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PhD. Thesis

STUDY OF THE EFFECTS OF DIFFERENT STRESSORS ON CYANOTOXIN PRODUCTION THROUGH METABOLOMICS AND METAGENOMIC

BY

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"The only way I can tell that a new idea is really important is the feeling of terror that seizes me."

JAMES FRANCK
Nobel Prize in Physics 1925

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ABSTRACT

Cyanobacteria are the most primitive group of organisms thriving on the earth's surface for millions of years. During this course of time, they have adapted, modified and evolved regarding the environmental niches. They have turned out to be one of the most scientifically used organisms, whether it can be for pharmacological causes, ecological causes or anything non-ecological. Cyanobacteria are green, photosynthetic bacteria that produce most of their food and important metabolites themselves. This PhD Thesis focuses on growing *Anabaena flos aqua*, a marine cyanobacterium, under different stress conditions, mimicking natural stimuli and producing a diverse range of secondary metabolites; developing and optimizing a framework for producing more secondary metabolites at lab conditions and facilities; and finally testing the extract/fractions for cell viability in lethal pancreatic cancer model.

Anabaena flos aquae were grown in variations in BG11 media, especially in various iron levels (as iron is one of the key factors in the production of secondary metabolites such as siderophore). The variation in iron concentration alone does produce novel varieties of siderophores, including Synechobactin C16:1, Synechobactin oxyC14, Desacetyl-synechobactin A, Desacetyl-Synechobactin C14, Deoxysynechobactin C14 and Deoxysynechobactin C14:1.

Within this PhD Thesis workflow, we came up with establishing a framework called a 4-stage pipeline and validated it using the model organism, *Anabaena flos aquae* UTEX 1444, as a prototypical organism for increased siderophore expression/production. Our framework takes less time, is less expensive, and can be elaborated to any microorganism,

making it a demanding strategy over the other approaches explained by researchers over the past few years, such as the OSMAC (One Strain Many Compound) framework. Studying the genetic orientation of the model organism (*Anabaena variabilis*), discovering the siderophore operons and analysing the promoters are all the initial stages of the pipeline. The reporter vectors were prepared, cloned with siderophore coding regions and inserted in *E.coli* to justify the gene expression under various environmental conditions such as temperature, pH, salinity, iron levels, citrate, sugars and amino acids such as lysine (all suggested to us after analysing the promoter region). The screening was achieved using as little as 50 µl sample, providing a quick and economical platform for testing media variations. Following this, the best culture conditions were used to optimize the culture conditions of *Anabaena flos-aquae* UTEX1444 for siderophore production in the laboratory. The produced siderophores were identified and quantified using mass spectroscopy (MS)-based molecular networking. Assuming that the structurally related compounds share similar fragmentation patterns, we discovered novel siderophores and in increased quantities. Schizokinen, Synechobactin A and Synechobactin C14 being the majority, especially in the culture with 3µM of ferric ammonium citrate (0-5 µM) confirming the bell-shaped connection between the amount of Fe^{3+} and siderophore production suggested by our in-vitro pipeline.

In the end, we tested the fractions rich in siderophore (composition as stated in the above study) for cell viability in the PDAC (Pancreatic ductal adenocarcinoma) SUIT2 cell line. The three fractions, namely Butanol fraction, Methanol fraction, and Aqueous fraction, showed lower cell proliferation at the initial concentrations and noticeable cell death at high concentrations such as 25 µg/ml for Butanol Fraction, 25 µg/ml for Methanol Fraction and 250 µg/ml for Aqueous Fraction. To know more about the cell death

pathway, we combined our extract and fractions with ferroptosis (a form of cell death regulated by the presence of iron) inducers and inhibitors. Erastin was used as a ferroptosis inducer (standard) and showed cell death, and its activity was successfully reversed by using four different anti-ferroptosis drugs (inhibitors). For various fractions, we got mixed results as the ferroptotic inhibitors failed to rescue the viability, hence suggesting a different pathway of ferroptosis, i.e. cell death. Future work includes testing the hallmarks for ferroptosis, such as GPX4 level, GSH activities, ROS levels and the amount of lipid peroxidation in the cells.

INTRODUCTION

In recent times, the metabolites that are used in treating various diseases have shown to have diminished effects on humans, creating a pharmacological niche to develop more metabolites naturally from the sources readily available around us. Marine cyanobacteria (CB) are one such source of organisms that have been studied intensively throughout the years for the production of novel secondary metabolites. CB are the most ancient group of photosynthetic microorganisms which demonstrate the highest range of physiological adaptation for colonising in various ecological niches; they are able to develop some specific physiological mechanisms that help them react to different types of environmental stressors. They have been associated with a variety of ecological and health impacts for hundreds or even thousands of years. In some cases, blooms can be observed when higher levels of pollutants can lead to cyanobacteria blocking sunlight from reaching underwater grasses, making them unable to photosynthesise and consuming oxygen in the water; also, abundance in anthropogenic waste leads to fish kills and dead zones occur, producing surface scum and odours, interfering with organisms that filter water standing on their feeding process [1].

CBs, when in blooms, can cause both beneficial and harmful effects on the health of humans, whether in freshwater and marine recreational areas and have a negative impact to a considerable extent. Apart from being health hazards, cyanobacterial blooms pose significant problems in relation to food chains due to possible bioaccumulation of cyanotoxins [2]. They release potent neurotoxins, such as microcystins, that are harmful. On the other hand, they represent an undiscovered source of novel compounds profitable for drug discovery. The marine CB has also encouraged the development of synthetically prepared analogues with

enhanced biological activity and pharmacokinetics, such as dolastatin, cryptophyte, curation, etc. [3].

The research group I am part of for my PhD has strong experience working with cyanobacteria and the genes responsible for secondary metabolite production. The key objective of my PhD PhD Thesis is to investigate the effect of different environmental factors that affect secondary metabolite production in the model cyanobacteria, such as *Anabaena flos aquae*, and their environmental impact on the food chain. The study is extended by testing these cyanobacterial extracts, rich in metabolites for cell viability in PDAC (pancreatic ductal adenocarcinoma).

From the literature, the strain of Anabaena is said to produce a variety of metabolites by minutely changing the media's proportions. The studies have previously stated the capacity of Anabaena to secrete the necessary metabolites to the environment and utilise it for various purposes such as Iron acquisition, prevention from unknown bacterial invasion, cell membrane maintenance, growth, colony protection and so on [4]–[7]. This PhD Thesis focuses on enhancing the media in such a composition, allowing Anabaena to produce Siderophore (a highly valued pharmaceutical molecule) [8].

Siderophore, a product of microbes, acts as an iron carrier that binds to metal ions, forming a relatively more straightforward complex to transport back to the cell with the scarcity of iron. Once the siderophore-metal complex enters the body and reaches the cell, the iron is then freed by hydrolytic or reductive mechanisms. Citric acid metabolites bind with Fe^{3+} to form complexes to increase iron's bioavailability. These iron chelators both maintain cellular Iron levels and fulfil additional biological functions, such as structuring microbial communities, that have been reported for pathogenic siderophore producers [9].

The structures of siderophores in cyanobacteria are pretty diverse, and it has been stated that several hundred different structures exist. Schizokinen, first discovered in *Anabaena* sp. (PCC-

7120), were the first to be reported. The functional groups chelating ferric iron are highly reserved across catechols and hydroxamates, even though the inclusive geometry of siderophores differs from species to species. Some pathogens can use sleuth siderophores to get inside the immune systems of mammals [10]. As a result of the capacity of these siderophores to quickly ingest Fe (III) from mammalian cells, shown in a study of siderophore production, siderophores are significant virulence factors for the pathogens that cause these diseases. Because of this, proteins involved in the production of siderophores can potentially be targets for therapeutic medicines [9], [11].

These molecules are used for various purposes, including environmental and medicinal. Highly encouraging progress has been made in medicine, and siderophores are now commonly utilized in treating iron overload illnesses. Antibiotics-siderophores conjugates are undergoing clinical testing to circumvent bacterial resistance and enhance treatment for infectious diseases. Siderophores might potentially be used to block metalloenzymes, which is a field that still needs a significant amount of study since many metalloenzymes are involved in the pathology of illness. Siderophores have a wide variety of potential applications within the area of environmental research. Given that they have been found to be beneficial to the health of plants and fish, they are used in agriculture and aquaculture [9], [10]. The secretion of siderophores is the most prevalent mechanism for iron scavenging in the microbial world, as it has been noted that almost all known bacterial species produce them.

In this context, the PhD Thesis aimed in the first stage to discover the composition of the media, allowing us to produce novel secondary metabolites that could be used as lead compounds in drug discovery. Later, attention was brought to the need to develop a framework allowing easier, more economical and quicker production of the siderophore, which was hence justified in developing a 4-stage pipeline with successful validation using our model organism *Anabaena flos aquae*. The isolated Anabaena fractions rich in siderophore (composition similar

to the above sections' findings), extracted in laboratory conditions, were used as a potential drug in cancer treatment and determining the cell death pathways (work ongoing and yet to be published).

The results obtained can be summarized in three main sections:

1. Culture condition generating novel secondary metabolites (siderophores)
2. 4-stage pipeline as an ideal strategy for siderophores' production
3. Siderophore-rich Anabaena extract's role in PDAC (pancreatic ductal adenocarcinoma)

The results obtained during my PhD have been reported in the two papers already published:

1. Kundu, K., Teta, R., Esposito, G., Stornaiuolo, M., & Costantino, V. (2023). A Four-Step Platform to Optimize Growth Conditions for High-Yield Production of Siderophores in Cyanobacteria. Metabolites, 13(2), 154.
2. Teta, R., Esposito, G., Kundu, K., Stornaiuolo, M., Scarpato, S., Pollio, A., & Costantino, V. (2022). A Glimpse at Siderophores Production by *Anabaena flos aquae* UTEX 1444. Marine Drugs, 20(4), 256.

During my PhD, I have presented three posters and given two talks while attending conferences nationally and internationally. Attached below is the list of such activities:

1. POSTER at conferences
    a. Testing Secondary Metabolites from *Anabaena flos aquae* for Cell Viability in Pancreatic Cancer at 20 years of Conway Festival, UCD Conway Institute, Dublin, Ireland.
    b. A Four-Step Platform to Optimize Growth Conditions for High-Yield Production of Siderophores in Cyanobacteria at 5° Workshop: I CHIMICI PER LE BIOTECNOLOGIE at University of Naples Federico II, Department of Chemistry, Napoli, Italy.
    c. A Multidisciplinary Approach to The Study of Siderophores from Cyanobacteria at the 7th Conference University Cooperation in The New Challenges for Sustainable Development, CUCS, Naples, Italy.
2. TALK at conferences
    a. Valorization of Toxic Cyanobacteria: Boosting Siderophore Production at Cyano-2022, Helmholtz-Zentrum für Umweltforschung – UFZ, Leipzig, Germany.
    b. Study different stressors' effects on cyanotoxins production through metabolomics and metagenomics at ISSNP, Summer School-Virtual Meet, 4th edition, Naples, Italy.

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Chapter 1

Marine Cyanobacteria: An Evolutionary History

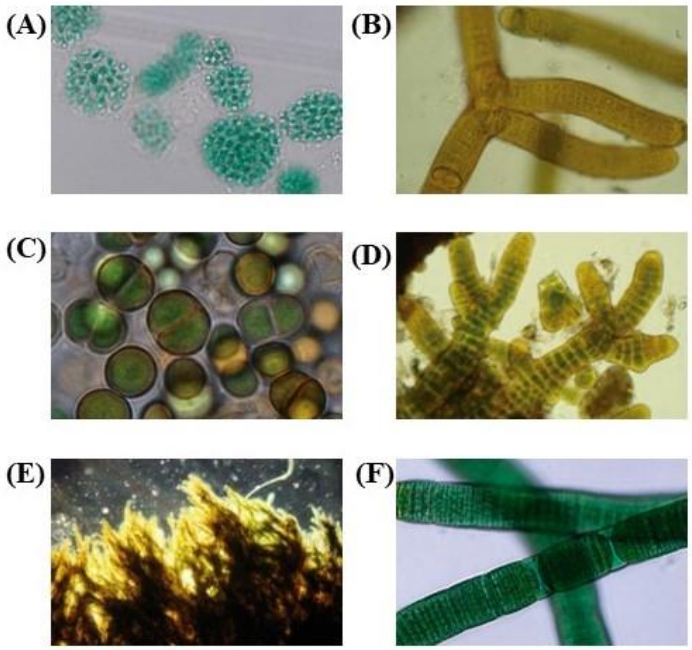
1. Introduction

Marine Cyanobacteria, commonly called blue-green algae, belong to the category of gram-negative eubacteria [1]. The cellular architecture of cyanobacteria exhibits a remarkable similarity to that of bacteria, as it lacks a visible nucleus or organelles enclosed by membranes. Regarding their biology, cyanobacteria have a cell wall made of peptidoglycan and are usually covered by a mucilagenous sheath. The cell membrane, as a rule, is situated within the confines of the cell wall. In the realm of cellular dimensions, it is worth noting that cyanobacteria cells, as a rule, tend to exhibit larger sizes in comparison to most of their bacterial counterparts. The sizes of these organisms can exhibit a considerable range, spanning from 1 μm for unicellular varieties to exceeding 30 μm for multi-cellular species [2]. In morphological observation, one can discern three fundamental forms: the unicellular form, the filamentous form devoid of heterocysts, and the filamentous form replete with heterocysts. Heterocysts represent a distinctive cellular entity endowed with the remarkable capacity to engage in nitrogen fixation. These cells are said to have the potential to enhance viability in nitrogen-deprived habitats [3].

Cyanobacteria exhibit the remarkable ability of photo-autotrophy, where they use light energy, predominantly via chlorophyll-a, to facilitate the process of photosynthesis. This produces cellular carbon while also emitting oxygen. In addition, some species also habitually display homotrophic growth by using organic compounds as a carbon source [4]. During the course of their evolution, cyanobacteria have developed a unique ability

to incorporate and store large amounts of phosphorus and nitrogen from surrounding environments. These unique features appeared 1,000,00 million years ago, allowing cyanobacteria to thrive in demanding environments [5], [6] Some cyanobacteria also have the impressive ability to produce proteinaceous gas vacuoles, allowing them to float on the surface of water. Such organisms are buoyant because their gas vacuoles have a cylindrical shape with conical end caps [7]. In addition, cyanobacteria may also exhibit sliding motility [8], [9]. Thanks to their diverse physical and biological characteristics, they can be found in various wastewater, freshwater, terrestrial, and marine environments. Cyanobacteria exhibit many shapes and sizes, including single-celled organisms, adherent forms, mats, and filamentous colonies (Figure 1.1, A-F).

Figure 1.1: Cyanobacterial Variety (A) Coccoid Cyanobacteria, Elongated Cells- *Aphanothece*
(B) Heterocytous Filament Showing Double False Branching- *Scytonema*
(C) Coccoid Cyanobacteria, Hemispherical Cells- *Chroococcus*
(D) Heterocytous Filament Showing True Branching- *Stigonema*
(E) Heterocytous Filaments Forming Fascicles- *Dapisostemon*
(F) Homocytous Filament- *Lyngbya*



Cyanobacteria find their classification within the esteemed Kingdom Monera (Prokaryota), specifically the Division Eubacteria, and are further categorised as the class Cyanobacteria. Nonetheless, deliberations persist about classifying superior taxonomic assemblages, namely orders, families, genera, and species. The wrong classification of historical records, especially for blue-green algae (Cyanophyta) under botanical codes, is because early identification and classification methods relied too much on the shape of cells. Knowledge from molecular methods such as SSU rRNA gene (small-subunit ribosomal RNAs) sequencing has strengthened existing evidence for many commonly recognizable taxonomic principles. However, it must be recognized that there are indeed differences among these various approaches. The taxonomic validity of cyanobacteria with filaments possessing heterocysts, such as *Anabaena cylindrica*, needs to be clarified. It is worth noting that cyanobacteria encompass considerable diversity, boasting an estimated count of approximately 150 genera, which, in turn, encompasses around 2000 distinct species [10].

Cyanobacterial Harmful Algal Blooms, commonly referred to as CHABs, represent a matter of considerable concern arising from the proliferation of cyanobacteria. The blooms above present a grave peril to the integrity of aquatic environments and the availability and quality of water sources for human consumption and agricultural purposes. Furthermore, they exert detrimental effects on fishery production and the delicate balance of aquatic and freshwater ecosystems [11]. The potential peril arises from the genesis of cyanotoxins, which can engender adverse consequences and taint water reservoirs and downstream water systems upon their liberation [12]. At present, it has been ascertained that approximately 40 distinct species exist, stemming from 20 distinct species of cyanobacteria that have been duly recognised as capable of producing toxins.

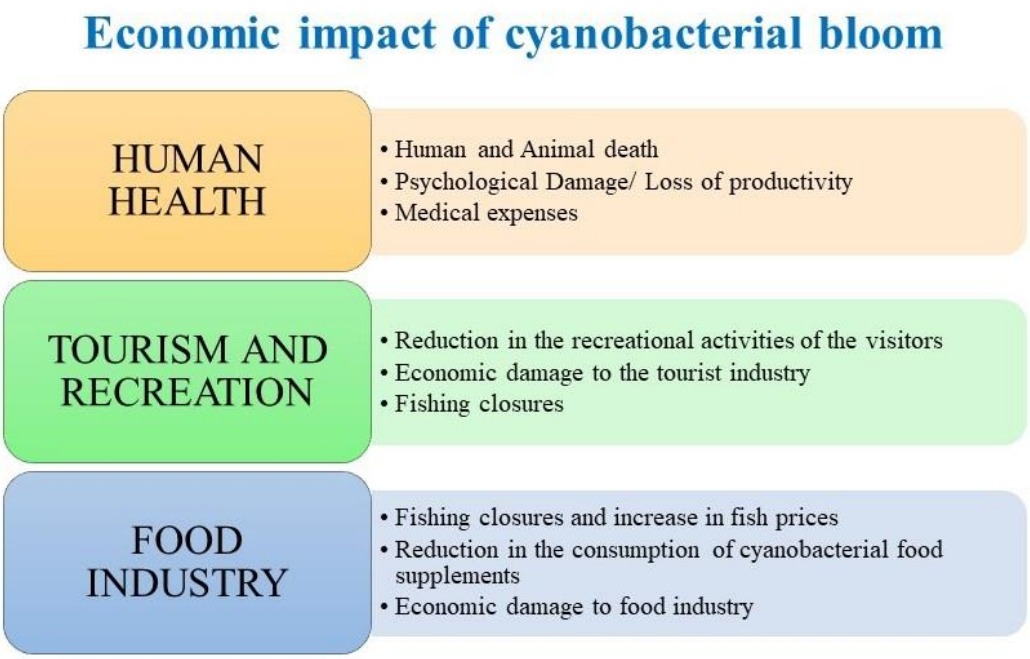
The toxins manifest diverse structures and toxicities, thereby necessitating their classification according to their respective chemical compositions. The categories above encompass alkaloids, lipopolysaccharides (LPS), and cyclic peptides [13]. In terms of their mechanism of action, these substances can be classified into several categories:

1. Hepatotoxins, which include microcystin (MC) and nodularin.
2. Cytotoxins like cylindrospermopsin (CYN).
3. Dermotoxins, which encompass lyngbyatoxin (LA).
4. Neurotoxins such as anatoxin-a (ATX-a), BMAA, and saxitoxin (STX).
5. Irritant toxins, specifically lipopolysaccharides.

About the pathways of poisoning, it is noteworthy that intoxication may transpire via dermal contact, inhalation, or alternative non-oral routes of exposure. Nevertheless, it is worth noting that the predominant mode of poisoning commonly observed is typically attributed to the act of ingesting water or food that has been contaminated [14]. Indeed, well-documented reports exist that provide evidence of aquatic organisms exhibiting the development of resistance towards cyanotoxins [15]. The organisms above possess the remarkable ability to amass said compounds via ingestion or through the absorption of dissolved toxins via their integumentary system. The organisms above, which exhibit resistance to toxins, fulfil a pivotal function in the propagation of cyanotoxins within the food chain, thereby giving rise to instances of human poisoning. It is worth noting that certain cases of such poisoning have tragically culminated in fatal consequences [16], [17]. Cyanotoxins possess the inherent capacity to accumulate within plants' cellular structures after exposure to surface irrigation water that has been tainted with

contamination. The toxins can be stored within many consumable plant components, encompassing fruits, roots, and leaves [18].

Figure 1.2: Effect of cyanobacterial bloom on the economy



Cyanotoxins, with their diverse array of targets and multiple avenues of exposure, present a substantial danger to public health [19]. Algae blooms can profoundly impact an entire ecology, lowering the variety and number of marine species and the amount of flora. This type is known by the acronym CHAB (Contents-Harmful Algal Blooms). Therefore, these changes significantly impact (Figure 1.2) the complex interrelationships of all aspects of our food world. In addition, the scope of these events will also gradually enlarge. As they expand in size over time, it could even lead to the creation of areas where oxygen levels decline and living creatures suffer tremendous losses (anoxic zones) [20]. Each year, there are countless news reports of animals dying from eating freshwater containing cyanotoxins. The mortalities encompass a diverse array of animal species, encompassing domesticated companion animals and livestock [21]. Owing to the inherent

unpredictability and intricate variables at play, the capacity to forecast harmful algal blooms (CHABs) remains constrained promptly and reliably. Consequently, this limitation poses a significant obstacle to effectively mitigating adverse consequences for human and animal populations.

In recent years, there has been a noticeable rise in the application of advanced methods for nano-structural and molecular studies within the realm of taxonomic classification. The assessment of cyanobacteria diversity has predominantly relied on this method. These methods clarify many patterns, adjustments, and phylogenetic connections within multiple clusters initially lacking in understanding. The use of molecular methods involves applying 16S rRNA gene sequencing, which is widely recognised as a standard practice, especially for identifying genera. Specific clusters of sequencing of 16S rRNA genes exhibit resemblances to established cyanobacterial taxa. They can be distinguished by observing their distinct phenotypic characteristics [22], [23]. Numerous species form vital symbiotic associations, including macro- and micro-eukaryotes [24], [25]. In the course of life's evolution on this planet, cyanobacteria were indispensable for oxygenic photosynthesis, and their emergence was an important event in history [26]. In their chosen evolutionary niche, cyanobacteria have been among the most important species to dominate many critical ecological processes. They play pivotal roles in nitrogen (N) and carbon cycles. According to some estimates, cyanobacteria are responsible for 20-30 % of organic carbon on earth using photosynthesis and fixation in living organisms [27], [28].

Cyanobacteria have some special biological features indispensable to the Earth's nitrogen cycle. Nitrogenase enzymes are used to convert nitrogen (N_2) into ammonia (NH_3) [29]. The nitrogenase enzyme complex in cyanobacteria is easily destroyed by oxygen, so they

have a mechanism to separate the fixing of carbon dioxide and nitrogen fixation. Certain cyanobacteria have evolved special cells called heterocysts. During photosynthesis, the heterocysts enclose a nitrogenase (nitrate reductase) enzyme within pockets to protect it from deactivation by oxygen [30].

From a biotechnological standpoint, the availability of abundant CO_2 and their capacity to thrive in high salinity conditions contribute to their sustainability, thereby reducing the need for competition over drinking water. In addition, marine cyanobacteria exhibit resilience to environmental changes due to their adaptation to natural habitats with fluctuating temperature, nutrient availability, salinity, and light intensity. This characteristic could prove advantageous in industrial settings where slight variations in growth parameters may occur. Although many species have a circadian rhythm that helps them adapt to changes in light exposure through complex regulatory processes, certain species have developed the ability to thrive in dark environments [31]. These species have demonstrated remarkable adaptability and expanded metabolic flexibility without light. The evolution of cyanobacteria has been dramatically influenced by the wide range of marine environments they inhabit. It is worth noting that a strong connection exists between certain species' unique characteristics and their specific habitats [32].

The first marine members of this group, including one *Synechococcus* and three *Prochlorococcus* strains, were successfully sequenced because of their compact genomes and prevalent existence in oceanic ecosystems [33]. While these new studies were starting, there has been a consistent and gradual rise in the accessibility of genome sequences associated with marine cyanobacteria. Comparative genomics research has offered significant insights into the adaptive strategies employed by cyanobacteria in their marine environment. Earlier research has shown that cyanobacteria have developed

specific processes to adapt to salinity, allowing them to actively remove inorganic ions from their cells. This strategy, known as the salt-out technique, can also be used to produce such compounds. In addition, these organisms may increase cellular osmolarity by storing matching solutes, that is, small organic molecules capable of reducing cytosol viscosity [34]. Compensable solute makeup varies significantly, depending on the habitat or descent in question. The corresponding solutes, trehalose, glucosyl glycerol and sucrose, have also been detected in marine species. While *Prochlorococcus* relies primarily on the compatible solute of sugar (sucrose and glucosyl glycerate), *Crocosphaera watsonii* has only one such substance, trehalose. Marine *Synechococcus*, str. WH7803 can collect glucosyl glycerol and may possibly also have the ability to gather glucosyl glycerate [35], [36].

Moreover, it can absorb glucosyl glycerol, sucrose, and its own metabolism-related substances, such as glycine betaine. These compounds are noteworthy for being factors of osmoregulation related to the cell cycle and affected by various environmental conditions [37]. *Trichodesmium* Perhaps because of this, an initial analysis did not yield any unique genetic code responsible for generating the proper complementary solutes. The mere mention of human-like behaviour has instilled fear in many people's hearts and triggered rapid response measures behind closed doors [34]. Nuclear Magnetic Resonance (NMR) and liquid chromatography coupled with mass spectroscopy were used to analyse the compound structure of a quaternary ammonium compound within it, which was identified as being N,N,N-trimethyl homoserine betaine or homoserine.

The influence of nutrient availability on marine cyanobacteria development stands out as a significant environmental aspect to consider. In the uppermost layer, covering approximately 75% of the earth’s ocean, marine productivity is restricted by nitrogen (N)

deficiencies, while in specific regions, limitations in iron (Fe) or phosphorus (P) are the primary factors [38] Cyanobacteria have implemented three fundamental approaches to address this challenge in response to a shortage of inorganic nitrogen. These strategies encompass

1. engaging in the process of atmospheric nitrogen fixation,
2. reducing the size of their cellular structures and genome to minimise their nitrogen requirements and
3. harnessing organic nitrogen sources to meet their nutritional needs.

Numerous morphological and physiological adaptations have been developed in response to the initial approach, focusing on overcoming the main challenge of N2 fixation, which is the sensitivity of nitrogenase to oxygen. It is hypothesised that the potential barrier to the increase of atmospheric oxygen during the Proterozoic Eon could be linked to the inhibitory impact exerted by oxygen on nitrogenase.

Contrasted to typical vegetative cells, they are larger, have a thicker cell wall preventing gas from leaking, and contain less chlorophyll. The *Trichodesmium* genus, characterised by its lack of heterocysts, predominantly engages in diurnal nitrogen fixation. However, it must be stressed that, unlike oxygenic photosynthesis, this process takes place at diverse locations and times. Specialised cells called "diazocytes" are where nitrogenase is most often found within the trichomes. The diazocytes in question exhibit a lack of granulation and demonstrate a diminished presence during the early hours of the day. Yet, their frequency progressively rises as the day progresses towards the midday period [39]. The presence of inorganic nitrogen exerts a harmful influence on the frequency of diazocytes. Compared to *Trichodesmium*, the time division for photosynthesis and N_2 fixation is more

pronounced in the unicellular organism *Crocosphaera watsonii*. Photosynthesis, a vital biological process, is known to transpire exclusively during the daily period when sunlight is available to fuel the intricate biochemical reactions involved. Conversely, the intriguing phenomenon of N_2 fixation, which holds paramount importance in the realm of nitrogen cycling, intriguingly unfolds during the night hours, when environmental conditions are conducive to this intricate process. Non-heterocystous cyanobacteria that engage in nitrogen fixation possess the remarkable capability to synthesise distinct hopanoid lipids. The lipids mentioned above assume a pivotal function in the prevention of oxidation of nitrogenase, effectively constraining the diffusion of molecular oxygen (O_2) across their respective membranes, and the mechanism holds significant importance within the context of surface oceanic waters that exhibit complete saturation with molecular oxygen (O2) [40]–[43].

An alternative approach involves significantly decreasing cell size, providing them with a specific advantage in nutrient uptake by increasing their volume-to-surface ratio. This phenomenon is evident in the marine pico-cyanobacteria Synechococcus, known for their average one μm of cell diameter [44]. The phenomenon in question is further demonstrated in numerous lineages of *Prochlorococcus*, wherein the mean cellular diameter measures approximately 0.7 μm. The observed decrease in cellular dimensions was concomitant with a noteworthy streamlining of the genetic material. The genomes of *Prochlorococcus* experienced a reduction in size of approximately 30% in comparison to their ancestral counterparts, which was achieved through a comprehensive process of streamlining [45]–[47]. The third approach, commonly employed in tandem with either of the methodologies, entails integrating naturally occurring nitrogen sources, such as amino acids, sugars, and urea. The capacity to assimilate organic carbon and nutrients

through a light-induced mechanism known as "photoheterotrophy" has prompted numerous scholars to reassess the conventional perception of marine cyanobacteria as exclusive photoautotrophs [48], [49].

Cyanobacteria encounter challenges during their adaptation to the marine environment, specifically related to the diverse light conditions they encounter in the water. The water molecule's resonance patterns formed five distinct spectral niches, each characterised by a specific range of wavelengths [50]. These niches include the red niche found within murky coastal water bodies and the violet niche observed within open ocean waters characterised by low nutrient levels. Cyanobacteria are commonly characterised by possessing expansive exterior light-harvesting complexes referred to as phycobilisomes. These complexes comprise a central core composed of allophycocyanin, encircled by six to eight rod-like structures. The rod-shaped structures display various combinations of phycobiliproteins, such as phycocyanin (PC), either alone or in conjunction with phycoerythrin-I (PEI), phycoerythrin-II, and/or phycoerythrocyanin (PEC) [49], [51]. The absorption characteristics of these phycobiliproteins exhibit variability based on the specific kind and relative quantities of covalently linked phycobilins [52]. Throughout the evolution, during which cyanobacteria have successfully inhabited virtually every available light-accessible marine environment, these organisms have emerged as highly intriguing subjects, drawing significant attention and becoming the focal point of vibrant and dynamic scientific exploration.

Cyanobacteria provide substantial advantages in sustainable agriculture and environmental stewardship. These microorganisms possess various capabilities, including nitrogen fixation, breaking down organic waste, purifying pollutants, and producing vital bioactive compounds crucial for plant development. Cyanobacteria can

bolster soil quality, foster plant growth, and decrease production expenses by complementing effective crop management approaches when integrated into agricultural methods [53]. Conventional agricultural methods, reliant on chemical fertilisers and intense practices, contribute to amplified production costs, exploitation of resources, and environmental degradation [54]. Implementation of sustainable agricultural methods that are cost-efficient, eco-friendly, and conducive to long-term food security is vital. Employing cyanobacteria in soil and environmental management yields economic advantages, prevents pollution, improves nutrient cycling, and rejuvenates soil fertility. Applying cyanobacteria in agroecosystems manages pollutant spread, enhances nutrient availability, stimulates plant growth, and enhances soil conditions [55].

The formation of different cyanobacterial secondary metabolites allows them to adapt to a variety of ecologies [56]. These metabolites have both photoprotective and antioxidant properties and many pharmacologically promising secondary metabolites, such as potent antifungals or antibiotics, anti-inflammatory drugs, and even cancer medicines [57]. In addition to its outstanding biotechnological technique, cyanobacteria have a relatively simple growth style, are environmentally friendly, and easily sustain economic feasibility. This has attracted the attention of such industries as cosmetics, pharmaceuticals, and food [58].

They can thrive in environments that are typically unbearably dry or subject to high-frequency radiation. Cyanobacteria rely remarkably on adaptability, enabling them to adjust themselves even as temperatures drop past freezing levels and they become more susceptible [59]. Cyanobacteria secrete extracellular polysaccharides (EPS) in order to resist desiccation. The extracellular polymeric substances (also known as EPS) also protect the bacteria from environmental stresses such as high temperatures or freezing.

Photosynthetic cyanobacteria can produce certain substances, such as MAAs (cycloamino acid-based compounds). These possess protective properties against UVR damage. Cyanobacteria are photoautotrophs, get their energy from light and have a photosynthetic system including chlorophyll, carotenoids and xanthophyllic pigments. Phycobiliprotein pigments (PBPs, PCP), including phycocyanin PC and phycoerythrin PE in cyanobacteria. These highly specialised pigments collect an ideal amount of solar radiation and produce visible colours [60], [61].

Cyanobacteria are able to withstand dehydration through the secretion of EPS (extracellular polymeric substances). These show a varying range of extracellular polymeric matter (EPS), which can appear either as covalently bound or adhesively attached to the surface of cells [59]. They can serve as sheath, cap and mucilage on the cells or be released outside. The genus *Cyanothece*, a type of cyanobacteria that predominates in the marine environment, has received notoriety for its role as a huge producer of EPS (extracellular polymeric substances). The evolution of salt tolerance in certain cyanobacteria living under severe conditions has dramatically stimulated the secretion of extracellular polymeric substances (EPS). This causes a massive increase in the extracellular polymeric substance (EPS) mucus layer, accumulating water and preventing membranes from drying [62].

In the course of evolution, cyanobacteria have developed a natural immunity to alleviate damage from high radiation levels, so they can thrive in areas with solid sunshine because of this adaptability [63]. Therefore, the researchers have produced compounds that can prevent the deleterious effects of exposure to excess solar radiation. One of the cyanobacteria's fascinating traits is that they have multiple mechanisms for protecting themselves from ultraviolet radiation (UVR) --means of prevention, protection and repair

to put a stop or reduce its adverse consequences on their cells. For example, natural substances with UV absorbing properties--including mycosporine-like amino acids (MAA) commonly referred to as “microbial sunscreens” and the compound scytonemin (SCY), found in cyanobacteria have attracted quite a bit of recognition for their ability to shield from ultraviolet radiation star wards [64], [65].

Cyanobacteria, the most rudimentary photosynthetic organisms, exhibit remarkable morphological, physiological, and metabolic attributes. The organisms above, which have adeptly acclimated to diverse ecological niches, possess compounds such as EPS, MAAs, SCY, and polyphenols that manifest notable antioxidant properties. In addition to its primary role in moisturising the skin, EPS has garnered acknowledgement for its notable antioxidant characteristics. An investigation [66] pertains to the genus *Nostoc*, explicitly focusing on the species *Nostoc sp*. The experimental findings indicate that EPS displays a dose-dependent antioxidant activity when examined in a controlled environment, specifically in vitro. These results are particularly noteworthy as they demonstrate significant efficacy in scavenging the superoxide anion and hydroxyl radical, which are highly reactive species. The antioxidant properties of MAAs and SCY in cyanobacteria are noteworthy. Multiple studies have been conducted, demonstrating the efficacy of these substances in capturing free radicals and exhibiting antioxidant properties, both in vivo and in vitro. More precisely, the compound known as SCY has been observed to exhibit a reduction in intracellular reactive oxygen species (ROS) levels, thereby offering a protective mechanism against oxidative stress induced by ultraviolet radiation [67].

The significance lies in the antioxidant potential of polyphenols derived from cyanobacteria strains, specifically *Leptolyngbya sp*. and *Calothrix sp*. The efficacy of these polyphenols’ hinges upon the phenolic compounds that have been extracted and

their corresponding quantities [68]. Moreover, it is worth noting that many investigations have emphasised the remarkable antioxidant characteristics exhibited by various strains of cyanobacteria, namely *Synechocystis sp*. BASO444 and *Synechocystis sp*. BASO673. These investigations helped us know about the copious presence of phenolic compounds within these organisms, further accentuating their significance in this context [69]. The attribution of antioxidant potential can be ascribed to many compounds, including pigments, namely phycobiliproteins (PBPs), with a specific emphasis on PC and PE [70], [71]. In conjunction with phenolic compounds, the pigments enhance the thermal stability of antioxidant properties within cyanobacterial extracts, allowing them to be utilised as constituents in cosmeceutical products [72]. The study [73], elucidates the multifaceted antioxidant properties exhibited by cyanobacteria, thereby indicating their promising potential as feasible alternatives for incorporation within the cosmetics industry.

2. Applications

The attainment of authentic sustainability within a flourishing agroecosystem is of utmost importance for conserving natural biodiversity and perpetuating complex and multiple ecosystems. This idea provides the necessary backing and durability to achieve sufficient food production commensurate with the escalating global populace. Also, it aims to ensure economic feasibility and enhanced safety for both the human and livestock populations. First and foremost, the discourse currently revolves around contemporary environmental issues. The financial burden of procuring costly chemical pesticides and fertilisers poses a formidable challenge to those in developing countries, particularly for poor farmers.

Furthermore, it’s worth noting that these diligent agricultural practitioners have conveyed their concerns about various environmental issues. In the present circumstance, it is worth noting that cyanobacteria possess the potential to exert a significant influence on the expansion of soil's natural nitrogen and carbon levels, alongside their capacity to enhance phosphorus accessibility to plants. Cyanobacteria can proficiently degrade diverse environmental contaminants, heavy metals, pesticides, and compounds derived from oil. These readily accessible bio-agents capture and store carbon dioxide, thereby contributing to climate change mitigation. This is achieved through various processes, including photosynthesis and biological calcification. Moreover, these organisms are an exceptional reservoir of a wide array of bioactive compounds that exhibit remarkable antagonistic properties.

The field of sustainable agriculture presents a notable prospect for the progression of bio-agents, specifically cyanobacteria. Bio-agent utilisation has the potential to augment the nutrients in the soil significantly and proficiently regulate pest and disease populations, thereby leading to a notable reduction in agricultural expenditures [74]. Nevertheless, it is imperative to conduct further research to delve into the untapped potential of cyanobacteria to attain sustainable farming methods and fulfil ecological goals in the forthcoming years. Considering the decline in the productivity and health of soils resulting from the escalation of lifestyle choices, safeguarding environmental sustainability presents a formidable challenge for the foreseeable future. Cyanobacteria, or blue-green algae, exhibit various applications in agriculture, environmental sustainability, and other fields. Their remarkable versatility and environmentally friendly nature render them highly valuable as bio-agents. Considerable emphasis should be placed on augmenting their efficacy within agriculture and its associated sectors.

Henceforth, expeditiously attending to salient concerns about using cyanobacteria to attain enhanced outcomes is of utmost importance.

Moreover, molecular biology methods have significantly contributed to the understanding and progress of resilient and ecologically sustainable agroecosystems. Based on the current state of research about the utilisation of cyanobacteria for the synthesis of valuable compounds, particularly in the realm of food supplements, it becomes apparent that there remains considerable scope for further advancements in this domain. Future research should prioritise enhancing performance in beneficial cyanobacteria to generate superior-quality food and fuel commodities. Simultaneously, it is imperative to ascertain their capacity for rapid growth and resilience in arduous environmental circumstances. This should be undertaken in conjunction with the progress of product advancements. The factors above are pivotal in effectuating the shift from laboratory-based investigations to the realm of extensive and lucrative bio-fuel manufacturing, guaranteeing the endurance of agricultural practices, ecological systems, and environmental progress.

The utilisation of genetic manipulations presents a viable avenue for enhancing cyanobacteria's efficacy in sustainable farming and environmental applications [75]. Though the field of genetic engineering to enhance biofuel manufacturing using cyanobacteria is presently in its nascent stages of development, in the forthcoming era, the augmentation of cyanobacteria via genetic and metabolic modification is anticipated to yield noteworthy advancements in bolstering the economic dimensions of bio-fuel production facilitated by cyanobacteria. Cyanobacteria possess the inherent capability to undergo genetic alterations that promise to augment their growth and optimise their efficiency in photosynthesis. The modifications above can result in an enhanced yield of biomass, heightened carbohydrate and lipid productivity, increased tolerance to

temperature, and a decrease in both photo-oxidation and photo-inhibition [76]. However, the transition from the lab to field conditions may pose challenges, as one must consider many factors, such as the social significance of the research, the potential political influence it may have, and the necessity to adhere to regulatory standards. Furthermore, it is of utmost importance to comprehensively examine contamination matters when contemplating utilising closed-photo bioreactors as a viable alternative for open ponds.

2.1.The Extreme Cyanobacteria

Cyanobacteria are blue-green algae known to be gram-negative prokaryotes. These microorganisms can perform oxygenic photosynthesis and obtain nitrogen from the atmosphere through fixation. Thriving in various aquatic habitats such as lakes, ponds, or any waterways, these microorganisms exhibit impressive adaptability in challenging circumstances like thermal springs, saline waters, freezing temperatures, and dry deserts. They can withstand temperatures ranging from 45 to 70°C, pH values as low as 4-5, and a range of 7.5 to 10 [77]–[79]. Employing their ability to endure severe environments can improve salt-affected soils by reducing salt levels, increasing P, N and C content, and improving soil moisture. The growth of cyanobacteria shows a significant impact on soil aggregation and the enhancement of water permeability. This is especially advantageous for improving the quality of barren land with poor structure [80].

2.2.Bio-fertilizers from Cyanobacteria

Cyanobacteria have the ability to fix atmospheric nitrogen individually or in symbiosis with plants like the cycads, water fern Azolla, and Gunnera. These cyanobacteria own a specialised type of cells called heterocysts, which play a crucial role in nitrogen fixation. The process utilises an enzyme known as nitrogenase, which transforms molecular N_2

into different forms of nitrogen, such as ammonia [81]. The potency to fix nitrogen is not confined to heterocysts cyanobacteria alone but is also found in various single-celled and filamentous groups. Approximately 20 to 30 kg Nha^{-1} of nitrogen and organic matter is converted into the soil [82]. Cyanobacteria are used in paddy fields as a nitrogen fertiliser substitute in various Asian countries such as China, Vietnam, and India [83]. This practice helps improve the availability of nitrogen in paddy fields. In addition, cyanobacteria improve the availability of phosphorus in soil by breaking it into insoluble organic phosphates and hydroxyapatite, making them soluble and motile. After absorbing inorganic phosphate for nutrition, the PO_4^{3-} released after the death of cells becomes readily available to plants and other life forms [84], [85].

2.3.Biocontrol by Cyanobacteria

Cyanobacteria generates a diverse range of active compounds that possess the potential to eliminate a broad spectrum of microorganisms, such as bacteria, algae, and viruses [86]. These can be categorized into various categories: amides, polyketides, alkaloids, fatty acids, lipopeptides, and indoles [87] They are capable of secreting compounds that inhibit algae growth and disrupt the development of pathogens by impacting their physiological and metabolic activities [86]. The reduction of numerous plant diseases has shown the effectiveness of these cyanobacterial components. Compounds such as Fischerellin, derived from *Fischerella muscicola*, have been found to possess antifungal properties against various plant pathogenic fungi [88], [89]. One exciting example is *Nostoc muscorum*, a cyanobacteria that exhibits antifungal properties against soil fungi associated with damping off. Findings indicate that effective cyanobacterial strains have the potential to act as bio-control agents, assisting in enhancing agricultural productivity [90]. The potential use of cyanobacterial metabolites for sustainable agriculture products

has been investigated. However, most studies have been conducted in controlled laboratory environments rather than in real-world agricultural settings. Therefore, further investigation is necessary to ascertain the practical use of cyanobacteria as bio-control agents for different plant diseases.

2.4.Restoration of Salt-Affected Soils by Cyanobacteria

Cyanobacteria exhibit the potential to restore salt-affected, dry soils. Traditional chemical techniques, such as applying gypsum, sulphur, or excessive irrigation to mitigate the effects of salt-affected lands, are not economically viable or environmentally sustainable. These soils, classified as alkaline and/or saline, have decreased productivity due to excess salts. This leads to impermeability and low fertility, resulting in reduced agricultural output. Soils with high pH and sodium content, known as alkaline soils, experience limited hydraulic conductivity [91]. On the other hand, saline soils contain many soluble salts, which can hinder water absorption by plant roots. Research suggests that cyanobacteria can restore such lands by creating a protective layer that retains organic matter and moisture while converting sodium clay into a more advantageous state. Cyanobacteria enhance soil permeability, aeration, and physicochemical properties by solubilizing nutrients, reducing pH levels, electrical conductivity, and improving hydraulic conductivity. In dry areas with compact, infertile, and water-deficient soils, the capacity of cyanobacteria to bind soil particles and improve nutrient levels, specifically carbon and nitrogen, is advantageous. Some specific types of cyanobacteria can withstand elevated salt concentrations, aiding soil enhancement by producing compounds that bind soil particles and improve moisture retention [92]. Using cyanobacteria exhibits the potential to address the issues related to inadequate soil quality in arid and semi-arid areas, which could potentially enhance soil fertility and promote plant growth.

2.5.Bioremediation by Cyanobacteria

Cyanobacteria have distinct benefits in bioremediation because of their ability to perform photosynthesis, convert atmospheric nitrogen, and adjust to polluted surroundings [93]. By accumulating or breaking them down, they show potential in dealing with different environmental pollutants, such as pesticides, crude oil, toxic metals, and industrial contaminants [58]. Cyanobacteria can absorb metals and reproduce quickly, making them a promising option for removing toxins from industrial waste. This introduces an economically viable approach for treating wastewater generated by industries and can assist in the advanced treatment of municipal and agro-industrial wastewater, which can help decrease the negative impacts of eutrophication and metal contamination on marine ecosystems [94]. While genetic engineering presents opportunities to improve the biodegradation abilities of cyanobacteria, there are still considerable challenges regarding regulations and environmental considerations that hinder their extensive application in bioremediation in real-world settings [95]. Further investigation is required to tackle these concerns and harness the prospect of designed cyanobacteria as practical bioremediation tools.

2.6.Plant Development Inducing Cyanobacteria

Cyanobacteria secrete substances that promote plant growth outside their cells, including hormones such as gibberellins, abscisic acids, and cytokinin, auxin [58], [96], [97]. Other substances in this category include vitamin B, amino acids, antibiotics, and toxins [98]. Most research conducted on the beneficial impact of cyanobacteria on plant growth, specifically in relation to paddy crops, has shown that introducing cyanobacteria can improve the germination of rice seeds and promote the growth of roots and shoots.

Furthermore, the combination of cyanobacteria and wheat increased the dry weight of roots and chlorophyll levels.

2.7.Bioenergy from Cyanobacteria

During anaerobic digestion, the (C/N) Carbon to Nitrogen ratio is an essential factor in producing (CH_4) methane [99]. Cyanobacteria have a superior protein content, resulting in a low C/N ratio. As a result, they release significant quantities of ammonia during anaerobic digestion, which negatively affects microorganisms responsible for methane production. For example, *Spirulina maxima* is a type of algae with a protein content ranging from 60% to 71%. This alga produces elevated levels of ammonia, reaching up to 7000 mgL^{-1}. These high ammonia levels can harm methanogens, which are known to be sensitive to ammonia. Nevertheless, these bacteria can adjust to elevated levels of ammonia. It is proposed that to increase the amount of CH_4 produced, co-digest cyanobacterial biomass (which has a low C/N ratio) and plant residue materials with high levels of carbon, like maize straw, which have higher C to N ratios. With a C/N ratio of 20 to l, there was an increase in the amount of methane produced by as much as 61.69 % [100].

Cultivating cyanobacteria, which are environmentally friendly organisms, can effectively generate biofuels at various scales. This process requires less space and can be carried out in diverse water conditions, such as contaminated or unused water. The amount and quality of cyanobacterial biomass may be manipulated using physicochemical treatments, allowing the production of high-quality biofuels. Using cyanobacteria to produce biofuel has several benefits, such as lower freshwater consumption than conventional crops, development, the utilisation of wastewater for the generation of biomass, and lower

emissions of greenhouse gases. This makes it a more sustainable and cost-effective approach than conventional agriculture farming.

2.8.Addressing Climate Change with Cyanobacteria

The attribution of Cyanobacteria's capacity to sequester CO_2 has led to its gaining increased attention as a potential solution for mitigating the impact of escalating atmospheric CO_2 levels. Cyanobacteria, indeed, assume a noteworthy role within the intricate process of photosynthesis, making a substantial contribution to the overall conversion of solar energy and carbon dioxide assimilation [101]. Cyanobacteria, fascinating microorganisms, demonstrate a carbon dioxide fixation rate that is roughly 10 to 50 times quicker than what is observed in their terrestrial plant counterparts. Henceforth, employing biological agents is believed to be one of the most viable methods for reducing the concentrations of atmospheric carbon dioxide (CO_2) and, consequently, assisting in the amelioration of prospective global warming [102]. The cyanobacterial biomass possesses the capacity to store CO_2 as organic molecules, which can subsequently be employed in various applications. Cyanobacteria assume a notable function in organic matter and nitrogenous component composition within paddy field soils [103].

The renewed focus on maximising the use of cyanobacterial CO_2 fixation in photobioreactors has garnered significant attention as a potentially promising strategy for reducing CO_2 emissions. In recent decades, many studies have been conducted on the approach of carbon dioxide sequestration [104]. Photobioreactors, in contrast to open-pond systems, possess distinct advantages owing to their capacity to furnish heightened environmental regulation and optimise the utilisation of space and volume [105]. These

variables play a pivotal role in augmenting the productivity of cyanobacteria, optimising the utilisation of land, enhancing water use efficiency by mitigating losses due to evaporation, and improving the efficiency of the harvesting process. Moreover, in the event that they are deemed appropriate, it is plausible to utilise genetically altered cyanobacterial strains in a manner that does not engender any disturbance to the encompassing ecosystem.

Cyanobacteria can capture carbon dioxide (CO_2) from exhaust gases and subsequently store it as precipitated calcium carbonate ($CaCO_3$/$CaHCO_3$) through the complex procedures of photosynthesis and calcification. In a multitude of lacustrine, terrestrial, and marine ecosystems, one can observe the presence of substantial quantities of calcium [106]. The utilisation of agricultural drainage water, saline water from oil extraction, or brines, such as saline water from geological CO_2 injections, can be employed by halophilic cyanobacteria or sources of calcium for the purpose of facilitating the calcification process. This process of accelerated calcification can perhaps be increased through the supply of calcium with gypsum or silicate minerals and even combined with biological rapid weathering [56]. Nevertheless, we must still seek to find and define the various species of cyanobacteria that have been found to assimilate carbon dioxide in large amounts when raised under conditions where global temperature and concentrations of CO_2 are increased. The investigation of the impact of elevated carbon dioxide (CO_2) levels, specifically those observed in exhaust gases, on the calcification process warrants attention. Furthermore, it is imperative to investigate potential approaches for the automation of the photosynthetic apparatus and light systems in cyanobacteria that are grown either in photobioreactors or open ponds. The augmentation of one's understanding pertaining to the intricate biochemical and genetic mechanisms

underlying the process of carbon dioxide sequestration by cyanobacteria can be significantly amplified by the application and integration of sophisticated genetic engineering techniques.

2.9.Reducing Methane Emissions with Cyanobacteria

Cyanobacteria exhibit remarkable potential in mitigating anthropogenic global warming from the release of greenhouse gases [107]. Cyanobacteria possess the inherent capability to mitigate the release of methane (CH_4) from inundated rice fields throughout the various stages encompassing production, transportation, and consumption. Bioagents, such as methanotrophs, have the potential to fulfil a pivotal function in the mitigation of significant quantities of potent and harmful greenhouse gases (GHGs), specifically CH_4, originating from diverse ecosystems' soils. This can be achieved through their symbiotic interaction with cyanobacteria.

Some have also proposed that cyanobacteria could be used to increase the oxygenation level of paddy fields by tapping into their rhizosphere. This, in turn, might help increase methanotrophs 'methane-providing activity. In addition, similarly beneficial biological agents can somewhat alleviate environmental engineers from the global warming potential of flooded rice fields. This is in addition to their inherent ability to facilitate the fixation of atmospheric nitrogen in the soils of said paddy fields. Cyanobacteria, through photosynthesis, have the remarkable ability to liberate oxygen (O_2) into inundated soils. The oxygen can be liberated into the soil, engendering an aerobic milieu deemed unfavourable to produce CH_4 [108]. Synchronously, cyanobacteria liberate oxygen, a phenomenon that has the potential to augment the populace and functionality of aerobic

methane-oxidising bacteria, commonly referred to as methanotrophs, within inundated paddy soils.

Consequently, this process leads to an elevation in methane oxidation, denoted as CH_4. The utilisation of organic amendments such as farmyard manure in conjunction with cyanobacteria holds the potential to not only augment paddy yield but also exhibit a plausible capacity to mitigate methane (CH_4) emissions during the cultivation of paddy, in contrast to the exclusive application of farmyard manure [74]. Using cyanobacteria has been shown to be a viable approach to mitigating methane emissions while maintaining optimal rice crop productivity. One conceivable pragmatic resolution for mitigating the global warming ramifications of inundated paddy ecosystems and fostering nitrogen fixation is the utilisation thereof [108]. A prospective strategy for enhancing crop productivity and mitigating long-term CH_4 emissions in agricultural fields involves the promotion of a more diverse assemblage of microorganisms, including cyanobacteria and methanotrophs, within paddy fields. The usage of cyanobacteria and their inherent capacity to serve as a viable alternative to nitrogen fertilisers presents many advantages, encompassing cost-efficiency, ecological compatibility, and a more secure methodology for rehabilitating depleted land [109]. Furthermore, the practice above has the possibility to effectively safeguard the enduring diversity of methanotrophic while concurrently facilitating the process of methane consumption [79]

2.10. Supplementing Foods with Cyanobacteria

Diverse forms of cyanobacteria, from capsules to tablets to liquid forms, are readily available within the commercial sphere as dietary supplements intended for human ingestion [110]. Those items under consideration for nutritional value enhancement

include pasta, treats, candies, drinks, and chewing gums [111]. These substances can function as a supplementary means of nourishment or as a storehouse of naturally occurring pigments to enhance the visual appeal of food products [112]. Spirulina, scientifically known as *Arthrospira*, is prominent in the spectrum of cyanobacterial strains harnessed for human consumption, owing to its unique nutritive properties and copious protein composition [113]. In numerous nations, including but not limited to Southern America and Southeast Asia, it is a prevailing practice to incorporate specific cyanobacterial species, such as *Anabaena, Nostoc*, and *Spirulina*, into the human diet as a viable source of sustenance. Raceway ponds or sophisticated photobioreactors are the two main methods used to develop *Arthrospira platensis*, often called *Spirulina platensis*. It is then packaged and marketed as flakes, pills, capsules, or powders. They are rich in thiamine, beta-carotene, and riboflavin and contain more than 60% proteins. The scientific community widely recognises and acknowledges that this source is indeed one of the most abundant and plentiful providers of the essential nutrients known as vitamin B12 [114]. Owing to its considerable nutritional composition and inherent digestive properties, this substance is frequently employed to supplement one's diet. Studies [115], [116] have demonstrated that Spirulina possesses a comprehensive array of preventive and healing nutrients.

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Chapter 2

Cyanobacterial Secondary Metabolites

1. Introduction

The capacity to carry out oxygenic photosynthesis is another of the characteristics, making the cyanobacteria the only gram-negative bacterium with this capability. Their oxygen-generating capacity is expressed by their effect on diversity in Earth's evolutionary record of life. Cyanobacteria exist in both unique environments, such as terrestrial and marine ecosystems, such as seawater or brackish water. Indeed, the adaptability of this type of bacteria is renowned. They may either live solitary lives or join up with other organisms, that is, in symbiosis with marine invertebrates, higher plants, and cyanolichens [1]. Moreover, the cyanobacteria can biosynthesise a wide variety of secondary metabolites. Thus, their flexibility is limitless. In addition to the production of toxins, cyanobacteria are also known for producing certain substances with several important properties. These include antimicrobial, antifungal, cytotoxic properties and anti-tumour, anti-inflammatory, or protease-inhibiting activities. The fantastic potential for bioactive compounds is an input to new discoveries in the pharmaceutical and cosmetics industries [2].

The diversity of cyanobacteria's secondary metabolites corresponds to their variety of bioactivities. This plasticity can be explained as a survival strategy adopted by nature for these bacteria to adapt better; it is their 'placebos'. The diversity of species indicated, on the one hand, that cyanobacteria were widely distributed and could evolve in many different directions. The cyanobacterial secondary metabolites include antifungal,

antibacterial, antiviral, and cytotoxic behaviour. Many bioactive molecules have medicinal importance as potential anticancer agents, immunomodulators, or protease inhibitors, even if their natural tasks are still incomplete. The inherent duality exhibited by bioactive molecules highlights the significant import of cyanobacteria into the environment while also highlighting their enormous potential in the pharmaceutical industry. By investigating the sequencing of cyanobacterium genomes, researchers have discovered a wide range of different and new variables in the genes responsible for producing bioactive proteins [3]. The substances that make up the proteins in question include ribosomal and non-ribosomal peptides and complex hybrid entities formed by the combination of polyketides and peptides. Genetic analysis helps to understand the characteristic capabilities demonstrated by cyanobacteria and presents opportunities for future use in a variety of fields.

The previous studies performed have witnessed significant progress in the field of separation and purification methods over the last ten years. Techniques such as High-Performance Liquid Chromatography (HPLC), chiral HPLC, and hyphenated methods have been notably responsible for this progress. Aside from several spectroscopic techniques, these advances include X-ray crystallography, 2D-Nuclear Magnetic Resonance, and electronic circular dichroism. The technological developments mentioned above have made it easier to accurately determine the molecular architectures of a small amount of different chemical materials. Consequently, we have witnessed the emergence of a series of complex secondary metabolites originating from cyanobacterial assemblages, which exhibit complex structural compositions. The mentioned progress represents a significant achievement in understanding and describing these entities. This has paved the way for further research in this field [2].

2. Chemical Classes

Many genera of cyanobacteria, such as *Lyngbya, Nostoc, Microcystis, Symploca*, and *Moorea*, are known for their exceptional ability to produce a wide range of complicated and biologically significant secondary metabolites. Many review articles exploring bioactive substances derived from cyanobacterium have been published recently. This body of scientific research has examined some cyanobacteria genera in addition to this taxonomic group's broad spectrum [4]–[6]. The main objective of this chapter is to provide a broad collection of various categories of bioactive secondary metabolites found in cyanobacteria from various environmental environments. Furthermore, we shall investigate these separate substances' biological and pharmacological functions as judged appropriate for our research.

