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Synthetic Investigations Towards the Preparation of Novel Tetracycline Antibiotics

By
Jennifer Farrell



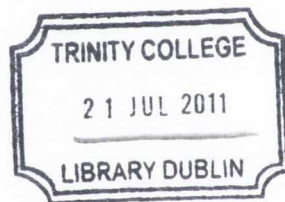
A thesis submitted to the University of Dublin, Trinity
College for the degree of Doctor of Philosophy

School of Chemistry

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University of Dublin

Trinity College



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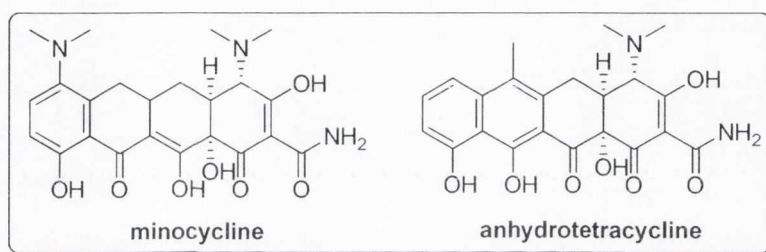
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Abstract

This research initially concerned itself with the synthesis of a dimeric compound of tetracycline antibiotics linked by a flexible tether. Tetracycline antibiotics have been in use since their discovery in the 1940s but widespread resistance has become a problem, limiting their clinical use. We propose that a dimeric compound of tetracycline antibiotics would be capable of circumventing the most ubiquitous resistance mechanism, the tetracycline efflux proteins. Tetracycline antibiotics minocycline and anhydrotetracycline were chosen for use in this study.



The chemistry of minocycline and anhydrotetracycline has been investigated in detail in this study and a number of novel tetracycline analogues have been produced. Varying linker structures for the dimer were also explored including acyl, amino, azido, alkynyl and organostannane compounds. A number of novel organostannane variants were also produced. The reactivity and stability of organostannanes in a variety of reactions were studied as part of this process.

The reactivity of anhydrotetracycline in Pd(0) coupling reactions with organostannanes was explored and it was found that the Stille reaction was not amenable for use with anhydrotetracycline analogues. An unexpected method of producing vinylphosphonium salts was revealed during the course of the Pd(0) coupling study, which is believed to proceed through a radical mechanism.

The halogenation of minocycline analogues at position 8 was examined in addition to the use of a 1,2,3-triazole moiety at position 9 for the production of new minocycline derivatives. The potential of a 1,2,3-triazole moiety at position 9 in anhydrotetracycline was explored and the reactivity of anhydrotetracycline towards acylation was also investigated.

The reactivity of substituted minocycline and anhydrotetracycline analogues in the Huisgen reaction and Cu(I) catalysed cycloaddition of alkynes and azides was investigated

and it was determined that anhydrotetracycline was unstable under the necessary reaction conditions.

Finally, a general methodology for the production of anhydrotetracycline analogues with greater ability to traverse cell membranes was successfully examined and a small number of novel anhydrotetracycline compounds of this nature were prepared.

Abbreviations

13-CPTC	13-Cyclopentylthio-5-hydroxy-6-deoxy tetracycline
4-DMAP	4-dimethylaminopyridine
6-DMDOT	6-Demethyl-6-deoxytetracycline
6-DOT	6-Deoxytetracycline
aatRNA	Aminoacyl transfer RNA
AD	Alzheimer's disease
AIBN	2,2-azobis(2-methylpropionitrile)
AIDS	Acquired immunodeficiency virus
ALS	Amyotrophic lateral sclerosis
AscOH	Ascorbic acid
BBB	Blood-brain barrier
CA-MRSA	Community acquired MRSA
Cap-1	Caspase-1
Cap-3	Caspase-3
COX-2	Cyclooxygenase-2
Dbi	Dibenzylidene acetone
DEAD	diethylazidocarboxylate
DIBAL	Diisobutylaluminium hydride
DIPEA	Diisopropylethylamine
DMF	N,N-dimethylformamide
DMG-DMOT	9- <i>N,N</i> -Dimethylglycylamido-6-demethyl-6-deoxytetracycline
DMG-MINO	9- <i>N,N</i> -Dimethylglycylamidomincycline
DMPU	<i>N,N</i> -Dimethylpropylene urea
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual of mental disorders-IV
ESBL	Extended spectrum beta-lactamase
FDA	Food and drugs administration
GTP	Guanosine triphosphate
HA-MRSA	Hospital acquired MRSA
HD	Huntington's disease
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
IC ₅₀	Concentration at which 50% inhibition is observed
IN	Integrase protein

iNOS	Inducible nitric oxide synthase
LDA	Lithium diisopropylamine
mCPBA	<i>m</i> -Chloroperbenzoic acid
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MR <i>E. coli</i>	Multidrug resistant <i>Escherichia coli</i>
mRNA	Messenger RNA
mRS	Modified Rankin scale
MRSA	Multidrug resistant <i>Staphylococcus aureus</i>
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	<i>N</i> -bromosuccinimide
NBSH	Nitrobenzenesulfonyl hydrazide
NIS	<i>N</i> -iodosuccinimide
PANSS	Positive and negative syndrome scale
PD	Parkinson's disease
PPI	Pre-pulse inhibition
RNA	Ribonucleic acid
RP-MPLC	Reverse phase medium pressure chromatography
RPP	Ribosomal protection protein
TBAF	Tetrabutylammonium fluoride
TBDMS	<i>t</i> -butyldimethylsilyl
TBDMSCl	<i>t</i> -butyldimethylsilyl chloride
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TMSN ₃	Trimethylsilylazide
tRNA	Transfer RNA
UHDRS	Unified Huntington's disease rating scale
UK	United Kingdom
USA	United States of America
VRSA	Vancymycin resistant <i>Staphylococcus aureus</i>
WHO	World health organisation

Chapter 1

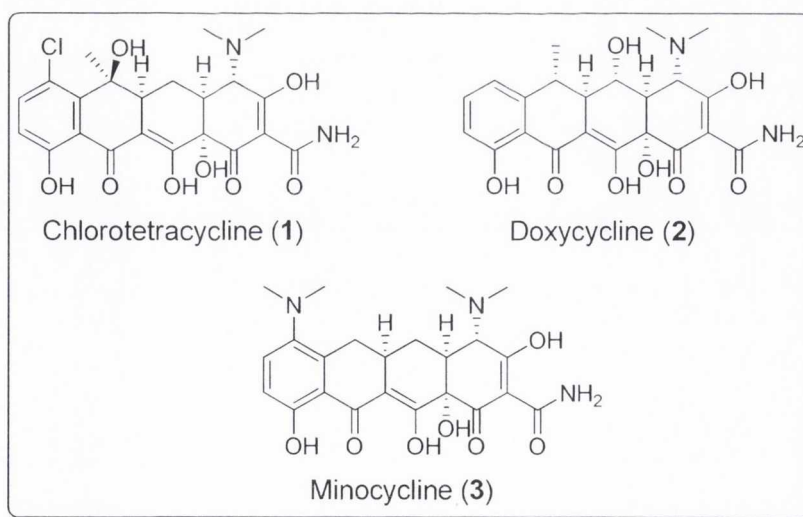
Introduction

1 Introduction

1.1 Tetracycline antibiotics: a historical perspective

In 1947 Duggar reported the discovery of a new antibiotic, which he named aureomycin, that would come to revolutionise the treatment of infection over the next six decades.² This antibiotic, now generally known as chlorotetracycline (Figure 1.1), was isolated by Duggar and his collaborators from *Streptomyces aureofaciens*, and its inherent antimicrobial properties were notable. Chlorotetracycline (**1**) was the first antibiotic in what would later become a much larger class of tetracycline drugs, including both naturally occurring compounds and semi-synthetic derivatives. The class has been divided into two separate groups, the so called ‘typical’ tetracyclines which exhibit a bacteriostatic effect, such as tetracycline, doxycycline (**2**) and minocycline (**3**) (Figure 1.1), and the ‘atypical’ tetracyclines which exhibit a bactericidal effect, such as anhydrotetracycline (**4**), chelocardin (**5**) and 6-thiatetracycline (**6**) (Figure 1.2). These compounds display a wide range of activity against both Gram-positive and Gram-negative bacteria, as well as protozoa,³ and a diverse spectrum of activity pertaining to other non-microbe related diseases (discussed in Section 1.6.2).

Figure 1.1 Typical tetracyclines



Since the discovery of this class of compounds, there have been persistent efforts to gain a better understanding of their mechanisms of action, improve upon their naturally occurring

antibacterial potency and also to circumvent the inevitable resistance mechanisms that have evolved over time^{4, 5} It is generally accepted that it is the overuse of such broad spectrum antibiotics in human and veterinary medicine, as well as their use as growth promoters in animal feeds in the USA since the 1950's, that has lead to the extensive and widespread resistance to these antibiotics that we observe today.⁶ This, however, has spurred research into the genetic mechanisms of bacterial resistance which in turn has inspired the development of new tetracyclines, such as the glycylcyclines, which are active against tetracycline resistant species.⁷

1.2 Cellular uptake and mechanisms of action

1.2.1 Typical tetracyclines

Typical tetracyclines are known to be rapidly absorbed by susceptible bacteria in a partially energy dependent manner.⁸ A model explaining the energy dependent nature of the accumulation of tetracyclines has been generally accepted and much research has provided supporting evidence indicating the model to be accurate.⁸⁻¹¹ In the case of Gram-negative bacteria, the tetracycline passes through the outer cytoplasmic membrane through the porins OmpF and OmpC as a cationic magnesium chelated complex, $[\text{Tc-Mg}]^+$, a fact supported by studies proving that mutated bacteria lacking these porin channels are much less susceptible to tetracyclines.⁹⁻¹¹ The cationic $[\text{Tc-Mg}]^+$ complex is attracted across the outer membrane by the Donnan potential and accumulates inside the periplasm where it dissociates, generating the uncharged tetracycline. This species is weakly lipophilic and can, as such, diffuse across the inner cytoplasmic membrane where it again forms a magnesium chelate complex due to a higher intracellular pH and an increased concentration of divalent metal ions, in particular magnesium.¹² Similarly, it is the neutral tetracycline species that is presumed to transfer across the cell membrane of Gram-positive bacteria. The accumulation of tetracycline is dependent on the ΔpH element of the proton motive force, explaining the partially energy dependent nature of the process.¹³ The antibacterial properties of tetracycline have also been shown to be related to intracellular pH and concentration of Mg^{2+} ions and it is probable that the active species within the bacterial cell is the $[\text{Tc-Mg}]^+$ complex.¹⁴

Since the 1950s the reversible inhibition of protein synthesis by tetracyclines has been known to be primarily responsible for their bacteriostatic activity.¹⁵ However, the details of

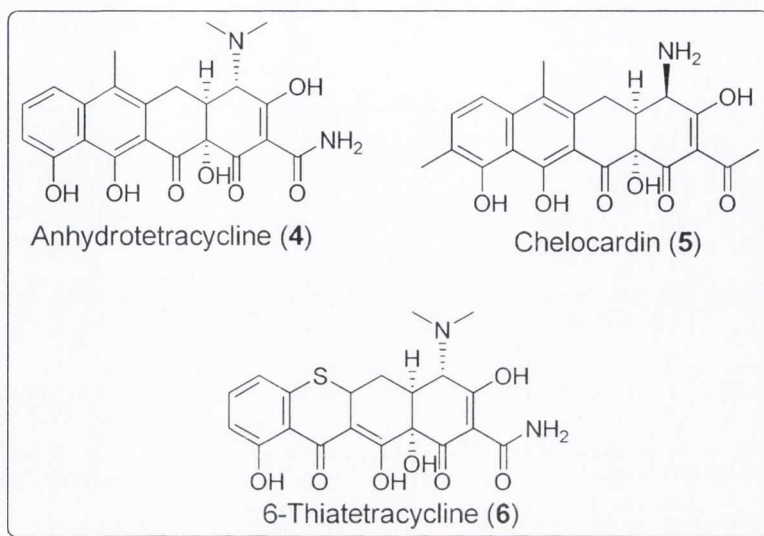
the mechanism by which this occurs have proved more difficult to elucidate. A number of studies have since proven that tetracycline binds in a reversible manner to the bacterial ribosome. One high-affinity binding site has been located on the 30S subunit of the bacterial ribosome, primarily at the ribosomal protein S7,¹⁶ and a number of low affinity binding sites have been located on both the 30S and 50S subunits.¹⁷ Binding of tetracycline at these sites has been shown to weaken aminoacyl-tRNA binding to the ribosome^{18,19} and this is considered to be the origin of the inhibition of protein synthesis. This reasoning is supported by the close proximity of the S7 protein on the 30S subunit to the aminoacyl-tRNA binding site on the bacterial ribosome and it is believed that steric hindrance by tetracycline elicits its effect.²⁰

This class of tetracyclines are therapeutically useful due to their ability to distinguish between mammalian and microbial cells. The selectivity arises from the weak inhibition of mammalian protein synthesis, due to differences between the small subunits of mammalian and bacterial ribosomes²¹ and poor accumulation of tetracyclines in mammalian cells.²²

1.2.2 Atypical tetracyclines

The discovery of atypical tetracyclines followed that of typical tetracyclines, the first of the class being chelocardin, in 1962.²³ Considerable research was undertaken on this new class of bactericidal tetracycline antibiotics, containing chelocardin (**5**), 6-thiatetracycline (**6**) and anhydrotetracycline (**4**) (Figure 1.2, *vide infra*), to elucidate a mechanism of action.²⁴⁻²⁶ It was found that both chelocardin and 6-thiatetracycline were poor inhibitors of protein synthesis in cell-free translation studies.²⁵ Enzyme leakage studies performed by Olivia *et al.* proved that the cytoplasmic membrane was in fact the target for atypical tetracyclines and that the interaction of these compounds with the cell membrane results in cell lysis.²⁴ Several theories were proposed for the mechanism of cell lysis but it was Chopra *et al.* who finally proposed, with significant evidence, that a disruption of cell membrane potential was the trigger for cell autolysis.

Figure 1.2 Atypical tetracyclines



It is known that typical tetracyclines exist in two free base forms; a low energy, electroneutral, lipophilic species that is predominant when crossing the cell membrane, and a high energy, zwitterionic, hydrophilic species that is important for interaction with the bacterial ribosome.²⁷ However, it has been shown that chelocardin, and presumably all atypical tetracyclines, exist in only the non-ionised lipophilic form regardless of the nature of their surrounding medium.²⁵ This provides an explanation for the observed affinity of atypical tetracyclines for the bacterial cell membrane, as it is likely that these compounds become trapped within this membrane and elicit their bactericidal effect as such. This, of course, provides no method for distinction between bacterial cells and mammalian cells. In the only study of its nature to be performed, healthy human volunteers were administered with 6-thiatetracycline and considerable adverse effects in the volunteers were noted, in particular with respect to the central nervous system.²⁸ This evidence, along with the much lower LD₅₀ values of 6-thiatetracycline and chelocardin in murine studies²⁸ (murine intraperitoneal injection: LD₅₀ tetracycline²⁹ = 120 mg kg⁻¹, LD₅₀ chelocardin³⁰ = 88 mg kg⁻¹) has resulted in the atypical tetracyclines being discarded as potential therapeutic agents to date.

1.3 Multidrug resistant bacteria

Multidrug resistant bacteria are becoming more prevalent in today's society. This can be explained in part by the extensive use of antibiotics in human medicine, prophylaxis and as growth promoting agents in agriculture,³¹ with noted cases demonstrating the ability of resistant bacteria to pass from animals to humans by contact or through food products.^{22, 32} Furthermore, bacterial resistance determinants are often passed on in sets by a single resistance plasmid, which complicates the treatment of the resultant infections.⁶ These plasmids can code for a mechanism which ensures they are genetically enduring, as they code for a stable toxin and unstable antitoxin. Therefore, if the plasmid is somehow lost, the bacterial cell will fail to produce antitoxin and succumb to the remaining toxin.³³ Two of the most infamous and clinically challenging multidrug resistant bacteria, MRSA (multidrug resistant *Staphylococcus aureus*/methicillin resistant *Staphylococcus aureus*) and MR E. coli (multidrug resistant *Escherichia coli*), are discussed in Sections 1.3.1 and 1.3.2 below.

1.3.1 MRSA

Staphylococcus aureus is a Gram-positive, facultatively anaerobic bacterium that is a common member of skin flora.³⁴ It is responsible for a variety of ailments ranging from minor skin infections to life threatening illnesses such as meningitis, toxic shock syndrome, endocarditis and bacteraemia.³⁵ In the past thirty years the frequency of colonisation and infection caused by this bacterium has increased significantly and *Staphylococcus aureus* has become the most common cause of nosocomial pneumonia and the second most common cause of nosocomial bloodstream infections.³⁶ Members of the penicillin class are the first line of treatment indicated for *Staphylococcus aureus* infections, although cephalosporins are advised for patients with β -lactam allergies, as there is a much lower rate of resistance to these β -lactam antibiotics.³⁵

MRSA is a multidrug resistant strain of *Staphylococcus aureus*. It was first documented by medical practitioners in 1961 in the UK,³⁷ but has since become a worldwide issue. Between 2001 and 2004 the prevalence of MRSA colonisation in the general population (USA) increased from 0.8% to 1.5%.³⁸ More recently a UK study revealed that 1.3% of hospital patients tested had acquired MRSA.³⁹ In 2007, Klein *et al.*⁴⁰ published a study which claimed that the number of MRSA infections treated in hospitals had doubled from 1999 to

2005 and in 2005 it was the cause of nearly 19,000 hospital stay related deaths in the USA alone.⁴¹ In 2006, a study in the UK reported the percentage mortality over thirty days for patients contracting MRSA as 34%.⁴² Currently the rate of MRSA infection in Ireland is one of the highest in Europe, with treatment costing the state €23 million per annum.⁴³

MRSA infections are sub-categorised into CA-MRSA (community acquired MRSA) and HA-MRSA (hospital acquired MRSA). CA-MRSA is more virulent and a more frequent cause of infection, however HA-MRSA (commonly referred to as the “hospital superbug”) results in more severe infections and holds a greater spectrum of resistance.⁴⁴ Currently vancomycin and teicoplanin, members of the glycopeptide antibiotic family, are recommended for the treatment of HA-MRSA and while this treatment was sufficient for many years, in 2002 the first fully vancomycin resistant strains (VRSA) emerged.⁴⁵ The rapid emergence of resistance in *Staphylococcus aureus* demonstrates its ability to deal with newer antibiotics. It is now feared that VRSA may become as wide-spread as MRSA and currently only linezolid and daptomycin are reserved as last defence antibiotics.^{46, 47}

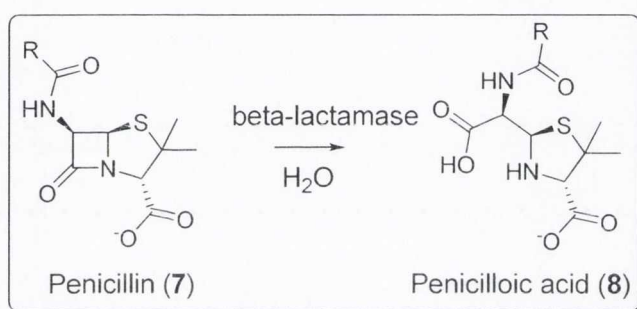
1.3.2 ESBL producing *Enterobacteriaceae*

Enterobacteriaceae are a family of Gram-negative, facultatively anaerobic, rod-shaped bacteria, made up of plant and animal parasites, saprophytes and commensals. Most are motile and are found in plants and animals, soil and water.⁴⁸ This family contain many common genera including *Escherichia*, *Klebsiella*, *Enterobacter* and *Salmonella*.⁴⁸ They generally harmless gut flora but some species and strains cause infection in humans, most notably *Escherichia coli* and *Klebsiella pneumoniae*,⁴⁹ which cause a variety of conditions including gastroenteritis, urinary tract infections, sepsis and meningitis.⁵⁰ The treatment of Gram-negative bacterial infections is challenging due to the presence of a bacterial outer membrane which confers inherent resistance to some antibiotics.⁵¹ These infections have presented further difficulties with the emergence of extended spectrum β -lactamase (ESBL) producing variants.⁵²

Many Gram-negative bacteria possess a naturally occurring chromosomally mediated β -lactamase that catalyses the ring opening hydrolysis of the β -lactam ring of β -lactam antibiotics and renders them inactive (Figure 1.3, *vide infra*). These enzymes were known to exist before the discovery of the first β -lactam antibiotic, therefore development of these

enzymes is most likely due to bacterial evolution in the presence of β -lactam generating soil microbes.⁵³ Over the last thirty years new β -lactam antibiotics, such as cephalosporins, have been introduced but, predictably, new β -lactamase enzymes conferring resistance to these have emerged and proliferated. Organisms which express β -lactamases contribute to the failure of first line clinical antibiotic treatment, as resistance plasmids often code for several resistance factors, including aminoglycoside, chloramphenicol and tetracycline resistance.⁵⁴

Figure 1.3 The action of beta-lactamases



The epidemiology of ESBL-producing enterobacteriaceae, particularly *E. coli* strains, is now similar to that of MRSA: the first cases being documented in nosocomial isolates with subsequent appearance of similar isolates in the community.⁴⁹ The intravenous administration of carbapenem antibiotics is currently useful for the treatment of ESBL-producing enterobacteriaceae, however it is accepted that research into new agents is necessary as resistance may well emerge.⁵² Tetracycline analogues that circumvent the resistance spectrum of ESBL-producing enterobacteriaceae could provide a new therapeutic option, as this class of drugs possesses an excellent activity profile against many Gram-negative bacteria.

1.4 Resistance mechanisms

1.4.1 Introduction to protein biosynthesis

Protein biosynthesis is the process by which proteins are synthesised from the genetic codes in DNA. The synthesis is split into two stages; transcription, which produces RNA from DNA, and translation, which converts RNA to proteins.⁵⁵

The first stage of transcription involves the binding of an enzyme, RNA polymerase, and other proteins, called transcription factors, to the promoter region of the DNA to be transcribed. RNA polymerase then moves along the DNA helix, separating the strands as it does so and pairing the DNA bases with RNA nucleotides to form messenger RNA (mRNA). The mRNA peels away from the DNA as it is formed and transcription is halted when RNA polymerase reaches the termination site on the DNA. In eukaryotes the mRNA is synthesised within the confines of the nuclear envelope and then transported out into the cytoplasm for translation. In prokaryotes both transcription and translation occur within the cytoplasm.⁵⁵

During the process of translation mRNA is bound by the ribosome, which subsequently moves along the mRNA 'translating' the DNA derived information. The cell interprets the mRNA by reading the codons (three RNA bases) using transfer RNA (tRNA). The function of tRNA is to transfer amino acids from the cytoplasm, where reserves of all twenty are stored, to the ribosome. tRNA is composed of a protein which is folded in such a way that a three base anticodon is located at one end and an amino acid attachment site at the other. As the tRNA moves through the cytoplasm, it binds its amino acid at the attachment site to form aminoacyl tRNA (aatRNA). When aatRNA reaches the ribosome the anticodon hydrogen bonds with the appropriate codon on the mRNA at the A-site of the ribosome (Figure 1.4, *vide infra*), which is so called as it is the site of binding for aatRNA. A component of the large ribosomal subunit then catalyses the formation of a peptide bond between the newly arrived amino acid in the A-site and the amino acid attached at the P-site (peptidyl t-RNA binding site). The amino acid in the newly forming protein dissociates from its tRNA and the ribosome/mRNA complex shifts, moving the free tRNA from the P-site to E-site (exit site), the aatRNA at the A-site to the P-site and the next codon of the mRNA into the unoccupied A-site. The free tRNA in the E-site then leaves the ribosome where it can bind another amino acid for incorporation into the protein. With the A-site empty the cycle can start again. This translation process continues until a termination codon reaches the A-site. A protein called a release factor binds to the termination codon resulting in the release of the newly formed protein from the ribosome and the dissociation of the ribosome/mRNA complex.⁵⁵

1.4.2 Resistance transfer

It has been well documented that resistance genes can be transferred from one bacterial cell to another, in the form of resistance vectors such as recombinant plasmids or chromosomes, by means of conjugation.⁶ These plasmids are highly maintained during binary fission to the extent that some bacteria contain a “killer” protein which is activated if the plasmid is lost or incomplete.⁵⁶ In 1998, a significant example of the spread of bacterial resistance was highlighted by Wright *et al.*,⁵⁷ who demonstrated amino acid sequence homologies of 61-64% for three proteins conferring vancomycin resistance in different bacterial genera. These proteins, VanH, VanA and VanX, were found in vancomycin resistant enterococci and vancomycin producing streptomyces bacteria, *Streptomyces toyocaensis* and *Amycolatopsis orientalis*. The orientation of these genes in the enterococci and streptomyces strains were also found to be identical, leading this research group to conclude that the origin of vancomycin resistance in enterococci was most likely a direct transferral of genes from the streptomyces family. Vancomycin resistant bacteria are now considered to be one of the most challenging group of bacteria to treat clinically, in particular, vancomycin resistant *Staphylococcus aureus* has been highlighted as a potential epidemic threat.⁵⁸

Resistance genes are often organised into an integron: a bacterial apparatus containing a gene which codes for an integrase, an enzyme which catalyses the introduction of resistance genes downstream from a strong promoter.⁵⁹ Once incorporated into an integron, resistance genes can be organised into an operon and up to eight resistance genes can be included in a single integron.⁶⁰ Integrons are usually inserted into larger transposons, which then facilitate the transfer of resistance genes between the plasmids and chromosomes of different bacteria by horizontal gene transfer.⁶¹

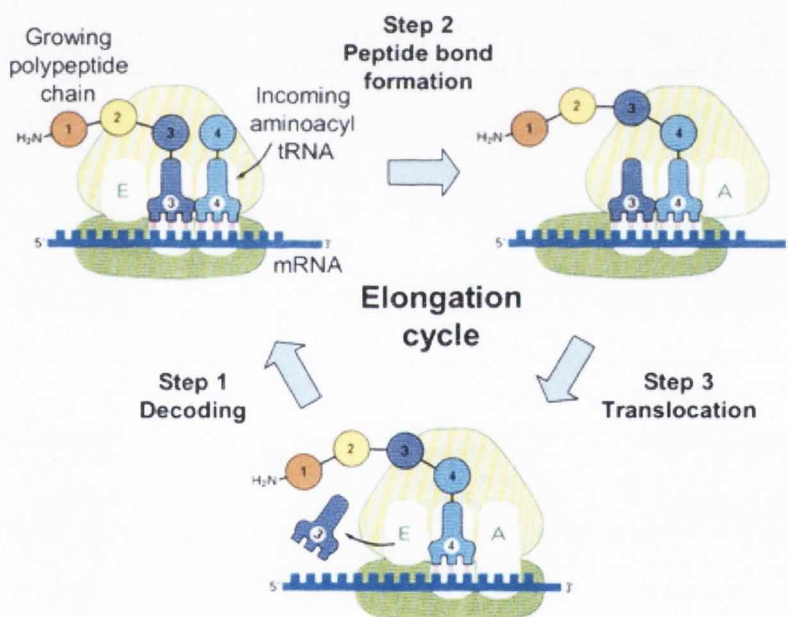
1.4.3 Ribosomal protection

Protection of the ribosome from the action of tetracyclines is an important strategy in drug resistance. There are currently eight known members of this class of tetracycline ribosomal protection proteins (RPPs) of which Tet(O) and Tet(M) are the most widely studied. RPPs are broadly dispersed throughout the bacterial world and it is generally accepted that all eight members have similar mechanisms of action.⁴

Tet(M) and Tet(O) are soluble cytoplasmic proteins with 75% sequence homology.⁶² Genes coding for Tet(O) are known to be located on a transferrable resistance plasmid and, similarly, genes coding for all RPPs are located on genetic components which are easily passed from one bacterial cell to another *via* horizontal gene transfer.⁴ The exact mechanism of action of RPPs is somewhat disputed,⁶³ however it is known that Tet(O) and Tet(M) can displace tetracycline from the bacterial ribosome in a GTP (guanosine triphosphate) dependent manner which increases the K_d from 5 μ M to 30 μ M.^{64, 65}

A mechanism of action for Tet(O) was proposed by Spahn *et al.* in 2001.⁶⁶ They proposed that, in the presence of tetracycline, the bacterial ribosome is prevented from progressing through the various states of its elongation cycle (Figure 1.4, Section 1.4.2.1)⁶⁷ due to the steric interference of the A-site by the tetracycline.

Figure 1.4 Ribosomal elongation cycle^a



^a Taken from http://xray.bmc.uu.se/~kurs/BiostrukfunkX2/practicals/practical_4/practical_4.html. [21/11/2010]⁶⁷

The steric interference at the A-site is relieved by the presence of Tet(O), which binds to the ribosome and releases tetracycline so that the elongation cycle may continue. This proposed order of events, *i.e.* the initial binding of tetracycline to the ribosome and its subsequent release by Tet(O), is supported by the fact that Tet(O) cannot bind to a ribosome with an occupied A-site. Spahn *et al.*⁶⁶ suggested that Tet(O) prompts the release of tetracycline by

binding at an allosteric site and contacting the base histidine-34. This in turn induces a disturbance in this helix of the ribosome which causes the release of tetracycline. Successive dissociation of Tet(O) from the ribosome allows protein synthesis to continue.

1.4.4 Enzymatic inactivation of tetracycline

To date there has been only marginal investigative research into this mechanism of resistance with relation to tetracyclines, perhaps because it is not as ubiquitous as other mechanisms. What is known though, predominantly through the work of Speer *et al.*, is that the only example of this form of resistance is coded for by the gene *tet(X)*.⁶⁸ This gene produces a cytoplasmic protein, which has some homology with other oxidoreductases and is considered to catalyse the autooxidation of tetracycline.⁶⁸ The protein, of approximate size 44kD, is capable of chemically modifying tetracyclines to inactive analogues in the presence of NADPH and oxygen.⁶⁹

1.4.5 Efflux proteins

The mechanism of action of the tetracycline efflux proteins is the most studied of the resistance mechanisms. These proteins have been shown to be coded for by genes belonging to the major facilitator superfamily (MFS) and are membrane bound proteins which efflux tetracycline-metal cation complexes from the cytoplasm,⁷⁰ coupled with the influx of a proton against the concentration gradient, thus reducing the intracellular concentration of the compound. The genes coding for efflux proteins are found across a wide spectrum of Gram-positive and Gram-negative bacteria⁴ and are preceded upstream in most cases by repressor genes coding for a repressor protein. The strict regulation of expression of these genes is discussed in Section 1.4.5.1.

In most cases the efflux proteins are capable of exporting tetracyclines from the cell, but not in the case of minocyclines or glycylcyclines. However, the protein Tet(B), which is found only in Gram-negative bacteria, is capable of expelling tetracycline and minocycline from the cell but not the glycylcyclines.^{14, 71} In the case of Tigecycline (marketed as Tygacil® by Pfizer) this effect has been attributed to steric hindrance caused by the presence of a bulky *t*-butylglycylamido group.^{71, 72} To date no natural mutation in efflux proteins has arisen which allows the export of glycylcyclines from the cytoplasm yet it is generally

acknowledged that it is merely a matter of time. Studies performed by Rothstein *et al.* in the late 1990s involving the deliberate mutation of the *tet(A)* and *tet(B)* genes have shown these mutations to be a distinct possibility in nature, as their laboratory-mutated proteins were capable of effluxing glycylicyclines from the bacterial cell.⁷³

The efflux proteins are approximately 46 kDa membrane-bound proteins consisting of lipophilic α -helices that are embedded in the lipid bilayer, and hydrophilic cytoplasmic and periplasmic loops. They have been divided into six separate groups determined by amino acid sequence identity. Group 1 contains Tet(A)-Tet(E), Tet(G), Tet(H), Tet(Z), Tet(I), Tet(J) and Tet(30).⁷⁴ These proteins have amino acid sequence homology of 41-78% and their respective repressor proteins have amino acid sequence homology of 37-88%. Studies have predicted these proteins to have 12 transmembrane α -helices connected by cytoplasmic loops between helices 6 and 7.^{75, 76} Group 1 proteins are found only in Gram-negative bacteria with the exception of Tet(Z) which is found solely in Gram-positive strains and is the first Gram-positive efflux protein discovered which is regulated by a repressor protein.⁷⁴ The efflux proteins are embedded in the lipid bilayer, with the lipophobic amino acid loops extending out into the periplasm and cytoplasm, and have been shown to couple the export of a $[\text{Tc-Mg}]^+$ complex from the cell with the import of a proton.⁷⁶ Mutations in the N- or C- termini of the efflux proteins of Gram-negative bacteria have been shown to eliminate resistance, suggesting that residues across the entire protein are imperative for its function and loops 2/3 and 10/11 have been shown to be important for the energy dependent portion of the efflux mechanism.⁷⁷ Evidence from genetic mutation studies suggests that these loops cooperate to form a proton pump.⁷⁸ The Gram-negative efflux genes are related to large plasmids which often carry genes conferring resistance to other antibiotics, heavy metal resistance genes and toxins.⁶ Therefore, selection for any of the above mentioned genes results in selection for all resistance mechanisms, and multiple drug resistant bacteria.

Group 2 efflux proteins, Tet(L) and Tet(K), are found largely in Gram-positive bacteria. They have 58-59% amino acid identity and are predicted to have 14 transmembrane α -helices responsible for conferring resistance against tetracycline and chlorotetracycline (**1**). The efflux genes are located on small plasmids which are easily transferred directly to other bacteria or integrated into larger plasmids or chromosomes of other bacteria.

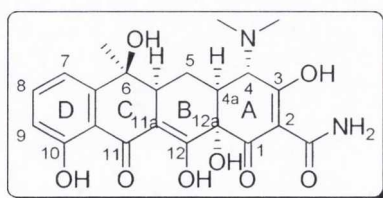
Efflux proteins from groups 3 to 5 are much smaller in number and are also less common. They contain proteins of both 12 and 14 transmembrane α -helices conformations.

Group 6 contains only one efflux protein, which is believed to use ATP rather than the proton gradient as a source of energy.⁴

1.4.5.1 Regulation of efflux proteins

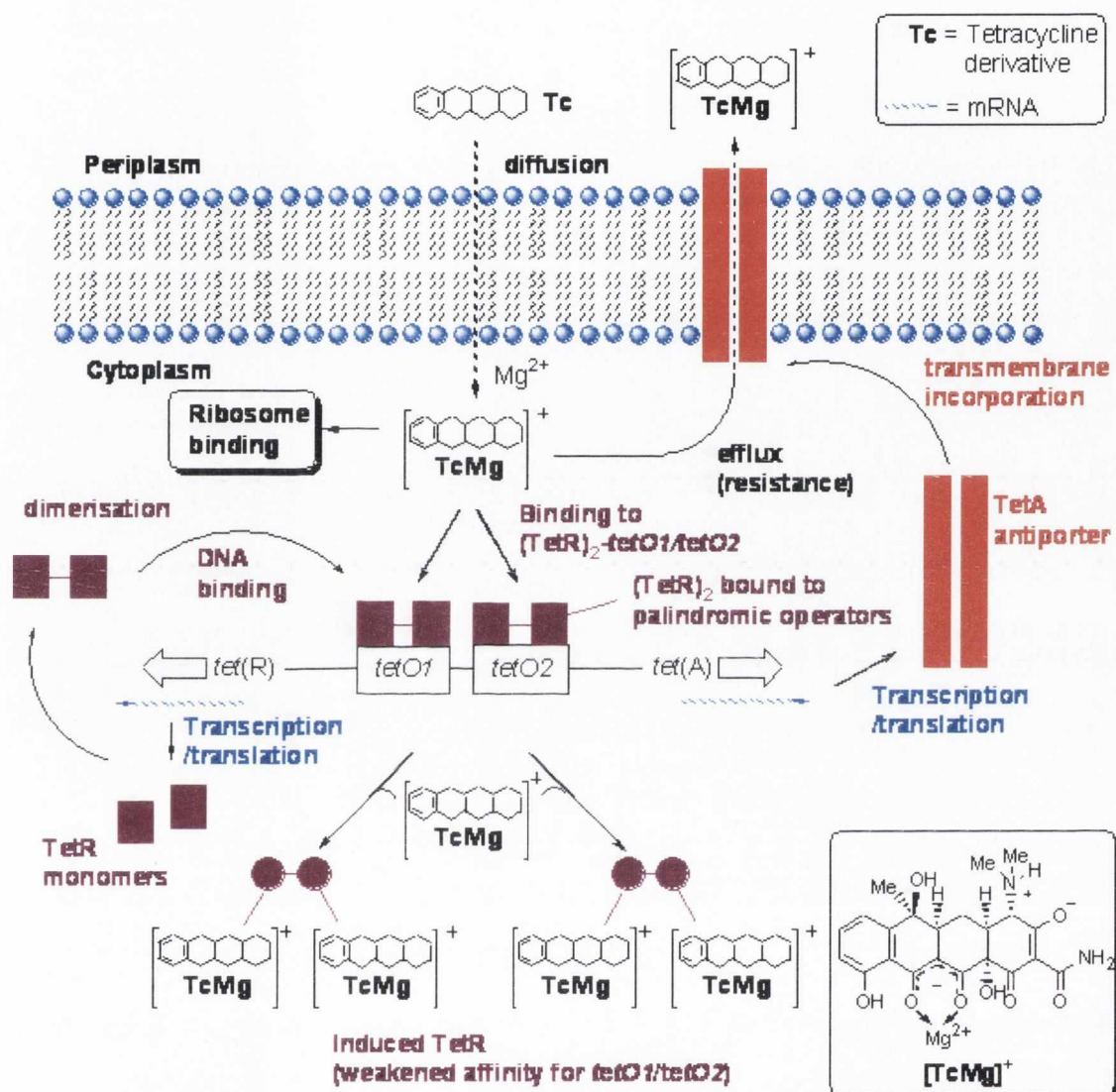
The expression of efflux proteins in bacterial cells is controlled by the regulatory protein Tet(R) and tetracycline. In the absence of tetracycline, the regulatory protein is bound tightly ($K_{\text{association}} = 10^{11} \text{M}^{-1}$) to the regulation site on the bacterial DNA, *tetO1* and *tetO2* (which contain palindromic nucleotide sequences)⁷⁹ as a homodimer, Tet(R)₂. The Tet(R)₂ homodimer binds two adjacent major grooves in the DNA of the aforementioned regulation site.⁸⁰ This Tet(R)₂-DNA complex will subsequently be referred to as [TetR-*tetO*]. This binding represses the transcription upfield of the monomers of Tet(R) itself and downfield of the Tet(A) efflux protein. When tetracycline enters the cell and forms a magnesium chelate complex, [Tc-Mg]⁺, Tet(R)₂ is released from the DNA. This occurs as two [Tc-Mg]⁺ complexes bind to [TetR-*tetO*] with high affinity ($K_{\text{association}} = 10^9 \text{M}^{-1}$)⁸¹ enforcing conformational changes upon the dimer which reduce its binding affinity for *tetO1* and *tetO2* by between 6 and 10 orders of magnitude,⁷⁹ thus liberating it from the bacterial DNA.⁸² The binding of [Tc-Mg]⁺ to [TetR-*tetO*] occurs in two channels of the regulatory domain of Tet(R)₂. Tc enters the binding site with the A ring (Figure 1.5) head on as hydrogen bonds form between its functional groups and the amino acids in this site. This binding results in a conformational change which closes a 'sliding door' mechanism behind the tetracycline to further increase the association of the complex.⁸³ The complex of [Tc-Mg]⁺ and Tet(R)₂ is additionally stabilised by two chains of eight water molecules in each of the Tet(R) monomers, which help to prevent re-association of the released Tet(R)₂ complex with its binding domain in the bacterial DNA.⁸⁴

Figure 1.5 Ring nomenclature and numbering in tetracyclines



The dissociation of [TetR-*tetO*] caused by [Tc-Mg]⁺ results in the transcription and translation of the Tet(A) efflux protein downfield, which is then incorporated into the membrane of the bacterium conferring tetracycline resistance upon it. In this way the bacterial cell is protected from the action of [Tc-Mg]⁺, as its affinity for the 30s subunit of the bacterial ribosome ($K_{\text{association}} = 10^6 \text{ M}^{-1}$)⁸⁰ is 1000-fold lower than its affinity for [TetR-*tetO*]. When the tetracycline has been effluxed from the cell, Tet(R)₂ again complexes with the bacterial DNA to form [TetR-*tetO*], preventing further translation of Tet(A). In this way the presence of tetracycline itself induces the bacterium to express its resistance genes, while the absence of tetracycline in the cell represses the resistance genes.^{4, 85} Figure 1.6 presents a schematic representation of the regulation processes.⁸⁶

Figure 1.6 Regulation of expression of TetA^a



^a Based upon scheme by W. Saenger *et al*⁸⁰

1.5 Combating resistance

In developed countries, between 5% and 10% of patients admitted to hospitals develop a nosocomial infection, the leading causes of which in bloodstream infections include coagulase-negative staphylococci, *S. aureus* and enterococci with respective mortality rates of 21%, 25% and 34%.⁸⁷ Among these strains 75% of coagulase-negative staphylococci and 41% of *S. aureus* present with methicillin resistance (MRSA) and 60% of *E. faecium* cases are

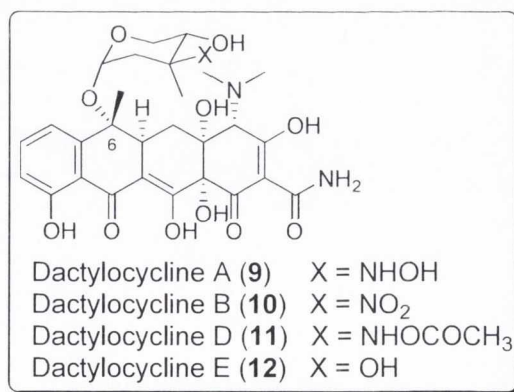
complicated by vancomycin resistance.⁸⁷ The incidence of mortality related to these types of bloodstream infections doubles from 32% to 64% if an active antibiotic is not administered in the first 24 hours (statistics acquired in 2004). It is clear then why the need for new antibiotic agents is urgent in today's society, as despite the ongoing advances in various medical fields multidrug resistant hospital and community acquired infections still account for an unreasonable percentage of deaths per annum.

Improved understanding of the resistance mechanisms discussed in Section 1.4 has led to a great deal of research into the development of new compounds which are capable of overcoming microbial resistance. While a great deal of research has been carried out on a broad spectrum of agents overcoming various types of bacterial resistance, this section aims to highlight only the most pertinent discoveries to date relating to tetracycline resistance.

1.5.1 Dactylocyclines

In 1992 Andersen *et al.* reported the discovery of a new class of tetracycline glycosides, the dactylocyclines, which they isolated from fermentation broths of *Dactyloporangium* sp.⁸⁸ The general structure of dactylocyclines is shown in Figure 1.7 below. This class are the first naturally occurring tetracyclines isolated with an inversion of the typical stereochemistry at C-6.

Figure 1.7 Dactylocyclines



A further analogue, dactylocycline C, was isolated but the structure of the sugar moiety could not be determined. All analogues were found to be active against tetracycline-sensitive and tetracycline-resistant Gram-positive bacteria but were only weakly active against Gram-

negative bacteria. The activity of all five dactylocyclines was tested against tetracycline-sensitive and tetracycline-resistant *S. aureus* strains in an assay determining the zone of growth inhibition for a given dosage, 50 µg.⁸⁹ This allows dactylocyclines to be rated in terms of activity for this test. It was found that the most active isolate was dactylocycline D which produced the largest zone of inhibition with respect to tetracycline-resistant *S. aureus* and the second largest zone of inhibition for tetracycline-sensitive *S. aureus*.

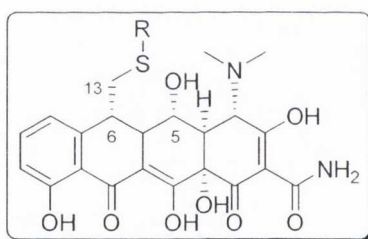
While these indications are promising, dactylocyclines have not been introduced for clinical use despite being highlighted as being potentially important antibacterial agents.⁸⁹

1.5.2 Efflux pump inhibitors

Over the past twenty years there has been much research into mechanisms to circumvent the activity of the Tet family of efflux proteins. This work initially aimed to improve the clinical efficacy of existing tetracycline antibiotics; a sensible strategy when one considers the cost of the discovery and development of new antibiotic agents with activity against resistant bacteria.

In 1993 Nelson *et al.*⁹⁰ reported the discovery of a new tetracycline analogue with the ability to reverse efflux pump resistance. The general structure of these analogues is shown in Figure 1.8 below.

Figure 1.8 **General structure of 13-thio substituted 5-hydroxy-6-deoxytetracyclines**



The tetracycline analogues synthesised were tested for their ability to inhibit the uptake of tritium-labelled tetracycline into everted vesicles derived from resistant bacteria (*E. coli* D1-209) expressing a Tet efflux protein.⁹⁰ Methacycline was chosen as the standard in these assays as it was the starting material from which the new analogues were synthesised. The synthesis of these analogues is discussed in Section 1.7.2. It was found that when R = phenyl

or benzyl a considerable decrease in the IC_{50} values resulted with respect to tetracycline. The order of activity for the aromatic series synthesised was found to be 13-(benzylsulfanyl) ($0.7 \mu M$) > 13-(benzylthio) ($1.4 \mu M$) $\geq$ 13-[(4-halophenyl)thio] (Cl = $1.6 \mu M$, Br = $1.4 \mu M$) > 13-(phenylthio) ($6.9 \mu M$) $\gg$ tetracycline ($13.2 \mu M$). These results demonstrated the dependence of inhibitory activity on the size, lipophilicity and polarity of the C-13 sulfur moiety.

Linear alkyl chains and alicyclic groups were also inserted at position R and the most active tetracyclines produced showed a dependency on chain length and the degree of branching. The most potent analogues generated in this series were the cyclopentyl and isopentyl derivatives which both displayed IC_{50} values of $0.3 \mu M$.

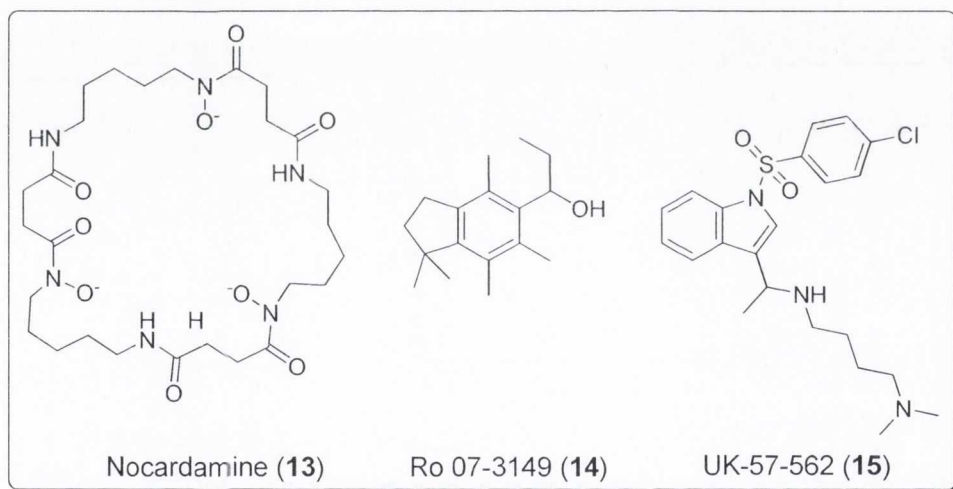
It is clear from these results that, in this series of 13-thio substituted 5-hydroxy-6-deoxytetracyclines, there is a direct correlation between the increase in lipophilicity of the R group and an increase in the inhibition of the efflux protein. Steric interactions also seem to play an important role and a lipophilic pocket of a distinct size in the efflux protein was suggested by this research group, with the cyclopentylthio moiety providing the optimal space-filling conditions.⁹⁰

In a subsequent paper published by Nelson and Levy⁹¹ the properties of the most active compound in the series, 13-cyclopentylthio-5-hydroxy-6-deoxytetracycline (13-CPTC), were examined by its administration to tetracycline-resistant *E. coli*. They demonstrated that 13-CPTC is a powerful inhibitor of Tet(B) and has a 6-fold higher affinity for the protein than tetracycline or doxycycline. 13-CPTC was also shown to be more active than doxycycline against strains expressing Tet(K) or Tet(L) efflux proteins as well as the Tet(M) ribosomal protection protein. It was also determined that co-administration of 13-CPTC and doxycycline (Figure 1.1) provided growth inhibition at minimum inhibitory concentrations (MIC's) well below those obtained for the individual compounds. Tet(A) and Tet(B) inhibition was 50 times greater when both compounds were administered than the MICs for doxycycline or 13-CPTC alone. This study also revealed that 13-CPTC exerts its inhibitory activity by competitively binding to the Tet proteins and not by interference with the proton motive force of the pump.

Another group of efflux pump inhibitors were also discovered in 1993 in an independent study by Rothstein *et al.*⁹² This group formulated assays that detected whether or not a given compound was a tetracycline and whether or not that compound was capable of inhibiting the TetA(B) and Tet(K) efflux pumps. One compound tested gave a positive

inhibitory response and was shown not to be a tetracycline. This compound was determined to be nocardamine (**13**, Figure 1.9), which strongly chelates Fe^{3+} . To determine whether nocardamine was unique in its ability to inhibit TetA(B) and Tet(K) or whether other siderophores (small, high-affinity iron chelating compounds secreted by microorganisms) would have the same effect, ferrichrome and ferrichrome A were subjected to the same testing. Both compounds were found to inhibit the Tet proteins, thus proving that the inhibition is not structure related but rather related to the ability of these three compounds to chelate Fe^{3+} . This finding was rationalised by the hypothesis that the siderophores disrupt the active efflux of tetracyclines by disturbing the proton gradient which bacteria use for energy in this process: as the electron transport chain (which uses iron as a co-factor) is essential in preserving the proton motive force.

Figure 1.9 **Efflux pump inhibitors**



Yamaguchi *et al.* discovered that Ro 07-3149 (**14**, Figure 1.8) was an inhibitor of Tet(K) expressed in *S. aureus*.⁹³ This compound, however, had no effect on Tet(K) proteins expressed in laboratory mutants of Gram-negative bacteria, presumably due to an inability to traverse the outer membrane.

UK-57-562 (**15**, Figure 1.8) was discovered by high-throughput screening for activity against Tet(C). It was subsequently revealed that this compound also inhibited Tet proteins (A), (B), (D), (K) and (L). SAR (Structure Activity Relationship) studies proved that the side

chain at position 3 of the indole ring was essential for activity and it was therefore suggested that disturbance of the bacterial membrane might be UK-57-562's mechanism of action.⁹⁴

While the research discussed in this section seems promising, further research is needed to develop tetracycline efflux pump inhibitors with low toxicity and high activity. This challenge is complicated further by the existence of a large number of Tet protein subclasses, which have a great deal of structural variation. It is unlikely that a single compound will be discovered with activity against all Tet proteins but possible that individual compounds could be designed for each specific efflux pump class. However, this alone would not be sufficient in combating tetracycline resistance in bacteria as the majority of resistant species also carry either ribosomal protection mechanisms and/or inactivating enzymes which would prevent tetracycline from exerting its antibiotic action.

1.5.3 Glycylcyclines

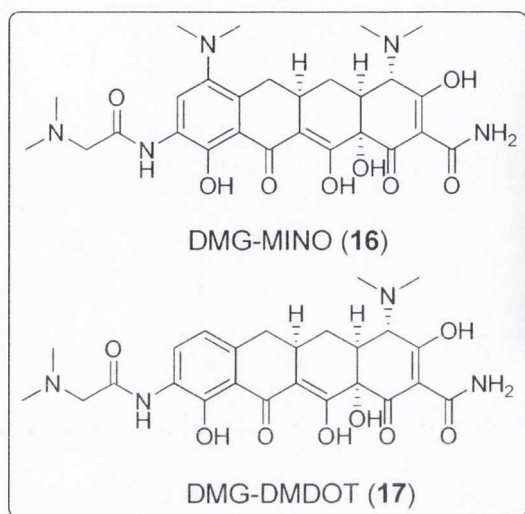
1.5.3.1 History and mechanism of action

Lederle Laboratories (currently Pfizer) have been involved in the development of tetracycline antibiotics since they discovered the class in 1947.² In 1988 they undertook a study to realise the potential of tetracyclines by designing analogues that were active against microorganisms exhibiting resistance to current tetracyclines, while retaining their potency with respect to tetracycline sensitive strains.⁷ This multi-disciplinary study investigated not only the bactericidal activity of the new analogues, but also their uptake by Gram-negative and Gram-positive bacteria, ribosome binding properties and ability to inhibit bacterial protein synthesis. Compounds modified at the D-ring (Figure 1.4, *vide supra*) were found to have new antibiotic properties.

The synthesis of a 9-amino series of various tetracyclines furnished the first lead compounds. It was of note that these compounds were active against *S. aureus* strains expressing the Tet(M) ribosomal protection protein, unfortunately they were inherently unstable under the testing conditions.⁹⁵ Subsequent research led to the discovery of the glycylcyclines; a series of tetracycline analogues substituted with a glycyamido moiety at position 9.⁹⁶ Initially, the most promising of these compounds were 9-(*N,N*)-dimethylglycyamidomincycline (DMG-MINO) (**16**) and 9-(*N,N*)-dimethylglycyamido-6-

demethyl-6-deoxytetracycline (DMG-DMDOT) (**17**, Figure 1.10, *vide infra*) which displayed higher activities against tetracycline resistant bacteria than any analogues previously tested.⁹⁶

Figure 1.10 **Dimethylglycylcyclines**



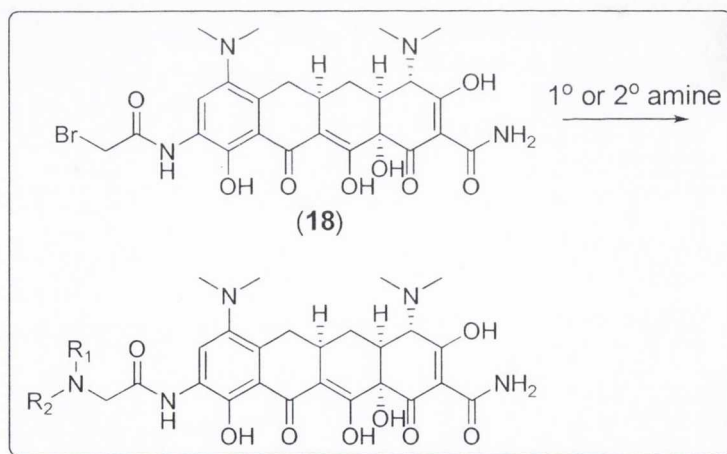
The range of activity of DMG-MINO (**16**) and DMG-DMOT (**17**) was unprecedented; the compounds were active against bacteria displaying the efflux pumps Tet(A), (B), (C), (D) and (K) and the ribosomal protection protein Tet(M). A series of biological tests performed by Testa *et al.* in 1993 demonstrated the extensive potential of these two compounds.⁹⁶ *In vitro* testing, using the agar dilution method, furnished MICs for DMG-MINO and DMG-DMOT against *E. coli* strains carrying Tet(A), (B), (C), (D) and an *S. aureus* strain carrying Tet(K) that were considerably lower than the MIC's of tetracycline and minocycline. In addition, both compounds had similar activities against Tet(M) resistant *E. coli* and *S. aureus* strains and tetracycline susceptible strains of the same, which were again significantly lower than the MIC's of tetracycline or minocycline. Rasmussen *et al.* reported that glycylcyclines were also outstanding inhibitors of bacterial protein synthesis⁹⁷ and it was proven that they presented a higher ribosomal binding affinity than earlier tetracyclines.⁹⁸ In addition to these findings, a study by Yamaguchi *et al.* concluded that DMG-DMOT (**17**) is not recognised by the Tet(B) antiport protein and it can be assumed that it is this property that allows glycylcyclines to overcome this form of bacterial resistance.⁹⁹ However, mutation studies performed on Tet(B) in 1994 demonstrated the possibility of the evolution of resistance to glycylcyclines, as

laboratory mutated proteins conferred a higher level of resistance to DMG-MINO (**10**) than the wild-type.¹⁰⁰ Fortunately, the promising *in vitro* activity of these compounds was mirrored by their *in vivo* activity against acute lethal infections in mice.⁷¹ However, a clinical drawback in the use of these compounds was noted; the compounds' efficiency when administered orally was significantly lower when compared with intravenous injection.⁷¹ Despite this slight disadvantage, the discovery of these compounds was a major advance in the field and represented a new hope for the treatment of lethal multi-drug resistant infections.

1.5.3.2 SAR studies

In 1999 an SAR study was published by Sum and Peterson, who had been working on the glycyclcycline project at Wyeth-Averest Research (formerly Lederle Laboratories).¹⁰¹ In this study a number of analogues of 9-glycyclamidominocycline (Figure 1.11) were synthesised and their antibacterial properties investigated.

Figure 1.11 General synthetic route to 9-glycyclamidominocycline analogues



The initial series of compounds was synthesised from 9-(2-bromo)acetamidominocycline (**18**, Figure 1.10, *vide supra*) by reaction with various secondary amines. The azetidine and piperidine derived compounds displayed activities similar to that of DMG-MINO (**16**), while the pyrrolidinyl compound exceeded its activity by up to a factor of four against resistant [Tet(C)] *E. coli* strains and up to a factor of two against resistant *S. aureus* [Tet(M)] strains. The conclusion was drawn that steric bulk associated with the glycyclamido moiety played a

role in their activity. Interestingly, imidazolyl, morpholinyl and thiomorpholinyl derivatives were inactive against Gram-negative bacteria and only mildly active against the Gram-positive bacteria tested. This pointed towards the requirement of a certain level of lipophilicity in the amino moiety. The activity of **18** was also tested for comparison and found to have good activity against Gram-positive bacteria but no activity against Gram-negative bacteria, indicating that the amino moiety is essential for the action of these compounds against Gram-negative bacteria.

A second series of compounds synthesised from the reaction of **18** with a range of primary amines was also designed. Biological testing of this series confirmed the relationship between activity against Tet resistant bacteria and lipophilicity; the methylamino derivative was significantly less active than the dimethylamino product (DMG-MINO). Increasing the lipophilicity of the molecule seemed to have a positive effect on the compounds' action against bacteria expressing all *tet* genes; however the limit was found to be a four carbon amine as the *n*-hexylamine and undecylamine derivatives exhibited decreased activity when compared with butylamine derived analogues.

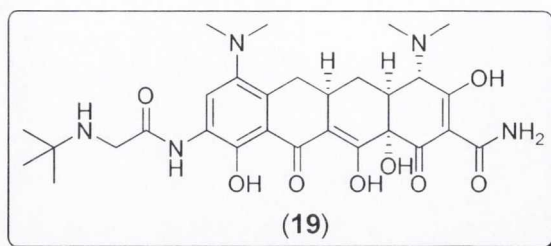
An optimal combination of lipophilic and steric considerations was discovered in the form of the *t*-butylamine derivative, GAR-936 (tigecycline), where R₁ = *t*-butyl and R₂ = H (Figure 1.11, *vide supra*). This compound was found to be the most active against a wide range of resistant bacteria expressing both efflux proteins and ribosomal protection proteins, examples of activity are shown in Table 1.1.¹⁰¹

Table 1.1 Activity of tigecycline against tetracycline resistant bacteria

Entry no.	Bacterium, resistance mechanism	MIC (µg/mL)
1	<i>E. coli</i> , Tet(D)	0.12
2	<i>E.coli</i> , Tet(M)	0.12
3	<i>S. aureus</i> , Tet(K)	0.5
4	<i>S. aureus</i> , Tet(M)	0.12

1.5.3.3 Tigecycline

Figure 1.12 Tigecycline (Tygacil)



Tigecycline (**19**, GAR-936) (Figure 1.12) was approved by the US FDA (Food and Drugs Administration) for use in human and veterinary medicine in June 2005 and was marketed by Pfizer under the trade name Tygacil®.¹⁰² Prior to this, extensive *in vitro* and *in vivo* testing was performed. The activity of tigecycline lies in its ability to inhibit bacterial protein synthesis by binding the 30S ribosomal subunit with high affinity, while remaining unaffected by efflux protein and ribosomal protection protein forms of resistance.^{101, 103, 104}

In an extensive study by Petersen *et al.*,¹⁰⁴ the antibacterial efficacy of tigecycline (**19**) were determined in tests against a wide-ranging distribution of bacteria, including strains bearing characterised resistance determinants and newer clinical isolates arising at the time. During *in vitro* tests against *E. coli* strains expressing Tet(A) and Tet(C) efflux proteins, tigecycline (**19**) was found to be the most active of any agent tested ($\leq 0.5 \mu\text{g/mL}$). It was also the most active, matching DMG-MINO (**16**), against *S. aureus* strains where resistance was the result of Tet(M) (ribosomal protection) expression, with MICs of 0.25-0.5 $\mu\text{g/mL}$.¹⁰⁴

When tested against emerging clinical isolates of MRSA, *S. pneumoniae* and *Enterococcus* strains, including vancomycin resistant isolates, tigecycline (**19**) was found to be the most active, MIC $\leq 0.5 \mu\text{g/mL}$. In general, this study found that with MICs of $\leq 1 \mu\text{g/mL}$ tigecycline (**19**) was more effective against the staphylococcal and enterococcal isolates tested than the clinically available alternatives; vancomycin, ciprofloxacin and erythromycin. When administered intravenously in a single dose *in vivo* (murine model), tigecycline (**19**) retained its *in vitro* activity, however it was found to be 60 times less active when administered orally.¹⁰⁴ With resistance among *S. pneumoniae*, MRSA and *Enterococcus* strains becoming increasingly frequent further assessment of tigecycline (**19**) was merited.

Clinical data obtained from a study of the treatment of complicated skin infections and complicated intra-abdominal infections, involving 800 and 1000 adults respectively, proved tigecycline (**19**) to be as effective as the leading drug combinations available at the time.¹⁰⁵ The advantages of tigecycline (**19**) over other equally efficient antibiotic combinations were deemed to be its relative safety with relation to major organ damage and its use as an alternative for patients with β -lactam allergies.¹⁰² And so, in 2005, Tygacil® was introduced to the market and currently remains a key line of defence against bacterial resistance. However, after only 2 years in clinical use, the identification of the first tigecycline resistant bacterial strains were reported.^{106, 107}

1.6 Current applications of tetracycline antibiotics

1.6.1 Human and veterinary medicine

Tetracyclines are used as antibiotics to treat a wide variety of infections in humans. In Europe, minocycline (**3**) is used for the treatment of acne vulgaris¹⁰⁸, while doxycycline (**2**) is approved for the treatment of a wide array of infectious diseases, including: respiratory tract infections, urinary tract infections, sexually transmitted diseases and ophthalmic infections.¹⁰⁹ Doxycycline (**2**) is also indicated as a prophylactic agent for the prevention of scrub typhus, travelers' diarrhea (enterotoxigenic *Escherichia coli*), leptospirosis, malaria and cholera.¹⁰⁹ Tigecycline (**19**), however, is reserved for the treatment of complicated infections of the skin and soft tissue and complicated intra-abdominal infections.¹¹⁰

Tetracyclines, such as oxytetracycline, doxycycline, chlorotetracycline and oxytetracycline, are widely used in veterinary medicine. Their main clinical indications include the treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis.³¹ They are approved for use in animal species such as beef cattle, pigs, sheep, goats, horses, dogs, cats, poultry, rabbit and fish; however they can only be administered intravenously in ruminants due to the destruction of ruminal microflora when given orally.³¹ The growth promoting properties of tetracyclines were discovered by Stockstad *et al.* in 1949. Consequently, they are widely applied at subtherapeutic levels in animal husbandry.⁴

1.6.2 Recent and prospective applications

The activity of tetracycline antibiotics is not restricted to their antimicrobial action alone. Several other advantageous properties of this class of compounds have been discovered over the past 60 years and there are currently over 130 clinical trials involving these compounds ongoing.¹¹¹ Clearly, with this number of varying trials, the tetracyclines are an extremely interesting class of compounds with significant application in medicinal chemistry. The subsections below highlight some of the potential uses of tetracyclines in the treatment of a wide array of diseases.

1.6.2.1 Neurological protection

In 1994 Clark *et al.* discovered a new and fascinating feature of tetracycline antibiotics: one member of the family, doxycycline (**2**), was capable of neurological protection.¹¹² This study provided evidence that the neurological function of rabbits was protected by doxycycline (**2**) when focal ischemia (a lack of blood flow to a particular region of the brain caused by the blockage of a cerebral blood vessel) was induced. A subsequent *in vivo* study by Yranheikki *et al.* involving global brain ischemia (a lack of blood flow to the entire brain, commonly caused by cardiac arrest) in rats proved that both doxycycline (**2**) and minocycline (**3**) had neuroprotective properties and inhibited microglial activation.¹¹³ Microglia are cells that act as an immune system in the central nervous system, removing infectious agents and prompting the cell death of compromised neurons by the induction of caspase-1 (Cap-1) and inducible nitric oxide synthase (iNOS). Overactivation of microglia is a common feature of neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD), leading to the compromise of neurons and the oligodendroglial cells that insulate them.¹¹⁴

Minocycline (**3**) is considered to be of more clinical importance with respect to neurological protection as it crosses the blood-brain barrier (BBB) at a rate that is five times higher than that of doxycycline (**2**).¹¹⁵ Minocycline (**3**) has also been shown to directly prevent tissue inflammation caused by ischemia, by inhibition of cyclooxygenase-2 (COX-2) and iNOS as well as indirectly prevent inflammation by inhibiting microglia.¹¹³ The inhibition of matrix metalloproteinases, that alter BBB permeability to allow T-cells into the central nervous system (CNS) where they degrade oligodendroglia, by minocycline (**3**) has also been

reported by Yong *et al.* in an effort to highlight the potential of minocycline (**3**) as a treatment for multiple sclerosis (MS).¹¹⁶

Minocycline (**3**) prevents cell apoptosis by blocking the release of pre-apoptotic factors and apoptosis-inducing factors from the mitochondria.¹¹⁴ These factors are a signal to the cell to induce cell death and are released during excitotoxic events (cell death due to overexposure of neuronal cells to glutamate receptor agonists). Excitotoxic events are features of many CNS diseases, such as AD, HD, PD, MS, and amyotrophic lateral sclerosis (ALS, motor neuron disease in UK).¹¹⁷⁻¹¹⁹ Interestingly, while minocycline (**3**) has been shown to have beneficial effects in several diseases of this type, phase III clinical trials using minocycline for ALS treatment revealed that it speeds up the progress of this disease rather than attenuating it.¹²⁰⁻¹²²

The diverse neuroprotective properties of minocycline (**3**) have made it a popular candidate for pre-clinical and clinical trials of incurable CNS diseases, such as AD, HD, PD, and MS.¹²³⁻¹²⁶ The results of these trials are summarised below.

1.6.2.1.1 Ischemia

Ischemia is the restriction of blood supply to a specific area of the body, resulting in either the damage or dysfunction of tissues. Ischemia in the CNS can be acute (rapid) or chronic (long-lasting). Minocycline (**3**) has been involved in a number of ischemia trials with both humans and animal models. In animal models of global ischemia it has been shown to increase the survival of neurons, attenuate microglial activation, and reduce CAP-1 levels.¹²⁷ In focal ischemia animal studies, minocycline (**3**) reduces apoptosis, improves neurologic outcomes,¹¹³ reduces microglial activation and protects schwann cells.^{128, 129} In human clinical trials of acute stroke, patients administered with minocycline (**3**) demonstrated an average lowering of the NIHSS (National Institute of Health stroke scale: a scale of 0-4 for assessing stroke patients at which 0 is allocated to a patient with minimal/no damage to neurological function and 4 is allocated to patients with severe damage) and mRS (modified Rankin scale: a scale of 0-6, whereby 0 represents no symptoms of stroke and 6 represents death) by reducing brain injury.

1.6.2.1.2 Alzheimer's disease

Alzheimer's disease (AD) is a terminal, progressive, neurodegenerative disease characterised by neuritic plaques composed of amyloid fibrils and neurofibrillary tangles which result in significant impairment of neurological functions and eventually death.¹³⁰ In rat and mouse models minocycline (**3**) has been shown to suppress the microglial production of necrotic factors, halt cognitive impairment and inhibit neuronal cell death.^{131, 132} *In vitro* testing on human microglial cells showed that the administration of minocycline (**3**) resulted in a significant reduction in the production of pro-inflammatory cytokines.¹³³ Despite these promising results, no human clinical trials involving minocycline with this disease as a target have been performed to date.

1.6.2.1.3 Huntington's disease

Huntington's disease (HD) is a genetic, terminal neurodegenerative disease. The early symptoms of the disease include involuntary twitches or movements, leading eventually to loss of motor control, dementia and death.¹³⁰ In murine models minocycline (**3**) has been shown to inhibit CAP-1 and CAP-3 expression and reduce iNOS activity, all of which signal cell death.¹³⁴ When administered in combination with pyruvate, minocycline (**3**) inhibits inflammation, oxidative damage and the loss of neurons.¹³⁵ In an open label clinical trial on 11 human volunteers, minocycline (**3**) was found to improve the UHDRS (unified Huntington's disease rating scale: which assigns values to patients on the basis of assessment of clinical features) after six months treatment and stabilise the rating over a three year period, so that no further deterioration was noted.¹³⁶ This study gives hope for the treatment of Huntington's disease, the normal pathology of which is continual deterioration until death, on average twenty years after diagnosis.¹³⁷

1.6.2.1.4 Parkinson's disease

Parkinson's disease (PD) is a degenerative movement disorder caused by the loss of neurons in the substantia nigra that affects 1% of the world's population over 60 years of age. Four main symptoms of the disease present as a resting tremor, rigidity, postural instability and bradykinesia (a slowed ability to start and continue movement).¹³⁸ Dementia is also a

common symptom of the disease and a study of end-stage patients found that over 80% were suffering with dementia.¹³⁹ In animal models of PD, minocycline (**3**) was shown to inhibit microglia, reduce the formation of neurotoxic nitrotyrosine and ease the rate of neurodegeneration.¹⁴⁰ Minocycline (**3**) is currently in phase II clinical trials for use in conjunction with creatine as a treatment of PD.¹⁴¹

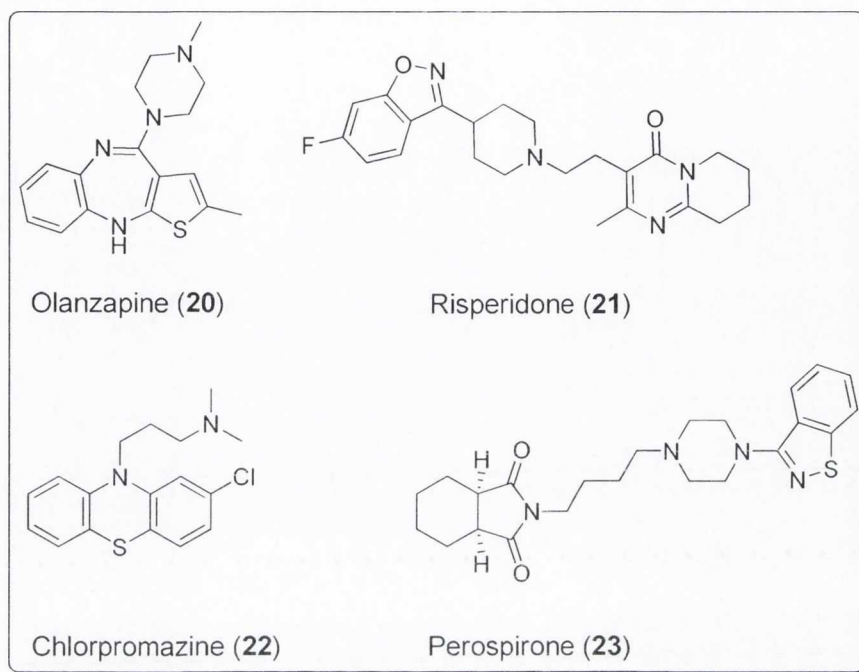
1.6.2.1.5 Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory disorder of the brain and spinal cord which leads to myelin and axon damage. The symptomatic effects of this degenerative disease include motor function, sensory, visual and autonomic nervous system damage with a mortality rate of about 65%.¹⁴² In a study in 2002 minocycline (**3**) was shown to inhibit matrix metalloproteinase activity and prevent T-cell migration to the CNS.¹¹⁶ Minocycline is currently in phase II clinical trials for the treatment of MS.^{143, 144}

1.6.2.2 Schizophrenia

Schizophrenia is defined as a heterogeneous clinical disorder characterised by severely impaired cognitive process, personality disintegration, affective disturbances and social withdrawal.¹³⁰ This disease affects 0.5-1.0 % of the general population and while current therapy using antipsychotics such as olanzapine (**20**), risperidone (**21**), chlorpromazine (**22**) and perospirone (**23**, Figure 1.13, *vide infra*) can be effective in attenuating or lessening the symptoms of the disease, resistance to antipsychotic drugs is reported in up to 25% of all cases.¹⁴⁵

Figure 1.13 Antipsychotic drugs



In 2007 Miyaoka *et al.* serendipitously discovered the beneficial effects of minocycline (**3**) on schizophrenia patients while treating their infections. They noticed a marked improvement in the schizophrenic symptoms of two male patients while they were being treated with antipsychotics and minocycline (**3**).¹⁴⁶ When the course of minocycline was completed the patients deteriorated to their prior states and upon re-administration of minocycline the symptoms again eased.¹¹⁵ Miyaoka *et al.* decided to investigate this phenomenon, building upon the work of Levkovitz *et al.*¹⁴⁷ and Hashimoto *et al.*¹⁴⁸ which had demonstrated in animal studies that minocycline (**3**) could reverse cognitive visual spatial memory deficits and sensorimotor gating effects induced *in vivo*¹⁴⁷ and could attenuate hyperlocomotion, PPI (pre-pulse inhibition) and lower extracellular dopamine when these symptoms were induced to mimic schizophrenia.¹⁴⁸ In a clinical trial undertaken by Miyaoka *et al.*,¹⁴⁹ twenty two patients whom were non-responsive or resistant to their current treatment were administered with minocycline (**3**) plus their current treatment for a period of four weeks. The PANSS (positive and negative syndrome scale) was used to evaluate the improvement in the patients, which ranged from 40% to 52% after four weeks treatment with minocycline (**3**) plus an atypical antipsychotic drug. In a larger study by the same investigators spanning twenty eight weeks,

patients treated with minocycline (**3**) plus an antipsychotic were shown to have greater improvement in cognitive functions, negative symptoms and depression and reduced weight gain related to antipsychotic drug treatment than those treated with an antipsychotic drug plus a placebo.¹⁵⁰

These studies clearly demonstrate the beneficial effects of minocycline (**3**) in the treatment of schizophrenia, especially with relation to negative symptoms, such as apathy, anhedonia and alogia, which were previously untreatable. The mode of action of minocycline (**3**) in schizophrenia has not yet been fully uncovered, however it is postulated that the prevention of apoptosis and inflammation (discussed in Section 1.6.2.1) could be involved as they are common features of neurodegeneration in schizophrenia.¹¹⁵

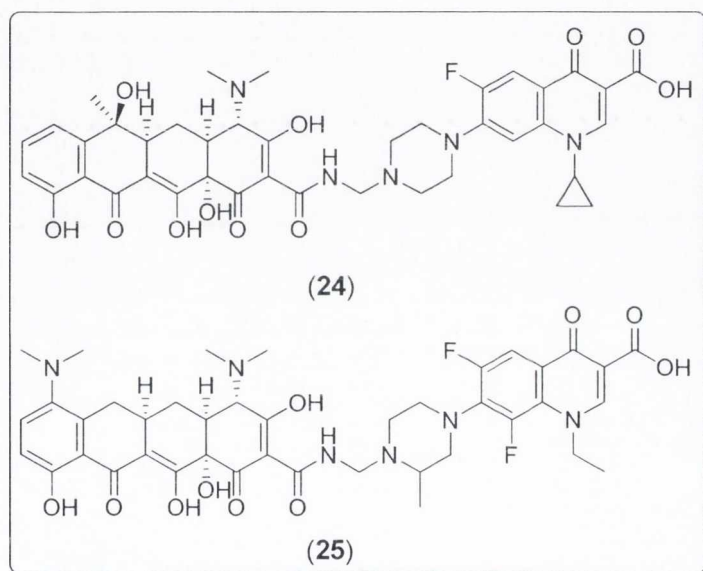
1.6.2.3 HIV

HIV (human immunodeficiency virus), causing AIDS (acquired immunodeficiency syndrome), is one of the most serious health problems the world faces today, particularly in third world and developing countries. The number of HIV sufferers worldwide has risen year on year since 1990 and although infection rates have slowed, due to increased education on the disease, they have by no means peaked.¹⁵¹ Data obtained for 2008 calculated that 33.4 million people are infected with the virus worldwide, of which 2 million cases were fatal that year alone.^{151, 152} Current therapy, using a combination of reverse transcriptase and protease inhibitors, has improved the prognosis for patients with HIV/AIDS; however significant side effects, cost, and the emergence of resistant viral strains complicates treatment. There is, as such, cause for the design of new anti-HIV therapies which might offer lower costs to patients, fewer side effects and circumvent resistance mechanisms.

