Integrated approaches for marine actinomycete biodiscovery

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ABSTRACT

This PhD project examined integrated approaches for marine actinomycete biodiscovery. A library of 120 actinomycete strains derived from the three Australian ascidians, *Symplesma rubra*, *Aplidium solidum* and *Polyclinum vasculosum*, was established in order to access new chemical diversity of underexplored marine ecological niches for natural product drug discovery. Specifically, the genera *Streptomyces* and *Micromonospora* were highly diverse and abundantly present, while fewer *Nocardia* and *Rhodococcus* and only one *Streptosporangium* representative were isolated. Only two isolates occurred in all three ascidians indicating species-specificity of actinomycetes in the ascidian. LC-MS/MS profiling of extracts obtained from the ascidians and their actinomycete associates revealed many overlapping ions between hosts and cultured isolates indicating that these compounds were likely to be synthesised by the microbial associates. Laboratory cultures of the actinomycetes displayed even more diverse metabolomes than those of their ascidian hosts; thus making ascidian-associated actinomycetes an excellent target for biodiscovery.

Reisolation of already known compounds is a major obstacle to natural product drug discovery; therefore, sophisticated dereplication approaches have to be employed in the early stages of discovery. To facilitate the dereplication, a new strain prioritisation approach using HSQC-TOCSY NMR spectra together with anti-plasmodial activity data was developed. This allowed for prioritisation of microbial strains that are more likely to produce diverse polyketide or peptide natural products. This approach was further validated through dereplication of mono- and co-culture extracts of four different microbial strains. HSQC-TOCSY NMR profiles of the extracts clearly showed co-cultivation induced changes in the microbial metabolomes.

Streptomyces sp. (USC-16018) was selected based on the strain prioritisation approach and large-scale fermentations led to the isolation of a new ansamycin polyketide, herbimycin G with weak anti-plasmodial activity (77 % at 40 μ M), as well as the known compounds elaiophyllin, and the four diketopiperazines, Cyclo-L-Pro-L-Leu, Cyclo-L-Pro-L-Phe, Cyclo-L-Pro-L-Val and Cyclo-L-Pro-L-Tyr. The crude NMR profile of the sediment-derived *Streptomyces* sp. (USC-636) revealed the presence of indole-associated signals and three tryptophan containing diketopiperazines, nasesezine A – C, were isolated. The new compound nasesezine C exhibited moderate anti-plasmodial activity (IC₅₀ 3.52 μ M).

Overall, this thesis highlights the immense chemical diversity of actinomycetes from marine sources, as well as the importance of sophisticated dereplication approaches and provides an alternative new solution for strain selection that has led to the discovery of new bioactive natural products with potential as new drug leads.

STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed) _____ (Date) 01/09/2017

Larissa Buedenbender

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ABBREVIATIONS

16S rRNA	16S ribosomal RNA subunit
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
clog <i>P</i>	Logarithm of its partition coefficient between n-octanol and water $\log(c_{\text{octanol}}/c_{\text{water}})$
COSY	Correlation Spectroscopy
CS	Corn starch media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECD	Electronic circular dichroism
ESI	Electrospray Ionization
EtOH	Ethanol
FDA	Food and Drug Administration
HMBC	Heteronuclear Multiple Bond Correlation
HRESIMS	High-Resolution Electrospray Ionisation Mass Spectrometry
Hsp90	Heat shock protein 90
HSQC	Heteronuclear Single Quantum Coherence/Correlation
HTS	High Throughput Screening
IC50	Half maximal Inhibitory Concentration
MA	Marine agar
MeOH	Methanol
MS	Mass Spectrometry
NCBI	National Center for Biotechnology Information
nM	Nano molar
NMR	Nuclear Magnetic Resonance
NSW	New South Wales
OMA	Oatmeal agar
OTU	Operational Taxonomic Unit
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PEP	Peptide
PK	Polyketide
PKS	Polyketide synthetase
PLS-DA	Partial least squares discriminant analysis
PoI	Peaks of Interest
QIIME	Quantitative Insights Into Microbial Ecology
QM	Queensland Museum
RNA	Ribonucleic acid
ROESY	Rotating frame nuclear Overhauser Effect spectroscopy
RPM	Revolutions per Minute
TDDFT	Time-dependent density functional theory
TOCSY	Total Correlation Spectroscopy
USC	University of Sunshine Coast
UV	Ultraviolet

ALL PAPERS INCLUDED ARE CO-AUTHORED

Acknowledgement of Papers included in this Thesis

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research (“Criteria for Authorship”), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output, and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:

- conception and design of the research project
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- Include in the list of authors only those who have accepted authorship
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- Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.

Included in this thesis are papers in Chapters 2 and 6 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details (if published or accepted for publication)/status (if prepared or submitted for publication) for these papers including all authors, are:

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CHAPTER 1 - INTRODUCTION

1.1. Microbial natural product in drug discovery

Nature provides an enormous resource for biodiscovery that has been exploited by the pharmaceutical industry. Natural products (also known as secondary metabolites) are a highly diverse group of small molecules derived from living organisms. Unlike primary metabolites such as amino acids, carbohydrates and fatty acids that maintain function and growth of an organism; secondary metabolites are specific to their producer(s) but not essential for survival.¹ The structural complexity of natural products is also closely linked to the ecological role of the producing organism and normally provides greater benefit in survival.^{2,3} Particularly, sessile or slow moving organisms rely more heavily on bioactive secondary metabolites, which act as defences, antimicrobials, allelochemicals, signalling molecules, UV protectants or feeding deterrents.^{4,5} The production of these compounds is energetically costly for the producing organism and thus they represent the most successful chemical structures, which form biologically friendly leads for drug development as they co-evolved with target sites in biological systems.^{1,6} Natural products are made by enzymes and thus present a greater number of chiral centres compared to synthetic compounds and their three-dimensional diversity aligns well with the majority of drugs on the market.² Technological advancements and the advent of new tools such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) have enhanced our understanding of natural products and aided the discovery of thousands of biologically active lead structures, drug candidates, and drugs to treat various diseases.⁷

Alexander Fleming's discovery of penicillin (**1**) from the fungus *Penicillium notatum* in 1928 and its subsequent development into an antibiotic in the 1940s was probably the most significant breakthrough in modern medicine, providing the foundation of drug discovery from microorganisms.⁸ The discovery of penicillin allowed for the treatment of bacterial infections, which until then were often fatal. Since then 120 natural products sourced from microorganisms, mainly from the bacterial order *Actinomycetales* (commonly termed actinomycetes), have found their way into the clinics as antimicrobials (i.e. rifamycin, **2**), anticancer drugs (i.e. actinomycin, **3**) and immunosuppressive agents (i.e. cyclosporin, **4**) (**Figure 1**).

Figure 1. Important microbial drugs: Penicillin G (1), rifamycin (2), actinomycin (3) and cyclosporin (4).

Of all drugs newly approved between 1981 and 2014, 26 % are natural products or natural product derived and 25 % are natural product inspired synthetic drugs.⁹ Of the natural product derived drugs, 30 % were isolated from microorganisms. This highlights the importance of natural products in drug discovery, particularly those from microbial sources.¹⁰ However, the time it takes from the discovery of a natural product until it becomes a marketed drug is very long and rediscovery of already known natural products has become more frequent. In the 1990's, high throughput screening (HTS) of combinatorial compound libraries became favoured by pharmaceutical companies as they are generally easier and cheaper to develop.¹¹ Yet, the surge did not last long and the number of Food and Drug Administration (FDA) approved drugs decreased once pharmaceutical companies began to focus on purely synthetic drugs.¹² Combinatorial compounds only occupy a very small area of chemical space, while natural products cover a much larger area of chemical space which aligns well with that of marketed drugs.² Consequently, natural products and their derivatives remain of immense importance for new drug discovery and development. Nonetheless, to continue the successful exploitation of natural products for drug development, new approaches to increase chemical diversity were needed.

One successful approach to enhance chemical diversity is to target organisms that have not previously been investigated. The high rediscovery of already known natural products from terrestrial bacteria has directed a focus to microorganisms from different ecological niches, particularly the marine environment, which covers 70 % of the earth's surface and provides a vast and highly biodiverse resource for biodiscovery.¹³ The underlying hypothesis in marine biodiscovery is that taxonomic diversity correlates with chemical diversity and in recent years increased research has targeted species from the marine environment, which are taxonomically distinct to terrestrial organisms.¹³ It is believed that the physiochemical properties of the marine environment, including pH, temperature, osmolarity, and pressure, as well as presence of uncommon halogenated functional groups result in natural products with enhanced bioactivities that have quite different properties compared to the terrestrial environment.^{13,14}

A second way to minimise rediscovery is to implement sophisticated dereplication approaches early in the drug discovery efforts. An example of this is pre-screening of microbial strains and extracts with either molecular or spectrometric techniques to avoid repeatedly isolating the same microbial natural products.¹⁵ Another approach to maximise the great potential of bacteria for natural product drug discovery is to diversify culturing conditions. Microorganisms produce natural products in response to environmental stresses; therefore, using a range of different culturing media as well as chemical, physical and biological elicitors can trigger the biosynthesis of new metabolites.¹⁶ In the following, more detail on the approaches to diversify natural product biodiscovery from marine actinomycetes is provided.

1.2. Marine actinomycetes

Actinobacteria are Gram-positive and morphologically diverse bacteria.¹⁷ Currently, intraclass relatedness of the class actinobacteria reveals the presence of nine orders (<http://www.bacterio.net/-classifphyla.html>, accessed 14-08-2017). Accordingly, in this thesis “actinobacteria” will only refer to the “actinomycetes” covering the members of the order *Actinomycetales*. Actinomycetes are often filamentous and present diverse colony morphologies, which according to their function can be divided into areal hyphae, substrate mycelium, and in agar cultures often produce diffusible pigments (**Figure 2**).^{18,19} They inhabit a wide range of habitats from arid deserts to deep ocean sediments and are responsible for the production of many antibiotics, anticancer agents and immunosuppressive compounds.^{20,21} They are known to have symbiotic associations with plants, insects, and marine invertebrates.²²⁻²⁵ Actinomycetes, among other natural product producing bacteria, are the most prolific suppliers of bioactive compounds to the pharmaceutical industry.²⁶

Figure 2. Common mycelial-forming actinomycete morphology on agar (colony cross section showing areal hyphae and substrate mycelium).¹⁹

Studies of marine actinomycetes date back to the 1940s, when the pioneers of marine microbiology, ZoBell and Rosenfeld, recognised the antimicrobial potential of marine microorganisms.²⁷ However, only in the last two decades, extensive research in marine microbiology and chemistry has led to the isolation of many marine actinomycete species and the appreciation of their biosynthetic potential for drug discovery. The species described belong to the genera *Dietzia*, *Streptomyces*, *Salinibacterium*, *Aeromicrobium*, *Williamsia*, *Verrucosipora*, *Kocurea*, and *Polaribacter*, as well as *Salinispora*, *Demequina*, *Salinibacterium*, *Sciscionella*, *Serinococcus* and *Micromonospora*. So far, only a small fraction of actinomycetes can be cultivated from the marine environment. True marine actinomycetes are generally difficult to culture in the laboratory, due to their special growth requirements.^{28,29} However, metagenomic data has revealed 10400 rRNA gene sequences of actinomycetes that are widely distributed throughout the marine environment; 16S rRNA genes have been isolated from the upper water column, different geological sediments as well as marine sponges, ascidians, and seaweeds.³⁰ Development of new cultivation techniques allowed isolation of marine actinomycetes, which have produced novel and structurally diverse chemistry.^{31,32} Examples of bioactive marine actinomycete metabolites are: salinipyrones (anti-tumour, **5**), proximicines (anti-tumour, antibiotic, **6**), daryamides (anti-tumour, **7**) or violaceins (anti-protozoal, **8**) (**Figure 3**).³³⁻³⁶ The most promising discovery was salinosporamide A (**9**) produced by two rare *Salinispora* species. The compound is biosynthesized by an unusual hybrid PKS/NRPS pathway and features a bicyclic ring structure with a chloroethyl group and is functionalised with a cyclohexene.³⁷ Extracts showed IC₅₀ values of less than 2 ng/mL against human colon carcinoma HCT-116 cells and was found to be a highly selective tumour cell growth inhibitor.^{38,53} Salinosporamide A, under the name Marizomib®, is currently undergoing clinical trial phase I in relapsed refractory multiple myeloma.^{39,40}

Figure 3. Examples of bioactive marine actinomycete metabolites: salinipyron (5), proximycin A (6), daryamide A (7), violacein (8), salinosporamide A (9), saliniketol (10) and arenamide A (11).

Marine actinomycetes, like their terrestrial counterparts, have an extraordinary large genome, which points towards a great potential to biosynthesize secondary metabolites with biological activity.⁴¹ The first full genome sequenced from an obligate marine actinomycete species was that of *Salinispora tropica* strain CNB-440, one of the producers of salinosporamide A. The genome is composed of an average G + C content of 69.5 % and 17 secondary metabolic biosynthesis gene clusters, thus devoting approximately 10 % of the genome to the production of natural products.⁴² Genome sequencing of the marine actinomycete *Salinispora arenicola* revealed 39 biosynthetic loci that produce secondary metabolites,⁴³ including saliniketals (10) and arenamides (11).^{44,45} However, it has become apparent that the majority of biosynthetic genes are silent under normal laboratory conditions or only low concentrations of natural products are produced.⁴⁶ Genome sequences of *Streptomyces coelicolor* and *Streptomyces avermitilis*, two extensively studied species, also revealed interesting biosynthetic gene clusters with the potential to produce new natural products that are not yet expressed under the laboratory conditions. Thus, it seems necessary for marine actinomycete drug discovery that new culturing techniques and genome-based natural product mining develop in parallel. The culture-dependent approach is still very important in order to study the expressed bacterial metabolome, and if successful allows rapid assessment of structure, novelty and bioactivity.⁴⁷ In contrast, the culture-independent approach will reveal the full biosynthetic potential of marine actinomycetes.

1.1.1. Ascidian-associated actinomycetes

Ascidians are sessile, filter-feeding marine invertebrates known to produce highly potent bioactive natural products.⁴⁸ These invertebrates are associated with a breadth of associated microorganisms and it has become evident that many of these bioactive metabolites are produced by an associated symbiont.⁴⁹ Didemnin B and ecteinascidin 743, two potent anti-tumour agents originally isolated from ascidians, have been proven to be of bacterial origin and similar evidence was found for about another 80 metabolites.⁵⁰ The associations between microorganisms and ascidians have been described as species-specific and can be of obligate or facultative nature.^{51,52}

Actinomycetes are commonly detected by molecular sequencing techniques in many ascidians.⁵³⁻⁵⁶ Most studies have been unable to conclusively confirm any functional role of ascidian-associated actinomycetes. In an ascidian from the *Microcosmus* genus, actinomycetes were in particular associated with the digestive tract and it was therefore suspected that they were ingested from the water column through feeding.⁵⁷ Actinomycetes play an important role in the digestion of recalcitrant organic matter in terrestrial invertebrates. Consequently, it could be possible that actinomycetes have a digestive functional role to play in ascidians that also feed on plankton containing high amounts of silica.

Furthermore, numerous bioactive compounds isolated from ascidians have remarkable similarities to actinomycete-derived metabolites (**Figure 4**). For instance, the ascidian antitumor compound, namenamicin (**12**), has striking structural similarity to calicheamicins (**13**) and esperamicins (**14**) both synthesised by actinomycetes.⁵⁸⁻⁶⁰ Several staurosporine derivatives (e.g. **15**), a structure class known to be synthesised by terrestrial and marine actinomycetes, have repeatedly been isolated from *Eudistoma* ascidians,⁶¹ pointing towards a functional role of staurosporine production by an associated actinomycetes strain.⁶²⁻⁶⁴ Cultures of ascidian-associated actinomycetes have produced unique and novel chemistry under laboratory conditions; however, most studies were again inconclusive in revealing their functional roles in the ascidian-actinomycete association. In the hunt for the true producers of namenamicin, He and co-workers isolated a halophilic *Micromonospora* species, which produced lomaiviticins A (**16**) and B, two novel dimeric diazobenzofluorene glycosides that exhibit potent antitumor properties.⁶³ A *Streptomyces* sp. strain isolated from *Aplidium lenticulum* at Heron Island, Australia, resulted in the discovery of the aromatic spiroketal polyketide griseorhodin A (**17**)⁶⁵ and *Streptomyces* sp. YM14-060 from an unidentified ascidian from Palau produced the new cytotoxic antibiotics, piericidins C7 and C8 (**18**).⁶⁶ Another ascidian derived *Micromonospora* sp. yielded a new antimicrobial alkaloid, diazepinomicin (**19**)⁶⁷; and peptidolipins B (**20**) –F with antibacterial properties were isolated from an ascidian-derived *Nocardia* species.⁶⁸

Recently, Zhang and co-workers isolated micromonohalimanes A and B (**21a/b**) from a *Micromonospora* sp. associated with the ascidian, *Symplegma brakenhielmi*; this is the first account of such halimane-type diterpenoids from *Micromonospora*.⁶⁹

Figure 4. Diverse secondary metabolites derived or potentially derived from ascidian-associated actinomycetes

Ascidian-associated actinomycetes are still an untapped source for natural product drug discovery, yet the few natural products that have been reported from ascidian-associated actinomycetes to date display an astonishing range of structural complexity, thus highlighting their potential for new drug discovery. The question whether actinomycetes play a functional role in the ascidian secondary metabolism is yet to be resolved. Much evidence points towards them playing a part in the metabolism, but more extensive studies, with newly developed culturing conditions that mimic ascidian-actinomycete associations more closely are needed to shed more light onto this mystery.

1.2. Potential and hurdles in marine (microbial) drug discovery

The re-supply of bioactive compounds derived from natural sources poses a major challenge, as natural populations of marine invertebrates are too small and often only minute amounts of the natural products are produced by the organisms.⁷⁰ Nonetheless, natural product drug discovery has come a long way and it is now an interdisciplinary field which combines traditional natural products chemistry, synthetic chemistry, microbial- and molecular biology, metabolomics and toxicology. The union of these disciplines has resulted in a number of success stories.

One of the most significant true marine drugs is the ascidian-derived anti-tumour agent trabectedin (**22**), marketed as Yondelis®. This compound was the first drug to be directly sourced from the marine environment. However the producer *Ecteinascidia turbinata* only yielded 0.0001 % of this compound.⁷¹ Five grams of the natural product were needed for clinical trials, and to produce this amount of compound, an unsustainably large quantity of tunicate biomass (5 tonnes) was required.⁷² Such extensive harvesting of the marine invertebrates can be restricted due to the general shortage of the marine organisms and can have adverse effects on the environment.⁷⁰ In the most extreme case, extinction of the target species could result.⁷³ Aquaculture and mariculture was then implemented to retrieve more biomass of that ascidian *Ecteinascidia turbinata*.⁷¹ However, yields of in-sea culturing are affected by environmental factors and often low, furthermore, diseases can spread easily; therefore, the pharmaceutical company PharmaMar needed to develop a new approach for full-scale production of the compound.⁷⁴ The structure of trabectedin was inherently similar to the base structures of safracins (**23**) and saframycines (**24**) both derived from terrestrial bacteria, indicating that **22** was of microbial origin (**Figure 5**).^{75,76} PharmaMar established a semisynthetic process starting with the bacterial metabolite safracin B.⁷¹ Total synthesis was also finally described, but the

semisynthetic approach provided higher yields as well as additional related compounds.^{71,77} In 2007, 38 years after the original discovery of the compound, Yondelis® was approved as treatment for soft tissue sarcoma.

Figure 5. Trabectedin (22) and related safracin A (23) and saframycine A (24)

Equally impressive was the story of how eribulin became the marketed drug Halaven® for metastatic breast cancer. Halichondrin B was originally isolated from a marine sponge and exhibited very potent activity against murine leukaemia cells. Again, the supply of sufficient material for clinical development was the limiting factor.⁷⁸ Total synthesis was achieved by Aicher and co-workers⁷⁹, which was the most complex synthesis ever achieved. Subsequent research identified the active pharmacophore, which led to the synthesis of a simplified macrocyclic ketone analogue named eribulin mesylate with the same level of activity.^{72,80} This success story was the work of many international collaborators from industry, academia, and government, ultimately resulting in the approval of Halaven® in 2010.⁷⁸

As it becomes more evident that microorganisms are synthesising many natural products, cultivation of those microorganisms and heterologous gene expression techniques, where genes are expressed in a host organism, hold valuable alternatives to chemical synthesis or extraction from marine invertebrate sources.⁷³ For example, the cytotoxic patellamides A (**25**) and C (**26**) (**Figure 6**), originally produced by the cyanobacterium *Prochloron didemni*, could be produced through heterologous gene expression in *E. coli*.⁵¹ While this approach seems very promising, at present it still has its constraints as not all genes will be expressed in all hosts, and it is likely that the target genes need specific chemical or environmental cues in order to be expressed.⁸¹ In conclusion, the supply problem is still the biggest hurdle to drug discovery from marine natural products, but examples like Yondelis® and Halaven® bring great hope to the natural product research community. Natural products possess many valuable and unique features and there is a continuous interest in the discovery of new organisms, especially from unusual ecological niches.⁸² New analytics and biotechnologies will further impel the field.

Figure 6. Patellamides A (25) and C (26) from ascidian-associated cyanobacteria

1.2.1. Dereplication efforts

Natural products are undoubtedly an important source of new drugs;⁹ however, biological screening, large-scale fermentation, isolation and structure elucidation are very resource- and time-intensive. Frequent rediscovery of already known natural products in the past decades has resulted in a decline in the use of natural products as sources for pharmaceuticals. Therefore, new analytical tools were required. In order to minimise rediscovery, efficient dereplication protocols have to be implemented early in natural product isolation efforts. Dereplication is the identification and elimination of known metabolites within samples that are targeted for new natural product discovery.⁸³ This is particularly important for microbial natural products, where microbial strains cannot easily be distinguished through morphological features and it is not guaranteed that strains that optically appear different also produce different secondary metabolites. Conversely, strains that look the same might be able to produce totally distinct metabolomes. Dereplication can either be performed at the level of the microorganism or the chemical extracts.

1.2.1.1. Taxonomic dereplication

Molecular techniques currently allow dereplication at the genetic level. For decades, cluster analysis has been utilised to identify and dereplicate bacterial species. 16S rRNA sequencing is a widely accepted technique to identify environmental bacteria by using a common gene that possesses the universal primer of all bacteria that is distinct to the small subunit rRNA of eukaryotic organisms.⁸⁴ Different microbial species have varying regions within the 16S rRNA sequence that, when matched up against 16S gene databases, can be utilized for identification. Even microorganisms that cannot be identified to the species level will at least be placed in a group of related organisms.⁸⁴ The American National Centre for Biotechnology Information (NCBI) offers an extensive open source database of nucleotide sequences and offers a basic

local alignment search tool (BLAST) that matches input sequences against all deposited sequences.⁸⁵ However, the ability to biosynthesise certain metabolites can vary even between different strains of the same taxonomic species;⁸⁶ therefore, culture dependent methods that pre-screen microbial crude extracts are more commonly utilised by natural product chemists to evaluate the true potential of each bioactive strain.

1.2.1.2. Chemical dereplication

Traditionally, sample selection was based on biological pre-screening of the crude extract and subsequent bioassay-guided fractionation was used to identify the bioactive metabolites in the sample.⁸⁷ This method is challenged by the repeated isolation of known natural products as it does not provide any chemical information about the active compounds in the extract. Recently, analytical spectroscopic and spectrometric data screening of the entire metabolome of an organism has become more popular in order to dereplicate samples, and more dereplication databases have emerged.^{15,88} Amongst others these include the Dictionary of Natural Products, AntiBase, MarinLit, GNPS and DEREPI-NT.⁸⁸⁻⁹¹

The two most common metabolomics profiling tools are LC-MS/MS and NMR profiling.⁹² The advantages of mass spectrometry are the high resolution and sensitivity, as well as the possibility to use MS/MS fragmentation. However, the technique is highly dependent on ionisation of compounds, is difficult to quantify and replicate between different instruments and thus not universally applicable.⁹² NMR techniques, on the other hand, have much lower resolution and sensitivity compared to MS, but NMR is non-selective, non-destructive, and quantitative, it requires minimal sample handling and undeniably its greatest advantage is that it provides structural information about the constituents of the extracts.⁹²

Metabolomics was originally aimed at studying the total metabolomic processes within organisms and mostly applied in the human health sector, but also plant research has advanced the field of metabolomics.⁹³ More recently its application has become appreciated in natural products drug discovery, particularly for the purpose of dereplication and mode of action studies.⁹³ Chemometric-metabolomics profiling approaches provide an overview of the expressed metabolites in extracts, reduce redundancy and can be used for large datasets without comprehensive fractionation. In chemometric-metabolomics approaches, the chemical profiles are subsequently analysed using multivariate statistics, such as Principle Component Analysis (PCA) or Partial Least Squares Discriminant Analysis (PLS-DA). PCA is an unsupervised method that reduces multivariate data to a few principle components, while PLS-DA is a supervised technique that determines the variation within a dataset based on classification labels.⁹⁴ These techniques produce a scores plot that allows visualisation of the spectral

variations of the samples. Samples containing the same or similar chemical profiles will cluster together while differing profiles will cluster away from each other.

Hou and co-workers⁹⁵ analysed a microbial collection of 47 samples using LC-MS and utilised PCA to prioritise chemically unique microbial strains for natural product discovery. They noted that one of the limiting factor to this analysis is that it is not readily applicable for large datasets and rather proposed a successive analysis of a smaller number of microbial isolates (20 – 50 at a time).⁹⁵ Even in their analysis of 47 isolate samples, over 25000 bucket variables derived from these samples and 74 principle components were needed to explain 98% of the variation in the dataset, indicating that the first two principle components most likely only accounted for a very small percentage of the variation in the data. Nonetheless, the group successfully selected a microbial strain that produced new natural products (**21a/b**, **Figure 4**).⁶⁹ MS-based metabolomics approaches are now more commonly used to aid natural product discovery.⁹⁶⁻¹⁰¹ LC-MS data is easily matched against databases; however, if a compound is a 'hit' that does not match the database, LC-MS data does not provide any information about the compound except for its molecular mass. More recently, molecular networking based on MS/MS fragmentation patterns that allows assignment of structural classes to unknown compounds has gained a lot of popularity.^{102,103}

In recent years, NMR advancement led to faster acquisition through stronger magnets and made this technique more powerful and applicable for metabolomics studies.^{82,104} Yet, only few natural products studies have used NMR-based metabolomics as a dereplication tool. Chen and co-workers¹⁰¹ used ¹H-NMR as well as LC-MS data for metabolomic profiling of sponge-associated actinomycetes. Thereby, ¹H-NMR data of 64 microbial isolates was binned into 0.01 ppm integral regions and the resulting peaks were analysed via PCA. Comparison to LC-MS PCA revealed some overlap between the two analytical methods, which both identified a *Streptomyces* sp. to contain unique chemistry.¹⁰¹ Schroeder and his co-workers used differential analysis of double quantum filtered correlation spectroscopy (DOF-COSY) spectra, which they graphically analysed based on stacking of spectral bitmaps to identify of unique peaks.¹⁰⁵ This was followed by detailed analysis of the unique signals and their corresponding spin-systems, which for a number of compounds was sufficient for their elucidation.¹⁰⁵ For more complex structures, additional HSQC, HMBC, and NOESY spectra were acquired, which identified two new natural products.¹⁰⁵

1.2.2. One strain many compounds (OSMAC) approach

Whole genome sequencing has revealed the true genetic potential of microorganisms; however, only a fraction of the biosynthetic genes are transcribed under laboratory conditions and many biosynthetic genes remain silent.^{81,106} Considering the functions of natural products in microbes, it is assumed that every natural product is a result of interactions of the organism with its environment.^{107,108} Manipulation of culture conditions with chemical or physical elicitors can exert stresses on the microbial culture and as a result lead to enhanced production of secondary metabolites. In 2002, Bode and co-workers¹⁰⁹ reported that through small changes such as media composition, aeration, culture vessel or the addition of enzyme inhibitors, large effects on the secondary metabolite production could be observed. Twenty different metabolites could be isolated from just one strain and they gave this approach the term “one strain – many compounds (OSMAC)”.¹⁰⁹ Nowadays, this approach is common practice and has resulted in the isolation of natural products with enhanced chemical diversity.

