Revisiting unexploited natural products in search of new antibacterial drug candidates: the case of the benzoisochromanequinone and dithiolopyrrolone classes

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

It has become vital to identify antibacterial drug candidates with novel modes of action to address the growing problem of antibiotic resistance. One way of rapidly accessing novel antibiotics is to revisit natural product classes that have previously been shown to possess antibacterial activity, but have never been clinically exploited. The recent introduction of the antibiotics daptomycin, retapamulin, and fidaxomicin to the clinic is a proof of concept that revisiting known of novel natural product scaffolds could achieve clinical benefit. In this thesis, the benzoisochromanequinone (BIQ) and dithiolopyrrolone (DTP) classes of natural products were revisited.

In chapter three, the BIQ class actinorhodin was subject to a detailed biological characterization. γ-actinorhodin was found to possess several requisite properties of a useful antibacterial drug candidate; it possessed potent bactericidal anti-Gram-positive activity, which included activity against MRSA and VISA strains (MIC_{90} of 2 μg/ml), was found to exhibit selective toxicity against prokaryotes, and displayed low resistance potential *in vitro*. Mode of action studies showed that γ-actinorhodin acts on the bacterial membrane in a manner distinct from other membrane-perturbing agents in clinical use. Preliminary studies of safety and efficacy of γ-actinorhodin *in vivo* showed potential promise for treatment staphylococcal infection.

In chapter four of this thesis, the DPT thiolutin was studied, with an emphasis on understanding its mode of antibacterial action. In *E. coli*, thiolutin demonstrated preferential inhibition of RNA synthesis in agreement with earlier studies. In *S. aureus*

however, while potent bacteriostatic activity was noted, thiolutin showed an unusual profile in radiolabel incorporation experiments with no inhibitory effect on any macromolecular biosynthetic pathway. Genetic analysis of mutants resistant to thiolutin revealed mutations in the *S.aureus* thioredoxin and in *E. coli* glutaredoxin redox systems components suggesting the involvement of these systems in the reductive activation of thiolutin. Inhibition of *E. coli* transcription and translation could not be detected *in vitro,* even in the presence of reducing agent suggested that RNA polymerase is not the primary target of thiolutin. The finding from this study and recent publication (Chan *et al.* 2017) suggest that DTPs exerts their antibacterial activity via a novel mode of action.

In summary, the findings of this study with thiolutin and γ-actinorhodin underscore the utility of revisiting unexploited natural product in the search for antibacterials with novel mode of action.

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Abbreviations

ATCC- American Type Culture Collection

ATP- adenosine triphosphate

BIQ- benzoisochromanequinone

CDC- Centers of Disease Control and Prevention

CFU- colony forming unit

CLSI- Clinical and Laboratory Standards Institute

CTAB- cetyltrimethylammonium bromide

DMSO- dimethylsulphoxide

DNA- deoxyribonucleic acid

DTP- dithiolopyrrolone

DTT- dithiothreitol

EDTA- ethylenediaminetetraacetic acid

FDA- Food and Drug Administration

HEPES- 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HTS- high throughput screening

ISP2- International Streptomyces Project medium no.2

LA- Lysogeny agar

LB- Lysogeny broth

MDR- multiple-drug resistant

MeOH- methanol

MHA- Mueller-Hinton agar

MHB- Mueller-Hinton broth

MIC- minimum inhibitory concentration

MIC_{90}- minimum inhibitory concentration required to inhibit the growth of 90% of isolates tested

MMS- macromolecular synthesis

MRSA- methicillin-resistant *Staphylococcus aureus*

MSSA- methicillin-sensitive *Staphylococcus aureus*

NADH-reduced nicotinamide adenine dinucleotide

NCTC- National Collection of Type Culture

OD- optical density

PBS-phosphate-buffered saline

PCR- polymerase chain reaction

pH- potential hydrogen

PMBN- polymyxine B nanopeptide

RNA- ribonucleic acid

ROS- reactive oxygen species

SDS- sodium dodecyl sulphate

SOD- superoxide dismutase

TCA- trichloroacetic acid

TE- Tris-EDTA

VISA- vancomycin-intermediate *Staphylococcus aureus*

v/v- volume per volume

WHO- World Health Organization

YM-yeast malt broth

Chapter one

Introduction

1.1. The impact of antibiotic discovery and the problem of antibiotic resistance

Antibiotics are one of the most successful forms of chemotherapy in the history of medicine. Following their initial discovery in the early twentieth century, it was widely believed that antibiotic use would lead to the eventual eradication of bacterial diseases (Aminov 2010). Antibiotics have contributed to an increase of 30 years in average life expectancy in developed countries, both as a direct consequence of treating infection and indirectly in their prophylactic use to prevent infection following invasive surgeries (Ventola 2015). However, the widespread use of antibiotics in the treatment of infectious diseases has provided the selection pressure necessary to drive the spread of antibiotic resistance (Davies and Davies 2010; Ventola 2015).

The increasing frequency of multidrug-resistant (MDR) and pan-resistant strains like those of the ESKAPE pathogen group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) is now a major cause of concern which requires immediate action (CDC 2013). A recent report estimated that by 2050, 10 million lives a year and the cumulative of US $100 trillion of economic output are at risk due to the rise of antibacterial resistant infections (O'Neill 2016). The problem has intensified following the emergence of resistance to the antibiotic

colistin, heralding a disturbing breach of the last group of antibiotics and an end to the last line of defense against multidrug-resistant Gram-negative bacterial infections (Cannatelli *et al.* 2016; Elnahriry *et al.* 2016; Liu *et al.* 2016; Yu *et al.* 2016; Zeng *et al.* 2016). The recent death of a patient in Nevada from an infection resistant to all available antibiotics in the US-including colistin has gained media attention, and led to public awareness about the current threat of bacterial resistance to antibiotics (Chen *et al.* 2017). This single case became a stark reminder of what a post-antibiotic era could look like, and highlighted the current complex problem of antibiotic resistance and the catastrophic consequences of failing to act.

It is crucial to identify new antibiotic classes which have a novel mode of action and exhibit antibacterial activity against pathogens which are resistant to the current arsenal of antibiotics. However, many of the pharmaceutical companies have abandoned their antibacterial and infectious disease discovery programs (Fischbach and Walsh 2009; Wenzel 2004; Silver 2011). Confronted with the scientific, economic, and regulatory challenges, the pharmaceutical sector seems incapable of responding to the threat of antibacterial resistance. The fact remains that better returns can be made in other areas of therapeutics (Silver 2011). Undoubtedly, discovering and developing new antibacterials is an expensive and lengthy process even with the right economic incentives in place (Projan and Youngman 2002). It is estimated that the research and development cost of a new antibiotic is approximately US $1.8 billion, and the time commitment is more than a decade (FDA 2004; Wenzel 2004). Additionally, bacterial resistance to antibiotics renders these compounds ineffective after a relatively short period of clinical use compared to other pharmaceuticals. The field of antibiotic discovery is suffering from 30 years ‘Discovery Void’ in which no new classes of antibiotics have successfully progressed from discovery to the

clinic (Silver 2011; Ooi and O'Neill 2017). The following section will briefly review the methods and strategies employed in the early stages of antibiotic discovery and the reasons to support a shift in strategies to find novel antibiotics to address the growing public health threat of antibiotic-resistant infections.

1.2. A brief history of antibacterial drug discovery- past and current trends

The earliest recorded antibacterial drug discovery programs, which involved screening of hundreds of chemicals and dyes for inhibitory activity, were effective. Prontosil (sulfanilamide) one of the first synthetic antibiotics, was discovered by such methods (Silver 2011). The serendipitous discovery of the first natural product, penicillin, in a laboratory in St. Mary's Hospital in London, UK, after a *Penicillium rubens* (later confirmed to be *Penicillium chrysogenum*) spore landed on a partially opened Petri dish (Fleming 1929). The introduction of penicillin to the clinic in the 1940s was soon followed by the discovery of a vast number of antibiotics from microbes, in particular from members of the Actinomycetes and fungi (Pelaez 2006; Lewis 2013) (Figure 1.1). The majority of antibacterial agents were discovered by systematic screening (an approach introduced by Selman Waksman) for antibacterial activity from soil-derived streptomycetes against a susceptible indicator microorganisms. This period (1940s-1960s) is now referred to as the ˝Golden Agę of antibiotic discovery (Lewis 2013; Schatz, Bugie and Waksman 1944). Numerous antibiotics discovered until the early 1970s reached the market, and were further improved by chemical modifications to increase their activity, lower their toxicity and overcome bacterial resistance mechanisms.

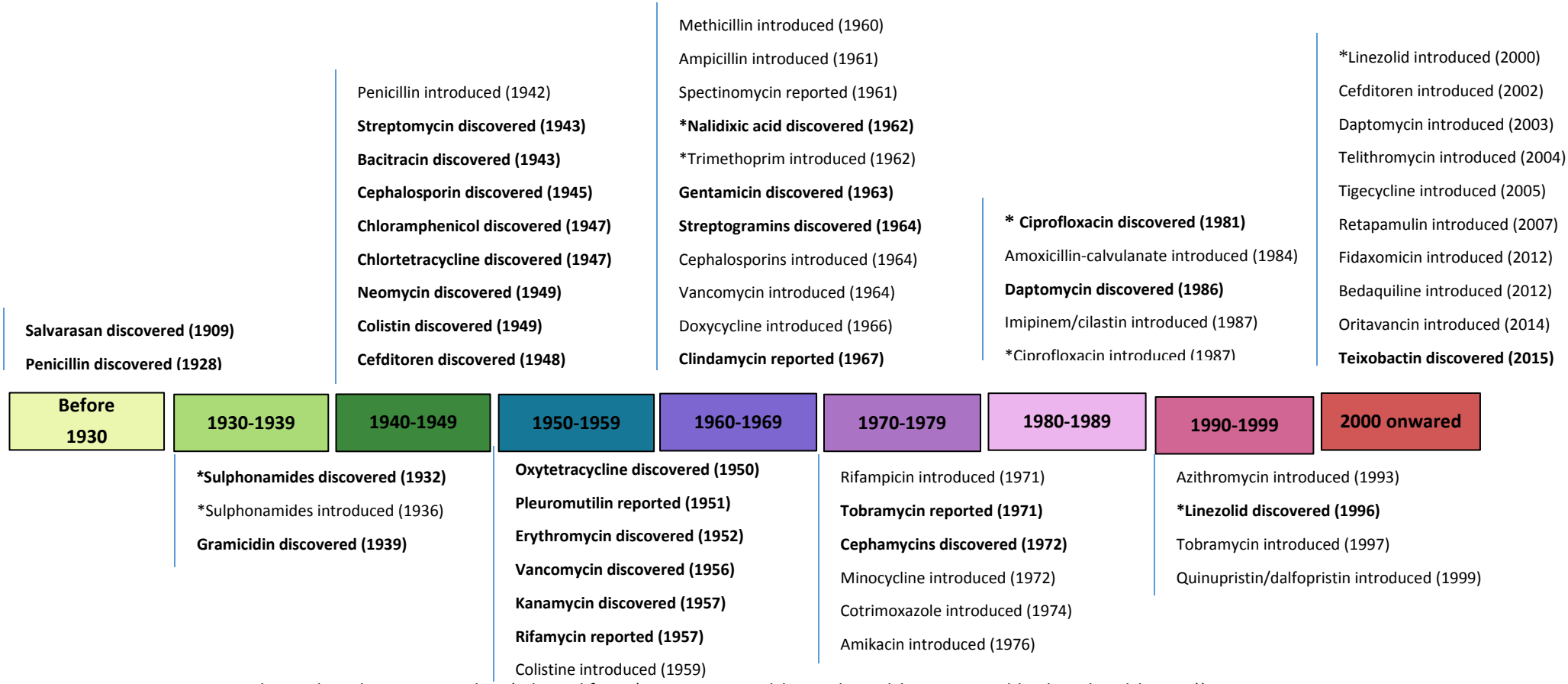


Figure 1.1. Antibiotic drug discovery timeline (adapted from (Lewis 2013; Walsh 2003b; Walsh 2003a; Fischbach and Walsh 2009))

* Synthetic compounds

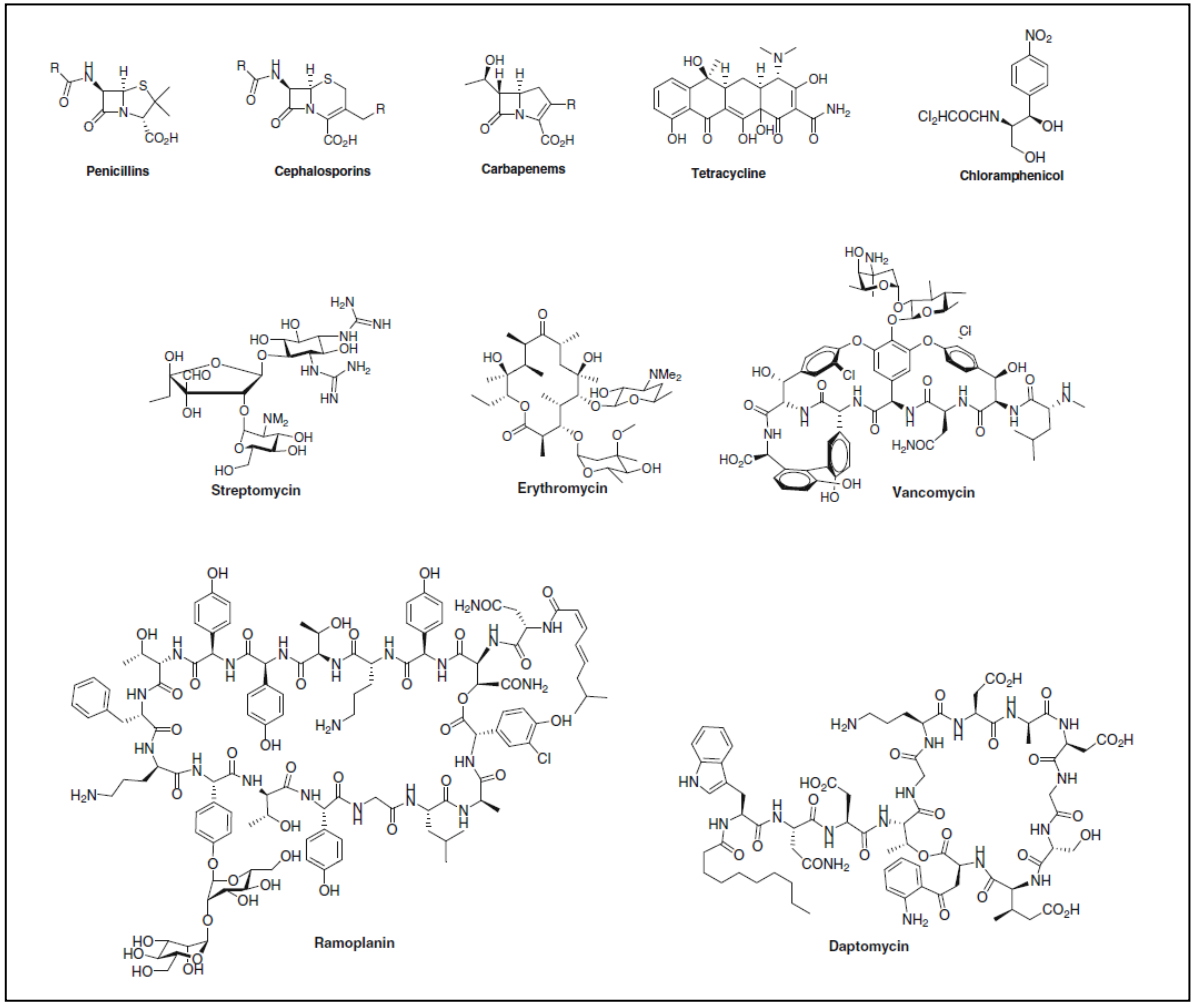
By the late 1970s, antibiotic discovery had slowed significantly. In 1977, a new platform for antimicrobial and antiviral drug discovery was proposed by Cohen, involving screening for inhibitors of specific enzymes involved in bacterial metabolism in a cell-free assay (Cohen 1977). This approach aimed to identify specific biochemical targets that were essential to bacterial survival and absent in humans to overcome issues such as host toxicity (Cohen 1977). At the time, competition amongst pharmaceutical companies towards introducing novel classes of antibiotics stimulated them to find a way to accelerate the drug discovery process. The advent of the high-throughput screening (HTS) approach in the 1980s, combined with the introduction of combinatorial chemistry, was a breakthrough (Payne *et al.* 2007; Silver 2011). At that point, major pharmaceutical companies abandoned natural products in favor of screening large libraries of synthetic molecules for antibacterial activity. This is mainly because of the lack of compatibility of traditional natural product extract libraries with HTS assays. Although many molecules discovered through HTS exhibited potent activity against specific cellular targets, they were often unable to penetrate the bacterial membrane or were inactivated *in vivo* (Payne *et al.* 2007; Silver 2011). Consequently, they lacked antibacterial activity.

The introduction of genomics in the mid-1990s also failed to herald a new era of antibacterial discovery as hoped for, even though the products of more than 160 genes were determined as essential for bacterial growth and therefore offered potential novel targets for new antibiotics. Between 1995 and 2001, 70 HTS campaigns on antibacterial targets run against the GlaxoSmithKline (GSK) compound collection resulted in very few hits. Most of these hits turned out to be equally toxic to both mammalian and bacterial cells, usually as a consequence of the indiscriminate cell membrane disruption, leading to disappointment with this approach (Payne *et al.* 2007; Lam 2007).

The rarity of novel antimicrobial scaffolds reaching the clinic has become evident over the last 40 years. Only the streptogramin mixture, dalfopristin/quinupristin (Manfredi 2005), the oxazolidone linezolid (Ford, Zurenko and Barbachyn 2001), the lipopeptide daptomycin (Carpenter and Chambers 2004), the ketolide telithromycin (Shi, Montay and Bhargava 2005), the pleuromutilin retapamulin (Parish and Parish 2008), and the macrocycle fidaxomicin (Cornely *et al.* 2012), can be considered new antibiotic classes. However, almost all of these classes are narrow spectrum, and potent antibacterial scaffolds active against Gram-negative pathogens have not been introduced. Through history, it can be concluded that the current absence of antibiotic productivity is mainly attributed to the poor choice of approaches to address the discovery of new antibiotics. The transition from screening natural products to combinatorial chemistry during the 1980s, combined with the switch from traditional screening to target based HTS may have led to the current lack of new scaffolds in drug development pipelines (Harvey, Edrada-Ebel and Quinn 2015). The answer to the current dilemma might be to return to the natural product whole cell screening platform.

It is a well-known fact that microbial natural products have been the source of almost all antibiotic scaffolds currently used in the clinical setting (Clardy, Fischbach and Walsh 2006; Walsh 2003a; Newman and Cragg 2016). To date, antibacterial discovery has yielded over a dozen distinct and clinically relevant chemical scaffolds, all of which are naturally derived excluding the oxazolidinones, quinolones, sulfonamides, and trimethoprim (Figure 1.2). Re-establishing natural products as the primary source of leads, coupled with using the current advanced screening strategies would offer a better opportunity to obtain novel and useful therapeutic agents (Berdy 2012; Milshteyn, Schneider and Brady 2014; Rutledge and Challis 2015). Although the larger pharmaceutical companies terminated their natural product

A



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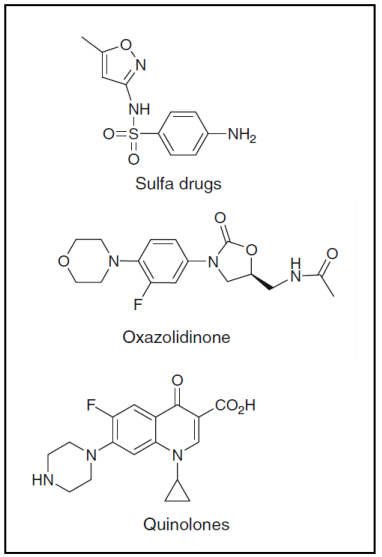


Figure 1.2. Representative classes of antibacterial agents of (A) natural product origin and (B) synthetic origin

programs in the 1990s, efforts from academic universities and start-up companies have continued in the area. However, owing to insufficient sources of suitable compounds to screen together with poorly validated discovery methodologies, repeated rediscovery of previously known compounds with limited antibiotic lead potential, dominates most efforts (Baltz 2006).

To address the challenges of discovering novel chemical scaffolds with desirable antibacterial properties from nature, process miniaturization is required to increase the throughput by a considerable measure over traditional screening. In addition, methods that can be monitored and improved over time are needed, to minimize the rediscovery of known antibiotics (Lok 2015; Baltz 2006). Two approaches can be employed for new classes of antibiotics to be introduced. The first is to search for new scaffolds in nature using new assays, and new cultivation methods and, in new niches to maximize the chance of finding novel compounds. The second approach, which is the scope of this research, is to revisit clinically unexploited natural products that showed some antibacterial promise and ascertain if the reasons for failure can be overcome. Each approach has its opportunities and challenges that will be briefly discussed in the following sections.

1.3. Approaches for developing new antibacterial agents

1.3.1. New antibiotic from under- and unexploited microbial sources

Most clinically useful antibiotics are produced by soil-dwelling microorganisms that form spores or resting structures; the actinomycetes, primarily *Streptomyces* spp., account for the production of more than 60% of the known antibiotics (Pelaez 2006; Demain and Sanchez 2009). In view of the fact that a vast number of bioactive metabolites from

Streptomyces spp. have already been identified, the likelihood of finding novel antimicrobial leads among conventional actinomycetes have substantially diminished owing to the rediscovery of known compounds (Singh 2014). Therefore, more current efforts are concentrated on less exploited genera in soil ecology, mostly non-streptomycetes actinomycetes or rare actinomycetes such as *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Microbispora*, *Micromonospora*, *Streptosporangium* and *Saccharopolyspora* (Lazzarini *et al.* 2000). Rare actinomycetes are strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of streptomycete strains (Jose and Jebakumar 2013). these strains are considered a source of promising natural products since they produced many useful antibiotics including the rifamycins (produced by *Amycolatopsis mediterranei)*, erythromycin (produced by *Saccharopolyspora erythraea),* teicoplanin (produced by *Actinoplanes teichomyceticus*), vancomycin (produced by *Amycolatopsis orientalis*), and gentamicin (produced by *Micromonopsora purpurea*) (Tiwari and Gupta 2012). A literature survey conducted by Lazzarini and others has revealed that out of more than 8,000 antimicrobial products described in the database, 16% of bioactive microbial products are produced by strains considered rare actinomycetes, increasing the relevance of this group as a source of new antibiotics (Lazzarini *et al.* 2001).

It has been established that the isolation of rare actinomycetes in the laboratory environment can be stimulated by pre-treatment of soil by drying and heating or adding antibiotics that inhibit the growth of competing bacteria, including fast-growing *Actinomycetes* (Subramani and Aalbersberg 2013). Additionally, developing methods such as ultrasonic treatments and high-frequency irradiation, have also supported selectivity for rare actinomycetes, allowing more fruitful screening (Subramani and Aalbersberg 2013). Past and present efforts in the isolation of rare actinomycetes have

generated a strain collection with more than 20,000 strains, showing that when selective isolation methods are established and comprehensively applied, some genera, such as *Actinomadura*, *Actinoplanes*, *Micromonospora*, *Microtetraspora*, are not, in fact, rare but can be recovered from many soil samples (Suzuki, Okuda and Komatsubara 1999; Hayakawa *et al.* 1996; Lazzarini *et al.* 2001). One chemical lead discovered in Biosearch Italia is the antibiotic GE2270 that selectively targets elongation factor Tu in bacterial protein synthesis, from the producing organism *Planobispora rosea* (Selva *et al.* 1991). Currently, an amide derivative of GE2270 (CB-06-01) is undergoing Phase II dose ranging clinical trial as a topical antibiotic for the treatment of mild to moderate acne (Cassiopea 2017).

Another approach to access and expand the chemical diversity of microbial secondary metabolite involves isolation of new microbial species (Singh and Pelaez 2008). Uncultivable microorganisms isolated from exploited habitats (Piddock 2015) and underexploited species found in extreme environments have been shown to produce a range of complex natural products with high biological activity (Mohammadipanah and Wink 2016). Growing bacteria that have previously not been cultured is a successful approach to finding unexploited microbial genera, and methods for this have been established (Kaeberlein, Lewis and Epstein 2002; Nichols *et al.* 2010). The principle of this approach is to grow microorganisms in their natural environment. This is done by introducing a marine sediment or soil sample diluted with agar between two semipermeable membranes and placing this diffusion chamber back into the natural environment. Compounds diffuse freely through the chamber, and microorganisms perceive it as their natural environment (D'Onofrio *et al.* 2010). Repeated re-inoculation from chamber to chamber results in the domestication of a considerable proportion of

uncultured microorganisms, which enables their subsequent growth in standard laboratory conditions (Bollmann, Lewis and Epstein 2007). NovoBiotic Pharmaceuticals (Cambridge, MA, USA) in collaboration with the University of Bonn in Germany and Northeastern University in USA has been using *in situ* cultivation for antibiotic discovery and recently reported the discovery of new antibiotic, teixobactin, from the newly discovered Gram-negative bacterium *Eleftheria terrae* with properties that minimize the development of bacterial resistance (Ling *et al.* 2015).