The integrase protein (IN), which catalyses the assimilation of HIV DNA into human chromosomes, is frequently the subject of study as it is crucial to the viral replication cycle and has no similar counterpart in humans. In 2007, Sriram *et al.* published the synthesis of a new series of tetracycline derivatives coupled to various fluoroquinolones which were active inhibitors of HIV-1 integrase.¹⁵³ Their study was built previous work by Neamati *et al.* which showed that some tetracycline derivatives were reasonably active IN inhibitors and that this inhibition was increased by bulk at the carboxamide moiety at position 2 (Figure 1.5, *vide supra*).¹⁵⁴ Sriram *et al.* synthesised new tetracycline analogues with a fluoroquinolone

attached at the piperazine moiety, and these compounds displayed a range of antimycobacterial and IN inhibition activities. The most active compounds produced, **24** and **25** (Figure 1.14), exhibited promising anti-HIV activities, $EC_{50} = 8.4 \mu\text{M}$ and $5.2 \mu\text{M}$ respectively, and low cytotoxicity. These tetracycline derivatives were also evaluated as antimycobacterial agents with relation to *Mycobacterium tuberculosis*, the number one cause of co-infection in patients with HIV-1.¹⁵⁵ Compound **24** afforded the lowest MIC ($0.2 \mu\text{M}$). This, coupled with its anti-HIV activity revealed **24** to be a promising new, dual action, anti-HIV tetracycline compound.

Figure 1.14 Anti-HIV tetracyclines



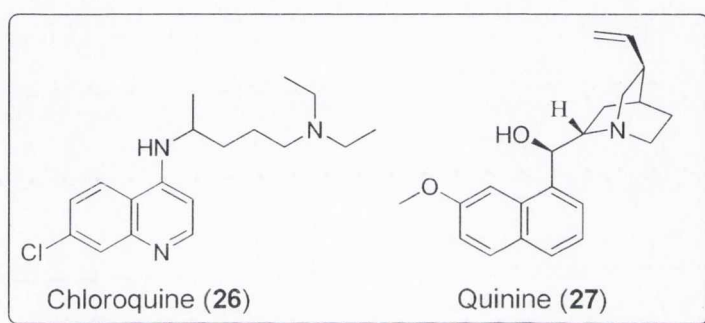
1.6.2.4 Malaria

Malaria is a parasitic disease caused by protists of the genus *Plasmodium*, of which five species cause infection in humans.¹⁵⁶ The parasites are transmitted to humans by the bite of infected female mosquitoes and within the first thirty minutes the *Plasmodium* parasite infects the hepatic system. Here it multiplies asexually, with a single parasite being able to produce thousands of progeny. These progeny are then released into the bloodstream where they infect erythrocytes and multiply further, bursting from the cell and continuing the cycle of infection.¹⁵⁶ This causes mild symptoms in the early stages of the disease, such as fever/chills, headache, fatigue and nausea and if untreated can lead to fatal complications including

cerebral infection, pulmonary oedema, anaemia, kidney failure, reduced liver function and 'blackwater fever' (a severe lack of functioning red blood cells).^{157, 158}

WHO (World Health Organisation) statistics for 2008 claim there are 243 million cases of malaria worldwide with 85% of cases being in the African region. Malaria was the cause of 863,000 deaths in 2008, 89% of which were in the African region.¹⁵⁹ Because of the prevalence of this disease in poorer countries, in particular sub-Saharan Africa, there is a great pressure to provide inexpensive and effective treatments.

Figure 1.15 **Anti-malarial drugs**

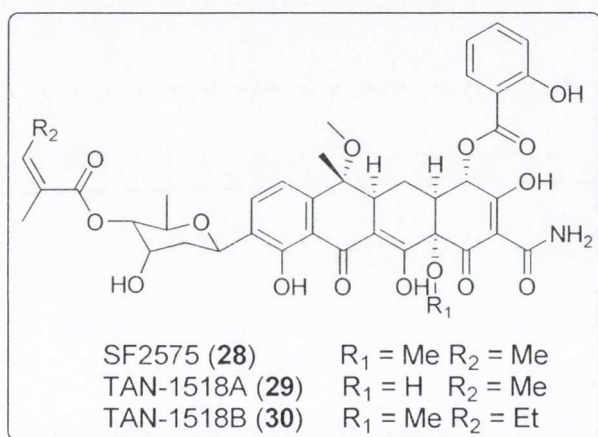


While chloroquine (**26**, Figure 1.15) has remained the treatment of choice for malaria since its discovery, there is a growing problem with resistance and the majority of malarial zones now harbour chloroquine resistant strains.¹⁶⁰ It has been known since 1987 that doxycycline (**2**) is a cheap, effective, low dose treatment for malaria¹⁶¹ and it is advised for use in uncomplicated malarias and chloroquine resistant malarias in conjunction with quinine (**27**, Figure 1.15). Doxycycline (**2**) can also be used as a prophylactic agent against malaria¹⁶² as it inhibits the genome replication of the apicoplast, an organelle which contains its own genome coding for ribosomal RNAs, tRNAs and proteins. The apicoplast houses enzymes involved in fatty acid synthesis, a pathway for isoprenoid biosynthesis and heme-biosynthetic pathways.¹⁶³ When treated with doxycycline (**2**), the progeny of malarial parasites express abnormal apicoplasts and cannot develop into viable parasites, thus preventing the progression of the disease in humans.¹⁶³

1.6.2.5 Cancer

In 1992 a unique tetracycline was isolated from *Streptomyces* sp SF2575. Hatsu *et al.* isolated this compound during a screening programme to find new antibiotics with anti-tumour activity.¹⁶⁴ The isolated tetracycline aglycon,¹⁶⁵ SF2575 (**28**, Figure 1.16), was found to have weak activity against Gram-positive bacteria and no activity against Gram-negative bacteria. It was, however, extremely potent against P388 leukaemia cells *in vitro*, $IC_{50} = 7.5$ ng/mL. When tested *in vivo* in a murine model using mice implanted with P388 leukaemia, intraperitoneal injection of SF2575 at 10 mg/kg resulted in a 91% increase in life span.

Figure 1.16 Anti-cancer tetracyclines



TAN-1518A (**29**) and TAN-1518B (**30**, Figure 1.16), isolated from *Streptomyces* sp AL-16012 in 1994,¹⁶⁶ were also found to possess anti-cancer activity. They specifically inhibited Topoisomerase I *in vitro*, a target currently used in anti-cancer therapies such as doxorubicin. In the case of TAN-1518A (**29**), inhibition of human HeLaS3 epitheloid carcinoma cell growth occurred at a concentration of 0.7 ng/mL. *In vivo* studies on murine Meth A fibrosarcomas using TAN-1518A (**29**) displayed its anti-tumour capabilities as it reduced tumour growth by 57% after 2 days (10 mg/kg daily administered intraperitoneally). These studies clearly show the potential of tetracycline aglycons such as these as new and potent anti-cancer agents.

1.7 Tetracycline chemistry

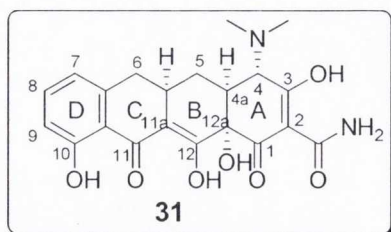
Over the last 50 years there has been a great deal of research into the chemistry of tetracycline antibiotics. A variety of modifications have been made to the naturally occurring structures in an attempt to make more active antibiotics, produce analogues that are active against resistant bacteria and tune their properties for the treatment of various ailments. While significant milestones have been achieved, it is important to note the dearth of chemical modifications that can be performed on this class of compounds when compared to any other. This is at least in part due to the inherent instability of many tetracycline compounds, the lack of reactivity in others and difficulties in purifying these structures.

This section is intended to highlight the most varied and important modifications that are possible within this class of chemicals and how these modifications have been used to advance the treatment of a wide range of disease by reviewing the literature since their discovery and patents published over the last twenty years.

1.7.1 D-Ring substitutions

Given the minimum structure of all tetracycline antibiotics (Figure 1.17), substitution at the D-ring by electrophilic aromatic substitution has been a popular method of producing tetracycline analogues. In the subsections below, the analogues are split into those substituted at positions 7 (Section 1.7.1.1), 8 (Section 1.7.1.2), 9 (Section 1.7.1.3) and 10 (Section 1.7.1.4).

Figure 1.17 **Sancycline: the minimum tetracycline structure required for activity**

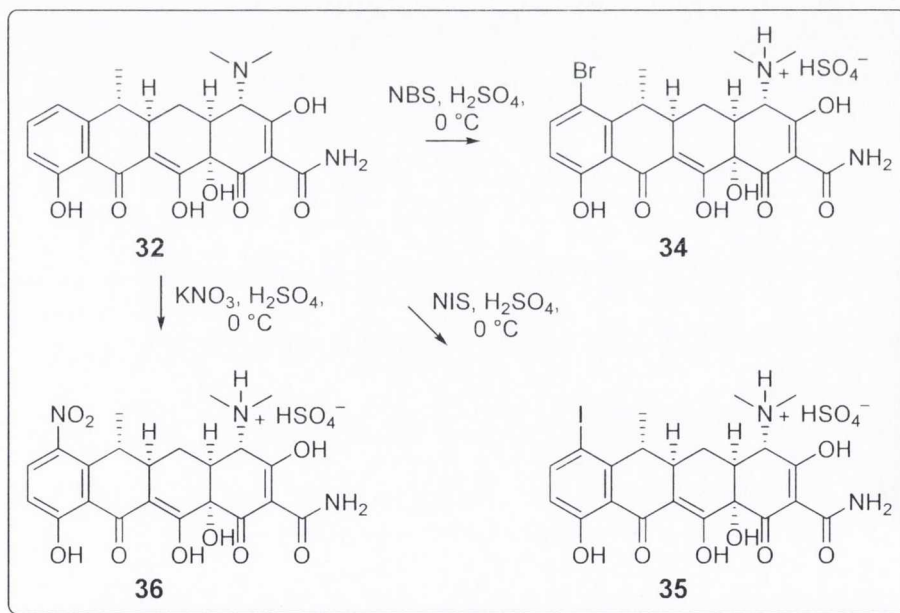


1.7.1.1 7-Substituted tetracyclines

In the early 1960's Boothe and co-workers embarked on a study of the electrophilic aromatic substitution of two similar tetracyclines, 6-deoxytetracycline (**32**, Scheme 1.1) and 6-demethyl-6-deoxytetracycline (**33**, Scheme 1.2, *vide infra*), to probe the chemical reactivity of the two and produce more active tetracycline analogues.¹⁶⁷⁻¹⁶⁹ The novel tetracyclines synthesised were tested for activity against *Staphylococcus aureus* and the activities collected by turbidimetric measurement. The reactions of 6-DOT (**32**) are summarised in Scheme 1.1.

The reaction of 6-DOT (**32**) with *N*-bromosuccinimide (NBS) or *N*-iodosuccinimide (NIS) yielded compounds **34** and **35** respectively (Scheme 1.1).¹⁶⁷ Compound **34** was found to be 1.4 times as active against *Staphylococcus aureus* than tetracycline.¹⁶⁹ The insertion of a nitro moiety furnished 7-nitro-6-DOT (**36**) which was 2.4 times as active as tetracycline (Scheme 1.1).¹⁶⁸

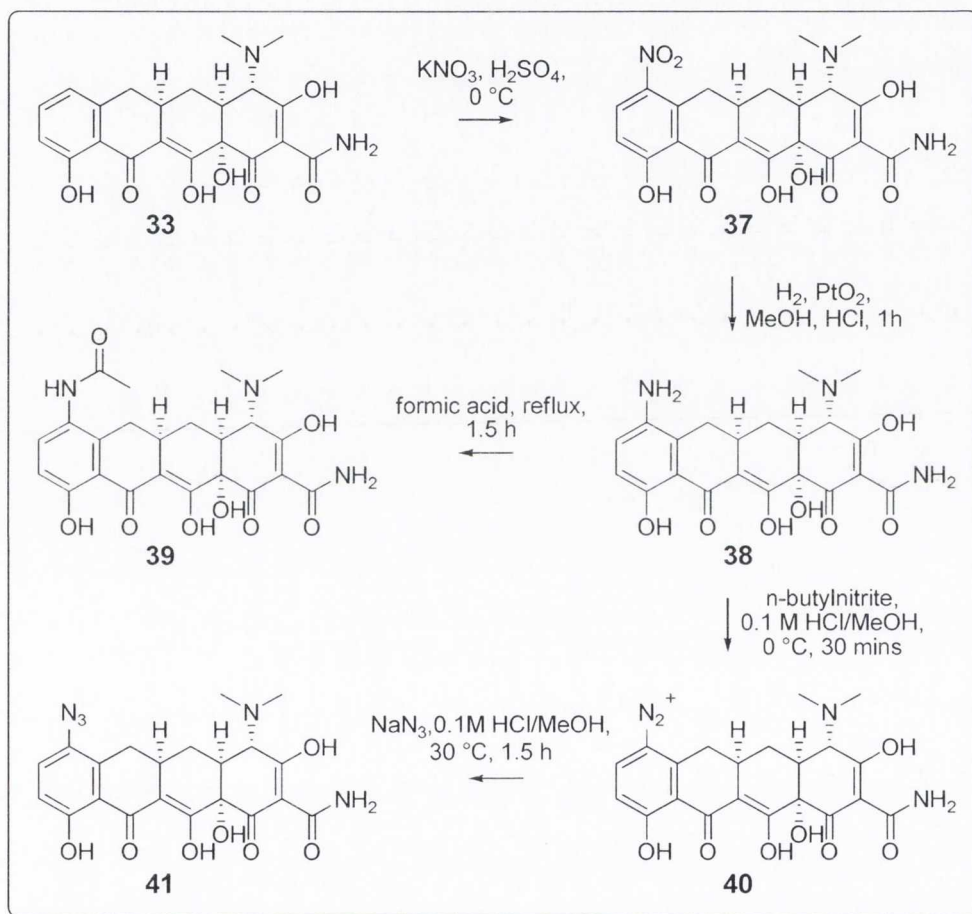
Scheme 1.1 7-Substituted 6-DOTs



Similar transformations were performed on 6-DMDOT (**33**) yielding more active analogues, with 7-nitro-6-DMDOT (**37**) displaying 6.4 times the activity of tetracycline.^{168, 169} Compound **37** was reduced by hydrogenation to yield amino-6-DMDOT (**38**) resulting in a considerable loss of activity. Acetylation of **38** produced **39** which displayed further reduced

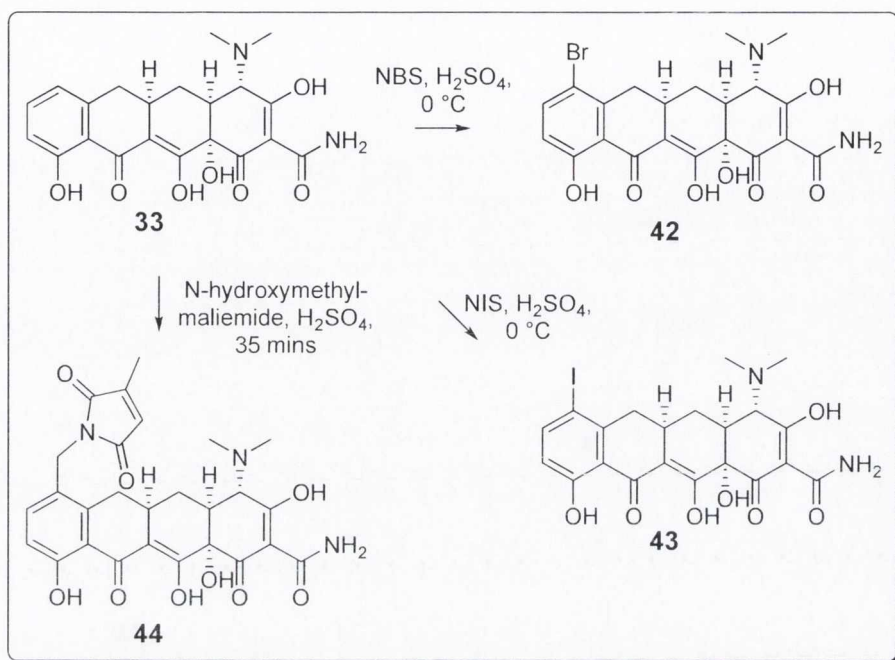
activity.¹⁶⁸ Compound **38** was converted to the diazo analogue **40** which was only 0.2 times as active as tetracycline. Further substitution with sodium azide furnished 7-azido-6-DMDOT (**41**), which was 1.5 times as active as tetracycline (Scheme 1.2).¹⁶⁹

Scheme 1.2 **7-Substituted 6-DMDOTs via the amino substituted intermediate**
31



Halogenation reactions using NBS and NIS were also performed yielding 7-bromo-6-DMDOT (**42**) and 7-iodo-6-DMDOT (**43**, Scheme 1.3, *vide infra*). Notably, 7-bromo-6-DMDOT (**42**, Scheme 1.3, *vide infra*) displayed 1.4 times the activity of tetracycline.^{168, 169} Boothe *et al.* reported the Tscherniaie-Einhorn reaction for imidomethylation of 6-deoxytetracyclines. Several analogues were produced, the most active of these being compound **44** (Scheme 1.3) which displayed seven times greater activity than tetracycline.¹⁷⁰

Scheme 1.3 **7-Substituted 6-DMDOTs**



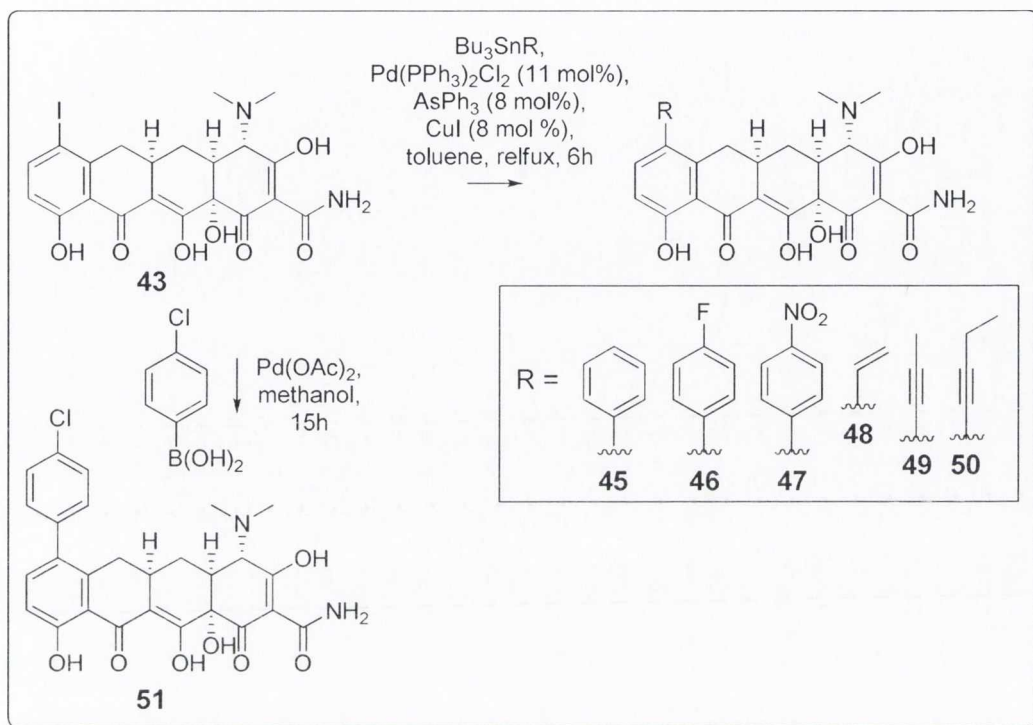
These studies provided useful insights into the SAR of deoxytetracyclines. In the case of both 6-DOT (32) and 6-DMDOT (33) electron withdrawing groups, notably bromo and nitro groups, improved antibiotic activity. It can be suggested tentatively therefore, that the insertion of electron withdrawing groups at position 7 in tetracyclines improves their antibiotic activity. Compound 44, however, does not conform to this trend, although it was not ascertained whether this compound behaved as a typical or atypical tetracycline. The relative activities of some of the compounds discussed in this section are displayed in Table 1.2 (*vide infra*) for clarity.

Table 1.2 **Activity of 6-DOTs and 6-DMDOTs vs. tetracycline**

Entry no.	Compound no.	% Activity vs. oxytetracycline
1	34 (Scheme 1.1)	140
2	36 (Scheme 1.1)	240
3	37 (Scheme 1.2)	640
4	40 (Scheme 1.2)	20
5	41 (Scheme 1.2)	150
6	42 (Scheme 1.3)	140
7	44 (Scheme 1.3)	700

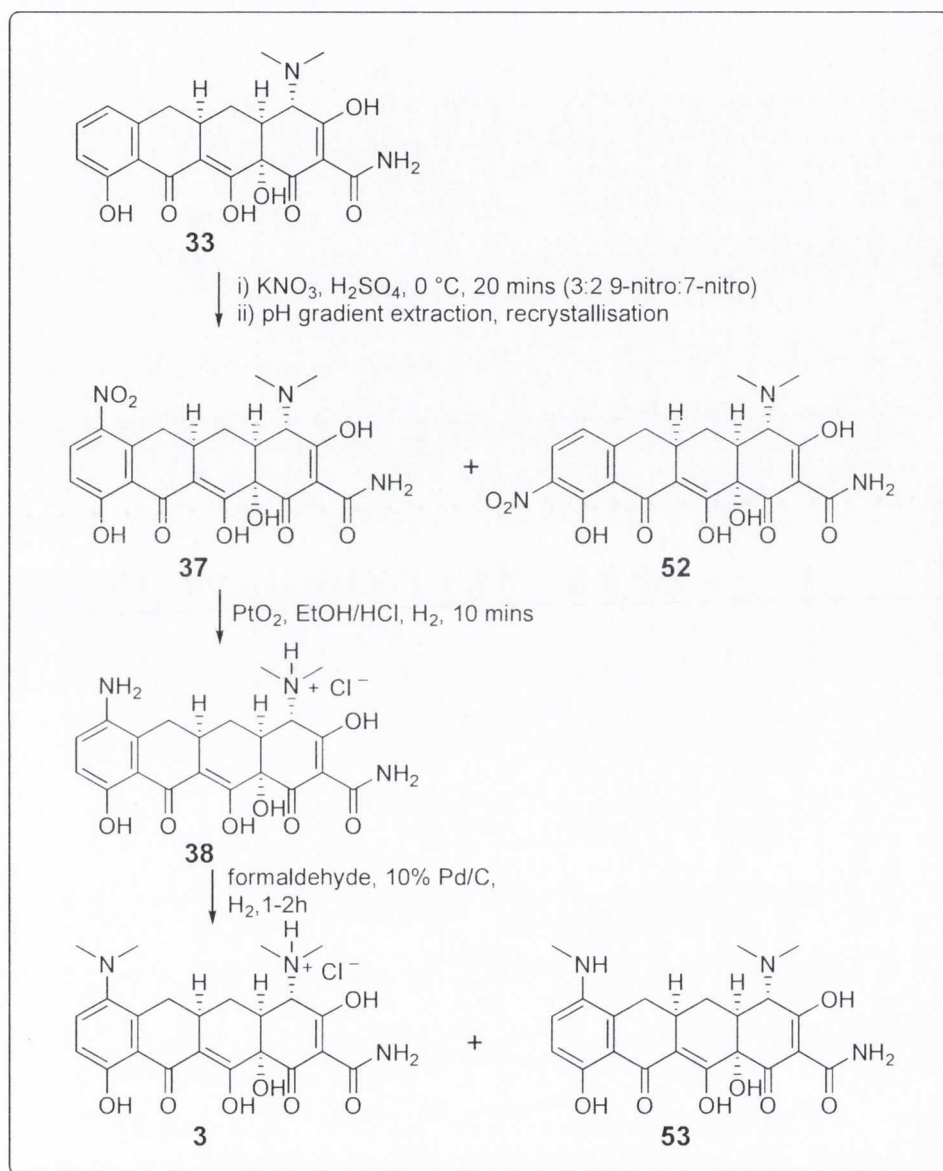
Starting from **43** (Scheme 1.3, *vide supra*), Koza produced a wide array of new tetracycline analogues using palladium catalysed cross coupling reactions. Employing both Stille and Suzuki coupling procedures with a variety of palladium catalysts Koza produced novel tetracycline analogues with new carbon-carbon bonds at position 7, despite the polyhydroxylated structure of the compound (Scheme 1.4).¹⁷¹ Compound **51** (Scheme 1.4) was found to be between 2 and 500 times more active than doxycycline against a variety of *S. aureus*, *E. coli* and *E. hirae* strains, with MICs of <1 µg/mL.¹⁷² The 7-ethynyl substituted series also produced some analogues with excellent antibiotic activity, in particular compounds **49** and **50** (Scheme 1.4). These compounds displayed excellent potencies against *S. aureus*, *E. coli* and *E. hirae*.

Scheme 1.4 **The use of Pd(0) catalysed cross coupling reactions to install functionality at position 7**

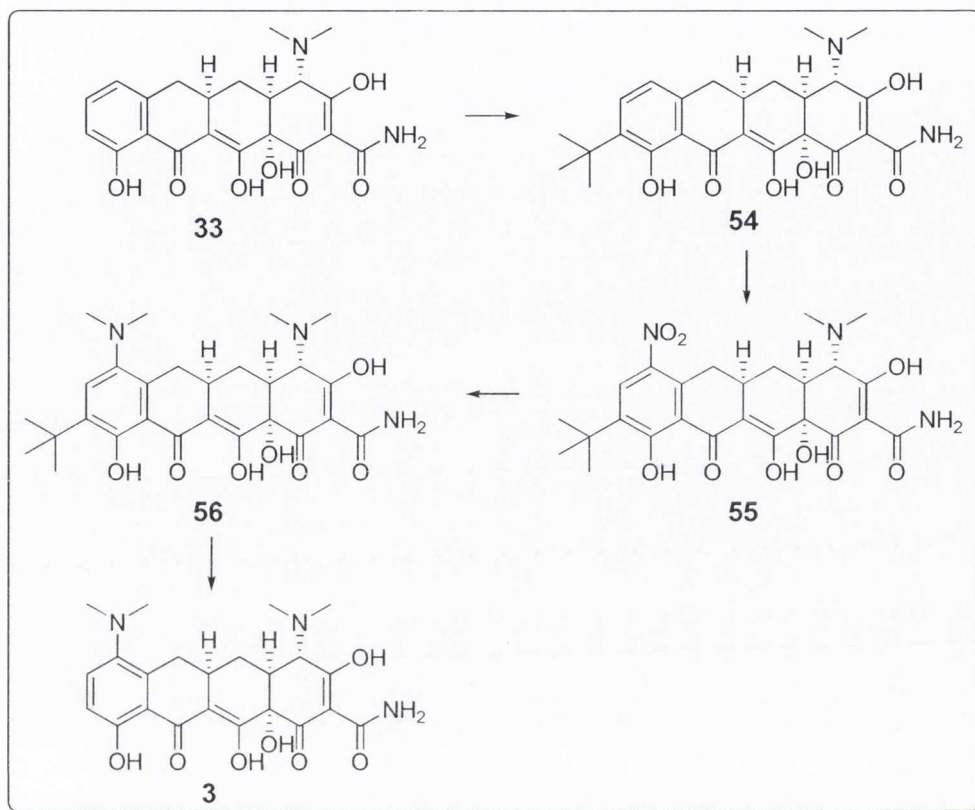


Koza and Nsiah published the synthesis of 7-amino substituted 6-DMDOT using similar chemistry.¹⁷³ The formation of aminostannanes *in situ*, by transamination of (*N,N*-dimethylamino)trimethyltin, and subsequent reaction with 7-iodo-6-DMDOT (**43**) under Stille coupling conditions afforded a variety of analogues. This procedure also provided an attractive route for the synthesis of minocycline (**3**) from 6-DMDOT in two steps (Scheme 1.7, *vide infra*), compared to the original three step synthesis previously used by The American Cyanamid Company (Scheme 1.5, *vide infra*),¹⁷⁴⁻¹⁷⁷ despite being non-regiospecific and requiring lengthy purifications, and the current synthesis used in industrial manufacturing (route details undisclosed, Scheme 1.6, *vide infra*).¹⁷³

Scheme 1.5 **Original route to minocycline designed by The American Cyanamid Company**¹⁷⁴⁻¹⁷⁷

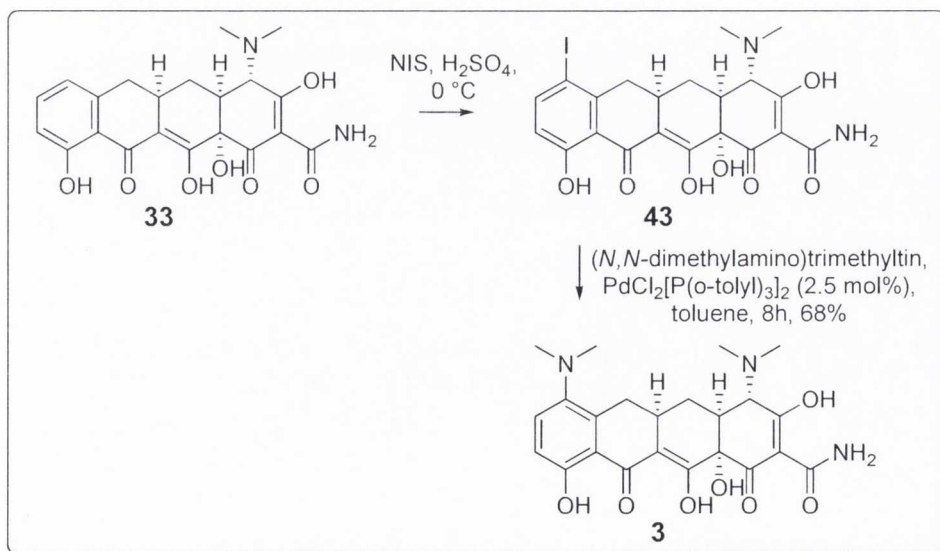


Scheme 1.6 Industrial route to minocycline currently used by Pfizer¹⁷³



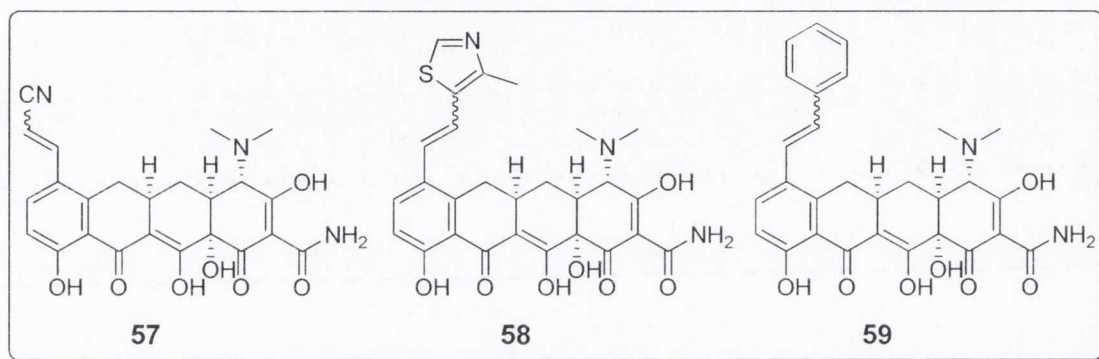
route details undisclosed

Scheme 1.7 Koza's route to minocycline (3)¹⁷³



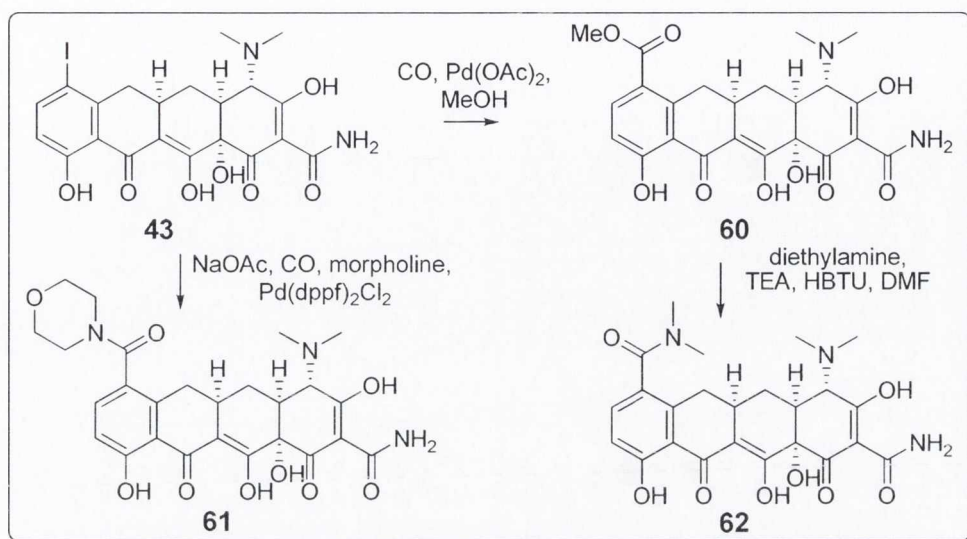
In 2003 Nelson *et al.*¹⁷⁸ reported the synthesis of a variety of 7-substituted 6-DMDOT analogues using palladium coupling chemistry. Novel analogues **57**, **58** and **59** (Figure 1.18) were obtained by reaction of **43** (Scheme 1.7, *vide supra*) with the appropriate olefin in the presence of Pd(PPh₃)₄, CuI, Pd(OAc)₂, and NEt₃ in acetonitrile at room temperature to 20 °C for 2 - 24 h. These three novel tetracycline compounds from the series displayed excellent activity against *S. aureus* and *E. hirae* strains during *in vitro* testing.¹⁷⁹

Figure 1.18 7-Vinyl derivatives of 6-DMDOT



Nelson *et al.* also reported a general procedure for the synthesis of 7-amido derivatives of 6-DMDOT *via* carbonylation with subsequent amide formation, albeit in low yields (Scheme 1.8, *vide infra*).^{172, 178}

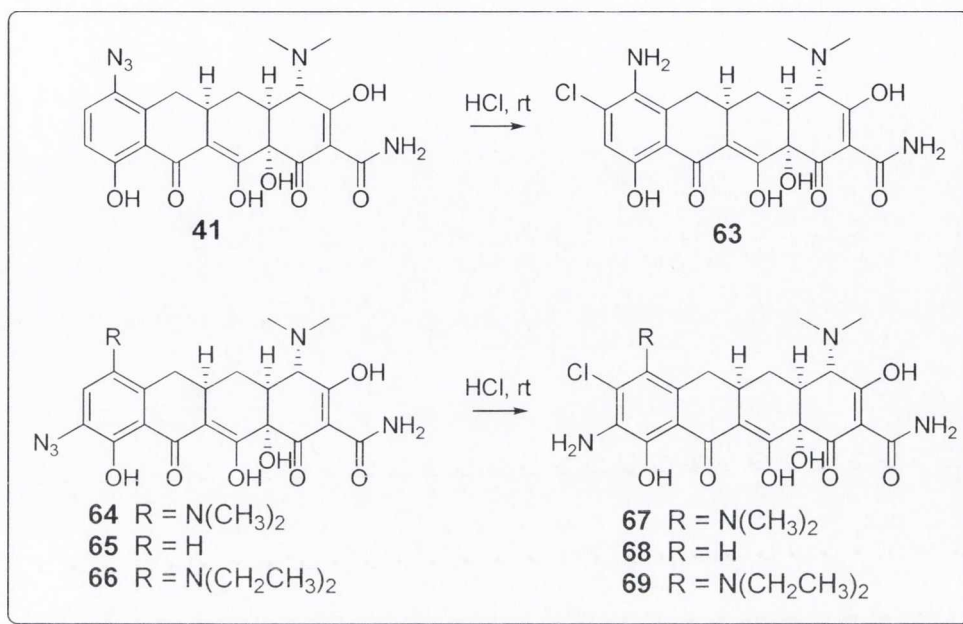
Scheme 1.8 **7-Carbonyl amide 6-DMDOTs**



1.7.1.2 8-Substituted tetracyclines

Given the *ortho/para* directing nature of the D-ring phenolic unit of tetracyclines, it is understandable that very little variation has been achieved at the 8-position. The first synthesis of 8-substituted tetracyclines from an intact tetracycline nucleus was not achieved until 1994, nearly 50 years after the compounds class' discovery. Sum *et al.* reported the synthesis of a series of 8-substituted tetracyclines by way of a rearrangement reaction from 7- and 9-azido tetracyclines (Scheme 1.9, *vide infra*).¹⁸⁰

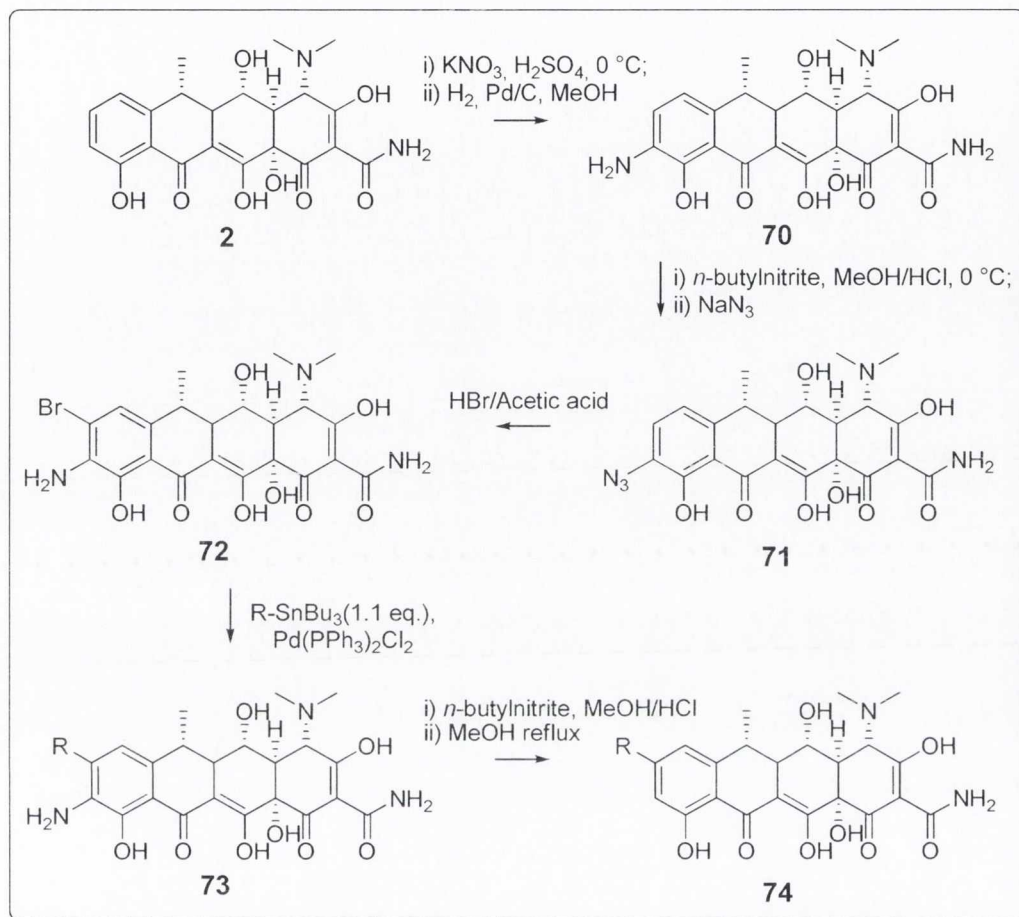
Scheme 1.9 Synthesis of 8-substituted tetracyclines



While the above method was applicable to a number of tetracyclines, the yield was greatly reduced (< 40%) when the minocycline derivative **64** was employed as the substrate for the rearrangement reaction. However, *in vitro* testing using the agar dilution method revealed the product of this reaction, **67** (Scheme 1.9), to be twice as active as minocycline against *S. aureus* strains expressing Tet(K) efflux proteins and Tet(M) RPP resistance, with MICs of 0.25 µg/mL and 1 µg/mL respectively. Gratifyingly, it completely inhibited the growth of *E. coli* strains expressing Tet(M) RPP's. During *in vivo* testing using an acute lethal infection in mice with *S. aureus smith*, **67** proved to be among the most potent compounds, displaying an ED₅₀ of 4-8 mg/kg.¹⁸¹

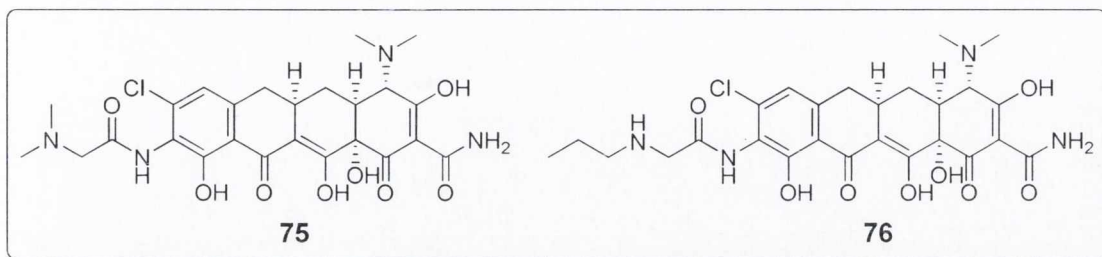
Subsequently, in 2000, Koza adapted the above procedure for the incorporation of a bromine moiety at position 8 of doxycycline (**2**), in an attempt to achieve higher yields in subsequent palladium catalysed reactions than those achieved with the 8-chloro series of tetracyclines produced by Sum *et al.*'s synthesis.¹⁸² Using the synthetic methodology depicted in Scheme 1.10 (*vide infra*), Koza prepared the first doxycycline (**2**) derivatives with a new carbon-carbon bond installed at position 8. While no biological data has been published to date on this series of compounds, this methodology allowed for the preparation of various aryl, alkenyl and alkynyl substituted tetracyclines which have been patented.^{183, 184}

Scheme 1.10 **Synthesis of 8-substituted doxycyclines**



Further compounds synthesised by Sum *et al.*, which possess a greater spectrum of activity than the previous series, were patented by the group.¹⁸⁵ Functionalisation of the 9-amino moiety of **67** and **68** (Scheme 1.9, *vide supra*) to yield various 8-halo-9-glycylamidotetracyclines (Figure 1.19, *vide infra*) furnished compounds active against bacteria expressing both efflux protein tetracycline resistance and RPP tetracycline resistance.

Figure 1.19 8-Substituted-9-glycylamidotetracyclines



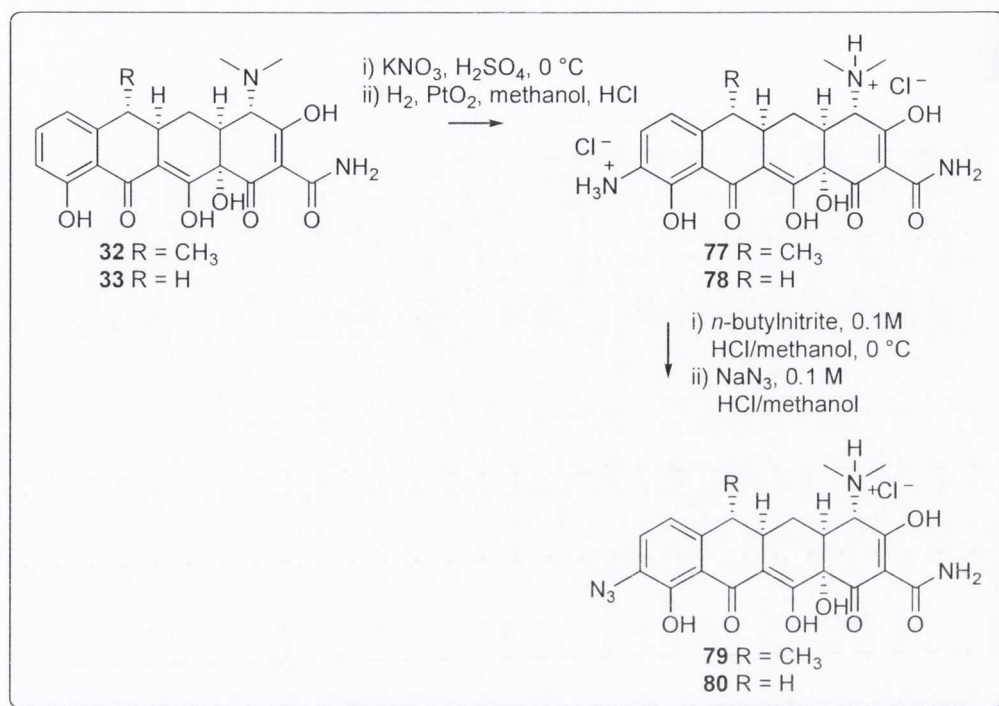
The activity of these compounds was measured by the agar dilution method. Compound **75** was found to be of particular potency, displaying a MIC of 0.25 $\mu\text{g/mL}$ against *S. aureus* expressing the Tet(M) RPP compared to the MIC of minocycline, 8 $\mu\text{g/mL}$.¹⁸⁵ Compound **76** was found to be 32 times more active against Tet(B) efflux protein resistant *E. coli* than minocycline, with a MIC of 0.5 $\mu\text{g/mL}$ versus 16 $\mu\text{g/mL}$ for minocycline.¹⁸⁵

A trend can be detected in the SAR of the 8-substituted tetracycline derivatives - the insertion of a chloro or bromo moiety at position 8 was found to increase the activity of the tetracycline compounds.

1.7.1.3 9-Substituted tetracyclines

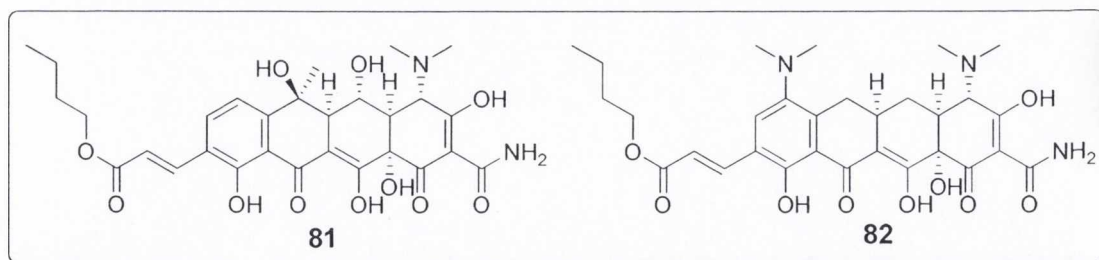
Substitution at position 9 has proved to be a popular method of producing tetracycline analogues, owing to the fact that substitution at this position has led to tetracyclines with improved activity. In the 1960's, studies by The American Cyanamid Company/Pfizer led to some simple, 9-substituted 6-DOTs and 6-DMDOTs which displayed varying levels of antibiotic potency.^{167-170, 186} Of the initial series produced, only one compound, **78** (Scheme 1.11, *vide infra*), was found to be more active than tetracycline. Compound **78** displayed an activity 1.7 times that of tetracycline.^{169, 187}

Scheme 1.11 **9-Substituted tetracyclines**



Nelson *et al.* later employed the chemistry outlined in Scheme 1.11 to produce diazo analogues of tetracyclines that would be reactive in palladium coupling chemistry.¹⁷² The aryl diazonium salt tetracyclines were either employed in Suzuki and Heck coupling reactions directly, or converted to iodo tetracyclines *via* Sandmeyer reactions, to produce 9-aryl and 9-enyl substituted tetracycline analogues, two of which are illustrated in Figure 1.20.

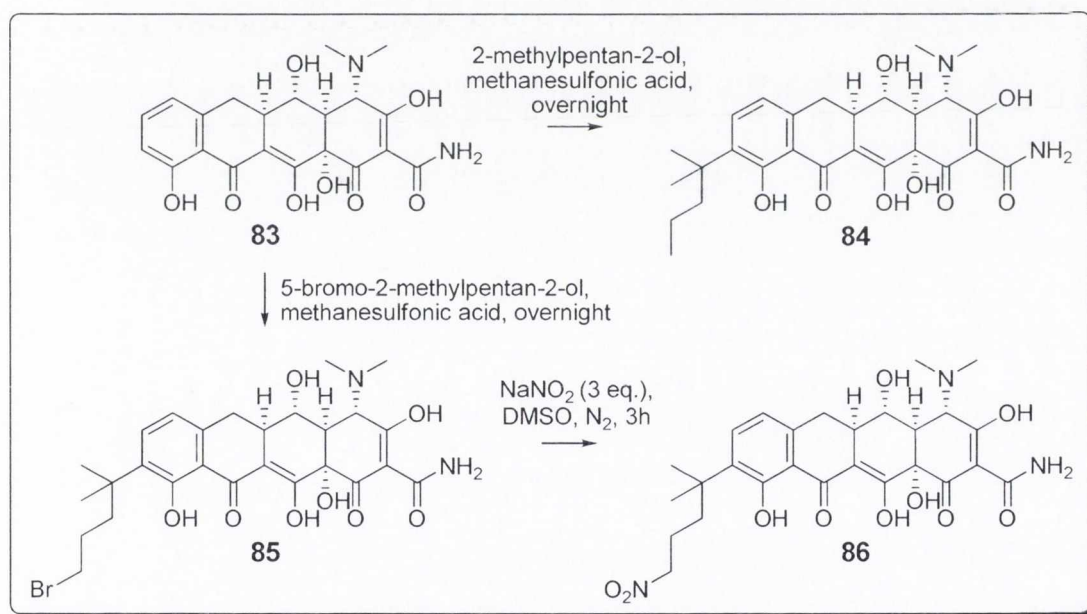
Figure 1.20 **Suzuki- and Heck-reaction derived tetracyclines**



The above compounds were tested for antibacterial activity *in vitro* using the broth dilution method. Compound **82** was found to be twice as active as doxycycline against MRSA, while **81** was found to be 16 times as active as doxycycline against the same strain (MICs: doxycycline = 6.25 µg/mL; **81** = 0.39 µg/mL; **82** = 3.12 µg/mL).¹⁷²

In 1999 Levy *et al.* patented the synthesis of a number of 9-alkyl substituted 5-hydroxytetracyclines which were active against tetracycline resistant and tetracycline sensitive bacteria.¹⁸⁸ The compounds were tested for antibacterial activity using the broth dilution method. The synthesis of these alkylated tetracycline analogues involved the reaction of 5-hydroxytetracycline (**83**) with an alkyl, tertiary alcohol in the presence of methanesulfonic acid to afford the 9-alkyl substituted 5-hydroxytetracycline (Scheme 1.12).

Scheme 1.12 Alkylation of 5-hydroxytetracycline



Of the series synthesised and tested, **84**, **85** and **86** were found to exhibit the most potent activity against tetracycline resistant *S. aureus* and *E. faecalis* strains. Compound **84** was found to be twice as active as doxycycline (**2**) against tetracycline sensitive *S. aureus*, with MIC values of 0.08 µg/mL and 0.195 µg/mL respectively, while **85** and **86** were found to be less active, displaying MICs of 0.39 µg/mL and 3.12 µg/mL.¹⁸⁸ When tested against tetracycline resistant strains, however, **84**, **85** and **86** were significantly more potent than

doxycycline in all cases. Both **84** and **85** were 16 times more active than doxycycline against MRSA (MICs: **84** and **85** = 0.39 µg/mL, doxycycline = 6.25 µg/mL) and 16 times more active than doxycycline against tetracycline resistant *E. faecalis* (MIC's: **84** and **85** = 0.78 µg/mL, doxycycline = 12.5 µg/mL). Compound **86** was found to be 8 times more active than doxycycline against MRSA, with a MIC = 0.78 µg/mL, and 16 times more active against tetracycline resistant *E. faecalis*, with a MIC = 0.78 µg/mL.¹⁸⁸ The activities of the above compounds are displayed in Table 1.3.

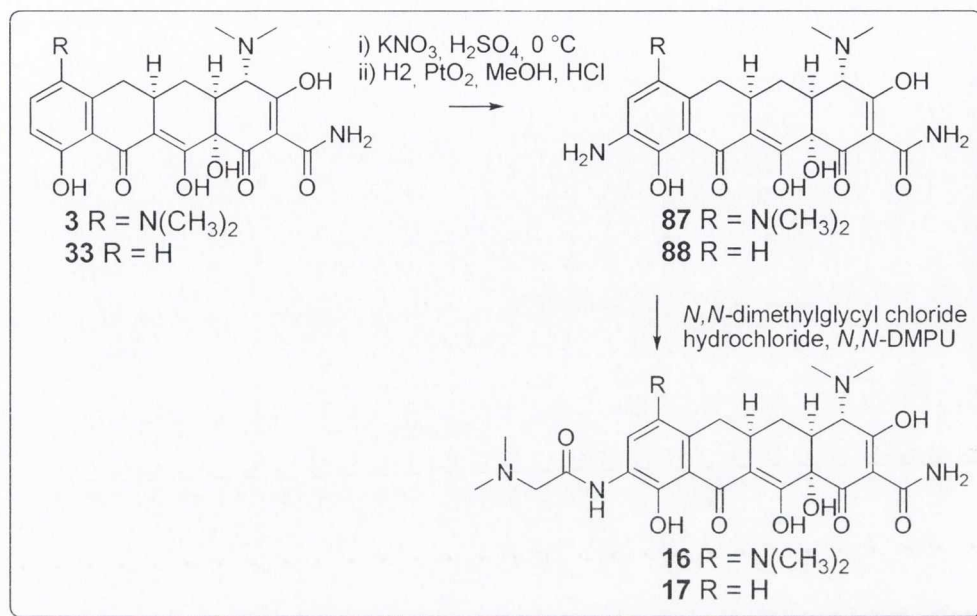
Table 1.3 **Activity of 9-alkylated 5-hydroxytetracyclines**

Entry no.	Strain	MIC (µg/mL)			
		84	85	86	Doxycycline
1	<i>S. aureus</i>	0.08	0.39	3.12	1.95
2	MRSA ^r	0.39	0.39	0.78	6.25
3	<i>E. faecalis</i> ^r	0.78	0.78	0.78	12.5

r = tetracycline resistant

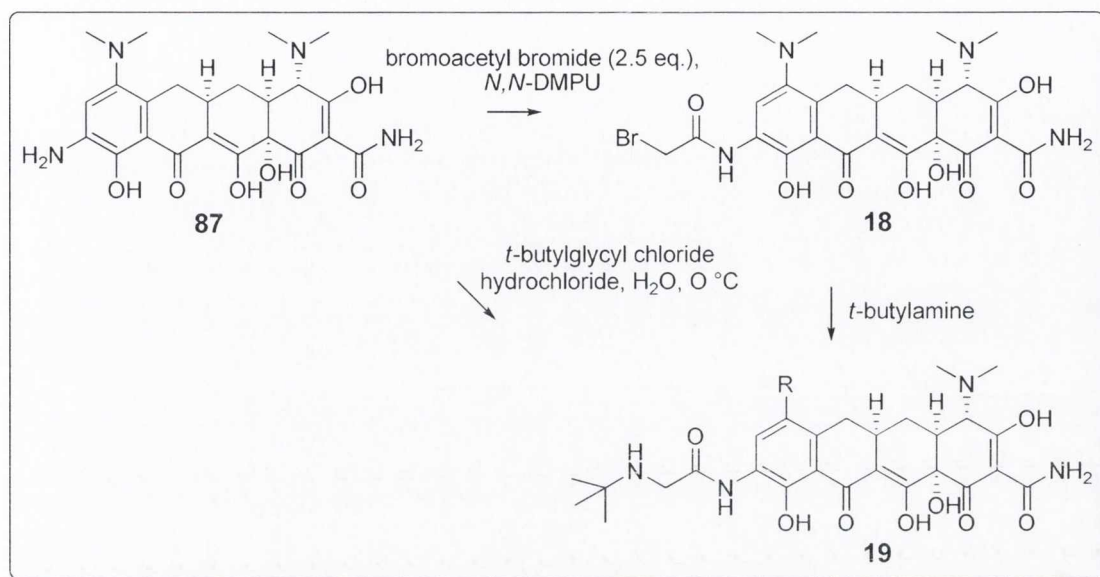
One of the most significant discoveries with respect to 9-substituted tetracyclines was the synthesis and evaluation of glycylicyclines. The activity of the glycylicyclines is discussed in Section 1.5.3. In 1994, Sum *et al.* published the synthesis of a series of 9-glycylamidotetracyclines, which they named glycylicyclines.⁹⁵ The synthesis of the two most active of the series, DMG-MINO (**16**) and DMG-DMDOT (**17**) (Figure 1.10, *vide supra*) is depicted in Scheme 1.13 (*vide infra*). Nitration and subsequent reduction afforded the 9-amino compounds which could then be condensed with a number of 2-substituted acetyl chlorides to afford the series.

Scheme 1.13 **Synthesis of glycylcyclines**



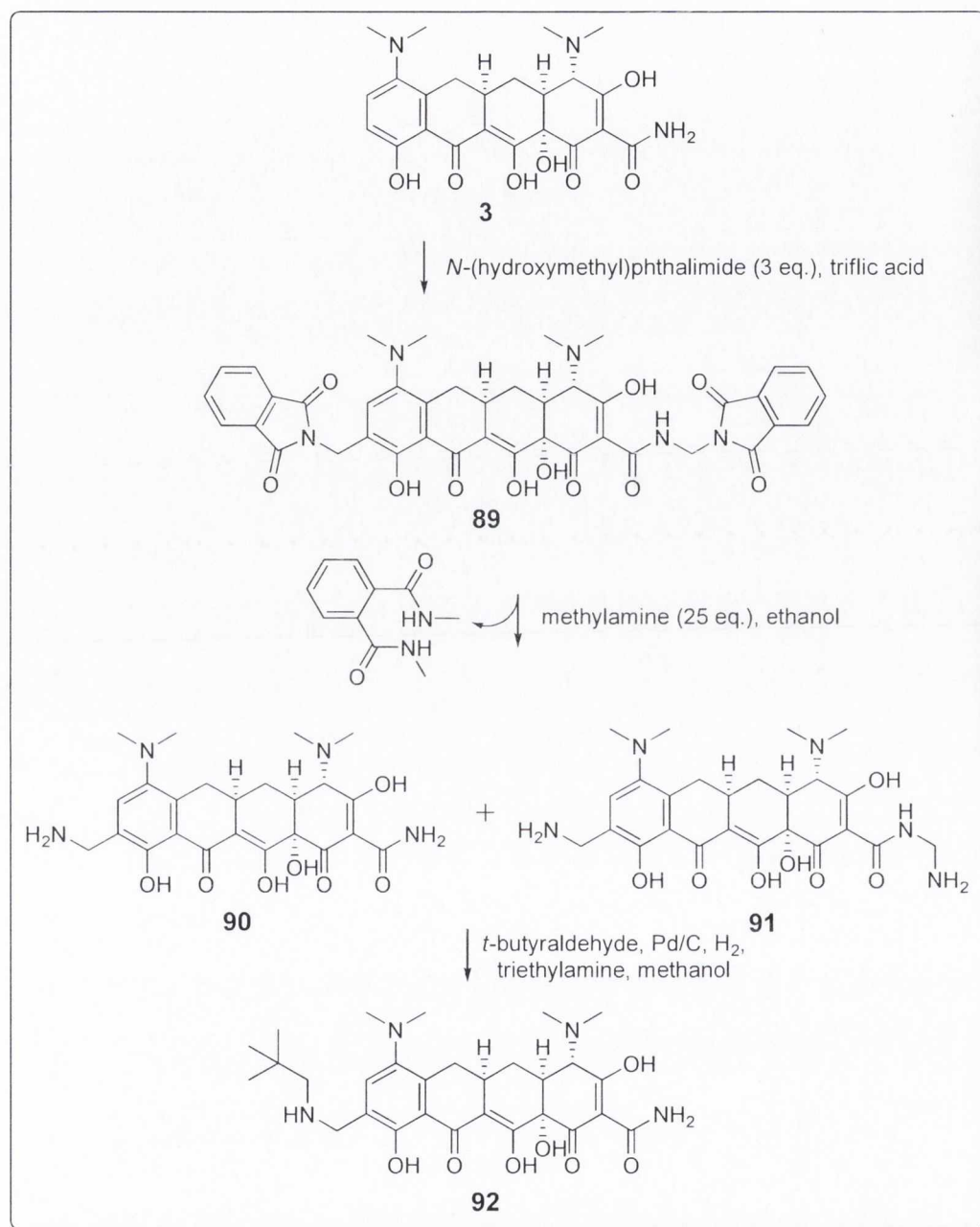
In 1999 Sum and Petersen reported the synthesis of GAR-936 (**19**), a glycylcycline which would later become known as tigecycline.¹⁰¹ Two alternative routes to **19** were proposed (Scheme 1.14, *vide infra*): the reaction of **87** with bromoacetyl bromide to yield **18**, which was then reacted with *t*-butylamine to afford the desired product **19** or the direct transformation of **87** to **19** by reaction with *t*-butylglycyl chloride hydrochloride. While both syntheses are patented by Wyeth¹⁸⁹, it was observed that the synthetic route 'c' (Scheme 1.14, *vide infra*) was less costly.¹⁰¹ A purification method combining pH gradient extraction, reverse phase HPLC and recrystallisation was devised and the synthesis was scaled up for manufacture of tigecycline (**19**) on an industrial scale.^{101, 189}

Scheme 1.14 **Alternative synthesis of tigecycline**



In 2002 a new class of tetracycline antibiotics was developed, with the synthesis of so called aminomethylcyclines; 9-aminomethyl substituted minocyclines. A joint effort involving Bayer and Paratek Pharmaceuticals Inc saw the development of PTK-0796 (BAY 73-7388) (**92**), now known as amadacycline (Scheme 1.15, *vide infra*), which exhibited a significant spectrum of activity against a range of multidrug resistant bacteria.¹⁹⁰ The synthesis of amadacycline from minocycline (**3**) is depicted in Scheme 1.15.

Scheme 1.15 Synthesis of amadacycline



Multiple reactive functional groups lead to numerous side products in this synthetic methodology. The conversion of **3** to **89** using 2-(hydroxymethyl)phthalimide leads to bis and tris substituted products, with the second and third equivalent of 2-(hydroxymethyl)phthalimide reacting with the phenol at position 10 and amide at position

2.¹⁹¹ Deprotection of the phthalimides with methylamine leads to a mixture of the desired product **90** and side product **91**, whereby the amide at position 2 is also substituted with methylamine. The de-aminomethylation of this side product and simultaneous reductive amination at position 9 provided the crude product **92** which required lengthy purification by reverse-phase HPLC (high pressure liquid chromatography), pH gradient extraction into dichloromethane and precipitation with a mixture of heptane and methyl-*t*-butyl ether. The overall process furnished amadacycline in 15% to 18% yield.¹⁹¹

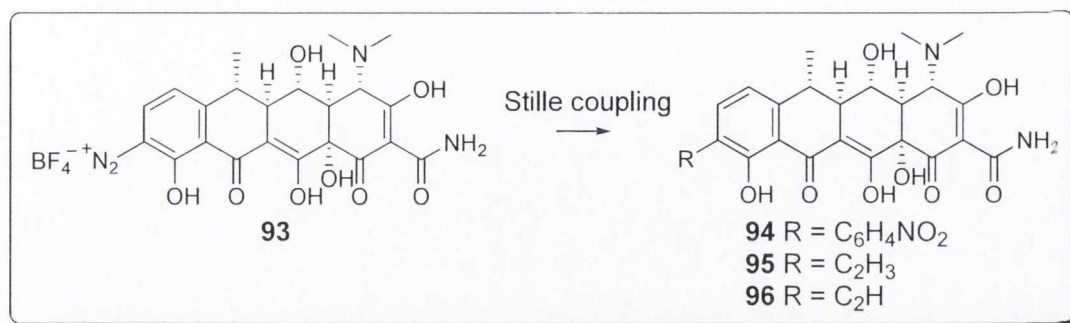
Amadacycline (**92**) is currently in clinical trials and is proving to be both potent and tolerant of administration with a number of other antibiotics. The MIC₉₀s (the concentration at which 90% of bacteria are killed) are compared to those of vancomycin (VAN), linezolid (LIN) and levofloxacin (LEV), all of which are currently indicated drugs for the treatment of multidrug resistant bacterial infections, in Table 1.4.¹⁹² Should this compound succeed in clinical trials it will certainly be useful against MRSA and VRE, for which there are currently few therapies.

Table 1.4 **Activity of amadacycline (92) vs. VAN, LIN, and LEV**

Entry no.	Strain	MIC ₉₀ (µg/mL)			
		92	VAN	LIN	LEV
1	MRSA	0.5	1.0	2.0	32.0
2	Efa VRE	0.5	>64.0	2.0	>64.0
3	Ef	0.5	2.0	2.0	32.0
4	Spn PENR	≥0.06	0.25	1.0	1.0
5	BHS	0.25	0.5	1.0	0.5

2002 also saw the development of Stille coupling procedures to synthesise new 9-substituted tetracycline derivatives which were more active than minocycline (**3**) and doxycycline (**2**) against MRSA.¹⁹³ The diazotisation of 9-aminodoxycycline provided 9-diazodoxycycline tetrafluoroborate (**93**) in quantitative yield. Compound **93** was used directly in Stille coupling reactions with organotin compounds to afford a series of doxycycline analogues which includes those depicted in Scheme 1.16.

Scheme 1.16 Stille coupling of 9-diazodoxycycline



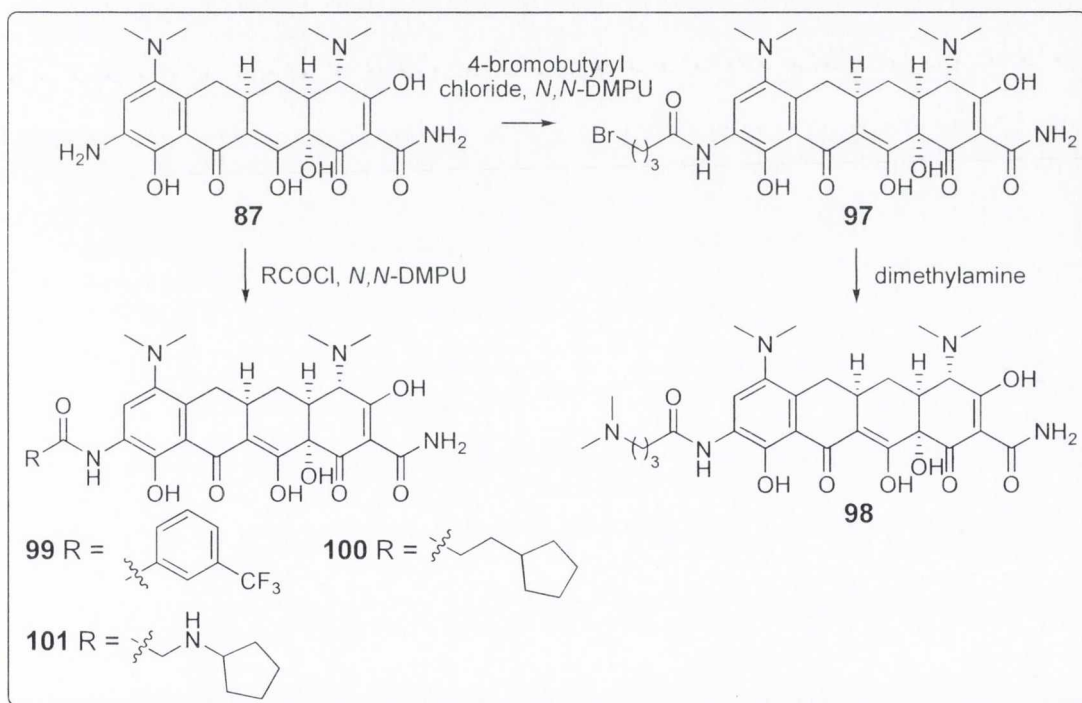
The tetrafluoroborate counterion was found to increase the yield of the reactions compared to the use of other counterions. This was attributed to the increased stability of the diazo salt compounds which resulted in reduced decomposition.

Compounds **94-96** were tested for activity against *S. aureus*, *E. faecalis* and MRSA. **94-96** were found to be equally or more active than doxycycline and minocycline against *S. aureus* (MICs: **94** = 3.12 µg/mL, **95** = 1.56 µg/mL, **96** = 1.56 µg/mL, doxycycline = >50 µg/mL, minocycline = 3.12 µg/mL). Compound **95** was found to be as active as minocycline against *E. faecalis* (MIC **95** and minocycline = <0.098 µg/mL). In tests against MRSA, however, all compounds were found to be more active than either minocycline or doxycycline (MICs: **94** = 0.39 µg/mL, **95** = <0.098 µg/mL, **96** = 0.39 µg/mL, doxycycline = 6.25 µg/mL, minocycline = 6.25 µg/mL). This publication provided another method for the development of tetracycline compounds with activity against tetracycline resistant bacteria.

In 2006, Sum *et al.* reported the synthesis of an array of acylamino and sulfonylamino derivatives of minocycline with promising activity against tetracycline resistant bacteria.¹⁹⁴ The compounds were tested for *in vitro* activity by the agar dilution method. The reaction of 9-aminominocycline (**87**) with various acyl chlorides in the presence of *N,N*-DMPU produced a series of compounds with good antibacterial potency (Scheme 1.17). Of the arylacylamino derivatives produced, **99** (Scheme 1.17, *vide infra*) displayed the highest activity with MICs of 0.5-1.0 µg/mL against *S. aureus* strains expressing Tet(M) RPP's and Tet(K) efflux proteins.¹⁹⁴ However, the arylacylamino derivatives had very low activity against all *E. coli* strains tested, suggesting that they are unable to penetrate the cell wall of Gram negative bacteria.

Compound **100** was found to be less active than compound **101** which suggests that the basic amine moiety is essential for broad spectrum activity.¹⁹⁴ Compound **98** was compared to DMG-MINO (**16**) (Figure 1.10, *vide supra*) to probe the importance of the chain length between the carbonyl and dimethylamino moieties. It was found that compound **98** was significantly less active than **16** against all *S. aureus* strains tested, e.g. compound **98** MIC = 8.0 µg/mL for Tet(M) resistant *S. aureus* compared with a MIC = 0.12 µg/mL for **16**. Compound **98** also exhibited a greatly reduced activity against Gram negative bacteria, e.g. compound **98** MIC = >32 µg/mL for Tet(B) resistant *E. coli* versus compound **16** MIC = 0.25 µg/mL.

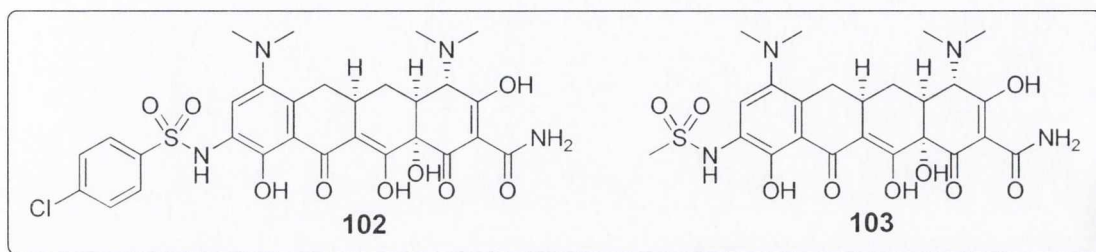
Scheme 1.17 **Synthesis of 9-acyl substituted minocyclines**



Sum *et al.* also reported a procedure for the synthesis of 9-sulfonylamino derivatives of minocycline (**3**). The treatment of 9-aminominocycline (**87**) with a range of sulfonyl chlorides in the presence of *N,N*-DMPU provided analogues which were purified by reverse phase HPLC and tested for antibacterial activity by the agar dilution method.¹⁹⁴ Compounds **102** and **103** (Figure 1.21, *vide infra*) displayed increased activity against *S. aureus* strains expressing Tet(M) RPP resistance when compared with minocycline. The MICs of compounds **102** and

103 against an *S. aureus* strain with Tet(M) resistance were 0.12 $\mu\text{g/mL}$ and 0.06 $\mu\text{g/mL}$ respectively, compared with 2 $\mu\text{g/mL}$ for minocycline.¹⁹⁴

Figure 1.21 **Sulfonylamino minocyclines**

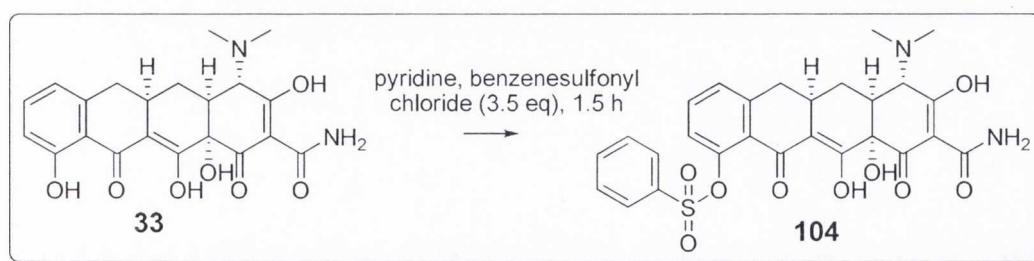


Interestingly, these compounds showed greatly reduced activity against Gram negative bacteria, most likely due to an inability to cross the cell wall. This study provided further insight into the SAR of minocyclines and their activity against Gram positive and Gram negative tetracycline resistant bacteria, while also provided a new method for the synthesis of sulfonylamino derivatives of tetracyclines.¹⁹⁴

1.7.1.4 10-Substituted tetracyclines

In 1963 Stephens *et al.* reported the synthesis of a 10-substituted 6-DMDOT (**33**, Scheme 1.18). Reaction of **33** with benzenesulfonyl chloride in the presence of pyridine provided **104** in 33% yield.¹⁸⁷

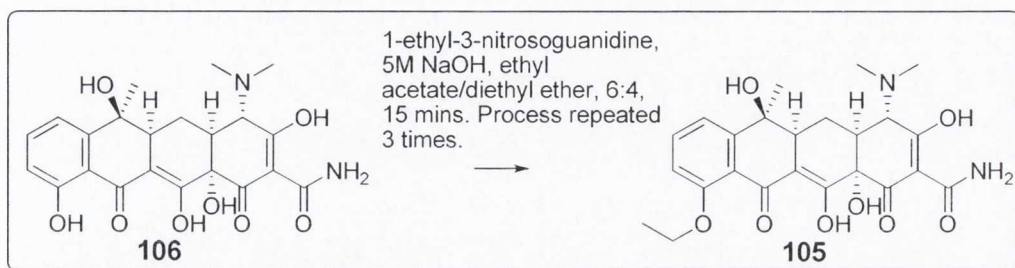
Scheme 1.18 **Synthesis of a sulfonyl 6-DMDOT**



The antibacterial activity of **104** was not evaluated; however, 10-substituted tetracyclines have been proven to have improved activity against resistant bacteria. In 2001 Bartzatt *et al.* reported the synthesis of an ethylated tetracycline compound which displayed activity against

tetracycline resistant *E. coli* bacterial cells.¹⁹⁵ The synthesis of this tetracycline analogue **105** was achieved by reaction of oxytetracycline (**106**) with diazoethane formed *in situ* from 1-ethyl-3-nitro-1-nitrosoguanidine and sodium hydroxide (Scheme 1.19, *vide infra*).