1.2.3. Elicitation of the microbial metabolome: community cultures

In the environment, microorganisms usually live in diverse microbial communities. Inter- and intraspecific interactions of microorganisms may stimulate enhanced natural product synthesis.¹⁰⁷ The specific mechanisms of these interactions are not fully resolved, although four different mechanisms have been proposed by Abdelmohsen and co-workers.¹¹⁰ Metabolite synthesis may be triggered through physical cell to cell interactions, small molecule mediated interactions, enzymes produced by one species that activate the metabolite precursor of another species. Alternatively, metabolite production could be made possible through gene transfer between two different species.¹¹⁰ Community cultures (co-cultures) of two or more different microorganisms intends to mimic such interactions in the laboratory.¹⁰⁶ Several studies have exploited co-cultivations on solid agar or mixed liquid fermentations to induce synthesis of bioactive natural products that are not expressed in standard pure cultures.¹¹¹⁻¹¹⁴ Cueto and co-workers¹¹⁴ demonstrated that mixed fermentations stimulated the production of a new compound, pestalone (**27**, **Figure 7**), in a marine *Pestalotia* species when co-cultured with an unidentified antibiotic-resistant marine bacterium. Pestalone, which is active against *Staphylococcus aureus* and *Enterococcus faecium*, was not detected in monocultures of the *Pestalotia* strain.¹¹⁴ While these techniques allow interactions between different members of the cultured communities that could trigger the production of promising lead structures for biomedical research, co-cultures are very dynamic and therefore difficult to reproduce. Furthermore, this technique still does not provide other variables of the source environment that in nature stimulate the production of secondary metabolites.¹⁰⁶

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Figure 7. Pestalone (27), a new natural product triggered through co-cultivation

1.3. Bioactivity target: the malaria parasite

Malaria is a vector-borne infectious disease, caused by a protozoan parasite from the genus *Plasmodium*, with *Plasmodium falciparum* causing the most severe form of human malaria. The malaria parasite was responsible for almost half a million human deaths in 2015 mostly from developing countries of Africa, Latin America and South-East Asia.¹¹⁵ Humans contract malaria when a female *Anopheles* mosquito feeds on human blood. As the mosquito takes a blood meal, *Plasmodium* sporozoites in its saliva are simultaneously injected into the body, which then travel to the liver.¹¹⁶ Here, they bind to negatively charged sugars and asexually reproduce to give rise to thousands of merozoites that invade red blood cells, where the intra-erythrocytic cycle begins.¹¹⁷ Within the red blood cells, merozoites develop into the early trophozoites, also known as the ring form. The mature trophozoites asexually multiply, ultimately forming 16 - 32 daughter merozoites. The production of daughter merozoites causes the red blood cell to rupture, releasing merozoites into the bloodstream. At this stage the disease manifests and the human host experiences symptoms of strong fevers and chills.¹¹⁸ Some merozoites then re-invade new red blood cells, repeating the cycle. Inside the red blood cells, a small portion of merozoites will form schizonts, producing male and female gametocytes. The gametocytes are transmitted back to a mosquito as it takes a blood meal and there they fuse to form oocysts, which divide into sporozoites. These are then transferred to the mosquito's saliva glands and the life cycle of the parasite begins again once the mosquito feeds on a new human's blood (**Figure 8**).

Figure 8. Life cycle of *Plasmodium* parasite¹¹⁸

The majority of drugs to treat malaria are natural products derived from plants. Quinine (28), chloroquine (29), artemisinin (30) and its derivatives, and antifolates are the most commonly used anti-plasmodial drugs, but resistance is emerging (**Figure 9**). All currently available antimalarial drugs have similar modes of actions: antifolate drugs target the folate biosynthesis pathway, quinoline-type compounds interfere with enzymatic conversion of toxic ferric haem into to a non-toxic form resulting in cell lysis of the parasite, and artemisinin blocks the production of phosphoinositol.¹¹⁹ Resistance to chloroquine, the cheapest and safest antimalarial drug, was first reported in Asia in 1957 and by 1970 chloroquine-resistance was spread throughout Asia, Africa, and South America. As a consequence, antifolate combination drugs were developed; however, the *Plasmodium* parasite developed resistance soon after their development. Artemisinin brought new hope to treat the disease and artemisinin mono and combination therapy proved effective against the parasite. In 2005, the World Health Organisation reported resistance to artemisinin at the Thailand-Cambodia boarder and advised withdrawal from monotherapy. The spread of artemisinin resistance presents a major threat to millions of people infected with malaria every year; new drugs with novel targets are urgently needed.

Figure 9. Anti-plasmodial natural products

Marine natural products have been a new focus on finding novel anti-plasmodial agents and also marine bacteria have yielded anti-plasmodial natural products.¹¹⁹ Salinosporamide A (**9**), from the marine actinomycete *Salinospora tropica* demonstrated potent activity against chloroquine-sensitive and resistant *P. falciparum* clones. In a mouse model, growth inhibition of the parasite was observed at very low doses of 130 $\mu\text{g/kg}$.¹²⁰ The long-chain bicyclic phosphotriester compounds salinipostins A (**31**) - K isolated from the marine-sediment derived *Salinospora* species exhibited anti-plasmodial activity, with salinipostin A having the lowest EC_{50} (50 nM) and high selectivity against mammalian cells.¹²¹ In a study by Na and co-workers¹²², a marine *Streptomyces* sp. strain was identified that produced an anti-plasmodial polyether (**32**) (0.145 to 0.29 μM). Abdehlmosen and co-workers¹¹⁹ reviewed marine natural products and their drug potential that have biological activity against drug-resistant pathogens. It was found that the majority of the marine natural products, including several marine microbial compounds and invertebrate compounds that are likely to be synthesised by a microbial symbiont, exhibit drug-like properties as they adhered to the Lipinski's rule of five. Fewer anti-plasmodial compounds showed good oral bioavailability; particularly the salinipostins were unfavourable due to their high number of rotatable bonds, thus these characteristics should be assessed thoroughly before pursuing development.¹¹⁹ Nonetheless, the authors state that new discoveries of natural products from the marine environment, particular actinomycetes and sponges, bring new hope for the treatment of drug-resistant infections.

1.4. Overview and aims of the thesis

The overarching aim of this thesis was to integrate and explore different biodiscovery approaches to maximise natural product discovery from marine actinomycetes (**Figure 10**). The thesis consists of seven chapters: a general introduction, five results chapters and a conclusion & outlook chapter. This thesis has been prepared in accordance with the Griffith University policy on inclusion of papers within a thesis. The results chapters 1 - 5 are written in form of manuscripts in preparation (Chapters 3 - 5), submitted (Chapter 2) or that have been accepted (Chapter 6) in peer-reviewed academic journals. Each of the chapters can stand alone and therefore, there is some degree of repetition among the introductions, methods, and results of these chapters.

In the light of the above presented introduction, it has become apparent that the major issue of microbial natural product drug discovery is the high rediscovery of already known compounds. To account for this issue, this PhD project focused in the first section on the isolation of actinomycetes associated with three Australian marine ascidians, an underexplored ecological niche, in order tap into new bacterial and chemical diversity. Section 2 looks at diversifying microbial natural products for drug discovery. It was envisaged that a new innovative metabolomics strain selection approach should then be implemented to identify actinomycete strains within the established strain library that are more likely to produce new bioactive metabolites and exclude redundant strains to minimise repeated isolation of the same compounds. Furthermore, the effect of co-cultivation on the chemical diversity of microbial extracts was evaluated using the newly developed metabolomics approach. Classic natural product chemistry isolation and structure elucidation approaches were then implemented in Section 3 to characterise new anti-plasmodial actinomycete natural products (**Figure 10**).

Figure 10. Integrated approaches to characterise new bioactive metabolites

The thesis specifically aims to make the following scientific contributions:

1. Establish an isolate library of ascidian-associated actinomycete strains and evaluate their chemical potential. (Chapter 2)
2. Develop a new dereplication/strain prioritisation approach that utilised two-dimensional NMR profiling and anti-plasmodial activity data to identify microbial strains that have greater potential to produce anti-plasmodial secondary metabolites. (Chapter 3)
3. Enhance chemical diversity through co-culturing and identify changes in the actinomycete secondary metabolome through 2D NMR profiling. (Chapter 4)
4. Isolate and elucidate new anti-plasmodial peptide and polyketide natural products from selected marine actinomycete strains. (Chapters 5 and 6)

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SECTION 1

Untapped ecological niche: Ascidian-associated actinomycetes

A powerful approach to enhance chemical diversity and discover novel natural products is to explore new sources of organisms that have not previously been investigated. Historically, microorganisms from the terrestrial environment have been a very successful source for biodiscovery and numerous compounds derived from terrestrial microorganisms have become marketed drugs. On the contrary, the marine environment contains an incredibly rich and vast biodiversity, most of which still remains to be uncovered. In Chapter 2 of this thesis, I set out to explore actinomycetes associated with the tree Australian ascidians, *Symplegma rubra*, *Aplidium solidum* and *Polyclinum vasculosum*, collected from Hastings Point in Northern New South Wales, Australia. This region, unlike the Great Barrier Reef, has not received a lot of attention for biodiscovery. Yet, it is shaped by intermixing of tropical waters coming from the north and temperate waters from the south making it a unique target for biodiscovery. Furthermore, the microbial communities associated with the three chosen ascidian species have not previously investigated. Through this work I wanted to answer the following questions: Are there any actinomycetes associated with these three ascidian species? What families of actinomycetes are associated with these ascidians and can they be cultured? Are the ascidian-associated actinomycetes a good source for natural product drug discovery and biotechnology?

CHAPTER 2 - Taxonomic and metabolite diversity of actinomycetes associated with three Australian ascidians

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L.Buedenbender designed the experiments; organised the sampling event, performed the experiments, conducted molecular work, analysed the microbiome, as well as microbiological and chemical data and wrote the manuscript; D. I. Kurtböke and A. R. Carroll are the supervisors of the PhD project and provided advice on the research design and feedback. M. Ekins identified the ascidian specimens to species level.

(Signed) _____ (Date) 01/09/2017

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2.1. Abstract

Actinomycetes are known to be the most prolific producers of biologically active metabolites. Here, we investigated the host species-specificity and related secondary metabolites of actinomycetes associated with three different Australian ascidians, namely *Symplegma rubra*, *Aplidium solidum* and *Polyclinum vasculosum*. Results indicated that while isolates from the genera *Streptomyces* and *Micromonospora* were highly diverse in the ascidian samples, only two culturable actinomycete OTUs overlapped between all of the ascidians, pointing to some degree of host species-specificity of the isolates and selective acquisition of microbial associates by the host from the surrounding environment. LC-MS/MS profiling of extracts obtained from the ascidians and their actinomycete associates revealed many overlapping ions between hosts and actinomycetes indicating that these compounds were likely to be synthesised by the microbial associates. Laboratory cultures of the actinomycetes displayed even more diverse metabolomes than those of their ascidian hosts; thus, making ascidian-associated actinomycetes an excellent target for natural product drug discovery and biotechnology.

Keywords: Actinobacteria, Actinomycetes, *Actinomycetales*; marine microbial diversity; ascidian-associated actinomycetes; secondary metabolites

2.2. Introduction

Ascidians (phylum: Chordata, sub-phylum: Tunicata) are soft-bodied, sessile, filter-feeding marine invertebrates that often rely on secondary metabolites for their defence.¹⁻³ These compounds often show potent biological activity and thus have been recognised for their pharmaceutical and biotechnological potential. Some striking examples include ecteinascidin marketed as Yondelis®, and others undergoing clinical trials are plitidepsin and lurbinectedin.⁴ Even though ascidians have immense potential for the discovery of bioactive metabolites, sustainable supply of these bioactive molecules from wild harvesting of these animals is difficult. This is because ascidians often live in large colonies of thousands of individuals, but are only a few millimetres thick.

Increasing evidence has shown that associated bacteria are the true producers of many of the ascidian's bioactive metabolites.^{5,6} Therefore, isolation of microbial associates provides a sustainable source of supply for the development of novel pharmaceutical drug leads, as it permits fermentation-based production of natural products rather than the harvest of enormous amounts of marine invertebrate biomass.^{7,8} The composition of the microbiome associated with the ascidian host has been shown to be species-specific, as well as influenced by the physiological environmental at the geographical location.^{9,10} Currents, preceding weather events,

and vicinity to urbanisations are some of the factors that can impact acquisition of the microbial associates by the invertebrate host during filter feeding. These factors ought to be considered when obtaining samples in order to identify the specificity of ascidian-associated bacteria that are producers of diverse bioactive metabolites found in the host.¹¹

Even though ascidians and microorganisms are known to associate, the true nature of the relationship between the bacteria and the ascidian host remain mostly unresolved. Obligate symbiosis has been confirmed between the alpha-proteobacterium, *Candidatus* Endoecteinascidia faulkneri and the ascidian *Lissoclinum patella*. The symbiont lives intracellularly in the blood cells of the host and is vertically transmitted over generations; as a result, the bacterium has lost its original shape and the genome has degraded to solely produce bioactive secondary metabolites.^{12,13} Symbiotic relationships have also been defined for the cyanobacterium, *Prochloron didemni*. However, in this case the symbionts are found extracellularly and the bacterium has been associated with nutrition of fixed carbon and the recycling of nitrogen.^{14,15} Additionally, genes responsible for the synthesis of highly toxic cyanobactins were identified in the *P. didemni* genome, but could not be found in the didemnid ascidian genome.^{5,16,17} Chemistry of didemnid ascidians often varies between animals; therefore, an ascidian species-specific combination of horizontal and vertical transmission of symbiotic bacteria has been proposed.^{16,18}

Actinobacteria have been reported in marine environments and in associations with ascidians.¹⁹⁻²¹ Current intraclass relatedness of the class Actinobacteria reveals the presence of nine orders (<http://www.bacterio.net/Actinomycetales.html>, accessed 14-08-2017). Accordingly, “Actinobacteria” in this paper will only refer to the “actinomycetes” covering the members of the order “Actinomycetales” in the historical sense. It will also include the newly created orders *Frankiales*, *Geodermatophiales*, *Kineosporiales* and *Micrococcales* (<http://www.bacterio.net/Actinomycetales.html>, accessed 14-08-2017) as they were part of this order until recently.²²

Actinomycetes are known as prolific producers of bioactive metabolites, and several bioactive secondary metabolites have been isolated from ascidian-associated actinomycetes.²³⁻²⁷ However, most studies are inconclusive in relation to revealing any functional role of ascidian-associated actinomycetes. In most cases, the actinomycete metabolites could not be detected in their ascidian host extracts, leading to the conclusion that actinomycetes do not play an important role in secondary metabolism symbiosis and their associations to ascidians might be of facultative nature where actinomycetes are horizontally transmitted from the environment.⁶ In contrast to these findings, several staurosporine derivatives, a structure class known to be synthesised by terrestrial and marine actinomycetes, have repeatedly been isolated from

Eudistoma ascidians,²⁸ pointing towards a functional role of staurosporine production by a symbiotic actinomycetes strain.²⁹

The Polyclinidae and Styelidae are two of three ascidian families from which the majority of bioactive natural products have so far been isolated, many of which are likely to be of microbial origin.³⁰ Yet, few attempts have been made to investigate their microbial associates. Herein, we selected *Aplidium solidum* and *Polyclinum vasculosum* both belonging to the same taxonomic family Polyclinidae, which are abundant species at Hastings Point in Northern New South Wales, Australia, as well as *Symplegma rubra* (Styelidae) from the same marine environment to explore species-specificity of their associated actinomycete diversity that had not previously been examined. As it is suggested that actinomycetes are horizontally acquired from the environment, we hypothesised that consequently their diversity should greatly overlap between the three different species sampled at the same location. In order to evaluate this hypothesis, we initially examined the overall microbial diversity associated with the three Australian ascidians to identify the presence of actinomycetes based on a culture-independent approach. We then focused our culturing efforts to isolate actinomycetes and conducted molecular networking of actinomycete extracts as well as ascidian extracts to depict their metabolic diversity and identify metabolites of the ascidian metabolome that are potentially synthesised by an actinomycete associate.

2.3. Materials and Methods

2.3.1. Site description and ascidian collection

Hastings Point is a rocky shore habitat that receives warm waters from the East Australian Current containing tropical marine invertebrate larvae as well as more temperate waters cycling back from the South.³¹ The intermixing of cold and warm waters results in a rich and unique biodiversity of marine invertebrates, predominantly ascidians, making the location a prime target for biodiscovery. For this study, three different ascidian specimens were collected. *Aplidium solidum* (QM G335842) and *Polyclinum vasculosum* (QM G335843), both belonging to the same taxonomic family Polyclinidae, were obtained on 13 July 2015 from rock pools at depths between 1 – 2 m at Hastings Point, New South Wales, Australia (28° 22' S, 153° 35' E). *Symplegma rubra* (QM G335841), family Styelidae, was collected by snorkel at a depth of 6 m at a rocky reef approximately 200 metres offshore from Hastings Point headland (Site 1, **Figure 1a**).

All three ascidians are colonial species with microsize tunics (approximately 1 - 4 mm thick). The samples were collected at low tide under a Scientific Collection Permit (Permit No: P09/0031-3.0) obtained from the New South Wales Department of Primary Industries. Ascidian samples were placed into separate plastic zip lock bags containing seawater and were transported to the laboratory on ice within 2 hours of sampling. About 1 - 16 g of ascidian tissue was exhaustively extracted with methanol to obtain a crude chemical profile of each specimen. Preserved ascidian specimens were deposited at the Queensland Museum, Brisbane (<http://www.qm.qld.gov.au/>) where they were identified taxonomically at the species level.

Figure 1. The Northern New South Wales coastline and *in-situ* aspects of the Hastings Point rock pools (a) Location of Hastings Point along the Northern New South Wales coastline and sampling sites;³² (b) Topography of the rocky reef at site 1; (c) *S. rubra*; (d) *A. solidium*; (e) *P. vasculosum*.

2.3.2. Culture-independent microbial diversity analysis

Three 1 mm³ cubes were cut out from different locations of each ascidian specimen to allow a representative sample of the whole organism. The tissue sections were washed with sterile seawater and microbial DNA was extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals). The extracted DNA was sent to the Australian Genome Research Facility (AGRF) for microbial diversity Illumina sequencing. The DNA samples were amplified using the primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) for the V3 and V4 regions of the 16S rRNA gene. Forward and reverse microbiome sequences were quality filtered with Trimmomatic and joined using PandaSeq.^{33, 34} In the software package Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1, the joined sequences of all three ascidian microbiomes were combined to one fasta file using the command `add_qiime_labels.py`.³⁵ The workflow script `pick_de_novo_otus.py` was used to pick *de novo* OTUs (Operational Taxonomic Units) based on 97% sequence similarity; one representative sequence for each OTU was selected and used to assign taxonomy; subsequently, OTU sequences were aligned to build a phylogenetic tree. The `summarize_taxa_through_plots.py` script was run to group OTUs to phylum, class and family levels; these tables were exported to Microsoft Excel to illustrate the phylum assignments as a bar chart. Alpha rarefaction plots were generated through `alpha_rarefaction.py`. The sequences of OTUs belonging to actinomycetes were individually searched against the BLASTn database to confirm family level assignments.

2.3.3. Culture-dependent actinomycete isolation and identification

The ascidian specimens were first subjected to surface sterilization by rinsing five times with sterile seawater to remove transient and loosely attached bacteria. Each ascidian was cut into small pieces of 5 mm³ that were briefly rinsed with 70 % EtOH and then again rinsed with sterile seawater. The ascidian tissue was homogenised with a sterile mortar and pestle with 10 mL of sterile seawater; the extract and ascidian tissue were transferred to a 15 mL Eppendorf tube and vortexed for 10 minutes. The supernatants were then diluted (1:2 and 1:20) and 250 µL were plated in duplicates on agar plates. Five different microbiological media; Difco marine agar 2216 (Difco Laboratories, USA), Starch Casein Agar (SCA; 10.0 g starch, 2.0 g K₂HPO₄, 2.0 g HNO₃, 2.0 g NaCl, 0.3 g casein, 0.05 g MgSO₄·7H₂O, 20.0 g agar),³⁶ Starch Agar (SA; 10.0 g glucose, 10.0 g potato starch, 10.0 g corn starch, 3.0 g yeast extract, 4.0 g peptone, 4.0 g beef extract, 33.3 g artificial sea salt, 20.0 g agar),³⁷ Corn Starch (CS; 20.0 g corn starch, 10.0 g glucose, 10.0 g peptone, 6.0 g yeast extract, agar 20.0 g),³⁷ *Nocardia histidans* medium (NH; 10.0 g yeast extract, 10.0 g glucose, 0.95 g Na₂HPO₄, 0.91 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 20.0 g agar),³⁸ were used for isolation of actinomycetes. All media were supplemented with 50 µg/mL cycloheximide and 50 µg/mL nystatin to prevent fungal growth as well as 25 µg/mL

nalidixic acid to inhibit the growth of background non-actinomycete bacteria. After 8 days of incubation at 28 °C, actinomycete colonies were picked from the plates and re-streaked on fresh Oatmeal Agar plates (OMA; 20 g oatmeal, 20 g bacteriological agar, 3 g yeast extract)³⁹ for purification and morphological investigation; this was repeated until pure cultures were obtained. For long term storage actinomycete isolates were kept in 20 % glycerol at -20 °C and -80 °C.⁴⁰

2.3.4. Molecular identification and phylogenetic analysis of actinomycete isolates

Actinomycete DNA was extracted from individual colonies using the DNeasy Blood and Tissue Kit (Qiagen, Germany) per the manufacturer's instructions for gram-positive microorganisms. PCR was performed to amplify the 16S rRNA gene, therefore, 4 µL of DNA extract were used as template in 40 µL solution containing 1 mM of forward primer 27F (AGAGTTTGATCMTGGCTCAG) and 1 mM of reverse 1492R (TACGGYTACC TTGTTACGACTT), 20 µL of TopTaq Master Mix (Qiagen, Germany), and 14 µL of sterile H₂O. Amplification was performed on a Thermocycler 100 (BioRad, USA) under following conditions: initial denaturation (10 min at 95 °C); followed by 25 cycles of denaturation (10 sec at 95 °C), primer annealing (15 sec at 55 °C), primer extension (1:20 min at 72 °C); and final extension (10 min at 72 °C). To test whether the amplification was successful, gel electrophoresis was conducted at 100 V for 45 minutes on 1.2 % agarose gel in 0.6 % TAE buffer stained with ethidium bromide. The presence of the 16S rRNA fragment was inspected under UV light using a Bio-Rad ChemiDoc XRS+ imaging system. Sequencing was performed by Macrogen Inc., Korea (<http://macrogen.com/eng/>). Actinomycete DNA sequences were pairwise aligned and trimmed in Geneious R6.1.8; the data was then searched against the National Center for Biotechnology Information (NCBI) nucleotide database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to find the closest relative based on %-identity and query cover scores. All associate sequences were multiple aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation).⁴¹ The best evolutionary model was determined based on the lowest BIC (Bayesian Information Criterion) and AIC (Akaike Information Criterion) values. A Maximum Likelihood phylogeny was constructed in MEGA 7.0.21 using the model GTR+I+G. The 16S rRNA sequence for *Bifidobacterium angulatum* was downloaded from NCBI and used as an outgroup. The robustness of the phylogenetic tree was evaluated by bootstrap analysis with 1000 re-samplings. Furthermore, the sequence data was imported into QIIME, *de novo* OTUs were established using pick_de_novo_otus.py based on 99.9% sequence similarity.

2.3.5. Metabolite diversity

The 120 actinomycete isolates were incubated in Corning® MiniBioreactors with 30 mL ASW-A (20.0 g soluble starch, 10.0 g glucose, 5.0 g peptone, 5.0 g yeast extract, 5.0 g CaCO₃ per litre of artificial seawater) at 28 °C and 250 rpm. After 2 weeks, the tubes were centrifuged at 8000 rpm for 10 minutes, and the liquid media supernatant was collected. Cells were washed with 15 mL chilled deionised water, vortexed and centrifuged at 8000 rpm and again supernatant was collected. Methanol (15 mL) was added to the harvested microbial cell pellet and the mixture was incubated on a rotational shaker for 2 h at room temperature and 200 rpm. The liquid phase was separated by centrifugation (8000 rpm, 5 min) and subsequently collected. The methanol extraction of the cells was repeated with rotation overnight. The combined extracts were filtered using a 0.22 µm-pore-size filter and dried under pressure (GeneVac Technologies HP-12). Methanol was chosen as the extraction solvent due to its amphiphilic characteristics that enable extraction of a very broad range of polar and non-polar metabolites.

Microbial and ascidian crude extracts were prepared at 2 mg/mL and analysed on a 6530 Accurate Mass Q-TOF spectrometer (Agilent Technologies) workstation coupled to an Agilent 1260 LC system using positive electrospray ionization. A Kinetex® C₁₈ 5 µm 100Å HPLC column (100 x 4.6 mm) was used under chromatographic conditions from 0 - 100 % ACN/ 0.1 % FA over 20 min. The Q-TOF MS was set to positive mode mass range *m/z* 100 – 1700, MS scan rate 3 spectra/sec, MS/MS scan rate 2/sec, fixed collision energies at 10, 20 and 40 keV. Source parameters were set to gas temperature 300 °C, gas flow 10 L/min, nebulizer 35 psig; and scan source parameters were: VCap 3500, nozzle voltage 1500 V, fragmentor 130 V, skimmer 65 V, and octopoleRFPeak 750 V.

Every fifth acquired sample was followed by a blank. At the beginning and end of the LC-MS/MS acquisition, a 10 µg/mL mixed pesticide standard (acidic and basic, Agilent Technologies) was tested as a quality control. Data was inspected for integrity in MassHunter software (Agilent Technologies). For data analysis, all MS/MS data was converted from Agilent MassHunter data files to mzXML file format using MSConvert. The data was uploaded to the GNPS server (gnps.ucsd.edu). A molecular network was created using the online workflow at GNPS. The data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra. Consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 4 matched peaks. Further edges between two nodes were kept in the network only if each of the

nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 4 matched peaks.⁴² The molecular network was visualised in Cytoscape 3.3.0., where all mass ions associated with the blanks and mixed pesticide standard were removed from the network. Nodes were coloured per actinomycete taxa group or ascidian group. The network figure was edited in Inkscape 0.92 freeware.