Of several alternative habitats than soil, the marine environment stand for a promising source of for new bioactive molecules (Ziemert *et al.* 2014; Zhao 2011). In contrast to terrestrial habitats, marine environment is a rich source of novel chemical classes, in which many marine natural products are characterized by the marine factors such as halogens (Wietz *et al.* 2013; Fenical 1993). Exploring the oceans has led to the discovery of new secondary metabolites isolated from algae, sponges or corals (Mehbub *et al.* 2014). However, recent studies have credited the production of many of these compounds to microbes, associated with the eukaryotic organism presumably thought to be the producer (Wilson *et al.* 2014). However, toxicity has to date been problematic for secondary metabolites derived from the sea, which has so far precluded their development as antibiotics (Buchanan *et al.* 2005).

Despite all the potentials of the approaches above, isolation of new natural product scaffolds remains a lengthy process that requires particular expertise (Shore and Coukell 2016). The task of identifying the main active components in microbial filtrates can be complicated even with advanced technology at hand (Lim and Hai-Meng 2013). Besides, it appears that the problem is more complicated than simply a need for more innovative screening. Most natural product scaffolds exhibit a range of challenges that often include

cytotoxicity, lack of solubility, high protein binding, and a lack of activity against Gram-negative bacteria (Silver 2011). Finding those rare novel natural products that are effective and nontoxic will require sustained and focused effort (Silver 2011; Lim and Hai-Meng 2013).

1.3.2. Revisiting abandoned natural product scaffolds

To evade some of the drawbacks of past and current attempts to identify novel antibacterial agents, re-evaluation of previously discarded natural products which have been reported previously to possess antibacterial activity may offer some potential (Chopra, Hesse and O'Neill 2002; Zahner and Fiedler 1995). Although a proportion of such compounds might have been assessed and excluded for development as they display unfavorable properties, it is also feasible that many might not have been carefully evaluated as antibacterial drug candidates by scientists with appropriate drug discovery, natural products, and medicinal chemistry expertise. The introduction of the antibiotics linezolid, daptomycin, retapamulin, and fidaxomicin to the clinic represents a proof of concept of that innovative revisiting of antimicrobial chemical matter with fresh eyes could achieve clinical benefit (Wright 2012; Silver 2011). None of these antibiotics represent truly novel classes of drugs, given that they have been discovered in decades past and either repurposed for human use or revived from shelved projects (Wright 2012).

The antimicrobial properties of the oxazolidinone scaffold were first discovered by researchers at DuPont in the 1970s and shown to be effective in the treatment of bacterial and fungal plant diseases (Robert B. Fugitt 1978), and for treating bacterial infections in mammals (Slee *et al.* 1987). In the 1990s, the scaffold was modified by researchers at

Pharmacia and Upjohn, (now part of Pfizer), using traditional medicinal chemistry efforts into the first-in-class drug Zyvox, which received FDA-approval in 2000 (Barbachyn and Ford 2003; Ford, Zurenko and Barbachyn 2001). Similarly, daptomycin was first isolated in 1987 from the soil actinomycete, *Streptomyces roseosporus,* by scientists at Eli Lilly from a soil sample from Mount Ararat (Turkey) (Debono *et al.* 1987). Phase I and II clinical trials were conducted in the late 1980s, and early 1990s, and resulted in the termination of the intravenous (IV) daptomycin clinical programs because of toxicological concerns (Woodworth *et al.* 1992). Daptomycin was then acquired by Cubist in the mid-1990s, who focused their efforts on developing formulation for oral and topical clinical indications to limit the systemic exposure and re-deployed with alternate dosing for the IV treatment, and received FDA approval in 2003 for the treatment of skin and skin structure infections caused by Gram-positive pathogens (Baltz, Miao and Wrigley 2005; Carpenter and Chambers 2004; Eisenstein, Oleson and Baltz 2010). The pleuromutilins were first discovered in 1951 (Kavanagh, Hervey and Robbins 1951), and used for years in veterinary medicine, but revisited by GlaxoSmithKline (GSK) and presented as semisynthetic retapamulin for topical human use, gaining FDA approval in 2007 (Parish and Parish 2008). Fidaxomicin has a similar history, identified by groups at Lepetit in 1975 as a poorly absorbed antibiotic. Optimer took advantage of the poor solubility of fidaxomicin to selectively target infections caused by the serious intestinal pathogen *Clostridium difficile*, and eventually brought to market (Poxton 2010).

It seems this approach is more likely to yield more rapid results and a greater number of new classes to the antibiotic pipeline to address the current antibiotic resistance crisis. However, antibiotic discovery has a long history and much of the published research is buried in old journal issues or out of print books, and other research (especially industrial)

never makes it to publication (Shore and Coukell 2016). Thousands of different natural products have been isolated from cultures of Gram-positive and Gram-negative bacteria and of filamentous fungi. However, only a small proportion of these have been used commercially as therapeutics (Kumar, Duraipandiyan and Ignacimuthu 2014). Shaping this body of research and making it accessible to investigators who need it is critical for advancing discovery. Valuable knowledge may include assemblies of screens that have been run before and information on past research programs. While plenty of these materials are widely available, what may be most beneficial is a justification of what made these projects unsuccessful, and why.

1.4. Preclinical evaluation of novel antibacterial agents

The development of novel antibacterial agents requires extensive preclinical evaluation to ensure that antibacterial activity is correlated with inhibition of a specific bacterial target with low, or no activity against eukaryotic homologues, before it will be approved for entry into the prolonged and costly process of clinical trials (O'Neill and Chopra 2004; Silver 2011). In addition, preclinical evaluation includes determining the antimicrobial spectrum of activity *in vitro*, and the level of selective bacterial inhibition. These studies provide a preliminary evaluation of therapeutic potential (O'Neill and Chopra 2004). One important consideration when characterizing the biological properties of a novel antibiotic is the necessity for the use of standardized methodology for susceptibility testing. Using standardized method (such as CLSI) will allow for susceptibility data to be reproducible and the efficacy of novel antibiotic to be assessed properly (Andrews 2001; CLSI 2012). Determination of MIC of an antibiotic should be done. For therapeutic concentrations, the

MIC of an antibiotic at the site of infection should be three to four times the MIC. Concentrations greater than this generally do not improve the therapeutic effect and tends to increase the likelihood of toxicity (Leekha et al., 2011).

Defining the mode of action of a novel compound is also an important phase of pre-clinical evaluation (O'Neill and Chopra 2004). The mode of action is the biochemical event by which the growth of a sensitive microorganism is inhibited as a result of the antibiotic interference with a target molecule essential for the survival of the host bacteria (Walsh 2003a). To date, the antibacterial therapeutic field is a target-poor environment. Despite thousands of molecules exhibiting antibiotic properties, there are essentially five major clinically validated antibacterial targets/pathways (Walsh 2003b; Hancock 2005; O'Neill and Chopra 2004). As shown in Figure 1.3, these include bacterial peptidoglycan/cell wall biosynthesis, bacterial protein synthesis, nucleic acid synthesis, folate biosynthetic pathway, and the most recent target of bacterial membrane integrity.

However, understanding many of the bacterial responses that occur as a consequence of the primary drug-target interactions remain incomplete. When the target identification of novel agents could not be addressed, the prospects for the development of these compounds will rely on elucidating their selectivity for prokaryotic organisms (O'Neill and Chopra 2004). The selective toxicity of antibiotics against bacteria is usually a consequence of the absence of their cellular targets in eukaryotes, are sufficiently different structure to an equivalent eukaryotic homologue, or are non-accessible.

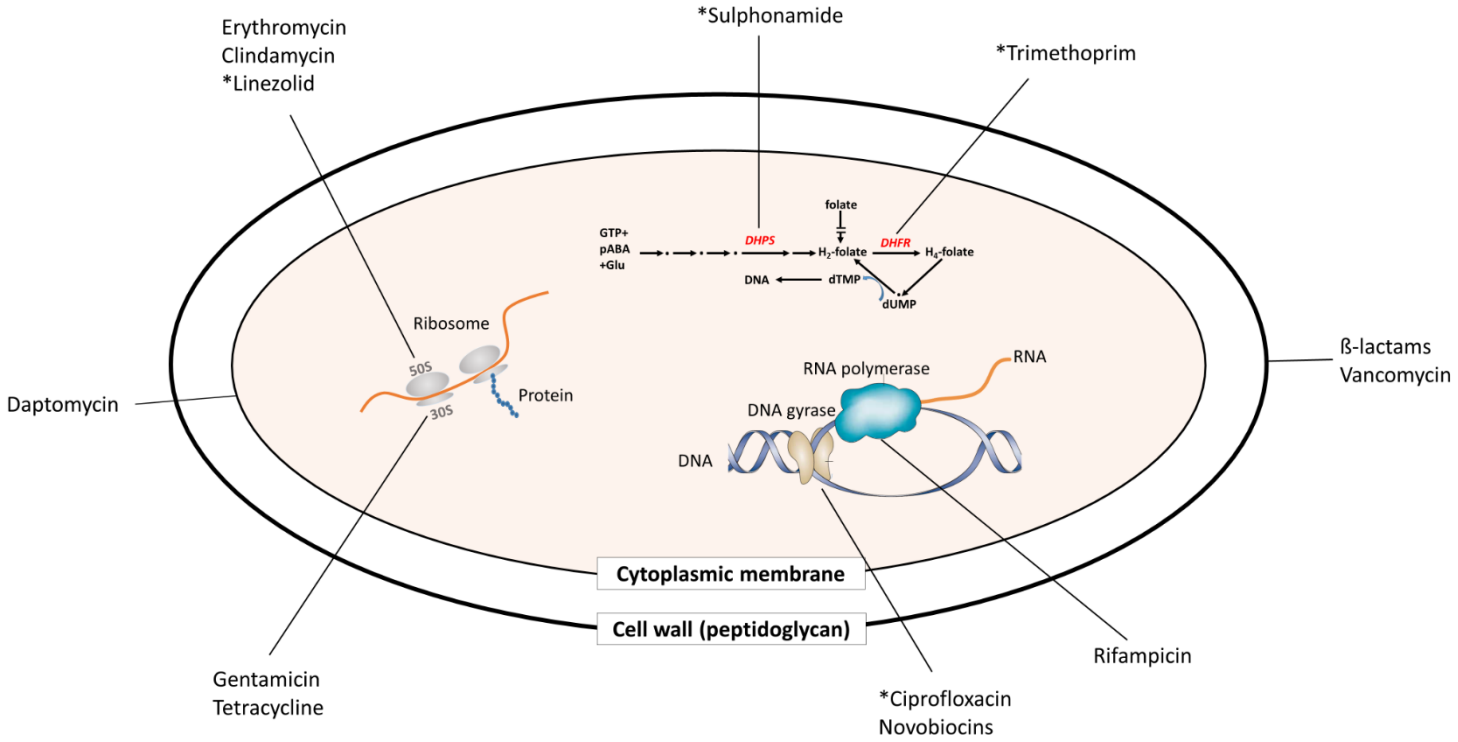


Figure 1.3. Schematic of the cellular targets of established antibacterial agents, grouped by target site (adapted from (Walsh 2003b))

*Synthetic compounds

There are a large number of established methods available to gain a full understanding of the mode of action, and the experiments described in Chapter 3 and Chapter 4 of this thesis, have been successfully used in earlier studies (Oliva *et al.* 2003; Oliva *et al.* 2004; Randall *et al.* 2013b; Ooi *et al.* 2013). Initial mode of action studies might include measurements of the effect of compounds on an essential bacterial macromolecular synthetic process through incorporation of radiolabelled precursors. This assay rapidly identifies the most sensitive biosynthetic pathway to inhibition by compounds and consequently provide a focus for more detailed experiments to be carried out (O'Neill and Chopra 2004). Compounds that damage the bacterial membrane simultaneously inhibit all biosynthetic pathways and membrane perturbation can be confirmed by measuring the ingress and egress of fluorescent dye and leakage of intracellular components (O'Neill *et al.* 2004). Before the introduction of daptomycin and telavancin for clinical use, membrane-based activities had been considered an undesirable property of a novel inhibitor, as some compounds may have promiscuous interaction with both prokaryotic and eukaryotic membranes (Hurdle *et al.* 2011). Agents with novel targets or those that affect essential bacterial components are desirable as resistance to these agents is less likely to arise by minor alterations to either existing resistance mechanisms or conserved structures. The development of resistance should be assessed during preclinical evaluation, as resistant mutants can provide insight into the cellular target of the compound.

1.5. Objective of the study

Due to the increasing prevalence of infections caused by multidrug-resistant bacteria and the lack of novel antibiotics in development, this research aimed to identify potential

candidates for use in the treatment of resistant bacterial infections. The lessons in bringing the most recent classes of antibiotics to market are substantial and exemplify the significance of revisiting old scaffolds from natural products. To avoid some of the drawbacks of past and current attempts to identify novel antibacterial compounds, studies were conducted with compounds that were already shown to have activity against whole-bacterial cells: the benzoisochromanequinone (BIQ) and dithiolopyrrolone (DTP) classes of natural products were revisited. Representative compounds from these classes would be isolated and purified from producing microorganisms unless commercially available. Compounds would be screened for activity against laboratory and clinically relevant Gram-positive and Gram-negative cultures.

Characterization of the mode of action is a critical aspect of novel drug development (O'Neill and Chopra 2004). Therefore, the time-kill kinetics and mode of action studies of compounds under investigation would be carried out. The development of resistance to agents would be explored, in order to estimate the chance of resistant developing in the clinic. Subsequently, the genetic basis of resistant mutants would be studied *via* genome sequencing and the contribution of any identified mutations will be confirmed. Additionally, the resistant mutants would be subjected to cross-resistance examination to provide further information regarding the cellular target of the compound. Finally, compounds with useful antibacterial activity would be assessed for bacterial specificity, effects on mammalian cells *in vitro,* and *in vivo* safety and efficacy in an experimental animal model.

Chapter two

Materials and methods

2.1. Strains and growth conditions

Laboratory strains of bacteria and yeast used in this study are listed in Table 2.1. Clinical isolates of *S. aureus* used for susceptibility testing were part of a culture collection belonging to the Antimicrobial Research Centre, University of Leeds. The actinorhodin overproducer strain *Streptomyces coelicolor,* L646 (kindly provided by K. McDowall, University of Leeds), was used as the source of γ-actinorhodin and was propagated on ISP medium 2 (Difco) containing 50 µg/ml apramycin at 30°C.

Bacteria (with the exception of *S. coelicolor* L646) were routinely cultured in Mueller-Hinton broth (MHB) and on Mueller-Hinton agar (MHA) (Oxoid Ltd, Cambridge, UK) for 24 h at 37°C, whilst *Candida albicans* was grown in Lysogeny broth (LB) and on Lysogeny agar (LA) (Oxoid) for 48 hours at 35°C.

Table 2.1: Strains used in this study

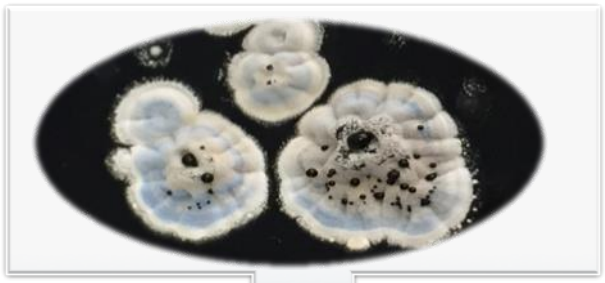
Strain	Genotype or description	Reference/ Source
Staphylococcus aureus		
SH1000	Laboratory strain derivative of strain 8325-4, containing functional *rsbU*	(Horsburgh *et al.* 2002)
KS100	SH1000 deficient in catalase KatA (*katA*::Tn*917* (*ery*))	(Cosgrove *et al.* 2007)
KC043	SH1000 deficient in catalase KatA and alkyl hydroperoxide reductase AhpC (*ahpC*::*tet katA*::Tn*917* (*ery*))	(Cosgrove *et al.* 2007)
MHKA	SH1000 deficient in superoxide dismutase SodA (*sodA::Tn917*)	(Karavolos *et al.* 2003)
MHKM	SH1000 deficient in superoxide dismutase SodM (*sodM::tet)*	(Karavolos *et al.* 2003)
MHKAM	SH1000 deficient in superoxide dismutase SodA and SodM (*soda::Tn917 sodM::tet*)	(Karavolos *et al.* 2003)
Escherichia coli		
BW25113	Derivative of *E.coli* K12 strain BD792 ($lacI^q$ $rrnB_{T14}$Δ$lacZ_{WJ16}$*hsdR514* Δ*araBAD*$_{AH33}$Δ*rhaBAD*$_{LD78}$)	(Baba *et al.* 2006)
Δ*acrAB*	Derivative of BW25113 deficient in AcrAB efflux pump (*acrAB::kan*)	(Baba *et al.* 2006)
Δ*tolc*	Derivative of BW25113 deficient in TolC efflux pump (*tolC:: kan*)	(Baba *et al.* 2006)
Δ*trxA*	Derivative of BW25113 deficient in thioredoxin 1 (*trxA::kan*)	(Baba *et al.* 2006)
Δ*trxB*	Derivative of BW25113 deficient in thioredoxin reductase (*trxB::kan*)	(Baba *et al.* 2006)
Δ*trxCA*	Derivative of BW25113 deficient in thioredoxin 2 (*trxC::kan*)	(Baba *et al.* 2006)
Δ*gshA*	Derivative of BW25113 deficient in glutamate-cysteine ligase (*gshA::kan*)	(Baba *et al.* 2006)
Δ*gshB*	Derivative of BW25113 deficient in glutathione synthase (*gshB*::kan)	(Baba *et al.* 2006)
Δ*grxA*	Derivative of BW25113 deficient in glutaredoxin 1 (*grxA::kan*)	(Baba *et al.* 2006)
Δ*grxB*	Derivative of BW25113 deficient in glutaredoxin reductase (*grxB::kan*)	(Baba *et al.* 2006)
Δ*grxC*	Derivative of BW25113 deficient in glutaredoxin 2 (*grxC::kan*)	(Baba *et al.* 2006)

Table 2.1: Continued

Strain	Genotype or description	Reference/ source
***S. epidermidis* ATCC 14990**	Control strain for *Staphylococcus epidermidis* isolated from nose	ATCC
***S. haemolyticus* ATCC 29970**	Control strain for *Staphylococcus haemolyticus* isolated from human skin	ATCC
***Streptococcus pyogenes* ATCC 19615**	Control strain for *Streptococcus* Group A isolated from pharynx of child following episode of sore throat	ATCC
***S. pneumoniae* ATCC BAA-255**	Non-virulent strain	ATCC
***Enterococcus faecalis* ATCC 29212**	Control strain for *Enterococcus faecalis* isolated from urine	ATCC
***E. faecium* 7634337**	Vancomycin resistant enterococci (VRE) strain	Leeds General Infirmary
***Klebsiella pneumoniae* NCTC 9145 (K25)**	Unencapsulated wild-type strain isolated from clinical specimen	NCTC
***Acinetobacter baumannii* ATCC 19606**	Control strain for *Acinetobacter baumannii* isolated from urine	ATCC
***Pseudomonas aeruginosa* ATCC 15692 *(*PAO1)**	Opportunistic strain of Pseudomonas isolated from infected wound	ATCC
Yeast		
Candida albicans CA-6	Azole-sensitive strain with normal sterol profile	(Martel *et al.* 2010)
Streptomyces coelicolor		
S. coelicolor L646	*S. coelicolor* M145 containing an integrating plasmid overexpressing wild-type *atrA*, which leads to hyper-production of actinorhodin	(Towle 2007)

2.2. Extraction and purification of γ-actinorhodin

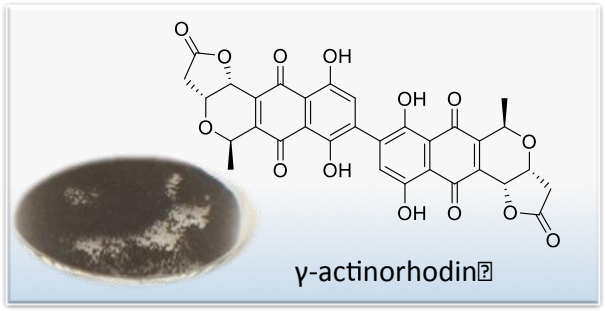
The method for extraction and purification was optimized in close cooperation with Sannia Farooque (School of Chemistry, University of Leeds, UK) to target the extraction of pure γ-actinorhodin (Figure 2.1.). *S. coelicolor* L646 was grown on ISP2 agar at 30°C for six days, at which point the medium turned deep blue in colour owing to secretion of actinorhodins. The agar was manually fragmented and the pigment extracted twice with ethyl acetate at 30°C for an hour with shaking (200 rpm). The resulting red extract was then concentrated on a rotary evaporator *in vacuo*, dissolved in chloroform, and washed with aqueous sodium carbonate solution (pH 10.8, 60 mL ×3). The blue aqueous layer was acidified with hydrochloric acid (6 M), and the crude red pigment was obtained by filtration. γ-Actinorhodin was then triturated from methanol (red precipitate, 125 mg, 19%). R_F 0.29 (1% MeOH in dichloromethane). All NMR experiments, interpretation of signals, and purity determination of γ-actinorhodin were performed by Sannia Farooq (School of Chemistry, University of Leeds). The identity and purity (95%) of γ-actinorhodin was assessed using mass spectrometry, 1-D NMR, 2-D NMR and infrared spectroscopy and analytical HPLC.



Production of dark blue diffusible pigment into ISP2 agar plates (4-6 days) (3 L)

- Extracted twice with ethyl acetate, filtered concentrated *in vacuo*, and dissolved in chloroform.
- Crude extract was subjected to alkyne wash, acidified and then recrystallized

650 mg of dried crude extract



Identified by ^{1}H NMR and characterised by 1-D, 2-D NMR, and MS

Figure 2.1. Overview of γ-actinorhodin extraction and purification

2.3. Antibacterial agents, chemicals, and kits

Antibacterial agents were from Sigma-Aldrich (Saint Louis, Missouri, USA) unless otherwise stated. Thiolutin (LKT Laboratories, Inc, Saint Paul, Minnesota, USA), holomycin (Santa Cruz Biotechnology, Inc, Heidelberg, Germany), vancomycin (LEK Pharmaceuticals, Ljublijana, Slovenia), cetyltrimethylammonium bromide (CTAB) (BDH Laboratory Supplies, Poole, UK), linezolid (Pfizer, Kalamazoo, Michigan, USA), ciprofloxacin (Bayer, Leverkusen, Germany), nisin (NBS Biologicals Ltd, Huntingdon, UK), daptomycin (Cubist Pharmaceuticals, Massachusetts, USA), and nisin (Duchefa Biochemie, Haarlem, Netherlands).

Radiolabelled chemicals were from PerkinElmer (Waltham, Massachusetts, USA), whilst 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], the Live/Dead *Bac*Light™ kit, and SYBR safe gel stain were from Invitrogen (Carlsbad, California, USA); unless otherwise stated, all other chemicals were from Sigma-Aldrich.

The PureElute™ bacterial genomic kit was from EdgeBio (Gaithersburg, MD, USA), Phusion® High-Fidelity PCR Kit was from New England Biolabs (Hitchin, UK), QlAquick PCR purification kit was from Qiagen (Manchester, UK). The *E. coli* S30 Extract System for Circular DNA kit, CytoTox-ONE™ Homogeneous Membrane Integrity Assay, and the PCR nucleotide mix were from Promega Corporation (Madison, USA). Unless otherwise stated, all other kits were from Sigma-Aldrich.

2.4. *In vitro* antimicrobial activity

2.4.1. Standard susceptibility testing

The Minimum Inhibitory Concentrations (MICs) of antibiotics against bacterial isolates was determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). Antifungal activity was assessed in essentially the same way, though used LB in place of MHB, and MICs were read after 48 hours' incubation at 35°C. Media was supplemented with 4 µg/ml polymyxin B nonapeptide (PMBN) to test the role of membrane permeability on antibiotic delivery against Gram-negative bacteria (Vaara 1992).

Following incubation, the MIC was defined as the lowest concentration of antibiotic that inhibited all visible growth. MIC determination with appropriate comparator antibiotics was undertaken to permit comparison of the antibacterial activity of the investigated natural products with existing clinical agents against the tester strains. Positive control (growth control) and solvent control were included in each experiment. Susceptibility testing was conducted on a minimum of three independent occasions to ensure reproducibility.

2.4.2. Time –dependent killing (time-kill) studies

Studies to determine the bactericidal activity were carried out on exponential-phase cultures using a method described elsewhere (Ooi *et al.* 2013). Briefly, bacteria were cultured to early exponential phase and diluted to a final density of 10^5-10^6 CFU/ml in MHB and challenged with antibacterial agents at 4x MIC. Time-kill experiments were also

performed with bacterial cells in non-growing states. Overnight cultures of SH1000 were harvested by centrifugation and cells were re-suspended in the spent supernatant to 10^5-10^6 CFU/ml before exposure to antibacterial agents.

Following antibiotic challenge, bacterial viability was monitored by plating cultures onto MHA, and enumerating colonies after incubation at 37°C for 18–24 h to allow plotting of $\log_{10}$ CFU/ml versus time. Bactericidal activity was defined as a reduction of ≥3 $\log_{10}$ CFU/ml relative to the initial inoculum (Petersen, Jones and Bradford 2007; CLSI 1999). All experiments were performed in three independent cultures and values are expressed as mean ± standard deviation (SD).