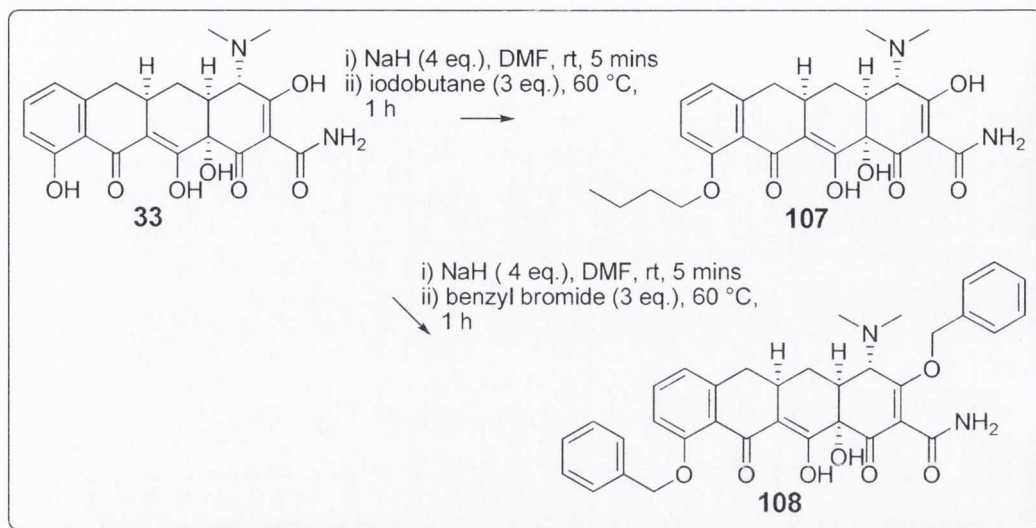
Scheme 1.19 Synthesis of ethylated oxytetracycline



Compound **105** was found to be active against tetracycline resistant XL1-blue *E. coli* cells, inhibiting 30% cell growth at a 12 µg/mL agar dilution. At the same concentration oxytetracycline (**106**) was found to be completely inactive.¹⁹⁵

In 2005, Bandarage *et al.* of Paratek Pharmaceuticals Inc. patented a similar synthetic methodology for the synthesis of 10-*O*-alkyl substituted 6-DMDOT (**33**), minocycline (**3**) and doxycycline (**2**) analogues.¹⁹⁶ Sodium hydride was used to deprotonate the phenolic position of compound **33** and subsequent addition of haloalkanes furnished the desired products, a selection of which are shown in Scheme 1.20. The same patent also introduced a method for benzylation at position 10 and position 3 (Figure 1.17, *vide supra*) to obtain a dibenzylated tetracycline analogue **108**.¹⁹⁶

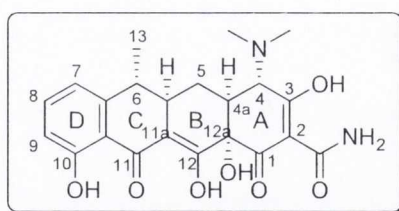
Scheme 1.20 **Synthesis of *O*-alkylated and *O*-benzylated 6-DMDOTs**



1.7.2 13-Thio substituted tetracyclines

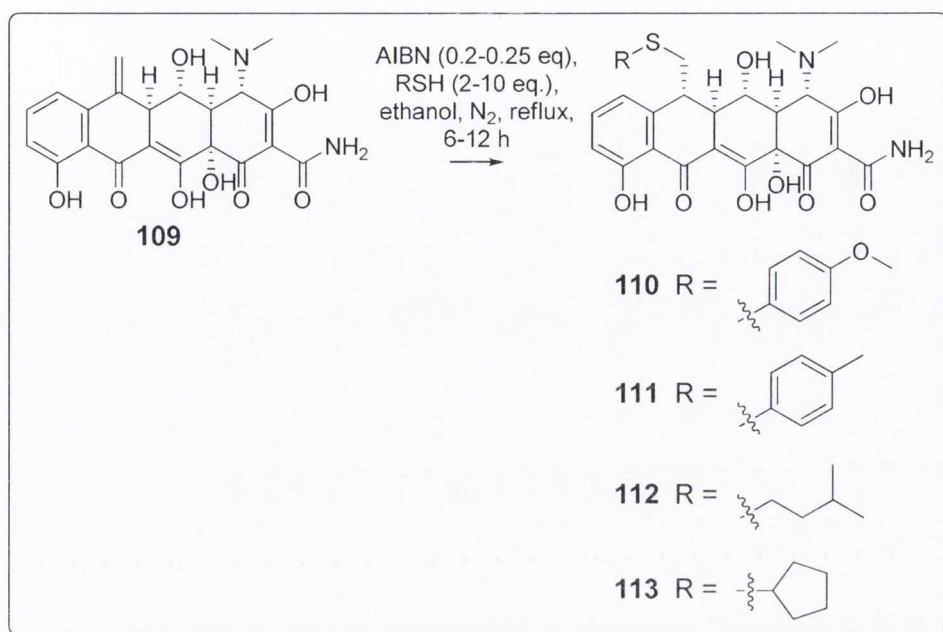
In tetracyclines where a methyl group is present at position 6, the methyl position can be numbered 13. There have been several studies on substitutions at this position and their effect on antibacterial activity.

Figure 1.22 **Position 13**



In 1993 Nelson *et al.* reported the synthesis of 13-thio substituted 5-hydroxytetracyclines.⁹⁰ The activity of these compounds as efflux pump inhibitors has been discussed in Section 1.5.2. The reaction of methacycline (**109**) suspended in ethanol, with 20-25 mol% 2,2-azobis(2-methylpropionitrile) (AIBN) in the presence of the appropriate thiol reagent produced compounds of the general structure depicted in Scheme 1.21.⁹⁰

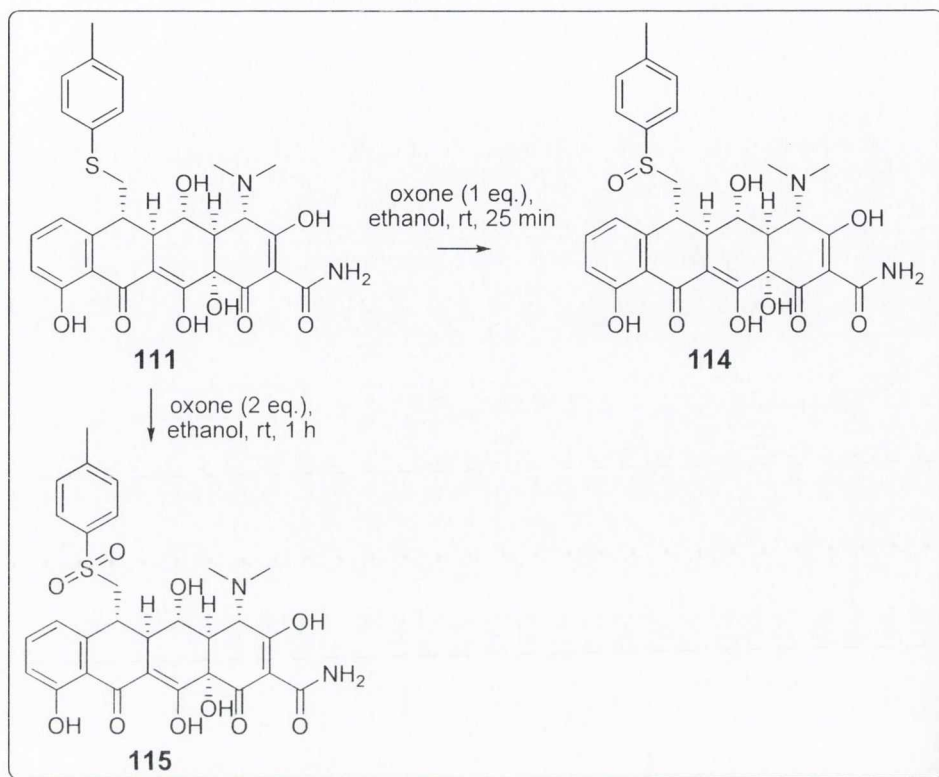
Scheme 1.21 **Synthesis of 13-thio substituted tetracyclines**



The highly reactive thiophenol reagents used in the synthesis of **110** and **111** were found to cause degradation of methacycline (**109**) under previously reported reaction conditions.¹⁹⁷ The number of equivalents of thiophenol used was found to be crucial, two equivalents of the thiophenol reagent reacted for 6 h furnished the desired products, albeit in low yields of 19-25%. Compounds **112** and **113** were produced in similar yields (22% and 15%) by reflux in ethanol for 6-12 h with up to 10 equivalents of the appropriate thiol.⁹⁰

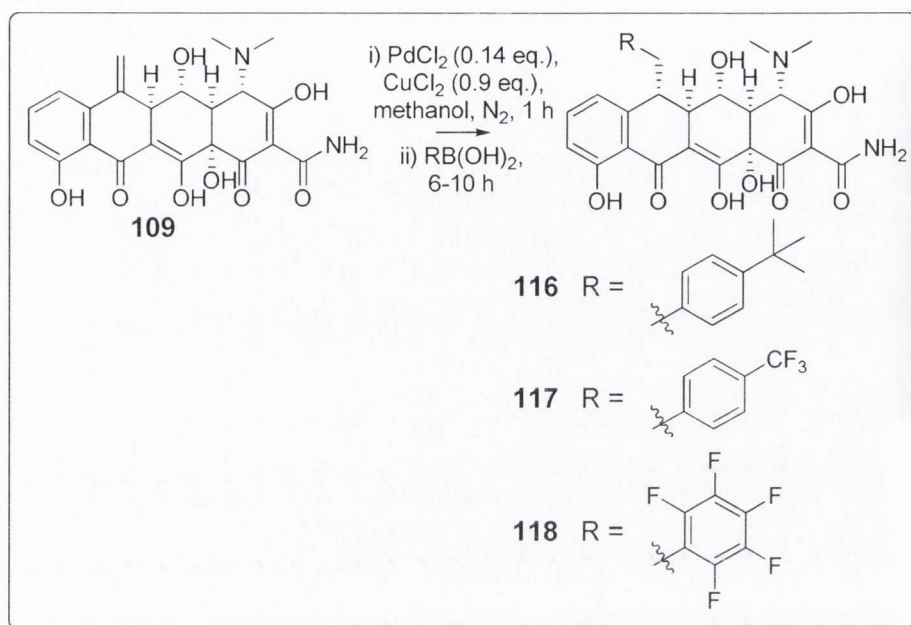
The oxidation of **111** was feasible by reaction with one equivalent of oxone in ethanol at room temperature for 25 mins. The sulfinyl product **114** was isolated in 35% yield after purification. Alternatively, the sulfonyl analogue of **111** could be produced by reaction with two equivalents of oxone in ethanol at room temperature for 1 h. The sulfonyl product **115** was isolated in 50% yield after purification (Scheme 1.22). All 13-thio substituted tetracycline analogues in the series were purified by a combination of pH gradient extraction, recrystallisation and reverse phase HPLC.⁹⁰ The 13-thio substituted tetracyclines were patented by Nelson and Levy in 1999.¹⁸⁸

Scheme 1.22 **Oxidation of tetracycline sulfides**



In 2003 Nelson *et al.* reported the synthesis of 13-substituted derivatives of tetracyclines, which were patented in 2004.^{178, 198} Palladium (II) coupling chemistry was employed successfully to produce new carbon-carbon bonds at position 13 in methacycline (**109**). Substituted phenyl analogues were synthesised by the reactions depicted in Scheme 1.23. Compound **116** was produced in 43% yield, an excellent yield in terms of tetracycline chemistry.¹⁷⁸ No yields were reported for the patented compounds **117** and **118** and all compounds were purified by reverse phase HPLC.¹⁹⁸

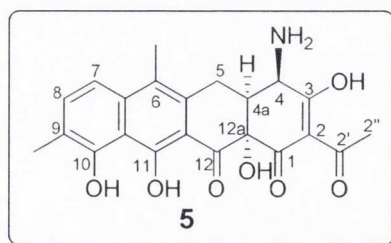
Scheme 1.23 Palladium couplings of methacycline



1.7.3 Atypical tetracyclines

There has been little research into the chemical transformations that can be performed on atypical tetracyclines as they are generally considered to be clinically useless due to their inability to distinguish between bacterial and mammalian cell targets.^{5, 28} In the 1970s and 1980s a number of studies were performed on the chemistry of chelocardin (5), the first of which was reported in 1977 by Chu *et al* of Abbott Laboratories.¹⁹⁹

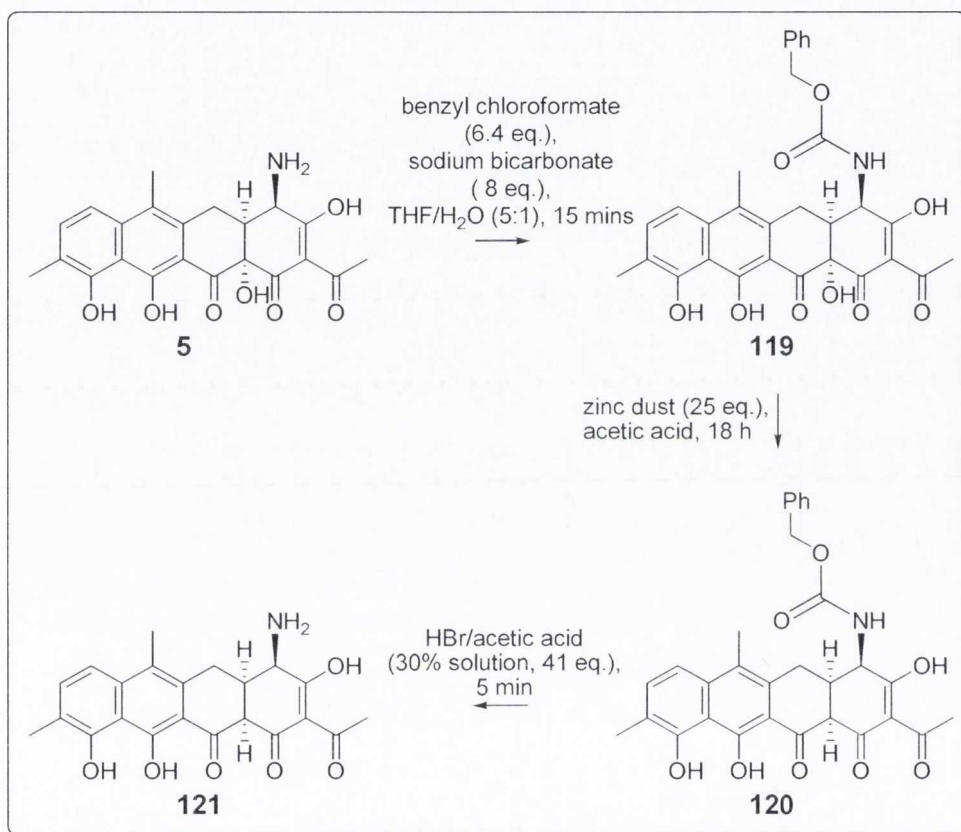
Figure 1.23 Assignment of numbers in the carbon skeleton in chelocardin



This group investigated the removal of the hydroxyl moiety at position 12a (Figure 1.23) and its affect on the antibacterial activity of chelocardin. It was known that the same modification

performed on oxytetracycline (**106**) (Scheme 1.19, *vide supra*) resulted in a 98% loss of activity against *S. aureus*.²⁰⁰ The desired dehydroxylated product, **121**, was obtained as a hydrobromide salt in 29% yield from **5** (Scheme 1.24).¹⁹⁹

Scheme 1.24 **Synthesis of 12a-deoxychelocardin**

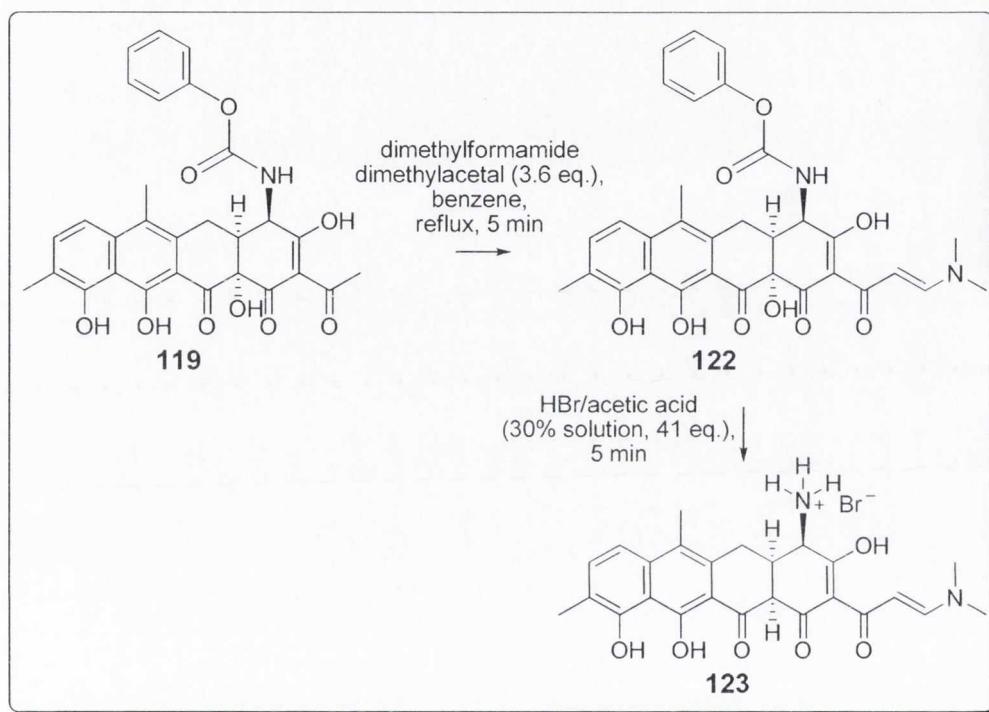


In contrast to the dehydroxylated oxytetracycline analogue, **121** retained its antibacterial activity compared to chelocardin (**5**).¹⁹⁹ This is most likely due to the fact that chelocardin does not have the same mechanism of action as oxytetracycline (**106**) and removal of a hydroxyl group would, if anything, increase its ability to lodge in the lipid bilayers of cell membranes.

The same group probed the importance of the methyl group at position 2'' (Figure 1.23, *vide supra*). It was found that substitution at this position and subsequent deprotection of the amine at position 4 to produce compound **123**, a hydrobromide salt, (Scheme 1.25) resulted in an extreme loss of activity. **123** Was weakly active against Gram positive bacteria

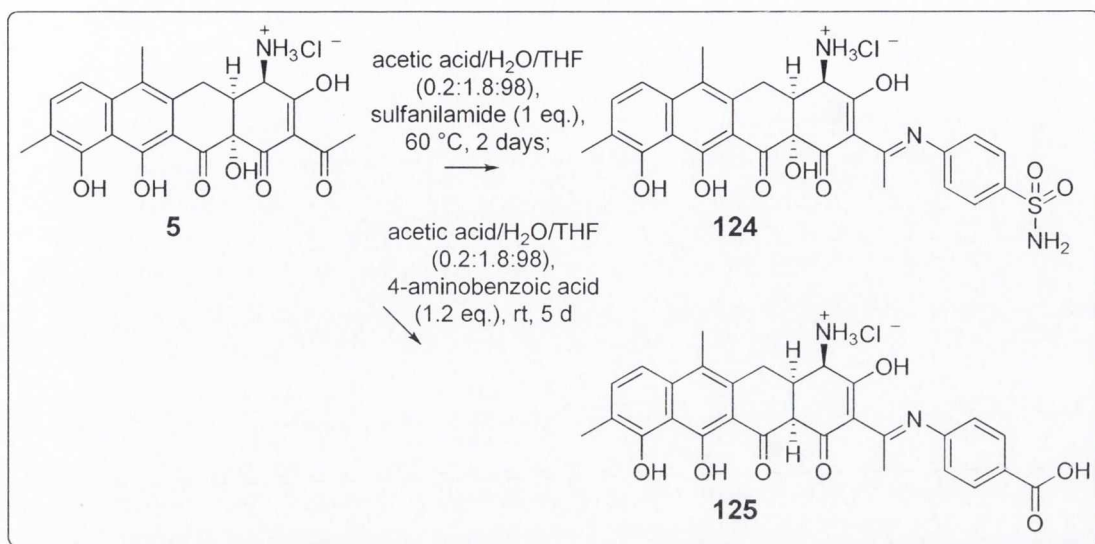
and inactive against Gram negative bacteria at concentrations up to 100 $\mu\text{g/mL}$.¹⁹⁹ It can be deduced that this loss of activity may be due to the addition of a tertiary amine group which can be partially protonated at physiological pH.

Scheme 1.25 C-2'' substitution of chelocardin



Abbott Laboratories also patented the synthesis of anilino derivatives of chelocardin (5) at position 2'.^{201, 202} Reaction of chelocardin with various aniline derivatives provides the desired secondary ketimine substituted chelocardin products. The series of compounds were tested for antibacterial activity using the agar dilution method and a number of moderately active analogues were identified, the two most active of which, **124** and **125**, are shown in Scheme 1.26.

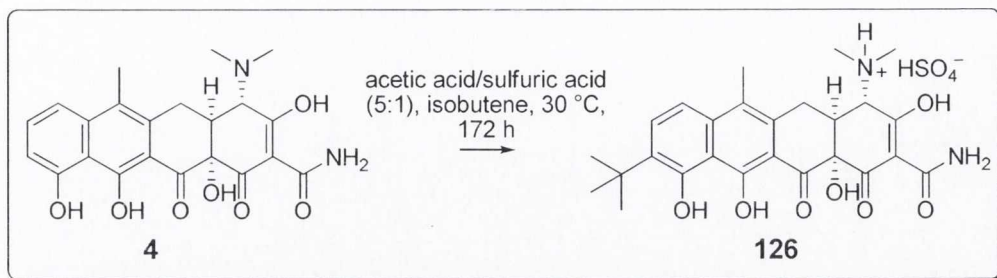
Scheme 1.26 Ketimino derivatives of chelocardin



124 and **125** displayed a MICs of 1.56 µg/mL against *Proteus vulgaris*. When tested against *Salmonella typhi*, **124** displayed a MIC of 0.78 µg/mL while **125** was less active, with a MIC of 1.56 µg/mL. The compounds were reported to have low oral toxicity and **124** was found to be more active than chelocardin *in vivo*.²⁰²

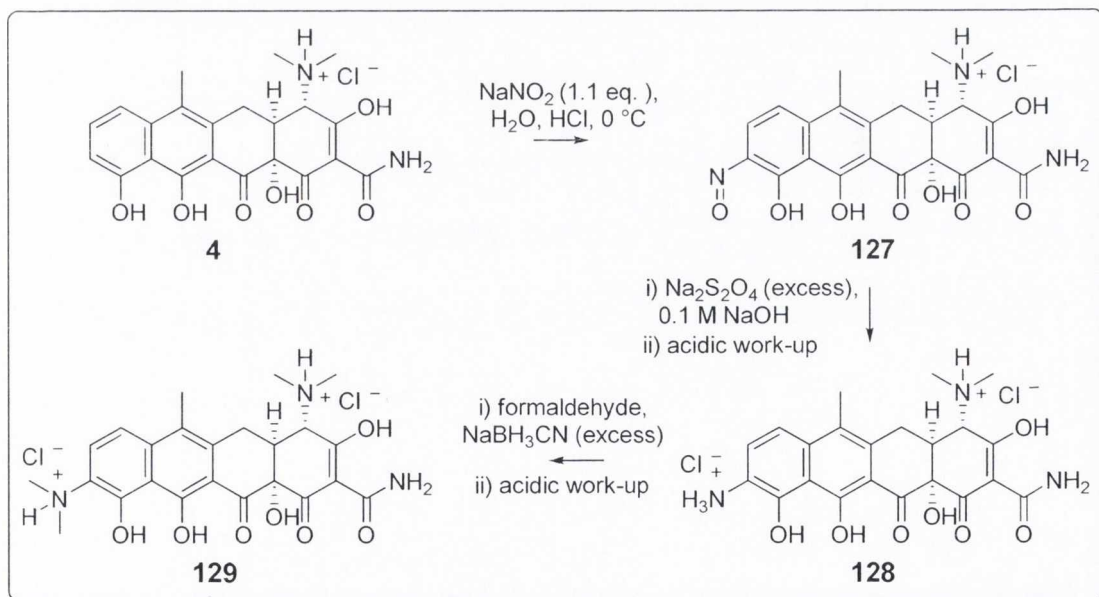
In 1957 Stephens *et al.* of Pfizer and Co. patented a method for the alkylation of anhydrotetracycline (**4**),²⁰³ similar to that depicted in Scheme 1.12 (*vide supra*) for the alkylation of 5-hydroxytetracyclines. The reaction of various electrophiles in the presence of strong acids afforded the 9-alkyl anhydrotetracycline series, as exemplified by compound **126** (Scheme 1.27, *vide infra*). **126** was shown to be the most effective compound produced in both *in vitro* and *in vivo* tests. **126** cleared 100% of infection with *Trichomonas vaginalis* in mice at 300 mg/kg administered dermally and was a successful anthelmintic agent against *Syphacia oblevata* (pin worm).²⁰³

Scheme 1.27 Alkylation of anhydrotetracycline



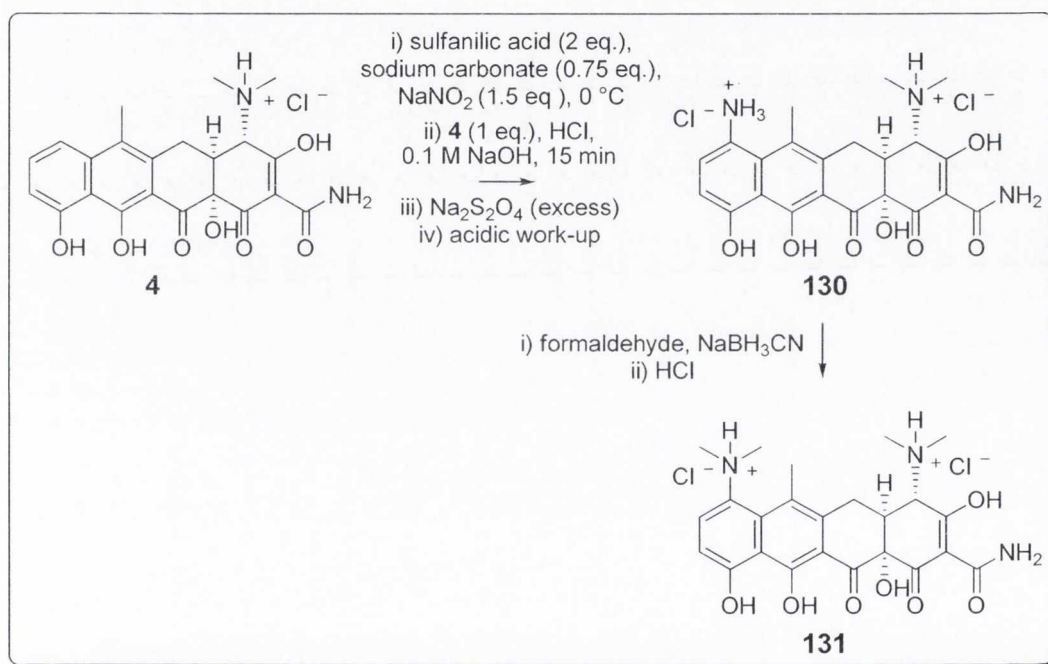
In 1984 Menachery and Cava reported the regiospecific synthesis of amino derivatives of anhydrotetracycline (**4**), a procedure which was to be much used in future syntheses.²⁰⁴ Their synthetic route to 9-amino substituted derivatives involved the nitrosation of anhydrotetracycline to **127** with subsequent reduction to the 9-amino anhydrotetracycline **128**. This compound was further functionalised by methylation of the amine to afford compound **129** (Scheme 1.28). The regiospecificity of this reaction may be explained by the stabilisation of the 9-nitroso compound **127** by tautomerisation to the *o*-quinone oxime.

Scheme 1.28 Synthesis of 9-dimethylamino anhydrotetracycline



The regiospecific synthesis of the 7-amino anhydrotetracycline (**130**) was also reported, employing a synthetic pathway through an azo dye which was then reduced to the amine (**130**), and subsequently methylated to compound **131** (Scheme 1.29).²⁰⁴ The alternative regioselectivity is directed by the synthetic route employed. As substitution through the azo dye coupled product of anhydrotetracycline and sulfanilic acid is directed to the less hindered *para* position, the product obtained after reduction of the azo dye is the 7-substituted compound **130** (Scheme 1.29).

Scheme 1.29 **Synthesis of 7-dimethylamino anhydrotetracycline**

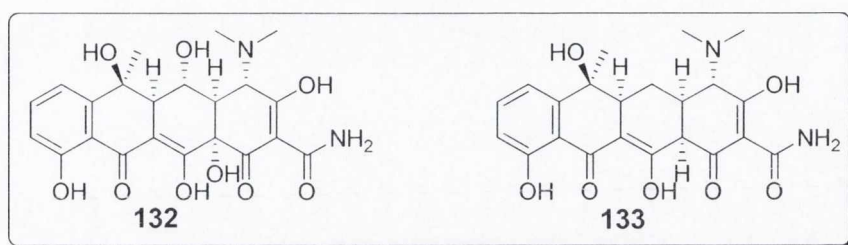


1.7.4 Total synthesis

Total synthesis provides a platform by which more varied tetracycline analogues can be produced, as it overcomes the restrictions of chemical reactivity and purification issues involved in the synthesis of tetracycline analogues from an intact tetracycline nucleus. While many strategies have been employed for the total synthesis of tetracyclines, most have proved to be extremely poor yielding. In 1968, Korst *et al.* published the total synthesis of 6-DMDOT (**33**, Scheme 1.2, *vide supra*) in 25 steps and 0.002% overall yield.²⁰⁵ In 1979 the total synthesis of 5-oxytetracycline (**132**, Figure 1.24, *vide infra*) was achieved by Muxfeldt *et al.*

in 22 steps but with an extremely low yield of 0.06%.²⁰⁶ One of the more successful routes designed was the synthesis of 12a-deoxytetracycline (**133**, Figure 1.24, *vide infra*) in 1996 by Stork *et al.*²⁰⁷ This route provided the desired product in 16 steps and 18% yield; however these compounds are of less interest as 12a-deoxytetracyclines possess very little antibacterial activity.²⁰⁰

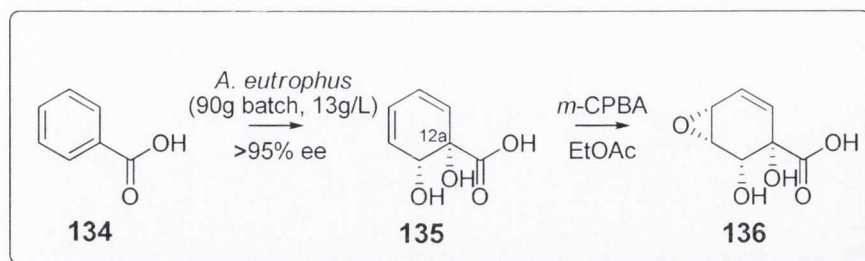
Figure 1.24 5-Oxytetracycline and 12a-deoxytetracycline



It was not until 2005 that a synthetic methodology was reported which provided useful antibacterial tetracycline analogues in higher yields. This synthetic route was developed by Myers *et al.* and afforded doxycycline (**2**) and 6-deoxytetracyclines with modified D rings from benzoic acid in 14 to 15 steps and 5-8% overall yields.²⁰⁸

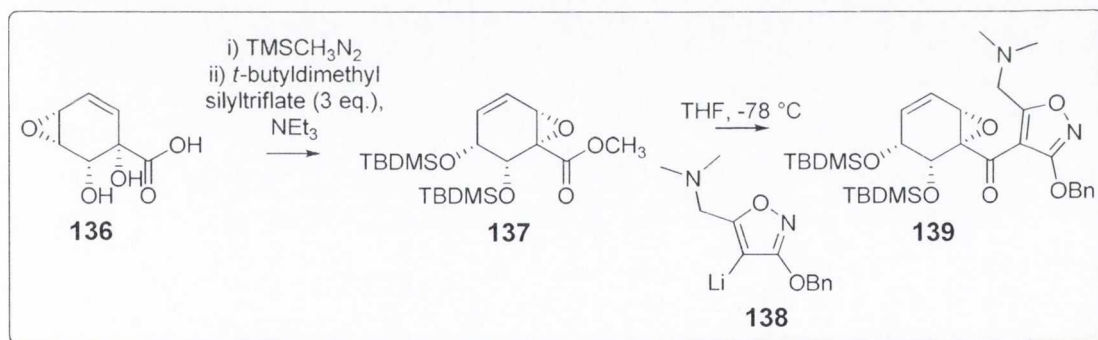
The synthesis of 6-deoxytetracyclines begins with the formation of the A and B rings of the tetracycline (Figure 1.5, *vide supra*). The first step in this synthesis involved the use of bacterial whole cells of *Alcaligenes eutrophus* to facilitate the enantioselective enzymatic dihydroxylation of benzoic acid (**134**) to **135** in 79% yield. Compound **135** contains the desired stereochemistry at what will become position 12a and fortuitously also directs the stereoselectivity of the successive reactions. **135** subsequently underwent a stereoselective oxidation with *m*-chloroperbenzoic acid (m-CPBA) to epoxide **136** in 83% yield (Scheme 1.30).²⁰⁸

Scheme 1.30 Dihydroxylation and epoxidation



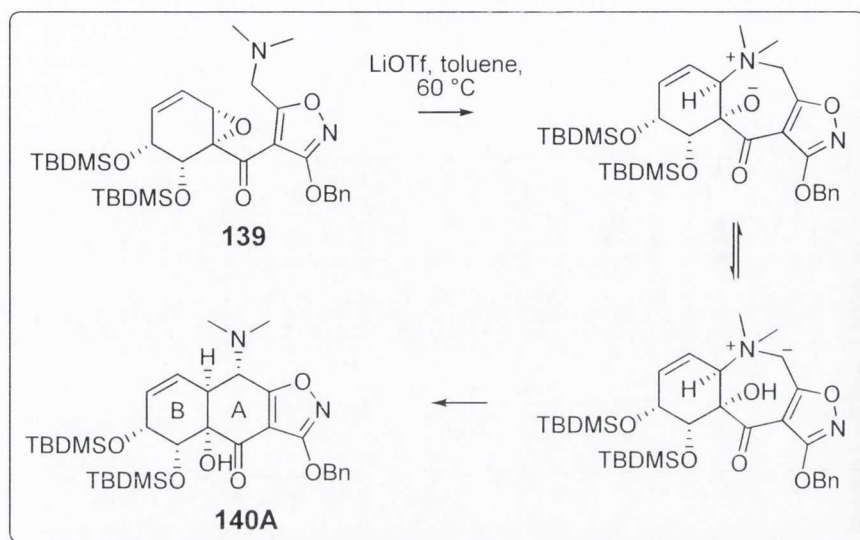
Compound **136** was converted to **137** by esterification using trimethylsilyldiazomethane followed by treatment with 3 equivalents of *t*-butyldimethylsilyl triflate to afford the desired product with protected alcohols and isomerised epoxide in 70% yield (Scheme 1.31). **137** was then reacted with organolithium reagent **138**, which had been deprotonated with *n*-butyllithium, in tetrahydrofuran (THF) to form **139** in 73% yield (Scheme 1.31).²⁰⁸

Scheme 1.31 TBS protection and reaction with organolithium reagent



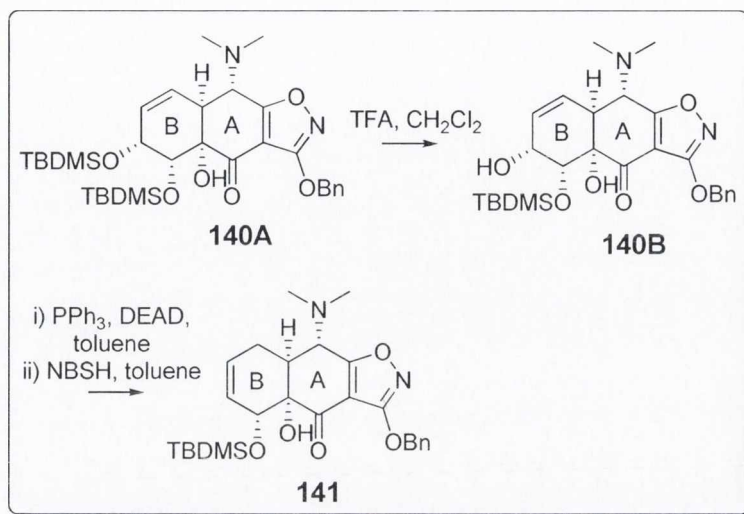
The ring closure of **139** to form the A and B rings was afforded by heating the compound to 60 °C in toluene in the presence of 5 mol% lithium triflate (Scheme 1.33, *vide infra*). Initially, the dimethylamino group attacks the epoxide and an ylide is formed. A subsequent proton transfer and Stevens rearrangement furnishes the desired 6-membered ring (Scheme 1.32).²⁰⁸

Scheme 1.32 Ylide formation and rearrangement



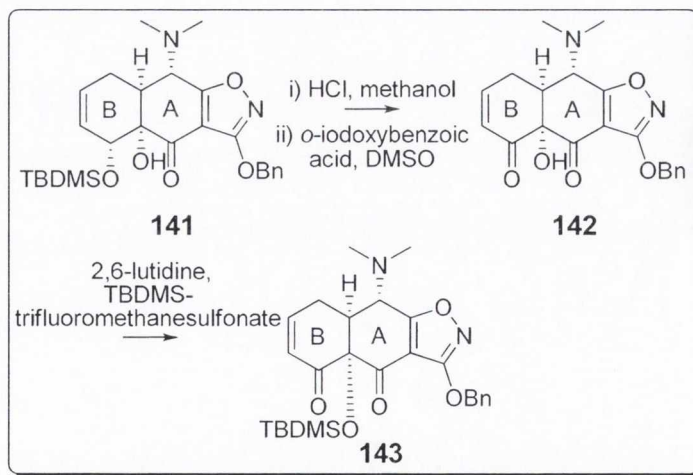
The deprotection of the allylic alcohol was then accomplished by reaction with trifluoroacetic acid (TFA) in CH_2Cl_2 to furnish the AB tricycle **140B** in 62% yield after column chromatography (Scheme 1.33). **140B** possessed the correct stereochemistry at the AB ring junction. The next step required the migration of the B ring double bond and reduction of the hydroxyl moiety. This was achieved by the reaction of **140B** with triphenylphosphine (PPh_3) and diethylazidocarboxylate (DEAD) in toluene with the successive addition of *o*-nitrobenzenesulfonyl hydrazide (NBSH) to afford **141** in 74% yield (Scheme 1.33).²⁰⁸

Scheme 1.33 Double bond migration and hydroxyl reduction



The silyl ether protecting group of **141** was removed with HCl and methanol and the resulting alcohol was oxidised with *o*-iodoxybenzoic acid to give **142**. Compound **142** was treated with *t*-butyldimethylsilyl trifluoromethanesulfonate and 2,6-lutidine to afford compound **143** in 66% yield over the three steps (Scheme 1.34).²⁰⁸

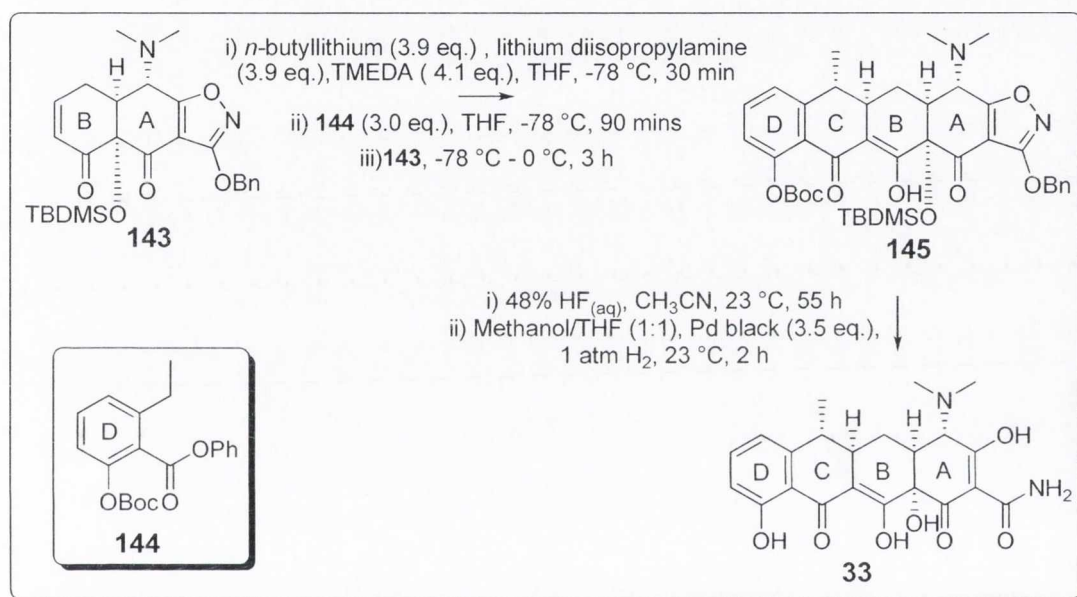
Scheme 1.34 Deprotection, oxidation and protection at 12a



Compound **143** was coupled to a range of D ring precursors to afford a series of substituted 6-deoxytetracyclines. This was performed in a Michael addition-Dieckmann reaction sequence that forms the C ring of the tetracycline and attaches the D ring concomitantly.²⁰⁸ Compound

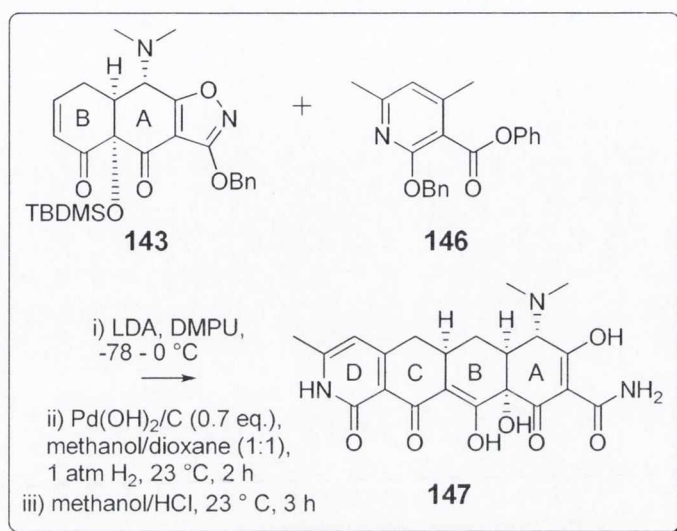
144 is reacted with lithium diisopropylamine (LDA) and *N,N,N,N*-tetramethylethylenediamine (TMEDA) at $-78\text{ }^{\circ}\text{C}$ to deprotonate the benzylic position, added to a reaction vessel containing **143** and allowed to warm to room temperature to afford **145** in 81% yield (Scheme 1.35). The addition of hydrofluoric acid and subsequent reduction of **145** afforded the desired product, 6-DOT (**33**).

Scheme 1.35 Michael addition, deprotection and reduction

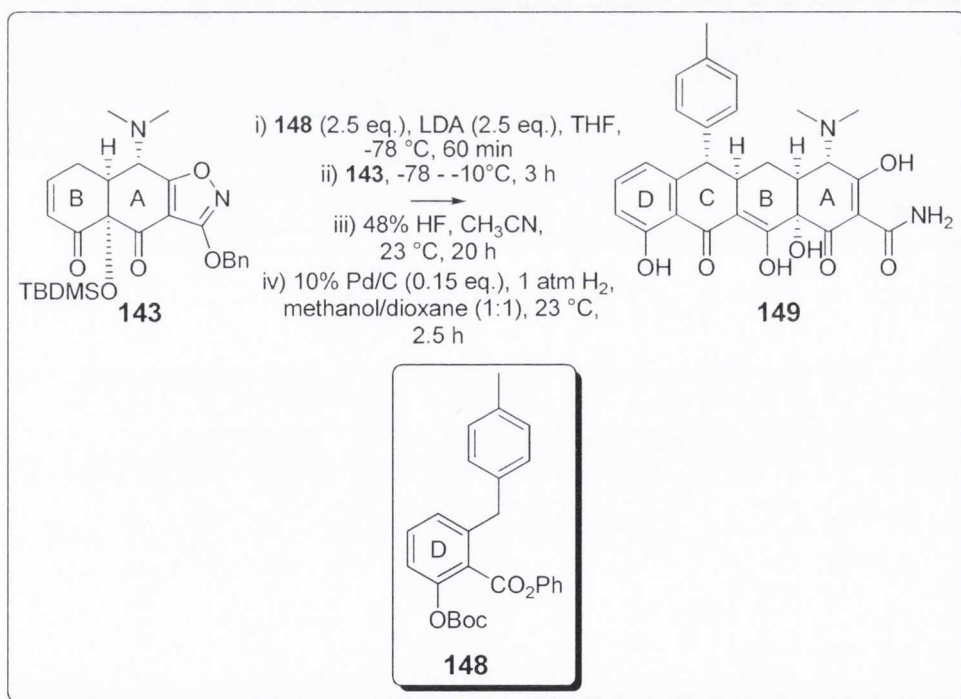


The final step in the synthetic sequence (Scheme 1.35) was modified to incorporate a range of substituted D rings. Pyridine analogues (**146**, Scheme 1.36, *vide infra*) could be used in place of **144** to afford heteroaromatic tetracyclines, *e.g.* compound **147** (Scheme 1.36, *vide infra*).²⁰⁸ In 2008 Sun *et al.* reported the modification of the synthesis to produce C ring substituted tetracyclines, *e.g.* compound **149** (Scheme 1.37, *vide infra*) and a wide range of pentacyclines, *e.g.* compound **152** (Scheme 1.38, *vide infra*).²⁰⁹

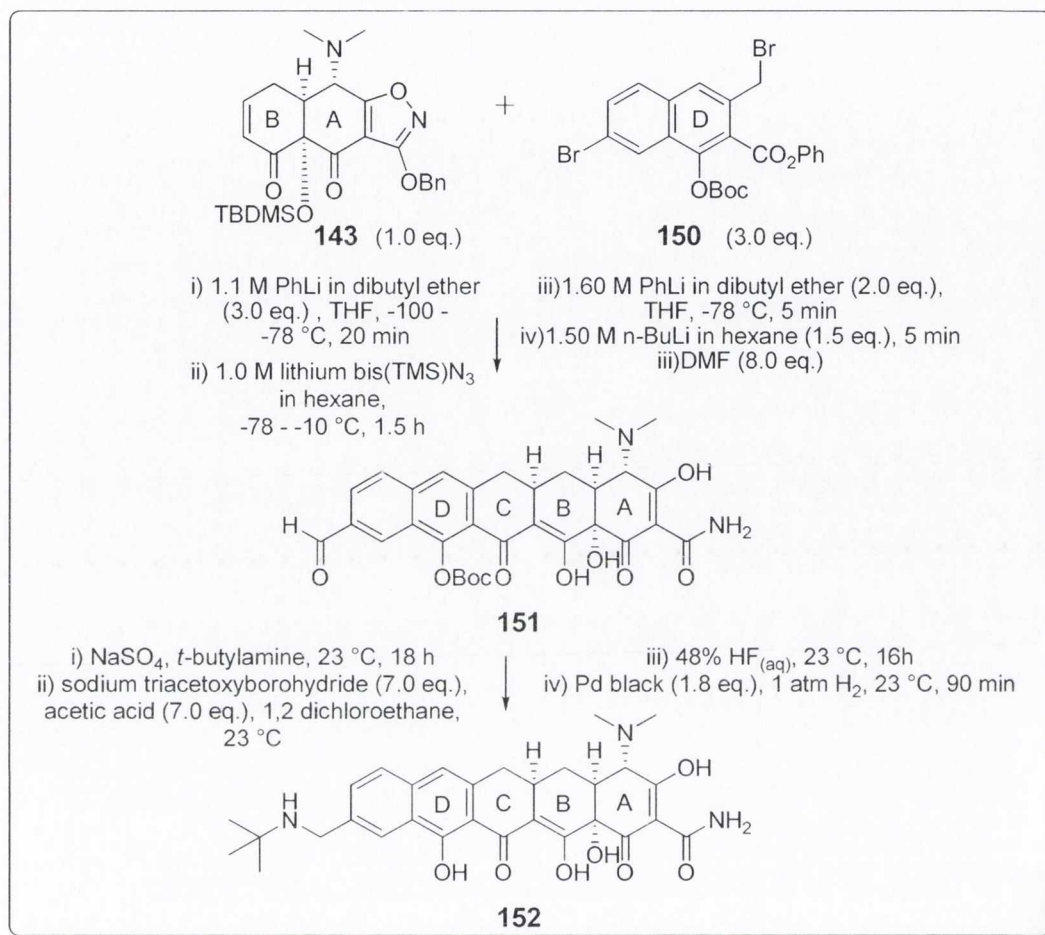
Scheme 1.36 Heteroaromatic tetracycline synthesis



Scheme 1.37 Synthesis of C ring substituted tetracyclines



Scheme 1.38 Pentacycline synthesis



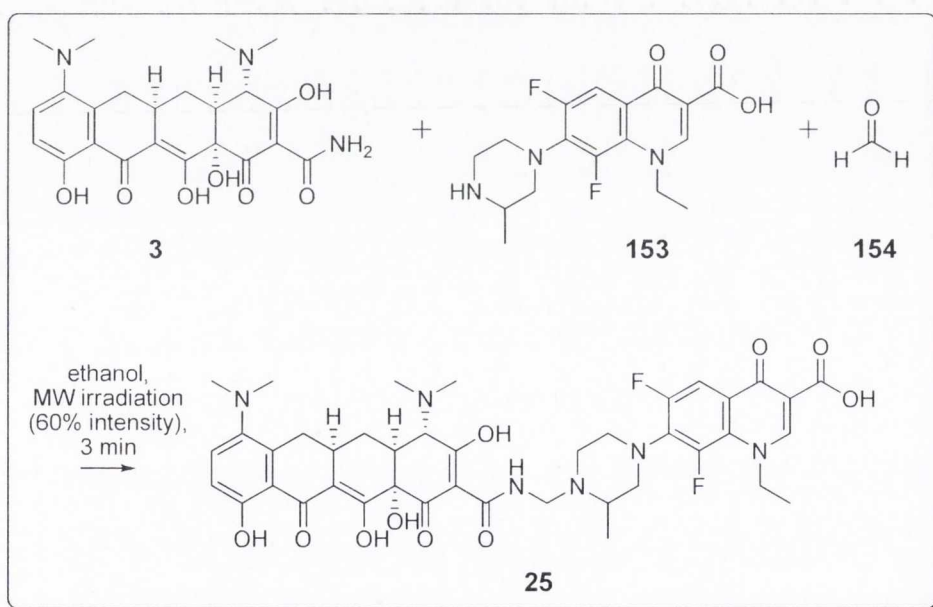
It is clear from the examples above that total synthesis provides the opportunity to produce a wider variety of tetracycline analogues than the conventional derivatisation of naturally occurring tetracyclines. Unfortunately, the syntheses are generally low yielding overall and require many intricate transformations and difficult purifications. However, the pentacyclines produced by Sun *et al.* in 2008 proved to be extremely active, with many analogues displaying activities of <0.06 – 1 µg/mL against a range of tetracycline sensitive and tetracycline resistant Gram positive and Gram negative bacteria.²⁰⁹ This adds value to the total synthesis approach and further research must be performed to exploit this synthetic methodology to the full.

1.7.5 Conjugated compounds containing a tetracycline

1.7.5.1 Tetracycline-fluoroquinolone conjugates

In 2007, Sriram *et al.* published the synthesis of Mannich base tetracycline-fluoroquinolone dimers that were active against HIV-1 integrase and *Mycobacterium tuberculosis*.¹⁵³ The activity of these compounds is discussed in Section 1.6.2.3. The series of dimers were synthesised by the Mannich reaction of varied tetracyclines and fluoroquinolones with microwave irradiation in the presence of formaldehyde. This methodology provided the conjugates in 41 – 78% yield without the need of further purification. The synthetic route is exemplified in Scheme 1.39, which shows the synthesis of the most active compound, **25**.

Scheme 1.39 **Synthesis of tetracycline-fluoroquinolone dimers**

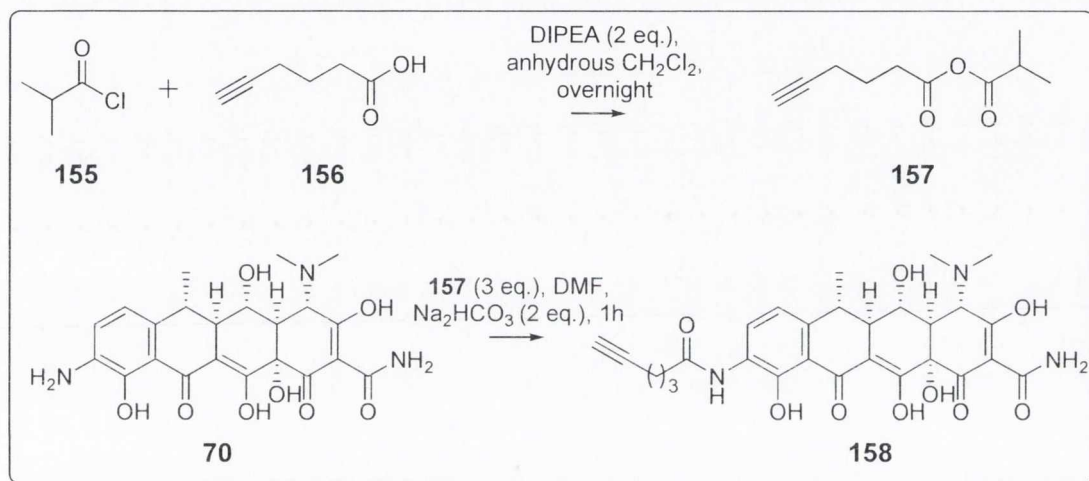


1.7.5.2 Doxycycline-amino acid conjugates

In 2010, Gmeiner *et al.* reported the synthesis of doxycycline-amino acid conjugates.²¹⁰ The purpose of the study was to design a compound that would specifically bind the revTet(R) protein, which displays the opposite phenotype to the Tet(R) protein (Section 1.4.5.1, *vide supra*); it binds to DNA to prevent transcription only in the presence of tetracycline compounds. The Tet(R) and revTet(R) system were implicated in gene regulation in bacterial

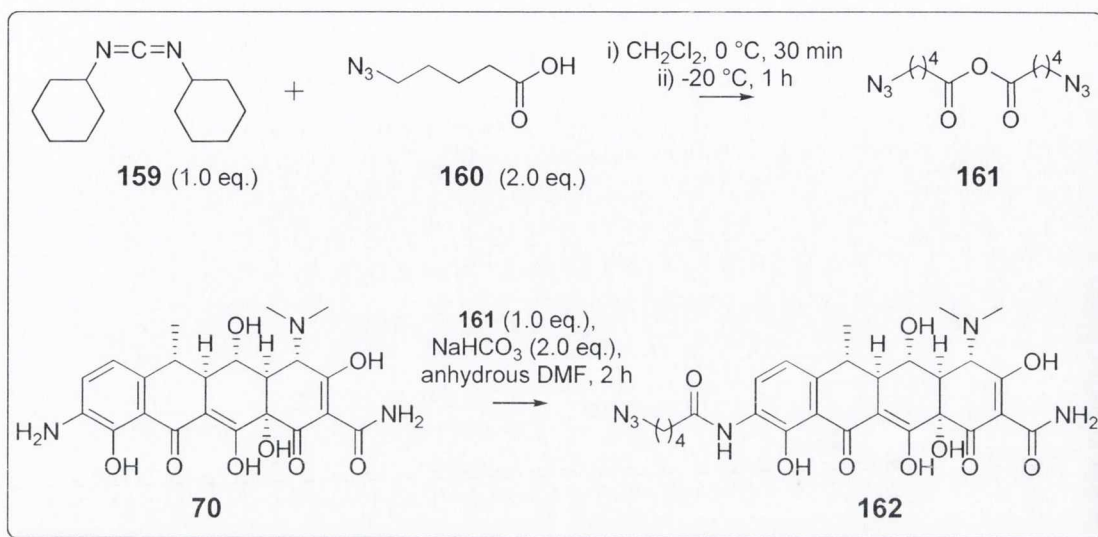
systems.²¹⁰ The substituted peptides and doxycycline analogues were linked *via* the Sharpless ‘click-chemistry’ triazole formation (Scheme 1.42, *vide infra*) with the alkynyl doxycycline derivative **158** (Scheme 1.40) or azido doxycycline derivative **162** (Scheme 1.41, *vide infra*) and a range of azido/alkynyl terminally functionalised peptides. The synthesis detailed in Scheme 1.40 involves the formation of the anhydride **157** which is reacted with **70** to furnish **158** in 23% isolated yield after purification by reverse phase HPLC.²¹⁰

Scheme 1.40 **Synthesis of 9-(5-hexynoylamino)doxycycline (150)**



An azido doxycycline derivative, **162**, was synthesised by the route depicted in Scheme 1.41, which also involves an anhydride formation. *N,N*-dicyclohexylcarbodiimide (**159**) was reacted with **160** to produce anhydride **161**, which was subsequently reacted with **70** to produce the azido functionalised doxycycline derivative **162** in 23% yield after purification by reverse phase HPLC (Scheme 1.41, *vide infra*).²¹⁰

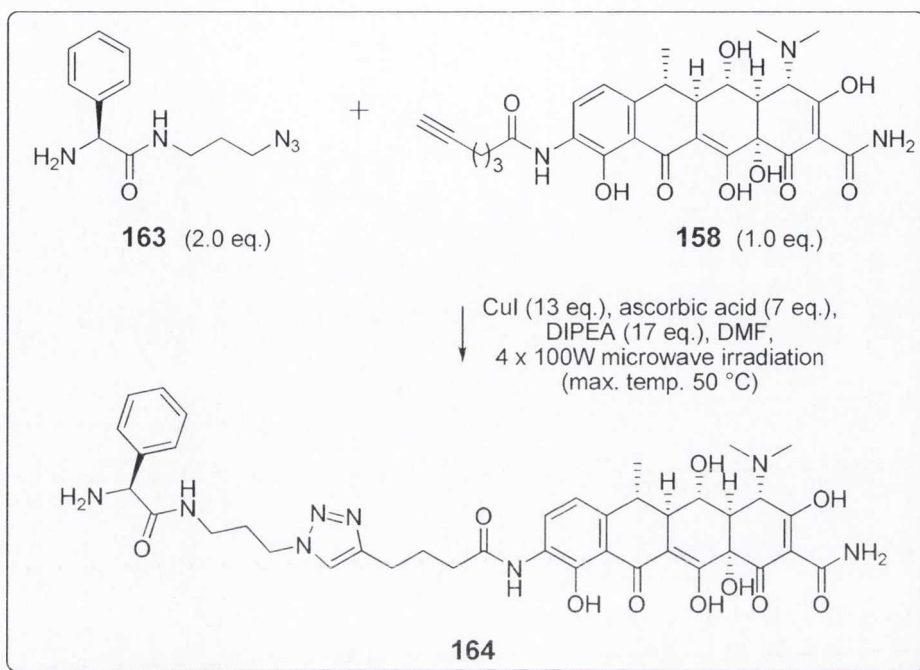
Scheme 1.41 **Synthesis of 9-(5-azidopentanoylamino)doxycycline (154)**



A series of conjugates were evaluated for binding specificity of revTet(R) over Tet(R) and **164** was found to be the most active conjugate. A general synthetic procedure was employed for the triazole formation of all the conjugates formed. This is exemplified in Scheme 1.42 (*vide infra*) by the synthesis of compound **164**, in 53% yield, from phenyl glycine derivative **163** and doxycycline derivative **158**.

13 Equivalents of the normally catalytically employed copper(I)iodide were necessary as peptides and tetracyclines are known to chelate monovalent and divalent metal ions.^{10, 211, 212} The choice of solvent was also found to be crucial. Freshly distilled, degassed, anhydrous DMF was chosen to dissolve all reagents and reactants. The instability of tetracyclines at high temperatures, extreme pHs and in the presence of light and oxidising agents was also taken into consideration. The microwave irradiation was performed in short intervals, with a maximum temperature of 50 °C and the reaction was cooled to -10 °C between each irradiation. These measures limited the degradation of the tetracycline during the reaction. All the conjugates synthesised were purified by reverse phase HPLC.²¹⁰

Scheme 1.42 Sharpless triazole formation of doxycycline-peptide conjugates

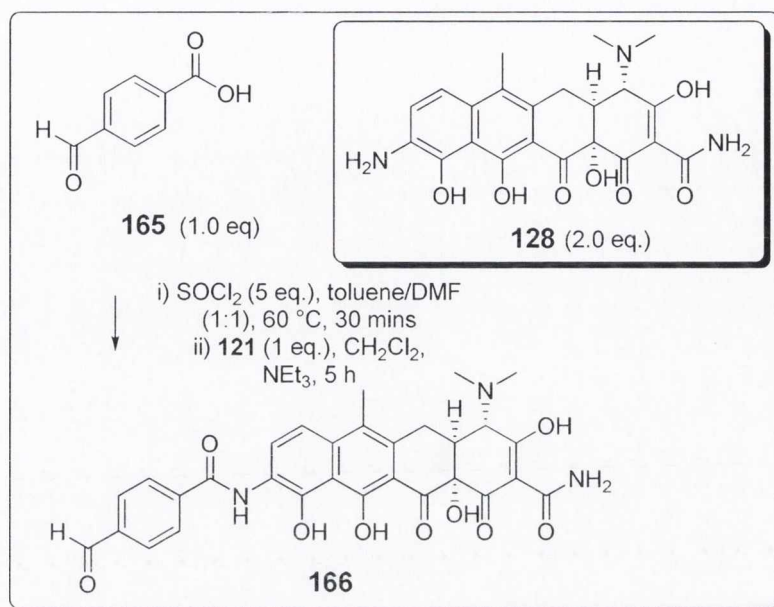


1.7.5.3 Anhydrotetracycline-peptide conjugates

In 2010, Gmeiner *et al.* reported the synthesis of anhydrotetracycline-peptide conjugates for the transactivation of gene expression.²¹³ Transactivation is an increased rate of gene expression which is induced by a transactivating protein. The conjugates synthesised contained an anhydrotetracycline (**4**) moiety, which has a high binding affinity for Tet(R) and revTet(R) regulation proteins, and the minimum transactivation sequence derived from the VP16 transactivation protein of the Herpes simplex virus, VP1 (amino acid sequence: DFDLDMLG). The aim of this study was to synthesise conjugates which would be powerful transactivating systems for use in gene regulation.²¹³

The synthesis of the most potent transactivating system produced, **168**, is depicted in Scheme 1.43 and Scheme 1.44 (*vide infra*).

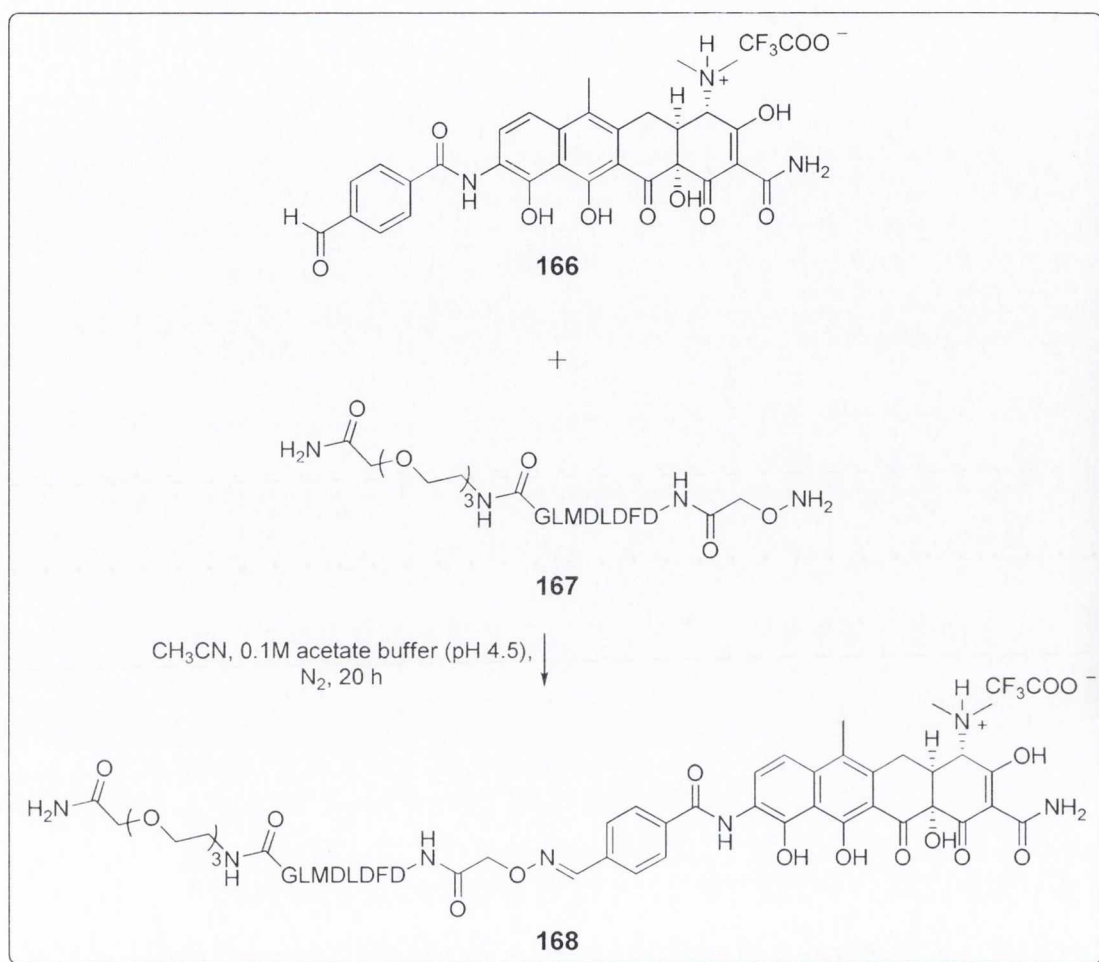
Scheme 1.43 Synthesis of anhydrotetracycline analogue 166



While the doxycycline conjugates synthetic pathway involved the coupling of an anhydride to the amino-tetracycline, the synthetic strategy was modified to prepare anhydrotetracycline conjugates. On this occasion, an acid chloride was produced from compound **165** and the crude product reacted with the amino-anhydrotetracycline to produce compound **166**, which was purified by reverse phase HPLC and isolated as a trifluoroacetic acid salt in 55% yield (Scheme 1.43).²¹³

Compound **166** was then conjugated to the polypeptide **167** by imine formation (Scheme 1.44, *vide infra*). The conjugated product, **168**, was again purified by reverse phase HPLC and two isomers obtained in 33% and 19% yields. Compound **168** was subjected to evaluation and proved to be the most active conjugate, inducing 4 times the expression of luciferase (the reporter protein) in HeLa cells than that of anhydrotetracycline (**4**). This study has shown that anhydrotetracycline-peptide conjugates may be useful compounds in the field of gene regulation.²¹³

Scheme 1.44 **Synthesis of anhydrotetracycline-peptide conjugate**



1.8 Structure activity relationships

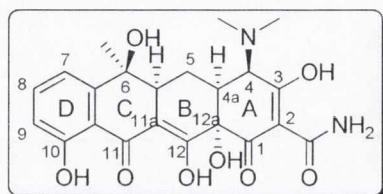
1.8.1 Antibacterial activity

In order to design new tetracycline analogues for use as antibiotics, there are a number of structure activity relationships that must be considered. These have been identified over the course of the last 60 years by the biological evaluation of chemically modified tetracycline analogues. Figure 1.5 (*vide supra*) depicts the numbering of the tetracycline nucleus and ring nomenclature used throughout this section.

To preserve antibacterial activity the 6 membered, fused, carbocyclic tetracycle must be maintained.⁴ The only known exceptions to this are the 6-thiatetracyclines; however they

are classed as atypical tetracyclines and are of less therapeutic value.²¹⁴ The naturally occurring stereochemistry at positions 4, 4a and 12a must be conserved, 4-*epi* tetracyclines have been particularly well studied and are known to be almost devoid of antibacterial activity (Figure 1.25).²¹⁵ It is, in fact, a common consideration in tetracycline chemistry to design routes which minimise the epimerisation of tetracyclines at this position, as pH 2-6 are known to increase the rate of epimerisation.²¹⁵

Figure 1.25 Ring nomenclature and numbering in 4-*epi* tetracyclines



The keto-enol system at positions 11, 11a and 12 are also essential for antibiotic activity, as are the enols at positions 1 and 3.⁴ This is, in part, due to their metal chelating abilities and it has been shown that the chelation of divalent metal ions is essential for antibacterial activity.^{216, 217} The carboxamide moiety at position 2 has been shown to be necessary for accumulation of tetracyclines within bacterial cells, and so removal of this group results in a loss of antibacterial activity.²¹⁸

On the D ring, substitutions that improve antibiotic activity include chloro, bromo, nitro or dimethylamino groups at position 7. The glycylcycline antibiotics display the greatest activity against tetracycline resistant bacteria when the side chain at position 9 is a *t*-butylglycylamido moiety, as in tigecycline (**19**, Figure 1.12, *vide supra*), so steric bulk at this position is essential for activity.¹⁰¹

In atypical tetracyclines it is the relative planarity of the B, C and D rings and overall lipophilicity of the compounds that is responsible for their bactericidal effect.⁴ Conformational studies on the atypical tetracycline anhydrotetracycline (**4**, Figure 1.2, *vide supra*) concluded that it exists in solution almost exclusively in an 'extended' more planar conformation, while oxytetracycline (**106**, Scheme 1.19, *vide supra*) exists predominantly in a 'twisted' less planar conformation.²¹⁹

1.8.2 Factors affecting binding of tetracycline to Tet(R)

In 1996, Hillen *et al.* performed a study on a variety of tetracycline analogues to determine their binding constants with the Tet(R) protein, *i.e.* their ability to induce the expression of the Tet efflux proteins.²²⁰ Anhydrotetracycline (**4**) exhibited the highest binding constant, 4 times higher than that of the next best compound, methacycline (**109**). Comparing oxytetracycline to substituted oxytetracyclines provided information on how substitution affects binding to Tet(R). The results of this study are summarised in Table 1.5 with the binding constants reported as $K_A \times 10^9$ (M^{-1}).

Table 1.5 Biding constants of tetracyclines and Tet(R)

Entry no.	Compound	$K_A \times 10^9$ (M^{-1})
1	anhydrotetracycline	100.00 ± 20.000
2	methacycline	24.00 ± 3.00
3	oxytetracycline	3.30 ± 1.300
4	5-hydroxyoxytetracycline	2.60 ± 0.900
5	4-de(dimethylamino)oxytetracycline	no binding
6	6-DOT	1.80 ± 0.800
7	6-Demethyloxytetracycline	0.17 ± 0.080
8	6-DMDOT	8.00 ± 4.000
9	9-amino-6-DMDOT	1.20 ± 0.700
10	9-amino-8-chloro-6-DMDOT	8.10 ± 3.700
11	4- <i>epi</i> -oxytetracycline	0.01 ± 0.002
12	7-chlorooxytetracycline	10.00 ± 5.000
13	DMG-DMOT	0.64 ± 0.110

A number of conclusions can be deduced from these data. In the first instance, the dimethylamino group at position 4 is essential for binding to the Tet(R) protein, as its removal results in a complete loss of binding (entry 5). The presence of a hydroxyl group at position 5 (entry 4), or an amino (entry 9) or dimethylglycyl group (entry 13) at position 9 results in a loss of activity. Removal of both the hydroxy and methyl groups together at position 6 (entry 8) results in an improvement in Tet(R) binding, as does the insertion of a chloro moiety at

either position 7 (entry 12) or position 8 (entry 10). This information is useful for the synthesis of tetracycline analogues which will be less likely to induce the resistance response in bacteria by binding to Tet(R) and allowing the transcription of DNA coding for efflux proteins.

1.8.2.1 The crystal structure of tetracycline bound to Tet(R)

In 1994, Hinrichs *et al.* reported the first analysis of a crystal structure of a tetracycline, 7-iodo-6-DMDOT (**43**) (Scheme 1.3, *vide supra*) with the Tet(R) protein.²²¹ This crystal structure identified the specific interactions between the individual moieties of the tetracycline and the amino acid residues of the Tet(R) protein's binding domain. This information substantiates the deductions made in Section 1.8.2 and the SAR information in Section 1.8.1.

An Mg^{2+} ion is chelated to **43** at the ketoenolate at positions 11 and 12 (Figure 1.5, *vide supra*). This Mg^{2+} is bound also by Histidine-100 and three water molecules to form an octahedral geometry, with the two of the water molecules also bound to Glutamic acid-147.²²¹ The amide oxygen at position 2 and negatively charged O^- at position 3 in **43** (see Figure 1.5/Figure 1.25 for numbering) are bound by two hydrogen bonds to Glutamine-116 and Histidine-64. The negatively charged O^- at position 3 and positively charged dimethylamine at position 4 are bridged by hydrogen bonding to the amide side chain of Asparagine-84.²²¹ This explains why the removal of the dimethylamino moiety at position 4 results in a greatly reduced ability of the tetracycline analogue to bind Tet(R). The hydroxyl group at 12a forms an aromatic hydrogen bond with phenylalanine-86. Valine-113 forms an unfavourable van der Waals interaction with the hydroxyl group at position 6,²²¹ which also agrees with the SAR and binding data suggesting that the removal of this group results in a greater binding affinity of the tetracycline analogue with Tet(R).

In 1999, Hinrichs *et al.* reported the crystal structure of DMG-DMDOT (**17**, Figure 1.10, *vide supra*) and the Tet(R) protein.⁸⁴ They ascertained that Methionine-177 forms a van der Waals interaction with the dimethylglycylamido side chain. This prevents the 'sliding door' of the entry channel, described in Section 1.4.5.1 (*vide supra*), from closing as it would in the presence of other tetracyclines. The hydrogen bond between the oxygen of the hydroxyl group at position 10 and Arginine-104 is weakened in this case also.⁸⁴ In addition, the interactions between amino acid residues within the Tet(R) protein are interrupted by the presence of **17**.

The π -stacking interaction that occurs between Histidine-151 and Arginine-104 in the complexes of Tet(R)/oxytetracycline (**106**) and Tet(R)/7-chlorotetracycline are disrupted in the complex of Tet(R)/**17** by a much weaker cation- π interaction between the positively charged amine of the dimethylglycylamido group of **17** and Histidine-15 of the Tet(R) protein.⁸⁴ There are also perturbations of the interactions between the Mg^{2+} ion chelated to **17** and the water molecules that would bind to form the octahedral complex, as in the example described by Hinrichs *et al.*^{84, 221} This reduces the overall binding of **17** to Tet(R) and it is deduced that the cause of this is the prevention of the water molecules from positioning themselves in the optimal manner due to disruption of the entrance to Tet(R) by the dimethylglycylamido side chain of **17**.⁸⁴ These effects combined explain the dramatic loss of binding affinity of **17** observed by Hillen *et al.*²²⁰

Chapter 2

Results and discussion: minocycline analogues and minocycline linkers

2 Results and discussion: minocycline analogues and minocycline linkers

2.1 Project aims

As discussed in the introduction, tetracycline antibiotics are important clinical compounds in combating bacterial infections. As such, it was decided to attempt to synthesise tetracycline antibiotic dimers in an effort to develop new analogues with activity against tetracycline resistant bacteria. In the first instance, the aim of the synthetic strategy was to develop dimers consisting of:

- i) a tetracycline analogue, with a high affinity for the Tet(R) regulation homodimer, capable of inducing the expression of efflux proteins in the cell and
- ii) a tetracycline analogue which is not a substrate for the efflux protein itself

In 1989, Eckert and Beck reported that the over-expression of the Tet(A) efflux protein resulted in cell death in *E. coli*.²²² The mechanism of cell death was investigated and characterised as a disruption of the Donnan potential of the cell membrane. It was thought that the dimers designed would induce the expression of resistance efflux genes and remain unsuitable substrates for the efflux protein. The inability of the efflux protein to expel the dimer from the cell would lead to over-expression of the efflux protein within the cell membrane, causing a disruption of the membrane potential leading to cell lysis and death. To the best of our knowledge this was the first attempt at using the bacterial cell's resistance mechanism against itself.

The potential candidates for inclusion in the dimers are shown in Figure 2.1 (*vide infra*) and their biological properties are listed in Table 2.1 (*vide infra*).

Figure 2.1 Drug candidates for inclusion in dimers

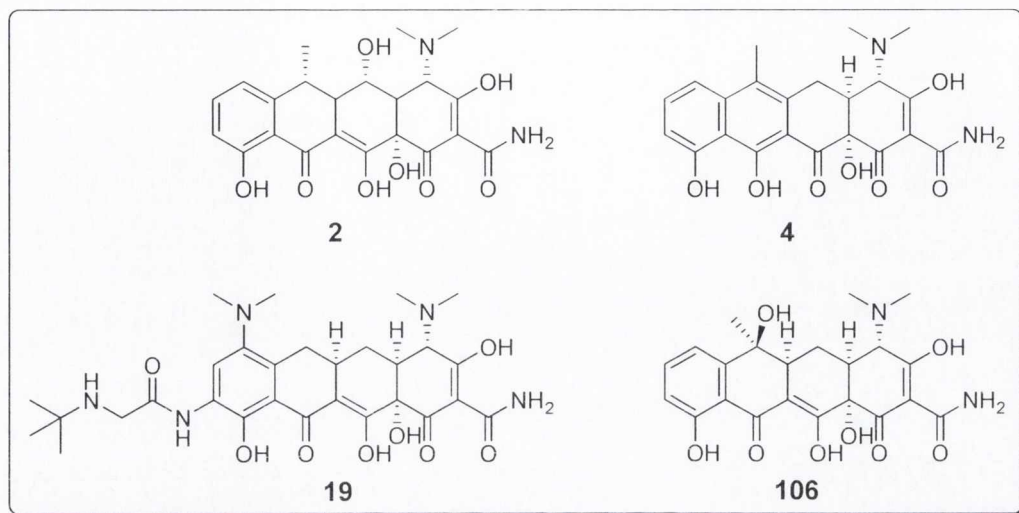


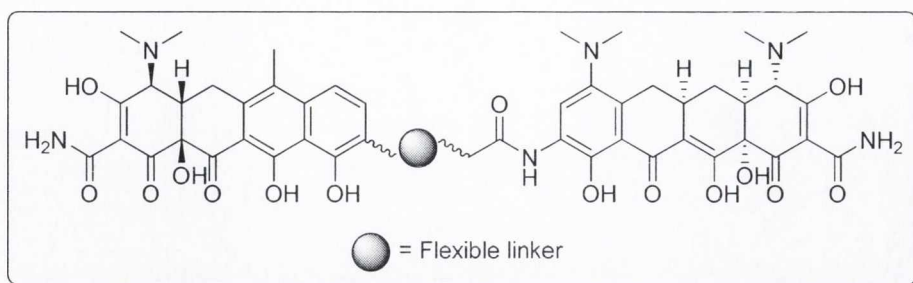
Table 2.1 Biological properties of drug candidates

Entry no.	Compound no.	Compound name	Typical/ Atypical	Tet(R) binding 10^9 M^{-1}	Tet(A) susceptible
1	2	Doxycycline	typical	1.5 ± 5.0^c	yes
2	4	Anhydrotetracycline	atypical	100.0 ± 20.0^b	-----
3	19	Tigecycline	typical	Poor ^a	no
4	106	Oxytetracycline	typical	3.3 ± 1.3^b	yes

^a Poor association with Tet(R). For comparison, a 6-demethyl-6-deoxy tetracycline analogue with the 9-dimethylglycylamido group has an association constant of $0.64 \pm 0.110 \times 10^9 \text{ (M}^{-1})^{220}$ ^b Reference ²²³. ^c Reference ²¹.

