GLYCOPEPTIDE RESISTANCE AND TAILORING ENZYMES

EXPLOITING GLYCOPEPTIDE TAILORING ENZYMES AS AN APPROACH TO OVERCOME RESISTANCE

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ABSTRACT

Microbial resistance to antibiotics has become a global problem and it is present not only in the clinic but in the community and throughout nature. There is a dire need for the discovery and creation of novel antimicrobials to combat life threatening infections with emerging resistant organisms. The glycopeptide antibiotic vancomycin is used as front line treatment for serious Gram-positive infections. Resistance to this drug is widespread in the environment and numerous resistant phenotypes are found in clinical pathogens. Three genes are essential for resistance, *vanHAX*, which are controlled by a two-component regulatory system VanR and VanS. Rather than rely on the discovery of a novel antibiotic and then wait for the emergence of inevitable resistance, previously described natural products such as those in the vancomycin class can be re-purposed to overcome resistance.

Here, glycopeptide resistance is found to be ancient and very diverse in the environment. The D-Ala-D-Lac ligase VanA is required for restructuring the glycopeptide target of aborning peptidoglycan pentapeptides. A *vanA* open reading frame from 30 000 yr old DNA was identified and the enzyme was shown to be as functional as comparable to modern day VanA homologs.

In the environment resistance is shown to be diverse and widespread. In the organism *Desulfitobacterium hafniense* Y51 VanH was shown as non-essential in conferring inducible resistance. Furthermore in the glycopeptide producer *Amycolatopsis balhimycina* harboring the classic *vanHAX,* a functional VanA homolog is described as an orphan gene outside of any recognizable gene cassette . Orthologonal approaches to overcome resistance are discussed.

Glycopeptides are generated from large biosynthetic gene clusters in the genomes of members of the Actinomycete family. In addition, they are modified by a number of different tailoring enzymes. Of particular interest is the glycopeptide A47934, which is 'aglyco', and provides a novel scaffold for combinatorial biosynthesis with tailoring enzymes found in other glycopeptide biosynthetic clusters. In addition, A47934 is sulfated- and the sulfotransferase StaL has been well characterized. StaL will transfer not only a sulfate group to A47934, but a sulfamide and fluorosulfonate group.

Focusing on additional tailoring enzymes, the biosynthetic cluster of the sulfated glycopeptide UK68597 was sequenced. This cluster has provided a resource for glycopeptide tailoring enzymes for use to modify the A47934 backbone. Sulfation was the first focus and the substrate promiscuity was exploited to expand the chemical diversity A47934 and vancomycin. This work has led to the discovery that glycopeptide sulfation will antagonize the activation and expression of *vanHAX*. A new sulfated vancomycin derivative was created with this same antagonizing activity. Of particular interest is the loss of resistance activation by vancomycin-SO_3 in the clinical pathogen *Enterococcus faecium* of the VanB phenotype (sensitive to teicoplanin while resistant to vancomycin). This phenotype allowed for a simple assay to be developed to measure VanS binding and activation of resistance genes in environmental and clinical organisms. Implications of these results and the further use of tailoring enzymes to modify glycopeptides to antagonize resistance will be discussed.

ACKNOWLDEGMENTS

First and foremost I must acknowledge the guidance and support from my supervisor Dr. Gerry Wright. Working with you and fellow members of the Wright lab, my like for science has turned into a true passion and love. Thank-you for encouragement when experiments were failing and pushing me to think creatively. To my colleagues, thank-you for making our working environment fun, light-hearted and supportive. I am lucky to have been surrounded by so many intelligent and passionate people from which have greatly contributed to parts of this thesis. I must pay special thanks to Dr. Kalinka Koteva for working so closely with me the last five years, we will always be “sisters in misery and joy”. My committee members Dr. Marie Elliot and Dr. Brian Coombes have provided countless pieces of advice and guidance in both my research and career development, thank-you. I would also like to acknowledge financial support from a CIHR Canada Graduate Doctorate Award.

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I would like to dedicate this work to my grandmothers Marie Steeves and Adela Kalan. I am thankful everyday to have both of you still in my life.

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LIST OF ABBREVIATIONS

ASTIV	Aryl sulfotransferase IV
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHI	Brain heart infusion
CLSI	Clinical and Laboratory Standards Institute
D-Ala-D-Ala	D-alanyl-D-alanine
D-Ala-D-Lac	D-alanyl-D-lactate
BLAST	Basic Local Alignment Search Tool
DHPH	3,5-dihydroxyphenylglycine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
EDTA	Ethylenediaminetetraactetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPG	Hydroxyphenylglycine
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
kPSI	Kilo pound per square inch
LB	Luria-Bertani
LC/ESI-MS	Liquid chromatography/Electro Spray Ionization Mass Spectrometry
Mb	Mega base
MIC	Minimum inhibitory concentration

MDR	Multi-drug resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
MWCO	Molecular weight cutoff
NMR	Nuclear Magnetic Resonance
NP	Natural product
NRPS	Non-ribosomal peptide synthetase
NRRL	Northern Regional Research Laboratory
PAP	3'-phosphoadenosine-5'-phosphate
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PK/LDH	Pyruvate kinase – lactate dehydrogenase
PNPS	*p*-nitrolphenylsulfate
PNPS-NH_2	*p*-nitrolphenylsulfamic acid
PNPS-F	*p*-nitrolphenylfluorosulfonate
RE	Restriction enzyme
Rf	Retention factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SET	Salt-EDTA-Tris
TLC	Thin-layer chromatography
VRE	Vancomycin-resistant *Enterococc*i
WAC	Wright Actinomycete Collection

1.0 Introduction

1.1 Natural Products

1.1.1 The Beginning:

Bioactive and naturally derived small molecules or natural products (NPs) play a seminal role in evolution. Our understanding of that role is still naïve; however, before the time of Charles Darwin and before we cared about evolution, humans have exploited NPs for their own use. Prior to 1500 B.C., the ancient Egyptians documented a pharmacopeia listing over 800 prescriptions containing mixtures of natural ingredients to treat a vast repertoire of ailments (5). Jumping ahead to 2000 years ago, in his famous book De Materia Medica, Dioscorides described a tea made from willow bark (Fig. 1.1) used to treat various types of pain and gout. Willow bark is the natural source of salicyclic acid and the scaffold of the highly successful pain medication aspirin. This encyclopedia describes numerous other natural remedies to treat conditions ranging from ulcers to urinary problems to the common cold and more (3). Dated to the same time period, Nubian mummies have been discovered with significant

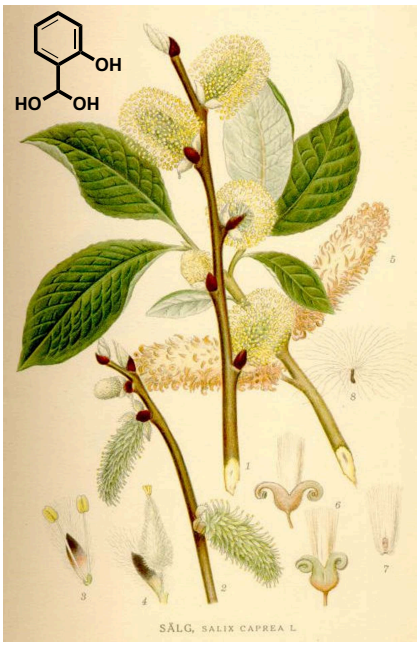


Figure 1.1: *Salix caprea*, common name goat willow and salicyclic acid (inset). Image taken from De Materia Medica (3)

quantities of the antibiotic tetracycline in their bones, thought to be deposited by ingestion of grains contaminated with tetracycline-producing streptomycete bacteria (6).

In North America, native groups have described over 2500 species of plants used medicinally, many of which have provided a foundation for modern pharmacotherapy. For example, taxol, one of todays most successful cancer drugs is derived from a natural source (the Pacific Yew tree), which has been described by the Tsimishian natives of British Columbia for use to treat internal ailments and cancer (7).

These examples pre-date modern medicine but still represent a relatively recent timeframe in human evolution, however there are controversial examples of early humans using plants for medicinal purposes. While the above examples exemplify documented uses of crude NPs, there is evidence to suggest the use of NPs as far back as 3350 B.C. One of most famous cases is the fungus of the Iceman. This frozen corpse discovered in Austria has been dated to between 3350 and 3100 B.C. Among everyday items discovered such as an axe and satchel, three distinct fungal specimens were fully preserved with the body (1). Two fruiting bodies of fungi were carefully mounted on leather straps while the third was intricately associated with a leather girdle bag containing flint tools (Fig. 1.2). Debate pertaining to the use of the fungus as tinder, a medicinal treatment, or both is apparent in literature (8-10), however it is important to consider that such a debate exists because the species of fungus discovered on the straps,

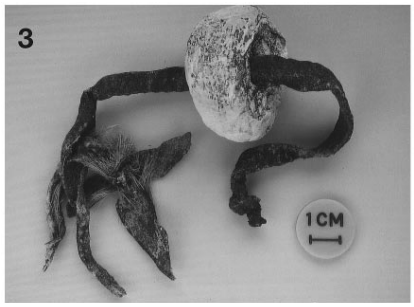


Figure 1.2: The iceman's fungi

Piptoporus betulinus, produces known pharmacologically active substances (1, 9, 11, 12).

The roots of modern medicine have grown from the experience of native cultural groups that utilize plants, fungi and bacteria to influence the human condition and as a result the vast majority of all pharmaceutically pure drugs are derived or designed in some part from a naturally occurring precursor.

1.1.2 The Recent Past:

In order to fully appreciate the infinite diversity of NPs one must look to the source, nature. Plants have been on earth far longer than humans (> 400 million years vs. 50 000 years for modern humans (13) thus it's reasonable to argue that plant NPs are produced for some benefit to the host and as it was stated famously "Nature does nothing uselessly" [Aristotle, 384-322 BC]. Scientists have identified what many of these uses are, for example nicotine in tobacco leaves is actually produced as a natural insecticide to the plant but humans have exploited it for other uses (14-16). Each of the instances cited above describe a natural product that has been harnessed by humans in a "non-natural" way, and recent data (which will be discussed throughout this thesis) have provided clues that there are not only new useful NPs to identify but their natural evolutionary roles are of profound ecological significance.

Most NPs are classified as secondary metabolites, which are traditionally categorized based on essentiality to the producing organism. Where primary metabolites are essential to cellular growth and regeneration, secondary metabolites are dispensable and often confer either a niche or evolutionary advantage. Plant-derived NPs have been the focus of discussion so far in this thesis and their contribution to medicine is immeasurable, however when

contemplating a drug discovery program it is often challenging to obtain appreciable quantities of the desired chemical without destroying large masses of plant material for extraction. This is particularly detrimental when the target plant is a slow growing and old organism like the Yew tree; 1500 trees were killed at the peak of demand (17). Supply becomes an issue and plant genetics are complicated when trying to manipulate plant tissue culture to produce the compound of interest in a laboratory.

Plants aren't the only organisms to produce bioactive molecules and indeed the enormous chemical diversity of biologically active NPs from different kingdoms of life fully began to be understood and exploited in an efficient manner with the discovery of an anti-Staphylococcal small molecule produced by a species of the *Penicillium* fungus. This famous discovery occurred in the laboratory of Sir Alexander Fleming in 1928 and he named the active substance "Penicillin" (18). Figure 1.3 was taken from his Nobel Laureate lecture, and one of the most striking features is the obvious inhibition of *Staphylococcus* but not *Bacillus violaceus.* His simple method of streaking two organisms on an agar plate to look for inhibition of growth is still commonly used today. Fleming's discovery has clearly changed how infectious disease is treated, however he was never able to produce the active substance in purity to deduce it's chemical nature. This has two important historical implications. First, it took nearly ten years before the compound was isolated by Dr. Norman Heatley working with Dr. Ernst Chain and Sir Howard Florey at the University of Oxford

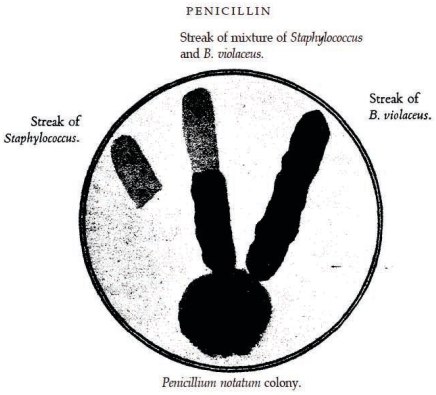


Figure 1.3: Activity of *P. notatum* fungus against *Staphylococcus*. Presented by Fleming in his Nobel lecture (2).

(19). It was at this time the first experiments in mice were done demonstrating *in vivo* efficacy, followed by successful treatment of human patients (20). The later success of penicillin is so well known its details won't be further discussed here in order to highlight the significant discoveries made during the time gap between discovery and clinical use of penicillin.

During the ten year gap between penicillin's discovery and application in medicine another scientist, Dr. Selman Waksman, was studying soil microbial communities and particularly key group of bacteria from the phyla Firmicutes and Actinomycetes. In 1939, one of Waksman's former graduate students discovered tyrothricin, a mixture of the antibiotics tyrocidin and gramicidin by directly adding pathogenic cells to soil samples and looking for death (21). Both of these agents are toxic when administered in mammals and since penicillin was shortly thereafter shown to be safe, it diminished the excitement surrounding the finding. Waksman however began to hypothesize that chemical warfare was ongoing in soil ecosystems and proposed the soil as a natural reservoir of antimicrobial agents. He defined the term "antibiotic" as a chemical agent produced by one microorganism to destroy another microorganism (22). His forward thinking completely changed the face of modern medicine and placed Actinomycetes and Firmicutes on the forefront in the battle of infectious disease . Directly looking to the soil and these bacteria for pathogen killing initially yielded results similar to the tyrothricin story, all were toxic in mammals. Still, Waksman wasn't discouraged and in 1943 Streptomycin was discovered (23). It was active against both Gram-positive and Gram-negative pathogens including the infectious agent of tuberculosis (TB) and more importantly was relatively non-toxic and systemically bioavailable in humans (24). Now in the

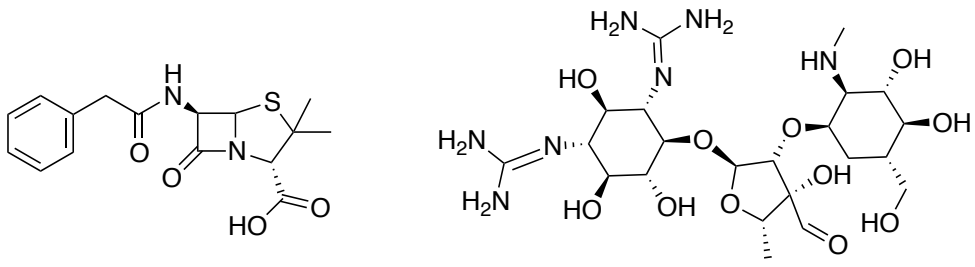


Figure 1.4: Chemical structures of the first two natural product antibiotics to be used clinically, penicillin and streptomycin

mid-1940s two chemical classes of antibiotics; penicillin and streptomycin (Fig.1. 4) became major products in the pharmaceutical industry (22).

Antibiotics have been heralded as the most important discovery of the twentieth century. However, it was the method of looking to fungus and then bacteria for chemically diverse, non-toxic NPs and then developing them into biological factories that has changed the face of drug discovery.

1.1.3 The Present:

Since the introduction of antibiotics nearly 70 years ago, our ability to cure bacterial infections has been taken for granted. The thirty years following the discovery of penicillin are often referred to as the “Golden Age” of antibiotic discovery in which most of the clinically relevant antibiotics used today were discovered. This includes numerous iterations of the ß-lactam class of molecules to which penicillin belongs, tetracyclines, aminoglycosides, macrolides, glycopeptides and others. The introduction of antibiotics was so impactful that the US Surgeon General announced in 1969 “We can close the books on infectious disease” (25). However in the present day the efficacy of many antibiotics has waned and further misappropriation of antibiotics is contributing to their demise.

Why was the surgeon general off the mark? To begin one must think about the natural context of antibiotics. Their success relies on the ability to target bacterial specific architectures. If a bacterium is producing a molecule targeted to kill another bacterium, how does it save itself? "Self-resistance" is the ability of a producing organism to evade the inhibitory action of its own antimicrobial and often resistance to antibiotics produced by others in its niche. Resistance to penicillin and streptomycin have been described since they were introduced (2, 18, 26). In environmental organisms it is common to find resistance to up to 14 different antibiotics and on average, resistance to 7-8 irrespective of whether they are a producer or not (27)! It is now understood that the environmental reservoir of antibiotic resistance genes is vast and both a natural and ancient phenomenon (28-30). Unfortunately multi-drug resistance (MDR) in clinical pathogens has also become the norm as emergence of multi-, extensively- and totally drug resistant pathogens is increasing (31-33).

Since the end of the Golden Age (~1960) an innovation gap has occurred with few novel antimicrobials being released for clinical use. Conversely, the –omics era has allowed a deeper understanding of NP biosynthesis and mechanisms of resistance. New technologies in DNA sequencing have driven down the cost resulting in a rapid expansion in the number of sequenced microbial genomes (34). From this genetic information we have learnt that bacteria of the *Streptomyces* genus have the capacity to produce not one or two bioactive natural products but on average 20-25! (35) Dissection of the biosynthetic pathways have taught us how these complex molecules are built and with that understanding, new synthetic biology approaches can be pursued to further expand the chemical diversity of natural products. The plethora of

resistance genes can be characterized to understand how they are regulated, what their function is, and how they interact with their cognate antibiotic.

Great advances have been made since the days of Fleming and Waksman 70 years ago. Their groundbreaking work has provided the foundation for future drug discovery. Antibiotic production and resistance are intimately associated and not only understanding but appreciating that association will facilitate the discovery and creation of new chemical entities with predictable resistance patterns. This thesis will explore the evolution of environmental resistance, characterize its function at a molecular level and use that information to design novel antibiotic derivatives focusing on the glycopeptide class of antibiotics as a model.

1.2 Glycopeptide Antibiotics

1.2.1 Chemical Diversity:

The glycopeptide antibiotics are a medically important class of natural products. Two major sub-classes, type I (vancomycin, with an N-terminal Leu-ß-hydroxy-3-chloroTyr-Asn tripeptide) and type II (teicoplanin, with an N-terminal hyroxyphenylGly-3-chloroTyr-3,5-dihydroxylphenylGly tripeptide) (Fig. 1.5) have been categorized based on their structural features (36). There are numerous members in this class of antibiotics but they all share common topographies including a heptapeptide backbone cross-linked by oxygenases yielding a cup-shaped scaffold.

Differences in this basic scaffold lie in the degree of modification by tailoring enzymes that glycosylate, halogenate, methylate, acylate, and sulfonate the antibiotic. Regardless of class or degree of modification all glycopeptides act by binding the terminal D-alanyl-D-alanine dipeptide in peptidoglycan precursors through a series of five hydrogen bonds (Fig. 1.5, inset). The bulky molecule efficiently arrests transglycosylation and transpeptidation reactions thereby blocking cell wall synthesis, which results in cell death (37, 38). Vancomycin and teicoplanin are the only members approved for clinical use but the erosion of their efficacy has been exemplified by outbreaks of resistant infections worldwide (39-43). Dalbavancin (Zeven, Durata Therapeutics) (44, 45), telavancin (Vibativ, Theravance) (46) and oritavancin (Medicines) (47) represent a class of second generation glycopeptide drugs targeted for treatment of serious Gram-positive infections such as vancomycin resistant *Enterococci* (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA). Each derivative shows improved

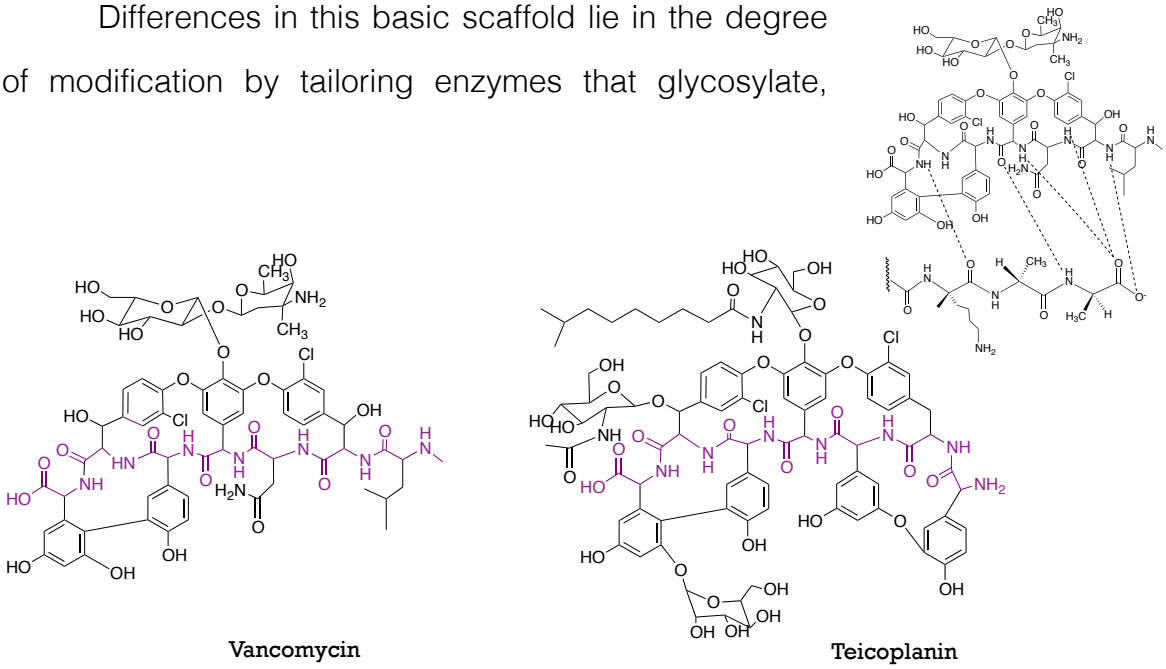


Figure 1.5: Structures of vancomycin and teicoplanin. The heptapeptide scaffold is highlighted. Inset demonstrates hydrogen bonding to target D-alanyl-D-alanine.

activity against VRE as the acyl chain is thought to anchor the drug in the membrane, increasing the local drug concentration at the site of action. Furthermore, Kim *et al*. (47) have shown that oritavancin has a dual mode of action binding both the D-Ala-D-Ala precursor and the pentaglycine bridge that gives the peptidoglycan layer its rigidity and strength in the *S. aureus* cell wall (47-49). As seen in figure 1.6, each of these molecules are lipoglycopeptides, semi-synthetic derivatives of teicoplanin, vancomycin and chloroeremomycin respectively, with N-acyl modifications at position 4 on the heptapeptide backbone. Of the three compounds described, televancin is the only one so far that has been clinically approved (2009) for complicated skin and skin structure infections (cSSSIs) primarily caused by MRSA (50). Unfortunately, VanA-type resistant enterococci are not susceptible to telavancin thus the glycopeptide resistance problem has not been resolved completely.

There have been a variety of approaches to modify glycopeptide antibiotics including multivalent vancomycins (51) and the use of tailoring enzymes such as the glycosyltransferases to expand and create new derivatives (52-55). Semi-synthetic approaches can limit the diversity

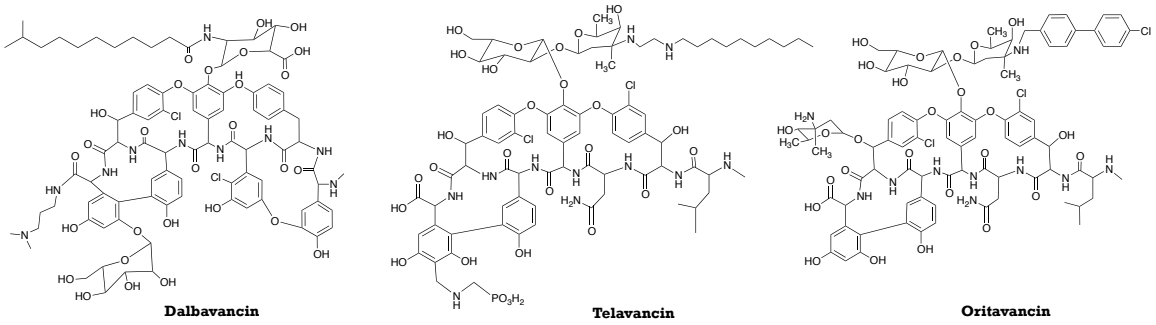


Figure 1.6: Structures of second generation glycopeptides Dalbavancin, Televancin and Oritavancin.

achievable as it is difficult to mimic natural product chemistry. Therefore, exploitation of the biosynthetic machinery in producing organisms is becoming an attractive approach.

1.2.2 Resistance:

Glycopeptide resistance involves cell wall reprograming by replacing the terminal D-alanyl-D-alanine with D-alanyl-D-lactate, resulting in loss of a single hydrogen bond between the antibiotic and cell wall precursor, and subsequent 1000-fold reduction in binding affinity. Three genes are vital to resistance: *vanH* encoding a D-lactate dehydrogenase, *vanA* encoding a D-Ala-D-Lac ligase and *vanX* encoding a D-Ala-D-Ala dipeptidase that prevents existing D-Ala-D-Ala from being incorporated. *vanHAX* are tightly regulated by a two-component regulatory system, VanR, a response regulator and VanS, a sensor kinase (38, 56, 57). The *vanHAX* cassette is widely dispersed in the environment and will be discussed in detail in chapter two. Importantly, glycopeptide resistant infection is a major nosocomial problem, with limited treatment options.

1.2.3 Biosynthesis:

Glycopeptides are made via large multi-subunit non-ribosomal peptide synthetases (NRPS) (36). In producing organisms, gene clusters typically contain most of the enzymatic machinery necessary to build a heptapeptide scaffold along with multiple tailoring enzymes that decorate the molecule. Contributing to the chemical diversity is the ability of NRPS modules to incorporate unusual amino acids such as hydroxyphenylglycine (HPG) or ß-hydroxytyrosine. Genes necessary for crafting them are contained within the biosynthetic clusters (58, 59). Anecdotal evidence has speculated that the frequency of glycopeptide discovery to be $1x10^{-5}$ (60), however new screening

approaches now estimate the frequency to be much higher, closer to $1x10^{-2}$ (private communication from Dr. Maulik Thaker, McMaster University). Furthermore, genome sequencing has allowed new clusters to be rapidly identified which further uncovers chemical diversity.

Of particular interest to us are uncharacterized modifications performed by novel tailoring enzymes found in glycopeptide biosynthetic clusters. Chapters three and four of this work provide evidence that alterations to the glycopeptide scaffold result in measurable changes in activity. This includes not only antimicrobial action but the apparent ability to modulate resistance. In order to maximally exploit these enzymes, a platform must be established to quickly evaluate the biochemistry and selectivity of each enzyme. The enzymes can be used in a combinatorial fashion to create new glycopeptide congeners.

Streptomyces toyocaensis NRRL 15009 produces the glycopeptide A47934 (sub-class II) and its biosynthesis has been well characterized (61).

