with ethyl acetate, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The resulting yellow or colorless oil was purified by flash silica column chromatography (1:99 methanol: dichloromethane) to yield methylated peptide.

4.3.5. Procedure for coupling using HATU

In a reaction vial, containing the dried *t*-Boc removed **2.48** (22 mg, 0.05 mmol, 1 eq), was added the acid (**2.42,** 12 mg, 0.05 mmol, 1 eq), DIPEA (26 µl, 0.15 mmol, 3 eq) and HATU (38 mg, 0.1 mmol, 2 eq) and dimethyl formamide (DMF). The reaction mixture was stirred at room temperature overnight. The reaction mixture was then dried using high

vacuum and purified by automated flash chromatography using Biotage KP-Sil SNAP Cartridge and methanol: dichloromethane mobile phase (1:99 to 6:94) to yield **2.5.**

4.4. Synthesis and characterization

4.4.1. *Methyl dimethyl-L-valyl-L-valinate* (**2.41**)

Following the procedure of peptide coupling described in section 4.3.1, compound **2.41** was isolated by flash silica column chromatography using 3% methanol in dichloromethane as a white solid. Yield: 436.93 mg, 88%. ^{1}H NMR (500 MHz, $CDCl_3$): 0.92-1.02 (12 H, m, 4xCH_3), 2.11 (1H, m, CH), 2.21 (1H, m, CH), 2.29 (6H, s, 2xCH_3), 2.50 (1H, d, CH), 3.74 (3H, s, CH_3), 4.57 (1H, q, CH), 6.88 (1H, d, NH); ^{13}C NMR (126 MHz, MeOD):15.7, 17.1, 18.4, 18.5, 27.2, 30.1, 41.1, 72.5, 165.9. 4.6.7.

4.4.2. *Dimethyl-L-valyl-L-valine* (**2.42**)

Following the procedure of ester hydrolysis described in section 4.3.2, compound **2.42** was isolated by flash silica column chromatography using 3% methanol in dichloromethane as a colorless solid. Yield: 280.3 mg, 65%. ^{1}H NMR (500 MHz, MeOD): 1.01 (9H, d, 3xCH3), 1.17 (3H, d, CH3), 2.24 (1H, m, CH), 2.42 (1H, bs, CH), 2.92 (6H, s, 2xCH3), 3.92 (1H, s, CH), 4.38 (1H, d, CH), 8.67 (1H, d, NH); ^{13}C NMR (126 MHz, MeOD): δ 15.5, 17.1, 18.4, 18.5, 27.2, 30.0, 41.1, 72.5, 72.5, 165.9.

4.4.3. *Methyl (tert-butoxycarbonyl)-L-isoleucyl-L-valinate* (**2.44)**

Following the general procedure of peptide coupling described in 4.3.1, compound **2.44** was synthesized and isolated by flash silica column chromatography using 15% ethyl acetate in hexane as a white solid. Yield: 430 mg, 83%; ^{1}H NMR (400 MHz, $CDCl_3$): 0.92 – 0.97 (12H, m, 4xCH_3), 1.17 (1H, m, CH), 1.46 (9H, s, 3xCH_3), 1.53 (1H, m, CH_2), 1.89 (1H, m, CH_2), 2.19 (1H, qd, CH), 3.76 (3H, s, CH_3), 3.96 (1H, m, CH), 4.57 (1H, dd, CH), 5.05 (1H, dd, NH), 6.36 (1H, dd, NH); ^{13}C NMR (126 MHz, CDCl3) : 11.3, 15.4, 17.7, 18.9, 24.8, 28.3, 31.1, 36.9, 52.0, 57.0, 59.3, 79.7, 155.8, 171.8, 172.1

4.4.4. *(Tert-butoxycarbonyl)-L-isoleucyl-L-valine* (**2.45**)

Following the general procedure of ester hydrolysis described in 4.3.2, compound **10** was obtained after drying as a white solid. Yield: 390 mg, 96%; ^{1}H NMR (400 MHz, $CDCl_3$): 0.88-0.99 (12 H, m, 4xCH_3), 1.16 (1H, m, CH), 1.45 (9H, s, CH), 1.56 (1H, m, CH_2), 1.83 (1H, m, CH_2), 4.05 (1H, t, CH), 4.62 (1H, dd, CH), 5.38 (1H, m, NH), 6.91 (1H, m, NH). ^{13}C NMR (126 MHz, MeOD) δ 10.7, 15.2, 17.3, 18.9, 24.9, 28.2, 28.3, 31.0, 36.3, 56.8, 59.2, 80.2, 156.4, 173.0, 174.3.

4.4.5. *Tert-butyl ((2S,3S)-3-methyl-1-(((S)-3-methyl-1-oxo-1-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)butan-2-yl)amino)-1-oxopentan-2-yl)carbamate* (**2.47**)

Following the general procedure of peptide coupling described in 4.3.1, compound **2.47** was synthesized and isolated by flash silica column chromatography using 25% ethyl acetate in hexane as a white solid. Yield: 356.6 mg, 50%; $[\alpha]_D^{27}$ -35.50 (c 0.9, DCM); ^{1}H NMR (400 MHz, $CDCl_3$): 0.79-0.96 (12H, m, 4xCH3), 1.15 (1H, m, CHH), 1.47 (9H, s, 3xCH3), 1.54 (1H, m, CHH), 1.90 (1H, m, CH), 2.13 (1H, m, CH), 3.31 (2H, dd, CH2), 3.95 (1H, t, CH), 4.40 (1H, dd, CH), 5.43 (1H, d, NH), 5.65 (1H, dd, CH), 6.64 (1H, d, NH), 7.10 (1H, m, CH, aromatic ring), 7.22 (4H, m, 4xCH, aromatic ring), 7.45 (1H, dd, NH), 7.78 (1H, d, CH, aromatic ring) ; 13C NMR (126 MHz, CDCl3): 10.7, 15.2, 17.3, 18.9, 24.9, 28.2, 31.0, 36.3, 56.8, 59.2, 80.2, 156.4, 173.0, 174.3. MS: m/z calcd for [M+, $C_{27}H_{40}N_4O_4S$+]: 516.28; found: 516.7.

4.4.6. *Tert-butyl methyl((2S,3S)-3-methyl-1-(methyl((S)-3-methyl-1-(methyl((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)-1-oxobutan-2-yl)amino)-1-oxopentan-2-yl)carbamate* (**2.48**)

Following the procedure described for methylation in *4.3.4*., compound **2.48** obtained as a colorless oil. Yield: 73.5 mg, 82%. $[\alpha]_D^{27}$ -264.0 (c 0.3, DCM) NMR data are assigned on the basis of 2D correlations. ^{13}C data are extracted from HSQC and HMBC spectra; % ^{1}H NMR (500 MHz, $CDCl_3$): Mixture of rotamers; major rotamers: 0.70 (6H, m, 2xC*H*$_3$), 0.72 (6H, m, 2xC*H*$_3$), 1.45 (9H, s, 3xC*H*$_3$), 2.01 (1H, m, C*H*), 2.30 (1H, m,

CH), 2.46 (3H, s, C*H*$_3$), 2.70 (3H, s, C*H*$_3$), 2.87 (3H, s, C*H*$_3$), 3.30 (1H, m, CH*H*), 3.69 (1H, d, C*H*H), 4.58 (1H, d, C*H*), 5.05 (1H, d, C*H*), 6.62 (1H, d, C*H*), 7.27 (2H, d, C*H*, aromatic ring), 7.37 (4H, m, aromatic ring), 7.77 (1H, d, aromatic ring). ^{13}C NMR: 10.9, 14.8, 16.8, 18.9, 20.0, 28.3, 29.7, 29.4, 29.9, 35.9, 54.3, 58.1, 58.5, 77.3, 120.1, 128.6, 129.4, 142.6, 169.5, 170.2, 170.6; [M+Na$^+$, $C_{30}H_{46}N_4O_4NaS^+$]: 581.76; found: 580.7.

4.4.7. *(2S,3S)-2-(2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-N,3-dimethyl-N-((S)-3-methyl-1-(methyl((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)-1-oxobutan-2-yl)pentanamide* (naranjamide, **2.5)**.

Following the procedure described for HATU coupling, compound **5** was obtained as a white solid with an overall yield of 5%. $[\alpha]_D^{27}$ -506.7 (c 0.12, DCM) MS data and comparison of proton NMR with that of natural naranjamide are given in figure **4.9** and table **4.1**.

4.4.8. *((S)-3-methyl-1-oxo-1-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)butan-2-yl)carbamate* (**3.14**).

Following the general procedure of peptide coupling described in section 4.3.1, compound **3.14** was synthesized. The impure mixture was a yellow oil. Successive column purification using Biotage KP-Sil SNAP Cartridge and methanol: dichloromethane (1:10) yield fractions with the target molecule and some impurities. All fractions were recombined and then purified as a white solid by manual flash silica column using 2 % methanol in

dichloromethane. Yield: 179.8 mg, 89%. ^{1}H NMR (500 MHz, $CDCl_3$): 0.83-0.92 (6H, m, 2xCH_3), 1.46 (9H, s, 3xCH_3), 2.12-2.21 (1H, m, CH), 3.32 (2H, t, CH_2), 3.94 (1H, t, CH), 4.95 (1H, bs, NH), 5.65 (1H, dd, CH), 6.75 (1H, bs, NH), 7.10 (2H, d, 2xCH, aromatic ring), 7.22-7.26 (4H, m, 4xCH, aromatic ring), 7.76 (1H, d, CH, aromatic ring)

4.4.9. *Tert-butyl ((2R,3S)-3-methyl-1-oxo-1-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)pentan-2-yl)carbamate* (**3.15**)

Following the general procedure of peptide coupling described in section 4.3.1, compound **3.14** was synthesized and isolated by flash silica column chromatography using 3% methanol in dichloromethane. Yield: 116.7 mg, 56%.

4.4.10. *(S)-2-(dimethylamino)-3-methyl-N-((S)-3-methyl-1-(((S)-3-methyl-1-oxo-1-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)butan-2-yl)amino)-1-oxobutan-2-yl)butanamide* (**3.8**)

Following the general procedure of peptide coupling described in section 4.3.1, compound **3.8** was synthesized and isolated by flash silica column chromatography using 0.5% to 2% methanol in dichloromethane. Yield: 41.1 mg, 65%.

4.4.11 *(2S,3S)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-3-methyl butanamido)-3-methyl-N-((S)-2-phenyl-1-(thiazol-2-yl)ethyl)pentanamide* (**3.9**)

Following the general procedure of peptide coupling, compound **3.9** was synthesized and purified as a white solid by flash column chromatography using 5% methanol in dichloromethane. Yield: 15.9 mg; 25%. ^{1}H NMR (500 MHz, $CDCl_3$): 0.80-

085 (6H, m, 2xCH_3). 0.90-0.98 (9H, m, 3xCH_3), 1.04 (3H, d, Ch_3), 1.28 (2H, m, obscured, CH_2), 1.92 (1H, m, CH), 2.13 (1H, m, CH), 2,29 (6H, s, 2xCH_3), 2.54 (1H, d, CH), 3.32 (2H, d, CH_2), 4.21 (1H, t, CH), 4.35 (1H, dd, CH), 5.65 (1H, dd, CH), 6.39 (1H, d, NH), 6.97 (2H, m, b, 2xNH), 7.12 (2H, d, 2CH, aromatic ring), 7.21-7.27 (4H, m, 4xCH, aromatic ring), 7.74 (1H, d, CH, aromatic ring)

4.4.12. *Synthesis of (S)-2-(dimethylamino)-3-methyl-N-((S)-3-methyl-1-oxo-1-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)butan-2-yl)butanamide* (**3.10**)

Following the general procedure of peptide coupling described in 4.3.1, compound **3.10** was synthesized and purified as a white solid by flash column chromatography using 5% methanol in dichloromethane. The total amount of the synthesized compound was not recorded.