Potential therapeutic targets were designed including, tigecycline (**19**), which is not a substrate for the efflux protein, and anhydrotetracycline (**4**), which has a high binding affinity for the Tet(R) regulation homodimer and is therefore capable of strong induction of the expression of the Tet efflux proteins. These two tetracyclines were to be linked *via* a relatively flexible tether (Figure 2.2, *vide infra*), the exact structure of which was variable (Figure 2.3, *vide infra*).

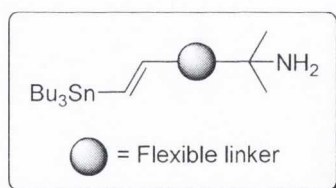
Figure 2.2 General dimer structure



A minocycline (**3**) incorporating the 9-*t*-butyl group could mimic the antibiotic action of tigecycline (**19**). Anhydrotetracycline (**4**) has an extremely high association constant with Tet(R). It was expected that this dimer would induce the over-expression of the efflux protein without being removed from the cell, thus causing cell lysis and death.

The linker was to be attached to the anhydrotetracycline (**4**) moiety by a palladium catalysed cross coupling reaction, such as the Suzuki, Heck or Stille reaction. The Stille reaction was deemed to be the most suitable choice of cross coupling reaction as it does not require the presence of a base (tetracycline compounds can be unstable in the presence of a base). The tigecycline mimic was to be synthesised *via* an S_N2 reaction between the *t*-butylamine moiety of the linker (Figure 2.3) and a (2-chloro)acetyl functionalised analogue of minocycline (**3**).

Figure 2.3 General linker structure



Since it has been shown that antibiotic dimers are often effective in the treatment of resistant bacteria,²²⁴ it was hoped that these linked tetracyclines would show therapeutic promise by exhibiting increased *in vitro* activity relative to their respective monomers.

The linker was to be attached at position 9 in both tetracyclines. There are a number of reasons why it is necessary to link the antibiotics at this position:

- i) This position, *ortho* to the phenol group, is relatively reactive in electrophilic aromatic substitution (EAS) chemistry.
- ii) SAR information shows that a number of varied substitutions at this position are tolerated and, in some cases, increase antibiotic activity.²²⁵
- iii) The orientation of tetracycline when bound to Tet(R):
 - a) Figure 2.4 (from the Brookhaven database) shows the binding site of 2 tetracycline molecules within the Tet(R)₂ dimer.
 - b) Figure 2.5 illustrates the orientation of each tetracycline within the binding pocket of the protein. The yellow arrow indicates position 9 in the tetracycline structure. It is clear from the crystal structure that the tetracycline D-ring is held near the exterior of the binding pocket, thus it is reasonable to attach the long linker at position 9 as there is a relatively open space available and disruption of the protein-ligand interactions would be minimised.

Figure 2.4 **Two tetracycline molecules bound within the Tet(R) dimer**

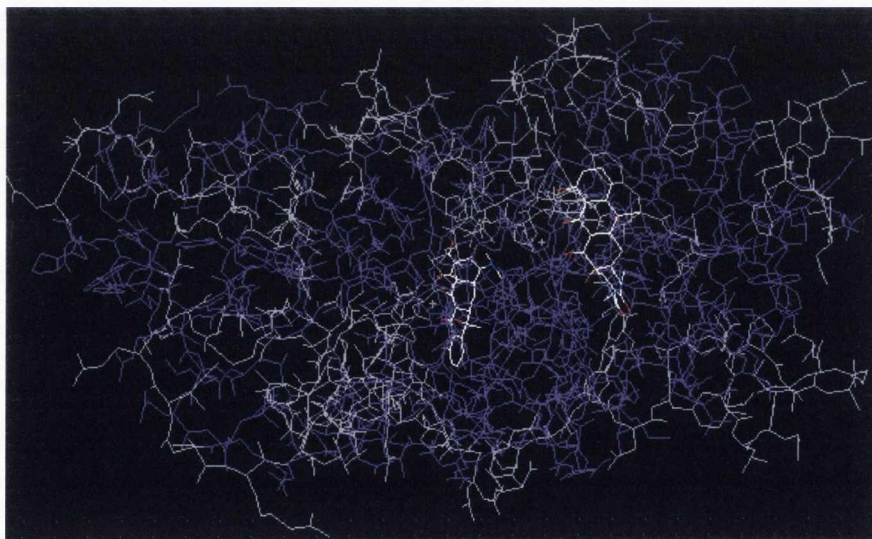


Figure 2.5 **The orientation of tetracycline within the binding pocket of Tet(R)**



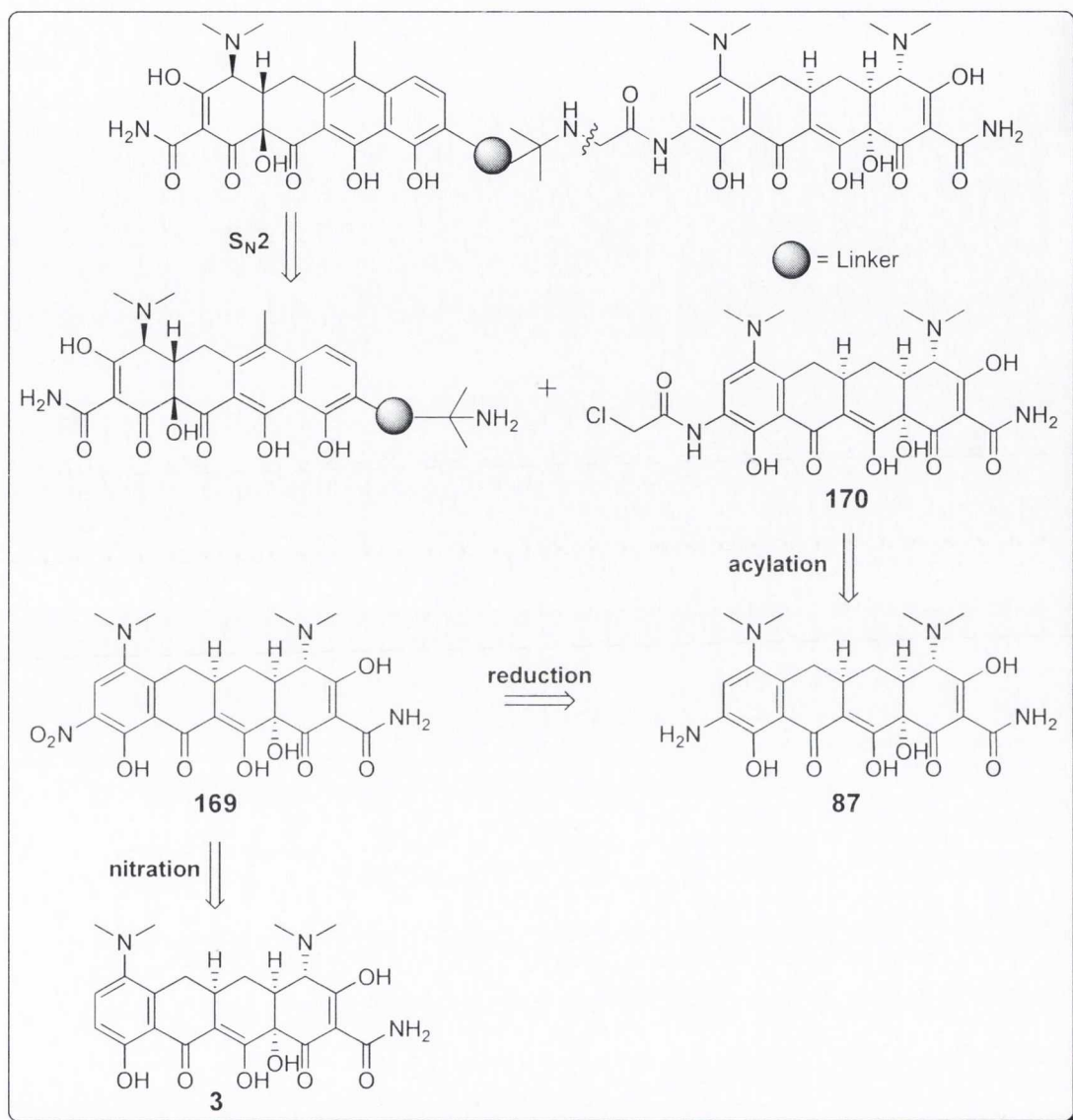
Glycylcycline derivatives of tetracyclines have previously been shown to possess excellent *in vivo* activity against resistant strains of bacteria and SAR studies have shown that long-chain aliphatic moieties are tolerated at position 9.²²⁵ The crystal structure of DMG-DMOT (**33**) clearly shows that the glycylamido moiety of the compound is threaded out along the open space at the entry of the binding pocket and it was hypothesised that our linkers would behave in the same way if linked at position 9.⁸⁴

The antibiotic chelocardin differs from anhydrotetracycline (**4**) by a methyl group at position 9 and is a more effective antibacterial agent.²²⁶ This raises the possibility that a long-chain alkyl group at position 9, which is the proposed method of linkage, may be tolerated with respect to the activity of anhydrotetracycline (**4**).

2.2 Retrosynthesis of minocycline dimer half

The general structure of the target tetracycline dimer is shown in Scheme 2.1 (*vide infra*) along with the retrosynthetic analysis of the minocycline dimer half.

Scheme 2.1 Retrosynthesis of minocycline dimer half



2.3 Synthetic route 1

2.3.1 Foreword

It was expected that the synthesis of the minocycline analogues would be relatively facile; however when undertaking the project we were unaware of the purification and instability issues that we would face. Tetracycline compounds are known to be unstable in the presence

of excessive heat, light, base and oxidising agents.²²⁷⁻²²⁹ It is also known that tetracyclines epimerise at a range of pH's, most rapidly between pHs 2 and 7.²¹⁵

Papers published on complicated syntheses of tetracycline derivatives have included the monitoring of reactions and purification of products by reverse-phase HPLC or reverse-phase preparative TLC (as the compounds are not suitable for normal phase chromatography),^{182, 193, 213, 230} which was unfortunately not available during the course of this research. Recrystallisation and pH gradient extraction were the only methods of purification available during the first two years of the project, after which time a reverse phase medium pressure liquid chromatography (RP-MPLC) system was installed in our department.

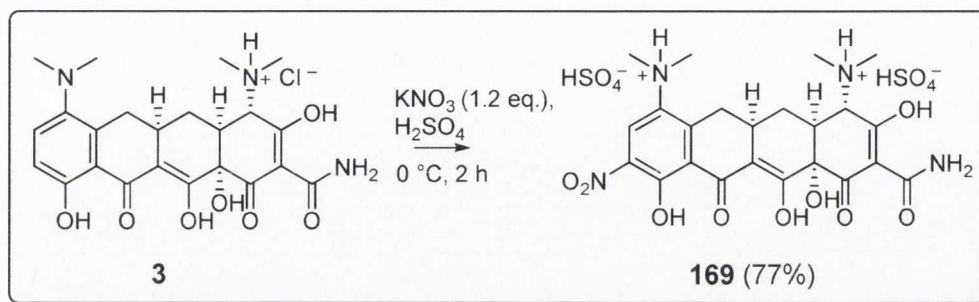
The majority of the tetracycline transformations performed have also been published without experimental reaction conditions and, as such, it was necessary to devise reaction conditions *ab initio* for previously reported compounds. This is primarily because the vast majority of tetracycline research is carried out by industrial institutions *e.g.* Pfizer Inc. and Paratek Pharmaceuticals, and discoveries are patented without the disclosure of characterisations or experimental detail.

The synthesis of tetracycline analogues proved to be challenging due to a number of unforeseen problems associated with the chemical and physical properties of these molecules. In a number of cases these problems proved to be insurmountable despite considerable synthetic effort. The methodologies used and alternative strategies investigated are discussed in detail in the results and discussion chapters.

2.3.2 Synthesis of 9-(2-chloro)-acetamidominocycline

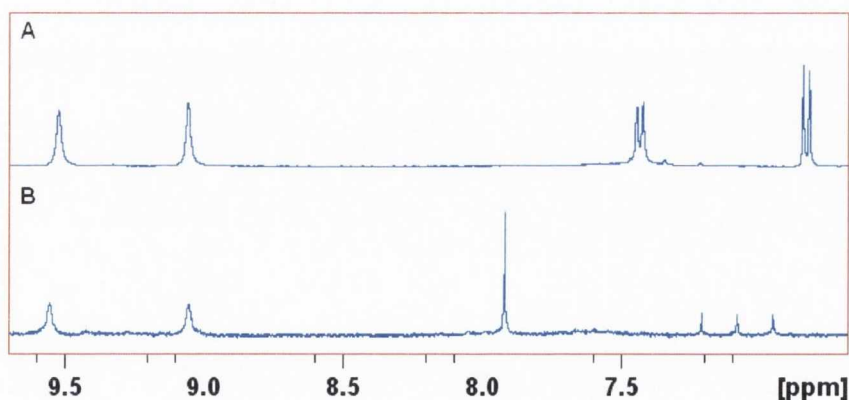
The first step in this synthetic process (Scheme 2.1, *vide supra*) is the nitration of minocycline hydrochloride (**3**) with 95-97% H₂SO₄ and KNO₃ to afford 9-nitrominocycline disulfate (**169**) in good yield (Scheme 2.2, *vide infra*). Isolation and purification of this compound proved to be troublesome due to its deliquescent nature. Recrystallisation attempts using methanol, ethanol and diethyl ether proved unsuccessful. Triturating the compound in diethyl ether proved to be the most useful method of purification.

Scheme 2.2 Synthesis of 9-nitrominocycline disulfate (169)



The product was identified as **169** using NMR spectroscopy and HRMS techniques. The assignment of ^1H NMR spectroscopic data of analogues of minocycline (**3**) was aided using the NMR spectroscopic work of Nilges *et al.*²²⁹ ^1H NMR spectroscopy revealed the disappearance of one of two aromatic resonance signals, observed at 6.84 and 7.44 ppm in the spectrum of the starting material (**3**), and the appearance of the aromatic resonance signal of the product (**169**) as a singlet, shifted significantly downfield (to 7.92 ppm) with respect to the aromatic resonance signals of **3** (Figure 2.6). This indicated the presence of an electron withdrawing group on the aromatic ring which, due to the directing effects of the phenolic moiety of **3**, would be at position 9. HRMS confirmed the presence of the desired molecular ion (Found: 503.1755, Requires: 503.1778) and the product was confirmed to be **169**.

Figure 2.6 Aromatic regions of ^1H NMR spectra of **3 and **169****



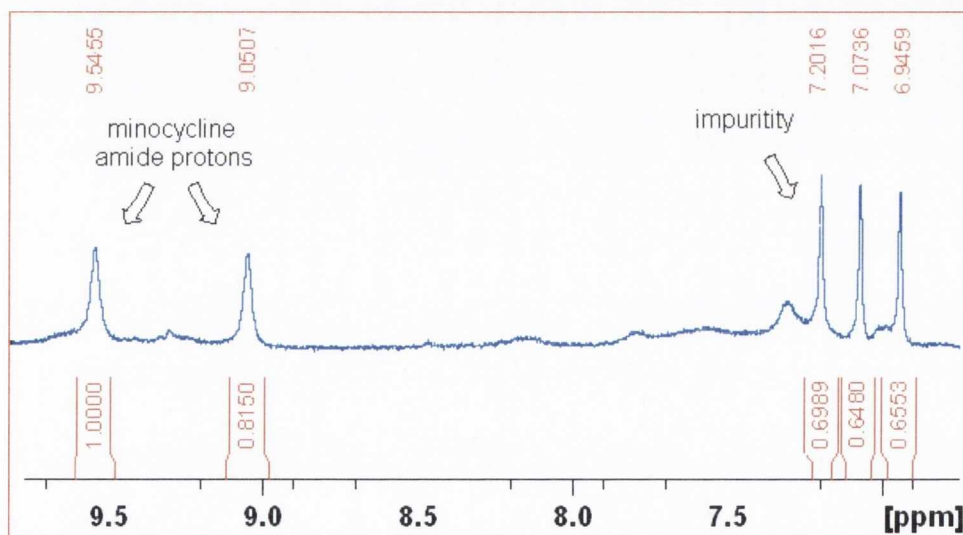
A = Section of the ^1H NMR spectrum of **3**; B = Section of the ^1H NMR spectrum of **161**.

An unidentified impurity was observed in the ^1H NMR spectrum of the product, appearing as three singlets at 6.96, 7.08 and 7.21 ppm. This impurity was found to be present in the

commercially available starting material minocycline hydrochloride (**3**) also, and increased if the compound was not stored below 4 °C under an atmosphere of argon, away from light. The impurity was removed by careful precipitation of minocycline hydrochloride from a minimum volume of methanol by the dropwise addition of diethyl ether at 0 °C. This process was subsequently performed before using minocycline hydrochloride in reactions and all further synthetic analogues of minocycline were stored at -18 °C under an atmosphere of argon.

It was decided to attempt to ascertain the identity of the impurity by using HRMS and NMR spectroscopic methods. The impurity was separable from minocycline analogues by RP-MPLC using 10% acetonitrile in Millipore water as the mobile phase, during which it had a very short retention time (less than 1 minute/column volume). It appeared as 3 singlets in spectra taken in DMSO- d_6 , between 6.9 and 7.5 ppm, each integrating for 1 with respect to each other, separated by 0.13 ppm (51 Hz) each (Figure 2.7, *vide infra*).

Figure 2.7 ^1H NMR spectrum of impurity (in DMSO- d_6)



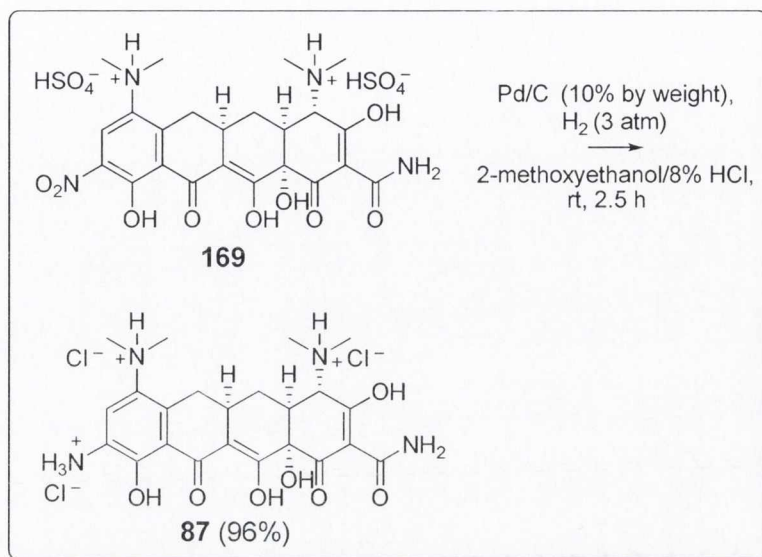
The resonance signals corresponding to the impurity were not connected to any other signals in ^1H NMR spectra when TOCSY and NOE experiments were performed. This compound was found to be inorganic and protic in nature, as it displayed no resonance signals in ^{13}C NMR or HSQC experiments and in ^1H NMR peaks disappeared with the addition of D_2O . As this same impurity was seen in a number of subsequent reactions involving tetracyclines and a wide variety of other reagents, it was inferred that the impurity must be produced by the

decomposition of tetracyclines. With this in mind, we propose that the impurity may be a quaternary ammonium salt, as splitting patterns of this nature can be seen in ^1H NMR spectra of quaternary ammonium salts due to interactions between ^{14}N and ^1H .²³¹ Unfortunately, the molecular ion could not be observed by mass spectrometry as the mass of the molecule was below the limits of detection on our system. However, this observation became extremely useful in later investigations as the decomposition product had a distinctive pattern of resonance signals and so when it was detected in spectra during the monitoring of reactions it was known the minocycline analogue was decomposing under the reaction conditions. Studying the decomposition products of the tetracyclines could also be interesting from a pharmacokinetic point of view, as this impurity would subsequently be observed in reactions performed between ambient temperature and 40 °C over the course of this research.

9-Nitrominocycline dihydrosulfate (**169**) was easily reduced by catalytic hydrogenation using 3 atm of H_2 , 2-methoxyethanol/8% HCl (40:1) and 10% Pd/C (Scheme 2.3, *vide infra*) to furnish the desired product (**87**) as a trihydrochloride salt, which required no further purification, in 96% yield (Scheme 2.3).

This reaction was initially performed with the inclusion of H_2SO_4 in the solvent mixture to furnish the trihydrosulfate salt of the amine. It was observed after the synthesis of this product that it was extremely hygroscopic and therefore difficult to isolate and purify. This was a concern in terms of the use of the product in future reactions. It had been observed in earlier syntheses performed at the beginning of this research project that, in general, the hydrochloride salts of tetracycline derivatives were less hygroscopic than their corresponding hydrosulfate salts. With this in mind, it was decided to produce the trihydrochloride salt of 9-aminominocycline (**87**) instead, by including 8% HCl in the solvent system. Gratifyingly, the hydrochloride salt of compound **87** was noticeably less hygroscopic and easier to handle. The hydrochloride salt of **87** was chosen as the starting material for all future reactions.

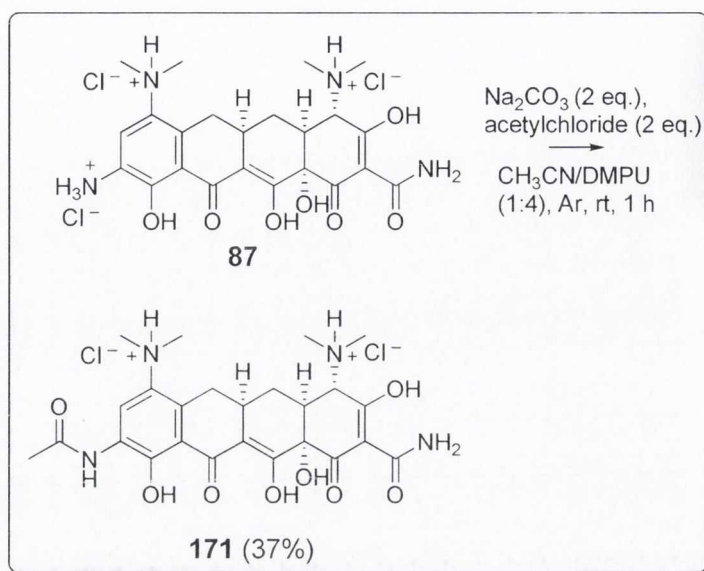
Scheme 2.3 Synthesis of 9-aminominocycline trihydrochloride (87)



The next step in the synthetic pathway required the installation of a (2-chloro)acetamido functionality to amine **87**. The synthesis of 9-acetamidominocycline dihydrochloride (**22**) was well described by Sum *et al.*²³² and it was decided to synthesise this compound first to ensure that the reaction conditions were reliable (Scheme 2.4). If the reaction conditions proved to be reliable they could be modified to afford the synthesis of the (2-chloro)acetamido functionalised minocycline derivative. This reaction would allow for the introduction of a ‘synthetic handle’ at position 9 in minocycline that would allow for further modification at this centre *via* S_N2 reactions.

Compound **171** was synthesised from the reaction of **87** with 2 equivalents of acetyl chloride and 2 equivalents of sodium carbonate at room temperature for 1 hour (Scheme 2.4, *vide infra*). After purification by pH gradient extraction from water and formation of the hydrochloride salt by the addition of hydrogen chloride in anhydrous diethyl ether, compound **171** was afforded as a dihydrochloride salt in a moderate yield (37%). This yield is comparable to that of the literature procedure (Scheme 2.4, *vide infra*).

Scheme 2.4 Synthesis of 9-(acetamido)-minocycline (171)



The procedure was subsequently adapted for the use of chloroacetyl chloride as the acylating agent. This reaction furnished 9-(2-chloroacetamido)minocycline dihydrochloride (**170**), after purification by pH gradient extraction from water and formation of the hydrochloride salt by the addition of hydrogen chloride in anhydrous diethyl ether, in an acceptable yield, 27% (Scheme 2.5). The necessity of the aqueous work up becomes apparent when examining ^1H NMR spectra taken *in situ* from the reaction (Figure 2.8, *vide infra*).

Scheme 2.5 Synthesis of 9-(2-chloro)acetamidominocycline (170)

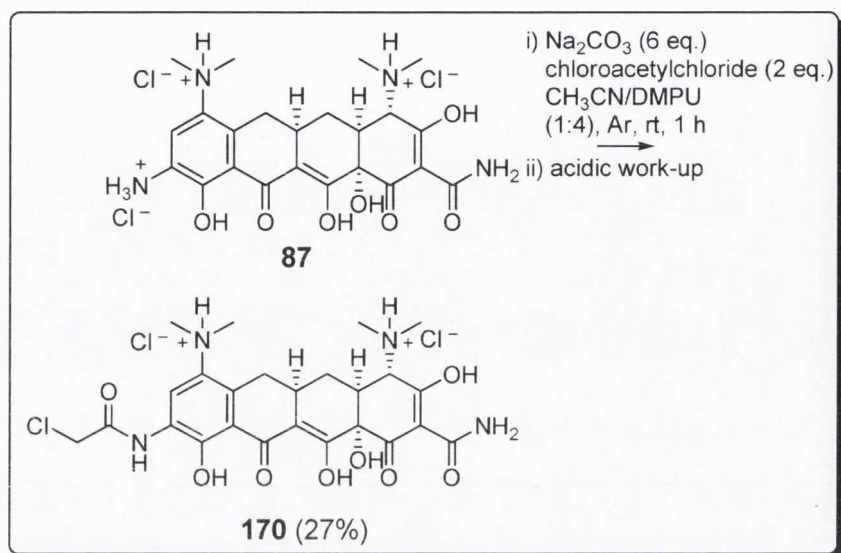
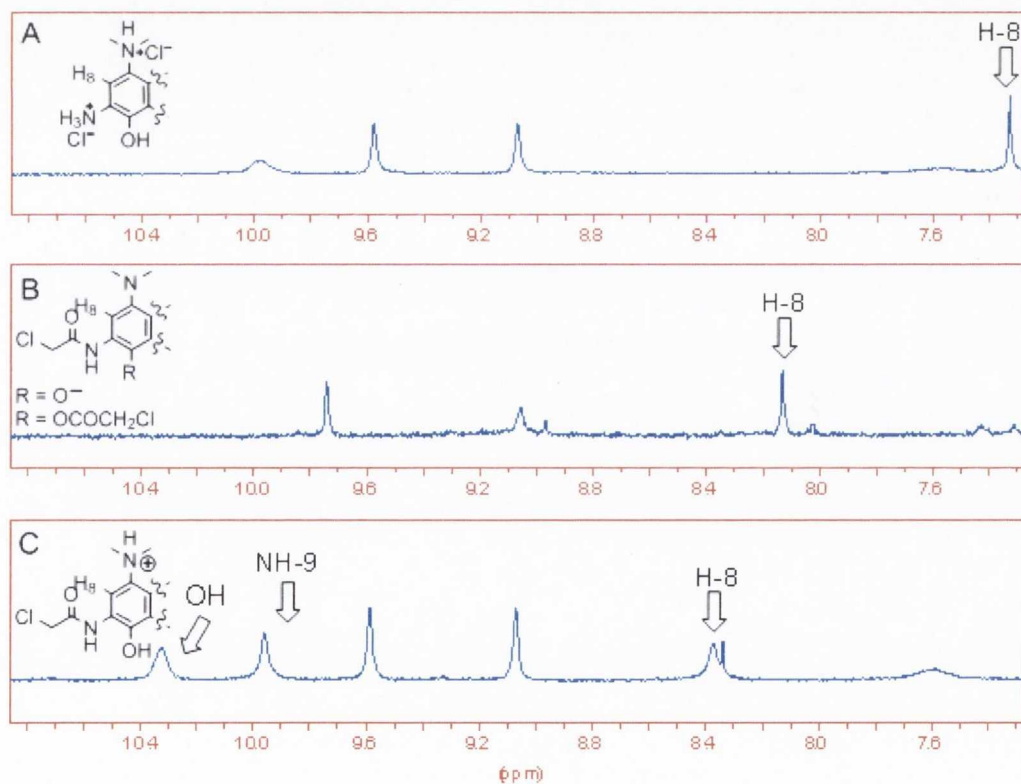


Figure 2.8 ^1H NMR analysis of acetylation reaction



A = Compound **80**; B = *in situ* spectrum: acylation of **80** using chloroacetyl chloride; C = Compound **170**

Spectrum B, shown above in Figure 2.8, was taken from an acylation reaction of **87**, 50 minutes after the addition of 2 equivalents of chloroacetyl chloride, under the previously stated reaction conditions. It was observed that the signal corresponding to H-8 had shifted as expected, however, gross simplification of the structure was observed, with the loss of protic hydroxy signals. This may be due to acylation of a hydroxy group, or deprotonation of the hydroxy groups by the base (as there are six equivalents of dibasic carbonate in the reaction). After aqueous work-up, the signals reappeared in the ^1H NMR spectrum of the product (Figure 2.8, spectrum C). It was determined that the intermediate compound in this reaction may be a *poly*-acylated compound, due to the number of reactive hydroxyl groups in compound **87**. During aqueous work-up however, any esters would be cleaved, furnishing the desired *mono*-acylated product, 9-(2-chloro)acetamidominocycline (**170**, Scheme 2.5, *vide supra*).

Attempts were made to increase the yield of the reaction, as ^1H NMR analysis of the reaction showed the conversion of **87** to **170** to be >99%. However, this proved to be difficult due to the nature of the purification method, pH gradient extraction. 73% of the compound was lost between the aqueous layer and impure fractions. The pure product was extracted from water at pH 3.5 - 4.5 but **170** was also extracted along with other impurities at higher and lower pH values. Brine was added to the aqueous layer also to encourage the extraction; however not all of the product could be forced into the organic layer at the desired pHs. This resulted in the yield for the synthesis of **170** from minocycline (**3**) being just 20% over the three steps; however given that the yields in reported tetracycline chemistry are comparably low,²³² the yield was accepted as it could not be improved.

At this point a methodology was in place for the synthesis of the precursor, **170**, to the minocycline dimer half and so attention was turned to the synthesis of the linkers.

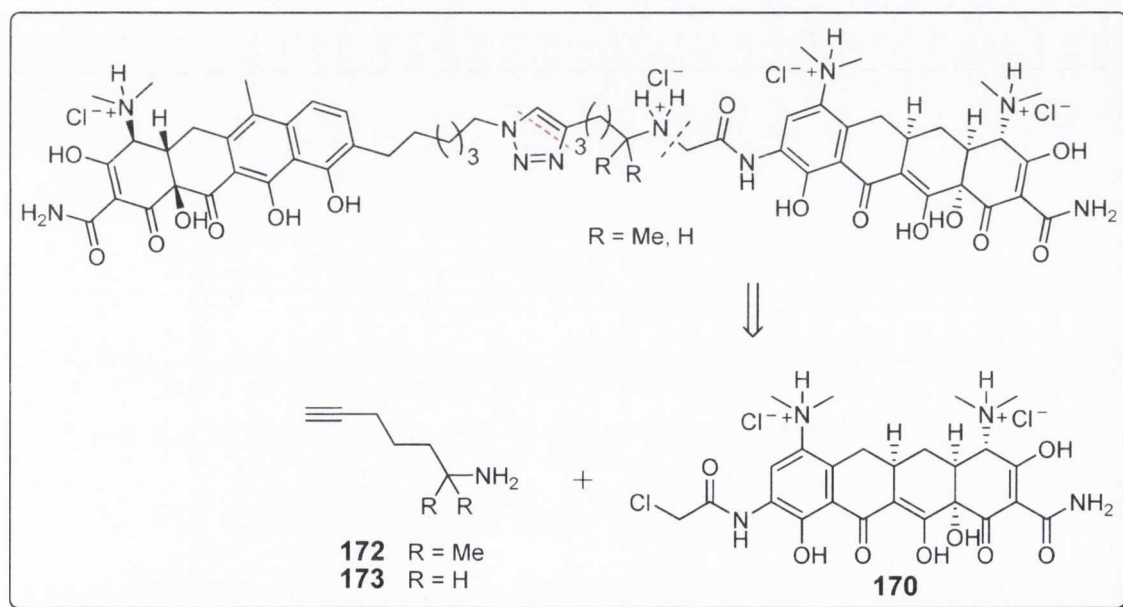
2.3.3 Synthesis of minocycline linker 1 (**172**)

The initial design of the linker involved the synthesis of a complete linker including an organostannane at one end and a tertiary amine at the other. However, it was decided early on that a more versatile route would be to synthesise separate, shorter linkers for each tetracycline. These linkers would first be attached to the individual tetracyclines and subsequently connected to each other to form the dimer. This avoided complications

involving attempting to functionalise one end of a linker molecule without destroying a reactive centre at the other.

The two dimer halves, with linkers attached, would be connected using ‘click chemistry’ to provide the complete dimer. The Sharpless copper catalysed cycloaddition reaction of alkynes and azides was chosen as the method to connect the dimer as it is known to be versatile, high yielding and this reaction avoided any regioselectivity issues which could be encountered when using tetracyclines, which contain a number of varied functional groups.²³³ A diagrammatic representation of the dimer design is depicted in Scheme 2.6, with the revised route to the minocycline dimer half (right of the red hatched line). The synthesis of the anhydrotetracycline half of the dimer is discussed in Chapter 3.

Scheme 2.6 Revised dimer structure and retrosynthesis minocycline dimer half from **170**

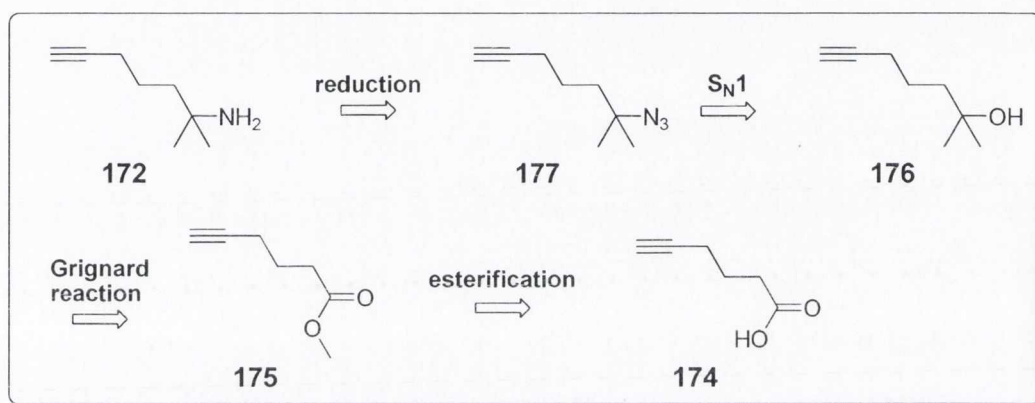


Two linkers, containing a terminal alkyne for the cycloaddition reaction and an amine for connection to **170** via an S_N2 reaction, were designed for the minocycline dimer half. This would allow the synthesis of two different dimers which could be compared during *in vitro* testing. Where R = methyl, it was hoped that the analogue would mimic the antibiotic action of tigecycline (**19**). Where R = H, it was hoped that the analogue would mimic the action of

DMG-MINO (**16**). Both analogues have been shown to be active against bacteria expressing efflux protein resistance mechanisms (Section 1.5.3, *vide supra*).

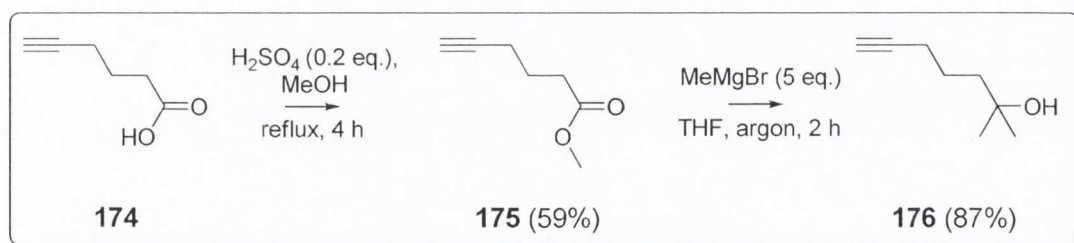
Initially work was undertaken to produce the linker to mimic tigecycline, where R = methyl, compound **172** (Scheme 2.6). A retrosynthesis of **172** to commercially available 5-hexynoic acid (**174**) was designed (Scheme 2.7, *vide infra*).

Scheme 2.7 Retrosynthesis of **172** to **174**



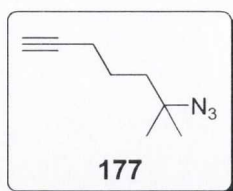
Starting from **174**, an esterification reaction was performed using concentrated H₂SO₄ and methanol to yield compound **175** in 59% yield after purification by Kugelrohr distillation (Scheme 2.8).

Scheme 2.8 Synthesis of **176** from **174**



Ester **175** was reacted in a Grignard reaction using 5 equivalents of methylmagnesium bromide to furnish **176** in 87% yield after column chromatography. The proceeding step in the synthesis was the formation of azide **177** (Figure 2.9) from tertiary alcohol **176**.

Figure 2.9 **Desired azide 177**



It was envisaged that this step could be performed using simple, well known S_N1 chemistry; however this was not the case and the substitution reaction proved to be problematic. The reaction was attempted initially using H_2SO_4 and sodium azide in a biphasic system of aqueous and chloroform layers with alcohol **176**. Table 2.2 lists some of the reaction conditions attempted.

Table 2.2 **Azide formation with Brønsted acid**

Entry no.	Eq. of 176	Eq. of NaN_3	% H_2SO_4 (aq)	Reaction time (h)
1	1	2	48	5
2	1	2	97	5
3	1	10	48	3

Disappointingly, none of the reaction conditions attempted afforded the desired product. In all cases the starting alcohol was converted to another unidentified product; however this product did not display the expected resonance signals of **177** when 1H NMR analysis was performed. Analysis of the reaction corresponding to entry 2 above revealed resonance signals in the region of 4 - 5 ppm. Since these peaks may have been associated with the formation of an alkene due to competition with the elimination reaction over the substitution reaction, it was decided to use 10 equivalents of sodium azide and to lower the acid strength to encourage the desired transformation to **177**. Unfortunately, this did not furnish the azide product and IR analysis of the crude products of the reaction revealed no azide stretching absorbances.

Reactions involving the use of a Lewis acid were attempted to encourage the formation of the desired azide, **177**. Zwierzak *et al.* had previously reported the synthesis of a number of tertiary azides with the use of a tertiary alcohol, borontrifluoride diethyletherate

(BF₃.Et₂O) and trimethylsilylazide (TMSN₃) in benzene at 30 °C.²³⁴ A summary of the reaction conditions attempted can be seen in Table 2.3 (*vide infra*).

Table 2.3 **Azide formation with Lewis acid**

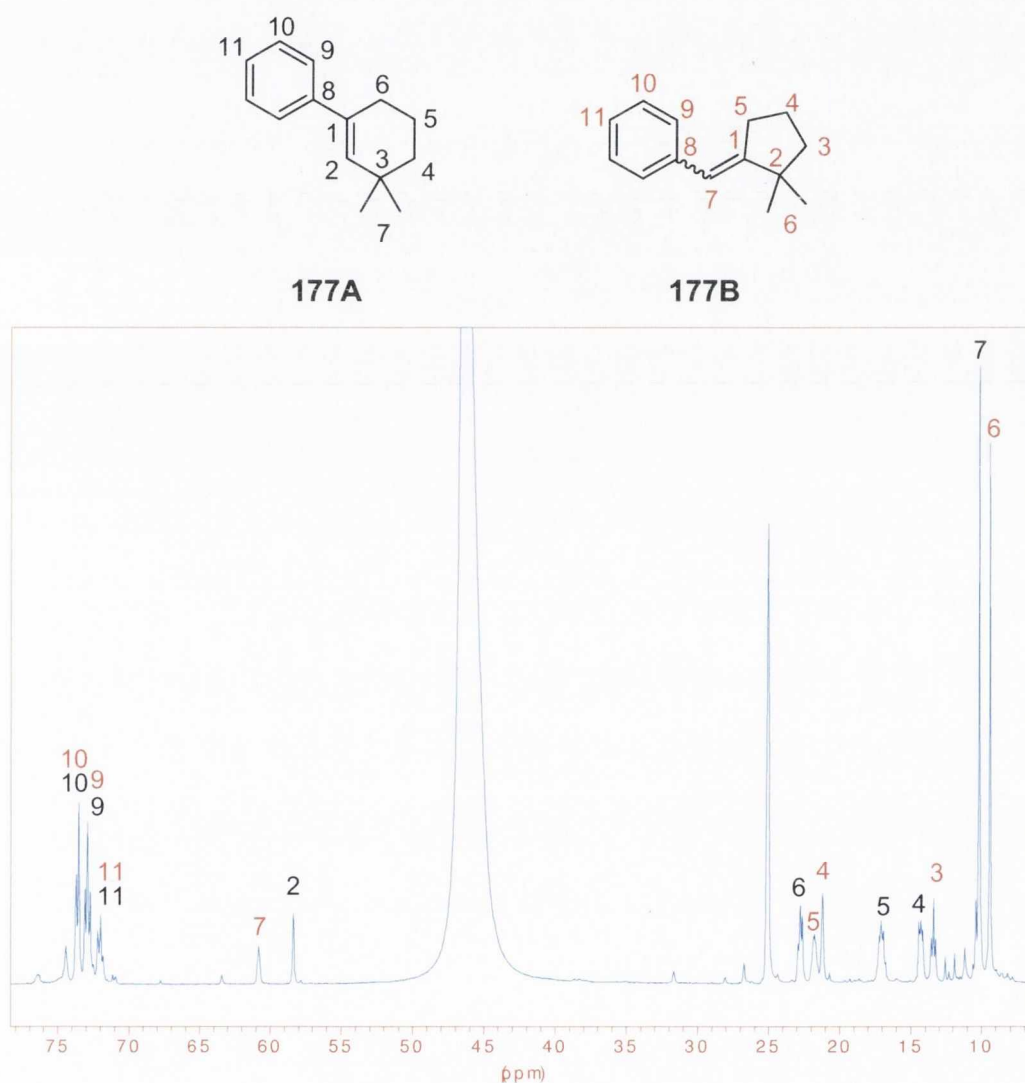
Entry no.	Eq. of 174	Eq. of TMSN ₃	Eq. BF ₃ .Et ₂ O	Reaction time (h)
1	1	1.2	1.2	24
2	1	2	1.2	18
3	1	2.4	2.4	24

Initially following their exact procedure, entry 1 was attempted. Disappointingly, this reaction did not furnish compound **177**. As had been seen with the reactions using Brønsted acids, all of the starting alcohol **176** had been consumed, but compound **177** had not been produced. Two products were observed by TLC (eluent 10% ethyl acetate in hexane) with R_f values of 0.65 and 0.85 indicating the loss of the hydroxyl group. Entry 2 employed 2 equivalents of trimethylsilylazide to encourage azide formation over the unwanted reaction; however this approach failed. A final attempt was made, entry 3, using twice the equivalents of trimethylsilylazide and borontrifluoride diethyletherate reported by Zwierzak *et al.* but to no avail.

Kugelrohr distillation of the crude reaction obtained from entry 3, Table 2.3, provided a clearer view of the products of the reaction. One fraction obtained was shown to contain two very similar compounds which were not separable from each other and were analysed by NMR and IR spectroscopy. ¹H-¹H correlation spectroscopy confirmed the presence of two separate compounds. These compounds will be referred to as **177A** and **177B** and were characterised using NMR spectroscopy, IR spectroscopy and HRMS. The ¹H NMR spectrum and determined structures of **177A** and **177B** are displayed in Figure 2.10 (*vide infra*). NMR spectra agree with the suggested structures, which were determined using ¹H, TOCSY, HSQC, HMBC, ¹³C and DEPT experiments. HRMS analysis provided the desired molecular ion (Found: 186.1414, Requires: 186.1409). IR spectral analysis confirmed the absence of an azido or alkynyl moiety in the structures. The geometry of the double bond in **177B** could not be confirmed by an NOE experiment between the methylene at position 5 and the aromatic proton at position 9 as the chemical shifts of the methylenes at positions 4 and 5 in **177B** and

the methylene at position 6 in **177A** were too similar. However, the most likely geometry of the double bond is that where the benzene ring is in a *trans* position with respect to the dimethyl group of the 1,1-dimethyl-2-methylenecyclopentane ring due to the possibility of steric clashing in the *cis* orientation.

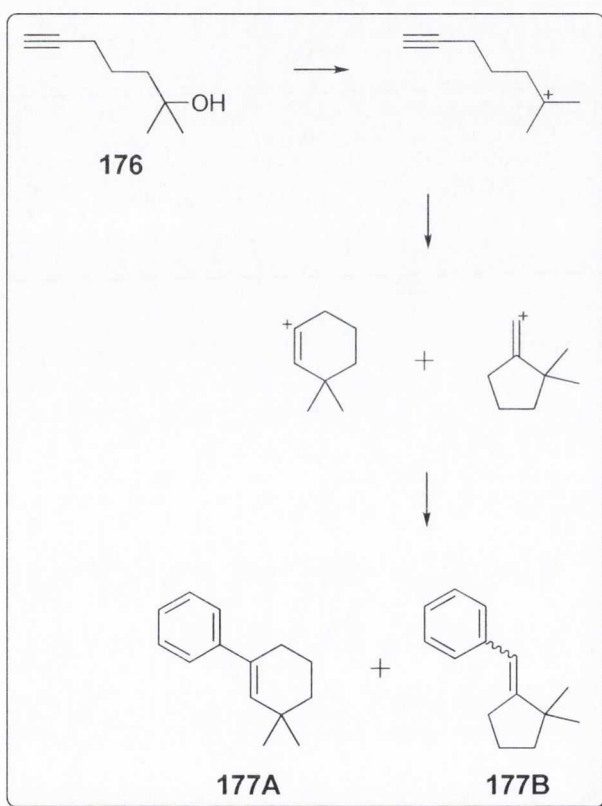
Figure 2.10 Suggested structures of **177A** and **177B** and ^1H NMR spectrum of both



The formation of products **177A** and **177B** is proposed to proceed by the mechanism depicted in Scheme 2.9 (*vide infra*). It is proposed that the formation of the tertiary cation proceeds as

expected, however the intramolecular attack by the π -system of the alkynyl moiety must proceed faster than the intermolecular attack by the azide. This provides the sp^2 cationic intermediates. Since these intermediates are formed by 6-*exo-trig* and 5-*exo-trig* cyclisations, they are both highly favoured. The reaction proceeds by the electrophilic aromatic substitution of benzene to provide the products **177A** and **177B**. The benzene ring is then re-aromatised by the loss of a proton, which could be abstracted by the hydroxide ion produced during the formation of the sp^2 cation.

Scheme 2.9 Proposed mechanism for the formation of **177A** and **177B**



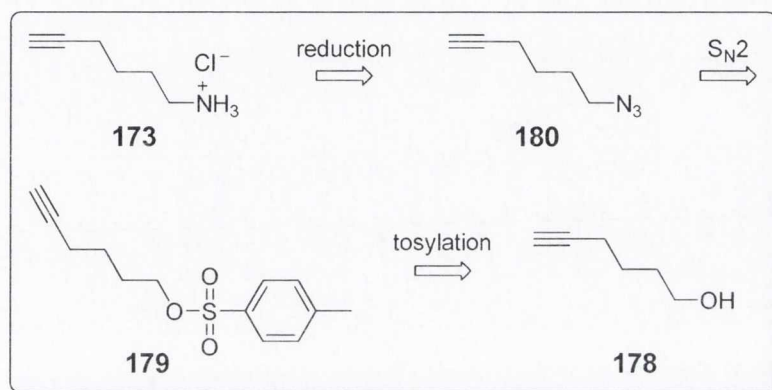
Having attempted a number of unsuccessful reactions to form azide **177**, it was decided to concentrate on the synthesis of the linker for the DMG-MINO mimic, **173** (Scheme 2.10, *vide infra*) as it was thought that this synthesis would prove to be facile.

2.4 Synthetic route 2

2.4.1 Synthesis of minocycline linker 2 (173)

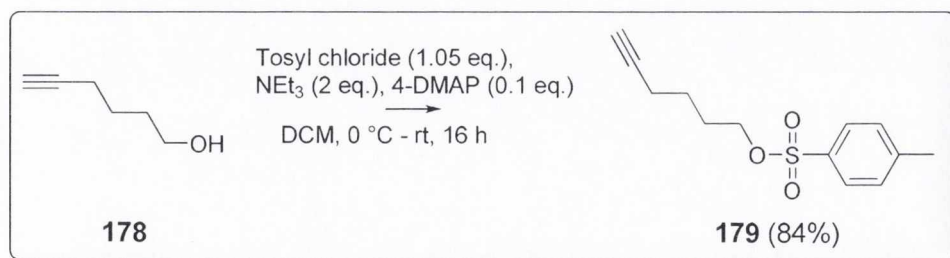
A retrosynthetic analysis of amine **173** to alcohol **178** is illustrated in Scheme 2.10.

Scheme 2.10 Retrosynthesis of amine **173** to alcohol **178**



The first step in the synthesis was the conversion of hex-5-yn-1-ol (**178**) to its tosylated analogue **179** (Scheme 2.11). This conversion was performed using tosylchloride and NEt_3 , with the addition of 4-dimethylaminopyridine (4-DMAP) in catalytic amounts. The product was isolated in 84% yield after purification by column chromatography using a mobile phase of 10% ethyl acetate in hexane.

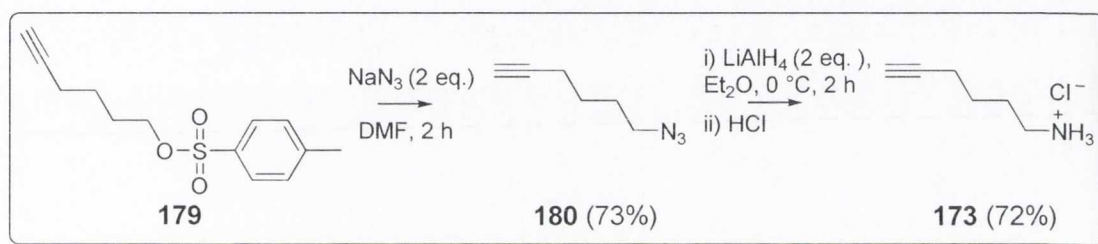
Scheme 2.11 Conversion of alcohol **178** to **179**



Contrary to the functionalisation of the tertiary alcohol **176**, the azidation of the primary alcohol **179** was readily achieved using sodium azide in DMF (Scheme 2.12, *vide infra*). **180** required no further purification and the loss of yield is attributed to the low boiling point of

the molecule. The reaction mixture was diluted with diethyl ether and the DMF removed by aqueous extraction. The diethyl ether was subsequently distilled from the product by fractional distillation. Azide **180** was subsequently reduced to amine **173** using lithium aluminium hydride in anhydrous diethyl ether. When the reaction was deemed to have reached full conversion (TLC analysis) the unreacted lithium aluminium hydride was quenched with water and sodium hydroxide (40% w/v) and the reaction filtered. Hydrogen chloride in anhydrous diethyl ether was then added to the solution of the amine to afford the conversion to hydrochloride salt **173**. The compound was isolated as a salt, in 72% yield, to avoid the issue of volatility and because it would be sensible to handle and store a solid compound. The yield of **173** from starting material **178** was 44% over the three steps.

Scheme 2.12 **Synthesis of minocycline linker 2 (173) from 179**



The components required to synthesise the minocycline dimer half, amine **173** and 9-(2-chloroacetamido)-minocycline **170** (Scheme 2.5), were now in hand and attention was turned to the optimisation of the $\text{S}_{\text{N}}2$ reaction to ensure that only the mono-alkylated product would be produced.

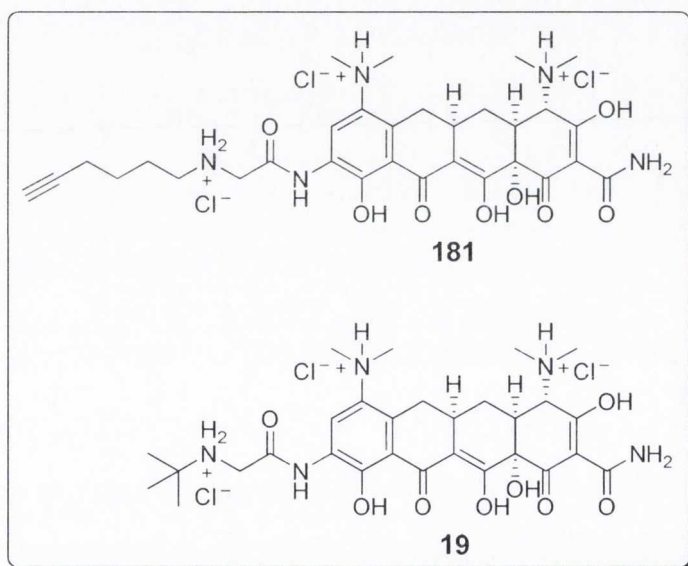
2.4.2 $\text{S}_{\text{N}}2$ linkage: mono- vs. bis-alkylation

The $\text{S}_{\text{N}}2$ reaction of (2-bromo)acetamidominocycline (**18**, Figure 1.11) with various amines had been previously reported by Sum *et al.*¹⁰¹ in the research which had led to the discovery of tigecycline. However, experimental reaction conditions were not provided for any of the analogues produced. It was therefore necessary to optimise reaction conditions for the $\text{S}_{\text{N}}2$ reaction of **170** with primary amines before performing the reaction to produce the minocycline dimer half **181** (Figure 2.11, *vide infra*). It was decided to use a commercially available amine, *t*-butylamine, to produce tigecycline trihydrochloride (**19**, Figure 2.11, *vide infra*) and optimise the $\text{S}_{\text{N}}2$ reaction conditions concurrently. This was advantageous on two

fronts: the optimisation of the reaction to produce tigecycline trihydrochloride (**19**) was necessary for future work involving the derivatisation of tigecycline (discussed in Section 2.6) and it was postulated that the optimised reaction conditions could be applied to the S_N2 reaction of **173** and **170** to produce the minocycline dimer half, **181**.

The optimisation of the S_N2 reaction of **170** using a commercially available amine, *t*-butylamine, was advantageous as the production of **173** from **178** yielded only 44% overall. Complications relating to *bis*-alkylation of the amine **173** (Scheme 2.12, *vide supra*) were envisaged and so the optimisation of the reaction before using valuable amine **178** was advisable.

Figure 2.11 Minocycline dimer half 1 (**181**) and tigecycline trihydrochloride (**19**)



A reaction was performed to test the theory that *bis*-alkylation of amine **173** could occur despite the bulk of the alkylating agent, **170**. Compound **170** was reacted with 1 equivalent of *t*-butylamine and sodium hydrogen carbonate in DMPU (Scheme 2.13, *vide infra*). The crude product was obtained by precipitation of the reaction medium into 2-propanol/diethyl ether (1:4) and filtration of the precipitate under argon. The ¹H NMR spectrum of the crude product is displayed in Figure 2.12 (*vide infra*), with *bis*-alkylation of the amine indicated by

resonance signals integrating for the presence of two minocycline moieties with respect to the *t*-butylamino signals.

Scheme 2.13 **Bis-alkylation of *t*-butylamine with 170**

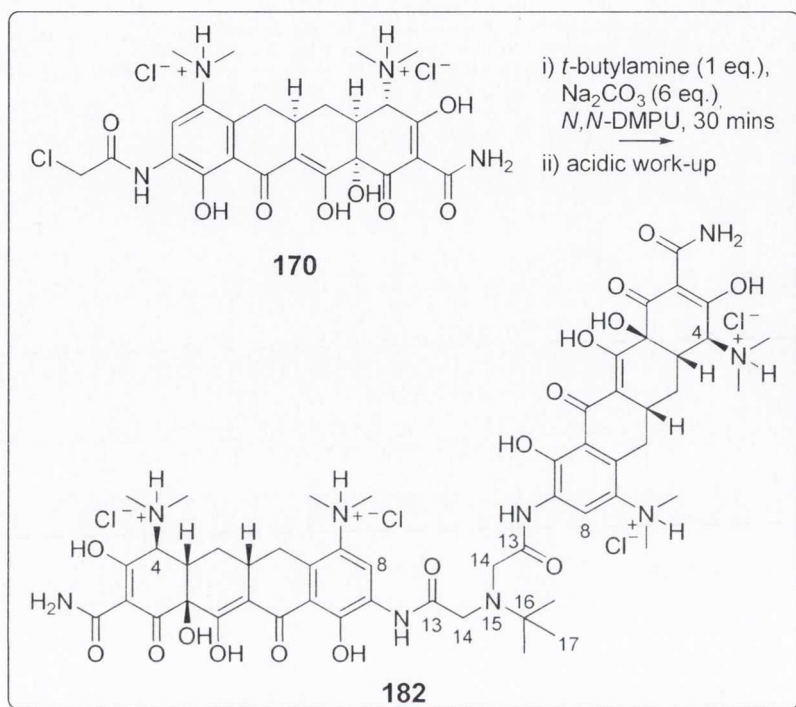
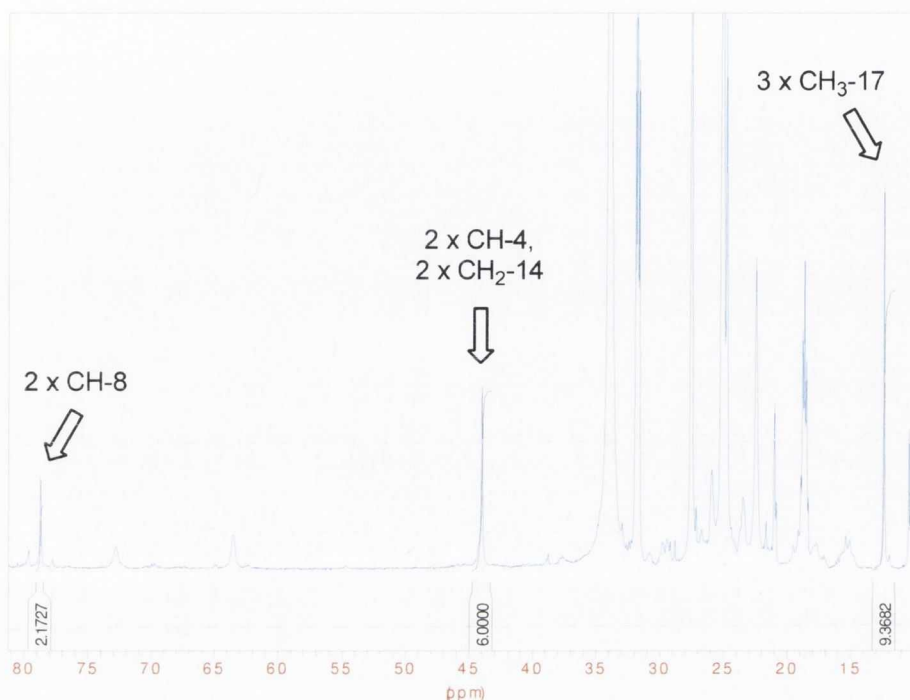
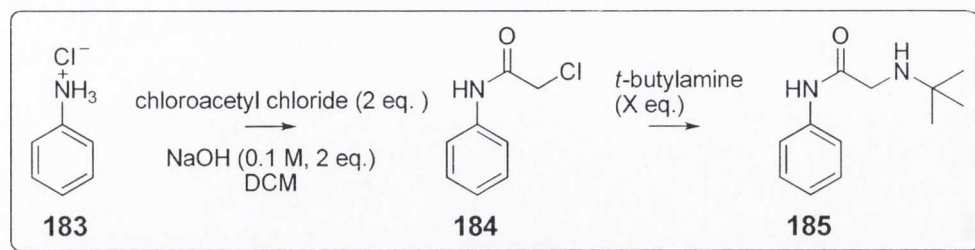


Figure 2.12 ^1H NMR spectrum of crude **182**



It was envisaged that the product of this reaction could prove to be a *bis*-alkylated amine. This proved to be the case as amine **182** was observed by ^1H NMR spectroscopy. It was decided therefore to use a large excess of *t*-butylamine in the synthesis of **19** to circumvent the issue of over-alkylation. Since compound **170** was time consuming to produce and the reaction yields during its production were low, *N*-(2-chloroacetyl)-aniline (**184**, Scheme 2.14) was chosen as a model compound for test alkylation reactions to determine the minimum number of molar equivalents of amine required to produce a mono-alkylated product. Compound **184** was prepared from the reaction of aniline, **183**, with chloroacetyl chloride (Scheme 2.14).

Scheme 2.14 Synthesis of model compound 184 and 185



Model compound **184** was then subjected to a number of test reactions to determine the minimum number of molar equivalents of amine required to ensure that only the mono-alkylated product, **185**, would be produced and to ensure full conversion of the starting material to product. It was necessary to devise reaction conditions that would provide quantitative conversion of starting material to product as tetracycline derivatives are extremely difficult to separate from each other by pH gradient extraction and recrystallisation is only feasible in some cases. A representative sample of the test reactions attempted for the conversion of **184** to **185** are shown in Table 2.4 (*vide infra*). Entries indicate whether or not reactions provided quantitative conversion after 24 h.

Table 2.4 Alkylation reactions with test compound 184 and *t*-butylamine

Entry no.	Eq. of 184	Eq. of <i>t</i> -butylamine	full conversion
1	1	4	x
2	1	5	x
3	1	7	x
4	1	15	√ ^a
5	1	10	√

a = after 19 h

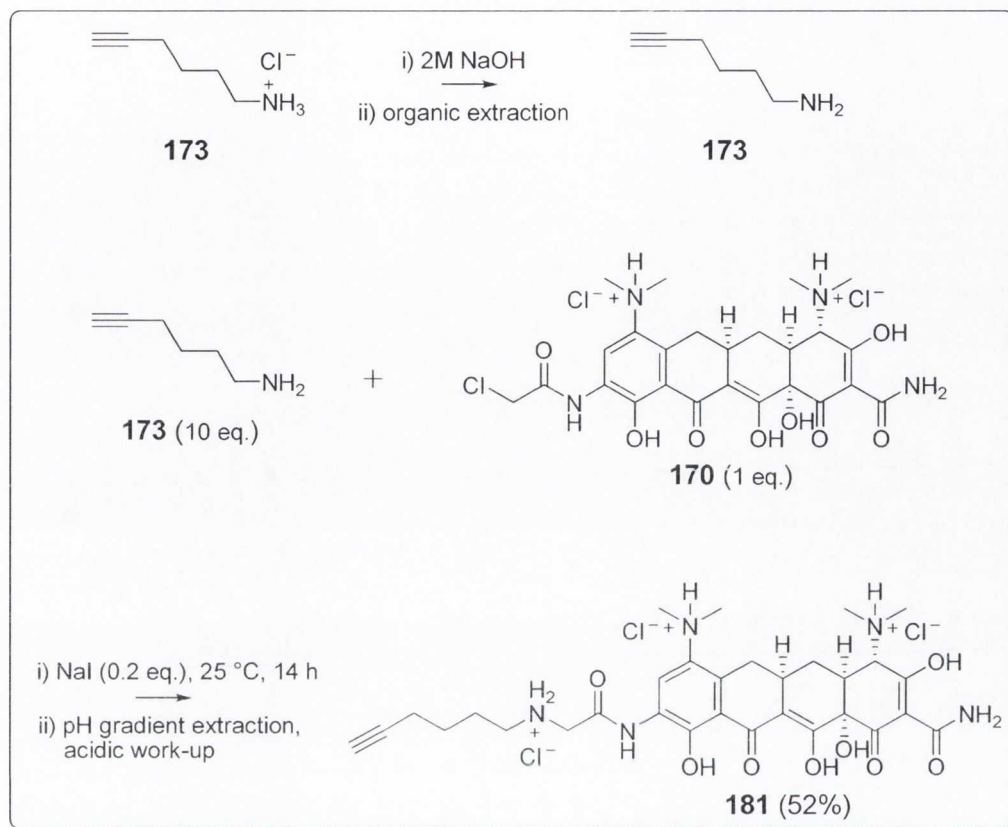
The reactions were monitored for up to 24 h as tetracyclines can prove unstable in solution over long periods of time. Long reaction times in this case would also be inadvisable as tetracyclines can degrade and epimerise in basic media. Previous reactions using fewer than four equivalents of amine had resulted in *bis*-alkylation of the amine or mixtures of *mono*- and *bis*-alkylated products. Initial reactions had also employed anhydrous acetonitrile/anhydrous *N,N*-DMPU (1:4) as a solvent system and sodium carbonate as a base but the reaction times were greatly increased in these cases, as such, *t*-butylamine was employed as a reactant, base

and solvent for the reaction. Entries 1-3 provided mono-alkylated products but were incomplete after 24 hours. Entry 4, resulted in full conversion of **184** to **185** after 19 h. An attempt to reduce the equivalents of amine required was successful and reaction conditions employing ten equivalents of amine (entry 5) were chosen to proceed.

2.4.3 Synthesis of the minocycline dimer half (**181**)

It was expected that the optimised conditions from the test reactions would afford the quantitative conversion of compound **170** to the desired minocycline derivative, **181**. It was decided to employ catalytic amounts of sodium iodide in the reaction in an attempt to reduce the reaction time necessary. Gratifyingly, this endeavour proved successful: **170** was quantitatively converted to **181** in the presence of 10 equivalents **173**, and sodium iodide in 14 hours (Scheme 2.15). The free-base amine **173** was obtained from its hydrochloride salt by dissolution in sodium hydroxide and extraction of the free-base amine into diethyl ether. The diethyl ether was removed from **173** by fractional distillation at 35 °C.

Scheme 2.15 **Synthesis of minocycline dimer half 1 (181)**

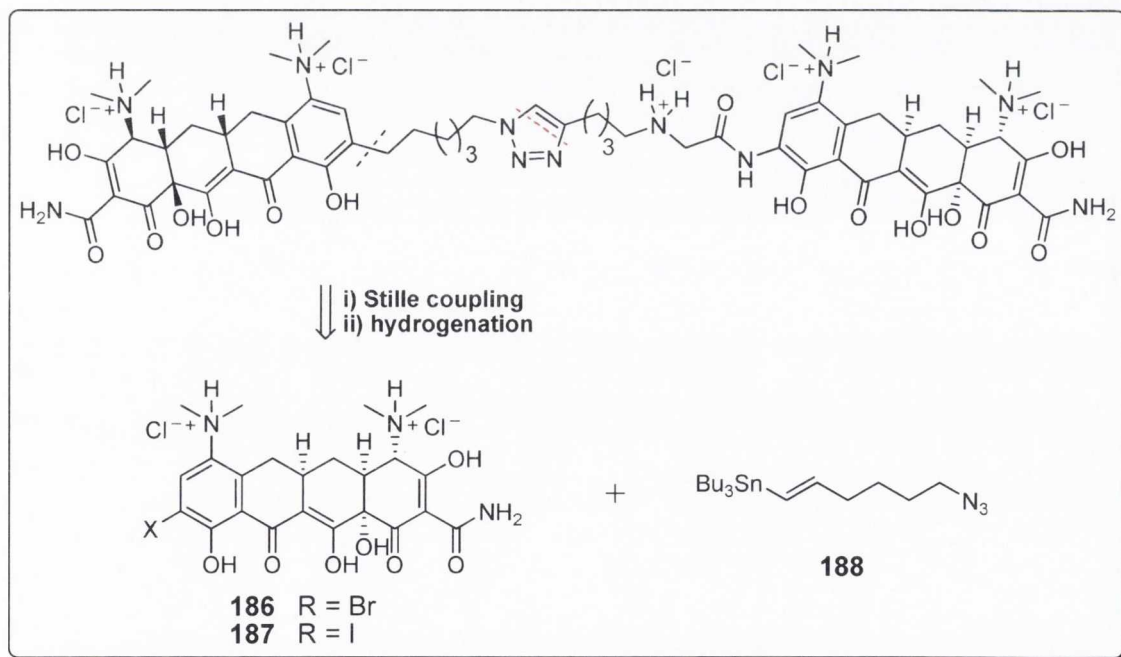


After 14 hours the reaction was subjected to an acidic work up and the crude product was purified by pH gradient extraction. This afforded **181** in the organic extracts taken from pH 4.5 – 5.0. These extracts were acidified with hydrogen chloride in diethyl ether (2.0 M) to furnish the trihydrochloride salt of **181** in 52% yield. 3.5 equivalents of unreacted amine **173** were also recovered at a basic pH range (8 – 11). This provided a useful means of recovering the excess amine. A viable synthetic route had now been designed for the production of **181**, albeit in only 10% yield (with respect to minocycline (**3**)). Minocycline analogue **181** contained a terminal alkyne moiety for linkage with an appropriate azido functionalised anhydrotetracycline analogue, thus attention was turned to the synthesis of the anhydrotetracycline dimer half, discussed in Chapter 3.

2.4.4 Halogenation of minocycline

In 2007, Arimoto *et al.* synthesised vancomycin dimers that displayed activity against vancomycin resistant enterococci.²²⁴ Therefore, it seemed possible that a minocycline/minocycline dimer could have activity against resistant bacteria. As such, a synthetic route was designed to prepare a minocycline derivative which could be linked to minocycline dimer half 1 (**181**) *via* a copper catalysed cycloaddition reaction. This synthetic route required the preparation of minocycline derivatives halogenated at position 9. These compounds were to be used in Stille couplings with vinyl stannanes to install linkers onto the minocycline structural core. A diagrammatic representation of the dimer and a retrosynthesis of minocycline dimer half 2 (left of the red hatched line) is depicted in Scheme 2.16. The synthesis of organotin compound **188** is discussed in Chapter 3.

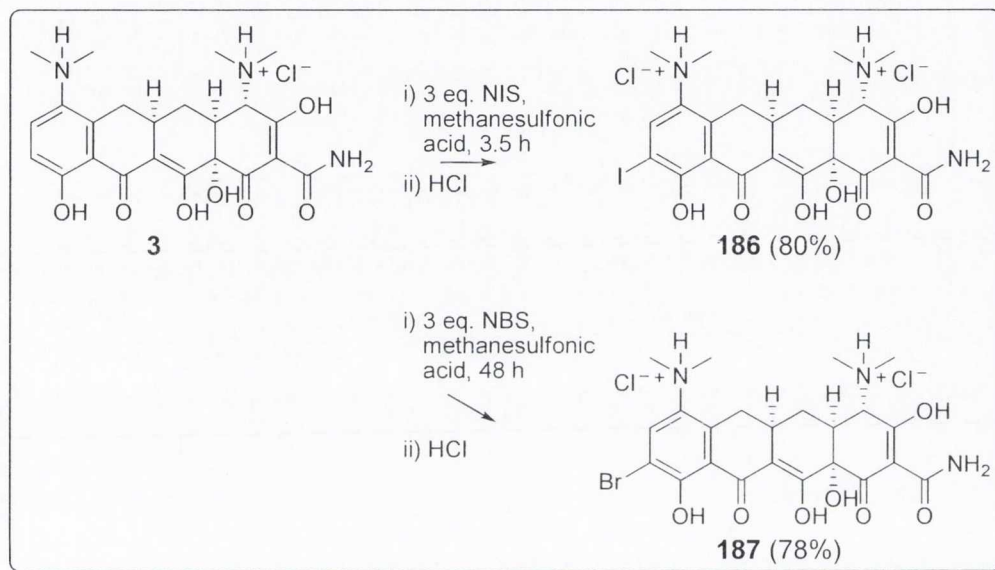
Scheme 2.16 Minocycline-minocycline dimer and retrosynthesis of minocycline dimer half 2



The synthesis of 9-iodominocycline **186** proved to be relatively facile following a procedure reported by Neslon *et al.*,¹⁷⁸ using minocycline hydrochloride and NIS. The procedure was

adapted to the use of NBS and produced the heretofore unreported 9-bromominocycline, **187**. The halogenated compounds **186** and **187** were obtained by reactions in methanesulfonic acid with greatly varied reaction times, 48 hours for the bromo- analogue **187** vs. 3.5 hours for the iodo- analogue **186** (Scheme 2.17, *vide infra*). Both compounds were obtained in good yield.

Scheme 2.17 **Synthesis of 9-halo minocyclines**



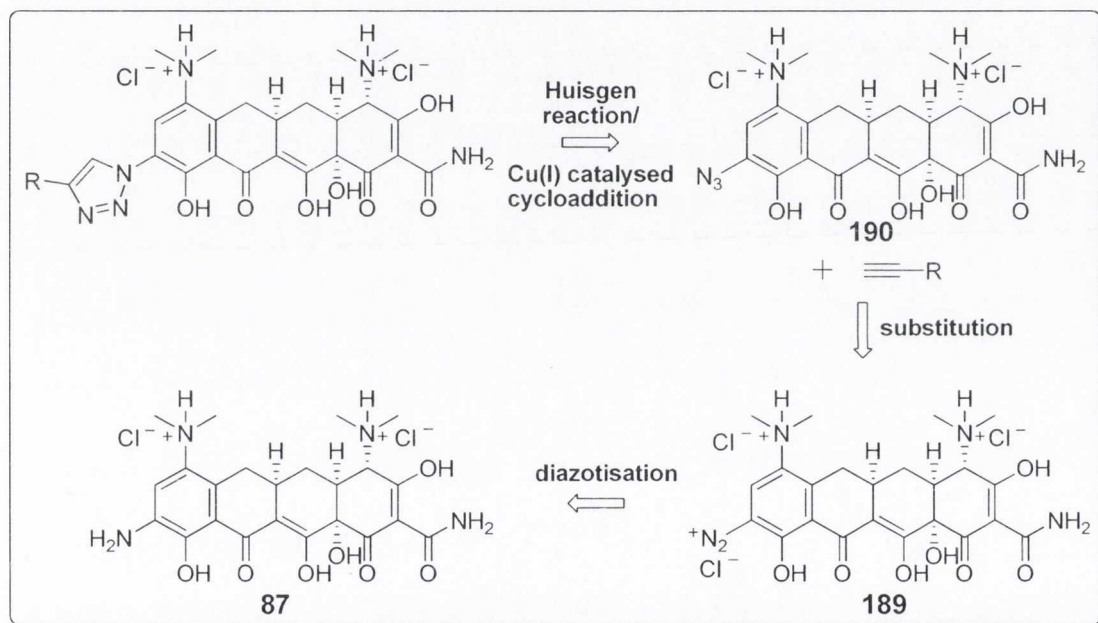
2.4.5 Conclusions

At this point a methodology had been developed for the synthesis of a novel minocycline derivative, minocycline dimer half 1 (**181**) which could be linked to *via* its terminal alkyne to an appropriately modified anhydrotetracycline or minocycline analogue to produce a tetracycline dimer. While the yield from minocycline (**3**) to **181** was very low, 10%, a number of synthetic and purification difficulties had been overcome to maximise the yield. Novel and previously reported 9-halo minocyclines had also been produced with a view to synthesising a minocycline/minocycline dimer. With these molecules in hand, attention was turned to investigating methodologies to prepare novel classes of minocycline derivatives.

2.5 Click chemistry with 9-Azidominocycline

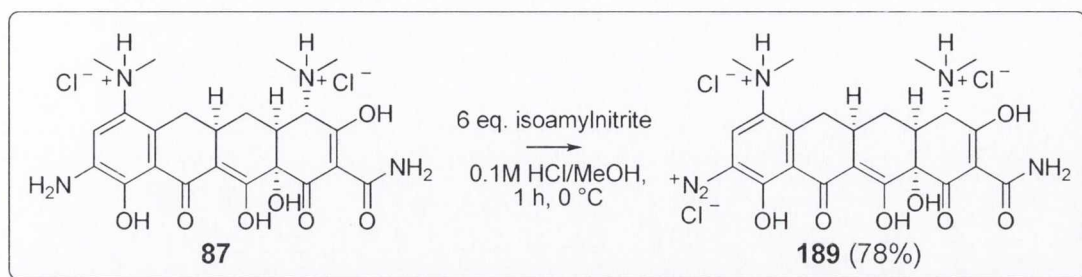
A viable synthetic route to produce novel minocycline analogues was designed. This route required the synthesis of 9-azidominocycline (**190**, Scheme 2.18, *vide infra*) which could be functionalised using a range of alkynes *via* the Huisgen reaction or a copper catalysed cycloaddition reaction. This approach to the synthesis of analogues could provide novel active antibiotic compounds. A retrosynthesis of the 9-triazole substituted minocycline analogues to compound **87** is depicted in Scheme 2.18.