Euler-Venn diagrams were generated with eulerAPE v3 ([http:// www.eulerdiagrams.org/eulerAPE](http://www.eulerdiagrams.org/eulerAPE)) to depict the numbers of unique and shared actinomycete OTUs between the three ascidians based on the data obtained through culture-independent and culture-dependent approaches. Similarly, shared secondary metabolite mass ions of the three ascidians as well as their cultured ascidian-associated actinomycetes were analysed. Thereby, any primary metabolites that were identified against the GNPS database were excluded from the Euler-Venn diagrams.

2.4. Results

2.4.1. Microbial diversity associated with three Australian ascidians

We evaluated the microbial diversity of Australian ascidians using a culture-independent approach targeting ascidian genera, whose microbiomes had not previously been studied in breadth. The samples were collected at an intertidal habitat at Hastings Point, New South Wales, Australia and based on morphological traits the specimens were taxonomically assigned to *Symplegma rubra* (**Figure 1c**), *Aplidium solidum* (**Figure 1d**) and *Polyclinum vasculosum* (**Figure 1e**).

16S rRNA gene Illumina sequencing of the V3 - V4 region resulted in a total of 287758 sequences for the three ascidians. After quality filtering, 253962 sequences remained for analysis and 13945 OTUs were determined based on 97% sequence similarity. Species richness was established through alpha rarefaction and compared between the three ascidian samples. From the rarefaction curves (**Figure 2a**) it was apparent that *S. rubra*, which was the only sample collected at the small rocky reef approximately 100 meters offshore, had a more diverse microbial community than the other two ascidian species, *P. vasculosum* and *A. solidum*, which were obtained from sheltered intertidal rock pools.

Nineteen different bacterial phyla were observed and all three ascidian samples were dominated by Proteobacteria (54 - 58%), followed by Cyanobacteria, which showed a larger variation between 11 - 35%. All ascidians contained a fraction of unassigned phyla; specifically, in *P. vasculosum* this fraction had a high contribution of 28% of the overall bacterial diversity. The phylum Actinobacteria was present in all three ascidians; however, in a far smaller portion, with the highest percent observed in *S. rubra* being 1.91%, while *A. solidum* and *P. vasculosum* both had values below 1% (**Figure 2b**). Within the phylum Actinobacteria, the order *Actinomycetales* is of particular interest due to their ability to produce many bioactive metabolites. Therefore, we further inspected the order *Actinomycetales* to the family level (**Table 1**). Sequences of the taxonomic families Corynebacteriaceae, Microbacteriaceae, Nocariaceae and Streptomyetaceae were detected in all three ascidians, yet at the OTU level (97%) only two OTUs were shared between all three ascidians (**Table 1** and **Figure 3a**). Thermomonosporaceae and Mycobacteriaceae had the highest number of OTUs, but sequences were only detected in *S. rubra* and *P. vasculosum*. In these two ascidians, the natural product producing families Micromonosporaceae and Streptosporangiaceae were discovered, as well as Kineosporiaceae. *A. solidum* exhibited the lowest diversity of actinomycete families; while Actinomycetaceae and Micrococcaceae were unique to this ascidian, Pseudonocardiaceae OTUs were shared with *S. rubra*. Four OTUs that could not be identified to family level unambiguously were obtained from *A. solidum* and *P. vasculosum*. All other observed actinomycetes family OTUs were unique to only one ascidian (**Table 1**).

Figure 2. Microbial diversity analysis (a) Average alpha rarefaction curve showing that *S. rubra* contained the highest microbial diversity, while *A. solidum* had the least diverse microbial community; (b) Percent diversity of taxonomic phyla assigned to three Australian ascidian species.

Table 1. Summary of *de novo* OTUs representing the taxonomic order “Actinomycetales”.

No of OTUs	Family	Ascidian host
1	Acidothermaceae	<i>Polyclinum vasculosum</i>
1	Actinomycetaceae	<i>Aplidium solidum</i>
1	Cellulomonadaceae	<i>Polyclinum vasculosum</i>
2	Corynebacteriaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i> , <i>Aplidium solidum</i>
1	Gordoniaceae	<i>Symplegma rubra</i>
2	Intrasporangiaceae	<i>Polyclinum vasculosum</i>
1	Kineosporiaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i>
5	Microbacteriaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i> , <i>Aplidium solidum</i>
1	Micrococcaceae	<i>Aplidium solidum</i>
3	Micromonosporaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i>
6	Mycobacteriaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i>
5	Nocardiaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i> , <i>Aplidium solidum</i>
1	Promicromonosporaceae	<i>Polyclinum vasculosum</i>
1	Pseudonocardiaceae	<i>Symplegma rubra</i> , <i>Aplidium solidum</i>
4	Streptomycetaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i> , <i>Aplidium solidum</i>
3	Streptosporangiaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i>
6	Thermomonosporaceae	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i>
4	Ambiguous assignment	<i>Symplegma rubra</i> , <i>Polyclinum vasculosum</i>

Figure 3. Microbial and secondary metabolite overlaps between the three Australian ascidians in terms of (a) detectable actinomycete OTUs (b) culturable actinomycete OTUs (c) molecular ions (d) associate metabolites. (Note: primary metabolite database hits were excluded from this diagram).

2.4.2. Culture-dependent actinomycete diversity

Once the presence of actinomycete sequences in the different ascidians was confirmed through culture-independent techniques, traditional culture-dependent microbiological techniques were used to obtain the culturable actinomycete diversity. This resulted in the isolation of 120 diverse actinomycete-like isolates. Phylogenetic analysis was used to confirm their taxonomy. Therefore, the 16S rRNA gene was amplified for all culturable actinomycete-like isolates and subsequently sequenced. All sequences were amplified successfully for more than 1200 bp using the 27F and 1492R universal primers. The 120 sequences were searched against the NCBI 16S ribosomal RNA database for bacteria and archaea and sequences of their closest phylogenetic relative were obtained from the database and included in the phylogenetic analysis. One hundred and eighteen strains exhibited 99% and above similarities to previously described actinomycete strains. Lower sequence similarities were observed for *Streptomyces* isolates USC-16002 (98%) and USC-16062 (97%), both isolated from the *S. rubra*, suggesting that these are potential candidates of novel actinomycete taxa.

The maximum likelihood analysis was used to estimate the phylogenetic relationship of the ascidian-associated actinomycete isolates and revealed four major clades with high statistical support (bootstrap values of 98-100%). This included a clade for Micromonosporaceae, Streptosporangaceae, Streptomyceae and a Nocardia clade that further branched into well-supported *Nocardia* and *Rhodococcus* genus clades (**Figure 4**).

The three ascidian specimens yielded a total of 120 actinomycete isolates originating from five different genera: *Micromonospora* (58%), *Streptomyces* (34%), *Nocardia* (5%), *Rhodococcus* (2%) and *Streptosporangium* (1%) (Table S1). Four actinomycete-specific isolation media and one generic commercially available marine agar were chosen for isolation. The selection of media had a notable influence on the number of isolates recovered. Starch Casein Agar (SCA) allowed the highest recovery of isolates and a total of 60 actinomycetes were obtained, predominantly from the genus *Micromonospora*. The highest actinomycete diversity was recovered from Corn Starch (CS) media; however, it appeared most selective for *Streptomyces* species. Starch Agar (SA) was highly selective for *Micromonospora* species and yielded 20 *Micromonospora* isolates and only one *Streptomyces* species. NH media only recovered one *Nocardia* sp. isolate. A low number of actinomycetes (6 isolates) were obtained from Marine Agar (MA); but interestingly, the only *Streptosporangium* isolate (USC-16119) was recovered from this media (**Figure 5a**).

Figure 4. Phylogenetic analysis of the actinomycete isolates in relation to their closest relatives. (*Bifidobacterium angulatum* (AB489094) was used as an out-group sequence to root the tree) Isolates obtained from *S. rubra* are indicated through (a), *A. solidum* isolates (b) and *P. vasculosum* (c).

The recovery of actinomycetes varied between the three ascidian samples. *S. rubra* yielded the highest number (80) and diversity of isolates with representatives of all five isolated actinomycete genera, *Micromonospora*, *Streptomyces*, *Nocardia*, *Rhodococcus* and *Streptosporangium* (**Figure 5b**). *A. solidum* and *P. vasculosum*, which were both collected from the shore rock pools, yielded a similar number of isolates, 19 and 21 respectively, but much lower numbers and diversity in comparison to *S. rubra*, which was collected from the rocky reef 100 m offshore (**Figure 1a**). The isolation success, therefore, reflects the observed actinomycete distribution detected through molecular analysis

Figure 5. Numbers and taxonomic diversity of actinomycetes (a) isolated from five different isolation media; (b) derived from three Australian Ascidiens.

As indicated in the phylogenetic tree, several of the isolated actinomycetes appeared to be identical; therefore, the QIIME toolbox was utilised to determine culturable actinomycete OTUs, which were compared between three ascidian samples. The 120 actinomycete sequences clustered into a total of 78 OTUs based on 99.9% sequence similarity (**Appendix I, Table IIA**). **Figure 3** depicts the microbial and metabolite overlaps between the three Australian ascidians. Interestingly, only two microbial OTUs were shared between all three ascidian specimens. This suggests that the isolated actinomycete associates were mostly unique to their ascidian host. Specifically, the *Streptomyces*, except for one OTU (comprised for one isolate from *S. rubra* and one from *A. solidum*), showed no overlap between ascidian samples (Table S1). The numbers of detectable and culturable actinomycetes OTUs shared between the ascidians appeared mostly proportional, only shared OTUs between *S. rubra* and *P. vasculosum* were higher with ten shared OTUs compared to the two cultured OTUs (**Figure 3a/b**). Interestingly, the two actinomycete OTUs shared between all three ascidians that were defined based on the culture-independent methods belonged to the families of Streptomycetaceae and Nocardiaceae. We compared the sequences from culture-independent and dependent approaches and found that they well aligned, but none of the sequences matched 100 %. However, 16S sequence fragments in the culture independent analysis were maximal 465 bp long and of lower quality, while sequences of 1200 bp were recovered from the cultured isolates, making definite conclusions about shared OTUs difficult.

2.4.3. Metabolite diversity

LC-MS/MS data was collected for the three ascidian hosts as well as extracts of the actinomycete associates. In contrast to the microbial diversity, *A. solidum* had the highest secondary metabolite diversity (186 molecular ions) followed by *S. rubra* (73 ions) and *P. vasculosum* had the lowest ion diversity (55 ions). Thirteen molecular ions were found in all three ascidian specimens (**Figure 3c**). The metabolite diversity of the ascidian-associated actinomycetes reflected the diversity of the microbial diversity and *S. rubra*-associated actinomycetes by far had the highest abundance of molecular ions (**Figure 3d**). For visualisation, a molecular ion network was constructed (**Figure 6**). After removal of ions present in blanks and standards, a total of 1532 parent ions were observed in the samples and represented as nodes coloured by taxa where edges indicate significant pairwise alignment between nodes. The network shows several taxa-specific node clusters; specifically, *Streptomyces* and *Micromonospora* display a diverse plethora of unique molecular ion clusters. With 842 detected ions, *Streptomyces* exhibited the richest metabolite diversity followed by 681 ions detected in *Micromonospora* isolates. *Nocardia* showed several unique clusters but had most ion overlap with *Micromonospora*. Only one *Streptosporangium* isolate was included in the analysis, yet its metabolite diversity is noteworthy with a total of 91 molecular ions detected of which 20 were unique and 73 coincided with the *Micromonospora* ions. Seventy-four metabolites could be identified through MS/MS spectra matches against the GNPS database (Table S2). The majority of the database matches present common primary metabolites, such as nucleotides, sugars, amino acids, fatty acids, vitamins and cell membrane components (phosphatidylcholines), which are of less interest for biotechnological or pharmaceutical studies. Furthermore, several linear and cyclic dipeptides, as well as other previously described bioactive secondary metabolites were identified (**Figure 6a**). Surugamide A and leupeptin, both protease inhibitors previously isolated from *Streptomyces* sp.,^{43,44} which could be annotated to *Streptomyces* specific clusters (**Figure 6b/c**). Rakicidin A and B were attributed to a small cluster of only three nodes shared between several *Micromonospora* and the *Streptosporangium* isolates (**Figure 6d**); these compounds were previously reported to exhibit cytotoxic and anti-tumor properties.⁴⁵ Additionally, the common actinomycete siderophore for iron acquisition, desferrioxamine B, was linked to a shared actinomycete cluster (**Figure 6e**). The remaining great majority of ions in the networks could not be assigned against the GNPS database.

Figure 6. Ascidian-associated actinomycete molecular network: (a) overall molecular network, where node colour represents different actinomycete taxa and ascidian hosts; networks with identified primary metabolites are highlighted with grey ellipses. Identified secondary metabolite networks of (b) leupeptin; (c) surugamide A; (d) rakicidin A and B; (e) desferrioxamine B.

Of great interest were the nodes that overlapped between ascidian hosts and their microbial associates, as these nodes are likely to be compounds that are produced by a microbial associate of the ascidians. Only one detected ion with a molecular mass of 479.9 Da was identified as an oxobutanoic acid derivative and was found in all three ascidians and *Micromonospora* and *Nocardia* associates. Ninety-one molecular ions coincided between *S. rubra* and its actinomycete associates. Whilst *P. vasulosum* had 74 overlapping ions, and in *A. solidum* 52 ions matched the associates. *Micromonospora* and *Streptomyces* isolate metabolomes showed the greatest overlap and thus appear to contribute to the ascidian metabolome. Of great interest were the nodes that overlapped between ascidian hosts and their microbial associates, as these nodes are likely to be compounds that are produced by a microbial associate of the ascidians. Only one detected ion with a molecular mass of 479.9 Da was identified as an oxobutanoic acid derivative and was found in all three ascidians and *Micromonospora* and *Nocardia* associates. Ninety-one molecular ions coincided between *S. rubra* and its actinomycete associates. Whilst *P. vasulosum* had 74 overlapping ions, and in *A. solidum* 52 ions matched the associates. *Micromonospora* and *Streptomyces* isolate metabolomes showed the greatest overlap and thus appear to contribute to the ascidian metabolome. *Rhodococcus* had only 8 ions that also were found in ascidian hosts, suggesting that *Rhodococcus* may have been acquired by chance from the environment and probably does not play a role in these ascidians.

2.5. Discussion

In this study, we compared the microbiomes and metabolomes of three Australian ascidian species collected from the same geographical location at Hastings Point, NSW, Australia. To date, scientists still rely on culture-dependent methodologies in order to study the expressed secondary metabolome and its biological activity; however, this approach might miss out on unculturable microbial strains. Therefore, we initially determined the microbial diversity associated with the three ascidians based on a culture-independent approach and then compared the findings with the culturable actinomycete diversity. We further intended to provide insight into the functional role of actinomycetes in associations with ascidians and connect the results to the production of bioactive secondary metabolites.

At the phylum level, the overall microbial diversities and distributions associated with the three Australian ascidian specimens were comparably similar. Other culture-independent ascidian microbiome studies from different geographical regions, including Asian-Pacific, South Pacific, and the Caribbean reported similar phylum distributions, where Proteobacteria were generally reported as the most dominant phylum and also Cyanobacteria were commonly found in high abundance.^{9,17,19,46-48} Actinobacteria were usually reported in lower numbers in

these studies. However, low numbers of actinobacterial sequences could potentially be attributed to poor DNA extraction and primer biases of the V3 - V4 region of the 16S rRNA gene.⁴⁹ Particularly, for Gram-positive bacteria, DNA extractions have been proven difficult and most commercial DNA extraction kits require specific cell lysis pre-treatments of the samples, which could be a reason that higher numbers of Gram-negative bacteria were reported.⁴⁹

The isolation media were targeted at mycelium forming actinomycete genera, this included *Streptomyces*, *Micromonospora*, and *Nocardia*, which are renowned to produce bioactive secondary metabolites. As detected in the culture-independent sequencing analysis, we successfully isolated highly diverse *Streptomyces* species strains from all ascidian samples. *Micromonospora* species were also obtained in high diversity and abundance from all three ascidians, while only *S. rubra* and *P. vasculosum* showed Micromonosporaceae OTUs in the culture-independent analysis. Other actinomycete resembling family members, including Gordoniaceae, Corynebacteriaceae, and Micrococcaceae colonies were also observed on isolation plates, confirming culture-independent data; however, due to time constraints were not targeted for isolation. Streptosporangiaceae sequences were identified in *S. rubra* and *P. vasculosum*, but could only be recovered from *S. rubra*, which could have been affected by the choice of isolation media. *Nocardia* and *Rhodococcus* were isolated in very low numbers and displayed little diversity that might be due to their transitory characteristics within the ascidian hosts. Similarly, the chosen isolation media are commonly used for a broad range of actinomycete taxa and may not have been selective enough to culture these genera.

It has been proposed that microbial diversity of ascidians is stable and species-specific, but it also has a location-specific aspect where microorganisms are acquired from the local environment.^{9,10} Actinomycetes are commonly found in the marine as well as the terrestrial environment. In fact, it is commonly believed that the order is of terrestrial origin and its spores have been introduced to the marine environment as wash off and then adapted to the saline conditions.⁵⁰⁻⁵³ Hence, it is likely that actinomycetes are facultative symbionts, meaning that they are acquired from the environment and thus should be location-specific rather than host specific.^{10,54} Nonetheless, actinomycetes still present a valuable target species for biodiscovery, especially as it is proposed that as they adapt to the marine environment this adaptation also leads to a change in their expressed secondary metabolome.^{50, 53, 55-56} If actinomycete associations are facultative, then actinomycete diversity of ascidians sampled at the same location and time should greatly overlap. The study on these three different ascidians collected at nearby geographic locations showed otherwise; most culturable actinomycete isolates were unique to only one ascidian. Only two cultured actinomycete OTUs were shared between the three different ascidian species; it is noteworthy that these strains were fairly abundant, pointing towards a location factor. *S. rubra*, which was sampled at the small rocky reef approximately

200 meters offshore, had a more diverse microbial community based on Illumina sequencing data as well as culturable actinomycete diversity compared to the other two ascidian species. It might be possible that *S. rubra* receives more microorganisms through oceanic currents and terrestrial waters from the river mouth of Cudgara Creek adjacent to the sampling site (**Figure 1**). *A. solidum* and *P. vasculosum* were obtained from rock pools just off the mainland that provide more shelter from oceanic currents. Additionally, *A. solidum* incorporate sand internally into their tissues as they form hard rigid colony structures;⁵⁷ therefore, the low microbial diversity associated with *A. solidum* might be attributed to its tissue being significantly higher in sand content, allowing for less living tissue per standard volume. Since, the overlap of actinomycete isolates between the different ascidian species sourced from the same physiological environment was not found to be significant; these findings support the argument that the acquisition of actinomycetes is selective. It has to be taken into account that these conclusions were established based on the actinomycetes that could be derived from the chosen isolation media and solely three nearby collected specimens and further studies would be required to confirm these conclusions.

Polyclinidae after Didemnidae is the ascidian family that has yielded most reported natural products.³⁰ Numerous secondary metabolites (138) from the genus *Aplidium* are listed in the Dictionary of Natural Products (<http://dnp.chemnetbase.com>, accessed 30-08-2017), while only three and one natural products are listed for *Symplegma* and *Polyclinum*, respectively. These reports fit the diversity of molecular ions associated with three ascidians in this study. We investigated whether metabolite diversity of the three Australian ascidians was correlated with the host's microbial diversity. Our results show that the microbial diversity did not reflect the chemical diversity. *S. rubra*, which contained an impressive diversity of microbial associates based on culture-independent as well as culture-dependent approaches, had lower chemical diversity by LC-MS/MS analysis than the other two ascidian specimens. While *A. solidum* exhibited the highest metabolite diversity, low numbers of microbial sequences and culturable actinomycetes were recovered. Similarly, Tianero *et al.* found no correlation between microbial and chemical diversity nor biological activity, but rather specific interactions with bioactive secondary metabolite producers.⁹

To gain an overview whether there were any secondary metabolites that can be attributed to actinomycete associates isolated from these ascidians we acquired LC-MS/MS profiles of all ascidians and their cultured actinomycete associates. Many nodes overlapped between ascidians and actinomycetes indicating that these compounds are potentially produced by microbial associates. Generally, ions in a molecular network are all closely related and therefore it is likely that as soon as there is one ion match between actinomycete and its host that all compounds within this network are of microbial origin. The molecular network indicates that

actinomycetes derived from Australian ascidians contain a highly diverse metabolome that in this case even exceeds that of the ascidians and targeting actinomycete associates thus proves a valuable approach for natural product drug discovery and aids to overcome the supply problem. Even more so, as bacterial cultures can be manipulated in the laboratory, by using different fermentation media or using different chemical or physical elicitors to activate silent gene clusters in order to further induce secondary metabolite diversity.⁵⁸

In this study, we presented a rich taxonomic as well as metabolite diversity of actinomycetes associated with ascidians which both appear species specific. The geographic and taxonomic scope was however limited to only three ascidian species from one habitat, therefore we regard this work as a small contribution to the bigger picture. Extended knowledge of diversity and distribution of actinomycetes in the marine environment will contribute further to a deeper understanding of their ecological function and will aid towards the design of improved biodiscovery strategies. The next steps in this work will be to identify new secondary metabolites that could not be assigned from the molecular network analysis and thus maximise the great potential of actinomycetes for natural product drug discovery.

2.6. Conclusions

This study has shown that the ascidians, *S. rubra*, *A. solidum* and *P. vasculosum*, harbour a range of diverse actinomycetes, which we successfully identified with culture independent methods and subsequently isolated using culture dependent techniques. The actinomycete metabolomes, specifically for *Streptomyces* and *Micromonospora* isolates, were highly diverse and thus present a good target for natural product drug discovery.

2.7. References

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SECTION 2

Tackling the issue of rediscovery and dereplication

Natural products provide an immense wealth of structural complexity; however, natural product isolations and screening require a lot of time and effort. Furthermore, rediscovery of already known compounds is a major issue in this field of research. In Chapter 2, I focused my microbial isolation efforts on deriving actinomycetes from marine ascidians, a mostly untapped ecological source of biodiscovery, with the intention of enhancing the chemical diversity of natural products. This resulted in an actinomycete library of 120 diverse isolates. Thus, the challenge was to decide which isolates were most valuable for compound isolations. Recently, MS-based metabolomics approaches have been implemented for microbial strain prioritisation and several references were provided in the introduction to the thesis. Mass spectrometry is a very powerful analytical tool with high sensitivity and resolution; however, it does not provide a lot of insight about structural components in an extract. Therefore, it was my aim to develop a new approach using 2D NMR profiling for microbial strain selection which is presented in Chapter 3. In particular, I intended to investigate the questions, whether HSQC-TOCSY profiles can be used as a profiling tool to detect presence of specific structural classes in microbial extracts without the need of fractionation. Subsequently, whether this technique can be used as an approach to detect similarities and differences in microbial extracts and therefore be used for microbial strain dereplication.

Co-cultivation is a culturing method that can enhance chemical diversity as a result of interspecies interactions. In Chapter 4, four different microbial strains were co-cultured and the developed strain prioritisation approach (described in Chapter 3) was applied to dereplicate the obtained mono- and co-culture extracts. This provided further validation for the new 2D NMR profiling method.

CHAPTER 3 - HSQC-TOCSY NMR profiling: using informative metabolomics to prioritise microbial strains for natural product drug discovery

STATEMENT OF CONTRIBUTION TO CO-AUTHORED PAPER IN PREPARATION

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The author this thesis, L. Buedenbender, developed the approach, performed microbial fermentations, extractions, chemical profiling; data analysis and prepared the manuscript. Leesa Habener assisted in revising the manuscript for important intellectual content. The co-authors T. Grkovic, D. I. Kurtböke and A. R. Carroll are the supervisors of the PhD project and provided advice on the research design and feedback. S. Duffy and V. M. Avery performed anti-plasmodial activity testing of the crude extracts.

(Signed) _____ (Date) 01/09/2017

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3.1. Abstract

Microbial natural products are still a promising source for drug leads due to their unique structural diversity and the possibility of laboratory based fermentations rather than harvesting organisms from the environment. However, rediscovery of already known natural products poses a great challenge and therefore, it is important to incorporate effective dereplication protocols early in natural product isolation efforts. A systematic approach for microbial strain prioritization for natural product discovery based on HSQC-TOCSY NMR profiles combined with biological activity of crude extracts was developed. NMR data provides structural information of the microbial extracts and can be used to target specific functional groups of natural products. The use of HSQC-TOCSY experiments offered increased chemical shift resolution by spreading the structural information onto two dimensions and thus permitted assessment of unfractionated extracts. Here, the utility of this approach was demonstrated to aid the selection of microbial strains with unique NMR profiles and potent anti-plasmodial activity from a library of 119 ascidian-associated actinomycete extracts that were prioritised for further natural product discovery investigations.

3.2. Introduction

Natural products and their derivatives continue to play an important role in the discovery and development of new therapeutical agents due to their unique structural diversity and chirality.¹ Microorganisms represent one of the most important sources for new medicines. Furthermore recent evidence has shown that a significant number of invertebrate-derived natural product drug leads are actually produced by a microbial symbiont of the host species from which the natural product was originally isolated.² With this in mind, microbial libraries, particularly derived from the marine environment, are expanding rapidly. Strains for drug discovery were traditionally selected based on morphology and/or biological activity of their crude extracts. This was then followed by bioassay-guided fractionation to isolate new bioactive molecules. The problem herein is that two microbial isolates could visually appear different but produce the same secondary metabolites, or alternatively appear the same morphologically yet produce different metabolites. Such aspects, as well as genuine duplication of isolates within a microbial strain library, have to be considered and thus new and innovative approaches are evolving to dereplicate cultured strains in order to overcome repeated re-isolation of the same metabolites.

Chemometric-metabolomics profiling approaches provide an overview of the expressed metabolites in extracts and aim to reduce redundancy. The two most common metabolomics

profiling tools are LC-MS/MS and NMR profiling. The advantages of mass spectrometry are the high resolution and sensitivity, as well as the possibility to use MS/MS fragmentation. However, the technique is highly dependent on the ability of a compound to ionise and it is difficult to quantify and replicate the obtained results using different instruments and thus the technique is not universally applicable. NMR techniques, on the other hand, have much lower resolution and sensitivity compared to MS, but NMR is non-selective, non-destructive, and quantitative, it requires minimal sample handling and undeniably its greatest advantage is that it provides structural information about the constituents of the extracts. In recent years, NMR advancement has led to faster acquisition through optimised probes and stronger magnets, as well as automation and thus making this technique more powerful and more applicable for metabolomics studies. For this reason, NMR profiling was chosen as the preferred analytical technique in this study.

Microbial crude extracts are made up of potentially hundreds of metabolites, resulting in an immense signal overlap in the 1D ^1H -NMR spectrum. Therefore, implementation of 2D NMR techniques can increase chemical shift resolution by spreading the detected signals across two dimensions and thereby permitting assessment of unfractionated crude extracts. Two-dimensional NMR experiments, such as TOCSY and HSQC, have previously been used for metabolite profiling of crude extracts.^{3,4} Herein, this chapter of the thesis aims to (1) develop the use of HSQC-TOCSY spectral profiles for characterisation of polyketide and peptide chemical diversity; (2) utilise HSQC-TOCSY spectral profiles in combination with biological activity to prioritise microbial strains from a library of 119 actinomycete crude extracts for natural product isolation efforts and (3) select a promising microbial strain to discovery bioactive natural products.

