SCREENING RARE FILAMENTOUS ACTINOBACTERIA FOR NOVEL ANTIMYCOBACTERIAL COMPOUNDS

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DECLARATION

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Abstract

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (*M.tb). According to the World Health Organization (WHO), TB is one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent. There were an estimated 1.2 million TB deaths among HIV negative people in 2019 and an additional 208 000 deaths among HIV positive people.

Although there are drugs that can cure TB, drug-resistant TB continues to be a public health threat. In 2019, there were about half a million new cases of rifampicin-resistant TB (of which 78% had multidrug-resistant TB). Novel drugs need to be isolated and developed to combat this spread of drug resistance.

Historically, natural product screening has been an effective tool in anti-tuberculosis drug discovery. Examples include rifampicin, derived from rifamycin which was originally isolated from the bacterium *Amycolatopsis rifamycinica* and streptomycin originally isolated from *Streptomyces griseus*. Both these bacteria belong to the phylum *Actinobacteria.* Due to this history, filamentous actinobacteria were selected for screening of novel antimycobacterial compounds. However, the focus in this investigation was on species from understudied genera, the so-called “rare actinobacteria” that have not been extensively explored for antimycobacterial compounds – to increase the likelihood of discovering novel antimycobacterial compounds.

The actinobacteria were assessed for antimycobacterial properties using *Mycobacterium aurum* A+ by the overlay method. *M. aurum* has been shown to be a suitable indicator for *Mycobacterium tuberculosis*, is safer to work with, and more amenable to high-throughput screening.

Once active strains were identified by the overlay method, the zone of inhibition was excised, and the compounds were extracted using ethyl acetate. The extracts were analysed with a high-resolution mass spectrometer, and their mass spectral data were analysed using Global Natural Products Social (GNPS) molecular networking methodology. Molecular networking allowed for the dereplication of known compounds

early in the drug discovery process. Strains *Amycolatopsis circi* S1.3^{T}, Actinomadura strain M2 grown in ISP2 medium and *Kribbella speibonae* SK5 produced active fractions. These compounds could not be matched to any known compound in the GNPS database and, therefore, could be novel antimycobacterial compounds.

List of Abbreviations

AFB	Acid-fast bacteria
AIDS	Acquired Immune Deficiency Syndrome
BCG	Bacille Calmette-Guerin
BLA	Biologics License Application
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus Disease of 2019
CYP3A4	Cytochrome P450 3A4
DSMZ #553	German Culture Collection medium #553
FDA	Food and Drug Administration
G-C percentage	Guanine-Cytosine content
GNPS	Global Natural Products Social Molecular Networking
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
IND	Investigational New Drug
ISP2	International *Streptomyces* Project medium #2
JCM #61	Japan Collection of Microorganisms medium #61
M.tb	*Mycobacterium tuberculosis*
MDR/RR-TB	Multidrug-resistant TB or rifampicin-resistant TB
MDR-TB	Multidrug-resistant TB
MIC	Minimal Inhibitory Concentration
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OD_{600}	Optical density measured at a wavelength of 600 nm

QTOF	Quadrupole Time of Flight
RR-TB	Rifampicin-resistant TB
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2 causative agent of the coronavirus disease 2019
TB	Tuberculosis
TLC	Thin Layer Chromatography
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant TB
ZOI	Zone of Inhibition

Chapter 1: Literature Review and Project Proposal

1.1 Introduction

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (M.tb) (Ellner, 2012). TB can also be caused by the closely related species *Mycobacterium africanum* and *Mycobacterium bovis,* but *M. tuberculosis* causes most TB cases (Loeffler, 2007). *M. tuberculosis* was first isolated in 1882 by the microbiologist Robert Koch (Cambau & Drancourt, 2014). The bacteria usually infects the lungs (pulmonary TB), but can spread to other parts of the body. *M. tuberculosis* transmission is airborne (Centers for Disease Control and Prevention (CDC), 2019; World Health Organisation (WHO), 2018).

There are two types of TB conditions: active TB, which causes the disease, and latent TB infection. *M. tuberculosis* multiplies in the body of a person with active TB, and the infected person displays signs and symptoms indicative of TB disease. According to the CDC (2019), pulmonary TB disease symptoms include:

- A bad cough that lasts for three weeks or longer;
- Coughing up blood or sputum; and
- Chest pains.

Other symptoms, as stated in CDC (2019), may include:

- fatigue
- weight loss
- loss of appetite
- chills
- fever
- night sweats

Symptoms of extrapulmonary TB disease depends on which part of the body is infected.

In latent TB, the individual's immune system prevents the pathogen from multiplying, and the person is no longer infectious. With latent TB, the infected

person does not show any symptoms, but live bacteria persist in their body, which means that active TB can recur (CDC, 2019).

Latent TB becomes active TB when *M. tuberculosis* overcome the defences of the immune system. Approximately 5–10% of people with latent TB will develop active TB. The risk of developing TB disease is considerably higher for people with a weakened immune system such as HIV positive people than for people with normal immune systems (CDC, 2019).

1.2 Pathogenesis and Transmission

The cycle of pulmonary TB infection starts with *M. tuberculosis* aerosol droplets being released from an infected human. A portion of 1–10 bacilli are scattered through the air, making the risk of transmission likely since 1–5 organisms are sufficient to cause an infection (Ellner, 2012; LabCE, 2019). *M. tuberculosis* is transmitted through the air when a person breathes in the droplet nuclei containing *M. tuberculosis*.

The droplet nuclei navigate through the mouth or nasal passages to the upper respiratory tract and bronchi to the alveoli of the lungs (Mandal, A., 2019). Only aerosol droplets of less than 5 µm make it to the alveoli when inhaled. These droplets may contain 1–3 bacilli per droplet. Larger droplets are trapped in the upper respiratory system by mucus and ciliary action (Fernandez Tena & Casan Clara, 2012). Successful transmission is affected by an assortment of conditions, including proximity and length of contact with a person with the TB disease and the immune-competency of the person infected with *M. tuberculosis* (Churchyard, 2017; Mathema, 2017; Turner *et al*., 2017).

In the lungs, *M. tuberculosis* is quickly phagocytised by alveolar macrophages that most often can kill the entering bacteria (Urdahl, 2011). If the bacilli survive this first line of defence, they start actively replicating in macrophages (Wolf, 2007) and invade the underlying alveolar epithelium.

Here, monocytes from neighbouring blood vessels are recruited to form a granuloma, as the immune system endeavours to control the disease (LabCE, 2019).

Inside the granuloma, a core of infected macrophages is encompassed by foamy macrophages, mononuclear phagocytes, and lymphocytes (LabCE, 2019). The outcome is a fibrous capsule with expanded foam macrophages, presumed to make the commonplace caseous debris in the centre of the granuloma.

Despite the appearance of successful immunological containment, infection can occur if the caseous centre disintegrates and cavitates to discharge thousands of *M. tuberculosis* bacilli into the airway. If this occurs, the damaged lungs produce a cough that contains the infectious droplet nuclei containing *M. tuberculosis* which get transmitted into the air, completing the cycle.

With the onset of the host immune response, active bacterial replication is inhibited by adaptive immune cells and dispersal is thought to be controlled by the granulomas, which prevent bacterial dissemination to extrapulmonary sites. Despite the fact that the immune response against *M. tuberculosis* can be successful in controlling the primary infection, the bacterium is rarely completely destroyed. In this way, *M. tuberculosis* is first among bacterial pathogens in its capacity to establish and maintain latency (Glickmana & Jacobs, 2001; Sia & Rengarajan, 2019).

Studies in animal models and humans have not yet yielded a comprehensive picture of why immunity fails to eradicate the pathogen (Ernst, 2012).

1.3 Epidemiology

According to the WHO (2020), TB is one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent. About a fourth of the global population is infected with *M. tuberculosis* and thus at risk of developing TB. Globally, an estimated 10 million individuals became sick with TB in 2019, a number that has been relatively steady since 2015. According to Sulis *et al*. (2014), TB is mostly a poverty-related disease: this can explain its uneven distribution in

different population groups. Well-characterised vulnerable groups include individuals living with HIV disease, prisoners, homeless people, migrants/refugees, and substance or heavy alcohol users.

Individuals living with HIV/AIDS are at extremely high risk of TB because of the combined effects of immunological impedance related to this disease and frequent co-existence of deprived social conditions.

There were an estimated 1.2 million TB deaths among HIV negative people in 2019 and an additional 208,000 deaths among HIV positive people (WHO, 2020). According to UNAIDS (2020), 690,000 people died from AIDS-related illnesses in 2019, which means that those also infected with TB disease, account for 30% of all HIV/AIDS-related deaths, a significant percentage.

The development of the SARS-CoV-2 pandemic may further complicate the management of TB disease by causing disturbances to health services. If the number of individuals with TB who are identified and treated decreases by 25–50% over a period of three months, there may be an increase of 0.2–0.4 million TB deaths in 2020 alone (WHO, 2020). TB affects people of all ages and sexes; however, men account for most of the TB burden. In 2019, men accounted for 56% of all TB cases. Women accounted for 32% and children below the age of 15 years accounted for 12% of all TB cases. Most TB cases in 2019 were in South-East Asia (44%), Africa (25%), and the Western Pacific (18%). The global TB incidence rate slowly declined from 1997 to 2001, with an increase in 2001 due to the rising number of cases among HIV-infected patients in Africa (Sulis *et al*., 2014).

1.4 TB Prevention, Diagnosis and Treatment

According to TB Alert (2019), TB can be prevented by using the live vaccine Bacille Calmette-Guérin (BCG). The vaccine is prepared from a strain of the weakened bovine tuberculosis bacillus, *Mycobacterium bovis*. The BCG vaccine is presently the main authorised vaccine against TB and has been in use since 1921. It is one of the most widely used vaccines worldwide. Yet, regardless, we see around 10

million new instances of TB every year, a demonstration of the BCG's constrained viability in adults. The BCG is:

- 80% effective in preventing TB for 15 years
- more effective against complex forms of TB in children
- of restricted viability in individuals beyond 35 years old
- less successful when given in equatorial regions because of significant levels of naturally occurring environmental mycobacteria

Despite the fact that TB is a preventable and curable disease, the inability to detect the disease early is one of the significant challenges in TB control. TB diagnostic tests with low sensitivity developed more than a century ago are still in use today (Zijenah, 2018).

The diagnosis of latent TB infection is established by a positive result on either a tuberculin skin test (TST) or an interferon-γ (IFN-γ) release assay (IGRA), in the absence of active TB.

Active TB is diagnosed on the basis of a combination of epidemiological (e.g., travel to, or residence in, a high prevalence area, previous TB), clinical (e.g. a cough lasting longer than 2–3 weeks, fever, night sweats, weight loss), radiographic (e.g. infiltrates, fibrosis, cavitation), microbiological (e.g. positive sputum smear or culture), and histopathologic (e.g. caseating granuloma) features. Patients who are strongly suspected of having clinical TB infections on the basis of clinical criteria should undergo chest radiography. Patients with chest radiographic findings suggestive of pulmonary TB should submit three sputum specimens, preferably obtained on different days, for acid-fast bacteria (AFB) smears and culture (Sia & Wieland, 2011). The presence of acid-fast-bacilli on a bronchoscopic smear or alternative specimen usually indicates TB sickness. Acid-fast microscopy is simple and fast; however, it can generate an inaccurate TB diagnosis because some acid-fast-bacilli are not *M. tuberculosis.* Therefore, a biological sample from the patient is cultured to substantiate the diagnosis. A positive culture for *M. tuberculosis* confirms the diagnosis of TB disease. Culture

examinations ought to be completed on all specimens, regardless of AFB smear results (CDC, 2019).