2.5. Mode of action studies

2.5.1. Macromolecular synthesis (MMS) assay

MMS assays were performed in 96-well microtiter plates with an assay volume of 200 µl per well. The effect of the investigated compounds and control agents was monitored in early exponential phase cultures (10^8 CFU/mL in LBB) of *S. aureus* and *E. coli* by following the incorporation of radiolabeled precursors into TCA-precipitable material (Greenwood and Gentry 2002). The radioactive precursors [methyl-^{3}H] thymidine (70–95 Ci/mmol), [5, 6-^{3}H] uridine (31–56 Ci/mmol), [3,4-^{3}H(N)] glutamine (20–50 Ci/mmol), [1,2-^{14}C] acetic acid (45-60 mCi/mmol), and ^{14}C (U)-glycine (>80 mCi/mmol) were used to monitor the synthesis of DNA, RNA, protein, fatty acid and peptidoglycan, respectively.

Cultures were grown to early exponential phase (OD_{600} of 0.2) in LB. Culturs were labled by the addition of radiolabel precursors at 1µCi/ml for 10 minutes at 37 °C.. Samples were incubated for 10 minutes, and incorporation was terminated by the addition of (1:1) of

TCA and cooling on ice for 60 minutes to allow for the precipitation of macromolecules (these cultures correspond to experimental time zero). The remainder of each culture was then treated with test compounds at 4x their respective MIC. After 10 min of incubation, 100 µl of the treated culturs was mixed with an equal amount of 10% TCA and kept on ice for 60 min. TCA precipitates were collected on MultiScreen Filter Plates using a MultiScreen Vacuum Manifold (Millipore), washed twice with TCA and acetic acid, dried, and radioactivity counted in a Plate CHAMELEON™V scintillation counter after adding 25 µl of MicroScint 20 scintillation fluid (Perkin Elmer, Waltham, MA) to each well of the filter plates. For each set of experiments, antibiotics known to inhibit all pathways were included. The data were expressed as a percentage of incorporation into a drug-free control as described previously (Hilliard *et al.* 1999). All experiments were performed in at least three independent cultures. Statistical significance of data was detemined by applying a student's t-test using GraphPad Prism virsion 5 (GraphPad Software, USA). Differences were considered significant if *p ≤0.05.

2.5.2. *In vitro* transcription/translation assays

The *E. coli* S30 extract system for circular DNA (Promega) was used according to the manufacturer's instructions with some modification. An optimized quantity of S30 extract was used. Briefly, 8 µL of S30 extract, 8 µL of S30 pre-mix, 2.5 µL of mixture of amino acids, and 1 µg/µL pBESTluc™ DNA were added in a total volume of 25 µL reaction. Antibiotics (1 µL) were pre-incubated with or without pBESTluc™ DNA for 10 minutes in accordance with assay requirements. Reactions were incubated at 37°C for 1 hour, and the level of transcription/translation was quantified by the addition of a saturating

concentration of luciferase assay reagent and subsequent measurement of luminescence using a Flustar Optima plate reader (BMG labtech, Ortenberg, Germany).

2.5.3. Membrane perturbation assays

*2.5.3.1. Assessment of bacterial membrane integrity using Bac*Light™

The LIVE/DEAD *Bac*Light bacterial viability kit was used to assess membrane integrity of bacteria re-suspended in water following exposure to antibacterial agents at 4× MIC for 10 minutes (Hilliard *et al.* 1999). *S. aureus* SH1000 was grown to OD_{600} 0.5-0.6. Volumes of cultures were washed in sterile deionized water and re-suspended to twice the volume. Cells were incubated with 5% SDS or test compounds at 4x MIC for 10 minutes at 37°C with aeration and then washed and re-suspended in water. A volume of 50 µl of the bacterial suspension was added to the corresponding wells of the 96-well plate containing 150 µl of a 1:1 (v/v) mixture of two dyes; SYTO® 9 (green fluorescent) and propidium iodide (PI) (red fluorescent), was diluted 300-fold from a stock solution provided by the manufacturer and mixed into sterile dH_2O in diminished light. The content of each well was mixed thoroughly and incubated in the dark for 15 minutes at room temperature. Fluorescence was then measured in a Multidetection Microplate Reader (*FLUOstar Omega*, BMG LABTECH, and Offenburg, Germany) at an excitation of 485 nm and emission of 520 nm or 620 nm. The ratio of green to red fluorescence was determined and normalized against a drug-free control. Percentage membrane integrity of antibiotic/ inhibitor- treated SH1000 was expressed as the ratio of the green: red relative to cultures treated with 5% SDS. Compounds were considered membrane damaging if they caused >

30% loss of membrane integrity. All experiments were performed in three independent cultures and values were expressed as mean ± standard deviation (SD).

2.5.3.2. Measurement of cytoplasmic membrane potential using $DiSC_3(5)$

The membrane potential of bacterial cells following exposure to antibacterial agents over a 3 hour period at 4 x MIC was monitored using the fluorescent dye $DiSC_3(5)$, as previously described (Higgins *et al.* 2005). Briefly, cultures of *S. aureus* SH1000 were grown to exponential phase (OD_{600} of 0.2), washed twice and re-suspended in 5 mM HEPES/glucose buffer. For membrane potential measurements, cells were incubated with 0.1 M KCl and 2 µM $DiSC_3(5)$ for 30 minutes at 37°C to allow uptake of dye. Subsequently, cultures were exposed to compounds at 4x MIC and samples removed at appropriate time points. Cells were pelleted, and 1 ml of supernatant was mixed with 1 ml of DMSO. The pellet was lysed in DMSO for 10 minutes and was then added to an equal volume of HEPES/glucose buffer. Extracellular and intracellular fluorescence was measured on a LS 45 luminescence spectrometer (ParkenElmer) at an excitation of 622 nm and an emission of 670 nm. Membrane potential was calculated using the Nernst equation

$$\Delta\Psi = -\frac{RT}{F}\ \mathrm{In}\left(\frac{\text{Intracellular DiSC3(5)}}{\text{Extracellular DiSC3(5)}}\right)$$

and was expressed as a percentage of the initial value. $\Delta\Psi$ is the membrane potential in mV, R is the universal gas constant, T is the absolute temperature, and F is the Faraday constant. All experiments were performed in three independent cultures and values are expressed as mean ± standard deviation (SD).

2.5.3.3. Measurement of potassium (K+) leakage

For ionic leakage experiments, cells resuspended in HEPES and glucose buffer were exposed to compounds at 4x MIC at 37°C with aeration for 3 hours. Samples (5 mL) were removed at regular intervals, filtered through a sterile 0.2 µm filter and stored at -20°C until they could be analyzed by flame atomic absorption spectroscopy. The positive controls for potassium leakage experiments were cells that had been boiled for 10 minutes prior to filtration. Potassium standards (0.05, 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5, and 3 µg/mL) were made in HEPES and glucose buffer, and a PerkinElmer A Analyst 100 spectrometer was used to generate standard curves from which ionic contents of samples were calculated (Hobbs *et al.* 2008). All experiments were performed in three independent cultures and values are expressed as mean ± standard deviation (SD).

2.6. Resistance studies

2.6.1. Generation of spontaneous antibiotic resistant mutants and mutation frequencies

S*. aureus* SH1000 and *E. coli* BW25113 were used in the selection for antibiotic-resistant mutants. Bacterial cultures were grown aerobically at 37°C in until they reached the late logarithmic phase of growth (OD_{600nm}~ 1). The cultures were serially diluted in PBS, and aliquots spread onto selective MHA containing the tested antibiotic at 4xMIC, and onto non-selective MHA plates, and both sets of plates incubated at 37°C. Colony counts were made after 24 hours incubation on non-selective media and after 48 hours on selective plates. The frequency of mutation was expressed as the number of resistant mutants

recovered as a fraction of the viable count (O'Neill, Cove and Chopra 2001). Resistant mutant MICs was determined and then stored in aliquots in 8% glycerol at -80^{o}C.

2.6.2. Selection of resistance by repeated exposure method

For compounds where mutants were not identified in mutation frequency determinations, attempts were made to select resistant mutants using the extended spectrum MIC method (Friedman, Alder and Silverman 2006). Cultures of *S. aureus* SH1000 were challenged with antibacterial agents using broth MIC methodology with the exception that the dilution series had smaller increments between concentrations. Following 24 hour incubation at 37°C, the MIC was recorded, and cells from the well containing the highest concentration of drug that permitted growth were used as the inoculum for the next MIC. The process was repeated for up to 20 passages, during which cells were continually exposed to selective compounds. Putative resistance was confirmed by standard broth MIC methodology.

2.7. Cytotoxicity assays

2.7.1. *In vitro* human cell cytotoxicity assay

For cytotoxicity testing, HK-2 cells, a human proximal tubular cell line from healthy kidney tissue (ATCC CRL-2190), were obtained from the American Type Culture Collection and maintained in high-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 1% of Penicillin-Streptomycin combo solution and 10% fetal bovine serum. Cells were cultured at 37°C in 5% CO_2 saturated air. Culture media were replaced every two

days. Cells were passaged when they reached 80% confluency. HK-2 cells were seeded in 96-well flat bottom plates at a density of 1.2 x 10^4 cells/well with 200 µl of DMEM medium without serum (37°C, 5% CO_2 atmosphere). After 24 hours of culturing, cells were exposed to antibiotics for 6 hours. The cytotoxicity effects of antibiotics on HK-2 cells were evaluated by measuring the release of lactate dehydrogenase (LDH) enzyme and determination of the adenosine 5' triphosphate (ATP) released from a suspension of viable somatic cells. Using the CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega, USA) and bioluminescent somatic cell assay kit (Sigma-Aldrich, UK), activity was measured according to the manufacturer's protocol.

2.7.2. *In vivo Galleria mellonella* killing assay

Wax moth larvae (*G. mellonella*) were purchased from Livefood UJ Ltd (Rooks Bridge, Somerset, UK) and were maintained on wood chips in the dark at 14°C. They were stored for no longer than two weeks. Bacterial infection of *G. mellonella* is as essentially described elsewhere (Wand *et al.* 2011), with antibiotic treatment of *G. mellonella* was as described by Peleg (Peleg *et al.* 2009). Concentrations of antibiotic injected into *Galleria* were based on the average weight and volume calculations, and took into account the increase in volume caused by bacterial and antibiotic injections. Data were analyzed using the Mantel-Cox method using Prism Software Version 6 (GraphPad, San Diego, CA, USA). All experiments and data analysis were performed by Matthew Wand and Charlotte Hind (Public Health England, Salisbury, UK).

2.8. Molecular genetic analysis

2.8.1. Preparation of genomic DNA

Genomic DNA was prepared from *E. coli* BW25113 and *S. aureus* SH1000 using the PurElute™ bacterial genomic kit (EdgeBio, Gaithersburg, MD, USA) in accordance with manufacturer's instructions, with the exception that for *S. aureus*, following resuspension in spheroplast buffer, cells were exposed to lysostaphin (40 µg/ml) and incubated at 37°C for 1 hr, before continuing with the manufacturer's protocol. Purified DNA samples were resuspended in 100 µl of TE buffer. The DNA purity and concentration was determined using a NanoPhotometer™ Pearl nano spectrophotometer (Implen GmbH, Munich, Germany).

2.8.2. Amplification of DNA by polymerase chain reaction (PCR)

Oligonucleotides primers for PCR were designed using Oligo software (Molecular Biology Insight Inc., West Cascade, Colorado, USA) and ordered from Eurofins MWG operon (Ebersberg, Germany). PCR reactions were carried out in a Techne Thermal Cycler (TC-3000) (Bibby Scientific Ltd, Staffordshire, UK) using Phusion® High-Fidelity PCR Kit, according to the manufacturer's protocol. Thermal cycling conditions consisted of initial denaturation at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing for 30 seconds, and extension at 72°C for 30 seconds/kb. A final extension cycle at 72°C for 7 minutes and reactions then held at 4°C until required. The annealing temperature was optimized for each reaction based on the primer's melting temperature using Phusion Tm calculator. Approximate DNA concentrations were

determined from measurements of absorbance at $OD_{260\ nm}$ using NanoPhotometer™Pearl (Implen GmbH, Munich, Germany). The resultant amplicons were visualized using agarose gel electrophoresis (section 2.8.3), purified and their DNA sequence determined.

2.8.3. Agarose gel electrophoresis and DNA purification

Agarose gel electrophoresis was performed in a Bio-Rad electrophoresis cell (Bio-Rad, Hemel Hempstead, UK) to determine the presence and size of PCR products alongside a 1 kb molecular weight ladder marker (Promega). Gels contained 0.8% agarose (Geneflow LTD, Fradley, UK) in TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0, and were stained with SYBR safe stain (Molecular Probes, Invitrogen; Paisley, UK). DNA samples containing 1/5 volume 5x loading buffer were run at 90 volts for 30 minutes. The gel images were captured by the Gene-Genius Bio-Imaging-System (Syngene, Frederick, MD) using the GeneSnap Program (Syngene). DNA fragments required for further applications were extracted from gels and purified using MinElute gel extraction kit from Qiagen according to the manufacturer's protocol.

2.8.4. Sanger sequencing

The DNA sequence of purified PCR fragments was determined by Beckman Coulter Genomics (Beckman Coulter, Inc, Buckinghamshire, UK) using appropriate oligonucleotide primers. Sequence data were aligned with reference sequence using Sequencher 4.8 software (Gene Codes, Michigan, USA).

2.8.5. Next generation DNA sequence determination

Samples of total chromosomal DNA from two spontaneous thiolutin resistant mutants (ThioR1 and ThioR2) were sent to the Leeds Clinical Molecular Genetics Centre (St, James' Hospital, University of Leeds) and nucleotide sequences determined using the Illumina sequencing-by-synthesis method (2x250 paired-end reactions) on a MiSeq platform. Mutations within the chromosome of the resistant mutants were identified using probability based variant detection software in CLC genomics workbench, version 6 (CLC Bio, Cambridge, Massachusetts, USA), using the nucleotide sequence of *S. aureus* SH1000 as the reference.

Chapter three

Biological characterization of γ-actinorhodin

3.1. Abstract

The dimictic BIQ, actinorhodin, was not pursued as an antibacterial drug candidate at the time of its discovery and remains poorly characterized. Work reported in this chapter was undertaken to evaluate comprehensively the potential of actinorhodin as antibacterial drug candidate. Here, γ-actinorhodin, a member of the actinorhodin class was studied. A novel method was developed to isolate and purify γ-actinorhodin and the antibacterial properties, mode of action, and the potential of γ-actinorhodin, as an antistaphylococcal agent were evaluated. γ-actinorhodin demonstrated potent anti-Gram-positive activity with an MIC_{90} of 2 µg/ml against methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA), but lacked activity against Gram-negative pathogens. γ-actinorhodin at a concentration of 4x MIC was bactericidal and caused a rapid decrease in the cellular incorporation of RNA, DNA, and protein precursors in whole cells, as a consequence of perturbation of the cytoplasmic membrane. Evidence was also obtained that γ-actinorhodin stimulates the production of ROS, which appears to contribute to the compound's antibacterial effect. γ-actinorhodin was found to exhibit selective toxicity; it lacked activity against yeast and displayed a high selective index in studies using mammalian cells. γ-actinorhodin did not cause overt toxicity in an animal model (*Galleria*

melonella), and demonstrated modest degree of protection following administration to animals infected with *S. aureus* USA300. With the low resistance potential, in addition to the evidence for *in vivo* efficacy, γ-actinorhodin could potentially be useful for treating drug-resistant strains like MRSA and VISA and this compound warrant further investigation as candidate antistaphylococcal agent.

3.2. Introduction

The benzoisochromanequinones (BIQs)- also referred to in the literature as pyranonaphthoquinones or isochromanequinones-, represent a large class of aromatic polyketide natural products mainly isolated from bacteria and fungi (Brimble, Duncalf and Nairn 1999; Donner 2013). The common structural framework of these molecules consists of a *bis*-tricyclic system incorporating pyran, quinone, and benzene rings in a core scaffold (Figure 3.1). Their carbon skeletons are biosynthesized by polyketide synthases (PKS), ketoreductases, aromatases and cyclases through sequential biosynthetic reactions. Subsequently, these carbon skeletons undergo structural modifications steps including oxygenation, dimerization, glycosylation, and methylation resulting in a substantial diversity in the structure and biological activity for members of this class (Moore and Hertweck 2002).

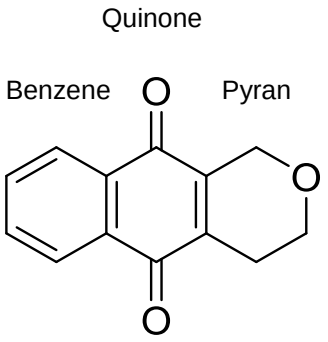


Figure 3.1. The basic structure of the BIQ natural products

Many members of the BIQ class have been found to exhibit a variety of pharmacological activities such as antibacterial, antifungal, antimalarial, antiviral, and anticancer properties (Brimble, Duncalf and Nairn 1999). However, to date, none of these compounds has advanced to clinical application.

3.3. The actinorhodin family

Actinorhodin is a dimeric BIQ that was first described by Brockmann and Pini in the late 1940s (Brockmann and Pini 1947). Actinorhodin isolation was followed by the detection of closely related symmetrical and asymmetrical congeners (α, ß, γ, δ, and ε-actinorhodins) (Bystrykh *et al.* 1996), all isolated from *Streptomyces* (Figure 3.2). Because of the symmetrical structure of actinorhodin in which two BIQ units are coupled via a C–C bond, its regulation, and biosynthesis has drawn considerable attention (Taguchi *et al.* 2015; Gorstallman *et al.* 1981; Rudd and Hopwood 1979) and its characteristic blue pigment has been widely employed as a phenotypic marker for the functional replacement in pathway-specific regulation in streptomycete research (Khosla, Ebertkhosla and Hopwood 1992; Khosla *et al.* 1993; Sherman *et al.* 1992).

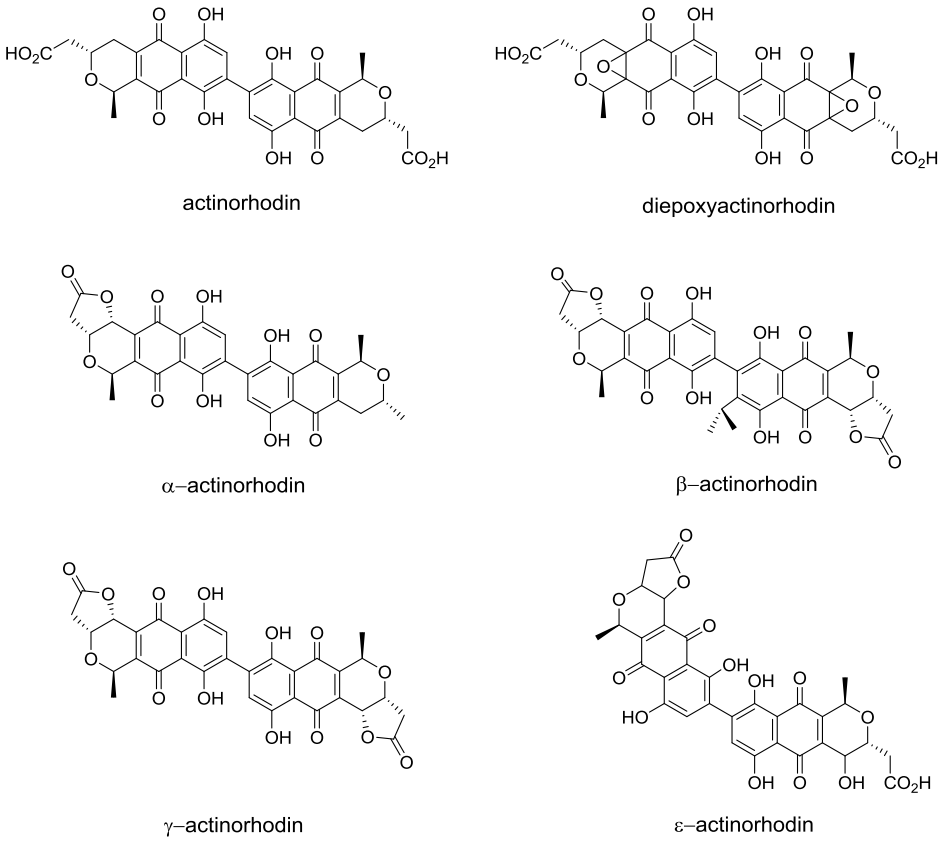


Figure 3.2. Actinorhodin and structurally related pigments produced by various strains of Streptomyces**.**

It was not until the late 1970s when the inhibitory effect of actinorhodin at high concentrations against some Gram-positive bacteria including *S. aureus* was recognized (Wright and Hopwood 1976). The reported weak activity of actinorhodin (estimated MIC of 25-30 µg/ml against *S. aureus*) in contrast with antibiotics in use may be responsible for the termination of interest of this novel compound as an antibiotic candidate at the time. Apart from this reported antibacterial activity, its antibacterial properties have not been followed up, and its mode of antibacterial action remains unknown. A recent study proposed that actinorhodin acts as an organocatalyst, catalyzing the conversion of L-ascorbic acid and L-cysteine with the concomitant production of hydrogen peroxide (H_2O_2), and suggested that it might kill bacteria by catalyzing the production of toxic levels of H_2O_2 (Nishiyama *et al.* 2014). However, further studies will be required to confirm this idea.

3.4. Aims and objectives

Here we revisited the BIQ scaffold to comprehensively evaluate its potential to form a basis for a new antibacterial class. For this purpose, the purification and full characterization of a single, active extracellular form of actinorhodin were attempted in close cooperation with chemists. Using the existing structural information of the actinorhodin family, a simplified method for purification was developed. The spectrum of activity of the pure compound was assessed, with a particular emphasis on its anti-staphylococcal activity against a number of clinical isolates. Killing kinetics were also determined, and the mechanism of its antibacterial activity and any detrimental effects of the compound on eukaryotic cells was investigated. Finally, to assess the potential for the compound to be used clinically, the rate of resistance development was also explored. To

determine the bacterial specificity of γ-actinorhodin using *C. albicans* as a model eukaryote, followed by evaluating the activity against human cell line and *in vivo* infection model.

3.5. Results and discussion

3.5.1. Isolation and purification of γ-actinorhodin

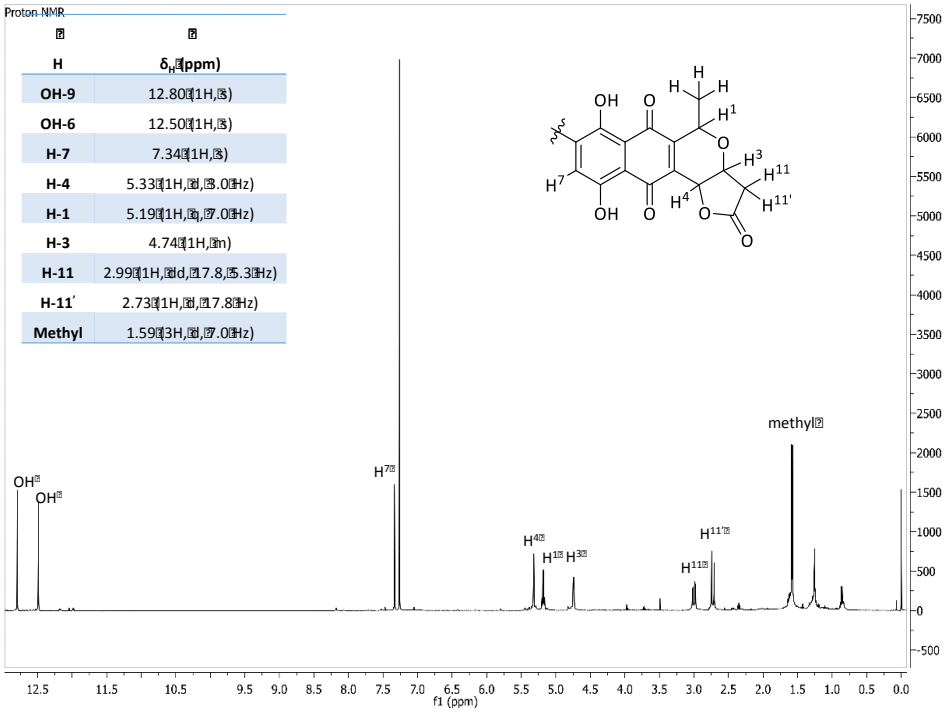
We attempted to isolate and purify a single active species of actinorhodin from *S. coelicolor* to explore the biological properties of this family of antibiotics. *S. coelicolor* L646 strain was used for this purpose. This strain contains an integrated plasmid that overexpresses wild-type *atrA,* leading to hyperproduction of actinorhodins relative to the wild-type strain (M145) (Uguru *et al.* 2005). In initial studies, several different media (YM broth, ISP2 agar, and R5 broth/agar) were tested to identify the most suitable for the growth of *S. coelicolor* L646 and the production of the blue pigments. ISP2 agar was found to best support the growth of *S. coelicolor* L646 and the production of diffusible blue pigments (Figure 3.3), and was therefore used in subsequent studies.

Figure 3.3. *Streptomyces coelicolor* L646 colonies on ISP2 agar medium. After 4-6 days at 30°, dark blue diffusing pigment developed around the colonies and blue droplets appeared on the colony surface.

Experiments to isolate actinorhodin following published methods (Bystrykh *et al.* 1996; Wright and Hopwood 1976) was unsuccessful. Poor solubility and the lack of antibacterial activity were the major issues experienced during purification. According to the structure of actinorhodin, the dicarboxylic acid moiety should render the molecule polar (Sannia Farooq, personal communication). However, when the blue pigment was extracted from agar with water followed by acidification and extraction with ethyl acetate, actinorhodin could not be detected by LC-MS and NMR. Additionally, no antibacterial activity of the crude ethyl acetate extract was noticed. Mass-directed and UV-directed fractionation by HPLC under several conditions was attempted, though proved unsuccessful. Each condition tested to purify actinorhodin revealed that the extract is more complex than initially thought, judging from the number of bands observed on TLC chromatograms (data not shown), indicating several pigmented compounds with actinorhodin-like characteristics (e.g. pH-responsive colour changes). After more than a year of trial and error to isolate actinorhodin, we came to the conclusion that the HPLC approach for purification of actinorhodin was not suitable. I failed to attain material with sufficient purity to perform chemical characterization and biological evaluation.