Scheme 2.18 Retrosynthesis of 9-triazole substituted minocyclines to **87**



The first step in the synthesis of these compounds was the diazotisation of **87** to diazonium salt **189**. This was prepared in a straightforward manner using 0.1M HCl in methanol and six equivalents of isoamyl nitrite at 0 °C (Scheme 2.19).

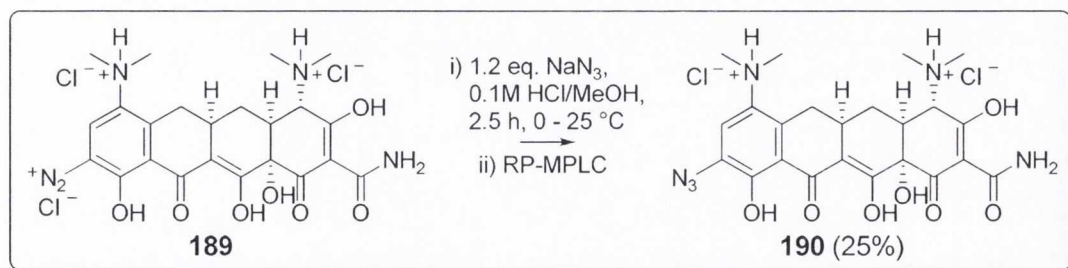
Scheme 2.19 Synthesis of 189



The diazonium salt **189** was isolated by precipitation from diethyl ether at 0 °C and filtration under argon. The filtration was performed quickly to minimise decomposition of **189** as it warmed. ¹H NMR confirmed the formation of a new product; however the solvent, CD₃OD, required cooling to below 0 °C before use and the sample analysed as soon as it was dissolved. This precaution was taken as previously, a gas (N₂) had evolved when preparing ¹H NMR samples of this compound and analysis showed that the sample had decomposed to minocycline (**3**). IR analysis of **189** confirmed that the diazo salt had been formed by the presence of the diazo stretch at 2185 ν/cm⁻¹. Compound **189** was stored at -18 °C under argon, away from light.

189 was then reacted with sodium azide in the presence of 0.1 M HCl in methanol at 0 - 25 °C to afford the azido analogue **190** in 96% crude yield (Scheme 2.20). The crude product required purification by reverse phase chromatography as it contained 15% minocycline (**3**), the decomposition product of **189**. Compound **190** was recovered in 25% yield after purification by RP-MPLC.

Scheme 2.20 Synthesis of 9-azidominocycline (190)



Compound **190** was subsequently reacted under a variety conditions in an effort to produce triazole analogues. Initially, heptyne was chosen as a reactant as it was expected that the resonance signals in the ^1H NMR spectra would be easy to track, in particular the alkynyl proton signal at 1.96 ppm, since they would not overlap with the signals of **190**. A summary of the first series of reaction conditions employed is presented in Table 2.5.

Table 2.5 **1st series of trizole formation reactions**

Entry no.	Eq. 190	Eq. Heptyne	Eq. CuI	Solvent	Temp. (°C)	min/h
1	1	1	0.2	H ₂ O	100 (MW) ^b	30 min
1	1	1	0.2	H ₂ O/DMF ^a	80 (MW) ^b	10 min
3	2	1	0.2	DMF	100	48 h

a = 1:1 mixture; b = MW: microwave irradiation

Entry 1 represents the first reaction conditions attempted. Microwave irradiation was employed as the benefits of microwave irradiation in this reaction had been well reviewed.²³⁵ After 30 minutes of microwave irradiation at 100 °C the reaction was worked up, with the CuI removed by filtration through celite, and analysed by ^1H NMR. Compound **190** was found to be decomposed. The signal relating to CH-8 had disappeared and the characteristic three singlets around 7 ppm were visible. It was decided that perhaps Millipore water was not a suitable solvent for the reaction as it did not dissolve heptyne and therefore the reaction could have been prevented from occurring. Alternatively, decomposition of the starting materials or product could have occurred.

Entry 2 relates to the same reaction conditions but with the use of Millipore water and DMF as solvents in a 1:1 ratio. The reaction temperature was lowered to 80 °C and the reaction time reduced to 10 minutes in an attempt to avoid decomposition of **190**. Disappointingly, after work up and separation of the reaction components by RP-MPLC, no new tetracycline analogue was observed and the starting material **190** was recovered.

At this point it was considered that using an excess of azide **190** might encourage formation of the triazole product. 2 equivalents of **190** were employed (entry 3) and DMF used as solvent to ensure all starting materials, including copper iodide, were dissolved. The reaction was not performed in the microwave as it was considered that microwave irradiation may have been contributing to the decomposition of **190**. The reaction was heated

conventionally to 100 °C (the maximum temperature when the boiling point of heptyne is considered) and fitted with a reflux condenser to ensure no loss of heptyne occurred. The reaction was allowed to proceed for 48 hours after which time the copper iodide was removed and the DMF removed by lyophilisation. Disappointingly, ¹H NMR analysis revealed that the desired triazole product had not been formed. The azide **190** had in fact decomposed to its most basic structure, minocycline (**3**).

Around this time, Gmeiner *et al.* reported the first catalysed triazole formation reaction involving tetracycline derivatives.²³⁰ Their procedure involved the linkage of a doxycycline (**2**) derivative with a linker at position 9 containing a terminal azide and various alkyne substituted amino acid analogues. 13 equivalents of copper iodide, which is normally used in catalytic amounts, were required to afford the triazole products in low yields, around 20%. The large excess of copper iodide was required due to the number of chelation sites in the tetracycline structure and the amino acids. They also commented that the use of a system of 17 equivalents of diisopropylethylamine (DIPEA) and 7 equivalents of ascorbic acid (AscOH) was required to ensure that oxidation of the copper iodide did not occur. A universal solvent was chosen, dry, degassed DMF, to ensure all starting materials were dissolved and microwave irradiation was performed at a maximum of 100 W and 50 °C to avoid decomposition of the tetracycline derivative. The reactions were performed in cycles of microwave irradiation and subsequent cooling to -10 °C a number of times to avoid decomposition of the tetracycline. An excess of the alkyne was used to attempt to drive the reaction. With these considerations in mind, a new set of experiments were designed to attempt to synthesise our triazole functionalised minocycline derivatives. 3-Butynol was chosen as the next reactant as it is more polar than heptyne and it was felt that the choice of solvents would be less restricted by its use. During the course of these reactions it was discovered that azide **190** decomposed after 5-6 weeks, despite storage at -18 °C, under argon, away from light. It was therefore necessary to keep batches of **190**, stored as before, for a maximum of 3-4 weeks. This made it difficult to carry out full methodology screening if the 'click' conditions as **190** had to be prepared freshly and purified a number of times. Unless otherwise stated copper iodide was used as the source of Cu(I). 13 Equivalents of Cu(I) were used in all reactions. A representative sample of the reaction conditions attempted are summarised in Table 2.6.

Table 2.6 **2nd series of triazole formation reactions**

Entry	Eq. 190	Eq.	Solvent	Temp. (°C)	min/h
3-butynol					
1 ^a	1	2	DMF	50	5 x 1 min
2	1	2	DMSO-d ₆	50	5 x 1 min
3	1	2	MeOH	30	5 x 1 min
4	1	2	D ₂ O/DMPU ^b	50	4 x 1 min
5 ^c	1	2	H ₂ O	50	5 min

a = 17 eq. DIPEA, 7 eq. AscOH used also; b = 3:1 mixture; c = 14 eq. CuSO_{4(aq)} and 28 eq. Sodium ascorbate as Cu(I) source.

Entry 1 was performed under the conditions described by Gmeiner *et al.*²³⁰ Unfortunately, on our system the reported literature procedure proved unsuccessful due to decomposition of **190**. Also, the reaction was difficult to analyse as the AscOH heavily obscured the region of interest in the ¹H NMR spectrum and streaked during RP-MPLC through all fractions. It was therefore decided to perform the reactions without these additives so that they could be monitored by ¹H NMR. Gmeiner *et al.* had not encountered this issue as their reactions were monitored and purified by reverse phase HPLC, a technique that was not available in our laboratory.

Entry 2 was designed to enable easy monitoring of the reaction by ¹H NMR spectroscopy by using a deuterated universal solvent. The reaction was cooled to -10 °C between each 1 minute microwave irradiation (50 W). This reaction provided useful information. ¹H NMR spectroscopic analysis of the reaction after 3 x 1 minute cycles confirmed that no reaction had taken place between **190** and 3-butynol: the alkynyl proton was still visible in the ¹H NMR spectrum at 2.28 ppm in the same proportion as it had been before the reaction began. After a further 2 x 1 minute cycles compound **190** had decomposed, and the 3-butynol signals were still clearly visible.

Entry 3 was performed in a solvent that **190** was known to be stable in, methanol. The heating of the reaction was also lowered to 30 °C to deter decomposition. Unfortunately, under these conditions, the copper iodide was not particularly soluble and after 5 x 1 minute cycles (50 W) no reaction had occurred and **190** was recovered.

Entry 4 was again designed to monitor the reaction by ^1H NMR, with the addition of 20% DMPU to facilitate solubility in a tetracycline friendly solvent, D_2O . The reaction was heated to 50 °C as no reaction had occurred at 30 °C. After 5 x 1 minute cycles of MW irradiation (with cooling to -10 °C between each) no reaction had occurred. At this point it was considered that perhaps copper iodide was not a suitable choice of Cu(I) source for this system due to its insoluble nature.

Copper sulfate and sodium ascorbate were used as a source of Cu(I) in entry 5 as both are water soluble. This complicated the work up of the reaction, however, as Cu(II) is known to bind with a very high affinity to tetracyclines¹⁰ and sodium ascorbate was difficult to remove from the reaction as it is soluble in the same solvents as **190** and streaked on RP-MPLC. Condensing the reaction in methanol to afford the precipitation of sodium ascorbate and filtering was necessary to remove the majority of sodium ascorbate. The copper-**190** chelate was dissociated by vigorous stirring of the chelate in 35% HCl/methanol for 18 hours. After 5 minutes microwave irradiation at 50 °C and work up of the reaction for analysis, it was found that no reaction had occurred and **190** was recovered.

2.5.1 Conclusions

Copper catalysed cycloaddition reactions between an alkyne and an azide, normally a very robust and reproducible ligation methodology, was found to be ineffective for our system. Gmeiner *et al.* had reported that all triazole formations had been completed within 5 minutes using the reaction conditions they described.²³⁰ In our system either no reaction had occurred, or **190** had decomposed. It was decided that the use of this reaction to produce novel analogues of minocycline was inadvisable and it is tentatively suggested that perhaps the proximity of the azido moiety to the tetracycline, especially given its position *ortho* to a phenolic moiety, was interfering with the reaction's progress. In the case of the analogues synthesised by Gmeiner *et al.* the azido functionality had been spaced from the tetracycline by a linker. This did not deter us from the synthesis of the tetracycline dimers by triazole formation however, as in that case both the alkyne and azide would be removed from the tetracycline core by the linker.

2.6 Tigecycline and attempted functionalisations

It was considered that in conjunction with the synthesis of tetracycline dimers, a single tetracycline analogue could be designed which would bear the two characteristics of the dimers designed: the ability to induce the expression of the efflux proteins while not being a suitable substrate for efflux, in order to cause overexpression of the efflux protein and therefore cell death. Tigecycline (**19**) is not a substrate for Tet efflux proteins, however, it displays poor binding affinity for the Tet(R) regulation protein. Since strong binding with Tet(R) is necessary to induce effective expression of the efflux protein, it was thought that modifications to the tigecycline structure could positively influence its binding affinity.

Figure 2.13 A tigecycline analogue with increased affinity for Tet(R)

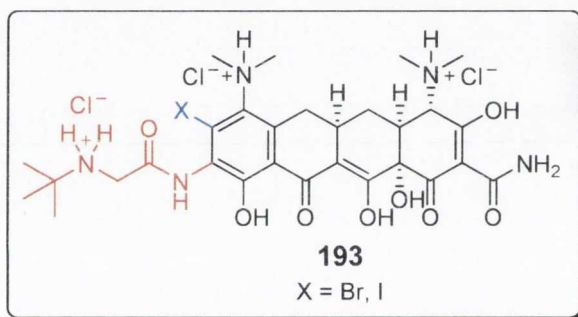
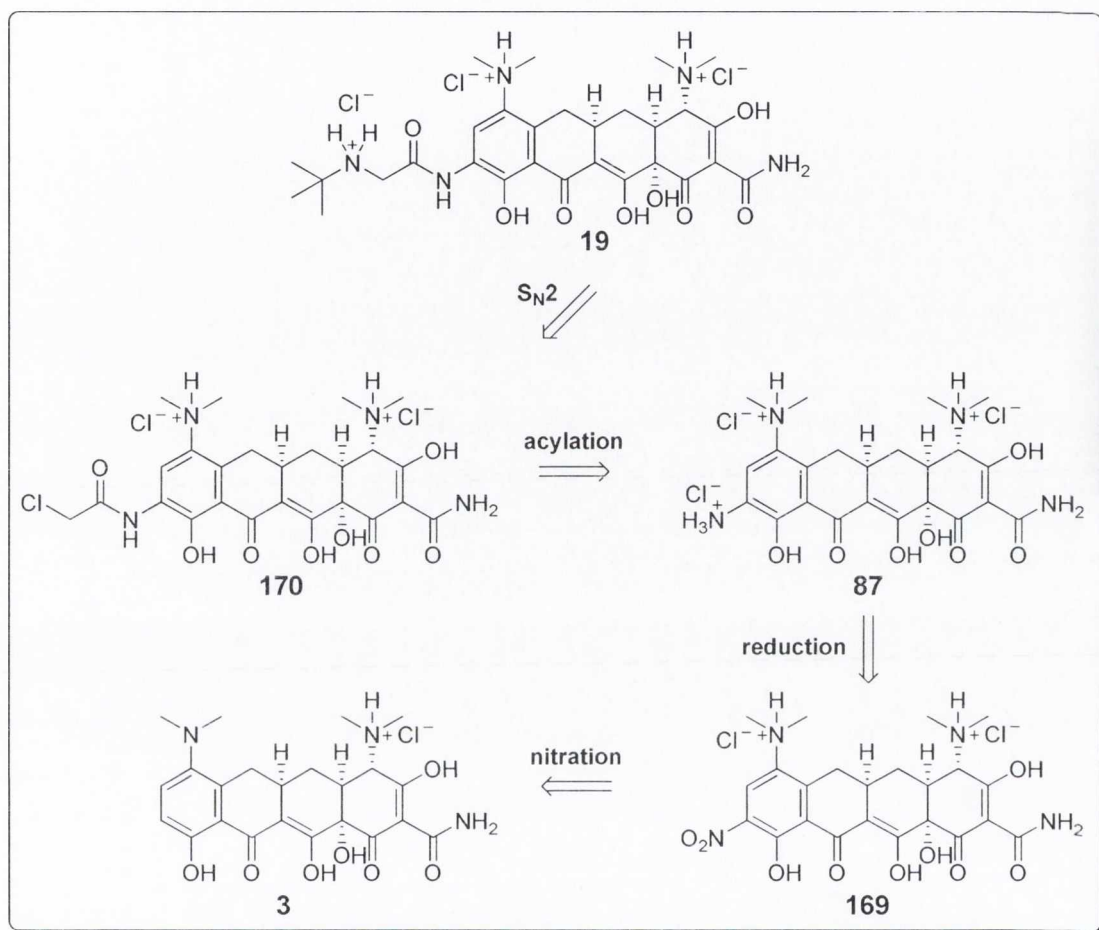


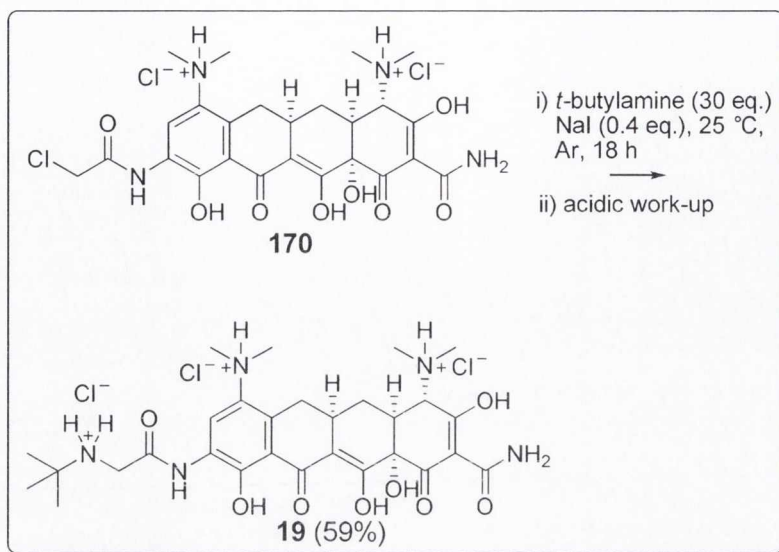
Figure 2.13 displays a tigecycline analogue with the potential for increased binding to the Tet(R) regulation protein. The *t*-butylglycylamido moiety, which prevents the analogue from being expelled from bacterial cells by the efflux proteins, is highlighted in red. The halogen moiety at position 8 is highlighted in blue. It was postulated that the introduction of a halogen at this position in the molecule could increase its binding affinity for the Tet(R) regulation protein (and therefore increase the rate of induction of expression of the efflux proteins) as the introduction of a chloro moiety at position 8 at in 9-amino-DMDOT increased its affinity for the Tet(R) protein by a factor of 7 (*vide supra* Section 1.8.2).²²⁰ It was hypothesised that similar modifications could have the same effect on the binding affinity of tigecycline (**19**) and therefore bestow the same activities as the dimers designed to a monomeric tetracycline analogue. A retrosynthesis of tigecycline is presented in Scheme 2.21.

Scheme 2.21 Retrosynthesis of tigecycline (19)



Tigecycline (**19**) was synthesised from the reaction of **170** with a large excess of *t*-butylamine at 25 °C for 18 hours. A work-up involving extraction of the crude product from water at pH 7.2 and subsequent acidification of the organic extracts with HCl in anhydrous diethyl ether (2.0 M) afforded **19** in good yield (Scheme 2.22, *vide infra*).

Scheme 2.22 **The synthesis of tigecycline (19) from 170**



A series of experiments were performed to attempt to introduce a halogen at position 8 in tigecycline (**19**) to increase its binding affinity for the Tet(R) protein and as such increase its ability to induce the expression of the efflux protein. This could be performed at various stages of the synthesis (Scheme 2.21, *vide supra*) on 9-nitrominocycline (**169**), 9-aminominocycline (**87**) or tigecycline itself (**19**), as once installed the halogen moiety should prove unreactive under the conditions necessary to produce tigecycline. The reaction conditions to **170** had been previously designed for the synthesis of the minocycline dimer half and so only the final step in the synthesis needed to be explored. If a halogen was introduced at position 8 in **169** catalytic reduction to the amine would no longer be amenable as the Pd could insert into the carbon-halogen bond. However, other methods of reduction such as the use of tin (II) chloride and HCl could be employed, as had been employed by Xing *et al.* for the reduction of 2-bromonitrobenzene to 2-bromoaniline.²³⁶ If chelation of the tin (II) species to minocycline occurred it was envisaged that the chelate would be dissociated by stirring in methanol and HCl, as had been previously performed (Section 2.5). It was also envisaged that it would be possible to use azide **190** to introduce a halogen at position 8 employing reaction conditions described by Koza.¹⁸² While it was understood that substitution at this position would be troublesome due to the deactivated nature of the D ring, it was thought that since position 8 was the only available aromatic position for substitution that the

reaction conditions could be optimised to encourage the synthesis of the desired product. The desired product was considered to be significant enough that even small conversions to the product would be accepted.

Initial studies involved the substitution of **169**. A representative sample of the reaction conditions employed are summarised in Table 2.7 and the desired product depicted in Scheme 2.23.

Scheme 2.23 Halogenation of 169 at position 8

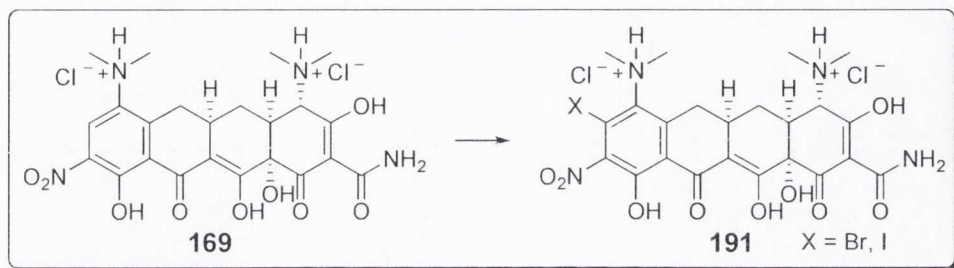


Table 2.7 Exploration of the halogenation of 169

Entry	Halogen donor	Eq. Halogen donor	Solvent	Temp. (°C)	Time (h)
1	NIS	3	MSA ^a	rt-40	72
2	NIS	6	MSA	rt	216
3	NIS	16	MSA	rt	17
4	HBr	9	AcOH	rt-35	24
5	Br ₂	1	AcOH	rt	24
6	NBS	5	H ₂ SO ₄	rt	24
7 ^b	NBS	3	H ₂ SO ₄	rt	24

a = MSA: methanesulfonic acid; b = **169** Was employed as its free-base amine

Entry 1 summarises the reaction of **169** with 3 equivalents of NIS. Methanesulfonic acid was chosen as it is known to be a good ionising solvent. After 24 hours no reaction was observed and the reaction was heated to 40 °C. The reaction was monitored by ¹H NMR spectroscopy by comparing the integration of the CH-8 aromatic proton with that of other protons in **169**: tracking the disappearance of CH-8 by ¹H NMR spectroscopy was the best way to monitor the reaction as NMR spectroscopy was the only available method of evaluation. The reaction was

monitored intermittently and after 72 hours the starting material was found to have decomposed, the formation of **191** had not been observed after this time.

Entries 2 and 3 were performed without heat in attempts to prevent decomposition of the starting material. The use of 6 equivalents of NIS resulted in no reaction after 9 days stirring at room temperature and no decomposition of the starting material was observed by ^1H NMR spectroscopy (entry 2). The use of 16 equivalents of NIS at room temperature resulted in the degradation of the starting material within 17 hours, as evidenced by 3 singlets between 6.9 and 7.5 ppm in the ^1H NMR spectrum and the disappearance of the peaks relating to CH-4, CH₂-5 and CH₂-6 (entry 3). It was now evident that heat and/or excess NIS may have been causing the decomposition of **169**; it was decided to screen an array of different halogen sources.

9 equivalents of HBr in acetic acid were employed at room temperature for 20 hours followed by heating to 35 °C for 4 hours (Entry 4). After 20 hours no reaction had occurred to form **191**, and after heating, the starting material was found to decompose (analysed by ^1H NMR).

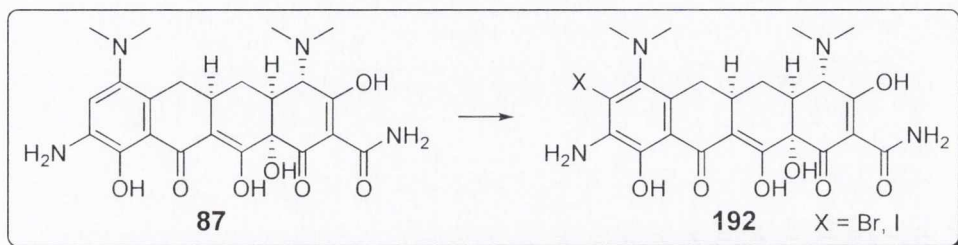
As it appeared that heating the reactions was causing decomposition, the subsequent reactions were performed at room temperature. Molecular bromine was used in only one equivalent as these conditions are reasonably harsh and we were somewhat concerned about possible addition to the olefin at position 2-3 in the A ring, despite its deactivated appearance. After 24 hours, **169** was found to be decomposing to a number of compounds resulting in multiple aromatic singlets in the ^1H NMR spectrum (entry 5).

The bromination of **169** was attempted using NBS in sulfuric acid (entry 6), as Saiganesh *et al.* had reported the bromination of deactivated aromatics using these conditions around the same time.²³⁷ 5 equivalents of NBS were employed since the compound had been shown to be stable when 6 equivalents of NIS were used in the reaction described by entry 2. Unfortunately, after 24 hours, **169** was found to be decomposed.

Entry 7 represents a reaction where the free-base amine of **169** was employed in the reaction. It was hypothesised that removal of the ammonium salt on the ring would bestow greater reactivity towards EAS. After 24 hours stirring at room temperature with 3 equivalents of NBS, since the use of 5 equivalents previously had resulted in decomposition of **169**, the desired product, **191**, was not observed and **169** was found to have decomposed (determined by ^1H NMR).

Under the reaction conditions tested, **169** was found to be either unreactive or unstable in all cases, and as such it was decided to attempt the bromination of 9-aminomincycline (**87**) in its trihydrochloride salt and free base forms to investigate if it would prove more stable and reactive under halogenating conditions (Scheme 2.24).

Scheme 2.24 Halogenation of 87 (free base or trihydrochloride salt)



Compound **87** was subjected to the reaction conditions reported by Saiganesh *et al.*²³⁷ in both trihydrochloride salt form and free base form (Table 2.8). It was thought that the protonated amine at position 9 in the trihydrochloride would be more stable but deactivate the ring towards substitution, while the free base amine at position 9 would activate the ring but prove to be less stable.

Table 2.8 Bromination of 87

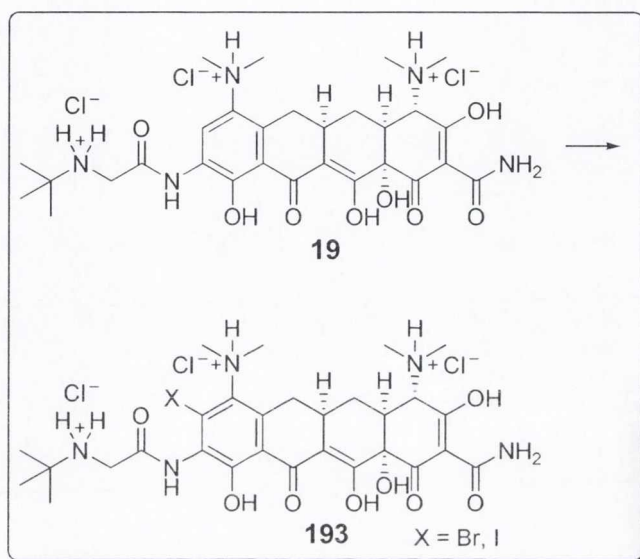
Entry no.	3 HCl salt	Free base	Eq. NBS	Solvent	Temp (°C)	Time (h)
1	√	---	1.5	H ₂ SO ₄	0-rt	2
2	---	√	1.5	H ₂ SO ₄	0-rt	2

Unfortunately neither of the reaction conditions provided the right compromise between reactivity and stability. Entry 1 resulted in no reaction and entry 2 resulted in decomposition of the starting material within 2 hours.

It was hoped that tigecycline (**19**) itself might provide the correct reactivity as the ring would be less deactivated than in the case of **169** and **87** in their hydrochloride salt forms. Compound **19** was reacted with 1.5 equivalents of NBS in H₂SO₄ (Scheme 2.25, *vide infra*) as per Saiganesh *et al.*²³⁷ After 24 hours the ¹H NMR spectrum displayed the aromatic resonance signal relating to the starting material **19**, and 5 other aromatic singlets along with the disappearance of several of the starting material protons. It was clear at this point that,

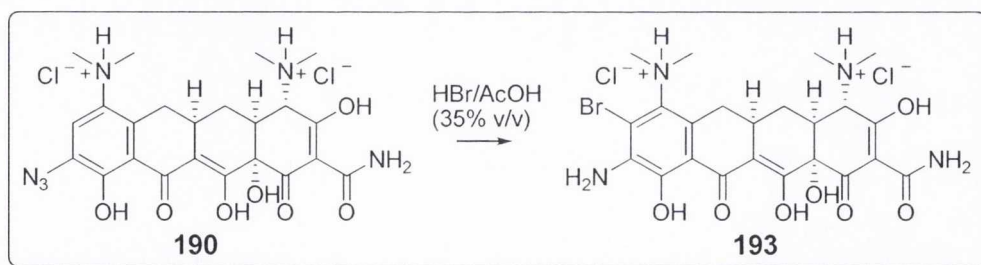
unfortunately, **19** was an unsuitable choice of reactant as there were clearly a number of sites available for halogenation and the disappearance of a number of indicative tetracycline peaks indicated the destruction of the tetracycline core.

Scheme 2.25 Halogenation of tigecycline



A final effort to product a C-8 substituted tigecycline employed the reaction conditions reported by Koza in 2000.¹⁸² These reaction conditions were optimised for use with doxycycline (**2**) but it was hoped they would also be useful for converting **189** to a C-8 substituted amine (Scheme 2.26).

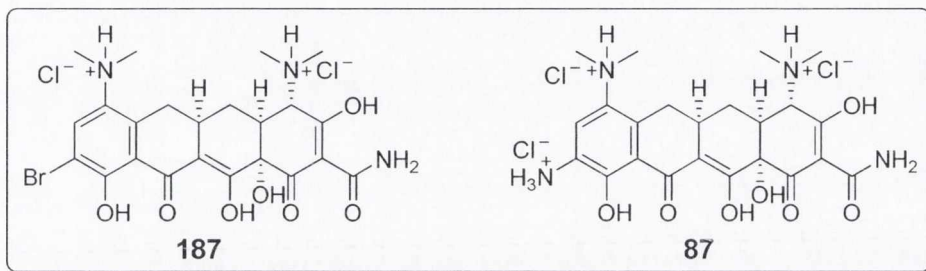
Scheme 2.26 Halogenation of 189



Compound **190** was reacted with HBr in acetic acid (35% v/v) as solvent and reactant at 0.18 M with respect to **190** for 1 hour at 0 °C. After this time a new product had been produced,

however the desired halogenation/reduction had not occurred. Mass spectral analysis ruled out the possibility of the new product being the desired product (Scheme 2.26), brominated minocycline (as decomposition of the azide **190** to minocycline (**3**) had been observed previously) or aminominocycline (by reduction of the azide) (Scheme 2.27).

Scheme 2.27 Possible undesired products of halogenation of **190**



2.6.1 Conclusions

Disappointingly, no reaction conditions could be found to provide a C-8 halogenated minocycline compound which could be converted to a C-8 halogenated tigecycline analogue. Although the halogenation of minocycline at position 9 had proceeded smoothly (Section 2.4.4, *vide supra*) it was apparent that the deactivated nature of position 8, *meta* to a phenolic moiety on a deactivated aromatic ring, was preventing the desired reaction from occurring. Despite the exhaustion of numerous halogenation methods and varying the reaction conditions and starting materials the tetracycline compounds proved either unstable, unreactive or a competing reaction occurred. This method of analogue production was not possible under the reaction conditions attempted and so efforts were concentrated on the synthesis of the tetracycline dimers.

2.7 Chapter conclusions

After concentrated efforts, one of the desired linkers, **177** (Scheme 2.6), could not be obtained by the retrosynthesis designed. Under the required reaction conditions, only cyclic products **177A** and **177B** were produced (Section 2.3.3). However, linker **173** (Scheme 2.6) was synthesised in 44% yield over three steps from the commercially available starting material **178** (Section 2.4.1).

Attempts at producing a small library of 9-triazole substituted minocyclines had proved unsuccessful (Section 2.5). While the requisite novel precursors were designed and synthesised, it had been demonstrated that the desired triazole products could not be formed by Huisgen reaction or the 'click chemistry' Cu(I) catalysed version of the same. The starting material **190** was found to be unstable under the required reaction conditions.

An 8-halotigecycline analogue had also proven unobtainable (Section 2.6). Despite extensive experimentation attempting halogenations of each tigecycline intermediate (Scheme 2.21) all compounds proved to be either unreactive or unstable under a variety of halogenating reaction conditions.

However, the chemical reactivity and stability of minocycline (**3**) had been extensively researched and novel minocycline analogues had been produced. A viable synthetic route to the minocycline dimer half **181** had been designed and optimised to afford a 10% yield of **181** from minocycline (**3**) (Section 2.3.2 and Section 2.4.3). The issues of over-alkylation of the amine by **181** had been researched and overcome (Section 2.4.2), with the resultant reaction between **181** and **183** being performed in an economical manner whereby 39% of the unreacted excess of **183** could be recovered from the reaction. A method of pH gradient purification of **181** had been designed and provided the compound in excellent purity if performed with care. This allowed for continuation of the synthesis of tetracycline dimers by focussing on the synthesis of the anhydrotetracycline dimer half.

The direct halogenation of minocycline (**3**) had been achieved to produce 9-halo minocyclines **186** and **187** in good yield. This allowed for the future synthesis of minocycline/minocycline dimers by Pd(0) catalysed coupling chemistry (Section 2.4.4)

Finally, a method for determining the stability of minocycline analogues during reactions using ^1H NMR spectroscopy had also been determined, by the observation of a common decomposition product which was proposed to be a quaternary ammonium salt in light of the available NMR spectroscopic evidence.

Chapter 3

Results and discussion:

Anhydrotetracycline

analogues,

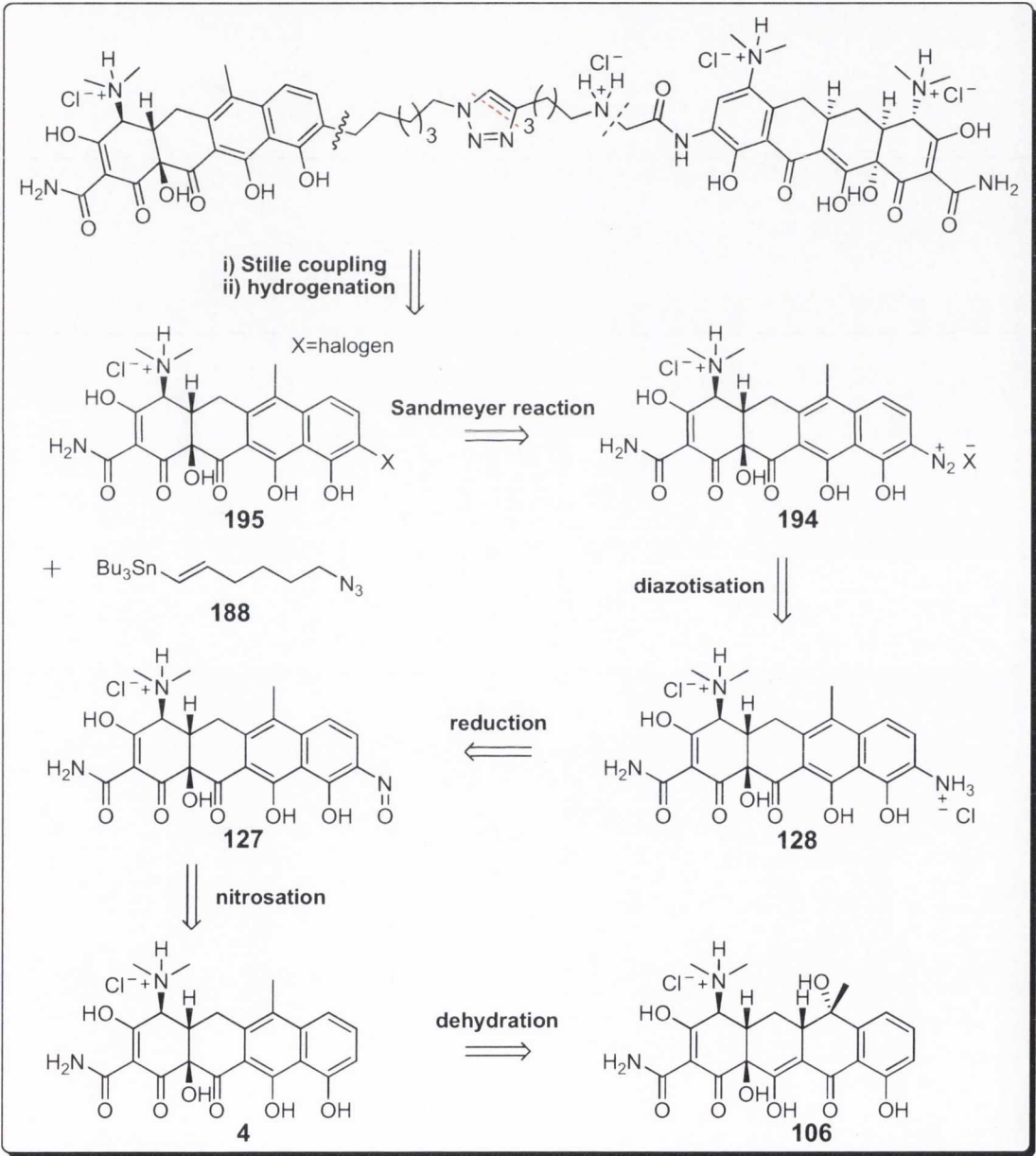
anhydrotetracycline linkers

and click chemistry linkage

3 Results and discussion: Anhydrotetracycline analogues, anhydrotetracycline linkers and click chemistry linkage

3.1 Synthetic route 1: Pd(0) coupling for connection of the linker

Scheme 3.1 Retrosynthetic route 1 of anhydrotetracycline dimer half to oxytetracycline (106)

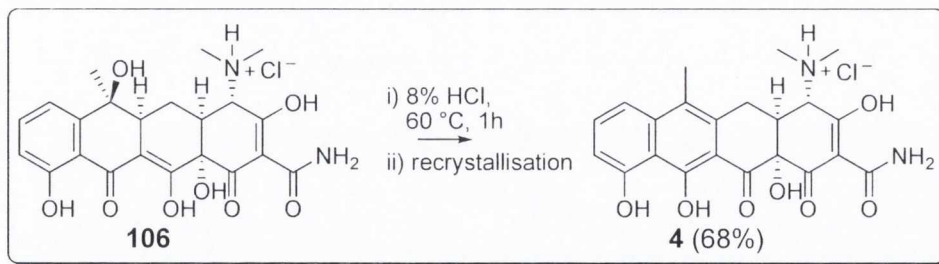


The retrosynthesis of the anhydrotetracycline dimer half (left of the red hatched line) from oxytetracycline (**106**) is depicted in Scheme 3.1 (*vide supra*). The final step involved connection of the linker **188** by a Stille coupling reaction. It was anticipated that the reactions chosen in the retrosynthesis would proceed smoothly as all are known to be reliable reactions in aromatic chemistry. However, due to the difficulties faced in the synthesis of the minocycline analogues it was necessary to take precautions when storing and handling the analogues experimentally. There has been limited literature data published in relation to synthetic experimentation involving anhydrotetracycline: two synthetic papers, in 1984²⁰⁴ and 2010,²¹³ and a small number of patents that were filed between 1950 and 1980.^{238,203} Thus, reaction conditions that had been successful for other members of the tetracycline class were tailored for use with anhydrotetracycline or reaction conditions were designed *ab initio*.

3.1.1 Synthetic route 1: Synthesis of 9-diazoanhydrotetracycline

The first step in the synthesis of the anhydrotetracycline dimer half (Scheme 3.1, *vide supra*) involved the dehydration of **106** using HCl (8% v/v) at 60 °C to afford anhydrotetracycline (**4**, Scheme 3.2). The crude product was then recrystallised from a mixture of methanol and diethyl ether at 0 °C to afford pure anhydrotetracycline hydrochloride (**4**) in 68% yield.

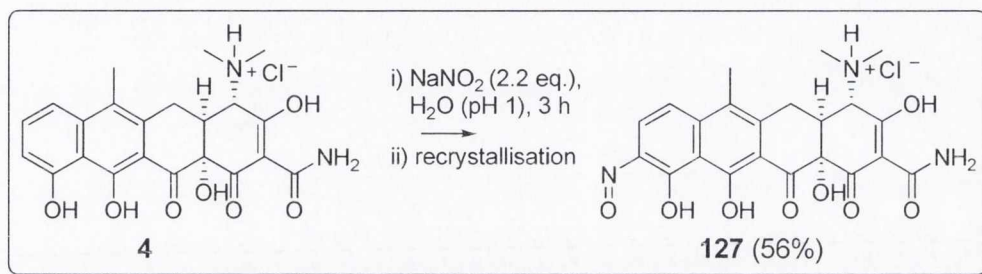
Scheme 3.2 **Synthesis of 4 from 106**



Gratifyingly, anhydrotetracycline hydrochloride (**4**) proved to be a relatively stable material and, as such, could be stored in bulk (under argon at -18 °C). Menachery and Cava had previously reported the synthesis of **127** (Scheme 3.3, *vide infra*) from **4** in 98% yield using nitrous acid at 0 °C.²⁰⁴ Unfortunately, this procedure proved to be difficult to reproduce and in spite of numerous attempts such a high yield could not be obtained. Compound **4** was found to be difficult to solubilise in water and when dissolved in water at pH 1 formed a gelatinous

residue. The reaction of **4** with sodium nitrite in Millipore water at pH 1, using HCl (35% v/v), afforded full conversion of **4** to **127**, however the product did not precipitate from the reaction as had been reported. Instead, the addition of copious amounts of brine, approximately equivolume to the reaction itself, was required to force the precipitation of **127**.

Scheme 3.3 Synthesis of **127** from **4**



Compound **127** was found to be extremely hygroscopic and difficult to filter from the reaction medium. The crude, wet **127** was recrystallised from a mixture of methanol and diethyl ether at 0 °C to afford the desired product in 56% yield.

Notably, an interesting feature of 9-nitrosoanhydrotetracycline hydrochloride (**127**) is its instability. It is well documented that *o*-hydroxynitrosobenzene can readily tautomerise to form oximes that can then be hydrolysed to the corresponding ketone, however it was found that in the case of **127**, the compound decomposed to anhydrotetracycline hydrochloride (**4**) in a matter of weeks if left open to air. The mechanism of this reaction has not been investigated, however storing the compound under Ar at -18 °C appears to slow this decomposition process, although not avert it completely.

A number of different conditions were screened in order to reduce **127** to 9-aminoanhydrotetracycline dihydrochloride (**128**). Considerable experimentation was required to furnish the desired product in a reasonable yield (*vide infra*). The reported method used by Menachery and Cava²⁰⁴ (using $\text{Na}_2\text{S}_2\text{O}_4$ as the reducing agent in 0.1 M NaOH) proved to be extremely unreliable, despite repeated experiments. After numerous experimental attempts involving variation of the concentration of the reaction, the number of equivalents used and the solvent system, the reported method was abandoned.

The non-catalytic methods of reduction attempted are summarised in Table 3.1 (*vide infra*) alongside the required reducing agents (Figure 3.1, *vide infra*). Several different

methods were used in an attempt to purify the products from these reactions. Recrystallisation from methanol, methanol/diethyl ether mixtures, hot ethanol, hot methanol/chloroform mixtures and hot ethanol/chloroform mixtures were all attempted. Recrystallisation from methanol/diethyl ether at 0 °C was found to be the most expedient method.

Figure 3.1 Reducing agents employed in the reduction of 127 to 128

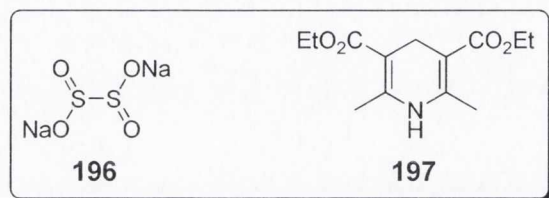


Table 3.1 Reduction of 127 to 128

Entry	Reducing agent	Eq. reducing agent	Solvent	Reaction time (min/h)	Conversion to 128 ^a	Isolated yield
1	196	1.2	0.1M NaOH	18 h	100	11 ^d
2	196	2	Buffer ^b	18 h	100	29 ^d
3	196	2	Buffer ^b	20 min	---	56 ^d
4	197	2	THF/MeOH ^c	28 h	50	50

a = Conversion determined by ¹H NMR spectroscopy; b = Buffer at pH 4.75; c = 1:1 mixture; d = Yields quoted are highest achieved. Reactions were performed several times.

In the case of entry 1 the disparity between conversion and isolated yield can be explained by the method of extraction. Because this reaction was performed in an aqueous medium, it was necessary to extract the product as its free-base by lowering the pH of the reaction mixture to 4.5 (the estimated pK_a of the new amine group) using aqueous HCl, and extracting with chloroform, as per the procedure by Menachery and Cava.²⁰⁴ It was clear after a number of attempts that this was not an efficient method of isolating the product.

It was decided to perform the reaction at a pH equivalent to the pK_a of the new amine in the product. Using this method the free-base product could be extracted directly with chloroform without changing the pH of the reaction medium. Initial attempts using this method yielded disappointing results (entry 2, Table 3.1). After several endeavours involving

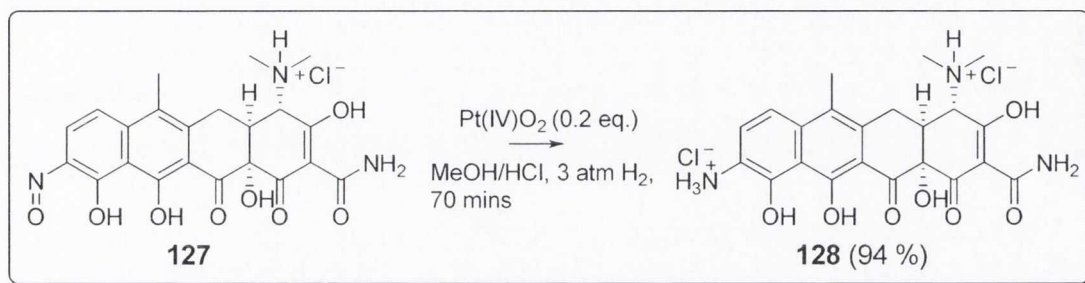
variation of reaction time, it became apparent that the free base amine product was decomposing *in situ* to anhydrotetracycline (**4**) over time. This was confirmed by ^1H NMR spectroscopic studies, as the percentage of **128** would decrease over time and **4** would increase. This was evident by the reduction in intensity of the two resonance signals corresponding to the two aromatic protons of **128** coupled with the appearance of the augmenting three resonance signals corresponding to the three aromatic protons of **4**. With this knowledge in hand, it was decided to repeat the procedure using a biphasic system of buffer solution at pH 4.75 and chloroform. If the reaction was stirred vigorously it was assumed that the amine product would be extracted into the chloroform as it was produced. Gratifyingly, this proved to be the case when the reaction was performed in a separating funnel and agitated manually (entry 3, Table 3.1, *vide supra*). The chloroform layer containing the product was separated several times and replaced with fresh chloroform over the course of the reaction. The extraction of the free base amine product into the chloroform was evident as a colour change to deep red was observed in the chloroform layer after agitation.

Despite the relative success of this experiment, compared with previous experiments, it was felt that there were other reliable reduction methods available that would perhaps furnish better results. It was decided to use the Hantzsch ester to attempt to reduce the aromatic nitroso group (entry 4, Table 3.1, *vide supra*). Frustratingly, after 28 hours the reaction only provided 50% conversion. It was decided to abandon the reaction at this point as it was not efficient with respect to time.

At this stage a catalytic reduction strategy appeared to be the most promising methodology available. Compound **127** was reduced by hydrogenation catalysed by Pt (IV) oxide (20 mol%) at 3 atm for 70 mins in methanol/HCl, which allowed the isolation of **128** in excellent yield by precipitation into diethyl ether (Scheme 3.4, *vide infra*). As was observed in previous reduction experiments, product yield was found to be highly dependent on reaction time. At the given concentration, 0.01 M, if the reaction was allowed to proceed for 90 mins, the amine product **128** would begin to decompose to anhydrotetracycline hydrochloride (**4**). If the reaction proceeded for less than 70 mins conversion of **127** to **128** would be incomplete and the compounds proved difficult to separate and yields were therefore greatly reduced. The optimal reaction time was determined to be 70 minutes, as 100% conversion occurred at this time and no decomposition to **4** was observed. Unfortunately, due to the restrictions of the

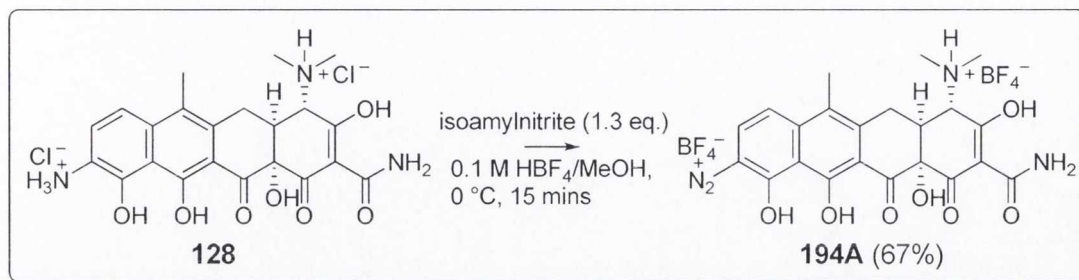
hydrogenation apparatus with respect to the volume of the reaction, only 250 mg of **120** could be reduced to **128** as a time, however this methodology was still the most attractive as the yield was almost quantitative (94%).

Scheme 3.4 Catalytic reduction of **127** to **128**



Amine **128** was then to be converted to **194A** (Scheme 3.5) by diazotisation using isoamyl nitrite in HBF_4 /methanol at -10°C . The tetrafluoroborate counterion was chosen as it is known to provide more stable diazonium salts when compared to chloride or bromide counterions. The reaction was allowed to proceed for 15 minutes, with the appearance of a deep red colour after 1 minute indicating the formation of the diazonium salt. Due to the inherent instability of **194A** in solution and at temperatures above -10°C , ^1H NMR spectroscopic analysis was performed in CD_3OD which had been cooled to -10°C . This provided a spectrum of the tetracycline derivative and IR analysis confirmed the formation of the diazonium salt with the presence of the characteristic strong diazo peak at 2136 v/cm^{-1} . Compound **194A** proved to be much less stable than the diazonium salt analogue of minocycline, **189**. Therefore, it was necessary to reduce the reaction temperature to -10°C and precipitate the product from the reaction medium into diethyl ether, maintaining the temperature at -10°C . The compound was then stored at -18°C under argon for a short period before use.

Scheme 3.5 Diazotisation of 128 to 194A

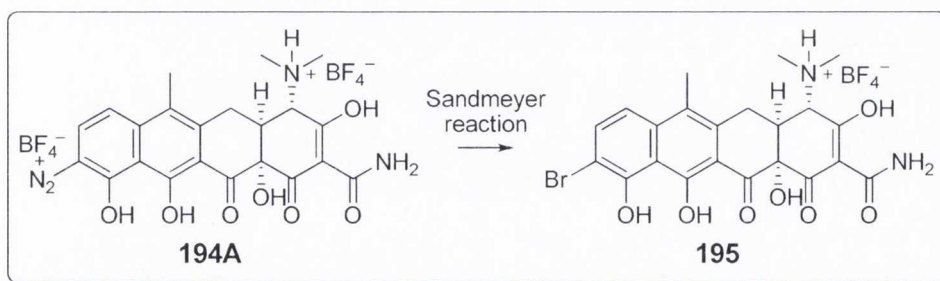


The next step in the synthetic sequence required the insertion of a halogen moiety at position 9 by way of a Sandmeyer reaction.

3.1.1.1 Sandmeyer reaction

The conversion of aromatic diazonium salts to halo moieties *via* the Sandmeyer reaction is a well known method of producing aromatic halides.²³⁹ It was expected that this methodology would afford compound **195** in good yields such that it could be subsequently used in palladium coupling reactions (Scheme 3.6). This synthetic route was chosen over a direct halogenation of the aromatic ring as the major product of direct halogenation reactions was expected to be that where the ring was halogenated *para* to the phenolic moiety.

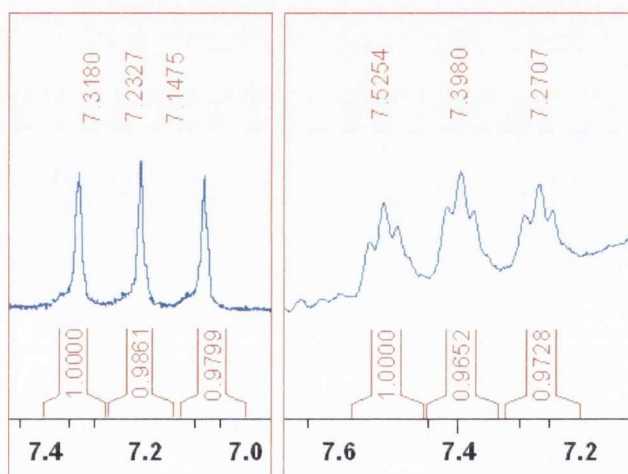
Scheme 3.6 Sandmeyer reaction of 194 to afford 195



Initially, **194A** was reacted with 1 equivalent of copper bromide and 10 equivalents of potassium bromide in 0.1M HCl. The reaction was firstly held at 0 °C for 1 hour and then heated to 30 °C for 1 hour. After 1 hour ¹H NMR spectroscopic analysis revealed that the reaction had not proceeded to any measurable extent and that a small amount of decomposition of **194A** had occurred, after 2 hours the decomposition of **194A** was complete.

Interestingly, the decomposition of the anhydrotetracycline products appeared to proceed in a similar fashion to that of the minocyclines. Three peaks integrating for 1 with respect to each other appeared between 7 and 7.5 ppm in the ^1H NMR spectrum (in DMSO-d_6), separated by 0.13 ppm (51 Hz) each, exactly as those observed in the minocycline decomposition spectra (Figure 2.9, *vide supra*). However, in the case of anhydrotetracyclines, these peaks were observed as either multiplets or singlets. Figure 3.2 (*vide infra*) is taken from the ^1H NMR spectrum of the Sandmeyer reaction as described above. It is again postulated that the detectable decomposition product is a quaternary ammonium salt, due to the characteristic splitting pattern observed.

Figure 3.2 **Observed decomposition of anhydrotetracyclines in ^1H NMR spectra (in DMSO-d_6)**



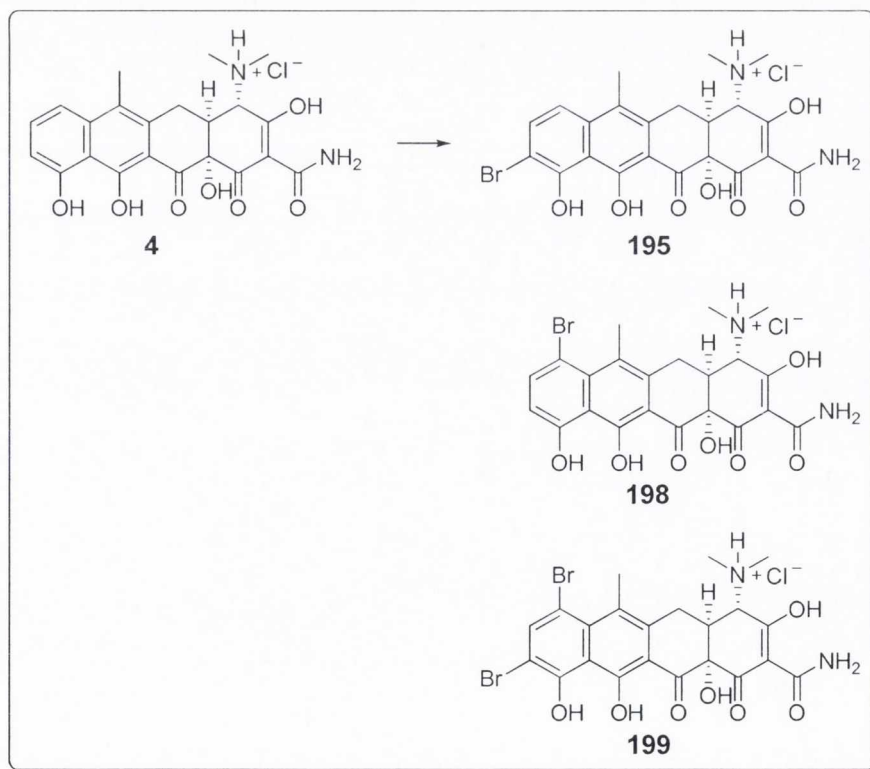
It was decided to repeat the reaction again using copper bromide and HBr , reliable and frequently used reagents for Sandmeyer transformations,²³⁹ in an attempt to enable the reaction to proceed and prevent decomposition of the anhydrotetracycline occurring. Compound **194A** was reacted with 1 equivalent of copper bromide in HBr (40% v/v as solvent and reagent). These were added to **194A** at $-10\text{ }^\circ\text{C}$ and the reaction was subsequently heated to $100\text{ }^\circ\text{C}$ for 1 hour. Unfortunately, no reaction was observed and after 1 hour complete decomposition of **194A** had occurred, as evidenced by the 3 peaks in the ^1H NMR spectrum as shown in Figure 3.2.

A final attempt using the same conditions outlined above was employed but the reaction was halted after 30 minutes. Unfortunately **194A** appeared to be degraded and no reaction was observed. The Sandmeyer reaction was deemed unsuitable at this point as it was clear that **194A** was not stable under the range of reaction conditions employed to afford the desired product.

3.1.2 Synthesis of 9-bromoanhydrotetracycline

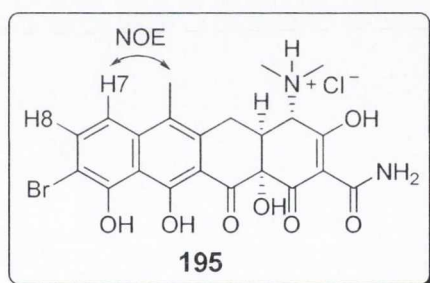
Since at this time RP-MPLC was made available in the laboratory, it was decided to attempt to synthesise 9-bromoanhydrotetracycline (**195**) directly from anhydrotetracycline (**4**). Although regioselectivity issues were foreseen (Scheme 3.7, *vide infra*), it was hoped that the isomers could be separated by RP-MPLC. It was envisaged that the major isomer would prove to be that where bromination would occur at the position *para* to the phenolic group of the D ring, however we were interested to see how much of the product, if any, would prove to be the desired *ortho*-halogenated product **195**.

Scheme 3.7 Bromination of anhydrotetracycline (**4**)



Anhydrotetracycline (**4**) was reacted with 2.1 equivalents of NBS in sulfuric acid for 4 hours. ^1H NMR spectroscopic analysis revealed that only one monosubstituted aromatic product had been produced, accounting for 71 mol% of the crude reaction mixture. In addition, an aromatic singlet was observed, accounting for 29 mol% of the crude reaction mixture, which corresponded to the dibrominated product **199** (observed by ^1H NMR spectroscopy and HRMS). These compounds were separable by RP-MPLC using a mobile phase of 10% acetonitrile in 0.1M HCl/H₂O. NOE analysis was performed to elucidate whether the monobrominated product was the *ortho*- (**195**) or *para*-substituted (**198**) isomer. It was assumed that if only one monobrominated isomer had been produced that it would be the undesired *para*-substituted isomer. Much to our surprise, it proved to be the *ortho*-substituted compound, **195**. NOE analysis confirmed a response from H-7 when the methyl group was irradiated, thus proving the structure of the compound (Figure 3.3).

Figure 3.3 NOE experimentation with **195**



This was extremely unexpected as, although there is a methyl group in proximity to the *para* position, it was not envisaged that it would be sufficient to provide such regiocontrol over the reaction.

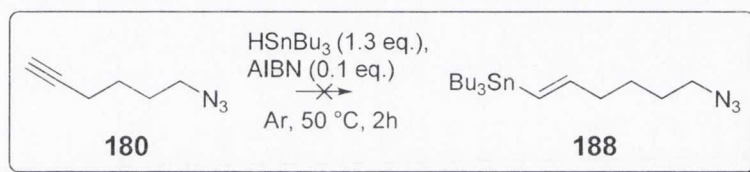
While this was a positive result, **195** proved to be unstable during RP-MPLC purification and only 3% yield was recovered from the column. This was not sufficient as an isolated yield, as the amount of **195** that would have to be produced in order to gain reasonable amounts to use in palladium coupling reactions was impractical. Recrystallisation was also not feasible due to the percentage of impurities present. However, since Koza and Nsiah had reported the use of a diazonium doxycycline derivative in Stille couplings,¹⁹³ it was decided to investigate the use of diazonium salt **194A** in Stille couplings to install the anhydrotetracycline linker.

3.1.3 Synthesis of organotin linkers

3.1.3.1 Route 1 to compound 188

The organotin linker **188** (Scheme 3.8, *vide infra*) could be synthesised in a number of ways. Initially, it was considered that the product could be attained directly from azide **180** via the radical addition of tributyltin hydride. Unfortunately, this reaction did not proceed as expected and a mixture of products was generated. Montevecchi *et al.* had reported that tributyltin hydride could preferentially reduce azides in the presence of alkynes²⁴⁰ and this was considered to be one of the competing reactions taking place.

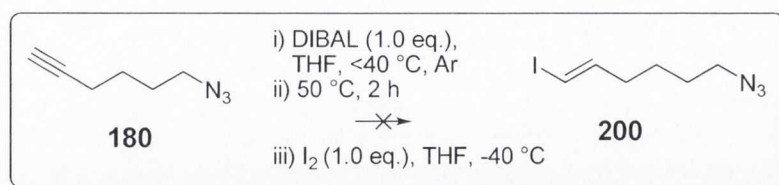
Scheme 3.8 Radical stannylation of **180** to **188**



Attempts to separate the products provided a useful piece of evidence: vinyl organostannanes are not stable in the presence of silica gel. The compounds were found to streak on TLC and so column chromatography furnished mixed fractions of destannylated compounds. If the stannylation reaction was to be useful it would have to convert the starting material to product in near quantitative yield or provide products which could be separated by distillation.

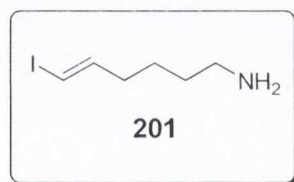
It was thought that the issue of chemoselectivity could be overcome by using reliable reactions. The regioselective iodination of the double bond²⁴¹ and subsequent stannylation^{242, 243} would afford the desired product, **188**. The first step in this synthesis involved the formation of a vinyl allane, which would be produced regioselectively to give the *E*-allane, followed by its conversion to the *E*-iodoalkene. This was to be performed using diisobutylaluminium hydride (DIBAL) and iodine crystals in THF (Scheme 3.9).

Scheme 3.9 Iodination of 180 to 200



Isolation of the reaction product was conducted *via* decomposition of diisobutylallane in sulfuric acid and extraction of the product into organic solvent. However, ¹H NMR spectroscopic analysis provided evidence that the reaction had not proceeded as expected. The methylene signal alpha to the azide group had shifted significantly upfield, indicating that perhaps the azide had been reduced to an amine during the reaction. IR analysis confirmed that the azide had indeed been reduced and as such it was deduced that the product obtained was not **200** as expected, but in fact **201** (Figure 3.4).

Figure 3.4 Product of the iodination of 180



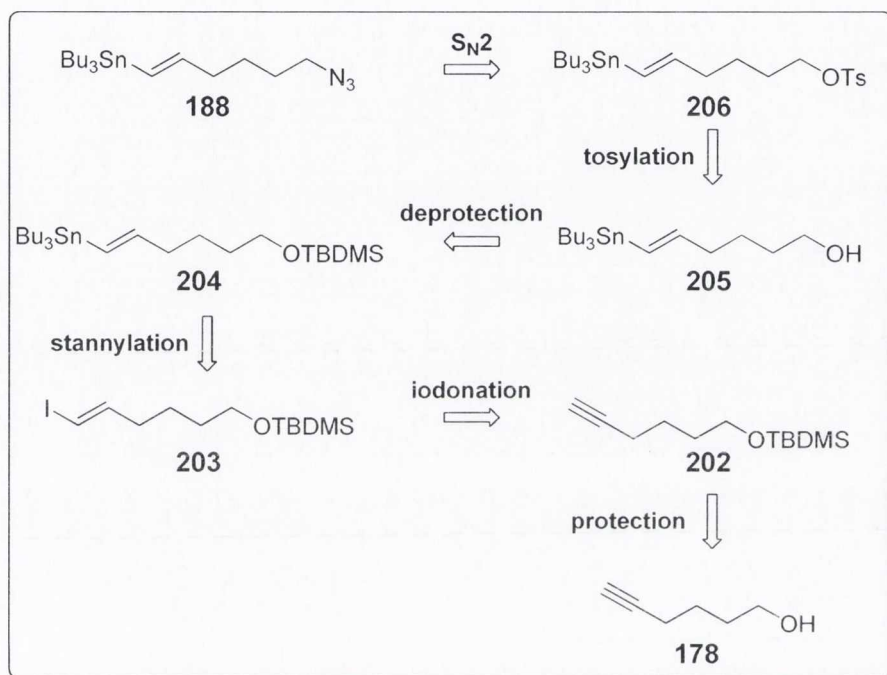
Since the chemoselectivity issues had not been averted as planned, it was necessary to design a new route which would circumvent these issues.

3.1.3.2 Route 2 to compound 188

A second route to the desired compound was designed, starting from an available material, 5-hexyn-1-ol (**178**). It was decided to use the halogenation method again as this reaction can be performed to yield solely *E*-geometry in the olefin. This would provide a choice of two linkers for the dimer: one with a double bond with geometry that still allows the linker to thread through the protein in a straight manner and also the hydrogenated linker. This would probe how the flexibility of the linker affected dimer activity. The retrosynthesis of this route is depicted in Scheme 3.10 (*vide infra*). It was hoped that protection of the alcohol would

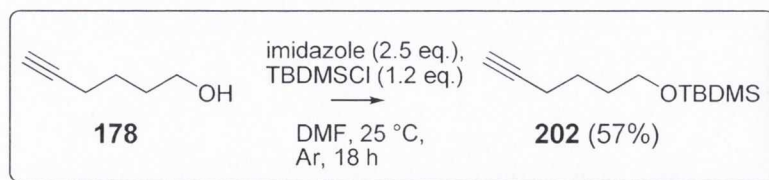
allow the clean formation of the vinyl iodide. The introduction of the azide group in the last step would avoid chemoselectivity issues between the azide and alkynyl moieties.

Scheme 3.10 Retrosynthetic route 2 to organotin linker **188**



The first step in the synthesis involved the protection of the alcohol **178** with a *t*-butyldimethylsilyl group (TBDMS). This was afforded using *t*-butyldimethylsilyl chloride (TBDMSCl) and imidazole in DMF. Compound **202** was obtained in 57% yield after column chromatography (Scheme 3.11).

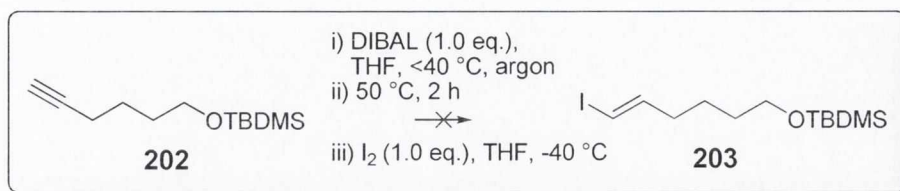
Scheme 3.11 TBDMS protection of **178** to afford **202**



The next reaction of this synthetic sequence required the conversion of **202** to iodoalkene intermediate **203** (Scheme 3.12, *vide infra*) using the reaction conditions previously described

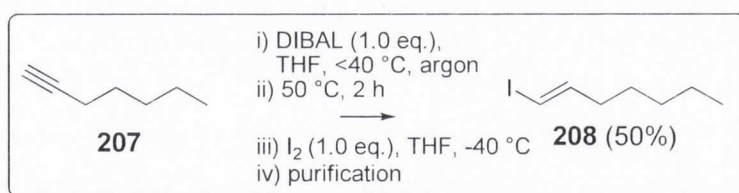
for the iodination of **180**. Unfortunately these conditions provided no reaction at all and the starting material **202** was recovered.

Scheme 3.12 Regioselective iodination of 202



Since the iodination had occurred in the same reaction when **180** was employed as a reactant (Scheme 3.9, *vide supra*), albeit with concurrent reduction of the azide group, a test reaction was undertaken using heptyne as the starting material (Scheme 3.13). Under identical reaction conditions to those applied to **202**, heptyne (**207**) was converted fully to **208** and obtained in 50% yield after purification by flash column chromatography. NOE experimentations confirmed the geometry of double bond to be solely the desired *E*-geometry. It was concluded that the OTBDMS moiety of alkyne **202** prevented the reaction from occurring.

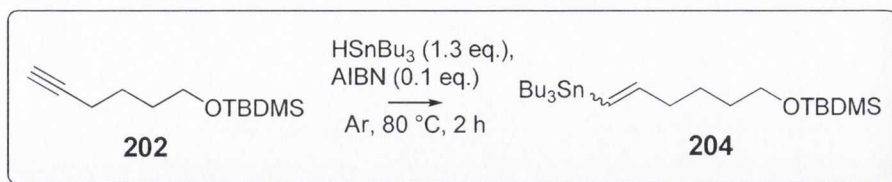
Scheme 3.13 Regioselective iodination of heptyne (207)



It was decided at this point to divert from iodination of the alkyne and directly stannylate the alkynyl bond using a radical stannylation method. It was envisaged that the major isomer provided would be that of the *E*-olefin and that optimisation of the reaction conditions would afford regiocontrol over the reaction to provide solely *E*-geometry in the olefin.

Compound **202** was heated in the presence of tributyltin hydride and AIBN under an atmosphere of argon to afford vinylstannane **204** (Scheme 3.14, *vide infra*). 20% of the product was produced as the *Z*-isomer and 80% as the *E*-isomer (as determined by ¹H NMR and NOE spectroscopic experiments).

Scheme 3.14 Radical stannylation of 194 to furnish 204

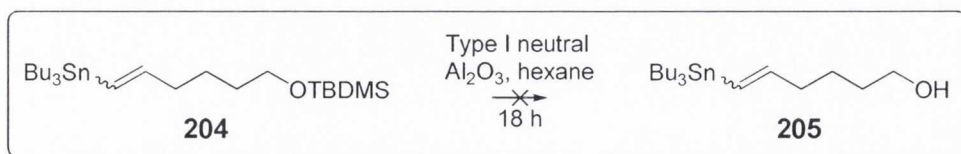


Frustratingly, this product proved to be extremely unstable in DMSO-d_6 and CDCl_3 and decomposed to a destannylated olefin over the time necessary to perform ^{13}C NMR experiments. Assignments of the ^1H NMR spectral data in the experimental section are therefore based upon knowledge of the expected shifts of peaks, splitting patterns, coupling constants and data obtained from the synthesis of subsequent, more stable, vinylstannanes.

As expected, compound **204** was not stable on silica and 2-D TLC indicated decomposition of the compound. In addition, an attempt at Kugelrohr distillation of the compound was unsuccessful. Given the instability of **204** and the difficulties in purification by distillation methods, it was decided to take the crude product of approximately 87% purity (with 13% unreacted HSnBu_3 as impurity) forward to the next reaction in the hope that the product of this reaction would prove to be more stable than that previously obtained. It was also hoped that since the product of this reaction would be an alcohol, the distillation would be more successful in this case.

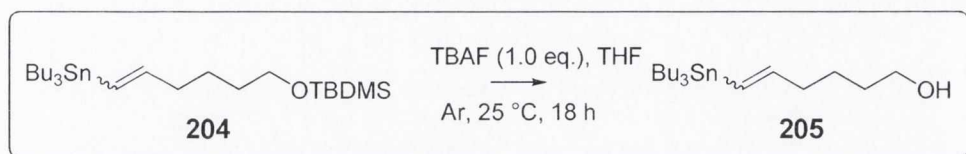
The next step in the synthesis entailed the removal of the TBDMS protecting group of silyl ether **206**. Initially, mild conditions were attempted due to the sensitivity of the vinylstannyl moiety. Feixas *et al.* reported the deprotection of TBDMS protected alcohols using neutral aluminium oxide in hexane.²⁴⁴ Unfortunately, this reaction did not proceed as desired and decomposition of **204** occurred, as was evident from the disappearance of signals relating to vinyl protons in the ^1H NMR spectrum (Scheme 3.15).

Scheme 3.15 Deprotection of 204 with Al_2O_3



A second attempt using the traditional deprotection method of tetrabutylammonium fluoride (TBAF) in THF was undertaken (Scheme 3.16). It was expected that the fluoride ion might also destannylate compound **204**; gratifyingly, this did not occur and **204** was converted cleanly to compound **205**.

Scheme 3.16 Deprotection of **204** with TBAF

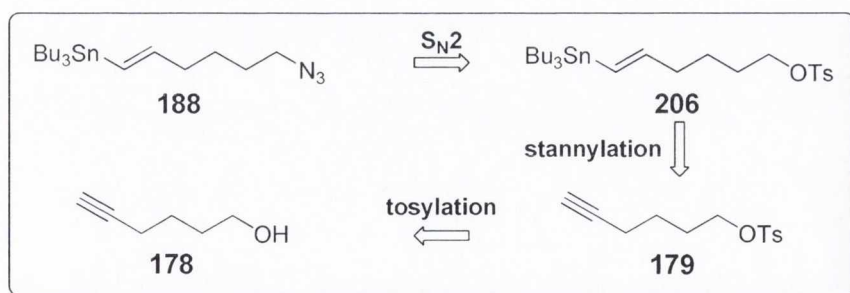


This was an extremely encouraging result, however, it was realised that a better approach may lie in the radical addition of tributyltin hydride to the tosylated alcohol **179** (Scheme 3.17) that had been prepared previously for the synthesis of minocycline linker 2 (Section 2.4.1, *vide supra*). This approach, if successful, would remove two steps from the synthesis of **188** and avoid the preparation and handling of the most unstable compound, **204**.

3.1.3.3 Route 3 to compound **188**

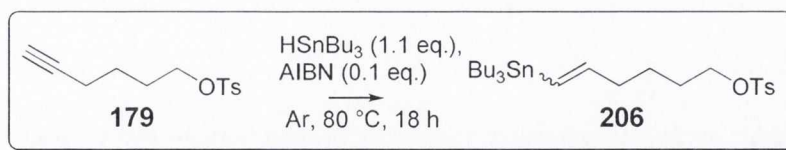
The revised retrosynthesis of **188** is depicted in Scheme 3.17. The first step in the forward synthesis had already been performed (Section 2.4.1, *vide supra*).

Scheme 3.17 Retrosynthetic route 3 to organotin linker **188**



The second step in the forward synthesis was performed using the reaction conditions applied to the TBDMS protected alcohol **204** (Scheme 3.14, *vide supra*). The tosylated alcohol **179** was reacted with 1 equivalent of tributyltin hydride and 0.1 equivalent of AIBN at 80 °C under an atmosphere of argon for 18 hours (Scheme 3.18, *vide infra*).

Scheme 3.18 Radical addition of HSnBu₃ to 179



After 18 hours, however, the reaction was found to have reached only 50% conversion (determined by ¹H NMR spectroscopy). The reaction was repeated with the use of 2 equivalents of tributyltin hydride. After 24 hours the reaction was found to have reached 100% conversion to the desired, novel product with a ratio of 1:4 *Z:E* isomers. However, the issue of removing the excess tributyltin hydride remained. It was postulated that the reaction could be driven to 100% conversion using only 1 equivalent of tributyltin hydride if performed using microwave irradiation as a source of energy.

Two reactions were initially performed, varying only the reaction time. 1 equivalent of **179**, 1 equivalent of tributyltin hydride and 0.1 equivalents of AIBN were heated in a microwave reaction vessel at 100 °C for 30 mins and 1 hour, respectively. Both reactions were found to have reached full conversion and the ratio of *Z:E* isomers remained at 1:4 in both cases. Table 3.2 summarises the various reaction conditions used in the microwave assisted synthesis of **206** from **179** in attempts to improve the ratio of the desired *E*-isomer over the undesired *Z*-isomer.

In each case 1 equivalent of **179** and tributyltin hydride were used and 0.1 equivalent of AIBN. The reactions were performed in the absence of solvent. The product, **206**, was found to be stable to RP-MPLC and hence this method of purification was employed where necessary.

Table 3.2 Screening of reaction conditions to afford control over *Z:E* ratio

Entry no.	Temperature (°C)	Reaction time (mins)	<i>Z:E</i> ratio
1 ^a	100	60	1:4
2 ^a	100	30	1:4
3 ^a	100	15	1:4
4 ^a	50	30	1:4

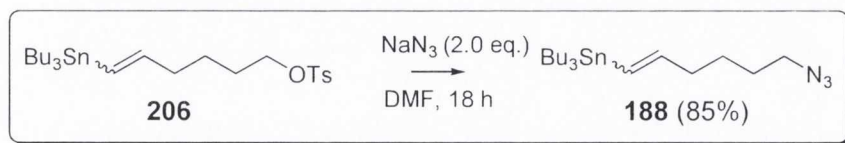
a = full conversion observed by ¹H NMR spectroscopy

It was evident after some experimentation that neither reducing the reaction time nor lowering the reaction temperature had any effect on the ratio of isomers obtained. The regioselective iodination of the triple bond as employed for compounds **180** (Scheme 3.9, *vide supra*) and heptyne (Scheme 3.13, *vide supra*) could not be employed in this case as the tosylate moiety of **179** would activate the compound towards S_N2 reactions with iodine.