In South Africa, as stated in the 2014 South African National TB Management Guidelines (Department of Health, 2014), people who have bacteriological confirmation of TB, either by GeneXpert, AFB smear or TB culture should be treated for the disease. Individuals with clinical signs and symptoms of TB are also started on TB treatment as are individuals with radiological evidence (with or without bacteriological confirmation).

The first-line TB drugs in the guideline include rifampicin, isoniazid, pyrazinamide and ethambutol. Dose administration is dependent on the body mass of the patient. Standard TB treatment is carried out for six months, divided into two phases: intensive phase (two months) and a continuation phase (four months). In the intensive phase, all four drugs are administered daily. In the continuation phase, only rifampicin and isoniazid are administered daily. Treatment may be extended to nine months if the infection is considered severe, with the continuation phase lasting for seven months.

The treatment regime for patients who also have HIV infection may be modified, with rifampicin typically being replaced by rifabutin for patients on the antiretroviral atazanavir. Rifampicin is replaced by rifabutin in patients taking the protease inhibitor atazanavir because atazanavir is primarily metabolised by CYP3A4 and rifampicin is a very strong inducer of CYP3A4. Administration of rifampicin together with atazanavir may lead to drug-drug interactions where subtherapeutic concentrations of atazanavir are produced (Finch *et al*., 2002; Kolars *et al*., 1992; Mallolas, 2007). This further complicates coinfection of HIV and TB which is already dangerous due to the fact that the weak immune system caused by HIV results in a more severe case of TB infection than in people with a healthy immune system (AIDSinfo, 2020).

1.5 Mycobacterium tuberculosis drug resistance

M.tb can, and has, become resistant to the antimicrobial drugs used to cure the disease. Multidrug-resistant TB (MDR-TB) is TB infection that does not respond to at least isoniazid *and* rifampicin, the two most powerful anti-tuberculosis drugs (WHO, 2018). According to the WHO (2020), drug-resistant TB continues to be a public health threat. In 2019, there were approximately half a million new cases of rifampicin-resistant TB (of which 78% had multidrug-resistant TB). Globally in 2019, 3.3% of new TB cases and 17.7% of previously treated cases had MDR-TB or rifampicin-resistant TB (RR-TB). Detection of MDR/RR-TB requires bacteriological confirmation of TB and drug resistance testing using rapid molecular tests, culture methods, or sequencing technologies.

The WHO recommends that patients with confirmed isoniazid-resistant, but rifampicin-susceptible TB be put on rifampicin, ethambutol, pyrazinamide, and levofloxacin for six months. Patients with RR-TB and MDR-TB should be placed on the second-line TB drugs (Table 1). Treatment requires a course of second-line drugs (Table 1) for at least 9 months and up to 20 months, supported by counselling and monitoring for adverse events (WHO, 2019).

Table 1: Second-line TB drugs for treatment of rifampicin resistant and multi drug resistant tuberculosis

Group A	Group B	Group C
		Ethambutol
Levofloxacin/Moxifloxacin	Clofazimine	Delamanid
		Pyrazinamide
Bedaquiline		Imipenem cilastatin/Meropenem
	Cycloserine/Terizidone	Amikacin/Streptomycin
Linezolid		Ethionamide/Prothionamide
		P-aminosalicyclic acid

All three Group A drugs and at least one Group B drug should be administered as part of the treatment regimen for MDR/RR-TB. This is recommended by WHO to ensure that the treatment begins with at least four drugs that are likely to be effective (WHO, 2019). The recommendation also ensures that at least three drugs are administered for the rest of the treatment after bedaquiline administration has been stopped. It is recommended that both Group B drugs are taken if only one or two of the Group A drugs are included in the regimen. If treatment cannot consist of Group A and B drugs alone, Group C drugs are to be included to complete the regimen.

In certain nations, such as the Russian Federation, it is getting progressively more difficult to treat MDR-TB. Treatment alternatives are constrained and costly, prescribed medication is not always accessible, and patients experience many adverse effects from the medication (WHO, 2018). TB is more common in countries where many people live in absolute poverty (TB Alert, 2020), and the costliness of the treatment makes it harder for MDR-TB to be treated effectively. In some cases, even more severe drug-resistant TB may develop. Extensively drug-resistant TB, (XDR-TB), is a form of multidrug-resistant TB. It is characterised by additional resistance to antitubercular drugs used to treat MDR-TB, i.e., XDR-TB responds to even fewer available medicines. XDR-TB has been detected in 117 nations around the world. The two reasons why MDR-TB continues to develop and spread, are mismanagement of TB treatment and person-to-person transmission of the MDR-TB. Inappropriate or incorrect use of the prescribed TB drugs such as, prematurely discontinuing the medication due to the lengthy treatment regimen or side effects or use of ineffective formulations, can result in the development of drug-resistant strains. The drug-resistant strains can then be passed on to the next person, particularly in crowded places such as prisons and hospitals through the air (WHO, 2018).

The increase in drug resistance has led to an increased need for novel antitubercular drugs to combat MDR and XDR *M. tuberculosis.* Historically, natural products have been a rich source of antimycobacterial drugs and therefore are an ideal source to search for new compounds.

1.6 Natural products and their role in drug discovery and development

1.6.1 Drug discovery and development

Drug discovery and development together are the complete process of identifying a new drug and bringing it to market (Nature, 2020). Sources for discovery of a possible new chemical entity include natural products, computational medicinal chemistry, and chemical libraries (Katiyar *et al*., 2012).

Before a drug can reach the patient market, it must go through rigorous testing to determine whether it is safe, effective at treating the condition it was developed for and to ascertain the correct dosage and appropriate administration route (Lansdowne, 2020). Figure 1 shows a summary of the drug discovery and development process.

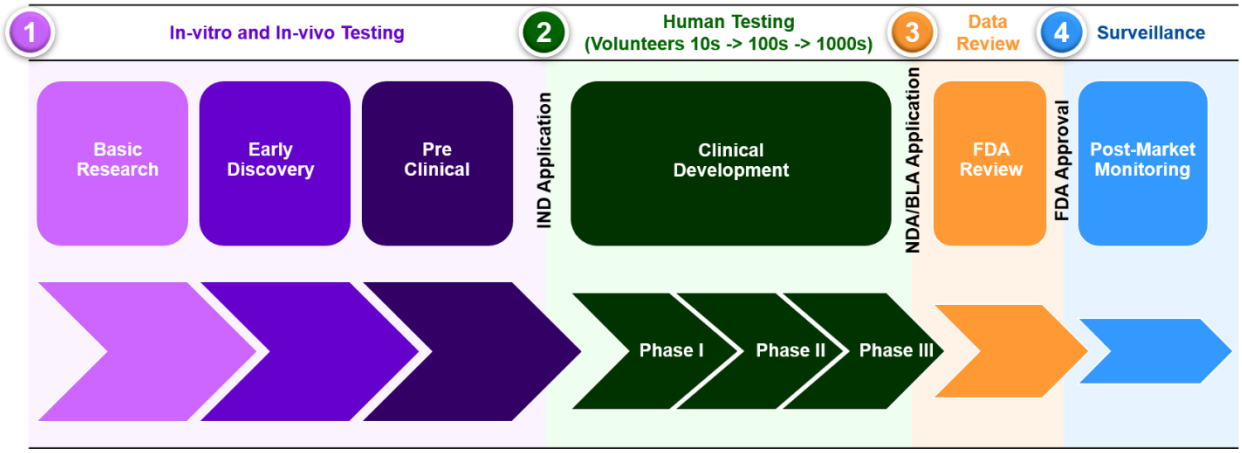

Figure 1. Drug discovery and development process. IND means Investigational New Drug; BLA means Biologics License Application; FDA is an example of a regulatory body and stands for Food and Drug Administration (NorthEast Biolab, 2020).

Drug discovery involves screening hits, medicinal chemistry, and optimisation of hits to reduce potential drug side effects. Efficacy or potency, metabolic stability, and oral bioavailability are also improved in this step of the drug discovery and development process (NorthEast Biolab, 2020).

Early drug discovery includes target identification and validation, hit identification and validation, hit-to-lead and lead optimisation, and candidate selection. A target can be defined as an enzyme, receptor, channel, or other cellular structure whose activity can be altered by a drug, resulting in a therapeutic effect (Lansdowne, 2020). A hit is a compound which has the desired activity in a compound screen and whose activity is confirmed upon retesting (Hughes *et al*., 2011). A lead is a compound that shows the potential of acting against a drug target and may initiate the development of new chemical entities (Seema, 2018).

Early drug discovery is followed by preclinical testing, where important information about a drug candidate's efficacy and safety is investigated before it is tested in human subjects. The tests are conducted *in vitro* and *in vivo* (Lansdowne, 2020; NorthEast Biolab, 2020).

Next in the drug discovery and development process is clinical development which includes phase I, II, and III clinical trials aimed at determining the investigational new drug's safety, it's efficacy, information on dosage, and to monitor adverse events (Lansdowne, 2020).

If a drug candidate passes the clinical development stage, after the clinical trials, a regulatory body reviews the collected data to determine whether the drug can be released to the market for use by the public. An example of one such regulatory body is the USA's FDA. Following drug approval, the regulatory body continues to monitor the drug's safety as it is being used by the public, a stage known as post-market monitoring (FDA, 2018).

This research project falls under the "early drug discovery" of the drug discovery and development process because it consists of screening actinobacteria for compounds with antimycobacterial properties and searching for novel compounds.

1.6.2 Benefits and limitations of natural products as a source of drug discovery

Natural compounds are any substances naturally produced by a living organism. For the purpose of this project, a natural product will be defined as a pharmacologically or biologically active chemical compound, which is found in

nature and produced by a living system (Krause & Tobin, 2013; Webster's revised unabridged dictionary, 2019). Natural products can be considered as such even if they can be prepared by a synthetic approach. Most natural products are produced by secondary metabolism and have played a significant role in drug discovery. Secondary metabolites are organic molecules that have no primary function directly involved in the normal growth, development, or reproduction of the producing organism, but increase its competitiveness within its environment (Zahner, 1979). They may be unique to the producing organism (Sarka *et al*., 2006). Some of the main classes of secondary metabolites include terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, non-ribosomal polypeptides, and enzyme cofactors (McMurry, 2015; Thirumurugan *et al*., 2018).

Secondary metabolites serve:

(i) as antimicrobial compounds to inhibit surrounding microorganisms, amoebae, plants and insects, to reduce the level of competition;
(ii) as metal transporting agents;
(iii) as signalling agents between microbes and plants, nematodes, insects, and higher animals;
(iv) as sexual hormones;
(v) as differentiation effectors (Demain & Fang, 2000).

Natural product screening used to be the main source of new drug compounds; however, pharmaceutical companies reduced their research investment in natural products for drug discovery from the late 20th century. The challenge which led to the reduction of natural products drug discovery was the repetitive rediscovery of previously known natural products (Roemer, 2011). This is known as dereplication. The concern was that existing discovery models were incapable of delivering novel lead compounds (Jansen, 2014). Natural product screening was a slow and costly process that did not match the pace of high-throughput screening, and overtime combinatorial chemistry was favoured over natural product screening.

Combinatorial chemistry involves the generation of a large array of structurally-diverse compounds, called a chemical library, through systematic, repetitive and

covalent linkage of various building blocks (Liu *et al*., 2017). High-throughput screening is a method used to identify hits from compound libraries that may become leads for medicinal chemistry optimisation (Entzeroth *et al*., 2009). This methodology could be automated, which enables the analysis of a series of compounds in a short time (Szymański *et al*., 2012).