Hence, we pursued a collaboration with a skilled chemist specialized in natural products to develop a method for actinorhodin purification. Since HPLC failed to provide a pure compound, it was suggested to take advantage of the pigmentation of the compound and attempt to separate a single form using TLC. When the crude ethyl acetate extract was chromatographed on TLC [solvent: Dichloromethane-Methanol 9:1 (v/v)], a major, well-separated purple band with R_f 0.29 was collected and found to be γ- actinorhodin by LC-MS and ^{1}H NMR (Figure 3.4). This presumptive γ-actinorhodin showed good activity against *S. aureus* SH1000, with MIC of 1 µg/ml. To generate sufficient amount of material for biological evaluation, a modification of the extraction method to target the isolation

A



B

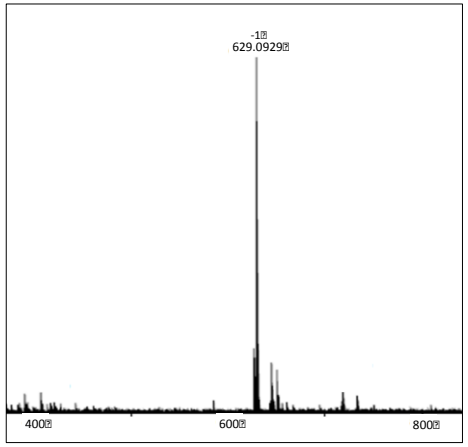


Figure 3.4. Initial identification of γ-actinorhodin by (A) ^{1}H NMR and (B) mass spectrometry (MS)

of γ-actinorhodin was established. γ-actinorhodin is significantly less polar than actinorhodin due to the presence of lactone substituent (Sannia Farooq, personal communication). It was expected that extracting the blue pigment directly from the agar using an organic solvent of moderate polarity (such as ethyl acetate) would recover γ-actinorhodin, while more polar contamination would remain associated with agar. ^{1}H NMR analysis of the crude ethyl acetate extract confirmed the presence of γ-actinorhodin (*ca*. 20%) alongside unknown impurities.

Subsequent purification was facilitated by exploiting the key functional group (lactone ring). It was expected that at high pH (>10), γ-actinorhodin would ring-open to afford hydroxycarboxylate (the salt of actinorhodinic acid), which would have high water solubility (Sannia Farooq, personal communication). Hence, the crude extract then washed with aqueous sodium carbonate (pH 10.9) and resulted in a blue aqueous solution. This was then acidified with hydrochloric acid (6 M) to reform the lactone (Figure 3.5), and to neutralize any salts formed. The red precipitate was then washed with methanol to afford γ-actinorhodin as red solid in 19% yield.

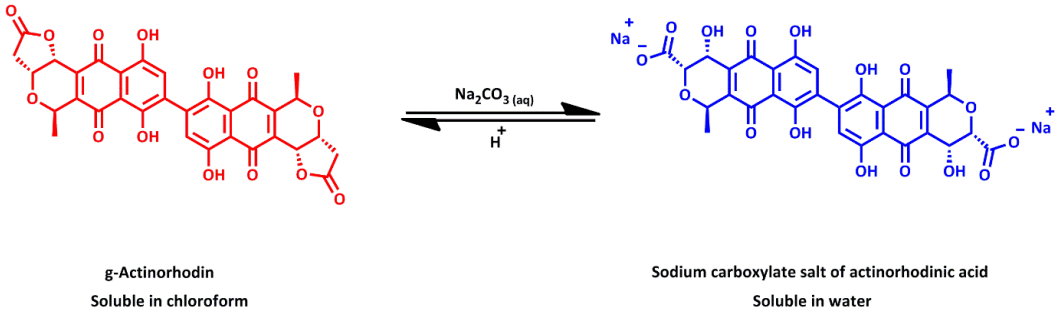


Figure 3.5. Proposed acid-base catalyzed interconversion of the cyclized and ring-opened form of γ-actinorhodin

1-D and 2-D NMR spectroscopy were utilized to confirm the structure of γ-actinorhodin. All NMR experiments, interpretation of signals, and purity determination were performed

by Sannia Farooq (School of Chemistry, University of Leeds). The identity and purity (95%) of γ-actinorhodin was assessed using mass spectrometry, 1-D NMR, 2-D NMR and infrared spectroscopy and analytical HPLC (Appendix A1-A8).

3.5.2. Antibacterial activity of γ-actinorhodin

γ-actinorhodin MICs were determined for a number of clinically important Gram-positive and Gram-negative bacteria using standard CLSI broth microdilution methods. γ-actinorhodin exhibited potent antibacterial activity against Gram-positive pathogens, exhibiting MIC values of 1-2 µg/ml. However, it lacked useful antibacterial activity against Gram-negative pathogens, with MICs of >256 µg/ml against representative Enterobacteriaceae and non-fermentative bacilli (Figure 3.6, A).

The antistaphylococcal activity of γ-actinorhodin was further investigated against a panel of clinical *S. aureus* isolates (70 isolates) including MRSA and VISA strains. The *in vitro* susceptibility results for all the strains tested are in Appendix B. The MIC_{90} of γ-actinorhodin was 2 µg/ml, with MIC of γ-actinorhodin ranging from 1-4 µg/ml (Figure 3.6, B). γ-actinorhodin therefore possess as potent antibacterial activity that includes two of the ESKAPE pathogens (*S. aureus* and *E. faecium*), with MIC values against members of these species, falling within the comparable range for systemically administrated antibacterial drugs in current clinical use. The MIC values for the clinically useful antibiotics, rifampicin, and fusidic acid for systemic use is often <1 µg/ml against *S. aureus.* Whereas, vancomycin and linezolid, extremely useful antibacterial agents, display higher MIC values (e.g. values of 4 µg/ml) against susceptible *S. aureus* strains (O'Neill and Chopra 2004).

A

Bacterial strains	MIC (µg/ml)
S. aureus SH1000	1
S. epidermidis ATCC 14490	1
S. haemolyticus 41207	1
S. pyogenes ATCC 19615	1
S. pneumoniae ATCC R6	1
E. faecalis ATCC 29212	2
E. faecium 7634337	1
E. coli BW25113	>256
K. pneumoniae K25	>256
A. baumannii ATCC 19606	>256
P. aeruginosa PO1	>256

B

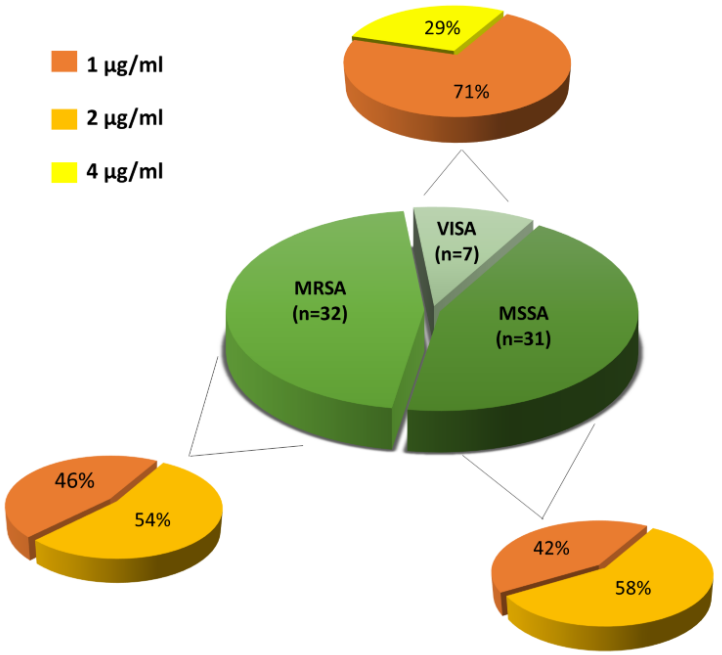


Figure 3.6. *In vitro* antibacterial activity of γ-actinorhodin**.** (A) MICs of γ-ACT against a panel of Gram-positive and Gram-negative bacteria (B) Distribution of *S. aureus* clinical isolates and MICs for γ-actinorhodin in MSSA, MRSA, and VISA clinical isolates (n = 70). All MICs were determined by broth microdilution method according to CLSI.

The antistaphylococcal activity of γ-actinorhodin shown here is significantly greater than that previously published for actinorhodin. The original study that reported on the antibacterial activity of this class estimated an antistaphylococcal MIC of 25-30 μg/ml by agar diffusion (Wright and Hopwood 1976). Several explanations could account for this apparent discrepancy. In the original study, the MIC value was estimated using agar plug diffusion method which involves growing the antibiotic producer strain on its appropriate culture medium, and then an agar-plot is cut and placed on the surface of another agar plate and then overlaid with the test microorganism (Wright and Hopwood 1976). In this study, however, γ-actinorhodin appeared to have significantly reduced activity against *S.aureus* SH1000 (MIC of 16 μg/ml) when incorporated into agar medium. Therefore, it is likely that the choice of culture media is responsible for the significant difference in the MICs obtained. However, it is also feasible that different actinorhodin analogs may demonstrate various levels of antibacterial activity. The weak activity originally reported for actinorhodin could be the reason why this class has not been further evaluated in respect of its therapeutic potential over the last 40 years. This is interesting, considering that other potentially interesting/ useful antibiotic classes might be overlooked due to similar points of technicality.

Insusceptibility to antibiotics in Gram-negative bacteria often results from the permeability barrier offered by the outer membrane, or the action of the broad substrate range efflux pumps, such as AcrAB-TolC (Poole 2002). It has been demonstrated previously in our laboratory that most antistaphylococcal agents are active against Gram-negative bacteria when allowed to reach their target (Randall *et al.* 2013a). To explore the basis for the poor activity of γ-actinorhodin against Gram-negative bacteria, γ-actinorhodin susceptibility determinations were conducted against *E. coli* BW25113 in the presence of an outer-membrane permeabilising agent (PMBN), and against derivatives of BW25113 deleted for components of the major efflux transporter, AcrAB-TolC. γ-

actinorhodin appeared more active against *E.coli* in the presence of PMBN (MIC of 16 μg/ml), while no change in the activity against *E.coli* strains lacking AcrAB or TolC (MICs of >256 μg/ml). At sub-inhibitory concentrations, PMBN, known to cause disruption and/or disorganization of the outer membrane, and enhanced antibiotic penetration (Sahalan and Dixon 2008). The results suggest that the limited activity of γ-actinorhodin against Gram-negative bacteria is a result of limited ingress across the outer membrane to reach its target site.

3.5.3. Evaluation of bacterial killing by γ-actinorhodin

To characterize the antibacterial activity of γ-actinorhodin further, killing kinetics were evaluated beside established antibiotics. The addition of vancomycin (at 4x MIC) to early-exponential-phase cultures of *S. aureus* SH1000 caused a reduction in cell viability of approximately 3 $\log_{10}$ CFU/ml after 6 hours (bactericidal), while tetracycline showed <1 $\log_{10}$ drop in cell viability over 24 hours (bacteriostatic) (Figure 3.7). γ-actinorhodin (at 4x MIC) demonstrated modest killing activity, causing approximately 1 $\log_{10}$ drop in cell viability over 6 hours and 3.3 $\log_{10}$ drop at 24 hours, indicating that it is bactericidal (Figure 3.7).

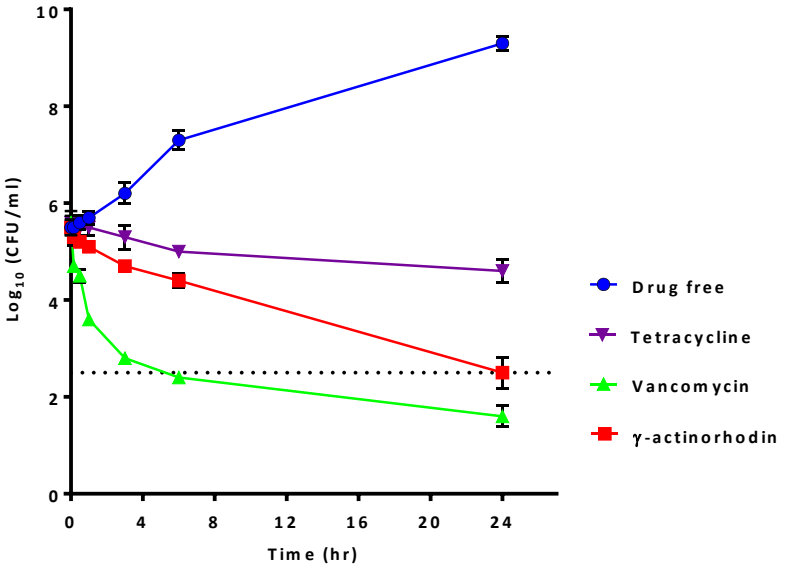


Figure 3.7. Evaluation of killing action of γ-actinorhodin and comparator agents at 4x MIC on exponential cultures of *S. aureus* SH1000 over 24 hours in MHB. The dotted line represents 3 log reductions. Values shown are the means of at least three independent experiments, each of which had two technical replicates. Error bars represent standard deviations from the mean.

During infection, bacteria often encounter unfavorable conditions that lead to periods of limited growth in which the organisms enter a quiescent state and persist within the host (Kolter, Siegele and Tormo 1993). Slow- or non-growing bacteria often display tolerance to antibiotics that are active against rapidly dividing cells, and this contributes to the prolonged treatment periods required to resolve persistent infections (Coates *et al.* 2002). Consequently, antibacterial drugs that retain bactericidal activity under growth-arrested conditions may have clinical advantages over those that do not display such activities. To determine whether γ-actinorhodin possesses this characteristic, stationary phase cultures of *S. aureus* SH1000 were recovered after 24 hours' growth, and the bacteria recovered by centrifugation and resuspended at 10^5 CFU/mL in the spent growth medium from these cultures. Similar to vancomycin, γ-actinorhodin bactericidal activity was abolished against stationary phase *S. aureus* SH1000 at 4x MIC (Figure 3.8).

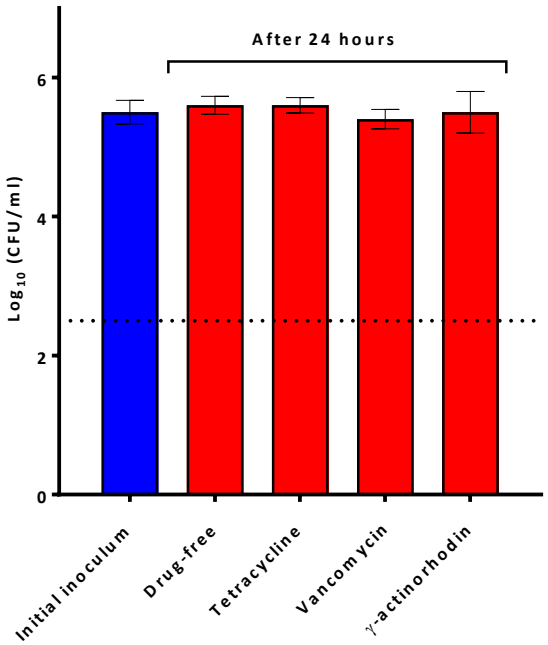


Figure 3.8. Evaluation of killing action of γ-actinorhodin and comparator agents at 4x MIC on stationary-phase cultures of *S. aureus* SH1000 after 24 hours in nutrient-depleted MHB. The dotted line represents 3 log reductions. Values shown are the means of three independent experiments, each of which had two technical replicates. Error bars represent standard deviations from the mean.

3.5.4. *In vitro* selective toxicity of γ-actinorhodin

To provide a preliminary assessment of selective prokaryotic toxicity, the MIC of γ-actinorhodin was determined against the eukaryotic microorganism, *C. albicans*. No activity was observed, even at the highest concentration tested (256 µg/ml) (Figure 3.9, A). Although some BIQs has been reported to possess antifungal activity (Brimble, Duncalf and Nairn 1999), this was not the case for γ-actinorhodin, suggesting that γ-actinorhodin exhibits prokaryotic selectivity in its action.

To further explore γ-actinorhodin's selectivity, the effect of γ-actinorhodin on human kidney 2 (HK-2) cells was evaluated by monitoring the release of lactate dehydrogenase (LDH) as an indicator of membrane integrity loss and the intracellular ATP levels as an indicator of cell viability. No LDH release was detected at any concentration tested (EC_{50} of >256 µg/ml) (Figure 3.9, B). However, γ-actinorhodin caused a reduction in ATP content

at the higher concentrations tested (EC_{50} of ~128 µg/ml) (Figure 3.9 C). Thus, whilst some cytotoxic effects were observed for γ-actinorhodin, these occurred at concentrations >50-fold higher than those required to achieve complete inhibition of bacterial growth, indicating that γ-actinorhodin does indeed exhibit prokaryotic selectivity.

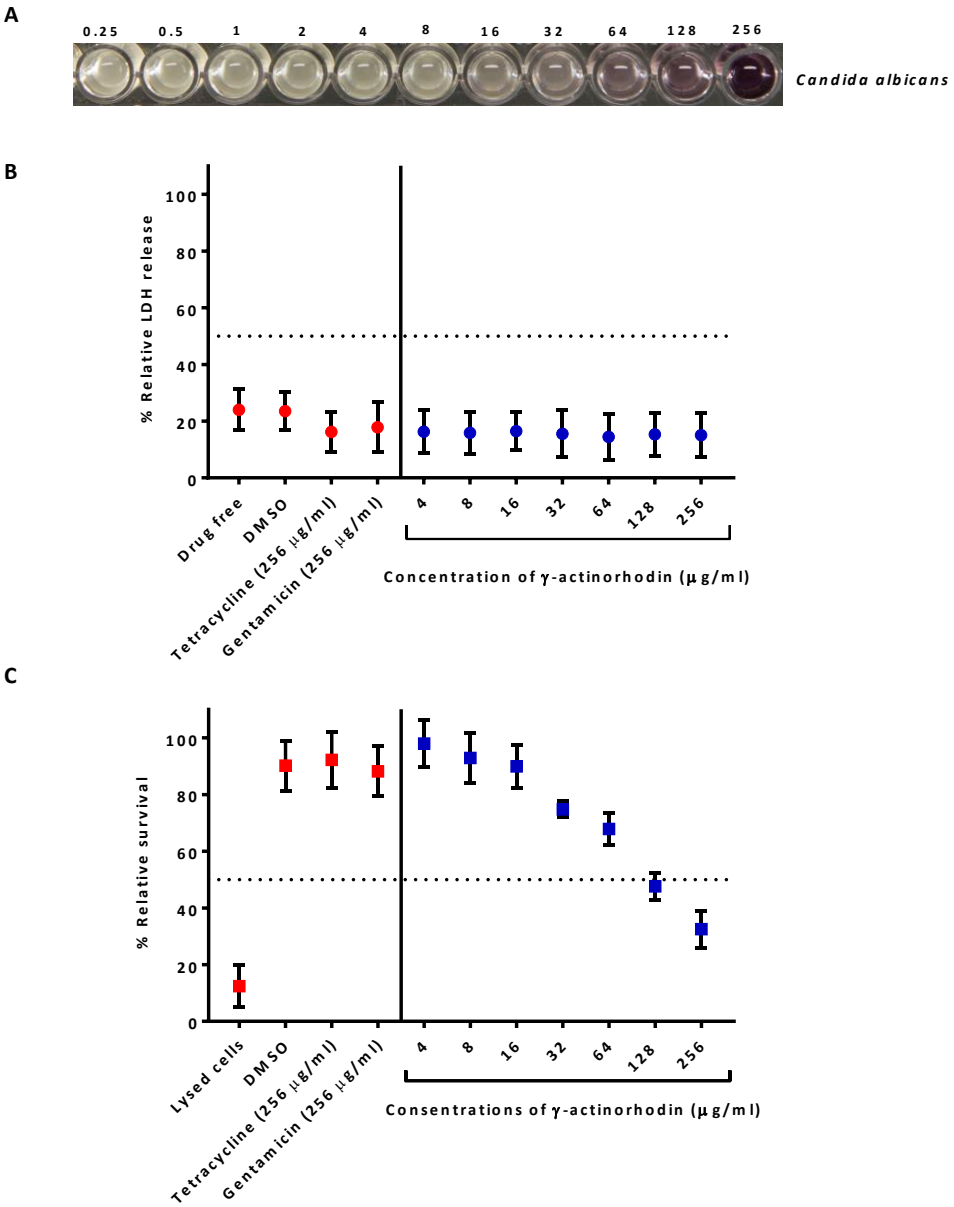


Figure 3.9. Selectivity and in vitro cytotoxicity of γ-actinorhodin**.** (A) The effect of γ-actinorhodin against *C. albicans*. The *in vitro* effect of γ-actinorhodin and control antibiotics tetracycline and gentamicin on the membrane integrity of the human kidney proximal tubular (HK-2) cells as determined by (B) the release of lactate dehydrogenase (LDH) and (C) the cellular ATP levels.

3.5.5. Investigation into the antibacterial mechanism of action of γ-actinorhodin

3.5.5.1. Effect of γ-actinorhodin on macromolecular biosynthesis

Monitoring the ability of an antibacterial compound to inhibit biosynthesis of cellular macromolecules can provide insight into its mode of action (O'Neill and Chopra 2004). Antibacterial agents in clinical use commonly exert their activity through inhibition of specific macromolecular biosynthesis pathways (O'Neill and Chopra 2004). To evaluate the effects of γ-actinorhodin on macromolecular biosynthesis, incorporation of radiolabeled precursors into macromolecules was compared with inhibitors known to specifically inhibit DNA, RNA, protein, fatty acid, and cell wall biosynthetic pathways. At 4x MIC γ-actinorhodin caused substantial and comparable inhibition of all five biosynthetic pathways in 10 minutes, with no evidence for preferential inhibition of a single biosynthetic process (Figure 3.10). This non-specific inhibition of all macromolecular biosynthetic pathways is indicative of disruption to energy producing metabolic pathways, and is a characteristic profile observed for compounds that perturb the bacterial membrane (O'Neill *et al.* 2004; Hurdle *et al.* 2011; Hobbs *et al.* 2008; Oliva *et al.* 2004).

Membrane perturbation can be considered an undesirable characteristic in potential chemotherapeutic candidates, as compounds that damage the bacterial membrane frequently also disrupt the structure of mammalian plasma membranes (Payne *et al.* 2007). Since it was demonstrated that γ-actinorhodin is not compromising the integrity of mammalian cell (Figure 3.9, B), therefore, it was predicted that the non-specific activity observed in MMS experiments is bacteria-specific.

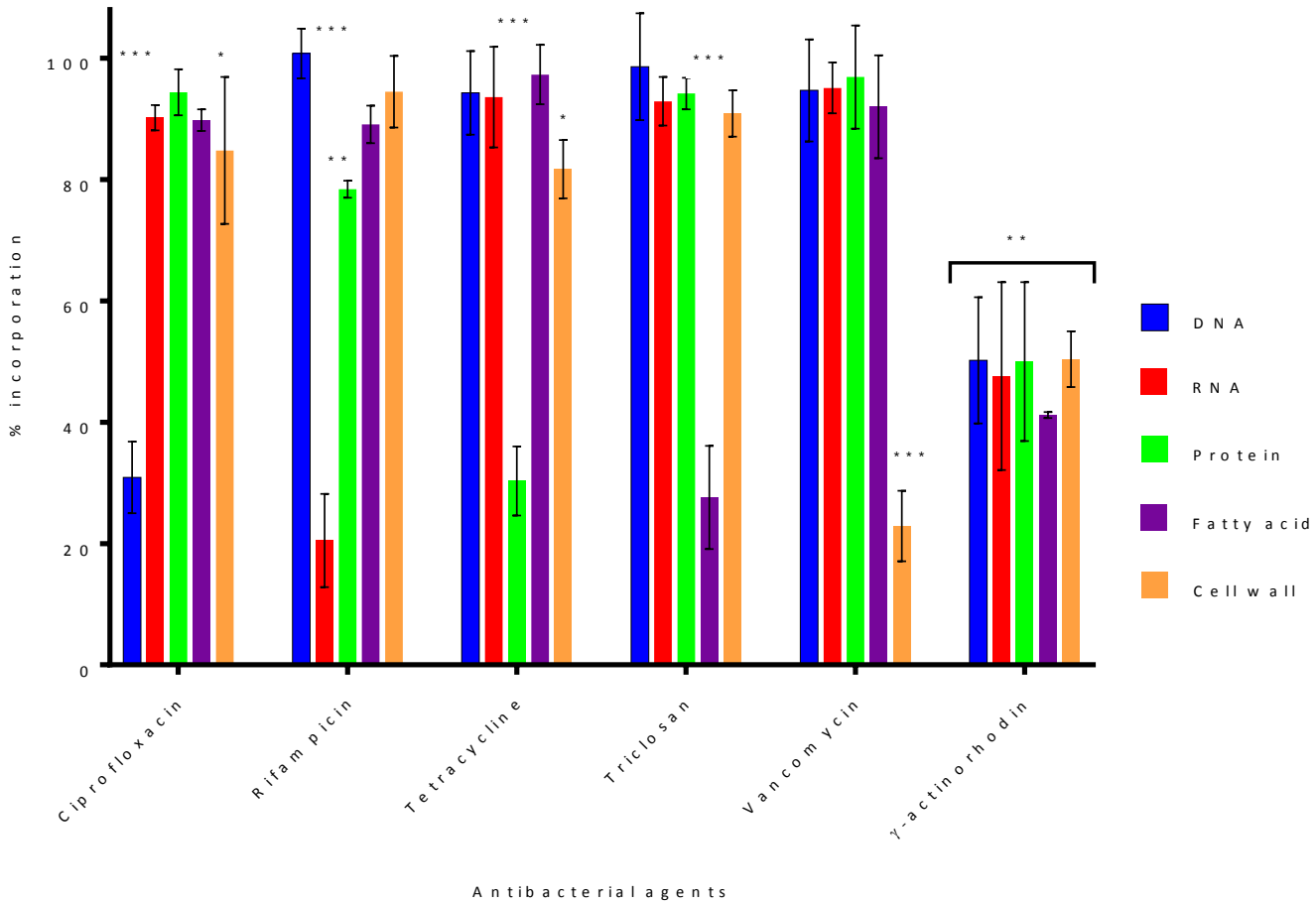


Figure 3.10: Effect of γ-actinorhodin and comparator agents on DNA, RNA, protein, fatty acid, and cell wall biosynthesis in *S. aureus* SH1000**,** as measured by incorporation of radiolabeled precursors**.** Values represent the percentage incorporation relative to drug-free controls. Values shown are the means of three independent experiments, each of which had two technical replicates. Error bars represent standard deviations from the mean. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to untreated control).