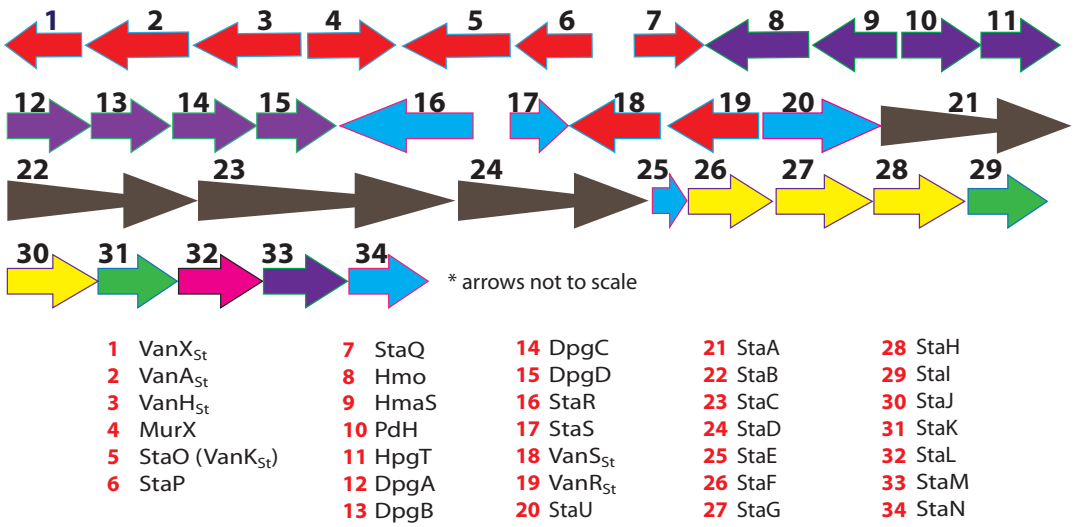


Figure 1.7: Biosynthetic gene cluster for A47934 produced by *S. toyocaensis.* Adapted from (4)

Figure 1.7 is a cartoon of the biosynthetic genes required for A47934 production in *S. toyocaensis*. A47934 is 'aglyco' but sulfated, and provides a novel scaffold for combinatorial biosynthesis work (62, 63). Genetic manipulation of *S. toyocaensis* has been optimized in our lab, and we are able to quickly and efficiently delete and introduce new genes into the chromosome. This has provided an optimal platform for a synthetic biology approach toward expanding the chemical diversity of glycopeptide antibiotics. Work by Baltz and colleagues (64) and more recently Brady *et. al*. (65) have already established a proof of principle and we wish to further develop this system by engineering *S. toyocaensis* for maximal production and straightforward combinatorial biosynthesis.

A solid framework has been established for this method which will be discussed in detail in chapter 3. Seemingly small changes to a chemical scaffold can result in significant changes in bioactivity and desirable properties. The work described here outlines the diversity and function of glycopeptide resistance and provides novel evidence that resistance can be overcome using tools at hand without discovery of a brand new class of antibiotic.

1.3 Rationale and Scope of This Work

1.3.1 Evaluating Glycopeptide Resistance in the Environment

Between 2007 and 2012, the use of vancomycin in the clinic changed status from "drug of last resort" to "frontline treatment" in serious Gram-positive infection. Outbreaks of VRE in hospital wards have been repeatedly reported and little work on second generation glycopeptides has been accomplished since telavancin, ortitavancin and dalbavancin were described. At the same

time, the rise of genome sequencing has identified the true ubiquity of glycopeptide resistance in the environment. Gene annotation is largely based on the closest hit in a BLAST search often resulting in incorrect annotations. We believe it is important to verify the true function of annotated resistance genes to gain a better understanding of how widespread such genes exist in the bacterial population. This will aide in proposing both the evolutionary history and perhaps advantages of harboring such genes. Furthermore, there seems to exist an idea that antibiotic resistance arose solely from human use and misuse of antibiotics after their introduction. This misconception is understood by those who closely study natural products and antibiotic resistance, but there has never been concrete data to support the theory that resistance has been present since before humans discovered antibiotics and evolved in parallel with the biosynthesis of antibiotics.

Our approach is simple. First, look to ancient DNA for the presence of glycopeptide resistance genes. Second, biochemically characterize genes annotated as *vanA* to confirm their function. This in conjunction with a bioinformatics analysis to examine the context of resistance in genomes will provide a deeper understanding of the molecular mechanisms of regulation and inspire ideas to overcome clinic resistance.

1.3.2 Expand the Chemical Diversity of Glycopeptide Antibiotics

As discussed earlier, there are groups that have worked on creating derivatives of naturally occurring glycopeptides. These approaches have been based on modifying a single aspect of glycopeptide biosynthesis. For instance,

libraries of semi-synthetic vancomycin analogs have been reported using a technique referred to as glycorandomization (66). Out of 50 derivatives, only one showed improved activity relative to the parent vancomycin and the change in MIC was only two-fold better. Rather than approaching diversification with a brute force tactic, a rational hypothesis driven method will be ideal for identifying a larger percentage of molecules with improved activity. By focusing on the glycopeptide tailoring enzymes, the effect of specific spatial modifications will allow the prediction of favorable combinations. Combinatorial biosynthesis with tailoring enzymes on the desulfated scaffold of A47934 (DSA47934) is the ideal platform on which to develop this idea. Moreover, while each modification is directed, they are "natural" in the sense that we are using the enzymes on substrates they would encounter in nature. Sulfation of glycopeptides is a desirable starting point as the role of antibiotic sulfation has yet to be deduced. Little work has been done to determine the prevalence of glycopeptide sulfation, partially due to the lack of sequenced biosynthetic clusters. Resources available from the Wright Actinomycete Collection (WAC) have led to the identification of multiple glycopeptide producing organisms. This collection is an instrumental resource for collecting a diverse array of unique tailoring enzymes for this and future work. The aim will be to first establish a sound platform for combinatorial work in a host organism (*S. toyocaensis*) and to focus on characterization of sulfation.

1.3.3 Glycopeptide Modification to Modulate Resistance

A serendipitous discovery during this project was the inability of A47934 to induce resistance to teicoplanin in *Streptomyces coelicolor* while DSA47934 could. Could it be that sulfation prevents binding to VanS and the expression of

vanHAX? It has been established that the VanS sensor kinase responds directly to the presence of a specific glycopeptide (57), resulting in the various phenotypes described ($VanS_{Sc}$ responds to vancomycin but not teicoplanin). The molecular details of this interaction have not been uncovered. If sulfation prevents binding to VanS then this could serve as a survival strategy for the producing organism when nutrients are scare amongst close neighbors harboring *vanHAX.* If so, then evasion of resistance altogether is a unique strategy in the design of second generation glycopeptides. Most of the focus in the literature has been on the interaction of vancomycin with the cell wall with little regard to the interaction of the regulatory mechanism for resistance. Exploring this interaction fills a major gap in glycopeptide research and provides new avenues for drug discovery.

Chapter 2

CHARACTERIZING GLYCOPEPTIDE RESISTANCE: ENIVRONMENTAL DIVERSITY AND EVOLUTION

This chapter contains material in published in the following articles:

Kalan, L., Ebert, S., Kelly, T., and Wright, G. 2009. A non-canonical vancomycin resistance cluster from *Desulfitobacterium hafniense* Y51. Antimicrobial Agents and Chemo. 53(7):2841-2845

For this work I cloned, purified and performed biochemical analysis of all ligases reported. I carried out the qRT-PCR analysis of *vanA* gene expression and wrote the manuscript with my supervisor Dr. Gerry Wright.

D'Costa, V.D., King, C.E., Kalan, L., Morar, M., Sung, W., Schwarz, C,. Froese, G., Zazula, G., Calmels, F., Debruyne, G., Golding, B., Poinar, H.N., and Wright, G.D. 2011. Antibiotic Resistance is Ancient. Nature 477:457-61

For this work I had four putative ancient *vanA* genes synthesized and purified the enzymes, and characterized their biochemical functions. I helped with editing of the manuscript.

2.0 Chapter 2

2.1 Introduction

Antibiotic resistance is the ultimate antagonist to arguably the most important medical discovery of the 20th Century. Resistance to penicillin was identified before it achieved wide clinical use (2) and since then resistance to all classes of antibiotics has been described after clinical introduction. The cycle of introduction of new antibiotics or their derivatives following the emergence of resistance has characterized antibiotic use and discovery over the past half-century (29). For example, clinicians turned increasingly to the glycopeptide antibiotic vancomycin following the emergence of highly antibiotic (in particular ß-lactam) resistant strains of *Staphylococci* plaguing hospitals in the mid-80s (67, 68). Glycopeptides do not pass the cell membrane or bind a specific protein target but instead they bind to the murein sacculus and its precursors of Gram-positive cells. This property led to the belief that resistance would not develop without interfering with an essential cellular process; cell division. Moreover, lack of a protein target meant that mutations conferring resistance would not be easily selected for.

This was a time when the medical community thought infectious disease had been “beat” and only few saw the potential grave consequences if antibiotic resistance continued to develop. It wasn’t until nearly three decades after its discovery that vancomycin resistance was discovered in species of enterococci (69, 70), taking the scientific and medical communities by surprise (71). This

was partially because although vancomycin was discovered in 1953, it wasn't heavily used until the 1970s. Secondly, resistance to glycopeptide antibiotics is a highly complex mechanism requiring a tightly regulated operon of genes that specifically respond to different glycopeptide molecules in the surrounding

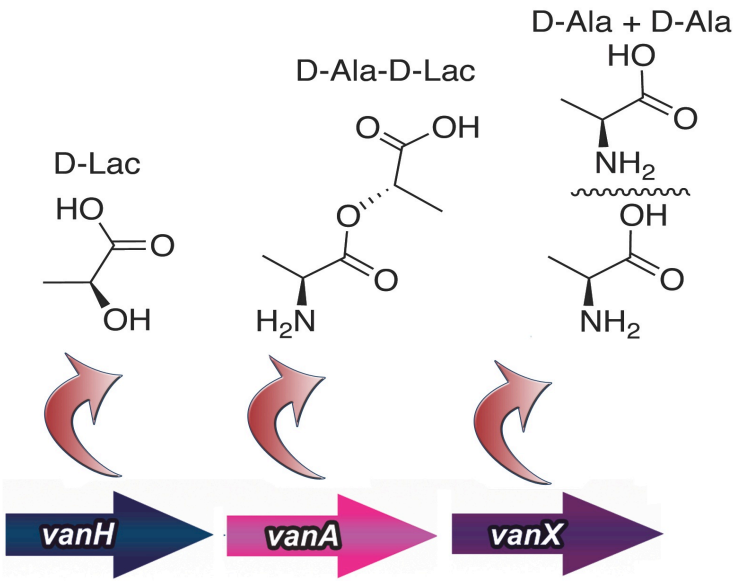


Figure 2.1: Function of VanHAX. VanH is a D-lactate dehydrogenase. VanA a D-Ala-D-Lac ligase and VanX a D-Ala-D-Ala dipeptidase.

environment (38, 72, 73). Glycopeptide resistance is fascinating because the cell re-structures the growing peptidoglycan chains to terminate with the depsipeptide D-alanyl-D-lactate rather than D-alanyl-D-alanine. Formation of both di-peptide and depsi-peptide precursors leads to redundant cellular activity that intuitively leads to fitness cost, hence the tight regulation. Maintaining the extra genetic information seems to be worthwhile because introduction of D-lactate results in formation of an ester rather than amide bond and subsequent loss of a hydrogen bond between the glycopeptide and pentapeptide chain. This coupled with

electronic repulsion between a backbone carbonyl oxygen of the glycopeptide with the ester oxygen render the cell highly vancomycin resistant. Three genes are vital to resistance; *vanH* encoding a D-lactate dehydrogenase, *vanA* encoding a D-Ala-D-Lac ligase and *vanX* encoding a D-Ala-D-Ala di-peptidase that prevents existing D-Ala-D-Ala from being incorporated (Fig. 2.1). This cassette is usually accompanied by a two-component regulatory system (*vanS/R*) that controls transcription.

Although vancomycin has only been in clinical use since the 1950s, this mechanism is not a newly evolved phenomenon. Rather, genes encoding resistance are ubiquitous in the environment and found not only in glycopeptide producing organisms but other environmental microbes (74). Historically vancomycin resistance has been described as the canonical *vanHAX* in both pathogenic and producing organisms (Fig 2.2). We set out to determine how this gene cassette evolved by asking how old it is and does variability in gene organization and essentiality exist? It is clear that resistance will only continue

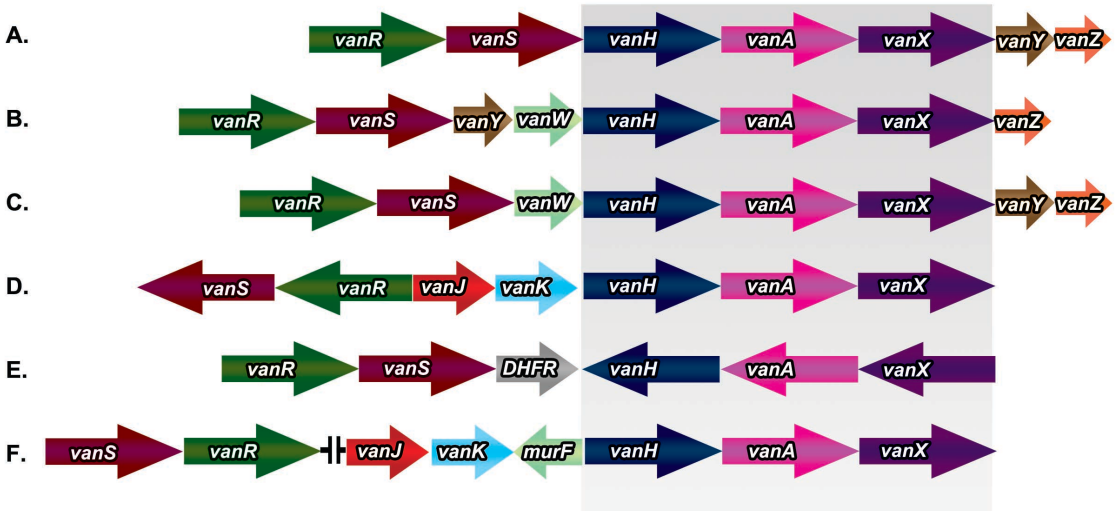


Figure 2.2: Conserved arrangement of glycopeptide resistance clusters. A. VREA, B. VREB, C. *Paenibacillus paparius*, D. *Streptomyces coelicolor,* E. *Amycolatopsis teicomyceticus*, F. *Streptomyces toyocaensis*,

to evolve and spread thus it is critical to fully understand the natural evolution and presence of glycopeptide resistance genes in the environment. Though second generation glycopeptide drugs have been introduced, we predict resistance will arise, unless we continue to expand the chemical diversity of glycopeptides and maintain alternative options.

2.2 Materials and Methods

2.2.1 Cloning, expression and purification of VanA and Ddl homologs

Desulfitobacterium hafniense Y51 strain and genomic DNA were generously provided by Dr. Masatoshi Goto and Dr. Kensuke Furukawa (Kyushu University, Fukuoka, Japan). Cultures were maintained in m-ISA medium consisting of 1% tryptone peptone, 0.35% sodium lactate, 0.05% Na_2SO_3, 0.2% $MgSO_4 \bullet 7H_2O$, 0.05% iron (III) ammonium, and 0.001% resazurin, pH 7.2 and incubated at 30°C.

Cloning of the putative D-Ala-D-Lac and D-Ala-D-Ala ligase genes from *D. hafniense* Y51 was achieved by PCR amplification of the genes DSY3690 and DSY1579 respectively from genomic DNA. Primers were designed to amplify 1.1 kB fragments from *D. hafniese* Y51 genomic DNA. Primers included *attB* sites to facilitate cloning using the Gateway® technology (Invitrogen, Carlsbad, CA). The specific primers used for DSY3690 are: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATCGGTTGAAAATCGCA-3', 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCATCCTATCGGCA-3'

For DSY1579 the specific primer sequences are:: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTYYCATATGATGACACGGCAAAAGATTATTATTC-3', 5'-GGGACCACTTTGTACAAGAAAGCTGGGTYAAGCTTCTAACGGGATATTTTCCGGG-3'

The resulting PCR products were inserted into the pDEST17 (Invitrogen, Carlsbad, CA) destination vector containing a His6 tag for ease of downstream purification. Gene integrity was confirmed by DNA sequencing. Plasmids were propagated in *Escherichia coli* TOP10' cells and subsequently used to transform *E. coli* BL21 (DE3) Rosetta (Novagen, Darmstadt, Germany) cells for high level protein expression under control of the T7 promoter. For $VanA_{Ao}$, $VanA_{Dh,}$ and Ddl_{Dh} overexpression, cells were propagated in 1 L of Luria-Bertani broth until the optical density at 600 nm reached 0.6. Protein expression was induced by addition of isopropyl ß-D-1-thiogalactopyranoside to a final concentration of 1 mM and incubation of cultures at 16°C for 18 hours. Cells were harvested and washed in 0.85 % (w/v) NaCl before resuspension in 10 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride, 1 mM DNAse I, pH 8.0). Cells were lysed by four passes through a French pressure cell at 1250 psi and cell debris removed by centrifugation at 27 000 x g for 30 minutes. The supernatant was collected and purified enzyme was obtained using Nickel-NTA immobilized metal affinity chromatography (Qiagen, Valencia, CA). Fractions containing the purified enzyme were pooled and dialysed against 50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, pH 8.0 at 4°C.

Two putative D-Ala-D-Lac ($VanA_{Ab}$ and $pVanA_{Ab}$) and D-Ala-D-Ala ligase (Ddl_{Ab}) genes from *A. balhimycina* (kindly provide by Dr. Evi Stegmann) in addition to four complete ancient *vanA* ($VanA_{A1-4}$) sequences (the consensus

sequences from four distinct sequence groups apparent in an initial subset of 20 clones) were synthesized with codon optimization for expression in *E. coli* and incorporated 5' NdeI and 3' HindIII restriction enzyme sites (GenScript, Piscataway, NJ, USA). The resulting genes were sub-cloned from pUC57 into the expression vector pET28a with an N-terminal His6 tag for downstream purification. Constructs were confirmed by sequencing and subsequently propagated in *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) for expression. The three *A. balhimycina* constructs and two ancient *vanA* constructs (designated $vanA_{A2}$ and $vanA_{A4}$) produced enough soluble protein for enzyme analysis. High-level protein expression was achieved by growing cells in 1 L of Luria-Bertani broth to an optical density of 0.6 at 600 nm. Protein expression under the T7 promoter was induced by addition of isopropyl ß-D-1-thiogalactopyranoside to a final concentration of 1 mM, followed by incubation at 16°C for 16-18 hours. Post-harvest, cells were washed in 0.85% NaCl (w/v) and the pellet stored at -20°C or prepared for protein purification. Enzymes were purified as His6 tagged proteins as above with the following modifications. Cell pellets were resuspended in lysis buffer containing 50 mM HEPES, 500 mM NaCl, 20 mM imidazole, 10 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, 1 mM Dnase, pH 7.5 and lysed by three passes on a continuous cell disruptor (Constant Systems Ltd., Daventry, UK) at 30 kPSI. After elution in imidazole from a Nickel-NTA immobilized metal affinity column fractions containing pure protein were pooled and dialysed into 50 mM HEPES, 150 mM NaCl, pH 7.5 at 4°C.

E. coli BL21(DE3) harboring pET28b$vanA_{Ao}$ from the vancomycin producer *Amycolatopsis orientalis* C329.2, was prepared as previously

described (37, 75) and purified as a His6 tagged protein as described above. *E. coli* W3110 harboring pTB2 for expression of the D-Ala-D-Ala ligase DdlB was previously reported (76).

2.2.3 Ddl assays

For qualitative determination of ligase substrate specificity, initial enzymatic characterization was carried out using the pyruvate kinase/lactate dehydrogenase coupled assay to monitor ADP formation (77). Amino and hydroxy acid substrate specificity was determined by thin-layer-chromatography and using radiolabelled substrates. [U-^{14}C]-L-Alanine was isomerized to a racemic mixture of [^{14}C]-L/D-Alanine with one unit of *Bacillus stearothermophilus* alanine racemase (SigmaAldrich). Ligase reactions contained 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 40 mM KCl, 6 mM ATP, 2 µM enzyme, 0.1 µCi [U-^{14}C]-L/D-Ala, 1 mM D-Ala and 10 mM D-X substrate. Reactions were quenched with 50% methanol and applied onto a cellulose TLC plate (SigmaAldrich). The plates were developed in 12:3:5 butanol:acetic acid:water, dried overnight and exposed to a phosphor-storage imaging screen. The screens were imaged using a Typhoon™ variable mode imager and relative radioactive intensity was quantified using ImageQuant 5.2 software.

Michaelis-Menten kinetics were determined by using the software program GraFit version 4.0.21 (Erithacus software) and initial rates were determined using the non-linear least squares method and equation 1 (78).

$$v = (k_{cat}/E_t)[S]/(K_M + [S]) \quad [1]$$

2.2.4 RNA preparation

Cultures of *D. hafniense* Y51 were grown in m-ISA media (79) for 24 hours and diluted 1/100 into m-ISA containing 0 µg/mL or 125 µg/mL vancomycin and incubated at 30°C for 24 or 48 hours. Cultures were harvested by centrifugation for 15 minutes at 3000 x g and the pellets resuspended in 1 mL of RNAprotect bacterial reagent (Qiagen, Valenica, CA). After subsequent centrifugation, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the enzymatic lysis and proteinase K digestion of bacteria before purification of total RNA following the manufacturers instructions. An on-column DNase I digestion was performed in addition to a post elution DNase I digestion and additional column purification. RNA was quantified using a NanoDrop ND-1000 spectrophotometer and analyzed for genomic DNA contamination by PCR analysis.

2.2.5 Expression analysis

Reverse transcription was carried out with 0.3 µg of DNase I treated RNA and 200 ng of random hexamers to generate cDNA using the Superscript III RT kit (Invitrogen, Carlsbad, CA). The samples were incubated at 25°C for 5 minutes prior to 50°C for 45 minutes followed by inactivation at 70°C for 15 minutes. One microlitre of the RT reaction was used for real-time PCR with SYBR green in a SmartCycler system (Cepheid, Sunnyvale, CA). The following primers were designed to amplify a 293 bp $vanA_{Dh}$ product and a 270 bp ddl_{Dh} product in a 25 µL reaction volume. $vanA_{Dh}$ primers are 5'-TTCTTCTTGGCGGCATACTT-3' and 5'-ATCTGGTGTTTCCCGTTCTG-3' while ddl_{Dh} primers are 5'-GTGAAGAACGGGGAAAATCA-3' and 5'-CAATCCGGAGAACATGAGGT-3'. The results were normalized by the $2^{-\Delta\Delta C_T}$

method (80) to 16S *rRNA* expression using the primers 5'-AGGCCTTCGGGTTGTAAAGT- 3' and 5'-ATACCCAGTTTCCGATGCAG-3' to amplify a 237 bp product. Products were analyzed on a 1% agarose gel to confirm a single product was amplified.

2.2.6 Antibiotic susceptibility testing

D. hafniense Y51 susceptibility tests for vancomycin and teicoplanin using disc-diffusion assays were performed according to CLSI methods (81). In addition, the minimum inhibitory concentration (MIC) for vancomycin was determined using Etest gradient strips (Biomérieux, Marcy l'Etoile, France). The organism was grown on purchased Brucella agar with 5% sheep blood, vitamin K and hemin supplements (PML Microbiologicals, Wilsonville, OR,) and the manufacturer's protocol was followed for MIC determination.

2.3 Results and Discussion

2.3.1 VanA is an ancient enzyme

DNA extracted from Late Pleistocene permafrost sediment, dated at approximately 30,000 calendar years was analysed for *vanHAX* resistance elements (28). Full-length *vanA* gene sequences were identified and to ascertain whether the sequences were indeed functional and did not represent PCR artifacts or pseudo-genes, we synthesized four open reading frames from the 40 H1AX/H2AX sequences (28). Two of these generated soluble proteins in *E. coli* suitable for purification to homogeneity. Enzymatic characterization indicated that these ligases were indeed D-alanyl-D-lactate-specific (Fig. 2.3), and analysis revealed steady-state kinetic parameters consistent with contemporary enzymes derived from both the clinic and the environment (Table

2.1). These results clearly show that the *vanHAX* genes identified in the ancient samples encode enzymes capable of genuine antibiotic resistance. This study allows us to conclude that glycopeptide resistance is a natural and old phenomenon but does not provide clues as to how this cluster came together. To answer those questions we can look to the known reservoir of glycopeptide resistance genes found in the environment today.

2.3.2 Prevalance of VanA homologs in the environment

When glycopeptide resistance was first described in clinical pathogens it wasn't expected to also be widespread in the environment. Advances in next generation sequencing technology have not only facilitated an exponential rise in whole genome sequencing but have created data sets that allow a more comprehensive analysis of evolutionary relationships and diversity of specific protein products. For example, a BLAST search for VanA homologs revealed two predicted D-Ala-D-X ligase genes in the genome of the anaerobic bacterium *D. hafniense* Y51. The first, DSY1579, is a predicted D-Ala-D-Ala ligase (Ddl_{Dh}) while the second, DSY3690, is homologous to VanA-like D-Ala-D-Lac ligases ($VanA_{Dh}$). The ddl_{Dh} gene is located proximal to other genes predicted to encode peptidoglycan assembly proteins (e.g. DD-carboxypeptidase). Conversely, $vanA_{Dh}$ is clustered with *murF, vanX*, *vanK*, and *vanW* homologues and a predicted *vanSR* two component regulatory system (Fig 2.6). We hypothesized that this novel *vanAWKmurFX* cluster may encode an alternative glycopeptide resistance cluster rather than the canonical *vanHAX* found in VRE, glycopeptide producers, and other environmental bacteria. As noted above, VanX is a D-Ala-D-Ala hydrolase required to eliminate constitutively produced D-Ala-D-Ala. VanK (82, 83) is a FemX protein that catalyzes the cross linking

reaction of peptidoglycan terminating in D-Ala-D-Lac (where native FemX enzymes will not), while MurF adds the D-Ala-D-Lac depsipeptide to the growing chain (84). VanW on the other hand is a protein of unknown function often associated with VanB phenotype resistance clusters (conferring resistance to vancomycin but not teicoplanin) (Fig. 2.2, 2.6). Bateman *et al*. hypothesized that VanW may be important in localizing other vancomycin resistant proteins to unlinked peptidoglycan based on the presence of a G5 domain in the C-terminus, which may bind to N-acetylglucosamine residues (85).

Another example of unusual glycopeptide resistance lies in the genome of the balhimycin producer *Amycolatopsis balhimycina*. This organism does possess the canonical *vanHAX* genes however this cassette lies 2 Mb from the biosynthetic cluster, which contains a *vanRS* pair. Furthermore, a second orphan *vanA* homolog is present in addition to the expected D-Ala-D-Ala ligase clustered with cell wall biosynthetic enzymes. The *vanHAX* cassette does not appear to be regulated by VanRS but instead is constitutively expressed (personal communication from Hans-joerg Frasch, University of Tübingen, Tübingen, Germany) while the orphan *vanA* ($vanA_{Ab2}$) is only transcribed during balhimycin production indicating transcriptional regulation expected of the *HAX* cluster however, a VanR binding site is absent. If the single copy $vanA_{Ab2}$ is linked to biosynthesis then they were likely acquired at the same time whereas the acquisition of *vanHAX* may have been at a different time in the evolutionary history of this species. It is interesting that the organism maintains two functional D-Ala-D-Lac ligases and a D-Ala-D-Ala ligase, only one of which is transcriptionally tied to antibiotic biosynthesis.

In order to experimentally verify the predicted biochemical activities of our collection of predicted D-Ala-D-X ligase genes, each was overexpressed as

His6-tag fusions in *E. coli*, and purified by immobilized metal affinity chromatography by standard methods yielding pure proteins.