4.4.13. *(2S,3S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-3-methyl-N-((S)-2-phenyl-1-(thiazol-2-yl)ethyl)pentanamide* (**3.11)**

Following the general procedure of peptide coupling described in 4.3.1, compound **3.11** was synthesized and purified as a white solid by flash column chromatography using 5% methanol in dichloromethane. Yield: 20.7 mg; 39%. ^{1}H NMR (500 MHz, $CDCl_3$): 0.85-0.92 (9H, m, 3xCH_3), 1.02 (3H, d, CH_3), 1.09-1.16 (1H, m, CH*H*), 1.44-1.54 (1H, m, C*H*H), 1.86-1.93 (1H, m, CH), 2.11 (1H, dt, CH), 2.23 (6H, s, 2xCH_3), 2.48 (1H, d, CH), 3.30 (2H, d, CH_2), 4.34 (1H, dd, CH), 5.62 (1H, dd, CH), 6.87 (1H, d, NH), 6.90 (1H, d,

NH), 7.10 (2H, dd, 2CH, aromatic ring), 7.18-7.24 (4H, m, 4xCH, aromatic ring), 7.75 (1H, d, CH, aromatic ring)

4.4.14. *Synthesis of (S)-2-(dimethylamino)-3-methyl-N-((S)-2-phenyl-1-(thiazol-2-yl)ethyl)butanamide* (**3.12**)

Following the general procedure of peptide coupling described in 4.3.1, compound **3.12** was synthesized and purified as a white solid by preparative HPLC. Yield: 17.3 mg; 54%. ^{1}H NMR (500 MHz, $CDCl_3$): 0.68 (3H, d, CH_3), 0.92 (3H, d, CH_3), 2.19- 2.05 (1H, m, CH), 2.16 (6H, s, 2xCH_3), 2.43 (1H, d, CH), 3.30 (1H, dd, CH*H*), 3.39 (1H, dd, C*H*H), 5.73 (1H, dd, CH), 6.97 (1H, d, NH), 7.18-7.28 (4H, m, 6xCH, aromatic ring), 7.77 (1H, d, CH, aromatic ring).

Due to loss of data, spectroscopic information for **3.8**, **3.10,** and **3.15** are not presented here.

4.5. Bioassays

4.5.1. Chagas disease bioassay

Trypanosoma cruzi bioassays were performed using a colorimetric method, and the inhibition of parasite growth was assessed by the expression of the reporter gene for β-galactosidase (β-Gal) in the recombinant Tulahuen clone C4 of *T. cruzi* from The American Type Cell Collection (ATCC). Assays were performed in duplicates on the amastigote stage, which is the intracellular form of the parasite infecting African green monkey kidney

(Vero) cells, exposed during 120 h to different concentrations (10, 2, and 0.4 μg/mL) of the test compounds at 37°C under an atmosphere of 5% CO_2 in air. The resulting color from the cleavage of chlorophenol red-β-D-galactoside (CPRG) by β-Gal expressed by the parasite was measured at 570 nm. The concentration that inhibited 50% expression of β-Gal (IC_{50}) was calculated by log regression of the obtained optical density values and compared with the untreated controls. Nifurtimox was used as a positive control (IC_{50} 2-5 μg/ml).

4.5.2. Malaria bioassay

The antiplasmodial activity was evaluated using a fluorometric method based on the detection of parasite DNA with the fluorochrome PicoGreen. The chloroquine-resistant strain Indochina W2 (a generous gift of The Walter Reed Army Institute of Research, Silver Spring, MD) of *Plasmodium falciparum* was used for the crude extract and subsequent fractions. For the synthetic product, the chloroquine-sensitive HB3 strain obtained from the (Malaria Research and Reference Reagent Resource Center (MR4, Manassas, VA, USA) was used. All initial screenings were performed at 10 μg/mL. The IC_{50} value was calculated by normal regression of the resulting inhibition percentages at 0.08, 0.4, 2, and 10 μg/mL. Parasites were maintained *in vitro* by a modification of the method of Trager and Jensen,[17] synchronizing the parasites with alanine, as described in Almanza *et al.*[18]

Chloroquine was used as a positive control (IC_{50} 80-100 nM for W2 and 15-26 nM for HB3).

4.5.3. Leishmania bioassay

Axenically grown (cell-free) amastigotes of *Leishmania donovani* WR2801, a kind gift from Max Grogl (Experimental Therapeutics Division, Walter Reed Army Institute of Research, Silver Spring, MD, USA), were used to assess parasite growth and survival. Samples were tested in duplicates at 10 μg/mL. The results were expressed as a percentage of growth inhibition (GI) compared to controls. Samples that showed over 70% GI were considered active and were then assayed at four different concentrations (0.08, 0.4, 2, and 10 μg/mL) to determine IC_{50} values. Amphotericin B was used as a positive control with an IC_{50} value response of 80-120 ng/ml.

4.5.4. MCF-7 cell line assay

The MCF-7 mammalian breast cancer cell line was obtained from ATCC. On the day before the bioassay, 5×10^3 cells were seeded in a final volume of 100 μl/well in 96-well plates and incubated with RPMI-1640 supplemented with gentamicin (0.05 mg/ml), L-glutamine (GIBCO; 2 mM), $NaHCO_3$ (4.6 mM), HEPES buffer (25 mM), and FBS (10%) at 37°C. For each bioassay, 100 μl of the extract was diluted in culture media, added to the cells, and incubated for 48 h at 37°C. Cells were fixed with tricholoroacetic acid

(50%), and treated with sulphorhodamine B (0.4%), which was allowed to react for 15-30 min at 22°C. The cells were then rinsed with acetic acid (1%), dried, and treated with Tris-HCl (10 mM; pH 7) for 15 min. Color intensity was read at 570 nm. The positive control used was adriamycin (normal IC_{50} value is approximately 20-50 nM).

4.6 Spectroscopic data for isolated and synthetic naranjamide

4.6.1 High-resolution ESI-MS spectrum of natural compound 2.5

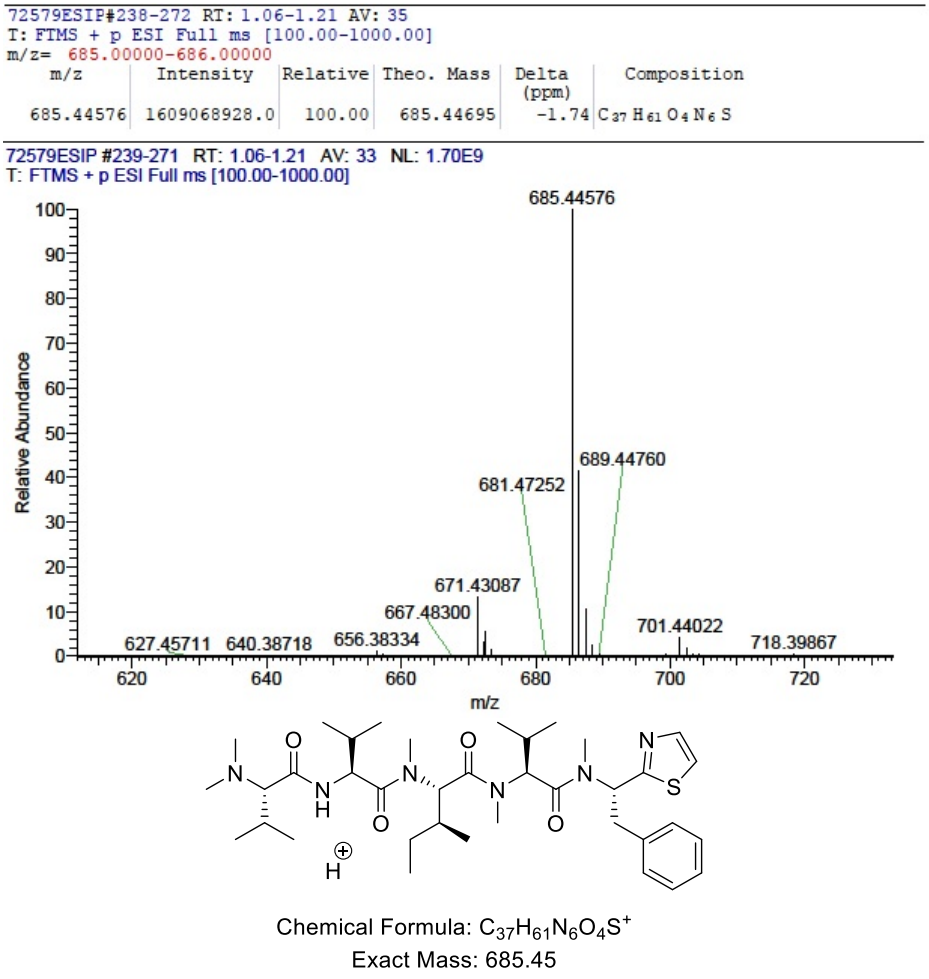
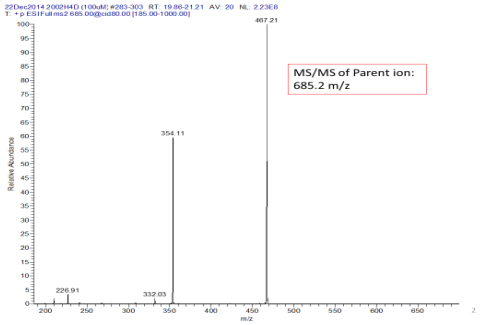
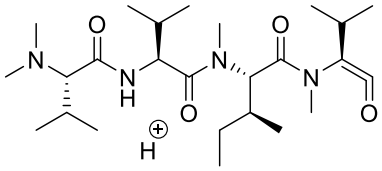


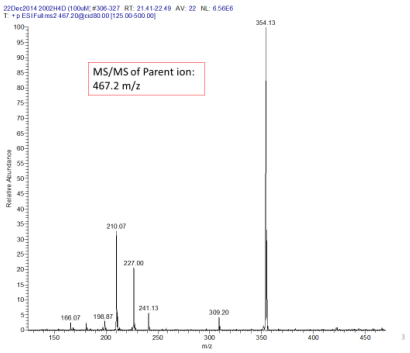
Figure 4.1: High resolution ESI-MS spectrum of natural compound **2.5** HPLC separation

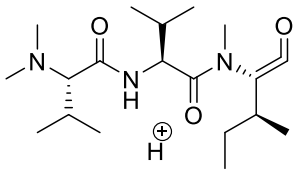
4.6.2 MS-MS fragmentation of natural compound 2.5



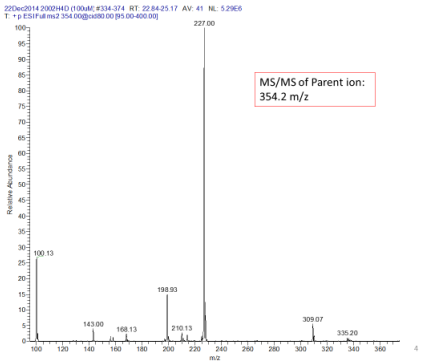


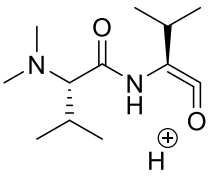
Chemical Formula: $C_{25}H_{47}N_4O_4^+$
Exact Mass: 467.36