3.5.5.2. Activity of γ-actinorhodin on the S. aureus membrane

The non-specific inhibition of macromolecular biosynthetic pathways are characteristic of compounds that mediate their antibacterial effects through action on the cytoplasmic membrane (Ooi *et al.* 2010; O'Neill *et al.* 2004). Therefore, it was sought to examine more directly whether γ-actinorhodin exerts effects on the membrane of *S. aureus* SH1000. Using the potentiometric probe molecule $DiSC_3$ (5), γ-actinorhodin was shown to cause a substantial (>60%) loss of membrane potential in 10 minutes, an effect comparable to that observed for the known membrane-active agents, nisin and CTAB (Figure 3.11 A). In order to determine whether membrane perturbation following exposure to γ-actinorhodin was extensive enough to allow leakage of intracellular ions, leakage of potassium ions was measured. In contrast to control agents, however, γ-actinorhodin appears to exert relatively subtle effects on the membrane that are insufficient to permit leakage of intracellular ions from the cell (Figure 3.11 B). Several antibacterial agents in use as drugs (daptomycin, telavancin), antiseptics (CTAB) or food preservatives (nisin) possess a mode of action that involves membrane depolarization. However, in contrast to γ-actinorhodin, these agents additionally cause gross physical perturbation of the membrane, leading to detectable leakage of intracellular contents (O'Neill *et al.* 2004). Thus, the action of γ-actinorhodin on the bacterial membrane is apparently distinct from that of other membrane-perturbing agents in use.

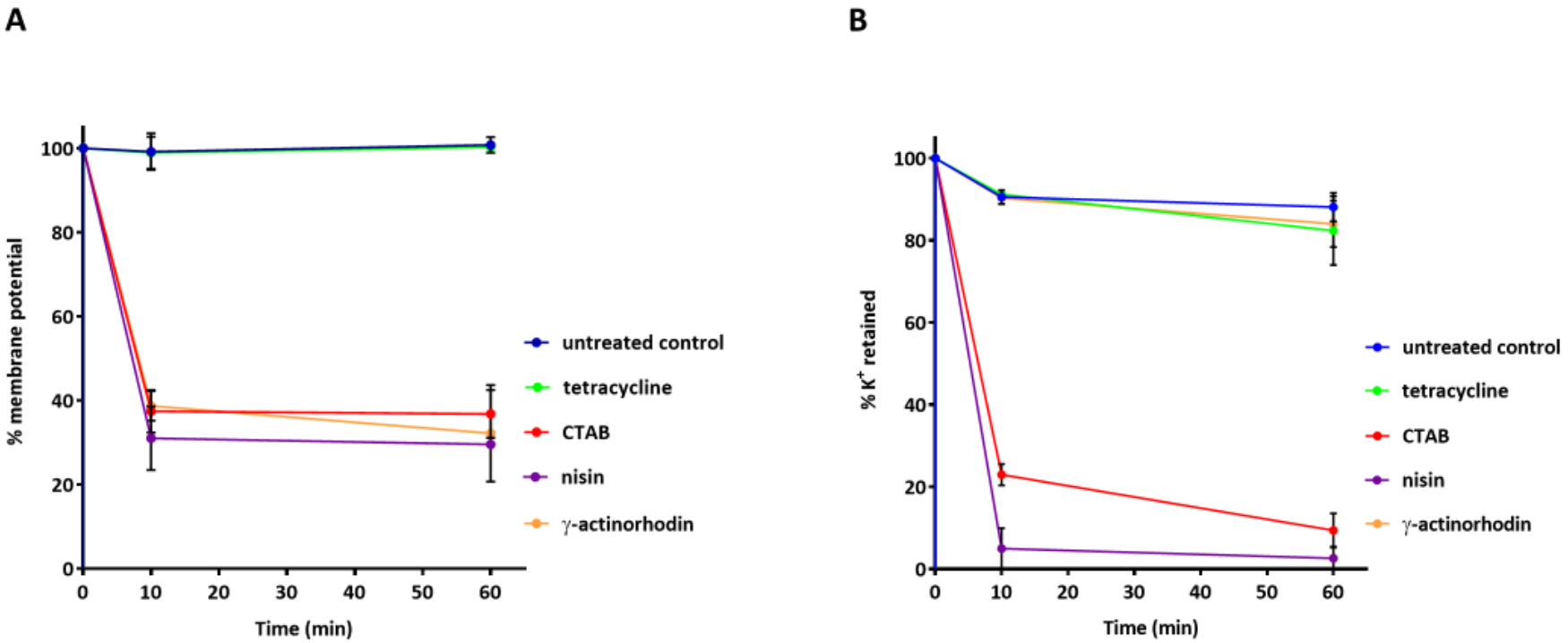


Figure 3.11. Effect of γ-actinorhodin and comparator agents on (a) membrane potential of *S. aureus* SH1000 as measured by $DiSC_3$ (5) and (b) leakage of K^+ ions. Values shown are the means of three independent experiments, each of which had two technical replicates. Error bars represent standard deviations from the mean.

The introduction of daptomycin and telavancin into clinical use has regenerated interest in developing compounds with membrane-based mode of actions (Higgins *et al.* 2005). Agents which perturb membrane structure, or damage the function of multiple membrane-bound respiratory enzymes, have been suggested to be particularly efficacious against persistent infections harboring dormant or slow-growing bacterial cells. In addition, membrane-damaging agents have a very low potential for resistance development, which makes them attractive chemotherapeutic candidates, provided that they also display acceptable bacterial specificity and low toxicity (Hurdle *et al.* 2011).

3.5.5.3. Effect of γ-actinorhodin on reactive oxygen species (ROS) generation

It has been proposed that some bactericidal agents act via disturbing membrane-associated electron transport chain, leading to disruption of bacterial metabolism and the respiratory chain which causes the formation of lethal ROS (Kohanski *et al.* 2007). A recent study established that actinorhodin is capable of catalyzing oxidation reactions *in vitro*, an observation that directed the authors to suggest that the antibacterial properties of actinorhodin might result from the production of toxic levels of H_2O_2 (Nishiyama *et al.* 2014). In potential support of this idea, they observed modest restoration of staphylococcal growth in the presence of actinorhodin when cultures were supplemented with catalase (Nishiyama *et al.* 2014).

To explore a potential role for reactive oxygen species (ROS) in the antibacterial mode of action, the effect of γ-actinorhodin on strains of *S. aureus* defective in the major components of the ROS protection response to examine whether they exhibited greater susceptibility to the action of the antibiotic (Table 3.2). No change in the MIC of γ-actinorhodin was observed against an SH1000 derivative (KS100) lacking the major

catalase enzyme, KatA, or against a strain (KC043) completely devoid of catalase activity as a consequence of lacking both KatA and the enzyme alkyl hydroperoxide reductase (AhpC) (Cosgrove *et al.* 2007). However, a substantial (8-fold) increase in susceptibility to γ-actinorhodin was observed for a strain of SH1000 lacking the major superoxide dismutase enzyme, SodA (Table 3.2), and a further 2-fold increase in γ-ACT susceptibility for a strain concurrently lacking the other staphylococcal SOD, SodM (Table 3.2). By contrast, none of these strains exhibited a significant increase in susceptibility (>2-fold) to several comparator antibacterial agents, including those whose MOA involves action on the membrane (Table 3.2).

Table 3.2: effect of γ-actinorhodin against ROS protection defective mutants

	MIC values (μg/ml)						
	γ-act	**Dap**	**CTAB**	**Clof**	**Van**	**Cip**	**Rif**
SH1000 (wild type)	1	1	1	4	1	0.25	0.016
SK100 (*katA::Tn917* (*ery*))	1	1	1	2	1	0.12	0.016
KC034 (*ahpC::tet katA::Tn917* (*ery*))	1	1	1	0.5	1	0.25	0.016
MHKA (*sodA::Tn917*)	0.12	1	1	1	1	0.12	0.016
MHKM (*sodM::tet*)	1	1	1	1	1	0.12	0.016
MHKAM(*sodA::Tn917 sodM::tet*)	0.06	0.5	1	0.5	1	0.12	0.007

γ-act= γ-actinorhodin; Dap= daptomycin; CTAB= cetyltrimethylammonium bromide; Clof= clofazamine; Van= vancomycin; Cip= ciprofloxacin; Rif= rifampicin

The dramatic sensitization to γ-actinorhodin observed in *S. aureus* strains lacking superoxide dismutases, enzymes that constitute the major cellular defense against superoxide, strongly implicates this radical in the mode of action of γ-actinorhodin. Sensitization to γ-actinorhodin was mainly associated with loss of SodA, an enzyme that has been linked specifically with protection against internally-generated oxidative stress in *S. aureus* (Clements, Watson and Foster 1999), implying that γ-actinorhodin prompts the generation of endogenous, rather than exogenous, superoxide.

It seems probable that antibacterial mechanisms observed for γ-actinorhodin (membrane depolarization and ROS production) share a common cause, and indeed both effects could be explained by γ-actinorhodin-mediated interference with the electron transport chain. Based on the findings of this study, we hypothesize that γ-actinorhodin mediates oxidative damage to one or more components of the electron transport chain, which in turn acts both to comprise the bacterium's ability to maintain a polarized membrane and yields a source of free electrons that drive the generation of superoxide.

3.5.6. Attempts to select resistance to γ-actinorhodin

To assess the propensity for γ-actinorhodin to select resistance, saturated cultures of *S. aureus* SH1000 were plated onto MHA agar containing γ-actinorhodin at multiples of the MIC (4x -128x) as determined by broth microdilution method. Confluent growth was observed on agar containing up to 32X MIC γ-actinorhodin, an observation that can be explained by the finding that the antibacterial activity of γ-actinorhodin becomes substantially attenuated upon incorporation into agar. Although a small number of colonies appeared on agar containing 64-128x MIC of γ-actinorhodin after 48 hours' incubation, these colonies retained full susceptibility to γ-actinorhodin upon MIC determination.

Subsequently, we examined whether resistance to γ-actinorhodin could be selected upon an extended passage in the presence of the compound. Serial passage of *S. aureus* SH1000 in the presence of sub-MIC levels of γ-ACT over a period of 20 days also produced the same negative result (Figure 3.12 and appendix C). The inability to select for γ-actinorhodin resistance in *S. aureus* strains over a prolonged period point toward that the emergence of γ-actinorhodin resistant by endogenous mechanisms is likely to be rare.

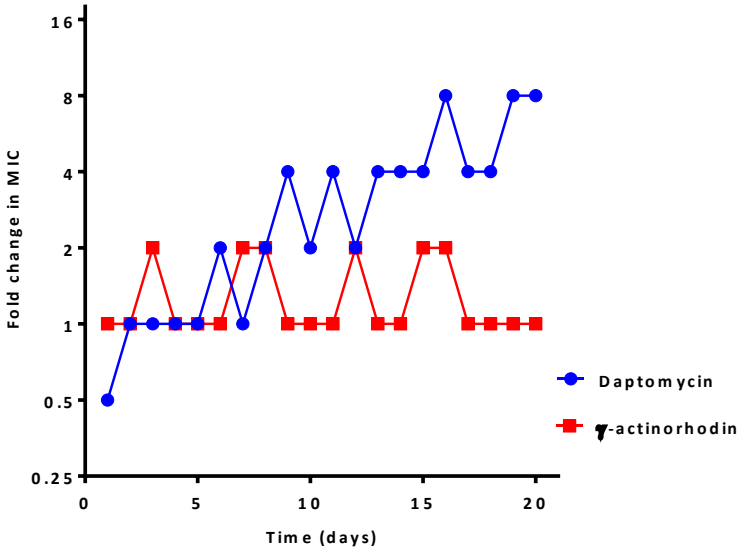


Figure 3.12. Resistance acquisition during Serial passage experiments with *S. aureus* SH1000 in the presence of sub-MIC levels of γ-actinorhodin and daptomycin. The data is representative of 3 independent experiments.

3.5.7. *In vivo* toxicity and antibacterial efficacy of γ-actinorhodin

For a compound to be developed as a therapeutic agent for systemic administration, it is required to evaluate its efficacy in resolving infection (O'Neill and Chopra 2004). Usually, this is accomplished using a mammalian model. However, such experiments are expensive, time-consuming, and necessitate full ethical consideration. Therefore, economically and ethically more acceptable invertebrate models of infection have been introduced, including the larvae of the greater wax moth *Galleria mellonella*. *In vivo* efficacy of γ-actinorhodin in protecting *G. mellonella* larvae from killing by *S. aureus* (USA300) was tested. To determine the lethal concentrations of γ-actinorhodin, the concentrations of 50 mg/kg, 20 mg/kg, 5 mg/kg, and 1 mg/kg were injected in *G. mellonella* larvae, and the percentage survival was recorded over 120 hours. The concentration of 50 mg/kg led to the death of 20% (low toxicity) of the population after 120 hours while 100% survived at concentrations of 20 mg/kg, 5 mg/kg, and 1 mg/kg. The

concentrations proving to be nonlethal to the larvae presented the following results when injected in *G. mellonella* infected by *S. aureus* USA300: 20 mg/kg led to the death of 85% after 48 hours, 5 mg/kg led to the death of 97% after 72 hours, and 1 mg/kg led to the death of 80% *G. mellonella* after 120 hours. As shown in Figure 3.13, the control group (infected with *S. aureus* USA300 without any treatment) led to the death of 100% of the larvae after 96 hours.

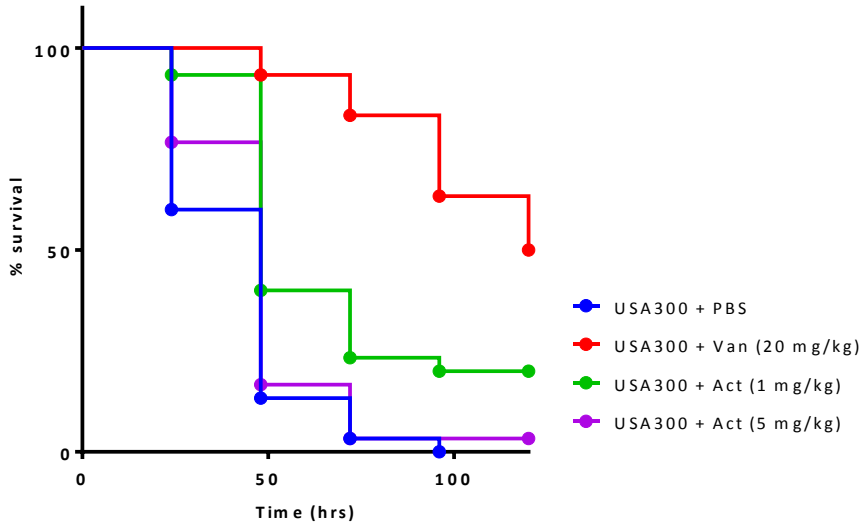


Figure 3.13. Single dose treatment (30 min post-infection, ten larvae per group) with γ.actinorhodin and vancomycin in *G. mellonella* protection model using *S. aureus* USA300**.**

Therefore, γ-actinorhodin appears to possess some therapeutic potential, though further studies will be required to understand why the compound not only fails to improve survival of infected *G. melonella* when administered at higher concentrations but beyond a certain level, actually acts to reduce it. Potentially, while not clearly cytotoxic on its own at the concentrations applied, γ-actinorhodin might compromise the immune response in *G. melonella*, to facilitate the progress of the infection. Whether such a phenomenon is also evident in higher organisms remains to be established, but even if that does transpire

to be the case, that need not rule γ-actinorhodin from further consideration as a candidate topical agent to prevent and treat staphylococcal disease.

3.6. Conclusions

Given the growing problem of antibiotic resistance, it seems practical to consider drug sources that were previously abandoned. One of the most critical challenges in the natural product research is to obtain material of sufficient purity to assess its biological activity. This study highlights the necessity of strong collaboration between a biologist and a chemist to obtain the desired active compounds efficiently. Sufficient quantities of the pure γ-actinorhodin were thereby recovered to permit characterization and preclinical evaluation. The BIQ γ-actinorhodin demonstrated potent, bactericidal anti-Gram-positive activity against a number of bacterial species including MRSA and VISA, but lacked activity against Gram-negative pathogens as a result of the permeability barrier presented by the outer membrane. This study indicates a complex mode of action for γ-actinorhodin that is distinct from other antibacterials in clinical use. Our findings suggest that the antibacterial mode of action of γ-actinorhodin involves rapid dissipation of membrane potential, prompting a complete shutdown of macromolecular biosynthesis, and eventually, cell death. γ-actinorhodin possesses many of the requisite properties of a useful antibacterial drug candidate as it showed evidence for *in vivo* efficacy and γ-exhibits extremely low resistance potential. The findings of this study highlight the utility of re-visiting unexploited natural product in the search for novel antibacterial drug candidates, and a comprehensive re-evaluation of such compounds is now warranted.

Chapter four

Investigations into the antibacterial mode of action of thiolutin

4.1. Abstract

In the present study, the antibacterial activity of thiolutin was evaluated against a panel of clinically relevant bacterial pathogens along with deciphering the mode of action in Gram-positive and Gram-negative bacteria. Thiolutin exhibited potent, broad-spectrum activity against *S. aureus* and *E. coli* and also demonstrated useful antibacterial activities against other clinically significant bacterial pathogens tested. Using a combination of macromolecular synthesis, membrane integrity assessments, and time-kill assays it was shown that *S. aureus* inhibition by thiolutin was likely due to specific drug target interaction rather than nonspecific membrane disruption. However, the establishment of target pathway/s in this organism was not yet known. In contrast, inhibition of RNA synthesis of *E. coli* was pronounced after 10 minutes exposure to thiolutin. Spontaneous resistance to thiolutin occurred at a frequency of 1.38 (± 1.28) x 10^{-8} and 8.32 ± (1.02) x 10^{-7} in *S. aureus* and *E. coli*, respectively. Genetic analysis of isolated mutants revealed missense mutations in the *S. aureus* genes encoding thioredoxin (*trxA*) and thioredoxin reductase (*trxB*), and nonsense mutations in *E. coli* γ-Glutamyl cysteine synthetase *(gshA*) gene. Thioredoxin/ glutaredoxin systems are likely to be involved in the intracellular

reductive activation of thiolutin and resistance to this class is resulted from inactivation of reduction pathways.

4.2. Introduction

The dithiolopyrrolone (DTP) class of natural products possess a broad spectrum of biological activity including antibacterial, antifungal, and anticancer activities (Seneca, Kane and Rockenbach 1952; Li *et al.* 2014). DTPs are characterized by the possession of a unique DTP nucleus with two sulfur atoms (Figure 4.1). These compounds were initially isolated from *Streptomyces sp*. and, subsequently have also been found to be produced by other organisms (Qin *et al.* 2013). This group of compounds includes aureothricin, thiolutin, holomycin and xenorhabdins (Umezawa H. 1948; Eisenman 1956; Celmer and Solomons 1955; Von Daehne *et al.* 1969; McInerney *et al.* 1991). The thiomarinols are another group of antibiotic with a DTP ring which has the polyketide antibiotic, pseudomonic acid, linked to it (Shiozawa, Shimada and Takahashi 1997; Fukuda *et al.* 2011). The DTP class of molecules has attracted numerous research groups not only because of their biological activities, but also owing to the chemical logic of disulfide bond formation and the molecular machinery of their biosynthesis (Li *et al.* 2014).

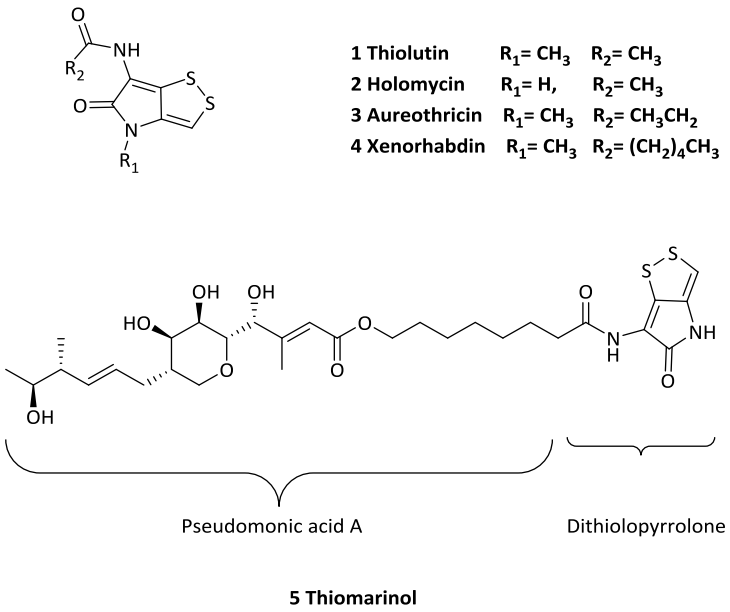


Figure 4.1. Some of the naturally occurring DTP antibiotics**.** 1 thiolutin, 2 holomycin, 3 aureothricin, 4 xenorhabdin, and thiomarinol 5

Among the DTPs, thiolutin is the most extensively studied member in respect of antimicrobial activities. Thiolutin has been shown to possess a broad spectrum of biological activity, demonstrating inhibatory action against bacteria (Khachatourians and Tipper 1974a), yeast (Tipper 1973), fungi (Gopalkrishnan and Jump 1952; Deb and Dutta 1984), parasites (Iwatsuki *et al.* 2010), and insects (Cole and Rolinson 1972) and selective anticancer activity (Jia *et al.* 2010; Minamiguchi *et al.* 2001). Interestingly, however, the activity of the structurally closely related compound, holomycin, appears to be restricted to prokaryotes (Oliva *et al.* 2001).

4.2.1. Mode of action of the DTPs

It has been widely accepted that DTPs inhibit RNA synthesis, and early studies on the mode of action of thiolutin appeared to confirm that it act as an inhibitor of RNA synthesis in yeast (Jimenez, Tipper and Davies 1973; Tipper 1973) and bacteria

(Khachatourians and Tipper 1974a; Khachatourians and Tipper 1974b). However, the mechanism underlying the observed inhibition of RNA biosynthesis by DTPs is yet to be clarified.

In *Saccharomyces cerevisiae,* rapid preferential inhibition of RNA synthesis in the presence of low concentrations of thiolutin was observed following radiolabel incorporation experiments (Jimenez, Tipper and Davies 1973) and inhibition of the all three RNA polymerases in cell-free assays confirmed the target of thiolutin in yeast (Tipper 1973). In a more recent study using DNA microarray (Grigull *et al.* 2004), it was proposed that thiolutin mediated inhibition of RNA synthesis in *S*. *cerevisiae* results from chelation of ions rather than from direct drug-target interaction. This proposal was based on a comparison of global transcript stability profiles following chemical inhibition of transcription to *rpb1-1* mutants (temperature-sensitive mutant in the catalytic subunit of RNA polymerase II) by five inhibitors including thiolutin. It was shown that the effects of thiolutin and 1, 10-phenanthroline (metal chelator that most likely inhibits Pol II by sequestering magnesium) were most similar to *rpb1-1*, leading the authors to propose that thiolutin may inhibit RNA synthesis in a manner similar to 1,10-phenanthroline (Grigull *et al.* 2004).

In contrast to the observations in yeast, inhibition of RNA synthesis by thiolutin in *E. coli* could only be demonstrated in whole-cell incorporation experiments and not in a cell-free assay (Khachatourians and Tipper 1974b). A study on holomycin revealed a similar outcome, shedding doubts on RNA polymerase as the primary target of thiolutin in bacteria (Oliva *et al.* 2001). Other mechanisms of action have also been proposed for the DTPs. In one study, glucose utilization and respiration in *E. coli* were shown to be inhibited by thiolutin (Bergmann 1989), while in another study in *S. typhimurium,* the bacterial membrane was implicated as one site of action of thiolutin (Joshi and

Chakravorty 1977). Potantially, the DTP scaffold might influence multiple pathways that contribute to its overall antimicrobial effect.