2.3.4 Steady state kinetics of D-Ala-D-Ala/Lac Ligases

We performed steady state kinetic analyses to quantify the enzyme activity of $VanA_{Dh}$, $VanA_{Ab}$, and $VanA_{Ab2}$. Purified ligases were kinetically characterized for utilization of D-Ala, D-Lac, and ATP by monitoring the change in absorbance at 340 nm with the coupled pyruvate kinase-lactate dehydrogenase continuous ADP release assay. The results indicate (Table 2.1) that D-Lac is the preferred substrate for all three putative VanA homologs where the K_M is 40-70 times lower compared to D-Ala. The *D. hafniense* $VanA_{Dh}$ has a catalytic efficiency (k_{cat}/K_M) 42-fold higher for D-Lac under the same conditions while the *A. balhimycina* enzymes were a significant 176 and 121-fold higher in

Table 2.1: Kinetic characterization of D-Ala-D-X ligases using the coupled pyruvate kinase/lactate dehydrogenase (PK/LDH) assay.

Enzyme	Product	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
	D-Ala-D-Ala	22 ± 2.6	0.58 ± 0.03	2.6×10^1
$VanA_{Dh}$	D-Ala-D-Lac	0.53 ± 0.06	0.61 ± 0.02	1.1×10^3
	ATP	0.013 ±0.001	0.65 ± 0.01	4.7×10^4
	D-Ala-D-Ala	0.26 ± 0.061	0.82 ± 0.036	1.7×10^2
Ddl_{Dh}	D-Ala-D-Lac	0.80± 0.081	0.24 ± 0.023	3.1×10^3
	ATP	0.018 ±0.002	0.18 ± 0.0054	3.8×10^4
	D-Ala-D-Ala	-	-	1.7×10^1
$VanA_{Ab}$	D-Ala-D-Lac	0.13 ± 0.0098	0.38 ± 0.0078	3.0×10^3
	ATP	0.06 ± 0.041	0.38 ± 0.011	6.7×10^3
	D-Ala-D-Ala	-	-	2.8×10^1
$pVanA_{Ab}$	D-Ala-D-Lac	0.13 ± 0.014	0.43 ± 0.014	3.4×10^3
	ATP	0.12 ± 0.0092	0.40 ± 0.014	3.4×10^3
	D-Ala-D-Ala	32 ± 3.5	0.82 ± 0.036	2.6×10^1
$VanA_{A2}$	D-Ala-D-Lac	0.22 ± 0.061	0.24 ± 0.023	1.1×10^3
	ATP	0.085 ±0.0092	0.18 ± 0.0054	2.2×10^3
	D-Ala-D-Ala	35 ± 3.2	0.29 ± 0.012	8.3×10^1
$VanA_{A4}$	D-Ala-D-Lac	0.55 ± 0.042	0.13 ± 0.0041	2.4×10^2
	ATP	0.043 ± 0.0034	0.1 ± 0.0035	2.5×10^3

k_{cat}/K_M . Ddl_{Dh} was unable to use D-Lac as a substrate and the k_{cat}/K_M for D-Ala is comparable to the k_{cat}/K_M for D-Lac utilization by $VanA_{Dh}$ ($3.1x10^3$ and $1.1x10^3$ $M^{-1}s^{-1}$ respectively). Interestingly, saturable conditions for D-Ala were not achievable for either $VanA_{Ab}$ or $VanA_{Ab2}$ even at concentrations exceeding 100 mM D-Alanine. The putative D-Ala-D-Ala ligase identified in the *A. balhimycina* genome was not functional under all conditions tested.

The enzymes bind two molecules of D-amino/hydroxy acids in two distinct binding sites (76). The first is always D-Ala thus we are measuring the kinetic parameters for the second binding site using the coupled assay.

Although the active site discriminates which hydroxy amino acid it prefers, in the case of VanA enzymes some D-Ala-D-Ala is concurrently being formed in addition to D-Ala-D-Lac. Unfortunately, it is difficult to resolve the rate and the effect on D-Ala-D-Lac formation using the spectrophotometric coupled assay. The markedly large increase in k_{cat}/K_M is a good indicator of the discrimination for D-Lac in the active site of the enzyme; however, this assay is unable to quantify directly the formation of D-Ala-D-Lac. Therefore, a direct TLC assay involving radiolabelled substrates was employed.

2.3.5 Substrate specificity of recombinant D-Ala-D-X ligases

The amino acid specificity of each ligase was qualitatively determined using [U-^{14}C]-D-Ala and unlabeled D-amino and D-hydroxy acid substrates followed by separation of the products by thin-layer chromatography on a cellulose TLC plate. DdlB, the D-Ala-D-Ala ligase from *E. coli* and $VanA_{Ao}$, the D-Ala-D-Lac ligase from the vancomycin producer *A. orientalis* C329.2, were used as positive controls. DdlB is only able to produce D-Ala-D-Ala, while $VanA_{Ao}$ catalyzes D-Ala-D-Lac synthesis. Although the amino acid sequence

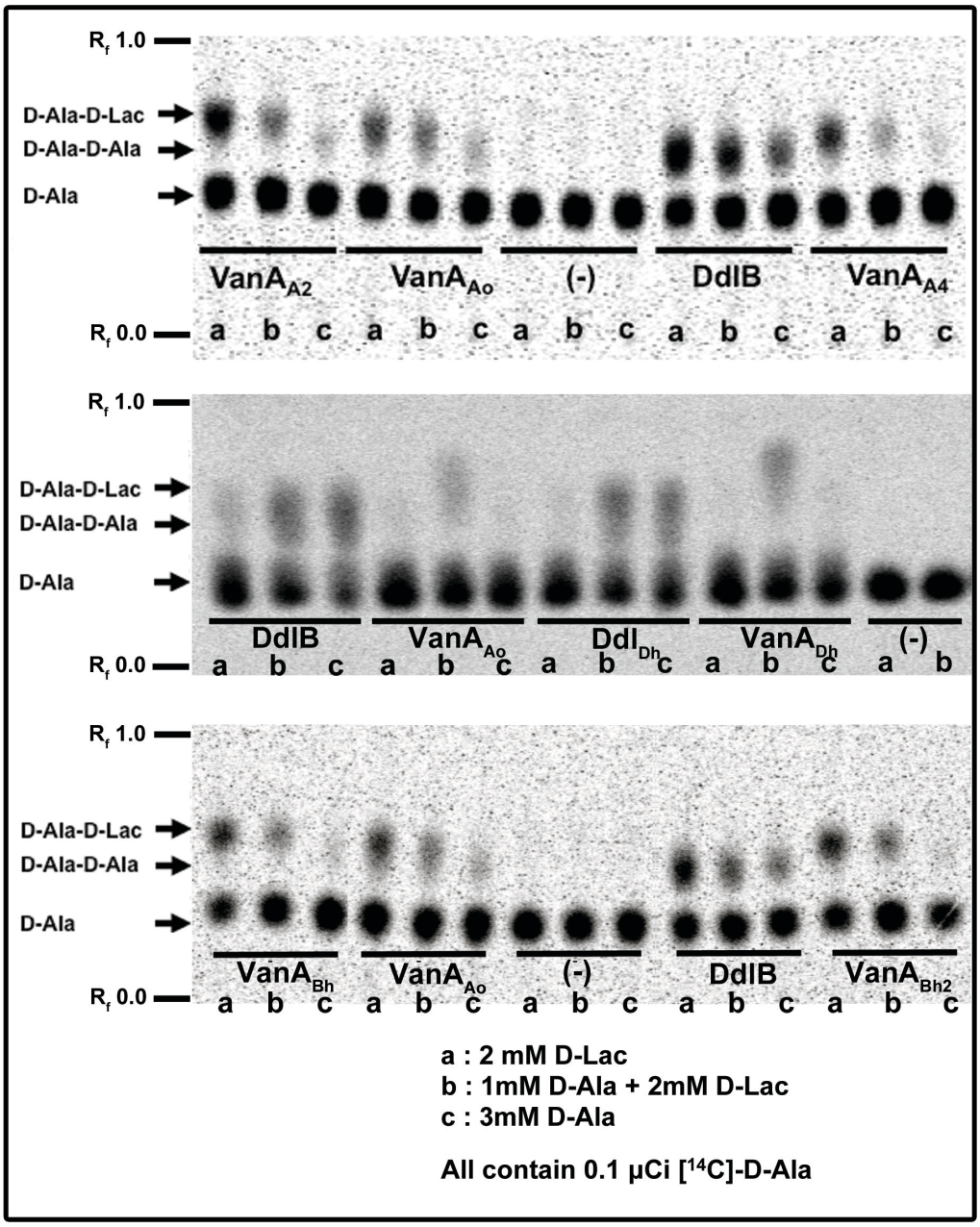


Figure 2.3. Substrate specificity of D-Ala-D-X ligases. Substrate specificity was examined using [U-^{14}C]-D-Ala and either D-Ala or D-Lac. The products of each reaction were separated by TLC and exposed to a phosphor-storage screen. All reactions contain 0.1 uCi [U-^{14}C]-D-Ala . The top blot represents ancient VanAs, the middle *D. hafniense* Y51 enzymes and the bottom *A. balhimycina* enzymes. The middle panel differs as follows: a) 10 mM D-Ala; b) 1 mM D-Ala, 10 mM D-Lac or c) 10 mM D-Lac.

identity is only 34%, Ddl_{Dh} exhibits activity similar to that of *E. coli* DdlB,

producing only D-Ala-D-Ala even in the presence of excess (10-fold) D-Lac. In contrast, the conserved amino acid identity between $VanA_{Dh}$ and $VanA_{Ao}$ is 67% and $VanA_{Dh}$ shows substrate specificity similar to $VanA_{Ao}$, producing only D-Ala-D-Lac (Table 2.1, Fig. 2.3). $VanA_{Ab}$ and $VanA_{Ab2}$ are 71.7% identical to each other and 75.2% and 75.4% identical to $VanA_{Ao}$ respectively. As expected both enzymes have TLC profiles comparable to $VanA_{Ao}$ and selectivity for D-lactate. Even with this more sensitive assay, the D-Ala-D-Ala ligase from *A. balhimycina* was not active. Figure 2.3 clearly illustrates that formation of D-Ala-D-Lac is preferred over D-Ala-D-Ala for each predicted VanA while the predicted D-Ala-D-Ala ligases produce only D-Ala-D-Ala identified by shift in the retention factor (Rf) between the depsipeptide and dipeptide.

The two D-D ligases in *D. hafniense* Y51 clearly show different substrate specificities indicating that $VanA_{Dh}$ is able to produce D-Ala-D-Lac, an observation consistent with our hypothesis that *vanAWmurFKX* may encode an alternate glycopeptide resistance cluster. *A. balhimycina* possess two functional D-Ala-D-Lac ligases however the activity of its D-Ala-D-Ala ligase is still unclear since we were unable to prepare active protein. An amino acid alignment of the $DdlI_{Bh}$ with closely related enzymes from other organisms was

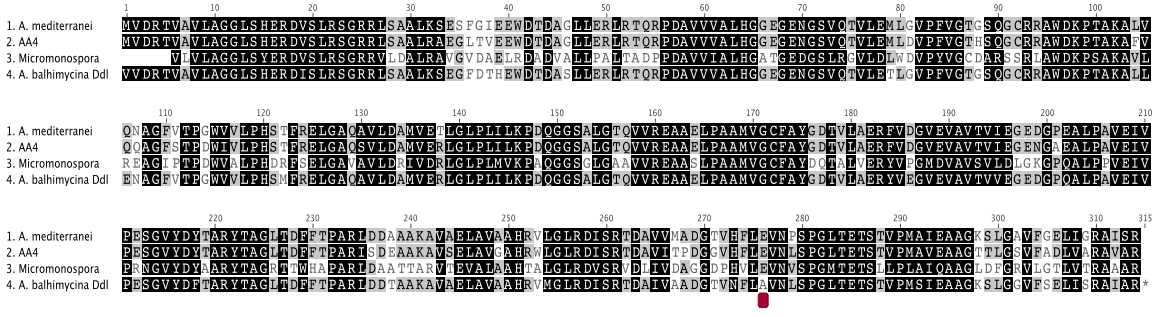


Figure 2.4: Amino acid alignment of D-Ala-D-Ala ligases from *Amycolatopsis mediterranei*, *Streptomyces* sp. AA4, *Micromonospora* sp. L5, and *Amycolatopsis balhimycina*. Red box indicates the position of the non-conserved alanine in $DdlI_{Bh}$.

performed (Fig. 2.4). A conserved ATP binding glutamate residue appears as an alanine in Ddl_{Bh} (highlighted in figure 2.4) Site-directed mutagenesis was performed to correct the mutation (A276E) and the enzyme was assayed again. Gain of function was not observed.

2.3.6 D. hafniense Y51 is vancomycin resistant

D. hafniense Y51 was found to be resistant to vancomycin as indicated by no inhibition of growth around a 5 µg vancomycin paper disc while only intermediately resistant to teicoplanin indicated by a 40 mm zone of inhibition around a 30 µg disc. The minimum inhibitory concentration for vancomycin was determined using Etest gradient strips and found to be 64 µg/mL.

2.3.7 *vanA*$_{Dh}$ expression levels during growth in vancomycin

The induction of vancomycin resistance by *vanA*$_{Dh}$ was examined by real-time PCR. Relative RNA levels were normalized to 16S rRNA expression and it was determined that *vanA*$_{Dh}$ expression is increased 181 and 256 fold after 24

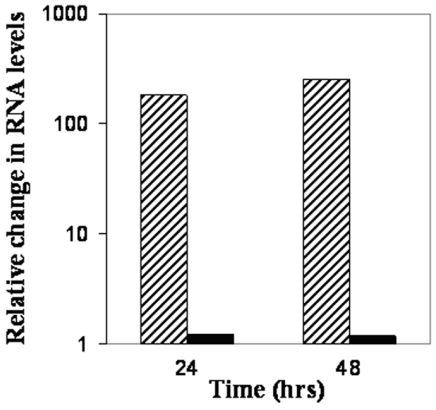


Figure 2.5. Change in expression levels of *vanA*$_{Dh}$ (striped bars) and *ddl*$_{Dh}$ (shaded bars) after growth in 125 µg/mL vancomycin. Relative RNA levels are normalized to 16S rRNA levels after 24 and 48 hours of growth.

and 48 hours incubation in vancomycin respectively. Furthermore, ddl_{Dh} RNA levels remained constant regardless of growth in the presence or absence of vancomycin. The fold change of ddl_{Dh} upon addition of vancomycin was only 1.19 and 1.23 after 24 and 48 hours growth respectively (Fig. 2.5).

2.4 Summary and conclusions.

Glycopeptide resistance is ancient. This study unequivocally confirms that resistance pre-dates clinical use of antibiotics and that it is a natural phenomenon. Furthermore, glycopeptide resistance is diverse and widespread. We have identified a glycopeptide antibiotic resistance cluster with novel gene organization in the dehalorespiring organism *D. hafniense* Y51. This cluster includes predicted *vanA* and *vanX* genes as well as additional auxiliary genes predicted to be required for inducible glycopeptide resistance. Biochemical analysis of recombinant $VanA_{Dh}$ confirms that it is a functional D-Ala-D-Lac ligase. Although, a D-lactate dehydrogenase (*vanH*) was not part of this new cluster, four annotated D-isomer specific 2-hydroxyacid dehydrogenase genes (DSY0996, DSY1673, DSY3442, and DSY4020) are present in the genome of *D. hafniense* Y51. The organism exhibits high level resistance to vancomycin even though these dehydrogenases have less than 30% identity to VanH, and therefore at least one of these gene products can provide the requisite D-Lac substrate for $VanA_{Dh}$.

In the case of *A. balhimycina* the situation is more complex. Dr. Evi Stegmann and her group at the University of Tübingen are actively working on uncovering the regulation of glycopeptide resistance and biosynthesis of balhimycin in this organism. They are looking for auxiliary D-isomer specific

dehydrogenases and examining the activity to compliment the $VanA_{Bh2}$ activity in a *vanHAX* mutant strain. Furthermore through the use of genetic manipulation they are attempting to understand how these enzymes interact with each other. The information uncovered in this study indicates that glycopeptide resistance is more complicated than previously thought. Rather than a "on/off" function for survival, these enzymes appear to have auxiliary roles in cellular metabolism.

Studying annotated resistance genes, particularly in the context of a new vancomycin resistance cluster expands our understanding of the genetic diversity that encompasses the vancomycin resistome (Fig. 2.6), which includes all the genes in pathogenic and environmental bacteria that can give rise to glycopeptide resistance (86). The presence of this new vancomycin resistance

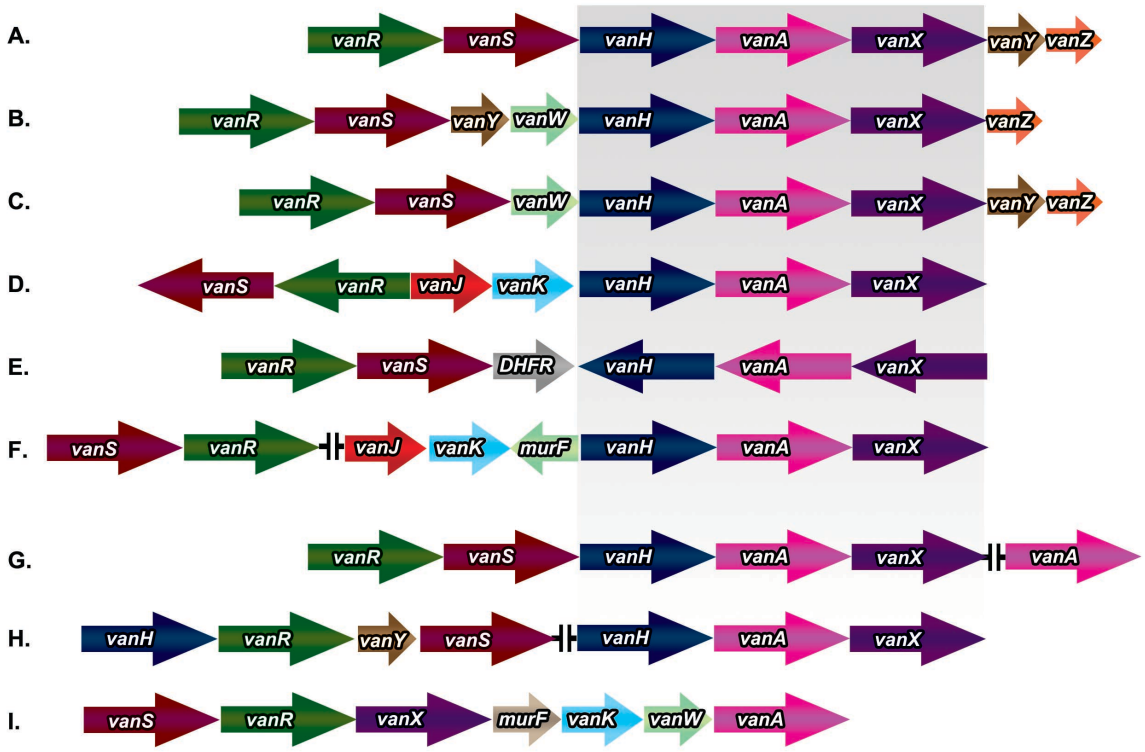


Figure 2.6: Diversity of glycopeptide resistance clusters discovered in this work. A. VREA, B. VREB, C. *Paenibacillus paparius*, D. *Streptomyces coelicolor,* E. *Amycolatopsis teicomyceticus*, F. *Streptomyces toyocaensis*, G. *Amycolatopsis balhimycina*, H. *Actinoplanes* sp. ATCC 53555, I. *Desulfitobacterium hafniense* Y51.

cluster gives rise to a number of unanswered questions including: why do *D. hafniense* harbor these genes (is it antibiotic resistance or some physiological advantage for D-Ala-D-Lac terminating cell walls?); is this gene cluster more wide spread in other organisms?; and can this new cluster be mobilized into pathogenic bacteria like *vanHAX*? While the answers to these questions require further study, what is clear is that continued microbial genome sequencing is revealing the remarkable depth of the antibiotic resistome within microbial populations across the globe. Looking to environmental organisms containing the building blocks of the highly successful modern resistance clusters we find in clinical pathogens will provide clues to how they evolved. For example, *A. balhimyinca* also harbors a *vanY* encoding for a D-Ala-D-Ala carboxypeptidase within the balhimycin biosynthetic cluster. If the *vanHAX* acquisition event followed the evolution or capture of the balhimycin biosynthesis cluster, then VanA and VanY likely work together in the absence of VanX. Eventually *vanHAX* would come together as a highly functioning gene unit and later *vanY* before movement into organisms like *Enterococcus*. The capacity for microorganisms to readily exchange genetic information results in a high rate of evolution on a relatively rapid timescale compared to higher organisms.

Chapter 3

ACCESSORIZING AN ANTIBIOTC: EXPANDING GLYCOPEPTIDE CHEMICAL DIVERSITY

Acknowledgments must be made to a number of people. First Nick Waglechner for help in assembling the *Actinoplanes* sp. ATCC 53533 genomic data. Dr. Kalinka Koteva for her help in chemical synthesis and small molecule purification and Dr. Maulik Thaker for help with *Streptomyces* genetics.

3.0 Chapter 3

3.1 Introduction

Glycopeptide resistance is ancient and diverse, tightly regulated and chemospecific. Along with our understanding of biosynthesis, this knowledge provides a foundation for expanding the chemical diversity of this class of antibiotics. It has been well documented that modification of the glycopeptide scaffold results in changes to antimicrobial activity. For example, Boger and colleagues found that increasing hydrophobicity by protecting the phenol groups on vancomycin with methyl ethers improved antimicrobial activity nearly 100-fold in *Enterococcus faecalis* VanB strains (87). Many groups have focused on the effects of glycosylation (53, 88-91) and more specifically the promiscuous nature of the glycosyltransferases to accept different glycopeptide backbones. Both combinatorial biosynthesis and protein engineering have led to libraries of novel glycosylated vancomycin derivatives (53, 55, 88, 89). Furthermore, halogenation is important for binding to acyl-D-alanyl-D-alanine and thus contributes to antimicrobial activity (92). The substrate of glycopeptide halogenases is predicted to be the nascent heptapeptide chain attached to the non-ribosomal-peptide machinery and due to the difficulty in characterizing this reaction mechanism, these enzymes represent an underexplored class of tailoring enzymes (93, 94). As will be discussed in chapter 4, sulfation on A47934 imparts favorable properties modulating the VanS sensor kinase, while regio-specific effects of sulfation on other residues remain to be explored.

With new glycopeptide biosynthetic gene cluster sequences becoming available, the repertoire of tailoring enzymes imparting previously uncharacterized modifications is also increasing. Here we describe complimentary approaches leading to expanded chemical diversity of the glycopeptide antibiotics. Sulfotransferases (Stfs) are the main focus, and similar to glycosyltransferases they accept different glycopeptide backbone molecules. Our focus is on A47934, produced by *Streptomyces toyocaensis* (Fig. 3.1), where we exploit the substrate promiscuity of the Stf StaL (62) yielding both a sulfamate and fluorosulfonate derivative *in vitro*.

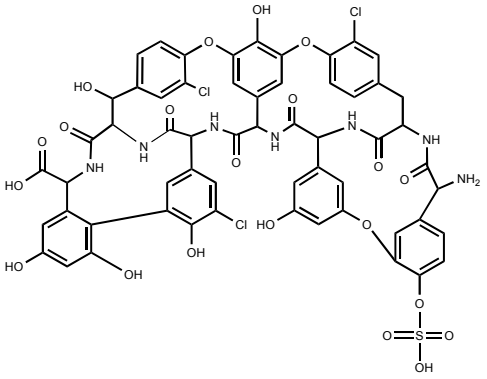


Figure 3.1: Structure of A47934

In vivo, we have created a platform to genetically engineer *S. toyocaensis* in a combinatorial fashion with glycopeptide tailoring enzymes from newly sequenced clusters. A47934 is 'aglyco-' and therefore represents a minimal teicoplanin scaffold on which to build new molecules. Each tailoring enzyme can be introduced into the chromosome of both the wild-type and *staL* deletion genetic backgrounds as either a single insertion, or combination of multiple enzymes (eg. glycosyltransferase + sulfotransferase).

While the vast majority of groups focus on *in vitro* modification with purified enzymes, there are drawbacks to this system. A47934 must be purified from *S. toyocaensis* mycelia in very high quantities (mgs) for full compound characterization. Unlike vancomycin, A47934 is not very water soluble resulting in use of dimethylsulfoxide (DMSO) or other solvents that may inhibit enzymatic

activity. Moreover, the enzymes are not generally highly efficient *in vitro* and a reaction can take up to 72 hours to reach completion, and often times the enzyme fails to recognize the substrate. In the cellular environment, large multi-enzyme complexes synthesize glycopeptides and there may be co-factors or other protein interactions occurring at a molecular level that cannot be readily mimicked *in vitro*. Genetic engineering of a glycopeptide producer is very attractive and precedent of *S. toyocaensis* as a model host for glycopeptide recombineering exists (64, 65). These studies introduce cosmids or other mobile elements containing glycopeptide biosynthetic enzymes into *S. toyocaensis* and screen for derivatives of A47934. Here, we take a more direct approach and clone each gene of interest into a vector that allows stable integration into the chromosome. The resulting derivatives are purified directly after fermentation and assayed for activity.

The initial effort of the *in vivo* system was to explore the function of sulfation in the context of polysulfation using a library of Stfs we have collected in house and from our colleague Dr. Sean Brady (95). We then focused on the tailoring enzymes of the glycopeptide UK68597 produced by *Actinoplanes* sp. ATCC 53533.

Finally, the role of glycopeptide sulfation will be discussed and a novel sulfated vancomycin derivative will be described. This work will lead into further studies evaluating the interaction between sulfated molecules and the glycopeptide VanS sensor kinase controlling expression of resistance.

3.2 Materials and Methods

3.2.1 Isolation of genomic DNA and 454-pyrosequencing

Actinoplanes sp. ATCC 53533 genomic DNA was isolated using a salting out procedure. Briefly, cells from 50 mL of culture were resuspended in 5 mL lysis buffer (Qiagen DNAeasy kit, Valencia) and 5 mL SET buffer (96). The cells were homogenized with a glass homogenizer, pelleted by centrifugation and resuspended in 10 mL lysis buffer. Lysozyme (2.5 mg/mL) and mutanolysin (10 µg/mL)was added to the cell suspension followed by incubation at 37°C overnight. After overnight incubation, 4 µg proteinase K and 600 µL of 10% sodium dodecyl sulfate (SDS) was added to the emulsion and incubated at 55°C for ~3 hours. Five milliliters of 5M NaCl was added and mixed thoroughly by inverting the tube. 10 mL chloroform was added and incubated at room temperature on a rocking platform for 30 minutes. The solution was centrifuged at 4150 rpm for 15 minutes and the upper aqueous phase was transferred to a 50 mL falcon tube. Isopropanol was added (0.6 volumes) and mixed by inversion to precipitate the DNA. Genomic DNA was spooled using a sealed Pasteur pipette and washed in 70% ethanol. After air drying the DNA was dissolved in 200-500 µL water. The gDNA yield and quality was assessed using a NanoDrop spectrophotometer and submitted for library preparation and sequencing on the Roche 454 pyrosequencer. Library preparation was performed by Christine King, (McMaster metagenomic center). Raw sequence data was assembled using MIRA software (6 passes) (97) (Ch. 6, Table A1) and refined using Geneious Pro version 5.5.3 (98).

3.2.2 Cloning of tailoring enzymes

3.2.2.1 Construction of pSET152_LK1 and 2

pSET152_LK1 was constructed by PCR amplification of *the ermEp** (99) promoter from the plasmid pMC500 [pUC57 with the following modification to the MCS - MfeI - BglII – KpnI - to – XbaI – ermEp*- EcoRI – EcoRV – BamHI – tmmr(rev) – BglII – HindIII] kindly donated by Dr. Marie Elliot. The 3' primer contained extra sequence with EcoRV-NdeI-EcoRI-EcoRV-MfeI restriction enzyme (RE) sites. The promoter was cloned into the EcoRV/EcoRI sites of the pSET152 MCS with a 5' blunt end and 3' MfeI overhang resulting in loss of both the native EcoRV and EcoRI sites. pSET152_LK2 was constructed by introducing additional RE sites into the MCS after subcloning Teg13, 14 and Auk20 engineered with 5'-NdeI and 3'-KpnI-BglII-MfeI-EcoRI sites. Each open reading frame was synthesized (GenScript, Piscataway, NJ, USA) with codon optimization for expression in *Streptomyces* sp. and sub-cloned into the 5'-NdeI and 3'-EcoRI RE sites of pSET152_LK1. A kanamycin resistant version of each construct was generously made by Dr. Maulik Thaker (Ch 6., Method A1).

