Identification of Anti-tubercular Compounds in Marine Organisms from Aotearoa

by

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Abstract

Tuberculosis (TB) is responsible for more than one million human deaths per year globally, more than any other disease caused by a bacterial pathogen. Although effective treatments exist, the long duration of the current treatment regimen as well as associated drug toxicities can lead to patient non-compliance. This has resulted in the spread of drug resistance which is highlighted by the isolation of extensively drug-resistant strains of *Mycobacterium tuberculosis* complex in all regions of the globe. The management of drug-resistant TB cases requires an extended treatment duration of 18 months minimum post sputum-culture conversion, using drugs that are more frequently linked to adverse side-effects. Therefore, there is a need to identify new drugs that shorten treatment time, and also exhibit better tolerance in patients.

This thesis describes the screening of crude marine extract libraries for the identification of novel anti-tubercular compounds. The libraries consisted of extracts from New Zealand marine organisms that were screened for growth inhibitory activity using a surrogate TB model organism *M. smegmatis*, and subsequently were validated against *M. tuberculosis*. Bioassay-guided fractionation led to the identification of two new compounds as well as a mixture of known compounds. In addition to anti-mycobacterial activity, the spectrum of activity of the isolated compounds was also investigated.

Fractionation of the liver extract of the blobfish *Psychrolutes microporosus* led to the identification of a mixture of four common fatty acids as the major bioactive components. Testing each of the fatty acids individually identified palmitic acid as the most active component against *M. tuberculosis* H37Ra. This is at odds with current understanding of the fatty acid parameters required for bioactivity in terms of chain length and degree of unsaturation.

Bioassay-guided fractionation of a *Latrunculia* sp. sponge extract led to the isolation of a novel taurinated terpene rimarikiamide A (**98**). This compound was initially believed to be responsible for the observed anti-mycobacterial activity, however, further purification led to partitioning of the anti-mycobacterial activity away from **98**. The taurinated terpene rimarikiamide A was nevertheless tested for activity in

mammalian cell lines and was found to exhibit higher levels of growth inhibition towards HL-60 cells with respect to HEK cells.

Working with *Xiphophora chondrophylla*, a New Zealand brown alga, led to the identification of the amino alcohol 3-*epi*-xestoaminol C (**156**) a stereoisomer of a known compound, as the active principle. This compound displayed the same level of activity in all microorganisms tested, however it proved to be more active against HL-60 cells. To further investigate the mode of action of **156**, chemical genetics was employed to indicate the relevant genetic networks that the compound interacts with. This led to the identification of expected pathways such as lipid metabolism as well as cytoskeletal organization, which have been linked to this class of compounds. Furthermore this work also identified new processes such as RNA catabolism, protein targeting and phosphorylation in addition to several unknown processes.

98

156

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Abbreviations

A	Acetone.
AU	Absorbance units.
br	Broad.
CDI	1,1' Carbonyldiimidazole.
CFU	Colony forming units.
d	Doublet.
DAD	Diode array detector.
DCM	Dichloromethane.
DMAP	4-(Dimethylamino) pyridine.
dd	Doublet of doublets.
ddd	Doublet of doublet of doublets.
DIOL	2,3-Dihydroxy-propoxypropyl-derivatised silica gel.
DMF	Dimethylformamide.
DMSO	Dimethylsulfoxide.
DOTS	Directly observed therapy short course.
EDCI	<i>N</i> -(3-Dimethylaminopropyl)- <i>N</i> '-ethylcarbodiimide hydrochloride.
EGFR	Epidermal growth factor receptor.
ESBL-E	Extended-spectrum beta-lactamase producing Enterobacteriaceae.
EtOAc	Ethyl acetate.
FACS	Fluorescence activated cell sorting.
FAME	Fatty acid methyl esters.
FFA	Free fatty acid.
GC-FID	Gas chromatography with flame ionisation detection.
GFP	Green fluorescent protein.
H	Hexanes.
H37Rv	virulent disease causing strain of <i>M. tuberculosis</i> .
H37Ra	avirulent non-pathogenic strain of <i>M. tuberculosis</i> .
HEK	Human embryonic kidney.
HIP	Haploinsufficiency profiling.
HTS	High-throughput screen.
HOP	Homozygous deletion profiling.

HP20/HP20ss	Poly(styrene-divinylbenzene) stationary support.
HRESIMS	High-resolution electrospray ionisation mass spectrometry.
HSQC	Heteronuclear single-quantum coherence (^1H to ^{13}C).
IA	Inactive.
IHD	Index of hydrogen deficiency.
IPTG	Isopropyl β -D-1-thiogalactopyranoside.
LB	Luria-Bertani broth.
LOPAC	Library of Pharmacologically Active Compounds.
MDR	Multi-drug resistant.
MeCN	Acetonitrile.
MeOH, M	Methanol.
MIC	Minimum inhibitory concentration.
MIC ₉₀	Minimum inhibitory concentration at which 90% inhibition is observed.
MNP	Marine natural product.
MOA	Mechanism of action.
MQ	Milliq purified distilled water.
MRSA	Methicillin resistant <i>Staphylococcus aureus</i> .
MS	Mass spectrometry.
MTPA	α -Methoxy- α -trifluoromethylphenylacetic acid.
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5(diphenyltetrazolium bromide).
NA	Not achievable.
OADC	(0.06% oleic acid, 5% BSA, 2% Dextrose, 0.85% NaCl).
PBS	Phosphate-buffered saline.
PC-2	Physical containment level 2.
PKC	Protein kinase C.
PKS	Polyketide synthase.
PLA2	Phospholipase A2.
PSDVB	Poly(styrene-divinylbenzene).
RFU	Relative fluorescence units.
SAR	Structure-activity relationship.
SC	Synthetic complete broth.
SDS	Sodium dodecyl sulphate.
SGA	Synthetic genetic array.

SLAM	Synthetic lethality analysed by microarray.
TB	Tuberculosis.
TDR	Totally drug resistant.
TI	Therapeutic index.
TOCSY	Total correlation spectroscopy (^1H to ^1H).
VUW	Victoria University of Wellington.
XDR	Extensively drug resistant.

List of publications

Nathaniel Dasyam, Andrew B. Munkacsi, Nazmi H. Fadzilah, Dinindu S. Senanayake, Ronan F. O'Toole, Robert A. Keyzers. Identification and bioactivity of 3-*epi*-xestoaminol C isolated from the New Zealand brown alga *Xiphophora chondrophylla*. *Journal of Natural Products*, **2014**, *ACCEPTED*, DOI: 10.1021/np500171z.

Expected

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Chapter 1 Introduction

1.1 Tuberculosis

1.1.1 Disease

Mycobacterium tuberculosis was first identified as the cause of tuberculosis (TB) by Robert Koch in 1882 [1]. TB is an ancient disease, with evidence of its persistence through time recorded in the remains of 3000 year old ancient Egyptian populations [2]. The earliest evidence of humans infected with TB can be dated back to a 9,000 year old Neolithic settlement in the Eastern Mediterranean [3].

TB primarily affects the lungs, with common symptoms such as fever, night sweats, fatigue, coughing, in severe cases bloody and weight loss [4]. Without appropriate treatment 70% of pulmonary TB infections are terminal [5]. Extra-pulmonary TB is a form that infects any part of the body other than the lungs including the skin, the central nervous system and the reproductive organs [6]. The most severe form of extra-pulmonary TB is miliary TB, which occurs when bacteria infiltrate blood circulation and can affect multiple organs [7]. This form of the disease has a high mortality rate, 15-20% in children and 25%-50% in adults [7].

1.1.2 Disease statistics

While the overall mortality rates associated with TB is decreasing, in 2012 8.6 million cases of TB and 1.3 million deaths due to TB were reported by the World Health Organisation (WHO) [8]. Controlling this disease is hindered by the latent form of TB, which is currently believed to affect a third of the world population [9]. In latent TB infection bacilli stay dormant and do not cause active disease, individuals with this form of TB have a 10% risk of developing active TB during their lifetime, each of whom will go on to infect 10-15 people [9]. In New Zealand (NZ) a total of 661 cases of TB were identified in 2010 [10]. NZ has a much higher incidence of TB (6.6 per 100, 000) compared to Australia and the United States of America [10]. Among cases of NZ born patients over 50% were of Maori ethnicity compared to 19% for patients of European ethnicity [10].

1.1.3 Mycobacteria

Mycobacteria are rod shaped non-motile bacteria that can be differentiated using acid fast staining. The acid fastness is unique, due to the high mycolic acid content in the cell walls of mycobacteria, which retain the carbol-fuchsin dye after decolourising with acidic alcohol [11, 12]. These bacteria are widespread in diverse environments ranging from soil to water [13], and are generally categorised into two groups, fast-growing and slow-growing. Fast growing mycobacteria show defined colonies on agar within seven days of inoculation [14]. These include *M. smegmatis*, *M. fortuitum* and *M. abscessus*, which are responsible for post-surgical as well as skin and soft tissue infections [15]. This group also includes *M. phlei* and *M. aurum*, which are generally considered as non-infectious mycobacteria, although there have been reported cases of these strains causing disease in immunocompromised patients [16, 17].

Slow-growing mycobacteria take longer than seven days to show visible growth [14], this group includes *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. leprae*, which belong to the *Mycobacterium tuberculosis* complex (MTB complex), referring to a group of mycobacteria that can cause tuberculosis in either human or animal hosts. Other slow growers include *M. avium*, *M. kansasii* and *M. marinum* all of which cause varying types of skin lesions [15].

Details of two species of mycobacteria that were utilised in this research are described below.

1.1.3.1 *Mycobacterium smegmatis*

This species of mycobacteria is widespread in soil [18], although generally considered to be non-pathogenic it can be an opportunistic pathogen resulting in post-surgical infections [19]. Due to its low pathogenicity as well fast replication time, this species is the work horse for mycobacterial research [20]. The ease of genetic manipulation as well as similarities in drug susceptibility to pathogenic mycobacteria make *M. smegmatis* an appropriate model organism for studying clinically relevant mycobacteria [21, 22].

1.1.3.2 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis H37Rv and H37Ra were both derived from their virulent parent strain H37 [23]. As such, H37Ra shares a high similarity to the virulent strain H37Rv, although as a result of 53 insertion and 21 deletion events, H37Ra has a genome 8,445 bp larger than H37Rv [23]. In addition, the genome of H37Ra differs even further due to 76 single nucleotide variance in 32 genes. These genetic variances are believed to account for the avirulence of H37Ra, the difference in colony morphology and decreased survival inside macrophages as well as under anaerobic conditions [23]. Although H37Rv is considered the standard laboratory strain, the lack of pathogenicity of H37Ra makes it an ideal model of TB that can be used in PC2 facilities.

1.1.4 Disease and pathogenesis

TB spreads through aerosols when an infected individual coughs, spits or sneezes [9]. Upon entry into the lung, bacilli are engulfed by resident alveolar macrophages, although research has also identified that other phagocytic cells such as dendritic cells and neutrophils are also responsible for the uptake of bacteria [24]. This process is mediated through several different receptors that recognise and bind to mycobacterial cell wall components [24]. *M. tuberculosis* can modulate the host immune response in multiple ways, one of which is preventing the maturation of the phagosome [24]. Further modulation by bacilli ensure that necrosis rather than apoptosis is induced in infected host cells, which recruits more non-infected macrophages and facilitates further replication of bacilli [24].

The uptake of bacilli and migration of infected macrophages to a different site is followed by the concomitant formation of granulomas, which is a clear sign of tuberculosis pathology in patients [24]. Within a granuloma, activated macrophages and other inflammatory cells destroy everything including host tissue in order to control intracellular growth of bacilli, this is termed caseous necrosis [25]. It should be noted that not all mycobacteria are destroyed within granulomas, instead a state of balance between the host defence and pathogen's progress is met [24]. By recruiting immune cells the host is able to control the spread of bacteria, while the bacilli are able to use the newly recruited immune cells to replicate [24].

The connection of caseous necrotic tissue to the airway within a patient is what leads to active TB. The change from anoxic conditions to an oxygen rich environment leads to an increase in bacterial growth and eventually patient mortality if left untreated [25]. When caseous necrotic tissue that contains dormant bacilli are released into the airway, bacilli are able to spread to other areas of the lung and start replicating extracellularly [25]. It is at this point where the disease becomes contagious, as bacteria are now able to be spread when infected individuals cough or sneeze [26]. Newly exposed bacteria are taken up by macrophages, through necrosis these infected macrophages eventually destroy neighbouring tissue and create a new area of caseous tissue. Cycles of increased infection followed by resolution forms scar tissue, causing permanent damage to the lung leading to mortality [25]. The ability of *M. tuberculosis* to modulate the immune system and proliferate within immune cells highlights the reason behind the resilience of the bacteria through time. Fortunately, effective treatments exist to manage a TB infection.

1.2 Current treatment regimen

Direct observed therapy short course (DOTS) is the current standard treatment for TB. DOTS lasts for six months to treat drug susceptible TB and can take up to 20 months to treat multi-drug resistant TB (MDR-TB) [27]. DOTS can be either universal or selective; in selective DOTS the treatment can be self-administered, whereas in universal DOTS the entire course of treatment is monitored by health professionals [28]. A recent study comparing the two methods has indicated that universal DOTS results in far less occurrence of drug resistant TB arising within patients, with isolates from selective DOTS being five times more likely to be resistant [28].

DOTS consists of an intensive and a continuation phase. The intensive phase lasts for two months, using a combination of pyrazinamide, rifampicin, ethambutol and isoniazid. This is followed by four months of the continuation phase using a combination of isoniazid and rifampicin [27].

Rifampicin (**1**) is a bactericidal drug that inhibits transcription through its interaction with the DNA dependent RNA-polymerase β subunit [29]. Pyrazinamide (**2**) is believed to disrupt the membrane potential and decrease intracellular pH, leading to inactivation of many important cellular pathways [30]. Ethambutol (**3**) works to inhibit arabinosyl

transferases that are important for cell wall synthesis [31], while isoniazid (**4**) inhibits cell wall synthesis as well, but does so by inhibiting fatty acid synthesis [32]. The use of a combination of drugs with different modes of action reduces the risk of drug resistance arising as well as limits the chance of relapse among patients [33, 34].

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Patient non-adherence, poor treatment management due to lack of funding, overburdened health systems as well as socioeconomic factors all contribute to ineffective application of DOTS and consequently the rise of drug resistance [35, 36]. The treatment of tuberculosis is compounded by the effects of Human Immunodeficiency Virus (HIV) [35]. HIV positive patients are more likely to develop TB and have a higher rate of mortality [37]. Drug-drug interactions are the main issue when treating co-infected individuals. The use of rifampicin becomes problematic in situations where patients are taking either protease inhibitors or non-nucleoside reverse transcriptase inhibitors for HIV, as well as receiving DOTS [38]. Rifampicin causes an increase in CYP3A4, a cytochrome P450 enzyme, which decreases the plasma levels of the anti-retroviral drugs, thus limiting their activity [39]. These types of complex drug-drug interaction must be addressed and stress the need for more effective treatments.

Table 1.1 Current first and second line anti-tuberculosis drugs

Mechanism of action	
First-line drugs	
Isoniazid (4)	Inhibits mycolic acid synthesis by targeting Enoyl-[acyl-carrier-protein] reductase
Rifampicin (1)	Inhibits transcription by targeting RNA polymerase beta subunit
Pyrazinamide (2)	Inhibits translation and trans-translation, acidifies cytoplasm targets S1 component of 30S ribosomal subunit.
Ethambutol (3)	Inhibits arabinogalactan biosynthesis by targeting arabinosyl transferases
Second-line drugs	
Para-amino salicylic acid (5)	Inhibits folate biosynthesis by inhibiting dihydropteroate synthase
Streptomycin (6)	Inhibits protein synthesis by inhibiting S12 and 16S rRNA components of the 30S ribosomal subunit
Ethionamide (7)	Inhibits mycolic acid biosynthesis by targeting Enoyl-[acyl-carrier-protein] reductase
Ofloxacin (8)	Inhibits DNA supercoiling by targeting DNA gyrase and topoisomerase
Capreomycin (9)	Inhibits protein synthesis forming an interbridge B2a between the 30S and 50S ribosomal subunits.
Kanamycin (10)	Inhibits protein synthesis by targeting the 30S ribosomal subunit
Amikacin (11)	Inhibits protein synthesis by targeting the 30S ribosomal subunit
Cycloserine (12)	Inhibits peptidoglycan synthesis by targeting D-alanine racemase and ligase

Tuberculosis treatment drugs and their primary mechanism of action. Table adapted with permission from Cole *et al.* [40].

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1.2.1 Vaccination

At present, the only way to prevent a TB infection is the use of the live attenuated strain *M. bovis* BCG (Bacillus Calmette-Guerin) as a vaccine [41]. It is derived from virulent *M. bovis*, which primarily infects cattle [42]. Thirteen years of passaging cultures of *M. bovis* led to developing BCG and the vaccine has been shown to reduce the risk of TB by 50% [42, 43]. Furthermore, BCG also decreases the risks of developing more harmful forms of TB such as miliary TB and TB meningitis [7]. Until a more effective vaccine can be developed chemotherapeutics continue to be the most effective control for TB.

1.3 Drug resistance

The discovery of **6** in 1943 was believed to herald the end of this persistent disease [44], however this was not to be, with resistance evident as early as the first clinical trial [44]. TB is now established all around the world and drug resistant forms of the disease are widespread [9]. Drug resistance can occur through many pathways including drug inactivation, modification of the drug target, drug efflux pumps or by using alternative pathways to overcome drug mediated effects [45](Table 1.2). In addition, the high mycolic acid content of mycobacterial cell walls protect the bacilli by decreasing the permeability to chemotherapeutic agents [46].

Table 1.2 Common mechanisms of drug resistance to first line anti-TB drugs

Drug	Mechanism of resistance
Isoniazid	Mutation within the gene <i>katG</i> [47], through its peroxidase and catylase activity KatG is normally responsible for the activation of the pro-drug [48]. Other mutations such as <i>ahpC</i> and <i>inhA</i> have also been linked to isoniazid resistance [47].
Rifampicin	Resistance is linked to mutations in the <i>rpoB</i> gene at codon positions 531, 526 and 516, which affects the binding of rifampicin to its target binding site in the β subunit of RNA polymerase [49].
Pyrazinamide	As the exact target has not been identified, the consequent mechanism of pyrazinamide resistance has not been fully elucidated, although mutations within the <i>pncA</i> gene, which is believed to activate the pro-drug, have been implicated in resistance [50].
Ethambutol	Mutation within the <i>embCAB</i> operon, with mutations in <i>embB</i> , which encodes for an arabinosyl transferase, accounting for most cases of resistance [51].

The current definitions of drug resistance in TB include: mono-resistance, polydrug resistance, rifampicin resistance, multi drug resistance and extensive drug resistance [52]. Mono-resistance refers to resistance against only one first-line anti-TB drug, while polydrug resistance is resistance to more than one first-line drug that does not include rifampicin or isoniazid [52].

Multi-drug resistant TB (MDR-TB) refers to strains resistant against multiple drugs but has to include resistance to rifampicin and isoniazid [52]. For extensive drug resistant TB (XDR-TB), in addition to the resistance profile of MDR-TB, the strain should also

display resistance to any fluoroquinolone and at least one of the second-line injectable drugs [52]. Rifampicin resistance includes any strain that displays resistance to rifampicin and may include mono, poly, multi or extensive drug resistant strains [52].

Compared to the cure rate for drug susceptible TB (85-87%) the cure rate for MDR-TB is much lower at 48% [8]. Reports of MDR-TB cases started to rise in the 1990s [53, 54]. Approximately 450,000 cases of MDR-TB were reported in 2012, 9.6% of which were XDR-TB [8]. Recently, the emergence of a new strain unofficially termed totally drug resistant TB (TDR-TB) has been reported from Iran, India and Italy, this further highlights the threat that TB still imposes upon society [55-57]. TDR-TB has displayed resistance to all first and second line drugs, making a TDR-TB infection nearly impossible to cure with current drugs. The need for a new classification for this emerging strain has not yet been deemed necessary by the WHO [58].

MDR-TB strains were first reported in the 1990s [59], their required treatment uses drugs that are more expensive and have more adverse side effects [60]. XDR-TB was first detected in strains of TB collected between 2000-2004 [59]. This form of the disease is even more difficult to cure and if patients are immunocompromised they have much higher chance of mortality, which can be as high as 98% [61]. With a treatment efficacy as low as 20% for XDR-TB, patients have a much lower chance for survival [8]. In New Zealand, there have been a total of 28 MDR- TB cases in the past 13 years, as well as one reported case of XDR-TB in 2010 [62].

MDR and XDR-TB as well as the emergence of TDR-TB stress the need for better treatments for TB now more than ever before. The promising drug candidates listed below highlight the response to address the need for new and more effective drugs. These include the new class of anti-mycobacterial compounds, PA-824 (**13**) and OPC-67683 (**14**), nitroimidazole drugs, which are in Phase II and III clinical trials, respectively [40]. These drugs are active against both replicating and non-replicating bacteria. When these drugs undergo reduction, damaging reactive nitrogen species are produced, resulting in the observed inhibitory activity [63].

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SQ-109 (**15**) is an ethylenediamine analogue of ethambutol (**3**), with a differing mode of activity. The current proposed target for this drug is the trehalose monomycalate transporter, which supplies mycolic acid during cell wall synthesis [64].

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PNU-100480 (**16**), an oxazolidinone, is a derivative of linezolid that inhibits protein synthesis by binding to the 23S rRNA within the 50S ribosomal subunit [65]. This drug is currently under Phase II clinical trials [40].

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The final example highlights a drug that has passed through the drug development process and has gained orphan drug status to treat MDR and XDR-TB. The diarylquinoline TMC-207 (**17**, bedaquiline) inhibits the ATP-synthase subunit c and was approved by the FDA in December 2012 [66, 67].

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Even with the wealth of novelty in the current pipeline [40], the high attrition rate of new candidate drugs undergoing the drug approval process, requires a constant supplement to the pipeline with new potent compounds [68]. Natural products can provide a source of such compounds.

1.4 Natural product chemistry

Natural product chemistry tends to focus on the identification of novel secondary metabolites [69, 70]. By definition, these metabolites are not essential for the viability of the organism, rather they may aid the organism by providing a competitive advantage [71]. It has been postulated that some of these compounds may be produced by symbiotic bacteria, as is the case for bryostatin-1, which is currently under clinical trials for its role as an anti-cancer agent and also as a treatment for Alzheimer's disease [69]. The bryostatins were first isolated from the bryozoan *Bugula neritina*, however the genes for the biosynthesis of this compound can be identified in the symbiotic bacterium *Candidatus Endobugula sertula* [72]. In plants, some compounds isolated are produced by endophytic fungi, as in the case of taxol [73]. The biogenesis of these metabolites usually employ alternative pathways to primary metabolism such as the non-ribosomal peptide synthetase or polyketide synthetase pathways [74]. These pathways are open to modifications by molecular biology tools and can also be used as genetic markers to identify microbes that may be potential sources of unique compounds [69, 75].

1.4.1 Terrestrial sources

Throughout history natural products have provided treatments for ailments with the earliest record for the use of natural remedies being documented on clay tablets from Mesopotamia dating back to 2600 BC [76]. Morphine represents the first commercial

pure drug and penicillin the first antibiotic [76]. Approximately 74% of all approved antibacterial drugs identified in the 1981-2010 time frame originated from natural products [77]. This highlights the wealth of natural products and reveals their potential for the future.

1.4.1.1 Natural products as anti-TB drugs

The potential of natural products for the treatment of TB can be highlighted by the fact that many of the current TB drugs that have been used for decades originated as a result of natural product research. Streptomycin, the first drug that was used to fight TB, was isolated from *Streptomyces griseus* [78]. Isoniazid and pyrazinamide are examples of two very effective first line drugs, which were identified due to their structural similarities to the natural product nicotinamide that demonstrated anti-tubercular effects [79]. Rifamycin, a polyketide isolated from the bacterium *Amiclatopsis rifamycinica*, provided a lead to rifampicin, a gold standard anti-tubercular compound that is currently used for its bactericidal activity [80, 81].

p-Aminosalicylic acid, a second line drug, was a product of research that observed the natural product salicylic acid increased oxygen uptake in *M. tuberculosis*; this led to screening of analogues of salicylic acid to identify competitive inhibitors in the pathway that salicylic acid was interacting with [82]. Cycloserine, another second line drug, is a cell wall synthesis inhibitor and was first isolated from *Streptomyces orchidaceus* [83, 84]. The second line aminoglycoside drug kanamycin, a protein synthesis inhibitor, which later provided the lead for the protein synthesis inhibitor amikacin, was isolated from *Streptomyces kanamyceticus* [85]. Capreomycin, a cyclic peptide often grouped with the aminoglycosides, is another protein synthesis inhibitor that was isolated from *Streptomyces capreolus* [86].

Given the fact that more than nine of the current anti-tubercular drugs have originated from natural products, it is appreciable that they provide a beneficial source for future anti-tubercular drugs. However considering rifampicin, the most recent first line anti-TB drug, was discovered nearly 55 years ago [87], there is a significant demand to find new and more effective drugs.

1.4.2 Marine environment

Scientists have searched the terrestrial environment for antibiotics since the serendipitous discovery of penicillin in 1928 [88]. Researching un-investigated environments may prove useful in identifying novel chemical scaffolds to provide leads for efficacious drugs. In 2011, 1152 new compounds were reported from marine organisms [89]. The marine environment contains organisms producing an array of distinctive molecules, which can be illustrated by the abundance of such structures reported by researchers [89].

From the coasts of New Zealand, specific lipids isolated from the green lipped mussel may provide an effective treatment for asthma and other inflammatory diseases [90, 91]. Peloruside A (**18**) was isolated from the sea sponge *Mycale hentscheli* collected in the Marlborough Sounds of New Zealand. This metabolite shows anti-mitotic activity, making it of interest in the study for anti-cancer therapies [92].

Another interesting discovery was from *Conus magus*, a cone snail that paralyses its prey using a poison tipped barb. From this poison, a painkiller called ziconotide (**19**), was developed and is currently marketed as Prialt for the treatment of chronic pain. This was the first marine drug to be approved by the FDA [93]. From *Ecteinascidia turbinata*, a sea squirt, a compound ecteinascidin 743 (**20**) that PharmaMar has brand-named Yondelis[®] has gained approval from the European Medicines Agency for its anti-tumor activity against soft-tissue sarcomas and ovarian cancer [69].

A more recent addition to the list of approved drugs that have been derived from the marine environment is Eribulin (halaven, **21**), which gained approval for the treatment of metastatic breast cancer [94]. This compound is a synthetic analogue of halichondrin B originally isolated from the Japanese sponge *Halichondria okadai* [95].

More recently, Jensen and Fenical have worked on a marine microbiology discovery program and have discovered a group of obligate marine actinomycetes belonging to the genus *Salinospora* [96]. There are now well above 2000 strains of this genus identified [97]. With this wealth of biodiversity one can imagine the potential to identify unique new compounds. This potential is reinforced by the fact that there are currently 13 marine chemical entities at different stages of clinical trials, with the majority focusing towards treatments for cancer [98].

1.4.2.1 Marine natural products as anti-tubercular agents

In literature pertaining to the TB field, it seems that most active marine extracts tend to come from sea sponges, most probably due to the fact these organisms contain vast numbers of symbionts [99]. Some novel structures that have been identified to inhibit mycobacteria include homopseudopteroxazole (**22**), a diterpene alkaloid isolated from the Caribbean gorgonian octocoral *Pseudopterogorgia elisabethae* [100]. Halicyclamine A (**23**), obtained from an Indonesian sea sponge *Haliclona* sp. has shown bactericidal activity against *M. bovis*, *M. smegmatis* and *M. tuberculosis* [101]. From the Caribbean sponge *Svenzea zeai*, two abeo-sterols, parguesterol A (**24**) and B (**25**) were isolated with reported MIC values of 7.8 and 11.2 $\mu\text{g/mL}$ against *M. tuberculosis* H37Rv as well as reported IC_{50} values of 52 $\mu\text{g/mL}$ in Vero cells for both compounds [102].

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Pseudopyronines A (**26**) and B (**27**), isolated from a marine *Pseudomonas* sp F92S91 [103], displayed inhibitory activity against *M. tuberculosis*, with pseudopyronine B a greater inhibitor of InhA, a fatty acid biosynthesis enzyme [104]. Manzamine A (**28**) isolated from a *Haliclona* sp. sponge [105], and manzamines E (**29**) and F (**30**) isolated

from an Okinawan *Xestospongia* sp sponge [106], displayed respective MIC values of 1.53, 3.76 and 2.56 $\mu\text{g/mL}$ against *M. tuberculosis* H37Ra [107].

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29 R = H

30 R = OH

The marine fungus *Aspergillus versicolor* was isolated from a marine sediment from the Bohai Sea, China, which led to the identification of brevianamide S (**31**), this compound displayed an MIC of 6.25 $\mu\text{g/mL}$ against BCG [108]. Trichoderins A (**32**), A1 (**33**) and B (**34**) were isolated from a *Trichoderma* sp., a marine sponge-derived fungus with MIC values of 0.12, 2.0 and 0.13 $\mu\text{g/mL}$ respectively against *M. tuberculosis* H37Rv [109]. Abyssomicin C (**35**) and atrop-abyssomicin C (**36**), isolated from a marine *Verrucosipora* strain AB-18-032 [110], displayed MIC values of 3.6 and 7.2 μM against *M. tuberculosis* H37Rv [111].

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Considering the growth of marine microbial research, it is believed that scientists have only studied a small fraction of this valuable environment for structurally novel compounds [69]. The function of these compounds could be vital by providing cures or bio-probes to ascertain important information on biological processes [93].

1.4.3 Screening for natural products

1.4.3.1 Bioassay-guided fractionation

The classical approach to natural product isolation has been bioassay-guided fractionation [112]. The serendipitous isolation of penicillin heralded the era of bioassay-guided fractionation, an approach that has never ceased to be a valuable resource for finding bioactive compounds. This approach involves screening crude extracts for activity, following which fractionation is performed on an active sample. The subsequent fractions are tested for bioactivity, the most active fractions are further purified and tested again, and the process is repeated in iterative fashion until the bioactive compounds have been isolated. The purities of the fractions are monitored using spectroscopic methods. This approach requires a set drug target or desired biological activity or a pathway to investigate, with a well-designed and optimised assay. These assays may be simple whole cell assays looking for growth inhibition of clinically relevant pathogens [69], or more complex biochemical assays that may require extensive optimizations prior to screening [113]. This technique is considered destructive as each bioassay requires the expenditure of compound. Another common issue with this classical approach is that it often leads to the isolation of known compounds, especially if the source of the extract has been explored in earlier research [114]. Furthermore this approach can lead to novel compounds being completely ignored as they maybe inactive in the assay system being employed. In some cases, compounds with detergent-like or promiscuous activity are identified as hits and are undesired as they are often cytotoxic and do not make ideal drug leads [115]. In rare cases, activity may be due to two or more compound working in synergy or with additive effects, further fractionation in such situations can lead to a loss of activity. The strength in this approach lies in the fact that a bioactivity of interest is able to be pursued throughout all stages of isolation. To avoid the issue of re-isolation, this approach can be complimented with spectroscopic analysis to prevent further fractionation of extracts with chemical profiles of known compounds [114].

Figure 1.1 Bioassay-guided fractionation

The basic approach to bioassay-guided fractionation for novel compounds.

1.4.3.2 Structure-guided isolation

An alternative approach to bioassay-guided fractionation is structure-guided isolation. This approach is often employed in groups where applying a bioassay after each point of fractionation is often not feasible either due to lack of expertise or lack of appropriate facilities. The robustness of this approach can be observed by the fact that throughout the fractionation process, the compound of interest can be tracked using either nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography coupled to mass spectroscopy (LC-MS) or high performance liquid chromatography linked to a diode array detector (DAD-HPLC). In essence, the bioassay is replaced by an analytical instrument such as a NMR spectrometer and rather than bioactivity, the chemical signatures of unusual secondary metabolites are tracked. When dealing with small levels of compound, highly sensitive spectrometers are required. The downfall of this approach is that since activity was never the primary goal, large masses of compound must be sacrificed to different assays in order to identify the compound's bioactivity. If the compound of interest has been isolated in low quantities this may not allow for it to

be studied in enough suitable assays. This expenditure of compound in multiple assays can be circumvented if the compound is used in an assay that can monitor its effects on a genome wide scale, such as the *Saccharomyces cerevisiae* deletion mutant library [116].