Although we have seen a decline in research aimed at exploiting natural products for drug development, over time, it has become apparent that chemical libraries cannot match the chemical diversity of natural products (Liu *et al.,* 2017). Natural products are still an ideal source for new chemical entities because they provide exceptional efficacy and selectivity. This is thought to be due to their distinct and diverse structures which allow for very specific binding and, therefore, a strong response and reduced toxicity (Cragg & Newman, 2013).

Secondary metabolites come from a limited number of parent molecules as primary metabolites, receptors, enzymes, transporters and regulatory proteins. This means that these molecules have co-evolved to interact with one another and subsequently, natural products perform better as drugs (Ginsburg & Deharo, 2011). Their diversity increases the chance of finding novel molecular skeletons due to their diverse biological functions and functionalities not limited by human imagination (Beutler, 2009; Shen, 2015).

Natural products have been a rich source of drugs for decades. Over 60% of the medications that are in the market are derived from natural products (Molinari, 2009). Examination of medical indications by the source of compounds has shown that natural products and related drugs are used to treat 87% of all classified human diseases (Chin *et al*., 2006). From 1981 to date, 80% of small molecule anticancer drugs are natural product-based, with 53% being either natural products or their derivatives. Among the 20 approved small molecule new chemical entities in 2010, half of them are natural products (Scripps, 2019).

Natural products or their pharmacophores may also be used as building blocks for chemical libraries resulting in improved chemical libraries (Brohm *et al.*, 2002). Combinatorial chemistry may be used to supplement natural product drug

development. By modifying the structure to create mechanistically new, inherently active natural compounds, the absorption, transport and uptake may be more effective *in vivo* (Berdy, 2005).

Natural products may be produced by plants, such as the antimalarials quinine and artemisinin the animal kingdom such as insulin, and by microorganisms which have produced several antibiotics such as penicillin and streptomycin (Demain, 2009; Veeresham, 2012; Waksman, 1970).

Current challenges to the use of natural products for drug discovery include the difficulty of purifying unknown compounds and elucidating their biological mechanisms (Thomford *et al*., 2018). Technologies such as high-performance liquid chromatography, nuclear magnetic resonance spectroscopy, mass spectrometry (MS), microfluidics, and computational algorithms have mitigated these challenges and allowed for the determination of chemical components of natural products and their utilisation in drug discovery (Wang *et al*., 2018; Zhang *et al*., 2018). Another issue with natural product drug discovery is the difficulty in cultivating certain species that produce these natural products, but this may be overcome by the huge potential of genetic engineering (Berdy, 2005).

1.7 Actinobacteria for drug discovery

Actinobacteria are well-known as a rich source of antibiotics. For decades these microbes have been exploited for the development of novel drugs. *Actinobacteria,* is a phylum of Gram-positive bacteria, mostly aerobic, with a high G-C percentage, and a complex life cycle. They are widely distributed across both terrestrial and aquatic ecosystems but are mainly found in soil (Sharma *et al*., 2014). *Actinobacteria* represent one of the largest taxonomic units among the 18 major lineages currently recognised within the *Bacteria* domain (Ludwig *et al*., 2012). The filamentous members are known to produce antibiotics under adverse environmental conditions indicated by the presence of aerial hyphae.

The era of microbial drug discovery began with Alexander Fleming in 1928 when he discovered penicillin, which he isolated from the fungus *Penicillium notatum*.

The screening of actinomycetes by Waksman's group in the 1940s led to the discovery of actinomycin in 1940, streptothricin in 1942, and, most notably, streptomycin (anti-TB drug) in 1944. This, in turn, prompted the extensive screening of actinomycetes extracts, primarily against pathogenic bacteria (Katz & Baltz, 2016). Acquisition of these microbes was, and is, facilitated by their abundance in soil.

Some of the antibiotics that have been isolated from *Actinobacteria* include but are not limited to amphotericin, nystatin, chloramphenicol, gentamycin, erythromycin, vancomycin, tetracycline, novobiocin, and neomycin.

Vancomycin is important in treating multiple-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). Rifamycins, cycloserine and kanamycin, all important in the treatment of TB, are also produced by actinobacteria (Dowling, 2004; Drugbank, 2020). Actinobacteria are also used as plant growth-promoting agents, biocontrol tools, biopesticide agents, antifungal compounds, for biocorrosion, and as a source of agroactive compounds (Sharma *et al*., 2014).

From the 22,500 biologically-active compounds that have been obtained so far from microbes, 45% are produced by *Actinobacteria*, 38% by fungi, and 17% by unicellular bacteria (Demain & Sanchez, 2009; Bérdy, 2005). The filamentous species produces over 10,000 bioactive compounds; 7,600 derived from *Streptomyces* and 2,500 from the so-called rare actinomycetes. Rare actinomycetes are actinobacteria for which the isolation frequency by conventional methods is lower than the *Streptomyces* abundance and include, but are not limited to, *Actinomadura, Actinoplanes, Amycolatopsis, Actinokineospora, Kibdelosporangium, Microbispora, and Nonomuraea* (Lazzarini *et al*., 2000; Tiwari & Gupta, 2013). Figure 2 shows the key findings and dates of discovery of antibiotics. The *Streptomyces* genus produced more than half of the key antibiotic discoveries (de Lima Procópio *et al*., 2012).

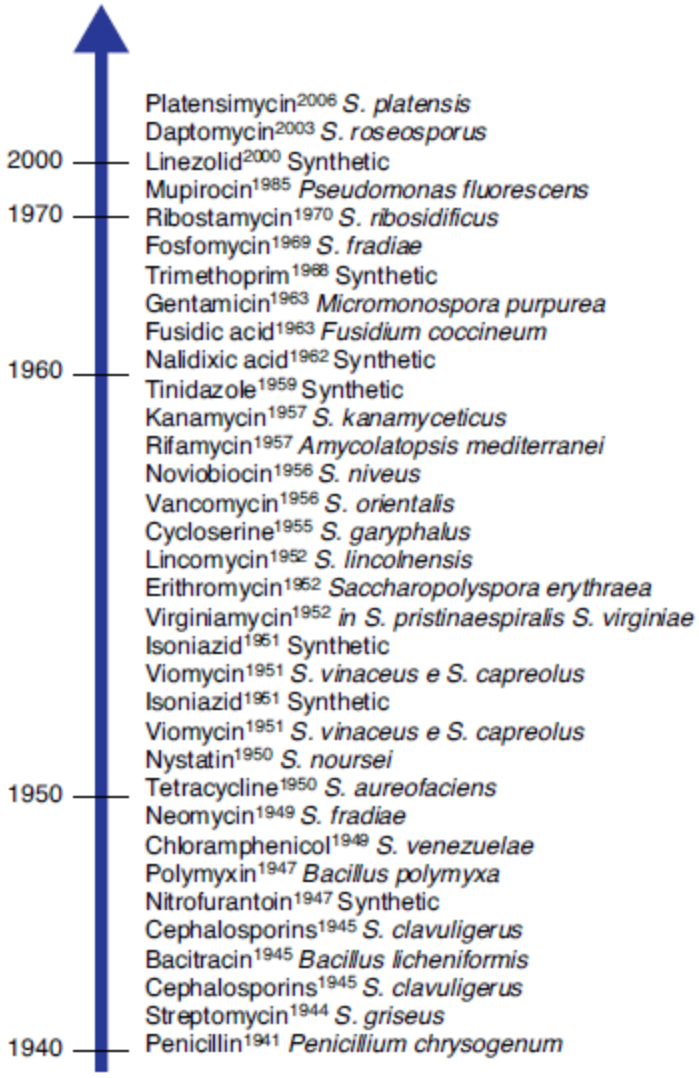

Figure 2. Key findings and dates of antibiotics. The streptomyces species produced the majority of the key findings (de Lima Procópio *et al*., 2012)**.**

1.8 Rare actinobacteria for drug discovery

With the decline in the discovery of novel compounds for drug development and increased drug resistance, it would be beneficial to continue investigating the less explored species of the *Actinobacteria* phylum. History (Figure 2) has shown that these bacteria are a rich source of drugs; however, the focus has been mainly on the *Streptomyces* genus.

The rare actinobacteria were selected as the focus of this investigation as they have been shown to be potential sources of antibiotics as can be seen in the examples below in Table 2, despite not being explored as extensively as *Streptomyces*. A decline in novel bioactive compounds produced by *Streptomyces* has been seen since the most common compounds have been discovered (Manteca & Yagüe, 2019). The less explored actinobacteria species may produce novel antibiotics. Certain research groups have already started studying the less explored species and have found potential sources of new drugs.

Bundale *et al*. (2019) discovered three potent compounds which were tested against *Bacillus subtilis* MTCC441. The first compound was isolated from *Micromonospora auratinigra* MMS16 and showed activity against *B. subtilis* with a minimum inhibitory concentration (MIC) of 8.00 µg/mL. The compound was identified as chloroanthraquinone, first reported from *M. auratinigra*. MMS16, identified as a member of the family *Micromonosporaceae*, produced MMS16B analysed to be a novel bafilomycin analogue. The MIC of the compound was found to be 7.00 µg/mL against *B. subtilis*. KCR3 identified as *Kocuria kristinae* produced a novel antimicrobial peptide with antibacterial and antifungal activity, a first for *K. kristinae.* The compound was found to be active against *B. subtilis*, *Escherichia coli,* and *Candida albicans.*

Hussain *et al*. (2017) isolated 10 actinobacteria strains with metabolites antagonistic against the standard H37Rv strain of *M. tuberculosis* with their MICs ranging from 0.20–250 µg/mL. Of particular note was a compound isolated from the rare actinobacteria *Lentzea violacea* AS08 with a MIC of 3.90 µg/mL against *M. tuberculosis* strain H37Rv.

Table 2 shows examples of bioactive secondary metabolites isolated from some rare actinobacteria, as stated in Ding *et al*. (2019), strongly suggesting that they are a viable source for novel potential drug candidates.

Table 2: Examples of bioactive compounds produced by rare actinobacteria

Compound	Source	Activity
Pradimicin-IRD	*Amycolatopsis sp.*	Antimicrobial activity against many bacteria such as *Staphylococcus aureus* (MIC = 3.10 µg/mL). It also displayed strong cytotoxicity against HCT-116 colon carcinoma cells & melanoma cells (Bauermeister *et al*., 2019).
Dipyrimicin A	*Amycolatopsis sp.*	Showed strong antimicrobial activity against *Saccharomyces cerevisiae*, *Kocuria rhizophila* & *Escherichia coli*. It also showed cytotoxicity against HeLa 3S, A549, THP-1, Jarkat & HL-60 cells with ≤ 5.00 µM (Izuta *et al*., 2018).
Actinomadurol	Actinomadura strain KC191	Antibacterial activity against *S. aureus*, *Proteus hauseri* & *K. rhizophila* (MICs = 0.39-0.78 µg/mL) (Shin *et al*., 2016).
Eight 3-oxyanthranilic acid derivatives	*Actinomadura sp.* BCC27169	Two compounds showed antitubercular activity (MIC = 50.0 µg/mL), and one compound showed cytotoxicity against KB cells with an IC_{50} of 18.6 µg/mL (Intaraudon *et al*., 2014).
Nonomuric acid	*Nonomuraea rhodomycinica* NR4-ASC07	Antimalarial activity IC_{50} = 8.00 µg/mL (Supong *et al*., 2019).
7-deoxy-13-dihydrocarminomycinone (known compounds)	*Nonomuraea rhodomycinica* NR4-ASC07	Activity against *M. tuberculosis* (MIC = 50.0 µg/mL) & *Bacillus cereus* (MIC = 12.5 µg/mL) (Supong *et al*., 2019).
A4 & A5	*Saccharothrix* SA198	Antifungal: *Mucor ramannianus* (MIC = 5.00-1.00 µg/mL); *Aspergillus carbonarius* (MIC = 10.0-2.00 µg/mL) & *Penicillium expansum* (MIC = 2 µg/mL) (Boubetra *et al*., 2013).
Philipimycin	*Actinoplanes philippinensis* MA7347	Antimicrobial against Gram-positive bacteria including MRSA (MIC = 0.02-1.00 µg/mL). *In vivo* mouse model of *S. aureus* infection: ED_{50}= 8.40 mg/kg. (Zhang *et al*., 2008).