3.3. Results & Discussion

3.3.1. Determination of microbial culturing and chemical extraction protocol

For the discovery of microbial natural products, it is of great importance to determine a suitable culture medium and metabolite extraction protocol. On the one hand, it is important to select a media type that offers enough nutrients for the culture to grow, but on the other hand it should not heavily contaminate the extracts with primary metabolites. Similarly, the extraction protocol should maximise the amount of extract, but should also be specific for the natural product containing part of the metabolome. Therefore, a range of culturing media and extraction techniques were compared to determine the most appropriate protocol for natural products

discovery (**Figure 1**). Initially, a random selection of six different actinomycete strains was cultured on solid GYM + ASW and OMA agar plates as well as in liquid ASW-A media. The proton spectra of EtOAc extracts of both GYM + ASW and OMA solid media showed measurable yields of secondary metabolites. MeOH was also trialled as an extraction solvent, however, resulted in increased media elements in the extracts; particularly sugars and fatty acids contributed to the broadening and overlap of signals in ^1H -NMR spectra. In liquid cultures, the actinomycete mycelia were separated from the media by centrifugation and cells were extracted in MeOH. The liquid media phase was extracted by adsorbing onto C_{18} silica gel, which was subsequently eluted with 20 % and 100 % MeOH. The liquid media extracts contained only minute quantities of secondary metabolites and in the proton spectra, the metabolites of interest were shadowed by larger signals attributed to media components. Cell extracts revealed interesting secondary metabolite chemistry but overall resulted in significantly lower yields compared to those obtained through solid agar culture extractions. Consequently, solid GYM + ASW media was chosen for the cultivation of the entire actinomycete library.

Figure 1. Evaluation of culturing and extraction methods for actinomycete isolate USC-16022. Normalised ^1H -NMR spectra for each culturing and extraction condition, where f1 represents proton chemical shift in ppm. (a) EtOAc extraction of solid GYM + ASW medium. (b) EtOAc and MeOH extraction of solid OMA medium. (c) MeOH extract of cells from liquid culture. (d) C_{18} silica gel adsorption of liquid medium with C_{18} silica gel eluted with 20% MeOH. (e) C_{18} silica gel adsorption of liquid medium with C_{18} silica gel eluted using 100% MeOH.

3.3.2. Identification of regions and peaks of interest in 2D HSQC-TOCSY NMR spectra

Microorganisms are capable of producing secondary metabolites of immense structural complexity (**Figure 2**). In bacteria, the majority of secondary metabolites are derived from polyketide synthase and non-ribosomal peptide synthases.⁵ These pathways utilise large protein complexes and have been studied extensively in the past two decades.⁶ The increased knowledge of biosynthetic pathways has opened the door for new development of sequence-based approaches to natural product drug discovery. However, these techniques are not commonly available in natural products chemistry laboratories; therefore, an approach to utilise 2D HSQC-TOCSY NMR profiles to characterise polyketide and peptide chemical diversity was developed.

A HSQC-TOCSY NMR experiment is a 2D TOCSY experiment that is resolved into the carbon dimension. This shows through bond HSQC correlations of a proton to the carbon that it is attached to as well as further protonated carbons within the same spin system.⁷ In the traditional sense, this experiment is particularly useful for the elucidation of structures such as peptides, and to some extent polyketides, that typically display many overlapping proton signals. Spin fragments of these two structural classes occupy very specific chemical shift regions in HSQC-TOCSY spectra.

Figure 2. Examples of marine actinomycete derived polyketides and peptides: arenicolide A (**1**)⁸, saliniketol A (**2**)⁹, rifamycin B (**3**)¹⁰, lucentamycin A (**4**)¹¹ and cyclomarlin A (**5**)¹²

The most characteristic features of polyketides are carbon-carbon chains that contain carbonyls on alternating carbons (**Figure 2**). These carbonyls are often reduced to alcohols or dehydrated to yield tri-substituted double bonds.¹³ The carbons adjacent to the hydroxy group display a characteristic chemical shift of δ_C 60.0 to 80.0 ppm.¹⁴ A HSQC-TOCSY spectrum allows observation of correlations between the methyl-protons, that generally have chemical shifts ranging from δ_H 0.5 – 1.2 ppm, into the carbon at δ_C 60.0 – 80.0 ppm at the α -position to the alcohol into (**Figure 3**). Both the α -proton (δ_H ~1.70 ppm) and the β -proton (δ_H ~1.50 ppm) display correlations into the carbon at δ_C 60.0 – 80.0 ppm. However, their peaks are generally less intense compared to those of methyl-protons. The utilisation of these the long-range HSQC-TOCSY correlations of the proton (δ_H 3.0 – 4.0 ppm) bonded to the hydroxy carbon (δ_C 60 – 80 ppm) are very useful to distinguish these signal from the similar correlations observed in hydroxy methines of sugars. Sugars are common by-products in microbial crude extracts, and occupy a region in the two-dimensional spectra that is prone to overcrowding of signals (**Figure 3**). The polyketide region of interest can be extended to include other typical polyketide moieties; for instance, when ethers or methoxy-groups are positioned next to the methyl, this results in a downfield shift of the adjacent carbons to δ_C 75 – 80 ppm. Further downfield shifts to approximately δ_C 100 ppm are indicative of the presence of dioxy-groups (**Figure 3**). Originally, the use of HMBC spectra was proposed, which would show correlations into the unprotonated carbons α to ketones, for the identification of polyketides in crude extracts; however, HMBC experiments are not as sensitive as HSQC-TOCSY experiments and would take an enormous amount of time for acquisition.

A similar approach can be taken to identify regions of interest in the spectra that are characteristic of peptides. If a deuterated NMR solvent is used, such as DMSO- d_6 that allows visualisation of exchangeable protons, characteristic HSQC-TOCSY correlations from an amide proton to α - and β -carbons can be observed. Amide protons typically display chemical shifts of δ_H 6.0 – 10 ppm and the absence of 1H - ^{13}C HSQC correlations.¹⁴ In HSQC-TOCSY spectra, correlations through the spin system to carbons with shifts ranging from δ_C 10 – 80 ppm can be observed. Examples of partial peptide structures that depict such correlations are shown in **Figure 3**. Indoles are specific cases where HSQC-TOCSY correlations from the indole proton (δ_H 10 ppm) to aromatic carbon with resonances of approximately δ_C 125 ppm can be detected.¹⁴ Other heteroaromatic resonances occur even further downfield; imidazoles show a characteristic N-H resonances of δ_H 13.5 ppm that show correlations to aromatic carbons resonating at δ_C 135 ppm.¹⁴

Figure 3. Regions of interest: HSQC-TOCSY spectrum highlighting chemical shift (ppm) regions that correspond to specific compound classes. Carbon chemical shifts are represented on f1 (ppm) and proton chemical shifts on f2 (ppm). Regions of interest include peptides (PEP), alkaloids, heteroaromatics, olfenic signals (DB), aromatics, polyketides (PK), sugars, and fatty acids (FA).

3.3.3. Integrating metabolomics tools for microbial strain prioritisation from a library of 119 actinomycete extracts

Metabolomics uses analytical chemometric techniques to identify and quantify the total metabolic processes of an organism.¹⁵ Thereby, chemical profiling data is analysed using multivariate statistics that allows detection of differential metabolites, identification of unique samples, and reduction of chemical redundancy.¹⁶ The theoretical workflow commences with microbial crude extracts that are then chemically profiled using spectroscopy (NMR) or spectrometry (MS) and are most often also screened for biological activity (**Figure 4**). The combined data is then statistically analysed to prioritise unique and chemically diverse samples for natural product isolations. Principal component analysis (PCA) and Partial Least Squares –

Discriminatory Analysis (PLS-DA) are the two routinely used multivariate statistics in metabolomics.¹⁷ These techniques reduce large numbers of observed variables to fewer principal components that describe the variation between samples.¹⁸

Figure 4. Workflow for metabolomics guided strain selection

The adapted metabolomics approach (**Figure 4**) was applied to dereplicate the acquired HSQC-TOCSY spectra for an actinomycete collection of 119 diverse isolates. One GYM + ASW agar culture plate per isolate was prepared and extracted in EtOAc after 2 weeks of incubation at 28 °C. HSQC-TOCSY profiles were obtained and the crude extracts were tested for anti-plasmodial activity. Cross-peaks were picked from the regions of interest (detailed in **Figure 3**) and the data was exported to Excel, peaks were aligned by implementing an in-house macro allowing for a bin-threshold of 0.04 ppm in the proton dimension and 0.4 ppm in the carbon dimension. This resulted in a presence/absence matrix of a total of 1405 unique peaks from the 119 samples. This data is not actually suitable for PCA; and consequently, Principal Coordinate Analysis (PCoA) was chosen which can be used with similarity measures that are more suited for presence/absence data. The data was also subcategorised based on the partial structures derived from the HSQC-TOCSY polyketide (PK) and peptide (PEP) regions of interest, to reduce the numbers of observations in the multivariate analysis. When all peaks of interest (PoI) were included in the analysis, no clear clustering was observed in the PCoA plot, but there was a trend in the data based on the genus taxonomic level and the majority of active

samples were observed to cluster closer together (**Figure 5a/b**). However, the first two principal coordinates accounted for 12 % of the variation in the data, which indicates that the data set is comprised of very diverse samples. PCoAs were then individually performed for the polyketide and the peptide subcategories, which reduced the number of PoIs to 172 and 291, respectively (**Figure 5c-f**).

In the PCoA of polyketide PoIs, five clusters were observed (**Figure 5c**) and specific structural fragments could be associated to these clusters. The clusters 1, 2 and 3 all contain specific CH₃-CHOR fragments, while cluster 4 had very specific correlations from an upfield proton at δ_H 1.23 ppm to a downfield carbon at δ_C 155 ppm that is likely to be associated with a olefinic fragment. The first two dimensions the PCoA explained only 16 % of the variation in the data, which essentially alludes to the high chemical diversity of the actinomycete collection. The remaining samples in cluster 5 contained a breadth of additional polyketide fragments with rather different HSQC-TOCSY profiles (Appendix II, Figure IIB contains PCoA plot with sample labels). In terms of natural products discovery a collection displaying chemical diversity is a desirable feature. There was no correlation found between samples that showed anti-plasmodial activity and bioactive samples were present in each cluster of the polyketide analysis. However, samples that clustered on top of each other were found to contain the same or very similar polyketide resonances. Therefore, the PCoA was considered to be a useful tool for dereplication of identical samples. Nonetheless, HSQC-TOCSY spectra should always be consulted to confirm that two or more samples are identical, especially if the percent variance in the PCoA is low.

Three main clusters were observed for the principal coordinate analysis of the PEP PoI (**Figure 5e**). Cluster 1 contained only *Micromonospora* isolates that all had a specific HSQC-TOCSY correlation from δ_H 6.16 ppm to δ_C 91.8 ppm in common; this correlation is likely to be an acetal type correlation and does not actually belong to a peptide. The various isolates in cluster 2 shared a correlation at δ_H 6.58 ppm/ δ_C 75.9 ppm, while occurrence of another 38 PEP PoIs varied between the samples. From the broad cluster 3, it was not possible to discern any true correlations between the samples, neither inspection of the bioactive samples that all appeared to correlate revealed any particular PoI that contributed to their clustering (**Figure 5f**).

Figure 5. PCoA plots of HSQC-TOCSY data of 119 actinomycete stains; (a) for all PoI by genus (b) for all PoI by activity (c) for all PK by genus (d) for all PK by activity (e) for all PEP by genus (f) for all PEP by activity

Of the 119 actinomycete isolates screened for anti-plasmodial activity, 18 demonstrated above 85 % inhibition against *Plasmodium falciparum* at a tested concentration of 0.2 µg/mL. Examination of respective HSQC-TOCSY spectra allowed detailed insight about the structural constituents of their extracts. First, all peaks of interest (PoI) were tallied, which averaged at 44 peaks for all active isolates (**Figure 6**). The highest number of PoI (88) was detected in the extract of *Micromonospora* sp. USC-16041 and it was clearly evident that *Streptomyces* sp. strains USC-16007 and USC-16025 metabolites were below the NMR detection limit, with only five and zero PoIs observed in the HSQC-TOCSY spectra, respectively. Such information is equally valuable as it reflects the lower ability of these strains to produce high amounts of secondary metabolites under the given culture conditions. It also suggests that the potency of the active compounds present in these strains are likely to be high. However further culture optimisations would be required for drug discovery efforts, therefore, these strains were not prioritised.

Figure 6. Account of HSQC-TOCSY peaks of interest (PoI) for each bioactive isolate (PK = polyketide; PEP = peptide; HA = heteroaromatic, DB = olefinic)

Prioritisation of microbial strains for natural product drug discovery was first based on biological activity of the samples and high secondary metabolite signal diversity (total PoI) under the given culturing conditions was considered favourable, then also specific structural classes such as polyketides and peptides could be targeted. Lastly, PCoA was used to dereplicate the selected isolates to ensure that unique profiles were prioritised. Based on these criteria the order of prioritisation of isolates within a collection of 119 microbial isolates that are more likely to produce bioactive polyketide or peptides are detailed in **Table 1**. For natural product isolations, *Streptomyces* sp. USC-16018 was the number one choice if isolation of

bioactive polyketides was desired. The strain, when grown on GYM + ASW solid media, had a total of 63 PoI associated with secondary metabolites, of which 20 diverse peaks were associated with polyketide-type compounds. In the PCoA, the isolate was positioned in ‘cluster 1’ but well away from any other sample (**Figure 5c**). USC-16041 and USC-16022 also scored high in the strain prioritisation approach (**Table 1**). Both isolates actually had higher total PoI diversity compared to USC-16018, however, their scores for polyketide and peptide associated PoI were lower. USC-636 and USC-16008 were better candidates for the isolation of peptides.

Table 1. Order of strain prioritisation based on bioactivity, high PK and PEP diversity and uniqueness of HSQC-TOCSY profile

Isolate	Genus	% inhibition ^a	total PoI	PK PoI	PEP PoI	PK+ PEP	Order of prioritisation
USC-16018	<i>Streptomyces</i>	94	63	20	9	29	1
USC-16041	<i>Streptomyces</i>	93	88	13	6	19	2
USC-16008	<i>Streptomyces</i>	96	61	13	9	22	3
USC-16022	<i>Streptomyces</i>	71	70	15	5	20	4
USC-636	<i>Streptomyces</i>	84	33	4	14	18	5
USC-16016	<i>Streptomyces</i>	99	48	11	3	14	6
USC-16003	<i>Streptomyces</i>	96	25	11	1	12	7
USC-16013	<i>Streptomyces</i>	83	47	4	8	12	8
USC-16101	<i>Streptomyces</i>	94	35	8	0	8	9
USC-16017	<i>Streptomyces</i>	99	35	5	2	7	10
USC-16005	<i>Streptomyces</i>	89	74	3	2	5	11
USC-16115	<i>Streptomyces</i>	84	52	4	2	6	12
USC-16004	<i>Streptomyces</i>	92	66	1	3	4	13
USC-16107	<i>Micromonospora</i>	99	50	4	0	4	14
USC-16048	<i>Micromonospora</i>	97	18	1	3	4	15
USC-16000	<i>Streptomyces</i>	83	13	2	0	2	16
USC-16007	<i>Streptomyces</i>	94	5	1	1	2	-
USC-16025	<i>Streptomyces</i>	84	0	0	0	0	-

^a %-inhibition against *P. falciparum* at 2.0 µg/mL

3.3.4. Discovery of bioactive natural products from selected strains

As a proof of concept, the microbial strain that exhibited the highest polyketide peak diversity was selected to investigate whether polyketide compounds could in fact be isolated from this strain. Fermentation of *Streptomyces* sp. strain USC-16018 was scaled up to 55 agar plates and extraction with EtOAc yielded 1.6 g of crude material. This extract was separated by reversed phase HPLC and natural product isolations were initially guided by ¹H NMR analysis of fractions. Isolations were guided by selecting fractions containing ¹H resonances between δ_H 4.00 and 6.00 ppm indicative of oxygenated and olefinic protons potentially associated with polyketides. Short HSQC-TOCSY experiments (8 scans = 15 minutes) of the selected fractions

were acquired to confirm the presence of polyketides in the fractions. Fraction 46 was identified to contain a pure polyketide and a full set of two-dimensional NMR experiments (^1H - ^1H COSY, ^1H - ^{13}C HSQC, and ^1H - ^{13}C HMBC) was obtained to elucidate the structure. The compound in fraction 46 turned out to be the known antibiotic dimer elaiophylin (6), which was previously reported to exhibit antibiotic and anti-plasmodial properties.^{19,20} The full structure elucidation of 6, as well as that of a new polyketide compound from the selected strain, is described in Chapter 5. It was of interest to confirm the presence of signals associated with this molecule in the original HSQC-TOCSY crude extract spectra of USC-16018. Nineteen elaiophylin-associated correlations were observed in the crude HSQC-TOCSY spectra (**Figure 7**).

Several indole-containing peptides with anti-plasmodial activity were previously isolated from the *Streptomyces* sp. strain USC-636.²¹ This isolate was included in the HSQC-TOCSY analysis as a control actinomycete strain and ranked fifth in the HSQC-TOCSY prioritisation approach. In comparison to the other active strains, USC-636 exhibited the most diverse

Figure 7. Elaiophylin correlations (labelled with ^1H , ^{13}C chemical shifts in ppm) observed in the crude HSQC-TOCSY spectra of strain USC-16018 (f1 representing ^{13}C chemical shifts in ppm and f2 representing ^1H chemical shift in ppm; HSQC-TOCSY correlations are shown in bold).

peptide-associated HSQC-TOCSY peaks in the crude extract. Cross-peaks at δ_H 10.78 and 10.82 ppm / δ_C 123.7 ppm clearly identified the indole group found in naseaeazines A - C (**7 - 9**, **Figure 8**). In all three structures the indole of one subunit was reduced to an indoline and correlations at δ_H 7.11/ δ_C 123.4 ppm could be observed in the crude spectrum.

Figure 8. Naseaeazine correlations (labelled with ^1H , ^{13}C chemical shift in ppm) observed in the crude HSQC-TOCSY spectra of strain USC-636 (f1 representing ^{13}C chemical shift in ppm, and f2 representing ^1H chemical shift in ppm; HSQC-TOCSY correlation are shown in bold).

3.3.5. Detection limit of HSQC-TOCSY NMR spectra

The compounds described above were isolated in low quantities (<1% of the crude extract), yet they could be detected in the HSQC-TOCSY spectra with 16 scans and 128 increments on a 800 MHz NMR spectrometer equipped with a cryoprobe. In order to establish the actual detection limit of the HSQC-TOCSY profiles, a concentration curve was prepared using the commercially available polyketide antibiotic rifampicin (**10**, **Figure 9**). HSQC-TOCSY spectra

were acquired under the same parameters as used for the microbial crude extracts. Average peak intensities for PK PoI were established for 5.0, 2.5, 2.0, 1.5, and 1.0 mg/mL rifampicin (**Table 2**). Long-range HSQC-TOCSY correlations from the upfield methyls to the polyketide region of interest could be detected at concentrations as low as 1.5 mg/mL and the peak intensity of these polyketide fragments was used to establish a concentration curve (**Figure 9**). At 1.0 mg/mL (0.2 mg), only one-bond HSQC correlations of upfield protons were detected. One agar plate of an actinomycete culture on averaged yielded 15 mg of extract and 0.3 mg of this would equate to a 1.3 % yield, which is a realistic yield for secondary metabolites in crude extracts.

Figure 9. Rifampicin (**10**) concentration curve and corresponding HSQC-TOCSY spectra (800 MHz, 200 μ L DMSO- d_6 , NS=16) (a) 5.0 mg/mL (b) 2.5 mg/mL (c) 1.0 mg/mL

Table 2. Average PK PoI %-intensities (relative to DMSO) for rifampicin standards at 5.0, 2.5, 2.0, 1.5, and 1.0 mg/mL

Concentration (mg/mL)	%-intensity at $\delta_H -0.26 / \delta_C 73.4$	%-intensity at $\delta_H 1.03 / \delta_C 73.4$	%-intensity at $\delta_H 0.83 / \delta_C 72.0$	Average peak %-intensity
5.0	1.2	0.50	0.69	0.79
2.5	0.46	0.15	0.12	0.24
2.0	0.072	0.067	-	0.070
1.5	0.059	0.050	-	0.055
1.0	0.046	0.046	-	0.046
0.5	-	-	-	0.00

3.3.6. Limitations and considerations

A limitation of this approach is the requirement of a NMR spectrometer with a high-field strength magnet. The experiments in this study were performed on a Bruker 800 MHz NMR spectrometer equipped with a cryoprobe and on average the HSQC-TOCSY approach proved useful. Acquisition using the same parameters on a 500 MHz NMR spectrometer did not provide enough sensitivity. Even when acquired on the 800 MHz spectrometer, extracts with low yields (< 4 mg of crude extract) did not show many correlations in the spectra. The trade-off when using a lower frequency magnet is the increased number of scans required to obtain the same spectrum sensitivity. Depending on the number of isolates to be screened, increased acquisition time might be a feasible option in order to improve detection. Reduction of the relaxation delay between scans to 0.8 seconds further enables shortening of acquisition time without overly compromising the sensitivity of the spectrum (except of protons with long spin-lattice relaxations - T_1).²² Another way to overcome the issue of low detection would be the use of Shigemi NMR tubes that require smaller amounts of NMR solvent and therefore result in increased concentration of the sample.

The advantage of working with microorganisms is that their secondary metabolism can be manipulated through small modifications of the culture media.²³ The one-strain-many-compounds (OSMAC) principle is commonly used to diversify chemical extracts of microorganisms. HSQC-TOCSY spectral profiling is a useful way to determine which cultivation media and conditions are most successful at triggering secondary metabolite production. **Figure 10** represents how cultivation of *Streptomyces* sp. strain USC-16022 on GYM + ASW compared to OMA media produced a greater diversity of secondary metabolites. It appears that GYM + ASW media particularly triggered the synthesis of peptides and a lot more aromatic peaks can be observed. A further solution to enhance peak detection by NMR would be media enrichment with ^{13}C -labelled sugars, amino acids or propionate.

Figure 10. Representation of OSMAC for strain USC-16022 on (a) GYM + ASW and (b) OMA extracts

3.4. Conclusion

A new approach to detect specific natural product structural fragments in HSQC-TOCSY NMR spectra of microbial crude extracts was developed. These HSQC-TOCSY profiles together with biological activity and subsequent dereplication through PCoA was used to prioritise microbial strains that have a higher potential to produce bioactive secondary metabolites. The successful application of this technique was exemplified through the selection of a promising microbial strain USC-16018 from a library of 119 actinomycete crude extracts that lead to the isolation of a polyketide with potent anti-plasmodial activity. This adds to the repertoire of strain selection/dereplication approaches and it is the first time that HSQC-TOCSY NMR spectra have been used for this purpose. These findings highlight the great potential of NMR for chemical profiling.

3.5. Experimental section

3.5.1. Sample collection and microbial strain isolations

One hundred and twenty actinomycete strains were isolated from three Australian ascidians, *Symplesma rubra*, *Aplidium solidum* and *Polyclinum vasculosum* as previously described in Chapter 2. One marine sediment derived actinomycete isolate (USC-636) with previously assessed chemistry was also included in the analysis.²¹

3.5.2. Establishment of microbial extract library

One hundred and twenty-one actinomycete isolates were incubated on both oatmeal agar (OMA; 20 g oatmeal, 20 g bacteriological agar, 3 g yeast extract, 1 L deionised H₂O) and glucose yeast malt agar plates (GYM + ASW; adapted from DSMZ Medium 65 https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium65.pdf) for 2 weeks at 28 °C. Two of actinomycete isolates (USC-16072 and USC-16114) could not be revived from the glycerol stocks and were excluded from the analysis. After two weeks of incubation, the agar cultures were transferred to a 100 mL Schott bottle and suspended in EtOAc, terminating the growth of the culture and commencing chemical extraction. The culture plates were blended with a conventional kitchen stick blender and left on a rotary shaker at 200 rpm overnight. The EtOAc extracts were decanted into clean test tubes and dried under vacuum in a centrifugal evaporator (GeneVac Technologies HT-12). The extracts were resuspended in MeOH, filtered (0.2 µm), and again dried under vacuum. Yields of crude extract per culture plate varied

between 0.8 – 68 mg. For NMR spectroscopy, the unfractionated extracts, as well as media blanks, were suspended in 200 μ L DMSO- d_6 and transferred to 3 mm NMR tubes.

The ^1H NMR data was acquired under following parameters: number of scans 16, ^1H 90° pulse 8.4 μ sec, interscan relaxation delay 0.8 sec, spectral width 20.0294 ppm, transmitter centre 6.175 ppm, O1 4940.62 (acquisition time 58 sec). HSQC-TOCSY spectra were obtained at: number of scans 16, ^1H 90° pulse 8.4 μ sec, interscan relaxation delay 0.8 sec, increments 128, spectral width 16.0235 ppm, transmitter centre 6.000 ppm, O1 4800.6 Hz, F1 spectral width 170 ppm, F1 transmitter centre 80.000 ppm, spinlock mixing time 0.06 μ sec.

3.5.3. Anti-plasmodial testing

P. falciparum parasites (3D7 and Dd2 strains) were grown in RPMI 1640 supplemented with 25 mM HEPES, 5 % AB human male serum, 2.5 mg/mL Albumax II, and 0.37 mM hypoxanthine. Initially, the parasites were subjected to two rounds of sorbitol synchronisation. After which ring stage parasites were exposed to the microbial crude extracts at concentrations of 200, 20, 2, and 0.2 μ g/mL in 384-wells imaging microplates (PerkinElmer CellCarrier), as previously described.²⁴ Plates were incubated for 72 h at 37 °C, 90 % N_2 , 5 % CO_2 , 5 % O_2 , following incubation parasites were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI), and imaged using an Opera QEHS micro-plate confocal imaging system (PerkinElmer). Images were analysed as previously described by Duffy and Avery.²⁴

3.5.4. Data processing and Multivariate analysis

In MestReNova 11.0, proton spectra were corrected for phase and baseline and referenced to the DMSO- d_6 solvent peak (δ_{H} 2.50 ppm). For processing of HSQC-TOCSY spectra, t2 noise was reduced, apodisation was set to 0.00° sine bell, 10.00 Hz Gaussian and 50.0 % Sine Bell II and Sine Square II in the f2 dimension and to 90° sine bell in f1; spectra were again referenced to the DMSO- d_6 solvent peak in both dimensions (δ_{H} 2.50 ppm and δ_{C} 39.51 ppm). Cross peaks were picked by zooming in to the regions of interest setting peak picking to manual threshold with the peak picking options set to 4.00 sensitivity and maximum number of peaks 1000 and dragging the cursor over the spectra. For polyketide signals this included the region from δ_{H} 0.00 – 2.00 ppm and from δ_{C} 60.0 – 160.0 ppm, for peptides from δ_{H} 6.00 – 14.00 ppm and δ_{C} 10.0 – 100.0 ppm, and for heteroaromatics and double bonds with electron withdrawing atoms between δ_{H} 5.00 – 14.00 ppm and δ_{C} 135 – 160 ppm. If several spectra are selected, MestReNova allows peak picking to be performed in parallel on all selected spectra. All 2D peaks were exported as text files and peak data was tabulated in Excel (Microsoft Office 2010). Using an Excel macro, peaks between samples were aligned allowing for an error threshold of

0.04 ppm for proton shifts and 0.4 ppm for carbon chemical shifts. A Dice similarity matrix was then established and used for Principal Coordinate Analysis (PCoA).