Figure 1.2 Structure-guided isolation.

The principles of structure-guided isolation of novel compounds.

1.4.4 Structure-activity relationships and mechanism of action studies

After isolation of a novel compound, the next stage of the drug discovery process involves structure-activity relationship (SAR) and mechanism of action (MOA) studies. While SAR studies are vital in pinpointing the pharmacophore responsible for bioactivity within a compound, MOA studies answer the important question of how a compound causes an observed activity.

SAR involves chemical modification of the lead compound to generate analogues that are then tested in the assay of interest. In addition to identifying functionalities crucial for bioactivity, it could also identify functional groups of the molecule that are open for modification in order to improve activity or other properties (such as solubility) when compared to the parent compound [113].

One approach for MOA studies involves attaching the drug of interest to sepharose beads and using affinity chromatography on cell extracts to purify proteins that bind to the protein-bound solid phase support [117]. Alternatively, genetic approaches can also be used with the underlying commonality of using or generating mutant strains. One approach involves genome sequencing of resistant mutants to identify mutations conferring drug resistance [118]. This is generally followed by validation assays, which can involve restoring normal functionality of the mutated gene in order to see a return in sensitivity to the compound of interest [51]. Another approach involves overexpressing the drug target to either purify protein to perform biochemical assays or to induce phenotypic effects on drug sensitivity [66]. As some compounds may have no specific target or may target an essential gene, no specific mutants will be detected and this is a limitation to this approach.

If the target is unknown a genome-wide approach can be employed, which involves screening of the compound of interest against mutant libraries in order to find mutants that interact with the drug. Following the identification of mutants, validation through dose response curves is performed, which allows for the top mutants to be chosen. These mutants are then sequenced to identify the specific gene that has been mutated. Once this information is inferred, a genetic network can then be established to identify which pathways may be perturbed by the compound of interest. This approach generates an immense amount of data which can then be used to carry out follow up validation and rescue assays to study the effects of each mutation.

1.4.4.1 *Saccharomyces cerevisiae* and chemical genetics

Saccharomyces cerevisiae (baker's yeast) is an important tool for mode of action studies by chemical genetics [119]. Yeast can exist in diploid or haploid states, and extensive genetic manipulation has led to generation of libraries of homozygous diploid deletion mutants of non-essential genes, heterozygous diploid knock-outs that include essential genes and haploid libraries for the two mating types of yeast [120]. Each knockout mutant has its gene replaced by a kanamycin resistance gene flanked by a 20 nucleotide long sequence unique for each mutant similar to barcodes [121]. Flanking these 'barcodes' are two lengths of nucleotides at the 5' and 3' ends. These nucleotide

sequences are identical and allow for the use of just one set of primers to bind to and amplify the sequence in between [121].

These libraries have led to the development of multiple approaches for MOA studies, such as haploinsufficiency profiling (HIP), homozygous deletion profiling (HOP), synthetic lethality analysed by microarray (SLAM) and synthetic genetic array (SGA) [122].

HIP involves knocking out one of the two genes that code for a particular function within the yeast [123]. Having one less copy results in a decreased amount of the gene product, therefore strains are more sensitive to inhibitors that target that particular gene [123]. This approach is carried out in a pooled format where the mutants are grown in a liquid culture in either the presence or absence the drug, following which polymerase chain reaction amplification of the two different pools of mutants and microarray analysis of the oligomers allows for the identification of mutants that are most sensitive to the drug [124]. This approach allows inferences to be made on the actual drug target as the strains used in this approach are heterozygous knock outs and include essential genes.

HOP involves treating the library of mutants with drug followed by microarray analysis to identify an expression profile. These are compared to the expression profile with homozygous knockouts, which as a consequence are non-essential gene knock-outs. The closest matching deletion profiles allow for conclusions to be drawn as to which pathways the drug of interest is interacting with [125].

SLAM and SGA use haploid strains of yeast and work on the principle of synthetic lethality. This is the phenomenon where two mutations on their own have no effect on viability, however if the two mutations exist in the same cell then this results in cell death. In this way, profiles have been generated for libraries that have an additional “query” mutation introduced to identify synthetic lethal genetic interactions [122]. SLAM exploits the use of the unique tags by performing the experiment in a pooled format and analysing for the absence of drug exposed mutants using microarray analysis [126]. SGA monitors the growth of each mutant on agar and identifies growth defects in mutants exposed to drug [127]. Both approaches assume a drug can replace the role of a genetic mutation. Should this hold true, then synthetic lethal profiles observed for the drug exposed mutants should be possible to match to the profile of a query gene’s

interactions. Using this approach conclusions can be drawn on pathways the drug interacts with or perturbs.

1.5 Research goals

The aims of this research were to isolate novel anti-mycobacterial compounds from marine organisms. This would be achieved through:

Aim I: Detecting anti-mycobacterial activity from screens of New Zealand marine organism extract libraries.

Aim II: Purification and identification of anti-mycobacterial bioactives using bioassay-guided fractionation and spectroscopic structural characterization.

Aim III: Determination of the mechanism of action via studies of novel anti-*Mycobacterium tuberculosis* inhibitors.

Chapter 2 Materials and Methods

2.1 Media and culture conditions

2.1.1 LB broth

LB broth (Duchefa Biochemie low salt) was made up as per instructions on the container, 20 g to 1 L of ddH_2O , autoclaved at 121 °C for 30 min and stored at room temperature before use.

2.1.1.1 Media supplements

Additional antibiotics and supplements were added as required for certain strains of bacteria and have been listed when used in later sections.

Antibiotics were purchased from Sigma and were dissolved in an appropriate solvent or ddH_2O at desired concentrations and filtered through a 0.22 μm syringe filter. Antibiotic stock solutions were aliquoted into microfuge tubes and stored at -20 °C.

Isopropyl- β -D-thiogalactopyranoside (IPTG) was made up with ddH_2O at 100 mg/mL filtered through a 0.22 μm syringe filter and stored at -20 °C.

D-Arabinose and D-xylose were made up to a 100 mg/mL and a 10% w/v solution respectively in ddH_2O , filtered through a 0.22 μm syringe filter and stored at -20 °C until use.

Glycerol was made up to 50% (v/v) with ddH_2O , sterilised in an autoclave at 121 °C for 30 min and was added to media when required, prior to autoclaving.

Tween 80 was made up to 10% (v/v) with ddH_2O and sterilised in an autoclave at 121 °C for 30 min. This was added to media when required prior to autoclaving.

Sodium oleate was made by adding 12.5 mL of 2 M NaOH and 6.0 mL oleic acid to 481.5 mL of ddH_2O . This solution was filtered through a 0.22 μm millipore filter, stored at room temperature and the container was wrapped in aluminium foil.

OADC enrichment supplement for 7H9 media was made by adding 8.5 g NaCl to 925 mL ddH_2O that was stirred until the salt has dissolved, 50 g of bovine serum albumin Fraction V was then added as well as 20 g of dextrose and 50 mL of a 1% sodium oleate solution. The pH was adjusted to 7.0 and this solution was filtered through a 0.22 μm millipore filter and stored at 4 °C.

2.1.1.2 LB broth for *M. smegmatis*

LB broth (section 2.1.1) was prepared with additional supplements 0.1% (v/v) Tween 80 and D-arabinose 100 µg/mL and autoclaved at 121 °C for 30 min. After the broth had reached room temperature, kanamycin sulphate was added to achieve either a final 50 µg/mL or 20 µg/mL concentration. This was done in a sterile PC2 laminar flow hood following which media was stored at 4 °C. When making media to culture electro-competent cells or carry out transformations, D-arabinose and antibiotics were omitted from media, which was stored at room temperature before use.

2.1.2 7H9 media

Middlebrook 7H9 media was made as per instructions on the container (4.7 g per 900 mL) and autoclaved at 121 °C for 30 min. After the broth had cooled to room temperature it was supplemented with 10% (v/v) OADC (section 2.1.1.1). This was done in a sterile PC2 laminar flow hood following which media was stored at 4 °C.

2.1.3 Tissue culture media

RPMT-1640 media was purchased from Invitrogen and supplemented with 0.22 µm filtered 10% FCS and 1% of Pen/Strep (100 units/mL of penicillin and 100 units/mL of streptomycin). Handling of media was carried out in a sterile PC2 laminar flow hood and stored at 4 °C until use.

2.2 Phosphate buffered saline

Phosphate buffered saline (PBS) solution was made by adding 8 g of NaCl, 0.2 g of KCl, 1.42 g of Na₂HPO₄ and 0.27 g of KH₂PO₄ to 900 mL of ddH₂O. The pH of the solution was adjusted to 7.0 using 1 M solutions of HCl and NaOH, and the final volume made up to 1.0 L before autoclaving the solution at 121 °C for 30 min. The solution was stored at room temperature.

2.3 Staining procedures

Cells were stained to observe the purity of culture using the methods describe below. Dyes and staining solutions were purchased from BD Biosciences.

2.3.1 Gram staining

A 10 μ L aliquot of culture was smeared and air dried onto a slide. The slide was covered in Gram Crystal Violet solution for one minute and washed with water, following which the slide was covered in Stabilized Gram Iodine solution for one minute. The slide was then washed with water and decolourised using Gram Decolourizer for 10 sec. Finally Gram safranin was used to cover the slide for 30 sec and washed off with water and air dried. Cover slips were placed on slides and slides observed under 100 x oil immersion using a light microscope.

2.3.2 Acid fast staining

A 10 μ L aliquot of culture was smeared and air dried onto a slide. The slide was covered in TB Carbol Fuchsin KF solution for five minutes and washed with water, following which the slide was decolourised using TB Decolorizer solution for 10 sec. Finally TB Brilliant Green solution was used to counter stain the slide for 30 sec and washed off with water and air dried. Cover slips were placed on slides and slides were observed under 100 x oil immersion using a light microscope.

2.3.3 Fluorescent microscopy

Green Fluorescent Protein (GFP) expressing cells (5 μ L) were prepared on a slide and coverslip and observed under a 100 x oil immersion using a fluorescein isothiocyanate (FITC) filter in an Olympus[®] Research Microscope AX70 Provis, equipped with an OlympusR DP70 Digital Microscope Camera.

2.4 Transformation of mycobacteria

2.4.1 Plasmids

Table 2.1 Plasmids used in this study

Plasmid	Characteristics	Selection	Reference
pLL192_hsp60	Constant expression of GFP.	Strep 50 μ g/mL, Kan 50 μ g/mL	[128]
pCG79	Contains the Tn611 transposon shuttle vector and a temperature sensitive mycobacterial origin of replication	Kan 20 μ g/mL	[129]

2.4.2 Creating electro-competent *Mycobacterium smegmatis*

Mycobacterium smegmatis, mc²155 (ATCC700084); was the strain of mycobacteria used in this research and was sourced from Dr. Desmond Collins, AgResearch Limited, New Zealand. *M. smegmatis* was streaked out onto LB agar plates and grown for 3-5 days until colonies appeared. A loop full of colonies were then used to inoculate 10 mL of LB broth (section 2.1.1.2) and incubated at 37 °C in a shaking incubator at 200 rpm overnight. This culture was then sub-inoculated into 100 mL of LB broth (section 2.1.1.2). Growth was measured once every 1.5 h using optical density (OD) measured at 600 nm. Once an OD₆₀₀ between 0.5-1.0 absorbance units (AU) was achieved, cells were incubated on ice for 20 min and centrifuged at 2700 x g at 4 °C for 15 min. After discarding the supernatant, cells were washed and pelleted a further two times with 50 mL and 20 mL of ice cold 10% glycerol with a 15 min incubation on ice in between. After pelleting cells a third time, cells were re-suspended in 4 mL of ice cold 10% glycerol and 200 µL aliquots of the competent cells were stored in 1.5 mL tubes at -80 °C.

2.4.2.1 Transformation of *Mycobacterium smegmatis*

To electroporation cuvettes 200 µL of electro-competent (e⁻) cells (section 2.4.2) and 200 µL of 10% glycerol was added. To the e⁻ cells 5 µL of either the plasmid pLL192_hsp60 or pCG79 was added. As a negative control 5 µL of MQ H₂O was added. Cells were electroporated at the following settings, voltage: 2500 V, capacitance: 25 C, resistance: 1000 Ω, cuvette width: 2 mm. Following electroporation a 600 µL aliquot of LB broth without antibiotics (section 2.1.1.2) was added to the cuvettes immediately and cells were transferred into sterile 1.5 mL centrifuge tubes. Cells were allowed to recover overnight at 37 °C at 200 rpm. The following day cells were plated out onto LB agar with appropriate antibiotics (Table 2.1). Appearance of colonies only for the mycobacteria carrying plasmids following 2-3 days of incubation indicated a successful electroporation. Colonies were then inoculated in LB broth for mycobacteria (section 2.1.1.2) with appropriate antibiotic (Table 2.1). The overnight cultures were then tested for purity using acid fast staining and fluorescence using a fluorescent microscope (section 2.3.2 & 2.3.3). Freezer stocks of cultures were then made by adding 600 µL of cells to 400 µL of 50% glycerol, following which cells were stored at -80 °C.

2.5 Bacteria and mammalian cell line culturing

Table 2.2 Mycobacteria used in this study

Strain	Use
<i>M. smegmatis</i>	
pLL192_hsp60	Library screening and dose response assays,
pCG79	Transposon mutant library screening
<i>M. tuberculosis</i>	
H37Ra ATCC25177	Dose response assays

2.5.1 Culturing of *Mycobacterium smegmatis*

Freezer stocks of *M. smegmatis* carrying either the pLL192_hsp60 or pCG79 plasmid were streaked out on LB agar with an appropriate drug and incubated for 48 h at 37 °C. Following the appearance of colonies a 5 µL inoculating loop was used to scoop bacteria into 10 mL of LB broth (section 2.1.1.2). After growth incubation for 24 h these cultures were checked for purity using either acid fast staining or fluorescence (section 2.3.2 & 2.3.3) before being used for bioassay.

2.5.2 *Mycobacterium tuberculosis* H37Ra

M. tuberculosis H37Ra ATCC25177 was grown on 7H10 plates for 3-4 weeks at 37 °C, after which colonies were picked and cultured in 7H9 media (section 2.1.2). After approximately 14 days, cells from these cultures were checked for purity using acid fast staining (section 2.3.2) following which they were used for bioassay.

2.5.3 *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Table 2.3 Fast growing bacterial strains

Strain	Characteristic	Culture conditions	Use
<i>S. aureus</i>			
ATCC25923	Clinical isolate, no resistance marker	LB	Dose response assays
Newman	Expresses GFP, erythromycin (Em) resistance	LB, 0.5% (w/v) xylose, Em 50 µg/mL	Library screening
<i>E. coli</i>			
ATCC25922	Clinical isolate, no resistance marker	LB	Dose response assays
DH5α/ pOT11	IPTG induced expression of GFP, chloramphenicol (Chl) resistance	LB, Chl 25 µg/mL, IPTG (100 µg/mL)	Library screening
<i>P. aeruginosa</i>			
PAO1	Opportunistic human pathogen, no resistance marker	LB	Dose response assays

Freezer stocks of cultures were streaked onto LB agar (Duchefa Biochemie) with appropriate antibiotics (Table 2.3). After incubating the plates overnight a 5 µL inoculating loop was used to scoop colonies into 10 mL of LB broth with appropriate antibiotics and supplements (Table 2.3). Cultures were incubated overnight in a 37 °C shaking incubator at 200 rpm. Gram staining was used to check the purity of the cultures (section 2.3.1) which were used to for bioassay.

2.5.4 HL-60 cells

The HL-60 cells used in this research were supplied by Dr. John Miller, VUW; and were sourced from Dr. Graeme Findlay, University of Auckland. HL-60 cells were thawed out from frozen stocks and transferred to 15 mL tubes containing 9 mL of RPMI-1640 supplemented media (section 2.1.3). Cells were pelleted at 300 x g for 5 min and the supernatant was discarded. The pellet was then resuspended in 10 mL of media and transferred to a 25 cm³ flask. Cells were incubated at 37 °C and 5% CO₂ and after reaching approximately 80% confluence cells were pelleted at 300 x g. Cells were

then resuspended in 5 mL of media and 10 μ L of cells were added to 10 μ L of trypan blue to count the number of viable cells using a haemocytometer.

2.5.5 HEK cells

Human embryonic kidney-293 (HEK) cells were supplied by Dr. Bronwyn Kivell VUW; and were sourced from (ATCC, Manassas, VA, USA). HEK cells were thawed as stated above and cultured in 10 mL of media (section 2.1.3) until they had reached ~80% confluence. Following this, media was discarded from the flask and cells were trypsinised with 1 mL of trypsin for 2-3 min, media was then added to the flask to deactivate the trypsin. After centrifuging cells for 5 min at 300 x g, the media was decanted out and the cells were resuspended in 5 mL of media. Cell counts were performed as stated above (section 2.5.4).

Freshly cultured HL-60 and HEK cells were used to set up assays described in this research. This was done to avoid any issues that may arise from using cells that have undergone several passages.

2.5.6 Marine extract libraries

The NIWA library was a four plate library donated by Drs Vicky Webb and Sally Anderson. The library was generated as a nutraceutical library, which contained extracts of vertebrate as well as invertebrates collected from New Zealand waters, and did not include algae. Marine organisms were dissected and extracts were generated of each organ. The library plates contained either water, ethanol or ethylacetate extracts and comprised unknown masses per well.

The VUW MNP library was generated in Dr. Rob Keyzers laboratory using extracts of invertebrates, which also included algae. Samples were collected by Dr. Peter Northcote from New Zealand coastal waters and were extracted in methanol. Extracts were dried down and redissolved to generate solutions of known concentrations after which aliquots were transferred to a 96 well plate to generate a library plate of 50 μ g per well. The locations from where samples were collected are on record, and voucher samples were generated from each sample used to generate the library.

2.6 Assay protocols

2.6.1 Assay measurements

Fluorescence and OD measurements of assays were measured using a PerkinElmer EnSpire, 2300 multilable reader. OD was measured at 600 nm taking an average of 16 points per well (4x4 circle) for mycobacteria, and a single point per well for all other cells. GFP fluorescence was measured at excitation (ex): 488 nm and emission (em): 597 nm taking an average of 16 points per well (4x4 circle) for mycobacteria, and a single point per well for all other cells. GFP was used as a measure of bacterial viability, as only live cells would be expected to express GFP and cells inhibited in growth will not.

After GFP and OD was measured, a 30 μ L aliquot of a 0.02 % (w/v) solution of resazurin were added to each well of the assay plates. After 2 h for *M. smegmatis*, or 20 min for *S. aureus*, *E. coli*, and *P. aeruginosa*, fluorescence of resazurin was measured at ex: 530 nm and em: 590 nm taking a single point measurement per well. For *M. tuberculosis* H37Ra plates were incubated for 24 h before measuring resazurin fluorescence. This additional method to measure growth was also used to add further support for measuring inhibition. Resazurin is a dye that is reduced by actively metabolizing bacteria and in the process goes from a non-fluorescent blue to a fluorescent pink, which can be exploited as a measure of bacterial viability [130].

2.6.2 Screening of marine extract libraries

Marine extract libraries were resuspended in DMSO and aliquoted in 96-well plates with 50 μ L of broth with a maximum final concentration of 2.5% (v/v) DMSO. To this 50 μ L of either *M. smegmatis*, *S. aureus*, or *E. coli* were added to wells to achieve a final OD₆₀₀ of 0.01 AU. Screening was carried out such that 80 extracts were screened per 96-well plate. In the wells of column 1, 100 μ L MQ H₂O was added to maintain a humid environment and prevent evaporation and column 12 comprised of a set of controls in duplicate: media alone, media+cells, media+cells and solvent, media+cells and a positive control (ethambutol 250 μ M or tetracycline 250 μ M) (Figure 2.1). Plates were sealed using breathable SealPlate® plate seals (Interlab) and incubated for 96 h at 37 °C in a shaking incubator at 200 rpm.

Figure 2.1 General set up of a 96-well plate for screening marine extract libraries

Following the assay, plates were measured for GFP and resazurin fluorescence as described in section 2.6.1.

2.6.3 Bacteriostatic assay

Samples were serial diluted two fold in appropriate media in 96-well plates to test the range of concentrations at which they were active. To the outer 36 wells of a 96-well plate, 100 μ L of MQ was added and to the inner 60 wells except those of column two, 50 μ L of media was added. The wells of column two were made up to a final volume of 100 μ L by adding the appropriate amounts of media and sample to give an initial two fold higher starting concentration of extract. Following this 50 μ L of the mixture was aspirated from the wells of column two and pipetted to column three, this step was repeated until the wells of column 11 and the final 50 μ L was discarded. To these plates, 50 μ L of cells were added to achieve a final OD₆₀₀ of 0.01 AU. Plates were sealed and incubated for 96 h at 37 °C in a shaking incubator at 220 rpm.

As a positive control for inhibition, every assay had either rifampicin for *M. smegmatis* or tetracycline for *S. aureus*, *E. coli*, and *P. aeruginosa*, starting at 250 μ M. Negative controls included media alone and cells grown without the presence of any inhibitors. Solvent controls were also included. Plates were measured for GFP, OD and resazurin fluorescence as described in section 2.6.1.

2.6.4 Bacteriostatic assay set up in *M. tuberculosis* H37Ra

Sample dilutions and plate set up was identical to section 2.6.3, using 7H9 media with supplements (section 2.1.2). This was followed by addition of cells to plates to achieve a final OD₆₀₀ of 0.005 AU, after which plates are sealed and incubated for 7 days at 37 °C at 200 rpm. Streptomycin starting at 25 μ M was used as a positive control. Following incubation, plates are unsealed in a laminar flow hood and 30 μ L of a 0.02 % (w/v) of resazurin was added to each well and plates were resealed and incubated a further 24 h at 37 °C in a stationary incubator. Resazurin fluorescence was measured as described in section 2.6.1.

2.6.5 Stationary phase assay protocol in *M. tuberculosis* H37Ra

Following the method described by Bassett *et al.* cultures of *M. tuberculosis* H37Ra were grown for 30 days, pelleted for 10 min at 2700 x g and resuspended in a volume of sterile PBS (section 2.2) to a final OD₆₀₀ of 1.0 AU. Plate set up was identical to section 2.6.3 using sterile PBS. To the plates, 50 μ L of the diluted cells were added to achieve a final OD₆₀₀ of 0.5 AU. Plates were sealed and incubated for 96 h at 37 °C at 200 rpm after which 30 μ L of a 0.02 % (w/v) of resazurin was added to each well and plates were resealed [131]. Resazurin fluorescence was measured as described in section 2.6.1.

2.6.5.1 Statistics for library screening

Z-factors were calculated for each assay and used as a measure of the validity of the screens. The range of the Z-factor can be between 0 – 1, where 0 indicates an invalid assay due to overlap between positive and negative controls and 1 indicates a perfect assay. Values of 0.5 or above are considered acceptable.

$$Z = 1 - \frac{3 (\sigma_{\text{positive}} + \sigma_{\text{negative}})}{|\mu_{\text{positive}} - \mu_{\text{negative}}|}$$

Figure 2.2 Z- factor calculation

μ represents the mean of the positive or negative controls and σ is the standard deviation of the positive and negative controls.

2.6.5.2 Reporting inhibitory values

Where possible, IC₅₀ values that have been reported are based on calculated values after using the stats software package GraphPad Prism V. 5.00 for Windows. As sigmoidal curves were not possible in all cases of dose response assays, MIC₉₀ values have been listed. MIC₉₀ values reported throughout this thesis are based on the lowest concentration at which 90% or greater growth inhibition is observed directly in all wells of the biological replicates. Minimum inhibitory concentration (MIC) values in this thesis are the concentration at which $\geq 98\%$ inhibition is observed in all wells of the biological replicate.

2.6.6 HL-60 MTT assay

Samples were serially diluted two-fold in RPMI media using the same method as described in section 2.6.3, following which 50 μL of cells at 2×10^5 cells/mL were then added to plates to achieve a final cell number per well of 1×10^5 cells/mL. Plates were incubated for 48 h at 37 °C and 5% CO₂. Internal controls included solvent controls, media with cells and media alone. After 48 h, 20 μL of MTT (5 mg/mL in PBS) was added to each well and plates were wrapped with cling wrap and incubated for 2 h. Following incubation with MTT, 100 μL of solubiliser (45% DMF, 10% SDS at pH 4.5)

was added to all wells and plates were incubated overnight. The next day, OD of plates was measured at 570 nm.

2.6.7 HEK MTT assay

Cells were diluted to 1×10^5 cells/mL; 100 μ L of cells was then added to the inner 60 wells of a 96-well plate, and plates were then incubated for 24 h. The following day, 100 μ L of media was taken out of each initial well and the volume of that well was made up to 200 μ L with the appropriate amount of desired drug. From this initial well, 100 μ L was taken out and added to the next well, after thorough pipetting, 100 μ L was taken out from this second well and the process was repeated to achieve the desired number of serial dilutions. Internal controls were incorporated as stated in the protocol of HL-60 cells in section 2.6.6. Plates were incubated for 72 h at 37 °C and 5% CO₂. After incubation, MTT and solubiliser was added to plates as stated above in section 2.6.6.

2.7 Chemistry protocols

Reversed-phase column chromatography was performed using Supelco Diaion[®] HP20 and HP20ss poly(styrene-divinylbenzene) (PSDVB) resins. Normal-phase column chromatography was performed using Supelco Discovery[®] DSC-DIOL functionalised silica: 3-(2,3-dihydroxy-propoxy)-propyl-silica (Diol).

To perform HPLC two systems were used, a Rainin Dynamax SD-200 solvent delivery system with 25 mL pump heads and a Varian Prostar 335 photodiode array detector for UV/Vis detection. The second HPLC system was an Agilent Technologies 1260 Infinity HPLC equipped with a quaternary pump, a thermostated column compartment and diode array detector (DAD). Following the DAD, a Quicksplit[™] flow splitter directs 5% of the flow to an Agilent 380-evaporative light scattering detector (ELSD) with 95% of the flow directed towards collection.

The columns used in this research were reversed-phase, either a C18 analytical (Phenomenex Prodigy, 4.6 x 250 mm, 5 μ m particle size) or semi-preparative (Phenomenex Prodigy, 10.0 x 250 mm, 10 μ m particle size) columns.

HPLC-grade solvents were used for all work (purchased from Fisher Scientific) except for hexanes and dichloromethane, which were Optima[®] grade. Water was distilled prior to use and for HPLC the water was further filtered through a membrane with a pore size of 0.45 μm before use. The solvent compositions used are all reported as the % v/v. TLC analyses were performed using Machery-Nagel Polygram[®] Sil G/UV₂₅₄ plates and visualised under UV light (254 nm and 350 nm). TLC plates were then visualised by dipping in 5% conc. H₂SO₄ in methanol (v/v) followed by 0.1% vanillin in ethanol (w/v) and heated for analysis.

NMR spectra were obtained using a Varian DirectDrive spectrometer equipped with a triple resonance HCN cryogenic probe operating at 600 MHz for ¹H and 150 MHz for ¹³C nuclei. ¹⁹F spectra were acquired using a Varian Inova 300 MHz NMR at 280 MHz. The chemical shifts of ¹H and ¹³C spectra were referenced to the residual solvent peaks (CDCl₃: δ_{C} 77.0, δ_{H} 7.26; CD₃OD: δ_{C} 49.0, δ_{H} 3.31; d₆ DMSO: δ_{C} 39.5, δ_{H} 2.50). All NMR solvents were purchased from Aldrich with the exception of deuterated methanol, which was purchased from Cambridge Isotope Laboratories Inc.

NMR quantifications of masses less than 1 mg were performed with an internal nitromethane (CH₃NO₂) standard and using the acquisition parameters described by West [132].

HRESIMS results were obtained from an Agilent 6530 Q-TOF mass spectrometer equipped with an Agilent 1260 HPLC for solvent delivery utilising a JetStream[™] electrospray ionisation source in positive and negative ion modes. Water and acetonitrile (MeCN) solvents were both made up as 5 mM solutions with ammonium formate.

For sponge identification, the organic matter was dissolved by immersing a small piece of sponge (1 cm³) in a boiling tube and adding concentrated HNO₃ to cover the sample. Spicules that remained after this were then washed by suspending them in water and then centrifuging them to allow the water to be decanted. This process was repeated twice and the third time a MeOH wash was carried out. The spicules were then

visualised using an Olympus® Research Microscope AX70 Provis equipped with an OlympusR DP70 Digital Microscope Camera and these images were compared with descriptions in the literature to classify the sponge.

2.8 General NMR data acquisition protocols

¹H:	number of scans 64 d1 relaxation delay 0.01 s Acquisition time 6.08 s Spectral width 0.0 ppm - 9.0 ppm
¹³C:	number of scans 19776 d1 relaxation delay 0.01 s Acquisition time 1.73 s Spectral width -10.0 - 230.0 ppm
¹⁹F:	number of scans 52 d1 relaxation delay 1.00 s Acquisition time 1.00 s Spectral width -200.0 – 30.0 ppm
COSY:	number of scans 1 number of increments 512 d1 relaxation delay 2.0 s Acquisition time 0.25 s Spectral width 0.0 ppm - 9.0 ppm

HSQC:

- number of scans 8
- number of increments 512
- d1 relaxation delay 1.5 s
- Acquisition time 0.19 s
- Spectral width 0.0 ppm - 9.0 ppm ¹H dimension
- Spectral width 0.0 ppm – 160.0 ppm ¹³C dimension

HMBC:

- number of scans 16
- number of increments 512
- d1 relaxation delay 0.8 s
- Acquisition time 0.25 s
- Spectral width 0.0 ppm - 9.0 ppm ¹H dimension
- Spectral width 0.0 ppm – 160.0 ppm ¹³C dimension

1D NOE:

- number of scans 128
- d1 relaxation delay 1.5 s
- Acquisition time 3.04 s
- Spectral width 0.0 ppm - 9.0 ppm
- Mixing time 0.5 s

1D TOCSY:

- number of scans 3000
- d1 relaxation delay 1.5 s
- Acquisition time 3.40 s
- Spectral width -2.0 ppm – 14.0 ppm
- Mixing time 0.2 s

2D ROESY:	number of scans 16
	number of increments 1024
	d1 relaxation delay 1.5 s
	Acquisition time 0.15 s
	Spectral width 0.0 ppm - 8.0 ppm
	Mixing time 0.5 s

2.9 Column chromatography

2.9.1 Cyclic loading

To carry out cyclic loading, biomass samples were cut into approximately 3 cm³ pieces and allowed to steep in methanol (MeOH) overnight. The MeOH from the first steep was decanted into a container and fresh MeOH was poured onto the sample which was allowed to steep again overnight. Column beds of HP20 (reversed-phase polystyrene-divinylbenzene copolymer) were set up based on the principle that 1 kg of sponge has 8 g of extractable compounds, and approximately 25 mg of extractable compounds can bind onto 1 mL of HP20. Extracts were filtered through Büchner funnels with celite and filter paper, and extract was passed through the column at a flow rate of 4 mL/min. Following this, a two-fold dilution of the eluent was carried out with H₂O, this was then passed through the column again. Dilution of eluent was carried out by adding small volumes of H₂O at a time, with care taken to avoid unwanted precipitation. If precipitation occurred, the solution was passed through the column again, before adding the rest of the volume of H₂O. These cycles of dilution and passing the solution through the column were repeated until a final MeOH concentration of 12.5% was passed through the column. Columns were eluted with 20%, 40%, 60%, 80% and 100% mixtures of acetone in water. The 100% acetone fraction was evaporated under reduced pressure, while the other fractions were subject to backloading.