Chemical Formula: $C_{19}H_{36}N_3O_3^+$
Exact Mass: 354.28





Chemical Formula: $C_{12}H_{23}N_2O_2^+$
Exact Mass: 227.18

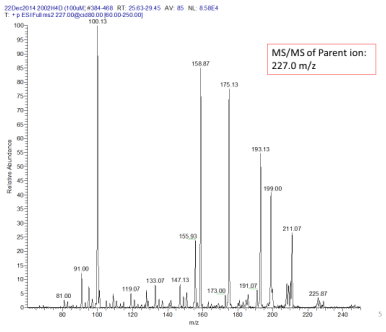


Figure 4.2: MS-MS fragmentation of natural compound **2.5** via ion trap

4.6.3 NMR data of natural compound 2.5

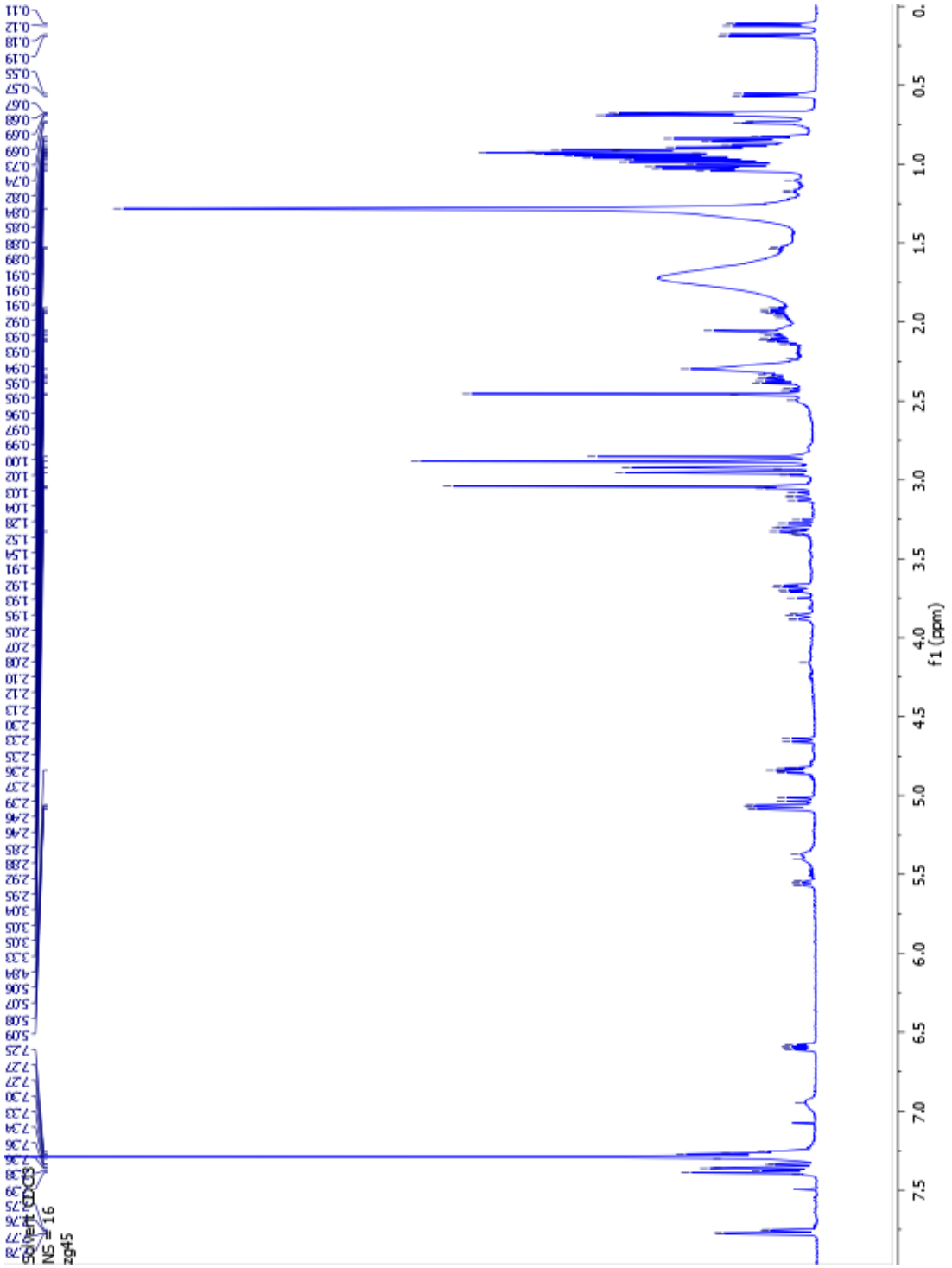


Figure 4.3: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of natural compound **2.5**

