Bioactive Molecule Discovery:

Medicines Derived from Streptomyces

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I. Introduction

Medicines have been derived from natural products since the beginning of mankind. Modern scientific methods have allowed for systematic approaches to identify and test new natural products. What was once considered herbal remedies from plant sources is now seen as a possible treasure trove of new bioactive molecules. Traditionally, herbal medicines were passed down through generations and differ widely across cultures, but the idea is the same: natural products (from plants, animals, and minerals) serve to promote the well-being of an individual.[1] Although history is full of examples of medicines that were neither therapeutically useful nor good ideas, the future of natural products as medicines proves to be promising.

A. A Brief History of Natural Products Used as Medicines

First isolation of morphine occurred in 1804 by Friedrich Sertürner and is widely considered to be the first natural product isolation from poppy seeds (**Figure 1**).[2] Morphine was also the first natural product to become commercially available, and was released to the public by Merck in 1826.[3] Soon, the demand for morphine grew and a total laboratory synthesis method was viewed as important since an average poppy seed only produces 25 μg of morphine. A standard dose is 10-30 mg.[4] So, in order to obtain enough for one minimum dose of morphine, it would take 400 poppy seeds or 0.12 g of poppy seeds. Finally, in 1952 by Marshall D. Gates, Jr. successfully created morphine in the lab. It consisted of 31 steps and produced a yield of 0.06%.[2]

B. Importance of Bioactive Molecule Discovery

Bioactive molecule discovery is an important industry in the United States, accounting for roughly two-thirds of the drugs approved each year by the Federal Drug Administration

(**Figure 2**). This field is often responsible for identifying new drug classes, in addition to new drugs within existing classes. Scientists are always looking to expand the frontier into uncharted territory. One frontier that has proven itself is the world of bacteria. For decades bioactive molecules have been extracted from bacteria. In contrast to the terrestrial bacteria, marine sediment is a rich and relatively untapped reservoir of novel bioactive products.[5] Over 15,000 structurally diverse bioactive molecules with an astounding assortment of bioactivities have been identified from marine environments since the 1970s.[6] Specifically, the genus *Streptomyces* has supplied roughly two-thirds of the clinically relevant antibiotics since the establishment of modern medicine.

Figure 1. Poppy seed plant, pods, and seeds.

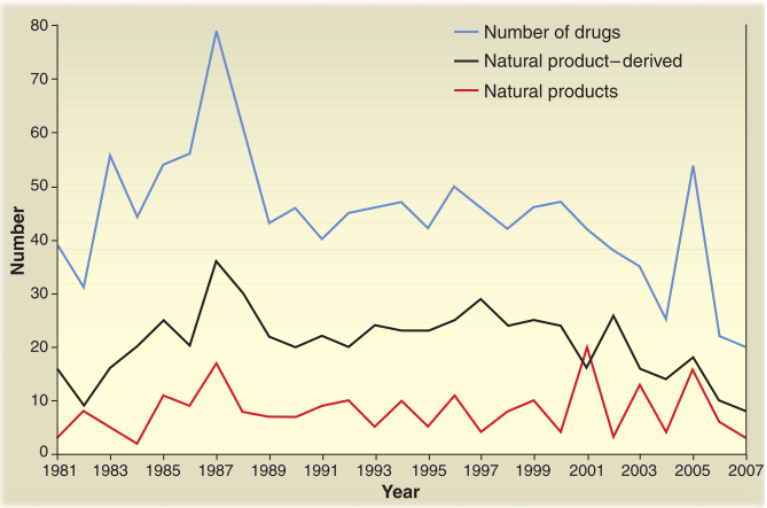

Figure 2. The number of drugs approved in the US between 1981 and 2007.[7]

C. Streptomyces and their Bioactive Molecule Synthesis

Streptomyces has approximately 550 known species. These bacteria are aerobic, Gram-positive, filamentous (**Figure 3**), and are known for giving dirt its signature smell. In fact, the molecule responsible for this odor is geosmin (**Figure 4**), which is a secondary metabolite

extracted from *Streptomyces*. Additionally, this is the characteristic smell of rain. Most people assume it is actually due to the rain itself, but in fact, it is the bacterium's response to increased water in its immediate surroundings. Geosmin is also the first terpene to be isolated from *Streptomyces*. As of September 2001, over 7600 bioactive molecules (more specifically, secondary metabolites) have been discovered and tested from the genus *Streptomyces*.[8]

Secondary metabolites are biosynthetic compounds that the organism synthesizes but are not essential for basic metabolic processes, such as growth and reproduction. Nevertheless, many secondary compounds function as signal molecules, used to control the producer's metabolism. Additionally, another function attributed to these secondary metabolites is a suppression of competing microorganisms in the environment, which offers the antibiotic-producing bacteria an advantage in competing for nutrients.