2.9.2 Backloading

Backloading was employed to avoid issues with bumping and long evaporating times when drying down solvent mixtures that contain large volumes of water. HP20 acetone fractions were diluted two fold with ddH_2O before passing them through a smaller HP20 column. Columns were eluted with three column volumes of MeOH and acetone, which was collected in a round bottom flask and evaporated under reduced pressure. The mass in the flask was dissolved in a small amount of a solvent mix of 4:1 MeOH: dichloromethane (DCM), which was then transferred to a pre-weighed glass sample vial. The vial was then evaporated *in vacuo* to obtain the absolute dry weight.

2.9.3 HP20ss chromatography

Columns were set up using the same principle for HP20 of 25 mg of extractable material binding to 1 mL of resin, therefore for example for 100 mg of a sample that needed to be fractionated, 4 mL of beads was used. This ‘loaded HP20ss’ was then transferred on the top of a larger column bed of HP20ss which was approximately three times the volume of the loaded HP20ss. Columns were eluted with appropriate solvent mixtures as described in the relevant methods sections at a flow rate of 1.5 mL/min.

2.9.4 DIOL column chromatography

Columns of the normal phase DIOL stationary phase (2,3-dihydroxy-propoxypropyl-derivatised silica gel.), were set up similarly to HP20ss columns, however the loading capacity estimate used was 10 mg of compounds bind onto 1 mL of material. Columns were eluted with appropriate solvent mixtures as described in the relevant methods sections at a flow rate of 1.5 mL/min.

2.9.5 LH20 chromatography

For all LH20 size exclusion columns run, 100 mL of resin were soaked in appropriate running solvents overnight before setting up the column. Flow rates for all columns were maintained at approximately 0.2 mL/min. Fractions were collected in 10 mL test tubes using an automatic fraction collector.

Chapter 3 Screening and validations

3.1 Background

In the preliminary research described in this chapter, libraries of crude marine extracts were screened to identify potential new sources of antitubercular activity. Two marine extract libraries were screened in 96-well plate format. The first was kindly donated by the National Institute of Water and Atmospheric Research (NIWA), with the second generated at VUW. For the NIWA library, the sample mass per well was unknown and varied between extracts. Validation as well as furthering of “hit” extracts with observed bioactivity depended on re-collection and re-extraction of the marine specimens. In contrast, the VUW marine natural product (MNP) library was generated in house with a constant mass per well across all crude extracts. Furthermore, biological samples were readily available should any be identified as hits.

As stated earlier, drug resistance in TB poses a serious health problem. In addition to this, general bacterial resistance to antibiotics pose a problem that must be addressed. A recent report by the Centers for Disease Control and Prevention estimated approximately 2 million people are infected with drug resistant bacteria leading to 23,000 deaths in America [133]. In NZ the rate of methicillin resistant *S. aureus* is 26.1 per 100 000, and 195.7 per 100 000 for extended-spectrum β -lactamase producing Enterobacteriaceae in 2012 [134, 135]. In order to address drug resistance there is a need to identify new more effective antibiotics. This section highlights current problems with screening natural products as well as some approaches used to identify novel antibiotics.

3.1.1 Current issues when screening for new antibiotics

High-throughput screening (HTS) of natural products for antibiotics is not the most actively pursued research avenue by pharmaceutical companies [115]. There are certain aspects associated with screening of natural extracts for antibiotic activity that deter pharmaceutical companies from this field of research. First, accessibility to the samples proves a major hurdle, as it is often restricted due to intellectual property concerns of the governments involved [114, 136]. Increased extinction of species that are sources of

structural novelty due to human activities adds further restraints in sourcing material for drug discovery [137]. Drug companies need reliable access to the natural resource as well as patent control of any potential drug, to ensure ownership for the product upon which they have invested time and resources. In addition, seasonal variation and environmental changes are known to affect the production of metabolites of interest; this majorly affects reproducibility of natural product isolation during scale-up processes [138].

Provided such obstacles are overcome, the complexities of natural products chemistry are still left to be considered. For example, in a crude fraction it is possible that bioactivity may be due to a mixture of two or more compounds [114]. Following a rigorous purification process, the bioactive metabolite could be present in very low amounts, or be unstable. The presence of multiple stereogenic centres complicates matters even more, not only for structural elucidation but in the downstream process of identifying an economically viable synthetic route [114]. In such cases, structural elucidation of the metabolite can be even more complex, requiring uncommon NMR experiments or chemical derivatisation.

Re-isolation is another common issue encountered when screening natural products, where HTS that focus on activity alone may result in the re-isolation of so-called “promiscuous” known compounds, making the investigation redundant. For example, streptomycin is produced by ~ 1% of all actinomycetes, subsequently there is a high chance that projects focusing on soil bacteria will lead to the isolation of this well-known compound [139]. Repeat isolations of known compounds is a waste of both time and resources, a further disincentive for pharmaceutical companies to invest in this temperamental field [71].

The final test for a potential drug is how it performs in clinical trials. In total, the development of a new drug can take up to 15 years and cost up to a billion dollars [113, 140]. The high standards of regulatory authorities result in a high attrition rate during clinical trials; this means a vast amount of time and resources are devoted to drug candidates that may eventually fall out of the development pipeline [141].

Further reasons behind the waning interest of large pharmaceutical companies in antibacterial research is that there is a better return on investment in other disease research areas [115]. Antibiotics are taken for a short period of time and cure the disease,

however treating chronic disease provide a better return as patients have to consume the medication for the remainder of their life [142, 143]. Furthermore the expiry of patents for many drugs means that big Pharma have to compete with generic manufacturers [144]. For these reasons antibacterial research is no longer in vogue with Pharma, resulting in a decrease of commercially available, structurally unique antibiotics.

It is mainly due to cost cutting that recent screens have tended to focus on libraries comprising known chemical scaffolds, which has resulted in a lack of structurally diverse antibiotics [143, 145]. To avoid resistance, combinatorial synthesis provides a powerful tool, by modifying a parent compound to introduce variation and improve activity [146]. However, the fact remains that it is still the same parent scaffold that is being used and there are certain limits as to how many modifications can be made to each compound [143]. As known chemical scaffolds tend to show less complexity compared to natural products, screening libraries of known chemical entities has decreased the number of novel antibiotics [147]. This is particularly concerning given the increasing reports of antibiotic resistance [148].

3.2 Screening natural products

Having noted the obstacles natural product research faces, this avenue should not be viewed as a dead end. The role of natural products in human health has been crucial for thousands of years [76]. Plant derived natural products have been especially crucial and the WHO estimate that a majority of the global population still rely on traditional medicines [76]. Anti-infective agents such as the β -lactams, tetracyclins, aminoglycosides, macrolides and streptogramins all play crucial roles in controlling various bacterial infections, and they all are a result of natural product research [76]. The majority of current antibiotics were discovered during the 'golden era' of antibiotic research spanning from the 1930s to the 1970s [149]. Following this timeframe the number of new classes of antibiotics dwindled, until the 2000s where five new classes of antibiotic were approved (linezolid, daptomycin, retapamulin, fidaxomicin, and bedaquiline) [149]. Of the 49 compounds under clinical development as antibiotics, 25 are of synthetic origin and 22 are of natural origin including peptide derived compounds [149]. Though these numbers may seem unfavourable for natural products, if we look at structural novelty, there are only a total of six novel truly synthetic compounds compared to 11 of natural product origin [149]. These examples illustrate the

effectiveness of natural products as a source of chemical diversity, even when the number of companies actively engaged in antibiotic research has drastically declined [149].

There needs to be a clear reason for more focus in this field. There is now a slow rise in incentive for the field of antibiotic research, the US Generating Antibiotic Incentives act provides an automatic priority review and a 5-7 year market exclusivity for antibiotics that target qualifying pathogens [149]. The Innovative Medicines Initiative-New Drugs for Bad Bugs is a \$280 million dollar fund to identify new antibiotics that target Gram-negative bacteria [149]. These highlight important first steps and will require further commitments which will help towards drawing more research into this vital field.

3.2.1 Overcoming the challenges in natural product research

As stated earlier, approximately 74% of all approved antibacterial drugs identified between 1981-2010 originated from natural products [77]. Natural products therefore still remain a vital source for novel therapeutics [77]. Though the field remains temperamental, it is crucial that innovative methods are developed to uncover the yet undiscovered bioactive compounds that already exist, a few such methods are described below.

Cubist Pharmaceuticals have developed a method to screen multiple soil fermentation broths against strains of bacteria that are resistant to known antibiotics, to avoid isolating known compounds [139]. This approach involves screening more than 10^7 strains of actinomycetes a year, using Ca^{2+} -alginate macro-droplet beads in which bacteria are grown to produce secondary metabolites. In order to avoid re-isolating known compounds, these cultures are screened for inhibitory activity against an *E. coli* strain modified to be resistant to many known antibiotics [139].

Another technique, developed by Merck, employed the use of two strains of *S. aureus*, one wild-type and the other expressing anti-sense mRNA for FabF, an enzyme essential for bacterial fatty acid biosynthesis. The latter strain was sensitised towards fatty acid biosynthesis inhibitors. The two strains were then screened against a library of natural extracts and the hits specific to the anti-sense expressing mutant led to the eventual isolation of two unique compounds, platensimycin A (**37**), an inhibitor of FabF fatty

acid acyl carrier protein synthase II [150], and platencin (**38**), a dual FabF/FabH inhibitor [151].

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Bugni *et al.* developed methods that involved the application of hyphenated techniques to allow for the fractionation, analysis and preparation of semi-crude extracts for screens. HPLC coupled with mass spectrometry followed by the collection of compounds in HTS plates allowed for the semi-purification and identification of novel compounds with anti-tumor activity against breast cancer. This technique also separated the compounds from general cytotoxic compounds, which would be observed using traditional bioassay-guided techniques [152].

Genome mining is another method that can be employed to identify structurally diverse compounds. This technique is generally applied when dealing with secondary metabolites of microbial origin. It entails genome sequencing, followed by bioinformatics profiling to identify pathways involved in production of unique molecules. Using this method salinosporamide K (**39**) was discovered from *Salinospora pacifica* [153]. Additionally, cryptic biosynthetic pathways can be identified, and compounds that are not normally produced can be induced through genetic manipulation. By inducing the constant expression of a regulatory gene within the polyketide synthase (PKS) gene cluster of *Streptomyces ambofaciens*, stambomycins A-D, which were not produced under normal culture conditions, were able to be isolated [154].

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These examples highlight natural products and the importance they play in identifying the next generation of bioactive molecules. Two common screening approaches towards drug discovery are described below.

3.2.1.1 Whole cell screening

Whole cell screening involves measuring the growth of bacteria in the presence of potential inhibitors. Optical density (OD), green fluorescent protein (GFP) fluorescence, as well as dyes that measure metabolism such as resazurin, are often employed as measures of growth [130, 155]. Whole cell screening has led to the identification of many current anti-tubercular drug candidates, such as PA-824 (**13**), a nitroimidazofuran [156] and TMC-207 (**17**), a diarylquinoline that has gained approval for the treatment of MDR-TB [118]. The benefit of this approach is that factors such as cell wall penetration, pro-drug activation and drug efflux pumps can be accounted for. However, the pitfall of this approach is that it is unable to account for general cytotoxicity, which is a parameter that must be evaluated at some stage. Modification of screening conditions can also be used to replicate conditions that represent *in vivo* environments [155]. For example, a macrophage infection model can be used to replicate the pathogenesis of TB and allow for screening and identification of inhibitors active under these conditions [157]. In this model, toxicity of the compounds towards eukaryotic cells can be accounted for, however the mode of the action of the drug still has to be elucidated.

3.2.1.2 Targeted screening

The genomic era spurred the idea of target screening; as genomes of pathogenic bacteria were sequenced it allowed for the identification of essential genes, and therefore new targets to guide the search for new antibiotics [115].

Enzyme/biochemical assays allow for the screening of inhibitors of a particular cellular process. Though effective, these assays are unable to account for the complexities of a whole cell [158], and the compound may not be active when tested in whole cell assays [159]. SQ-641 (**40**), a drug currently in pre-clinical development to treat tuberculosis, was discovered using a screen to target phospho-*N*-acetylmuramyl-pentapeptide-translocase. It was later also found to inhibit growth of *Mycobacterium smegmatis* [40, 160].

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Targeted screens also include anti-sense screens as described earlier in the isolation of platensimycin A [150]. This method, which involves the controlled expression of anti-sense RNA of a desired gene, makes the strain sensitive to inhibitors that target the gene of interest.

3.2.1.3 Anti-tubercular drug screening at VUW

The assay protocols developed in our laboratory were established by Drs. Miller and O'Toole. The general method involved a liquid screen of chemical libraries of known compounds such as the LOPAC, Spectrum Collection, and the NIH Diversity and Natural Products Set II libraries against *M. smegmatis*. By using GFP and OD, bacterial growth was measured, followed by validation of hits using dose response curves. In this protocol, culture conditions were altered in order to identify compounds that were active under various starvation conditions that emulated tuberculosis pathogenesis [155]. The 96-well plate liquid assay to screen for anti-mycobacterial activity is widely used and a well-established method [161, 162] .

As stated screening libraries of known compounds introduces very little variation in chemical structures. Diverse chemical scaffolds are a priority when dealing with diseases as imposing as TB [163]. Natural products have provided, and will continue to be, a source of structural novelty and the focus of this thesis was to utilise this powerful resource to identify such compounds.

Applying this assay protocol, the current project involved screening NZ marine extract libraries for anti-mycobacterial activity against *M. smegmatis*. The strains of *M. smegmatis* employed were modified to express a GFP gene and also included a kanamycin resistance gene, which allows for the selective growth of this strain and hence reduces contamination risks by other bacteria [155]. *M. smegmatis* offers the advantage of a faster doubling time and poses a low health risk compared to the slower growing pathogenic mycobacteria. The extract libraries used were generated from New Zealand marine vertebrates and invertebrates. Following the identification of hit extracts, validation of anti-mycobacterial activity was performed in *M. tuberculosis* H37Ra. Following the selection of samples, a bioassay-guided approach was implemented focusing on the identification of potentially novel anti-mycobacterial compounds.

3.3 Methods

3.3.1 Marine extract library screening

Libraries were screened as described in section 2.6.2, with specific details for the NIWA library and VUW MNP library mentioned here. Six 96-well plates of a pre-existing marine extract library donated from NIWA were re-suspended in DMSO (500 μ L). From these mother plates, daughter plates were created containing 100 μ L aliquots of dissolved extract and were used to screen for activity. As mentioned earlier, the library from NIWA was generated with an unquantified and varying amount of extract present in each well. To screen the library, 2.5 μ L aliquots from each well of a daughter plate were added to 50 μ L of media, followed by 50 μ L of bacteria. The VUW MNP library was screened such that the final concentration of crude extracts was 100 μ g/mL per well. The VUW MNP library was screened against *M. smegmatis*, *S. aureus* and *E. coli*. The final OD for bacteria used in screens was 0.01 AU.

3.3.2 Validation

Hits identified from screens were validated using dose response assays as described in sections 2.6.3 and 2.6.4. In brief, aliquots of crude extracts were diluted two-fold in appropriate media for either *M. smegmatis* or *M. tuberculosis* H37Ra, resulting in a 50 μ L final volume of media and extract. Bacterial cultures were diluted to ODs of 0.02 AU for *M. smegmatis* or 0.01 AU for *M. tuberculosis* H37Ra with 50 μ L aliquots added

to each well. For each validation assay, plates with a dilution series of a solvent control and a known anti-tubercular drug (either streptomycin or rifampicin) as positive control were included.

3.4 Results

Results of screening the NIWA marine extract library and VUW MNP library are depicted below in graphical format (Figure 3.1 and 3.2). Growth inhibition is represented using GFP measurement on the X axis and resazurin fluorescence on the Y axis. A hit was defined as greater than 80% inhibition of growth as indicated by using both GFP and resazurin. This is indicated by hits that fall within the black squares in both scatter plots. Z-factors were calculated for these screens with values above 0.5 considered statistically acceptable [164]. Following screening results, tables depict the activity of the validated hits from both screens. As sigmoidal curves were not possible for these crude extracts, MIC₉₀ values have been listed.

Figure 3.1 *M. smegmatis* screening results from the NIWA library

Scatterplot depicting the growth inhibitory effects of all extracts from the NIWA library against *M. smegmatis* pLL192_hsp60. Hits (indicated by asterisks) fall within the 80% cut off indicated by black square. The NIWA library screen was performed in triplicate, error bars have been excluded from the scatter plots to allow for better visualisation of data. The Z-factors calculated for the NIWA screens were $Z = 0.92$ (GFP) and $Z = 0.59$ (resazurin).

Figure 3.2 *M. smegmatis* screening results from the VUW MNP library

Scatterplot depicting the growth inhibitory effects of all extracts from the VUW MNP library against *M. smegmatis* pLL192_hsp60. Hits (indicated by asterisks) fall within the 80% cut off indicated by black square. Of the three VUW MNP library plates two screened in duplicate and one was in a single screen as this plate was used to optimise screening concentrations. Error bars have been excluded from the scatter plots to allow for better visualisation of data. The Z-factor for the VUW MNP screens were $Z = 0.90$ (GFP) and $Z = 0.78$ (resazurin).

Table 3.1 Top hits from NIWA Library

Sample	MIC ₉₀ (µg/mL) vs. <i>M. tuberculosis</i> H37Ra
<i>Lipkius holthuisi</i> (shrimp body without carapace)	600
Starfish intestine	400
Red star fish intestine	250
<i>Paralomis hystrix</i> (spiky crab intestines)	400
<i>Hoplostethus mediterraneus</i> (silver roughy intestines)	400
<i>Psychrolutes marcidus</i> (blob fish intestines)	400
<i>Psychrolutes marcidus</i> (blob fish liver)	600
<i>Neolithodes brodiei</i> (red crab intestines)	1350

Validation of the hits from the NIWA library against *M. tuberculosis* H37Ra using dose response curves, MIC₉₀ values are the lowest concentration of extract tested that displayed $\geq 90\%$ growth inhibition.

Table 3.2 Top hits from VUW MNP library

Sample	MIC ₉₀ (µg/mL) vs. <i>M. tuberculosis</i> H37Ra
PTN 3_37C, unknown Tongan black ascidian	200
PTN 3_40B, unknown Tongan purple ascidian	NT
PTN 2_50B, unknown Hen and Chicken Islands (Northland) Brown sponge	200
PTN 2_45H, unknown Tutakaka sponge	IA
PTN 2_45I, unknown Tutakaka sponge	IA
MNP_0999, <i>Haliclona</i> sp. sponge	NT**
PTN 2_56C, <i>Latrunculia</i> sp. sponge	6.25
PTN 2_93D, <i>Xiphophora condrophylla</i>	200

NT: not tested in *M. tuberculosis* H37Ra, ** MIC₉₀ in *M. smegmatis* 25 µg/mL, IA: MIC₉₀ not achievable at the highest concentration tested (200 µg/mL). Validation of the hits from the VUW MNP library against *M. tuberculosis* H37Ra using dose response curves, MIC₉₀ values are the lowest concentration of extract tested that displayed $\geq 90\%$ growth inhibition.

Figure 3.3 Spectrum of activity and overlap of hits from VUW MNP library screen.

Table 3.3 Hits further investigated

Sample	Species	MIC ₉₀
Red Crab	<i>M. smegmatis</i>	1% v/v
	<i>M. tuberculosis</i> H37Ra	1350 µg/mL
Hen and Chicken islands brown sponge PTN 3_50B	<i>M. smegmatis</i>	62.5 µg/mL
	<i>M. tuberculosis</i> H37Ra	≥ 200 µg/mL
<i>Psychrolutes marcidus</i>	<i>M. smegmatis</i>	1% v/v
	<i>M. tuberculosis</i> H37Ra	600 µg/mL
<i>Latrunculia</i> sp.* PTN 2_56C	<i>M. smegmatis</i>	100 µg/mL
	<i>M. tuberculosis</i> H37Ra	6.25 µg/mL
<i>Xiphophora chondrophylla</i> PTN 2_93D	<i>M. smegmatis</i>	≥200 µg/mL
	<i>M. tuberculosis</i> H37Ra	≥ 200 µg/mL

Comparison of activity of hit extracts in two species of mycobacteria using dose response curves, MIC₉₀ values are the lowest concentration of extract tested that displayed ≥ 90% growth inhibition. *Based on comparisons of MIC₉₀ values, this extract shows a specificity for *M. tuberculosis* H37Ra.

3.5 Discussion

The results from the NIWA library screening performed against *M. smegmatis* are provided in Figure 3.1 and for the VUW MNP library in Figure 3.2. GFP and resazurin fluorescence measurements were used as a measure of cell replication. OD was not used as measurements become unreliable due to the coloured nature of crude extracts, which contribute to OD measurements. In the NIWA library screen it is noted that many data points are negative values, where the resulting growth is higher with respect to control wells, indicating the possibility of growth promotion by the extracts in question. As the Z-factors calculated for the NIWA screens were above 0.5 this screen can be considered statistically acceptable (Figure 3.1) [164].

As stated earlier, the NIWA library was generated with an unknown mass of extract per well and as such, the library of extracts was dissolved in a constant solvent volume (500 μ L DMSO). Following this the library was screened at a set (2.5% v/v) concentration of extract per well. This concentration was chosen as this is the highest concentration of DMSO that *M. smegmatis* can tolerate without solvent effects inhibiting cell growth. The unknown mass per well posed a risk of overestimating the activity of extracts that were present at higher masses in some wells than others. Once hits were identified, they were validated in dose response assays. These hits were validated by drying 100 μ L aliquots of the dissolved extract to identify the dry mass (masses ranged between 10-100 mg) before re-dissolving samples to perform dose response curves. Table 3.1 indicates the validation of the top hits from the NIWA library against *M. tuberculosis* H37Ra.

During optimisation of screening the VUW MNP library, no hits were observed with activity against *M. smegmatis* at 10 or 20 μ g/mL. However, at 100 μ g/mL, inhibitory activity was observed for some extracts, therefore this was used as the screening concentration. As previously stated the VUW MNP library was generated with a constant mass for each extract per well, and library screens were subsequently performed at the defined 100 μ g/mL concentration. The Z-factors for the VUW MNP screens were above 0.5 and therefore this screen can also be considered statistically significant (Figure 3.2).

The VUW MNP library consisted of three library plates, RAK18_52A/B, 53A/B and 53C/D. RAK18_52A/B was used to optimise screening concentrations for the assay and

due to this it was possible to only screen this plate only once rather than in duplicate as was done for RAK18_53A/B and 53C/D. As it so happened, the most active hits identified were those from RAK18_52A/B. These hits were validated in dose response assays against *M. tuberculosis* H37Ra and the MIC₉₀ values are listed (Table 3.2). It should be noted that these validations allowed for false positives to be excluded due to the lack of activity observed in this phase of testing. A possible reason for these false positives may be due to the fact that these extracts may be active against *M. smegmatis* and not *M. tuberculosis* H37Ra. An alternative reason for these false positive could be due to the fact they were all picked from the library plate that was screened only once; therefore there was a higher chance of false positives coming through due to random growth in wells. Fortunately the validation of hits allowed for the exclusion of false positives at an early stage, which avoided investing time into samples that may eventually fall out at later stages of the project.

Using the in-house libraries not only enabled for optimization of screening concentration but also allowed for screening the library in duplicate, as well as against *S. aureus* and *E. coli*. This allowed for the identification of samples that were specifically active against mycobacteria. Figure 3.3 identifies two such samples that showed specific activity against *M. smegmatis*. The two specific hits from the VUW MNP library screen were unfortunately not available in sufficient masses to allow for bioassay-guided fractionation.

Towards achieving the goal of Aim I, Table 3.3 identifies the top hits selected after considering sample availability as well as activity. PTN 2_50B (Hen and Chicken Islands brown sponge) was collected near the Hen and Chicken Islands, New Zealand, and was stored frozen. The crude extract had an MIC₉₀ of 62.5 mg/mL against *M. smegmatis*. This sponge (250 g) was extracted twice in MeOH (450 mL) and cyclic loaded onto HP20 and the column eluted using 20%, 40%, 60%, 80% and 100% acetone in water. One of the most active fractions ND4_29C (280 mg) displayed an MIC₉₀ of 25 µg/mL, and was further fractionated using HP20ss (40 mL column), with mixtures of 20%, 40%, 60%, 70%, 80%, 90% and 100% methanol in water and finally, 100% acetone, used to elute the column. This led to fraction EJA_94D (33.6 mg) with an MIC₉₀ of 12.5 µg/mL, which was purified using semi-preparative reversed-phase C18 HPLC with 70% MeOH and 0.1% formic acid under isocratic conditions to give ND4_36I (2.3mg) with an MIC₉₀ of 50 µg/mL. As this sample did not show any major

improvement in activity and due to its low mass, it was not pursued any further in order to focus on other samples.

Bioassay-guided fractionation work on the liver of *Psychrolutes marcidus* (Blob fish, collected by NIWA) led to the identification of a mixture of fatty acids as the bioactive component. GC-MS analysis was used to analyse and identify the major components of the bioactive fraction, following which those fatty acids that were commercially available were purchased and tested for bioactivity. This work is discussed in further detail in Chapter 4.

Working with the *Latrunculia* sp. sponge, collected near Rimariki Island led to the isolation of a novel taurinated terpene. Based on the bioactivity of the initial fractionation, this taurinated terpene was believed to be the component responsible for the observed anti-mycobacterial activity. However, final purification of the bioactive fraction using size exclusion chromatography resulted in the separation of the taurinated terpene from the anti-mycobacterial component. This component was not isolated in a mass sufficient enough to allow for structure elucidation. Details of this work are further elaborated in Chapter 5.

Bioassay-guided fractionation of a brown alga *Xiphophora chondrophylla*, collected near the Hen and Chicken Islands, led to the isolation of a new 1-deoxysphingoid. This compound has an MIC₉₀ of 65.1 µM against *S. aureus*, *E. coli*, *M. smegmatis* and *M. tuberculosis* H37Ra. However, the bioactivity of the 1-deoxysphingoid was more potent versus mammalian cell lines. Therefore, the *Saccharomyces cerevisiae* gene knock-out library was used to identify pathways that were involved in the mechanism of action of this compound. Further details of this work are described in Chapter 6.

Chapter 4 Bioactive fatty acids

from *Psychrolutes marcidus*

4.1 Background

Following a screen against the NIWA library, the liver extract of the blob fish *Psychrolutes microporosus* was identified as being active. After subsequent validations in dose response assays, this sample was chosen to be pursued further. Upon collection of more specimens by NIWA, the livers were dissected and extracted to allow for isolation of any bioactive compounds responsible for inhibitory activity. This led to the identification of a mixture of fatty acids as the source of bioactivity.

4.1.1 Fatty acids

Fatty acids are ubiquitous in nature and play important roles within a cell such as energy reserves in the form of triglycerides, or in the cell membrane as phospholipids. Through the action of enzymes such as lipases, lipids can be cleaved from their polar head groups (such as sugars or phosphates), releasing free fatty acids [165]. It has been reported that free fatty acids (FFAs) possess a range of activities including antibacterial, anti-fungal, and cytotoxic properties [165]. Within biological systems, chain lengths ranging from 10 to 28 carbons are common for fatty acids [165]. When there is one or more double bonds within the carbon chain, they are classed as unsaturated fatty acids. In humans, FFAs act as an innate defence against pathogens on skin and mucosal surfaces and it is believed that there are approximately 10-15 µg of FFAs per square centimetre of skin [165, 166]. Their antibacterial action on the skin may be the result of direct killing of bacteria, or by decreasing the pH on the skin surface, making conditions unfavourable for bacterial growth [167, 168].

Antimicrobial FFAs from a number of sources such as algae, plants and animals have been isolated using bioassay-guided fractionation [169-172]. FFAs are also believed to be the active component of some traditional remedies [171, 172].

A general trend in literature indicates that antibacterial activity of FFAs depends on the carbon chain length, degree of unsaturation as well as the positioning and geometry of the double bond, with a *cis* geometry favouring more potent activity [173-175]. Most active FFAs have chain lengths of either 14 or 16 carbons [173]. Correlations have been observed identifying unsaturated FFAs as more active compounds compared to saturated FFAs of similar chain length [173]. In addition, a free carboxyl group is believed to be essential for activity [173, 176]. Gram-positive bacteria are more

susceptible to growth inhibition in the presence of FFAs compared to Gram-negative bacteria, however it should be noted that most research has only focused on Gram-positive bacteria [175, 177, 178].

4.1.1.1 Nomenclature of fatty acids

Fatty acids are named using set abbreviated nomenclature; the number of carbons in the chain is first stated followed by the number of double bonds. For example, palmitic acid, a saturated fatty acid with a chain of 16 carbon atoms, would be C16:0, whereas palmitoleic, which has the same chain length but one double bond is C16:1. In addition, the bond geometry is also stated. Therefore, for palmitoleic acid, C16:1, *cis*-9 would be used, which identifies a 16 carbon long chain, with one *cis*-double bond at the 9th carbon.

4.1.1.2 Mechanism of action

While there has been extensive literature published on the effects of FFAs, only the mechanisms that would lead to the most detrimental effects on a cell will be discussed. While the exact mechanisms of action are not fully elucidated, it has been proposed that FFA activity is potentiated in multiple ways. The presence of a hydrophilic polar head and a hydrophobic tail makes these molecules amphiphilic. Therefore, they can cause cell lysis by affecting membrane permeability as with detergents [179, 180]. Oleic acid has been shown to increase membrane fluidity in *S. aureus* [181].

In altering membrane permeability, FFAs can affect processes integral to cell viability such as the electron transport chain and enzyme activity. Direct interaction with electron carriers, which disrupts the electron transport chain, or alteration of membrane fluidity, can also perturb cell homeostasis in bacteria [182, 183].

FFAs are believed to uncouple oxidative phosphorylation, which is based on research focusing on mitochondria and has been used to draw comparisons to antibacterial activity [165, 184, 185]. This is achieved by disrupting the proton motive force generated by the electron transport chain, which is essential for ATP synthase to convert ADP to ATP [186]. FFAs have been shown to directly interact with the permeability transition pore within the mitochondrial membrane, therefore uncoupling oxidative phosphorylation [187]. Further disruption of this process is believed to occur via the dissociation of the proton from the acid group of a FFA within the cytosol, thus

decreasing the pH within the cytosol. This is facilitated via anion transporters within the mitochondrial membrane [188].

An alternative mechanism proposed for the activity of FFAs includes the direct interaction and inhibition of enzymes, with reports of unsaturated FFAs being more potent than saturated FFAs [189, 190]. Within the literature, FFAs have also been linked to inhibition of fatty acid biosynthesis, which is essential for maintaining membrane integrity within bacteria [191, 192].

The varying susceptibility of bacteria to FFAs is believed to be due to the differences in permeability of bacterial cell walls, which alters FFA access to the inner membrane [165]. Some bacteria are able to resist inhibition by FFAs, by modifying their cell wall to make it more charged and therefore more hydrophilic and unfavourable for FFA binding [193]. *S. aureus* has been shown to upregulate genes involved in cell wall synthesis in the presence of FFAs [183]. The presence of carotenoids allows for membrane stability, negating the effects of FFA. Strains of *S. aureus* with high levels of carotenoids have been reported to display more resistance to FFAs compared to strains with lower levels [181].

4.2 Methods

4.2.1 Extraction of material

Blob fish were dissected and livers extracted. Livers (200 g) were cut into 1 cm³ sized pieces and soaked in methanol (~ 200 mL). Following overnight extraction, the methanol was decanted into a conical flask and the liver material was re-extracted with the same volume of methanol. The two extracts were treated separately and filtered using a Büchner funnel and Celite. The second extract was passed through an 80 mL HP20 column followed by the first. The eluents were combined and diluted two-fold with H₂O before passing through the same column again. This was repeated until the original MeOH extract was diluted to 12.5% and passed through the column. Following cyclic loading, the column was eluted with 240 mL of 20% and 40% acetone in water followed by 60% and 100% acetonitrile in water, and finally 100% acetone was used to strip the column.