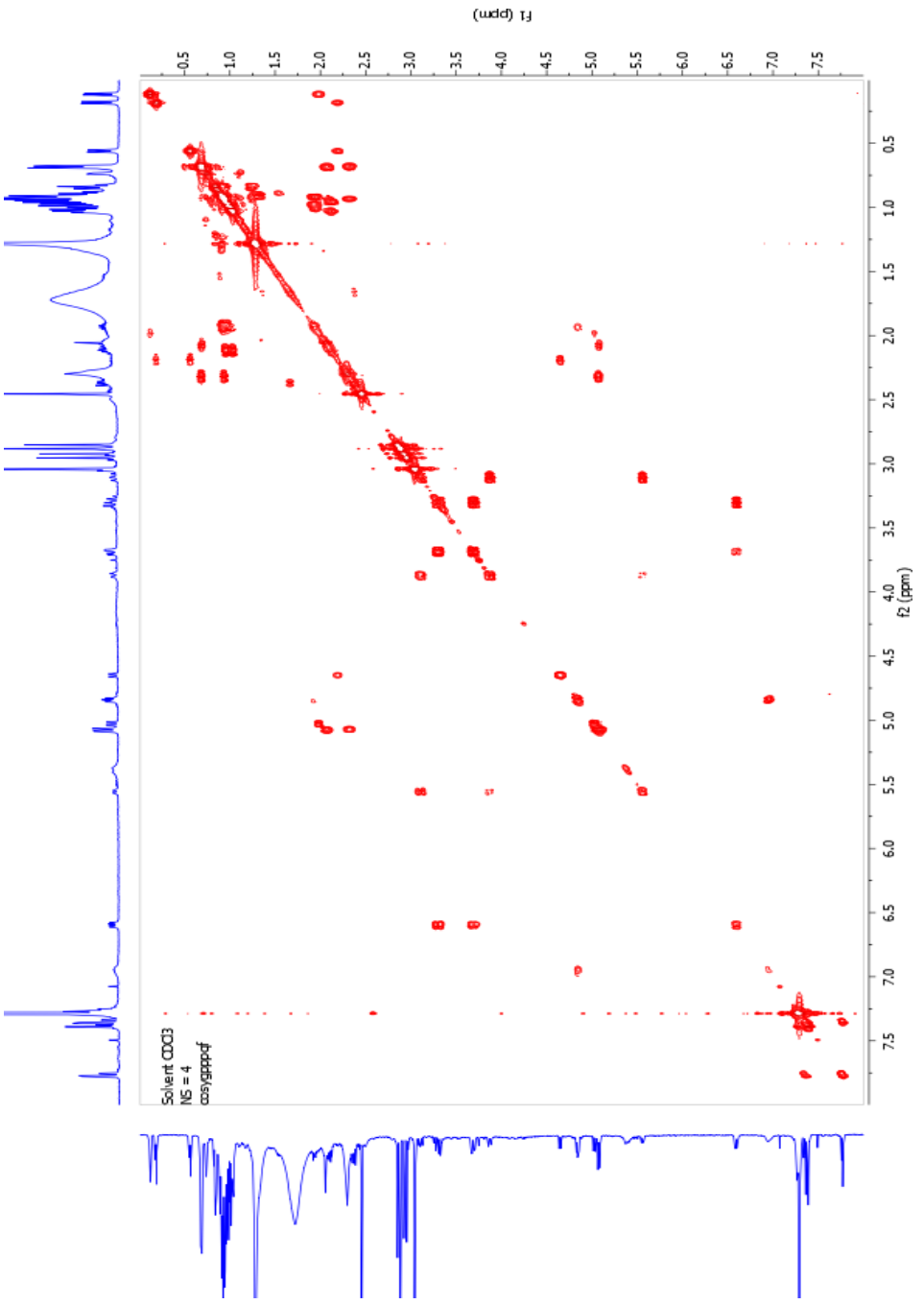


Figure 4.4: COSY spectrum ($CDCl_3$, 500 MHz) of natural compound **2.5**

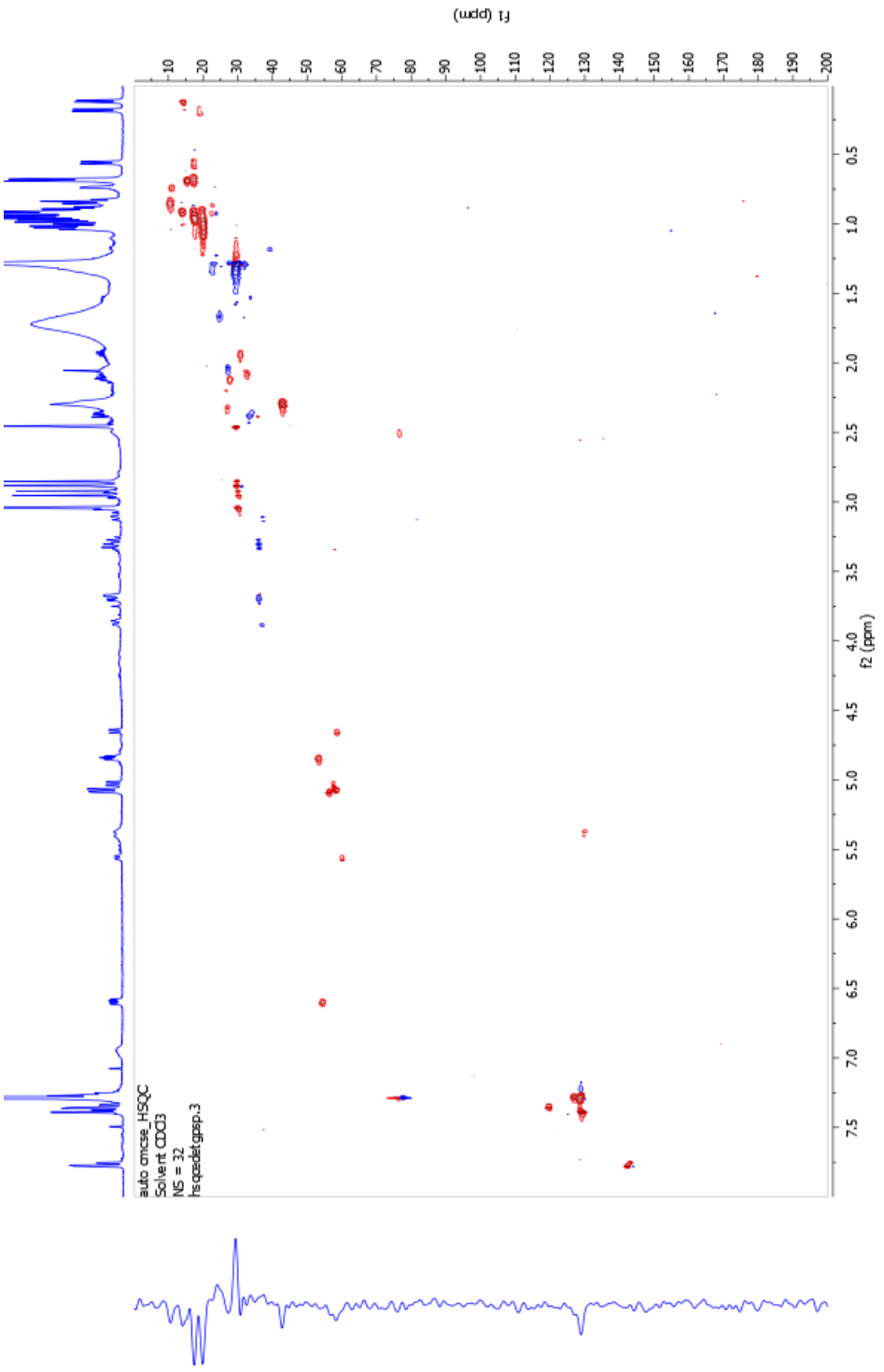


Figure 4.5: HSQC spectrum ($CDCl_3$, 500 MHz) of natural compound **2.5**

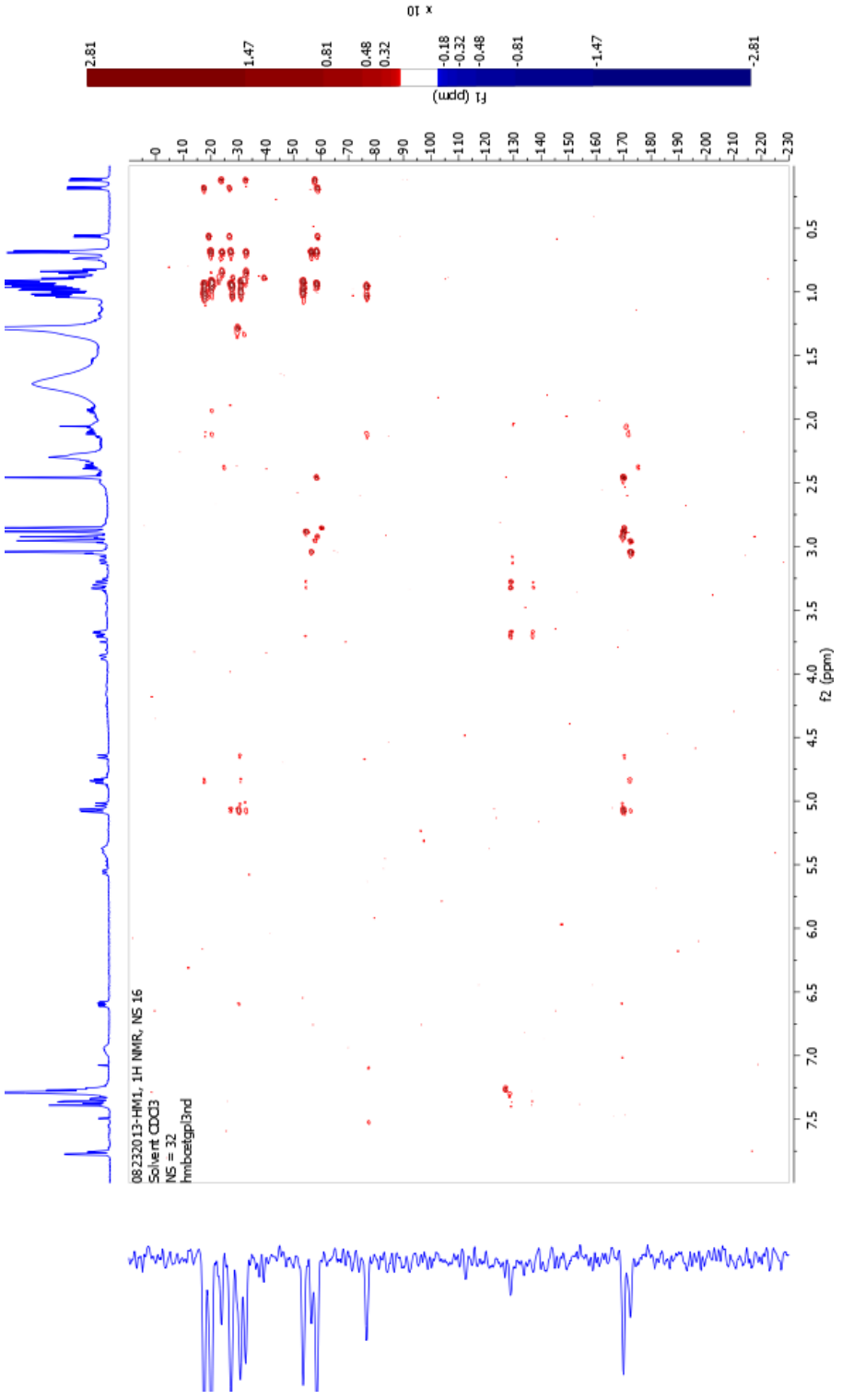

x 10
2.81
1.47
0.81
0.48
0.32
-0.18
-0.32
-0.48
-0.81
-1.47
-2.81
f1 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
f2 (ppm)
08232013-HM1, 1H NMR, NS 16
Solvent CDCl3
NS = 32
hmbcetgpl3nd

Figure 4.6: HMBC spectrum ($CDCl_3$, 500 MHz) of natural compound **2.5**

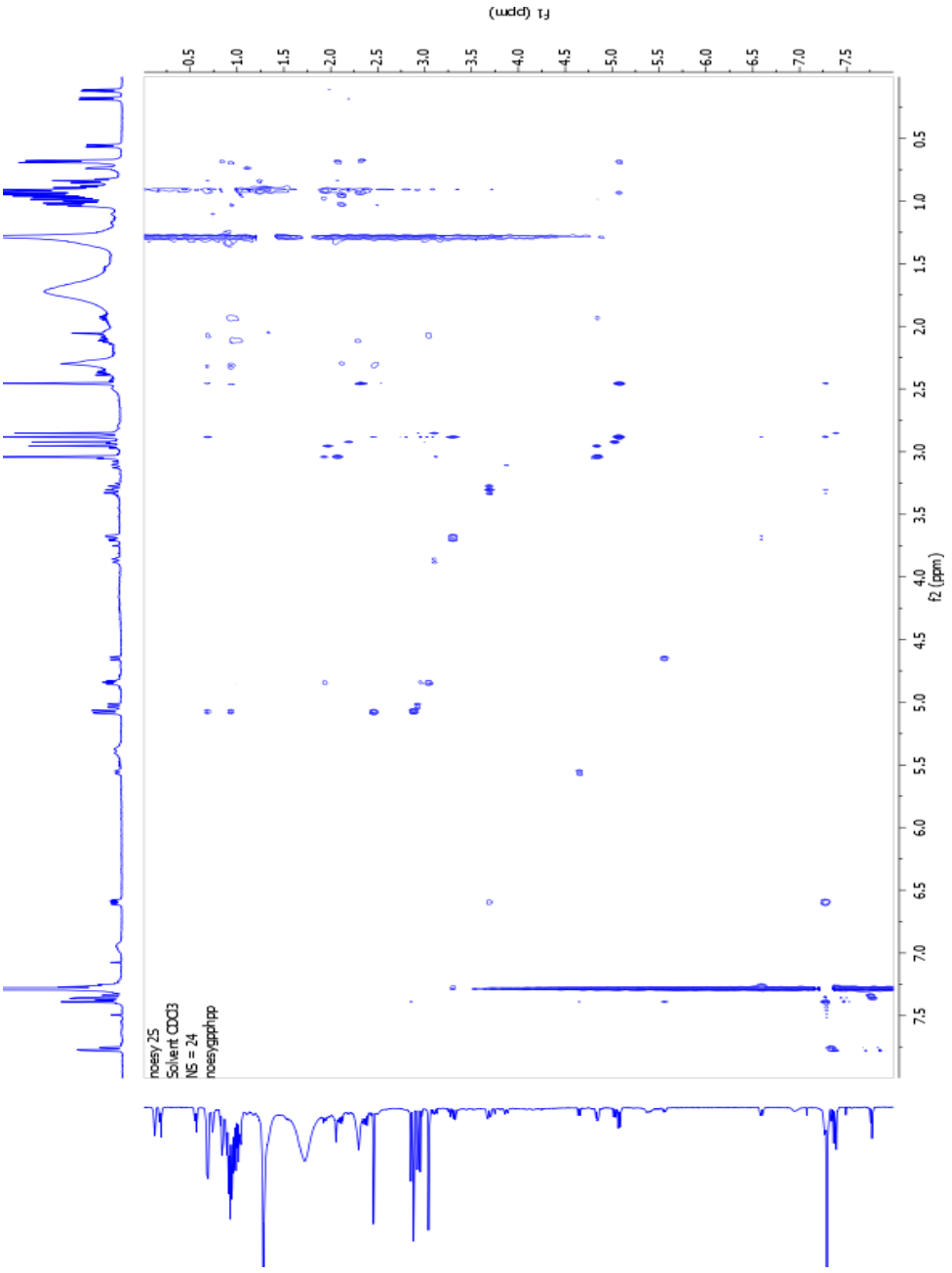


Figure 4.7: NOESY spectrum ($CDCl_3$, 500 MHz) of natural compound **2.5**.