The biosynthesis of secondary metabolites in microorganisms is usually limited. To be usable for the commercial production of secondary metabolites, high-yielding strains need to be selected through multiple mutations of the strain´s genetic material, optimization of culture conditions and genetic engineering. Today's poppy plants are genetically modified to increase morphine production by ~50%.

It was long thought that this bacteria genus was not a terpenoid producer. However, advances in genome sequencing, discussed later, have discovered several enzymes, including terpene synthases, which has led to increased terpenoid discovery.[9] *Streptomyces* has been responsible for some of the most common drugs in the last 20 years, such as Amphotericin, Neomycin, Streptomycin, Tetracycline, Ivermectin, Bleomycin. This genus of bacteria extends filaments up through the dirt toward the open air or water (**Figure 5**).

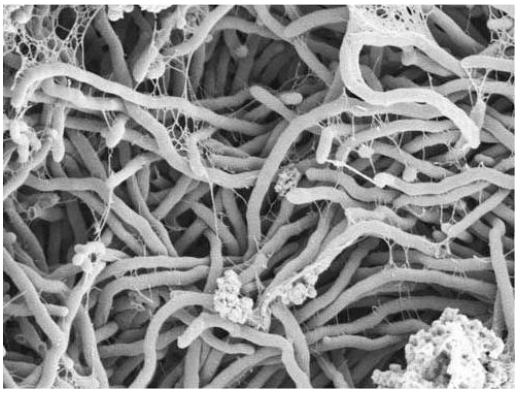

Figure 3. Scanning electron micrograph of *Streptomyces* strain CNH365.[10]

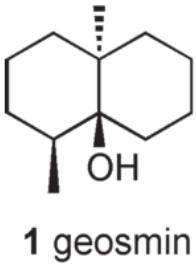

Figure 4. Structure of geosmin.

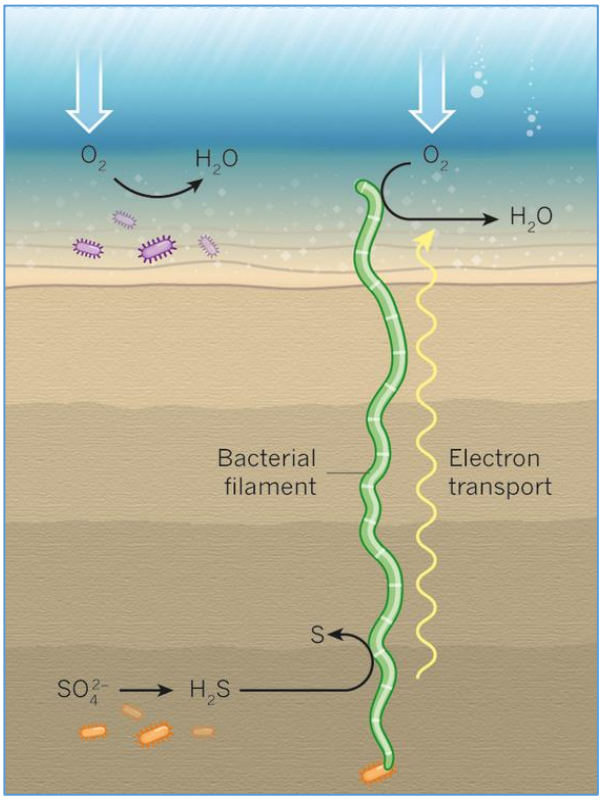

Figure 5. Filamentous *Streptomyces* bacterium in marine sediment.