From the 100% acetonitrile fraction (12.6 grams of oil, MIC₉₀ 2% v/v), 100 mg was further fractionated using a 10 mL DIOL column, eluting with 30 mL of 100% hexanes, 75%, 50%, 25% mixtures of hexanes in EtOAc, 100% EtOAc, 75%, 50%, 25% mixtures of EtOAc and MeOH, 100% MeOH and column strip of 50% MeOH and H₂O. Only the 100% hexanes and the column strip were collected in bulk, whereas the other eluents were collected in test tubes. Test tubes were dried and combined based on charring (5% H₂SO₄ in MeOH) of TLC (running condition 4:1:2 butanol: acetic acid:water). From the resulting fractions, ND4_43B (116.2 mg, MIC₉₀ 250 mg/mL) was separated by LH20 size exclusion chromatography using 50% MeOH/ 50% DCM. This gave ND5_96L as the active fraction (2.5 mg MIC₉₀ 250 µg/mL).

4.2.2 Cyclic loading and column purification

Scheme 4.1 Isolation scheme of fatty acids from *Psychrolutes marcidus*

4.2.3 GC-MS analysis of fatty acids

A common method to analyse fatty acids is to derivatise them and generate fatty acid methyl esters (FAMES), which are then analysed via GC-MS [194]. Fatty acid methyl esters were generated by adding 1 mL of a 10% solution of boron trifluoride in methanol to 100 µg of ND5_96L. This mixture was heated for 10 minutes at 90 °C, followed by liquid-liquid partitioning with water and chloroform three times. The chloroform layer was dried for analysis by GC-MS (Integrated Bioactive Technologies group, Callaghan Innovation). Chloroform (45 mL) was added to the sample and analysis was conducted on a Shimadzu QP2010 GC-MS/FID, using a Thermo TGWaxA column (30 m, 0.25 mm i.d., 0.25 µM film), with isothermal conditions at 210 °C, and the injector temperature set at 280 °C using a 50:1 split. Helium was used as the carrier gas. Mass spectrometry fragmentation patterns (electron impact ionisation at 70 eV in positive ion mode) as well as equivalent chain length values were used to identify peaks.

4.2.1 Fatty acids

The following fatty acids identified using GC/MS, were sourced from Sigma: eladic acid, petroselenic acid, oleic acid, *cis*-vaccenic acid, *trans*-vaccenic acid, linoleic acid, palmitic acid and palmitoleic acid; all fatty acids were of $\geq 99.0\%$ purity. These fatty acids were either tested individually or in mixtures of two, three or four. Fatty acid mixtures were generated using equal proportions of each fatty acid within each respective mixture.

4.3 Results

Using the methodology described in section 2.6, the results of the dose-response assays for the active fraction as well as fatty acids and their mixtures are listed below. Graphs were generated using GraphPad Prism v.5.0. Resazurin dye was used to measure bacterial metabolism after each assay was run, and fluorescence measurements (RFU) were used as a measure of growth. OD was used as measure of growth for the HL-60 and HEK MTT assay as well as the *S. cerevisiae* assay. As stated earlier, where possible, IC₅₀ values have been listed, however as sigmoidal curves were not achievable for all data, MIC₉₀ values have been listed as well.

4.3.1 GC/MS results

Table 4.1 GC/MS profile of FAMES in ND5_96L

Chain length	ND5_96L FAME	
	ECL (16,18)	% FA
	13.84	0.1
14:0	13.97	0.34
15:0	14.99	0.27
	15.5	0.17
16:0	16.00	11.58
16:1 n-9	16.27	2.05
16:1 n-7	16.33	12.25
16:1 n-5	16.45	0.19
17:0	16.51	0.34
	16.60	0.22
	16.69	0.18
	16.93	0.19
17:0	17.00	0.14
17:1	17.29	1.64
	17.41	0.29
	17.49	0.37
18:0	18.00	1.83
18:1 n-9	18.25	52.25
18:1 n-7	18.32	7.77
	18.44	0.44
	19.26	0.19
	19.30	0.47
20:1	20.16	0.28
20:1	20.20	2.05
Phthalate	20.72	3.91
	22.57	0.49

Fatty acid composition of ND5_96L determined by FAME analysis. Estimated chain length (ECL) values indicate the retention time of each fatty acid on the GC column in relation to their saturated fatty acid counterparts.

4.3.2 Bioactivity

ND5_96L vs. stationary phase *M. tuberculosis* H37Ra

Figure 4.1 ND5_96L vs. stationary phase *M. tuberculosis* H37Ra

Dose response curve of ND5_96L using stationary phase assay conditions. RFU units were used as a measure of growth on the Y-axis. +/- value indicates standard error. MIC₉₀ value is two-fold greater in stationary phase cells compared to actively growing cells. (n=1, triplicate)

A

B

Figure 4.2 ND5_96L vs. mammalian cell lines

MTT assay of ND5_96L against HL-60 (A) and HEK cell lines (B). The HEK cell line has been used as an indication of general cytotoxicity. OD was used as a measure of growth on the Y-axis. NA not achievable, +/- value indicates standard error. (n=1, duplicate)

Table 4.2 Broad- spectrum activity of active mixture of fatty acids

Microorganism	Activity
<i>S. aureus</i>	-
<i>E. coli</i>	-
<i>P. aeruginosa</i>	-
<i>M. smegmatis</i>	+
<i>M. tuberculosis</i> H37Ra	+
<i>S. cerevisiae</i>	-
HL60 cells	+
HEK cells	-

(+) indicates activity, (-) indicates no observed activity. Activity is defined by $\geq 90\%$ growth inhibition observed at the highest concentrations tested (500-1000 $\mu\text{g/mL}$).

Table 4.3 Bioactivity profile of individual fatty acids

Name	Chain length	MIC ₉₀ ($\mu\text{g/mL}$)
Palmitic acid	C16:0	250
Palmitoleic acid	C16:1	500
Petroselenic acid	C18:1, cis-6	≥ 1000
Oleic acid	C18:1, cis-9	≥ 1000
Eladic acid	C18:1, trans-9	500
<i>cis</i> -vaccenic acid	C18:1, cis-11	500
<i>trans</i> - vaccenic acid	C18:1, trans-11	500
Linoleic acid	C18:3, cis-9,12,15	NA

MIC₉₀ values of fatty acids against *M. tuberculosis* H37Ra.

Table 4.4 Comparison of MIC₉₀ values of artificial mixtures against *M. tuberculosis* H37Ra

Name	MIC ₉₀ ($\mu\text{g/mL}$)
<i>cis</i> mix	250
<i>trans</i> mix	125
ND5_96L	250

Comparison of activities of the mixtures of the four fatty acids with either *cis* or *trans*-vaccenic acid to ND5_96L.

Table 4.5 Bioactivity of fatty acid mixtures vs. *M. tuberculosis* H37Ra

Name	Chain lengths	IC ₅₀ (µg/mL) ± std. err.
Palmitic acid	C16:0	66 +/- 1.1
Palmitoleic acid	C16:1, cis-9	274 +/- 1.1
Eladic Acid	C18:1, trans-9	301 +/- 1.1
<i>trans</i> -vaccenic acid	C18:1, trans-11	306 +/- 1.1
<i>cis</i> -vaccenic acid	C18:1, cis-11	166 +/- 1.1
Palmitic/palmitoleic	C16:0/ C16:1, cis-9	51 +/- 1.1
Palmitic/eladic	C16:0/C18:1, trans-9	50 +/- 1.1
Palmitic/ <i>trans</i> -vaccenic	C16:0/C18:1, trans-11	48 +/- 1.1
Palmitic/ <i>cis</i> -vaccenic	C16:0/ C18:1, cis-11	102 +/- 1.1
Palmitoleic/eladic	C16:1, cis-9/C18:1, trans-9	73 +/- 1.1
Palmitoleic/ <i>trans</i> -vaccenic	C16:1, cis-9/C18:1, trans-11	64 +/- >1
Palmitoleic/ <i>cis</i> -vaccenic	C16:1, cis-9/ C18:1, cis-11	104 +/- 1.3
Eladic/ <i>trans</i> -vaccenic	C18:1, trans-9/ C18:1, trans-11	71 +/- 1.2
Eladic/ <i>cis</i> -vaccenic	C18:1, trans-9/C18:1, cis-11	113 +/- 1.0
Palmitic/palmitoleic/eladic	C16:0/C16:1, cis-9/C18:1, trans-9	60 +/- 1.1
Palmitic/palmitoleic/ <i>trans</i> - vaccenic	C16:0/ C16:1, cis-9/C18:1, trans-11	68 +/- 1.1
Palmitic/palmitoleic/ <i>cis</i> -vaccenic	C16:0/C18:1, trans-9/C18:1, cis-11	125 +/- >1
Palmitic/eladic/ <i>trans</i> -vaccenic	C16:0/C18:1, trans-9/C18:1, trans- 11	69 +/- 1.1
Palmitic/eladic/ <i>cis</i> -vaccenic	C16:0/C18:1, trans-9/ C18:1, cis-11/	79 +/- 1.1
Palmitoleic/eladic/ <i>trans</i> - vaccenic	C16:1, cis-9/C18:1, trans-9/C18:1, trans-11	72 +/- 1.1
Palmitoleic/eladic/ <i>cis</i> -vaccenic	C16:1, cis-9/C18:1, trans-9/C18:1, cis-11	81 +/- 1.3
<i>trans</i> -vaccenic mix of the original	C16:0/C16:1, cis-9/C18:1, trans-9/ C18:1, trans-11	76 +/- 1.1
<i>cis</i> -vaccenic mix of the original	C16:0/C16:1, cis-9/C18:1, trans-9/ C18:1, cis-11	102 +/- 1.1

4.4 Discussion

4.4.1 Fractionation and isolation

An initial crude fractionation was performed using HP20 (PSDVB) column chromatography (Scheme 4.1). Based on the anti-mycobacterial activity of the fractions generated, the 100% acetonitrile fraction was chosen for the next stage of purification, using DIOL normal phase chromatography. Based on its profile, the 75% hexanes / EtOAc fraction was selected to undergo the final stage of size exclusion chromatography using LH20 gel. Based on the bioactivity as well as the ^1H NMR spectra of the preceding fractions, ND5_96L was identified as the most potent fraction, however it was apparent that it was still a mixture of fatty acids.

4.4.2 Structural elucidation

The initial method used to identify the structure of the bioactive compound was NMR spectroscopy. Using NMR, it was concluded that the most pure and active fraction, ND5_96L, was a mixture of C-18 fatty acids. Therefore, a range of C-18 fatty acid standards were purchased, spectra were acquired and these were compared to ND6_96L. Based on ^1H and ^{13}C NMR data, it was initially concluded that the sample was a mixture of oleic and *cis*-vaccenic acids (Figure 4.3 to 4.7).

Figure 4.3 Comparison of ^{13}C NMR (CDCl_3 , 150 MHz) spectra of ND5_96L (top), *cis*-vaccenic (middle) and oleic acids (bottom) at full spectral width.

Figure 4.4 Comparison of ^{13}C NMR (CDCl_3 , 150 MHz) spectra of ND5_96L (top), *cis*-vaccenic (middle) and oleic acids (bottom) at a specific range of interest.

Figure 4.5 Comparison of ^1H NMR (CDCl_3 , 600 MHz) spectra of ND5_96L (top), *cis*-vaccenic (middle) and oleic acids (bottom) at full spectral width.

Figure 4.6 Comparison of ^1H NMR (CDCl_3 , 600 MHz) spectra of ND5_96L (top), *cis*-vaccenic (middle) and oleic acids (bottom) at a specific range of interest.

Figure 4.7 Comparison of ^1H NMR (CDCl_3 , 600 MHz) spectra of ND5_96L (top), *cis*-vaccenic (middle) and oleic acids (bottom) at a specific range of interest.

Testing the C-18 fatty acid standards identified MIC_{90} values of 1000 $\mu\text{g/mL}$ for oleic acid and 500 $\mu\text{g/mL}$ for *cis*-vaccenic acid (Table 4.3). The lack of correlation of the activities of the standards to ND5_96L (MIC_{90} 250 $\mu\text{g/mL}$) could be due to two factors; either the fatty acids in the mixture had not been correctly identified, or the fatty acids need to be present together in a certain ratio to be active. To address the issue of misidentification of the appropriate fatty acids, the mixture was methylated with a 10% solution of BF_3 in methanol to generate the corresponding fatty acid methyl esters (FAMES) for analysis by GC-MS. The FAME analysis identified the mixture as eladic (C18:1 *trans*-9) (52.3%), vaccenic (C18:1 *cis* or *trans*-11) (7.8%), palmitic (C16:0) (11.6%) and palmitoleic (C16:1 *cis*-9) (12.3%) acids (Table 4.1). This means the initial conclusion of the active mixture as a binary mixture of fatty acids was incorrect.

Given vaccenic acid can exist in two different geometries, two sets of mixtures were generated for bioassay testing, one using *cis*-vaccenic acid while the other used *trans*-vaccenic acid combined with the other three acids. All possible combinations of two or

three fatty acids, in addition to the individual fatty acids, were tested for their bioactivity against *M. tuberculosis* H37Ra (Table 4.5).

4.4.3 Bioactivity

In theory, the mixture of four fatty acids should be approximately as active as ND5_96L and based on the bioassay data this is the case with an MIC₉₀ of 125 µg/mL for the *trans*-vaccenic acid mixture and an MIC₉₀ of 250 µg/mL for the *cis*-vaccenic acid mixture (Table 4.4). These values are consistent with the observed activity of the ‘natural mixture’ ND6_96L (250 µg/mL), and based on this it could be concluded that the natural mixture contains *cis*-vaccenic acid.

Testing the fatty acids individually identified palmitic acid as the most biologically active with an IC₅₀ of 66 µg/mL (Table 4.5). When comparing the IC₅₀ value of palmitic acid (C16:0) to palmitoleic acid (C16:1) a four-fold difference is observed (Table 4.5), which is very unusual as it is generally believed that unsaturation of the alkyl chains improves antibacterial activity [173-175].

When comparing the anti-mycobacterial activities of the fatty acids tested in combinations, the most effective mixtures were those that contain two fatty acids, where one of the fatty acids is palmitic acid (Table 4.5). When comparing activities of *cis* vs. *trans* FFAs it is hard to draw a conclusion, as only two fold differences were observed when comparing either IC₅₀ or MIC₉₀ values. Surprisingly, when *trans* C18 fatty acids were mixed with palmitic acid in a 1:1 ratio, roughly a six-fold improvement in activity was observed (see Table 4.5).

This result seems slightly unusual as it is generally believed that *trans*-fatty acids should display lower activity compared to *cis*-fatty acids. What seems more unusual is that the activities of the *trans* FFAs are enhanced by a saturated FFA [173-175]. The improvement in activity with palmitic acid is also observed in combination with palmitoleic acid, a C16 *cis*-fatty acid with a shift in IC₅₀ from 274 µg/mL to 68 µg/mL.

The data (Table 4.5) illustrates that palmitic acid, a C16 fatty acid, is more active compared to C18 fatty acids. Furthermore, based on these results, it seems that this saturated C16 fatty acid is more active than unsaturated fatty acids. Research studying the effects of FFAs on fast growing mycobacteria has identified that as chain length

increased, activity decreased for saturated FFAs [171]. In addition, unsaturated FFAs tend to have better inhibitory activity with C16 chain lengths preferred over C18, however if C18 chains contained two double bonds the activity was comparable to that of the unsaturated C16 FFA [171]. As the aforementioned study focused on fast growing mycobacteria, a more relevant analogous comparison looking at slow growing mycobacteria identified a 2-4 fold improvement in MIC values for palmitic acid (C16) compared to unsaturated fatty (C18) acids [170].

Figure 4.1 illustrates the activity profile of the ND5_96L against stationary mycobacteria and mammalian cell lines. For the bacteriostatic assay, the highest concentration used was 250 µg/mL and at this concentration 90% inhibition of growth was observed. When analysing activity against stationary phase mycobacteria, ideally higher concentrations of drugs or samples should be used, as it is believed mycobacteria are more tolerant to inhibition by drugs in this state [195]. This can be illustrated in the drop in activity of streptomycin in a bacteriostatic assay (MIC 0.39 µM) compared to a stationary phase assay (MIC not achievable) (Appendix A). This assay showed that ND5_96L had an MIC₉₀ of 500 µg/mL (Figure 4.1), which is two-fold greater when compared to the bacteriostatic assay. Studying the activity of the mixture in HL-60 and HEK cells identified the mixture was two-fold more active in HL-60 cells than HEK cells when comparing MIC₉₀ values (Figure 4.2).

4.4.3.1 Palmitic acid and mycobacteria

Based on the activity of fatty acids analysed, it was observed that palmitic acid had the most prominent activity. This section highlights work relating to palmitic acid and mycobacteria. One of the earliest studies in this area identified growth inhibitory effects at higher concentrations of the fatty acid [196]. However, when non-toxic concentrations were used, the saturated acid was incorporated into cells. Following assimilation of all the palmitic acid within the growth medium, further doses of non-inhibitory concentrations of palmitic acid could be added to the media, which was in turn consumed. This assimilation process was dependent on aerobic conditions [196].

Studying the effects of complexing fatty acids with detergent to reduce their toxicity identified that when Triton WR1339 was added in a 2.5:1 ratio, the toxicity of palmitic acid was reduced. This was due to the detergent sequestering the fatty acids, making less available to inhibit bacterial growth [197]. Testing the effects of fatty acids in the

absence of the detergent against *M. tuberculosis* identified inhibition of growth by oleic and palmitic acids at 2 µg/mL [197]. This inhibitory concentration is inconsistent with the work described here and that of others as stated below. In our assay the MIC (an observed maximum of 2% growth) was 1 mg/mL, with greater than 90% growth observed at the lowest tested concentration, 7.8 µg/mL.

The effects of palmitic acid at non-inhibitory concentrations on the metabolism of *M. avium* identified that this fatty acid is incorporated to form lipids, a majority of them being triglycerides [198]. A follow-up study focusing on the effects of the fatty acid on cell division identified that the acid increased the formation of clear virulent colonies compared to the cultures starved of palmitic acid, which selected for opaque non-virulent colonies [199].

A study of the anti-mycobacterial component of the hexane extract of *Humulus lupulus* identified palmitic, stearic and oleic acids as the major components. The growth inhibitory effect of these fatty acids on *M. fortuitum* identified an MIC of 512 µg/mL for palmitic acid using a broth microdilution assay, however no activity was observed against *M. smegmatis*, *M. phlei* or *M. aurum*. In the same study oleic and linoleic acids proved more active with MIC's ranging from 4-16 µg/mL [200]. The reported activity for palmitic acid in the same study is more consistent with the study described here. However, the activities of oleic and linoleic acids do not match the results of this thesis with more than 90% growth observed at concentrations ranging from 4-16 µg/mL.

A similar study focusing on a native South African herbal remedy made from the root extracts of *Pelargonium reniforme* and *P. sidoides* identified *in vitro* activity from the hexanes fractions. Bioassay-guided fractionation identified that a mixture of fatty acids was responsible for this activity. Testing of the saturated and unsaturated components found an MIC of 256 µg/mL against *M. aurum* for palmitic acid, which was inactive against *M. smegmatis*, *M. fortuitum*, *M. abscessus* or *M. phlei*. This study also identified unsaturated fatty acids as being more active than saturated fatty acids. They reported oleic acid MIC's ranging from 4-256 µg/mL, linoleic acid MIC's ranging from 2-32 µg/mL and palmitoleic acid with MIC's ranging from 4-16 µg/mL for the different species of mycobacteria [171].

Bioassay-guided isolation focusing on the anti-mycobacterial activity of a red alga, *Polysiphonia virgata*, identified a mixture of fatty acids from the dichloromethane extract as being responsible for activity. Studying the effect of individual fatty acids identified that the most active fatty acids were again unsaturated fatty acids with oleic acid having an MIC of 25 µg/mL, and linoleic, myristic and lauric acids having MICs of 50 µg/mL against *M. tuberculosis*. Testing the acids against a strain of MDR-TB identified the same MIC values for linoleic, myristic and lauric acids, with oleic acid reported as inactive in this strain. This paper reported palmitic acid as inactive [201].

A more recent study on the bioactivity of the hexane extract of fruit peel of *Citrus aurantifolia* (Mexican lime) identified fatty acids as the components responsible for activity, with MICs in the range of 25-50 µg/mL for palmitic acid against *M. tuberculosis* H37Rv and three other mono-resistant strains of TB. Interestingly, this paper reported that the unsaturated fatty acids linoleic and oleic acid were slightly less active with MICs ranging from 50-100 µg/mL. This study complements the results presented in this thesis, with the saturated acids identified as more active than the unsaturated, although the reported MIC for palmitic acid by the authors matches more closely to the observed IC₅₀ of 66 µg/mL described in this thesis [170].

4.4.4 General summary

In general, current literature has suggested that unsaturated fatty acids tend to be more active than saturated ones. However, this work demonstrates that when looking at the activities of the fatty acids, palmitic acid, a C16 saturated fatty acid, displays greater activity than the C16 and C18 unsaturated fatty acids that have been tested.

Also worth noting is the current view that *cis* fatty acids tend to be more active than *trans* fatty acids. When comparing the activities of *cis*-vaccenic to *trans*-vaccenic acids, this holds marginally true. However, the combinations of palmitic and *trans*-vaccenic acids gave the most effective IC₅₀ value of all the different mixtures tested with a two-fold improvement in activity compared to the mixture of palmitic and *cis*-vaccenic acids.

One trend that emerges from the literature is that unsaturated fatty acids tend to be more active against mycobacteria [171, 200, 201]. Assessing the inhibitory effects of fatty acids on mycobacteria has identified that for saturated FFAs, once the chain length

There are conflicting views of the activity of palmitic acid against mycobacteria, nonetheless, this work identified fatty acids as moderate to weak inhibitors of mycobacteria, with palmitic acid causing the most inhibition.

The image shows a steroid molecule with a complex side chain at the C-17 position. The side chain consists of a 3-methylbutyl group attached to a 2-sulfoethyl group. The steroid core has two substituents, R₁ and R₂, at the C-3 and C-14 positions, respectively. The definitions for these substituents are provided below the main structure:

$R_1 = \text{OH}$

$R_2 = \text{H}_3\text{N}^+ \text{---} \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}^+\text{H}_2 \text{---} \text{CH}_2\text{CH}_2\text{CH}_2\text{N}^+\text{H}_2 \text{---}$

$R_1 = \text{OSO}_3\text{Na}$

$R_2 = \text{---} \text{CH}_2\text{CH}_2\text{CH}_2\text{N} \text{---}$

82

Chapter 5 Rimarikiamide A from ***Latrunculia* sp.**

5.1 Background

Screening the VUW MNP library identified the crude extract of a *Latrunculia* sponge (PTN_256C) as an active inhibitor against *M. smegmatis* and *S. aureus* at the screening concentration of 100 µg/mL. The subsequent validation of the crude extract in *M. tuberculosis* H37Ra found it to be more active in H37Ra (MIC₉₀ = 6.25 µg/mL), compared to *M. smegmatis* (MIC₉₀ = 100 µg/mL) (Table 3.3). Bioassay-guided fractionation of this sponge led to the identification of a novel taurinated terpene with activity against cancer cells *in vitro*, in addition to other fractions with anti-mycobacterial activity. This chapter describes the bioactivity, isolation and structure elucidation of the taurinated terpene. Furthermore this chapter gives an overview of taurinated marine natural products as well as known biological activities linked to the compounds.

Taurinated marine natural products are common as the presence of taurine in marine organisms is ubiquitous [208, 209]. The bioactivity reported for most of the compounds is generally anti-cancer due to the common use of anti-tumor assays in screening for novel compounds. Using SciFinder® and MarinLit [210], a literature search was performed to identify published work on taurinated marine natural products. From this search it was evident that sponges predominate as a major source of taurinated compounds. Most instances of taurinated marine natural compounds tend to involve taurine groups linked to a long alkyl chain or long fatty acids. The focus of this literature review will be on marine natural products that have a taurine group attached via an amine functionality. Compounds where taurine has been completely incorporated into the backbone of the compound will not be discussed. The compounds discussed below are grouped based on the common functionalities and carbon skeletons they share.

5.1.1.1 Taurinated fatty acid derivatives

The taurinated compounds grouped in this class are either conjugates of fatty acids and taurine, or a major portion of their structure is derived from a fatty acid. A recent report highlighted the isolation of 2-heptadec-11-enamidoethanesulfonic acid (**43**), from a South China Sea sponge *Axinella* sp. [211]. The authors also re-isolated two known compounds 2-octadec-7-enamidoethanesulfonic acid (**44**) and 2-palmitamidoethanesulf-

onic acid (**45**). Compound **45** was first isolated from a South China sea sponge *Haliclona* sp. [212]; there has been no reported bioactivity for any of these compounds.

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Structurally related are two acylated taurine fatty acid derivatives, (all-*Z*)-2-(icosa-5,8,11,14,17-pentaenoylamino)ethane sulfonic acid (**46**) from a star fish *Certonardoa semiregularis*, and **47** isolated from the sponge *Erylus nobilis* [213]. These two compounds were isolated guided by a brine shrimp lethality assay. An *N*-acyl taurine (**48**) was isolated from a sea urchin *Glyptocidaris crenularis* with a reported LD₅₀ of 222 µg/mL in a brine shrimp assay [214].

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Irciniasulfonic acid B₁ (**49**) and B₂ (**50**), a mixture of two taurinated fatty acids isolated from a Japanese marine sponge *Ircinia* sp., restored sensitivity to vincristine in resistant cancer cells [215]. Taurolipids A (**51**), B (**52**), and C (**53**), isolated from the protozoa *Tetrahymena* [216], have been reported to inhibit *Clostridium perfringens* sialidase enzyme (20 mU) by 50% at 140 and 110 pM for **51** and **52**, respectively [217]. This enzyme cleaves sialic acid from the cell surface and glycoproteins and has been implicated in virulence of bacteria [218].

51 R₁ = R₂ = H

52 R₁ = H, R₂ = OH

53 R₁ = R₂ = OH

Taurospongina A (**54**), an acetylenic lipid isolated from the Okinawan sponge *Hippospongia* sp., was shown to inhibit DNA polymerase β and human immunodeficiency virus (HIV) reverse transcriptase [219]. Under normal settings, DNA

polymerase β plays a role in base excision repair, however, overexpression of variants of this enzyme have been linked to cancer [220].

54

Carteriosulfonic acids A-C (**55-57**), isolated from a *Carteriospongia* sp. sponge collected in the Philippines, were shown to inhibit the kinase GSK-3 β , which is part of the Wnt signalling pathway. This pathway is involved in several human diseases including cancer [221, 222].

Tauro-pinnaic acid B (**58**), a novel taurinated fatty acid, was isolated from the Okinawan bivalve *Pinna muricata*. This compound displayed an IC₅₀ of 90 μ M against cytosolic phospholipase A2, in a search for potential anti-inflammatory compounds [223].

58

5.1.1.2 Bromopyrrole containing compounds

This next set of seven taurinated compounds all contain a bromopyrrole functionality, with all but one compound also featuring guanidine. Tauroacidins A (**59**) and B (**60**), isolated from the Okinawan sponge *Hymeniacidon* sp., displayed inhibitory activity against EGF receptor kinase and *c-erbB-2* kinase each with IC₅₀ values of 20 μ g/mL [224]. The increased activity or overexpression of these kinases has been linked to different types of cancers [225].

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Mauritamide A (**61**), was isolated from the Fijian sponge *Agelas mauritiana* [226], and congeners mauritamide B-D (**62-64**) were isolated from the Indonesian sponge *A. linnaei* [227]. Mauritamide D (**64**) lacks the commonly found guanidine moiety. None of the mauritamides have any bioactivity reported to date.

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Taurodispacamide A (**65**), isolated from a Mediterranean sponge *A. oroides*, displayed anti-histamine activity when tested in the guinea pig ileum assay [228]. Nagelamide H (**66**), isolated from an *Agelas* sp., exhibited antibacterial activity against *Micrococcus luteus* and *Bacillus subtilis* with respective MIC's of 16.7 and 33.3 $\mu\text{g/mL}$, in addition an IC_{50} of 46 μM against protein phosphatase 2A was also reported [229].

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5.1.1.3 Aromatic compounds

The taurinated compounds listed in this section contain aromatic functionalities as part of their structure. 2-[(Phenylacetyl)]aminoethanesulfonic acid (**67**), a conjugate of phenylacetic acid and taurine, was isolated from *Colobometra perspinosa*, an Australian crinoid, with a reported GI_{50} of 195 μM against the MCF-7 breast-pleural effusion

adenocarcinoma cell line [230]. Pyridinebetaine B (**68**) was isolated from a Caribbean sponge, *Agelas dispar* [231].

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Secoadociaquinones A (**69**) and B (**70**), isolated from a *Xestospongia* sp. sponge from the Philippines, showed inhibitory activity against topoisomerase II [232]. This enzyme creates double strand breaks when uncoiling DNA during replication. Consequently, its activity is more prominent in actively replicating cells, such as cancer cells. As such, many cancer therapeutics target this enzyme [233].

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Cribrastatin 7 (**71**), a novel taurinated isoquinoline derivative with an IC_{50} of 45 $\mu\text{g/mL}$ against human colon carcinoma (HCT 116) cells, was reported from a *Petrosia* sp. sponge, also collected in the Philippines [234].

71

The heteroaromatic alkaloids nakijiamine C (**72**) and D (**73**), were isolated from an Okinawan sponge *Suberites* sp., with no reported bioactivity from these compounds [235]. Microxine (**74**), a taurinated purine derivative isolated from an Australian sponge *Microxina* sp., was reported to have inhibitory activity against cdc2 kinase with an IC₅₀ of 13 μ M [236]. This enzyme is involved in the cell cycle and its entry into mitosis [237].

72

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A trichostatin analogue, JBIR-110 (**75**) was reported from the marine sponge derived *Streptomyces* sp. strain RM72, with a reported IC₅₀ value against HDAC1 of 74 μ M [238]. HDACs are involved in epigenetic activity; the enzyme is involved in condensing chromatin and therefore altering gene expression [239]. These types of inhibitors have been studied as potential anti-cancer agents [239]. From the sponge *Leucetta microhaphis*, the alkaloid (9*E*)-clathridine 9-*N*-(2-sulfoethyl) imine (**76**), was isolated [240].

75

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5.1.1.4 Miscellaneous taurinated compounds

Mycosporine-taurine (**77**) was isolated from the sea anemone *Anthopleura elegantissima* in a study targeting compounds responsible for providing UV protection [241]. From the Okinawan ascidian *Didemnidae* sp., a novel chlorinated polyketide macrolide biselide D (**78**) was reported [242].

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5.1.1.5 Taurinated terpenoids

This section highlights taurinated terpenes. Terpenes are linear or cyclised hydrocarbon chains comprised of repeating isoprene units. (+)-4β-*N*-Methenetauryl-10β-methoxy-1β,5α,6β,7β-aromadendrane (**79**) and (-)-4β-*N*-methenetauryl-10β-methoxy-1β,5β,6α,7α-aromadendrane (**80**), isolated from a South China Sea gorgonian coral *Melitodes squamata*, displayed inhibitory activity against *B. subtilis* and *M. luteus* at

25 μg in a disk diffusion assay [243]. The bicyclic terpenoids, clathrimides A (**81**) and B (**82**), isolated from the sponge *Clathria compressa* [244], showed no activity against human embryonic stem cells (BG02), Gram-positive (*Staphylococcus aureus* including methicillin-resistant *S.aureus*) or Gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) bacteria.

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Spongodine D (**83**), contains a pyridine functionality with inhibitory activity against human synovial phospholipase A2 (PLA2), and was isolated from a Vanuatu sponge *Spongia* sp. [245]. PLA2 is responsible for the release of free fatty acids, which are then metabolised to produce pro-inflammatory mediators [246]. Phorbasin D (**84**), a diterpene-taurine conjugate, was isolated from the *Phorbas* sp., Australian sponge. This compound was tested and reported as inactive against lung (A549) and breast (HT29 and MDA-MB-231) cancer cell lines [247].

83

84

Gukulenin E (**85**), a diterpene pseudo-dimer, was isolated from *Phorbas gukulensis*, a Korean sponge, with a reported LC₅₀ of 0.32 and 0.11 μ M against K562 and A549 cell lines, respectively [248].