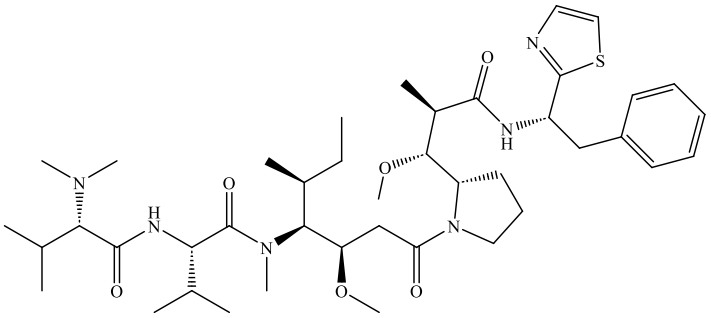
2.1. The Linear Peptides

Initially found in the sea hare (the species from which it takes its name) *Dolabella auricularia*, Dolastatin 10 (Figure 2.1(A)) has since been isolated in crude form from lipophilic extract of intracellular molecules gathered between dynamic cells and walls made up by *Symploca sp.* VP 642 belongs to the Palau area [7]. Symplostatin 1 (Figure 2.1(B)) is the chemical compound that was surface active for a specific *Symploca hydnoides Kutzing & Gomont*, belonging to UOG strain VP37. This organically extracted substance can act as an indispensable ingredient in cosmetic products. This was a particular strain obtained from an area close to Guam [8]. Subsequent studies confirmed that Symplostatin 1 and Dolastatin 10 are, in fact, constituents of the lipid extract obtained from *Symploca sp.* VP 642. On exposure to KB cells, a specific lineage of epidermoid carcinoma cells, a compound known as Symplostatin 1 demonstrated cytotoxic

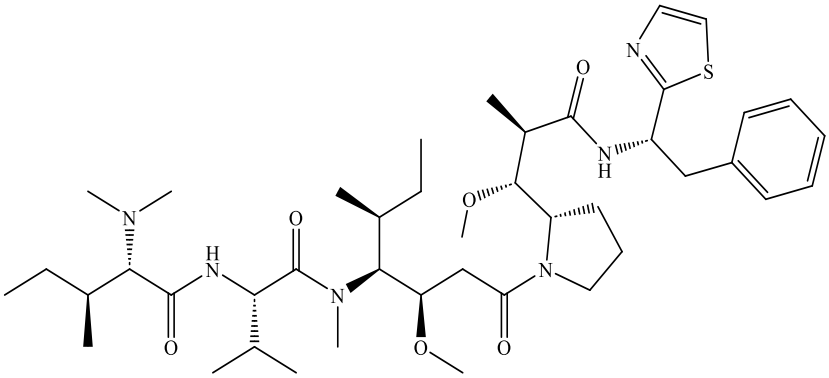
properties. The measured IC50 value indicates the concentration at which 50% of the cells were impacted, which was found to be 0.3 ng/ml. According to further studies, the modus operandi of this entity was very similar to that of dolastatin 10.

Figure 2.1- Chemical Structure

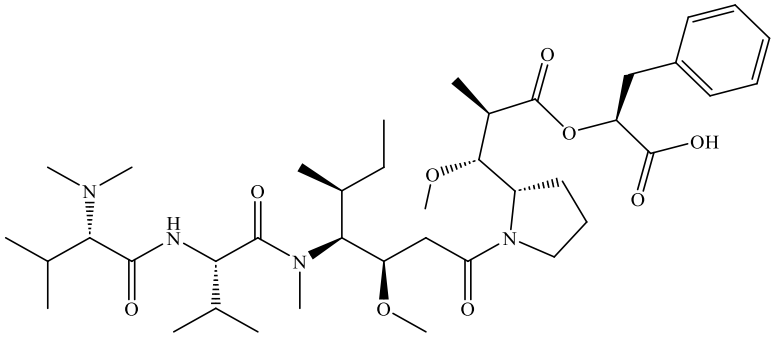
(A) Dolastatin 10



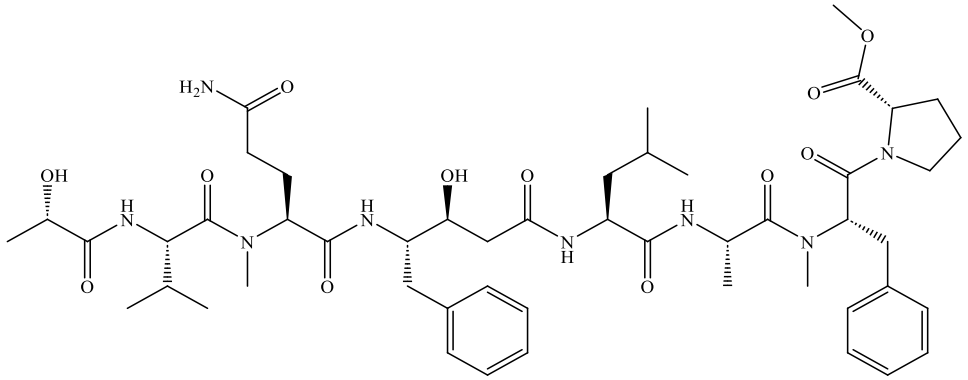
(B) Symplostatin 1



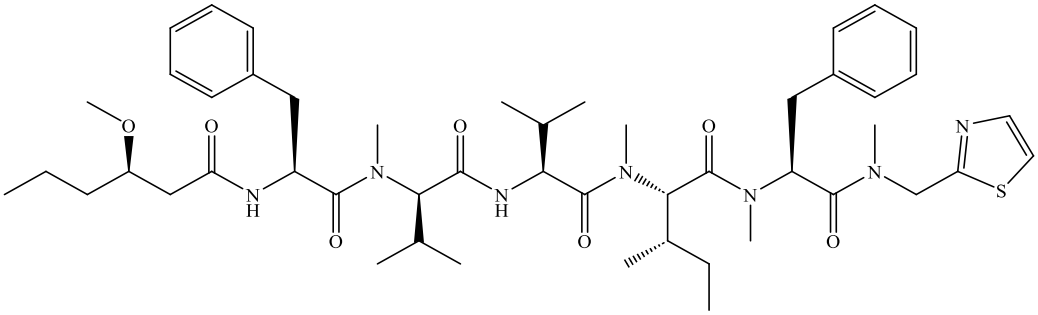
(C) Symplostatin 3



(D) Tasiamide B



(E) Micromide



A similar one, Symplostatin 3 (Figure 2.1(C)), was extracted from *Symploca sp.* VP 452, a marine cyanobacterium found in the Oahu region of Hawaii. Symplostatin 3 showed notable cytotogenic effects on KB and LoVo cells; both classified as HCAC cells. The IC50 values for KB cells were 3.9 nM, and those for LoVo cells were 10.3 nM. Interestingly, Symplostatin 3 did not cause cellular microfilament loss in A-10 cells despite its ability to induce microtubule depolymerization. The effects of the microtubules were remarkably similar to those induced by dolastatin 10 and symplostatin 1. Despite this, it was able to induce the division of the nucleus into micronuclei [9]. A unique residue known as 4-amino-3-hydroxy-5-phenylpentanoic acid is what distinguishes oligopeptide tasiamide B. It has been discovered that the extract derived from *Symploca*

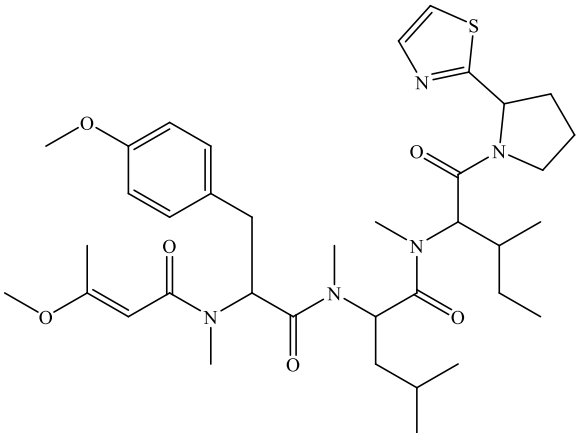
sp. (NIH304), a marine cyanobacteria that was obtained from the region of Palau, contains essential information. The compound known as Tasiamide B (Figure 2.1(D)), demonstrated notable cytotoxicity on KB cells in the laboratory, as demonstrated by an IC50 of 0.8 μM [10].

Researchers have discovered a new amalgam of peptide and polyketide, called Micromide (Figure 2.1(E)), in an effort to pursue diversification. The marine cyanobacterium *Symploca sp*. VP727, was the source of the compound under investigation [11]. Despite this, it is essential to highlight that Micromide demonstrated extremely low cytotoxicity when tested on KB cells. These results show the remarkable variety of compounds derived from cyanobacteria. Each of these compounds has distinct cytotoxic properties and mechanisms of action. The complexity of marine cyanobacteria is still a field of research that offers opportunities for the advancement of new drugs.

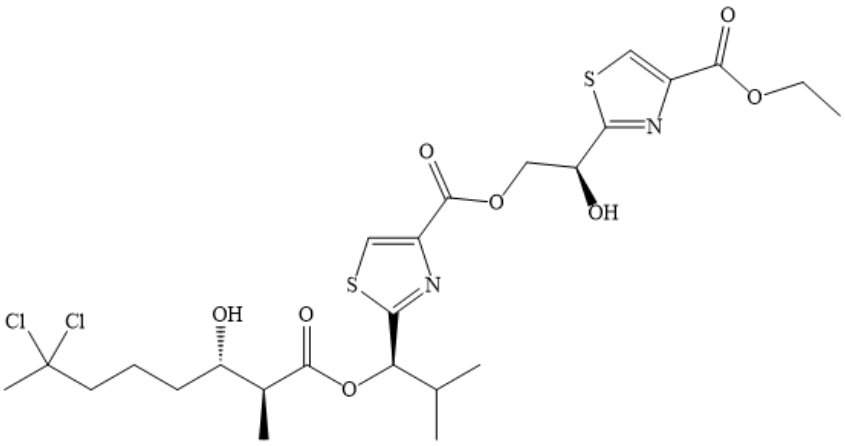
Lyngbyapeptin A (Figure 2.1(F)) is the initial member of an additional class of amino acid-based tetrapeptides, and has been isolated from *Lyngia bouillonii*, which grows around Papua New Guinea [12]. Consequently, when the compound mentioned above and a lipophilic extract obtained from *L. majuscula*, were both subjected to analysis, it was discovered that each contained one or another of these substances. Research has also shown that Lyngbyapeptin A lacks any aesthetic, bacteriostatic or fungistatic effects and possesses no protease-inhibiting activity [13]. The nature of such a thing can only be all the more enigmatic as a result. The *L. majuscula*, discovered in shallow waters of Alotau Bay on the island of New Ireland off Papua New Guinea, produces linear lipopeptides lyngbyabellins F and lyngbyabellins I; lyngbyapeptin I exhibited significant cytotoxicity against Neuro-2a cells. This is confirmed by the recorded LC_{50} (lethal concentration), or fatal level, of 62 % at a concentration of only 0.7 μM [14]. The statement successfully

highlights the wide range of biological activities that can be performed by cyanobacterial compounds. In experimentation using Lyngbyabellin M (Figure 2.1(G)), which is derived from the marine cyanobacterium *Moorea bouillonii* found in Palmyra Atoll (USA), no apparent cytotoxic effects were found on H-460 lung cancer cells [15]. The statement highlights the complex differences in the bioactivity of substances derived from cyanobacteria.

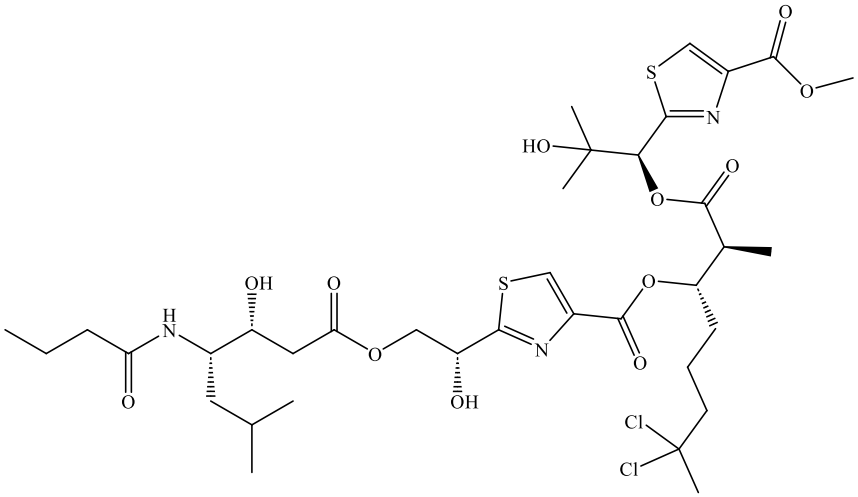
(F) Lyngbyapeptin A



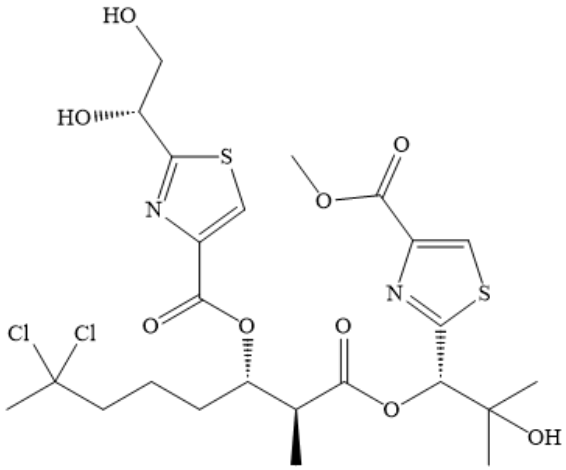
(G) Lyngbyabellin M



(H) Lyngbyabellin P



(I) Lyngbyabellin O



The extract is derived from the *Okeania sp*. isolated from the Red Sea. This extract also shows the introduction of Lyngbyabellins O (Figure 2.1(H)) and Lyngbyabellins P (Figure 2.1(I)). The compounds displayed a decisive antifouling action on the larvae of *Amphibalanus amphitrite* barnacle. In addition, Lyngbyabellin P was also discovered to have cytotogenic effects on MCF-7 breast cancer cells. Most importantly, from the concentration required for 50 % inhibition of cell growth, he was able to calculate that it

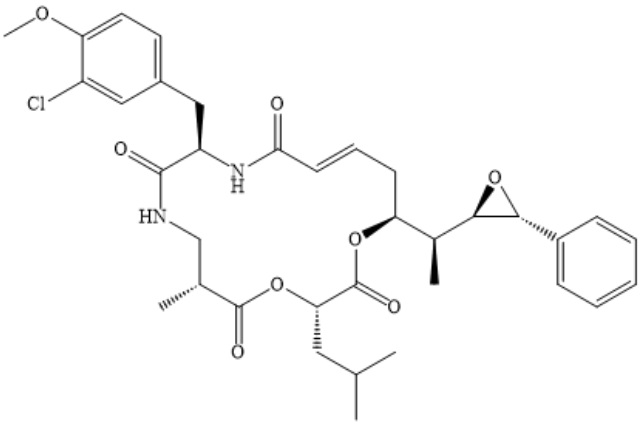
should be about 9 μM [16]. There are also good opportunities for its use in cancer treatment. These cyanobacterial compounds are all very peculiar in their activities and behaviour. With constant research, they reveal some mysterious secrets of marine nature. The deeper the examination of their inherent properties, the more obvious it becomes that these substances offer a broad spectrum of potentials. It can be the source of new therapeutic drugs and increase our knowledge about marine ecologies. For this reason, it belongs to a type of organism for which we have great hope in exploring its other potential applications.

2.2. The Cyclic Peptide

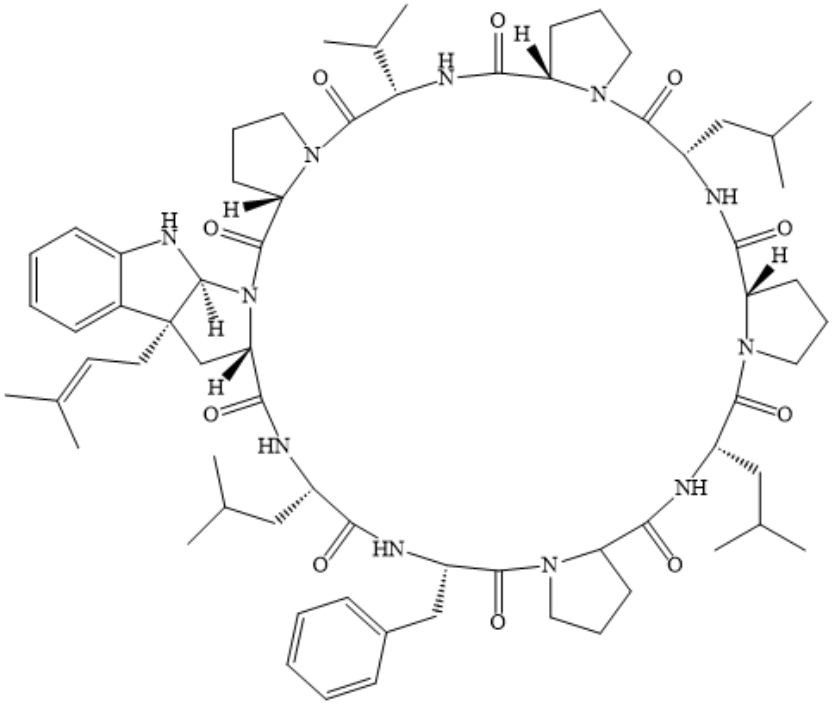
The group of macrocyclic depsipeptides known as cryptophycins consists of 16 parts. Two hydroxyl acids are responsible for setting off another kind of derivative. It may be 5-hydroxy-6-methyl-8-phenylocta-2,7-dienoic acid or its derivative 7,8-epoxy. Compounds with a methyl attached to carbon 3 are known as 2-hydroxyvaleric acid, and those in which the alcohol group is on chain four, compound specifications. It can improve its structural stability by combining two amino acid components, 3-(3-chloro-4-methoxy-phenyl) alanine and 3-amino-2-methylproprionic acid. This adaptability also explains why they have become popularly known as 'cryptophycins' in view of the fact that they are famous for being highly toxic to yeast from the species *Cryptococcus* [17]. In one case, Cryptophycin-1 (Figure 2.2(A)) caused programmed cell death in neoplastic cells during experiments conducted in a controlled laboratory environment. Furthermore, it prevented the assembly of microtubules effectively. We have discovered another remarkable member of the renowned cryptophycin family from the same source. In particular, researchers have discovered cryptophycins-176, cryptophycins-175 and cryptophycin-46 [18].

Figure 2.2- Chemical Structure

(A) Cryptophycin 1



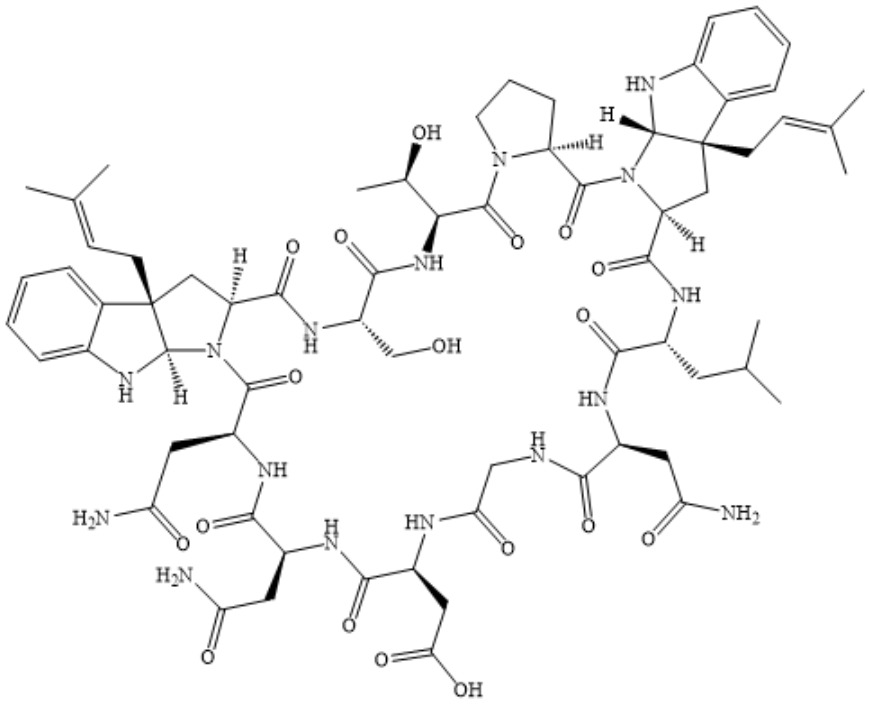
(B) Trikoramide A



While the focus on the beach area of Trikora on the prestigious island of Bintan in the captivating nation of Indonesia, an intriguing discovery has emerged. It has been determined that the marine cyanobacterium *Symploca hydnoides,* which is scientifically

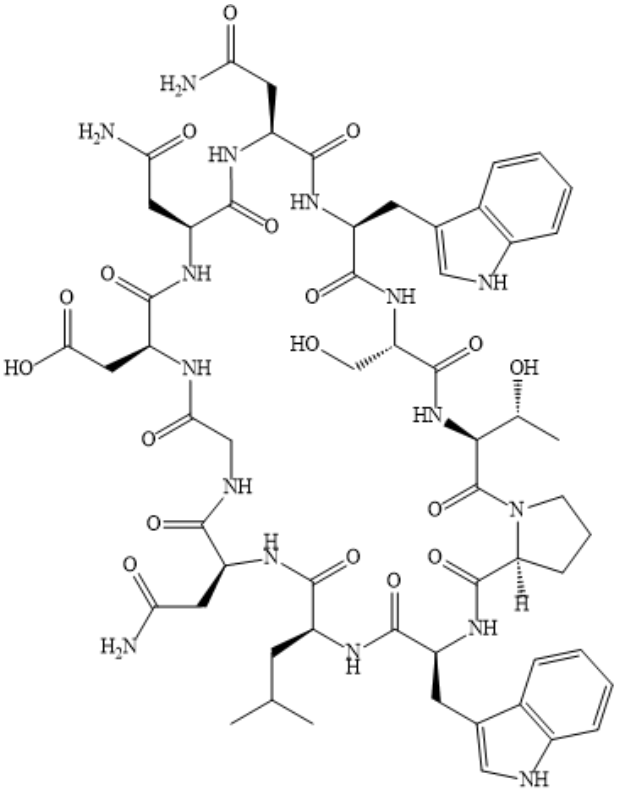
known as Trikoramide A (Figure 2.2(B)), has demonstrated a remarkable ability to synthesize a cyclic decapeptide of utmost significance. The substance demonstrated its cytotoxicity against MOLT-4 cells, a group of T lymphocytes involved in acute lymphoblastic leukaemia, and AML2 cells, which are related to acute myeloid leukaemia. Despite this, the observed results did not demonstrate any apparent cytotoxicity towards MCF-7 cells, a cell type that is often associated with breast adenocarcinoma. Furthermore, trikoramide A has been shown to have a limited ability to inhibit quorum sensing [19]

(C) Kawaguchipeptin A



The *Microcystis aeruginosa* (NIES-88), a microorganism isolated from the Lake Kawaguchi bloom, transformed cycloaminase into undecapeptide Kawaguchipeptin A (Figure 2.2(C)) and Kawaguchipeptin B (Figure 2.2(D)). *Staphylococcus aureus* was tried as an antibacterial test, and the peptides showed potent activity against it [20].

(D) Kawaguchipeptin B



The wide range of compounds that are being extracted from cyanobacteria continues to captivate the scientific community. These compounds are attractive because of two things: their complex structures and the various biological activities they exhibit. The mentioned discoveries expand our knowledge of marine ecosystems and offer prospects for possible uses in medicine and other fields.

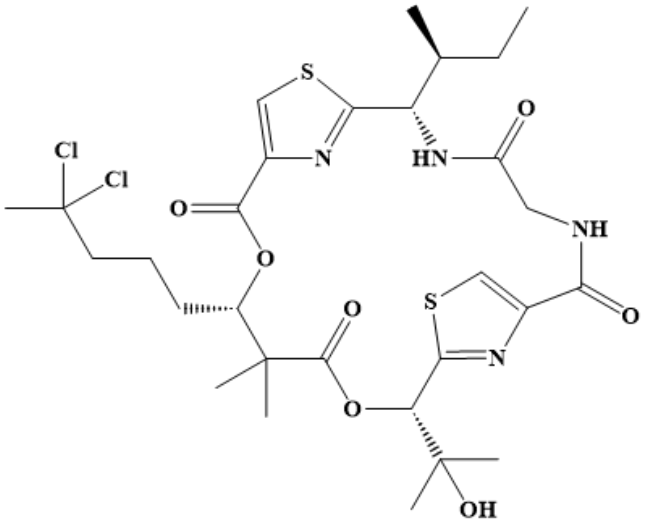
2.3. Cyclic Lipopeptides

One interesting lipopeptide group is lyngbyabellins, which have a structure composed of one C-7 chlorinated 2-methyloctanoic acid and two thiazole rings. It is reported that the series itself originated from lyngbyabellin A (Figure 2.3(A)), a marine cyanobacterium extracted from *L. majuscula* off of Guam. In fact, this specific compound helps to break

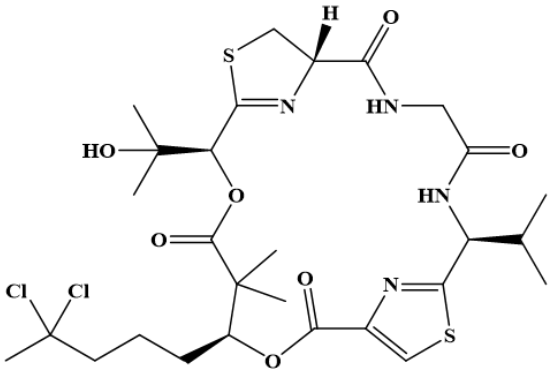
down microfilaments [13]. Further investigation into the organism listed above revealed that lyngbyabellin B (Figure 2.3(B)) has a specific power, for its in vitro cytotoxicity does not decrease to as great an extent as it had with a closely related compound. Lyngbyabellin B, of the lipopeptide family, has shown promising properties. Against both KB and LoVO cells, the IC50 value is 0.1 ug/mL and 0.83 ug/mL [13]. A Researcher once studied *L. majuscula* in the shallow waters of Papua New Guinea. Thus, this study concentrated on the lyngbyabellins E (Figure 2.3(C)), lyngbyabellins G (Figure 2.3(D)) and lyngbyabellins H (Figure 2.3(E)). The above compounds have proved to be very toxic in vitro against mouse neuroblastoma cell line Neuro-2a cells and mammalian lung tumour NCI-H460. Here, the LC_{50} values were obtained within a 0.2-4.8 μM range [14]. Such values reflect the concentration at which half of the cells show an effect. This discovery has helped shed light on the bioactivity of lyngbyabellin.

Figure 2.3 Chemical Structure

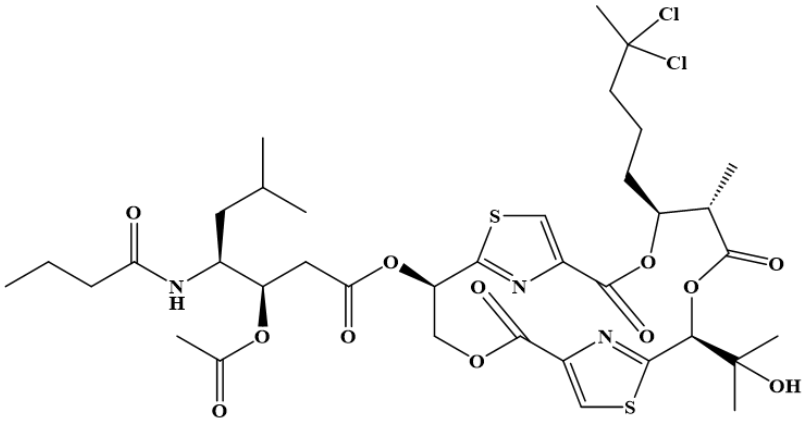
(A) Lygbyabellin A



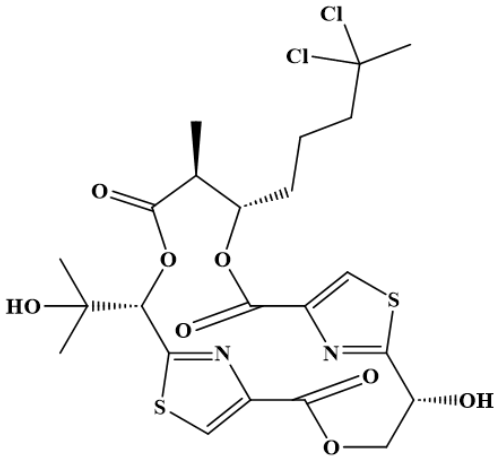
(B) Lygbyabellin B



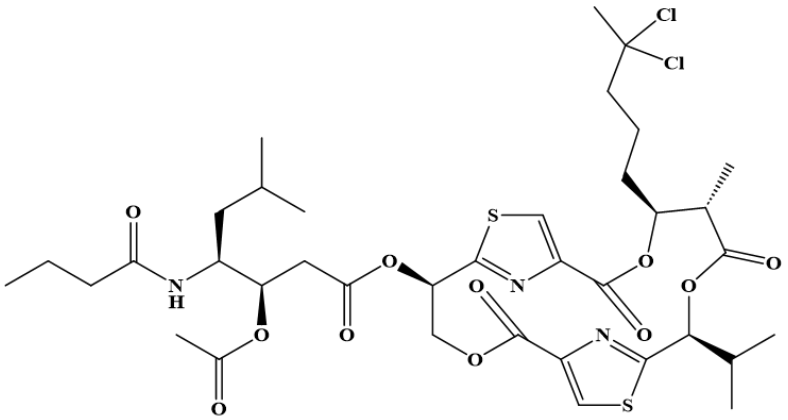
(C) Lygbyabellin E



(D) Lygbyabellin G



(E) Lygbyabellin H



The marine cyanobacterium *M. bouillonii,* which was obtained from Strawn Island, Palmyra Atoll (USA), helped the lyngbyabellin family grow. The various other derivatives such as lyngbyabellin H, K, L, N, and 7-epi-lyngbyabellin L were successfully isolated. Lyngbyabellin N is a compound that has demonstrated notable cytotoxicity against colon cancer cells of the HCT116 line. The compound shows an IC50 value of 40.9 nM [15]. The above mentioned observation supports the idea that the Lyngbyabellin family has anti-neoplastic solid qualities.

Furthermore, during the expedition, the meticulous researchers made remarkable discoveries about a variety of chemical substances. For instance, lyngbyabellin J was extracted from the *L. bouillonii*, which was recovered in Guam years ago. Lyngbyabellin J has displayed comparatively little cytotoxic effects against HeLa cervical carcinoma cells and H29 colorectal adenocarcinoma [21]. which can be considered one of the reasons that there is such a wide variety of behaviours among lipopeptides. When the distinctive characteristics of each lyngbyabellin compound are explained, the complete understanding of these lipopeptides, particularly their cytotoxic properties and their

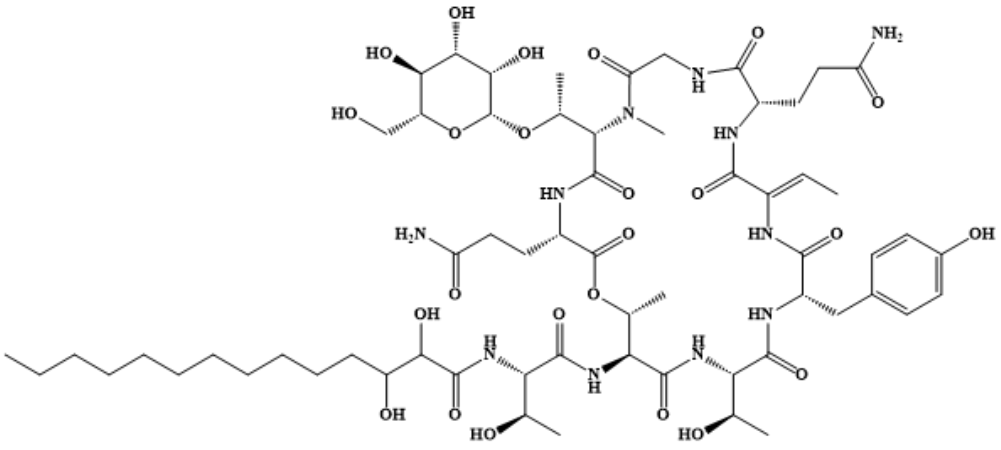
possible therapeutic uses in cancer treatment, becomes increasingly complex. The mentioned discoveries not only broaden our understanding of complicated natural processes, but also highlight the possible therapeutic applications of these substances.

2.4. Glycosylated lipopeptide

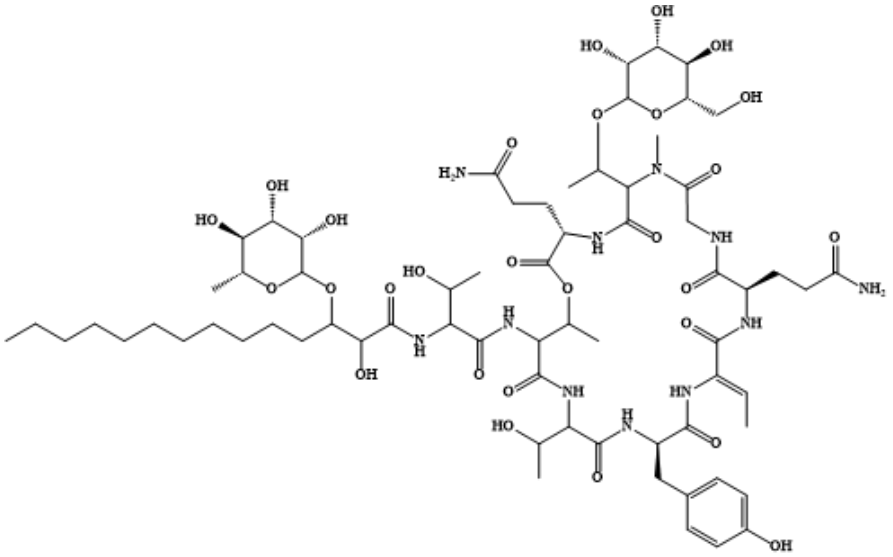
Hasallidins are a fascinating group of cyclic glycosylated lipopeptides that possess immense complexity and incorporate certain dihydroxy fatty acids as particular structural characteristics. The bonding of these different sugars with the hassallidin family is known as glycosylation. Sugars like pentose, hexoses, deoxyhexoses and N-acetyl hexosides are used in the biochemical process. This discovery was purely a byproduct of serendipity, as both hassallidins A (Figure 2.4(A)) and hassallidins B (Figure 2.4(B)) were extracted from the microorganism *Hassalia sp*., which is cultivated in Orrido Clough at Bellano in Italy. The organisms used to culture epilithic cyanobacteria there belong to this species. Importantly, what marks the difference between hassallidin B and A is that in addition to a rhamnose molecule linked with its lipid through hydrogen bonds, it also possesses an additional uronic acid. The serial dilution trials observed for antifungal effects against *Candida albicans* or *Aspergillus fumigatus* were quite good [22], [23].

Figure 2.4- Chemical Structure

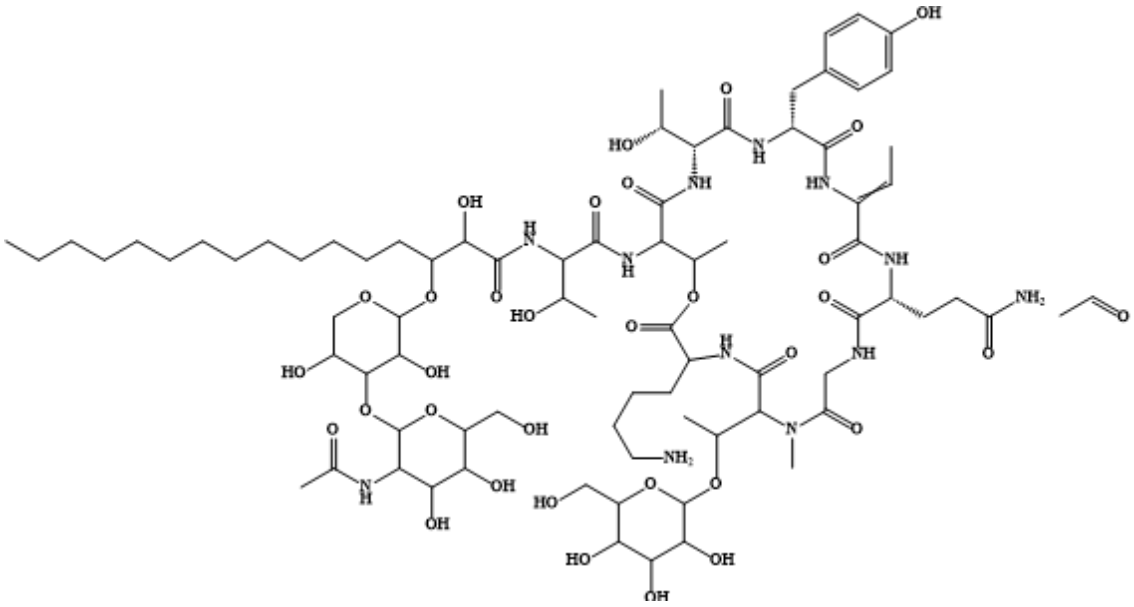
(A) Hassallidin A



(B) Hassallidin B

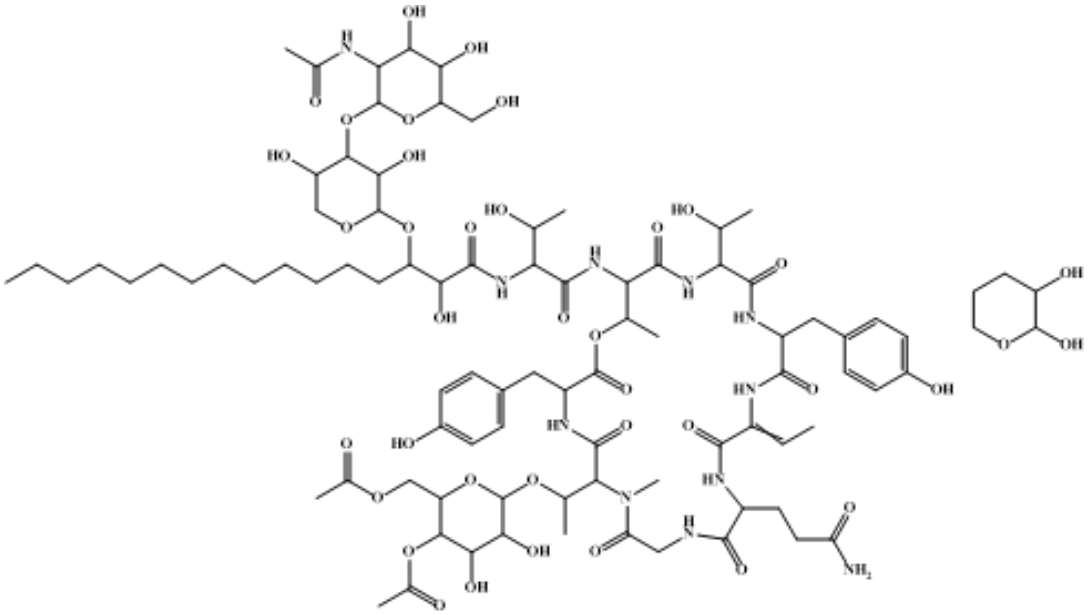


(C) Hassallidin C



At least 40 structural variables [24] come to light in the hassallidin biosynthesis pathway of *Anabaena sp.* SYKE748A. In particular, this is the finding of several substances referred to as hassalidins C (Figure 2.4(C)) and hassallidins D (Figure 2.4(D)). The main focus of scientific research is the identification and purification of D-hassallidins obtained from the cultivated cyanobacterium *Anabaena sp*. UHCC 0258. First, this research aimed to discover the effects of hassallidins D on mammalian and fungal cellular elements. A substance known as Hasssallidin D has the ability to alter membrane cells, causing lytic or necrotic cell death.

(D) Hassallidin D



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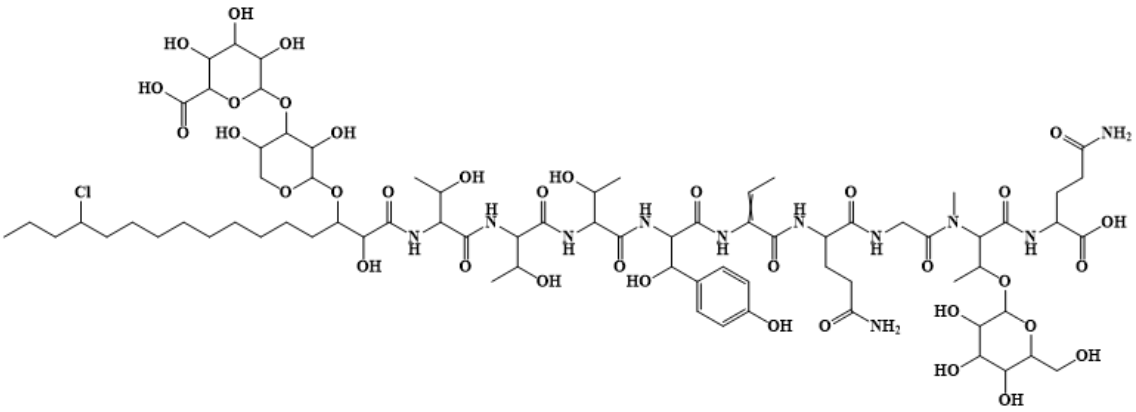
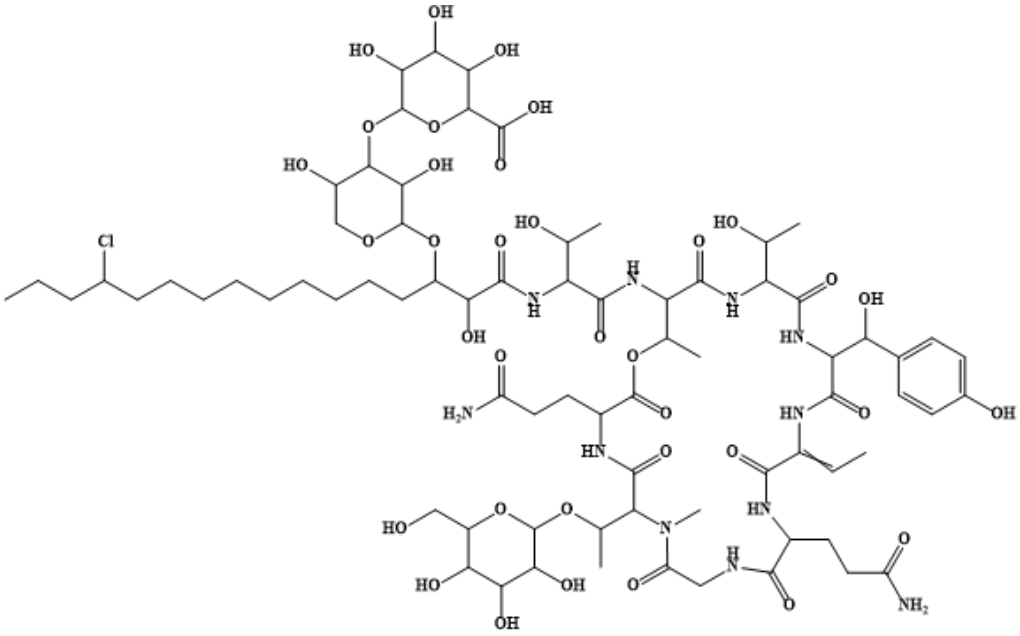
(E) Balticidin A

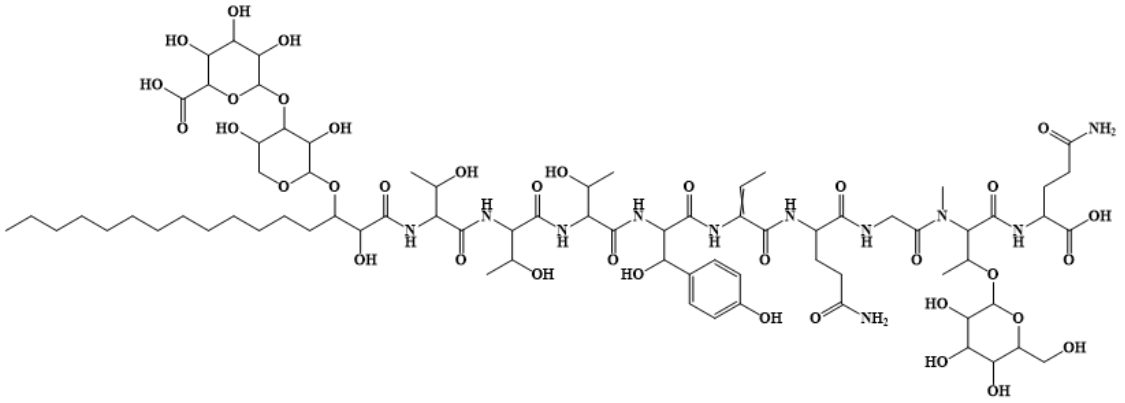


(F) Balticidin B

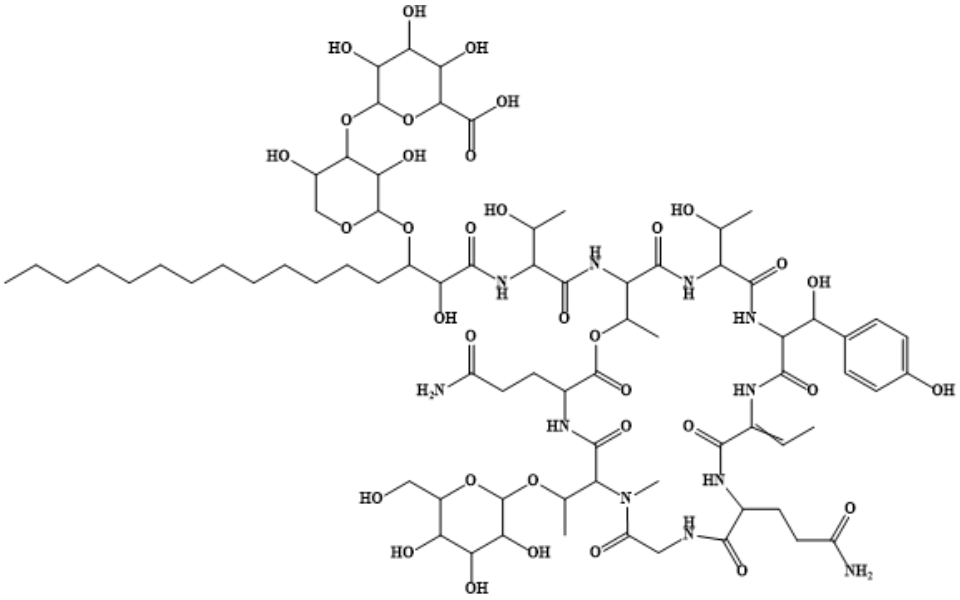


The above mentioned phenomenon is well documented because it manifests itself in the quick disintegration of the external membranes. Hassallidin D has a strong affinity with membranes containing sterols, as demonstrated by the artificial liposomal membrane assay results. By incorporating computational membrane modelling, the importance of cholesterol in facilitating the incorporation of hassallidin into the membrane was confirmed.

(G) Balticidin C



(H) Balticidin D



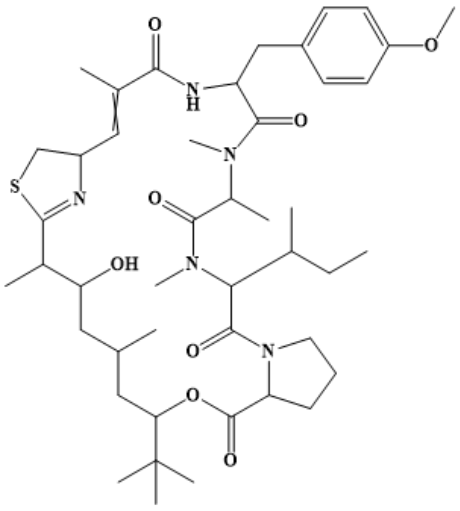
Furthermore, it is essential to note that two lipopeptides, balticidins A (Figure 2.4(E)) and balticidins C (Figure 2.4(G)), have demonstrated great importance because they do not have cycles [25]. These substances are remarkable with their cyclic opposites, balticidins B (Figure 2.4(F)) and balticidins D (Figure 2.4(H)). According to agar diffusion tests, the test substances showed antifungal properties against *Candida maltosa*. The derivation process was used to obtain the compounds in question. These were obtained from the Bio33 strain of the marine cyanobacterium *Anabaena cylindrica* [26].

2.5. Peptide Polyketide Hybrid

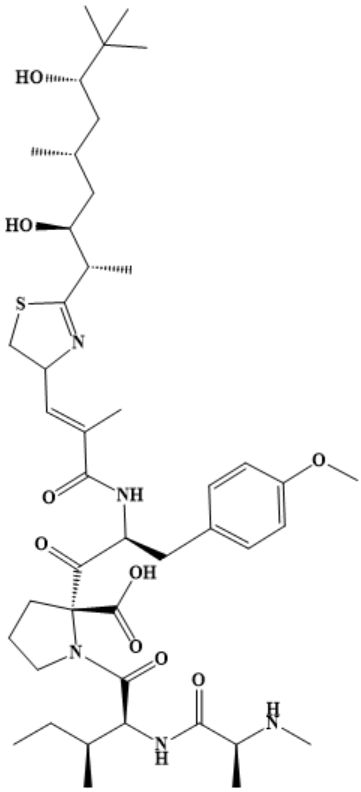
We mention cyanobacteria, one of nature's rarely regarded groups but have outstanding features. They possess a variety of cyclopeptides with peptide-and polyketide cores that exhibit many marvelous natural phenomena. Another extraordinary product of the sea is a compound known as apratoxin A (Figure 2.5(A)), which has been extracted from marine cyanobacterium *L. majusculum*. It was first noticed in Finger's Reef of Apra Harbor, one of the most respected harbours in Guam. The apratoxin A, has a highly complex molecular composition of enormous potential. A polyketide component and methylated amino acids are part of this architecture.

Figure 2.5- Chemical Structure

(A) Apratoxin A

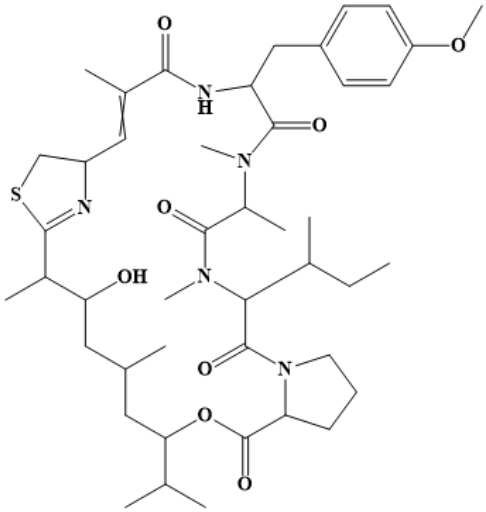


(B) Apratoxin B

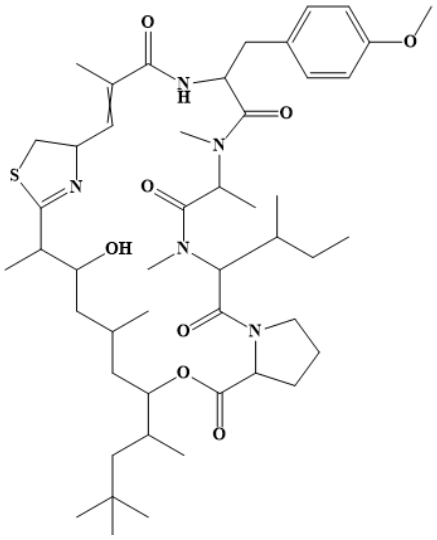


In laboratory tests, Apratoxin A demonstrated strong cytotonic properties against KB and LoVo cancer cells. According to these results, Apratoxin A could be a promising treatment [27]. Extensive research led to the discovery of apratoxins B (Figure 2.5(B)) and apratoxins C (Figure 2.5(C)), originating from *Lyngbya sp*. VP 417, obtained from the same tropical area. When tested on KB and LoVo cancer cells, apratoxin C demonstrated similar cytotoxicity to that of its predecessor, apratoxin A. Importantly, the activity of apratoxin B decreased modestly [28]. The variety of apratoxin demonstrates the dynamic nature of these cyanobacterial compounds.

(C) Apratoxin C



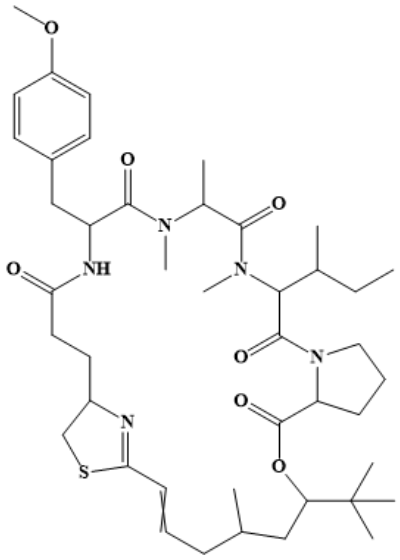
(D) Apratoxin D



The extraction of apratoxin D (Figure 2.5(D)) in samples of *L. majuscula* and *L. sordid* were collected in the tropical areas of Papua New Guinea. Although it has an amino acid structure similar to its competitors, Apratoxin D features a distinct polyketide moiety. When tested in the laboratory on H-460 lung cancer cells, the substance in question demonstrated significant cytotoxic activity [29]. The above mentioned discovery is a valuable addition to the ongoing study of the remarkable qualities demonstrated by

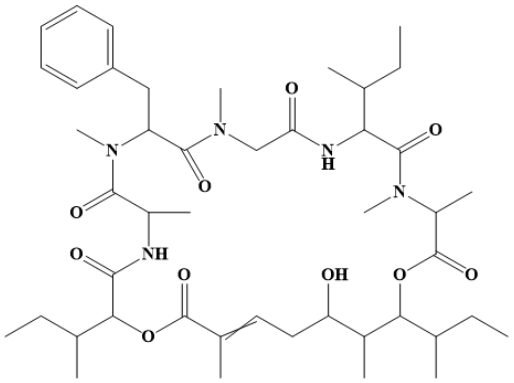
cyanobacteria. Unexpectedly, Apratoxin E (Figure 2.5(E)), a compound of great scientific interest, showed that this compound from *L. bouillonii* had a cytotoxic effect on human colon adenocarcinoma (HT-29), cervical carcinoma (HeLa) and osteosarcoma variant cells U2OS, which are used as test subjects in cancer research experiments [30].

(E) Apratoxin E

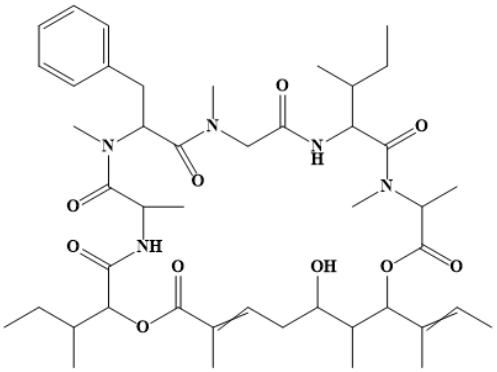


An intriguing marine cyanobacterium called *L. majuscula* has been discovered in another place, specifically in the Western lagoon, Singapore. Previously, the mentioned cyanobacterium demonstrated the ability to produce a class of substances called cyclic hybrid peptide-polyketides. It has been possible to isolate and characterize these compounds, which have been identified as lagunamides A (Figure 2.5(F)), lagunamides B (Figure 2.5(G)), and lagunamides C (Figure 2.5(H)). The group of substances mentioned has demonstrated notable antimalaria capabilities against *Plasmodium falciparum* and has demonstrated a moderate anti-swarming capacity against *Pseudomonas aeruginosa PA01* [31], [32]. When tested on murine leukemia P388 cells, laguanamide A and B demonstrated significant cytotoxic effects. In contrast, C lagunamides have demonstrated remarkable activity against a variety of cancer cells.