1.9 Conclusion

With the rise and spread of drug resistance, novel drugs are required to combat TB. New treatments need to be shorter to promote compliance and effectiveness against all strains of *M. tuberculosis*. The understudied rare actinobacteria have the potential to provide these much-needed drugs and warrant further study. The issue of dereplication which led to the decline in natural product drug discovery in

the past was addressed by the use of molecular networking, a tool that relies on the observation that structurally similar molecules share similar MS/MS fragmentation (Yang *et al*., 2013). This technique allows accurate library matching to known compounds which dereplicates active compounds before extensive study is undertaken and can be used to identify novel analogues based on fragmentation pattern matching. This subsequently leads to the acceleration of natural-product-based drug discovery (Quinn *et al*., 2017).

1.10 Scope of the Study

Aim: The aim of this project was to screen, evaluate, and dereplicate active antimycobacterial compound(s) produced by rare filamentous actinobacteria.

The research questions that this project aims to answer are as follows:

- Do the selected strains of rare filamentous actinobacteria produce antimycobacterial compounds?
- Are the antimycobacterial compounds produced novel compounds?
- Can known antimycobacterial compounds be identified following their extraction from the zone of inhibition (ZOI) and analyses by molecular networking?

Objectives:

- To culture strains of rare filamentous actinobacteria and novel, highly-active *Streptomyces* under six different media conditions.
- Screen the cultured strains for *in vitro* antimycobacterial activity against *Mycobacterium aurum* A+ using the plate overlay technique.
- Reproduce any activity observed during the screening under the same growth conditions.
- Extract the ZOI of active cultures.
- Analyse the active crude extract by molecular networking metabolomic approaches through the Global Natural Products Social Molecular Networking database (thus, dereplicate the samples and identify known antibiotics).
- Select actinobacteria that produce unknown antimycobacterial compounds for further study.

Chapter 2: Screening for antimycobacterial activity

2.1 Introduction

Actinobacteria from the Meyers' culture collection from Dr Paul Meyers' lab in the Department of Molecular and Cell Biology at the University of Cape Town, were assessed for antimycobacterial properties using *M. aurum* A+ by the overlay method (Wood *et al*., 2007).

The actinobacteria were initially grown in liquid media to cultivate the bacteria from stock cultures kept at -20ºC. The liquid cultures were shaken during cultivation because actinobacteria are mostly aerobic organisms which require oxygen to grow and produce antibiotics (Iwai & Omura, 1982). Shaking the liquid cultures ensured that they were homogenous and well-aerated.

M. aurum A+ was used to test for production of antimycobacterial properties because it has an antibiotic-susceptibility profile similar to that of *M. tuberculosis* and is safer to work with as it is non-pathogenic (Gupta, 2012). *M. aurum* A+ also grows faster than *M. tuberculosis* (Gumbo *et al.*, 2009; Musuka *et al*., 2013; Namouchi *et al*., 2017). *M. aurum* A+'s faster growth rate means that more bacteria can be screened in less time.

Antimycobacterial activity of each culture was detected using the overlay method. The overlay method begins with culturing the test strain in the centre of an agar plate following stab inoculation under sterile conditions. After sufficient growth is observed, sloppy agar containing *M. aurum* A+ is overlayed onto the agar plate. If the test strain produces antimycobacterial compounds that can diffuse through the agar, the growth of *M. aurum* A+ will be inhibited. This inhibition produces a cleared zone known as the zone of inhibition (ZOI) (Hudzicki, 2009). This method was used because it is relatively inexpensive with minimal resource requirements (Hockett, 2017), and it can also be excised for further study. The ZOI is measured as the area of the zone of clearing minus the area of the test strain and is typically measured in mm^2. The size of the ZOI is directly proportional to the antimycobacterial strength of the test strain.

The test strains were stab inoculated on six different media for the overlay method to attempt to provide optimal growing conditions, enabling the selection of the best media for optimal growth and antibiotic production. It is beneficial to provide multiple growth conditions because one single strain can produce various secondary metabolites depending on the cultivation conditions. Factors that may influence the production of secondary metabolites include nutrient content, temperature, and rate of aeration. This environment-dependent production of metabolites is known as one strain many active compounds, also known as OSMAC (Romano *et al*., 2018). Conditions of growth, including media recipe, incubation temperature, and duration of incubation, were previously shown in the Meyers lab to be optimal for antibiotic production (Soshankana, 2017).

Seventeen actinobacteria strains were tested for antimycobacterial activity from seven different genera, namely: *Streptomyces, Nonomuraea, Microbispora, Micromonospora, Actinomadura, Amycolatopsis,* and *Kribbella*. Although the focus of this project was on non-Streptomyces species, novel *Streptomyces* that showed antimicrobial potential in other investigations were also studied.

Actinobacteria strains that showed antimycobacterial activity in the initial screen were grown again in all the media they were active in for reproducibility and excision of the ZOI. This time, one strain was stab inoculated per plate, and the ZOI was recorded.

Excision and extraction of the agar in the ZOI allowed close study of the compounds present at the site of inhibition. This is hypothesised to simplify the identification of the active antimycobacterial compounds as there are hypothesised to be fewer background compounds present. The agar extraction was performed using the mid-polar solvent, ethyl acetate. Ethyl acetate was used because it will extract matching mid-polar compounds which are predicted to make better oral drugs (Lipinski *et al*., 2001). Oral drugs are the better option for treating TB because it is a disease of poverty (Oxlade & Murray, 2012), and oral drugs are an economical solution (Cyriac & James, 2014). TB treatment requires extensive treatment times, making convenience essential and oral administration is relatively

easier (Homayun, 2019). A patient can take a tablet at home or anywhere as instructed by a healthcare professional and does not need special skills to take a tablet. However, administration through an intravenous injection, for example, will require the patient to go into a designated healthcare centre which may reduce adherence.

2.2 Methodology

2.2.1 Cultivation of actinobacteria

Glycerol stock solutions (15%) (*v/v*) of the test strains were cultured in 15 mL of sterile International *Streptomyces* Project medium #2 (ISP2) (Shirling & Gottlieb, 1966) liquid medium in a 250 mL Erlenmeyer flask at 30°C while shaking. After sufficient growth was reached (3–5 days), the strains were Gram-stained and streaked for single colonies on sterile ISP2 agar plates to determine purity. The streaked plates were left to grow at 30ºC until they produced aerial mycelium, which was approximately 7 days.

2.2.2 Cultivation of *M. aurum* A$^+$

M. aurum A+ glycerol stocks (15%) (*v/v*) solution was inoculated in 5 mL in a sterile 10 mL universal tube of 2YT liquid media and left to grow for seven days at 37ºC, while shaking, in preparation for the first overlay test method. Subsequently, *M. aurum* A+ was streaked out each week on sterile 2YT agar plates and incubated at 37ºC to maintain the culture.

2.2.3 Stab Inoculations for Overlay Method

Single colonies from the streak plates were picked up with sterile toothpicks and stab inoculated aseptically in six different media as listed below. All media was autoclaved to make it sterile. Three to four strains were initially stab inoculated per plate. Overlay experiments were repeated with all active strains, stab inoculated individually under the same media conditions, to reproduce activity. The bacteria

were incubated at 30ºC for 8–9 days. This growth period accommodated for secondary metabolite production during exponential or stationary phase (Ripa *et al*., 2009).

Media used for stab inoculations:

- Difco Middlebrook 7H9 agar (Becton Dickinson) which was modified with 10 mM glucose
- ISP2 agar
- Czapek solution agar (Atlas, 2004)
- Japan Collection of Microorganisms medium #61(JCM #61) (Ara & Kudo, 2007)
- German Culture Collection (DSMZ) medium #553 (German Culture Collection, 2007)
- Bennett's agar medium (Atlas, 2004)

2.2.4 Overlaying with *M. aurum* A+

M. aurum A+ culture picked from a streak plate was inoculated in 5 mL of 2YT broth media in a 10 mL sterile universal tube and left to grow at 37ºC with shaking, overnight prior to the day of the overlay. Four to five loopfuls were used as the initial inoculum. On the day of the overlay, the purity of the *M. aurum* A+ culture was checked by Gram staining and streaking for single colonies on 2YT agar. If the culture was pure, the overlay method could proceed. The optical density of the culture at 600 nm (OD_{600}) was measured using a Beckman DU®-64 spectrophotometer. To measure the optical density, the *M. aurum* A+ culture was first diluted as follows: 250 µL of culture in 750 µL of 2YT media (1:4). The measured OD_{600} was then multiplied by 4 to get the actual optical density of the culture. The optical density was used to determine the volume of culture to add to 6 mL of 2YT sloppy agar (2YT with 0.7% bacteriological agar) using the equation:

OD_{600} x Volume of culture (µL) = 160.

This formula ensured that a consistent concentration of *M. aurum* A+ was used across experiments. The 2YT sloppy agar was prepared in a Schott bottle then warmed up until it reached boiling point in a microwave to dissolve the agar. The sloppy agar was then divided into 6 mL aliquots and autoclaved to ensure sterility. To avoid harming the culture, after autoclaving the sloppy agar was allowed to cool down enough that it did not set. This was determined by being able to hold the tube against the wrist for 10 seconds. The calculated amount of *M. aurum* A+ culture was added to sterile sloppy agar and mixed by gentle vortex. The sloppy agar was then poured on the plates with stab inoculated actinobacteria strains and incubated for 2 days at 37ºC; care was taken not to wash off any spores. Results were read after 2–3 days by checking whether a ZOI around the test strain was present or not.

Initial overlay results were recorded as positive if a ZOI was present or negative if none were observed. When the overlay experiments were repeated on individual plates to reproduce antimycobacterial activity, the ZOI was measured. The antimycobacterial activity was then assessed as very weak (ZOI < 100 mm^2), weak (100–1000 mm^2); moderate (1001–2000 mm^2); strong (2001–3000 mm^2), or very strong (> 3000 mm^2) (Pelser, 2018; Wood *et al*., 2007).

The strains that showed moderate to very strong activity when stabbed inoculated individually were tested again using the same methods as before to assess whether or not the results were reproducible. Antimycobacterial activity was observed again in all the strains that were tested to assess reproducibility.

2.2.5 Agar extraction of the zone of inhibition

To study and identify the active antimycobacterial compounds present, the agar containing the ZOI was extracted using a modified version of the method used by Oppong-Danquah *et al*. (2018). The ZOI was excised with a spatula in approximately 1 cm x 1 cm pieces and macerated in a beaker with a spatula. One hundred millilitres of ethyl acetate was added to the agar pieces, stirred and was

left to shake on a Labnet™ Orbit 1000 multipurpose shaker at 120 rpm for 30–60 minutes. The agar was then filtered using two coffee filters (1 x 4 House of Coffee).