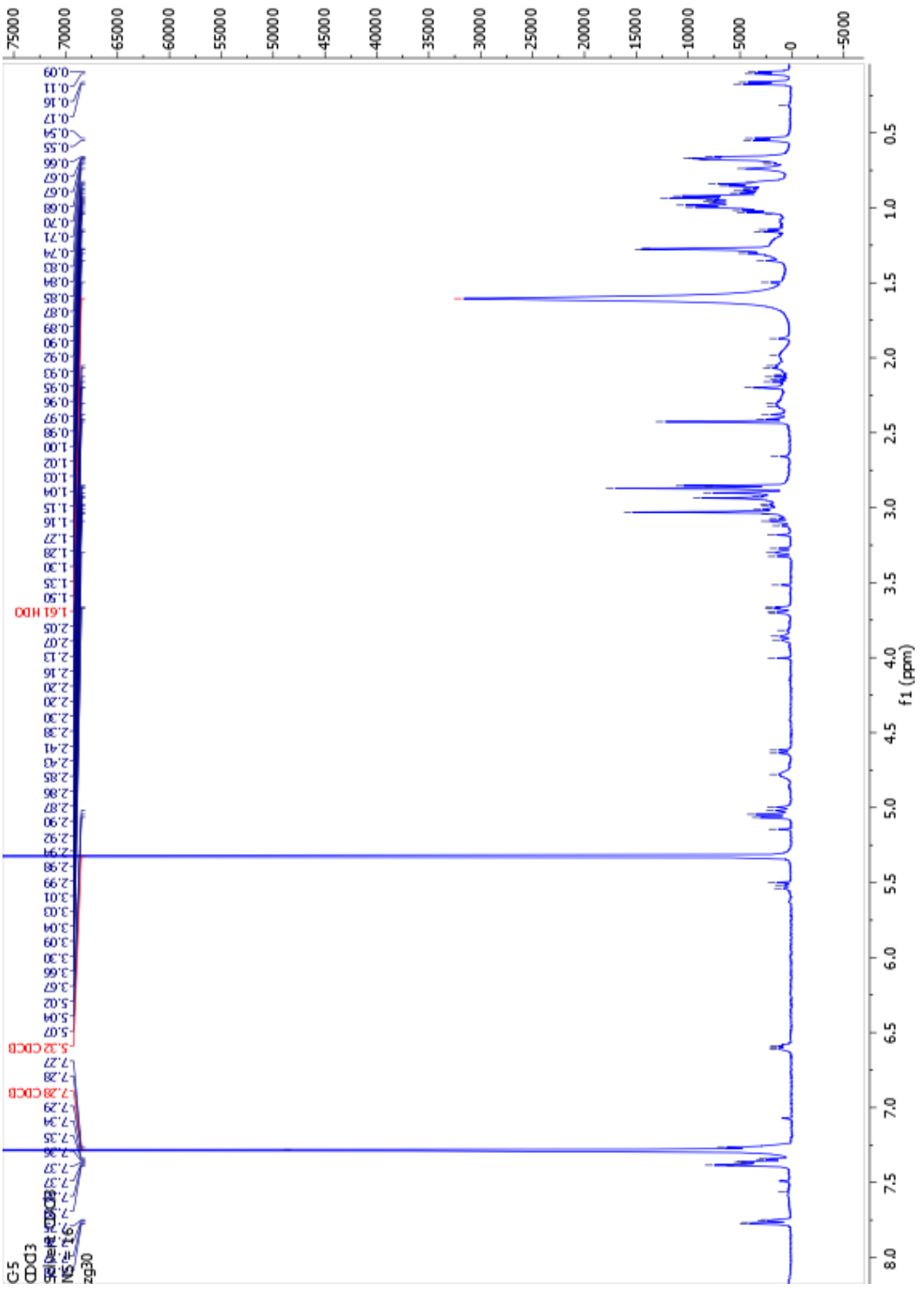


Figure 4.8: ^{1}H spectrum ($CDCl_3$, 500 MHz) of synthetic compound **2.5**

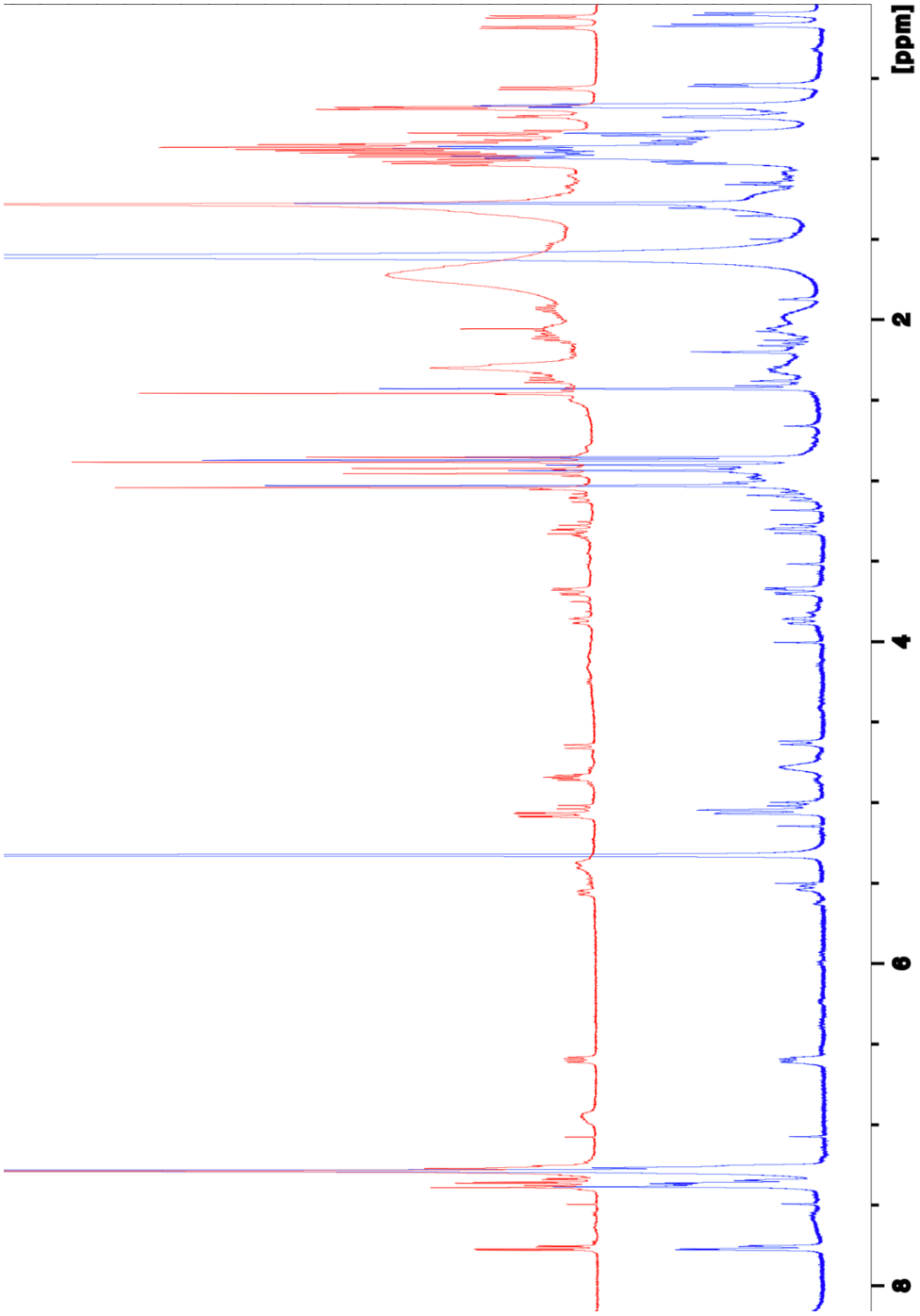


Figure 4.9: Stacked ^{1}H NMR spectra of natural and synthetic **2.5**

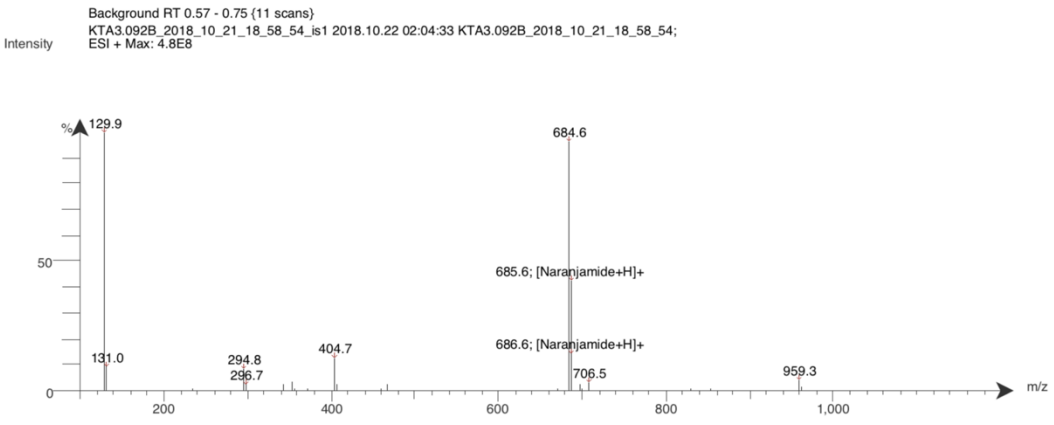


Figure 4.10: MS spectrum of synthetic compound **2.5**.

Table 4.1: Comparison of ^{1}H NMR data of natural and synthetic naranjamide

C/H No.	**δ_H of natural naranjamide (multiplicity, *J* in Hz)**	**δ_H of synthetic naranjamide**
2		
4	7.77 (d, 3.3)	7.77 (d, 3.3)
5	7.36 (d, 3.3)	3.36 (d, 3.3)
6	6.60 (dd, 11.8, 4.8)	6.60 (dd, 11.6, 4.9)
6a	3.69 (dd, 15, 4.9) 3.30 (dd, 15, 11.8)	3.69 (dd, 15.1, 4.9) 3.29 (dd, 15.0, 11.6)
1′		
2′, 6′	7.27	7.26
3′, 5′	7.39	7.38
4′	Obscured	
7a	2.87	2.87
8		
9	5.06 (d, 2.7)	5.06 (dd, 10.8, 1.9)
9a	2.32 (m)	2.31 (m)
9b, 9c	0.69, 0.93 (m)	0.68, 0.96 (m)
10a	2.46 (s)	2.43 (s)
11		
12	5.07 (dd, 10.8, 2.7)	5.06 (dd, 10.8, 1.9)

12a	2.07 (m)	2.07 (m)
12b	Obscured	
12c		
12d		
13a	3.04 (s)	3.03 (s)
14		
15	4.84 (m)	4.82 (m)
15a	1.94 (m)	1.95 (m)
15bc	0.92, 1.01 (m)	
16 NH	6.95	obscured
17		
18	2.49	
18bc	2.30 (s)	2.39 (s)
19	2.12	
20	0.95	
21	1.03	

Chapter V: Conclusion

Nature directly or indirectly inspires and reveals the solution of all problems, and science provides the way to make these insights tangible, practical, useful, and meaningful. If we take away nature's contribution from the history of drug discovery and development, it would have been still in its primitive stage. Nature is, by far, the richest contributor to drug discovery research. From ancient times to the present day, search for disease remedies in nature has been continued. Unfortunately, not all severe diseases get the required emphasis. Neglected tropical diseases (NTDs) have been known for centuries, and some of these lethal diseases are severely limited in terms of therapeutic options. The most vulnerable population to NTDs are the impoverished community of Africa and Asia, which do not promise a significant financial return for a successful NTD drug discovery campaign. Thus, most pharmaceutical companies are less interested in developing drugs for NTDs. No wonder that diseases like Chagas has only two drugs in the market, which are far from patient compliant, effective therapeutic options. Our effort led to the discovery of a compound that inhibited the growth of both malaria and the Chagas parasite. Various potent antimalarial drugs are available in the market, and yet malaria is one of the deadliest diseases on this planet. Though the total number of infected population has declined in recent years due to vector control and other preventive measures, malaria's severity is still monumental. The emergence of resistant pathogens has necessitated the discovery of new

antimalarials. Unlike malaria, Chagas disease is not widespread but still require new drugs with improved safety and efficacy since it lacks a favorable treatment option. Both malaria and Chagas disease are NTDs that require extensive drug discovery research in both academia and industry.

The discovery of marine natural products as a source of bioactive compounds introduced researchers to a new stockpile of structurally intriguing molecules. Successful efforts have been made to take these molecules from the ocean to the shelves of the clinic. And, efforts should be continued to yield the maximum output from this drug source. Billion-year-old marine cyanobacteria are among the most attractive sources among marine species that produce a plethora of bioactive molecules. Inherently, compounds from marine organisms are extremely potent since when released in their neighboring aquatic environment, despite dilution to a considerable extent, they have to function properly. Thus, marine cyanobacteria promise the discovery of highly potent bioactive molecules. The importance of NTD drug development and the potential of marine cyanobacteria as the drug source led to approaches that resulted in the discovery of a cyanobacterial secondary metabolite.

The compound was given the name naranjamide. It is a pentapeptide with multiple *N*- methylation of the amide nitrogen and contains two unnatural amino acids at the *C*-terminus and the *N*- terminus. From the knowledge of cyanobacterial biosynthetic

machinery, we can assume that naranjamide is biosynthesized via non-ribosomal peptide synthase and *N*- methyltransferase pathway.

The discovery of naranjamide and its bioactivity, led us to attempt the total synthesis of the natural product. In our attempt, we synthesized synthetic naranjamide, but the chiral integrity of the molecule was lost. One possible reason for this could be using a strong base in one of the reactions to synthesize a reaction intermediate. Interestingly, this synthetic version was also active against malaria and the Chagas parasite. Thus, our next logical approach was to explore the structure-activity relationship of naranjamide. In doing so, we synthesized a series of non-methylated analogs, where the peptide length was gradually reduced, one amino acid at a time. We found that removing all *N*- methyl groups of amide nitrogen made the analogs malaria selective, though we significantly lost the potency. The analogs also elucidated critical information about the neighboring adjacent amino acid of dolaphenine. We saw an improvement of activity when this amino acid is isoleucine instead of valine. This observation leads to the hypothesis that the target may have a hydrophobic binding pocket adjacent to the dolaphenine binding pocket and can be accessed by the isoleucine side chain.

In this attempt of naranjamide synthesis, the stereo-configuration was compromised. It will be compelling to see how a stereochemically pure synthetic naranjamide behaves in bioassays. Other structural modifications are required to build up

a complete story of naranjamide and its requirements for activity and selectivity. Synthesis of more naranjamide, will also allow researchers to elucidate its mechanism of action. Once a target is known, an *in silico* process may lead to more potent analogs through the use of molecular modeling for design. Also, target identification will allow us to explore the key features that determine the selectivity between the Chagas and malaria parasites. This project utilized both natural product chemistry and medicinal chemistry approaches to discover a potential lead for maria and Chagas disease, and obtained results can act as a good starting point for further research to complete the story of naranjamide.

References

1. Zhong, G.; Wan, F. An outline on the early pharmaceutical development before Galen. *Zhonghua Yi Shi Za Zhi* **1999,** *29*, 178-182.

2. Majumdar, S. K.; Corpus Hippocraticum 'on the sacred disease'. *Bull. Indian Inst. Hist. Med. Hyderabad* **1998,** *28*, 111-118.

3. Staub, P. O.; Casu, L.; Leonti, M. Back to the roots: A quantitative survey of herbal drugs in Dioscorides' De Materia Medica (ex Matthioli, 1568). *Phytomedicine* **2016,** *23*, 1043-1052.

4. Thakar, V. J. Historical development of basic concepts of Ayurveda from Veda up to Samhita. *Ayu.* **2010,** *31*, 400-402.

5. Cragg, G. M.; Newman, D. J. Natural Product Drug Discovery in the Next Millennium. *Pharm. Bio.* **2001,** *39* (Supp1 1), 8-17.

6. Dias, D. A.; Urban, S.; Roessner, U. A Historical Overview of Natural Products in Drug Discovery. *Metabolites* **2012,** *2*, 303-336.

7. Schiff, P. L. Opium and Its Alkaloids *Am. J. Pharm. Edu.* **2002,** *66*, 186-194.

8. Ji, H.-F.; Li, X.-J.; Zhang, H.-Y. Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? *EMBO reports* **2009,** *10*, 194-200.