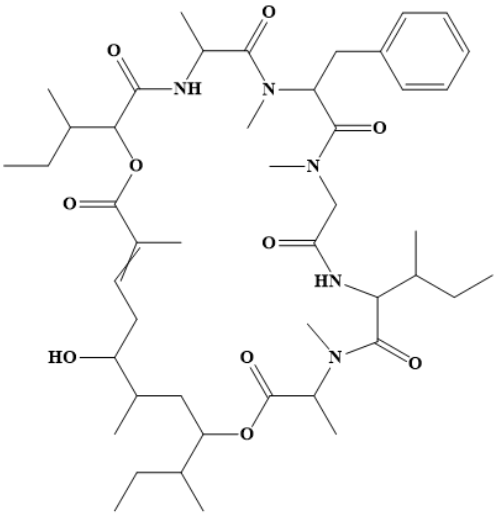
(F) Lagunamide A



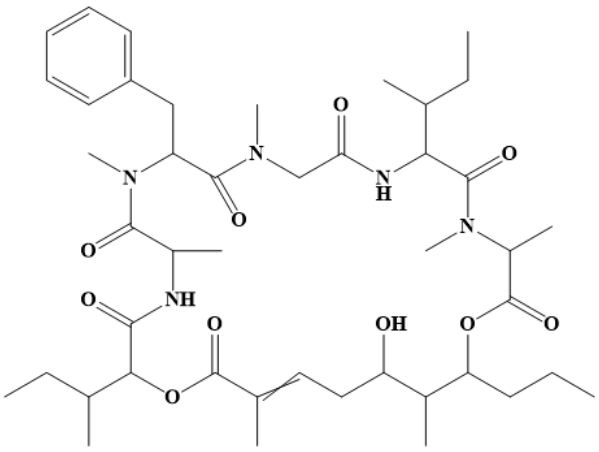
(G) Lagunamide B



(H)Lagunamide C



(I) Lagunamide D



With the unexpected discovery of lagunamide D (Figure 2.5(I)) within the cyanobacterial tufts at Loggerhead Key in the prestigious region of Florida, USA. The compound showed cytotoxic solid activity on A549 cells, causing cell death through the activation of caspases 3/7 [33]. In the field of cyanobacterial compounds, each new discovery helps to make the story more complete, showing not only their different mechanical characteristics but also their possible applications in medicine and other related fields. Invariably, the

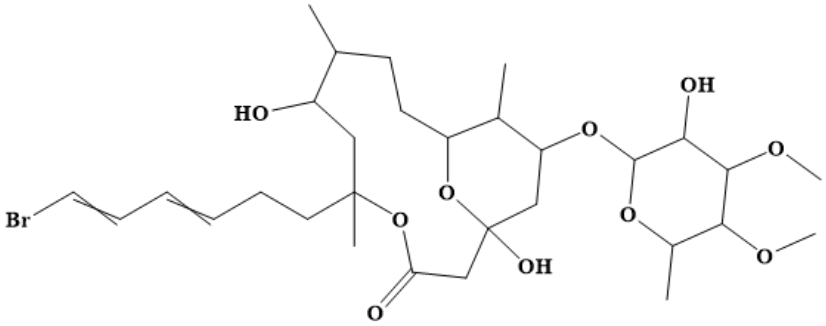
tiny organisms in question command attention and inspiration, providing us with a glimpse into the depths of natural biochemical ingenuity.

2.6. Macrocyclic Polyketides

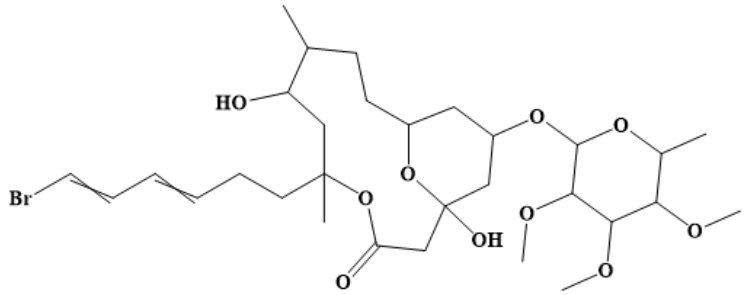
A captivating compound called lyngbyaloside reveals its compelling tale in the mysterious domains of marine cyanobacteria. A macrolide with 16 components with bromine is the compound under investigation. A compound known as 2,3,4-tri-O-methyl-6-deoxy-α-mannopyranoside is closely related to the material in question. The compound in question was unexpectedly found within the barriers of *L. bouillonii*, a microorganism native to the remote island of Laing in Papua New Guinea [12]. An analogue of the present compound, Lyngbyaloside B (Figure 2.6(A)), was accidentally discovered in a lipophilic extract derived from *Lyngbya sp.* NIH309, an animal that lives in the surrounding waters of the Ulong Channel in Palau. The substance in question has a distinctive bromine characteristic. When tested on KB and LoVo cells, the compound known as Lyngbyaloside B demonstrated a modest level of cytotoxicity. For KB cells, the IC50 values were approximately 4.3 μM and for LoVo cells, approximately 15 μM [28].

Figure 2.6- Chemical Structure

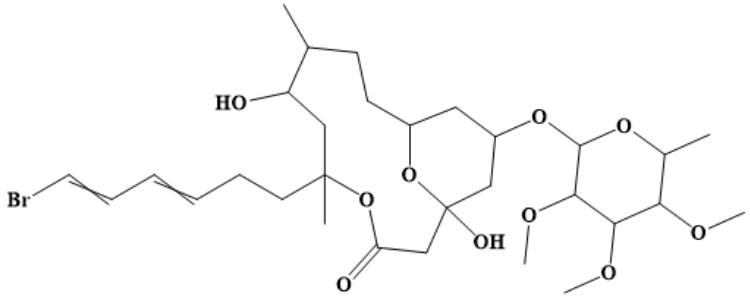
(A) Lyngbyaloside B



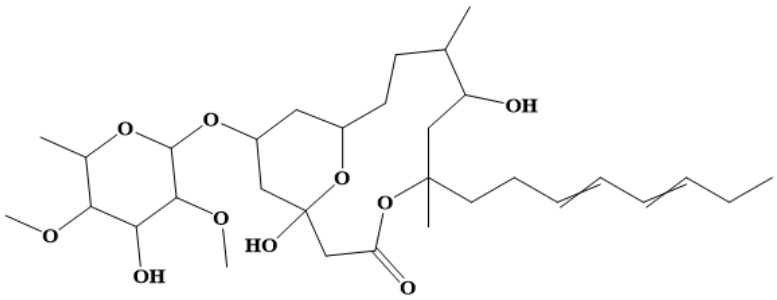
(B) 18E-Lyngbyaloside C



(C) 18Z-Lyngbyaloside C



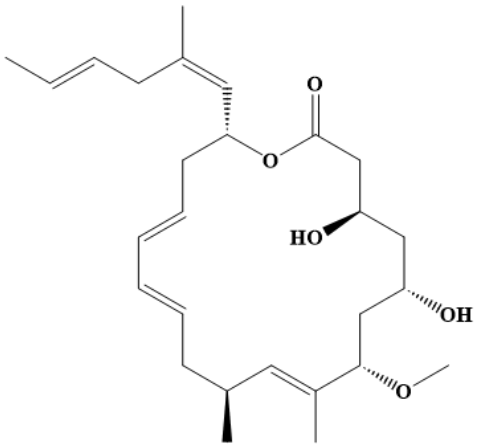
(D) Lyngbouilloside



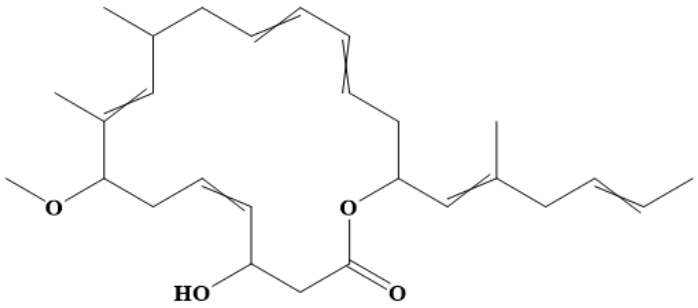
Amazing 2-epi-lyngbyaloside and its regioisomers 18E- and 18Z- lyngbyaloside C (Figure 2.6 (B and C)) were successfully isolated on the island in China. A profound natural phenomenon When moving into awe-inspiring artistic manifestations discovered worldwide, this remarkable location yields abundant inspiration. Their various chemical compositions displayed mild to moderate cytotoxicity when tested on HT 29 and HeLa cell lines [21]. Lyngbouilloside (Figure 2.6(D)) is a glycosidic macrolide that was accidentally discovered in the marine cyanobacterium *L. bouillonii* species. However, the source of this species can be traced back to the Papua New Guinea region. These

compounds differ because they obtain energy from bromine, although this particular macrolide does not draw its power from there. That compound, lyngbouilloside had a low degree of cytotoxicity toward the Neuro-2a neuroblastoma cells. The IC50 of the tested substance was 17 uM [34], which seems promising for suppressing cell growth.

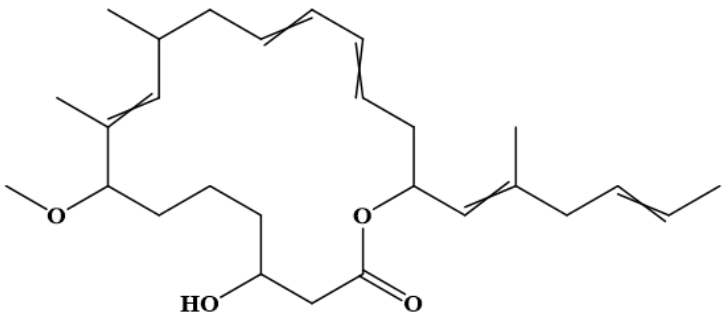
(E) Biselyngbyolide A



(F)Biselyngbyolide B



(G) Biselyngbyolide C



The investigation of eight-membered macrocyclic polyketides and their glycosylated counterparts marks the ongoing progression of the *lyngbya* saga. In the coastal areas of Okinawa Prefecture, Japan, the compound biselyngbyaside 1 was successfully extracted from the organism *Lyngbya sp*. using a bioassay-guided fractionation technique. This brought about a significant response. Notably, this polyketide showed its potency when 39 various cancer cell lines were treated. Compared to chemo drugs currently on the market, at a concentration of 0.60μM, it effectively inhibited growth by half (GI50) [35]. It is a cognate of biselyngbyolide A (Figure 2.6(E)), which has been found in the marine world around Tokunoshima Island. The above experiment demonstrated its ability to induce considerable cell death in HeLa S3 and HL6O cells [36]. When increases were made through the addition of biselyngbyasides B (Figure 2.6(F)), biselyngbyasides C (Figure 2.6(G)) and biselyngbyasides D to these symphonies (altering their colour), samples showed a high degree of inhibitory impact on proliferation. Eventually, they caused HeLa S3 or HL60 cells to die. The research team led by Suenaga conducted an in-depth investigation into the history of *Lyngbya sp*., which was found on Ishigaki Island, Japan. The researchers discovered biselyngbyolides C, E and F, which were previously unknown, along with congeners they had already identified. In this speech, a mixture of synergistic substances prevented the proliferation of HeLa S3 and HL60 cells [37]. Notably, biselyngbyaside C has demonstrated additional capabilities to reduce endoplasmic reticulum stress and induce the death of particular HeLa cells.

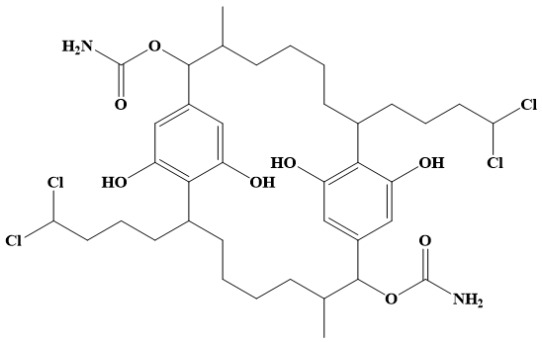
2.7. Carbamidocyclophanes

In the captivating area of cyanobacteria, a compelling story about a group of substances called paracyclophane derivatives unfolds. The story offers a gripping and engaging adventure exploring the complicated processes of biosynthesis and the resulting

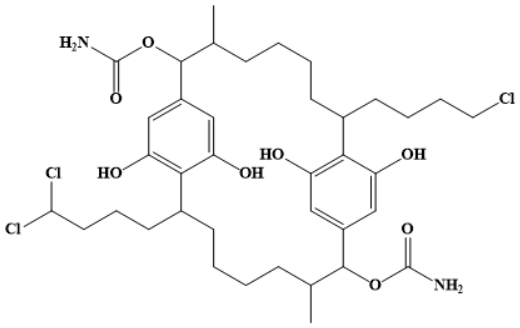
biological activities. The taxonomic group known as Nostoc includes those who have exceptional chemical ability and are recognized for the complex synthesis of these exciting molecules. A study showed the existence of carbamidocyclophanes A–E (Figure 2.7 (A-E)) in the cultivated environment of *Nostoc sp.* strain CAVN10, first found in North Vietnam's soil. The compounds mentioned above demonstrated antibacterial solid properties against *S. aureus* ATCC6538. They are distinguished by the presence of chlorine or bromine atoms on their side chains [38].

Figure 2.7- Chemical Structure

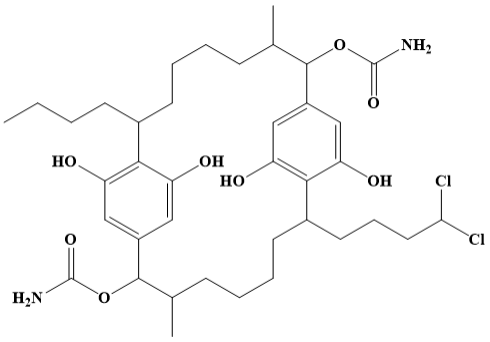
(A) Carbamidocyclophane A



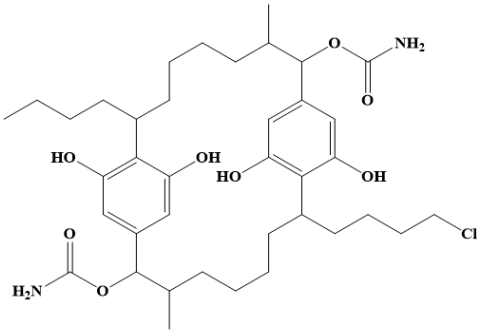
(B) Carbamidocyclophane B



(C) Carbamidocyclophane C



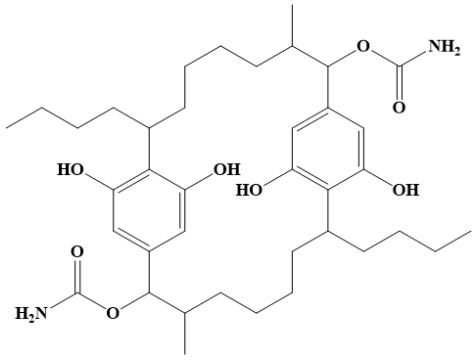
(D) Carbamidocyclophane C



As carbamidocyclophanes F and G gracefully entered, joining their fellow counterparts A, B, and C on the grand stage, the melodious composition persisted. The compounds mentioned above were found within the bounds of Des Plaines, Illinois. The antimicrobial regimen used by the subjects under investigation was evaluated for its efficacy against

the well-known pathogens *Staphylococcus aureus* and *Enterococcus faecalis*. The minimum inhibitory value (MIC) was found to be 0.1 μM and 0.2 μM, respectively, indicating the low concentration necessary to stop the growth of these microorganisms. In the borders of cancerous cell lineages, the notable ability of Carbamidocyclophanes F and G to prevent cellular proliferation has been demonstrated [39].

(E) Carbamidocyclophane E



The remarkable ability to produce carbamidocyclophanes H-L of *Nostoc sp*. strain CAVN2 has attracted attention. In this study, we discover a variety of chemicals called carbamidocyclophane M-U. When tested against *M. tuberculosis*, the mentioned compounds show a remarkable range of MIC values of 1.1 to 6.8 uM. Furthermore, they have MICs of 0.1–1.5 uM and are resistant to many varieties of *Staphylococcus* [40]. Their contribution to cyanobacterial chemistry is enormous. The paracyclophane derivatives found in the Nostoc organism show remarkable antibacterial and cytotoxic properties, captivating us with their fine biosynthetic craft.

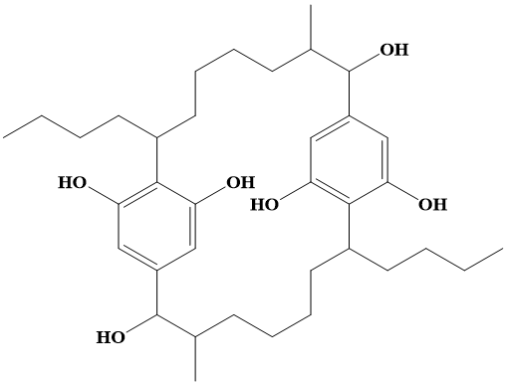
2.8. Cylindrocyclophanes

Terrestrial cyanobacterium *Cylindrospermum licheniforme* [41] was found to produce three different strains, yielding Cylindrocyclophanes A-F (Figure 2.8 (A-F)). Scientific

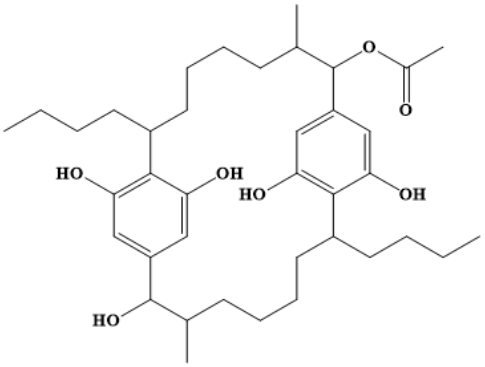
investigation revealed that there are actually several subdifferentions, cylindrocyclophanes A1-A4, C1-C3 and F4.

Figure 2.8- Chemical Structure

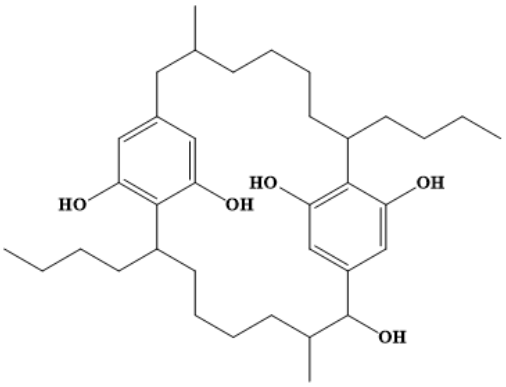
(A) Cylindrocyclophane A



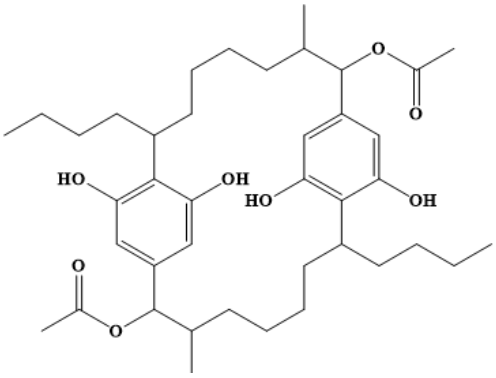
(B) Cylindrocyclophane B



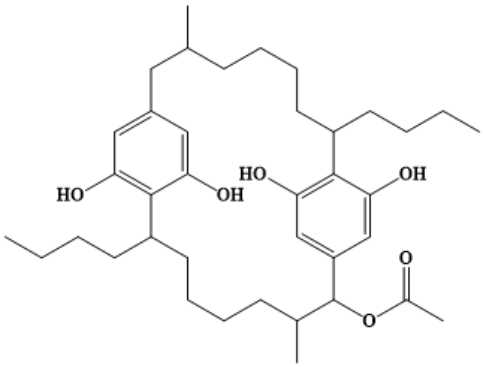
(C) Cylindrocyclophane C



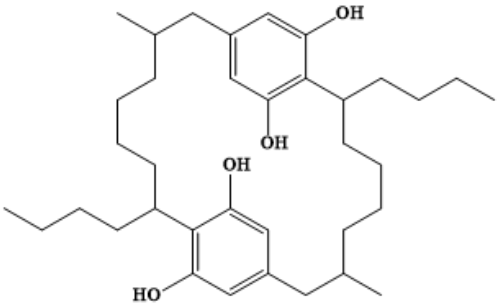
(D) Cylindrocyclophane D



(E) Cylindrocyclophane E



(F) Cylindrocyclophane F



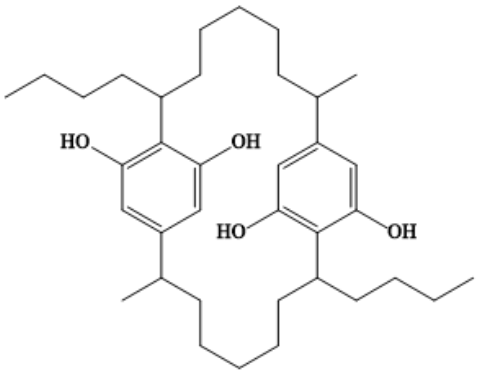
Furthermore, the bacterial culture extract of *Nostoc sp*. yielded many compounds. The stain UIC 10022A evolved from an algal community found in the soil of Chicago. Their collection included carbamidocyclophanes A, C, F and AB4. Many studies have also demonstrated that several different substances may be inhibitors of the 20S proteasome. Those compounds include carbamidocyclophanes A, C and F; cylindrocyclophane type I (A1 through A4); cy- limenaphothetype type II (C2 through AB6) [42]. This latest discovery highlights the great multitude of components in nature and their possible role as biological functions.

2.9. Merocyclophanes

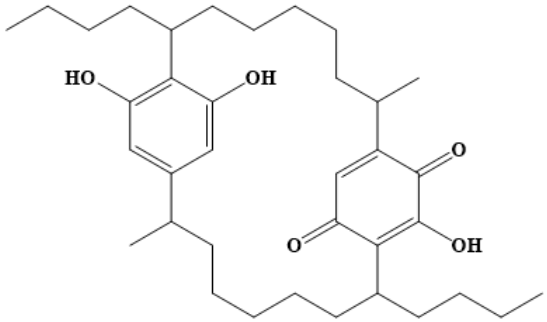
Nostoc sp. extract of the culture filtrate has yielded merocyclophanes A (Figure 2.9(A)) and merocyclophanes B (Figure 2.9(B)). Strain UIC 10O62, which, on the other hand, was clipped from a sample taken in Michigan. Interestingly, these two chemicals have also recently been found to prevent the growth of colon cancer (HT-29 cancers) in adults. IC50 values were calculated to be 3.3 and 1.7 μM, respectively [43] for the two compounds tested on clearance activity in human cells with EhcO phosphate esterase deletion syndrome being studied by these drug makers elucidates how they often overlook painstaking efforts patients go through trou panting themselves before getting out of.

Figure 2.9- Chemical Structure

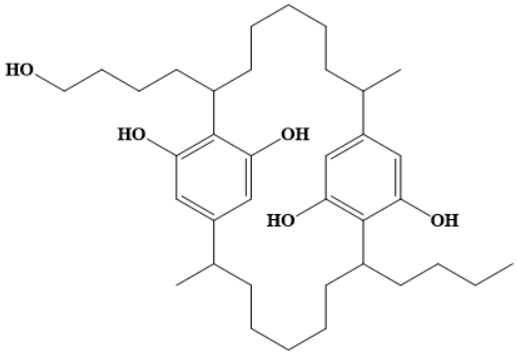
(A) Merocyclophane A



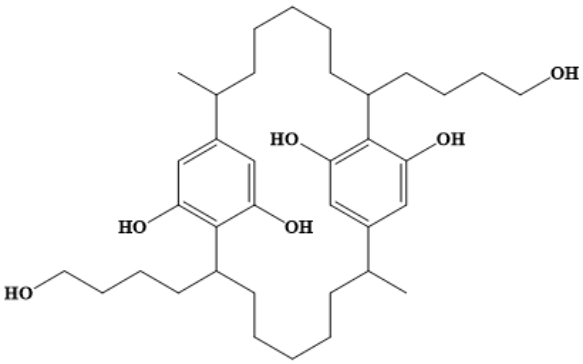
(B) Merocyclophane B



(C) Merocyclophane C



(D) Merocyclophane D



Another extended investigation was conducted that finally discovered two chemicals, called merocyclophanes C (Figure 2.9(A)) & merocyclophanes D (Figure 2.9(A)). Scientists detected the presence of these compounds in culture filtrate of freshwater variety of *Nostoc sp*. strain UIC 10110. Among these, the IC50 values of merocyclophanes C and merocyclophanes D against MDA-MB 435 were at levels comparable to those seen many times before with antiproliferative agents (1.6 and 0.9 μM), suggesting that both have a suppressive effect on tumor growth in humans cells as well [44].

3. Secondary Metabolites in Drug Discovery

The cyanobacteria are gram-negative, taxonomically belonging to the group of photoautotrophic prokaryotes. These organisms appear to have extraordinary skill in oxygenic photosynthesis. In the scientific world, they are called cyanoprokaryota, cyanophyta and blue-green bacteria. They also produce another pigment called c-phycocyanin (C -PC), which is involved in photosynthesis, the complex series of reactions whereby light receptors convert sunlight into chemical energy. The oldest living thing on Earth, Cyanobacteria, has an existence stretching back 3.3-3.5 billion years [45]. The fossil record shows that they once thrived in many ecosystems worldwide. Advanced internal membranes in these organisms facilitate the movement of electrons when they photosynthesize and respire. Even though their cells contain bacterial chromatin, they have complex internal structures similar to eukaryotes. Myxophycean starch, cyanophycin for taxonomic purposes, the group whose members are cyanobacteria do not have food storage.

Microorganisms like cyanobacteria, for example, can show amazing water oxidation during photosynthesis. This process relies on utilising Photosystem I and II, with an organization that is Z-type. This process also requires the use of Photosystem I and II, which are arranged in a Z scheme. The above example is impressive. Though these do not lack oxygen, organisms of this type can rely only on Photosystem I as they do for purple bacteria in anaerobic environments (lacking O2). Also, photosynthesis and respiration are both tied together by the exact electron transport mechanism [46]. In general, cyanobacteria feed mainly through photoautotrophy. Despite this, it is essential to underline that some of the species in this group exhibit a fascinating phenomenon

known as photoheterotrophy. In this way, they can use glucose as carbon and energy, especially in situations where light is challenging to obtain.

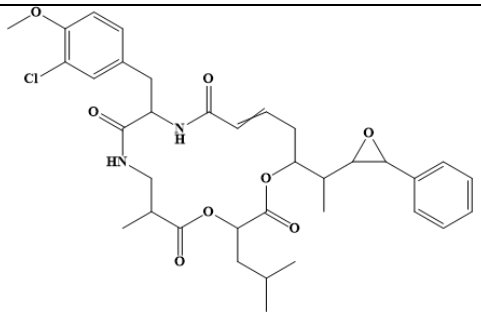
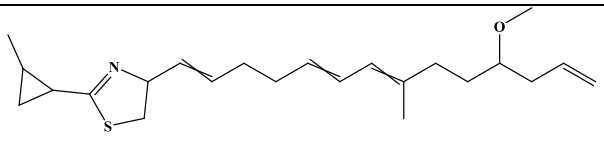
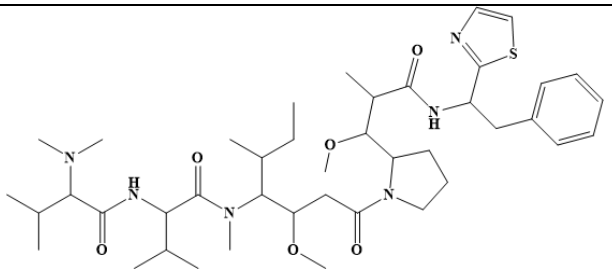
The morphology of cyanobacteria is very varied and includes unicellular and multicellular forms, such as colonial and filamentous. Typically, the above-mentioned structures are covered by a mucilaginous or gelatinous membrane, the composition of which varies depending on environmental circumstances. *Nostoc,* a filamentous cyanobacterium that is very interesting for scientific research, has the remarkable ability to form oval colonies that can have a diameter of up to three or four centimetres. Three different cell morphologies are observed in some filamentous cyanobacteria: thick-walled heterocysts, climate-resistant akinetes, and vegetative cells. In their natural habitat, cyanobacteria are extremely widespread. They live in a variety of environments, including not only rocky substrates, soil, aqueous environments, and even the atmosphere. It is not uncommon to see cyanobacterial species in symbiotic relationships with different plants or animals, although it is common for cyanobacterial species to inhabit their surroundings without limitations.

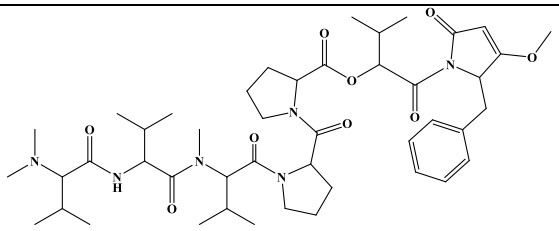
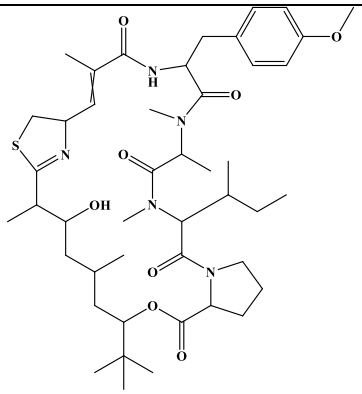
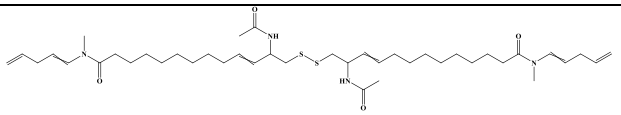
3.1. Anticancer Activity

The increasing resistance exhibited by tumor cells towards current therapies such as vinca alkaloids and taxanes has led to a growing demand for novel anticancer drugs. In the field of cancer chemotherapy, the existence of this resistance presents significant obstacles. Furthermore, the increased occurrence of new cancer manifestations, such as glioblastoma, highlights the need for new treatment methods. Despite progress in many areas, cancer remains a leading cause of mortality worldwide. This fact underlines how important it is to carry out effective and simplified interventions. Cryptophycins, a class

of substances derived from cyanobacteria, are widely recognized as highly effective in fighting cancer [47]. Cryptophycin 1 (Table 1), has demonstrated a notable increase in effectiveness compared to older anticancer drugs such as taxol and vinblastine. Its power increases 100 to 1000 times, indicating a notable improvement in its therapeutic abilities. The fact that the chemical analogue cryptophycin-52 (LY 355073) has been used in clinical trials is fundamental. Despite this, it is sad to note that the results of the trials demonstrated relatively low efficacy. Currently, two notable entities called cryptophycins 249 and 309 are present among the second-generation candidates [48]. The candidates above have demonstrated excellent stability and solubility in liquid solutions, making them potentially suitable for clinical trials.

Table 2.1- Anticancer Compounds from Cyanobacteria

S. No.	**Secondary Metabolite**	**Structure**	**PubChem CID Identifier**
1.	Crytophycin 1		6438401
2.	Curacin A		5280967
3.	Dolastatin 10		9810929

4.	Dolastatin 15		9918978
5.	Apratoxin A		6326668
6.	Somocystinamide A		11115357

Curacin A (Table 1), which was first found within the walls of *L. majuscula*, has encountered some obstacles resulting from its insoluble nature. At this moment, the derivatives mentioned are the subject of preclinical trials as possible candidates for future anti-neoplastic treatments [49]. Initially found within the marine gastropod *Dolabella auricularia,* Dolostatin 10 (Table 1) has demonstrated notable efficacy as an antiproliferative. It was later recognized as a compound created by cyanobacteria. Despite this, it is essential to remember that the clinical trial of this particular drug encountered several difficulties, leading to its interruption and the onset of peripheral neuropathy. TZT-1027, also known as auristatin PE or soblidotin, is a very promising alternative that has demonstrated high efficacy in human xenograft models [50].

Dolastatin 15 (Table 1) is a member of the dolastatin family that has exhibited a remarkable ability to inhibit cell proliferation. Despite this, due to its complex molecular

structure, poor production efficiency and limited solubility in water, the compound encountered numerous obstacles during clinical trials. In phase I clinical trials, the analogue Cematodin (LU-103793), which is soluble in aqueous solutions, has demonstrated promising characteristics. As a result of this success, ILX-651 (also known as Synthadotin)—a third-generation equivalent in phase II trials [51].

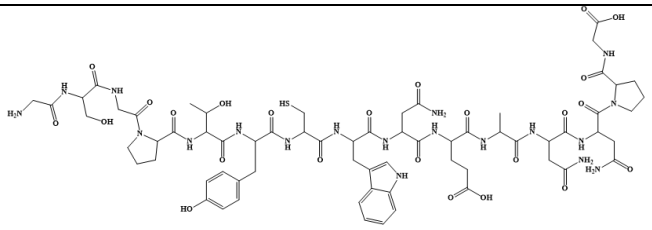
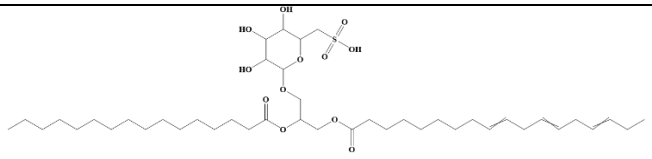
Apratoxin A (Table 1), is a cyclodepsipeptide, from *Lyngbya sp*., which is native to China. It has been observed that the photosynthetic pigment C-PC may be used due to its anti-inflammatory and anticancer properties. This indicates that it may effectively prevent or treat cancer [52]. Tolyporphin, which is derived from *Tolypothrix nodosa*, showed remarkable photosensitizing activity and was shown to be more effective than typical photodynamic treatments when dealing with tumours [53]. The organism *Lyngbya majuscula* provided the compound known as Somocystinamide A (ScA) (Table 1). In particular, this organism can remarkably hinder both angiogenesis and the proliferation of malignant cells within a tumour. Furthermore, it has been demonstrated that this phenomenon can induce the death of both tumours and angiogenic endothelial cells [54]. It is worth noting in this article that numerous other cyanobacterial metabolites have promising anticancer properties, though they are not discussed here. Despite this, it is essential to investigate deeper into the details of how they work.

3.2. Antiviral Activity

Acute and chronic viral infections such as dengue and AIDS (acquired immune deficiency syndrome) have become significant threats to public health. Hence, developing effective, safe antiviral drugs against them is essential. However, the approved treatment for treating infected individuals as highly active antiretroviral therapy (HDART) -has its own

limitations. For example, they cite the possibility of potential toxicity, development of significant viral resistance, and problems in eliminating vital viruses. In vitro experiments have shown that all three kinds of cyanobacterial compounds have significant antiviral properties.

Table 2.2- Antiviral Compounds from Cyanobacteria

S. No.	Secondary Metabolites	Structure	PubChem CID Identifier
1.	Scytovirin N		139587544
2.	Sulfoglycolipid		6450969

Polysaccharides spirulan and Ca-spirulan are probably most deserving of attention for their studies on antiviral effects. These polysaccharides come from Spirulina sp., a well-known species of cyanobacteria. Chemicals extracted from cyanobacteria have been shown to be effective in fighting against a host of viral strains, including influenza virus and human immunodeficiency virus 1 and 2. Most importantly, it is essential to remember that the efficacy of these three sulfated polysaccharides has been shown in their ability to prevent viruses from attaching themselves and fusing with their host cells. It is this same substance that suppresses the reaction of CD4 + lymphocytes (found in people affected by AIDS) with uninfected CD4 + cells. This functional capability is essential for promoting virus transmission. That is because the first set provides relatively reduced

anticoagulant effects, making them ideal as an alternative to other sulfated polysaccharides in treating viral diseases [55]. Nostoflan is a polysaccharide acid extracted from microorganisms, which is why it is called *Nostoc flagelliforme*. It has potent antiviral activity against the Herpes simplex virus-1 [56].

Cyanovirin-N and scytovirin, two carbohydrate-binding proteins, are currently under development for use as exceptionally effective antiviral drugs. Cyanovirin-N is a polypeptide consisting of 101 amino acids from the organism *Nostoc ellipsosporum* [57]. The phenomenon in question has shown strong antiviral properties against the human immunodeficiency virus (HIV) and other members of the lentivirus family in both living organisms and controlled laboratory conditions. This effect has been observed even in situations where the substance is present at exceptionally low levels within the nanomolar range. Scytovirin (Table 2) is extracted from an organism called *Scytonema varium* [58] and contains a polypeptide chain of 95 amino acids. Studies have shown that the complex can successfully bind to the envelope glycoproteins (gp120, gp160 and gp41) of HIV, effectively neutralising it. However, it has been verified that the neutralization effect persists even at a shallow concentration level. Nuclear magnetic resonance (NMR) shows that scytovirin comprises two distinct domains. Furthermore, the first domain was found to have antiretroviral activity against HIV, similar to scytovirin [59].

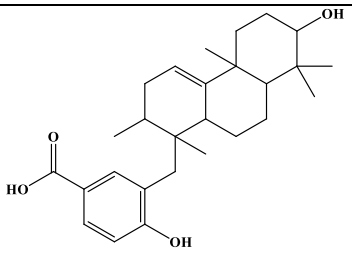
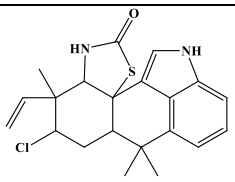
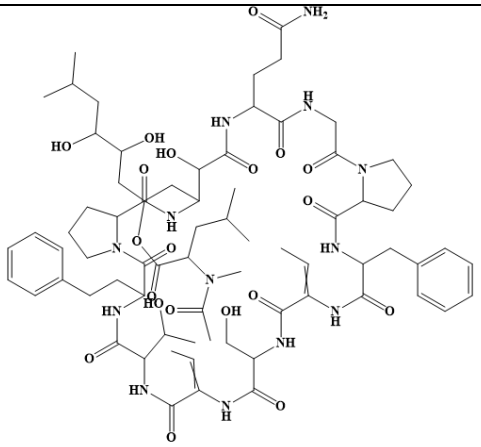
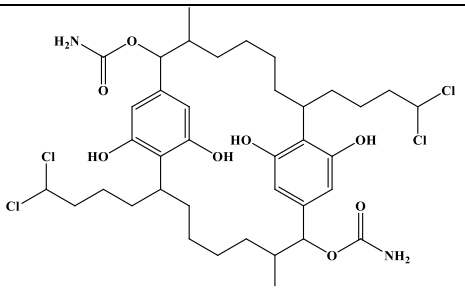
Furthermore, cyclic peptides that have shown antiviral properties include natural depsipeptide-based drugs such as ichthyopeptins A and ichthyopeptins B obtained from *Microcystis ichthyoblabe* [60] have proved effective counters to the influenza virus to type A. The IC50 value, which indicates the concentration at which a specific activity is inhibited by 50%, was established to be 12.5 g ml–1. Sulfoglycolipids (Table 2), obtained from cyanobacteria, also have the ability to hinder the activity of HIV reverse

transcriptase and DNA polymerases [61]. The discovery highlights the opportunity to use cyanobacterial compounds to create effective antivirals that can selectively attack a variety of viruses. The above statement is paramount in our fight against the viral threats plaguing the entire world.

3.3. Antibacterial Activity

In view of the increase in the presence of resistant bacteria, the growing danger of diseases that cannot be cured becomes increasingly worrying. The proliferation of bacteria that are resistant to various drugs, mainly those responsible for infections in healthcare settings such as *Staphylococcus aureus*, methicillin-resistant, vancomycin-resistant, and *Enterobacteriaceae* that produce AmpC-beta-lactamase, represents an issue of great concern worldwide [62]. The advancement of new antibiotics that can effectively address bacterial infections is critical due to the severity of this problem. Scientists are currently looking for antibacterial qualities in cyanobacterial extracts. The potential antimicrobial effect of the extracts has been carefully examined [63]. Despite these actions, so far, only a small number of antimicrobials derived from cyanobacteria have undergone in-depth analysis of their mechanical properties.

Table 2.3- Antibacterial Compounds from Cyanobacteria

S. No.	Secondary Metabolites	Structure	PubChem CID Identifier
1.	Noscomin		10410225
2.	Hapalindole T		21671523
3.	Pahayokolide A		70697949
4.	Carbamidocyclopane A		16210902

Noscomin (Table 3) [64], which comes from the plant *Nostoc commune*, is an example of these fortifying substances. According to the research results, the examined substance has

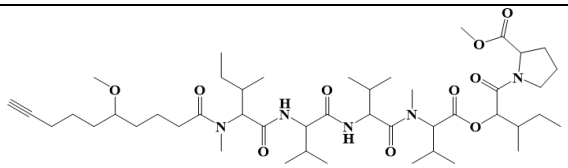
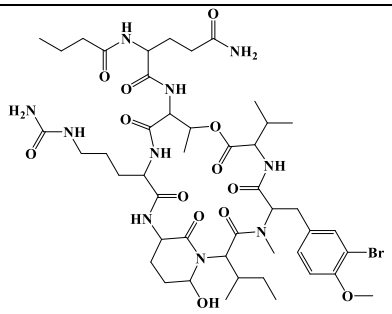
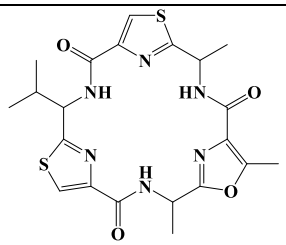
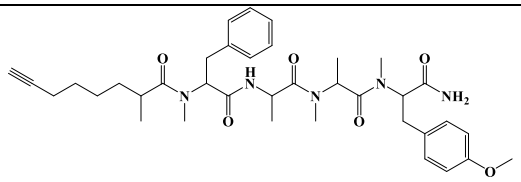
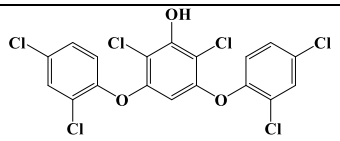
antimicrobials that fight *Bacillus cereus* (MIC of 32 parts/million), *Staphylococcus epidermidis* (MIC of 8 parts/million) and *Escherichia coli* (MIC of 128 parts/million). This effect that has been observed is similar to that which occurs with well-known drugs. Another study examined the effectiveness of an Anabaena extract in fighting bacteria resistant to vancomycin, particularly *S. aureus*. The MIC of the extract was between 32 and 64 ug/ml. Furthermore, it is essential to highlight that the process of extracting nine distinct ambiguines from *Fischerella sp*. [65] led to intriguing results regarding its antimicrobial capabilities. It is important to note that Ambiguine-I isonitrile was more effective than streptomycin in fighting *Bacillus subtilis* and *Staphylococcus albus*. According to the prestigious research on *Micrococcus lacustris*, two new norbietane compounds have been discovered. Thanks to their exceptional antimicrobial properties, these substances fight a variety of bacteria, such as *S. epidermidis, S. aureus, S. Typhi, B. subtilis, V. cholerae, E. coli, B. cereus,* and *K. pneumoniae* [38]. The findings highlight the natural ability of cyanobacterial compounds to be an essential resource for the ongoing search for effective antibiotics to fight bacterial infections.

3.4. Antiprotozoal Activity

Worldwide, more than a billion people are affected by tropical ailments caused by various types of parasites, including *Plasmodium, Trypanosoma, Leishmania, Schistosoma* and others [66]. The figures were reported by the prestigious World Health Organization. The emergence of drug resistance within these unicellular entities causes difficulties in the management of these diseases, such as malaria [67] and leishmaniasis. Despite this, progress in creating effective and affordable therapies has been relatively slow. An International Co-operative Biodiversity Group is currently conducting screenings of extracts obtained from various sources, including terrestrial and marine, to enhance the

expeditious advancement of drug discovery endeavours pertaining to these ailments. The most recent result of the research work led to the discovery of five distinct categories of antiprotozoal substances derived from cyanobacteria.

Table 2.4- Antiprotozoal Compounds from Cyanobacteria

S. No.	**Secondary Metabolites**	**Structure**	**PubChem CID Identifier**
1.	Viridamide A		25111596
2.	Symplocamide A		44448139
3.	Venturamide A		11225555
4.	Dragomabin		16737469
5.	Ambigol C		5276614

Indeed, it is crucial to highlight the discovery of the alkaloid nostocarboline [68] within the ramifications of *Nostoc sp.* 78-12 A. In particular, this alkaloid has demonstrated a remarkable ability to fight different types of parasites, including *Leishmania donovani*, *Plasmodium falciparum, Trypanosoma cruzi* and *Trypanosoma brucei*. Its promising potential as a protease inhibitor is demonstrated by its IC50 values, which show a voltage range from 0.5 to 0.194 μM. Furthermore, it is important to highlight that Aerucyclamide C, which is derived from the organism *Microcystis aeruginosa* PCC 7806, has demonstrated remarkable efficacy in combating *T. brucei* [69]. Despite this, it is important to highlight that aerucyclamide B, which was previously identified, demonstrated significant antimalarial effects against *Plasmodium falciparum*. Its IC50 values are within the submicromolar range, indicating this. In another research project, a group of six unknown acyl proline derivatives were accidentally discovered during an investigation.

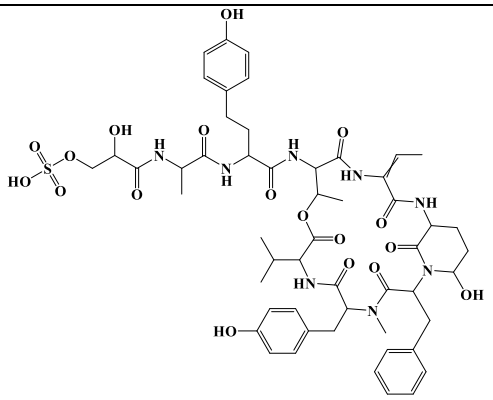
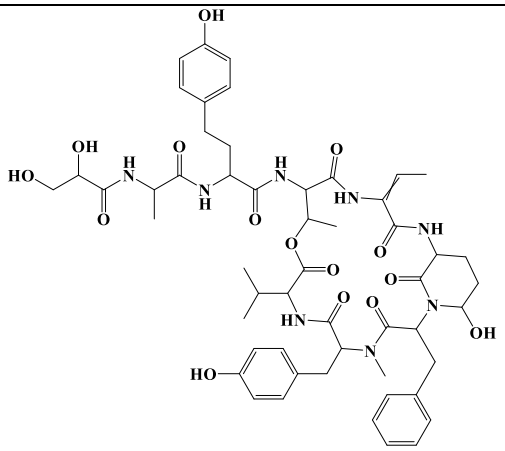
3.5. Other Activities

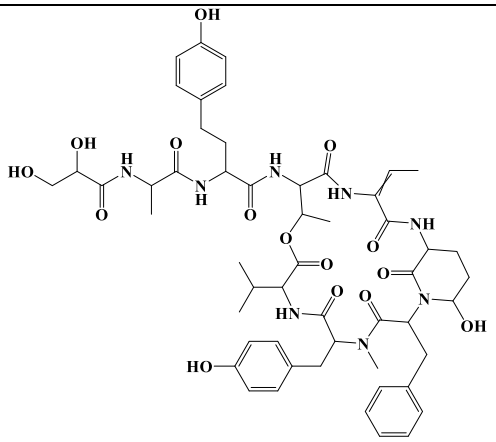
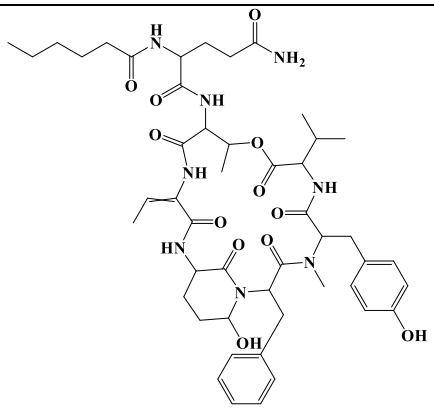
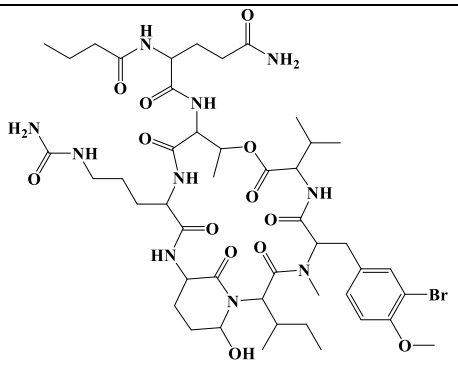
In the pharmaceutical field, the identification of new protease inhibitors presents an enormous prospect. Many of these protease inhibitors that are derived from cyanobacteria, such as *aeruginosins microginins,* and *cyanopeptolins*, have been extensively studied and documented. For instance, these have been successfully used to treat hypertension, while serine protease inhibitors like cyanopeptolin have been used with great success in the pharmacological treatment of a number of conditions, including but not limited to viral infections and asthma.

In recent research [70] two particular kempopeptins that show a remarkable ability to inhibit the protease have been identified. The cyclodepsipeptide Kempopeptin A comes from *Lyngbya sp*. When subjected to the protease enzyme elastase, the compound in

question showed an IC50 of 0.32 μM. In contrast, kempopeptin B had a notable inhibitory effect on trypsin, as demonstrated by its IC50 of 8.4 μM. The immunomodulatory effects of Spirulina have been the subject of numerous scientific research. Numerous scientific studies have shown that Spirulina products have the ability to influence the immune system in various ways. Increased macrophage activity, production of antibodies and cytokines, facilitation of proliferation of NK cells, and stimulation of T and B cells are some of the effects mentioned [71].

Table 2.5- Protease Inhibitors from Cyanobacteria

S. No.	Secondary Metabolites	Structure	PubChem CID Identifier
1.	Lyngbyastatin 4		16104920
2.	Lyngbyastatin 5		23657136

3.	Lyngbyastatin 6		23657138
4.	Lyngbyastatin 7		23657291
5.	Symplocamide A		44448139

Spirulina is generally considered safe by all, but it is essential to remember that some strains of cyanobacteria may contain cytotoxic metabolites, such as *microcystin* [72]. The substances mentioned have the natural ability to damage the lymphocyte and hinder the functioning of the membrane-bound leucine aminopeptidase. As a result, this has the potential to negatively impact the body's ability to process and present antigens. The first record on the immunotoxicity of an extract derived from a cyanobacterial bloom, focusing

on microcystin, was created. In mice that were immunized with T-dependent antigens, there was a decrease in lymphoproliferation caused by lipopolysaccharide and a decrease in the number of cells responsible for antibody production. Engagement with the cyanobacterial bloom extract resulted in an immunosuppressive condition, as demonstrated by evident changes in the complex cytokine network [73].

4. Siderophores

Synthesizing siderophores ('iron carrier' in Greek) is a common biological strategy bacteria, fungi, and plants use to transport iron. Siderophores are natural iron chelators with a high affinity for ferric iron. Their molecular weight is between 400 and 2000 Da. These molecules were developed to address the scarcity of iron [74]-[75], with their primary function being iron sequestration through the formation of soluble iron III complexes. One of the crucial functions of these entities involves endowing organisms with the capacity to harness iron species that are not readily soluble. Moreover, deleterious microorganisms actively acquire iron from proteins present within the host organism [76], [77]. The continuous revelations about novel structures indicate the persistent allure surrounding siderophores [78], manifesting in the insight of over 500 distinct siderophores [79].

Maintaining iron homeostasis emerges as a matter of tremendous significance for all organisms, given that the abundance of ferric iron can negatively affect cellular integrity. Consequently, a series of stringent control mechanisms have been implemented to regulate the concentration of iron effectively [80]. Comparably, bacteria can actively regulate their internal iron concentrations by synthesising siderophores in response to the prevailing iron levels in their immediate environment. Repressor proteins play a pivotal

role in this biological process, as they serve to hinder the activation of genes associated with iron absorption mechanisms, including the synthesis of siderophores, under conditions where enough iron is present within the cytoplasm. Fur, also known as the Ferric Uptake Regulator, is pivotal in governing iron-dependent repression in gram-positive bacteria and gram-negative. In contrast, it is noteworthy that high-guanine-cytosine gram-positive bacteria rely on the DtxR (diphtheria toxin regulators) family of repressor proteins for regulating iron-related pathways, employing a comparable mechanism [81].

Bacteria, fungi, and plants exhibit a diverse array of mechanisms for the synthesis of siderophores. The better chemical assessment of their activity stems from the fact that most bacteria produce siderophores mainly via non-ribosomal peptide synthetases (NRPSs) [82]. Enzymes also display a remarkable potential for assembling several different catalytic units into an assembly line. The modular domain architecture makes the efficient generation of novel structures possible [83], which enables the incorporation of different substrates into the final siderophore structure through the contribution of each module [84], [85]. While it is indeed true that NRPS-dependent synthesis is a prevalent phenomenon [79], it is imperative to acknowledge that the assembly of specific siderophores also necessitates the involvement of NRPS-independent synthetases (NIS). This alternative pathway holds equal significance in the grand scheme of siderophore synthesis [83]. Mugineic acid, the predominant siderophore in plants, is synthesised from nicotinamine under iron deficiency conditions.

When an organism perceives diminished iron concentrations within its environment, it proceeds to secrete siderophores to acquire this essential element. However, these molecules are remarkable because they have an excellent scavenging capacity and can

specifically bind to iron III to form ferri-siderophore. Later, the highly ordered structure is distinguished and brought into the cell component. Considerable investigation has been carried out on the intricate matter of ferri-siderophore complex transportation within the realm of gram-negative bacteria. The outer membrane comprises receptors with a pronounced affinity towards the iron-siderophore complex.

Obtaining iron has indeed been a key to survival in the course of mammalian evolution. Many species of microorganisms have also been discovered to possess transport systems which help in the absorption of external siderophores [86]. However, do not forget that there are already recorded examples of species from other kingdoms where the target fungi are incapable of synthesizing siderophores [87]. However, these fungi can also take advantage of such molecules when other organisms expel them. However, these fungi can also take advantage of such molecules when they are expelled by other organisms. However, it is precisely these fungi that are able to use and absorb them when they leave other microorganisms. Nonetheless, the fungi, which act as higher scavengers in nature's food chain, can absorb and make use of these molecules when other microorganisms sear them off. This reveals a vital survival mechanism designed to secure sufficient iron, even in adverse situations [79]. In addition, ongoing research is now trying to probe into the phenomenon of some strains of bacteria which can absorb siderophores but do not produce them. A similar phenomenon is the concept of "cheating" -cheated iron.

Siderophores exhibit a greater affinity for Fe^{3+} than for Fe^{2+}. In scientific circles, Fe^{3+} is thoroughly familiar--it has an intensely strong Lewis acid property, which gives it a remarkable ability to form complexes bound with electron-pair donors. As electron donors, siderophores use oxygen moieties that have negative charges to chelate iron (III) very strongly [88]. For the most part, Siderophores have an octahedral composition, and

they bind iron in a hexadentate arrangement that is thermodynamically advantageous [89], [90]. This approach can be seen in the design of single complexes. Chelating agents with a hexadentate structure have higher formation constants (Kf) than siderophores with 1:2 or 2:3 stoichiometries [79]. The former has a bidentate, and the latter tetradentate complex. In examining the world of siderophores, one cannot help but be awed by their expansive variety. However, amidst this diversity, it is intriguing that these compounds also share certain features. Furthermore, they employ similar methods regarding their ability to bind iron (III). Siderophores, a class of molecules, can be categorised into 3 groups based on their iron-binding modules. These groups include carboxylates, hydroxamates, and catecholates. However, it is essential to note that certain siderophores can incorporate multiple iron-binding constituents, thereby warranting their classification as mixed types [91], [92].

Carboxylates are chemical compounds that are derived from citric acid. They include siderophores, such as staphyloferrin A, which are synthesised by different species of *Staphylococcus* [93]. The siderophores are functional groups, including hydroxyl and carboxylate donor moieties. It is generally observed that carboxylates exhibit diminished efficacy in the process of iron scavenging when the pH is maintained at a neutral level. However, it is essential to note that carboxylate-type siderophores exhibit their advantageous properties primarily in environments with elevated acidity levels. This is due to their ability to demonstrate a higher degree of effectiveness when compared to hydroxamates and catecholates, which tend to remain in a protonated state [82]. Consequently, this specific siderophore confers a notable advantage to microorganisms that reside within acidic environments.

Hydroxamates constitute yet another significant class within the category of chemical compounds. Within this classification, one can find a diverse array of desferrioxamine variants, including but not limited to DFO-B and DFO-E. These specific variants are known to be synthesised by various species of the *Streptomyces genus* [94]. Moreover, it is worth noting that the predominant types of fungal siderophores that have been discovered are hydroxamates. These hydroxamates are distinguished into 3 well-defined categories focusing on the structural organization: ferrichromes, coprogenes, and fusarinines [95]. Hydroxamates, like catecholates, can generate chelate rings consisting of five members. Nevertheless, it should be noted that their affinities for iron are comparatively lower.

Considerable research has been diligently undertaken to investigate the catecholate group, predominantly observed within the bacterial domain. Several notable examples of siderophores can be observed in the microbial world. One such instance is enterobactin, which *Escherichia coli* and *Pseudomonas aeruginosa* synthesised. Another significant siderophore is salmochelin, a glycosylated derivative produced by various Salmonella species. Lastly, bacillibactin is a siderophore synthesised by different *Bacillus* species [96]. The siderophores belonging to the catecholate type exhibit a remarkable affinity for ferric iron, owing to their ability to form chelate rings comprising five members. Enterobactin *is* a compound characterised by a trilactone backbone composed of three catechol moieties [79].

The presence of two or more such components give rise to mixed-type siderophores. The most commonly mentioned example is the iron-binding compound yersiniabactin. The bacterium *Yersinia pestis* releases a compound called yersiniabactin. However,

researchers believe Yersiniabactine's ability to adhere to iron is due, in part only, to the oxazoline, carboxylate and phenolate groups as well as a thiazole group. The siderophore parabactin is the most familiar example; its structure appears highly complex due to rings of oxazoline and catechol groups [82]. Mycobactin is secreted by a kind of bacteria called Mycobacterium tuberculosis. Also, oxazoline rings are characteristic of compounds such as carboxymycobactin. In addition, the group also consists of mixed-type siderophores such as aerobactin and petrobactin. Another pathogenic orally transmitted bacterium is *Escherichia coli*, which produces the substance Aerobactin. The components of aerobactin are hydroxamate and carboxylate groups [97]. Marinobacter hydrocarbonoclasticis is a bacterium synthesising petrobactin, combining the catechol and carboxyl components [98]. Besides pyoverdine, the mixed type siderophores also have hydroxamate and catecholate components, which are produced by fluorescent species of *Pseudomonas* as well [99].

All the siderophores show a distinct correlation between pH levels and complex formation. Iron and proton (H+) ions have been observed in a competitive relationship if we consider natural environments. With the small amount of siderophores available, this interaction naturally takes place [100]. An elevated pKa value serves to intensify the degree of proton competition. When carrying out an extensive study, the de-protonation constants of freed pyoverdine and its (III) complex, which are generated by the microorganism *Pseudomonas putida* ATCC 33015, were determined. This exhibits the ability to synthesise the ferric complex by utilising three distinct chelating groups: α-hydroxycarboxylic acid, hydroxamate, and catecholate. The researchers observed the formation of the iron (III)-pyoverdine complex under acidic conditions. In that order, the earliest contact was discovered to occur with hydroxamate, the subsequent interaction

being with α-hydroxycarboxylic acid moieties and catecholates. The observation of iron coordination was found to be limited to conditions of neutral pH exclusively. A consequence of the disturbance brought about by protons was the development of the pFe (pM) scale [101].