The ethyl acetate was added to a separating funnel with 100 mL of milli-Q water, mixed vigorously and allowed to separate. The addition of water served to remove salts and other polar contaminants that could affect MS analysis (Oppong-Danquah *et al*., 2018). After sufficient separation of the ethyl acetate from the water, the ethyl acetate layer was transferred into a clean beaker and left to dry in a fume hood overnight. This wash step was repeated. The next day, the extracts were reconstituted in ethyl acetate and transferred into a clean pre-weighed plastic tube.

2.3 Results & Discussion

2.3.1 Screening for antimycobacterial activity

A total of 17 actinobacterial strains were screened for antimycobacterial activity from seven different genera including *Streptomyces, Nonomuraea, Microbispora, Micromonospora, Actinomadura, Amycolatopsis,* and *Kribbella* using the agar overlay method described previously. Of the 17 strains tested, 10 displayed antimycobacterial activity, as shown in Table 3. Of the 12 non-*Streptomyces* actinobacteria tested, five were active. These included *Actinomadura* strain M2, *Actinomadura* strain M27, *Amycolatopsis circi* S1.3^{T}, *Kribbella speibonae* SK5, and *Nonomuraea* strain QMC26.

Table 3: Screening of Actinobacteria under companion growth for antimycobacterial properties.

List of strains tested against *M. aurum* A+ under different nutrient conditions.

Test Strain	Middlebrook	Czapek	DSMZ #553	Bennett's	ISP2	JCM #61
Actinomadura napierensis B60^T	-	-	-	-	-	-
Actinomadura rudenti HMC1T	-	-	-	-	-	-
Actinomadura strain M2	+	+	+	+	+	-
Actinomadura strain M27	-	+	+	+	+	+
Amycolatopsis circi S1.3^T	+	-	-	+	-	-
Kribbella speibonae SK5	-	-	-	+	+	-
Microbispora SMA-HA3	-	-	-	-	-	-
Micromonospora equina Y22T	-	-	-	-	-	-
Micromonospora sp. Muiz A55	-	-	-	-	-	-
Micromonospora tulbagliae TVU1	-	-	-	-	-	-
Nonomuraea QMC26	+	+	+	+	+	-
Nonomuraea sp. SMA-BO4	-	-	-	-	-	-
Streptomyces isolate 268	-	+	-	-	+	-
Streptomyces strain PR10	-	+	+	+	+	-
Streptomyces strain PR3	+	+	+	+	+	+
Streptomyces strain No 15	+	+	-	+	+	-
Streptomyces strain 1J1	-	+	+	-	-	-

'-' no antimycobacterial activity and '+' antimycobacterial activity observed.

It is interesting to note that out of the four tested *Actinomadura* strains, two of them were active in five of the six media they were tested in. Furthermore, one of the two that were not active, *Actinomadura napierensis* B60^T, has been previously shown to produce antimycobacterial compounds (Cook *et al*., 2005) and should be retested for antimycobacterial activity. This suggests that the *Actinomadura* genus may be rich in antibiotic production and is worth exploring extensively. There is evidence from literature that supports this trend. Euanorasetr *et al*. (2015) isolated strain 2EPS from soil and characterisation by physiological, chemical, and genetic analysis indicates that 2EPS belongs to genus *Actinomadura*. This strain was found to produce extracts with activity against Gram-positive bacteria, *Clostridium*

perfringens S107 and *Clostridium difficile* 630. Furthermore, a novel antibacterial compound CCp1 was isolated by a different group from the fermentation broth of *Actinomadura* sp. AL2 and was found to have activity against both Gram-positive and Gram-negative bacteria (Bhattacharjee *et al*., 2017). Another example is pradimicins. Pradimicins are polyketide antibiotics with antifungal activity produced by *Actinomadura hibisca* P157-2. (Kim *et al*., 2007).

The *Amycolatopsis* genus has also been seen to have the potential to produce aromatic polyketide antibiotics (Everest & Meyers, 2011). The authors found that the novel strains S1·3^T, SE(8)3^T and S3·6^T have antibiotic biosynthetic genes, strain S1·3^T being one of the strains that were found to be active against *M. aurum* A+ in this project. Another example of an antibiotic produced by an *Amycolaptosis* species is the clinically used rifamycin. Rifamycin is an antibiotic used to treat several types of bacterial infections, including tuberculosis, *Mycobacterium avium* complex, leprosy, and Legionnaires' disease and is produced by the actinobacterium *Amycolatopsis rifamycinica* (McHugh, 2011; The American Society of Health-System Pharmacists, 2020).

Although *Nonomuraea* QMC26 was active in all but one of the six media that it was tested on, it showed weak activity; therefore, it was not investigated further. However, the *Nonomuraea* genus, which is largely unexplored, has been shown to produce relevant antibiotics. *Nonomuraea gerenzanensis* ATCC 39727 is one such example, which produces the glycopeptide antibiotic (GPA) A40926 – the precursor of the clinically-relevant antibiotic dalbavancin, approved by the FDA in 2014 for the treatment of acute skin infections caused by multi-drug resistant Gram-positive pathogens (Yushchuk, 2020).

It is worth noting that all active rare actinobacteria were active in Bennett's medium, which could mean that this medium may be ideal for rare actinobacteria cultivation.

All five of the tested *Streptomyces* strains displayed antimycobacterial activity. The active strains included *Streptomyces* Isolate 268, *Streptomyces* strain No. 15, *Streptomyces* strain PR3, *Streptomyces* strain PR10, and *Streptomyces* strain 1J1. As discussed previously, *Streptomyces* are well-known producers of antibiotics.

From the initial overlay tests, it appeared that the *Streptomyces* species tended to produce greater ZOIs in Czapek media. This media is not rich in nutrients and bacteria are known to produce antibiotics under stressful conditions such as nutrient starvation (Martin & Demain, 1980) which could explain this observation. JCM #61 did not induce any antimycobacterial compounds production from the test strains. Middlebrook media was also not favourable as only one strain was able to display antimycobacterial activity, and even that activity was very weak.

2.3.2 Reproduction of antimycobacterial activity

The active bacteria were stab inoculated individually in the media they showed activity in, and the ZOI were measured. Table 4 shows the measurements of the ZOI.

Table 4: The zones of inhibition of each active strain under different cultivation and isolated inoculation conditions

Strains	**Area of Zone of Inhibition (mm^2)**					
	Middlebrook	**Czapek**	**DSMZ #553**	**Bennett's**	**ISP 2**	**JCM #61**
Actinomadura strain M2	204.1	2 238.8	204.1	423.9	241.8	N/A
Actinomadura strain M27	N/A	-	628.0	-	-	-
Amycolatopsis circi S1.3^T	-	N/A	N/A	1 648.5	N/A	N/A
Kribbella speibonae SK5	N/A	N/A	N/A	3 476.0	3815.1	N/A
Streptomyces isolate 268	N/A	1 020.5	N/A	N/A	-	N/A
Streptomyces strain PR10	N/A	1347.1	-	1 406.7	1 548.0	N/A
Streptomyces strain. No 15	N/A	-	1 177.5	1 020.5	593.5	N/A
Streptomyces strain 1J1	N/A	-	1 849.5	N/A	N/A	N/A

Actinomadura strain M2 and *Kribbella speibonae* SK5 were the only actinobacteria for which activity was observed in both the combined and single inoculation runs.

Actinomadura strain M2 had the strongest activity in Czapek medium with a ZOI above 2,000 mm^2 which is indicative of strong activity.

The other *Actinomadura* strain M2 stab inoculations showed weak activity. It was particularly difficult to grow the *Actinomadura* strain M27 on individual plates, and as a result, activity was only observed in 1 of the 5 media it was previously observed to be active in. Even that activity was weak with a ZOI of 628 mm^2 (Table 4). *Kribbella speibonae* SK5 showed the strongest activity of all the tested actinobacteria with ZOIs above 3000 mm^2, indicating very strong activity. *Amycolatopsis circi* $S1.3^T$ showed no activity when repeated as a single stab inoculation in Middlebrook medium.

The lack of correlation in production of ZOIs between the combined and isolated growth attempts may be explained by the phenomenon of bacterial bioactive secondary metabolite production activating in response to the presence of other bacteria in the community (Slattery, 2001). A possible explanation for this is that the bioactive metabolite is produced to give the strain a competitive advantage over the other strains in an environment with depleting nutrients. Therefore, the strains that produced bioactive compounds when they were stab inoculated as three or four different strains per plate may have been responding to a competitive environment that was absent with individual inoculation.

The reproducibility of the obtained results was assessed. Comparison of the ZOI from both experiments can be found in table 5. The mean area of ZOI and standard deviation are also given in table 5.

Table 5: Comparison of two overlay method experiments to assess the reproducibility of results. These experiments were both individual inoculation runs.

Strain (medium it was grown in)	**Area of ZOI (mm^2) Experiment 1**	**Area of ZOI (mm^2) Experiment 2**	**Mean Area of ZOI (mm^2)**	**SD (mm^2)**	**% SD**
Actinomadura strain M2 (Czapek)	2239	2967	2603	515	20
Amycolatopsis circi S1.3^T (Bennett's)	1649	1206	1428	313	22
Kribbella speibonae SK5 (Bennett's)	3476	3733	3605	182	5
Kribbella speibonae SK5 (ISP2)	3815	2044	2930	1252	43
Streptomyces strain PR10 (Bennett's)	1407	2572	1990	824	41
Streptomyces strain PR10 (Czapek)	1347	1849	1598	355	22
Streptomyces strain PR10 (ISP2)	1548	1143	1346	286	21
Streptomyces strain No. 15 (DSMZ #553)	1178	1849	1001	28	3
Streptomyces strain No. 15 (Bennett's)	1021	980	1514	474	31
Streptomyces strain 1J1 (DSMZ #553)	1849	2035	1942	132	7

Of the 10 overlay tests repeated, four results, *Kribbella speibonae* SK5 grown in ISP2, *Streptomyces* PR10 grown in Bennett's, *Streptomyces* strain No. 15 grown in Bennett's, and *Streptomyces* strain 1J1 fell into a different antimycobacterial strength category when comparing the areas of ZOI (very weak, weak, moderate, strong, very strong) however, the difference wasn't extreme. i.e. strains moved just one category up or down (e.g. from moderate to strong). Therefore, the results were considered reproducible and could be studied further.

2.4 Conclusion

Based on the screening results observed, *Kribbella speibonae* SK5, *Actinomadura* strain M2, *Amycolatopsis circi* S1.3^T, *Streptomyces* strains PR10, 1J1, No. 15, and

isolate 268 warrant further study. Each strain displayed moderate to very strong activity repeatedly and could produce novel antimycobacterial compounds. To determine if any of these strains were producing known antimycobacterial compounds, the zones of inhibition were analysed by high-resolution MS and GNPS molecular networking.

Chapter 3: Spot Bioautography and Molecular Networking Analysis of Active Extracts

3.1 Introduction

One of the aims of this project was to determine if high-resolution MS analysis of the extracted zones of inhibition could be used to identify known compounds from active samples identified using the overlay method. If this was shown to be possible, then it would allow rapid dereplication of active strains at the site of inhibition, without extensive cultivation and separation. If no known active compounds could be identified, it would suggest that the samples may be novel and warrant further study.