3.5.5. Determination of HSQC-TOCSY spectra detection limit

Rifampicin (SigmaAldrich) standards were prepared at 5.0, 2.5, 2.0, 1.5, and 1.0 mg/mL in DMSO-*d*₆ and transferred to 3 mm NMR tubes. HSQC-TOCSY spectra were acquired under the same parameters as stated above for the microbial crude extracts. Peaks in the polyketide region of interest (δ_{H} -0.50 – 2.00 ppm and δ_{C} 60 – 155 ppm), as well as the DMSO solvent peak (δ_{H} 2.50/ δ_{C} 39.51) were picked in MestReNova 11.0 and exported to Excel. For each concentration standard, the average peak intensity of polyketide associated peaks were determined relative to the DMSO solvent peak and plotted as a concentration curve.

3.6. References

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CHAPTER 4 - Detecting chemical diversity: HSQC-TOCSY NMR spectra show co-cultivation-induced secondary metabolite production

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The author this thesis, L. Buedenbender, designed and performed all experimental work, data analysis and prepared the manuscript. The co-authors D. I. Kurtböke and A. R. Carroll are the supervisors of the PhD project and provided advice on the research design and feedback.

(Signed) (Date) 01/09/2017

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4.1. Abstract

Co-cultivation of different microbial strains can activate biosynthetic gene clusters that remain silent under standard laboratory fermentations involving mono-cultures. Therefore, the co-culturing technique has great potential for natural product drug discovery. However, innovative methods are still needed to evaluate the success of any co-cultured fermentation end product. Herein, the effective application of HSQC-TOCSY NMR spectra and subsequent PCoA for the detection of induced secondary metabolites in co-cultures between *Streptomyces* sp. (USC-16018), *Micromonospora* sp. (USC-16046), *Nocardia* sp. (USC-16096) and *Staphylococcus aureus* (ATCC 157293) is described.

4.2. Introduction

Rediscovery of already known compounds is a major issue in microbial natural product drug discovery. In recent years, progress has been made in developing more efficient dereplication approaches to minimise rediscovery. In parallel, whole genome sequencing of microorganisms has revealed their immense potential to produce secondary metabolites, yet the majority of biosynthetic genes remain silent under common laboratory culturing conditions.¹⁻³ Therefore, increased research has focused on optimising culturing methods in order to activate the silent biosynthetic gene clusters.⁴⁻⁶ The OSMAC (one-strain-many-compounds) strategy, which uses chemical and physical stressors in the media that trigger production of diverse secondary metabolites, has become widely implemented.⁷ Another effective approach to induce chemical diversity is to co-cultivate two or more microbial strains.⁸ In nature, microorganisms occur in dynamic communities and thus co-culture allows for interspecific interactions that resemble natural microbial ecosystems more closely and trigger the production of cryptic metabolites.⁹ Numerous examples of successful co-cultivation induced natural products, most often polyketides and non-ribosomal peptides, have been published and reviewed in the literature.^{6,10-14} Such examples highlight the potential of co-culturing for natural product drug discovery, however, effective methods for the detection of metabolite induction through co-cultivation are still necessary to evaluate their success.

Chemometric-profiling approaches have been used to assess the induction of microbial metabolites through co-cultures.¹² Hyphenated methods such as LC-PDA or LC-MS are often used, but are dependent on the presence of chromophores or the ability of the natural products to ionise. NMR fingerprinting techniques have also been reported, by which the crude extract of mono- and co-cultures were fractionated and ¹H NMR spectra of the pure cultures profiles were compared to those of the co-culture.¹⁰ The strengths of NMR profiling is that it provides insight into structural components of the extract, it is highly reproducible and provides universal detection.¹⁵ A technique

using 2D HSQC-TOCSY NMR profiles to prioritise microbial strains that have higher potential to produce drug-like natural products was reported previously (Chapter 3). The advantage of using 2D NMR profiles is that chemical resolution is improved by spreading the structural information over two dimensions and consequently allows assessment of unfractionated crude extracts. Herein, the effectiveness of this approach to identify changes in the metabolite diversity induced through co-cultivation was investigated.

4.3. Results and Discussion

For co-cultivation, four strains all originating from different species, namely *Streptomyces* sp. (USC-16018), *Micromonospora* sp. (USC-16046), *Nocardia* sp. (USC-16096) and *Staphylococcus aureus* (ATCC 157293), were chosen in order to assess a broader diversity of microbial strains. Liquid fermentation was favoured over solid agar cultures as mixed fermentations can more easily be upscaled, while on agar cultures the interactions between the two strains are generally highly localised and therefore compound isolation becomes more challenging as often low quantities of the compounds are produced.¹² Mono- and co-cultures were fermented in liquid ASW-A media and after two weeks of incubation, the microbial cells were separated from the media and extracted in MeOH. The liquid media phases were partitioned with EtOAc to extract the metabolites that were diffused into the media. The crude extracts of the mono- and co-cultures were profiled using 2D HSQC-TOCSY NMR experiments. The advantage of this approach is that metabolite changes such as *de novo* production, and up- or down-regulation of metabolites in crude extracts can readily be detected in the spectra without fractionation of the crude extract. NMR further provides insights about the structural classes in the extract. This is particularly valuable for untargeted analysis, where a chemical profile is first established to assess chemical diversity within a sample and subsequent chemometric analysis can be used to identify uniqueness and similarities in the metabolome and reduce redundancy within the set of samples.

Visual inspection of the mono-culture profiles of the MeOH cell extracts depicted notable differences in microbial metabolomes at the genus level. The *Micromonospora* sp. (USC-16046) displayed resonances of high intensity associated with peptides that were not detected in the other three mono-cultures. Resonances associated with polyketide fragments were observed in the *Nocardia* sp. (USC-16096) mono-culture as well as numerous peptide-associated NMR signals and diverse aromatic resonances. The *Staphylococcus* species (ATCC 157293) profile appeared chemically less diverse but indicated the presence of unique polyketide signals and *Streptomyces* sp. (USC-16018) had the least diverse mycelia extract (**Figure 1**). In contrast, the EtOAc extract of the

Streptomyces sp. mono-culture showed the most complex secondary metabolite resonances in the HSQC-TOCSY NMR spectrum with particular correlations from a proton at δ_H 11.07 ppm to carbons at δ_C 111.9 ppm and δ_C 123.1 ppm indicating the presence of a heteroaromatic compound (**Figure 2**). The other three EtOAc mono-culture extracts had almost identical HSQC-TOCY profiles (**Figure 2**).

Comparison of the HSQC-TOCSY spectra of the MeOH cellular mono- and co-culture extracts showed clearly that the co-culture between *Micromonospora* sp. (USC-16046) and *S. aureus* (ATCC 157293) had triggered *de novo* secondary metabolite production. Numerous aromatic resonances not observed in the mono-cultures of the two strains as well as several peptide-associated peaks could be identified in the HSQC-TOCSY NMR profile (**Figure 1**). Additional polyketide fragment resonances were observed in the co-culture of *Streptomyces* sp. (USC-16018) and *Nocardia* sp. (USC-16096) (**Figure 1**). The EtOAc extract of the co-culture between *Streptomyces* sp. (USC-16018) and *S. aureus* (ATCC 157293) indicated that heteroaromatic compounds had diffused into the culture media (correlations of δ_H 8.08 and 8.16 to δ_C 152.8) (**Figure 2**).

Figure 1. HSQC-TOCSY NMR profile matrix of mono- and co-cultures of MeOH mycelia extracts from four different microbial strains
(dark teal = MeOH extracts of mono-cultures, light teal = MeOH extracts of co-cultures)

Figure 2. HSQC-TOCSY NMR profile matrix of mono- and co-cultures of liquid media extracts from four different microbial strains
(black = EtOAc extracts of mono-cultures, grey = EtOAc extracts of co-cultures)

A chemometric metabolomics approach using multivariate analysis was implemented in order to dereplicate the dataset and identify significant correlations and differences in the secondary metabolomes. As described in the strain selection approach (Chapter 3), peaks were picked from the HSQC-TOCSY NMR spectra regions of interest that were associated with polyketide, peptide, double bond, aromatic and heteroaromatic structure fragments. After removal of resonances found in the media control, this resulted in a total of 253 HSQC-TOCSY cross-peaks associated with secondary metabolites. Peaks between the samples were aligned using an in-house Excel macro. Dice similarities were calculated and the newly generated correlation matrix was used for Principal Coordinate Analysis (PCoA, **Figure 3**). The first two principal coordinates (F1/F2) were able to explain 46.6 % of the variation within the dataset.

Clear separation of the EtOAc liquid media extracts and the MeOH cell mycelium extracts could be observed (**Figure 3**). Furthermore, the mono-cultures of *Streptomyces* sp. (USC-16018) (S) and its co-cultures with *Micromonospora* sp. (USC-16046) and *Nocardia* sp. (USC-16096) (SM and SN) clustered away from the remaining EtOAc culture extracts. Visual inspection of their HSQC-TOCSY NMR profiles revealed that they were almost identical and dominated by *Streptomyces* sp. (USC-16018) metabolites. The remaining liquid media extracts grouped closely together in the PCoA plot (**Figure 3**); therefore, *Streptomyces* sp. (USC-16018) metabolites in the co-culture with *S. aureus* (ATCC 157293) should be suppressed. These observations were further validated through comparison of the HSQC-TOCSY NMR profiles (**Figure 3**).

Figure 3. PCoA plot showing differences between MeOH and EtOAc extracts of mono- and co-cultures (S = *Streptomyces* sp., M = *Micromonospora* sp., N = *Nocardia* sp., Sa = *Staphylococcus aureus*)

The differences in secondary metabolites of the MeOH extracts were not as obvious to derive from the PCoA plot; therefore, separate PCoA plots for MeOH and EtOAc extracted cultures were generated (**Figure 4**). Based on the position of the mono-cultures compared to the co-culture it could be determined which of the two strains was the more dominant in the culture (**Figure 4a**). The co-culture of *Micromonospora* sp. (USC-16046) and *S. aureus* (ATCC 157293) (MSa) was distinct to both mono-cultures reiterating the uniqueness of this co-culture previously observed in the HSQC-TOCSY profile.

Figure 4. PCoA plot showing differences between (a) MeOH mycelia extracts and (b) EtOAc liquid culture media extracts (S = *Streptomyces* sp., M = *Micromonospora* sp., N = *Nocardia* sp., Sa = *Staphylococcus aureus*)

The HSQC-TOCSY NMR profiling approach proved to be very useful for the assessment of co-cultures. Not only did the profiles provide insight about *de novo* induction of secondary metabolites but in some cases also their suppression. The *Micromonospora* sp. (USC-16046) peptide resonances, which were dominant in all other MeOH co-cultures with the species, were highly suppressed in the co-culture with *S. aureus* (ATCC 157293), while aromatic resonances were significantly enhanced. Furthermore, it was clearly evident that the microorganisms diffuse secondary metabolites into the media that significantly differ from the metabolites found in their mycelia. Therefore, it is valuable to consider a range of extraction methods and solvents to obtain a complete spectrum of secondary metabolites in the culture.

Observations about the success and what type of structural fragments were found in the extracts could be made with minimal sample preparation in very little time as these 2D NMR experiments took only half an hour to acquire. The integration of chemometric analysis can be a useful guide to detect unique samples. Especially, in larger datasets where sometimes similarities or differences in the

NMR spectra cannot be identified solely by visual inspection. In a co-culture setting, chemometric metabolomics analysis is more suitable than in the strain selection approach (Chapter 2) where the samples were extremely diverse. All co-culture will be related to the mono-cultures of the strains and thus better “fit” in the statistical multivariate analysis.

Only few examples have been reported that utilise NMR profiles for the assessment of co-cultures.^{10,16-18} Wu and co-workers, implemented a ¹H-NMR-based metabolomics approach to study metabolomic changes in a *Streptomyces*-fungus co-culture and found significant differences between mono- and co-culture extracts, but only in combination with UHPLC-TOF-MS they were able to identify the compound classes.¹⁷ The HSQC-TOCSY profiling approach here presented is the first account where 2D NMR profiling was used on co-cultivations. Due to the increased chemical shift resolution through spreading of the detected signals across two planes in 2D NMR spectra, assessment of unfractionated crude extracts was permitted and immediately revealed the structure type of the induced secondary metabolites.

In the future, it will be interesting to pursue large-scale fermentation of the co-culture between *S. aureus* (ATCC-157293) and either *Micromonospora* sp. (USC-16046) and or *Streptomyces* sp. (USC-16018) in order to identify the co-culture induced *de novo* produced secondary metabolites.

4.4. Experimental section

4.4.1. Microbial fermentations

Streptomyces sp. (USC-16018), *Micromonospora* sp. (USC-16046), *Nocardia* sp. (USC-16096) and were isolated from the Australian ascidian *Sympylegma rubra* (Chapter 2). *Staphylococcus aureus* (ATCC-157293) strain was obtained from Mr Joshua Hayton (Griffith University, Gold Coast). Each strain obtained from 20 % glycerol stocks was fermented in 15 mL Eppendorf tubes containing 4 mL of brain heart infusion broth (Thermo-Fischer) and incubated at 28 °C and 200 rpm for 4 days. Subsequently, 0.5 mL of each culture were individually transferred to 50 mL Cornig MiniBioreactors with 20 mL ASW-A (20.0 g soluble starch, 10.0 g glucose, 5.0 g peptone, 5.0 g yeast extract, 5.0 g CaCO₃ per litre of 186 artificial seawater) and kept at 28 °C and rotation (200 rpm) again for 4 days. For the co-cultivation experiment, 0.8 mL of each strain was separately inoculated with 0.8 mL of each of opponent strain in 100 mL conical flask containing 60 mL ASW-A and kept at rotation (150 rpm) at 28 °C for 2 weeks. In parallel, one mono-culture of each of the four microbial strains was also incubated under the same conditions as controls.

4.4.2. Preparation of microbial extracts

After fermentation, the cellular mycelium was separated from the culture media by filtration through refillable cellulose tea bags. The harvested cell mycelia were washed with deionised water and subsequently exhaustively extracted in MeOH. The liquid media phases were transferred into a 250 mL separation funnels and twice extracted with 60 mL EtOAc. All extracts were dried under vacuum (GeneVac Technologies HP-12), resuspended in a small portion of MeOH, then filtered using a 0.22 μ m-pore-size filter and subsequently dried. Yields per culture varied between 4 – 72 mg.

For NMR spectroscopy, the unfractionated extracts, as well as an ASW-A media blank, were suspended in 200 μ L DMSO- d_6 and transferred to 3 mm NMR tubes. The ^1H NMR data was acquired under following parameters: number of scans 16, ^1H 90° pulse 8.4 μ sec, interscan relaxation delay 0.8 sec, spectral width 20.0294 ppm, transmitter centre 6.175 ppm, O1 4940.62. HSQC-TOCSY spectra were obtained at: number of scans 16, ^1H 90° pulse 8.4 μ sec, interscan relaxation delay 0.8 sec, increments 128, spectral width 16.0235 ppm, transmitter centre 6.000 ppm, O1 4800.6 Hz, F1 spectral width 170 ppm, F1 transmitter centre 80.000 ppm, spinlock mixing time 0.06 sec.

In MestReNova 11.0, proton spectra were corrected for phase and baseline and referenced to the DMSO solvent peak (2.50 ppm). For processing of HSQC-TOCSY spectra, t2 noise was reduced, apodization was set to 0.00° sine bell, 10.00 Hz Gaussian and 50.0 % Sine Bell II and Sine Square II in the F2 dimension and to 90° sine bell in F1; spectra were again referenced to the DMSO solvent peak (δ_{H} 2.50 ppm and δ_{C} 39.51 ppm). Cross peaks were picked by zooming into the regions of interest setting peak picking to manual threshold with the peak picking options set to 4.00 sensitivity and maximum number of peaks 1000 and dragging the cursor over the spectra.; for polyketide specific peaks from 0.00 to 2.00 ppm in the proton dimension and from 60.0 to 160.0 ppm in the carbon dimension, for peptides from 6.00 ppm – 14.00 ppm (F2) and 10.0 to 100.0 ppm (F1) and heteroaromatics and double bonds with electron withdrawing atoms between 5.00 – 14.00 ppm (F1) and 135 – 160 ppm (F2). If several spectra were selected, MestReNova allowed peak picking to be performed in parallel on all selected spectra. All 2D peaks were exported as text files and peak data was tabulated in Excel. Using an Excel macro, peaks between samples were aligned allowing for an error threshold of 0.04 ppm for proton shifts and 0.4 ppm for carbon chemical shifts. A Dice similarity matrix was then established and used for Principal Coordinate Analysis (PCoA), which was visualised in OriginPro.

4.5. References

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SECTION 3

Marine actinomycetes as a source of new anti-plasmodial compounds

Malaria is a highly infectious and potentially deadly disease caused by parasites from the genus *Plasmodium*. Due to the evolution of drug resistance in the parasite, it is still of high importance to find new drugs to treat this disease. This section focuses chemical investigation on marine actinomycetes as a source for anti-plasmodial compounds. The application of the new HSQC-TOCSY NMR profiling approach presented in Chapter 3 indicated that *Streptomyces* sp. (USC-16018) contains a wealth of polyketide associated NMR signals in its crude extract, which also exhibited 97 % inhibition of *P. falciparum* at a concentration of 0.2 µg/mL. Chapter 5 of this thesis documents the isolation and structure elucidation of a new polyketide with weak anti-plasmodial activity, as well as isolation of five known natural products.

In Chapter 6, isolation of another new and moderately anti-plasmodial natural product from *Streptomyces* sp. (USC-636) is described. This actinomycete strain was previously isolated from marine sediment by my supervisor D. Ipek Kurtböke. I pursued chemical investigations of this isolate in the early stages of this PhD project and it benefited as a proof of concept to determine the potential of actinomycetes as producers of anti-plasmodial molecules.

CHAPTER 5 - HSQC-TOCSY profiling directed discovery of anti-plasmodial polyketide compounds in marine *Streptomyces* species (USC-16018)

STATEMENT OF CONTRIBUTION TO CO-AUTHORED PAPER IN PREPARATION

This chapter is a co-authored paper in preparation for submission. The bibliographic details of the co-authored paper, including all authors, are:

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The author of this thesis, L. Buedenbender fermented the microbial strain, isolated, and elucidated the natural products and prepared the manuscript. All of the theoretical calculations for the ECD spectra presented were the work of Luke P. Robertson. Dr Leonardo Lucantoni, from “Discovery Biology” of the Griffith Institute for Drug Discovery performed biological activity testing. The co-authors D. I. Kurtböke and A. R. Carroll are the supervisors of the PhD project and provided advice on the research design and feedback.

(Signed) (Date) 01/09/2017

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5.1. Abstract

Chemical investigations of the EtOAc extract of the ascidian-derived *Streptomyces* sp. (USC-16018) yielded a new ansamycin polyketide, herbimycin G (**1**) another known macrocyclic polyketide, elaiophyllin (**2**) and four known diketopiperazines (**3** – **6**). The structures of the compounds were elucidated based on 1D/2D NMR and MS data. The absolute configuration of **1** was established by comparison of experimental and predicted ECD spectra, calculated using TDDFT. Anti-plasmodial activities were tested for the natural products against chloroquine sensitive (3D7) and chloroquine resistant (Dd2) *Plasmodium falciparum* strains; the two polyketides (**1** - **2**) caused significant inhibition of > 75 % against both parasite strains and while **2** was highly cytotoxic, the new compound (**1**) showed no cytotoxicity and good water solubility.

5.2. Introduction

Malaria is a vector borne disease, induced by protozoan parasites of the genus *Plasmodium*, and has caused millions of deaths worldwide.¹ The life cycle of the malaria parasite involves two hosts. The disease is contracted when an *Anopheles* mosquito feeds on human blood and simultaneously injects sporozoites into the blood stream. The sporozoites are then carried to the liver, where they asexually reproduce to give rise to thousands of merozoites that spread through the body via the blood stream.^{2,3} At this intraerythrocytic stage, the disease manifests and the human host experiences strong fevers and chills.⁴ Within the red blood cells, merozoites develop into the early trophozoites also known as the ring form. The mature trophozoites asexually multiply forming schizonts to finally produce male and female gametocytes. The gametocytes are transmitted back to a mosquito as it takes a blood meal and there they fuse to form oocysts, which divide into sporozoites. These are then transferred to the mosquito's salivary glands and the life cycle of the parasite begins again once the mosquito feeds on a new human's blood. Drug resistance against the currently available malaria treatments is a major concern and therefore new drugs with novel targets are urgently needed.

When the malaria parasite changes from mosquito to human hosts, it experiences immense heat stress and specific heat shock proteins are highly expressed in *Plasmodium* species, particularly during the intraerythrocytic stage of their life cycle.⁵ Recently, anti-plasmodial drug development has been focused on heat shock protein 90 (Hsp90) inhibitors. Benzoquinone ansamycin compounds, such as geldanamycins and herbimycins, produced by *Streptomyces* sp. strains, are known to exhibit specific inhibition of Hsp90. In vitro studies have found that geldanamycin inhibits the growth of chloroquine-sensitive and chloroquine-resistant

Plasmodium falciparum strains (IC_{50} 20 nM).⁶ Other geldanamycin derivatives were reported to be effective against *Plasmodium yoelii* in mouse models when injected in repeated doses of 300 nM/mouse and treated mice appeared to develop immunity against reinfection.⁷ Thus, ansamycin analogues appear to be good candidates for anti-plasmodial drugs. Due to the low water solubility and high liver toxicity of geldanamycin, it is of great interest to find new water soluble and non-cytotoxic Hsp90 inhibitors to treat malaria infections.

Actinomycetes are the main class of bacteria known to produce ansamycins. In order to increase structural diversity, the presented study focused microbial isolations on underexplored ecological niches and 120 actinomycetes from Australian ascidian species were isolated. A new approach utilising two-dimensional HSQC-TOCSY NMR profiles and biological activity data was previously described (Chapter 3). This allowed targeting of polyketide-producing strains based on identified regions of interest in the NMR spectrum that corresponded to correlations from upfield methyl proton resonances (δ_H 0.50 – 2.00) into carbon resonances between δ_C 60–80 ppm (Appendix III: Figure IIIA), which were associated with polyketide-type compounds. Based on this approach *Streptomyces* sp. strain USC-16018 was selected because its crude extract HSQC-TOCSY NMR profile indicated the presence of several polyketide containing compounds and the extracts also exhibited 97 % inhibition of *Plasmodium falciparum* at 0.2 μ g/mL. The strain was fermented on a larger scale and purification of the EtOAc extract derived from this culture resulted in the isolation of two anti-plasmodial polyketides, herbimycin G (**1**), a new geldanamycin analogue, and the known antibiotic elaiophylin (**2**), as well as four known diketopiperazines (**3** - **6**).

5.3. Results and discussion

Fermentations were scaled up on 55 culture plates for two solid media, OMA and GST, which after extraction with EtOAc yielded 1.6 g and 0.8 g of crude extract respectively. The crude extracts were separated on a reversed-phase C_{18} HPLC column and 60 fractions were collected and analysed by 1H NMR. NMR-guided isolation led to the identification of two polyketides (**1** and **2**) and four diketopiperazine compounds (**3-6**).

Herbimycin G (**1**) was isolated as a dark brown solid with a UV maximum at 245 nm. A sodium adduct ion at m/z 602.3111 $[M+Na]^+$ obtained from HRESI-QTOF-MS together with 1H and ^{13}C NMR data suggested that **1** had a molecular formula of $C_{30}H_{46}N_2O_9$. The 1H NMR spectrum of **1** in DMSO- d_6 had resonances that could be assigned to an amide proton at δ_H 8.02 (d, J = 9.4 Hz), five olefinic proton resonances at δ_H 7.13 (dd, J = 12.2 Hz, , 1.2 Hz), δ_H 6.65 (ddd, J = , 3.3 Hz, 1.9 Hz, 1.4 Hz), δ_H 6.48 (ddd, J = 12.1 Hz, 10.9 Hz, 1.4 Hz), δ_H 5.65 (dd,

$J = 10.9$ Hz, 7.9 Hz) and δ_{H} 5.20 (d, $J = 7.7$ Hz), and an oxygenated methine at δ_{H} 4.68 (dddd, $J = 10.6$, Hz 5.1 Hz, 4.7 Hz, 2.5 Hz) that correlated to a hydroxyl group at δ_{H} 5.56 (d, $J = 5.1$ Hz) in the COSY spectrum. A further five oxygenated methines were observed at δ_{H} 4.56 (dd, $J = 7.9$ Hz, 1.4 Hz), δ_{H} 5.16 (s), δ_{H} 3.15 , δ 3.33 and δ_{H} 4.15 (d, $J = 1.4$ Hz), and four methoxy-resonances occurred between δ_{H} $3.13 - 3.35$ ppm. The ^1H NMR spectrum contained an additional three methines at δ_{H} 4.55 , δ_{H} 2.45 , δ_{H} 1.51 , two methylene pairs δ_{H} 1.55 and δ_{H} 1.41 as well as δ_{H} 1.96 and δ_{H} 2.26 , and four methyl signals δ_{H} 1.84 (d, $J = 1.2$ Hz), δ_{H} 1.62 (s), δ_{H} 0.94 (d, $J = 6.7$ Hz) and δ_{H} 0.69 (d, $J = 6.7$ Hz). The ^{13}C and HSQC NMR data showed 30 carbon resonances (**Table 1**).