The influence of the redox potential on the degree of stability of ferri-siderophore complexes is a crucial factor that must be considered. The redox potential known as E° of the Fe 3+ and Fe 2+ coupled in an aqueous solution is commonly observed to be approximately +770 millivolts (mV). However, it's noteworthy that variations in the redox potential may appear due to the process of iron chelation. Iron (II) complexes, exemplified by Fe-phenanthroline, typically exhibit elevated redox potentials in comparison to the iron (III) and (II) couple [82]. The consequence of this phenomenon is diminished accessibility of iron (II) in its capacity as a reductive agent. In contrast, it is worth noting that in cases where ferri-siderophore complexes exhibit stability, the observed redox potentials undergo a notable shift towards the negative end of the spectrum, with values ranging from -350 to -750 mV. This observation implies that the chelated form of iron (III) exhibits limited reactivity towards most biological reducing agents [97].

Controlling the E° of complexes between iron (III) and siderophores is paramount for two key reasons. First and foremost, iron contributes as an initiator in redox reactions, including the Haber-Weiss cycle that generates reactive oxygen species. The generation of deleterious hydroxyl radicals is attributed to a favourable redox potential within an aerobic environment [88]. As an illustrative instance, it is worth noting that the redox potential of iron (III)-EDTA is measured to be -110 mV, a value that is notably greater in

magnitude when compared to the redox potentials exhibited by ferri-siderophore complexes. It is of considerable importance to acknowledge that EDTA has garnered recognition for its remarkable capacity to facilitate the production of hydroxyl radicals [102].

Moreover, it hinders iron liberation via redox-facilitated mechanisms at sites apart from the designated cellular receptor or intracellular milieu. Iron is released from the combination, according to the present idea, since iron (III) is reduced to iron (II). The siderophores have a reduced affinity for ferrous iron than ferric iron and thus promote the release of iron II from this compound. Nonetheless, these powerful ferri-siderophore complexes also bring a negative redox potential, requiring particular historicity (such as lowering the pH) to become reduced, thus explaining this reduction. It is observed that when the redox potential comes into contact with an acidic environment, it has a considerable tendency to shift toward higher and more positive values. The iron is released by a control reduction of carboxylates and hydroxamate with redox potentials near -350 mV. The former have higher negative redox potentials and so are more easily reduced without first being degraded by an enzyme or chemically altered. This is possible for catecholates up to iron numbers [99].

The number of gene clusters present in cyanobacterial genomes has been observed, and their specific functions have not yet been fully explained. The topic of discussion in the above-mentioned article was the phenomenon of silent gene clusters being activated as a result of iron deprivation. The biosynthesis of cyanochelins, a type of significant lipopeptide distinguished by the presence of -hydroxy aspartate, was triggered by the previous activation event. The lipopeptides in question are extremely important because

they are essential in facilitating iron absorption. In science, it is commonly recognized that a single strain is capable of producing different molecules under different environmental conditions. As the notable reference explains, the OSMAC strategy is based on the essential need to modify parameters for cultivation, such as metal ions, nutrient composition, temperature and aeration level. This method is intended to induce the synthesis of biomedically relevant compounds and start the activation of quiescent biosynthetic gene clusters.

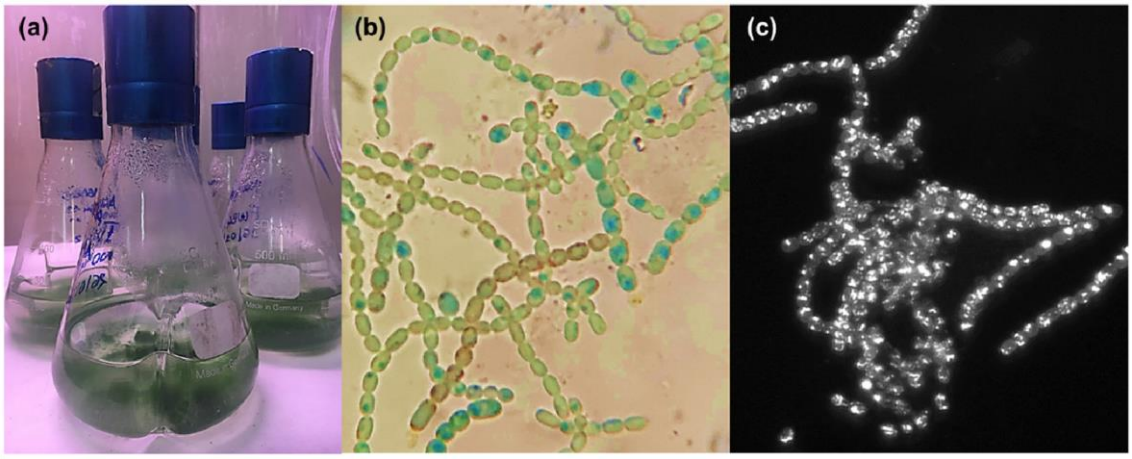
For this chapter, we thoroughly examined the metabolic profile of the *Anabaena flos-aquae* strain UTEX 1444 during for the potential use of cyanobacteria as a source of bioactive compounds. This was done under conditions that have both a deficit and an excess of iron. According to our in-depth study, we found that synechobactin exist. There are a total of seven variables in this collection that have not been recognized so far. These variables were exclusively found in extracts obtained from cultures that had undergone the "zero-Iron (III) treatment" process.

5. Result and Discussion

5.1. Cultivation and Extraction of *Anabaena flos-aquae* UTEX 1444

In this study, the *Anabaena flos-aquae UTEX* 1444 strain, obtained from Prof. A. Pollio's collection (Università degli Studi di Napoli Federico II), was cultivated using a BG11 culture medium. The media was supplemented with different concentrations of Fe^{3+}, derived from ferric ammonium citrate (Figure 2.10).

Figure 2.10 Cultures of *Anabaena flos-aquae* UTEX 1444. Microscopic observation of 0 µM Fe^{3+} (#1) culture with (b) optical microscope and (c) fluorescence microscope (RFP filter).



Here, the aim was to investigate how various cultures (cultures number one to six) related to their relative Fe^{3+} concentrations at values of 0 µM Fe^{3+} (#1), 5 µM Fe^{3+} (#2), 10 µM Fe^{3+} (#3), 20 µM Fe^{3+} (#4), 60 µM Fe^{3+} (#5), and 100 µM Fe^{3+} (#6). In our laboratory, cultures #1-6 were extracted using a standard procedure, which included sonication, centrifugation, and vortexing to separate pellets from the liquid supernatant. Pellets were extracted with mixtures of MeOH and CHCl3, then the supernatants were extracted with BuOH. Subsequently, all extracts were subjected to liquid chromatography coupled with

high-resolution mass spectrometry (LC-HRMS). In this study, molecular networking analyses, as previously described, were used to perform the process of dereplication of high-resolution mass spectrometry (HRMS) data.

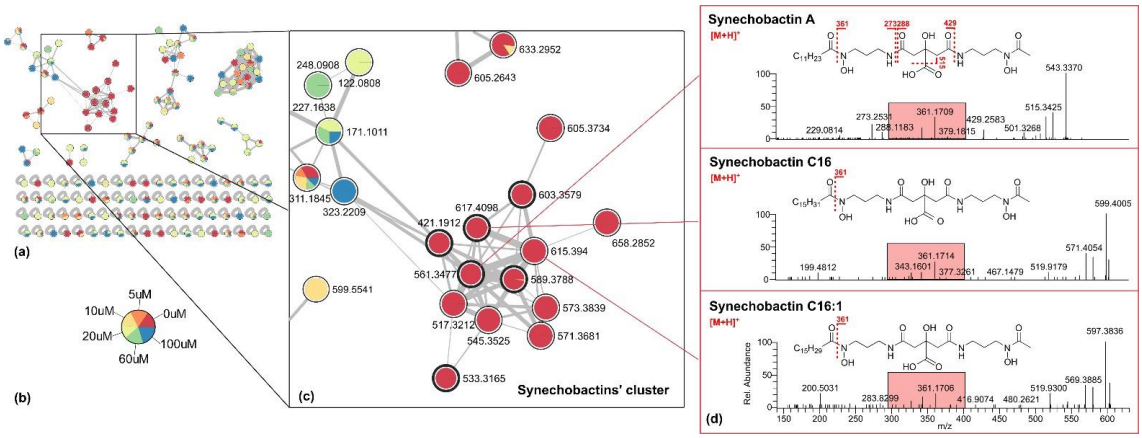
5.2.Molecular Networking and Synechobactins' Identification

The LC-HRMS analysis using the LTQ Orbitrap instrument to examine extracts obtained from cultures #1-6. In data-driven purchases, MS2 scans are initiated for the 10 most potent ions found in the full MS scan. The MZmine 2.53 was used for pre-processing LC-MS data to remove isotopic peaks, find adducts and quantify. For each culture, butanol extract, representing their respective metabolomes, appears to be the most important extract, according to the preliminary analysis of MZmine data. Consequently, the butanol extracts from cultures #1-6 were the focus of data processing and molecular networking. The GNPS, known as the Global Natural Products Social Molecular Networking website, was reached by sending the global.mgf file contained MS2 data from the butanol extracts number 1–6 and the quantitation table obtained through mzMine.

A Feature-Based Molecular Network (FBMN) was created (https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=664d019a6b52486d880014bb1e16bd69 (accessed on 12 November 2021)). This study used the Cytoscape version 3.9.0 program to visualize the molecular networks. This thesis presents a qualitative-quantitative network analysis of butanol extracts from #1-6 (Figure 2.11 (A)), which includes 165 characteristics organized into 10 clusters. The data can be viewed visually by representing each node in the network like a pie chart displaying the amount of compound present in all cultures with various levels of iron (Figure 2.11 (B)). In a group

of 10 nodes, the composition is examined. It turns out there are 23 nodes, with only 13 coming from the no iron (III) culture (Figure 2.11 (C)).

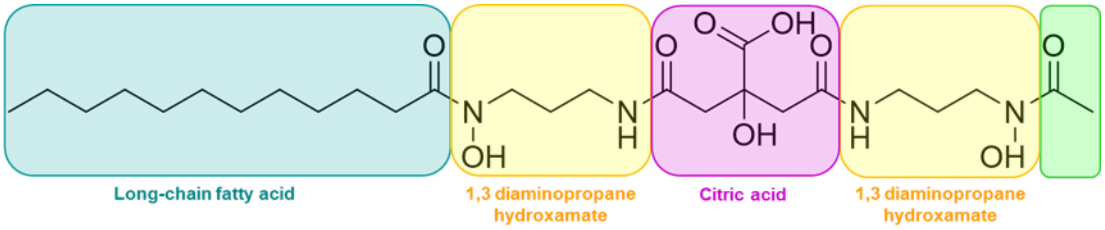
Figure 2.11. Molecular networking and HRMS/MS analysis of extracts of #1–6 cultures of *Anabaena flos-aquae* UTEX 1444. (a) The molecular network was obtained by combining the LC-HRMS/MS analyses of all the #1–6 culture extracts. Nodes are labelled with parent mass. (b) Nodes are represented as a pie chart showing the source culture of the compound ([Fe^{3+}] = 0 µM (red), 5 µM (orange), 10 µM (dark yellow), 20 µM (yellow), 60 µM (green), 100 µM (blue)). (c) A cluster of synechobactin is present almost exclusively in zero iron (III) culture (0 µM, red). (d) HRMS/MS analysis and fragmentation pattern of synechobactin A, C_{16} and the new synechobactin $C_{16:1}$.



A distinctive feature of these nodes is the absence of matches with compounds known in the GNPS library. The ion at 52.9117 amu difference $[M + 56Fe - 3H]^+$, which indicates the presence of iron-adducts in the ESI-HRMS spectra of these substances, suggests that the $[M + H]^+$ ion that was observed is in its apo form and is not dependent on iron. This observation indicates the presence of compounds that chelate iron. Among the options presented, 6 nodes were classified as synechobactin (Figure 2.11 (D)) and (Table-6), particularly hydroxamate-type siderophores. The two 1,3-diaminopropane and one citric acid units form the basis of synechobactin (Figure 2.12). First, diaminopropane is subjected to N-acetylation and N-hydroxylation processes, which lead to the creation of the hydroxamate group followed by the variations in the degree and length of unsaturation

of the fatty acid tail, focused on the N-hydroxylation and acylation of the second diaminopropane unit. The 1st node of the sequence is schizokinen (apo $[M + H]^+$, m/z 421.1934, $C_{16}H_{29}O_9N_4^+$), a symmetric siderophore. In this compound, the two amino terminals of the two diaminopropane units are acetylated.

Figure 2.12- Structure of Siderophore.



In contrast, the siderophore synechobactin A (apo $[M + H]^+$, m/z 561.3477, $C_{26}H_{49}O_9N_4^+$) has a dodecanoyl chain. In the ESI-HRMS2 spectrum of synechobactin A, there is a particular fragment that is distinguished by the simultaneous absence of the acyl residue ($C_{12}H_{22}O$, 182.1661 amu) and a water molecule, probably coming from citrate, at m/z 361.1709 ($[M - RCOH - H_2O + H]^+$, $C_{14}H_{25}O_7N_4^+$). Within each synechobactin, the presence of this fragmentation serves as a diagnostic element that provides valuable clues to the molecular characteristics of the compound. Two additional fragments were found as a result of the slow removal of acetyl and water from the acetamide side. The first fragment had a mass of 60.0206 amu and an m/z value of $[M + H]^+$ 501.3267. It has the chemical formula $C_{24}H_{45}O_7N_4^+$. The second fragment had a mass of 132.0894 amu and an m/z value of $[M + H]^+$ 429.2582. It has the chemical formula $C_{21}H_{37}O_7N_2^+$. These fragments showed that there was an acetyl group on the second hydroxamate unit. Analysing the characteristic fragmentation patterns of the synechobactin within the cluster makes it possible to identify all of them. A common fragment ion at m/z 361.17 is

observed in the ESI-HRMS2 spectra of synechobactin B (3), synechobactin C_{14} (4), and synechobactin C_{16} (5). This is due to the removal of particular fatty acid units. The amu 172.1465 (H_2O and $C_{10}H_{18}O$), 228.2078 (H_2O and $C_{14}H_{26}O$), and 256.2404 (H_2O and $C_{16}H_{30}O$) are lost with these units.

Table 2.6- Synechobactins in *Anabaena flos-aquae* UTEX 1444. The new variants are highlighted in Purple.

No.	**Name**	***apo* [M + H]$^+$**	**Molecular Formula**
1.	Skizokinen	421.1934	$C_{16}H_{29}O_9N_4^+$
2.	Synechobactin A	561.3477	$C_{26}H_{49}O_9N_4^+$
3.	Synechobactin B	533.3165	$C_{24}H_{45}O_9N_4^+$
4.	Synechobactin C_{14}	589.3788	$C_{28}H_{53}O_9N_4^+$
5.	Synechobactin C_{16}	617.4098	$C_{30}H_{57}O_9N_4^+$
6.	**Synechobactin $C_{16:1}$**	**615.3940**	**$C_{30}H_{55}O_9N_4^+$**
7.	**Synechobactin oxyC_{14}**	**605.3734**	**$C_{28}H_{53}O_{10}N_4^+$**
8.	**Synechobactin oxy$C_{14:1}$**	**603.3579**	**$C_{28}H_{51}O_{10}N_4^+$**
9.	**Desacetyl-synechobactin A**	**517.3212**	**$C_{24}H_{45}O_8N_4^+$**
10.	**Desacetyl-synechobactin C_{14}**	**545.3525**	**$C_{26}H_{49}O_8N_4^+$**
11.	**Deoxysynechobactin C_{14}**	**573.3839**	**$C_{28}H_{53}O_8N_4^+$**
12.	**Deoxysynechobactin $C_{14:1}$**	**571.3681**	**$C_{28}H_{51}O_8N_4^+$**

Compound 6, which has the molecular formula $C_{30}H_{55}O_9N_4^+$, had a molecular weight of 2 atomic mass units lower than synechobactin C_{16} (5). This discrepancy indicates the existence of a double bond in compound 6. The peak found at m/z 361.1706 in the MS2 spectrum 6 supports the association of additional unsaturation with the fatty acid tail. The loss of the unsaturated fatty acid $C_{16}H_{30}O_2$ is equivalent to this peak. What still needs to

double bond was arranged has yet to be clarified. It is important to note that only rhizobactin (C_{10}) has the structure of synechobactin with an unsaturated acyl chain. This indicates that synechobactin $C_{16:1}$ (6) is a novel and distinct compound. Compound 7 was recognized as a novel synechobactin oxyC_{14}. Removing 244.2045 amu (H_2O and $C_{14}H_{26}O_2$) from the pseudo molecular ion revealed a hydroxy acyl (tetradecanoyl) chain. Compound 8 is a derivative of synechobactin C_{14} and has a molecular ion peak of 603.3579 at m/z. A fragment ion at m/z 361.1702 ($C_{14}H_{25}O_7N_4^+$) is formed due to the loss of 242.1879 amu, corresponding to removing H_2O and $C_{14}H_{24}O_2$. The ion fragment in question corresponds to unsaturated hydroxytetradecanoic acid. Despite this, the extra hydroxyl groups' and double bonds' precise arrangements and locations have yet to be determined.

Dihydroxamate-citrate moiety is common in compounds 1–8, with the only difference being the number of fatty acid residues. In contrast, the polar part is modified in compounds 9–12. The pseudomolecular ions of dodecanoic acid and tetradecanoic acid are cleavaged by synechobactin A at m/z 517.3212 (9) and 545.3525 (10). The fragment ion $C_{12}H_{21}O_6N_4^+$ (m/z 317.1459) is formed due to the mentioned cleavages and is in accordance with the non-acetylated hydroxamate and citrate backbone. The presence of the peak at m/z 429.2601 in the MS2 spectrum of compound 9 confirms the structure. This peak is related to the loss of the 3-(hydroxyamino) propan-1-amine unit (88.0634 amu, $C_3H_8ON_2$), which is also seen in synechobactin A's MS2 spectrum. The gap of 15.9948 amu detected between synechobactin C_{14} (4) and the ion at m/z 573.3839 (compound 11) can be attributed to the presence of an oxygen atom. Removal of the tetradecanoic acid from pseudomolecular ion number 11 leads to the appearance of a peak at m/z 345.1772 ($C_{14}H_{25}O_6N_4$), indicating that the dihydroxamate-citrate segment of the

compound does not contain an oxygen atom. The m/z 328.1508 peak ($C_{14}H_{22}O_6N_3$) that is seen in both the MS2 spectra of synechobactin C_{14} (4) and deoxyC_{14} (11) is associated with the fragment (citryl) N-(3-aminopropyl)-N-hydroxyacetamide. This indicates that the nitrogen of the other hydroxamate does not have a hydroxyl group. Furthermore, compound 12's second hydroxamate unit is dehydrated, indicating the presence of deoxy synechobactin C_{14} in the dehydrated form. In the synechobactin cluster, the thirteenth node is the iron bounded ion of synechobactin oxyC_{14}, which has a mass-to-charge ratio of 658.2852 ($C_{28}H_{50}O_{10}N_4Fe^+$).

6. Experimental set-up

6.1. *Anabaena flos-aquae* UTEX 1444: Strain, Cultivation and Extraction

The genus Anabaena BORY ex BORN. et FLAH. 1888 encompasses filamentous, nonbranched species, which differ in the form of cells, coiling of trichomes, and shape and size of akinetes. Anabaena species exhibit a wide range of strain-specific variability, and environmental conditions mainly affect cell dimensions and the size of heterocysts [103]. The species *A. flos-aquae* Brébisson ex Bornet et Flahault 1888 is a free-floating organism whose coiled filaments can be solitary or irregularly assembled, with narrow mucilaginous sheath. Cells are usually spherical to barrel-shaped, whereas akinetes can be cylindrical or elliptical and heterocysts are usually spherical [104]. *Anabaena flos-aquae* is diffused in freshwater environments worldwide and is responsible for blooms that can have noxious effects on other organisms due to the excretion of toxic metabolites, as anatoxins [105]. From a taxonomical point of view, *A. flos-aquae* is a combined morphological group, in that an alternation of regularly and irregularly coiled filaments is observed for the same strain in laboratory cultures; moreover, vegetative cell shape and size, along with heterocysts and akinetes dimensions, were largely variable among different populations. Considering that the type species of the genus Anabaena, *A. oscillarioides* Bory ex Bornet et Flahault 1888 is a benthic type, a study proposed to transfer the planktic Anabaena types under the genus *Dolichospermum*, designating *A. flos-aquae* as the type species of the genus.

The strain *Anabaena flos-aquae* UTEX 1444 was grown in BG-11 medium containing (per liter): 2.21 mg of Na_2EDTA, 12 mg of Citric Acid, 1 g of $NaNO_3$, 80 mg $K_2HPO_4 \cdot 3H_2O$, 150 mg of $MgSO_4 \cdot 7H_2O$, 52.8 mg of $CaCl_2$, 34.2 mg of $NaCO_3$, 2 mL of

Nitsch solution (H_2SO_4, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, H_3BO_3, $Na_2MoO_4 \cdot 2H_2O$, $CoCl_3 \cdot 6H_2O$), 500 µL of $NiSO_4$ $(NH_4)_2$ $SO_4 \cdot 6H_2O$ (0.1 mM stock), 200 µL of Na_2SeO_4 (0.1 mM stock). Cultures were grown in six different concentrations of iron supplemented as ferric ammonium citrate in BG11 medium; serial dilutions from a mother ferric ammonium citrate solution (100 µM) were prepared in order to get the following final concentrations: 0 µM Fe^{3+} (#1), 5 µM Fe^{3+} (#2), 10 µM Fe^{3+} (#3), 20 µM Fe^{3+} (#4), 60 µM Fe^{3+} (#5), and 100 µM Fe^{3+} (#6). All cultures were made in triplicates and were incubated at room temperature (18–25 ◦C) for 30 days. Cultures were observed under an optical microscope (OPTECH, Biostar B3) and a fluorescence microscope (iRiS Digital Cell Imaging System–Logos Biosystems, Boston, MA, USA); then, they were vortexed, sonicated (ARGO Lab Digital Ultrasonic Cleaner, Model-DU 32, Carpi, Italy) at 20 KHz for 10 min at 23 ◦C, and centrifuged (HERMLE Labortechnik, Model-Z36HK, Wehingen, Germany) at 10,000 rpm for 5 min at 25 ◦C to separate the solid pellets from the liquid supernatant. Pellets were extracted with organic solvents as follows: MeOH (100%, 0.3 L), MeOH/$CHCl_3$ (1:1, 0.3 L), and $CHCl_3$ (100%, 0.3 L). All the extracts were paper filtered and concentrated under vacuum, yielding (average weights): 9.6 mg (#1), 6.6 mg (#2), 6.3 mg (#3), 9.0 mg (#4), 7.2 mg (#5) and 9.3 mg (#6) of MeOH extract; 2.3 mg (#1), 3.5 mg (#2), 4.3 mg (#3), 3.4 mg (#4), 1.2 mg (#5) and 2.5 mg (#6) of MeOH/$CHCl_3$ extract; 7.6 mg (#1), 0.5 mg (#2), 1.7 mg (#3), 0.6 mg (#4), 1.3 mg (#5) and 0.3 mg (#6) of the $CHCl_3$ extracts. Supernatants were extracted with BuOH. The BuOH phases were concentrated under vacuum affording to 3.5 mg (#1), 13.3 mg (#2), 30.3 mg (#3), 45.6 mg (#4), 40.3 mg (#5) and 40.6 mg (#6) of extract. Each extract was resuspended in MeOH (100%, 5 mg/mL) for the subsequent LC-HRMS analyses.

6.2. LC-HRMS Analyses and Molecular Networking

LC-HRMS experiments were performed using a Thermo LTQ Orbitrap XL highresolution ESI mass spectrometer (Thermo Fisher Scientific Spa, Rodano, Italy) coupled to an Agilent model 1100 LC system (Agilent Technologies, Cernusco sul Naviglio, Italy), which included a solvent reservoir, in-line degasser, binary pump, and refrigerated autosampler. A 5-µm Kinetex C18 column (50 × 2.10 mm), maintained at room temperature, was eluted at 200 mL min−1 with H_2O (supplemented with 0.1% HCOOH) and MeOH, using gradient elution. The gradient program was as follows: 10% MeOH for 3 min, 10%→100% MeOH for 30 min, 100% MeOH for 7 min. Mass spectra were acquired in positive ion detection mode. MS parameters were a spray voltage of 4.8 kV, a capillary temperature of 285 ◦C, a sheath gas rate of 32 units N2 (ca. 150 mL/min), and an auxiliary gas rate of 15 units N2 (ca. 50 mL/min). Data were collected in the data-dependent acquisition (DDA) mode, in which the first, the second, up until the tenth most intense ions of a full-scan mass spectrum were subjected to high-resolution tandem mass spectrometry (HRMS/MS) analysis. The m/z range for data-dependent acquisition was set between 100 and 2000 amu. HRMS/MS scans were obtained for selected ions with CID fragmentation, isolation width of 2.0, normalized collision energy of 35, Activation Q of 0.250, and activation time of 30 ms. Data were analyzed using Thermo Xcalibur software.

Raw files were imported into MZmine 2.53 open-source software [106]. The mass detection was performed on raw data and exact masses with mass level 1 and centroided masses with mass level 2, by keeping the noise level at 1000. Chromatograms were built using an ADAP module with a minimum height of 1000, and m/z tolerance of 0.001 (or 5 ppm). Peak alignment was performed using the Join aligner algorithm (m/z tolerance at

0.005 (or 5 ppm), absolute RT tolerance at 0.2 min). [M + Na – H], [M + K – H], [M + Mg − 2H], [M + NH_3], [M − Na + NH_4], [M + 1, ^{13}C], [M − ^{35}Cl + ^{37}Cl]+, [M + ^{56}Fe − 3H]+ adducts were filtered out by setting the maximum relative height at 100%. Peaks without associated MS/MS spectra were finally filtered out from the peak list. Clustered data were then exported to a .mgf file for GNPS, while chromatographic data, including retention times, peak areas, and peak heights, were exported to a .csv file. A Feature-Based Molecular Network was generated on GNPS' online platform [107], with the following parameters: the parent mass tolerance and MS/MS fragment ion tolerance were set both at 0.05 Da, the cosine score at above 0.5, and matched peaks above 5. Spectra were retained only if the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries using a cosine score above 0.7 and at least 6 matched peaks. The molecular network was visualized using Cytoscape software.

7. Conclusion

Cyanobacteria are known for their particular adaptations that help them handle extreme conditions. In particular, this group represents a significant source of bioresources that still needs exploring. These bioresources have the potential to naturally produce valuable products that are of great importance for both biotechnology and the environment. This study was focused on its bioactive metabolite siderophores, and whether the *Anabaena flos- aquae strain* UTEX 144 can be a renewable resource to produce these compounds. The metabolome of UTEX 1444, when cultivated under conditions measuring both iron deficiency and an excess, will be examined in this study.

The growth of the strain under an iron-depleted environment demonstrated its ability to produce siderophores. In our study, organic extracts (#1-6) were quickly replicated using a molecular networking analysis. A distinct group of metabolites that were unique to zero iron (III) culture was successfully demonstrated in this study. This technique made the rapid identification of the siderophore cluster possible. It made the rapid identification of synechobactin and its new variations, which are based on their distinct fragmentation patterns. Presenting the fragmentation patterns of synechobactin A, C16, and the recently discovered synechobactin C16:1, a high-resolution MS/MS analysis, demonstrated a practical method for diagnosing any extract. Structural and typological characteristics of cyanobacterial siderophores are poorly documented in previous literature.

This study discovered six different varieties of synechobactin that had not previously been identified. These variants present particular modification in the acylated chain composition (hydroxylated, unsaturated) and in the hydroxamate-citrate backbone. This

report provides the first evidence of the ability of UTEX 1444 to synthesize a variety of synechobactin. Anabaena UTEX 1444 has demonstrated its ability to produce eleven varieties of saturated and unsaturated fatty acids, which could be the ultimate step in biosynthesis. Furthermore, modifications were made to the hydroxamate-citrate backbone, broadening the range of chemical variables observed in synechobactin discovered so far.

Our forthcoming research will focus on establishing UTEX 1444 as a prototypical organism for increased siderophore expression/production, considering the wide range of applications, from ecological investigation to drug discovery, that can benefit from the ability of siderophore production to scavenge ferric ions and chelate toxic metals. This study contributes to the exploration of natural molecules that have the potential to improve both environmental and human health sustainably. This is achieved by introducing new techniques for producing, describing and researching siderophores using a reliable bioinformatic approach. This study adheres to the principle of "Do no significant harm". It concentrates on the critical issue of addressing the "supply" challenge in investigating new bioactive compounds derived from marine organisms, per EU directives.

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Chapter 3

Development of 4-Stage Pipeline for Siderophores' Production

1. Introduction

One Strain Many Compound (OSMAC) approach was first developed by Professor Zeeck [1]. According to Zeeck, OSMAC has revolutionized our understanding of how microorganisms regulate their secondary metabolic pathways and has more useful information as such. This approach ensures that by studying strains, students can understand how cells provide a lot of options for particular genes. The particular strain is beneficial for researchers to study how different environmental changes lead to the production of secondary metabolites, which in turn impact community behaviour. This will improve how we understand ecosystems and life itself by a long shot.

The role of secondary microbial metabolites has been crucial, as they are the major source for new drugs to be discovered. Typically, for the traditional investigation of microorganisms, valuable compounds are extracted and distinguished. These methods are not giving fruitful results because many of the already discovered microbial secondary metabolites are being found again. During the fermentation process, it is known that a big part of these gene clusters is deactivated by some means [2]. Each strain, even though it produced multiple compounds under certain circumstances, there is only a limited number of them displayed behind the growth conditions. Over the past 20 years numerous studies have exposed that when there are changes ensured in cultivation-oriented parameters,

mainly physical and chemical factors related to habitat, it leads to altering gene regulation mechanisms, which collectively influence how microorganisms produce a determined set of secondary metabolites [3]. There have to be several alternative strategies through which the cellular processes of the cultivating agent can adapt and then modify themselves according to a given stimuli. These approaches have the added advantage that the gene clusters and regulatory pathways do not need to be known beforehand [4].

2. Approachable Techniques

To fully utilize microbial populations and target their biosynthetic gene clusters, researchers can apply different techniques, such as gene regulation and inducing mutations through the recombinant DNA technique. Added steps could involve different nutrient substances, tiny elements, and things like heat and cold levels (for example, the pH balance or how hot it is). there might be a need for more growth-stimulating chemicals, such as low amounts of medicine that kills harmful bacteria, but not all of them at once mixed with other small molecules used to help cells talk between themselves. Also, working with multiple types of small living things and the use of factors that change how genes are controlled can fit into the OSMAC rule as well. [5]–[7]. The OSMAC framework is established on the idea of "one strain many compounds." it emphasises that using various cultivation techniques will produce the best results.

2.1. Metagenomics

Enhancing the production of a secondary metabolic pathway using metagenomics can increase or decrease certain compounds' quantity. In order to minimize the synthesis of certain components, some different approaches could be implemented [8]. The pathway can reduce the expression of specific mRNA levels by a gene silencing technology or by

increasing enzyme inhibitory proteins [9]. There are still other methods, which include increasing the target compound's breakdown or redirecting the path to a competing way. The target is to up-regulate a few compounds in the usual producing species or transfer some portion of the pathway to other plants or microorganisms. There's also a keen interest in developing new compounds that are not naturally produced by plants. In order to increase the production of compounds, two common approaches have been adopted. The genes' expression level is increased and decreased by some predefined methods to avoid any competitive pathway and to lower the breakdown of the products. Another function of regulatory genes is to change the control of bi-product synthesis.

2.2.Variations in Nutrient Composition

2.2.1. Role of Carbon

Having to examine the potential effects and impacts that different nutrients might put on an organism, it is also vital to first assess the different carbon options available for uptake, and how they affect secondary growth [10]. Regulation of genes through carbon catabolite has been a widely accepted and known method in the microbial world. Another important paper [11] talked about how gene transcription in *S. coelicolor* is regulated on a global scale. The impact of nutrients coming from different sources has been studies in this research. It is particularly targeted at appraising the amounts of carbon and phosphorous, or nitrogen in addition to discerning their consequences. Many researchers and studies aim to improve marine microbes to produce important metabolites in raised quantities. These same ways are also useful for creating unique and different metabolites. Research [12] has been done to check how the OSMAC strategy can improve the production of compounds from a fungus called *Spicaria elegans*. The strain that was previously acquired from marine waste in China has the ability to produce a specific types of toxins,

as studied during previous research. The experiment was carried out to ferment starch as the major substrate involved some specific strains. Different studies have shown that there is a significant change in the production of secondary metabolites. This contained 5 newly recognized aspochalasins, named spicochalasin. In the meantime, they have discovered two known aspochalasins. When provided with spicochalasin, HL-60 leukemic cells showed an average cytotoxic effect. Scientists found that this compound is produced from the same gene responsible for producing cytochalasin when grown using glucose. An example shows the involvement of primary and secondary metabolism that leads towards change in metabolite profiles.

2.2.2. Role of Nitrogen

The entrance of nitrogen (which is needed for cell development) into the body needs to be studied in detail, like that of other important substances that enter the cells. This fact becomes more significant if the amino acids are the source of nitrogen. Antibiotic (PKS-NRPS hybrids) and nonribosomal (NRPS) peptide systems incorporating amino acid substrates have now been recognized as important players in the microbial world. Also, we need to be mindful of the fact that during the stationary phase, there is a delicate balance between cell growth and metabolites used. It has been proven through studies that how excess metabolites are being produced and grown by using marine-derived fungi [13]. During the fermentation process, fungal cells that are bio-mass ones produce a positive change in the way microorganisms fight to eliminate other microorganisms. This is seen only when yeast extract or peptone is added as an additional nitrogen source. The high concentration levels of nitrogen sources can reverse the scenario and enhance the proliferation of carbon during limited source levels, leading to cell sustainability. This clearly had a bad impact on the production of antibiotics. Conversely, when there is a

limited amount of nitrogen within the source, this will eventually result in greater growth of secondary metabolites and lower cell development within the tissue [13].

Through previous studies, it has been identified that, for diatom *Skeletonema marinoi,* anti-cancer and antibacterial products against nitrogen are sensitive to concentration rather than the specific source. Growth of human skin cancer cells was unable to take place, and certain germs couldn't be produced because there wasn't enough nitrogen. The actions were not found present as the diatoms were grown in media rich in high-nutrient [14]. Another study [15] concluded that nitrogen availability significantly affects secondary metabolite production. They gained insights from the research that low levels of nitrogen did not do any good to the microalga *Eustigmatos sp*. but instead hindered its growth capabilities in comparison with adequate nitrogen levels to high concentrations of it globally.

2.2.3. Role of Phosphorus and sulphur

The impact of phosphorus concentrations in microbial secretion has been studied broadly; however, less emphasis has been given to sulphur. The link between sulphur sources and sulphur metabolites is exemplified by the antibiotics containing sulphur. The research on β-lactam producing fungi depicts that specific kind of amino acids is essentially involved in the induction and regulation of final antibiotic production [16]. The sulphur-rich compounds, which include dithiolopyrrolones, are produced more in the presence of cysteine and cystine with *Saccharothrix algeriensis* as chosen [17]. The final synthesis of antibiotics involves sulphur-containing compounds such as cysteine and methionine, which are provided directly from the amino acids. Bacteria of the marine environment that generate an amazingly potent antibiotic (tropodiethetic acid) also show parallel

outcomes. Studies have shown that the *Roseobacter clade* can make sulphur compounds and tropodiethetic acid. Marine algae are compatible with this solute named dimethylsulfoniopropionate (DMSP), which mainly contains sulphur, and it increases the production of secondary metabolites and exopolymeric material [18].

Marine microbiology studies have been rare that evaluate how secondary metabolite production is affected by phosphorous concentration. Studies conducted earlier showed that phosphates can affect the synthesis of secondary metabolites in marine algae [19]. Certain antibiotically active constituents are manufactured by some types of microalgae if there is a rise in the phosphate levels. Similarly, phosphorous limitations increase the production of saxitoxins that paralyze shellfish in the *Alexandrium minutum* [20]. The degree to which antibiotics are produced is determined by the level of phosphorous within marine bacteria. Researchers have discovered that the shipworm bacterium produces antibiotics when it attains phosphorous from phosphate in little amount. The level of Phosphorous in marine generators, and the quality of media has not been checked or processed systematically. Even though these situations highly impact how secondary metabolites are produced in the *Streptomyces* and affect antibiotic production for marine microbes [21].

2.2.4. Role of Trace Elements

The biochemical compositions are very different in both marine and terrestrial ecosystems. A large number of halogens and various others are found in the ocean; this is a clear difference between oceans and freshwaters. Species within the marine system exhibit a large, varied diversity among each other, indicating multiple differences towards several morphological, physiological, and reproductive properties within itself. Organic

halogenated compounds are rare in land-based systems, except for marine environments, where they make up the bulk of an estimated 5000 organohalogen compounds [22]. With time, the levels of trace elements in culture media are changed but these alternations are consistent. Especially in halogen concentration. Through this efficient practice, unique social methodologies were recognized. The use of 1mM NaBr solution to grow *Aspergillus flavipes* ended with exciting results. The discovery identified six cytochalasans, while the remaining two are still unnamed (Flavichalasine N and O) [23]. This is the first documentation of nitrogen-oxygen heterocycle cytochalasan. In the lab tests, all of these elements have shown strong anti-cancer abilities. Marine fungus *Isaria felina* were studies past by researchers in which they found that have powerful molecules that can help to combat against cancer. Molecules were formed when KBr was brought into the cell [24]. The extraction of novel brominated anthraquinones and some non-halogenated ones was obtained due to adding NaBr into the solution. It is possible to develop different chemicals by changing the origin of halides. Thus, these novel compounds belong to variations of these molecules. An example is a water-related fungus known as *Trichoderma sp.* TPU199 is a pigment that was extracted from the red alga. In the medium of growth, it took halide ions from sodium compounds and made a specific synthesized chemical [25].

2.2.5. Role of Uniformity of the Media

There are differences seen in the amount of secondary metabolites produced not only on different like nutrient media and solidified media but also when variations occur between solid and liquid media. In *Streptomyces*, interference in the production of antibiotics was studied in *Streptomyces sp.* USC-633, by making use of OSMAC strategy. It was observed from a particular study that there are 11 different carbon sources producing

antibiotics in the presence and absence of seawater. They observed the effects of trace elements in every formulation and how effective they are, including single amino acids. To prepare the media, agar was included in two forms of media; solid and liquid [26]. Agar plugs were used from solid media samples, and liquid culture broths spots in the well were evaluated. In order to evaluate antimicrobial potential, different bacterial strains were chosen that are gram positive and gram negative. The activity of *E. coli* ATCC 13706 and *E. faecalis* was observed when the agar gels were used despite the expected outcome. In both paper-disc diffusion and the well-in-the-spot technique, *E. coli* ATCC 13706 was the most effective among all species used. Later on, this study obtained certain organic extracts after detecting the activities from agar samples.

The NMR was used to analyze the chemical fractionation of the extracts. The difference was observed between the spectra of nuclear magnetic resonating spectrums amongst both the liquid broth and solid extracts. There were some components in the liquid medium with no antibiotic activity, which were not found in the agar. Elsewhere, the researchers identified different secondary metabolite profiles in *Penicillin adametsiodes* AS-53, when they examined potato-dextrose-broth (PDB) liquid and rice solid culture media [27]. From the organic extracts of specific source material, a new spiroquinazoline compound called N-formyllaptin A was obtained. Further new bisthiodiketopiperazines (adamatizines A and B) were also discovered from the same source material alongside some previously recognized meroterpenes A few specific chemical compounds were isolated from the solid-state fermentation, which are not found in the liquid state fermentation. While being tested, the Adametizine A showed both general and specific cytotoxic effects in a brine shrimp lethality assay. There is a wide range of antimicrobial actions that are being exhibited by adametizine A and B.

Focused on the similar pathway, scientists experimented using OSMAC by making use of a certain type of fungi *Penicillium sp.* F23-2. It was noticed that there were significant changes in some metabolites when the strain moved from liquid media to solid rice-based made media. Five new penycicclone compounds A, B, C, D and E with similar characterstics are successfully extracted by using solid medium. There is a notable antibacterial effect found out in newly discovered compounds against *S. aureus* [28]. It is obvious that the application of intricate media could greatly impact the production of microbial metabolites. It is important to study the strain *Streptomyces sp*. While the experiment [29] of *Streptomyces* sp. YB104 has been exposed to different media; its outcomes will never be the same because this yeast comes from the deep waters and is considered to have unique outputs.

2.3. Variation in Physical Parameters

2.3.1. Temperature

The heat at which things grow affects how fast cells multiply and also secondary metabolism. Research showed that *A. saccharicola* grew quicker at 30 °C. However, they saw that this fungus made the most secondary metabolite at 25 °C [13]. Another similar research looked into how temperature affects the making of an antibiotic that can fight MRSA. The antibiotic medicine was made by an organism known as *Pseudoalteromonas piscicide* PG-02. The scientists did tests at different heat levels and discovered that the best temperature for getting the top antibiotic amounts was 28 °C [30]. A study [31] done using diatoms confirmed that different molecules with altering bioactivities were made at certain temperature levels. This research grew five kinds of diatom species in two different light and heat situations. The aim was to watch the making of molecules. These molecules have many good features, like being anti-oxidant, fighting diabetes and cancer,

boosting immunity, and stopping infections. The test experiments done on a small scale found out the temperature range for each kind. The low temperatures went from 3.3 °C to 5.6 °C, and the high ones varied between 6.6 °C and 9.0 °C.

The extracts from *Attheya longicornis*, which were only found to have bioactivities, came from growths done at high temperatures. The only biological activity found in *Chaetoceros furcellatus* was discovered in extracts made at low temperatures. The *Porosira glacialis* showed traits that fight cancer and diabetes in both hot and cold temperatures. However, it only showed traits of an antioxidant when grown in cold temperatures. In the end, *Skeletonema marinoi* showed abilities to fight oxidation in both cool and warm settings. But, the anti-cancer features were only found after the creature was grown in scorching temperatures. A very interesting case is about the fungus type called *Penicillium raistrickii* JH-18. It was found in salty soil near the seacoast. This type showed the ability to make a few new compounds, raistrickiones A–E, when the media's temperature was dropped from 28 °C to 15 °C [32]. Still, different metabolites have shown they can be created at a broad range of temperatures in certain cases. Another study was done that watched the making of a protein antibiotic named andrimid by a cold-resistant type called *Serratia proteamaculans*. This making was seen over a heat level from 4 to 20 °C [33].

2.3.2. Ventilation, Shaking and Aeration, Glassware, etc.

The latest study shows that the shape/size of the glassware used for fermenting, especially when they copy certain conditions that help biofilms to grow, can significantly affect the making of secondary metabolites in tiny sea life forms. Even though we know that many microbes make special chemicals and their exact role in nature is not scientifically proven,

it is accepted that these can produce antibiotics. It has been linked with stopping the growth of other nearby small life forms either by winning over them or maybe even through talking between cells [34]. A group of scientists did well in showing many findings. These show a link between various kinds of ships, ways of living, and the making of secondary metabolites in different tiny organisms. A different study was done where they made a flask shaped like a conical, cylindrical flask, plus an extremely low-speed spinning disc bioreactor [35]. To copy the beach area where a *Shewanella sp*. A method was used where the disc would turn once each day. It was discovered. This disc will be under liquid for 12 hours and above it for another twelve, copying the specific environment.

The kind of container used for growing can also affect the amount of air in biotech machinery. This is a usual method to boost the growth of tiny organisms and then increase the making of extra substances. The method of aeration mostly means shaking containers or putting air in through an underwater tool to boost the amount of oxygen mixed with water and fill up any low levels of it. Still, research [36]. showed that the water bacteria called *Lyngbya majuscula* made more lipopeptide when the air was moved around above the water in the container used for the study. It is important to note they did not use stirring of the container to increase levels of oxygen mixed in water. A new research [37] was done recently on the sea-based *Streptomyces sp.* CNQ-525 clearly showed how the level of oxygen affects making secondary metabolites. In this study, when hypoxia happened, it caused a change from making a pharmacological molecule- napyradiomycin to creating 8-aminoflaviolin. This is a middle compound in the process of building up napyradiomycin. The writers of the research suggested that 8-aminoflaviolin, a substance with redox action, might work as an outside electron transporter. In experiment batches,

they usually control aeration by changing the speed of shaking. It has been seen that this change can affect the making of secondary substances. As said before, producing TDA is an example of what the *Roseobacter* group does [38].

2.3.3. Salt Concentration, pH and Osmotic pressure

Different physical and chemical conditions control the various places in the ocean where tiny sea creatures live. For example, seawater is made up of water upto 96.5% and 3.5% different things like salts, various gases that are mixed in the water, substances from living things, and particles that do not dissolve. You should know that almost 87% of the salts dissolved in seawater are NaCl. The saltiness of the sea is usually about 3 to 3.5 percent. However, it can change in different settings. In very salty small areas like saltwater pools, the saltiness can be more than usual. In places with mangroves, the saltiness might be less. So, we can guess that making tiny life forms' waste in the ocean has grown to properly adjust to many different situations. One of the first recorded cases showed how saltiness affected secondary metabolite production. This case involved four different isolates of *actinomycetes*. Interestingly, only one sample kept the ability to make bioactive secondary metabolite even without seawater [39].

A research [40] looked into how osmolarity and saltiness influence the making of secondary metabolites in a land-loving, salt-tolerant fungus known as *Aspergillus aculeatus*. They uncovered that other important molecules like secalonic acid D, aspergillusol, aculene C were increased in the fungus when it was salty. The making of aspergillusol especially seemed linked with the fungus being able to handle saltiness from sea water or sea salts. They found out that adding seawater or salts to the place where salt-tolerant fungi grow could likely lead to more production of secondary substances.

High salt stress is known to play a key role in the creation of new antimicrobial substances. This comes from *Aspergillus terreus* PT06-2, a fungus found in marine sediment [41]. When this type was raised in different salt amounts, it made twelve familiar elements and three fresh substances named terremides A and B as well as terrelactone A. Growing in very salty conditions led to more output of secondary metabolites overall and especially sparked the making of two new types.

Usually, sea places are a bit more basic, so it is good to watch and change the pH when using sea germs to find NPs. A study looked at how pH affects the making of secondary metabolites. The research discovered that a type of *Streptomyces* in the sea makes antibiotics. Biofilms influenced it, and there was a link between creating biofilms and the pH level of the growth liquid [42]. When testing culture liquids for things that kill germs, they noticed there were no activities seen in a sour environment with a pH of 4.0. Still, we saw the power to kill germs between pH levels of 9.0 and 10.0; with most strength was at a pH level of 9.0. In a similar research, found that making the pH a little bit more basic was listed as the best condition for creating an anti-MRSA antibiotic by the strain *Pseudoalteromonas piscicida* PG-02 [30].

2.4. Other factors such as Co-cultivation

Growing different tiny life forms together is an exciting way used to boost the making of SMs and wake-up sleeping gene groups. Even though the OSMAC method was first thought of without directly thinking about co-culture plans [1]. In nature, tiny organisms meet changes in non-living physical and chemical elements. They also have many relationships with and among different kinds of creatures. Cells can be impacted by molecules that spread quickly or in direct touch with other cells. These meetings can

affect cell activities that boost competition and health, resulting in the making of bioactive secondary metabolites. Over thirty years ago, important discoveries were made about the increased hostile behaviours shown by different types of ground fungi when they were grown together [43]. These impacts relied on the creation of various signal particles (secondary metabolites), which were noticed by the nearby strain. So, the nearby strain saw a significant increase in making anti-fungal substances. A research group [44] dug deeper into these findings by taking out a new antibiotic named pestalone from a sea fungus grown in the presence of bacteria. In the last few years, different plans that depend on chemical signals, like low levels of antibiotics, have been tried out using the same interaction method. These farming methods are now more popular and provide a hopeful way to find secondary metabolites. Usually, the method of mixing different types of bacteria and/or fungi is often used in co-cultivation. However, new research has shown that adding bacteriophages can also affect the creation of secondary metabolites. The event was recorded in *Geobacillus sp.* E263, a type gotten from a deep-water hot spring in the sea. This special type showed it could make a unique cancer-fighting substance, but only when the GVE2 virus was present [45].

3. Anabaena flos aquae

Records from 1965 and after [46] showed the first writing about *Anabaena flos-aquae*. They looked at how this plant could be grown for business purposes and demonstrated a way to get a clean group of *Anabaena flos-aquae* A-37 that is by reducing the level of basic nitrogen. This team deeply looked into how *A. flos-aquae* A-37 loses ions through a full study of the electrolysis method. Another study [47], [48] on how cells make large sugar molecules showed that fructose can take the place of carbon dioxide gas. Moreover, some complex sugars were effectively separated into different parts. Usually, this kind

does well at the start of summer. However, we know from notes that *A. flos-aquae* also exists during winter time too [49]. We used connection and main parts study (PCA) to check the likenesses between two different kinds of blue-green algae (*Anabaena flos-aquae* and *Aphanizomenon flos-aquae*) seen at different deepness levels in a lake. We used Principal Component Analysis (PCA) to study the part of light that goes from 1750 to 900 cm^{-1}. Loading plots were used to find the exact parts that helped tell samples apart. The main differences between types are in their protein, nucleic acid and phosphorus compound builds. Differences in deepness did not cause any major changes to the food conditions for the Anabaena group. Many studies have also been done on the makeup of the green pigment protein mix.

The kinds *of Anabaena* species found in water are diverse, and they include about 80 different types. These types look distinct from each other, but all belong to a group known as cyanobacteria. This specific type includes a bunch of string-like cyanobacteria. They are known for being able to make nitrogen stable and create poisonous substances. There are over 40 kinds of cyanobacteria that come from about 20 different groups. These include *Anabaena, Microcystis, Aphanizomenon, Cylindrospermopsis*, and *Oscillatoria*, among many more. These blue-green bacteria can make different kinds of poisons. These poisons are all called cyanotoxins. These poisons show many different structures and roles. Cyanobacteria cause different problems with water quality. This includes making toxins and having things that taste bad or smell funny [50]. Cyanotoxins are usually sorted by how they affect living things. This can include nerve poisons like anatoxins and toxins that cause muscle paralysis, liver-damaging ones such as microcystins or nodularins, and cell-killing molecules like cylindrospermopsin; there may also be some with irritating features called lipopolysaccharides (LPS).

Some types of harmful algae growth in water can create adverse effects. The worst type of poisoning ever happened in Brazil back in 1996. This was because the water used to make a treatment mix was not cleaned well enough for safe use, harmfully affecting patients who were getting their blood cleaned by a machine called hemodialysis. There was a significant increase in cyanobacteria, like *Anabaena, Microcystis*, and *Cylindrospermopsin*, in the place where we store water for drinking. In just 20 months, a group of 131 people getting treatment called hemodialysis had terrible things happen to their liver and digestive system. Sadly, 76 of these patients did not make it. They found high amounts of *microcystis* and *cylindrospermopsin*, which are toxic substances produced by cyanobacteria, in the liver [51], [52]. Cyanobacteria have gone to different places all over the world, such as North America, Australia, Canada, and Europe [50].

The group Anabaena was named by BORY and is also known as BORY ex BORN. et FLAH. 1888 comes from order Nostocales and Family of Nostocaceae. They can look like thin threads (threads alone or in loose groups) or in big piles on the base, generally with twisted and knotted parts, sometimes even tightly coiled like a screw. It's not often you will see them arranged more-or-less straightly from each other. They are sometimes covered in sticky, clear and often watery coats. These have a row of hairs on them that may look like beads or snail shells when seen from different angles, always having the same ends next to each other. The cells are tube-shaped, like a barrel or round. They can be shorter than their width or longer. The colour is light blue-green to dark green, and some have tiny air pockets inside them, while others do not but might contain grainy stuff at times. The end cells are stretched out, shaped like cones, cone-rounded or round but not empty inside. Sometimes, they can be round or oval in shape, like a ball. Other times, they may look longer and are usually a bit larger than normal plant cells. Akinetes are

round, oval or long and thin. They can be alone or in a group lined up with each other. In between new cells, they always grow paraheterocytic, where they're close to heterocysts on both sides of them, but sometimes slightly far away from those points too [53].

3.1. Anabaena and Siderophore Making

The making of siderophores has been linked to the seen growth of *Anabaena flos-aquae*, as well as other kinds like *Anabaena sp*. type 6411 and *Anabaena cylindrica*, along with the freshwater green-bluish bacteria *Anacystis nidulans, Microcystis aeruginosa, Nostoc muscorum* and *Lyngbya sp*. It has been discovered that the sea-based cyanobacteria *Agmenellum quadruplicatum* and *Coccochloris elabens* are also capable in making their own siderophores. Most of these bacteria-produced compounds that grab iron seem to have hydroxamate parts, but the only one with a known chemical buildup is from *Anabaena sp*. strain 6411 and is named schizokinen. It is important to know that schizokinen has been seen in the ground of rice fields. This probably came from cyanobacteria, which are tiny living things found in soil. Schizokinen is a mix made from a Citric acid that holds two particular parts called hydroxamate groups and one hydroxycarboxylate group. It works like a magnet for iron by holding onto it using these useful parts in the citrate section of the substance. The showing of how *Anabaena sp.* strain 6411 uses siderophores to take in iron has only been seen in this type of bacteria, which is different from other types of cyanobacteria. It was found that the body's way of collecting iron showed a particular liking for rust-coloured schizokinen [54]. This liking was noticed to be stronger when the living thing grew in a place where there wasn't much iron available. On the other hand, we found that having metabolic harm in the system slowed down how iron was taken up.

Recently, the pharmacological industry has started to care about siderophores that could be used as good ways to produce drugs and protect farming and fish-growing areas from metal trash in the environment [55]. The potential pharmaceutical molecule, siderophores can stop metal-eating enzymes and have shown to help prevent hardening of the arteries in people. There are also studies on how well foods and food extras with a siderophore can help to cut back on too much iron in people's bodies. This is important because having too much iron might cause stress that harms the heart. In new research, scientists have been looking into using bonded antibiotics with siderophores to improve treatment against infections [56].

The different types of industries that want siderophores all have one problem - they struggle to get them from tiny living things made artificially in labs. Normally, the amount of these useful things created this way is very small and only show up when checked with special tools. But, big improvements in how much is produced can be reached by making the right conditions for tiny living things to grow [57]. So far, we have used the One Strain Many Compounds (OSMAC) method to find out the best physical and chemical conditions for making a lot of siderophores. The second way is to do tests with different growth materials in order to grow the particular type that makes siderophores. In the end, we compare how much siderophore is made in small-sized cultures that hold between 20-50 mL. But this way takes a lot of money and time. Making siderophores in blue-green bacteria needs 30 days of growing them before we can pick the final product. So, it's not right for quick testing and making it automatic [58].

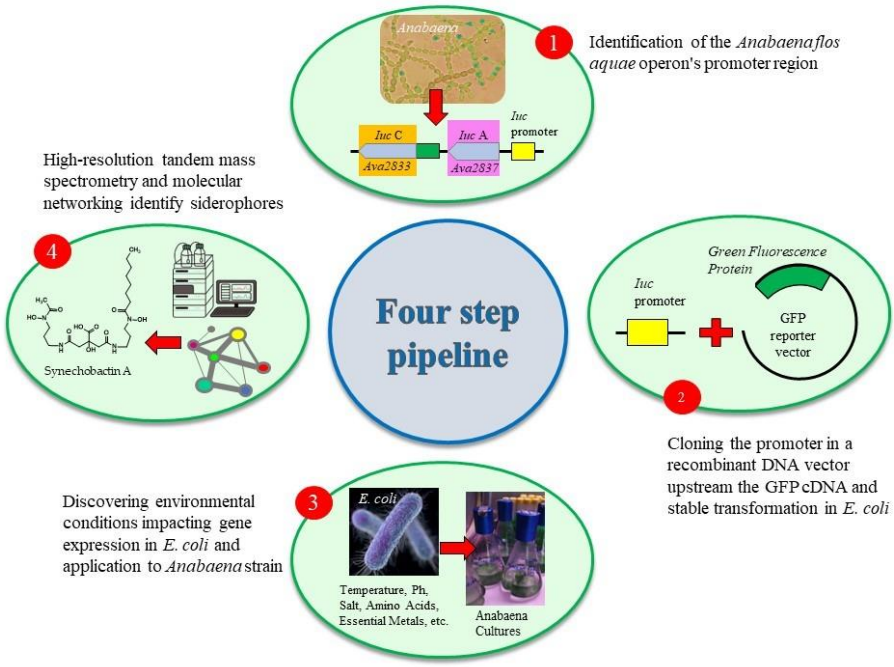
We suggest a different pipeline system to quickly find the growth factors that increase siderophore production. The reason for this four-step process is based on the truth that (A) the enzymes needed to make siderophores are often found together in a single operon,

and so under only one promoters' control switch, plus (B) these operons can be really closely managed at transcriptional levels when they work as part of making new stuff by how much iron there might or might not be around, negative feedback of the product, variation in heat and cold, and the density and composition of the media. The four-step pipeline (Figure 3.1) includes

1. Identification of the operon: the cartoon describes the arrangement of *Iuc* operon in *Anabaena flos aquae* UTEX 1444 (namely *A. variabilis* ATCC 29413) with the *Iuc* promoter, *IucA* and the *IucC* genes
2. Cloning of *Iuc* promoter upstream the cDNA coding for GFP in the reporter vector *Iuc*-GFP and testing of conditions activating GFP expression in transformed *Iuc*-GFP *E.coli*
3. Documenting of the growth factors affecting the expression of the gene in *Escherichia coli* and their application to the cultivation of the Anabaena strain;
4. Analysing cell cultures via dereplication by the combined use of high-resolution tandem mass spectrometry with molecular networking

To test our system, we started with how it is used to find the growth factors and outside conditions that, at a cell's writing level, influence the making of siderophores. We tested our model organism called *Anabaena flos aquae* UTEX1444, or just *Anabaena variabilis* for short (also known as *Thrichormus variabilis* in some guides). The test results helped find out that things like heat, how acidic something is or isn't and the amount of salty stuff in a solution, along with iron and citrate levels, can all impact what's called siderophore making at its basic trascriptional step.