The primary method used to identify known compounds was through Global Natural Product Social molecular networking. Molecular networking is a visualisation strategy for untargeted MS (Quinn *et al*., 2017). MS-based molecular networking relies on the observation that structurally similar molecules share similar tandem mass spectrometry (MS/MS) fragmentation patterns (Yang *et al*., 2013). Molecular networking organises the MS/MS data as a relational spectral network, thereby mapping the chemistry that was detected in an MS/MS-based metabolomics experiment (Quinn *et al*., 2017). Metabolomics is the comprehensive analysis of metabolites in a biological specimen (Clish, 2015). Metabolomics is a powerful technique since the underlying biochemical function and cell/tissue state are directly reflected by metabolites and their concentrations. Thus, metabolomics best represents the molecular phenotype (EMBL-EBI, 2020). GNPS is an online open-access knowledge base for the sharing of raw, processed, and identified MS and MS/MS data from a community of researchers around the world (Wang *et al*., 2016).

As mentioned before, the majority of the pharmaceutical industry has reduced their reliance on natural product-based drug discovery programs partly due to the costs behind high rates of rediscovery in the late stages of the isolation process (Yang *et al*., 2013). GNPS can be used to dereplicate known compounds by comparing

the obtained mass spectra from the extracts to those of compounds in the GNPS database. This saves time, effort, and cost by eliminating known compounds early in the drug discovery process. The summary of the molecular networking process is described in Yang *et al*. (2013). The first step is to collect MS/MS spectra; then, a molecular network is generated using cosine scores, which measure relatedness in MS/MS spectra and can be visualised using Cytoscape, a tool designed to visualise correlations of large data sets (Nguyen *et al*., 2013; Smoot *et al*., 2011 & Watrous *et al*., 2012). Finally, the molecular network and MS/MS data are screened against a library of known compounds. MS/MS data allows a very accurate library search, as each fragmentation pattern is unique. While certain compounds share the same precursor mass, each compound, and its isomers, have a unique MS/MS spectrum.

3.2 Methodology

3.2.1 Spot bioautography

M. aurum A+ cultures were inoculated as described in section 2.2.2 and allowed to grow overnight at 37°C while shaking. Culture purity was determined by Gram staining and streaking for single colonies. The OD_{600} was measured, and the culture was diluted with sterile 2YT media such that it had an OD_{600} = 0.5. This was to ensure the cell concentration was controlled for each experiment.

Extracts were reconstituted into ethyl acetate to a concentration of 20 mg/mL. Samples were tested at 100 µg/mL by spotting them onto silica gel 60F aluminium TLC strips from Merck at a volume of 5 µL. The diluted bacterial indicator was dabbed onto the plates with sterile cotton pads. These plates were placed in a humidified container and were incubated at 37°C overnight. Following incubation, 1% MTT was sprayed onto the plates which were left to incubate for approximately 30 minutes before observing the TLC plates for antibiotic activity.

3.2.2 High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

A High-Performance Liquid Chromatography (HPLC) gradient method developed by Ms Alicia Evans at the Division of Clinical Pharmacology, University of Cape Town, was used for HPLC-QTOF analysis. The method has been routinely used for drug screening, separating a wide range of molecules with different polarities. It starts at 2% organic and ends at 98% organic phase, with a flow rate of 600 µL/min and a run time of 18 minutes. The aqueous mobile phase used consisted of 1 mM ammonium formate in water, and the organic mobile phase was 0.5% formic acid in methanol. A Kinetex® Evo C18 LC column (5 µm, 100 Å, 50 mm x 2.1 mm), with a column protector was used. All solvents were sonicated for 10 minutes before use to remove bubbles.

The instrument used was an AB Sciex® X500R quadrupole linked to a time of flight (QTOF) mass spectrometer coupled to an AB Sciex® Exion LC system. Spectral data were obtained using information-dependent acquisition. Samples were scanned from 50–1200 Da. All methods, batches, and data were processed using OS Sciex® v1.2. The declustering potential was 80 V, the curtain gas was at 25 pounds per square inch (psi), the ion spray voltage was 5,500 V, and the source temperature was 450°C. Ion source gas 1 was 45 psi, and ion source gas 2 was 55 psi. The collision energy was 10 eV for the MS scans and ramped from 15 to 35–50 eV for MS/MS scans. The information-dependent acquisition intensity threshold was 50 cps.

3.2.3. Molecular Networking

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data were filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. The precursor ion mass tolerance was set to 2.0 Da and an MS/MS fragment ion tolerance of 0.5 Da. A network was then created where edges were filtered to have a cosine score above 0.65 and more than 5 matched peaks. Further, edges between two nodes were kept

in the network if, and only if, each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra, and library spectra were required to have a score above 0.65 and at least 5 matched peaks (Wang *et al*., 2016).

3.3 Results and Discussion

3.3.1 Method Development

To determine if extraction of the ZOI and analysis by molecular networking provided the same results as traditional bioassay-guided fractionation, two comparative studies were undertaken.

The molecular networks from *Streptomyces* strains PR3 and PR10 samples grown in ISP2 medium were compared to results obtained in previous studies. In an antimalarial screening project, Dr Daniel Watson (Division of Clinical Pharmacology, University of Cape Town) identified valinomycin and a number of analogues as the active compounds produced by *Streptomyces* strain PR3 when cultured in ISP2 liquid media following extraction and fractionation by solid-phase extraction.

In an antibacterial screening project, Ms Joanna Chan (Division of Clinical Pharmacology, University of Cape Town) identified actinomycin D and a number of analogues as the active compounds produced by *Streptomyces* strain PR10 when cultured in liquid ISP2 media following extraction and fractionation by solid-phase extraction. The results obtained from these two studies were compared to the extracted ZOIs produced by strain PR3 and PR10 using the agar overlay method (Table 6). The main parameters being evaluated from the GNPS data are the mass difference of the spectra being compared and the cosine scores.

The mass difference between the paired spectra must be 0 or equal to a common MS adduct to be confirmed as a match.

A cosine score is the measure of vector similarities which measures the relative intensities of the fragment ions and the precursor *m/z* difference between the paired spectra (Watrous *et al*., 2012). The closer the cosine score is to 1, the more similar the two compounds whose spectra are being compared. For the purpose of this project, a cosine score of 0.75 or above was considered high.

Table 6: The GNPS results for known active compounds from *Streptomyces* strain PR3 and PR10 using traditional bioassay-guided fractionation and the ZOI extraction method.

Bioassay-guided fractionation						
Strain	**Media**	**Match**	**Cosine score**	**Library *m/z***	**Mass difference (Da)**	**GNPS Link**
Streptomyces strain PR3	ISP-2	Valinomycin	0.77	1111.63	0.216	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=01c4504a863a4919b5566e0a269a9e34
Streptomyces strain PR10	ISP-2	Actinomycin D	0.87	1255.34	0.004	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7d67374ad2d0428cb7bab9f50751ad16
ZOI Extraction Method						
Strain	**Media**	**Match**	**Cosine score**	**Library *m/z***	**Mass difference (Da)**	**GNPS Link**
Streptomyces strain PR3	ISP-2	Valinomycin	0.90	1111.63	17.04	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e69d14e32f0f4dfb8f0da1a736e74482
Streptomyces strain PR10	ISP-2	Actinomycin D	0.77	1255.34	0.002	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=bdd644d234e7470d971327c51bf8e10f

The above results match very well, both valinomycin and actinomycin D detected in the previous studies were detected with a high cosine score and a low mass difference in the ZOI extracts. The valinomycin match in the ZOI extraction method differs by 17.04 Da, which represents an ammonium adduct. Valinomycin is commonly detected as an ammonium adduct with an *m/z* of 1128.63 (Paulo *et al.,*2019).

These results suggest that the extraction directly from the ZOI is valid and can yield the same results as traditional bioassay-guided fractionation with fewer steps and a simpler extraction method. Furthermore, this method ensures that the antimicrobial agents at the site of inhibition, and therefore those most likely responsible for the antimycobacterial activity, are extracted. The method allows for the rapid screening of strains under many different conditions and identification of active compounds present at the site of inhibition.

3.3.2 Spot Bioautography

To assess whether the ZOI extracts retained their antimycobacterial activity, a few of the extracts were tested using the spot bioautography technique. This technique employs chromatography in detecting antimicrobial compounds from a mixture of compounds (Rios *et al*., 1988; Wagman & Bailey, 1969). The extracts are spot inoculated on thin-layer chromatography (TLC) plates, and the bacterial indicator culture is dabbed onto the extract-inoculated TLC plates. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is used as a stain because MTT reduction assay gives an indication of bacterial survival as it involves the conversion of MTT by viable cells into a purple-coloured formazan product. An active compound is identified by the lack of the purple colour in a clear ZOI (Kuete *et al*., 2017).

Spot bioautography of the 10 selected ZOI extracts revealed no antibacterial activity against *M. aurum* A+ at 100 µg/ml. The zones of inhibition are not clear and distinct as they should be. This reveals that the samples contain low concentrations of the active compound (Figure 3). This suggests that, while this method can be used to identify active compounds, the samples cannot be used in bioactivity studies.

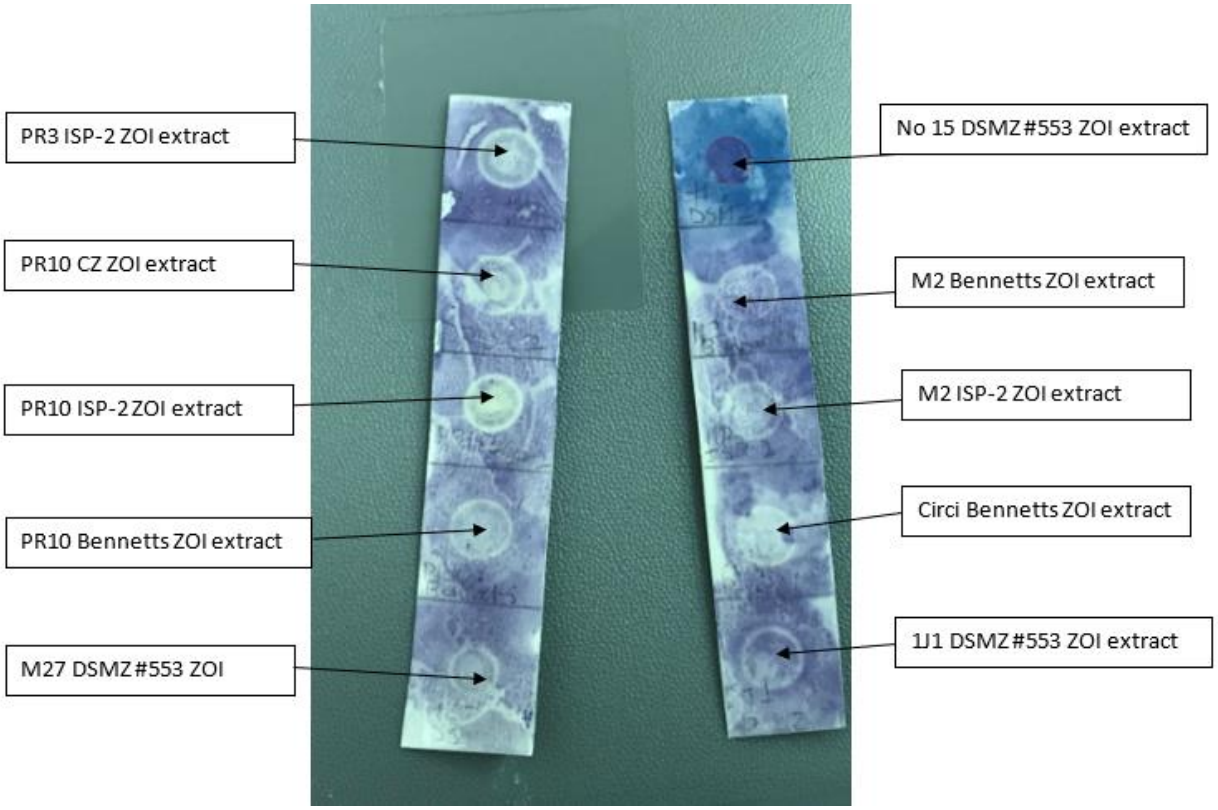

Figure 3: Spot bioautography of selected ZOI extracts against *M. aurum* 2YT at 100 µg/mL

3.3.3 Molecular Networking

In a molecular network, the nodes or vertices represent a mass spectrum from an individual molecule, and the edges represent relationships between the nodes, in this case, the cosine score or relatedness (Grennan, 2014). The length of the edge does not affect the relatedness. Figure 4 shows an example of a molecular network created from the *Actinomadura* strain M2 extract grown in Bennett's medium.