85

5.1.1.5.1 Meroterpenes

Meroterpenes are compounds that are of mixed biogenesis and contain a terpene attached to an aromatic functionality. Aplidone C (**86**) was isolated from the Mediterranean ascidian *Aplidium conicum* and had no reported activity [249]. Siphonodictyal B1 (**87**) was isolated from a Caribbean sponge, *Aka coralliphagum*, with reported inhibitory activity against *S. aureus* at 12 μ g/mL and cytotoxic activity against L929 mouse fibroblasts at 10 μ g/mL [250]. Nakijiquinone R (**88**), a

sesquiterpenoid quinone, was isolated from an Okinawan sponge belonging to the family Spongiidae. This compound displayed greater than 99% and 52% inhibition against EGFR and HER2 kinases at 1 mM, respectively [251].

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88

Melemeleones A (**89**) and B (**90**) were isolated from the Solomon Islands sponge *Dysidea avara*. Melemeleone B displayed an approximate IC_{50} of 28 μ M in an assay against the pp60^{V-src} PTK, a tyrosine kinase [252]. Tyrosine kinase inhibitors are important in the treatment of cancer progression [253]. An anti-inflammatory sesquiterpene dysidine (**91**), was isolated from a *Dysidea* sponge from Vanuatu. This compound was shown to inhibit the activity of human synovial PLA2, with an IC_{50} of 2.0 μ M [254].

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5.1.1.6 Taurinated sterols

The next set of six compounds are taurinated sterols, which have all been isolated from starfish. From *Myxoderma platyacanthum*, (92) and (93) were isolated [255]. (20*R*,22*E*,24*R*,25*S*)-24-Methyl-3 β ,6 α ,8,15 β -tetrahydroxy-5 α -cholest-22-en-27-oic acid (94), was isolated from *Astropecten triseriatus* [256]. Minutoside B (95), isolated from *Anasterias minuta*, had inhibitory activity against *Aspergillus flavus* at concentrations between 20-60 μ g/mL in a disk diffusion assay [257]. Novaeguinosides B (96) and D (97), were isolated from *Culcita novaeguineae*, with reported IC₅₀ values of 7.9 and 9.5 μ M against K-563 and BEL-7402 cell lines for 96 and 4.6 and 4.1 μ M for 97 [258].

94 $R_1 = R_2 = H$ $R_3 = OH$, Δ sat.

$R_2 = H$ $R_3 = OH$, Δ sat.

96 $R_1 = SO_3Na$

$R_3 = H$, Δ

97

5.2 Methods

5.2.1 Extraction of sponge material

Latrunculia sp. (350 g), was cut into $\sim 1\text{ cm}^3$ sized pieces and extracted twice in methanol (450 mL). The two extracts were treated separately and filtered using a Büchner funnel and Celite. The second extract was passed through an HP20 column (140 mL) followed by the first. The eluents were combined and diluted two-fold with H₂O before passing through the same column again. This was repeated until the original MeOH extract was diluted to 12.5% and passed through the column. Following this cyclic loading, the column was eluted with 420 mL volumes of 20%, 40%, 60%, 80% and 100% acetone in water. The 40% fraction was further fractionated using a 15 mL column of HP20ss resin, the column was eluted with 45 mL volumes of 50%, 60%, 70%, and 80% MeOH and H₂O solvent mixtures as well as 100% MeOH and 100% acetone. Following this, the 80% MeOH/H₂O fraction was further purified using reversed-phase C18 semi-preparative column (Phenomenex Prodigy, 10.0 x 250 mm, 10 μm particle size), 35% acetonitrile/H₂O at 4 mL/min. From the resulting fractions, ND4_34D and E were pooled together to give rimarikiamide A (**98**), (200 μg) (Scheme 5.1). Quantification of samples lower than 1 mg was performed as described in section 2.7.

Based on this initial isolation, a repeat isolation was attempted, using the remaining sponge sample. As the structure of rimarikiamide A had already been elucidated from the first isolation, it was evident that the lack of a chromophore would make a repeat isolation via HPLC problematic. Therefore size exclusion chromatography using LH20 was attempted using a 100 mL column, eluting with 100% MeOH to give rimarikiamide A (**98**) (133.7 μg).

5.2.2 Isolation schemes for rimarikiamide A

Scheme 5.1 Original isolation scheme for rimarikiamide A (98) from *Latrunculia* sp.

Scheme 5.2 Optimised isolation scheme for rimarikiamide A (98**) from *Latrunculia* sp.**

5.3 Results

Using the methodology described in section 2.6, the results of the dose-response assays for rimarikiamide A are listed below. Graphs were generated using GraphPad Prism v.5.0. Resazurin dye was used to measure bacterial metabolism after each assay was run and RFU measurements were used as a measure of growth. OD was used as measure of growth for the HL-60 and HEK MTT assays. Where possible IC₅₀ values have been listed, however as sigmoidal curves were not achievable for all data MIC₉₀ values have been listed.

5.3.1 Antimicrobial activity

Figure 5.1 Loss of anti-mycobacterial activity of rimarikiamide A

(A), dose response curve illustrating the loss of activity of rimarikiamide A. (B), dose response curve of the active fraction ND4_47D. RFU units were used as a measure of growth.

(n=1, duplicate)

5.3.2 Mammalian cell line assays

Figure 5.2 Rimarikiamide A vs human cell lines

MTT assay of ND6_96L against HL-60 (A) and HEK cell lines (B). The HEK cell line was used as an indication of general cytotoxicity. OD was used as a measure of growth.

(n=1, duplicate)

5.4 Discussion

5.4.1 Fractionation and isolation

Guided by bioassays against *M. tuberculosis* H37Ra, an initial crude fractionation using HP20 (PSDVB) column chromatography was performed, with the 40% acetone/H₂O fraction taken to the next stage of purification (Scheme 5.1). HP20ss (PSDVB) column chromatography was used to further purify this fraction culminating with reversed-phase C18 semi-preparative HPLC to purify the final bioactive fraction rimarikiamide A (200 µg) (Scheme 5.1).

To isolate more compound, the second half of the sponge was extracted and fractionated to the point of collecting the HP20ss 80% MeOH/H₂O fraction (Scheme 5.2). As we could not rely on HPLC to guarantee the isolation of the compound due to the lack of a suitable chromophore, LH20 size exclusion chromatography was employed for the next stage of purification. Following size exclusion chromatography, the fractions of the second work up of the original sponge were combined based on TLC, and tested for activity (Scheme 5.2). This identified that the taurinated-compound initially believed to be responsible for activity was not the source of anti-mycobacterial activity, which can be observed when comparing the dose response curve of rimarikiamide A (**98**) to ND4_47D (Figure 5.1).

5.4.2 Structural elucidation

Negative ion HRESIMS was used to detect an [M-H]⁻ ion at m/z 428.2469, which was suitable for the molecular formula C₂₂H₃₉NO₅S (calcd m/z 428.2471, Δ = 0.0002), requiring four double bond equivalents. Both ¹H and multiplicity edited HSQC NMR spectra in d₆-DMSO revealed that 36 of the 39 protons were attached to 18 carbons, including five methines (δ_C 124.5, 124.2, 121.3, 76.0, 29.8), eight methylenes (δ_C 50.7, 43.2, 39.3, 36.3, 35.5, 33.9, 26.2, 24.5), and five methyls (δ_C 25.6, 19.4, 17.6, 16.1, 11.3). The remaining four carbons in the molecule were quaternary carbons, which included an ester or amide carbonyl (δ_C 171.0) and three olefinic carbons (δ_C 137.4, 134.9, 130.6). The three remaining protons were therefore exchangeable. The presence of an ester or amide and three olefinic methines accounted for all the double bond equivalents required by the molecular formula, therefore the molecule must be linear.

COSY and HMBC correlations established the connectivity for most of the structure. The terminal methyls (CH₃-16: δ_C 25.6, δ_H 1.63); (CH₃-20: δ_C 17.6, δ_H 1.55) showed HMBC correlations to the olefinic quaternary carbon C-15 (δ_C 130.6) and its neighbouring olefinic methine C-14 (δ_C 124.2, δ_H 5.06). This was further supported by allylic COSY correlations from H₃-16 and H₃-20 to H-14. H-14 in turn showed a COSY correlation to the methylene proton resonances of C-13 (δ_C 26.2, δ_H 2.00), which in turn correlated to methylene C-12 (δ_C 39.3, δ_H 1.91). This established the first substructure below (Figure 5.3).

Figure 5.3 Key COSY and HMBC correlations establishing substructure 1

HMBC correlations from the methyl CH₃-19 (δ_C 16.1, δ_H 1.54) to the olefinic methine C-10 (δ_C 121.3, δ_H 5.04), quaternary C-11 (δ_C 134.9) and methylene C-12 helped further extend the substructure. This was further supported by HMBC correlations from C-12 to C-10, C-11 and C-19. COSY correlations observed from H-10 to H₂-9 (δ_C 33.9, δ_H 2.08), H₂-9 to the C-8 methine (δ_C 76.0 δ_H 3.77) and finally an allylic COSY correlation from H₂-12 to the olefinic methine H-10 helped extend the substructure (Figure 5.4).

Figure 5.4 Key COSY and HMBC correlations establishing substructure 2

The chemical shifts of the C-8 methine (δ_C 76.0, δ 3.77) were characteristic of a carbinol centre. Fortuitously a COSY correlation from H-8 to an exchangeable proton of an alcohol group (δ_H 4.59) further supported the presence of this functional group (Figure 5.5).

Figure 5.5 Key COSY and HMBC correlations establishing substructure 3

The substructure was extended by HMBC correlations from methyl CH₃-18 (δ_C 11.3 δ_H 1.51), to C-8, quaternary alkene C-7 (δ_C 137.4) and olefinic methine C-6 (δ_C 124.5 δ_H 5.22), and given the H₃-18 is a singlet, it must be attached to C-7. This was supported by HMBC correlations from H₂-9 to C-7. COSY correlations from H₃-18 to the olefinic methine C-6, from H-6 to the methylene CH₂-5 (δ_C 24.5 δ_H 1.95), and from H₂-5 to CH₂-4 (δ_C 36.3 δ_H 1.13, 1.26), supported by HMBC correlations from H-5 to C-4, C-6 and C-7 allowed the addition of a further subunit to the proposed substructure (Figure 5.6).

Figure 5.6 Key COSY and HMBC correlations establishing substructure 4

HMBC correlations from the methyl doublet CH₃-17 (δ_C 19.4 δ_H 0.82) to the methine CH-3 (δ_C 29.8 δ_H 1.82), methylenes CH₂-4 and CH₂-2 (δ_C 43.2 δ_H 1.81, 2.02) in addition to COSY correlations from H₃-17 to H₂-2 and H-3 and from H₂-2 to H-3 extended the established alkyl chain. This was further supported by HMBC correlations from H-3 to C-4 and C-17 as well as from H₂-2 to C-3, C-4 and C-17 (Figure 5.7).

Figure 5.7 Key COSY and HMBC correlations establishing substructure 5

Further HMBC correlations from H₂-2 and H-3 to the C-1 carbonyl (δ_C 171.0), in addition to the HMBC correlations of the C-21 methylene (δ_C 35.5 δ_H 3.27), to C-1, helped assign the position of the carbonyl carbon. The chemical shifts of CH₂-21 and of C-1 (δ_C 171.0) were indicative of an amide functionality, supported by a COSY correlation from H₂-21 to the exchangeable amide proton (δ_H 7.68). HMBC correlations

from the exchangeable amide proton to C-1 and COSY correlation from H₂-21 to H₂-22 (δ_{C} 50.7 δ_{H} 2.50) further extended the carbon skeleton (Figure 5.8).

Figure 5.8 Key COSY and HMBC correlations establishing substructure 6

The substructure established to this point accounted for all the carbons required by the molecular formula, leaving SO₃H. As all branching termini had been accounted for, the SO₃H group must be attached to C-22 (δ_{C} 50.7), which would account for the downfield shift of this carbon resonance.

To confirm the geometry of the alkenes, a 2D ROESY experiment was performed, to determine the spatial positions of the methyls. The chemical shifts of the olefinic methyls C-18, C-19 and C-20 (δ_{C} 11.3, 16.1 and 17.6) were indicative of *E* rather than *Z* geometries, which would have approximate chemical shifts of 25.0 ppm [259], this further confirmed the proposed *E* geometry for the alkenes in rimarikiamide A (**98**) (Figure 5.9). The planar structure of **98** was therefore as shown in Figure 5.9.

Figure 5.9 Key ROESY correlations establishing the alkene geometry of rimarikiamide A (98**).**

Table 5.1 ^1H (600 MHz) and ^{13}C (150 MHz) NMR data ($\text{d}_6\text{-DMSO}$) for rimarikiamide A (98).

98

Position	δ_{C}	$^1J_{\text{CH}}$ (Hz)	δ_{H} (J in Hz)	COSY	HMBC
1	171.0				
2a	43.2	122	1.81 m, 1H	2b,17	1,3,4,17
2b		120	2.02 m, 1H	2a,3,17	1,3,4,17
3	29.8	122	1.82 m, 1H	2b,17	1,4,17
4a	36.3	121	1.13 m, 1H	4b,5	
4b		122	1.26 m, 1H	4a,5	
5	24.5	122	1.95 m, 2H	4,6	4,6,7
6	124.5	152	5.22 (t, 7.0 Hz, 1H)	5,18	8,18
7	137.4				
8	76.0	142	3.77 (dd, 4.1, 6.5 Hz, 1H)	8-OH,9	
9	33.9	122	2.08 (t, 6.7 Hz, 2H)	8,10,19	7,8,10,11
10	121.3	153	5.04 m, 1H	9,12,19	12,19
11	134.9				
12	39.3	122	1.91 s, 2H	10,13	10,11,13,14,19
13	26.2	113	2.00 m, 2H	12,14,16,20	12,14,15
14	124.2	151	5.06 m, 1H	13,16,20	
15	130.6				
16	25.6	125	1.63 s, 3H	13,14,20	14,15,20
17	19.4	120	0.82 (d, 6.1 Hz, 3H)	2a,b,3	2,3,4
18	11.3	123	1.51 s, 3H	6	6,7, 8
19	16.1	125	1.54 s, 3H	9,10	10,11,12,
20	17.6	126	1.55 s, 3H	14,16	14,15,16
21	35.5	142	3.27 m, 2H	1-NH,22	1,22
22	50.7	134	2.50 m, 2H	21	21
1-NH			7.68 (t, 5.8 Hz, 1H)	21	1
8-OH			4.59 (d, 4.0 Hz, 1H)	8	9

Unfortunately, a lack of material at this point prevented establishing the configuration of the C-8 alcohol and C-3 methyl positions. The distance between C-8 and CH_3 -17 as well as the flexible nature of the chain prevented the relative configuration from being established. Thus, the configurations of C-3 and C-8 remain unknown.

One possible method to elucidate the configuration is through the use of electronic circular dichroism (ECD). When looking at the structure of **98** the two chiral centers (C-3 and C-8) give rise to four possible diastereomers as part of two enantiomeric pairs (*RR/SS* and *RS/SR*). The ECD curves could be predicted for each of the four possible configurations and compared to the actual curve of **98**. This would allow us to deduce the absolute configuration of the natural product [260]. However as there is no CD instrument available at VUW this approach was not possible. It should also be noted that calculating ECD spectra can lead to erroneous data when dealing with flexible molecules, where different conformers can give enantiomeric spectra [261]. Therefore the best way to elucidate the absolute configuration would be through the synthetic route.

5.4.3 Biogenesis

As rimarikiamide A is a novel taurinated diterpene, a biogenesis of (**98**) is proposed below. The two main building blocks to the terpenes are dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which could be products of the mevalonate pathway or the mevalonate-independent pathway [262]. DMAPP and IPP are condensed together by geranyl diphosphate synthase to give geranyl diphosphate (GPP) [262]. GPP and IPP are then condensed by farnesyl diphosphate synthase to generate farnesyl diphosphate (FPP), which is further condensed with IPP to give geranylgeranyl diphosphate (GGPP) [262]. GGPP is proposed to be hydrolysed to generate the alcohol geranylgeraniol, which is oxidised to produce the carboxylic acid. At this point a thioesterase could act upon the carboxyl to generate a thioester. The next step could involve the reduction of the double bond closest to the carbonyl via a reductase. Following this the oxidation of the C-8 carbon after the second isoprene unit could occur, introducing the hydroxyl functionality. The last two aforementioned steps could also occur in the opposite order, without a directed study there is no clear way of stating which step comes first. A final taurination step where taurine replaces the thioester linked enzyme is proposed to generate **98** (Figure 5.10). Compared to all the described taurinated terpenoids in section 5.1.1.5, **98** is a simple linear diterpene, without any cyclisation or any aromatic constituents. The simplicity of the molecule would mean a synthetic route should be fairly plausible, which would allow for the absolute configuration to be resolved.

Figure 5.10 Proposed biogenesis of rimarikiamide A (98).

5.4.4 Reported compounds from *Latrunculia* sp.

To date, a range of compounds have been isolated from *Latrunculia* sponges of these, four classes emerge as the most common, these are the latrunculins, trunculins, discorhabdins and callipeltins.

The latrunculins are a class of actin depolymerizing agents [263]. Latrunculin A (**99**) and B (**100**), isolated from *Latrunculia magnifica*, later re-classified as *Negombata magnifica* [264], have displayed cytotoxic effects on cell lines at sub-micromolar concentrations [265]. Studies indicate **99** binds to G-actin monomers [266], and both **99** and **100** have been shown to modulate actin stress filaments, altering the cytoskeleton and affecting cell morphology [267].

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Trunculins A (**101**) and B (**102**) were isolated from an Australian *Latrunculia* sp. later re-classified as either *Dicarnus* or *Sigmaceptrella* sp. [264], and were active against *Bacillus subtilis* as well as *Saccharomyces cerevisiae* [268].

101 R = H

102 R = CH₃

The discorhabdins belong to a class of compounds termed pyrroloiminoquinones, these molecules have displayed various bioactivities such as antibacterial, anti-viral, anti-malarial, cytotoxic and anti-tumour activity [269]. The highly potent low micro- to nano-molar level of activities of these compounds made them of high interest, however, the lack of selectivity of these compounds prevented them progressing as anti-cancer agents [269]. These compounds have also displayed activity against *M. tuberculosis* in addition to a range of Gram-positive and Gram-negative bacteria [269]. Below is the structure of discorhabdin C (**103**) the first reported discorhabdin, isolated from a New Zealand *Latrunculia* sp. [270]. For further information on this class of compounds, please refer to two extensive reviews by Hu *et al.* and Antunes *et al.* [264, 269].

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Callipeltins F-I are peptides isolated from *Latrunculia* sp. from Vanuatu which have been reported to have anti-*Candida* activity at 1 mM [271]. Below is the structure of Callipeltin F (**104**).

104

As these four classes of molecule represent the four most common classes of compounds isolated from *Latrunculia* sp., it is possible that very low levels of any of these compounds could be responsible for the anti-tubercular activity. To identify if any of these compounds were present in the bioactive fractions, ^1H NMR signals of ND4_47D+E were compared to identify signature resonances to indicate the presence of any of these compounds. However, none of the diagnostic signals for any of these compounds were identifiable in the ^1H NMR spectra of the active fraction (Figure 5.11).

Figure 5.11 ^1H NMR (CD_3OD , 600 MHz) spectrum spectra of ND4_47D+E.

5.4.5 Bioactivity

With only minimal amounts of **98** remaining, and with no anti-mycobacterial activity linked to this compound, it was decided to investigate the effects of this compound in mammalian cell line assays. Comparison of IC_{50} data from the HL-60 and HEK cell line assays indicated an interesting result. HL-60 cells were chosen to represent cancer cells, while HEK cells were chosen to exhibit the general mammalian cytotoxic effects of the compound. The effectiveness of compounds can be judged by their therapeutic index

(TI), which is the general cytotoxicity divided by the desired activity. Therefore, the IC_{50} value in HEK cells (61.1 μ M) divided by the IC_{50} in HL-60 cells (9.8 μ M) would identify a rough therapeutic index of 6 for rimarikiamide A (Figure 5.2). This is indicative of specificity for *in vitro* anti-cancer activity compared to generic toxicity. TI is an indicator of the potential of drug candidates [272]. High TI's are generally ideal at the early stages of drug development to avoid progression of candidates that may fall out further downstream of the development process [272]. As a compound may have an ideal TI value *in vitro*, *in vivo* higher levels of the compound may be required, which could lead to undesired side effects, thus over interpretation of the TI value should be cautioned [272]. Compounds can be modified to allow for improvements in the TI. However the lack of sufficient compound meant that any further work such as mechanism of action studies could not be performed.

5.4.6 Future work

To carry out further studies of the bioactivity of rimarikiamide A, more compound will be required, and identifying an efficient route for synthesis will aid in this avenue of research. However, before any future work can be done, the relative and absolute configuration of the molecule must be elucidated. Pursuing the synthetic route to generate two of the possible four stereoisomers and comparing optical rotation data and NMR spectra of the synthesized compound to that of the natural product will confirm the planar structure and will establish the configuration of the natural product. These studies are currently underway in our laboratory.

Chapter 6 3-*Epi*-xestoaminol C
from *Xiphophora chondrophylla*.

6.1 Background

Screening the VUW_MNP extract library also identified a brown alga *Xiphophora chondrophylla* as a hit, which displayed inhibitory activity against all three species of bacteria that were screened (Figure 3.3). Since the crude extract displayed similar levels of activity against *M. tuberculosis* H37Ra (MIC₉₀ ≥200 µg/mL) as *M. smegmatis* (MIC₉₀ ≥200 µg/mL) (Table 3.3), the fractionation process was guided by assays against *M. smegmatis*. As stated earlier, the strain of *M. smegmatis* used allows for faster assay times and the kanamycin resistance marker allows for antibiotic use to overcome contamination issues. Once the bioactive compound was isolated, the goal would be to validate it against *M. tuberculosis* H37Ra and, should it prove potent enough, its activity against clinical isolates would also be analysed. The compound's cytotoxic activity against mammalian cell lines would also be studied to evaluate its potential as an anti-tubercular drug. This work led to the isolation of a new 1-deoxysphingoid, a class of molecules also known as 2, 3 amino alcohols. This chapter discusses the isolation and structure elucidation of the new amino alcohol as well as early mechanism of action studies. In addition, an overview of marine derived amino alcohols as well as known biological activities linked to these compounds is also described.

6.1.1 Marine derived amino alcohols

Amino alcohols or 1-deoxyshpingoids are a class of compounds that contain hydroxyl and amino functional groups on hydrocarbon chains of a lipid. Their isolation has predominantly been associated with tunicates [273-282]. The range of activities these compounds have displayed include anti-cancer, antimicrobial and anti-fungal activities [273-282]. The first reported isolation of an aliphatic amino alcohol was from a *Xestospongia* sp. sponge [283] followed by the isolation of xestoaminols from another *Xestospongia* [284]. Of all the reports to date, the isolation of aliphatic amino alcohols from marine algae is unprecedented.

6.1.1.1 Alkyl chains with varying levels of unsaturation

This section describes amino alcohols with varying alkyl chain lengths and unsaturation levels, which represent the most common class of amino alcohols. The first reported isolation of aliphatic amino alcohols was in 1988, with 2-(*S*)-aminotetradecan-5,7-dien-3-(*S*)-ol (**105**) and 2-(*S*)-aminotetradecan-5,7-dien-3-(*R*)-ol (**106**) from a Papua-New Guinea *Xestospongia* sp. sponge. These compounds possessed inhibitory activity against *Candida albicans* at 19 µg/mL in a disk diffusion assay [283]. Similarly another group of aliphatic amino alcohols, xestoaminols A-C (**107-109**) were isolated from a Fijian *Xestospongia* sp.. Compound **107** displayed activity against the bacteria *S. aureus*, *S. pyogens* and *E. coli*, and the fungi *C. albicans* and *Trichophyton mentagrophytes* in disk diffusion assays at 100 µg/mL. In addition, both **107** and **109** displayed extremely weak inhibitory activity in a reverse transcriptase assay at 1 mg/mL [284].

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(2*S*, 3*R*)-2-Aminododecan-3-ol (**110**) was reported from the ascidian *Clavelina oblonga* and displayed anti-fungal activity against *C. albicans* with an MIC of 0.7 µg/mL [273]. Crucigasterins 277, 275 and 225 (**111-113**) were first isolated in 1993 from a Mediterranean tunicate *Pseudodistoma crucigater* with moderate activity reported against L1210, *B. subtilis* and *S. cerevisiae* [277].

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Crucigasterins A-E (**114-118**) were also isolated from *P. crucigater*, with **115** displaying the best activity of 50 µg/mL against *E. coli* and *C. albicans*, while **118** was active at 100 µg/mL against *E. coli* [276].

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Closely related to the crucigasterins are the haloaminols A-C (**119-121**), isolated from a *Haliclona* sponge from the Great Barrier Reef, which have been reported to inhibit the growth of *E. coli*, *B. subtilis*, *C. albicans*, *T. mentagrophytes* and *C. resinae* [285]. The researchers also identified haloaminol D (**122**) isolated as the *N*-acetyl derivative.

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Obscuraminols A-F (**123-128**), also closely related to the crucigasterins and haloaminols, were isolated from the ascidian *Pseudodistoma obscurum*. These compounds were reported to be active against P388, A-549 and HT-29 cell lines with IC_{50} values of 0.5, 0.2 and 0.5 $\mu\text{g/mL}$ respectively [278]. Haloaminol D and Obscuraminol A are in fact the same compound, however **122** was initially isolated as the *N*-acetyl derivative only four months prior to the isolation of **123**.

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Clavaminols A-F (**129-134**) were isolated from the ascidian *Clavelina phlegrae*. The most potent of these aliphatic amino alcohols, **129**, had an IC₅₀ value of 5 µg/mL against a gastric tumour cell line (AGC cells), lung cancer cell line (A-549) and a breast cancer cell line (T47D). In addition, **129** was shown to inhibit cell growth by inducing apoptosis in the G1-S phase of the cell cycle in Jurkat cells [275].

The final example in this class of amino alcohols, spisulosine or ES-285 (**135**), has drawn much interest. Initially isolated from the marine mollusc *Spisula polynyma*, this compound has drawn major interest due to its activity against solid tumours such as colorectal, renal and prostate tumors, amongst others, for which it progressed to phase 1 clinical trials [286-289].

135

The first trial indicated doses ranging from 4-128 mg/m² were manageable and showed no dose limiting toxicities. However, at 256 mg/m² one of the four patients receiving treatment displayed neurotoxicity, a side effect responsible for the fatality of one of the

three patients treated at 200 mg/m². Overall this study reported that at 128 mg/m² patients achieved desired pharmacokinetic and pharmacogenetic changes [289].

In all the clinical trials, only moderate anti-tumor activity was observed, in addition to side effects which included elevated hepatic and neural toxicities, anaemia, lymphopenia, asthenia and nausea. This led to the discontinuation of the clinical development of this compound [286-288].

The mode of action for **135** has not been fully elucidated, although the research to date proposes that the compound may work through multiple pathways. The induction of cell death *in vitro* has been shown to occur as a result of disruption of actin cell fibres; this destabilization of the cytoskeleton has been shown to not affect the microtubules of cells [290]. The activation of caspase 3 and 12, the phosphorylation of p53 and the activation of isoforms of protein kinase C (PKC) have all been shown to induce atypical cell death [291]. Further work in a prostate cancer cell line PC-3 focusing on the effects of spissulosine on PKC indicated the activation of the isoform PKC ζ [292], increasing ceramide levels, which has been linked to induction of apoptosis [293-295]. The most recent research has focused on how ES-285 affects ceramide levels in MDA MB 468 breast cancer cells. Treating cells with the compound, as well as analogues, caused an increase in *N*-C18 1-deoxyceramide levels, indicating ES-285 as the preferred substrate for acylation [296], a quality that identifies this compound as a probe for ceramide synthase activity.

6.1.1.2 Cyclised amino alcohols

This section describes the limited number of amino alcohols that contain cyclic structures. The pseudodistomins A (**136**) and B (**137**), isolated from the tunicate *Pseudodistoma kanako* in 1986, represent the first amino alcohols to be reported. These cyclic piperidine alcohols inhibited the growth of mouse leukaemia cell lines L1210 with IC₅₀ values of 2.5 and 0.4 μ g/mL, and L5178Y with values of 2.4 and 0.7 μ g/mL, respectively [280, 297]. Pseudodistomin C (**138**) was later isolated from *P. kanako*, with reported activity against the L1210 cell line and a human epidermoid carcinoma (KB cells) with IC₅₀ values of 2.6 and 2.3 μ g/mL, respectively. Shortly after, pseudodistomins D-F (**139-141**) were reported from the ascidian *P. malgrava* [281]. In addition to the re-isolation of **137** and **138**, the authors reported the activity of **137-141**

against *S. cerevisiae* at a range of concentrations between 110-200 $\mu\text{g/mL}$ in a zone of inhibition assay [281].

Containing a fused bicyclic ring as part of the linear chain, amaminols A (**142**) and B (**143**) were reported from a tunicate belonging to the family Polyclinidae, with IC_{50} values of 2.1 $\mu\text{g/mL}$ against P388 murine leukaemia cells [274].

6.1.1.3 α , ω -Double headed amino alcohols

This section covers compounds with amino alcohol polar head groups at both ends of the chain. Leucettamols A (**144**) and B (**145**) were isolated from the sponge *Leucetta microraphis*, with moderate activity reported against *B. subtilis* [298]. Leucettamol A was recently reported to inhibit the interaction of Ubc13 with Uev1A,

with an IC_{50} of 50 $\mu\text{g/mL}$. This inhibition is believed to increase p53 levels and therefore makes an ideal target for anti-cancer agents, [299].

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BRS1 (**146**), isolated from an Australian calcareous sponge, is very closely related to the leucettamols, with reported inhibition of PKC activity with an EC_{50} of 98 μM [300]. The final example for compounds belonging to this group is rhizocalinin B (**147**), isolated from a North-Western Australian sponge *Oceanapia* sp. with no reported bioactivity [301].

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In addition to having the bis-amino alcohol head groups, the compounds listed below also contain carbohydrates as part of their structure. Rhizochalin (**148**) was isolated from a Madagascan sponge *Rhizochalina incrustata* with reported inhibitory activity against *S. aureus* [302]. Rhizochalinin A (**149**), also isolated from *R. incrustata*, is a sphingolipid aglycone that has anti-leukemic activity against human monocytic THP-1 cells, with a reported IC_{50} of 7.5 μM [303]. Rhizochalin B (**150**) was isolated from a

North-Western Australian sponge *Oceanapia* sp. [301], while rhizochalins C (**151**) and D (**152**) were isolated from *R. incrustata* [304]. There has been no reported activity for rhizochalins B-D.

148

149

Isorhizochalin (**153**) is an epimer of rhizochalin also isolated from *R. incrustata*, although no bioactivity has been linked to this compound [305].

153

Oceanapiside (**154**), another sphingolipid, was isolated from the South Australian sponge *Oceanapia phillipensis*. This compound varies from the rhizochalins near the terminus that contains the glucose, with true sphingosine head-group unlike that found in the rhizochalins. Oceanapiside has a reported MIC of 10 $\mu\text{g/mL}$ against *Candida glabrata* [306].

154

Calyxoside (**155**) isolated from a *Calyx* sponge is a sphingolipid that was reported to have inhibitory activity in rad 52 and 51 knock outs of *S. cerevisiae* strains at 62 and 36 $\mu\text{g/mL}$, respectively, in a zone of inhibition assay. In addition, it has reported activities against the HFF, MRC-5, SW480, HT-29, Saos-2, DLD-1 and H460 cell lines with IC_{50} values between 3-20 $\mu\text{g/mL}$ [307]. It also contains a formal sphingosine head.