D. Genome Mining

The process of genome mining has become increasingly popular in recent years, with the improvement of gene sequencing methods.[11] Genome mining is responsible for the characterization of human physiological processes, the identification of new drug targets in human pathogens, and the discovery of new chemical species from natural sources.[12] Researchers search through genomic databases and identify areas of DNA which code for an organism's secondary metabolisms. Once a sequence is identified, one can modify the bacteria to produce more of the metabolite, usually utilizing feedback loops that "turn on" the production of the molecule. The basic premise is that if an organism can manufacture a molecule, it is probably going to cause biological activity.[13]

The basic method for genome mining typically involves computer programs which are designed to utilize sequencing data and propose a molecule. This happens in a few steps: first the sequence is analyzed and an enzyme structure is anticipated, second, a molecule catalyzed by the enzyme is predicted. **Figure 6** provides a visual representation of these steps. These methods are highly automated and can usually be completed with only a few clicks of a mouse button. Many structures can be quickly analyzed with these computer programs (such as BLAST and THREADER), which makes this option popular with pharmaceutical companies who are interested in high volume throughput results in order to maximize profits.[14]

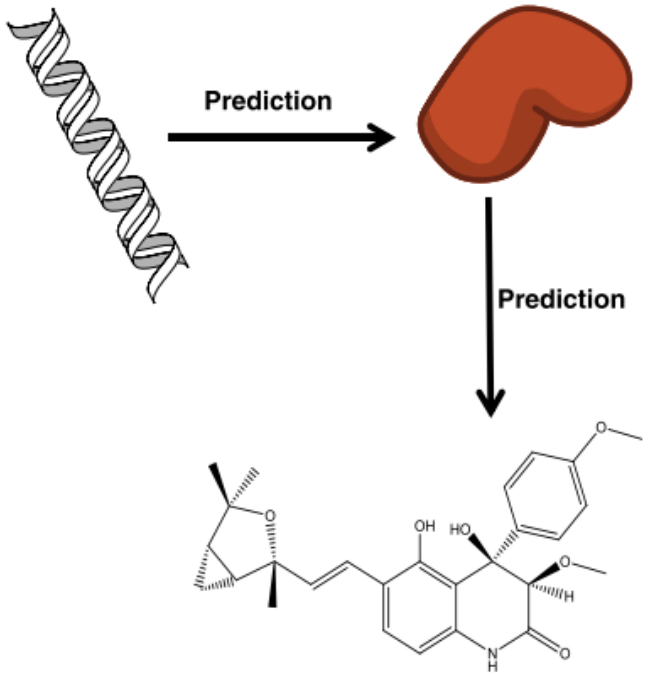

Figure 6. Bioinformatics approach to molecule discovery.[15]

Genome mining is a multifaceted approach with many options for analysis (**Figure 7**). Many different tools can be used to elucidate biosynthetic pathways in microorganisms, each with its own benefits and limitations. Prediction of physic-chemical properties relies heavily on

bioinformatic data. Once a new enzyme is proposed, researchers estimate the precursors and, according to the enzyme activity, propose a molecule and infer its properties. This method offers the benefit of attempting multiple pathways in a fairly short amount of time, without requiring the use of materials and specialized equipment. Gene knock-out/comparative metabolic profiling offers a "snapshot" of the organism's metabolism, in an effort to obtain a better understanding of what is happening in the organism at that time. Generally, an organism can be changed (e.g. temperature, chemical environment, and genetic manipulation) and the metabolic products can be monitored. The primary benefit of performing these tests is understanding the organism as a whole, which can guide researchers in future decisions about secondary metabolites. Limitations are generally caused by lengthy experiments in which several samples are taken over time and each sample must be analyzed for metabolites.

Heterologous gene expression/comparative metabolic profiling is an extension of the previous method. In addition to the previously mentioned method, a comparison is used between the organism's gene sequence and a known gene sequence. Similarities and differences are analyzed to provide a more focused course of experiments, which translates to a benefit of decreased experiment time due to a reduced number of pathways that need to be identified. *In vitro* reconstitution involves mimicking the biological processes, essentially, in a test tube. Metabolites are able to be more easily analyzed, as concentrations may be increased in order to produce a higher yield of target compounds. However, a true understanding of the organism is sacrificed.