Figure 3.1- 4-stage pipeline.



In the same way, by looking at how siderophore promoters work, we found a never-before-seen citrate-sensitive part that changes a gene's writing down the process needed for making siderophores in *Anabaena flos aquae*. To make sure the process is correct, we check the growth factors found in our lab-grown *Anabaena flos aquae* UTEX 1444 cultures. We study these parts by looking at their extracts and identifying siderophores using a method that relies on high-resolution mass spectrometry joined with liquid chromatography-based molecular networking.

4. Result and Discussion

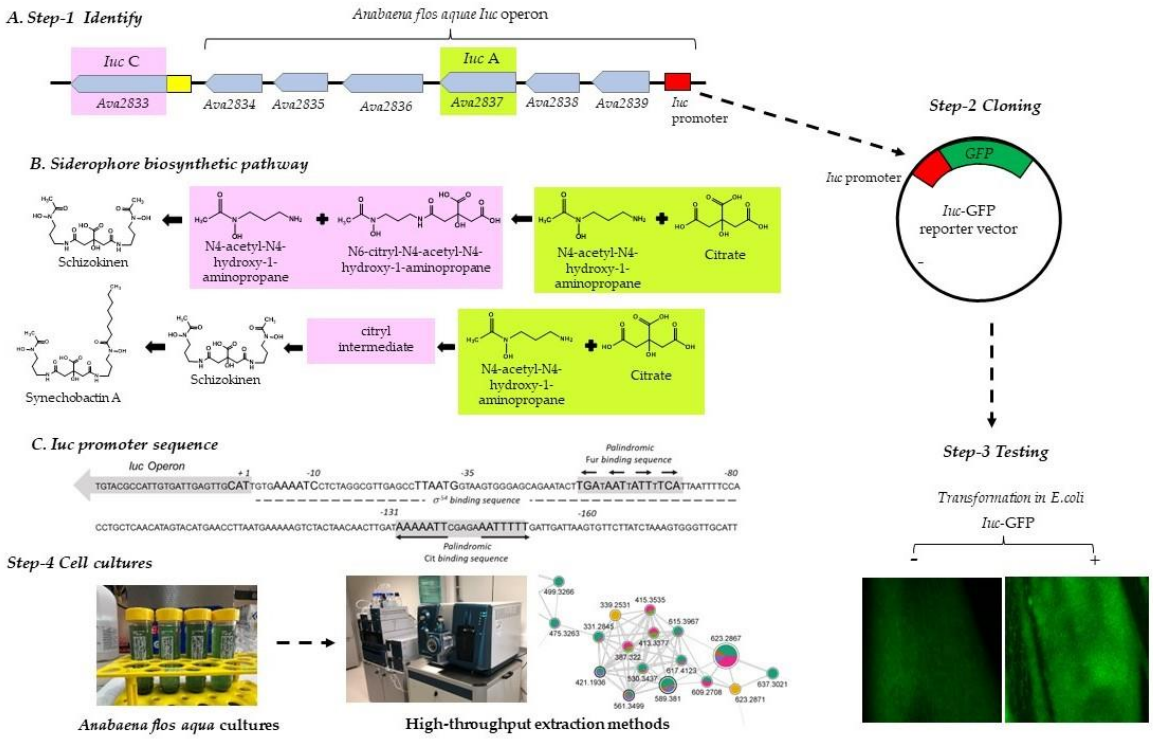
4.1. Identification of the promoter region of the operon *Iuc* in *Anabaena flos aquae*.

In the previous chapter, we shared that a type of bacteria called *Anabaena flos aquae* UTEX 1444 makes several similar chemicals (like synechobactin, schizokinen, aerobactin and rhizobactin 1021) when there's not enough iron around [59]. So far, the amount of these siderophores produced by *Anabaena flos aquae* has been little. Schizokinen is a clear hydroxamate siderophore with a strong structure. It is made of specific freshwater Anabaena species [60]. This happens when two parts of N4-hydroxy-1-aminopropane connect to citrate at spots 1 and 3 in citric acid. Schizokinen is a middle step in making Rhizobactin 1021, which is another type of siderophore. They both use the same enzyme-making system to be produced. This last part also helps in making two similar siderophores called synechobactin and aerobactin [61].

Usually, non-ribosomal peptide synthetase (NRPS) makes siderophores. However, in the case of schizokinen, rhizobactin 1021, synechobactin, and aerobactin backbones, these are formed by NRPS-independent siderophore synthetase (NIS) [62]. The making of schizokinen in the NIS involves the proteins *IucA* and *IucC* (Figure 3.2 (A)). *IucA* attaches the first N-acetyl-N-hydroxylysine to a carboxylic group of citric acid, yielding N-citryl-N-acetyl-N-hydroxylysine. *IucC* helps add the second part, N-acetyl-N-hydroxylysine, to the carboxylic group of N-citryl-N-acetyl-N-hydroxylysine, making schizokinen (Figure 3.2 (A)) [63].

The step one of the pipeline included the recognition of NIS operon in the genome of *Anabaena flos aquae* UTEX 1444 (similar to *A. variabilis* ATCC 29413). GenBank assigns the *IucA/IucC* operon from locus *Ava_2833* to locus *Ava_2839* (Figure 3.2 (A)) in a ~12.5 Kilobase long sequence on the complementary strand of Anabaena chromosome (genomic region 3,506,499 to 3,518,085).

Figure 3.2. (**A**): 4-step pipeline in detail: **Step 1**: Identification of the operon: the cartoon describes the arrangement of *Iuc* operon in *Anabaena flos aquae* UTEX 1444 (namely *A. variabilis* ATCC 29413) with the *Iuc* promoter (red box), *IucA* (green region) and the *IucC* (pink region) genes highlighted; **Step 2**: Cloning of *Iuc* promoter (red box) upstream the cDNA coding for GFP (green box) in the reporter vector *Iuc*-GFP ; **Step 3**: testing of conditions activating GFP expression in transformed *Iuc*-GFP *E.coli.* The photographs show *E.Coli* transformed (+) or not (-) with *Iuc*-GFP. **(B)** Biosynthesis reactions leading to schizokinen and synechobactin A with highlighted those catalyzed by *IucA* and *Iuc* in green and pink, respectively. **(C)** *Iuc* promoter sequence showing the first ATG (+1) of the *Iuc* operon and the σ^{54} binding region. Predicted Fur and Cit binding sequences are highlighted in gray, **(D)** analyzing cell cultures via dereplication by the combined use of high-resolution tandem mass spectrometry with molecular networking.



We used an Operon mapper [64] to study the area and find operons, units for transcription, and promoter sections. The software says that the protein sequences made by certain genes, *IucA*, *Ava_2836*, *Ava_2835* and *IucC* are like parts of other proteins involved in making a substance called rhizobactin siderophore. *RhbC* (*IucA*), *RhbD* (Acetyltransferases, including N-acetylases of ribosomal proteins), *RhbE* (Lysine/ornithine N-monooxygenase) and *RhbF* (*IucC*) (Supplementary Figure 1) [65]. *Ava_2839* and *Ava_2838* codes for an aminobutyrate aminotransferase and a Glutamate decarboxylase, respectively. Instead, *Ava_2834* was discovered to be like a theoretical protein of unknown use.

A single operon is predicted covering from *Ava_2839* to *Ava_2834* and incorporates *IucA* but not *IucC,* which, on the contrary, seems to be a separate transcription unit (Figure 3.2 (A)). The *Iuc* operon presents a ~670 basepairs long promoter region allocated upstream of the *Ava_2839* coding sequence (Figure 3.2 (C)). The promoter was checked using the prediction tool BacPP [66]. The software confirmed the presence in the first 160 bp of the promoter of a binding region for sigma factors *σ54* (promoting transcription of genes as a consequence of metabolite deprivation) as well as a conserved palindromic binding sequence for *Fur* proteins- inhibiting transcription of genes in the presence of iron [67] (Figure 3.2 (C)).

4.2. Construction of the reporter vector for Iuc promoter activity

A DNA fragment corresponding to the 160 bp long operon promoter region (genomic region 3,517,420-3,517,580) was synthesized *in vitro* and ligated into the vector *pCDNA3.1(+) E-GFP*, between restriction sites for the endonucleases *BglII* and *HindIII* to create an expression vector (referred to as *Iuc-GFP* (Figure 3.2 (A), step 2)). The

cloning steps took out any other start sequence from the carrier that might have changed how bacteria showed *GFP*. To make it easier to clone, two matching parts that fit with *BglII* and *HindIII* were put at the start and end (*5'* and *3'*) of the *Iuc* promoter. The *Iuc* - *GFP* reporter vector was then transformed in *E.Coli* for the downstream steps of the pipeline (Figure 3.2 (A), step 3). Correctness of the DNA sequence was confirmed upon sequencing of the full vector. Compared to untransformed *E.Coli* cells, *Iuc-GFP* cells were endowed with a brighter intracellular green fluorescence, as shown in microscopy (Figure 3.2), suggesting a basal activity of the *Iuc* promoter in *E.Coli*.

4.3. Screening of the environmental conditions promoting Iuc promoter activation

The screening of the growth conditions stimulating *Iuc* promoter activity was achieved by measuring intracellular *GFP* fluorescence. The multiple Spectrofluorometer used to measure *GFP* intensity required very small culture volumes (50 µL) and allowed the simultaneous testing of multiple growth parameters with high reproducibility as a result of the several technical and experimental replicates

Table 3.1. Results of the optimization of GFP expression from the reporter vector *Iuc-GFP* in *E.coli*.

Parameter	**Range tested**	**Optimal value**	**Fold Induction at optimal value[1]**
Temperature (˚C)	23-37	29	9.1 ± 1.7
pH	6.0-8.0	7.5	3.8 ± 0.2
NaCl g/L	8.0-10.0	9.5	2.8 ± 0.2
Fe^{3+} (µM)	0-52	39.5	41.3 ± 0.7
Fe^{2+} (µM)	0-92	37	10.2 ± 0.6
Citrate (mM)	0-52	2.6	74.2 ± 1.5
Glucose (g/L)	0-52	2	26.3 ± 1.1
Lysine (mg/L)	0-5	0.75	5.3 ± 0.1

[1]fold induction at optimal value = Intracellular *GFP* fluorescence measured in *E. coli* at the indicated optimal condition and normalized to *GFP* fluorescence measured in normal growth condition [supplemented LB Medium]**.** Values are reported as mean + S.D. (n=5 replicates)

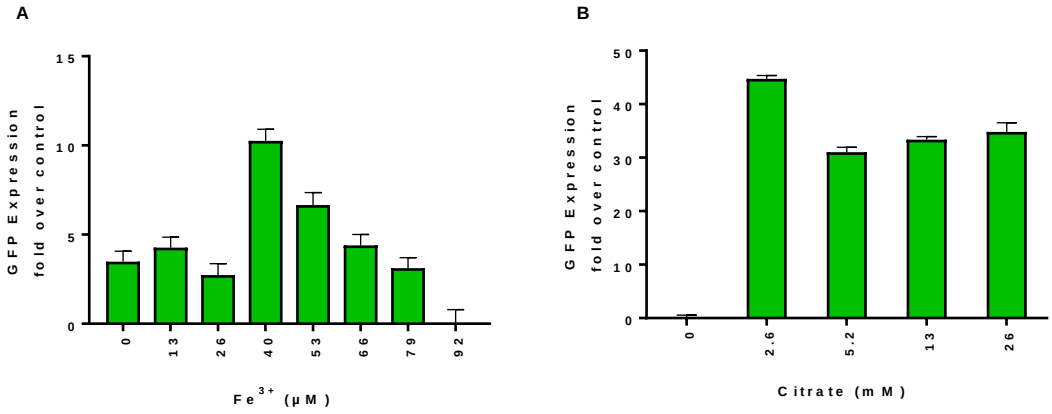
The Table 3.1 reports the tested parameters, the range of testing, the optimal condition suggested by the screening and the fold induction of *GFP* fluorescence achieved at the optimal condition compared to normal growth conditions. As a result of the screening, we identified optimal *GFP* expression from *Iuc-GFP* in *E. Coli*: optimal temperature 29°C, growth in Luria Broth Medium at pH 7.5 and supplemented with Glucose (2g/L), NaCl (9.5 g/L) , Fe^{3+} 39.5 µM (or 37 µM Fe^{2+}), 2.6 mM Citrate and 0.75 mg/L of Lysine.

In cyanobacteria, the production of siderophore is upregulated under iron limitation. The *Iuc* operon is transcriptional regulated by Fe^{3+} ions, thanks to cis-acting transcription repressors belonging to the *Fur* family [67]. In the presence of iron, *Fur* proteins bind and repress the *Iuc* operon. In conditions of iron limitation, *Fur* proteins detach from the promoter allowing cyanobacterial RNA polymerase to transcribe the operon. As shown in (Figure 3.2 (C)), a detailed analysis of the promoter region of *Iuc* of *Anabaena flos aquae* confirmed the presence of the Fur responsive element in this strain. One common strategy to increase the yield of in vitro produced siderophore is to cultivate cyanobacteria in iron depleted media [59]. However, it is notoriously difficult to determine for each specific cyanobacteria the optimal Fe^{3+} concentration since iron is essential for many other bacterial processes.

Indeed, as shown in (Figure 3.3 (A) and Table 3.1), in our recombinant system, iron's ability to control the *Iuc* promoter of *Anabaena flos aquae* is preserved, with intracellular *GFP* expression depending on Fe^{3+} concentration. The relation between Fe^{3+} concentration and *GFP* production is described by a bell-shaped curve with optimal Fe^{3+} concentration for *GFP* in the micromolar range. Citrate is one of the substrates of *IucA*. As many other substrates, it is a limiting factor for the activity of the enzyme. However,

the results of Table 3.1 suggest that citrate can as well control the operon *Iuc* at the transcriptional level, and thus regulate the expression of *IucA*.

Figure 3.3. Activation of the *Iuc* promoter expressed as GFP expression from *Iuc*-GFP at the indicated Fe^{3+} (A) and citrate (B) concentrations. Fold induction refers to intracellular GFP fluorescence measured in *E. coli at the indicated conditions and normalized to GFP fluorescence measured in normal growth conditions*. Values are reported as mean + S.D. (n=5 replicates)



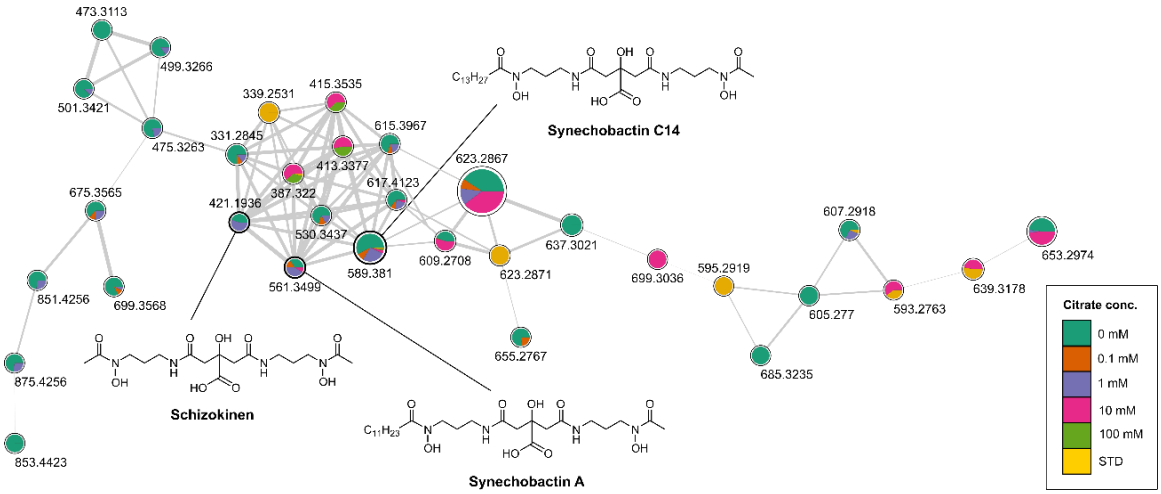
Indeed, as shown in (Figure 3.3 (B) and Table 3.1), increased concentrations of citrate stimulate *Iuc* promoter activity and augment GFP expression. It has been already reported the existence of citrate responsive elements in microbial chromosomes and of the existence of regulators of citrate metabolism, like the proteins belonging to the family of *Cit* promoters [68] from *Enterococcus faecalis*. In the presence of citrate, *Cit* proteins bind to *cis*-acting sequences located upstream of genes and operons, usually those involved in citrate utilization. As shown in (Figure 3.2 (C)), detailed analysis of the promoter region of *Iuc* revealed the presence of hypothetical citrate responsive element similar to those recruiting *CitO*.

4.4. Optimization of culture conditions of *Anabaena flos-aquae* UTEX1444 for siderophore production

To finally prove the eligibility of our four-step pipeline as a platform to identify the growth condition increasing the yield of siderophores production in *Anabaena flos aquae* UTEX 1444, we cultivated the strain in different media, modified in accordance with the screening results shown in Table 3.1. For siderophore identification and quantitation, we used MS-based molecular networking [69], a powerful metabolomic tool that allows complete metabolic profiling of the species under study. MS-based molecular networking is a robust and less time-consuming bioinformatic tool for MS/MS data analysis and dereplication of complex natural matrixes. It assumes that structurally related compounds share similar fragmentation patterns and provides a visual representation of structural relationships between compounds in the extracts, as revealed by MS/MS data. The MS/MS spectra of the compounds in the extracts are aligned, the peaks in common between the various spectra are identified (the so-called peaks match), and based on the alignments, a score is assigned (cosine score). The higher the scoring, the more similar the spectra are. In a molecular network, each MS/MS spectrum is represented as a node, labelled with the parent mass, and the relatedness between compounds (cosine score) is represented by edges. Molecular networking presents high scalability, and it allows an easy evaluation (in a single visual network) of multiple datasets [70], avoiding a huge amount of complex data, always difficult to manage, as well as the capture and quantification of molecular analogues. Moreover, it enables a facilitated quantitative comparison of the desired metabolites between the samples, dispensing from the laborious peak-by-peak analyses, as in standard LC-MS data.

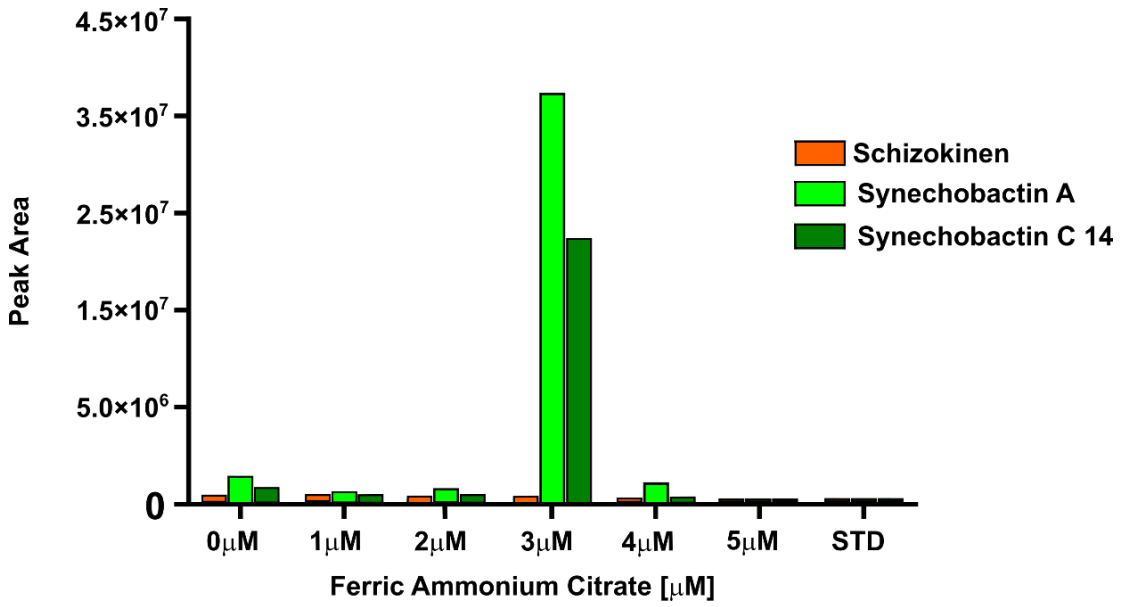
Differently from *E. coli*, *Anabaena flos aquae* UTEX 1444 cultures are usually maintained at 28 to 29 °C and the FWBG11growth medium has a pH of 7.5 and physiological osmolarity. This reveals that the temperature, pH and osmolarity at which this strain grows best already correspond to those leading to the highest transcription of the *Iuc* operon (Table 1). We thus maintained fixed these parameters and modified growth media varying Fe^{3+} and citrate concentration. Till now [59], the culture medium used for siderophore production was Fe^{3+}deprived and contains 31 µM/L of citric acid. We thus verified the effect of different iron and citrate concentration on the yields of siderophore produced. As first, six cultures (no added citrate, 0 mM (final (citrate) 31 µM, #1), 0.1 mM ((citrate) 131 µM, #2), 1 mM ((citrate) 1.031 mM #3), 10 mM ((citrate) 10.031 mM, #4), 100 mM ((citrate) 100.031 mM #5), STD ((citrate) 31 µM in the presence of Fe^{3+}, #6) were extracted and analysed through LC-HRMS and molecular networking [59]. In the comprehensive molecular network of *Anabaena* #1-6 cultures' extracts (**Figure 3.4**), the arrangement of the cluster containing schizokinen and the known synechobactins A and C_{14} showed an uneven distribution of such siderophores in the different cultures, with a clear prevalence in those with the lowest concentrations of citrate (31 to 131 µM of citrate/L) and in the absence of Fe^{3+}

Figure 3.4 Cluster containing synechobactin in the molecular network obtained combining the LC-HRMS/MS analyses of all the extracts of *#1-6* cultures of *Anabaena flos-aquae* UTEX 1444. Nodes are labelled with parent mass and are represented as a pie chart with colour coding showing the source culture of the compound ([Citrate] = 0 mM, 0.1 mM, 1 mM, 10 mM, 100 mM, STD, standard citrate concentration). The size of the nodes corresponds to the sum of precursor ion intensities.



Combining all the information coming from this study and our previous study [31], a new set of cultures was prepared, in which the concentration of ferric ammonium citrate in FWBG 11 varied from 0 µM to 5 µM. In contrast, sodium citrate has been supplemented to each culture in the amount necessary to compensate for the removed ferric ammonium citrate.

Figure 3.5. LC-HRMS quantitation of schizokinen (*m/z* 421.1936, orange bars), synechobactin A (*m/z* 561.3499, green bars) and synechobactin C_{14} (*m/z* 589.3810, blue bars) in *Anabaena flos-aquae* UTEX1444 cultures (FWBG) containing 0 µM, 1 µM, 2 µM, 3 µM, 4 µM, 5 µM and 23 µM (STD condition) ferric ammonium citrate.



The extracts of the above cultures were subjected to LC-HRMS. Figure 3.5 shows the results of this analysis: at a concentration of 3 µM Fe^{3+} production of synechobactin A and synechobactin C_{14} is sharply increased, confirming the bell-shaped correlation between the amount of Fe^{3+} and siderophore production suggested by our in-vitro pipeline.

5. Experimental set-up

5.1. *Anabaena flos aquae* Genome

The genome of *Anabaena variabilis* ATCC 29413 was retrieved from GenBank (Accession: CP000117.1, National Library of Medicine, NIH) (https://Www.Ncbi.Nlm.Nih.Gov/Nuccore/CP000117.1 (accessed on 1 November 2022)). The *Iuc* operon was analysed by means of an Operon Mapper to identify operon coordinates, operon-gene pairs, and ORF coordinates and predict gene and protein sequences. The promoter region of the *Iuc* operon was analysed by using the software BacPP (Http://Www.Bacpp.Bioinfoucs.Com/Home (accessed on 17 August 2022)).

5.2. Bacterial Cultures

Anabaena flos aquae UTEX 1444 gifted by prof. Antonio Pollio (Department of Biology, Unina, Italy) was cultured in FW BG11 medium [freshwater BG11, 1L of the solution contains: Na_2EDTA - 1 mg, Citric Acid - 6 mg, $NaNO_3$- 500 mg, $K_2HPO_4.3H_2O$- 40 mg, $MgSO_4.7H_2O$- 75 mg, $CaCl_2$- 26.4 mg, $NaCO_3$- 17.1 mg, $NiSO_4$ $(NH_4)_2$ $SO_4.6H_2O$ - 250 µL of 0.1 mM stock solution, Na_2SeO_4- 100 µL of 0.1 mM stock solution, Nitsch Solution - 1 mL (where 100 mL solution contains: concentrated H_2SO_4 - 0.5 mL, $MnSO_4.H_2O$ - 2.29 mg, $ZnSO_4.7H_2O$ - 0.5 g, $CuSO_4.5H_2O$ - 15.9 mg, $Na_2MoO_4.2H_2O$ - 0.025 g, H_3BO_3 - 0.5 g, and, $CoCl_3.6H_2O$ - 0.135 g)] fortified with vitamin mix [100 mL of 10X solution contains: nicotine acid - 100 mg, PABA - 10 mg, biotin - 1 mg, thiamine - 251 mg, vitamin B12 - 1 mg, folic acid - 1mg, inositol - 1 mg, Calcium-pantothenate - 100 mg]. Culture was grown in culture media with six different concentrations of citrate supplemented as sodium citrate. The concentration of sodium citrate was determined by making a series of

dilutions from a 100 mM parent solution to get the following concentrations such as 0 mM (#1); 0.1 mM (#2); 1 mM (#3); 10 mM (#4); 100 mM (#5) and Standard STD (#6). Another serial dilution was made for growing the cultures in Ferric Ammonium Citrate for the concentration of 0 μM to 5 μM. Triplicates of all cultures were cultivated at the temperature of 29˚C for 30 days. *E. coli* was grown in Luria Broth [Bacto tryptone - 10 g/L; Bacto yeast - 5 g/L; NaCl - 10 g/L) at pH 7.0 (NaOH) at 37°C with gentle shaking.

5.3. Cloning of Iuc-GFP vector

The viable *E.coli.* Cells were made competent by the heat shock method that uses sudden extreme temperature exposure and a calcium-rich environment, making the cell walls permeable to surroundings. These competent cells were transformed by taking up the 6.1 bp long bacterial plasmid pcDNA3.1(+) eGFP (Supplementary Figure 2)) in the presence of continuous heat and cold shocks and calcium chloride that counteracts the electrostatic repulsion between the plasmid DNA and bacterial cell membrane and facilitates the transformation. The transformed cells were grown in the Luria Broth Agar Plates [composition- Batotrypton- 10g/L; Bacto-yeast- 5g/L; NaCl- 10g/L, Bacto Grade Agar- 15g/L] supplemented with antibiotic ampicillin. The plasmid pcDNA3.1(+) eGFP has an antibiotic resistance gene that allows selective growth in the presence of ampicillin and promotes the growing culture's purification. The transformed cells are grown at 37˚C for 12-16hrs with the gentle shaking of 250 rpm.

The DNA of the overnight-grown transformed cells were extracted following a Midiprep method. The procedure begins with the phase separation of the 10ml culture sample by centrifuging at 4˚C, 4000rpm for 10 mins. The pallet is taken and treated with resuspension buffer, lysis buffer and precipitation buffer to remove all the extracellular

materials. The precipitated liquid is loaded in the miditubes and is washed with wash buffer. The elution buffer binds with the genetic materials and passes through the miditubes. This eluate is secured in a sterile tube and is washed using Isopropanol and 70% ethanol. The remaining genetic material was dried, suspended in water and was qualified and quantified using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometers.

The DNA obtained from the previous step was digested with restriction enzymes. The plasmid pcDNA3.1(+) eGFP has various restriction sites, and we have used 2 of those sites, BGLII at 192 bp and HIND III at 1091 bp, to cut the DNA. BGLII cuts 5ˈ-Aˈ GATCT- 3ˈ and 3ˈ-TCTAGˈA- 5ˈ whereas HIND III cuts 5ˈ-Aˈ AGCTT- 3ˈ and 3ˈ-TTCGAˈA- 5ˈ producing sticky ends called single-stranded overhangs having unique sequences. The efficiency of the digestion was confirmed by an electrophoresis gel run. The digested fragment of DNA was separated from the cut piece by the fragment purification method. A second electrophoresis gel run was performed with soft agarose (0.8% agarose), and the run was performed for 45 mins. After a clear separation run of the fragment and the cut piece in the gel, the gel containing the fragment area only was sliced careful and weighed and ground in the Eppendorf. The groundmass of gel and fragment was purified with binding buffer, wash buffer and elution buffer, and the precipitated eluate with the fragment piece of DNA having sticky ends was secured in Eppendorf. The desired fragment's quality and quantity were determined using NanoDrop™ One Microvolume UV-Vis Spectrophotometers.

As the next part of the study, an insert was designed to fit into the DNA fragment. For facilitating the safe and sure bindings, the Insert had identical end sequences to that of the single-stranded overhangs. The siderophore promoter upstream IUCA was redesigned

using the software into a 120bp-long sequence. The upstream sequence of Insert 5 ' - > 3 ' -

GATCTAAATCTCATTCTCTCTGTGTCTCTGCGTGAGCAAAAAAATAGGAGAG AGGGAGAGCGAACAACTCTCCTCACTCCCCAACATTAAATAACTITCACTCAA ACCATACACTAGACA (430 ug) and the downstream sequence 5 ' - > 3 ' - AGCTTGTCTAGTGTATGGTTTGAGTGAAAGTTATTTAATGTTGGGGAGTGAGG AGAGTTGTTCGCTCTCCCTCTCTCCTATTTTTGCTCACGCAGAGACACAGAG AGAATGAGATTTA (654 ug) respectively, was annealed together using SSC buffer [concentration- Sodium Chloride and Sodium Citrate at pH 7.0] by RT-PCR at the temperature ranging from 35˚C to the highest of 95˚C for 2hrs forming an Insert (dimer) and this was confirmed by electrophoresis gel run, comparing it with the loading dye as a standard.

The fragment and the Insert were ligated together to form a recombinant vector. The ligation procedure used prolonged heat exposure time with sudden cold shocks for the fragment and inserted it to stick together at the digested site. This new recombined vector was transformed in *E.coli.* and was plated in the Luria Broth agar plates supplemented with the antibiotic ampicillin. The recombinant cells were grown overnight at 37˚C for 12-16hrs with the gentle shaking of 250 rpm. The DNA was extracted by the miniprep method. The procedure begins with the phase separation of the 2ml culture sample in Eppendorf by centrifuging at 4˚C, 3000rpm for 5 mins. A pallet is taken and treated with resuspension buffer, lysis buffer and precipitation buffer with continuous heat and cold shocks to remove all the extracellular materials. The precipitated liquid is loaded in the minitubes and washed with wash buffer. The pallet remains were dried, suspended in water, and was qualified and quantified using a Thermo Scientific™ NanoDrop™ One

Microvolume UV-Vis Spectrophotometers. The *E.coli.* Cells with the recombinant DNA were used as a tool to test various possible environmental stimuli such as temperature, pH; salt concentrations, Fe^{3+}; Fe^{2+}; citrate; glucose and lysine. The DNA expressions for each of the stimuli were measured via a spectro-fluorometer. The amount of GFP measured was directly related to the gene expression for siderophore.

5.4. Spectrofluorimetric measurement of GFP expression

Aliquots (50 µL) of *Iuc-GFP* expressing *E.Coli* culture were challenged for *Iuc* promoter activity and *GFP* expression in a 96 or 354-well black optiplate (Perkin Elmer, Waltham, USA). Intracellular *GFP* fluorescence was measured in a Perkin Elmer Envision 2105 Multiplate reader (Perkin Elmer) as already reported [71], using the inbuilt monochromator and with the following parameters: λ excitation 488 nm, λ emission 509 nm, and monochromator cut off 360 nm. After the *GFP* measurement, aliquots were transferred in transparent Optiplate (Perkin Elmer, Waltham, USA) for nephelometric measurement (λ= 600 nm). This second measurement indicates the total number of bacterial cells in each well and was used for normalization. Normalized *GFP* expression is reported as the ratio between intracellular *GFP* fluorescence and absorbance of the culture at 600 nm ± SD.

5.5. LC-HRMS and molecular networking of the extracts

LC-HRMS and molecular networking were performed according to [72] with slight modifications. A Thermo LTQ Orbitrap XL high-resolution ESI mass spectrometer coupled to an Agilent model 1100 LC system was used to perform LC-HRMS experiments. A 5-µm Kinetex C18 column (50 × 2.10 mm), maintained at room temperature, was eluted at 200 mL min^{-1} with H_2O (supplemented with 0.1% HCOOH)

and MeOH, using gradient elution. The gradient program was as follows: 10% MeOH for 3 min, 10%→100% MeOH for 15 min, and 100% MeOH for 12 min. Mass spectra were acquired in positive ion detection mode. Data were collected in the data-dependent acquisition (DDA) mode, in which the fifth most intense ions of a full-scan mass spectrum were subjected to high-resolution tandem mass spectrometry (HRMS/MS) analysis. The m/z range for data-dependent acquisition was set between 150 and 2000 amu. HRMS/MS scans were obtained for selected ions with CID fragmentation, isolation width of 2.0, normalized collision energy of 35, Activation Q of 0.250, and activation time of 30 ms. Data were analyzed using Thermo Xcalibur software.

Pre-processing of .raw files was performed through MZmine 2.53 [73]. The mass detection was performed on raw data and exact masses with mass level 1 and centroided masses with mass level 2, by keeping the noise level at 10000. Chromatograms were built using an ADAP module with a minimum height of 10000, and m/z tolerance of 0.01 (or 20 ppm). Peak alignment was performed using the Join aligner algorithm (m/z tolerance at 0.01 (or 10 ppm), absolute RT tolerance at 0.3 min). [M+Na–H], [M+K–H], [M+Mg−2H], [M+NH_3], [M-Na+NH_4], [M+1, ^{13}C], [M-^{35}Cl+^{37}Cl]$^+$, [M+^{56}Fe-3H]$^+$ adducts were filtered out by setting the maximum relative height at 100%. Peaks without associated MS/MS spectra were finally filtered out from the peak list. Clustered data were then exported to a .mgf file for GNPS, while chromatographic data, including retention times, peak areas, and peak heights, were exported to a .csv file. A Feature-Based Molecular Network [59] was generated on GNPS' online platform, with the following parameters: the precursor ion mass tolerance was set to 0.05 Da and the MS/MS fragment ion tolerance to 0.05 Da, cosine score above 0.7 and more than 5 matched peaks. For

GNPS' library search a cosine score of 0.7 and at least 6 matched peaks were set. The molecular network was visualized using Cytoscape software [74].

6. Conclusion

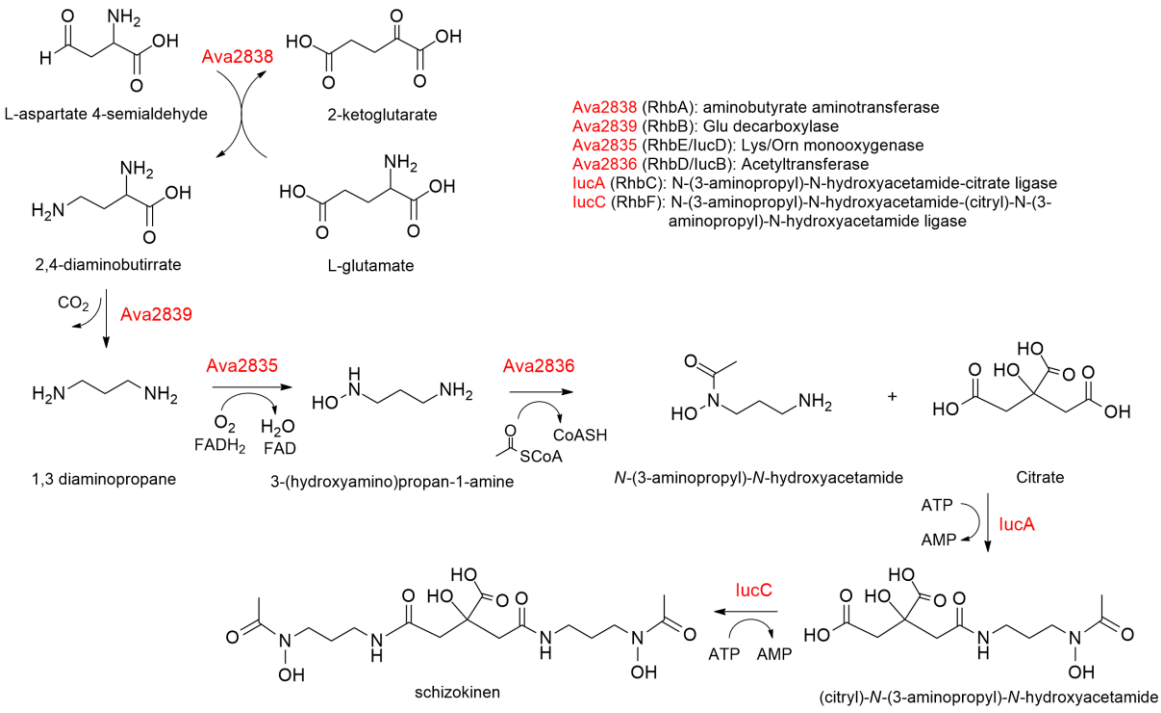
Microorganisms make siderophores, special molecules that can grab iron. They release these in their surroundings to take up iron inside cells and keep cell balance steady. They can be used to clean up polluted natural areas in an eco-friendly way and also to find new medicines. Here, we suggest a four-step pipeline to research and improve growth conditions for boosting the output of siderophores in cyanobacteria.

This pipeline is validated with the *Anabaena flos-aquae* UTEX 1444 and in chapter 2, it has been shown to make a group of similar chemicals (including schizokinen, rhizobactin, synechobactin and aerobactin) when there's not enough iron. By using the OSMAC method, we must change many environmental factors to find the best growth conditions for making siderophores. But, thinking about the time it takes *Anabaena flos aquae* to grow and how much culture space is needed for a useful amount of biomass, using OSMAC strategy would be slow and costly. Here, we used a smaller amount of liquid (50 μL needed for our way versus 50 mL needed in OSMAC) to quickly spot if temperature, acidity level or saltiness impacts the growth of something important or manipulate genes to produce something useful. This helps us make more records about what is called siderophore, which includes two types - schizokinen and synechobactin A and C_{14}. This quick system could be easily automated and used on more types of blue-green bacteria to increase the creation of any iron-grabbing or other nature-acting substances. Moreover, the fourth part of this process, called molecular networking, proves to be very useful in quickly sorting out any kind of real-world sample. In the end, by finding that citrate helps

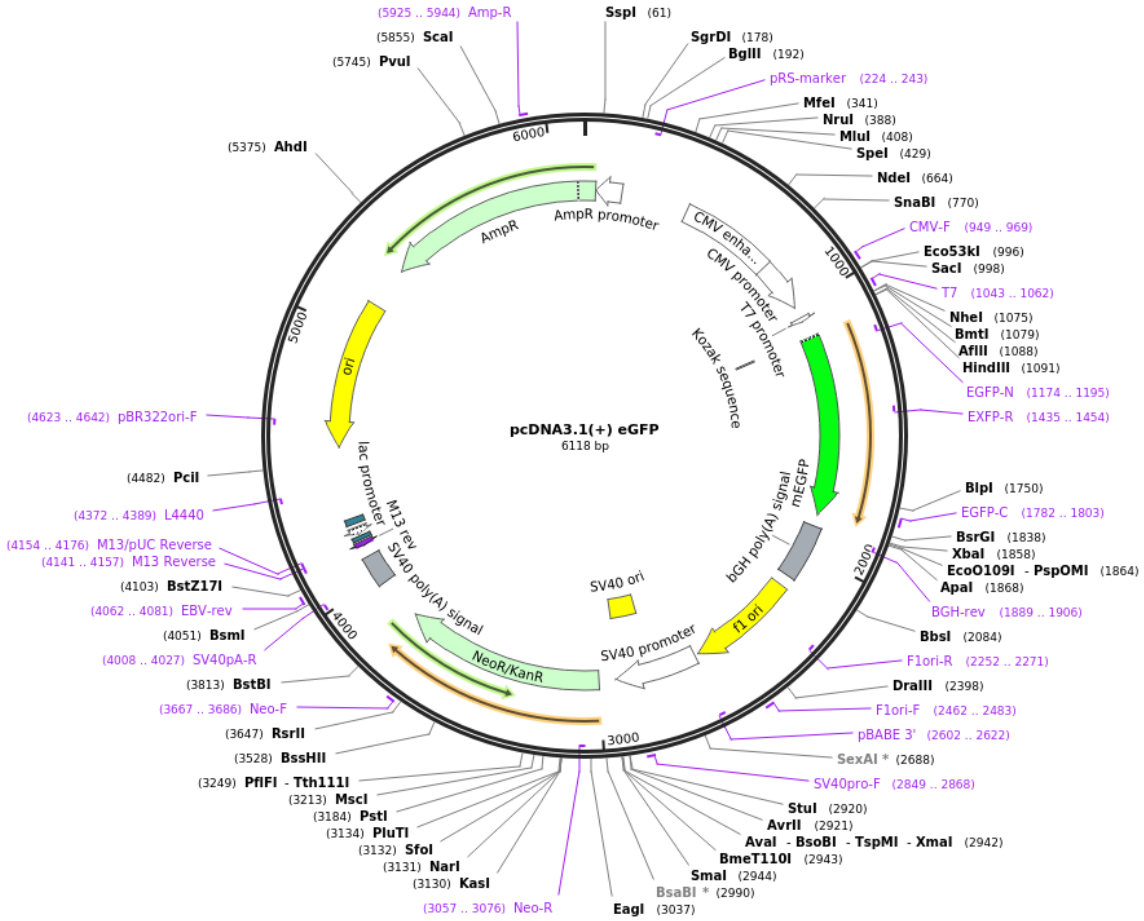
with growth and affects siderophore production at a writing level in DNA, this system lets us find something new. We saw for the first time how an area before the *IUC* promoter part changes because of citrate signals. It made us understand more about genes controlling the siderophore-making process in cyanobacteria.

Supplementary Figure

Supplementary Figure 1- Reaction catalysed by *IucA* and *IucC*.



Supplementary Figure 2- The Plasmid pcDNA3.1(+) eGFP from E. coli.



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Chapter 4

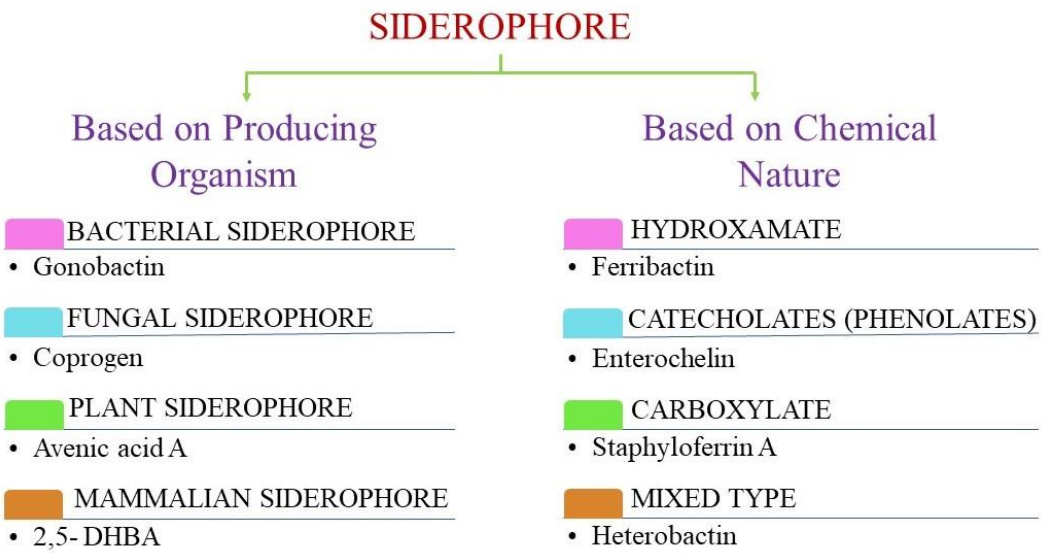
Secondary Metabolites in Anticancer Drug Research

Small molecules called siderophores that chelate iron (III) are produced and released by microorganisms. This chapter initially explores how siderophores provide both gram positive/negative bacteria with iron and identifies the participating proteins. Developing Trojan horse antibiotics, treating iron overload and using fluorescent chemosensors are examples of potential applications for the binding-capable solid iron (III) chelators, which are discussed here.

1. Classification of Siderophore

Many different classification systems for siderophores have been put forward by various scientists. However, it is clear that a satisfactory and internationally accepted form of classification for these compounds needs to be established [1], [2].

Figure 4.1- Siderophore classification.



1.1. Bacterial Siderophore

Bacteria are usually known for their ability to produce siderophores as the only observable extracellular form of these substances [3]. Both Gram-negative and positive bacteria possess the potential of making siderophores when iron is insufficient in quantity [4], [5]. There is also the representative example of *Pseudomonas stutzeri* CCUG 36651, which shows a particularly notable finding. These organisms are capable of producing and excreting four different ferrioxamine siderophores by the influence of molecular oxygen [6]. As far as anaerobes and aerobes are concerned, both have what they need to make siderophore under stress. However, it is worth noting that the detection of any one of these ferrioxamine siderophores depends on an atmosphere containing oxygen.

Escherichia coli is a type of Gram-negative bacterium that stands in high regard among scientists for its ability to produce siderophores. This microorganism is constantly passed into the intestine. It also has a unique ability to endure rich oxygen conditions. *Escherichia coli*, which is part of the Enterobacteriaceae family and thrives in warm-water environments, produces a siderophore called enterobactin. One type of siderophore, called a catechol siderophorone, has turned out to be very special. It binds Fe^{3+} ions with an affinity higher than that exhibited by other similar compounds ever tested before. Enterobactin is a cyclic triserin lactone; it has three catechol groups [3].

However, Gram-positive actinobacteria including *Streptomyces* produce siderophores in varying forms among a wide range of desferrioxamine G, B and E. Mycobacteria, as represented by the genus Mycobacterium, are responsible for both the onset and development of tuberculosis. These microorganisms are capable of extracellularly producing siderophores such as carboxymycobactins and exochelins [7]. *Pseudomonas*

fluorescens, a bacterium of notable significance, assumes a pivotal role in the synthesis and secretion of a hydroxamate siderophore known as ferritin. In contrast, *Neisseria meningitidis* and *Neisseria gonorrhoeae* can synthesise hydroxamate-type siderophores, referred explicitly to as gonobactin and nocobactin, respectively [8].

Agrobacterium *tumefaciens* and *Paracoccus denitrificans* are the organisms responsible for synthesising linear catecholate siderophores, specifically agrobactin and parabactin [9]. The strain DM4 of *Rhizobium meliloti* has been identified as the primary agent responsible for synthesising and secretion of the carboxylate-type siderophore, commonly referred to as rhizobactin [10]. Cyanobacteria, in addition, fulfil a significant function in the synthesis of siderophores. These compounds encompass dihydroxy mate-type siderophores, namely schizokinen and Michelin H, which are ubiquitously present in diverse cyanobacterial species [11]. *Anabaena oryza*, a cyanobacterium of notable interest, is frequently encountered within the confines of paddy fields. The organism possesses the capacity to synthesise siderophores, which are intrinsic bioactive molecules facilitating the sequestration of cadmium metal ions [12].

1.2. Fungal Siderophore

Fungi have intracellular and extracellular siderophores which are responsible for transportation of ferric ions [13]. For the most part, it is generally agreed that fungi are capable of producing siderophores in two chemical classifications--carboxylates and hydroxamates. Large amounts of analytical work have been done to produce siderophores in *Aspergillus nidulans* and *Aspergillus fumigatus*, considering them as a group with 55 closely related variants. In the organism *Aspergillus nidulans*, one can observe secretion of both ferricrocin and ferrihordin [14]. In fact, *Laccaria laccata* and *L. bicolor* both

contain the siderophore linear fusigen (an ester-containing compound) in low quantities [15].

In addition, it is known that these fungi can produce compounds such as ferricrocin, coprogen, and triacetyl fusarinine. *C. Trichoderma* species generate coprogens and *Fusarium* varieties fusigen. There are also coprogens and fusigen, all of which belong to the family of hydroxamate-type siderophores [16], [17]. The results reveal that *T. harzianum* has a higher percentage of hydroxamate and carboxylate-type siderophores compared with more Trichoderma species such as *Trichoderma asperellum*, *Trichoderma viride* or *Trichoderma longibrachiatu* [18]. In addition, many different strains of *ericoid mycorrhizal* fungi (associated with fusigen) seem capable of producing Ferricrocin [19]. The ectendo-mycorrhizal fungus (ectomycorrhiza) is the little organism that enter into an entirely reciprocal association with plant roots. It has indeed been observed in the organisms *Wilcoxina rehmii* and the ectomycorrhizal fungus *Cenococcus geophilum* that they can synthesise siderophores, specifically ferricrocin. [20]. In addition, recent research has revealed a unique kind of siderophore- basidiochrome- found in fungi capable of establishing symbiotic relationships with the intricate root systems developed by orchids. These fungi are found only within the genera *Ceratobasidium* and *Rhizoctonia* [21].

1.3.Mammalian Siderophore

Studied in the FL5.12/EC- 24p3 murine pro-B lymphocyte cell lines, which are dependent upon interleukin-3 (IL - 3) [26], have been those mammalian siderophores that bear structural and functional similarities to bacterial siderophores [22]. It has been found that the lipocalin 24p3 proteins in mammals are capable of binding enterobactin. The

Enterobactin, on the other hand, has the ability to form a complex with iron ions intracellularly. The compound referred to as 2,5 DHBA, which is found within the biological organisms known as mammals, demonstrates comparable structural attributes to the iron-binding constituent of bacterial enterobactin, namely 2,3 DHBA. The mammalian siderophore exhibits the remarkable capability to form a binding interaction with 24p3, even without iron. This particular mechanism aids in the intricate process of binding trivalent iron (Fe (III)) and effectively encourages the uptake of said iron by the mitochondria [23].

The short-chain dehydrogenase reductase BDH2 has been identified as the human homolog of EntA protein. An enzyme of great importance, BDH2 playing a major role in catalysing its precursor within the human body 2,3 DHBA. It is, however, of great significance in the complex process of synthesizing 24p3-associated mammalian siderophores that are absolutely essential to their subsequent iron-carrying ability [22]. It has been suggested that cellular iron concentration increases as a result of BDH2 ablation within cells. In addition, this results in the breakdown of iron-regulated promoter 1 (IRP1) regulatory protein, increased concentrations of aconitase and ROS generation at an accelerated rate. The latter eventually leads to apoptosis caused by oxidative stresses. These outcomes indicate that BDH2 is an essential regulator of iron homeostasis.

1.4. Plant Siderophore

A class of compounds occurring frequently in plant species within the Poaceae botanical family is represented by phytosiderophore (PS). It belongs to a famous mugineic acid family and is indeed capable of forming complexes with iron (Fe-PS) [24]. This intricate structure is distinguished by the full complement of precisely six binding sites. The

siderophores actually have a pair of amine-N moieties, countered by two carboxylate-O groups and one alpha-hydroxycarboxylate site. As a result, the configuration produces an octahedral arrangement that results in secure coordination of the central iron (III) atom.

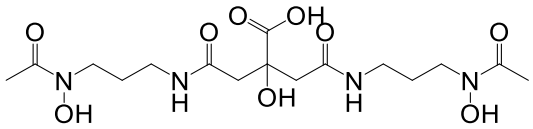
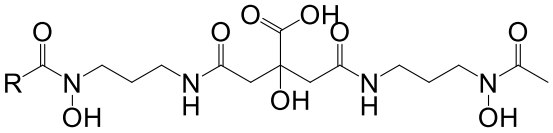
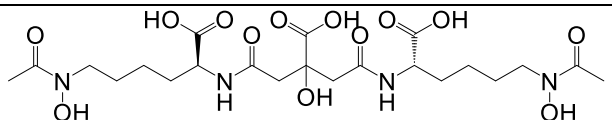
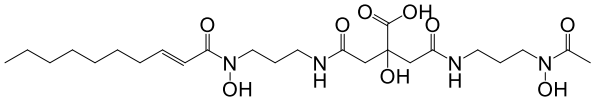
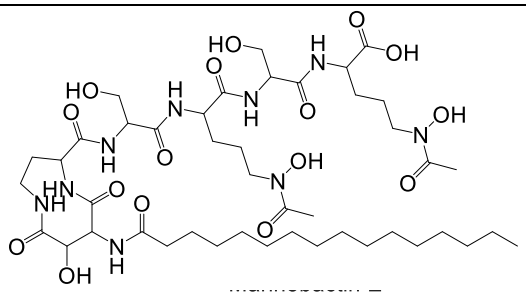
1.5. Hydroxamate Siderophore

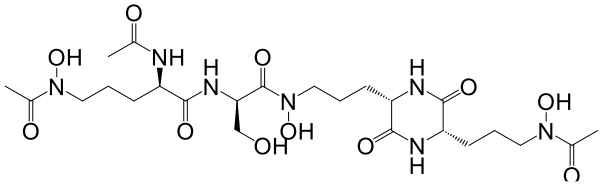
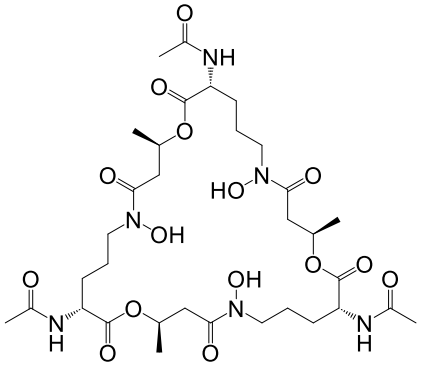
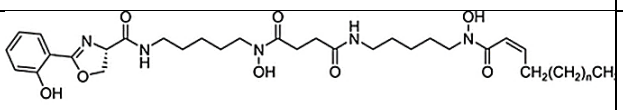
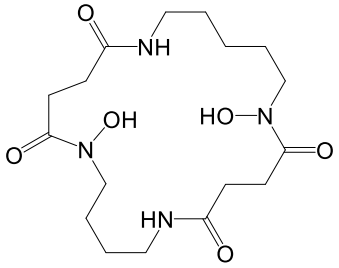
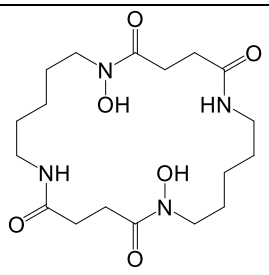
Among the most common class of siderophore found in the marine environment are hydroxamate-type ones, which contain alkylamine groups that have been modified through hydroxylation and acylation. Two hydroxamate siderophores that have been described in detail are schizokinen [25] and synechobactin [26]. Their structures are similar to aerobactin and rhizobactin 1021, which also fall into the dihydroxamate-type of siderophores [27]. First, in *Bacillus megaterium* (ATCC 19213), schizokinen, a citrate-containing dihydroxamate, was found. It is also produced by certain strains of *Anabaena* sp. in freshwater environments. Schizokinen's structure is formally condensed with the carboxy groups of citric acid at positions 1 and 3 as N-(3-aminopropyl)-N-hydroxyacetamide. The long-term stability of hydroxamate-type siderophores depends on various factors, including the number of hydroxamates, the denticity of the siderophore, and its structural features, whether it is linear or cyclic. It is worth mentioning that amphiphilic siderophores such as schizokinen and synechobactin demonstrate similar stability to non-amphiphilic siderophores (Table 4.1).

Siderophore-metal complexes have two main functions: to capture necessary metals from the surroundings or to reduce the harmful effects of excessive metals within cells. Based on the hard-soft acid-base theory, the interaction between metals and siderophores relies on the compatibility between hard oxygen atoms and soft metals like Zn (III) and Cu (II).

Hydroxamate siderophores, which are highly skilled at copper binding, show promise in preventing copper toxicity [28].

Table 4.1- Hydroxamate siderophores

S. No.	Siderophore	Structure	PubChem CID Identifier
7.	Schizokinen	 Schizokinen	3082425
8.	Synechobactin A, B, C, C9, C11, C13, C14 and C16	 Synechobactin, where R can be- A= $C_{11}H_{23}$ B=C_9H_{19} C=C_7H_{15} C11=$C_{10}H_{21}$ C13= $C_{12}H_{25}$ C14=$C_{13}H_{27}$ C16=$C_{15}H_{31}$	21778355-56-57
9.	Aerobactin		123762
10.	Rhizobactin		71581041
11.	Marinobactin E		21775723

12.	Erythrochelin		102171016
13.	Vicibactin		101941096
14.	Nocardichelins A and B	 Nocardichelin A (1): n = 11 Nocardichelin B (2): n = 9	[29]
15.	Avaroferrin		102234583
16.	Bisucaberin		133971

An example of this is when the hydroxamate siderophore derived from *Anabaena flos-aquae* hinders the development of *Chlamydomonas reinhardtii* through copper chelation, resulting in competition between microorganisms [30]. Heavy-metal sequestration is one of the broader applications of siderophore-metal complexes. It is worth mentioning that siderophores derived from *Synechococcus elongate* BDU 130911 have the ability to bind

with uranium, even when there is an abundance of iron. This suggests that these siderophores play a crucial role in detoxifying heavy metals. Likewise, *Anabaena oryzae* produces a hydroxamate-type siderophore which can chelate Cd2+ in rice-paddy fields when there is an overabundance of iron [12]. Their high metal binding functions have caught the attention of both pharmacy and medical science, with suggestions that they may be useful in relieving conditions where metals are imbalanced [31]. In addition, a number of recent research studies have studied and analysed siderophores produced by various mammalian cells [22].