It has been suggested that the DTPs are prodrugs that require enzymatic activation to exert their action (Oliva *et al.* 2001) in which the structural characteristics of DTP disulfide-bridged heterocycle might offer some justification (Li *et al.* 2014). By analogy, it was proposed that DTPs may inhibit RNA synthesis in bacteria in a manner similar to gliotoxin, an inhibitor of poliovirus RNA synthesis that possesses a similar disulfide bridge (Figure 4.2). Gliotoxin's activity requires intracellular reduction of the disulfide bond leading to more active dithiol groups which can react with the thiol groups of cellular proteins (Scharf *et al.* 2010; Jones and Hancock 1988).

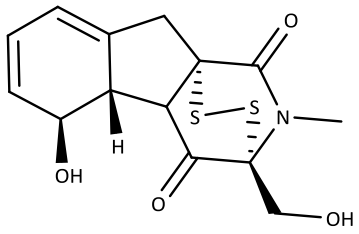


Figure 4.2. The structure of the natural product mycotoxin, gliotoxin, containing a disulfide bond

In support of this hypothesis, Li and coworkers has found an accumulation of modified intermediates of mono- and di-*S*-methylation- in a *ΔhlmI* mutant holomycin producer, *Streptomyces clavuligerus*, in which the gene for disulfide bond formation was deleted (Li *et al.* 2012). This led the authors to suggest that S-methylation is a "backup plan" to protect the producer from the toxicity of the reactive dithiol groups in the reduced intermediates.

4.2.2. Use of staphylococci to investigate DTP mode of action

E.coli has been used as a model system for almost all studies of the mode of action of DTPs and their mechanism of action against Gram-positive bacteria has not been explored. Gram-positive bacteria lack the outer membrane of Gram-negative and possess a considerably thicker peptidoglycan layer (Silhavy, Kahne and Walker 2010). These differences in cell envelope are known to affect antibiotic uptake (Silhavy, Kahne and Walker 2010; Pages, James and Winterhalter 2008). Other differences between Gram-positive and Gram-negative that may influence antibiotic action include stress responses, efflux pumps, and biochemical target availability. It would, therefore, be interesting to explore the antibacterial activity and mode of action of this class of antibiotics against Gram-positive bacteria.

4.2. Aims and objectives

Work described in this chapter sought to gain a better understanding of DTP antibacterial activity and mode of action. The spectrum of antibacterial activity and prokaryotic specificity of thiolutin were investigated. Killing kinetics were determined and the mechanism of antibacterial activity studied against the Gram-positive pathogen, *S. aureus*. Resistant mutants were selected and characterized to further our understanding of the mode of action and resistant mechanisms. Due to the unusual profile observed against *S. aureus*, mode of action and resistance studies were additionally performed against the Gram-negative bacteria *E. coli* for comparison. Although the work in this chapter originally intended to use holomycin given the reported greater degree of bacterial selectivity (Oliva *et al.* 2001), thiolutin was used instead due to the unavailability of holomycin at the commencement of the study.

4.4. Results

4.4.1. *In vivo* antibacterial activity of thiolutin

The activity of thiolutin was determined against a range of Gram-positive and Gram-negative bacteria by a standard broth microdilution procedure (Table 4.1). Thiolutin exhibited potent, broad-spectrum activity against *S. aureus* and *E. coli,* in agreement with previous reports (Seneca, Kane and Rockenbach 1952; Oliva *et al.* 2001; Li *et al.* 2014). Thiolutin also showed useful antibacterial activity against clinically relevant non-fermentative Gram-negative bacilli, *A. baumannii,* and *P. aeruginosa.*

Table 4.1 *In vivo* activity of thiolutin against range of pathogens

Bacterial strain	**MIC (μg/ml)**
	Thiolutin
***S. aureus* SH1000**	2
***S. epidermidis* ATCC 14490**	2
***S. haemolyticus* 41207**	2
***Streptococcus pyogenes* ATCC 19615**	2
***S. pneumoniae* ATCC R6**	2
***Enterococcus faecalis* ATCC 29212**	4
***E. faecium* 7634337**	4
***E. coli* BW25113**	1
BW25113+PMBN (4μg/ml)	0.5
BW25113-*ΔacrAB*	0.5
BW25113-*ΔtolC*	1
***Klebsiella pneumoniae* K25**	8
***Acinetobacter baumannii* ATCC 19606**	64
***Pseudomonas aeruginosa* PAO1**	64

Evaluating the effect of compounds in the presence of an outer membrane permeabilizer and efflux pump inactivation on antibacterial resistance bacteria is worthwhile, since the results may provide an indication concerning resistance mechanisms employed by Gram-negative bacteria against the compound under investigation (O'Neill and Chopra 2004). A modest increase in susceptibility to both antibiotics was observed for *E.coli* when their intrinsic resistance mechanisms were artificially compromised (Table 4.1).

4.4.2. Evaluation of bacterial killing by thiolutin in *S. aureus*

Previous reports demonstrated that the DTPs thiolutin and holomycin exhibit a bacteriostatic action against *E. coli* (Oliva *et al.* 2001; Khachatourians and Tipper 1974a). To characterize the antibacterial activity of thiolutin against Gram-positive bacteria, killing kinetics were evaluated beside comparator antibiotics against *S.aureus* SH1000. The addition of vancomycin (at 4x MIC) to early-exponential-phase cultures of *S.aureus* SH1000 caused a reduction in cell viability of approximately 3 $\log_{10}$ CFU/ml after 6 hours (bactericidal), while tetracycline showed <1 $\log_{10}$ drop in cell viability over 24 hours (bacteriostatic). The addition of 8 µg/ml of thiolutin (equivalent to 4x MIC as determined by the microdilution method) resulted in cessation of growth (within 10 minutes), but no loss of viability (Figure 4.3). Thus, thiolutin exhibited a bacteriostatic response against *S. aureus,* consistent with the previous report on thiolutin against *E. coli* (Khachatourians and Tipper 1974a).

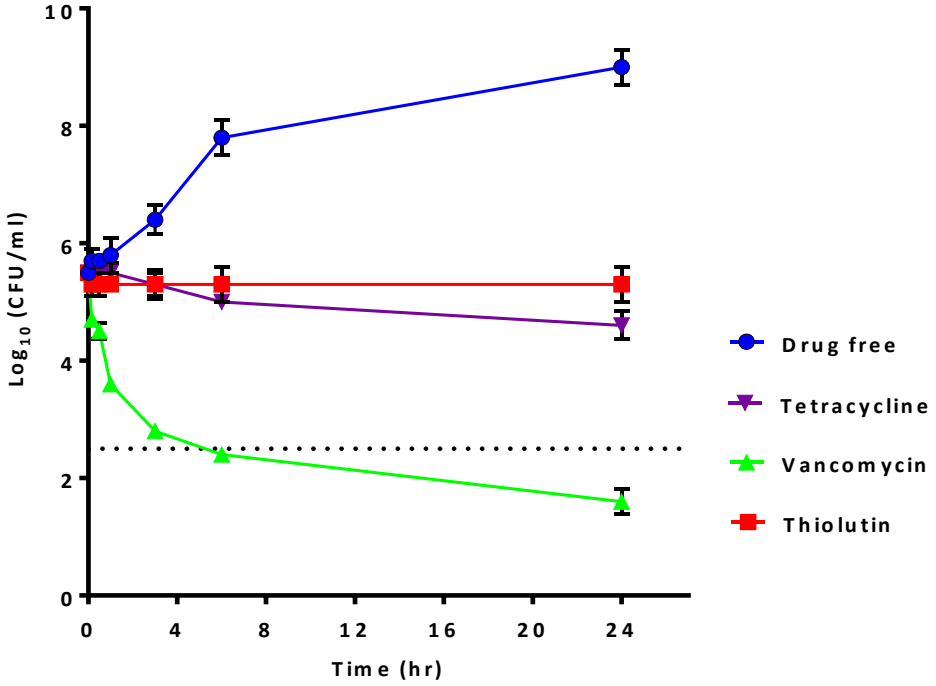


Figure 4.3. Evaluation of killing action of thiolutin and comparator agents at 4x MIC on exponential cultures of *S. aureus* SH1000 over 24 hours in MHB. The dotted line represents 3 log reductions. Values shown are the means from at least three independent experiments, each of which had two technical replicates. Error bars represent standard deviations from the mean.

4.4.3. Investigation into the antibacterial mechanism of action of thiolutin against *S. aureus*

4.4.3.1. Effect of thiolutin on staphylococcal biosynthetic pathways

The effect of thiolutin on macromolecule biosynthesis in *S. aureus* SH1000 was explored by monitoring the incorporation of radiolabeled precursors into macromolecules. This experiment was run over a fixed period of only 10 minutes to reveal direct effects caused by antibiotic before secondary effects become prominent. Interestingly, no significant inhibition by thiolutin was observed for any of the biosynthetic pathways, whereas

control experiments with known antibiotics produced the expected inhibitory effects (Figure 4.4).

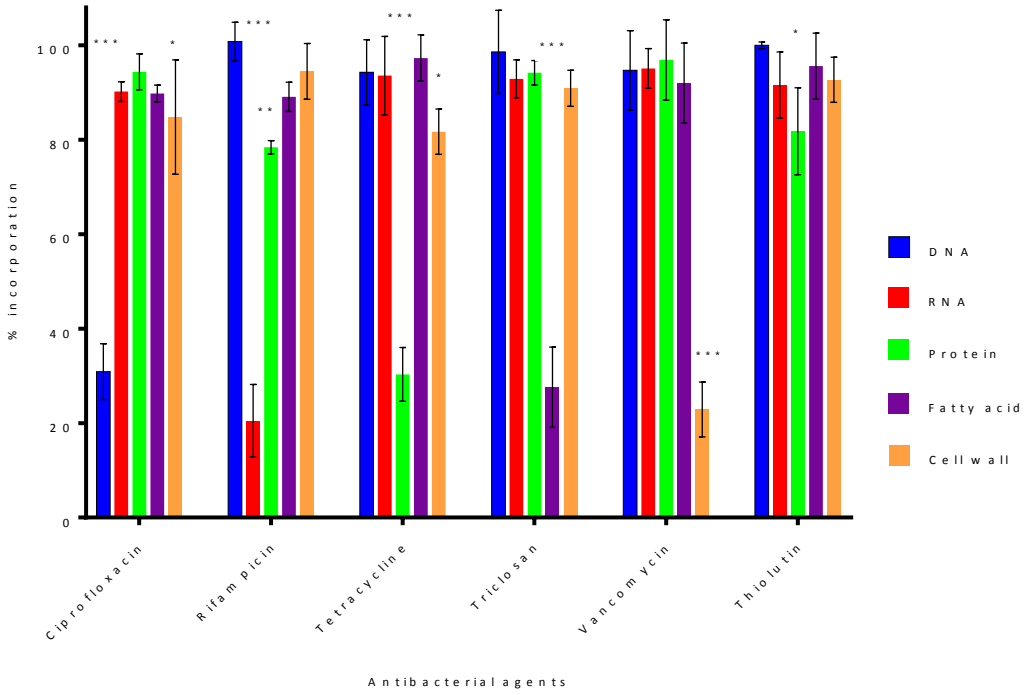


Figure 4.4. Effect of thiolutin and comparator agents on DNA, RNA, protein, fatty acid, and peptidoglycan biosynthesis in *S. aureus* SH1000, as measured by incorporation of radiolabeled precursors. Values represent the percentage incorporation relative to drug-free controls. Values shown are the means from at least three independent experiments, each of which had two technical replicates. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to untreated control) Error bars represent standard deviations from the mean. (The control data is the same from figure 3.10)

Studies on the mode of action of thiolutin and holomycin against *E. coli* have suggested that these antibiotics inhibit RNA synthesis (Jimenez, Tipper and Davies 1973; Tipper 1973; Khachatourians and Tipper 1974a; Oliva *et al.* 2001). Here, in contrast, only a minor effect on staphylococcal RNA biosynthesis was observed (inhibited by ~11% in ten minutes) particularly when compared with rifampicin (inhibited by ~80% in ten minutes), a known RNA biosynthesis inhibitor. The inability to show inhibition of any staphylococcal macromolecular synthetic pathway in the 10 minutes assay window suggested that the compound may be slow acting in *S. aureus* in comparison to *E. coli*.

Given the above results, I examined whether specific inhibition of RNA biosynthesis by thiolutin could be demonstrated when the exposure time was extended beyond 10 minutes. As shown in Figure 4.5, inhibition of staphylococcal RNA biosynthesis increased to ~20 % and ~50 % after 20 and 30 minutes' exposure to thiolutin, respectively. However, this seems to be a secondary effect since inhibition of RNA synthesis was also observed when *S. aureus* cultures were exposed to ciprofloxacin and tetracycline, antibiotics that are known to specifically inhibit DNA synthesis and protein synthesis, respectively.

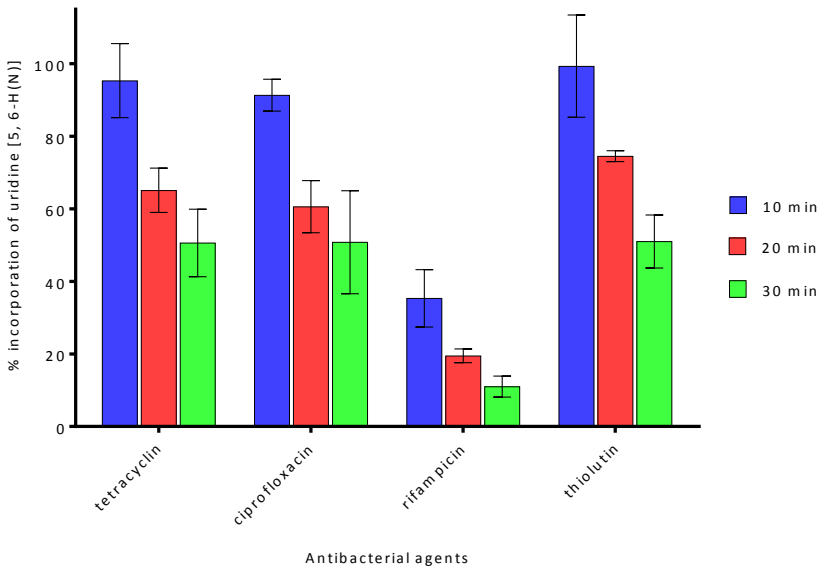


Figure 4.5. Effect of thiolutin and comparator agents on RNA biosynthesis in *S. aureus* SH1000, as measured by incorporation of radiolabeled precursors uridine [5, 6-H(N)] at different time points. Values represent the percentage incorporation relative to drug-free controls. Values shown are the means from three independent experiments, each of which had three technical replicates. Error bars represent standard deviations from the mean.

4.4.3.2. Effect of thiolutin on the staphylococcal cytoplasmic membrane

Antibacterial agents usually exert their activity by targeting a single biosynthetic pathway or by non-specific action on the membrane. Since MMS experiments failed to show a preferential pathway for thiolutin mode of action in *S. aureus*, I investigated whether

thiolutin exerts effects on the staphylococcal membrane. Using the *Bac*Light™ assay, thiolutin exhibited no effect on membrane integrity, similar to the negative control compound, tetracycline (a protein synthesis inhibitor). By contrast, the known membrane damaging agents nisin and CTAB reduced membrane integrity substantially within 10 minutes (Figure 4.6 A). Since this assay may not detect subtle changes in membrane potential or perturbation occurring over longer time periods, the membrane-potential sensitive dye $DiSC_3(5)$ was also employed. No changes in the staphylococcal membrane potential were detected after 1 hour of exposure to thiolutin at 4x MIC (Figure 4.6 B). Although the mode of action against *S. aureus* therefore remains to be established, the results of these membrane damaging assays imply that thiolutin does not act on the membrane.

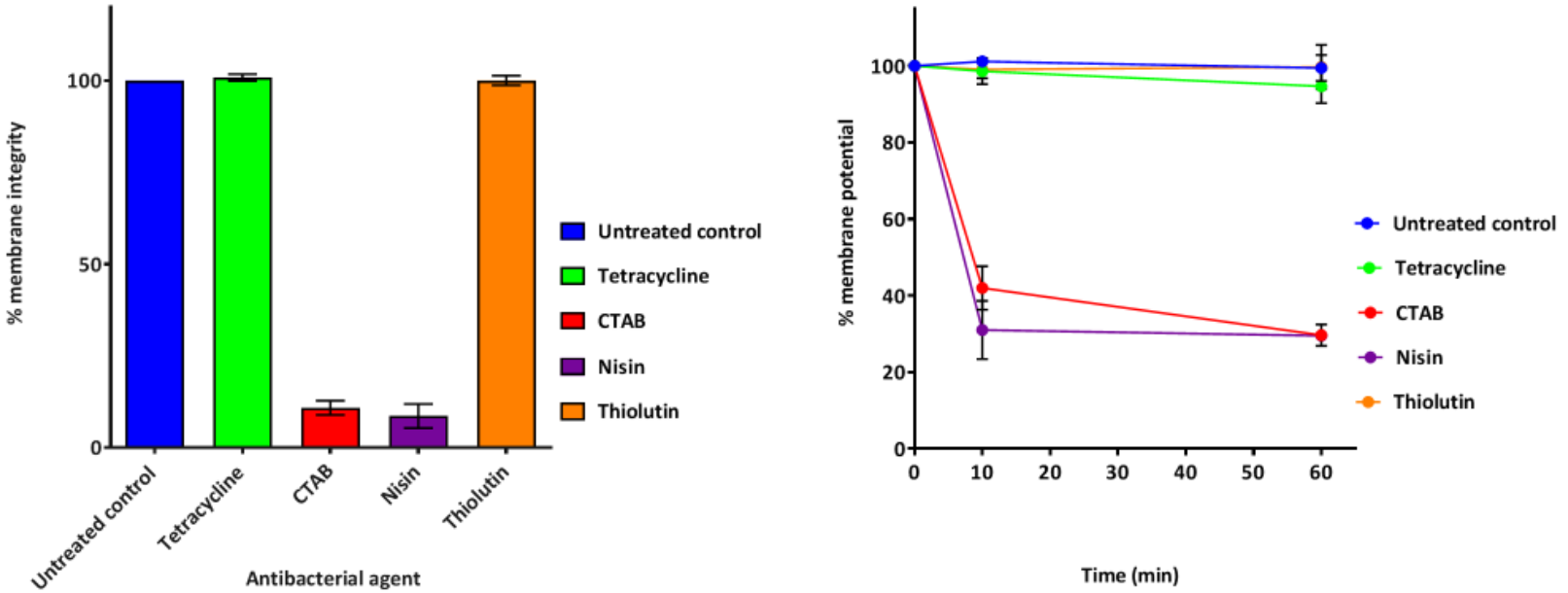


Figure 4.6. Effect of thiolutin and comparator agents on (a) membrane integrity of *S. aureus* SH1000 as measured by *Bac*light™ and (b) membrane potential of *S. aureus* SH1000 as measured by $DiSC_3$ (5). Values shown are the means of three independent experiments, each of which had three technical replicates. Error bars represent standard deviations from the mean.

4.4.3.3. Resistance studies with thiolutin

The propensity to select resistance to thiolutin was examined previously in *E. coli* and *S. typhimurium* (Joshi and Chakravorty 1977; Sivasubramanian and Jayaraman 1976). The aim here was to determine the frequency of selection of thiolutin resistance by mutation in *S. aureus* and to attempt to identify the antibacterial target of the compound by identification of mutational changes. The resistance potential of thiolutin was evaluated by plating saturated cultures of *S. aureus* SH1000 onto agar containing the compound at 4x MIC; spontaneous resistance to thiolutin occurred at a frequency of 1.38 (± 1.28) x 10^{-8}. Comparator agents gave mutation frequency measurements in agreement with published values; for both rifampicin and fusidic acid, resistant mutants arose with frequency around 10^{-8} (O'Neill, Cove and Chopra 2001). Nine spontaneous thiolutin-resistant mutants (named SA-TR1 - SA-TR9) were picked at random from selection plates and their MICs determined (MICs of 4-8 μg/ml). Subsequent attempts to select higher level thiolutin resistance by repeated exposure to escalating, subinhibatory concentrations of thiolutin in *S. aureus* were unsuccessful.

To explore whether thiolutin resistance affords cross-resistance to other antibacterial agents, susceptibility to a range of compounds was determined. No cross-resistance was observed with ampicillin (MIC of 0.5 μg/ml), gentamicin (MIC of 0.25 μg/ml), tetracycline (MIC of 0.5 μg/ml), rifampicin (MIC of 0.016 μg/ml), ciprofloxacin (MIC of 1 μg/ml), and vancomycin (MIC of 2 μg/ml). This suggests that these antibiotics interact with different pathways or components of staphylococci than thiolutin.

4.4.3.4. Genetic characterization of thiolutin resistant strains

Performing whole genome sequencing is one way to identify the genetic loci participating in the observed reduction in thiolutin susceptibility. The complete genome sequence of

two thiolutin resistant mutants SA-TR1 (MIC of 4 μg/ml) and SA-TR2 (MIC of 8 μg/ml) were determined using the Illumina Miseq platform and compared to the genome of the parental strain *S. aureus* SH1000. Mutations in genes coding for thioredoxin (*trxA*) (SAOUHSC_01100) in strain SA-TR1 and thioredoxin reductase (*trxB*) in strain SA-TR2 (SAOUHSC_00785) were detected. Subsequently, PCR was used to search for mutations at these loci in the remaining *S. aureus* mutants. A 654 bp amplicon of *trxA* and a 1348 bp amplicon of *trxB* were generated by PCR using the primers 5'-GCCAAGCGAAGGTGGATTTG-3'(forward) and 5'-TCCCACCATTCTCAAGTGGTA-3' (reverse) and 5'-TGAGCCATATATCGGTGCGTT-3' (forward) and 5'-CTGTCCCACTCCCATAAAGTT-3' (reverse), respectively. Sequencing of these amplicons revealed in several cases missense mutations associated with thiolutin resistance in either the *trxA* gene or the *trxB* of *S. aureus* (Table 4.2). However, as shown in table 4.2, mutations could not be detected in five strains in which full reversions in the MICs occurred for three mutants (SA-TR3, SA-TR7, and SA-TR8) after plating in the absence of thiolutin.

Table 4.2: Missense mutations associated with thiolutin resistance in *S. aureus* SH1000

Mutants	**MIC (μg/ml)**	***trxA*** **DNA seq/Amino acid change**	***trxB*** **DNA seq/Amino acid change**
SH1000 (WT)	2	-	-
SA-TR1	4	GAT→GGT /Asp_{68}→ Gly_{68}	-
SA-TR2	8	-	GCT→GGT/ Ala_{289}→Val_{289}
SA-TR3	2	-	-
SA-TR4	8	-	GGA→AGA/ Gly_{128}→Arg_{128}
SA-TR5	4	GAT→GTT/Asp_{68}→Val_{68}	-
SA-TR6	4	-	-
SA-TR7	2	-	-
SA-TR8	2	-	-
SA-TR9	4	-	-

Thioredoxin/thioredoxin reductase is one of the major cellular oxidation-reduction (redox) control systems found in all species from Archebacteria to man (Arner and Holmgren 2000). In *S. aureus*, thioredoxin, with a dithiol/disulfide active site (CGPC), plays an essential roles in maintaining cellular redox homeostasis and cell survival. It also provides reducing power for key reductive enzymes such as ribonucleotide reductases, and methionine sulfoxide reductases (Arner and Holmgren 2000). According to the usual enzymatic reactions of the thioredoxin system (Figure 4.7), it is reasonable to deduce that this system is accountable for activating thiolutin *via* reduction of disulphide bridge resulting in a sulfhydryl reactive group that exerts the biological effect by performing a thiol-disulfide exchange with target protein/s.

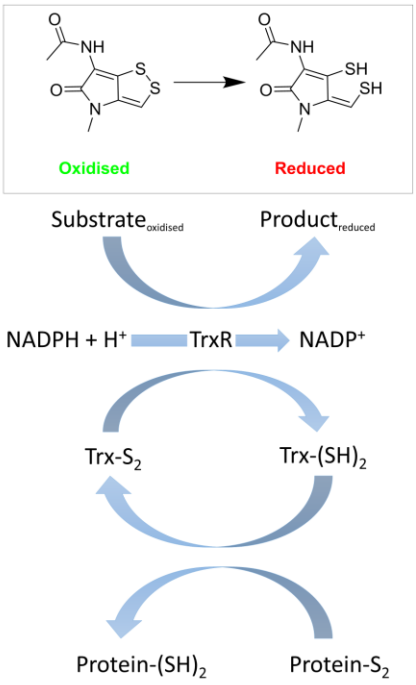


Figure 4.7. The mechanism of disulphide reduction catalyzed by the thioredoxin system**.** Thioredoxin reductase (TrxR) catalyzes the reduction of the active site disulfides in Trx using NADPH. Reduced thioredoxin [Trx $(SH)_2$] reduces oxidized proteins and consequently oxidized [Trx $(S)_2$]. Oxidized thioredoxin is in turn reduced by thioredoxin reductase (TrxR) with NADPH as a source of reducing equivalents. Thioredoxin reductase may have substrates other than thioredoxin (adapted from (Arner and Holmgren 2000))

Changes in redox regulation leads to oxidative stress (ROS generation) in the cell that can damage essential cellular components (Green and Paget 2004). To explore a potential role for ROS in the antibacterial mode of action, the effect of thiolutin on strains of *S. aureus* defective in the main components of the ROS protection response were examined to determine whether they exhibited greater susceptibility to the action of the antibiotic. No change in the MIC of thiolutin was observed against KS100 (SH1000 derivative lacking the major catalase enzyme, KatA), KC043 (SH1000 derivative completely devoid of catalase activity as a consequence of lacking both KatA and the enzyme alkyl hydroperoxide reductase [AhpC]), MHKA (SH1000 lacking the major superoxide dismutase enzyme, SodA), MHKM (SH1000 lacking the SodM), or MHKAM (SH1000 double mutant deficient in both SodA and SodM), when compared to *S. aureus* SH1000.