Table 1. NMR data for herbimycin G (**1**) (800 MHz in DMSO-*d*₆, δ in ppm)

Position	δ_C	δ_H (multiplicity, <i>J</i> in Hz)	COSY	HMBC	ROESY
NH		8.02 (d, 9.4)	20	1, 20	3, 19a
1	171.5				
2	133.6				
3	127.5	7.13 (dd, 12.2, 1.2)	4	1, 3, 5, 22	
4	126.4	6.48 (ddd, 12.2, 10.9, 1.4)	3,5	2, 6	
5	135.4	5.65 (dd, 10.9, 7.9)	4,6	4	
6	78.1	4.56 (dd, 7.9, 1.4)	5,7	5	7
6-OCH ₃	56.5	3.20 (s)			6, 24
7	78.0	5.16 (s)	6	8	
7-OCONH ₂	156.5	6.66, 6.30			
8	133.0				
9	129.4	5.20 (d, 7.7)	10	23	
10	33.5	2.45, m	9,11, 24	8, 11	
11	83.8	3.15, m	10,12	12-OCH ₃ , 13	
11-OCH ₃	59.8	3.35 (s)		11	12, 24
12	82.3	3.33, m	11,13		11-OCH ₃
12-OCH ₃	56.5	3.19 (s)		12	13a
13a	33.8	1.41 (dddd, 14.2, 9.7, 3.4, 3.4)			12, 14
13b		1.55, m			
14	36.6	1.51, m	13,15, 25		
15	77.6	4.15 (d, 1.4)	14	13, 15-OCH ₃ , 16, 17, 25	13a, 14
15-OCH ₃	57.2	3.13 (s)		15	
16	134.0				
17	149.9	6.65 (ddd, 3.3, 1.9, 1.4)		15, 16, 19, 21	
18	65.1	4.68 (dddd, 10.6, 5.1, 4.7, 2.5)	17,19		19b
18-OH		5.56 (d, 5.1)		17, 18, 19	19a
19a	39.2	1.96 (dddd, 14.1, 11.8, 10.6, 2.5)	18	18, 20	NH, 18-OH
19b		2.26 (dddd, 11.8, 4.9, 4.7 1.9)	18	17, 18, 20, 21	18, 20
20	53.2	4.55 (ddd, 14.1, 9.4, 4.9)	NH,19	19, 21	19b
21	197.1				
22	12.9	1.84 (d, 1.2)		1, 2, 3	
23	14.2	1.62 (s)		7, 8, 9	
24	17.2	0.94 (d, 6.7)	10	9, 10, 11	11-OCH ₃
25	13.9	0.69 (d, 6.7)	14	13, 14, 15	

(s – singlet, d – doublet, m – multiplet)

HMBC correlations confirmed the assignment of these spin systems and were used to elucidate the linkage points of the molecule. It was established that the oxygenated methines, H-6, H-11, H-12 and H-15, were all next to *O*-methyls (**Table 1**). The HMBC correlation of the exchangeable proton at δ_H 8.02 to a carbonyl resonance at δ_C 171.5 was consistent with an amide. $^3J_{CH}$ correlations into the same carbonyl were observed from the olefinic proton H-3 and the methyl at δ_H 1.84/ δ_C 12.9 (H-22) and $^2J_{CH}$ correlations from both these proton resonances into quaternary carbon resonance at δ_C 133.6 suggested that this carbon was *alpha* to the amide carbonyl. HMBC correlations from the methyl at δ_H 1.62 to C-7 (δ_C 78.0), C-9 (δ_C 129.4) and a quaternary carbon resonating at δ_C 133.0 (C-8) established the connection of the first and second described spin systems through C-8. H-11 showed HMBC correlations to a carbon resonating at

δ_C 82.3 (C-12) and H-12 to C-11 (δ_C 83.8) that were very hard to discern due to immense overlap in this region, but provided sufficient evidence for a linkage between C-11 to C-12. H-15 (δ_H 4.15) showed an additional two unassigned HMBC correlations to the olefinic carbon C-17 (δ_H 149.9) as well as a quaternary carbon at δ_H 134.0, linking the spin systems at C-16 with the last spin system. The far downfield double bond resonance of C-17 indicated an electron withdrawing group *beta* to the double bond proton and a strong HMBC correlation from H-17 to a carbon at δ_C 197.1 determined it to be a carbonyl group. As the downfield methylene proton (H-19b) also showed a correlation to the carbonyl resonance, it was concluded that this fragment consisted of a six-membered 4-hydroxyl-cyclohexenone system. An additional carbon resonance at δ_C 156.5 was clearly visible in the carbon spectrum; however, it showed no HMBC correlations. The chemical shift of this carbon was appropriate for a carbamate and the observation of an additional two heteroatom substituted protons at δ_H 6.66 and δ_H 6.30 supported this assignment.⁸ The downfield chemical shift of the oxygenated proton at δ_H 5.16 (H-7) suggested that the carbon C-7 was the point of attachment of the carbamate group. With this remaining fragment assigned, the final structure was consistent with the sodium adduct ion m/z 602.3111 ($M + Na^+$) as well as the degrees of hydrogen deficiency calculated from the molecular formula.

An examination of the literature indicated that this compound was an analogue of geldanamycin and had an identical macrolactam ring to the structures of the herbimycin analogues A, D, E and 17,19-dimethylthioherbimycin A.⁹⁻¹¹ Previously reported geldanamycines and herbimycines contained a 2,6-disubstituted-p-benzoquinone ring or a hydroquinone ring bonded to the macrolactam, whereas the newly described compound, herbimycin G (**1**), contains a 2,6-disubstituted 4-hydroxyl-cyclohexenone ring and therefore has an additional two stereogenic centres. Consequently, a ROESY spectrum was obtained in order to elucidate the relative configuration of the nine stereogenic centres in **1**. ROESY correlations between H-6 (δ_H 4.57) and H-7 (δ_H 5.16) showed that the two protons were *cis* to each other, indicating that the O-methyl at C-6 and the carbamate group attached to C-7 were on the β -face of the molecule. Based on ROESY exchange (antiphase to the ROESY correlations) correlations the exchangeable carbamate NH₂ protons could be assigned as δ_H 6.30 and δ_H 6.66. A weak long range correlation between H-7 and the methyl protons H-24 at δ_H 0.94 established the relative configuration of the two spin system relative to C-7. The methyl protons at δ_H 0.94 also correlated to the methoxy protons at δ_H 3.35, and H-13a (δ_H 1.41), H-14 (δ_H 1.51) and H-15 (δ_H 4.15) were assigned to the α -face of the molecule. A weak ROESY correlation from H-15 to H-18 (δ_H 4.68) allowed determination of the configuration of the 4-hydroxyl-cyclohexenone system relative to the macrolactam ring. Further strong correlations between H-18 and H-19b (δ_H 2.26), which in turn correlated to H-20 (δ_H 4.55), indicated their position to be on the α -face.

The hydroxyl group, H19a (δ_{H} 1.96) and the amide proton (δ_{H} 8.02) faced in the β -direction. No COSY correlation was observed between H-17 and H-18, indicating that the two protons were at an angle close to 90° and small long range couplings ($J = 1.8$ Hz) between H-17 and H-19b suggested “W” arrangement. ROESY correlations from the methyl resonating at δ_{H} 0.69 to δ_{H} 1.96 indicated that the macrolactam folded over the 4-hydroxyl-cyclohexenone. The two-dimensional structure was therefore established and redrawn in Maestro 2016 to perform a Monte-Carlo Multiple Minimisation (MCM) search using the OPLS2005 force field to generate the energy minimised conformation of **1** (**Figure 1a**). The previously observed ROESY correlations were compared to the energy-minimised 3D structure of **1**, confirming its relative configuration. The absolute configuration of **1** was determined by comparison of its experimental and predicted electronic circular dichroism (ECD) spectra, calculated using time-dependent density functional theory (TDDFT). A conformational search using MacroModel was performed on the energy minimised structure of **1** using the force field parameters as above with extended torsional sampling options. All structures within a 10.0 kJ/mol energy window were saved, generating 13 conformers. Geometry optimisation and Gibbs free energy calculations using GAUSSIAN16 at the B3LYP/31G(d) energy level was then performed on each of the conformers. Following this, the ECD spectra of each of the geometry optimised conformers was calculated using GAUSSIAN16 at the same theoretical level. A Boltzmann-weighted ECD spectrum was then calculated using SpecDis 1.7. The experimental ECD spectrum of **1** showed good agreement with the calculated ECD of (6*S*,7*S*,10*R*,11*R*,12*S*,14*S*,15*R*,18*S*,20*R*)-**1** (**Figure 1b**).

Figure 1. (a) Key ROESY correlations of 1. (b) Comparison of the experimental ECD spectrum of 1 with those calculated at B3LYP/6-31G(d) level

The configuration of the macrolactam ring is consistent with herbimycin analogues previously reported in the literature, but herbimycin G (**1**) is the first example of an ansamycin compound that contains a 2,6-disubstituted 4-hydroxyl-cyclohexenone ring. A total of eleven herbimycin analogues, including herbimycin G, have now been reported from *Streptomyces* sp. strains (Appendix III: Figure IIIH).⁹⁻¹⁴ Benzoquinone ansamycines have been identified as potent inhibitors of the Hsp90, an important cancer target and more recently a novel target for anti-malaria drugs. Geldanamycin had reached clinical trials as an anti-cancer drug, however, the compound had low water solubility and exhibited high liver toxicity. As a result, a number of synthetically modified geldanamycin analogues were made and among those tanespimycin (17-AAG) advanced to phase three of anticancer clinical trials for multiple myeloma.¹⁵ In herbimycin G (**1**) the benzoquinone has been reduced to a 4-hydroxyl-cyclohexenone and thus potentially has better water solubility. Therefore, we assessed the $\text{clog}P$ and other Lipinski parameters of all known herbimycins, as well as a number of related ansamycin compounds (**Figure 2**, Appendix III: Table IIIA). Compared to geldanamycin, most herbimycin analogues, except for herbimycin D, F and TAN-420E, exhibited similar or lower $\text{clog}P$ values. Herbimycin G (**1**) and TAN-420B had the lowest $\text{clog}P$, 0.68 and 0.63, respectively, indicating better water solubility. The molecular weights of all assessed herbimycin and geldanamycin compounds were non-compliant with the Lipinski rule of five that uses four physiochemical properties (molecular weight < 500 Da, partition coefficient $\text{clog}P$ < 5, hydrogen bond donors < 5 and hydrogen bond acceptors < 10) to assess drug-likeness.¹⁶ Macrocycles frequently violate the rule of five, but nonetheless have been successful as clinical leads (i.e. rifamycin) or candidates (i.e. erythromycin) and have made their way to the market.¹⁷ This is due to their high binding affinity, stability and specificity for a molecular target.¹⁷

Figure 2. Analysis of $\text{clog}P$ values of herbimycins and related ansamycin compounds

In addition to the new herbimycin analogue, GST extracts from the *Streptomyces* sp. strain USC-16018 yielded the known polyketide, elaiophylin (**2**), and four diketopiperazines (**3** - **6**). Obtained spectroscopic data for **3** - **6**, including NMR spectra (**Table 2**) and specific rotations, were compared to the literature and Cyclo-L-Pro-L-Leu (**3**), Cyclo-L-Pro-L-Phe (**4**) Cyclo-L-Pro-L-Val (**5**) and Cyclo-L-Pro-L-Tyr (**6**) were identified.^{18,19} Additionally, a positive LR-ESI-MS pseudo-molecular ion at m/z 1024.65 was observed and the molecular formula for **2** was established to be $C_{54}H_{88}O_{18}$. Based on 1H , COSY, HSQC and HMBC spectra, a partial structure was elucidated containing 44 protons, 27 carbons and 10 oxygen atoms (**Table 3**). This only accounted for half the number of carbons and hydrogens, suggesting that **2** was a symmetric dimer. Comparison of this NMR data with carbon data documented in the literature identified **2** as the known antibiotic compound elaiophylin.²⁰ Several *Streptomyces* species are known to produce elaiophylin and geldanamycin compounds. Recently, complete genome mining of four geldanamycin producing *Streptomyces* sp. strains identified up to 56 putative biosynthetic gene clusters and found highly similar geldanamycin and elaiophylin gene clusters in all strains.²¹ Geldanamycin and herbimycin have the same ansamycin carbon skeleton, it is therefore assumed that both biosynthetic pathways exhibit very similar PKS activity. In fact, Rasher and co-workers²² proposed that both compounds are “assembled from the 3-amino-5-hydroxybenzoic acid (AHBA) starter unit by successive condensation of two-carbon building blocks in seven chain-elongation steps that use one malonyl, four methylmalonyl and two methoxymalonyl extender units” and structural differences are introduced through enzymatic modifications post PKS assembly.²² Such findings yet again highlight the biosynthetic potential of *Streptomyces* species. It is very likely that *Streptomyces* sp. strain USC-16018 under different laboratory conditions will be able to produce a wide range of additional secondary metabolites.

Table 2. NMR data for 3 – 5 (500 MHz in DMSO-d₆, δ in ppm)

position	3		4		5		6	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		168.0		165.0		165.9		165.0
3	3.38, 3.38	44.5	3.27, 3.38	44.6	3.26, 3.35	44.4	3.36, 3.40	44.2
4	1.83, 1.83	22.0	1.73	21.0	1.79, 1.87	22.0	1.72, 1.84	21.7
5	1.90, 2.13	27.0	1.42, 2.01	27.8	1.84, 2.13	27.6	1.40, 2.00	27.6
6	4.19	59.9	4.06	58.5	4.11	58.0	4.05	58.9
7		171.0		169.0		171.1		169.0
8	8.01		7.99		7.97		7.86	
9	4	52.3	4.35	56.0	3.92	59.5	4.26	55.7
10	1.37, 1.76	37.0	3.02, 3.06	35.0	2.35	27.3	2.93, 2.93	35.6
11	1.87	23.3		137.0	0.85	16.4		127.0
12	0.86	21.2	7.27	129.0	1.01	18.2	7.05	131.0
13	0.88	22.0	7.26	127.0			6.69	115.0
14			7.19					156.0
15			7.26	127.0			6.69	115.0
16			7.27	129.0			7.05	131.0

Table 3. NMR data for 2 (800 MHz in DMSO-d₆, δ in ppm)

position	δ_{H}	δ_{C}
1,1"		167.1
2,2"	5.07	121.0
3,3"	6.82	144.7
4,4"	6.12	130.4
5,5"	5.65	144.8
6,6"	2.48	40.7
7,7"	5.08	75.4
8,8"	1.78	36.0
9,9"	3.77	69.2
10,10"	1.57	42.6
11,11"		99.2
12,12"	0.96, 2.25	36.4
13,13"	3.78	68.8
14,14"	1.05	48.1
15,15"	3.76	65.6
16,16"	1.04	18.9
17,17"	0.96	15.3
18,18"	0.78	9.0
19,19"	0.86	6.8
20,20"	1.36, 1.60	18.7
21,21"	0.78	9.0
1',1'''	4.91	91.9
2',2'''	1.38	32.9
3',3'''	3.37	65.0
4',4'''	3.38	70.0
5',5'''	3.75	66.0
6',6'''	1.08	17.0

With the aim to discover marine actinomycete-derived anti-plasmodial compounds, compounds **1** - **5** were screened for activity against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strains of *Plasmodium* (Table 4). Elaiophylin (**2**) inhibited both the 3D7 and Dd2 parasites with similar potency, $IC_{50} = 778$ nM and 598 nM, respectively. Anti-plasmodial activity (IC_{50} 214 nM) against the 3D7 strain was previously reported in the literature, which was 3.6 fold lower than here presented.²³ Herbimycin G showed no cytotoxicity and significant parasite inhibition of above 77 % for 3D7 at 40 μ M. Against the chloroquine-resistant strain Dd2, **1** showed 82 % inhibition, which was similar to the observed activity for **2**, however, the curve did not plateau at the highest tested dose and therefore no IC_{50} could be determined (Figure 3). The remaining compounds were devoid of antimalarial or cytotoxic activity, while the activity of the reference compounds was in the expected range (Table 4).

Table 4. Anti-plasmodial activity and cytotoxicity of compounds 1 – 6

Compound	% inhibition at 40 μ M (IC_{50} in nM)		
	3D7	Dd2	HEK-293
Herbimycin G (1)	77.2	81.7	IA
Elaiophylin (2)	96.6 (777.9)	86.1 (598.5)	101.9 (1445)
Cyclo-Pro-Leu (3)	45.9	39.0	IA
Cyclo-Pro-Phe (4)	IA	IA	IA
Cyclo-Pro-Val (5)	IA	IA	IA
Cyclo-Pro-Tyr (5)	IA	IA	IA
Artesunate	99.6 (0.9)	98.4 (1.3)	50.6
Chloroquine	98.8 (10.0)	96.5 (87.9)	40.9
DHA	99.9 (0.4)	98.0 (0.6)	37.9
Puromycin	99.0 (148.9)	99.1 (114.4)	102.6 (1409.5)
Pyrimethamine	98.7 (4.7)	23.2	68.4
Pyronaridine	99.9 (7.4)	97.8 (8.3)	98.7 (2825.5)

Figure 3. Dose-response curve for 1 against 3D7, Dd2 and HEK-293 in duplicates

These results show that marine actinomycetes are still a valuable source for the discovery of new secondary metabolites. Several herbimycin, geldanamycin and other ansamycin compounds have been reported previously from terrestrial and marine *Streptomyces* species. To our knowledge, the new compound, herbimycin G (**1**), is the first example of such a compound containing a 2,6-disubstituted 4-hydroxyl-cyclohexenone ring. Herbimycin G exhibited weak anti-plasmodial activity with no cytotoxicity. We investigated the physiochemical properties of reported herbimycins and related analogues and found that **1** had a lower *clogP* compared to the other herbimycin analogues, potentially related to the different ring structure, suggesting good bioavailability. As herbimycin G did not show cytotoxicity against HEK293, it appears promising to test this compound for other biological activity, such as anti-tumour, in the future.

5.4. Experimental section

5.4.1. General Experimental Section

Extracts were dried with a rotary evaporator (Christ Rotational Vacuum Concentrator – RVC 2-25 CD plus). NMR spectra were recorded at 25 °C on a Bruker BioSpin GmbH 800 MHz spectrometer equipped with a cryoprobe. The ^1H and ^{13}C chemical shifts were referenced to the DMSO- d_6 solvent peak at δ_{H} 2.50 and δ_{C} 39.5, respectively. Standard parameters were used for obtained 2D NMR spectra, including gCOSY, gHSQC, gHMBC and ROESY. HRESI-QTOFMS were recorded on a 6530 Accurate Mass Q-TOF (Agilent Technologies) workstation using positive electrospray ionisation, with mobile phase ACN/H₂O containing 0.1% formic acid. A LaChrom Merck Hitachi L7100 series pump equipped with a Hitachi L7450 PDA detector was used for HPLC separations. Prior to HPLC separation, the microbial crude extract was adsorbed onto Alltech Davisil 35- 75 μm 150 Å C₁₈. All separations were performed with a Thermo Hypersil-Keystone Betasil 5 μm Å C₁₈ HPLC column (21.2 mm $\times$ 150 mm). Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra were obtained on a Shimadzu UV-1800 UV/vis spectrophotometer. CD spectra were recorded on a JASCO J-715 spectropolarimeter. EtOAc used for extractions was Honeywell HPLC grade. All solvents used for HPLC, UV, and $[\alpha]_{\text{D}}$ -measurements were Scharlau HPLC grade. For MS, Fisher Scientific LC/MS grade acetonitrile was used and H₂O was Millipore Milli-Q PF filtered.

5.4.2. Biological material

Streptomyces strain USC-16018 was isolated from the ascidian *Sympylegma rubra* collected on July 15, 2015, Hastings Point, NSW, Australia. The ascidian specimen was identified by Merrick Ekins (Queensland Museum). The *Streptomyces* strain was isolated using selective

media and identified via 16S rRNA sequencing (as described in Chapter 2). The 16S sequence was deposited in GenBank (accession number MF773774).

5.4.3. Fermentation, Extraction and Isolation

Strain USC-16018 was fermented on 55 OMA (20 g oatmeal, 20 g bacteriological agar, 3 g yeast extract, 1 L deionised H₂O) and 55 GST (5 g glucose, 2 g starch, 3 g beef extract, 5 g yeast extract, 5 g tryptone, 4 g CaCO₃, 4 g NaCl, 1 g NaSO₄, 0.5 g KCl, 2 g MgCl, 0.5 g KH₂PO₄, 20 g bacteriological agar) agar plates for 2 weeks at 28 °C. Culture plates were exhaustively extracted by shaking in ethyl acetate. The extracts were dried under reduced vacuum to yield 1.6 g of dark brown gum for OMA cultures and 0.8 g of light red gum for GST cultures. Each of the extracts was dissolved in a small amount of MeOH and C₁₈ silica gel was added in a 1:2 ratio. The solvent was evaporated and the extract adsorbed on the C₁₈ was transferred to a refillable HPLC guard column (10 mm x 20 mm) which was attached to a C₁₈ Betasil HPLC column. At a flow rate of 9 mL/min, isocratic HPLC conditions of 10 % aqueous MeOH were initially employed for 10 min, followed by a linear gradient over 40 min from 10 % to 100 % MeOH, finishing with another 10 min isocratic conditions at 100 % MeOH. Sixty 1-minute-fractions were collected and analysed by ¹H NMR spectroscopy. Of the OMA extract, fractions 31- 35 were combined and further purified by eluting with a gradient from 25 to 60 % aqueous MeOH over 40 min to yield 0.9 mg of compound **1**. Fraction 46 and 26 of the GST extract HPLC separation yielded 0.6 mg and 0.7 mg of compound **2** and **3** respectively. Fractions 20 – 25 were combined and repurified to yield 1.2 mg of compound **4** and 2.1 mg of compound **5**. 0.9 mg of compound **2** were also detected in the OMA extract.

Herbimycin G (**1**): C₃₀H₄₆N₂O₉; brown solid; [α]_D + 8.6 (c 0.007 g/100 mL, MeOH), UV (MeOH) λ_{max} (log ε) 245 (1.93) nm; ¹H NMR (800 MHz, DMSO) and ¹³C NMR (200 MHz, DMSO) data, **Table 1**; (+)-HR-ESIMS *m/z* 601.3111 [M + Na]⁺ (calculated for C₃₀H₄₆N₂NaO₉, 601.3096); all 1D and 2D NMR spectra in Appendix III.

Elaiophylin (**2**): C₅₄H₈₈O₁₈; ¹H NMR (800 MHz, DMSO) and ¹³C NMR (200 MHz, DMSO) data, **Table 3**; (+)-LR-ESIMS *m/z* 1024.6 [M + H]⁺ (calculated for C₅₄H₈₈O₁₈, 1024.60).

Cyclo-L-Pro-L-Leu (**3**): C₁₁H₁₈N₂O₂; yellow solid; [α]_D -3.1(c 0.02 g/100 mL, MeOH); UV (MeOH) λ_{max} (log ε) 277 (0.71) nm, 329 (-0.01) nm; ¹H NMR (800 MHz, DMSO) and ¹³C NMR (200 MHz, DMSO) data, **Table 2**.

Cyclo-L-Pro-L-Phe (**4**): C₁₄H₁₆N₂O₂; yellow solid [α]_D - 1.76 (c 0.008 g/100 mL, MeOH), UV (MeOH) λ_{max} (log ε) 216 (1.58) nm; ¹H NMR (800 MHz, DMSO) and ¹³C NMR (200 MHz, DMSO) data, **Table 2**.

Cyclo-L-Pro-L-Val (**5**): $C_{10}H_{14}N_2O_2$; yellow solid; $[\alpha]_D - 1.3$ (c 0.01 g/100 mL, MeOH); UV (MeOH λ_{max} (log ϵ) 215 (1.72) nm; 1H NMR (800 MHz, DMSO) and ^{13}C NMR (200 MHz, DMSO) data, **Table 2**.

Cyclo-L-Pro-L-Tyr (**6**): $C_{14}H_{16}N_2O_3$; yellow solid; $[\alpha]_D - 1.3$ (c 0.01 g/100 mL, MeOH); UV (MeOH λ_{max} (log ϵ) 215 (1.72) nm; 1H NMR (800 MHz, DMSO) and ^{13}C NMR (200 MHz, DMSO) data, **Table 2**.

5.4.4. Molecular modelling calculations

The generation of conformers was performed using Schrödinger MacroModel 2016 using the protocol reported by Willoughby and co-workers.²⁴ First-principles calculations based on density functional theory (DFT) were carried out to optimize the atomic structures at the B3LYP/6-31G(d) level with the GAUSSIAN 16 suite of programs.²⁵ Electronic transition and rotational strength were calculated using the time-dependent DFT (TDDFT) method at the same theoretical level. The solvent effect in MeOH solution was considered during all calculations using the Polarizable Continuum Model (PCM).²⁶ Boltzmann-weighted UV and ECD were calculated using the freely available software SpecDis²⁷ and GaussSum²⁸ using a sigma/gamma value of 0.25 eV. Lipinski parameters were calculated in ChemBio3D Ultra 14.0.

5.4.5. Biological activity testing

P. falciparum parasites (3D7 and Dd2 strains) were grown in RPMI 1640 supplemented with 25 mM HEPES, 5% AB human male serum, 2.5 mg/ml Albumax II, and 0.37 mM hypoxanthine. Parasites were subjected to two rounds of sorbitol synchronization before undergoing compound treatment. Ring stage parasites were exposed to the experimental compounds in 384-wells imaging microplates (PerkinElmer CellCarrier), as previously described.²⁹ Plates were incubated for 72h at 37 °C, 90% N₂, 5% CO₂, 5% O₂, then the parasites were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI), and imaged using an Opera QEHS micro-plate confocal imaging system (PerkinElmer). Images were analysed as previously described.²⁹

Human Embryonic Kidney cells (HEK293) were maintained in DMEM medium supplemented with 10% FBS. HEK293 cells were exposed to the compounds in TC-treated 384-wells plates (Falcon) as previously described.³⁰ Plates were incubated for 72h at 37 °C, 5% CO₂, then the media was removed from the wells and replaced with an equal volume of 44 μ M resazurin. After an additional 5-6 hours incubation at standard conditions, the total fluorescence (excitation/emission: 530 nm/595 nm) was measured using the Envision plate reader (PerkinElmer).

Raw data was normalized using the in-plate positive and negative controls to obtain normalized % inhibition data, which was then used to calculate IC₅₀ values, through a 4 parameter logistic curve fitting in GraphPad Prism. The experiments were carried out in two independent biological replicates, each consisting of two technical replicates.

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CHAPTER 6 - Naseseazine C, a new anti-plasmodial dimeric diketopiperazine from a marine sediment derived *Streptomyces* sp.

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The author this thesis, L. Buedenbender, performed microbial fermentations, extraction, isolation and structure elucidation of the natural products; and prepared the manuscript. The co-authors T. Grkovic, D. I. Kurtböke and A. R. Carroll are the supervisors to the PhD project and provided advice on structure elucidation and feedback to the manuscript. S. Duffy and V. M. Avery performed anti-plasmodial activity testing of the isolated compounds.

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6.1. Abstract

A new dimeric diketopiperazine, naseseazine C, and the known naseseazines A and B were isolated from solid culture extracts of an Australian marine sediment derived *Streptomyces* species. Naseseazine C displayed a new C-6'/C-3 linkage between the two diketopiperazine subunits and showed moderate activity against the chloroquine-sensitive malaria parasite, *Plasmodium falciparum* (3D7 strain).

6.2. Introduction

Naseseazines A and B were originally isolated from the Fijian marine sediment derived actinomycete *Streptomyces* (CMB-MQ030) and reported to possess diketopiperazine skeletons derived from tryptophan and either proline or alanine.¹ The unique feature of the structures is their dimeric nature, with the two diketopiperazine units joined by a bond between the dihydroindole C-3 carbon of one monomer and the aryl C-7' carbon of the second monomer. Since their initial discovery, the two naseseazines have been the target of total synthesis and in 2011 Kim and Movassaghi successfully synthesised the proposed structures.² However, the spectroscopic properties of these compounds differed from those of the natural products and based upon this observation diastereomers at C-11, C-15, C-11' and C-15' were also made. These compounds possessed identical spectroscopic properties to naseseazines A and B and therefore allowed the structures (including absolute configurations) of the natural products to be corrected (**1-2**, **Figure 1**). Additionally, a regioisomer named *iso*-naseseazine B (**3**, **Figure 1**) was also synthesised. This compound differed from naseseazine B through a regiochemical shift of the bond linking the two monomers to C-6'/C-3 (rather than C-7'/C-3). Very recently another molecule also named *iso*-naseseazine B (**4**, **Figure 1**) from a Yellow Sea sediment-derived *Streptomyces* species was reported.³ This compound has been proposed to differ from naseseazine B by the inversion of chirality at the two stereogenic centres C-2 and C-3. In the course of our studies to identify new actinomycete derived compounds from Australian marine environments, we isolated a molecule with the same spectroscopic properties as that reported for **4**. However, interpretation of 2D NMR spectroscopic data led us to the conclusion that the proposed structure reported for **4** was incorrect. This manuscript describes the isolation of **5** and structural revision of compound **4**; we have named the new compound naseseazine C (**5**).