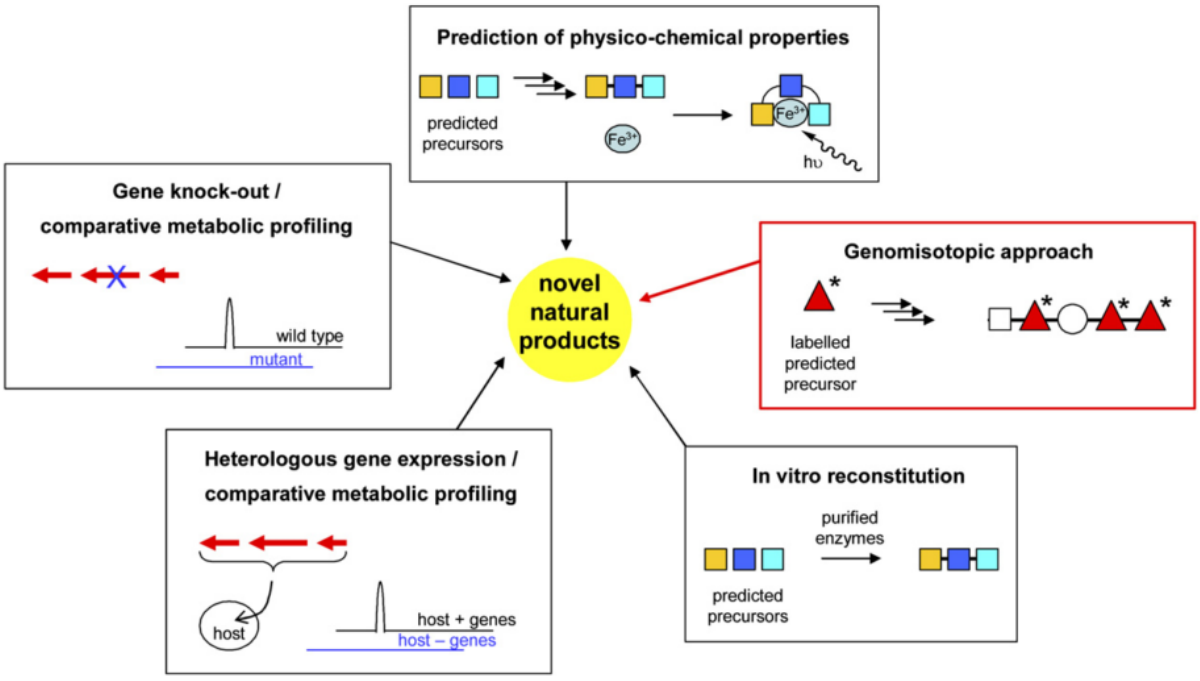

Figure 7. Schematic representations of different strategies for the isolation of new natural products by genome mining.[16]

E. Marine Sediments and Terrestrial Soils

The composition of marine sediment is highly variable, which means the amount of bacteria present is dependent upon the depth of sediment and water, distance from the mainland, and temperature. There can be up to 160 million bacteria per cubic centimeter in the sediment, under the best conditions.[17] For those strains that call terrestrial soil home, the main criteria for dense bacteria populations is a moderate amount of decaying plants. *Streptomyces* feed on the decomposing matter, which aids the process. Considering the abundance of this genus of bacteria and the ability to cultivate many of the strains in a laboratory setting, it is an ideal way to discover bioactive products.[3]

The high variability in habitat leads to a diverse population of bacteria, from which we can isolate these bioactive molecules. In spite of the variety of their structures, bioactive secondary metabolites are synthesized from simple building units used in living organisms for the biosynthesis of cellular structures. These units include amino acids, acetate, isoprene, sugars, nucleotides, etc. With this in mind, an overview of the discovery process will be presented.

II. Discovery Process

A. Introduction

The process of bioactive molecule discovery is a multistep process that can span months. In short, the steps include sample collection, isolation, purification, characterization, bioactivity screening, and synthesis. Even though several thousands of compounds isolated from bacteria having some biological activity are known, new substances are still sought by pharmaceutical companies, in order to combat new diseases, new infections and even an increase in antibiotic resistance. The probability of finding a new molecule that would be usable as a new antibiotic or another biologically active compound is low, so a large number of bacteria must be screened. A rough estimation says that about 100,000 microorganisms are screened for the presence of biologically active compounds per year.[18] Modern screening techniques are highly automated. Companies even produce screening kits. Typically, the specific methods for isolation, purification and screening are not published. This manuscript will briefly describe some of the more common methods in each category, utilizing *Streptomyces* found in marine sediment as an example (**Figure 8**).

Figure 8. A sample of marine sediment containing a highly-filamentous *Streptomyces* strain.