As such, it was decided to use compound **206** as a mixture of isomers for the synthesis of the final linker, **188**. This would eliminate the possibility of having the anhydrotetracycline dimer half linked *via* a double bond also, as the geometry of the *Z* isomer would not provide the correct orientation for the linker to thread out along the binding pocket of the Tet(R) regulation protein (Section 2.1, *vide supra*). Also, the isomers could prove extremely difficult to separate given the earlier issues encountered with regards to purification and stability of tetracycline compounds. It was decided to employ the synthetic procedure involving the reduction of the double bond once the linker was attached to the anhydrotetracycline analogue. This eliminated the need to separate the *E*- and *Z*-isomers of **188** as once attached to the anhydrotetracycline analogue and reduced the two isomers would provide the same dimer half. Also, anhydrotetracycline had proven stable under catalytic reduction conditions.

Compound **206** was reacted with 2 equivalents of sodium azide in DMF to produce the desired linker **188** in 85% yield (Scheme 3.19, *vide infra*). Compound **188** required no further purification and its structure was proven by disappearance of the tosyl signals in ¹H NMR spectra and the appearance of the characteristic azide peak at 2093 ν/cm^{-1} by IR analysis. This route provided the desired, novel product, **188**, in 33% yield over 3 steps.

Scheme 3.19 **Synthesis of 188 from 206**



3.1.3.4 Conclusions

Over the course of the synthesis of **188** a number of novel organotin compounds had been produced. A synthetic route had been developed which avoided chemoselectivity issues

between the alkynyl bond and the azide in the desired product and accounted for the chemical instability of the organotin compounds. All organotin compounds were stored at -18 °C, under an atmosphere of argon, to avoid decomposition.

With the synthesis of linker **188** and its coupling partner, diazo anhydrotetracycline **194**, complete attention was turned to the Pd catalysed coupling of the two components *via* the Stille reaction.

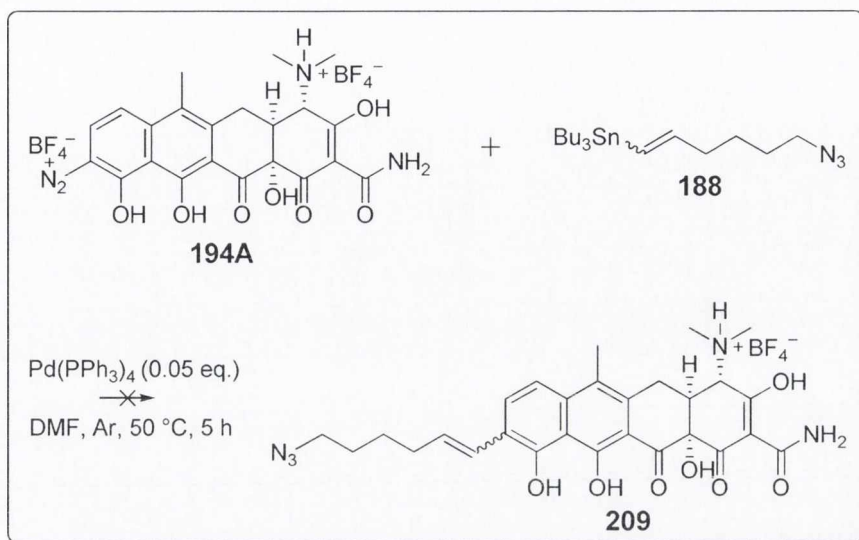
3.1.4 Stille couplings

3.1.4.1 Stille reactions with **194** using palladium tetrakis

All solvents used for palladium coupling reactions were distilled anhydrously and degassed using the freeze-pump-thaw method.

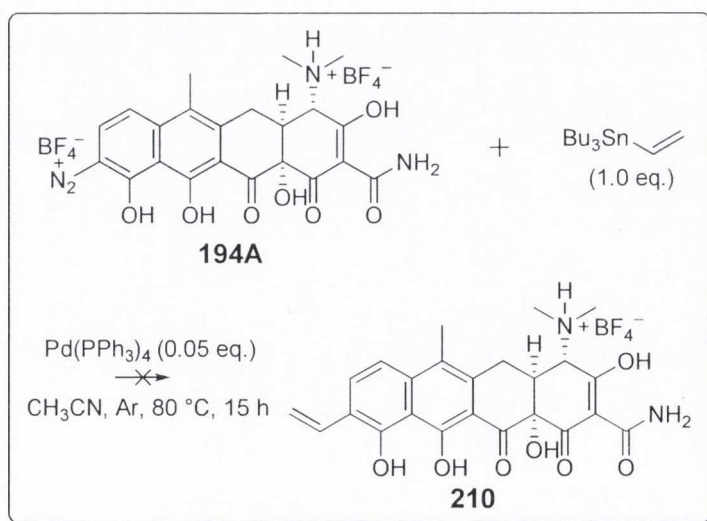
The first Stille reaction performed with compound **194A** and compound **188** employed 1 equivalent of each coupling partner and 0.05 equivalents of palladium tetrakis in DMF at 0.2 M concentration (Scheme 3.20). After 5 hours no formation of the desired product, **209**, was observed by ¹H NMR spectroscopy and the reaction was worked up for further analysis. The reaction was diluted with methanol and added to diethyl ether dropwise. The resultant precipitate was analysed by ¹H NMR spectroscopy. No signals were observed in the vinyl region and upon examination of the crude in DMSO-d₆ the characteristic anhydrotetracycline decomposition peaks were observed.

Scheme 3.20 Stille reaction 1 with 194A and 188



It was decided at this time that, since compound **188** had proved difficult to synthesise, it would be advisable to perform optimisation of the Stille reaction with compound **194A** and tributylvinyltin. The reaction conditions could then be applied to the reaction involving **188** and **194A**. The resulting product, compound **210** (Scheme 3.21), was expected to give rise to a similar aromatic region in ^1H NMR spectra to that of *o*-vinylphenol and this would be helpful in monitoring the reaction.

Scheme 3.21 Stille reaction 1 with diazonium salt 194A and tributylvinyltin

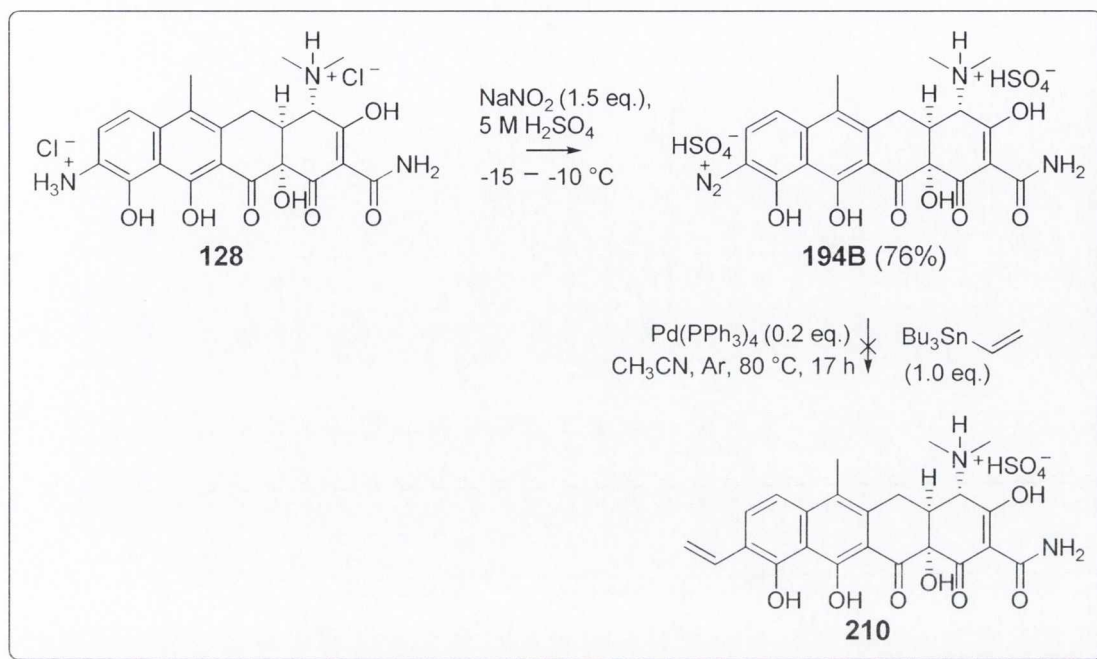


Initially a reaction was designed using 1 equivalent of **194A** and 1 equivalent of tributylvinyltin (Scheme 3.21). Acetonitrile was employed as the solvent and 0.05 equivalents of palladium tetrakis were used. The reaction was performed at 0.1 M concentration with respect to **194A**. Acetonitrile was chosen as the solvent in this case as the use of DMF complicates reaction work up and can obscure the region of interest in a ^1H NMR spectrum. The reaction temperature was raised as no reaction was observed in the previous attempt and it was felt that reflux might be necessary to encourage product formation. After 15 hours of reflux in acetonitrile, a ^1H NMR spectrum of the crude reaction mixture revealed new resonance signals at 6.3 and 7.0 ppm with a similar splitting pattern to that found in styrene ^1H NMR spectra. This was encouraging and the reaction was filtered to remove the catalyst and the solvent was removed under reduced pressure. The crude mixture was subjected to RP-MPLC in an attempt to separate the desired product from the remaining triphenylphosphine and tributylvinyltin. Unfortunately, no tetracycline fraction was recovered, and while the peaks at 6.3 and 7.0 ppm did appear in one fraction, it was clear that they were not associated with a tetracycline moiety. This unidentified compound will heretofore be referred to as compound **211**. It was deduced that compound **194A** had decomposed under the reaction conditions.

Hydrosulfate salts of diazo compounds have been reported to be stabilised by protection of the diazo moiety by the sulfate. With this in mind, it was decided to prepare the hydrosulfate diazonium salt compound **194B** (Scheme 3.22, *vide infra*) and test its use in a Stille reaction to evaluate if it would provide protection against the degradation of the tetracycline during the reaction.

Compound **194B** was formed by the treatment of amine **128** with excess sodium nitrite 5 M sulfuric acid (Scheme 3.22). Reaction of these components at $-14\text{ }^\circ\text{C}$ for 15 minutes provided full conversion of **128** to **194B**, as evidenced by a deep red colour forming in the solution. The compound was precipitated into diethyl ether at $0\text{ }^\circ\text{C}$ and quickly collected by filtration under argon. The compound was stored under argon at $-18\text{ }^\circ\text{C}$ to avoid decomposition of the diazo moiety.

Synthesis of hydrosulfate diazonium salt 194B and Stille reaction

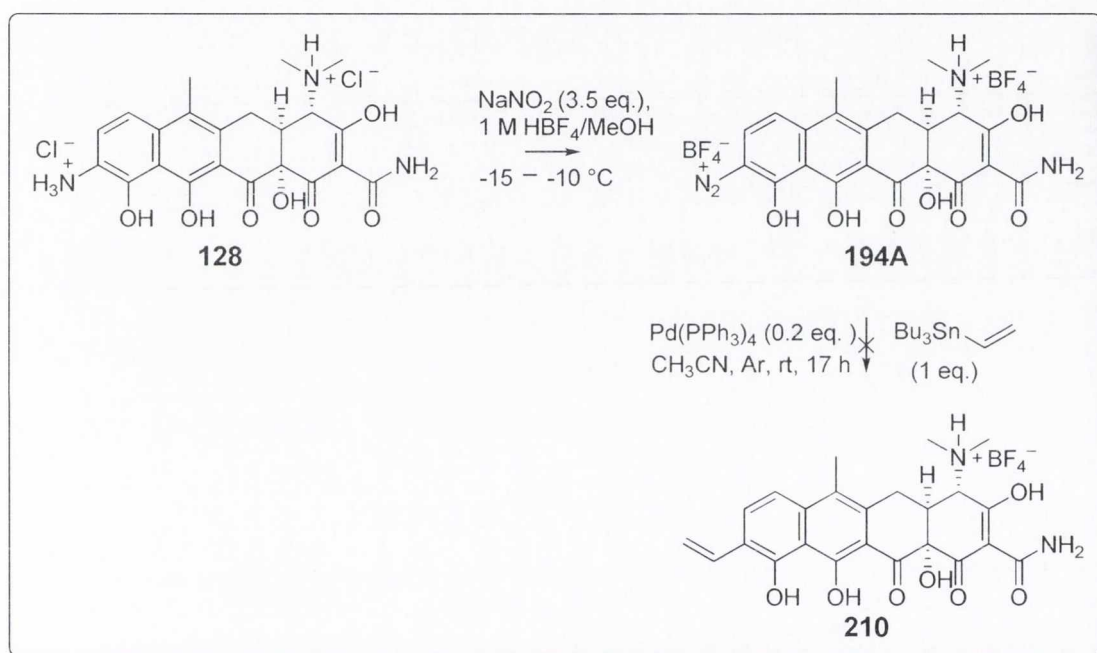


Compound **194B** was subsequently refluxed in acetonitrile with 1 equivalent of tributylvinyltin in the presence of 0.2 equivalents of palladium tetrakis. The reaction was monitored by ^1H NMR spectroscopy, however, after 16 hours no reaction had been observed and degradation of **194B** was observed, as evidenced by the three characteristic resonance signals in the ^1H NMR spectrum separated by 51 Hz each. Since the reported literature had only employed tetrafluoroborate diazonium tetracycline salts and the change of counterion from **194A** to **194B** had provided no detectable protection of the compound, it was decided to return to the use of compound **194A** for further reactions.

Koza *et al.* had previously reported the synthesis of 9-substituted doxycycline (**2**) derivatives *via* Stille coupling chemistry.¹⁹³ In this procedure, the diazonium salt tetracycline analogue was prepared and directly used in the subsequent Stille reaction. It was decided to follow this procedure in an attempt to couple compound **194A** and tributylvinyltin (Scheme 3.23, *vide infra*). The first step in the reaction sequence involved the formation of the diazonium salt **194A** from compound **128**. This reaction was performed between -10 °C and -15 °C to avoid decomposition of the diazo moiety. According to the reported procedure the

solvent was subsequently removed by evaporation at reduced pressure and while no exact conditions were given it was felt that it would be best to perform this operation at room temperature due to the unstable nature of the diazonium salt. Compound **194A** was then dissolved in acetonitrile under an atmosphere of argon and added to the reaction flask containing the other reaction components *via* syringe. After 17 hours stirring in acetonitrile the Stille reaction was halted and the reaction solvent removed at reduced pressure.

Scheme 3.23 **Reaction of 128 to 210 via Koza *et al.*'s procedure for doxycycline derivatives**



The reported literature procedure involved the use of reverse phase preparative thin layer chromatography.¹⁹³ Unfortunately, this method was not available and so the crude reaction was split into two portions and worked up separately. The first portion was dissolved in water at pH 3 (to ensure full protonation of **210**) and washed with CH_2Cl_2 diethyl ether to remove organic impurities. Evaporation at reduced pressure and ^1H NMR spectroscopic analysis of the organic extract revealed the formation of compound **211**. The same treatment of the aqueous extract revealed compound **211** and the characteristic peaks associated with anhydrotetracycline degradation. While all of the tributylvinyltin had been consumed, no

evidence of **210** could be found by ^1H NMR or high resolution mass spectrometry (HRMS). The second portion of the crude reaction was purified *via* by RP-MPLC. The first fraction obtained during the purification process proved to be the characteristic decomposition product of anhydrotetracycline. The second fraction provided compound **211** and the third fraction proved to contain triphenylphosphine oxide and a small amount of an undetermined *n*-butyl compound.

Since the initial attempt at employing the procedure set out by Koza *et al.* had failed, it was decided to attempt the reaction again, as Koza *et al.* had reported the use of palladium acetate and not palladium tetrakis. It was initially considered that the change of catalyst would not adversely affect the reaction, however, as the initial reaction had failed, it was repeated employing one equivalent of tributylvinyltin, one equivalent of **194A** (prepared freshly as per the procedure)¹⁹³ and 0.2 equivalents of palladium acetate in acetonitrile. The reaction was heated at reflux for 4 hours after which diazonium salt **194A** was found to have decomposed (determined by ^1H NMR spectroscopy) with no visible formation of the desired product.

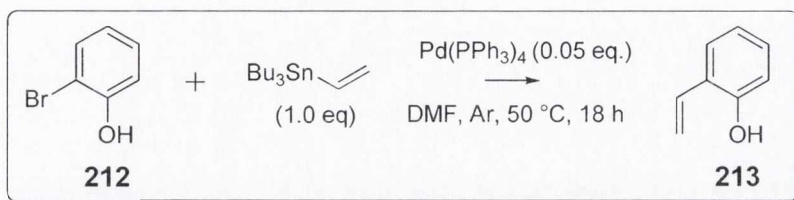
It was it was postulated that the phenolic group *ortho* to the diazo moiety in compound **194A** may have caused an adverse interference during the reaction process by chelation to the palladium and it was considered that this could be the process affecting the progress of the reaction. A number of test reactions were designed and model compounds employed to investigate this theory, as the synthesis of **194A** was laborious and it was felt that test reactions would be better employed on simpler compounds that were easy to produce.

3.1.4.2 Test reactions to optimise Stille conditions

Given that an unexpected product (**211**) was forming during Stille couplings with the anhydrotetracycline analogues, a number of test reactions were devised to investigate and optimise reaction conditions before proceeding with further reactions on anhydrotetracycline analogues. Initially, *o*-bromophenol (**212**) was chosen as a model compound and subjected to the same Stille coupling reaction conditions as was compound **194A**. This reaction was performed to investigate if the *o*-phenolic moiety was affecting the progression of the reaction, as bromobenzene is known to react in Stille couplings. After 18 hours, the reaction was found to contain approximately 10% of the product, 2-vinylphenol (**213**) (Scheme 3.24, *vide infra*), as determined by ^1H NMR spectroscopy using an internal standard. Although the

conversion was low, it was encouraging that the reaction was progressing. This disproved the theory that an *o*-phenolic moiety could prevent the desired reaction from occurring entirely.

Scheme 3.24 Test reaction 1 with **212** and tributylvinyltin

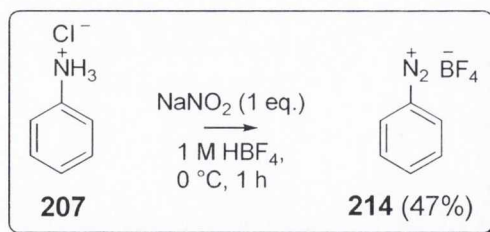


To ensure that the homocoupling of tributylvinyltin was not occurring preferentially over the formation of the desired products under our reaction conditions, a reaction was performed using only tributylvinyltin and palladium tetrakis. The two reaction components were refluxed in acetonitrile for 19 hours and no homocoupled product was observed and the tributylvinyltin remained unreacted. This eliminated the possibility that homocoupling was preventing the desired Stille coupling reaction between **194A** and tributylvinyltin from taking place.

It was decided to explore whether or not the use of an aromatic diazonium compound was inhibiting these reactions. Although aromatic diazonium compounds have previously been shown to be useful in Stille couplings,^{193, 245} it was decided to employ diazobenzene tetrafluoroborate (**214**) in our reaction conditions.

Compound **214** was produced from the reaction of aniline hydrochloride (**215**) with 1 equivalent of sodium nitrite in 1M HBF_4 (aq) (Scheme 3.25). The reaction was stirred for 1 hour at 0 °C and the product, **214**, was collected by precipitation and filtration.

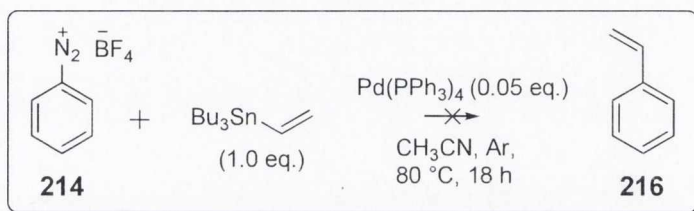
Scheme 3.25 Synthesis of diazobenzene (**214**) from aniline hydrochloride (**215**)



Compound **214** was reacted with tributylvinyltin in the presence of 0.05 equivalents of palladium tetrakis in the hope of producing styrene (**216**, Scheme 3.26, *vide infra*). This

reaction however produced no visible amounts of styrene (by ^1H NMR spectroscopy and TLC evaluation) but did produce the same signals in the vinyl region, at 6.3 and 7.0 ppm, as had been seen previously in the reaction of **194A** and tributylvinyltin (Scheme 3.21, *vide supra*). This indicated the production of compound undesired by-product **211**.

Scheme 3.26 Test reaction with **214** using palladium tetrakis



Copper iodide has been shown to enhance the rate of Stille coupling reactions and this effect has been well documented.²⁴⁶ The rate of reaction is particularly enhanced in cases where the palladium ligand is triphenylphosphine. As such, it was decided to attempt the Stille coupling reaction of **214** and tributylvinyltin with the addition of 0.1 equivalents of copper iodide. The reaction was performed at 80 °C in acetonitrile using 1 equivalent each of compound **209** and tributylvinyltin. The reaction was performed in the same manner as test reaction depicted in Scheme 3.26. However, after 1 hour there was evidence (^1H NMR spectroscopy) of the production of styrene (**216**) and vinylphosphonium salt **211** in a 2:1 ratio. After 18 hours, all of the tributylvinyltin had been consumed and, while the yields of **216** and **211** produced had increased, the ratio remained the same. Thus, it was encouraging that the addition of copper iodide had resulted in the formation of the desired product, **216**, despite the concurrent production of by-product **211**.

To investigate if reaction temperature had any effect on the production of **216** over **211** the reaction depicted in Scheme 3.26 was repeated, however, with the addition of 1 equivalent of copper iodide but reacted at an ambient reaction temperature (instead of refluxing at 80 °C). After 1 hour, however, there was no evidence of the formation of styrene (**216**); only a small amount of what appeared to be compound **211** could be observed by ^1H NMR spectroscopy.

3.1.4.2.1 Ascertaining the identity of compound **211**

It was deemed prudent that the identity of compound **211** should be ascertained given its appearance in a variety of the Stille reactions performed using tributylvinyltin. It was clear that the undetermined impurity was not the homocoupled product, 1,3-butadiene, and must therefore be a vinylic species given the peaks present in the ^1H NMR spectrum (Figure 3.5). Gratifyingly, **211** could be separated from the crude reaction mixture using RP-MPLC. It was characterised by NMR spectroscopy, mass spectrometry and NOE experiments, which revealed two separate sets of vinyl signals, two double doublets and a multiplet in each set (Figure 3.6, *vide infra*).

Figure 3.5 ^1H NMR spectrum of **211**

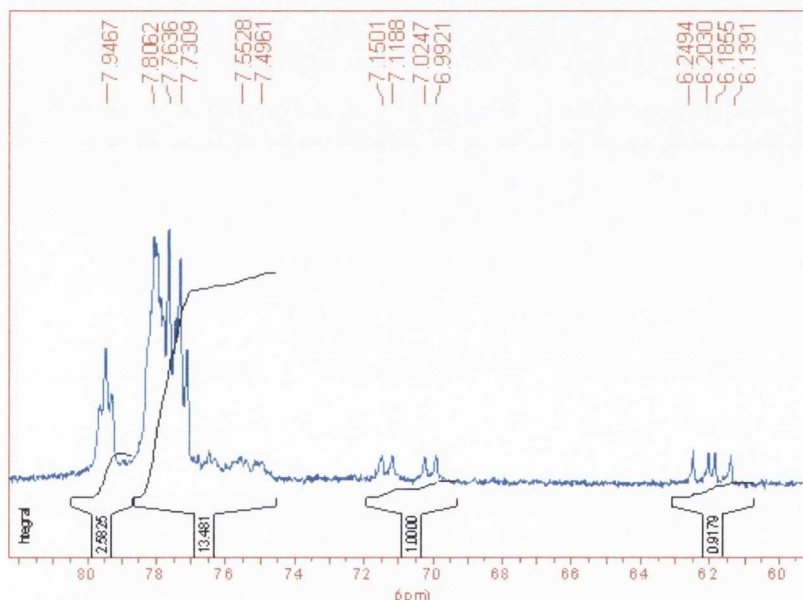
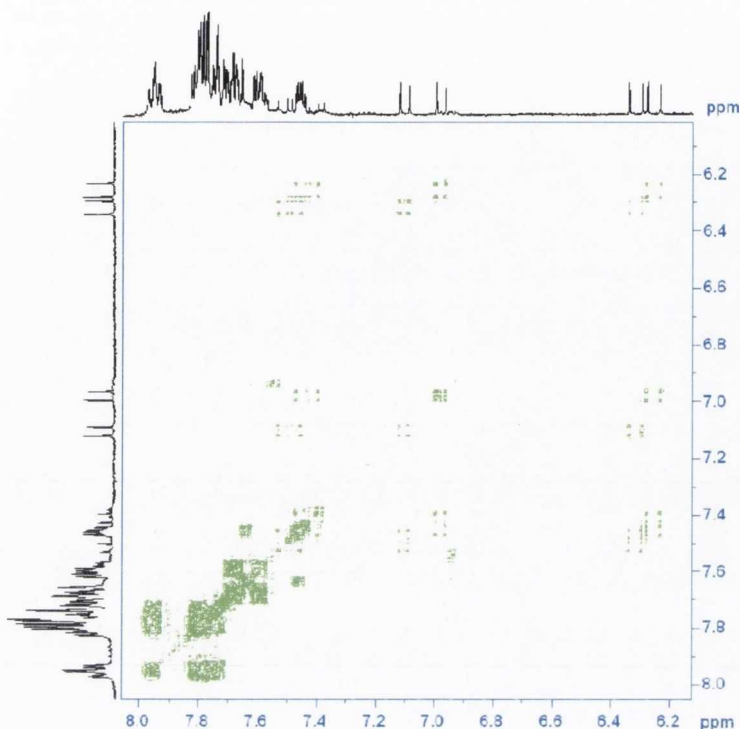


Figure 3.6 2-D NOE experiment of **211**



The anomaly of the detection of two sets of spin systems for the vinylic compounds in the NOE is unexplained and not commented upon in the literature examples of spectra. It may be that there are in fact two spin systems in the compound, but it is more likely to be due to an error in the way the data is collected for NOE making it appear that there are two spin systems due to the large coupling constants between ^1H and ^{31}P . Given the presence of aromatic signals in the compound and the large coupling constants in the doublets (Figure 3.5, *vide supra*), it was considered that compound **211** could be triphenyl(vinyl)phosphonium tetrafluoroborate, depicted in Figure 3.7.

Figure 3.7 **Proposed structure of 211**



This proved to be the case and **211** as depicted in Figure 3.7 and its tetrafluoroborate counterion were detected separately by mass spectrometry. The ^1H NMR spectrum (Figure 3.5) agrees almost exactly with the ^1H NMR spectrum of triphenyl(vinyl)phosphonium bromide.²⁴⁷ The vinylic proton alpha to the phosphonium ion is shifted upfield in compound **211**, compared with triphenyl(vinyl)phosphonium bromide, however, the splitting pattern and coupling constants of all protons agree. ^{31}P NMR spectroscopy of **211** provided a resonance peak at 19.1 ppm, compared with that reported for triphenyl(vinyl)phosphonium bromide, 19.3 ppm.²⁴⁸

When the reaction conditions were examined further, reference was found in the literature warning against the use of palladium catalysts with triphenylphosphine ligands and arenediazonium tetrafluoroborate salts as they can form radical compounds as shown in Scheme 3.27.²⁴⁹

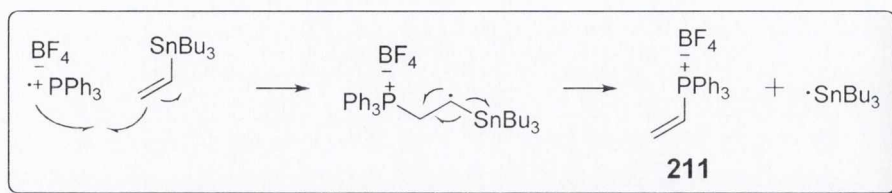
Scheme 3.27 **Formation of triphenyl phosphine radical**



It is tentatively suggested that perhaps the formation of **211** begins with this process. Subsequently, the triphenylphosphine radical could react with the tributylvinyltin to produce **211** and a tributyltin radical (Scheme 3.28), which is known in the radical addition of tributyltin hydride to alkynes. The tributyltin radical could then be easily transformed to tributyltin hydride or a tributyltin dimer. This mechanism is also supported by the fact that compound **211** was not formed when tributylvinyltin and palladium tetrakis, in the absence of

an aryldiazonium compound, were reacted in acetonitrile to determine if homocoupling was occurring.

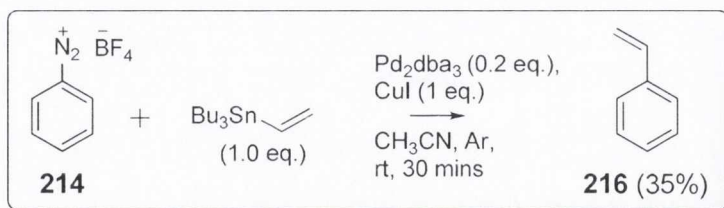
Scheme 3.28 Proposed mechanism of formation of **211**



3.1.4.3 Stille reactions with compound **194** and test reactions using Pd_2dba_3

Since it appeared that the use of palladium tetrakis was causing the formation of compound **211**, it was decided to use Pd_2dba_3 (dba = dibenzylidene acetone) as a source of palladium (0). The change of catalyst was investigated in the coupling of **214** and tributylvinyltin (Scheme 3.29).

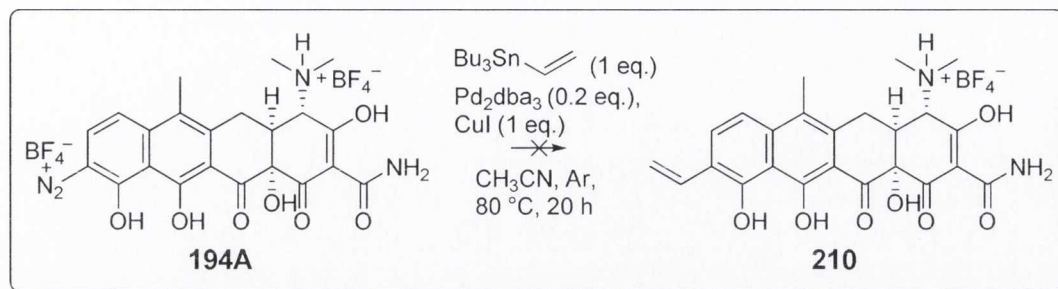
Scheme 3.29 Test reaction with **214** using Pd_2dba_3



Using 0.2 equivalents of Pd_2dba_3 and one equivalent of copper iodide, styrene (**216**) was afforded in 35% yield after 30 minutes at ambient temperature (determined by ^1H NMR spectroscopy using 2,5-diphenylfuran as an internal standard). With the change of catalyst having proven to be so effective, it was decided to return to the use of compound **194A**.

Having determined the successful coupling of diazobenzene (**214**) and tributylvinyltin, compound **194A** was reacted under identical reaction conditions in an attempt to prepare compound **210** (Scheme 3.30).

Scheme 3.30 **Reaction 1 of 194A and tributylvinyltin with Pd₂dba₃**



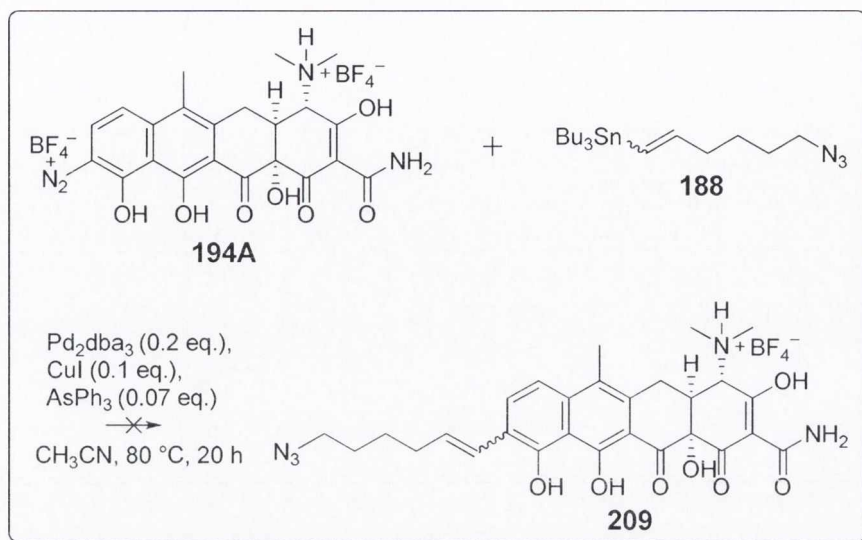
Disappointingly, ^1H NMR spectroscopic analysis showed no product formation after 20 hours and RP-MPLC separation of the reaction mixture provided the characteristic decomposition product of anhydrotetracycline.

In 2000, Koza reported an additional Stille reaction procedure for the coupling of 8-halo substituted doxycyclines which incorporated the use of copper iodide and triphenylarsane as additives to the reaction.¹⁸² Koza reported 93% yield after 6 hours of reflux (using preparative reverse phase TLC for purification).

We attempted this procedure for the coupling of **194A** and tributylvinyltin, with 0.1 equivalents of copper iodide, 0.07 equivalents of triphenylarsane and 0.2 equivalents of Pd_2dba_3 . After 20 hours of reflux, no product formation was observed in the reaction of **194A** and tributylvinyltin and RP-MPLC revealed decomposition of the starting material, **194A**.

To ensure that the lack of reaction between compound **194A** and tributylvinyltin was not being caused by the tributylvinyltin itself, or that the product of the reaction was not unstable and therefore decomposing after production, the reaction was attempted using Pd_2dba_3 with compound **194A** and organotin linker **188** (Scheme 3.31, *vide infra*). The reaction conditions reported by Koza were employed.¹⁸²

Scheme 3.31 Stille reaction between **194A** and **188** using Pd_2dba_3



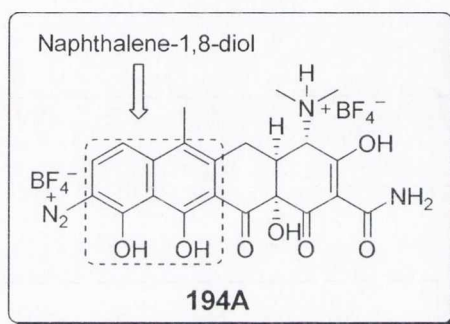
The reaction was monitored intermittently by means of ^1H NMR spectroscopy. Disappointingly, no vinylic signals were observed at any stage during the reaction and, after 2 hours, the disappearance of peaks relating to the tetracycline core of **194A** had begun and the decomposition of **188** by destannylation was evident. After 20 hours no product formation was observed by ^1H NMR spectroscopy and further decomposition of **188** had occurred.

3.1.4.4 Conclusions

It was clear at this point that the Stille reaction would not tolerate **194A** or **194B** as a substrate. Reaction conditions that had proceeded smoothly when diazobenzene (**214**) was employed were unsuccessful when applied to **194A** or **194B**. Despite exhaustive efforts varying reaction conditions, catalysts, diazonium counterions and reaction additives no successful coupling reaction was ever observed when **194A** or **194B** were employed as substrates. Reaction conditions that were reported to be successful when employed with doxycycline (**2**) derivatives were unsuccessful when compound **194A** was used. It is noteworthy that although Koza *et al.* reported the use of the Stille reaction in journal articles,^{182, 193} when the research was patented the Stille reaction was not once included and the coupling procedure reported was modified to Suzuki- or Heck-coupling conditions only.^{172, 183}

The issue of the unreactive nature of compounds **194A** and **194B** in Stille coupling procedures may be related to the instability of the diazonium salt moiety, although this would appear less likely when the success of similar reactions with diazobenzene (**214**) are considered. Another hypothesis to explain the lack of reaction progress when **194A** or **194B** were employed would be that the naphthalene-1,8-diol moiety of the C and D rings of the **194A** and **194B** (Figure 3.8) could, in theory, provide excellent chelation to palladium and prevent the reaction from proceeding.

Figure 3.8 Naphthalene-1,8-diol moiety of **194A**



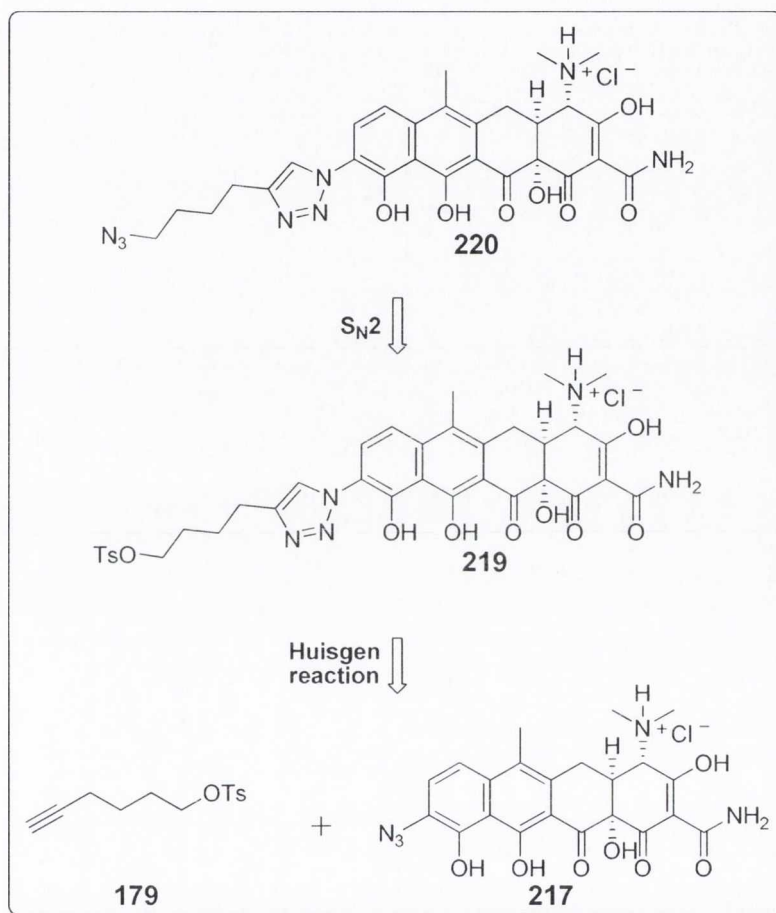
It was decided at this stage that a new synthesis for the anhydrotetracycline dimer half would have to be designed. This was extremely disappointing given the considerable effort that had been put into this original synthesis, however, it was evident that a carbon-carbon bond could not be formed at position 9 in anhydrotetracycline under the available reaction conditions. This necessitated new ways of linking anhydrotetracycline to the minocycline dimer half (**181**, Scheme 2.15, *vide supra*) and thus new methods of linkage were explored and new linkers designed.

3.2 Synthetic route 2: Triazole formation for connection of the linker

It was considered at this point in the project that an anhydrotetracycline dimer half linked at position 9 by a 1,2,3-triazole could be synthesised (Scheme 3.32, *vide infra*). This work was conducted concurrently with the endeavours to generate a 9-triazole substituted minocycline analogue (previously outlined in Section 2.5, *vide supra*). A retrosynthesis which allowed for an anhydrotetracycline dimer half containing a terminal azide for linkage with the terminal

alkyne moiety of minocycline dimer half **181** (Scheme 2.12, *vide supra*) was designed (Scheme 3.32, *vide infra*).

Scheme 3.32 Retrosynthetic route 2 of anhydrotetracycline dimer half (**220**)

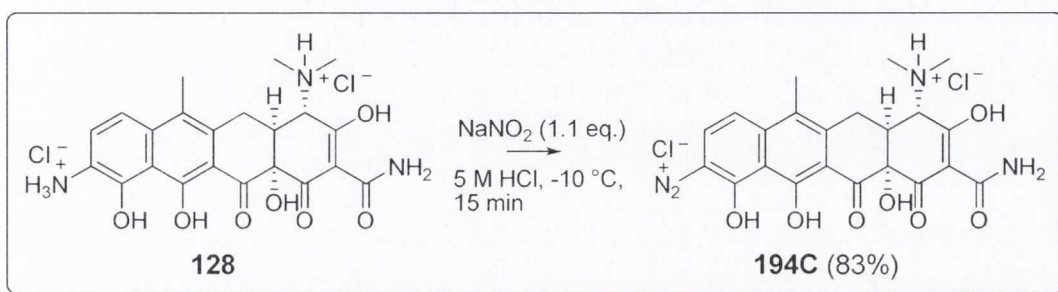


Compound **179**, originally synthesised for the formation of minocycline linker **173**, could be employed as a linker in this case to produce the triazole intermediate. This tosylated intermediate **219** could then be transformed into the desired anhydrotetracycline dimer half **220** via S_N2 substitution with sodium azide in DMF. It was felt that the anhydrotetracycline core should be stable to these reaction conditions and that no competing reaction would occur.

The first step in this synthesis would be the formation of 9-diazoanhydrotetracycline **194C**. It was decided to use a diazonium salt with a chloride counterion so as to mimic the conditions which proved successful in the formation of the analogous minocycline derivative

(**190**, Scheme 2.16, *vide supra*). Compound **194C** was synthesised from compound **128** by reaction with 1.1 equivalents of sodium nitrite in 5M HCl_(aq) at -10 °C (Scheme 3.33, *vide infra*).

Scheme 3.33 **Synthesis of diazonium salt 194C from 128**



The reaction was complete after 15 minutes and the product was collected by means of precipitation into a 7:10 mixture of 2-propanol/diethyl ether at 0 °C. The compound was analysed by IR and ^1H NMR spectroscopy. The ^1H NMR spectrum proved the diazo product **194C** to be less stable than its analogous minocycline derivative (**190**, Scheme 2.16, *vide supra*), as decomposition was observed in CD_3OD over the course of multiple NMR scans. However, it was discovered that the decomposition product was not anhydrotetracycline (**4**) itself, but another monosubstituted anhydrotetracycline analogue, most likely produced by attack of water on the cation that would be produced by the decomposition of the aryl diazonium salt at position 9. Figure 3.9 is a ^1H NMR spectrum of the aromatic resonance signals of both **194C** and the undetermined decomposition product.

formation and for this reason the attempted synthesis of **217** was not pursued further. This decision was taken upon consideration that, in general, the anhydrotetracycline analogues had proved to be considerably more unstable than their minocycline counterparts.

3.2.1 Conclusions

Two disparate attempts to determine suitable reaction conditions for the synthesis of an azido functionalised anhydrotetracycline had proved unsuccessful. Given the extensive experimentation that was undertaken in the formation of the triazole-containing minocycline compound (which inevitably proved unfruitful), it was decided not to pursue the synthesis of an azido functionalised anhydrotetracycline. The connection of a linker to anhydrotetracycline by way of a triazole moiety was not feasible, as the formation of azide **217** was unsuccessful and it appeared that triazole formation at this position could not be achieved. This was a disappointing result and accordingly attention was turned towards the design of a new method of linkage and also set of linkers.

3.3 Synthetic route 3: Amide formation for connection of the linker

At this stage, it was evident that not only would the choice of reaction for introduction of the linker to the anhydrotetracycline core need to be a reliable reaction, but also the conditions under which this reaction took place would also have to be extremely mild, with little chance of competing side-reactions occurring. The two connection strategies chosen previously had also been based upon reliable reactions but it was evident that the use of anhydrotetracycline in chemical transformations was an enormously challenging undertaking as it was proving to be a very unstable and recalcitrant compound which was, in addition, extremely difficult to purify.

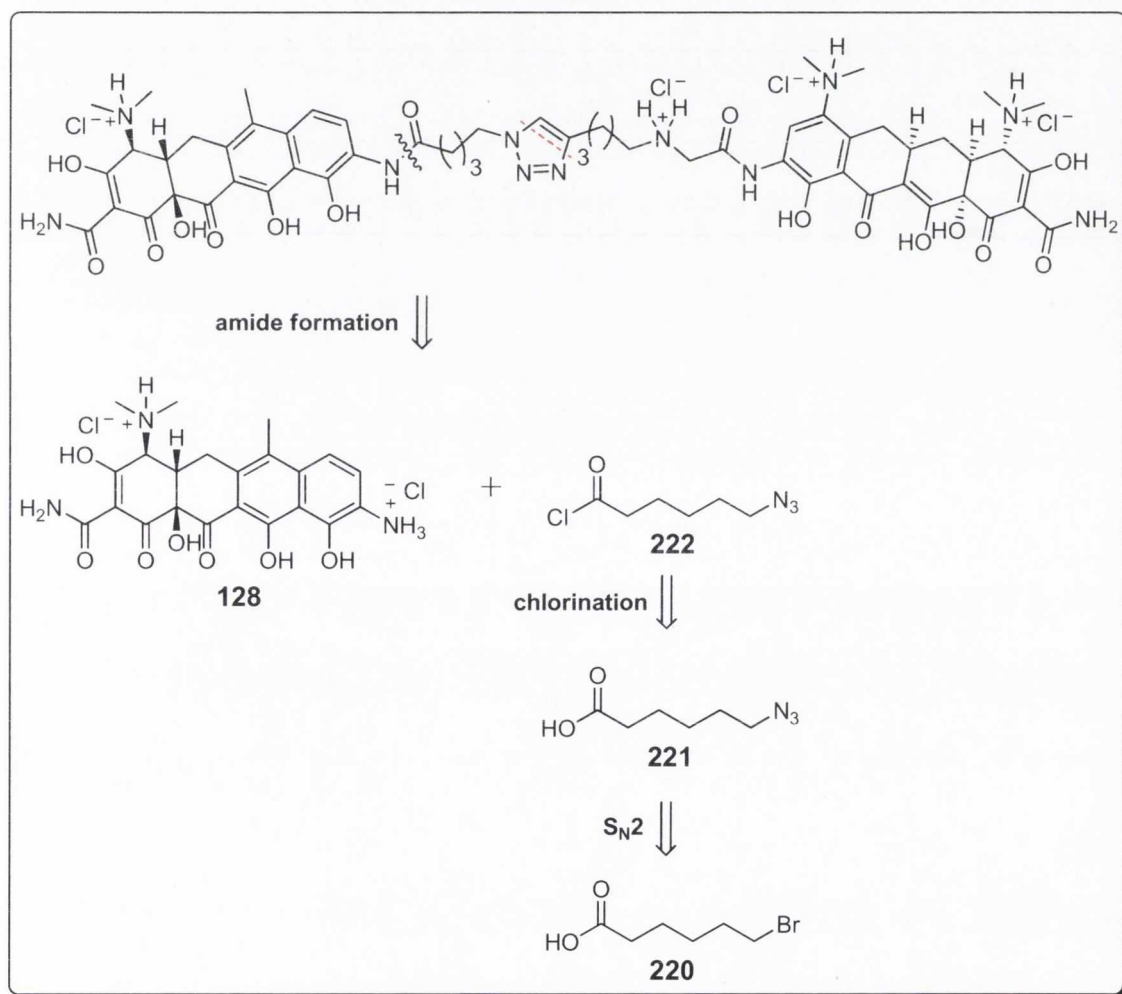
The best option available at this point was to use an amide formation reaction to connect a linker to the anhydrotetracycline core. This was advantageous on three fronts:

- 1) A synthesis for the starting material, 9-aminoanhydrotetracycline (**128**) had already been performed.
- 2) The amide formation could be performed at low temperatures (anhydrotetracycline analogues had previously proved unstable at high temperatures).

- 3) Since it was intended for anhydrotetracycline to enter bacterial cells, the increased polarity and hydrophilicity provided by the amide group could only deter the anhydrotetracycline half of the dimer from lodging in the cell membrane as anhydrotetracycline (**4**) itself had been proven to do.²⁶

To this end, a new anhydrotetracycline dimer half (Scheme 3.35, left of the red hatched line, *vide infra*) and linker were designed, the retrosynthesis of which is depicted in Scheme 3.35 (*vide infra*). The synthesis of the minocycline dimer half (right of the red hatched line) is described in Section 2.4.3 (*vide supra*).

Scheme 3.35 Retrosynthetic route 3 of anhydrotetracycline dimer half

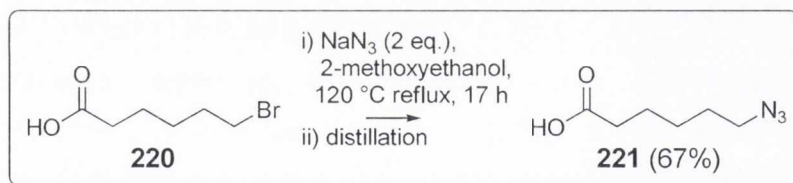


3.3.1 Acid chloride linker synthesis

The first step in the forward sense of the synthesis of an azido functionalised acid chloride linker was the substitution of 6-bromohexanoic acid (**220**) with sodium azide to produce 6-azidohexanoic acid (**221**). This was afforded through the use of 1 equivalent of bromo-containing **220** and 2 equivalents of sodium azide to furnish azide **221** in 67% yield after purification (Scheme 3.36, *vide infra*). Initially, column chromatography was attempted to purify the product but **221** was found to streak on silica and the recovered yield was low. Distillation at reduced pressure provided a more efficient method of purification.

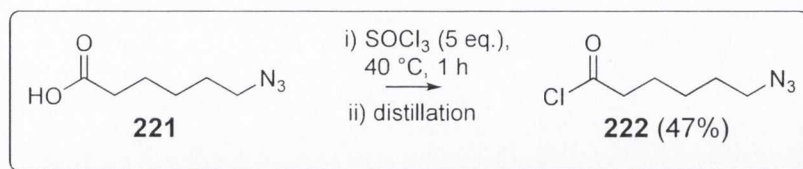
Confirmation of formation of the desired product was provided by ^1H NMR spectroscopy which exhibited an upfield shift of the signal corresponding to the methylene group alpha to the azide group of **221** when compared to the chemical shift of the signal of the methylene group alpha to the bromine atom in **220**. In addition, an IR spectrum of **221** contained the characteristic azide stretch at 2092 v/cm^{-1} .

Scheme 3.36 **Synthesis of linker intermediate 221 from compound 220**



Compound **221** was subsequently converted to acid chloride **222** by reaction with thionyl chloride. 5 Equivalents of freshly distilled thionyl chloride were heated in the presence of **221** at $40\text{ }^\circ\text{C}$ for 1 hour (Scheme 3.37) and after this time the excess thionyl chloride was removed by distillation. A black oil remained in the reaction vessel as the crude product which was distilled by Kugelrohr distillation at atmospheric pressure to provide the acid chloride linker, 6-azidohexanoyl chloride (**222**) in 47% yield.

Scheme 3.37 **Synthesis of linker 222 from carboxylic acid 221**

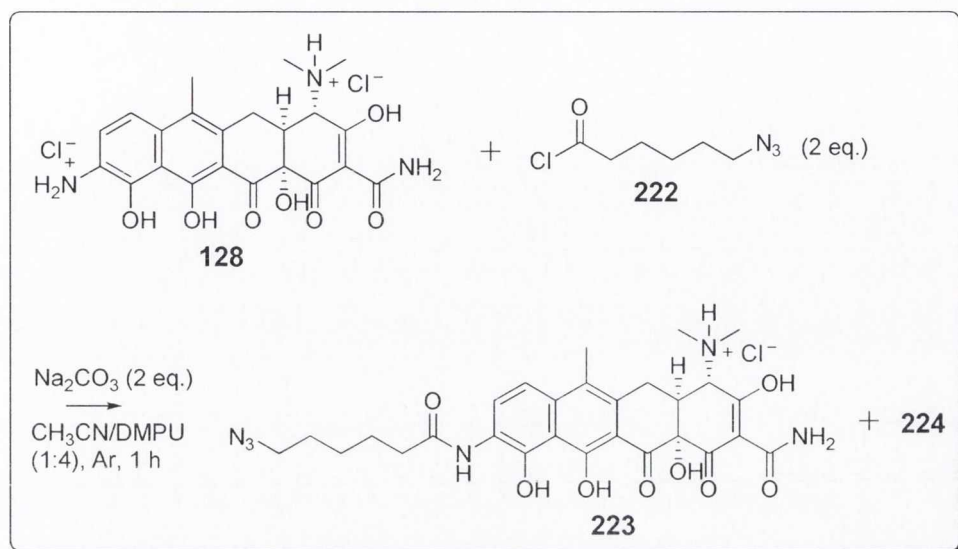


With linker **222** in hand, attention was turned towards formation of the amide moiety from compound **222** and 9-aminoanhydrotetracycline **128**.

3.3.2 Connection of compound **128** and linker **222**

It was considered that the reaction between the two linkage components **128** and **222** would proceed in the same manner as did the formation of the minocycline dimer half precursor **170** (Scheme 2.12, *vide supra*). The reaction conditions employed were based upon those that had proved successful for the synthesis of dimer half **181**, however, the reaction of **128** and **222** was initially performed at 0°C as a precautionary measure against the degradation of the anhydrotetracycline starting material and product. Amine **128** was reacted with 2 equivalents of sodium carbonate and 2 equivalents of acid chloride **222** (which was diluted 1/16 v/v in 1:4 anhydrous acetonitrile:DMPU for addition) in a solvent system of 1:4 anhydrous acetonitrile/DMPU at 0°C for 1 hour (Scheme 3.38). This reaction did indeed furnish the desired product **223**; however, an additional unknown tetracycline compound was also produced which will be referred to as by-product **224**.

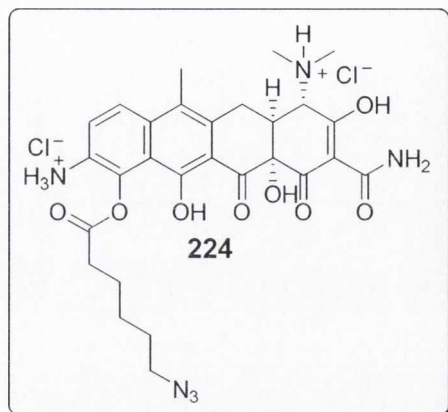
Scheme 3.38 **Reaction 1 to form anhydrotetracycline dimer half 222**



Tetracycline **223** and by-product **224** were formed in an approximately 3:2 ratio. The undetermined by-product **224** exhibited two aromatic resonance signals in conjunction with the other resonance signals associated with the anhydrotetracycline core in the ^1H NMR spectrum and appeared otherwise to be very similar in structure to **223**. The two products were separated by RP-MPLC for analysis. Compound **223** eluted from the column first whereas **224** required a lower polarity mobile phase and exhibited a higher retention time.

High resolution mass spectral analysis of the two compounds revealed that products **223** and **224** exhibited identical exact masses. This evidence, coupled with the ^1H NMR spectroscopic data (it was noteworthy that the shifts of the aromatic peaks were more similar to that of aminotetracycline **128** than amide **223**) and higher retention time of compound **224** on RP-MPLC supported the deduction that the undesired product was an ester product, most likely formed by attack of the phenolic moiety at position 10 on the acid chloride. The suggested structure of compound **224** is depicted in Figure 3.10 (*vide infra*), although this structure was not confirmed further than is reported in this section due to the constraints of time. Ester **224** was not useful for the formation of tetracycline dimers as the orientation of the linker would not allow it to thread out of the binding pocket of the Tet(R) regulation protein as was required.

Figure 3.10 Suggested structure of the undesired product (**224**) of the acylation of **128** with **222**



It is necessary to note that neither of the amido-anhydrotetracycline products **223** and **224** were entirely stable under RP-MPLC conditions. Some of the recovered products from the columns contained the inorganic decomposition product of anhydrotetracycline. This decomposition was found to take place during the purification stage rather than over the course of the reaction.

Optimisation of the reaction conditions was necessary in order to avoid the formation of undesired by-product **224** and consequently improve the yield of **223** and also assist with the purification. It was initially considered that the equivalents of base used in the reaction procedure could influence the formation of one product over another. The pKa of the aniline moiety would be expected to be ~4.5. The pKa of the phenolic moiety at position 10 in oxytetracycline (**106**, Scheme 3.2, *vide supra*) is known to be 7.8, however, this would be significantly lowered in anhydrotetracycline analogues such as **128**, due to increased conjugation provided by the naphthalene-1,8-diol moiety. This could provide an explanation for the concurrent formation of **223** and **224**.

As such, parallel experiments were performed using 1 molar equivalent (entry 1, Table 3.3, *vide infra*) and 0.5 molar equivalents (entry 2, Table 3.3, *vide infra*) of (dibasic) sodium carbonate. The reaction conditions were otherwise retained as before, with the use of 1 equivalent of **128** and two equivalents of **222** (in a solution of anhydrous acetonitrile/DMPU) with the base in 1:4 anhydrous acetonitrile/DMPU at -10 °C. During these reactions **222** was added dropwise over 15 minutes.

Table 3.3 **Effect of the equivalents of base/temperature on the reaction of 128 and 222**

Entry no.	Eq. 128	Eq. 222	Eq. Na ₂ CO ₃
1	1	2	1 ^a
2	1	2	0.5 ^a

a = full conversion after 20 mins

Gratifyingly, both reactions provided solely product **223**, with quantitative conversion after 20 minutes. The reaction work-up required acidification of the crude reaction with HCl in anhydrous diethyl ether (2.0M) and precipitation of the reaction into diethyl ether. The crude product was then purified using RP-MPLC to afford pure **223** in an excellent yield (80%). NMR, mass spectrometry and IR spectroscopy experiments confirmed the formation of the desired product.

3.3.3 Conclusions

The new procedures designed allowed for the synthesis of novel anhydrotetracycline dimer half **223**, *via* compound **128**, in 27% overall yield from oxytetracycline (**106**). The synthesis of **223** was significant as compounds of this nature, and chemistry of a challenging nature such as this, had not been previously reported in the literature. Both dimer halves, **223** and **181**, were now in hand and attention was to be turned to the Huisgen reaction to afford a tetracycline dimer.

3.4 Tetracycline analogues for comparison

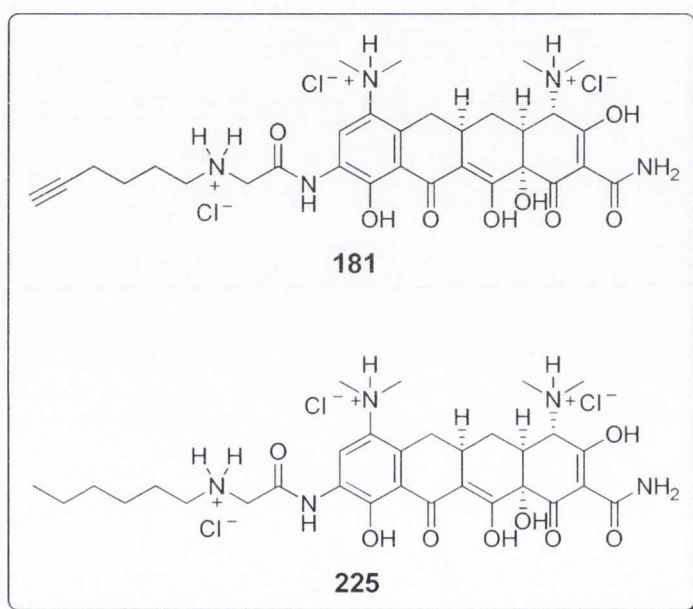
Tetracycline analogues of the nature presented in this research had not been synthesised or tested for *in vitro* activity previously. Thus, it was decided that analogues of minocycline (**3**) and anhydrotetracycline (**4**) must be designed to determine if the activity that might be exhibited *in vitro* was the result of the entire dimeric tetracycline compound or if the presence of the linker on a tetracycline core was responsible for activity.

The individual dimer halves **181** and **223** would also be tested for *in vitro* activity, however, it was felt that minocycline (**3**) and anhydrotetracycline (**4**) analogues with long chain aliphatic moieties should also be synthesised. This decision was taken as, individually, the dimer halves possessed terminal alkyne and azido groups that are quite different in

chemical nature to the triazole linker which would be present in the dimeric structure. The terminal groups could also, in theory, be chemically altered *in vitro*. Therefore, it was decided that derivatives of **3** and **4** with long chain unsubstituted moieties connected in a similar way to that of the individual linkers should be synthesised for comparison *in vitro* testing.

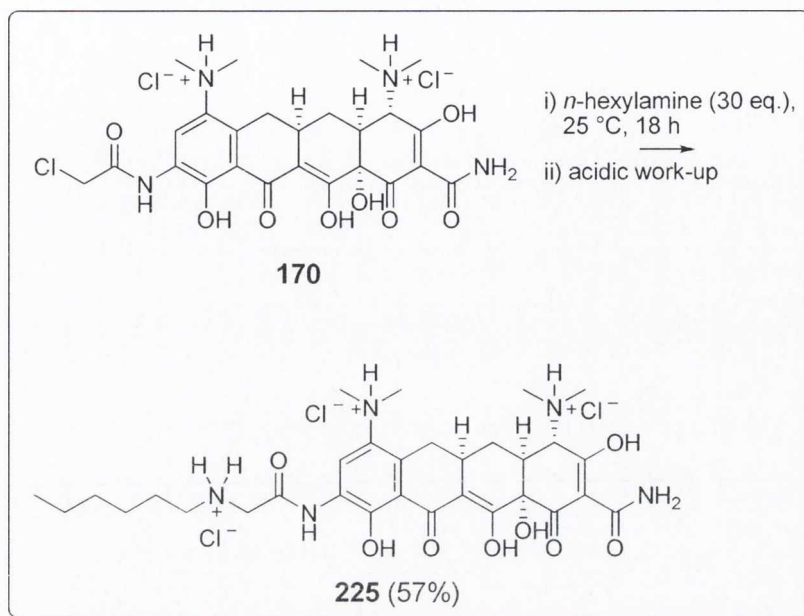
The minocycline derivative **225** was designed for comparison with minocycline dimer half **181**, both structures are illustrated below in Figure 3.11.

Figure 3.11 Minocycline dimer half **181** and analogue **225** for comparison *in vitro*



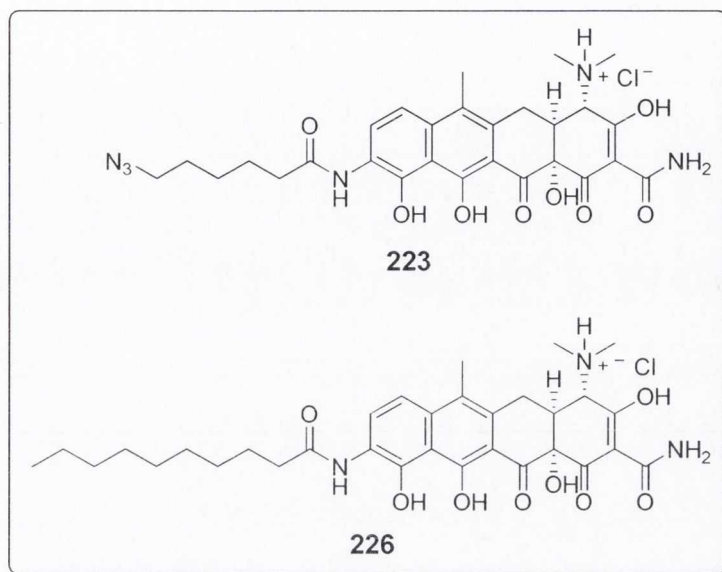
225 Was prepared with relative ease from **170** using a large excess of *n*-hexylamine as solvent, reactant and base (Scheme 3.39, *vide infra*). The reaction was quenched using methanol and the reaction solvent was evaporated at reduced pressure. The crude product **225** was subsequently extracted as its free-base from Millipore water at pH 7.2 with CH₂Cl₂. Acidification of the organic extract using HCl in anhydrous diethyl ether (2.0 M) and evaporation of the solvent at reduced pressure afforded the pure product, **225**, in 57% yield.

Scheme 3.39 Synthesis of minocycline analogue **225** from **170**



A similar, novel anhydrotetracycline species, **226**, was designed for comparison with anhydrotetracycline linker **223** and the tetracycline dimer. Both structures are depicted in Scheme 3.40.

Scheme 3.40 Anhydrotetracycline dimer half **223** and **226** for comparison *in vitro*

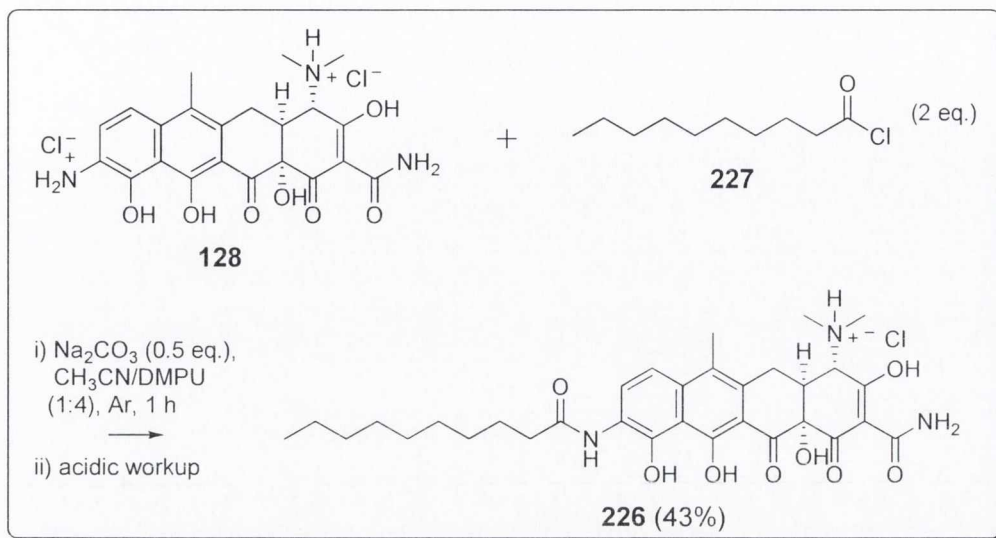


It was envisaged that the long chain anhydrotetracycline derivative **226** could be produced from amino anhydrotetracycline **128**. Amine **128** was reacted with 0.5 equivalents of sodium carbonate and 2 equivalents of decanoyl chloride (**227**) in anhydrous acetonitrile/DMPU (1:4) at 0 °C for 1 hour (Scheme 3.41). **227** Was added slowly over 15 minutes as a solution in acetonitrile/DMPU (1:4) to administer regiocontrol over the reaction, and in this case, the *O*-acylated compound was not observed. The crude product was precipitated into diethyl ether and triturated with diethyl ether to remove the excess reagent **227**.

Initial attempts at purification of the crude compound by RP-MPLC were unsuccessful. RP-MPLC was employed to purify **226** using a mobile phase of acetonitrile/Millipore water (gradient 10%-70%). Disappointingly, the compound was found to decompose during the purification process.

The reaction was therefore repeated and the crude product was subjected to a pH dependent extraction. **226** Was dissolved in Millipore water at pH 7.2 and extracted with CH₂Cl₂. Acidification of the organic extracts with HCl in anhydrous diethyl ether (2.0 M) and evaporation of the solvent at reduced pressure afforded the pure product, **226**, as its hydrochloride salt in 43% yield.

Scheme 3.41 **Synthesis of anhydrotetracycline analogue 226 from 128**



3.5 Click chemistry: the cycloaddition reaction of alkynes and azides

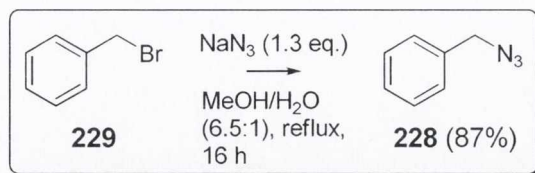
With the anhydrotetracycline dimer half **223** and minocycline dimer half **181** successfully synthesised, it was necessary to find reaction conditions under which these two compounds could be connected *via* the synthesis of a triazole: the ‘click chemistry’ Cu(I) catalysed cycloaddition reaction.^{233, 250} It was envisaged that despite the previous failure of ‘click chemistry’ on azide **190** (Section 2.5, *vide supra*), that the reaction of **181** and **223** would proceed as the alkyne and azide moieties were both in a terminal position on flexible linkers and therefore more accessible for Cu(I).

Given the difficulty of synthesis and time required to synthesise and purify **223** and **181**, it was decided to use test compounds to optimise conditions which were considered to be most likely to be transferrable to the use of tetracycline analogues.

3.5.1 Test reactions to optimise conditions which were transferrable to tetracyclines

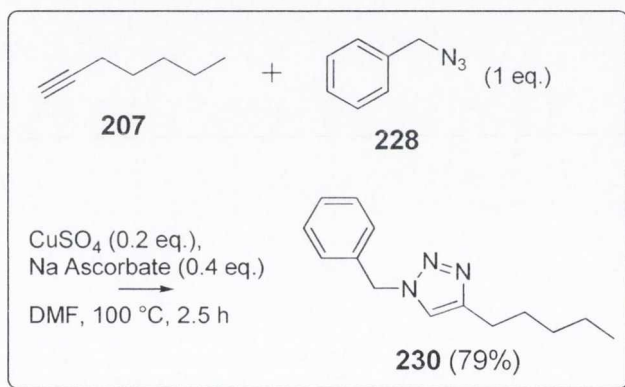
Initially, heptyne (**207**) and benzyl azide (**228**) were used as test compounds. Azide **228** is easily synthesised from benzyl bromide (**229**) in excellent yield (Scheme 3.42).

Scheme 3.42 Synthesis of 228 from 229



Compounds **207** and **228** were tested in a catalysed cycloaddition reaction using 0.2 equivalents of copper sulfate and 0.4 equivalents of sodium ascorbate at 100 °C for 2.5 hours in DMF (as both **223** and **181** would be soluble in this solvent). This afforded 79% conversion of **228** to **230** (Scheme 3.43, yield determined by ^1H NMR spectroscopy).

Scheme 3.43 Catalysed triazole formation between 201 and 228 in DMF



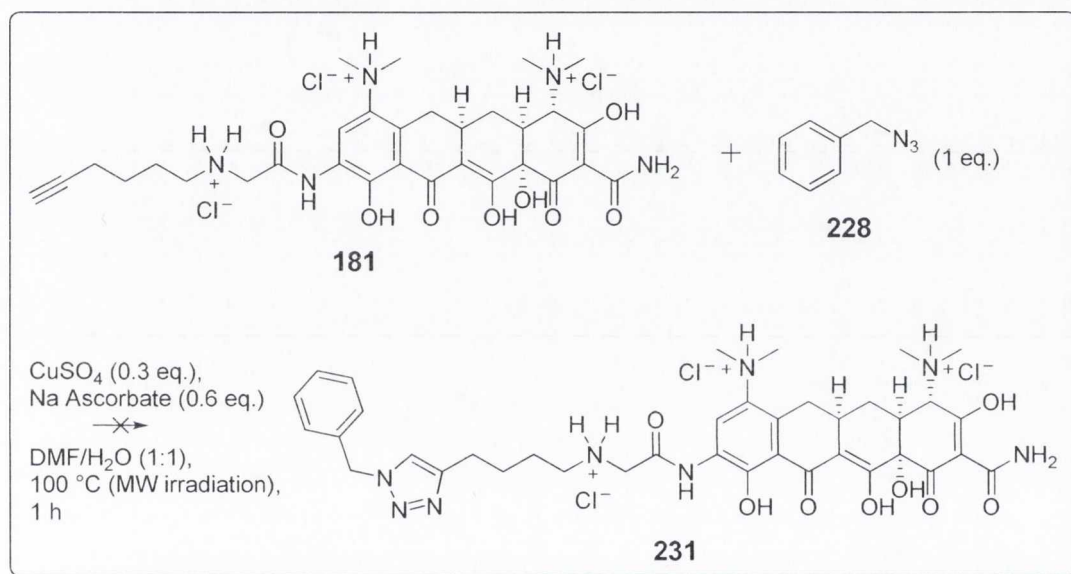
It was decided to explore if microwave irradiation would reduce the reaction time or increase conversion, as tetracyclines are known to be instable at high temperatures over long periods of time. Thus, the reaction as depicted in Scheme 3.43 was repeated with microwave irradiation at 100 °C. After 1 hour the reaction had reached 59% conversion (determined by ^1H NMR spectroscopy). The reaction time was not extended as it was predicted that the tetracycline analogues would not be stable to microwave irradiation for long periods of time, however it was noted that the rate of reaction had increased.

A third variant of the reaction displayed in Scheme 3.43 was performed using a solvent system of DMF/Millipore water (1:1) with microwave irradiation at 100 °C for 1 hour. After this time the reaction was determined to have reached 85% conversion (^1H NMR

spectroscopy). This change in solvent system seemed promising in terms of conversion and so a reaction involving minocycline dimer half **181** and **228** was performed.

Minocycline analogue **181** was reacted with **228** in the presence of 0.3 equivalents of copper sulfate and 0.6 equivalents of sodium ascorbate in DMF/Millipore water (1:1). This reaction mixture was subjected to microwave irradiation at 100 °C for 1 hour (Scheme 3.44, *vide infra*).

Scheme 3.44 Test reaction 1 with **181** and **228** using microwave irradiation



Disappointingly, the desired triazole product **231** was not observed and unreacted **228** was isolated from the crude reaction mixture. Compound **181** appeared to have decomposed under the reaction conditions, as peaks relating to the alkynyl side chain were detected in the ^1H NMR spectra of organic extracts from the crude reaction mixture, but without the accompanying resonance signals indicating the minocycline core structure. It was deduced that microwave irradiation for this length of time was the cause of the decomposition of **181** and endeavours began to determine reaction conditions which would afford the triazole with shorter reaction times.

At the time that this research was being performed, Gmeiner *et al.* published the synthesis of triazole functionalised doxycycline (**2**) derivatives.²³⁰ For the reasons discussed

in Section 2.5, these reaction conditions were taken as a guideline for our subsequent reactions, in particular in relation to the use of 13 equivalents of a Cu(I) source.

To eliminate the possibility that it was the minocycline analogue that was preventing the desired reaction from occurring, by chelation of the copper (as it possesses a dimethylamino moiety which doxycycline does not) test reactions involving **228** and 3-butyne-1-ol (**232**) were performed in the presence of minocycline analogue **225** (Scheme 3.39). Analogue **225** was chosen as it possessed the general linker structure, with amide and secondary amine functionalities, of **181** but did not possess a moiety which could be involved in a competing reaction. The choice of alkyne was altered to **232** as it was more hydrophilic and would therefore behave more like a tetracycline analogue, in terms of solubility, than **126**. A summary of the reaction conditions tested is presented in Table 3.4. The general reaction scheme is displayed in Scheme 3.45 (*vide infra*).

Both entries 1 and 2 (Table 3.4) were performed using 1 equivalent of **228**, 2 equivalents of **232** and 1 equivalent of **225**. Copper sulfate and sodium ascorbate (2 mole:1 mole) were employed as the source of Cu(I). Conversion to triazole **233** (Scheme 3.45) was determined by ¹H NMR spectroscopy.

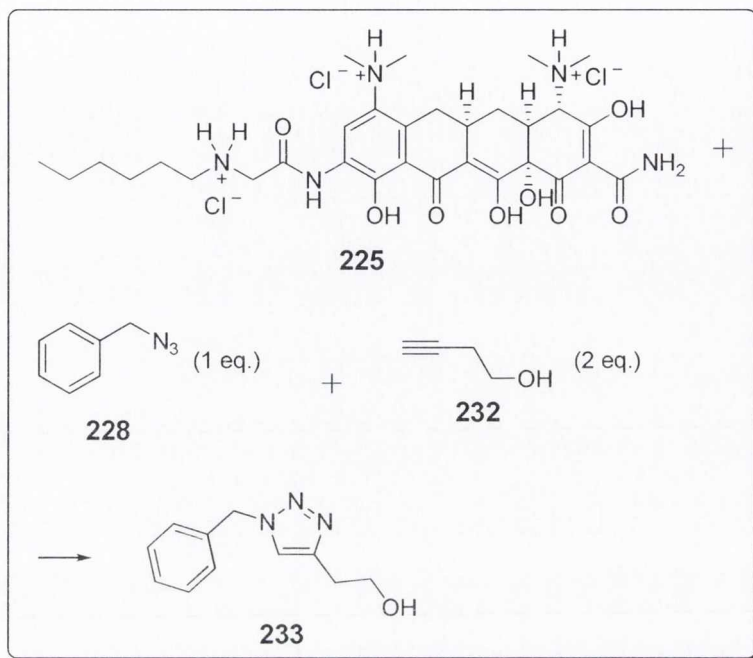
Table 3.4 Huisgen reactions in the presence of minocycline analogue **225**

Entry no.	Eq. Cu(I)	Solvent	Temperature	Reaction time	Conversion
				(h)	(%)
1	0	EtOH	80 °C	16.5	0
2	13	EtOH/H ₂ O ^a	rt	0.5	100

a = 1:1 mixture

Entry 1 summarises a classic Huisgen reaction. It was deemed pertinent to explore this route, although it is reported that reaction times can be quite long,^{233, 250} as the absence of copper in the reaction would be advantageous due to the excellent chelating ability of tetracyclines with Cu(II) ions.¹⁰ Disappointingly, after 16.5 hours, no product (**233**, Scheme 3.45, *vide infra*) had been observed, although encouragement was taken from the fact that **225** had not decomposed in any observable amount (by ¹H NMR spectroscopy) under the reaction conditions.