3.2.2.2 Construction of pSET152_LK1 integration vectors

Cloning of Auk10, 14, 21 and 23 from *Actinoplanes* sp. ATCC 53533 was achieved by PCR amplification of the genes from genomic DNA. Primers were designed with 5' NdeI and 3' EcoRI restriction enzyme sites (Ch. 6,Table A.2) The resulting PCR products were cloned into pSET152_LK1 for integration into *Streptomyces* sp. chromosome. Gene integrity was confirmed by DNA sequencing. Plasmids were propagated in *E. coli* TOP10' cells and subsequently used to transform *E. coli* ET12567/pUZ8002 for conjugation with *S. toyocaensis*.

3.2.3 Streptomyces toyocaensis conjugation

pSET152_LK1[Auk10,14,20,21,23, Teg13 and 14] were integrated into the chromosome of wild-type *S. toyocaensis* and the *staL* deletion mutant following the protocol outlined in Practical Streptomyces Genetics (p.249-250) (96).

3.2.4 Synthesis of vancomycin-sulfate

3.2.4.1 Purification of Teg14

The Teg14 Stf sequence was kindly provided by Dr. Sean Brady and was synthesized with codon optimization for expression in *E. coli* with incorporated 5' NdeI and 3' HindIII restriction enzyme sites (GenScript, Piscataway, NJ, USA) in the expression vector pET28a with an N-terminal His6 tag for downstream purification. Constructs propagated in *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) and high-level protein expression was achieved by growing cells in 1 L of Luria-Bertani broth to an optical density of 0.6 at 600 nm. Protein expression under the T7 promoter was induced by addition of isopropyl ß-D-1-thiogalactopyranoside to a final concentration of 1 mM, followed by incubation at 16°C for 16-18 hours. Post-harvest, cells were washed in 0.85% NaCl (w/v) and the pellet stored at -20°C or prepared for protein purification. Enzymes were purified as His6 tagged fusions as follows: cell pellets were resuspended in lysis buffer containing 50 mM HEPES, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 mM DNase, pH 7.5 and lysed by three passes on a continuous cell disruptor (Constant Systems Ltd., Daventry, UK) at 30 kPSI. After elution in imidazole from a Nickel-NTA immobilized metal affinity column fractions containing pure protein were pooled and stored at -20 °C in 10% glycerol.

3.2.4.2 Enzymatic synthesis of vancomycin-sulfate

Vancomycin-sulfate was prepared from vancomycin purchased from Sigma Aldrich (St. Louis, MO, USA). Briefly, vancomycin-aglycone was prepared by acid hydrolysis (50 mg vancomcyin in 2 mL of trifluoroacetic acid incubated at 40°C for 3 hours). The reaction was diluted 10X in water and dried by lyophilization followed by purification on a 13 g reverse phase C18 flash column (Teledyne Isco, Lincoln, Nebraska, USA). The aglycone eluted at ~50% acetonitrile.

Stfs reactions were set up as follows: 8 mg purified vancomycin-aglycone, 3.75 mg 3'-phosphoadenosine-5'-phosphosulfate (PAPS), 16 µg Teg14 (95), 50 mM HEPES pH 7.5 and water to 3 mL. The reaction was incubated at 30°C for 30 hours. The reaction was then passed through a 10,000 MWCO filter to remove enzyme and fresh Teg14 was added and incubated for an additional 48 hours. The reaction went to completion and vancomycin-SO_3 was subsequently purified on a 5.5 g C18 gold reverse phase flash column (Teledyne Isco, Lincoln, Nebraska, USA). Fractions containing vancomycin-SO_3 were pooled, dried by lyophilization and stored at -20 °C.

3.2.3 Purification of glycopeptide antibiotics

A47934 and derivatives were purified from *S. toyocaensis* and *S. toyocaensis* Δ*staL* (62) cells after fermentation in Streptomyces Antibiotic Media (96) for 6 days. The mycelia were harvested and the glycopeptide antibiotic was extracted with 1 mL 1% NH_4OH per gram wet weight of cell paste. The extract was retained and dried by lyophilization followed by re-suspension in 1/20th the volume dH_2O and applied to a 26 g reverse phase C18 gold (Teledyne Isco, Lincoln, Nebraska, USA) column and purified by flash chromatography with a

linear gradient of 0-100% acetonitrile. Fractions were monitored by liquid chromatography-mass spectrometry (LC/MS) and antibiotic-containing fractions were pooled and lyophilized. For A47934 the pooled fractions were applied to a 5.7 g SAX anion exchange column (Teledyne Isco, Lincoln, Nebraska, USA) whereas desulfo(DS)A47934 was applied to a 4.3 g reverse phase C18 column (Teledyne Isco, Lincoln, Nebraska, USA).

3.2.4 Determination of antibiotic activity

Minimum inhibitory concentration (MIC) values for antibiotics vs. VREA (*E. faecalis* clinical isolate), VREB (*E. faecalis* ATCC 51299) and *Bacillus subtilis* 1A1 were determined according to CLSI protocols (100). Human plasma was obtained from Sigma-Aldrich (P9253) (Oakville, ON, CA) and used in a 50% concentration according to the protocol in (101, 102). *S. coelicolor* MICs were determined on solid phase media in 96-well plate format inoculated with 5 µL of a 1/25 dilution from thawed spore stocks. The plates were incubated at 30°C for 72 hours and scored visually by eye for growth.

3.2.5 Sulfotransferase enzymatic assays

Activity of glycopeptide sulfotransferases were examined using a coupled assay followed by LC/MS analysis. Reactions contained 50 mM HEPES pH 7.5, 1 mM DTT, 1 mM p-nitrophenolsulfate (PNPS) or *p*-nitrophenolsulfamate (PNPS-NH_2) or *p*-nitrophenolfluorosulfonate (PNPS-F), 1 mM 3'-phosphoadenosine-5-phosphate (PAP), A47934 or DSA47934 and arylsulfotransferase IV (ASTIV) (103). Reactions were incubated at room temperature overnight and analysed by ESI-LC/MS or (Qtrap 2000, Applied Biosytems) ESI-microTOF (Bruker).

3.2.6 Synthesis of *p*-nitrophenol fluorosulfonic acid

Synthesis of PNPS-F was accomplished in two steps following protocols described in literature and the scheme described in figure 3.3. First disuluryl fluoride was made as described in (104) followed by reaction with 4-nitrophenol (PNP) (105) to yield a yellow oil confirmed to be PNPS-F by ESI-LC/MS and NMR (Ch. 6. Appendix 7)

3.3 Results and Discussion

3.3.1 Creating A47934 derivatives with altered sulfate donors

Modification of the glycopeptide scaffold via the activity of tailoring enzymes can be accomplished by different approaches. One path is to exploit the activity of Stfs to transfer sulfate analogs, such as a sulfamide, rather than a sulfate group, providing a unique chemical handle for semi-synthesis of derivatives. A coupled assay (103, 106) was used to transfer the sulfamide group to DSA47934 (Fig. 3.2) from *p*-nitrophenol sulfamate (PNPS-NH_2). The enzyme ASTIV is an aryl sulfotransferase isolated from rat liver, that can regenerate the sulfate donor (PAPS) from PAP and *p*-nitrophenyl sulfate (PNPS) (103, 106).

The advantages of this assay are two-fold: 1) PAPS is costly while PAP and PNPS are not and, 2) PNPS-NH_2 was previously synthesized in our lab to use as starting material for the sulfamidation reaction. Indeed, StaL is able to transfer this sulfamate derivative to desulfated-A47934 (DSA47934) (data not shown) however the efficiency is rather low. In fact, numerous attempts at scaling up the reaction to produce enough A47934-NH_2 for characterization did not lead to increased yields. It was concluded that this reaction is not efficient and not worth further pursuit. Instead, we set out to try a different analog that better mimics the sulfate group. *p*-nitrophenyl fluorosulfonate (PNPS-F) was synthesized as the two-step chemical synthesis described in figure 3.3.

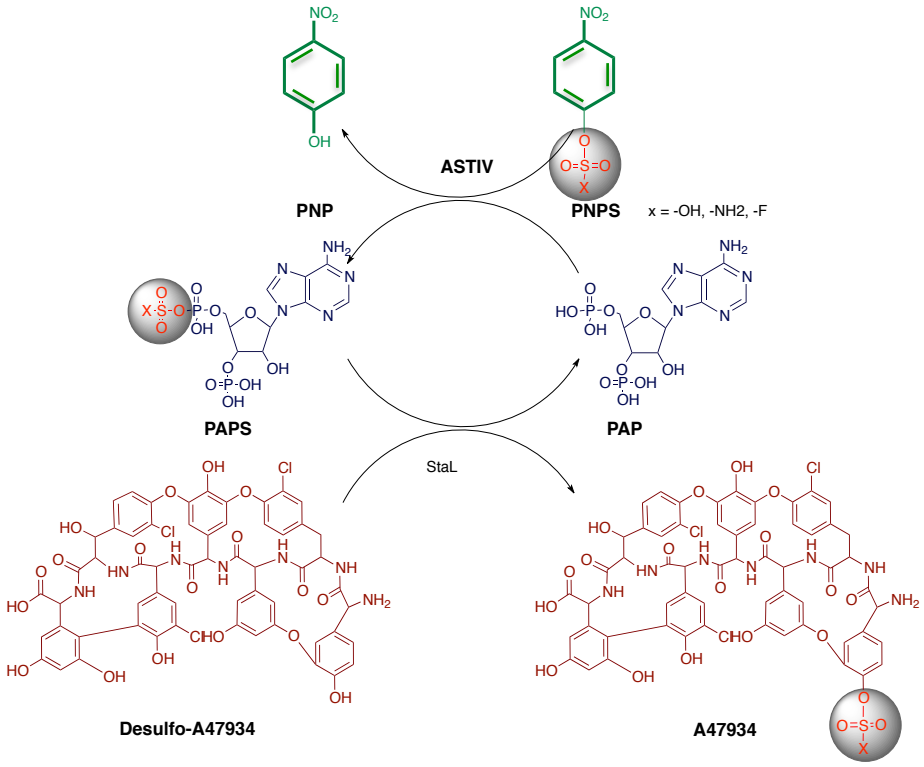


Figure 3.2: Coupled assay to generate A47934 congeners. PNPS-X is used as the sulfur donor and PAPS-X is generated by aryl sulfotransferase IV (ASTIV)

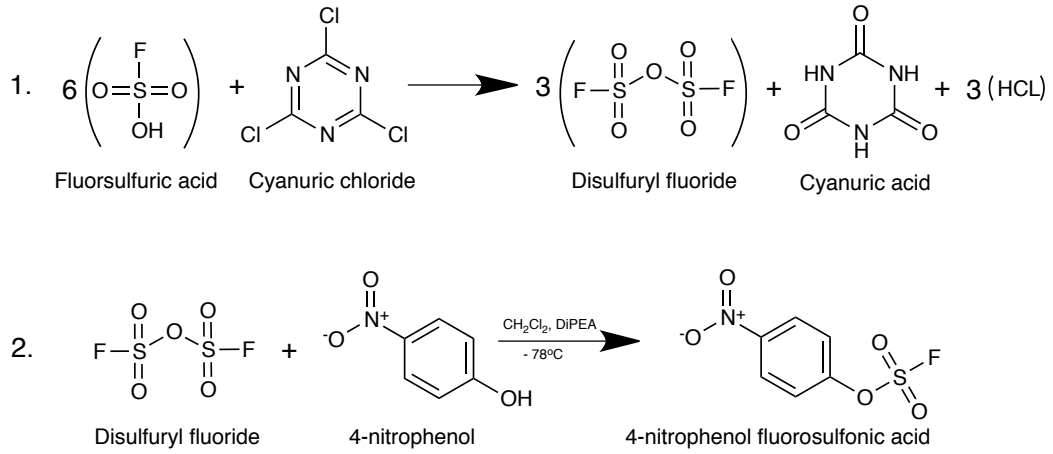


Figure 3.3: Synthetic scheme for synthesis of 4-nitrophenol fluorosulfonic acid to be used as a substrate for glycopeptide sulfotransferases

This substrate exists as non-water-soluble oil, resulting in difficulty optimizing reaction conditions. It was determined that the coupled-assay with PNPS could proceed in a maximum DMSO concentration of 20%, therefore 20% DMSO was included in reactions with PNPS-F to aide in dissolution with the reaction buffer. After several attempts we were able to observe a product by ESI-LC/MS with the expected *m/z* ratio of 1311, consistent with formation of the desired product. Unfortunately this result was not reproducible upon scaling up and subsequent attempts to replicate the initial conditions were not successful. While the production of either a sulfamidated or fluorosulfonated derivative would have yielded interesting avenues to pursue combinatorial synthesis, we decided to focus on the effect of sulfation and other modifications such as glycosylation and halogenation.

First, the regio-specific effects of glycopeptide sulfation were explored to build a platform on which to readily practice combinatorial biosynthesis of A47934 using the tailoring enzymes found in *Actinoplanes* sp. ATCC 53533.

3.3.2 The tailoring enzymes of *Actinoplanes* sp. ATCC 53533

Two naturally sulfated glycopeptides have been described in the literature, UK68597 (*Actinoplanes* sp. ATCC 53533) (107) and A47934, the latter of which has been well characterized in our laboratory. Unlike A47934, UK68597 (Fig. 3.4). is glycosylated and uniquely chlorinated on residue 1 hydroxyphenylglycine (HPG). A 454-pyrosequencing approach was used to obtain a draft sequence of the whole genome and the entire biosynthetic cluster for UK68597 was identified on a ~350 kB contig by searching for a homolog of glycopeptide stfs Each open reading frame was identified and annotated, yielding a 95 kB span of DNA (Fig. 3.5, Table 3.1).

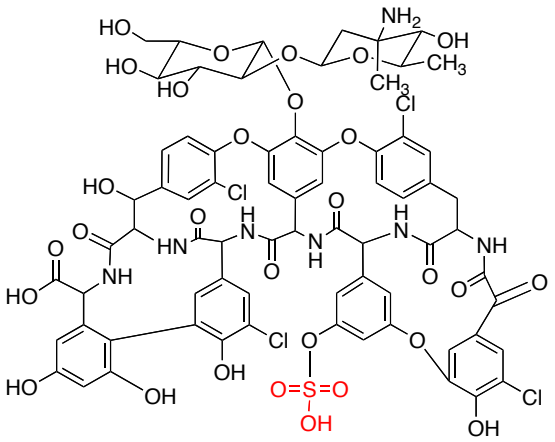


Figure 3.4: Structure of UK68597

The Stf (Auk20) is clustered with other tailoring enzymes and the overall organization of the genes is consistent with other glycopeptide biosynthetic clusters (108). Unique features include a resistance cluster in the organization of *vanSYxxRH* where 'x' represents a protein of unknown function and interestingly another gene cassette organized as *vanRHAX* was identified on a separate contig elsewhere in the chromosome that appears to be clustered with peptidoglycan biosynthetic genes. UK68597 is glycosylated on R4 (HPG) with both glucose and 4-*epi*-vancosamine. Typically two glycosyltransferases will perform these actions, however three are found in the UK68597 cluster. Finally, two halogenases were identified in the cluster however it is difficult to discern

the specificity of each based solely on sequence, therefore biochemical testing will be required.

Actinoplanes sp. ATCC 53533 is not readily amenable to genetic manipulation so we elected to move the tailoring enzyme encoding genes into a new host, *S. toyocaensis*. As previously mentioned, *S. toyocaensis* produces A47934, which is an ideal scaffold for creating novel glycopeptide congeners. It is sulfated, however it is not glycosylated, thus providing multiple residue attachment points accessible for enzymatic manipulation. Furthermore, we have a strain deficient in the Stf gene *staL* and can purify the DSA47934 for use with this approach (62). *S. toyocaensis* is amenable to genetic engineering with the tailoring enzymes of interest to produce the desired glycopeptides by fermentation. Integration of each gene or combination of genes into the chromosome results in stable expression without the need for additional selection during growth. At the same time, each enzyme can be purified *in vitro* if further biochemical characterization is desired.

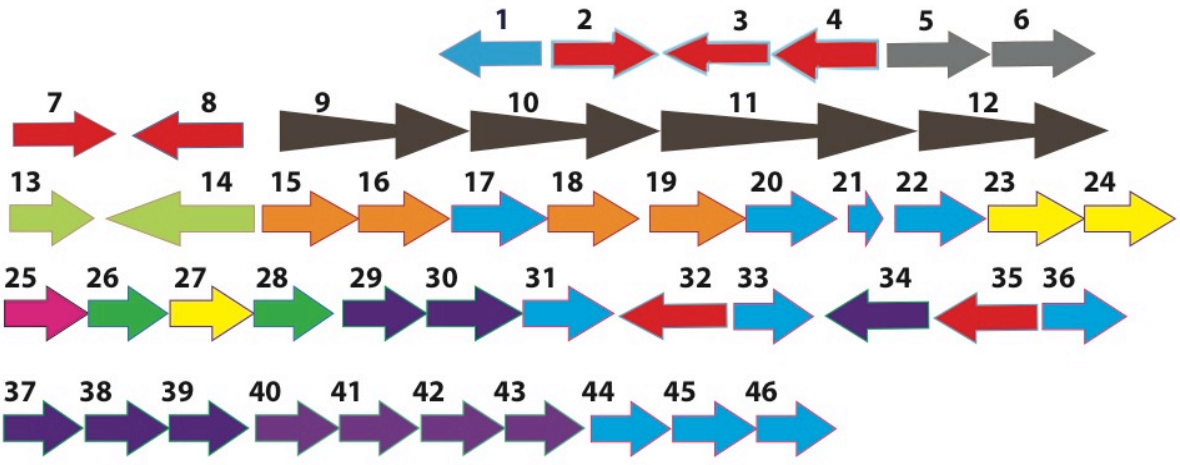


Figure 3.5: Biosynthetic gene cluster for UK68597 produced by *Actinoplanes* sp. ATCC 53533. DNA sequence was obtained by 454 pyrosequencing. Gene annotation in Table 3.1. Arrows are not to scale.

Table 3.1: Summary of open reading frames and predicted protein function encoded by the UK68597 biosynthetic gene cluster.

Orf	Protein	Start (bp)	Stop (bp)	Length	Predicted function
1	Auk 1	7258	8142	885	Phytanoyl CoA dioxygenase
2	DdlAuk	8357	9304	948	D-Ala-D-Ala ligase
3	VanS	9337	10452	1116	VanS
4	VanY	10649	11077	429	VanY-partial
5	Auk 2	11290	13062	1773	Teg 18
6	Auk 3	13119	14213	1095	Dyp-Type peroxidase
7	VanR	14294	14986	693	VanR
8	VanH	15002	16054	1053	VanH
9	Auk 4	16515	22769	6255	NRPS Module 1-2
10	Auk 5	22826	26875	4050	NRPS Module 3
11	Auk 6	27313	38856	11544	NRPS Modules 4-6
12	Auk 7	39641	45175	5535	NRPS Module 7
13	Auk 8	45177	47057	1881	Thymidylyltransferase
14	Auk 9	46943	48307	1365	Methyltransferase
15	Auk 10	48430	49653	1224	Glycosyltransferase
16	Auk 11	49719	50936	1218	Glycosyltransferase
17	Auk 12	51130	52185	1056	Aminotransferase
18	Auk 13	52272	53576	1305	NDP hexose 2,3 hydratase
19	Auk 14	53676	54824	1149	Glycosyltransferase
20	Auk 15	54946	56919	1974	ABC transporter
21	Auk 16	57037	57246	210	MbtH like protein
22	Auk 17	57359	58534	1176	ABC transporter
23	Auk 18	58599	59711	1113	Monooxygenase
24	Auk 19	59965	60897	933	Monooxygenase
25	Auk 20	60939	61778	840	Sulfotransferase
26	Auk 21	61882	63351	1470	Halogenase
27	Auk 22	63478	64677	1200	Monooxygenase
28	Auk 23	64739	66079	1341	Halogenase
29	Auk 24	66170	67681	1512	Non-haem dioxy
30	Auk 25	68007	69053	1047	DAHP synthase
31	Auk 26	69736	70680	945	Transcriptional regulator
32	Auk 27	70756	72375	1620	StaM-dioxygenase
33	Auk 28	72596	73324	729	siderophore
34	Hmo	73345	74664	1320	Hmo
35	Auk 29	75203	77506	2304	Lux R regulator
36	Auk 30	77723	79222	1500	Ion anti-porter
37	HpgT	79401	80654	1254	HpgT
38	HmaS	81041	82117	1077	HmaS
39	Pdh	82120	83529	1410	Pdh
40	DpgA	83704	84810	1107	DpgA
41	DpgB	84804	85472	669	DpgB
42	DpgC	85469	86770	1302	DpgC
43	DpgD	86863	87567	705	DpgD
44	Auk 31	87739	88880	1142	Hypothetical protein
45	Auk 32	89056	90996	1941	Hypothetical protein
46	Auk 33	90973	93002	2030	MerR regulator

We first focused on sulfation and the Stf (Auk20) was amplified from *Actinoplanes* sp. ATCC 53533 genomic DNA and cloned into a pET28a vector for expression in *E. coli* and *in vitro* characterization. At the same time the open reading frame was synthesized, along with two other Stfs of interest Teg 13 and 14 (95) with codon optimization for *Streptomyces* sp.. Sites of sulfation are highlighted in figure 3.8.

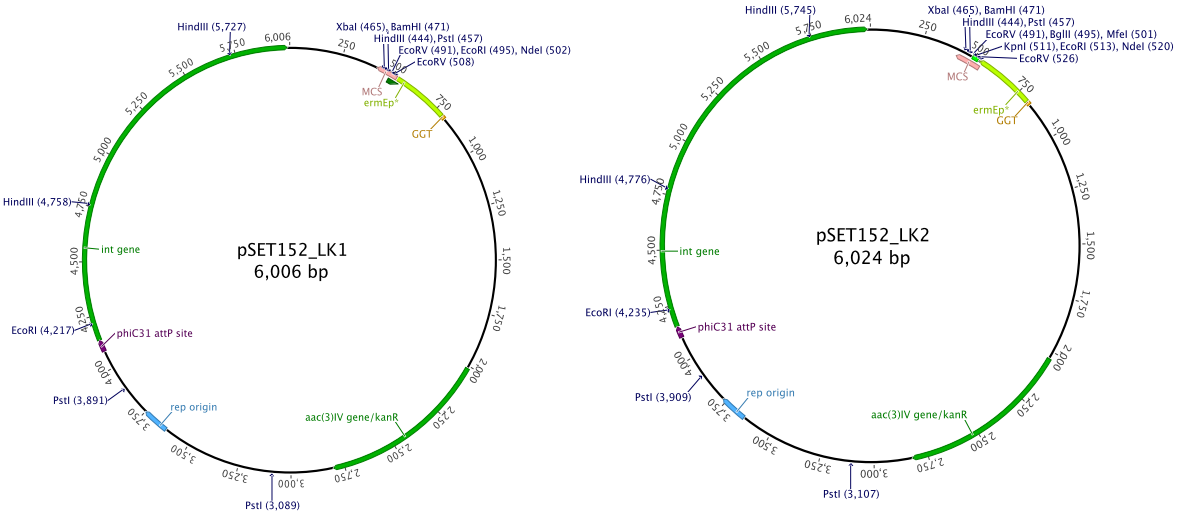


Figure 3.6: Cloning vectors pSET152_LK1/2 for integration into the chromosome. The parent vector pSET152 was engineered with the constitutive promoter *ermEp** and an expanded MCS as labeled. Each exist with either apramycin or kanamycin selection.

Each gene was cloned into the pSET152 vector engineered with the constitutive promoter (*ermEp**) (99) (Fig. 3.6) for integration into the *S. toyocaensis* genome. Chromosome integration was confirmed by PCR and strains examined for A47934-analog production.

The substrate specificity of Auk20 was determined by *in vitro* assays and analysis by LC/MS. A47934 was not a substrate for this enzyme but DSA47934 is. Teg13 and 14 sulfate both A47934 and DSA47934. With this information, integration of each Stf gene into the genomes of both the wild-type and *staL* deletion strains of *S. toyocaensis* was carried out.

Di-sulfated-A47934 was detected in cell extracts from both *S.toyocaensis::Teg13 and 14* (Fig. 3.7). Overall efficiency differed for Teg 13 and 14, for example di-sulfated A47934 was the primary ion detected in the Teg 13 cultures while a mixed population of mono- and di-sulfated A47934 was detected in the Teg14 cultures. Nonetheless, the fermentation volumes were scaled up for purification and downstream characterization. A47934 was the only glycopeptide detected in the *S. toyocaensis::auk20* cultures.

All attempts at scaling up the fermentations and purifying the di-sulfated products were unsuccessful. This included culture volumes of 50 mL to reproduce the original results and volumes ranging from 50-500 mL. It is unclear why this was occurring, however anecdotal evidence and personal observations have shown that *S. toyocaensis* production of A47934 is temperamental. Future work will be discussed subsequently, however gene expression analysis would provide some insight into molecular activities.

This work is ongoing as part of a larger combinatorial and synthetic biology based platform our laboratory wishes to develop. A fundamental element to this platform is an understanding of the regio-specific effects of modification and the ecological outcomes of the type of modification (ie.

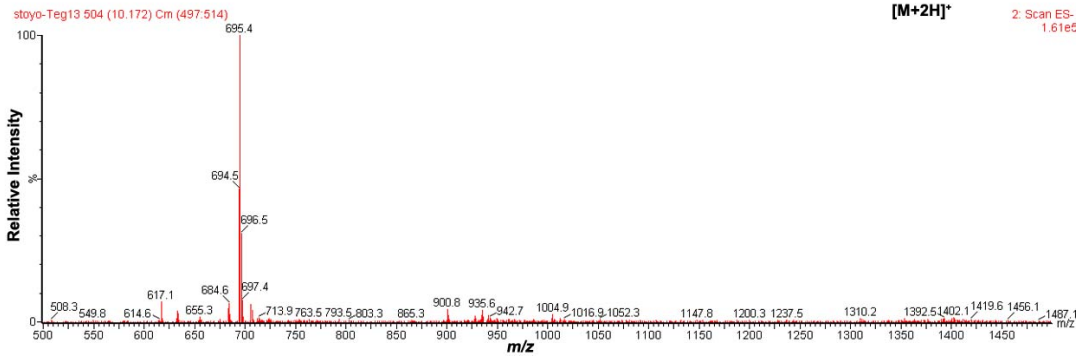


Figure 3.7: LC/MS analysis of *S. toyocaensis::Teg13* cell extraction. 695 corresponds to M+2H in the negative ion mode.

glycosylation vs. sulfation). Working with the Stf's in our library the biological function of sulfation has begun to be revealed.