A marine model organism of *Synechococcus sp*. PCC 7002, synechobactin is an amphiphilic hydroxamate siderophore [32]. It is the only known siderophore of its type whose structure has been characterised. Synechobactin has similar features to schizokinen. However, it differs by having a saturated fatty acid tail connected to one of its two alpha-hydroxamate groups. The length of the fatty acid trail differs, leading to the formation of synechobactin A (C_{12}), synechobactin B (C_{10}), and synechobactin C (C_8). The presence of an even number of carbons in the chain suggests that there is flexibility in incorporating fatty acid tails, which enables the existence of additional variations such as C_{11}, C_{13}, and so on, although they are not as frequently found.

The noteworthy characteristic of the synechobactin-iron complex is its photoreactivity, which is also observed in other siderophores based on alpha-hydroxy-carboxylic acid. Upon exposure to light, the iron-synechobactin A complex remains intact with its iron content, while the citrate component undergoes decarboxylation to produce 3-ketoglutarate [33]. In certain instances, the photoreduction of Fe^{3+} to Fe^{2+} is facilitated by siderophores derived from other bacteria. Although schizokinen lacks observed photoreactivity, it is functionally similar to siderophores like aerobactin in acquiring iron

from the surroundings. Further investigation is needed to determine the impact of synechobactin's photoreactivity on the import of siderophores into cyanobacterial cells, despite the structural similarities observed between schizokinen, aerobactin, and synechobactin.

Siderophores, as is commonly observed, have a propensity to form robust complexes with ferric ions (Fe (III)), thereby exhibiting notable thermodynamic formation constants (β). Tris-catecholate Enterobactin, for example, has a formation constant of up to 10^{49}, which is more than the formation constant of EDTA, a regularly used metal ion complexing agent, which is 10^{25}. The formation constants of Tris hydroxamate siderophores exhibit considerable significance as their values ascend to approximately 10^{30}. When ligands undergo complete deprotonation under fundamental conditions, it has been observed that catecholate siderophores demonstrate a higher affinity for Fe (III) when compared to hydroxamates. The observed range of log β values exhibits a notable magnitude of up to 19 units, implying that the catecholate-Fe complex's concentration surpasses that of the hydroxamate-Fe complex by 10^{19} under identical experimental circumstances [34].

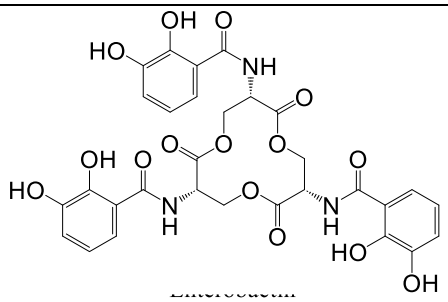
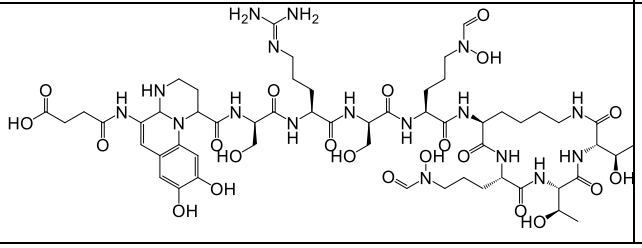
Several different methods can be used to detect hydroxamate-type siderophores. The Neilands spectrophotometric assay was first used [8]. In analytical chemistry, ESI-MS (electrospray ionisation mass spectrometry) is a technique frequently used to determine structures for hydroxamate siderophores.

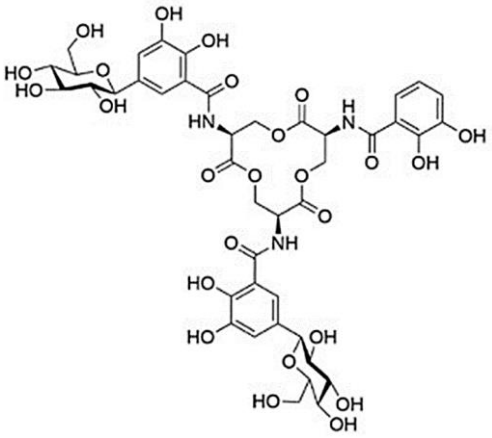
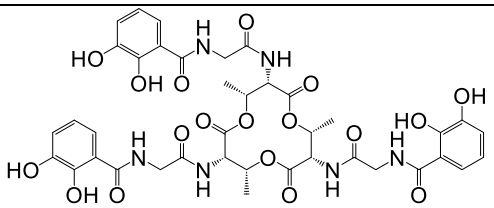
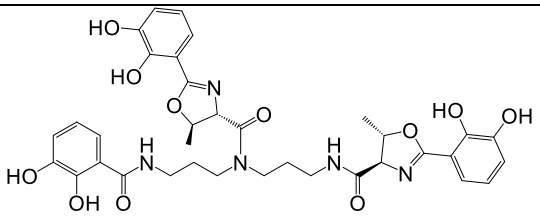
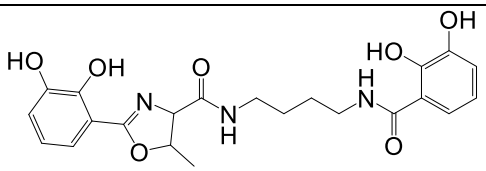
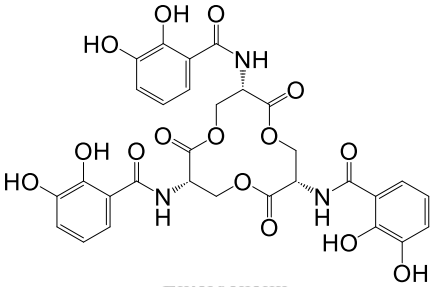
1.6. Catecholate Siderophore

The catecholate class and the hydromicoscinate subclass of siderophores are generated mainly by particular bacteria. It is the two oxygen atoms of the catecholate moiety that play a significant role in chelation with iron. As a result of this interaction, an octahedral

hexadentate complex is formed. For example, *Salmonella typhimuriumure*, *Klebsiella pneumoniae* and *Escherichia coli* are all bacteria capable of producing one specific compound named enterochelin [35]. These structures tend to demonstrate a strong affinity for ferric ion (Fe^{3+}), becoming one of its most firm and long-lived companions. However, this interaction has an abnormally high binding constant (K) of 1,052 M^{-1} because enterochelin's vigorous interaction with iron can be utilized to precisely determine even low levels of the element in many environmentally relevant samples. A group of bacteria exists that exclusively synthesise catecholate siderophores, whereas another group of bacteria is capable of producing mixed siderophores, wherein catecholate serves as one of the constituent components. It is interesting to note that *Erwinia carotovora* exclusively engages in the synthesis of catecholate siderophores, whereas certain *Pseudomonas* species exhibit the ability to produce mixed siderophores comprising both hydroxamates and catecholates (Table 4.2) [9].

Table 4.2- Catecholates Siderophore

S. No.	Siderophore	Structure	PubChem CID Identifier
3.	Enterobactin		34231
4.	Pyoverdine		5289234

5.	Salmochelins		[36]
6.	Bacillibactin		125349
7.	Vibriobactin		441167
8.	Photobactin		136778174
9.	Enterochelin		34231

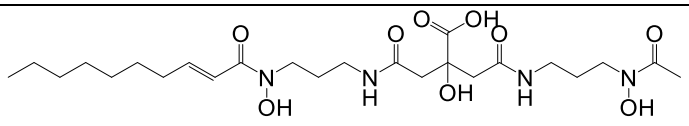
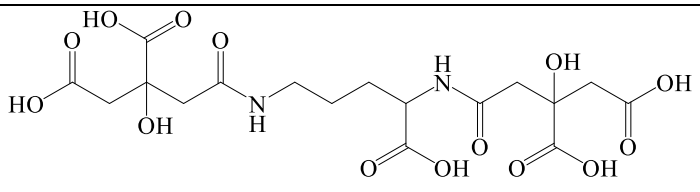
A multitude of methodologies exist at one's disposal for the purpose of discerning catecholate siderophores. The spectrophotometric assay by Neilands [8] is widely used in the field. In the present methodology, it is observed that the catecholate siderophore

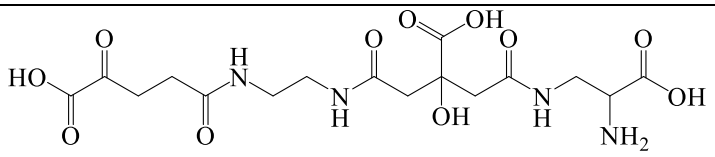
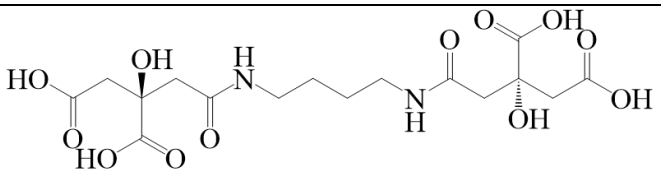
undergoes a reaction with $FeCl_3$, forming a complex exhibiting a distinct wine-coloured hue. This complex exhibits its maximum absorbance at a wavelength of 495 nm [8]. High performance liquid chromatography (HPLC) coupled with diode array detection (DAD) and electrospray ionization mass spectrometry (ESI-MS) can readily identify catecholates. Also, the O-CAS assay method developed for measuring catechol siderophores represents a valid and highly effective way of doing so [37].

1.7. Carboxylate Siderophore

Carboxylate-type of siderophores are primarily generated by *Staphylococcus* and *Rhizobium* microorganisms as well as *Mucorales* fungi. And through judicious use of hydroxyl and carboxyl functional groups, it shows a tendency to bind iron. The strand of *Rhizobium meliloti* DM4 is among the most noticeably recognised, for it produces a carefully studied carboxylate siderophore which has earned itself an appropriate name: Rhizobactin (Table 4.3).

Table 4.3- Carboxylates Siderophore

S. No.	Siderophore	Structure	PubChem CID Identifier
5.	Rhizobactin		71581041
6.	Staphyloferrin A		3035516

7.	Staphyloferrin B		46926216
8.	Rhizoferrin		197392

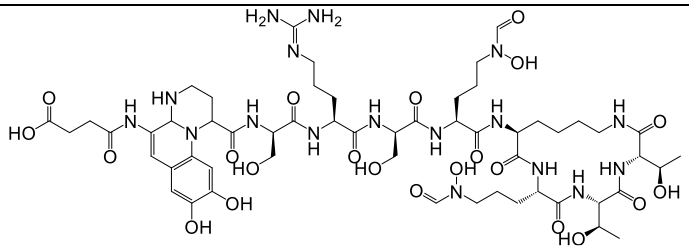
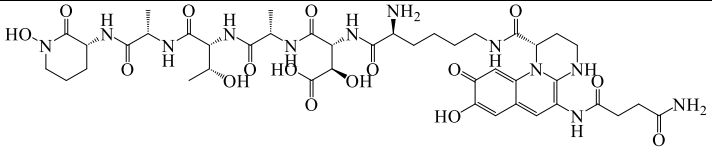
One representative example is rhizobactin. Its composition includes dihydroxylcarboxylic acid groups and ethylendiamine dicarboxylic acids. These groups are known for their outstanding efficiency at chelating iron. The carboxylate type siderophore *Staphyloferrin A* is renowned for its surprising degree of hydrophilicity. In the world of *S. hyicus* DSM 20459, microorganisms responded when their environment featured a decrease in iron levels. It is not only the one which produces it that benefits from this role [38].

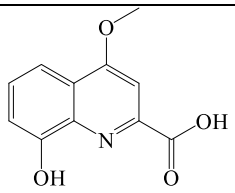
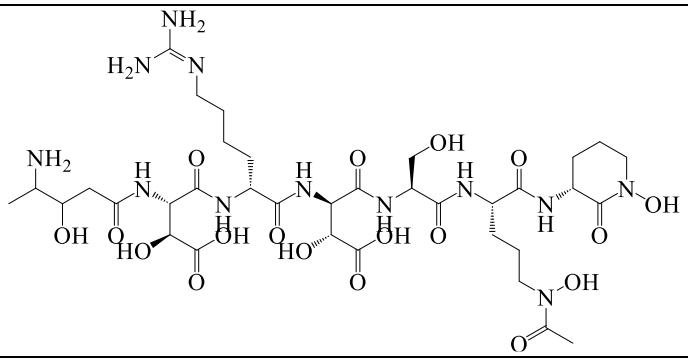
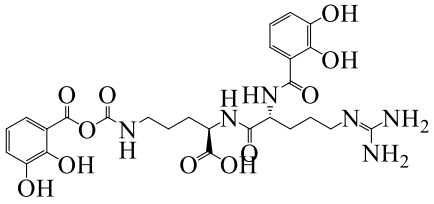
Thus, a spectrophotometric test can be used to detect carboxylate siderophores. This involves the preparation of a copper complex and its careful examination for absorption peaks, with wavelengths situated within the 190 to 280 nm range [39]. Again, the O-CAS assay is a reliable technique for distinguishing carboxylate siderophores. Using high-performance liquid chromatography and MS- mass spectrometry methods has led to recent breakthroughs that have improved the structural recognition of carboxylate-type siderophores [40], [41].

1.8. Mixed-Type Siderophore

Mixed-type siderophores (Table 4.4) are defined into this category because they possess multiple iron-chelating groups. *Pseudomonas* strains exhibiting inherent fluorescence can synthesise a diverse array of siderophores collectively referred to as pyoverdines [42], [43]. The dicarboxylic acid (or its corresponding amide) is linked to the chromophore, and the quinoline chromophore, a peptide, characterises the structure of all pyoverdines. While the peptide exhibits a certain level of constancy within bacteria belonging to the identical strain, it is plausible to observe variations when comparing diverse strains and species. As an illustrative instance, it has come to light that *Pseudomonas fluorescens* possesses the capability to produce three distinct variants of pyoverdines, specifically pyoverdine, pyoverdine A, and pyoverdine 0 (commonly referred to as ferribactin) [44]. Other species within the Pseudomonas genus, namely Pseudomonas syringae, possess the remarkable capability of synthesising pyoverdine siderophores. Moreover, it has been duly documented that the microorganism known as *Pseudomonas aureofaciens* exhibits the ability to synthesise pyoverdine siderophores [45].

Table 4.4- Mixed-Type Siderophores

S. No.	Siderophore	Structure	PubChem CID Identifier
6.	Pyoverdines		5289234
7.	Pseudobactin		5486206

8.	Quinolobactin		46910770
9.	Pacifibactin		145720824
10.	Mirubactin		59051490

The first single case of siderophore was obtained from the bacterium *Azotobacter vinelandii*, known as the famous azotobactin belonging to the pyoverdine type [46]. NMR techniques were used to establish the pyoverdine structure in *A. vinelandii*. The present analysis has exhibited some interesting optical characteristics: absorption at 380 nm and fluorescence at 500 nm [47]. Within Pseudomonas B_{10}, pseudobactin A and pseudobactin both possess the essential characteristic of having quinoline derivatives within their molecular structure [48]. There is broad consensus within the scientific community that *Pseudomonas fluorescens* has an unparalleled ability to produce many siderophores. Such agents include quinolobactin, enantio-pyochelin, pseudomonins and ornicorrugatin. *Pantoea eucalypti* M91 is a microorganism that can synthesise siderophores similar to pyochelin and pyoverdine under alkaline conditions [49].

The fact that the siderophores have antifungal properties is of great significance. A siderophore synthesised by many species of *Pseudomonas*, pyoverdine has received much attention in scientific research because its antifungal properties are particularly noteworthy. It promotes competition for this vital ingredient by the microorganisms around it so that pathogenic microbes will not be able to spread. This phenomenon results from the lower affinity for Fe (III) shown by fungal siderophores compared with their bacterial counterparts [50]. One significant discovery is that of an indispensable function found in *Pseudomonas fluorescens* WCS374r (Psb 374), which confers siderophore-mediated resistance on rice plants afflicted with *Magnaporthe oryzae* [51]. The prevailing consensus posits that pyoverdins are pivotal in the intricate biological regulation of phytopathogenic microorganisms within the rhizosphere. These compounds can establish stable complexes with iron that is present within the soil matrix. As a result, this renders the iron inaccessible to detrimental microorganisms that inhabit the rhizosphere, thereby impeding their capacity to utilise said iron [52].

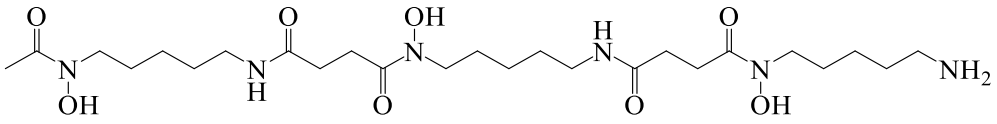
2. Chemistry of siderophore

It is widely acknowledged that siderophores exhibit greater affinities for iron (III) as compared to iron (II). The attempt to formulate ligands that demonstrate a preference for iron (II) over other noteworthy divalent cations of biological importance, such as copper (II), manganese (II), zinc (II), and nickel (II), poses a substantial and daunting obstacle. In contrast, the selectivity concerning iron (III) becomes more simplified due to the limited presence of biologically relevant trivalent cations, primarily inert cobalt (III). Therefore, one may deduce that a ligand possessing precision towards trivalent metals shall inevitably display an affinity for favouring iron in diverse biological conditions [53].

Although aluminium (III) is commonly present in water and soils, its ability to compete successfully with iron for siderophore binding is relatively limited. The observed difference in affinities displayed by most hexadentate siderophores can be linked to the significant difference in atomic radius between the iron (III) cation, which measures 0.65 Å and the aluminium (III) cation, which measures 0.54 Å. In particular, it has been observed that these siderophores exhibit a significantly greater affinity for iron (III) than for aluminium (III). To interpret, it is important to note that the logarithmic affinity constants associated with desferrioxamine B (Figure 4.2(A)) are documented as 30.6 and 21.4. The detailed investigation carried out explored the details of iron (III) and chelation characteristics [54].

Figure 4.2- Chemical Structure

A- Desferrioxamine B

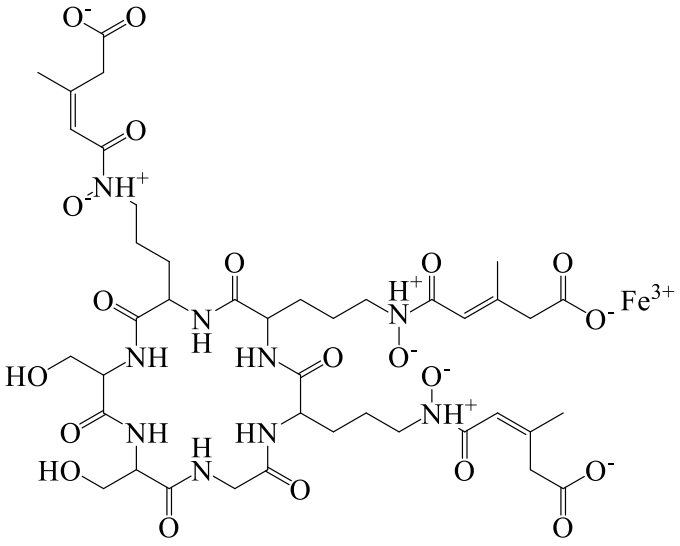


2.1. Iron (III) Selectivity

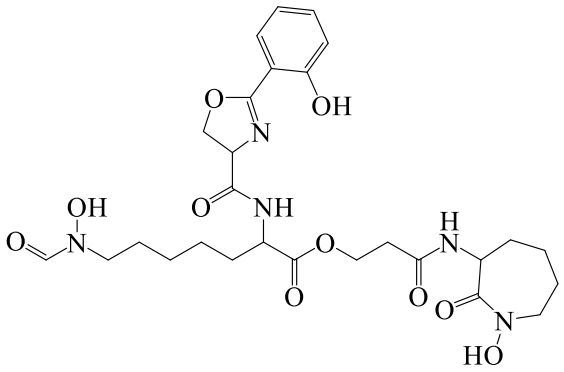
The molecules with a negative charge, specifically oxygen atoms, possess the most remarkable propensity for donating electrons to iron (III). The potency of the association between these entities escalates in direct proportion to the amplification of the charge magnitude of oxygen. Siderophores commonly use charged oxygen atoms as electron donors, with the prevalent geometric configuration being octahedral in nature. This configuration facilitates the envelopment of the iron centre by six ligands, effectively mitigating any potential repulsion among said ligands. In its inherent nature, the octahedral field fosters the formation of iron (III) species that exhibit high-spin

configurations, which possess a notable degree of thermodynamic stability. Certain siderophores possess the remarkable capability to modify the octahedral field by incorporating sulphur or nitrogen as donor atoms. Nevertheless, it is essential to note that these modifications generally decrease the binding capacity towards iron (III) ions. The structures of siderophores encompass three primary bidentate groups, specifically α-hydroxycarboxylate, hydroxamate, and catechol. These groups demonstrate an apparent predilection for iron (III). The rationale underlying the robust affinity of Catechol towards iron (III) can be attributed to the presence of its two ortho-phenolate moieties [54]. The charge density of these atoms is notably substantial, as indicated by the corresponding pKa values.

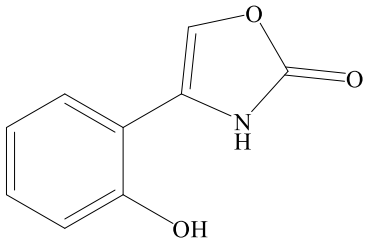
B- Ferrichrome A



C- Mycobactin



D- Hydroxyphenyloxazolone



Like the amide bond, the hydroxamate group exhibits a pair of resonance structures, whereby one yields a notable charge buildup carbonyl group positioned on the oxygen atom. Various modifications can be discerned in diverse siderophores, such as ferrichrome A (Figure 4.2 (B)) and mycobactin (Figure 4.2 (C)). The α-hydroxycarboxylate moiety, characterised by its elevated pKa value, demonstrates a pronounced affinity towards iron (III). The remarkable selectivity exhibited by the α-hydroxycarboxylate moiety towards iron (III) can be attributed to the inherent propensity of iron (III) to outcompete protons for binding to alkoxide functionalities, particularly when juxtaposed with other biologically relevant cations [55]. The pKa values of the hydroxyl function, precisely 10.05 and 11.3 in this particular instance, are effectively diminished due to the presence of intramolecular H-bonding within the conjugated anion, as exemplified in relation to rhizoferrin [56], [57]. Hydroxyphenyloxazolone (Figure 4.2 (D)) exhibits the ability to establish coordination bonds with iron (III) through the utilisation of the nitrogen atom

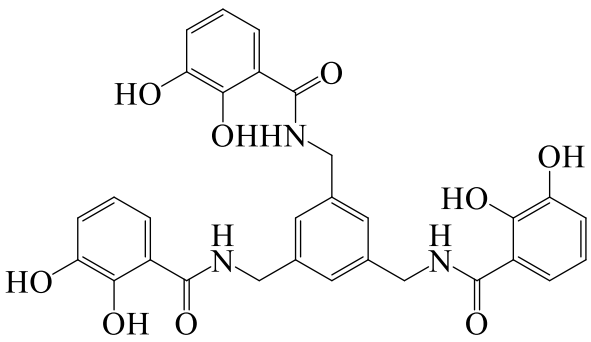
and the phenolate anion found within the oxazolone ring, parallel to its function in mycobactin [58].

2.2. Siderophore and Iron (III) Complex Stability

Determining the long-term stability of siderophore and Iron (III) complexes depends upon evaluating the energies associated with the donor-acceptor bonds. It is crucial to consider both the enthalpy and entropy contributions when assessing this stability. The coordinated movement of water molecules during the change to hexadentate complexes from tris (bidentate) elicits a discernible influence on entropy, which can be characterised as positive. The examination of the synthetic tricatechol ligand MECAM (Figure 4.3 (A)) and tris (N,N-dimethyl-2,3-dihydroxybenzamide) iron (III) yields a prominent observation, as evidenced by their individual formation constants of 40.2 and 45.9, respectively [59], [60].

Figure 4.3- Chemical Structure

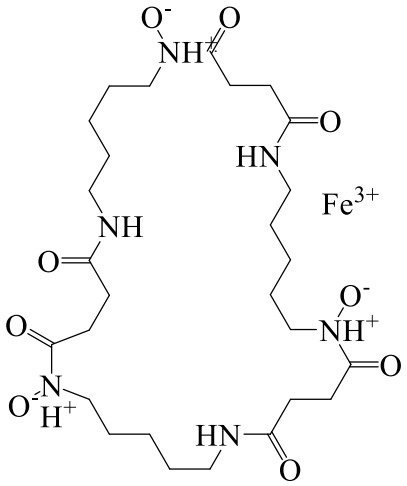
A- MECAM



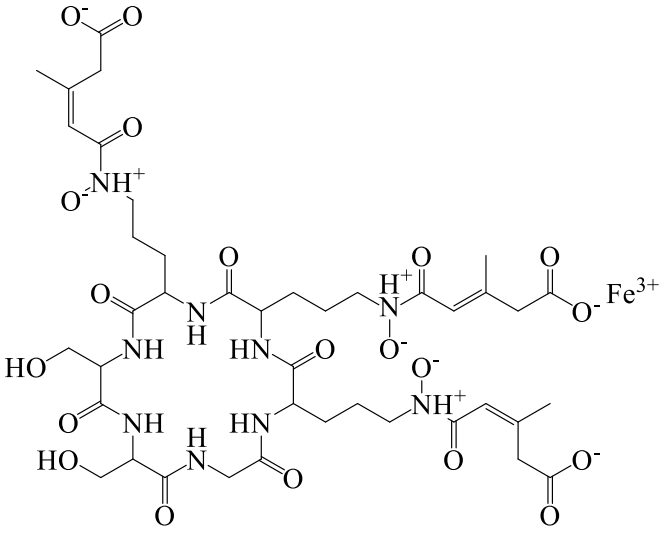
The hydroxamate ligands, too, demonstrate analogous patterns, albeit with a less pronounced differentiation. In the realm of chemical complexes, it is worth noting that ferrioxamine E (Figure 4.3 (B)) and tris (acetohydroxamic acid) exhibit log formation constants of 28.3 and 32.5, respectively. The formation of hexadentate complexes, while indeed resulting in a favourable increase in entropy, can also give rise to molecular strain,

thereby leading to unsuitable enthalpic contributions. This phenomenon has been duly noted in the case of ferrichrome (Figure 4.3 (C)), with a logarithmic stability constant (logKf) of 29.1 [60].

B- Ferrioxamine E



C- Ferrichrome



Certain hexadentate siderophores exhibit robust affinity for iron (III) owing to the inherent genetic predisposition of the ligand prior to its complexation with the ferric cation. Siderophores that possess six binding sites demonstrate a minimal rate of iron dissociation when subjected to a neutral pH environment, thereby facilitating a gradual

interchange of iron among two siderophores. The capacity of tri- and bidentate siderophores to experience kinetic modifications suggests their role in the solubilisation of iron and facilitation of its transfer to hexadentate siderophores, which exhibit lower reactivity. This intricate process ultimately enables the precise delivery of iron to the organism responsible for its production.

2.3. Siderophores' denticity

The shifts in entropy linked to the creation of complexes exhibit a more pronounced influence when employing hexadentate ligands, in contrast to bidentate ligands. Consequently, hexadentate ligands demonstrate an elevated affinity constant. The manifestation of this phenomenon is contingent upon the degree of concentration, specifically in the context of iron (III) complexes that have reached full maturation. Consequently, the values of [Fe] (and, by extension, the pFeIII values) are contingent upon the quantity of the unbound ligand, denoted as L. The degree of dependence can be classified as follows:

- Bidentate hydroxamate siderophores exhibit a reliance level of 3.
- Tetradentate hydroxamate siderophores demonstrate a reliance level of 1.5.
- Hexadentate hydroxamate siderophores display a reliance level of 1.

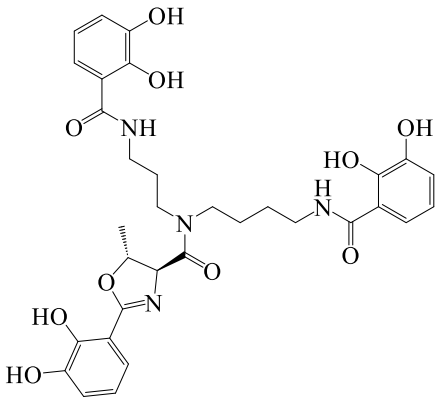
It is of considerable importance to note that the pFeIII value exhibits a noteworthy connection with the amounts of bidentate ligands [61]. This observation serves to underscore the advantageous scavenging capacity exhibited by hexadentate siderophores, particularly in instances where their concentrations are relatively diminished.

2.4. Geometry of Siderophore

Mononuclear complexes are commonly generated through the coordination of iron (III) with bidentate and hexadentate ligands. However, it is noteworthy that the utilisation of tri- and tetradentate ligands can lead to the coordination of iron (III) ions by facilitating the creation of bi-nuclear and multinuclear complexes [62]. The predominant form of siderophore encountered in nature is the hexadentate structure, known for its inclusion of catechol and hydroxamate functionalities affixed to both linear and cyclic frameworks. Including coordinating ligands within cyclic structures is of utmost importance, as these ligands exhibit a unique integration of coordinating properties.

Figure 4.4- Chemical Structure

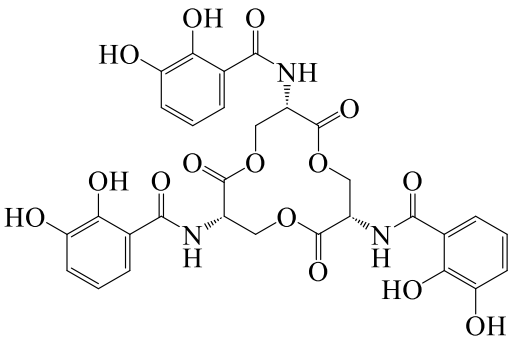
(A)Agrobactin



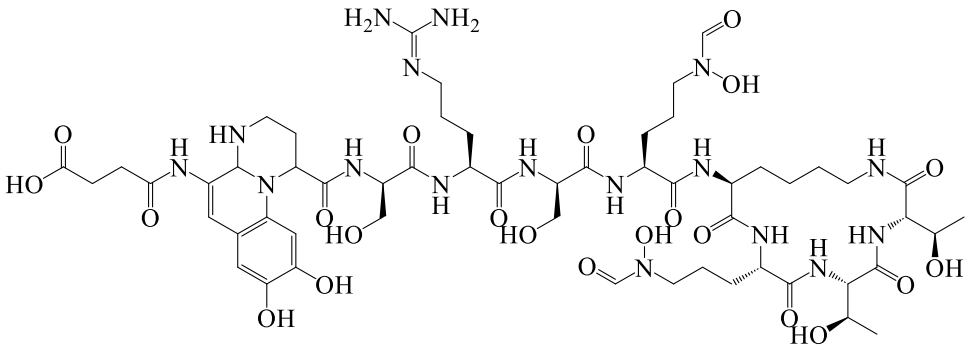
While the present text offers a constrained depiction of siderophore structures, it is worth noting that a diverse array of closely affiliated structures has been extensively documented in the scientific literature. These structures frequently arise from the same organism. In the context of chemical compounds, it is interesting to note that ferrioxamines boast a collection of 20 compounds that bear striking similarities. Similarly, agrobactin (Figure 4.4 (A)) possesses a trio of compounds with common

characteristics. On the other hand, desferriferrichromes showcase a assemblage of 21 compounds that exhibit resemblances [53].

(B).Enterobactin



(C).Pyovendins



Furthermore, Enterobactin (Figure 4.4 (B)) encompasses a set of 5 compounds that can be classified as similar. In a similar vein, mycobactin (Figure 4.2 (C)) manifest an array of 13 compounds that share commonalities. Lastly, it is worth noting that pyoverdins (Figure 4.4 (C)) present a compilation of 62 compounds that can be classified as similar. Some of these structures contain D- and L-amino acids in their peptide sequences, which are the most effective octahedral iron (III) binding sites and are resistant to lytic enzymes. The reason that siderophores are more stable is because the N- and C-terminal blocked peptides, as well as cyclic peptides, provide protection against their being broken down by lytic or peptidase enzyme activity. Moreover, some siderophores are amphiphilic as

they have one or two fatty acyl chains attached to the framework of the molecule. Siderophores possessing four coordinating atoms have the capacity to manifest themselves in both linear and cyclic configurations. Conversely, siderophores characterised by 2-3 coordinating atoms typically exhibit structures of lesser intricacy.

2.5. Iron (III)-Siderophore Complexes' Stereochemistry

The symmetrical bidentate ligand, like catechol, can form a tris-chelate, which could lead to the existence of an enantiomeric pair. When contemplating the properties of an asymmetric ligand, such as hydroxamate, it is imperative to acknowledge the potential isomers that develop. These isomers can be classified into two categories: geometric isomers, which include Δ-cis/trans and optical isomers. It is worth noting that stereochemical constraints frequently hinder the formation of cis isomers in various hexadentate hydroxamates. The stereochemical properties of certain hexadentate siderophores are indeed quite remarkable. Among these, ferrioxamines deserve special attention as they exhibit a unique characteristic across other recognised siderophores: a lack of optical activity. So, it is clear that this group of molecules does not have a clear preference for any one type of coordination propeller [58].

2.6. Iron (III)-Siderophore Redox Chemistry

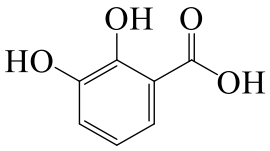
Due to their distinctive structural composition, siderophores exhibit a markedly heightened affinity towards iron (III) compared to iron (II). These attributes are of utmost significance in facilitating their capacity to selectively adhere to iron in the presence of other metallic elements within the biosphere. The degree of selectivity pertaining to iron (III) compared to iron (II) is intricately linked to the redox potential, whereby a negative potential signifies a heightened level of selectivity. A redox potential that exhibits a

significantly negative value, specifically below -400 mV, holds considerable significance due to its ability to effectively hinder the iron (III) complex from participating in redox-cycling activities. Consequently, the generation of deleterious hydroxyl radicals in oxygen-rich surroundings is circumvented. The utilisation of catechol-dependent siderophores is associated with the manifestation of highly unfavourable redox potentials. A clear correlation can be observed between the pFeIII values of these potentials. Bidentate iron complexes exhibit a comparatively less negative potential than their hexadentate counterparts [61]. When subjected to acidic conditions, it is possible for the redox potential to exhibit a decrease in negativity [63].

3. Biosynthesis of Siderophore

Synthesising a siderophore requires not only the correct assembly of suitable ligands, but they must also be inserted into much larger molecular structures which can firmly grasp onto free ferric ions. The procedure is to add different spacer groups into the structural framework and create an octahedral linking site. The biosynthetic binding of spacer groups and ligands occurs usually by way of amide or ester bonds. But before we consider the enzymes involved in catalysing these reactions, it is first necessary that our attention be turned to reviewing how different ferric-chelating groups evolved.

Figure 4.5- 2,3-dihydroxybenzoic acid



One of the precursors commonly used in the biosynthesis of siderophores is 2,3-dihydroxybenzoate (DHBA), whose structure contains catechol. DHBA, or 2-3-dihydroxybenzoic acid (Figure 4.5), is a product of the shikimate pathway. This

biosynthetic pathway is important to produce aromatic amino acids (AA) and other essential compounds in plants, bacteria, and fungi. In this pathway, chorismate—a crucial intermediate in the process—is an important branching point, so downstream products can be formed. From this fork comes DHBA, so it is an important molecule in the shikimate pathway. An enzyme known as isochorismate synthase has a key role in catalysing the reaction whereby oxygen atoms break away, leaving carbon behind. The process involves substitution by water at each position on chorismate via a 1,5-double SN2 displacement mechanism. In other words, the final product undergoes hydrolytic cleavage of its enolpyruvyl moiety. The moiety undergoes a later conversion process by an alcohol dehydrogenase to produce aromatic catechol [64]. Hydroxamate residues are frequently derived from the amino acid constituents of lysine or ornithine. In a biphasic procedure, a monooxygenase that relies on flavin adenine dinucleotide-FAD as a cofactor undertakes the oxidation of the amino group present in the side chain. The oxidation process leads to the generation of an intermediate species known as hydroxylamine. Following this, the hydroxylamine undergoes acylation through the catalytic action of an acyl-coenzyme A transferase. Upon careful comparison, one can discern that the procedure for synthesising α-hydroxycarboxylates exhibits a lesser degree of complexity. Citrate, which contains an α-hydroxycarboxylate group, is one of the compounds frequently employed in making siderophores. Its role in the biosynthesis of aerobactin provides an example. Furthermore, α-ketoglutarate can be converted and aspartic acid hydroxylated to supply it with alternative building blocks for the construction of its own α-hydroxycarboxylates [65], [66]. Salicylate is the most frequently occurring ligand in bacterial siderophores and usually combines with a nitrogen heterocycle agent, like

thiazoline or oxazoline. Except for one instance, the basis of salicylate is derived by way of eliminating pyruvate from isochorismate [67].

The synthesis of the siderophore backbone involves the assistance of two different types of enzymes: nonribosomal peptide synthetases (NRPS) and NRPS-independent siderophore (NIS) proteins distinguished by their large size and modular structure are nonribosomal peptide synthetases (NRPS). Such a structural feature falls entirely in the line of an enzymatic logic that is based on thiotemplate principles [68]. From a biochemical point of view, the modules within NRPS are clearly differentiated in terms of function. Each module attaches one amino acid to the developing peptide chain. The adenylation (A) domain plays the role of striction guard, carefully ensuring that only those perfectly matched substrates are selected to be integrated. ATP is used to get the proper amino acid underway and then forms a covalent bond with the resulting acyladenyate. The key docking site is in the peptidyl carrier protein (PCP). It needs to be phosphopantetheinyated in order to activate a thiol group capable of binding amino acids. Intrinsic chain elongation is the mechanism by which a condensation (C) domain causes an amide bond to be formed between the peptidyl-S-PCP component of products from the module there before and the aminoacyl S PCP structure in the current module [69]. For this subsequent attack, a receiver substrate (aminoacyl-S-PCP) is used to translocate the peptide chain. Once the condensation reaction is complete, peptidyl-S-PCP undergoes another transformation and becomes ready for a fresh cycle of elongation. The job is done by the C domain of that module mentioned above [68].

Supplementary domains included within NRPS modules increase the structural divergence in siderophores of NRPs origin. These additional domains cooperate with the necessary C, A and PCP domains to provide functionality to simple molecules. An

instance would be a methyltransferase (MT) domain or an epimerisation (E) domain, which changes the organisation of amino acid (AA) monomers situated on their carbon atoms at the alpha position. D-amino acids are essential in the increased proteolytic resistance of a peptidic siderophore, and they make an enormous contribution to conformational changes, which allows ferric ions to bind. The C domain may be replaced by a cyclisation (Cy) domain in specific modules that are partial to cysteine, serine and threonine. Formation of the amide bond Then, when the Cy domain, endowed with dual functionality herein, executes an intramolecular cyclodehydration mechanism, one obtains thiazoline or oxazoline rings. These heterocycles also undergo further redox modifications through their catalytic domains' reductase or oxidase activity. This is the final stage: another domain (a thioesterase or TE), now attached to an enzyme at a defined site, performs hydrolysis of the thioester-bound compound from this carrier. Another possible outcome is the release of a reductive chain [70].

Unlike the nonribosomal peptide synthetases (NRPS), NIS synthetases constitute independent enzymes, with existing intermediates serving as their substrates. Acyl adenylates are formed using an ATP-driven mechanism to convert a specific carboxylic acid compound. Despite similarities in their mechanisms for activating substrates, the visible structural differences between NIS synthetases and those of adenylation domains on NRPSs are apparent [71]. Examining sequences has provided insights indicating that NIS synthetases can be classified into three principal families, primarily distinguished by their predilections for carboxylic acid substrates. The enzymes of type A exhibit a specialisation towards the metabolism of citric acid, while those of type B are known to utilise α-ketogluaric acid.

4. Applications of Siderophores

Siderophores have remarkable versatility in that they become compounds capable of scavenging free iron within its environment. They play an essential part in regulating the balance of microbial micronutrient homeostasis. Humanity has made use of these molecular entities' natural potentials and exploited them to significant effect in various medical and non-medical applications. Progress in medicine should be noted: siderophores are now used routinely within clinical settings to treat iron overload disorders [69]. In this attempt, the main goal is to solve bacterial resistance and improve anti-infective therapy. Further research is needed to employ the siderophores as inhibitors of metalloenzymes, which are involved in all forms of disease pathology. Within the field of environmental studies, siderophores have numerous applications. These organisms have been demonstrated to be important in supporting the overall health and reproduction of fish and plants [69].

4.1. Role in Iron Chelation Therapy

Iron must be carefully regulated as an essential component of biological processes; otherwise, excessive accumulation causes serious health problems. The human body has the capacity to retain from 3 g (0.1 oz) up to as much as 4 g of iron in an estimated amount. This iron is mostly bound, associating with porphyrin in the heme-containing proteins like haemoglobin (blood) and myoglobin (muscle) or Fe-S complexes containing cysteineyl sulphur. The vital role of transferrin is to transport mobilised iron rapidly and efficiently from its storage sites in various tissues. The low solubility of iron presents an enormous obstacle to the control of homeostasis, which necessitates careful modulation of uptake by physiological losses [72]. Therefore, maintaining a routine flow of blood

infusions poses a great danger in promoting iron overload [73]. The redox-active non-transferrin bound plasma iron can act as a catalyst for the generation of reactive oxygen species through Fenton reactions. The ensuing cellular dysfunction and failure can cause significant organ damage, leading to death. Iron chelation therapy eliminates excess plasma-load iron and is frequently used to treat transfusion-dependent individuals [74].

Desferrioxamine B (DFO) is a siderophore (hydroxamate-based) and the first technique used in the treatment of iron overload, which was employed extensively since the 1960s [75]. Furthermore, it has been included in the World Health Organisation's (WHO) list of its most essential medications. Because the oral bioavailability of DFO is low, it usually has to be given intravenously or subcutaneously. It has a short plasma half-life, requiring frequent daily dosing [74]. Therefore, there have been significant strides toward enhancing pharmaceutical agents for clinical use. In particular, deferasirox (DFX) and deferiprone (DFP) provide good examples. Both synthetic iron chelators are taken orally, and their half-life is longer than that of DFO [74]. Additionally, the cellular permeability has been enhanced. It should be noted, however, that the combined treatment approach using DFO and DFP has already been proven superior to either therapy alone. This integrated strategy has brought patient mortality rates down dramatically [76]. It is postulated that this hypothetical synergistic effect involves a shuttle mechanism, in which DFP promotes the elimination of intracellular iron only for it to be transported by DFO on exit from cells first into and then out of urine or faeces [77]. Deferitazole, a novel iron-chelator, is undergoing phase 1 and 2 clinical trials [78]. It is believed to have the potential for a wider therapeutic window compared to the existing iron chelators, DFP and DFX. Significantly, the structure of deferitazole was derived from the naturally existing siderophore desferrithiocin [79], [80].

4.2. Role as an Antibiotic Carrier

Sideromycin can be classified as a group of antibiotic conjugates wherein the active compound is chemically bonded to a siderophore. This refers to the utilisation of naturally occurring transporter systems by siderophores, thereby facilitating their ingress into bacterial cells [81]. Instances of naturally transpiring sideromycin. Those compounds employ an intelligent delivery system, commonly known as a Trojan horse strategy, to significantly decrease the concentrations of inhibition in antibiotics [82], [83]. The process of antibiotic cleavage from the siderophore moiety upon uptake is of paramount importance for its activation. The inherent benefit of this particular characteristic, combined with its capacity to surmount bacterial resistance, has been a driving force behind the synthetic development of novel antibiotics utilising siderophores as a foundation.

The primary emphasis lies in the novel synthesis of sideromycin, which involves an unmatched fusion of antibiotic and siderophore components. The employed methodology enables the advancement of targeted therapeutic agents by incorporating carefully chosen antibiotics and siderophores specific to the pathogen as carrier moieties. A special case where the mycobactin–artemisinin conjugate [84], a synthetic derivative of mycobactin T, secreted by *M. tuberculosis*, facilitates targeted entry for the pathogen while functioning as the antibiotic pharmacophore [85]. When these factors come together, they effectively stop the growth of *M. tuberculosis* because they make it easier for iron to get to and from the cells of the mycobacterium.

In the context of bacterial resistance, scholars have investigated the potential of siderophore–drug conjugates as a therapeutic approach for managing *Pseudomonas*

aeruginosa infections. Using natural siderophores derived from Pseudomonas aeruginosa, specifically pyoverdines, as carriers facilitates the application of antibiotics, such as ampicillin, to combat this bacterium that would otherwise exhibit resistance [86]. The development of successful conjugates was achieved through the covalent linkage of ampicillin and amoxicillin with a constructed tris-catecholate siderophore, which imitated Enterobactin [87]. These conjugates demonstrate interesting *in vitro* efficacies against diverse strains of *Pseudomonas aeruginosa* [88].

Despite the presence of promising exemplars, it is essential to acknowledge the existence of various challenges. Certain artificial sideromycins exhibit reduced activity compared to unbound antibiotics, potentially as a result of modified interactions with transporters, altered iron transportation into the bacterial periplasm, or inadequate release of antibiotics upon cellular uptake [82], [89]. Moreover, the expeditious emergence of resistance presents a cause for concern. In order to tackle this issue, there has been a proposal to develop siderophore-antibiotic conjugates that incorporate mixed ligand groups. This approach aims to counteract the development of resistance caused by transport-related mechanisms by enabling the entry of these conjugates into bacterial cells through various outer membrane transporters [90]. Although sideromycins have not been implemented in clinical practice, two β-lactam antibiotics, namely S-649266 and BAL30072, have undergone clinical trials with Siderophore-conjugated properties. S-649266 has advanced to phase III [91], while BAL30072 has progressed to phase I [92]. These advancements serve to underscore the considerable potential of sideromycins in the field of clinical applications.

4.3. Role as Inhibitors of Metalloenzymes

Metalloproteins comprise approximately 50% of the total population of naturally occurring proteins and exhibit indispensable functions. Alteration in their normal functioning has been associated with pathological conditions such as infections, inflammation, and cancer [93]. Metalloprotein inhibitors frequently employ metal binding groups, as demonstrated by the inclusion of such groups in FDA-approved pharmaceuticals like vorinostat [94] and zileuton [95]. Due to their wide range of structural variations and ability to selectively bind to Fe^{3+} ions, Siderophores have emerged as highly promising contenders for the development of pharmaceutical agents aimed at modulating the activity of metalloproteins.

Matrix metalloproteinases (MMPs), enzymes containing zinc and playing a key role in various cellular procedures, have garnered significant attention as potential therapeutic targets owing to their implication in cancer and inflammatory diseases [96], [97]. Microbial siderophores, such as DFO, rhodotorulic acid, and ferrichrome have been found to inhibit the activity of matrix metalloproteinase-2 (MMP-2) [98]. This is especially true in the cases of tumour cell invasion and periodontitis. This inhibitory effect of MMP-2 was also observed in pyoverdine-type siderophores produced by several strains from the genus *Pseudomonas* [98]. Another antimetastatic siderophore, Myxochelin A, has been shown to have anti-tumour effects by blocking the two enzymes, MMP-2 and MMP-9, that promote metastasis [99].

4.4.Role in Soil Bioremediation

Siderophore-assisted bioremediation is a phenomenon that has attracted much attention from researchers due to its impressive potential capabilities in cleaning up contaminated

soils [100]. Many methods have been devised, exploiting the ability of many siderophores to show metal-binding characteristics beyond iron. Some bacterial species can synthesize siderophores in the presence of different heavy metals and judge from this their ability to dissolve and make those same metals bioavailable. Technical applications using bioreactors have made it possible to capitalise on this capability and to extract and separate solubilised heavy metals from the soil matrix [101]. An excellent example of this is provided by *Cupriavidus metallidurans* [101], which has the capability to produce staphyloferrin B, a citrate siderophore [101]. This microorganism has been shown to reduce significantly the heavy metal concentration in soil samples treated in a bioreactor. This notable success presents robust evidence that the potential for siderophores in soil remediation is enormous [102], [103].

Soil remediation techniques like excavation used in the past have been shown to be effective, but they are not environmentally friendly or economical. In soil remediation, the diffuse bioaugmentation techniques are both feasible and less invasive substitutes for *in situ* bioremediation. The current approach involves the intentional application of microorganisms to soil that has been polluted with metals, considering aspects such as their ability to resist metals, whether they can grow in contaminated soils, and how effective they are in mobilising metal ions [104]. Thus, the encapsulation of cells within beads provides increased resilience against potential competitors and predators. With its more considerable survival ability (ability to process metals), it is better able to protect itself from danger in various forms than living organisms or animal life can do. Combining bioaugmentation and phytoextraction has been highly influential in cleaning soil polluted with pollutants [104]. The siderophore-producing rhizosphere bacteria,

which help plants grow and accumulate metals within the context of this integrated methodology, play a key role [105].

Siderophores are critical for enhancing the efficiency of phytoremediation, because they promote plant biomass and facilitate concentrations of heavy metals in plant tissues [106]. Rhizosphere bacteria with the ability to produce siderophores play a crucial role in increasing iron availability to plants. These, thus, can effectively fulfill plant requirements for iron where heavy metals have polluted soils. Moreover, these siderophores can also interact with other metallic elements besides iron and increase the total metal uptake by plants [107]. Plant biomass increases because siderophore-producing rhizosphere bacteria indirectly affect plant growth processes like reducing ethylene and oxidative stress. The good solubility of heavy metals, the effectual transportation into plants via siderophores and a reduction in metal concentrations inside the soil environment all show their enormous potential for use within phytoremediation strategies.

4.5. Role in Removing Petroleum Hydrocarbon from Marine Environment

When hydrocarbons are introduced into marine ecosystems, it creates an environmental dilemma, requiring the implementation of effective measures to cope with petroleum contamination in oceanic areas. Consequently, bioremediation is now a viable and practical means to abate the adverse consequences of oil pollution in marine settings [108]. Siderophores assume a pivotal function in the process of hydrocarbon biodegradation by facilitating the provision of iron to the microorganisms actively engaged in said process [109]. The pace of hydrocarbon degradation is directly influenced by the supply of essential nutrients, specifically nitrogen, phosphorus, and iron. Numerous studies consistently underscore the augmented iron demands exhibited by

hydrocarbon-degrading bacteria, specifically *Alcanivorax borkumensis*, in instances where hydrocarbons are utilised as the primary carbon source [110]. The enzymes responsible for the oxidation of alkanes, particularly AlkB, are essential in the oxidation route of medium and long-chain alkanes. These enzymes depend on iron, which helps explain the increased demand for iron during the degradation of hydrocarbons [111], [112].

The presence of hydrocarbons exerts a substantial influence on the composition and organisation of the microbial community within the impacted regions. Studies conducted after the occurrence of the Deepwater Horizon oil rig explosion 2010 have brought to light a discernible decrease in the microbial diversity within the oil plume [113]. The primary reason for this is that these belong to species which have a high level of skill in utilising alkanes. An analysis of the metagenomic dataset showed that not only is n-alkane degradation complete with all genetic elements related to iron acquisition present and adequately ordered, but its relative abundance was also among the highest. An abundant microorganism of aquatic environments polluted with oil, *Alcanivorax borkumensis* [114], is capable of synthesising amphibactins and pseudomonines [115]. These compounds, known as siderophores, are essential in maintaining the efficacy of degrading hydrocarbons by helping them handle limited supplies of iron [115]. In particular, *Marinobacter hydrocarbonoclasticus* (a microorganism famous for its ability to degrade oils) can synthesise petrobactin and petrobactin sulfonate, two compounds essential to oil-degrading abilities which have been documented eight or nine times from the sea floor [116], [117]. Some *Vibrio* species which use hydrocarbons have been found to produce siderophores known as amphibactins, possessing an inherent character of being amphiphilic and may function as biosurfactants [109]. It is supposed that siderophores

can contribute to the degradation of alkanes in two different ways. Firstly, they are able to help microorganisms which catabolise hydrocarbons by making it easier for them to acquire iron. Second, because this type of compound has pore-forming properties and enhances emulsification oil dissolution at the producer level, it increases.

4.6. Role as Biocontrol of Algal Blooms

Regarding environmental application, it is generally understood that siderophores can be used to control the problem of toxic algal blooms. The proliferation of harmful algae can be effectively impeded through the sequestration of iron by siderophores. A study investigates the effects of microbial siderophores, specifically ferrioxamine and ferrichrome, on the growth pattern of thirteen different species of red tide algae that are known to be highly prevalent [118]. The efficacy of Fe-ferrichrome was found to be lacking across all species examined. Notably, only a limited subset of 3 species demonstrated discernible growth when ferrioxamine complexed with Fe. While certain microalgae, namely *Skeletonema costatum* and *Chattonella antiqua*, have been observed to synthesise their own iron-complexing ligands, the extent to which picoeukaryotic algae, such as *Ostreococcus*, engage in the utilisation of microbial siderophores remains unconfirmed [119], [120]. In diatoms, such as *Thalassiosira*, the mechanism of reductive iron removal exhibits similarities to that observed in Saccharomyces cerevisiae [121]. However, the presence of siderophores induces a gradual uptake of iron, which may consequently result in the imposition of iron limitation on eukaryotic algae [122].

Conversely, utilising photoreactive siderophores, possessing an α-hydroxycarboxylate moiety, augment the bioaccessibility of iron within the microbial consortium [69]. These molecules in various freshwater, marine, and soil ecosystems demonstrate interesting

photoreactive characteristics [123]. Upon exposure to solar radiation, the Fe^{3+}-siderophore complex experiences expeditious photooxidative cleavage, resulting in the liberation of ferrous iron into the surrounding milieu. With the reciprocal exchange of resources such as iron and carbon, phytoplankton can form cooperative relations with marine bacteria. This intriguing phenomenon indicates that photoreactive siderophores could play a crucially defining role in shaping the composition of microbial communities and their dynamics within this ecological setting. The suggestion is made to test whether a specific bacterial strain can produce siderophores and has the potential as a biocontrol agent against algal blooms. Nonetheless, carefully evaluating the complex factors and possible repercussions of introducing siderophores prior to any practical applications is essential [124].

4.7. Role as a Plant Growth Promoter

This has been very often discussed in view of phytoremediation and the role that siderophore-producing rhizosphere bacteria play in stimulating plant growth. The bacteria here function as biofertilisers through the provision of iron to plants, a correlation substantiated by a multitude of empirical investigations [125]. A study revealed that maize's and sunflowers' iron nutrition was enhanced when cultivated in non-sterile environments [126]. This improvement was attributed to the presence of bacterial siderophores. Furthermore, it has been reported that the application of *Pseudomonas* strain GRP_3 resulted in the alleviation of chlorotic symptoms and a subsequent increase in chlorophyll levels in mung beans [50]. Significantly, another study has developed a strain of *Pseudomonas fluorescens* ATCC 13525 that exhibits an overproduction of siderophores [127]. Furthermore, this particular mutant demonstrated a notable capacity

for cold resistance, thereby augmenting its viability for utilisation in agricultural contexts within distinct climatic conditions.

The efficacy of biopesticides [125] derived from siderophore-producing rhizosphere bacteria is enhanced by their ability to exclude plant pathogens via the mechanism of iron deprivation competitively [128], [129]. This biocontrol mechanism is substantiated by a multitude of studies. The research made an observation regarding the impact of introducing a *Pseudomonas* strain or its siderophore into soil that is conducive to disease. They found that this intervention resulted in a transformation of the soil, rendering it disease-suppressive in opposition to plant pathogenic fungi [128]. The disease-suppressive effect exhibited a decrease in magnitude upon the introduction of iron additions, thereby highlighting the criticality of restricted siderophore production. Investigations involving mutants of *Pseudomonas sp.* having altered siderophore production have revealed a remarkable link between more significant amounts of it and improved biocontrol activity against *Ralstonia solanacearum*, the bacterium responsible for wilt disease [130].

Siderophore-producing bacterial strains, namely *Pseudomonas fluorescens* LPK2 and *Sinorhizobium fredii* KCC5, which were obtained from the rhizosphere, exhibited notable growth-inhibitory properties against the plant pathogenic fungus *Fusarium udum* [131]. Although the suppression of wilt disease by siderophore knock-out mutants of *Pseudomonas putida* WCS358 suggests the presence of other plant protection mechanisms, it is essential to note that siderophores continue to be a viable and economically viable approach for promoting plant growth and disease prevention [132]. The proposition is to integrate diverse biocontrol mechanisms with siderophores in order to achieve maximum effectiveness, taking into account various factors such as pH, which

plays a significant role in the siderophore-induced iron deprivation of pathogens [129]. The inherent environmentally conscious and economically advantageous characteristics of siderophores establish them as invaluable instruments for augmenting botanical proliferation and ameliorating botanical ailments, with prospective enhancements attainable via the optimisation of siderophore synthesis in the soil through the utilisation of diverse mineral and carbon substrates [133].

4.8. Role as Biocontroller for Aquatic Pathogens

The escalation in fishing practices in recent periods has been observed to concurrently align with a surge in disease outbreaks instigated by diverse pathogens [134]. In response to this matter, probiotics have emerged as a viable and gentle modality for the prevention of diseases. The principal mechanism of action employed by probiotic bacteria entails the synthesis and secretion of siderophores, thereby inducing iron sequestration and subsequent deprivation for pathogenic microorganisms. Iron plays a key role in the pathogenicity along with intercellular communication of various pathogens [135].