B. Sample collection

Marine sediment collection is generally done by a diving team or by dragging a collection device behind a boat. A diving team can be more selective regarding the location of the sample, such as between rocks or in underwater caves, however, an increased cost is associated with this. The research team will weigh their options and choose which best suits their needs. Farneside A (**Figure 9**) from *Streptomyces* strain CNT-372 was isolated from a sediment sample collected in July 2008 at a depth of 5m off Nacula Island in the Yasawa Island chain, Fiji.[19] A new *Streptomyces* strain was discovered (strain CNH-189) from a near-shore marine sediment collected off Oceanside, California, which produced actinarone.[20]

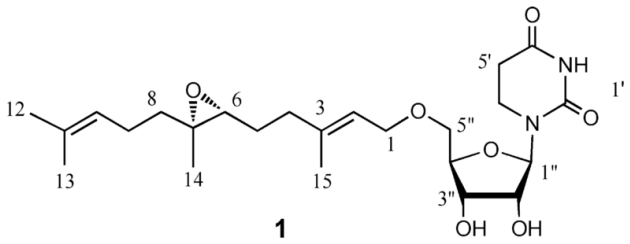

Figure 9. Structure of farneside A.

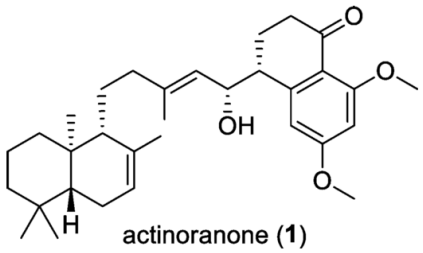

Figure 10. Structure of actinoranone.

Acquiring a terrestrial sample is much easier. One may simply walk to the nearest patch of dirt. However, to obtain a quality sample, researchers generally seek a forested area where many fallen trees can be found. An overly dry environment is usually avoided, as well as extreme temperatures, though metabolites from extremophiles are something of interest to researchers, although it may not be practical for experimentation. Once the sample has been collected, the specific location and site information is recorded, including date, temperature, and any other pertinent information that the researchers deem as potentially important.

C. Isolation and Purification

After the bacteria have been removed from the sediment and other impurities, usually a detergent is applied to break down cell walls. Also, cellular contents are usually suspended in a phosphate buffer to aid in solubility of molecules of interest. The sample is then shaken in order to promote homogenization, in preparation for separation. Typically, a sample is first filtered to remove any large debris or other large molecules, such as complex proteins, which can be removed if a pressurized filtration system is used with controlled pore sizes. Considering the numerous compounds that are present in biological samples, traditional column chromatography is usually not the first choice in separation. Once filtration is complete, the sample is separated into smaller aliquots and run through a high-performance liquid chromatography instrument with a mass spectrometer attached. Generally, the instrument will have limitations on the molecular weight of molecules, which can aid in narrowing the number of compounds that must be characterized. Size exclusion chromatography can also assist with this problem. In the case of oligomycin A (**Figure 11**) (an antibiotic extracted from *Streptomyces diastaticus)*, flash chromatography was utilized with prepackaged RP-18 cartridges.[21]

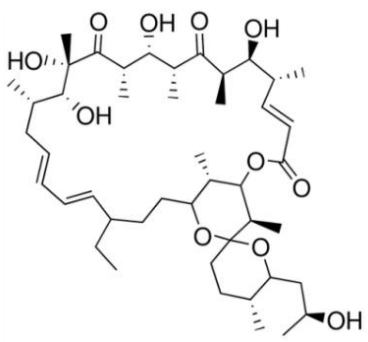

Figure 11. Structure of oligomycin A.

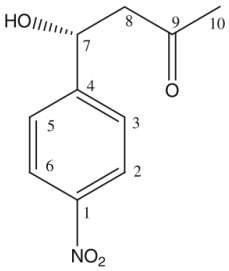

Figure 12. Structure of 4-hydroxy-4-(4-nitrophenyl)butan-2-one.