9. Colchicine (marketed as Colcrys) Information. https://www.fda.gov/drugs/postmarket-drug-safety-information-patients-and-providers/colchicine-marketed-colcrys-information#:~:text=Healthcare%20professionals%20prescribe%20the%20FDA,and%20additional%20drug%20interaction%20information (accessed Nov 14, 2019).

10. McLendon, K; Preuss, C. V. Atropine. In *StatPearls*; StatPearls Publishing: Florida , 2020.

11. Newman, D. J. Natural Products as Leads to Potential Drugs: An Old Process or the New Hope for Drug Discovery? *J. Med. Chem.* **2008,** *51*, 2589-2599.

12. Atanasov, A. G.; Waltenberger, B.; Pferschy-Wenzig, E.-M.; Linder, T.; Wawrosch, C.; Uhrin, P.; Temml, V.; Wang, L.; Schwaiger, S.; Heiss, E. H.; Rollinger, J. M.; Schuster, D.; Breuss, J. M.; Bochkov, V.; Mihovilovic, M. D.; Kopp, B.; Bauer, R.; Dirsch, V. M.; Stuppner, H. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* **2015,** *33*, 1582-1614.

13. Calixto, J. B. The role of natural products in modern drug discovery. *An. Acad. Bras. Ciên. 91* (Suppl 3), **2019,** No. e20190105, doi: 10.1590/0001-3765201920190105.

14. Newman, D. J.; Cragg, G. M. Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* **2016,** *79*, 629-661.

15. US Fodd and Drug Administration. https://www.fda.gov/about-fda/fda-basics/what-does-fda-do. (accessed Nov 15, 2019)

16. Newman, D. J.; Cragg, G. M. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J. Nat. Prod.* **2020,** *83*, 770-803.

17. Patridge, E.; Gareiss, P.; Kinch, M. S.; Hoyer, D. An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Discovery Today* **2016,** *21*, 204-207.

18. Shen, B. A New Golden Age of Natural Products Drug Discovery. *Cell* **2015,** *163*, 1297-1300.

19. Wetzel, S.; Schuffenhauer, A.; Roggo, S.; Ertl, P.; Waldmann, H. Cheminformatic Analysis of Natural Products and their Chemical Space. *CHIMIA Int. J. Chem.* **2007,** *61*, 355-360.

20. Feher, M.; Schmidt, J. M. Property Distributions: Differences between Drugs, Natural Products, and Molecules from Combinatorial Chemistry. *J. Chem. Info. Comp. Sci.* **2003,** *43*, 218-227.

21. Ahmed, K. T.; Lax, N. C.; Tidgewell, K. J. Central Nervous System Modulators from the Oceans. In *Marine Biomedicine*, 1st ed.; Baker, B. J., Ed.; CRC Press: Florida, 2015; pp 247-278.

22. Tambo, E.; Khater, E. I. M.; Chen, J.-H.; Bergquist, R.; Zhou, X.-N. Nobel prize for the artemisinin and ivermectin discoveries: a great boost towards elimination of the global infectious diseases of poverty. *Infect. Dis. Poverty* **2015,** *4*, 58-58.

23. Caly, L.; Druce, J. D.; Catton, M. G.; Jans, D. A.; Wagstaff, K. M. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. *Antivir. Res.* **2020,** *178*, 104787.

24. Ōmura, S., Microbial metabolites: 45 years of wandering, wondering and discovering. *Tetrahedron* **2011,** *67*, 6420-6459.

25. Tu, Y. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. *Nat. Med.* **2011,** *17*, 1217-1220.

26. Martins, A.; Vieira, H.; Gaspar, H.; Santos, S. Marketed Marine Natural Products in the Pharmaceutical and Cosmeceutical Industries: Tips for Success. *Mar. Drugs* **2014**, *12*, 1066-1101.

27. Blunt, J. W.; Carroll, A. R.; Copp, B. R.; Davis, R. A.; Keyzers, R. A.; Prinsep, M. R., Marine natural products. *Nat. Prod. Rep.* **2018,** *35*, 8-53.

28. MarinLit. http://pubs.rsc.org/marinlit (accessed July 16, 2019).

29. van Santen, J. A.; Jacob, G.; Singh, A. L.; Aniebok, V.; Balunas, M. J.; Bunsko, D.; Neto, F. C.; Castaño-Espriu, L.; Chang, C.; Clark, T. N.; Cleary Little, J. L.; Delgadillo, D. A.; Dorrestein, P. C.; Duncan, K. R.; Egan, J. M.; Galey, M. M.; Haeckl, F. P. J.; Hua, A.; Hughes, A. H.; Iskakova, D.; Khadilkar, A.; Lee, J.-H.; Lee, S.; LeGrow, N.; Liu, D. Y.; Macho, J. M.; McCaughey, C. S.; Medema, M. H.; Neupane, R. P.; O'Donnell, T. J.; Paula, J. S.; Sanchez, L. M.; Shaikh, A. F.; Soldatou, S.; Terlouw, B. R.; Tran, T. A.; Valentine, M.; van der Hooft, J. J. J.; Vo, D. A.; Wang, M.; Wilson, D.; Zink, K. E.; Linington, R. G. The Natural Products Atlas: An Open Access Knowledge Base for Microbial Natural Products Discovery. *ACS Cent. Sci.* **2019,** *5*, 1824-1833.

30. Abdelmohsen, U. R.; Bayer, K.; Hentschel, U., Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat. Prod. Rep.* **2014,** *31*, 381-399.

31. Zhou, X.; Liu, J.; Yang, B.; Lin, X.; Yang, X. W.; Liu, Y. Marine natural products with anti-HIV activities in the last decade. *Curr. Med. Chem.* **2013,** *20*, 953-973.

32. Villa, F. A.; Gerwick, L. Marine natural product drug discovery: Leads for treatment of inflammation, cancer, infections, and neurological disorders. *Immunopharmacol. Immunotoxicol.* **2010,** *32*, 228-237.

33. Mayer, A. M. S. The Global Marine Pharmaceutical Pipeline. http://marinepharmacology.midwestern.edu/ (accessed July 10, 2020).

34. Kijjoa, A.; Sawangwong, P. Drugs and Cosmetics from the Sea. *Mar. Drugs* **2004,** *2*, 73-82.

35. Paintsil, E.; Cheng, Y.-C. Antiviral Agents. In *Encyclopedia of Microbiology,* 3rd ed,; Schaechter, M. Ed.; Academic Press: Oxford, 2009; pp 223-257.

36. Lichtman, M. A. A historical perspective on the development of the cytarabine (7days) and daunorubicin (3days) treatment regimen for acute myelogenous leukemia: 2013 the 40th anniversary of 7+3. *Blood Cells Mol. Dis.* **2013,** *50*, 119-130.

37. B.M., O., ω-Conotoxin MVIIA: From Marine Snail Venom to Analgesic Drug; In *Drugs from the Sea*, Fusetani, N., Ed.; Karger: Basel, 2000; pp 74-85.

38. Jia, J.; Chen, R.; Jia, Y.; Gu, H.; Zhou, Q.; Chen, X. Convergent Formal Synthesis of Ecteinascidin 743. *J. Org. Chem.* **2019,** *84* (21), 13696-13706.

39. Incalci, M.; Galmarini, C. M. A Review of Trabectedin (ET-743): A Unique Mechanism of Action. *Mol. Cancer Ther.* **2010**, *9*, 2157-2163.

40. Dybdal-Hargreaves, N. F.; Risinger, A. L.; Mooberry, S. L. Eribulin Mesylate: Mechanism of Action of a Unique Microtubule-Targeting Agent. *Clin. Cancer Res.* **2015,** *21*, 2445-2452.

41. Gerwick, William H.; Moore, Bradley S. Lessons from the Past and Charting the Future of Marine Natural Products Drug Discovery and Chemical Biology. *Chem. Bio.* **2012,** *19*, 85-98.

42. Martins, A.; Vieira, H.; Gaspar, H.; Santos, S., Marketed Marine Natural Products in the Pharmaceutical and Cosmeceutical Industries: Tips for Success. *Mar. Drugs* **2014**, *12*, 1066-1101.

43. Altmann, K.-H. Drugs from the Oceans: Marine Natural Products as Leads for Drug Discovery. *CHIMIA Int. J. Chem.* **2017**, *71*, 646-652.

44. Luesch, H.; Moore, R. E.; Paul, V. J.; Mooberry, S. L.; Corbett, T. H. Isolation of dolastatin 10 from the marine cyanobacterium Symploca species VP642 and total stereochemistry and biological evaluation of its analogue symplostatin 1. *J. Nat. Prod.* **2001**, *64*, 907-910.

45. Stal, L. J. Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytol.* **1995,** *131*, 1-32.

46. Hedges, S. B.; Chen, H.; Kumar, S.; Wang, D. Y.; Thompson, A. S.; Watanabe, H. A genomic timescale for the origin of eukaryotes. *BMC Evol. Biol.* **2001,** *1*, no 4, DOI: 10.1186/1471-2148-1-4.

47. Büdel, B. Cyanobacteria: Habitats and Species. In *Plant Desiccation Tolerance*; Lüttge, U.; Beck, E.; Bartels, D. Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2011; pp 11-21.

48. Singh, R. K.; Tiwari, S. P.; Rai, A. K.; Mohapatra, T. M. Cyanobacteria: an emerging source for drug discovery. *J. Antibiot. (Tokyo)* **2011,** *64*, 401-412.

49. Tidgewell, K.; Clark, B. R.; Gerwick, W. H. The Natural Products Chemistry of Cyanobacteria. In *Comprehensive Natural Products II*; Liu, H.-W.; Mander, L. Eds.; Elsevier: Oxford, 2010; pp 141-188.

50. Rague, A. L.; Parker, S.-A. J.; Tidgewell, K. J. Evaluating Marine Cyanobacteria as a Source for CNS Receptor Ligands. *Molecules* **2018**, *23*, no 2665, DOI: 10.3390/molecules23102665.

51. Karl, G.; Cyril, P. Secondary Metabolites from Cyanobacteria: Complex Structures and Powerful Bioactivities. *Curr. Org. Chem.* **2008**, *12*, 326-341.

52. Dittmann, E.; Neilan, B.; Börner, T. Molecular biology of peptide and polyketide biosynthesis in cyanobacteria. *App. Microb. Biotech.* **2001**, *57*, 467-473.

53. Volk, R.-B. Screening of microalgae for species excreting norharmane, a manifold biologically active indole alkaloid. *Microb. Res.* **2008**, *163*, 307-313.

54. Wase, N. V.; Wright, P. C. Systems biology of cyanobacterial secondary metabolite production and its role in drug discovery. *Exp. Opi. Drug. Disc.* **2008**, *3*, 903-929.

55. Xue, Y.; Zhao, P.; Quan, C.; Zhao, Z.; Gao, W.; Li, J.; Zu, X.; Fu, D.; Feng, S.; Bai, X.; Zuo, Y.; Li, P. Cyanobacteria-derived peptide antibiotics discovered since 2000. *Peptides* **2018**, *107*, 17-24.

56. Mi, Y.; Zhang, J.; He, S.; Yan, X. New Peptides Isolated from Marine Cyanobacteria, an Overview over the Past Decade. *Mar. Drugs* **2017,** *15*, no 132, DOI: 10.3390/md15050132.

57. Vijayakumar, S.; Manogar, P.; Prabhu, S. Potential therapeutic targets and the role of technology in developing novel cannabinoid drugs from cyanobacteria. *Biomed. Pharmacother.* **2016**, *83*, 362-371.

58. Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuinman, A. A.; Boettner, F. E.; Kizu, H.; Schmidt, J. M.; Baczynskyj, L.; Tomer, K. B.; Bontems, R. J. The isolation and structure of a remarkable marine animal antineoplastic constituent: dolastatin 10. *J. Am. Chem. Soc.* **1987**, *109*, 6883-6885.

59. Bai, R.; Pettit, G. R.; Hamel, E. Dolastatin 10, a powerful cytostatic peptide derived from a marine animal. Inhibition of tubulin polymerization mediated through the vinca alkaloid binding domain. *Biochem. Pharmacol.* **1990**, *39*, 1941-1949.