155

6.2 Methods

6.2.1 Extraction of algae

Xiphophora chondrophylla (300 g) was extracted twice with methanol (MeOH, 400 mL) that was cyclic loaded onto a 120 mL HP20 column, following which the column was eluted with 360 mL mixtures of 20%, 40%, 60%, 80% and 100% acetone in water. Except for the 100% acetone, all other fractions were back loaded onto 60 mL columns and eluted with 180 mL of MeOH followed by 180 mL of acetone, fractions were dried down *in vacuo* and transferred to pre-weighed vials. Based on bioactivity, the 40% acetone fraction was further pursued, half of the mass (350 mg) was fractionated using a 40 mL HP20ss column, eluting with 120 mL of 30%, 40%, 50%, 60%, 80% and 100% mixtures of MeOH in H₂O; the 30%-60% fractions were collected in test tubes. Guided by bioassays, test tubes 43-46 (50% MeOH fractions, 17.8 mg) were fractionated a further three times using LH20 size exclusion chromatography using 90% MeOH/H₂O. The final fractionation was performed using a 1 mL DIOL column and eluted with 3 mL volumes of 0%, 25%, 50%, 75% and 100% mixtures of ethyl acetate (EtOAc) in hexanes followed by 25%, 50%, 75% and 100% MeOH in EtOAc, finally the column was stripped with 50% and 75% H₂O in MeOH to give 3-*epi*-xestoaminol C (**156**) (0.9 mg).

Based on the results of the initial isolation, an optimised method was used on the second half of the 40% acetone fraction. The remaining 40% HP20 fraction (350 mg) was separated on a 30 mL DIOL column, eluting with 90 mL volumes of 0%, 25%, 50%, 75% and 100% mixtures of EtOAc in hexanes followed by 25%, 50%, 75% and 100% MeOH in EtOAc, finally the column was stripped with 50% H₂O in MeOH. The 0%, 25% and 50% EtOAc in hexanes and 25%, 50%, 75% and MeOH in EtOAc elutions were collected in test tubes. Test tubes 47-50 (25% MeOH/ EtOAc) were pooled together (248.1 mg) and separated further on a 25 mL DIOL, eluting with 75 mL volumes of the same solvent mixtures for the earlier column. ¹H NMR identified test tubes 6-13 to contain 3-*epi*-xestoaminol C, the combined mass (185 mg) was dissolved in dichloromethane and partitioned against 1 M HCl, the organic layer was collected and dried, the acidic aqueous layer was then neutralised with 1 M NaOH and this was partitioned against dichloromethane again. Following the HCl and NaOH washes, a

total of 117 mg was isolated, which was divided into thirds and fractionated on three DIOL columns using the same eluting conditions for all columns, 0%, 50% and 100% mixtures of EtOAc in hexanes (collected in bulk) followed by 25%, 50% and 100% MeOH in EtOAc (collected in test tubes), finally the column was stripped with 50% H₂O in MeOH. This afforded a total of 58 mg of 3-*epi*-xestoaminol C (**156**) (test tubes 5-7).

3-*Epi*-xestoaminol C: $[\alpha]_D^{25}$ -6.19 (*c* 0.42, CHCl₃); IR $\nu_{\max}$ 3367, 2954, 2871, 1602 cm⁻¹; CD₃OD NMR data see Table 6.2; ¹H NMR (CDCl₃, 600 MHz) δ 3.65 (1H, t, *J* = 8.5 Hz, H-3), 3.32 (1H, m, H-2), 1.56 (1H, m, H-4b), 1.53 (1H, m, H5-b), 1.40 (1H, m, H-4a), 1.38 (3H, d, *J* = 6.6 Hz, H-1), 1.35 (1H, m, H-5a), 1.27 (2H, m, H-13), 1.25 (14H, m, H-6 to H-12), 0.88 (3H, t, *J* = 7.0 Hz, H-14); ¹³C NMR (CDCl₃, 150 MHz) δ 73.0 (C-3), 53.3 (C-2), 33.6 (C-4), 32.1 (C-12), 29.88-29.52 (C-6 to C-11), 25.5 (C-5), 22.9 (C-13), 15.8 (C-1), 14.3 (C-14) ; HRESIMS *m/z* 230.2467 [M+H]⁺ (calcd for C₁₄H₃₂NO, 230.2478).

156

6.2.2 Isolation schemes for 3-*epi*-xestoaminol C (156)

Scheme 6.1 Original isolation scheme for 3-*epi*-xestoaminol C (156) from *Xiphophora chondrophylla*.

Scheme 6.2 Optimised isolation scheme for 3-*epi*-xestoaminol C (156) from *Xiphophora chondrophylla*.

6.2.3 Derivatisation reactions

6.2.3.1 Oxazolidinone derivative

3-*Epi*-xestoaminol C (**156**) (3 mg) was stirred with 1,1' carbonyldiimidazole (CDI, 3 mg, 1.4 equiv.) for 24 h in DCM. Monitoring via TLC (running conditions 1:1 MeOH:EtOAc) showed the reaction had not gone to completion, therefore further CDI (7 mg, 3.3 equiv.) was added to the reaction, after 2 h the reaction had gone to completion. The reaction mixture was partitioned against H₂O three times and the organic layer was dried, yielding 4.9 mg. This sample was purified using a 1 mL DIOL column eluting the column with 3 mL volumes of 50% hexanes in ethyl acetate which afforded oxazolidinone ND5_83A (**157**, 2.8 mg, 93% yield).

157

Oxazolidinone (**157**): $[\alpha]_D^{25}$ -43.3 (*c* 0.18, CHCl₃); IR $\nu_{\max}$ 3285, 2922, 2853, 1747, 1541 cm⁻¹; ¹H NMR (CDCl₃, 600 MHz) δ 5.18 (1H, s, NH), 4.09 (1H, ddd, *J* = 7.9, 6.4, 4.8 Hz, H-3), 3.57 (1H, quin, *J* = 6.2 Hz, H-2), 1.71 (1H, m, H-4b), 1.62 (1H, m, H-4a), 1.48 (1H, m, H-5b), 1.37 (1H, m, H-5a), 1.28 (2H, m, H-13), 1.27 (3H, d, *J* = 6.2 Hz, H-1), 1.26 (12H, m, H-6 to H-11), 1.25 (2H, m, H-12), 0.88 (3H, t, *J* = 7.0 Hz, H-14); ¹³C NMR (CDCl₃, 150 MHz) δ 158.97 (C-15), 84.4 (C-3), 53.7 (C-2), 34.3 (C-4), 32.1 (C-12), 29.76-29.46 (C-6 to C-11), 25.0 (C-5), 22.84 (C-13), 20.82 (C-1), 14.3 (C-14); HRESIMS *m/z* 256.2264 [M+H]⁺ (calcd for C₁₅H₃₀NO₂, 256.2271)

6.2.3.2 Acetylation

3-*Epi*-xestoaminol C (10 mg) was reacted with a 1:1 mixture of pyridine and acetic anhydride (3 mL) for 3 h at room temp and monitored via TLC. The reaction evaporated *in vacuo* and dissolved in DCM (20 mL) following which the organic layer was partitioned against 1 M HCl, 1 M NaOH and H₂O, yielding 7 mg of diacetyl-3-*epi*-xestoaminol C (**158**, 70% yield).

158

Diacetyl-3-*epi*-xestoaminol C (**158**): $[\alpha]_D^{25}$ -23.0 (*c* 0.1, CHCl₃); IR $\nu_{\max}$ 3297, 2923, 2854, 1740, 1652, 1460, 1373 cm⁻¹; ¹H NMR (CDCl₃, 600 MHz) δ 5.53 (1H, d, *J* = 9.3 Hz, NH), 4.85 (1H, ddd, *J* = 7.6, 5.9, 4.1 Hz, H-3), 4.20 (1H, dqd, *J* = 9.3, 6.8, 4.1 Hz, H-2), 2.09 (3H, s, OAc, H-18), 1.99 (3H, s, NAc, H-16), 1.52 (2H, m, H-4), 1.28 (2H, m, H-13), 1.27 (2H, m, H-5) 1.25 (2H, m, H-12) 1.24 (12H, m, H-6 to H-11), 1.10 (3H, d, *J* = 6.8 Hz, H-1), 0.87 (3H, t, *J* = 7.0 Hz, H-14); ¹³C NMR (CDCl₃, 150 MHz) δ 171.1 (C-17), 169.6 (C-15), 76.6 (C-3), 47.4 (C-2), 32.1 (C-12), 31.7 (C-4), 29.78-29.5 (C-6 to C-11), 25.4 (C-5), 23.6 (C-16), 22.8 (C-13), 21.2 (C-18), 18.7 (C-1), 14.3 (C-14); HRESIMS *m/z* 314.2693 [M+H]⁺ (calcd for C₁₈H₃₆NO₃, 314.2690)

6.2.3.3 Monodeacetylation

Diacetyl-3-*epi*-xestoaminol C (**158**, 7 mg) was reacted with 5 mg of KCO₃ in MeOH (3 mL) at 0 °C for 30 min followed by room temperature for 16 h. The reaction was monitored via TLC (10% MeOH 90% DCM), following which the reaction mixture was evaporated and re-dissolved in EtOAc (15 mL). This was partitioned against H₂O three times to yield 4.7 mg of *N*-acetyl-3-*epi*-xestoaminol C (**159**, 67% yield).

159

N-Acetyl-3-*epi*-xestoaminol C (**159**): $[\alpha]_D^{25}$ -6.3 (*c* 0.16, CHCl₃); IR $\nu_{\max}$ 3477, 2924, 2854, 1652 cm⁻¹; ¹H (CDCl₃, 600 MHz) δ 5.68 (1H, d, *J* = 8.54 Hz, NH), 3.97 (1H, dqd, *J* = 8.6, 6.8, 3.2 Hz, H-2), 3.53 (1H, q, *J* = 4.4, 3.7 Hz, H-3), 2.00 (3H, s, NAc, H-16), 1.44 (1H, m, H-4), 1.41 (1H, m, H-5b), 1.34 (1H, m, H-5a), 1.29 (2H, m, H-13), 1.25

(14H, m, H-6 to H-12), 1.19 (3H, d, $J = 6.5$ Hz, H-1), 0.88 (3H, t, $J = 7$ Hz, H-14); ^{13}C (CDCl_3 , 150 MHz) δ 170.3 (C-15), 75.0 (C-3), 49.1 (C-2), 34.6 (C-4), 32.1 (C-12), 29.8-29.5 (C-6 to C-11), 25.8 (C-5), 23.7 (C-16), 22.9 (C-13), 18.5 (C-1), 14.3 (C-12) HRESIMS m/z 272.2581 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{16}\text{H}_{34}\text{NO}_2$, 272.2584)

6.2.3.4 R and S- Mosher's esters

Two aliquots of *N*-acetyl-3-*epi*-xestoaminol C (**159**) (1.5 mg each) were treated with EDCI (7.3 mg, 8.6 equiv) and DMAP (1.41 mg, 2.1 equiv) dissolved in dry DCM under argon. To each flask 9.02 mg aliquots of either (*R*)-(+)- α -methoxy- α -trifluoromethylphenylacetic acid or (*S*)-(-)- α -methoxy- α -trifluoromethylphenylacetic acid (MTPA) (7 equivalents) were added. Reactions were monitored via TLC over 22 h (10% MeOH, 90% DCM). Upon completion, reaction mixtures were washed with 0.01M HCl followed by 0.01 M NaOH and finally H_2O before performing HPLC purification of the *R* (**160**) and *S* esters (**161**) using a C18 analytical column (Phenomenex Prodigy, 4.6 x 250 mm, 5 μm particle size) at a flow rate of 1 mL/min (5 min at 30% MeCN, 15 min ramp to 100% MeCN, 5 min at 100% MeCN, 2 min ramp down to 30% MeCN, 5min hold at 30% MeCN) to give *R* (**160**, 0.6 mg) and *S* (**161**, 1.2 mg) esters.

160

R-ester (**160**): $[\alpha]_{\text{D}}^{25}$ 0 (c 0.04, CHCl_3); IR ν_{max} 3378, 2924, 2854, 1746, 1651 cm^{-1} ; ^1H NMR (CD_3OD , 600 MHz) δ 7.55 (2H, m, H-24), 7.45 (2H, m, H-22), 7.44 (1H, m, H-23), 5.5 (1H, s, NH), 5.11 (1H, ddd, $J = 7.6, 5.6, 4.6$ Hz, H-3), 4.17 (1H, qd, $J = 6.9, 5.5$ Hz, H-2), 3.54 (3H, s, OMe, H-19), 1.83 (3H, s, NAc, H-16), 1.64 (2H, m, H-4), 1.34 (3H, m, H-5b, H-6), 1.31 (2H, m, H-5a, H-13b), 1.29 (1H, m, H-13a), 1.28 (12H, m, H-

7 to H-12), 0.99 (3H, J = d, 6.9 Hz), 0.90 (3H, t, J = 7 Hz, H-14); ^{13}C NMR (CD_3OD , 150 MHz) δ 172.7 (C-15), 167.5 (C-17), 133.4 (C-18), 130.9 (C-23/25), 129.5 (C-24), 128.7 (C-22/26), 125.8 (C-21), 86.1 (C-20), 79.7 (C-3), 56.1 (C-19), 47.7 (C-2), 33.1 (C-12), 31.5 (C-4), 30.75-30.4, 26.1 (C-6 to C-11), 30.48 (C-5), 23.8 (C-13), 22.6 (C-16), 16.8 (C-1), 14.5 (C-14); HRESIMS m/z 488.2979 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{26}\text{H}_{41}\text{F}_3\text{NO}_4$, 488.2982).

161

S-ester (**161**): $[\alpha]_D^{25}$ -17.5 (c 0.13, CHCl_3); IR ν_{max} 3335, 2925, 2854, 1746, 1651 cm^{-1} ; ^1H NMR (CD_3OD , 600 MHz) δ 7.55 (2H, m, H-24), 7.44 (3H, m, H-22, H-23), 5.5 (1H, s, NH), 5.11 (1H, ddd, J = 8.0, 6.4, 4.0 Hz, H-3), 4.2 (1H, quin, J = 6.8 Hz, H-2), 3.55 (3H, s, OMe H-19), 1.9 (3H, s, NAc, H-16), 1.59 (1H, m, H-4b), 1.54 (1H, m, H-4a), 1.32 (1H, m, H-13b), 1.29 (1H, m, H-13a), 1.28 (2H, m, H-12), 1.27-1.19 (12H, m, H-6 to H-11), 1.15 (2H, m, H-5), 1.13 (3H, d, J = 6.9 Hz, H-1), 0.90 (3H, t, J = 6.9 Hz, H-14); ^{13}C NMR (CD_3OD , 150 MHz) δ 172.7 (C-15), 167.5 (C-17), 133.5 (C-18), 130.9 (C-23/25), 129.5 (C-24), 128.5 (C-22/26), 125.8 (C-21), 85.9 (C-20), 79.5 (C-3), 56.2 (C-19), 48.1 (C-2), 33.1 (C-12), 31.3 (C-4), 30.74-30.3 (C-6 to C-11), 25.7 (C-5), 23.8 (C-13), 22.7 (C-16), 17.0 (C-1), 14.5 (C-14); HRESIMS m/z 488.2979 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{26}\text{H}_{41}\text{F}_3\text{NO}_4$, 488.2982)

6.2.4 Sensitive mutant screen

A random mutant library with 32 x 384-well plates comprising of 7680 Tn611 *M. smegmatis* transposon mutants was generated by Richard Campen (VUW). These mutants were pinned into corresponding assay plates containing 100 μL LB Kan₂₀

(section 2.1.1.2) and incubated for 72 h, these plates were then sub-inoculated into corresponding LB Kan₂₀ plates for a further 24 h, following which the 24 hour cultures were pinned into plates with LB Kan₂₀ and 2 µg/mL of 3-*epi*-xestoaminol C (**156**). These plates were incubated for 48 h after which 20 µL of 0.03% resazurin (w/v) was added to plates and measured for fluorescence after 2 h or colour change was visible (from blue to purple/pink).

Hits were mutants that identified growth sensitivity, % growth was measured for each well. Using Microsoft Excel the wells with the most sensitivity to growth were ranked to identify the top 120 mutants. These mutants were split into half and were picked from library wells and grown in 100 µL volumes in two 96-well plates. After culturing plates for 72 h, 70 µL of 80% glycerol was added to these plates to generate freezer stocks of these cultures to a final concentration of 33% glycerol. For screening, the glycerol plates were pinned into 100 µL of LB Kan₂₀ to grow for 48 h, these cultures were sub-inoculated into fresh 100 µL LB Kan₂₀ plates and grown for a further 24 h. These cultures were pinned into plates containing LB Kan₂₀ and 2 µg/mL of 3-*epi*-xestoaminol C (**156**) and incubated for 48 h after which OD measurements as well resazurin measurements were taken. From this screening out of the top 60 hits, 17 hits were validated in dose response assays and from the second 60 hits, 14 hits were validate in dose response assays.

6.2.5 Resistant mutant generation and screen protocol

A pool of random Tn611 mutants was generated using a modification of the methods described by Gilhot and Perez [129, 308]. In brief, these mutants were generated as follows: a pcG70 vector carrying the Tn611 transposon was electroporated into *M. smegmatis* mc²155 following which cells were plated onto LB Kan₂₀ plates at 30 °C. Colonies were picked and grown in LB Kan₂₀ at 30 °C for 48 h at 200 RPM. This allows for the replication and generation of a large pool of transposon mutants. These cultures were transferred to a 41 °C incubator for 24 h, this ceases transposition events. Glycerol was then added to cultures to achieve a final concentration of 30% in order to generate freezer stocks of mutants. For the control strain the plasmid pRC10 was electroporated into *M. smegmatis* mc²155 and glycerol stocks were made. Following

electroporation, colony forming units (CFUs) were counted in order to have an accurate gauge of the number of cells plated out in a 100 μ L aliquot of cells.

Agar plates were made up to either 10, 7.5 or 3.75 μ g/mL of 3-*epi*-xestoaminol C (**156**), following which 100 μ L of transposon mutants were plated out. This was performed in duplicate. Control strains of pRC10 were also plated out.

6.2.6 G2-M block assay

In brief, using 24-well plates 1A9 cells were diluted to a final cell concentration of 1×10^5 in a final volume of 500 μ L. The drug concentrations used were IC₉₉, IC₅₀ and IC₁₀ to allow for analysis at concentrations at which complete death, 50% cell death and a concentration at which no major inhibition occurs. Caution was taken not to exceed the final DMSO concentration of 0.5% per well to avoid inhibition of cell growth due to solvent effects. Cells were incubated with drug over 16 h at 37 °C 5% CO₂ before labelling cells with propidium iodide and performing fluorescence activated cell sorting (FACS) analysis.

6.2.7 *S. cerevisiae* bioassay methods

6.2.7.1 Yeast strains, media and chemicals.

The *Saccharomyces cerevisiae* deletion library (Open Biosystem) was generated from strain BY4741 (MATa haploid: *his3 Δ 1 leu2 Δ met15 Δ ura3 Δ 0*). The yeast deletion library was stored in -80 °C and grown in yeast extract (10 g/L)-peptone (20 g/L)-dextrose (20 g/L) (YPD) agar. 3-*Epi*-xestoaminol C (**156**), was dissolved in DMSO to give a 500 μ g/mL working stock before use.

6.2.7.2 Screening for 3-*epi*-xestoaminol C sensitivity and resistance in liquid medium.

Replications and inoculation of yeast cells were run using the Singer RoToR HDA[®]. Yeast knockout mutants were tested in 96-well plates containing Synthetic Complete (SC) (1.7g/L yeast nitrogen base, 1 g/L monosodium glutamate, 2 g/L amino acid mixture) broth media in each well. Yeast were incubated for 20 h at 30 °C and then inoculated into 96-well plates containing 200 μ L SC with 0.8 μ g/mL final concentration

of 3-*epi*-xestoaminol C (**156**), and the appropriate volume of DMSO (solvent control). Plates were sealed with gas permeable seals and incubated for 16 h at 30 °C. Optical density (OD) was measured at 590 nm (OD₅₉₀) using Perkin Elmer EnVision 2102 Multilabel Reader, the 16th hour OD₅₉₀ was normalised to the OD₅₉₀ at 0th hour. The screen was done in triplicate, with the % residual growth of drug treated cells measured in comparison to DMSO treated cells using Microsoft Excel.

Hits were selected using a *t* test with a Bonferroni correction adjusting the *p* value to 0.017. Using this selection criterion, sensitive strains that displayed less than 70% residual growth and resistant strains that had greater than 130% growth in comparison to DMSO controls were chosen for validation. Using the same selection criterion, these hits were validated again in triplicate to identify the hits for generating a network map.

6.3 Results

6.3.1 Bioassay data

Using the methodology described in section 2.6, the results of the dose-response assays for 3-*epi*-xestoaminol C (**156**) are listed below. Graphs were generated using GraphPad Prism v.5.0. Resazurin dye was used to measure bacterial metabolism after each assay was run, and fluorescence measurements (RFU) were used as a measure of growth. OD was used as measure of growth for the HL-60 and HEK MTT assays. OD measurements were also used for all *S. cerevisiae* assays. Where possible IC₅₀ values have been listed, and as stated earlier as sigmoidal curves were not achievable for all data, MIC₉₀ values have been listed as well.

6.3.1.1 Antimicrobial assays

Table 6.1 3-*Epi*-xestoaminol C SAR work

Compound	MIC ₉₀ (µg/mL) in <i>M. tuberculosis</i>
	H37Ra
3- <i>epi</i> -xestoaminol C (156)	15
Oxazolidinone (157)	≥ 60
Diacetyl-3- <i>epi</i> -xestoaminol C (158)	IA
<i>N</i> -acetyl-3- <i>epi</i> -xestoaminol C (159)	IA
<i>R</i> -Mosher's ester (160)	IA
<i>S</i> -Mosher's ester (161)	IA

Bioactivity of derivatives of 3-*epi*-xestoaminol C against *M. tuberculosis* H37Ra using growth inhibition assays. IA: inactive at the highest concentration tested (60 µg/mL).

Figure 6.1 Antimicrobial activity of 3-epi-xestoaminol C (156).

Antimicrobial activity of 3-epi-xestoaminol C (156) against a range of microorganisms, using growth inhibition assays, compound has been diluted two fold before addition of cultures. Assay durations ranged from 16 h for *E. coli*, *S. aureus* and *S. cerevisiae* to 7 days for *M. tuberculosis* H37Ra. Percentage growth signal inhibition on the Y-axis is relative to controls measured. NA: not achievable. (n=1, triplicate)

3-*epi*-xestoaminol C vs. stationary phase *M. tuberculosis* H37Ra

Figure 6.2 Stationary phase assay

Inhibitory effects of 3-*epi*-xestoaminol C (**156**) against stationary phase cultures of *M. tuberculosis* H37Ra. Compound was serially diluted two fold in PBS before 30 day old cultures washed and re-suspended in PBS were added to plates. (n=1, triplicate)

6.3.1.2 Mammalian cell line assays

A

B

Figure 6.3 3-*epi*-xestoaminol C (156**) vs. mammalian cell lines**

The bioactivity of 3-*epi*-xestoaminol C (**156**) in HL-60 (**A**) and HEK cell lines (**B**). The HEK cell line has been used as a representative of general cytotoxicity. OD was used as a measure of growth. (n=1, duplicate)

Figure 6.4 Network map of hits from yeast chemical genetics screening of 3-epi-xestoaminol C (156).

6.4 Discussion

6.4.1 Fractionation and isolation

PTN2_93D, a brown algae identified as *Xiphophora chondrophylla*, was observed as being active against all three species of bacteria screened using the VUW MNP library. As the crude extract displayed similar levels of activity against *M. tuberculosis* H37Ra and *M. smegmatis*, *M. smegmatis* was used for the subsequent bioassays. As the 40% acetone fraction from the initial HP20 fractionation had the highest mass and displayed the best MIC₉₀ of 50 µg/mL, it was taken to the next stage of purification. Half of the mass of this fraction was separated using HP20ss and based on the activity profiles of test tubes 43-46 (15.6 µg/mL, 17.8 mg) and 47-54 (15.6 µg/mL, 78.2 mg), they were further pursued. Attempts to purify the 78.2 mg fraction using reversed phase (C18) HPLC did not achieve any separation. Guided by bioassays, the 17.8 mg fraction was separated a further three times using LH20 size exclusion chromatography. Ultimately using a DIOL column for the last purification led to the identification of an amino alcohol 3-*epi*-xestoaminol C (**156**) as the compound responsible for bioactivity.

In order to isolate more of the active compound to elucidate its relative and absolute configurations, a more refined isolation scheme was employed to separate the second half of the 40% acetone fraction (Scheme 6.2). Testing the purified compound using the optimised method identified a consistent MIC₉₀ of 15 µg/mL in all microbes tested (Figure 6.1).

6.4.2 Structural elucidation

HRESIMS analysis indicated an [M+H]⁺ adduct ion at *m/z* 230.2467, suitable for the formula C₁₄H₃₂NO (calcd for 230.2478), and having no double bond equivalents. The ¹H, ¹³C and multiplicity-edited HSQC NMR spectra in CD₃OD (Table 6.2) revealed 32 protons attached to 14 carbons, including two methyls (δ_C 16.1 and 14.5), ten methylenes (δ_C 34.7, 33.1, 30.8-30.5, 26.4, 23.8) an oxymethine (δ_C 73.2) and an aminomethine (δ_C 53.5).

Figure 6.8 TOCSY correlations

From a literature search using MarinLit [210] and SciFinder[®], the carbon connectivity was the same as the known amino alcohol xestoaminol C (**109**), first reported by Jiménez and Crews from a *Xestospongia* sp. sponge [284]. However, further analysis of the ¹H NMR spectrum of **156** (CDCl₃) identified several significant differences to that reported by Crews for **109** (**156**: 1.38 ppm, H₃-1; and 3.65 ppm, H-3; **109**: 1.15 ppm, H₃-1; 3.95 ppm, H-3) [284]. One possible explanation for this shift in resonance could be a different configuration, therefore these findings warranted further investigation into the compound isolated.

To identify the relative stereochemistry of the amino alcohol, it was reacted with 1,1'-carbonyldiimidazole (CDI) to generate the oxazolidinone derivative (**157**). The coupling constant between H-2 and H-3 was approximately 6.5 Hz, diagnostic of a *trans* relationship between the protons of the five membered ring system [274]. In comparison a *cis* relationship would have a coupling constant of approximately 7.3 Hz.

The *trans* relationship of these protons can be accounted for if the amine and hydroxyl group were *syn* to each other in the acyclic system. To confirm the *trans* relationship in the oxazolidinone, a 1D-NOE experiment was performed, irradiating H-1, H-2, H-3, H-4a and H-4b. Irradiating H-3 enhanced H-1 while irradiation of H-4a and b led to the excitation of H-2, confirming the *trans* relationship in the cyclic system. This therefore indicated that **156** has a *syn* relationship between NH₂-2 and OH-3, which differed from xestoaminol C (**109**) that has a *trans* relationship.

Figure 6.9 NOE correlations of oxazolidinone 157.

With enough evidence to support a different relative configuration in **156** to **109**, Mosher's method was used to solve the absolute configuration, which was carried out in three stages. Amino alcohol **156** was first treated in a 1:1 mixture of pyridine and acetic anhydride to acetylate the amine and hydroxyl groups, generating the diacetyl derivative (**158**). Following acetylation, the hydroxyl group was deprotected using base hydrolysis with KCO_3 in MeOH to afford *N*-acetyl-3-*epi*-xestoaminol C (**159**), which was treated with *R* and *S*- MTPA acids to yield the *R* (**160**) and *S* (**161**) Mosher's esters.

158 $\text{R}_1 = \text{R}_2 = \text{Ac}$

159 $\text{R}_1 = \text{Ac}$ $\text{R}_2 = \text{H}$

160 $\text{R}_1 = \text{Ac}$ $\text{R}_2 = R\text{-MTPA}$

161 $\text{R}_1 = \text{Ac}$ $\text{R}_2 = S\text{-MTPA}$

When analysing the changes in ^1H chemical shifts of the Mosher's esters in CDCl_3 , an interesting result was observed (Figure 6.10). Under normal circumstances, the $\Delta\delta$ shifts flanking the ester would either be solely positive or negative depending on the positioning of the phenyl ring. However, a mixture of shifts was observed at the amino terminal end of the compound (Figure 6.11). Hydrogen bonding between the carbonyl group of the ester and the amide proton may have caused a conformational change from that required to adequately study each side, as evidenced by the different couplings observed for H-3, indicated by different multiplicities (Figure 6.10). This proposed intramolecular bonding could directly affect the shielding and deshielding effects of MTPA, obscuring interpretation of data.

Figure 6.10 Change in multiplicity of the oxymethine ^1H NMR resonance for *R*-ester (160, top panel) and *S*-ester (161, bottom panel) in CDCl_3 (600 MHz).

Figure 6.11 $\Delta\delta$ ^1H NMR data of MTPA esters 160 and 161 in CDCl_3 (600 MHz).

In order to disrupt the effects of intramolecular hydrogen bonding, NMR analysis was performed in the polar deuterated solvent CD₃OD. As expected, the oxymethine resonance no longer varied between the *R* and *S*-esters and both had roughly the same multiplicity (Figure 6.12). Furthermore, comparison of ¹H NMR data from the two esters indicated that the intramolecular bonding was no longer causing a misinterpretation of the data (Figure 6.15).

Figure 6.12 ¹H NMR oxymethine resonance for *R*-ester (160, top panel) and *S*-ester (161, bottom panel) in CD₃OD (600 MHz).

Analysing the multiplicities in CD₃OD (Figure 6.12), it can be observed that they match that of the *S*-ester in CDCl₃ (Figure 6.10), and therefore it can be deduced that the *R*-ester was the cause of the issues in interpretation due to intramolecular bonding.

Using Mosher's method, and performing the analysis in the appropriate deuterated solvent provided unequivocal evidence for the absolute stereochemistry of 3-*epi*-xestoaminol C. The $\Delta\delta$ values on either side of the ester were either solely positive or solely negative (Figure 6.13), and based on the $\Delta\delta$ shifts, it was possible to elucidate the absolute structure as 2-*S*-aminotetradeca-3-*S*-ol. Figures 6.14 and 6.15 illustrate the shifts observed in the ¹H NMR spectra of the *R* and *S*-esters.

160 R = *R*-MTPA

161 R = *S*-MTPA

Figure 6.13 $\Delta\delta$ ^1H shifts between MTPA esters 160 and 161 of *N*-acetylated xestoaminol C in CD_3OD .

Figure 6.14 Oxy- and aminomethine region, ^1H NMR spectra for the *R*-ester (160, top panel) and *S*-ester (161, bottom panel) in CD_3OD (600 MHz)

Figure 6.15 Aliphatic region, ^1H NMR spectra for the *R*-ester (160, top panel) and *S*-ester (161, bottom panel) in CD_3OD (600 MHz).

To further confirm these results, ^{19}F NMR analysis of the esters was performed as originally proposed by Mosher [309, 310]. In this method it was proposed that if the phenyl ring was on the same side as the most bulky substituent of the ester, then coplanarity is lost resulting in a shift of the CF_3 from the deshielding zone to the shielding zone of the carbonyl (Figure 6.16).

Figure 6.16 Steric effects influencing ^{19}F NMR shifts

In this figure the phenyl ring of *S*-MTPA ester is on the same side as the most bulky substituent. This results in the loss of coplanarity and the resulting shift in sterics moves CF_3 to the shielding zone of the carbonyl.

Based on the ^1H NMR shifts observed from the *R* and *S*- MTPA esters (Figure 6.13), it is expected that for the *R*-ester the phenyl ring is on the same side as the *N*-acyl group. If this holds true, the steric interaction of the phenyl ring and the bulky *N*-acyl group would cause the CF_3 to resonate further up-field [311]. Consequently, the resulting shift in the *S*-ester would be at higher ppm, and this is exactly what was observed (Figure 6.17).

Figure 6.17 ^{19}F NMR shifts (280 MHz) in CD_3OD for the *R*-ester (160, top panel) and *S*-ester (161, bottom panel) in CD_3OD .

Table 6.2 ^1H (600 MHz), ^{13}C (150 MHz) NMR data of **3-*epi*-xestoaminol-C (156)** (CD_3OD).