3.3.3 Regio-specific effects of glycopeptide sulfation

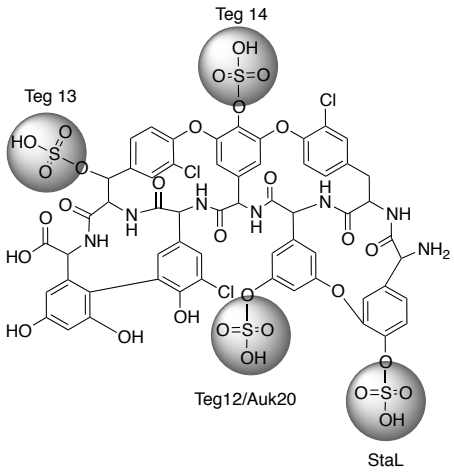


Figure 3.8: Regiospecificity of 5 sulfotransferases. Teg12, 13 and 14 come from metagenomic sequencing. StaL sulfates A47934 and Auk20 from the UK68597 biosynthetic cluster

Sulfation may have previously been thought of as a rare glycopeptide modification, but sulfation itself is not a rare phenomenon and is implicated in many important biological reactions in all kingdoms of life (109-114). As already discussed, as the number of sequenced glycopeptide biosynthetic clusters from the environment grows so does the family of glycopeptide Stfs (65, 95). The role of glycopeptide sulfation on antimicrobial and pharmacological properties of glycopeptide molecules will be investigated.

It has been documented that in the case of A47934, sulfation causes an increase in MIC, albeit a relatively small (two-fold increase) (62). We hypothesized that sulfation, while compromising activity *in vitro*, could impart favorable properties to the glycopeptide heptapeptide scaffold such as improved solubility or decrease in protein binding *in vivo*. The bioavailability of A47934 and DSA47934 in the presence of human plasma was therefore examined. Surprisingly, presence of the sulfate on A47934 caused a 16-fold

increase in the MIC against *Bacillus subtilis* and *S. aureus* (Table 3.2) while DSA47934 had an 8-fold increase in MIC. This effect may be regio-specific as there have been reports of sulfation by Teg13 on residue 6 (ß-hydroxytyrosine on the heptapeptide scaffold) having no effect on antimicrobial activity, albeit in the absence of plasma (95).

Table 3.2: Minimum inhibitory concentrations in ug/mL for DSA47934 and A47934 +/- human plasma.

Test organism	DSA47934	DSA47934 + plasma	A47934	A47934 + plasma
B. subtilis	<0.25	2	1	16
S. aureus	<0.25	2	1	16

It has already been established that each Stf will sulfate DSA47934. Interestingly, two of the five Stfs (Teg13 and 14) associated with type II class sulfation, transfer a sulfate to residues common to both class I and II glycopeptide (Fig. 3.8). Sulfation by Teg13 on residue 6 (ß-hydroxytyrosine) and Teg14 on residue 4 (HPG) occurs where glycosylation is often documented in other glycopeptide antibiotics. We asked if these two enzymes were specific for type II glycopeptides like A47934, or if they are able to accept vancomycin as a substrate.

Vancomycin aglycone was prepared and reactions were set up with PAPS, Teg13 and Teg14. Teg13 did not sulfate vancomycin or the aglycone however in the reaction containing Teg14 and vancomycin aglycone a new peak appeared when analysed by ESI-LC/MS with a *m/z* of 1222, corresponding to the expected mass of sulfated vancomycin (Fig. 3.9). Antimicrobial activity was assayed by both the Kirby-Bauer disk assay (Fig. 3.10) and determination of MIC (Table 3.3). It appears that sulfation significantly diminishes activity compared to both vancomycin and the aglycone molecule. The MIC for a sensitive strain of *B.*

subtilis was 64 µg/mL compared to <0.5 µg/mL and 2 µg/mL for vancomycin and vancomycin-aglycone respectively (Table 3.3).

There have been reports (115) that the pseudoaglycone (loss of one rather than both sugars) of vancomycin is detrimental to activity (116) and our results further support the importance of enzymatic modification at this position on the molecule. Glycopeptide Stfs have specific residues on the heptapeptide they sulfate and this particular enzyme appears to maintain regio-specificity regardless of the class of glycopeptide.

While sulfation of HPG_4 on the vancomycin scaffold does not appear to impart favorable antibiotic characteristics, this study has provided insight

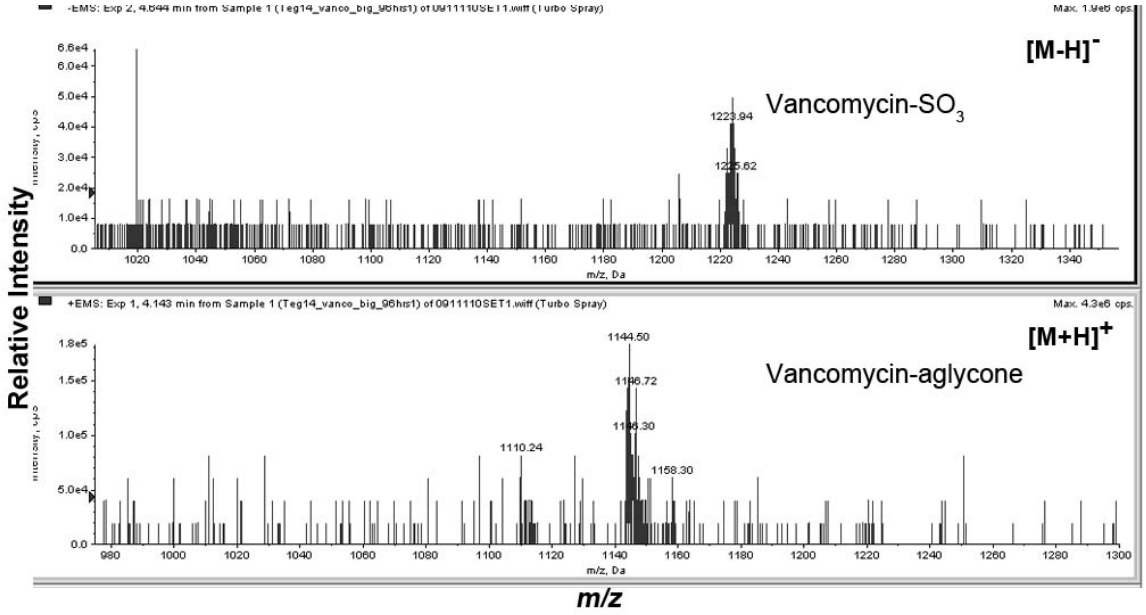
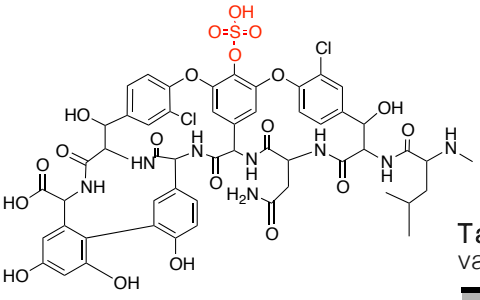


Figure 3.9: ESI-LC/MS trace of a reaction with Teg14 and vancomycin-aglycone. In the top window is unreacted vancomycin-aglycone with a m/z of 1144. The middle window shows an ion with a m/z of 1222, the expected mass of vancomycin-SO_3 and in the bottom window is the LC trace

into the role of glycopeptide modification. Modification of this residue appears to be important, although is not involved in binding the cell wall target. Previous studies have shown glycosylation improves antibiotic activity but replacement (115) with a negatively charged sulfate group essentially abolishes activity. Enzymatic inactivation of antibiotics is well known for many classes of antibiotics. In this particular instance, sulfation by Teg14 could serve as a



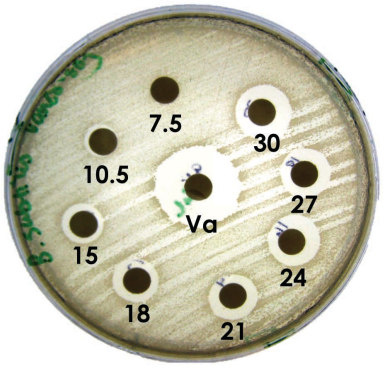


Table 3.3: Minimum inhibitory concentrations in µg/mL for vancomycin, vancomycin-aglycone and vancomycin-SO_3.

Test Organism	Vancomycin	Vancomycin aglycone	Vancomycin-SO_3
B. subtilis	<0.5	2	64
VRE VanA	>256	>256	>256
VRE VanB	64	256	>256

Figure 3.10: Kirby-Bauer disk diffusion assay for Vancomycin-SO_3. (Structure shown above) Test organisms is *Bacillus subtilis* 1A1. Numbers indicate µg antibiotic applied.

detoxifying strategy for the host organism while sulfation of other residues on the heptapeptide might impart properties yet to be characterized.

3.4 Conclusions

Glycopeptide antibiotics are known to be modified by various means, such as halogenation, methylation, and hydroxylation in addition to glycosylation

and sulfation, however the roles of each have not been well studied. Systematic characterization of not only each tailoring enzyme, but also regio-specificity of modifications supports hypothesis- and activity-driven drug design. Tailoring enzymes constitute a 'toolbox' to accessorize the glycopeptide scaffold in a manner that nature evolved to create novel "unnatural" natural products with improved activity against resistant organisms by either overcoming resistance or evading it all together.

This work can be expanded to include directed protein engineering and complete re-construction of biosynthetic clusters. For instance, in the A47934 biosynthetic cluster a negative regulator exists (StaS). Deletion of this gene results in overproduction of A47934 (data not shown). Combining such methods of deletions/insertions of new enzymes and biosynthetic machinery will generate infinite possibilities of derivatives and greatly expand the chemical diversity of this class of antibiotic. Preliminary work and suggestions for future effort will be discussed in detail in chapter 5.

Chapter 4

SULFATION EVADES VANS MEDIATED RESISTANCE

This chapter contains material in preparation for publishing entitled:

Kalan, L., Perry, J., Koteva,K., Thaker,M., and Wright, G.D., 2012. Sulfation Evades VanS Mediated Resistance. *Manuscript in preparation for submission*

For this work I purified the glycopeptides and sulfotransferase Teg14. I made the vancomycin aglycone and did the enzymatic reactions to create vancomycin sulfate. I performed the ITC, dimerization, and MIC experiments. I analyzed all of the data and wrote the manuscript.

I would like to acknowledge Dr. Kalinka Koteva, Dr. Julie Perry and Dr. Maulik Thaker for their contribution to this work. This was truly a collaborative project spawned from a brainstorming session and creative discussion.

4.0 Chapter 4

4.1 Introduction

Antimicrobial drug resistance is a global concern. Drug resistant infectious organisms such as methicillin resistant *Staphylococcus aureus* (MRSA) are a major public health problem. Glycopeptide antibiotics vancomycin and teicoplanin are used as front-line therapy to treat serious Gram-positive infections such as MRSA, but glycopeptide resistant organisms have emerged in response. A problem of particular significance is the spread of vancomycin resistant *Enterococci* (VRE) in hospitals and other health care facilities. The genes that confer glycopeptide resistance in VRE have been passed to MRSA on several occasions, presenting a even greater threat due to the increased virulence and drug resistance of this organism.

The strategy employed by organisms like VRE to achieve vancomycin resistance is via modification of the drug target. This is accomplished through the protection of a tightly regulated five gene cassette: *vanRSHAX.* Vancomycin binds the terminal lysyl-D-alanyl-D-alanine of peptidoglycan and its precursors on the exterior of the cell. Consequently, the growth of cell wall peptidoglycan by transglycosylation along with vital cross-strand bridging catalyzed by transpeptidases are impaired leading to a weakened cell wall, inhibition of cell division and death. The *vanHAX* genes encode enzymes that re-engineer peptidoglycan precursors to terminate in D-alanyl-D-lactate rather than D-alanyl-D-alanine; this change disrupts glycopeptide antibiotic binding by elimination of a key drug-binding hydrogen bond, resulting in a subsequent 1000-fold

decrease affinity (Fig. 4.2) (38, 56, 72). Expression of these genes is under the genetic control of a two-component regulatory system consisting of the response regulator VanR and the membrane spanning receptor His kinase VanS (57, 117).

Glycopeptides are categorized according to their core heptapeptide backbone and fall into two general classes: type I (vancomycin, with an N-terminal Leu-ß-hydroxy-3-chloroTyr-Asn tripeptide) and type II (teicoplanin, with an N-terminal hyroxyphenylGly-3-chloroTyr- 3,5-dihydroxylphenylGly tripeptide) (36, 118) (Fig. 4.1). Within each class, individual antibiotic structures differ based on the degree and nature of modification of the backbone by glycosylation, halogenation, methylation, acylation, and sulfation. Resistant phenotypes are classified based on which molecules the organism remains

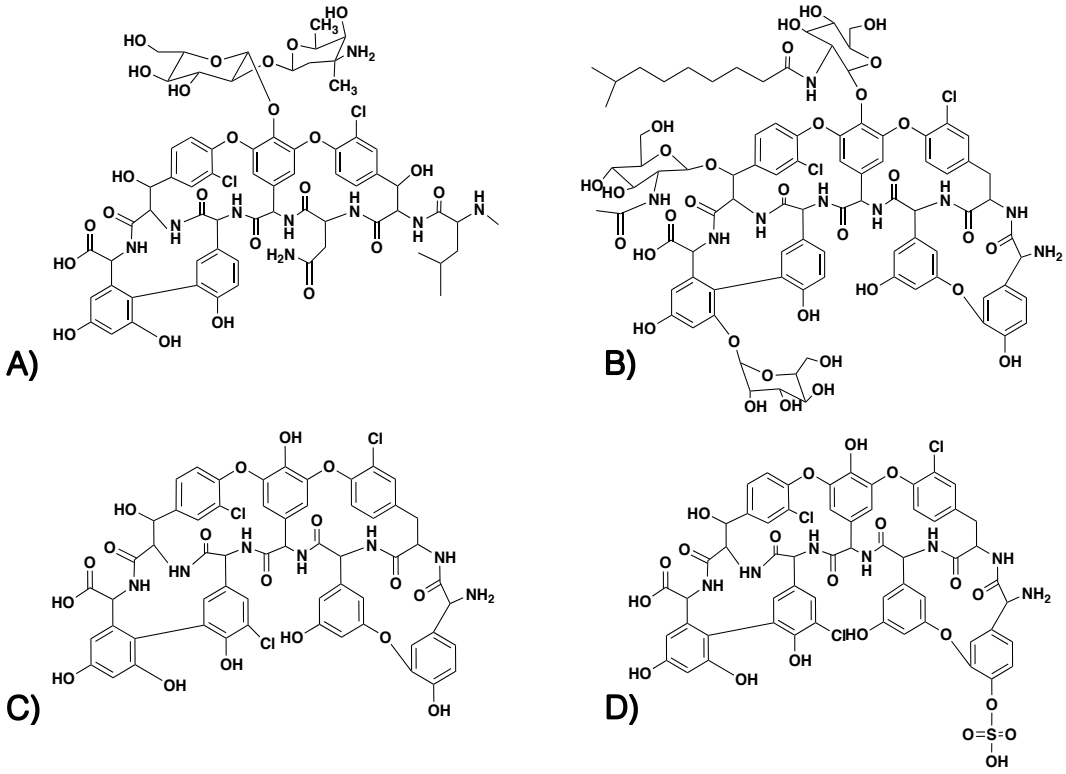


Figure 4.1: Glycopeptide structures. A) Vancomycin. B) Teicoplanin. C) DSA47934. D) A47934

sensitive to. For example, the VRE VanA (VREA) is resistant to both vancomycin and teicoplanin while VRE VanB (VREB) is resistant to vancomycin but sensitive to teicoplanin. We are interested in exploiting this phenotype of differential induction as a strategy to find novel glycopeptides active against glycopeptide resistant organisms. The actinomycete *Streptomyces coelicolor* is an excellent model for this system as it does not produce glycopeptides yet it harbors a *vanRSKJHAX* cassette with a 'VanB' phenotype (119, 120).

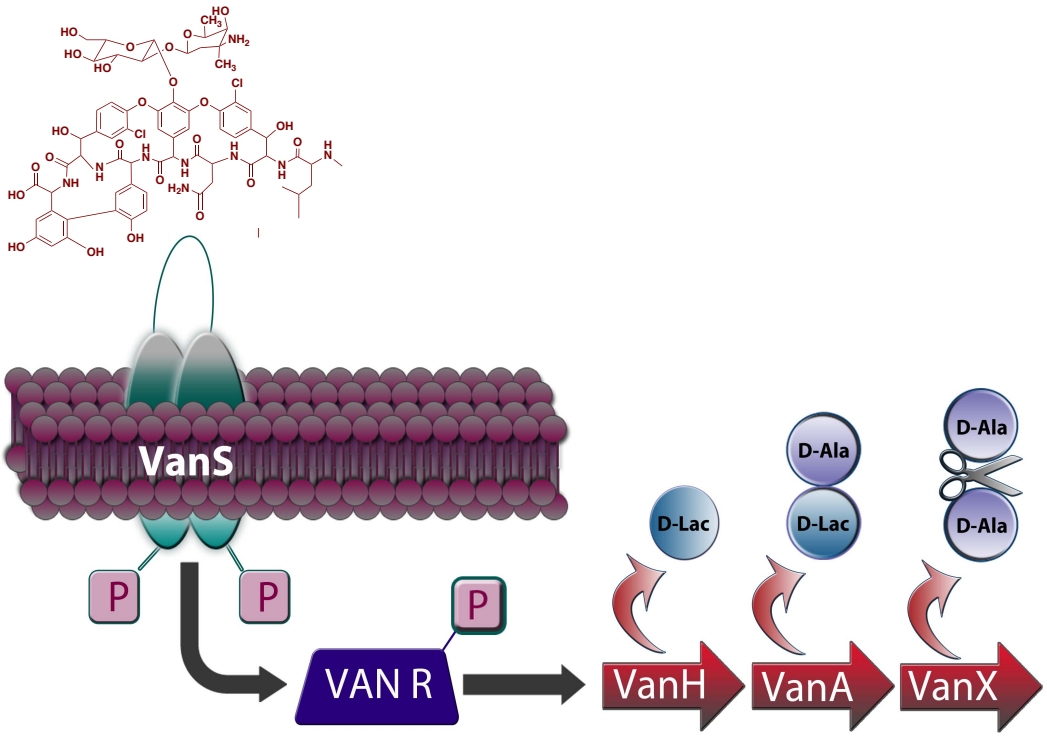


Figure 4.2: Regulation and mechanism of glycopeptide resistance. Vancomycin binds $VanS_{Sc}$ causing self-phosphorylation and dimerization. $VanS_{Sc}$ phosphorylates VanR which activates transcription of *vanHAX*. VanH, A, and X enzymes restructure peptidoglycan precursors to terminate in D-Ala-D-Lac rather than D-Ala-D-Ala. This leads to a 1000-fold decrease in vancomycin binding and high-level resistance.

Natural product modification by sulfation is relatively uncommon and only two sulfated glycopeptides have been described in the literature, both are Type II glycopeptides with sulfate addition to phenolic hydroxyls (4, 107). UK68597 is

sulfated on residue 3 (3,5-dihydroxyphenylGly) (107), while A47934 is sulfated on the N-terminal 3-hydroxyphenylGly (61, 62). A47934 is the most studied and is active against VREB but not *S. coelicolor.* Recently, environmental metagenome sequencing has uncovered a number of glycopeptide sulfotransferases with varying regio-specificity of compound modification, indicating glycopeptide sulfation may be more widespread (65, 95). Previous work has shown that sulfation actually decreases the antimicrobial activity of the molecule, regardless of the residue sulfated (62, 65, 95).

Why would nature evolve the mechanism of sulfation if the effect of the modification were a reduction in biological activity? We hypothesized that sulfation of glycopeptides may alter their affinity for VanS and serve as a strategy to evade resistance. Therefore we set out to deduce the role of glycopeptide sulfation in both mechanism of action and induction of resistance. We report that while sulfation does not affect cell wall binding, it has a profound effect on recognition by VanS in *S. coelicolor* ($VanS_{Sc}$). The affinity of desulfo-A47934 (DSA47934) for $VanS_{Sc}$ does not significantly differ from that of A47934, while low levels of DSA47934 are able to induce resistance to teicoplanin when A47934 does not. This work suggests a role for sulfation in next-generation glycopeptide synthesis and directed modifications to both improve antibiotic activity and evade resistance by lack of VanS binding and expression of *vanHAX*.

4.2 Materials and Methods

4.2.1 Isolation and purification of glycopeptides

A47934, DSA47934, vancomycin-sulfate and vancomycin-aglycone were obtained and purified as described in chapter 3.

4.2.2 Isothermal titration calorimetry (ITC)

ITC was used to monitor the interaction of antibiotics with cell wall precursors as previously described (57) with the following modifications: 1000 μM N,N-diacetyl-Lys-D-Ala-D-Ala tripeptide solution was placed in the calorimetric syringe and 50 μM A47934 or DSA47934 in 20 mM sodium citrate buffer pH 5.1 was placed in the cell.

4.2.3 Antibiotic activity

Minimum inhibitory concentration (MIC) values for antibiotics vs. a *Enterococus faecalis vanA* clinical isolate (VREA), *Enterococus faecalis* ATCC 51299 (VREB) and *Bacillus subtilis* 1A1 were determined according to CLSI protocols (100). *S. coelicolor* MICs were determined on solid phase media in 96-well plate format inoculated with 5 μL of a 1/25 dilution from thawed spore stocks. The plates were incubated at 30°C for 72 hours and scored visually

Disk assays to examine induction of resistance to teicoplanin in clinical isolates were performed by plating a lawn of spores (10 μL spore stock diluted in 20 μL of 10% glycerol) on Bennetts agar plates containing 0.5 μg/mL teicoplanin or no drug and placing 6 mm paper disks on the lawn. Each glycopeptide was spotted onto a paper disk at quantities of 0.05, 0.5 or 7.5 μg. The plates were incubated at 30 °C for 72 hours. For VREB, an overnight lawn of VREB grown on a brain heart infusion (BHI) agar plate and was diluted in 0.85%

NaCl to an optical density 600 nm of 0.08-0.1. The dilution was swabbed three times on a BHI agar plate and the glycopeptides applied to paper disks as described above.

4.2.4 Gene expression analysis

Transcriptional analysis of *vanA* expression in *S. coelicolor* exposed to glycopeptide antibiotics was conducted by real-time reverse transcription (RT)-PCR. Cells were inoculated at 1/25 dilution from thawed 2 x 10^4 spore stocks onto Bennett's agar containing vancomycin, A47934 and DS-A47934 (all at concentrations of 0.1, 0.25 and 1 µg/ml) alone and in combination with teicoplanin (0.1, 0.25, 0.5 µg/ml) in 12-well plates. Cells were grown at 30°C for 72 hours, and harvested by gently scrapping cells off the surface of the agar using wooden sticks. Total RNA was extracted from harvested cells using the RNeasy Mini RNA extraction kit (Qiagen, Valencia, CA, USA) according the manufacturer's directions for Gram-positive bacteria. Contaminating genomic DNA was removed from RNA samples using RNase-free DNase (Fermentas, Burlington, ON, CAN), which was subsequently inactivated by heat/EDTA according to the manufacturer's directions. DNA-free RNA samples were subjected to reverse transcription using the SuperScript® VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR reactions were carried out using Phusion® High Fidelity DNA polymerase and 1x SYBR Green I DNA stain (Invitrogen, Carlsbad, CA, USA) in a BioRad C1000 Thermocycler. Cycling conditions were as follows: 98°C for 30 sec., (98°C for 10 sec., 54°C for 30 sec., 72°C for 10 sec.) x 45 cycles, 72°C for 10 minutes. A standard curve was plotted with cycle threshold (Ct) values obtained from amplification of known quantities of cDNAs. The standard curve was used to determine the efficiency

(E) of primer set binding and amplification using the formula $E=10^{-1/slope}$. Comparison of the expression of *vanA* gene between Bennett's medium without antibiotic (control) and in the presence of antibiotic (condition) was determined using the formula:

$$Ratio=(E_{vanA})^{\Delta Ct(control-condition)}/(E_{16SrRNA})^{\Delta Ct(control-condition)}$$

The 16S rRNA gene was used as internal reference. All assays were performed in triplicate with RNA isolated from three independent experiments.

4.2.6 Determination of dimerization constants

Dimerization constants were determined using the method described in (121) with the following modifications. Each antibiotic was analyzed on a Bruker micro-TOF ESI instrument. Each molecule was serially diluted 1 in 2 ranging from 1 µg/µL to 8 ng/µL and the ability to dimerize was recorded at each concentration.

4.3 Results and Discussion

4.3.1 DSA47934 and A47934 induction of teicoplanin resistance:

VanS receptor kinases tightly regulate *vanHAX* expression by responding to individual glycopeptide molecules (57). In *S. coelicolor*, vancomycin induces resistance via activation of $VanS_{Sc}$. On the other hand, teicoplanin is unable to trigger this induction and as a result *S. coelicolor* is sensitive to this antibiotic (MIC 0.5 µg/mL). However, in the presence of vancomycin, *S. coelicolor*

becomes resistant to high levels of teicoplanin because of vancomycin's ability to induce the *vanHAX* resistance cassette via $VanS_{Sc}$ (Fig. 4.2). We exploited this induction of teicoplanin resistance phenotype as a biological indicator of $VanS_{Sc}$ binding and activation by other glycopeptides. Combinations of teicoplanin with vancomycin, A47934 and DSA47934 in solid media were used to quantify the

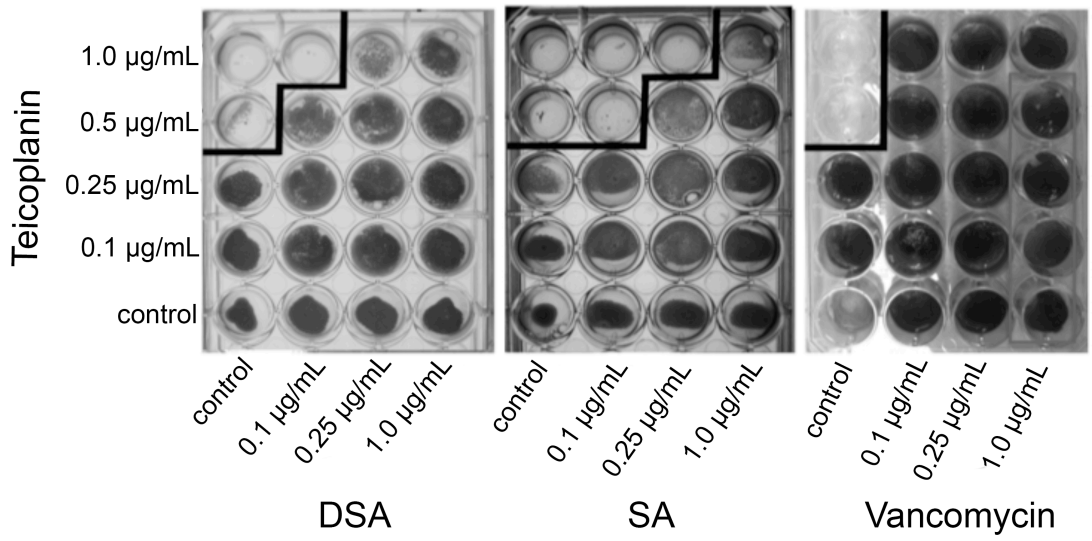


Figure 4.3: Solid-phase checkerboard analysis of *S. coelicolor* with teicoplanin in combination of DSA47934 (DSA), A47934 (SA) and vancomycin. On the Y-axis is the concentration of teicoplanin in each row and in the X-axis the concentration of DS-,A47934 or vancomycin in each column. Boxes represent concentrations chosen for qRTPCR analysis.

ability of these three glycopeptides to antagonize the intrinsic teicoplanin antibiotic activity against *S. coelicolor* (Fig. 4.3).