Figure 1. Naseseazine compounds

6.3. Results and discussion

The *Streptomyces* sp. (USC-636) (GenBank Accession Number KX379154) was isolated from marine sediment obtained on the Sunshine Coast, QLD, Australia. 16S rRNA sequencing revealed the close relationship of the isolate to the previously isolated *Streptomyces* sp. (AMS662) from sediments collected in the western Gulf of California. *Streptomyces* isolate (USC-636) was cultivated on CYC agar (DSMZ 550, www.dsmz.de). For further chemical analysis, fifty two agar plates were incubated for two weeks at 28 °C and then extracted with ethyl acetate. The ^1H NMR spectrum of the extract revealed unusual aromatic and alkaloid moieties and on the basis of these observations the sample was selected for further investigation. The ethyl acetate extract was purified by HPLC using $\text{H}_2\text{O}/\text{MeOH}$ to yield 0.05 mg of naseseazine C (**5**), as well as 0.1 mg and 0.08 mg of the known compounds naseseazines A (**1**) and B (**2**) respectively.

Naseseazine C (**5**) was isolated as an optically active yellow oil $[\alpha]_{\text{D}} -18.5$.⁴ A pseudomolecular ion at m/z 565.2576 obtained from HRESI-QTOFMS indicated **5** had the same molecular formula $\text{C}_{32}\text{H}_{32}\text{N}_6\text{O}_4$ as naseseazine B. The NMR data, however, showed distinct differences, particularly in the resonances of the aromatic region (**Table 1**).

Table 1. NMR data for nasesezine C (5) (800 MHz in DMSO-*d*₆, δ in ppm)

Position	δ_H , multiplet, (<i>J</i> in Hz)	δ_C	HMBC	ROESY
1	6.56, s		3, 4	2
2	5.62, s	81.1	3, 4, 9	1, 12a, 5', 7'
3		59.7		
4		134.1		
5	7.16, dd, (7.4, 1.2)	125.8	9	5', 12b
6	6.61, td, (7.4, 1.0)	118.1	4, 8	
7	6.98, td, (7.7, 1.2)	128.1	5, 9	
8	6.65, dd, (7.8, 1.5)	111.4	4, 6	
9		149.3		
10				
11	4.16, m	60.1		12a/b
12a	2.69, m	40.0	3, 4, 13	2, 11, 5', 7'
12b	3.04, m		2	11
13		165.3		
14				
15	4.29, t	58.5		17b
16		169.1		
17a	1.94, m	27.4	16	15, 18b
17b	2.14, m			
18a	1.81, m	22.5		
18b	1.91, m			19b
19a	3.35, m	44.7		
19b	3.42, m			
1'	10.82, d (2.4)		3', 4', 9'	2', 8'
2'	7.17, d (2.4)	124.8	3', 4', 9'	1', 12a'
3'		109.6		
4'		127.1		
5'	7.62, d, (1.8)	114.6	3, 3', 7', 9'	2, 5, 12a
6'		134.8		
7'	7.03, dd, (8.5, 1.8)	119.2	3, 5', 9'	2, 12a
8'	7.22, d, (8.5)	110.4	4', 6'	1'
9'		135.9		
10'	7.70, s		13'	11'
11'	4.30, m	55.1		10', 15'
12'a	3.08, m	25.4	3'	
12'b	3.21, m		13'	
13'		166.0		
14'				
15'	4.08, m	58.8		11', 17'b
16'		165.6		
17'a	1.44, m	27.7		
17'b	2.01, m			
18'a	1.61, m	21.9		
18'b	1.69, m		13'	
19'a	3.28, m	44.7		
19'b	3.40, m			

The ¹H NMR spectrum of the compound was well resolved and showed 28 resonances. Eight resonances were observed in the aromatic region, with two different benzene ring systems assigned from analysis of proton coupling constants and COSY spectra. Five additional proton spin systems (CHNH, two CH₂CH and two CH₂CH₂CH₂CH) could be assigned on the basis of

^1H and COSY spectral analysis. Correlations observed in the HSQC and HMBC spectra indicated that these proton spin systems were part of tryptophan/proline derived diketopiperazine moieties and were almost identical to those present in nasesezazine B. This suggested that nasesezazine C was a diketopiperazine dimer closely related to **2**. The *ortho* coupled aromatic proton at δ_{H} 7.22 (H-8') correlated to an upfield aromatic carbon resonating at δ_{C} 110.4 in the HSQC spectrum suggesting that this carbon was *ortho* to an electronegative substituent which indicated that it could be assigned to C-8'. Raju and co-workers reported a similar chemical shift (δ_{C} 110.3) for the carbon at position 8' of nasesezazine B (**2**).¹ However, since H-8' was *ortho* to a protonated aromatic carbon, nasesezazine C (**5**) was likely to be a regio-isomer of **2**. HMBC correlations from the two *meta* coupled aromatic protons, H-5' and H-7' and the indole NH proton to a quaternary carbon at δ_{C} 135.9 (C-9') further indicated that the indole was substituted at C-6' (**Figure 2**). This was clearly different to the substitution pattern found in nasesezazine B, suggesting that **5** had a C-3/C-6' linkage between the two subunits. HMBC correlations from H-5' and H-7' to a carbon at δ_{C} 59.7 (C-3) confirmed this assignment (**Figure 2**). By comparison structure **4** proposed by Xiong and co-workers showed intense $^4J_{\text{CH}}$ HMBC correlations from δ_{H} 7.64 (H-8', *meta* coupled doublet) to δ_{C} 109.6 (C-3') and from δ_{H} 7.12 (H-6', *ortho*, *meta* coupled doublet of doublets) to C-8a' and no $^3J_{\text{CH}}$ correlations from H-6' or H-8' to C-4'.³ These correlations are highly unlikely in an indole system since $^4J_{\text{CH}}$ couplings are typically small (< 1 Hz) while $^3J_{\text{CH}}$ couplings are typically large (> 8 Hz). The HMBC correlation from δ_{H} 7.64 to δ_{C} 109.6 is really only plausible if this proton is attached to C-5' and since this proton is a *meta* coupled doublet, the carbon *ortho* to it (C-6') must be the point of attachment to subunit A (**Figure 2**). Therefore, the structure **4**, presented by Xiong and co-workers wrongly assigned a C-3/C-7' linkage as shown in **Figure 2** since their NMR data clearly supported a molecule (**5**) with a C-3/C-6' linkage.

Figure 2. Key HMBC correlations observed for the indole fragments of **4** (600 MHz in MeOH-d_4) and **5** (800 MHz in DMSO-d_6). (Dashed arrows represent $^4J_{\text{CH}}$ correlations.)

Correlations observed in a ROESY experiment were used to assign the relative configuration of the six stereogenic centres in **5** (**Figure 3**). ROESY correlations between H-2 (δ_{H} 5.62) and the aromatic protons H-5' (δ_{H} 7.62) and H-7' (δ_{H} 7.03) clearly showed that H-2 and the indole in subunit B were *cis* to each other. Further correlations from H-2 to H-12a (δ_{H} 2.96), and from H-12a to H-5' and H-7', indicated that H-12a was also on the β face of subunit A. The H-11 (δ_{H} 4.16) resonance at showed ROESY correlations to H-12b (δ_{H} 3.04) and H-15 (δ_{H} 4.29) indicating that these three protons were on the α -face of subunit A (**Figure 3**). Protons at H-11' (δ_{H} 4.27) and H-15' (δ_{H} 4.06) also had a mutual ROESY correlation indicating that they were on the same face of the molecule (**Figure 3**). Based upon this evidence naseseazine C was assigned as the C-3/C-6' epimer of the synthetic compound *iso*-naseseazine B (**3**). The reported structure of **4** was proposed to differ from **2** by epimerisation at C-2 and C-3 based upon the same ROESY evidence outlined above, however, because the linkage between the two subunits was missassigned the overall structure was incorrect.

Figure 3. Relative configuration for 5 based on observed ROESY correlations

The revised structure of naseseazine C (**5**) belongs to a rare class of dimeric 2,5-diketopiperazines. The naseseazines along with pestalazines and asperazine are the only known examples of such dimeric diketopiperazines from a natural source and to the best of our knowledge **5** is the first natural product with a C-6'/C-3 linkage. Many diketopiperazine dimers have potent biological activities including antibacterial, anti-fungal, anti-viral, anti-blood-clotting, and anti-tumour functions.⁵ Xiong and co-workers³ reported minor anti-fungal activity against fluconazole-resistant *C. albicans* for **4**, but no other biological activity was observed in their assays. In bioassays performed by Raju and co-workers,¹ no anti-microbial activity could be determined for **1** and **2** against *E. coli*, *S. aureus*, *B. subtilis* and *C. albicans*. Neither was any activity detected against cancer cell lines of gastric adenocarcinoma, neuroblastoma, erythroleukemia, or colorectal adenocarcinoma.¹

Many diketopiperazines contain indole units derived from tryptophan. Such functional groups have often been associated with anti-plasmodial activity; in particular *bis*-indole alkaloids with a molecular weight higher than 400 g/mol have shown strong anti-malarial activity.⁶ Also, Brevicompanine B was reported to be active against the *Plasmodium falciparum* (IC₅₀ 95 mM);⁷ this compound has the same substructure as nasesezine A. Thus biological assessment targeting the malaria parasite was pursued. Compounds **1**, **2** and **5** were tested in three separate experiments for their activity to inhibit the growth of a chloroquine sensitive strain of *Plasmodium falciparum* (3D7) as described by Duffy and Avery.⁸ Neither **1** nor **2** showed activity against the malaria parasite at a dose of 20 µM. Nasesezine C (**5**), however, displayed moderate anti-plasmodial activity with an average IC₅₀ value of 3.52 µM (± 1.2). This suggests that the C-6'/C-3 linkage between the two subunits and/or the change in regiochemistry in subunit A could be responsible for the enhanced bioactivity.

In conclusion, three 2,5-diketopiperazines were isolated from a marine sediment derived *Streptomyces* strain. Nasesezines A and B were previously described, while nasesezine C displayed a new C-6'/C-3 linkage; this is the first time that this family of compounds was investigated for anti-plasmodial activity. These results showed that marine actinomycetes can be a potential source for anti-plasmodial compounds and studies are currently ongoing to discover other *Plasmodium* inhibiting natural products.

6.4. Experimental section

6.4.1. General Experimental Procedures

Extracts were dried with a rotary evaporator (Christ Rotational Vacuum Concentrator - RVC 2-25 CD plus). Optical rotations were measured on a JASCO P-1020 polarimeter for Nasesezine A, B and C. For the new compound nasesezine C, a UV spectrum was obtained on a Shimadzu UV-1800 UV/vis spectrophotometer. NMR data for nasesezine A and B were obtained on a Varian 600 MHz Unity INOVA spectrometer. All NMR spectra for nasesezine C were recorded at 25 °C on a Bruker BioSpin GmbH 800 MHz spectrometer. The ¹H and ¹³C chemical shifts were referenced to the DMSO-*d*₆ solvent peak at δ_H 2.50 and δ_C 39.5, respectively. Standard parameters were used for 2D NMR spectra obtained, including gCOSY, gHSQC, gHMBC and ROESY. HSESI-QTOFMS were recorded on a 6530 Accurate Mass Q-TOF (Agilent Technologies) workstation using positive electrospray ionization, with mobile phase ACN/H₂O containing 0.1% formic acid. A LaChrom Merck Hitachi L7100 series pump equipped with a Hitachi L7450 PDA detector was used for HPLC separations. Prior to HPLC separation the microbial crude extract was adsorbed Alltech Davisil 35- 75 µm 150 Å C₁₈. All

separations were performed with a Thermo Hypersil-Keystone Betasil 5 μ m Å C₁₈ HPLC column (21.2 mm × 150 mm). EtOAc used for extractions was Honeywell HPLC grade. All solvents used for HPLC, UV, and [α]_D were Scharlau HPLC grade. For MS, Fisher Scientific LC/MS grade acetonitrile was used and H₂O was Millipore Milli-Q PF filtered.

6.4.2. Microbial isolation

Streptomyces species (USC-636) was isolated from near shore marine sediments samples collected in Maroochydore, Sunshine Coast, QLD, Australia. Starch-casein agar supplemented with antifungal agents cycloheximide and nystatin (75 ppm) and nalidixic acid (25 ppm) to prevent the growth of non-actinomycete taxa was used for the isolation.^{9, 10} The isolate was identified via the PCR method using the QIAGEN HotStarTaq® Multiplex PCR Kit and the primers B27F and 1492R.¹¹ The PCR products were sequenced at Macrogen Inc. (South Korea, <http://www.macrogen.com/>). 16S rRNA sequences were subsequently deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) (GenBank accession number KX379154). A phylogenetic tree was constructed using these sequences with bootstrap analysis using 1000 data re-samplings.^{12,13} Sequences were also checked for taxonomic classifications using the Greengenes ‘classifier’ (Figure 4).¹⁴

Subsequent Scanning Electron Microscopy imaging conducted at Central Analytical Research Facility operated by the Institute for Future Environments of the Queensland University of Technology, Brisbane, Australia revealed the spiral spore chains of the isolate (Figure 5).

Figure 4. Phylogenetic tree of the *Streptomyces* isolate (USC-636) in relation to its nearest relatives (*Anaerolinea thermolimosa* AB109437 was used as an out-group sequence to root the tree).

Figure 5. Electron micrograph of the *Streptomyces* isolate (USC-636) (bar indicates 10 μ m)

6.4.3. Extraction and Isolation

Fifty-two agar plates containing microbial cultures were blended with a conventional kitchen blender and repeatedly extracted in ethyl acetate (EtOAc) in a sonic bath. The extract was dried with a rotary evaporator under vacuum. The residue (373 mg) was adsorbed onto C₁₈ silica gel (1:1) and loaded into a refillable HPLC guard column (10 mm x 20 mm) attached to the HPLC system and eluted with a linear solvent gradient from 90% H₂O/10% MeOH to 100% MeOH at a flow rate of 9 mL/min over 60 minutes. Sixty one-minute fractions were collected. UV spectra were obtained at 210 nm, 254 nm, 280 nm, 320 nm and 380 nm. Fractions containing similar signals were combined. The naseseazines eluted in four fractions around 37% MeOH as a mixture of compounds. These fractions were recombined and separated further using refined solvent gradients (10% to 50 % MeOH and 10% to 40 % MeOH) until naseseazine A (0.1 mg), B (0.03 mg) and C (0.05 mg) were obtained as pure fractions.

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6.6. References

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CHAPTER 7 - Conclusion and future outlook

The work of this thesis was aimed to integrate and explore different biodiscovery approaches to maximise natural product discovery from marine actinomycetes. The findings presented in this thesis highlight the potential of marine actinomycete in natural product drug discovery. Natural products present the most successful ecologically adapted metabolites that have evolved to interact with specific biological targets and therefore make effective leads for drug discovery. Microbial natural products, specifically from actinomycetes, offer an unmatched structural diversity and offer a sustainable source of supply. In the past numerous actinomycete metabolites have made their way as drugs to the pharmaceutical market. However, the isolation and elucidation of natural products is time consuming and rediscovery of already known compounds is a major challenge. Consequently, interdisciplinary approaches that connect molecular and microbiology, bioinformatics, metabolomics and analytical chemistry are needed for successful natural product drug discovery.

Untapped ecological niche: Ascidian - actinomycete associations

In order to expand chemical diversity of secondary metabolites there has been an urge to isolate new microbes from new ecological niches, particularly the marine environment. Oceans cover 70 % of the earth's surface and provide a vast, untapped and diverse resource for bioprospecting. As seawater is low in nutrients many marine microorganisms live in associations with marine invertebrates such as sponges and ascidians, which have historically been excellent sources for biologically active metabolites. Increasing evidence shows that many natural products isolated from marine invertebrates are actually biosynthesised by their microbial symbionts and recent natural product discovery programs targeting marine invertebrate associates have revealed an array of previously undiscovered metabolites.

In chapter 2, the actinomycete diversity associated with three different Australian ascidian species was examined based on culture-dependent and independent techniques. A broad diversity of 120 natural product producing actinomycetes, mainly from the genera *Streptomyces* and *Micromonospora*, were isolated. Interestingly, very little overlap of actinomycete associates between the three ascidians was observed. This suggested that actinomycete associations with ascidians may be species-specific. One *Streptomyces* sp. strain isolate had a 99.2 % identical 16S rRNA sequence to an isolate that was previously derived from marine sediments, it was concluded that actinomycetes are most likely selectively acquired by the ascidian host from the environment. Further studies will be needed to confirm this hypothesis. A GNPS molecular ion

network was established in order to evaluate the metabolite potential of ascidian-associated actinomycetes and to identify compounds of the ascidian host that are produced by a microbial isolate. Overlap between ascidian hosts and associated *Streptomyces*, *Micromonospora*, *Streptosporangium* and *Nocardia* isolates was observed for several ions; while *Rhodococcus* did not appear to play a role in the ascidians' metabolomes. The actinomycete MS metabolite profiles, specifically for *Streptomyces* and *Micromonospora* isolates, were highly diverse and thus present a good target for natural product drug discovery.

Tackling the issue of rediscovery and dereplication

Rediscovery of already known natural products poses a great challenge to natural product drug discovery. Therefore, it is important to incorporate effective dereplication protocols early in natural product isolation efforts. In Chapter 3, a new NMR-based metabolomics approach for microbial strain prioritisation was presented. HSQC-TOCSY NMR spectra were used to characterise polyketide and peptide diversity in microbial crude extracts. These spectra were subsequently analysed in combination with anti-plasmodial activity data to systematically prioritise microbial strains for natural product discovery that have higher potential to produce bioactive metabolites. NMR is an extremely powerful analytical tool and, in contrast to MS, it provides structural information of compounds in the microbial extracts and can be used to target specific groups of natural products. Moreover, the use of two-dimensional NMR experiments offered increased chemical shift resolution by spreading the structural information onto two dimensions and thus permitted assessment of unfractionated extracts. The HSQC-TOCSY NMR experiment was proven to be much more sensitive compared to HMBC NMR experiments, and HSQC-TOCSY spectra could be acquired in about 30 minutes.

The application of HSQC-TOCSY NMR experiments for the purpose of microbial strain selection (Chapter 3) as well as for assessment of different culture conditions (Chapter 4) proved to be very valuable. Out of 119 microbial isolates, *Streptomyces* sp. (USC-16018) was selected based on its anti-plasmodial activity and diverse HSQC-TOCSY NMR peaks associated with secondary metabolites, in particular polyketides, which were subsequently isolated. This was the first time that HSQC-TOCSY spectra were utilised in this manner, and compared to MS or 1D NMR profiling techniques, the new approach readily revealed the presence and abundance of polyketides in the extracts.

Marine actinomycetes as a source of new anti-plasmodial compounds

The HSQC-TOCSY NMR spectrum of the ascidian-associated *Streptomyces* sp. USC-16018 indicated presence of a broad diversity of polyketide-associated cross-peaks. Subsequent large scale fermentation and preparative HPLC resulted in the purification of a new ansamycin polyketide, herbimycin G, as well as the known natural products, elaiophyllin, Cyclo-L-Pro-L-Leu, Cyclo-L-Pro-L-Phe, Cyclo-L-Pro-L-Val and Cyclo-L-Pro-L-Tyr. Ansamycin polyketides have previously been identified from terrestrial and marine actinomycetes, with geldanamycin proceeding to advanced clinical trials as an anti-tumour drug. Many ansamycin compounds act as Hsp90 inhibitors. When the *Plasmodium* parasite changes host, heat shock proteins including Hsp90 are highly expressed in the parasite and thus Hsp90 inhibitors have been proposed as a good drug target for anti-malarial drugs. Therefore, herbimycin G was tested against a chloroquine-sensitive and chloroquine-resistant *P. falciparum* strain and caused significant inhibition (> 75 %) of both parasite strains (Chapter 5).

A new dimeric diketopiperazine was isolated from the marine sediment derived *Streptomyces* sp. USC-636. Naseseazine C was a new regio-isomer of naseseazine B, which was previously isolated from a marine Fijian sediment-derived actinomycetes species. *Iso*-naseseazine B isolated from a Yellow Sea sediment-derived *Streptomyces* sp. published in the literature was proposed to be a stereoisomer of naseseazine B. However, when comparing the spectroscopic data published with our experimental data, we found that they were identical. However, indisputable evidence of the NMR spectra indicated a new C-6'/C-3 linkage between the two diketopiperazine subunits of the compound, leading to the conclusion that the reported structure in the literature was in fact incorrect. In Chapter 6, the full isolation and structure elucidation of the new compounds was described. These findings were also published as a peer-review article in the journal *Tetrahedron Letters*. We also screened naseseazines A - C against *P. falciparum* and found that naseseazine C was active, while A and B were not. This difference in activity may be associated with the different C-6'/C-3 linkage.

Remarks and future directions

The aims of the thesis were successfully met and a library of 120 ascidian-associated actinomycetes was established. Based on the newly established strain prioritisation approach, *Streptomyces* sp. USC-16018 was selected and a new anti-plasmodial polyketide was isolated. Overall, this thesis highlights the immense chemical diversity of actinomycetes associated with marine invertebrates, as well as the importance of sophisticated dereplication approaches and provides an alternative new solution for strain selection that has led to the discovery of new

bioactive natural products with potential as new drug leads. Another 14 actinomycete isolates had interesting HSQC-TOCSY profiles and exhibited above 80 % inhibition against *P. falciparum*. Large scale fermentation of the bioactive strains and natural product isolation efforts will be pursued in the future. As observed in Chapter 3, co-cultivation often results in enhanced chemical diversity of the extracts and *de novo* synthesis of secondary metabolites. As such, co-cultivations will be implemented in the fermentation process of the selected actinomycete strains.

Actinomycetes are prolific producers of bioactive metabolites. However, it was noted that even though new anti-plasmodial compounds were isolated, the core structures of both naseaeazine C and herbimycin G were previously reported in actinomycete extracts. The culture-independent approach taken to uncover the microbial diversity of the ascidians revealed that actinomycetes only account for less than 2 % of the microbiome and there remains a huge diversity of unculturable and thus untapped microbial families. Less effort has been made to isolate unculturable microorganisms and thus still represent an untapped source for biodiversity. In earlier stages of this PhD project, a device for *in situ* isolation of marine microorganisms was designed, but due to time constraints its implementation could not yet be realised (Appendix V). The so-called iChip contains numerous miniature diffusion chambers in which individual cells of environmental bacteria can be isolated simultaneously. This approach has successfully been used for isolation of soil bacteria and has resulted in the isolation of a novel bacterium that produced the novel antibiotic teixobactin. For the future, it is envisaged that the modified version of the iChip will be implanted into living marine invertebrate tissue in order to isolate new symbiotic bacteria *in situ* that will yield novel natural product scaffolds.