no.	δ_C, #H.[b]	δ_H (mult, J (Hz))	HMBC
1	39.3, CH_2	1.85,[c] 0.94 dt (13.5, 3.6)	3, 18
2	25.4, CH_2	1.55, m 1.29, m	4, 10
3	42.3, CH_2	1.42, m 1.16[c]	5
4	32.7, C		
5	50.4, CH	1.16, m	7
6	23.7, CH_2	1.97, m 1.86, m	7, 8
7	122.2, CH	5.38, br s	6, 8, 19
8	135.0, C		
9	54.2, CH	1.61, m	11, 12
10	36.7, C		
11	25.4, CH_2	1.55, m 0.78, m	8, 13
12	42.1, CH_2	2.24, m 2.01, m	9, 13, 14
13	139.5, C		
14	126.8, CH	5.25, d (8.5)	15, 20
15	70.4, CH	4.47, dd (8.5, 8.5)	8′
16	32.5, CH_3	0.86, s	3, 4, 5, 17
17	21.0, CH_3	0.90, s	3, 4, 5, 16
18	12.8, CH_3	0.78, s	1, 5, 9, 10
19	21.5, CH_3	1.71, s	7, 8, 9
20	15.6, CH_3	1.63, s	12, 13, 14
1′	197.3, C		
2′	115.5, C		
3′	162.7, C		
4′	97.2, CH	6.50, d (2.0)	2′, 5′, 6′
5′	164.4, C		
6′	107.5, CH	6.56, d (2.0)	2′, 4′, 5′, 7′, 8′
7′	150.4, C		
8′	45.3, CH	2.97, m	15, 2′, 6′, 7′, 9′, 10′
9′	23.8, CH_2	2.12, m	1′, 7′
10′	36.3, CH_2	2.44, ddd (18.0, 11.0, 8.0) 2.59, dt (18.0, 4.5)	1′, 2′, 8′, 9′
11′	54.9, CH_3	3.83, s	3′
12′	55.0, CH_3	3.87, s	5′

Figure 13. NMR data for the elucidation of actinarone structure.[20]

D. Characterization

Mass spectrometry is generally done first, simply because it is part of the isolation and purification step. After this is done, researchers focus on a more narrow range of molecular weights which are indicative of bacterial secondary metabolites. Nuclear magnetic resonance (NMR) is usually performed, as well as crystallography to generate an Oak Ridge Thermal

Ellipsoid Plot (ORTEP). Occasionally UV-Vis circular dichroism is employed to aid in chiral identification. If this is not enough to confirm chirality, ligand exchange liquid chromatography can be used to separate and identify molecules with varying chirality. For 4-hydroxy-4-(4-nitrophenyl)butan-2-one (**Figure 12**) (a cytotoxic compound isolated from terrestrial *Streptomyces collinus*) was characterized by ^{1}H and ^{13}C NMR spectra.[22] Also, for highly cyclic structures, NMR data is indispensable. In the case of actinarone (previously introduced), data was collected and analyzed using ^{1}H, ^{13}C, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond correlation spectroscopy (HMBC) (**Figure 13**), rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY), and heteronuclear long-range couplings (HETLOC[10]).[20] Clearly, the amount of effort required to identify some of these multi-ring structures is great.

E. Bioactivity Screening

Generally, the first type of screening is done with target microorganisms (such as *Staphylococcus aureus, Sarcina lutea, Klebsiella pneumoniae, Salmonella gallinarium, Pseudomonas spp., Bacillus subtilis,* and *Candida albicans)* in well-plates with the specific extracted molecule applied. Reaction to the extract is monitored for the suppressed growth of the microorganism. Any promising compounds are tested further utilizing enzyme assays and sometimes well-plates with viruses. A new molecule, anthracimycin (**Figure 14**), discovered in *Streptomyces* strain CNH365, was found to significantly restrict the growth of anthrax (*Bacillus anthracis*). Utilizing a broth microdilution assay and metabolic labeling experiments, in which an organisms metabolites are monitored via fluorescent labeling and spectroscopy, it was found that anthracimycin inhibits DNA and RNA synthesis.[10]

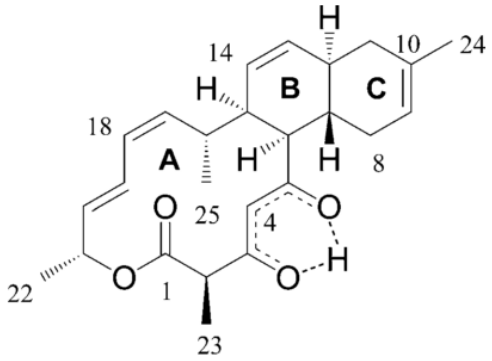

Figure 14. Structure of anthracimycin.

F. Synthesis and Genome Mining

Molecules that show bioactivity against target microorganisms are investigated to figure out how the bacteria produce the metabolite. With advances in gene sequencing and a greater understanding of how to isolate genes based on function, recently more and more biosynthetic pathways have been elucidated using these techniques. A researcher can isolate a gene that encodes for enzymes that catalyze the synthesis, for example, a typical scheme for biosynthesis of novel compounds merochlorin A-D is shown in **Figure 14**. In this figure, each code next to an arrow has been identified using genome mining as being responsible for that step in biosynthesis. As an example, the mcl23 gene codes for an aromatic prenyltransferase gene (which transfers the long prenyl group to the aromatic site for attachment).