Vancomycin was able to induce teicoplanin resistance within a range of 0.1 – 1 µg/mL as previously reported (73, 120) (Fig. 4.3). The same range of DSA47934 also allowed growth on a teicoplanin concentration of > 0.5 µg/mL, while a 10-fold higher concentration of A47934 (1 µg/mL) was required for growth on teicoplanin. The difference between DSA47934 and A47934 in their ability to induce resistance to teicoplanin is intriguing given that the only distinction between the two molecules is a single sulfate modification of the

phenolic hydroxyl group of the N-terminal 4-hydroxylphenylGly. Moreover, this structural difference is not reflected in the MIC of each molecule. In fact, *S. coelicolor* is resistant to A47934 with a MIC of 512 µg/mL and a MIC of 256 µg/mL for DSA47934.

4.3.2 Sulfation does not affect target affinity:

As mentioned above, A47934 consistently presented a 2-4 fold greater MIC than DSA47934 against susceptible bacteria (Table 4.1); in other words sulfation decreases antibiotic activity. One possibility for this observation is that sulfation decreases the affinity of the molecule for the target acyl-D-Ala-D-Ala due to electrostatic repulsion or steric hindrance. We used isothermal titration calorimetry (ITC) to determine the dissociation constant (K_D) of vancomycin, DSA47934 and A47934 for $N_{\alpha,}N_{\varepsilon}$-Diacetyl-Lys-D-Ala-D-Ala. K_D values for these

Table 4.1 Summary of MIC in µg/mL, ITC binding constants, and k_{dim} data

	Vancomycin	DSA47934	A47934	Teicoplanin	Vancomcyin-SO_3
VRE A MIC	> 256	>256	>256	256	> 256
VRE B MIC	64	1-2	4	0.5 - 1	> 256
S. coelicolor MIC	512	256	512	4	N.D
B. subtilis MIC	< 0.5	0.25	1	< 0.25	64
ITC µM	2.94	10.4	4.76	N.D	N.D
$K_{dimerization}$	2.7×10^2 M^{-1}	2.6×10^2 M^{-1}	N.A	1.6×10^3 M-1	2.2×10^3 M-1

antibiotics differed only 2-3-fold (2.94, 10.4 and 4.76 µM respectively), indicating no significant difference in affinity for the drug target. Although this *in vitro* assay isn't a direct representation of physiological conditions it does allow us to conclude that sulfation does not compromise antibiotic affinity for the target. These results are consistent with interpretation of changes in antibiotic

activity and resistance profiles are a result of *vanHAX* expression and not of altered target affinity.

The physiochemical properties of each glycopeptide molecule was further examined by monitoring their ability to form dimers in solution. Vancomycin and other Type I glycopeptides have been reported to dimerize 'back-to-back' (122, 123) and this property is postulated to enhance its antimicrobial activity. On the other hand, teicoplanin has not been reported to form dimers. This difference prompted us to explore whether dimer formation correlates with induction of resistance.

Mass-spectrometry was used to determine the dimerization constants (K_{dim}) for the four glycopeptides in this study (Table 4.1). Vancomycin dimers were readily quantified and at higher concentrations the molecule appeared to

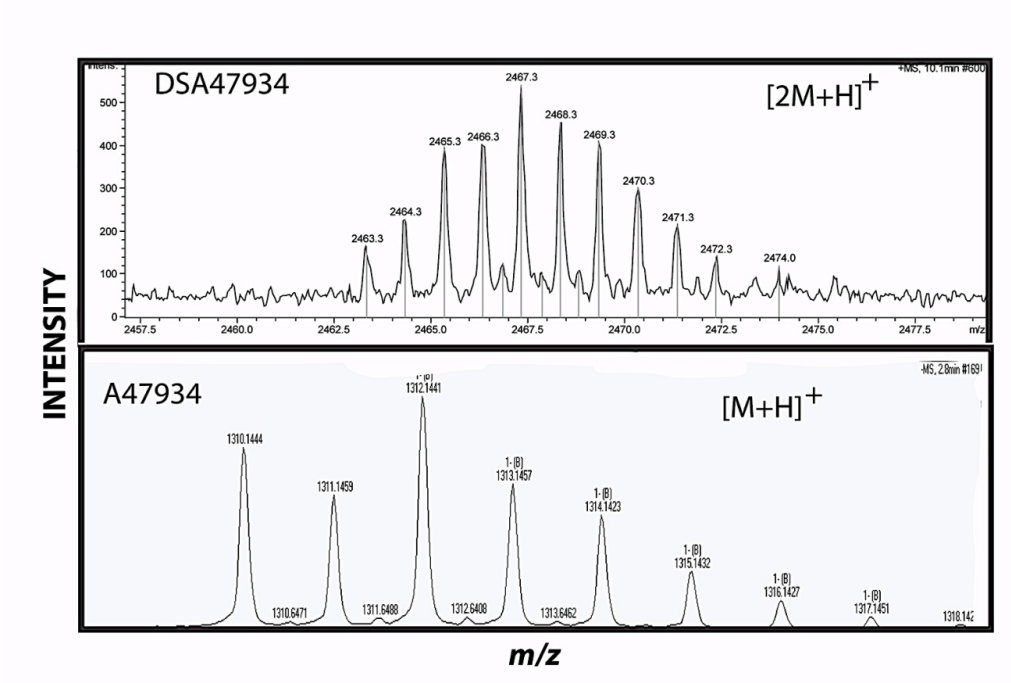


Figure 4.4: *m/z* of DSA47934 (MW: 1232) as a dimer and A47934 (MW: 1312) as a monomer.

oligomerize forming higher order complexes. DSA47934 exhibited similar properties as vancomycin however it appeared that a critical concentration of 0.25 µg/mL was required. On the other hand, the sulfated antibiotic A47934 remains in a monomeric species even at concentrations reaching 4 mg/mL (Fig. 4.4). Surprisingly, teicoplanin dimers were observed in our mass spectrometry assay in a manner positively correlated to concentration making this the first report of teicoplanin dimer formation (Ch 6, Fig. A1). Vancomycin-SO_3 formed dimers but higher order complexes were not observed as with vancomcyin (Ch 6. Fig. A1). 'Back-to-back' and 'face-to-face' dimers resulting in tetramers has been described in the literature for vancomycin (122) and balhimycin (124) supporting our observations. While the full effect of dimerization is still not clearly understood in terms of antibiotic action, in the context of our studies, the formation of higher order oligomers (n > 2) correlates with activation of $VanS_{Sc.}$. It should also be considered that dimer formation is observed in solution with pure glycopeptide and the conditions may not necessarily mimic cellular binding.

4.3.4 Glycopeptide sulfation depresses transcription of *vanA*

To directly address the impact of antibiotic sulfation on *vanHAX* gene expression, we determined the effect of each antibiotic on levels of *vanHAX* transcripts in *S. coelicolor* at concentrations of glycopeptide antibiotic that induce resistance to 0.5 µg/mL teicoplanin, and analyzed them by quantitative RT-PCR. Vancomycin served as a positive control and a dose dependent effect on *vanA* expression was observed: 0.1 µg/mL and 1 µg/mL increased expression of *vanA* 1.76 and 20.3 fold respectively. Curiously, DSA47934 induced *vanA* expression was inversely correlated to dose but nevertheless

stimulated the greatest fold-increase in *vanA* expression with nearly a 70-fold increase compared to the control in the presence of 0.1 µg/mL DSA47934 and 9-fold in the presence of 1 µg/mL DSA47934. In keeping with our hypothesis that sulfation prevents the induction of resistance, 0.1 and 1 µg/mL A47934 had a modest 2.35 and 4.32-fold increase and appears to be the weakest inducer of teicoplanin resistance of all three molecules tested. These results imply that not only is the desulfo form of the antibiotic a more powerful inducer of *vanHAX* than the sulfated form, but that low concentrations of DSA47934 are more powerful inducers of resistance than higher concentrations (Fig. 4.5).

These results indicate that sulfation can serve as a strategy of glycopeptide producing organisms to overcome acquired resistance in non-producer neighbors. To examine the ability of each molecule to induce resistance to teicoplanin, *vanA* expression was monitored when 1 µg/mL of each molecule was in combination with a growth inhibiting concentration of 0.5 µg/mL

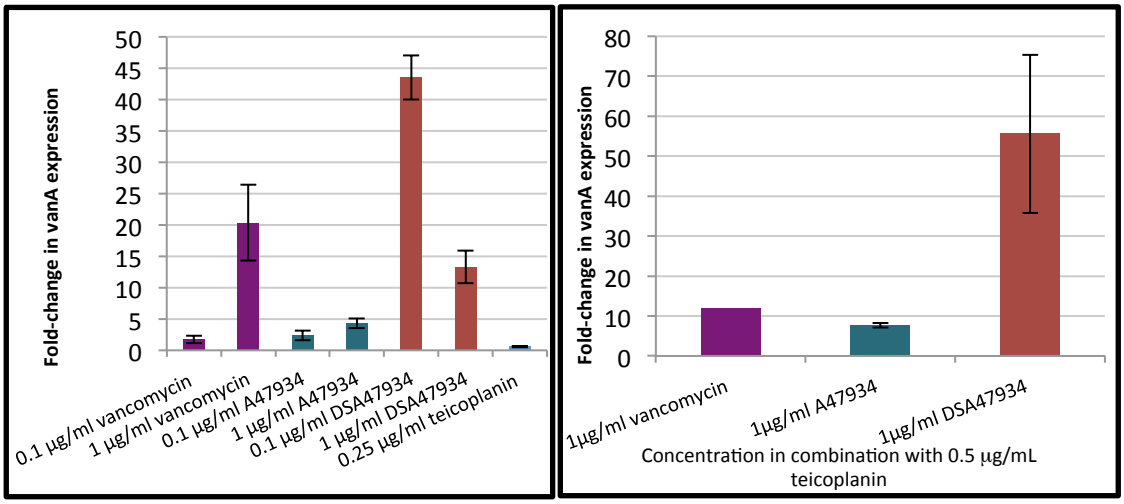


Figure 4.5: qRTPCR analysis of *S. coelicolor vanA* upon exposure to DSA47934, A47934, vancomycin and teicoplanin alone (left panel) and in combination with 0.5 µg/mL teicoplanin (right panel). Data is normalized to 16S RNA levels.

teicoplanin. Vancomycin and A47934 resulted in a 9.45 and 7.66-fold increase in expression respectively. Once again, DSA47934 still triggered the largest increase in expression of 55.6-fold compared to a no antibiotic control. Although the sulfated A47934 was still able to induce resistance, presumably via a VanS mediated event, 10-fold more antibiotic is required compared to the desulfated form and even then the downstream transcriptional effect is only 10% of DSA47934 at the same concentration (Fig. 4.5).

Sulfation therefore effectively masks the antibiotic toward organisms that have acquired the VanB phenotype. This is intriguing in the context of several reports that suggest that antibiotics behave as signaling molecules at low concentrations (125). An organism that produces a non-sulfated antibiotic (DSA47934), risks triggering induction of resistance at low, sub-lethal concentrations. As a result, sulfation becomes an attractive modification to reduce induction of resistance.

4.3.5 Sulfation masks resistance in *vanB* type environmental isolates

Our observations support sulfation as a strategy to evade resistance in *S. coelicolor*, but is this phenomenon apparent in other species of non-glycopeptide producing *Streptomyces* that are vancomycin resistant? A collection of such strains that have been isolated from various geographically distinct soil samples in our lab were tested on teicoplanin and treated with either DSA47934 or A47934. Consistent with *S. coelicolor*, DSA47934 induced resistance to teicoplanin with much higher sensitivity than A47934. While induction was still observed with the sulfated molecule, it was only at the highest concentration tested as expected (Fig. 4.6, Table 4.2). Interestingly, in some

strains both DS- and A47934 acted as an antibiotic when administered alone yet still induced resistance to teicoplanin when in combination.

4.3.6 Evasion of resistance by sulfation is not regio-specific

A47934 is of the sub-class II type of glycopeptides and while a great tool for studying interactions in the laboratory, it is not approved for use in the clinic. Furthermore, we are curious if the site of sulfation on the heptapeptide backbone impacts the ability to modulate resistance. Vancomycin-sulfate (SO_3), described in chapter 3 has a greatly diminished activity (MIC of 64 µg/mL vs. 0.5 µg/mL against *B. subtilis*) and VREB remains resistant to it. Still, the ability to bind VanS and induce resistance to teicoplanin was questioned. A panel of environmental isolates resistant to vancomycin, was tested and the results are indisputable. Both vancomycin and the aglycone clearly induce resistance to teicoplanin in a dose dependent manner, while vancomycin-SO_3 does not. At the highest quantity of vancomycin-SO_3 a small zone of growth is observed, indicating induction but at levels only comparable to the bottom quantities of vancomycin (Fig. 4.6, Table 4.2). Sulfation of vancomycin may diminish the antimicrobial

Table 4.2: Relative ability of different glycopeptides to induce resistance to 0.5 µg/mL teicoplanin in environmental vancomycin resistant isolates of the VanB phenotype. Strain 1401 is of VanA phenotype and serves as a control. +, ++, and +++ denote degree of activation of resistance.

Strain	A47934 Induction	DSA47934 Induction	Vancomcyin Induction	Vancomycin - SO_3 Induction
WAC 1380	++	+++	++	+
WAC 1386	++	+++	++	+
WAC 1387	++	+++	N.A	N.A
WAC 1391	++	+++	+++	+
WAC 1397	++	+++	+++	+
WAC1401	N.A	N.A	Teico R	Teico R

activity, but it also reduces binding of the sensor kinase VanS. This activity could have important implications if it is also present in clinical pathogens. It is hypothesized that resistance entered the clinic from the environment, therefore regulation should be analogous (28, 75, 126).

VREB infections are a serious nosocomial problem. Both DS- and A47934 are active against VREB and thus neither induce resistance to teicoplanin. Vancomycin however, will induce resistance. The induction assay was performed on VREB with vancomycin, the aglycone and sulfated molecule.

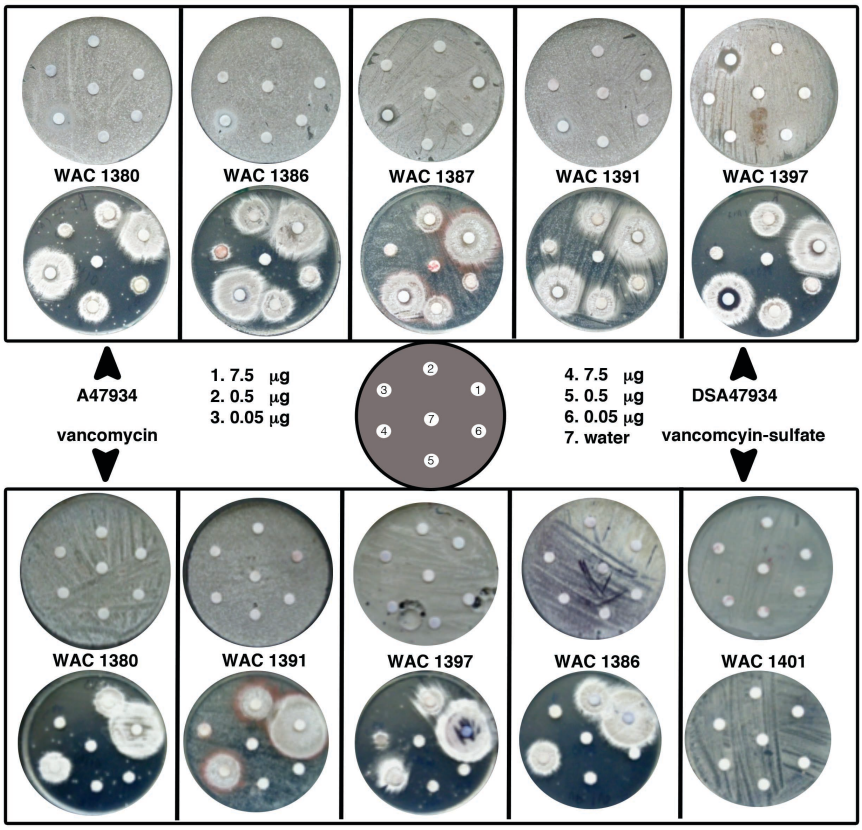


Figure 4.6: Teicoplanin induction assay. Top panel: A47934 and DSA47934 applied to VanB type isolates in the absence of teicoplanin (upper) and in the presence of 0.5 µg/mL teicoplanin (lower). Bottom pane: Vancomycin and vancomycin-sulfate applied to VanB type isolates in the absence of teicoplanin (upper) and in the presence of 0.5 µg/mL teicoplanin (lower). Growth in the lower panel indicates induction of resistance. Numbers indicate quantity of glycopeptide in µgs.

Figure 4.7 displays three concentrations of each antibiotic. Albeit vancomycin-sulfate induces a small zone of growth on the teicoplanin plates, relative to both vancomycin and the aglycone it is not significant. This observation has lead to many conclusions and further questions.

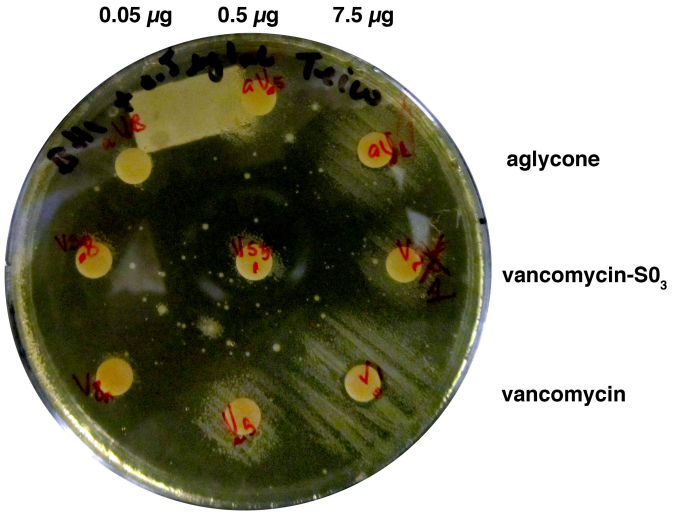


Figure 4.7: Induction of teicoplanin resistance in VRE VanB phenotype by the vancomycin aglycone, vancomycin-SO_3 and vancomycin. Quantity of antibiotic per disk is across the horizonatal and antibiotic applied on the vertical. Induction of teicoplanin resistance via VanS binding indicated by zone of growth around disk.

Sulfation depresses activation of resistance presumably by lack of/or non-productive binding of VanS. This is true in both environmental bacteria and successful pathogenic organisms. Sulfation may make the molecules worse antibiotics but in the case of A47934 only a two-fold change in MIC is observed. An MIC of 64 µg/mL for vancomycin-sulfate is still not high level resistance (ie. > 256 µg/mL). Thus, if we further modify the scaffold, will antimicrobial activity while be restored while still blocking stimulation of resistance?

Recently, Boger and colleagues describe a study in which they "redesign" glycopeptide antibiotics to improve binding to the D-Ala-D-Lac PG precursors(127). Their approach is unique in that they have reconstructed the

backbone of vancomycin and specifically the binding pocket, rather than modification to the parent compound. While they have successfully described molecules with improved binding efficiency, the approach involves total synthesis of the natural product and is not feasible to apply on an industrial scale. Other groups have targeted the binding to D-Ala-D-Lac via the more traditional approach of backbone modification either synthetically or with modifying enzymes as we have. From this body of work, nothing of large significance [i.e. new molecules to be developed as drugs] has come to light.

Blocking resistance rather than overcoming the mechanism of resistance is a novel approach to fight resistant organisms. Understanding the molecular interactions will only further develop this idea. Using nature's toolbox of glycopeptide modifying enzymes is cost-effective and a better mimic of natural evolution in nature.

4.4 Conclusions

The role glycopeptide sulfation has not previously been elucidated. A47934 is not only sulfated but is lacking in glycosylation, a modification proposed to enhance antimicrobial activity. Sulfation does not affect the binding affinity of the molecule to its target D-Ala-D-Ala but inducible resistance mediated by VanRS is significantly repressed relative to the desulfated molecule. Furthermore, addition of sulfate appears to block dimerization of the glycopeptide which may also result in altered binding to the $VanS_{Sc}$ sensor peptide loop. This study has confirmed unequivocally that sulfation of A47934 does not significantly affect antimicrobial activity however it serves to depress the activation of resistance in non-glycopeptide producing organisms like *S. coelicolor.*

Sulfated vancomycin also depresses activation of VanS in both environmental strains and clinical pathogens exhibiting the VanB phenotype. Sulfation of vancomycin is more detrimental to antimicrobial activity than A47934, however we have a collection of tailoring enzymes that can be used in a combinatorial fashion to find the right permutation of modifications. For example, the vancomycin type antibiotic balhimycin is glycosylated on residue 6 (ß-hydroxytyrosine). The glycosyltransferase from this biosynthetic cluster can potentially be used to glycosylate vancomycin-SO_3 to improve dimerization activity and binding to the cell wall. Conversely, it may not have an effect but the key will be to test different combinations in a systematic fashion to find the ideal activity.

This rational approach to drug discovery is easily scalable and methods developed in our lab have provided a large reservoir of glycopeptide biosynthetic clusters to harvest our enzymes from. Chapter 3 described a platform that is amenable to an *in vivo* synthetic biology approach while at the same time the biochemistry of each enzyme can be deduced *in vitro*. The glycopeptide class of antibiotics remains one of the most important classes of antibiotics used clinically. Both the biosynthesis and resistance mechanisms are natural occurrences of which their evolution and usefulness in the environment is still not deeply understood. Looking to the producing organisms and their neighbors will allow us to modulate Nature's method.

Chapter 5

SUMMARY AND FUTURE DIRECTIONS

5.0 Chapter 5

5.1 Summary

Natural product antibiotics have had great success in human medicine. The evolution and diversity of glycopeptide antibiotics was investigated to exemplify the intimate link between biosynthesis in the environment and widespread resistance. First, glycopeptide resistance was found to be ancient. Full length *vanA* open reading frames were identified from 30,000 year old permafrost cores. Synthesis of two genes yielded soluble active protein. Biochemical analysis confirmed that each is in fact a D-Ala-D-Lac ligase with enzymatic activity consistent with contemporary VanA homologs. In this study each *vanA* gene was amplified to include the boundry between *vanHA* and *vanAX* indicating that this classic gene organization that was first described in the clinic is also ancient.

Looking to the environment for homologs of VanA we have shown that functional glycopeptide resistance can be non-canonical and diverse across many different genera. In glycopeptide producing organisms VanA D-Ala-D-Lac ligases can be found stand-alone while a second copy occurs in the context of *vanHAX* within the same chromosome. This situation in *Amycolatopsis balhimycina* is the first example of a VanA homolog found in such an arrangement. Work with our collaborators indicates that this resistance is also not inducible but constitutively expressed; another unusual feature and an indication of a biological role perhaps not exclusively linked to glycopeptide production and self-resistance.

The environmental organism *Desulfiobacterium hafniense* Y51 harbors a *vanSRAWKmurFX* gene cassette that is both inducible by vancomycin and confers high level drug resistance. Presumably in the absence of VanH D-lactate can be obtained from other sources. Interestingly though, this is the only example of a resistance cassette containing *vanK* and *murF* homologs outside of the *Streptomyces* genus. *D. hafniense* Y51 has a low G+C chromosome (47%) compared to *Streptomyces coelicolor* for example with a G+C content of 72%. While there is no evidence of horizontal gene transfer in the *D. hafniense* Y51 chromosome surrounding the resistance cassette, it can be speculated that this organization likely evolved prior to modern day clinically relevant cassettes found in species like *Enterococcus*. This is because VanK is a FemX protein required for cross-linking nascent peptidoglycan chains ending in D-Ala-D-Lac as the native FemX will not accommodate the depsi-peptide. This is not an issue in pathogenic resistant strains. Regardless, in most cases resistance is tightly regulated and responds specifically to the presence of glycopeptide.

Glycopeptide molecules themselves are diverse but as resistance spreads, increasing the chemical diversity of this class has become a priority. The sulfotransferase (Stf) StaL, responsible for sulfating A47934 was shown to transfer not only a sulfate but both a sulfamate and fluorsulfonate *in vitro* to DSA47934 resulting in the sulfamidated and fluorosulfonated derivatives being observed. Although we were unable to scale up synthesis of these molecules, this is the first example of exploiting glycopeptide Stf activity with the potential to introduce novel moieties amenable to downstream synthesis.

To further expand the chemical diversity of glycopeptides a toolbox of tailoring enzymes was collected. A new glycopeptide biosynthetic cluster was identified by whole genome sequencing using 454 technology. The product of

the cluster UK68597 is glycosylated, sulfated and uniquely chlorinated. Three glycosyltransferases, a sulfotransferase and two halogenases were harnessed from this sequence information. A cloning vector for integration into the genome of *Streptomyces* sp. bacteria was modified with a promoter for constitutive expression. The multiple-cloning site was reconstructed to facilitate a "plug and play" type system where tailoring enzymes can be introduced alone or in tandem. Two di-sulfated A47934 derivatives were produced by *S. toyocaensis in vivo* with the Stfs Teg13 and Teg14 integrated in the chromosome. *In vitro,* Teg14 was used to create a completely novel sulfated derivative of vancomycin.

The role of sulfation in facilitation of VanS coordinated resistance was established. Both the sulfated A47934 and vancomycin derivative do not induce expression of resistance genes which allow resistance to teicoplanin in the normally sensitive organism *S. coelicolor.* This activity was also observed in a panel of environmental actinomycete isolates that are resistant to vancomycin but do not produce a glycopeptide. Vancomycin-SO_3 has depressed binding of $VanS_B$ in *Enterococcus faecium* ATCC 51299 VanB phenotype as demonstrated by the inability to induce teicoplanin in this pathogen while both vancomycin and the aglycone derivative do. These findings have great implications in design of second-generation glycopeptide antibiotics. Evasion of resistance rather than overcoming the resistant phenotype has become a strategy in glycopeptide chemical biology.

5.2 Future Directions

The role of sulfation and collection of glycopeptide sulfotransferases for combinatorial biosynthesis of glycopeptides has been discussed in chapters 3

and 4. It has been established by these studies that a single sulfation event on a glycopeptide backbone will modulate resistance but at the same time is detrimental to antimicrobial activity. To truly approach expansion of chemical diversity efficiently a systematic approach must be taken. As discussed in chapter 3, a collection of tailoring enzymes has been collected that can be expanded.

First, regio-specific effects of modification should be examined. For example, the two species of disulfated A47934 observed in chapter 3 must be examined for not only antimicrobial activity but physio-chemical properties such as ability to bind D-Ala-D-Ala/Lac, dimerization constants and affinity for VanS. The teicoplanin induction assay with *S. coelicolor* is a great tool we have used for the search of resistance evasion. The first steps in this goal are to confirm the structural properties and region of sulfation on A47934 by NMR and MS-MS methods. Then large scale fermentations and purification must be accomplished. The advantages to this are reproducibility once optimal expression conditions have been established. Shake flasks can have much variability with dissolved oxygen (DO) and agitation when scaling up volumes, while a fermenter provides a controlled environment for DO and even pH.

Each of the tailoring enzymes from the UK68597 biosynthetic gene cluster, two glucosyltransferases (Auk10,14) two halogenases (Auk21,23) and the [1]sulfotransferase (Auk20) have been cloned into both the *in vitro* expression vector pTIP-QC1 for expression in *Rhodococcus erythropolis* L88 and the modified pSET152_LK1 vector for *in vivo* expression in *S. toyocaensis*. The purified enzymes can be used to determine whether both DSA47934 and

[1] Auk20 was cloned into a pET28a vector for *in vitro* expression in *E. coli*.

A47934 are substrates *in vitro* in either a step-wise or combinatorial fashion. This is important for downstream troubleshooting the *in vivo* expression if the expected products are not observed. *In vitro,* Auk20 will only sulfate DSA47934 therefore it is not surprising to find only a singly sulfated species (A47934) in the *S. toyocaensis::Auk20* strain.