Scheme 3.45 **General reaction scheme of Huisgen reactions of 228 and 232 in the presence of minocycline analogue 225**



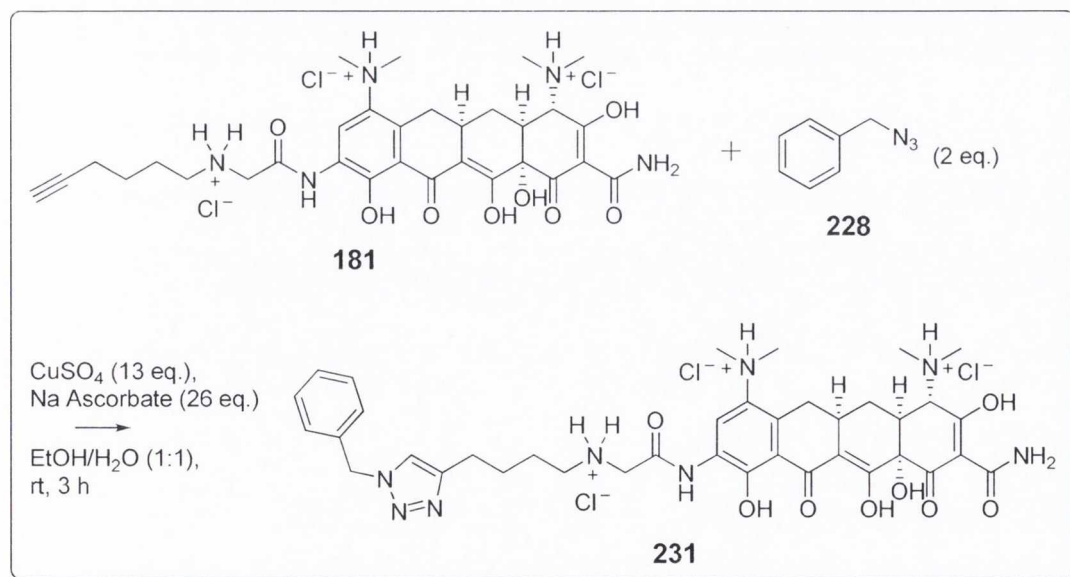
Entry 2 (Table 3.4, *vide supra*) summarises the reaction depicted in Scheme 3.45 whereby 13 equivalents of Cu(I) were used to catalyse the reaction. The solvent system was altered to EtOH/Millipore water (1:1) to facilitate the dissolution of all reagents. After only 30 minutes, the reaction of **228** and **232** to form triazole **233** had afforded 100% conversion to the desired product (determined by ^1H NMR spectroscopy). Triazole **233** was extracted from the reaction using CH_2Cl_2 and analysed ^1H NMR spectroscopy and HRMS confirmed the formation desired product. With this reaction having proved that the minocycline analogue should not interfere with the progression of a triazole formation, **181** was employed in reactions with **228** once more.

Scheme 3.46 (*vide infra*) depicts a Cu(I) catalysed cycloaddition reaction with minocycline analogue **181**, 2 equivalents of **228** and 13 equivalents of Cu(I). The reaction was performed in EtOH/Millipore water (1:1) and stirred at ambient temperature for 3 hours. After this time, a resonance signal at 5.5 ppm in the ^1H NMR spectrum of the reaction appeared which integrated for two protons with respect to the aromatic region of the spectrum. This signal was believed to be representative of the benzylic methylene signal in the desired

product, **231**. Signals representative of the minocycline core structure were also observed but appeared to be dampened in the ^1H NMR spectrum. This transpired to be due to chelation of Cu(II) to the tetracycline core. Previously, Gmeiner *et al.* had reported the dissociation of tetracycline-Cu(II) complexes with overnight stirring in HCl/MeOH, however, this was not ideal given the length of time needed to dissociate the complex. Thus, since it was known that sulfur ions have an extremely high affinity for copper ions, sodium sulfide was added to the samples taken from the reaction and after 1 minute stirring a black precipitate (copper sulfide) was observed. This was a much more efficient method of dissociating the tetracycline-Cu(II) complex and no adverse effects on the tetracycline core were observed (by ^1H NMR spectroscopy).

When this technique was being employed, great care was taken with respect to safety as it was evident that the formation of H_2S could occur. Therefore, all samples were flushed with nitrogen using a two neck flask with a nitrogen inlet to bubble through the solution, and an outlet which bubbled through bleach to oxidise any H_2S which might be liberated. The copper sulfide was also treated with bleach when filtered from the samples.

Scheme 3.46 Test reaction 2 with **181** and **228** using 13 equivalents of Cu(I) at ambient temperature



The reaction mixture was treated with sodium sulfide and HCl in anhydrous diethyl ether and filtered through celite to remove the copper sulfide. However the ^1H NMR spectrum still exhibited greatly broadened signals, indicating interference of the data collection by a paramagnetic ion. Therefore, the reaction crude was dissolved in methanol and centrifuged and the supernatant decanted and dried. This work up procedure was used in all future reactions involving tetracycline analogues and copper compounds.

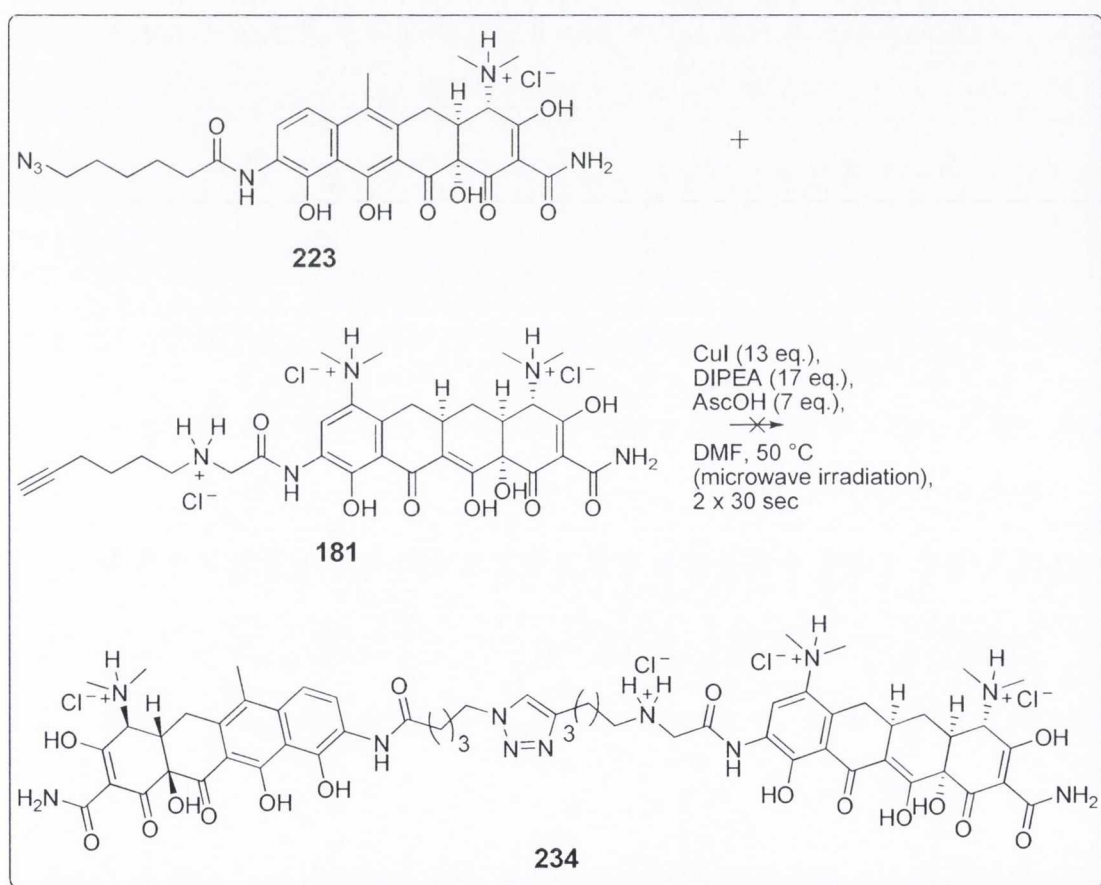
This provided an encouraging crude ^1H NMR spectrum, with a singlet at 8.55 ppm integrating as 1 proton (the resonance signal for C-8 of minocycline), a multiplet at 7.37 ppm integrating as 6 protons (the 5 aromatic protons of the benzyl moiety and the triazole proton) and a singlet at 5.59 ppm which integrated for 2 protons, the benzylic methylene. The signals relating to CH-4 and the methylene alpha to the amide of the minocycline structure were also visible. However the spectrum was still unclear below 4 ppm due to the presence of other impurities obscuring signals. As such it was difficult to observe the resonance signals relating to the aliphatic chain or the remainder of the minocycline core. HRMS of the crude reaction mixture, using a MALDI⁺ technique, provided a positive response for the desired molecular ion ($M + H$, Found: 743.3492, Requires: 743.3517), however, due to insufficient mass obtained from the reaction, **231** was not isolated from the crude product by MPLC.

The reaction was repeated using identical reaction conditions to those reported by Gmeiner *et al.*²³⁰ Minocycline analogue **181** and 2 equivalents of **228** were reacted in the presence of 13 equivalents of copper iodide, 17 equivalents of DIPEA and 7 equivalents of AscOH in anhydrous, degassed DMF. The reaction was irradiated in a microwave reactor for two cycles of 30 seconds at 50 °C with cooling of the reaction to -10 °C between cycles. The DMF was removed at reduced pressure and the crude reaction mixture subjected to the same work up procedure as described for the previous reaction. This provided a crude product with a the same resonance signals in ^1H NMR as those seen in the previous reaction crude. HRMS using ESI⁺ revealed the presence of the desired molecular ion ($M + H$, Found: 743.3518, Requires: 743.3517) indicating the formation of the desired product.

3.5.2 Cycloaddition reactions of minocycline dimer half **181** and anhydrotetracycline dimer half **223**

Following on from the success of these reaction conditions, it was decided to attempt the copper catalysed reaction of anhydrotetracycline dimer half **223** and minocycline dimer half **181** (Scheme 3.47, *vide infra*) using a large excess of copper. It was expected that the desired product would be easier to purify given that it would be very different from any other component in the reaction and would lend itself well to purification by RP-MPLC.

Scheme 3.47 Copper catalysed triazole formation of **234** from analogue **223** and minocycline analogue **181**



The crude reaction mixture was worked up using the procedure outlined previously to provide only 15 mg of crude (from an initial 21 mg **223** and 26 mg **181**). The loss of mass was 186

worrying but no tetracycline compounds were found to be present in any of the removed filtrate or solvent from the work up. The crude reaction product was analysed by ^1H NMR spectroscopy. The ^1H spectrum of the crude was not as expected. Exhibited in the spectrum were large broad peaks covering the region between 6 ppm and 10 ppm. The product was not the desired triazole **234** and HRMS confirmed that no molecular ion or expected fragments could be found, even when copper chelates were considered.

It was decided to attempt the reaction using 2 equivalents of azide **223** and 1 equivalent of alkyne **181** to encourage product formation, however the ^1H NMR spectrum provided the same resonance signals as were seen in the previous reaction. A final attempt using 2 equivalents of alkyne **181** and 1 equivalent of azide **223** was attempted using identical reaction conditions, but with no success.

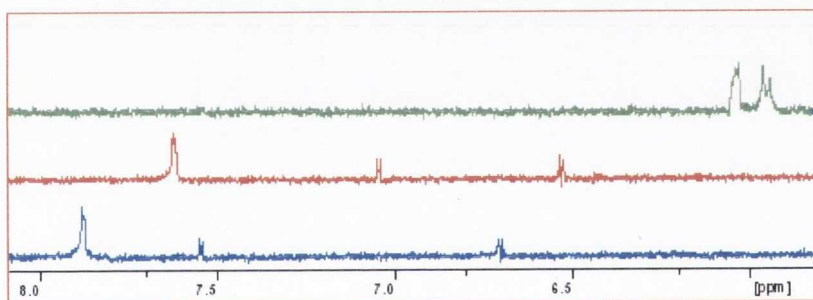
It was postulated that perhaps a change in solvent would provide more positive results, as DMF is known to decompose at elevated temperatures. It was also decided at this time to heat reactions conventionally as it was clear that **223** was not stable towards microwave irradiation, even for short periods of time. Water has been reported to be an excellent solvent choice for the performance of thermal Huisgen reactions and copper catalysed cycloadditions of alkynes and azides.^{233, 251} With this and the water solubility of tetracyclines in mind, the reaction of **181** and **223** was performed in aqueous medium. The reactions were performed with only 1 equivalent of **181** and **223** as previously no positive influence had been observed by the use of excess of either reagent and, given their difficulty of synthesis and purification, it was advisable to utilise stores of the compounds prudently.

Alkyne **181** and azide **223** were reacted in a classical Huisgen reaction by reflux in water for 16.5 hours. The reaction was monitored by ^1H NMR, and with no additives in the reaction it was easy to see the progress. After 16.5 hours, no amount of the desired triazole **234** had been observed and the decomposition of **223** to the classic anhydrotetracycline decomposition product (multiplets at 7.29ppm, 7.42ppm and 7.55 ppm in the ^1H NMR spectrum) was complete. No peaks relating to the original anhydrotetracycline analogue **223** were observed in the ^1H NMR spectrum after 16.5 hours, although minocycline analogue **181** appeared to have survived the reaction conditions intact.

It was hoped that, although compound **223** appeared to be extremely unstable, the reaction could be encouraged to occur before decomposition took place and that perhaps the desired product **234** would prove to be more stable. The reaction was therefore repeated in

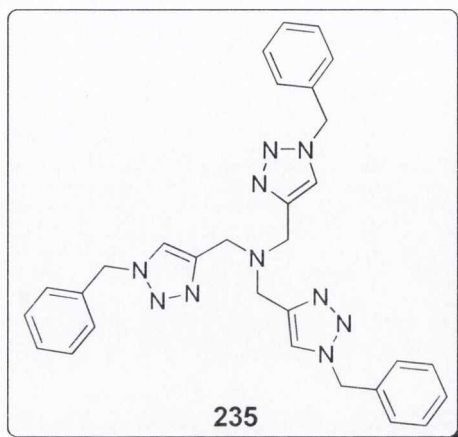
refluxing water for 16.5 hours with the addition of 13 equivalents of copper sulfate and 26 equivalents of sodium ascorbate. The reaction was analysed initially by ^1H NMR spectroscopy. The ^1H NMR spectrum contained resonance signals for many protons in the aromatic region, none of which related to compounds **234** or **223**. 1-D TOCSY experiments revealed three spin systems in the crude mixture: two spin systems of 3 aromatic resonance signals, integrating for 1 with respect to each other, each and one spin system of 2 aromatic multiplets which integrated for 1 with respect to each other (Figure 3.12). It was clear that these resonance signals could not be related to the desired product **234** and HRMS confirmed that the desired molecular ion was not present in the crude products of the reaction. Due to the constraints of time, the identities of the unknown products of this reaction were not ascertained.

Figure 3.12 Aromatic region of the Huisgen reaction of **223** and **181**: ^1H NMR resonance signals obtained by 1-D TOCSY experiments



A final reaction procedure was attempted to encourage the synthesis of the desired triazole product **225**. It was reported that ligands could be added to the traditional ‘click chemistry’ triazole formation reactions to prevent oxidation of the Cu(I) ions to Cu(II) in solution. Wang *et al.* reported the use of TBTA (**235**, Figure 3.13) as an additive of this nature for the optimisation of triazole formation reactions. It had also been reported that Cu(I) source $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$ was an excellent choice of catalyst for the cycloaddition reactions of alkynes and azides and when employed at ambient temperature these reactions were high yielding.²³⁵ Thus, having exhausted other available options, reactions were designed using $\text{Cu}(\text{CH}_3\text{CH})_4\text{PF}_6$ and TBTA (**235**)²⁵² for the cycloaddition reaction of **223** and **181**.

Figure 3.13 The structure of TBTA



Three reactions were attempted using these conditions, the conditions of which are summarised in TABLE (*vide infra*). All reactions were performed using 1 equivalent of **223** and **181** with Millipore water as solvent.

Table 3.5 Cycloaddition reactions of **223 and **181** using $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$ and TBTA (235)**

Entry no.	Eq. 235	ubunit of $\text{f}_3\text{CN})_4\text{PF}_6$	Temperature	Reaction time (h)
1	26	13	100 °C	20.5
2	26	13	50 °C	20.5
3	26	13	rt-40 °C	139

Entry 1 summarises the reaction of **223** and **181** using the new catalyst/ligand system at 100 °C. It was hoped that at such high temperatures the reaction time would be short. The reaction was monitored by ^1H NMR spectroscopy and after 20.5 hours no product formation had been observed and **223** was found to have decomposed to the characteristic anhydrotetracycline decomposition product (^1H NMR spectroscopy: resonance signals at 7.29 ppm, 7.42 ppm and 7.55 ppm). The reaction was concurrently performed at 50 °C but no reaction was observed after 20.5 hours and **223** was found to be decomposed (entry 2).

Since $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$ had been used successfully at ambient temperature,²³⁵ it was decided to perform the final attempt at a cycloaddition reaction of **223** and **181** under the reaction conditions summarised in entry 3. The reaction was monitored intermittently by ^1H

NMR spectroscopy and after 115 hours no change had been observed in the NMR spectrum. The reaction was therefore heated to 40 °C. After 24 hours at 40 °C resonance signals relating to the tetracycline core and aromatic region of **223** began to disappear indicating that decomposition was occurring (as the desired reaction would not have such a marked effect on the aromatic region of the **223**).

3.5.3 Conclusions

Test reaction conditions had been explored carefully using both model compounds and minocycline dimer half **181** to optimise the reaction conditions. It was discovered that **181** was not stable to high reaction temperatures for periods of 1 hour under microwave irradiation.

The possibility that the inherent structure of the tetracyclines chosen was preventing the reaction from occurring was eliminated by performing test reactions in the presence of a tetracycline with similar structural features to **232** and **181** but without a moiety that would prove reactive in a cycloaddition.

A reliable method for the work up of reactions involving tetracyclines and copper ions had been devised: the use of sodium sulfide to quickly dissociate tetracycline-Cu(II) complexes without decomposition of the tetracycline. A Cu(I) catalysed cycloaddition reaction was successfully performed between minocycline dimer half **181** and **228** to form triazole product **231** using two sets of reaction conditions involving ambient temperature and 1 minute of microwave irradiation.

Disappointingly, it was demonstrated that the cycloaddition reaction of minocycline dimer half **181** and anhydrotetracycline dimer half **223** was not progressing under currently available conditions. Reaction conditions reported by Gmeiner *et al.*²³⁰ for use with doxycycline (**2**) derivatives proved unsuccessful when employed to **223** and **181** despite attempts at using excesses of both reagents. Traditional conditions using conventional heating of the Huisgen cycloaddition reaction were performed, however, **223** proved unstable under these conditions. Cu(I) catalysed reactions of **223** and **181** using conventional heating did not provide the desired product **234**. Finally, reaction conditions which provided the most successful and current literature examples of Cu(I) catalysed azide/alkyne cycloaddition reactions, with the addition of TBTA (**226**) to prevent oxidation of the Cu(I) and the use of

the most successful catalyst $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_4$, were employed in the reaction of **223** and **181** but with no product formation observed. Reactions at ambient temperature provided no reaction after over 100 hours, and heating decomposed anhydrotetracycline analogue **223** within an hour with no formation of product **234**.

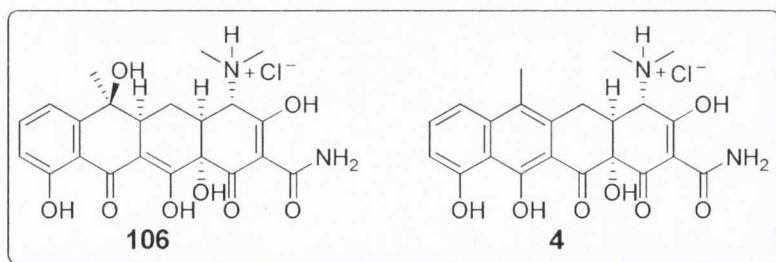
It was realised at this point that, owing to the inherent instability of the anhydrotetracycline analogue **223**, the desired series of tetracycline dimers would not be possible to produce using this methodology. However, given the amount of investigation that we had performed on the chemistry of tetracycline antibiotics, it was felt that a new procedure should be devised to produce a novel series of tetracyclines which could be eventually tested *in vitro*.

3.1 Anhydrotetracycline analogues for cell entry

3.1.1 Project introduction

In 2010 Kishony *et al.* published a study on the effects of oxytetracycline (**106**, Figure 3.14) and its decomposition products on the selection of *E.coli* cells for their tetracycline sensitive (Tet^s) or tetracycline resistant (Tet^r) states.²⁵³ The research proved that the main decomposition product of **106** was anhydrotetracycline (**4**, Figure 3.14). They incubated 1:1 mixtures of Tet^r and Tet^s cells with solutions of **106** in various stages of its decomposition process to **4**. The incubated cells could then be tested to determine if the Tet^r or Tet^s variant had survived and propagated successfully.

Figure 3.14 Oxytetracycline (**106**) and anhydrotetracycline (**4**)



It is known that in the absence of a tetracycline the Tet^s state is favoured and that in the presence of a tetracycline the Tet^r state is favoured.⁴ The research of Kishony *et al.* provided

useful insights into the effect of the decomposition products of **106** on the selection. They found that in the presence of **106** which was fully decomposed to **4**, the Tet^s state was favoured. They also discovered that the Tet^r state could be inverted to the Tet^s state in the presence of **4**. The selection of the Tet^r state in the presence of **106** was found to be a short lived phenomenon, with the Tet^s state being favoured over longer time periods. However, the selection for the Tet^s state was found to be a more lasting effect.

The findings of this research are of paramount importance when considered in the context of this work. **4** Could only effect the selection of Tet^s over Tet^r states if, at some stage, it was able to penetrate the cell membrane and bind to the Tet(R) regulation protein. This was a significant finding given that previously it was thought that **4** was solely lodged in the hydrophobic cell membrane when administered.²⁶ Also, the fact that **4** resulted in the selection of the Tet^s state proved our initial hypothesis correct: **4** could indeed cause the over-expression of efflux proteins and cell death if it gained entry to the cell. This would quickly prove detrimental to the cell lines and so the resultant selection for the Tet^s state would occur. This is the only manner in which **4** could cause selection of Tet^s over Tet^r as binding to cell membranes could not induce this effect, given that the affinity for the membrane should be equal in each state.

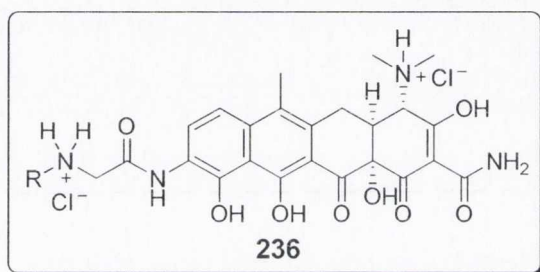
The inversion of the Tet^r state to the Tet^s state by **4** was also an interesting anomaly when considered in a therapeutic setting. If analogues of **4** could be produced which would have a greater ability to enter the bacterial cell, they could in theory transform a Tet^r infection to a Tet^s infection and, since this inversion was found to be exhibited as a long-lasting effect, the infection could be treated with typical tetracyclines. Additionally, the analogues of **4** capable of entering the cell would not cause the cytotoxic effects related to **4**, owing to its preferential lodgement in cell membranes that has rendered it useless therapeutically to this date.

With this information in mind, it was decided to produce a methodology for the synthesis of analogues of **4** that would possess a greater ability to traverse the cell membrane. These analogues could then be active against Tet^r bacteria in the manner that it was initially hoped that the dimers would exert activity: by the induction of the overexpression of the efflux proteins which leads to cell lysis.

3.1.2 The design of anhydrotetracycline analogues for cell entry and their retrosynthesis

It was initially considered that minor modifications, such as sulfonation, of the D-ring of **4** would result in anhydrotetracycline derivatives with higher hydrophilicity and a greater ability to cross the bacterial cell membrane. However, initial investigations revealed **4** to be unreactive under sulfonation conditions. As such, it was decided to synthesise analogues of the general structure displayed in Figure 3.15 (*vide infra*).

Figure 3.15 General structure of anhydrotetracycline analogues for cell entry

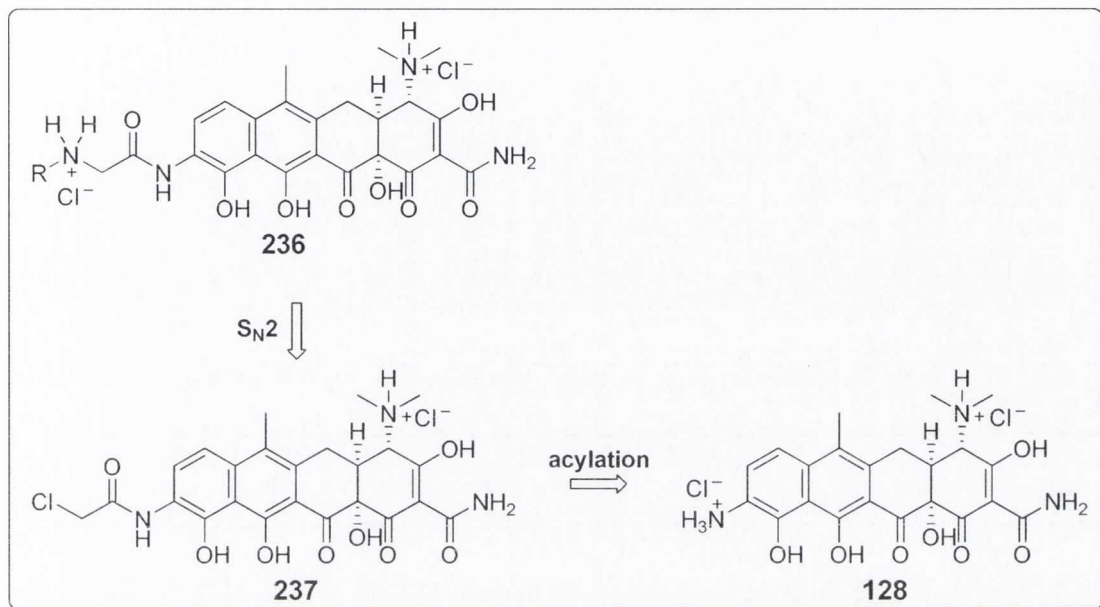


This general structure (**236**) was considered to be advantageous for cell entry on two fronts:

- 1) The second amide moiety would provide hydrophilicity to the analogues, thus rendering them less likely to be preferentially trapped in the cell membrane
- 2) The addition of a second aliphatic amine, which would most likely be protonated at physiological pH, would provide further hydrophilicity to the analogues and the addition of a second charge would drastically reduce the affinity of the variants for the hydrophobic lipid bi-layer surroundings of the cell membrane

Given what had been learned about the chemistry and instability of **4**, a retrosynthetic route from the general structure depicted in Scheme 3.48 to **128** was designed which allowed for mild reaction conditions and did not require excessive heating.

Scheme 3.48 Retrosynthesis of the general anhydrotetracycline analogue structure to **128**



3.1.3 The synthesis of anhydrotetracycline analogues for cell entry

The first step in the forward sense of the synthesis was the acylation of **128** with chloroacetyl chloride to afford **237**, a general scheme for which is depicted in Scheme 3.49 (*vide infra*). As regioselectivity issues involving the acylation of **128** had been encountered previously (Section 3.3.2), a number of small-scale reactions were undertaken to investigate the effect of base and temperature on this reaction. The reaction conditions initially attempted relate to Scheme 3.49 and are summarised in Table 3.6. All reactions were performed using 1 equivalent of **128** and 2 equivalents of chloroacetyl chloride in anhydrous DMPU/anhydrous acetonitrile (4:1) under an atmosphere of argon.

Scheme 3.49 **General procedure for the synthesis of intermediate 237 from 128**

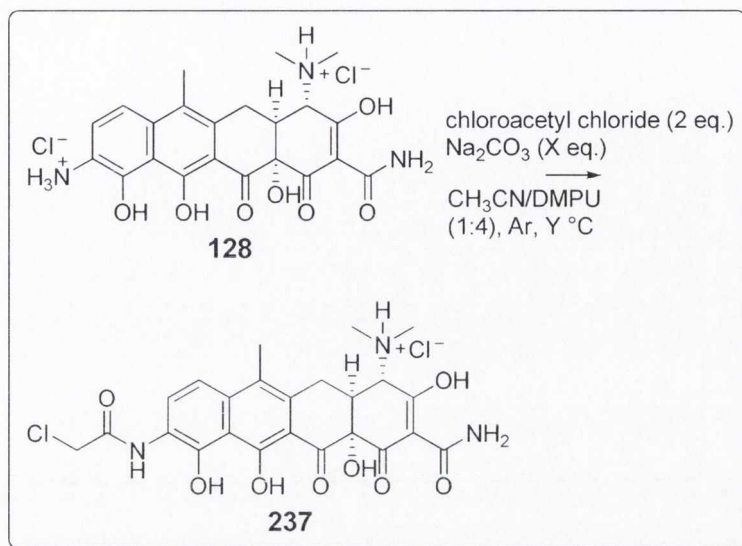


Table 3.6 **Initial acylation reactions with chloroacetyl chloride and 128**
(relating to Scheme 3.49)

Entry no.	Eq. Na_2CO_3 (X)	Temperature (Y)	Reaction time (min)
1	4	rt	30
2	2	rt	30
3	4	0 °C	30
4 ^a	2	0 °C	30

a = Full conversion to desired product (**237**)

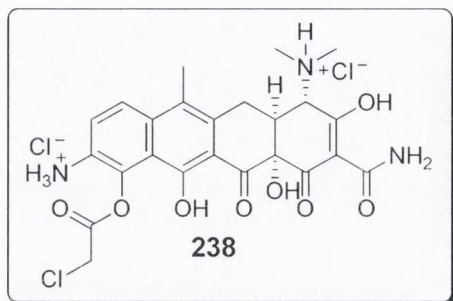
Entries 1 and 2 (Table 3.6) both furnished the desired product but in conjunction with a by-product of a similar nature. Entry 3 also furnished the desired product and a smaller amount of the undesired product. Entry 4 afforded only the desired product, with quantitative conversion to **237** after 30 min.

The reaction conditions summarised in entry 4, Table 3.6 and Scheme 3.49 were used in a large scale synthesis of **237** from **128**. Disappointingly, despite reproducing the conditions carefully, a 50/50 mixture of **237** and the undesired product were obtained. The two products were separable by RP-MPLC using a gradient of 10-30% acetonitrile/Millipore water and HRMS confirmed that they were of the same exact mass. Given this information,

the pattern of resonance signals in the ^1H NMR spectrum and previous acylation experience, the undesired product was deduced to be ester **238** (Figure 3.16)

Figure 3.16

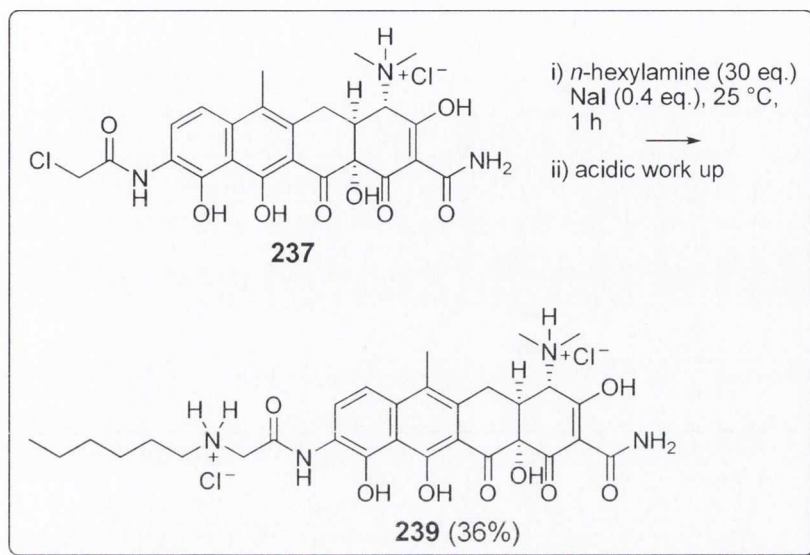
Undesired product of the acylation of 128



It was considered that the reaction temperature would have to be lowered for large scale synthesis and that reducing the equivalents of sodium carbonate used might also be advantageous. Therefore, two further reactions using 1.0 and 0.5 equivalents of sodium carbonate at a reaction temperature of $-10\text{ }^{\circ}\text{C}$ were attempted. After 20 min, the reactions were analysed by ^1H NMR spectroscopy and found to have afforded only the desired product in both cases. It was decided to use the methodology with 0.5 equivalents of sodium carbonate at $-10\text{ }^{\circ}\text{C}$ for large scale synthesis. Gratifyingly, the large-scale synthesis also produced solely the desired amide **237**.

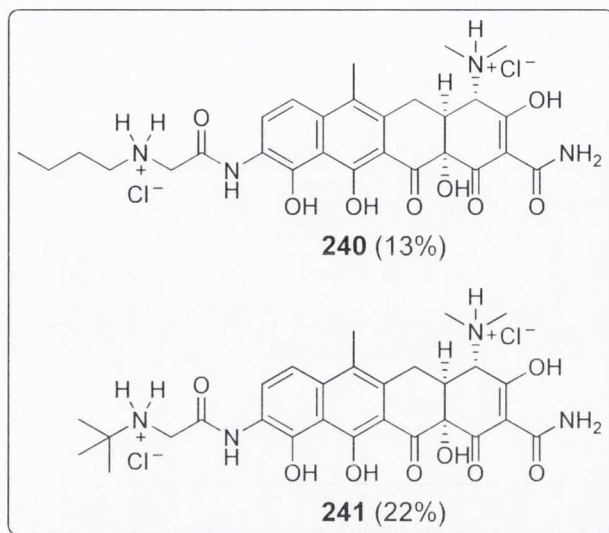
The next step in the forward synthesis required the reaction of **237** with various primary amines to afford the desired general structure depicted in Scheme 3.48. The first amine chosen for reaction was *n*-hexylamine. Compound **237** was reacted with 0.4 equivalents of sodium iodide and 30 equivalents of *n*-hexylamine at $25\text{ }^{\circ}\text{C}$ for 1 h (Scheme 3.50). After this time methanol was added to the reaction and the solvent and unreacted *n*-hexylamine were removed by evaporation at reduced pressure. It was decided that since the crude compound was quite pure, and given that previous analogues of anhydrotetracycline, such as **226** (Scheme 3.41), had proved unstable during RP-MPLC, the products would be purified by a pH dependent extraction from water. The crude product was dissolved in water at pH 7.2 and extracted into CH_2Cl_2 /methanol (4:1). The organic extracts were then acidified with HCl in anhydrous diethyl ether (2.0 M) and the solvent removed at reduced pressure to afford pure **239** in 36% yield.

Scheme 3.50

Synthesis of anhydrotetracycline analogue **239** from **237** and *n*-hexylamine

The same synthetic procedure was followed for the reaction of **237** with *n*-butylamine and *t*-butylamine to afford compounds **240** (13% yield) and **241** (22% yield), respectively (Figure 3.17). The straight chain anhydrotetracycline **240** was afforded in 1 h reaction time, however, the bulkier variant **241** required overnight reaction (18 h) to reach completion. Both reactions were worked up exactly as per that of **239**.

Figure 3.17 Anhydrotetracycline analogues **240** and **241** for cell entry



n-Butylamino derivative **240** was chosen for synthesis as it was felt that that presence of the shorter alkyl chain would be more advantageous for cell entry. The *t*-butylamino containing product **241** was chosen to mimic tigecycline (**19**, Figure 1.12, *vide supra*) in the hope that after entering the cell, it would not be removed from the cell by the efflux resistance proteins.

The same methodology was screened for the reaction of **237** with secondary and tertiary amines. It was hypothesised that the reaction of secondary amines with **237** could provide tertiary amine products which would be protonated at physiological pH. **237** Was reacted with 30 equivalents of pyrrolidine, as it is a relatively unhindered secondary amine, in the presence of 0.4 equivalents of sodium iodide at 25 °C for 24 hours. Unfortunately, after this time no product formation was detected by ¹H NMR spectroscopy and HRMS did not detect the desired molecular ion.

The reaction conditions were concurrently employed in the reaction of **237** with triethylthylamine. Although the proceeding of this reaction seemed unlikely, the resultant quaternary amine product would be an interesting product for *in vitro* testing and so **237** was reacted with 30 equivalents of triethylamine and 0.4 equivalents of sodium iodide at 25 °C for 24 hours. After this time no product formation was observed by ¹H NMR spectroscopy and the desired molecular ion was not detected by HRMS.

It appeared that the synthetic methodology in place was only amenable to the use of primary amines in reactions with **237**. However, this was not discouraging as the resultant

products were anhydrotetracycline analogues which would possess an extra positive charge at physiological pH and would therefore be more likely to gain entry into bacterial cells, bind to the Tet(R) regulation protein causing the overexpression of efflux proteins, and therefore result in cell death.

3.2 Conclusions and future work

The chemistry of minocycline (**3**) and anhydrotetracycline (**4**) has been well explored over the course of this work. The synthesis of tetracycline dimers *via* the chosen route was examined and monomer analogues for comparison were produced. The synthesis of a precursor to a minocycline analogue (**181**) which could be reacted with a variety of amines has been prepared and linked to the minocycline linker **173**. This provided a minocycline containing a terminal alkyne (**181**) for functionalisation *via* cycloaddition reactions with azides. It had been demonstrated that 9-azidominocycline (**190**) was unreactive under Cu(I) catalysed cycloaddition conditions with alkynes and that a spacer unit between the minocycline core and alkyne group was necessary for the reaction to occur. The functionalisation of minocycline at position 8 did not proceed even though a large number of reaction conditions were performed on a number of different 9-substituted minocycline analogues. It was also determined that reaction conditions which were successful when 9-substituted doxycycline analogues were employed were not transferrable to 9-substituted minocyclines.

The desired transformation of 9-diazoanhydrotetracycline salts **194A**, **194B** and **194C** to the corresponding 9-haloanhydrotetracyclines was not feasible under the Sandmeyer conditions employed due to the inherent instability of anhydrotetracycline analogues. The regioselective bromination of anhydrotetracycline (**4**) at position 9 was possible, but the resultant compound was found to be unstable under available purification conditions. A number of novel organostannane compounds were synthesised for use as linkers with diazoanhydrotetracyclines, however, it was found that anhydrotetracycline was unreactive under the required Stille coupling conditions. An unexpected route to vinylphosphonium salts was unearthed during this study.

A modified acid chloride linker (**222**) was produced and connected regioselectively to the anhydrotetracycline core, producing anhydrotetracycline analogue **223**. **223** And **181** were tested under a variety of Huisgen and Cu(I) catalysed cycloaddition reaction conditions but

the reaction could not be rendered viable due to the instability of **223** at mildly elevated temperatures, for example 40 °C. However, it was found that **181** was amenable to cycloaddition reactions with various azides under these conditions and reaction conditions for the successful production of derivatives of this nature were devised. Due to the constraints of time, a minocycline-minocycline dimer was not produced, although it appears that this would be possible if an appropriate linker were designed and synthesised. **181** could be used in future to provide new triazole analogues of minocycline by reaction with appropriate azides.

A novel idea for the synthesis of anhydrotetracycline variants which could bestow therapeutic use on this heretofore unused member of the tetracycline family was explored and a synthetic route amenable to the use of a number of amine compounds was put in place. Three novel tetracycline analogues of this nature were produced and gratifyingly the general synthetic procedure set in place proved successful. Due to the constraints of time, a larger number of compounds of this general structure were not produced but it was envisaged that this route could be employed to link amino acid compounds to the anhydrotetracycline core to aid the compounds' ability to traverse the cell membrane. This hypothesis is supported by the recent work of Gmeiner *et al.*, who managed to produce anhydrotetracycline-peptide conjugates capable of cell entry.²¹³

Chapter 4

Experimental

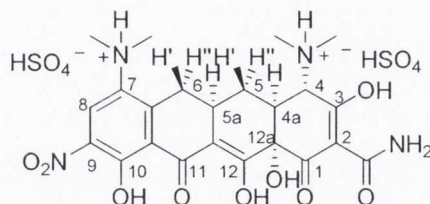
4 Experimental section

4.1 General

Melting points were determined using a standard melting point apparatus and are uncorrected. Decomposition temperature was judged to have been reached when crystals and solids began to turn brown/black. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with a universal ATR sampling accessory. Proton nuclear magnetic resonance (NMR) spectra were recorded on: Bruker Avance III 400 MHz, Bruker DPX400 400 MHz and Bruker Avance II 600 MHz spectrometers (^1H NMR spectra were recorded at 400.23 MHz, 400.13 MHz and 600.13 MHz respectively). Chemical shifts are reported in ppm relative to residual non-deuterated solvent and coupling constants (J) are quoted in Hertz. ^{13}C NMR spectra were recorded on the previously mentioned instruments (^{13}C NMR spectra were recorded at 100.64 MHz, 100.61 MHz & 150.9 MHz, respectively) with total proton decoupling. ^{31}P NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer at 161.98 MHz. HSQC, HMBC, TOCSY and NOE NMR experiments were used to aid assignment of NMR peaks. A Waters micromass LCT-tof mass spectrometer was used in ES positive and ES negative modes for electrospray mass spectrometry. Electron impact mass spectra were determined on a Quatro-II mass spectrometer in the EI mode. Mass spectra were recorded in CSCB Trinity College Dublin. Microwave reactions were performed in a Discover microwave system with variable temperature and wattage. Flash chromatography was performed using Merck Kiesegel 60 (art. 9385) and aluminium oxide 90, standardised (activity II-III). Reverse-phase chromatography was performed on a Biotage-SP4 Flash MPLC system using SNAP C-18 cartridges (12g and 60g). Merck precoated Kiesegel 60F₂₅₄ and alumina (neutral, type E) were used for thin-layer chromatography and slides were visualised by UV irradiation, KMnO_4 , or phosphomolybdic acid staining. Tetrahydrofuran was distilled over sodium-benzophenone ketyl radical before use. CH_2Cl_2 , Et_3N , benzene and acetonitrile were distilled over calcium hydride before use. Anhydrous DMPU and anhydrous diethyl ether were sourced from Sigma Aldrich. Minocycline hydrochloride was sourced from Waterstone Technology and was recrystallised from a MeOH/diethyl ether mixture before use. Unless otherwise stated, all chemicals were obtained from commercial sources and used as received. *Epi*-tetracyclines exhibit inverted stereochemistry at C-4.

4.2 Chapter 2

4.2.1 9-Nitrominocycline disulfate (169)



A solution of minocycline hydrochloride (**3**) (249 mg, 0.50 mmol) in 95 % H_2SO_4 (aq) (3.5 mL) was cooled to 0 °C in a 10 mL round bottom flask. Potassium nitrate (62 mg, 0.61 mmol) was added in two aliquots over a 30 min period and stirred for 2 h. The reaction mixture was added to a 100 mL beaker containing diethyl ether (20 mL) at 0 °C and a yellow precipitate was observed. The precipitate was collected by filtration under Ar and purified by MPLC on a SNAP-12g KP-C18-HS cartridge (mobile phase: acetonitrile/Millipore water (gradient 10%-50%)). 10% H_2SO_4 (aq) was added to the product fractions and the solvent evaporated at reduced pressure. The remaining solid was dissolved in a minimum volume of methanol and added dropwise to diethyl ether (20 mL) at 0 °C. The resulting precipitate was collected under Ar to furnish **169** as a disulfate salt (269.9 mg,) in 77% yield. M.p. 186-190 °C (dec.)

δ_{H} (400 MHz, DMSO-d_6): 9.57 (s, 1H, NH_2), 9.07 (s, 1H, NH_2), 7.99 (s, 1H, CH-8), 4.23 (app. s, 1H, CH-4), 3.38 (dd, 1H, J 14.0, 7.0, $\text{CH}_2\text{-H-6''}$), 3.25 (m, 1H, CH-5a), 3.35 (m, 7H, 2 x $\text{CH}_3\text{-NMe}_2\text{-7}$, CH-4a), 2.65 (s, 6H, 2 x $\text{CH}_3\text{-NMe}_2\text{-4}$), 2.40 (m, 1H, $\text{CH}_2\text{-H-6'}$), 2.23 (m, 1H, $\text{CH}_2\text{-H-5''}$), 1.53 (m, 1H, $\text{CH}_2\text{-H-5'}$)

δ_{C} (100 MHz, DMSO-d_6): 193.8 (q), 192.0 (q), 186.8 (q), 175.8 (q), 171.9 (q), 150.6 (q), 144.2 (q), 136.1 (q), 122.2 (CH-8), 118.3 (q), 108.4 (q), 95.0 (q), 74.0 (q), 68.0

(q), 62.1 (CH-4), 44.1 (2 x CH₃; NMe₂-7), 41.9 (CH₃-NMe₂-4), 40.1 (CH₃-NMe₂-4), 34.4 (CH-4a), 33.7 (CH-5a), 33.3 (CH₂-5), 29.9 (CH₂-6)

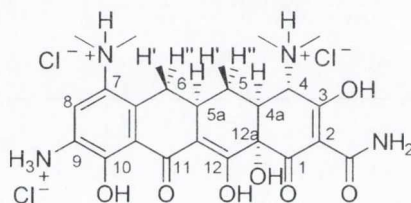
HRMS (*m/z* ESI+):

Found: 503.1755 (M + H C₂₃H₂₇N₄O₉ Requires: 503.1778)

ν/cm^{-1} (neat):

3048 (O-H phenolic stretch), 1531 (N=O stretch), 1176, 1042, 760

4.2.2 9-Aminominocycline trihydrochloride (**87**)



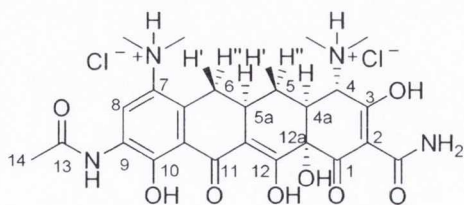
Compound **169** (706 mg, 1.01 mmol) was dissolved in 2-methoxyethanol (15 mL) and 8% HCl_(aq) (1.5 mL) and stirred for 3 h with 10 % Pd/C (70 mg, 0.066 mmol) under H₂ at 3 atm. The catalyst was removed by filtration through a pad of celite and the filtrate evaporated to dryness. The solid was then dissolved in a minimum volume of methanol and added dropwise to diethyl ether (400 mL) at 0 °C resulting in the formation of a yellow precipitate. The precipitate was collected by filtration under Ar and dried *in vacuo* to furnish the amine product **87** as a trihydrochloride salt in 96% yield (564 mg, 0.970 mmol). M.p. 230-233 °C (dec.)

δ_{H} (400 MHz, CD₃OD):

8.02 (s, 1H, CH-8), 4.18 (app. s, 1H, CH-4), 3.64-3.62 (m, 1H, CH₂-H-6''), 3.38 (s, 6H, 2 x CH₃-NMe₂-7), 3.26 (m, 1H, CH-5a), 3.09 (s, 3H, CH₃, NMe₂-4), 3.06 (m, 1H, CH-4a) 2.99 (s, 3H,

	CH ₃ -NMe ₂ -4), 2.65-2.57 (m, 1H, CH ₂ -H-6'), 2.43-2.40 (m, 1H, CH ₂ -H-5''), 1.70 (m, 1H, CH ₂ -H-5')
δ_C (100 MHz, CD ₃ OD):	194.1 (q), 192.0 (q), 186.5 (q), 174.9 (q), 172.8 (q), 155.3 (q), 135.3 (q), 130.8 (q), 121.4 (CH-8), 121.1 (q), 117.2 (q), 107.7 (q), 94.9 (q), 74.2 (q), 69.2 (CH-4), 46.4 (2 x CH ₃ -NMe ₂ -7), 42.0 (CH ₃ -NMe ₂ -4), 40.7 (CH ₃ -NMe ₂ -4), 34.4 (CH-4a), 34.1 (CH-5a), 33.4 (CH ₂ -5), 29.3 (CH ₂ -6)
HRMS (<i>m/z</i> ESI+):	Found: 473.2039 (M + H C ₂₃ H ₂₉ N ₄ O ₇ Requires: 473.2036)
ν/cm^{-1} (neat):	3356 (O-H phenolic stretch), 3048 (N-H amide stretch), 2950 (C-H aromatic stretch), 2950, 2624, 1638 (C=O amide stretch), 1597, 1459, 1356, 1176, 1045, 997, 941, 918, 849, 762, 736, 687, 659

4.2.3 9-Acetamidominocycline dihydrochloride (171)



A 10 mL round bottom flask was placed under an atmosphere of Ar and charged with compound **87** (150 mg, 0.26 mmol), anhydrous sodium carbonate (165 mg, 1.56 mmol), anhydrous acetonitrile (1 mL) and *N,N*-DMPU (4 mL). Acetyl chloride (40 μ L) was added

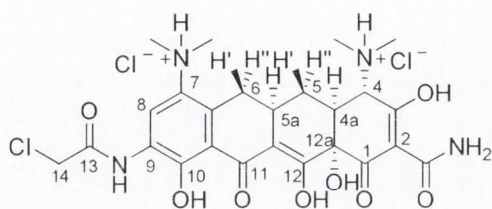
dropwise *via* syringe and the reaction stirred at ambient temperature for 1 h. Methanol (200 μ L) was added to the reaction vessel *via* syringe and the reaction mixture was filtered and added dropwise to diethyl ether/isopropanol (1:10, 110 mL) to furnish the crude product as a yellow/green precipitate (119 mg). The crude product was purified by pH gradient extraction from aqueous solution into chloroform and the HCl salt produced by bubbling anhydrous HCl directly through the chloroform solution. The yellow solid product **171** (57 mg, 0.097 mmol) was obtained in 37% yield by filtration under Ar. ^1H NMR data agree with the referenced literature.²³²

δ_{H} (400 MHz, DMSO- d_6): 14.65 (br. s, 1H, OH-12), 12.20 (br. s, 1H, OH-10), 10.35 (br. s, 1 H, OH), 9.58 (app. br. s, 2H, NH_2 -2, NH-9), 9.07 (s, 1H, NH_2 -2), 8.36 (s, 1H, CH-8), 4.36 (s, 1H, CH-4), 3.09 (m, 1H, CH_2 -H-6'') 2.91 (m, 7 H, CH-5a, 2 x CH_3 -NMe $_2$ -7), 2.75 (s, 6H, 2 x CH_3 -NMe $_2$ -4), 2.33 (m, 1H, CH-4a), 2.23 (m, 1H, CH_2 -H-6'), 1.75 (m, 1H, CH_2 -H-5'), 1.49 (m, 1H, CH_2 -H-5')

HRMS (m/z ESI+): Found: 514.2139 (M + H C $_{25}$ H $_{31}$ N $_4$ O $_8$ Requires: 515.2142)

ν/cm^{-1} (neat): 3328 (O-H phenolic stretch), 3027 (C-H aromatic), 2925 (C-H alkyl stretch), 2871 (C-H alkyl stretch), 2672, 1590 (C=O amide stretch), 1527, 1452, 1406, 1370, 1318, 1280, 1252, 1213, 1142, 1125, 1054, 999, 940, 918, 892, 851, 822, 806, 789, 736, 710

4.2.4 9-(2-chloroacetamido)Minocycline dihydrochloride (**170**)



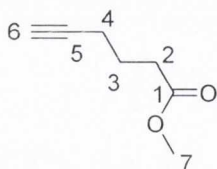
Compound **87** (650 mg, 1.12 mmol) and anhydrous sodium carbonate (712 mg, 6.72 mmol) were added to a 50 mL round bottom flask. The flask was evacuated and placed under an atmosphere of Ar. Anhydrous acetonitrile (3.9 mL) and anhydrous *N,N*-DMPU (15.6 mL) were added to the flask *via* syringe and the mixture stirred until **87** was dissolved. A solution of chloroacetyl chloride (18 μ L, 25 mg, 2.24 mmol) in anhydrous *N,N*-DMPU (210 μ L) and anhydrous acetonitrile (60 μ L) was added dropwise to the reaction mixture over 30 mins. After stirring the reaction for a further hour, methanol (200 μ L) was injected and the reaction stirred for 5 mins. The reaction was filtered through cotton wool and added dropwise to 1:10 2-propanol:diethyl ether. HCl in anhydrous diethyl ether (2.0 M, 4 mL, 8.0 mmol) was added and the precipitated crude product was collected and purified by pH gradient extraction into chloroform. The dihydrochloride salt was formed by the addition of anhydrous HCl in diethyl ether (2.0 M, 2 mL, 4.0 mmol) and the product collected by filtration under Ar, affording the dihydrochloride salt **170** as a yellow powder (188 mg, 30 μ mol) in 27% yield. M.p. 200-203 $^{\circ}$ C (dec.)

δ_{H} (400 MHz, DMSO- d_6):

14.66 (s, 1H, OH-12), 12.17 (s, 1H, OH-10), 10.31 (s, 1H, OH), 9.95 (s, 1H, NH-9), 9.58 (s, 1H, NH₂-2), 9.07 (s, 1H, NH₂-2) 8.36 (s, 1H, CH-8), 7.59 (s, 1H, OH) 4.44 (s, 2H, CH₂-14), 4.34 (app. s, 1H, CH-4), 3.50 (m, 1H, CH₂-H-6''), 3.03 (m, 8H, CH-4a, CH-5a, 2 x CH₃-NMe₂-4), 2.88 (m, 6H, 2 x CH₃-NMe₂-7), 2.33 (m, 1H, CH₂-H-6'), 2.22 (m, 1H, CH₂-H-5''), 2.07

	1.48 (m, 1H, CH ₂ -H-5')
δ_C (100 MHz, DMSO-d ₆):	194.2 (q), 192.5 (q), 187.6 (q), 176.0 (q), 173.3 (q), 172.3 (q), 166.0 (q), 152.7 (q), 133.1 (q), 132.0 (q), 126.4 (q), 121.1 (CH-8), 116.2 (q), 108.6 (q), 74.6 (q), 68.3 (CH-4), 46.1 (2 x NMe ₂ -7), 43.7 (CH ₂ -14), 41.9 (CH ₃ , NMe ₂ -4), 41.6 (CH ₃ , NMe ₂ -4), 34.8 (CH-4a), 34.3 (CH ₂ -5), 31.7 (CH-5a), 30.2 (CH ₂ -6)
HRMS (<i>m/z</i> ESI+):	Found: 549.1760 (M + H C ₂₅ H ₃₀ ClN ₄ O ₈) Requires: 549.1752)
ν/cm^{-1} (neat):	3339 (O-H phenolic stretch), 2689, 1804, 1622 (C=O amide stretch), 1589, 1560, 1529, 1461, 1350, 1260, 1226, 1203, 1145, 1125, 1051, 1033, 1017, 988, 920, 894, 852, 824, 808, 726

4.2.5 Methyl hex-5-ynoate (175)



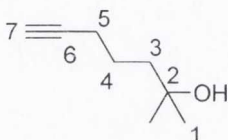
A round bottom flask was charged with **174** (2.00 mL, 2.03 g, 18.1 mmol), methanol (36 mL) and 97% H₂SO₄ (200 μ L, 3.58 mmol) and the reaction stirred. A white precipitate was noted. The reaction was heated at reflux for 4 h and the solvent subsequently removed by evaporation at reduced pressure. The resulting solid was suspended in CH₂Cl₂ (40 mL) and washed with saturated sodium hydrogen carbonate (40 mL). The aqueous layer was washed with CH₂Cl₂ (20 mL) and the organic extracts combined and the solvent evaporated at reduced pressure to provide the crude product as a yellow oil. The crude product was distilled

by Kuglerrohr distillation at 200 mbar and 95 °C to provide the pure product, **175** (1.35g, 10.7 mmol) in 59% yield. ¹H NMR data agree with the referenced literature.²⁵⁴

δ_{H} (400 MHz, CDCl_3): 3.70 (s, 3H, CH_3 -7), 2.49 (t, 2H, J 7.4, CH_2 -2), 2.29 (dt, 2H, J 7.0, 2.5, CH_2 -4), 1.99 (t, 1H, J 2.5, CH-6), 1.87 (m, 2H, CH_2 -3)

HRMS (m/z ESI+): Found: 127.0763 ($\text{M} + \text{H}$ $\text{C}_7\text{H}_{11}\text{O}_2$ Requires: 127.0759)

4.2.6 2-Methylhept-6-yn-2-ol (**176**)



A 50 mL round bottom flask was dried, evacuated and placed under an atmosphere of Ar. To this was added methylmagnesium bromide in anhydrous diethyl ether (3.0 M, 17.8 mL, 53.5 mmol) and anhydrous THF (15 mL). The solution was cooled to 0 °C and a solution of compound **175** (1.35 g, 10.7 mmol) in anhydrous THF (10 mL) was added *via* syringe over 30 mins. The reaction was allowed to stir and warm to room temperature over 2 h. Millipore water (30 mL) was added to the reaction and the product was extracted with diethyl ether (4 x 100 mL). The solvent was removed from the organic extracts by evaporation at reduced pressure and the crude product purified by column chromatography (mobile phase: 20 % ethyl acetate/hexane) to provide the pure product, **176**, (1.18g, 9.32 mmol) in 87% yield. NMR spectroscopic data agree with the referenced literature.²⁵⁵

δ_{H} (600 MHz, CDCl_3): 2.25 (m, 2H, CH_2 -5), 1.99 (t, 1H, J 2.5, CH-7), 1.63 (m, 4H, 2 x CH_2 , CH_2 -3, CH_2 -4), 1.44 (s, 1H, OH), 1.28 (s, 6H, 2 x CH_3 -1)

δ_{C} (150 MHz, CDCl_3): 84.2 (q, C-6), 70.6 (q, C-2), 68.3 (CH-7), 42.7 (CH_2 -3), 29.1 (2 x CH_3 -1), 20.3 (CH_2 -4), 18.7

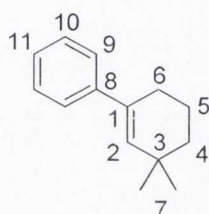
209

(CH₂-5)

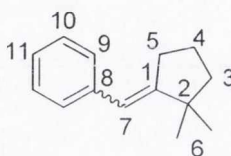
HRMS (*m/z* ESI+):

Found: 127.1122 (M + H C₈H₁₅O Requires:
127.1123)

4.2.7 (3,3-dimethylcyclohex-1-enyl)Benzene (177A) and [(2,2-dimethylcyclopentylidene)methyl]Benzene (177B)



177A



177B

Anhydrous benzene (85 mL), TMSN₃ (2.27 mL, 1.99 g, 17.26 mmol) and **176** (1.09g, 8.64 mmol) were added *via* syringe to a dry round bottom flask under an atmosphere of Ar. The mixture was heated to 30 °C and borontrifluoride diethyletherate (2.17 mL, 2.45 g, 17.28 mmol) was added *via* syringe. The reaction was allowed to cool to ambient temperature and stirred for 24 h. The reaction was poured into Millipore water (200 mL) and the organic phase separated and washed with sodium hydrogen carbonate (10% w/v, 100 mL). The organic extract was dried over magnesium sulfate and the solvent was evaporated at reduced pressure. The crude product was distilled by Kugelrohr distillation. Compounds **177A** and **177B** were isolated at 40 °C and 40 mbar as a 1:1 mixture.

177A:

δ_H (400 MHz, CDCl₃):

7.36 (app. d, 2H, J 7.6 Hz, 2 x CH-10), 7.29 (t, 2H, J 7.6 Hz, 2 x CH-9), 7.20 (t, 1H, J 7.6 Hz, CH-11), 5.84 (s, 1H, CH-2), 2.28 (t, 2H, J 6.0, CH₂-6), 1.71 (m, 2H, CH₂-5), 1.43 (m, 2H, CH₂-4), 1.02 (s, 6H, 2 x CH₃-7)

δ_C (100 MHz, $CDCl_3$): 141.7 (q, C-1), 134.7 (CH-2), 134.6 (q, C-8), 128.9 (2 x CH-9), 126.6 (2 x CH-11), 124.7 (2 x CH-10), 36.2 (CH₂-4), 31.7 (q, C-3), 29.7 (2 x CH₃-7), 26.6 (2 x CH₃-6), 19.5 (CH₂-5)

HRMS (m/z EI⁺): Found: 186.1414 (M^+ C₁₄H₁₈ Requires: 186.1409)

ν/cm^{-1} (neat): 3380, 1765, 1666 (alkenyl C=C stretch), 1634, (alkenyl C=C stretch) 1575, 1489, 1453, 1424, 1391, 1346, 1197, 1087, 984, 932, 890, 857, 832 (aromatic C-H bend), 802, 777, 717

177B:

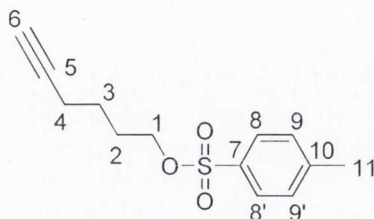
δ_H (400 MHz, $CDCl_3$): 7.36 (app. d, 2H, J 7.6 Hz, 2 x CH-10), 7.29 (t, 2H, J 7.6 Hz, 2 x CH-9), 7.20 (t, 1H, J 7.6 Hz, CH-11), 6.09 (m, 1H, CH-7), 2.18 (m, 2H, CH₂-5), 2.12 (m, 2H, CH₂-4), 1.35 (t, 2H, J 6.5, CH₂-3), 0.94 (s, 6H, 2 x CH₃-6)

δ_C (100 MHz, $CDCl_3$): 135.0 (q, C-1), 134.6 (q, C-8), 128.9 (2 x CH-9), 126.6 (2 x CH-11), 124.7 (2 x CH-10), 123.0 (CH-7), 40.5 (CH₂-4), 34.15 (CH₂-3), 29.0 (q, C-2), 28.1 (CH₂-6), 22.8 (CH₂-5)

HRMS (m/z EI⁺): Found: 186.1414 (M^+ C₁₄H₁₈ Requires: 186.1409)

ν/cm^{-1} (neat): 3380, 1765, 1666 (alkenyl C=C stretch), 1634, (alkenyl C=C stretch) 1575, 1489, 1453, 1424, 1391, 1346, 1197, 1087, 984, 932, 890, 857, 832 (aromatic C-H bend), 802, 777, 717

4.2.8 Hex-5-ynyl *p*-methylbenzenesulfonate (**179**)



A 50 mL round bottom flask, under a positive atmosphere of Ar, was charged with 5-hexyn-1-ol (325 μ l, 289 mg 3.0 mmol), 4-dimethylaminopyridine (37 mg, 0.3 mmol) and *p*-toluenesulfonyl chloride (601 mg, 3.15 mmol). The mixture was cooled to 0 $^{\circ}$ C and anhydrous CH_2Cl_2 (15 mL) was injected *via* syringe, followed by distilled NEt_3 (835 μ l, 606 mg, 6.0 mmol). The reaction was allowed to warm to ambient temperature and stir for 16 h, after which time it was diluted with CH_2Cl_2 (15 mL) and washed with 8% $\text{HCl}_{(\text{aq})}$ (15 mL). The organic layer was separated and washed with saturated sodium hydrogen carbonate (10 mL) and brine (15 mL). The solvent was evaporated at reduced pressure to give 1.062 g crude product which was adsorbed onto silica. The crude was purified by column chromatography (mobile phase 10% ethyl acetate/hexane) followed by recrystallisation from diethyl ether to furnish **179** (691 mg, 2.50 mmol) as a white crystalline solid in 84% yield. M.p. 32-34 $^{\circ}$ C. ^1H NMR spectroscopic data agree with the referenced literature.²⁵⁶

δ_{H} (400 MHz, CDCl_3): 7.80 (d, 2H, J 8.3, H-8, H-8'), 7.36 (d, 2H, J 8.3, H-9, H-9'), 4.07 (t, 2H, J 6.4, CH_2 -1), 2.47 (s, 3H, CH_3 -11), 2.18 (dt, 2H, J 7, 2.5, CH_2 -4), 1.94 (t, 1H, J 2.5, H-6), 1.80 (m, 2H, CH_2 -2), 1.57 (m, 2H, CH_2 -3)

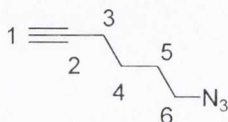
δ_{C} (125 MHz, CDCl_3): 144.3 (q, C-7), 132.6 (q, C-10), 129.4 (CH-9, CH-9'), 127.4 (CH-8, CH-8'), 82.9 (q, C-5), 69.5 (CH_2 -1) 68.5 (CH-6), 27.3 (CH_2 -2), 23.7 (CH_2 -3), 21.2 (CH_3 -11), 17.3 (CH_2 -5)

HRMS (m/z ESI+):

Found: 275.0715 (M + Na C₁₃H₁₆NaO₃S

Requires: 275.0718)

4.2.9 6-Azidohept-1-yne (180)



A 100 mL round bottom flask was charged with compound **179** (1.081 g, 4.29 mmol), *N,N*-dimethylformamide (35 mL) and sodium azide (558 mg, 8.58 mmol) and the reaction stirred for 2 h. Diethyl ether (35 mL) was added and the organic layer washed with Millipore water (5 x 35 mL) and brine (35 mL). The diethyl ether was distilled from the product by fractional distillation (product volatile) affording **180** as a clear oil (386 mg, 3.13 mmol) in 73 % yield. ¹H NMR spectroscopic data agree with the referenced literature.²⁴⁰

δ_H (600 MHz, CDCl₃):

3.34 (t, 2H, J 7.0, CH₂-6), 2.27 (dt, 2H, J 7.0, 2.6, CH₂-3), 2.00 (t, 1H, J 2.6, H-1), 1.77 (m, 2H, CH₂-5), 1.64 (m, 2H, CH₂-4)

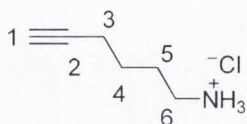
δ_C (150 MHz, CDCl₃):

83.9 (C-2), 68.9 (CH-1), 51.0 (CH₂-6), 27.8 (CH₂-5), 25.5 (CH₂-4), 18.0 (CH₂-3).

HRMS (m/z ESI+):

Found: 124.0878 (M + H C₆H₁₀N₃ Requires: 124.0875)

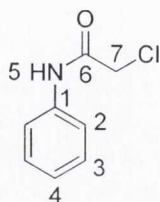
4.2.10 6-Aminohexy-1-yne hydrochloride (**173**)



Lithium aluminium hydride (1.707g, 44.98 mmol) was added to a 250 mL round bottom flask and the reaction vessel flushed with Ar. A positive atmosphere of Ar was applied and anhydrous diethyl ether (80 mL) was added *via* syringe, the system cooled to 0 °C and compound **180** (2.770g, 22.49 mmol) added *via* syringe. The reaction was stirred at 0 °C for 1.5 h and allowed to warm to ambient temperature. The reaction was diluted with diethyl ether (80 mL) and Millipore water (550 μ L). A 40 % solution (w/v) of sodium hydroxide (aq) (227 μ L) and Millipore water (1.7 mL) were added and the mixture stirred for 15 mins. Magnesium sulfate was added to the mixture and it was allowed to stir for a further 15 mins. The contents of the reaction vessel were then filtered and HCl in anhydrous diethyl ether (2.0 M, 20 mL, 40 mmol) was added to furnish the desired product **173** (2.163g, 16.19 mmol), in 72 % yield, as a white solid precipitate which was collected by filtration. NMR spectroscopic data agree with the referenced literature.²⁵⁷

δ_{H} (600 MHz, DMSO- d_6):	8.10 (br. s, 3H, NH_3^+), 2.83 (t, 1H, J 2.6, H-1), 2.77 (m, 2H, CH_2 -6), 2.19 (dt, 2H, J 7.0, 2.6, CH_2 -3), 1.64 (m, 2H, CH_2 -5), 1.49 (m, 2H, CH_2 - 4)
δ_{C} (150 MHz, DMSO- d_6):	84.5 (q, C-2), 72.1 (CH-1), 38.7 (CH_2 -6), 26.6 (CH_2 -5), 25.3 (CH_2 -4), 17.7 (CH_2 -3)
HRMS (m/z ESI+):	Found: 98.0972 (M + H $\text{C}_6\text{H}_{12}\text{N}$ Requires: 98.0970)

4.2.11 *N*-(chloroacetyl)Aniline (**184**)



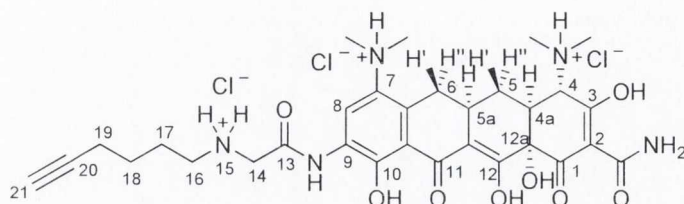
A round bottom flask was charged with **183** (2.00g, 15.4 mmol), 1M NaOH (31 mL, 31 mmol) and CH₂Cl₂ (31 mL). Chloroacetyl chloride (2.45 mL, 3.48 g, 30.8 mmol) was dissolved in CH₂Cl₂ (5 mL) and added to the reaction vessel. An exothermic reaction was noted and a gas was evolved. The reaction was allowed to proceed for 10 mins with vigorous stirring. The organic layer of the reaction was separated and the solvent removed at reduced pressure to furnish the desired product, **184** (2.617 g, 15.4 mmol) as a white solid in >99% yield. M.p. 132-135 °C. ¹H NMR spectroscopic data agree with the referenced literature.²⁵⁸

δ_{H} (400 MHz, DMSO-d₆): 10.31 (s, 1H, NH-5), 7.59 (app. d, 2H, J 7.5, 2 x CH-3), 7.34 (app. t, 2H, J 7.5, 2 x CH-2), 7.08 (t, 1H, J 7.5, CH-4), 4.26 (s, 2H, CH₂-7)

δ_{C} (100 MHz, CDCl₃): 162.2 (q, C-6), 134.8 (q, C-1), 127.3 (CH), 123.5 (CH), 118.3 (CH), 41.0 (CH₂-7)

HRMS (*m/z* EI⁺): Found: 169.0292 (M + H C₈H₈NOCl Requires: 169.0294)

4.2.12 9-[2-(hex-5-ynylamino)]Acetamidominocycline trihydrochloride (**181**)

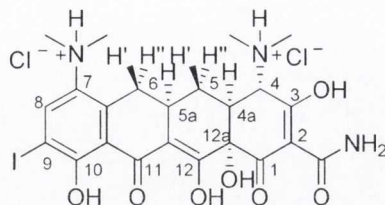


173 (228 mg, 1.7 mmol) Was dissolved in a minimum volume of NaOH (2M) and the free base amine extracted with diethyl ether (3 x 10 mL). The solution of free base **173** was transferred to a 50 mL round bottom flask and the solvent removed by fractional distillation at 50 °C. To the round bottom flask containing **173** as its free base was added NaI (20 mg, 0.13 mmol) and compound **170** (100mg, 0.17 mmol). The reaction vessel was flushed with Ar and heated to 25 °C for 18 h. Methanol (10 mL) was added to the reaction and the solution was added dropwise to diethyl ether (100 mL) forming a precipitate. HCl in anhydrous diethyl ether (2.0 M, 10 mL, 20 mmol) was added to the mixture resulting in further precipitation. The precipitate contained 9-[2-(hex-5-ynylamino)]acetamidominocycline trihydrochloride (**181**) and 6-aminohex-1-yne hydrochloride (**173**). This crude mixture was dissolved in water and the compounds separated and purified by pH gradient extraction (from pH 1 to 13 in increments of 0.5 pH units) into chloroform. The hydrochloride salts were formed by the addition of HCl in anhydrous diethyl ether (2.0 M) to each fraction, providing recovered **173** as its hydrochloride salt (77 mg, 0.58 mmol) and pure **181** as its trihydrochloride salt as a yellow solid (60 mg, 0.088 mmol) in 52% yield. M.p. 118-120 °C (dec.). Nitrogen NMR assignments were extracted from HSQC experiments between ^1H and ^{15}N .

δ_{H} (400 MHz, DMSO- d_6): 14.64 (s, 1H, OH-12), 12.19 (s, 1H, OH-10), 10.58 (s, 1H, OH), 10.30 (s, 1H, NH-9), 9.58 (s, 1H, NH₂-2), 9.34 (br. s, 2H, NH₂⁺-15), 9.07 (s, 1H, NH₂-2), 8.35 (s, 1H, CH-8), 7.55 (s, 1H,

	OH), 4.37 (app. s, 1H, CH-4), 4.04 (s, 2H, CH ₂ -14), 3.55 (m, 1H, CH ₂ -H-6''), 3.05 (m, 1H, CH-4a) 2.99 (m, 3H, CH-5a, CH ₂ -16), 2.88 (m, 12H, 4 x CH ₃ -NMe ₂ -4, NMe ₂ -7), 2.83 (t, 1H, J 2.6, CH-21), 2.37 (m, 1H, CH ₂ -H-6'), 2.27 (m, 1H, CH ₂ -H-5''), 2.22 (dt, 2H, J 6.9, 2.6, CH ₂ -19), 1.77 (m, 2H, CH ₂ -17), 1.52 (m, 3H, CH ₂ -H-5', CH ₂ -18)
δ_C (125 MHz, DMSO-d ₆):	194.0 (q), 192.8 (q), 187.6 (q), 175.7 (q), 173.2 (q), 172.2 (q), 165.1 (q), 132.4 (q), 125.5 (q), 120.7 (CH-8), 116.3 (q), 108.2 (q), 95.6 (q), 79.5 (q), 74.8 (q), 74.3 (q), 71.9 (CH-21), 68.0 (CH-4), 48.7 (CH ₂ -14), 46.9 (CH ₂ -16), 34.7 (CH-4a), 34.2 (CH-5a), 45.5 (2 x CH ₃ , NMe ₂ -7), 41.8 (CH ₃ , NMe ₂ -4), 41.2 (CH ₃ , NMe ₂ -4), 34.4 (CH ₂ -5), 29.8 (CH ₂ -6), 25.5 (CH ₂ -18), 25.1 (CH ₂ -17), 17.8 (CH ₂ -19)
δ_N (600 MHz, DMSO-d ₆):	124.0 (NH, amide-9), 109.4 (NH, amide-2) , 38.7 (NH ₃ ⁺ -15)
HRMS (<i>m/z</i> ESI+):	Found: 610.2860 (M + H. C ₃₁ H ₄₀ N ₅ O ₈ Requires: 610.2877)
ν/cm^{-1} (neat):	3356 (O-H phenolic/C-H alkyne stretch), 2113 (terminal alkyne C $\equiv$ C stretch), 1617 (C=O amide/ketone stretch), 1594, 1542, 1466, 1363, 1266, 1208, 1183, 1128, 1103, 1054, 1021, 980, 944, 916, 894, 805

4.2.13 9-Iodominocycline dihydrochloride (**186**)



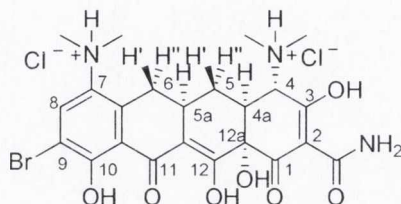
A round bottom flask was charged with minocycline hydrochloride (**3**) (250 mg, 0.506 mmol) and methanesulfonic acid (1.7 mL). NIS (228 mg, 1.28 mmol) was added in 3 aliquots over a 2 hour period and the reaction stirred for 3.5 h. The reaction was quenched by the addition of ice-cold water (16.7 mL) containing sodium thiosulfate (152 mg, 0.61 mmol), this was allowed to stir with the reaction mixture for 30 mins. The aqueous reaction mixture was extracted with ethyl acetate and the aqueous layer poured onto a saturated sodium hydrogen carbonate solution (8.3 mL) containing 1-butanol (2.5 mL). The aqueous layer was then extracted with 1-butanol (2 x 10 mL) and the organic layers combined, washed once with brine and the solvents evaporated at reduced pressure. The resulting solid was suspended in methanol and gaseous HCl bubbled through the suspension until the solid dissolved. The methanol was evaporated at reduced pressure and the solid product triturated with methyl-*t*-butylether. The yellow/brown solid product **186** (266 mg, 0.405 mmol) was collected by filtration under Ar and washed with diethyl ether and hexane to furnish **186** in 80% yield. M.p. 186-188 °C (dec.). ¹H NMR spectroscopic data agree with the referenced literature.²⁵⁹

δ_{H} (400 MHz, CD₃OD):

8.44 (s, 1H, CH-8), 4.22 (s, 1H, CH-4), 3.43-3.38 (dd, 1H, J 15.5, 4.0, CH₂-H-6''), 3.23 (m, 1H, CH-5a), 3.10 (s, 3H, CH₃-NMe₂-7), 3.05 (ap. s, 1H, CH-4a), 3.01 (s, 3H, CH₃-NMe₂-7), 2.72 (s, 6H, 2 x CH₃-NMe₂-4), 2.57-2.50 (m, 1H, CH₂-H-6'), 2.40-2.37 (app. d, 1H, CH₂-H-5'') 1.74-1.65 (m, 1H, CH₂-H-5')

HRMS (*m/z* ESI+):

Found: 584.0916 (M + H. C₂₃H₂₇N₃O₇I)

4.2.14 9-Bromominocycline dihydrochloride (**187**)

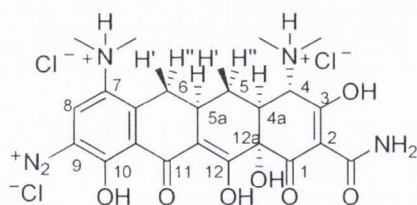
A 25 mL round bottom flask was charged with minocycline hydrochloride (**3**) (200 mg, 0.405 mmol) and methanesulfonic acid (1.3 mL). NBS (228 mg, 1.28 mmol) was added in 3 aliquots over a 2 hour period and the reaction stirred for 48 h. The reaction was quenched by the addition of ice-cold water (13.3 mL) containing sodium thiosulfate (119 mg, 0.48 mmol), this was allowed to stir with the reaction mixture for 30 mins. The aqueous reaction mixture was extracted with ethyl acetate and the aqueous layer poured onto a saturated sodium hydrogen carbonate solution (6.7 mL) containing 1-butanol (2.0 mL). The aqueous layer was then extracted with 1-butanol (2 x 10 mL) and the organic layers combined, washed once with brine and the solvents evaporated at reduced pressure. The resulting solid was suspended in methanol and gaseous HCl bubbled through the suspension until the solid dissolved. The methanol was evaporated at reduced pressure and the solid product triturated with methyl-*t*-butylether. The yellow/brown solid product **187** (192 mg, 0.316 mmol) was collected by filtration under Ar and washed with diethyl ether and hexane to furnish **187** in 78% yield. M.p. 172-176 °C (dec.)