As previously discussed, genome mining has become an indispensable tool for researchers hoping to elucidate new bioactive molecules.[23] *Streptomyces coelicolor* is the most commonly studied among actinomyces strains. One such analysis of genetic sequence led to the discovery of a section of the sequence which coded for previously unknown nonribosomal peptide synthetase. This, coupled with metabolic profiling, potential precursors were selected

and incubated with the new synthetase. Researchers isolated coelichelin from the assay, and later found it to contain bioactive properties.[24]

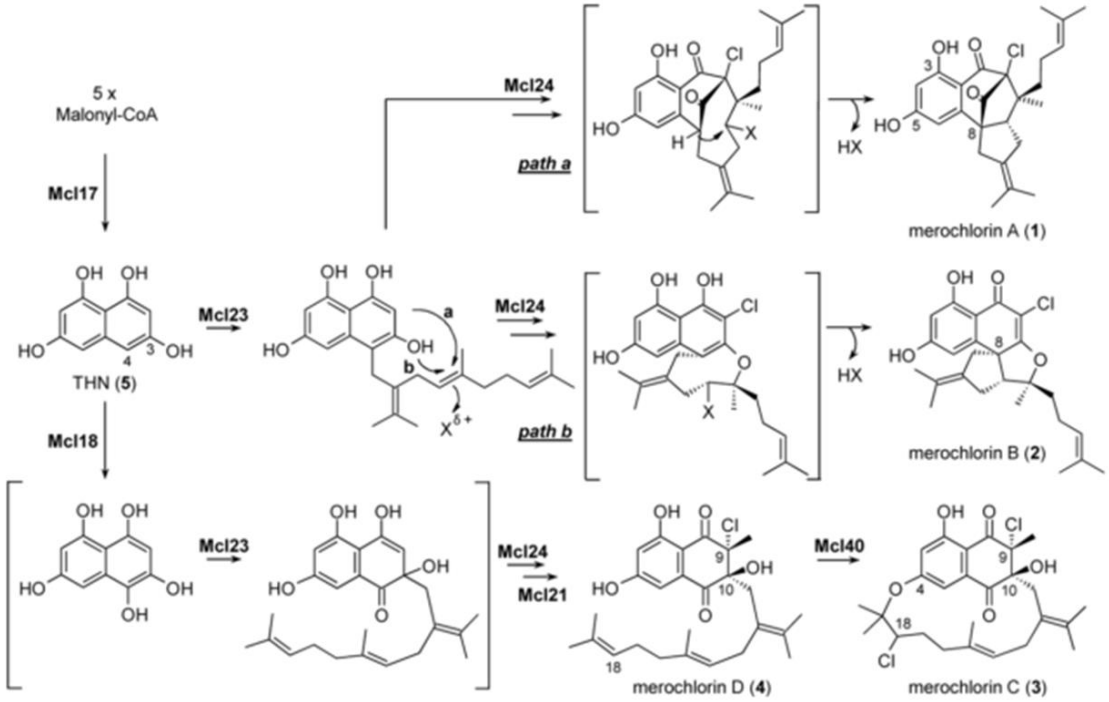

Figure 15. Total biosynthesis of merochlorins A-D via enzymatic pathways discovered by genome mining.[25] Compounds **1** and **2** were found to have high bioactivity against methicillin-resistant *Staphylococcus aureus*.

Finally, a complete lab synthetic mechanism is sought, in order to upscale production of the molecule of interest. This is especially important for molecules whose natural abundance is low. Often a preliminary step is to genetically modify the bacteria strain to increase production of the metabolite of interest. **Figure 15** shows a complete synthesis of merochlorin A. The steps include aromatization, alkylation, chlorination, and oxidative cyclization. In this synthesis, five steps were reported, with an overall yield of 0.06%.