The third glycosyltransferase (Auk11) is expected to transfer the 4-*epi*-vancosamine to glucose attached to the residue 4 HPG. This sugar is not readily available, therefore the enzymes required for biosynthesis of 4-epi-vancosamine will need to be cloned as a continuous fragment containing all three glycosyltransferases (Auk 8-13, Table 3.1, Ch.3) and integrated into *S. toyocaensis.*

From here each single modification derivative can be purified from fermentation broths and assayed for activity and desirable properties as discussed above. Modifications of interest can then be combined either by cloning two or more genes into the pSET_LK vector or integration of the second gene into a pseudo-*att* site in the chromosome.

5.2.1 Preliminary work

Table 1 summarizes the progress achieved thus far. Each tailoring enzyme from the UK68597 cluster has been integrated into the wild-type *S. toyocaensis* chromosome and examined for production of A47934 derivatives. Unfortunately, A47934 was the only glycopeptide detected in both broth and cellular extraction from the strains *of S. toyocaensis::Auk10,14,21* and *23.* It is possible that the sulfated A47934 is not a substrate for the enzymes or that the genes are not being expressed. In the case of the halogenases, redundancy

is expected for one of them as UK68597 and A47934 share three common halogenation sites. Bioinformatic analysis of the amino acid sequences does uncover any significant differences to discern the difference in halogenation pattern which is why the *in vitro* analysis will be key.

Table 5.1: Summary of tailoring enzymes in pipeline and progress to date.

Gene	Vector	Integration	Fermentation and LC/MS	Product observed?	*In vitro* expression	Active enzyme?
Auk 10 - Gtf	-pSET_LK -pTip-QC1	Yes –*wt*	Yes	No	Yes	In progress
Auk14 - Gtf	-pSET_LK -pTip-Qc1	Yes - *wt*	Yes	No	Yes	In progress
Auk21 - Hal	-pSET_LK -pTip-QC1	Yes - *wt*	Yes	No	Yes	Need substrate
Auk23 - Hal	-pSET_LK -pTip-QC1	Yes - *wt*/Δ*staL*	Yes	No	Yes	Need substrate
Auk20 - Stf	-pSET_LK -pET28a	Yes - *wt*/Δ*staL*	Yes –wt	No	Yes	Yes - DS
Teg13 - Stf	-pSET_LK -pET28a	Yes - *wt*	Yes-wt	Yes	Yes	Yes - both
Teg14 -Stf	-pSET_LK -pET28a	Yes - *wt*	Yes –wt	Yes	Yes	Yes - both
Auk 8-13	In progress	-	-	-	-	-

Integration of each gene into *S. toyocaensis* Δ*staL* is currently still in progress. Ex-conjugants have been picked for Auk20 and 23. The remaining genes are being pursued. For this strain the kanamycin resistant vector is being

used. While a clean deletion of *staL* was made (removal of the apramycin resistance cassette used to disrupt the gene) a different resistant marker was still desirable to exclude the possibility of any contaminating apramycin resistant spores and false positives.

5.2.3 *In vitro* expression and enzyme activity

Initially the two glucosyltransferases were appearing as insoluble protein in an *E. coli* expression system hence the move to a *Rhodococcus* based system. Expression yields are much lower however optimization has led to soluble protein for Auk 10 and activity assays are underway. Auk 14 is still being investigated. This work is being carried out by Dr. Grace Yim.

The two halogenases have been purified as soluble protein and assayed for activity with the single amino acids hydroxyphenylglycine and tyrosine. Chlorination was not observed however literature suggests that halogenation likely occurs while the peptide is still attached to the NRPS before cross-linking occurs (93, 94). Glycopeptide halogenases have not been characterized biochemically and are therefore of interest. Chlorination has been reported as an important modification for activity (92), but the effects of different sites and multiple chlorination events have not been uncovered. The characterization of these enzymes will be carried out as outlined below. First, *S. toyocaensis*::*Auk21* and *23* should be further examined. A newly halogenated A47934 has not been detected in fermentation broths but it could be produced as a minor product and not readily detectable. Then the relative expression of each integrated halogenase gene should be examined by RT-PCR. Secondly, the fermentation broth and cell extracts can be assessed for new chlorinated intermediates of A47934 compared to the wild-type strain.

Finally, the biochemical characterization of the halogenases will be achieved by solid-phase synthesis of the A47934 backbone. The peptide will be linked to a solid support to mimic the cellular acyl-carrier protein. Each species can be made (i.e. di-, tri-, tetra-…heptapeptide) and reacted with the halogenase to determine which is the optimal substrate and timing of halogenation during biosynthesis. The peptide synthesis scheme is outlined in chapter 6 figure A2 and A3. This work is being undertaken by undergraduate student Irene Tang.

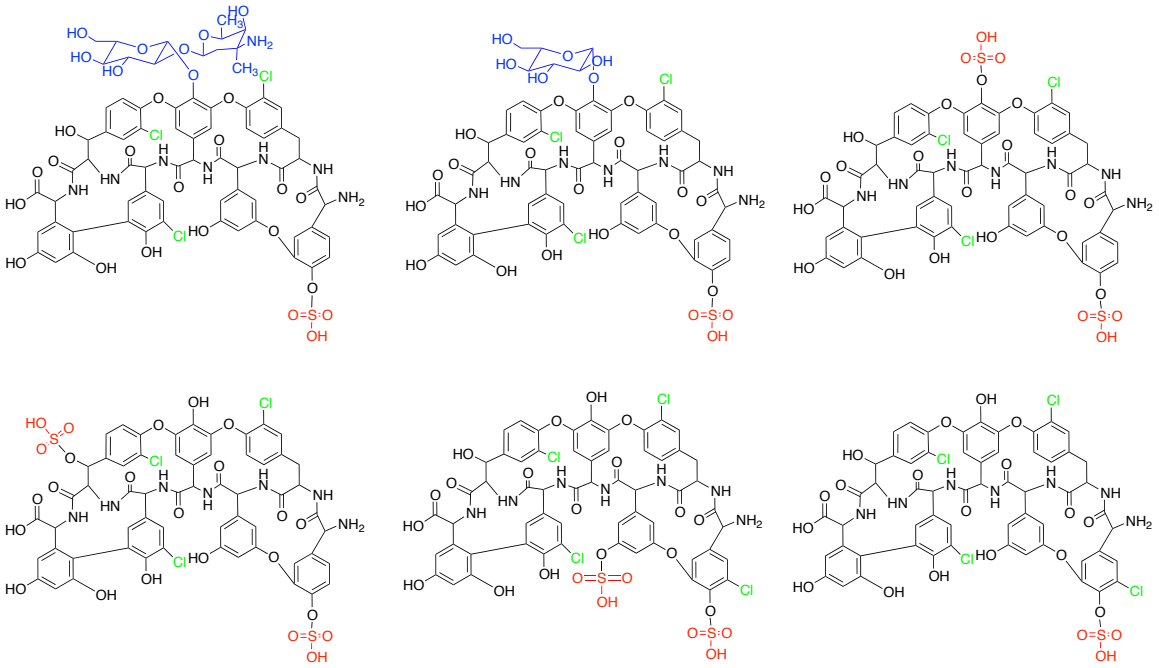


Figure 5.1: Potential A47934-derivates achievable by combinatorial biosynthesis with tailoring enzymes.

Together, this work provides a foundation for directed combinatorial biosynthesis of glycopeptide antibiotics. Utilizing the tailoring enzymes of the UK68597 cluster along with Stfs already in our toolbox will expand the chemical

diversity of glycopeptide antibiotics to include some of the molecules shown in figure 5.1.

Looking into the future, the library of tailoring enzymes can be expanded as long as new biosynthetic clusters are sequenced. The cost of both sequencing and synthesis of DNA is continually dropping (128-130). One can imagine if this trend continues we can begin to move away from a traditional genetic recombineering approach described here. To be more specific, synthesis of entire biosynthetic clusters designed with the building machinery and tailoring enzymes of interest, can be achieved (130). These clusters can then be introduced into genome minimized hosts engineered for optimal overproduction of introduced pathways, thus creating a biological factory producing a designer drug. This allows complete design, regulation and production of natural products to be controlled. For a review specific to antibiotic producing organisms see (131).

Concluding Remarks

Natural product small molecules have played a seminal role in evolution. From ancient times to the present day humans have exploited these molecules made by plants, fungi and bacteria for therapeutic uses. It was only very recently that we have been able to isolate in a pure form the active ingredients from the above sources. Since then, the intricate biosynthesis executed in nature has been unraveled and we can begin to use that knowledge to our advantage. In the case of antibiotics, each active molecule has a conjugate resistance mechanism that evolved alongside. Harnessing this information to

create “new-old” or accessorized antibiotics allows for predictive activity and prevention of downstream resistance.

5.3 Bibliography:

1. Peintner U, Poder, R., and Pumpel, T., (1998) The iceman's fungi. *Mycology Research* 102(20):1153-1162.
2. Fleming A (1945) Penicillin - Nobel Lecture. *Nobel Lectures, Physiology or Medicine 1942-1962*.
3. Osbaldeston TA, and Wood, R.P.A (2000) Dioscorides De Materia Medica. in *BEING AN HERBAL WITH MANY OTHER MEDICINAL MATERIALS* (IBIDIS Press cc, Johannesburg, South Africa).
4. Pootoolal J*, et al.* (2002) Assembling the glycopeptide antibiotic scaffold: The biosynthesis of A47934 from Streptomyces toyocaensis NRRL15009. *Proc Natl Acad Sci U S A* 99(13):8962-8967.
5. Bryan CP & Joachim H (1930) *The Papyrus Ebers* (G. Bles, London,) pp xl, 167 p.
6. Bassett EJ, Keith MS, Armelagos GJ, Martin DL, & Villanueva AR (1980) Tetracycline-labeled human bone from ancient Sudanese Nubia (A.D. 350). *Science* 209(4464):1532-1534.
7. Moerman DE (1998) *Native American Ethnobotony* (Timber Press, Inc., Portland, Oregon).
8. Capasso L (1998) 5300 years ago, the Ice Man used natural laxatives and antibiotics. *Lancet* 352(9143):1864.
9. Poder R & Peintner U (1999) Laxatives and the Ice Man. *Lancet* 353(9156):926.
10. Tunon H & Svanberg I (1999) Laxatives and the Ice Man. *Lancet* 353(9156):925-926.
11. Schlegel B, Luhmann U, Hartl A, & Grafe U (2000) Piptamine, a new antibiotic produced by Piptoporus betulinus Lu 9-1. *J Antibiot (Tokyo)* 53(9):973-974.
12. Wangun HV, Berg A, Hertel W, Nkengfack AE, & Hertweck C (2004) Anti-inflammatory and anti-hyaluronate lyase activities of lanostanoids from Piptoporus betulinus. *J Antibiot (Tokyo)* 57(11):755-758.
13. Clarke JT, Warnock RC, & Donoghue PC (2011) Establishing a time-scale for plant evolution. *New Phytol* 192(1):266-301.
14. Soloway SB (1976) Naturally occurring insecticides. *Environ Health Perspect* 14:109-117.
15. George J, Bais HP, & Ravishankar GA (2000) Biotechnological production of plant-based insecticides. *Crit Rev Biotechnol* 20(1):49-77.
16. Yamamoto I (1965) Nicotinoids as insecticides. *Adv Pest Control Res* 6:231-260.

17. Renneberg R (2007) Biotech History: Yew trees, paclitaxel synthesis and fungi. *Biotechnol J* 2(10):1207-1209.
18. Fleming A (1929) On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. injluenzae. *British]ournal of Experimental Pathology,* 10:226-236.
19. Hamilton-Miller JM (2004) Appreciation. Dr. Norman Heatley. (Translated from eng) *The Journal of antimicrobial chemotherapy* 53(5):691-692 (in eng).
20. Florey HW (1944) Penicillin: A Survey. *Br Med J* 2(4361):169-171.
21. Dubos RJ & Hotchkiss RD (1941) The Production of Bactericidal Substances by Aerobic Sporulating Bacilli. *J Exp Med* 73(5):629-640.
22. Hopwood DA (2007) *Streptomyces in nature and medicine : the antibiotic makers* (Oxford University Press, Oxford ; New York) pp viii, 250 p., 258 p. of plates.
23. Jones D, Metzger HJ, Schatz A, & Waksman SA (1944) Control of Gram-Negative Bacteria in Experimental Animals by Streptomycin. *Science* 100(2588):103-105.
24. Smith DG & Waksman SA (1947) Tuberculostatic and tuberculocidal action of streptomycin. *J Bacteriol* 54(1):67.
25. Shnayerson M & Plotkin MJ (2002) *The killers within : the deadly rise of drug-resistant bacteria* (Little, Brown and Co., Boston) 1st Ed p 328 p.
26. Waksman SA, Reilly HC, & Schatz A (1945) Strain Specificity and Production of Antibiotic Substances: V. Strain Resistance of Bacteria to Antibiotic Substances, Especially to Streptomycin. *Proceedings of the National Academy of Sciences of the United States of America* 31(6):157-164.
27. D'Costa VM, McGrann KM, Hughes DW, & Wright GD (2006) Sampling the antibiotic resistome. *Science* 311(5759):374-377.
28. D'Costa VM*, et al.* (2011) Antibiotic resistance is ancient. *Nature* 477(7365):457-461.
29. Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5(3):175-186.
30. Brown MG & Balkwill DL (2009) Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb Ecol* 57(3):484-493.
31. Loewenberg S (2012) India reports cases of totally drug-resistant tuberculosis. *Lancet* 379(9812):205.
32. Fournier PE*, et al.* (2006) Comparative genomics of multidrug resistance in Acinetobacter baumannii. *PLoS Genet* 2(1):e7.
33. Bush K & Fisher JF (2011) Epidemiological expansion, structural studies, and clinical challenges of new beta-lactamases from gram-negative bacteria. *Annu Rev Microbiol* 65:455-478.

34. MacLean D, Jones JD, & Studholme DJ (2009) Application of 'next-generation' sequencing technologies to microbial genetics. *Nat Rev Microbiol* 7(4):287-296.
35. Bentley SD*, et al.* (2002) Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature* 417(6885):141-147.
36. Nicolaou KC, Boddy CN, Brase S, & Winssinger N (1999) Chemistry, Biology, and Medicine of the Glycopeptide Antibiotics. *Angew Chem Int Ed Engl* 38(15):2096-2152.
37. Marshall CG, Lessard IA, Park I, & Wright GD (1998) Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob Agents Chemother* 42(9):2215-2220.
38. Bugg TD*, et al.* (1991) Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry* 30(43):10408-10415.
39. Hoshuyama T, Moriguchi H, Muratani T, & Matsumoto T (2008) Vancomycin-resistant enterococci (VRE) outbreak at a university hospital in Kitakyushu, Japan: case-control studies. *J Infect Chemother* 14(5):354-360.
40. Yang KS*, et al.* (2007) Predictors of vancomycin-resistant enterococcus (VRE) carriage in the first major VRE outbreak in Singapore. *Ann Acad Med Singapore* 36(6):379-383.
41. Liu Y, Cao B, Gu L, & Wang H (2011) Molecular characterization of vancomycin-resistant enterococci in a Chinese hospital between 2003 and 2009. *Microb Drug Resist* 17(3):449-455.
42. Bourdon N*, et al.* (2011) Changing trends in vancomycin-resistant enterococci in French hospitals, 2001-08. *The Journal of antimicrobial chemotherapy* 66(4):713-721.
43. Zhanel GG*, et al.* (2008) Characterization of methicillin-resistant Staphylococcus aureus, vancomycin-resistant enterococci and extended-spectrum beta-lactamase-producing Escherichia coli in intensive care units in Canada: Results of the Canadian National Intensive Care Unit (CAN-ICU) study (2005-2006). *Can J Infect Dis Med Microbiol* 19(3):243-249.
44. Chen AY, Zervos MJ, & Vazquez JA (2007) Dalbavancin: a novel antimicrobial. *Int J Clin Pract* 61(5):853-863.
45. Bailey J & Summers KM (2008) Dalbavancin: a new lipoglycopeptide antibiotic. *Am J Health Syst Pharm* 65(7):599-610.
46. Barcia-Macay M, Mouaden F, Mingeot-Leclercq MP, Tulkens PM, & Van Bambeke F (2008) Cellular pharmacokinetics of telavancin, a novel lipoglycopeptide antibiotic, and analysis of lysosomal changes in cultured eukaryotic cells (J774 mouse macrophages and rat embryonic fibroblasts). *J Antimicrob Chemother* 61(6):1288-1294.

47. Kim SJ*, et al.* (2008) Oritavancin exhibits dual mode of action to inhibit cell-wall biosynthesis in Staphylococcus aureus. *J Mol Biol* 377(1):281-293.
48. Kim SJ, Cegelski L, Preobrazhenskaya M, & Schaefer J (2006) Structures of Staphylococcus aureus cell-wall complexes with vancomycin, eremomycin, and chloroeremomycin derivatives by 13C{19F} and 15N{19F} rotational-echo double resonance. *Biochemistry* 45(16):5235-5250.
49. Zou Y, Brunzelle JS, & Nair SK (2008) Crystal structures of lipoglycopeptide antibiotic deacetylases: implications for the biosynthesis of a40926 and teicoplanin. *Chem Biol* 15(6):533-545.
50. Damodaran SE & Madhan S (2011) Telavancin: A novel lipoglycopeptide antibiotic. *J Pharmacol Pharmacother* 2(2):135-137.
51. Li L & Xu B (2005) Multivalent vancomycins and related antibiotics against infectious diseases. *Curr Pharm Des* 11(24):3111-3124.
52. Baltz RH, Brian P, Miao V, & Wrigley SK (2006) Combinatorial biosynthesis of lipopeptide antibiotics in Streptomyces roseosporus. *J Ind Microbiol Biotechnol* 33(2):66-74.
53. Losey HC*, et al.* (2001) Tandem action of glycosyltransferases in the maturation of vancomycin and teicoplanin aglycones: novel glycopeptides. *Biochemistry* 40(15):4745-4755.
54. Kruger RG*, et al.* (2005) Tailoring of glycopeptide scaffolds by the acyltransferases from the teicoplanin and A-40,926 biosynthetic operons. *Chem Biol* 12(1):131-140.
55. Park SH*, et al.* (2009) Expanding substrate specificity of GT-B fold glycosyltransferase via domain swapping and high-throughput screening. *Biotechnol Bioeng* 102(4):988-994.
56. Bugg TD, Dutka-Malen S, Arthur M, Courvalin P, & Walsh CT (1991) Identification of vancomycin resistance protein VanA as a D-alanine:D-alanine ligase of altered substrate specificity. *Biochemistry* 30(8):2017-2021.
57. Koteva K*, et al.* (2010) A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor. (Translated from eng) *Nat Chem Biol* 6(5):327-329 (in eng).
58. Chen H, Tseng CC, Hubbard BK, & Walsh CT (2001) Glycopeptide antibiotic biosynthesis: enzymatic assembly of the dedicated amino acid monomer (S)-3,5-dihydroxyphenylglycine. *Proceedings of the National Academy of Sciences of the United States of America* 98(26):14901-14906.
59. Hubbard BK, Thomas MG, & Walsh CT (2000) Biosynthesis of L-p-hydroxyphenylglycine, a non-proteinogenic amino acid constituent of peptide antibiotics. *Chemistry & biology* 7(12):931-942.

60. Baltz RH (2007) Antimicrobials from Actinomycetes: Back to the Future. *Microbe* 2(3):125-131.
61. Pootoolal J*, et al.* (2002) Assembling the glycopeptide antibiotic scaffold: The biosynthesis of A47934 from Streptomyces toyocaensis NRRL15009. (Translated from eng) *Proceedings of the National Academy of Sciences of the United States of America* 99(13):8962-8967 (in eng).
62. Lamb SS, Patel T, Koteva KP, & Wright GD (2006) Biosynthesis of sulfated glycopeptide antibiotics by using the sulfotransferase StaL. *Chem Biol* 13(2):171-181.
63. Shi R*, et al.* (2007) Crystal structure of StaL, a glycopeptide antibiotic sulfotransferase from Streptomyces toyocaensis. *J Biol Chem* 282(17):13073-13086.
64. Solenberg PJ*, et al.* (1997) Production of hybrid glycopeptide antibiotics in vitro and in Streptomyces toyocaensis. *Chem Biol* 4(3):195-202.
65. Banik JJ, Craig JW, Calle PY, & Brady SF (2010) Tailoring enzyme-rich environmental DNA clones: a source of enzymes for generating libraries of unnatural natural products. *J Am Chem Soc* 132(44):15661-15670.
66. Fu X*, et al.* (2003) Antibiotic optimization via in vitro glycorandomization. *Nat Biotechnol* 21(12):1467-1469.
67. Kristinsson KG, Fenton P, & Norman P (1987) Control of epidemic methicillin-resistant Staphylococcus aureus. *Lancet* 1(8527):274-275.
68. Kirst HA, Thompson DG, & Nicas TI (1998) Historical yearly usage of vancomycin. *Antimicrob Agents Chemother (Bethesda)* 42(5):1303-1304.
69. Moellering RC, Jr. (2006) Vancomycin: a 50-year reassessment. *Clin Infect Dis* 42 Suppl 1:S3-4.
70. Leclercq R, Derlot E, Duval J, & Courvalin P (1988) Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *N Engl J Med* 319(3):157-161.
71. Leclercq R, Derlot E, Weber M, Duval J, & Courvalin P (1989) Transferable vancomycin and teicoplanin resistance in Enterococcus faecium. *Antimicrob Agents Chemother (Bethesda)* 33(1):10-15.
72. Arthur M*, et al.* (1992) Evidence for in vivo incorporation of D-lactate into peptidoglycan precursors of vancomycin-resistant enterococci. *Antimicrob Agents Chemother* 36(4):867-869.
73. Hong HJ, Hutchings MI, & Buttner MJ (2008) Vancomycin resistance VanS/VanR two-component systems. *Adv Exp Med Biol* 631:200-213.
74. Guardabassi L, Christensen H, Hasman H, & Dalsgaard A (2004) Members of the genera Paenibacillus and Rhodococcus harbor genes homologous to enterococcal glycopeptide resistance genes vanA and vanB. *Antimicrob Agents Chemother* 48(12):4915-4918.

75. Marshall CG & Wright GD (1998) DdlN from vancomycin-producing Amycolatopsis orientalis C329.2 is a VanA homologue with D-alanyl-D-lactate ligase activity. *J Bacteriol* 180(21):5792-5795.
76. Zawadzke LE, Bugg TD, & Walsh CT (1991) Existence of two D-alanine:D-alanine ligases in Escherichia coli: cloning and sequencing of the ddlA gene and purification and characterization of the DdlA and DdlB enzymes. *Biochemistry* 30(6):1673-1682.
77. Wampler DE & Westhead EW (1968) Two aspartokinases from Escherichia coli. Nature of the inhibition and molecular changes accompanying reversible inactivation. *Biochemistry* 7(5):1661-1671.
78. Leatherbarrow RJ (1992) GraFit (Erithacus Software Ltd.), 4.0.21.
79. Nonaka H*, et al.* (2006) Complete genome sequence of the dehalorespiring bacterium Desulfitobacterium hafniense Y51 and comparison with Dehalococcoides ethenogenes 195. *J Bacteriol* 188(6):2262-2274.
80. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.
81. CLSI (2007) *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* (Wayne, PA) 7 ed. Approved standard M11-A7 Ed.
82. Hong HJ, Hutchings MI, Hill LM, & Buttner MJ (2005) The role of the novel Fem protein VanK in vancomycin resistance in Streptomyces coelicolor. *J Biol Chem* 280(13):13055-13061.
83. Hong HJ*, et al.* (2004) Characterization of an inducible vancomycin resistance system in Streptomyces coelicolor reveals a novel gene (vanK) required for drug resistance. *Mol Microbiol* 52(4):1107-1121.
84. El Zoeiby A, Sanschagrin F, & Levesque RC (2003) Structure and function of the Mur enzymes: development of novel inhibitors. *Mol Microbiol* 47(1):1-12.
85. Bateman A, Holden MT, & Yeats C (2005) The G5 domain: a potential N-acetylglucosamine recognition domain involved in biofilm formation. *Bioinformatics* 21(8):1301-1303.
86. Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5(3):175-186.
87. Crane CM*, et al.* (2010) Synthesis and evaluation of selected key methyl ether derivatives of vancomycin aglycon. *J Med Chem* 53(19):7229-7235.
88. Oberthur M*, et al.* (2005) A systematic investigation of the synthetic utility of glycopeptide glycosyltransferases. *Journal of the American Chemical Society* 127(30):10747-10752.
89. Losey HC*, et al.* (2002) Incorporation of glucose analogs by GtfE and GtfD from the vancomycin biosynthetic pathway to generate variant glycopeptides. *Chemistry & biology* 9(12):1305-1314.

90. Sun B*, et al.* (2001) Hybrid glycopeptide antibiotics. *Journal of the American Chemical Society* 123(50):12722-12723.
91. Zhang C*, et al.* (2006) Exploiting the reversibility of natural product glycosyltransferase-catalyzed reactions. *Science* 313(5791):1291-1294.
92. Harris CM, Kannan, R., Kopecka, H., and Harris, T.M. (1985) The Role of the Chlorine Substituents in the Antibiotic Vancomycin: Preparation and Characterization of Mono- and Didechlorovancomycin. *Journal of the American Chemical Society* 107:6652-6658.
93. Puk O*, et al.* (2002) Glycopeptide biosynthesis in Amycolatopsis mediterranei DSM5908: function of a halogenase and a haloperoxidase/perhydrolase. *Chemistry & biology* 9(2):225-235.
94. Puk O*, et al.* (2004) Biosynthesis of chloro-beta-hydroxytyrosine, a nonproteinogenic amino acid of the peptidic backbone of glycopeptide antibiotics. *J Bacteriol* 186(18):6093-6100.
95. Banik JJ & Brady SF (2008) Cloning and characterization of new glycopeptide gene clusters found in an environmental DNA megalibrary. *Proc Natl Acad Sci U S A* 105(45):17273-17277.
96. Kieser T, Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A. (2000) *Practical Streptomyces Genetics* (John Innes Foundation, Norwich, England) 2nd Ed.
97. al. Ce (1999) Genome Sequence Assembly Using Trace Signals and Additional Sequence Information Computer Science and Biology: . *Proceedings of the German Conference on Bioinformatics (GCB)*:45-56.
98. Drummond AJ AB, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious v5.4).
99. Bibb MJ, Janssen GR, & Ward JM (1985) Cloning and analysis of the promoter region of the erythromycin resistance gene (ermE) of Streptomyces erythraeus. *Gene* 38(1-3):215-226.
100. Clinical and Laboratory Standards Institute. (2005) Clinical and Laboratory Standards Institute : [document]. (Clinical and Laboratory Standards Institute, Wayne, Pa.), p v.
101. McKay GA*, et al.* (2009) Impact of human serum albumin on oritavancin in vitro activity against enterococci. *Antimicrob Agents Chemother (Bethesda)* 53(6):2687-2689.
102. Beer J, Wagner CC, & Zeitlinger M (2009) Protein binding of antimicrobials: methods for quantification and for investigation of its impact on bacterial killing. *Aaps J* 11(1):1-12.
103. Burkart MD & Wong CH (1999) A continuous assay for the spectrophotometric analysis of sulfotransferases using aryl sulfotransferase IV. *Analytical biochemistry* 274(1):131-137.