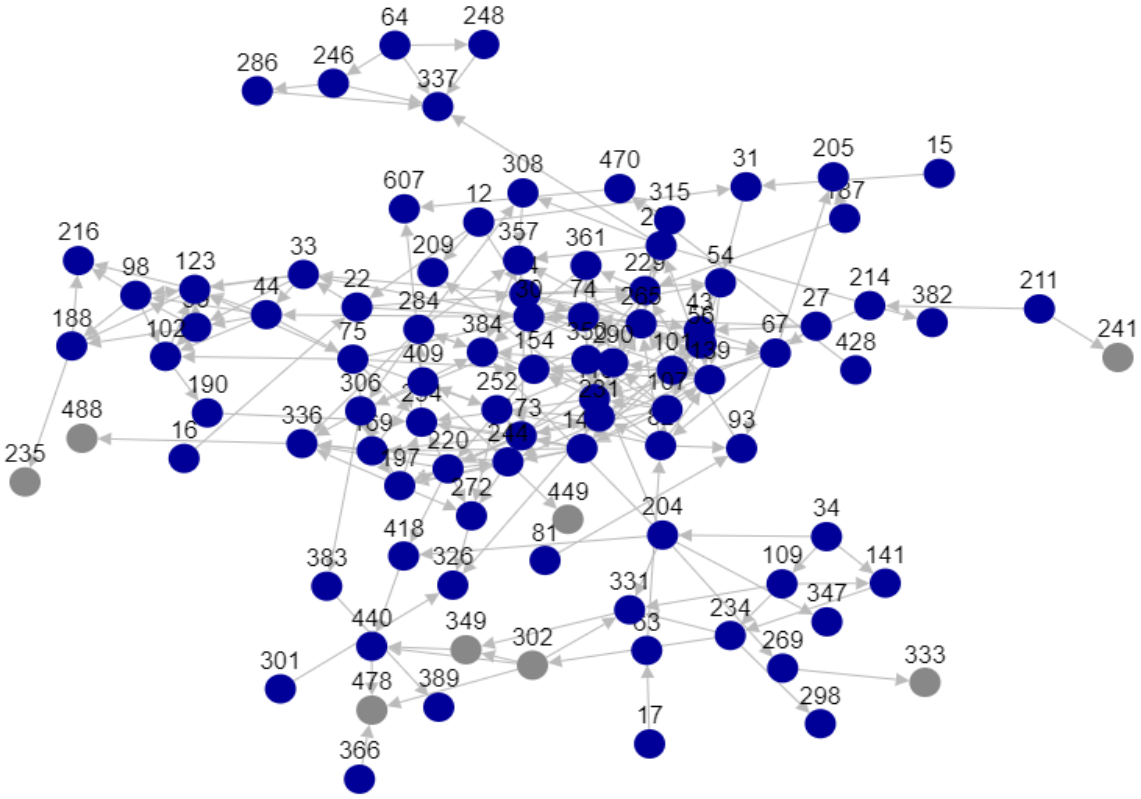

Figure 4*:* Molecular network of *Actinomadura* strain M2 extract grown in Bennett's medium created using the GNPS platform.

Molecular networking analysis was performed on all eight active extracts. *M. aurum* A+ was overlaid on uninoculated media plates, excised and extracted to prepare blanks. These blanks could then be used to identify background signals in active samples and aid identification of active compounds. Only signals present in the active extracts and not in the blanks would warrant further study.

Two strains produced antimicrobials with no match on the GNPS database, *Amycolatopsis circi* S1.3^{T} and *Kribbella speibonae* SK5. Both these strains showed moderate to strong antimycobacterial activity (Table 5), particularly *Kribbella speibonae* SK5. Furthermore, the *Amycolatopsis* genus has been previously reported to display activity against *M. tuberculosis*. Amycolatopsins A and C, isolated from *Amycolatopsis* sp. MST-108494, were found to reach IC_{50} values of 0.50–7.00 µg/mL (Khalil *et al*., 2017). A species of the *Kribbella*

genus, *Kribbella* MI481-42F6, produces antifungal compounds called kribellosides A-D. These alkyl glyceryl ether compounds were found to inhibit the activity of RNA 5'-triphosphatase from *Saccharomyces cerevisiae in vitro* with IC_{50} of 5.00–8.00 µM and showed antifungal activity with MICs ranging from 3.12–100.00 µg/mL against *S. cerevisiae* (Igarashi *et al*., 2017*).* This suggests that these genera could be a viable source of bioactive compounds and that *Amycolatopsis circi* S1.3^T and *Kribbella speibonae* SK5 may be producing novel antimycobacterial compounds and are worth studying further.

For the rest of the strains, one or more matches were identified in the GNPS database. The strains were found to produce different bioactive compounds in different media. For most of the extracts, except for actinomycin D presented in Table 8 there were noticeable mass differences. These differences could be due to different mass adducts presented, or the signals represent analogues of the identified compound. Further study would be warranted to determine which is the case for each. The GNPS matches for the *Actinomadura* strains M2 and M27 are listed in Table 7 below.

Table 7: GNPS matches for the active *Actinomadura* strains

Strain	Media	Match	Cosine score	Library *m/z*	Mass difference (Da)	GNPS Link
Actinomadura strain M2	Bennett's	Rifapentine	0.78	877.46	5.759	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5d260befb92e4fecaea08f3ad2285237
Actinomadura strain M2	Czapek	Acarbose	0.76	663.28	55.868	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=7b575e5a35824bf4a967031f691a53c2
		Bonactin	0.67	418.28	30.371	
Actinomadura strain M2	DSMZ #553	Rifabutin	0.66	847.45	24.251	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c7e2465ac4c8484f9da4c03b2f7c9f49
		Acarbose	0.75	663.28	55.890	
		Bonactin	0.67	418.28	29.690	
Actinomadura strain M2	Middlebrook	Acarbose	0.75	663.28	15.881	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=3b561a243956434ba8fdb8e478e5885a
		Rifapentine	0.73	877.46	7.773	
		Streptomycin	0.90	614.30	6.844	
Actinomadura strain M27	DSMZ #553	Rifapentine	0.79	877.46	5.760	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ff2a85bd61464f5d9acecbe637e90970

Actinomadura strains M2 and M27, both produced compounds whose mass spectra matched that of the TB drug rifapentine. *Actinomadura* strain M2 also matched with rifabutin in DSMZ #553 media. Rifapentine is a semisynthetic derivative of rifampicin (Donald & McIlleron, 2009). Rifabutin is also a semisynthetic antibiotic that is similar in structure and activity to rifampicin and rifapentine (National Center for Biotechnology Information, 2020). Rifabutin, rifapentine, and rifampicin belong to a class of antibiotics known as rifamycins. The cosine scores for the rifapentine hits produced by *Actinomadura* strain M27 and *Actinomadura* strain M2 in Bennett's are high – as seen in Table 7. But, there is a mass difference of 5.760 *m/z* for *Actinomadura* strain M27, and 5.759 and 7.773 *m/z* for *Actinomadura* strain M2. These results suggest that these species could be producing a rifamycin or a compound similar in structure to a rifamycin. Since rifamycins are active against M.tb, this compound is recommended for further study.

No significant match for *Actinomadura* strain M2 grown in ISP2 was found in the GNPS database; this could either mean that the strain is producing a novel compound in this medium, or the bioactive compound being produced was produced in small quantities, or that it is unstable and therefore was not detected by the mass spectrometer. Either way, *Actinomadura* strain M2 grown in ISP2 warrants further study.

A compound similar in structure to acarbose was produced by all the tested strains in multiple different media (ISP2, Czapek, DSMZ #553 & Middlebrook) except for *Actinomadura* strain M27. This means that this compound is produced in several growth conditions and its production pathway may be common amongst actinobacteria or at least *Actinomadura* and *Streptomyces* species. Acarbose is an alpha-glucosidase inhibitor which decreases intestinal absorption of carbohydrates and is used as adjunctive therapy in the management of type 2 diabetes (National Center for Biotechnology Information, 2020). This secondary metabolite is known to be isolated from the culture broth of various *Actinoplanes* strains (Geen *et al*., 1996).

The mass difference of acarbose for strain M2 in Middlebrook and *Streptomyces* strain 1J1 and strain No. 15 was 15.881 Da, 15.939 Da, and 15.945 Da respectively, which suggests that there could be a molecular oxygen difference between the library hit and query. The matches to acarbose for *Streptomyces* strain PR10 (in ISP2), *Streptomyces* Isolate 268, *Actinomadura* strain M2 (grown in Czapek) and *Actinomadura* strain M2 (grown in DSMZ #553) had a mass difference of 16.140 Da, 18.347 Da, 55.868 Da, and 55.890 Da respectively. Further study is required to determine the analogue structure. Considering that acarbose is a known drug but has never been reported for any kind of antimicrobial activity, it is unlikely that the acarbose-related compound is inhibiting *M. aurum* A+.

Bonactin, isolated from *Streptomyces* sp. BD21-2, has been shown to have antibacterial and antifungal activity (Schumacher *et al*., 2003). In this project, a compound possibly similar to bonactin was produced by only two species, *Actinomadura* strain M2 in two different media (Czapek and DSMZ #553) and *Streptomyces* strain No. 15 grown in Bennett's medium. Therefore, this compound was produced under different conditions, both rich and low in nutrients, and there is no indication of growth condition preference. Interestingly, the mass difference between the query and library spectra for the bonactin hits in *Actinomadura* strain M2 Czapek and DSMZ #553 samples is 30.4 Da and 29.7 Da respectively, suggesting that the same analogue may be produced under each condition.

Bonactin has no reported antimycobacterial activity. This could mean that this compound could be further tested for antimycobacterial properties or the strain is producing another antimycobacterial compound which was not identified through the GNPS database and, therefore, could be a novel compound.

It is worth noting that the *Actinomadura* strain, M2 grown in Czapek only matched to acarbose and bonactin. This could mean that one of these identified compounds could have antimycobacterial properties or, if neither are active, due to the strain's strong activity, it is recommended that it be studied further to determine what compounds it is producing.

Compounds produced by the *Streptomyces* species and their GNPS matches are shown in Table 8 below.

Table 8: GNPS data for the *Streptomyces*

Different strains producing the same compounds under different growth conditions.