	δ_{C}	δ_{H} (J in Hz)	COSY	HMBC
1	16.1, CH_3	1.27 (d, 6.7 Hz)	2	2,3,7
2	53.5, CH	3.09 (quin, 6.8 Hz)	1,3	1,3,4
3	73.2, CH	3.44 (ddd, 8.5, 7.0, 3.0 Hz)	2,4a,b,	1,4,5
4a	34.7, CH_2	1.40, m	3,4b	3,5
4b		1.56, m	3,4a	5,9
5a	26.4, CH_2	1.39, m		2,3
5b		1.54, m		6
6-11	30.5-30.8, CH_2	1.30, m		
12	33.1, CH_2	1.29, m	14	13
13	23.8, CH_2	1.31, m	14	12,14
14	14.5, CH_3	0.91 (t, 7.0 Hz)	12,13	12,13

6.4.3 Compounds from the genus *Xiphophora*

Algae belonging to the genus *Xiphophora* have not been extensively studied in the field of marine natural products chemistry. The majority of studies on this genus are marine ecological studies focusing on topics such as ammonia, urea and nitrate uptake, or phylogenetics as well as surveys of the species of algae from different intertidal zones. However, there has been one reported study that involved the isolation of a *Penicillium* sp. fungus from *Xiphophora gladiata*, which led to the isolation of two 2-pyridones (**162** and **163**) [312].

162

163

The isolation of **162** and **163** was a result of the research group's focus on compounds isolated from fungi, and therefore the metabolites of the algae were not investigated. As *X. gladiata* belongs to the same genus as *X. chondrophylla*, it is

possible that amino alcohols may also be present in *X. gladiata*, however this remains to be investigated.

The amino alcohols xestoaminol A-C (**107-109**) were first reported by Jiménez and Crews as stated earlier. To elucidate their configurations, xestoaminol A was derivatised to form an oxazolidinone, the coupling constant between H-2 and H-3 was approximately 7.5 Hz, suggestive of a *cis* relationship between the protons of the five membered ring system. Based on these results, Jiménez and Crews proposed the *2S**, *3R** stereochemistry for xestoaminol A. The proposed *2S**, *3R** stereochemistry for xestoaminol C (**109**) was based on similarities of the C-2 and C-3 carbons chemical shifts of xestoaminol C to xestoaminol A [284]. As stated earlier in section 6.4.2, the relative stereochemistry of 3-*epi*-xestoaminol C (**156**) was also elucidated by generating the oxazolidinone derivative and the coupling constant measured was 6.5 Hz, which indicated a *trans* relationship between H-2 and H-3 in **157** [274]. Sata *et al.* have previously used differences in coupling constant to elucidate the relative configuration of two epimers, amaminols A (**142**) and B (**143**) which differ at the 2-position [274]. It should be noted that the absolute configuration of **109** was elucidated as *2S*, *3R* for the two stereogenic centers, 11 years later using Mosher's method [278].

The research described in this thesis applied established and accepted methods to elucidate the relative and absolute configurations of 3-*epi*-xestoaminol C. Sata *et al.* as well as Jiménez and Crews formed the oxazolidinone derivative of their respective amino alcohols and then measured the coupling constants between the oxymethine and aminomethines. Sata *et al.* measured the differences between two different epimers and showed that a coupling constant of 6.5 Hz indicated a *trans* relationship and 7.3 Hz indicated a *cis* relationship within the oxazolidinone ring [274]. This translates to the opposite when considering the respective linear forms, i.e. a *cis* relationship for the oxazolidinone indicates a *trans* relationship for the underivatised form.

Garrido *et al.* applied Mosher's method for elucidating the absolute stereochemistry xestoaminol C by [278]. With a few minor differences the same approach was applied to elucidate the absolute configuration of **156**. Garrido *et al.* identified an *R* configuration for the C-3 center, thus elucidating the absolute configuration of

xestoaminol C as 2*S*,3*R* [278]. Using the methods detailed, the compound isolated from *X. chondrophylla* was identified as the C-3 epimer of xestoaminol C.

Amino alcohols or 1-deoxysphingoids, tend to be isolated from sponges and ascidians, which makes this the first report of the isolation of this class of compounds from algae. The closest reports from marine algae involve the isolation of sphingosine derivatives from the red alga *Laurencia nidifica* and the green alga *Ulva fasciata* [313, 314]. This research has therefore identified the presence of sphingoids from brown algae for the first time.

6.4.4 Bioactivity

The crude extract of *X. chondrophylla* had an MIC₉₀ value of 200 µg/mL, and after purification the pure compound had an MIC₉₀ of 15 µg/mL (65.1 µM), an overall 13-fold improvement in activity. This activity is consistent in all species of bacteria studied (Figure 6.1), except *Pseudomonas aeruginosa* which was resistant to the highest concentration tested (60 µg/mL). In addition to the growth inhibition assay, the compound was tested against stationary phase *M. tuberculosis* H37Ra, yielding an MIC₉₀ of 65.1 µM in this assay as well (Figure 6.2). This hints to the possibility of a generic mechanism of action, possibly a detergent-like activity, which is not unexpected when considering the structure of the compound, the polar head and non-polar tail, which make for an ideal membrane disruptor.

When the compound was investigated for its activity against mammalian cell lines the results proved more interesting. Comparison of the HL-60 (IC₅₀ 8.8 µM) to the HEK (IC₅₀ 18.0 µM) data proves a slight specificity of the compound towards the HL-60 cancer cell line compared to the HEK cell line, which was used as a control to measure general toxicity (Figure 6.3). Though the therapeutic index of this compound is 2, which is very low and far from ideal, this may still warrant future studies to investigate this compound. Furthermore, the availability of **156** in such a high mass as well as the observed activity against *S. cerevisiae* allowed profiling of its mechanism of action using yeast chemical genetics, which may shed light on the current understanding of how 1-deoxysphingoids cause their observed activity.

When studying the effects the compound has on 1A9 cells transitioning from G2 to M-phase, 3-*epi*-xestoaminol C (**156**) does not inhibit this stage of the cell cycle (this

work was done with the help of Dr. Arun Kanakkanthara, Appendix A, page 173). The mechanism of action to induce cell death could be via alternative ways mentioned earlier for ES-285, such as disruption of actin cell fibres causing the destabilization of the cytoskeleton of the cell [290], activation of caspase 3 and 12, the phosphorylation of p53 and activation of isoforms of protein kinase C (PKC), inducing atypical cell death [291], or alternatively by causing ceramide induced apoptosis via activation of PKC ζ [292-295].

6.4.4.1 SAR study

Table 6.1 displays the results of the basic SAR work done on 3-*epi*-xestoaminol C. Testing oxazolidinone derivative **157** against *M. tuberculosis* H37Ra resulted in a drop in the MIC₉₀ to 60 μ g/mL. This suggests that the polar head is crucial for bioactivity. The diacetyl and *N*-acetyl derivatives generated for carrying out Mosher's analysis were also tested for activity. This indicated that modification to both the NH and OH resulted in a loss of activity with a maximum of 80% inhibition observed at the highest tested concentration of 60 μ g/mL. Solely modifying the NH via the addition of an acetyl group also resulted in complete loss of activity. A clear trend is therefore noted, where any modifications to the polar head group causes a decrease in activity. This loss of activity is further highlighted by the bioactivity results of the Mosher's esters with both showing a lack of activity at the highest concentration tested (60 μ g/mL) (Table 6.1).

6.4.5 Mechanism of action

In order to elucidate the mechanism of action of this compound in mycobacteria, 3-*epi*-xestoaminol C was screened against a library of random transposon mutants. The top 120 mutants were selected and testing them for sensitivity followed by validation of 31 mutants using dose response curves did not identify any mutants that were more sensitive to the compound. This may indicate that either this compound targets an essential gene, which would explain why no specific mutant was identified, or alternatively that this compound acts as a detergent, which could also explain its activity against a range of microbes.

In an alternative approach, the generation of resistant mutants was attempted. This involved the generation of random transposon mutants that were grown on plates at a

range of concentrations for over four weeks. However, growth was only observed at the non-inhibitory concentration, with no growth of mutants observed at concentrations above 7.5 µg/mL.

6.4.5.1 Chemical genetics

To gain further understanding of how the compound interacts in a eukaryotic system, the powerful tool of yeast genetics was used to identify pathways that this compound interacts with to generate the observed biological effect. A synthetic genetic array (SGA) was performed in a liquid assay system optimised to allow for minimal use of drug. The chemical genetics results described in this section were obtained by Nazmi H. Fadzilah.

Chemical genetics employs a library containing 4800 non-essential gene deletion mutants of *Saccharomyces cerevisiae* (baker's yeast).[315] The library was screened at the IC₂₀ concentration as this ensures for bioactivity as well as allowing for the identification of mutants that are the most sensitive to drug treatment. A dose response assay in *S. cerevisiae* identified an IC₅₀ of 17.1 µM, and an IC₂₀ of 3.5 µM. The library of non-essential mutants was grown for 16 h in the presence of 3-*epi*-xestoaminol C at the IC₂₀ concentration. Hits were selected using a *t* test with a Bonferroni correction adjusting the *p* value to 0.017. Using this selection criterion, sensitive strains which displayed less than 70% residual growth and resistant strains that had greater than 130% growth in comparison to DMSO controls were chosen for validation. This identified 805 hits, using the same selection criterion validation of the 805 hits had identified 28 mutants of significance (two of which were of dubious annotation and therefore excluded).

This resulted in 12 networks representing 19 genes involved in known pathways and one network with seven genes as part of unknown pathways (Figure 6.4). The largest number are genes were involved in unknown processes, followed by genes involved in lipid metabolism (AIM6, PLB3, CRC1, SPS19 and OPI1), and actin cytoskeleton organization (AKL1, RHO2 and SLA1) (Figure 6.4).

As sphingolipids are believed to regulate lipid metabolism, it is expected that this process should be affected by 3-*epi*-xestoaminol C [316]. Another process that was expected to appear in our network map was actin cytoskeleton organization, as it has

been previously suggested that ES-285 prevents the formation of actin stress fibres by decreasing the activity of Rho proteins [290]. Our results correlate with the possible mechanisms of action reported for ES-285.

Furthermore, as this type of study has not been performed before for 1-deoxysphingoids, it sheds further light on other possible pathways that may be involved in the mechanism of action of 1-deoxysphingoids. These new pathways include RNA catabolism, protein targeting and phosphorylation, amongst others (Figure 6.4). Studying how 1-deoxysphingoids interact with these new and unknown pathways may allow for a better understanding of the mechanism of action of these compounds. In addition, **156** may also help in identifying what role these unknown pathways play within cells. Interestingly, mutants in the ceramide synthase pathways did not appear in this screen, this may be due to the statistical cut off used to identify hits. It would therefore be interesting to pick individual mutants involved in ceramide synthesis and study the phenotypic effects when they are exposed to 3-*epi*-xestoaminol C (**156**).

6.4.6 Future directions

As the HPLC attached to an ELSD was available for use near the end of this study, a repeat isolation assisted by the ELSD may help in purifying 3-*epi*-xestoaminol C in fewer and less laborious steps. As the 1-deoxysphingoids have been linked to alteration of ceramide levels as was the case for ES-285, it would be interesting to study the effect of **156** on mutants involved in this pathway. Studying the effects of compounds with the same configuration, however with varying levels of saturation would also be of interest.

Chapter 7 Concluding remarks

Marine natural products research has led to the isolation of a plethora of novel compounds. The recent improvement in culturing methods of microbes once believed to be unculturable as well as genome mining methods is sure to lead to the identification of more unique compounds. Dereplication issues are a common problem in this field, the isolation of known compounds is a frustrating waste of time and resources that can be overcome using unique isolation methods. However, when pursuing bioactivity, the goal is often to isolate the bioactive, which may be a known compound.

The research described in this thesis employed a classic approach of bioassay-guided fractionation using whole cell assays. Whole cell assays are better suited for screening of crude extract as the complex nature of crude extracts can often interfere with more sensitive targeted biochemical assays.

The NIWA and VUW MNP libraries were screened to select extracts with potentially new anti-tubercular compounds. These screens led to the identification of a number of hits of which five were chosen due to availability, activity, and amount.

Due to time constraints as well as loss of activity, eventually three samples were pursued. As stated before, a common issue encountered is re-isolation of known compounds, which was observed with one sample that resulted in the isolation of common fatty acids from the active extract of blob fish liver.

Another common issue often encountered is low sample mass for the isolated compound. The fractionation of the *Latrunculia* sponge led to isolation of a novel compound, however the compound responsible for anti-mycobacterial activity was unable to be purified and identified. This compound was present at such low levels that it was impossible to elucidate any structural information. Furthermore, even rimarikiamide A (**98**) was not isolated in sufficient amounts to allow for the elucidation of relative and absolute configuration. The next step will be to synthesize two of the four possible stereoisomers and compare optical rotation and NMR data to the natural product to confirm the absolute configuration of the natural product, as well as further investigate its bioactivity.

The isolation work performed on the brown algae *Xiphophora chondrophylla* led to the isolation of a new compound, 3-*epi*-xestoaminol C (**156**) this compound was equally active against all strains of bacteria except *Pseudomonas aeruginosa*.

Xestoaminol C (**109**) was first isolated from a *Xestospongia* sp. sponge. The spectral data reported by Jiménez and Crews correlated very closely to the data of **156**, with the exception of a signature methyl doublet and oxymethine. The relative stereochemistry of the amino alcohol was identified by locking the compound's amine and alcohol moieties in an oxazolidinone ring followed by measuring the coupling constant between the amino- and oxymethine, as well as from 1D NOE experiments. Generating the Mosher's esters allowed characterisation of the absolute configuration of the compound; this validated the fact that this compound has a different configuration from **109**, and is therefore a new compound. The bioassay data generated from this work and crude SAR analysis identified the polar head as crucial for activity.

This compound proved to be a better cytotoxic agent towards HL-60 cells. Therefore the next logical step was to study the effects of this compound on eukaryotic cells. A model organism that allows for genome wide analysis at VUW was the *S. cerevisiae* gene deletion set. This was employed to perform SGA studies which led to the identification of 13 categories that represented pathways 3-*epi*-xestoaminol C interacts with, two of which were expected, one represented unknown pathways and the others were novel. This study therefore highlights cellular processes yet to be implicated in the mechanism of action of 1-deoxysphingoids, which will require further studies on the mutants identified. As 1-deoxysphingoids have also been shown to affect ceramide levels within cells, studying how mutants involved in this pathway interact with 3-*epi*-xestoaminol C may also be of value to gain a better understanding of these compounds.

The *X. chondrophylla* project saw the progress of a sample from the crude stages through to a pure compound, following which derivatives were made to elucidate the absolute stereochemistry, which aided in performing SAR studies. Finally, the SGA work in *S. cerevisiae* ensured the overarching three goals of my thesis were achieved.

It is interesting to note that all the compounds isolated through this work share similar features. These compounds all have polar head groups and long hydrophobic chains, which may be due to the assay used in this approach.

Looking at the activities of palmitic acid and 3-*epi*-xestoaminol C against *M. tuberculosis* H37Ra, it is apparent that a polar head group is important for compounds such as these. Although the exact reason for this was not investigated, the presence of a polar head as well as a non-polar tail may allow for better solubility of the compound, which would allow for more of it to be in solution and would aid in traversing both hydrophilic and hydrophobic environs. Furthermore the amphiphilic nature of the compound may allow for better permeation through the bacterial cell wall or alternatively cause lysis of cells due to detergent type activity.

When looking at chain length it seems that C14-16 carbon chains may be effective as observed with the activity of 3-*epi*-xestoaminol C and palmitic acid. It would be interesting to study the effects of other C14 fatty acids to better understand the effects of chain lengths. When looking at level of unsaturation it can be observed that having saturated chains is more favoured.

If this project were to be redesigned and performed again, a better possible approach could include performing the library screens directly against *M. tuberculosis* H37Ra. Due to the increased bioactivity observed in the crude extract of *Latrunculia* sp. sponge against *M. tuberculosis* H37Ra, the implied specificity may hint at the possibility of other specific hits being overlooked. In addition, a counter screen could be introduced by screening the extract against HEK cells. Although crude extracts may have higher toxicity due to the possibility of higher levels of surfactants present, it may allow for a better selection of hits to be made.

Appendices

Appendix A:

NIWA library screening raw data

Table 7.1 Raw data of NIWA library screen vs. *M. smegmatis*

Plates 12, 13 and 14 were kindly donated by NIWA and screened for anti-mycobacterial activity. Top hits highlighted in yellow, positive control in red and negative control in green. Wells are represented as either P12, 13 or 14 followed by the well number for plates 12, 13 and 14 respectively. Standard error (std error) values are also listed for each sample.

Well number	GFP average	GFP std error	Resazurin average	Resazurin std error
P12C11	95.29507	0.785983	86.38034	3.157086
P12E9	95.23894	0.81616	86.08682	2.949949
Positive	94.17888	0.805538	86.0707	1.28972
P12D2	95.44557	0.774508	85.98647	2.898718
P12B11	95.22699	0.795667	85.63803	3.24214
P12H5	95.29462	0.798398	84.61087	2.82355
P12A5	95.18794	0.741449	84.50358	2.231667
P12H8	94.91964	0.883313	84.38737	3.549344
P12F4	94.65777	0.900614	83.94367	3.177821
P14D8	79.84463	10.39383	79.52913	6.688682
P12C5	92.33131	3.153455	74.69969	9.574227
P14A10	76.53547	13.60222	74.00939	12.16117
P12H2	65.38177	30.07546	72.72987	10.19009
P14C8	74.31914	15.79287	72.54576	14.13008
P14F6	85.78419	4.406677	71.18628	16.53152
P12E7	80.54696	14.88215	70.23149	13.03872
P12G7	88.10912	7.205349	70.19471	12.21514
P13C4	86.06836	9.197341	69.88816	17.19673
P12G11	91.05706	4.254647	67.37275	14.86076
P12B4	82.64904	12.74172	66.18606	17.17667
P12D11	49.1333	45.78911	66.1028	11.47069
P12B10	79.7254	15.5422	64.57238	15.22279
P12E1	73.53752	21.89476	63.24637	20.81483
P14G1	87.61734	4.389685	62.15067	8.500751
P14G4	87.94448	1.672556	60.93931	19.99167
P13C3	88.19733	7.070043	60.48392	25.30041
P13F3	80.15213	15.23033	59.29562	27.22728
P14H3	89.10644	1.622557	59.17182	26.97998
P14B1	51.06214	40.52924	47.83945	23.17653
P13F2	61.09333	33.45879	45.45379	24.96453
P12D4	57.49756	32.63582	39.50918	33.59945
P12D6	63.57766	28.34269	38.3046	35.8992

P14A7	70.86429	9.036251	36.84229	27.03343
P13G3	69.56712	24.46582	35.55058	31.69946
P14C9	49.30522	23.41664	33.04114	28.47672
P12D5	68.03184	21.56482	32.43605	35.22354
P14D11	-15.7199	42.80098	29.93673	2.796792
P12C6	70.14362	12.3702	28.00977	34.27149
P12A9	82.31134	8.054148	26.9769	33.4664
P13B2	71.13187	23.20145	26.87892	34.10814
P14C7	53.16032	12.48884	25.55587	8.495419
P14G5	63.19724	11.91114	25.42668	22.88006
P14B8	47.98046	16.84785	25.13695	7.053966
P14E7	49.87826	15.84849	25.01259	1.765235
P14E8	3.435204	12.42514	24.75312	11.46179
P14D6	68.49973	11.54012	24.21785	1.913252
P14E6	53.46062	23.30951	23.97877	5.462849
P14B7	47.95174	13.52469	23.912	16.88196
P14G10	31.32469	21.25331	22.74761	3.867035
P14G7	36.47978	32.63412	21.95137	1.840131
P14E4	51.67138	20.94187	21.71609	2.734935
P14E11	19.13935	24.94786	21.40771	6.166837
P14F10	-8.16547	48.91745	21.11393	6.880446
P14C6	50.46009	22.35922	21.05689	3.309809
P14E9	44.116	16.96219	20.98442	11.91788
P13B5	46.76197	48.55091	20.52903	65.74664
P14E5	-10.3081	33.59287	20.29615	8.892144
P14F8	27.96623	26.44523	19.7249	4.788833
P14F4	21.3651	36.61031	19.7137	6.165707
P14G8	27.28424	34.13498	19.39925	2.782101
P14B2	60.67015	19.44923	19.18749	4.301774
P14C2	64.68015	13.99315	18.60105	6.759065
P12H9	75.27249	13.65901	18.58306	38.66039
P14C5	60.94756	9.250122	17.6841	2.371108
P14G6	-2.55169	48.03772	17.61007	6.378801
P14G2	14.21108	34.27734	17.41164	15.3916
P14A8	33.91814	17.58041	17.33406	3.371426
P14F5	37.62318	36.35582	17.25284	4.997432
P14G3	27.32602	35.76232	17.24924	3.812852
P14F11	28.84561	29.12361	17.21426	5.242239
P14B5	56.666	10.1577	17.19392	6.036702
P12F5	69.06763	11.45544	17.10833	30.20712
P12D10	70.23466	14.68935	17.00887	37.72978
P13F10	56.6338	14.76691	16.75626	20.25039
P14C10	56.85615	7.524538	16.68879	1.451631
P14C12	69.56004	2.807874	16.60262	18.89024
P13D5	69.32286	11.68947	16.27412	16.58991
P14D5	72.3954	1.938687	15.82492	9.756835

P12G12	29.23471	35.89494	15.61437	4.148292
P14C4	45.91544	28.8453	15.39723	5.21136
P14E1	15.81121	23.72353	15.35797	11.95728
P14H9	37.37998	27.72498	15.33101	4.833102
P13E5	74.45022	2.457501	15.32738	18.02214
P14F3	22.31453	27.11177	15.21476	6.446794
P13E10	57.1752	22.91158	14.78389	15.13841
P13B7	60.70775	9.274539	14.6302	23.13163
P13D8	69.07614	9.93776	14.34612	20.50569
P14E2	39.19593	26.29932	14.34283	7.568437
P14C1	18.0416	24.64411	13.96777	14.86596
P13C5	78.82951	4.784819	13.96105	19.45644
P13G5	69.64096	20.74357	13.85236	38.74775
P14C3	41.04125	26.34051	13.28868	8.387458
P13B1	44.52683	43.27951	13.23391	21.78682
P14D2	53.9266	12.1028	13.13169	6.563468
P14B12	39.33416	28.3577	13.02601	11.31142
P13G4	82.65762	4.760891	12.9056	16.7919
P14D3	37.14536	25.56468	12.75301	5.807382
P13E6	74.49791	5.151318	12.69926	21.36707
P14H8	41.57048	23.14885	12.29052	4.769722
P14B9	29.04049	6.906667	12.2193	10.47905
P13C7	78.14485	7.030314	12.09265	20.67555
P12D8	31.1107	39.33485	11.93888	28.49429
P13C8	64.07387	10.25899	11.73318	24.61139
P13F7	62.11512	9.044507	11.68964	14.1738
P14F9	15.18114	27.34339	11.55868	2.146023
P14D7	61.08407	2.7064	11.54734	19.75837
P14H12	47.13213	14.99895	11.54464	14.61622
P13A8	61.05464	7.360603	11.5295	22.66638
P14B3	32.47928	18.86657	11.50742	1.90807
P14H6	21.26094	27.86608	10.82551	4.592332
P13A4	66.99853	11.48356	10.52092	22.08381
P13E3	78.61078	4.938423	10.43951	22.96065
P13G6	52.02614	6.498841	9.989098	20.71243
P13B11	54.63023	16.13409	9.873841	21.80595
P14D12	28.13839	20.54754	9.866753	15.01883
P13B3	57.62392	12.70165	9.59344	21.62048
P14E12	31.85651	18.16397	9.459336	5.571199
P12F2	16.11191	16.22787	9.288915	14.8254
P13G8	64.9466	6.885534	9.151431	23.37667
P13D2	65.69508	7.682957	9.033771	21.50588
P14H5	8.680589	33.75608	8.947887	3.791927
P13D10	59.33892	19.75762	8.860935	19.83009
P14B10	42.78492	17.36829	8.721749	8.617814
P13D9	67.43655	11.37215	8.599439	26.26022

P13H6	64.21682	11.16812	8.413744	21.63533
P12A8	64.69136	10.25722	8.397333	20.10236
P12C7	62.57825	10.58206	8.321585	30.69887
P13C10	67.70048	9.27671	8.225073	23.17331
P12A11	54.71581	9.358774	8.057547	20.18418
P14H1	53.34001	4.460681	8.033757	21.58946
P13H11	75.21667	10.63116	7.962502	43.87446
P14A2	7.278837	13.2975	7.830901	9.951199
P13F8	50.79972	10.11439	7.669997	19.27028
P14H4	39.53698	10.33974	7.567364	6.568749
P13F5	63.48538	8.849496	7.222446	17.27257
P13H7	53.19039	16.20456	7.126441	17.85725
P13H5	61.16707	3.049284	7.115328	18.58506
P14D1	38.08819	34.61624	6.814165	13.30402
P13F9	55.4294	5.015927	6.763839	21.76425
P13G10	55.28635	14.76988	6.726579	15.25053
P12C3	48.60107	8.431397	6.590149	5.760599
P13B10	57.43152	11.83799	6.363667	21.5196
P13D6	65.51006	19.01638	6.350273	21.98976
P12E2	71.85173	4.66917	6.344223	24.93408
P12D3	71.1574	8.609529	6.30302	28.15031
P13C9	69.56654	10.48855	6.297048	16.53759
P12C10	68.00201	9.616971	6.231508	19.17401
P13E11	76.31375	3.70551	6.045135	23.55996
P12E5	68.61907	6.330934	5.557262	21.98866
P12A6	57.71936	1.301973	5.064177	17.58
P14C11	10.60718	21.81412	4.982768	10.29458
P13E2	61.23323	16.43361	4.822049	22.9139
P14A5	-16.4687	48.64646	4.746931	17.85379
P13D7	70.92203	6.410861	4.41082	17.24668
P13E7	61.03934	19.52656	4.409845	24.14269
P13B4	79.59598	4.024533	4.342918	25.83868
P12F3	44.8628	36.53872	4.22125	36.011
P13D1	24.00031	31.74321	4.153307	5.733589
P12E8	79.68616	1.521917	4.14307	26.70217
P14F2	14.52617	26.40959	4.131409	24.03614
P14F12	48.08661	7.866409	3.837718	16.40139
P13E12	23.79505	33.47606	3.778476	15.28476
P14B4	33.99386	13.52558	3.422502	14.01213
P14A11	11.06668	16.55673	3.297886	7.922972
P12B5	52.1512	38.33371	3.127009	41.4558
P12H10	81.29384	4.204137	2.823713	28.91615
P14A12	30.34584	19.23776	2.793087	6.21532
P12B6	54.66647	9.987044	2.664445	25.24767
P14H2	13.92118	27.26741	2.573717	9.559425
P12F10	79.05507	1.653811	2.544383	24.63217

P13B6	59.98612	11.40872	2.488611	22.66604
P13H4	33.22263	11.54821	2.4225	16.63882
P13F1	40.5449	7.502826	2.131612	11.72879
P12F6	52.24805	15.40515	2.119675	11.85613
P13E8	35.6204	19.22529	2.092663	27.56439
P13F4	68.62508	5.490216	2.042568	22.91815
P12C4	63.50569	8.143879	1.884164	25.27153
P13A5	62.4339	3.883837	1.85076	41.17196
P13B9	59.88139	10.3023	1.752514	31.54771
P12A3	35.56098	7.305887	1.672668	13.25954
P12A10	52.13727	10.13055	1.435534	17.48855
P13H10	67.46127	6.511313	1.431417	16.00071
P13G11	41.55106	23.23634	1.134434	20.43297
P12D7	61.20281	16.86063	1.081377	26.45987
P13D3	80.97955	1.981237	1.068096	26.38719
P13C6	76.1625	3.490828	1.031579	24.7379
P12B8	51.07312	14.2143	0.986961	22.11304
P13C2	78.06332	4.960391	0.933395	25.76688
P13C11	53.87485	8.696173	0.789122	18.37219
P12D9	53.58877	6.771584	0.703301	23.69749
P13E9	57.01774	6.927957	0.675407	21.78598
P14H10	19.79333	29.65136	0.662297	7.325898
P14A1	52.57091	3.656583	0.616609	26.74211
P12H4	34.7322	17.46375	0.574882	28.7213
P12C2	30.27995	11.51482	0.393493	11.53775
P13H9	62.3895	2.493845	0.064536	15.2876
P13G9	62.55288	13.77852	0.001952	21.21672
P12E3	47.98199	23.28462	-0.7108	16.20891
P12E11	73.93991	6.194231	-0.9781	22.24723
P12H7	65.96385	12.26945	-1.00993	23.9661
P12B1	69.93618	8.805625	-1.12423	17.49904
P12B7	53.76263	2.034971	-1.13119	19.9671
P14F7	33.10686	5.167287	-1.28679	21.95373
P14B11	68.00783	7.936257	-1.29373	28.44095
P14A3	0.583484	15.13281	-1.37645	22.08034
P14A4	9.124315	35.75554	-1.41311	12.09699
P12E6	62.86929	18.59965	-1.56159	18.83752
P14H11	21.08003	26.82509	-1.62077	3.884184
P13H2	29.7638	18.18381	-1.72577	21.89342
P14G11	11.23583	15.66973	-1.7408	4.112302
P12B9	54.0754	1.867384	-2.42683	18.78046
P13D11	22.45504	2.907022	-2.48612	28.57463
P12C8	55.44219	10.50015	-2.57503	15.96577
P13H8	75.14213	7.815239	-2.59829	21.20335
P14D10	28.18139	11.26894	-2.62771	16.64255
P12H6	60.53709	4.946292	-2.65674	16.26725

Negative	-1.6E-15	1.04E-15	-2.74431	2.744315
P13F6	12.81342	38.15991	-2.82903	22.87259
P12F8	72.08113	6.79015	-3.06318	23.90158
P14A9	2.276	10.23496	-3.19931	19.05033
P14A6	12.33946	15.24049	-3.2443	16.26517
P14E10	7.978985	19.19814	-3.48289	19.87775
P13H1	48.59927	19.5299	-3.65957	17.3299
P12G9	76.25165	2.058681	-3.67557	26.36751
P14F1	32.11258	30.8922	-4.10634	16.84287
P14D9	24.72489	6.711745	-4.26301	23.80922
P12B12	34.76547	39.41461	-4.27627	9.297359
P12F7	70.16464	7.793636	-4.33108	16.42393
P12G2	60.79392	20.11073	-4.41381	21.2987
P13A9	48.55745	11.63737	-5.04958	8.261878
P14G12	43.73585	32.16446	-5.19446	23.06536
P12G8	69.28146	7.119817	-5.33807	27.94992
P14G9	25.99625	19.62383	-5.50316	17.5543
P14B6	42.80862	3.91153	-5.75594	27.53277
P13G7	46.48067	17.87033	-5.83064	25.21456
P14E3	11.85072	6.362463	-5.90241	20.92926
P13A10	66.15942	8.439018	-6.01168	25.72006
P13H3	12.10762	25.54477	-6.23194	21.08624
P14H7	-7.08517	17.56731	-6.30445	20.40175
P12G4	75.53395	3.990711	-6.44333	25.79118
P12B2	51.80511	11.88245	-6.79739	19.93671
P12G3	78.85571	3.758784	-6.98983	25.07222
P12G6	74.16993	4.408778	-7.33904	27.2574
P12G10	74.40513	4.569056	-7.37694	27.74614
P12C9	51.26161	14.80368	-7.39038	37.42863
P13A11	36.57503	20.32198	-7.66617	23.99213
P12E4	53.62801	11.61941	-7.94973	8.836291
P13E4	65.3128	14.90984	-8.39176	23.83041
P13F11	66.52103	1.827431	-8.51001	25.53271
P13B12	70.02164	4.281268	-8.9044	2.921295
P12H3	45.39753	39.3663	-9.0057	14.54912
P12H11	69.3944	9.255719	-9.23519	26.14936
P14D4	18.07649	29.50043	-9.58947	24.40286
P12G5	75.83877	3.947214	-10.0791	28.68455
P13A12	47.13184	0.562767	-10.547	8.076361
P13B8	69.55005	1.087336	-10.9057	15.64739
P13E1	72.67067	9.20003	-11.3864	9.612788
P13G12	56.09426	9.868374	-11.6851	7.481183
P12F11	70.29524	1.079568	-12.1406	25.01625
P12A4	57.60193	10.34467	-12.5505	9.768548
P13A3	49.4022	15.62358	-13.9029	28.20824
P12B3	61.98406	11.2399	-14.0016	30.86147

P13A7	34.23005	14.97206	-14.9375	25.28122
P12A2	50.93835	8.329339	-15.6527	31.23382
P12F9	42.43112	15.97481	-17.0093	15.5608
P13A6	62.16428	4.181207	-17.1228	44.78536
P13A2	47.25156	10.37642	-17.8695	28.98473
P12E12	56.76999	7.364205	-18.9406	3.793946
P13A1	46.68483	2.310021	-19.1867	13.46318
P13F12	76.88013	0.700357	-19.2921	10.16751
P12E10	29.92259	37.12848	-19.8402	12.0464
P13D12	55.02205	12.21098	-20.6057	13.36992
P13C12	47.46493	29.56519	-20.8498	6.125715
P12H1	48.19581	9.363045	-23.1262	24.56357
P13H12	12.01996	17.51483	-23.9993	23.24625
P13G2	16.64483	19.67116	-26.3061	32.82964
P12F12	44.35305	12.37506	-28.4174	16.9287
P12F1	47.2474	11.47322	-29.6369	14.3363
P12A7	44.48769	14.79392	-31.7808	6.799194
P12G1	69.45505	3.245193	-31.8402	7.978208
P12C12	62.69667	16.04693	-31.9423	11.03574
P13D4	61.82762	12.19567	-33.2304	36.61669
P12D1	48.25377	20.44374	-33.4047	12.65876
P12C1	65.50274	7.063881	-37.7655	16.60946
P12A12	38.54574	8.3305	-39.7585	8.580804
P12H12	12.05098	24.78073	-45.1262	40.06738
P12D12	51.24517	6.066775	-49.5603	27.39543
P13G1	38.28933	19.71935	-51.7756	22.4385
P12A1	23.71434	11.1765	-54.5519	54.92307
P13C1	28.31176	33.08247	-57.5063	58.08748

VUW MNP library screening raw data

Table 7.2 Raw data of VUW MNP library screen vs. *M. smegmatis*

Top hits highlighted in yellow, positive control in red and negative control in green. Plates RAK18_52A/B, 53A/B and 53C/D were screened against *M. smegmatis* as stated earlier RAK18_52A/B was not screened in duplicate therefore no standard deviation (std dev) value are listed for extracts from this plate. Std dev is used instead of standard error as standard error values is applicable for triplicate data.