104. Booth HS & American Chemical Society. (1939) *Inorganic syntheses* (McGraw-Hill Book Co., New York) p v.
105. Roth GP, and Fuller, C.E. (1991) Palladium Cross-Coupling Reactions of Aryl Fluorosulfonates: An Alternative to Triflate Chemistry. *J. Org. Chem* 56:3493-3496.
106. Burkart MD, Izumi M, & Wong CH (1999) Enzymatic Regeneration of 3'-Phosphoadenosine-5'-Phosphosulfate Using Aryl Sulfotransferase for the Preparative Enzymatic Synthesis of Sulfated Carbohydrates. *Angewandte Chemie* 38(18):2747-2750.
107. Skelton NJ, and Williams, D.H. (1990) Structure Elucidation of the Novel Glycopeptide Antibiotic UK-68,597. *J. Org. Chem* 55:3718-3723.
108. Donadio S, Sosio M, Stegmann E, Weber T, & Wohlleben W (2005) Comparative analysis and insights into the evolution of gene clusters for glycopeptide antibiotic biosynthesis. *Mol Genet Genomics* 274(1):40-50.
109. Hummerjohann J, Laudenbach S, Retey J, Leisinger T, & Kertesz MA (2000) The sulfur-regulated arylsulfatase gene cluster of Pseudomonas aeruginosa, a new member of the cys regulon. *J Bacteriol* 182(7):2055-2058.
110. Kertesz MA (2000) Riding the sulfur cycle--metabolism of sulfonates and sulfate esters in gram-negative bacteria. *FEMS Microbiol Rev* 24(2):135-175.
111. Schelle MW & Bertozzi CR (2006) Sulfate metabolism in mycobacteria. *Chembiochem : a European journal of chemical biology* 7(10):1516-1524.
112. Bick JA & Leustek T (1998) Plant sulfur metabolism--the reduction of sulfate to sulfite. *Curr Opin Plant Biol* 1(3):240-244.
113. Falany CN (1997) Enzymology of human cytosolic sulfotransferases. *Faseb J* 11(4):206-216.
114. Falany CN (1991) Molecular enzymology of human liver cytosolic sulfotransferases. *Trends Pharmacol Sci* 12(7):255-259.
115. Oh TJ, Kim DH, Kang SY, Yamaguchi T, & Sohng JK (2011) Enzymatic synthesis of vancomycin derivatives using galactosyltransferase and sialyltransferase. *J Antibiot (Tokyo)* 64(1):103-109.
116. Nagarajan R (1993) Structure-activity relationships of vancomycin-type glycopeptide antibiotics. *J Antibiot (Tokyo)* 46(8):1181-1195.
117. Wright GD, Holman TR, & Walsh CT (1993) Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in Enterococcus faecium BM4147. *Biochemistry* 32(19):5057-5063.
118. Kahne D, Leimkuhler C, Lu W, & Walsh C (2005) Glycopeptide and lipoglycopeptide antibiotics. *Chem Rev* 105(2):425-448.

119. Hong HJ*, et al.* (2004) Characterization of an inducible vancomycin resistance system in Streptomyces coelicolor reveals a novel gene (vanK) required for drug resistance. *Mol Microbiol* 52(4):1107-1121.
120. Hong HJ, Hutchings MI, Hill LM, & Buttner MJ (2005) The role of the novel Fem protein VanK in vancomycin resistance in Streptomyces coelicolor. *The Journal of biological chemistry* 280(13):13055-13061.
121. mirgorodskaya OA, Olsufyeva, E.N., Kolume, D.E., Joergensen, T.J.D., Roepstorff, P., Pavlov,A.Y., Miroshnikova, O.V., and Preobrazhenskaya, M.N. (2000) The Dimerization of Semisynthetic Eremomycin Derivatives Studied by the Electrospray Ionization Mass Spectrometry and Its Effect on Their Antibacterial Activity. *Russian Journal of Bioorganic Chemistry* 26(8):566-574.
122. Nitanai Y*, et al.* (2009) Crystal structures of the complexes between vancomycin and cell-wall precursor analogs. *J Mol Biol* 385(5):1422-1432.
123. Schafer M, Schneider TR, & Sheldrick GM (1996) Crystal structure of vancomycin. *Structure* 4(12):1509-1515.
124. Lehmann C, Bunkoczi G, Vertesy L, & Sheldrick GM (2002) Structures of glycopeptide antibiotics with peptides that model bacterial cell-wall precursors. *J Mol Biol* 318(3):723-732.
125. Goh EB*, et al.* (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proceedings of the National Academy of Sciences of the United States of America* 99(26):17025-17030.
126. Marshall CG, Broadhead G, Leskiw BK, & Wright GD (1997) D-Ala-D-Ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin-resistance ligases VanA and VanB. *Proc Natl Acad Sci U S A* 94(12):6480-6483.
127. James RC, Pierce JG, Okano A, Xie J, & Boger DL (2012) Redesign of glycopeptide antibiotics: back to the future. *ACS Chem Biol* 7(5):797-804.
128. Metzker ML (2010) Sequencing technologies - the next generation. *Nat Rev Genet* 11(1):31-46.
129. Zhang J, Chiodini R, Badr A, & Zhang G (2011) The impact of next-generation sequencing on genomics. *J Genet Genomics* 38(3):95-109.
130. Ellis T, Adie T, & Baldwin GS (2011) DNA assembly for synthetic biology: from parts to pathways and beyond. *Integr Biol (Camb)* 3(2):109-118.
131. Medema MH, Breitling R, Bovenberg R, & Takano E (2011) Exploiting plug-and-play synthetic biology for drug discovery and production in microorganisms. *Nat Rev Microbiol* 9(2):131-137.

Chapter 6

APPENDICES

Chapter 6

APPENDIX 1:

Actinoplanes sp. ATCC 53533 genome statistics

Table A1: Assembly results from 454 sequence data of *Actinoplanes* sp. ATCC 53533.

Total number of reads	Total contigs	N50	Largest contig	Total number of base pairs
434270	431	65128	267166 bp	13034595 bp

APPENDIX 2

Method A1: Creating the kanamycin resistant pSET152_LK

pSET152_LK1 and 2 were constructed by recombination. Briefly, the neomycin/kanamycin resistance cassette was amplified using PCR from the pIJ8600kanR plasmid (created by Dr. Xiao Dong Wang). The following primers were used: PLK_Forward 5'-GCAGAGCGAGGTATGTAGGCGG-3' and PLK_Reverse: 5"-GAGCTGGTGAAGTACATCACCG-3'. The PCR cycling program was 94°C, 30s; 55°C, 15s; 72°C, 72s for 30 cycles. The amplicon and plasmid (pSET152_LK) were co-electroporated into *E. coli* BW25113 strain and plated at 30°C. The plasmid was isolated from the transformants and retransformed into *E. coli* ET12567/pUZ8002 strain and selected on Cam/Kan/Amp. (Note; the pUZ8002 with ampicillin marker was used).

APPENDIX 3:

Teicoplanin and vancomycin-SO_3 dimerization

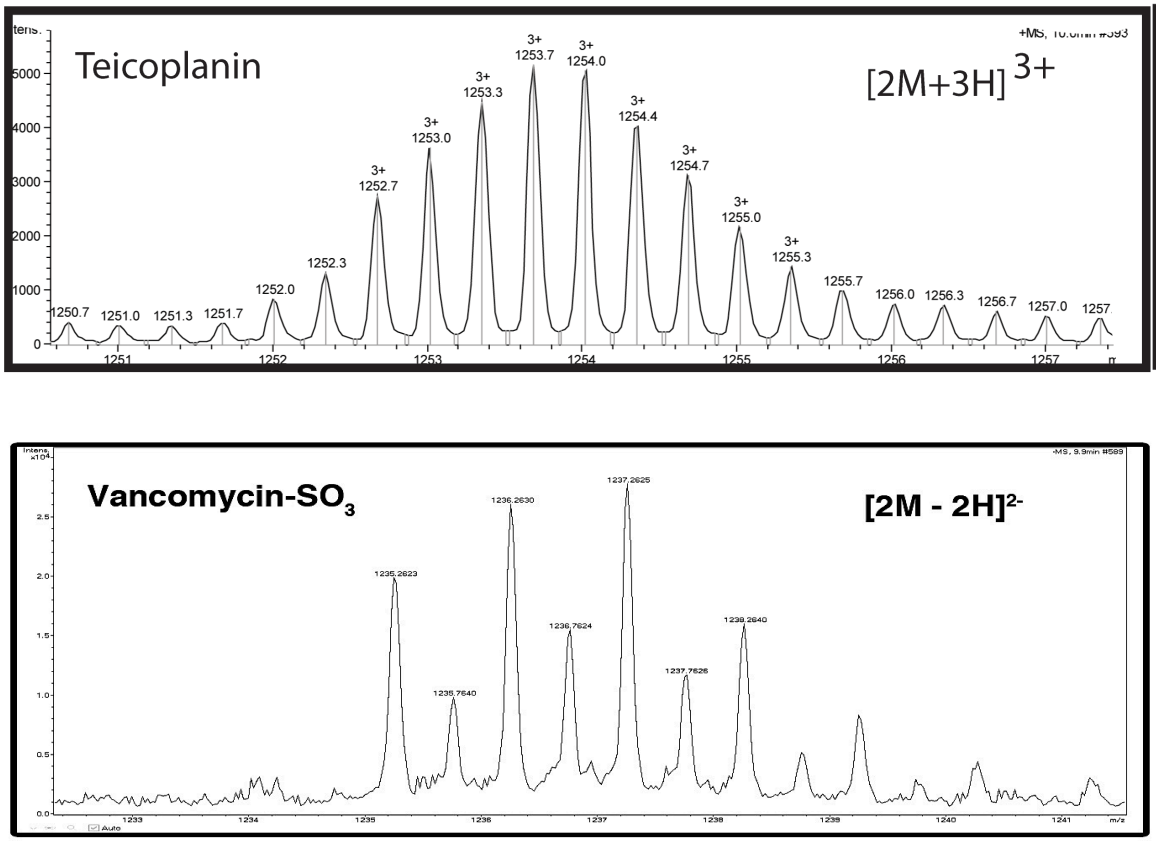
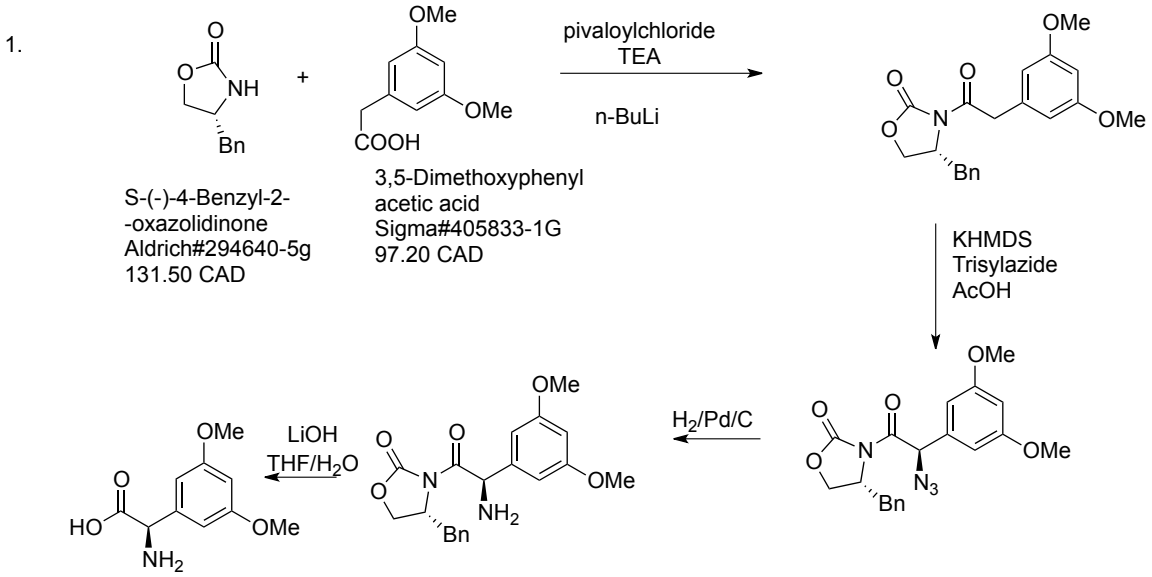


Figure A1: Teicoplanin (top panel) and vancomycin-SO_3 (bottom panel) dimerization determined by micro-TOF LC/MS. Mass of teicoplanin is 1880 Da. The dimer *m/z* is 3760 Da and was also observed in the same trace but is not shown here. Mass of vancomycin-SO_3 is 1220 Da, shown here is the doubly charged dimer with a *m/z* of 1440 Da.

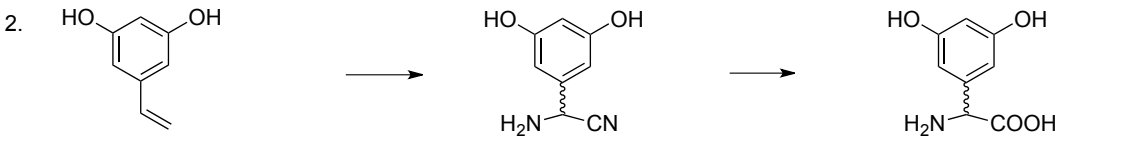
APPENDIX 4

Synthesis of DHPG



from 3g of 3,5-Dimethoxyphenylacetic acid- 1g final product

Ref: Evans et al. Tetrahedron lett. 33(9), 1992, 1189-1192



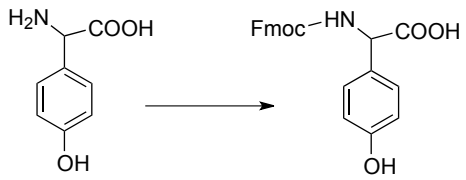
Ref: Bioorganic and Medicinal Chemistry Lettres, 2010 (20), 392-397.

Figure A2: Two alternate schemes for synthesis of dihydroxyphenyl glycine. Original references are included under each scheme.

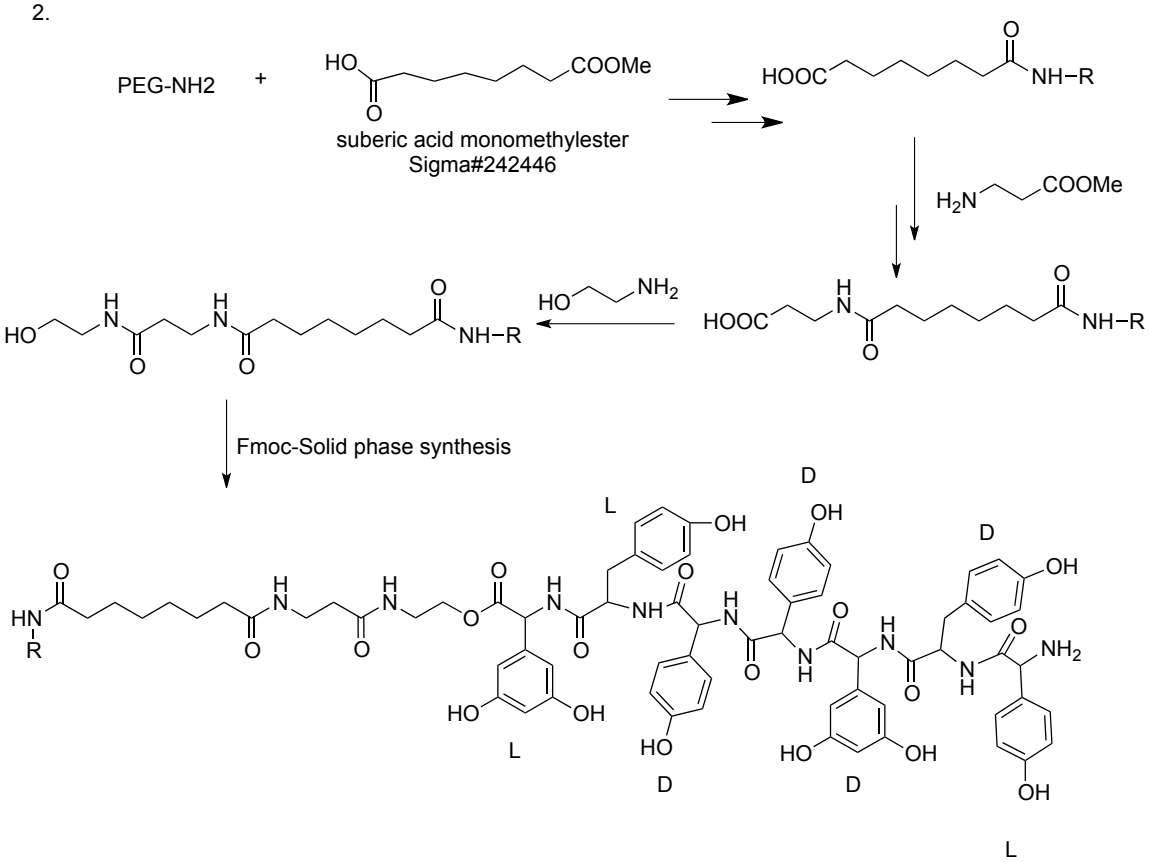
APPENDIX 5:

Synthesis of the linear A47934 heptapeptide

1. Fmoc protection of Hpg (L- and D-)



2.



Notes: D and L-Hpg we have in the lab and we will Fmoc-protect them;
Fmoc-D-Tyr(tBu)- Sigma# 47319

Figure A3: Scheme for solid phase peptide synthesis of the A47934 heptapeptide backbone.

APPENDIX 6

Cloning UK68597 tailoring enzymes – primer set

Table A1: Primer names, sequence and melting temperature used for cloning UK68597 tailoring enzymes.

Sequence Name	Sequence	Tm
Auk10_F_NdeI	5'-CATATGATGCGTGTGTTGTTGTCGACGT-3'	61.0
Auk10_R_EcoRi	5'-TCTAGAATTCTCACACAGCGACGGCAGG-3'	63.2
Auk11_F_NDeI	5'-CATATGATGCGTGTGTTGTTGACAACGTG-3'	60.2
Auk11_R_EcoRI	5'-TCTAGAATTCTCACACGGAAACGGCAGG-3'	61.7
Auk14_F_NdeI	5'-CATATGGTGTTGTTCTGGTCGTGTGGACC-3'	62.4
Auk14_R_EcoRI	5'-TCTAGAATTCTCACACGGAGACAGCCGG-3'	62.8
Auk20_F_NdeI	5'-CATATGATGACACGGATCTCATGGATC-3'	56.9
Auk20_R_EcoRI	5'-TCTAGAATTCTCACTCGTCATAACCGAATTG-3'	57.9
Auk13_R_EcoRI	5'-TCTAGAATTCCATGTGTCAGCGCGGCGACAGCCC-3'	69.2
Auk21_F_NdeI	5'-CATATGGTGGAAGAGTTCGATGTGGTGGT-3'	61.6
Auk21_R_EcoRI	5'-TCTAGAATTCTCACGCGGGATGGTAGGG-3'	62.6
Auk23_F_NdeI	5'-CATATGGTGGACATGCCGGTTGAG-3'	60.0
Auk23_R_EcoRI	5'-GAATTCTCATGATCGAATAGCTTCCGG-3'	57.3

Appendix 7

NMR data for PNPS-F

Identification of PNPSF: The NMR data from ^{1}H NMR, ^{13}C, COSY, HSQC, and HMBC were used to assign all ^{1}H and ^{13}C NMR chemical shifts and the structure was thus confirmed. NMR experiments were carried on a Bruker Avance III 700 MHz NMR spectrometer equipped with a 5 mm QNP cryoprobe and operating at 700.17 MHz for ^{1}H and 176.06 MHz for ^{13}C. Chemical shifts were referenced to the residual solvent signals dichloromethane (DCM $-d_2$) (5.32 ppm for ^{1}H and 54.0 ppm for ^{13}C).

^{1}H- NMR- 8.36 (m, 2H); 7.56 (m, 2H). ^{13}C-NMR: 154.2, 148.1; 126.7; 122.70.

^{1}H NMR spectra of PNPS-F

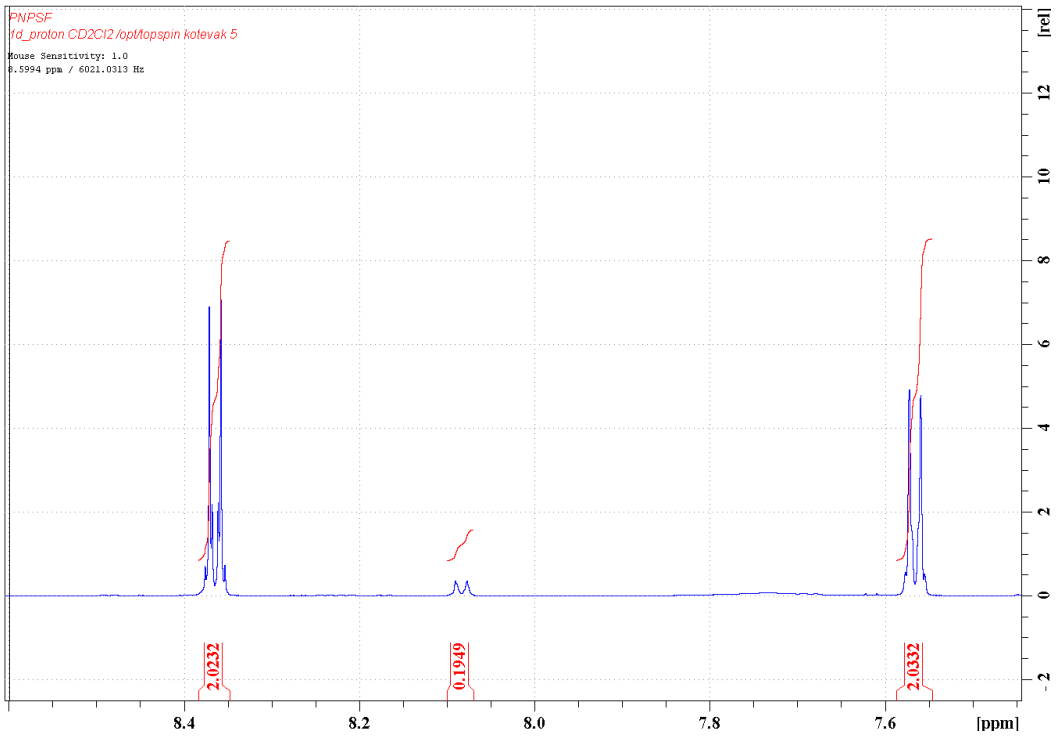


Figure A4: ^{1}H NMR spectra of PNPS-F. The first peak group with a chemical shift of ~8.35 ppm corresponds to NO_2. The middle peak group with a chemical shift of 8.1 ppm is PNP and the peak group at 7.5 ppm is SO_2F.

COSY spectra

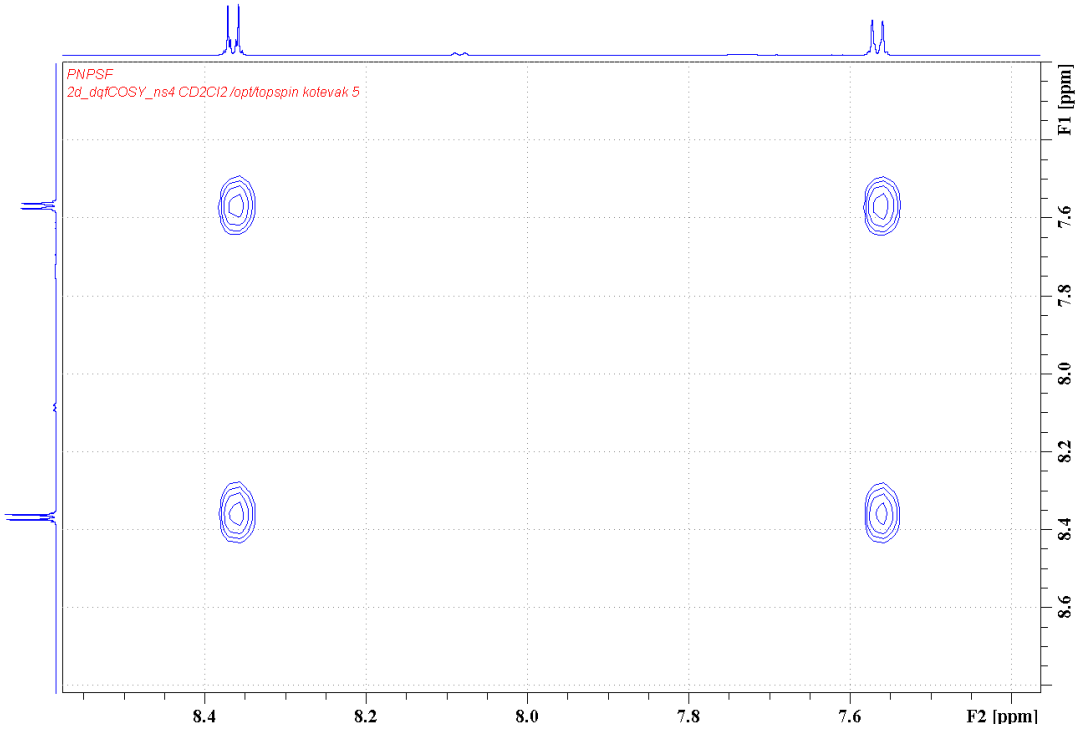


Figure A5: COSY 2D correlation spectra.

HSQC spectra

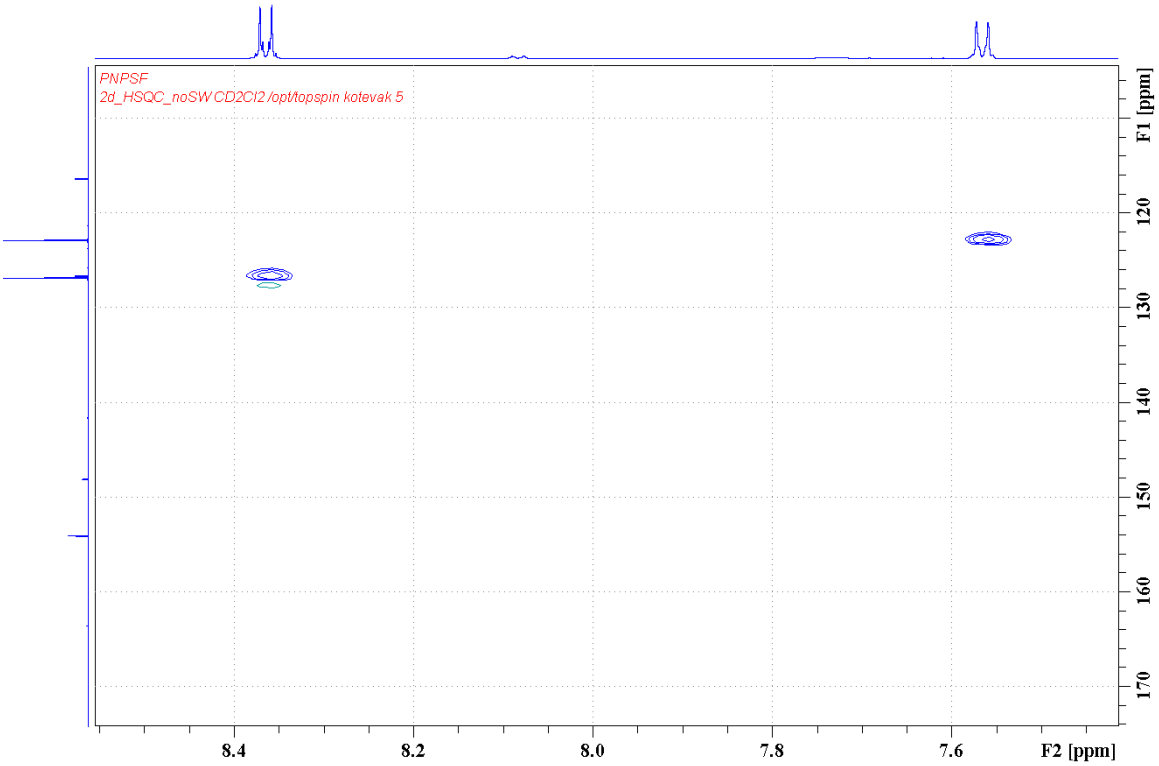


Figure A6: Heteronuclear single-quantum correlation (HSQC) spectra.