60. Kalemkerian, G. P.; Ou, X.; Adil, M. R.; Rosati, R.; Khoulani, M. M.; Madan, S. K.; Pettit, G. R. Activity of dolastatin 10 against small-cell lung cancer in vitro and in vivo: induction of apoptosis and bcl-2 modification. *Cancer. Chemother. Pharmacol.* **1999**, *43*, 507-515.

61. Pettit, G. R.; Srirangam, J. K.; Barkoczy, J.; Williams, M. D.; Durkin, K. P.; Boyd, M. R.; Bai, R.; Hamel, E.; Schmidt, J. M.; Chapuis, J. C., Antineoplastic agents 337. Synthesis of dolastatin 10 structural modifications. *Anticancer. Drug. Des.* **1995**, *10*, 529-544.

62. Newman, D. J.; Cragg, G. M. Current Status of Marine-Derived Compounds as Warheads in Anti-Tumor Drug Candidates. *Mar. Drugs* **2017**, *15*, no 99, DOI: 10.3390/md15040099.

63. Senter, P. D.; Sievers, E. L. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nature Biotech.* **2012**, *30*, 631-637.

64. Sachs, J.; Malaney, P. The economic and social burden of malaria. *Nature* **2002**, *415*, 680-685.

65. Neglected Tropical Disease. https://www.who.int/features/qa/58/en/ (accessed Feb 05, 2020).

66. About Malaria. https://www.cdc.gov/malaria/about/index.html (accessed Feb 07, 2020).

67. Kumar, S.; Bhardwaj, T. R.; Prasad, D. N.; Singh, R. K., Drug targets for resistant malaria: Historic to future perspectives. *Biomed. Pharmacother.* **2018,** *104*, 8-27.

68. The top 10 causes of death. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (accessed June 15, 2020).

69. World Health Organization. *World malaria report 2019*; World Health Organization: Geneva, 2019.

70. Flannery, E. L.; Chatterjee, A. K.; Winzeler, E. A. Antimalarial drug discovery — approaches and progress towards new medicines. *Nat. Rev. Microb.* **2013,** *11*, 849-862.

71. Institute of Medicine (US) Committee. Antimalarial Drugs and Drug Resistance. In *Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance*; Arrow, K. J.; Panosian, C. B.; Gelband, H. Eds. The National Academies Press: Washington, DC, 2004; pp 252-297.

72. Antony, H. A.; Parija, S. C. Antimalarial drug resistance: An overview. *Trop. Parasitol.* **2016**, *6*, 30-41.

73. Achan, J.; Talisuna, A. O.; Erhart, A.; Yeka, A.; Tibenderana, J. K.; Baliraine, F. N.; Rosenthal, P. J.; D'Alessandro, U. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Mal. J.* **2011,** *10*, no 144, DOI: 10.1186/1475-2875-10-144.

74. Lu, G.; Nagbanshi, M.; Goldau, N.; Mendes Jorge, M.; Meissner, P.; Jahn, A.; Mockenhaupt, F. P.; Müller, O. Efficacy and safety of methylene blue in the treatment of malaria: a systematic review. *BMC Medicine* **2018**, *16*, no 59, DOI: 10.1186/s12916-018-1045-3.

75. Vinetz, J. M.; Clain, J.; Bounkeua, V.; Eastman, R. T.; Fidock, D. Chemotherapy of Malaria. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 12 ed.; Brunton, L. L. Ed. The McGraw-Hill Companies, Inc.: New York 2011; pp 1021-1048.

75. Fattorusso, E.; Taglialatela-Scafati, O. Marine Antimalarials. *Mar. Drugs* **2009,** *7*, 130-152.

77. Peach, K. C.; Linington, R. G. New innovations for an old infection: antimalarial lead discovery from marine natural products during the period 2003–2008. *Future Med. Chem.* **2009**, *1*, 593-617.

78. Yousaf, M.; El Sayed, K. A.; Rao, K. V.; Lim, C. W.; Hu, J.-F.; Kelly, M.; Franzblau, S. G.; Zhang, F.; Peraud, O.; Hill, R. T.; Hamann, M. T. 12,34-Oxamanzamines, novel biocatalytic and natural products from manzamine producing Indo-Pacific sponges. *Tetrahedron* **2002**, *58*, 7397-7402.

79. El Sayed, K. A.; Kelly, M.; Kara, U. A.; Ang, K. K.; Katsuyama, I.; Dunbar, D. C.; Khan, A. A.; Hamann, M. T. New manzamine alkaloids with potent activity against infectious diseases. *J. Am. Chem. Soc.* **2001,** *123*, 1804-1808.

80. Yousaf, M.; Hammond, N. L.; Peng, J.; Wahyuono, S.; McIntosh, K. A.; Charman, W. N.; Mayer, A. M.; Hamann, M. T. New manzamine alkaloids from an Indo-Pacific sponge. Pharmacokinetics, oral availability, and the significant activity of several manzamines against HIV-I, AIDS opportunistic infections, and inflammatory diseases. *J. Med. Chem.* **2004,** *47*, 3512-3517.

81. Rao, K. V.; Santarsiero, B. D.; Mesecar, A. D.; Schinazi, R. F.; Tekwani, B. L.; Hamann, M. T. New manzamine alkaloids with activity against infectious and tropical parasitic diseases from an Indonesian sponge. *J. Nat. Prod.* **2003,** *66*, 823-828.

82. Tan, L. T. Bioactive natural products from marine cyanobacteria for drug discovery. *Phytochem.* **2007,** *68*, 954-979.

83. Salvador-Reyes, L. A.; Luesch, H. Biological targets and mechanisms of action of natural products from marine cyanobacteria. *Nat. Prod. Rep.* **2015**, *32*, 478-503.

84. Barzkar,N.; Jahromi, S.T.; Poorsaheli, H.B.; Vianello, F. Metabolites from marine microorganisms, micro, and macroalgae: Immense Scope for Pharmacology. *Mar. Drugs* **2019**, *17*, no 464, DOI: 10.3390/md17080464.

85. Linington, R. G.; Clark, B. R.; Trimble, E. E.; Almanza, A.; Ureña, L.-D.; Kyle, D. E.; Gerwick, W. H. Antimalarial Peptides from Marine Cyanobacteria: Isolation and Structural Elucidation of Gallinamide A. *J. Nat. Prod.* **2009**, *72*, 14-17.

86. McPhail, K. L.; Correa, J.; Linington, R. G.; González, J.; Ortega-Barría, E.; Capson, T. L.; Gerwick, W. H. Antimalarial Linear Lipopeptides from a Panamanian Strain of the Marine Cyanobacterium Lyngbya majuscula. *J. Nat. Prod.* **2007**, *70*, 984-988.

87. Balunas, M. J.; Linington, R. G.; Tidgewell, K.; Fenner, A. M.; Ureña, L.-D.; Togna, G. D.; Kyle, D. E.; Gerwick, W. H. Dragonamide E, a Modified Linear Lipopeptide from Lyngbya majuscula with Antileishmanial Activity. *J. Nat. Prod.* **2010**, *73*, 60-66.

88. Tripathi, A.; Puddick, J.; Prinsep, M. R.; Rottmann, M.; Tan, L. T. Lagunamides A and B: cytotoxic and antimalarial cyclodepsipeptides from the marine cyanobacterium Lyngbya majuscula. *J. Nat. Prod.* **2010,** *73*, 1810-1814.

89. Tripathi, A.; Puddick, J.; Prinsep, M. R.; Rottmann, M.; Chan, K. P.; Chen, D. Y.; Tan, L. T. Lagunamide C, a cytotoxic cyclodepsipeptide from the marine cyanobacterium Lyngbya majuscula. *Phytochem.* **2011**, *72*, 2369-75.

90. Linington, R. G.; Gonzalez, J.; Urena, L. D.; Romero, L. I.; Ortega-Barria, E.; Gerwick, W. H. Venturamides A and B: antimalarial constituents of the panamanian marine Cyanobacterium Oscillatoria sp. *J. Nat. Prod.* **2007**, *70*, 397-401.

91. Shao, C.-L.; Linington, R. G.; Balunas, M. J.; Centeno, A.; Boudreau, P.; Zhang, C.; Engene, N.; Spadafora, C.; Mutka, T. S.; Kyle, D. E.; Gerwick, L.; Wang, C.-Y.; Gerwick, W. H. Bastimolide A, a Potent Antimalarial Polyhydroxy Macrolide from the Marine Cyanobacterium Okeania hirsuta. *J. Org. Chem.* **2015,** *80*, 7849-7855.

92. Parasites - American Trypanosomiasis (also known as Chagas Disease). https://www.cdc.gov/parasites/chagas/ (accessed July 08, 2019).

93. Chagas disease (also known as American trypanosomiasis). https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis) (accessd August 21, 2019).

94. Pérez-Molina, J. A.; Molina, I. Chagas disease. *Lancet* **2018,** *391*, 82-94.

95. Chatelain, E. Chagas disease research and development: Is there light at the end of the tunnel? *Comp. Struct. Biotechnol. J.* **2017,** *15*, 98-103.

96. Bern, C.; Messenger, L. A.; Whitman, J. D.; Maguire, J. H. Chagas Disease in the United States: a Public Health Approach. *Clin. Microb. Revs.* **2019,** *33*, e00023-19, DOI: 10.1128/CMR.00023-19.

97. Jones, A. J.; Grkovic, T.; Sykes, M. L.; Avery, V. M. Trypanocidal Activity of Marine Natural Products. *Mar. Drugs* **2013,** *11*, 4058-4082.

98. Regalado, E. L.; Tasdemir, D.; Kaiser, M.; Cachet, N.; Amade, P.; Thomas, O. P., Antiprotozoal steroidal saponins from the marine sponge Pandaros acanthifolium. *J. Nat. Prod.* **2010**, *73*, 1404-1410.

99.Swain, S. S.; Paidesetty, S. K.; Padhy, R. N. Antibacterial, antifungal and antimycobacterial compounds from cyanobacteria. *Biomed. Pharmacother.* **2017**, *90*, 760-776.

100. Hayashi, S.; Ohashi, K.; Mihara, S.; Nakata, E.; Emoto, C.; Ohta, A. Discovery of small-molecule nonpeptide antagonists of nociceptin/orphanin FQ receptor: The studies of design, synthesis, and structure-activity relationships for (4-arylpiperidine substituted-methyl)-[bicyclic (hetero)cycloalkanobenzene] derivatives. *Eur. J. Med. Chem.* **2016**, *114*, 345-364.

101. Chopra, K.; Arora, V. An intricate relationship between pain and depression: clinical correlates, coactivation factors and therapeutic targets. *Expert Opi. Ther. Targets* **2014**, *18*, 159-176.

102. Pink, R.; Hudson, A.; Mouries, M. A.; Bendig, M. Opportunities and challenges in antiparasitic drug discovery. *Nat. Rev. Drug Disc.* **2005**, *4*, 727-740.

103. García-Reynaga, P.; Vannieuwenhze, M. S. Biomimetic Synthesis of Cbz-(*S*)-Dolaphenine. *Tetrahedron Lett.* **2012**, *53*, 4989-4993.

104. Roxin, A.; Zheng, G. Flexible or fixed: a comparative review of linear and cyclic cancer-targeting peptides. *Future Med. Chem.* **2012**, *4*, 1601-1618.

105. Sharma, A.; Kumar, A.; Abdel Monaim, S. A. H.; Jad, Y. E.; El-Faham, A.; de la Torre, B. G.; Albericio, F. *N*-methylation in amino acids and peptides: Scope and limitations. *Biopolymers* **2018**, *109*, e23110, DOI: https://doi.org/10.1002/bip.23110

106. Fischer, E. Ueber einige Derivate des Glykocolls, Alanins und Leucins. *Ber. Dtsch. Chem. Ges.* **1902,** *35*, 1095-1106.