Many of the intensive efforts in searching for suitable probiotic partners to employ have already brought together to be potential. *Pseudomonas* strains, closely related but with different mechanisms of action when confronted by the pathogenic *Flavobacterium psychrophilum* have been observed in another study [136], [137]. The mechanisms included siderophore production and immunostimulation on fish. Under these circumstances, *Bacillus cereus* was shown to effectively exhibit competitive exclusion against the pathogenic *Aeromonas hydrophilia* and cause a state of iron deprivation within the pathogen. In aquaculture, bacillus species JB-1, *Aeromonas sobria* and the *Pseudoaltermonons* have been shown to possess good potential as siderophore producers

[138]. Not surprisingly, some potential pathogens toxic to fish are able to take iron from their victims through siderophores and therefore create a more serious challenge for probiotic exclusion.

Fish pathogens have thus been found to contain genes coding for siderophore biosynthesis and transport on transmissible plasmids [139]. This phenomenon has been observed to encourage the proliferation of these pathogens. The horizontal transfer of genes can add complications to the exclusion of pathogens by probiotics [140]. Many variables are involved in the efficacy of probiotic bacteria as a way to protect fish from disease. These include factors such as affinity constants for rival siderophores and possible other paths for action. Using probiotics in aquaculture displays a competitive edge when compared to the addition of vaccines or antibiotics. This discernible advantage has been critical to the increasingly widespread use of probiotics within this field.

4.9. Role as an agent to recover rare earth metals

The rare earth elements (REEs), among which the 15 lanthanides also belong, have many properties unique in all material science. As requirements increase from a variety of advanced technological fields such as computing, catalysis and renewable energy, these unusual characteristics are coming into play more than ever before [141]. The mining of rare earth elements (REEs), with a focus on the region around Baotou in Inner Mongolia, is mainly at China's mercy and under its control [142]. However, the conventional extraction procedures, which rely on chemical leaching, lack specificity and cause severe environmental pollution. Siderophores represent an entirely novel and green approach for extracting rare earth elements (REEs) [143], [144]. Thus far, investigations have focused on a widespread siderophore desferrioxamine where they hope to measure its binding

affinity towards rare earth elements (REEs) [144]. The effectiveness of desferrioxamine in the leaching process was proved, with mobilisation rates coming to 60 % for lithium and 40 % for molybdenum, respectively. In general, the stability of REE-desferrioxamine is high. However, it must be remembered that interferences may arise from competing cations such as Fe^{3+} when complexes are formed. Tweaking specific parameters, such as concentration of H^{+}, temperature and the selected or useable siderophores, can dramatically improve both selectivity and efficiency. The critical and intricate matter of metal separation within the leach liquor requires further investigation. Its practical application depends greatly on how this is handled [144].

5. Siderophore and cell death

The complicated relationship between iron and cancer explains why there are so many different variations in how iron is regulated or metabolized across various cancer types. Health status and the associated disorders significantly affect physiological iron metabolism, particularly concerning its role in cancer development [145]. In cases of solid neoplasms locally confined at the site of malignancy, iron accumulation is often seen. The transcriptomic analyses thus far have been aimed mainly toward tissues, but some interesting points to note include the existence of mitochondrial and cytosolic Fenton reactions in various cancer types. These reactions are due to excess ferrous iron (Fe^{2+}) and hydrogen peroxide (H_2O_2) [146]. A variety of interesting phenomena, such as cytosolic Fenton reactions with their proposed consequences, are included in the suggested outcomes [147], [148]. These include the possibility of raising intracellular pH, enhancing nucleotide synthesis and glycolytic ATP generation, and producing highly reactive hydroxyl radicals (•OH). Such intermediates are thought to contribute intimately to kick-starting and perpetuating inflammatory responses, metabolic aberrations,

disruption of signalling pathways associated with cell death or survival, and impairment of the integrity of lipid species within biological membranes [148]

Colorectal cancer, hepatocellular carcinoma, breast cancer, and ovarian cancer are characterised by elevated levels of transferrin receptors and aberrant expression of various proteins associated with iron transport [149]. Through the strategic manipulation of various variables, such as the inhibition of the transferrin receptor or the reduction of intracellular iron levels, notable advancements have been observed in the field of mitigating the proliferation of malignant cells, impeding the expansion of tumours, and diminishing the incidence of metastatic events. On the contrary, it is worth noting that a positive correlation exists between elevated levels of iron within cellular structures and heightened expansion in colorectal cancer cells [150]

Iron intake can impact the fortunes of sinister cells by altering iron availability to them, enhancing the uptake of that metal into microorganisms through siderophores, changing the composition of gastrointestinal flora, and creating higher levels of metal toxicity [151]. As a result, the iron fortification process enhances enterobacteria at the expense of *bifidobacteria* and *lactobacilli*. The density of harmful *Escherichia coli* also becomes elevated within children's microbiota [152]. Siderophore and dietary iron are emphasised as highly needed for bacterial proliferation, which may be associated with the onset of cancer.

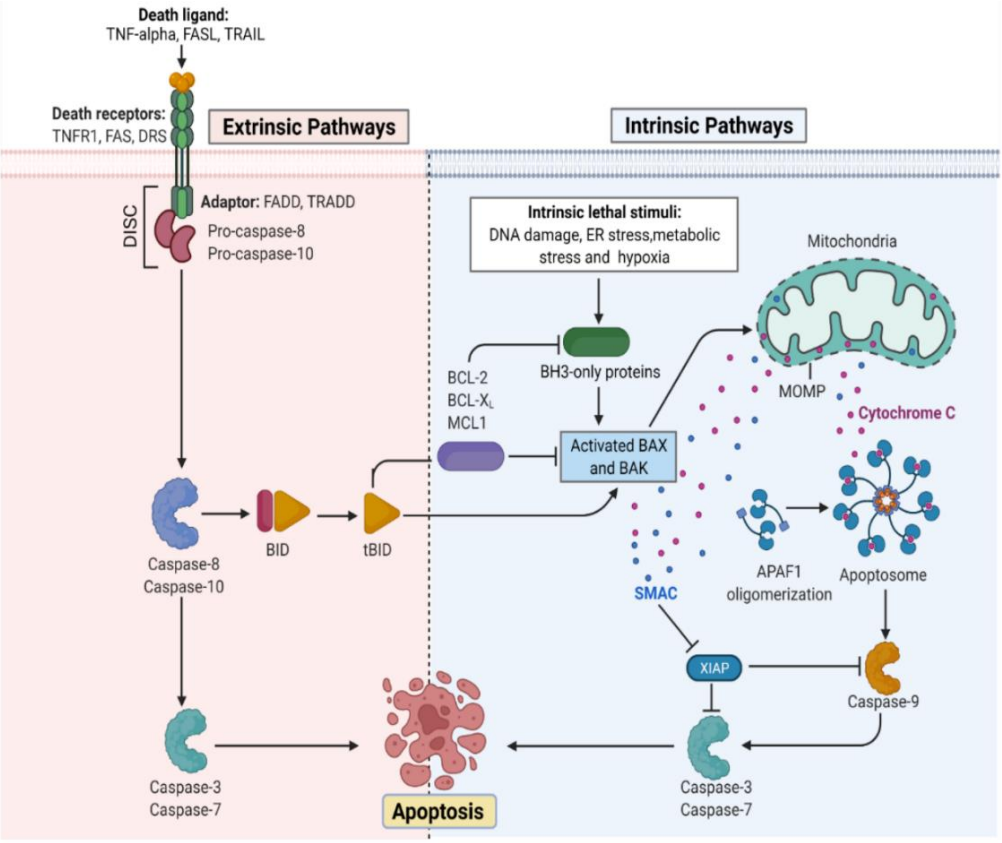
5.1. Siderophore and Apoptosis

PCD, or programmed cell death (also known as apoptosis, cellular suicide), is a central mechanism for maintaining the homoeostasis of cells and enabling their survival in multicellular organisms. It profoundly affects all kinds of different cellular metabolisms,

including the immune response to viral pathogens and histological changes accompanying embryonic organogenesis. Because apoptosis is a highly complex, tightly regulated form of programmed cell death capable at once of both preventing cells from going awry and promoting cancer progression (not to mention its role in the development of neurodegenerative disorders), it has been implicated as one pathogenic factor or another. Oversight of the process by which cells die is essential for their survival. Research conducted in the mid-1980s has provided strong evidence in favour of this idea that cellular death is not passive and indeed involves many factors highly regulated by genes.

In contrast to other forms of cell death, apoptosis is a methodical process which preserves the structure and integrity of the plasma membrane. Apoptosis (Figure 4.6) itself was later discovered in several different species, from yeast through to rats and humans. The process differs from other types of cell death called necrosis, where the breakdown of the plasma membrane is much more severe and often activates inflammatory responses. The phenomenon of necrosis will only appear in certain extreme circumstances, such as injury from stroke-like events or the presence of harmful substances. In consequence, the plasma membrane is disturbed. Apoptosis is one of the most fundamental biological phenomena, representing a typical, essentially intrinsic mechanism for programmed cell death in humans.

Figure 4.6- The multiple routes of programmed cell death. Reproduced from Wani *et al.* [153].



The insight of siderophores within neoplastic growths and the microbial community inhabiting bodies afflicted with malignancies have not been provided with substantial scientific proof. A preliminary inquiry [154] was undertaken with the purpose of evaluating whether considering the sputum microbiome could be a means to diagnose and monitor the progression of lung cancer. The results indicate that, in fact, four people from within this cohort were indeed diagnosed as having lung cancer over a one-year period; on the other hand, six others did not develop any characteristics suggestive of cancer [154]. The main purpose of the research was to establish the variations in bacterial species composition between subjects who had manifested lung cancer and those who had not. Between the two cohorts, seven different bacterial species were found to be expected. In

particular, within this set, five of these species demonstrated remarkable increases in abundance among those diagnosed with lung cancer. One of these species, *Streptococcus viridans*, emerged in the analysis with an exceptionally high average percentage abundance among patients. Patients who developed lung cancer exhibited notably elevated levels of *Streptococcus intermedius, Granulicatella adiacens, Streptococcus viridans, Mycobacterium bovis,* and *Mycobacterium tuberculosis* [155]

It has been discovered that the microorganisms *Mycobacterium bovis* and *Mycobacterium tuberculosis* can secrete mycobactin siderophores to obtain iron [156]. The investigation [154] discovered that the level of iron siderophore receptors was markedly higher in patients with lung cancer than those without. The finding was based on a metagenomic sequencing technique and rigorous functional alignment analysis. This understanding of disease progression requires that we understand the upregulation in the sputum microbiome's iron siderophore receptors for people with lung cancer. Because of the relationship between excessive iron and the pathogenesis of lung cancer, this is a critical point. The following is one of the only scientific efforts ever undertaken to find resident siderophores or their target receptors in reference to cancer [157]. Going forward, future studies must do a more detailed dissection and explanation of these molecules in order for us to better understand the complex interplay between tumours and the microbiome.

5.1.1. Siderophore in apoptosis

Deferoxamine (DFO), a bacterial siderophore, holds a prominent position in the field of cancer therapy research [158] having undergone comprehensive examination. This tri-hydroxamate hexadentate siderophore exhibits water solubility. Notably, this compound is naturally synthesised by various species belonging to the *Streptomyces* genus [158].

Deferoxamine, in addition to its therapeutic applications, is readily accessible in the commercial market for the purpose of managing conditions characterised by an excessive accumulation of iron within the body [159]. In instances pertaining to leukaemia, it has been duly noted that the administration of Deferoxamine (DFO) has exhibited a propensity to augment the process of apoptosis within controlled laboratory environments. This augmentation is achieved through the upregulation of the tumour suppressor gene known as gene *TP53*, alongside *Bax* and *Fas*, the pro-apoptotic genes. At the same time, it also evokes a decrease in the expression of the anti-apoptotic gene *Bcl-2* [160]. Either alone or in combination with doxorubicin chemotherapy, the employment of DFO affords notable efficacy in arresting breast tumour growth within xenograft mouse models [161]. The combination of doxorubicin with siderophores has been shown to reduce, by as much as half or more, the cardiotoxicity caused by doxorubicin. This is an example of the possible beneficial implications of siderophores as supplementary therapeutic agents within chemotherapy [161]. Besides, the administration of DFO (deferoxamine) in a mouse model of subcutaneous xenograft HCC (hepatocellular carcinoma) during the early period when tumours are forming shows either a decrease or temporary stabilisation in their size. This is because intracellular iron concentration has declined [155].

Although DFO's anti-cancer effect has been reliably validated in preclinical studies, it is essential to emphasise that clinical tests involving patients with various cancers have produced conflicting results. This has meant that many trials have been called off as a result of the difficulties encountered in identifying suitable participants. Clinical trials were first conducted on paediatric patients with recurrent neuroblastoma receiving continuous intravenous infusions of DFO (deferoxamine). The study's findings indicate

that the selected dosage and interval proved inefficacious when utilised as an independent therapeutic approach. Furthermore, it was observed that elevated dosages yielded notable deleterious consequences. Another study which focused on children who have been diagnosed with unresectable neuroblastoma, examined a range of outcomes, including instances of complete remission following surgical intervention. However, it is worth noting that a more recent study undertaken on adult individuals afflicted with advanced hepatocellular carcinoma (HCC), who had previously exhibited no discernible response to hepatic arterial infusion chemotherapy, has demonstrated a noteworthy 20% overall response rate, along with a cumulative survival rate of 1-year [162]. The implications of these findings suggest that the effectiveness of DFO treatment may be somewhat constrained. Among the entirety of registered clinical trials pertaining to the subject matter of siderophores and iron chelation therapy in the context of cancer, it is noteworthy to mention that a mere four trials have been identified wherein DFO was employed as the intervention.

The study [163], which focused its attention on leukaemia and myelodysplastic syndrome, noticed notable impediments in the recruitment of participants. These obstacles were primarily attributed to the inherent complexities associated with the administration of the treatment within the confines of the participants' own abodes. Currently, the HCC trial is in the recruitment phase, whereas the pancreatic cancer trial has reached its conclusion or been prematurely terminated.

A presently under investigation bacterial siderophore is desferrithiocin (DFT) [79]. This is a naturally occurring siderophore obtained from *Streptomyces antibioticus* DSM. *In vitro*, it already has shown potential anti-neoplastic activity against those hepatocellular

carcinomas (HCCs) [164]. However, it must be pointed out that the use of DFT in chelation therapy is regarded as extreme due to its severe nephrotoxicity.

Enterobactin [165], a compound produced by the Gram-negative *Enterobacteriaceae* bacteria, has received attention for its anti-cancer capabilities. Despite this limited scientific evidence, laboratory studies have shown that Enterobactin can restrain the production of ROS and disrupt the balance in their labile iron pool [166]. The interesting compound enterobactin can very selectively attach to iron in cancerous cell lines. Its exceptional quality is that it can produce cytotoxicity in cell lines with rapid proliferation. When the probability of enterobactin-induced toxicity is reduced in noncancerous cells, levels also increase intracellularly. However, low levels of Lcn2 (Lipocalin 2 is a Protein Coding gene) within malignant cells indicate that there may have been a decrease in the ratio between bound and unbound enterobactin, which could enhance its cancer therapeutic properties [166]. Nonetheless, it is necessary to point out that Lcn2 levels are generally high in various cancer types. Thus, this observation may constrain the applicability of these results to various types of tumour.

The probiotic strain *Lactobacillus casei* has produced a compound with antibacterial effects known as ferrichrome [167]. It has demonstrated considerable promise as an anti-tumour agent. An investigation into the possible lethal effects of unbound ferrichrome examined both stomach and colon cancer cell lines as well as xenograft mice models [167]. The results have shown that free ferrichrome possesses antitumor activity by activating the pro-apoptotic pathway, which has been named explicitly c-Jun N-terminal (JNK)-DNA damage–inducible transcript 3 (DDIT3) [167]. In fact, when bound to iron, ferrichrome does not possess the same incredible ability to stunt tumour growth. This observation strongly indicates that the precise location responsible for its anti-tumoral

activity is probably at some point involved in chelating iron. In a xenograft model of colorectal cancer (CRC) [168], it was found that unbound ferrichrome also exhibited equal effectiveness in suppressing tumour activity. It was achieved through up-regulation of the tumour suppressive gene DDIT3. Crucially, it was noted that this activity of free ferrichrome had no observable effect on serum iron levels in the mice studied.

A recent study [169], entitled three bacterial siderophores affect proliferation of both malignant and non-malignant human/mouse cell lines, included deferoxamine B (DFO), mycobactin S (MBS) along with exochelin-MS (Exo-MS) in consideration. Exo-MS is a water-soluble compound which showed fascinating characteristics. It has also been seen to produce growth-inhibiting effects on mouse cancer cell lines. On the other hand, this compound does not seem to produce any detectable effect on human cancer cell lines. DFO, an alternative water-soluble siderophore, has been observed to exhibit growth-inhibitory properties when tested on both leukaemia cancer cells and mouse and human breast. Meanwhile, it has been duly noted that MBS, a siderophore that exhibits lipid solubility, has demonstrated an adverse effect on the viability of hepatic carcinoma cells in humans. The effect was not noticed when utilising DFO or Exo-MS methodologies. The proposition put forth by the authors posits that the enhancement of efficacy in combating tumour cells may be achieved through the amalgamation of water-soluble siderophores with molecules of lipids.

Bacterial-derived molecules possessing siderophore-like characteristics, namely Amamistatin A and B derived from the actinomycete *Nocardia asteroids*, have exhibited notable inhibitory effects on the proliferation of various human tumour cell lines [170]. This is because these molecules can chelate iron and thus have the same structure as mycobacterial siderophores, which may possess antitumor activity. Also, Amamistatins

have shown great promise as inhibitors of histone deacetylase (HDACs), a property linked with the prevention of tumorigenesis [171], [172].

The chemical element gallium has been the recipient of considerable attention in cancer research: it behaves equally well as a siderophore scavenger. Siderophores are aromatic compounds small enough to pass through cell membranes and which might be thought of as iron transporters on behalf of living cells [173], [174]. This unique characteristic of gallium gives it the ability to suppress pathogenic bacteria, which signifies a potential response in oncology. It shows anticancer characteristics toward such tumour entities by causing cell death within human lymphoma cells through stimulating the pro-apoptotic *Bax*, and inducing reactive oxygen species (ROS) formation in mitochondria [175]. An agent that has been licenced by the FDA for the treatment of malignancies such as non-Hodgkin's lymphoma and gallium nitrate has been the subject of clinical studies, and the findings have been seen to be encouraging [176]. Nevertheless, it is of utmost significance to acknowledge that the utilisation of said substance may give rise to plausible complexities, encompassing but not limited to the occurrence of lack of iron and anaemia [177]. The continuous administration of gallium nitrate possesses the inherent capacity to give rise to the emergence of resistance. Consequently, ongoing investigations are being carried out pertaining to alternative gallium compounds, including gallium maltolate. This compound has exhibited a notable capacity to hinder the proliferation of lymphoma cells that have developed resistance towards gallium nitrate [178].

There is an ongoing investigation regarding various bacterial siderophores and their analogues. Nevertheless, it is imperative to acknowledge that at present, exclusively three compounds, namely DFO, deferasirox (a compound of synthetic origin), and deferiprone (likewise a synthetic compound), are presently engaged in clinical trials. Deferasirox is

often favoured over deferoxamine (DFO) due to its advantageous oral administration modality. Clinical trials primarily focus on systemic iron overload conditions, such as beta-thalassemia, leukaemia, and myelodysplastic syndrome [179]. Nevertheless, it is imperative to acknowledge that the representation of solid tumours in the existing literature needs to be revised. Regrettably, we find ourselves confronted with a solitary investigation pertaining to hepatocellular carcinoma (HCC) and another solitary investigation concerning pancreatic cancer [179]. It is disheartening to note that neither of these studies has yielded any discernible outcomes thus far. The disparity observed in preclinical investigations, wherein siderophores are examined for their potential in tumour therapy while disregarding the possibility of systemic iron overload, underscores the imperative need for targeted chelators to avert the occurrence of systemic iron deficiency and its associated complications.

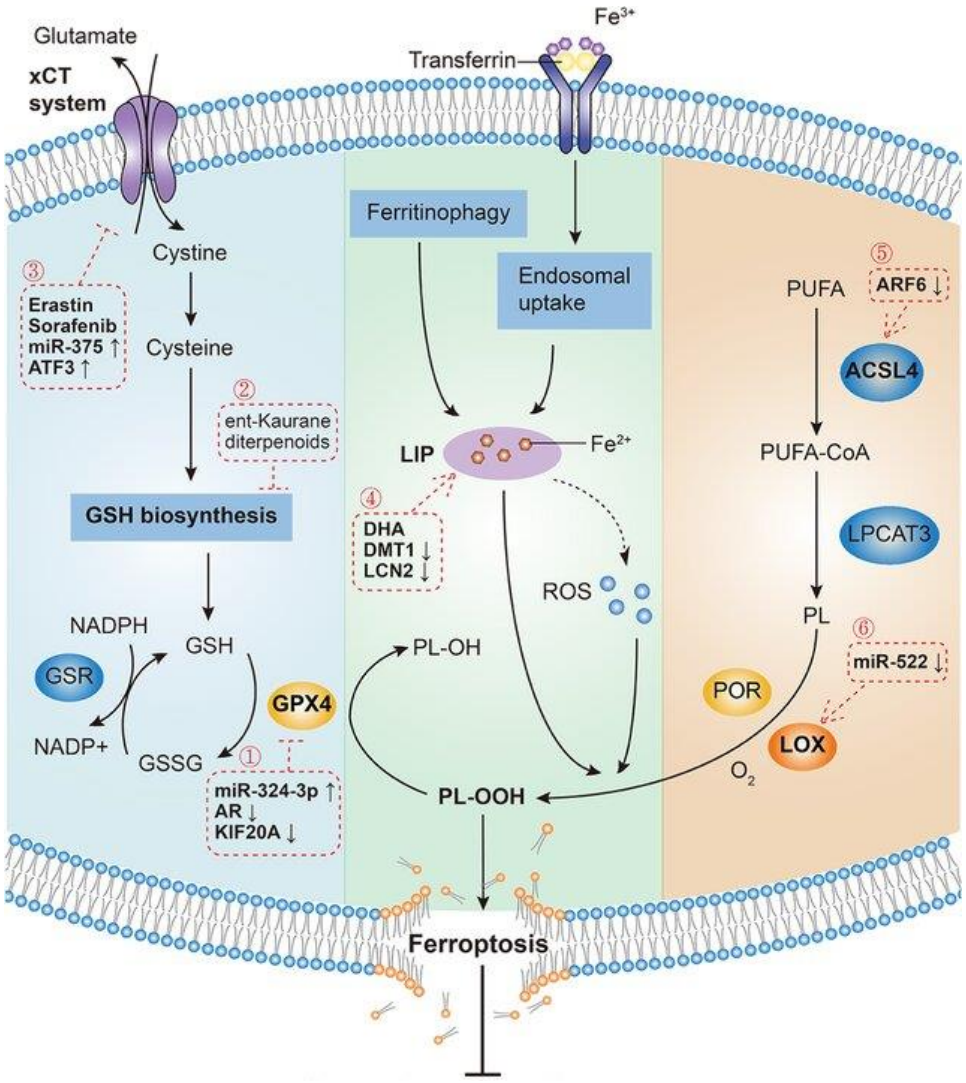
5.2. Siderophore and Ferroptosis

Ferroptosis is a type of regulated cell death predicated upon the presence of Fe- Iron, which was initially identified by Dr Brent Stockwell and colleagues in 2012 [180]. The phenomenon under consideration exhibits a marked dissimilarity in its morphological characteristics, biochemically mediated processes, and genetic foundation when compared with other widely recognised mechanisms of cellular death, such as apoptosis, necrosis, and autophagy [181]. In contradiction to the intricate pathways and BCL2-associated X protein (*BAX*)- the indispensable pro-death effector proteins upon which apoptosis relies, ferroptosis-regulated cell death is incited by the removal/deletion of glutathione (GSH) or due to inactivation of GPX4 activity [180]. In addition, ferroptosis is associated with unique changes in the morphology of mitochondria [182]. These changes include the destruction of structural integrity and a reduction in the size of

mitochondria, which show condensed membrane densities and breakdown of outer membranes [183].

Only through the results of many research studies involving cell death on a macro level is it possible to understand that autophagy plays a vital role in ferroptosis early on (Figure 4.7). This function entails the stimulation of ferritin degradation through a process called NCOA4-mediated ferritinophagy, eventually producing labile iron [184], [185].

Figure 4.7- Mechanisms controlling ferroptosis. [186]



Nonetheless, it is worth pointing out that in the case of ferroptosis induction (especially at its early stages), a central regulatory mechanism such as autophagy may take on far

less weight. The main reason why this phenomenon of ferroptosis is brought about, however, lies in an excessive accumulation of lipid peroxidation byproducts that is intrinsically connected to the iron presence itself. Ferroptosis is a type of cell death that can be avoided by using lipophilic based antioxidants, such as ferrostatin-1 as well as α-tocopherol, in combination with iron chelating compounds such as deferoxamine. These substances serve to distinguish ferroptosis from alternative modalities of cellular demise. System Xc- (which is a central regulator of ferroptosis in cancers) [180] and GPX4 [187] are indeed recognised as pivotal regulators, exerting a substantial influence on diverse biological processes. It is worth noting that specific compounds, namely erastin and (1S, 3R)-RSL3, have been discovered to elicit the function of ferroptosis by proficiently impeding the functionality of said regulators [187].

The phenomenon of cellular demise assumes a pivotal function in normal ontogeny, the preservation of homeostasis within the organism, and the fortification against aberrant cellular proliferation pathologies, notably including neoplastic conditions such as cancer. Erastin, a chemical compound renowned for its ability to induce ferroptosis, has exhibited promising potential in enhancing the effectiveness of chemotherapy medications, specifically in select cancer cell populations. This proposition posits the potential for a valuable therapeutic strategy. The pivotal involvement of GPX4 and system Xc- can be observed in the facilitation of growth [188] and sustenance of renal cell carcinomas, B cell-derived lymphomas, as well as a distinct subset of TNBC cell lines. This suggests that the initiation of ferroptosis holds great potential as a viable strategy in combating cancer, particularly in tumours that manifest these distinct metabolic attributes. This is, of course, about the tumour suppressant p53, a protein frequently aberrated in cancerous cells. Since ferroptosis is initiated via p53, it has been found that if one can inhibit system

Xc-, which gets turned on with activation of p53, then this will have some impact upon or prevent neoplastic growth [189]. In addition, it has been shown that ferroptosis can be stimulated in many cancer cells with the help of FDA-approved clinical drugs such as sorafenib, and sulfasalazine. This indicates that ferroptosis-induced cell death in preclinical and clinical settings can be a viable approach [180].

5.2.1. Ferroptosis Mechanism

Extensive research has been conducted to uncover new pathways that regulate ferroptosis, the mechanisms centred on GPX4 were first identified. Furthermore, pathways not reliant on GPX4 have been identified, which added to our theoretical knowledge of the three main pathways that lead to ferroptosis initiation: that mediated by GPX4, that involving iron metabolism, and that involving lipid metabolism.

5.2.1.1.GPX4-regulated pathway

The initiation of ferroptosis is intricately associated with the reduction in GPX4 activity, a phenomenon that transpires via two distinct mechanisms. The suggested approach involves the impediment of system Xc- (such as through the utilisation of erastin), thereby leading to the indirect suppression of GPX4 [180], [190]. System Xc- functions as a glutamate/cystine antiporter, thereby facilitating the importation of cystine from the extracellular environment while concurrently exporting glutamate from the intracellular milieu [191]. Cysteine, derived from cystine, serves as an intermediate for the biosynthesis of glutathione (GSH). The activity of GPX4 is completely dependent on the availability of glutathione as an obligatory co-substrate. It uses its phospholipid peroxidase activity to reduce lipid peroxides. Therefore, as a result of the small molecule inhibiting system Xc-, glutathione (GSH) becomes depleted. This culminates in the

silencing of GPX4, producing lethal lipid peroxides [180], [186], [187], [190]. Therefore, this chain of events provides the impetus for ferroptosis to begin. Thus, γ-GCS (which regulates GSH biosynthesis) can act as a sufficient trigger for inducing ferroptosis simply by being inhibited. For example, this is seen when the giving of buthionine sulfoximine reduces γ-GCS.

Another molecular pathway related to ferroptosis is triggered by the direct suppression of GPX4. This goal can be realised by either reducing its support or functionality deterioration. The typical ferroptotic inducer is the commercially available (1S, 3R)-RSL3 [187]. This compound displays spectacular activity in suppressing GPX4 enzymatic function through selective covalent binding to its active site selenocysteine. A compound recently discovered is FIN56, which can decrease the level of GPX4 and cause ferroptosis. However, a complete understanding of this phenomenon's exact mechanism still needs to be done. Nevertheless, it is conceivable that a relationship might be found between this event and the enzymatic activity of acetyl-CoA carboxylase [192]. Furthermore, the suitability of small interfering RNA (siRNA) to genetically repress GSHPX4 expression has also been proven valuable in enabling an increase of lipid reactive oxygen species (ROS) as well as a subsequent ferroptotic cell demise by complex means [187]. From a long-term perspective, GPX4 plays the role of the central regulator in this complex web, constituting one stage towards ferroptosis. Because the production of lipid ROS, which depends on iron and triggers the death of cells in ferroptosis, occupies a central position within this complex series of events preceding ultimate demise, understanding how iron metabolism interacts with free fatty acid peroxidation is essential to make sense out of it all.

5.2.1.2. Fe Metabolism Pathway

The Iron responsive element binding protein 2 (IRESB) or IRP2 is a critical genetic factor that controls ferroptosis induced by erastin in Calu-1 and HTM080 cell lines [180]. One essential component of this mechanism is the IREB2 gene. This regulates internal cellular as well as systemic iron levels throughout the human body. The intracellular process by which ferritin is digested through autophagy requires the protein NCOA4. This provides a rationale for the occurrence of ferroptosis [184]. Decreasing cellular intracellular levels of iron and lipid peroxidation byproducts, MDA, are the main mechanisms behind this suppression. In addition, the suppression of lysosomal function causes a weakening impact on ferroptosis. Transferrin (TF) is a vital serum protein that binds to iron and transports it into the body's cells through a process called transferrin receptor-mediated endocytosis. The contribution of TF has been recognised in cases such as serum-dependent cell death under low cystine [193]. In addition, it has a key role in the induction of ferroptosis-regulated cell demise. In particular, the ability to induce ferroptosis can be seen when iron-loaded transferrin (TF) invades the cellular environment by vectoring through transferrin receptor 1 (TfR1).

Heat Shock Protein Family B 1, or HSPB1 for short, prevents erastin-induced ferroptosis [194]. This downregulation of TfR1-mediated iron uptake can be explained by the effects on HSPB1, a protein responsible for maintaining stable actin cytoskeletons. With the delay of HSPB1 expression, transferrin endocytosis and recycling speeds decrease [195].

Heme oxygenase 1 (HO-1) plays a dual responsibility in the regulation of ferroptosis related events. In HT-1080 fibrosarcoma cells, the induction of ferroptosis by erastin involves a significant role for HO-1 expression at an elevated level. However, this carries

the potential to aid in giving iron supplements, which would assist ferroptosis [196]. On the other hand, there is an opposing scholarly investigation which suggests that when the knockdown of heme oxygenase-1 is coupled to treatment using erastin/sorafenib, growth inhibition in HCC cells increases [194]. In different pathological contexts, the responsibility of heme oxygenase-1 in ferroptosis is somewhat inconsistent. However, it is necessary for accurately investigating the precise underlying mechanisms that produce this phenomenon. Its effect on promoting the regulation of ferroptosis affects PHKG2 in a favourable manner. Through inhibition of its expression, it may show potential to become an iron chelator [197]. Further investigation of this issue is needed to flesh out in detail how PHKG2 participates in regulation of iron metabolism.

5.2.1.3. Lipid Peroxidation Pathway

ROS is the main lipid reactive oxygen species accumulated in ferroptosis. As long as an iron chelator or appropriate antioxidant is used to increase membrane stability [180]. Erastin-triggered ferroptosis is linked to the production of ROS by NADPH oxidases (NOXs). The pharmacology-based inhibition of NOXs and the pentose phosphate pathway (PPP- key to produce NADPH) resulted in successful attenuation of erastin-induced ferroptosis. However, it is noteworthy that the suppression of NOXs or PPP only afforded a partial defence against erastin-stimulated ferroptosis in HT-1080 cells. This observation is most apparent when these cells are treated with higher concentrations of erastin. These variations in results can be explained by differences between cell lines so that we may infer that the NOX/PPP pathway probably operates afterwards and is not an initial factor. Moreover, we must also recognise that in addition to NOXs, the process of lipid oxidation on cellular membranes is another mechanism by which reactive oxygen species (ROS) are generated.

Within the fatty layer of the cellular membrane, there is an obvious reduction in the presence of PUFA, namely arachidonic acid. Subsequent to the administration of erastin in HT-1080 cells, increased levels of prostaglandins such as PGE2 or linoleate are observed [198]. In fatty acid synthesis, it is essential to recognise that various regulators and paths are also involved. In this complex process, certain key players appear of special note in particular. These include glutaminolysis, citrate synthase and acetyl-CoA carboxylaes among others. These are viewed as necessary conditions for carrying out successfully the complicated procedure termed ferroptosis [192]. ACSL4 favours the esterification of amino acids (AA), and LPCAT3 is responsible for introducing AA into phospholipid membranes. In this way, lysoPC is converted into PC [199]. Genes such as ACSF2 are especially important in helping to produce the necessary membrane lipid polyunsaturated fatty acids (PUFA). Therefore, this process contributes to the formation of ferroptosis in that it stimulates lipid peroxidation and ROS generation.

Lipoxygenases are a key player in ferroptosis. Their role is to aid the dioxygenation process, in which membrane lipid PUFAs undergo a series of transformations [197]. This reaction is also part of ferroptosis' production of precious fatty acid hydroperoxides. While aminocaproic acid was effective in delaying the neurodegenerative process, Zileuton [95], understood to be a pharmacological agent acting as an inhibiting factor against 5-lipoxygenase has been found at times to have strong neuroprotective capability. Generally speaking, the change of fatty acid hydroperoxides into corresponding alcohol is brought about through GPX4 participation. This obstacle is the deactivation of GPX4 in this specific framework for ferroptosis. Fatty acid hydroperoxides which accumulated inside the cell, can be converted into cytotocin lipid peroxy radicals by iron-mediated Fenton reactions. Elevated oxidative degradation of membrane lipid polyunsaturated fatty

acids (PUFAs) leads to accumulations, respectively, in lysophosphatidylcholine (lysoPC) and specific PUFA types. This is a characteristic feature [198]. Accompanying the resistance to inhibition of system Xc- is an upregulation of AKR1C. Up-regulation exerts an essential function in the elimination of cytotoxic oxidative breakdown products from polyunsaturated fatty acids (PUFA), such as malondialdehydes (MDA) and 4-hydroxynonenals (4-HNE) [190]. However, the complete elucidation of just how precisely it is that GPX4 inhibition actually leads to ferroptosis still remains an unanswered question.

5.2.2. Siderophore in Ferroptosis

Microorganisms secrete non-ribosomal peptides known as siderophores to get iron from the surroundings. The mammalian immune system produces lipocalins, secretory proteins that sequester siderophores generated by invasive bacteria in response to bacterial infections. By doing this, the bacteria cannot get iron from the host. Lipocalins do, however, perform other roles, such as delivering iron bound to siderophores to host cells. A study compared the human lipocalin or LCN2 expression in an average WRL-68 cell line and hepatocellular carcinoma (HepG2) cell lines. The viability of LCN2 binding with fungal siderophores was assessed using computational analysis. The results imply that ferroptotic cell death may be induced by the fungus siderophore tri acetyl fusigen from *A. nidulans*. This is shown by siderophore-induced increases in ROS, membrane damage, and modifications in the activity of antioxidative enzymes in malignant cells, with no appreciable variations seen in normal cells. As a result, this research provides opportunities for investigating new approaches, particularly siderophore-mediated activation of ferroptosis, as a possible hepatocellular carcinoma (HCC) therapy. In

contrast to conventional treatments, this method little endangers healthy cells, suggesting that it might be a safer option [200].

The protein lipocalin 2 controls the body's iron balance by binding to siderophores. Many forms of tumours show increased expression of Lipocalin 2, yet it is unclear how this protein contributes to the progress of tumours. *In vitro* and *in vivo* investigations using colon cancer cell lines show that overexpression of Lipocalin 2 results in resistance to the chemotherapeutic medication 5-fluorouracil. This resistance is explained by the prevention of ferroptosis. By lowering intracellular iron levels and increasing the production of glutathione peroxidase 4 and xCT, a part of the cysteine glutamate antiporter, lipocalin 2 prevents ferroptosis. In cells overexpressing Lipocalin 2, higher levels of ETS1 are associated with greater xCT levels. Reduced chemo-resistance and transformation *in vitro*, as well as decreased tumour growth and chemo-resistance in xenograft animal models, are the outcomes of using a monoclonal antibody to inhibit Lipocalin 2 activity [201].

6. Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC) remains as the mighty adversary in the realm of therapeutics, above all due to its inherent resistance against current treatment methods and tendency early on during the convalescent stage for the development of further resistances. Although the rate of incidence as a portion of newly diagnosed cancer cases is only 3.2 %, it cannot be denied that pancreatic ductal adenocarcinoma (PDAC) exerts enormous pressure on the mortality statistics for cancers in general. In fact, 8% of all cancer-related deaths around the world are due to PDAC [202]. There are several contributing factors that have precipitated a progressive increase in the incidence of

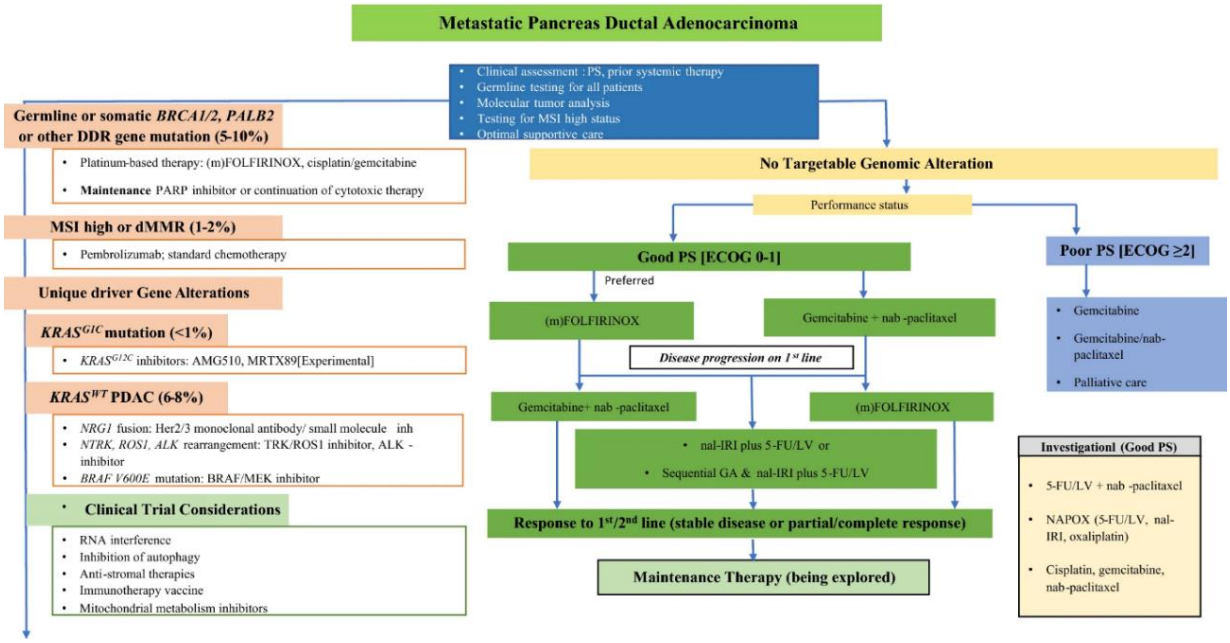
pancreatic cancer. These factors encompass the prevailing obesity epidemic, the presence of diabetes mellitus, the influence of lifestyle and environmental elements, as well as the notable rise in life expectancy and the heightened occurrence of cancer among younger cohorts [203].

Between the years 2007 and 2016, a discernible upward trend in the prevalence of pancreatic cancer has been observed. In a discernible manner, it is noteworthy to highlight that there has been a consistent yearly increase of 0.7% within the White population, juxtaposed with a comparatively lower growth rate of 0.3% observed within the Black population. Contrary to the prevailing trend of a 27% decline in cancer incidences since 1991, this observation stands in stark contrast. Early-onset pancreatic cancer (EOPC), a variant of pancreatic ductal adenocarcino, manifests in individuals below the age of 50, constituting approximately 5.7-9.5% of the total PDAC cases [204], [205]. The diagnosis of this condition often occurs at an advanced stage, thereby presenting significant challenges in terms of surgical intervention or the management of metastasis. Whilst there exists no discernible discrepancy in the overall survival rates when compared with instances that arise in subsequent stages of life, scholarly investigations suggest a plausible proclivity towards the emergence of advanced maladies. The trend may be correlated with the escalating prevalence of obesity among the younger adult population.

The lack of genomic data pertaining to early-onset pancreatic cancer (EOPC) patients is a matter of concern. Nevertheless, a recent investigation has yielded noteworthy findings, revealing that a substantial proportion, specifically 29%, of the examined EOPC patients exhibited pathogenic germline mutations. These mutations encompass *BRCA1/2*, *CHEK2*, *PALB2*, *ATM*, *MLH1*, and *MSH3*. By means of somatic profiling, it has been ascertained that a notable 16% of tumours have been categorised as *KRAS* wild-type,

thereby signifying the existence of actionable mutations within a distinct subset of tumours (Figure 4.8). The median overall survival for the entire cohort comprising 450 patients diagnosed with early onset pancreatic cancer (EOPC) was observed to be 16 months. In the cohort of individuals afflicted with metastatic pancreatic ductal adenocarcinoma (PDAC), it has been observed that the median overall survival stands at an approximate duration of 11.3 months.

Figure 4.8- Current approaches to treat metastatic pancreatic cancer. [203]



While it is true that there has been a modest rise in overall survival rates, particularly in the 5-year survival rate, which has increased from 6% in 2014 to approximately 10% in 2023, it is important to note that the prognosis for individuals with metastatic pancreatic ductal adenocarcinoma (PDAC) continues to be unfavourable. Metastatic pancreatic ductal adenocarcinoma (PDAC) constitutes a substantial portion, precisely 53%, of all diagnoses. Regrettably, the prognosis for this advanced stage of the disease remains disheartening, as it is accompanied by a mere 3% survival rate over a span of five years.

Based on empirical observations derived from real-life circumstances, it has been ascertained that the act of receiving medical intervention engenders a notable tripling in the likelihood of survival when juxtaposed with the absence of any form of treatment [203]. This remark points out that the right policies must be put in place. The increased survival rates of patients are due to improvements made in supportive care, interventional biliary procedures, infection control and implementation of treatment. In a small but distinct subpopulation of patients with pancreatic ductal adenocarcinoma (PDAC), the discovery of actionable mutations in genes involved in repairing DNA damage and somatic mutation convertible to target therapy, along with improved understanding of tumour microenvironment and immune system pathophysiology gives hope.

The most crucial step in understanding the resistance PDAC presents to therapeutic treatments, or the significant differences in survival outcomes among PDAC patients with advanced tumours at a single hospital and of different responses shown by individual cancer cells towards specific treatment modalities is to first obtain an all-around knowledge of its pathology. The PDAC onset proceeds in three stages. The first stage [206] involves the start of tumour growth triggered by changes in proto-oncogenes, which occur several years before observable traits become visible. Pancreatic cancer cases exhibit a more extended latency period, which is attributable to the relatively sluggish rate of cellular reproduction. The following stage [207] shows a noticeable expression of clonal expansion. This process involves the selection of driver and passenger mutations to come together, along with a further clonal heterogeneity enhancement. The last stage is the introduction of neoplastic cells into an altered microenvironment, where they undergo a continuous process of dissemination. The spread of this phenomenon is probably limited to a small population of cells with high resistance.

Anatomic imaging of localised and locally advanced pancreatic ductal adenocarcinomas (PDACs) have identified three subgroups that histopathological examinations have revealed. This classification is essentially determined by the composition of immune cells within the microenvironment surrounding tumours. Moreover, it is important to note the presence of an immune-rich subtype that contains a high density of T and B cells as well as low concentrations of Tregs. Lastly, we arrive at the immune-exhausted subtype. This category can be further divided into two separate subpopulations (3). The first subgroup is characterised by high levels of PD-L1, while the second shows microsatellite instability. This particular subtype with a strong immune response offers a greatly improved prognosis. Moreover, in fact, there are many mutations of genes related to DNA damage repair: ATM and STK11 [208]. These mutations offer hopeful potential in the development of targeted therapies as well as immune-based interventions.

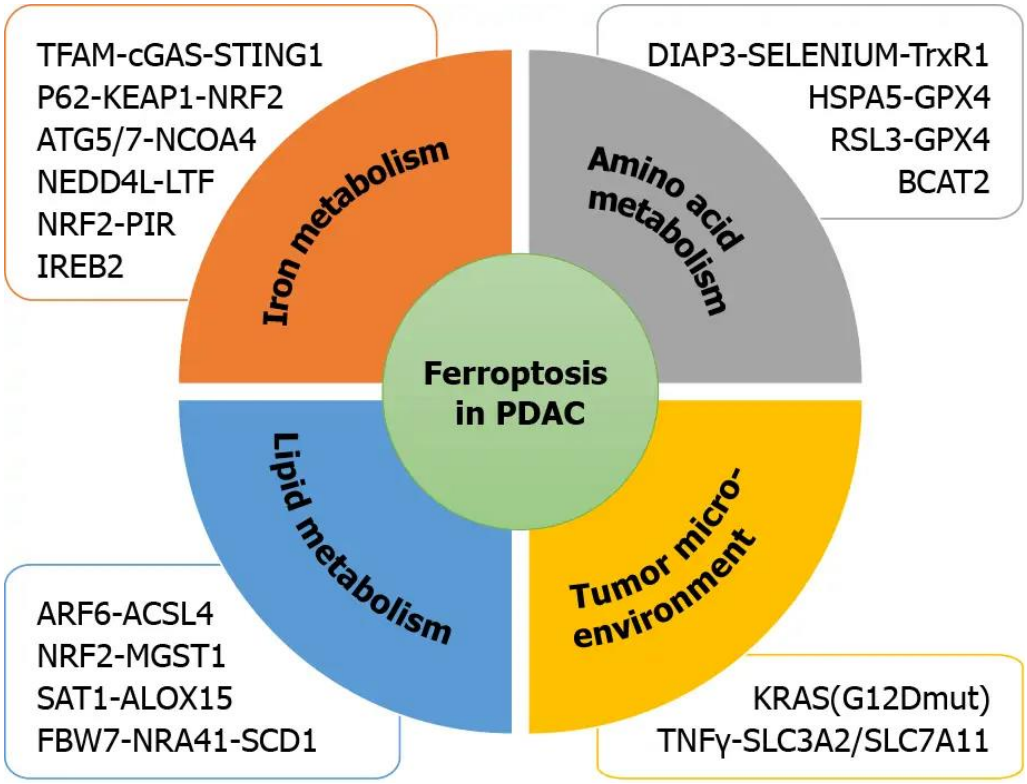
PDAC's genetic makeup encompasses what are known as somatic mutations in critical genes such *KRAS*, *CDKN2A*, *TP53* and *SMAD4*. The most common mutations that have been found among cases are changes in the *KRAS* gene, accounting for 90-94 % of all observed incidence. Apart from the *KRAS* gene, exceedingly worthy of note are mutations discovered on *TP53* and *CDKN2A*. Also noteworthy is a change in *SMAD4*. A simultaneous gene amplification also occurs in a subgroup of patients having precisely 4% with KRAS mutation [209]. Components of the PDAC microenvironment include a dense extracellular matrix (ECM), stromal cells, and immune cells. The myofibroblasts form pancreatic stellate cells and help produce the thick extracellular matrix (ECM) that serves as a powerful barrier to the tumour [210]. Hyaluronic acid, commonly referred to as HA, assumes a pivotal role in the modulation of pressure levels, thereby potentially impeding the optimal delivery of pharmaceutical substances. The limited presence of

intra-tumoral effector T cells contribute to the phenomenon of immune evasion. Moreover, the accumulation of immune-suppressive cells gives rise to supplementary challenges in the realm of immune surveillance [211].

6.1. PDAC and Ferroptosis

At present, the primary systemic treatment for pancreatic ductal adenocarcinoma (PDAC) is therapeutics based on 5-fluorouracil and gemcitabine. Unfortunately, the general and rapid development of chemical resistance warns us not to be overly optimistic.

Figure 4.9- Mechanisms of ferroptosis regulation. [212]



As a result of recent research (Figure 4.9), we have learned that ferroptosis is closely related to pancreatic ductal adenocarcinoma (PDAC). Accordingly, interest in investigating whether it would be possible to provoke ferroptosis as a novel approach for overcoming these many difficulties of PDAC has increased. This novel approach attempts

to avoid the drawbacks of traditional chemotherapy drugs while at the same time improving treatment results for people suffering from pancreatic ductal adenocarcinoma (PDAC).

6.1.1. Iron Metabolism in PDAC

To help in the transportation of extracellular ferric ions (Fe^{3+}) into cells, there is one kind of receptor on the cellular membrane called transferrin receptor 1, which can bind with transferrin. The first step involves the entry into a cellular environment of this conjugate via endocytosis. In addition, it should be noted that the six transmembrane epithelial antigen belonging to prostate 3 (STEAP3) greatly influences Fe^{3+} as well as Fe^{2+}. This transformation is significant because only after this can these ions be transported out of the endosome and into the cytoplasm. The divalent metal ion transporter 1 (DMT1) [213] enables this transportation. Intracellular Fe^{2+} species can be bound up as ferritin or exist unbound.

Excess Fe^{2+} inside the cells causes a Fenton reaction. This chemical process involves a reaction between ferrous ions (Fe^{2+}) and hydrogen peroxide (H_2O_2), forming ferric ions (Fe^{3+}) and more powerful free radicals, the hydroxyl. The hydroxyl radicals, previously discussed, belong to a class of compounds called reactive oxygen species, capable of causing impairment by destroying protein, lipid and DNA. In so doing, they also affect the efficiency with which cellular membranes perform their normal functions, such as permeability control or recognition behaviour. This phenomenon is virtually fatal to the cellular entities [194]. The Fenton reaction plays a key role in this complex phenomenon called ferroptosis. Its principal role lies in its constant production of reactive oxygen species, hydroxyl radicals and peroxy free radicals [214]. But iron ions function as a

catalyst. They tend to expedite the generation of reactive oxygen species, especially in the cell systems, which is particularly true about tumour cells.

Ferritin is a protein complex consisting of two components, ferritin heavy chains (FHC) and ferritin light chains (FLC). According to an investigation, IREB2 is found capable of increasing FHC and FLC expression, thus delaying ferroptosis [215]. Lactotransferrin (LTF) is one of the proteins in a group known as transferrin. This process is regulated under the jurisdiction of neural precursor cell-expressed developmentally downregulated 4-like (NEDD4L) [216]. NEDD4L promotes the degradation of LTF protein. It thus prevents intracellular iron accumulation, thereby blocking ferroptosis brought on by oxidative cell death in pancreatic ductal adenocarcinoma (PDAC). In addition, it should be pointed out that Lipocalin-2 (LCN 2) can bind with siderophores to serve as an iron carrier. This complicated process profoundly affects the delicate balance of intracellular and extracellular iron concentrations. Some academic research has shown that LCN2 is an inhibitor of invasion and angiogenesis in pancreatic ductal adenocarcinoma (PDAC) [217].

6.1.2. Amino acid and GSH metabolism in PDAC

This specialised transporter is called system XC-, and with its help, cysteine and glutamic acid can be transported across cellular membranes. Its structure is a heterodimer, containing the glycosylated heavy chain (SLC3A2) and non-glycosylated xCT (SLC7A11) [218]. This interconnection is possible because there are disulfide bonds present. Cystine is converted to cysteine by a reduction mechanism, which plays an important role in glutathione (GSH) biosynthesis and also determines the extent of subsequent lipid peroxidation. Glutathione, known as GSH detoxifies this toxic oxygen

by acting as an electron donor. In the presence of glutathione peroxidase 4 (GPX 4), a specific enzyme and collateral cofactor required for its function, it participates in electron transfer from phospholipids to molecular oxygen. This reduces harmful lipid [187].

Cells can take up cysteine actively through the alanine-serine-cysteine system. This system has been considered as an inhibitor of ferroptosis [219]. It The synthesis of cystine can occur from methionine by means of trans-sulfuration. The XC system plays key role in providing cystine needed for cystein biosynthesis to the cells. Thus, the start of ferroptosis really occurs by stopping this step. It will eventually lead to a decrease in intracellular cysteine levels and damage GPX4's ability to repair lipids. It has been well documented that substances like erastin, sulfasalazine and sorafenib extensively suppress the activity of system XC- transport. So, it is this inhibition that gives us the fascinating phenomenon of ferroptosis.

Comprehensive research [220] inquiry was undertaken to examine the consequences of system XC- on ferroptosis and so forth. As the main enzyme involved in metabolizing sulphur amino acids, BCAT2 has now been proven by this study to be a significant player. However, in the laboratory, BCAT2 is proven to have a key responsibility in regulating intracellular glutamate concentration in-vitro and in-vivo. The system of action is to antagonize systems XC-(-inhibition). Doing this protects pancreatic ductal adenocarcinoma (PDAC) cells from the destructive process of ferroptosis. BCAT2 thus plays the role of regulator suppressing ferroptosis when sorafenib synergises with sulfasalazine in its induction.

RSL3 is an inhibitor of GPX4 that works by acting as an alternative small molecule. It accumulates lipid reactive oxygen species (ROS), which then initiates the process of

ferroptosis. Thus, through the use of a selenocysteine residue, selenium increases the antiferroptotic activity of GPX4. Selenium incorporated in the process of selenoprotein biosynthesis exemplifies through enzyme thioredoxin reductase 1 (TrxR1) a most important function, that is, direct reduction of hydroperoxides. For instance, some studies [221] have shown that diaphanous homolog 3 (DIAPH3) is powerfully related to PDAC tissues. The presence of these proteins also involves increased levels of selenium and is related to the expression correlation between them. Therefore, the overexpression of TrxR1 induced by DIAPH3 leads to increased cellular levels of ROS.

6.1.3. Lipid Metabolism in PDAC

The fatty acids are said to play a crucial role in the esterification process of the formation of membrane phospholipids in lipid metabolism. PUFAs are more susceptible to oxidation than the other two, SFAs and MUFAS. When molecular oxygen reacts with the phospholipids in a cellular membrane, it creates an intermediate substance called phospholipid hydroperoxide (or PL-OOH). This hydroperoxide reacts with lipids when it comes into contact with ferrous ions (Fe^{2+}), subsequently inducing a chain of reactions known as lipid peroxidation [222]. The breakdown products of phospholipid hydroperoxide have a significant impact on the integrity, shape and even permeability characteristics of the cell membrane. This eventually results in the cell's demise. Enzymes, namely lysophosphatidylcholine acyltransferase 3 (LPCAT3), long-chain acyl-CoA synthetase 4 (ACSL4), and arachidonate lipoxygenase (ALOX), assume pivotal roles in the facilitation of lipid peroxidation reactions [199], [223].

Through a variety of mechanisms, lipid peroxides are responsible for destroying cells. In the early stages, it can be seen that these high-energy substances begin to decompose.

These then yield reactive oxygen species (ROS). This then reinforces the phenomenon of lipid peroxidation. In addition, it is also noteworthy that lipid peroxides can cause changes to the chemical nature of the membrane. These changes consist of thickening, bending and perforation. Thus, this releases harmful substances and disrupts metabolism intracellularly. Also, during lipid peroxidation, by-products harmful to the body are formed, such as 4-hydroxy-2-nonenal and malondialdehyde. [223].

6.1.4. Tumor microenvironment in PDAC

The tumour micro-environment, which consists of the cancer cells themselves and blood vessels as well as the extracellular matrix and immune cells, affects how successful treatment will be. According to reports, nano-inducers of ferroptosis can take the iron out of intercellular spaces and reintroduce it into cells. Also, it has been demonstrated that ferroptosis in tumour cells might be initiated by increasing FHC and reducing GSH to raise the intracellular ROS level [224]. The perfluorocarbon nanodroplets loaded with near-infrared photosensitizer IR780 serve as an extra inducer. The efficacy of such inducers depends on the microenvironmental differences between tumour and normal tissues, including immune system activity, pH levels and oxygenation.

For photodynamic treatment, a perfluorocarbon-based oxygen source is used to generate ROS within tumour tissues [225]. Although standard wisdom has it that CD8+ T cells bring about cellular death, recent studies have indicated that tumour cells experience a considerable enhancement in ferroptosis-potentiating lipid peroxidation when immunotherapy awakens their CD8+ T cells. In different ways, subgroups of tumour microenvironment macrophages are sensitive to ferroptosis. M1-type resting macrophages seem to be anti-tumorigenic. By activating fatty acid oxidation through

signal transducer and activator of transcription 3, the oncogenic $KRAS^{G12D}$ in pancreatic ductal adenocarcinoma (PDAC) turns macrophages into M2-like pro-tumour cells. This is believed to play an important role in the interaction between cancer cells and macrophages. Ferroptosis also makes cancer cells experience oxidative stress, which leads to the secretion of $KRAS^{G12D}$ proteins [226]. A new approach to cancer treatment that takes advantage of the interaction between inducers and the intricate tumour microenvironment is targeting the tumour microenvironment to improve ferroptosis in PDAC cells.

Here in this chapter, the cell death pathway of pancreatic cancer (SUIT2 cell line) is determined using cyanobacterial metabolites and metabolite-rich fractions. The cell viability is compared with the standards, inhibitors, and inducers of apoptosis/ferroptosis.