4.4.4. Investigation into the antibacterial mechanism of action of thiolutin in *E. coli*

4.4.4.1. Effect of thiolutin on E. coli biosynthetic pathways

Due to the conflicting results obtained with *S. aureus,* mode of action studies were undertaken in *E. coli* to resolve this conflict and allow us a better understanding of thiolutin antibacterial mode of action. Selecting mutants resistant to thiolutin in *E. coli* and characterizing their properties would also be a useful approach to gain an understanding of the possible target. Firstly, the ability of thiolutin to specifically inhibit RNA synthesis in *E. coli* BW25113 was investigated. Thiolutin at 4x MIC was found to preferentially inhibit the RNA biosynthesis in *E. coli* BW25113 in 10 minutes (Figure 4.8). A moderate inhibitory effect on DNA and protein biosynthesis was also observed, though this could be an effect secondary effect to inhibition of transcription.

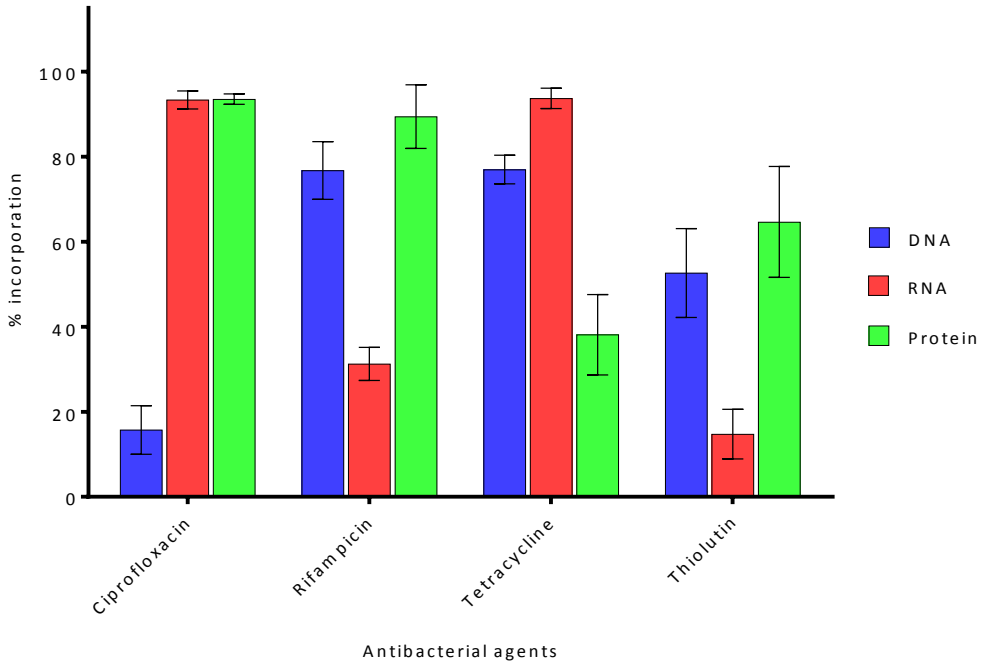


Figure 4.8. Effect of thiolutin and comparator agents on DNA, RNA, and protein biosynthesis in E. coli BW25113, as measured by incorporation of radiolabeled precursors. Values represent the percentage incorporation relative to drug-free controls. Values shown are the means from at least three independent experiments, each of which had three technical replicates. Error bars represent standard deviations from the mean.

4.4.4.2. Resistance studies with thiolutin in E. coli

As indicated previously in this study, the low level of resistance to thiolutin in *S. aureus* mutations were linked to amino acid alterations found in regions in *trxA* and *trxB* genes. In *E. coli*, two pathways use NADPH to reduce disulfide bonds that form in some cytoplasmic enzymes during catalysis: the thioredoxin system, which consists of thioredoxin reductase and thioredoxin, and the glutaredoxin system, composed of glutathione reductase, glutathione, and three glutaredoxins (Figure 4.9) (Toledano *et al.* 2007).

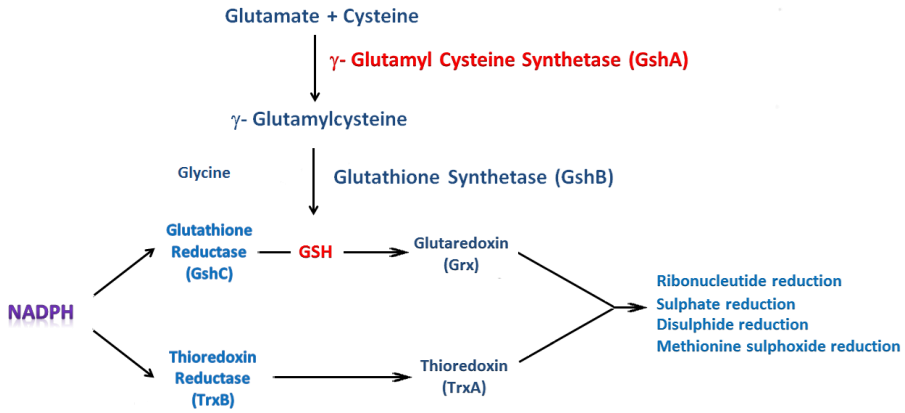


Figure 4.9. Components of *E coli* thioredoxin and glutaredoxin systems**.** Adapted from (Toledano *et al.* 2007)

To explore a potential role of the thiol redox system in *E. coli*, strains of keio knockout collection of mutants lacking in the main components of the thiol redox system were screened for reduced susceptibility to thiolutin (Table 4.3). It is worth mentioning, however, that the existence of the listed mutations was not actually validated.

Table 4.3: Effect of thiolutin on *E. coli* K12 mutants defective in components of thioredoxin and glutaredoxin redox systems

Strain	Thiolutin MIC (µg/ml)
BW25113	1 µg/ml
BW25113 *ΔtrxA*	2 µg/ml
BW25113 *ΔtrxB*	2 µg/ml
BW25113 *ΔtrxC*	2 µg/ml
BW25113 *ΔgshA*	4 µg/ml
BW25113 *ΔgshB*	2 µg/ml
BW25113 *ΔgrxA*	2 µg/ml
BW25113 *ΔgrxB*	2 µg/ml
BW25113 *ΔgrxC*	2 µg/ml

A four-fold reduction in susceptibility to thiolutin was observed in the strain of BW25113 lacking gene *gshA*, which encodes for γ-glutamylcysteine ligase, an enzyme that catalyze

the first step of glutathione (GSH) biosynthesis pathway (Masip, Veeravalli and Georgioui 2006). By contrast, only a two-fold increase in thiolutin MIC was observed against the other strains tested (Table 4.3). Subsequently, *E. coli* BW25113 resistant mutants with low-level resistance to thiolutin were generated at a frequency of 8.32 (± 1.02) x 10^{-7} in an attempt to confirm the role of *gshA* in resistance to thiolutin. Six resistant colonies were picked and confirmed to exhibit 2-4-fold greater resistance to thiolutin than *E. coli* BW25113.

A 1997 bp *gshA* fragment from mutants resistant to thiolutin alongside with the parental strain *E. coli* BW25113 were amplified using the primers 5'-GCTATGGTGCAGTCACGCTAT-3' (forward) and 5'-GGGTATGATCGACTGTGAAGC-3' (reverse). Sequencing analysis revealed that four of the six mutants contained an alteration in *gshA*. In three strains, gshA apparently become inactivated by a nonsense mutation. In another mutant (E-TR2), a deletion of 50 nucleotides has occurred (Table 4.4) which could cause a premature termination to of translation as a consequence of frameshift mutation. Another consequence of a frameshift mutation to be considered is that the expression of downstream genes in the operon (*yqaA* (inner membrane protein) and *yqaB* (fructose-1-phosphatase)) might be altered. Since the operon organization of these genes is yet to be identified, we had to consider polar effects on downstream genes by deletion of 50 pb.

Table 4.4: Mutations associated with thiolutin resistance in *E.coli* BW25113

Mutants	**MIC (µg/ml)**	***gshA* (γ-glutamylcysteine ligase) DNA sequence/Amino acid change**
E-TR1	4	TCA→TAA/ Ser_6→Stop
E-TR2	4	Deletion
E-TR3	2	-
E-TR4	4	CAA→TAA/ Gln_{209}→Stop
E-TR5	2	-
E-TR6	4	CAA→TAA/ Gln_{209}→Stop

4.4.4.3. In vitro activity of thiolutin in an E. coli transcription/translation system

The results obtained from both *S. aureus* and *E. coli* support the idea that DTPs are prodrugs that require activation in the cell to exert their biological activity (Oliva *et al.* 2001; Li *et al.* 2012). To establish whether reducing conditions were required for thiolutin to work, I utilized a cell-free, *in vitro E. coli* transcription/translation system to monitor the effect of thiolutin on the inhibition of the enzymes involved in these pathways in the presence of reducing agent dithiothreitol (DTT) (section 2.5.2 in material and method chapter).

To the best of my knowledge, only two studies to date have demonstrated an *in vitro* inhibitory effect of thiolutin on yeast RNA polymerases (Jimenez, Tipper and Davies 1973; Tipper 1973). In these studies, inhibition of *S. cerevisiae* RNA polymerases was only established following pre-incubation of the enzyme with thiolutin in the absence of DNA. That led the authors to propose that the target site on RNA polymerase only accessible to thiolutin before interaction with DNA, and that thiolutin mode of inhibition is by interfering with functional polymerase-DNA interaction (Tipper 1973; Jimenez, Tipper and Davies 1973).

Here, however, no inhibition was observed when 4 µg/ml of thiolutin pre-incubated with the enzyme mixture for 10 minutes in the presence of 1mM DTT. This concentration of thiolutin is corresponded to the 4xMIC of the antibiotic for *E. coli* strain BW25113. In contrast to the non-observed inhibition displayed by thiolutin, fusidic acid was a potent inhibitor of *E. coli* T/T system *in vitro* with 90% inhibitory concentrations of approximately 2 µg/ml. Due to the limited time available, no further investigations were carried out.

4.5. Discussion

Studies on the mechanism of antibacterial action of thiolutin have been limited to the Gram-negative bacteria *E. coli*. Early studies on the thiolutin mode of action suggested that it might act as an inhibitor of RNA synthesis based on whole-cell experiments and not in a cell-free assay (Jimenez, Tipper and Davies 1973; Tipper 1973; Khachatourians and Tipper 1974a). Here, I started the investigation of the mode of thiolutin action on the Gram-positive bacteria *S. aureus.* The inability to show inhibition of any staphylococcal macromolecular pathway (Figure 4.4) suggestes a new target in this organism.

At the time of writing this chapter, Bo Li of the University of North Carolina, Chapel Hill, and coworkers published their findings on the DTP mode of action (Chan *et al.* 2017). They revealed that holomycin and thiolutin, along with gliotoxin exert their antimicrobial activity by interfering with cellular metal homeostasis in *E. coli*. They used a chemical genomic screen in *E. coli* K-12 to profile global drug–gene interactions. By probing growth profiles of a mutant library in the presence of sub-inhibitory concentrations of thiolutin, holomycin, and gliotoxin resulted in the detection of genes imparting fitness (Chan *et al.* 2017; Shiver *et al.* 2016). The clustering of the dataset revealed that gene deletion mutants in the uptake of extracellular iron and zinc were sensitized to all three compounds, but not to rifampicin or actinomycin D (inhibitors for transcription), compared to the wild type. Instead, the sensitivity profiles of these DTPs and gliotoxin were more similar to the metal chelator EDTA (Chan *et al.* 2017). In addition, an observation of less than 20% inhibition of purified *E. coli* RNA polymerase by high concentration of reduced holomycin (~20 μg/ml) in comparison to complete inhibition by rifampicin, led the authors to conclude that RNA polymerase is unlikely to be the primary antimicrobial target of holomycin in *E. coli* (Chan *et al.* 2017).

Based on the findings of this study, the authors proposed a model for the mode of action of DTPs (Figure 4.10). Holomycin is internalized by the target bacterium in the disulfide form, and reduced intracellularly to red-holomycin in which disrupt zinc homeostasis in two possible ways; (1) the reduced holomycin limits the zinc availability by chelating the free zinc and/or (2) the reduced holomycin removes zinc from a subset of zinc-dependent enzymes which may consequently impact glucose utilization, RNA synthesis, and respiration (Chan *et al.* 2017).

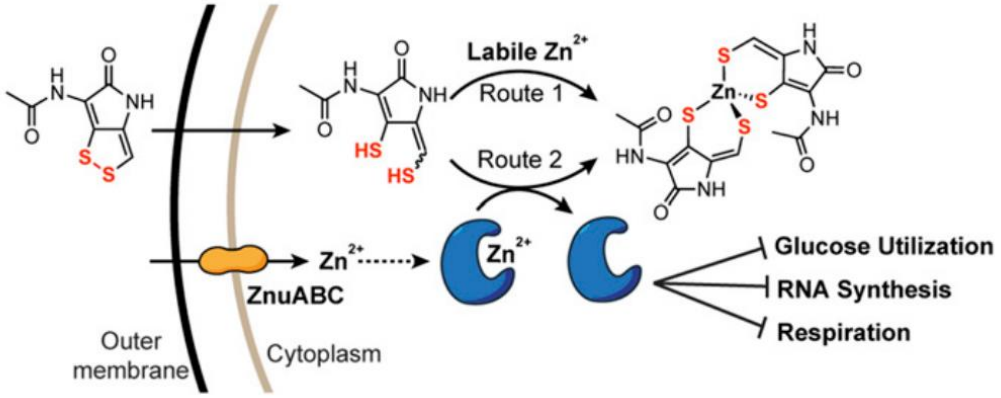


Figure 4.10. A model for the mechanism of action of DTPs as proposed by (Chan *et al.* 2017)

Although it was suggested that DTPs exert their indirect RNA inhibition by chelating metals upon their reduction intracellularly in *E. coli*, this seems not to be the case in *S. aureus*. Based on the findings of this study, the immediate cessation of growth in *S. aureus* cultures upon thiolutin exposure (Figure 4.3) suggests that intracellular reduction of thiolutin is not likely to be delayed in comparison with *E. coli.* Furthermore, if metal chelation is the prime mode of DTPs action upon reduction, one would expected to observe inhibition of multiple macromolecular synthesis pathways, since zinc-domains are a vital component of several

proteins including DNA and RNA polymerases, proteases, and ribosomal components (Panina, Mironov and Gelfand 2003). Therefore, the failure to demonstrate *in vitro* inhibition of any of the major biosynthesis pathways in *S. aureus* indicates a different mode of DTPs action in Gram-positive bacteria that needs further investigation.

5.4. Conclusions

Given the growing problem of antibiotic resistance, it is practical to consider antibacterial compounds that are yet unexploited. The DTP holomycin demonstrated better activities against all bacteria tested and high level of prokaryotic specificity than thiolutin. Thiolutin has revealed an unusual mechanism against Gram-positive bacteria that could be linked to differences in stress responses in comparison to Gram-negative bacteria. While the mode of action against *S. aureus* remains to be established, it is clear that this antibiotic does not directly cause growth inhibition through non-specific action on the membrane. The antibacterial activity of thiolutin appears to require reductive activation by the predominant redox system/s in the bacterial cell, and low-resistance to this class can results from inactivation of their reductive pathways. Further studies are required for further clarifying the antibacterial mode of DTPs action.

Chapter five

General conclusions and future works

5.1. General conclusions

The increasing trend of infections caused by multi-drug resistant pathogens has led to concerns that we are soon to enter a post-antibiotic era. Resistance to the last resort antibiotic, colistin, has already been detected in various parts of the world (O'Neill 2016). In one recent case, a 70-year-old woman died from septic shock in Nevada, after test showed 26 antibiotics-including colistin- could not have treat her bloodstream infiction (Chen *et al.* 2017). It is more vital now than ever to identify new antibiotic classes with a novel mode of action to address growing global resistance to antibacterial.

Nearly all antibiotics introduced into clinical practice are derived from a limited number of chemical scaffolds, mostly are natural products that discovered over 40 years ago (Cole 2014).The most recent introduction of new classes of antibiotics daptomycin (2003), retapamulin (2007), and fidaxomicin (2012) into clinic resulted from revisiting known scaffolds that failed to be developed as antibiotic candidates beforehand. The concept of revisiting of known natural product scaffolds, if carefully applied, could yield rapid results and a great number of new classes to the antibiotic pipeline to address the current antibiotic resistance crisis.

This thesis contains the first report of biological characterization of the dimeric BIQ γ-actinorhodin. The work presented in Chapter Three has made a significant contribution to our understanding of the previous report on the level of antibacterial activity of

actinorhodin, and suggests that re-evaluation of antibiotic classes that have been reported to exhibit weak activity using non-standard methods could potentially identify useful antibiotics. γ-actinorhodin's selective potent activity against MRSA and VISA strains, novel mode of action, low resistance potential, and the preliminary evidence of *in vivo* efficacy, suggests that actinorhodin scaffold may have potential as an antistaphylococcal drug candidate.

In addition, this thesis has made a contribution to the understanding of the mode of DTPs action (Chapter Four). In particular, it has been shown that the mode of action differs between Gram-positive and Gram-negative bacteria. The genetic analysis of mutants resistant to thiolutin has revealed genes involved in resistance to DTPs (thioredoxin system in *S. aureus* and glutaredoxin system in *E. coli*) and suggests their involvement in the activation of the DTP antibiotics by reduction *in vivo*, in agreement with a recent report (Chan *et al.* 2017). In addition, the failure to demonstrate inhibition of *E. coli* RNA polymerase or other enzymes required for transcription/translation *in vitro*, alongside the failure to demonstrate *in vivo* inhibition of any of the major biosynthesis pathways in *S. aureus,* indicates a novel mode of antibacterial action warrants further investigation.

This study therefore highlights the utility of revisiting unexploited natural product, and will hopefully; this study will encourage renewed efforts in revisiting unexploited natural product in the search for novel antibacterial drugs.

5.2. Future works

Although it was established that γ-actinorhodin at low concentration exerted some protection against infection by *S. aureus* USA3000 in an *in vivo* model, a progression of infection in *G. melonella* was observed at higher concentrations (Chapter Three). It seems that high concentrations of γ-actinorhodin retard the ability of *G. melonella's* immune

cells (haemocytes), leading to the progression of infection rather than protection. Future study could gain insight into this by extracting haemocytes from *G. melonella* larvae, and determining their ability to kill bacteria *in vitro* following exposure to different concentrations of γ-actinorhodin (Fallon et al., 2011). If the killing activity of haemocytes vanished/ or weakened after exposure to low concentration of γ-actinorhodin, it can be considered as topical agent for use in the treatment of chronic wounds and surgical site infections, especially since γ-actinorhodin is not toxic on its own at high concentration. The design of this experiment would reflect this situation (*in vitro* and *in vivo* infected wound model).

While it has been established that γ-actinorhodin interacts with components of the membrane (Chapter Three), the precise nature of this interaction is as of yet undefined. To provide further insight into the membrane component that γ-actinorhodin interacts with, staphylococcal cell ghosts could be generated, comprising cell envelops produced by releasing the cytoplasmic material through a channel in the cell envelope. A simple method to generate *S. aureus* ghosts was established by using the MIC of sodium hydroxide (NaOH) and the development of a transmembrane lysis channel in the cell envelop, which can be visualized by scanning electron microscopy (Vinod et al., 2015). In combination with methodology to reseal bacterial ghosts using membrane vesicles, cell ghosts could be infused with fluorescent dye and exposed to γ-actinorhodin. If no leakage of dye is induced, this may indicate that an energized membrane is required for the interaction. This could distinguish between agents that disrupt the function of components embedded within the membrane as opposed to those that simply destructure the membrane.

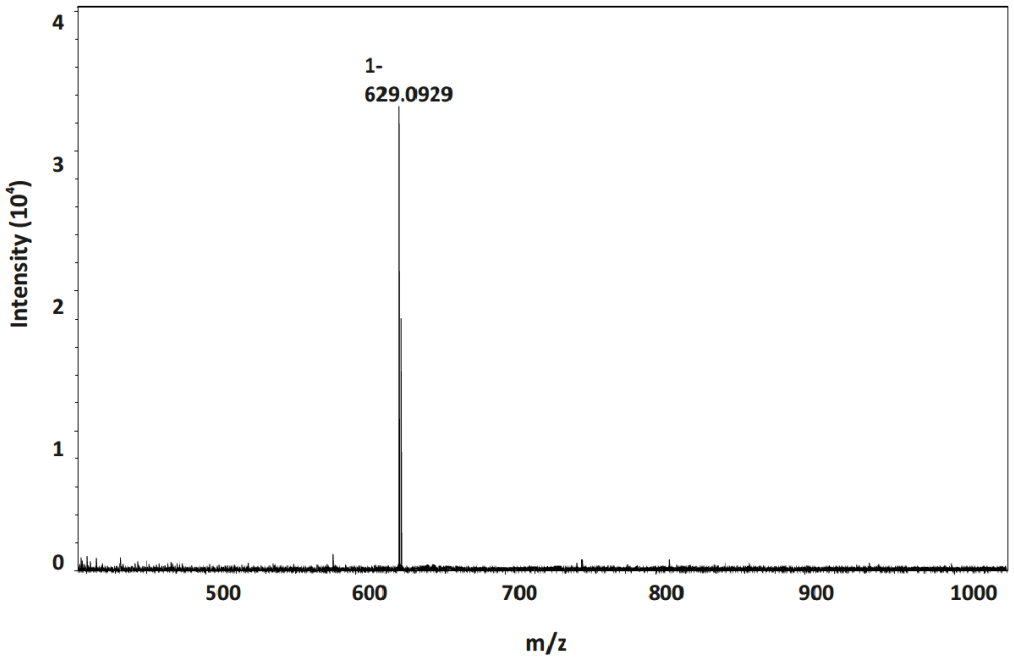
Interestingly, apart from the reported lethal dose values- (LD_{50} 25 mg/kg, oral) and (LD_{50} 25 mg/kg subcutaneous)- in mouse, its toxicological properties have not been thoroughly investigated (http://www.drugfuture.com/toxic/q51-q357.html). Only one preliminary

short report was found in literature on the antimicrobial effectiveness of thiolutin in the treatment of tinea capitis (fungal infection of the scalp) (Franks 1952) in which sixteen cases were treated by application twice daily of an ointment containing 0.2% of thiolutin. All cases were cured after an average of six weeks, except two cases involving *Microsporum audouini* infections. It was reported in the study that almost all patients treated exhibited a strong inflammatory reaction two to three weeks after administration of the ointment. However, after the treatment was stopped for seven to ten days, thiolutin ointment was reapplied, and no significant adverse effects was observed for the reminder of the treatment period. Indeed, the lack of thiolutin specificity may have been encountered in the development as an antimicrobial. This single report highlights the therapeutic potential of this class of antibiotics in the treatment of difficult to treat infections. It is clear that the prospect for development of this class will depend upon elucidating their precise mode of action in bacteria and determining whether they are truly specific for prokaryotic organisms. Thiolutin and holomycin, both likely share the same mode of action. However, some differences between the two include the selectivity ratios were observed (Oliva et al. 2001). Judging from this, holomycin should be a better choice for future studies and therapeutic use.

The literature has provided evidence that suggesting the involvement of the polyamines, spermidine and putrescine, in the control of RNA synthesis of *E. coli* in which 90% of spermidine exist as polyamine-RNA complexes (Lightfoot and Hall, 2014). Therefore, I hypothesize that thiolutin- in a reduced form- may interact with polyamines and inhibit of RNA synthesis by indirectly affecting the regulation of RNA synthesis. Further investigation of the role of polyamines in the mode of RNA inhibition by DTPs in *E. coli* is therefore warranted.