107. Curtius, T. Ueber die Einwirkung von Chlorbenzoyl auf Glycocollsilber. *J. Prak. Chem.* **1881**, *24*, 239-240.

108. Bodanszky, M. *Principles of Peptide Synthesis*; Springer: Berlin, 2012.

109. Pierce, J. G.; Gordon, S.; Du Vigneaud, V., Further distribution studies on the oxytocic hormone of the posterior lobe of the pituitary gland and the preparation of an active crystalline flavianate. *J. Biol. Chem.* **1952**, *199*, 929-940.

110. Tuppy, H. The amino-acid sequence in oxytocin. *Biochim. Biophys. Acta.* **1953**, *11*, 449-450.

111. Vigneaud, V. d.; Ressler, C.; Swan, C. J. M.; Roberts, C. W.; Katsoyannis, P. G.; Gordon, S. The synthesis of an octapeptide amide with the hormonal acitivity of oxytocin *J. Am. Chem. Soc.* **1953**, *75* (19), 4879-4880.

112. Du Vigneaud, V.; Ressler, C.; Trippett, S., The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. *J. Biol. Chem.* **1953**, *205*, 949-57.

113. Jursic, B. S.; Zdravkovski, Z. A Simple Preparation of Amides from Acids and Amines by Heating of Their Mixture. *Syn. Comm.* **1993**, *23*, 2761-2770.

114. Al-Warhi, T. I.; Al-Hazimi, H. M. A.; El-Faham, A. Recent development in peptide coupling reagents. *J. Saudi. Chem. Soc.* **2012,** *16*, 97-116.

115. Albeiicio, F.; Chinchilla, R.; Dodsworth, D. J.; Nájera, C. New trends in peptide coupling reagents. *Org. Prep. Proc. Int.* **2001,** *33*, 203-303.

116. Tipper, D. J.; Strominger, J. L. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proc. Natl. Acad. Sci.* **1965,** *54*, 1133-1141.

117. Wise, E. M., Jr.; Park, J. T. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proc. Natl. Acad. Sci.* **1965,** *54*, 75-81.

118. Clayden, J.; Greeves, N.; Warren, S. *Organic Chemistry*. Oxford University Press: Oxford, UK, 2012.

119. Patrick, G. L., *An Introduction to Medicinal Chemistry*. Oxford University Press: Oxford, 2013.

120. Novel Drug Approvals for 2019. https://www.fda.gov/drugs/new-drugs-fda-cders-new-molecular-entities-and-new-therapeutic-biological-products/novel-drug-approvals-2019 (accessed May 19, 2020).

121. Beuckmann, C. T.; Ueno, T.; Nakagawa, M.; Suzuki, M.; Akasofu, S. Preclinical in vivo characterization of lemborexant (E2006), a novel dual orexin receptor antagonist for sleep/wake regulation. *Sleep* **2019,** *42*, 1-19.

122. Rappas, M.; Ali, A. A. E.; Bennett, K. A.; Brown, J. D.; Bucknell, S. J.; Congreve, M.; Cooke, R. M.; Cseke, G.; de Graaf, C.; Doré, A. S.; Errey, J. C.; Jazayeri, A.; Marshall, F. H.; Mason, J. S.; Mould, R.; Patel, J. C.; Tehan, B. G.; Weir, M.; Christopher, J. A. Comparison of Orexin 1 and Orexin 2 Ligand Binding Modes Using X-ray Crystallography and Computational Analysis. *J. Med. Chem.* **2020**, *63*, 1528-1543.

123. Roughley, S. D.; Jordan, A. M. The Medicinal Chemist's Toolbox: An Analysis of Reactions Used in the Pursuit of Drug Candidates. *J. Med. Chem.* **2011,** *54*, 3451-3479.

124. El-Faham, A.; Albericio, F. Peptide Coupling Reagents, More than a Letter Soup. *Chem. Rev.* **2011**, *111*, 6557-6602.

125. Han, S.-Y.; Kim, Y.-A., Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron* **2004**, *60*, 2447-2467.

126. Sheehan, J. C.; Hess, G. P., A New Method of Forming Peptide Bonds. *J. Am. Chem. Soc.* **1955**, *77*, 1067-1068.

127. König, W.; Geiger, R. Eine neue Methode zur Synthese von Peptiden: Aktivierung der Carboxylgruppe mit Dicyclohexylcarbodiimid unter Zusatz von 1-Hydroxy-benzotriazolen. *Chem. Ber.* **1970**, *103*, 788-798.

128. Carpino, L. A.; El-Faham, A.; Albericio, F. Efficiency in Peptide Coupling: 1-Hydroxy-7-azabenzotriazole vs 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine. *J. Org. Chem.* **1995**, *60*, 3561-3564.

129. Carpino, L. A. 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. *J. Am. Chem. Soc.* **1993**, *115*, 4397-4398.

130. Carpino, L. A.; Xia, J.; El-Faham, A. 3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene. *J. Org. Chem.* **2004,** *69*, 54-61.

131. C. Spetzler, J.; Meldal, M.; Felding, J.; Vedsø, P.; Begtrup, M., Novel acylation catalysts in peptide synthesis: derivatives of N-hydroxytriazoles and *N*-hydroxytetrazoles. *J. Chem. Soc., Perkin Trans.* **1998**, *10*, 1727-1732.

132. Subirós-Funosas, R.; Prohens, R.; Barbas, R.; El-Faham, A.; Albericio, F. Oxyma: an efficient additive for peptide synthesis to replace the benzotriazole-based HOBt and HOAt with a lower risk of explosion. *Chemistry* **2009,** *15*, 9394-403.

133. Chen, S.-H.; Farina, V.; Vyas, D. M.; Doyle, T. W.; Long, B. H.; Fairchild, C. Synthesis and Biological Evaluation of C-13 Amide-Linked Paclitaxel (Taxol) Analogs. *J. Org. Chem.* **1996**, *61*, 2065-2070.

134. Sarantakis, D.; Teichman, J.; Lien, E. L.; Fenichel, R. L., A novel cyclic undecapeptide, Wy-40,770, with prolonged growth hormone release inhibiting activity. *Biochem. Biophys. Res. Comm.* **1976**, *73*, 336-342.

135. Sheehan, J.; Cruickshank, P.; Boshart, G. Notes- A Convenient Synthesis of Water-Soluble Carbodiimides. *J. Org. Chem.* **1961**, *26*, 2525-2528.

136. Izdebski, J.; Pachulska, M.; Orlowska, A. *N*-Cyclohexyl-*N'* -isopropylcarbodiimide: a hybrid that combines the structural features of DCC and DIC. *Int. J. Pep. Prot. Res.* **1994**, *44*, 414-419.

137. Gawne, G.; Kenner, G. W.; Sheppard, R. C. Acyloxyphosphonium salts as acylating agents. Synthesis of peptides. *J. Am. Chem. Soc.* **1969**, *91*, 5669-5671.

138. Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. Reactifs de couplage peptidique I (1) - l'hexafluorophosphate de benzotriazolyl N-oxytrisdimethylamino phosphonium (B.O.P.). *Tetrahedron Lett.* **1975**, *16*, 1219-1222.

139. Montalbetti, C. A. G. N.; Falque, V. Amide bond formation and peptide coupling. *Tetrahedron* **2005**, *61*, 10827-10852.

140. Dourtoglou, V.; Ziegler, J.-C.; Gross, B. L'hexafluorophosphate de *O*-benzotriazolyl-*N,N*-tetramethyluronium: Un reactif de couplage peptidique nouveau et efficace. *Tetrahedron Lett.* **1978**, *19*, 1269-1272.

141. Sanchez, L. M.; Lopez, D.; Vesely, B. A.; Della Togna, G.; Gerwick, W. H.; Kyle, D. E.; Linington, R. G. Almiramides A-C: discovery and development of a new class of leishmaniasis lead compounds. *J. Med. Chem.* **2010**, *53*, 4187-4197.

142. Chatterjee, J.; Rechenmacher, F.; Kessler, H. *N*-Methylation of Peptides and Proteins: An Important Element for Modulating Biological Functions. *Angew. Chem. Int. Ed.* **2013**, *52*, 254-269.

143. Di Gioia, M. L.; Leggio, A.; Malagrinò, F.; Romio, E.; Siciliano, C.; Liguori, A. *N*-Methylated *α*-Amino Acids And Peptides: Synthesis And Biological Activity. *Mini Rev. Med. Chem.* **2016**, *16*, 683-90.

144. Flores, C.; Fouquet, G.; Moura, I. C.; Maciel, T. T.; Hermine, O. Lessons to Learn From Low-Dose Cyclosporin-A: A New Approach for Unexpected Clinical Applications. *Front. Immuno.* **2019**, *10*, no 588, DOI: 10.3389/fimmu.2019.00588.

145. Kumawat, M. K. Thiazole Containing Heterocycles with Antimalarial Activity. *Curr Drug Discov. Technol.* **2018**, *15*, 196-200.

146. Chhabria, M. T.; Patel, S.; Modi, P.; Brahmkshatriya, P. S. Thiazole: A Review on Chemistry, Synthesis and Therapeutic Importance of its Derivatives. *Curr. Top. Med. Chem.* **2016**, *16*, 2841-2862.

147. Welker, M.; Von Döhren, H. Cyanobacterial peptides — Nature's own combinatorial biosynthesis. *FEMS Microb. Rev.* **2006**, *30*, 530-563.

148. Clayton, J. Chagas disease 101. *Nature* **2010,** *465*, S4-S5.

149. Craik, D. J.; Fairlie, D. P.; Liras, S.; Price, D. The Future of Peptide-based Drugs. *Chem. Bio. Drug. Des.* **2013**, *81*, 136-147.

150. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J., Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug. Deliv. Rev.* **1997**, *23*, 3-25.

151. Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisky, M. Synthetic therapeutic peptides: science and market. *Drug Discov. Today* **2010**, *15*, 40-56.

152. Simmons, T. L.; Engene, N.; Ureña, L. D.; Romero, L. I.; Ortega-Barría, E.; Gerwick, L.; Gerwick, W. H. Viridamides A and B, lipodepsipeptides with antiprotozoal activity

from the marine cyanobacterium Oscillatoria nigro-viridis. *J. Nat. Prod.* **2008**, *71*, 1544-1550.

153. Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H. N-Methylation of Peptides: A New Perspective in Medicinal Chemistry. *Acc. Chem. Res.* **2008**, *41*, 1331-1342.

154. Sunil, K.; Ranjana, A., Thiazole: A Privileged Motif in Marine Natural Products. *Mini-Rev Org Chem* **2019**, *16*, 26-34.

155. Sharma, P. C.; Bansal, K. K.; Sharma, A.; Sharma, D.; Deep, A. Thiazole-containing compounds as therapeutic targets for cancer therapy. *Eur. J. Med. Chem.* **2020**, *188*, no. 112016. DOI: https://doi.org/10.1016/j.ejmech.2019.112016.

156. Lefèvre, F.; Rémy, M.-H.; Masson, J.-M. Alanine-stretch scanning mutagenesis: a simple and efficient method to probe protein structure and function. *Nuc. Acids Res.* **1997**, *25*, 447-448.

157. Hummel, G.; Reineke, U.; Reimer, U., Translating peptides into small molecules. *Mol. Biosyst.* **2006**, *2*, 499-508.

158. Qian, Z.; Rhodes, C. A.; McCroskey, L. C.; Wen, J.; Appiah-Kubi, G.; Wang, D. J.; Guttridge, D. C.; Pei, D. Enhancing the Cell Permeability and Metabolic Stability of Peptidyl Drugs by Reversible Bicyclization. *Angew. Chem. Int. Ed.* **2017**, *56*, 1525-1529.

159. Rezai, T.; Yu, B.; Millhauser, G. L.; Jacobson, M. P.; Lokey, R. S. Testing the conformational hypothesis of passive membrane permeability using synthetic cyclic peptide diastereomers. *J. Am. Chem. Soc.* **2006**, *128*, 2510-2511.

160. Borthwick, A. D. 2,5-Diketopiperazines: Synthesis, Reactions, Medicinal Chemistry, and Bioactive Natural Products. *Chem. Rev.* **2012**, *112*, 3641-3716.