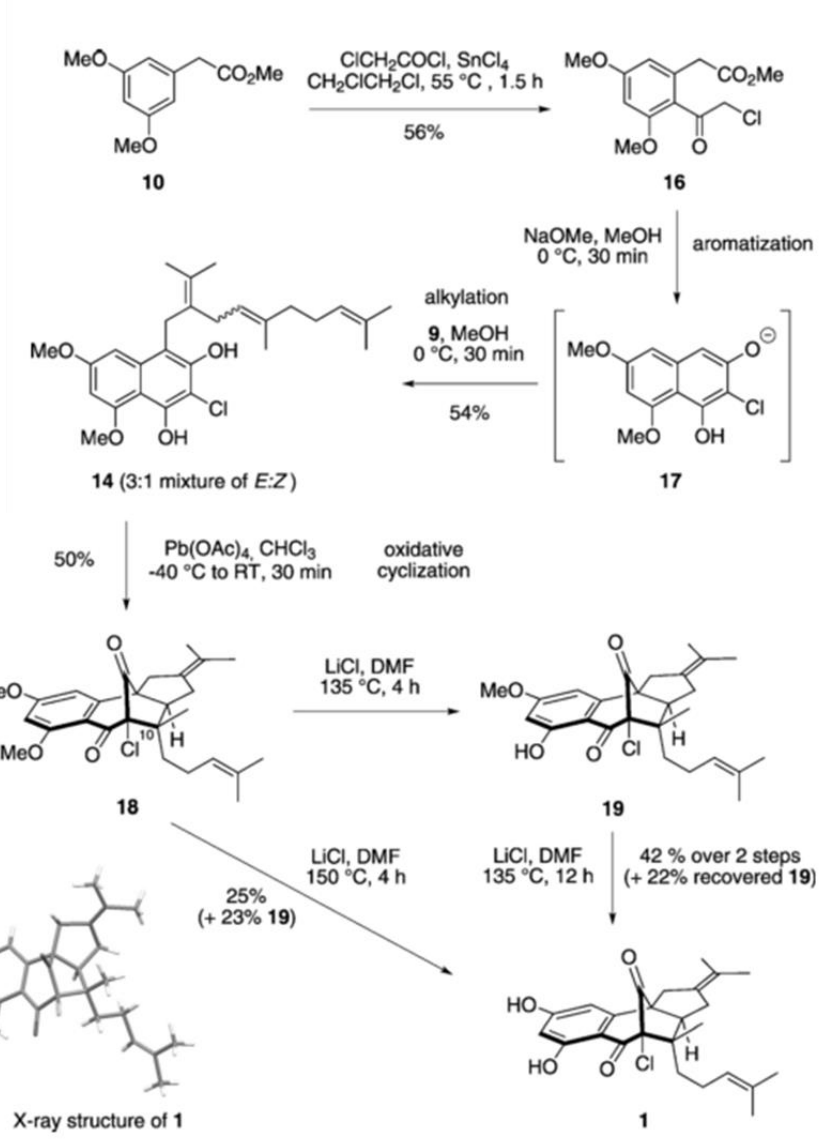

Figure 16. Total biomimetic synthesis of merochlorin A.[26]

III. Conclusion

In order to exploit biological activity of naturally-occurring compounds, it is vital to understand the mechanisms which underlie the effects of different bioactive products. Research in this field is focused on just that. The diversity of natural products is so vast, that one could consider nature as a never-ending supply of medicines, just waiting to be discovered. Many new

technologies have allowed this field to advance rapidly in recent years, especially genomics. Since the discovery of morphine more than 200 years ago, researchers have changed modern medicine with the ability to discover and synthesize bioactive compounds.

Through the discovery process, natural products are transformed from crude product to pure compound. The bioactivity of these molecules is examined after thorough characterization that can take months. Isolation and purification methods can be very complex and intense. Streptomyces bacteria, whether they are found in terrestrial and marine environments, can provide a new frontier to the field of bioactive molecule discovery. The terpenoids discovered by genome mining are numerous and inspires researchers to look for more.

IV. Outlook

The field of bioactive molecule discovery has grown in the last decade, thanks to advances in genome sequencing methods, which can uncover "silent pathways" to molecules yet undiscovered.[27] Silent pathways are the pathways in an organism that exist to produce a secondary metabolite, but the metabolite itself has not been isolated in a sample. There has even been speculation on the ability to recover extinct organisms in order to screen their metabolites for bioactivity. Considering the vast quantity of living species has been estimated between 2 and 100 million, the volume of possible biosynthetic products remaining to be examined is vast. Systems biology could eventually map the probable metabolic products for most species. Also, just as synthetic chemists utilize an array of reagents and known mechanisms, synthetic biologists could do the same, only using enzymes and other metabolic pathways.

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