RAK18_52 A/B represented as RAK1852 followed by the respective well number.

RAK18_53A/B represented as RAK18A followed by the respective well number.

RAK18_53C/D represented as RAK18C followed by the respective well number.

Well number	GFP average	GFP std dev	Resazurin average	Resazurin std dev
RAK1852D5	82.51134		86.33764	
RAK1852D7	82.29852		84.95481	
RAK1852G6	81.28371		84.82935	
RAK1852H7	81.93423		84.08974	
RAK1852B5	82.48722		83.98016	
RAK1852G2	83.40519		83.86051	
RAK1852D8	82.61491		83.76624	
RAK18CH8	88.25275	2.492621	80.87763	0.734711
RAK18AD7	87.31818	0.149184	78.43139	1.641461
Positive	86.92569	2.794045	78.12862	5.72208
RAK18AA11	85.72099	0.54014	74.28695	0.237072
RAK18AE6	78.32324	8.843107	52.21352	27.78748
RAK1852G12	78.8809		41.2392	
RAK18CG8	62.80922	24.57021	39.83496	45.13486
RAK1852G1	62.75679		31.20559	
RAK18CC10	60.35443	13.82721	26.96934	34.5404
RAK1852G9	43.6266		22.82428	
RAK18CF10	54.17289	13.87771	21.88638	7.693616
RAK1852G7	31.59895		21.81058	
RAK18CD7	49.98409	15.10035	21.58811	1.318869
RAK1852G3	44.64637		21.58543	
RAK1852E9	37.43063		21.04569	
RAK1852C9	61.86191		20.79466	
RAK1852H12	56.28451		20.73401	
RAK18CF7	39.27054	16.71114	19.8724	7.734189

RAK18CD6	43.65879	12.61971	18.84471	7.782954
RAK1852F8	41.61507		18.39029	
RAK18CC4	38.4603	6.25646	18.30972	0.086892
RAK1852G8	47.00231		17.86712	
RAK18CE4	47.10365	16.12104	17.40278	4.279494
RAK18AD6	46.50234	15.32	17.22348	9.320529
RAK18CD11	39.32563	8.246603	16.76528	4.730476
RAK18CE6	45.73882	18.35168	16.57323	10.01583
RAK1852G11	43.14526		16.37191	
RAK18CG5	25.97592	39.92709	16.26847	0.591971
RAK18CB7	34.60375	6.208976	15.99004	1.254972
RAK18CB5	28.84004	23.9779	15.88827	0.04278
RAK18AE7	61.49888	6.629442	15.85789	9.75534
RAK18CC5	44.09384	5.974945	15.66845	3.851974
RAK1852F9	30.20816		15.56943	
RAK1852C7	41.64167		15.36025	
RAK18CF5	37.79198	29.84378	15.2449	4.505259
RAK18CE11	48.44091	2.063985	14.87257	8.80086
RAK18CC9	43.08292	9.864415	14.75548	1.130829
RAK18CD3	25.37132	12.49168	14.58015	6.087665
RAK18CF3	40.54543	18.34584	14.36066	4.67791
RAK18CD4	38.59718	9.979407	14.35189	12.69731
RAK18CB6	41.31467	9.555635	14.2968	2.334622
RAK1852F3	18.98356		14.04147	
RAK18CG3	38.91149	14.82204	14.03498	4.153189
RAK18AB5	37.5093	19.65451	13.80519	4.430898
RAK1852D9	-0.42405		13.5773	
RAK1852F2	-2.52531		13.38082	
RAK18CE3	32.87118	5.11551	13.25755	4.092863
RAK18CE10	44.37084	8.91691	13.24639	8.578496
RAK1852G10	43.68512		13.20478	
RAK18AC4	39.07579	11.82997	13.03168	3.172523
RAK18AF6	46.50283	10.27338	12.94231	2.156692
RAK18CG6	31.17505	4.672351	12.92744	1.885358
RAK18CF9	38.95533	21.41062	12.8467	4.86981
RAK18AG1	41.24539	29.82615	12.77045	15.59249
RAK18AC8	37.92471	17.9055	12.66563	3.207292
RAK18CF8	44.56399	14.30857	12.66233	5.333487
RAK1852H2	26.73171		12.65593	
RAK18CE8	40.45081	6.147955	12.595	11.34503
RAK18CB11	29.96592	0.952737	12.5565	3.123463
RAK18CG7	33.10349	19.54638	12.31101	0.764271
RAK18CC6	48.80099	9.638707	12.03279	8.855017
RAK1852H5	34.93849		11.8828	
RAK18AB8	44.77955	12.36526	11.82948	1.666298
RAK18CB4	37.25737	12.81713	11.82043	2.358022

RAK1852A9	13.5793		11.48219	
RAK18CE5	40.57995	16.65375	11.30892	11.64201
RAK18CC1	54.5389	6.189958	11.183	2.062172
RAK18CB9	24.35392	5.51662	11.14215	1.17628
RAK18CE2	35.62878	3.671658	11.06653	5.279631
RAK1852C4	44.99575		10.87405	
RAK18CB8	42.4589	12.07647	10.72786	0.043497
RAK1852C8	25.35723		10.70004	
RAK18CG2	23.10204	24.08118	10.5254	2.620404
RAK18AD11	6.681053	31.8454	10.50397	10.6675
RAK1852G4	43.38859		10.45328	
RAK18AD8	42.50065	1.771551	10.39133	7.766189
RAK18CE7	44.62251	22.53877	10.26418	2.268317
RAK18AF7	48.19767	16.46289	10.04269	9.697508
RAK18CF11	32.25211	3.666043	9.943542	8.772544
RAK18CC11	23.73454	13.20087	9.925965	1.371286
RAK18CC8	39.47905	20.25609	9.833364	3.769109
RAK1852H3	35.3471		9.791177	
RAK18CB10	35.93593	14.53557	9.557172	6.739276
RAK18CG10	35.993	26.48967	9.384845	1.807702
RAK18CA7	27.20761	11.24038	9.379337	4.720824
RAK18CH10	30.17527	13.92102	9.182157	11.48433
RAK18CA6	25.0981	34.55846	9.099836	3.663083
RAK18CC7	35.07491	30.47901	9.030643	1.813343
RAK1852B7	54.14604		8.948965	
RAK1852A10	28.73898		8.904786	
RAK18AH4	26.33717	19.60195	8.819939	14.93681
RAK18CH2	15.53336	2.192368	8.771624	6.578632
RAK1852D4	34.93919		8.519383	
RAK18AC9	7.212093	12.72296	8.512579	1.67748
RAK18CD8	36.16599	5.225469	8.494863	9.418876
RAK18CF1	43.94552	12.94351	8.365288	6.558098
RAK18AG6	25.03923	0.789772	7.868625	2.268652
RAK1852G5	34.29895		7.867839	
RAK1852E7	51.68121		7.853403	
RAK18AG7	46.76334	24.68317	7.746715	4.128095
RAK1852H11	23.93061		7.725226	
RAK18CA3	29.31859	13.31378	7.569492	3.033543
RAK18CB2	16.42576	1.2311	7.452216	2.652107
RAK18CH7	33.19343	11.03563	7.299778	8.886059
RAK18AA3	37.28179	14.72419	7.222739	1.578026
RAK18AH3	31.27687	5.289814	7.057244	3.935705
RAK1852E4	37.31997		7.03425	
RAK18CG4	34.74121	15.33369	6.984139	1.921967
RAK18CF2	21.73172	11.09809	6.981507	10.75625
RAK1852A3	23.35777		6.957131	

RAK18CH5	33.66798	14.86288	6.942604	6.816235
RAK18CA5	34.44111	15.19794	6.931589	3.247933
RAK18CG9	28.45219	11.31215	6.843277	0.070078
RAK18AF3	22.40652	7.444122	6.70158	13.23768
RAK1852H4	43.34248		6.668901	
RAK18AG5	43.12163	4.664573	6.612338	6.895605
RAK18CA1	60.67739	5.464107	6.417738	11.39781
RAK18AH6	24.60597	15.1173	6.369117	6.603738
RAK18CA4	27.5695	22.09493	6.350291	6.073261
RAK18AH7	45.46937	0.591618	6.31376	10.67808
RAK18CE9	14.61035	40.4074	6.299608	1.603988
RAK18CE1	63.42	7.432334	6.105656	9.977392
RAK18CC12	53.13311	1.716488	6.056883	2.523299
RAK1852E3	33.95454		6.035378	
RAK18AG12	37.4429	20.45551	5.979747	0.954379
RAK18AB12	35.50482	23.77472	5.956387	11.27223
RAK1852F10	9.737147		5.861956	
RAK18AE5	57.3575	8.644206	5.832018	8.076328
RAK1852H8	10.38767		5.810317	
RAK1852H9	36.52259		5.784546	
RAK1852B9	28.5822		5.656369	
RAK18CA10	26.64784	7.684994	5.635112	2.967397
RAK18AE11	34.31452	19.35108	5.589142	0.43874
RAK18CA8	27.08561	1.497708	5.542429	1.66355
RAK18AF12	32.5208	16.52901	5.528628	3.723113
RAK1852F11	30.86223		5.4191	
RAK18CA2	1.223262	45.65745	5.287721	3.660894
RAK18CD2	23.35751	1.332938	5.193092	1.669427
RAK18CD9	41.33375	23.41621	5.167979	2.879564
RAK18CD5	19.13102	1.057542	5.105608	13.0501
RAK18CF6	34.90949	10.74338	4.936552	4.301336
RAK18AC6	37.26106	8.716831	4.838043	0.262285
RAK18CB3	17.34306	19.77998	4.796711	8.537323
RAK18AF5	37.94824	10.5327	4.79034	2.841401
RAK18AD9	1.142218	7.697591	4.786374	1.883591
RAK1852D10	33.72469		4.608182	
RAK18CD10	30.58667	4.002616	4.415699	12.47103
RAK18AG3	40.74942	17.08258	4.354143	0.981454
RAK1852A12	36.23474		4.341587	
RAK18CG12	48.34821	1.777344	4.185734	2.055925
RAK18CA12	45.63538	3.622087	4.169079	1.060737
RAK18CH9	26.98868	1.196705	3.994208	6.954887
RAK18CA9	26.46577	10.15487	3.902579	1.996798
RAK1852F6	31.71352		3.816351	
RAK18CD1	42.90091	12.06179	3.642459	8.126461
RAK1852B10	32.85283		3.559221	

RAK18AE4	36.00865	13.30036	3.5313	3.035798
RAK18CA11	35.08207	0.156968	3.511606	4.793009
RAK18CC3	9.339796	21.06663	3.308806	3.413138
RAK18CE12	56.49319	9.311254	3.257728	5.234882
RAK1852D11	28.21366		3.247062	
RAK18AH5	34.5343	10.56111	3.244301	2.190558
RAK1852A11	13.27461		3.212668	
RAK18CG11	20.58392	3.106348	3.18032	6.079504
RAK18CH6	2.026747	2.656611	2.974406	7.80542
RAK18CG1	35.21885	13.69045	2.922377	0.335708
RAK18AB3	33.19032	11.31146	2.831559	1.151677
RAK18CH4	22.10564	4.257631	2.784111	3.676249
RAK18AE10	40.97388	0.586597	2.702233	5.538246
RAK1852H10	32.09944		2.6427	
RAK18AC12	26.08682	15.27571	2.619875	11.35434
RAK18AG2	23.3445	17.59309	2.45287	0.261224
RAK1852B1	19.16339		2.449645	
RAK18CB12	36.54129	5.752983	2.440765	5.117247
RAK18CC2	12.15135	17.3535	2.310045	6.849242
RAK18AE2	35.09948	8.516478	2.083469	6.950927
RAK18AA10	39.61308	5.143171	2.025051	5.119686
RAK18AF2	13.06663	6.515053	1.903731	10.461
RAK18AD10	19.20469	8.58081	1.706661	4.976488
RAK18CH11	25.4635	18.03673	1.700377	11.90163
RAK18CH3	12.98827	25.94547	1.631092	0.233987
RAK18AE3	29.61926	30.09737	1.377858	11.51169
RAK18AA9	9.107801	17.83364	1.223838	7.841813
RAK18AB9	16.14871	7.001322	1.171749	4.285827
RAK18AG11	25.67891	3.957708	0.992288	4.036634
RAK1852C6	53.59519		0.78379	
RAK1852H6	32.59602		0.78379	
RAK18AH2	6.720081	23.87637	0.692042	0.39497
RAK1852F4	20.25943		0.64108	
RAK18AA5	26.95119	0.39597	0.537887	0.175219
RAK1852A1	19.12317		0.464357	
RAK1852A5	7.751871		0.42377	
RAK1852A2	31.78198		0.265171	
RAK18AD3	6.729815	9.866928	0.181802	1.809977
RAK18AE12	37.37279	20.09251	0.056468	0.418971
Negative	-3E-15	5.59E-15	2.84E-15	3.89E-15
RAK18AF11	26.33552	12.44472	-0.05612	7.400697
RAK18AF1	24.00114	6.522307	-0.18053	4.742422
RAK18AC3	25.21636	14.04352	-0.25865	0.38762
RAK18AE1	27.51207	10.3161	-0.28578	4.21642
RAK18CD12	49.20714	0.853602	-0.4331	4.111115
RAK18AF4	27.20402	14.37533	-0.5966	20.17942

RAK18AF9	18.09764	24.6521	-0.67438	11.77507
RAK18AB6	46.21676	0.334301	-0.75874	11.49639
RAK18AD1	29.46976	18.97909	-0.86904	1.072651
RAK18AA6	39.8147	0.584787	-0.9598	2.391276
RAK18AG4	37.55022	0.01991	-1.01646	3.350942
RAK18AB10	24.1238	11.79857	-1.02203	10.6011
RAK18AD12	21.25354	7.169368	-1.2628	1.676767
RAK18AA7	39.52037	13.7623	-1.35869	0.724543
RAK1852E1	32.45254		-1.50761	
RAK18AA1	35.75929	21.90377	-1.51005	5.884542
RAK18AE8	41.74214	9.733895	-1.52283	15.52196
RAK18AE9	-0.41931	54.80276	-1.56743	20.57051
RAK1852A6	24.64428		-1.81056	
RAK18AH9	18.17687	43.73469	-1.96815	2.740514
RAK18AB4	30.54428	13.07372	-1.99401	1.181503
RAK1852B6	35.49998		-2.00201	
RAK1852F1	34.28081		-2.12521	
RAK1852A7	14.05921		-2.18027	
RAK1852E6	38.86789		-2.38712	
RAK18AD5	22.01684	37.56784	-2.4592	14.40564
RAK1852H1	20.79624		-2.56741	
RAK18AD2	28.86165	16.17891	-2.70755	0.700842
RAK18AG9	18.95324	19.94399	-2.72205	14.41937
RAK18AH10	5.739257	0.799466	-2.80551	0.622771
RAK18AC10	15.20642	2.990794	-2.84881	14.5402
RAK18AD4	22.78329	8.821005	-2.86509	8.614111
RAK18AA8	32.99255	5.673311	-2.87797	11.01093
RAK18CF4	48.18199	13.88986	-3.05744	5.139073
RAK18AB11	22.3611	14.16701	-3.17642	3.068798
RAK18AH1	28.21666	16.50828	-3.24822	6.158713
RAK18AA12	34.7098	14.96365	-4.01141	6.415065
RAK18CH12	32.50313	9.541595	-4.07263	9.542489
RAK18AF10	25.91422	14.70727	-4.1228	1.729383
RAK18AF8	34.45813	5.627901	-4.21142	16.53235
RAK1852E8	2.674997		-4.43389	
RAK18AH12	32.71475	10.57416	-4.47234	0.615571
RAK18AC11	27.25304	0.372096	-4.86194	0.409552
RAK18CB1	17.40274	16.69361	-4.92256	13.15581
RAK18AG10	19.18288	16.19178	-5.16962	0.978594
RAK18AC1	25.46507	8.67867	-5.29521	7.674458
RAK1852D12	19.96291		-5.34074	
RAK1852C1	46.85089		-5.60544	
RAK18AC5	28.45948	8.570964	-6.33864	19.13271
RAK1852A4	9.629672		-6.43095	
RAK18AA4	18.58409	1.197894	-6.63394	2.948968
RAK1852C2	39.57375		-6.73817	

RAK18AB1	24.96809	10.69279	-6.89769	0.527962
RAK18AC2	24.18161	8.432652	-7.29601	6.497312
RAK18AB7	33.56221	29.0952	-7.36759	20.2746
RAK18AH11	-6.39996	24.00148	-7.50226	14.69246
RAK18AB2	33.68643	9.540991	-7.86444	9.194541
RAK18CH1	22.20906	11.19038	-8.68446	1.955926
RAK18AA2	7.372359	9.452771	-9.21463	2.771314
RAK18AC7	15.8392	39.01105	-9.67571	20.9994
RAK1852F12	26.92394		-10.3629	
RAK18AH8	27.36875	10.97076	-10.8755	17.81443
RAK18CF12	23.06386	14.98629	-11.641	0.434402
RAK1852E2	59.21263		-20.1123	
RAK1852E10	32.41725		-24.7939	
RAK1852D1	66.17376		-25.3368	
RAK1852F5	46.0063		-25.8884	
RAK1852B11	33.04011		-28.8941	
RAK18AG8	45.88341	15.66059	-29.7372	16.09452
RAK1852B8	53.89066		-29.9097	
RAK1852D6	62.61566		-30.8714	
RAK1852A8	42.47381		-32.3404	
RAK1852D2	64.59845		-33.3008	
RAK1852B2	54.27409		-33.5476	
RAK1852C5	59.30343		-33.5801	
RAK1852D3	64.28844		-33.8877	
RAK1852B3	40.79783		-34.6267	
RAK1852F7	62.8487		-35.4226	
RAK1852C3	62.76392		-35.9771	
RAK1852E5	62.48796		-37.2181	
RAK1852B4	62.5174		-38.3427	
RAK1852C10	58.65113		-38.7962	
RAK1852E11	58.51209		-39.3114	
RAK1852E12	31.53805		-41.141	
RAK1852C11	39.84829		-41.1927	
RAK1852B12	59.0764		-52.5119	
RAK1852C12	57.55604		-53.5649	

Increased resistance of stationary phase mycobacteria

A) dose response curve of streptomycin in a growth inhibition assay. **B)** dose response curve of streptomycin against stationary phase H37Ra. 99% inhibition is observed at 0.4 μ M of streptomycin in log phase H37Ra (**A**), however at this concentration there is still 50% growth observed in stationary phase bacteria (**B**). Graph generated using GraphPad Prism v.5.0. It is noted that the graph shape for **B** is not sigmoidal, however it is still evident that compared to log phase bacteria, stationary phase bacteria are more resistant to inhibition. This is made evident when observing the fact the even at the starting concentration of 200 μ M approximately 10 times more than that used for log phase cells, there is still 15% residual metabolism measure.

G2-M block assay

Sample	Ungated	apoptotic	G0	G2-M	other	S	total	G2-M % <i>compared to total</i>
	<i>Count</i>	<i>Count</i>	<i>Count</i>	<i>Count</i>	<i>Count</i>	<i>Count</i>	<i>Count</i>	
Specimen_001_Tube_001.fcs	10341	2038	4551	1999	8303	1616	8139	24.6
Specimen_001_Tube_002.fcs	10414	2473	5176	1455	7941	1168	7805	18.6
Specimen_001_Tube_003.fcs	10428	2411	4613	1705	8017	1562	7875	21.7
Mean	10394	2307	4780	1720	8087	1449	7940	
SD	46.7	235	344	272	191	245	176	

The samples show no significant difference at any concentration of **156**

Appendix B: Fatty acid mixture from *Psychrolutes marcidus*

^1H NMR (CDCl_3 , 600 MHz) spectrum of fatty acid mixture from the MeOH liver extract of *Psychrolutes marcidus*

^{13}C NMR (CDCl_3 , 150 MHz) spectrum of fatty acid mixture from the MeOH liver extract of *Psychrolutes marcidus*

GC/MS Chromatograms of ND5_96L FAME analysis

Full GC trace of ND5_96L FAME (Integrated Bioactive Technologies group, Callaghan Innovation).

Zoom in of relevant section of GC trace of ND5_96L FAME, with relevant labels for peaks of interest (Integrated Bioactive Technologies group, Callaghan Innovation).

Appendix C: NMR spectra of rimarikiamide A (98)

^1H NMR (DMSO- d_6 , 600 MHz) spectrum of rimarikiamide A (98)

^{13}C NMR (d_6 DMSO, 150 MHz) spectrum of rimarikiamide A (**98**)

gCOSY NMR (DMSO- d_6 , 600 MHz,) spectrum of rimarikiamide A (**98**)

Multiplicity-edited HSQC NMR (DMSO- d_6 , 600 MHz,) of rimarikiamide A (**98**) showing methylenes in blue and methyls and methines in red.

gHMBC NMR (DMSO- d_6 , 600 MHz,) spectrum of rimarikiamide A (**98**).

2D ROESY NMR (DMSO-d₆, 600 MHz,) spectrum of rimarikiamide A (**98**).

2D ROESY NMR (DMSO- d_6 , 600 MHz,) spectrum of rimarikiamide A (**98**) specific range of interest.

2D ROESY NMR (DMSO- d_6 , 600 MHz,) spectrum of rimarikiamide A (**98**) specific range of interest.

Appendix D: NMR spectra of 3-*epi*-xestoaminol C (**156**)

^1H NMR (CD_3OD , 600 MHz) spectrum of 3-*epi*-xestoaminol C (**156**).

^{13}C NMR (CD_3OD , 150 MHz) spectrum of 3-*epi*-xestoaminol C (**156**).

gCOSY NMR (CD₃OD, 600 MHz) spectrum of 3-*epi*-xestoaminol C (**156**).

Multiplicity-edited HSQC NMR (CD_3OD , 600 MHz) spectrum of 3-*epi*-xestoaminol C (**156**) showing methylenes in blue and methyls and methines in red.

gHMBC NMR (CD₃OD, 600 MHz) spectrum of 3-*epi*-xestoaminol C (**156**).

1D TOCSY NMR spectrum (CDCl_3 , 600 MHz, 200 ms spin-lock mixing time) of 3-*epi*-xestoaminol C (**156**). Irradiation of H-3 (δ_{H} 3.65) and H-4/5 (δ_{H} 1.55) resulted in TOCSY transfer of energy to H₃-14 (δ_{H} 0.88), indicated by circles in the bottom two panels. Irradiating H₃-14 resulted in TOCSY transfer of energy to H-4/5, indicated by a circle in the top panel.

^1H NMR (CDCl_3 , 600 MHz) spectrum of oxazolidinone (**157**).

^{13}C NMR (CDCl_3 , 150 MHz) spectrum of oxazolidinone (**157**).

1D NOE NMR spectra (CDCl_3 , 600 MHz) spectrum of oxazolidinone (**157**). Irradiating H-2 (δH 3.57) resulted in NOE transferr to H-4a (δH 1.62) and H-4b (δH 1.71) indicated by the circle in the top panel. Irradiating H-3 (δH 4.09) resulted in NOE transfer to H3-1 (δH 1.27) indicated by the circle in the bottom panel.

^1H NMR (CDCl_3 , 600 MHz) spectrum of diacetyl-3-*epi*-xestoaminol C (**158**).

^{13}C NMR (CDCl_3 , 150 MHz) spectrum of diacetyl-3-*epi*-xestoaminol C (**158**).

¹H NMR (CDCl₃, 600 MHz) spectrum of *N*-acetyl-3-*epi*-xestoaminol C (**159**).

^{13}C NMR (CDCl₃, 150 MHz) spectrum of *N*-acetyl-3-*epi*-xestoaminol C (**159**).

^1H NMR (CD_3OD , 600 MHz) spectrum of the *R*-Mosher's ester (**160**).

^{13}C NMR (CD_3OD , 150 MHz) spectrum of the *R*- Mosher's ester (**160**).

^1H NMR (CD_3OD , 600 MHz) spectrum of the *S*- Mosher's ester (**161**).

^{13}C NMR (CD_3OD , 150 MHz) spectrum of the *S*-Mosher's ester (**161**).

Appendix E: Chemical genetics mutant hits

Top 28 *S. cerevisiae* mutants identified for chemical genetic profiling.

Residual Growth	P-values	ORF	Gene Name	Description
64.9	0.000	YNL202W	SPS19	Peroxisomal 2,4-dienoyl-CoA reductase; auxiliary enzyme of fatty acid beta-oxidation; homodimeric enzyme required for growth and sporulation on petroselineate medium; expression induced during late sporulation and in the presence of oleate
57.0	0.003	YOL011W	PLB3	Phospholipase B (lysophospholipase) involved in lipid metabolism; hydrolyzes phosphatidylinositol and phosphatidylserine and displays transacylase activity in vitro; PLB3 has a paralog, PLB1, that arose from the whole genome duplication
40.6	0.004	YJL216C	IMA5	Alpha-glucosidase; specificity for isomaltose, maltose, and palatinose, but not alpha-methylglucoside; member of the IMA isomaltase family; not required for isomaltose utilization, but Ima5p overexpression allows the ima1 null mutant to grow on isomaltose
60.5	0.004	YDR032C	PST2	Protein with similarity to a family of flavodoxin-like proteins; induced by oxidative stress in a Yap1p dependent manner; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies; protein abundance increases in response to DNA replication stress; PST2 has a paralog, RFS1, that arose from the whole genome duplication
43.2	0.004	YNL090W	RHO2	Non-essential small GTPase of the Rho/Rac family of Ras-like proteins; involved in the establishment of cell polarity and in microtubule assembly
64.8	0.005	YDR257C	RKM4	Ribosomal lysine methyltransferase; specific for monomethylation of Rpl42ap and Rpl42bp (lysine 55); nuclear SET-domain containing protein
57.4	0.006	YDR193W	YDR193W	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data
45.3	0.006	YDR183W	PLP1	Protein that interacts with CCT (chaperonin containing TCP-1) complex; has a role in actin and tubulin folding; has weak similarity to phosducins, which are G-protein regulators
62.65	0.007	YLR361C-A	YLR361C-A	Putative protein of unknown function
46.8	0.008	YBR059C	AKL1	Ser-Thr protein kinase; member (with Ark1p and Prk1p) of the Ark kinase family; involved in endocytosis and actin cytoskeleton organization
61.1	0.013	YDR272W	GLO2	Cytoplasmic glyoxalase II; catalyzes the hydrolysis of S-D-lactoylglutathione into glutathione and D-lactate; GLO2 has a paralog, GLO4, that arose from the whole genome duplication
58.5	0.013	YNL235C	YNL235C	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; partially overlaps the verified ORF SIN4/YNL236W, a subunit of the mediator complex
69.4	0.014	YBL007C	SLA1	Cytoskeletal protein binding protein; required for assembly of the cortical actin cytoskeleton; interacts with proteins regulating actin dynamics and proteins required for endocytosis; found in the nucleus and cell cortex; has 3 SH3 domains
43.8	0.016	YIL010W	DOT5	Nuclear thiol peroxidase; functions as an alkyl-hydroperoxide reductase during post-diauxic growth

61.5	0.017	YDR183W	PLP1	Protein that interacts with CCT (chaperonin containing TCP-1) complex; has a role in actin and tubulin folding; has weak similarity to phosducins, which are G-protein regulators
63.6	0.019	YBL008W	HIR1	Subunit of the HIR complex; HIR is a nucleosome assembly complex involved in regulation of histone gene transcription; contributes to nucleosome formation, heterochromatic gene silencing, and formation of functional kinetochores
69.1	0.019	YBR276C	PPS1	Protein phosphatase; has specificity for serine, threonine, and tyrosine residues; has a role in the DNA synthesis phase of the cell cycle
61.3	0.025	YAL064C-A	TDA8	Putative protein of unknown function; null mutant is sensitive to expression of the top1-T722A allele; not an essential gene
62.9	0.026	YDL237W	AIM6	Putative protein of unknown function; required for respiratory growth; YDL237W is not an essential gene
66.5	0.029	YJL191W	RPS14B	Protein component of the small (40S) ribosomal subunit; required for ribosome assembly and 20S pre-rRNA processing; mutations confer cryptopleurine resistance; homologous to mammalian ribosomal protein S14 and bacterial S11; RPS14B has a paralog, RPS14A, that arose from the whole genome duplication
35.3	0.029	YHL020C	OPI1	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; involved in telomere maintenance; null exhibits disrupted mitochondrial metabolism and low cardiolipin content, strongly correlated with overproduction of inositol
61.8	0.030	YBL017C	PEP1	Type I transmembrane sorting receptor for multiple vacuolar hydrolases; cycles between the late-Golgi and prevacuolar endosome-like compartments
65.6	0.031	YOR300W	YOR300W	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; overlaps with verified gene BUD7/YOR299W; mutation affects bipolar budding and bud site selection, though phenotype could be due to the mutation's effects on BUD7
50.1	0.033	YPR042C	PUF2	mRNA-binding protein; member of the PUF protein family, which is defined by the presence of Pumilio homology domains that confer RNA binding activity; preferentially binds mRNAs encoding membrane-associated proteins; PUF2 has a paralog, JSN1, that arose from the whole genome duplication
54.6	0.034	YOR100C	CRC1	Mitochondrial inner membrane carnitine transporter; required for carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria during fatty acid beta-oxidation
69.1	0.037	YMR105W-A	YMR105W-A	Putative protein of unknown function
48.4	0.046	YKL053W	YKL053W	Dubious open reading frame; unlikely to encode a functional protein, based on available experimental and comparative sequence data; partially overlaps verified ORF ASK1
46.8	0.049	YBR071W	YBR071W	Protein of unknown function found in the cytoplasm and bud neck; mRNA expression may be regulated by the cell cycle and/or cell wall stress; overexpression of YBR071W affects endocytic protein trafficking

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