APPENDIX I – *for Chapter 2*

Table IA – Ascidian-associated actinomycete collection

Isolate-ID	OTU	Ascidian host	Isolation media	16S rRNA identification	GenBank accession #
USC16000	denovo32	<i>S. rubra</i>	SCA	<i>Streptomyces</i> sp.	MF773756
USC16001	denovo11	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773757
USC16002	denovo69	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773758
USC16003	denovo19	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773759
USC16004	denovo49	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773760
USC16005	denovo43	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773761
USC16006	denovo0	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773762
USC16007	denovo28	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773763
USC16008	denovo68	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773764
USC16009	denovo57	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773765
USC16010	denovo38	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773766
USC16011	denovo10	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773767
USC16012	denovo11	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773768
USC16013	denovo24	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773769
USC16014	denovo34	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773770
USC16015	denovo4	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773771
USC16016	denovo25	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773772
USC16017	denovo29	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773773
USC16018	denovo19	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773774
USC16019	denovo48	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773775
USC16020	denovo48	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773776
USC16021	denovo30	<i>S. rubra</i>	SCA	<i>Streptomyces</i> sp.	MF773777
USC16022	denovo29	<i>A. solidium</i>	CS	<i>Streptomyces</i> sp.	MF773778
USC16023	denovo73	<i>A. solidium</i>	CS	<i>Streptomyces</i> sp.	MF773779
USC16024	denovo27	<i>P. vasculosum</i>	SCA	<i>Streptomyces</i> sp.	MF773780
USC16025	denovo18	<i>P. vasculosum</i>	SCA	<i>Streptomyces</i> sp.	MF773781
USC16026	denovo63	<i>P. vasculosum</i>	CS	<i>Streptomyces</i> sp.	MF773782
USC16027	denovo74	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773783
USC16028	denovo67	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773784
USC16029	denovo67	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773785
USC16030	denovo5	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773786
USC16031	denovo46	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773787
USC16032	denovo67	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773788
USC16033	denovo70	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773789
USC16034	denovo67	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773790
USC16035	denovo23	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773791
USC16036	denovo52	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773792
USC16037	denovo1	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773793
USC16038	denovo71	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773794
USC16039	denovo16	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773795
USC16040	denovo52	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773796
USC16041	denovo12	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773797

Table IA – Ascidian-associated actinomycete collection (*continued*)

Isolate-ID	OTU	Ascidian host	Isolation media	16S rRNA identification	GenBank accession #
USC16042	denovo51	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773798
USC16043	denovo61	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773799
USC16044	denovo56	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773800
USC16045	denovo70	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773801
USC16046	denovo33	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773802
USC16047	denovo37	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773803
USC16048	denovo23	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773804
USC16049	denovo51	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773805
USC16050	denovo52	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773806
USC16051	denovo37	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773807
USC16052	denovo70	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773808
USC16053	denovo50	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773809
USC16054	denovo67	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773810
USC16055	denovo61	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773811
USC16056	denovo8	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773812
USC16057	denovo26	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773813
USC16058	denovo67	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773814
USC16059	denovo52	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773815
USC16060	denovo70	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773816
USC16061	denovo13	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773817
USC16062	denovo31	<i>S. rubra</i>	SCA	<i>Streptomyces</i> sp.	MF773818
USC16063	denovo47	<i>S. rubra</i>	SCA	<i>Streptomyces</i> sp.	MF773819
USC16064	denovo53	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773820
USC16065	denovo67	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773821
USC16066	denovo66	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773822
USC16067	denovo72	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773823
USC16068	denovo36	<i>A. solidium</i>	SCA	<i>Streptomyces</i> sp.	MF773824
USC16069	denovo17	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773825
USC16070	denovo67	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773826
USC16071	denovo72	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773827
USC16072	denovo70	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773828
USC16073	denovo70	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773829
USC16074	denovo70	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773830
USC16075	denovo70	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773831
USC16076	denovo41	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773832
USC16077	denovo39	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773833
USC16078	denovo55	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773834
USC16079	denovo58	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773835
USC16080	denovo22	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773836
USC16081	denovo67	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773837
USC16082	denovo3	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773838
USC16083	denovo67	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773839
USC16084	denovo9	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773840
USC16085	denovo2	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773841
USC16086	denovo51	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773842
USC16087	denovo9	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773843

Table IA – Ascidian-associated actinomycete collection (continued)

Isolate-ID	OTU	Ascidian host	Isolation media	16S rRNA identification	GenBank accession #
USC16088	denovo61	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773844
USC16089	denovo53	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773845
USC16090	denovo37	<i>P. vasculosum</i>	SA	<i>Micromonospora</i> sp.	MF773846
USC16091	denovo7	<i>A. solidium</i>	CS	<i>Nocardia</i> sp.	MF773847
USC16092	denovo20	<i>S. rubra</i>	CS	<i>Rhodococcus</i> sp.	MF773848
USC16093	denovo7	<i>S. rubra</i>	CS	<i>Nocardia</i> sp.	MF773849
USC16094	denovo14	<i>S. rubra</i>	CS	<i>Rhodococcus</i> sp.	MF773850
USC16095	denovo42	<i>S. rubra</i>	SCA	<i>Nocardia</i> sp.	MF773851
USC16096	denovo45	<i>S. rubra</i>	SCA	<i>Nocardia</i> sp.	MF773852
USC16097	denovo64	<i>S. rubra</i>	SCA	<i>Nocardia</i> sp.	MF773853
USC16098	denovo75	<i>A. solidium</i>	SCA	<i>Streptomyces</i> sp.	MF773854
USC16099	denovo76	<i>A. solidium</i>	SCA	<i>Streptomyces</i> sp.	MF773855
USC16100	denovo6	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773856
USC16101	denovo21	<i>S. rubra</i>	SA	<i>Streptomyces</i> sp.	MF773857
USC16102	denovo52	<i>S. rubra</i>	SA	<i>Micromonospora</i> sp.	MF773858
USC16103	denovo35	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773859
USC16104	denovo70	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773860
USC16105	denovo40	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773861
USC16106	denovo61	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773862
USC16107	denovo54	<i>P. vasculosum</i>	SCA	<i>Micromonospora</i> sp.	MF773863
USC16108	denovo59	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773864
USC16109	denovo61	<i>A. solidium</i>	SCA	<i>Micromonospora</i> sp.	MF773865
USC16110	denovo38	<i>S. rubra</i>	CS	<i>Streptomyces</i> sp.	MF773866
USC16111	denovo62	<i>S. rubra</i>	SCA	<i>Micromonospora</i> sp.	MF773867
USC16112	denovo70	<i>S. rubra</i>	CS	<i>Micromonospora</i> sp.	MF773868
USC16113	denovo60	<i>P. vasculosum</i>	MA	<i>Streptomyces</i> sp.	MF773869
USC16114	denovo67	<i>S. rubra</i>	MA	<i>Micromonospora</i> sp.	MF773870
USC16115	denovo44	<i>S. rubra</i>	MA	<i>Streptomyces</i> sp.	MF773871
USC16117	denovo4	<i>S. rubra</i>	MA	<i>Streptomyces</i> sp.	MF773872
USC16118	denovo4	<i>S. rubra</i>	MA	<i>Streptomyces</i> sp.	MF773873
USC16119	denovo77	<i>S. rubra</i>	MA	<i>Streptosporangium</i> sp.	MF773874
USC16120	denovo7	<i>A. solidium</i>	NH	<i>Nocardia</i> sp.	MF773875

Appendix Ia: Ascidian-associated actinomycete library (USC-University of Sunshine Coast)

Streptomyces sp. isolates:

USC16000

USC16001

USC16002

USC16003

USC16004

USC16005

USC16006

USC16007

USC16008

USC16009

USC16010

USC16011

USC16012

USC16013

USC16014

USC16015

USC16017

USC16018

USC16019

USC16020

Appendix Ia: Ascidian-associated actinomycete library (USC-University of Sunshine Coast)

Streptomyces sp. isolates:

USC16020

USC16021

USC16022

USC16023

USC16024

USC16025

USC16026

USC16027

USC16062

USC16098

USC16099

USC16100

USC16101

USC16113

USC16115

USC16117

USC16118

Streptosporangium sp.: USC16119

Appendix Ia: Ascidian-associated actinomycete library (USC- University of Sunshine Coast)

Micromonospora sp. isolates:

USC16028

USC16029

USC16030

USC16031

USC16032

USC16033

USC16034

USC16035

USC16036

USC16037

USC16038

USC16039

USC16040

USC1642

USC16043

USC16044

USC16045

USC16046

USC16047

USC16048

Appendix Ia: Ascidian-associated actinomycete library (USC- University of Sunshine Coast)

Micromonospora sp. isolates:

USC16049

USC16050

USC16051

USC16052

USC16053

USC16054

USC16055

USC16056

USC16057

USC16058

USC16059

USC16060

USC16061

USC16064

USC16065

USC16066

USC16067

USC16068

USC16069

USC16070

Appendix Ia: Ascidian-associated actinomycete library (USC- University of Sunshine Coast)

Micromonospora sp. isolates:

USC16071

USC16073

USC16074

USC16075

USC16076

USC16077

USC16078

USC16079

USC16080

USC16081

USC16082

USC16083

USC16084

USC16085

USC16086

USC16087

USC16088

USC16089

USC16090

USC16102

Appendix I: Ascidian-associated actinomycete library (USC-University of Sunshine Coast)

Micromonospora sp. and *Nocardia* form isolates:

USC16103

USC16104

USC16105

USC16106

USC16107

USC16108

USC16109

USC16111

USC16112

USC16091

USC16092

USC16093

USC16094

USC16095

USC16096

USC16097

USC16120

Table IB. Molecular ion table – GNPS library hits

Parent Mass	RT (min)	RT Stderror	LibraryID	<i>S. rubra</i>	<i>A. solidum</i>	<i>P. vasculosum</i>	<i>Symplegma _associate</i>	<i>Aplidium _associate</i>	<i>Polyclinum _associate</i>	<i>Streptomyces</i>	<i>Micromonospora</i>	<i>Streptosporangium</i>	<i>Nocardia</i>	<i>Rhodococcus</i>
495.899	11.83	0.54	1-Palmitoyl-sn-glycero-3-phosphocholine	12	19	11	0	0	3	0	3	0	0	0
479.9	11.48	0.60	4-[5-[[4-[5-[acetyl(hydroxy)amino]pentylamino]-4-oxobutanoyl]-4-	25	5	15	9	2	4	0	14	0	1	0
481.929	14.96	4.70	LysoPAF	10	5	1	0	0	0	0	0	0	0	0
411.325	15.58	0.33	NCGC00180497-02!	4	4	0	0	0	5	0	5	0	0	0
474.831	12.60	3.21	SildenafilCitrate	0	2	0	1	0	2	0	3	0	0	0
127.055	1.92	0.01	Thymine	0	2	0	0	0	0	0	0	0	0	0
137.054	1.41	0.02	Hypoxanthine	0	1	0	3	1	2	0	4	2	0	0
105.076	7.46	2.50	Benzaldehyde	0	1	0	1	1	1	1	2	0	0	0
482.369	11.12	4.59	LysoPAF	1	1	0	0	0	0	0	0	0	0	0
261.14	2.44	0.35	gamma-L-Glutamyl-L-leucine	0	0	0	179	43	89	39	208	4	32	3
261.029	3.54	0.10	cyclo(Phe-4-Hyp)	0	0	0	127	20	57	88	94	1	7	2
211.077	3.32	1.02	Cyclo(Pro-Leu)	0	0	0	93	20	24	47	72	1	6	0
247.063	1.84	0.10	gamma-L-Glutamyl-L-valine	0	0	0	64	16	33	22	60	3	14	2
227.075	3.10	0.11	cyclo(L-Leu-L-4-Hyp)	0	0	0	63	18	35	50	52	0	5	0
254.174	1.73	0.02	Pheniramine formamide	0	0	0	55	11	13	39	35	1	1	0
426.648	3.96	0.35	Leupeptin	0	0	0	29	0	0	29	0	0	0	0
1128.73	17.45	2.26	6,18,30-trimethyl-3,9,12,15,21,24,27,33,36-nona(propan-2-yl)-1,7,1	0	0	0	28	0	0	28	0	0	0	0
245.026	4.53	0.03	cyclo(L-Phe-D-Pro)	0	0	0	23	1	8	8	20	0	0	0
309.798	1.97	0.10	Bestatin	0	0	0	20	6	6	15	11	0	4	0
426.277	9.15	0.13	1-tetradecanoyl-sn-glycero-3-phosphoethanolamine	0	0	0	18	1	7	20	6	0	0	0
585.34	2.29	0.03	Desferrioxamine B + Al	0	0	0	18	4	3	0	23	2	0	0
912.889	7.61	0.08	Surugamide_A	0	0	0	15	0	3	18	0	0	0	0
182.091	1.52	0.03	Tyrosine	0	0	0	15	0	1	9	6	0	1	0
188.08	2.76	0.06	Metrazoline	0	0	0	14	0	3	8	6	0	3	0
227.15	3.14	0.12	cyclo(L-Leu-L-4-Hyp)	0	0	0	13	2	6	8	11	1	0	0
132.11	1.45	0.62	Leucine	0	0	1	13	3	4	11	6	0	1	1
197.138	3.10	0.01	cyclo(L-Va-L-Pro)	0	0	0	13	0	1	4	9	0	1	0
260.828	2.46	0.25	gamma-L-Glutamyl-L-leucine	0	0	0	12	6	7	1	16	0	4	0
293.046	1.13	0.02	Vanillylnonanamide	0	0	0	10	0	1	9	1	0	1	0
220.13	2.16	0.06	Vitamin B5	0	0	0	10	0	0	10	0	0	0	0
143.091	1.04	0.01	Ectoine	0	0	0	8	2	1	5	1	0	4	0
169.084	3.59	0.03	Norhamane	0	0	0	6	1	2	3	4	0	0	0
403.758	14.39	0.44	2-(14-methylpentadecanoylamino)-3-phenylpropanoic acid	0	0	0	6	0	1	7	0	0	0	0
621.434	12.89	0.02	Rakicidin B	0	0	0	6	1	0	0	5	2	0	0
487.18	7.02	0.01	(3R)-4-[6-[(2R,4R,5S,6R)-4,5-dihydroxy-6-methyloxan-2-yl]-1,5-dih	0	0	0	6	0	0	6	0	0	0	0
118.094	1.32	0.25	Betaine	0	0	0	6	0	0	4	1	0	1	0
310.865	2.23	0.01	gamma-Glutamyltyrosine	0	0	0	6	0	0	0	6	0	0	0
124.047	1.38	0.01	Vitamin B3	0	0	0	5	0	3	2	5	0	0	0
150.068	1.63	0.31	Methionine	0	0	0	5	2	0	0	0	0	5	0
260.887	3.57	0.13	cyclo(Phe-4-Hyp)	0	0	0	5	0	0	2	3	0	0	0
181.607	1.52	0.03	L-Tyrosine	1	0	1	4	0	1	2	3	0	0	0
166.014	1.93	0.14	Phenylalanine	0	0	0	4	0	6	5	5	0	0	0
426.074	9.25	0.09	1-tetradecanoyl-sn-glycero-3-phosphoethanolamine	0	0	0	4	0	2	4	2	0	0	0
597.22	10.24	0.01	(3R)-4-[6-[(2S,4aS,5aR,9R,9aR,10aR)-2,9-dimethyl-3-oxo-4,4a,5a,6,	0	0	0	4	0	0	4	0	0	0	0
298.112	2.53	0.02	5'-Methylthioadenosine	0	0	0	4	0	0	2	2	0	0	0

Table IB. Molecular ion table – GNPS library hits (*continued*)

parent mass	RT (min)	RT Stderror	LibraryID	<i>S. rubra</i>	<i>A. solidum</i>	<i>P. vasculosum</i>	<i>Symplegma _associate</i>	<i>Aplidium _associate</i>	<i>Polyclinum _associate</i>	<i>Streptomyces</i>	<i>Micromonospora</i>	<i>Streptosporangium</i>	<i>Nocardia</i>	<i>Rhodococcus</i>
429.333	3.94	0.09	Leupeptin hemisulfate salt	0	0	0	4	0	0	4	0	0	0	0
261.136	3.50	0.03	cyclo(Phe-4-Hyp)	0	0	0	3	0	1	2	2	0	0	0
146.101	1.06	0.01	Glutamic acid	0	0	0	3	3	0	0	6	0	0	0
296.325	4.68	0.05	methyl 2-[(3-formamido-2-hydroxybenzoyl)amino]-3-hydroxybutyrate	0	0	0	3	0	0	3	0	0	0	0
296.29	4.65	0.03	methyl 2-[(3-formamido-2-hydroxybenzoyl)amino]-3-hydroxybutyrate	0	0	0	3	0	0	3	0	0	0	0
267.614	1.72	0.20	Adenosine	2	0	0	2	0	0	1	1	0	0	0
426.387	15.20	0.18	Ehmozine	0	0	0	2	1	2	3	1	0	0	0
153.786	16.47	1.50	Acetamide	0	0	0	2	0	2	2	2	0	0	0
132.11	1.21	0.18	L-Leucine	0	0	0	2	2	1	3	2	0	0	0
267.261	1.61	0.04	Adenosine	0	0	0	2	1	1	1	1	1	0	0
227.147	3.15	0.10	cyclo-[L-(4-hydroxy-Pro)-L-leu]	0	0	0	2	0	1	1	2	0	0	0
526.969	1.10	0.09	Polysaccharide Hexose x3	0	0	0	2	0	1	2	1	0	0	0
364.857	1.06	0.09	sucrose	0	0	0	2	0	1	1	2	0	0	0
178.095	2.19	0.01	3-Amino-3-(4-hydroxyphenyl)propionate	0	0	0	2	0	0	2	0	0	0	0
257.222	6.42	0.01	5-beta-Androstane-3-alpha	0	0	0	2	0	0	2	0	0	0	0
211.118	2.03	0.02	cyclo(L-Leu-L-Pro)	0	0	0	2	0	0	1	1	0	0	0
205.119	1.94	1.22	Tryptophan	0	0	0	2	0	0	1	0	0	1	0
205.107	2.75	0.08	Tryptophan	0	0	0	2	0	0	2	0	0	0	0
152.066	1.39	0.28	Xanthine	3	0	0	1	0	0	0	1	0	0	0
144.089	3.23	0.22	Quinolin-4-ol	0	0	0	1	2	2	1	4	0	0	0
227.149	3.25	0.02	cyclo(L-Leu-L-4-hydroxy-Pro)	0	0	0	1	0	1	1	1	0	0	0
247.141	1.88	0.07	gamma-L-Glutamyl-L-valine	0	0	0	1	0	0	0	1	0	0	0
194.572	2.15	0.53	Caffeine	12	0	0	0	0	0	0	0	0	0	0
429.023	4.81	0.03	8-desoxyenterocin	0	0	0	0	0	12	12	0	0	0	0
521.431	11.18	0.55	1-Oleoyl-sn-glycero-3-phosphocholine	0	0	2	0	0	4	0	4	0	0	0
245.139	4.55	0.02	cyclo(L-Phe-D-Pro)	0	0	0	0	1	1	1	1	0	0	0
592.93	14.85	0.02	Pheophorbide A	0	0	3	0	0	0	0	0	0	0	0
154.165	16.51	2.59	Xanthine	0	0	1	0	1	0	1	0	0	0	0
154.167	18.16	0.04	L-Histidine	0	0	0	0	1	0	1	0	0	0	0

Figure IIBa. PCoA plot of HSQC-TOCSY all PoI data of 119 actinomycete stains labelled with their isolate number

Figure IIBb. PCoA plot of HSQC-TOCSY PK PoI data of 119 actinomycete stains labelled with their isolate number

Appendix II—for Chapter 3

Figure IIA. HSQC-TOCSY NMR spectra of actinomycete crude extracts (DMSO- d_6 ; 800 MHz; USC-isolate number given in the top left corner of spectra)

Figure IIA. HSQC-TOCSY NMR spectra of actinomycete crude extracts (*continued*) (DMSO- d_6 ; 800 MHz; USC-isolate number given in the top left corner of spectra)

Figure IIA. HSQC-TOCSY NMR spectra of actinomycete crude extracts (*continued*) (DMSO- d_6 , 800 MHz; USC-isolate number given in the top left corner of spectra)

Figure IIA. HSQC-TOCSY NMR spectra of actinomycete crude extracts (*continued*) (DMSO- d_6 , 800 MHz; USC-isolate number given in the top left corner of spectra)

Figure IIA. HSQC-TOCSY NMR spectra of actinomycete crude extracts (*continued*) (DMSO- d_6 , 800 MHz; USC-isolate number given in the top left corner of spectra)

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Figure IIA. HSQC-TOCSY NMR spectra of actinomycete crude extracts (*continued*) (DMSO-*d*₆, 800 MHz; USC-isolate number given in the top left corner of spectra)

APPENDIX III – Appendix for Chapter 4

Figure IIIA. HSQC-TOCSY spectrum of USC-16018 EtOAc crude extract ($\text{DMSO}-d_6$, 800 MHz)

Figure IIIB. ^1H NMR spectrum of herbimycin G (1) ($\text{DMSO}-d_6$, 800 MHz)

Figure IIIC. ¹³C NMR spectrum of herbimycin G (1) (DMSO-*d*₆; 800MHz)

Figure IIID. COSY NMR spectrum of herbimycin G (1) (DMSO-*d*₆; 500MHz)

Figure IIIE. HSQC NMR spectrum of herbimycin G (1) (DMSO- d_6 , 800MHz)

Figure IIIF. HMBC NMR spectrum of herbimycin G (1) (DMSO- d_6 , 800MHz)

Figure IIIG. ROESY NMR spectrum of herbimycin G (1) (DMSO- d_6 ; 800MHz)

Table IIIA. Physiochemical parameters of herbimycins and related ansamycin compounds

Compound	MW	clogP	HBA	HBD	RB	TPSA
HerbA	574.289	2.1592	8	2	2	152.48
HerbB	530.2628	2.156	7	3	4	154.25
HerbC	560.2734	1.396	8	3	5	163.48
HerbD	647.2877	2.505	8	4	6	165.67
HerbE	590.2839	2.107	9	3	6	172.71
HerbF	544.2785	3.297	7	3	5	138.57
HerbG	578.3203	0.6762	8	3	6	155.64
HerbA-2	666.264	1.977	8	2	8	152.48
HerbB-2	548.2734	0.985	8	4	4	174.48
HerbB-3	546.3577	1.011	8	4	4	174.48
TAN420A	548.2734	1.178	8	6	4	180.8
TAN420B	546.2577	0.634	8	4	4	174.48
TAN420E	576.3047	2.703	8	4	6	158.8
HeronA	651.319	1.544	8	4	6	167.67
Geld	560.2734	2.0573	8	3	5	163.48
17-AAG	585.305	3.019	8	4	7	166.28
Reblast	546.2941	3.305	7	4	5	149.57
MacI	514.2679	2.962	6	3	3	145.02

Figure IIH. Herbimycin analogues and related ansamycines

Figure IIIH. Herbimycin analogues and related ansamycines (cont.)

APPENDIX IV for Chapter 6

Figure IVA. ¹H spectrum of nasesezine C (5) in DMSO-*d*₆

Figure IVB. ¹³C NMR spectrum of nasesezine C (5) in DMSO-*d*₆

Figure IVC. COSY spectrum of naseseazine C (5) in DMSO-d₆

Figure IVD. HSQC spectrum of naseseazine C (5) in DMSO-d₆

Figure IVE. HMBC spectrum of naseseazine C (5) in DMSO- d_6

Figure IVF. ROESY spectrum of naseseazine C (5) in DMSO- d_6

Figure IVG. ^1H NMR spectrum of naseseazine A (1) (600 MHz, methanol- d_4)

Table IVA. NMR data for naseseazine A (600 MHz, methanol- d_4)

Position	δ_{H} , multiplet, (J in Hz)	Raju <i>et al.</i> ^1H NMR
2	5.83, s	5.83, s
3		
4		
5	6.86, d, (7.5)	6.85, d, (7.4)
6	6.67	6.67, t, (7.5)
7	7.05, t	7.05, t, (7.2)
8	6.69, d (8.0)	6.69, d, (7.6)
9		
10		
11	4.47, dd, (7.5, 8.7)	4.64, dd, (8.4, 7.4)
12a	3.36, m	3.26, m
12b	2.61, dd, (9.6, 13.8)	3.26, m
13		
14		
15		4.15, q, (6.9)
16		
17	1.38, d, (6.7)	1.38, d, (6.9)
18		
2'	7.11, s	7.11, s
3'		
4'		
5'	7.57, d, (8.7)	7.57, d, (8.4)
6'	7.04, d, (8.7)	7.02, d, (8.4)
7'		
8'	7.40, d, (1.8)	7.40, s
9'		
10'		
11'	4.40, broad t, (4.4)	4.39, broad t, (4.5)
12'a	3.28	3.28, m
12'b	3.3	3.30, m
13'		
14'		
15'	3.97	3.97, dd, (10.8, 6.6)
16'		
17'a	0.95, m	0.93, m
17'b	1.96, m	1.97, m
18'a	1.42, m	1.43, m
18'b	1.66, m	1.66, m
19'a	3.26, m	3.24, m
19'b	3.42, m	3.42, dt, (11.8, 8.1)

Figure IVH. ^1H NMR spectrum of nasesezine B (2) (600 MHz, methanol- d_4)

Table IVB. NMR data for nasesezine B (600 MHz, methanol- d_4)

Position	δ_{H} , multiplet, (J in Hz)	Raju <i>et al.</i> ^1H NMR
2	5.83, s	5.85, s
3		
4		
5	6.85, dd, (8.1, 1.2)	6.84, dt, (7.2, 0.9)
6	6.66, td, (7.7, 1.1)	6.68, t, (7.6)
7	7.04, td, (7.7, 1.1)	7.06, td, (7.6, 1.3)
8	6.67, d, (7.7)	6.69, d, (7.6)
9		
10		
11	4.74	4.75, dd, (10.2, 8.7)
12a	2.59, dd, (14.1, 9.9)	2.59, dd, (13.8, 10.2)
12b	3.25, m	3.27, m
13		
14		
15	4.32, dd, (7.1, 9.7)	4.33, dd, (9.5, 7.1)
16		
17a	2.27, m	2.11, m
17b	2.10, m	2.28, m
18a	1.97, m	1.95, m
18b	2.00, m	2.00, m
19a	3.45, m	3.44, m
19b	3.48, m	3.49, m
2'	7.10, s	7.12, s
3'		
4'		
5'	7.56, d, (8.5)	7.58, d, (8.4)
6'	7.02, dd, (8.5, 1.7)	7.03, dd, (8.4, 1.8)
7'		
8'	7.39, d, (1.7)	7.41, d, (1.4)
9'		
10'		
11'	4.38, broad t (4.6)	4.40, broad t, (4.7)
12'a	3.27, m	3.28, m
12'b	3.33, m	3.32, m
13'		
14'		
15'	3.97, ddd, (11.4, 6.9, 1.7)	3.99, ddd, (11.4, 6.6, 1.6)
16'		
17'a	0.93, m	0.92, m
17'b	1.96, m	1.97, m
18'a	1.43, m	1.44, m
18'b	1.66, m	1.67, m
19'a	3.24, m	3.24, m
19'b	3.43, m	3.43, m

Table IVC. Anti-plasmodial activity of naseeseazine A-C and chloroquine

	Expt 1	Expt 2	Expt 3		
Naseeseazine A	47% at 20 uM	35% at 20 uM	28% at 20 uM		
Naseeseazine B	52% at 20 uM	15% at 20 uM	IA at 20 uM		
	IC ₅₀ uM	IC ₅₀ uM	IC ₅₀ uM	Average IC ₅₀ uM	stdev
Naseeseazine C	4.87	3.06	2.62	3.52	1.19
Chloroquine*	0.0118	0.00495	0.00754	0.0081	0.0035

*positive control

APPENDIX V - *In situ* iChip isolation techniques

The isolation chip (iChip) is an effective version of diffusion chambers that can also be deployed *in situ*.¹ The iChip consists of hundreds of miniature diffusion chambers of approximately 1 mm diameter in a central plate, which is dipped into a microbial suspension in molten agar.¹ Semi-permeable membranes cover the central plate allowing diffusion of nutrients into the chamber, but restrict the movement of the cells to the outside environment. Two supporting plates are screwed to the central plate providing sufficient pressure to seal the isolation (**Figure VA**).¹ This device allows simultaneous isolation of environmental bacteria. Initially, an environmental sample, i.e. sediment, is diluted so that approximately one bacterial cell is delivered to each miniature chamber, the device is then sealed with semi-permeable membranes between central and side plates, and incubated back in the natural environment where the sample was taken from originally. Once a colony of sufficient cells is produced, it is likely that the previously uncultured isolates are able to grow *in vitro*. This isolation approach has recently led to a major breakthrough in microbial research as the novel bacterium *Eleftheria terrae* could be isolated from soil samples.² This bacterium is the producer of the antibiotic teixobactin (**22**), the first antibiotic discovered in the last 30 years without detectable resistance.² To date, no marine bacteria have been isolated with the iChip, this was probably due to the so far limited incubation time of only two weeks.³ However, it should be possible to isolate marine bacteria with optimised iChip conditions in the future.

iChip design and assembly

The software SolidWorks was used to design the iChip assembly. Ten iChips consisting of hydrophobic plastic polyoxymethylene were laser cut by Griffith School of Engineering. The central plate, 40.5 mm x 20.25 mm x 2 mm, is supported by two symmetrical side plates of the same dimensions 40.5 mm x 20.25 mm x 2 mm. All plates have six rows of fifteen 1 mm diameter through holes (2.25 mm from centre to centre) eventuating to 90 wells per iChip.

All plates will be sterilised in ethanol, followed by drying in a laminar flow hood and rinsed in particle-free DNA- grade water prior to the sampling event. The assemblies will be transported in sterile plastic bags. A solution of the growth media will be supplemented with 100 µg/mL cycloheximide to prevent fungal growth and injected with diluted sponge extract after autoclaving. Therefore, approximately 1.5 µL of the microbial infused media will be dispensed into the iChip wells. Next, semi-permeable polycarbonate membranes of 0.03µm pore

size are applied on each side of the central plate and fixed tightly by screwing the side plates to the central plate; excess membrane material will be cut off. The assembly will subsequently be incubated in living sponges for a minimum of 4 weeks; thereupon the iChip will be cut out of the sponge tissue, placed in sterile seawater and transported back to the laboratory. In the laboratory, the central plate will be examined under a compound microscope for colony counts. The well contents of the iChips will be streaked onto agar plates to test the ability of the microorganisms to grow outside the iChip and for colony purification.

Figure VA. iChip prototype

- (1) Lewis, K.; Epstein, S.; D'Onofrio, A.; Ling, L. L. *The Journal of antibiotics* **2010**, 63, 468.
- (2) Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schäberle, T. F.; Hughes, D. E.; Epstein, S. *Nature* **2015**.
- (3) Hameş-KocabaŞ, E. E.; Ataç, U. *Journal of microbiological methods* **2012**, 88, 342.