δ_{H} (400 MHz, CD_3OD):

8.33 (s, 1H, CH-8), 4.23 (s, 1H, CH-4), 3.44-3.40 (ap. dd, 1H, J 15.6, 2.5, $\text{CH}_2\text{-H-6''}$), 3.25 (m, 1H, CH_2 , H-5a), 3.11 (s, 3H, $\text{CH}_3\text{-7-NMe}_2$), 3.05 (s, 1 H, CH-4a), 3.01 (s, 3H, $\text{CH}_3\text{-7-NMe}_2$), 2.72 (s, 6H, 2 x CH_3 , 4-NMe₂), 2.56 (m, 2H, CH_2 , H-6', CH_2 , H-5''), 1.74-1.60 (m, 1H, CH_2 , H-5')

δ_C (100 MHz, CD ₃ OD):	194.1 (q), 192.2 (q), 187.7 (q), 175.9 (q), 173.3 (q), 172.3 (q), 156.8 (q), 136.8 (q), 132.04 (CH-8), 117.3 (q), 109.5 (q), 108.6 (q), 95.7 (q), 74.5 (q), 68.2 (CH-4), 45.8 (2 x CH ₃ , NMe ₂ -7), 42.0 (CH ₃ , NMe ₂ -4), 41.3 (CH ₃ , NMe ₂ -4), 34.9 (CH-4a), 34.4 (CH-5a), 30.7 (CH ₂ -5), 23.3 (CH ₂ -6)
HRMS (<i>m/z</i> ESI+):	Found: 536.1006 (M + H C ₂₃ H ₂₇ N ₃ O ₇ Br) Requires: 503.1032)
ν/cm^{-1} :	3667 (O-H phenolic stretch), 3185 (N-H amide stretch), 3058 (N-H amide/C-H aromatic stretch), 1771 (C=O ketone stretch), 1702 (C=O ketone stretch), 1611, 1580, 1429, 1357, 1294, 1248, 1177, 1043, 987, 920, 851, 811, 778, 729

4.2.15 9-Diazaminocycline chloride dihydrochloride (**189**)



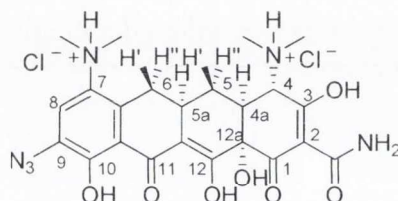
Compound **87** (200 mg, 0.335 mmol) was dissolved in 0.1 M HCl in methanol (7 mL, 0.7 mmol) with stirring and cooled to -10 °C. Isoamyl nitrite (280 μ L, 244 mg, 2.07 mmol) was added dropwise and the reaction stirred for 1 h. To the reaction was added dropwise diethyl ether and the resulting precipitate was collected by filtration under Ar to afford compound **189** (155 mg, 0.261 mmol) in 78% yield. Due to the instability of the diazo moiety of the compound, only ¹H NMR spectroscopy (solvent below 0 °C) and IR spectroscopy could be employed for characterisation.

δ_H (600 MHz, CD ₃ OD):	8.39 (s, 1H, CH-8), 4.15 (app. s, 1H, CH-4), 3.39 (m, 1H, CH ₂ -H-6''), 3.25 (m, 1H, CH-5a), 3.10 220
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(s, 6H, 2 x CH₃-NMe₂-7), 3.08 (s, 3H, CH₃-NMe₂-4), 3.00 (m, 4H, CH₃-NMe₂-4, CH-4a), 2.53 (app. t, 1H, CH₂-H-6'), 2.33 (m, 1H, CH₂-H-5''), 1.65 (app. q, 1H, CH₂-H-5')

ν/cm^{-1} : 3354 (O-H phenolic stretch), 3067 (C-H aromatic stretch), 2185 (N $\equiv$ N stretch), 1664 (C=O amide stretch), 1635, 1522, 1471, 1411, 1358, 1298, 1177, 1147, 938, 858, 671

4.2.16 9-Azidominocycline dihydrochloride (**190**)

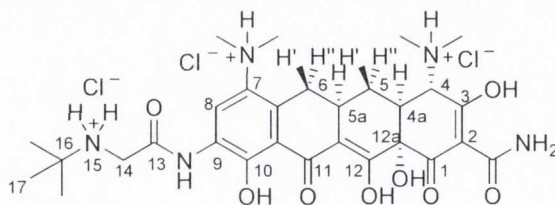


Compound **189** (155 mg, 0.261 mmol) was added to a round bottom flask and dissolved in HBF₄ in methanol (0.1 M, 3.1 mL, 0.31 mmol) with stirring at 0 °C. Sodium azide (20 mg, 0.313 mmol) was added to this solution and the reaction heated at 25 °C for 2.5 h. The reaction was added dropwise to diethyl ether and the resulting precipitate collected by filtration under Ar to furnish the crude product (590 mg) in 96% yield. The crude product was purified by MPLC on a 12 g KP-C18-HS column (mobile phase: acetonitrile/Millipore water (gradient 10%-50%)) in 100 mg portions to provide **190**. The dihydrochloride salt was formed by the addition of 8% HCl (aq) to afford **190** (37 mg, 0.065 mmol) in 25% yield. M.p. 139 °C (dec.)

δ_{H} (600 MHz, CD₃OD): 7.61 (s, 1H, CH-8), 4.18 (app. s, 1H, CH-4), 3.39 (m, 1H, CH₂-H-6''), 3.30 (s, 6H, 2 x CH₃-NMe₂-

	7), 3.18 (m, 1H, CH-5a), 3.05 (m, 7H, NMe ₂ -4, CH-4a), 2.53 (app. t, 1H, CH ₂ -H-6'), 2.37 (app. d, 1H, CH ₂ -H-5''), 1.69 (app. q, 1H, CH ₂ -H-5')
δ_C (125 MHz, CD ₃ OD):	193.1 (q), 191.9 (q), 186.8 (q), 174.7 (q), 173.3 (q), 156.7 (q), 131.5 (q), 130.0 (q), 129.1 (q), 118.7 (q), 117.1 (CH-8), 108.0 (q), 95.1 (q), 74.6 (q), 69.7 (CH-4), 46.5 (2 x CH ₃ -NMe ₂ -7), 42.2 (CH ₃ -NMe ₂ -4), 41.0 (CH ₃ -NMe ₂ -4), 34.7 (CH-4a), 33.8 (CH ₂ -5), 32.1 (CH-5a), 29.7 (CH ₂ -6)
HRMS (<i>m/z</i> ESI+):	Found: 497.1789 (M - H C ₂₃ H ₂₅ N ₆ O ₇ Requires: 497.1785)
ν/cm^{-1} :	3306 (O-H phenolic stretch), 3024 (N-H amide/C-H aromatic stretch), 2119 (N ₃ -stretch), 1663 (C=O amide/ketone stretch), 1610, 1587, 1553, 1454, 1355, 1334, 1286, 1221, 1180, 1126, 1068, 1019, 990, 939, 892, 844, 822, 805, 788, 761

4.2.17 Tigecycline trihydrochloride (19)



Compound **172** (210 mg, 0.36 mmol) and sodium iodide (21 mg, 0.14 mmol) were added to a 5 mL round bottom flask and placed under an atmosphere of Ar. *t*-Butylamine (1.13 mL, 10.75 mmol) was injected *via* syringe and the reaction heated to 25 °C and stirred for 18 h.

Methanol (1 mL) was added followed by removal of the solvent by evaporation at reduced pressure. The residue was dissolved in Millipore water (1.26 mL) and the pH adjusted to 7.2 using 32% HCl_(aq). The solution was transferred to a separating funnel and MeOH (3 mL) and CH₂Cl₂ (8 mL) were added. The organic layer was separated and the product extracted from the aqueous layer with CH₂Cl₂ (3 x mL). The organic extracts were combined, HCl in anhydrous diethyl ether added (2.0 M, 1.0 mL, 2.0 mmol) and the solvent removed by evaporation at reduced pressure to furnish the desired product **19** (146 mg, 0.21 mmol) as a brown solid in 59% yield. M.p. 228-230 °C (dec.) (Patent: 232 °C)²⁶⁰

δ_H (400 MHz, DMSO-d₆): 14.62 (br. s, 1H, OH-12), 12.26 (s, 1H, OH-10), 10.69 (s, 1H, OH), 10.30 (s, 1H, NH-9), 9.60 (s, 1H, NH₂-2), 9.26 (br. s, 2H, NH₂⁺-15), 9.06 (s, 1H, NH₂-2), 8.41 (s, 1H, CH-8), 7.53 (br. s, 1H, OH), 4.47 (app. s, 1H, CH-4), 4.04 (app. s, 2H, CH₂-14), 3.60 (m, 1H, CH₂-H-6''), 3.07 (m, 1H, CH-5a), 2.89 (m, 13H, CH-4a, 4 x CH₃-NMe₂-4, NMe₂-7), 2.36 (m, 1H, CH₂-H-6'), 2.27 (m, 1H, CH₂-H-5''), 1.45 (m, 1H, CH₂-H-5'), 1.32 (s, 9H, 3 x CH₃-17)

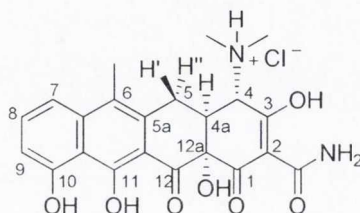
δ_C (125 MHz, CD₃OD): 193.7 (q), 192.3 (q), 187.5 (q), 175.7 (q), 172.2 (q), 165.6 (q), 160.6 (q), 151.5 (q), 132.0 (q), 125.6 (q), 120.8 (CH-8), 115.9 (q), 108.1 (q), 95.3 (q), 74.0 (q), 67.9 (CH-4), 56.5 (q, C-16), 43.1 (CH₂-14), 45.5 (2 x CH₃-NMe₂-7), 41.8 (CH₃-NMe₂-4), 40.7 (CH₃-NMe₂-4), 35.0 (CH₂-5), 34.7 (CH-5a), 31.5 (CH-4a), 29.6 (CH₂-6), 25.1 (3 x CH₃-17)

HRMS (*m/z* ESI⁺): Found: 586.2887 (M + H. C₂₉H₄₀N₅O₈ Requires: 586.2877)

ν/cm^{-1} : 3354 (O-H phenolic stretch), 2968, 1980, 1615 (C=O amide stretch), 1592 (N-H bend), 1536, 1437, 1367, 1279, 1201, 1126, 1054, 1020, 993,

4.3 Chapter 3

4.3.1 Anhydrotetracycline hydrochloride (**4**)



Compound **106** (4.990 g, 11.23 mmol) was dissolved in 8% HCl_(aq) (29 mL) in a 250 mL round bottom flask and stirred at 60 °C for 1 h. Ice-cold water (90 mL) was added to the reaction vessel and the orange precipitate was collected by filtration. The precipitate was washed with water at pH 4.5 to give an orange solid. The crude product was purified by recrystallisation at 0 °C from a mixture of methanol and diethyl ether to give **4** as an orange/yellow crystalline solid (3.548 g, 7.67 mmol) in 68% yield. M.p. 224 °C (dec.) (Lit. 219-222 °C). ¹H NMR data agree with the referenced literature.²⁶¹

δ_{H} (400 MHz, CD₃OD): 7.60 (app. t, 1H, J 8.3, CH-8), 7.47 (d, 1H, J 8.0, CH-9), 6.89 (d, 1H, J 8.0, CH-7), 4.29 (d, 1H, J 4.0, CH-4), 3.64 (dd, 1H, J 17.6, 5.0, CH₂-H-5''), 3.44 (m, 1H, H-4a), 3.09 (s, 7H, 2 x CH₃-NMe₂, CH₂-H-5'), 2.44 (s, 3H, Me)

δ_{C} (150 MHz, CD₃OD): 199.1 (q), 192.4 (q), 186.4 (q), 173.1 (q), 163.4 (q), 158.2 (q), 139.1 (q), 132.7 (CH-8), 129.4 (q), 122.3 (q), 115.0 (CH-9), 112.0 (q), 110.9 (CH-7), 108.2 (q), 96.8 (q), 76.4 (q), 68.3 (CH-4), 42.5 (CH₃-NMe₂), 41.9 (CH₃-NMe₂), 36.0 (CH-4a), 28.6 (CH₂-5), 12.9 (CH₃)

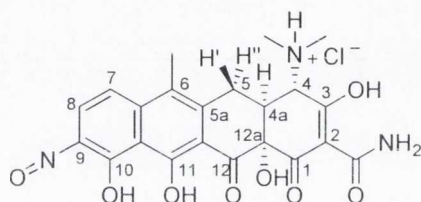
HRMS (*m/z* ESI+): Found: 427.1517 (M + H. C₂₂H₂₃N₂O₇ Requires: 224

427.1505)

ν/cm^{-1} (neat):

3344 (O-H phenolic stretch), 1628 (C=O amide stretch), 1543, 1392, 1323, 1239, 1133, 1072, 934, 805

4.3.2 9-Nitrosoanhydrotetracycline hydrochloride (**127**)



Compound **4** (500 mg, 1.08 mmol) and distilled water (18 mL) were added to a 50 mL round bottom flask containing a stirring bar. The pH of the reaction mixture was lowered to 1 using 32% HCl. The solution was cooled to 0 °C, NaNO₂ (153 mg, 2.22 mmol) added and the reaction stirred at 0 °C for 3 h. When the reaction had been determined to have reached completion (¹H NMR spectroscopy), brine was added until a precipitate was observed. This precipitate was then collected by filtration, and recrystallised at 0 °C from a mixture of methanol and diethyl ether to give **127** as a deep red solid (297 mg, 0.603 mmol) in 56% yield (of which 3% is *epi*-9-nitrosoanhydrotetracycline hydrochloride).²⁰⁴ M.p. 187 °C (dec.)

δ_{H} (400 MHz, DMSO-d₆):

14.43 (s, 1H, OH-12a), 10.41 (s, 1H, OH-10), 9.61 (s, 1H, NH₂), 9.31 (s, 1H, NH₂), 7.35 (d, 1H, J 10.5, CH-8), 7.21 (d, 1H, J 10.5, CH-7), 4.37 (app. s, 1H, CH-4), 3.34 (m, 3H, CH-4a, CH₂-H-5', CH₂-H-5''), 2.94 (s, 6H, NMe₂), 2.30 (s, 3H, CH₃)

δ_{C} (125 MHz, DMSO-d₆):

193.6 (q), 187.3 (q), 186.0 (q), 172.4 (q), 163.3 (q), 150.9 (q), 147.2 (q), 138.5 (q), 126.1 (q), 225

125.9 (CH-8), 119.3 (q), 117.7 (CH-7), 113.9 (q),
98.6 (q), 76.5 (q), 66.5 (q), 48.6 (CH-4), 43.5
(CH₃-NMe₂), 41.1 (CH₃-NMe₂), 36.1 (CH-4a),
29.8 (CH₂-5), 13.8 (CH₃)

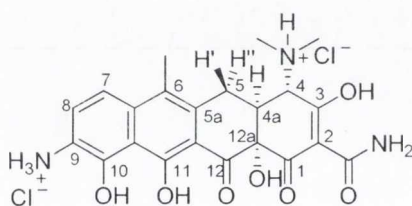
HRMS (*m/z* ESI+):

Found: 456.1427 (M + H. C₂₂H₂₂N₃O₈ Requires:
456.1407)

ν/cm^{-1} (neat):

3053 (O-H phenolic stretch), 1557 (N=O
stretch), 1395, 1033, 987, 917

4.3.3 9-Aminoanhydrotetracycline dihydrochloride (**128**)



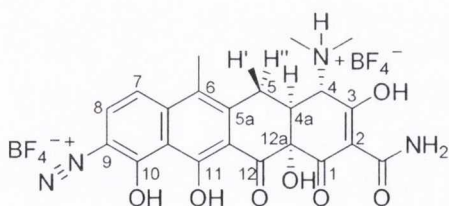
A Parr apparatus hydrogenation vessel was charged with **127** (150 mg, 0.30 mmol), methanol (30 mL), 32 % HCl (200 μL) and platinum (IV) oxide (15 mg, 0.06 mmol). This mixture was reacted under H₂ at 3 atm for 70 mins. The catalyst was removed by filtration through celite. The filtrate was evaporated to *ca.* 20 % of its original volume and added dropwise to diethyl ether at 0 °C. The resulting yellow precipitate was collected by filtration under Ar and washed several times with diethyl ether to give the amine product **128** as a dihydrochloride salt (144.7 mg, 94%). M.p. 246 °C (dec.)

δ_{H} (400 MHz, CD₃OD):

7.71 (d, 2H, J 9.0, CH-8), 7.66 (d, 2H, J 9.0, CH-7), 4.35 (d, 1H, J 2.6 CH-4), 3.72 (dd, 1H, J 17.5, 4.7, CH₂-H-5''), 3.51 (m, 1H, CH-4a), 3.11 (m, 7H, CH₂-H-5', NMe₂), 2.51 (s, 3H, CH₃)

δ_C (125 MHz, CD ₃ OD):	200.3 (q), 192.5 (q), 186.6 (q), 173.0 (q), 162.0 (q), 150.6 (q), 138.6 (q), 131.9 (q), 126.4 (CH-8), 123.1 (q), 116.1 (CH-7), 113.1 (q), 112.3 (q), 109.5 (q), 96.8 (q), 76.6 (q), 68.3 (CH-4), 42.8 (CH ₃ -NMe ₂), 41.6 (CH ₃ -NMe ₂), 35.8 (CH-4a), 29.3 (CH ₂ -5), 13.0 (CH ₃)
HRMS (<i>m/z</i> ESI+):	Found: 442.1609 (M + H. C ₂₂ H ₂₄ N ₃ O ₇ Requires: 442.1614)
ν/cm^{-1} (neat):	3356 (O-H phenolic stretch), 3048 (N-H amide stretch), 2950 (N-H amide/C-H aromatic stretch), 2950, 2624, 1638 (C=O amide stretch), 1597, 1459, 1356, 1176, 1045, 997, 941, 918, 849, 762, 736, 687, 659

4.3.4 [9-Diazoanhydrotetracycline hydrotetrafluoroborate]⁺ BF₄⁻ (194A)



General procedure A:

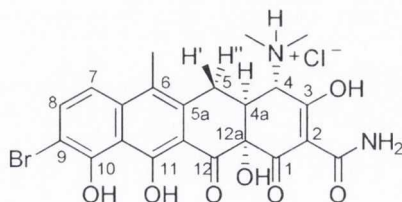
Compound **128** (242 mg, 0.10 mmol) was added to a 5 ml round bottom flask containing HBF₄ in methanol (0.1 M, 9 mL, 0.9 mmol) at -10 °C. To this, isoamyl nitrite (82 μ L, 0.13 mmol) was added and the reaction was stirred at -10 °C for 15 mins. The reaction mixture was

then poured into a conical flask containing diethyl ether (50 mL) at 0 °C. The resulting precipitate was collected by filtration under Ar to afford the desired product, **194A**, as a brown solid (198 mg, 0.067 mmol) in 67% yield. Due to the unstable nature of the diazo moiety of the compound, only ^1H NMR (solvent below 0 °C, some decomposition of diazo in solution) and IR spectroscopy could be employed for characterisation. Since the compound was not stable in solution, the assignment of the ^1H NMR spectrum is based on that of compound **128**. **194A** was used without further purification.

δ_{H} (400 MHz, CD_3OD): 8.06 (d, 1H, J 9.2, CH-8), 6.55 (d, 1H, J 9.2, CH-7), 4.20 (app. s, 1H, CH-4), 3.19 (m, 9H, $\text{CH}_2\text{-H-5'}$, $\text{CH}_2\text{-H-5''}$, CH-4a, 2 x $\text{CH}_3\text{-NMe}_2$), 2.26 (s, 3H, CH_3)

ν/cm^{-1} (neat): 3342 (OH-phenolic stretch), 2136 ($\text{N}\equiv\text{N}$ stretch), 1558, 1419, 1180, 1000, 958, 899, 811, 666

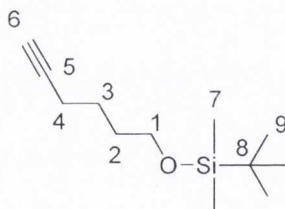
4.3.5 9-Bromoanhydrotetracycline chloride (**195**)



Compound **4** (250 mg, 0.540 mmol) was dissolved in 97 % H_2SO_4 (7.5 mL) at 0 °C in a 25 mL round bottom flask containing a stirring bar. To this was added NBS (200 mg, 1.11 mmol) and the reaction stirred at 0 °C for 2.5 h. The reaction was added dropwise to ice-cold diethyl ether (200 mL) to facilitate precipitation of the product which was then collected by filtration under Ar. The crude product was purified by MPLC on a 12g KP-C18-HS column (mobile phase: 20% acetonitrile/0.1 M $\text{HCl}_{(\text{aq})}$) to yield **195** as a yellow solid (8 mg, 0.015 mmol) in 3% yield. M.p. 184 °C (dec).

δ_{H} (400 MHz, CD_3OD):	7.75 (d, 1H, J 8.0, CH-8), 7.35 (d, 1H, J 8.0, CH-7), 4.28 (app. s, 1H, CH-4), 3.63 (m, 1H, CH_2 , H-5''), 3.50 (m, 1H, CH-4a), 3.10 (app. br. s, 7H, NMe_2 , CH_2 , H-5'), 2.42 (s, 3H, CH_3)
δ_{C} (125 MHz, CD_3OD):	199.7 (q), 192.1 (q), 186.2 (q), 172.8 (q), 161.4 (q), 153.4 (q), 137.5 (q), 135.1 (CH-8), 130.1 (q), 122.3 (q), 115.8 (CH-7), 112.0 (q), 108.7 (q), 104.0 (q), 96.5 (q), 76.1 (q), 68.1 (CH-4), 42.7 ($\text{CH}_3\text{-NMe}_2$), 41.2 ($\text{CH}_3\text{-NMe}_2$), 35.3 (CH-4a), 28.8 ($\text{CH}_2\text{-5}$), 12.6 (CH_3)
HRMS (m/z ESI+):	Found: 505.0602 (M + H $\text{C}_{22}\text{H}_{22}\text{BrN}_2\text{O}_7$) Requires: 505.0610
ν/cm^{-1} (neat):	3324 (O-H phenolic stretch), 1981, 1617, 1460, 1396, 1317, 1227, 1057, 992, 940, 812, 670

4.3.6 *tert*-Butyl(hex-5-ynyloxy)dimethylsilane (202)

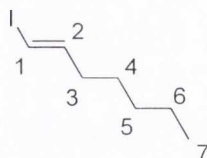


Compound **178** (1.31 mL, 1.17 g, 11.9 mmol), imidazole (2.07 g, 30.40 mmol) and *t*-butyldimethylsilyl chloride (2.20 g, 14.59 mmol) were added to a round bottom flask which was subsequently evacuated and charged with an atmosphere of Ar. Anhydrous *N,N*-dimethylformamide (50 mL) was added *via* syringe and the reaction heated to 25 °C and stirred for 18 h. The reaction was diluted with Millipore water (50 mL) and extracted with diethyl ether (3 x 50 mL). The organic layer was dried over magnesium sulfate, filtered and

the solvent removed at reduced pressure to provide the crude product, which was subsequently purified by column chromatography (mobile phase: 5% ethyl acetate/hexane) to furnish the desired product, **202**, (1.48 g, 6.97 mmol) in 57 % yield. ^1H NMR spectroscopic data agree with the referenced literature.²⁶²

δ_{H} (600 MHz, $\text{CDCl}_3\text{-d}_6$):	3.66 (t, 2H, J 6.1, $\text{CH}_2\text{-1}$), 2.24 (dt, 2H, J 2.6, 7.0 $\text{CH}_2\text{-4}$), 1.97 (t, 1H, J 2.6, CH-6), 1.63 (m, 4H, $\text{CH}_2\text{-2}$, $\text{CH}_2\text{-3}$), 0.92 (s, 9H, 3 x $\text{CH}_3\text{-9}$), 0.08 (s, 6H, 2 x $\text{CH}_3\text{-7}$)
δ_{C} (150 MHz, $\text{CDCl}_3\text{-d}_6$):	84.5 (q, C-5), 68.2 (CH-6), 62.6 ($\text{CH}_2\text{-1}$), 31.8 ($\text{CH}_2\text{-2}$), 25.9 (3 x $\text{CH}_3\text{-9}$), 25.0 ($\text{CH}_2\text{-3}$), 18.3 (q, C-8), 18.2 ($\text{CH}_2\text{-4}$), -5.3 (2 x $\text{CH}_3\text{-7}$)
HRMS (m/z ESI+):	Found: 213.1765 (M + H $\text{C}_{12}\text{H}_{25}\text{OSi}$ Requires: 213.1767)

4.3.7 *E*-(1-iodo)Hept-1-ene (208)

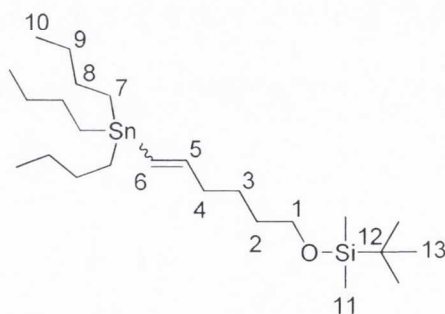


Heptyne (250 μL , 184 mg, 1.91 mmol) and anhydrous hexane (500 μL) were added *via* syringe to a round bottom flask under an atmosphere of Ar. The reaction was cooled to 0 $^{\circ}\text{C}$ and 1.0 M diisobutylaluminium hydride in toluene (1.90 mL, 1.9 mmol) was added *via* syringe. The reaction was heated to 50 $^{\circ}\text{C}$ for 2 h. The solvent was removed *in vacuo* (≥ 0.1 mbar) and anhydrous THF (1 mL) added to the vinylalane. The solution was cooled to -40 $^{\circ}\text{C}$ and a solution of iodine crystals (484 mg, 1.91 mmol) in anhydrous THF added *via* syringe. The reaction was allowed to warm to ambient temperature after the addition of iodine was complete. The diisobutylalane $[\text{Al}(\text{C}_4\text{H}_9)_2]$ was decomposed by the addition of 20% H_2SO_4 until the evolution of isobutane had ceased. The reaction was poured onto cold 20% H_2SO_4 (2

mL) and extracted with hexane (3 x 5 mL). The organic extracts were combined and washed with sodium thiosulfate solution (10% w/v, 15 mL), saturated sodium hydrogen carbonate (15 mL) and brine (15 mL). The organic extract was dried over magnesium sulfate, filtered and the solvent removed at reduced pressure to furnish the crude product which was purified by column chromatography (mobile phase: 15% ethyl acetate/hexane). The desired product **208** was obtained as a clear oil (215 mg, 0.96 mmol) in 50% yield after purification. The *E* geometry was confirmed by NOE experiments. NMR spectroscopic data agrees with the referenced literature.²⁶³

δ_{H} (600 MHz, DMSO- <i>d</i> ₆):	6.55 (m, 1H, J 7.2, 14.3, CH-1), 6.00 (dt, 1H, J 1.4, J 14.3, CH-2), 2.08 (app. dq, 2H, J 1.4, J 7.2, CH ₂ -3), 1.42 (m, 2H, CH ₂ -4), 1.32 (m, 4H, CH ₂ -5, CH ₂ -6), 0.92 (t, 3H, J 7.1, CH ₃ -7)
δ_{C} (150 MHz, CDCl ₃ - <i>d</i> ₆):	147.1 (CH-1), 74.3 (CH-2), 36.0 (CH ₂ -3), 31.1 (CH ₂ -5), 28.0 (CH ₂ -4), 22.4 (CH ₂ -6), 14.0 (CH ₃ -7)
HRMS (<i>m/z</i> EI+):	Found: 224.0067 (M ⁺ C ₇ H ₁₃ I Requires: 224.0062)

4.3.8 *tert*-Butyldimethyl[6-(tributylstannyl)hex-5-enyloxy]silane (**204**)



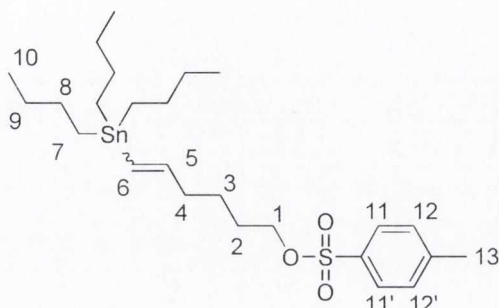
Compound **202** (265 mg, 1.25 mmol) and AIBN (21 mg, 0.125 mmol) were added to a round bottom flask and placed under an atmosphere of Ar. Tri-*n*-butyltin hydride (435 μ L, 471 mg,

1.62 mmol) was added *via* syringe and the reaction heated to 80 °C and stirred for 2 h. The reaction was diluted with CH₂Cl₂ (20 mL) and washed with Millipore water (20 mL). The aqueous layer was washed with diethyl ether (2 x 20 mL) and the organic extracts combined and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent evaporated at reduced pressure to furnish the crude product, **204** (736 mg), in 87% purity (13% unreacted tri-*n*-butyltin hydride remained). **204** was obtained as a 4:1 mixture of E/Z isomers (as confirmed by NOE experiments). **204** Was unstable in CDCl₃-d₆ and DMSO-d₆ over the time required to perform ¹³C NMR spectroscopy and unstable under the HRMS conditions available. ¹H NMR spectroscopic data agree with the referenced literature.²⁶⁴

δ_{H} (600 MHz, CDCl₃): 6.53 (dt, 0.2H, J 7.0, 12.4, Z-CH-5), 5.97 (dt, 0.8H, J 5.5, 18.9, E-CH-5), 5.89 (app. d, 0.8H, J 18.9, Z-CH-6), 5.81 (dt, 0.2H, J 1.0, 12.4, Z-CH-6), 3.63 (t, 2H, J 6.5, CH₂-1), 2.17 (m, 1.6H, E-CH₂-4), 2.06 (m, 0.4H, Z-CH₂-4), 1.65 (m, 2H, CH₂-2), 1.52 (m, 8H, CH₂-3, 3 x CH₂-8), 1.35 (m, 6H, 3 x CH₂-9), 0.95 (m, 24H, 3x CH₃-10, 3 x CH₂-7, 3 x CH₃-13), 0.08 (s, 6H, 2 x CH₃-11)

ν/cm^{-1} (neat): 2943 (C-H alkyl stretch/C-H alkenyl stretch), 2932 (C-H alkyl stretch/C-H alkenyl stretch), 2856 (C-H alkyl stretch), 1491, 1372, 1267, 1251, 1134, 962, 918, 860, 739, 638

4.3.9 6-(tributylstannyl)Hex-5-enyl *p*-methylbenzenesulfonate (**206**)



Compound **179** (760 mg, 3.01 mmol), AIBN (49 mg, 0.30 mmol) and tri-*n*-butyltin hydride (810 μ L, 876 mg, 3.01 mmol) were added to a microwave reaction vessel, the vessel sealed and heated to 100 $^{\circ}$ C for 15 mins. After this time the reaction was diluted with diethyl ether (3 mL) and washed with Millipore water (2 x 3 mL) and brine (2 x 3 mL). The organic layer was dried over magnesium sulfate, filtered and the solvent evaporated at reduced pressure. The crude product was purified by MPLC on a 12 g KP-C18-HS column (mobile phase: 50 % acetonitrile/methanol) to furnish the pure product, **206**, as a 4:1 mixture of *E/Z* isomers (695 mg, 1.38 mmol) in 46 % yield.

δ_{H} (600 MHz, DMSO- d_6):

7.78 (d, 2H, J 8.4, H-11, H-11'), 7.48 (d, 2H, J 8.4, H-12, H-12'), 6.40 (dt, 0.2H, J 7.0, 12.4, Z-CH-5), 5.83 (m, 1.6H, *E*-CH-5, *E*-CH-6), 5.76 (app. d, 0.2H, J 12.4, Z-CH-6) 4.0 (t, 2H, J 6.3, CH₂-1), 2.43 (s, 3H, CH₃-13), 2.02 (app. dd, 1.6H, J 7.0, 15.0 *E*-CH₂-4), 1.93 (app. dd, 0.4H, J 7.0, 15.0 Z-CH₂-4), 1.55 (m, 2H, CH₂-2), 1.45 (m, 2H, CH₂-8), 1.28 (m, 4H, CH₂-3, CH₂-9), 0.85 (m, 6H, CH₃-10, CH₂-7)

δ_{C} (150 MHz, DMSO- d_6):

149.1 (*E*-CH-5), 149.0 (Z-CH-5), 145.3 (q), 130.7 (CH-12, CH-12'), 133.0 (q), 128.5 (Z-CH-

6), 128.1 (CH-11, CH-11') 128.0 (Z-CH-6), 71.2 (CH₂-1), 36.9 (Z-CH₂-4), 36.3 (Z-CH₂-4), 29.0 (CH₂-8), 28.1 (CH₂-2), 27.1 (CH₂-9), 24.4 (CH₂-3), 21.6 (CH₃-13), 14.0 (CH₃-10), 9.4 (CH₂-7)

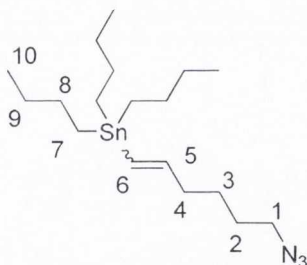
HRMS (*m/z* ESI+):

Found: 567.1966 (M + Na. C₂₅H₄₄NaO₃SnS
Requires: 567.1931)

ν/cm^{-1} (neat):

2955 (C-H alkyl stretch/C-H alkenyl stretch),
2925 (C-H alkyl stretch/C-H alkenyl stretch),
2871 (C-H alkyl stretch), 2853 (C-H alkyl stretch),
1599 (C=C aromatic bend), 1496, 1464, 1364, 1307, 1291, 1189, 1178, 1098, 1072, 1044, 1020, 989, 959, 935, 865, 814 (C-H aromatic bend), 783, 735, 689, 663 (C-H aromatic bend)

4.3.10 6-(tributylstannyl)azidohex-5-ene (188)



Compound **206** (357 mg, 0.646 mmol) and sodium azide (213 mg, 1.29 mmol) were added to a round bottom flask containing *N,N*-dimethylformamide (6.5 mL). The reaction was stirred for 18 h and subsequently diluted with diethyl ether (6.5 mL) and washed with Millipore water (5 x 10 mL) and brine (10 mL). The organic layer was dried over magnesium sulfate, filtered and evaporated at reduced pressure to provide the desired product, **188**, as a 4:1

mixture of *E/Z* isomers (228 mg, 0.549 mmol) in 85 % yield. The compound required no further purification.

δ_{H} (600 MHz, CDCl_3 -d6): 6.51 (dt, 0.2H, *J* 7.0, 12.4, 1H, *Z*-CH-5), 5.94 (m, 1.8H, *E*-CH-5, *E*-CH-6), 5.86 (dt, 0.2H, *J* 1.0, 12.4, *Z*-CH-6) 3.30 (t, 2H, *J* 6.9, CH_2 -1), 2.20 (m, 1.6H, *E*- CH_2 -4), 2.09 (app. ddd, 0.4H, *J* 1.0, 7.0, 14.5, *Z*- CH_2 -4), 1.64 (m, 2H, CH_2 -2), 1.52 (m, 8H, CH_2 -3, 3 x CH_2 -8), 1.34(m, 6H, 3 x CH_2 -9), 0.92 (m, 15H, 3x CH_3 -10, 3 x CH_2 -7)

δ_{C} (150 MHz, CDCl_3 -d6): 148.6 (*E*-CH-5), 148.2 (*Z*-CH-5), 128.3 (*Z*-CH-6), 128.2 (*E*-CH-6), 51.4 (CH_2 -1), 37.2 (*E*- CH_2 -4), 36.4 (*Z*- CH_2 -4), 29.1 (CH_2 -3), 27.9 (CH_2 -2), 27.3 (CH_2 -8), 17.5 (CH_2 -9), 13.7 (CH_3 -10), 9.4 (CH_2 -7)

HRMS (*m/z* ESI+): Found: 414.1916 (*M* + *H* $\text{C}_{18}\text{H}_{38}\text{N}_3\text{Sn}$ Requires: 414.1931)

ν/cm^{-1} (neat): 2955 (C-H alkyl stretch/C-H alkenyl stretch), 2926 (C-H alkyl stretch/C-H alkenyl stretch), 2871 (C-H alkyl stretch), 2855 (C-H alkyl stretch), 2093 (N_3 stretch), 2035, 1647, 1496, 1455, 1439, 1415, 1388, 1253, 1180, 1150, 1100, 1063, 989, 960, 864, 660

4.3.11 Diazobenzene tetrafluoroborate (**214**)



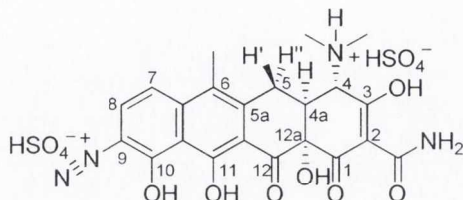
Compound **215** (500 mg, 3.86 mmol) was dissolved in HBF_4 (1 M, 50 mL, 50 mmol) and cooled to 0 °C. NaNO_2 (270 mg, 3.91 mmol) was added and the reaction stirred at 0 °C for 1 h. The reaction was added dropwise to 7:10 isopropanol/diethyl ether and the product precipitated as a white solid. **214** Was collected by filtration (351 mg, 1.83 mmol) in 47% yield. M.p. 83-86 °C. ^1H NMR data agree with the referenced literature.²⁶⁵

δ_{H} (400 MHz, DMSO-d_6): 8.67 (d, 2H, J 7.8, 2 x CH-2), 8.26 (t, 1H, J 7.3, CH-4), 7.99 (app. t, 2H, J 7.8, 2 x CH-3)

HRMS (m/z ESI+): Found: 105.0457 (M^+ $\text{C}_6\text{H}_5\text{N}_2$ Requires: 105.0453)

ν/cm^{-1} (neat): 3110 (aromatic C-H stretch), 3017 (aromatic C-H stretch), 2296 (N_2^+ stretch), 1815, 1570, 1462, 1312, 1291, 1185, 1172, 1024, 990, 944, 756, 665

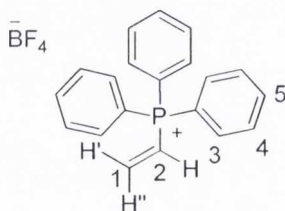
4.3.12 [9-Diazoanhydrotetracycline hydrosulfate]⁺ HSO₄⁻ (**194B**)



General procedure A was followed using compound **128** (50 mg, 0.097 mmol), NaNO₂ (10 mg, 0.146 mmol) and 5 M H₂SO₄ (750 μL, 3.75 mmol) to afford compound **194B** (48 mg, 0.074 mmol) in 76% yield.

δ_H (400 MHz, CD₃OD): 7.69 (app. s, 1H, CH-8), 6.54 (app. s, 1H, CH-7), 4.22 (app. s, 1H, CH-4), 3.18 (m, 9H, CH₂-H-5', CH₂-H-5'', CH-4a, 2 x CH₃-NMe₂), 2.31 (s, 3H, CH₃)

4.3.13 Triphenyl(vinyl)phosphonium tetrafluoroborate (**211**)

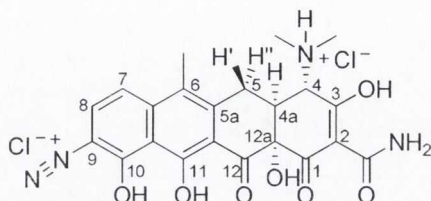


A round bottom flask was charged with **128** (101 mg, 0.196 mmol) and HBF₄ in methanol (1 M, 1.6 mL, 1.6 mmol), stirred and cooled to -15 °C. NaNO₂ (47 mg, 0.68 mmol) was added and the reaction stirred between -15 °C and -10 °C for 15 min. The reaction solvent was evaporated at reduced pressure and the resulting solid triturated with diethyl ether (3 x 10 mL). The crude product (**196A**) was dried *in vacuo* and placed under an atmosphere of Ar. To

this was added anhydrous, degassed acetonitrile (8 mL). The solution was injected *via* syringe to a reaction vessel under an atmosphere of Ar containing Pd(PPh₃)₄ (45 mg, 0.039 mmol) and anhydrous, degassed acetonitrile (7 mL). Tri-*n*-butylvinyltin (57 µL, 62 mg, 0.195 mmol) was injected *via* syringe to the reaction vessel and anhydrous, degassed acetonitrile (1 mL) added to afford the full transfer of reagents to the reaction vessel. The reaction was stirred at ambient temperature for 17 h and 2,5-diphenylfuran (21.6 mg, 0.0980 mmol) added as an internal standard to determine % yield by ¹H NMR spectroscopy. **211** Was determined to be present in 20% yield. The reaction solvent was evaporated at reduced pressure and the crude product split in two portions. 50% of the crude was purified by MPLC on a 12g KP-C18-HS column (mobile phase: acetonitrile/Millipore water (gradient 10%-90%)), which afforded the pure product **211**. 50% of the crude was partitioned between Millipore water (pH 3, 20 mL) and CH₂Cl₂ (20 mL) and diethyl ether (3 x 20 mL). **211** was found present in both aqueous and organic extracts. No isolated yield was obtained. ¹H NMR data agree with the referenced literature.²⁴⁷

δ _H (400 MHz, DMSO-d ₆):	6.19 (app. dd, 1H, J 18.0, J 25.4, CH ₂ , CH-1'), 7.10 (app. dd, 1H, J 12.6, 50.0, CH ₂ , CH-1''), 7.56 (m, 1H, CH-2), 7.77 (m, 12H, 3 x CH-3, 3 x CH-4), 7.95 (t, 3H, J 7.3, 3 x CH-5)
δ _P (162 MHz, DMSO-d ₆):	19.1
HRMS (<i>m/z</i> ESI+):	Found: 289.1140 (M ⁺ C ₂₀ H ₁₈ P Requires: 289.1146)

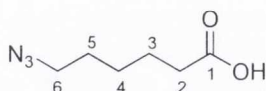
4.3.14 [9-Diazoanhydrotetracycline hydrochloride]⁺ Cl⁻ (**194C**)



General procedure A was followed using compound **128** (100 mg, 0.194 mmol), NaNO₂ (14.8 mg, 0.214 mmol) and 5 M HCl (1.5 mL) to afford compound **194C** (73 mg, 0.161 mmol) in 83% yield.

δ_H (400 MHz, CD₃OD): 7.99 (d, 1H, J 9.1, CH-8), 6.52 (d, 1H, J 9.1, CH-7), 4.24 (app. s, 1H, CH-4), 3.18 (m, 9H, CH₂-H-5', CH₂-H-5'', CH-4a, 2 x CH₃-NMe₂), 2.27 (s, 3H, CH₃)

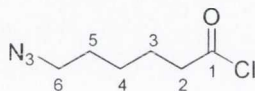
4.3.15 6-Azidohexanoic acid (**221**)



A 1 L round bottom flask was charged with **220** (8.016g, 41.09 mmol), sodium azide (5.343g, 82.19 mmol) and 2-methoxyethanol (320 mL). The reaction was refluxed at 120 °C with stirring for 17 h. The reaction was cooled, diluted with CH₂Cl₂ (320 mL) and washed with Millipore water (200 mL) and 0.1M HCl (aq) (100 mL). The organic layer was dried over magnesium sulfate, filtered and the solvent removed at reduced pressure to furnish the crude product as a yellow oil. The crude product was distilled at reduced pressure providing the desired product **221** (4.158g, 27.73 mmol) in 67% yield. The NMR data agree with the referenced literature.²⁶⁶

δ_{H} (400 MHz, CDCl_3):	3.31 (t, 2H, J 6.9, CH_2 -6), 2.41 (t, 2H, J 7.4, CH_2 -2), 1.68 (m, 4H, CH_2 -5, CH_2 -3), 1.47 (m, 2H, CH_2 -4)
δ_{C} (100 MHz, CDCl_3):	178.6 (q), 50.8 (CH_2), 33.3 (CH_2), 28.1 (CH_2), 25.7 (CH_2), 23.7 (CH_2)
HRMS (m/z ESI-):	Found: 156.0768 (M - H $\text{C}_6\text{H}_{10}\text{N}_3\text{O}_2$ Requires: 156.0773)

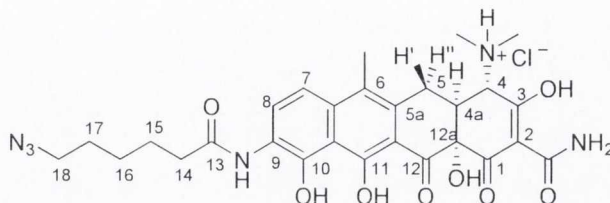
4.3.16 6-Azidohexanoyl chloride (**222**)



Compound **221** (1.08g, 6.88 mmol) was added to a 25 mL round bottom flask fitted with a condenser and drying tube. Freshly distilled thionyl chloride (2.5 mL, 4.1 g, 34 mmol) was added to the reaction vessel slowly *via* pipette. The reaction was heated to 40 °C and stirred for 1 h. The thionyl chloride was distilled from the reaction mixture and the remaining crude product distilled at reduced pressure. The desired product, **222**, was obtained as a colourless oil (2.296g, 13.07 mmol) in 47% yield. The NMR spectroscopic data agree with the referenced literature.²⁶⁷

δ_{H} (400 MHz, CDCl_3):	3.32 (t, 2H, J 6.8, CH_2 -6), 2.94 (t, 2H, J 7.4, CH_2 -2), 1.17 (m, 4H, CH_2 -5, CH_2 -3), 1.47 (m, 2H, CH_2 -4)
δ_{C} (100 MHz, CDCl_3):	173.2 (q), 50.6 (CH_2), 46.4 (CH_2), 28.0 (CH_2), 25.1 (CH_2), 24.1 (CH_2)
HRMS (m/z ESI+):	Found: 133.0394 (M - N_3 $\text{C}_6\text{H}_{10}\text{ClO}$ Requires: 133.0406)

4.3.17 9-(6-azido)Hexanamidoanhydrotetracycline hydrochloride (223)



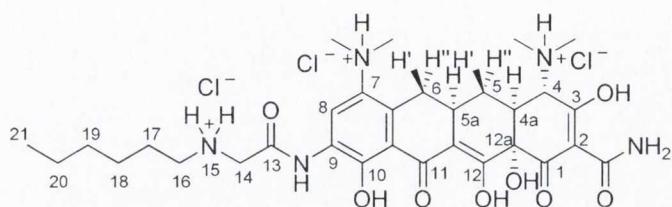
A 50 mL round bottom flask was charged with anhydrous sodium carbonate (21 mg, 0.194 mmol) and compound **128** (200 mg, 0.389 mmol) and placed under an atmosphere of Ar. Anhydrous acetonitrile (1.3 mL) and anhydrous *N,N*-DMPU (5.2 mL) were added *via* syringe and the solution cooled to -10 °C. A solution of compound **222** (137 mg, 0.778 mmol) in anhydrous *N,N*-DMPU (880 µL) and anhydrous acetonitrile (220 µL) was added dropwise *via* syringe over 15 min and a further 500 µL anhydrous acetonitrile used to afford the complete transfer of the acid chloride. The reaction was allowed to stir at -10 °C for 20 mins after which time methanol (2 mL) was added and the reaction stirred for a further 5 mins as it warmed to room temperature. The reaction was acidified with anhydrous hydrogen chloride in diethyl ether (2.0 M, 2 mL, 4.0 mmol) and added dropwise to diethyl ether (50 mL) at 0 °C to afford precipitation of the product. The supernatant was decanted and the compound washed with diethyl ether (2 x 50 mL). The product was filtered under Ar, washed with diethyl ether and purified by MPLC on a SNAP-12g KP-C18-HS cartridge (mobile phase: acetonitrile/Millipore water (gradient 10%-50%)). The product fractions were acidified with 8% HCl (aq) and the solvent removed at reduced pressure. The solid was dissolved in a minimum volume of methanol, precipitated into diethyl ether (50 mL) and collected by filtration under Ar. The product was dried under high vacuum to yield **223** (180 mg, 0.310 mmol) as a yellow solid in 80% yield (of which 4% is *epi*-9-(6-azido)hexanamidoanhydrotetracycline hydrochloride). M.p. 144-150 °C (dec.). Nitrogen NMR assignments of amides were extracted from HSQC experiments between ¹H and ¹⁵N.

δ_{H} (400 MHz, CD₃OD):

8.22 (d, 1H, J 9.1, CH-8), 7.42 (d, 1H, J 9.1, CH-

	7), 4.32 (s, 1H, CH-4), 3.65 (m, 1H, CH ₂ -H-5''), 3.44 (m, 1H, CH-4a), 3.37 (m, 2H, obscured by methanol peak, CH ₂ -18), 3.11 (m, 7H, CH ₂ -H-5', NMe ₂), 2.54 (m, 2H, CH ₂ -14), 2.43 (s, 3H, CH ₃), 1.79 (m, 2H, CH ₂ -15), 1.68 (m, 2H, CH ₂ -17), 1.54 (m, 2H, CH ₂ -16)
δ_C (100 MHz, CD ₃ OD):	199.1 (q), 192.3 (q), 186.3 (q), 172.9 (q), 172.6 (q), 163.3 (q), 146.9 (q), 135.1 (q), 128.3 (q), 127.0 (CH-8), 122.1 (q), 121.0 (q), 114.1 (CH-7), 111.3 (q), 108.1 (q), 96.4 (q), 76.0 (q), 67.9 (CH-4), 50.5 (CH ₂ -18), 42.33 (CH ₃ -NMe ₂), 41.1 (CH ₃ -NMe ₂), 35.6 (CH ₂ -14), 34.1 (CH-4a), 29.2 (CH ₂ -5), 27.8 (CH ₂ -17), 25.6 (CH ₂ -16), 24.6 (CH ₂ -15), 12.4 (CH ₃)
δ_N (600 MHz, DMSO-d ₆):	123.0 (NH-9), 112.5 (NH-2)
HRMS (m/z ESI+):	Found: 581.2339 (M + H C ₂₈ H ₃₃ N ₆ O ₈ Requires: 581.2360)
ν/cm^{-1} (neat):	3327 (O-H phenolic stretch), 2938 (C-H stretch), 2098 (N ₃ stretch), 1597, 1513, 1404, 1318, 1232, 1139, 1090, 985, 897, 817, 708

4.3.18 9-[2-(hexylamino)]Acetamidominocycline trihydrochloride
(225)



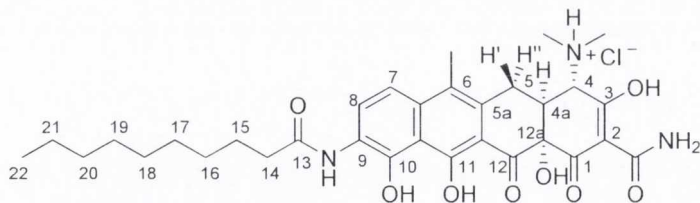
Compound **170** (100 mg, 0.17 mmol) and NaI (10 mg, 0.067 mmol) were added to a 50 mL round bottom flask and placed under an atmosphere of Ar. *n*-Hexylamine (680 μ L, 520 mg, 5.14 mmol) was added *via* syringe and the reaction heated to 25 $^{\circ}$ C and stirred for 18 h. Methanol (2 mL) was added and the reaction evaporated at reduced pressure to a solid. The crude product was dissolved in methanol (3 mL) and Millipore water (6 mL) and the pH of the solution lowered to 7.2 using 8% HCl (aq). The product was extracted with CH₂Cl₂ (3 x 8 mL) and the organic extracts combined. HCl in anhydrous diethyl ether (2.0 M, 400 μ L, 0.8 mmol) was added resulting in precipitation of the product as a trihydrochloride salt. The solvent was removed at reduced pressure to afford **225** as a trihydrochloride salt (70 mg, 0.097 mmol) in 57% yield. M.p. 89 $^{\circ}$ C (dec.)

δ_{H} (400 MHz, DMSO-d₆):

14.62 (s, 1H, OH-12), 12.30 (s, 1H, OH-10), 10.52 (s, OH), 10.34 (s, 1H, NH-9), 9.61 (s, 1H, NH₂-2), 9.32 (s, 2H, NH₂⁺-15), 9.06 (s, 1H, NH₂-2), 8.39 (s, 1H, CH-8), 7.54 (s, 1H, OH), 4.39 (s, 1H, CH-4), 4.04 (t, 2H, J 5.2, CH₂-14), 3.53 (m, 1H, CH₂-H-6''), 3.05 (m, 1H, H-4a), 2.96 (m, 3H, H-5a, CH₂-16), 2.88 (m, 12H, 4 x CH₃-NMe₂-4, NMe₂-7), 2.35 (m, 1H, CH₂-H-6'), 2.27 (m, 1H, CH₂-H-5''), 1.67 (m, 2H, CH₂-17), 1.49 (m, 1H, CH₂-H-5'), 1.29 (m, 6H, CH₂-18, CH₂-19, CH₂-20), 0.88 (m, 3H, CH₃-21)

δ_{C} (125 MHz, DMSO- d_6):	194.2 (q), 192.7 (q), 187.5 (q), 175.5 (q), 173.4 (q), 172.3 (q), 165.1 (q), 132.7 (q), 132.3 (q), 125.3 (q), 121.2 (CH-8), 116.3 (q), 108.5 (q), 95.6 (q), 74.3 (q), 68.3 (CH-4), 66.1 (CH ₂ -14), 47.3 (CH ₂ -16), 45.5 (2 x CH ₃ , NMe ₂ -7), 41.7 (CH ₃ , NMe ₂ -4), 41.3 (CH ₃ , NMe ₂ -4), 34.7 (H-4a), 34.2 (CH ₂ -5), 31.8 (H-5a), 31.1 (CH ₂ -19), 29.9 (CH ₂ -6), 25.8 (CH ₂ -18), 25.6 (CH ₂ -17), 22.2 (CH ₂ -20), 14.1 (CH ₃ -21)
ν/cm^{-1} (neat):	3355 (O-H phenolic stretch), 2928 (C-H alkyl stretch), 1538 (N-H bend), 1468, 1263, 1208, 1051
HRMS (m/z ESI+):	Found: 614.3187 (M + H C ₃₁ H ₄₄ N ₅ O ₈ Requires: 614.3190)

4.3.19 9-Decanamidoanhydrotetracycline hydrochloride (**226**)



Anhydrous sodium carbonate (5.0 mg, 0.049 mmol) and compound **128** (50 mg, 0.097 mmol) were added to a 10 mL round bottom flask containing a stirring bar under an atmosphere of Ar. Anhydrous acetonitrile (320 μL) and anhydrous *N,N*-DMPU (1.3 mL) were added *via* syringe and the solution cooled to 0 $^{\circ}\text{C}$. A solution of decanoyl chloride (39 μL , 35.8 mg, 0.19 mmol) in anhydrous *N,N*-DMPU (480 μL) and anhydrous acetonitrile (120 μL) was added dropwise *via* syringe over 15 min and the reaction stirred at 0 $^{\circ}\text{C}$ for 1 h. Methanol (2 mL) was added to the reaction and stirred for 5 min. The mixture was then acidified with

hydrogen chloride in diethyl ether (2.0 M, 200 μ L, 0.4 mmol) and added dropwise to diethyl ether (200 mL) to facilitate precipitation of the crude product. The crude product was triturated with diethyl ether (3 x 20 mL) and dissolved in water at pH 7.2. Extraction of the free base product into CH_2Cl_2 (3 x 10 mL) and acidification of the organic extracts with HCl in anhydrous diethyl ether (2.0 M, 200 μ L, 0.4 mmol) followed by evaporation of the solvent at reduced pressure, furnished **226** a brown solid (13 mg, 0.021 mmol) in 43% yield. M.p. 79 $^\circ\text{C}$ (dec.)

δ_{H} (600 MHz, CD_3OD):

8.26 (d, 1H, J 9.0, CH-8), 7.49 (d, 1H, J 9.0, CH-7), 4.31 (app. s, 1H, CH-4), 1.21 (m, 1H, $\text{CH}_2\text{-H-5''}$), 3.45 (m, 1H, CH-4a), 3.10 (app. br s, 7H, $\text{CH}_2\text{-H-5'}$, 2 x $\text{CH}_3\text{-NMe}_2\text{-4}$), 2.51 (t, 2H, J 7.8, $\text{CH}_2\text{-14}$), 2.46 (s, 3H, CH_3), 1.76 (m, 2H, $\text{CH}_2\text{-15}$), 1.45 (m, 2H, $\text{CH}_2\text{-16}$), 1.41 (m, 2H, $\text{CH}_2\text{-17}$), 1.34 (m, 8H, $\text{CH}_2\text{-18}$, $\text{CH}_2\text{-19}$, $\text{CH}_2\text{-20}$, $\text{CH}_2\text{-21}$), 0.91 (t, 2H, $\text{CH}_3\text{-22}$)

δ_{C} (150 MHz, $\text{CD}_3\text{OD-}d_6$):

199.2 (q), 192.5 (q), 186.5 (q), 173.6 (q, C-13), 173.0 (q), 162.9 (q), 157.4 (q), 147.4 (q), 135.6 (q), 128.7 (q), 127.6 (CH-8), 122.5 (q), 121.5 (q), 114.6 (CH-7), 111.8 (q), 96.7 (q), 76.3 (q), 68.3 (CH-4), 42.6 ($\text{CH}_3\text{-NMe}_2\text{-4}$), 41.4 ($\text{CH}_3\text{-NMe}_2\text{-4}$), 36.2 ($\text{CH}_2\text{-14}$), 36.0 (CH-4a), 31.5 ($\text{CH}_2\text{-21/20}$), 29.04 ($\text{CH}_2\text{-18/19}$), 28.95 ($\text{CH}_2\text{-17}$), 28.85 ($\text{CH}_2\text{-18/19}$), 28.76 ($\text{CH}_2\text{-16}$), 28.7 ($\text{CH}_2\text{-5}$), 25.5 ($\text{CH}_2\text{-15}$), 22.2 ($\text{CH}_2\text{-21/20}$), 12.9 ($\text{CH}_3\text{-22}$), 12.7 (CH_3)

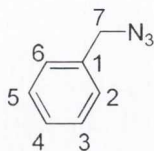
HRMS (m/z ESI+):

Found: 596.2969 (M + H. $\text{C}_3\text{H}_{42}\text{N}_3\text{O}_8$ Requires: 596.2972)

ν/cm^{-1} (neat):

3365 (O-H phenolic stretch), 2970, 2495, 1637 (C=O amide stretch), 1591, 1436, 1230, 1099, 990

4.3.20 Benzyl azide (**228**)

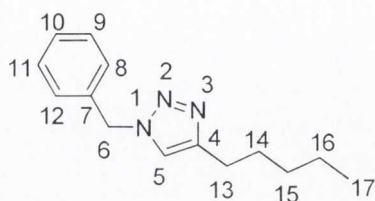


A round bottom flask was charged with **229** (2.00 mL, 2.88 g, 16.84 mmol), sodium azide (1.39 g, 21.34 mmol), methanol (12.4 mL) and Millipore water (1.9 mL) and heated at reflux (100 °C) for 16 h. The reaction was allowed to cool to room temperature and Millipore water (5 mL) was added. The product was extracted into diethyl ether (3 x 50 mL) and the organic extracts combined and washed with a saturated solution of calcium chloride (50 mL). The organic extract was dried over magnesium sulfate and the solvent evaporated at reduced pressure to afford **228** (1.95 g, 14.7 mmol) in 87% yield.

δ_{H} (400 MHz, CDCl_3):
7.39 (m, 5H, CH-2, CH-3, CH-4, CH-5, CH-6),
4.37 (s, 2H, CH_2 -7)

ν/cm^{-1} (neat):
3092 (aromatic C-H stretch), 3065 (aromatic C-H stretch), 2174 (N_3 stretch), 1463, 1462, 1389, 1235, 1162, 1177, 1041, 986, 775, 668

4.3.21 1-benzyl-4-pentyl-1-*H*-1,2,3-triazole (**230**)

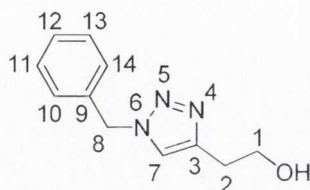


A microwave reaction vessel was charged with **207** (34.0 μ L, 25.0 mg, 0.260 mmol), **228** (32.5 μ L, 34.6 mg, 0.260 mmol), CuI (15 mg, 0.078), DMF (1.3 mL) and Millipore water (1.3 mL) and irradiated in a microwave reactor for 1 h at 100 $^{\circ}$ C. 1 H NMR spectroscopy deemed the conversion to **230** to be 85%. **230** Was extracted into diethyl ether (10 mL) and washed with Millipore water (3 x 5 mL). The organic extract was dried over magnesium sulfate and evaporated at reduced pressure to provide **230** (35 mg, 0.15 mmol) in 59% yield. 1 H NMR spectroscopic data agree with the referenced literature.²⁶⁸

δ_{H} (400 MHz, CDCl_3): 7.31 (m, 5H, CH-8, CH-9, CH-10, CH-11, CH-12), 7.20 (s, 1H, CH-5), 5.49 (s, 2H, CH₂-6), 2.68 (t, 2H, J 7.7, CH₂-13), 1.64 (m, 2H, CH₂-14), 1.32 (m, 4H, CH₂-15, CH₂-16), 0.88 (t, 3H, J 6.9, CH₃-17)

HRMS (m/z ESI⁺): Found: 230.1652 (M + H C₁₄H₂₀N₃ Requires: 230.1657)

4.3.22 2-(1-benzyl-1*H*-1,2,3-triazol-4-yl)ethanol (**233**)



A 10 mL round bottom flask was charged with **225** (20 mg, 0.028 mmol), **228** (3.5 μ L, 3.8 mg, 0.028 mmol) in ethanol (31.5 μ L) and **232** (4.3 μ L, 4.0 mg, 0.056 mmol) in ethanol (38.7 μ L). To this was added CuSO₄ (91 mg, 0.364 mmol) and sodium ascorbate (144 mg, 0.728 mmol) and ethanol/Millipore water (1:1, 1 mL). It was noted that not all of the CuSO₄ and sodium ascorbate dissolved. The reaction was allowed to proceed for 30 min after which time triazole **233** was extracted into CH₂Cl₂ and the solvent evaporated at reduced pressure to provide **233** (13.6 mg, 0.0177 mmol) in 63% yield. ¹H NMR spectroscopic data agree with the referenced literature.²⁶⁹

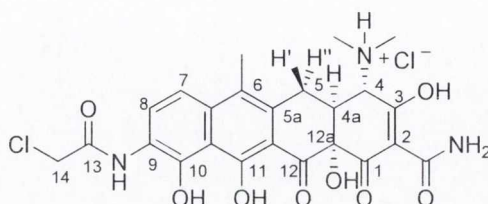
δ_{H} (400 MHz, CD₃OD):

7.40 (app. br. s, 6H) 5.71 (br. s, 2H), 2.94 (m, 2H), 2.73 (app. br. s, 2H)

HRMS (m/z ESI+):

Found: 226.0961 (M + Na C₁₁H₁₃N₃ONa
Requires: 226.0956)

4.3.23 9-(2-chloro)Acetamidoanhydrotetracycline hydrochloride (235)



Compound **128** (200 mg, 0.389 mmol) was added to a 10 mL round bottom flask containing anhydrous sodium carbonate (21 mg, 0.194 mmol) and placed under an atmosphere of Ar. To this, anhydrous *N,N*-DMPU (5.2 mL) and anhydrous acetonitrile (1.3 mL) were added *via* syringe. The reaction was cooled to -10 °C and a solution of chloroacetyl chloride (62 µL, 0.778 mmol) in anhydrous *N,N*-DMPU (800 µL) and anhydrous acetonitrile (200 µL) was added dropwise *via* syringe. After 20 mins, methanol (1.5 mL) was added and the reaction stirred for a further 5 mins. Hydrogen chloride in anhydrous diethyl ether (2.0 M, 0.5 mL, 1.0 mmol) was added and the reaction mixture precipitated into diethyl ether (200 mL). The supernatant was decanted and the solid washed with diethyl ether (2 x 200 mL). The solid was collected by filtration under Ar and purified by MPLC on a 12 g SNAP KP-C18-HS cartridge (mobile phase: acetonitrile/0.1 % HCl in Millipore water (gradient 10%-50%)) to furnish the desired product, **235**, as a mustard coloured solid in 22% yield (48 mg, 0.09 mmol). M.p. 140-145 °C (dec.)

δ_{H} (400 MHz, DMSO- d_6):

9.82 (s, 1H, NH-9), 9.65 (s, 1H, NH₂-2), 9.24 (s, 1H, NH₂-2), 8.25 (d, 1H, J 9.0, CH-8), 7.54 (d, 1H, J 9.0, CH-7), 4.44 (s, 2H, CH₂-14), 4.39 (s, 1H, CH-4), 3.60 (m, 1H, CH₂-H-5''), 3.45 (m, 1H, CH-4a), 2.93 (app. s, 7H, CH₂-H-5', 2 x CH₃-NMe₂-4), 2.43 (s, 3H, CH₃)

δ_{C} (100 MHz, DMSO- d_6):

199.7 (q), 192.6 (q), 186.6 (q), 173.1 (q), 165.8 (q), 162.7 (q), 157.5 (q), 146.7 (q), 135.5 (q), 129.1 (CH-8), 125.8 (q), 121.53 (q), 114.8 (CH-249

7), 111.6 (q), 108.7 (q), 96.9 (q), 76.5 (q), 68.4 (CH-4), 43.8 (CH₂-14), 42.6 (CH₃-NMe₂), 41.7 (CH₃-NMe₂), 36.0 (CH-4a), 29.0 (CH₂-5), 13.0 (CH₃)

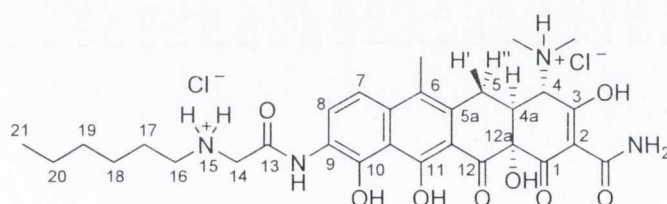
HRMS (*m/z* ESI+):

Found: 518.1337 (M + H C₂₄H₂₅ClN₃O₈)
Requires: 518.1330)

ν/cm^{-1} (neat):

3334 (O-H phenolic stretch), 1597, 1403, 1320, 1231, 1089, 987

4.3.24 9-(2-hexylamino)Acetamidoanhydrotetracycline dihydrochloride (**239**)



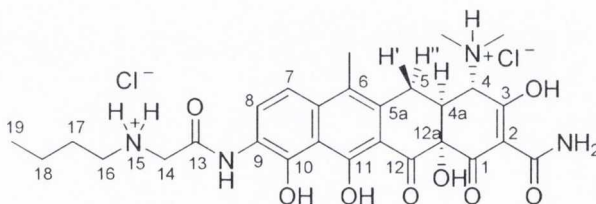
A 10 mL round bottom flask was charged with **235** (20mg, 0.036 mmol) and NaI (2mg, 0.013 mmol) and placed under an atmosphere of Ar. *n*-Hexylamine (143 μL , 110 mg, 1.08 mmol) was added *via* syringe. The reaction was heated to 25 °C and stirred for 1 h. Methanol (1 mL) was added and the reaction concentrated at reduced pressure and dissolved in a minimum volume of water at pH 7.2. The free base product was extracted into a mixture of methanol/CH₂Cl₂ (1:4) and the hydrochloride salt formed by the addition of hydrogen chloride in anhydrous diethyl ether (2.0 M, 100 μL , 0.2 mmol) to furnish the desired product, **239**, as a brown solid (8 mg, 0.13 mmol) in 36% yield. M.p. 220-223 °C (dec.)

δ_{H} (400 MHz, CD₃OD):

8.40 (d, 1H, J 8.0, CH-8), 7.54 (d, 1H, J 8.0, CH-7), 4.38 (app. s, 1H, CH-4), 4.14 (s, 2H, CH₂-14), 3.66 (m, 1H, CH₂-H-5''), 3.46 (m, 1H, CH-250

	4a), 3.11 (m, 9H, CH ₂ -16, 2 x CH ₃ -NMe ₂ -4, CH ₂ -H-5'), 2.49 (s, 3H, CH ₃), 1.79 (m, 2H, CH ₂ -17), 1.43 (m, 6H, CH ₂ -18, CH ₂ -19, CH ₂ -20), 0.97 (t, 3H, J 6.0, CH ₃ -21)
δ_C (150 MHz, DMSO-d ₆):	200.5 (q), 193.3 (q), 187.1 (q), 173.8 (q), 164.3 (q), 163.2 (q), 147.7, (q), 136.3 (q), 129.7 (q), 127.4 (CH-8), 123.2 (q), 121.2 (q), 115.3 (CH-7), 112.4 (q), 109.2 (q), 97.4 (q), 76.9 (q), 68.9 (CH-4), 48.8 (CH ₂ -14), 47.5 (obscured by DMSO, CH ₂ -16), 43.2 (CH ₃ -NMe ₂), 41.8 (CH ₃ -NMe ₂), 36.4 (CH-4a), 31.4 (CH ₂ -19), 29.3 (CH ₂ -5), 26.2 (CH ₂ -18), 26.1 (CH ₂ -17), 22.4 (CH ₂ -20), 13.3 (CH ₃), 8.2 (CH ₃ -21)
HRMS (<i>m/z</i> ESI+):	Found: 583.2768 (M + H C ₃₀ H ₃₉ N ₄ O ₈ Requires: 583.2768)
ν/cm^{-1} (neat):	3336 (O-H phenolic stretch), 2507, 1571 (N-H bend), 1398, 1317, 1231, 1097, 954, 800

4.3.25 9-[2-(*n*-butylamino)]Acetamidoanhydrotetracycline dihydrochloride (240)



A 10 mL round bottom flask was charged with **235** (20mg, 0.036 mmol) and NaI (2mg, 0.013 mmol) and placed under an atmosphere of Ar. *n*-Butylamine (107 μL , 79.2 mg, 1.08 mmol) was added *via* syringe and the reaction heated to 25 °C and stirred for 1 h. Methanol (1 mL)

was added and the reaction concentrated at reduced pressure and dissolved in a minimum volume of water at pH 7.2. The free base product was extracted once into a mixture of methanol/dichloromethane (1:4, 30 mL), once into CH₂Cl₂ (30 mL) and once into chloroform (30 mL). The hydrochloride salt was formed by the addition of HCl in anhydrous diethyl ether (2.0 M, 100 μ L, 0.2 mmol) to the combined organic layers. The solvent was removed at reduced pressure to furnish the desired product, **240**, as a brown solid (3 mg, 0.05 mmol) in 13% yield. M.p. 195-200 °C (dec.)

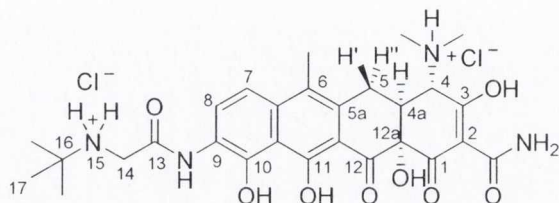
δ_{H} (400 MHz, CD₃OD): 8.39 (d, 1H, J 9.0, CH-8), 7.55 (d, 1H, J 9.0, CH-7), 4.32 (app. s, 1H, CH-4), 4.17 (s, 2H, CH₂-14), 3.68 (m, 1H, CH₂-H-5''), 3.46 (m, 1H, CH-4a), 3.15 (t, 2H, J 7.8, CH₂-16), 3.11 (m, 7H, CH₂-H-5', 2 x CH₃-NMe₂) 1.78 (m, 2H, CH₂-17), 1.50 (m, 2H, CH₂-18), 1.04 (t, 2H, J 7.4, CH₃-19)

δ_{C} (150 MHz, CD₃OD): 200.7 (q), 193.1 (q), 187.6 (q), 173.8 (q), 173.6 (q), 164.4 (q), 147.8 (q), 136.4 (q), 129.7 (q), 127.4 (CH-8), 123.2 (q), 121.2 (q), 115.3 (CH-7), 112.3 (q), 109.1 (q), 97.4 (q), 77.0 (q), 68.9 (CH-4), 48.6 (CH₂-14), 47.4 (obscured by CD₃OD, CH₂-16), 43.3 (CH₃-NMe₂), 42.0, (CH₃-NMe₂), 36.5 (H-4a), 29.4 (CH₂-5), 28.1 (CH₂-17) 19.8 (CH₂-18), 13.3 (CH₃), 12.9 (CH₃-19)

HRMS (m/z ESI+): Found: 555.2462 (M + H C₂₈H₃₅N₄O₈ Requires: 555.2455)

ν/cm^{-1} (neat): 3376 (O-H phenolic stretch), 2963 (C-H alkyl stretch), 1624 (C=O amide stretch), 1594 (N-H bend), 1469, 1402, 1260, 1226, 1104, 919, 802, 746

4.3.26 9-[2-(*t*-butylamino)]Acetamidoanhydrotetracycline dihydrochloride (241)



A 10 mL round bottom flask was charged with **235** (20mg, 0.036 mmol) and NaI (2mg, 0.013 mmol) and placed under an atmosphere of Ar. *t*-Butylamine (113 μ L, 78.6 mg, 1.08 mmol) was added *via* syringe and the reaction heated to 25 $^{\circ}$ C and stirred for 18 h. Methanol (1 mL) was added and the reaction concentrated at reduced pressure and dissolved in a minimum volume of water at pH 7.2. The free base product was extracted once into a mixture of methanol/ CH_2Cl_2 (1:4, 30 mL), once into CH_2Cl_2 (30 mL) and once into chloroform (30 mL). The hydrochloride salt was formed by the addition of hydrogen chloride in anhydrous diethyl ether (2.0 M, 100 μ L, 0.2 mmol) to the combined organic layers. The solvent was removed at reduced pressure to furnish the desired product, **241**, as a brown solid (5 mg, 0.008 mmol) in 22% yield. M.p. 195-200 $^{\circ}$ C (dec.)

 δ_{H} (400 MHz, CD_3OD):

8.45 (d, 1H, J 9.1, CH-8), 7.58 (d, 1H, J 9.1, CH-7), 4.28 (app. s, 1H, CH-4), 4.13 (s, 2H, CH₂-14), 3.65 (m, 1H, CH₂-H-5''), 3.46 (m, 1H, CH-4a), 3.08 (m, 7H, 2 x CH₃-NMe₂-4, CH₂-H-5'), 2.50 (s, 3H, CH₃), 1.46 (s, 9H, 3 x CH₃-17)

 δ_{C} (150 MHz, DMSO- d_6):

200.3 (q), 194.1 (q), 193.0 (q), 175.6 (q), 173.6 (q), 164.5 (q), 147.6 (q), 136.3 (q), 129.7 (q), 127.3 (CH-8), 123.1 (q), 121.3 (q), 115.3 (CH-7), 112.4 (q), 109.2 (q), 97.2 (q), 76.9 (q), 68.9 (CH-4), 53.8 (q), 43.4 (CH₂-14), 43.3 (CH₃, NMe₂), 41.7 (CH₃, NMe₂), 36.4 (CH-4a), 29.7

(CH₂-5), 24.9 (3 x CH₃-17), 13.2 (CH₃)

HRMS (*m/z* ESI+): Found: 555.2462 (M + H C₂₈H₃₅N₄O₈ Requires: 555.2455)

ν/cm^{-1} (neat): 3347 (phenolic O-H stretch), 2963 (alkyl C-H stretch), 1597, 1403, 1320, 1260, 1030, 789, 707

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