Strain	Media	Match	Cosine score	Lib m/z	Mass difference	GNPS link
Streptomyces Isolate 268	Czapek	Acarbose	0.68	663.28	18.347	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=4d9703a2d0374dc3954b41f1aba4b323
Streptomyces strain no.15	Bennett's	Bonactin	0.71	418.28	11.693	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=33f69101f5444c0fa0dbb4b0aca1b611
Streptomyces strain No. 15	DSMZ #553	Cycloheximide	0.68	282.17	62.020	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=f0247c710e5e49adb90e6b8e529b09ef
		Brefeldin-A	0.68	281.18	6.002	
Streptomyces strain *no*.15	ISP2	Acarbose	0.74	663.28	15.945	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c45a43a3e178400188a54350ec58597d
		Anisomycin	0.67	288.12	42.216	
		Cycloheximide	0.69	282.17	64.280	
Streptomyces strain PR10	Bennett's	Actinomycin D	0.86	1255.64	0.001	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ee70d619584e4843b55de9c8b500bfb7
		Anisomycin	0.72	304.10	19.772	
Streptomyces strain PR10	Czapek	Actinomycin D	0.87	1255.64	0.002	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=76e5f378185c43e7969f9643f01281b2
		Anisomycin	0.72	304.10	19.767	
Streptomyces strain PR10	ISP2	Acarbose	0.75	663.28	16.140	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=bdd644d234e7470d971327c51bf8e10f
		Actinomycin D	0.77	1255.64	0.002	
		Brefeldin-A	0.71	281.18	20.026	
		Cycloheximide	0.70	282.17	6.015	
Streptomyces strain 1J1	DSMZ #553	Acarbose	0.65	663.28	15.939	https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5d62e7e6957e47bc972f915b33f5a4e4
		Saliniketal A	0.66	396.27	60.203	

One of the compounds produced by *Streptomyces* strain, No. 15 and *Streptomyces* strain PR10 grown in DSMZ #553 and ISP2, matched to cycloheximide. Cycloheximide is an antibiotic produced by the bacterium *Streptomyces griseus*. It has a role as a bacterial metabolite, a protein synthesis inhibitor, a neuroprotective agent, and an anticoronaviral agent (National Centre for Biotechnology Information, 2020). There was a mass difference of 62.020 Da and 64.280 Da between the library and the compounds produced by *Streptomyces* strain No. 15. For strain PR10, the mass difference was lower at 6.015 Da. This means that the compound identified is not cycloheximide, but possibly an analogue. The compound would have to be evaluated further to

elucidate its structure. The media that the compound grew in were ISP2 and DSMZ #553, both of which are rich media.

No known antimycobacterial compounds were detected in *Streptomyces* strain 1J1 and *Streptomyces* strain. No. 15 grown in Bennett's and DSMZ #553 media, and *Streptomyces* Isolate 268.

Brefeldin-A, a compound that exhibits a wide range of antibiotic activity, is known to be produced by a fungus; *Penicillium brefeldianum* (DrugBank, 2021), however, it matched to the spectra of compounds produced by *Streptomyces* strain No. 15 (in DSMZ #553) and *Streptomyces* strain PR10 (in ISP2) samples, both rich media. Due to the mass differences, the matched compound cannot be brefeldin-A, but may prove to be an analogue. None of the blanks' mass spectra matched with brefeldin-A; therefore, this could be the first report of this compound being produced by *Streptomyces.* Brefeldin-A inhibits protein secretion in mammalian and other eukaryotic cells by interfering with the function of the Golgi apparatus (Sigma-Aldrich, 2021). This compound may be used to study cell processes which depend upon intracellular protein transport.

Streptomyces strain No. 15 grown in ISP2 and *Streptomyces* strain PR10 grown in Bennett's and Czapek produced compounds similar in structure to anisomycin. Interestingly, the compounds produced by *Streptomyces* strain PR10 had the same mass difference, indicating that the strain is producing the same compound under the two different conditions. Anisomycin has been shown to activate c-Jun N-terminal kinase (JNK) which induced selective apoptotic killing of M.tb-infected human macrophages, although this points more to cytotoxicity than antibacterial activity (Schaaf, 2017). According to the life sciences company Sigma-Aldrich, anisomycin is primarily an antiprotozoal agent with little antibacterial or antifungal activity (Sigma-Aldrich, 2020). It is, therefore, likely that the anisomycin-related compound is not responsible for the inhibition of *M. aurum* growth.

As discussed previously, strain PR10 was shown to produce actinomycin D under all conditions. The results had very low or no mass differences strongly suggesting actinomycin D is present. Actinomycin D has been previously shown to have antimycobacterial activities (Alvirez-Freites *et al*., 2002; Chen *et al*., 2012).

A compound similar in structure to saliniketal A was produced by *Streptomyces* strain 1J1. Saliniketal A is a bicyclic polyketide known to be produced by another rare filamentous actinobacteria, *Salinispora arenicola* (Jensen *et al*., 2007). This compound inhibits the enzyme EC 4.1.1.17 ornithine decarboxylase, which is thought to be a drug target for cancer treatment because it is a key enzyme in polyamine biosynthesis that is required for cell proliferation (MetaCyc, 2020). The pathway to the production of saliniketal has been hypothesized to be linked to rifamycin B biosynthesis, as indicated in Figure 5. As mentioned earlier, rifamycins are a class of drugs used to treat illnesses such as TB. Therefore, saliniketal A may be responsible for the antimycobacterial activity observed from strain 1J1. The mass difference of 60.203 Da for saliniketal A suggests that this compound is an analogue of saliniketal A.

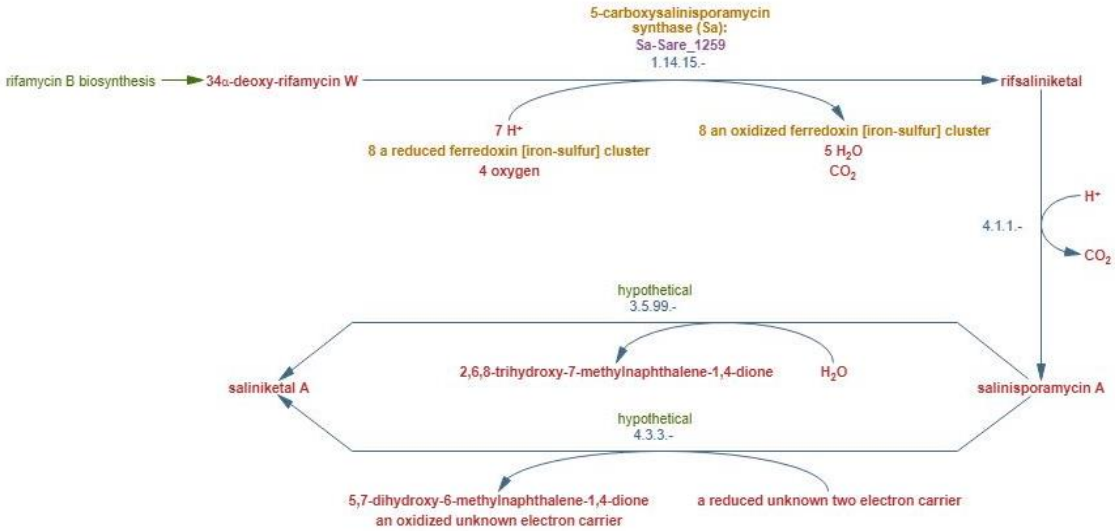

Figure 5: MetaCyc Pathway: saliniketal A biosynthesis

3.4 Conclusion

As mentioned before, many pharmaceutical research units abandoned the study of natural compounds for drug discovery. One of the reasons for this was the issue of rediscovering known compounds. Because the compounds couldn't be identified early in the drug discovery process, time and money were wasted. The extraction and analysis of the ZOI by molecular networking allows for the dereplication of known compounds early in the drug discovery process, allowing novel active compounds to be prioritized for extensive study. Without molecular networking, the cultures would have had to be grown in larger scales, extracted, purified and then the structure would have to be elucidated by multiple spectrometric methods and, only after the structure elucidation, would we know what the compound was. This method, however, makes it possible to extract the compounds directly from the ZOI, analyse them by high-resolution MS and use the mass spectra to identify whether the compounds being produced are known compounds or possibly novel analogues. Although spot bioautography showed the extracts were not active, the spectrometric results from extracted ZOIs can be used to guide larger scale bioassay-guided fractionation studies to isolate novel compounds.

Based on the results discussed in this project and literature, evidence shows that the rare actinobacteria are suitable sources of antimycobacterial compounds. The answer to the question of whether these antimycobacterial compounds are novel is not definitive, but, based on the GNPS results received, only one match, actinomycin D, could be considered accurate, the rest had too big a mass difference. This warrants further analysis of the active strains because this could mean that the compounds being produced have not been previously identified.

Chapter 4: Conclusion

4.1 Research Summary

The objectives that were set for this research have been met. Actinobacteria from seven genera were screened for production of compounds with antimycobacterial properties. The bacteria were grown in six different media conditions and with four of those conditions inducing the production of antimycobacterial compounds – ISP2, Bennett's, DSMZ #553, and Czapek medium. The results were shown to be reproducible by growing and screening the selected active bacteria again using the same conditions.

The ZOI were extracted using ethyl acetate, and the extracts were tested for activity using spot bioautography and analysed using high-resolution MS and molecular networking. Several matches were found for the extracts in the GNPS molecular networking database; however, with the exception of actinomycin D, the matches are unlikely to be accurate due to the significant calculated mass differences between the query and library spectra.

From this investigation, the strains to prioritise for further study are *Actinomadura* strains M2, *Amycolatopsis circi* S1.3^{T}, *Kribbella speibonae* SK5 and *Streptomyces* strain 1J1. The compounds related to bonactin and acarbose could also be extracted by using bioassay-guided fractionation and tested again against *M. aurum* to determine whether they are active against it or not. If these are active, they could be studied further against *M. tuberculosis*. If the bonactin and acarbose-related compounds are not found to be active against *M. aurum*, then at least one of the strains that produce these compounds ought to be studied further as this could be an indication that the strains are producing another compound which was not identified using GNPS due to its novelty.

Streptomyces strain No. 15 grown in Bennett's media had only one match – bonactin. For this reason, this strain could be the strain of choice to study for assessing the activity of the bonactin-related compound. *Actinomadura* strain, M2 grown in Czapek had the highest cosine score for acarbose; therefore, this strain

could be the strain of choice for further analysis of the activity of the acarbose related compound.

4.2 Limitations

The use of the GNPS database for dereplication is not perfect. Other public and private spectral libraries such as Dictionary of Natural Products or AntiBase need to be used to confirm hit results. Other spectrometric techniques such as ultraviolet or infrared absorbance and nuclear magnetic resonance analysis could be utilised as well.

4.3 Future prospects

Taking into consideration the literature discussed here as well as the results of the experiments, it can be concluded that rare actinobacteria may produce novel antimycobacterial compounds and should be explored further. The promising strains can be grown again at a larger scale using the same growth conditions and active compounds extracted using bioassay-guided fractionation. The fractions can then be tested against *M. tuberculosis*, and active hits can be studied closely. The strains that warrant further study include the very active *Kribbella speibonae* SK5 and Actinomadura M2. Seeing that not a single match was found for ZOI extracts from *Amycolatopsis circi* S1.3^{T}, this strain also ought to be explored more closely as this could mean that the strain is producing an unknown compound.

4.4 Contributions

The overlay method has been in use for years in microbiology labs, but the extraction of compounds from the ZOI for analyses is novel. The use of the GNPS database for dereplication while not novel, is a modern technique that is continuously growing and proving useful. This method plays a crucial part in rapidly detecting known compounds early in the drug discovery process, and it is relatively easy and inexpensive to perform.

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