7. Result and Discussion

7.1.MTT and cell viability in SUIT2 cells

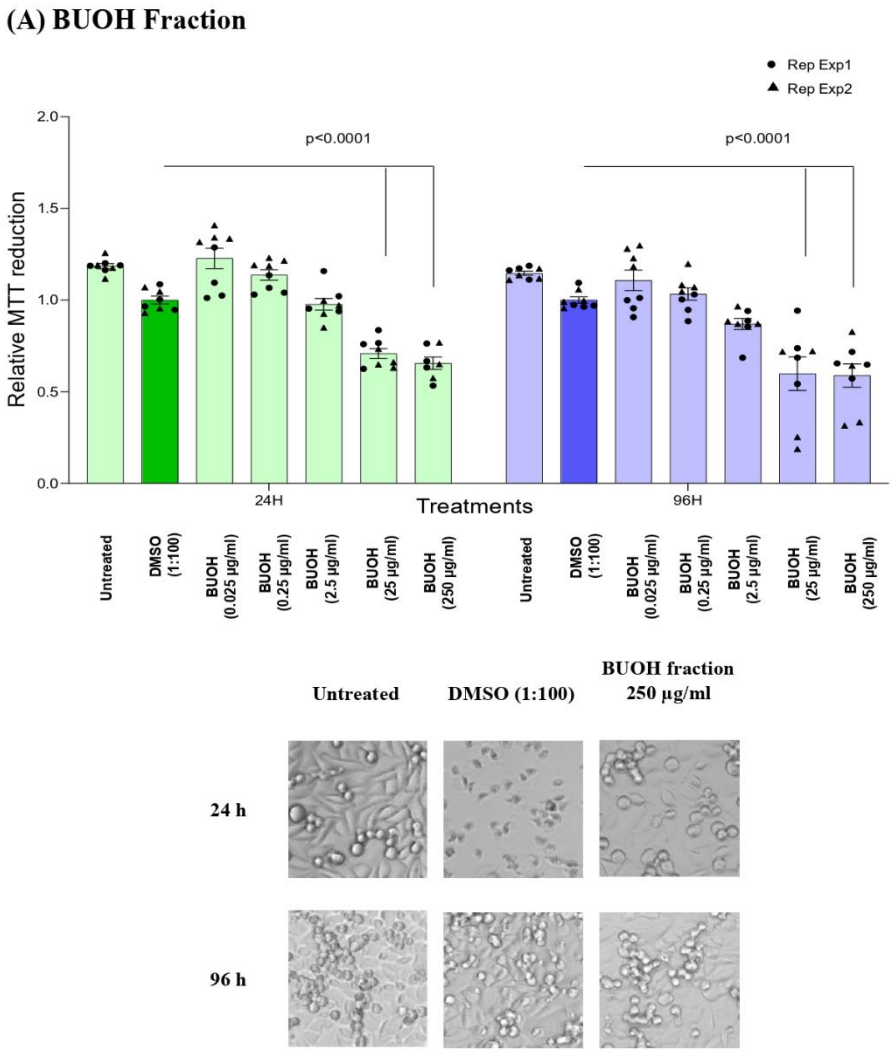
The MTT assay was performed to determine the cell viability and death patterns in the Pancreatic ductal adenocarcinoma (PDAC) SUIT2 cell line. The cells platted at 15,000 cells/well were treated with (A) BuOH fraction, (B) MeOH fraction, (C) Aqueous Fraction, (D) Erastin, (E) Deferoxamine (DFO), (F) Deferasirox (D'ROX), (G) Trolox, and (H) Ferrostatin.

Studies have shown that the siderophore and related compounds produced by microorganisms have always been reported and given attention. The Deferoxamine secreted by *Streptomyces* spp has shown the alteration in viability for leukaemia [160], [169], breast cancer [161], [169], [227], hepatocellular carcinoma [155], [162], [169], [228], gastric cancer [229], ovarian cancer [227], etc. The compound DFCAF (considered a DFO complex), secreted by *Streptomyces* spp, has also been reported to show anti-cancer properties toward cancer stem cells [230]. The *Streptomyces antibioticus* DSM secreted a siderophore named Desferrithiocin, showing its activity towards decreasing cell viability in hepatocellular carcinoma [164]. Another study reported the cell viability potential of Enterobactin, secreted by a variety of organisms- *E. coli, Salmonella enterica*, *Shigella dysenteriae* and *Klebsiella pneumoniae* on monocyte-derived cancer cells [166].

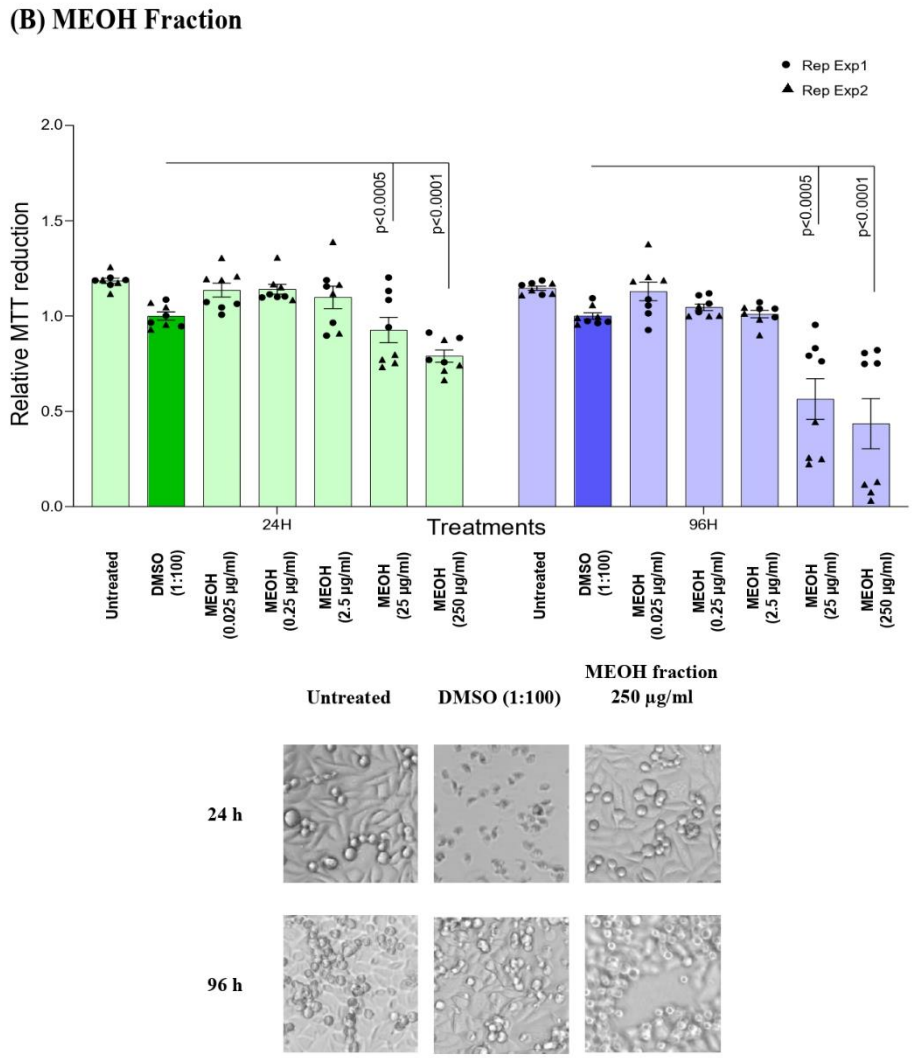
Other studies showed the effect of Ferrichrome, secreted by *Lactobacillus casei*, on gastric [167] as well as colon cancer [168], [231]. Breast cancer, leukaemia, and liver cancer were shown to be treated with Exochelin-MS and Mycobactin S secreted by *Mycobacterium smegmatis* [169]. The two siderophores secreted by *Nocardia asteroids*

[170], namely Amamistatin A and Amamistatin B, are reported to show their anti-cancer activity towards leukaemia, breast cancer, and lung and stomach cancer.

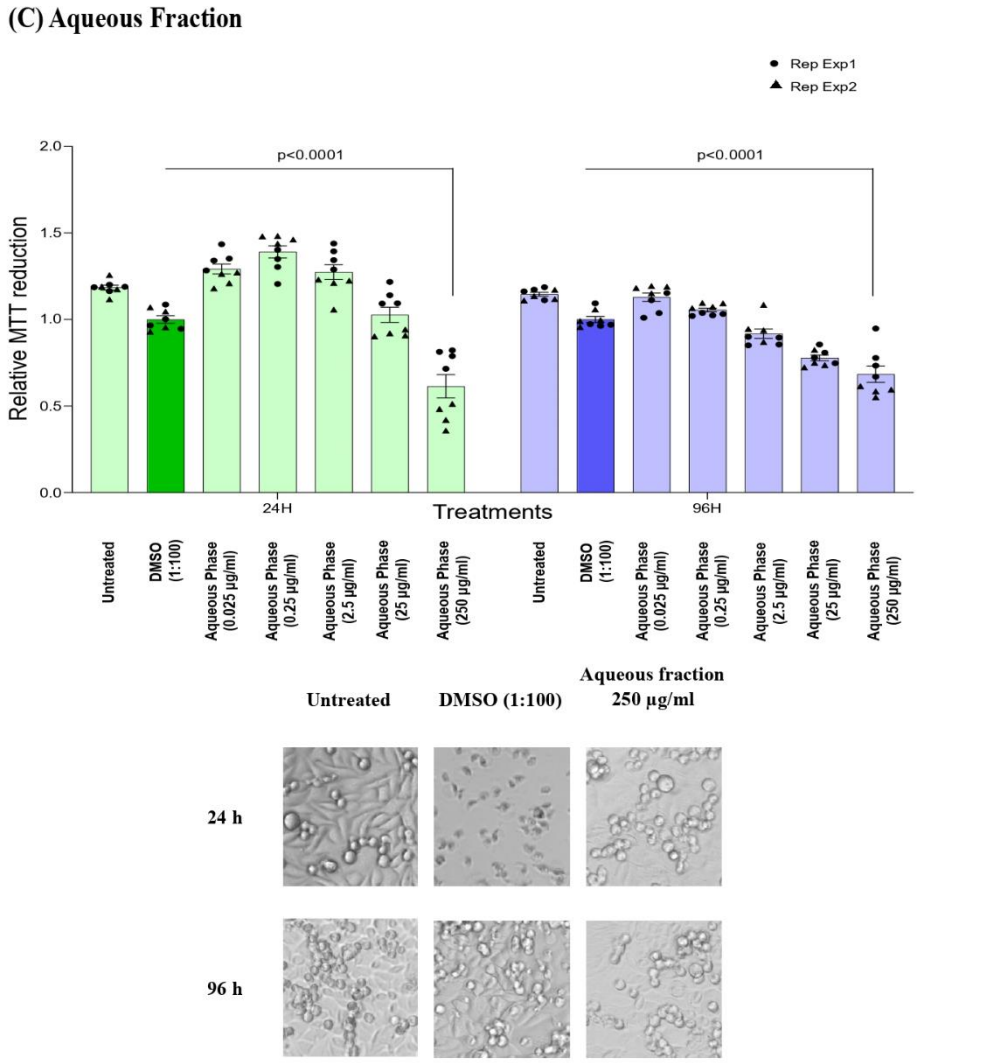
Figure 4.10- Cell viability for (A) BuOH fraction, (B) MeOH fraction, (C) Aqueous Fraction, (D) Erastin, (E) Deferoxamine (DFO), and (F) Deferasirox (D'ROX). (n= 4-8; Experiments= 1-2; p <0.001; Mean ± SD).



The BuOH fraction, with DMSO 1:100 as vehicle control, range 250 µg/ml (Figure 4.11 (A)), is seen to support cell proliferation when used at a lower concentration until 2.5 µg/ml for 24h and 0.25 µg/ml for 96h and induces cell death properties when used at a concentration from 25µg/ml for both 24h and 96h time points.



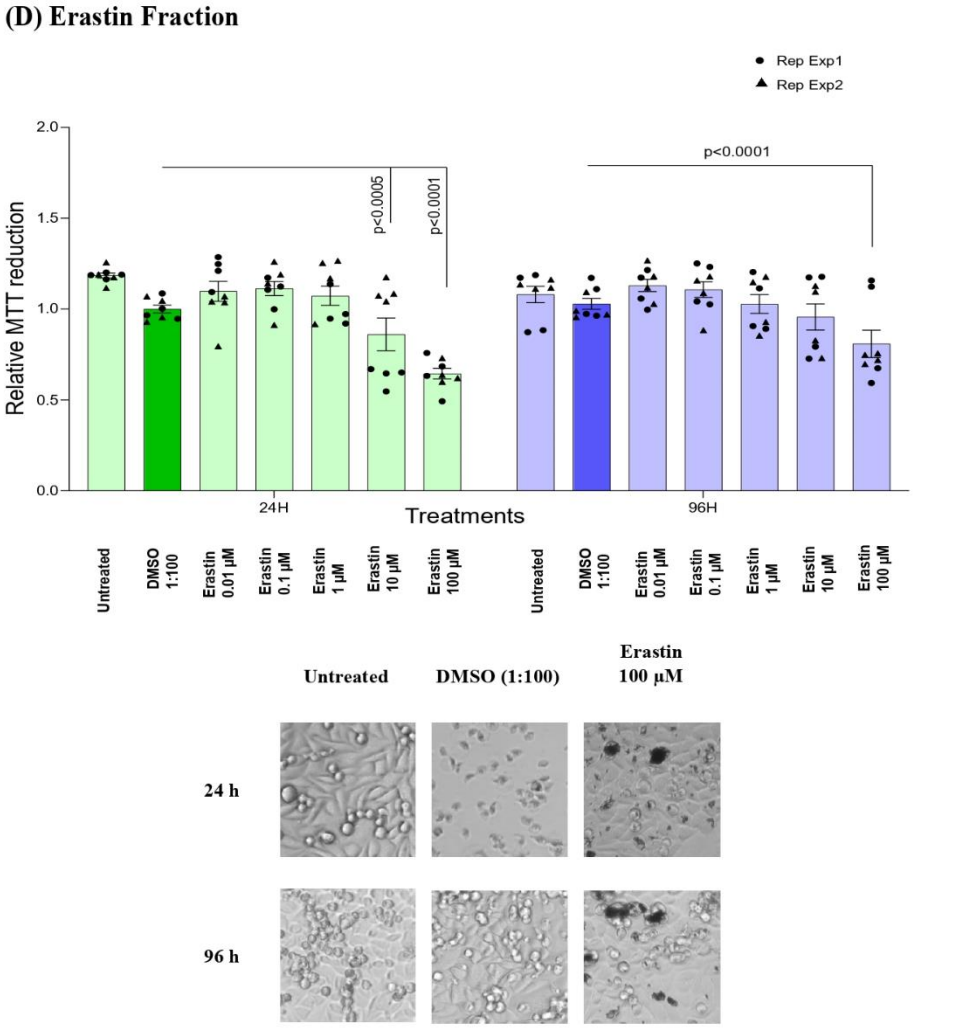
A similar pattern is seen in the MeOH fraction, range 250 µg/ml, (Figure 4.11 (B)), which is seen to support cell proliferation when used at a lower concentration of 2.5 µg/ml for 24h and 96h and induces cell death properties when used at a concentration from 25µg/ml for both 24h and 96h time points, with DMSO 1:100 as vehicle control.



The MTT assay shows cell viability for the Aqueous phase, with a range of 250 µg/ml (Figure 4.11 (C)), showing contradictory results for the two-time points. For 24h time point, at a concentration up to 25 µg/ml, an increase in cell proliferation is seen, and cell death is only predicted at 250 µg/ml. For time point 96h, at a concentration of up to 2.5 µg/ml, an increase in cell proliferation is seen; at 25 µg/ml, it induces slow growth, and at 250 µg/ml, it induces cell death, with DMSO 1:100 as a vehicle control.

Previously, the siderophore has been seen to follow a ferroptotic cell death pathway, so to analyse the cell death pathway in this study, we have utilised ferroptotic drugs that are

readily available in the market and have been reported and validated. Firstly, the compound Erastin, known for its activity to initiate ferroptosis via increased lipid peroxidation, is used as a positive control for ferroptosis.



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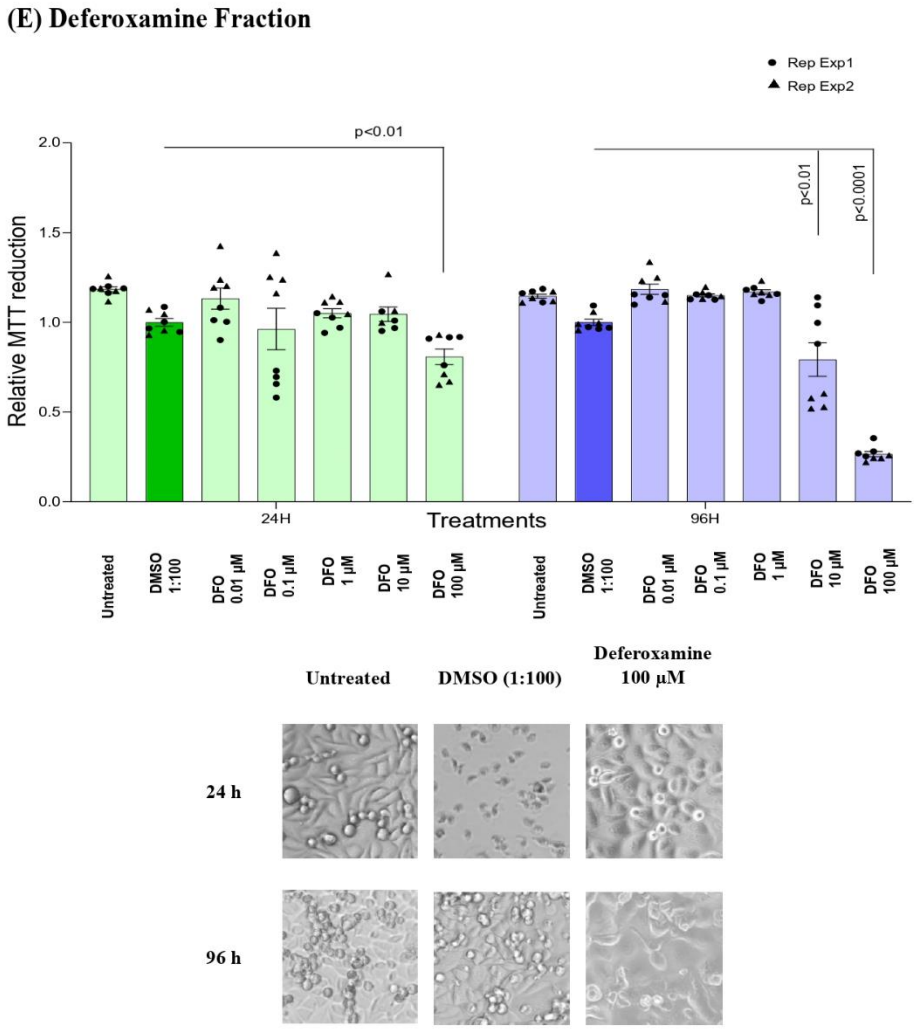
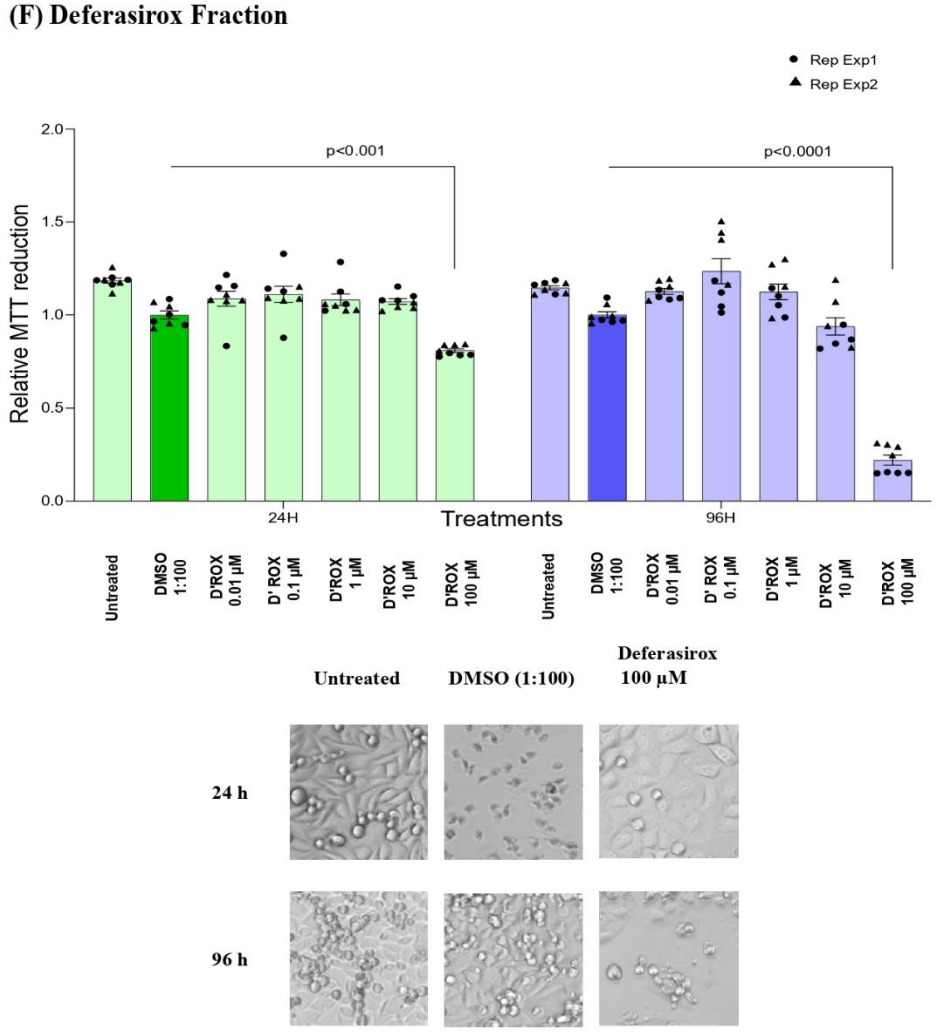
Erastin (Figure 4.11 (D)), when used within the range of 0-100 µM, showed a subtle decrease in cell viability and induced cell death at the concentration of 100 µM for both 24h and 96h time points, with DMSO 1:100 as vehicle control. Unlike the study by Consoli *et al*. [232], where the response to erastin (range 0-50 µM) was significantly

different in the chosen breast cancer cell lines. Specifically, cell viability was greatly decreased after 48 h of treatment in MDA-MB 231 compared with MCF-7. Since the cell lines used in the studies are different then the SUIT2 cell line used in this study could be the potential reason for the variability of results.



For validating the ferroptotic model, we further treated cells with known ferroptosis inhibitors such as Deferoxamine (DFO), Deferasirox (D'ROX), Trolox, and Ferrostatin. Deferoxamine (DFO) (Figure 4.11 (E)), in the range of 0-100 µM, showed a difference

in viability for both time points, such as for 24h, it decreased cell proliferation at 100 µM. In contrast, it decreased cell proliferation at 10 µM for 96h time point.

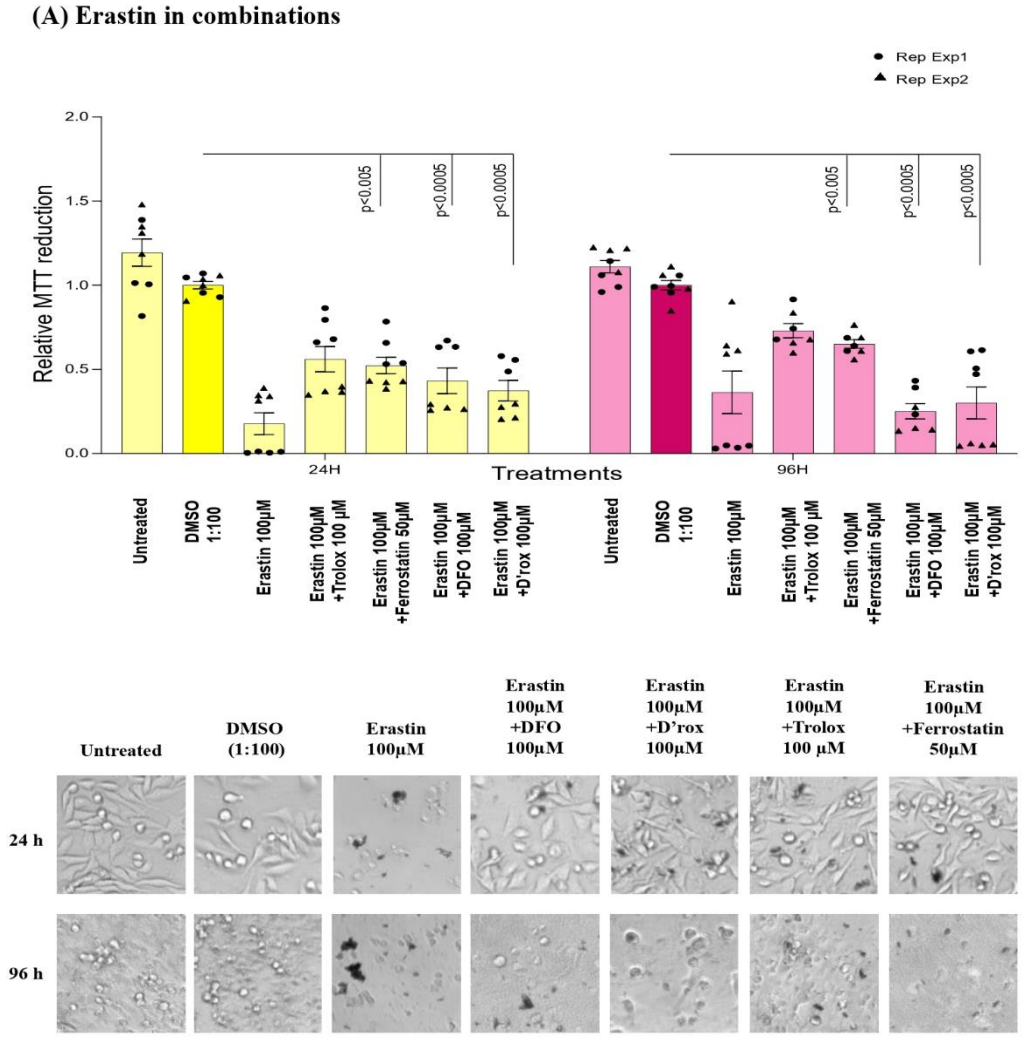


The same pattern was observed in Deferasirox (D'ROX) (Figure 4.11 (F)), in the range of 0-100 µM, showing cell death at 100 µM for both 24h and 96h time points. Trolox, an antioxidant that scavenges cytosolic ROS, and ferrostatin, a potential Inhibitor of ferroptosis, both were tested for the SUIT2 cell line in the concentration range of 100 µM and 50 µM for the latter one, showed no significant role in influencing cell viability (Supplementary Figure (1 and 2)). DMSO 1:100 was vehicle control for all for inhibitors.

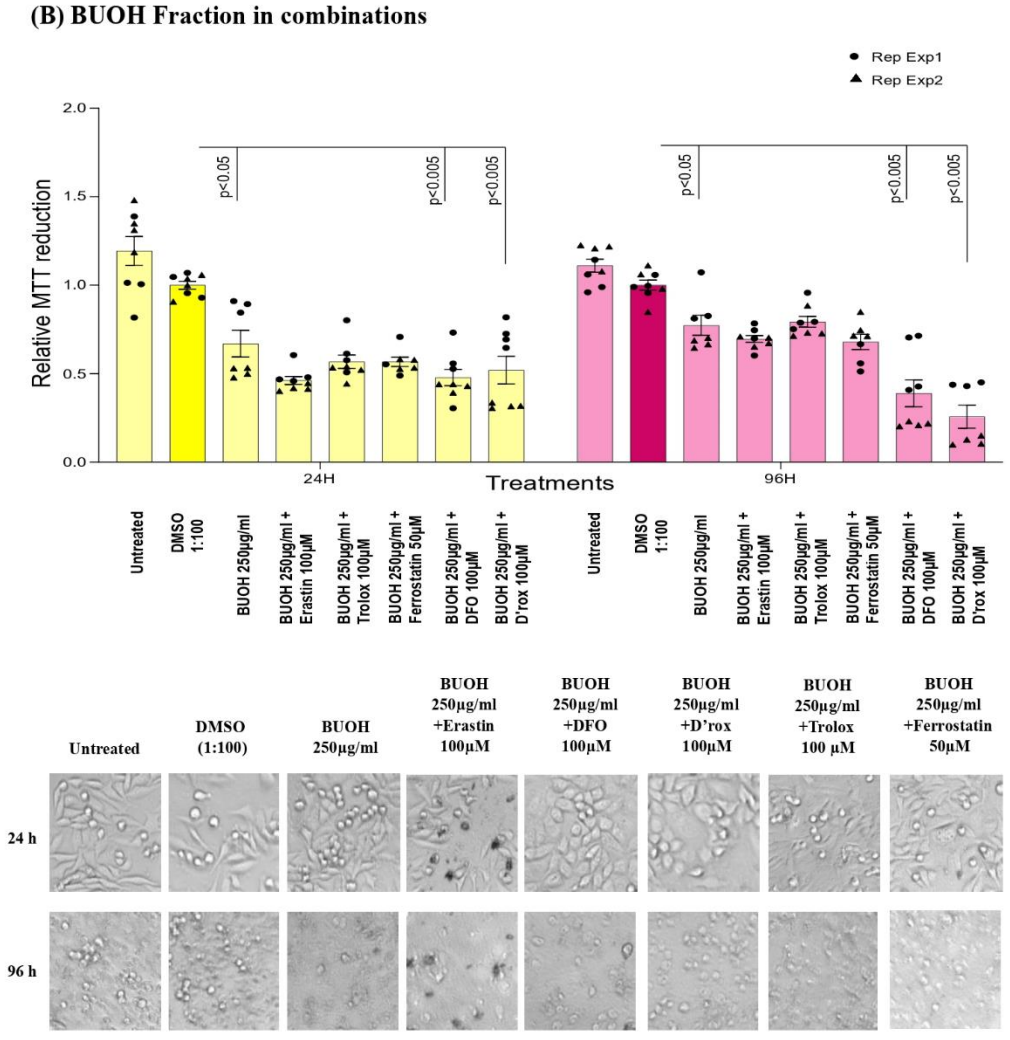
7.2. MTT and cell viability in SUIT2 cells for combination studies

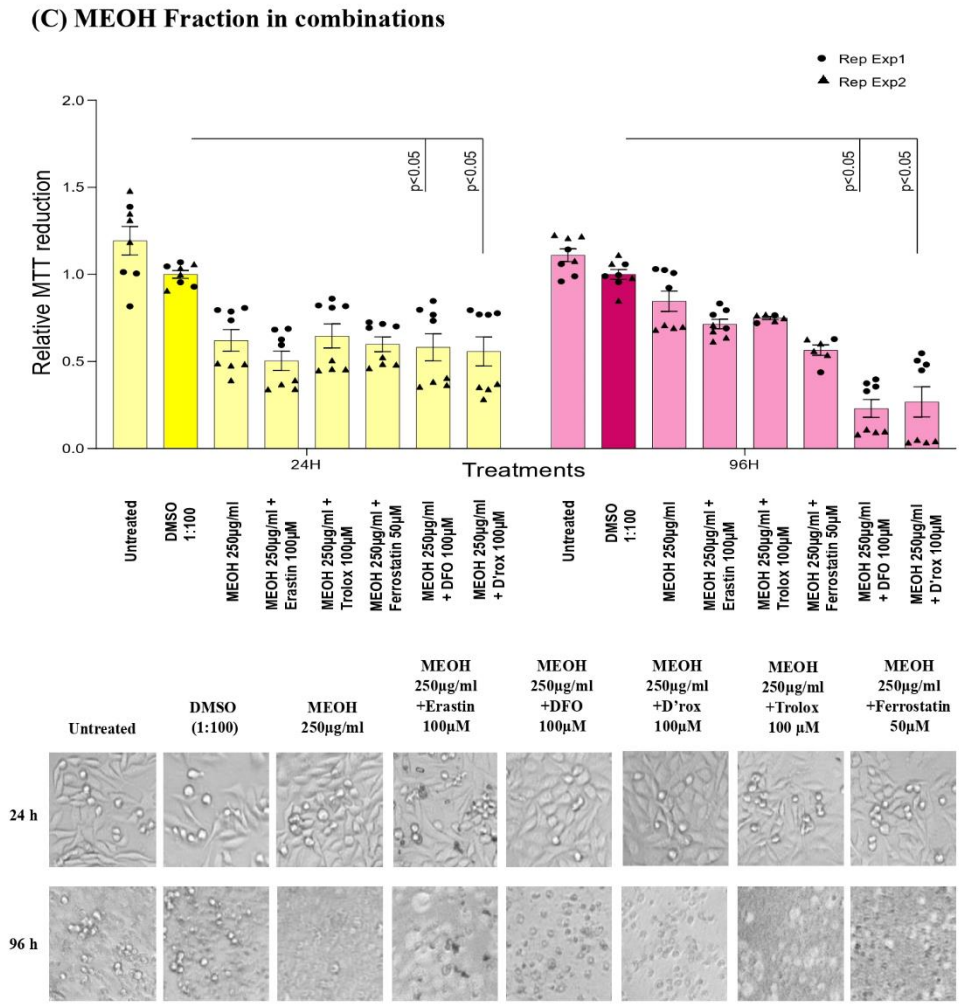
To analyse the cell death pathway of siderophore and related fractions, we performed cell studies in combination with ferroptotic inducers and inhibitors. To rescue the effect of ferroptosis inducers, we combined erastin with all four inhibitors and tested them. These results served as the benchmark for when we test siderophore with these inducers and inhibitors.

Figure 4.11- Cell viability in combinations for (A) Erastin, (B) BuOH fraction, (C) MeOH fraction, and (D) Aqueous Fraction. (n= 4-8; Experiments= 1-2; p <0.001; Mean ± SD).

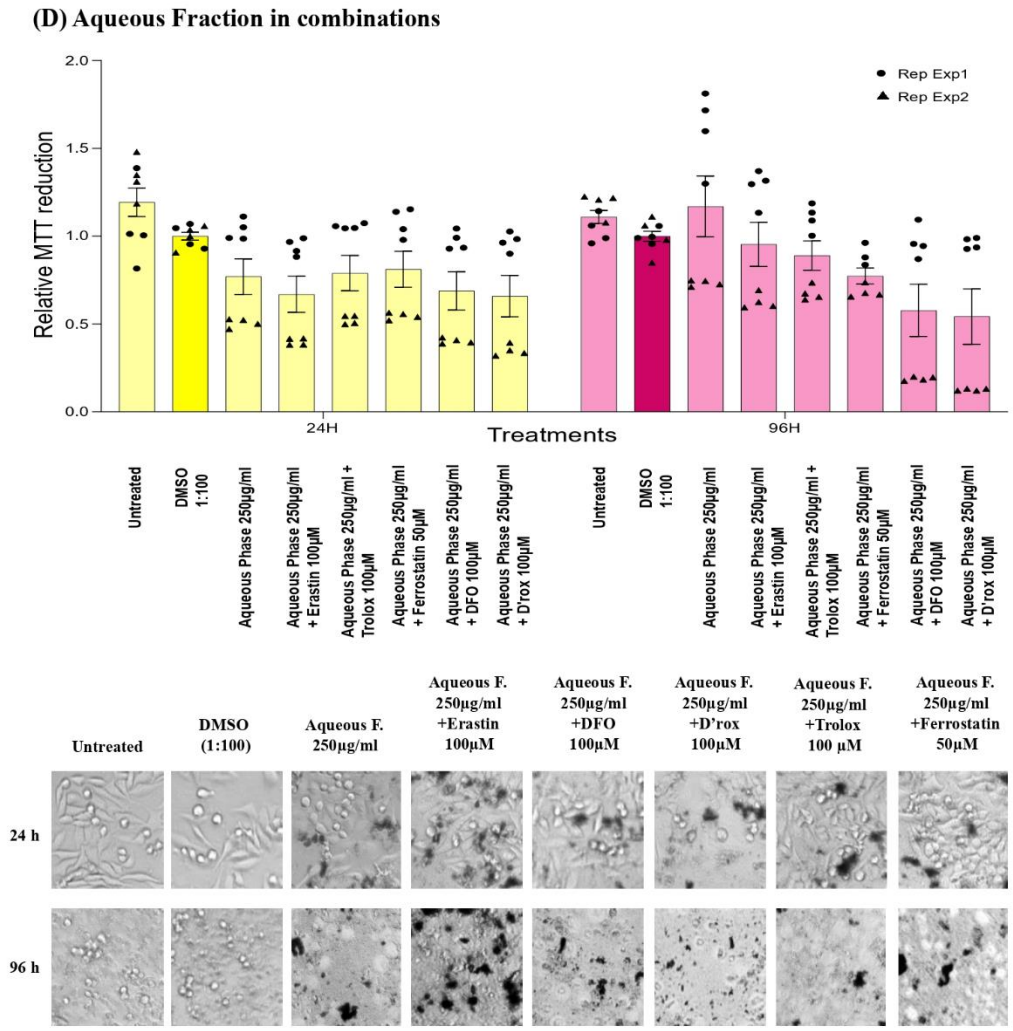


The erastin at the concentration of 100 µM was tested in combination with DFO 100µM, D'rox 100 µM, Trolox 100 µM, and Ferrostatin 50 µM showed a successful reverse in the ferroptotic activity (Figure 4.12 (A)).





When the same idea was applied to the BuOH fraction (Figure 4.12 (B)) at the concentration of 250 µg/ml, the MeOH fraction (Figure 4.12 (C)) at the concentration of 250 µg/ml, and the Aqueous fraction (Figure 4.12 (D)) at the concentration of 250 µg/ml was combined with erastin 100 µM/ml, DFO 100µM/ml, D'rox 100 µM/ml, Trolox 100 µM/ml, and Ferrostatin 50 µM/ml to reverse the cell death, the result showed that the ferroptotic inducer and inhibitors failed to rescue the viability, hence suggesting no ferroptosis or different pathway of cell death.



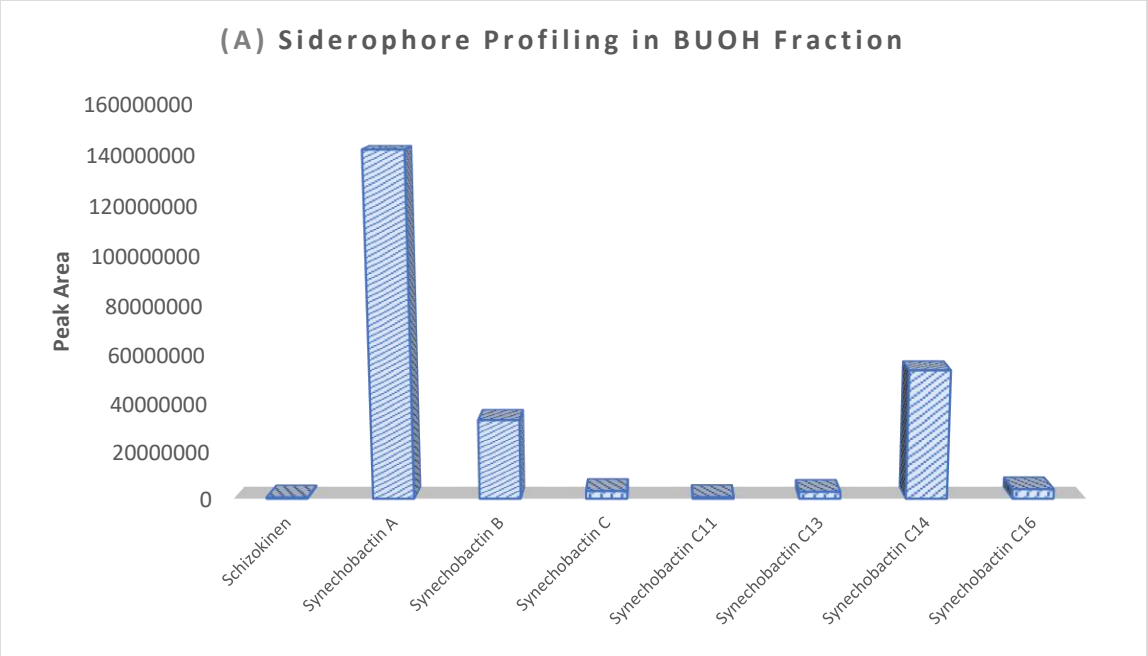
However, further studies are ongoing to state whether the siderophore-rich extract possesses ferroptotic properties or not. The data will be published; however, it is not included in this thesis report.

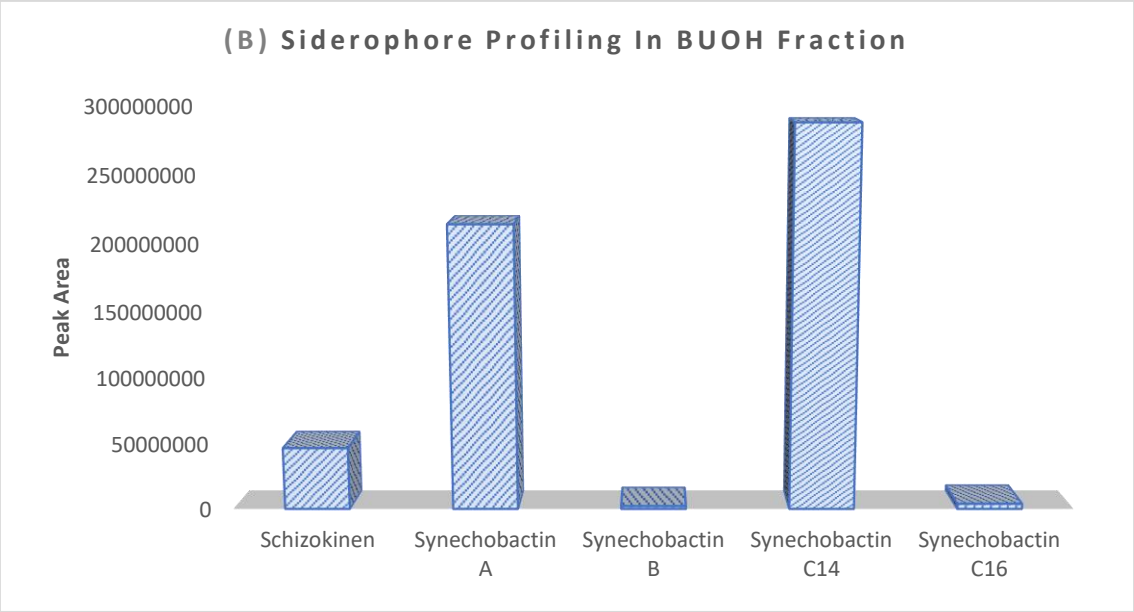
8. Experimental Set-up

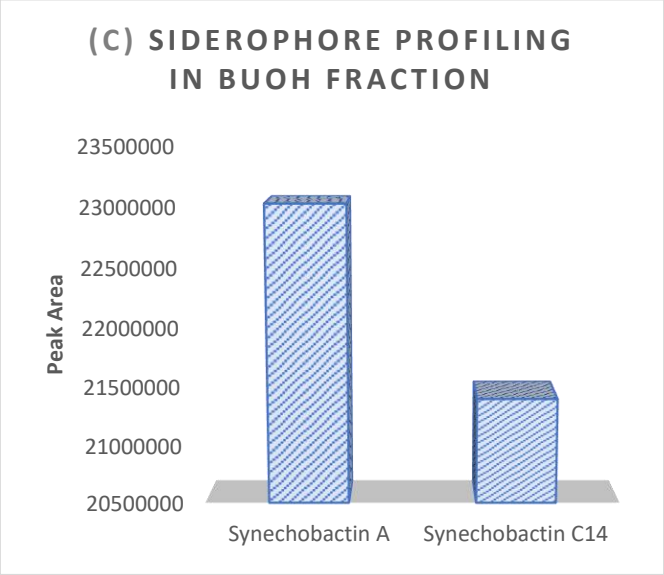
8.1.Treatments

The treatments, such as Butanol Fraction, Methanol Fraction, and Aqueous Fraction, rich in siderophore, were all obtained from the solvent extraction procedure of *Anabaena flos aqua*, profiled, and dried under vacuum. The siderophores in the extract are similar to the ones stated in the previous chapters. The Figure- 4.10 (A-C) shows the composition of each extract following preliminary LCMS data.

Figure 4.12- Siderophore profiling in (A) Butanol Fraction, (B) Methanol Fraction, and (C) Aqueous Fraction.







The Ferroptosis inducer- Erastin (initiates ferroptosis via increased lipid peroxidation) was provided by Dr Breandan Kennedy's lab at Conway Institute, University College Dublin, Dublin, Ireland. The Ferroptosis Inhibitors such as Deferoxamine (Iron chelator), Deferasirox (Iron Chelator), Trolox (Antioxidant, scavenges cytosolic ROS), and Ferrostatin were provided by Dr Breandan Kennedy and Dr Valentina Tonelotto.

8.2. Cell Cultures

The SUIT2 cell line generously provided by Dr Stephen Thorpe (University College Dublin, Dublin, Ireland) was grown in 96 well plates seeded 15,000 cells/cm^2. Dulbecco's Modified Eagle's Medium- low glucose was supplemented with 10% FBS and 1% PenStep (Penicillin-Streptomysin). After 48 hours of culturing, treatments were added. Butanol Fraction from 0-250 µg/ml, Methanol Fraction from 0-250 µg/ml, Aqueous Fraction from 0-250 µg/ml, Erastin from 0-100 µM, Deferoxamine from 0-100 µM, Deferasirox from 0-100 µM, Trolox from 0-100 µM, and Ferrostatin from 0-50 µM. DMSO was used as vehicle control and Hydrogen Peroxide as positive control. The 2 sets of cells were maintained for this study, one at 24 h cell viability and the other for 96h cell viability. Each study had 4 biological replicates, and some studies were repeated thrice.

8.3. MTT assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were plated in 96-well plates, at a density of 15,000 cells/well, in a total volume of 200 µl of complete medium. After treatment for 24 h and 96 h, 100 µl of MTT (5 mg/ml in PBS) was added to each well, and the cells were incubated for 2 h at 37 °C. Dimethylsulfoxide (DMSO) was added to each well after the removal of the MTT, and the plates were shaken to dissolve the formazan for 10 min at 37 °C. The absorbance reading of each well was determined using a microtiter plate reader (SpectraMax® M3, San Jose,CA, USA) at the wavelength of 540 nm. All experiments have four biological replicates for each condition. The optical density of the cells incubated with culture medium alone was considered no reduction and

used as the blank. Differences between groups were determined by analysis of variance (ANOVA) and were considered statistically significant at $p < 0.005$.

8.4. Combination Studies

To determine whether the Anabaena Fraction promoted ferroptosis, they were studied in combination with ferroptosis inducers and ferroptosis inhibitors. Erastin was used as standard and was combined with Deferoxamine, Deferasirox, Trolox, and Ferrostatin. Whereas Butanol Fraction, Methanol Fraction and Aqueous Fraction were combined with Erastin, Deferoxamine, Deferasirox, Trolox, and Ferrostatin. DMSO was used as vehicle control, and hydrogen peroxide was used as positive control. The SUIT2 cell line was grown in 96 well plates seeded with 15,000 cells/well. Dulbecco's Modified Eagle's Medium- low glucose was supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. After 48 hours of culturing, treatments were added. The 2 sets of cells were maintained for this study, one at 24 h post-treatment addition and the other for 96h post-treatment addition. Each study had 4 biological replicates, and some studies were repeated thrice.

8.5. Statistical Analysis

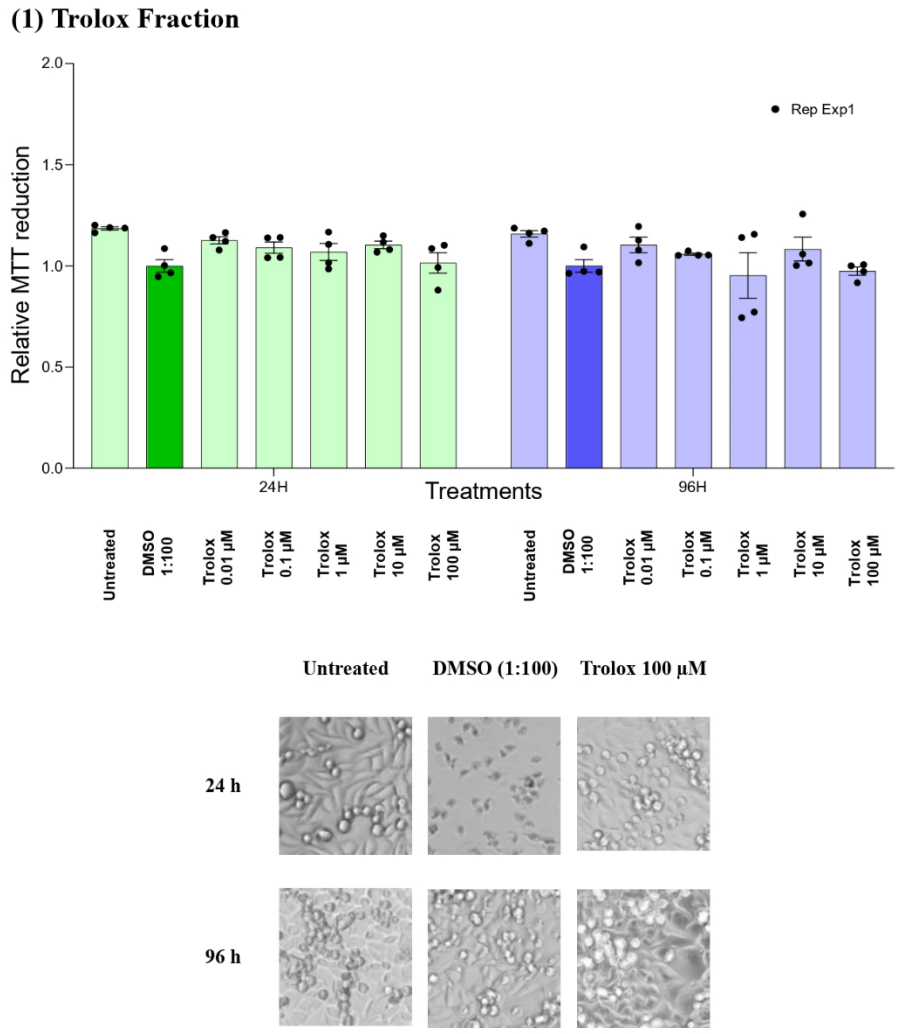
Statistical analysis was realized with GraphPad Prism version 10.0 software (GraphPad Software LLC, Boston, MA, USA). Differences between groups were determined by analysis of variance (ANOVA) and were considered statistically significant at $p < 0.005$.

9. Conclusion

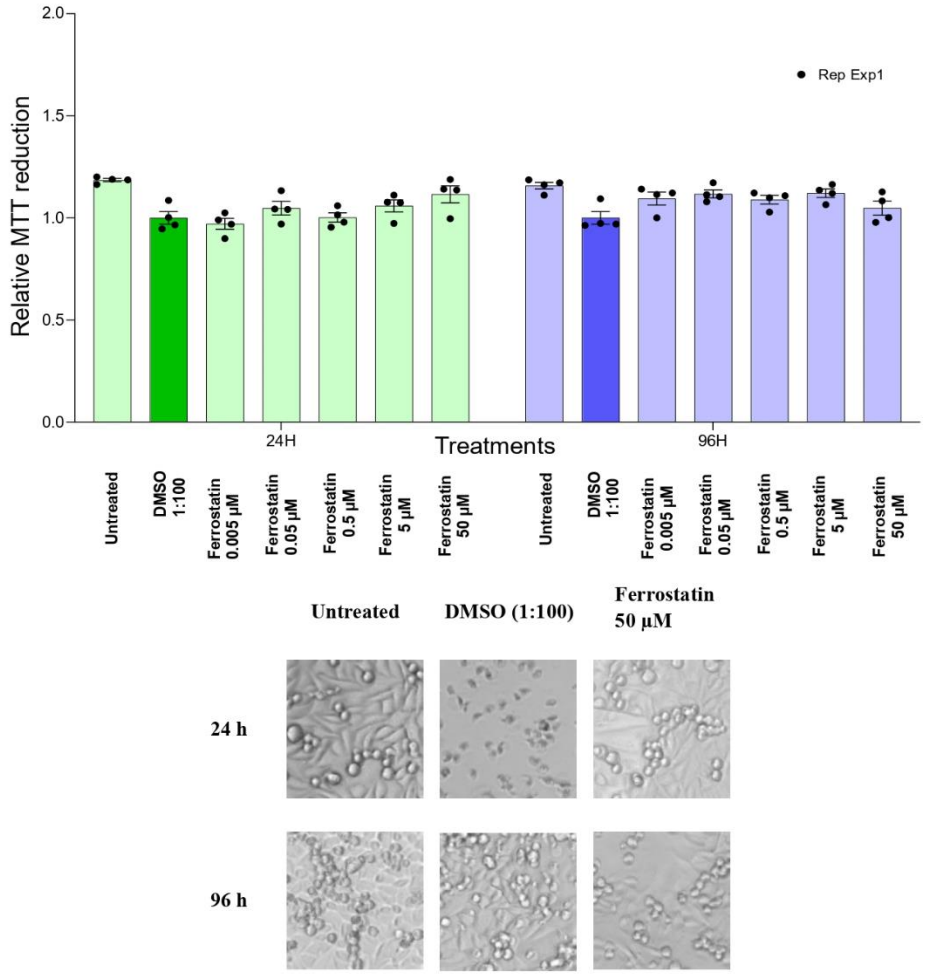
The tests were conducted on siderophore-rich fractions (similar in compositions as stated in the previous chapter) of the *Anabaena flos aquae* for the cell viability in the PDAC (Pancreatic ductal adenocarcinoma) cell line, SUIT2, and showed that the fractions- Butanol Fraction, range 0-250 µg/ml; Methanol Fraction, range 0-250 µg/ml; Aqueous Fraction, range 0-250 µg/ml, exhibited lower cell proliferation at the initial concentrations and noticeable cell death at high concentrations such as 25 µg/ml for Butanol Fraction, 25 µg/ml for Methanol Fraction and 25 µg/ml at 24h and 250 µg/ml for 96h for Aqueous Fraction. To learn more about the cell death pathway, we combined our extract and fractions with ferroptosis inducers and inhibitors. Erastin was used as a ferroptosis inducer and showed cell death, and its activity was successfully reversed by using four different anti-ferroptosis drugs. For the various fractions, we got mixed results as the ferroptotic inhibitors failed to rescue the viability, hence suggesting a different pathway of cell death induction to ferroptosis. The possible reason for having no rescuing effect in the SUIT2 can be no pretreatment with the inhibitor prior to adding the ferroptotic or anti-ferroptosis drugs. The experiment has to be repeated to address the timing of Inhibitor addition related to fractions. The future work includes looking at the mechanism of cell death via multiple assays targeting new pathways, using additional cell models as different calls behave differently and repetition of experiment to optimize the results.

Supplementary Figure

Supplementary Figure 1- Cell viability for (1) Trolox and (2) Ferrostatin, (n= 3-4; Experiments= 1; p <0.001; Mean ± SD).



(2) Ferrostatin Fraction



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CONCLUSION

For my 3.5 years of PhD journey and thesis work, I studied *Anabaena flos aquae*, various media conditions the bacteria require to produce novel secondary metabolites. During the study, I found that using variations in media, growing the strain under an iron-depleted environment, alone does produce novel secondary metabolites, such as new varieties of siderophores, including Synechobactin C16:1, Synechobactin oxyC14, Desacetyl-synechobactin A, Desacetyl-Synechobactin C14, Deoxysynechobactin C14 and Deoxysynechobactin C14:1. However, the growth was slow, time-consuming, costly and needed intense maintenance.

Following this preliminary knowledge and results, we decided to establish *Anabaena flos aquae* UTEX 1444 as a prototypical organism for increased siderophore expression/production, considering the wide range of applications, from ecological investigation to drug discovery, which can benefit from the ability of siderophore production to scavenge ferric ions and chelate toxic metals. We successfully developed a 4-stage pipeline and validated it with our model organism, *Anabaena flos-aquae* UTEX 1444. We initially followed the OSMAC framework, but due to its various drawbacks, we worked and developed a better framework that takes less time, is less expensive and can be elaborated to any microorganism. The main advantage of our 4-stage pipeline was the lower work volumes (of 50 µl) rather than the previously mentioned in the idea OSMAC strategy (of 50 ml). The genes coding for siderophores were discovered and studied, and the promoter regions were analysed. The reporter vector was prepared, cloned with siderophore coding regions and inserted in E.coli to justify the gene expression under various environmental conditions. The screening was achieved using as

little as 50 µl sample, providing a quick and economical platform for testing media variations. Following this, we used the best culture conditions to optimize the culture conditions of *Anabaena flos-aquae* UTEX1444 for siderophore production in the laboratory. The produced siderophores were identified and quantified using mass spectroscopy (MS)-based molecular networking. Assuming that the structurally related compounds share similar fragmentation patterns, we discovered novel siderophores and in increased quantities. In the end, we saw for the first time how the promoter part changes because of various cultural conditions, such as the presence of citrate. It made us understand more about genes controlling the siderophore-making process in cyanobacteria.

For the last part, in collaboration with Dr Stephen Thorpe, Conway Institute, University College Dublin, Ireland, we tested various fractions of *Anabaena flos aquae* extract for the cell viability in PDAC (Pancreatic ductal adenocarcinoma) SUIT2 cell line. The fraction showed lower cell proliferation at the initial concentrations and noticeable cell death at high concentrations. To know more about the cell death pathway, we combined our extract and fractions with ferroptosis (a form of cell death regulated by the presence of iron) inducers and inhibitors. We got mixed results, and due to a shortage of time and sources, we concluded the thesis here. Our further work includes doing western blotting with GPX4 and 4-HNE to confirm that cell death is indeed regulated by ferroptosis. Further, we are testing the cultures for GSH levels, Lipid Peroxidation, Ros activities, etc., known as hallmarks of ferroptosis, to conclude our work. The work is ongoing to claim our hypothesis.

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-Karishma Kundu

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