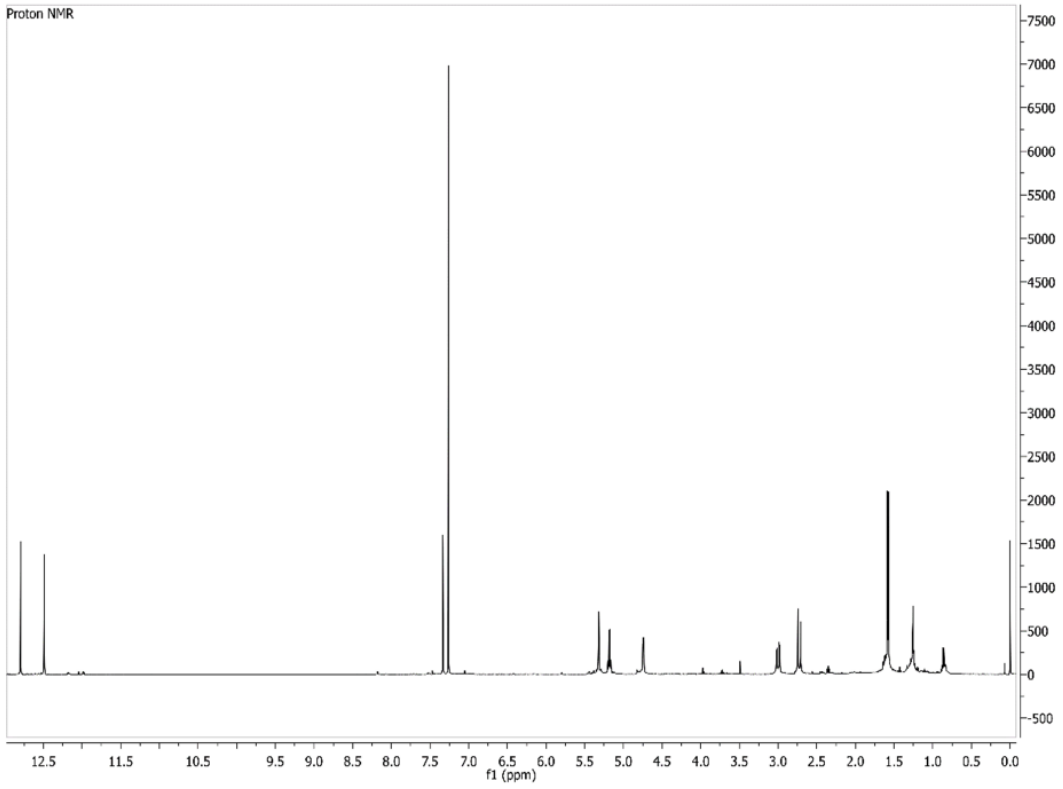
Appendices

Appendix A1



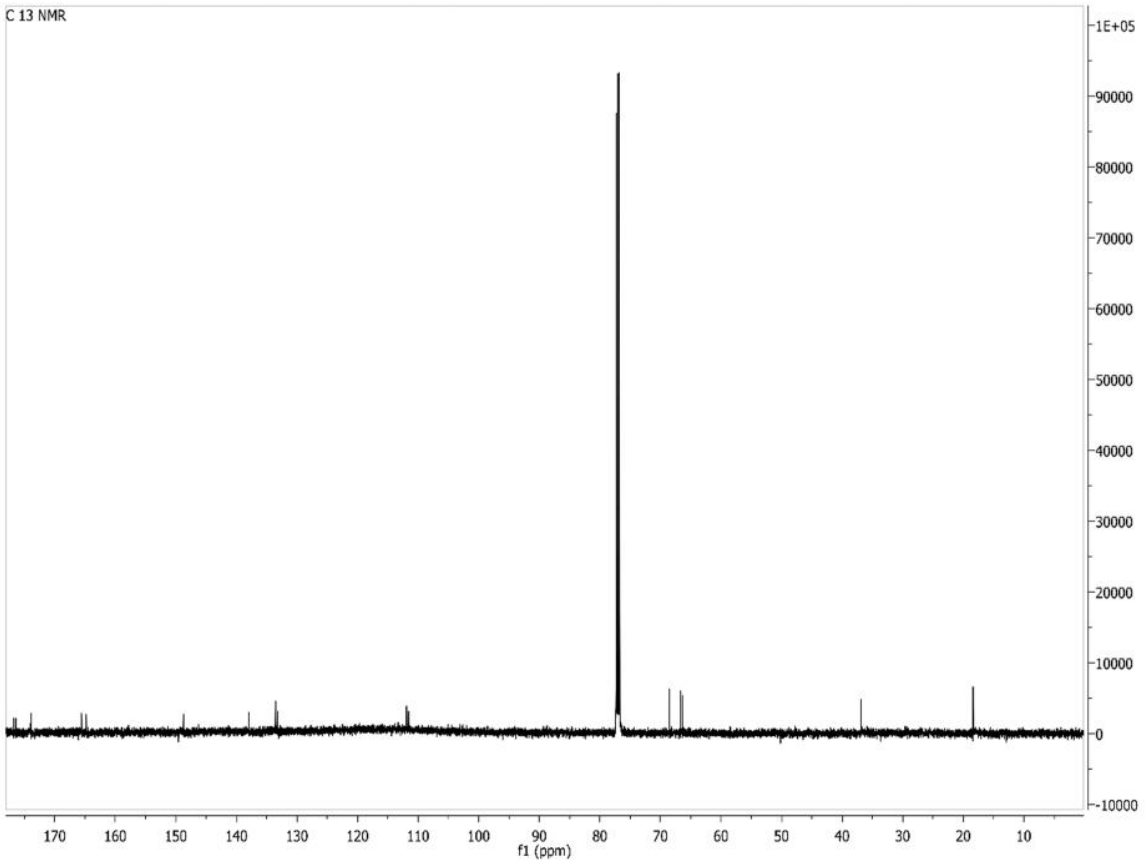
The accurate mass spectrum for γ-actinorhodin.

Appendix A2



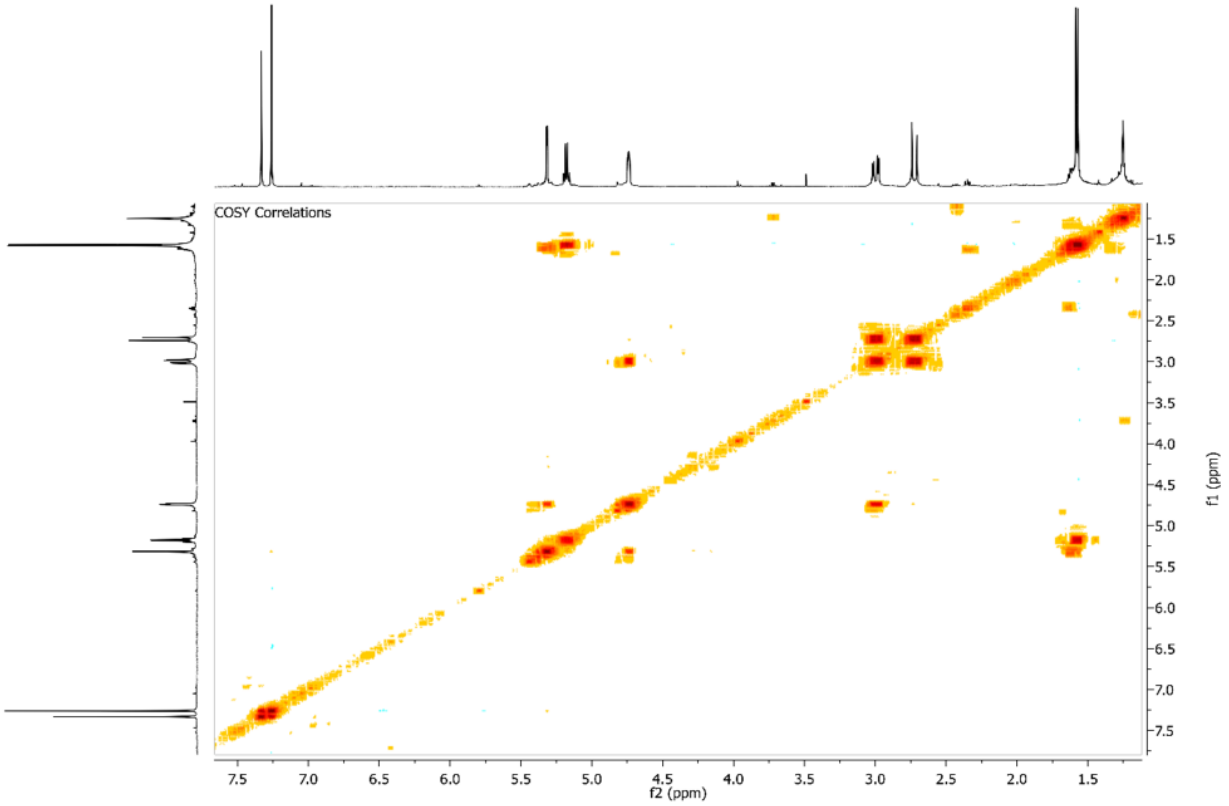
^{1}H NMR spectrum for γ-actinorhodin

Appendix A3



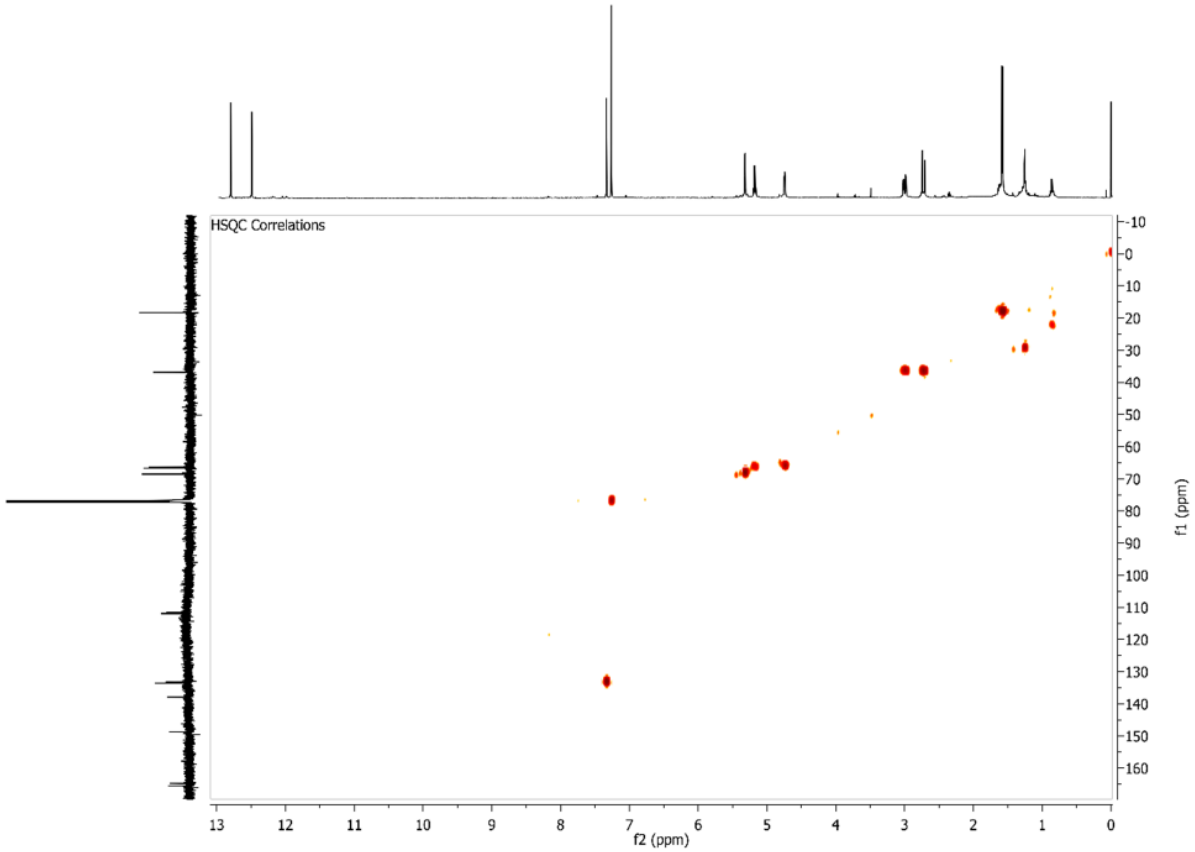
^{13}C spectrum for γ-actinorhodin.

Appendix A4



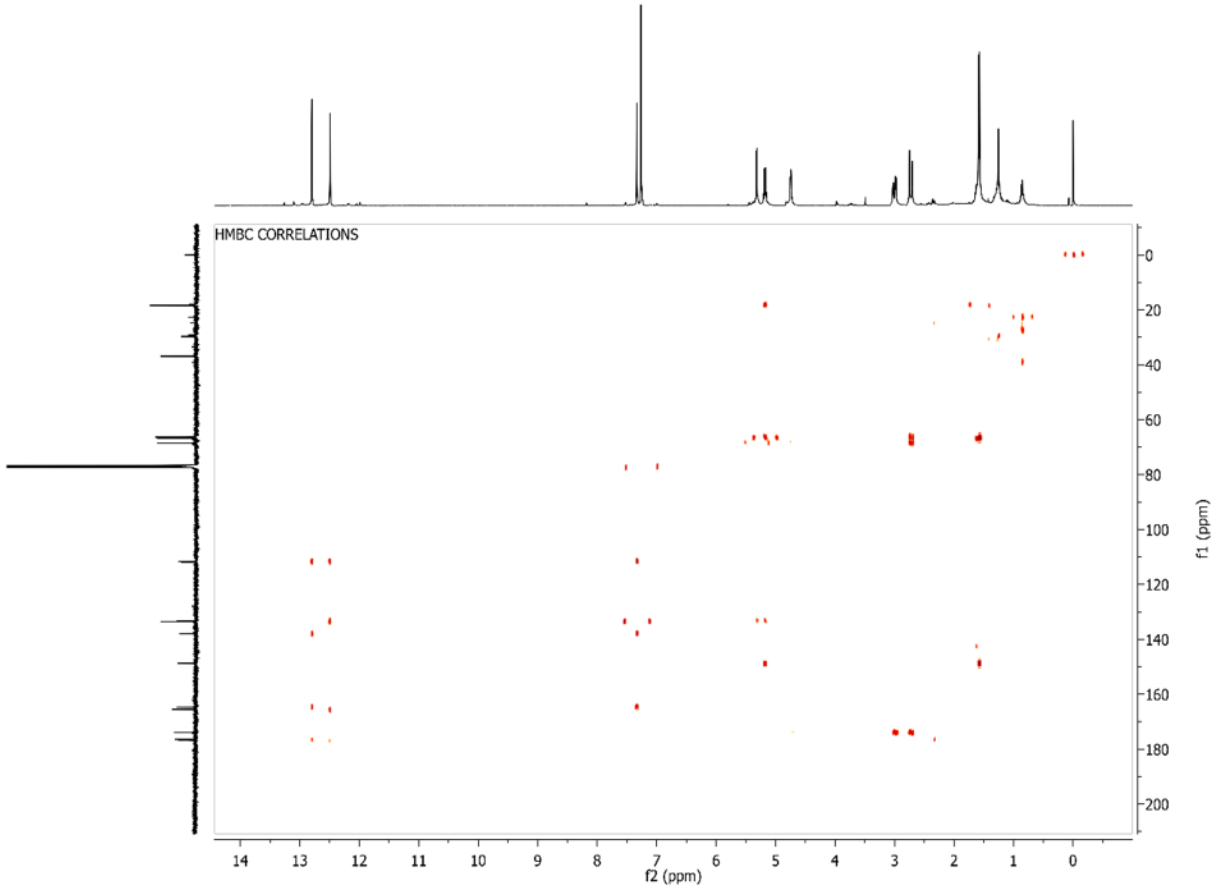
^{1}H-^{1}H COSY spectrum for γ-actinorhodin.

Appendix A5



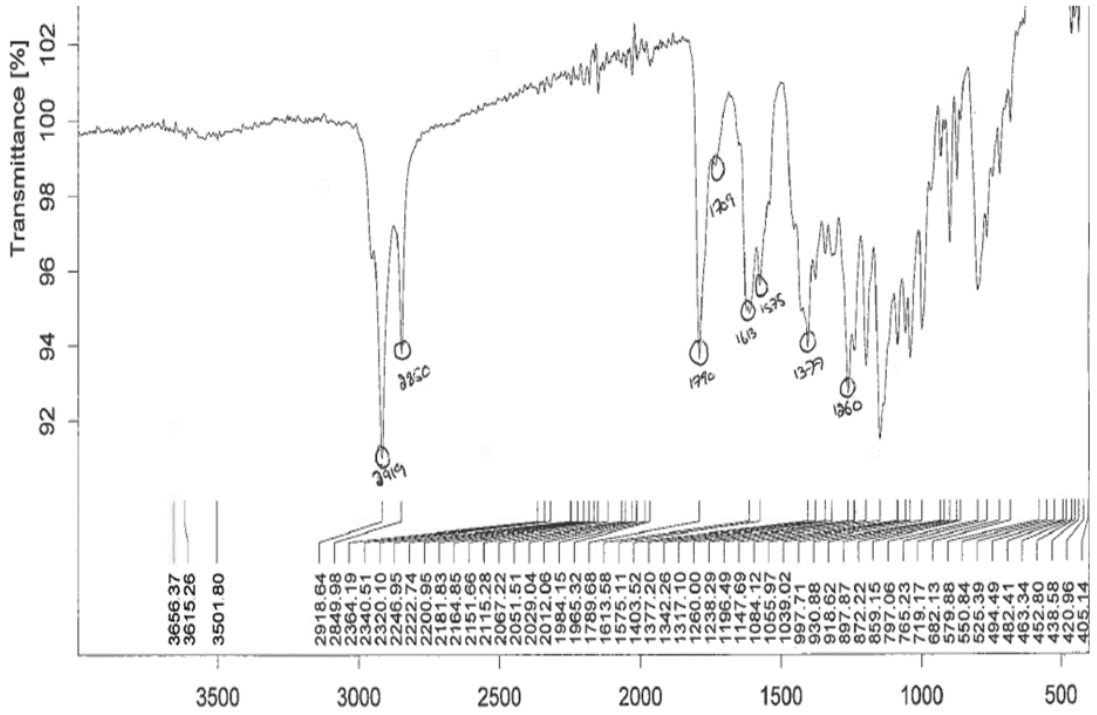
^{13}C-^{1}H HSQC spectrum for γ-actinorhodin.

Appendix A6



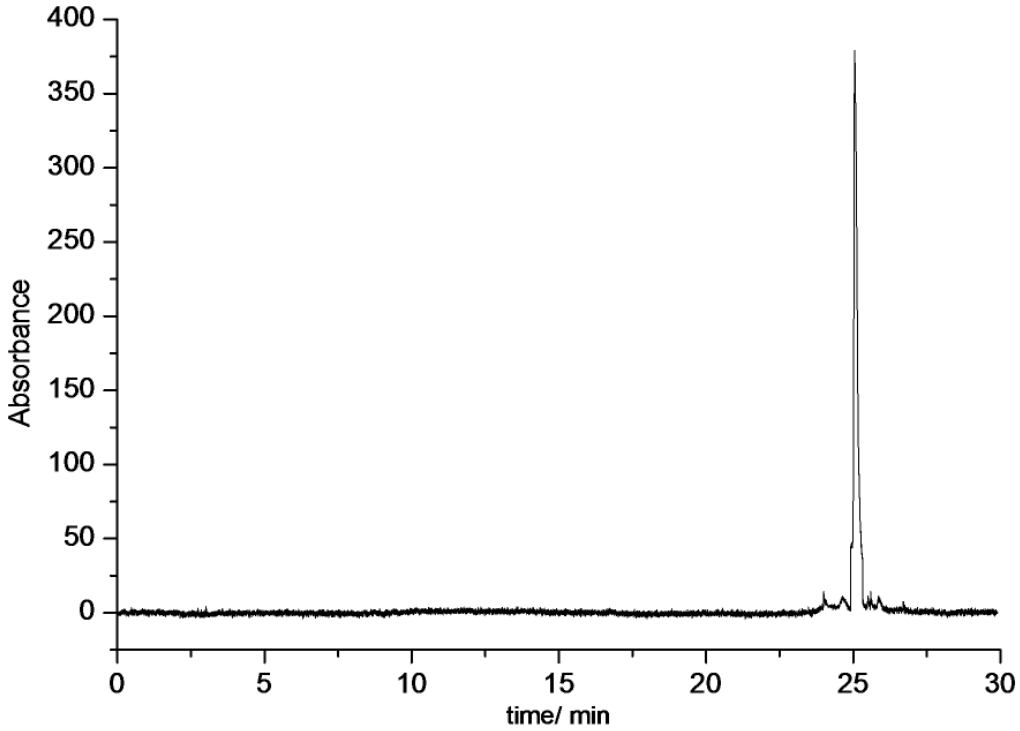
^{13}C-^{1}H HMBC spectrum for γ-actinorhodin

Appendix A7



IR spectrum for γ-actinorhodin.

Appendix A8



HPLC chromatogram recorded for γ-actinorhodin in acetone at 520 nm

Appendix B

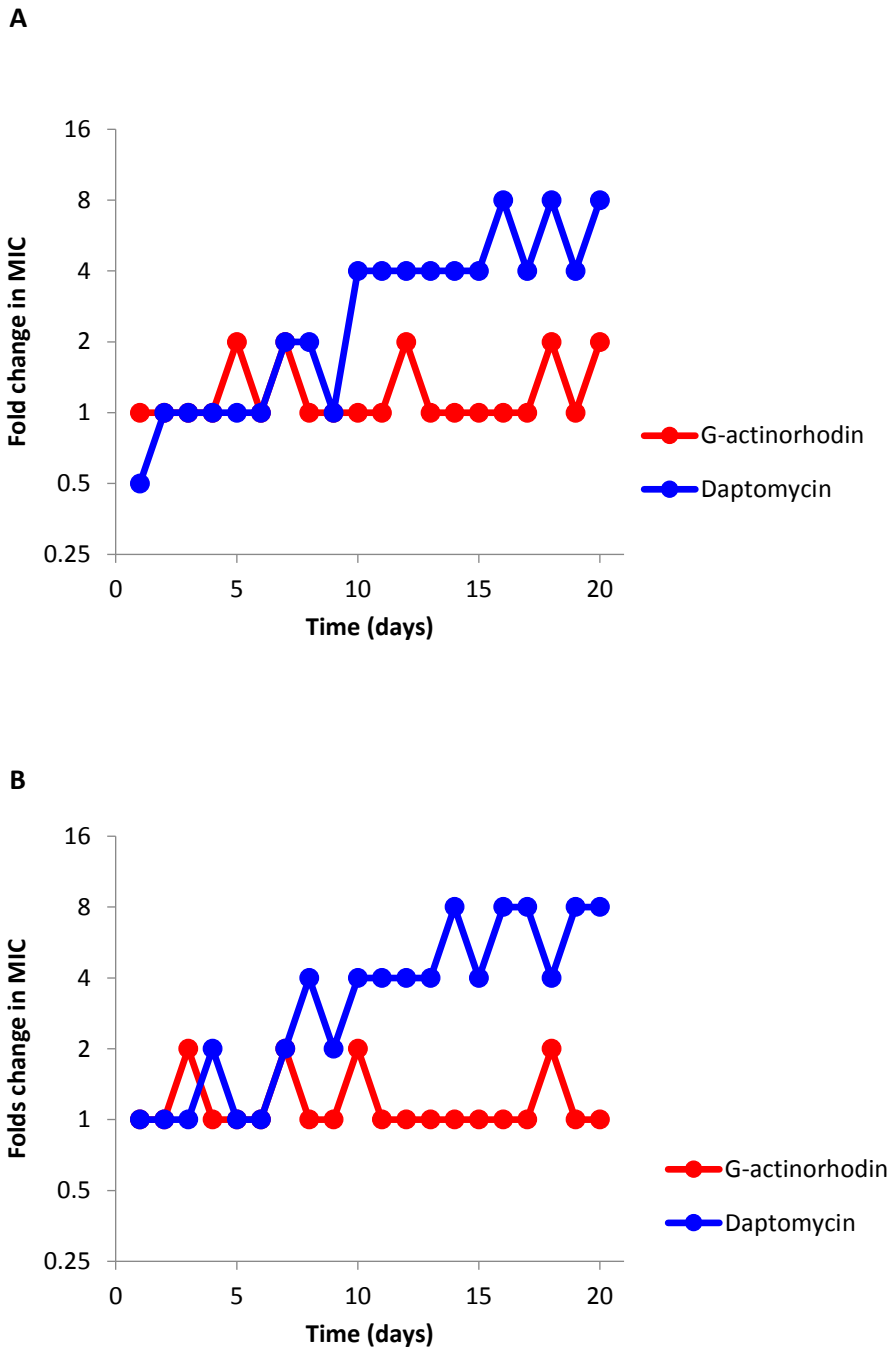
MICs of γ-actinorhodin against a collection of S. aureus clinical isolates

Strain name	Antibiotic susceptibility	MIC (μg/Ml) γ-actinorhodin
G1	MSSA	1
G2	MSSA	2
G4	MSSA	1
G5	MSSA	2
G6	MSSA	1
G9	MSSA	1
G10	MSSA	1
G11	MSSA	2
G12	MSSA	1
G13	MSSA	1
G15	MSSA	2
G16	MSSA	2
G17	MSSA	1
G18	MSSA	1
G19	MSSA	2
G20	MSSA	1
G22	MSSA	1
G23	MSSA	1
G24	MSSA	1
G25	MSSA	2
G26	MSSA	1
G27	MSSA	2
G28	MSSA	2
G29	MSSA	1
G30	MSSA	1
G31	MSSA	1
G32	MSSA	2
G33	MSSA	1
G34	MSSA	2
G35	MSSA	2
Oxford	MSSA	2

Strain name	Antibiotic susceptibility	MIC (µg/Ml) γ-actinorhodin
R24	MRSA	2
R25	MRSA	1
R26	MRSA	2
R27	MRSA	1
R28	MRSA	2
R29	MRSA	1
R30	MRSA	1
R31	MRSA	1
R32	MRSA	2
R33	MRSA	1
R34	MRSA	1
R35	MRSA	2
R36	MRSA	2
R37	MRSA	2
R38	MRSA	2
R39	MRSA	2
R40	MRSA	2
12232	MRSA	2
12233	MRSA	1
W71	MRSA	1
W74	MRSA	2
W80	MRSA	2
W82	MRSA	1
W85	MRSA	2
W96	MRSA	2
W97	MRSA	1
W98	MRSA	1
W99	MRSA	2
EMRSA 15	MRSA	2
EMRSA 16	MRSA	2
EMRSA 17	MRSA	2

Strain name	*Antibiotic susceptibility*	*MIC (µg/Ml)* γ-actinorhodin
VISA 2	VISA	2
Mu3	VISA	4
New jersey	VISA	2
Mu50	VISA	4
V99	VISA	2
Michigan	VISA	2
VISA 26	VISA	2

Appendix C



Resistance acquisition during Serial passage experiments with *S. aureus* SH1000 in the presence of sub-MIC levels of γ-actinorhodin and daptomycin from two experiments A and B.

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